UNIVERSIDADE FEDERAL DO RIO DE JANEIRO

RESPOSTAS ADAPTATIVAS NA INTERAÇÃO *DAPHNIA*-CYANOBACTERIA: COMPORTAMENTO ALIMENTAR E DEFESAS QUÍMICAS INDUZIDAS

MAURO CESAR PALMEIRA VILAR

2020

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INSTITUTO DE BIOFÍSICA CARLOS CHAGAS FILHO

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Tese de Doutorado apresentada ao Programa de Pós-Graduação em Ciências Biológicas (Biofísica), Instituto de Biofísica Carlos Chagas Filho – Universidade Federal do Rio de Janeiro, como requisito à obtenção do título de Doutor em Ciências

Orientadora: Dra. Sandra Maria Feliciano de Oliveira e Azevedo

Universidade Federal do Rio de Janeiro

Centro de Ciências da Saúde

Instituto de Biofísica Carlos Chagas Filho

Rio de Janeiro

2020

Vilar, Mauro Cesar Palmeira.

Respostas adaptativas na interação *Daphnia*-Cyanobacteria: comportamento alimentar e defesas químicas induzidas. Mauro Cesar Palmeira Vilar. – Rio de Janeiro: UFRJ / Centro de Ciências da Saúde, Instituto de Biofísica Carlos Chagas Filho, 2020.

229 f.: il.; 31 cm.

Orientadora: Sandra Maria Feliciano de Oliveira e Azevedo.

Tese (doutorado) -- UFRJ, / Centro de Ciências da Saúde, Instituto de Biofísica Carlos Chagas Filho, Programa de Pós- Graduação em Ciências Biológicas (Biofísica), 2020.

Referências: f. 152-165.

1. Cianobactérias. 2. Fitoplâncton. 3. Zooplâncton. 4. Evolução Biológica. 5. Microcistinas. 6. Biofísica - Tese. I. Azevedo, Sandra Maria Feliciano de Oliveira e. III. UFRJ, CCS, Instituto de Biofísica Carlos Chagas Filho. IV. Título.

Ficha catalográfica elaborada pela equipe de Referência da Biblioteca Central do Centro de Ciências da Saúde da Universidade Federal do Rio de Janeiro.

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MAURO CESAR PALMEIRA VILAR

TESE DE DOUTORADO SUBMETIDA À UNIVERSIDADE FEDERAL DO RIO DE JANEIRO VISANDO A OBTENÇÃO DO GRAU DE DOUTOR EM CIÊNCIAS.

APROVADA POR:

RIO DE JANEIRO, 14 DE OUTUBRO DE 2019.

DRA. SUSANA FRASES CARVAJAL (DOUTOR – UFRJ)
COORDENADORA DO CURSO DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS (BIOFÍSICA)

DRA. SANDRA MARIA FELICIANO DE OLIVEIRA E AZEVEDO (DOUTORA – UFRJ) – ORIENTADORA

DRA. LUCIA HELENA SAMPAIO DA SILVA (DOUTORA – UFRJ) - REVISORA

DR. JEAN REMY DAVEE GUIMARÃES (DOUTOR – UFRJ)

DRA. MARIÂNGELA MENEZES (DOUTOR – UFRJ)

DR. RENATO CRESPO PEREIRA (DOUTOR – UFF)

AGRADECIMENTOS

Esta tese é mais que as pesquisas aqui relatadas. É sobre as pessoas que passaram na minha vida durante os últimos quatro anos e me deram suporte para que eu seguisse nessa caminhada até o fim. Portanto, mais que justo que eu demonstre aqui a minha gratidão.

Agradeço ao Programa de Pós-graduação em Ciências Biológicas (Biofísica) do Instituto de Biofísica Carlos Chagas Filho (IBCCF/UFRJ) onde tive o privilégio de cursar o Doutorado e aprender com Mestres que me ensinaram com um elevado nível de excelência. À Universidade Federal do Rio de Janeiro por ter me acolhido e me tornado parte da sua história. Parafraseando o Dr. Carlos Chagas Filho, "aqui se ensina porque se pesquisa". Agradeço também ao Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) pela concessão da bolsa e taxa de bancada.

Gratidão à valiosa e privilegiada orientação que recebi durante o Doutorado. À minha orientadora, Professora Sandra Maria Feliciano de Oliveira e Azevedo, de quem eu tenho um orgulho e admiração imensos, e foi a principal motivação para eu deixar o meu Nordeste e vir tentar a carreira no Rio de Janeiro. Sou muito grato por todo aprendizado como Cientista e cidadão; pela confiança depositada, oportunidade de desenvolver o meu potencial e fazer parte da família LETC. Não posso deixar de mencionar as dicas de culinária que eu sempre escutei atento, sobretudo a receita da farofa de cebola que desde então é item indispensável nos churrascos. Como ela mesma diz: "aqui a formação é completa! Eu ensino do laboratório à cozinha". Muitíssimo obrigado, chefa! Para mim foi e sempre será uma honra!

Também gostaria de agradecer imensamente ao Dr. Aloysio da Silva Ferrão-Filho que, mesmo que extra-oficialmente, desempenhou um papel fundamental na orientação desta tese. Gratidão pela confiança, pelos ensinamentos e pelo suporte. Para mim foi um privilégio poder trabalhar diretamente com um Cientista o qual eu admiro e que posso dizer que também foi um dos pilares da minha formação no mundo das cianobactérias. Estendo também os meus agradecimentos aos seus alunos Júlia Vianna, Gabriele dos Reis e Michael Ribas, pelo apoio e colaboração.

Um agradecimento mais que especial à Professora Valéria Magalhães que esteve comigo em todos os momentos durante esses quatro anos. Gratidão imensa pelo apoio, conselhos e ensinamentos valiosos; risadas, viagens, vinhos e costelinhas maravilhosos; e por ser essa pessoa próspera e iluminada que me deu suporte nos momentos difíceis.

Com ela eu aprendi Kabbela (Kabbalah por tabela, haha) e brincadeiras à parte, posso dizer que ela me ensinou a ser uma pessoa melhor. Gratidão pela sua amizade, môbem! Muito obrigado! Também estendo meus agradecimentos à Professora Ana Beatriz F. Pacheco pelos ensinamentos em Biologia Molecular, sua visão crítica e espírito jovem que são inspiradores.

À família LETC e demais amigos que me receberam no Rio de Janeiro: Iamê Guedes, Roberta Fernandes, Carolina Miranda, Ricardo Guedes, Roberta Guedes, Daniel Neves, Suema Branco, Sabrina Calado, Isabel Rocha, Raquel Moraes, Andreia Gomes, e às minhas raposinhas Flávia Muniz, Adriana Carvalho e Ricardo Rogers. Também não posso deixar de mencionar os meus alunos de Iniciação Científica os quais eu tive a oportunidade de orientar, compartilhar um pouco de conhecimento e aprender com cada um em sua singularidade. O meu muito obrigado à Gabriela Santos, Thiago Rodrigues e Luan Silva. Sem o suporte de vocês, certamente este trabalho não seria o mesmo!

Aos meus amigos pernambucanos: Josivan Soares, Felipe Cavalcante, Catarina Ramos, Aline Spíndola, Mayara Pessoa, Mayara Barbosa, Erika Santana e William Xavier pelo carinho, paciência e, sobretudo, pelos momentos de distração, risadas, desabafos que tornaram a distância RJ-PE mais curta. Estendo meus agradecimentos também ao querido Diego Rogaciano e meu *roomie* Leandro Silvino pelo carinho e companhia fundamentais para suportar todo caos da Pandemia da COVID-19 a qual tornou a escrita da tese um desafio maior do que costuma ser. Eu amo muito cada um de vocês!

Agradeço imensamente à minha família pelo amor, confiança e suporte dado desde quando eu decidi me tornar um Biólogo. À minha mãe Carminha, minhas avós Berenita e Ivonete, e meu avô Rosemiro (*in memoriam*) por terem sido os alicerces para que eu construísse a pessoa que sou hoje. Ao meu pai Walter Vilar por sempre se mostrar orgulhoso de mim. Às amadas tias Rita Palmeira, Rosinete Palmeira, Verônica Silva, Ceça Silva, Valéria Vilar e Verônica Vilar pelas palavras de conforto e encorajamento que sempre me motivaram a seguir essa jornada. Ao meu irmão Murilo e primos, em especial à minha prima Adriana pelo seu carinho, apoio e por ser um exemplo de altruísmo. Dedico a cada um de vocês esse título de Doutor! Muito obrigado, de coração!

Por fim, agradeço a Deus e todas as forças boas do Universo pelas pessoas maravilhosas que cruzaram o meu caminho durante essa jornada. Obrigado por cuidar sempre de mim e daqueles que eu amo. Dedico este trabalho a todos vocês!

“Para ser grande, sê inteiro: nada Teu exagera ou exclui. Sê todo em cada coisa. Põe quanto és no mínimo que fazes. Assim em cada lago a lua toda brilha, porque alta vive.”

Fernando Pessoa

Em dedicatória à minha família, meus amigos e Mestres que sempre acreditaram em mim.

RESUMO

VILAR, Mauro Cesar Palmeira - Respostas adaptativas na interação *Daphnia*-Cyanobacteria: comportamento alimentar e defesas químicas induzidas. Tese (Doutorado em Ciências Biológicas – Biofísica) – Instituto de Biofísica Carlos Chagas Filho, Universidade Federal do Rio de Janeiro, Rio de Janeiro, 2020.

Florações de cianobactérias são fenômenos recorrentes em corpos d'água eutrofizados e nas últimas décadas têm se intensificado devido a mudanças climáticas extremas. A dominância de cianobactérias e produção de cianotoxinas têm sido atribuídas a muitos fatores abióticos, como temperatura e nutrientes. Entretanto, o papel das interações biológicas ainda é pouco explorado, sobretudo na relação predador-presa com o zooplâncton, sendo a predação considerada uma das maiores pressões de seleção do fitoplâncton. Assim, esta tese teve como objetivo avaliar as respostas recíprocas na interação *Daphnia*-cianobactéria a fim de contribuir para o conhecimento da dinâmica eco-evolutiva do fitoplâncton e zooplâncton em ambientes aquáticos eutrofizados. No primeiro capítulo foi avaliado o comportamento alimentar do cladócero nativo de ambientes temperados *Daphnia similis*, e a espécie neotropical *D. laevis* submetidas a dietas simples e mistas compostas de alimento nutritivo e cianobactérias tóxicas (MCs e STXs) em diferentes proporções. Houve uma diminuição acentuada na taxa de filtração de *D. similis*, mas não para *D. laevis* mensurada pelo biovolume e clorofila-α. Por outro lado, observou-se uma redução significativa na taxa de ingestão de alimento nutritivo relacionada ao aumento na proporção de cianobactérias na dieta para ambas as espécies, as quais exibiram um comportamento alimentar generalista. Nos segundo e terceiro capítulos foi avaliada a defesa química nas cianobactérias *Microcystis aeruginosa* NPLJ-4 (MC+) e *Raphidiopsis raciborskii* T3 (STX+) induzida por infoquímicos de *D. gessneri*, além dos custos subjacentes à resposta de defesa através de análise de parâmetros de crescimento e fotossíntese. Ambas as cianobactérias apresentaram um aumento significativo na produção de cianotoxinas induzido por infoquímicos do zooplâncton, mas nenhuma variação morfológica, sugerindo uma resposta de defesa química induzida. Para *R. raciborskii*, o aumento na produção de saxitoxinas ocorreu subsequente à sobrerregulação de genes (*sxt*U e *sxt*I) da biossíntese de saxitoxina. Por outro lado, nenhum custo na fotossíntese foi registrado para ambas cianobactérias, mas uma redução pontual no crescimento de *R. raciborskii* T3. No quarto capítulo foi avaliada a resposta transgeracional das defesas antioxidantes e enzima de biotransformação (GST) em *D. gessneri* submetida a diferentes dietas compostas de *R. raciborskii* tóxica e algas verdes com perfis distintos de ácidos graxos. Neste estudo, a qualidade da dieta em relação à disponibilidade de itens alimentares nutritivos governou a resposta antioxidante transgeracional às cianobactérias produtoras de saxitoxina em *D. gessneri*. Esta tese destaca aspectos comportamentais e fisiológicos como respostas adaptativas na interação *Daphnia*-cianobactéria contribuindo para um melhor entendimento da dinâmica eco-evolutiva do plâncton.

