UNIVERSIDADE FEDERAL DE MINAS GERAIS
Instituto de Ciências Biológicas
Programa de pós-graduação em Ecologia, Conservação e Manejo da Vida Silvestre

Mehrzad Zare

THE ROLE OF NUTRIENTS (PHOSPHORUS AND NITROGEN) IN MODULATING SAXITOXIN GENE EXPRESSION IN *RAPHIDIOPSIS RACIBORSKII* (CYANOBACTERIA): laboratory experiments and field observations

Belo Horizonte
2023

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Orientadora: Profa. Dra. Alessandra Giani
Co-orientadora: Dra. Juliana S. M. Pimentel

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"The role of nutrients (phosphorus and nitrogen) in modulating saxitoxin gene expression in Raphidiopsis raciborskii (Cyanobacteria): laboratory experiments and field observations"

MEHRZAD ZARE

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RESUMO

O desenvolvimento de florações de cianobactérias em sistemas aquáticos é principalmente causado pela disponibilidade de nutrientes, especialmente nitrogênio e fósforo. Quando as florações são dominadas por espécies potencialmente tóxicas, os efeitos nocivos da carga de nutrientes tornam-se particularmente relevantes. Algumas das cianotoxinas recorrentes com maior potencial em corpos d'água são as saxitoxinas. As saxitoxinas (STXs) são alcalóides neurotóxicos produzidos por dinoflagelados marinhos e algumas espécies de cianobactérias filamentosas de água doce. Entre as espécies tóxicas de cianobactérias, *Raphidiopsis* (anteriormente *Cylindrospermopsis*) *raciborskii* é uma espécie amplamente distribuída e cada vez mais registrada em sistemas de água doce em todo o mundo. É particularmente preocupante porque as cepas em algumas áreas geográficas são capazes de produzir toxinas com implicações para a saúde humana e animal. Os estudos desta espécie aumentaram rapidamente nas últimas duas décadas, especialmente no hemisfério sul, onde as cepas tóxicas são predominantes. Esta espécie apresenta uma grande flexibilidade mesmo sob condições de nutrientes baixos e variáveis, ajudada pela sua considerável tolerância fisiológica. Existem dois tipos de toxinas produzidas por *R. raciborskii*: cilindrospermopsinas (CYNs) e saxitoxinas (STXs). Cepas de *R. raciborskii* encontradas em ecossistemas sul-americanos são caracterizadas pela produção de saxitoxina e seus análogos (Paralytic Shellfish Poisoning, PSP), e os fatores que desencadeiam sua produção não foram ainda elucidados. Neste estudo, foi examinado o efeito do estresse de nitrogênio (N) e fósforo (P) na saxitoxina e seus análogos produzidos em *R. raciborskii*.

O objetivo deste estudo foi dividido em duas categorias:

1) O efeito da disponibilidade de nitrogênio e fósforo na expressão gênica da saxitoxina e na produção intracelular das toxinas em *R. raciborskii*, realizado em experimentos de laboratório (*in vitro*).

2) A estimativa da expressão gênica da saxitoxina em *R. raciborskii* e sua relação com a disponibilidade de fósforo e nitrogênio no reservatório da Pampulha (Brasil), realizada em experimentos ambientais, de campo (*in situ*).

Em experimentos de laboratório (*in vitro*), a PCR quantitativa por transcrição reversa (RT-qPCR) foi utilizada para investigar a resposta transcricional do gene *sxtA4* à limitação de nitrogênio e fósforo em duas cepas tóxicas de *R. raciborskii*. Os resultados mostraram que o gene *sxtA4* foi em

geral regulado positivamente quando as cepas cresciam sob menor concentração de nutrientes. Correlações foram observadas entre transcritos dos genes *sxtA4* (gene *sxt*), *ntcA* (regulador global de nitrogênio) e *nifH* (fixação de N2) em condições N-limitadas. Além disso, foi identificada uma correlação significativa entre os genes *sxtA4* e *pstS* (gene que codifica a proteína periplasmática de ligação ao fosfato) em condições limitadas por P. A concentração de toxina foi medida por Cromatografia Líquida de Alta Eficiência (HPLC). Sob limitação de N, a concentração total de saxitoxina intracelular foi mais alta com concentração de 10% de N em ambas as cepas; quase similar às alterações na expressão do gene *sxtA4*, que na cepa 186 foi maior com limitação de 10%, enquanto na cepa 36 com limitação de N de 1%. No entanto, a quantidade de toxinas intracelulares totais diminuiu significativamente sob limitação de P em ambas as cepas, o que foi contrário à transcrição do gene *sxtA4* que mostrou aumento significativo sob limitação de P em comparação ao controle (P mais elevado) em ambas as cepas. Este estudo mostrou que a limitação de nutrientes tem efeito na produção total de toxinas intracelulares, mas embora a limitação de ambos os nutrientes induza maior expressão gênica, a redução de N pode levar a uma maior produção de toxinas intracelulares, enquanto a redução de fósforo pode resultar em redução de toxinas. Estes são aspectos importantes a serem considerados quando se trata do manejo de corpos d'água dominados por cianobactérias.

Em experimentos ambientais de campo (*in situ*), os resultados mostraram que as limitações de P e N estavam significativamente relacionadas à abundância de transcritos dos genes *ntcA* e *pstS*, como esperado, mas também de *sxtA4*. A expressão desses genes aumentou significativamente com a diminuição das concentrações de N e P. Além disso, foi observada uma correlação significativa entre a biomassa de *R. raciborskii* e a expressão do gene *sxtA4*. Este estudo mostrou que em um reservatório hipereutrófico com florescimentos persistentes de cianobactérias, mudanças sazonais na concentração de P e N podem ser consideradas fatores críticos não apenas para o desenvolvimento do florescimento de cianobactérias, mas também para a regulação de sua toxicidade.

Concluindo, no presente estudo, por meio de experimentos de laboratório e medições de campo, encontramos que a limitação de nutrientes foi um importante estressor na regulação e produção de toxinas em *R. raciborskii*. Estudos de laboratório e de campo são ferramentas importantes para compreender o papel dos fatores ambientais sobre essas toxinas, bem como o impacto potencial

das toxinas no meio ambiente. Os resultados obtidos neste trabalho também fornecem alguns insights sobre a presença do gene *sxt* e de toxinas intracelulares em cepas de *R. raciborskii*.
Palavras-chave: cianobactérias; cianotoxinas; *Raphidiopsis raciborskii*; expressão gênica; saxitoxina; limitação de nutrientes; eutrofização.

ABSTRACT

The development of cyanobacterial blooms in aquatic systems is widely promoted by nutrient availability, especially nitrogen and phosphorous. When blooms are dominated by toxigenic species the harmful effects of nutrient loading become particularly relevant. Some of the most potential recurrent cyanotoxins in water bodies are the saxitoxins. Saxitoxins (STXs) are alkaloid neurotoxins produced by marine dinoflagellates and some species of freshwater filamentous cyanobacteria. Among cyanobacterial toxic species, *Raphidiopsis* (formerly *Cylindrospermopsis*) *raciborskii* is a widespread species increasingly being recorded in freshwater systems around the world. It is of particular concern because strains in some geographic areas are capable of producing toxins with implications for human and animal health. Studies of this species have increased rapidly in the last two decades, especially in the southern hemisphere where toxic strains are prevalent. This species has a high level of flexibility under low and variable nutrient conditions, helped by its considerable high physiological tolerance. There are two types of toxins produced by *R. raciborskii*: cylindrospermopsins (CYNs) and saxitoxins (STXs). *R. raciborskii* found in South American ecosystems is characterized by the production of saxitoxin and analogs (Paralytic Shellfish Poisoning, PSP), for which the factors that trigger their production have not been elucidated. In this study, the effect of nitrogen (N) and phosphorus (P) stress on saxitoxin and its analogs produced in *R. raciborskii* was examined.
The aim of this study was divided in two categories:

1) The effect of nitrogen and phosphorus availability on saxitoxin gene expression and intracellular toxin production in *R. raciborskii,* which was carried out in laboratory experiments (*in-vitro*).

2) Estimating saxitoxin gene expression in *R. raciborskii* and its relationship with phosphorus and nitrogen availability in Pampulha reservoir (Brazil), which was carried out in environmental (field) experiments (*in situ*).

In laboratory experiments (*in-vitro*), reverse transcription-quantitative PCR (RT-qPCR) was used to investigate the transcriptional response of the *sxtA4* gene to nitrogen and phosphorus limitation in two toxic *R. raciborskii* strains. Our results showed that the *sxtA4* gene was generally upregulated when growing under lower nutrient concentration. Correlations were observed among transcripts of *sxtA4* (*sxt* gene), *ntcA* (global nitrogen regulator) and *nifH* (N_2 fixation) genes in N-

limited conditions. In addition, a significant correlation was identified between *sxtA4* and *pstS* (coding for phosphate-binding periplasmic protein) genes in P-limited conditions. Toxin concentration was measured by High Performance Liquid Chromatography (HPLC). Under N limitation, total intracellular saxitoxin concentration was highest at 10% N-concentration in both strains; almost in line with the changes in *sxtA4* gene expression, which in strain 186 was higher at 10% limitation, whereas in strain 36 at 1% N- limitation. However, the amount of total intracellular toxins significantly decreased under P limitation in both strains, which was contrary to the *sxtA4* gene transcription that showed significant increase under P limitation as compared to control (high P) in both strains. This study showed that nutrient limitation has an effect on total intracellular toxin production, but while limitation of both nutrients induced higher gene expression, N reduction might lead to higher intracellular toxin production, whereas reducing phosphorus could result in toxin reduction. These are important aspects to be considered when dealing with management of cyanobacteria dominated water bodies.

In environmental (field) experiments (*in situ*), our results showed that that P and N limitations were significantly related to the transcript abundance of *ntcA* and *pstS* genes, as expected, but also of *sxtA4.* The expression of these genes significantly increased with decreasing N and P concentrations. Moreover, we observed a significant correlation between *R. raciborskii* biomass and the expression of *sxtA4* gene. This study showed that in a hypereutrophic reservoir with persistent cyanobacteria blooms, seasonal changes of P and N concentration may be considered critical factors not only for cyanobacteria bloom development but also for the regulation of their toxicity.

In conclusion, in the present study, via laboratory experiments and field measurements, we found that nutrients' limitation was an important stressor in the regulation and production of toxins in *R. raciborskii*. Laboratory and field studies are important tools to understand the role of environmental factors on these toxins as well as the potential impact of the toxins in the environment. The results obtained in this work provide some insights about the presence of SXT gene and intracellular toxins in *R. raciborskii* strains.

Keywords: cyanobacteria; cyanotoxins; *Raphidiopsis raciborskii*; gene expression; saxitoxin; nutrient limitation; eutrophication.

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1- GENERAL INTRODUCTION

Cyanobacteria, also known as 'blue-green algae,' are prokaryotes regarded as the oldest oxygenic photosynthetic organisms on earth, with the fossil records of ~3.5 billion years (Schopf 2002; Tomitani et al. 2006; Hess, 2011; Boopathi and Ki., 2014; Wittmann and Liao, 2016; Fig. 1). Cyanobacteria may cause a multitude of water quality concerns. Among these concerns is the potential risk of toxin production, since cyanotoxins in drinking water reservoirs and in recreational water pose a serious risk to human health but also to ecosystem functioning (Sabart et al., 2015).

The most studied cyanotoxin is the hepatotoxic microcystin (MC), which consists of more than 240 structural variants and is produced by several cyanobacterial genera (Spoof and Catherine, 2016; Bouaïcha et al., 2019), including *Dolichospermum* (basionym *Anabaena*) (Wacklin, et al., 2009), *Microcystis* and *Planktothrix* (Moraes et al., 2021).

Another important cyanotoxin is saxitoxin (STX), a neurotoxin that occurs in more than 50 variants in dinoflagellates and cyanobacteria (Wiese et al., 2010; Moraes et al., 2021), and 15 of these variants have so far not been detected in cyanobacteria (Wiese et al., 2010). The STX-producing freshwater cyanobacteria genera include, among others, *Raphidiopsis* (previously known as *Cylindrospermopsis*) (Aguilera et al., 2018), *Dolichospermum*, and *Aphanizomenon* (Moraes et al., 2021). The cyanobacterial species *Raphidiopsis raciborskii* has gained considerable attention due to its toxicity, bloom formation capacity, and invasiveness into temperate climates (Antunes et al., 2015; Abreu et al., 2018).

In the last decades, nutrient enrichment of both freshwater and marine systems by anthropogenic activities promoted the growth of cyanobacteria, and increased the incidence of harmful algal blooms (Paerl and Paul, 2012; Suikkanen et al., 2013; Boopathi and Ki., 2014). Several environmental factors, such as nutrient availability, rainfall, water temperature, water retention time may control the population structure of cyanobacteria, as well as the presence of toxic strains and their cyanotoxin production (Moraes et al., 2021; Giani et al., 2022). However, specific factors that stimulate and/or control the production of cyanotoxin by cyanobacteria remain to be determined (Neilan et al., 2013). Hence, understanding how the dynamics of toxic strains are regulated under different nutrient and environmental conditions is crucial for predicting and controlling cyanobacterial bloom toxicity (Lei et al., 2021).

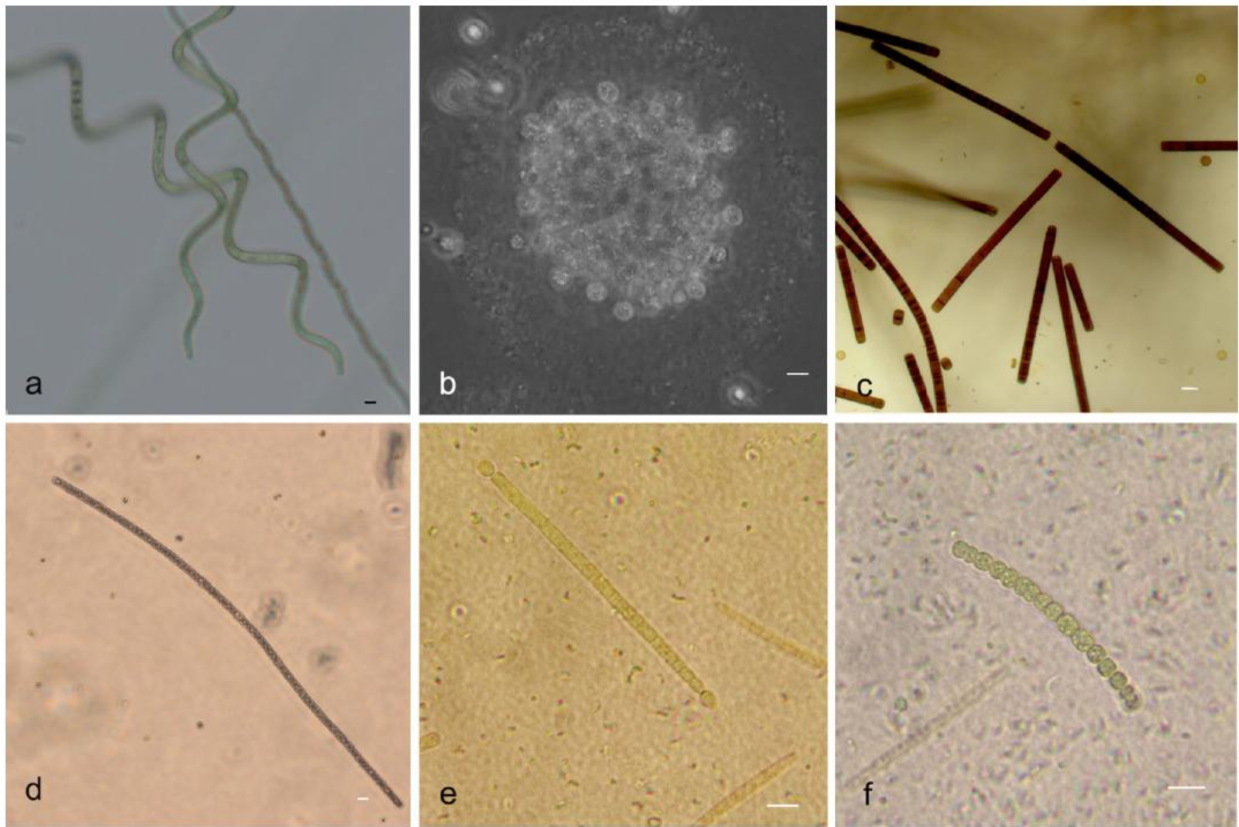


Fig. 1 Some examples of different cyanobacteria morphologies. **a** *Arthrospira* sp.; **b** *Microcystis botrys*; **c** *Lyngbya* sp.; **d** *Planktothrix rubescens*; **e** *Raphidiopsis* (=*Cylindrospermopsis*) *raciborskii*; **f** *Nostoc* sp. *Scale bar* 10 µm except **c** *Lyngbya* sp. = 30 µm. Figure adapted from (Buratti et al., 2017).

1.1 Cyanotoxins' classification

Cyanotoxins are generally organized according to their methods of action and they consist of hepatotoxins, neurotoxins, and dermatotoxins (aplysiatoxin, lyngbyatoxin-a) (Fig. 2). Cyanobacterial hepatotoxins include microcystin (MCs), nodularin (NODs) and cylindrospermopsin (CYN). The latter has both cytotoxic and neurotoxic potentials (Kaebernick and Neilan, 2001; Kaplan et al., 2012; Corbel et al., 2014;). Anatoxins (ATXs) and saxitoxins (STXs) are neurotoxins which are produced by various cyanobacterial species (Pearson et al., 2010) (Fig. 2). When cyanobacterial cells lyses, the toxins can be released at high concentrations into the environment (Kaplan et al., 2012). Anatoxin-a(s) is a unique organophosphate natural toxin that inhibits cholinesterases (Matsunaga et al., 1989). Recently, because it was easily confused with

other cyanobacterial toxins, such as anatoxin and homoanatoxin, which have different structures and distinct mechanism of action, Fiore et al. 2020 proposed changing the name to guanitoxin (GNT), linking the name to the structural characteristic of the guanidine group present in the molecule.

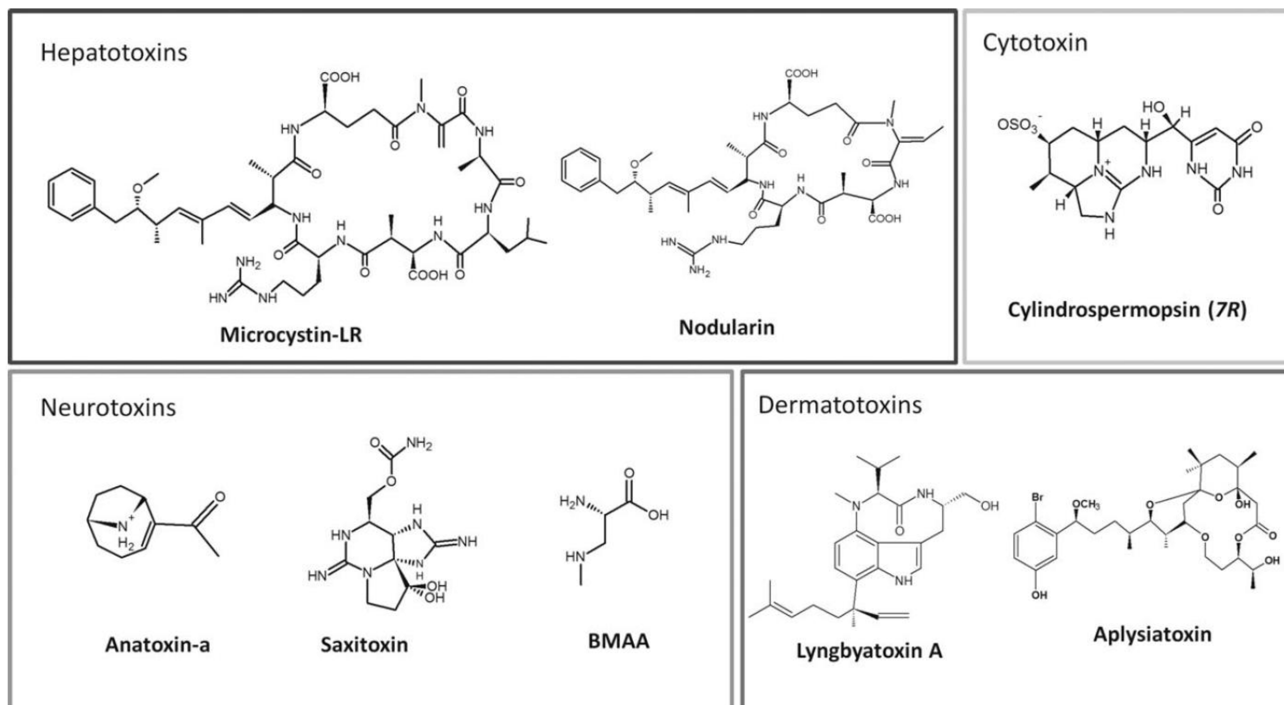


Fig. 2 Representative chemical structures for cyanobacterial toxin families. Figure adapted from (Dittmann et al., 2013).

1.1.1 - Saxitoxin (STX) and Its Analogs: the Paralytic Shellfish Toxins (PST)

These natural alkaloids (STXs) are also called PST toxins (Paralytic Shellfish Toxins), and are a family of more than 57 congeners or analogs (Wiese et al. 2010). Poisoning with PSTs can cause a serious and sometimes fatal disease known as paralytic shellfish poisoning (PSP) or saxitoxin pufferfish poisoning (SPFP) (Landsberg et al., 2006; Wiese et al., 2010). PSTs are mostly associated with marine dinoflagellates (eukaryotes) and freshwater cyanobacteria (prokaryotes), which form extensive blooms around the world. PST producing dinoflagellates belong to the genera *Alexandrium, Gymnodinium* and *Pyrodinium* whilst production has been identified in several

cyanobacterial genera including *Anabaena, Cylindrospermopsis, Aphanizomenon Planktothrix* and *Lyngbya* (Lefebvre et al., 2008; Wiese et al., 2010). The molecular formula of saxitoxin (STX) is C10H17N7O4 (Molecular Weight = 299) and is composed of a 3,4-propinoperhydropurine tricyclic system. A dose of approximately 1 mg of the toxin from a single serving of contaminated shellfish is fatal to humans (Schantz., 1957; Wiese et al., 2010). PSTs perform by inhibition of voltage-gated channels in vertebrates, such as sodium, calcium and potassium channels (Wang et al., 2003; Vico et al., 2016; Fig. 3).

