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FATORES BIÓTICOS E ABIÓTICOS ASSOCIADOS A FLORAÇÕES DE CIANOBACTÉRIAS

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Tese de doutorado submetida à Universidade Federal do Rio de Janeiro visando a obtenção do grau de doutor em Ciências

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1. Diversidade molecular. 2. 16S rRNA. 3. Microcistinas. 4. Variabilidade Intraespecífica. 5. Bactérias heterotróficas

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Folha de Aprovação


EN LA VIDA HAY QUE IR SIEMPRE PARA ADELANTE, ENRIQUETA...
¿POR QUÉ? ¿QUÉ HAY ADELANTE?
MÁS VIDA.
PROFUNDO, FELLINI. PROFUUUNDO

Macanudo

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Resumo

GUEDES, Iamê Alves – Fatores bióticos e abióticos associados a florações de cianobactérias. Tese (Doutorado em Ciência Biológicas/ Biofísica) – Instituto de Biofísica Carlos Chagas Filho, Universidade Federal do Rio de Janeiro, Rio de Janeiro, 2017.

Florações de cianobactérias ocorrem em ambientes de água doce em todo o mundo, principalmente como resultado da eutrofização. Em contraste com fatores abióticos, o papel das interações bióticas na dinâmica de florações é menos explorada. As vantagens adaptativas de cianobactérias são insuficientes para explicar a prevalência de uma espécie em detrimento de outra em um período de floração, o que pode estar relacionado a estratégias específicas e interações com outros componentes da comunidade planctônica. Este estudo tem como objetivo avaliar a influência de fatores bióticos e abióticos sobre a sucessão de espécies de cianobactérias durante uma floração. O reservatório do Funil que foi utilizado como modelo é um reservatório tropical eutrófico no qual vem sendo observadas florações de cianobactérias nos últimos 20 anos. No primeiro capítulo, apresentamos uma visão integrada de uma floração mista de cianobactérias revelando mudanças temporais na dominância de gêneros de cianobactérias, bem como na comunidade de bactérias heterotróficas associadas. As principais filos foram Cyanobacteria e Proteobacteria, seguidos de Actinobacteria, Bacteroidetes, Verrucomicrobia e Planctomycetes. O primeiro período da floração foi caracterizado por altas abundâncias de *Microcystis* e Bacteroidetes. O segundo período foi dominado por *Synechococcus* e C. *raciborskii*, juntamente com Planctomycetes. Tanto correlações positivas quanto negativas foram observadas entre certos taxa de cianobactérias e bactérias heterotróficas, apontando para possíveis associações ecologicamente significativas. No segundo capítulo, abordamos a estrutura populacional dos principais gêneros de cianobactérias envolvidos na floração, explorando a diversidade intraespecífica das sequências do 16SrDNA. Enquanto *Microcystis*, *Pseudanabaena* e *Cylindrospermopsis* foram caracterizados pela dominância de um genótipo, *Synechococcus* e *Dolichospermum* apresentaram maior número de genótipos esporádicos. Microcistina (MC) e potenciais células produtoras de MC foram detectadas em todas as amostras, mas não houve correlação significativa entre MC e genótipos. Os resultados sugerem que as populações destes gêneros são estruturadas de diferentes maneiras, o que pode estar relacionado a adaptações para persistir durante a floração. Como no reservatório as concentrações de fósforo (P) estão diminuindo ao longo dos anos, investigamos a resposta das duas principais espécies de cianobactérias à restrição de P. Assim, no terceiro capítulo, testamos cinco linhagens de *C*. *raciborskii* e *M*. *aeruginosa*, avaliando crescimento, eficiência fotossintética, atividade de fosfatase alcalina e taxa de absorção máxima, sob privação de P. Todas as linhagens foram capazes de crescer, manter a atividade fotossintética e ativar a fosfatase alcalina, apontando para sua capacidade de tolerar a privação de P. O nível de variação intraespecífica impede generalizações e reforça a ideia de que a diversidade fisiológica de cianobactérias é subestimada em estudos baseados em apenas uma ou poucas linhagens. Em suma, este trabalho destaca a importância dos fatores bióticos na ecofisiologia das cianobactérias.

Palavras-chave: microcistina, sequenciamento de nova geração, diversidade molecular, variabilidade intraespecífica.

Abstract

GUEDES, Iamê Alves – Biotic and abiotic factors related to cyanobacterial blooms. Tese (Doutorado em Ciências Biológicas/ Biofísica) – Instituto de Biofísica Carlos Chagas Filho, Universidade Federal do Rio de Janeiro, Rio de Janeiro, 2017.

Cyanobacterial blooms occur in freshwater environments around the world, mainly as result of eutrophication. In contrast to abiotic factors, the role of biotic interactions on cyanobacterial bloom dynamics is less explored. Cyanobacterial adaptive advantages are insufficient to explain the prevalence of one species over another in a bloom period, which may be related to specific strategies and interactions with other components of the plankton community. This study aims to evaluate the influence of biotic and abiotic factors on the succession of cyanobacterial species during a bloom. The Funil reservoir was used as model, it is a eutrophic tropical reservoir in which cyanobacterial blooms have been reported over the last 20 years. In the first chapter, we present an integrative view of a mixed cyanobacterial bloom approaching temporal shifts in the dominance of cyanobacteria genera as well as in the associated heterotrophic bacteria community. The major bacteria phyla were Cyanobacteria and Proteobacteria, followed by Actinobacteria, Bacteroidetes, Verrucomicrobia, and Planctomycetes. The first period of the bloom was characterized by high abundances of *Microcystis* and Bacteroidetes. The second period was dominated by *Synechococcus* and *C. raciborskii*, together with Planctomycetes. Both significant positive and negative correlations were observed between certain cyanobacterial and heterotrophic bacteria taxa, pointing to potential ecologically significant associations. In the second chapter, we addressed the population structure of the main cyanobacterial genera involved in the bloom, exploring the intraspecific diversity from 16S rRNA gene sequences. While *Microcystis*, *Pseudanabaena* and *Cylindrospermopsis* were characterized by the dominance of one genotype, *Synechococcus* and *Dolichospermum* presented a higher number of sporadic genotypes. Microcystin (MC) and potentially MC producing cells were detected in all samples but no significant correlation between MC and genotypes were found. The results suggest that the populations of these genera are structured in different ways which can be relevant as an adaptation to persist during the bloom. Since in the reservoir phosphorous (P) concentrations are decreasing over the years we investigated the response of the two main cyanobacterial species to P restriction. So, in the third chapter, we tested five strains of *C. raciborskii* and *M. aeruginosa*, measuring Growth, photosynthethic efficiency, alkaline phosphatase activity, and maximum uptake rate under phosphorus deprivation. All strains were able to grow, maintain photosynthetic activity, and activate alkaline phosphatase, pointing to their ability to tolerate P deprivation. The level of intraspecific varation precludes generalization and reinforce that the physiological diversity of freshwater cyanobacteria is underscored in studies based in only one or few strains. Altogether, this work highlights the importance of biotic factors in the ecophysiology of cyanobacteria.

Keywords: microcystin, Next generation sequencing, Molecular diversity, intraspecific variability,.

Sumário

Introdução

Cianobactérias

O filo Cyanobacteria é um grupo altamente diversificado de bactérias fototróficas que produzem oxigênio a partir da fotossíntese. Este grupo possui uma longa história evolutiva e evidências fósseis indicam que já eram abundantes há mais de 2,5 bilhões de anos, podendo ter surgido há 3,5 bilhões de anos (Schopf, 2000). Acredita-se que descendam dos primeiros organismos primitivos capazes de produzir oxigênio através da fotossíntese e possuem papel fundamental na produção primária global (Carey *et al.*, 2012).

Este grupo apresenta uma variedade de características estruturais e metabólicas que lhe confere grande plasticidade adaptativa, podendo ser encontrado em praticamente qualquer ambiente. Habitam majoritariamente ambientes aquáticos, mas também podem ser encontradas colonizando solos e rochas, onde desempenham importante papel nos processos funcionais do ecossistema e na ciclagem de nutrientes. Podem ainda participar de associações simbióticas (Paerl & Otten, 2013a). As cianobactérias apresentam grande variação morfológica, podendo ser unicelulares, coloniais ou filamentosas e estão classificadas em aproximadamente 150 gêneros e mais de 2000 espécies (Palinska & Surosz, 2014). Possuem clorofila-*a*, carotenóides e ainda outros pigmentos acessórios proteicos como as ficobilinas: ficocianina, ficoeritrina e aloficocianina. Também apresentam uma série de adaptações, descritas a seguir, que as permitem sobreviver em ambientes em que outros microrganismos fotossintetizantes não são capazes de sobreviver (Yoo *et al*., 1995). Vários gêneros de cianobactérias possuem a capacidade de fixar nitrogênio atmosférico (Gallon, 1992), estocar fósforo em vesículas intracelulares (Gonzalez-Esquer *et al.*, 2016) e acumular ferro e outros metais. Outra adaptação importante presente em algumas espécies são os acinetos, células de resistência que permitem que as cianobactérias sobrevivam em momentos desfavoráveis, mesmo na ausência de luz, e depois regenerem em melhores condições. A presença de vacúolos gasosos é outra característica adaptativa importante que permite que algumas cianobactérias flutuem na coluna d' água e explorem de forma otimizada a energia luminosa (Padisák, 2004).

Florações de cianobactérias

As florações de cianobactérias, resultado do crescimento massivo de células desses microrganismos na coluna d'água, culminam em grandes alterações na qualidade da água. Entre seus efeitos está a diminuição da transparência da água, o que pode suprimir o crescimento de macrófitas e outras espécies fitoplanctônicas, afetando negativamente habitats de invertebrados e peixes. Outro aspecto importante é a decomposição das florações de cianobactérias por bactérias heterotróficas, levando à diminuição ou até mesmo esgotamento do oxigênio e consequentemente causando a mortandade de peixes (Otten & Paerl, 2011). As florações também levam à diminuição da diversidade ecológica, alterações nas cadeias alimentares, com potenciais efeitos na ciclagem de nutrientes e diminuição da diversidade de espécies planctônicas (Paerl, 2008).

Embora florações de cianobactérias venham sendo reportadas na literatura há quase 140 anos (Francis, 1878), nas últimas décadas, a frequência, duração e intensidade dessas florações aumentaram, tanto em ambientes marinhos como de água doce (Lürling *et al.*, 2017; O'Neil *et al.*, 2012). Além de profundas mudanças ecológicas no ambiente, as florações de cianobactérias podem ser formadas por espécies potencialmente produtoras de toxinas, o que se torna um sério problema de saúde pública, principalmente em mananciais de abastecimento (Dittmann *et al.*, 2013).

Vários fatores relacionados ao estabelecimento e persistência de florações de cianobactérias são bastante explorados e conhecidos (Figura 1) (Paerl, 2008). O enriquecimento de nutrientes dos ambientes aquáticos é sem dúvida o fator mais fortemente associado ao aparecimento e persistência das florações de cianobactérias (Paerl & Otten, 2013b). Tradicionalmente, o fósforo tem sido considerado o principal elemento limitante ao crescimento das cianobactérias e altas concentrações desse elemento favorecem florações, especialmente no caso daquelas formadas por espécies fixadoras de nitrogênio, que podem suprir a sua demanda por este outro elemento chave através da fixação do nitrogênio atmosférico (Downing *et al.*, 2001).

Temperaturas elevadas também favorecem gêneros formadores de florações de superfície, por estes possuírem taxas máximas de crescimento em temperaturas

acima de 25ºC (Lürling *et al.*, 2013; Paerl & Otten, 2013b). A temperatura é um dos fatores ambientais que mais afetam o crescimento das microalgas e cianobactérias, uma vez que influencia diretamente processos metabólicos relacionados à fotossíntese e outras vias biossintéticas (Robarts & Zohary, 1987; Davidson, 1991; Cole & Jones, 2000). Embora a variação anual da temperatura nos trópicos não seja tão grande quanto nas regiões temperadas, a ocorrência de cianobactérias em muitos sistemas brasileiros tem sido relacionada a períodos de temperaturas mais elevadas (Soares *et al.*, 2013; Branco & Senna, 1994; Bouvy *et al.*, 2000; Huszar *et al*., 2000; Marinho & Huszar, 2002).

A dominância das cianobactérias também está associada a algumas outras condições ambientais características, tais como: regime de mistura com estratificação duradoura da coluna d'água (Reynolds, 1987) ou diária (constância ambiental) (Ganf, 1974); reduzida razão Zona eufótica/Zona de mistura (Jensen *et al*., 1994); pH elevado com baixa disponibilidade de CO_2 (Caraco & Miller, 1998).

Contudo, por ser um grupo muito diverso, os diferentes gêneros e espécies de cianobactérias apresentam requerimentos bastante distintos. Certas características que permitem que algumas espécies dominem em alguns ambientes aquáticos não são compartilhadas por todo o táxon. Por exemplo, a fixação de nitrogênio só pode ser realizada por espécies pertencentes à ordem *Nostocales* e por um pequeno grupo de cianobactérias marinhas não heterocitadas, como as espécies do gênero *Trichodesmus* (Dolman *et al.*, 2012). Portanto, generalizações acerca de características do grupo devem ser interpretadas com cautela, e estudos mais detalhados explorando a fisiologia dos diferentes gêneros e espécies são importantes.

As principais espécies de cianobactérias formadoras de florações tóxicas em ambientes de água doce são pertencentes aos gêneros *Dolichospermum, Aphanizomenon, Cylindrospermopsis, Microcystis* e *Planktothrix* (Paerl, 2008). No Brasil, *Dolichospermum*, *Cylindrospermopsis* e *Microcystis* são os principais formadores de florações. A dominância de cada um desses gêneros é bastante expressiva em reservatórios, especialmente os localizados em regiões de maior densidade populacional, com maior impacto antrópico (Huszar *et al.*, 2000; Soares *et al.*, 2013). Apesar de apresentarem traços importantes em comum, como aerótopos e potencial produção de toxinas, os três gêneros parecem apresentar diferentes preferências ambientais que afetam sua ocorrência. Enquanto *Dolichospermum* e *Microcystis* estão mais associados a temperaturas elevadas e estabilidade da coluna

d´água, com dominância em períodos quentes e chuvosos, *Cylindrospermopsis* apresenta sucesso durante períodos secos, com maior mistura da coluna d´água ou então ocorre em dominância perene (Soares *et al.*, 2013).

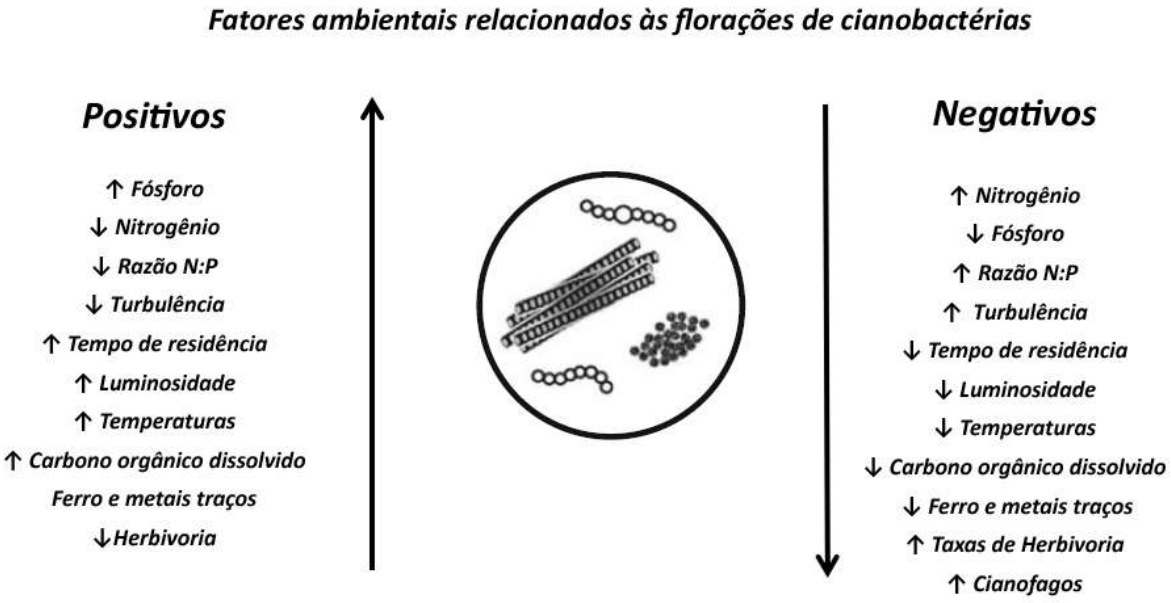


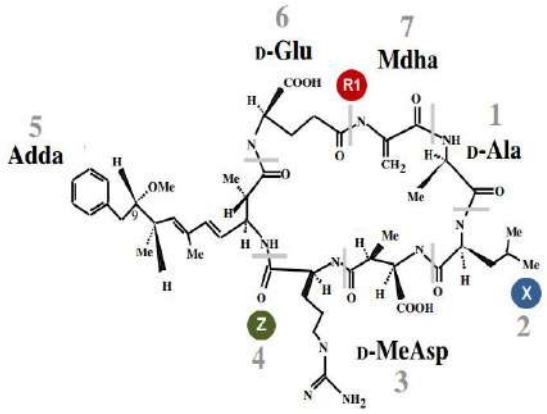
Figura **1**- Lista de fatores ambientais positivos e negativos relacionados às florações de cianobactérias. ↑indicam fatores que favorecem as florações de cianobactérias e ↓ indicam condições desfavoráveis ao crescimento de cianobactérias. (Modificado de Paerl & Otten, 2013)

Microcistinas

A maioria das florações de cianobactérias tóxicas reportada em todo o mundo é constituída por espécies produtoras dos cianopeptídeos microcistina e nodularina. (Dittman *et al*., 2013). Microcistinas são heptapeptídeos cíclicos que possuem uma estrutura básica comum (Figura 2). Sua estrutura química geral consiste de 5 D-aminoácidos conservados e 2 L-aminoácidos variáveis. Além da maior parte dos aminoácidos da molécula ser de isomeria D, pouco comum em estruturas biológicas, há dois aminoácidos raros: 3-amino-9-metoxi- 2,6,8-trimetil-10-fenildeca-4,6-ácido dienóico (ADDA) e N-metildehidroalanina (Mdha) (Harada et al., 1996; Soares, 2009b). A variação estrutural da microcistina pode ocorrer em todos os sete aminoácidos, no entanto, mais substituições ocorrem nas duas regiões variáveis de L-aminoácidos, nas posições X e Z (Figura 2). Essas variações tornam a molécula mais ou menos

hidrofílica e resulta em diferentes toxicidades. Mais de 80 variantes de microcistina já foram descritas, variando no grau de metilação, hidroxilação, sequência peptídica e toxicidade (Welker & Dohren, 2006).

As microcistinas são predominantemente produzidas por cianobactérias dos gêneros *Microcystis, Planktothrix e Dolichospermum* (Sivonen & Jones, 1999), apesar de já terem sido descritas em gêneros terrestres, como *Nostoc* e *Hapalosiphon* (Oksanen *et al*., 2004) e bentônicos *Phormidium* (Izaguirre *et al*., 2007) e outros gêneros planctônicos como *Pseudoanabaena e Synechococcus* (Boopathi & Ki, 2014; Paerl & Otten, 2013a).



Microcistina	X	Z	R1
MC-LR	L-Leu	L-Arg	CH_3
MC-RR	L-Arg	L-Arg	CH_3
MC-YR	L-Tyr	L-Arg	CH_3

Figura 2 - Estrutura da molécula da microcistina (MC). Os aminoácidos X e Z e o grupo R1 são variáveis conforme tabela à direita. Os aminoácidos que formam a molécula de MC estão numerados de 1 a 7 (adaptado de Tanabe *et al*., 2009)

Nem todas as linhagens de um gênero possuem a capacidade de produzir toxinas e portanto, linhagens tóxicas e não tóxicas podem coexistir no mesmo ambiente. Inicialmente, a distribuição variável da capacidade de síntese de microcistina entre as linhagens de um gênero era atribuída à transferência lateral de genes (Schwabe *et al.,* 1988), mas estudos filogenéticos vieram a demonstrar que a presença de genes para produção de toxinas é uma característica ancestral das cianobactérias e que linhagens não tóxicas aparecem devido a mutações e perda parcial ou completa destes genes (Rantala *et al.,* 2004). Portanto, pode-se supor que um número maior de gêneros de cianobactérias do que os descritos até agora pode apresentar a capacidade de produção de microcistina (Dittman *et al,* 2013).

Atualmente, métodos baseados na detecção da presença de genes relacionados à produção de toxinas são bastante utilizados para indicar o potencial de produção de microcistina por cianobactérias no ambiente (Sivonen & Borner 2008).

Fatores bióticos associados a florações de cianobactérias

Apesar de haver um amplo consenso de que o enriquecimento de nutrientes em corpos d'água a partir de fontes antropogênicas (urbanas, agrícolas e industriais) tem promovido a expansão e persistência de florações, também se considera que estes são eventos complexos, resultantes de múltiplos fatores que ocorrem simultaneamente. Portanto, aprofundar o conhecimento dos fatores abióticos e bióticos associados às florações de cianobactérias é de fundamental importância para seu manejo e prevenção.

A dinâmica de florações, no que se refere a interações dentro da comunidade planctônica, é bem menos explorada que os efeitos de fatores abióticos. Excetuando-se a herbivoria, o impacto das interações entre espécies de cianobactérias e do parasitismo ainda são pouco esclarecidos.

A dominância de cianobactérias em sistemas de água doce tem sido relacionada principalmente ao controle ascendente, especialmente pelo fato do grupo possuir alguns importantes traços funcionais que minimizam a pressão de herbivoria (Carey *et al.*, 2012). Como exemplo, algumas cianobactérias produzem metabólitos secundários conhecidos como cianotoxinas (incluindo alguns com efeitos neurotóxicos ou hepatotóxicos em mamíferos) e diversos outros compostos menos conhecidos, com efeitos letais e sub-letais ao zooplâncton (Leflaive & Ten-Hage, 2007). Cianobactérias também são deficientes em esteróis e os ácidos graxos polinsaturados de cadeia longa (PUFAs), vitais para regular a função de células animais (Muller-Navarra *et al.*, 2000). Por fim, a formação de colônias ou filamentos oferece proteção contra a herbivoria, por entupir o aparelho de filtração dos animais (DeMott *et al.*, 2001). Portanto, o efeito da herbivoria sobre populações de cianobactérias em muitos casos é considerado moderado (Ferrão-Filho & Kozlowsky-Suzuki, 2011).

Além da herbivoria, interações entre cianobactérias e bactérias heterotróficas também podem desempenhar um papel importante no estabelecimento e manutenção

das florações (Bagatini *et al.*, 2014; Gerphagnon *et al.*, 2015). Contudo, apesar da alta abundância de bactérias e vírus nos corpos d'água, a sua importância como potenciais reguladores de populações de cianobactérias ainda é pouco explorada (Bechette *et al.*, 2013;Suttle et al., 2017; Yoshida et al.,2012). Cianobactérias e bactérias heterotróficas podem estabelecer diversos tipos de interação, vantajosos ou desvantajosas para os organismos envolvidos. Além disso, as cianobactérias podem interagir com bactérias de vida livre, mas também podem manter associações estreitas com bactérias aderidas em sua superfície celular ou na mucilagem. Esse micro-habitat circundante que pode favorecer a colonização é chamado de ficoesfera (Dziallas & Grossart, 2011; Louati *et al.*, 2015).

