

Influence of environmental variables on saxitoxin yields by *Cylindrospermopsis raciborskii* in a mesotrophic subtropical reservoir

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ABSTRACT

Saxitoxins are a class of toxins produced by at least two groups of evolutionarily distant organisms (cyanobacteria and dinoflagellates). While the toxicity of these toxins is relatively well characterized, to date little is known about their drivers and ecological functions, especially in lower latitude tropical and subtropical freshwater ecosystems. In the present study, we aimed to obtain a better understanding of the main drivers of saxitoxin concentrations in aquatic environments. We investigated the relationships among saxitoxin concentrations in a mesotrophic subtropical reservoir dominated by the cyanobacteria *Cylindrospermopsis raciborskii* with physical, chemical and biological water variables. The highest saxitoxin concentrations were $0.20 \mu\text{g}\cdot\text{L}^{-1}$, which occurred in the samples with the highest densities of *C. raciborskii* (maximum of $4.3 \times 10^4 \text{ org}\cdot\text{mL}^{-1}$) and the highest concentration of dissolved nutrients (nitrate from 0.2 to $0.8 \mu\text{g}\cdot\text{L}^{-1}$, orthophosphate from 0.3 to $8.5 \mu\text{g}\cdot\text{L}^{-1}$). These correlations were confirmed by statistical analyses. However, the highest saxitoxin relative concentrations (per trichome) were associated with lower *C. raciborskii* densities, suggesting that saxitoxin production or the selection of saxitoxin-producing strains was associated with the adaptation of this species to conditions of stress. Our results indicate that *C. raciborskii* toxin yields vary depending on the enrichment conditions having potential implications for reservoir management.

Key words | artificial eutrophication, cyanobacteria, cyanotoxins, subtropical reservoir

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INTRODUCTION

Saxitoxins and their derivatives are neurotoxic alkaloids with Na^+ and Ca^{2+} channel affinity that affect the generation and transmission of nerve impulses in metazoan muscle and nerve cells and ion transport in bacteria (Wiese *et al.* 2010; McCusker *et al.* 2012). These toxins, which belong to the paralytic shellfish toxin group, are synthesized by two groups of evolutionarily distant organisms from different domains: Cyanobacteria (Prokarya) and Dinoflagellates (Eukarya).

In marine environments, intoxication by saxitoxins (SXT) occurs through the ingestion of fish and, especially,

of filter-feeding mollusks, which concentrate toxins as a result of the consumption of toxic organisms (Derby & Aggio 2011). Acute intoxication can cause death by respiratory failure. Histopathological changes in the cerebellum and liver damage have also been observed in mice (Band-Schmidt *et al.* 2010). The effects of chronic exposure to these substances have not been clearly determined in humans (Picot *et al.* 2011).

Despite the existing knowledge on saxitoxin toxicology, the biological and ecological importance of saxitoxins remains unclear, as well as the main environmental drivers

of their production. However, the synthesis of saxitoxins by two distinct groups and the evolution of the genes responsible for their complex metabolism seem to indicate that the toxins are probably linked with ecological advantages for their producers (Wiese *et al.* 2010; Murray *et al.* 2011; Dittmann *et al.* 2013; Hackett *et al.* 2013).

Cyanobacteria growth and dominance in reservoirs are associated with anthropogenic eutrophication processes. Several genera of Cyanobacteria are potential producers of saxitoxins in inland water environments. Such toxins may affect ecosystem services and become a relevant public health issue by affecting the water supply for human consumption (Pomati *et al.* 2004; Soares *et al.* 2013). Although there are no reports of any acute human contamination with saxitoxins through water supply, the presence of toxic algal strains in inland water environments has been reported to exert a large influence on aquatic communities and food chains, affecting zooplankton and fish, and thus changing the ecosystem metabolism (Ferrão-Filho *et al.* 2010, 2011; da Costa *et al.* 2013). *Anabaena* sp., *Aphanizomenon* sp., *Cylindrospermopsis* sp., *Lyngbya* sp., *Planktothrix* sp. (Wiese *et al.* 2010), and more recently *Scytonema* sp. (Smith *et al.* 2011) and *Tolypothrix* (Belykh *et al.* 2016) are some of the known genera of potentially saxitoxin-producing cyanobacteria.