Although PST (also called STX) biosynthesis is complex and unique, the producing organisms from two kingdoms, including certain species of marine dinoflagellates and freshwater cyanobacteria, are able to produce these toxins, apparently by the same biosynthetic route (Shimizu, 1993; Kellmann et al., 2008). The saxitoxin biosynthetic gene cluster (*sxt*) was recognized in cyanobacteria, which provided new insight into the biosynthesis of saxitoxin and its analogs (Kellmann et al., 2008; Mihali et al., 2009).

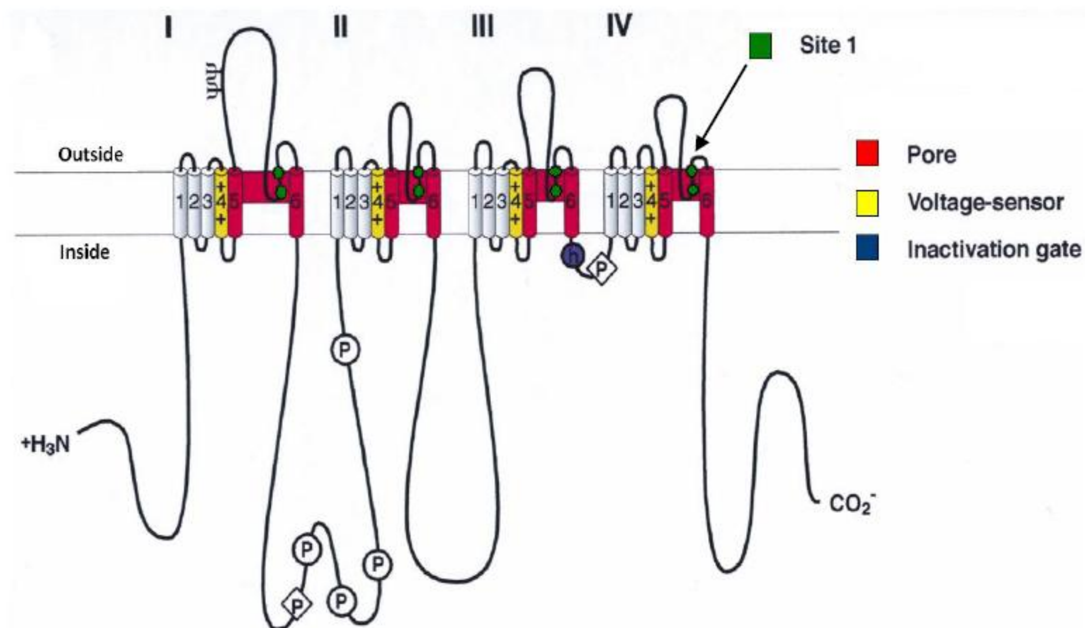


Fig. 3 The proposed transmembrane arrangement of the α-subunit of Na^+ channels. The pore is represented in red, the voltage sensors in yellow and the inactivation gate in blue. PSP is mediated by the interaction and blockage of Site 1 by STX. Figure adapted from (Cestèle and Catterall, 2000; Wiese et al., 2010).

The saxitoxins (STXs) are classified according to their structural variations in the three main groups, namely the carbamoyl, decarbamoyl, and sulfocarbamoyl derivatives. The carbamoyl group (STXs) includes STX, neosaxitoxin (Neo) and the gonyatoxins (GTX 1–4) (Dittmann et al., 2013; Mesquita et al., 2019). In addition, decarbamoyl variants of these analogs also exist, including decarbamoyl-saxitoxins (dcSTX, dcneoSTX), decarbamoyl-gonyautoxins (dcGTXs 1–4), and the 13-deoxy-decarbamoyl derivatives (doSTX, doGTX 2,3) (Wiese et al., 2010). In *Cylindrospermopsis raciborskii* (=*R. raciborskii*) T3 several analogs of STX have been reported in different combinations, including saxitoxin, carbamoyl saxitoxin (STX, NEO, GTX2/3), decarbamoyl saxitoxin (dcSTX) and N-sulfocarbamoyl (C1/2, B1) derivatives (Soto-Liebe et al., 2010).

The name saxitoxin was derived from the butter clam, *Saxidomus giganticus*, from which the toxin was first isolated (Vahle, 2013). Saxitoxins are heat stable and water soluble (Van der Merwe, 2014). They are tasteless and odorless, and are not destroyed by cooking (Trevino, 1998). Saxitoxins can be accumulated in freshwater fish such as tilapia (Galvao et al., 2009; Van der Merwe, 2014).

The cyanobacteria reported in the literature able to produce STXs are as follows: *Anabaena circinalis* and *Aphanizomenon.* (Chorus and Bartram, 1999); *Anabaena lemmermannii* (Kaas and Henriksen, 2000); *Raphidiopsis raciborskii* (=*C. raciborskii*) (Lagos et al., 1999); *Lyngbya wollei* (Chorus and Bartram, 1999; Carmichael et al., 1997) and *Planktothrix* (Pomati et al., 2000).

R. raciborskii has been considered an invasive species (Padisak, 1997; Figueredo et al., 2007; Abreu et al., 2018), and blooms have been reported in several countries. Toxic strains are recorded in Asia, Australia, and New Zealand to produce cylindrospermopsin (Wood and Stirling, 2003 ; Rzymski and Poniedziałek., 2014; Antunes et al., 2015; Rigamonti et al., 2018). Strains of *R. raciborskii* have also been reported to produce saxitoxins in Brazilian waters (Lagos et al., 1999; Molica et al., 2002; Ferrão-Filho et al., 2007; Restani and Fonseca, 2014; Reis 2019).

1.2 *Raphidiopsis raciborskii*

Raphidiopsis raciborskii (Woloszynska) (Aguilera et al. 2018), a filamentous diazotrophic cyanobacterial species, belongs to the order Nostocales, family Nostocaceae (Gugger et al., 2005; Pagni et al., 2020; Werner et al., 2020), with heterocysts and akinetes (Hoff-Risseti et al., 2013; Ling Zheng et al., 2023; Fig. 4). The species *Raphidiopsis raciborskii* until recently known and described in the publications as *Cylindrospermopsis raciborskii* (Wolozyn´ska) Seenayya & Subba, stands out as a bloom forming, potentially toxic, N_2 -fixing heterocytous and invasive cyanobacterium of apparently subtropical-tropical origin. This cyanobacterium is observed spreading into waterbodies and rivers in temperate regions (Padisák, 1997, Briand et al. 2004, Stüken et al. 2006; Werner et al., 2020), helped by its considerable high physiological tolerance (Briand et al. 2004, Sinha et al. 2012). Its global distribution is extremely concerning, not only due to blooms cause a decreasing in diversity in the aquatic ecosystem, but also due to this species can produce harmful toxins such as cylindrospermopsin and saxitoxin (Lagos et al. 1999, Mohamed 2007, Li et al. 2001, McGregor et al. 2011). The potential to produce either cylindrospermopsin (CYN) or saxitoxins (STXs) by *R. raciborskii* is associated with its geographical distribution (Mesquita et al., 2019). Some Australian, Asian, and New Zealand strains of *R. raciborskii* produce CYNs (Wood and Stirling, 2003 ; Rzymski and Poniedziałek., 2014; Antunes et al., 2015; Zare and Bahador, 2015; Rigamonti et al., 2018), whereas South American strains produce STXs (Piccini et al. 2011; Hoff-Rissetti et al., 2013; Lopes et al., 2017,); no isolates have yet been reported with the potential to produce both kinds of toxins at the same time (Haande et al., 2008; Vico et al., 2016).

R. raciborskii strains can have straight or coiled morphology (Burford et al., 2016; Willis et al., 2019), and characterized by presenting isopolar trichomes – solitary, cylindrical cells with minor attenuation towards the ends, and slight or no constriction at the cross walls (Pagni et al., 2020). This species form terminal heterocytes with akinetes which located internally within the trichomes (Alster et al., 2010; Komarek, 2013; Wojciechowski et al., 2016; Guiry & Guiry, 2019; Hauer & Komárek, 2019). Moreover, reproduction is by fragmentation of the trichome or by akinete formation (Pagni et al., 2020).

Taxonomy of *R. raciborskii* has been reviewed many times (Marques et al., 2022). Initially, it was described by Wolozyn´ska (1912) as *Anabaena raciborskii*. Afterwards, the genus *Anabaenopsis*

for individuals with terminal heterocytes created by Miller 1923, and *Anabaena raciborskii* was changed to *Anabaenopsis raciborskii*. Furthermore, the investigation that *Anabaenopsis raciborskii* had a distinct heterocyte differentiation process from all other *Anabaenopsis* species caused the creation of a new genus, *Cylindrospermopsis* (Seenayya & Subba Raju, 1972). Lately, according to the phylogeny and secondary structure of the 16S 23S ribosomal RNA intergenic spacer region, Aguilera et al. (2018) suggested the unification of *Cylindrospermopsis* and *Raphidiopsis* in a single genus. Even before their publication, the taxonomic validity of the two genera had been comprehensively discussed (Moustaka-Gouni et al. 2009, Stucken et al. 2010, Wu et al. 2011, Li et al. 2016), especially due to their major difference was only the presence or absence of heterocytes. Following Aguillera et al., 2018 study, the name *Raphidiopsis* was accepted according to the taxonomic rules of prior description of the genus and principle of priority.

Raphidiopsis raciborskii is a planktonic cyanobacterium known worldwide for forming cyanobacterial harmful algal blooms (cyanoHABs). CyanoHABs may have chronic toxic effects, in humans and other animals (Codd and Bell, 1984; Falconer and Fitzgerald, 1999). This cyanobacterium is increasingly found in tropical and temperate environments of both the Northern and Southern Hemispheres and is considered exotic and invasive (McGregor and Fabbro, 2000; Briand et al., 2004; Jones and Sauter, 2005; Kokociński et al., 2010; Antunes et al. 2015; Babanazarova et al., 2015). Furthermore, it is common in fresh waters, but it also occurs in swamps and slightly brackish waters (1.5-2%) (Padisák 1997, Antunes et al., 2015). In the 1990's, there was an expansion of the species in Brazil, probably due to increasing eutrophication (Tucci and Sant'Anna, 2003). It is now ubiquitous in both tropical and subtropical regions of the country. Most work on this species (including toxicology) is from blooms of Brazilian waters (e.g. Lagos et al. 1999, Bouvy et al. 2000, Molica et al. 2002, Costa et al. 2006, Gemelgo et al. 2008, Hoff-Risseti et al. 2013; Werner et al., 2020).

Many factors have been proposed for the success of *R. raciborskii* globally, such as buoyancy (Ramberg, 1984; Padisák, 1997), N_2-fixation (Padisák, 1997; Burford et al., 2006), low light tolerance (Padisák, 1997), high affinity for ammonia uptake (Burford et al., 2006), resistance to grazing (Padisá k, 1997), tolerance of a wide range in temperature (Briand et al., 2004), P-uptake and storage capacity (Isvánovics et al., 2000). Since *R. raciborskii* is ecologically and toxicologically important, information about biological mechanisms associated with cyanotoxins

produced by *R. raciborskii* in reservoirs and lakes can be important to offer better insight for control and management of cyanotoxins.

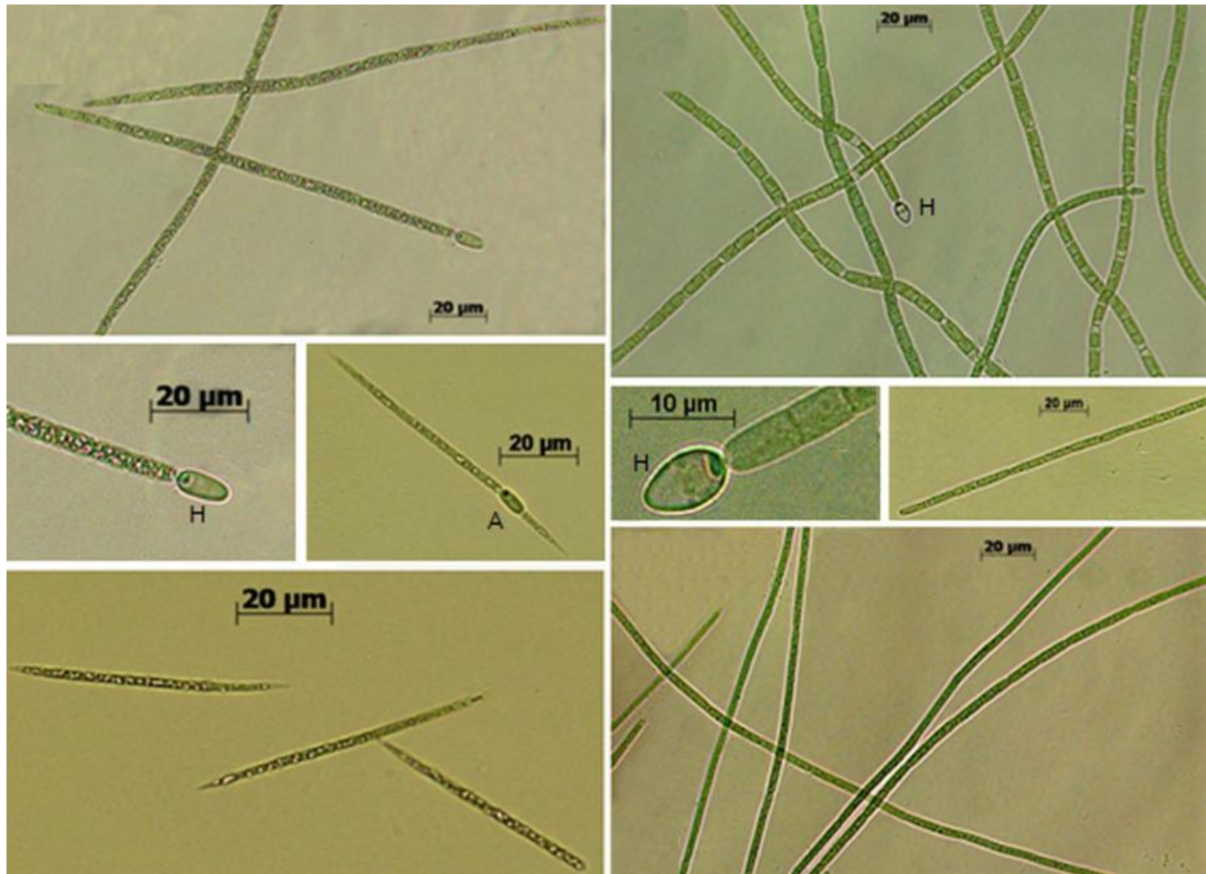


Fig. 4 *R. raciborskii* filament morphologies showing details of heterocyte **(H)** and akinete **(A).** Figure adapted from (Hoff-Risseti et al., 2013).

1.3 Environmental stress

Toxin producing cyanobacteria can tolerate various environmental stresses but it has also been suggested that they produce elevated levels of toxins under stressful conditions (Bouvy et al., 1999; Briand et al. 2002; Kurmayer, 2011; Zilliges et al., 2011; Sinha et al. 2012; Solovchenko et al 2020). Environmental factors such as nutrients, temperature, light intensity, pH, ultraviolet radiation, wind, trace metals, salinity, and pollutants can influence the growth of the cyanobacterial species and their cyanotoxin production (Neilan et al., 2013). Therefore, understanding the

influence of environmental stress on toxin production is crucial for the management of cyanotoxins in waterbodies affected by algal blooms.

Different environmental factors have been proposed as the modulating forces for PSP synthesis in *R. raciborskii*, and several studies have tried to elucidate the ecological role of these toxins as chemical defense, chemical signaling or N storage mechanisms (Murray et al., 2011a; Neilan et al., 2012). The invasion success of *R. raciborskii* was mainly ascribed to its phenotypic plasticity and growth optimization under different environments (Bonilla et al., 2012; Soares et al., 2013; Amaral et al., 2014; Burford et al., 2016; Wu et al., 2022). *R. raciborskii* is tolerant to low and high light intensity and a wide range of temperatures (Bonilla et al., 2012; Tonetta et al., 2015; Bonilla et al., 2016; Dokulil, 2016; Wojciechowski et al., 2016; Antosiak et al., 2020). This species has flexible strategies to use various forms of nitrogen (N), such as accumulating nitrogen as cyanophycin (Lu et al., 2022) and switching to N_2 fixation under N depletion (Moisander et al., 2012; Williset al., 2016; Stucken et al., 2014). With a strong affinity for phosphate, *R. raciborskii* is capable of absorbing and storing a lot of phosphorus (P) (Amaral et al., 2014; Wu et al, 2009). After depletion of inorganic P, dissolved organic phosphorus can be used by *R. raciborskii* through the secretion of alkaline phosphatase (ALP) (Wu et al, 2012; Shi et al., 2022). In addition, it has been suggested that this species has a competitive advantage over other bloom-forming cyanobacteria under N- or P-limited conditions (Wu et al, 2012; Bai et al., 2020; Jia et al., 2020).

The influence of nutrient availability on *sxt* gene expression has been addressed for *Raphidiopsis brookii* D9, a species that is closely related to *R. raciborskii* (Moustaka-Gouni et al., 2009), by comparing the expression of *sxt* genes when the strain was grown under alternative N regimes (Stucken et al., 2014). The results from that study suggested that in *R. brookii* D9 the type of N-source is not directly related with toxin production (Stucken et al., 2010). Similarly, it is still not clear if and how nutrients play a role in CYN production by *R. raciborskii*. For example, in natural phytoplankton assemblages, *R. raciborskii* was shown to increase CYN production under P sufficiency and independently of N additions (Burford et al., 2014), while other studies with cultures showed no effect on CYN production (Willis et al., 2015). Conversely, in other species such as *Aphanizomenon ovalisporum*, P deprivation was shown to induce CYN synthesis, which induced alkaline phosphatase secretion by other phytoplankters (Bar-Yosef et al., 2010). There is, however, less information currently available regarding *R. raciborskii* PSP production and

environmental nutrient concentrations. In spite of the increasing number of publications about *R. raciborskii* physiology and ecology, there are only few studies that have addressed transcription regulation by nutrient depletion (Stucken et al., 2014; Vico et al., 2016).

1.4 Nitrogen (N) and Phosphorus (P) in aquatic environments

Anthropogenic loading of nitrogen (N) and phosphorus (P) to freshwater and coastal marine systems, is a global environmental problem (Smith, 2003; Smith and Howarth, 2006) that generates social and financial costs for human populations (Pretty et al., 2003; Dodds et al., 2009).

The observation that cyanobacteria increase with eutrophication has been recognized for several decades (Hakanson et al,.2007; Dolman et al., 2012). One of the more unpleasant consequences of eutrophication is an increase in the occurrence of unsightly, odorous, and sometimes toxic cyanobacterial blooms (Chorus and Bartram, 1999; Paerl et al., 2011; Dolman et al., 2012). Thus, control of cyanobacterial blooms and cyanotoxins is a major concern for management of aquatic ecosystems.

For a long time, phosphorus was considered the primary nutrient limiting the development of eutrophication and cyanobacterial biomass (Schindler, 1974; Schindler, 1977) despite there being considerable evidence that N limitation is also a common and widespread phenomenon in lakes (Elser et al., 1990; Elser et al., 2007). Nitrogen received less attention in freshwater systems than phosphorus and the role that nitrogen may play in controlling eutrophication is less studied, and in particular its influence on the taxonomic composition of phytoplankton (Lewis and Wurtsbaugh, 2008; Sterner, 2008). Recently, however, there has been a renewed surge of interest and advocacy for nitrogen or dual nitrogen and phosphorus control (Paerl and Scott, 2010; Scott and McCarthy, 2010). Advocates of phosphorus control of eutrophication argue that phosphorus reductions also reduce harmful cyanobacterial blooms caused by nitrogen-fixing species by altering the N:P ratio in favor of other non-nitrogen fixing species (Barica et al., 1980; Tilman et al., 1982; McQueen and Lean, 1987; Stockner and Shortreed, 1988; Jeppesen et al., 2005; Schindler et al., 2008). Alternatively, the P-limitation paradigm also predicts that blooms of N-fixing cyanobacteria are made worse if nitrogen levels are reduced without also decreasing phosphorus concentrations (Schindler, 1977; Smith, 1983). These predictions have received some support from lake survey

data and whole-lake experiments (Barica et al., 1980; Findley and Kasian, 1987; Schindler, 1977; Schindler et al., 2008; Chislock et al., 2014).

1.5 Nutrients and *R. raciborskii*

The growth of *R. raciborskii*, in particular its ability to form blooms, is reported to be influenced by the concentrations of some nutrients that are typically observed in eutrophic environments (Yang et al., 2017). The biomass changes of most species of cyanobacteria are related to nutrient content in aquatic ecosystems (Pagni et al., 2020)

Tolerance to low environmental nitrogen concentrations is related to the presence of specialized cells called heterocytes in several cyanobacterial species as *R. raciborskii*. These cells have a thick cell wall providing an anaerobic intracellular environment. This is required for fixation of atmospheric nitrogen (N_2) and allows *R. raciborskii* to survive in environments with dissolved nitrogen deficiency (Padisák, 1997; Moustaka-Gouni et al., 2007; Wiedner et al., 2007; Everson et al., 2011; Antunes et al., 2015; Barros et al., 2015; Burford et al., 2016).

Tolerance to low phosphorus concentration is due to a higher absorption capacity and affinity of some cyanobacteria and *R. raciborskii* compared to other photoautotrophic organisms. This suggests that *R. raciborskii* is opportunistic with respect to dissolved inorganic phosphorus (Isvánovics et al., 2000). *R. raciborskii* has been observed in aquatic environments with wide variation of phosphorus concentrations (Burford & Davis, 2011; Wu et al., 2012; Wojciechowski, 2013; Burford et al., 2016).

