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Influence of Environmental Factors on Occurrence of Cyanobacteria and Abundance of Saxitoxin-Producing Cyanobacteria in a Subtropical Drinking Water Reservoir in Brazil

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Abstract: Blooms of cyanobacteria are frequent in Brazilian water reservoirs used for drinking water. The warning for the presence of potential toxin-producing cyanobacteria is typically based on time-consuming microscopy, rather than specific molecular detection of toxic genes in cyanobacteria. In this study, we developed a quantitative PCR assay for the detection of cyanobacteria producing the neurotoxin saxitoxin (STX). The assay targets the *sxtA* gene in the *sxt* gene cluster. Potential and dominant STX-producers in the Itupararanga reservoir were the genera *Raphidiopsis*, *Aphanizomenon* and *Geitlerinema*. Numbers of the *sxtA* gene varied from 6.76×10^3 to 7.33×10^5 cells mL^{-1} and correlated positively with SXT concentrations in the water. Concentrations of STX and the *sxtA* gene also correlated positively with TN:TP ratio and pH, but correlated negatively with inorganic nutrients and turbidity, confirming that regulation of the SXT production was impacted by environmental variables. In contrast, the occurrence of another cyanotoxin, microcystin, did not correlate with any environmental variables. The developed qPCR assay was found to be a rapid and robust approach for the specific quantification of potential STX-producing cyanobacteria and should be considered in future investigations on toxic cyanobacteria to provide an early warning of potential toxin episodes.

Keywords: Itupararanga reservoir; toxic cyanobacteria; cyanotoxins; qPCR; *sxtA*

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1. Introduction

Cyanobacteria may cause a multitude of water quality concerns. Among these concerns is the potential risk of toxin production, since cyanotoxins in drinking water reservoirs and in recreational water pose a serious risk to human health but also to ecosystem functioning [1]. The most studied cyanotoxin is the hepatotoxic microcystin (MC), which consists of more than 240 structural variants and is produced by several cyanobacterial genera [2,3], including *Dolichospermum* (basionym *Anabaena*) [4], *Microcystis* and *Planktothrix*. Another important cyanotoxin is saxitoxin (STX), a neurotoxin that occurs in more than 50 variants, and 15 of these variants have so far not been detected in cyanobacteria [5]. The STX-producing freshwater genera include, among others, *Raphidiopsis* (basionym *Cylindrospermopsis*) [6], *Dolichospermum*, and *Aphanizomenon*.

The physiological and ecological function of cyanotoxins in cyanobacteria is not clear, but some researchers believe that the toxins have a protective function against zooplankton grazing, or are related to interspecies competition for resources, or may improve the cell physiology [7,8]. However, specific factors that stimulate and/or control the production of

cyanotoxin by cyanobacteria remain to be determined [9]. Similarly, the fact that the same cyanobacterial strain is capable of synthesizing more than one toxic metabolite makes it even more difficult to understand a cause-effect relationship [10].

The identification and quantification of cyanobacteria by traditional microscopy is not adequate for the identification of toxic populations, since toxic and nontoxic strains are morphologically identical [11]. For the detection of toxin-producing strains, PCR-targeting genes that encode specific toxins may be a fast and useful approach [12]. If the number of potentially toxin-producing cyanobacteria is needed, e.g., in environmental studies, quantitative PCR (qPCR) can be applied and serve as a powerful and sensitive tool [13–17].

In Brazil, relatively few studies have focused on molecular approaches for the detection of toxic genes for early warning of cyanotoxin episodes, despite the frequent occurrence of cyanobacterial blooms [12,18–21]. In most cases, traditional PCR was used to detect the presence of potentially cyanotoxin-producing strains, but the application of qPCR-targeting genes in the MC synthesis was performed by Lorenzi [19], Pimentel and Giani [11] and Guedes et al. [22] to quantify the *mcyA* gene in Salto Grande reservoir, the *mcyD* gene in Furnas reservoir and the *mcyB* in Funil reservoir, respectively. For production of SXT by cyanobacteria, no qPCR approaches targeting genes in the *sxt* cluster have so far been applied in Brazilian freshwaters to our knowledge.

The Itupararanga reservoir is located in a subtropical urbanized region of São Paulo State, Brazil, and is one of the most important reservoirs of ecological and economic interests in the state. Water from the reservoir serves many purposes, such as a source of drinking water for approximately one million people, for hydroelectric power and for irrigation of crops [23]. The reservoir has suffered from water quality degradation due to growing urbanization, agricultural practices and discharge of untreated wastewater, especially in the tributaries [24–26]. Studies have classified this reservoir as meso-eutrophic [27,28]. Increased nutrient levels have not only contributed to the eutrophication process, they have also introduced a risk of water quality deterioration due to the proliferation of cyanobacteria [29–31].

In an attempt to better understand the dynamics of toxin-producing cyanobacteria in the Itupararanga reservoir, we related concentrations of STX and MC to the abundance of known toxin producers, as well as to important environmental parameters (nutrients, turbidity and pH). In parallel, to obtain actual numbers of STX-producing cells in the reservoir, we developed a qPCR approach that targets the *sxtA* gene in the *sxt* gene cluster. Finally, we hypothesize that numbers of the *sxtA* gene correlate with concentrations of STX in the water and can be used for early warning of health risk by saxitoxins.

2. Materials and Methods

2.1. Study Area and Sampling

Environmental samples were collected at the riverine and dam zones in the Itupararanga reservoir located in São Paulo State, Brazil (Figure 1). The main characteristics of the reservoir and sampling sites are shown in Table S1. Sampling was carried out in May, August, October 2017 and January 2018. Water samples were collected at two depths (100%—surface and 1%—lower limit of euphotic zone) determined by the photosynthetically active radiation (PAR, μE m^{-2} s^{-1}) using a light sensor (LI-1400 DataLogger, sensitivity of 400–700 nm, LI-COR Biosciences, Lincoln, NE, USA).

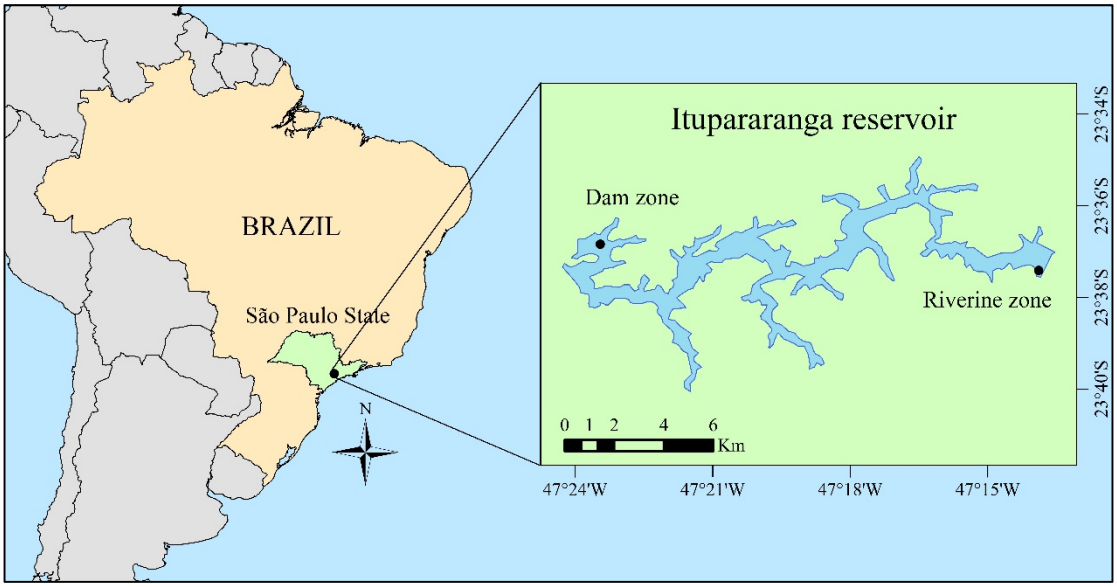

Figure 1. Sampling sites in the Itupararanga reservoir, São Paulo State, Brazil. Itupararanga riverine zone (23°37′24.8″ S, 47°13′53.4″ W); Itupararanga dam zone (23°36′50.7″ S, 47°23′27.3″ W).