Associações entre bactérias heterotróficas e cianobactérias podem ser positivas, por exemplo, uma relação trófica que favorece o aumento da população de bactérias via secreção de matéria orgânica pelas cianobactérias e/ou o estímulo do crescimento de cianobactérias. Isso foi demonstrado em alguns estudos, como em Shen et al. (2011), em que culturas axênicas de *M. aeruginosa* apresentaram crescimento mais lento quanto comparado a culturas não axênicas. Também no caso de *Anabaena* spp, em que foi observado um aumento da fixação de nitrogênio e crescimento da cianobactéria após a adição de bactérias na cultura (Paerl, 1978). Além desses exemplos, Sher et al. (2011) observaram que diversas cepas de *Alpha* e *Betaproteobacteria* podem favorecer o crescimento de *Prochlorococcus* através da produção de compostos difusíveis e Salomon *et al.* (2003) detectaram três linhagens relacionadas a *Shewanella* que estimularam o crescimento de *Nodularia spumigena.*

As relações entre bactérias heterotróficas e cianobactérias podem ainda ser negativas, pela competição por nutrientes, produção de substâncias antagonistas e parasitismo (Gerphagnon *et al.*, 2015). A lise de várias espécies de cianobactérias por bactérias foi relatada pela primeira vez em 1967 (Shilo, 1967) e, posteriormente as linhagens bacterianas foram descritas como pertencentes principalmente a *Myxobacteria* e *Cytophaga* (Rashidan & Bird, 2001). Essas bactérias causam a lise ligando-se diretamente à superfície da cianobactéria e secretando substâncias difusíveis capazes de hidrolisar a parede celular (Sudo et al., 1972). Em trabalhos mais recentes, o isolamento de várias linhagens capazes de lisar cianobactérias, demonstrou uma alta diversidade, incluindo pelo menos 33 gêneros pertencentes a Proteobacteria (19), Bacteroidetes (5), Actinobacteria (5) e Firmicutes (4). No entanto,

a relevância da capacidade de lise dessas bactérias no ambiente ainda precisa ser elucidada (Wichelen *et al.*, 2016).

Logo, apesar de estudos com linhagens isoladas já terem demonstrado claras associações entre determinados grupos taxonômicos de bactérias heterotróficas e de cianobactérias, indicando que essas interações sejam importantes não só para a dinâmica das populações microbianas mas também para ciclos biogeoquímicos, o real impacto dessas interações no ambiente natural ainda não foi elucidado (Bagatini *et al.*, 2014; Eiler & Bertilsson, 2004; Li *et al.*, 2015; Louati *et al.*, 2015).

Por outro lado, os taxa participantes de interações em populações naturais começam a ser mais bem conhecidos por meio de estudos mais recentes que acompanharam florações de cianobactérias e descreveram em paralelo variações na comunidade fitoplanctônica e na comunidade de bactérias heterotróficas, taxonomicamente muito variadas (Li *et al.*, 2015; Rooney-Varga *et al.*, 2005; Woodhouse *et al.*, 2015). Na última década, utilizando técnicas de biologia molecular, como bibliotecas de clones, DGGE (*denaturing gradient gel electrophoresis*) e mais recentemente NGS (*next generation sequencing*), vários estudos descreveram a diversidade de bactérias que acompanham florações, principalmente as do gênero *Microcystis* (Eiler & Bertilsson, 2004; Li *et al.*, 2012, 2015, 2011; Steffen *et al.*, 2012; Woodhouse *et al.*, 2016). De uma forma geral, filos como *Proteobacteria*, *Bacteroides*, *Actinobacteria* e *Verrucomicrobia* aparecem com abundância relativa maior durante florações de cianobactérias (Eiler & Bertilsson, 2004; Woodhouse *et al.*, 2016). Recentemente Tromas et al, 2017 sugeriram que a composição da comunidade bacteriana poderia ser utilizada para prever o estabelcimento de florações, já que eles identificaram algumas OTUs de bactérias como biomarcadoraes de florações. Mais especificamente, a familia Cytophagaceae e a ordem Chthoniobacterales foram associadas a presença de florações. Porém, ainda não é claro se a composição da comunidade bacteriana que acompanha um evento de floração de cianobactérias é dependente da(s) espécie(s) de cianobactéria presente(s), se segue padrões temporais e/ou espaciais, ou se seria determinada pelos mesmos fatores abióticos que influenciam a composição da população de cianobactérias.

Diversidade genética e fisiológica de cianobactérias

O estudo da diversidade molecular de bactérias iniciou-se nos anos 90 e revolucionou o conhecimento acerca da diversidade de espécies e da estrutura de comunidades bacterianas, revelando a alta diversidade presente nessas comunidades, já que apenas cerca de 1% das bactérias são cultiváveis. A utilização de ferramentas moleculares, como a construção de bibliotecas de clones seguida de sequenciamento de genes marcadores, hibridização *in situ* e DGGE e, mais recentemente, sequenciamento em larga escala, possibilitou um rápido avanço do conhecimento nesta área (Case et al., 2007).

Contudo, no caso de cianobactérias grande parte da taxonomia ainda é baseada em caracteres morfológicos, o que já não se aplica aos outros grupos bacterianos. Segundo Oren & Ventura (2017), pouquíssimas descrições de novos gêneros e espécies são publicadas no *International Journal of Systematic and Evolutionary Microbiology*, a publicação oficial para o registro de novos taxa procarióticos e a nomenclatura e classificação de cianobactérias ainda não é completamente organizada. Esta situação tem evidenciado a necessidade de métodos mais confiáveis de classificação e vem promovendo o uso de uma abordagem polifásica, combinando dados morfológicos e moleculares (Komárek 2016, Gkelis et al. 2005). Assim, atualizações e a revisão da sistemática de cianobactérias vêm sendo realizadas (Anagnostidis & Komarek, 1985, Komarek & Anagnostidis, 1999, 2005, KomaIsk, 2013), com a inclusão de abordagens tanto bacteriológicas quanto botânicas. A nomenclatura se baseia em critérios taxonômicos botânicos, mas também utiliza informações bacteriológicas e moleculares (Komarek & Anagnostidis).

As dificuldades relacionadas a definições taxonômicas em cianobactérias também foram destacadas por Palinska & Surosz (2014), como o emprego de conceitos tradicionais de espécies (Mayr, 1982), o elevado número de linhagens e ecotipos e a grande diferença entre linhagens cultiváveis e populações naturais, como por exemplo na morfologia.

De fato, este é um problema mais generalizado pois não há um conceito de espécie para procariontes amplamente aceito e a classificação de isolados em determinadas espécies baseia-se em medidas de semelhança fenotípica ou genotípica. Os métodos atuais para a definição de espécies procarióticas são

inadequados e insuficientes face aos níveis de diversidade que estão sendo descobertos na natureza (Stacke-Brandt et al., 2002). Na ausência de um consenso, no caso de estudos de caracterização de sequências de DNA a prática mais frequentemente utilizada para organizar a diversidade bacteriana é agrupar sequências com base na similaridade, geralmente considerando um locus conservado. A definição mais amplamente empregada é a que microrganismos da mesma espécie apresentam mais de 97% de identidade em sequências derivadas de genes 16S rRNA. Contudo, agrupamentos assim definidos reconhecidamente englobam isolados diversos no conteúdo do genoma, fisiologia e ecologia (Koeppel & Wu, 2013).

Assim, uma outra abordagem proposta para a definição de espécies seria buscar diferenças ecológicas, genômicas ou fenotípicas que suportassem as divisões derivadas de métodos moleculares (Palinska & Surosz, 2014). Cohan (2001, 2002, 2004) e Godreuil et al. (2005) propuseram que as espécies bacterianas poderiam ser divididas em unidades mais restritas, incorporando o conceito de ecotipo. Ecotipos são definidos como populações que ocupam o mesmo nicho ecológico (Palinska & Surosz, 2014). Idealmente, cada ecotipo formaria um agrupamento distinto, cujas sequências divergem em média menos do aquelas derivadas de outros ecotipos. Além disso, espera-se que cada ecotipo seja identificável como um grupo monofilético. Apesar do conceito de ecotipo fornecer uma base racional para a definição de espécies em bactérias, sua aplicação ainda não é bem estabelecida (Palinska & Surosz, 2014). Este conceito avançou em alguns grupos de cianobactérias nos quais ecotipos já foram definidos, como *Synechococcus* e *Phrochlorococcus*, mas para a maioria de cianobactérias de água doce ainda não há este tipo de distinção (Giovannoni & Stingl, 2005; Melendrez *et al.*, 2011).

Por outro lado, enquanto a diversidade de ecotipos ainda não vem sendo estudada em populações naturais, como florações, a presença de uma ampla diversidade de genótipos de uma mesma espécie já é bem descrita (Miller & McMahon, 2011; Pobel *et al.*, 2012; Sabart *et al.*, 2010). Usualmente, uma floração de cianobactérias é formada por diversas linhagens de um ou mais gêneros, apesar de uma ou poucas linhagens poderem predominar em algum estágio. Somente a identificação microscópica não fornece informação acerca da presença, diversidade ou abundância de linhagens ou de sua toxicidade (Saker *et al*, 2009). Técnicas

moleculares baseadas na análise de sequências gênicas têm se mostrado valiosas neste contexto e têm usado como alvos mais frequentes regiões (inter) gênicas de loci de 16S rDNA e regiões intergênicas de genes de ficocianina (*cpcBA*) (Briand *et al.*, 2009; Miller & McMahon, 2011; Neilan *et al.*, 1995a). Desta forma, pode-se avaliar a composição e a dinâmica de populações de cianobactérias temporal e espacialmente, e ainda tentar correlacionar tal dinâmica a fatores ambientais, físicos e químicos ou biológicos. Durante o desenvolvimento de florações, por exemplo, pode-se indicar se um, poucos, ou muitos genótipos são selecionados em diferentes momentos ou locais no ecossistema (Briand *et al.*, 2009; Sabart *et al.*, 2009, 2010, 2015)

Uma lacuna importante nesse tipo de estudo de populações naturais, porém, é atribuir significado à diversidade de genótipos revelada e, ainda mais difícil, correlacioná-la com a presença de ecotipos na população. Uma vez que ecotipos são caracterizados por adaptações específicas a condições ambientais e, portanto, por traços fenotípicos, estes aspectos são investigados a partir de linhagens isoladas. Isso gera um outro problema, pois durante décadas, diferenças entre linhagens dentro de espécies de cianobactérias foram reconhecidas em diferentes aspectos, tais como morfologia, taxa de crescimento, respostas a nutrientes e produção de toxinas, mas as causas da variabilidade observada muitas vezes são desconhecidas, podendo resultar de variação genética, plasticidade fisiológica ou mesmo de critérios inadequados para definição de espécies. Por exemplo, na espécie *Cylindrospermopsis raciborskii*, apesar da baixa variabiliade genética, linhagens apresentam grandes diferenças na morfologia, bem como nas taxas de crescimento, resposta à nutrientes e produção de toxinas (Piccini *et al.*, 2011; Willis *et al.*, 2016; Xiao *et al.*, 2017). O fato é que a variabilidade intraespecífica ainda é esquecida ou subestimada em estudos que procuram entender a biologia de espécies de cianobactérias e, mesmo aqueles que utilizam diferentes linhagens, o fazem com um número muito reduzido e geram generalizações sobre a espécie (Burkholder & Glibert, 2009). Este é o caso de *Microcystis aeruginosa*, uma espécie com ampla variabilidade genética já reconhecida (Wilson *et al.*, 2005), contudo a maioria dos estudos com foco na fisiologia utiliza somente uma ou poucas linhagens (Wilson *et al.*, 2006).

Florações de cianobactérias no Reservatório do Funil, Resende (RJ)

O Reservatório do Funil (Figura 3) é formado pelo represamento do Rio Paraíba do Sul no município de Resende (RJ). O uso do solo e ocupação industrial e urbana contribuíram para que este reservatório sofresse acelerado processo de eutrofização. Como reflexo, este reservatório apresenta frequentes florações de cianobactérias registradas desde o início da década de 90. Diversos estudos realizados no reservatório, inclusive por parte do nosso grupo de pesquisa, nos últimos 20 anos, relatam a dominância de três gêneros de cianobactérias *Microcystis, Cylindrospermopsis* e *Dolichospermum* (Ferrão Filho *et al*, 2009, Soares *et al*, 2009, Rocha, 2012, Guedes, 2013, Rangel, 2014). Esses três gêneros são também os principais formadores de florações no Brasil, e geram ainda mais interesse por serem potencialmente produtores de toxinas (Huszar e Silva,1999, Soares *et al*, 2013). Diversos estudos têm relacionado fatores responsáveis por seu sucesso e coexistência em diferentes sistemas aquáticos brasileiros, como tamanho, habilidade em controlar a posição na coluna d'água e toxicidade (Rangel *et al.*, 2016; Soares *et al.*, 2013).

Em particular quanto a *C. raciborskii* e *M. aeruginosa,* essas duas espécies coexistem em diversos corpos d'água (Dantas et al., 2011 e Miller e McMahon, 2011, Soares et al. 2009, Guedes, 2013, Soares et al. 2013). A coexistência e dominância de uma ou outra espécie sugere a ocorrência de competição direta por recursos e que possíveis mecanismos de regulação por trás dele possam ter sido desenvolvidos (Rzymski et al., 2014; Soares et al., 2009)

A competição entre essas duas espécies já foi investigada em relação a fatores específicos, como disponibilidade de luz, fósforo e alelopatia (Rzymski et al., 2014, Marinho et al., 2013; Mello et al., 2012). Marinho et al., 2013, estudando duas linhagens de *C. raciborskii* e duas de *M. aeruginosa*, demonstrou que as linhagens de *C. raciborskii* eram melhores competidoras por fósforo. Dolman et al., 2012, em um amplo estudo incluindo 102 lagos alemães, observou que *C. raciboskii* podia alcançar biomassas relativamente altas em lagos que apresentavam elevadas concentrações de nitrogênio e baixa disponibilidade de fósforo, se comparada a *Microcystis* e outras espécies. No caso do Reservatório do Funil, nos últimos anos uma drástica redução de fósforo vem sendo observada, devido à melhoria do saneamento na bacia

hidrográfica do Paraíba do Sul e à construção de duas Pequenas Centrais Hidrelétricas a montante do reservatório (Rangel *et al*., 2012; Rangel, 2014). Embora tenha havido uma diminuição das biomassas fitoplanctônicas, florações de cianobactérias ainda são frequentes nesta condição, principalmente nos meses de verão. No entanto, a contribuição relativa de *M. aeruginosa* diminui significativamente, enquanto que a de *C. raciborskii* aumentou.

Apesar dos diversos estudos já realizados neste reservatório e com linhagens isoladas de *M. aeruginosa* e *C. raciborskii*, ainda não foi esclarecido como e por que ocorre a sucessão temporal entre essas espécies. Portanto, o Reservatório do Funil representa um excelente ambiente para realizar um estudo de caso acerca desta problemática. Neste contexto, a dinâmica de coexistência e substituição de espécies e/ou linhagens dos três principais gêneros de cianobactérias tóxicas do Brasil poderá ser explorada levando-se em consideração o efeito de variáveis abióticas e bióticas, como a influência da comunidade bacteriana, além das interações interespecíficas de cianobactérias. Resultados nesta linha serão úteis não só para um melhor entendimento da dinâmica de florações de cianobactérias, como para sugerir possíveis alternativas para o manejo e prevenção destes eventos.

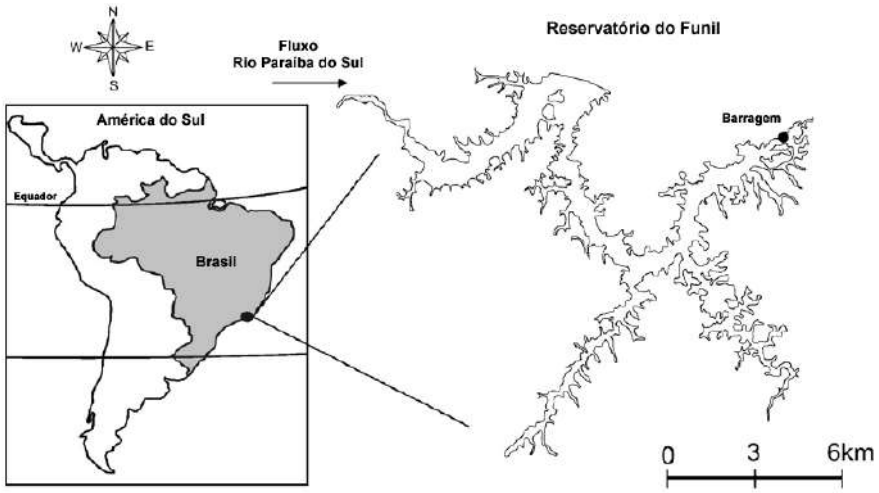


Figura 3– Localização do Reservatório do Funil. (modificado de Soares *et al*, 2009)

Objetivo geral

Considerando que os três principais gêneros de cianobactérias tóxicas encontrados no Brasil - *Microcystis, Cylindrospermopsis e Dolichospermum* - coexistem e alternam-se em dominância em diversos ambientes aquáticos, este estudo tem como objetivo avaliar a influência de fatores bióticos e abióticos neste fenômeno, utilizando com modelo o Reservatório do Funil (RJ).

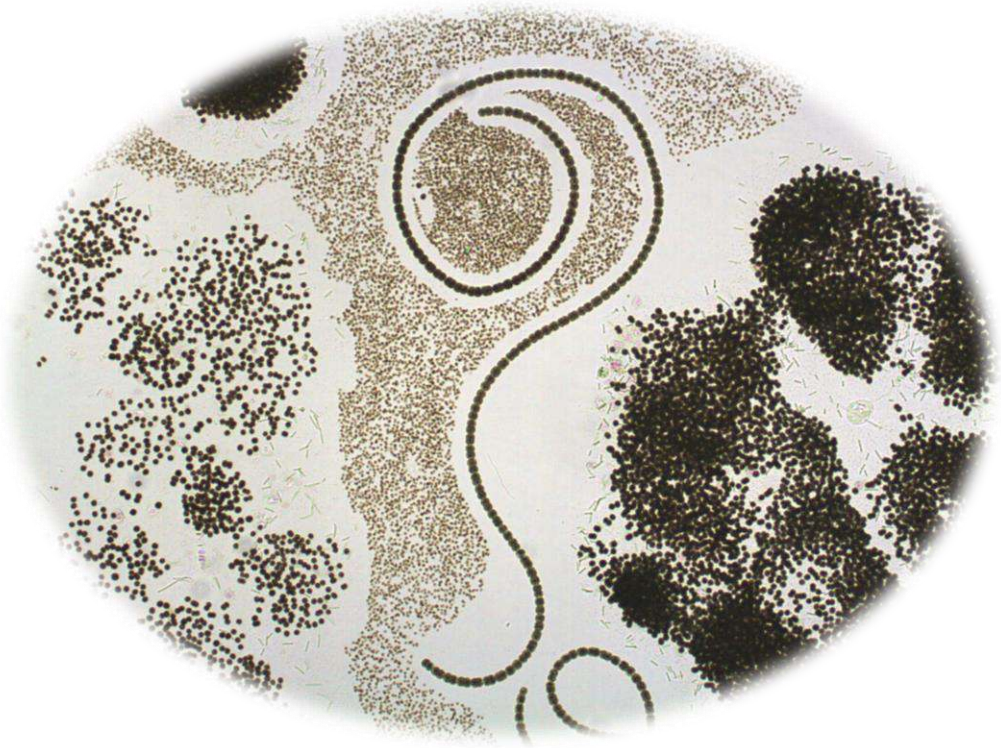
Objetivos específicos

- Avaliar a dinâmica de cianobactérias e sua relação com fatores abióticos ao longo de um período de floração.
- Avaliar a diversidade de bactérias heterotróficas durante um período de floração de cianobactérias e investigar a possível correlação entre a dinâmica de populações bacterianas e a dinâmica de populações de cianobactérias.
- Avaliar a diversidade intraespecífica de cianobactérias durante um período de floração e a possível correlação com a variação na concentração de microcistina.
- Avaliar, através de experimentos em laboratório com linhagens isoladas do Reservatório do Funil, a resposta de *Microcystis aeruginosa* e *Cylindrospermopsis raciborskii*, sob condições de limitação de fósforo.

Para responder os objetivos propostos, essa tese de doutorado foi elaborada em três capítulos organizados na forma de manuscritos que serão submetidos para publicação.

Capítulo 1

Close link between cyanobacterial dominance and associated bacterioplankton



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Close link between harmful cyanobacterial dominance and associated bacterioplankton in a tropical eutrophic reservoir

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Abstract

Cyanobacteria tend to become the dominant phytoplankton component in eutrophic freshwater environments during warmer seasons. However, general observations of cyanobacterial adaptive advantages in these circumstances are insufficient to explain the prevalence of one species over another in a bloom period, which may be related to particular strategies and interactions with other components of the plankton community. In this study, we present an integrative view of a mixed cyanobacterial bloom occurring during a warm, rainy period in a tropical hydropower reservoir. We used high-throughput sequencing to follow temporal shifts in the dominance of cyanobacterial genera and shifts in the associated heterotrophic bacteria community. The bloom occurred during late spring-summer and included two distinct periods. The first period corresponded to *Microcystis aeruginosa* complex (MAC) dominance with a contribution from *Dolichospermum circinale*; this pattern coincided with high water retention time and low transparency. The second period corresponded to *Cylindrospermopsis raciborskii* and *Synechococcus* spp. dominance, and the reservoir presented lower water retention time and higher water transparency. The major bacterial phyla were primarily Cyanobacteria and Proteobacteria, followed by Actinobacteria, Bacteroidetes, Verrucomicrobia, and Planctomycetes. Temporal shifts in the dominance of cyanobacterial genera were associated with physical features of the water (retention time and transparency) and with shifts in the associated heterotrophic bacteria. The MAC bloom was associated with a high abundance of Bacteroidetes, and positive correlations among Bacteroidetes OTUs (Cytophagaceae and Chitinophagaceae) and the most abundant *Microcystis* OTU were observed. Additionally, a strong positive correlation (r = 0.72) was found between a *Microcytis* OTU and an unclassified OTU of Cytophagales. In the second bloom period, Planctomycetes increased in relative abundance, and four OTUs (three from the genus *Planctomyces*) were positively correlated with *Synechococcus* and *C. raciborskii* OTUs. Our results suggest specific interactions of the main cyanobacterial genera with certain groups of the heterotrophic bacterial community; the results suggest that considering biotic interactions may lead to a better understanding of the shifts in cyanobacterial dominance.

Keywords: *16S rDNA*; *Microcystis*, *Synechococcus*, *Cylindrospermopsis*, cyanobacterial bloom; microbial community, Illumina

1. Introduction

Cyanobacterial blooms occur in freshwater environments around the world, mainly as a result of eutrophication (Rigosi et al., 2014). These events cause deleterious environmental and socioeconomic effects, impacting the ecosystem due to disruption of the food webs and thus potentially decreasing biodiversity. Cyanobacterial blooms also impair the use of water by human populations (Paerl and Paul, 2012). During the last few decades, an expansion of cyanobacterial blooms has been recorded, and global warming is expected to further exacerbate this situation (Paerl, 2008; Visser et al., 2016).

Potentially harmful cyanobacteria tend to become the dominant phytoplankton component in eutrophic freshwater environments, especially during warmer seasons (Paerl and Huisman, 2008). Indeed, the main reported abiotic drivers of cyanobacterial blooms are increased nutrient loading (nitrogen and phosphorus) and rising temperatures (Kosten and Huszar, 2012; Lürling et al., 2017). Some bloom-forming cyanobacteria have efficient nutrient uptake and storage abilities and can use both inorganic and organic N and P pools (Harke and Gobler, 2013; O'Neil et al., 2012; Paerl and Paul, 2012). Generally, cyanobacteria will grow faster at higher temperatures compared to other phytoplankton groups, although growth rates can be differentially affected depending on the species considered (Lürling et al., 2013; Paerl, 2008; Paerl and Paul, 2012). Rising temperatures also intensify vertical stratification, and in this situation some bloom-forming cyanobacteria can be benefited due to their buoyancy ability (Kruk et al., 2010). Cells can accumulate at the surface and shade underlying layers, thus outcompeting other phytoplankton groups through light limitation (Harke et al., 2016; Reynolds, 2006). Additionally, buoyant cyanobacteria can access nutrients from deeper waters when epilimnion concentrations are diminished (Graham et al., 2016). These general observations, however, are insufficient to explain the prevalence of one species over another in a bloom period, which may be related to particular adaptive strategies and interactions with other components of the plankton community (Wood et al., 2017; Woodhouse et al., 2016).