Analyses of *R. raciborskii*'s environmental requirements have demonstrated its great ability to survive in different conditions. The ecological success of *Raphidiopsis* can be attributed to ability to tolerate a range of environmental conditions and its ecophysiologic plasticity, or the existence of ecotypes with different preferences and environmental tolerances (Berger et al., 2006; Bonilla et al., 2012), as it has been noted by several authors (Chonudomkul et al., 2004; Fuentes et al., 2010; Bonilla et al., 2012; Gomes et al., 2013; Soares et al., 2013; Pierangelini et al., 2014b; Antunes et al., 2015; Wojciechowski et al., 2016; Casali et al., 2017; Fabre et al., 2017; Xiao et al., 2017). (Pagni et al., 2020). Due to its tolerance and increasing dispersal in many waterbodies, a better understanding of this cyanobacterium will have implications for the control of the species

and the toxins that it produces, as well as complementing the knowledge about the cyanotoxin function in aquatic ecosystems.

2- OBJECTIVES AND HYPOTHESES

This thesis was divided in two chapters, which objectives and hypotheses are described below.

Chapter 1: laboratory experiments (*in-vitro*)

- The effect of nitrogen and phosphorus availability on saxitoxin gene expression and intracellular toxin production in *Raphidiopsis raciborskii* (Cyanobacteria).

  General objective:

  - Given the critical importance of N and P supplies and metabolism in growth, bloom development and regulation of cyanotoxin production in aquatic environments, this study aimed to compare the effects of limited N and P sources in saxitoxin production by *R. raciborskii*.

  Specific objectives:

  - How nutrients (nitrogen and phosphorus availability) can affect toxin production by *R. raciborskii.*
  - In order to obtain deeper insights into the ecophysiology of SXT production by *R. raciborskii*, we investigated the effect of nitrogen and phosphorus availability on STX production by assessing *sxt* gene expression and intracellular toxin concentrations, as well as some genes related to nutrient metabolism: *ntcA* (global nitrogen regulator), *nifH* (N_2 fixation), and *pstS* (coding for phosphate-binding periplasmic protein).

  Hypotheses:

  1- Lower concentration of phosphorus and nitrogen will stimulate the expression of genes related to P and N metabolism;

  2- At the same time, lower concentration of phosphorus and nitrogen will increase the expression of genes related to the production of saxitoxin.

  3- Lower concentration of phosphorus and nitrogen will also increase the production of intracellular saxitoxin.

Chapter 2: Environmental (field) experiments (*in situ*)

- Estimating saxitoxin gene expression in *Raphidiopsis raciborskii* (Cyanobacteria) and its relationship with phosphorus and nitrogen availability in Pampulha reservoir (Brazil)

General objective:

- The ability of *R. raciborskii* to grow in different nutrient concentration ranges highlights the importance of understanding how nutrients can affect toxin production. With the increasing presence of this species globally, knowledge about the environmental factors affecting STX production will help to understand the dynamics of the toxin in aquatic ecosystems. A better understanding will be very useful when dealing with the management of saxitoxins in aquatic environments.

Specific objectives:

- How environmental nitrogen and phosphorus availability can have an effect on the expression of genes involved in the biosynthesis of saxitoxin by *Raphidiopsis raciborskii* in Pampulha reservoir (Brazil) and nutrient metabolism.
- Although diverse environmental factors such as nutrients, temperature and light intensity have been suggested as triggers for the production of cyanobacteria toxins in reservoirs, the ecological role of SXT in *R. raciborskii* has yet to be clarified, and this study tried to understand environmental nutrient stress conditions on genes expression related to saxitoxin produced by *Raphidiopsis raciborskii.*

Hypotheses:

1- It will be possible to measure the response of genes related to *R. raciborskii* N and P metabolism from field samples;

2- Response to nutrient metabolism, therefore to the environmental nutrient availability, will affect the expression of genes related to toxin production in *R. raciborskii*.

Chapter 1

The Effect of Nitrogen and Phosphorus Availability on Saxitoxin Gene Expression and Intracellular Toxin Production in *Raphidiopsis raciborskii* (Cyanobacteria)

Abstract

Raphidiopsis raciborskii (= *Cylindrospermopsis raciborskii*) is a diazotrophic cyanobacterium, causing harmful blooms in freshwater systems worldwide. Strains of *R. raciborskii* found in South America are characterized by the ability to produce saxitoxin (STX or Paralytic Shellfish Toxin, PST) and its analogs. The availability or scarcity of essential nutrients have the potential to change *R. raciborskii* toxin content. This study examined the effect of nitrogen (N) and phosphorus (P) stress on saxitoxin and its analogs produced by *R. raciborskii*. Reverse transcription-quantitative PCR (RT-qPCR) was used to investigate the transcriptional response of the *sxtA4* gene under nitrogen and phosphorus gradients in two toxic *R. raciborskii* strains. Our results showed that the *sxtA4* gene was generally upregulated when growing under lower nutrient concentration. Correlations were observed among transcripts of *sxtA4* (*sxt* gene), *ntcA* (global nitrogen regulator) and *nifH* (N_2 fixation) genes in N-reduced conditions. In addition, a significant correlation was identified between *sxtA4* and *pstS* (coding for phosphate-binding periplasmic protein) genes in P-reduced conditions. Toxin concentration was measured by High Performance Liquid Chromatography (HPLC). Under N reduction, total intracellular saxitoxin concentration was highest at 10% N-concentration in both strains; almost in line with the changes in *sxtA4* gene expression, which in strain 186 was higher at 10% N, whereas in strain 36 at 1% N. However, the amount of total intracellular toxins significantly decreased under P reduction in both strains, which was contrary to the *sxtA4* gene transcription that showed significant increase under lower P as compared to control (high P) in both strains. This study showed that decreasing nutrients had an effect on intracellular toxin, but while reduction nutrients induced higher gene expression in both N and P experiments, N reduction might lead to higher intracellular toxin production, whereas reducing phosphorus could result in intracellular toxin reduction. These results pointed to important aspects to be considered when dealing with management of cyanobacteria dominated water bodies.

Keywords: cyanotoxins; cyanobacteria; Nostocales; RT-qPCR; nutrient limitation.

1. Introduction

Raphidiopsis raciborskii, previously known as *Cylindrospermopsis raciborskii*, is a filamentous freshwater cyanobacterium with terminal heterocytes, belonging to the order Nostocales, family Nostocaceae (Gugger et al., 2005; Pagni et al., 2020; Werner et al., 2020). The species *Raphidiopsis raciborskii* can potentially produce secondary metabolites, called cyanotoxins, such as cylindrospermopsin (CYN) and saxitoxins (STXs), which can be extremely harmful to humans and animals (Carmichael et al., 2001; Guzm_an-Guill_en et al., 2015; Lopes et al., 2017). The ability to produce either cylindrospermopsin (CYN) or saxitoxins (STXs) by *R. raciborskii* is related to its geographical distribution (Mesquita et al., 2019). Some Australian, Asian, and New Zealand strains of *R. raciborskii* produce CYNs (Wood and Stirling, 2003 ; Rzymski and Poniedziałek., 2014; Antunes et al., 2015; Zare and Bahador, 2015; Rigamonti et al., 2018), while South American strains produce STXs (Piccini et al. 2011; Hoff-Rissetti et al., 2013; Lopes et al., 2017,); no isolates have yet been described with the ability to produce both kinds of toxins simultaneously (Haande et al., 2008; Vico et al., 2016). Saxitoxins are also found in marine dinoflagellates and are responsible for events of human poisoning by consumption of seafood, such as shellfish and fish, which accumulate these toxins (Ibelings and Chorus, 2007; Etheridge, 2010; Lopes 2017). The molecular formula of saxitoxin (STX) is $C10H17N7O4$ (Molecular Weight = 299) and is composed of a 3,4-propinoperhydropurine tricyclic system. A dose of around 1 mg of STX from a single serving of contaminated shellfish is fatal to humans (Schantz., 1957; Wiese et al., 2010).

Saxitoxin (STX) and its 57 analogs are a large group of natural neurotoxic alkaloids, widely known as the paralytic shellfish toxins (PSTs). Poisoning with PSTs can cause a serious and sometimes fatal disease known as paralytic shellfish poisoning (PSP) or saxitoxin pufferfish poisoning (SPFP) (Landsberg et al., 2006; Wiese et al., 2010). PSTs perform by inhibition of voltage-gated channels in vertebrates, such as sodium, calcium and potassium channels (Wang et al., 2003; Vico et al., 2016). Although PST biosynthesis is complex, all producer organisms seem to use the same biosynthetic route (Shimizu, 1993). The saxitoxin biosynthetic gene cluster (*sxt*) was recognized in cyanobacteria, which provided new insight into the biosynthesis of saxitoxin and its analogs (Kellmann et al., 2008; Mihali et al., 2009).

The *sxt* gene cluster within each organism contains a core set of genes putatively responsible for the biosynthesis of STX. However, the gene profile between each cluster differs, resulting in the production of a different suite of STX analogs by each organism (Gallacher et al., 1997; Prol et al., 2009; Wiese et al., 2010). In cyanobacteria, biosynthesis of STX is catalyzed by several enzymes otherwise rare in microbial metabolism (Wiese et al., 2010). The core PST biosynthetic gene, *sxtA*, is thought to have a chimeric origin and is putatively responsible for the initiation of STX biosynthesis, catalysing 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; Moustafa et al., 2009).

The saxitoxins (STXs) are classified according to their structural variations in the three main groups, namely the carbamoyl, decarbamoyl, and sulfocarbamoyl derivatives. The carbamoyl group (STXs) includes STX, neosaxitoxin (Neo) and the gonyatoxins (GTX 1–4) (Dittmann et al., 2013; Mesquita et al., 2019). In addition, decarbamoyl variants of these analogs also exist, including decarbamoyl-saxitoxins (dcSTX, dcneoSTX), decarbamoyl-gonyautoxins (dcGTXs 1–4), and the 13-deoxy-decarbamoyl derivatives (doSTX, doGTX 2,3) (Wiese et al., 2010). In *Cylindrospermopsis raciborskii* (=*R. raciborskii*) T3 several analogs of STX have been reported in different combinations, including saxitoxin, carbamoyl saxitoxin (STX, NEO, GTX2/3), decarbamoyl saxitoxin (dcSTX) and N-sulfocarbamoyl (C1/2, B1) derivatives (Soto-Liebe et al., 2010).

As *R. raciborskii* strains produce toxins, concerns related to water quality issues are increasing as its range or dominance has expanded from tropical into temperate regions. One reason for the success of *R. raciborskii* is its potential to dominate under variable nutrient conditions, for example, even at low concentrations of phosphorus (Bonilla et al., 2012; Dolman et al., 2012; Amaral et al., 2014; Willis et al., 2017) and of dissolved nitrogen (Présing et al., 1996; Koma´rkova et al., 1999; Dokulil and Teubner, 2000; McGregor and Fabbro, 2000; Vico et al., 2016).

Phosphorus (P) is one of the essential and major macronutrients needed for growth of phytoplankton, therefore P depletion in aquatic ecosystems is usually suggested to decrease phytoplankton blooms (Armstrong, 1999; Willis et al., 2017). Even though *R. raciborskii* has more often been described in eutrophic environments (Antunes et al., 2015; Burford et al., 2016; Pagni et al., 2020), it was also observed that it can still grow where P concentrations are near detection

limits, thanks to its ability of rapid uptake and P storage in the cell (Istavánovick et al. 2000; Burford & O'Donohue, 2006; Burford et al., 2006; Posselt et al., 2009; Prentice et al., 2015; Willis et al., 2017). *R. raciborskii* is also able to produce cytoplasmic polyphosphate granules, demonstrating high phenotypic plasticity and considerable ability to thrive under low-phosphate conditions (Amaral et al., 2014; Rigamonti et al., 2018).

Additionally, as a facultative diazotroph, *R. raciborskii* can alternatively use nitrogen (N) by atmospheric N_2 fixation, aside from assimilation of several forms of dissolved inorganic N compounds in the water (Sinha et al 2014). This characteristic allows the species to thrive in systems with low concentrations of dissolved N showing competitive advantage against other planktonic species (Harris and Baxter, 1996; McGregor and Fabbro, 2000; Dokulil and Teubner, 2000; Spröber et al., 2013).

Thus, the species shows a number of strategies for succeeding under nitrate- or phosphate deprived conditions, which makes predictions regarding the effect of nutrients on toxin production more challenging and important to investigate.

Cyanobacterial toxin production can be affected by several environmental and physical parameters, such as light, temperature, phosphorous, nitrogen, pH and trace metals (Rigamonti et al., 2018; Wang et al., 2018; Vico et al., 2016; Van der Westhuizen and Eloff, 1985; Song *et al*., 1998; Sivonen, 1990; Lukac and Aegerter, 1993). Among these factors, the effect of nutrients has been tested, for example, on the production of the hepatotoxic toxin microcystin in *Microcystis* and some studies showed that lower nutrient levels could promote an increase in toxin production (Ginn & Neilan, 2011; Pimentel & Giani, 2014; Alexova et al., 2016), suggesting a potential function of this toxin when producing species grow under stress conditions (Neilan et al. 2013). Authors have also discussed potential roles of cyanotoxins as evolutionary adaptations for competitive advantages and improved physiological functions for their producers in the environment (Holland & Kinnear, 2013).

Saxitoxins belong to a different group of cyanotoxins than microcystin, therefore in order to obtain deeper insights into the ecophysiology of SXT production by *R. raciborskii*, we investigated the effect of nitrogen and phosphorus availability on STX production by assessing *sxt* gene expression and intracellular toxin concentrations. Based in the previous studies cited above and under the assumption that STX would be related to nutritional conditions, we hypothesized that nutrient

deprivation (either nitrogen or phosphorus) would induce the expression of the genes involved in toxin synthesis, resulting in a higher production of STX in *R. raciborskii*. To follow the effect of N and P reduction on the species physiology, known-response genes were selected as markers. For nitrogen, the global nitrogen regulator NtcA activates the genes which are responsible for assimilation and uptake of alternative nitrogen sources (Herrero et al., 2001; Stucken et al., 2014), and for this reason it has been used as a good marker in studies following response to nitrogen limitation (Lindell, and Post., 2001; Pimentel and Giani, 2014). Additionally, the expression of the *nifH* gene, which encodes a subunit of the nitrogenase enzyme in nitrogen fixing cyanobacteria was evaluated. The presence of *nifH* transcripts under N limitation is an indication of *R. raciborskii* ability to fix atmospheric N_2. Finally, for phosphorus, the P-specific transport system (Pst) consists of a periplasmic Pi-binding protein (PstS) and sensitively responds to P deficiency (Wang et al., 2018), therefore the expression of the *pstS* gene was used to assess phosphorus deprivation.

The ability of *R. raciborskii* to grow at different range of nutrient concentrations indicate the importance to understand how nutrients can affect toxin production. As the presence of this species increases globally, the knowledge about environmental factors influencing STX production will help to understand toxin dynamics in aquatic ecosystems. Understanding this response will be extremely useful when dealing with cyanobacterial bloom management in aquatic environments.

2. Materials and methods

2.1 Culture conditions and experimental design

Two *R. raciborskii* toxic strains (strains UFMG-36 and UFMG-186) were obtained from the Cyanobacteria and Algae Culture Collection of the Phycology Laboratory of the Universidade Federal de Minas Gerais (Belo Horizonte, Brazil). Previous analyses in the laboratory (data not published) observed that both strains were toxic and that strain 186 was slightly more toxic than strain 36, thus they were selected to compare the response of strains with different toxin contents. Strains were originally isolated from Brazilian waterbodies (strain 36 from Lagoa Santa Lake and strain 186 from Pampulha Reservoir). Prior to the experiments, *R. raciborskii* was maintained in

WC medium (Guillard and Lorenzen.,1972), photoperiod of 12h light:12h dark cycles, around 40 µmol photons m^{-2} s^{-1} irradiance, and temperature at 22 ±1 °C.

For the experiments, different nutrient treatments were used by modifying nitrate ($NaNO_3$) and phosphate (K_2HPO_4) concentration in regular WC medium; when decreasing the amount of $NaNO_3$ and K_2HPO_4, they were replaced by the equivalent amount of NaCl and KCl, respectively, for maintenance of ion balance. For the nitrogen (N) experiments, strains were grown under the original nitrate concentration in WC medium, and two nitrogen-reduced conditions: medium and low N concentration (1/10 and 1/100 dilution of $NaNO_3$, respectively). A similar procedure was performed for the phosphorous (P) experiments: the original phosphate concentration in WC medium, and two phosphate-reduced conditions: medium and low P concentration (1/10 and 1/100 dilution of K_2HPO_4, respectively).

To ensure an acclimatization period, *R. raciborskii* strains were inoculated in the three different nutrient levels and grown under these conditions for about one week. After that, cultures were gently filtered through a cellulose membrane (0.8 µm pore size, MFS-Micro Filtration Systems) to remove all residual nutrients and the cyanobacterial cells retained on the filter were resuspended in 350 ml fresh medium, at the three experimental nutrient concentrations, and were allowed to grow for two days. Thereafter, subsamples were inoculated in triplicate in 250 ml at their respective concentration, and the experiment started. The experiments were finalized after 6 days, during the exponential growth phase. Several 20 mL aliquots of each replicate were filtered throughout glass fiber filters (GF-1, Macherey-Nagel). Filters for chlorophyll and toxin measurements were kept frozen at -20ºC until analysis. Filters for RNA extraction were kept in TRIzol (Invitrogen) at -80ºC. Furthermore, 2 ml aliquots of each sample were harvested and fixed with Lugol solution (0.5%) to estimate cell numbers. Cell counting was done in a Fuchs Rosenthal hemocytometer. All treatments were performed in triplicate (three independent subcultures for each treatment, growing under the same experimental conditions). All steps of the experimental procedure were performed under sterile conditions.

2.2 Chlorophyll *a* measurement

Chlorophyll *a* concentration was measured according to Nusch (1980). Chlorophyll *a* was extracted from the frozen filters by hot ethanol and measured in a spectrophotometer (PerkinElmer) at a 665-nm wavelength.

2.3 RNA extraction and cDNA synthesis

The filters of cyanobacterial cells were immersed in TRIzol (Invitrogen) and kept at -80ºC. For the RNA extraction, samples were quickly deep-frozen in liquid nitrogen and mechanically grounded by a homogenizer. Afterwards, the RNA was extracted according to TRIzol manufacturer's recommendations. Total RNA was suspended in 30 µL of DEPC-H_2O, and RNA was treated with 0.5 µL of DNase (Promega) at 37°C for 30 min. In addition, 1.4 µL EDTA 0.1 molar was added. The reaction was stopped by heating 10 min at 75°C. RNA was quantified using a Qubit Assay (Invitrogen). The extracted RNA was subjected to reverse transcription to produce DNA copies (cDNA) with a High Capacity kit (Applied Biosystems) with RT random primers. Concentrations and PCR cycling conditions were established according to the manufacturer's recommendations.

2.4 Quantitative PCR (qPCR)

The obtained cDNA was used for RT-qPCR to quantify the relative abundance of *sxtA4, ntcA, nifH* and *pstS* transcripts. Real-time PCR was applied using a StepOne system (Applied Biosystems) with 1 µL of cDNA each sample, 0.3 µL of each primer (10 pmol μL^{-1}), 5 µL of Power SYBR green I (Applied Biosystems), and sterile Milli-Q water for a final volume of 10 µL. The reactions were performed in four replicates, and specifications for the PCR cycle followed the manufacturer's guidelines. We measured the transcript levels of genes involved in saxitoxin gene (*sxtA4*), global nitrogen regulator (*ntcA*), N_2 fixation (*nifH*), and the gene coding for the PstS phosphate-binding protein (*pstS)*. Specific primers which were used in this study are described in Table 1. The new primer was designed with help of Primer-BLAST tools (NCBI) to amplify 132-bp products for the *pstS*. As in previous studies, the primer described for 16S rRNA was used as a housekeeping gene (Kaebernick et al., 2000; Wu et al., 2013; Pimentel and Giani., 2014; Marques et al., 2022). Relative quantification of the *sxtA4, nifH, ntcA* and *pstS* target genes was calculated

with 16S rRNA as reference gene and was represented as the change in transcription, which was calculated according with the formula Log_2 $(2^{-\Delta\Delta Ct})$, where $\Delta\Delta C_T$ = ΔC_T target gene – ΔC_T control, and results were compared to the control conditions. As reported by Pfaffl (2001) and Bustin et al., (2009) threshold cycle values (ΔC_T= C_T target gene – C_T housekeeping gene) were used for correlation analyses among *sxtA4, nifH, ntcA* and *pstS* genes.

The analysis of the fluorescent melting curve was carried out to recognize the amplification melting temperature of the single PCR products in the samples, by steadily rising the temperature from 70°C to 95°C at a rate of 0.1°C S^{-1}. Fluorescence intensity data were gathered constantly and were converted to melting peaks employing the LightCycler software (StepOne Software, version 2.0).

Table 1. Amplification primers used for qPCR

Primer	Type[a]	Sequence 5′–3′	Amplicon size (bp)	Function	Reference
sxtA4 **sxtA4**	F R	GGACTCGGCTTGTTGCTTC CCAGACAGCACGCTTCATAA	200	*sxtA* gene (Initiation of STX biosynthesis)	(Hoff-Risseti et al., 2013)
ntcA **ntcA**	F R	TGCGGTGGAATTGCTCTCTT CTGTTTGCAGAATCCGCGAG	113	Global nitrogen regulator	(Marques et al., 2022)
nifH **nifH**	F R	CGTAGGTTGCGACCCTAAGGCTGA GCATACATCGCCATCATTTCACC	297	N_2 fixation	(Gugger et al., 2005)
pstS **pstS**	F R	AAGCTGGGACGGTATTTGGGGG TACCAAACGTCCTATGCGCC	132	Coding for phosphate-binding periplasmic protein	(This study)
16S-rRNA **16S-rRNA**	F R	AGAAAAGAGGTTTACGACCCAAGAGC TGAAAGATTTATTGCCTGGAGATGAGC	267	Housekeeping gene	(Wu et al., 2013)

[a] F: forward; R: reverse.

