UNIVERSITY OF SÃO PAULO
SÃO CARLOS SCHOOL OF ENGINEERING

MUNIQUE DE ALMEIDA BISPO MORAES

Spatial and temporal variability of cyanobacteria in two subtropical reservoirs:
community composition, molecular and cyanotoxin analyses

Variabilidade espaço-temporal de cianobactérias em dois reservatórios subtropicais:
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Doctoral thesis presented at São Carlos School of Engineering, University of São Paulo in partial fulfillment of the requirements for the Degree of Doctor in Science: Hydraulic and Sanitation Engineering.

Supervisor: Profa. Tit. Maria do Carmo Calijuri

CORRECTED VERSION (VERSÃO CORRIGIDA)

São Carlos

2020

Ficha catalográfica elaborada pela Biblioteca Prof. Dr. Sérgio Rodrigues Fontes da EESC/USP com os dados inseridos pelo(a) autor(a).

Moraes, Munique de Almeida Bispo
M827s Spatial and temporal variability of cyanobacteria in two subtropical reservoirs: community composition, molecular and cyanotoxin analyses / Munique de Almeida Bispo Moraes; orientadora Maria do Carmo Calijuri. São Carlos, 2020.

Tese (Doutorado) - Programa de Pós-Graduação em Engenharia Hidráulica e Saneamento e Área de Concentração em Hidráulica e Saneamento -- Escola de Engenharia de São Carlos da Universidade de São Paulo, 2020.

1. Itupararanga reservoir. 2. Lobo reservoir. 3. Quantitative PCR. 4. Saxitoxin. 5. sxtA gene. 6. Microcystin. 7. mcyE gene. I. Título.

Eduardo Graziosi Silva - CRB - 8/8907

FOLHA DE JULGAMENTO

Candidata: Bacharela **MUNIQUE DE ALMEIDA BISPO MORAES.**

Título da tese: "Variabilidade espaço-temporal de cianobactérias em dois reservatórios subtropicais: composição da comunidade, análises moleculares e de cianotoxinas ".

Data da defesa: 24/07/2020.

Comissão Julgadora: **Resultado:**

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To my family, with love and gratitude.

ACKNOWLEDGEMENTS

First, I would like to acknowledge God to be present in my life, giving me health, wisdom, and strength to conclude one more step in my career.

I am grateful to my supervisor, Professor Maria do Carmo Calijuri, for being an example of character, a great supervisor, for sharing her knowledge, for believing and trusting my project, for giving me the freedom to pursue all my ambitions, for the excellent guidance, dedication, respect, and friendship.

I would like to extend my huge gratitude to my supervisor at the University of Copenhagen, Professor Niels Ole Gerslev Jørgensen, for his wisdom and respect, for sharing his knowledge and ideas with me, and for making my stay there so welcoming and one of the best experiences of my life.

This doctoral thesis could not have been done without the generous assistance of numerous researchers who shared their knowledge, expertise, and the hard work on our field samplings. I thank the laboratory technician Adriana Poli Miwa for her patience, teachings, assistance in collection planning and laboratory analysis and, above all, for her friendship. Many thanks to Professor Marli de Fátima Fiore, to the laboratory technician Renata Beatriz Cruz, to PhD Janaína Rigonato and to PhD Bruno Souza, from CENA/USP, for their collaboration, help and support during the molecular work.

I thank the PhD Raju Podduturi and the laboratory technicians, Susanne Iversen and Ulla Rasmussen from University of Copenhagen for all the help, cooperation and for all the fun moments we had together during my research internship abroad. Thanks to PhD Louise Schlüter of DHI Environment and Water, for all her assistance during pigment analysis. I thank the field technician Mr. Waldomiro Antônio Filho (Miro) for the incredible and essential support in the water collections and Mr. Benedito Patracon (Benê), official driver of the field trips. Thank you so much for all the help and for sharing fun moments together.

I am also grateful for the opportunity to develop my doctoral study with a great group (Laboratório de Biotoxicologia em Águas Continentais e Efluentes - BIOTACE), that made my workdays much more enjoyable. In particular, I thank Raphaella, Flavia, Wesley and Lucas, for their advice, information exchange and for all the fun moments we had together, mainly during the coffee time. A special thanks to my officemate Adriana at University of Copenhagen, for being my friend in Denmark and sharing such great moments and emotions with me.

My gratitude also refers to the Professors and staff of the Department of Hydraulic and Sanitation Engineering (SHS/EESC/USP), in particular to Maria Auxiliadora de Campos Altieri

Pin (Sá) and Priscila Carla Toselli de Almeida (Pri), for their administrative assistance.

I thank both members of my PhD defense committee, Professor Ana Teresa Lombardi, Professor André Cordeiro Alves dos Santos, Professor Marli de Fátima Fiore and Professor Viviane Moschini Carlos, for the constructive criticisms.

I do not have enough words to thank my parents Moraes and Neuci, who have always been on my side through the years, backing up all my decisions, taking care of me, helping me overcome the challenges. I thank my brother Henrique, my sister Rafaela, my sister-in-law Daniella, my nephew and godson Pedro Henrique, my niece Manuella and my boyfriend Gabriel for always being by my side, encouraging me, believing me throughout this process, and celebrating all my big and small accomplishments. I love you all!

The development of this doctoral thesis was feasible due to the specific financial support from São Paulo Research Foundation (FAPESP, grant numbers 2015/21191-4, 2016/09405-1 and 2018/00394-2).

Finally, I would like to acknowledge the São Carlos School of Engineering at University of São Paulo and the Department of Plant and Environmental Sciences at University of Copenhagen for the infrastructure and technical support.

“What we know is a drop, what we don't know is an ocean.”

Isaac Newton

RESUMO

MORAES, M. A. B. **Variabilidade espaço-temporal de cianobactérias em dois reservatórios subtropicais:** composição da comunidade, análises moleculares e de cianotoxinas. 2020. Tese (Doutorado) - Escola de Engenharia de São Carlos, Universidade de São Paulo, São Carlos, 2020.

Cianobactérias potencialmente tóxicas em reservatórios de abastecimento de água representam um sério risco à saúde, pois podem liberar cianotoxinas na água. Entre as cianotoxinas produzidas pelas cianobactérias estão a microcistina (MC - hepatotoxina) e a saxitoxina (STX - neurotoxina). Considerando que essas duas toxinas são recorrentes nos sistemas hídricos brasileiros, o principal objetivo desta pesquisa de doutorado foi avaliar o efeito de variáveis ambientais na composição, abundância e toxicidade de comunidades de cianobactérias nos reservatórios subtropicais de Itupararanga e do Lobo (São Paulo, Brasil) a fim de gerar informações para auxiliar no monitoramento adequado desses ecossistemas aquáticos e minimizar os riscos à saúde pública. Amostras de água foram coletadas em duas profundidades na cabeceira e na barragem de ambos os reservatórios em maio, agosto e outubro de 2017 e janeiro de 2018. Ocorrências de cianobactérias potencialmente produtoras de saxitoxina e microcistina foram determinadas por análise microscópica e ensaios quantitativos de PCR através da detecção dos genes *sxtA* e *mcyE*, respectivamente. Concentrações de MC e STX foram determinadas através do teste ELISA. A presença de cianobactérias potencialmente produtoras de MC e STX em ambos os reservatórios foi confirmada pela detecção dos genes *mcyE* e *sxtA*, respectivamente. Dentre os gêneros de cianobactérias identificados, A*phanizomenon*, *Geitlerinema*, *Phormidium* e *Raphidiopsis* foram considerados como os potenciais produtores de MC e STX nos reservatórios. O biovolume de cianobactérias no reservatório de Itupararanga foi maior que no reservatório do Lobo. Em janeiro de 2018, o biovolume de cianobactérias na barragem do reservatório de Itupararanga constituiu mais de 60% do biovolume total de fitoplâncton, com predominância de *Raphidiopsis raciborskii*. No reservatório do Lobo, a maior contribuição das cianobactérias foi de 12% na barragem em maio de 2017. Concentrações de MC e STX foram detectadas nos dois reservatórios subtropicais. Na maior parte do período de estudo, as concentrações das cianotoxinas estiveram abaixo do limite máximo permitido pelo Ministério da Saúde para água de abastecimento público. No entanto, as concentrações de MC excederam o limite permitido em água potável para consumo humano em duas amostras (maio de 2017 na barragem do reservatório do Lobo), representando um risco para a população local. A ocorrência de toxinas nos reservatórios foi influenciada por diferentes

variáveis ambientais, no entanto, os resultados mostraram que os nutrientes desempenharam um papel importante na produção de MC e STX, uma vez que a limitação de nitrogênio e fósforo pode ter sido responsável pelo aumento da produção de toxinas. Os genes *mcyE* e *sxtA* foram diretamente correlacionados às concentrações de microcistina e saxitoxina em ambos os reservatórios, sugerindo que o qPCR associado ao ELISA / LC-MS pode ser usado como uma ferramenta rápida e confiável para monitorar a ocorrência de cianobactérias tóxicas em amostras ambientais.

Palavras-chave: Reservatório de Itupararanga. Reservatório do Lobo. PCR quantitativo. Saxitoxina. Gene *sxtA*. Microcistina. Gene *mcyE*.

ABSTRACT

MORAES, M. A. B. **Spatial and temporal variability of cyanobacteria in two subtropical reservoirs:** community composition, molecular and cyanotoxin analyses. 2020. Thesis (Doctorate) - São Carlos School of Engineering, University of São Paulo, São Carlos, 2020.

Toxic cyanobacteria in public water supply reservoirs represent a serious health risk since they can release cyanotoxins into the water. Among the cyanotoxins produced by cyanobacteria are microcystin (MC - hepatotoxin) and saxitoxin (STX - neurotoxin). Considering that these two toxins are recurrent in Brazilian water systems, the main objective of this doctoral research was to assess the effect of environmental variables on the composition, abundance and toxicity of cyanobacterial communities in the subtropical reservoirs Itupararanga and Lobo (São Paulo State, Brazil) in order to generate information to help on the proper monitoring of these aquatic ecosystems and minimize the risks to the public health. Water samples were collected in the riverine and dam zones from both reservoirs at two depths in May, August and October 2017 and January 2018. Occurrences of potentially saxitoxin- and microcystin-producing cyanobacteria were determined by microscope analysis and quantitative PCR assays through the detection of *sxtA* and *mcyE* genes, respectively. Concentrations of MC and STX were measured through ELISA. Presence of microcystin- and saxitoxin-producing cyanobacteria in both reservoirs was confirmed by the detection of *mcyE* and *sxtA* genes, respectively. Among the identified genera of cyanobacteria, *Aphanizomenon*, *Geitlerinema*, *Phormidium* and *Raphidiopsis* were considered as the potential producers of MC and STX in the reservoirs. The cyanobacterial biovolume in Itupararanga reservoir was higher than in Lobo reservoir. In January 2018, the cyanobacterial biovolume in the dam zone of Itupararanga reservoir constituted more than 60% of total phytoplankton biovolume, and the most dominant cyanobacterium was *Raphidiopsis raciborskii*. In Lobo reservoir the highest contribution of cyanobacteria was 12% in the dam zone in May 2017. Microcystin and saxitoxin concentrations were detected in the two subtropical reservoirs. For most of the study period, cyanotoxin concentrations were below the maximum limit allowed by the Ministry of Health for public water supply. Nevertheless, MC concentrations exceeded the limit allowed in drinking water for human consumption in two samples (May 2017 in the dam zone of Lobo reservoir), posing a risk to the local people. The occurrence of toxins in the reservoirs was influenced by different environmental variables, but the results showed that nutrients played an important role in their occurrence since the nitrogen and phosphorus limitation appeared to be responsible for the increase in toxin production. The *mcyE* and *sxtA* genes were directly correlated with

microcystin and saxitoxin concentrations in both reservoirs, suggesting that qPCR associated with ELISA / LC-MS can be used as a fast and reliable tool to monitor the occurrence of toxic cyanobacteria in environmental samples.

Keywords: Itupararanga reservoir. Lobo reservoir. Quantitative PCR. Saxitoxin. *sxtA* gene. Microcystin. *mcyE* gene.

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LIST OF ABBREVIATIONS, ACRONYMS AND SYMBOLS

<	Less than
>	Greater than
≤	Less than or equal to
≥	Greater than or equal to
°C	Degree Celsius
µE m^{-2} s^{-1}	Microeinstein per square meter per second
µg chl *a* L^{-1}	Microgram of chlorophyll *a* per liter
µg L^{-1}	Microgram per liter
µL	Microliter
µS cm^{-1}	Microsiemens per centimeter
ADDA	3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid
ALPI	Aquatic Life Protection Index
ANEEL	Brazilian Electricity Regulatory Agency
Apha	*Aphanocapsa*
Aphaniz	*Aphanizomenon*
Biomass_cyano	Cyanobacterial biomass determined by pigment analysis
BLAST	Basic Local Alignment Search Tool
bp	Base pair
C. furcoides	*Ceratium furcoides*
CBH-SMT	Sorocaba and Middle Tietê River Basin Committee
CBH-TJ	Tietê-Jacaré Basin Committee
CCA	Canonical correspondence analysis
CCMA-UFSCar	Collection of Freshwater Microalgae Cultures of Federal University of São Carlos
cells mL^{-1}	Cells per milliliter
CETESB	Environmental Agency of the State of São Paulo
CHEMTAX	Chemical Taxonomy
chl	Chlorophyll
Ct	Threshold cycle
D-Glu	D-glutamic acid
DIN	Dissolved inorganic nitrogen
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide triphosphates
DO	Dissolved oxygen
Doli	*Dolichospermum*

EC	Electrical conductivity
ELISA	Enzyme-linked immune-assay
ESI	Electrospray ionization
FAPESP	São Paulo Research Foundation
Geit	*Geitlerinema*
h	Hour
HPLC	High-performance liquid chromatography
km	Kilometer
km^2	Square kilometer
LC-MS	Liquid chromatography - mass spectrometry
LC-MS/MS	Liquid chromatography - tandem mass spectrometry
LETC	Laboratory of Ecophysiology and Toxicology of Cyanobacteria
Limno	*Limnothrix*
m	Meter
$m^3\ s^{-1}$	Cubic meter per second
max.	Maximum
MC	Microcystin
mcy	Microcystin biosynthesis (*mcy*) gene cluster
$mg\ L^{-1}$	Milligram per liter
$MgCl_2$	Magnesium chloride
min	Minute
mM	Millimolar
$mm^3\ L^{-1}$	Cubic millimeter per liter
MRM	Multiple reaction monitoring
NCBI	National Center for Biotechnology Information
nd	Not detected
NH_4^+-N	Ammonium
NIES	National Institute for Environmental Studies
nm	Nanometer
NO_2^--N	Nitrite
NO_3^--N	Nitrate
NRPS	Nonribosomal peptide synthetase
ns	Not significant
NTU	Nephelometric turbidity units
p	Probability value
PAR	Photosynthetically active radiation

PAST	Paleontological statistics software package
PC	Principal component
PCA	Principal component analysis
PCC	Pasteur Culture Collection
PCR	Polymerase chain reaction
pH	Potential for hydrogen
PKS	Polyketide synthase
pmol	Picomole
Pseud	*Pseudanabaena*
PSPs	Paralytic shellfish poisons
PVDF	Polyvinylidene difluoride
qPCR	Quantitative polymerase chain reaction
R. brookii	*Raphidiopsis brookii*
R. raciborskii	*Raphidiopsis raciborskii*
R^2	Coefficient of determination (R-square)
Raphi	*Raphidiopsis*
rho	Spearman's rank correlation coefficient
rpm	Revolutions per minute
s	Second
SCCAP	Scandinavian Culture Collection of Algae and Protozoa
SPE	Solid phase extraction
SRP	Soluble reactive phosphorus
STX	Saxitoxin
sxt	Saxitoxin biosynthesis (*sxt*) gene cluster
SYBR	Synergy Brands, Inc.
Syne	*Synechocystis*
T_m	Melting temperature
TN	Total nitrogen
TN:TP	Total nitrogen and total phosphorus ratio
TP	Total phosphorus
TSI	Trophic State Index
Turb.	Turbidity
U μL^{-1}	Units per microliter
UFRJ	Federal University of Rio de Janeiro
UFSCar	Federal University of São Carlos
UGRHI	Water Resources Management Unit

USA	The United States of America
Water temp.	Water temperature
WHO	World Health Organization
WQI	Water Quality Index
Z_{eu}	Lower limit of euphotic zone

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CHAPTER 1 - GENERAL INTRODUCTION

1 Background and objective

Cyanobacteria are prokaryotic microorganisms found globally in both inland waters and coastal and marine systems (Merel et al, 2013; Buratti et al., 2017). Across varying environmental conditions, cyanobacteria can produce harmful secondary metabolites called cyanotoxins, which possess various physicochemical properties, molecular structures, and toxicological mechanisms of action (Calijuri et al., 2006; Koreivienė et al., 2014; Pearson et al., 2016; Wood et al., 2017). Occurrence of cyanotoxins in surface waters has been reported worldwide (Merel et al., 2013; Soares et al., 2013; Ndlela et al., 2016; Aguilera et al., 2018b; Buratti et al., 2017).

In a review of studies published on cyanobacterial toxins around the world, Merel et al. (2013) observed that microcystin (MC) alone represents approximately half of the studies published while a quarter of the literature available has emphasis on saxitoxin (STX). In Brazil, the occurrence of these two cyanotoxins is well documented, with MC being the most prevalent and STX the second most studied cyanotoxin (Bittencourt-Oliveira, 2003; Anjos et al., 2006; Moschini-Carlos et al., 2009; Pimentel and Giani, 2013; Bittencourt-Oliveira, 2014; Guedes et al., 2014; Borges et al., 2015; Brentano et al., 2016; Calado et al., 2017; Casali et al., 2017; Schlüter et al., 2018; Walter et al., 2018; Lorenzi et al., 2019; Oliveira et al., 2019).

Occurrence of cyanobacteria and their toxins results in various water quality problems and causes severe economic damage by impairing water supplies, recreational and fisheries activities. Furthermore, elevated exposure to cyanotoxins through food and water can be fatal to both humans and wildlife (Tucci and Sant'Anna, 2003; Yunes et al., 2005; Calijuri et al., 2006; Sant'Anna et al., 2008). The first documented human deaths from cyanotoxins occurred following intravenous exposure in a dialysis clinic in 1996 in Caruaru city, Brazil. After hemodialysis sessions, 116 patients with chronic kidney problems had symptoms as visual disturbances, nausea and vomiting. Among the patients, 100 patients developed acute liver failure and 76 patients died. Tests carried out after the event detected the presence of microcystin and cylindrospermopsin in the water used to wash the dialyzer (Carmichael, 2001; Azevedo et al., 2002).

Despite the emerging concern with respect to multiple routes of environmental exposure and economic losses caused by these contaminants, few water quality criteria and regulations for exposure to cyanotoxins exist. Considering the high toxicity of cyanotoxins, the Ministry of Health in Brazil announced an Ordinance 2914/2011 (Brasil, 2011) to establish

responsibilities and procedures related to surveillance and control of the water quality for human consumption and drinking standards. The regulation requires weekly analysis of cyanotoxins if the number of cyanobacteria at the point of water collection exceeds 20,000 cells mL^{-1}. Likewise, the maximum allowable limits for microcystins and saxitoxins were set to 1.00 $\mu g\ L^{-1}$ and 3.00 $\mu g\ L^{-1}$, respectively. The regulation stress that contamination of the water supply by cyanotoxins is of concern to public health authorities and water resources, but it also demonstrates the importance of understanding the factors that stimulate cyanobacterial blooms and cyanotoxins production, as these events can alter the odor and taste of the water, as well as causing public health problems and ecological imbalances in the aquatic environment.

As development of cyanobacterial blooms is related to environmental factors, many studies have focused on the relationship between the occurrence of potentially toxic cells and/or cyanotoxins production and physicochemical variables. Several studies have shown relationships between intensification of the trophic state, levels of nitrogen and/or phosphorus, and low TN:TP ratio with the formation of cyanobacterial blooms and the presence of cyanotoxins, such as microcystins (Fernandes et al., 2009; Davis et al., 2009; Te and Gin, 2011; O'Neil et al., 2012; Srivastava et al., 2012; Horst et al. 2014; Mariani et al., 2015; Sinang et al., 2015; Casali et al., 2017). However, while many potentially toxic cyanobacteria are associated with the intensification of the eutrophication process, Glibert and Burkholder (2011) stated that cyanobacteria may respond differently to environmental changes. Therefore, the challenge is to determine which species are actually related to eutrophication and to understand why some in particular proliferate under specific nutrient conditions and produce toxins.