In contrast to abiotic factors, the role of biotic interactions on cyanobacterial bloom dynamics has been less explored. Diverse biotic factors such as grazing, predation, parasitism and mutualism influence cyanobacterial biomass through interactions with other plankton components such as protozoans, zooplankton, bacteria, and viruses (Ger et al., 2016; Gerphagnon et al., 2015; Paerl and Otten, 2013; Steffen et al., 2017), interactions between cyanobacteria and heterotrophic bacteria can be positive due to the exchange of nutrients and oxygen, which can benefit both microorganisms (Bagatini et al., 2014; Gerphagnon et al., 2015). Interactions may also be negative, as for cyanolytic bacteria(Osman et al., 2017; Wichelen et al., 2016). During cyanobacterial blooms, bacteria can be found directly attached to cyanobacterial cells or adjacent to them, occupying the cyanobacterial phycosphere (Louati et al., 2015). In many cases, these associations were reported under laboratory conditions, but several recent studies in natural environments have provided evidence of the close relation between cyanobacterial biomass and associated heterotrophic

bacteria (Cai et al., 2013; Cheng et al., 2011; Dziallas and Grossart, 2011; Eiler and Bertilsson, 2004; Li et al., 2011, 2015; Louati et al., 2015; Parveen et al., 2013; Steffen et al., 2012; Wilhelm et al., 2011; Woodhouse et al., 2016; Wu et al., 2007). Moreover, specific associations between some cyanobacterial genera and heterotrophic bacteria have recently been reported (Bagatini et al., 2014; Louati et al., 2015), pointing to a possible connection of those bacteria attached to cyanobacteria and their participation in cyanobacterial bloom dynamics. Some authors extend this view and suggest that microbial communities of distinct taxonomic composition can play similar functional roles in bloom events (Steffen et al., 2012). Clearly, the complexity of these microbial interactions is still little explored, and their impact in the ecophysiology of cyanobacteria is underestimated.

High-throughput sequencing has revolutionized microbial ecology in recent years, particularly through the characterization of metagenomes. By following temporal variation in the composition of a community, it is possible to reveal correlations and to infer interactions among taxa (Stubbendieck et al., 2016). This application can be explored to better understand cyanobacterial species dynamics during a bloom, and the accompanying bacterial community can be seen as an integral and essential part of these events.

In this study, we present an integrative view of a mixed cyanobacterial bloom occurring during a warm, rainy period in a tropical hydropower reservoir; we used high-throughput sequencing to follow temporal shifts in the dominance of cyanobacterial genera and the associated heterotrophic bacterial community. We also looked for correlations between temporal shifts in the bacterial community and abiotic factors.

2. Materials and Methods

2.1 Sampling

Water samples were collected in the Funil Reservoir (22°30'S, 44°45'W), Rio de Janeiro, Brazil, from October 2013 to March 2014. This is a eutrophic reservoir with an area of 40 km^2, a volume of 8.9 x 10^6 m^3, maximum and medium depth of 70 and 22 m, respectively, and average residence time of 41.5 days (Rangel et al., 2012; Soares et al., 2009). The region in which the reservoir is located typically presents warm, rainy conditions during summer and cold, dry conditions during winter. The sampling period encompassed the end of spring and the summer.

Samples were collected from two locations: one in the central part of the reservoir (point 1) and the other near the dam (point 2). From one to three samples per month were obtained from the

integrated euphotic zone (determined as 2.7 times the Secchi disk depth; Cole, 1994) for a total of 22 samples. Water temperature and pH were measured using a Yellow Spring multiparametric probe (model 600 QS), and the water transparency was determined using a Secchi disk. Water temperature and pH values were measured at 0.5-m intervals to the end of the euphotic zone, and average values are presented. Volumes of 0.3 to 1 L of water (depending on the phytoplankton density) from the integrated euphotic zone were filtered (Whatman GF/F, 0.7 µm) to collect cells and then stored at -20 °C for DNA extraction.

2.2 Nutrient analysis

Aliquots of water were filtered (Whatman GF/F, 0.7 µm), and the filtrates were stored at

-20 °C in polypropylene bottles until the analysis. The soluble reactive phosphorus (SRP), ammonium, nitrate, nitrite and total phosphorus (TP) were measured using flow injection analysis (FIAlab 2500) according to the manufacturer's instructions (FIALab Instruments Inc., Seattle, Washington).

2.3 Quantitative analysis of phytoplankton

Aliquots of the integrated water samples were stored in amber glass vials with Lugol's solution. Phytoplankton abundance was determined by the Utermöhl method (Utermöhl, 1958) using an inverted optical microscope (Olympus BX-51). The biovolume (mm^3 L^{-1}) was estimated by multiplying the density of each species by the average volume of its cells (Hillebrand et al., 1999).

2.4 DNA extraction and *16SrDNA* amplification

DNA was extracted from cells collected in filters (Whatman GF/F, 0.7 µm) using the Power Soil DNA Isolation Kit (Mo Bio) according to the manufacturer's instructions. DNA samples were quantified using a fluorimeter (Qubit, Thermo Fisher Scientific). Amplification of the v3-v4 region of *16S rDNA* genes was performed with the primers S-D-Bact-0341-b-S-17F (5'-CCTACGGGNGGCWGCAG-3') and S-D-Bact-0785-a-A-21R (5'-GACTACHVGGGTATCTAATCC-3') (Klindworth et al., 2013) containing the appropriate adaptors for sequencing in the Illumina platform. Amplifications were performed in a 25 µl reaction mixture containing 12.5 µL of HiFi HotStart ReadyMix (KAPA Biosystems), 0.2 µM of each primer and 12.5 µg of DNA. The PCR program included an initial denaturation at 95 °C for 3

min followed by 25 cycles of amplification (95 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s) and a final step of 72 °C for 5 min. Products were purified using magnetic beads (Agencourt AMPure XP, Beckman Coulter) and subjected to a second PCR to incorporate dual indices, as described in the 16S Metagenomic Sequencing Library Preparation Protocol for the Illumina MiSeq System. The size and quality of DNA in the final libraries were verified on a Bioanalyzer 2100 (Agilent) using a Bioanalyzer DNA 1000 chip (Agilent Technologies). After quantitative PCR with a KAPA Library Quantification Kit for Illumina (KAPA Biosystems), samples were normalized and pooled for sequencing.

2.5 DNA sequencing and data analysis

Sequencing was performed in a MiSeq platform (Illumina) using the MiSeq Reagent Kit v3 (2 x 300 base pairs) according to the manufacturer's instructions. Files were recovered (.fastq), and paired-end reads were joined using mothur v.1.35.1 (Schloss et al., 2009). Sequences are available for download via the NCBI short read archive under BioProject PRJNA406945. The following criteria were used to eliminate low-quality reads: average quality (window size = 50) < 30, length 460 base pairs, presence of ambiguous characters ('N'), homopolymer < 8. The remaining reads were aligned using the SILVA database, trimmed and filtered. Then, sequences were preclustered with diff = 4. Chimeras were detected using UCHIME (Edgar et al., 2011) and excluded. Taxonomic classification was carried out using the RDP database (Release 11) with a confidence threshold of 80%. Sequences not assigned as Bacteria or classified as Chloroplast or Mitochondria were discharged. Singletons and doubletons were removed, and the number of sequences in the 22 samples was normalized to the same number of sequences. Sequences were then used as input to generate a distance matrix and clustered into operational taxonomic units (OTUs) at the sequence similarity cutoff of 97%. Species richness and Shannon diversity index were calculated in mothur. Taxonomic assignment of OTUs was performed using Greengenes (version 13_5).

The OTU relative abundances in the samples were used to generate an ordination plot by nMDS (non-metric multidimensional scaling) based on Bray-Curtis similarity coefficients. The limnological parameters and cyanobacterial cell counts with significant differences between the periods (t-test, $p < 0.001$) were plotted together with the nMDS. Statistical significance of sample grouping was tested with PERMANOVA. These analyses were performed on package Past3 (Hammer et al., 2001). To identify the major OTU contributors to grouping differentiation (periods), we used a similarity percentage analysis (SIMPER) (Clarke, 1993). Pearson correlation was used to test the degree of association among limnological variables with cyanobacterial cell counts and cyanobacterial OTUs and to test the association between specific cyanobacterial OTUs and heterotrophic bacterial OTUs (considering only those that contributed at least 0.2% to the total of sequences). We considered significant correlations those with $p < 0.001$

3. Results

3.1 Limnological characterization

During the study period, Funil Reservoir was characterized by turbid, dynamic and slightly eutrophic conditions (Table 1). Water transparency varied between 0.25 and 2 m, with lower values between October and early January. Water retention time ranged between 23.7 and 38 days, with lower values beginning in mid-January, coinciding with the increase in precipitation. The pH was generally alkaline, ranging between 7.1 and 10.9, with highest values on January and February. Nitrogen concentrations were always above the potentially limiting concentration for phytoplankton growth (>100 μg L^{-1}), while SRP concentrations were below the detection limit (<2 μg L^{-1}) in almost all samples, indicating strong phosphorus limitation during most of the studied period (Table 1).

3.2 Cyanobacterial dynamics

Microscopic analysis revealed that cyanobacteria were the dominant (96.2 to 99.8%) component of phytoplankton all over the sampling period; 23 cyanobacterial species were identified (Supplementary Table 1). The most abundant species were *Microcystis* spp. (referred herein as *Microcystis aeruginosa* complex, MAC)*, Dolichospermum circinale, Cylindrospermopsis raciborskii, Pseudanabaena mucicola, Synechococcus nidulans* and *Synechocystis aquatilis* (Figure 1). An MAC bloom was apparent from October to mid-January with the co-occurrence of *D. circinale* and *P. mucicola*. Beginning in mid-January, *C. raciborskii* dominated the phytoplankton community together with *D. circinale*, *S. aquatilis*, MAC and other cyanobacteria. Although the cyanobacterial biomass differed between the two sampling points during the period, the relative contribution of the cyanobacterial species was similar in the two locations (Figure 1).

The cyanobacterial community composition assessed by *16S rDNA* sequencing revealed a somewhat different pattern for the mixed bloom. The main cyanobacteria genera identified were *Microcystis, Dolichospermum, Cylindrospermopsis, Pseudanabaena* and *Synechococcus* (Figure 2). *Microcystis* dominated from October to end of January, as also observed with microscopy, but the dominance of *C. raciborskii* was not as evident. Instead, a *Synechococcus* predominance followed the decay of the *Microcystis* bloom. This pattern was evident in the two sampling points despite some differences in specific dates.

3.3 Microbial community structure

The metagenomic sequencing of the bacterioplankton community resulted in a total of 2,547,075 sequences (101,883 per sample) that clustered into 6,452 OTUs (3% dissimilarity). Rarefaction curves showed an excellent coverage (Figure S1) for all samples. The bacterial richness varied from 613 to 2038 OTUs, and Shannon indices varied from 3.2 to 5.0 (Table 1).

Analysis of the whole microbial community showed that Cyanobacteria and Proteobacteria were the dominant phyla followed by Actinobacteria, Bacteroidetes, Verrucomicrobia, and Planctomycetes (Figure 3). During the *Microcystis* (MAC) bloom, a positive correlation was observed between Chroococcales abundance and Cytophagales ($r = 0.38$, $p = 0.01$). On the other hand, the abundance of several orders was negatively correlated with Chroococcales: Rhizobiales ($r = -0.49$, $p = 0.01$), Planctomycetales ($r = -0.6$, $p = 0.003$), Acidimicrobiales ($r = -0.61$, $p = 0.002$), Verrucomicrobiales ($r = -0.5$ $p = 0.01$) and an unclassified order of Chloroflexi ($r = -0.45$, $p = 0.02$). During the dominance of Synechococcales, a positive correlation was observed with Planctomycetales ($r = 0.44$, $p = 0.04$), and negative correlations were observed with Proteobacteria ($r = -0.43$, $p = 0.04$) and Cytophagales ($r = -0.55$, $p = 0.007$) (Figure 3). The relationship between all samples was assessed using an NMDS based on the bacterial community distribution at OTUs level (3% similarity). The ordination showed clustering of the samples according to the two periods. The difference between these periods was supported by PERMANOVA analysis ($F = 3.19$, $p= 0.0001$). Period 1 corresponded to samples from October to mid-January (*Microcystis* dominance), and period 2 from mid-January to March (*Synechococcus* dominance) (Figure 4). For spatial variability, there was no significant difference between the sampling points ($F = 1.13$, $P = 0.29$).

The distinction between these two periods was also supported by some limnological parameters and cyanobacterial microscopic counts. Water retention time and transparency showed significant differences between periods one and two ($t(20) = 2.8$, $p<0.01$ and $t(20) = -4.9$, $p<0.01$ respectively). *Microcystis* spp. and *C. raciborskii* cell counts were significantly different between periods ($t(16) = 3.2$, $p<0.01$ and $t(16) = -3.29$, $p<0.01$, respectively) (Figure 4).

Regarding the main contributors to the distinction between the two periods, SIMPER analysis revealed that OTUs assigned as *Synechococcus* and *Microcystis* accounted for 12.75% and 10.13% of the variability, respectively (Table 2). Considering other OTUs with over 1% relative abundance, 11 orders also contributed to the variability of the two periods, although their combined impact was less than 2% (Figure 5).

3.4 Associations of biotic and abiotic factors during the bloom

The data pointed to a clear distinction of a *Microcystis* dominated bloom followed by a *Synechococcus* dominated period. The *16S rDNA* sequencing and microscopy data revealed the correlations among the cyanobacterial taxa. *Microcystis* was positively correlated with *Dolichospermum* (both for microscopy counts and *16SrDNA* data). *C. raciborskii* microscopy counts were positively correlated with *Synechococcus* (*16SrDNA*), while *Pseudanabaena*, which was a contributor to the transition between periods 1 and 2, was negatively correlated with *Microcystis* and positively with *C. raciborskii* (Table 2).

Considering abiotic factors, *Microcystis* microscopic counts (MO) were positively correlated with water retention time and were negatively correlated with water transparency; *C. raciborskii* (MO) was negatively correlated with water transparency; *Dolichospermum* (MO) was negatively correlated with DIN and water transparency; *Synechococcus* (OTU) was positively correlated with temperature and retention time and was negatively correlated with water transparency (Table 2).

In Period 1, significant correlations were demonstrated between *Microcystis* (OTU) and other bacterial taxa, including four positive correlations (Cytophagaceae, Chitinophagaceae, CL500-15, and Nitrospira) and one negative correlation (Burkholderiales) (Table 3). The *Dolichospermum* OTU, which co-occurred with *Microcystis,* showed positive correlations with Sinobacteraceae, Bacillales, Chitinophagaceae, Novosphingobium, and Comamonadaceae (Table 3).

In Period 2 dominated by *Synechococcus*, correlations were demonstrated between this cyanobacterial OTU and Cytophagales, Planctomyces and Chloroflexi. (Table 3). In this same period, the *C. raciborskii* OTU was positively correlated with *Roseococcus*, Pirellulaceae, *Mycobacterium*, *Vibrio*, *Hydrogenophaga*, *Planctomyces*, Oxalobacteraceae and Alphaproteobacteria (Table 3).

4. Discussion

In this study, we investigated the bacterial community coupled with a mixed cyanobacterial bloom (initially dominated by *Microcystis* and *Dolichospermum*, followed by *C. raciborskii* and *Synechococcus*) occurring in a tropical reservoir during late spring-summer, combining microscopy with metagenomics. Temporal shifts in the dominance of bloom-forming cyanobacterial genera were associated with physical features of the water and with shifts in the associated heterotrophic bacteria. Our results indicate specific interactions of the main cyanobacterial genera with some

components of the heterotrophic bacterial community, yielding insights about biotic associations during a cyanobacterial bloom in a tropical system.

4.1 Cyanobacterial dynamics

For the last several decades the Funil Reservoir has experienced cyanobacterial blooms, mainly during the summer, dominated by *Microcystis*, *Dolichospermum* and *Cylindrospermopsis* (Guedes et al., 2014; Rangel et al., 2016; Soares et al., 2013), which are also the major bloom-forming genera globally (Antunes et al., 2015; Harke et al., 2016; O'Neil et al., 2012). The succession of a bloom dominated by *Microcystis* and *Dolichospermum* by the dominance of *C. raciborskii* during the summer has been reported for several years in this system, and it has been more tightly associated with temperature and physical variables than with nutrient availability (Soares et al., 2012). Thus, the stratification of the water column and the reservoir residence time are important abiotic factors associated with the bloom dynamics (Rangel et al., 2016; Soares et al., 2009). In the present study, the *Microcystis* bloom was associated with high retention time and low water transparency. Similarly, previous studies in this reservoir have associated the dominance of *Microcystis* with a more stable and prolonged period of thermal stratification (Rangel et al., 2016; Soares et al., 2009, 2012). *M. aeruginosa* is considered a species adapted to high light intensities (Robarts and Zohary, 1987); it can thus form blooms on the surface. As a consequence, *Microcystis* can outcompete other species by reducing available light for nonbuoyant phytoplankton competitors (Harke et al., 2016). Indeed, in the initial period of the bloom, *Microcystis* accounted for 89% of the total cyanobacterial density. In addition, this species is sensitive to mixing and turbulence (Reynolds, 2006), which can explain the decline of its abundance in the middle of January (Period 1), when precipitation increased and retention time decreased.

After the decay of the *Microcystis* bloom, *C. raciborskii* and *Synechococcus* were the dominant cyanobacteria. Opposite to the former species, the latter two species were positively correlated with lower retention time and higher water transparency. Previous investigations in this reservoir have associated *C. raciborskii* blooms with periods of water column mixing (Soares et al., 2009, 2012). This species is considered to tolerate both stratified and mixed conditions and can dominate in mixed periods (Berger et al., 2006; Bonilla et al., 2012; Soares et al., 2013). This is likely due to the ability of cells to photoadapt to dark and fluctuating light conditions (O'Brien et al., 2009).

The period of *C. raciborskii* dominance was coupled with the high relative abundance of *Synechococcus* (shown by *16S rDNA* sequences). This has never been reported for this reservoir, since previous studies characterized the phytoplankton community by microscopy only. Picoplanktonic cells (size 0.2-2 μm) seem to be generally subquantified by the Utermöhl method,

while other techniques such as fluorescence microcopy and flow cell cytometry are more efficient for their quantification (Callieri, 2007). The high relative abundance of *Synechococcus* in conditions of higher water transparency and low retention time is in accordance with the high light requirement reported for this genus (Reynolds et al., 2002), which together with its high growth rate can explain its rise just after the senescence of the *Microcystis* bloom.

4.2 Microbial community dynamics

Changes in the composition of the bacterioplankton community can be linked to phytoplankton blooms (Li et al., 2015; Woodhouse et al., 2016; Xing et al., 2007) , and recent studies noted that the bacterial community associated with cyanobacteria can be genera specific, since different DOM qualities and quantities can be produced by different cyanobacteria (Bagatini et al., 2014; Louati et al., 2015) . In the present study, we found a clear distinction between the heterotrophic bacteria associated with the *Microcystis* bloom and those present during the *Synechococcus*/*C. raciborskii* dominance period. However, we did not observe a correlation between cyanobacterial abundance and diversity of the microbial community. A decrease in microbial diversity during a cyanobacterial bloom has been described before, especially for *Microcystis* blooms (Cheng et al., 2011; Li et al., 2015; Louati et al., 2015; Niu et al., 2011) . This can be related to direct competition for resources or to inhibitory activities of cyanobacteria affecting heterotrophic bacteria (Ostensvik et al., 1998). However, other studies suggested that bacterial communities accompanying cyanobacterial blooms can be as diverse as non-bloom communities (Eiler and Bertilsson, 2004; Tromas et al., 2016; Woodhouse et al., 2016).

In this study, the major observed bacteria phyla were Cyanobacteria and Proteobacteria followed by Actinobacteria, Bacteroidetes, Verrucomicrobia, and Planctomycetes (Figure 4). Actinobacteria is one of the most frequent phyla in freshwater environments and is reported as highly abundant in different lakes from oligotrophic to eutrophic systems (Newton et al., 2011). This phylum has not been found in physical association with Cyanobacteria but can be a major part of the bacterioplankton community during phytoplankton blooms (Newton et al., 2011; Steffen et al., 2017). On the other hand, Bacteroidetes, Verrucomicrobia and Planctomycetes frequently reach high relative abundance during cyanobacterial blooms (Li et al., 2015; Parulekar et al., 2017; Steffen et al., 2017; Woodhouse et al., 2016). In this study, Verrucomicrobia ranged from 1 to 18% of relative abundance. Bacteria from this phylum are described as able to degrade algal polysaccharides and organic matter, and so can be favored by high cyanobacterial abundances (Bagatini et al., 2014; Parulekar et al., 2017; Woodhouse et al., 2016) . The relative abundance of Bacteroidetes also increased during the bloom, and other studies have shown that some taxa in this phylum, such as *Sphingobacteria* and *Flavobacteria*, are often found in high abundance after phytoplankton bloom decay either adjacent or attached to phytoplankton. In the present study, the most abundant

Bacteroidetes taxa were Cytophagales and Saprospirales, and higher abundances were associated with the *Microcystis* bloom. Planctomycetes can be abundant in nutrient-enriched waters (Woebken et al., 2007), which frequently have a high abundance of phytoplankton. Other studies also reported higher densities of Planctomycetes after diatom (Morris et al., 2006) or cyanobacterial blooms (Eiler and Bertilsson, 2004), suggesting a possible association of this phylum with phytoplankton. For example, it has already been observed that Planctomycetes prefer to remain attached rather than be free-living (Allgaier and Grossart, 2006). The functional roles of these associations between cyanobacteria and heterotrophic bacterial taxa and are still unclear but likely will be revealed from studies exploring the microbial community functional response during a bloom and the links to environmental conditions (Steffen et al., 2012, 2017).

Previous studies that evaluated an entire year in this reservoir defined the cyanobacteria bloom period as October to March (Guedes et al., 2014; Rangel et al., 2016; Soares et al., 2009) . Thus, in the period evaluated here, cyanobacterial cell density was always high, so we could not distinguish between situations with and without a bloom. The temporal analysis of the variation in the composition of the bacterial community was related to the abundance of the main cyanobacterial genera that dominated the phytoplankton community. In this late spring-summer bloom, two periods could be distinguished; the first period was characterized by higher retention time, low transparency and dominance of *Microcystis*. This period was also characterized by the high abundance of Bacteroidetes and positive correlations were observed among Bacteroidetes OTUs (specifically unclassified OTUs of Cytophagaceae and Chitinophagaceae) and the most abundant *Microcystis* OTU. Particularly, a strong positive correlation ($r = 0.72$) was found between the *Microcystis* OTU and an unclassified OTU of Cytophagales. Some bacteria of the genus *Cytophaga* are described as predatory agents in freshwater and marine systems (Daft and Stewart, 1971; Imai et al., 1993; Kirchman, 2002; Rashidan and Bird, 2001). In a previous study, Daft and Stewart (1971) first described a *Cytophaga* strain whose dynamics closely followed *Microcystis* dynamics; the species was able to terminate a bloom. Rashidan and Bird (2001) found a close relationship between the abundance of *Anabaena* sp. and a lytic strain of *Cytophaga*; they suggested that one reason for the decay of the *Anabaena* bloom was the lysis induced by this *Cytophaga* strain. In the present study, the positive correlation between a Cytophagaceae OTU and the most abundant *Microcystis* OTU does not directly point to predation. We can speculate that the lytic capacity of these bacteria affects other cyanobacteria but not *Microcystis*, that the lytic capacity differentially affects various strains of *Microcystis*, or that such predatory activity is not sufficient to decrease this population of cyanobacteria. Moreover, in a broad study that followed the microbial community of Lake Champlain by for 8 years using *16S rDNA* sequencing, Tromas et al. (2016) defined *Cytophaga* as a bloom biomarker. Altogether, the strong correlation found between *Cytophaga* and *Microcystis* in the present study indicates that *Cytophaga* can be an important biotic factor contributing to the prevalence of the *Microcystis* bloom in the Funil Reservoir.

The second period of the summer bloom was characterized by the dominance of *Synechococcus* (*16S rDNA*) and *C. raciborskii* (MO), which coincided with lower retention time and less turbidity. In this period, Planctomycetes increased in relative abundance, and four Planctomycetes OTUs (three from the genus *Planctomyces*) ware positively correlated with *Synechococcus* and *C. raciborskii*. Specifically, we observed a strong correlation between *Planctomyces* and *Synechococcus* (r = 0.72). Few studies have investigated the possible association between strains of Planctomycetes and Cyanobacteria. Cai et al. (2013) observed an increased abundance of Planctomycetes associated with *Microcystis* colonies and pointed to a possible role of Planctomycetes in the degradation of sulfated polysaccharides produced by cyanobacteria. In a field study, Ruber et al. (2017) observed high Planctomycetes relative abundance (up to 25%) in a lake dominated by *Synechococcus*, and Woodhouse et al., (2016) also found associations between *Planctomyces* and Cyanobacteria.

