Débora Monteiro Brentano

A RELAÇÃO DOS FATORES ABIÓTICOS E DA DENSIDADE DE *Cylindrospermopsis raciborskii* (CYANOPHYCEAE) NA CONCENTRAÇÃO DE CIANOTOXINAS E ESTRUTURAÇÃO DA COMUNIDADE ZOOPLANCTÔNICA EM UMA LAGOA COSTEIRA SUBTROPICAL

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Por

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RESUMO

As relações entre variáveis abióticas, densidade de *Cylindrospermopsis raciborskii* e concentração de cianotoxinas foram estabelecidas em um lago costeiro subtropical (Lagoa do Peri – Florianópolis/SC – Brasil). O efeito da variação da concentração de toxinas no ambiente natural e a variação da densidade de *C. raciborskii* sobre população do zooplâncton (Cladocera) foi testado "in vitro"; e, investigou-se potenciais variáveis preditoras de cianotoxinas para este manancial que abastece 100 mil habitantes. Foram coletadas mensalmente amostras de água da Lagoa do Peri (Julho/2013 a Setembro/2014) e analisadas a composição do fitoplâncton, cianotoxinas dissolvidas e intracelulares e variáveis limnológicas. Oito grupos de cianotoxinas (microcistinas, nodularina, microginina, cyanopeptolinas, anabaenopeptinas, anatoxinas, cilindrospermopsina e saxitoxina) foram analisados usando cromatografia líquida associada à espectrometria de massa ionizante (MS/MS). Essas mesmas amostras foram testadas para toxicidade utilizando *Daphnia magna*. A relação entre a concentração de STX e as variáveis físicas, nutrientes e clorofila-a foi analisada utilizando um conjunto de dados de 45 meses de monitoramento (Março/2007 a Agosto/2014). A comunidade fitoplantônica foi dominada por cianobactérias (97,4% indivíduos por volume), composta por *C. raciborskii* (55,6%; 1,0 a 3,8 x 10^4 ind.mL-1) e *Limnothrix* sp. (41,8%; 0,7 a 3,6 x 10^4 ind.mL-1), ambas abundantes durante todo o período de estudo. Altas temperaturas e nutrientes (ortofosfato, nitrogênio inorgânico dissolvido (NID) e nitrogênio total) favoreceram a densidade de *C. raciborskii*. Apenas STX foi encontrada na Lagoa do Peri em baixa concentração (0,013±0,007 µg.L-1) e intracelularmente. A densidade de *C. raciborskii* teve relação significativa positiva com a concentração de STX na Lagoa do Peri, mas o poder explicativo é de apenas 20% sugerindo não ser um preditor robusto da concentração da toxina no sistema. Já as variáveis abióticas condutividade elétrica e a concentração de NID apresentaram maior poder explanatório (49%) na variação da concentração de STX "in situ". Testes de toxicidade aguda podem ser potencialmente utilizados em triagem preliminar de STX no ambiente natural. Foi observada uma boa correlação linear (84%) entre a concentração de STX no ambiente e o efeito no zooplâncton em testes "in vitro". A toxicidade aguda observada para o zooplâncton é melhor explicada pela variação da concentração de STX (amostra bruta – relação marginalmente significante, $p = 0,09$, pseudo - $R2 = 0,18$; e amostra sonicada – relação significante, $p<0,05$, pseudo - R2 = 0,16) que pela densidade de *C. raciborskii* (relação não significante, p>0,05, pseudo - R2 = 0,012). O efeito de intoxicação aguda devido a ingestão de *C. raciborskii* contendo STX é uma característica definitiva na estruturação da comunidade do zooplâncton, uma vez que seleciona o zooplâncton capaz de coexistir com esta cianobactéria no ecossistema. O mecanismo em que variáveis abióticas regulam a variação da concentração de STXs intracelular em *C. raciborskii* e a consequente intoxicação aguda do zooplâncton por herbivoria sugere uma estruturação do tipo "bottom-up" para este ambiente.

Palavras-chave: bottom-up, cianobactéria, comunidade fitoplantônica, gerenciamento de lagos, testes ecotoxicológicos, saxitoxinas

ABSTRACT

The abiotic factors and the density of *Cylindrospermopsis raciborskii* (Cyanophyceae) related with cyanotoxins concentration and the zooplankton effects in a subtropical coastal lake

The cyanobacterium *Cylindrospermopsis raciborskii* produces toxins including saxitoxins (STXs). The main aim was to understand the relation among abiotic variables, cyanotoxin concentration and *C. raciborskii* density. These relations are very important to manage reservoirs and understand aquatic community structure, but have been few explored *in situ*. We tested the effect of toxin concentration and *C. raciborskii* density of natural water samples on a zooplankton population (Cladocera) *in vitro*. In addition, we investigated some abiotic variables as potential predictors of cyanotoxin concentration in the Peri Coastal Lake (Santa Catarina Island, Brazil). This lake has been historically dominated by *C. raciborskii* and supplies potable water for about 100,000 local citizens. Water samples was collected monthly between July 2013 and September 2014 being analyzed for phytoplankton composition, intracellular and dissolved cyanotoxins, and limnological variables. A suite of eight cyanotoxin groups (microcystins, nodularin, microginin, cyanopeptolins, anabaenopeptins, anatoxins, cylindrospermopsin, and saxitoxin) was targeted using liquid chromatography tandem (MS/MS) mass spectrometry with electrospray ionization. These samples were also used in ecotoxicological tests using *Daphna magna*. The relationship among STX concentration and physical variables, nutrients and chlorophyll-a (chl-a) was analyzed using a dataset of 45-month monitoring period (March/2007 to August/2014). The phytoplankton community was dominated by cyanobacteria (97.4% individual per volume), comprised of *C. raciborskii* (55.6%, 1.0 to 3.8 x 10^4 ind.mL^{-1}) and *Limnothrix* sp. (41.8%, 0.7 to 3.6 x 10^4 ind.mL^{-1}), both abundant during the entire study period. High temperatures and nutrients (orthophosphate, dissolved inorganic nitrogen - DIN and total nitrogen - TN) favored the *C. raciborskii* density. STX was the only cyanotoxin found in the Peri Coastal Lake, in low concentration (0.013±0.007 µg.L^{-1}), and concentrated intracellularly. *C. raciborskii* density had a significant positive relation with STX concentration, but explained only 20% of the variation in STX concentration. This suggests that *C. raciborskii* density alone is not a reliable predictor of STX concentrations in this system. However, the abiotic variables as electrical conductivity and DIN concentration provided the greatest explanatory power (49%) for STX concentration *in situ*. The acute toxicity tests can potentially be used in preliminary screening of STX in the natural environment. We observed a good linear correlation (84%) between STX concentration in the lake with effect on zooplankton in the *in vitro* tests. The acute toxicity observed on the zooplankton is better explained by the variation in STX concentration (whole water samples - marginal significant relation, $p = 0.09$, pseudo - $R^2 = 0.18$; and sonicated water samples - significant relation, $p < 0.05$, pseudo - $R^2 = 0.16$) than by the *C. raciborskii* density (no significant relation, $p > 0.05$, pseudo - $R^2 = 0.012$). The acute intoxication effect of grazing upon STX-containing *C. raciborskii* is a defining feature in zooplankton community structuration, as this selects for zooplankton that are able to coexist with toxic *C. raciborskii* in this ecosystem. The abiotic variables have a role on the regulation of intracellularly STX concentration in *C. raciborskii* and there is acute intoxication in the zooplankton as consequence of the herbivory, so the bottom-up processes seems to drive community structure in Peri Coastal Lake.

Keywords: bottom-up, cyanobacteria, ecotoxicological test, lake management, phytoplankton community, saxitoxin

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SUMÁRIO

1. INTRODUÇÃO

As florações de cianobactérias são um problema crescente no mundo todo. Cianobactérias produtoras de toxinas podem prejudicar seriamente a qualidade da água, ameaçando a saúde humana, bem como os recursos ambientais (Chorus e Bartram 1999, Chorus 2005, Burch 2008). Cianobactérias produzem metabólitos secundários tóxicos e a maioria deles são bioativos, como hepatotoxinas, neurotoxinas, ou irritantes ao contato (Chorus 2001, Merel et al. 2013). No Brasil, os gêneros com maior número de espécies tóxicas são *Microcystis* e *Anabaena*, sendo que *Microcystis aeruginosa* e *Cylindrospermopsis raciborskii* são as cianobactérias mais dispersas (Sant'Anna et al. 2008).

Dentre tais gêneros a espécie *Cylindrospermopsis raciborskii* (Woloszynska) (Seenayya e Subba Raju 1972), originalmente identificada em regiões tropicais/subtropicais, é cada vez mais encontrada em regiões temperadas. Os crescentes relatos de florações de *C. raciborskii* ao redor do mundo sugere a expansão geográfica deste organismo (Dokulil e Mayer 1996, Padisák 1997, Sinha et al. 2012, Fastner et al. 2003). Atualmente, exceto na Antártica, há registros de *C. raciborskii* em todos os continentes (Tabela 1).

Tabela 1: Registros de *Cylindrospermopsis raciborskii* ao redor do mundo.

Continentes	Autores que registraram *C. raciborskii*
África	Mohamed 2007, Haande et al. 2008
Ásia	Chonudomkul et al. 2004, Wu et al. 2010
América do Norte	Hamilton et al. 2005, Chapman e Schelske 1997
América do Sul	Bouvy et al.1999, Bonilla et al. 2012
Europa	Dokulil e Mayer 1996, Manti et al. 2005, Hindak e Moustaka 1988, Borics et al. 2000, Fastner et al. 2003, Stucken et al. 2006, Saker et al. 2003
Oceania	Hawkins et al. 1985, McGregor e Fabbro 2000, Stirling et al. 2001

Efeitos nocivos nos seres humanos causados por *C. raciborskii* foram observados pela primeira vez em "Palm Island", na Austrália, em 1979, onde um surto de hepatoenteria ocorreu numa população, que havia sido abastecida com a água de um reservatório que apresentava florações de cianobactérias (Bourke et al. 1983). Nas investigações posteriores isolou-se uma estirpe de *C. raciborskii*, que demostrou hepatotoxicidade grave a ratos e, portanto, tornou-se o organismo suspeito causador do surto (Hawkins et al. 1985). Mais tarde, um alcalóide incomum, cilindrospermopsina (CYN), constituído por uma porção guanidinica combinada com hidroximetiluracil foi isolada a partir desta cepa (Ohtani et al. 1992).

A cianotoxina CYN é um alcalóide guanidínico, inibidor da síntese proteica que necrosa as células do fígado provocando o acúmulo de gordura neste órgão (Terao et al. 1994). Banker et al. (2001) a descrevem como um alcalóide tricíclico de baixo peso molecular (415 Da) que contém uma guanidina, juntamente com uma porção de uracila potencialmente responsável pela toxicidade (Fig. 1).

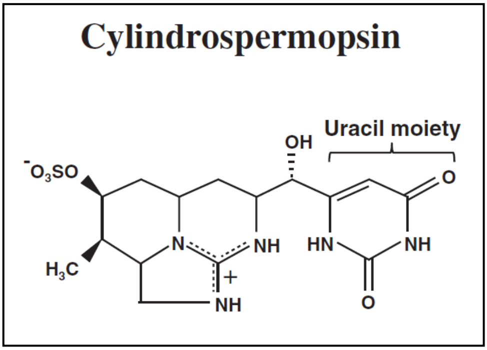
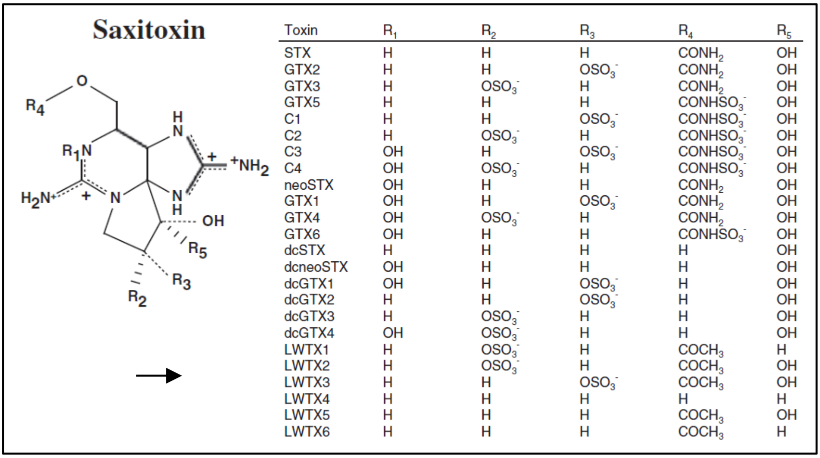


Figura 01: Fórmula molecular de cilidrospermopsina. Fonte: Modificado de Merel et al. (2013).

Hawkins et al. (1997) haviam sugerido a existência de outros análogos de CYN produzidos por *C. raciborskii*. Banker et al. (2000) explicitam que a orientação diferente do grupo hidroxila perto da porção uracila gera a 7-epicilindrospermopsina e, a ausência do grupo OH (substituído por H), gera a deoxicilindrospermopsina, um análogo não tóxico (Li et al. 2001, Norris et al. 1999).

Provou-se a mutagenicidade provocada por CYN através de evidências “in vitro” e observou-se evidências para a sua carcinogenicidade “in vivo” (Humpage et al. 2000, Falconer e Humpage, 2001). A CYN apresenta potencial de bioacumulação, o qual foi demonstrado em peixe (Saker e Eaglesham 1999), moluscos (Saker et al. 2004) e encontrou-se CYN em tecidos de cladóceros, ainda que a bioacumulação não tenha sido evidente (Nogueira et al. 2004). Em mamíferos, provoca lesão necrótica generalizada, que afeta especialmente órgãos como fígado, rins, pulmões, baço, intestino, timo e coração (Chorus e Bartmann 1999).

Surpreendentemente, cepas brasileiras de *C. raciborskii* demonstraram-se produtoras de saxitoxinas (Lagos et al. 1999, Molica et al. 2002, 2005). Saxitoxinas são compostos tricíclicos que podem ser não-sulfatados, isoladamente sulfatado ou duplamente sulfatados, com peso molecular variando de 241 a 491 Da (van Apeldoorn et al. 2007) (Fig. 2). As saxitoxinas bloqueiam os canais de sódio na membrana do axônio (Kao and Levinson 1986), causando interrupção da condução do impulso nervoso, levando à paralisia e a morte por insuficiência respiratória (van Apeldoorn et al. 2007). Ademais, também age nos canais de cálcio e potássio interferindo no sistema cardíaco (Carmichael 1994, Wang et al. 2003, Su et al. 2004). Saxitoxinas podem ser altamente tóxicas para os animais e, dependendo da dose, letais para humanos (Landsberg 2002).



Toxin	R_1	R_2	R_3	R_4	R_5
STX	H	H	H	$CONH_2$	OH
GTX2	H	H	OSO_3^-	$CONH_2$	OH
GTX3	H	OSO_3^-	H	$CONH_2$	OH
GTX5	H	H	H	$CONHSO_3^-$	OH
C1	H	H	OSO_3^-	$CONHSO_3^-$	OH
C2	H	OSO_3^-	H	$CONHSO_3^-$	OH
C3	OH	H	OSO_3^-	$CONHSO_3^-$	OH
C4	OH	OSO_3^-	H	$CONHSO_3^-$	OH
neoSTX	OH	H	H	$CONH_2$	OH
GTX1	OH	H	OSO_3^-	$CONH_2$	OH
GTX4	OH	OSO_3^-	H	$CONH_2$	OH
GTX6	OH	H	H	$CONHSO_3^-$	OH
dcSTX	H	H	H	H	OH
dcneoSTX	OH	H	H	H	OH
dcGTX1	OH	H	OSO_3^-	H	OH
dcGTX2	H	H	OSO_3^-	H	OH
dcGTX3	H	OSO_3^-	H	H	OH
dcGTX4	OH	OSO_3^-	H	H	OH
LWTX1	H	OSO_3^-	H	$COCH_3$	H
LWTX2	H	OSO_3^-	H	$COCH_3$	OH
LWTX3	H	H	OSO_3^-	$COCH_3$	OH
LWTX4	H	H	H	H	H
LWTX5	H	H	H	$COCH_3$	OH
LWTX6	H	H	H	$COCH_3$	H

Figura 02: Fórmulas moleculares dos análogos de saxitoxinas. Fonte: Modificado de Merel et al. (2013).

Diante do potencial de produção de cianotoxinas por *C. raciborskii*, ecossistemas aquáticos com ocorrência desta espécie são alvo de investigação científica. As pesquisas nestes ambientes são conduzidas visando salvaguardar a saúde pública (WHO 1998, Newcombe et al. 2010), uma vez que muitos mananciais servem para fins nobres, como abastecimento público. Pesquisas também são conduzidas buscando compreender como a estruturação da comunidade nestes ambientes está relacionada a presença da cianobactéria ou produção de cianotoxinas (Burns 1987, Hawkins 1988, Hawkins e Lampert 1989, Leonard e Paerl 2005). O presente trabalho está inserido neste contexto de investigação e foi desenvolvido em um lago com dominância de *C. raciborskii*.

1.1 ÁREA DE ESTUDO

O estudo foi conduzido na Lagoa Costeira do Peri (Figura 3), localizada entre as latitudes Sul 27°42’59” e 27°46’45” e as longitudes Oeste 48°30’33” e 48°31’59”. Ela está 3m acima do nível do mar, com o qual se conecta através de um canal de sentido único lagoa→mar (Canal Sangradouro). A lagoa apresenta área superficial de 5,7 km^2, comprimento máximo de 4 km, largura média de 1,7 km, profundidade média de 4,2 m e profundidade máxima de 11 m. Os riachos Cachoeira Grande e Ribeirão Grande são os principais tributários da lagoa, a qual tem seu volume calculado em 21,2 milhões de metros cúbicos de água (Laudares-Silva 1999, Oliveira 2002).

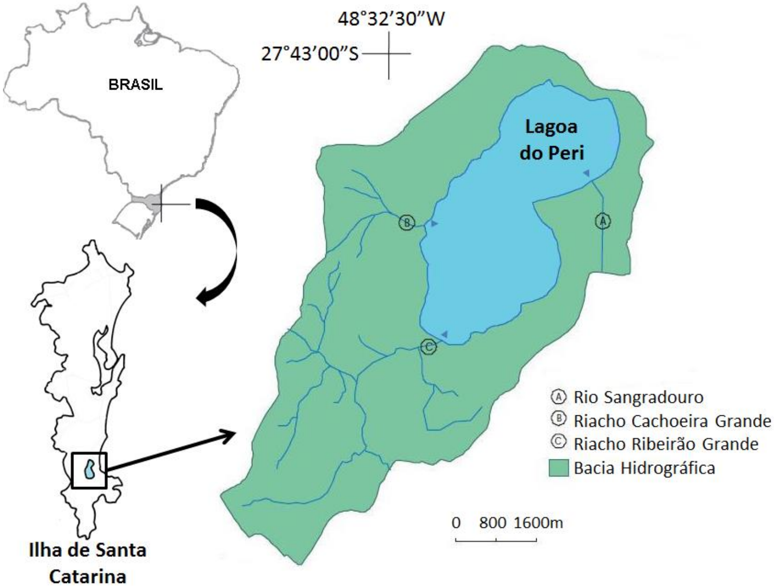


Figura 03: Mapa e localização geográfica da Lagoa do Peri, Ilha de Santa Catarina, Brasil.

Essa lagoa costeira subtropical de água doce apresenta natureza turbulenta, típica de ambientes polimíticos e a zona eufótica geralmente é menor que a zona de mistura, sendo, portanto, limitada por luz (Laudares-Silva 1999). A lagoa não apresenta estratificação térmica e os nutrientes se distribuem homogeneamente na coluna d'água (Hennemann 2010). Quanto ao grau de trofia, foi classificada como oligotrófica para a concentração de nutrientes e meso-eutrófica para a transparência e concentração de clorofila *a* (Hennemann e Petrucio 2011).

A comunidade fitoplantônica na Lagoa do Peri apresenta dominância histórica da cianobactéria *Cylindrospermopsis raciborskii* (Woloszinska) (Seenayya e Subba-Raju 1972), sendo o primeiro registro de 1996 (Laudares-Silva, 1999). Apesar desta característica, este manancial abaste mais de 100 mil pessoas na Ilha de Santa Catarina desde 2000 (Pereira e Zanin 2012). Amostragens pontuais da água em trabalhos exploratórios analisando STXs registraram a presença de STX (saxitonina), Neo-STX (neo-saxitoxina), GTX-1 (goniautoxina 1), GTX-2, GTX-3, GTX-4, GTX-5, dcGTX-2 (decarbamoil goniautoxina 2) e dcGTX-3 (Grellmann 2006, Melo Filho 2006, Mondardo 2009, Machado 2011, Machado e Sens 2012).