2.2. Environmental Variables

The variables pH, dissolved oxygen (DO, mg L^{-1}), electrical conductivity (EC, µS cm^{-1}), turbidity (NTU) and water temperature (°C) were determined on-site using a HANNA probe (HANNA HI9829, Limena, Italy). Water transparency (m) was determined using a Secchi disk and the lower limit of euphotic zone (Z_{eu}, m) was determined as 1% of PAR. Samples for dissolved nutrients (soluble reactive phosphorus—SRP, nitrate—NO_3^--N, nitrite—NO_2^--N and ammonium—NH_4^+-N) were filtered through 0.7 µm-pore-size glass fiber filters (GE Healthcare Life Sciences, Whatman, MA, USA) and the filtrates were kept frozen at −20 °C until analysis. The filters were used for the analysis of chlorophyll *a* (chl *a*) and were kept frozen at −20 °C until analysis according to methodology by Nusch [32]. The concentrations of total N (TN) in unfiltered water were determined from sum of Kjeldahl-N and content of nitrite and nitrate, while total *P* (TP) was determined according to APHA procedure [33]. Nutrient analyses were performed in triplicate by spectrophotometric methods according to APHA [33]. The trophic state index of the sampling sites was calculated considering the annual geometric means of TP and chl *a* [34].

2.3. Phytoplankton Community

For microscopic phytoplankton analysis, samples were Lugol-fixed for subsequent identification and quantification by the Utermöhl method [35] using an inverted microscope (Olympus CK2) at 400× magnification and sedimentation chambers of 2, 5, or 10 mL, depending on the phytoplankton density in each sample. Sedimentation time was 3 h cm^{-1} [36]. A counting limit was established by a species-rarefying curve and until reaching 100 individuals (cell, filament, colony and coenobium) of the most common species. Phytoplankton taxa were identified according to specialized literature. Mean cell volume of each taxon (n = 20) was calculated based on geometric models [37,38]. For taxa with relative abundance <10%, mean cell volumes were obtained from the literature. Biovolumes were estimated by multiplying the density of each taxon by its mean cell volume.

Water for the analysis of phytoplankton pigments was filtered through 1.2 µm-pore-size glass fiber filters (GE Healthcare Life Sciences, Whatman, MA, USA) that were kept frozen at −20 °C until pigment extraction. Pigments on the filters were extracted in 3 mL 95% acetone with vitamin E acetate as an internal standard. Initially, the filters were sonicated in an ice-cool sonication bath for 10 min, extracted further at 4 °C for 20 h,

and then mixed using a vortex mixer for 10 s. Filters and cell debris were removed from the extracts by filtration through 0.2 µm Teflon syringe filters. Pigment analysis was carried out in a high-performance liquid chromatography system (HPLC) according to Schlüter et al. [39,40] using the Van Heukelem and Thomas method [41]. The HPLC system was calibrated using 22 pigment standards from DHI Lab Products (Table S2). The biomass of the individual phytoplankton groups, detected by the identified pigments, was calculated by CHEMTAX software *v*.1.95 [42] in units of chlorophyll *a* (µg chl *a* L^{-1}). The initial pigment/chl *a* ratios used in the CHEMTAX software originated from Schlüter et al. [40,43].

2.4. Cyanotoxins

Cyanotoxins were extracted from 50 mL untreated water samples by triplicate freeze-thaw cycles, including freezing at −80 °C for minimum 1 h and sonication at 37 °C for 5–10 min to lyse the cyanobacteria cells. Total saxitoxin (STX) and total microcystin (MC) concentrations were measured by enzyme-linked immune-assay (ELISA), using commercial kits (Analytical System Inc., Beacon, NY, USA) according to procedures by the manufacturer. In the assay, cyanotoxin and cyanotoxin-protein analogues immobilized on the plate compete for binding sites of antibodies in solution. Between washing steps, an antibody-HRP label was added, followed by a color-generating substrate. Color intensities of duplicate samples were measured in an ELISA reader (Expert Plus, ASYS Hitech, Eugendorf, Austria) at 450 nm.

2.5. DNA Extraction, Primer Design and PCR of the sxtA Gene

For DNA extraction, water samples were immediately filtered through 0.22 µm-pore-size mixed cellulose ester membranes (GE Healthcare Life Sciences, Whatman, MA, USA), and the filters were kept frozen at −20 °C until processing. Total genomic DNA was extracted from filters using the DNeasy PowerWater kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. Concentrations of DNA were quantified using a NanoDrop 2000c spectrophotometer (Thermo Scientific, Wilmington, NC, USA) [44]. The DNA purity was determined by the ratio of absorbance at 260 nm to absorbance at 280 nm. Further, the DNA quality was visualized in 1% agarose gel stained with GelRed™ Nucleic Acid Gel Stain (Biotium Inc., Fremont, CA, USA) using a gel documentation system (Bio-Rad Gel Doc™ 2000, Bio-Rad Laboratories, Hercules, CA, USA).

For quantification of SXT-producing cyanobacteria, the qPCR approach by Al-Tebrineh et al. [15] targeting the *sxtA* gene in *Anabaena circinalis* and other cyanobacteria was chosen. Unfortunately, repetitive tests showed that multiple amplicons were produced by the primer set, as shown in Figure S1. Therefore, a new primer set for qPCR was designed.

Nucleotide sequences of the saxitoxin synthetase gene *sxtA* in the selected strains *Dolichospermum circinale* AWQC131C (EU629179.1), *Aphanizomenon flos-aquae* NH-5 (EU629175.1) and *Raphidiopsis raciborskii* T3 (EU629178.1) were obtained from GenBank database and clustered using ClustalW [45]. Regions of high similarity were identified and chosen for primer design using the Primer3Plus [46]. The developed primer pair *sxtA*-cyano-F (5′-TTATGAAGCGTGCTGTCTGG-3′) and *sxtA*-cyano-R (5′-TCTGCCGACATGGAATACAC-3′) was tested for specificity (in silico PCR, Table S3) by NCBI Primer-BLAST against the non-redundant database. The dimer prediction was verified with OligoAnalyzer 3.1 (Integrated DNA Technologies Inc., Coralville, IA, USA). The primer set designed for conventional PCR and qPCR assays produced fragments of 153 bp size.

Conventional PCR was performed in triplicate in a Mastercycler Pro thermal cycler (Eppendorf, Hauppauge, NY, USA) with 50 µL total reaction volume containing: 1X Buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 1 µL (10 pmol μL^{-1}) of each primer (*sxtA*-cyano-F and *sxtA*-cyano-R), 0.05 U μL^{-1} Taq polymerase (VWR International, LLC, Radnor, PA, USA) and 2 µL of template DNA. The thermal cycle program consisted of an initial preheating for 2 min at 95 °C, followed by 35 cycles, each consisting of 30 s at 95 °C, 30 s at 60 °C and 30 s at 72 °C, and a final extension step for 5 min at 72 °C. The amplified PCR products were visualized on 2% agarose gel stained with GelRed™ Nucleic Acid Gel Stain together with

a 2-Log DNA Ladder (New England BioLabs Inc., Ipswich, MA, USA) using the Bio-Rad Gel Doc™ 2000 gel documentation system.