2.5 Intracellular toxin measurements

2.5.1 STXs sample processing

The analysis of carbamoyl group (STXs) includes STX, neosaxitoxin (Neo), gonyatoxins (GTX 1–4), and decarbamoyl saxitoxin (dcSTX) were performed by High Performance Liquid Chromatography (HPLC) according to Oshima (1995) and Van et al. (2011). Saxitoxin extraction was performed with a 0.05 M acetic acid solution, through sonication of the samples and successive

centrifugation at 10,000 rpm. The procedure was performed three times, to ensure a good extraction of the material. Saxitoxin analyses were done by HPLC on a Waters Alliance 2695 system coupled with a fluorescence detector (Waters 2475), and equipped with a reversed phase C8 silica-based column (Waters Symmetry). The chromatographic procedure consisted of elution under isocratic conditions, using two different mobile phases (0.8 mL min^{-1}) for each toxin group (Oshima, 1995; Van et al., 2011). The mobile phase was composed of 2 mM sodium heptanesulfonate, 30 mM phosphoric acid and 5 ml of acetonitrile for each 100 ml of solution (pH 7.1). Post-column derivatization was carried out in an oven at 85°C, with the oxidizing reagent made of 7 mM periodic acid and 50 mM dipotassium phosphate (pH 9.0) and the acidifying reagent of 0.5 M acetic acid. The identification and quantification of compounds was performed using the fluorescence detector with excitation at 330 nm and emission at 390 nm. Peaks were identified and quantified by comparison with certified analytical standards of saxitoxins obtained from CIFGA (Spain).

2.6 Statistical analyses

Statistical analyses were performed by using SPSS software (version 26). Analysis of variance (ANOVA) and two-tailed Levene's test for equality of variances were applied to analyze differences of the relative gene expression and intracellular toxins between treatments under different nutrient concentrations. Contrast analysis was used to verify differences among treatments. Significant difference was accepted when P values were ≤ 0.05. Regression analyses were performed between ΔC_T *sxtA4* and the known-response genes *nctA, nifH and pstS*. To increase the number of samples and the significance of the correlation and to test for general trends, all measured values for each nutrient concentration were pooled together (all replicates and experimental repetitions).

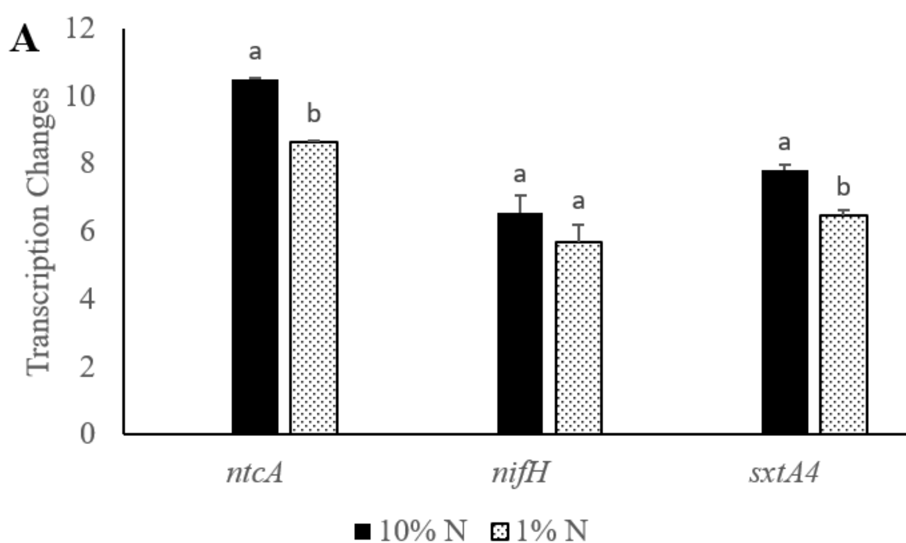
3. Results

3.1 Gene response under nitrogen and phosphorus gradient

The treatment with the highest nutrient concentration (control) was always used as a reference for the quantification of the gene expression of the treatments with reduced nutrients: medium concentration (10% N or P) and low concentration (1% N or P).

The *ntcA* gene showed an increase in its expression in all nitrogen limited conditions (Fig. 1, A and B). For strain 186, the gene was upregulated in both N concentrations ($P < 0.001$), while in strain 36 a significant increase in the expression level was observed under the lower N concentration ($P \leq 0.003$) (Fig. 1, B).

In both strains (186 and 36) was also observed a significant increase in *nifH* gene expression in both conditions of decreased nitrogen as compared with the control (strain 186 $P \leq 0.002$; strain 36 $P < 0.001$).



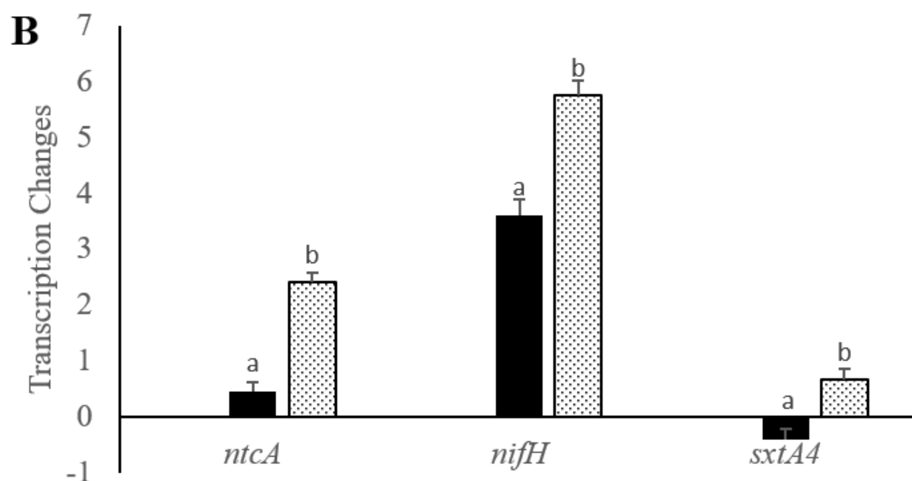
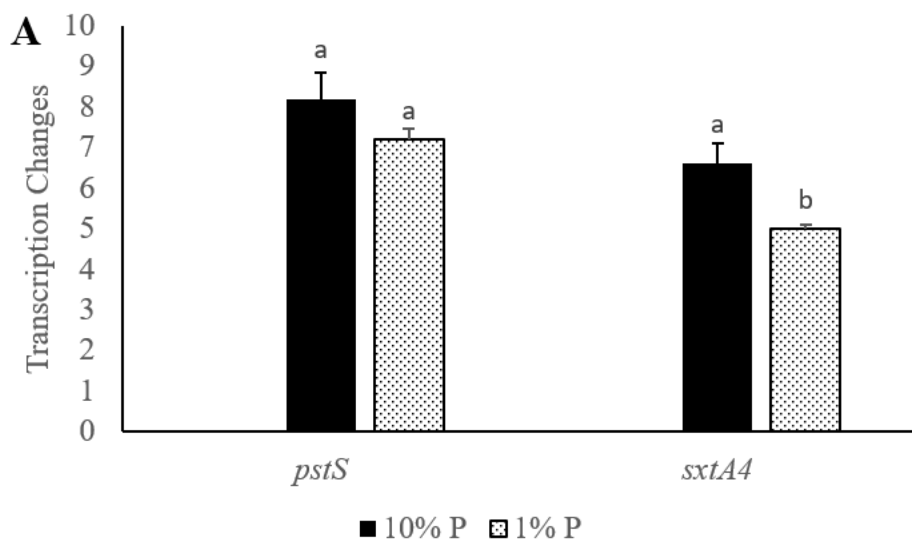


FIG 1. Fold change (log_2 relative quantification) of *ntcA, nifH* and *sxtA4* genes expression in the nitrogen (N) assay for two cyanobacterial strains (A: 186, and B: 36). The original N concentration (100% N) was used as control (indicated by 0 on the y axis) to estimate changes in gene expression under decreased concentration: medium (10%) and high (1%) N. Different letters above the bars indicate significant differences for each gene ($P \leq 0.05$), when the two treatments were compared to the control and among themselves. Whiskers indicate standard errors.



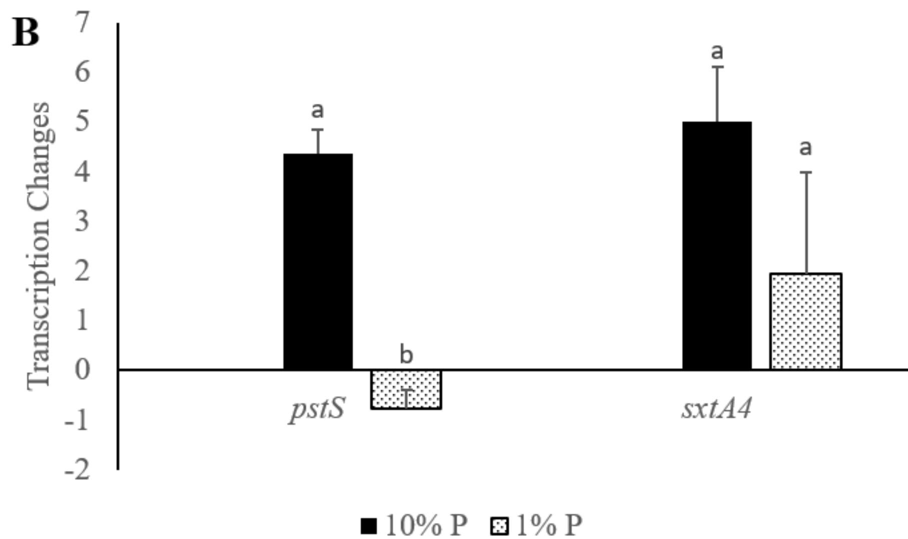


FIG 2. Fold change (log_2 relative quantification) of *pstS* and *sxtA* genes expression in the phosphorus (P) assay for two cyanobacterial strains (A: 186, and B: 36). The original P concentration (100% P) was used as control (indicated by 0 on the y axis) to estimate changes in gene expression under decreased concentration: medium (10%) and high (1%) P. Different letters above the bars indicate significant differences for each gene ($P \leq 0.05$), when the two treatments were compared to the control and among themselves. Whiskers indicate standard errors.

The *sxtA4* gene expression showed changes under all nitrogen conditions. In strain 186, it had a significant increase ($P < 0.001$) under both N concentrations as compared with the control (Fig. 1, A), while in strain 36 a significant increase ($P < 0.05$) was only observed under at 1% N concentration, when nitrogen was reduced 100 times compared to the control condition (Fig. 1, B).

In general, the analysis of *ntcA*, *nifH* and *sxtA4* transcription changes showed that the genes were upregulated in both reduced N concentrations (10% N and 1% N). The regulation pattern was similar for the three genes in strain 186 (Fig. 1, A), with higher expression at the medium N concentration. Whereas in strain 36, the expression of these genes was significantly higher in the lower N concentrations (1% N) (Fig. 1, B). These results showed that while different *R. raciborskii* strains showed similar trends to the stress conditions by increasing the expression of the target genes under N limitations, there were differences in the response related to the level of nutrient concentration able to promote the highest transcriptional change.

In strain 186, the *pstS* gene was upregulated in both P concentrations compared to the control ($P \leq 0.001$) (Fig. 2A). However, in strain 36, the expression of *pstS* gene only increased under medium P concentration (10% P) ($P < 0.05$), while was downregulated at 1% P (Fig. 2B).

Regarding *sxtA4* gene expression in the phosphorus reduction experiment (Fig. 2A and B), although both strains (186 and 36) showed noticeable increase of *sxtA4* gene expression in both P reduction levels, a significant higher expression of *sxtA4* gene was observed in the medium P concentration (10% P), when phosphorus was reduced 10 times, compared with control condition (strain 186 $P = 0.005$; strain 36 $P < 0.02$). At 1% P, the *sxtA4* gene was still upregulated, however less.

As a whole, the *ntcA, nifH* and *pstS* genes showed an increase in expression in the experiments, as expected under nitrogen and phosphorus reduction. The analysis of *sxtA4* gene expression also showed general trends of upregulation under these nutrient conditions, suggesting that nutrient stress may promote toxin production.

Correlation analyses of gene expression between pair of genes, *ntcA* and *sxtA4, nifH* and *sxtA4* and *ntcA and nifH* (Fig. 3A, B, C) for the N experiments, as well as between *sxtA4 and pstS* for the P experiments (Fig. 3D) showed high correlations between all pairs of genes. The R^2 values were always close to or above 0.7 demonstrating that there is a significant correlation between nutrient diminution and the expression of the *sxtA4* gene. The highest correlation was found between *ntcA* and *sxtA4* (R^2 value= 0.979) indicating how changes in the nitrogen metabolism had a strong effect on saxitoxin production (Fig. 3A).

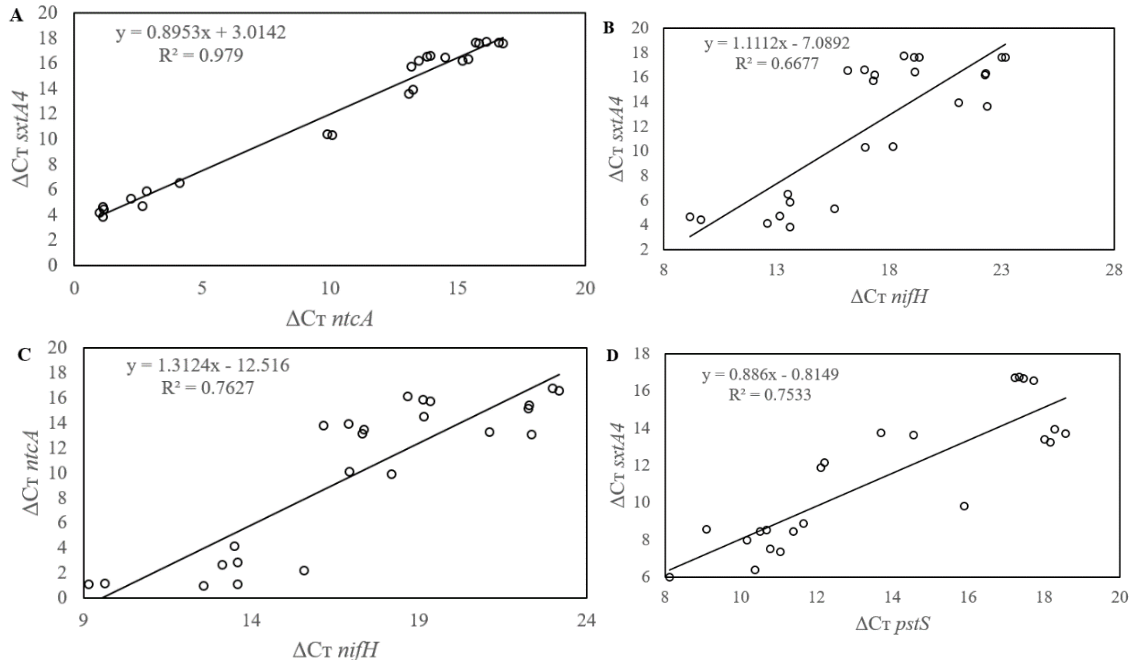


FIG 3.

Regression analyses between ΔC_T values of the expression of **(A)** *ntcA* and *sxtA4*, **(B)** *nifH* and *sxtA4*, **(C)** *ntcA* and *nifH* genes for all experimental data obtained from the nitrogen experiments and **(D)** *pstS* and *sxtA4* genes for all experimental data obtained from the phosphorus experiments.

3.2 Intracellular toxins and chlorophyll *a*

The analyses performed by HPLC on the two strains detected the existence of toxins belonging to the carbamoyl group (STXs) and included saxitoxin (STX), neosaxitoxin (NeoSTX), gonyautoxins (GTX 1–4), and decarbamoyl saxitoxin (dcSTX).

In the nitrogen experiment, strain 186 showed the presence of only two gonyautoxin variants (GTX2 and GTX3) (Fig. 4A), while five different toxins were identified in strain 36 (GTX1, GTX2, GTX3, NeoSTX and dcSTX) (Fig. 4B). Although the amount of GTX2 and GTX3 in strain 186 was higher in medium N concentrations (10% N), statistical analysis showed non-significant differences between control and the two N conditions ($P > 0.05$). On the other hand, in strain 36, we observed that the toxin profile changed according to the N level. For example, GTX1 was not detected in the control but significantly increased in both N concentrations ($P < 0.05$), especially at 1% N ($P < 0.05$). NeoSTX was only identified at 10% N.

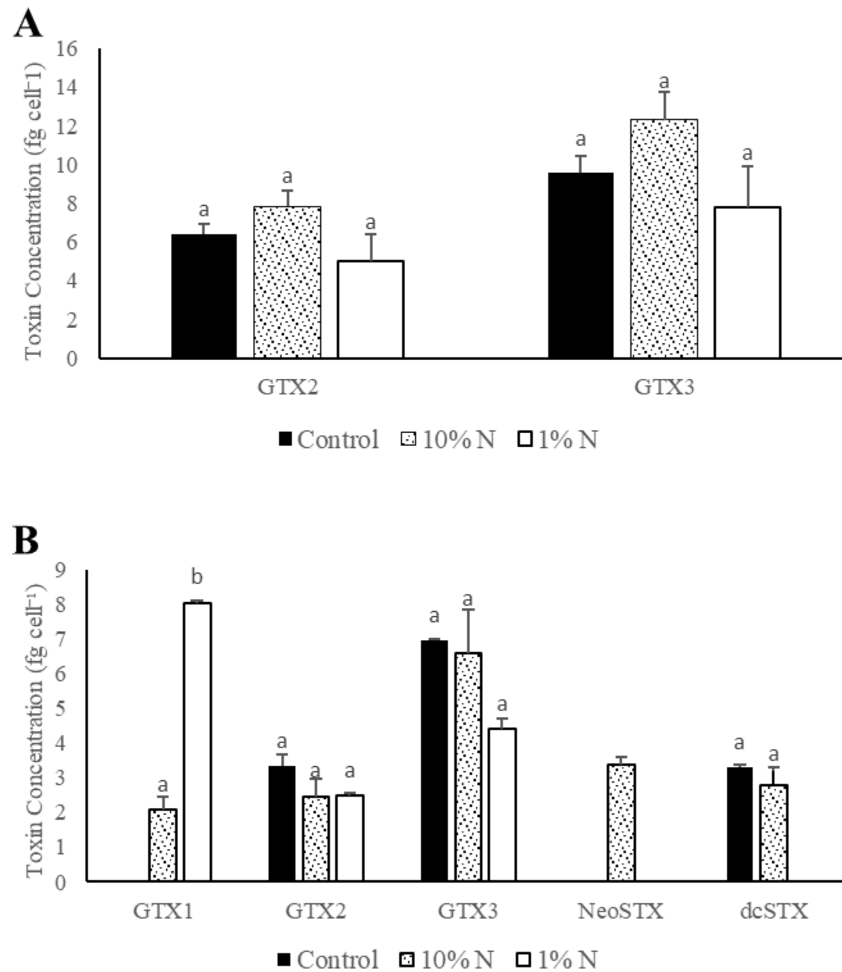


FIG 4. Toxin concentrations per cell for strains 186 (A) and 36 (B) at three nitrogen (N) concentrations: original N concentration (control: 100% N), medium (10%) and low (1%) N. Different letters above the bars indicate significant differences ($P \leq 0.05$) among treatments. Values represent average. Whiskers indicate standard errors.

And production of dcSTX was suppressed at 1% N. However, approximately the same amount of GTX2 and GTX3 toxins was produced in all N treatments and there were no significant differences among the N treatments ($P > 0.05$).

In the phosphorus experiment, GTX2, GTX3, GTX4 and STX were identified in strain 186 (Fig. 5A); GTX2 significantly decreased in both P-reduced treatments as compared with the control (*P*

< 0.05). GTX3 was produced in all P treatments with no significant differences treatments (P > 0.05). GTX4 was only detected after P reduction, at 10% and 1%, there was no significant difference between the two. Also, SXT was produced only at 1% P treatment. On the other hand, GTX1, GTX2, GTX3 and dcSTX toxins were detected in strain 36 (Fig. 5B). GTX1 was only produced in both P-limited treatments, but there was no significant difference between treatments (P > 0.05). Whereas, the production of GTX2, GTX3 decreased significantly in both P-limited treatments as compared with control treatment (P < 0.05). Furthermore, dcSTX did not show significant difference among P treatments.

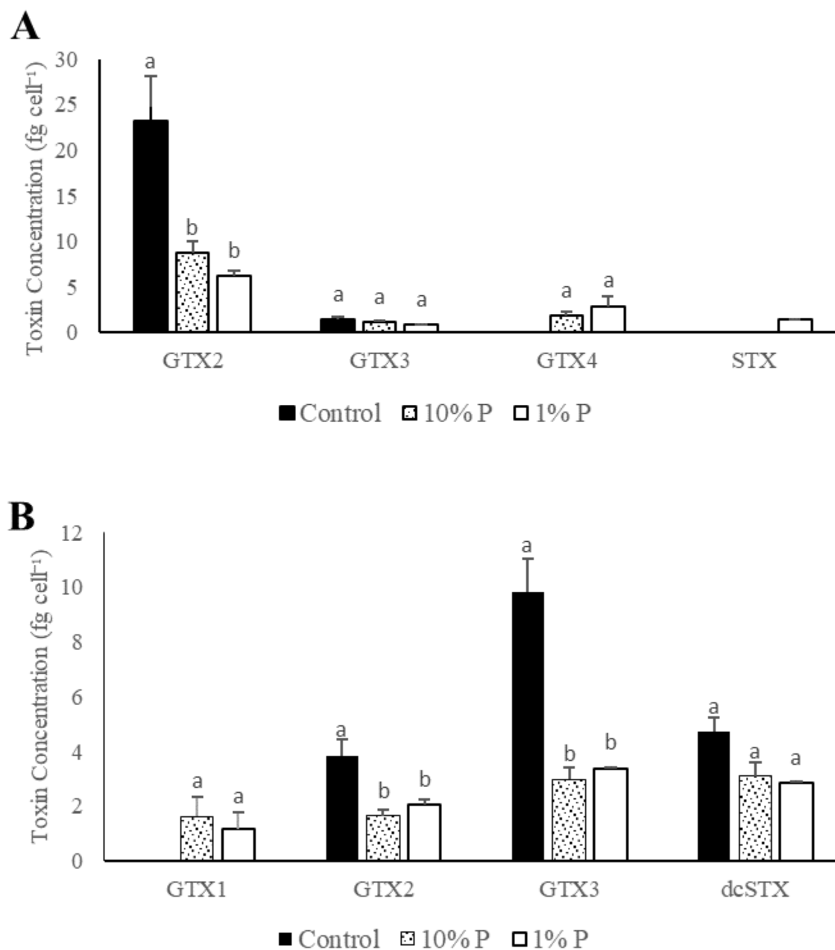


FIG 5. Toxins concentration per cell for strains 186 (A) and 36 (B) at three phosphorus (P) concentrations: original P concentration (control: 100% P), medium (10%) and high (1%) P. Different letters above the bars indicate significant differences ($P \leq 0.05$) among treatments. Values represent average. Whiskers indicate standard errors.