In contrast to what has been reported for *Microcystis*, little is known about bacterial groups directly associated with *C. raciborskii*. Limei et al. (2009) reported four bacterial strains associated with a cultivated *C. raciborskii* strain, along with three Burkholderiaceae (Betaproteobacteria) and one Sphingobacteriales (Bacteroidetes). Interestingly, in our study a strong positive correlation (r = 0.73) was observed between *C. raciborskii* and *Hydrogenophaga*, a Burkholderiaceae genus, as also described by Limei et al. (2009)

While other studies have analyzed the bacterial community associated with blooms of cyanobacteria by considering factors associated with the rise and decay of blooms, some investigations, including the present study, focused on variations that occur during a bloom period; this allowed us to detail not only the influence of cyanobacteria biomass but also the possible favoring of different bacterial communities by different cyanobacterial species (or *vice versa*). This approach is a first step to understanding the mechanisms associated with the shift of the dominance of cyanobacteria genera, which until now have been based almost solely on interactions that involve cyanobacteria alone such as direct competition or allelopathy. Future studies under laboratory conditions can simulate the interactions among the cyanobacterial and bacterial genera described here, evaluating possible synergistic or antagonist relationships, with the potential to develop biocontrol tools for cyanobacterial blooms.

Conclusion

Our study has provided insights on the bacterial communities associated with bloom-forming cyanobacteria in tropical systems. Temporal shifts in the dominance of cyanobacteria genera were associated not only with physical features of the water (retention time and transparency) but also with shifts in the associated heterotrophic bacteria. Our results suggest specific interactions of the

main cyanobacterial genera with certain groups of the heterotrophic bacterial community, but further studies exploring the microbial community functional and environmental conditions are needed to better understand the ecophysiological role of these associations.

Author Contributions

IG conceived the study design, conducted the sampling, performed sequencing and data analysis and wrote the paper. CR conducted the bioinformatic analysis. LR conducted sampling, phytoplankton quantification and analyzed data. LH analyzed data and edited the manuscript. PB provided counseling in sequencing and edited the manuscript. SA conceived the study design, analyzed data and edited the manuscript. AP conceived the study design, performed sequencing and data analysis and wrote the paper. All authors approved the final submitted manuscript.

Conflict of Interest Statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be considered a potential conflict of interest.

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Table 1 – Limnological variables and abiotic parameters associated with the collected samples. 1 and 2 corresponds to sampling points 1 (central part of the reservoir) and 2 (dam). RT, retention time (days); Secchi, water transparency (m); Temp, water temperature (°C); DIN, dissolved inorganic nitrogen (µg L^{-1}); SRP, soluble reactive phosphorous (µg L^{-1}); TP, total phosphorous (µg L^{-1}); Cyano, cyanobacterial biomass (mg L^{-1}), H'- Shannon index, S -Bacterial Richness

	Oct-30		**Nov-27**		**Dec-9**		**Dec-23**		**Jan-9**		**Jan-23**		**Jan-30**		**Feb-20**		**Feb-26**		**Mar-12**		**Mar-26**	
Points	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
RT	35	35	28	28	33	33	38	38	36	36	31	31	28	28	23	23	24	24	29	29	36	36
Secchi	0.9	0.7	0.7	0.7	0.25	0.8	0.35	0.9	0.4	0.7	0.7	0.8	0.8	0.9	1.1	1.4	1.3	1.6	1.0	1.2	1.4	2.0
Temp	24.5	24.3	24.9	24.9	27.9	27.4	26.9	26.6	29.1	28.8	28.9	28.4	28.7	29.2	28.0	27.9	28.7	28.6	28.5	27.7	27.5	28.9
pH	7.1	8.7	7.5	8.2	8.3	9.4	9.2	8.8	10.5	10.1	10.4	10.4	10.1	10.3	7.9	8.0	9.9	10.9	7.3	8.7	7.2	6.6
DIN	1079	1062	858	695	1038	265	402	437	435	453	307	435	665	597	700	829	171	505	507	485	437	577
SRP	28.7	<2.0	<2.0	<2.0	27.0	<2.0	<2.0	<2.0	12.0	<2.0	<2.0	<2.0	<2.0	<2.0	31.3	<2.0	<2.0	<2.0	<2.0	18.3	33.4	<2.0
TP	44.8	19.0	10.7	8.5	40.5	40.9	25.2	6.3	36.3	10.5	7.4	9.4	6.1	6.8	42.8	16.6	25.6	15.0	3.5	45.9	56.3	26.0
Cyano	1.0	2.0	3.0	3.0	5.0	7.0	6.0	5.0	8.0	4.0	8.0	4.0	3.0	2.0	2.0	1.0	3.0	1.0	1.0	2.0	1.0	2.0
H'	4.5	4.0	4.7	4.5	3.6	5.0	5.0	3.6	4.6	4.3	4.2	4.8	3.3	3.6	4.5	4.0	4.0	4.3	4.6	4.7	3.2	4.9
S	1718	997	1488	1347	613	973	801	702	1222	998	662	1170	1074	1706	2038	1781	1037	960	1241	1314	1835	1664

Table 2 - Values of Pearson correlations (r) among cyanobacterial taxa considering both 16S rDNA sequencing (OTU) and microscopy data and limnological parameters (*****p<0.05, ****** p<0.001). RT, retention time (days); Secchi, water transparency (m); Temp, water temperature (oC); DIN, dissolved inorganic nitrogen (ug L-1); SYN, *Synechococcus;* MIC, *Microcystis;* DLC, *Dolichospermum;* PSD, *Pseudoanabaena;* CR, *C. raciborskii*. *Microcystis* spp., *C. raciborskii, D. circinale*, microscopy count of these species

	Temp	RT	DIN	Secchi	*Microcystis spp*	*C.raciborskii*	*D. circinalis*	SYN OTU	MIC OTU	DLC OTU	PSD OTU
RT	-0.33										
DIN	-0.56	-0.04									
Secchi	0.16	-0.31	-0.17								
Microcystis spp	-0.03	0.65**	-0.17	0.70**							
C. raciborskii	0.26	-0.41	-0.11	0.50*	-0.36						
D. circinale	0.19	0.42	-0.56*	-0.47*	0.63**	-0.26					
SYN OTU	0.50*	0.72**	-0.22	0.52**	-0.54	0.63**	-0.25				
MIC OTU	-0.29	0.40	0.01	-0.49*	0.20	-0.33	-0.05	-0.39			
DLC OTU	0.16	0.38	-0.40	-0.34	0.62**	-0.03	0.76**	-0.04	0.03		
PSD OTU	0.25	-0.44*	0.01	0.45*	-0.51*	0.45*	-0.31	0.41	-0.58**	-0.31	
CR OTU	0.12	-0.10	-0.14	-0.01	-0.02	-0.31	0.32	0.12	-0.03	0.23	-0.43

Table 3 – Significant (p<0.001) Pearson correlations (r>0.5) among the main cyanobacterial OTUs and heterotrophic bacteria. Taxonomic assignment according to Greengenes databases. (p) Phyla; (c) Class; (o) Order; (f) Family; (g) Genus.

	OTUs	*r*
Microcystis	Cytophagaceae	0.72
	Pirellulaceae (f)	-0.55
	Betaproteobacteria (c)	-0.54
	CL500-15 (o)	0.54
	Pseudanabaena (g)	-0.58
	Aquirestis (g)	-0.53
	Chloroflexi (p)	-0.56
	Chitinophagaceae (f)	0.60
	Betaproteobacteria (c)	-0.63
	Limnohabitans (g)	-0.62
	Flavobacterium (g)	-0.59
	Burkholderiales (o)	-0.62
	Hyphomicrobium (g)	-0.59
	Actinomycetales (o)	-0.57
	Pirellulaceae (f)	-0.56
	Solirubrobacterales (o)	-0.59
	Nitrospira (g)	0.58
Synechococcus	Cytophagales (f)	-0.51
	Pirellulaceae (f)	-0.50
	Methylosinus (g)	-0.53
	Planctomyces (g)	0.72
	Chloroflexi (p)	0.72
	Prosthecobacter (g)	0.75

	Planctomyces (g)	0.67
	Limnohabitans (g)	0.51
	Comamonadaceae (f)	0.71
	Enterobacteriaceae (f)	0.58
	Solirubrobacterales (o)	0.73
Dolichospermum	Sinobacteraceae (f)	0.55
	Bacillales (o)	0.63
	Chitinophagaceae (f)	0.68
	Proteobacteria (p)	0.74
	Opitutus (g)	0.68
	Proteobacteria (p)	0.67
	Inhella (g)	0.70
	ACK-M1 (f)	0.53
	Cyclobacteriaceae (f)	0.61
	Chitinophagaceae (f)	0.64
Cylindrospermopsis	*Roseococcus (g)*	0.77
	OD1 (p)	0.62
	Mycobacterium (g)	0.54
	Vibrio (g)	0.55
	Pirellulaceae (f)	0.71
	Hydrogenophaga (g)	0.73
	Planctomyces (g)	0.54
	Oxalobacteraceae (f)	0.55
	Alphaproteobacteria (c)	0.66
	Armatimonadaceae (f)	0.58

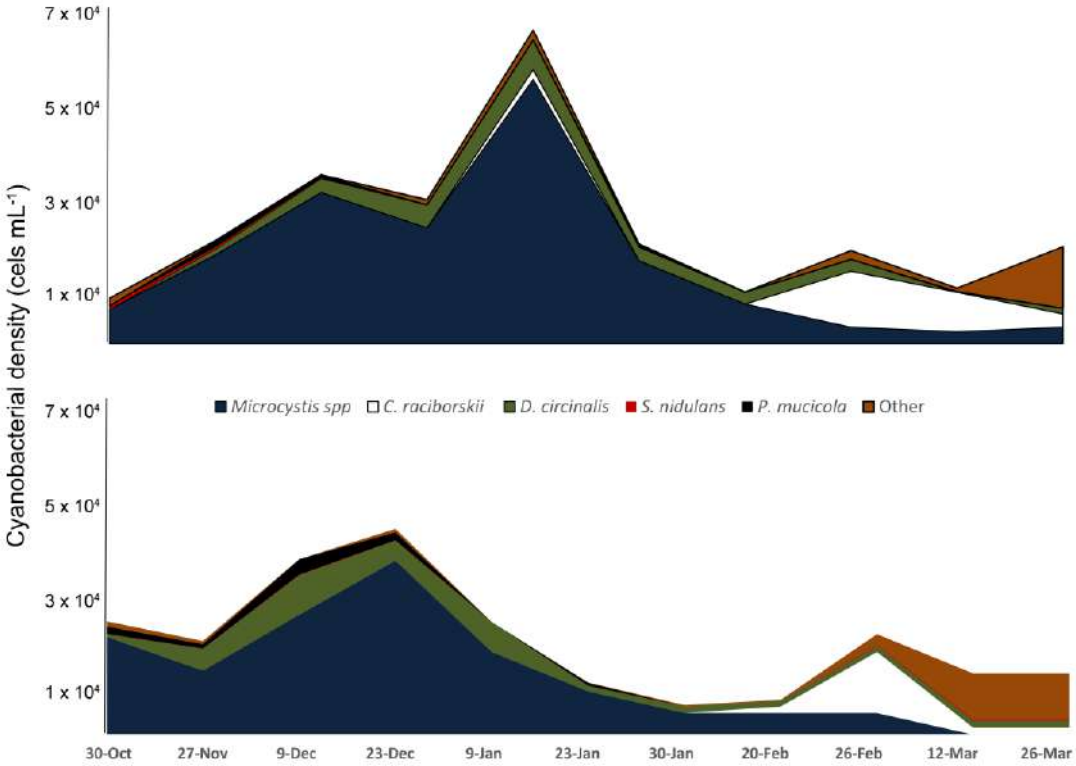


Figure 1 – Variation of cyanobacteria density from October 2103 to March 2014 in two locations of Funil reservoir (A) point 1, central part, (B) point 2, near the dam.

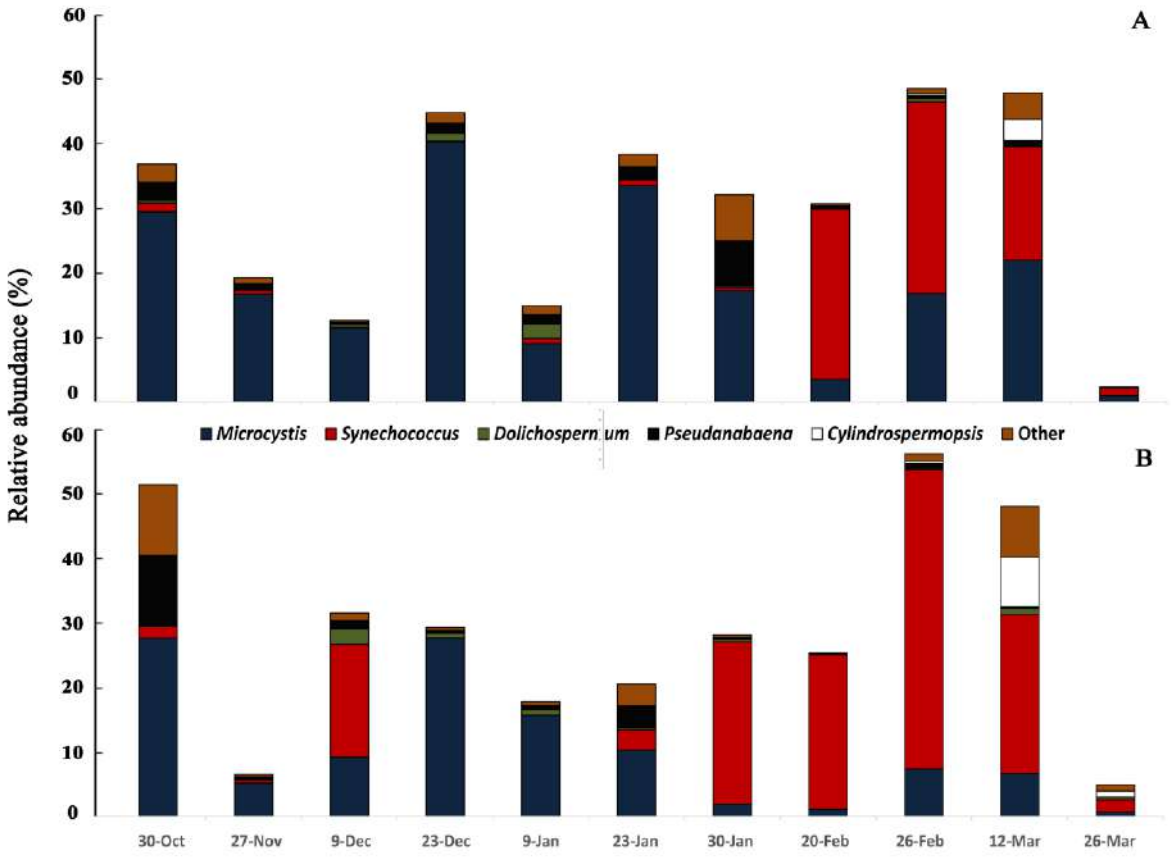


Figure 2 - Variation of cyanobacterial community accessed by 16S rDNA sequencing from October 2103 to March 2014 in two sampling points of Funil reservoir (A) point 1, central part, (B) point 2, near the dam.

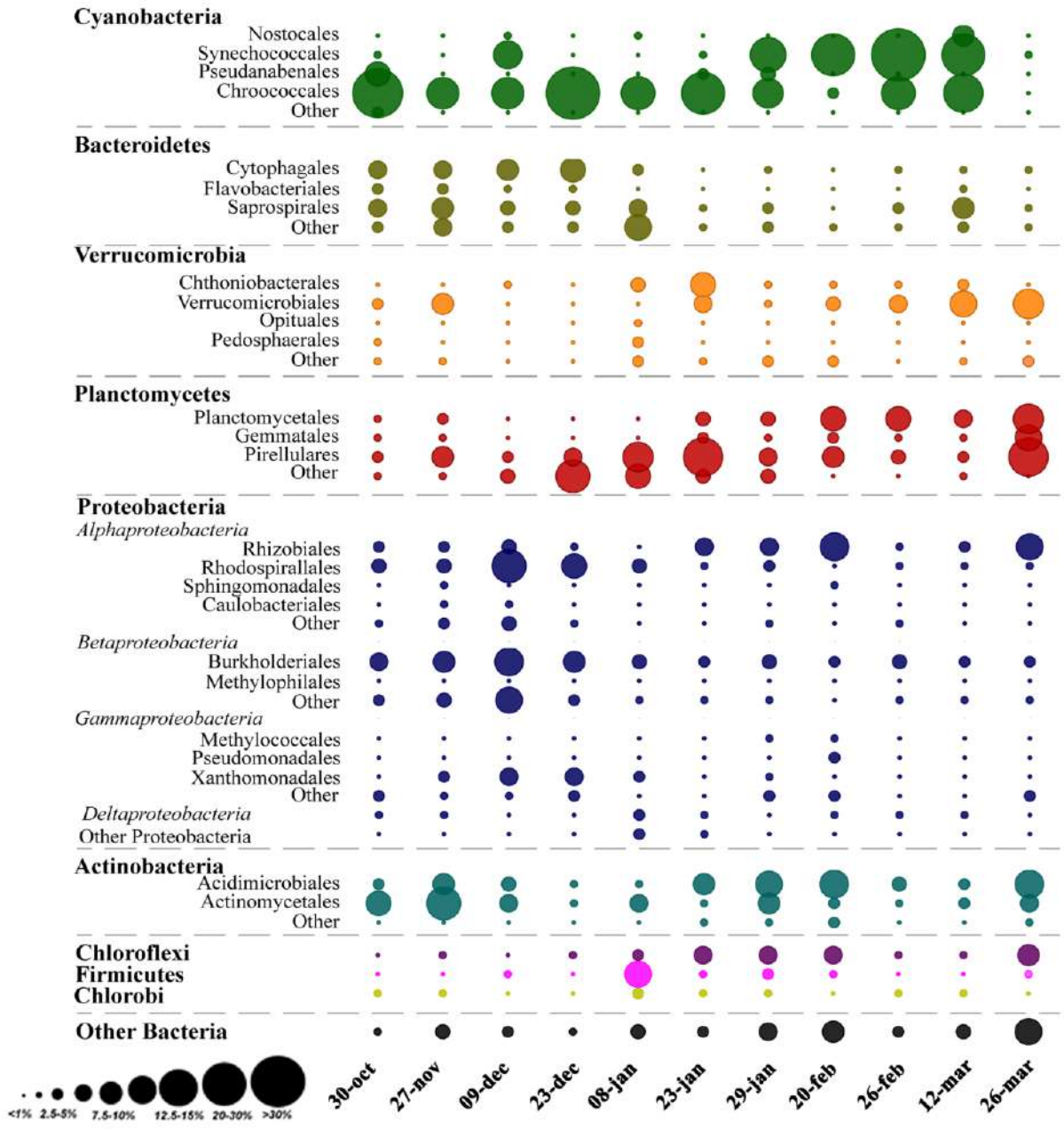


Figure 3 – Relative abundance of OTUs classified as Order and Phyla across the sampling period. The area of the bubbles represents the relative abundance of OTUs (average values of the two sampling points). The color of the bubbles indicates the Phylum to which the OTUs were assigned.

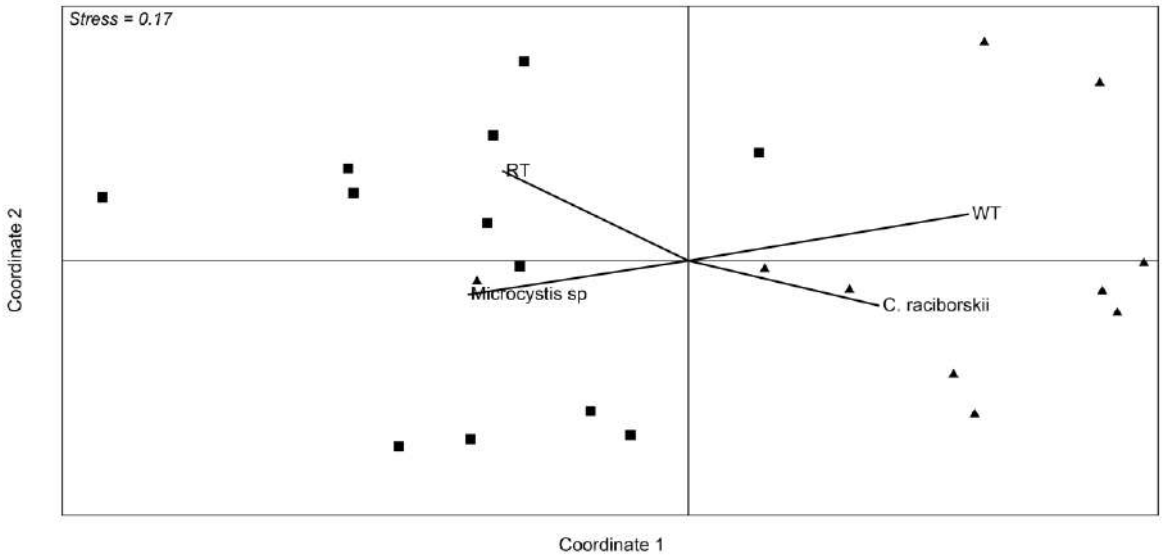


Figure 4 – Non-metric multidimensional scaling ordination based on Bray-Curtis similarity ofdata from OTU abundance in the samples from the two sampling points. Squares correspond to samples from October 2013 to mid January 2014, and triangles correspond to samples from the end of January 2014 to March. Vectors are environmental variables and cyanobacterial microscopic counts that were significantly different between the two defined periods ($p<0.01$).