Cylindrospermopsis raciborskii is prevalent in eutrophic environments worldwide, often dominating the phytoplankton communities of many tropical and temperate reservoirs (Wood *et al.* 2014; Antunes *et al.* 2015; Burford *et al.* 2016). *C. raciborskii* was recorded in Brazil in the last century (Lagos *et al.* 1999). Most *C. raciborskii* strains found in Brazil produce saxitoxin derivatives, whereas North American and Australian strains can produce cylindrospermopsin (hepatotoxin) and European strains produce other yet undescribed neurotoxins (Falconer 2005). Burford *et al.* (2016) correlated the saxitoxin production to conditions of high salinity and hardness, specifically in strains from South America. According to these authors, the success of *C. raciborskii* should be attributed to its great ability for photoadaptation and to survive in situations of low and variable concentrations of N and P.

Various hypotheses have been proposed for the biological and ecological functions of cyanotoxins and algal toxins, including allelopathy issues (Figueredo *et al.* 2007),

protection against predation and an increase in the competitive ability as a secondary metabolite (Holland & Kinnear 2013). However, little information is available regarding the action of saxitoxins on the physiology of cyanobacteria and their role in the population dynamics of these organisms in inland water environments.

Various different strains can coexist in the same environment and in some cases both toxin-producing and nonproducing strains coexist (Burford *et al.* 2014; Sinha *et al.* 2014; Willis *et al.* 2016). This observation shows that saxitoxin is important to increase the survival and competitive ability of the species, but it is not essential.

The present study aimed to relate the saxitoxin concentrations with physical, chemical, and biological (phytoplankton community) variables. This is expected to improve the knowledge about their drivers and ecological functions, especially in lower latitude subtropical freshwater ecosystems.

MATERIAL AND METHODS

The Itupararanga Reservoir is located in the most densely populated and economically developed region of Brazil (Southeast Region, State of São Paulo – Figure 1). The reservoir is medium sized and shallow by Brazilian standards (maximum depth: 23 m, average depth: 7.8 m, area: 20.9 km², hydraulic retention time: 112–749 days), and it is undergoing a gradual eutrophication process as a result of the disposal of untreated domestic sewage in its headwaters and diffuse pollution from agriculture in the surrounding area (Beghelli *et al.* 2012).

The reservoir is classified as mesotrophic and is polymictic with rare periods of mixing that usually occur in the winter (from June to August). The phytoplankton community, particularly in the deeper zones and closer to the dam, is dominated by cyanobacteria species, especially *Cylindrospermopsis raciborskii* (Woloszynska) Seenayya and Subba Raju (Cunha & Calijuri 2016b).

Sixty-one water samples from the integrated water column were collected at three different time scales: seasonal (dry and rainy), over 10 consecutive days, and every 4 hours over two daily variations. Water samples were taken at various depths (100, 50, 10, 1% in the euphotic zone