1.2 DOMINÂNCIA DE *Cylindrospermopsis raciborskii*, FATORES ABIÓTICOS E PRODUÇÃO DE CIANOTOXINAS

Fatores abióticos influenciam o crescimento e taxa fotossintética de *Cylindrospemopsis raciborskii*. Diversos experimentos laboratoriais tentam elucidar as condições ótimas de crescimento deste organismo conforme descrito abaixo.

C. raciborskii apresenta taxas positivas de crescimento de 15 a 35ºC; rápida supressão do crescimento acima de 40ºC; e, crescimento ótimo entre 25 a 35ºC (Briand et al. 2004) e 25 a 30ºC (Saker e Griffiths 2000). A intensidade de luz ótima para crescimento evidenciada por Shafik et al. (2001) em laboratório é de 80 µmol photons m^{-2} s^{-1}.

O incremento de HCO_3^- e pH em cultivos sob luz forte (100 µmol photons (PAR) m^{-2} s^{-1}), resulta em efeito positivo na taxa de crescimento. Entretanto, as mesmas condições não favorecem o crescimento em cultivos sob luz fraca (20 µmol photons (PAR) m^{-2} s^{-1}; Holland et al. 2012).

C. raciborskii aumentou as taxas de crescimento em concentrações de fósforo (P) superiores a 0,05 mg.L^{-1} (Wu et al. 2012). Entretanto, de acordo com Isvanóvics et al. (2000), *C. raciborskii* regula o seu metabolismo a fim de reduzir o estresse de limitação por P e compensar a deficiência deste, incrementando a atividade das enzimas catalase e fosfatase extracelulares. A cinética de absorção indicou que *C. raciborskii* é oportunista em relação ao P, armazenando-o após um pulso de saturação (Isvanóvics et al. 2000). Posteriormente observou-se que em condições de deficiência de fosfato, *C. raciborskii* apresenta otimização do crescimento quando exposta a curtos períodos de flutuação de fosfato no meio, revelando a flexibilidade fisiológica desta espécie em adaptar-se ao fosfato disponível (Amaral et al. 2014).

Em condições abundantes de fósforo (> 5mg L^{-1} PO_4^{3-}), a quantidade e a forma de nitrogênio disponíveis não interferem na concentração de biomassa em cultivos de *C. raciborskii* (Spröber et al. 2003). Esta espécie tem ampla faixa de tolerância a diferentes razões N:P e tende a dominar a comunidade fitoplantônica desde razões muito baixas (7:1) a altas (122:1) (Chislock et al. 2014). Diferentes concentrações de amônio não afetam o crescimento da espécie, uma vez que, a demanda de nitrogênio não suprida pelo nitrogênio presente na água é obtida pela fixação do N_2^- atmosférico (Spröber et al. 2003).

Os trabalhos em condições controladas de laboratório, como os citados acima, permitem o conhecimento do efeito isolado dos fatores abióticos sobre a cianobactéria. Poucos trabalhos dedicaram-se a relacionar os fatores abióticos com o crescimento de *C. raciborskii* “in situ”. Por exemplo, a dominância desta espécie em diferentes sistemas de água doce no Brasil foi relacionada a períodos de seca, ambientes polimíticos e com altas concentrações de fósforo (Soares et al. 2013). Gomes et al. (2013) observaram, também no Brasil, que a ocorrência de *C. raciborkii* não está associada a qualquer variável limnológica, incluindo fósforo. A espécie ocorre não só em sistemas eutróficos, mas também em sistemas oligotróficos e mesotróficos. Para estes autores, os fatores abióticos que parecem mais envolvidos na contribuição do desenvolvimento de *C. raciborskii* são altas temperaturas, altos valores de pH, alcalinidade e condutividade. Yamamoto et al. (2013) e Berger et al. (2006) também encontraram forte correlação entre o crescimento de *C. raciborskii* e a alta temperatura em ambiente natural.

O conhecimento sobre a relação entre fatores abióticos e produção de toxinas também é escasso e predominam os experimentos laboratoriais. Saker e Griffiths (2000) observaram, em laboratório, forte correlação negativa entre a quantidade de cilindrospermopsina produzida e temperatura.

Para as saxitoxinas, temperaturas entre 19 e 25ºC não tiveram qualquer influência na produção dos análogos STX, GTX2 e GTX3, exceto que a 25ºC a cepa C10 também produziu dcSTX (Castro et al.

2004). Contudo, observa-se que a estabilidade de degradação da toxina no meio extracelular é maior a 19ºC (50 dias) que a 25ºC (30 dias) (Castro et al. 2004).

As concentrações de STX em experimentos laboratoriais foram maires sob altas razões de N:P (Chislock et al. 2014). Ainda, altas concentrações de íons (Na^+, K^+, Mg^{2+}) na água parecem estar relacionadas com à produção de STX (Pomati et al. 2004, Kellmann e Neilan 2007, Carneiro et al. 2013).

Os diversos trabalhos desenvolvidos ainda não encerraram a questão sobre a relação entre fatores abióticos, densidade de *C. raciborskii* e cianotoxinas, especialmente explorando dados de campo. O reconhecimento de um padrão nesta relação contribuirá para esclarecer quão relevante esta cianobactéria é na dinâmica do sistema aquático. Na legislação brasileira, a densidade de cianobactéria é utilizada como alerta para monitoramento das concentrações de toxinas (Portaria MS 2914 de 12/12/2011, Brasil 2011) e limitante para o uso de um recurso hídrico para fins de abastecimento público (CONAMA 357 de 17/03/2005, Brasil 2005). Portanto, identificar qual o poder explicativo que a densidade de cianobactérias tem sobre a concentração de cianotoxinas é uma informação de alta relevância para saúde pública. Finalmente, identificar variáveis abióticas como preditores da concentração de cianotoxinas é desafiador diante da complexidade das interações entre elas e os sistemas aquáticos. Todas estas informações seriam extremamente úteis no manejo de mananciais de abastecimento público, visando servir de alerta, uma vez que o custo de análise de cianotoxinas é alto (Harada et al. 1999, Newcombe et al. 2010). A relação entre estas três variáveis - fatores abióticos, densidade de *C. raciborskii* e concentração de cianotoxinas - foi explorada neste trabalho, com dados coletados em campo, procurando esclarecer a relevância desta relação em um ambiente natural.

1.3 *Cylindrospermopsis raciborskii* E ESTRUTURAÇÃO DA COMUNIDADE ZOOPLANCTÔNICA

Diversos estudos têm sido conduzidos no sentido de compreender o efeito das florações de cianobactérias nas comunidades zooplanctônicas. Dentre os impactos ecológicos de cianobactérias em comunidades aquáticas, destaca-se as alterações na estrutura da comunidade de zooplâncton. Há mudança na composição de espécies de grandes crustáceos, como cladóceros e copépodes, para uma comunidade dominada por pequenos cladóceros e microzooplâncton (Leonard e Pearl 2005).

Especificamente, na presença de filamentos de *C. raciborskii*, há redução proporcional na abundância de espécies de cladóceros grandes devido a uma diminuição na taxa de filtração (Hawkins e Lampert 1989). De particular interesse são os efeitos sobre os microcrustáceos *Daphnia* spp., potenciais herbívoros de cianobactérias planctônicas. *Daphnia* spp. são representantes comuns da ordem Cladocera, um grupo chave de organismos em sistemas de água doce e a perturbação de suas populações pode ter efeitos em toda a cadeia alimentar aquática (Rohrlack et al. 2003).

Além do efeito mecânico, que pode ser o fator responsável pelas mudanças na estruturação da comunidade zooplanctônica, cladóceros são afetados pelos metabólitos tóxicos (cianotoxinas) de *C. raciborskii* (Nogueira et al. 2004). Em bioensaios de toxicidade, cladóceros nativos do Brasil (*Moina micrura* e *Daphnia gessneri*) mostraram-se extremamente sensíveis a presença de cianotoxinas (Ferrão-Filho et al. 2009), com respostas coincidentes com os meses nos quais observou-se maior biomassa de cianobactérias e níveis de toxinas mais elevados no Reservatório do Funil/RJ. Entretanto, estudos com organismos do zooplâncton expostos a cianobactérias e suas toxinas usando material coletado em campo são insuficientes na literatura (Sotero-Santos et al. 2006).

Na Lagoa do Peri, sabe-se que a comunidade zooplantônica é dominada por rotíferos (75%) e composta por cládoceros (25%) de pequeno porte (*Bosmina hagmanni* e *Bosmina frey*). Registrou-se declínio na densidade de cladóceros nos meses com aumento da concentração de clorofila-*a* (Gerzson 2011). Contudo, não há dados sobre o potencial efeito de cianobactérias ou cianotoxinas no zooplâncton exposto a amostras deste ecossistema.

Cascatas tróficas (Paine 1980) apresentam o efeito resultante de interações fortes ou fracas que interferem na abundância dos organismos de determinada espécie em um ecossistema. Na estruturação de uma comunidade, a hipótese de Hairston et al. (1960) assume que os predadores controlam a abundância dos herbívoros e acabam, indiretamente, controlando os produtores, em um mecanismo de “cima para baixo” (“top-down”). Murdoch (1966) considera que produtores têm um papel de regulação da herbivoria. Ou seja, há um controle “de baixo para cima” (“bottom-up”), em que espécies de níveis tróficos superiores teriam sua abundância regulada pela disponibilidade de recursos. Para Murdoch (1966), o fato de os herbívoros não esgotarem os recursos alimentares (produtores) deve-se ao fato de que nem todos os produtores são recurso alimentar disponível. Os produtores possuem adaptações como espinhos ou produção de substâncias tóxicas ou não palatáveis que evitam a predação ou fornecem alimento pobre em nutrientes (Hartley e Jones 1977); o que levaria a limitação dos herbívoros. Estas forças de regulação (“top-down” e “bottom-up”) podem alterar-se sazonalmente (Sinclair, 1975) e ambas explicariam a estruturação das comunidades (Estes et al. 2011).

Hong et al. (2013) sugerem que a persistência de *C. raciborskii* no ambiente aquático pode ser promovida pela preferência do zooplâncton em predar outras espécies do fitoplâncton, com perda significativa do controle “top-down” que tem como consequência o aumento da abundância de *C. raciborskii*. Por outro lado, o efeito tóxico observado no zooplâncton (Ferrão-Filho et al. 2009, 2010) pode indicar efeito direto da regulação “bottom-up”.

Neste trabalho, procurou-se compreender quais as forças que regulam a estruturação da comunidade zooplanctônica num ecossistema dominado por *C. raciborskii*. Buscou-se identificar entre a concentração de toxina (intra e extracelular) e a densidade de cianobactérias na Lagoa do Peri, qual teria maior efeito sobre uma população de Cladocera “in vitro” com consequente potencial de regular a comunidade zooplanctônica “in situ”.

Além desta abordagem de investigação ecológica, a observação do efeito de cianobactérias e cianotoxinas sobre o zooplâncton pode apresentar um importante papel no manejo de mananciais de abastecimento público. Bioensaios com *Daphnia magna*, por exemplo, são apontados como ferramentas úteis na avaliação de cianotoxinas devido à sua relativa simplicidade, baixo custo e capacidade preditiva que podem auxiliar na adoção de medidas de gestão (Ács *et al.* 2013). Entretanto, conforme pontuado por Ferrão-Filho et al. (2010), há necessidade de adaptação dos protocolos para teste padrão com *Daphnia* spp. para predição de cianotoxinas em ambientes naturais, os quais, no Brasil, são definidos pela Associação Brasileira de Normas Técnicas (ABNT 2009). Neste sentido, este trabalho avaliou se um organismo-teste padrão (*Daphnia magna*) responde à variação da concentração de cianotoxinas no ambiente natural, antecipando questões que podem auxiliar na gestão de um manancial como a Lagoa do Peri.

2. HIPÓTESES

No contexto do estado da arte apresentado, as seguintes hipóteses foram testadas por este estudo:

i. A densidade de *C. raciborskii* apresenta correlação positiva com a concentração de cianotoxinas na Lagoa do Peri.
ii. A relação de dependência entre a concentração de cianotoxinas e fatores abióticos (temperatura, relação N:P e concentração iônica) observada “in vitro” pode ser observada também em um ambiente natural dominado por *C. raciborskii*.
iii. A comunidade zooplanctônica (cladóceros) sofre efeito agudo causado pela presença de *C. raciborskii* e/ou pelas toxinas produzidas, indicando regulação “bottom-up” na estruturação da comunidade.
iv. Os testes ecotoxicológicos têm potencial indicativo da presença de cianotoxinas em um ambiente natural, sendo úteis em triagens preliminares.

3. JUSTIFICATIVA E OBJETIVOS

A Lagoa do Peri é um ambiente túrbido, bem misturado, limitado por nutriente e luz (Komárková et al. 1999, Laudares-Silva 1999, Greellmann 2006). Apesar disto, esta lagoa não apresenta limitações aparentes para o desenvolvimento de *C. raciborskii*, o que nos leva a pensar que os mecanismos da estruturação da comunidade aquática podem ser determinados pela densidade de cianobactérias e/ou pela concentração de cianotoxinas. Por este motivo, a estruturação da comunidade zooplanctônica neste ambiente é objeto de investigação ecológica.

O Parque Municipal da Lagoa do Peri (PMLP) é uma das oito áreas de proteção ambiental da Ilha de Santa Catarina e foi criado para preservar o maior manancial de água doce da ilha – a Lagoa do Peri, que vem sendo utilizada pela Companhia Catarinense de Águas e Saneamento (CASAN), desde o ano 2000, para abastecimento da população local. O PMLP foi regulamentado pela Lei Municipal 1.828/81, decretado pela Lei nº 091/82 e está localizado no sudeste da ilha de Santa Catarina com aproximadamente 1.500 hectares de área florestal (CECCA 1997).

Florações de cianobactérias produtoras de toxinas em reservatórios oferecem risco real de contaminação (Pádisak 1997). Diferentes cepas de *C. raciborskii* podem produzir cianotoxinas diferentes. Há registros de cepas da Austrália e Nova Zelândia produzindo cilindrospermopsinas (McGregor e Fabbro 2000, Pádisak 1997), cepas da América do Sul produzindo saxitoxinas (Molica et al. 2002), cepas da América do Norte que não foram investigadas minuciosamente para a presença de compostos tóxicos (Neilan et al. 2003) e pelo menos uma estirpe de Portugal demonstrou ser tóxica em bioensaio com ratos, mas nenhuma das cianotoxinas conhecidas foi identificada (Saker et al. 2003).

Considerando a) a Lagoa do Peri como um importante manancial de abastecimento para Ilha de Santa Catarina; b) a dominância de cianobactérias, especialmente *Cylindrospermopsis raciborskii* neste ambiente; c) o potencial efeito tóxico de cianotoxinas sobre humanos e comunidade biótica; d) que a estrutura da comunidade aquática pode ser alterada pela presença de cianobactérias/cianotoxinas; e) e, que a simples presença de cianobactérias não é condição determinante para a presença de cianotoxinas; o objetivo geral deste trabalho foi:

Contribuir com a compreensão da relação dos fatores abióticos e da densidade de *Cylindrospermopsis raciborskii* com a concentração de cianotoxinas em um ambiente natural e deste fenômeno com a estruturação da comunidade zooplantônica (cladóceros).

3.1 OBJETIVOS ESPECÍFICOS

Determinar se STX e CYN são produzidas por cianobactérias presentes na Lagoa do Peri, SC, Brasil e suas concentrações num período de um ano;

Verificar se as cianotoxinas presentes neste ecossistema aquático estão no meio intracelular (fitoplâncton) ou extracelular (dissolvidas na água);

Determinar a relação entre fatores abióticos, concentração de cianotoxinas e densidade de *Cylindrospermopsis raciborskii* "in situ";

Verificar o potencial que fatores abióticos e a densidade de *C. raciborskii* têm como preditores de cianotoxinas na Lagoa do Peri;

Verificar se há relação positiva entre a concentração de cianotoxinas "in situ" e o efeito sobre o zooplâncton (imobilidade) em testes de toxicidade aguda com *Daphnia magna*;

Descobrir qual fator apresenta maior efeito deletério sobre o zooplâncton (cladóceros): a densidade de *C. raciborskii* ou a concentração de toxinas, discernindo o que é determinante no suposto controle "bottom up" em um ambiente natural dominado por *C. raciborskii* produtora de toxinas.

4. CAPÍTULO 1

What is the density effect of *Cylindrospermopsis raciborskii* in the concentration of Saxitoxin in a freshwater system?

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What is the density effect of *Cylindrospermopsis raciborskii* in the concentration of Saxitoxin in a freshwater system?

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Abstract

Cylindrospermopsis raciborskii is expanding its distribution and produces toxins including saxitoxins (STXs), a prevalent toxin in Brazilian drinking water reservoirs. Efforts to predict and prevent human exposures to cyanotoxins often rely on measures of fitoplankton cell density. However, it is not clear how cell density relates to actual STXs levels. Our aim in this work was to understand how *C. raciborskii* density relates to variations of STX, and whether this is potentially a predictor of STX, within Peri Coastal Lake, a drinking water supply for 100,000 citizens. This lake has been historically dominated by *C. raciborskii.* Water samples collected from Peri Coastal Lake between July 2013 and September 2014 were analyzed for phytoplankton composition, intracellular and dissolved cyanotoxins, and limnological factors. The STX was targeted using liquid chromatography tandem (MS/MS) mass spectrometry with electrospray ionization. Also, a suite of seven other cyanotoxin groups (microcystins, nodularin, microginin, cyanopeptolins, anabaenopeptins, anatoxins and cylindrospermopsin) was analysed. The phytoplankton community was dominated by cyanobacteria (97.4%), comprised of *C. raciborskii* (55.6%, 1.0 to 3.8 x 10^4 ind.mL^{-1}) and *Limnothrix* sp. (41.8%, 0.7 to 3.6 x 10^4 ind.mL^{-1}), both abundant during the entire study period. High temperatures and nutrients (orthophosphate, DIN and TN) favored *C. raciborskii* density. STX was the only cyanotoxin found in the Peri Coastal Lake during the study period (0.013±0.007 µg.L^{-1}), and it was concentrated intracellularly. *C. raciborskii* density explained only 20% of the variation in STX concentration in the lake. This suggests that *C. raciborskii* density alone is not a reliable predictor of STX concentrations in this system, which could have implications for monitoring strategies in other environments.

Key-words: coastal lake; cyanobacteria; cyanotoxin; drinking water; lake management; phytoplankton community.

1. *Introduction*

Cylindrospermopsis raciborskii (Woloszynska) (Seenayya and Subba Raju 1972) is a filamentous, diazotrophic, planktonic cyanobacterium (Order Nostocales). This species has expanded its distribution in recent decades from tropical/sub-tropical habitats to other ecoregions such that it now has a global distribution (Wiedner et al. 2007; Bonilla et al. 2012; Sinha et al. 2012). Thus, it is an invasive species of global ecological importance (Antunes et al. 2015; Padisák 1997).

C. raciborskii has evolved a number of adaptations that corroborate its great ecological success tolerating a wide range of environmental conditions (Padisák 1997). Some strains of *C. raciborskii* are capable of producing one or more toxins. These include cylindrospermopsins (Ohtani et al. 1992, Li et al. 2001) and saxitoxins (Lagos et al. 1999, Molica et al. 2002, Molica et al. 2005).

Cylindrospermopsin (CYN) has been shown to inhibit protein synthesis, induce oxygen radical formation, and cause DNA strand breaks (Runnegar et al. 1994, 2002; Looper and Williams 2004; Humpage et al. 2005). Its pathological effects appear to be localized to the liver and kidneys (Humpage and Falconer, 2003). Saxitoxins (STXs) interferes with the function of voltage-gated ion channels, such as neuronal sodium channels (Kao and Levinson 1986), as well as calcium and potassium channels (Carmichael 1994, Wang et al. 2003, Su et al. 2004), resulting in subsequent muscular paralysis and depression of cardiac output. In this context, the presence of *C. raciborskii* in water supplies used by humans or livestock is potentially harmful.

Recent studies (Figueredo et al. 2013; Gomes et al. 2013; Soares et al. 2013; Tonetta et al. 2013; Yamamoto et al. 2013) have attempted to clarify the relationship between environmental variables and *C. raciborskii* density in the natural ecosystem. This is critical for managing water quality in lakes, particularly those used as water source for drinking water production. The practical problems associated with high cyanobacterial biomass are the potential health threats due to toxins present within cells (Chorus and Bartram 1999). As such monitoring of cyanobacterial biomass is used as an indicator for the presence of toxins by water supply companies in Brazil and is often used to avoid human health problems (WHO 1993, 1996, 1998). However, the timing of biomass maximums and toxin concentration maximums are not necessarily coincident (Chorus and Bartram 1999; Ibelings et al. 2015), considering *e.g.* the long breakdown reaction of the toxins (Jones and

Negri 1997) or the toxigenic composition of the bloom (Molica and Azevedo 2009; Beversdorf et al. 2015).