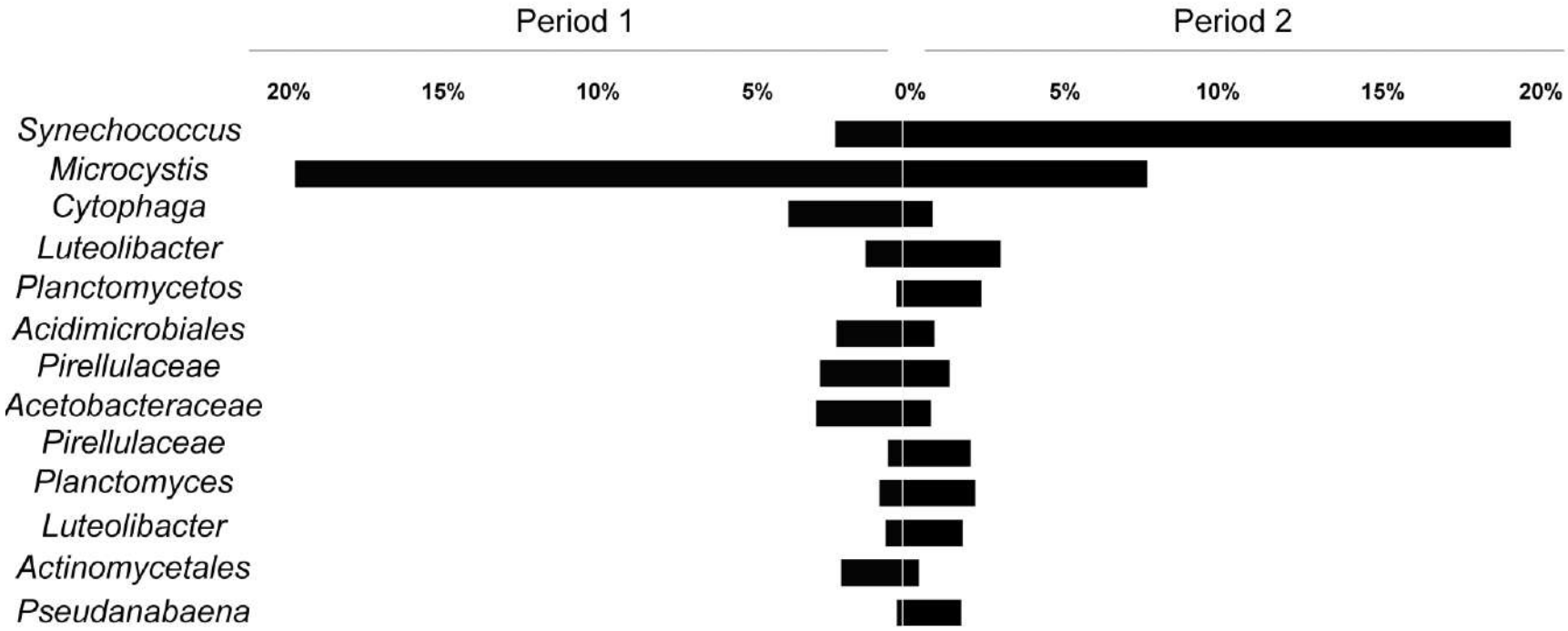
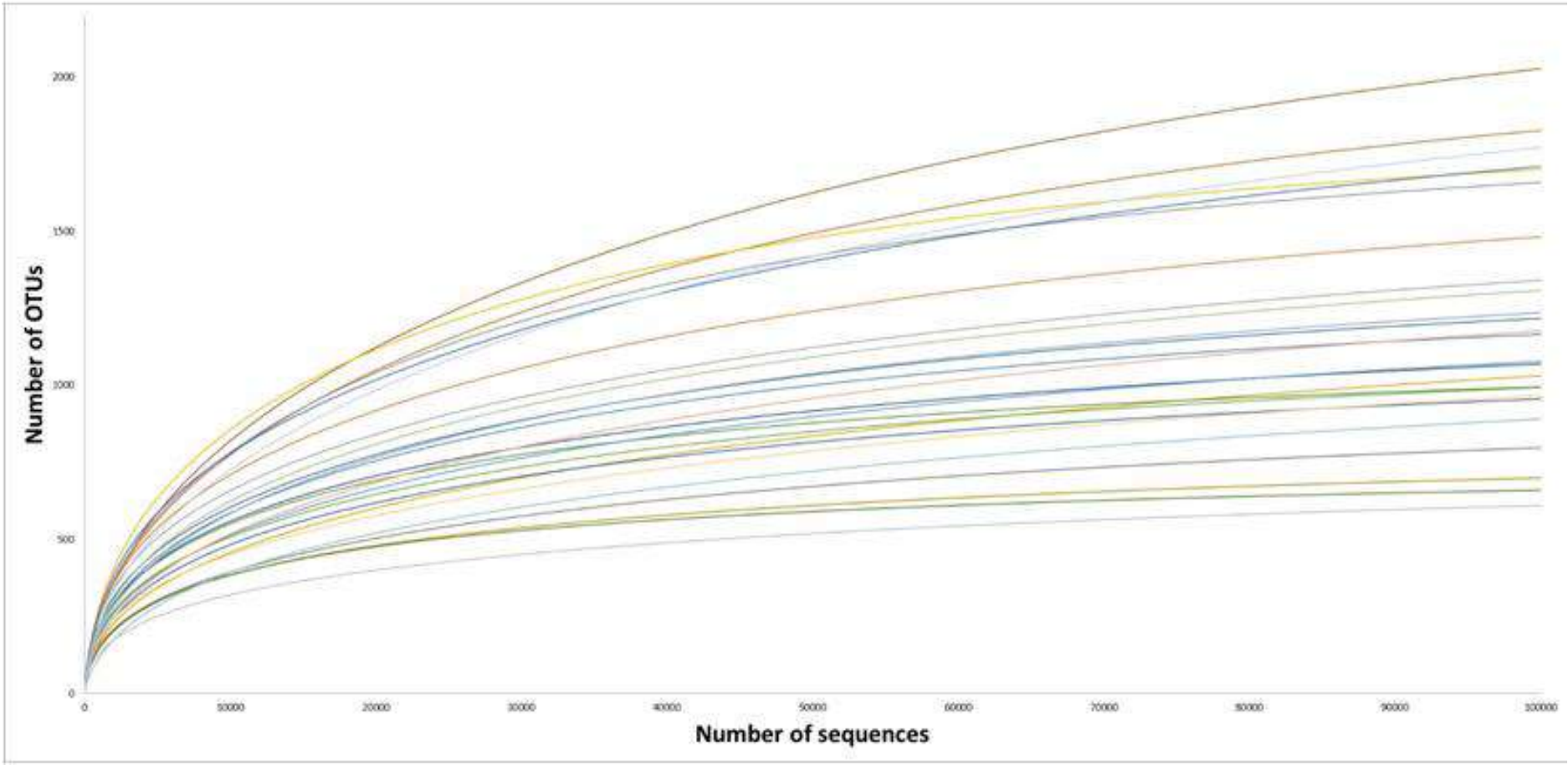


Figure 5 – Average relative contribution of OTUs in the two defined periods (Period 1 from October to mid January and Period 2 from mid January to march). The selected OTUs contributed to at least 2% for the differentiation between the periods (SIMPER analysis)



Supplementary Figure 1 - Rarefaction curves of 16S rDNA sequences from all samples.

Supplementary Table 1- Cyanobacterial species identified by microscopic analysis

Aphanocapsa delicatissima
Aphanocapsa elachista
Aphanocapsa incerta
Aphanothece sp.
cf Coelomorum tropicale
cf Coelosphaerium evidenter-marginatum
Cyanodiction imperfectum
Cyanogranis ferruginea
Cylindrospermopsis raciborskii
Dolichospermum circinale
Merismopedia tenuissima
Microcystis aeruginosa
Microcystis panniformis
Sphaerocavum brasiliense
Planktolyngbya limnetica
Planktothrix isothrix
Pseudanabaena mucicola
Radiocystis fernandoi
Snowella lacustris
Sphaerocavum brasiliense
Synechococcus nidulans
Synechocystis aquatilis

Capítulo 2

Population structure of harmful cyanobacteria in a tropical reservoir

Short Communication a ser submetida ao periódico *Microbiology*

Population structure of harmful cyanobacteria in a tropical reservoir

Abstract

In this study, we addressed the cyanobacterial diversity, using the 16S rRNA gene marker, during a bloom period in a tropical reservoir focusing in the population structure of each cyanobacterial genus and its relationship with microcystin (MC) concentrations. *Microcystis* bloom occurred from October to mid-January, with *Dolichospermum* and *Pseudanabaena* also present as minor contributors. From mid-January on, *Synechococcus* dominated, together with *Cylindrospermopsis*, *Dolichospermum* and *Microcystis.* From NGS data, an relevant intraspecific variability in different cyanobacterial groups was observed suggesting that populations are structured in different ways. While *Microcystis*, *Pseudanabaena* and *Cylindrospermopsis* were characterized by the dominance of one genotype, *Synechococcus* and *Dolichospermum* presented a higher number of sporadically genotypes. MC and potentially MC producing cells were detected in all samples but no significant correlation between MC and genotypes were found. The investigation of population dynamics can contribute to understand how blooms persist and can reveal different strategies related to the succession of the species involved.

Keywords: *Microcystis; Synechococcus; Pseudanabaena; Dolichospermum; Cylindrospermopsis; 16S rRNA*

Short Communication

Harmful algae blooms are recognized a serious issue in freshwater ecosystems worldwide, since they and can alter the aquatic food web and be toxic to wildlife, domestic animals and humans (O'Neil *et al.*, 2012; Paerl & Otten, 2013; Srivastava *et al.*, 2013). The main bloom forming cyanobacterial genera are *Microcystis*, *Dolichospermum*, *Plankthotrix* and *Cylindrospermopsis* and they can occur in mixed blooms in which other genera can participate with minor contribution such as *Pseudoanabaena* and *Synechococcus* (Acinas *et al.*, 2009; Antunes *et al.*, 2015; Bertos-fortis *et al.*, 2016; Harke *et al.*, 2016; Soares *et al.*, 2013). Among several bioactive secondary metabolites produced in these blooms, microcystins are the most commonly reported cyanotoxins and can be produced by strains of diverse genera, such as *Microcystis Dolichospermum,* and *Planktothrix*, among others (Dittmann *et al.*, 2013).

In the last decade several studies were reported trying to understand the process of bloom development and toxin production by studying the genotypic composition of cyanobacterial natural populations (Bozarth *et al.*, 2010; Briand *et al.*, 2008, 2009; Kim *et al.*, 2006; Liu *et al.*, 2016; Pobel *et al.*, 2012; Sabart *et al.*, 2009, 2015). In general, these studies questioned if the establishment of a cyanobacterial species in a bloom can be explained by the dominance of one or a few prevalent genotypes/ecotypes or by occurrence of many genotypes with varying frequencies over the bloom course.

Although some studies have described the genotype dynamics of *Dolichospermum* (former *Anabaena*) (Liu *et al.*, 2014) and *Plankthotrix* blooms (Briand *et al.*, 2008), most have focused on *Microcystis* (Briand *et al.*, 2009; Liu *et al.*, 2016; Wang *et al.*, 2012; Xu *et al.*, 2011). *Microcystis* population structure has been extensively investigated using diverse approaches including cloning-sequencing, denaturing gradient gel electrophoresis (DGGE), and other molecular methods based on 16S rRNA, 16S–23S rRNA ITS, PC-IGS and *mcy* genes (Kardinaal *et al.*, 2007; Kim *et al.*, 2010; Sabart *et al.*, 2009; Wang *et al.*, 2012; Ye *et al.*, 2009). In general, these studies have shown that only a few genotypes dominate the blooms in a given ecosystem, and in some cases a spatial-temporal distribution of genotypes was observed (Briand *et al.*, 2009; van Gremberghe *et al.*, 2011; Guedes *et al.*, 2014a; Kim *et al.*, 2010; Sabart *et al.*, 2009; Tanabe *et al.*, 2009). Another question frequently posed in these

investigations is whether changes in genotype composition can be associated with microcystin concentration, but in this case the findings have been controversial.

More recently, next-generation sequencing (NGS) has been increasingly used in the study of microbial diversity. NGS has the advantage to provide a large number of sequences, allowing a broader and deeper analysis of the genotypic diversity and also the characterization of the community composition encompassing several groups of cyanobacteria, including picoplankton (Bertos-fortis *et al.*, 2016). However, such approach is still poorly applied in context of cyanobacterial blooms, especially in the case of mixed blooms.

Mostly, these studies have used 16S rRNA gene amplicons with subsequent grouping of sequences into operational taxonomic units (OTUs) based on a 97% sequence identity threshold. This approach is used, in part, to mitigate effects of error rates from high-throughput sequencing technologies. However, OTUs defined at 97% can throw out informative sequence variation and can group together ecologically distinct populations (Coleman et al., 2006; Hunt et al., 2008; Denef et al., 2010; Shapiro and Polz, 2014). Tighter thresholds such as 99 or 100% have been proposed to better explore intraspecific variability (Dall'agnol *et al.*, 2012; Ruber *et al.*, 2017).

In this study, we addressed the cyanobacterial diversity, using the 16S rRNA gene marker, during a bloom period in a tropical reservoir, focusing in the population structure of each cyanobacterial genus and its relationship with microcystin production.

Water samples were collected in the Funil reservoir (22º30'S, 44º45'W), Rio de Janeiro, Brazil, from October 2013 to March 2014. Samples were collected from two locations: one in the central part of the reservoir (point 1) and the other near the dam (point 2). From one to three samples per month were obtained from the integrated euphotic zone (determined as 2.7 times the Secchi disk depth), totalizing 22 samples. Water samples were filtered (Whatman GF/F 0.7 µm) to collect cells and then stored at -20ºC for DNA extraction. For the determination of microcystin concentrations, one liter of raw water was lyophilized and extracted three times with 30 mL of methanol:butanol:water (5:20:75) as described by (Barco *et al.*, 2005). The determination of the concentration of the MC variants LR, RR, YR, LA, LW and LF was performed on the LC–MS–MS API 3200 Q-trap (Applied Biosystems) following the conditions described in Guedes et al., 2014. DNA was extracted from cells collected in

filters using the Power Soil DNA Isolation Kit (MoBio), according to the manufacturer's instructions. For the detection of potentially toxic cyanobacteria cells, PCR was performed using the primers and conditions described for the amplification of *mcyD* (Pimentel & Giani, 2013). For the evaluation of cyanobacteria diversity, amplification of the v3-v4 region of 16S rRNA genes was performed with the primers described by Klindworth *et al.* (2013) containing the appropriate adaptors for sequencing in the Illumina platform. Amplifications were performed in 25 µl reactions, following the conditions described in the 16S Metagenomic Sequencing Library Preparation Protocol for the Illumina MiSeq System. PCR products were purified using magnetic beads (Agencourt AMPure XP, Beckman Coulter) and subjected to a second PCR to incorporate dual indices. Sequencing was performed in a MiSeq plataform (Illumina) using the MiSeq Reagent kit v3 (2 x 300 base pairs) according to the manufacturer's instructions. Files were recovered (.fastq) and paired-end reads were joined using Mothur v.1.35.1(Schloss et al., 2009). The following criteria were used to eliminate low quality reads: average quality <30, length (window size=50), < 460 base pairs, presence of ambiguous characters ('N'). The remaining reads were aligned with SILVA database and a pre clustering at 1% was performed. Chimeras were detected and excluded by UCHIME (Edgar *et al.*, 2011). Taxonomic classification was carried out using the RDP database (Release 11) with a confidence threshold of 80%. Sequences not assigned as Bacteria or classified as Chloroplast or Mitochondria were discharged. Singletons and dubletons were removed and the number of sequences in the 22 samples was normalized creating subsamples. Sequences from the normalized samples were used as input to generate a distance matrix. Sequences classified as Cyanobacteria were clustered at the sequence similarity of 97, 99 or 100%, defining Operational taxonomic units (OTUs). To avoid sequencing artifacts only those sequences contributing > 0.01% of total sequences were considered. Taxonomic assignment of OTUs was performed using GreenGenes (version 13_5).

Possible correlations between the relative abundance of cyanobacterial OTUs and microcystin concentrations were evaluated using the Pearson correlation coefficient, with $p<0.05$.

The cyanobacterial bloom occurred during a spring-summer period and the associated limnological variables have been described previously in another study (Guedes et al., in preparation). A *Microcystis* bloom was detected from October to mid-January, with

Dolichospermum and *Pseudanabaena* also present as minor contributors. From mid-January on, the cyanobacteria dominance was shared by genera *Synechococcus*, *Cylindrospermopsis*, *Dolichospermum* and *Microcystis*. This spatial-temporal dynamics was derived from the relative contribution of 16S rRNA gene OTUs, according to their taxonomic assignment (grouped at 97% identity). Similar patterns were observed in the two sampling points, despite some differences in specific dates (Figure 1). Microcystin was detected in all samples (-LR, -RR and -YR) as well as potentially microcystin producer cells (*mcyD*+) (Table 1).

In order to detail the genetic diversity associated with each genus, OTUs were defined with different identity thresholds. In general, using a 97% identity cutoff, the presence a single OTU was observed for all genera and prevailed during most of the sampling period (Table 2). When applying more strict cutoffs, we identified higher OTU numbers, pointing to an even larger intraspecific variability (Table 2).

For *Microcystis*, with a 97% threshold, just one OTU was identified, while using a 99% cut-off 18 OTUs were defined (Table 2). Nevertheless, in both cases a single generalist OTU was dominant in all samples. On the other hand, when applying an identity threshold of 100%, a distinct pattern was revealed, with the alternation of three main OTUs over time (Figure 1). Thus, *Microcystis* population structure was marked by the presence of few and prevalent genotypes, together with numerous rare genotypes. As already pointed, *Microcystis* is the most studied genus regarding genotype dynamics during blooms (Bozarth *et al.*, 2010; Briand *et al.*, 2009; Guedes *et al.*, 2014a; Pobel *et al.*, 2012; Xu *et al.*, 2011). Although different loci (16S-23S rDNA ITS, cpcBA, 16SrRNA) and different thresholds for sequence grouping have been used and a relatively small number of sequences was considered (maximum of hundreds, usually based on sequence cloning), the general idea that came out from these studies was that multiple genotypes coexist and are replaced over time, including major ones and a variety of less-common genotypes, which is in accordance with our results. This situation suggests a balancing selection process, allowing the maintenance of a high diversity in these populations, as already pointed (Bozarth *et al.*, 2010; Briand *et al.*, 2009; Liu *et al.*, 2016; Pobel *et al.*, 2012; Sabart *et al.*, 2015; Xu *et al.*, 2011).

The second most abundant cyanobacteria genus was *Synechococcus*, which also showed the highest OTU numbers, irrespective of the identity threshold applied (Table

2). Using the identity cutoff of 97% a single OTU was revealed while grouping at 99%, three to four genotypes alternated over time. When the 100% identity cutoff was considered a higher number of genotypes (104) was revealed which occurred sporadically. Such a high genotypic diversity has already been pointed in this genus, with freshwater populations being even more diverse than those from marine ecosystems (Dall'Agnol et al., 2012, Sánchez-Baracaldo, et al., 2008). Some recent studies using NGS have confirmed the high genetic diversity of this group. For example, Ruber et al. (2017) described 49 OTUs (grouped at 99%) in four lakes and Bertos-fortis *et al.* (2016) reveled 46 OTUs (grouped at 98%) in 118 samples from the Baltic Sea.

The *Dolichospermum* population was composed of one OTU applying a 97% cut-off, two OTUs with a 99% and twenty with a 100% cut-off. In the case of OTUs with 99% identity, we identified the co-dominance of the same two generalist genotypes over time. Applying a 100% cut-off two genotypes also co-dominated in each sample, however their identity changed over time, characterizing them as opportunists. In any case, the pattern of co-dominance of two genotypes was present in all samples. Very few articles described the temporal-spatial dynamics of *Dolichospermum* blooms (Li *et al.*, 2016). The only study that followed the temporal dynamics of *Dolichospermum* genotypes (using 16S-23S ITS) also detected two major genotypes together with a high number of rare ones (116 genotypes) (Liu *et al.*, 2014).

In the case of *Pseudanabaena*, both using a 97% and a 99% cut-off, a single generalist genotype was revealed. However, considering 100% identity, a succession of opportunistic genotypes was detected. The diversity of *Pseudanabaena* is poorly known and only isolated strains have been compared. ITS sequencing revealed a relatively high nucleotide diversity (3.8%) and length variability and this locus was more diverse than 16S or 23S rRNA, for which all the sequences were grouped with 99% identity (Acinas *et al.*, 2009). To our knowledge, no study has described the genetic diversity of this genus during a bloom.

Differently from other genera, *Cylindrospermopsis* OTUs were not present in all samples. This genus presented the smaller number of genotypes and even applying a 100% cutoff only six genotypes were detected. In the samples with the highest number of OTUs, a single dominant genotype was observed. There is a consensus that

Cylindrospermopsis has lower genetic diversity in comparison with other cyanobacteria genera, but this is idea is mostly derived from studies with isolates from different continents (Haande *et al.*, 2008). Studies describing genotypes from natural samples are extremely scarce. For example, Miller & McMahon, (2011) studying four eutrophic lakes, distinguished only three *cpcBA* genotypes while Guedes et al. (2014) identified seven *cpcBA* genotypes from a total of 30 sequences. No study using 16S rDNA sequences was reported.

The possible correlation between microcystin concentration and the most abundant OTUs from the potential toxin producing genera (at 97, 99 and 100% cut-offs) was tested and no significant relation was found. Possibly, MC production is the result of the contribution of several different 16S rRNA genotypes, which is consistent with the fact that the presence of *mcyD* gene copies was detected all over the tested period. Some studies evaluated if the selection of specific genotypes was associated with changes in the toxicity of *Microcystis* populations and concluded that such a link did exist (Bozarth *et al.*, 2010; Briand *et al.*, 2009; Kim *et al.*, 2006; Pobel *et al.*, 2012). However, these conclusions must be viewed with caution, first because the ability to produce microcystin is dependent on the presence of the *mcy* cluster, which has a polyphyletic distribution in *Microcystis* and other genera (Otsuka *et al.*, 1999). Second, the sequence identity of a marker gene, such as 16S rRNA or ITS, does not necessarily distinguish ecotypes. Although in the case of *Synechococcus* some clades, defined by ITS, could be related to specific ecotypes, even in this genus this is not true for all ITS-based groups (Giovannoni & Stingl, 2005; Melendrez *et al.*, 2011). In other genera, like *Microcystis*, 16S rRNA variants cannot be used to discriminate ecologically distinct populations (Berry *et al.*, 2017).

With the advent of NGS, microbiome studies using 16S rRNA gene amplicons are increasing. The analysis of the generated sequences includes their clustering based on DNA similarity, defining OTUs. However, recent studies have questioned the biological significance of these groups. In fact, regardless of the cut-off used, OTUs are not monophyletic and 16S rRNA gene variable regions do not reflect phylogenetic distance or ecologically identity (Berry *et al.*, 2017; Koeppel & Wu, 2013). On the other hand, this approach offers a quick way to generate a comprehensive view of the microbial diversity in the context of whole communities and may detail the structure of populations in an unprecedented way.

In this study, from NGS data, we revealed intraspecific variability in different cyanobacterial groups, suggesting that populations are structured in different ways. The investigation of population dynamics can contribute to understand how blooms persist and can reveal different strategies related to the succession of the species involved. However, the ecological relevance of the presence of different genotypes remains to be investigated, with studies exploring diverse sets of isolated strains coupling additional genomic data and relevant physiological traits.

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Table 1 – Microcystins concentrations (µg L^{-1}) and detection of potentially toxic cells in collected samples from Funil Reservoir.

	Oct-30		**Nov-27**		**Dec-9**		**Dec-23**		**Jan-9**		**Jan-23**		**Jan-30**		**Feb-20**		**Feb-26**		**Mar-12**		**Mar-26**	
Sampling points	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
MC-LR	4.72	2.20	2.03	0.29	1.31	1.57	4.10	0.05	0.08	0.12	0.02	0.01	0.20	0.24	0.71	0.76	1.62	1.06	1.19	2.10	0.52	0.49
MC-RR	0.09	0.03	0.05	0.40	0.29	0.09	1.57	0.00	0.02	0.02	0.01	0.00	0.04	0.06	0.51	0.29	0.90	0.51	0.11	0.00	0.03	0.03
MC-YR	0.11	0.03	0.04	0.29	0.12	0.07	0.32	0.00	0.01	0.01	0.00	0.00	0.01	0.02	0.15	0.13	0.24	0.19	0.03	0.00	0.01	0.01
Total MC	4.92	2.26	2.12	0.98	1.72	1.73	5.99	0.05	0.10	0.15	0.04	0.02	0.25	0.32	1.37	1.17	2.75	1.76	1.33	2.11	0.56	0.53
mcyD	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

Table 2 – Number of OTUs using three distinct identity thresholds and the number of sequences of the main cyanobacterial genera in the Funil Reservoir

OTUs	97%	99%	100%	Number of sequences
Microcystis	1	18	30	409417
Synechococcus	3	23	104	267507
Pseudanabaena	1	3	7	41675
Dolichospermum	1	2	20	14769
C. raciborskii	1	3	6	13460

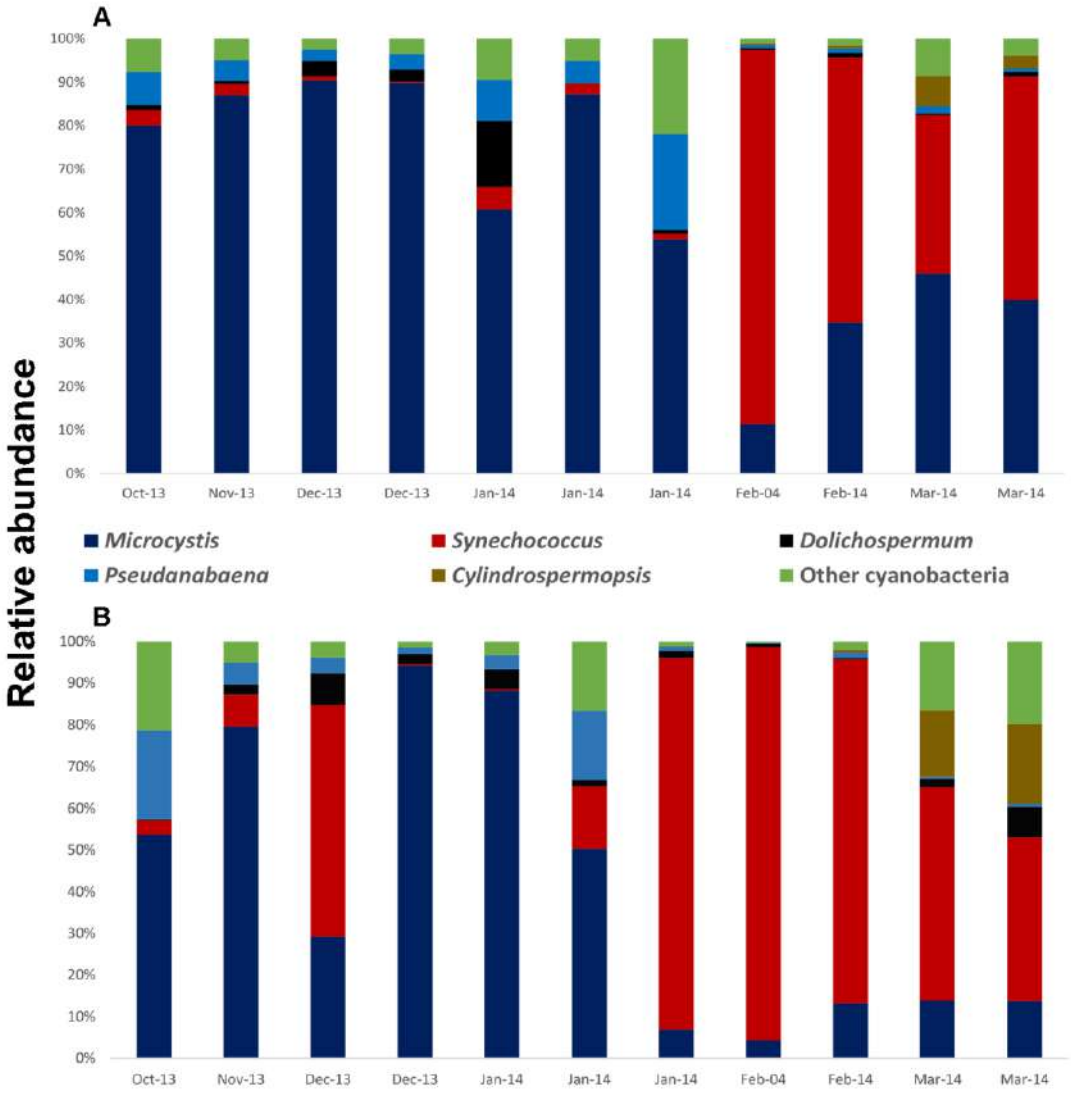


Figure 1 – Relative abundance of cyanobacterial populations accessed by 16S rDNA sequencing from October 2103 to March 2014 in two sampling points of Funil reservoir (A) point 1, central part, (B) point 2, near the dam