Environmental changes causing higher temperatures and changing pH values, light intensities, stratification and water levels, as well as other abiotic and biotic factors (interactions with other aquatic organisms) may control the development of cyanobacteria and alter concentrations of cyanotoxins (Nalewajko and Murphy, 2001; Paerl and Huisman, 2008; Winder and Sommer, 2012; Harke et al., 2016; Marmen et al., 2016; Visser et al., 2016; Yang et al., 2016; Paerl, 2018; Bui et al., 2018). Davis et al. (2009) observed that a higher water temperature (13 to 30 °C) increased the density of toxic *Microcystis* and also the copy number of the *mcyD* gene per cell (*mcyD* is a gene involved in the synthesis of microcystins in cyanobacteria), meaning that blooms with a greater cyanotoxin concentration in the aquatic ecosystem are generated. Although the aforementioned studies provide valuable information on environmental conditions that are most likely to result in high toxin concentrations, no consensus has emerged to date, since contradictory results are reported. In some cases, no relationship between physicochemical variables and cyanotoxin production was observed

(Pimentel and Giani, 2013; Ngwa et al., 2014; Wood et al., 2017).

Most cyanotoxins are synthesized by groups of multimodular enzymes known as polyketide synthase (PKS) and nonribosomal peptide synthetase (NRPS), or NRPS/PKS hybrids, which catalyze the formation of the chemical structure of cyanotoxins (Méjean and Ploux, 2013; Micallef et al., 2014). Considering that NRPS and PKS enzymes are capable of using a wide diversity of substrates in the toxin synthesis, a large number of different chemical structures can be obtained through these enzymatic systems, resulting in the diversity of cyanotoxins currently known (Valério et al., 2010).

Saxitoxin and its analogs form a group of neurotoxic carbamate alkaloids, also known as paralytic shellfish poisons (PSPs) due to their occasional occurrence in marine mollusks and crustaceans in which the toxins are known to bioaccumulate (Wiegand and Pflugmacher, 2005). These cyanotoxins are produced by the marine dinoflagellate genera *Alexandrium*, *Gymnodinium* and *Pyridinium*. Cyanobacteria belonging to the genera *Aphanizomenon*, *Raphidiopsis* (basionym *Cylindrospermopsis*) (Aguilera et al., 2018a), *Lyngbya, Planktothrix*, and *Dolichospermum* (basionym *Anabaena*) (Wacklin et al., 2009) do also synthesize this neurotoxin. The STX gene cluster consists of up to 33 genes and has been described for some cyanobacteria (*Lyngbya wollei*, *Aphanizomenon* sp, *Dolichospermum circinale* AWQC131C, *Raphidiopsis raciborskii* T3 and *Raphidiopsis brookii* D9). Organization of the genes differs, and the presence or absence of certain genes seems to explain the diversity of toxin profiles among STX-producing species (Mihali et al., 2011; D'Agostino et al., 2016). In order to confirm the function of *sxt* genes, the correlation between the presence of each gene and the saxitoxin analog produced by cyanobacterial species has been discussed (Mihali et al., 2011; Soto-Liebe et al., 2010).

Microcystins are cyclic heptapeptides that comprise a diverse group with more than 240 structurally similar congeners (Spoof and Catherine, 2017; Bouaïcha et al., 2019). The hepatotoxins are synthesized by species from different genera of cyanobacteria, including *Microcystis*, *Oscillatoria*, *Planktothrix*, *Nostoc*, *Dolichospermum* and *Anabaenopsis*. The microcystin gene cluster involved in the synthesis of microcystins in cyanobacteria was first described for the strain *Microcystis aeruginosa* PCC 7806. The gene cluster consists of ten genes (*mcyA-J*) that are arranged in two operons transcribed in opposite directions, *mcyA-C* and *mcyD-J* (Tillett et al., 2000; Méjean and Ploux, 2013). The organization of operons and genes varies in the cyanobacterial genera in which they have been described.

With the identification of the groups of genes responsible for the synthesis of microcystins (Rouhiainen et al., 2004) and saxitoxins (Kellmann et al., 2008), several

quantitative polymerase chain reaction (qPCR) assays targeting genes encoding cyanotoxins have been developed to detect and quantify potential toxin-producing cyanobacteria directly from an environmental sample (Moreira et al., 2014; Pacheco et al., 2016). qPCR assays allow determination of the proportion and abundance of a given species, as well as the proportion of toxic and non-toxic genotypes within a cyanobacteria bloom, e.g. in response to environmental factors (Pacheco et al., 2016; Gaget et al., 2017). Successful application of qPCR methods based on the *sxt* and *mcy* genes for detection of saxitoxin- and microcystin-producing cyanobacteria has been demonstrated in some studies (Kurmayer and Kutzenberger, 2003; Al-Tebrineh et al., 2010, 2012; Murray et al., 2011; Antonella and Luca, 2013; Zhang et al., 2014; Fortin et al., 2015; Savela et al., 2015; Pereyra et al., 2017). However, in Brazilian water bodies qPCR methods have only been performed in few studies to quantify densities of toxic cyanobacteria (Pimentel and Giani, 2013; Guedes et al., 2014).

In Brazil, there is a historical documentation of blooms and contamination of reservoirs by cyanotoxins, especially by the potentially toxic genera *Microcystis*, *Dolichospermum*, *Raphidiopsis*, *Oscillatoria*, *Planktothrix* and *Aphanizomenon* (Bittencourt-Oliveira et al., 2014). Altogether, twenty genera and more than forty species of cyanobacteria are known for their toxic potential (Leite, 2008; CETESB, 2013). The genus *Microcystis* includes several bloom-forming and cyanotoxin-producing species and is one of the most studied genera of toxic cyanobacteria (Sant'Anna et al., 2011). However, in Brazilian reservoirs cylindrospermopsin and saxitoxin produced by the genus *Raphidiopsis* have also been observed (Anjos et al., 2006; Bittencourt-Oliveira et al., 2014; Borges et al., 2015; Brentano et al., 2016). A recent study reported that saxitoxin-producing cyanobacterial blooms commonly occur during drought periods in the Northeast region of Brazil, and that STX might have acted as a co-insult to Zika virus infection in Brazil (Pedrosa et al., 2020). These authors also observed that STX may act synergistically with Zika virus, even at concentrations considered to be safe by Brazilian authorities (<3.00 μg L^{-1}). This fact has raised public health concern regarding the consequences of arbovirus outbreaks happening in areas with droughts and/or frequent occurrence of freshwater cyanobacterial blooms.

The Itupararanga and Lobo reservoirs, areas of investigation of the present doctoral research, were classified as supereutrophic and eutrophic in the period from 2013 to 2015, respectively (Marafão, 2016). In the same period, in both reservoirs a dominance of the potentially toxic cyanobacterium *Raphidiopsis raciborskii* and presence of saxitoxin were observed (Tundisi et al., 2015; Beghelli et al., 2016; CETESB, 2013). In a study carried out in the Itupararanga reservoir in 2011, Casali et al. (2017) found positive and significant

correlations between STX concentrations, *Raphidiopsis raciborskii* densities and dissolved nutrient concentrations. The authors concluded that variations of *R. raciborskii* toxin yields depend on the enrichment conditions, indicating potential implications for the reservoir management. Thus, determination of which environmental conditions that are most likely to control cyanobacterial bloom and subsequent toxin production is of great interest to water managers and natural resource professionals (Paerl, 2018). Considering this scenario, the main objective of this doctoral research was to assess the effect of environmental variables on the composition, abundance and toxicity of cyanobacterial communities in two subtropical reservoirs, Itupararanga and Lobo, in order to generate information to help on the proper monitoring of these aquatic ecosystems and minimize the risks to the public health.

2 Study area

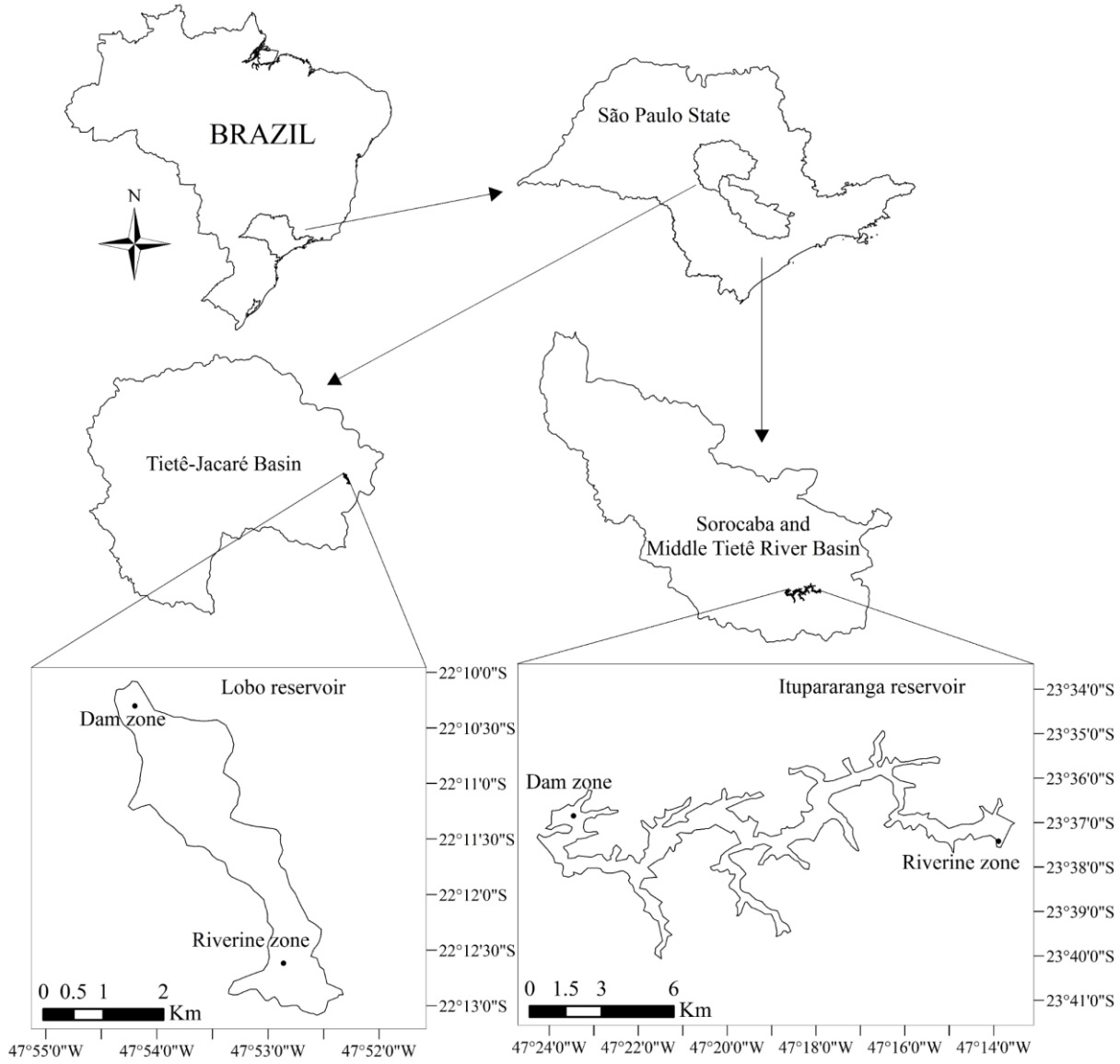
For the present study, two subtropical reservoirs were investigated, Itupararanga reservoir (Votorantim, São Paulo State, Brazil) and Lobo reservoir (Itirapina, São Paulo State, Brazil) (Figure 1.1). Both reservoirs present similar characteristics in relation to the eutrophication process. The main characteristics of the reservoirs are shown in the Table 1.1.

2.1 Sorocaba and Middle Tietê River Basin

The Sorocaba and Middle Tietê River Basin (UGRHI 10 - tenth Water Resources Management Unit) is located in the central-southeastern portion of the São Paulo State (Figure 1.1). The basin covers an area of 53 municipalities, of which 34 are based in its territory and 19 have only rural portions, and its main rivers are Sorocaba, Tietê, Sorocabuçu and Sorocamirim. The drainage area of the basin is 11,829 km² with an estimated population of 2.1 million inhabitants (10.2% of the inhabitants are concentrated in the rural region) (CBH-SMT, 2019). According to the climate classification presented by Köppen and Geiger (1928), three climatic types are predominant in UGRHI 10: hot-humid climate with dry winter, hot-humid climate without dry season and temperate-humid climate without dry season.

Considering the entire territorial portion occupied by the basin, the predominant forms of land use are pasture (43.7%) and native vegetation (12.3%), followed by urban (6.1%), agricultural (5.5%) and forestry areas (1.2 %). Main industrial activities include textiles, food, chemistry, mechanics, electronics and agro-industries. Water is mainly used for public and industrial supply, generation of hydroelectric energy, irrigation of plantations, recreation and discharge of domestic and industrial effluents.

Figure 1.1 - Sampling sites in Lobo and Itupararanga reservoirs, São Paulo State, Brazil. Lobo riverine zone (22°12’36.9” S, 47°52’52.8” W); Lobo dam zone (22°10’18.5” S, 47°54’11.2” W); 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)



Source: author.

In 2018, the average flow rate of the surface water in the basin was determined to 107 m^3 s^{-1}, corresponding to an annual availability of 1,668.55 m^3 per inhabitant. Some municipalities experienced a water deficit, reducing the annual availability of water to about 1,500 m^3 per inhabitant. This reduction was caused by population growth and an increase of economic activity (CBH-SMT, 2019). In the same year, 27 locations in the basin were monitored and classified by the Environmental Agency of the State of São Paulo (CETESB). The classification characterized 4% of the locations as oligotrophic, 48% as mesotrophic, 14.5% as eutrophic, 14.5% as supereutrophic and 19% were classified as hypereutrophic. Relative to previous years, this basin showed a water quality deterioration and some locations

indicated contamination by domestic sewage (CETESB, 2018). The reduced water quality was caused by high loads of polluted water from the Metropolitan Region of São Paulo State by the Tietê River and the Metropolitan Region of Campinas by the Capivari River (population growth in the basin), in addition to the intensification of industrial and agricultural activities, inefficiency of sanitation (only 88% of domestic effluents generated in the basin were collected and 80% were treated in 2018), and suppression of forest fragments (CETESB, 2018).

Table 1.1 - Characteristics of Lobo and Itupararanga reservoirs

Characteristics	Itupararanga	Lobo
Geographic location	23°36'S, 47°23'W	22°15'S, 47°49'W
Year of operation	1914	1936
Basin area (km²)	936.5	227.7
Surface area (km²)	20.9	6.8
Altitude (m)	710	770
Average depth (m)	7.8	3.0
Maximum depth (m)	22.0	12.0
Maximum volume (10^6 m³)	286.0	22.0
Perimeter (km)	192.8	21.0
Maximum water flow (m³ s^{-1})	39.0	20.0
Water residence time (days)	250	32
Hydrographic basin	Sorocaba and Middle Tietê River	Tietê-Jacaré
Main tributaries	Sorocamirim and Sorocabuçu rivers	Itaqueri and Lobo rivers
Trophic state (2013-2015)	Eutrophic	Supereutrophic
*Trophic state (2017-2018)	Mesoeutrophic	Mesotrophic

Source: Delello (2008); Cunha (2012); Marafão (2016); *this study.

2.1.1 Itupararanga reservoir

The Itupararanga reservoir (Figure 1.1, Table 1.1) is formed by the Sorocamirim and Sorocabuçu rivers and located in the Alto Sorocaba sub-basin in the Sorocaba and Middle Tietê River Basin (UGRHI 10). The dam of the reservoir is located in the Votorantim municipality. The reservoir was built by the São Paulo Electric Company in 1912 (ANEEL, 2004) and initiated operation in 1914. Since 1974, the dam operation has been managed by the Brazilian Aluminum Company (Votorantim group). In addition to public supply, other usages of the water in this aquatic system are for irrigation, navigation, fishing and recreation. There is a significant diversity of land use around the reservoir encompassing rural activities (such as agriculture and cattle-grazing), mining industries, high-quality residential subdivisions, areas

with exposed soil, as well as forest areas (Cunha, 2012).

This reservoir has undergone an intense eutrophication process and was classified as mesoeutrophic due to the supply of nutrients by diffuse sources and loads from its tributaries, especially the Una and Sorocamirim rivers (Cunha, 2012; Marafão, 2016). In 2017, a dominance by potentially saxitoxin-producing cyanobacterium *Raphidiopsis raciborskii* was observed in the reservoir. In the same year, occurrence of STX was monitored at the point close to the dam and it was detected at all sampling times but in concentrations below the standard for potable water (3.0 µg L^{-1}). Although in low concentrations, these data show the deterioration of the water quality of this aquatic system (CETESB, 2018). In addition, from an assessment of the evolution of the trophic state over 15 years (2000-2014) of this reservoir, a model that predicts hyper-eutrophication in the coming years was obtained (Beghelli et al., 2016).

2.2 Tietê-Jacaré Basin

The Tietê-Jacaré Basin (UGRHI 13 - thirteenth Water Resources Management Unit) is located in the central region of São Paulo State (Figure 1.1) and covers, totally or partially, 34 municipalities, the main ones being Araraquara, Bauru, Jaú and São Carlos. Main rivers in the basin are the Médio-Tietê, Jacaré-Guaçu and Jacaré-Pepira. The drainage area of the basin is 11,749 km² with an estimated population of 1.5 million inhabitants (3.6% of the inhabitants are concentrated in the rural region) (CBH-TJ, 2018). According to the classification of Köppen and Geiger (1928), the climate consists of humid summers (October to March) and dry winters with intense winds (April to September).

The predominant forms of land use are variable crops (50.65%) and forest area (11.82%), followed by pasture (9.62%), forestry (6.77%), permanent crops (5.55%), urbanization (5.20%), rural area (4.74%), uncovered areas (2.41%), continental waters (1.62%), unidentified use (1.50%), and mining areas (0.14%). The main industrial activities encompass sugar and alcohol plants, foundries, sand mining and tanneries. Most of the industries contribute to pollution by diffuse sources into the basin.

In 2017, the average flow rate of surface water in the basin was 97.00 m^3 s^{-1} and the annual water consumption per inhabitant was 1,953.00 m^3 (CBH-TJ, 2018). CETESB monitors water quality at fifteen locations in the basin for the evaluation of the Water Quality Index (WQI), the Aquatic Life Protection Index (ALPI) and the Trophic State Index (TSI). Among these monitoring locations, two locations were considered to have a poor water quality according to WQI and five locations were considered to have a low water quality according to ALPI. Regarding the TSI, 27% of the locations were classified as oligotrophic, 60% as

mesotrophic, 6.5% as eutrophic and 6.5% as super-eutrophic. These data demonstrate the deterioration of the water quality in the basin. The main reason for the deterioration is a persistent discharge of domestic sewage, as illustrated by only 67% of domestic effluents being treated in 2018 at UGRHI 13 (CETESB, 2018).

2.2.1 Lobo reservoir

The Lobo reservoir (Figure 1.1, Table 1.1) is formed by the Itaqueri and Lobo rivers and is part of the Environmental Protection Area of the Corumbataí municipality (State Decree No. 20,960, June 8, 1983). The reservoir is located in the Jacaré-Guaçu sub-basin in the Tietê-Jacaré Basin (UGRHI 13) in the central region of the São Paulo State. In 1936, the reservoir was built by the S/A Central Elétrica de Rio Claro for the production of hydroelectric energy for local communities and industries that had settled in the region. According to Köppen and Geiger (1928), the region's climate is subtropical mesothermal and is controlled by masses of equatorial and tropical air, with periods of humid summers (October to March) and dry winters (April to September).

The reservoir can be divided into two distinct compartments: an upper, shallow compartment with a high abundance of macrophytes, and a lower, deeper, well-mixed and vertically homogeneous compartment. Changes in water quality due to an increased trophic state and also changes in the diversity of the biotic communities have been observed in the reservoir (Motheo, 2005). Water in the reservoir is used for irrigation of plantations, local public supply, generation of hydroelectric energy, fishing and recreation, but also for discharge of domestic and industrial effluents.

Initial studies in the Lobo reservoir classified this environment as oligo-mesotrophic with low nutrient concentrations (Tundisi et al., 1978; Calijuri and Tundisi, 1990). However, more recent studies have shown occurrence of increased nutrient concentrations (nitrogen and phosphorus), resulting in an increased eutrophication and appearance of bioindicator species found in more eutrophic systems (e.g., the cyanobacteria *Microcystis* sp. and *Raphidiopsis raciborskii* and the macrophyte *Pistia* sp.) (Motheo, 2005; Delello, 2008; Tundisi et al., 2015).