Palavras-chave: cianotoxinas, zooplâncton, fitoplâncton, infoquímicos, eco-evolução

ABSTRACT

VILAR, Mauro Cesar Palmeira - Adaptive responses in the *Daphnia*-Cyanobacteria interaction: feeding behavior and induced chemical defenses. Thesis (PhD in Biological Sciences - Biophysics) - Carlos Chagas Filho Biophysics Institute, Federal University of Rio de Janeiro, Rio de Janeiro, 2020.

Cyanobacterial blooms are recurrent phenomena in eutrophic water bodies and in the last decades have been intensified due to extreme climate changes. Much has been attributed to abiotic factors such as temperature and nutrients to the dominance of cyanobacteria and cyanotoxin production. However, the role of biological interactions is still little explored, especially in the predator-prey relationship with zooplankton, where predation is considered one of the strongest selection pressures on phytoplankton. Thus, this thesis aimed to evaluate the reciprocal responses in the *Daphnia*-cyanobacteria interaction in order to contribute to the knowledge of the eco-evolutionary dynamics of phytoplankton and zooplankton in eutrophic environments. In the first chapter, the feeding behavior of the native cladoceran from temperate inland waters *Daphnia similis* and the neotropical species *D. laevis* was exposed to simple and mixed diets composed of nutritious food and toxic cyanobacteria (MCs and STXs) in different proportions. There was a marked decrease in the filtration rate of *D. similis*, but not for *D. laevis* measured by both biovolume and chlorophyll-a data. On the other hand, there was a significant reduction in the rate of nutritious food ingestion related to the increase in the proportion of cyanobacteria in the diet for both species, which exhibited a generalist feeding behavior. In the second and third chapters, chemical defense in the cyanobacteria *Microcystis aeruginosa* NPLJ-4 (MC +) and *Raphidiopsis raciborskii* T3 (STX +) induced by *D. gessneri* info-chemicals was evaluated, in addition to the costs underlying the defense response through analysis of growth and photosynthesis. Both cyanobacteria showed a significant increase in cyanotoxin production induced by zooplankton info-chemicals, but no morphological variation, suggesting an induced chemical defense response. For *R. raciborskii*, the increase in the production of saxitoxins occurred subsequent to an up-regulation of STX-biosynthesis genes (*sxt*U and *sxt*I). On the other hand, no cost in photosynthesis was registered for both cyanobacteria, but a short reduction in the growth of *R. raciborskii* T3. In the fourth chapter, the transgenerational response of antioxidant defenses and biotransformation enzyme (GST) was evaluated in *D. gessneri* submitted to different diets composed of toxic *R. raciborskii* and green algae with different fatty acid profiles. In this study, the quality of the diet in relation to the availability of PUFAs-rich nutritious food items governed the transgenerational antioxidant response to the saxitoxin-producing cyanobacteria in *D. gessneri*. This thesis highlights feeding behavior and physiological aspects as adaptive responses in the *Daphnia*-cyanobacteria interaction contributing to a better understanding of the eco-evolutionary dynamics of plankton.

Keywords: cyanotoxins, zooplankton, phytoplankton, infochemicals, eco-evolution

SUMÁRIO

APRESENTAÇÃO DA TESE

A presente tese está estruturada em sete partes, sendo uma **Introdução geral** na qual é apresentado o estado da arte das cianobactérias e suas toxinas; a interação de cianobactérias e o zooplâncton, bem como as respostas recíprocas dessa interação, descritas como a aquisição de tolerância a cianobactérias pelo zooplâncton e as respostas de defesas induzidas em cianobactérias tóxicas, mediadas pelo predador. Em seguida, são apresentados os **Objetivos** geral e específicos da tese e as respectivas **hipóteses** que foram testadas.

As hipóteses desenvolvidas foram contempladas em quatro capítulos sendo o **Capítulo 1** referente ao comportamento alimentar de espécies de *Daphnia* submetidas à dieta de cianobactérias tóxicas. Nos **Capítulos 2 e 3** foram abordadas as defesas químicas induzidas pelo zooplâncton e seus possíveis custos para as cianobactérias tóxicas *Microcystis aeruginosa* e *Raphidiopsis raciborskii*, sendo nesta última espécie também avaliada a expressão de genes relacionados à biossíntese de saxitoxinas. Em seguida, no **Capítulo 4** foi avaliado o efeito do ambiente alimentar maternal na resposta transgeracional do sistema de defesas antioxidantes e biotransformação em *Daphnia gessneri.* Também é apresentada uma **Discussão geral** com uma reflexão integrada de todos os capítulos.

Subsequentemente, a partir dos principais resultados apresentados na presente tese, foram feitas as **Conclusões** as quais estão seguidas da lista de **Referências bibliográficas** consultadas para a elaboração da introdução e discussão geral. Por fim, no ítem **Anexos** consta o total de trabalhos apresentados e publicados em anais de congressos nacionais e internacionais, assim como os artigos científicos publicados durante o período de vigência do doutorado.

INTRODUÇÃO GERAL

Cianobactérias e cianotoxinas

Cianobactérias são microrganismos procariotos oxi-fotossintetizantes capazes de colonizar diversos habitats (WHITTON; POTTS, 2000). Nos corpos d'água esses microrganismos se distribuem na região pelágica (constituindo parte do fitoplâncton) ou aderidos a substratos (bentos e perifíton), podendo também ser encontrados em ambientes terrestres (aerofíticas). Considerando a longa história evolutiva (~3 bilhões de anos), as cianobactérias dispõem de estratégias adaptativas morfológicas e fisiológicas suportadas por um genoma variável que permite ao grupo ter flexibilidade para tolerar diversas pressões ambientais (ex.: flutuações na temperatura, CO_2, nutrientes, predação, etc.), o que explica o sucesso de diferentes gêneros em se dispersar e formar florações (ver LARSSON; NYLANDER; BERGMAN, 2011 e HUISMAN et al., 2018).

De forma geral, florações de cianobactérias são definidas como uma alteração visível na coloração da água causada pelo acúmulo de células em suspensão, sendo comuns em corpos d'água eutrofizados – ricos em nitrogênio e fósforo – e reportadas com frequência nas últimas décadas devido ao aumento na concentração de CO_2 atmosférico e mudanças climáticas extremas (O'NEIL et al., 2012; HUISMAN et al., 2018). Por serem componentes naturais do fitoplâncton, as cianobactérias estão na base da teia trófica aquática, contribuindo também para a transferência de energia nesses sistemas. Entretanto, diversos gêneros são capazes de produzir uma variedade de metabólitos secundários bioativos denominados cianotoxinas os quais, com base nos efeitos em vertebrados, são classificados como: hepatotoxinas, neurotoxinas, citotoxinas e lipopolissacarídeos irritantes à pele – endotoxinas.

Quanto às características químicas e mecanismo de ação, as hepatotoxinas são caracterizadas como penta- (nodularinas, NOD) ou heptapeptídeos (microcistinas, MCs) cíclicos **(Figura 1)** inibidores de enzimas fosfatases 1 e 2A (CARMICHAEL, 1992), sendo as MCs mais estudadas e reportadas para águas interiores. Mais de 100 variantes de MCs já foram descritas com peso molecular variando de 900 a 1100 Da e podem variar com base em diferentes combinações de aminoácidos nas posições 2 e 4 da molécula dentre outras mudanças químicas (ex.: metilação) (PUDDICK et al., 2015).

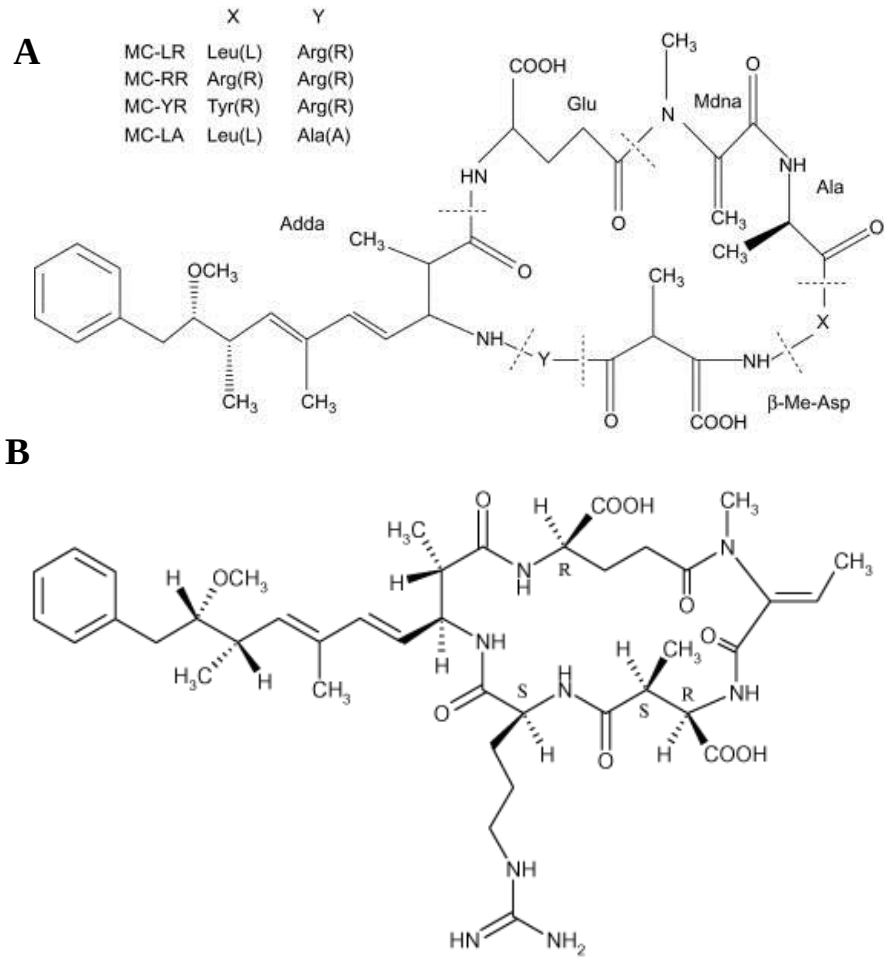

Figura 1. Estrutura geral da microcistina (A) e alguns dos análogos mais comuns (MC-LR, -RR, -YR e -LA) que variam principalmente de acordo com os aminoácidos na posição 2 (X) e 4 (Y), e estrutura geral da nodularina (B).