To test the specifity of the developed primer set, 4 saxitoxin-producing strains and 5 non-saxitoxin producing strains (Table S3) were analyzed with the PCR assay. Strains were grown at axenic conditions at 24 ± 1 °C, light intensity of 40 µmol photons m^{-2} s^{-1}, photoperiod of 12-h light/12-h dark, in ASM-1 medium [47]. The cultures were harvested during the exponential growth phase and filtered through 0.22 µm-pore-size mixed cellulose ester membranes under light vacuum. DNA was extracted as mentioned above and amplified with the PCR assay in triplicate. Selected PCR products were purified and sequenced (Eurofins Genomics Company, Ebersberg, Germany) to confirm the specificity of PCR amplification. Sequence identity was determined by BLAST of the NCBI database.

For quantification of the *sxtA* gene in the environmental samples, the qPCR assay was performed in triplicate by an AriaMx Real-Time PCR System (Agilent Technologies, Santa Clara, CA, USA) using 2 µL of template DNA, 0.4 µL of the *sxtA*-cyano-F and *sxtA*-cyano-R primers (10 pmol μL^{-1}), 10 µL of Brilliant III Ultra-Fast SYBR® Green qPCR Master Mix (Agilent Technologies, Santa Clara, CA, USA) and nuclease free water (Sigma-Aldrich, San Luis, MO, USA) for a final volume of 20 µL. The qPCR assay was carried out under the following cycling conditions: initial preheating at 95 °C for 3 min, followed by 40 quantification cycles, each consisting of 30 s at 95 °C, 30 s at 65 °C, 30 s at 72 °C and 10 s at 77.5 °C, and a melt cycle of 30 s at 95 °C, 30 s at 65 °C and 30 s at 95 °C. Fluorescence measurement of generated products was obtained at the end of each cycle at 77.5 °C. Melting curves analysis was performed at the end of each cycle to evaluate the specificity of the newly designed primer set.

To assess the abundance of the *sxtA* gene in the environmental samples, a standard curve based on cell densities of *Raphidiopsis raciborskii* T3 (obtained by direct microscopy counts) was established. Dilution series (from 1:10 to $1:10^7$) were performed from the initial DNA concentration of *R. raciborskii* T3. A standard curve was derived from the correlation between the DNA concentrations (in cell equivalents) and Ct (threshold cycle) values for each dilution.

2.6. Data Analysis

Data were tested for normality using the Shapiro-Wilk test. Canonical correspondence analysis (CCA) was carried out to evaluate the correlation between environmental variables and occurrence of potentially cyanotoxin-producing cyanobacteria genera, considering the sampling sites, months and depths. The environmental variables that showed the highest correlation with axes 1 and 2 ($r > 0.5$) were retained, whereas the variables that could cause multicollinearity were excluded. Data from the environmental variables were $\log(x + 1)$ transformed and the biovolume data were transformed by Hellinger transformation.

Considering the environmental variables, a Mann-Whitney test was performed to evaluate the differences between sites (riverine and dam zones) and depths (100% and 1% of PAR), while a Kruskal-Wallis test was conducted to evaluate the differences between months. Dunn's post hoc multiple comparison test was performed if significant differences were observed in the Kruskal-Wallis test ($p < 0.05$). To investigate the relationships between the *sxtA* gene, STX and MC concentrations and environmental variables, the Spearman's rank order correlation tests were performed ($p < 0.05$). Data were analyzed using the PAST version (4.06) [48] and STATISTICA version (13.5) (TIBCO Software Inc., Palo Alto, CA, USA).

3. Results

3.1. Phytoplankton Composition

Chromatographic detection of the cyanobacterial-specific pigments confirmed a general presence of cyanobacteria in the reservoir (Figure 2a). Zeaxanthin occurred in all samples, while echinenone, canthaxanthin and aphanizophyll were present in most samples. The presence of *euglenophytes* and *green algae* (including classes *Chlorophycceae, Trebouxio-*

phyceae and *Zygnematophyceae*) were identified by chl *b*, lutein, violaxanthin, and neoxanthin. Alloxanthin showed the presence of cryptophytes. *Diatoms* and *chrysophytes* were identified by the presence of fucoxanthin, chl c_2, and chl c_3, while peridinin revealed the presence of *dinoflagellates*. Microscopy analysis of the phytoplankton generally confirmed the presence of these groups (Figure 2b) (rho = 0.71, p = 0.002).