The first cyanobacterial bloom in the Lobo reservoir (caused by *Raphidiopsis raciborskii*) was observed in July 2014 (winter) and is the only bloom described in this environment (Tundisi et al., 2015). The authors attributed the occurrence of this bloom to an increase in the average water temperature during the winter, a low rainfall during the summer and an increased water residence time (approximately 60 days) to maintain the volume of water

for energy generation. In addition, the authors expressed concern on this bloom regarding the maintenance of the multiple uses of the reservoir, especially due to the observed production of saxitoxin and microcystin. Although in low concentrations in July 2014, a higher potential of cyanotoxins production might be expected in the future, because the reservoir is experiencing an accelerated process of eutrophication.

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CHAPTER 2 - CYANOBACTERIAL OCCURRENCE AND DETECTION OF SAXITOXIN AND MICROCYSTIN IN A SUBTROPICAL DRINKING WATER RESERVOIR

Abstract

Toxic cyanobacteria in public water supply reservoirs represent a serious health risk since they can release cyanotoxins into the water. Despite the frequent occurrence of cyanobacterial blooms in Brazilian water bodies, few studies focusing on molecular detection of toxic genes have been done. In this study, the occurrence of potentially saxitoxin- and microcystin-producing cyanobacteria, the detection of *sxtA* gene and the influence of environmental variables on cyanotoxin production were investigated in the Itupararanga reservoir. Microcystin was detected in low concentrations (max. 0.14 µg L^{-1}) and did not correlate with any environmental variables. Number of *sxtA* gene varied from 6.76×10^3 to 7.33×10^5 cells mL^{-1} and correlated positively with SXT concentrations (max. 0.23 µg L^{-1}) and biovolume of *Raphidiopsis raciborskii*, *Aphanizomenon* sp. and *Geitlerinema* sp. STX and *sxtA* gene were directly correlated with TN:TP ratio and pH, and negatively correlated with nutrients and turbidity, confirming the importance of local environmental variables in the overall regulation of saxitoxin production. The qPCR assay developed in this study was specific for quantification of saxitoxin gene and should be considered in future investigations on toxic cyanobacterial occurrences dynamics and in water quality monitoring to identify the risk of toxic cyanobacteria in subtropical reservoirs. However, considering that the monitoring of potentially STX-producing cyanobacteria in environmental samples by qPCR is scarce, more studies are still needed to evaluate the applicability of this method to monitor water samples aiming to infer the saxitoxin concentrations.

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

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 and recreational water pose a serious risk to human health and ecosystem functioning (Sabart et al., 2015). The most studied cyanotoxin is the hepatotoxic microcystin (MC), which consist of more than 240 structural variants and are produced by several cyanobacterial genera, including *Microcystis*, *Planktothrix* and *Dolichospermum* (basionym *Anabaena*, Wacklin et al., 2009)

(Spoof and Catherine, 2017; Bouaïcha et al., 2019). Another important cyanotoxin is saxitoxin (STX), a neurotoxin that occurs in more than 50 variants, and 15 of these variants have been detected so far in cyanobacteria (Wiese et al., 2010). The STX-producing freshwater genera include, among others, *Raphidiopsis* (basionym *Cylindrospermopsis*) (Aguilera et al., 2018), *Dolichospermum*, and *Aphanizomenon*.

The physiological and ecological function of cyanotoxins to 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 (Twiss and Gouvea, 2006; Valério et al., 2010; Holland and Kinnear, 2013). However, specific factors that stimulate and/or control the production of cyanotoxin by cyanobacteria remain to be determined (Calijuri et al., 2006). 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 (Ferrão-Filho and Kozlowsky-Suzuki, 2011).

Quantification of cyanobacteria by traditional microscopy is not adequate for the identification of toxic populations, since toxic and nontoxic strains are morphologically identical (Pimentel and Giani, 2013). Accurate quantification of potentially toxin-producing cyanobacteria can be performed by quantitative polymerase chain reaction (qPCR), which is a powerful and sensitive tool based on the genes involved in the cyanotoxin biosynthesis. Application of qPCR for quantification of potentially toxin-producing cyanobacteria has been reported in analysis of environmental samples (Al-Tebrineh et al., 2010, 2012; Murray et al., 2011; Zhang et al., 2014; Fortin et al., 2015; Gao et al., 2015; Savela et al., 2015, 2017; Crawford et al., 2017; McKindles et al., 2019).

In Brazil, few studies have focused on molecular detection of toxic genes, despite the frequent occurrence of cyanobacterial blooms (Bittencourt-Oliveira, 2003; Anjos et al., 2006; Lorenzi, 2008; Bittencourt-Oliveira et al., 2010; Lorenzi et al., 2015). In most studies, traditional PCR has been used for early-warning detection of potential microcystin-producing strains, but quantification of the cell numbers is not possible with this method (Sivonen, 2008). Only Lorenzi (2008), Pimentel and Giani (2013) and Guedes et al. (2014) have applied a qPCR method to quantify *mcyA* gene in Salto Grande reservoir, *mcyD* gene in Furnas reservoir and *mcyB* in Funil reservoir, respectively. Regarding quantification of *sxt* genes in Brazilian freshwaters, this methodology has to our knowledge so far not been used.

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 source of water supply for approximately one million people, for hydroelectric power and for irrigation of crops (Smith et al., 2005; Conceição et al., 2015). The reservoir has suffered from water quality degradation due to growing urbanization, agricultural practices and discharge of untreated wastewater, especially in the tributaries (Manfré et al., 2011; Beghelli et al., 2012; Taniwaki et al., 2013). Studies have classified this reservoir as mesoeutrophic (Pedrazzi et al., 2013; Rodrigues et al., 2019). Increased nutrient levels do not only contribute to the eutrophication process, they also introduce risk of water quality deterioration due to proliferation of cyanobacteria, as already observed in this reservoir (Cunha and Calijuri, 2011; Beghelli et al., 2016; Casali et al., 2017).

To better understand dynamics of toxin-producing cyanobacteria and production of cyanotoxins in Itupararanga reservoir, presence of potentially saxitoxin- and microcystin-producing cyanobacterial genera and concentrations of STX and MC were determined. The obtained results were related to environmental variables by statistical treatments to identify the main drivers controlling the occurrence of toxin-producing cyanobacteria. We also aimed at quantifying the *sxtA* gene by a new qPCR assay developed for estimation of potentially saxitoxin-producing cyanobacteria in environmental samples.

2 Material and methods

2.1 Study area and sampling

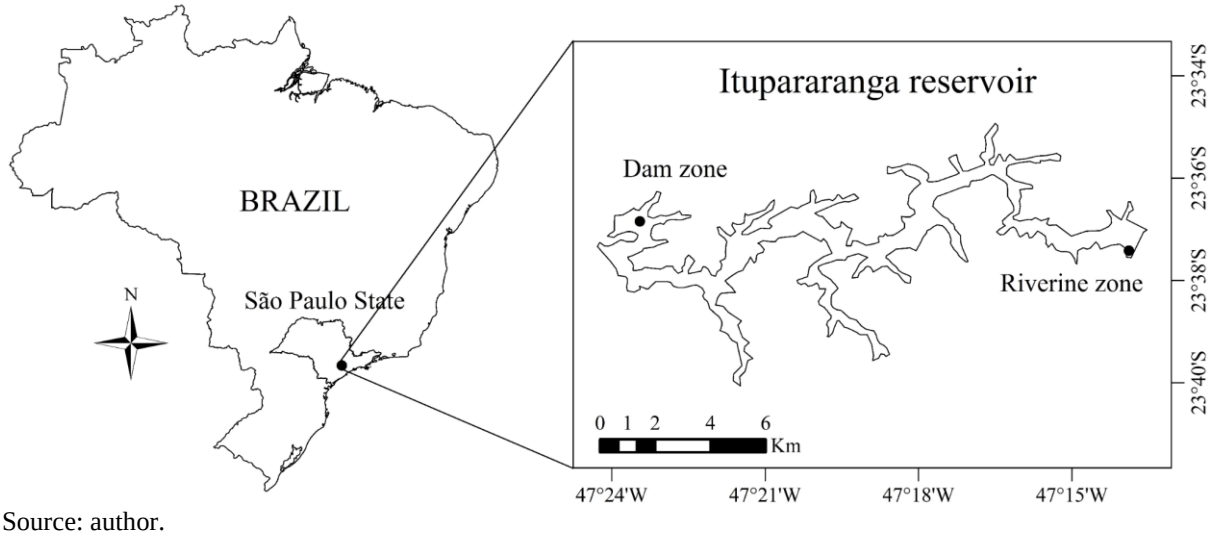
Environmental samples were collected at riverine zone and dam zone in the Itupararanga reservoir located in São Paulo State, Brazil (Figure 2.1). The main characteristics of the reservoir and sampling sites are shown in Table A.1. Sampling was carried out in May, August, October 2017 and January 2018. Water samples were collected at two depths (100% - surface and 1% - lower limit of euphotic zone) determined by the photosynthetically active radiation (PAR, $\mu E\ m^{-2}\ s^{-1}$) using a light sensor (LI-1400 DataLogger, sensitivity of 400-700 nm, LI-COR Biosciences, USA).

2.2 Environmental variables

The variables pH, dissolved oxygen (DO, $mg\ L^{-1}$), electrical conductivity (EC, $\mu S\ cm^{-1}$), turbidity (NTU) and water temperature (°C) were determined on-site using a HANNA probe (HANNA HI9829, 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 (Whatman, GE Healthcare Life Sciences, USA) and the filtrates were kept frozen at -20 ºC until analysis. The filters were kept frozen at -20 ºC until chlorophyll *a* (chl *a*) analyses according to methodology described by Nusch (1980). Samples for total nutrients (phosphorus - TP and nitrogen - TN) were collected and stored at -20 ºC. Nutrient analyses were performed as triplicates by spectrophotometric methods according to APHA (2005). The trophic state index (TSI) of the sampling sites was calculated considering the annual geometric means of TP and chl *a* (Cunha et al., 2013).

Figure 2.1 - 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)



Source: author.

2.3 Phytoplankton community

For microscopic phytoplankton analysis, samples were Lugol-fixed for subsequent identification and quantification following the Utermöhl (1958) method using an inverted microscope (Olympus CK2) at 400x 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} (Lund et al., 1958). A counting limit was established through the 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 (Hillebrand et al., 1999; Sun and Liu, 2003). For taxa with relative abundance <10%, mean cell volumes were obtained from literature. The biovolume was estimated by multiplying the density of each taxon by its mean cell volume.

Samples for pigment analysis were filtered through 1.2 µm-pore-size glass fiber filters

(Whatman, GE Healthcare Life Sciences, USA) that were kept frozen at -20 °C until pigment extraction. Pigments in phytoplankton on the filters were extracted in 3 ml 95% acetone with vitamin E acetate as an internal standard. The filters were sonicated in an ice-cool sonication bath for 10 min, extracted further at 4 °C for 20 h, and 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 into vials. Pigment analyses were carried out in a high-performance liquid chromatography system (HPLC) according to Schlüter et al. (2016, 2018) using the Van Heukelem and Thomas (2001) method. The HPLC system was calibrated using pigment standards from DHI Lab Products (Table A.2). The biomass in units of chlorophyll *a* (µg chl *a* L^{-1}) of the individual phytoplankton groups detected by the pigments was calculated by CHEMTAX software v.1.95 (Mackey et al., 1996). The initial pigment/chl *a* ratios used in the CHEMTAX software were from Schlüter et al. (2006, 2018).

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 (Beacon Analytical System Inc., 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, Austria) at 450 nm.

2.5 DNA extraction, primer design and PCRs for saxitoxin

For DNA extraction, water samples were immediately filtered through 0.22 µm-pore-size mixed cellulose ester membranes (Whatman, GE Healthcare Life Sciences, 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 by a NanoDrop spectrophotometer. The DNA purity was determined by the ratio of the absorbance measured at 260 nm to the absorbance measured at 280 nm. Besides, the DNA quality was visualized in 1% agarose gel stained with GelRed™ Nucleic Acid Gel Stain (Biotium Inc., USA) using a gel documentation system (Bio-Rad Gel Doc™ 2000 Bio-Rad Laboratories, USA).

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 the ClustalW (Thompson et al., 1994). Regions of high similarity were identified and chosen for primer design using the Primer3Plus (Untergasser et al., 2007). The developed primer pair *sxtA*-cyano-F (5'-xxxxGAAGCGxxxxGTCTGG-3') and *sxtA*-cyano-R (5'-TCTGCCxxxxxxGAATACAC-3') was tested for specificity (*in silico*, Table A.3) with the National Center for Biotechnology Information (NCBI) Primer-BLAST against the non-redundant database. The dimer prediction was verified with OligoAnalyzer 3.1 (Integrated DNA Technologies, Inc.). The primer set designed for conventional PCR and qPCR assays produced fragments of 153 bp size.

Conventional PCR was performed in triplicates in a Mastercycler Pro thermal cycler (Eppendorf, Hauppauge, 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.com) and 2 µL of template DNA. The thermal cycle program consisted of an initial preheating for 2 min at 95 ºC, followed by 35 cycles, each consisting of 30 s at 95 ºC, 30 s at 60 ºC and 30 s at 72 ºC, and a final extension step for 5 min at 72 ºC. The amplified PCR products were visualized on 2% agarose gel stained with GelRed™ Nucleic Acid Gel Stain (Biotium Inc., USA) together with a 2-Log DNA Ladder (New England BioLabs Inc., USA) using a gel documentation system (Bio-Rad Gel Doc™ 2000 Bio-Rad Laboratories, USA).

To test the specifity of the developed primer set, 4 saxitoxin-producing strains and 5 non-saxitoxin producing strains (Table A.4) were analyzed with the PCR assay. Strains were grown in axenic conditions at 24±1 ºC, light intensity of 40 µmol photons m^{-2} s^{-1}, photoperiod of 12-h light/12-h dark, in ASM-1 medium (Gorham et al., 1964). Cultures were harvested during exponential growth phase and filtered through 0.22 µm-pore-size mixed cellulose ester membranes under slight vacuum pressure. DNA was extracted as mentioned above and amplified with the PCR assay in triplicate. Selected PCR products were purified and sequenced (Eurofins Genomics Company, Germany) to confirm the specificity of PCR amplification. Sequence identity was determined by a Basic Local Alignment Search Tool (BLAST) of the National Center for Biotechnology Information (NCBI) database.

For quantification of *sxtA* gene in the environmental samples, the qPCR assay was performed in triplicate using an AriaMx Real-Time PCR System (Agilent Technologies, USA) with 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, USA) and nuclease free water (Sigma-Aldrich) for an end volume of 20 µL. The qPCR assay was carried out under the following cycling conditions: initial preheating at 95 ºC for 3 min, followed by 40 quantification cycles, each consisting of 30 s at 95 ºC, 30 s at 65 ºC, 30 s at 72 ºC and 10 s at 77.5 ºC, and a melt cycle of 30 s at 95 ºC, 30 s at 65 ºC and 30 s at 95 ºC. Fluorescence measurement of generated products was obtained at the end of each cycle at 77.5 ºC. Melting curves analysis was performed at the end of each cycle to evaluate the specificity of the newly designed primer set.

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

2.6 Statistical analysis

Data were tested for normality using 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 data were log(x+1) transformed and the biovolume data were transformed by Hellinger transformation.

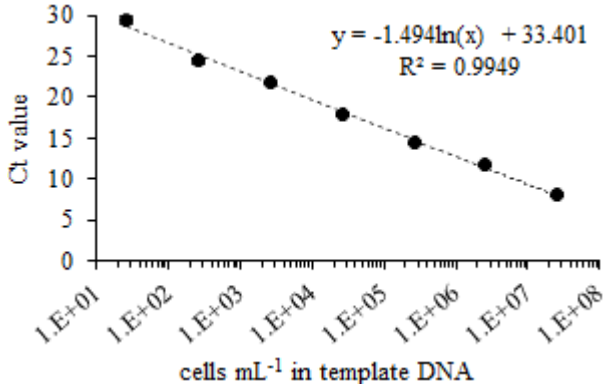
Considering the environmental variables, Mann-Whitney test was performed to evaluate the differences between sites (riverine and dam zones) and depths (100% and 1% of PAR), while Kruskal-Wallis test was conducted to evaluate the differences between months. Dunn's *post hoc* multiple comparison test was performed if significant differences were observed in the Kruskal-Wallis test ($p < 0.05$). To investigate the relationships between *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) (Hammer et al., 2001) and STATISTICA version (13.5) (TIBCO Software Inc., Palo Alto, USA).

3 Results

3.1 Efficiency and specificity of *sxtA*-cyano primer set and standard curve

The standard curve, established for the *sxtA*-cyano primer set using the saxitoxin-producing cyanobacteria *Raphidiopsis raciborskii* T3, showed a strong and significant linear regression between the DNA concentration (in cells per milliliter) and Ct values. The efficiency of the qPCR assay was 95% (R^2 = 0.99 and slope of -3.44). The detectable number of *sxtA* gene in the standard ranged from 26 to 2.6 × 10^7 cells mL^{-1} with Ct values between 8 and 30 (Figure 2.2). All the melting peaks were found at a temperature between 80.0 and 80.5 ºC indicating the presence of a single amplification product (Figure A.1).

Figure 2.2 - 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



Source: author.

The new primer set was able to detect the presence of the *sxtA* gene for all saxitoxin-producing strains tested, while seven strains were confirmed positive *in silico* PCR, indicating high coverage among different genera. As expected, the non-saxitoxin producing strains did not yield any PCR products (Table A.3). The results demonstrated that the qPCR assay was applicable to quantify potentially saxitoxin-producing cyanobacteria in both cultures and environmental samples.

3.2 Environmental variables

None of the environmental variables varied significantly between depths ($p > 0.05$). Only water temperature varied significantly between months ($p = 0.003$). If excluding dissolved oxygen, water temperature, ammonium and chlorophyll *a*, all the other variables varied significantly between sites ($p < 0.05$) (Table 2.1). Water transparency and Z_{eu} were lower in the riverine zone where the turbidity values were higher, reducing the light penetration in the water

column. The pH remained close to neutral in the riverine zone (6.71±0.24) and was higher in the dam zone (7.18±0.35). Dissolved oxygen concentrations varied between 3.18 and 8.49 mg L^{-1} in the riverine zone, and 6.21 and 8.62 mg L^{-1} in the dam zone. Electrical conductivity was higher in the riverine zone (83.50±12.78 µS cm^{-1}) than in the dam zone (68.13±3.82 µS cm^{-1}). Water temperatures peaked in October 2017 and January 2018 (22.52±0.73 °C and 24.16±1.02 °C, respectively) and were lowest in May and August 2017 (19.04±1.18 °C and 16.51±0.91 °C, respectively).

Table 2.1 - 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

Variables	Riverine zone (n = 8)	Dam zone (n = 8)	Spatial factor	Temporal factor
Water transparency (m)*	1.20 (0.90 - 1.60)	1.50 (1.20 - 1.90)	-	-
Z_{eu} (m)*	2.38 (2.00 - 3.00)	5.25 (4.50 - 7.00)	-	-
pH	6.71 (6.43 - 7.04)	7.18 (6.70 - 7.73)	0.010	ns
Dissolved oxygen (mg L^{-1})	5.91 (3.18 - 8.49)	7.13 (6.21 - 8.62)	ns	ns
Conductivity (µS cm^{-1})	83.50 (65.00 - 101.00)	68.13 (63.00 - 73.00)	0.014	ns
Turbidity (NTU)	10.65 (5.70 - 16.90)	5.25 (3.00 - 7.00)	0.003	ns
Water temperature (°C)	19.97 (15.47 - 25.17)	21.15 (17.10 - 24.70)	ns	0.003
Total phosphorus (µg L^{-1})	51.82 (38.70 - 70.02)	11.70 (8.90 - 15.19)	0.001	ns
Soluble reactive phosphorus (µg L^{-1})	5.64 (3.28 - 7.37)	nd	0.001	ns
Total nitrogen (mg L^{-1})	1.05 (0.83 - 1.34)	0.52 (0.38 - 0.67)	0.001	ns
Nitrate (mg L^{-1})	0.83 (0.70 - 1.04)	0.36 (0.32 - 0.42)	0.001	ns
Nitrite (µg L^{-1})	5.70 (nd - 12.59)	0.77 (nd - 2.18)	0.006	ns
Ammonium (µg L^{-1})	40.49 (15.86 - 81.67)	46.09 (nd - 144.77)	ns	ns
TN:TP	37 (24 - 60)	149 (56 - 237)	0.001	ns
Chlorophyll *a* (µg L^{-1})	27.65 (2.30 - 79.43)	20.03 (15.79 - 23.29)	ns	ns
Trophic state	Eutrophic	Mesotrophic	-	-

Source: author. *Water transparency and Z_{eu} (n = 4); TN:TP = total nitrogen and total phosphorus ratio; nd = not detected; ns = not significant.