Por sua vez, o grupo das neurotoxinas constitui toxinas que, apesar de compartilharem o mesmo potencial neurotóxico, se distinguem quimicamente **(Figura 2)** e quanto ao mecanismo de ação, sendo representadas pelas: saxitoxinas (STXs) (C-toxinas, goniautoxinas, saxitoxinas e LWTs – toxinas de *Microseria* (*Lyngbya*) *wollei*) – constitui cerca de 56 variantes de alcaloides guanidínicos que atuam no bloqueio de canais iônicos (Na^+ e Ca^{2+}) e modulam o padrão de bloqueio de canais de potássio (K^+) nas células (WIESE et al., 2010; CUSICK, SAYLER, 2013); β-metilamino-L-alanina (BMAA) – aminoácido agonista de receptores dos neurotransmissores glutamato e AMPA (Ácido alfa-amino-3-hidroxi-5-metil-4-isoxazol-propiônico) com potencial excitotóxico, também tendo sido relacionado à neurodegeneração devido à incorporação e modificação pós-traducional de proteínas (*protein misfolding*) (BANACK; CALLER; STOMMEL,

2010; GLOVER et al., 2014); anatoxina-a e homoanatoxina-a (ATX e HTX) são alcalóides constituídos de aminas bicíclicas que atuam como potentes agentes pré- e pós-sinápticos competindo com a acetilcolina como agonistas de receptores nicotínicos nas junções neuromusculares e no sistema nervoso central (BURATTI et al., 2017); guanitoxina (GNT) (anterior anatoxina-a(s)) é um organofosforado inibidor irreversível da acetilcolinesterase (AChE) que atua especificamente no sistema nervoso periférico, sendo cerca de 10 vezes mais tóxico que a ATX (FIORI et al., 2020).

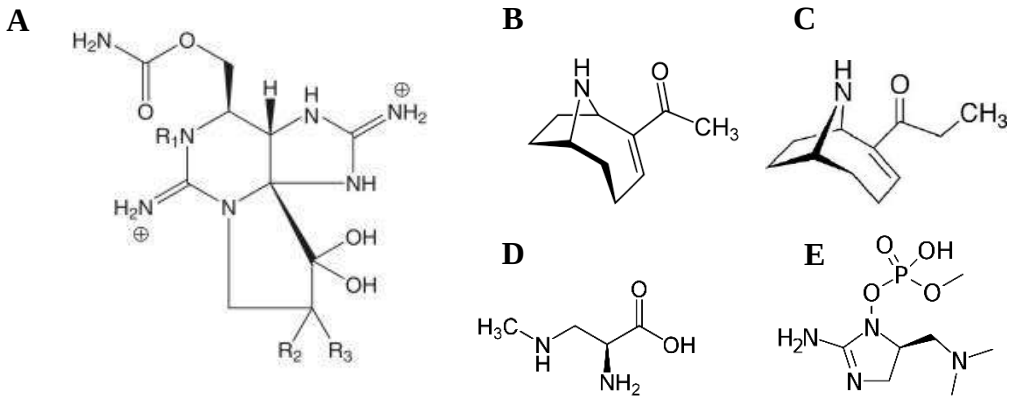

Figura 2. Estrutura geral das saxitoxinas (A) com análogos variando quanto à presença de hidroxilas na posição R1 e grupamentos sulfato (GTXs e C-toxinas) e acetato (LWTs) na R2 e R3; anatoxina-a (B), homoanatoxina-a (C), β-metilamino-L-alanina (D) e guanitoxina (E).

Na classe das citotoxinas está a cilindrospermopsina (CYN) e seus análogos (7-epicilindrospermopsina e 7-deoxicilindrospermopsina) que são alcalóides tricíclicos de potencial citotóxico cujo mecanismo de ação é a inibição da síntese proteica, mas também já são descritos efeitos genotóxicos, imunotóxicos e danos oxidativos (BURATTI et al., 2017; SCARLETT et al., 2020). Além disso, cianobactérias possuem na membrana externa do envelope celular, lipopolissacarídeos (LPS) que são endotoxinas características de bactérias gram-negativas e têm sido associadas a irritações na pele em consequência de respostas inflamatórias (DURAI; BATOOL; CHOI, 2015).

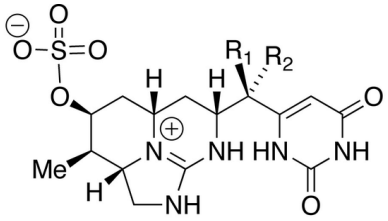

Figura 3. Estrutura geral da cilindrospermopsina e seus análogos que variam quanto à presença do radical hidroxila (-OH) nas posições R1 e R2.

Em sua maioria, as florações são formadas por espécies dos gêneros *Microcystis*, *Dolichospermum* e *Raphidiopsis* os quais são potencialmente produtores de cianotoxinas (SOARES et al. 2013). No gênero *Raphidiopsis,* destaca-se a espécie *Raphidiopsis raciborskii*, que em países da América do Sul tem sido reportada como produtora de STXs ou PSTs (*paralytic shellfish toxins*), enquanto em outros países há uma prevalência de linhagens produtoras de CYN. Esta espécie é considerada oportunista, estando seu predomínio associado à plasticidade de respostas a diferentes condições ambientais (ANTUNES; LEÃO; VASCONCELOS, 2015; BURFORD et al., 2016).

Além das cianotoxinas, as cianobactérias também podem produzir outros compostos bioativos, tais como cianopeptolinas, microviridinas, aeruginosinas e microgininas, os quais são denominados cianopeptídeos (JANSSEN, 2019). Esses têm recebido grande destaque devido ao seu potencial inibitório de serino-proteases digestivas, como tripsinas e quimiotripsinas, sendo amplamente descritos para gêneros também produtores de microcistinas como *Microcystis* (VON ELERT et al., 2005) e *Planktothrix* (KURMAYER et al., 2015). Em contrapartida, relatos da produção de cianopeptídeos por espécies produtoras de STXs, como *R. raciborskii* ainda são escassos. Silva-Stenico et al. (2011) foram pioneiros ao identificar cianopeptídeos nessa espécie ao detectar microgininas na linhagem tóxica *R. raciborskii* T3.

A dinâmica de cianotoxinas (e demais metabólitos) no ambiente ainda tem sido pouco compreendida, uma vez que o seu papel ecológico não está bem esclarecido. Possíveis vantagens adaptativas da produção de toxinas têm sido apontadas por Holland e Kinnear (2013) e Omidi, Esterhuizen-Londt e Pflugmacher (2018) os quais discutem a importância desses metabólitos para o sucesso competitivo, através de efeitos alelopáticos, toxicidade e persistência; e/ou ‘facilitadores fisiológicos’, no aprimoramento de atividades metabólicas, tais como eficiência fotossintética. Além da produção de metabólitos bioativos, a elevada versatilidade em atributos morfológicos como a formação de grandes filamentos e colônias, favorece a resistência à predação (GER et al., 2016; RANGEL et al., 2020). Em geral, juntamente a fatores abióticos, tais atributos são apontados por favorecer a dominância de cianobactérias. Por sua vez, tais fenômenos podem exercer mudanças não apenas na qualidade da água, mas sobretudo na estrutura e funcionamento desses ecossistemas, gerando perda de biodiversidade, afetando diretamente a comunidade de consumidores (ver AMORIM et al., 2019; 2020) e limitando o fluxo de energia nas teias tróficas aquáticas.

Interação Cianobactéria–Zooplâncton

Apesar de seu potencial toxicidade e baixo valor nutricional, as cianobactérias também representam uma importante alternativa à dieta do zooplâncton onívoro pois é um grupo representativo do fitoplâncton – produtores primários – e também promovem a transferência de energia na teia trófica aquática (AGRAWAL, 1998). No entanto, devido à pressão de herbivoria exercida pelo zooplâncton, o fitoplâncton evoluiu uma gama de atributos de defesa (VAN DONK; IANORA; VOS, 2011; PANČIĆ; KIØRBOE, 2018), sobretudo cianobactérias, devido à ampla versatilidade morfológica e química as quais têm sido atribuídas à dominância desse grupo em águas interiores eutrofizadas.

A hipótese mais aceita considera que a interação cianobactéria-zooplâncton é mantida por pressões evolutivas que definem um balanço entre a produção de metabólitos tóxicos como defesa química contra a predação, (LAMPERT, 1981; HANSSON et al. 2007) e a seleção de atributos de tolerância no zooplâncton (GUO; XIE, 2006; LI; JIANG, 2014). Assim, a interação predador-presa pode exercer uma pressão evolutiva que altera a aptidão de ambos indivíduos e levar à coevolução (PU; CORTEZ; JIANG, 2017). Tais *feedbacks* evolutivos podem não apenas alterar a frequência genotípica, mas também estabelecer respostas recíprocas em espécies fenotipicamente plásticas, como a produção de defesas anti-herbivoria no fitoplâncton.

Assumindo que há uma demanda energética na produção de atributos defensivos, a hipótese da defesa ótima (*optimal defense hypothesis* – ODH; McKEY, 1974), inicialmente baseada em plantas superiores, tem sido frequentemente aplicada para avaliar a relação custo-benefício das defesas (induzidas ou constitutivas), considerando seus impactos na aptidão (MELDAU; ERB; BALDWIN, 2012). Em 1974, McKEY analisou a distribuição de alcalóides em diferentes tecidos de plantas e concluiu que o armazenamento desses compostos defensivos estaria restrito a órgãos cuja presença é preponderante para o aumento do *fitness*. No entanto, embora as defesas no fitoplâncton sejam restritas a todo organismo, a suposição que defesas são benéficas, apesar dos custos associados ainda se mostra um princípio relevante a ser testado.

No final da década de 80 foi proposto o modelo PEG (*Plankton Ecology Group*; SOMMER et al., 1986) para descrever padrões sazonais da biomassa do plâncton em lagos eutróficos e oligotróficos. Neste modelo o alimento disponível para o zooplâncton é considerado apenas em termos de biomassa total do fitoplâncton, embora em lagos com elevada produtividade uma mudança sazonal dessa comunidade à predominância de

cianobactérias resulte em mudanças na qualidade nutricional, defesas químicas e atributos morfológicos da assembleia fitoplanctônica (SOMMER et al., 2012) afetando diretamente os consumidores. Mudanças em atributos do fitoplâncton já têm sido descritas por promover a diversificação coevolutiva do zooplâncton (PU; CORTEZ; JIANG, 2017), tendo um papel fundamental na dinâmica eco-evolutiva de consumidores-chave como os cladóceros (SCHAFFNER et al., 2019).

De forma geral, a taxa específica de ingestão do zooplâncton dulciaquícola mostra-se inversamente proporcional ao seu tamanho, a exemplo dos cladóceros que tendem a ter uma maior taxa de filtração, quando comparados a copépodos e rotíferos (PETERS; DOWNING, 1984). Estes últimos apresentam maior diversidade genética na natureza e uma menor eficiência de filtração que pode estar associada a um forrageamento seletivo, sendo indicado como uma característica-chave na manutenção desses organismos sob densas florações, ingerindo presas nutritivas e evitando a ingestão de partículas tóxicas ou inertes (GER et al., 2016). No entanto, essa seletividade tem como consequência um fraco controle *top-down* do fitoplâncton (SOMMER; SOMMER, 2006).