Since 2000, Peri Coastal Lake has supplied drinking water to approximately 100,000 inhabitants of Santa Catarina Island, in Southern Brazil. *C. raciborskii* has historically dominated this lake (Laudares-Silva, 1999; Grellmann, 2006; Tonetta et al,. 2013; Silveira, 2013) and is a species with toxin-producing potential. Understanding the ratio of intracellular-to-dissolved cyanotoxin can contribute to improvements in drinking water treatment strategies (Chorus and Bartram 1999; Newcombe et al. 2010; Zamyadi et al. 2012).

In this study, we relate the phytoplankton density (*C. raciborskii*) and the cyanotoxin concentration (STX). Considering the current literature, no previous work has linked phytoplankton density to the cyanotoxin concentration, which brings a comprehensive contribution. This study was the first long-term toxin monitoring study in Peri Coastal Lake, Brazil. We described the types, concentrations, and ratio of intracellular-to-dissolved cyanotoxins present there, what means a local important knowledge contribution.

2. *Methodology*

2.1 Study Area

Peri Lake (27°44’ S and 48°31’ W) is located in the Southeastern region of Santa Catarina Island, Brazil. The climate in the area is typically subtropical. The lake has a surface area of 5.07 km^2 and average and maximum depths of 4.2 m and 11.0 m, respectively. The volume is 21.2 million m^3 of water (Laudares-Silva 1999, Oliveira 2002). Peri Coastal Lake exhibits spatial homogeneity (horizontal and vertical) (Hennemann and Petrucio 2010; Tonetta et al. 2013). It is a freshwater coastal lake without marine influences (freshwater year-round), which makes it the main freshwater resource for Santa Catarina Island. The lake and surroundings (including almost the entire drainage basin) are within an environmentally protected area.

Peri Coastal Lake has been classified as oligotrophic based on total phosphorus and meso-eutrophic for water clarity and chlorophyll-a (chl-a), due to high cell densities of *C. raciborskii* and by the high recycling rates observed in tropical and subtropical water bodies (Hennemann and Petrucio 2010; Tonetta et al. 2013).

2.2 Limnological Factors

Peri Lake was sampled at the center of the lake (27º43’49.4’’S; 48º31’19.9’’W) where the depth is 9±1 m. Water was sampled at the depth of Secchi Disk extinction with a Van Dorn sampler. Monthly samples were taken over a 15-month period between July 2013 and September 2014. Sub-samples were filtered (glass microfiber filter AP40 - Millipore®) for analysis of chl-a and dissolved nutrients.

The air temperature was measured *in situ* with a mercury thermometer. The precipitation accumulated for seven days prior to the sampled day was calculated from data obtained from EPAGRI/CIRAM (Information Center for Environmental Resources and Hydrometeorology of Santa Catarina), measured using the station number 02748024.

In the field, water transparency was measured using the Secchi Disk method (Chapman 1992). Water temperature, pH, conductivity and dissolved oxygen were measured using a multiprobe YSI (Model 85). In laboratory, total alkalinity was determined by titration (Mackereth et al. 1978), and the concentrations of total nitrogen (TN) and total phosphorus (TP) were determined from unfiltered water samples (Valderrama 1981). Nitrite (Golterman et al. 1978), nitrate (Mackereth et al. 1978), ammonia (Koroleff 1976) and orthophosphate (Strickland and Parsons 1960) were determined from filtered water samples. Filtered water samples were preserved with phosphoric acid to determine dissolved organic carbon (DOC) concentrations (Shimadzu TOC-5000A). Chl-a was measured spectrophotometrically after extraction from filters with 90% acetone, and using a correction for phaeopigment (Lorenzen 1967).

2.3 Phytoplankton Analysis

The samples for phytoplankton analysis were fixed with 4% formaldehyde in the field immediately after sampling. Aliquots were sedimented with acetic Lugol’s solution and investigated using an inverted microscope according to the Utermöhl method (Hasle, 1978), in which 400 organisms (unicell, filament or colony) of the dominant species were counted. The density was estimated (Ros, 1979) and the biovolume of *C. raciborskii* was calculated from mean values of 30 individual measures (Hillebrand *et al.*, 1999; Sun and Liu, 2003). Dominant (>50% of total density) and abundant species (> mean density) were determined (Lobo and Leighton, 1986).

2.4 Cyanotoxin Extraction

Cyanotoxins were extracted from whole water, filtrate, and filtered biomass. Water samples were lyophilized to complete dryness. Lyophilized biomass was resuspended in 0.1% formic acid in water and subjected to three freeze-thaw cycles between -80 and 50 °C. Extracts were further disrupted in a sonicating water bath and centrifuged, collecting the supernatant for analysis using liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS). Toxins were extracted from filtered biomass in a similar fashion; however, the filters were not lyophilized. Formic acid in water (0.1%) was added to each filter in a sample tube. During thaw cycles, filters were disrupted in a sonicating water bath (45 °C) and shaked vigorously. $^{13}C_6$-phenylalanine was spiked into each sample prior to extraction as a surrogate standard for LC-MS/MS detection. The protocols were adapted from Harada (1988) and Selwood et al. (2007).

2.5 LC-MS/MS Analysis

Anatoxin-a (ATX; Sigma Aldrich), homoanatoxin-a (hATX; Abraxis), STX (National Research Council Canada), CYN (Abraxis), phenylalanine (Phe; no standard included in calibration) and $^{13}C_6$-phenylalanine were separated using a SeQuant ZIC-HILIC (zwitterionic hydrophilic liquid interaction chromatography) column (5 µm, 150 x 2.1 mm) with 60 mM formic acid in acetonitrile (B) and HPLC water (A). The LC gradient included column equilibration from 0-5 min at 60% B, a gradient from 60-20% B from 5-10 min, a step change to 60% B at 10.01 min, and column re-equilibration for a total of 14 min per run with a flow rate of 0.4 mL/min. $^{13}C_6$-phenylalanine was used to identify the retention time of native Phe and to discriminate from ATX, which have the same molecular weight and ion transitions, but different retention times (Supplemental Figure 1). Each analyte had two or three optimal ion transitions in a scheduled multiple reaction monitoring method with specific retention times and ion-specific parameters (Supplemental Table 1). General mass spectrometer parameters used are as follows: entrance potential: 10 mV; curtain gas: 10 psi; collision gas: high; ionspray voltage: 3500; source temperature: 600 °C; ion source gases 1 and 2: 70 psi.

Microcystins (MC-LR and MC-Dha^7-LR (NRC); MC-RR, MC-YR, MC-LA (Sigma Aldrich)), nodularin (NRC), anabaenopeptins (A, B; Marine Biotechnology in North Carolina (MARBIONC)), microginin 690 (MARBIONC), and cyanopeptolins (1020, 1041, 1007; (MARBIONC)) were separated using a Luna C18 column (Phenomoneex, 3 µm, 150 x 3 mm) with 0.1% formic acid and 5 mM ammonium formate in 95% acetonitrile (B) and HPLC water (A). Each sample was separated using an equilibration from 0-3 min at 30% B, gradient of 30-90% B from 9-15 min, step change back to 30% B at 15.01 min and a final five minutes for re-equilibration, resulting in a total run time of 20 minutes (Supplemental Figure 2). Additional MS parameters were as follows: entrance potential: 10 mV; curtain gas: 15 psi; collision gas: high; ionspray voltage: 5000; source temperature: 600°C; ion source gases 1 and 2: 70 psi. Compound-specific parameters for microcystins and other cyanopeptides are listed in Supplemental Table 2.

The LC-MS/MS method was optimized for each compound individually adjusting it to the mobile phase gradient and isocratic methods to achieve the best separations.

2.6 Statistical Analysis

The normal distribution of the climatological and limnological data and *C. raciborskii* density were tested by using Shapiro-Wilk's W test ($p>0.01$). Accumulated precipitation, orthophosphate, and TN:TP ratio were log-transformed to be adjusted to normal distribution. The other factors fit a normal distribution. The Pearson Correlation was used to observe the correlation among variables.

We developed a Generalized Linear Model (GLM) with gamma distribution, adjusted by the Akaike Information Criterion (AIC), assessing the relationship between environmental factors and *C. raciborskii* density within the study system. The factors used in this model were water temperature, pH, conductivity, alkalinity, DOC, orthophosphate, TP, dissolved inorganic nitrogen and TN.

The same statistical approach was used to define the relationship between STX concentration and *C. raciborskii* density, aiming to understand the degree to which *C. raciborskii* density explained the variance in STX concentration. This analysis was performed with a 15 month dataset plus two extra samplings made in the months of February and March of 2014.

The GLM method was used without data transformations and was selected because it presented a better residual analysis among the other approaches tested (Supplemental Figure 3 and 4). All statistical analyses were calculated using the R statistical computing environment (R Core Team 2015). The *MASS* package (Venables and Ripley 2002) was used for model selection based on the Akaike Information Criteria (Akaike, 1974) and a significance level of 0.05.

3. Results

3.1 Limnological Factors

Climatological variables showed oscillations in air temperature measured *in situ* reflecting the subtropical weather with distinct seasons. The maximum temperature was registered in summer (27.6°C on January 2014) minimum in winter (16.2°C on July 2013). The rainiest periods were in the spring (September) 2013 and summer (February) 2014, reaching 171.9 mm_{7d} and 76.3 mm_{7d}, respectively (Figure 1). The rain volume was not well distributed during the study period.

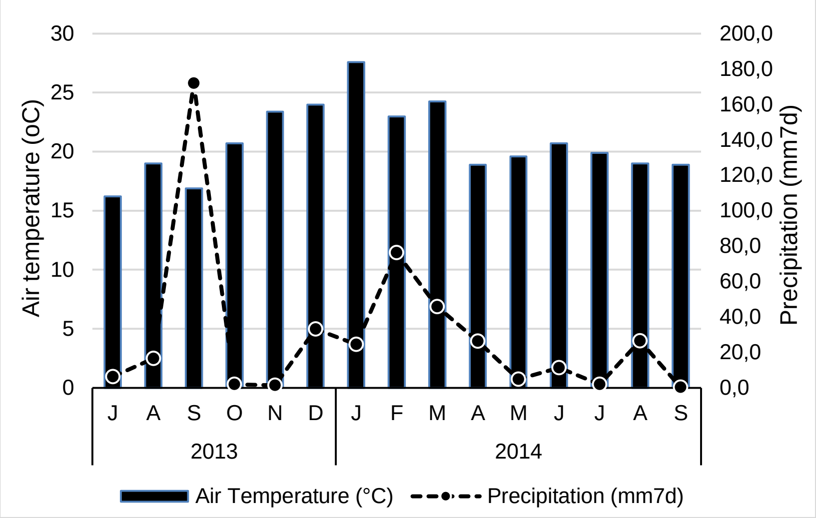

FIG. 1: Precipitation accumulated in the seven days prior and air temperature during sampling in the Peri Coastal Lake, from July 2013 to September 2014.

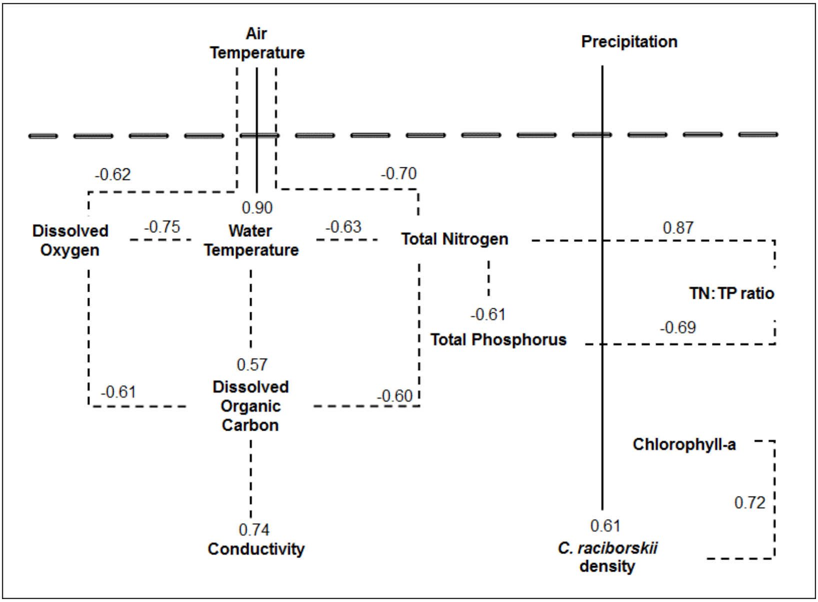

FIG. 2: Factors found to be well correlated in this study (Pearson Correlation - r>0.55, p<0.05) and the relationships among them.

Descriptive statistics for water sampled in Peri Coastal Lake from July 2013 to September 2014 with mean, standard deviation, minimum and maximum are shown in Table 1. Water temperature varied from 17.1 °C in August 2013 to 27.5 °C in January 2014. This temporal variation during the sampling period was strongly correlated with the air temperature (r=0.9) as usual (Figure 2). The water was well oxygenated during the entire study period (Table 2), and correlated negatively to water temperature (r=-0.75). DOC concentrations were relatively high, reaching 4.81±0.49 mg L^{-1}. Warmer periods were correlated with increasing DOC (r=0.57) and lower TN concentrations (r=-0.63). In addition, there is an inverse correlation between TN and DOC (r=-0.60). A high rate of primary production increases the DOC source due to the higher rate of cellular lysis and excretion of the community (Esteves 2011, Dunalska et al. 2012). Dissolved organic carbon from aquatic community excretions has been shown to increase also during periods of nutrient stress (Esteves, 2011). This processes linked with DOC also produced an increase of electrical conductivity in the water (r=0.74).

The sum means of nitrite, nitrate and ammonia (DIN= 5.34±2.85 μg L^{-1}) and orthophosphate concentrations (2.3±3.15 μg L^{-1}) were low reflecting the oligotrophic environment. Finally, *C. raciborskii* density was strongly correlated with both precipitation (r=0.61) and chl-a in Peri Coastal Lake (r=0.72).

TABLE 1. Descriptive statistics for water sampled in Peri Coastal Lake from July 2013 to September 2014 (n=15).

Variables	Mean	Std. Dev.	Minimum	Maximum
Transparency (m)	0.93	0.07	0.80	1.05
Water Temperature (°C)	22.01	3.83	17.10	27.50
pH	6.82	0.61	5.92	8.33
Electrical Conductivity (μS cm^{-1})	70.48	6.13	58.00	82.90
Dissolved Oxygen (mg L^{-1})	8.31	0.68	7.30	9.77
Alkalinity (mEq L^{-1})	0.14	0.02	0.10	0.18
Dissolved Organic Carbon (mg L^{-1})	4.81	0.49	4.27	5.97
Orthophosphate (μg L^{-1})	2.46	3.15	0.48	13.18
Total Phosphorus (μg L^{-1})	12.55	4.01	4.23	18.13
Nitrite (μg L^{-1})	0.13	0.22	0.00	0.90
Nitrate (μg L^{-1})	3.01	2.15	0.54	7.52
Ammonia (μg L^{-1})	2.48	2.02	0.00	6.40
Dissolved Inorganic Nitrogen (μg L^{-1})	5.34	2.85	1.69	12.36
Total Nitrogen (μg L^{-1})	437.56	299.52	12.01	998.79
TN:TP ratio	50.56	63.42	0.75	236.24
Chlorophyll-a (μg L^{-1})	33.54	14.32	17.09	54.47

3.2 Phytoplankton composition

The phytoplankton community was composed of five groups, with 10 freshwater taxa (two species and eight genera): Chlorophyta (4 taxa), Cyanobacteria (2 taxa), Bacillariophyta (2 taxa), Dinophyta (1 taxa) and Euglenophyta (1 taxa). Taxa and frequency of occurrence identified in counting chambers during all months are shown in Table 2.

TABLE 2: Phytoplanktonic species and frequency of occurrence (% of sampling days observed) observed in Peri Coastal Lake, from July 2013 to September 2014.

Taxa	**%**
Cyanobacteria	
Cylindrospermopsis raciborskii (Woloszynska) Seenayya and Subba-Raju	100
Limnothrix sp.	100
Dinophyta	
Peridinium sp.	7
Euglenophyta	
Trachelomonas sp.	7
Bacillariophyta	
Melosira sp.	53
Fragillaria sp.	7
Chlorophyta	
Ankistrodesmus sp.	40
Chlorella sp.	40
Monoraphidium irregulare (Smith) Komárková- Legnerová	93
Scenedesmus sp.	13

Cylindrospermopsis raciborskii and *Limnothrix* sp. were the most frequently detected Cyanobacteria species in Peri Coastal Lake during the sampling period, while *Monoraphidium irregulare* was the most frequently detected Chlorophyta species.

Cyanobacteria were the most representative group with 97.4% of total average density followed by Chlorophyta with 2.3%. Dinophyta, Bacillariophyta and Euglenophyta, together, had only 0.3% of total average density. Cyanobacteria were dominant during the entire study period, whose density ranged from 2.1×10^4 ind. mL^{-1} ($5x10^6$ μm^3. mL^{-1}) in August 2014 to 6.6×10^4 ind. mL^{-1} ($19x10^6$ μm^3. mL^{-1}) in August 2013 (Figure 3).

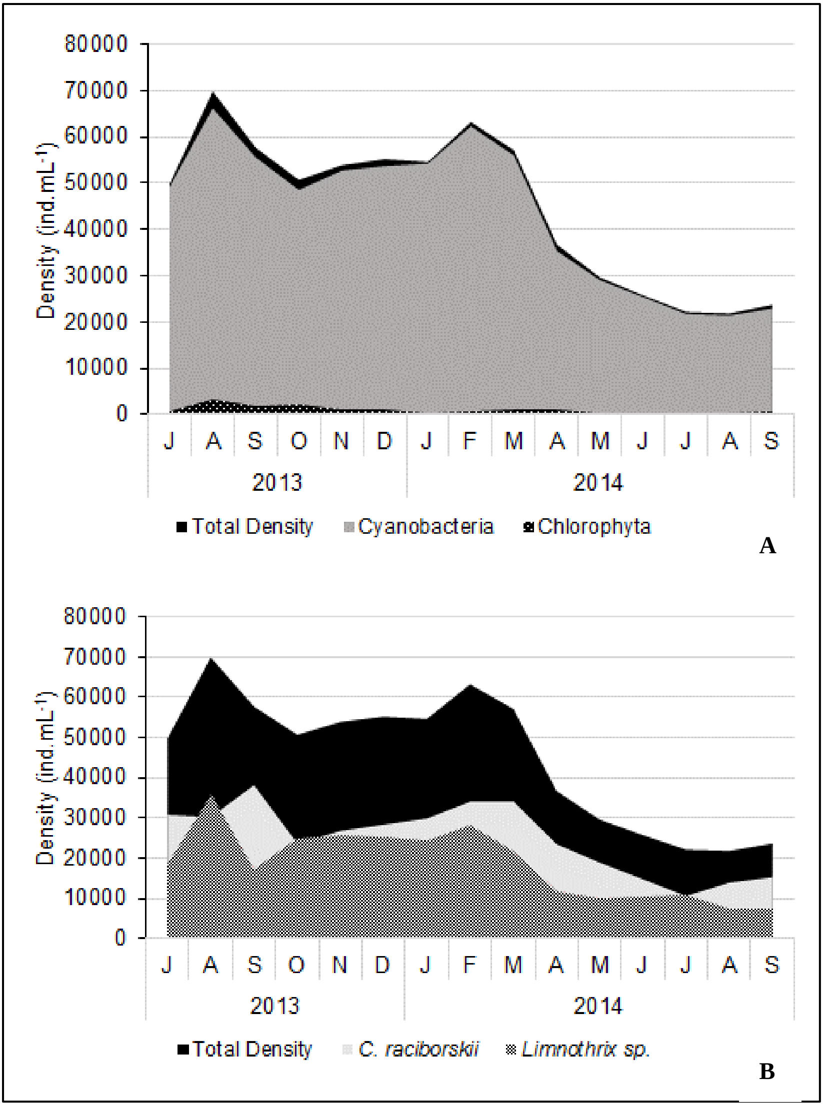

FIG. 3: (A) Density of total phytoplankton and most represented groups. (B) Density of total cyanobacteria and abundant species in Peri Coastal Lake from July 2013 to September 2014.

C. raciborskii was dominant seven of 15 months studied, in July and December 2013 and in February, April, May, August and September 2014. This species alone comprised 55.6% of the total phytoplankton community. The minimum and maximum density values recorded for this species were 1.0 x 10^4 ind.mL^{-1} (4x10^6 µm^3. mL^{-1}) on July 2014 and 3.8 x 10^4 ind mL^{-1} (1.3 x 10^7 µm^3 mL^{-1}) on September 2013. *C. raciborskii* and *Limnothrix* sp. were abundant in all months. *Limnothrix* sp. was dominant in August 2013 with a density of 3.6 x 10^4 ind.mL^{-1} (10x10^6 µm^3. mL^{-}1). *Limnothrix* sp. comprised 41.8% of the total phytoplankton community.