If excluding the ammonium, nutrient concentrations were higher in the riverine zone than in the dam zone. This result may indicate discharges of untreated domestic and/or industrial effluent along the tributaries. The average concentration of total phosphorus in the riverine zone (51.82±12.53 µg L^{-1}) was about 4.5 times higher than in the dam zone (11.70±2.01 µg L^{-1}). Soluble reactive phosphorus concentrations in the riverine zone varied from 3.28 to 7.37 µg L^{-1}, while in the dam zone were below the detection limit of the equipment (0.7 µg L^{-}

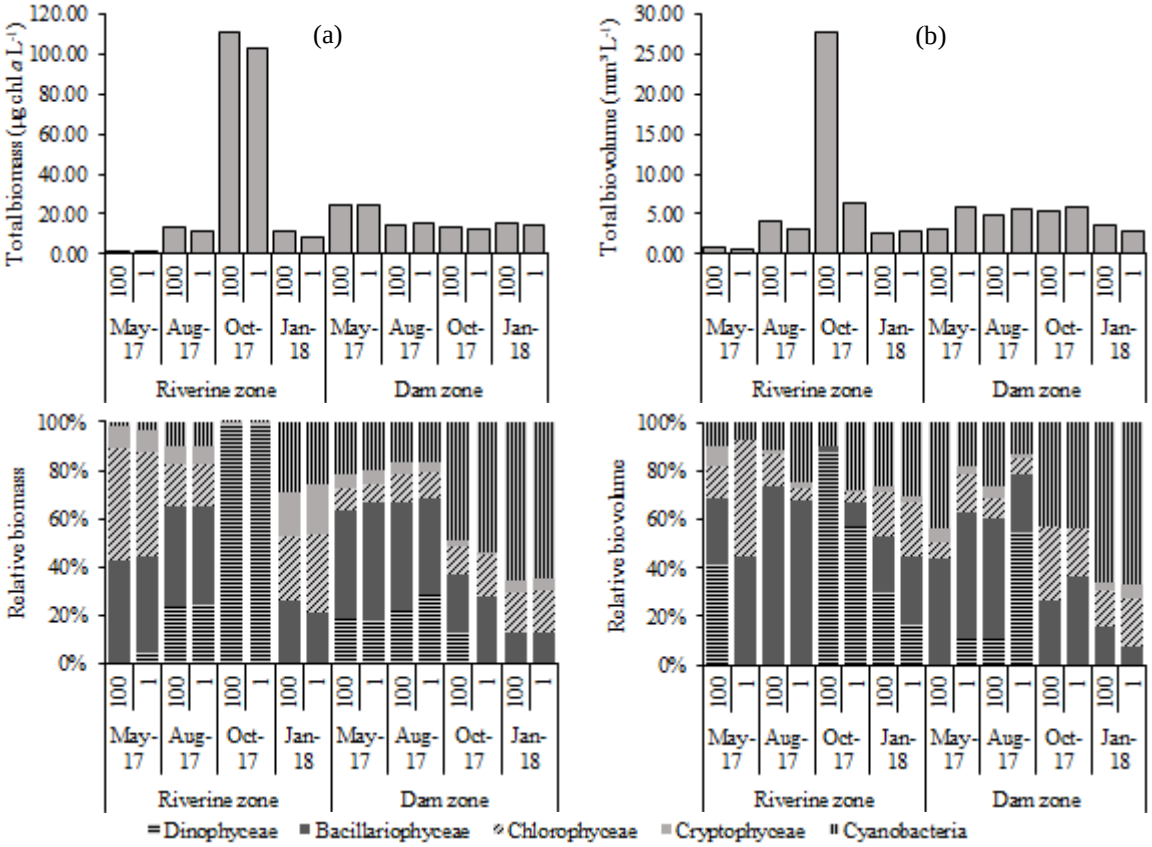
^{1}). Total nitrogen was mainly composed by nitrate (79% in the riverine zone and 69% in the dam zone). The mean concentration of nitrate in the riverine zone was 0.83±0.11 mg L^{-1}, while in the dam zone was 0.36±0.04 mg L^{-1}. The highest value of nitrite was detected in the riverine zone (12.59 µg L^{-1}), while in the dam zone the highest concentration was 2.18 µg L^{-1} (detection limit of the equipment = 0.5 µg L^{-1}). The highest ammonium (NH_4^+-N) concentration was detected in May 2017 in the dam zone (144.77 µg L^{-1}), however, in October 2017 and January 2018 in the same sampling site, NH_4^+-N was not detected in the water. The TN:TP ratios were higher in the dam zone (149±59), indicating a limitation by phosphorus. Concentrations of chlorophyll *a* varied between 2.30 and 79.49 µg L^{-1}, and the highest concentration was detected in October 2017 in the riverine zone where a bloom of dinoflagellates was observed. The trophic state index (TSI) classified the riverine zone as eutrophic and the dam zone as mesotrophic (Table 2.1).

3.3 Phytoplankton composition

The pigment analyses revealed the presence of cyanobacteria since echinenone, canthaxanthin, and aphanizophyll were detected in most samples, while zeaxanthin occurred in all samples. Presence of Chlorophyceae (including Trebouxiophyceae, Euglenophyceae, Chlamydophyceae and Zygnematophyceae) were identified by chl *b*, lutein, violaxanthin, and neoxanthin. Alloxanthin showed the presence of Chryptophyceae. Bacillariophyceae (including Chrysophyceae) were identified by presence of fucoxanthin, chl c_2, and chl c_3, while peridinin revealed the presence of Dinophyceae (Figure 2.3a). The results from microscopy generally confirmed the presence of these groups (Figure 2.3b) (rho = 0.71, p = 0.002).

Bacillariophyceae and Chlorophyceae were common in most samples and constituted an important fraction of the phytoplankton community. Chryptophyceae 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 2.3).

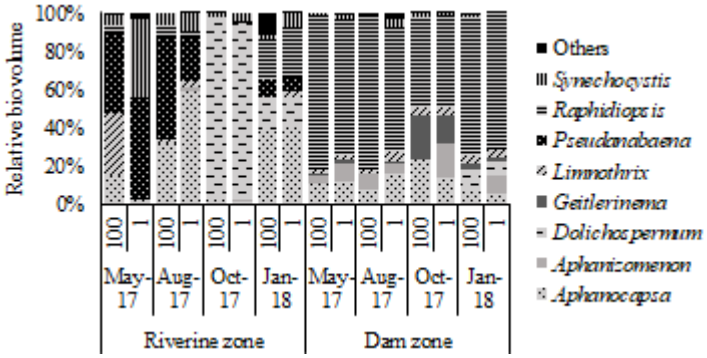
Figure 2.3 - (a) Total biomass (µg chl *a* L^{-1}) and relative biomass (%) of phytoplankton groups determined by the pigment method and (b) total biovolume (mm^3 L^{-1}) and relative biovolume (%) of phytoplankton groups in Itupararanga reservoir



Source: author. 100 = surface; 1 = lower limit of euphotic zone.

Regarding cyanobacteria, 22 species beloging to 16 genera were identified by microscopy with *Aphanizomenon*, *Aphanocapsa*, *Dolichospermum*, *Geitlerinema*, *Limnothrix*, *Pseudanabaena*, *Raphidiopsis*, and *Synechocystis* being the most abundant and frequent genera (Figure 2.4). Cyanobacteria were present at all samplings and varied significantly between sites ($p < 0.01$). While in the riverine zone the contribution of cyanobacteria ranged from 6 to 30% of all the phytoplankton community, in the dam zone cyanobacteria made up 43% (October 2017) and 67% (January 2018) (Figure 2.3b), and the most dominant cyanobacterium was *Raphidiopsis raciborskii* (Figure 2.4). 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 with the phytoplankton taxa identified in Itupararanga reservoir by microscopy analysis is presented in Table A.4.

Figure 2.4 - Relative biovolume (%) of cyanobacterial genera identified in Itupararanga reservoir

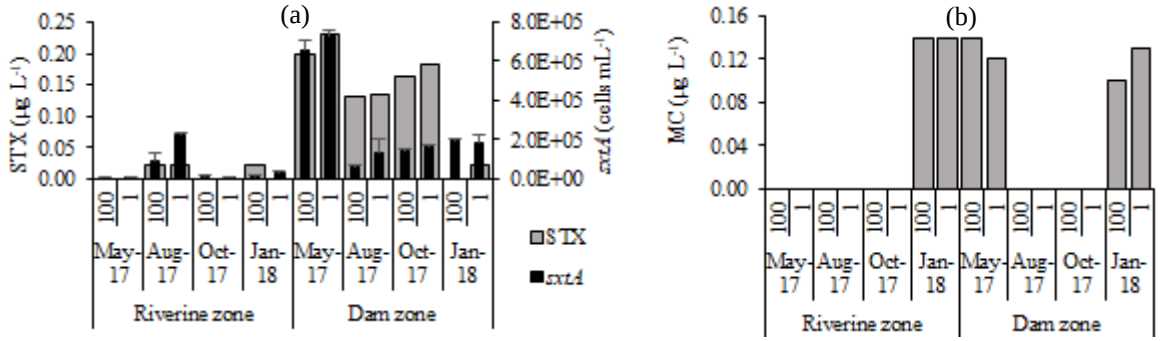


Source: author. Others = *Aphanothece*, *Chroococcus*, *Cuspidothrix*, *Merismopedia*, *Microcystis*, *Phormidium*, *Glaucospira* and *Limnococcus*; 100 = surface; 1 = lower limit of euphotic zone.

3.4 Cyanotoxins

The qPCR analysis confirmed the presence of *sxtA* genotypes in all samples, however, a spatial variation occurred ($p = 0.009$). The highest and lowest number of *sxtA* gene were observed in the dam zone in May 2017 (7.33×10^5 cells mL^{-1}) and in the riverine zone in May 2017 (6.76×10^3 cells mL^{-1}), respectively. Saxitoxin concentrations were also significantly higher in the dam zone than in the riverine zone ($p = 0.012$), and correlated positively with the *sxtA* gene (rho = 0.70, $p = 0.002$) (Figure 2.5a).

Figure 2.5 - (a) Number of *sxtA* gene (cells mL^{-1}) and saxitoxin concentration (STX, µg L^{-1}) and (b) microcystin concentration (MC, µg L^{-1}) in samples from two field stations, in four months and two depths in Itupararanga reservoir. Error bars provide standard deviations of triplicate samples



Source: author. 100 = surface; 1 = lower limit of euphotic zone.

Detection of total MC by ELISA showed average concentrations in the dam zone in May 2017 of 0.13 µg L^{-1}, and of 0.14 µg L^{-1} and 0.11 µg L^{-1} in the riverine and dam zones in January 2018, respectively, while no MC was detected in the other months (Figure 2.5b). MC concentrations did not vary significantly between sampling months, depths and sites ($p > 0.05$).

Potentially saxitoxin- and microcystin-producing genera were detected by microscopic analysis in all samples. Saxitoxin concentrations and *sxtA* gene were positively correlated with the biovolume of *Raphidiopsis*, *Geitlerinema* and *Aphanizomenon*, while *Dolichospermum* was

negatively correlated with STX (Table 2.2). Regarding to MC, the Spearman's rank order correlation tests did not show any significant correlations between the potentially microcystin-producing genera and the toxin concentrations throughout the study period ($p > 0.05$).

Table 2.2 - Spearman rank correlation coefficients between 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 highlighted 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**

Source: author.

3.5 Linkage between environmental variables, cyanobacteria and cyanotoxins

Correlation analyses suggested different variables influencing *sxtA* gene occurrence and STX production in Itupararanga reservoir (n = 16, Table 2.3). STX concentration and *sxtA* gene were positively correlated with cyanobacterial biomass and TN:TP ratio, and negatively correlated with turbidity, total phosphorus and soluble reactive phosphorus. STX was positively correlated with pH, and *sxtA* gene was negatively correlated with nitrate. None of the measured variables succeeded in explaining the microcystin production in the reservoir ($p > 0.05$).

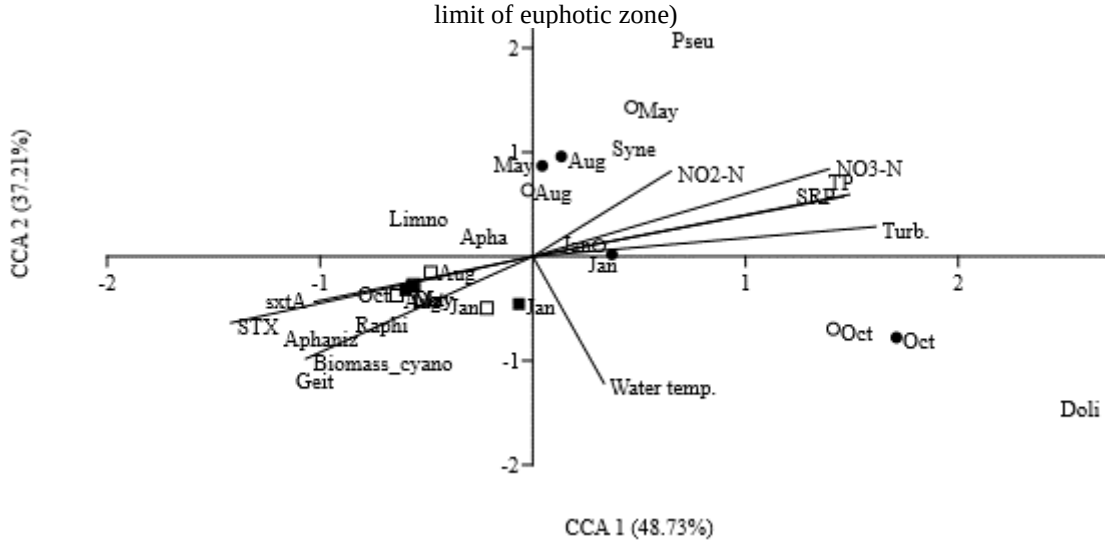
Table 2.3 - Spearman rank correlation coefficients between environmental variables and *sxtA* gene, saxitoxin (STX) and microcystin (MC) concentrations in Itupararanga reservoir (n = 16). Significant correlations ($p < 0.05$) are highlighted in bold

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

Source: author. 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 the separation of the sampling sites into two groups (riverine and dam zones), considering the biovolume data of cyanobacterial genera and environmental variables. 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 2.6).

Figure 2.6 - Canonical correspondence analysis (CCA) biplot of cyanobacteria biovolume and environmental variables in Itupararanga reservoir. The periods of sampling correspond to May (May 2017), Aug (August 2017), Oct (October 2017) and Jan (January 2018); 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)



Source: author. Water temp. = water temperature; Biomass_cyano = cyanobacterial biomass determined by pigment analysis; STX = saxitoxin; NO_2-N = nitrite; NO_3-N = nitrate; 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 biovolume, STX concentration and *sxtA* gene number 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 phosphorus, soluble reactive phosphorus and nitrate associated with the samples from riverine zone in October 2017 and January 2018. Nutrient concentrations were negatively correlated with STX, *sxtA*, *Raphidiopsis*, *Aphanizomenon* and *Geitlerinema*, that is, saxitoxin production was higher when nutrients were low. Biovolume of *Synechocystis* and *Pseudanabaena* were correlated with 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 2.6).

4 Discussion

4.1 Efficiency and specificity of the qPCR assay

Techniques that allow the rapid and reliable detection of potentially cyanotoxin-producing cyanobacteria in the environment can be extremely valuable. In the present study, a

SYBR Green based qPCR assay was developed for the quantification of *sxtA* gene (starting gene of saxitoxin-synthesis in cyanobacteria) in environmental samples from Itupararanga reservoir. Validation of the method by primer specificity, melting curve profiles and existence of only one amplicon clearly demonstrated the high sensitivity of the primer set, for both culture and environmental samples (Figure A.1).

Since it is very common to find several taxa of saxitoxin producing cyanobacteria in environmental samples (Fonseca et al., 2015; Lopes et al., 2015; Lu et al., 2019), high coverage primer set is required for their detection. The *sxtA*-cyano primer set was demonstrated to be specific to the *sxtA* gene allowing the detection of various cyanobacterial strains known as potentially saxitoxin-producers as demonstrated by the PCR and *in silico* PCR (Table A.3). To the best of our knowledge, the present study is the first report on *sxtA* gene quantification in environmental samples using qPCR assay in a Brazilian water body.

4.2 qPCR assay, cyanotoxin and cyanobacteria

Few studies have applied the qPCR for quantification of potentially saxitoxin-producing cyanobacteria in environmental samples by targeting the *sxtA* gene (Al-Tebrineh et al., 2010; 2012; Kramer et al., 2018; Chaffin et al., 2019). In most of them, the *sxtA* gene abundance obtained by qPCR correlated positively with the saxitoxin concentrations, as also observed in this study. However, no saxitoxin was measured in 6 samples from Itupararanga reservoir despite the *sxtA* gene were detected. There are three possible reasons for the differences between the *sxtA* gene and cyanotoxin concentrations. First, the *sxt* gene expression may be down-regulated resulting in a situation where the gene is detected but STX is not produced (Savela et al., 2015). Several studies have reported the presence of *sxt* genes in potentially STX-producing strains as *Dolichospermum*, *Anabaenopsis* and *Aphanizomenon* without apparent toxin production (Ballot et al., 2010; Ledreux et al., 2010; Casero et al., 2014; Cirés et al., 2014). Secondly, qPCR detected a small population of *sxtA*-containing cells that did not produce sufficient saxitoxin to be detected by ELISA. Besides, low levels of STX can be very difficult to quantify in environmental samples due to interfering compounds in the samples. Lastly, no STX was detected due to the toxin degradation in these samples. Nevertheless, the detection of *sxtA* gene in the samples from Itupararanga reservoir indicates that at least 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}. Al-Tebrineh et al. (2010), in a qPCR assay developed to quantify the saxitoxin-producing *Anabaena circinalis* in Australian water bodies, determined a high number

of *sxtA* gene in a eutrophic dam in New South Wales (2.91 x 10^9 copies mL^{-1}) associated with saxitoxin production. The same authors reported that STX was not detectable in the samples until *sxtA* gene exceeded 7.61 x 10^3 copies mL^{-1}. In this study, STX production was detected when *sxtA* gene exceeded 1.47 x 10^4 cells mL^{-1}. However, in two samples from January 2018 in the dam zone (surface) and in the riverine zone (lower limit of euphotic zone), no STX was detected even with a high number of *sxtA* gene (2.04 x 10^5 and 3.04 x 10^4 cells mL^{-1}, respectively), showing that qPCR cell densities may not be a good parameter to infer the toxin concentrations in the water body.

Spearman's rank order correlation analysis and CCA showed significant and positive correlations between the saxitoxin concentrations and *sxtA* gene numbers with the biovolume of *Raphidiopsis*, *Aphanizomenon* and *Geitlerinema*. With this result we can consider them as potentially saxitoxin-producing cyanobacteria in Itupararanga reservoir. However, it cannot be affirmed which cyanobacterial genera found in the water body were in fact responsible for the STX production, since the *sxtA* gene was detected in environmental samples that might have contained many other species, including picoplanktonic cyanobacteria, that could not be observed with the inverted microscope. To solve this problem and link a 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 (Moreira et al., 2014). Irrespective of applied approach, the cyanotoxins were detected by ELISA method in Itupararanga reservoir, indicating that one or more cyanobacterial strains were producing such metabolites.

4.3 Environmental variables and cyanobacteria

Several environmental factors, such as rainfall, availability of nutrients and water temperature, may play a role in the population structure of cyanobacteria, and in presence of toxic strains and their cyanotoxin production (Rantala et al., 2006; Fortin et al., 2010). In the present study, biovolume of *Raphidiopsis*, *Aphanizomenon* and *Geitlerinema* was higher in the dam zone and correlated with the lowest nutrient concentrations and highest TN:TP ratios. These results suggest that these genera appear to be better adapted to low-P nutrient environments. Similar results were reported by Casali et al. (2017) and Vargas et al. (2019) in studies investigating the STX production in *Raphidiopsis raciborskii*.

According to Kenesi et al. (2009), *R. raciborskii* is capable of growing faster and maintaining high biomass in phosphorus-limited environments, when inorganic nitrogen forms are available. In the dam zone, although the concentrations of phosphorus were low (<15.19 µg L^{-1}), nitrogen was available in nitrate form (0.32-0.42 mg 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 (Nalewajko and Murphy, 2001; Elliot et al., 2006; Qian et al., 2019).