Por outro lado, cladóceros são consumidores generalistas que desempenham um papel ecológico fundamental no controle do fitoplâncton dulcícola, sobretudo espécies de *Daphnia*. Apesar de serem grandes filtradores, dafinídeos tendem a ter capacidade reduzida na seleção das partículas que ingerem, sendo diretamente afetados por flutuações do fitoplâncton que levam a variações quali-quantitativas na dieta desses animais (KOUSSOROPLIS et al., 2017; FERRÃO-FILHO et al., 2019), embora já haja relatos de seletividade pequenos cladóceros (FERRÃO-FILHO et al., 2017). No entanto, embora tenham um genoma mais estável, cladóceros podem expressar diferentes fenótipos sob gradientes ambientais (plasticidade fenotípica), como variações no plâncton que culminem na predominância de cianobactérias tóxicas, podendo esses fenótipos adaptativos ser transferidos às gerações subsequentes *via* efeito materno (HAIRSTON et al., 2001; ORSINI et al., 2016). Já tem sido documentado que a evolução rápida desses consumidores em resposta à eutrofização e consequente ocorrência de florações nocivas pode ter implicações a nível ecossistêmico (CHISLOCK et al., 2019) como a supressão do desacoplamento trófico em virtude da dominância de cianobactérias no fitoplâncton. Portanto é importante entender a dinâmica eco-evolutiva na interação cianobactéria-zooplâncton a fim de gerar modelos cada vez mais robustos na predição de impactos na estrutura de teias tróficas em consequência da degradação dos corpos d'água.

Tolerância a cianobactérias em cladóceros

A persistência de algumas espécies do zooplâncton durante períodos de floração de cianobactérias tem demonstrado que estes animais dispõem de estratégias para a manutenção das populações (GER; HANSSON; LÜRLING, 2014). Registros da literatura têm evidenciado em vários cladóceros a evolução rápida da tolerância a cianobactérias **(Tabela 1)**, no entanto as respostas adaptativas podem variar entre clones (HAIRSTON et al., 2001; LEMAIRE et al., 2012; JIANG et al., 2013) e gêneros (GUO; XIE, 2006) da mesma ou de diferentes localidades. Assim, é importante distinguir entre adaptações baseadas na seleção natural daquelas baseadas na plasticidade fenotípica (GER; HANSSON; LÜRLING, 2014). Além disso, atributos do zooplâncton que conferem resistência a cianobactérias podem não apenas se manifestar em populações ativas, mas também em populações dormentes – ovos de resistência no sedimento (ORSINI et al., 2016).

Sabe-se que a plasticidade de respostas pode não apenas facilitar a expressão de fenótipos relativamente bem adaptados sob novas condições e permitir a uma população persistir; mas pode também afetar a eficiência e sucesso reprodutivo de organismos individuais e impactar nas próximas gerações (PIGLIUCCI et al., 2006; RICHARDS, 2006). Há mais de uma década Agrawal et al. (1999) já afirmavam que o ambiente parental prediz a qualidade do ambiente da progênie. Assim, esses organismos podem aumentar seu sucesso reprodutivo a partir de "efeitos maternais adaptativos", dotando sua prole de fenótipos que lidem com potenciais ameaças, tais como predação e substâncias nocivas, mantendo um *fitness* estável da população, mesmo em condições consideradas adversas. Assim, sugere-se que um aumento gradativo na biomassa de cianobactérias seja o gradiente ambiental que direcionará a comunidade à persistência de espécies ou clones (genótipos) tolerantes do zooplâncton, como ressaltado por Ger et al. (2014). Os autores afirmam que a ecologia e rápida evolução de adaptações selecionadas pela interação cianobactéria-zooplâncton provavelmente regularão a estrutura e função dos sistemas planctônicos em condições mais eutróficas.

Tais atributos de tolerância podem estar relacionados não apenas à eficiência do sistema de detoxificação, mas também a um aumento na expressão de enzimas digestivas, em contrabalanço à ingestão de peptídeos inibidores dessas enzimas. A sobrerregulação de genes associados à codificação de enzimas do sistema antioxidante, como a glutationa-S-transferase (ORTIZ-RODRIGUEZ; DAO; WIEGAND, 2012; WOJTAL-

FRANKIEWICZ et al., 2013; OEXLE et al., 2016; WANG et al., 2016), mecanismos de efluxo celular de compostos tóxicos (*MDR resistence ABC transporters*) e permeases (SCHWARZENBERGER et al., 2014) são alguns dos marcadores bioquímicos/celulares da tolerância a cianobactérias. Além disso, também são reportadas respostas em função do valor nutritivo da dieta, como o aumento na expressão de proteases tripsina e quimiotripsina, em resposta à presença de cianopeptídeos e ao elevado valor proteico das cianobactérias (SCHWARZENBERGER; ELERT, 2013), em detrimento da sobrerregulação de lipases induzida por dietas ricas em ácidos graxos essenciais (KOUSSOROPLIS et al., 2017). Dessa forma, supõe-se que a resposta plástica a variações na disponibilidade de alimento para o zooplâncton possa garantir um melhor funcionamento do sistema *detox* e aproveitamento da energia proveniente da dieta, podendo promover um bom estado fisiológico e maximização da aptidão.

Além da resposta genética, é importante considerar também que reguladores epigenéticos são de particular interesse para o estudo da plasticidade fenotípica, pois estudos prévios indicam a mudança do fenótipo em resposta a um gradiente ambiental com o estado *on/off* ou o nível de expressão quantitativa dos genes (MARDEN, 2008; MENZEL et al., 2011). A regulação epigenética representa a reprogramação do genoma para expressar um conjunto adequado de genes em células específicas em um dado momento da vida, a qual se constitui de mecanismos de metilação no DNA, acetilação de proteínas histonas, rearranjo da cromatina e da maquinaria de pequenos RNAs (GENG; GAO; YANG, 2013). Este mecanismo é particularmente importante para organismos com baixa diversidade genética, permitindo a adaptação a diferentes condições ambientais, sendo provavelmente uma resposta evolutiva favorável em períodos de estresse que durem um tempo inferior ao ciclo de vida.

Tais informações reforçam a hipótese da tolerância e a sua transferência transgeracional. Assim, supõe-se que populações naturais de cladóceros apresentem respostas recíprocas em função da composição e abundância do fitoplâncton, de forma que o aumento de espécies nocivas e não-palatáveis promovam a seleção de organismos com o metabolismo otimizado para esse tipo de dieta. Além disso, entender qual o *turning-point* nesse estado transcricional transiente dos genótipos que culminarão em fenótipos de maior valor adaptativo é fundamental para conhecer o papel ecológico e, sobretudo evolutivo, da expressão desses atributos funcionais em diferentes cenários ambientais.

Tabela 1. Espécies de cladóceros avaliados quanto à tolerância transgeracional a cianobactérias/cianotoxinas. MC-LR: Microcistina-LR; MC+: produtor de microcistina; MC-: não produtor de microcistina; BMAA: β-Metilamino-L-alanina. *mcy*: gene que codifica a microcistina

ESPÉCIE	CIANOBACTÉRIA / TOXINA PURIFICADA	REFERÊNCIA
Daphnia magna	*Microcystis aeruginosa* (NIVA-CYA 228/1 MC+)	Gustafsson e Hansson (2004)
	M. aeruginosa (NIVA-CYA 228/1 MC+ e NIVA-CYA 143 MC-)	Gustafsson, Rengefors e Hansson (2005)
	M. aeruginosa (UTEX LB 2063 e PCC 7806 *mcy*+ e *mcy Knockout* PCC 7806)	Schwarzenberger et al. (2009)
	M. aeruginosa (NIVA Cya 43 e PCC 7806 *mcy*+ e *mcy Knockout* PCC 7806	Schwarzenberger et al. (2010)
	M. aeruginosa (*mcy Knockout* PCC 7806)	Schwarzenberger, Kuster e Von Elert (2012)
	MC-LR purificada	Ortiz-Rodriguez, Dao e Wiegand (2012)
	M. aeruginosa (cepas PCC 7806 *mcy*+ e *mcy* gene *Knockout* PCC 7806)	Schwarzenberger & Von Elert (2013)
	M. aeruginosa (Cepas UTEX LB 2063; PCC 7806 *mcy*+ e *mcy Knockout* PCC 7806)	Schwarzenberger et al. (2014)
	M. aeruginosa PCC 7806 *mcy*+)	Lyu et al. (2015); (2016)
	BMAA	Faassen et al. (2015)
Daphnia carinata	*M. aeruginosa* (cepas PCC 7806 *mcy*+ e *mcy Knockout* PCC 7806)	Guo & Xie (2006)
	M. aeruginosa (FACHB-905 MC+)	Jiang et al. (2013)
Daphnia pulicaria	*M. aeruginosa* (UTEX 2667 MC+)	Sarnelle & Wilson (2005)
Daphnia pulex	*M. aeruginosa* (FACHB-905 MC+)	Li & Jiang (2014)
		Jiang et al. (2015)
Daphnia galeata	*M. aeruginosa* (MC+)	Hairston et al. (2001)
Moina micrura	*M. aerugnosa* (Cepas PCC 7806 *mcy*+ e *mcy Knockout* PCC 7806)	Guo e Xie (2006)
Moina macropora	Amostra de floração de *M. aeruginosa*	Alva-Martinez, Sarma e Nandini (2007)
Ceriodaphnia cornuta	*M. aerugnosa* (Cepas PCC 7806 *mcy*+ e *mcy Knockout* PCC 7806)	Guo e Xie (2006)
Ceriodaphnia dúbia;	*M. aeruginosa*	Alva-Martinez, Sarma e Nandini (2007)
Simocephalus vetulus	*M. aerugnosa* (FACHB-905 MC+)	Chen, Zhang e Jiang (2015)
Bosmina longirostris	*M. aerugnosa* (FACHB-905 MC+)	Jiang et al. (2014)

Defesas induzidas em cianobactérias como resposta à predação

De forma geral defesas induzíveis permitem aos organismos aproveitar os benefícios desse mecanismo, diminuindo os custos potenciais associados ao investimento de estratégias defensivas, quando não necessário (AGRAWAL et al., 1999). Van Donk, Ianora e Vos (2011) classificaram que as defesas induzidas no fitoplâncton podem ser desencadeadas por (i) sinais químicos associados ao dano mecânico ou lise celular em consequência da predação e (ii) infoquímicos (cairomônios) excretados pelo predador independente da dieta ou (iii) mediante uma dieta táxon-específica, estando o último relacionado a respostas recíprocas apenas em populações-alvo.

Em cultivos de laboratório, linhagens de cianobactérias tóxicas mantém a produção constitutiva de toxinas, o que por sua vez tem promovido questionamentos sobre outras funções além de defesa, como autorregulação e sinalização celular (ver OMIDI; ESTERHUIZEN-LONDT; PFLUGMACHER, 2018). No entanto, evidências do papel das cianotoxinas como defesa induzida pelo predador têm sido demonstradas como um aumento significativo na produção do metabólito relativo à sua produção basal (JANG et al., 2003; JANG; JUNG; TAKAMURA, 2007; JANG; HA; TAKAMURA, 2008; VAN GREMBERGHE et al., 2009; SAVIC et al., 2020). A maioria dos estudos concentram-se em espécies produtoras de MCs, como Jang et al. (2003) e Jang, Jung e Takamura (2007) e Jang, Ha e Takamura (2008) os quais têm reportado um aumento na produção de MCs por *M. aeruginosa* quando exposta direta e indiretamente (infoquímicos) a *Daphnia*. Por outro lado, o conhecimento de defesas químicas induzidas em cianobactérias produtoras de outras cianotoxinas (ex.: STXs e CYN) ainda é incipiente, sendo apenas registradas variações morfológicas em resposta ao zooplâncton (CERBIN; WEJNEROWSKI; DZIUBA, 2013; WEJNEROWSKI et al., 2018).