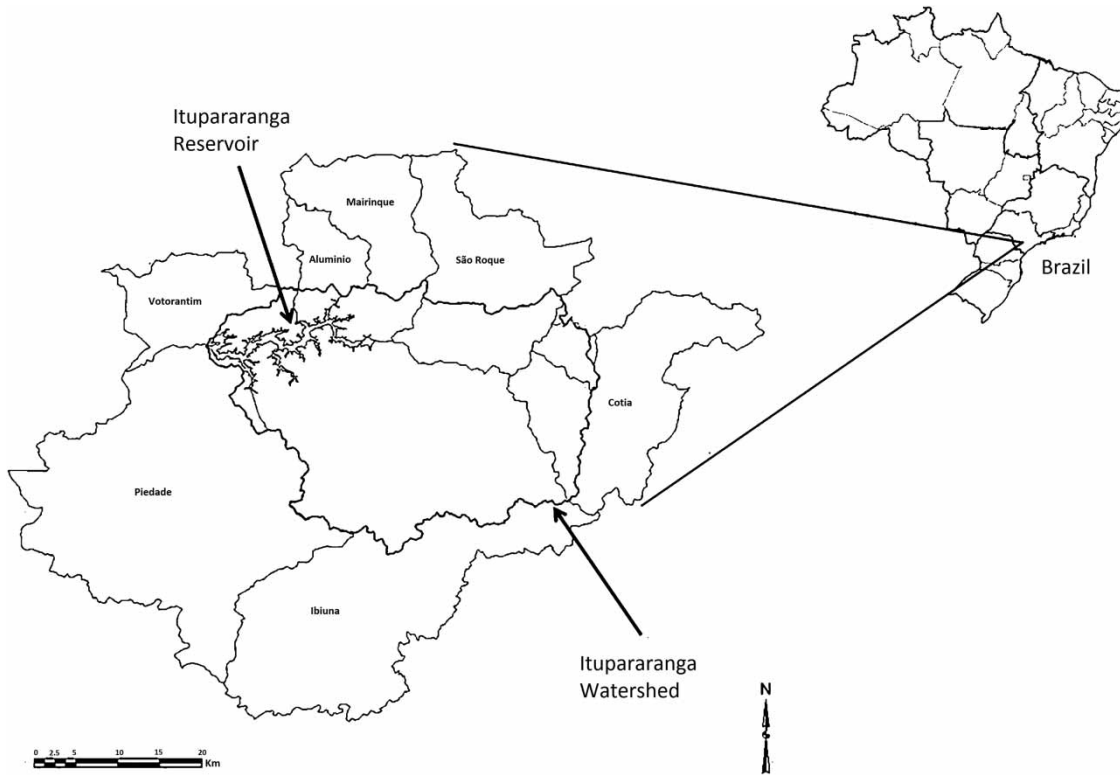


Figure 1 | Location of the hydrographic basin and the Itupararanga Reservoir. Modified from Secchin (2012).

and one in the aphotic zone), in daily samples (10 consecutive days) and in nictemeral samples (every 4 hours). For the physical and chemical data, the averages were weighted using the different depths. To analyze the concentration of saxitoxins, 100 mL aliquots of each depth were stored in a single vial producing a composite sample of the column. The samples were taken in February and July 2011 at two sampling sites in the deeper zone of the reservoir (two sets in the reservoir in the area closest to the dam, with 10 and 11 meters of depth).

The water temperature ($^{\circ}\text{C}$), electrical conductivity ($\mu\text{S}\cdot\text{cm}^{-1}$), dissolved oxygen (DO) ($\text{mg}\cdot\text{L}^{-1}$), and pH were measured *in situ* every 10 cm of the water column with a multiprobe (Yellow Springer, 556 MPS) and weighted averages were calculated for the water column. The analyses of the total and dissolved nutrients (phosphorus and nitrogen forms) and alkalinity were performed according to the APHA, AWWA & WPCF (2005) methodology. Chlorophyll *a* in filtered samples was determined according to Nusch (1980).

The quantitative analysis of phytoplankton was performed according to the sedimentation chamber method (Uthermöhl 1958). Total phytoplankton density was determined according to APHA, AWWA & WPCF (2005), the biovolume according to Hillebrand *et al.* (1999) and organic carbon according to Rocha & Duncan (1985).

The extraction of the total saxitoxin (extra and intracellular) from the water samples followed the methodology described (Törökné *et al.* 2004; Yılmaz *et al.* 2008). Afterwards, steps were taken to freeze and thaw the samples before the toxin quantification, allowing cell lysis to release intracellular toxin. Total saxitoxin determinations were carried out using the ELISA method (Enzyme Linked Immuno Sorbent Assay) based on the biochemical reactions among antigens and polyclonal antibodies of the toxin. Beacon plate kits (Beacon Analytical Systems Inc., ME, USA) were used for the reaction and the reading was made using a reader and microplate washer, Expert Plus and Atlantis models (ASYS Hitech, Eugendorf, Austria), respectively.

Descriptive statistics were calculated for the whole dataset (mean, maximum, minimum and standard deviation). The data were standardized according to Johnson & Wichern (2002) and a principal component analysis (PCA) was performed using 'Statistica' 2005 software. The selection and reduction of variables for PCA analysis was carried out through redundancy analysis.

RESULTS AND DISCUSSION

Monitoring saxitoxin production by *C. raciborskii* in freshwater aquatic systems is very important for water management and public health. However, the great adaptability to different environmental conditions, as well as the variability of responses of this species to these variations, make it difficult to control (Burford et al. 2016).

The phytoplankton community in the samples was dominated by only two species: *Monoraphidium contortum* (Thuret) Komárková-Legnerová and *Cylindrospermopsis*

raciborskii (Woloszynska) Seenayya and Subba Raju. These species accounted for between 100 and 54% of the relative abundance in terms of density ($\text{org}\cdot\text{mL}^{-1}$). The predominance of one or two species in the community composition indicates the great stability of the phytoplankton community of the studied reservoir (Flöder & Hillebrand 2012).

Monoraphidium contortum was more abundant than *C. raciborskii* in 50% of the samples and accounted for 10–74% of the community abundance, while the respective range for *C. raciborskii* was 7–83%. There was alternating dominance of the two species in the community in the two periods studied, considering density ($\text{org}\cdot\text{mL}^{-1}$). In the rainy season (summer), *M. contortum* predominated and in the dry period (winter) *C. raciborskii* was the most abundant (Figure 2).