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 if the dinoflagellate *Ceratium furcoides* outcompeted cyanobacteria in the reservoir. *C. furcoides* has been found in many Brazilian freshwater systems (Cavalcante et al. 2013; Jati et al. 2014; Moreira et al. 2015; Campanelli et al. 2017) and in the Itupararanga reservoir since 2010 (CETESB, 2011). Yet, few studies have dealt with the influence of *C. furcoides* on cyanobacterial population dynamics (Matsumura-Tundisi et al., 2010; Crossetti et al., 2018). 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 (Crossetti et al., 2018), and cause oscillation and reduction of cyanobacterial blooms in an urban lake (Silva et al., 2019). 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.

4.4 Environmental variables and cyanotoxins

Microcystin concentrations in the reservoir were generally low (max. 0.14 µg L^{-1}) and within the limit acceptable for the MC content in drinking water (≤1.00 µg L^{-1}) (Brasil, 2011), as recommended by the World Health Organization (WHO, 2011). Numerous studies reported that microcystin production could vary with the salinity (Jia et al., 2018; Vergalli et al., 2020), temperature (Bui et al., 2018; Walls et al., 2018), light intensity (Wiedner et al., 2003; Xie et al., 2016; Wang et al., 2019), pH (Francy et al., 2016; Huang et al., 2019), and nutrients (Lee et al., 2015; Singh et al., 2015; Mariani et al., 2015; Barros et al., 2019; Brandenburg et al., 2020). However, no significant correlations between environmental variables, MC

concentrations and potentially toxic genera were found in this study. Pimentel and Giani (2013) also did not find any correlations between environmental variables and MC production in Furnas reservoir (Minas Gerais, Brazil), and highlighted the necessity of further studies to understand the factors responsible for its occurrence in particular environmental conditions.

Saxitoxin concentrations and the number of *sxtA* gene were higher in the dam zone, negatively correlated with nutrients (TP, SRP and NO_3-N) and directly correlated with TN:TP ratio. The influence of nitrogen concentrations on saxitoxin production is controversial. According to Brentano et al. (2016), high STX concentrations in the aquatic system can be a result of increased dissolved inorganic nitrogen (DIN) which favor the growth of *R. raciborskii* population or improve cellular homeostasis due to a reduced metabolic demand, since the uptake of inorganic nitrogen requires less energy than N_2-fixation. On the other hand, Yunes et al. (2009), in an experimental investigation on saxitoxin production by *R. brookii*, observed a lower level of STX with increased concentrations of nitrogen. Therefore, further studies are still needed to provide a clear view about the influence of nitrogen on STX production.

Regarding phosphorus (P), an increase in saxitoxin production in samples with P-limitation was observed. In a study on the saxitoxin-producing dinoflagellate *Alexandrium* sp., Granéli and Flynn (2006) reported that the STX production in environments with low concentrations of phosphorus may indicate an adaptation to nutrient limitation. Frangópulos et al. (2004) stated that one of possible advantages of toxin production under P-limitation is to enhance interspecific competition by redirecting grazing pressure to non-toxic species. Vargas et al. (2019), in a laboratory investigation on STX production by *R. raciborskii* under different conditions of nutrients, reported that STX synthesis was higher under oligotrophic condition indicating a survivor strategy of the cyanobacteria and adaptation in the phosphorus-limited environment.

The saxitoxin production was higher in samples with high values of pH in the Itupararanga reservoir. In a study on pH effects on STX production it was observed that the intracellular STX concentrations increased in response to a rising pH, with the highest rate for pH > 9 (Pomati et al., 2004). According to these authors, STX plays a role in the maintenance of cyanobacteria homeostasis under alkaline pH and Na^+ stress conditions, which would confer an advantage to the STX producing cyanobacteria over the non-toxic cyanobacteria in environments with high values of pH or salt concentrations. These results indicate that STX production may be an adaptation to non-ideal conditions as reported by other studies (Holland and Kinnear, 2013; Casali et al., 2017; Vargas et al., 2019). The same authors stated that the toxin synthesis is related to the physiological and competitive advantages.

In the samples presenting turbidity values higher than 10 NTU, no saxitoxin was detected and the lowest numbers of *sxtA* gene were recorded, showing that potential saxitoxin-producing cyanobacteria (e.g., *Raphidiopsis*) may grow better in environments with low turbidity and high-water transparency. No data was found in literature regarding the effects of turbidity on STX production to confirm the results above. Some studies had reported a positive and significant correlation between MC production, *Microcystis* abundance, high values of turbidity, low water transparency and high concentrations of total suspended solids (Graham et al., 2004; Te and Gin, 2011; Cunha et al., 2018). However, *Microcystis* and *Raphidiopsis* present different environmental preferences as observed by Soares et al. (2013) and Guedes et al. (2018). Thus, it is not possible to use the finding on turbidity and MC to explain the occurrence of STX. Further studies are needed to better understand the role of turbidity on saxitoxin production.

4.5 Pigment analysis and qPCR assay as a tool for monitoring of toxic cyanobacteria

Deterioration of the water quality has caused increased occurrence of cyanobacteria and cyanotoxins in several reservoirs used for water supply, recreational and fisheries activities. Considering the potential risk to human health, since elevated exposure to cyanotoxin through consumption of contaminated drinking water or contaminated fish might be fatal (Christensen and Khan, 2020), reliable and fast methods that can specifically quantify toxin-producing cyanobacteria in the environment are needed.

In Itupararanga reservoir, the strong and significant positive correlation between cyanobacterial biomass and the number of *sxtA* gene demonstrated that pigment analysis associated with qPCR may be a useful tool to identify the risk of potentially toxic cyanobacteria occurrence in subtropical reservoirs. Similar results were observed by Schlüter et al. (2018) in a study of Brazilian reservoirs with aquaculture production and detection of microcystin. The authors stated that pigment analysis can be used to provide fast and reliable results for the early warning, the presence and potential risk of toxic cyanobacteria in freshwater reservoirs.

The biomass of phytoplankton groups determined by the pigment analysis correlated significantly with the biovolume 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, including the pico-sized cells that cannot be identified using standard microscopy methods, are included by the pigment method. Another advantage is that rare species in low densities can be identified by the pigment method but may be overlooked by

microscopy (Zapata, 2005; Schlüter et al., 2016; 2018). Despite these advantages, the pigment analysis will only detect phytoplankton groups, 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 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 (2008) 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 less equipment and reagents than the qPCR technique, the analyst's labor costs make 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 dam zone of Itupararanga reservoir was mainly characterized by the occurrence of saxitoxin-producing cyanobacteria, and the genera *Raphidiopsis*, *Aphanizomenon* and *Geitlerinema* were considered as the potential saxitoxin-producers in this ecosystem. The TN:TP ratios suggested that phosphorus plays an important role in the STX concentration, and high TN:TP ratios sustain STX production. These outcomes provide insights into effects of nutrients on toxin production contributing to the water resources management.

In this study, the qPCR results showed that the newly designed primer set *sxtA*-cyano enabled the specific detection and quantification of potentially saxitoxin-producing cyanobacteria in mixed phytoplankton communities in natural waters, with good sensitivity and over a broad quantification range. The characteristics cited above demonstrates that the qPCR method is a powerful tool to assist in monitoring and can be implemented in routine analysis. Therefore, the developed qPCR assay, associated with other methodologies as pigment analysis, will be useful for both managers of subtropical aquatic environments to identify the risk of toxic cyanobacteria and scientists to further study dynamics of toxic cyanobacteria.

Although records of cyanobacteria blooms and the occurrence of cyanotoxins are very common in Brazilian lakes and reservoirs, this study was the first one applying qPCR in a Brazilian water body to quantify the potentially saxitoxin-producing cyanobacteria.

Considering that the literature applying qPCR to monitor STX producing cyanobacteria in the environment is scarce, further studies are needed to evaluate the applicability of this method in different freshwater environments.

Acknowledgements

This study was financially supported by FAPESP - São Paulo Research Foundation (2016/09405-1). The first author thanks FAPESP for the doctorate and research internship abroad scholarships (2015/21191-4 and 2018/00394-2). We are very grateful to Professor Sandra Maria Feliciano de Oliveira e Azevedo from Federal University of Rio de Janeiro (UFRJ) for providing *Raphidiopsis* strains and to Professor Inessa Lacativa Bagatini from Federal University of São Carlos (UFSCar) for providing *Microcystis* strain that were critical for this study.

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CHAPTER 3 - ESTIMATING HEPATO- AND NEUROTOXIGENIC CYANOBACTERIA IN A SUBTROPICAL RESERVOIR BY QUANTITATIVE PCR

Abstract

Cyanobacteria and associated toxins are a major global water-quality issue, posing risk to public health since cyanotoxins can contaminate drinking and recreational waters. Between May 2017 and January 2018, we examined the cyanobacterial community in Lobo reservoir for abundance of *mcyE* and *sxtA* genes and quantified the MC and STX concentrations in the samples aiming to investigate the spatiotemporal dynamics of potentially toxin-producing cyanobacteria, as well as to gain insight into the environmental drivers associated with microcystin and saxitoxin occurrence. The *mcyE* gene varied from undetectable to 4.25×10^3 copies mL^{-1}, while *sxtA* gene was detected in all water samples (8.79×10^1 to 2.34×10^4 cells mL^{-1}). Microscope analysis confirmed the presence of potentially toxin-producing cyanobacteria. The *mcyE* and *sxtA* genes, as well as the toxins concentrations were associated with biovolume of *Geitlerinema*, *Phormidium* and *Raphidiopsis*. MC and STX concentrations were generally low, but in May 2017 the total MC concentrations were above 1.00 µg L^{-1} even with low cyanobacterial biovolume, i.e., the absence of a bloom in the water body did not indicate the absence of risk. MC and STX production were influenced by more than one environmental variable suggesting that their production is under multiple regulation, and was associated with stressful conditions, such as the nutrient limitation. The qPCR results showed that this molecular method can be used to estimate the potentially toxin-producing cyanobacteria in environmental samples and contribute to a reliable and fast risk evaluation protecting water users from hazards associated with toxic-cyanobacteria occurrence.

Keywords: Lobo reservoir; saxitoxin; *sxtA*; microcystin; *mcyE*; qPCR

1 Introduction

Occurrences of cyanobacteria and cyanotoxin production are becoming more common in freshwater systems causing many water qualities issues and posing risks to human and animal health (Zegura et al., 2011; Paerl, 2018; Facey et al., 2019; Subbiah et al., 2019). Monitoring and adequate management of cyanobacteria occurrence are essential steps to minimize their negative effects, particularly in freshwater systems used as a source for drinking water and for recreation. Efforts have been made to identify the drivers of cyanobacterial blooms. Ordinarily, eutrophication and climate changes have been considered the factors that play the most

significant role in promoting cyanobacteria dominance (Kosten et al., 2012; Rigosi et al., 2014; Cha et al., 2017). However, the role of environmental factors on the regulation of toxin production is still unclear.

Lobo is a subtropical reservoir located in São Paulo State, Brazil. The main uses of water in the reservoir are generation of hydroelectric energy, local public supply, irrigation of plantations, and recreation, contributing to the social and economic development of the surrounding area (Periotto and Tundisi, 2013). The reservoir was selected for an ecological research programme in 1971 (Tundisi and Matsumura-Tundisi, 2013). Since then, several studies were carried out in the system with different approaches and objectives. Regarding cyanobacteria and cyanotoxins, only one bloom caused by *Raphidiopsis raciborskii* (basionym *Cylindrospermopsis raciborskii*) (Aguilera et al., 2018) was reported in the system in July 2014 (Tundisi et al., 2015). Saxitoxin (STX) and microcystin (MC) productions were observed at the same time. Although STX and MC were detected in low levels, the authors suggested that a higher potential of cyanotoxin production may be detected in the future because the reservoir is experiencing a water quality deterioration due to the release of high loads of nutrients into the system.

Among the cyanotoxins produced by cyanobacteria, microcystin (hepatotoxin) and saxitoxin (neurotoxin) are the most reported worldwide (Merel et al., 2013). Microcystin and saxitoxin are synthesized by two gene groups known as *mcy* and *sxt*, respectively (Tillett et al., 2000; Rouhiainen et al., 2004; Kellmann et al., 2008; Mihali et al., 2009; Murray et al., 2011). The description of gene clusters responsible for cyanotoxins production led into the use of these genes as targets for polymerase chain reaction (PCR) and then quantitative polymerase chain reaction (qPCR). Thus, the quantification of potentially toxic cyanobacteria can be achieved by amplifying several genetic markers through qPCR allowing the differentiation between the toxic strains from the non-toxic strains, which would not be possible while considering only the morphology (Pacheco et al., 2016).

The *mcyE* gene plays an important role in the microcystin synthesis, encompassing the incorporation of the ADDA group (3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid) and D-Glu (D-glutamic acid) into the microcystin structure. The amino acids ADDA and D-Glu are consistent between microcystin variants and have a significant role in determining the toxicity of microcystins. Thus, the invariant nature of these amino acids makes the *mcyE* gene a reliable biomarker for detection of microcystin-producing cyanobacteria (Ngwa et al., 2014). Regarding saxitoxin production, the *sxtA* gene is responsible for the initiation of STX biosynthesis and appears to be carried by all saxitoxin-producing

cyanobacteria, catalyzing the incorporation of acetate to the enzyme complex and its subsequent methylation and Claisen condensation with arginine (Kellmann et al., 2008; Mihali et al. 2009; Murray et al., 2011). For the reasons stated above, *mcyE* and *sxtA* genes were chosen for detection of toxin-producing cyanobacteria in the environmental samples.

Cyanobacterial genera with potential for production of cyanotoxins have previously been identified in the phytoplankton community in Lobo reservoir (Tundisi et al., 2015 Marafão, 2016; Vicentin et al., 2018; Rodrigues et al., 2019) but molecular techniques for detection and quantification of microcystin- and saxitoxin-producing cyanobacteria in the aquatic system have not been applied. Therefore, we examined the cyanobacterial community in the reservoir between May 2017 and January 2018 for abundance of the *mcyE* and *sxtA* genes and quantified the MC and STX concentrations in the samples aiming to investigate the spatiotemporal dynamics of potentially toxin-producing cyanobacteria in the Lobo reservoir, as well as to gain insight into the environmental drivers associated with MC and STX occurrence.

2 Material and methods

2.1 Study sites and sampling

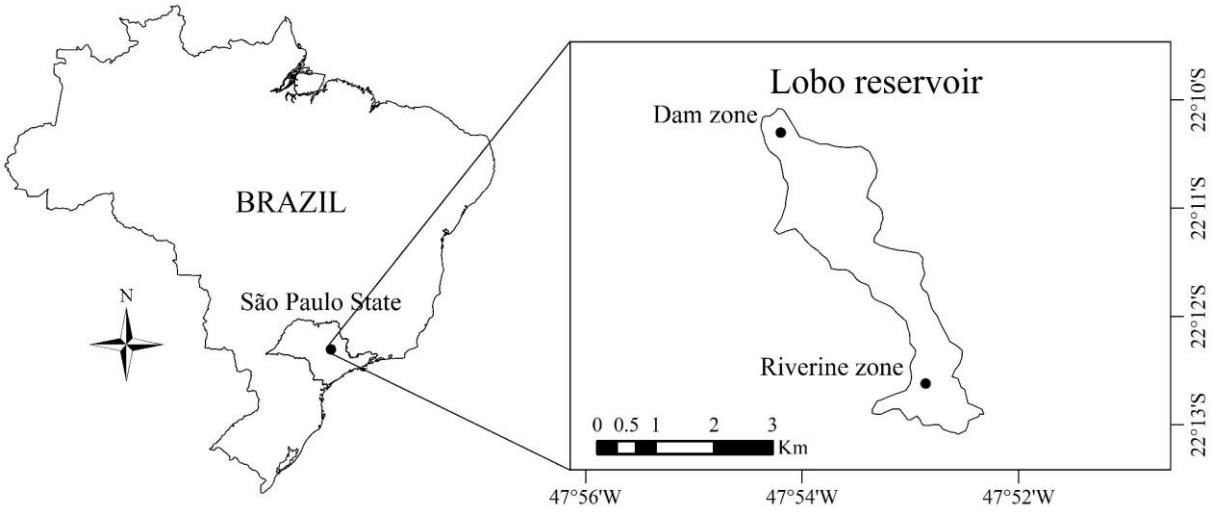
Water samples were collected at riverine and dam zones in the Lobo reservoir located in São Paulo State, Brazil (Figure 3.1). The main characteristics of the reservoir and sampling sites are shown in the Table B.1. Samplings were carried out in May, August, October 2017 and January 2018 at two depths (100% = surface; 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, USA).

2.2 Physicochemical variables

The profiles of 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, 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. Water samples were filtered through 0.7 µm-pore-size glass fiber filters (Whatman, GE Healthcare Life Sciences, USA). From the filters, chlorophyll *a* (chl *a*) was extracted with ethanol 80% (v/v) and the extracts were analyzed as described by Nusch (1980), and the filtered volume was kept frozen at -20 ºC until analysis of dissolved nutrients (soluble reactive phosphorus - SRP, nitrate - NO_3^--N, nitrite - NO_2^--N and ammonium - NH_4^+-N). Water samples for total nutrients

(phosphorus - TP and nitrogen - TN) were collected and stored at -20 ºC until analysis. The nutrients analyses were carried out using spectrophotometric methods in triplicates (APHA, 2005). The trophic state index (TSI) of the sampling sites (riverine and dam zones) was calculated considering the annual geometric means of chl *a* and TP (Cunha et al., 2013).

Figure 3.1 - Sampling sites in Lobo reservoir, São Paulo State, Brazil. Lobo riverine zone (22°12'36.9" S, 47°52'52.8" W); Lobo dam zone (22°10'18.5" S, 47°54'11.2" W)



Source: author.

2.3 Microscopic identification and enumeration

For phytoplankton analysis, samples were Lugol-fixed for subsequent identification and quantification in sedimentation chambers using an inverted microscope (Olympus CK2) at 400x magnification following the Utermöhl (1958) method. Phytoplankton taxa were identified according to specialized literature. A counting limit was established through the species-rarefying curve and until reaching 100 individuals (cell, filament, colony and coenobium) of the most common species. Twenty organisms of each taxa with relative abundance >10% were measured to estimate the mean cell volume based on the geometric models (Hillebrand et al., 1999; Sun and Liu, 2003). For taxa with relative abundance <10%, mean cell volumes were obtained from specialized literature. Cell densities were converted to biovolume by multiplying cell densities by the average taxa-specific cell volume.

2.4 DNA extraction and quantitative real-time PCR

For DNA extraction, water samples were immediately filtered through 0.22 µm-pore-size mixed cellulose ester membranes (Whatman, GE Healthcare Life Sciences, USA) and the filters were kept frozen at -20 °C until processing. Total genomic DNA was extracted from the filters using the DNeasy PowerWater kit (QIAGEN, Hilden, Germany) according to the

manufacturer's instructions. DNA concentrations were quantified by a NanoDrop spectrophotometer, and the DNA purity was determined by the 260/280 ratio. The DNA quality was visualized in 1% agarose gel stained with GelRed™ Nucleic Acid Gel Stain (Biotium Inc., USA) using a gel documentation system (Bio-Rad Gel Doc™ 2000 Bio-Rad Laboratories, USA).

The quantification of *mcyE* gene was carried out using the CyanoDTec Toxin Gene kit (Phytoxigene, Inc., USA). This kit is a molecular test based on quantitative real-time PCR. The analysis was performed according to the manufacturer's instructions using an AriaMx Real-Time PCR System (Agilent Technologies, USA) in a total volume of 20 µL per reaction. A standard of known toxin gene copy was assayed in serial dilutions to generate a standard curve encompassing four orders of magnitude (100 - 100,000 copies per reaction) for the target toxin gene. Each sample was run in duplicate. Cycling was initiated with preheating at 95 ºC for 2 min, followed by 40 quantification cycles, each consisting of 15 s at 95 ºC and 45 s at 60 ºC. Fluorescence measurement of generated products was obtained at the end of each cycle at 60 ºC. Gene copies in each reaction were calculated using the AriaMx Software, version 1.3 (Agilent Technologies, USA) and back-calculated to copies mL^{-1}.

A qPCR assay for *sxtA* gene using SYBR® Green developed by Moraes et al. (chapter 2) was adopted for use in this study. The qPCR assay was performed using the primer set *sxtA*-cyano-F (5'-xxxxGAAGCGxxxxGTCTGG-3') and *sxtA*-cyano-R (5'-TCTGCCxxxxxxGAATACAC-3'). The qPCR product was a 153 bp fragment. The qPCR reactions were run in triplicate in an AriaMx Real-Time PCR System (Agilent Technologies, USA). The final volume for each qPCR reaction was 20 µL, containing 2 µL of template DNA from standard or sample, 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, USA) and 7.2 µL of nuclease free water (Sigma-Aldrich). The qPCR assays were performed under the following cycling conditions: preheating at 95 ºC for 3 min, followed by 40 quantification cycles, each consisting of 30 s at 95 ºC, 30 s at 65 ºC, 30 s at 72 ºC and 10 s at 77.5 ºC, and a melt cycle of 30 s at 95 ºC, 30 s at 65 ºC and 30 s at 95 ºC. Fluorescence measurement of generated products was obtained at the end of each cycle at 77.5 ºC. Data analysis was done by the AriaMx Software, version 1.3 (Agilent Technologies, USA).