Defesas induzidas também podem se manifestar como variações morfológicas. Cianobactérias coloniais geralmente perdem esse fenótipo e assumem a forma unicelular quando isoladas e mantidas em cultivo, mas já tem sido documentada a formação *de novo* de colônias nesses microrganismos em resposta ao predador (ver YANG et al., 2006; XIAO; LI; REYNOLDS, 2018). Esta resposta também foi evidenciada em microalgas eucarióticas como a clorofícea *Scenedesmus* (LÜRLING, 2003; VERSCHOOR et al., 2004; WU et al., 2013) e *Chlamydomonas* (HERRON et al., 2019), sugerindo que talvez esse mecanismo tenha convergido evolutivamente entre espécies coloniais do fitoplâncton como resposta à predação pelo zooplâncton.

De forma geral, a formação de colônias tem sido avaliada em bactérias baseada na produção de SEPs (Substâncias Exopoliméricas) as quais são secretadas para manutenção da matriz polimérica do biofilme, bem como da mucilagem que pode promover a agregação de células na colônia (MARVASI; VISSCHER; MARTINEZ, 2010). Dois mecanismos de formação de colônia têm sido reconhecidos para a cianobactéria *Microcystis*: (i) divisão celular – as células permanecem unidas mesmo após a divisão, sendo as célula-filhas contidas em um envelope de SEPs que evita que se separem – e adesão celular (ii) – em que células se agregam *via* secreção de SEPs adesivas (ver XIAO; LI; REYNOLDS, 2018). O mecanismo molecular subjacente à formação de colônias em resposta ao zooplâncton na microalga eucariótica *Desmodesmus subspicatus* foi recentemente revelado por Roccuzzo et al. (2020) os quais identificaram uma via de sinalização envolvendo proteína quinase ativada por mitógeno (MAPK) onde a MAPK desencadeia a divisão celular e formação de colônia na alga verde. Os autores também reportaram a adesão célula-célula mediada pela exportação de carboidratos e proteínas com ligação dissulfeto para a formação matriz polimérica extracelular.

Alguns estudos como Roccuzzo, Beckermam e Pandhal et al. (2016) e Yasumoto et al. (2005, 2008) já têm evidenciado que a capacidade de formar colônia parece não ser perdida, sendo um fenótipo que pode ser induzido pela presença do zooplâncton a qual é sinalizada por infoquímicos (cairomônios). Essas substâncias foram caracterizadas quimicamente para *Daphnia* como surfactantes catiônicos (sulfatos alifáticos e sulfamatos) os quais podem interagir com a camada lipídica de membranas celulares. Também já foi possível caracterizar a copepodamida G; um infoquímico de natureza lipídica excretado por copépodos marinhos e capaz de induzir o aumento na produção de saxitoxinas em dinoflagelados (SELANDER et al., 2015, 2016).

Respostas na interação zooplâncton-cianobactéria também já têm sido demonstradas a nível molecular. Harke et al. (2017) avaliaram o transcriptoma da linhagem *M. aeruginosa* LE-3 exposta a *Daphnia* spp. e observaram um aumento significativo nos transcritos que codificam SEPs, além de outros relacionados ao crescimento e estresse, não observando variações significativas na síntese de cianotoxinas/peptídeos. Em contrapartida, Pineda-Mendonza, Zúñiga e Martínez-Jerónimo (2014) registraram um aumento na expressão do marcador da síntese de microcistinas (*mcy*A) em *M. aeruginosa* em resposta a infoquímicos de *D. magna*.

Supõe-se que devido às condições favoráveis estabelecidas no cultivo, além da ausência de interações biológicas (ex.: predadores; competição), alguns atributos funcionais possam ser perdidos temporariamente. Neste contexto, Van Donk, Ianora e Vos (2011) ressaltam que sob baixo custo de sobrevivência, tal característica tende a se manter indetectável, especialmente sob condições ótimas, como observado em laboratório. Dessa forma, é provável que tais características sejam consideradas defesas contra o predador, sejam elas constitutivas ou induzidas.

A formação de colônias e o aumento na produção de cianotoxinas são atributos apontados como defesas induzidas pelo zooplâncton. No entanto, já se tem conhecimento da cianotoxina estimulando a formação de colônias (GAN et al., 2012), o que sugere que talvez ambas respostas de defesa não sejam paralelas, mas ocorram subsequentemente. Além disso, nem sempre essas respostas se manifestam, sendo importante considerar a variação intraespecífica, como reportado por Van Gremberghe et al. (2009) os quais mostraram diferentes respostas no crescimento e produção de microcistinas para linhagens de *Microcystis* quando expostas indiretamente a *Daphnia*.

Abordagens considerando ambos eixos da resposta fenotípica recíproca já têm mostrado que existe diferença na resposta da presa e do predador com e sem histórico de interação prévia (AKBAR et al., 2017). Mudanças fenotípicas recíprocas no tempo ecológico podem ser (i) um determinante primário do fenótipo do organismo no ambiente (ex.: fitoplâncton colonial e filamentoso), (ii) o resultado da evolução em longo prazo onde o ambiente tem oscilado e (iii) um fator estabilizante de interações mutualísticas, podendo ser uma variação direcional no fenótipo de ambos, onde a exposição a certos sinais químicos ativa genes de forma dose-dependente (AGRAWAL, 2001). Lürling (2020) sugere que ambos rápida evolução e plasticidade fenotípica ocorram por causa de um *trade-off* entre as habilidades competitivas e defesas anti-herbivoria no fitoplâncton. Dessa forma, avaliar as respostas fenotípicas recíprocas do "eixo cianobactéria-zooplâncton" pode fornecer informações para o entendimento da pressão adaptativa que tais interações exercem nesses organismos, os quais devem investir em defesas químicas e morfológicas para a rápida evolução e manutenção dessas populações na natureza.

HIPÓTESES E OBJETIVOS

Objetivo geral

Investigar respostas adaptativas na interação *Daphnia*-cianobactéria.

Hipóteses

H_1 – Um forrageamento seletivo permite a persistência de populações de cladóceros menores sob elevada biomassa de cianobactérias.

Objetivo 1: Avaliar o comportamento alimentar em *Daphnia* spp. de diferentes tamanhos, submetidas à dieta de cianobactérias tóxicas.

H_2 – Cianobactérias tóxicas expostas indiretamente ao zooplâncton herbívoro aumentam a produção de toxinas como mecanismo de defesa química.

Objetivo 2: Investigar respostas de defesas químicas induzidas em linhagens de cianobactérias tóxicas sob exposição indireta (infoquímicos) ao zooplâncton tropical *Daphnia gessneri.*

H_3 – Populações de *Daphnia* pré-expostas a cianobactéria tóxica apesentam uma melhor resposta de detoxificiação em comparação a populações não expostas.

Objetivo 3: Avaliar o efeito de *Raphidiopsis raciborskii* tóxicas na resposta transgeracional do sistema de detoxificação de *Daphnia gessneri* como indicador da plasticidade fenotípica neste zooplâncton.

Os objetivos aqui apresentados serão abordados em quatro capítulos os quais constituirão publicações, sendo três manuscritos completos e um *short communication.*

3. RESUMO DOS MATERIAIS E MÉTODOS

3.1. *Cultivo e Manutenção dos organismos-teste*

Para esse estudo foram utilizadas linhagens da cianobactéria *Microcystis aeruginosa*, sendo NPLJ-4 (Lagoa de Jacarepaguá, RJ) a qual é potencialmente produtora de microcistina-LR e dos peptídeos cianopeptolina (inibidor de serino endopeptidases tripsina e quimiotripsina) e aeruginosina (SILVA-STENICO et al. 2011), a linhagem *Raphidiopsis raciborskii* CYRF-01 (Isolada do Reservatório do Funil, RJ) e T3 (produtora de microgininas, SILVA-STENICO et al. 2011) (Represa Billings, SP), sendo estas últimas potencialmente produtoras de saxitoxinas (COSTA et al. 2013; RANGEL et al. 2020). As cianobactérias foram cultivadas em meio ASM-1 (GORHAM et al. 1964) e mantidas sob temperatura de 24±0,5 °C, ~50 µmoles m^{-2} s^{-1} sob fotoperíodo de 12h.

Foram utilizadas as linhagens de algas verdes *Selenastrum caprocornutum*, *Ankistrodesmus stiptatus* ANRF-1 (isolada do reservatório do Funil-RJ) e *Chlamydomonas reinhardtii* CHLRN-1 (gentilmente cedida pelo CENPES/PETROBRÁS) como itens alimentares nutritivos para o zooplâncton. As microalgas foram cultivadas nas condições acima descritas.

Como representantes do zooplâncton, foram utilizados cladóceros da espécie *Daphnia laevis* (clone isolado da Lagoa de Ibirité, MG), *Daphnia similis* (gentilmente cedida pelo LABTOX – Fundação BioRio, RJ) e *Daphnia gessneri* BBR (isolada do reservatório Barra do Braúna, MG) e *D. gessneri* BA (isolada do reservatório Mucugê, BA). Os animais foram mantidos em meio RT (TOLLRIAN 1993) acrescido de 30% de água filtrada do reservatório do Camorim (Parque Estadual da Pedra Branca, RJ) e extrato húmico (*Microbe-lift® Amazon Black & Soft Water Conditioner*, USA) a 0,1%, sob pH 7,6; 23±1 °C, ~50 µmoles m^{-2} s^{-1}, fotoperíodo de 12h e alimentados com suspensão de células das algas verdes *Selenastrum capricornutum* e *Ankistrodesmus falcatus* ANRF-1 a uma concentração final de 500 µg C L^{-1} uma vez a cada dois dias.

3.2. *Desenho experimental*

3.2.1. *Comportamento alimentar de Daphnia* spp.

O comportamento alimentar de *Daphnia similis* e *D. laevis* clone Ibirité será avaliado em relação à dieta de diferentes proporções em biomassa das cianobactérias *Microcystis aeruginosa* (NPLJ-4), *Raphidiopsis raciborskii* (CYRF-01), e da alga verde *Selenastrum capricornutum*. Foram estabelecidos tratamentos simples (misturas com apenas 1

linhagem de cianobactéria) e mistos (misturas com as 2 linhagens de cianobactérias) com 50 e 90% de cianobactérias relativo à biomassa total. Os controles para testar o efeito das cianobactérias consistiram em tratamentos com alga verde como item alimentar. Os experimentos foram conduzidos durante 3 horas em placas de cultura de células (24 poços). Em todos os tratamentos (n=4) foram alocados dois indivíduos adultos por poço, em 3 mL de suspenção de células a uma concentração final de 400 µg C L^{-1}. Os controles para testar a pressão de herbivoria consistiram em poços sem animais.

Como parâmetros alimentares foram estimadas as taxas de filtração(*CR*, *clearance rate* – mL ind^{-1} h^{-1}) e de ingestão (*IR*, *ingestion rate* – µg C ind^{-1} h^{-1}), além da seletividade (*a* de Chesson) a partir de dados de clorofila-*α* (µg L^{-1}) e biomassa em carbono (µgC L^{-1}) (ver detalhes da análise no **Capítulo 1**).

3.2.2. *Efeitos de infoquímicos de <u>D. gessneri</u> BBR nas cianobactérias <u>R. raciborskii</u> e <u>M. aeruginosa</u>*

As linhagens de *R. raciborskii* T3 e *M. aeruginosa* NPLJ-4 foram utilizadas para avaliar a Ecofisiologia e resposta molecular das cianobactérias tóxicas na presença de infoquímicos do zooplâncton *D. gessneri* BBR, como resposta de defesa induzida pelo predador.