Despite the observed coexistence between these species, when the cell density data were analyzed, with a dominance of alternating periods, *C. raciborskii* biomass was on average 500 times greater than that of *M. contortum* and was the

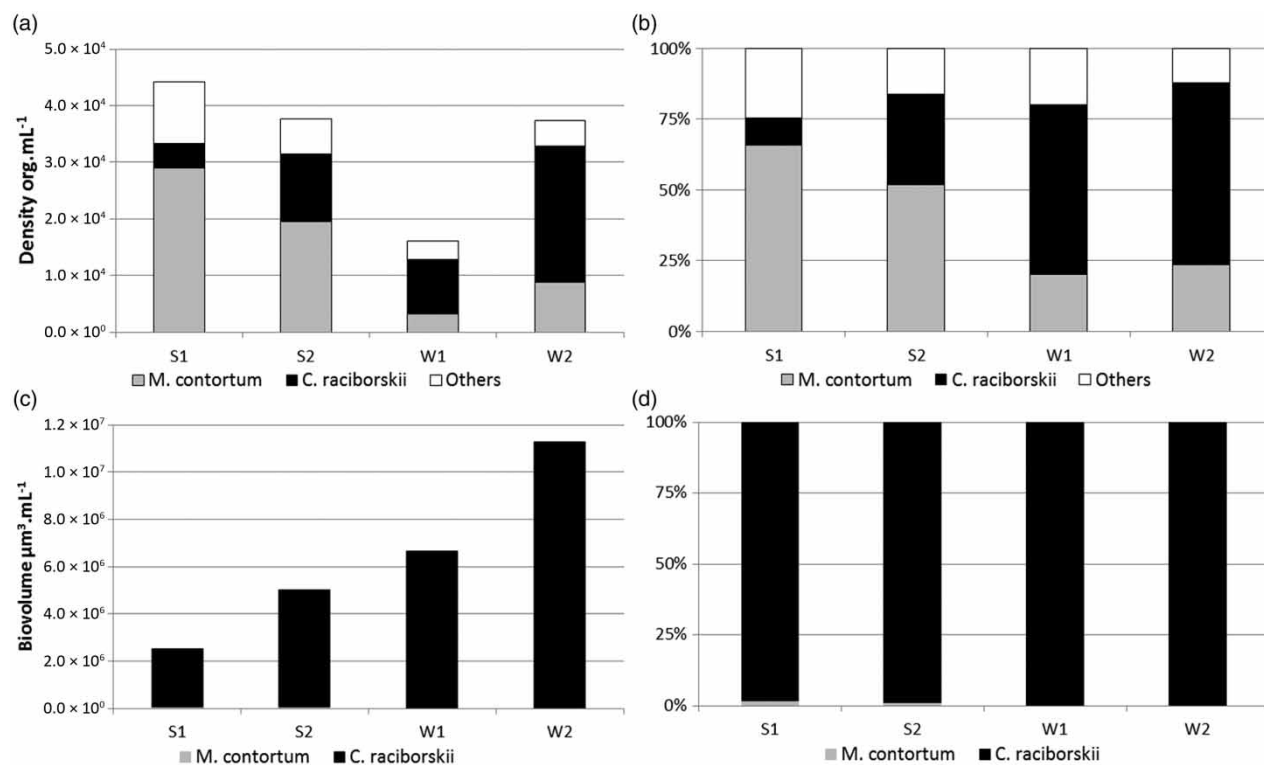


Figure 2 | (a) Variation of the mean of the phytoplankton organism densities ($\text{org}\cdot\text{mL}^{-1}$), (b) relative abundance by density, (c) average biomass of dominant species ($\mu\text{m}^3\cdot\text{mL}^{-1}$) and (d) relative abundance by biomass, where S1 = point 1 summer, S2 = point 2 summer, W1 = point 1 winter and W2 = point 2 Winter.

main contributor to the cyanobacteria dominance in the reservoir considering the cell volume and biovolume of *C. raciborskii*. The mean, minimum and maximum values, as well as the standard deviations for the quantified parameters are shown in Table 1.

The saxitoxin concentrations varied between 0.04 and 0.20 $\mu\text{g}\cdot\text{L}^{-1}$ and the highest concentrations were observed for the highest *C. raciborskii* densities ($r = 0.73$, $p < 0.001$). However, the saxitoxin concentration per trichome (TQ_{stx}) decreased when the *C. raciborskii* density ($r = -0.80$, $p < 0.001$) increased (Figure 3).