Orthophosphate (t= 4.2; p=0.002), temperature (t=3.2; p=0.009), DIN (t=-2.8; p=0.02) and TN (t=2.8; p=0.02) are the abiotic factors that best explained the variation in *C. raciborskii* density. These abiotic factors are responsible for 73% (pseudo R-squared= 0.73) of the variation in *C. raciborskii* biomass in the ecosystem studied (Figure 4). The strong linear correlation between accumulated precipitation and *C. raciborskii* density (r=0.61) shown above was reflected here. The orthophosphate increases in periods of higher rainfall may have fueled *C. raciborskii* growth among other sources (Figure 5).

3.3 Cyanotoxins and its relationship with C. raciborskii density

Of the cyanotoxins targeted, only STX was detected in Peri Coastal Lake during the study period with an average concentration of 0.013±0.007 µg L^{-1}. The maximum and the minimum values found were, respectively, 0.029 and 0.002 µg L^{-1}. All toxin detected was concentrated intracellularly. Dissolved STX in the water was not detected at a detection limit of 0.0025 µg.L^{-1}. Microcystins, nodularin, anabaenopeptins, cyanopeptolins, microginin, CYN, ATX and hATX were also not detected.

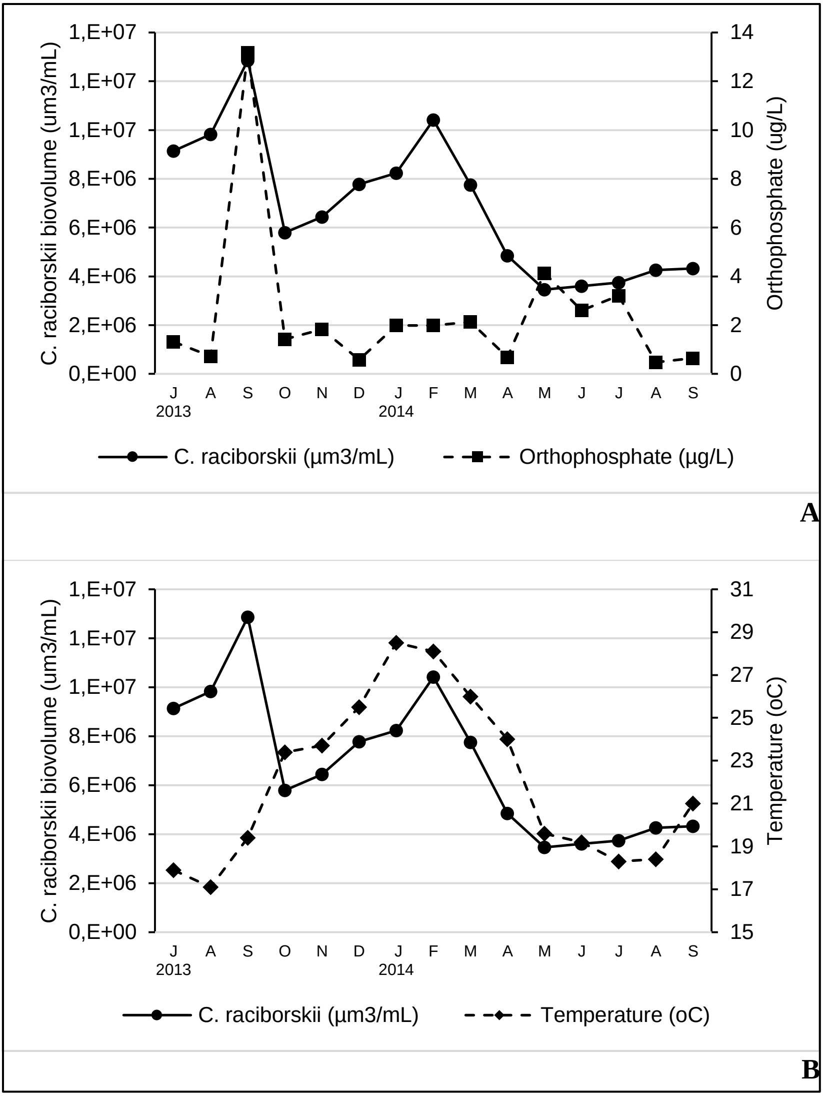

FIG. 4: Biovolume of *C. raciborskii* presented variation according the orthophosphate concentration (A) and water temperature values (B). These variables together with dissolved inorganic nitrogen (DIN) and total nitrogen (TN) explained 73% of the variation in the density of *C. raciborskii*.

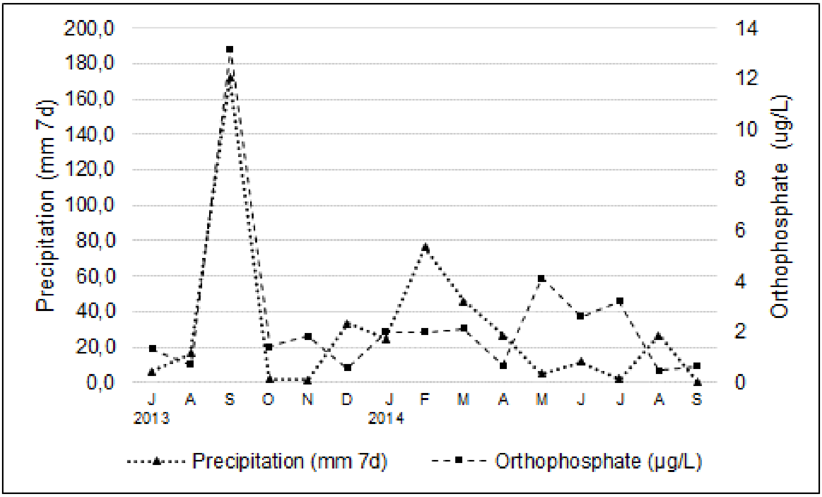

FIG. 5: Higher rainfall periods made the orthophosphate concentration increase in the Peri Coastal Lake during the study period (from July 2013 to September 2014), resulting in a good correlation between precipitation and *C. raciborskii* density.

Since measurable toxin concentrations were inside the cells, it is possible exists a dependent relationship between *C. raciborskii* density and the concentration of STX. However, only a weak, but significant relationship was observed between STX concentration and *C. raciborskii* biovolume (pseudo R-squared = 0.20) where the *C. raciborskii* biovolume (t=-2.15; p=0.05) explained only 20% of the variation in the concentration of STX (Figure 6A). Most notably in July 2014 a large increase in STX could not be explained by concomitant increases in the abundance of *C. raciborskii* and the inverse occurred in December of 2014 (Figure 6B).

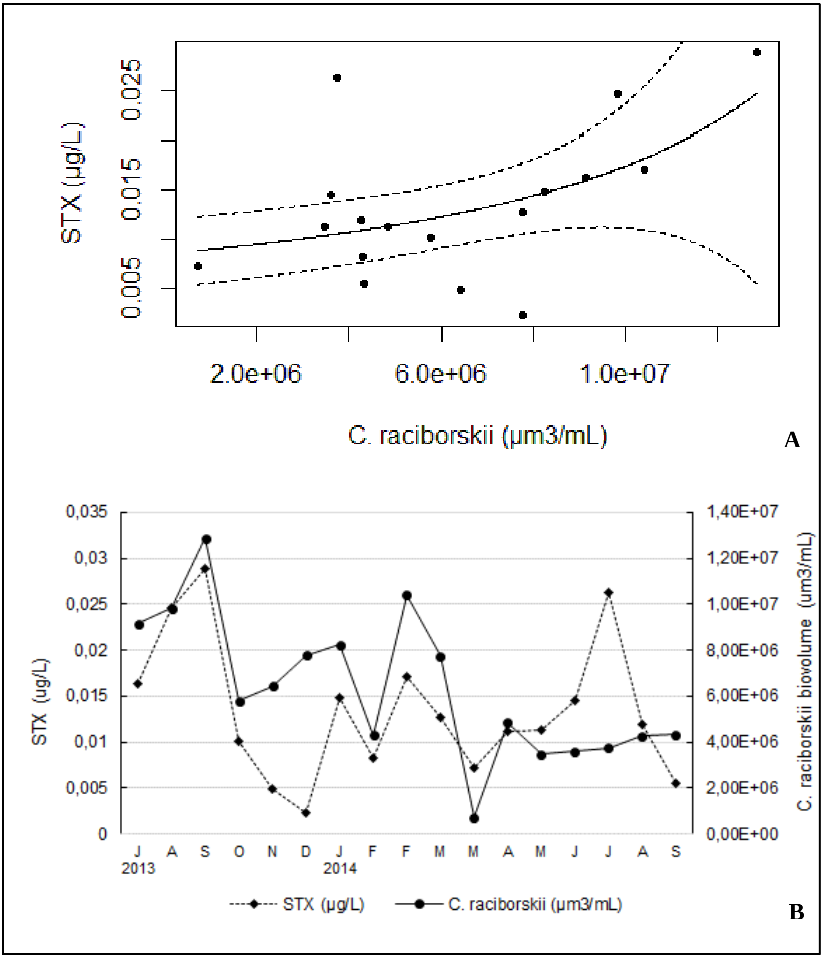

FIG. 6: (A) Relationship between *C. raciborskii* biovolume and the concentration of STX (July 2013 to September 2014). (B) Temporal variation of *C. raciborskii* biovolume (µm^3 mL^{-1}) and concentration of STX (µg L^{-1}), including two months in 2014 (February, March), in which two samples were taken, rather than a single monthly sample.

4. Discussion

Cyanobacteria were the most important group recorded during the study period in Peri Coastal Lake. *C. raciborskii* and *Limnothrix* sp. were the abundant species and the good correlation between chlorophyll-a and *C. raciborskii* reflects the dominance of this species during the sampling period.

Historically, *C. raciborskii* has been the dominant phytoplankton species in Peri Coastal Lake, as shown by the follow studies. In 1996, the maximum density recorded was less than 1.8 x 10^4 ind. mL^{-1}, corresponding to 56% of the total phytoplankton community (Laudares-Silva, 1999). In the following years maximum values corresponded to 8.5 x 10^4 ind. mL^{-1} (85%) in 2004 (Grellmann, 2006) and 2.2 × 10^5 ind. mL^{-1} in 2009-2011 (80%) (Tonetta *et al.* 2013). In this work, we recorded values lower than previously observed: maximum *C. raciborskii* density was 3.8 x 10^4 ind. mL^{-1}, corresponding to 55.6% of the phytoplankton community. In the 2011-2012 sampling season it was recorded that *C. raciborskii* was 62% of the phytoplankton community (Silveira, 2013) indicating a possible decay in its density since this year.

The density of *Limnothrix* sp. has been increasing in Peri Coastal Lake. The maximum density of 2.3 x 10^4 ind.mL^{-1} was recorded in September (2005), corresponding to 37% of the total phytoplankton density (Grellmann 2006). In this work, the maximum *Limnothrix* sp. density was recorded on August (2014) and the value was 3.6 x 10^4 ind.mL^{-1} (42%). *C. raciborskii* and *Limnothrix* sp. appeared together in 90% of the 47 reservoirs studied by Mcgregor and Fabro (2000). Increases in *Limnothrix* sp. density were linked with decreases and variability in *C. raciborskii* density by these authors. Similar situation could be occurring in Peri Coastal Lake. The *Limnothrix* sp. densisty is increasing affecting *C. raciborskii*.

The presence of *C. raciborskii* may modify the plankton structure, resulting in a substantial decrease in phytoplankton diversity (Bouvy et al. 1999). During almost two decades in which cyanobacteria have been the dominant phytoplankton group in Peri Coastal Lake, the current study observed the lower species richness (10 taxa) and the higher average cyanobacteria in the phytoplankton community (97.4%). Other studies in Peri Lake recorded 31 to 67 taxa and the average of cyanobacteria ranging from 83.5 to 88% (Grellmann, 2006; Tonetta *et al.* 2013; Silveira, 2013).

In this work, similar to others (Tóth and Padisák, 1986; Paerl, 1988; Townsend et al., 1996; Soranno 1997; Bouvy et al. 1999), physical conditions drove the cyanobacteria communities. The density of *C. raciborskii* was favored in Peri Coastal Lake by the increase in temperature during the studied period. In Peri Coastal Lake, Baptista and Nixdorf (2014) also showed that the influence of temperature seems to be the most important factor underlying abundance of *C. raciborskii.* It presented higher biovolume during the hot-rainy period (median = 22.7 mm^3 L^{-1}) than during the cold-dry period (18.6 mm^3 L^{-1}). Other authors have suggested that higher temperatures are an underlying factor in the appearance and the development of *C. raciborskii* (Komárková et al. 1999, Bouvy et al. 1999). This species can tolerate a wide range of climates but is favored by warmer temperatures (Bonilla et al. 2012).

The direct relationship between temperature and *C. raciborskii* biomass has been recorded in field observation and lab experiments. In a work that analyzed 39 reservoirs, higher temperature (ranged from 23.3 to 32.6°C) was related with higher percentages of *Cylindrospermopsis* (50% to 90%) (Bouvy et al., 2000). Using a dataset of 51 different aquatic systems, Gomes et al. (2013) also found *C. raciborskii* associated with high values of temperature. In a lab experiment testing two different *C. raciborskii* strains, both strains exhibited faster growth rates when grown under higher light intensity and temperature conditions (tested in a range of 21 to 31°C) (Bittencourt-Oliveira et al. 2012). In another study that investigated the effect of temperature (range, 20–35°C) on the growth of seven isolates of *C. raciborskii* grown in batch culture, maximum growth rates for all isolates occurred at temperatures between 25 and 30°C (Saker and Griffiths 2000).

Cyanobacteria can dominate in low nutrient conditions due to the high affinity for P, which allows them to outcompete other phytoplankton species (Carey et al. 2012). *C. raciborskii* is opportunistic in respect to P, since it displays high P-storage capacity (Isvánovics et al. 2000). However, an increase in rainfall intensity could promote an increased intake of nutrients and favor increased cyanobacterial biomass (Heisler *et al.* 2008). Padisák (1998) explained the bloom of *C. raciborskii* in a temperate zone (Lake Balaton) by the high precipitation with concomitant nutrient input. The coastal lakes located in this subtropical weather region are strongly subjected to climate variability, determining limnological conditions such as nutrient input (Kjerfve and Magill 1989). The coincident increase in the orthophosphate concentration and rainfall observed in this study, suggests the possible entrance of allochthon material in the nutrient-poor Peri Coastal Lake.

The feature of better growth under conditions of pulsed inputs of P, rapid uptake and the capacity of storage it (Wu et al. 2012; Amaral et al. 2014) provides to *C. raciborskii* an advantage over other phytoplankton, especially in lakes with low concentration of P (Posselt et al. 2009). In these environments, as is the Peri Lake case, the periodic P input that we observed could be one of the reasons why *C. raciborskii* keeps its dominance.

The combined effect of increasing temperatures and periods of heavy rain, which correspond to potential increases in P inputs, could signify better conditions for *C. raciborskii* to increase in biomass. This condition was also predicted in a mesocosm experiment in Peri Coastal Lake that proposed combined high temperatures and high P concentrations as the most worrying scenario to significantly augment phytoplankton biomass (Hennemman and Petrucio 2012).

Beside STX (Costa et al. 2006; Molica et al. 2002; Molica et al. 2005) and CYN (Bittencourt-Oliveira et al. 2011, Bittencourt-Oliveira et al. 2014), cyanotoxins that have previously been detected in Brazil include microcystins (Azevedo et al. 1994; Matthiensen et al. 2000) and anatoxin-a(s)-like toxin(s) (Monserat et al. 2001). Anatoxin-a has never been reported (Molica 2005). In the studied system, we only detected intracellular STX. However, release to the surrounding water can occur during cell senescence, death and lysis (Chorus and Bartram 1999). Since Peri Coastal Lake is used to supply potable water, it is advantageous to remove intact cyanobacterial cells prior to the water treatment process aiming to avoid the need for additional adsorptive (activated carbon) or oxidative (ozone or chlorine) toxin removal processes.

The STX concentration in Peri Coastal Lake (0.013±0.007 $\mu g.L^{-1}$) was low and probably below of the safe limit for human consumption. The value of 3μg L^{-1} was suggested as a maximum limit of STXs (saxitoxin equivalent) for human consumption, based on 60 Kg weight of an individual and a daily water consumption of 2 L (Fitzgerald et al. 1999). Brazilian Health Ministry’s resolution (MS 2914 of 12/12/2011) adopted 3μg L^{-1} Eq STX limit in terms of water quality for human consumption (Brazil 2011). Despite low STX concentrations, the water of Peri Coastal Lake presented a high cyanobacterial density (from 21×10^3 ind. mL^{-1} or $5x10^6$ μm^3. mL^{-1} to 66×10^3 ind. mL^{-1} or $19x10^6$ μm^3. mL^{-1}). The cyanobacterial density during the period of this study exceeded the safe levels for drinking water (< 2 x 10^3 cells. mL^{-1}) recommended by the World Health Organization (Chorus and Bartram 1999) and also the limit set by the Brazilian Environmental Ministry’s resolution (CONAMA 357 of 17/03/2005) that is ≤100 x 10^3 cell mL^{-1} or 10 x 10^6 μm^3 mL^{-1} (Brazil 2005).

The increase in the *C. raciborskii* biomass was not necessarily coincident with the increase in the STX concentration. There does not seem to be a consistent trend in STX concentration with cyanobacterial density. For example, Costa et al. (2006) and Molica et al. (2005) presented similar cell densities, but the relative STX concentrations are completely different (Table 3). Considering this and the data showed in this research, *C. raciborskii* density explained just part of the variation in concentration of STX. Other factors should be considered to explain the variation of STX concentration in the aquatic systems. In the case of Peri Coastal Lake, the concentration of STX analogues could be investigated and the STXs production by other species as well.

TABLE 3: Comparative between cyanobacteria density in blooms with abundance of *C. raciborskii* and the respective STX concentration among different aquatic ecosystems.

Location	Density of Cyanobacteria	Unit	STX concentration ($\mu g.L^{-1}$)	Authors
Greece	$4.7x10^6$ - $5.3x10^{11}$	ind mL^{-1}	0.4 – 1.2	Gkelis and Zaoutsos (2014)
Southern Brazil	$2.1x10^4$ - $6.6x10^4$	ind mL^{-1}	0.002 - 0.029	Current study
Northeastern Brazil	$3.4x10^4$ - $3.0x10^5$	cells mL^{-1}	0.052 (one sample)	Molica et al. (2005)
Northeastern Brazil	$9.1x10^4$ - $8.2x10^5$	cells mL^{-1}	3.14 (max value)	Costa et al. (2006)

Another relevant suggestion is there may be a waxing and waning of strains of quite different toxin quotas (i.e. toxin content per cell), or the toxin quotas may change in response to changes in environmental conditions (Chorus and Bartram 1999). In other words, the environmental conditions can regulate the abundance of the toxin producer strains or the production in the toxigenic strains (Molica and Azevedo 2009).

It has been previously shown that cyanobacteria strongly prefer stable environment conditions (Bouvy et al. 1999). In this context, the hypothesis that saxitoxin production is triggered in response to environmental changes has been explored for several laboratory studies (Castro et al. 2004; Carneiro et al. 2009, Chislock et al. 2014, Vico et al. 2016).

The genetic basis for toxin production has provided a framework for determining the environmental factors that regulate toxin production and therefore the factors that ultimately affect water quality (Neilan et al. 2013). Vico et al. (2016) tested the effect of nitrate availability on the relative transcript abundance of two genes (sxtU and sxtI) involved in different steps of the STX biosynthetic pathway. They showed that biosynthesis of saxitoxin and analogs in *C. raciborskii* is not related to nitrate. However, the role of STX production in *C. raciborskii* has been linked with the maintenance of homeostasis under salt stress condition (Pomati et al. 2003, Pomati et al. 2004, Ongley et al. 2015), and the pathway of STX production and exportation was demonstrated under high salt concentrations (Soto-Liebe et al. 2012; Ongley et al. 2015). It supports the hypothesis that toxin quotas may change in response to changes in environmental conditions.

The knowledge regarding environmental factors influencing STXs production (ecophysiology) would help to understand toxin dynamics in aquatic ecosystems (Vico et al. 2016) and it is interesting from a scientific viewpoint and critical for the risk assessment of contaminated water bodies (Neilan et al. 2013). Since *C. raciborskii* is currently expanding into temperate latitudes and forecasted to spread further in the future (Bonilla et al. 2012), the understanding about what triggers STXs production is necessary. A lack of relevant toxin data and lack of certainty for water quality monitoring to prevent human consumption of cyanobacterial toxins based on cyanobacterial density makes this need for understanding urgent in Brazil and in other countries where the cyanobacterial density is used to indicate the cyanotoxin concentration. Future work should include field and experimental studies testing the effect of abiotic factors over the STX concentration in lakes as Peri.

5. Conclusion

The only cyanotoxin found in the Peri Coastal Lake during the study period was intracellular STX. However, the density of *C. raciborskii* explains few of the variation of the STX concentration in the studied system and it is not a good predictor. Aiming to comply with the laws, the high cyanobacterial density may impair the use of Peri Coastal Lake as a source of drinking water, especially during hot-rainy periods.