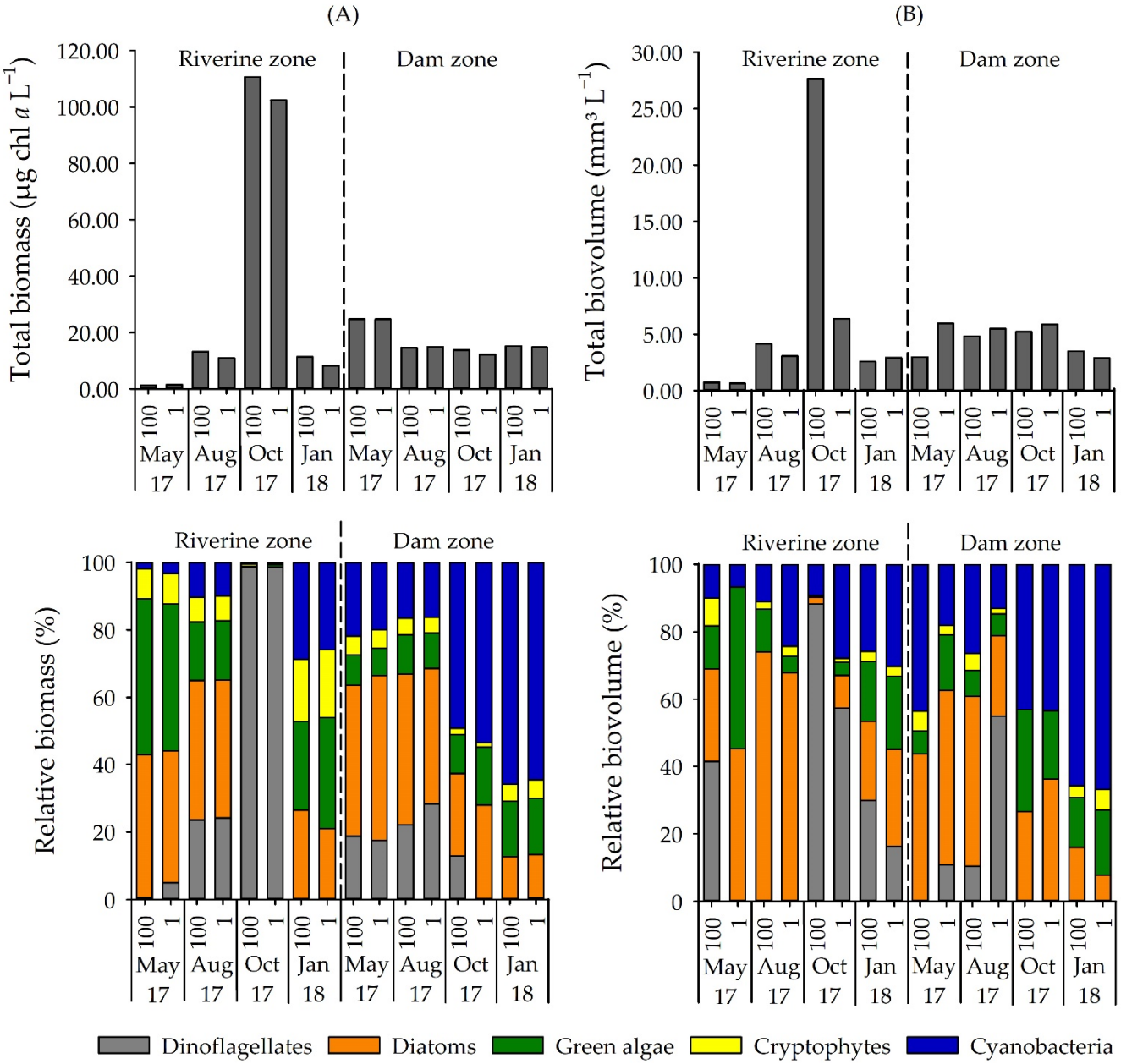

Figure 2. (**A**) Total biomass (µg chl *a* L^{-1}) and relative biomass (%) of phytoplankton groups determined by the pigment method and (**B**) total biovolume (mm^3 L^{-1}) and relative biovolume (%) of phytoplankton groups in Itupararanga reservoir. 100 = surface; 1 = lower limit of euphotic zone.

Diatoms and *green algae* were common in most samples and constituted an important fraction of the phytoplankton community. *Cryptophytes* never attained significant contribution in any of the samples. In October 2017 in the riverine zone, a bloom of dinoflagellates was observed (biomass of 105.08 µg chl *a* L^{-1} and biovolume of 24.43 mm^3 L^{-1}), and *Ceratium furcoides* was the dominant species. If excluding this occurrence, all the other samples

had a chl *a* biomass of less than 25 µg chl *a* L^{-1} and biovolume lower than 7.00 mm^3 L^{-1} (Figure 2).

Among *cyanobacteria*, microscopy identified 22 species belonging to 16 genera with *Aphanizomenon*, *Aphanocapsa*, *Dolichospermum*, *Geitlerinema*, *Limnothrix*, *Pseudanabaena*, *Raphidiopsis*, and *Synechocystis* being the most abundant and frequent genera (Figure 3). Cyanobacteria were present at all samplings but varied significantly between sites ($p < 0.01$). Based on biovolume, contribution of cyanobacteria to the phytoplankton community was 6–30% in the riverine zone but up to 43% (October 2017) and 67% (January 2018) in the dam zone (Figure 2b). The most dominant cyanobacterium was *Raphidiopsis raciborskii* (Figure 3). The analysis of group-specific pigments confirmed the high contribution of cyanobacteria in the dam zone during the warmer months (average of 52% in October 2017 and 65% in January 2018). A list of identified phytoplankton taxa in the reservoir is shown in Table S4.

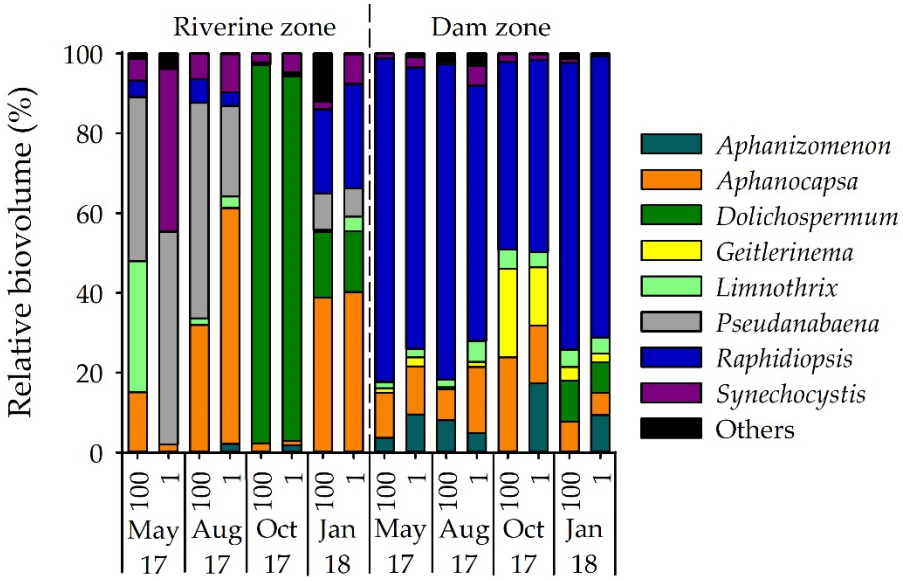

Figure 3. Relative biovolume (%) of cyanobacterial genera identified in Itupararanga reservoir. Others = *Aphanothece*, *Chroococcus*, *Cuspidothrix*, *Merismopedia*, *Microcystis*, *Phormidium*, *Glaucospira* and *Limnococcus*; 100 = surface; 1 = lower limit of euphotic zone.

3.2. Detection of STX Producers: Validation of the sxtA-Cyano Primer Set and Standard Curve

Using qPCR, the designed *sxtA* primer set produced a standard curve for the saxitoxin-producing cyanobacterium *Raphidiopsis raciborskii* T3 with a strong and significant linear regression between DNA concentration (as cells mL^{-1}) and Ct values. The efficiency of the qPCR assay was 95% ($R^2 = 0.99$ and slope of −3.44) for cell numbers between 26 and 2.6×10^7 cells mL^{-1} and Ct values between 8 and 30 (Figure 4). Peaks of melting point profiles occurred between 80.0 and 80.5 °C, indicating presence of a single amplification product (Figure S2).

The primer set detected the presence of the *sxtA* gene in all tested saxitoxin-producing strains. Seven strains were also confirmed positive in silico PCR, indicating a high coverage among different genera. As expected, the non-saxitoxin-producing strains did not yield any PCR products (Table S3). The results demonstrated that the qPCR assay was applicable to quantify potentially saxitoxin-producing cyanobacteria in both cultures and environmental samples. Since the number of *sxtA* copies per *R. raciborskii* T3 cell is unknown, numbers of *sxtA* genes in this study will be expressed in equivalents of *R. raciborskii* T3 cells.

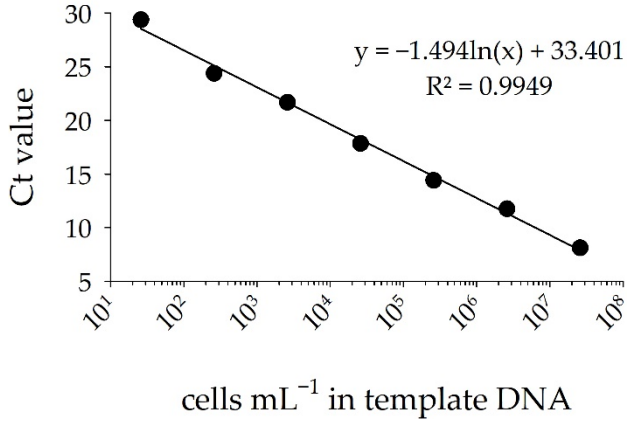

Figure 4. Standard curve of Ct values for tenfold dilutions of known DNA concentration of *Raphidiopsis raciborskii* T3 (cells mL^{-1}). Error bars provide standard deviations of triplicate amplifications.

3.3. Number of STX-Producing Cells and Concentrations of STX and MC

The qPCR analysis confirmed the presence of *sxtA* genotypes in all samples, but a clear spatial variation occurred ($p = 0.009$). The lowest number of *sxtA* genes occurred in the riverine zone in May 2017 (6.76×10^3 cells mL^{-1}), while the highest number was found for the dam zone in May 2017 (7.33×10^5 cells mL^{-1}) (Figure 5a).