The dominance of *C. raciborskii* was inversely proportional to that of *M. contortum* in the analyzed samples as these two species comprised most of the community and the highest saxitoxin concentrations were, thus, inversely proportional to the *M. contortum* densities ($r = -0.61$, $p < 0.001$).

A t-test was performed to compare these samples in which the majority of the density consisted of *C. raciborskii*

cells (>50% density, $n = 26$) with the remaining samples being $n = 30$. The total saxitoxin concentrations were significantly higher ($p < 0.00001$) in the samples where *C. raciborskii* predominated. However, although higher mean saxitoxin quota per trichome was observed in samples where the majority of the density did not correspond to *C. raciborskii* cells, the differences were not significant ($p = 0.060$).

Five strains with morphologies different from that of *C. raciborskii* had been previously isolated from the Itupararanga Reservoir, but only two of these strains were saxitoxin producers (Vargas 2012). This could explain the saxitoxin concentration observed in this reservoir alternating between producing and non-producing strains.

The observed lower saxitoxin concentrations per trichome in samples with higher *C. raciborskii* densities may indicate that the adaptive advantage conferred by saxitoxin production is less effective under optimal growth conditions and predominant in the community. This result could be due

Table 1 | Variation range (average, maximum, minimum and standard deviations) of the physical, chemical and biological data of samples ($n = 56$) collected at the Itupararanga Reservoir

Parameters	Average	Minimum	Maximum	Standard deviation
Temperature ($^{\circ}\text{C}$)	21.8	16.8	26.4	4.1
pH	7.1	2.9	8.9	0.9
DO ($\text{mg}\cdot\text{L}^{-1}$)	6.4	2.0	9.3	2.0
Conductivity ($\mu\text{S}\cdot\text{cm}^{-1}$)	64.3	27.8	96.1	9.2
Nitrate ($\mu\text{g}\cdot\text{L}^{-1}$)	0.5	0.2	0.8	0.1
Nitrite ($\mu\text{g}\cdot\text{L}^{-1}$)	0.5	0.0	1.8	0.5
Orthophosphate ($\mu\text{g}\cdot\text{L}^{-1}$)	3.2	0.3	8.5	1.8
Total Kjeldahl nitrogen ($\mu\text{g}\cdot\text{L}^{-1}$)	200	0	700	200
Total phosphorus ($\mu\text{g}\cdot\text{L}^{-1}$)	23.9	8.3	42.5	7.7
Turbidity (NTU)	9.8	3.1	13.4	1.7
Alkalinity ($\text{meq}\cdot\text{L}^{-1}$)	0.5	0.2	0.8	0.1
Total organic carbon ($\text{mg}\cdot\text{L}^{-1}$)	4.3	1.7	6.7	0.8
Chlorophyll <i>a</i> ($\mu\text{g}\cdot\text{L}^{-1}$)	27.6	6.6	46.4	10.7
Pheophytin ($\mu\text{g}\cdot\text{L}^{-1}$)	3.7	0.5	15.9	3.2
Saxitoxin ($\mu\text{g}\cdot\text{L}^{-1}$)	0.14	0.04	0.20	0.05
Saxitoxin per trichome (10^{-3} pg)	13.3	4.5	29.9	6.2
Total phytoplankton density ($\text{org}\cdot\text{mL}^{-1}$)	3.7×10^4	9.1×10^3	6.1×10^4	1.2×10^4
<i>M. contortum</i> density ($\text{org}\cdot\text{mL}^{-1}$)	1.7×10^4	1.1×10^3	4.1×10^4	1.1×10^4
<i>C. raciborskii</i> density ($\text{org}\cdot\text{mL}^{-1}$)	1.4×10^4	3.2×10^3	4.3×10^4	9.6×10^3
<i>M. contortum</i> biovolume ($\mu\text{m}^3\cdot\text{mL}^{-1}$)	2.9×10^4	1.6×10^5	1.8×10^5	2.8×10^4
<i>C. raciborskii</i> biovolume ($\mu\text{m}^3\cdot\text{mL}^{-1}$)	6.5×10^6	1.9×10^6	1.8×10^7	4.2×10^6