6. Acknowledgments

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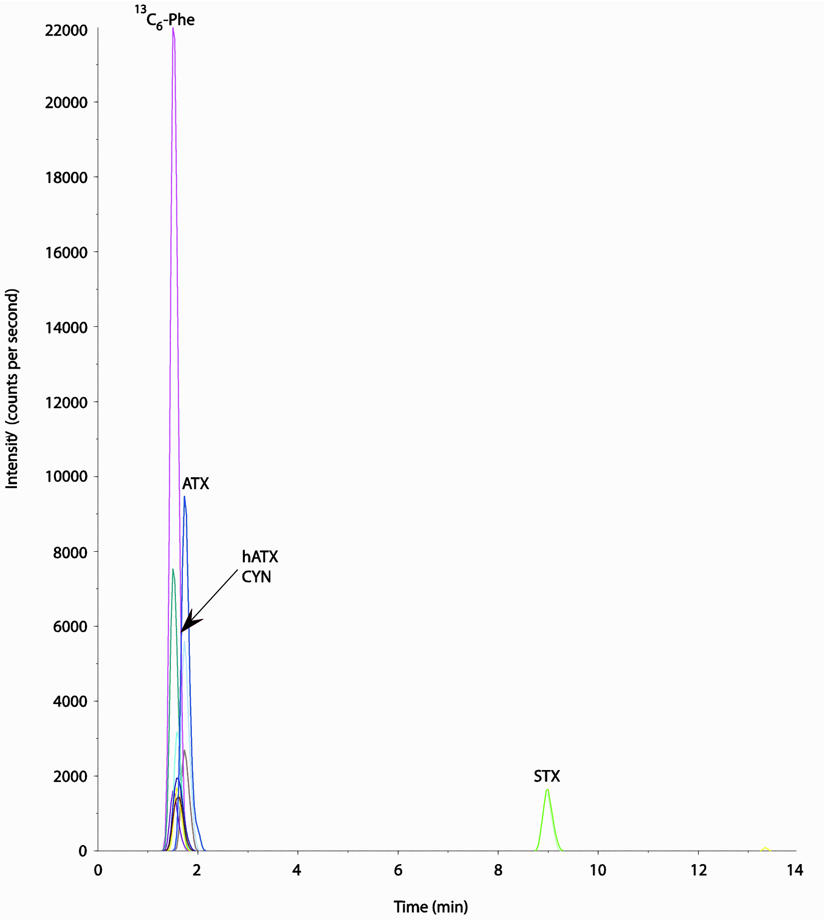
Supplemental Material

SUPPLEMENTAL TABLE 1: Specific MS/MS Parameters for Toxins Separated Using ZIC-HILIC Column.

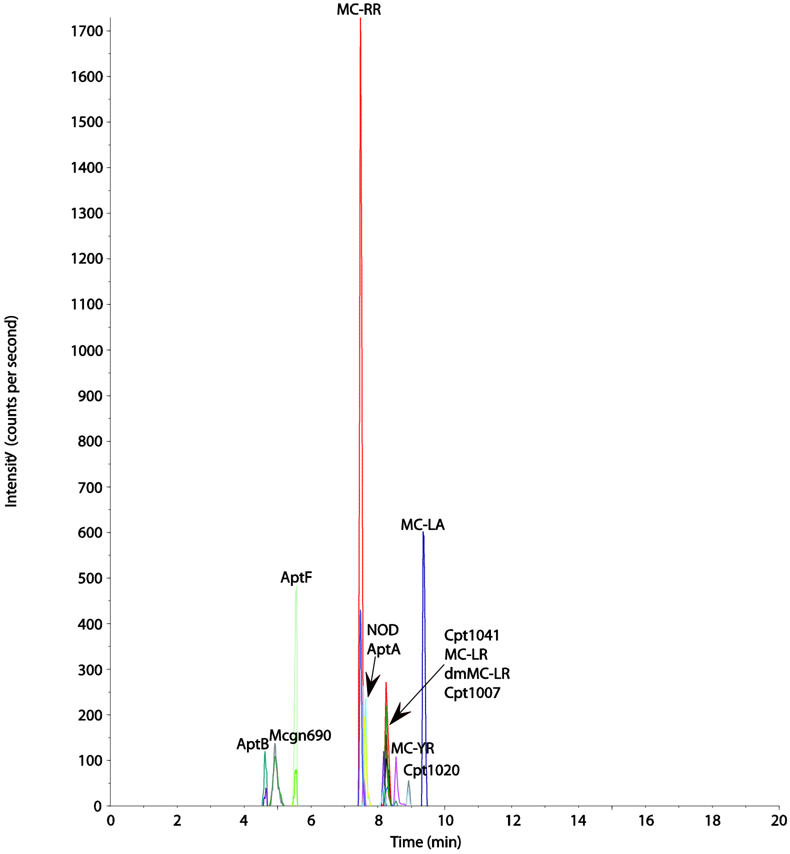
Toxin	**Parent Ion**	**Daughter Ion**	**Decluste-ring Potential**	**Collision Energy**	**Collision Cell Exit Potential**	**Retention Time (min)**
Anatoxin-a	166.150	131.300	46.000	25.000	25.000	1.74
	166.150	149.300	46.000	21.000	21.000	1.74
	166.150	107.200	46.000	25.000	25.000	1.74
Homoanatoxin-a	180.124	163.300	51.000	19.000	19.000	1.59
	180.124	145.300	51.000	23.000	23.000	1.59
Cylindrospermopsin	416.225	194.000	71.000	49.000	49.000	1.58
	416.225	336.200	71.000	31.000	31.000	1.58
Saxitoxin	300.145	282.100	101.000	25.000	25.000	8.98
	300.145	204.000	101.000	33.000	33.000	8.98
Phenylalanine	166.145	120.200	41.000	19.000	19.000	1.51
	166.145	103.200	125.000	53.000	53.000	1.51
$^{13}C_6$-Phenylalanine	172.145	126.200	41.000	19.000	19.000	1.51
	172.145	109.200	41.000	39.000	39.000	1.51
	172.145	83.100	41.000	57.000	57.000	1.51

SUPPLEMENTAL TABLE 2: Specific MS/MS Parameters for Toxins Separated Using C18 Column.

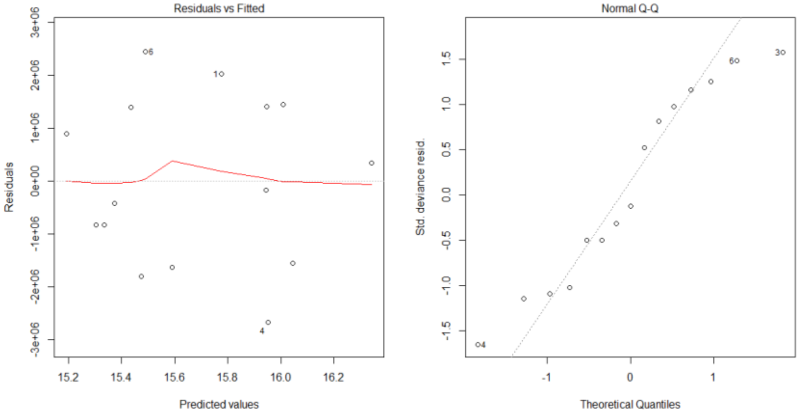
Toxin	Parent Ion	Daughter Ion	Declustering Potential	Collision Energy	Collision Cell Exit Potential	Retention Time (min)
Microcystin-LR	995.619	135.3	126	115	26	8.25
	995.619	127.1	126	115	26	8.25
Nodularin	825.522	103.2	116	83	8	7.57
	825.522	135.3	116	129	16	7.57
Microcystin-YR	1045.633	135.3	141	107	8	8.55
	1045.633	127.1	141	123	8	8.55
Microcystin-LA	910.617	776.4	106	27	8	9.37
	910.617	135.2	106	87	8	9.37
Microcystin-RR	520	135.1	81	43	8	7.48
	520	70.1	81	129	6	7.48
[Dha^7]-Microcystin-LR	981.531	135.3	126	101	22	8.25
	981.531	103.2	126	129	6	8.25
Anabaenopeptin B	837.544	201.4	106	57	14	4.66
	837.544	70	106	129	10	4.66
Anabaenopeptin F	851.757	201	121	53	12	5.61
	851.757	175.1	121	53	12	5.61
Anabaenopeptin A	844.532	84.3	81	129	14	7.63
	844.532	637.4	81	37	20	7.63
Cyanopeptolin 1007	1007.54	989.6	131	51	32	8.26
	1007.54	776.3	131	59	22	8.26
Cyanopeptolin 1041	1042.528	1024.5	131	51	28	8.18
	1042.528	828.3	131	51	34	8.18
Cyanopeptolin 1020	1021.6	989.6	131	57	32	8.92
	1021.6	776.4	131	63	22	8.92
Microginin 690	691.368	510.2	96	31	16	4.92
	691.368	343.1	96	37	10	4.92



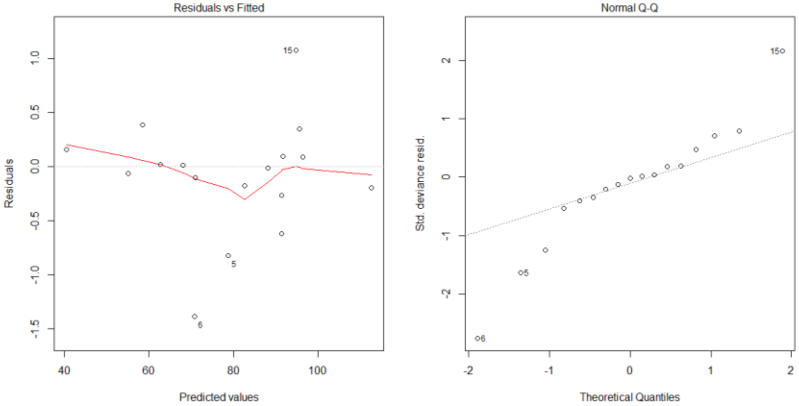
SUPPLEMENTAL FIG. 1: LC-MS/MS Chromatogram of standard reference materials (10 µg/L) separated on a ZIC-HILIC column used to detect saxitoxin (STX), anatoxin-a (ATX), homoanatoxin-a (hATX), cylindrospermopsin (CYN), and $^{13}C_6$-phenylalanine ($^{13}C_6$-Phe).



SUPPLEMENTAL FIG. 2: LC-MS/MS Chromatogram of standard reference materials (10 µg/L) separated on a C18 column used to detect microcystins (MC-LR, -YR, -LA, -RR, desmethyl (dm)-MC-LR), nodularin (NOD), anabaenopeptins (AptA, AptB), cyanopeptolins (Cpt1007, Cpt1041, Cpt1020), and microginin 690 (Mcgn690).



SUPPLEMENTAL FIG. 3: Equal variance and Normality of the residual of the model proposed to *C. raciborskii* density as dependent variable and environmental factors as independent variables.



SUPPLEMENTAL FIG. 4: Equal variance and Normality of the residual of the model proposed to STX concentration as dependent variable and *C. raciborskii* density as independent variables.

5. CAPÍTULO 2

Abiotic variables affect STX concentration in a meso-oligotrophic subtropical coastal lake dominated by *Cylindrospermopsis raciborskii* (Cyanophyceae)

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Abiotic variables affect STX concentration in a meso-oligotrophic subtropical coastal lake dominated by *Cylindrospermopsis raciborskii* (Cyanophyceae)

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Abstract

The cyanobacterium *Cylindrospermopsis raciborskii* is capable of producing toxins including saxitoxin (STX). Few studies have verified the influence of environmental variables on the production of STX and most have only been studied in the laboratory. The goal of this work was to identify the abiotic variables related to STX concentration *in situ*. The relationship among STX concentration and the physical variables, nutrients and chlorophyll-a (chl-a) concentration was examined in a meso-oligotrophic subtropical coastal lake dominated by *C. raciborskii*. A Generalized Linear Model was developed, incorporating all variables measured monthly over a 45-month monitoring period. Conductivity and dissolved inorganic nitrogen (DIN) concentration provided the greatest explanatory power for STX concentration *in situ*. Previous studies suggested that *C. raciborskii* cells exposed to stress associated with higher ionic concentrations appear to activate the biosynthesis of STX suggesting that STX can elicit changes cell permeability and may contribute to the homeostasis of this organism. An increase of DIN concentration results in a higher concentration of STX which may be related to a reduced metabolic demand, since the uptake of inorganic nitrogen requires less energy than N_2-fixation. Thus, increased DIN can favor the growth of *C. raciborskii* population or improve cellular homeostasis, both potentially increasing STX concentration in the aquatic system, which was observed through a delayed response pattern. The developed model, while providing only a moderate predictive power, can assist in the understanding of the environmental variables associated with increases in STX concentration, and in monitoring and minimizing the risks of toxic blooms.

Key-words: **cyanotoxins; drinking water; environmental protected area; management; toxic cyanobacteria; water supply**.

1. Introduction

The planktonic freshwater cyanobacterium *Cylindrospermopsis raciborskii* (Woloszynska) Seenayya and Subba Raju 1972 (Order Nostocales) is filamentous and diazotrophic. Originally identified in tropical/subtropical regions, *C. raciborskii* has increasingly been found in temperate regions (Padisák 1997) and is currently considered globally dispersed (Wiedner et al. 2007, Bonilla et al. 2012, Sinha et al. 2012). This cyanobacterium is considered an invasive species due to its successful global expansion, which was assisted by the ability of this species to tolerate a wide range of environmental conditions (Padisák 1997). There is great interest worldwide in this species because it produces toxic blooms that can seriously impact water quality, thus threatening human health (Chorus and Bartram 1999, Chorus 2005, Burch 2008).

The toxic secondary metabolites produced by *C. raciborskii* include the potent cylindrospermopsin (CYN) (Ohtani et al. 1992, Li et al. 2001) and saxitoxin (STX) (Lagos et al. 1999, Molica et al. 2002, Molica et al. 2005), which is the parent compound of more than 30 naturally occurring analogues (Kellmann et al. 2008). Furthermore, the gene locus that is responsible for the biosynthesis of STX in *C. raciborskii* has been described by Kellman et al. (2008).

Saxitoxins are a group of carbamate alkaloid neurotoxins that are non-sulfated (saxitoxins - STX), singly sulfated (gonyautoxins - GTX) or doubly sulfated (C-toxins). In addition, decarbamoyl variants and several new toxins have been identified in some species (Sivonen and Jones 1999). These compounds act by blocking voltage-gated sodium channels (Kao and Levinson 1986) and also interfere with voltage-gated calcium and potassium channels. Blockage of sodium channels results in the interruption of nervous conduction and subsequently causes muscular paralysis. Interference with voltage-gated calcium and potassium channels results in a depression of cardiac output (Carmichael 1994, Wang et al. 2003, Su et al. 2004). Thus, STX can be highly toxic and, depending on the dose, lethal to many species, including humans (Landsberg 2002).

The dominance of *C. raciborskii* in different freshwater systems in Brazil is related to dry periods, polymictic environments and high phosphorus concentrations (Soares et al. 2013). Positive relationships between species dominance and variables such as temperature, pH, alkalinity and conductivity have also been observed (Gomes et al. 2013). Though these studies suggest that environmental variables are related to the increase in biomass of *C. raciborskii* in freshwater systems, few studies have been performed to verify the influence of these environmental variables on the concentration of STX (Molica and Azevedo 2009). Laboratory-based studies have examined the influence of temperature (Castro et al. 2004), N:P ratio (Chislock et al. 2014), light intensity and quality (Carneiro et al. 2009), the concentration of ions in water and pH values (Pomati et al 2003a, Pomati et al 2004, Kellmann and Neilan 2007, Carneiro et al. 2011, Carneiro et al. 2013, Ongley et al. 2016) on STX production.

Considering the current literature, no work has linked abiotic variables to STX concentration produced by *C. raciborskii* in natural systems (*in situ*). New knowledge regarding which abiotic variables influence the STX concentration is needed in order to explain an ecologically important question. As the relationship of *C. raciborskii* with higher trophic levels supports the bottom-up theory (Murdoch 1966), the presence of this species can alter the trophic structure of freshwater systems (Leonard and Pearl 2005). Furthermore, it is necessary to put particular focus on determining the environmental conditions required for the biosynthesis of toxins on a species-by-species basis (Merel et al. 2013). Understanding the environmental variables associated with toxin concentration to determine the conditions influencing cyanotoxins production is critical for effective lake management and minimization of health risks associated with cyanotoxins (Xie et al 2012).

In this study, the following question was addressed: does a variation in abiotic variables determine the concentration of STX by *C. raciborskii* in the natural environment? The hypothesis is that if the biosynthesis of STX is activated in *C. raciborskii* by abiotic variables *in vitro,* this relationship will also be found *in situ*. The goal of this work was to identify which abiotic variables, if any, are related to STX concentration *in situ*.

2. Methodology

2.1 Study Area

Peri Coastal Lake (27°44’ S and 48°31’ W) is located in the southeastern region of Santa Catarina Island, Brazil. The climate in the area is typically subtropical. The lake has a surface area of 5.07 km^2 and average and maximum depths of 4.2 m and 11.0 m, respectively. The volume is 21.2 million m^3 of water (Laudares-Silva 1999, Oliveira 2002). Peri Coastal Lake exhibits spatial homogeneity both horizontally and vertically (Hennemann and Petrucio 2010; Tonetta et al. 2013). It is a coastal freshwater lake without marine influences (freshwater year-round), which makes it the main freshwater resource for Santa Catarina Island. Since 2000, the lake has supplied drinking water to approximately 100,000 inhabitants of Santa Catarina Island. The only other activity allowed in the lake is recreational swimming. The lake and surroundings (including almost the entire drainage basin) are within an environmentally protected area.

Peri Coastal Lake was classified as oligotrophic for nutrients concentrations and meso-eutrophic for transparency and chlorophyll-a (chl-a), which can be explained by the high densities of the cyanobacterium *C. raciborskii* and by the high recycling rates observed in tropical and subtropical water bodies (Hennemann and Petrucio 2010). The cyanobacterium *C. raciborskii* is dominant (80% of the phytoplankton community) with high density (23 to 220 × 10^3 ind mL^{-1}), which results in low occurrences of other species (Tonetta et al. 2013).

2.2 Limnological Variables

Peri Coastal Lake was sampled at a central point, with a maximum depth of 8.3±1.3 m, at the depth of Secchi Disk extinction (0.9±0.2 m). Samples were taken over a 45-month period between March 2007 and August 2014. Sub-samples were filtered (glass microfiber filter AP40 - Millipore®) for analysis of chl-a and dissolved nutrients.

The variables measured in the field were: water transparency (Secchi Disk; Chapman 1992), water temperature, pH, conductivity and dissolved oxygen (YSI probe-85). In the laboratory, total alkalinity was determined by titration (Mackereth et al. 1978), and the concentrations of total nitrogen and total phosphorus (Valderrama 1981) were determined from unfiltered water samples. Nitrite (Golterman et al. 1978), nitrate (Mackereth et al. 1978), ammonia (Koroleff 1976) and soluble reactive phosphorus (Strickland and Parsons 1960) were determined from filtered water samples. Chlorophyll-a was measured by extraction with acetone 90%, with a correction for phaeopigment (Lorenzen 1967).

2.3 STX data

Water from Peri Coastal Lake was sampled at the water uptake point of the supply system that treats and distributes water for the population. These samples were routinely taken weekly by the Catarinense Water Supply and Sanitation Company (CASAN), which is responsible for the system.

The samples were subjected to triple freezing and thawing to analyze the concentration of STX in the whole water. High-performance liquid chromatography (HPLC) analysis of saxitoxin was carried out on the initial 80% methanolic extract using an HPLC system (Shimadzu, Japan). Saxitoxin concentration was analyzed according to the post-column derivatization method (Oshima 1995). The chromatography was conducted at 30°C on a Luna® 4.6 x 250 mm column (Phenomenex, USA) filled with 5 µm C8 particles. After the chromatography, the column eluents were derivatized with buffered 50 nM periodic acid and stabilized with acetic acid using a two-piston LC 10® pump (Shimadzu, Japan). The peaks were detected on a RF 10Alx® fluorometer (Shimadzu, Japan). The system was calibrated with a standard obtained from the National Research Council, Canada. This method was used to analyze the water samples collected through March 2013 (n = 28). From April 2013 onward (n = 17), STX concentrations were analyzed by ELISA using the Microplate Kit developed and provided by Beacon Analytical Systems Inc. Only saxitoxin was analyzed, excluding other possible analogues. The detection limit in both methods was 0.01µg L^{-1}. All STX analyses were made by CASAN.

2.4 Statistical analysis

The weekly STX concentrations were used to calculate the monthly averages. The monthly values of the limnological variables were compared with the monthly averages calculated to obtain STX concentration for all of the months analyzed (n = 45).