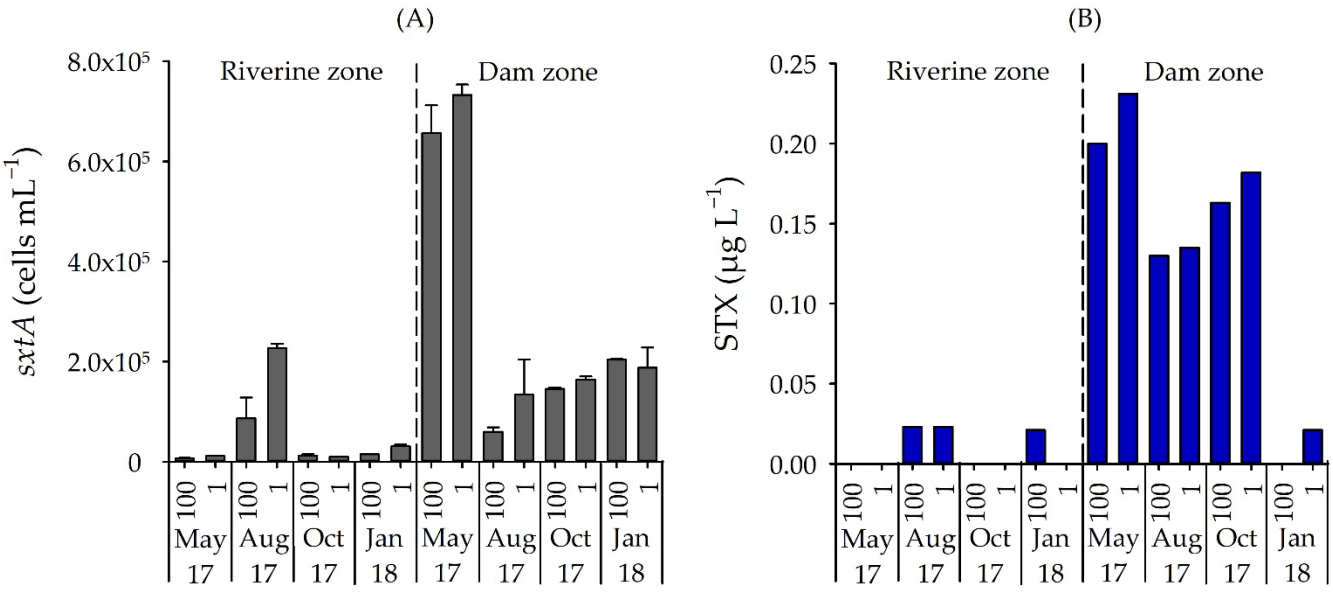

Figure 5. (**A**) Number of *sxtA* gene (cells mL^{-1}) and (**B**) STX concentration (µg L^{-1}) in samples from two field stations, in four months and two depths in Itupararanga reservoir. Standard deviations of triplicate samples shown. 100 = surface; 1 = lower limit of euphotic zone.

Concentrations of STX measured by ELISA were also significantly higher in the dam zone than in the riverine zone ($p = 0.012$) and, when detected in the water samples, ranged from 0.02 µg L^{-1} (riverine zone) to 0.23 µg L^{-1} (dam zone), but did not vary significantly between depths ($p > 0.05$) (Figure 5b). The STX concentrations correlated positively with the *sxtA* gene (rho = 0.70, $p = 0.002$).

STX was detected in the water when the number of *sxtA*-positive cells exceeded 1.47×10^4 cells mL^{-1}. However, in two dam surface samples from January 2018 and in all four samples from the lower limit of the eutrophic zone in the riverine zone, no STX was detected even with a high number of *sxtA* gene (2.04×10^5 and 3.04×10^4 cells mL^{-1}, respectively).

The presence of total microcystin (MC) was only detected in the riverine zone in January 2018 (0.14 µg L^{-1}) but occurred in both May 2017 and January 2018 in the dam zone at concentrations between 0.10 and 0.14 µg L^{-1} (Figure 6). MC concentrations did not vary significantly between sampling months, depths and sites ($p > 0.05$).

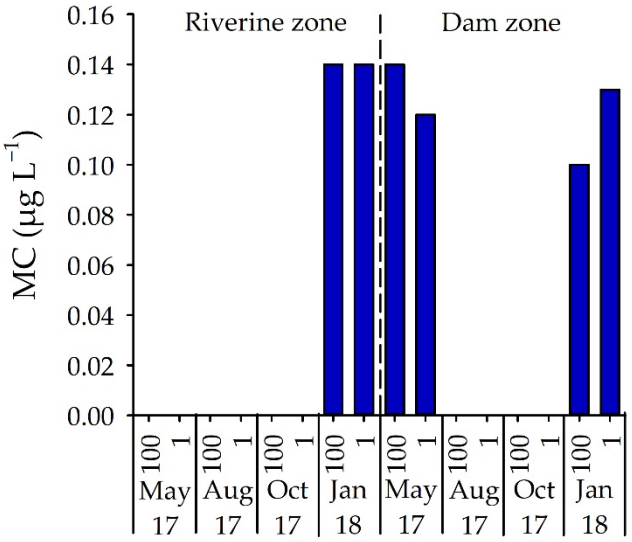

Figure 6. MC concentration (µg L^{-1}) in samples from two field stations, in four months and two depths in Itupararanga reservoir. 100 = surface; 1 = lower limit of euphotic zone.

Cyanobacterial genera with potential STX and MC production were detected by microscopy in all samples. STX concentrations and *sxtA* gene copy numbers correlated positively with the biovolume of *Raphidiopsis*, *Geitlerinema* and *Aphanizomenon*, while *Dolichospermum* was negatively correlated with STX (Table 1). As for MC, Spearman's rank order correlation tests did not show any significant correlations between potential microcystin-producing genera and toxin concentrations throughout the study period ($p > 0.05$).

Table 1. Spearman rank correlation coefficients between the biovolume of four potentially saxitoxin producing genera and *sxtA* gene and saxitoxin concentration (STX) in Itupararanga reservoir (n = 16). Significant correlations ($p < 0.05$) are shown in bold.

Variables	*Raphidiopsis*	*Geitlerinema*	*Aphanizomenon*	*Dolichospermum*
sxtA	**0.71**	**0.72**	**0.51**	−0.27
STX	**0.50**	**0.63**	**0.62**	**−0.65**

3.4. Linkage between Environmental Variables, Cyanobacteria and Cyanotoxins

The measured environmental variables showed that the riverine zone had more eutrophic conditions (higher nitrogen and phosphorus levels, higher chlorophyll *a* concentrations and higher turbidity) than the dam zone, as illustrated in Table S5.

Correlation analyses suggested different variables influencing *sxtA* gene occurrence and STX production in the reservoir (Table 2). STX concentrations and *sxtA* gene copy numbers were positively correlated with cyanobacterial biomass and TN:TP ratio, and negatively correlated with turbidity, TP and soluble reactive P. STX concentrations correlated positively with pH, and *sxtA* gene copy numbers were negatively correlated with nitrate. None of the measured variables succeeded in explaining the MC production in the reservoir ($p > 0.05$).

Table 2. Spearman rank correlation coefficients between environmental variables and *sxtA* gene, saxitoxin (STX) and microcystin (MC) concentrations in Itupararanga reservoir (n = 16). Significant correlations ($p < 0.05$) are shown in bold.