Figure 6 summarizes trends of total intracellular toxins concentration and chlorophyll *a* in each strain and treatment. As expected in photosynthetic autotrophs growing under nutrient reduction, chlorophyll *a* concentration decreased as the amount of nitrogen and phosphorus provided to the culture medium was reduced, confirming the occurrence of metabolic changes in the cells. In strain 186, a significant difference was found in chlorophyll *a* concentration between control and 1% N concentration ($P < 0.05$), while in strain 36, there was significant differences among all treatments ($P < 0.05$). In the phosphorus experiment, both strains showed significant and nearly similar decrease in both P concentrations as compared to the control (strain 186 $P= 0.001$; strain 36 $P \leq 0.001$). There was no significant difference between the two P reduced concentrations for both strains ($P > 0.05$).

In both strains, total saxitoxin concentration was higher and significantly different in medium N treatment (10% N) as compared with the other treatments (Fig. 6) and increased 1.3 times as compared with the control. In strains 36, toxins were also significantly higher at 1% N than in the control, but not higher than at 10%. However, in the phosphorus experiment, total intracellular toxins concentration significantly decreased around twofold in P limitations compared to the control, in both strains (Fig. 6). Because these results represent the sum of all toxin variants, they are slightly different from the individual toxins represented in previous figures. In summary, while chlorophyll *a* always decreased under nutrient stress, toxins showed some significant increase under certain N reduced conditions, but not under P limitation.

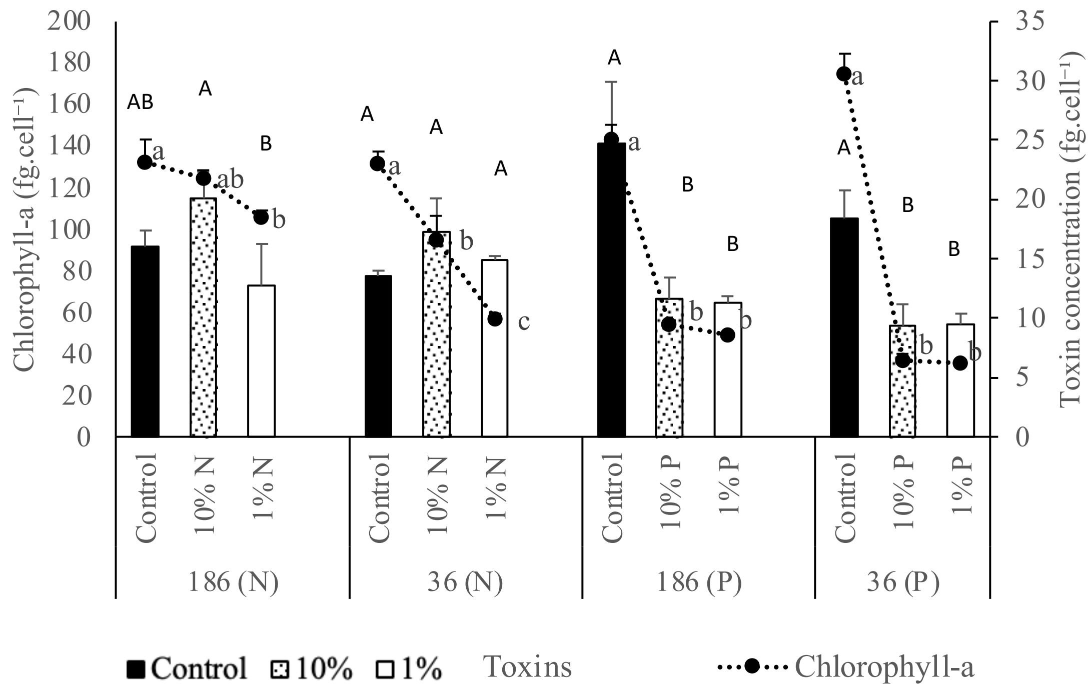


FIG 6. Chlorophyll-a and total toxins content per cell, for each strain (186 and 36) at three nutrient concentrations, in the nitrogen (N) and phosphorus (P) experiments. Different lowercase letters besides black circles indicate significant differences in chlorophyll concentration ($P \leq 0.05$); different capital letters above bar charts indicate significant differences in total toxins concentration ($P \leq 0.05$). Values represent average and were compared to the control and among themselves for each strain. Whiskers indicate standard errors (when not visible because very small).

4- Discussion

Environmental factors, such as nutrients, temperature, light and salt concentrations were previously studied for their influence on saxitoxin production (Dias et al., 2002; Castro et al., 2004; Pomati et al., 2004; Carneiro et al., 2009; Yunes et al., 2009; Boopathi & Ki., 2014). Based in previous observations of changes in toxin production in cyanobacteria strains submitted to stress conditions (Soto-Liebe et al., 2012; Stucken et al., 2014; Rigamonti et al., 2018; Mesquita et al., 2019), in this study we tested the hypothesis that expression of genes involved in saxitoxin synthesis would be upregulated when *R. raciborskii* strains were exposed to nutrient reduction, specifically to nitrogen and phosphorus. Our results were in general in line with this hypothesis, as gene expression of *sxtA4* increased in strains growing under reduced concentrations of nitrogen and phosphorus,

showing that *sxtA4* transcription can be influenced by nutrient stress. Additionally, the very good correlation observed between *sxtA4* and *nctA, nifH* and *pstS* gene transcription, which are directly related to nutrients' metabolism, confirmed the significant relationship of saxitoxin in this cyanobacterium to nitrogen and phosphorus limitation at the transcriptional level.

NtcA is a reliable marker to investigate nitrogen limitation, since it activates nitrogen-responsive genes (Lindell & Post., 2001). In cyanobacteria, nitrogen control is performed by NtcA at the transcriptional level (Herrero et al., 2001; Álvarez-Escribano et al., 2018). This protein is required for gene expression in ammonium and nitrate uptake pathways, and for heterocyte development as well (Wei et al., 1993; Wei et al., 1994). Under nitrogen deprivation, the *ntcA* gene is quickly induced and autoregulated (Wei et al., 1994; Ramasubramanian et al., 1996; Muro-Pastor et al., 2002). Lindell & Post (2001) reported that *ntcA* gene transcription in *Synechococcus* sp. strain WH7803 was enhanced when ammonium levels declined to 1 mM, while the increase of more than 1 mM ammonium caused a quick decrease in *ntcA* gene transcription. Furthermore, Lindell et al. (1998) using the same organism reported the highest level of *ntcA* transcription under conditions of nitrogen deprivation. Higher transcription of *ntcA* seems to imply lack of nitrogen (Lindell & Post 2001).

The common model of transcription which anticipated for NtcA (an activator regulatory protein) involves an enhancement in transcription. Afterwards, while manufacturing of the activator protein increases, transcription of the target protein is upregulated as well (Ginn et al., 2010). Ginn et al. (2010) reported that in *M. aeruginosa* PCC 7806 the expression of *mcyB* under nitrogen limitation was matched by the enhancement of *ntcA* expression, suggesting that microcystin production can be influenced by nitrogen levels. Pimentel & Giani (2014) also reported that nitrogen stress, either in the form of nitrate or ammonium, induced *ntcA* and *mcyD* expression in *Microcystis aeruginosa* strains, with direct correlation between these two genes. This correlation between *ntcA* and *mcyD* may be an effective indication of an intracellular function of microcystin (Ramasubramanian et al., 1996; Muro-Pastor et al., 2002; Ginn et al., 2010).

The *nifH* gene encodes the dinitrogenase reductase component of the nitrogenase enzyme complex and is conventionally used as marker for the process of N_2 fixation (Latysheva et al., 2012; Sinha et al., 2014; Marques et al., 2022). In Nostocales, the heterocytes are the only and individual sites for *nifH* transcription and NifH biosynthesis (Elhai & Wolk, 1990; Plominsky *et al*., 2013).

Marques et al. (2022) found that in *Raphidiopsis raciborskii* strains the transcription of *nifH, hetR* (regulatory gene involved in heterocyte differentiation) and *ntcA* genes and the quantity of heterocytes increased with nitrogen limitation. Therefore, it is possible that in the nitrogen-limited conditions, *R. racibroskii* cells may have obtained N from biological fixation.

P-specific transport system (Pst) consists of a periplasmic Pi-binding protein (PstS) and sensitively responds to P deficiency (Wang et al., 2018). Therefore, the expression of the *pstS* gene was used to assess phosphorus deprivation. Wang et al., (2018) reported that in *Dolichospermum flos-aquae*, a diazotrophic cyanobacterium, the transcription of *pstS* in the lack of P was significantly upregulated and 2.8 times higher in comparison to high P level conditions. Scanlan et al., (1997) showed that, in *Synechococcus* sp. strain WH 7803, Psts (phosphate-binding protein) was increased under lower PO_4^- concentration.

Environmental factors such as nitrogen and phosphate availability were found to be able to regulate saxitoxin both in dinoflagellates and cyanobacteria (Castro et al., 2004; Graneli & Flynn, 2006). Kellmann et al (2008) found two transcriptional factors, *sxtY* and *sxtZ*, related respectively to PhoU and OmpR. The phosphate (Pho) regulon plays a key role in phosphate homeostasis and part of it is encoded together with proteins of the phosphate-specific transport (Pst) system. Proteins related to PhoU are negative regulators of phosphate uptake, while proteins like OmpR may be part of the regulation of several metabolisms, for example nitrogen and osmotic balance (Kellmann et al., 2008). In a study on *R. raciborskii* MAGs (Metagenome-Assembled Genome) from Pampulha reservoir (Brazil), Laux et al. (2023) also observed that *sxtZ* and *sxtY* were placed together with the pho regulon, namely the Phosphate regulon sensor protein PhoR (*sxtZ*), and Phosphate transport system regulatory protein PhoU (*sxtY*). In their study, however, the complete saxitoxin cluster was not found in the same genomic region, keeping in mind that the use of MAG is not ideal to determine genomic arrangement.

Kellmann et al. (2008) suggested that saxitoxin production in *C. raciborskii* T3 could be regulated at the transcriptional level in response to phosphorous availability and other environmental factors. Pearson et al (2010) described a putative saxitoxin biosynthetic gene cluster (*sxt*) spanning from 25.7 to 35 kb, respectively in *C. raciborskii* T3 and *R. brooki* D9. The toxic profile of the strains seems to be controlled by the position and presence or absence of the genes in the cluster (Boopathi

and Ki, 2014). Boopathi and Ki stated that more research is needed on STXs, since little is still known about their gene regulation and pathways.

As mentioned before, we found a significant upregulation of *sxtA4* gene in the N and P depleted conditions relative to the control (high nutrient concentration). Similar findings were reported by Rigamonti et al. (2018) studying *Raphidiopsis* strains producing the cylindrospermopsin toxin (CYN) who observed a significant increase in *cyrA* gene expression in N-deprived conditions (−N+P) as compared to the control (+N+P). However, in the treatments with P deficiency, the relative expression of *cyrA* gene was downregulated, which is in contrast with our results regarding P reduction. They also reported that total CYN concentration was considerably higher in the −N+P treatment when compared to the control, in line with the change in gene expression, indicating that *cyrA* gene transcription and toxin synthesis were increased twofold (Rigamonti et al., 2018). This result is consistent with the investigation of Saker & Neilan (2001), who found highest CYN concentrations in cultures grown without a fixed N source, even though they did not measure gene expression. Furthermore, Yang et al. (2018) revealed higher CYN production at lower N levels, results that are in agreement with total intracellular toxin concentration observed under medium-N conditions (10% N). Nevertheless, Yang et al. did not address the induction of gene expression, and only assessed intracellular toxin in the biomass.

In our study, although the expression of the *sxtA* gene increased under phosphorus reduction and a correlation was found between saxitoxin gene expression and phosphorus limitation, the amount of total intracellular toxins showed a significant twofold decrease under P limitation. Burford et al. (2014, 2016) evaluated the influence of N and P concentration on cell division rates and CYN cell quotas in phytoplankton populations dominated by *R. raciborskii* and reported considerably higher toxins under high phosphorus concentration, in line with our finding regarding total intracellular toxins. Similarly, Bácsi et al. (2006) reported that starvation for phosphorous and sulfate caused a reduction of the cellular cylindrospermopsin (CYN) content in *Aphanizomenon ovalisporum,* while N starvation did not influence CYN content. On the other hand, Granéli & Flynn (2006) investigating *Alexandrinium tamarensis*, a saxitoxin-producing dinoflagellate, reported an increase in saxitoxin production in environments with phosphorus limitation, which probably imply that for these organisms saxitoxin production might be associated with the adaptation to nutrient limitation. Dinoflagellates are group of single-celled eukaryotes and usually considered algae, while

cyanobacteria are prokaryotes. Therefore, eukaryotes and prokaryotes might have various metabolic controls for saxitoxin production in similar stress conditions; which can be a reason that opposing results in *Alexandrium tamarensis* were reported as compared with *R. raciborskii* in phosphorus limitations. However, these studies did not address the induction of gene expression and mainly focused on intracellular toxin production. The current evidence regarding the role of nitrogen and phosphorus on STX production by *R. raciborskii* seems to seem contradictory in some studies, even though the majority indicates an increase of production or gene expression under limitation. As now, the role of saxitoxins in *R. raciborskii* remains to be elucidated, however the fact that under less nutrient availability STX is still produced or its production is even enhanced may point to a potential role in the cell metabolism when growing under stress conditions.

The effect of nitrogen availability on the intracellular toxin concentration was also similar in the two *R. raciborskii* strains under study, higher toxin content was observed in medium nitrogen concentration (10% N). However, total intracellular toxin concentration reduced significantly under phosphorus reduction and the effect of phosphorus availability was very similar in both strains. Furthermore, the chlorophyll *a* content per cell was significantly lower at the lower phosphorus concentration as compared to the higher phosphorus concentration. These physiological responses may indicate that *R. raciborskii* is able to save resources by modifying and adapting metabolic pathways and decreasing high energy-consuming physiological processes, like toxin production, under nutrient limited condition, a response also previously observed in the potentially toxic cyanobacterium *Microcystis aeruginosa* (Steffen et al., 2014; Pimentel & Giani, 2014).

Additionally, in the nitrogen experiment, total intracellular toxins concentration was higher in medium nitrogen treatment (10% N) in both strains, while some differences were observed on the gene transcription changes: strain 186 expressed more *sxtA4* genes in medium nitrogen treatment, whereas strain 36 expressed more *sxtA4* genes in low nitrogen treatment (1% N). However, in the phosphorus experiment, in both strains, the amount of total intracellular toxins significantly decreased by around half under phosphorus reduction, even though *sxtA4* gene was upregulated under P reduced conditions. The difference observed between the two nutrients seems to indicate that *R. raciborskii* is more resistant to N reduction than to P. Under N gradient, we observed that saxitoxin production responded to the increase of *stxA4* expression by increasing the amount of

toxin in the cells. But for P, even if the nutrient diminution induced *stxA4* upregulation, it seems that the lack of intracellular P hindered the production of toxins. The assumption is reinforced by the results recorded for chlorophyll *a* content per cell that were significantly and sharply reduced at lower phosphorus concentration as compared to nitrogen. This evidence indicates that phosphorus limitation could negatively influence *R. raciborskii* growth rate and toxin production more than nitrogen. It is possible that the potential ability of this species to fix atmospheric N_2 (Sinha *et al.,* 2014) helped to restrain the N reductions effects. These results may also suggest that a substantial phosphorus reduction in aquatic systems could have an effect not only controlling cyanobacterial blooms (Schindler et al 2016), but also reducing toxin production by this species, while a possible toxin increase could be expected for nitrogen.

5. Conclusions

The results obtained in this work provide some insights about the presence of SXT gene and intracellular toxins in *Raphidiopsis raciborskii* strains. Cyanobacterial toxins may be regulated by a variety of factors. In the present study we found that nutrients' reduction was an important stressor in the regulation and production of toxins in *R. raciborskii*. Laboratory experiments are important tools to understand the role of environmental factors on these toxins as well as the potential impact of the toxins in the environment. Field experiments and observations can be additionally used to confirm these findings and provide "*in situ*" supplementary information.

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Chapter 2

Estimating saxitoxin gene expression in *Raphidiopsis raciborskii* (Cyanobacteria) and its relationship with phosphorus and nitrogen availability in Pampulha reservoir (Brazil)

Abstract

During the last decades aquatic systems have been concerned with blooms and contamination by potentially toxic cyanobacteria. The relevance of monitoring cyanobacteria and their toxins is even higher in urban lakes and reservoirs due to their high toxicity to humans and animals and potential contact with the local population. Some of the most potential recurrent cyanotoxins in water bodies are the saxitoxins. Saxitoxins (STXs) are alkaloid neurotoxins produced by marine dinoflagellates and some species of freshwater filamentous cyanobacteria. Pampulha Reservoir is a tropical eutrophic and urban reservoir, located in the southeastern Brazil. *Raphidiopsis. raciborskii* is a cyanobacterium known for its potential to produce SXT and to be present and frequently dominant in the reservoir. The aim of this study was to measure the expression of gene related to the SXT production in *R. raciborskii* as well as genes related to nutrient uptake metabolism. Considering that phosphorus (P) and nitrogen (N) are main reasons of eutrophication of water bodies, this study examined the effect of environmental N and P stresses on saxitoxin gene expression in *R. raciborskii*. Environmental samples were collected once per month, from January to December 2021 for quantification of cyanobacteria and N and P concentration. Samples were also investigated for the presence and expression of *sxtA4* (*sxt* gene), *ntcA* (global nitrogen regulator) and *nifH* (N_2 fixation) and *pstS* (coding for phosphate-binding periplasmic protein) genes. Reverse transcription-quantitative PCR (RT-qPCR) was used to investigate the transcriptional response of the *sxtA4* gene in response to nitrogen and phosphorus changes in the environment. Our results showed that P and N concentrations was significantly related to the transcript abundance of *ntcA* and *pstS* genes, as expected, but also of *sxtA4*. The expression of these genes significantly increased with decreasing N and P. Moreover, in this study showed a significant correlation between *R. raciborskii* biomass and the expression of *sxtA4* gene. This study showed that in a hypereutrophic reservoir with persistent cyanobacteria blooms, seasonal changes of P and N concentration may be considered critical factors not only for cyanobacteria bloom development but also for the regulation of their toxicity.

Key words: Cyanotoxin; nutrient concentration; RT-qPCR; eutrophication; water quality.

1- Introduction

Cyanobacteria are a group of photoautotrophic prokaryotes regarded as the oldest oxygenic photosynthetic organisms on earth, and distributed in aquatic ecosystems around the world (Hess, 2011; Whitton, 2012; Chislock et al., 2013; Belykh et al, 2015; Visser et al., 2016). Cyanobacteria synthesize a variety of secondary metabolites including both highly beneficial to biomedical compounds and toxins which causing detrimental public health effects of humans and animals (Ibelings and Chorus, 2007; Burch, 2008; Smith et al., 2008; Al-Tebrineh et al., 2010; Belykh et al., 2015). The most well-known toxins produced by cyanobacteria are the hepatotoxic microcystins (MC) and neurotoxic saxitoxin (SXT) and its derivatives (Chorus and Bartram, 1999; Burch, 2008). During the recent decades various environmental problems related to the presence of cyanobacteria and cyanotoxins in water systems throughout the world have been investigated (Paerl and Paul, 2012; Steffen et al., 2012; Neilan et al., 2013).

Toxins which produced by cyanobacteria are famous to be harmful agents to animal and human health (Carmichael 1994; Jochimsen et al. 1998). Cyanobacterial toxins have high poisoning ability, besides chronic effects in human populations because of prolonged exposure to hepatic and neurotoxins (Suganuma et al. 1988, Falconer and Buckley 1989; Falconer 1991, 1996; Nishiwaki-Matsushima et al. 1992; Carmichael 1994; Ito et al. 1997, Ding et al. 1999; Cox et al. 2003; Clark et al. 2007).

Saxitoxin (STX) and its 57 analogs are a wide group of natural neurotoxic alkaloids, usually famous as the paralytic shellfish toxins (PSTs) due to their relation with seafood, mainly filter-feeding shellfish (Al-Tebrineh et al., 2010; Wiese et al., 2010). Since its initial discovery, 57 naturally occurring STX analogs have been recognized in several organisms, and they are also commonly known as PSTs (Paralytic Shellfish Toxins, Wiese et al., 2010). Poisoning with PSTs are the causative agents of paralytic shellfish poisoning (PSP) or saxitoxin pufferfish poisoning (SPFP) (Landsberg et al., 2006; Wiese et al., 2010), which is a toxin disease with both neurologic and gastrointestinal symptoms (Belykh et al., 2015) and it may lead to harmful public health effects

and considerable annual damage to the aquaculture industries and fishing (Al-Tebrineh et al., 2010). Saxitoxin acts as a selective blocker of sodium membrane channels of nerve and muscle cells, which prevents the conduction of electric impulses in irritated tissues, leading to the paralyses and death as a consequence of respiratory failure (Chorus and Bartram, 1999; Ibelings and Chorus, 2007; Deeds et al., 2008; Burch, 2008; Wiese et al., 2010).