2.5 Cyanotoxins analyses

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 (Beacon Analytical System Inc., 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, Austria) at 450 nm.

2.6 Statistical analysis

The data were tested for normality using Shapiro-Wilk test. Environmental variables were evaluated using principal component analysis (PCA) with a correlation matrix to characterize the sampling sites, months and depths and identify the variables that best differentiate them. Strong correlations were those with $r \geq 0.5$ with the ordination axes 1 or 2. The relationships between cyanotoxins genes, cyanotoxins production and environmental variables were assessed by Spearman's rank order correlations ($p < 0.05$). To obtain the significant factors that explained the occurrence of microcystin and saxitoxin in the reservoir, simple linear regression analysis was conducted. Environmental variables were log(x+1) transformed before analysis to meet the conditions of normality and homogeneity of variance in the residuals. Mann-Whitney test was carried out to evaluate the differences between sites (riverine and dam zones) and depths (100% and 1% of PAR) considering the environmental variables. Kruskal-Wallis test was conducted to evaluate the differences between months considering the environmental variables, and Dunn's *post hoc* multiple comparison test was performed if significant differences were observed in the Kruskal-Wallis test ($p < 0.05$). Data were analyzed using the STATISTICA version (13.5) (TIBCO Software Inc., Palo Alto, CA, USA) and PAST version (4.06) (Hammer et al., 2001).

3 Results

3.1 Environmental variables

The environmental variables pH, electrical conductivity (EC), dissolved oxygen (DO) and water temperature varied significantly between months ($p < 0.05$). Electrical conductivity, turbidity, total phosphorus, total nitrogen, nitrate and nitrite varied between months ($p < 0.05$) (Table 3.1). None of the environmental variables varied between depths ($p > 0.05$). The highest average pH value was observed in May 2017 (7.00±1.00) and the lowest in January 2018

(6.04±0.14). Regarding dissolved oxygen, the concentrations varied between 4.53 mg L^{-1} (January 2018) and 9.04 mg L^{-1} (May 2018). The highest mean value of EC was observed in January 2018 (30.00±3.74 µS cm^{-1}), while the lowest was observed in August 2017 (14.75±1.79 µS cm^{-1}). Turbidity was higher in the riverine zone (6.06±0.98 NTU) than in the dam zone (3.49±1.75 NTU), and the water transparency and Z_{eu} were lower in the riverine zone. The highest water temperature (24.21±0.93 °C) was observed in January 2018, while the lowest was observed in August 2017 (18.41±0.13).

Table 3.1 - 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 Lobo reservoir

Variables	Riverine zone (n = 8)	Dam zone (n = 8)	Spatial factor	Temporal factor
Water transparency (m)*	1.10 (1.00 - 1.20)	1.85 (1.20 - 2.30)	-	-
Z_{eu} (m)*	2.00 (2.00 - 2.00)	3.75 (2.50 - 5.00)	-	-
pH	6.59 (5.87 - 8.72)	6.38 (5.97 - 7.13)	ns	0.042
Dissolved oxygen (mg L^{-1})	6.27 (4.53 - 8.22)	6.67 (5.08 - 9.04)	ns	0.041
Conductivity (µS cm^{-1})	20.38 (15.00 - 36.00)	17.25 (13.00 - 28.00)	0.023	0.030
Turbidity (NTU)	6.06 (4.70 - 7.50)	3.49 (1.70 - 6.40)	0.014	ns
Water temperature (°C)	21.11 (18.21 - 23.80)	21.97 (18.38 - 25.00)	ns	0.005
Total phosphorus (µg L^{-1})	25.92 (19.75 - 37.85)	18.08 (14.48 - 22.74)	0.006	ns
Soluble reactive phosphorus (µg L^{-1})	2.36 (nd - 3.69)	1.28 (nd - 3.01)	ns	ns
Total nitrogen (mg L^{-1})	0.80 (0.62 - 0.92)	0.70 (0.53 - 0.89)	0.042	ns
Nitrate (mg L^{-1})	0.67 (0.59 - 0.75)	0.52 (0.41 - 0.57)	0.001	ns
Nitrite (µg L^{-1})	3.85 (1.79 - 4.96)	2.70 (1.62 - 3.94)	0.027	ns
Ammonium (µg L^{-1})	6.54 (nd - 18.55)	14.62 (nd - 28.34)	ns	ns
TN:TP	64 (30 - 100)	87 (42 - 124)	ns	ns
Chlorophyll *a* (µg L^{-1})	6.82 (3.95 - 8.88)	11.10 (3.95 - 27.30)	ns	ns
Trophic state	Mesotrophic	Mesotrophic	-	-

Source: author. *Water transparency and Z_{eu} (n = 4); TN:TP = total nitrogen and total phosphorus ratio; nd = not detected; ns = not significant.

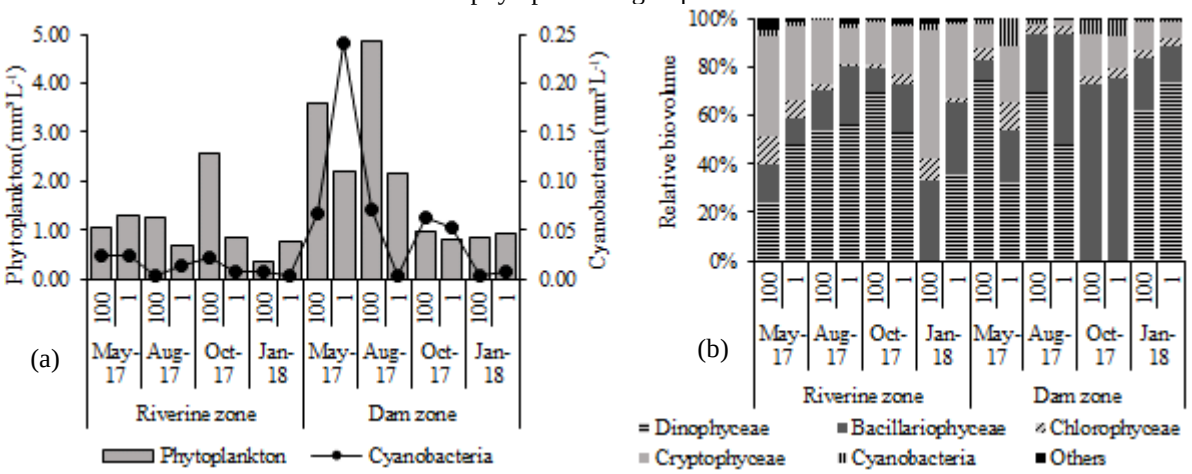
Total phosphorus (TP), soluble reactive phosphorus (SRP), total nitrogen (TN), nitrate (NO_3^--N) and nitrite (NO_2^--N) concentrations were higher in the riverine zone, which may be attributed to the discharge of untreated wastewater along the tributaries. Total phosphorus concentrations never exceeded 40.00 µg L^{-1}. The highest TP concentration was detected in October 2017 in the riverine zone (37.85 µg L^{-1}) and the lowest in August 2017 in the dam zone

(14.48 µg L^{-1}). Soluble reactive phosphorus and nitrite concentrations were always below 4.00 µg L^{-1} and 5.00 µg L^{-1}, respectively. In August and October 2017, mainly in the dam zone, SRP concentrations were frequently below the detection limit of the equipment (0.7 µg L^{-1}). Nitrate made up 83% of total nitrogen in the riverine zone and 74% in the dam zone. Ammonium concentrations ranged from undetectable in May 2017 in the riverine zone to a maximum of 28.34 µg L^{-1} in January 2018 in the dam zone. The average TN:TP ratio in the riverine zone was 64±22 and 87±29 in the dam zone, indicating a limitation by phosphorus in both sampling sites. Chlorophyll *a* (chl *a*) concentrations varied between 3.95 and 8.88 µg L^{-1} in the riverine zone, while in the dam zone the range was between 3.95 and 27.30 µg L^{-1}. However, the variation observed was not significantly different ($p > 0.05$). The trophic state index (TSI) classified both sampling sites as mesotrophic (Table 3.1).

3.2 Phytoplankton community

Cyanobacteria was not the dominant phytoplankton group in the Lobo reservoir (Figure 3.2a). The highest biovolume of cyanobacteria was observed in May 2017 in the dam zone (0.24 mm^3 L^{-1}) and constituted only 12% of all phytoplankton community. At the other sampling times, cyanobacteria made up less than 7% (Figure 3.2b).

Figure 3.2 - (a) Biovolume (mm^3 L^{-1}) of phytoplankton community and cyanobacteria group and (b) relative biovolume of phytoplankton groups in Lobo reservoir



Source: author. 100 = surface; 1 = lower limit of euphotic zone.

Fourteen species of cyanobacteria belonging to 10 genera were identified. Although in low proportion, potential microcystin- and saxitoxin-producing organisms were detected in all samples by microscopic analysis, belonging to the genera *Aphanizomenon*, *Aphanocapsa*, *Chroococcus*, *Geitlerinema*, *Microcystis*, *Phormidium*, *Pseudanabaena*, *Raphidiopsis* and *Synechocystis* (Table 3.2). Dinophyceae and Cryptophyceae were the most abundant groups of

phytoplankton in the Lobo reservoir, followed by Bacillariophyceae and Chlorophyceae (Figure 3.2b). A list with the phytoplankton taxa identified in Lobo reservoir by microscopy analysis is presented in Table B.2.

Table 3.2 - Biovolume ($mm^3\ L^{-1}$) of cyanobacterial genera identified in Lobo reservoir (riverine and dam zones) summarised as the mean values and ranges, and their average proportions (%) in total cyanobacterial biovolume

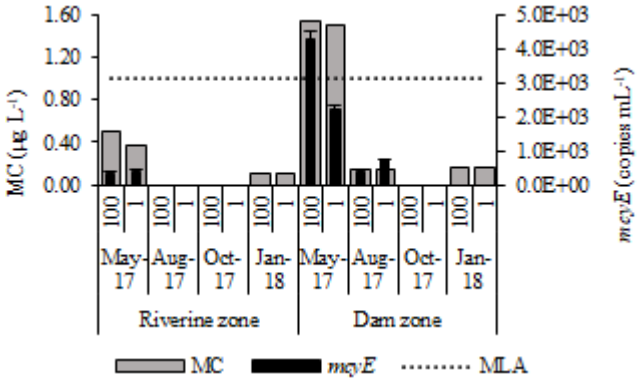
Genera	Riverine zone		Dam zone	
	Biovolume	%	Biovolume	%
Anathece	0.002 (nd - 0.011)	16.30	0.001 (nd - 0.004)	1.94
Aphanizomenon	0.002 (nd - 0.010)	17.00	0.002 (nd - 0.016)	3.19
Aphanocapsa	0.003 (0.001 - 0.009)	23.67	0.002 (nd - 0.004)	2.95
Chroococcus	0.001 (nd - 0.001)	3.11	0.001 (nd - 0.003)	1.64
Geitlerinema	0.001 (nd - 0.003)	3.23	0.006 (nd - 0.050)	9.96
Microcystis	nd	0.00	0.013 (nd - 0.052)	19.89
Phormidium	0.000 (nd - 0.002)	1.89	0.001 (nd - 0.004)	1.17
Pseudanabaena	0.001 (nd - 0.002)	6.61	0.001 (nd - 0.005)	2.10
Raphidiopsis	0.003 (nd - 0.018)	22.17	0.035 (nd - 0.163)	55.30
Synechocystis	0.001 (nd - 0.001)	6.02	0.001 (nd - 0.003)	1.86

Source: author. nd = not detected.

3.3 Cyanotoxins

The qPCR assay confirmed the presence of *mcyE* genotypes in Lobo reservoir in May 2017 (riverine and dam zones) and in August 2017 (dam zone). The number of *mcyE* gene ranged from undetectable to 4.25×10^3 copies mL^{-1}. The total MC concentrations ranged from undetectable to 1.54 µg L^{-1}. Microcystin concentrations correlated positively with the *mcyE* gene (rho = 0.75, $p < 0.001$) (Figure 3.3). Spearman's rank order correlation tests showed that MC production in Lobo reservoir was associated with the biovolume of *Geitlerinema* (rho = 0.55, p = 0.026) and *Phormidium* (rho = 0.66, p = 0.005). Although no correlation was observed between concentrations of microcystin and the biovolume of *Aphanocapsa* and *Synechocystis*, the possibility that these genera were also producing MC in the Lobo reservoir should not be ruled out.

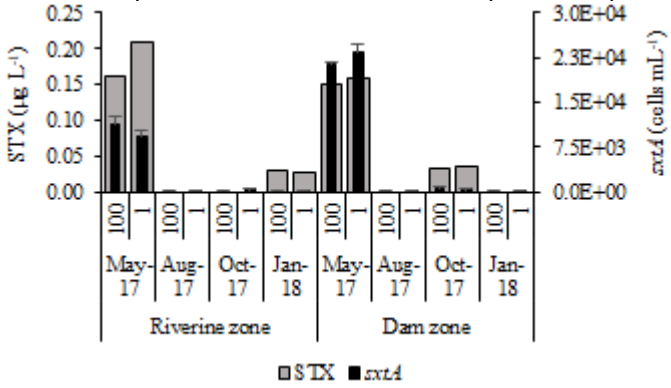
Figure 3.3 - Number of *mcyE* gene (copies mL^{-1}) and microcystin concentration (MC, µg L^{-1}) in Lobo reservoir. Error bars provide standard deviations of triplicate samples



Source: author. MLA (dotted line) = maximum limit of total MC allowed for human consumption in Brazil (Ordinance 2914/2011); 100 = surface; 1 = lower limit of euphotic zone.

Results from qPCR confirmed presence of the *sxtA* genotype in all water samples but a temporal variation occurred ($p = 0.017$). The number of *sxtA* gene in the Lobo reservoir during the sampling period ranged from 8.79×10^1 to 2.34×10^4 cells mL^{-1}. The highest and the lowest values were detected in May 2017 in the dam zone and in January 2018 in the riverine zone, respectively (Figure 3.4).

Figure 3.4 - Number of *sxtA* gene (cells mL^{-1}) and saxitoxin concentration (STX, µg L^{-1}) in Lobo reservoir. Error bars provide standard deviations of triplicate samples



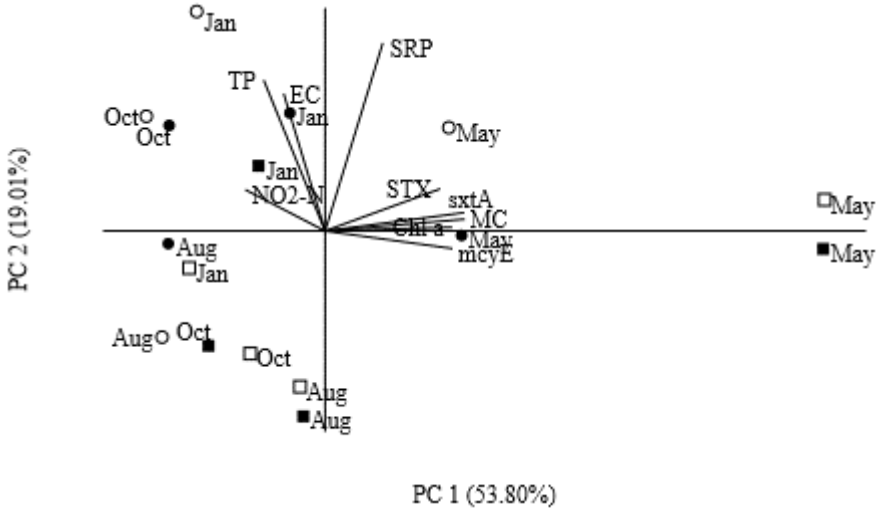
Source: author. 100 = surface; 1 = lower limit of euphotic zone.

Saxitoxin (STX) concentrations varied significantly between months ($p = 0.022$). When detected in the water samples, the total STX concentrations varied between 0.03 and 0.21 µg L^{-1}, and correlated positively with the *sxtA* gene abundance (rho = 0.74, $p < 0.001$) (Figure 3.4). Spearman's rank order correlation tests showed that STX production in Lobo reservoir was associated with the biovolume of *Phormidium* (rho = 0.64, $p = 0.008$) and *Raphidiopsis* (rho = 0.58, $p = 0.02$). Although no correlation was observed between concentrations of STX and the biovolume of *Geitlerinema* and *Synechocystis*, the possibility that these genera were also responsible for the saxitoxin production in the Lobo reservoir should not be ruled out.

3.4 Relationship between environmental variables, cyanobacteria and cyanotoxins

The principal component analysis (PCA) promoted a reduction from the 19 initial environmental variables to 9, accounting for 72.81% of the total variance in the two first axes (PC 1 = 53.80% and PC 2 = 19.01%). On the positive side of PC 1, microcystin, *mcyE* gene, saxitoxin, *sxtA* gene and chlorophyll *a* showed a strong and positive correlation with the axis associated with the samples from May 2017. Nutrient concentrations (TP and NO_2-N) and conductivity were related to October 2017 and January 2018, mainly in the riverine zone, and negatively correlated with STX, *sxtA*, MC, *mcyE* and chl *a* (Figure 3.5). Spearman's rank order correlation between PC 1 axis and cyanobacterial biovolume showed a moderate correlation (rho = 0.56; p = 0.02), indicating that cyanobacterial biovolume might be related to STX, *sxtA*, MC, *mcyE* and chl *a*.

Figure 3.5 - Ordination biplot by Principal Component Analysis (PCA) of the sample units generated from nine environmental variables in Lobo reservoir. The periods of sampling correspond to May (May 2017), Aug (August 2017), Oct (October 2017) and Jan (January 2018); 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)



Source: author. NO_2-N = nitrite; TP = total phosphorus; EC = electrical conductivity; SRP = soluble reactive phosphorus; STX = saxitoxin; MC = microcystin; Chl *a* = chlorophyll *a*.

Correlation analyses (n = 16, Table 3.3) suggested different variables influencing *mcyE* and *sxtA* genotypes and MC and STX production in Lobo reservoir. Microcystin concentrations and *mcyE* gene were positively correlated with chlorophyll *a* and negatively correlated with nitrite. The *mcyE* gene was negatively correlated with electrical conductivity and total phosphorus. Saxitoxin concentrations and *sxtA* gene were positively correlated with cyanobacterial biovolume. STX was positively correlated with soluble reactive phosphorus and *sxtA* gene was negatively correlated with nitrite.

Table 3.3 - Spearman rank correlation coefficients between environmental variables and microcystin (MC), *mcyE* gene, saxitoxin (STX) and *sxtA* gene in Lobo reservoir (n = 16). Significant correlations ($p < 0.05$) are highlighted in bold

Variables	MC	*mcyE*	STX	*sxtA*
Conductivity	-0.11	**-0.62**	-0.13	-0.40
Nitrite	**-0.62**	**-0.51**	-0.37	**-0.52**
Total phosphorus	-0.36	**-0.65**	-0.27	-0.36
Soluble reactive phosphorus	0.33	0.14	**0.51**	0.28
Chlorophyll *a*	**0.71**	**0.75**	0.20	0.21
Cyanobacterial biovolume	0.32	0.49	**0.55**	**0.69**

Source: author.

Linear regression analysis suggested an acceptable estimate of total microcystin by both chlorophyll *a* ($R^2 = 0.80$, $p < 0.001$) and *mcyE* gene ($R^2 = 0.86$, $p < 0.001$). Regarding saxitoxin, the best predictor was the *sxtA* gene ($R^2 = 0.68$, $p < 0.001$). Even though a moderate correlation was observed between cyanobacterial biovolume and saxitoxin, regression analysis indicated that cyanobacterial biovolume is not a good fit for the prediction of total SXT (Table 3.4).

Table 3.4 - Linear regression of total microcystin and saxitoxin to other measured variables (n = 16)

Variables	Microcystin		Saxitoxin	
	R^2 adj	p value	R^2 adj	p value
Chlorophyll *a*	0.80	<0.001	-	-
mcyE	0.86	<0.001	-	-
Cyanobacterial biovolume	-	-	0.23	0.04
sxtA	-	-	0.68	<0.001

Source: author.