Variables	pH	Turbidity	TP	SRP	NO_3^--N	TN:TP	Biomass_cyano
sxtA	0.39	**−0.63**	**−0.55**	**−0.61**	**−0.60**	**0.59**	**0.72**
STX	**0.57**	**−0.74**	**−0.58**	**−0.58**	−0.46	**0.62**	**0.51**
MC	0.20	−0.06	0.19	−0.04	0.09	−0.15	0.50

TP = total phosphorus; SRP = soluble reactive phosphorus; NO_3^--N = nitrate; TN:TP = total nitrogen and total phosphorus ratio; Biomass_cyano = cyanobacterial biomass determined by pigment analysis.

The ordination obtained in the canonical correspondence analysis (CCA) showed separation of the sampling sites into two groups (riverine and dam zones), with respect to biovolume of cyanobacteria and environmental variables (Figure 7). The first two axes accounted for 85.94% of the variance (CCA 1 = 48.73%; CCA 2 = 37.21%). The permutation based on the Monte Carlo test confirmed that the CCA model was significant ($p = 0.001$), as were the first two axes ($p = 0.001$; $p = 0.008$), that is, the correlation between the environmental variables and potentially cyanotoxin-producing genera was statistically significant for the first two axes indicating an association between matrices.

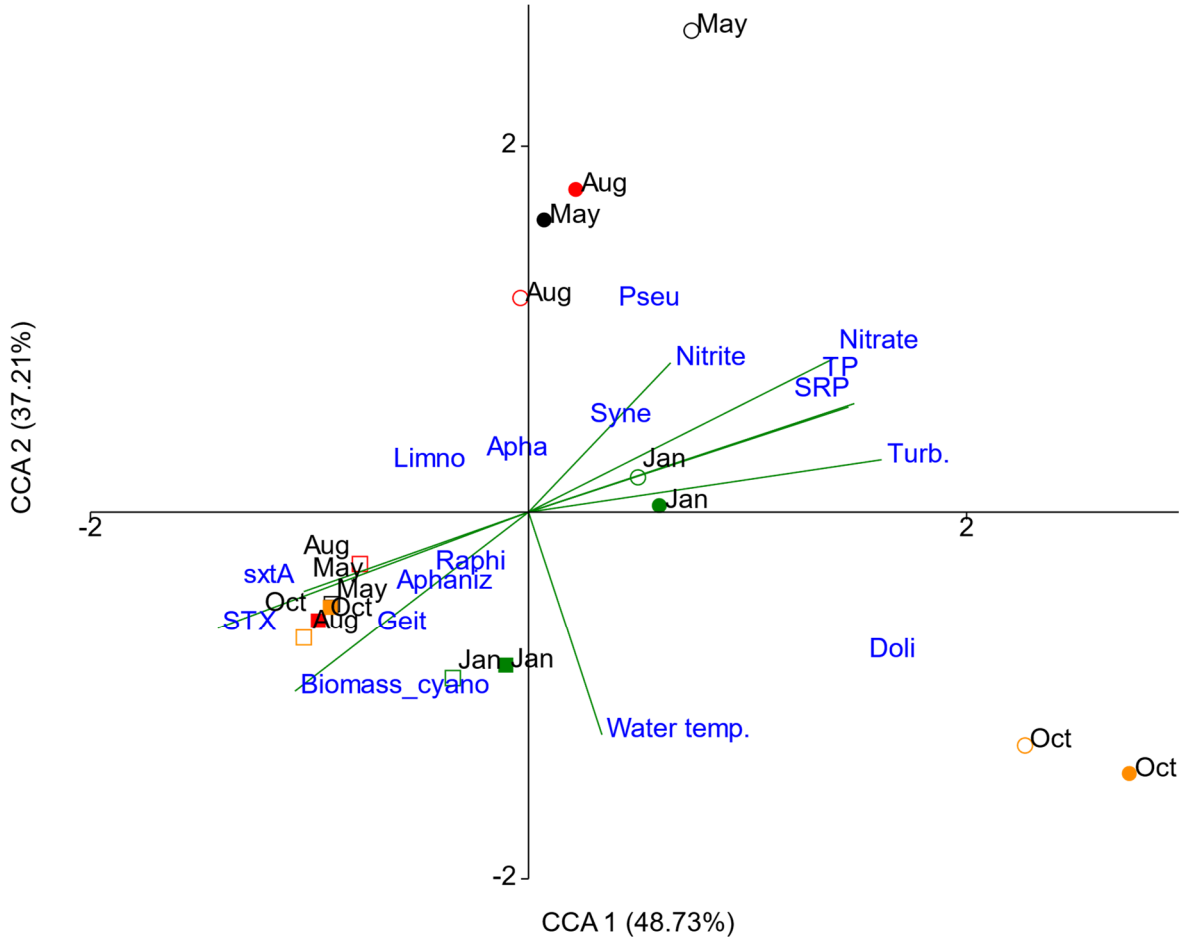

Figure 7. Canonical correspondence analysis (CCA) biplot of cyanobacteria biovolume and environmental variables in Itupararanga reservoir. The periods of sampling were May (May 2017, black), Aug (August 2017, red), Oct (October 2017, orange) and Jan (January 2018, green); the riverine zone is represented by dot and dam zone is represented by square; filled dot/square represents the depth 100 (surface); empty dot/square represents the depth 1 (lower limit of euphotic zone). Water temp. = water temperature; Biomass_cyano = cyanobacterial biomass determined by pigment analysis; STX = saxitoxin; SRP = soluble reactive phosphorus; TP = total phosphorus; Turb. = turbidity; Geit = *Geitlerinema*, Aphaniz = *Aphanizomenon*, Raphi = *Raphidiopsis*, Limno = *Limnothrix*, Apha = *Aphanocapsa*, Syne = *Synechocystis*, Pseud = *Pseudanabaena*, Doli = *Dolichospermum*.

Cyanobacterial biovolumes, STX concentrations and *sxtA* gene numbers appeared in the same quadrant as *Raphidiopsis*, *Geitlerinema* and *Aphanizomenon* in the dam zone, showing a strong correlation between these variables. On the positive side of CCA 1, turbidity showed a strong correlation with the axis, followed by total P, soluble reactive P and nitrate in the riverine zone in October 2017 and January 2018. Nutrient concentrations were negatively correlated with STX, *sxtA*, *Raphidiopsis*, *Aphanizomenon* and *Geitlerinema*, that is, the saxitoxin production was higher when nutrients were low. Biovolumes of *Synechocystis* and *Pseudanabaena* correlated in samples from May and August 2017 in the riverine zone, when the concentrations of nitrite were higher. The highest water temperatures (October 2017 and January 2018) correlated with the highest biovolume of *Dolichospermum*. The distribution of *Synechocystis*, *Pseudanabaena* and *Dolichospermum* in the riverine zone showed a temporal pattern. However, the distribution of the cyanobacterial group in the dam zone did not suggest a temporal pattern (Figure 7).

4. Discussion

4.1. Environmental Variables and Cyanobacteria

Deterioration of the water quality has increased the occurrence of cyanobacteria and their toxins in several reservoirs used for water supply, recreational and fishing activities. Therefore, there is a need to better understand population dynamics of cyanobacteria. Several environmental factors, such as nutrient availability, rainfall and water temperature, may control the population structure of cyanobacteria, as well as the presence of toxic strains and their cyanotoxin production [49,50]. In the present study, the cyanobacterial biovolume in the dam zone (dominated by *Raphidiopsis*) was highest in the riverine zone and correlated with the lowest nutrient levels and the highest TN:TP ratios. This suggests that *Raphidiopsis* is adapted to low-P nutrient environments, as also observed for *R. raciborskii* by Casali et al. [31] and Vargas et al. [51]. Supporting this, Kenesi et al. [52] showed that *R. raciborskii* had a higher growth performance in P-limited environments, but only when inorganic N was available. In the dam zone, the availability of P was low (<15.19 μg L^{-1}), but N was available (0.32–0.42 mg nitrate L^{-1}), which may have favored the growth of *R. raciborskii*.