The SXT synthesis of cyanobacteria and dinoflagellates is performed by a module multienzyme complex including polyketide synthase (PKS) and several enzymes with particular tasks (Belykh et al., 2015). The gene cluster encoding for saxitoxin biosynthesis (*sxt* genes) was initially observed in *R. raciborskii* (Wolosz.) Subba Raju strain T3 and also in *Anabaena circinalis* Rabenh. ex Born. et Fl. strain AWQC131C, *Aphanizomenon* sp. NH-5, *Raphidiopsis brookii* Hill strain D9 and *Lyngbya wollei* Farlow ex Gomont (Cusick and Sayler, 2013).

Saxitoxin is the most potentially toxic cyanotoxin, with a 50% lethal dose (LD_{50}) of 5 µg/kg (intraperitoneal injection) in mice (Carmichael, 1992). Moreover, LD_{50} of saxitoxin for mice in case of intravenous was 3.4 µg/kg (Chorus and Bartram, 1999). World Health Organization (WHO) maximum legal concentrations in water have not been developed for saxitoxin, although regional standards are established in several countries where often PSP incidents occur, for instance, in Brazil and Australia. Based on the EU standards, maximum legal saxitoxin concentration in shellfish used for food is 800 µg/kg shellfish weight (Chorus, 2012).

Although PST biosynthesis in cyanobacteria is a complex process, all producing organisms such as dinoflagellates and cyanobacteria seem to produce these toxins using the same pathway (Shimizu, 1993; Mihali et al., 2009). The saxitoxin biosynthetic gene cluster (*sxt*) was discovered in cyanobacteria, which reveal new insight into the biosynthesis of saxitoxin and its analogs (Kellmann et al., 2008; Mihali et al., 2009). A particular set of analogs can be detected from a single PST-producing organism, which is a direct consequence of the evolution of genes that exists inside the genome of the organism (Carmichael et al.,1997; Lagos et al.,1999; Ferreira et al.,2001 Llewellyn et al., 2001; Kellmann et al., 2008; Mihali et al.,2009).

The saxitoxins are usually categorized based on their structural diversity in the three major groups including: the carbamoyl, decarbamoyl, and sulfocarbamoyl derivatives. The carbamoyl group (STXs) includes saxitoxin (STX), neosaxitoxin (NeoSTX) and the gonyautoxins (GTX 1–4)

(Dittmann et al., 2013; Mesquita et al., 2019). The decarbamoyl variants of these analogs include decarbamoyl-saxitoxins (dcSTX, dcneoSTX), decarbamoyl-gonyautoxins (dcGTXs 1–4), and the 13-deoxy-decarbamoyl derivatives (doSTX, doGTX 2,3) (Wiese et al., 2010). In *R. raciborskii* T3 several analogs of STX have been observed in various combinations, namely saxitoxin, carbamoyl saxitoxin (STX, NEO, GTX2/3), decarbamoyl saxitoxin (dcSTX) and N-sulfocarbamoyl (C1/2, B1) derivatives (Soto-Liebe et al., 2010).

The most important factors contributing to increasing cyanobacterial blooms and the subsequent occurrence of cyanotoxins are considered the eutrophication of water bodies and global warming (Paerl and Paul, 2012; Belykh et al., 2015; Giani et al., 2020). However, specific factors that stimulate and/or control the production of cyanotoxin by cyanobacteria remain to be determined (Neilan et al., 2013). Although the causes of the blooms are becoming better understood, identifying how the dynamics of toxic strains are regulated under different nutrient and environmental conditions is very important to predict and control the toxicity of cyanobacterial blooms (Lei et al., 2021).

Previous investigations revealed that environmental factors such as nutrients, light, temperature, and salt concentrations can influence the growth of the cyanobacterial species and their cyanotoxin production (Kaebernick et al., 2000; Saker and Neilan, 2001; Dias et al., 2002; Castro et al., 2004; Pomati et al., 2004; Carneiro et al., 2009; Yunes et al., 2009; Neilan et al., 2013; Boopathi and Ki., 2014). In other studies, the effect of nutrient stress was investigated on the production of the hepatotoxic microcystin in *Microcystis* strains and some studies showed that lower nutrient levels could promote an increase in toxin production (Ginn and Neilan, 2011; Pimentel and Giani, 2014; Alexova et al., 2016), suggesting a potential function of this toxin when producing species grow under stress conditions (Neilan et al. 2013). Similarly, Yunes and colleagues (2009) highlighted the fact that in response to nitrogen availability, saxitoxin levels tended to decrease in the presence of higher concentrations of nitrogen in the species *Raphidiopsis brookii*. Furthermore, Rapala and colleagues (1993) reported that high temperatures reduced anatoxin-a levels in *Anabaena* and *Aphanizomenon* strains regardless of their growth rate. In addition, growth in nitrogen-free media elevated anatoxin-a levels. Moreover, Wang et al., (2018) observed that in *Dolichospermum flos-aquae*, a diazotrophic cyanobacterium, the transcription of *pstS* in the lack of P was significantly upregulated and 2.8 times higher in comparison to high P level conditions.

Because of the absence of winter and low temperatures, tropical hypereutrophic reservoirs may present cyanobacterial blooms during an entire year and some studies have been published describing this situation (Bouvy et al., 2000; McGregor and Fabbro, 2000; Pinto-Coelho and Bezerra-Neto, 2005; Silva et al., 2016; Figueredo et al, 2016; Araújo et al., 2018; Batista et al, 2018; Lei et al., 2019; Tilahun and Kifle, 2019; Lei et al., 2021; Moraes et al, 2021; Vanderley et al., 2021; Reiscet al., 2023). The present study is focused on a tropical reservoir (Pampulha Reservoir, Brazil) that has been suffering from increasing toxic cyanobacterial blooms for the previous decades. The reservoir is a tropical and urban lake which is located in the southeastern Brazil.

Pampulha reservoir was built in 1938 to provide water to the city of Belo Horizonte, capital of Minas Gerais State. Since the 1970s, its watershed has suffered the consequences of rapid urbanization and water quality deteriorated. In the early eighties the lake for using water supply had to be closed because of the emergence harmful cyanobacterial blooms (Pinto-Coelho, 1998). Various investigations carried out during the last decades which showed the high levels of eutrophication and emphasizes the cyanobacterial dominance in the reservoir (Pinto-Coelho 1998; Figueredo and Giani 2001; Figueredo et al., 2016; Silva et al., 2016; Barçante et al., 2020). However, Pampulha reservoir maintains a status as a main tourist spot and is recorded as one of the UNESCO's World Heritage sites due to its architectural and landscape importance in the city. Despite the risk of contamination, tourists and the local population are regularly observed around Pampulha reservoir, and fishing by locals is a common practice.

Cyanobacteria are always observed at high biomass in Pampulha reservoir and one of the most common and frequently dominant species is the Nostocales *Raphidiopsis raciborskii* (Silva et al 2016; Batista et al. 2018; Reis, 2019; Laux et al., 2023). This species is known to be able to produce saxitoxins in South-American strains (Lagos et al.,1999; Molica et al., 2005; Pomati et al., 2003; Ferrão-Filho et al., 2007; Piccini et al., 2011; Abreu et al., 2018).

Raphidiopsis raciborskii

Raphidiopsis raciborskii (Woloszynska) (Aguilera et al. 2018), a filamentous diazotrophic cyanobacterial species, belongs to the order Nostocales, family Nostocaceae (Gugger et al., 2005; Pagni et al., 2020; Werner et al., 2020), with heterocysts and akinetes (Hoff-Risseti et al., 2013; Ling Zheng et al., 2023; Fig. 4). The species *Raphidiopsis raciborskii* until recently known and described in the publications as *Cylindrospermopsis raciborskii* (Wolozyn´ska) Seenayya & Subba, stands out as a bloom forming, potentially toxic, N_2 -fixing heterocytous and invasive cyanobacterium of seeming subtropical-tropical origin. This cyanobacterium is found spreading into waterbodies and rivers in temperate regions (Padisák, 1997, Briand et al. 2004, Stüken et al. 2006; Werner et al., 2020), helped by its considerable high physiological tolerance (Briand et al. 2004, Sinha et al. 2012).

Its global distribution is extremely concerning, not only due to blooms cause a decrease in diversity in the aquatic environment, but also due to this species can produce harmful toxins such as cylindrospermopsin and saxitoxin (Lagos et al. 1999, Mohamed 2007, Li et al. 2001, McGregor et al. 2011). The potential to produce either cylindrospermopsin (CYN) or saxitoxins (STXs) by *R. raciborskii* is associated with its geographical distribution (Mesquita et al., 2019). Some Australian, Asian, and New Zealand strains of *R. raciborskii* produce CYNs (Wood and Stirling, 2003; Rzymski and Poniedziałek., 2014; Antunes et al., 2015; Zare and Bahador, 2015; Rigamonti et al., 2018), but South American strains produce STXs (Piccini et al. 2011; Hoff-Rissetti et al., 2013; Lopes et al., 2017,); no isolates have yet been reported with the potential to produce both kinds of toxins at the same time (Haande et al., 2008; Vico et al., 2016).

R. raciborskii strains can have straight or coiled morphology (Burford et al., 2016; Willis et al., 2019), and characterized by presenting isopolar trichomes – solitary, cylindrical cells with minor attenuation towards the ends, and slight or no constriction at the cross walls (Pagni et al., 2020). This species form terminal heterocytes with akinetes which located internally within the trichomes (Alster et al., 2010; Komarek, 2013; Wojciechowski et al., 2016; Guiry & Guiry, 2019; Hauer & Komárek, 2019). Moreover, reproduction is by fragmentation of the trichome or by akinete formation (Pagni et al., 2020). Its taxonomy has been reviewed many times. Initially, it was described by Wolozyn´ska (1912) as *Anabaena raciborskii*. Afterwards, the genus *Anabaenopsis*

for individuals with terminal heterocytes created by Miller 1923, and *Anabaena raciborskii* was changed to *Anabaenopsis raciborskii*. Furthermore, the investigation that *Anabaenopsis raciborskii* had a distinct heterocyte differentiation process from all other *Anabaenopsis* species caused the creation of a new genus, *Cylindrospermopsis* (Seenayya & Subba Raju, 1972). Lately, according to the phylogeny and secondary structure of the 16S 23S ribosomal RNA intergenic spacer region, Aguilera et al. (2018) suggested the unification of *Cylindrospermopsis* and *Raphidiopsis* in a single genus. Even before their publication, the taxonomic validity of the two genera had been comprehensively discussed (Moustaka-Gouni et al. 2009, Stucken et al. 2010, Wu et al. 2011, Li et al. 2016), especially due to their major difference was only the presence or absence of heterocytes. Following Aguillera et al., 2018 publication, the name *Raphidiopsis* was adopted according to the taxonomic rules of earlier description of the genus and priority principle. Moreover, the genus *Raphidiopsis*, and particularly the species *Raphidiopsis raciborskii*, is well known, because it has been introduced globally and it is represented in a plenty of cyanobacterial research publications.

R. raciborskii is one of the most successful bloom forming cyanobacteria in freshwater (Antunes et al., 2015; Burford et al., 2016). Many factors have been suggested for the global success of *R. raciborskii*, such as tolerance of a wide range in temperature (Briand et al., 2004), low light tolerance (Padisa´ k, 1997), buoyancy (Ramberg, 1984; Padisa´k, 1997), N_2-fixation (Padisa´k, 1997; Burford et al., 2006), high affinity for ammonia uptake (Burford et al., 2006), P-uptake and storage capacity (Isva´ novics et al., 2000) resistance to grazing (Padisa´k, 1997). Since *R. raciborskii* is ecologically and toxicologically important for public health, information about biological mechanisms associated with environmental stresses and cyanotoxins which produced by *R. raciborskii* in reservoirs and lakes can be showed better insight for controlling and management of cyanotoxins.

The main objectives of this study were: 1- to assess the expression of the gene related to saxitoxin production (*sxtA*), as well as some other genes known for their connection to nitrogen and phosphorus metabolism in the cell (*ntcA*, *nifH*, *pstS*); and 2- to evaluate how the expression of these genes could be linked to nitrogen (N) and phosphorus (P) concentrations during the seasonal cycle of one year. The primers used for these genes were previously tested for their specificity for *R. raciborskii* using laboratory cultures of cyanobacteria species. The use of this approach can show

important metabolic responses of cyanobacteria to eutrophication, and is necessary not only for monitoring but also for the understanding the influences of eutrophication on cyanotoxin levels.

2. Material and methods

2.1 Study site

Pampulha reservoir located in the northern part of the city of Belo Horizonte (Minas Gerais state) (19°51′09″S, 43°58′42″W), Brazil. It is considered as a hypertrophic (having the highest amount of biological productivity, "poor" water quality) reservoir which receives high loads of domestic sewage only partially treated. Pampulha reservoir has a surface area of around 1.8 km^2, volume of 9.9×10^6 m^3 and mean and maximum depth of about 5.1 m and 16.2 m, respectively (Figueredo et al., 2016, Silva et al., 2016; Barçante et al., 2020). The reservoir has eight branches, which Ressaca and Sarandi streams are the two most important and main contributors to external nutrient input into the reservoir. Ressaca and Sarandi streams are responsible for 70% of the total water inflow of the Pampulha reservoir (Torres et al., 2007). The climate of the region is recognized by two distinct seasons: cold and dry season between April and September, warm and rainy season between October and March.

2.2 Environmental samples

Environmental samples were collected during one year, from January to December 2021, in Pampulha reservoir. During the whole period, samples were collected once per month at a fixed sampling station located in the central region of the lake. The physical and chemical profiles of water temperature (°C) (WT), dissolved oxygen (mg L^{-1}) (DO), electric conductivity (µS cm^{-1}) (EC) and pH were measured in situ with a multi-parameter probe Yellow Springs Instruments-YSI, model 556. In addition, Secchi disk was used to measure water transparency. Water samples were collected by a Van Dorn sampler at 0.5 m depth. For phytoplankton analyses, samples were immediately preserved with Lugol's solution for subsequent identification and quantification. Water samples for the analysis of nitrite ($N\text{-}NO_2^-$), nitrate ($N\text{-}NO_3^-$), ammonium ($N\text{-}NH_4^+$) and soluble reactive phosphorus (SRP) were filtered on glass fiber filters (GF-1, Macherey- Nagel, 0.7 µm pore diameter) and frozen (-20 °C) until analysis. In addition, unfiltered samples were used for the analysis of total phosphorus (TP). Nutrients analyses were performed by colorimetric

techniques on a Perkin-Elmer spectrophotometer according to APHA (2005). Analyses were based on triplicate samples.

2.3 Chlorophyll *a* measurement

Chlorophyll *a* concentration was measured according to Nusch (1980). Analyses were based on duplicate samples. Chlorophyll *a* was extracted from the filters by hot ethanol and measured in a spectrophotometer (PerkinElmer) at a 665-nm wavelength.

2.4 Cyanobacteria identification and quantification

Identification and quantification of cyanobacteria species were done at 400X magnification using a ZEISS Axio Observer D1 inverted microscope according to Utermöhl (1958) sedimentation technique. To increase precision, cell counting was done until a minimum of 100 individuals of the dominant species were recorded, and subdominant species were used when dominants were extremely abundant.

2.5 Cyanobacteria strains

The strains used in this work were obtained from the "Cyanobacteria and Algae Culture Collection" of the Phycology Laboratory of the Universidade Federal de Minas Gerais (Belo Horizonte, Brazil). Saxitoxin and non-saxitoxin producing strains were chosen to test primer effectiveness: *Microcystis aeruginosa* (strain UFMG150), *Microcystis brasiliensis* (strain UFMG177), *Planktothrix agardhii* (strain UFMG236), *Planktothrix agardhii* (strain UFMG238), *Raphidiopsis raciborskii* (strain UFMG186 and UFMG36). Strains were grown in sterile conditions at 20°C, 75 μmol photons $m^{-2}s^{-1}$, photoperiod 12-h light/12-h dark, in WC medium (Guillard and Lorenzen 1972). Cultures were collected and filtered on glass fiber filters (GF-1, Macherey- Nagel, 0.7 μm pore diameter) under slight vacuum pressure and stored frozen (−20 °C) until DNA extraction.

2.6 DNA extraction

To test for primer efficiency, DNA was extracted from the frozen filters of the culture strains and environmental samples based on the protocol described by Kurmayer et al. (2003). Sucrose buffer, proteinase K and lysozyme were applied for cellular lysis and phenol-chloroform-isoamyl alcohol for DNA extraction. After that, DNA was resuspended and cleaned in 100% and 70 % ethanol, respectively. Furthermore, DNA was quantified using a NanoDrop spectrophotometer at 260/280 nm and its quality visualized in 1 % agarose gel stained with ethidium bromide.

2.7 Primer design and PCRs

Specific primers used in this study are described in Table 1. The selected strains were *Microcystis aeruginosa* (strain 150), *Microcystis brasiliensis* (strain 177), *Planktothrix agardhii* (strain 236), *Planktothrix agardhii* (strain 238), *Raphidiopsis raciborskii* (strains 186 and 36). The *sxtA* primers were tested in potentially saxitoxin-producing strains of our culture bank, namely *Raphidiopsis raciborskii* (strain 186 and 36) and *Planktothrix agardhii* (strain 236), *Planktothrix agardhii* (strain 238), and saxitoxin non-producing strains *Microcystis aeruginosa* (strain 150), *Microcystis brasiliensis* (strain 177).

Conventional PCR was applied on both cultures and environmental samples. PCR amplifications were cycled in a thermocycler (MyCycler, Bio-Rad) with 20 µL total reaction volumes containing: 2 µL Buffer IC 1X (Phoneutria), 1.5 mM $MgCl_2$, 0.4 µL DNTP, 0.2 µL Taq polymerase, 1.2 µL BSA, 0.5 µL (10 pmol μL^{-1}) of each primer (*sxtA4* F and *sxtA4* R) and 1 µL diluted DNA. The thermal cycle program (for *sxtA4* primers) consisted of an initial preheating of 94 °C for 5 min, followed by 35 cycles of 94 °C at 30 s, 61 °C at 30 s and 72 °C at 30 s and finally 72 °C at 7 minutes. The amplified PCR products were visualized on 1.2 % agarose gel (stained with ethidium bromide) together with a GeneRuler 100 bp DNA Ladder (Fermentas). The *ntcA, nifH* and *pstS* fragments were amplified using the conditions previously described by several authors (Turner et al., 1999; de la Torre et al., 2003; Gugger et al., 2005; Wu et al., 2013; Pimentel and Giani., 2014; Marques et al., 2022).

2.8 RNA extraction and cDNA synthesis

Environmental filters from samples collected in Pampulha reservoir, meant for RNA analyses, were submerged in TRIzol (Invitrogen) immediately after sampling and stored at -80ºC. For the RNA extraction, samples were quickly deep-frozen in liquid nitrogen and mechanically crushed by a homogenizer. Subsequently, the RNA was extracted as mentioned by TRIzol manufacturer's guidelines. Total RNA was suspended in 30 µL of DEPC-H_2O, and RNA was treated with 0.5 µL of DNase (Promega) at 37°C for 30 min and 1.4 µL EDTA 0.1 molar was added. The reaction was stopped by heating 10 min at 75°C. Afterwards, RNA was quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies). Furthermore, the extracted RNA was subjected to reverse transcription to produce DNA copies (cDNA) with a High Capacity kit (Applied Biosystems) with RT random primers.

2.9 Quantitative PCR (qPCR)

The obtained cDNA was applied for RT-PCR to quantify the relative abundance of *sxtA4, ntcA, nifH* and *pstS* transcripts. Real-time PCR was performed using a StepOne system (Applied Biosystems) with 1 µL of cDNA each sample, 0.3 µL of each primer (10 pmol μL^{-1}), 5 µL of Power SYBR green I (Applied Biosystems), and sterile Milli-Q water for a final volume of 10 µL. The reactions were performed in two replicates, and specifications for the PCR cycle followed the manufacturer's recommendations. The transcript levels of genes involved in saxitoxin gene (*sxtA4*), global nitrogen regulator (*ntcA*), N_2 fixation (*nifH*), and the gene coding for the PstS phosphate-binding protein (*pstS)* were measured. Primers are described in Table 1.

In addition, the analysis of the fluorescent melting curve was performed to detect the amplification melting temperature of the single PCR products in the samples, by gradually rising the temperature from 70°C to 95°C at a rate of 0.1°C S^{-1}. Fluorescence intensity data were collected continuously and were converted to melting peaks employing the LightCycler software (StepOne Software, version 2.0).

The measured Ct values were reported and transformed to the linear form using the term 2^{-Ct}.

The linear form is more accurate to represent the individual variation among reactions and avoids false representation of changes (Livak and Schmittgen, 2001).

Table 1. Amplification primers used in this study

Primer	Type[a]	Sequence 5′–3′	Amplicon size (bp)	Function	Reference
sxtA4 **sxtA4**	F R	GGACTCGGCTTGTTGCTTC CCAGACAGCACGCTTCATAA	200	*sxtA* gene (Initiation of STX biosynthesis)	(Hoff-Risseti et al., 2013)
ntcA **ntcA**	F R	TGCGGTGGAATTGCTCTCTT CTGTTTGCAGAATCCGCGAG	113	Global nitrogen regulator	(Marques et al., 2022)
QntcA **QntcA**	F R	TGCAGGGTTTGTCCTCGCGG CCCGGATGCCATCGGTGGTG	141	Global nitrogen regulator	(Pimentel and Giani., 2014)
nifH **nifH**	F R	CGTAGGTTGCGACCCTAAGGCTGA GCATACATCGCCATCATTTCACC	297	N_2 fixation	(Gugger et al., 2005)
pstS **pstS**	F R	AAGCTGGGACGGTATTTGGGGG TACCAAACGTCCTATGCGCC	132	Coding for phosphate-binding periplasmic protein	(This study)
16S- 356 **16S-1391**	F R	GTGGGGAATTTTCCGCAA GACGGGCGGTGWGTRCA	890	Housekeeping gene	(Turner et al., 1999; de la Torre et al., 2003)

[a] F: forward; R: reverse.