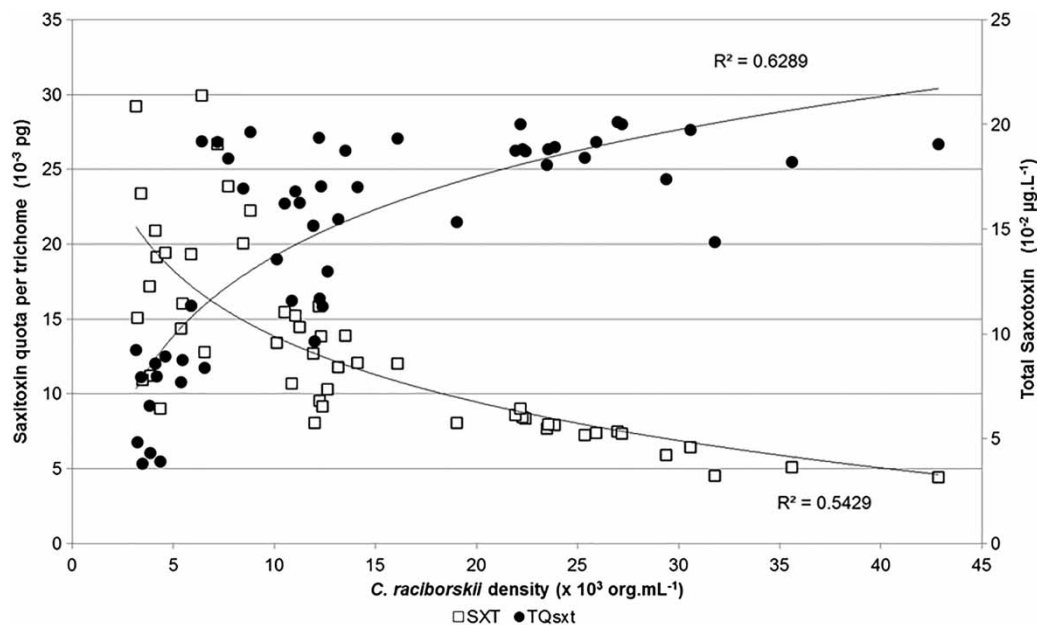


Figure 3 | Saxitoxin quota per trichome (TQsxt $\times 10^{-3}$ pg) and total saxitoxin concentration (SXT $\mu\text{g}\cdot\text{L}^{-1}$) for *C. raciborskii* density ($\text{org}\cdot\text{mL}^{-1}$).

to the production of secondary metabolites in bacterial physiology being linked to improved growth, but not necessarily to cell maintenance.

Willis *et al.* (2016) did not detect a relationship between cell size and filament length concerning saxitoxin quota in *C. raciborskii*. However, some studies have indicated that the size of cells or colonies is an important adaptive mechanism for increasing competitive potential (Yamamoto *et al.* 2011; Lopes *et al.* 2012). In our study, the mean *C. raciborskii* biovolume and the saxitoxin concentration per trichome were positively correlated ($r = 0.49$, $p < 0.001$). This result shows that, under conditions that promote the increase of in cell size, higher production of saxitoxin or selection of saxitoxin-producing strains also occurs, or even that saxitoxin-producing strains have larger cell sizes (Figure 4).

Burford *et al.* (2014) analyzed the effects of N and P concentrations on cell division and on the production of cylindrospermopsins in *C. raciborskii* in experiments with phytoplankton communities dominated by this species and obtained from tropical reservoirs. The amount of toxin per cell increased significantly in treatments where there was an increase in phosphorus without an increase in the rate of cell division. In this paper, positive and significant correlations were found between saxitoxin and DO ($r = 0.61$,

$p < 0.001$) and orthophosphate ($r = 0.57$, $p < 0.001$) concentrations, whereas negative correlations were found with the temperature ($r = -0.71$, $p < 0.001$) and pH ($r = -0.56$, $p < 0.001$) (Table 2).

The correlation of saxitoxin with oxygen and pH concentrations may be related to the growth of *C. raciborskii* biomass. In periods of high growth, an increase in oxygenation and a reduction in pH as a function of the photosynthetic process are commonly found. They are more a consequence than a cause of growth.

Cylindrospermopsis raciborskii was reported to be capable of growing faster and maintaining high biomass in phosphorus-limited environments when ammonium or nitrates were available (Kenesi *et al.* 2009). Cunha & Calijuri (2011a) reported phosphorus to be the nutrient that best explained the variation of the phytoplankton community dominated by *C. raciborskii* in the same ecosystem (Itaparanga Reservoir).