A principal component analysis (PCA) was conducted to determine the relationships among the following variables: temperature, pH, conductivity, dissolved oxygen, alkalinity, orthophosphate, total phosphorus (TP), DIN, total nitrogen (TN) and chl-a concentration. All variables were centered by their means and scaled them to unit standard deviations (z-scores), and then the PCA was built based on the correlation matrix (Gotelli and Ellison 2011).

A generalized linear model (GLM) was developed using the gamma distribution and the logarithmic link function to assess the relationships between environmental variables and STX concentration within the study system. The variables used as predictors in this model were the same as those used in the PCA, with the addition of total N and total P ratio (TN:TP). The problem of multicollinearity was addressed by first selecting variables that reduced the variance inflation factor (VIF). Variance inflation factor indicates how much of the variance or estimated standard deviation in an estimated coefficient increased because of its correlation with other variables in the model (Fox 2006). Only variables with $\sqrt{VIF} < 2$ were kept, which indicates that the estimated standard deviation was inflated by less than two times the expected value in the absence of collinearity. Next, a backward model selection procedure was applied based on Akaike information criterion (AIC). After finding models with the lowest AIC values, additional steps of model simplification were considered while $\Delta AIC < 4$ (Bolker 2008).

For model validation, residuals were inspected graphically both for the assumptions of normality and equal variance, and for residual temporal autocorrelation based on the Durbin-Watson test (Fox 2006). Because residual autocorrelation was detected for one-month lags, lagged variables were added and VIF and AIC-based model selection, and model validation procedures were executed again. The modeling procedures were computed in R (R Core Team 2015), using the package *car* for VIF analyses and residual inspection (Fox and Weisberg 2011) and package *MASS* for model selection based on AIC (Venables and Ripley 2002).

3. Results

Water temperatures in Peri Coastal Lake were between 17.1 and 28.9°C, which is characteristic of the typical subtropical climate. Water pH was approximately neutral, and the water was well oxygenated most of the time. The low conductivity observed indicated the lack of direct marine influence and low dissolved nutrient concentrations. In fact, the lake was very poor in nutrients (N and P) and presented a wide range of TN:TP ratios. High chl-a values, with large variation, were measured (maximum 58.74 µg L^{-1}, minimum 0.8 µg L^{-1}) (Table 1).

TABLE 1: Descriptive statistics for water sampled in Peri Coastal Lake over all months studied (n=45).

Variables	Mean	Std. Dev.	Minimum	Maximum
Water Temperature (°C)	22.74	3.72	17.10	28.90
pH	6.73	0.47	5.44	8.03
Conductivity (µS cm^{-1})	74.11	13.04	23.00	105.00
Dissolved Oxygen (mg L^{-1})	8.32	1.38	5.60	13.07
Alkalinity (mEq L^{-1})	0.12	0.05	0.05	0.19
Orthophosphate (µg L^{-1})	2.62	2.05	0.48	8.19
Total Phosphorus (µg L^{-1})	12.34	4.74	4.23	24.82
Nitrite (µg L^{-1})	0.32	0.27	0.002	0.90
Nitrate (µg L^{-1})	4.49	3.09	0.001	11.44
Ammonia (µg L^{-1})	9.42	8.62	0.001	30.64
Dissolved Inorganic Nitrogen (µg L^{-1})	13.42	9.82	1.69	41.09
Total Nitrogen (µg L^{-1})	648.93	275.24	12.01	1084.08
TN:TP ratio	67.47	45.26	0.75	236.24
Chlorophyll-a (µg L^{-1})	26.73	16.64	0.80	58.74

In the PCA analysis, the first two components explained 44.1% of the variation in the dataset (Fig. 1). The first axis (PC 1) accounted for 28.2% of the total variance; alkalinity, conductivity and dissolved inorganic nitrogen (DIN) were the main variables contributing to this result. The second axis (PC 2) explained only 15.8% of the data variation and was highly correlated with total phosphorus (TP), pH and dissolved oxygen (DO).

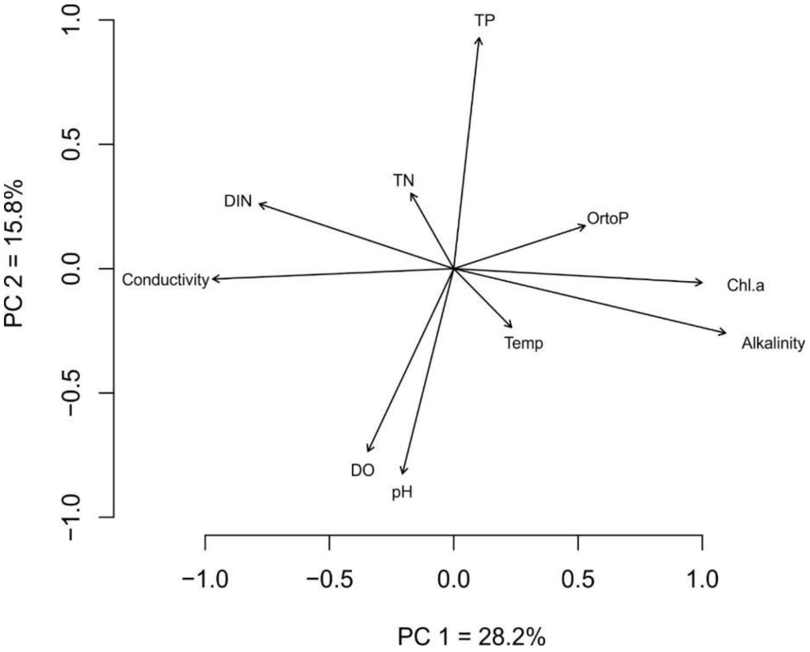

FIG. 1: Principal component analysis (PCA) showing the ordination of the measured environmental variables. Temp= temperature; DO= Dissolved Oxygen; OrtoP= Orthophosphate; TP= Total Phosphorus; DIN= Dissolved Inorganic Nitrogen; TN= Total Nitrogen; Chl-a= Chlorophyll-a.

For PC 1, conductivity was inversely related to alkalinity, which indicated the importance of a buffering system on regulating the concentration of ions in the water. The ordination analysis also showed that the dissolved inorganic nitrogen (DIN) explained much of the variation in the system. DIN and total nitrogen were inversely related to chl-a concentration. Additionally, it appears that the chl-a concentration had a stronger relationship to PC 1 than to PC 2. Thus, total phosphorus (TP), pH and dissolved oxygen (DO) were likely to have less of an impact on the phytoplankton community than were the variables associated with PC 1.

The average concentration of STX during the study period was 0.31±0.31 µg L^{-1}. The maximum value measured was 1.46 µg L^{-1}, and the minimum reached the limit of detection of the employed method (≤ 0.01 µg L^{-1}).

Variance inflation factor and Akaike information criterion model selection indicated that conductivity (t = 2.132; p=0.039), and pH (t = 1.736; p = 0.09), conductivity (t = 1.900; p=0.065) and DIN from previous month (t = 3.641; p = 0.0007) should be kept to explain STX concentration (AIC = -44.173; pseudo-R^2 = 0.552). Further simplification resulted in a model that included only conductivity (t= 3.961, p< 0.001) and DIN from the previous month (t= 3.889; p < 0.001; ΔAIC = 2.118). This model explained 49% of the STX variation (pseudo R^2=0.49; Fig. 2). Both variables had a positive effect on STX concentration and, in addition, the size of the effect of conductivity (β = 0.409 ± 0.103) and DIN from the previous month (β = 0.402 ± 0.10327) was very similar (calculated for centered and scaled variables).

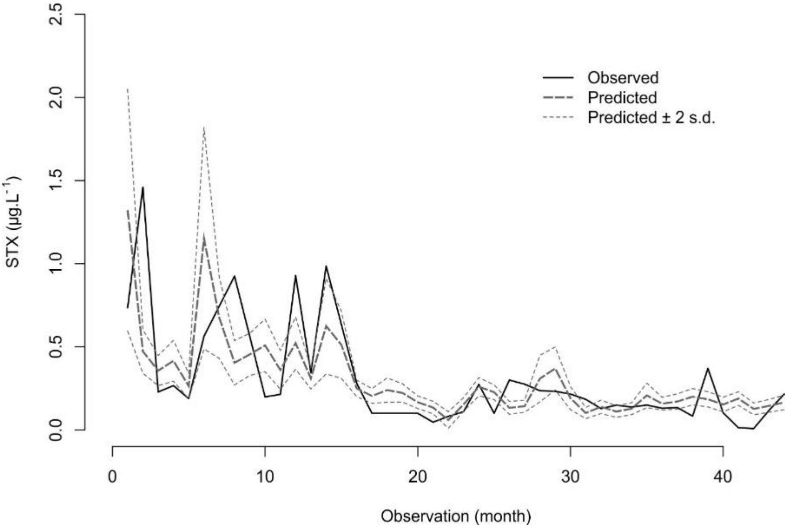

FIG. 2: Observed and predicted STX concentration as a function of conductivity and previous month DIN concentration during the study period.

4. *Discussion*

Although there are previous reports of abiotic variables driving changes in the concentration of STX in the laboratory and alter the abundance of *C. raciborskii* under natural conditions, evidence for abiotic control of STX concentration *in situ* was lacking. The results presented here help to fill this gap, suggesting that conductivity and DIN concentration have a strong explanatory power for the concentration of

STX in natural conditions in the lake studied. These results contribute to the understanding of which environmental variables are related to the concentration of STX produced by *C. raciborskii in situ*, thus connecting previous *in vitro* findings described in the literature.

Conductivity is directly related to the concentration of ions in an aqueous medium (Esteves 2011). Increases in conductivity in the Peri Coastal Lake were associated with an increased concentration of STX in this environment over a short period of time, pointing to a quick response to changing lake conditions. The probable mechanism underlying such correlation is that *C. raciborskii* cells activate the biosynthesis of STX as a response to stress caused by high ionic concentrations indicated by high conductivity; STX then alters cell permeability and contributes to organism homeostasis. In laboratory experiments, a decrease of an order of magnitude in Na^+ concentration corresponded to an order of magnitude decrease in intracellular STX levels (Pomati et al. 2003a), which suggests a possible role for this toxin in the maintenance of cyanobacterial homeostasis under Na^+ stress condition (Pomati et al. 2003a, Pomati et al. 2004). The Na channel-blocker effect elicited by STX can interfere with ion fluxes of both Na and K and affect the metabolism of bacterial cells as well as eukaryotic cells (Pomati et al. 2003b). Additionally, the exposure of *C. raciborskii* cultures to high concentrations of Mg^{2+} also increased STX production (Kelmann and Neilan 2007, Carneiro et al. 2013).

A strong upregulation of the STX biosynthesis gene (*sxtA*) and the cognate transporters, *sxtF* and *sxtM*, in *C. raciborskii* T3 has been observed under Na^+ stress (Ongley et al. 2016). Furthermore, the STX levels and localization showed significant variation under this stress conditions. Suggesting this toxin acts extracellularly as a protective mechanism to ensure homeostasis against extreme salt variation in the environment in other species of cyanobacteria. In *Raphidiopsis brookii* D9, PSP toxins (including STX) are exported directly in response to the presence of monovalent cations (Na^+, K^+) at least at elevated concentrations (Soto-Liebe et al. 2012).

The results of the current study showed an increase of STX concentration in a coastal lake dominated by *C. raciborskii* as a response to high conductivity, an indicator of ionic stress. In Australia, an extensive STX-producing bloom of *Anabaena circinalis* was associated with elevated water conductivity (Bowling and Baker 1996). Thus, both laboratory and field evidence suggest a very likely causal relationship to explain increases in STX concentration as a response to ionic stress.

Laboratory studies documented that isolated cyanobacteria typically produce paralytic toxins preferentially under conditions that are most favorable for growth (Sivonen and Jones 1999). This finding is in accordance with this study because conductivity was among the abiotic variables related to high biomass of *C. raciborskii* in the Brazilian aquatic system (Gomes et al. 2013).

The cyanobacterium *C. raciborskii* lives in waters with a large range of conductivity values and is able to tolerate high salinity concentrations up to 4200 $\mu S\ cm^{-1}$ (Padisák 1997). In fact, in Brazil, there are records of lakes dominated by *C. raciborskii* in which there is low conductivity, with values below 50 $\mu S\ cm^{-1}$ (Pedrosa et al. 1999) or around 100 $\mu S\ cm^{-1}$ (Souza et al. 1998). There are also records of high conductivity, with values greater than 1000 $\mu S\ cm^{-1}$ (Bouvy et al. 1999, Bouvy et al. 2000, Bouvy et al. 2003, Costa et al. 2009). In the Peri Coastal Lake, conductivity varied little during the assessed period and was normally very low. The data here presented suggest that in low conductivity environments, small oscillations of conductivity are likely able to active the metabolic pathway of STX synthesis by *C. raciborskii*. The high sensitivity of this mechanism could explain why this species presents phenotypic plasticity over a range of different conductivities, being strongly adapted to this condition.

Peri Coastal Lake is a nutrient-poor lake, with absolute low N, P and dissolved nutrients concentration (Tonetta et al. 2013). Low nutrient availability likely results from high recycling rates, well-oxygenated water and high assimilation by both phytoplankton and bacterial communities (Henemmann and Petrucio 2010).

The cyanobacterium *C. raciborskii* is not limited to waters with N-deficiency, it is a heterocytic blue-green alga (Padisák 1997). It is likely that the ability to proliferate under conditions of N-limitation is one that allows its dominance in Peri Coastal Lake. N_2 fixation is however energetically expensive and this process is dependent of light availability (Esteves 2011, Hoffmann et al. 2014). Therefore, cyanobacteria preferentially use inorganic nitrogen for growth, effectively using nitrate, nitrite and ammonium as nitrogen sources (Luque et al. 1994). In fact, N_2-fixation decreases in *C. raciborskii* as a result of a higher availability of ammonium or nitrate in culture experiment (Spröber et al. 2003). Nitrite uptake may occur by passive transport-diffusion of nitrous acid (Flores et al. 1987), mostly when under low pH, conditions similar to those found in Peri Coastal Lake. The uptake of inorganic nitrogen is independent of the energy provided by photosynthesis process (Garbisu et al. 1992), making this less energy-demanding than N_2-fixation.

Increasing DIN concentration results in higher growth rates of cyanobacteria, possibly due to the lower energetic expenses (Plominsky et al. 2014; Willis et al. 2015). The cyanobacterium *C. raciborskii* only produces heterocysts at the terminal ends of the trichomes and is thus limited to a maximum of two per trichome (Plominsky et al., 2013). This low number of heterocysts results in constraints to N transfer along the trichome especially in cells at central parts when N_2 fixed is used as the N source (Plominsky et al., 2014). This feature and considering the max N_2 fixed per heterocyst may explain the lower growth rate under DIN limited conditions; and, why N fixation is not sufficient to fulfill the cellular requirement to reach maximum growth rates (Willis et al. 2015).

The increase in DIN concentration may have resulted in an increase of STX concentration in the system studied because it provokes *C. raciborskii* bloom formation. Yet, DIN effects are slower than those found for conductivity: after an increase in DIN concentration, *C. raciborskii* populations grow and is followed by an increase of STX concentration.

Since *C. raciborskii* is a facultative diazotrophic, it likely spends energy in alternative metabolic processes when acquisition of N is secured from high environmental DIN concentration (Moisander et al., 2012). In addition, during a mesocosm experiment, STX concentrations were found to be higher under high N:P ratios (40:1 and 122:1; the lowest was 7:1; Chislock *et al.* 2014). Thus, the highest concentration of STX under high DIN can reflect both the increase in *C. raciborskii* density and the use of the extra energy in the improvement of its homeostasis through the cyanotoxin production. Highlighting that the inverse relationship between chl-a and DIN in the system, it is possible that the extra energy provided by the DIN available is diverted to the maintenance of homeostasis in preference to cellular proliferation.

The importance of temperature on STX production has been demonstrated *in vitro*. The production of STX by *C. raciborskii* C10 strain did not appear to vary over temperatures between 19 and 25 °C (Castro et al. 2004). Using another strain, T3, Kellman and Neilan (2007) found that neurotoxin production *in vitro* occurred over a temperature range of 23 to 38°C and was highest at 35°C. In this work a minimum temperature of 17°C and a maximum of 28.9°C were recorded. Reflective of the laboratory experiments, there was a tendency for increased STX concentration when temperatures increased in the study system (data not shown). The tendency was not found to be statistically significant. This may be due to the moderate variation in temperature that is typically observed in sub-tropical lakes and to the fact that the highest temperatures registered over the course of the study were lower than those potentially affecting STX concentration. In the sub-tropical lakes, temperature could be secondary to other abiotic variables (conductivity and nutrients).

Although the pH was found to be a crucial variable for explaining STX production *in vitro*, in the natural environment (*in situ*), this abiotic variable was not significant. Under experimental conditions, the intracellular STX concentrations of *C. raciborskii* T3 increased exponentially in response to rising pH of the culture medium, with the highest rate observed at pH >9 (Pomati et al. 2004). Additionally, *in vitro* neurotoxin production occurred over a pH range from 5 to 10, with a plateau between pH 7 and 9 (Kellman and Neilan 2007). The natural pH conditions observed in this study were close to neutral (6.73 ± 0.47) and were consistent with the plateau (between 7 and 9) described previously. This is likely why was not observed a correlation between STX concentration and pH.

Additional information regarding the relationship between STX concentration and the abiotic variables studied herein should be considered: i. the rate of STX degradation, with half-lives of this toxin being in the order of 1-10 weeks, requires more than three months for greater than 90% of breakdown (Jones and Negri 1997); ii. saxitoxin stability in the extracellular environment varies with temperature (Castro et al. 2004, Ho et al. 2012); and iii. the timing of maximum toxin concentration and maximum biomass are not necessarily coincident (Sivonen and Jones 1999). It would also be interesting to know the toxin quota, i.e., the amount (mass or moles) of toxin per cyanobacteria cell, or biovolume (Sivonen and Jones 1999). The density of *C. raciborskii* in the lake was not considered in this work due to a lack of data but would be an important variable to include in future research. Understanding the degree to which *C. raciborskii* density is correlated with increasing STX concentration is an intriguing question for future work.

5. ***Conclusion***

The *in situ* concentration of STX produced by *C. raciborskii* was related to abiotic variables. It was best explained by both a quick response to conductivity and a lagged response to DIN concentration in the aquatic system. As found in previous *in vitro* experiments, water ion concentration was directly related to STX concentration, probably because STX changes cell permeability and may contribute to homeostatic regulation of *C. raciborskii* under ionic stress condition. Available DIN means energy saving by avoiding the use of costly N_2-

fixation pathways. Thus, an increase of DIN concentration could result in *C. raciborskii* population expansion or improving its homeostatic regulation. In any event, higher DIN concentration could increase the STX concentration with a lagged effect. Although the model developed herein has just a moderate predictive power, these findings can help in the understanding of the environmental variables associated with STX concentration, thereby minimizing the risks associated with this toxin.

6. Acknowledgments

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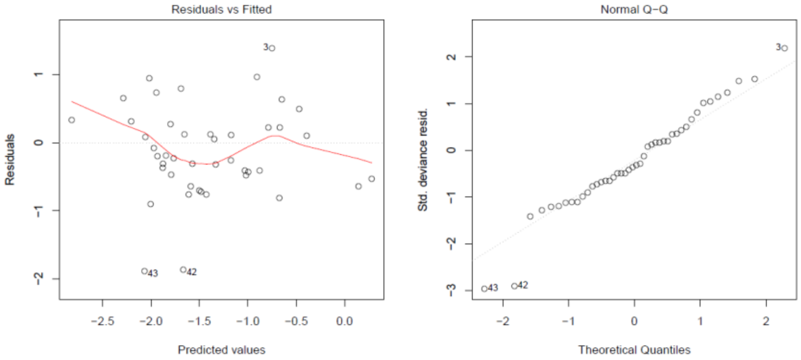
Supplemental Material

SUPPLEMENTAL TABLE 1: Durbin-Watson test for temporal autocorrelation in the model residuals. The null hypothesis is that the autocorrelation (ρ) is equal to zero for distinct lags Lag = 1: correlation of residuals with previous month; lag = 2: correlation of residuals with previous two months.

Lag	ρ	D-W Statistic	p-value
1	0.234	1.513	0.074
2	-0.097	2.077	0.731



SUPPLEMENTAL FIG. 1: Equal variance and Normality of the residual of the model proposed.