4 Discussion

4.1 Cyanobacteria and cyanotoxins

Only one cyanobacterial bloom has previously been observed in Lobo reservoir to date (Tundisi et al., 2015). The bloom occurred in July 2014 and was dominated by *Raphidiopsis raciborskii*. During this episode, the authors reported the detection of low levels of cyanotoxins in the reservoir and stated the importance of the water quality monitoring, considering future scenarios with an increase in cyanobacteria biomass and cyanotoxins production, since the reservoir is in a continuous process of enrichment by phosphorus and nitrogen. In the present

study, the phytoplankton community in Lobo reservoir was mainly characterized by the presence of dinoflagellates, diatoms, cryptophytes and chlorophytes. Although cyanobacteria have occurred in all the water samples, low biovolume was recorded. However, from the ten cyanobacterial genera observed in the reservoir, nine are known as potentially cyanotoxin-producing organisms.

Despite the presence of saxitoxin in the reservoir during the study period, the concentrations were below the indicated value for drinking water (3.00 μg L^{-1}), as also reported by Tundisi et al. (2015). Microcystin concentrations exceeding the limit of 1.00 μg L^{-1} allowed for human consumption according to the Brazilian Ministry of Health (Ordinance 2914/2011) (Brasil, 2011) were measured in May 2017 in the dam zone. Even though the cyanobacterial biovolume was low in the reservoir (<0.25 mm^3 L^{-1}) and no blooms occurred, this does not exclude a risk of cyanotoxin production and release into water. In a study on microcystin production in Lake Vancouver, Lee et al. (2015) reported that although *Microcystis* sp. abundance rarely exceeded one percent of the total cyanobacteria and was rarely detected in microscopic counts, the qPCR results indicated that the majority of the *Microcystis* population contained the *mcyE* gene, and the MC concentrations repeatedly exceeded WHO guidelines for drinking water.

The current Brazilian legislation (Ordinance 2914/2011) (Brasil, 2011) establishes that concentrations of MC and STX should be quantified weekly only if the cyanobacterial density exceeds the value of 20,000 cells mL^{-1}. However, considering the results observed in the Lobo reservoir, the cyanobacterial density should not be the only parameter for verifying the potential for cyanotoxins production in freshwater environments, since this method does not allow identifying the real toxic potential of the cyanobacterial community. To meet this need, we suggest performing a qPCR assay as it allows the quantification of potentially toxic cells. The qPCR method associated with the cyanobacterial density determined by microscopy will enable a better assessment of the real situation of the water body with respect to the possible occurrence of cyanotoxins, effectively assisting the water managers as a basis for decision-making.

The presence of MC in the Lobo reservoir being, in some samples, above the maximum limit allowed by Brazilian law, emphasizes the need for a long-term monitoring of water quality aiming to avoid a potential risk to the health of the local population. Acute exposure to microcystin can lead to liver failure and death (Falconer et al., 1999), and chronic exposure to small microcystin concentrations increases the risk of liver cancer, as reported by Hitzfeld et al. (2000). The authors noticed an increase in liver cancer cases in places with a MC concentration of 0.16 μg L^{-1} in Jiangsu Province (China).

Changes in microcystin and saxitoxin concentrations in the lower limit of the euphotic zone and on the surface did not show a clear pattern, suggesting that the presence of toxins did not differ between the two depths. Possibly, wind action and the shallow depth at the sampling sites (max. 2.5 m and 11.5 m in the riverine and dam zones, respectively) (Table B.1), resulted in a well-mixed water column. Statistical analyses indicated that MC production was related to the genera *Geitlerinema* and *Phormidium*, and the STX production was associated with the genera *Phormidium* and *Raphidiopsis* in the reservoir. However, it cannot be confirmed that these genera actually produced toxins in the reservoir, since the chemical and molecular analyses were performed on environmental samples that also included several other potential toxin-producing genera of cyanobacteria (Table 3.2).

4.2 Environmental variables and cyanotoxins

Cyanotoxins production in natural systems is a complex process that is regulated by several environmental factors and is not yet fully understood (Omidi et al., 2018; Kelly et al., 2019). In Lobo reservoir, our results showed that microcystin, *mcyE* and *sxtA* genes were indirectly correlated with nitrite concentrations, while STX correlated positively with soluble reactive phosphorus, and *sxtA* gene was negatively related to total phosphorus. Previous studies have demonstrated that nitrogen and phosphorus concentrations are significant parameters affecting microcystin and saxitoxin production, as well as the abundance of toxic genotypes (Yunes et al., 2009; Lee et al., 2015; Singh et al., 2015; Mariani et al., 2015; Barros et al., 2019; Vargas et al., 2019; Brandenburg et al., 2020), however the importance of each of these parameters may vary strongly between different environments.

In a laboratory study on microcystin production under nutrient stress conditions, Pimentel and Giani (2014) reported that nutrients (nitrogen and phosphorus) deprivation increased the MC production by promoting *mcyD* gene transcription, and suggested that the microcystin increase was linked to oxidative stress caused by severe nutrient limitation. An increase in the transcription of the *mcy* gene under conditions of nitrate starvation was also observed by Ginn and Neilan (2010). For saxitoxin, Cirés et al. (2017) observed in laboratory experiments with *Aphanizomenon gracile* that dissolved inorganic nitrogen (DIN) depletion induced an increase in saxitoxin production. Similar results on the influence of nitrogen on STX production were also reported in other studies (Dias et al., 2002; Yunes et al., 2009; Casero et al., 2014).

The saxitoxin concentrations correlated positively with soluble reactive phosphorus (SRP) in the Lobo reservoir, but the low SRP levels (<3.70 µg L^{-1}) may have affected the STX

production. Vargas et al. (2019) evaluated the effects of nutrient concentration on growth and STX production of *R. raciborskii* in laboratory tests and observed that STX concentrations were higher under oligotrophic condition (3.30 µg L^{-1}), indicating a survival strategy of the cyanobacteria and adaptation to a phosphorus-limited environment. Considering that the maximum SRP concentration in this study was similar to that used by Vargas et al. (2019), we can conclude that toxic cyanobacteria in the Lobo reservoir most likely were under a stressful condition which may explain the STX production in the environment.

The *mcyE* gene was significantly and negatively related to electrical conductivity (EC) in Lobo reservoir. Tao et al. (2012) applied a model to examine key factors affecting microcystin cellular quotas in Lake Taihu (China), and reported that conductivity explained 21% of the variation of MC cellular quotas in *Microcystis* and was the highest weighted parameter in the statistical model. Since conductivity is a parameter related to the ability of electric conduction of water and can indicate the ion concentration, including inorganic ions (e.g., nutrients), the authors explained that low values of EC can be an indication of suboptimal condition for growth (less nutrients), which may promote the MC production as a response to the nutrient limitation.

4.3 Monitoring of toxic cyanobacteria

In this study, MC concentrations and *mcyE* gene copy numbers, as well as the STX concentrations and *sxtA* gene abundance, were positively and significantly correlated in the reservoir. Detection of MC by ELISA and the *mcyE* molecular marker results were positive for the samples from May 2017 (both sampling sites) and August 2017 in the dam zone. However, the results of the two analytical approaches diverged in January 2018 (both sampling sites), when only the ELISA analysis yielded a positive result for microcystin. Considering that microcystins can persist in the water for several weeks (Jones and Orr, 1994; Lahti et al., 1997; Gagala and Mankiewicz-Boczek, 2012; Zastepa et al., 2014), this difference probably occurred as a result of cyanotoxin persistence in the reservoir even after the disappearance of toxic cyanobacteria, as also reported by Nimptsch et al. (2016). Regarding saxitoxin, although *sxtA* gene was detected in all samples indicating the presence of potentially-saxitoxin producing cyanobacteria in the reservoir during the study period, no STX was measured in half of the water samples. This might be due to (1) a down-regulation on the *sxt* gene expression (Savela et al., 2015), (2) the *sxtA*-containing cells detected by qPCR assay did not produce sufficient saxitoxin to be detected by ELISA (detection limit of 0.02 µg L^{-1}) and/or (3) no STX was detected due to the toxin degradation in these samples.

The direct relationship between cyanotoxins concentrations and gene copy numbers found in the reservoir agrees with reports from other studies (Davis et al., 2009; Rinta-Kanto et al., 2009; Al-Tebrineh et al., 2010, 2012; Te and Gin, 2011; Srivastava et al., 2012; Pimentel and Giani, 2013; Ngwa et al., 2014; Yu et al., 2014; Lee et al., 2015). However, in other cases, no correlations were found (Ye et al., 2009; Baxa et al., 2010; Martins et al., 2011; Oh et al., 2013; Guedes et al., 2014; Li, et al., 2014; Gao et al., 2015). This scenario may be attributed to the several methodological variations of qPCR (e.g., DNA extraction methods, target genes and primers), the qPCR derived parameters (e.g., gene copy number, toxic genotypes cell equivalents and proportion of toxic genotypes), the method for cyanotoxin quantification (e.g., ELISA, HPLC and LC-MS/MS), and the determination of intra- and extracellular fractions or only the intracellular fraction, as reported by Guedes et al. (2014) and Pacheco et al. (2016). These authors raised a concern regarding estimation of cyanotoxins concentrations in environmental samples based on the quantification of toxic positive genotypes, since the factors stated above can contribute to discrepancies.

Microcystin concentrations and *mcyE* gene copy numbers were significantly and positively related to chlorophyll *a* concentrations in the reservoir. Several studies have reported similar results and suggested that the monitoring of chlorophyll *a* could be used as a warning sign for the presence of microcystin in the water bodies (Graham et al. 2004; Rinta-Kanto et al. 2009; Ye et al. 2009; Te and Gin 2011; Gkelis et al. 2014; Shang et al. 2015; Singh et al. 2015). Cunha et al. (2018) used a 5-year time series of MC concentrations in 6 subtropical reservoirs to investigate the main factors related to toxin concentration and estimated thresholds for the prediction of toxin production. The authors found a chlorophyll *a* threshold of 13.00 μg L^{-1} for MC ≥0.10 μg L^{-1}, while the threshold for MC ≥1.00 μg L^{-1} was 39.80 μg L^{-1}. Compared to our results, the maximum MC concentration (1.54 μg L^{-1}) occurred even when chlorophyll *a* was lower than 28.00 μg L^{-1}. Cunha et al. (2018) stated that although chlorophyll *a* can be used as an indirect indicator of potential harmful blooms, correlations between toxic cyanobacteria and chlorophyll *a* may vary since cyanobacteria have pigments other than chlorophyll *a* (e.g., phycocyanin). Considering these findings, although chlorophyll *a* was strongly associated with MC production in the Lobo reservoir ($R^2 = 0.80$, $p < 0.001$), this variable may not be the best parameter to predict the microcystin toxic genotypes in the environment.

Ngwa et al. (2014) analyzed indicators of microcystin production under laboratory and field conditions. In laboratory cultures of *Microcystis* and *Planktothrix*, the chlorophyll *a* concentration was the best predictor of microcystin concentrations, followed closely by *mcyE* gene copy numbers and microscopic cell counts. In the environmental samples, *mcyE* gene copy

numbers were, on the contrary, the best indicators of MC concentrations, especially in water bodies comprising mixed assemblages of toxic and non-toxic cyanobacteria. Similar results were also reported by Davis et al. (2009). These authors found that *mcyD* gene in *Microcystis* was significantly correlated with microcystin concentrations in three lakes and one pond in the USA, being a better predictor of MC concentrations than total cell counts or chlorophyll *a* concentration. These findings reinforce the results found in our study since *mcyE* was considered the best predictor of potentially microcystin-producing cyanobacteria in Lobo reservoir ($R^2 = 0.86$, $p < 0.001$). In addition, chlorophyll-based monitoring focuses on biomass increases as indicative of risk, but the cyanotoxin content can be high even when the cyanobacterial biomass is low, and in this case, qPCR can be a better approach to detect potentially toxic strains and may serve as an early monitoring tool.

Although a moderate and significant correlation was observed between saxitoxin concentrations and cyanobacterial biovolumes ($rho = 0.55$, $p < 0.05$), a linear regression model calculation indicated that cyanobacterial biovolume was not a good fit for estimation of total saxitoxin in the reservoir ($R^2 = 0.23$, $p = 0.04$). The best predictor of potentially-saxitoxin producing cyanobacteria presence in the reservoir was the abundance of the *sxtA* gene ($R^2 = 0.68$, $p < 0.001$). Rapid estimates of the *sxtA* gene can be used to detect occurrence of potentially saxitoxin-producing cyanobacteria and aid in early warning, as corroborated by other studies (Al-Tebrineh et al., 2010, 2012). However, although the qPCR results in Lobo reservoir have shown that the *mcyE* and *sxtA* genes can be used as indicators of microcystin and saxitoxin concentrations, this relationship can vary in different water bodies and at specific times, since many environmental factors influence the toxin production. Thus, to assess the actual toxicity of the environmental samples, other analytical techniques have to be applied.

5 Conclusions

Microcystin and saxitoxin were common cyanotoxins in the Lobo reservoir throughout the study period, but both presence and concentrations varied temporally, with high values during the dry winter season. MC and STX concentrations were typically low, but in May 2017 the total MC concentrations exceeded 1.00 μg L^{-1}, despite a low cyanobacterial biovolume occurred. These results stress the importance of establishing a long-term monitoring of water quality for human consumption, since the high concentration of MC poses a risk to public health. Occurrence of MC and STX was influenced by more than one environmental variable, suggesting that microcystin and saxitoxin productions were under multiple regulation and possibly associated with stressful conditions, such as the nutrient limitation.

The quantitative PCR results showed that this molecular method can be used to estimate the presence of potentially toxin-producing cyanobacteria in environmental samples and contribute to a reliable and fast risk evaluation to protect water users from hazards associated with occurrence of toxic cyanobacteria. However, confirmation of actual presence of the cyanotoxin and determination of its concentration still depends on chemical analytical methods. Thus, qPCR in conjunction with ELISA or LC-MS is recommended for determining the toxicity of water samples and may be employed for efficient cyanobacteria monitoring in water bodies.

Acknowledgements

This study was financially supported by FAPESP - São Paulo Research Foundation (2016/09405-1). The first author thanks FAPESP for the doctorate and research internship abroad scholarships (2015/21191-4 and 2018/00394-2).

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CHAPTER 4 - GENERAL CONCLUSIONS AND RECOMMENDATIONS

Toxic cyanobacteria were detected in both Itupararanga and Lobo reservoirs during the sampling period. Nevertheless, cyanobacteria contribution to the total phytoplankton community was higher in Itupararanga reservoir. Among the potentially toxin-producing cyanobacteria genera detected in the reservoirs were *Aphanizomenon*, *Geitlerinema*, *Phormidium* and *Raphidiopsis*.

Besides the occurrence of potentially toxin-producing cyanobacteria, microcystin (MC) and saxitoxin (STX) were measured in both reservoirs. Although MC and STX concentrations were generally low, MC concentrations were above the limit allowed by the Ministry of Health for human consumption (<1.00 µg L^{-1}) in May 2017 in the dam zone of Lobo reservoir. These results reinforce the need of a long-term monitoring of the water quality aiming to avoid or minimize the risks to the public health.

Occurrences of MC and STX were influenced by more than one environmental variable in both reservoirs, however, nutrients appeared to be the main drivers on toxin production in the studied water bodies. Although nutrient limitation may restrict cyanobacterial growth, our results showed that nutrients limitation (nitrogen and phosphorus) may cause an increase in microcystin and saxitoxin production. In Itupararanga reservoir, the high TN:TP ratios sustained the STX production, indicating that the increase in toxin concentration may be a response to stressful conditions. These outcomes provide insights into effects of nutrients on toxin production, contributing to the water resources management.

In this study, we developed a new qPCR assay to detect the *sxtA* gene, which is a core gene in the saxitoxin biosynthesis. To the best of our knowledge, this was the first study applying a qPCR assay to target the *sxtA* gene in Brazilian drinking water reservoirs. The qPCR results showed that the newly designed primer set *sxtA*-cyano enabled the specific detection and quantification of potentially saxitoxin-producing cyanobacteria in the mixed phytoplankton communities from both reservoirs, with good sensitivity and over a broad quantification range. The qPCR assay characteristics demonstrated above are important for a monitoring application, since the method needs to be able to respond to a variable cyanobacterial population composition and cell densities in the environment.

The *sxtA* and *mcyE* genes were directly associated with the cyanotoxins measured in the reservoirs, being reliable indicators of the presence of potentially toxic genotypes in the aquatic systems. However, qPCR assays do not assess the toxin concentrations on environmental samples. Thus, for determining the toxicity in water bodies, analytical methods

(e.g., ELISA and LC-MS) are still needed to confirm the toxin production and measure its concentrations. Considering the co-occurrence of two different toxin genes (*mcyE* and *sxtA*) in the Lobo reservoir, we suggest that future research increasingly focus on multiplex qPCR assays, enabling the simultaneous amplification of two or more target genes in the same reaction using the same reagent mix.

The present study showed that the combination of different methods can be used to provide enhanced monitoring data for cyanobacteria management. For instance, the rapid and reliable detection of the cyanobacteria biomass by the pigment analysis associated with the quantification of potentially toxic cells by qPCR can be a valuable tool for the early warning of potential risk of toxic cyanobacteria in the water bodies.

The current Brazilian legislation establishes that concentrations of cyanotoxins should be quantified weekly only if the cyanobacterial density exceeds the value of 20,000 cells mL^{-1} (cyanobacterial bloom). However, considering the results observed in this study, the cyanobacterial density should not be the only criterion for verifying the potential for cyanotoxins production in freshwater systems, since this method is not sufficient to identify the real toxic potential of the cyanobacterial community. In this case, the qPCR assay would be a good complementary tool for the traditional methodology as it allows the quantification of potentially toxic cells. Thus, the qPCR method associated with the cyanobacterial density determined by microscopy could enable a better evaluation of the real situation of the water body regarding the possible occurrence of cyanotoxins, effectively assisting the water managers as a basis for decision-making.

This doctoral research contributed with information on toxic cyanobacterial population dynamics and on the main drivers involved in the cyanotoxin production in two subtropical reservoirs. However, the factors that influence the occurrence of toxic cyanobacteria may vary in space and time. Thus, it is recommended that more studies in several water bodies are developed in an attempt to better understand the toxic cyanobacteria dynamics under specific conditions, as well as the toxin production in different systems. For that, we believe that the qPCR assays performed in parallel with classical methods can enable the analysis of numerous samples in a relative short time, generating reliable results in order to improve the understanding of the environmental variables role on cyanobacteria toxicity in freshwater systems.

The process of elaboration of chapters 2 and 3 received great collaboration from co-authors to bring advances in the methodological delineation and discuss aspects regarding the occurrence of cyanobacteria and cyanotoxins in subtropical reservoirs. Confidently, it will be

valuable for other researchers aiming at studying toxic cyanobacterial population dynamics in different water bodies, as well as be useful for decision-makers of subtropical aquatic environments to identify the risk of toxic cyanobacteria during the water quality monitoring.

APPENDIX A - ITUPARARANGA RESERVOIR

Table A.1- Characteristics of Itupararanga reservoir and sampling sites (riverine and dam zones)

Characteristics	Itupararanga
Surface area (km²)	20.9
Maximum volume (10^6 m³)	286.0
Perimeter (km)	192.8
Maximum water flow (m³ s^{-1})	39.0
Water residence time (days)	250
Main tributaries	Sorocamirim and Sorocabuçu rivers
Hydrographic basin	Sorocaba and Middle Tietê River

Characteristics	Riverine zone	Dam zone
*Geographic location	23°37'24.8" S, 47°13'53.4" W	23°36'50.7" S, 47°23'27.3" W
*Maximum depth (m)	6.5	18.5
*Average depth (m)	5.0	16.5

Source: Cunha and Calijuri (2011); Cunha (2012); Beghelli et al. (2016); *this study.