Moisander *et al.* (2008) claim that *C. raciborskii* is a good competitor when higher concentrations of dissolved nutrients, especially nitrogen, are available. The influence of nitrogen concentrations on saxitoxin synthesis is controversial (Brentano *et al.* 2016; Burford *et al.* 2016; Vico *et al.* 2016). On the other hand, the increase in saxitoxin production in environments with phosphorus limitation was

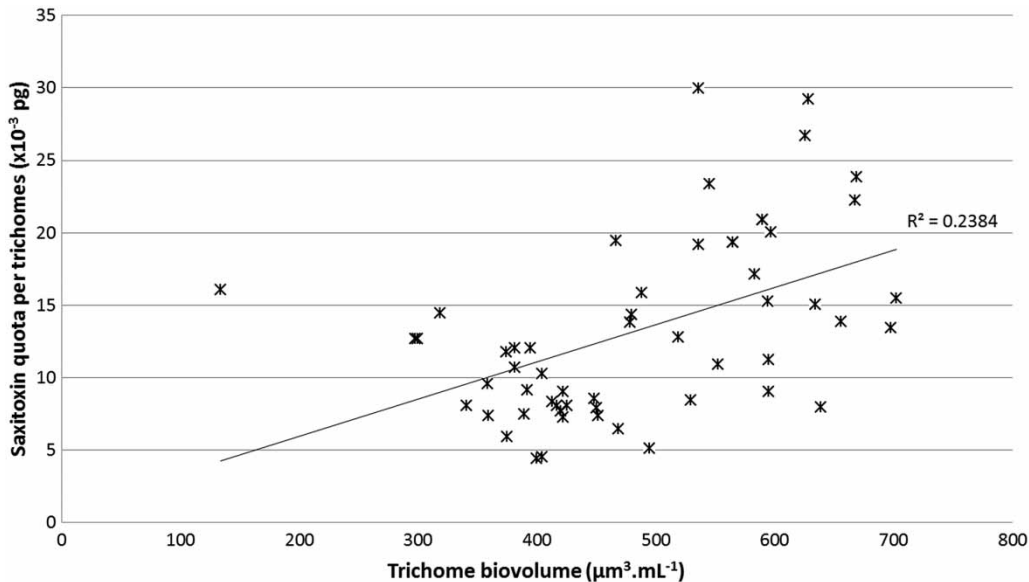


Figure 4 | Saxitoxin quota per trichome ($\times 10^{-3}$ pg) according to the mean *C. raciborskii* cell biovolume ($\mu\text{m}^3\cdot\text{mL}^{-1}$) in samples collected at the Itupararanga Reservoir.

Table 2 | Pearson's correlation coefficient (r) and significance (p) between environmental parameters and saxitoxin concentration for the 56 integrated samples from the Itupararanga Reservoir water column

Parameter	Correlation (r)	Significance (p)
Temperature	-0.71	0.000
DO	0.61	0.000
NO ₂	0.43	0.001
Orthophosphate	0.52	0.000
Total nitrogen	-0.38	0.004
Total phosphorus	-0.57	0.000
Alkalinity	-0.56	0.000
Total organic carbon	-0.40	0.002

observed in a study on *Alexandrium tamarensis*, a saxitoxin-producing dinoflagellate (Granéli & Flynn 2006), which may indicate that for these organisms the production of saxitoxin may be related to the adaptation to nutrient limitation.

Maileht et al. (2013) reported that the main determining factors of *C. raciborskii* distribution in European lakes were the water temperature, total phosphorus content and alkalinity. In the present study, however, higher *C. raciborskii* densities and higher saxitoxin concentrations were associated with lower temperatures. It should be considered that

the lowest temperature registered over the year in this subtropical environment was 16.8 °C, whereas in the European lakes studied by Maileht et al. (2013), temperatures were as low as 5 °C.

According to Briand et al. (2004) and Bonilla et al. (2012), *C. raciborskii* is tolerant to climates ranging from temperate to tropical. Many studies have suggested that the optimal temperature for these organisms ranges from 25 to 35 °C (Mehnert et al. 2010). However, Everson et al. (2011) observed high biomass in subtropical lakes at 19 °C for this species. Various authors hypothesized that the phenotypic plasticity of *C. raciborskii* may explain its expansion in temperate and tropical climates (Bonilla et al. 2012; Sinha et al. 2012; Wood et al. 2014; Burford et al. 2016).