Para obtenção do filtrado com os infoquímicos do zooplâncton foram estabelecidos cultivos em meio RT (ver **item 3.1.**) com indivíduos adultos a uma densidade de 60 ind L^{-1}. Após 96h os animais foram removidos e o meio esterilizado por filtração em membrana 0,22 µm (Whatman®) para obtenção do filtrado rico em infoquímicos de *D. gessneri*. A condição controle consistiu em filtrados de meios incubados sem zooplâncton.

Para avaliação da resposta das cianobactérias aos infoquímicos liberados pelo zooplâncton, foram feitos inóculos (~10^5 cels mL^{-1}) em erlenmeyers (n=3) de 1L com 600 mL de meio de cultura ASM-1 preparado em ambos filtrados (infoquímicos e controle). As culturas foram incubadas durante 6 dias ao longo dos quais foram feitas amostras a cada 2 dias análise de cianotoxinas (microcistinas e saxitoxinas), expressão de genes *sxt* (marcadores da produção de saxitoxinas), crescimento (células e biovolume), morfologia, produção de carboidratos (marcador de substâncias exopoliméricas) e fotossíntese (máximos rendimento quântico do fotossistema II e taxa de transporte de elétrons) (ver detalhes nos **Capítulos 2** e **3**).

3.2.3. *Efeito de diferentes dietas nas defesas antioxidantes de D. gessneri BA.*

Indivíduos de *D. gessneri* BA foram mantidos por duas gerações em tratamentos alimentares estabelecidos a partir da mistura das linhagens *A. stiptatus* ANRF-1 e *C. reinhardtii* CHLRN-1, sendo essas microalgas ricas em ácidos graxos poli-insaturados e saturados, respectivamente (MIRANDA et al. 2016); e a cianobactéria tóxica *R. raciborskii* T3. Com base no perfil de ácidos graxos (valor nutritivo) das linhagens, foram determinadas dietas de (i) qualidade alta (ANRF+CHLRN), (ii) média (CHLRN), (iii) baixa (T3+ANRF) e (iv) muito baixa (T3+CHLRN).

O experimento transgeracional foi iniciado com neonatos da geração parental (F_0, <24h) cultivados em meio RT enriquecido (ver **item 3.1.**) e dispostos em béqueres de 2 L a uma densidade de 50 ind L^{-1}. Os animais foram mantidos nas diferentes dietas até atingirem a fase adulta (12 dias) e indivíduos da 3ª ninhada (F_1) em cada tratamento foram subsequentemente expostos pelo mesmo intervalo de tempo a diferentes combinações de itens alimentares (palatável = ANRF+CHLRN e CHLRN; não-palatável = ANRF+CHLRN + T3 e CHLRN + T3) para avaliar o efeito do ambiente alimentar parental na resposta da prole à cianobactéria tóxica.

Adultos de ambos F_0 e F_1 foram separados para análise das enzimas GST, GPx e estimativa do nível de peroxidação lipídica (ver detalhes no **Cap. 4**) as quais foram usadas como parâmetro fisiológico da resposta às diferentes dietas, bem como da degradação de *ROS* (espécies reativas de oxigênio).

4. RESUMO DOS RESULTADOS

4.1. *Efeito de cianobactérias no comportamento alimentar de Daphnia spp.* **(Capítulo 1)**

Experimentos de curta duração para avaliar o impacto de dietas simples e mistas das cianobactérias tóxicas *Microcystis aeruginosa* NPLJ-4 e *Raphidiopsis raciborskii* CYRF-01 no comportamento alimentar de espécies de *Daphnia* foram analisados através de estimativas de biomassa e fluorescência da clorofila-α.

Foi possível estimar o efeito das dietas na taxa de filtração (*CR*, mL *Daphnia* h^{-1}) do zooplâncton através de ambos os dados de biomassa e clorofila-α uma vez que estes parâmetros estiveram correlacionados (ver **Fig. 1**). Dietas simples de cianobactérias causaram uma redução significativa na *CR* da espécie exótica *D. similis*, mas não na nativa *D. laevis* (ver **Fig. 2**). Não foram observados efeitos significativos de dietas mistas de cianobactérias na *CR* do zooplâncton.

Quando avaliada a taxa de ingestão (*IR*, μgC *Daphnia* h^{-1}) de alimento nutritivo (alga verde *Selenastrum capricornutum*), observou-se uma redução significativa em função do aumento na proporção de cianobactérias em ambas as dietas simples e mistas (ver **Fig. 3**), o que pode ser atribuído a uma capacidade de seletividade nula nos diferentes tratamentos alimentares (ver **Fig. 4**). Durante os ensaios os animais foram expostos a uma quota total de 0,021 $ng_{microcistinas}$/μgC e 0,03 $ng_{saxitoxinas}$/μgC nas dietas ricas em cianobactérias, respectivamente.

4.2. *Defesas induzidas em cianobactérias tóxicas* **(Capítulos 2 e 3)**

As cianobactérias tóxicas *Microcystis aeruginosa* NPLJ-4 e *Raphidiopsis raciborskii* T3 foram expostas a infoquímicos (cairomônios) do zooplâncton *Daphnia gessneri* durante 6 dias ao longo dos quais foram avaliadas respostas de defesas induzida através de parâmetros morfo-fisiológicos e moleculares.

Em ambas as linhagens de cianobactérias foi possível observar uma resposta de defesa química induzida por infoquímicos de *D. gessneri* a qual caracterizou-se como um aumento significativo na quota celular (ou por biovolume) de microcistinas em *Microcystis* (ver **Fig.4; Cap. 2**) e saxitoxinas em *Raphidiopsis* (ver **Fig. 3; Cap. 3**). Nesta última, o aumento na produção de saxitoxinas também esteve associado a um aumento significativo na toxicidade celular (ver **Fig. 4; Cap. 3**) e expressão relativa dos genes *sxt*I e *sxt*U (ver **Fig. 7; Cap. 3**).

A atividade fotossintética das cianobactérias não foi afetada por infoquímicos do zooplâncton. Quando avaliado o crescimento, *Microcystis* não foi afetada (ver **Fig. 1; Cap. 2**). Por outro lado, *Raphidiopsis* sofreu uma redução significativa no rendimento em biomassa e taxa específica de crescimento (ver **Fig. 1; Cap. 3**) quando incubada na presença dos infoquímicos.

A produção de carboidratos celulares (exopolissacarídeos) foi usada como *proxy* da produção de colônias por *Microcystis.* Este parâmetro não foi afetado pela presença de infoquímicos de *Daphnia*, assim como não foram registradas colônias nas culturas. Também não foram observadas alterações morfológicas em *Raphidiopsis* em resposta ao zooplâncton.

A concentração volumétrica de cianotoxinas (μg L^{-1}) nas culturas não sofreu efeito significativo do zooplâncton, com exceção da concentração de microcistinas excretadas por *Microcystis* (ver **Fig. 5B; Cap. 2)**. As concentrações de cianotoxinas intra- e

extracelulares detectadas neste estudo constituíram o *pool* total de toxinas produzidas na cultura e apresentaram um padrão similar ao crescimento celular ao longo do tempo de incubação. A partir desses dados foi possível calcular a taxa de produção de cianotoxinas nas diferentes condições experimentais com base na cinética de primeira ordem, em que observou-se uma relação 2:1 entre a produção de microcistinas (ver **Figs. 6-7; Cap. 2**) e saxitoxinas (ver **Fig. 6; Cap. 3**) e a divisão celular na presença de infoquímicos, indicando que a produção de toxinas esteve mais relacionada aos efeitos dos infoquímicos do que com a taxa de crescimento.

4.3. *Efeito da dieta sobre defesas antioxidantes em diferentes gerações de Daphnia gessneri* **(Capítulo 4)**

A resposta transgeracional de enzimas antioxidante e de biotransformação de *Daphnia gessneri* foi avaliada sob dietas com diferentes níveis de qualidade em relação à presença de cianobactéria e perfil de ácidos graxos.

Mudanças na qualidade da dieta afetaram significativamente a produção basal de enzimas de detoxificação em diferentes gerações do zooplâncton *D. gessneri*. A prole de fêmeas partenogenéticas mantidas sob dieta de alta qualidade nutricional apresentou uma maior atividade da glutationa-S-transferase (GST) quando exposta à cianobactéria *Raphidiopsis raciborskii* (ver **Fig 1; Cap. 4**). Indivíduos provenientes de fêmeas mantidas em dietas com qualidade variando de média a muito baixa não apresentaram um aumento significativo na resposta de biotransformação.

Por outro lado, quando avaliada resposta antioxidante pôde-se observar um efeito significativo da qualidade da dieta no que diz respeito à disponibilidade de item alimentar rico em ácidos graxos poli-insaturados. Observou-se um aumento transgeracional significativo na atividade basal da glutationa peroxidase (GPx) em proles de fêmeas partenogenéticas mantidas sob dieta composta da alga verde *Ankistrodesmus stiptatus* ANRF-01 (ver **Fig. 2; Cap. 4**). Esses animais apresentaram maior resposta à presença de cianobactérias tóxicas.

Apesar do aumento na GPx, os níveis de peroxidação lipídica (LPO) também aumentaram significativamente nas proles de fêmeas mantidas em dietas compostas de alga rica em ácidos graxos poli-insaturados, mesmo na presença de *R. raciborskii* tóxica (ver **Fig. 3; Cap. 4**), indicando uma falha na degradação das *ROS*, o que resultou no estresse oxidativo, independente da presença de cianobactérias na dieta.

CAPÍTULO 1

Feeding behavior in large and small-bodied *Daphnia* under single and mixed diets of toxic Cyanobacteria *Microcystis aeruginosa* and *Raphidiopsis raciborskii*

Manuscrito a ser submetido ao periódico *Food Webs*

Feeding behavior in large and small-bodied *Daphnia* under single and mixed diets of toxic Cyanobacteria *Microcystis aeruginosa* and *Raphidiopsis raciborskii*

Mauro Cesar Palmeira Vilar[1], Thiago F.C.P. Rodrigues[1], Gabriela S. Duarte dos Santos[1], Aloysio da Silva Ferrão-Filho[2], Sandra M. F. O. Azevedo[1]

[1]Laboratory of Ecophysiology and Toxicology of Cyanobacteria, Carlos Chagas Filho Biophysics Institute, Federal University of Rio de Janeiro, Rio de Janeiro - 21949-902, Brazil;

[2]Laboratory of Assessment and Promotion of Environmental Health, Oswaldo Cruz Institute, FIOCRUZ - Rio de Janeiro, 21040-360, Brazil

Corresponding author: Mauro Vilar (maurovilar@gmail.com)
Carlos Chagas Filho Biophysics Institute, Federal University of Rio de Janeiro, Rio de Janeiro 21949-902, Brazil
Phone: +55 21 39386648

Abstract

Cyanobacteria usually dominate phytoplankton in eutrophic water bodies as monospecific or mixed blooms. Moreover, these organisms are an important energy source for aquatic food webs in these environments, establishing a direct link with the zooplankton, especially omnivores such as cladocerans. In contrast, cyanobacteria can limit zooplankton grazing pressure as a consequence of their ability to produce toxic peptides and/or alkaloids, weakening that link. Nevertheless, some zooplankters survive under bloom conditions, and this ability has been related to a physiological tolerance allowing them to acquire energy from a cyanobacterial diet. However, the behavioral mechanisms on resisting cyanobacteria have been less explored. Thus, we aimed to investigate the feeding behavior of two species of *Daphnia* under a single and mixed diet of different toxin-producing cyanobacteria. The species *Daphnia similis* and *D. laevis* were maintained in RT medium and fed with green algae suspension until adulthood. The treatments consisted of mixtures of cell suspensions of the toxic cyanobacteria (M) *Microcystis aeruginosa* and (R) *Raphidiopsis raciborskii* and the green algae (S) *Selenastrum capricornutum* at the following proportions: control = 100% S; 1) 50% M; 2) 90% M; 3) 50% R; 4) 90% R; 5) 50% M+R and 6) 90% M+R at a total biomass of 400 μg C L^{-1}. The experiment was run in a 24-well plate (2.5 ml) with two animals/well, and feeding parameters were analyzed at the beginning and after 3 h. The use of different mixtures with an edible food source (green algae) allowed the potential for nutritional inadequacy and the influence of morphology or taste to be investigated in short-term grazing assays. There was a marked decrease in the feeding performance of *D. similis* but not for *D. laevis.* Overall, both displayed nonselective behavior and reduced feeding as cyanobacteria increased in food suspension.