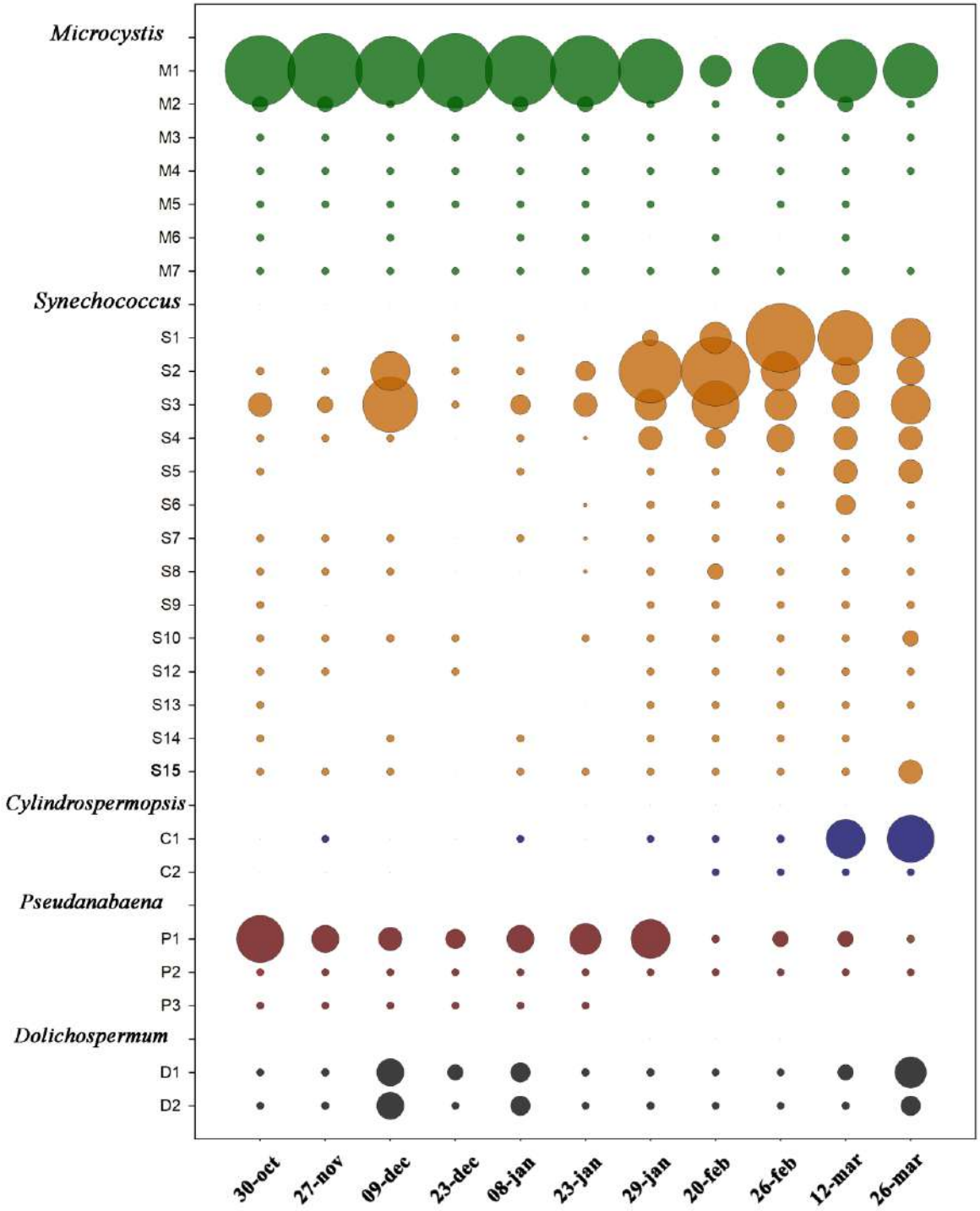
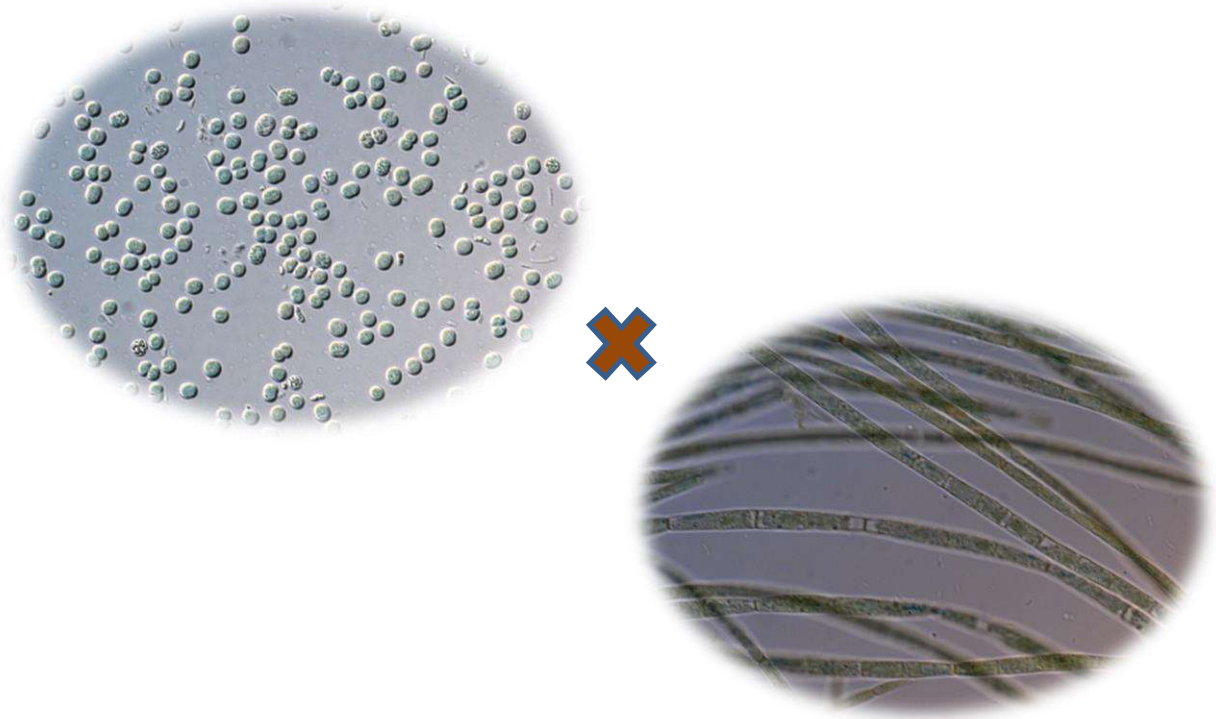


Figure 2- – Relative relative abundance of OTUs with identity thresholds of 99% classified as cyanobacteria Funil Reservoir from October 2103 to March 2014. The area of the bubbles represents the relative abundance of OTUs (average values of the two sampling points). The color of the bubbles indicates the genera to which the OTUs were assigned.

Capítulo 3

Intraspecific and interspecific variability in response to phosphorus depletion in the cyanobacteria *Microcystis aeruginosa* and *Cylindrospermopsis raciborskii*



Manuscrito a ser submetido ao periódico *Harmful Algae*

Intraspecific and interspecific variability in responses to phosphorus depletion in the cyanobacteria *Microcystis aeruginosa* and *Cylindrospermopsis raciborskii*

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Abstract

Phosphorus loading plays an important role in the occurrence of cyanobacterial blooms and the comprehension on how this nutrient affects the physiology of cyanobacteria is imperative to understand and manage blooms. *Microcystis aeruginosa* and *Cylindrospermopsis raciborskii* are cyanobacterial species that form potentially toxic blooms in freshwater ecosystems worldwide. Results from a number of studies have led to the idea that *C. raciborskii* is an opportunistic species regarding to the use of this nutrient. In order to test if *C. raciborskii* is a better competitor for phosphorus than *M. aeruginosa*, we tested five strains of each of these two species. Growth, photosynthethic efficiency, alkaline phosphatase activity, and maximum uptake rate under phosphorus deprivation were analyzed. Although differently affected by phosphorus deficiency, all strains were able to grow, maintain photosynthetic activity, and activate alkaline phosphatase, pointing to the ability of some *C*. *raciborskii* and *M*. *aeruginosa* strains to tolerate phosphorus stress. Taken together, these results support the well-known plasticity observed in these species. All tested parameters revealed a broad variation among strains with no significant difference between *M. aeruginosa* and *C. raciborskii.* Our results indicate that the level of intraspecific varation precludes generalization and the physiological diversity of many freshwater cyanobacteria is underscored in laboratory-based studies that use only one or few strains.

Keywords: alkaline phosphatase, cyanobacteria, phosphorus uptake, strains

1. Introduction

Eutrophication is a major water quality issue worldwide and phosphorus (P) enrichment of lakes is one of the most important keys to trigger and maintain cyanobacteria blooms (O'Neil *et al.*, 2012; Rangel *et al.*, 2012; Smith & Schindler, 2009). During progressive P-loading the phytoplankton biomass increases dramatically and the community often shifts towards cyanobacterial dominance (Watson *et al.*, 1997). The resulting cyanobacterial blooms may be comprised of different players, among which *Microcystis aeruginosa* and *Cylindrospermopsis raciborskii* are the most notorious ones, and insights in competitive interactions as well as responses to environmental conditions are crucial to improve our understanding of cyanobacterial dominance (Soares *et al.*, 2013). At the onset of blooms dissolved nutrient pools may be depleted (Wasmund, 1997) and particularly the capacity of cyanobacteria to generate phosphate from organic matter using exoenzymes may be a key factor in explaining their success (e.g.,(Stihl *et al.*, 2001). The exoenzymes activities may vary among populations of cyanobacterial species depending on P availability (Nausch *et al.*, 2004; Stihl *et al.*, 2001). Since many freshwater ecosystems can be P limited, P loading plays a role in the occurrence of many cyanobacterial blooms (Harke and Gobler, 2013). Given the fundamental importance of P in determining cyanobacterial biomass, the comprehension on how this nutrient affects the physiology of different species of cyanobacteria is imperative to understand and manage blooms.

Microcystis aeruginosa and *Cylindrospermopsis raciborskii* are cyanobacterial species that form potentially toxic blooms in freshwater ecosystems worldwide. The broad geographic distribution of these two species is explained by different evolutionary strategies. *C. raciborskii* is a filamentous diazotrophic cyanobacterium, initially isolated from a tropical site, but then described in all continents, being considered an invasive

species (Antunes *et al.*, 2015). This expansion is potentially harmful since strains are reported as producers of the hepatotoxin cylindrospermopsin or the neurotoxin saxitoxin (Lagos *et al.*, 1999; Molica *et al.*, 2002; Ohtani *et al.*, 1992). Phylogenetic analyses including representative strains collected worldwide revealed clustering according to geographic origin, and different hypotheses have been proposed for the global dispersion of this species (Gugger *et al.,* 2005; Haande *et al.,* 2008; Moreira *et al.,* 2011). Still, genotypes from diverse locations are highly related and the ecological success of *C. raciborskii* results from the combination of low genetic variability and great phenotypic diversity. The latter is reflected by the existence of diverse ecotypes with specific environmental adaptations (Bonilla *et al.*, 2012; Briand *et al.*, 2004; Chonudomkul *et al.*, 2004; Haande *et al.*, 2008; Piccini *et al.*, 2011).

M. aeruginosa is a unicellular species with the ability to form colonies, and one of the most common bloom-forming cyanobacteria in freshwater, with potential to produce the hepatotoxin microcystin (Dittmann *et al.*, 2013; O'Neil *et al.*, 2012). Evaluation of its global biogeography revealed that this species represents a homogenous taxon with no indication of distinct ecotypes (van Gremberghe *et al.*, 2011). A comparative genome approach indicated that the high adaptive capacity of *M. aeruginosa* is based on a high genomic plasticity that can account for its ecological success in various environments (Humbert *et al.*, 2013)

Some studies have described the ability of phosphorus uptake and storage of *C. raciborskii* (Bai *et al.*, 2014; Isvánovics *et al.*, 2000; Wu *et al.*, 2009) and *M. aeruginosa* (Harke *et al.*, 2012; Marinho & Azevedo, 2007; Olsen, 1989). However, only two studies have directly compared these two species regarding their behavior in situations of phosphorus limitation. Using two strains of each species, Marinho and collaborators (2013) found that although *C. raciborskii* has a lower phosphorus requirement if

compared to *M. aeruginosa*, this does not correspond to an advantage in competition with *M. aeruginosa*. Comparing one strain of each species, Wu and collaborators (2009) observed that *C. raciborskii* more effectivley uses and uptakes phosphate than *M. aeruginosa*. All together, these findings has led to the idea that *C. raciborskii* is an opportunistic species regarding to the use of this nutrient (Antunes *et al.*, 2015). However, such broad scale generalizations must be taken with caution, since it has already been shown that significant physiological differences exist between strains of the same species (Marinho *et al.*, 2013; Shen & Song, 2007; Willis *et al.*, 2016), and most of these studies were done with no more than two strains. Interpretations based on individual strains overlook the intraspecific variation and limit our understanding on ecologically relevant traits (Alexova *et al.*, 2011; Aryal *et al.*, 2014; Rocap *et al.*, 2003; Sandrini *et al.*, 2015; Willis *et al.*, 2016; Xiao *et al.*, 2017).

To get more insight in intraspecific and interspecific variability in ecophysiological traits related to low P environments, we examined growth, photosynthethic efficiency, alkaline phosphatase activity, and maximum P uptake rate of five *C. raciborskii* strains and five *M. aeruginosa* strains under phosphorus deprivation. This allowed us to test the hypothesis that *C. raciborskii* is a more opportunistic species and a better competitor for P than *M. aeruginosa*. In contrast to our expectation, considerable plasticity in both species, reflected in high intraspecific variability in traits, precludes generalizations on the performance on the species level.

2. Material and Methods

The experiments were performed with five *Cylindrospermopsis raciborskii* and five *Microcystis aeruginosa* strains. Strains were isolated from different Brazilian freshwater environments and were obtained from culture collections of Brazilian laboratories

(Supplementary Table 1). *M. aeruginosa* strains were grown as single cells, not in colonies. Cultures were not grown axenically, but regular microscopic inspection revealed that biomass of heterotrophic bacteria remained low. Cultures on the exponential growth phase were centrifuged (5,000×g, 10 min) in 50-mL centrifuge tubes. After washing and centrifugation with sterile WC medium twice, the cells were resuspended in WC medium .In all experiments, cells were grown in modified WC medium (Lurling & Beekman, 2006), at 24 °C and the incident light intensity was set at 40 μmol photons m^{-2} s^{-1}, with a light-dark cycle of 14:10 hours.

2.1 Characterization of the strains

For the characterization of the strains of both species, they were cultivated under the conditions described above. Morphology measurements were performed on cells collected from the exponential growth phase and immediately fixed in a mixture of freshly prepared 1% paraformaldehyde and 1% glutaraldehyde in 0.1 M phosphate buffer (pH 7.3, for 1 h at room temperature). The diameter (*M. aeruginosa* strains) and cell length and width (*C. raciborskii* strains) were measured in at least 70 cells of each strain with a Zeiss Axio Imager microscope (Carl Zeiss, Göttingen). The cell volume was calculated according Hillebrand et al. (1999).

2.2 cpcBA amplification and sequencing

DNA was extracted from cells collected from the exponential growth phase and extracted with Wizard@ Genomic DNA Purification kit (Promega). The extracted DNA was used as a template to amplify the *cpcBA* locus according to Neilan et al. (1995). Reactions contained a PCR buffer (Promega), 2.5 mM of $MgCl_2$, 0.5 U of GoTaq Flexi DNA polymerase (Promega), 0.2 mM of each deoxynucleotide triphosphate (Fermentas), 5 pmol of each forward (PCβF 5′ GGCTGCTTGTTTACGCGACA 3′) and

reverse (PCαR 5′ CCAGTACCACCAGCAACTAA 3′) primer and 50 ng of template DNA in a total volume of 20 µL. Amplifications were performed with an initial step at 94 °C for 5 min, followed by 40 cycles of 94 °C for 1 min, 50 °C for 1 min and 72 °C for 1 min, and a final step of 72 °C for 5 min. Amplification products were sequenced using the PCαR primer in reactions containing the Big Dye Terminator Cycle Sequencing Standard Kit Version 3.1 and analyzed using an ABI PRISM 3130 system (Applied Biosystems). Sequences are under submission to GenBank.

2.3 Cyanobacterial repeated-sequence PCR

C. raciborskii and *M. aeruginosa* HIP1 PCR amplifications were performed according to Saker and Neilan (2001) with modifications. Reactions contained primers HipCA and HipTG (10 pmol each), deoxynucleoside triphosphates (0.2 mM), $MgCl_2$ (2.5 mM), 5X Green GoTaq Reaction Buffer (4 µl), GoTaq polymerase (Promega) (0.1 U), cyanobacterial DNA (2 ng) and H_2O to a final volume of 20 µl. Thermal cycling consisted of a preincubation step of 5 min at 94°C, followed by 30 cycles of 30 s at 94°C, 20 s at 38°C, and 2 min at 72°C and a final elongation step at 72°C for 5 min. PCR products were separated by 1.2% agarose gel electrophoresis in Tris-acetate-EDTA buffer, and the gel was stained with ethidium bromide and photographed.

2.4 Phosphorus limitation experiments

Experiments with cyanobacteria subjected to phosphate deprivation (P-) and control replete P conditions (P+) were performed in 250 mL Kitasato flasks containing 100 mL of culture medium. This medium was either the standard WC medium (P+) or WC medium in which K_2HPO_4 was omitted and instead KCl was supplemented to prevent K deficiency (P-). Aliquots of *C. raciborskii* and *M. aeruginosa* collected from stock cultures in their exponential growth phase were centrifuged (5,000×g, 10 min) in 50-

mL centrifuge tubes. After washing and centrifugation with sterile P-free WC medium twice, the cells were resuspended in 100 mL of P-free WC medium (P-) or replete P WC Medium (P+). The experiment was performed in triplicates for 10 days, with sampling each two days. Samples were taken for the determination of the chlorophyll-*a* concentration, photosynthetic efficiency, biovolume determination and alkaline phosphatase activity. Chlorophyll-*a* concentration and the photosynthetic efficiency (quantum yield of photosystem II) were measured with the PHYTO-PAM phytoplankton analyzer (Heinz Walz GmbH, Effeltrich, Germany). Biovolume was measured with an automated cell counter (Casy Cell Counter, Schaerfe System GmbH, Reutlingen, Germany) with a 120-µm capillary, directly after sampling. Growth rates (day^{-1}) were calculated from the slope of the regression of natural log (biovolume) versus time until the 6^{th} day of cultivation.

2.5 Alkaline phosphatase activity

The alkaline phosphatase activity was determined using the colorimetric method with p-nitrophenyl phosphate (pNPP; Sigma) as the substrate, with a modified protocol from Ray et al.(1991). Hereto, 2 mL of culture was centrifuged at 3000 g for 5 mins and the pellet resuspended in 5nM p-nitrophenyl phosphate (Sigma) in P-free WC. After an incubation at the same culture conditions for one hour, the reaction was terminated by the addition of 1N NaOH. The samples were centrifuged and the supernatant was used to determine the ectophosphatase activity. Five nM pnPP without a sample was used as the control. Absorbance was read at 405 nm in a spectrophotometer and compared to the standard absorbance curve for p-nitrophenol (pNP). The enzyme activity was normalized with chlorophyll *a* concentration and expressed as fold change in relation to the control (P+ cultures).

2.6 Maximal Phosphorus Uptake Rates (Vmax)

To determine the maximum phosphorus uptake rate, the strains were starved of phosphorus after a 10 days culture on WC without phosphorus. To that, a pulse of phosphorus (10 µmol L^{-1} K_2HPO_4) was added and the inorganic phosphorus concentrations were measured each 10 min during 4 h. Samples for the monitoring of dissolved P concentrations were taken in triplicate after filtration with GF/C membranes and storage under -20ºC until the analysis. Concentrations of dissolved phosphate were determined with a continuous flow analyzer (CFA, Skalar Analytical BV). *Vmax* was determined as the initial linear slope of the curve of phosphorus concentrations vs. time.

2.7 Data analysis and statistics

Cell volume and maximum uptake rates among strains of *C. raciboskii* and *M. aeruginosa* were evaluated statistically running one-way ANOVAs in the tool pack SigmaPlot version 12.3 (Systat Software, Inc., San Jose, CA, USA). The ANOVAs were followed by pairwise multiple comparison procedures (Tukey test) to distinguish means that were significantly different ($p < 0.05$). The growth rates of *C. raciborskii* and *M. aeruginosa* under P+ and P- conditions were statistically evaluated by a two-way ANOVA with strain and P conditions as the fixed factors. The chlorophyll-*a* concentration and photosyntetic efficiency through time were evaluated by running a two-way repated measures ANOVA, while comparison of the responses of each strain separately under P- and P+ conditions were evaluated by running one-way repeated measures ANOVAs. The fold change of alkaline phosphatase activity of the ten strains was compared with a one-way repeated measures ANOVA (rmANOVA). After all rmANOVAs, homogeneous subgroups were defined by a Tukey *post hoc* comparison

at $p < 0.05$. All rmANOVAs were performed using the statistical package SPSS 24.0 (SPSS, Chicago, IL, USA).

3. Results

3.1 Characterization of C. raciborskii and M. aeruginosa strains

To assess the intraspecific variability of *C. raciborskii* and *M. aeruginosa*, we included five strains of each species. These strains were originally identified based on morphological traits, and here we further examined their diversity by assessing morphology, genetic profiles obtained by PCR for highly iterative palindromic repeats (HIP1) and by sequencing of the phycocyanine intergenic region (*cpcBA*).

The morphological characterization of the strains revealed significant differences in cell volume among *M. aeruginosa* strains ($F_{4,486} = 183.7$; $p < 0.001$) and *C. raciborskii* strains ($F_{4,297} = 19.8$; $p < 0.001$; Figure 1, Table 1). Three strains of *M. aeruginosa* showed similar mean cell volumes (MIRF, LEA12 and LEA13), while the other two (LEA4 and MIRS) had a significant smaller cell volume. Among *C. raciborskii* strains, CYRF had a significant higher cell volume than all the others (Table 1).

The *cpcBA* intergenic region sequences confirmed the morphological identification of the strains, with more than 99% of similarity with *M. aeruginosa* or *C. raciborskii cpcBA* sequences available in GenBank. However, we could not differentiate all the strains based on the *cpcBA* sequences, since some of them were identical. HIP1 PCR revealed three profiles among the five tested *C. raciborskii* strains and four profiles among the five tested *M. aeruginosa* strains (Figure 2).