2.10 Statistical analyses

Statistical analyses were carried out by applying SPSS, version 26 software. Analysis of variance (ANOVA) and two-tailed Levene's test for equality of variances were performed to analyze differences of the relative gene expression among all months during one year. Significant difference was accepted when *P* values were ≤0.05. Furthermore, contrast analysis was performed to compare differences among samples of months during one year. In addition, regression analyses were applied between 2^{-Ct} values of *sxtA4* and the other genes *nctA, nifH and pstS*. To increase the significance of the correlation and test for general trends, all measured values for each month were pooled together (all replicates and experimental repetitions). Linear regressions were also performed between each gene and *Raphidiopsis raciborskii* biovolume.

To investigate the major trends of the influence of environmental parameters on biotic variables, a redundancy analysis (RDA) was used following Borcard et al. (2011). Environmental parameters data were not transformed, except for *R. raciborskii* biovolume where log 10 (x + 1) was used to normalize the data. The statistical significance of the relationship was assessed by Monte Carlo permutation tests, under 999 permutations. The analysis was run by applying the R software and the vegan package (Oksanen et al., 2013; R Development Core Team, 2013). Environmental data

include: TP, SRP, NO_3 + NO2, NH_4^+ , water temperature, pH, while biotic data include: *R. raciborskii* biovolume, *sxtA4, ntcA, nifH, pstS* gene expression and chlorophyll-a concentration.

3. Results:

3.1 Validation of the *sxtA4* primer set by conventional PCR

PCR analyses were performed on the different strains to test if the primers were able to detect the presence of the *sxtA* gene in saxitoxin-producing *Raphidiopsis raciborskii* strains (strain 186, 182 and 36) as well as some other genes known for their connection to nitrogen and phosphorus metabolism in the cell (*ntcA*, *nifH*, *pstS*). The results indicated high efficiency and sensitivity for *Raphidiopsis raciborskii* strains. As expected, the saxitoxin non-producing strains from our culture collection (*Microcystis aeruginosa* (strain 150), *Microcystis brasiliensis* (strain 177), did not yield any PCR products. Potential saxitoxin producers as *Planktothrix agardhii* (strain 236) and *Planktothrix agardhii* (strain 238) also did not present amplification with the *sxtA4* primer set.

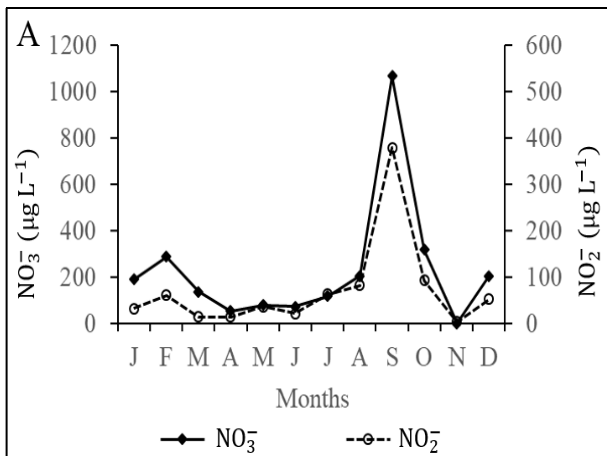
In addition, the primers detected the presence of the *sxtA* gene in all environmental samples collected throughout the year. The results demonstrated that the primer set was able to detect potentially saxitoxin-producing *R. raciborskii* in both cultures and environmental samples.

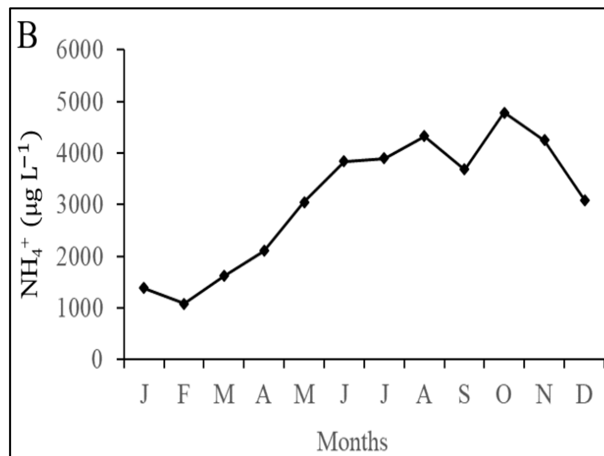
3.2 Environmental samples

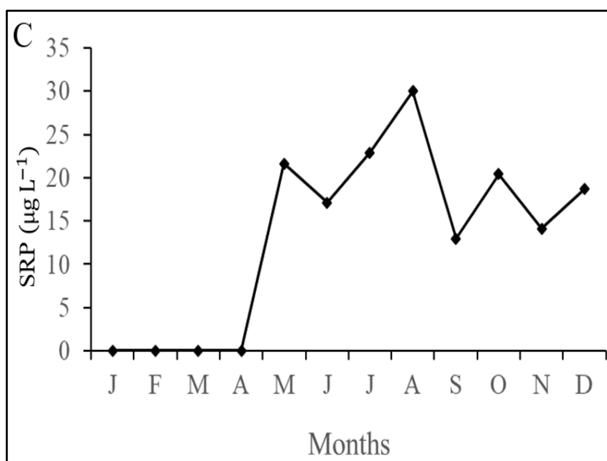
3.2.1 Nutrients and *Raphidiopsis raciborskii*

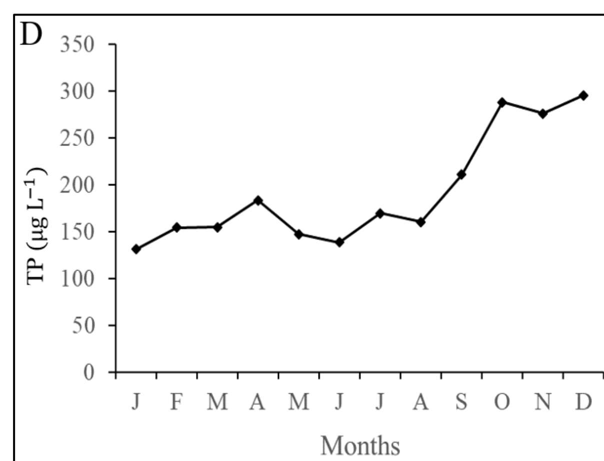
Figure 1 represents seasonal changes observed in nutrient concentrations (Fig. 1A - nitrate- NO_3 and nitrite-NO_2, 1B - ammonium- NH_4, 1C - soluble reactive phosphorus- SRP, 1D - total phosphorus-TP) and in the population biomass of *Raphidiopsis raciborskii* and chlorophyll-a concentration (Fig. 1E). Higher nutrient concentrations and chlorophyll were recorded in the second part of the year: August - October for NO_3 and NO_2, June - November for NH_4, September - December for TP, March-December for SRP, and September-December for chlorophyll. On the contrary, *R. raphidiopsis* presented higher biomass from January to June.

Additional data (pH, dissolved oxygen, water temperature, conductivity) can be found in the supplementary Table S1 (see table at the end of this chapter).


A
1200
1000
800
600
400
200
0
600
500
400
300
200
100
0
NO_3^- (μg L^{-1})
NO_2^- (μg L^{-1})
J F M A M J J A S O N D
Months
NO_3^-
NO_2^-


B
6000
5000
4000
3000
2000
1000
0
NH_4^+ (μg L^{-1})
J F M A M J J A S O N D
Months


C
35
30
25
20
15
10
5
0
SRP (μg L^{-1})
J F M A M J J A S O N D
Months


D
350
300
250
200
150
100
50
0
TP (μg L^{-1})
J F M A M J J A S O N D
Months

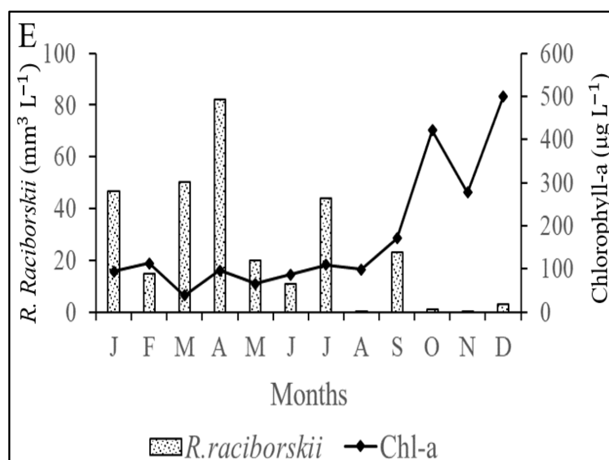

E
100
80
60
40
20
0
600
500
400
300
200
100
0
R. Raciborskii (mm^3 L^{-1})
Chlorophyll-a (μg L^{-1})
J F M A M J J A S O N D
Months
R.raciborskii
Chl-a

Fig 1. Variation of nutrient concentration (**A**: NO3 and NO2; **B**: NH4; **C**: SRP; **D**: TP) and *Raphidiopsis raciborskii* biovolume and chlorophyll-a (**E**) in Pampulha reservoir from January to December 2021.

3.2.2 Gene responses

A significant increase in *ntcA* gene expression was observed from March to April and from June to July, and again in September. The highest expression of the *ntcA* gene occurred in July ($P < 0.05$). While, the lowest expression of the *ntcA* gene expression was found in August to December (except September) ($P < 0.05$) as compared with other months (Fig. 2, A).

A similar pattern was observed for the *nifH* gene expression, but with higher values in July ($P < 0.05$), followed by June and September (Fig. 2, B). Low values were recorded in most months.

As for *ntcA*, *pstS* gene was upregulated from March to April, showed a decrease in May, and increased again in June July. The highest expression was in the April ($P < 0.05$), while, the expression of this gene was sharply declined in August to December (Fig. 2, C).

The RT-PCR analysis confirmed that the *sxtA4* gene was present and active throughout the year, but a clear variation occurred ($P < 0.05$). The lowest expression of the *sxtA4* gene was found in August to December (except September), while the expression of the *sxtA4* gene increased noticeably in the period from March to July. The highest expression of the *sxtA4* was recorded in July ($P < 0.05$) (Fig. 2, D).

In general, the analysis of *ntcA*, *nifH* and *sxtA4* transcription changes showed that the genes were considerably upregulated in July ($P < 0.05$), and some in April. Whereas, the expression of all genes decreased from August to December (except September). The similarity in changes observed in the population of *R. raciborskii* in the lake (Fig. 1E) is a proof that the genes were suitable to detect changes in its metabolism. We also observed lower expression of genes related to nutrient uptake (*ntcA* and *pstS*) during the months with higher N and P concentration (Fig. 1).

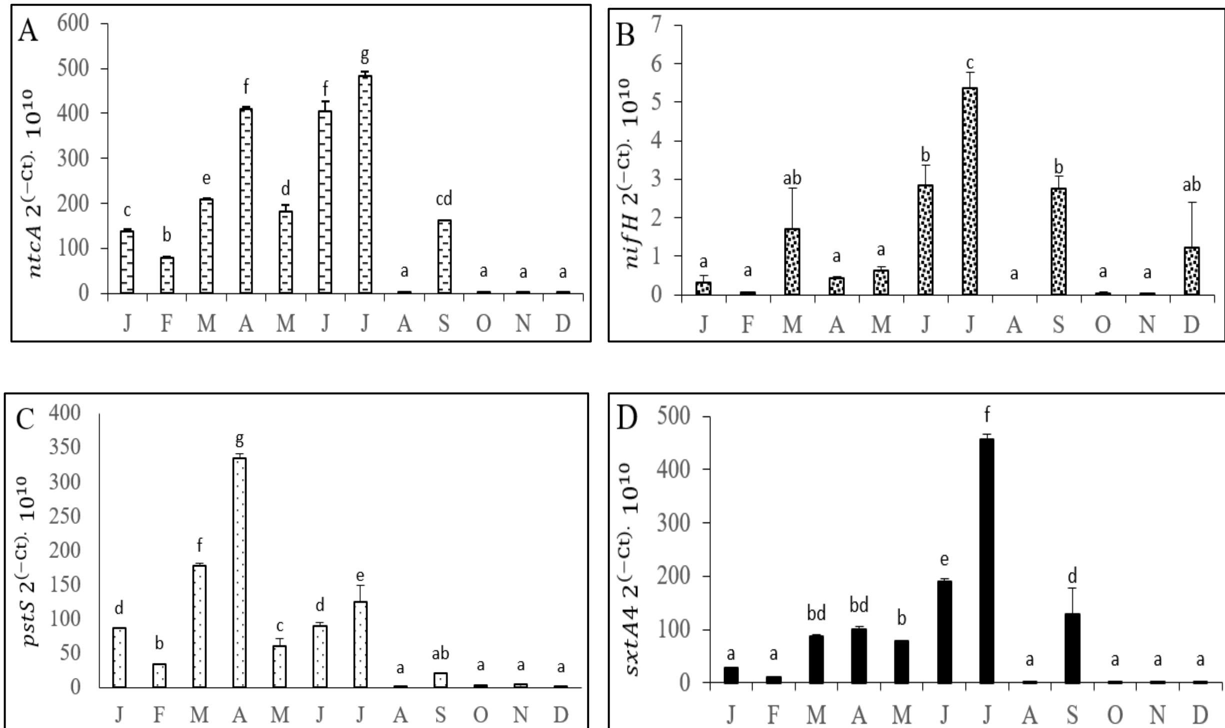


Fig 2. Relative quantification of *ntcA* **(A)**, *nifH* **(B)**, and *pstS* **(C), *sxtA4* (D)**genes expression in monthly samples throughout the year. Bars indicate standard errors. Different letters above the bars indicate significant differences ($P \leq 0.05$), when values were compared among themselves.

3.2.3 Correlation analyses between *R. raciborskii* and genes expression

Linear regression analysis was applied to test for correlation between *R. raciborskii* population and *ntcA*, *R. raciborskii and nifH, R. raciborskii and pstS,* and also between *R. raciborskii* and *sxtA4* genes expression in monthly samples throughout the year (Fig. 3 A, B, C, D). The analysis showed approximately high correlations between *R. raciborskii* and almost all genes, except for *nifH* gene. The R^2 values between *R. raciborskii* and *ntcA*, *4*, *R. raciborskii* and *pstS*, *R. raciborskii* and *sxtA* were above 0.7 implying that there is a significant correlation between the *R. raciborskii* and the expression of these genes, indicating a reliable connection between this species and gene responses including saxitoxin production (Fig. 3D). The lowest correlation was observed between *R. raciborskii* and *nifH* gene (R^2 value= 0.239).

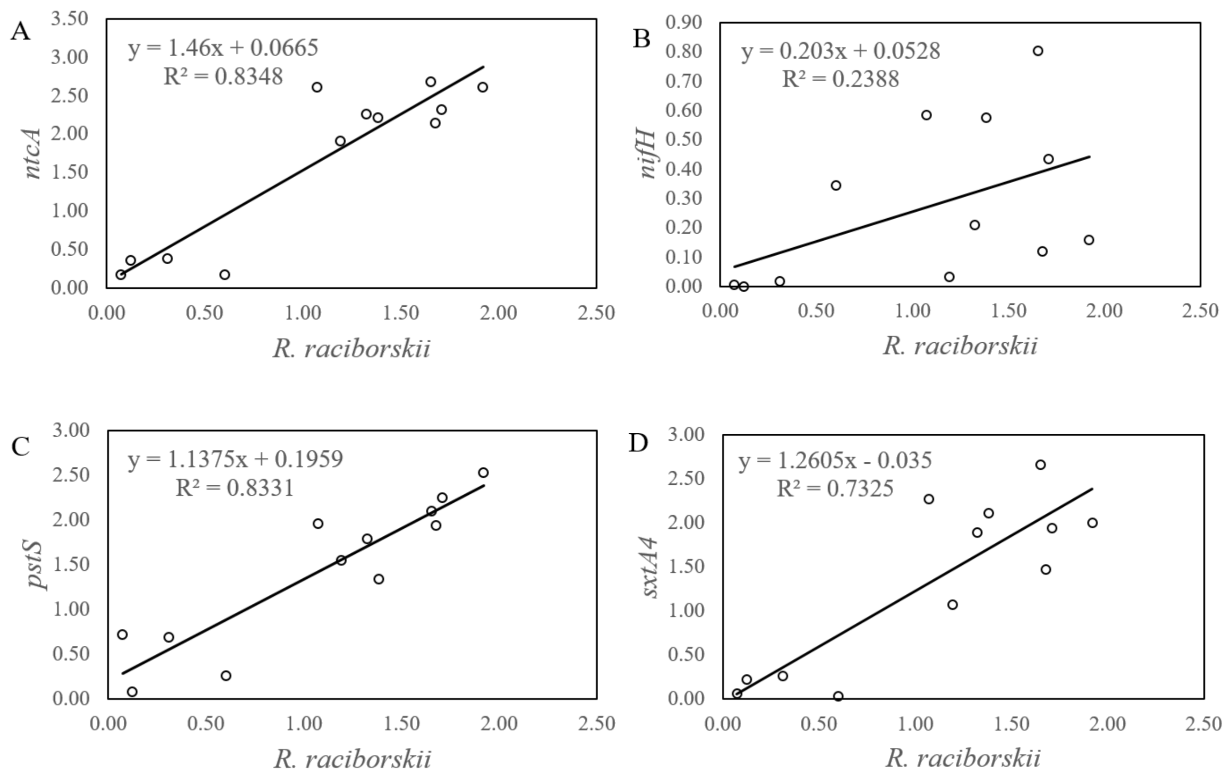


Fig 3. Correlation analyses between R^2 values of **(A)** *R. raciborskii* (biovolume - mm^3. L^{-1}) and *ntcA,* **(B)** *R. raciborskii* and *nifH*, **(C)** *R. raciborskii* and *pstS,* **(D)** *R. raciborskii* and *sxtA4* genes expression (2^{-Ct}) in monthly samples throughout the year.

3.2.4 Correlation analyses between genes' expression

Linear regression analysis was applied to test for correlation between expression of genes pair a pair, trying to observe if metabolic changes related to nutrient in the environment could affect saxitoxin production. Figure 4 represent correlations between *ntcA* and *sxtA4*, *pstS* and *sxtA4*, *nifH* and *sxtA4* (Fig. 4 A, B, C), and also between *pstS* and *ntcA, nifH* and *ntcA* (Fig. 4 D, E) genes expression in monthly samples throughout the year. The analysis showed high correlations between almost all pairs of genes. The R^2 values between *ntcA* and *sxtA4*, *pstS* and *sxtA4*, *pstS* and *ntcA* were close to or above 0.8 demonstrating that there is a significant correlation between the expression of the genes. The highest correlation was observed between *ntcA* and *sxtA4* (R^2 value= 0.939). Lower correlation was found between *nifH* and *sxtA4*, and also between *nifH* and *ntcA* (R^2 value=0.53 and 0.33, respectively).

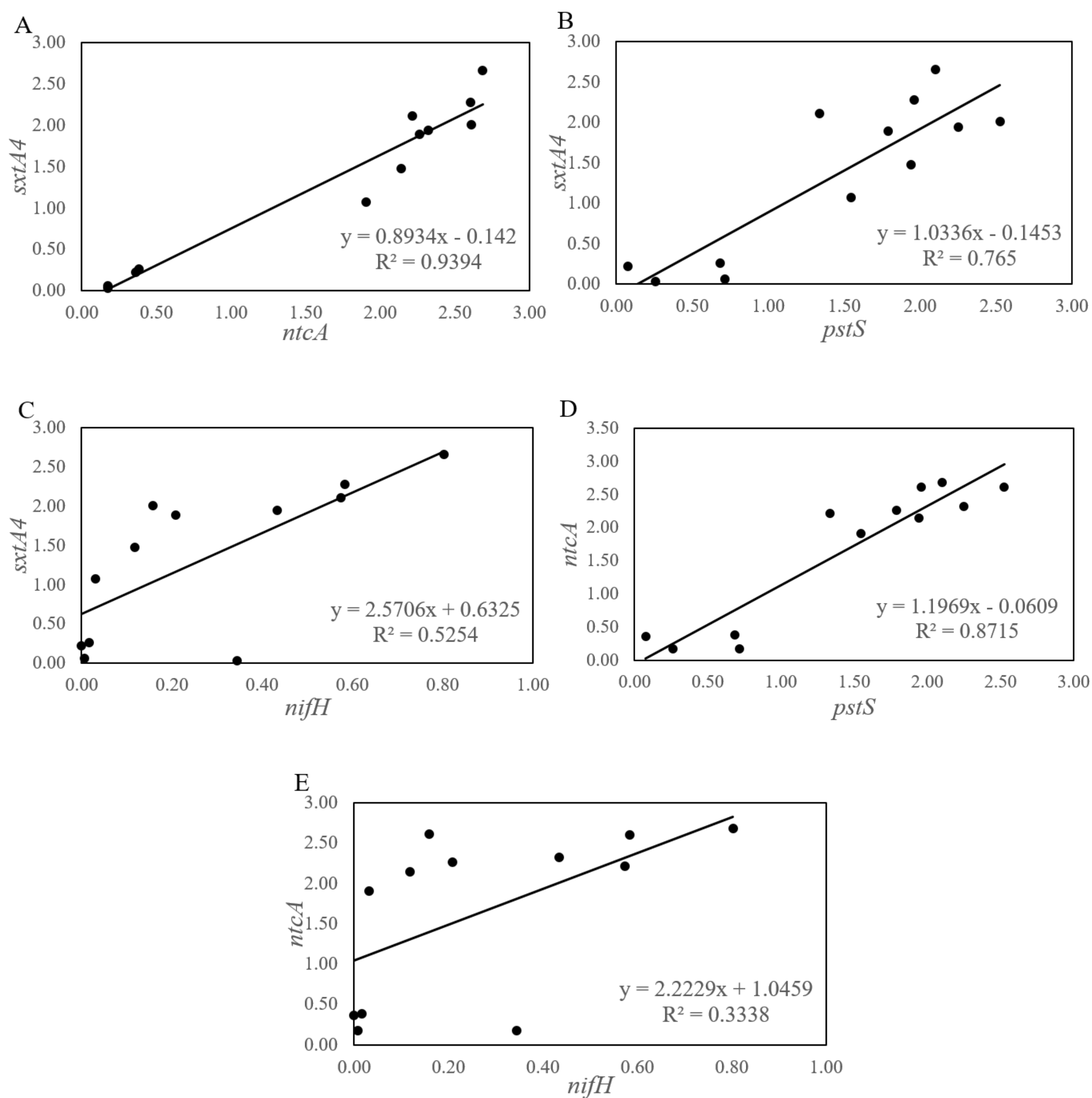


Fig 4. Correlation analyses between 2^{-Ct} values of the expression of **(A)** *ntcA* and *sxtA4*, **(B)** *pstS* and *sxtA4*, **(C)** *nifH* and *sxtA4,* **(D)** *ntcA and pstS,* **(E)** *ntcA and nifH* genes, in monthly samples throughout the year.