Environmental control of *Dolichospermum* (dominant biovolume among cyanobacteria in the riverine zone in October 2017) might be related to temperature, since the biomass peaked in October, when the water temperature was above 22 °C. An association between *Dolichospermum* and high-water temperatures has been reported in other studies, which showed optimum growth of *Dolichospermum* spp. at temperatures above 20 °C [53–55].

Besides the influence from environmental variables, composition and abundance of cyanobacteria may also have been affected by competition and interspecies relations between different phytoplankton groups. We speculate as to whether the dinoflagellate *Ceratium furcoides* outcompeted cyanobacteria in the reservoir. *C. furcoides* has been found in many Brazilian freshwater systems [56–59] and in the Itupararanga reservoir since 2010 [60]. Yet, few studies have dealt with the influence of *C. furcoides* on cyanobacterial population dynamics [61,62]. In the Itupararanga reservoir, the cyanobacterial biomass declined when *C. furcoides* occurred, and the lowest cyanobacterial biomass (<1.00 μg chl *a* L^{-1}) coincided with the riverine bloom of *C. furcoides* in October 2017. Invasion of *C. furcoides* has previously been shown to overturn the cyanobacterial dominance in a eutrophic tropical reservoir [61], and cause oscillation and reduction in cyanobacterial blooms in an urban lake [62]. These authors correlated the increased biomass of *C. furcoides* to a high-water transparency and high concentrations of N and P. Their observations appear to support the phytoplankton community modulations in the Itupararanga reservoir. Thus, in the riverine zone in October 2017, the water transparency was ≥1.20 m, and concentrations of TN and TP were 0.86 and 0.54 μg L^{-1}, respectively.

4.2. qPCR Assay, Cyanotoxin and Cyanobacteria

The qPCR assay developed in this study proved valid for the quantification of the *sxtA* gene in cyanobacteria. The *sxtA* gene is a core gene in the *sxt* gene cluster in STX-producing organisms [63]. Primer specificity, melting curve profile and the presence of only one amplicon clearly demonstrated a high sensitivity of the primer set for both cultures and environmental samples. Since several taxa of saxitoxin-producing cyanobacteria may occur in environmental samples [64–66], a high-coverage primer set is required for their detection. Our *sxtA* primer set showed specificity towards the *sxtA* gene, as demonstrated by both in vitro qPCR and in silico qPCR (Table S3). To the best of our knowledge, the present study is the first report on quantification of the *sxtA* gene in environmental samples by qPCR in a Brazilian water body. As mentioned earlier (Material and Methods, Section 2.5), testing of published *sxtA* primer sets [15] was not successful for amplification of the *sxtA* gene in our samples due to the formation of multiple amplicons. The resason for this is uncertain, but Zupancic et al. [67] report that amplification of the *sxtA* gene may be affected by geographical sequence variability.

The quantification of potentially saxitoxin-producing cyanobacteria in environmental samples by qPCR targeting the *sxtA* gene has been performed in few studies [14,15,68–70]. In most cases, the *sxtA* gene abundance correlated positively with STX concentrations, as also observed in our study. However, in the Itupararanga reservoir no STX was measured in some of the samples (surface of dam in January 2018 and lower limit of the euphotic stratum in the riverine zone), despite the *sxtA* gene being detected. Possibly, the *sxt* gene expression was down-regulated, resulting in the absence of STX production, as observed in a field study by Savela et al. [17] and in cultures of the potential STX-producing strain *Aphanizomenon* [71–73]. Alternatively, the qPCR-detected population was too small to produce sufficient amounts of STX for detection by ELISA. Nevertheless, the detection of the *sxtA* gene in the reservoir indicates that the genetic information necessary for the initiation of toxin synthesis was present.

The observed number of *sxtA* gene in the Itupararanga reservoir varied from 6.76×10^3 to 7.33×10^5 cells mL^{-1}. This range largely agrees with a recent study of Danish lakes in which 0.8×10^3 to 117×10^6 *sxtA* copies per mL were detected, except that a higher *sxtA* number was determined in the most eutrophic lake [69]. A high number of 2.91×10^9 copies mL^{-1} was measured by Al-Tebrineh et al. [15] in a quantitative assay for the saxitoxin-producing *Anabaena circinalis* in a eutrophic dam. The authors report that STX was not detectable in the water until the *sxtA* gene exceeded 7.61×10^3 copies mL^{-1}. In our study, STX production was detected when more than 1.47×10^4 *sxtA*-carrying cells mL^{-1} occurred, but as mentioned above, in 6 of 16 samples, no STX was detected even though a high number of the *sxtA* gene occurred (>3.0×10^4 *sxtA*-carrying cells mL^{-1}). This reinforces the need to understand the influence of environmental factors on the regulation of STX production. Additionally, knowledge on the number of the *sxtA* gene copies per cell is needed to determine accurate numbers of STX-producing cells. So far, a cell-specific *sxtA* copy number has only been determined for *A. circinalis* AWQC131C by Al-Tebrineh et al. [15], who report 3.58 *sxtA* copies per cell.

Spearman's rank order correlation analysis and CCA showed significant and positive correlations between STX concentrations and *sxtA* gene copies to biovolumes of *Raphidiopsis*, *Aphanizomenon* and *Geitlerinema*. Therefore, we consider these cyanobacteria as dominant saxitoxin-producing species in the reservoir. However, the actual cyanobacterial genus or genera being responsible for the STX production in the water cannot be determined, partly because the *sxtA* gene may have originated from species that were not observed by microscopy. To link a specific genus to saxitoxin (or microcystin) production, isolation of the cyanobacteria followed by a search for the target genes in each cyanobacterial strain would be needed [74].

4.3. Saxitoxin and Environmental Variables

Saxitoxin concentrations and number of *sxtA* gene copies were highest in the dam zone, correlated negatively with nutrients (TP, SRP and NO_3^--N) but correlated positively with the TN:TP ratio. The TN:TP ratio may indicate control of the STX production by available N, but the influence of N on saxitoxin production is controversial. According to Brentano et al. [75], dissolved inorganic nitrogen (DIN) may cause high STX concentrations in populations of *R. raciborskii*, since DIN favors growth or improves the cellular homeostasis due to a lower metabolic demand, because uptake of DIN requires less energy than N_2-fixation. In contrast, Yunes et al. [76] observed a reduced STX level by a culture of *R. brookii* when DIN concentrations increased. Therefore, further studies are needed to provide a clear view on the influence of N on the STX production.

Regarding phosphorus (P), our results showed an increase in saxitoxin concentrations when P-limitation was observed. For the STX-producing dinoflagellate *Alexandrium* sp., Granéli and Flynn [77] report that the STX production at low P levels may indicate an adaptation to nutrient limitation. Similarly, Vargas et al. [51] report a high STX production by *R. raciborskii* at oligotrophic conditions due to adaptation to low-P levels, which might serve as a survival strategy. Frangópulos et al. [78] state that toxin production under P-limitation enhances the interspecific competition by redirecting grazing pressure to non-toxic species. These reports indicate that the availability of P may control the STX production, but the specific mechanisms remain to be determined.