Considering all these traits almost all tested strains could be differentiated. However, *M*. *aeruginosa* and *C. raciborskii* strains that were isolated from the same location at

the same moment, showed similar profiles in the HIP1 PCR (LEA12-LEA13 and CYL1-CYL2) and also identical *cpcBA* sequences and cell volumes.

3.2 Physiological response of C. raciborskii and M. aeruginosa strains to phosphorus deprivation

To further explore the variability of *C. raciborskii* and *M. aeruginosa* strains, we investigated their physiological response to phosphate deprivation. All strains were able to grow for ten days in the absence of phosphorous, as derived from the biovolume and chlorophyll *a* measurements (Figure 3 and 5). The comparison of the growth rates (based on biovolume) of all strains in presence or absence of phosphorus (Figure 3) by two-way ANOVA yielded a significant treatment effect) ($F_{1,59}$ = 153.1, p < 0.001), a significant strain effect ($F_{9,59}$ = 9.13; p < 0.001) and a significant strain x treatment interaction effect ($F_{9,59}$ = 3.68; p = 0.002). With the exception of one strain (MIRS*)* all other *M. aeruginosa and C. raciborskii* strains showed significant lower growth rates under P deprivation. The variability among strains is such that homogeneous growth rate groups were formed with representatives of each species (Figure 3). Comparing the growth rates at the species level revealed that there are differences ($F_{3,59}$ =21.5; p < 0.001), but Tukey's test disclosed that only P-replete and P-deficient conditions differed and not species (Figure 4).

All tested strains expressed increasing chlorophyll *a* concentrations over time both in P+ and P- media (Figure 5). A two-way rmANOVA indicated a significant time effect ($F_{4,160}$ = 1753; p < 0.001). Chlorophyll-*a* concentrations were significantly higher in the presence than in the absence of P ($F_{1,40}$ = 844.7; p < 0.001), which was apparent from day 4 onwards in most strains (Figure 5) and reflected in a significant P-condition x time interaction effect ($F_{4,160}$ = 162.0; p < 0.001). Chlorophyll-*a* concentrations also

differed among strains ($F_{9,40}$ = 256.1; p < 0.001) and a significant P-condition x strain interaction was found ($F_{9,40}$ = 12.9; p < 0.001). The latter indicated that the effect of P-condition was strain dependent as can be seen from a marginal significant effect of P-condition on the course of chlorophyll-*a* concentrations in CYLP (Figure 5D; Supplementary Table 2), while the effect was much stronger in other strains (Figure 5). The Tukey *post hoc* test revealed a broad variation on the response of different strains, with seven homogeneous groups (Table 2).

The effect of P deprivation on photosystem II efficiency varied among strains (Figure 5). Two-way repeated measure ANOVA showed a significant difference between treatments ($F_{1,40}$ = 72.5; p < 0.001), between strains ($F_{9,40}$ = 205.4; p < 0.001) and indicated a significant treatment x strain interaction ($F_{9,40}$ = 4.95; p < 0.001). Analyzing the photosystem II efficiency over time for each strain under P- and P+ by rmANOVAs explained the significant interaction effect, because in half of the strains tested PSII-efficiency was significantly lower under P- than under P+ conditions, while for the other half there were no differences (Figure 5; Supplementary Table 2). The significantly reduced photosystem II efficiency in the absence of P (P-) was found in two *C. raciborskii* strains (CYL2 and T3) and in three *M. aeruginosa* strains (MIRF, LEA12 and LEA13). Five homogeneous subgroups were defined by a Tukey *post hoc* comparison (Table 2). With the exception of CYLP, photosystem II efficiency in all other *C. raciborskii* differed significantly from that in *M. aeruginosa* strains.

Enzymatic activation of alkaline phosphatase (AP) was observed under P-deplete conditions. (Figure 6). For all *C. raciborskii* strains increased AP activity was apparent from day 4 onwards. The increased AP activities were two to five times higher, yet also varied over time; for example, while CYRF maintained a relatively high activation throughout the experimental period, AP activity in CYL2 raised from day 6 onwards

(Figure 6A). In *M. aeruginosa*, the increase in AP activity under P deprivation varied broadly among strains and over time (Figure 6B). Significant differences between strains were observed ($F_{9,20}$ = 19.97; $p < 0.001$), but also a significant time effect ($F_{3,60}$ = 127.3; $p < 0.001$) and a time x strain interaction effect ($F_{3,60}$ = 14.8; $p < 0.001$). The great variation of *C. raciborskii* and *M. aeruginosa* strains in the alkaline phosphatase activity under P depletion is reflected in the six homogenous subsets defined by the Tukey *post hoc* comparison (Table 2). The most distinct response was observed in MIRF, that showed a pronounced activation at day 4 (a fold change of ~7 as compared to P replete conditions), which increased further at day 10 (Figure 6B). The other strains showed a lower AP activation (approximately 3 to 5 fold), and this remained so until the 10^{th} day.

In order to estimate the maximal uptake rate of P (V_{max}), strains were first starved for P and after a pulse of P addition (10 µM) the P concentration in the culture medium was measured. A great among strain variation in V_{max} values was observed (Figure 7). A one-way ANOVA on log transformed V_{max} values (to fulfil ANOVA requirements) indicate significant differences ($F_{9,29}$ = 104.9; $p < 0.001$) and a post hoc comparison yielded homogeneous groups comprised of representatives of both species (Figure 7).

4. Discussion

Five strains of *C. raciborskii* and *M. aeruginosa* were used to compare their physiological responses to P deprivation. Our results show that the intraspecific variability among strains was larger than the interspecific variability, when considering morphological, genotypic and physiological traits. Regarding the response to P deprivation the present results do not support the hypothesis that *C. raciborskii*, as a species, is a better competitor for phosphorus than *M. aeruginosa*.

The characterization of *C. raciborskii* and *M. aeruginosa* strains revealed differences in morphology, HIP1 PCR profiles and *cpcBA* sequences (*see* Table 1, Figure 1 and 2). In the case of strains of *M. aeruginosa* or *C. raciborskii* isolated from the same sampling location and date, these traits could not distinguish them. *C. raciborskii* strains presented similar or identical HIP1PCR profiles, indicating a low genetic variability as already noted (Saker & Neilan, 2001). This was also observed by Willis et al. (2016) who found identical HIP1PCR profiles among 24 *C. raciborskii* strains isolated from the same reservoir, although significant differences were reported in growth and cylindrospermopsin production. In contrast, *M. aeruginosa* strains showed a higher variability in HIP1PCR profiles as already found in previous studies (Bittencourt-Oliveira *et al.*, 2007; Wilson *et al.*, 2005).

The differences between strains in the cell volume were also more pronounced in *M. aeruginosa* than in *C. raciborskii*. Xiao et al. (2017) observed the opposite pattern when comparing four *M. aeruginosa* and eight *C. raciborskii* strains, but in their case cultivation time and conditions (different light intensities and temperatures) were different and *C. raciborskii* strains included straight and coiled morphologies.

To further explore this inter and intraspecific variability we tested physiological responses to P deprivation. In contrast to other studies that tested the abilities of strains growing in a medium with different phosphorus concentrations after a starvation period (Bai *et al.*, 2014; Harke & Gobler, 2013; Wu *et al.*, 2009), the present study explored the ability of *C. raciborskii* and *M. aeruginosa* strains to grow in P depletion directly after cultivation in P rich medium.

Growth was negatively affected by phosphorus deficiency, yet all strains were able to grow, which is in agreement with other studies on these species (Bai *et al.*, 2014; Harke

& Gobler, 2013; Wu *et al.*, 2012). The most likely explanation of sustained growth for 10 days, even when cells were placed in P free medium, is luxury consumption and internal storage of P. Polyphosphate accumulation is a common trait in Cyanobacteria (Gonzalez-Esquer *et al.*, 2016) and polyphosphate bodies have been described in *M. aeruginosa* (Shi *et al.*, 2003) and *C. raciborskii* (Noyma *et al.*, 2015). This storage could theoretically sustain 3 to 4 generations of growth in phosphate-deplete conditions (Droop, 1973; Morel, 1987), and probably is involved in the sustained growth observed even after 10 days in a phosphorus free medium.

Few studies compared strains of the same species in relation to P availability and even those studies that compared two strains detected variability (Marinho *et al.*, 2013). Using four unicellular *M. aeruginosa* strains, Shen and Song (2007) observed that growth rates were inhibited by 51 to 79% in low P concentrations (200 µg L^{-1}) . For *C. raciborskii*, Piccini et al. (2011) comparing two ecotypes showed that under low P concentrations (10 µM K_2HPO_4), their growth rate differed more than two fold. On the other hand, Willis et al. (2015) tested three *C. raciborskii* ecotypes and observed similar growth rates under low P (0-25 µM K_2HPO_4).

When comparing all strains of the same species together we found a broad variation in the growth response to low P. *C. raciborskii* showed a higher variability in the growth range and a greater intraspecific variation compared to the interspecific variation was observed. A recent work that also compared intraspecific variability with both *M. aeruginosa* and *C. raciborskii* strains, testing light intensity and temperature, also demonstrated a greater intraspecific variation than interspecific variation (Xiao *et al.*, 2017).

The response to P depletion was also investigated estimating the photochemical efficiency of the PSII reaction centers. In all P- strains a strongly reduced photosystem II efficiency was expected (Lippemeier *et al.*, 2003), because of expected lowered ATP synthesis and $NADP^+$/NADPH regeneration leading to photosystem II damage (Li & Sun, 2016). In contrast to our expectation, only in two *C. raciborskii* strain and in three *M. aeruginosa* strains photosystem II efficiency was reduced in the P- environment, but reductions were only 18% and 14-28%, respectively, and were apparent only on later times. Consequently, the transfer of the cells into a P deplete environment did not impose such a strong P stress over the 10 day incubation period that photosystem II damage occurred. Likewise, in the marine dinoflagellate *Prorocentrum donghaiense* 10 days in a low P environment did not affect the photosystem II efficiency (Li & Sun, 2016). When it comes to photosynthesis activities of *C. raciborskii* and *M. aeruginosa* in the absence of P, Bai and collaborators (2014), testing one strain, found that P depletion did not affect *C. raciborskii*. For *M. aeruginosa*, Shen and Song (2007) focused on morphology and found that unicellular *Microcystis* strains were more sensitive to P deficiency, while colonial strains showed no difference in photosynthetic efficiency when grown in low concentrations of P.

Varied responses were observed among the strains both for *C. raciborskii* and *M. aeruginosa* when photosystem efficiency was analyzed in low phosphorus conditions. Taken together, these results indicate that, at least for a restricted time, P depletion is not able to damage the photosynthetic activity, pointing to the ability of both *C. raciborskii* and *M. aeruginosa* strains to thrive in this condition. Altogether, these findings indicate that one species is not more susceptible to P limitation than the other.

In this work, more diverse patterns of temporal AP activation were observed for *M. aeruginosa* strains than for *C. raciborskii* strains. This has been pointed out in a

previous study comparing eight strains of *M. aeruginosa* (Shen & Song, 2007). Although the conservation of genes involved in the response to P limitation in *M. aeruginosa* indicates a universal molecular response (Harke *et al.*, 2012), at the physiological level the degree of AP activation can vary. In contrast, for *C. raciborskii*, although some studies have tested AP activity in varying P sources and concentrations (Wu 2011; Bai, 2104), to our knowledge, no study has compared different strains. Different efficiencies in AP activation may affect the fitness of strains and may be ecologically relevant to both species, to overcome P limitation and acquire P from organic compounds.

A great variation in Vmax values was observed between strains of the two species. In general, *C. raciborskii* showed higher maximum uptakes rates than *M. aeruginosa*, however this difference was not significant. Other studies also compared the maximum uptakes rates of these two species, with contradictory findings. Using two strains of each species, Marinho and collaborators (2013) found higher Vmax values for *M. aeruginosa*. In contrast, Wu (2009), using just one strain of each species observed that *C. raciborskii* had a higher phosphate uptake rate than *M. aeruginosa*. These findings, together with our data, do not support the general idea that *C. raciborskii* has greater P uptake ability than *M. aeruginosa* as mentioned in some studies (Istvanovics et al., 2000, Antunes et al., 2015).

Many freshwater ecosystems are frequently limited in inorganic phosphorus and even during blooms most of this element is already incorporated in the cells. Our results, as well as others found in the literature, show that *M. aeruginosa* and *C. raciborskii* are able to withstand P deprivation, supporting growth and photosynthetic activity, and this relies in luxury uptake of P, efficient storage of polyphosphate and activation of AP. Thus, management strategies based on phosphorous depletion must take in

consideration that these organisms can tolerate P limitation, and that the populations can be sustained for some time. Consequently, if these organisms remain in the water column after a P reduction intervention, for instance with a P adsorbent (e.g., Copetti et al., 2016), and the surface water receives a pulse of nutrients from run-off or other sources within 10 days or so, rapid regrowth of these cyanobacteria is expected. Therefore, P reduction interventions should either be executed when the cyanobacteria have not established yet, so prior to the blooming, or be combined with measures that actively reduce the biomass when blooms have established.

In part, the resilience of these species to nutrient limitation can be attributed to an extended plasticity conferred by numerous strains, with varying adaptive abilities that co-occur in the ecosystem. This study is a preliminary effort in showing the existing variability of these strains. Of note is the fact that working with five strains and a single parameter already provided data where the variability of these strains was evident. Further studies working with a larger number of strains and more than a single nutrient will more likely reveal an even greater variability among species. The physiological diversity of many freshwater cyanobacteria is underscored in laboratory-based studies and future efforts using an extended number of strains are imperative for a better understanding of their physiology.

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Figure captions

Figure 1- Microscopic image of C. raciborskii (up) and M. aeruginosa (bottom) strains. (A) CYL1, (B) CYL2, (C) T3, (D) CYLP, (E) CYRF T3 (F) MIRF, (F) MIRS, (G) LEA4, (H) LEA12, (I) LEA13. Scale bar = 5 µm

Figure 2 - HIP1 PCR profile of C. raciborskii and M. aeruginosa strains. HIP1 PCR revealed three profiles among the five tested C. raciborskii strains and four profiles among the five tested M. aeruginosa strains.

Figure 3- Growth rates of C. raciborskii and M. aeruginosa strains cultivated in the presence (P+) and absence of phosphorus (P-). Letters indicate homogeneous groups according to the Tukey test ($P < 0.05$)

Figure 4- Box plot of the growth rates of C. raciborskii and M. aeruginosa under the presence (P+) and absence of phosphorus (P-). Letters indicate homogeneous groups according to the Tukey test ($P < 0.05$)

Figure 5- Physiological parameters of the ten strains cultivated in the presence and absence of phosphorus. Bars represent Chlorophyll a concentration (white for cultures grown in the absence of phosphorus (P-) and grey for cultures grown in the presence of phosphorus (P+); Lines represent Photosystem II efficiency; Solid lines for P+ and dashed lines for P-. Left: C. raciborskii strains (A) CYRF, (B) CYL1, (C) CYL2, (D) CYLP, (E) T3; Right: M. aeruginosa strains (F) MIRS, (G) MIRF, (H) LEA12, (I) LEA13, (J) LEA4.

Figure 6- Fold change of Alkaline phosphatase activity for cultures grown in the absence(P-) of phosphorus. (A) C. raciborskii strains (B) M. aeruginosa strains.

Figure 7- Maximum uptake rate of phosphorus (Vmax) of M. aeruginosa and C. raciborskii strains after addition of a pulse of KH2P04 to starved cells. Letters indicate homogeneous groups according to the Tukey test ($P < 0.05$)

Table 1- Cell volume (means ± 1 SD) and *cpcBA* sequences of *Microcystis aeruginosa* and *Cylindrospermopsis raciborskii* strains . F- and P- values of one-way ANOVAs, while similar letters (a,b) indicate homogeneous groups that are not different at the P > 0.05 level.

M. aeruginosa strains	cell volume	*cpcBA* sequences
MIRS	30.6 ± 9.4 [a]	access number
MIRF	77.1 ± 19.4 [b]	
LEA12	71.2 ± 30.1 [b]	
LE13	77.4 ± 24.8 [b]	
LEA4	19.0 ± 6.9 [c]	
	$F_{4,486}$ = 183.7	
	p < 0.001.	
C. raciborskii strains	cell volume	cpcBA sequences
CYRF	65.1 ± 22.0 [a]	
CYL1	39.3 ± 11.3 [b]	
CYL2	50.5 ± 17.1 [b]	
CYLP	50.6 ± 16.7 [b]	
T3	43.1 ± 22.1 [b]	
	$F_{4,297}$ = 19.8;	
	p < 0.001	

Table 2- Statistical results of parameters obtained from the experiments with *M. aeruginosa* and *C. raciborskii* strains under P+ and P- conditions. Three different tests were performed, depending on the parameter. Two-way repeated measure ANOVA (Photosystem II efficiency and Chlorophyll a), Two way ANOVA (Growth rate) and One-way repeated measure ANOVA (Alkaline Phosphatase activity). Homogeneous subgroups were defined by a Tukey *post hoc* comparison at $p < 0.05$ and are indicated by similar symbols per column

Strains	Photosystem II efficiency	Chlorophyll *a*	Growth rate	Alkaline Phosphatase activity
CYRF	c	d	a	b,c,d,e
CYL1	e	t,g	c	d,e,f
CYL2	e	d,e	a	b,c,d
CYLP	b	a	a,b	b,c,d,e
T3	d	d,e	a,b	e,f
MIRS	b	b	a,b	a,b,c
MIRF	b	c	a	a
LEA12	a	e,f	a,b	f
LEA13	a	g	a	b,c
LEA4	b	c	b	b

ANOVA	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*
Treatment effect	844.7	<0.001	72.8	<0.01	146.7	<0.001	-	-
Strain effect	256.1	<0.001	205.3	<0.01	9.13	0.001	19.9	<0.001

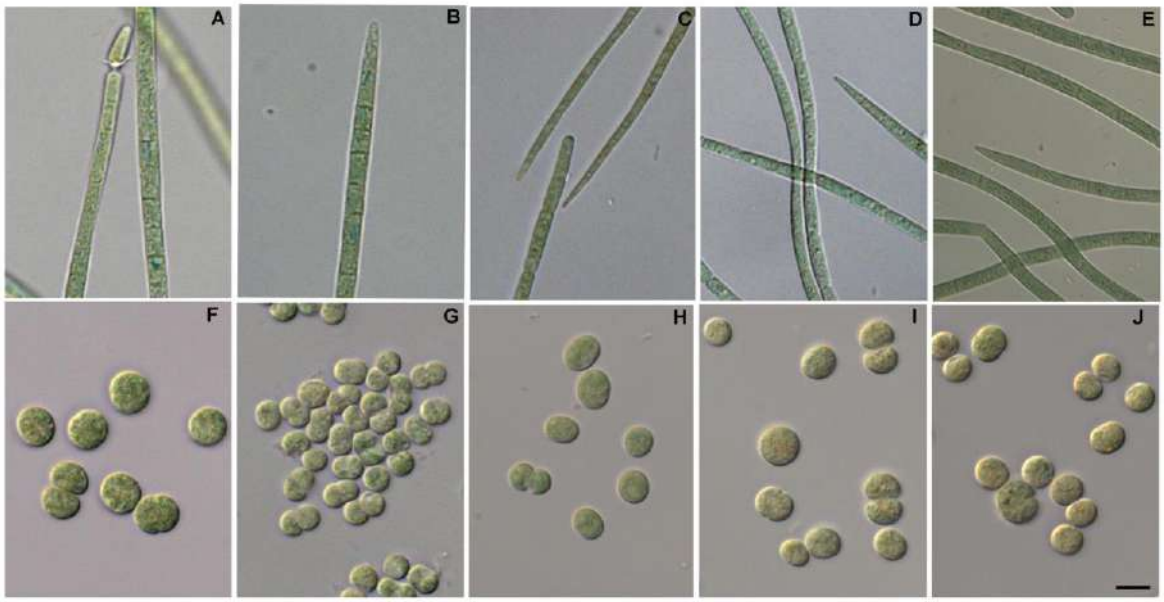
Supplementary Table

Supplementary Table 1. *Microcystis aeruginosa* and *Cylindrospermopsis raciborskii* strains used in this study, including their water body of isolation.

Strain	code	Origin	culture collection	Reference
M. aeruginosa MIRF01	MIRF	Funil Reservoir (22°30'S, 44°45′W) 2005	LETC	Mello *et al*., 2013
M. aeruginosa LEA-04	LEA4	Itumbiara Reservoir (18°24'S'; 49°05'W) 2005	LEFF	Marinho *et al.*, 2013
Microcystis sp. MIRS04	MIRS	Samuel Reservor (8°48'42"S, 63°25'5"W) 2010	LETC	-
M.aeruginosa LEA12	LEA12	Funil Reservoir (22°30'S, 44°45′W) 2013	LEFF	-
M. aeruginosa LEA13	LEA13	Funil Reservoir (22°30'S, 44°45′W) 2013	LEFF	-
C. raciborskii CYRF 01	CYRF	Funil Reservoir (22°30'S, 44°45′W) 2005	LETC	Mello *et al*., 2013
C. raciborskii CYLP01	CYLP	Paranoá Lake (15°47'19"S, 47°48'56"W) 1999	LETC	-
Cc. raciborskii T3	T3	Billings Reservoir(23°47'21"S, 46°36'48"W), 1996	LETC	Lagos et al, 1999
C. raciborskii CYL1	CYL1	Camorim Reservoir (22°5z'31.13"S, 43°26'45.63"W)	LEFF	-
C. raciborskii CYL2	CYL2	Camorim Reservoir ((22°57'31.13"S, 43°26'45.63"W)	LEFF	-

Supplementary Table 2- One-way repeated Measures ANOVAs comparing the course of the quantum yield of Photosystem II (PSII-Yield) and the chlorophyll-*a* concentration (Chl-*a*) in P+ and P- cultures per strains from start (T0) to the end (T10) of the experiment. Statistical significant differences ($p < 0.05$) are indicated in bold.