3.2.5 Redundancy analysis (RDA)

The results of the redundancy analysis (RDA) are represented in Fig. 5. The multivariate analysis was used to identify patterns between environmental and biotic parameters following their seasonal changes during the sampling period.

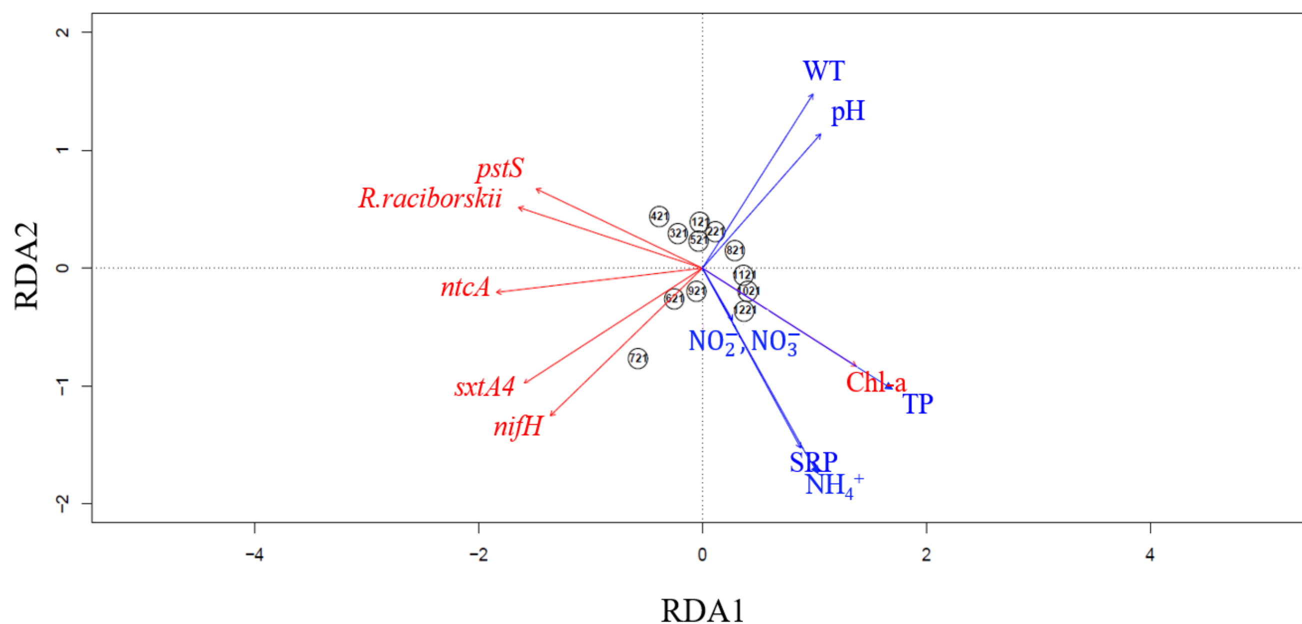


Fig. 5 Triplot redundancy analysis of the relationships observed between biotic parameters (red arrows) and abiotic parameters (blue arrows), and samples distribution (circles: the numbers inside the circles indicate month and year) in Pampulha reservoir (January to December, 2021). The arrows indicate the direction of increase. The angles between variables reflect their correlations (angles close to 90° indicate no correlation, angles close to 0° indicate a high positive correlation and angles close to 180° indicate a high negative correlation). WT = water temperature; pH = hydrogenation potential; TP = total phosphorus; SRP = soluble reactive phosphorus; NO_2^- = nitrite; NO_3^- = nitrate; NH_4^+ = ammonium; Chl-a= chlorophyll-a; *R. raciborskii* = *R. raciborskii* biomass; *ntcA, nifH, pstS* and *sxtA4* = genes' expression.

The RDA model was highly significant ($p < 0.007$) and the axes explained 70% of the variation (RDA1: 56%, RDA2: 14%). The analysis revealed a close correlation between *R. raciborskii* biomass and genes expression of *sxtA4, ntcA, nifH* and *pstS* to Axis 1 (See Table S2, at the end of this chapter). All these variables showed the same trend in increase, suggesting a connection with *R. raciborskii* as explanatory variable for the changes observed in gene expression. On the other hand, chlorophyll-a showed strong correlation with TP and both were more closely related to Axis 1. The other nutrients (NO_3^-+ NO_2^-, NH_4^+ and SRP) showed the same trend in increase. For the year under study, 2021, *R. raciborskii* was not the main species to contribute to chlorophyll, showing increase in an opposite direction. Water temperature (WT) and pH were more related to Axis 2 (Table S2) and showed the same direction as *R. raciborskii*. Furthermore, nutrient concentration and genes expression revealed negative correlation, which imply that the expression of genes considerably increased at lower concentration. This evidence indicated that diminshing nutrients could negatively influence *R. raciborskii* growth rate but increase *sxtA4* gene expression, while also affecting other genes related to metabolism.

4. Discussion

The incidence of cyanobacterial blooms in freshwater reservoirs is considered a global environmental problem. Notably, toxic cyanobacterial blooms have increased in frequency and severity in freshwater systems worldwide (Paerl and Paul., 2012; Steffen et al., 2012; Boopathi and Ki., 2014). Saxitoxin synthesis has been related to environmental factors and changes in saxitoxin production were observed and potentially linked to nutrients, light, temperature and salt content (Castro et al., 2004; Carneiro et al., 2009; Dias et al., 2002; Pomati et al., 2004; Yunes et al., 2009; Boopathi and Ki., 2014).

Phosphorous (P) and nitrogen (N) concentrations and their relative ratios play crucial roles in controlling the growth of cyanobacterial blooms (Rapala and Sivonen, 1998; Paerl and Paul, 2012; Wang et al., 2018; Giani et al. 2020). Despite the sequencing of diazotrophic cyanobacterial genomes in the last decade, the molecular response to P or N levels and their mutual effects are still quite unknown (Kaneko et al., 2012; Wang et al., 2018).

For a long period of time, P was considered the main nutrient that restricts the development of cyanobacterial biomass and eutrophication in freshwater ecosystems (Schindler, 1974; Schindler, 1977; Smith 2003), and therefore management efforts usually focus on P reduction (Smith 2003; Dolman et al., 2012), in spite of there being important evidence that N importance in lakes (Elser et al., 1990; Elser et al., 2007). Nitrogen received less notice in systems of freshwater than phosphorus and the function that nitrogen may play in controlling eutrophication is less investigated (Lewis and Wurtsbaugh, 2008; Sterner, 2008). Additionally, the influence of N concentration on saxitoxin synthesis is still a controversial subject among researchers (Brentano et al. 2016; Burford et al. 2016; Vico et al. 2016).

Following past studies of toxins production in cyanobacteria strains submitted to some stress conditions (Soto-Liebe et al., 2012; Pimentel & Giani, 2014; Stucken et al., 2014; Rigamonti et al., 2018; Mesquita et al., 2019), in the present study, we evaluated the hypothesis that the expression of the gene involved in saxitoxin synthesis would be upregulated in the saxitoxin-producing cyanobacteria *Raphidiopsis raciborskii* in Pampulha reservoir when exposed to some reduction in nutrients' concentration (specifically P and N). In this one year's field study, the expression of *sxtA4* showed a significant correlation with *ntcA* and *pstS* genes (Fig. 4) and the RDA analysis (Fig. 5) revealed an opposite trend between nitrogen and phosphorus nutrients and gene expression confirming that lower nutrient concentrations can upregulate the transcription of these genes. Thus, nutrient stress effect on genes' transcription can upregulate toxin production.

For the study of nitrogen regulation, NtcA is a reliable marker, since it activates nitrogen-responsive genes (Lindell & Post., 2001) and in cyanobacteria, nitrogen control is carried out by NtcA at the transcriptional level (Herrero et al., 2001; Álvarez-Escribano et al., 2018). NtcA protein is essential for gene expression in ammonium and nitrate uptake pathways, and for heterocyte development as well (Wei et al., 1993; Wei et al., 1994). The *ntcA* gene is induced quickly and autoregulated under lack of nitrogen (Wei et al., 1994; Ramasubramanian et al., 1996; Muro-Pastor et al., 2002).

Lindell & Post (2001) observed that *ntcA* gene transcription in *Synechococcus* sp. strain WH7803 was amplified when ammonium levels reduced at 1 mM, while an increase of more than 1 mM ammonium caused a quick downregulation in the *ntcA* gene. Moreover, Lindell et al., (1998) in the same organism, *Synechococcus* sp. strain WH 7803 observed the highest level of *ntcA* transcription

was obtained under lack of nitrogen conditions. Therefore, higher transcription of *ntcA* would imply nitrogen limitation (Lindell & Post 2001). The typical transcriptional pattern expected for NtcA (an activator regulatory protein) involves an increase in transcription. Subsequently, while the production of the activator protein is increased, the transcription of the target protein is also upregulated (Ginn et al., 2010). In addition, Ginn et al. (2010) observed that in *M. aeruginosa* PCC 7806 the expression of *mcyB* was induced under nitrogen limitation by increasing the expression of *ntcA* and they suggested that the production of microcystin could be affected by nitrogen levels.

Moreover, Pimentel & Giani (2014) observed that in *Microcystis aeruginosa* strains (Ma19 and Ma26), nitrogen (nitrate and ammonium) stress induced the expression of *ntcA* and *mcyD* with a direct correlation between these two genes. This correlation between *ntcA* and *mcyD* could suggest an intracellular function of microcystin. Since a variety of cyanobacterial genes revealed to be regulated by *NtcA*, it appears that *NtcA* responds not only to ammonium but also to the C/N ratio in the cell (Luque et al., 2004). The *ntcA* autoregulation is increased under nitrogen deprivation and also transcribed at a basal level in the presence of ammonium (Ramasubramanian et al., 1996; Muro-Pastor et al., 2002; Ginn et al., 2010).

The *nifH* gene is also related to nitrogen metabolism. It encodes the dinitrogenase reductase component of the nitrogenase enzyme complex and is conventionally used as marker for the process of N_2 fixation (Marques et al., 2022). The heterocytes are the only sites for *nifH* transcription and NifH biosynthesis in Nostocales (Elhai & Wolk, 1990; Plominsky *et al*., 2013). Marques et al. (2022) observed that in *Raphidiopsis raciborskii* (strains UFMG-40 and UFMG-188) the transcription of *nifH, ntcA* and *hetR* (regulatory gene involved in heterocyte differentiation) genes and the quantity of heterocytes increased with nitrogen deprivation Consequently, it is possible that at lower the nitrogen conditions, *R. racibroskii* may have obtained N from biological fixation. In the present study, *nifH* showed a relatively good correlation with *stxA* (R2= 0.53) and a close relationship between these two genes was also observed in the RDA analysis, suggesting that low N could induce *nifH* transcription, as expected in heterocystous cyanobacteria, but also induce saxitoxin production.

A large number of photosynthetic microorganisms, such as cyanobacteria, have evolved in nutrient-poor environments or environments with different nutrient concentrations (Solovchenko et al., 2020). Acquisition of phosphorus (P) is essential for growth of organisms since it is a major

element in biomolecules such as lipids and nucleotides (Karl 2014). Actually, phosphorus is essential nutrient for the cellular synthesis of nucleic acids and membrane phospholipids in addition for energy transfer through tri- and biphosphorylated nucleotides (Degerholm et al., 2006). At the same time, the accessibility of phosphorus in plenty of habitats may be scarce and/or fluctuating. Cyanobacteria developed a wide range of adaptations to cope with phosphorus deficiency (Solovchenko et al. 2020). Moreover, phosphorus eutrophication is often named among the root causes of harmful cyanobacteria blooms (Longhurst, 2001; Anderson et al., 2002).

Phosphorus can also affect gene regulation and potentially could also affect toxin production in cyanobacteria as reported in previous studies (Oh et al., 2000; Pimentel and Giani, 2014; Pereira et al. 2016; Wang et al., 2018). Previous studies observed that P deficiency inhibited cylindrospermopsin (CYN) production in *Chrysosporum (Aphanizomenon) ovalisporum* (Bácsi et al., 2006). Conversely, replete P supply was observed to increase CYN yield in *R. raciborskii* and cyanobacterial blooms samples (Mohamed and Al-Shehri, 2013; Burford et al., 2014). However, only intracellular CYN was assessed in these investigations, and the expression of CYN biosynthesis genes was not measured. On the other hand, an enhancement in the total CYN content was observed under P-depleted conditions, coupled with increased expression levels of the CYN biosynthesis genes (Bar-Yosef et al., 2010; Preußel and Fastner, 2014). Consequently, assessing the expression of toxin-biosynthesis genes under various P concentrations may be helpful to clarify the contradiction of these results.

In this study, we explored the P-dependent expression levels of the *pstS* and *sxtA4* by *R. raciborskii* in Pampulha reservoir. This species has the potential to grow even at low P concentrations, and this is related to the high P affinity and large P storage that has been previously reported for *R. raciborskii* (Istvánovics et al., 2000; Amaral et al., 2014). In the present study, we examined the effects of P stress on expression of genes related to SXT biosynthesis by using the P-regulated biomarker, *pstS*, to indicate P stress in the *Raphidiopsis raciborskii*. Under P-limiting conditions, the phosphate-specific transport (PST) system utilizes ATP-mediated transport and contains a high-affinity phosphate binding protein (PstS) to acquire P (Pereira et al., 2019). Generally, *pstS* applied as a biomarker for P stress in natural microbial populations (Fuller et al. 2005, Hung et al. 2013), *pstS* transcript abundance is considered to be a sensitive metric of P deprivation and adaptive responses to P availability. Molecular biomarkers like *pstS* can be used to assess when and where

P availability limits the growth of specific microorganisms (Pereira et al., 2019). Transcription of *pstS* in the *Synechococcus* species PCC7942, a model freshwater cyanobacterium, is regulated by phosphorus availability, and is activated when orthophosphate (Pi) falls below a threshold concentration that differs between organisms (Aiba and Mizuno 1994). Moreover, Pereira et al. (2016) reported that transcription of *pstS* in *Crocosphaera* (a unicellular N_2-fixing cyanobacterium) was upregulated within 1 h of exposure to low external Pi (Pereira et al. 2016). Reistetter et al. (2013) observed in the marine cyanobacterium *Prochlorococcus* MED4 II that the expression of three P uptake genes, *pstS*, the high-affinity phosphate-binding component of the phosphate transporter, *phoA*, an alkaline phosphatase, and porin PMM0709, were significantly increased (between 10- and 700-fold) under both P starvation and limitation. Expression of *pstS* was increased considerably under both P starvation and P limitation as compared with P-replete conditions (Reistetter et al. 2013). This was in the consistent with an increase in the maximal rate of PO_4^- uptake (V_{max}) under both P starvation and P limitation (Krumhardt *et al*., 2013). In another investigation, insignificant changes were revealed in the relative quantification of *mcyD* in *M. aeruginosa* PCC 7806 under P-replete, whereas P deficiency (N/P = 40:1) induces *mcyD* (microcystin synthetase gene) expression and microcystin synthesis (Kuniyoshi et al., 2013).

Furthermore, Wang et al., (2018) observed that in *Dolichospermum flosaquae*, a diazotrophic cyanobacterium, the transcription of *pstS* in the lack of P was considerably upregulated, up to 2.8 times, relative to high P level condition. Scanlan et al., (1997) reported that, on the same *Synechococcus* sp. strain WH 7803 cited above, Psts (phosphate-binding protein) was enhanced when PO_4^- reduced. Our results are also consistent with the investigation of Wang et al. (2018), who found that in *Dolichospermum. flosaquae*, *nifH* expression revealed a significantly negative relationship with NO_3^- /SRP ratio, although *phoD* and *pstS* significantly responded increasing their expression. Moreover, N played an influential function during P uptake. Noticeably, N addition considerably enhanced both *phoD* and *pstS* expression, suggesting that N availability may increase the ability of *D. flosaquae* to overcome P limitation and vice versa. They also reported that the expression of genes involved in P uptake, e.g., those involved in P-transport (*pstS*) and the hydrolysis of phosphomonoesters (*phoD*), was upregulated in cultures with lack of P. Interestingly, N addition increased not only the expression of these genes but also polyphosphate formation and alkaline phosphatase activity in lack of P cultures compared to the P-replete culture.

As mentioned before, in the present study we found a significant upregulation of *sxtA4*, *ntcA, pstS* genes with decreasing P and N. Similar findings were reported by Zare et al. (2023) studying saxitoxin producing *R. raciborskii* strains, under laboratory conditions, who found a significant upregulation of *sxtA4*, *ntcA* and *pstS* genes in the N and P depleted conditions relative to the control (high nutrient concentration).

These findings are in the line with Rigamonti et al. (2018) studying *Raphidiopsis* strains producing the cylindrospermopsin toxin (CYN) who reported a significant enhancement in *cyrA* gene expression in N-deprived conditions (−N+P) as compared to the control (+N+P). Nevertheless, in the treatments with P deprivation, the relative expression of *cyrA* gene was downregulated, which is in contrast with our results regarding P reduction. They also observed that total CYN concentration was considerably higher in the −N+P treatment when compared to the control, in line with the change in gene expression, indicating that *cyrA* gene transcription and toxin synthesis were enhanced two times (Rigamonti et al., 2018). This finding is consistent with the study of Saker & Neilan (2001), who reported highest CYN concentrations in cultures grown without a fixed N source, even though they did not measure gene expression. Moreover, Yang et al. (2018) found higher CYN production at lower N levels. However, Yang et al. did not address the induction of gene expression, and only evaluated intracellular toxin in the biomass.

The eco-physiological responses observed in this study complemented by previous studies help to improve our understanding about environmental nutrient stress such as N and P concentrations which can have effects on the expression of genes involved in the biosynthesis of saxitoxin in *Raphidiopsis raciborskii,* as well as helping to understand the dynamics of the toxin in aquatic ecosystems. A better understanding will be very useful when dealing with the management of saxitoxins in aquatic environments. The results presented here showed that environmental nutrient stress can influence the expression of genes involved in the biosynthesis of saxitoxin in *Raphidiopsis raciborskii*.

5. Conclusion

Nutrient management strategies frequently aimed at addressing only one nutrient may be successful in some reservoirs and lakes, but in the case of Pampulha reservoir in Brazil (and potentially other eutrophic and hypereutrophic reservoirs) certain cyanobacteria, including bloom forming and toxin-producing cyanobacteria, may become more dominant due to both P and N availability that favors their dominance. This study demonstrates and confirms the need for "knowing your reservoir", employing dual nutrient management strategies for phosphorus and nitrogen, and considering the mitigation strategies for limiting the production or dominance of cyanobacteria. Future field studies, including monitoring studies, and possibly more controlled experimental designs within the range of ecological plausibility, are needed to confirm these findings for other waterbodies. Furthermore, these results add to the evidence that phosphorous and nitrogen sources may modulate toxin-producing cyanobacteria even in existing phosphorous and nitrogen rich hypereutrophic reservoirs.

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SUPPLEMENTAL MATERIAL

Table S1. Additional parameters measured in Pampulha reservoir in 2021: hydrogenation potential (pH), dissolved oxygen (DO), water temperature (WT), and photic zone (Mean and standard deviation -SD- for each parameter).

Months	WT (°C)	pH	DO ($mg.l^{-1}$)	Photic Zone (m)
Jan	26.61	8.78	7.17	1.25
Feb	25.97	8.59	4.56	1.00
Mar	26.53	8.47	5.33	1.25
Apr	24.37	8.36	6.65	0.75
May	22.25	8.02	4.74	1.18
Jun	21.02	7.90	5.96	1.38
Jul	20.08	7.62	6.36	1.13
Aug	22.02	7.68	6.07	1.25
Sep	23.02	7.95	6.32	1.00
Oct	24.26	8.53	6.74	0.45
Nov	25.93	8.64	12.13	0.50
Dec	24.56	8.43	6.17	2.25
Mean:	23.88	8.25	6.52	1.11
SD:	2.20	0.39	1.93	0.47

Table S2**.** Redundancy analysis scores to Axis1 (RDA1) and Axis 2 (RDA2) for environmental and biotic variables.

Abiotic variable scores			Biotic variable scores		
Variables	**RDA 1**	**RDA 2**	**Variables**	**RDA 1**	**RDA 2**
NH_4^+	0.3100	**-0.7934**	*R.raciborskii*	**-0.8867**	0.3744
$NO_3^-.NO_2^-$	**0.8877**	-0.2149	*sxtA4*	**-0.8251**	-0.4987
SRP	0.2843	**-0.7460**	*ntcA*	**-1.0953**	-0.2046
WT	0.4168	**0.7442**	nifH	**-0.6829**	-0.5596
pH	0.4364	**0.5936**	*pstS*	**-0.8667**	0.2612
TP	**0.6697**	-0.4932	Chl-a	**0.8194**	-0.5605

WT = water temperature; pH = hydrogenation potential; TP = total phosphorus; SRP = soluble reactive phosphorus; NO_2^- = nitrite; NO_3^- = nitrate; NH_4^+ = ammonium; Chl-a= chlorophyll-a; *R. raciborskii* = *R. raciborskii* biomass; *ntcA, nifH, pstS* and *sxtA4* = genes' expression.

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