6. CAPÍTULO 3

Effect of STX concentration variation and *Cylindrospermopsis raciborskii* density in a natural freshwater system as factors affecting cladoceran

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Effect of STX concentration variation and *Cylindrospermopsis raciborskii* density in a natural freshwater system as factors affecting cladoceran

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Abstract

Cylindrospermopsis raciborskii influences the zooplankton community structure. The cyanobacterial density and toxin concentration are possible driving factors. We investigated the relationship between *C. raciborskii* density and STX concentration of natural samples to an observable effect (immobility) on a population of Cladocera. Secondarily, we verified the potential to use ecotoxicological tests to evaluate STX concentration in the natural environment. Peri Coastal Lake (27°84'40 S and 48°83'10 W), Brazil, was sampled monthly over a 15-month period (July 2013 to September 2014). The *C. raciborskii* density (biovolume) and cyanotoxin (analysed by LC – MS/MS) were related to the effect on a lab-cultured population of *Daphnia magna*. We performed acute ecotoxicological tests using the whole-, sonicated- and filtrated-lake water samples. The percentage of immobility in undiluted sample (100%) and the Toxicity Factor 48h (TF48h) for each treatment were the endpoints used. The STX concentration was related to the immobility of zooplankton subjected to the whole water samples (marginal significant relation, $p= 0.09$, pseudo - $R^2 = 0.18$) and sonicated water samples (significant relation, $p<0.05$, pseudo - $R^2 = 0.16$) over the months. The *C. raciborskii* density had less influence on the effect of the zooplankton's ability to prey this species (no significant relation, $p>0.05$, pseudo - $R^2 = 0.012$). The acute intoxication effect of grazing upon STX-containing *C. raciborskii* is a defining feature in the zooplankton community structure, as this selects for zooplankton that are able to coexist with a toxic *C. raciborskii* in the ecosystem. The TF48h (sonicated sample) showed a good linear relation (84%) with STX concentration in the lake. Hence, these tests may serve as a means to investigate STX concentration in the natural environment. However, standard protocols need to be established to enable the application of ecotoxicological tests as a preliminary screen for the presence of STX in water bodies.

Keywords: acute toxicity, ecotoxicological test, bottom-up, cyanotoxin, herbivory, STX predictor

1. *Introduction*

The cyanobacterium *Cylindrospermopsis raciborskii* induces changes in zooplankton community structure of natural water bodies. There is a reduction in the relative abundance of larger cladoceran species when *C. raciborskii* filaments are present (Burns, 1987; Hawkins, 1988; Hawkins and Lampert, 1989; Leonard and Paerl, 2005). The large cladoceran *Daphnia* are highly effective filter feeders that exert high grazing pressure on phytoplankton and form a major connection between primary producers and higher trophic levels (Lampert, 1987). Using this organism as a model, further studies suggested that *C. raciborskii* is a low nutrient food (Nogueira et al. 2006, Soares et al. 2009) that is difficult to be grazed upon (Hawkins and Lampert, 1989) by the zooplankton community, which could explain observed changes in community structure.

Further to the chronic effects of *C. raciborskii* consumption, the zooplankton community is also susceptible to the effects of toxins produced by this species. The toxic secondary metabolites produced include the potent cylindrospermopsin (CYN) (Ohtani et al. 1992, Li et al. 2001) and saxitoxins (STXs) (Lagos et al. 1999, Molica et al. 2002, Molica et al. 2005).

The cyanotoxins are located intracellularly or dissolved in the water due to cell lysis or excretion (Chorus and Bartram, 1999); both states have the potential to cause acute toxic effects. Zooplankton directly suffer from the presence of toxic cyanobacteria in water bodies, as they are primary consumers feeding on phytoplankton (Dao et al., 2010). The zooplankton is subjected to intoxication by ingestion, the route of exposure to the cyanotoxins is intracellularly. If the cyanotoxins are dissolved, laboratory experiments on cyanobacteria–zooplankton interaction have shown that exposure to cyanobacterial extracts, cells and purified toxins negatively affect the herbivorous community (Nogueira et al. 2006).

There are insufficient studies on zooplanktic organisms exposed to toxic algae and their toxins using field material collected during natural bloom events (Sotero-Santos et al., 2006). In the context of natural systems, previous work carried out in Peri Coastal Lake, Brazil, showed that the zooplankton community was comprised of Rotifera (75%) and Cladocera (25%) (Gerzson, 2011). The only Cladocera species detected were the small *Bosmina hagmanni* and *Bosmina frey*. Historically, *C. raciborskii* has been the dominant phytoplankton species in Peri Coastal Lake with high densities recorded (10^4 to 10^5 ind. mL^{-1} or 10^6 to 10^7 μm^3.

mL^{-1}) (Laudares-Silva 1999, Grellmann 2006, Tonetta *et al.* 2013, Silveira, 2013, Brentano et al. submitted). The cladoceran community density was inversely correlated with chlorophyll-a concentration (Gerzson, 2011). Additionally, the density of *C. raciborskii* explained 20% of the variation of STX concentration in the studied system during a 15-month monitoring period (Brentano et al. submitted).

Peri Coastal Lake is an important freshwater resource that supplies drinking water to approximately 100,000 inhabitants of Santa Catarina Island, Southern Brazil. The STX concentration recorded in this lake ranged from 0.013±0.007 µg.L^{-1} (2013-2014; n=15) (Brentano et al. submitted) to 0.31 ± 0.31 µg L^{-1} (2007-2014; n=45) (Brentano et al. 2016). However, higher STX concentrations (2.70 ± 0.24 µg L^{-1}) have been recorded in an exploratory study that analyzed two samples in February, 2010 and one in April, 2010 (n=3) (Machado, 2011). Critical to freshwater lake quality, particularly those used as a source of drinking water, is the level of cyanotoxins present (Chorus and Bartram 1999; Newcombe et al. 2010). Some studies have suggested the possibility of using aquatic organisms as a screening tool for the detection of cyanotoxins, especially STXs (Ferrão-Filho 2008, 2009, 2010); but these methods have received little attention from the scientific community (Sotero-Santos, 2006).

Considering that *C. raciborskii* can be directly related to the way in which the zooplankton community in Peri Coastal Lake is structured, this work was conducted to examine the cyanobacterial density and toxin concentration as the possible driving factors in this ecological phenomenon. We investigated the effect (immobility) of *C. raciborskii* density and STX concentration from natural samples (Peri Coastal Lake) on a laboratory cultured population of cladoceran (*Daphnia magna*). The secondary aim of this study was to evaluate the dataset created by ecotoxicological tests on a freshwater system that is dominated by cyanobacteria (Peri Coastal Lake). This was to determine the potential to use ecotoxicological tests to evaluate STX concentration in the natural environment.

2. *Methodology*

2.1 *Study area and sampling*

Peri Coastal Lake (27°84’40 S and 48°83’10 W) is located in the southeastern region of Santa Catarina Island, Brazil (subtropical climate). The lake has a surface area of 5.07 km^2 and average and maximum depths of 4.2 m and 11.0 m, respectively. The volume is 21.2 million m^3 of water (Laudares-Silva 1999, Oliveira 2002). Peri is a freshwater coastal lake, and the main aquatic resource for Santa Catarina Island. The lake and surroundings (including almost the entire drainage basin) are within an environmentally protected area (Hennemann and Petrucio 2010).

Peri Lake was sampled monthly over a 15-month period between July 2013 and September 2014, at the center of the lake (27º43’49.4’’S; 48º31’19.9’’W), where the depth is 9±1 m. Water was sampled at the depth of Secchi Disk extinction with a Van Dorn sampler. Sub-samples were both filtered (300 mL - glass microfiber filter AP40 - Millipore®), to obtain the particulate matter (filtered sample); and subjected to a sonication process during 15 min (Sonic Dismembrator model F60; output power 30 watts (RMS), frequency 50/60 Hz) aiming to lyse the *C. raciborskii* cells present in the sample and release the intracellular toxin (sonicated sample).

2.2 Ecotoxicological tests

Organisms

Stock cultures, maintained for 10 years at the Ecotoxicology Lab of the Federal Institute of Santa Catarina, Brazil, were used in acute toxicity tests. The cladoceran *Daphnia magna* Straus was cultured at 20±2º C with a 16-8h light-dark cycle, in Becker glass with M4 medium (Elendt and Bias, 1990) and with *Scendesmus subspicatus* as food *ad libitum* (ABNT, 2009). Prior to the experiments, females were separated to obtain one-day-old neonate born (24±2h) that were used as experimental animals.

Acute Ecotoxicological Tests

In order to: a) Discriminate between the alterations due to the density of *C. raciborskii* and STX concentration (whole sample) – when the *C. raciborskii* cells are intact; b) Observe the effect of toxic *C. raciborskii* lysate on the zooplankton (sonicated sample); and, c) Observe the effect of particulate-free lake water (filtered sample) on the zooplankton; we performed acute toxicity tests analyzing the proportion of immobilized organisms (%) in the undiluted water (100% treatment) after 48 h of exposure (ABNT, 2009). The experiment was conducted in 50 mL Becker glasses containing 25 mL sample. In each treatment (whole, sonicated and filtered sample), two replicate glasses received 10 neonates each. Test glasses were placed in an incubator at controlled light – temperature (20±2ºC) using a 16:8 h light–dark cycle. After 48 h the total number of immobilized organisms were counted. The Recovery Phase (Ferrão-Filho et al. 2008, 2010), when the immobilized zooplankton is transferred to a new suspension containing only the nutrition alga (*S. subspicatus*), was carried out to verify if the effect on the zooplankton was reversible. Following a 24 h recovery phase, the number of actively swimming and immobilized individuals was counted.

In order to investigate the potential of using an ecotoxicological test to predict the presence of STX we carried out the standard ecotoxicological tests. We used the same treatments for the Peri Costal Lake water sampled that in the first experiment: whole, sonicated and filtered sample. For each treatment, we prepared five test solutions. The basic design was to expose ten neonates of *Daphnia magna* in Becker glass with 25 mL of each test solutions in duplicate (twenty organisms per test solution in total). The test solution were prepared at concentrations of 0 (Control), 6.12, 12.5, 25, 50 and 100% of the Peri Costal Lake water sampled (whole, sonicated and filtered sample treatments) diluted in reconstituted water, the same used as control (ABNT, 2009). The reconstituted water characteristics are pH 7 - 8, hardness 125 - 225 $CaCO_3$ L^{-1}, conductivity 360-480 µS cm^{-1}. The test conditions were as described above. We used the different treatments to test the most appropriate exposure route to the zooplankton when the aim is to predict the STX concentration. We used as endpoint the Toxicity Factor 48 h (TF48h), defined as the lower dilution of the sample in which no deleterious effect (immobility) was observed on the organisms tested after 48 h.

2.3 Phytoplankton Analysis

The samples for phytoplankton analysis were investigated using an inverted microscope according to the Utermöhl method (Hasle, 1978). The density of *C. raciborskii* was estimated (Ros, 1979) and the biovolume of *C. raciborskii* was calculated from mean values of 30 individual measures (Hillebrand *et al.*, 1999; Sun and Liu, 2003). The minimum and maximum density values recorded for this species were 1.0 x 10^4 ind.mL^{-1} ($4x10^6$ $µm^3$. mL^{-1}) on July 2014 and 3.8 x 10^4 ind mL^{-1} (1.3 x 10^7 $µm^3$ mL^{-1}) on September 2013 (Brentano et al. submitted). The value of *C. raciborskii* biovolume of each month was used to relate with the effect in the zooplankton. The population of *C. raciborskii* from Peri Lake is STX producer as showed to the isolated strain LP2 (Miotto et al. submitted).

2.4 Cyanotoxin Extraction and LC-MS/MS Analysis of the natural samples

Cyanotoxins were extracted from the same sample submitted to the ecotoxicological tests. We extracted the cyanotoxins from whole water, filtrate, and filter-collected biomass and the concentration of STX was analyzed using LC-MS/MS as described in Brentano et al. (submitted).

The average STX concentration in Peri Lake between July 2013 and September 2014 was 0.013±0.007 µg L^{-1}. The maximum and the minimum values found were 0.029 µg L^{-1} (in September 2013) and 0.002 µg L^{-1} (in December 2013) respectively. All toxin detected was concentrated intracellularly from 300 mL of surface-collected filtered lake water, according previously studied. The STX concentration from each month was related to the immobility effect on the zooplankton during the same study period.

2.5 Statistical Analysis

Outlier data were removed using the Thompson Tau criteria. The distribution of *C. raciborskii* density, STX concentration and proportion of immobilized organisms subjected to whole- and sonicated-water were normality tested using the Shapiro-Wilk's W test (Shapiro and Wilk, 1965) (α>0.01).

We developed a Generalized Linear Model (GLM) with gamma distribution, assessing both, the relation between immobilized organisms and STX concentration; and the relation between immobilized organisms and *C. raciborskii* density. Additionally, we used the same statistical approach to determine the relation between the immobilized organisms subjected to the sonicated sample and STX concentration.

We tested the difference of the effect in the zooplankton subjected to the whole water and sonicated water (100% treatment) using the t test.

The immobilization data of the zooplankton in each dilution of the whole water sample and sonicated water sample were compared with the immobilization in the respectively controls and previous tested to the normal distribution. We used the One-way Analysis of Variance (ANOVA) followed by a Tukey-Kramer Multiple Comparisons Test for parametric data and Kruskal-Wallis Test follow by Dunn's Multiple Comparisons test for non-parametric data.

We used Linear Regression to establish a linear relation of STX concentration and the TF48h.

All statistical analyses were calculated using the R statistical computing environment (R Core Team 2015). The *MASS* package was used for model selection (Venables and Ripley 2002) and a significance level of 0.05 was set.

3. Results

3.1 Effect on the zooplankton due to *C. raciborskii* density and STX concentration

The immobility of the zooplankton subjected to the whole water presented a marginal relation with the STX concentration ($p = 0.09$, pseudo - $R^2 = 0.18$) and there was no significant relation with *C. raciborskii* density ($p>0.05$, pseudo - $R^2 = 0.012$). The immobility of the organisms subjected to the sonicated water had a significant relation with the STX concentration ($p<0.05$, pseudo - $R^2 = 0.16$) (Figure 1).

The effect on the zooplankton was the same when the *C. raciborskii* cells are lysed (sonicated sample) to when the cladoceran population was subjected to the intact *C. raciborskii* cells (whole water) ($t(20)= 0.93$, $p = 0.36$) (Figure 2). There was no effect on the zooplankton when the *C. raciborskii* cells were removed (filtered sample) from the water. The immobilization was irreversible in all tests, indicating mortality.

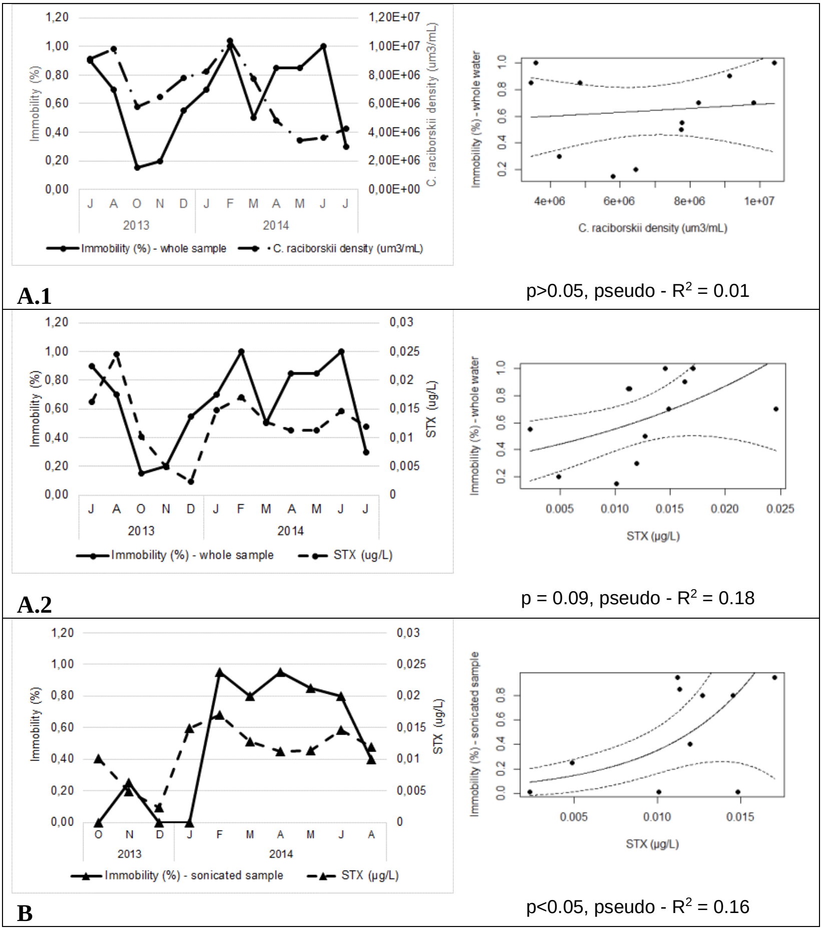

FIG. 1: A - The effect in the zooplankton subjected to the whole water. Temporal variation and the relation of the zooplankton immobility (%) with the *C. raciborskii* density (A.1) and STX concentration (A.2). B - The effect in the zooplankton subjected to the sonicated water. Temporal variation and the relation of the zooplankton immobility (%) with the STX concentration.

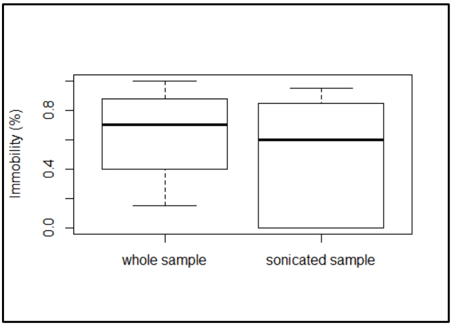

FIG. 2: The effect in the zooplankton (% of immobility) subjected to the whole water (0.64 ± 0.30) and sonicated water (0.50 ± 0.40). The exposure routes tested were not significantly different; t (20) = 0.93, p = 0.36.

3.2 Ecotoxicological test as predictor of STX concentration

The ecotoxicological tests performed with the whole water did not present a property curve dose-response; in this case, the TF48h was not determined. All treatments (dilutions 12.5% to 100%) were significantly different to the control ($p<0.05$ to the 12.5% treatment, $p<0.001$ to all others), with the exception of the 6.25% treatment that did not show a significant difference ($p>0.05$). The sample diluted 16 times (6.25%) presented the same effect as the control, but still induce acute toxicity (Figure 3A).

The dose-response curve resulting from the ecotoxicological tests performed with the sonicated sample was coherent. The 50% and 100% treatments presented difference when compared to the control ($p<0.05$), while the others (6.25, 12.5 and 25%) showed no difference ($p>0.05$) (Figure 3B). The sample diluted four times (25%) presented the same effect as the control, but still presented acute toxicity. The STX concentrations explained 84% of the variation in the TF48h during the study time ($p<0.001$; $R^2= 0.84$) (Figure 4).

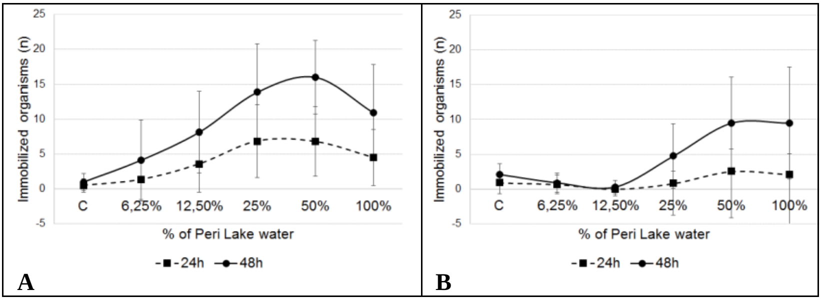

FIG. 3: Curve dose-response from the ecotoxicological tests performed with the whole sample (n=14) (A) and sonicated sample (n=13) (B). The graphs show the avarage and standard deviation of the study period samples, from July 2013 to September 2014.

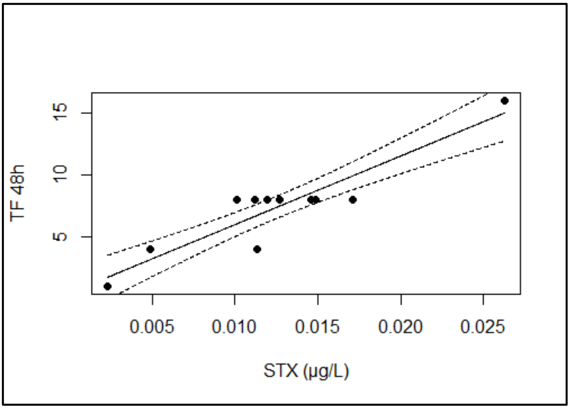

FIG. 4: Linear relation among the TF 48h and STX concentrations during the study time ($p<0.001$; R2= 0.84). The concentration of STX explained 84% of the variation in the TF48h.

4. Discussion

The STX concentration in Peri Coastal Lake was related to the effect on the zooplankton exposed to whole water samples. On the other hand, the density of *C. raciborskii* of these samples was not significantly related to immobility in the zooplankton tested.