Keywords: Zooplankton, foraging, cyanotoxins, HABs

Introduction

Cyanobacterial blooms are registered worldwide, and they are associated with eutrophication and intensified by extreme climatic changes (O'Neil et al. 2012). Once cyanobacteria dominate the phytoplankton community, they constitute, together with bacterioplankton, a primary source of energy to zooplankton in nutrient-rich water bodies. However, morphological traits, such as large colonies, filaments, the production of toxic metabolites and low nutritional quality are cyanobacterial attributes that can constrain zooplankton *fitness* and grazing performance (Gebrehiwot et al. 2019). Thus, it is suggested that these harmful microorganisms may disrupt aquatic ecosystem and lead to trophic uncoupling through adverse effects on filter feeder zooplankton, such as *Daphnia* (Mülller-Navarra et al. 2000).

On the other hand, the interaction between toxic cyanobacteria and zooplankton can also lead to shifts towards selection of adaptive traits within the life history of the animals, remaining those resistant (Ger et al. 2016). However, most of the studies on the cyanobacteria–zooplankton interaction has focused on temperate regions using large generalist zooplanktonic grazers, which rarely coexist with cyanobacteria. In tropical freshwater systems it is hypothesized that, due to the scarcity of large cladocerans and calanoids copepods, most of the top-down control of phytoplankton is promoted by small-bodied cladocerans and rotifers (Sarma et al. 2005; Severiano et al. 2018; Amorim et al. 2019). These zooplankters can break down filamentous cyanobacteria into small fragments in order to facilitate their ingestion (Bouvy et al. 2001; Gulati et al. 2001; Kâ et al. 2012) and/or forage on weakly attached cells around large colonies (e.g., *Microcystis* spp.). Nevertheless, although the feeding behavior of temperate crustaceans or rotifer zooplankton is well reported, data on tropical or subtropical zooplankton feeding is still scarce (Hart and Jarvis 1993; Hart 1998), making it difficult to explore this hypothesis.

Among cladocerans, small-bodied species are known to be more resistant to mechanical interference than large-bodied species because they have a relatively narrow gap in their carapace, which prevents filaments from entering the filtration chamber (Gliwicz and Siedlar 1980; Panosso and Lürling 2010). Therefore, it has been suggested that the food-gathering process is qualitatively different between *Daphnia* species that differ in body size due to the variation in the hydrodynamics of water flow through the filtering apparatus (Sikora and Dawidowicz 2017).

Eutrophic tropical freshwaters, especially those located in semiarid regions, commonly experience perennial cyanobacterial blooms throughout the year. For instance, some studies in tropical and sub-tropical regions have reported the co-dominance of *Microcystis* spp. and *Raphidiopsis raciborskii* (formerly *Cylindrospermopsis raciborskii*) forming dense surface blooms and representing more than 20% of the reported blooms (Soares et al. 2013; Moura et al. 2015). These cyanobacteria are potentially producers of microcystins (MCs) and saxitoxins (STXs) in South American (sub)tropical aquatic systems (Buratti et al. 2017), in addition to other bioactive cyanometabolites (Huang and Zimba 2019). Moreover, *R. raciboskii* is also potentially a producer of the cytotoxic alkaloid cylindrospermopsin (Kinnear 2010).

Microcystins are cyclic heptapeptide inhibitors of protein phosphatases 1 and 2A (PP1 and 2A) which disrupt the cell cytoskeleton due to hyperphosphorylation; they are usually regarded as a hepatotoxin (Buratti et al. 2017). On the other hand, saxitoxins comprise sulfated and nonsulfated neurotoxic carbamate alkaloids, which block voltage-gated sodium and calcium channels (Wiese et al. 2010). Overall, hepatotoxins and neurotoxins are the types of toxins most commonly found in freshwater ecosystems (Chorus and Bartram 1999); thus, aquatic organisms might be constantly exposed to complex mixtures of these metabolites.

The effects of single cyanotoxin (or cyanobacteria) in cladocerans are well documented on the literature, and they include reduced survivorship, growth, reproduction and swimming behavior (Tillmans et al. 2008; Ferrão-Filho and Kozlowsky-Suzuki 2011; Costa et al. 2013; Bownik 2016; 2017; Esterhuizen-Londt et al. 2016; Nandini et al. 2017; Ferrão-Filho et al. 2019). Additionally, cyanobacteria can affect zooplankton feeding efficiency by both physical (mechanical obstruction) and chemical impairments (Rangel et al. 2016, Fabre et al. 2017). Therefore, according to Ferrão-Filho et al. (2017), not only single toxic compounds but also combinations of toxins must be tested to better predict the effects of cyanobacteria on aquatic organisms as well as for health hazard risk assessment. Moreover, behavioral traits such as feeding and grazing are significant indicators for predicting the strength of the trophic interaction between phytoplankton and zooplankton (Chesney et al. 2019). Thus, we aimed to analyze the impact of single and mixed diets of toxin-producing *Microcystis aeruginosa* and *Raphidiopsis raciborskii* on the feeding responses of large- and small-bodied *Daphnia*

species. Our hypothesis is that a differential foraging depending on body size allows different *Daphnia* species to occur under cyanobacterial dominance.

Materials and Methods

Phytoplankton and zooplankton cultures

Two toxic cyanobacterial strains were used in this study: *Microcystis aeruginosa* (NPLJ-4), which is isolated from Jacarepaguá Lagoon-RJ and characterized as a producer of bioactive peptides such as [D-leu^1]microcystin-LR variants, cyanopeptoline and aeruginosin (PP1/2A and serine endopeptidases inhibitors) (Ferreira et al. 2010; Silva-Stenico et al. 2011), and *Raphidiopsis raciborskii* (CYRF-01), an STXs-producing strain isolated from Funil Reservoir-RJ (Costa et al. 2013; Rangel et al. 2016). Stock cultures were maintained in ASM-1 medium (Gorham et al. 1964) at 24±1°C, 50 µmol photons m^{-2} s^{-1} under a 12 h photoperiod. Under these conditions, *Raphidiopsis* had an average trichome length of 121±90.84 µm. The strain of *Microcystis* occurs mainly as unicell and a 2-cell coenobia with a mean cell diameter of 4.45±0.69 µm. Additionally, the green algae *Selenastrum capricornutum* was cultured under the same conditions and provided as an edible food source to zooplankton. Its population was dominated by unicells (mean length-width dimensions 13.80±1.56 × 3.18±0.66 µm).

The zooplankton consisted of the small-bodied native species *Daphnia laevis* (isolated from the eutrophic Ibirité Lake, MG) and the large-bodied exotic *Daphnia similis* (kindly provided by LABTOX - BioRio Foundation, RJ). *D. laevis* and *D. similis* achieve a maximum length of 1.8±0.03 and 2.2±0.03 mm, respectively. Both clones had been maintained in the laboratory for many generations before the study. The animals were kept in RT medium (Tollrian 1993) enriched with commercial 0.1% (2.25 mgC L^{-1}) humic extract (Microbe-lift® Amazon Black & Soft Water Conditioner, USA) at an initial pH of 7.6, 24±1 °C, 50 µmol photons m^{-2} s^{-1} and a 12 h photoperiod. The animals were fed a *S. capricornutum* cell suspension at a final concentration of 400 µgC L^{-1} once every two days.

Experimental setup

The grazing assay was performed to evaluate *Daphnia* spp. feeding behavior under different single and mixed diets combining the edible green algae and toxic

cyanobacterial strains. The food treatments consisted of mixtures of cell suspensions of toxic cyanobacteria and green algae:

(1) 100% green algae (100% *Selenastrum*)

(2) 50% cyanobacteria (50% *Microcystis* + 50% *Selenastrum*)

(3) 50% cyanobacteria (50% *Raphidiopsis* + 50% *Selenastrum*)

(4) 50% mixed (25% *Microcystis* + 25% *Raphidiopsis* + 50% *Selenastrum*)

(5) 90% cyanobacteria (90% *Microcystis* + 10% *Selenastrum*)

(6) 90% cyanobacteria (90% *Raphidiopsis* + 10% *Selenastrum*)

(7) 90% mixed (45% *Microcystis* + 45% *Raphidiopsis* + 10% *Selenastrum*)

Prior to grazing, the cell/trichome densities (unit per L^{-1}) were estimated in a Fuchs-Rosenthal hemocytometer and subjected to carbon biomass measurements according to Hillebrand et al. (1999) and Rocha and Duncan (1985). All food suspensions were provided at a total carbon biomass of 400 μg C L^{-1} without considering the nutritional differences among them. Thus, we assumed that despite the toxin content in cyanobacteria, both food sources have a comparable nutrition and digestibility profile.

The experiments were run in a 24-well plate and incubated for 3 hours to prevent the food particles from settling. In all treatments (n= 4) two adult individuals from each cladoceran species were distributed per well, which was filled with 2.5 mL of food suspension. The controls consisted of wells with no zooplankton. All zooplankton species were pre-starved for 2 h in RT medium without food to empty their guts prior to the beginning of the experiment.

Feeding behavior measurements

Before and after incubation, phytoplankton samples of the different treatments were analyzed for the chlorophyll-*a* content (PHYTO-PAM; Walz, Germany) and subsequently preserved in a mixture of 1% Lugol's iodine solution, stored in dark-cold conditions and subjected to carbon biomass quantification (Hillebrand et al. 1999; Rocha and Duncan, 1985).

The clearance rate (CR; mL ind^{-1} h^{-1}) was estimated from the differences in food concentration as algae and cyanobacterial biomass measured by both chlorophyll-*a* concentration and carbon biomass according to Lürling and Verschoor (2003) using the following equation: $\mathbf{CR=\{ln(Biomass_{Control} - Biomass_{Zoo})\}/\Delta t \times V/N}$ where Δt is the time of incubation (h), V is the total well volume (mL), and N is the number of animals/replicate.

The ingestion rate (*IR*, µg C ind^{-1} h^{-1}) was calculated as $\mathbf{IR = CR \times \sqrt{A_0 \cdot A_t}}$, where $\sqrt{A_0 \cdot A_t}$ is the geometric mean of the algal (edible or non-edible one) concentration over time such that A_0 is the initial food concentration (µgC L^{-1}) and A_t is the food concentration at the end of the experiment.