	PSII-Yeld		**Chl-a**	
Strain	$F_{1,28}$	p	$F_{1,28}$	p
MIRS	3.98	0.117	25.53	**0.007**
MIRF	23.63	**0.008**	507.61	**<0.001**
Lea12	25.51	**0.007**	173.28	**<0.001**
Lea13	58.17	**0.002**	370.28	**<0.001**
Lea04	6.03	0.070	290.62	**<0.001**
CYRF	5.29	0.083	163.78	**<0.001**
CYL1	6.17	0.068	45.07	**0.003**
CYL2	8.32	**0.045**	102.72	**0.001**
CYLP	7.69	0.05	8.3	**0.045**
T3	8.34	**0.045**	369.99	**<0.001**



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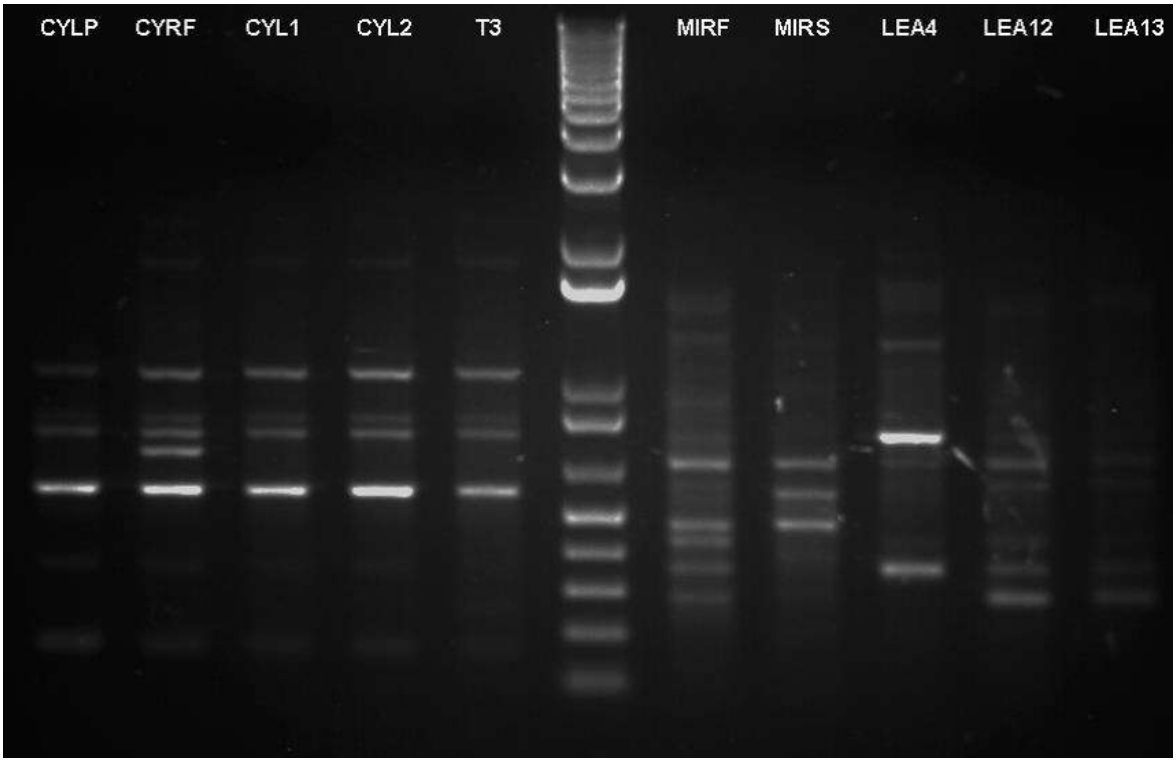

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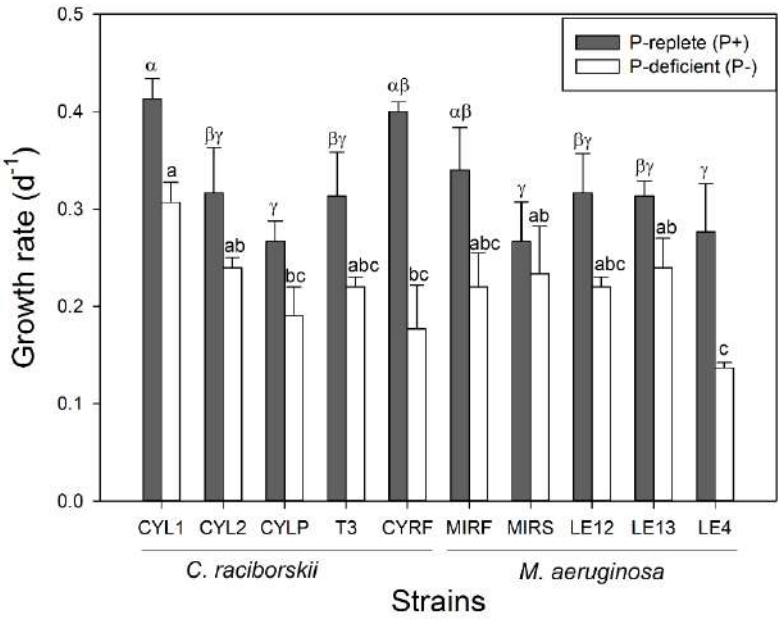

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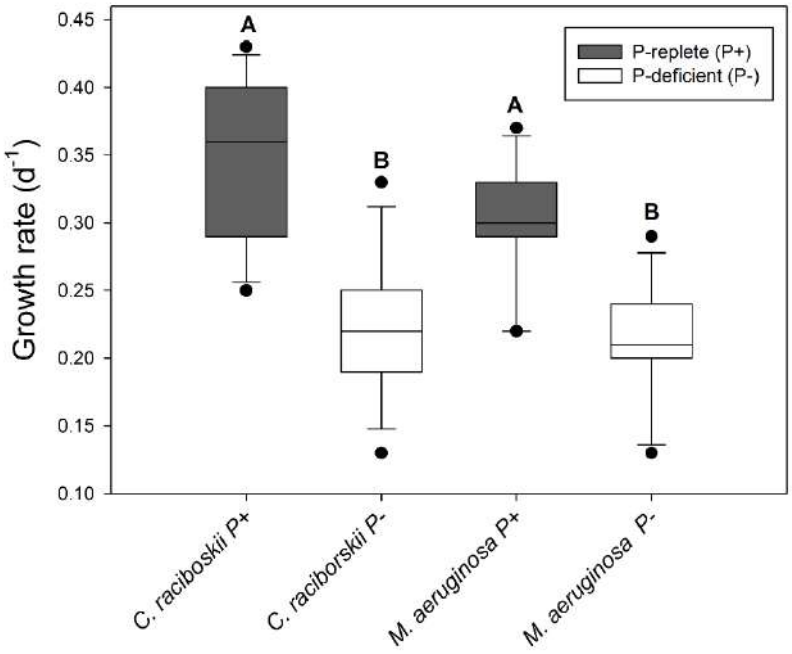

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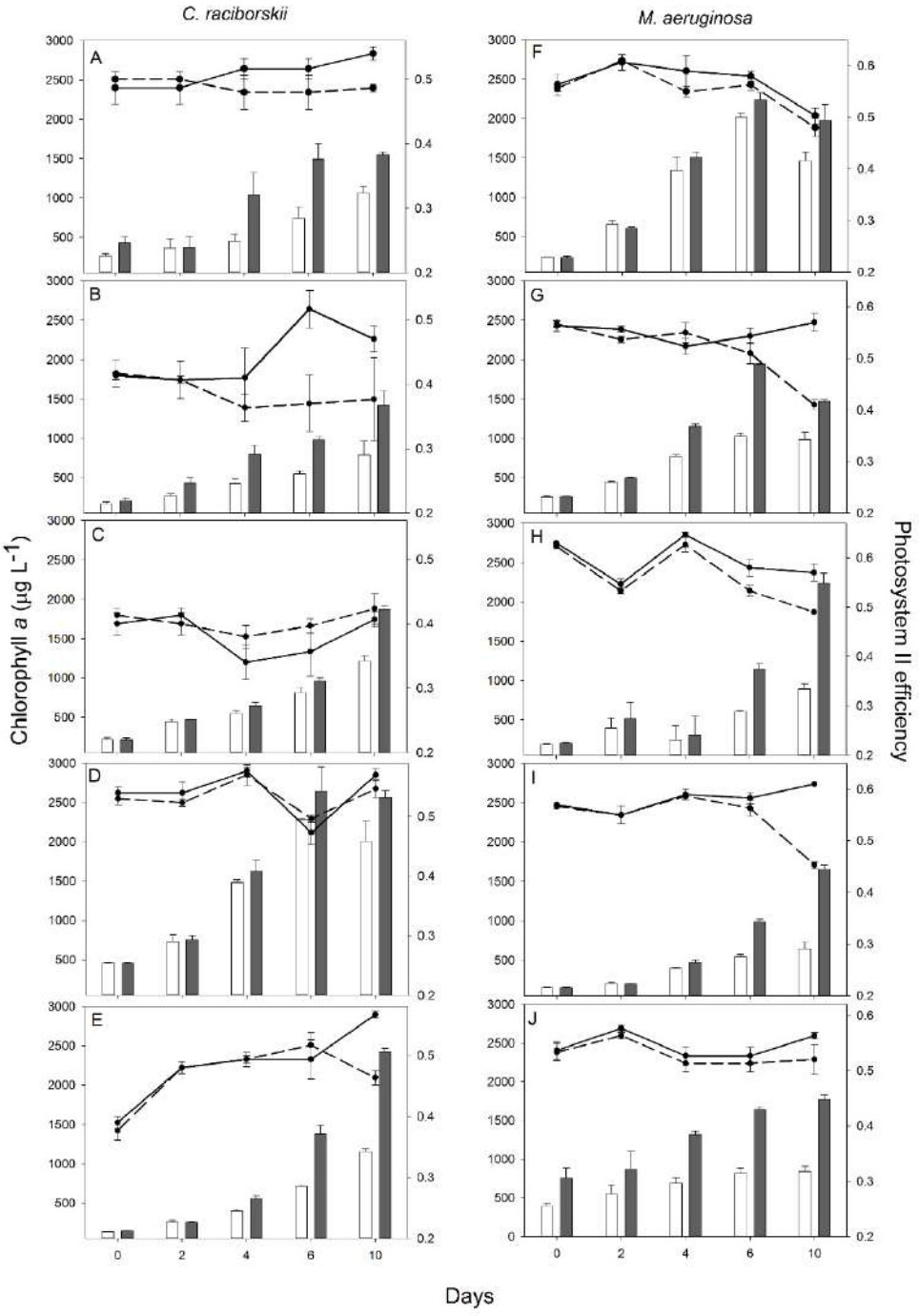

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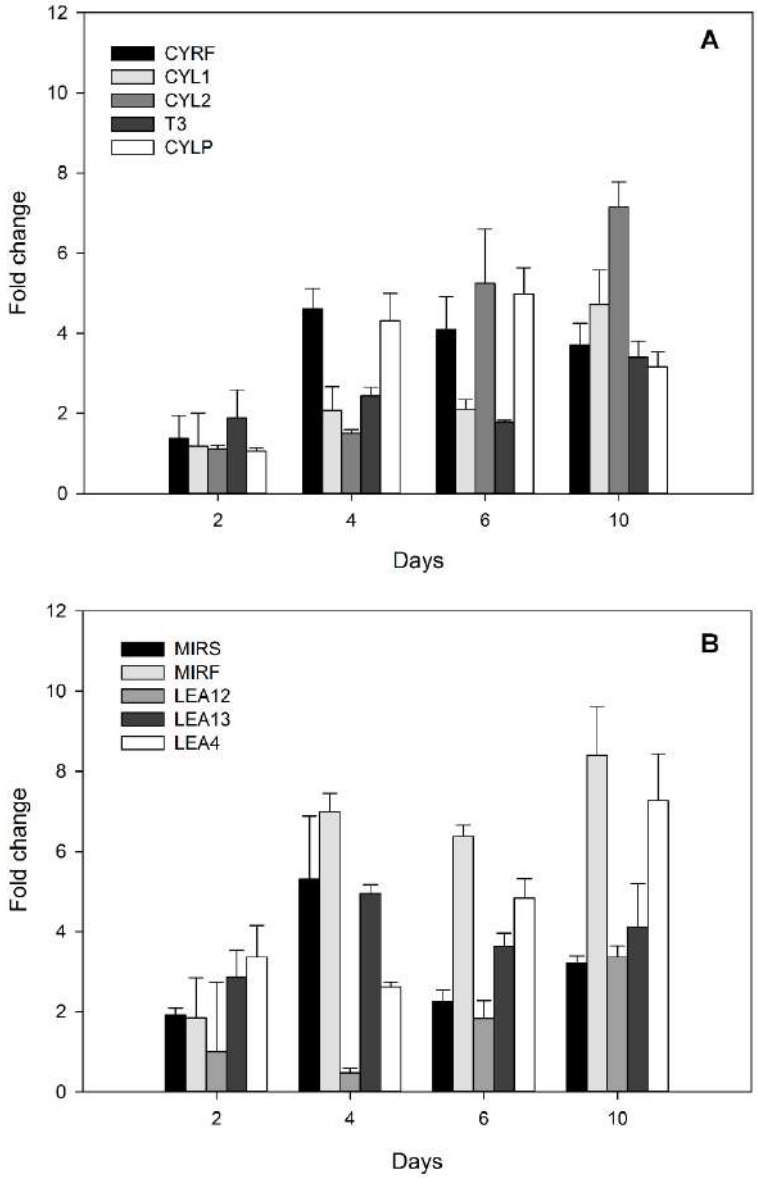

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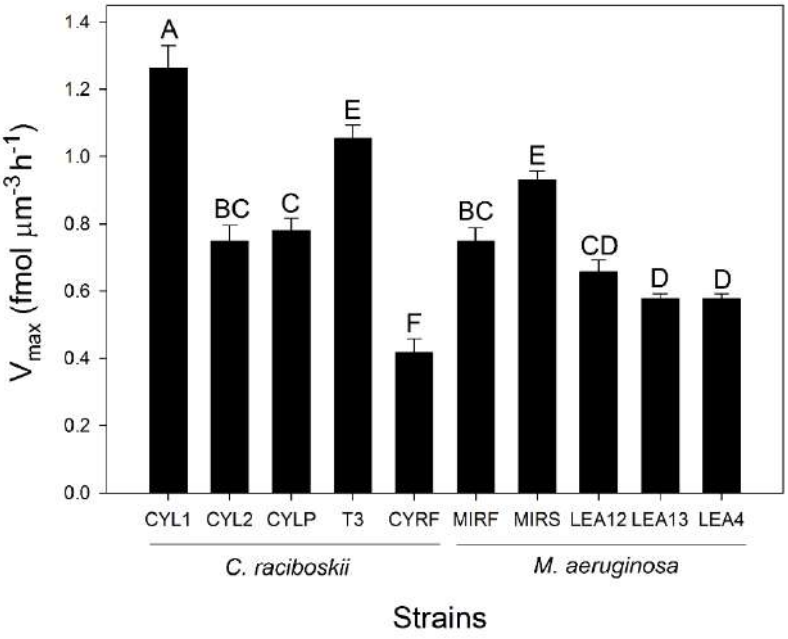

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Discussão Geral

Os resultados apresentados nesta tese ressaltam o papel de interações bióticas na dinâmica de florações de cianobactérias. O objetivo desta tese foi aprofundar o conhecimento sobre fatores associados a florações de cianobactérias tendo o reservatório do Funil como modelo, mas as questões aqui levantadas podem ajudar a compreendera dinâmica de florações de uma forma em geral.

No primeiro capítulo, a dinâmica de uma floração mista de cianobactérias foi avaliada juntamente com a dinâmica da comunidade de bactérias heterotróficas. A sucessão das diferentes espécies de cianobactérias durante o verão, principalmente a mudança da dominância de *M. aeruginosa* para *C. raciborskii*, já vem sendo observada no reservatório do Funil há muito tempo e esse fenômeno se repete anualmente (Ferrão-Filho *et al.*, 2009; Rangel *et al.*, 2016; Soares *et al.*, 2009). A influência de fatores abióticos, principalmente o tempo de retenção e a transparência, já vem sendo bem descrita como importante na sucessão de espécies neste reservatório. Interações alelopáticas entre *M. aeruginosa* e *C.racibosrkii* também já foram propostas como possível fator controlador, mas as observações foram feitas a partir de estudos em laboratório e o papel dessas interações neste reservatório ainda não é claro (Mello *et al.*, 2012). No presente estudo, novos componentes da comunidade foram considerados, incluindo a participação de *Synechococcus* como uma das principais espécies de cianobactérias e a comunidade de bactérias heterotróficas.

Synechococcus é um taxon de cianobactérias unicelulares presente em uma diversidade de ambientes, desde oceanos a ambientes de água doce (Callieri, 2007). Apesar do pequeno tamanho (<2µm) e portanto baixa contribuição em biomassa em relação a outros organismos fitoplanctônicos, sua participação na dinâmica fitoplanctônica não pode ser ignorada. A contribuição expressiva deste gênero nunca tinha sido reportada no Reservatório do Funil, possivelmente devido à dificuldade de quantificação pelo método de Uthermol, que é empregado usualmente nos estudos da comunidade fitoplanctônica. Uma questão que se coloca então é se o seu aparecimento após a floração de *M. aeruginosa* e concomitante à floração de *C. raciborskii* também segue um padrão anual e que fatores contribuem para sua abundância.

Esse é o primeiro estudo no Brasil que avalia a diversidade de bactérias heterotróficas associadas a uma floração de cianobactérias com o uso de NGS. Através dessa abordagem, foi possível observar os principais filos de bactérias associados à floração. *Proteobacteria*, *Bacteroidetes*, *Plantomycetes e Verrucromicrobia* foram os principais contribuintes para a comunidade e sua predominância já foi descrita durante florações de cianobactérias em outros ambientes (Li *et al.*, 2015; Te *et al.*, 2017; Woodhouse *et al.*, 2016). Além disso, foi possível revelar fortes associações entre determinadas OTUs de bactérias heterotróficas e diferentes gêneros de cianobactérias presentes durante a floração, como *Microcystis*, *Dolichospermum* e *Synechococcus*. Como destaque, a forte correlação entre *Microcystis* e *Cytophaga*. A ocorrência de linhagens de *Cytophaga* ao término de florações de *Microcytis* já foi reportada, assim como o isolamento e a caracterização de linhagens deste grupo capazes de causar a lise de cianobactérias em cultura (Rashidan & Bird, 2001). Apesar de co-ocorrências e correlações positivas entre abundâncias relativas de duas ou mais OTUs poderem indicar algum tipo de relação ecológica, tais inferências são apenas especulativas. Resultados de trabalhos como o presente, levantam hipóteses que devem ser testadas em cultivo a fim de investigar possíveis associações entre determinados grupos de bactérias heterotróficas e espécies de cianobactérias. Outra questão relevante a considerar é o significado ecológico de tais associações, uma vez que interações funcionais similares podem se estabelecer entre taxa distintos. Alguns estudos recentes apontam que apesar de uma alta variabilidade taxonômica, funcionalmente comunidades podem se manter estáveis (Louca *et al.*, 2016).

No segundo capítulo, a diversidade intraespecífica de populações de diferentes cianobactérias presentes no reservatório do Funil foi analisada. Aplicando-se diferentes níveis de porcentagem de identidade para distinção de OTUs (97, 99 e 100%) foi possível avaliar a flutuação de genótipos de cada população temporalmente.

Apesar de o gene 16S rRNA não ser o marcador mais utilizado para avaliar a diversidade intraespecífica de cianobactérias (Briand *et al.*, 2009; Miller *et al.*, 2013; Pobel *et al.*, 2012; Sabart *et al.*, 2015), foi possível observar pelo menos dois padrões entre os gêneros estudados. *Microcystis*, *Cylindrospermopsis* e *Pseudanabaena* foram caracterizados pela dominância de um genótipo em cada tempo amostral, e temporalmente foi observada uma sucessão de genótipos. Já *Synechococcus* e *Dolichospermum* foram caracterizados pela co-dominância de dois ou mais genótipos.

Usualmente, a diversidade de genótipos é avaliada em florações monoespecefíficas e este é o primeiro estudo que descreve a diversidade de genótipos em cinco gêneros de cianobactérias simultaneamente. Os resultados sugerem que os diferentes gêneros podem explorar diferentes estratégias adaptativas para manter suas populações durante a floração.

Além disso, também foi avaliada a relação entre a presença/abundância dos genótipos de gêneros potencialmente produtores de microcistina e a concentração desta toxina na água. Um estudo anterior neste reservatório avaliou simultaneamente a diversidade de genótipos (utilizando o locus *cpcBA*), a variação na abundância de genótipos tóxicos (*mcyB*+) e a concentração de microcistinas e não foram observadas correlações significativas entre os parâmetros avaliados (Guedes *et al.*, 2014). Assim como no presente estudo, não foi encontrada correlação positiva entre algum genótipo potencialmente produtor de microcistina e a concentração de microcistina na água. Deve-se considerar que a concentração de microcistina na água não depende apenas da biomassa de cianobactérias e da quantidade de células tóxicas, mas tabém da cota celular de microcistina, que pode ser influenciada por fatores ambientais (Pacheco *et al.*, 2016). Portanto, a falta de correlação entre a abundância de genótipos potencialmente tóxicos e a quantidade de microcistina é esperada.

Uma questão a ser levantada é que a delimitação de genótipos não possibilita a distinção de ecotipos. Com exceção de *Phroclorococcus* e *Synechococcus*, onde já foram reconhecidos alguns ecotipos que são suportados por determinados genótipos (com base em sequências 16S-23S ITS), para os outros grupos de cianobactérias essa correlação não foi estabelecida (Giovannoni & Stingl, 2005). Além disso, a definição de OTUs incorpora problemas, já que as sequências agrupadas em uma OTUs podem não corresponder a um único taxa. Em uma espécie de bactérias, linhagens diferentes podem evoluir em taxas diferentes e portanto, nenhum valor de corte universal irá capturar unidades equivalentes de diversidade em todas as linhagens bacterianas. Além disso, a definição de OTUs é baseada estritamente na identidade de sequências e informações filogenéticas não são levadas em consideração (Koeppel & Wu, 2013). Portanto, o real significado ecológico de um elevedo número de genótipos em uma população de cianobactérias precisa ser investigado.

No terceiro capítulo, a variabilidade intraespecífica de linhagens de *M. aeruginosa* e *C. raciborskii* foi avaliada, no que se refere à reposta à limitação de fósforo. Estudos anteriores levantaram a hipótese que *C. raciboskii* seria melhor competidora por fósforo que outras cianobactérias, incluindo *M. aeruginosa* (Antunes *et al.*, 2015). Contudo, grande parte desses estudos utilizou somente uma ou duas linhagens para a avliação dessa resposta (Bai *et al.*, 2014; Marinho *et al.*, 2013; Wu *et al.*, 2012). No presente estudo, foram utilizadas cinco linhagens de cada espécie, todas elas isoladadas de ambientes aquáticos brasileiros, incluíndo o Reservatório do Funil.

Apesar de algumas linhagens de *C. raciborskii* apresentarem maiores taxas de assimilação de fosfato, não foi observada diferença significativa entre as espécies, já que a variabilidade entre linhagens da mesma espécies foi expressiva. Estudos recentes têm demosntrado a grande variabilidade intraespecífica em traços fisiológicos de linhagens dessas espécies, mesmo quando isoladas de um mesmo corpo d'agua (Willis *et al.*, 2016; Xiao *et al.*, 2017). Esses estudos, incluindo o presente, demonstram a importância de se incluir muitas linhagens para conhecer a ecofisiologia de uma espécie e a dificuldade de extrapolar respostas obtidas a partir de uma linhagem para toda a espécie.

Uma observação interessante foi a expressiva capacidade de linhagens de *C. raciboskii* e *M. aeruginosa* de manter o crescimento por 10 dias, ainda que em menores taxas, na ausência de fósforo. Isso demonstra a capacidade de acúmulo de fósforo intracelular nessas espécies, possivelmente na forma de grânulos de polifosfato (Gonzalez-Esquer *et al.*, 2016). A capacidade de manter o crescimento, estocar fósforo intracelular e de produzir ectofosfatases pode explicar a persitência dessas espécies no Reservatório do Funil, mesmo com concentrações limitantes de fósforo dissolvido.

Essa tese de doutorado amplia o conhecimento da dinâmica de cianobactérias no Reservatório do Funil, uma vez que revela fatores que ainda não haviam sido considerados, como a participação da comunidade de bactérias heterotróficas e a importância da variabilidade intraespecífica nas populações dos principais gêneros formadores de florações neste ambiente. O desafio que permanece é atribuir significado ecológico a esses fatores bióticos aqui ressaltados na comunidade natural.

Conclusões

1- sse trabalho contribuiu com a investigação de mais um período de floração de cianobactérias no Reservatório do Funil e foi constatado um padrão semelhante ao já descrito em anos anteriores. Isso sugere que os fatores controladores dessa dinâmica continuem atuando, por mais que tenha havido diminuição da concentração de fósforo, alterações da hidrologia do reservatório e flutuações climáticas.

2- A associação da análise microscópica e molecular revelou, além do padrão conhecido, com dominância de *Microcystis*, *Cylindrospermopsis* e *Dolichospermum*, uma elevada contribuição de *Synechococcus*, associada ao período de dominância de *C. raciborskii*. Esta é a primeira vez que é relatada a contribuição significativa de *Synechococcus* , indicando que este pode ter um papel importante para a dinâmica de cianobactérias no reservatório do Funil.

3- Foi observada uma elevada diversidade de bactérias heterotróficas associada à floração de cianobactérias no Reservatório do Funil e essas podem contribuir por meio de interações bióticas para o estabelecimento e manutenção do padrão de substituição das principais espécies nesse reservatório.

4- Fortes correlações entre OTUs de cianobactérias e bactérias heterotróficas foram identificadas, indicando possíveis associações específicas, principalmente com *Microcystis* e *Synechococcus*.

5- Dois distintos padrões de estrutura de população foram identificados nos principais gêneros de cianobactérias potencialmete tóxicos, indicando diferentes estratégias adaptativas.

6- A concentração de microcistina na água não se correlacionou com a presença/abundância de nenhum genótipo específico.

7- Linhagens de *M. aeruginosa* e *C.raciborskii* apresentaram elevada diversidade nas suas respostas fisiológicas à privação de fósforo e portanto não foi possível definir que uma espécie é melhor competidora por esse recurso.

8- Linhagens de *M. aeruginosa* e *C.raciborskii* conseguiram manter o crescimento mesmo após 10 dias na ausência de fósforo, indicando que possuem mecanismos fisiológicos capazes de suportar a limitação desse macronutriente por um determinado período.

9- Em suma, este trabalho destaca aspectos variados da influência de fatores bióticos na ecofisiologia das cianobactérias

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Anexo 1

Como produção associada ao período de doutorado, foi publicada uma revisão intitulada “Is qPCR a reliable indicator of cyanotxin risk in freshwater?”