Regarding the deleterious effect that cyanobacteria induce on aquatic organisms, one of the main questions yet to be resolved is whether the observed inhibitory effects are due to the known cyanotoxins, other unidentified compounds, or the putative poor nutritional value of cyanobacteria (Sivonen and Jones 1999). It is generally accepted that cyanobacteria are an unsuitable food source for herbivorous zooplankton species (Nogueira et al. 2006). Feeding experiments using *C. raciborskii* as the main food source have shown a decrease in the filtering rate by *Daphnia* (Hawkins and Lampert 1989). Lower survival ratios and body length increases were observed for *Daphnia* individuals feeding on *C. raciborskii* than for those fed with a chlorophyte (Nogueira et al. 2006). *Daphnia magna* growth rates were depressed at feeds comprised of more than 75% *C. raciborskii* (Soares et al. 2009). However, in this study the chronic effect due to *C. raciborskii* density is of secondary importance, since intracellular STX concentration induced an acute effect on the zooplankton.

The *C. raciborskii* population from Peri Coastal Lake affected *D. magna*. We recorded up to 100% immobilization (February and June/2004) in the cladoceran population exposed to the whole sample. The effects of *C. raciborskii* on *Daphnia* seem to be strain-specific, and different *Daphnia* species are likely to differ in their sensitivities to this cyanobacterium (Panosso and Lürling 2010). Some works developed with a STX-producing *C. raciborskii* culture (strain CYRF01) did not show acute toxicity to *D. magna* (Soares et al. 2009), inducing only a decrease in swimming activity and in the clearance rate in *D. pulex* (Ferrão-Filho et al. 2007; Fabre et al. 2016). The clearance rate decreasing was observed also to the strains MVCC19 (Fabre et al. 2016). A reversible toxic effect of saxitoxins on *D. pulex* was found using *C. raciborskii* T3 (Ferrão-Filho et al. 2008) and CYRF01 (Ferrão-Filho et al. 2010). The immobilization was due to paralyzation in the muscles of the second antennae, which is responsible for the swimming movements of the animals. A similar effect was observed when zooplankton was subjected to the natural water from Funil Resevoir (RJ, Brazil) containing 2.5 ng Eq STX L^{-1} (Ferrão-Filho et al. 2008). There is still no consensus on *C. raciborskii* toxicity to *Daphnia* (Soares et al. 2009), but the isolation of *C. raciborskii* strains with significant variation in toxin cell quota isolated from a single lake sample (Willis et al. 2016) indicates that the interstrain diversity of this species may provide explanation to the toxicity of this species.

The absence of effect in the zooplankton subjected to the filtered samples confirms that the toxin found in Peri Coastal Lake is concentrated intracellularly and the amount of toxin dissolved is not enough to cause some effect. The same condition was found by Ferrão-Filho et al. (2009, 2010), testing the Funil Reservoir water samples. In this case, we can assume that the main exposure route of the zooplankton tested was the ingestion by grazing of intact *C. raciborskii* cells from the whole water, with STX uptake primarily in the digestive tract following digestion of cells (Rohrlack et al. 2005).

Daphnia can readily ingest filaments ≥1mm length (DeMott 1995) even if the filtering rates are reduced (Hawkins and Lampert 1989) and the filament length of *C. raciborskii* should not be regarded as a primary factor affecting daphnid grazing (Panosso and Lürling 2010). The ability to ingest larger prey increases linearly with *Daphnia* (Burns 1968, DeMott 1995) and *Bosmina longirostris* body size (Burns 1968). Supposedly, there is advantage of larger body size cracking open or slurping down large algae (DeMott, 1995). The intoxication of zooplankton by ingestion of intracellular toxin that we observed drives changes in the aquatic community. There is a shift in zooplankton size and community composition in nature, where the big cladoceran had an apparently negative response (Hansson et al. 2007). In St. Johns River System, Florida, Leonard and Paerl (2005) observed that when numbers of *C. raciborskii* were low or undetectable, zooplankton were more diverse and were comprised of larger species. Rotifers were the dominant zooplankton, and their numbers relative to other zooplankton increased as *C. raciborskii* concentrations increased. The Rotifers are dominant in Peri Coastal Lake (Gerzson, 2011) as well, where *C. raciborskii* is abundant throughout the year (Laudares-Silva 1999, Grellmann 2006, Tonetta *et al*. 2013, Silveira, 2013, Brentano et al. submitted). The genus *Bosmina* comprises 25% of the zooplankton community (Gerzson, 2011).

As with the genus *Daphnia*, *Bosmina* often feeds effectively on large algae including *Microcystis* (60-100 µm diameter; Jarvis et al. 1987) and large ciliates (40-120 µm length; Jack and Gilbert 1993). In this case, it is possible that *Bosmina* is recorded comprising the zooplankton in Peri Coastal Lake because the shift to smaller zooplankton in the community may not be a consequence of body size but also different methods of feeding (Hawkins and Lampert 1989). *Bosmina* uses a dual feeding mode and can be very selective (DeMott and Kerfoot 1982, Bogdan and Gilbert 1987) being less affected by filaments when compared with *Daphnia*, that have limited abilities to handle or reject individual particles (Gliwicz and Siedlar 1980).

Lampert (1987) concluded that food selection is primarily determined by particle diameter and shape. Rejection of particle size selection could be influenced by food concentration and alternative food (DeMott 1993, 1995). So, even if *Bosmina* is a good sorter, its mechanism may be less efficient with high *C. raciborskii* density. The inverse correlation between *Bosmina* density and Chl-*a* in Peri Coastal Lake (Gerzson, 2011) could reflect the inability to select the preferred food in conditions of high phytoplankton density or the lack of food alternatives, since *C. raciborskii* is frequently dominant in this ecosystem. Both conditions could induce intoxication of the cladoreans in Peri Coastal Lake by the intracellular STX of *C. raciborskii*; the same exposure route that was observed in this work causing acute toxicity on *Daphnia*.

Testing another exposure route, when the *C. raciborskii* cells were lysed (sonicated sample) dissolving the toxin in the water, we observed a significant relation between the STX concentration and the zooplankton immobility. It is not the natural condition found in Peri Coastal Lake, but simulates conditions of cellular lysis. Interestingly, the ingestion exposure route (intact cells) and the direct exposure route (lysed cells) had the same effect on the zooplankton. This observation confirms the effectiveness of grazing by this genus on the phytoplankton, and supports its role as a key species in the aquatic ecosystems (Rohrlack et al. 2003), connecting efficiently the trophic levels (Lampert 1987).

Considering the ecotoxicological test as predictor of STX concentration in the natural ecosystem, we found that the STX concentration in the Peri Coastal Lake samples explained 84% of the variation in the toxicity on zooplankton (TF48h) testing the sonicated sample (lysed cells, toxin extracted). Ferrão-Filho et al. (2010) found that saxitoxins content of seston showed significant relationship with Effective Time to immobilize 50% of the individuals (ET50) on *Daphnia pulex* ($R^2 = 0.87$), with this test being considered a good predictor of saxitoxins content of seston.

The possibility that other unanalyzed toxins from Peri Coastal Lake samples could improve the relation not explained by this work cannot be disregarded. Some exploratory studies recorded STX analogues (Neo-STX, GTXs and dcGTX) and the respective amount of equivalent STX (Eq STX) higher (4.54 ± 2.82 µg L^{-1}, n=5, Mondardo 2009; 4.57 ± 2.14 µg L^{-}1, n=8, Melo Filho 2006) than the STX amount (0.013±0.007 µg.L^{-1}, n=15, Brentano et al. submitted; 0.31 ± 0.31 µg L^{-1}, n=45, Brentano et al. 2016). New studies relating the toxicity on zooplankton and Eq STX are highly recommended, as the relationship between Eq STX concentration and toxicity may provide a better relationship than that of STX concentration alone. In addition, studies including other bioactive compounds, such as endotoxins (lipopolysaccharides), are also recommended to complement data on the toxicity and risks of cyanobacterial blooms (Sotero-Santos et al. 2006).

The results presented here suggest that standard ecotoxicological tests with *Daphnia magna* performed with the sonicated sample can potentially be used to predict STX concentration in the natural environment studied. As appointed by Ferrão-Filho et al. (2008, 2010), the rapid and sensitive response of cladoceran to *C. raciborkii* metabolites in water can be of great value in biomonitoring of water bodies, especially public water supply reservoirs dominated by this cyanobacterium. The analytical methods used to detect STX, such as High Performance Liquid Chromatography (HPLC), mass spectroscopy (MS), phosphatase assay (PPase) and Enzyme-Linked Immunosorbent Assay (ELISA), are expensive, requiring sophisticated equipment and qualified technicians (Harada et al. 1999, Ferrão-Filho et al. 2008, 2010, Newcombe et al. 2010). In this context, the utilization of ecotoxicological tests are suited to a preliminary screening for STX-contamination of water samples prior to further chemical analysis, but is not sufficiently sensitive to replace it.

The Brazilian Association of Technical Norms established two protocols to develop ecotoxicological tests with cladoceran, in Brazil. The NBR 12713 (ABNT 2009) and NBR 13373 (ABNT 2010), respectively to perform tests with *Daphnia* spp. and *Ceriodaphnia* spp. However, there are no protocols for testing the specific effects of cyanotoxins on freshwater cladocerans, with the only contribution in this direction being the procedures developed by Ferrão-Filho et al. (2008, 2009, 2010). In fact, the results presented here using the whole water from Peri Coastal Lake in the standard ecotoxicological tests, as described in ABNT (2009), clarified that this methodology must be adapted and standardized for the detection of STX when there are intact cyanobacterial cells in the sample. It will be necessary to take into account the specificities of the cyanotoxins and their mechanisms of action (Ferrão-Filho et al. 2010), as well as the mechanisms that drive their production.

In this work, we did not observe a dose-response standard curve to the whole water tested from Peri Coastal Lake. A lower concentration (50%) caused a larger effect (not statistical) on the zooplankton, when compared with the higher concentration (100%) tested. The reconstituted water used to dilute and test the Peri Coastal Lake samples has salts ($CaCl_2$, $MgSO_4$, KCl and $NaHCO_3$) that give it a high hardness and conductivity (ABNT 2009).

Brentano et al. (2016) found that water ion concentration was directly related to STX concentration in Peri Coastal Lake, suggesting that this abiotic factor could be driving the increase in STX concentration in this natural ecosystem. Previous laboratory work described in the literature already observed this relation. A direct relation between Na^+

concentration increase and increase of intracellular STX levels in *C. raciborskii* was proved in laboratory experiments (Pomati et al. 2003). Similar relation was also observed when this species was cultivated in Mg^{2+} high concentrations (Kelmann and Neilan 2007, Carneiro et al. 2013). Works using genetic basis (Soto-Liebe et al. 2012; Ongley et al. 2015) found the pathway for STX production and export stimulated under high salt concentrations. The role of STX has been linked with the maintenance of cyanobacterial homeostasis under salt stress condition (Pomati et al. 2003, Pomati et al. 2004).

Since the whole water tested in this work contained intact cyanobacterial cells, the results suggest that the effect observed in the dilution half sample and half reconstituted water is a consequence of the increase in STX concentration under the test conditions applied. In the 50% dilution condition, the *C. raciborskii* cells were in a higher salt concentration than in the lake and may have stimulated STX production and/or export during the test period (24-48h). This reasoning supports the literature and especially the results found to Peri Coastal Lake (Brentano et al. submitted) regarding conductivity playing an important role in driving STX variation in this system. In addition, the density of *C. raciborskii* seems to be less important that the STX concentration on the zooplankton effect. In the 50% concentration of whole water, the density of this cyanobacterium was halved yet the immobility zooplankton was larger.

When we compare the toxicity of sonicated and whole water, the first one is less toxic than the second one. The sonicated water diluted four times (25%) produced the same effect on the zooplankton as the control. In the case of whole water, it is necessary to dilute the sample 16 times (6.25%) to get the same result. We conclude that, the higher toxicity presented in the tests with whole water could be a consequence of an increase of STX concentration due to the reconstituted water used. Especially considering no difference in toxicity was observed in the undiluted (100% concentration) assays of both treatments (sonicated and whole water).

This work represents an important contribution to standardization of ecotoxicological tests for STX screening. The use of reconstituted water with high conductivity and hardness is not recommended when testing whole water from ecosystems where *C. raciborskii* (or any STX producer species) is dominant, due to its potential to, probably, stimulate higher STX production or extracellular STX. Instead, we recommend changing the reconstituted water used in the dilutions. Aqueous crude extract, as used by Sotero-Santos et al. (2006), or sonicated water sample, as used in this work, are options. The use of filtered lake water as controls (Ferrão-Filho et al. 2010) and maybe as water to dilute the sample and perform the test could be an option when there is intracellular STX present. The ecotoxicological tests have a potential to be used as an initial indicator of STX in natural ecosystems, however the method presented herein should be further optimized to improve the reliability of the results. Further work analyzing natural samples from aquatic environments dominated by cyanobacterium using this approach are welcome, and necessary, to improve the dataset and aid in the development of specific standard protocols.

5. Conclusion

The *C. raciborskii* density was secondarily important to the effect on the zooplankton that is able to prey this species, since there was acute effect related to STX concentration. The main exposure route and intoxication on the cladoceran tested was ingestion by grazing of this cyanobacterium. The toxic effect due to the intracellular STX of the *C. raciborskii* population present in Peri Coastal Lake exerts a selective pressure on which the zooplankton species are able to coexist with this STX-producing cyanobacterium, contributing to the structuration of the zooplankton community.

The standard ecotoxicological test using *Daphnia magna* performed with a sonicated water sample can be potentially used as a preliminary screening to predict STX concentration in the natural environment. Specific standard protocols should be developed to improve the reliability of the results and avoid the interference due to the mechanism of action or production of the cyanotoxins.

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6. CONCLUSÕES GERAIS

A densidade da cianobactéria *Cylindrospermopsis raciborskii* na Lagoa do Peri foi positivamente influenciada pelo aumento da temperatura e concentração de nutrientes, em especial o fósforo. Estas condições coincidiram com períodos quentes e chuvosos.

O aumento da densidade de *C. raciborskii* teve relação significativa positiva com a variação na concentração de STX na Lagoa do Peri (20%). Entretanto, os fatores abióticos (condutividade elétrica e concentração de nitrogênio orgânico dissolvido) tiveram poder explicativo maior na variação na concentração de STX neste ambiente (49%).

Observou-se pela primeira vez que o aumento da concentração de STX ocorre como resposta ao aumento da concentração iônica também no ambiente natural, ratificando este fato anteriormente observado apenas "in vitro".

Os testes ecotoxicológicos padronizados no Brasil com *Daphnia magna* tem potencial preditor da concentração de STX (84%) em ambiente natural dominado por *C. raciborskii*, desde que as amostras não sejam testadas com as células de cianobactérias intactas. Dentre os preditores testados (densidade de *C. raciborskii*, fatores abióticos e imobilidade de *D. magna*) o efeito sobre *D. magna* apresentou a melhor performance.

A água da Lagoa do Peri apresentou toxicidade aguda ao microcrustáceo *D. magna* e a via de intoxicação é a ingestão de *C. raciborskii*, devido a concentração intracelular de cianotoxina. O efeito sobre a população de Cladocera testada independe da densidade de *C. raciborskii*.

A comunidade aquática da Lagoa do Peri é regulada na direção dos produtores (fitoplâncton) aos herbívoros (zooplâncton) e estruturada pelo modelo "bottom-up". Sugere-se que o intrincado mecanismo entre fatores abióticos regulando a variação da concentração de STX intracelular em *C. raciborskii* e a consequente intoxicação aguda do zooplâncton por herbivoria é o que prioritariamente regula a densidade, ou mesmo a seleção dos organismos do zooplâncton, capazes de coexistir com esta cianobactéria e compor a comunidade zooplanctônica.

7. CONSIDERAÇÕES FINAIS E PERSPECTIVAS FUTURAS

A relação dos fatores abióticos e da densidade de *Cylindrospermopsis raciborskii* (Cyanophyceae) na concentração de saxitoxina na Lagoa do Peri pode ser sintetizada conforme descrito na figura 1. Também a relação da concentração de STX na lagoa sobre o efeito no zooplâncton (*Daphnia magna*) pode ser observado nesta figura. As relações encontradas indicam estruturação da comunidade de baixo para cima (“bottom-up”).

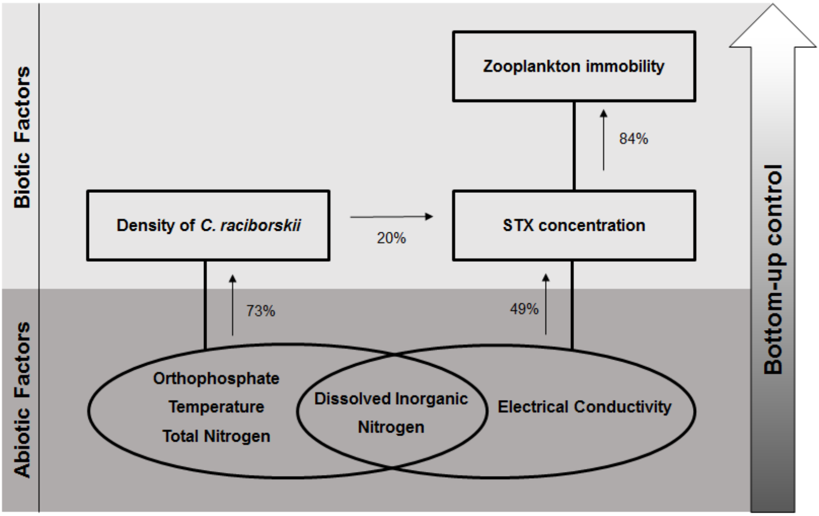

Figura 01: Modelo conceitual da Lagoa do Peri com base nos dados do presente estudo, sugerindo a estruturação da comunidade de baixo para cima (“bottom-up”). As setas indicam o poder de explicação das relações causais entre as variáveis.

O Parque Municipal da Lagoa do Peri é de extrema importância na preservação da qualidade da água da Lagoa do Peri e a criação do mesmo, na década de 80, foi uma decisão acertadíssima. Neste trabalho observamos que a densidade da população de *Cylindrospermopsis raciborskii* é favorecida pelo aumento de nutrientes no sistema aquático, cuja origem pode ser alóctone, associada a períodos de chuva. A concentração de STX também está positivamente relacionada com a concentração de nutrientes. Neste sentido, na bacia hidrográfica da Lagoa do Peri a cobertura do solo por vegetação nativa é necessária para manutenção da qualidade do manancial.

Observou-se forte relação entre aumento da concentração de sais e aumento da concentração de STX na Lagoa do Peri. Esta lagoa encontra-se próxima ao mar, o que nos remete a necessidade de monitoramento contínuo da salinidade na lagoa. Recomenda-se especial atenção nas interferências realizadas ao longo da costa nas praias da Armação e do Matadeiro, bem como em qualquer ponto ao longo do Canal Sangradouro, considerando a possibilidade de movimentação de águas no sentido mar – lagoa ou intrusão de cunha salina.

Com relação ao monitoramento da água da Lagoa do Peri, o Laboratório de Ecologia de Águas Continentais – LIMNOS da UFSC vem realizando mensalmente, desde 2007, o monitoramento de variáveis de qualidade de água desta lagoa. Contudo, há necessidade do monitoramento contínuo da comunidade fitoplantônica, minimamente da densidade de *C. raciborskii*. A Resolução CONAMA nº 357 (Brasil 2005) limita o uso de água com densidade de cianobactérias superior ou igual a 100×10^3 células mL^{-1} ou 10×10^6 μm^3 mL^{-1} para abastecimento humano. Durante este trabalho foi encontrado densidade de 21×10^3 ind. mL^{-1} ou 5×10^6 μm^3. mL^{-1} a 66×10^3 ind. mL^{-1} ou 19×10^6 μm^3. mL^{-1}, valores superiores ao limite estipulado por tal resolução. Ainda que a concentração de cianotoxina seja baixa, é de extrema importância o conhecimento da flutuação da população desta cianobactéria a longo prazo. Especialmente porque a modelagem da concentração de STX em função da densidade de *C. raciborskii*, como a que foi feito neste trabalho, pode apresentar maior precisão e acurácia utilizando dados de longo período.

Trabalhos futuros deverão avaliar STX e seus análogos nas amostras de água da Lagoa do Peri, levando em conta o equivalente de STX para comparações. Sugere-se a investigação da variação de concentração espacial de cianotoxinas (STX e análogos) tanto vertical quanto horizontalmente na Lagoa do Peri. Em termos de hidrodinâmica, existe fluxo na direção dos morros ao Rio Sangradouro o que pode implicar em variação espacial na concentração de toxinas.

Trabalhos de ecotoxicologia com espécies do zooplâncton nativo da Lagoa do Peri podem ser linhas de investigação interessante para complementar os resultados aqui obtidos com *Daphnia magna*. Investigação com microcosmos também é bemvinda.

Sugerimos a investigação sobre a diversidade genética de cepas de *C. raciborskii* existentes na Lagoa do Peri, bem como a investigação do potencial perfil de produção de toxinas que as mesmas apresentam.

Neste sentido, trabalhos futuros poderão relacionar a variação da concentração de STX e análogos e a observação da oscilação de diferentes cepas de *C. raciborskii*. Talvez devamos considerar a diversidade genética entre as cepas como uma variável mais relevante que apenas a densidade da população desta espécie na concentração de STX e seus análogos neste manancial.

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