Table A.2 - List of phytoplankton pigments included in HPLC analysis and taxonomic designations

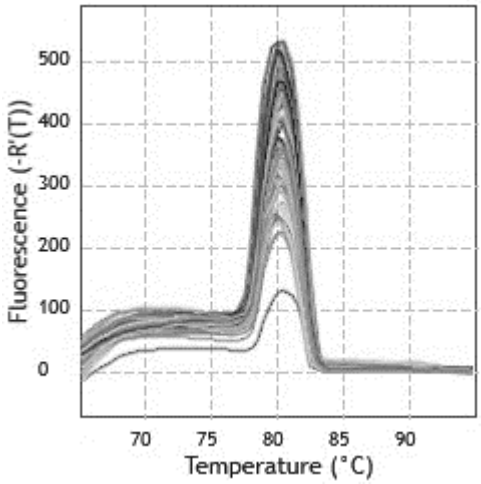
	Pigment	Designation
1	α-carotene	
2	Alloxanthin	Cryptophyceae
3	Antheraxanthin	Chlorophyceae
4	Aphanizophyll	Cyanobacteria
5	β-carotene	
6	Canthaxanthin	Cyanobacteria
7	Chlorophyll *a*	
8	Chlorophyll *b*	Chlorophyceae
9	Chlorophyll *c*2	
10	Chlorophyll *c3*	Bacillariophyceae + Chrysophyceae
11	Chlorophyllide *a*	
12	Diadinoxanthin	
13	Diatoxanthin	
14	Echinenone	Cyanobacteria
15	Fucoxanthin	Bacillariophyceae + Chrysophyceae
16	Lutein	Chlorophyceae
17	Myxoxanthophyll	Cyanobacteria
18	Neoxanthin	Chlorophyceae
19	Peridinin	Dinophyceae
20	Pheophytin *a*	
21	Violaxanthin	Chlorophyceae
22	Zeaxanthin	Cyanobacteria

Table A.3 - PCR and *in silico* PCR with cyanobacteria strains for investigation of specificity of the primer set *sxtA*-cyano on detection of *sxtA* gene

Strains	PCR	*In silico* PCR	Strain procured from
Aphanizomenon gracile K-0549	+		SCCAP, Denmark
Dolichospermum lemmermannii K-0584	+		SCCAP, Denmark
Microcystis aeruginosa CCMA-UFSCar 666	-		CCMA-UFSCar, Brazil
Microcystis aeruginosa PCC-7820	-		PCC, France
Microcystis wesenbergii NIES-107	-		NIES, Japan
Raphidiopsis raciborskii CYRF-1	+		LETC, Brazil
Raphidiopsis raciborskii T3	+		LETC, Brazil
Raphidiopsis raciborskii CYLP-1	-		LETC, Brazil
Raphidiopsis raciborskii NPCS-1	-		LETC, Brazil
Aphanizomenon gracile		+	
Aphanizomenon ovalisporum UAM287		+	
Dolichospermum circinale		+	
Dolichospermum flos-aquae ANA311E		+	
Lyngbya wollei		+	
Raphidiopsis raciborskii		+	
Heteroscytonema crispum		+	

Source: author. SCCAP = Scandinavian Culture Collection of Algae and Protozoa; CCMA-UFSCar = Collection of Freshwater Microalgae Cultures of Federal University of São Carlos; PCC = Pasteur Culture Collection; NIES = National Institute for Environmental Studies; LETC = Laboratory of Ecophysiology and Toxicology of Cyanobacteria of Federal University of Rio de Janeiro.

Figure A.1 - Melting curve for the *sxtA* primer set for standards and environmental samples showing a single peak (Tm = 80 - 80.5 °C) that indicates amplification of a single amplicon



Source: author. T_m = melting temperature.

Table A.4 - List of the phytoplankton taxa identified in Itupararanga reservoir

	Taxa	Riverine zone								Dam zone							
		May-17		Aug-17		Oct-17		Jan-18		May-17		Aug-17		Oct-17		Jan-18	
		100	1	100	1	100	1	100	1	100	1	100	1	100	1	100	1
	Bacillariophyceae	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
1	*Aulacoseira granulata* (Ehrenberg) Simonsen 1979	•		•	•			•	•			•					•
2	*Cyclotella meneghiniana* Kützing 1844	•	•	•	•	•	•	•	•	•	•					•	
3	*Discostella stelligera* (Cleve & Grunow) Houk & Klee 2004			•	•										•		•
4	*Encyonopsis* sp.	•	•				•					•					
5	*Fragilaria aquaplus* Lange-Bertalot & S. Ulrich 2014							•									
6	*Fragilaria grunowii* Lange-Bertalot & S. Ulrich 2014	•	•	•	•	•	•			•	•	•	•	•	•	•	•
7	*Fragilaria longifusiformis* (Hains & Sebring) Siver et al.						•	•	•						•		•
8	*Fragilaria spectra* P.D.Almeida, E.Morales & C.E.Wetzel 2016	•		•	•		•			•	•		•				
9	*Navicula* sp.	•	•														
10	*Pinnularia* sp.	•			•												
	Chlamydophyceae	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
11	*Chlamydomonas* sp. 1	•		•	•	•	•	•	•	•	•	•	•	•		•	•
12	*Chlamydomonas* sp. 2								•						•		
13	*Pandorina morum* (O. F. Müller) Bory 1826		•								•						
	Chlorophyceae	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
14	*Acutodesmus acuminatus* (Lagerheim) P. M. Tsarenko 2000										•						
15	*Ankistrodesmus fusiformis* Corda 1838						•		•	•	•	•	•	•	•	•	•
16	*Ankistrodesmus gracilis* (Reinsch) Korshikov 1953							•									
17	*Coelastrum microporum* Nägeli 1855								•								
18	*Crucigenia fenestrata* (Schmidle) Schmidle 1900			•													•
19	*Desmodesmus* sp.			•			•	•	•			•			•		•
20	*Eremosphaera* sp.									•							•
21	*Kirchneriella contorta* (Schmidle) Bohlin 1897			•	•		•		•				•		•		
22	*Kirchneriella lunaris* (Kirchner) Möbius 1894										•		•				
23	*Monoraphidium arcuatum* (Korshikov) Hindák 1970			•	•		•	•	•				•				•
24	*Monoraphidium contortum* (Thuret) Komárková-Legnerová 1969			•		•		•	•	•	•	•	•	•	•	•	•
25	*Monoraphidium irregulare* (G.M.Smith) Komárková-Legnerová 1969									•		•	•	•	•		•
26	*Monoraphidium komarkovae* Nygaard 1979			•			•	•		•		•	•		•	•	•
27	*Monoraphidium minutum* (Nägeli) Komárková-Legnerová 1969		•				•			•							
28	*Radiococcus* sp.	•	•	•	•	•	•	•	•	•	•	•	•				
28	*Tetraëdron incus* (Teiling) G.M. Smith 1926									•							•
30	*Treubaria* sp.											•					

	Taxa	Riverine zone								Dam zone							
		May-17		Aug-17		Oct-17		Jan-18		May-17		Aug-17		Oct-17		Jan-18	
		100	1	100	1	100	1	100	1	100	1	100	1	100	1	100	1
	Chrysophyceae	•	•	•	•	•	•	•	•			•				•	•
31	*Chromulina elegans* Doflein 1921	•	•	•	•	•	•	•	•								
32	*Dinobryon divergens* O.E. Imhof 1887							•	•							•	•
33	*Mallomonas* sp.							•									
34	*Synura* sp.			•					•			•					
	Cryptophyceae	•		•	•	•	•	•	•	•	•	•	•		•	•	•
35	*Cryptomonas brasiliensis* A. Castro, C.E.M. Bicudo & D. Bicudo 1992							•		•		•				•	
36	*Cryptomonas erosa* Ehrenberg 1832	•		•	•	•	•	•	•	•	•	•	•			•	•
37	*Rhodomonas lacustris* Pascher & Ruttner 1913			•	•		•	•	•						•		
	Cyanobacteria	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
38	*Aphanizomenon gracile* Lemmermann 1907				•		•			•	•	•	•		•		•
39	*Aphanocapasa incerta* (Lemmermann) G.Cronberg & Komárek 1994			•	•			•	•								
40	*Aphanocapsa delicatissima* West & G. S. West 1912			•						•		•	•		•		
41	*Aphanocapsa elachista* West & G.S. West 1894				•	•	•	•	•	•	•	•	•	•	•	•	•
42	*Aphanocapsa* sp.	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
43	*Aphanothece* sp.													•		•	
44	*Chroococcus* sp.														•		
45	*Cuspidothrix* sp.										•	•	•				
46	*Dolichospermum solitarium* (Klebahn) Wacklin, L. Hoffmann & Komárek 2009					•	•	•	•							•	•
47	*Geitlerinema* sp.				•			•		•	•	•		•	•	•	•
48	*Geitlerinema amphibium* (C.Agardh ex Gomont) Anagnostidis 1989							•		•	•	•	•	•	•	•	•
49	*Glaucospira* sp.				•			•	•			•	•				
50	*Limnococcus limneticus* (Lemmermann) Komárková, Jezberová, O. Komárek & Zapomelová 2010					•										•	•
51	*Limnothrix planctonica* (Woloszynska) Meffert 1988	•		•	•	•	•		•	•	•	•	•	•	•	•	•
52	*Merismopedia punctata* Meyen, nom. illeg. 1839															•	
53	*Merismopedia tenuissima* Lemmermann 1898	•	•						•								
54	*Microcystis protocystis* W.B. Crow 1923							•									
55	*Phormidium* sp.										•						
56	*Pseudanabaena catenata* Lauterborn 1915	•	•														
57	*Pseudanabaena limnetica* (Lemmermann) Komárek 1974	•	•	•	•	•	•	•	•								
58	*Raphidiopsis raciborskii* (Woloszynska) Aguilera, Berrendero-Gómez, Kastovsky, Echenique & Salerno 2018	•		•	•	•	•	•	•	•	•	•	•	•	•	•	•

	Taxa	Riverine zone								Dam zone							
		May-17		Aug-17		Oct-17		Jan-18		May-17		Aug-17		Oct-17		Jan-18	
		100	1	100	1	100	1	100	1	100	1	100	1	100	1	100	1
59	*Synechocystis aquatilis* Sauvageau 1892	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
	Dinophyceae	•				•	•	•	•		•	•	•				
60	*Ceratium furcoides* (Levander) Langhans 1925	•				•	•				•		•				
61	*Peridinium* sp.							•	•			•	•				
	Euglenophyceae	•	•	•	•	•	•	•	•		•	•	•	•	•		•
62	*Euglena* sp.	•				•		•	•		•		•				
63	*Phacus* sp.							•									
64	*Trachelomonas spinosa* A. Stokes 1890		•														
65	*Trachelomonas volvocina* (Ehrenberg) Ehrenberg 1834	•	•	•	•	•	•	•	•		•	•	•	•	•		•
	Trebouxiophyceae	•	•	•				•	•	•	•	•	•		•	•	
66	*Actinastrum* sp.	•															
67	*Chlorella* sp.			•				•	•	•		•					
68	*Closteriopsis longissima* (Lemmermann) Lemmermann 1899										•	•	•		•	•	
69	*Franceia* sp.																
70	*Golenkinia* sp.							•				•	•				
71	*Micractinium pusillum* Fresenius 1858	•	•						•				•				
72	*Oocystis* sp. 1		•	•				•	•								
73	*Oocystis* sp. 2			•													
	Zygnemaphyceae	•		•		•	•	•	•		•	•	•	•	•	•	•
74	*Closterium acutum* Brébisson 1848	•										•		•	•	•	
75	*Cosmarium* sp.															•	•
76	*Mougeotia* sp.			•		•	•	•	•		•	•	•			•	•
77	*Staurastrum chaetoceras* (Schröder) G.M.Smith 1924							•								•	•
78	*Staurastrum* sp.										•						

Source: author. 100 = surface; 1 = lower limit of euphotic zone; • = presence of the taxon at the sampling point.

APPENDIX B - LOBO RESERVOIR

Table B.1 - Characteristics of Lobo reservoir and sampling sites (riverine and dam zones)

Characteristics	Lobo
Surface area (km^2)	6.8
Maximum volume ($10^6 m^3$)	22.0
Perimeter (km)	21.0
Maximum water flow ($m^3 s^{-1}$)	20.0
Water residence time (days)	32
Main tributaries	Itaqueri and Lobo rivers
Hydrographic basin	Tietê-Jacaré

Characteristics	Riverine zone	Dam zone
*Geographic location	22°12'36.9" S, 47°52'52.8" W	22°10'18.5" S, 47°54'11.2" W
*Maximum depth (m)	2.5	11.5
*Average depth (m)	2.0	10.5

Source: Motheo (2005); Delello (2008); *this study.

Table B.2 - List of the phytoplankton taxa identified in Lobo reservoir

Taxa		Riverine zone								Dam zone							
		May-17		Aug-17		Oct-17		Jan-18		May-17		Aug-17		Oct-17		Jan-18	
		100	1	100	1	100	1	100	1	100	1	100	1	100	1	100	1
Bacillariophyceae		•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
1	*Aulacoseira distans* (Ehrenberg) Simonsen 1979	•		•	•	•	•	•	•	•	•	•	•	•	•	•	•
2	*Aulacoseira granulata* (Ehrenberg) Simonsen 1979	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
3	*Aulacoseira granulata var. angustissima* (O.Müller) Simonsen 1979						•					•	•	•		•	
4	*Cocconeis* sp.				•					•							
5	*Discostella stelligera* (Cleve & Grunow) Houk & Klee 2004	•	•	•			•	•	•	•		•	•			•	•
6	*Eolimna minima* (Grunow) Lange-Bertalot, nom. illeg. 1998													•			
7	*Eunotia sudetica* O. Müller 1898					•											
8	*Fragilaria* sp.															•	
9	*Gomphonema* sp.								•								
10	*Navicula* sp.	•															
11	*Pinnularia* sp.			•													
12	*Rhizosolenia* sp.	•	•				•			•		•	•	•	•		•
Chlamydophyceae		•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
13	*Carteria* sp.						•										
14	*Chlamydomonas* sp. 1	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
15	*Chlamydomonas* sp. 2	•	•		•	•		•	•	•	•	•	•		•	•	•
16	*Chlamydomonas* sp. 3	•					•			•		•					
17	*Chlamydomonas* sp. 4										•						
18	*Coccomonas* sp.															•	
Chlorophyceae		•	•	•		•	•	•		•	•	•	•	•	•	•	•
19	*Ankistrodesmus fusiformis* Corda 1838	•									•						
20	*Characium* sp.												•				
21	*Chlorolobion* sp.		•	•		•	•	•		•	•			•	•		•
22	*Choricystis minor* (Skuja) Fott 1976	•				•								•			
23	*Coelastrum* sp.	•															
24	*Desmodesmus denticulatus* (Lagerheim) S. S. An, T. Friedl & E. Hegewald 1999	•	•	•			•			•	•						
25	*Desmodesmus magnus* (Meyen) Tsarenko 2000		•				•					•					
26	*Desmodesmus* sp.		•														
27	*Eutetramorus fottii* (Hindák) Komárek 1979															•	•
28	*Eutetramorus planctonicus* (Korshikov) Bourrelly 1964									•	•	•	•				
29	*Eutetramorus* sp.						•										

	Taxa	Riverine zone								Dam zone							
		May-17		Aug-17		Oct-17		Jan-18		May-17		Aug-17		Oct-17		Jan-18	
		100	1	100	1	100	1	100	1	100	1	100	1	100	1	100	1
30	*Monoraphidium contortum* (Thuret) Komárková-Legnerová 1969		•	•								•					
31	*Monoraphidium minutum* (Nägeli) Komárková-Legnerová 1969											•					
32	*Monoraphidium nanum* (Ettl) Hindák 1980	•						•			•					•	
33	*Monoraphidium tortile* (West & G. S. West) Komárková-Legnerová 1969		•	•		•	•			•	•						
34	*Quadrigula* sp.						•										
35	*Scenedesmus arcuatus* (Lemmermann) Lemmermann 1899	•															
36	*Scenedesmus obtusus* Meyen 1829	•						•			•	•					•
37	*Scenedesmus quadricauda* (Turpin) Brébisson 1835														•		
38	*Scenedesmus* sp.					•							•	•	•		
39	*Stauridium tetras* (Ehrenberg) E. Hegewald 2005												•				
40	*Tetraëdron minimum* (A. Braun) Hansgirg 1889		•														
41	*Tetrallantos lagerheimii* Teiling 1916									•							
42	*Tetrastrum heteracanthum* (Nordstedt) Chodat 1895									•							
43	*Tetrastrum komarekii* Hindák 1977									•							
	Chrysophyceae	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	
44	*Chromulina elegans* Doflein 1921											•					
45	*Dinobryon bavaricum* Imhof 1890	•	•					•							•		
46	*Lagynion ampullaceum* (A. Stokes) Pascher 1912											•					
47	*Mallomonas* sp. 1								•							•	
48	*Mallomonas* sp. 2		•	•	•	•	•	•	•	•	•	•	•	•	•	•	
49	*Synura* sp.				•	•		•	•	•		•	•		•	•	
	Coleochaetophyceae				•												
50	*Chaetosphaeridium globosum* (Nordstedt) Klebahn 1893				•												
	Cryptophyceae	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
51	*Cryptomonas brasiliensis* A. Castro, C.E.M. Bicudo & D. Bicudo 1992	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
52	*Cryptomonas erosa* Ehrenberg 1832	•	•	•			•	•			•					•	•
53	*Cryptomonas marssonii* Skuja 1948									•							
54	*Cryptomonas ovata* Ehrenberg 1832	•	•	•					•	•				•	•		
55	*Cryptomonas tenuis* Pascher 1913		•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
56	*Rhodomonas lacustris* Pascher & Ruttner 1913							•		•	•	•		•	•		•
	Cyanobacteria	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
57	*Anathece* sp.	•				•	•	•	•		•			•	•	•	•
58	*Aphanizomenon gracile* Lemmermann 1907				•	•					•						
59	*Aphanocapsa delicatissima* West & G. S. West 1912	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•

	Taxa	Riverine zone								Dam zone							
		May-17		Aug-17		Oct-17		Jan-18		May-17		Aug-17		Oct-17		Jan-18	
		100	1	100	1	100	1	100	1	100	1	100	1	100	1	100	1
60	*Aphanocapsa elachista* West & G.S. West 1894														•		
61	*Chroococcus limneticus* Lemmermann 1898									•							
62	*Chroococcus minimus* (Keissler) Lemmermann 1904	•		•			•			•	•	•	•		•		
63	*Chroococcus minor* (Kützing) Nägeli 1849					•						•	•		•		
64	*Geitlerinema amphibium* (C.Agardh ex Gomont) Anagnostidis 1989		•							•							•
65	*Microcystis protocystis* W. B. Crow 1923										•	•					
66	*Phormidium* sp.		•							•	•						
67	*Pseudanabaena catenata* Lauterborn 1915	•	•	•	•						•	•					
68	*Pseudanabaena galeata* Böcher 1949									•							
69	*Raphidiopsis raciborskii* (Woloszynska) Aguilera, Berrendero-Gómez, Kastovsky, Echenique & Salerno 2018	•	•								•	•	•	•	•		
70	*Synechocystis aquatilis* Sauvageau 1892	•	•	•	•	•	•	•	•	•		•	•	•	•	•	•
	Dinophyceae	•	•	•	•	•	•		•	•	•	•	•			•	•
71	*Ceratium furcoides* (Levander) Langhans 1925			•		•	•		•		•	•	•			•	•
72	*Peridinium* sp. 1	•															
73	*Peridinium* sp. 2															•	
74	*Peridinium* sp. 3									•	•						
75	*Peridinium* sp. 4		•	•	•	•	•			•	•	•	•				•
	Euglenophyceae		•					•		•	•	•					•
76	*Euglena* sp.		•						•	•	•	•					
77	*Lepocinclis* sp.									•	•						
78	*Trachelomonas abrupta* Svirenko 1914		•														
79	*Trachelomonas curta var. subpunctata* Bourrelly 1950							•		•	•						
80	*Trachelomonas lacustris* Drezepolski 1925										•						
81	*Trachelomonas volvocina* (Ehrenberg) Ehrenberg 1834										•						•
	Klebsormidiophyceae			•													
82	*Elakatothrix* sp.			•													
	Trebouxiophyceae	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
83	*Chlorella minutissima*		•		•	•		•	•	•	•	•	•	•	•	•	•
84	*Chlorella vulgaris*	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
85	*Closteriopsis longissima* (Lemmermann) Lemmermann 1899		•				•			•	•	•					
86	*Dictyosphaerium pulchellum* H. C. Wood 1873	•	•	•	•	•	•	•	•	•	•	•	•	•	•		•
87	*Dictyosphaerium tetrachotomum* Printz 1914		•							•							
88	*Didymocystis fina* Komárek 1975	•	•	•	•	•	•			•	•	•	•	•	•	•	•

	Taxa	Riverine zone								Dam zone							
		May-17		Aug-17		Oct-17		Jan-18		May-17		Aug-17		Oct-17		Jan-18	
		100	1	100	1	100	1	100	1	100	1	100	1	100	1	100	1
89	*Micractinium pusillum* Fresenius 1858									•	•						
90	*Oocystis lacustris* Chodat 1897	•				•	•			•		•	•	•	•	•	•
91	*Oocystis solitaria* Wittrock 1879		•							•			•		•		
	Xanthophyceae	•															•
92	*Characiopsis* sp.	•															•
	Zygnemaphyceae	•	•									•					•
93	*Cosmarium* sp.		•														•
94	*Mougeotia* sp.	•															•
95	*Staurastrum paradoxum* Meyen ex Ralfs 1848											•					

Source: author. 100 = surface; 1 = lower limit of euphotic zone; • = presence of the taxon at the sampling point.