The PCA (Figure 5), performed using only the environmental parameters correlated with saxitoxin concentration, indicated that higher total saxitoxin concentrations were associated with lower temperatures, higher dissolved nutrient (NO₂ and orthophosphate) and DO concentrations, as well as higher densities of *C. raciborskii*. The highest saxitoxin quota per trichome is related to the lower densities of *C. raciborskii*, higher concentrations of total N and P (less available), dissolved organic carbon, nitrate and alkalinity, as well as higher temperatures and density of its major competitor (*M. contortum*). This indicates that the

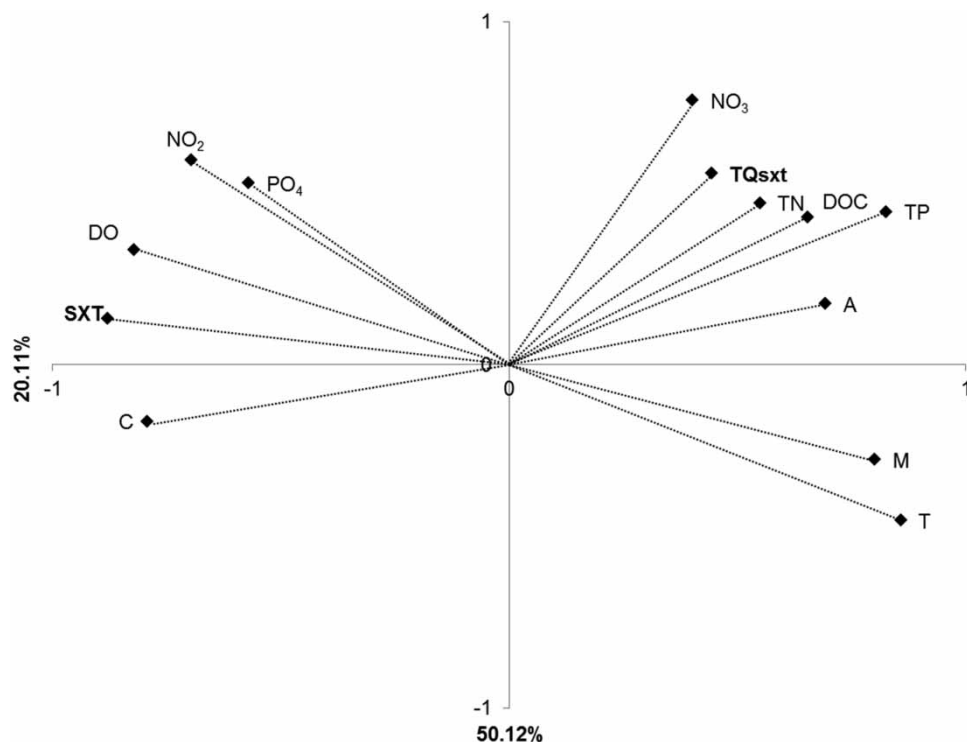


Figure 5 | Principal component analysis (PCA) including physical, chemical and biological standardized data: temperature (T), dissolved oxygen (DO), orthophosphate (PO₄), total phosphorus (TP), Kjeldahl total nitrogen (TN), pH, Alkalinity (A), nitrite (NO₂), nitrate (NO₃), dissolved organic carbon (DOC), *Cylindrospermopsis raciborskii* density (C), *Monoraphidium contortum* density (M), total saxitoxin concentration (SXT), and saxitoxin quota per trichome (TQsxt).

production of saxitoxin or selection of the producing strains may be an adaptation to non-ideal conditions or stress conditions.

In a laboratory study of competition between *C. raciborskii* and *M. contortum*, isolated from the same environment analyzed in this study, Vargas (2012) observed that the production of saxitoxin increased and there was a reduction in cell size under oligotrophic conditions, when compared to more eutrophic conditions. The authors explained that this decrease in cell size was due to the increase in area in relation to the cell volume, which would increase the nutrient absorption capacity.

Pomati et al. (2004) suggested that saxitoxin plays a role in the maintenance of cyanobacteria homeostasis under alkaline pH or Na⁺ stress conditions, which would confer an advantage in environments with higher salt concentrations. In this paper, no significant correlations were found between the total saxitoxin concentrations and saxitoxin quota with the conductivity or pH. Nevertheless, other conditions such as reduced availability of nutrients

or increased competition for resources, observed in this paper, can also be considered as stressful situations.

CONCLUSIONS

The analysis of the saxitoxin concentrations in the Ituparanga Reservoir indicated that higher total saxitoxin concentrations in the water column were associated with higher *C. raciborskii* densities and higher dissolved nutrient availability. The lowest concentrations of total saxitoxin were observed at higher temperatures, higher concentrations of organic matter and under the predominance of the competing species *M. contortum*. Nevertheless, the highest saxitoxin quota per trichome was observed in the periods with the lowest *C. raciborskii* density, where conditions are less ideal. This may indicate that the production of saxitoxin or the selection of toxic strains may be an adaptation to the stress condition.

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