In addition to access to N and P, the STX production appears to also be controlled by pH of the water. In the Itupararanga reservoir, a higher pH coincided with a high STX level. A similar relation was observed for intracellular STX concentrations in *R. raciborskii*, when pH increased to pH 9 or higher [79]. According to the authors, STX provides an advantage to STX-producing cyanobacteria over non-STX-producing cyanobacteria by maintaining homeostasis at alkaline pH and Na^+ stress conditions, e.g., at high salt concentrations. These results might indicate that STX production is an adaptation to non-ideal conditions as reported from other studies [8,31,51].

Water turbidity appeared to also affect the STX production in the reservoir, since no STX was detected above 10 NTU, also when the lowest numbers of *sxtA* gene were recorded. For microcystin (MC), some studies report a positive and significant correlation between MC production, *Microcystis* abundance, high turbidity, low water transparency and high concentrations of total suspended solids [80–82]. However, no published data are available to verify the effects of turbidity on STX production.

The presence of STX in the Itupararanga reservoir (up to 0.02 and 0.23 μg L^{-1} in the riverine and dam zones, respectively) appears slightly higher than concentrations measured in other eutrophic freshwaters, e.g., Lake Kabetogama in Minnesota, USA (up to 0.08 μg L^{-1}) [83] and a reservoir in Texas, USA (up to 0.05 μg L^{-1}) [84]. In the Brazilian Alagados reservoir (São Paulo State), highly variable concentrations of STX have previously been measured, ranging from 5 ng L^{-1} (spring) to 51 ng L^{-1} (autumn) in 2007–2008 [85], while Calado et al. [86] in 2013–2014 measured significantly higher concentrations of 0.36 μg L^{-1} (spring) to 5.3 μg L^{-1} (autumn). The dominant cyanobacterium in the reservoir was *R. raciborskii*, and blooms of this species was the likely source of the high STX concentrations [86]. Currently, there are no internationally accepted guidelines for maximum concentrations of STX for recreational or drinking water purposes, but concentrations of 0.2–1.5 μg L^{-1} [87] and 3 μg L^{-1} [88] have been suggested. Considering these concentrations, the presence of STX in the Itupararanga reservoir appears below critical levels.

4.4. Microcystin and Environmental Variables

Microcystin concentrations in the reservoir were generally low (max. 0.14 μg L^{-1}) and below the limit acceptable for MC content in drinking water (≤1.00 μg L^{-1}) [89], as also recommended by the World Health Organization [90].

Several environmental factors have been shown to control the MC production in cyanobacteria. Thus, microcystin production is reported to vary with salinity [91,92],

temperature [93,94], light intensity [95,96], pH [97,98] and nutrients [99,100]. However, in the Itupararanga reservoir, no significant correlations between environmental variables, MC concentrations and potentially toxic genera were found. This agrees with Pimentel and Giani [11] who did not find any correlations between environmental variables and MC production in the Furnas reservoir (Minas Gerais, Brazil), and highlighted the necessity of further studies to understand the factors responsible for its occurrence in particular environmental conditions.

4.5. Pigment Analysis and qPCR Assay as Tools for Monitoring of Toxic Cyanobacteria

Cyanotoxins constitute a potential risk to human health [101] and therefore there is a need for reliable and fast methods for the specific quantification of toxin-producing cyanobacteria in the environment. In the Itupararanga reservoir, the positive correlations between pigment-based biomass of cyanobacteria and *sxtA* copy numbers demonstrated that pigment analysis may serve as a useful tool to predict the risk of occurrence of potentially toxic cyanobacteria in subtropical reservoirs. Similar results were obtained by Schlüter et al. [40] in a study of Brazilian reservoirs with aquaculture production, where the presence of microcystin also correlated with pigment profiles. The authors stated that pigment analysis can provide fast and reliable data for early warning of risks of cyanobacteria and their toxins in freshwater reservoirs.

The pigment-based biomass of phytoplankton groups correlated positively with biovolumes determined by microscopy, showing that pigment profiles are a powerful tool in taxonomic analyses of phytoplankton. Pigment analyses have the advantage of being fast and reproducible, and no taxonomical expertise is required. Furthermore, all phytoplankton cells, also including pico-sized cells that are not identifiable by standard microscopy methods, are included by the pigment method. Another advantage is that rare species with low densities can be identified by pigment profiles, but may be overlooked by microscopy [39,40,102]. Despite these advantages, the pigment analysis will only detect phytoplankton groups and thus, for the identification of genera and species, microscopic analysis may still be necessary.

If the choice of methods for early warning and analysis of toxin episodes is the identification of potential toxin-producing cyanobacteria by Utermöhl technique or qPCR detection of toxin-encoding genes, calculation of the expenses indicates that qPCR may be a preferred method. Thus, Lorenzi [19] estimated costs of the Utermöhl technique to be about US$ 9 per sample, while expenses for analysis of one sample by qPCR amount to about US$ 8. Although quantification by the Utermöhl technique requires fewer equipments and reagents than the qPCR technique, the analyst's labor costs make the Utermöhl technique more expensive due to the low processing capacity. The qPCR technique has a higher cost of equipment and reagents, but the total costs, including the analyst's labor, are distributed among a larger number of samples analyzed. Therefore, it can be concluded that for rapid and large-scale monitoring, qPCR may prove more economically viable than cell counting by microscopy due to its greater sample processing capacity and less time for obtaining the results.

5. Conclusions

The application of the designed qPCR method showed that saxitoxin-producing cyanobacteria were permanently present among phytoplankton in the Itupararanga reservoir during the sampling period and led to measurable concentrations of STX in the water. Records of cyanobacterial blooms and the occurrence of cyanotoxins are common in Brazilian lakes and reservoirs, but this study was the first to apply qPCR for the detection of potentially STX-producing cyanobacteria. The correlation between numbers of the *sxtA* gene and concentrations of STX in the water showed that the qPCR assay can be used for the early warning of health risk by saxitoxin occurrence. The dominant STX producers in the reservoir appeared to belong to the genera *Raphidiopsis*, *Aphanizomenon* and *Geitlerinema*, and high TN:TP ratios and low availability of P seemed to sustain the STX production.

Results from the study can serve as valuable tools in understanding the effects of nutrients on toxin production and ensure that producers of STX are accurately quantified for better monitoring of adverse effects from blooms of cyanobacteria.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/w13121716/s1. Table S1. Characteristics of Itupararanga reservoir and sampling sites (riverine and dam zones) [29,30,103]; Table S2. List of phytoplankton pigments included in HPLC analysis and taxonomic designations; Table S3. PCR and in silico PCR with cyanobacteria strains for investigation of specificity of the primer set *sxtA*-cyano on detection of *sxtA* gene (+ and − indicate positive or negative PCR reaction, respectively); Figure S1. Agarose gel picture showing the PCR amplicons against 2-log ladder. The PCR was performed using primer pair reported by Al-Tebrineh et al. [15] for targeting the *sxtA* gene. DNA from several saxitoxin-producing cyanobacteria was used as template. As can be seen on the gel picture, a non-target amplification occurred for some strains when using the *sxtA* primer set by Al-Tebrineh et al. [15]; Figure S2. Melting curve for the *sxtA* primer set for standards and environmental samples showing a single peak (T_m = 80–80.5 °C) that indicates amplification of a single amplicon. T_m = melting temperature; Table S4. List of the phytoplankton taxa identified in Itupararanga reservoir; Table S5. Environmental variables summarised as the mean values and ranges, and statistical results (*p* values) of non-parametric Mann-Whitney test for spatial differences (riverine and dam zone) and non-parametric Kruskal-Wallis for temporal differences (months) of the environmental variables in Itupararanga reservoir.

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