To assess the food selection of *Daphnia* clones in both single and mixed food treatments, Chesson's selectivity index (α) was calculated (Chesson, 1983): $\boldsymbol{\alpha_i = (d_a/m_a)/((d_a/m_a) + (\underline{d_b}/m_b))}$, where *a* and *b* are the food types, *d* is the proportion of food removed in the diet, and *m* is the proportion of food available in the medium. In the mixed food treatment, the selectivity was calculated as the selectivity for edible algae considering each non-edible food in the mixture. Therefore, for the single and mixed food treatments, positive selectivity is assumed when α>0.5 (preference >50%) and α>0.33 (preference >33%), respectively.

Microcystin and Saxitoxins analysis

Cyanobacterial cultures were grown until the exponential phase and 200 mL samples were freeze-dried, weighed and then followed to toxin extraction.

Microcystins (MCs) produced by *M. aeruginosa* NPLJ-4 were extracted with 50% MeOH solution + 5% acetic acid, evaporated (up to 50% of total volume) in a thermostatic bath coupled to N_2 injection and subsequently purified in a solid-phase extraction cartridge (SPE) prior to injection.

The samples were analyzed on liquid chromatography equipment (Agilent model 1200) coupled to a mass spectrometer (Applied model 3200 Q-trap) (LC-MS). Ionization was performed using an Electronspray (ESI) source in positive mode. The analyses were performed under the following conditions: isocratic method with 50% acetonitrile with phase (A) as 0.5 mM ammonium acetate in milli-Q water + 0.1% formic acid and phase

(B) as 0.5 mM ammonium acetate in acetonitrile + formic acid 0.1%. Toxin separation was performed in a C18 column (Phenomenex®; 5 µ, 150 × 4.6 mm) at a flow rate of 1 mL/min for 90 min. Data are expressed as the toxin quota per biomass ($ng_{microcystin}$ μgC^{-1}).

Saxitoxin (STX) extraction was performed in 0.5 M acetic acid solution. The analysis was performed on a Shimadzu Class VP liquid chromatography system with a fluorescence detector (RF-10A XL). A reverse-phase C18 column (Lichrospher®; 150 mm x 4.6 mm; 5 µm - Merck) and a 20 µl loop injector were used. Chromatographic analyses were performed according to the post-derivatization method (Oshima, 1995) method using a mobile phase 2 mM sodium 1-heptanesulfonate in 30 mM ammonium phosphate and 5% acetonitrile at a flow rate of 0.8 mL min^{-1}. STXs were detected at a 330 nm excitation wavelength and 400 nm emission wavelength.

For the quantification of toxins, standard solutions of saxitoxin (STX) and neosaxitoxin (neoSTX) from the National Research Council (NRC) - Institute of Marine Biosciences (Canada) were used. The total saxitoxins content was expressed as the toxin quota per biomass ($ng_{saxitoxins}$ $\mu g\ C^{-1}$).

Data analysis

The clearance rate, ingestion rate and selectivity coefficient data were examined for normality and variance homocedasticity. Once the parametric premises were assumed, the data were compared using two-way ANOVA with food type and its proportion in diet as the fixed factors to assess the feeding performance of *Daphnia* spp.. Analyses were run in GraphPad Prism 6.0.

Results

After incubation, the biomass concentration in the controls (phytoplankton without zooplankton) remained unchanged. Therefore, all quantified data in this work were attributed to feeding or filtration by *Daphnia* species. Moreover, during the incubation in single and mixed diets of *Microcystis aeruginosa* NPLJ-4 and *Raphidiopsis raciborskii* CYRF-01, the animals were exposed to a toxin quota of 0.021 and 0.030 ng of total microcystin and saxitoxin per carbon biomass (ng_{toxin} μgC^{-1}), respectively.

The linear regression comparing the zooplankton clearance rate (CR) data estimated from the phytoplankton biomass derived from the biovolume measurement and the chlorophyll-a concentration indicated that the phytoplankton abundance measures displayed a similar pattern after grazing by *Daphnia similis* ($F_{(1,14)}$=0.2216; p=0.645; pooled slope= -0.0006232) and *D. laevis* ($F_{(1,14)}$=0.4003; p=0.5371; pooled slope= -0.008017) **(Fig. 1)**. These data reinforce the reliability of the results obtained by both estimates of phytoplankton abundance. Hence, the carbon biomass and chlorophyll-a concentration allowed for measuring the cyanobacterial impacts on zooplankton clearance and ingestion rate.

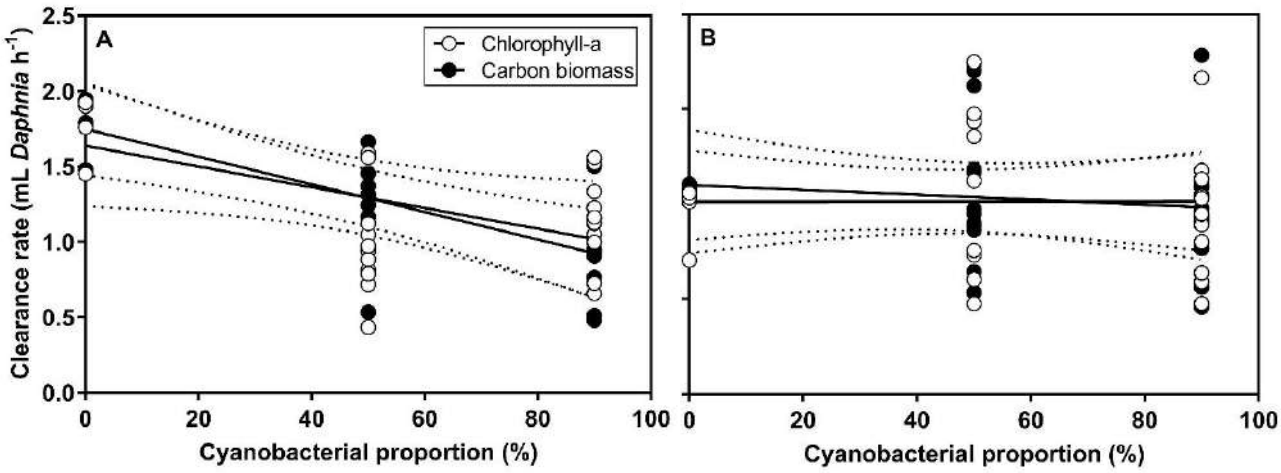

Figure 1. Linear regression between clearance rate data estimated from (white circle) chlorophyll-a and (black circle) carbon biomass removed by (A) *Daphnia similis* and (B) *Daphnia laevis*, and the different proportions of single and mixed cyanobacterial diets. Equations derived from the linear regressions: A= Ychl-a=-0.006879*X + 1.638; Ycarbon=-0.009156*X + 1.749 and B = Ychl-a=4.278-005*X + 1.011; Ycarbon=-0.001289*X + 1.10

A significant reduction in the CR of the large *D. similis* was observed but not in the small *D. laevis*. The statistical analysis indicated a significantly negative effect of cyanobacteria when provided to zooplankton as different carbon biomasses of single *M. aeruginosa* NPLJ-4 and *R. raciborskii* CYRF-01 (Two-way ANOVA, p<0.0001) but not from the mixed cyanobacterial diets **(Fig. 2; Suppl.)**.

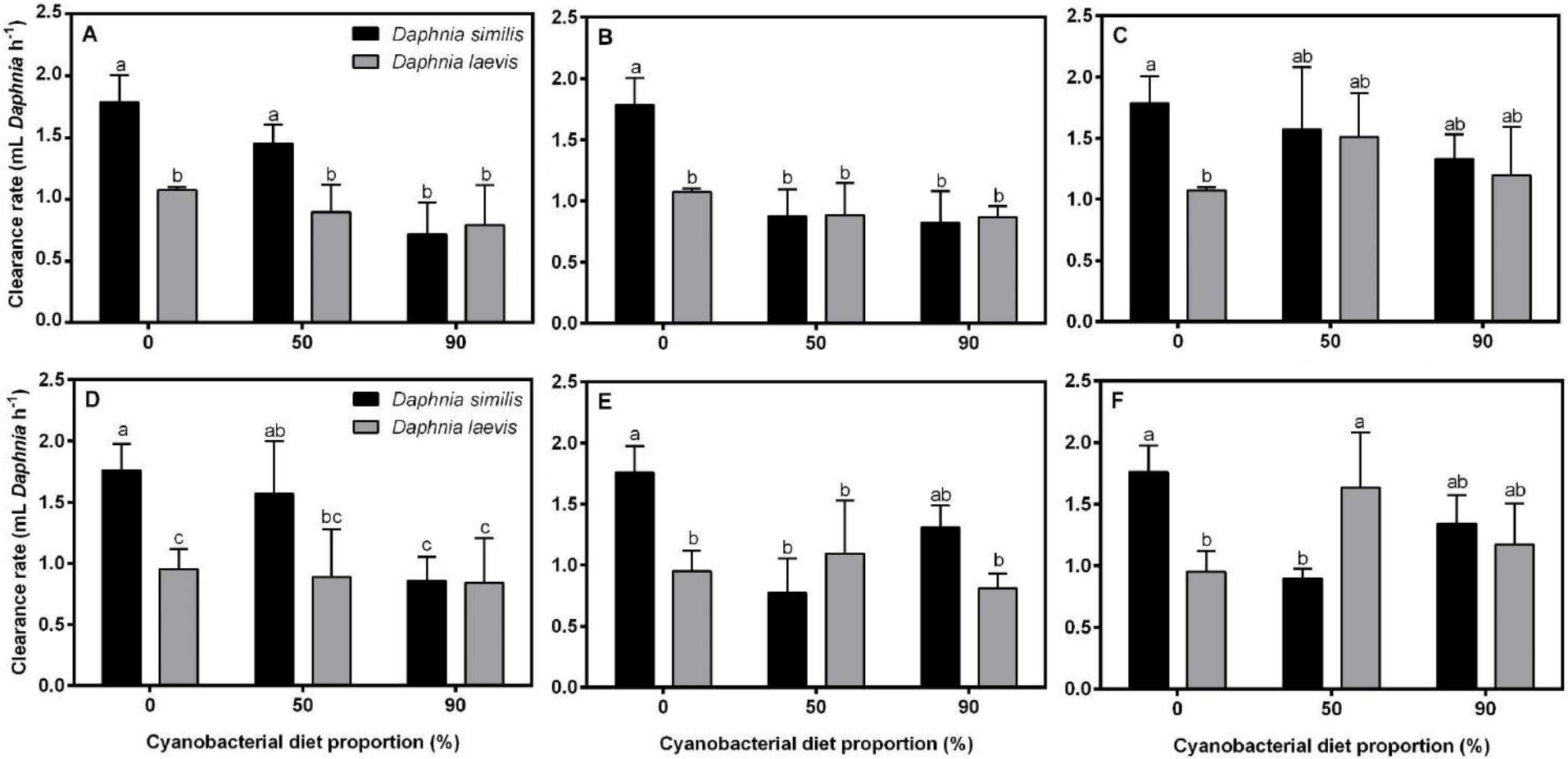

Figure 2. Clearance rate of (black bars) *Daphnia similis* and (grey bars) *Daphnia laevis* under different proportions of **(A;D)** *Microcystis aeruginosa* NPLJ-4, **(B;E)** *Raphidiopsis raciborskii* CYRF-01 and **(C;F)** mixed cyanobacterial diets measured by **(A-C)** carbon and **(D-F)** chlorophyll biomass, respectivelly. Different letters means statistical diferences (Tukey HSD test, $p<0.05$).

Overall, *Daphnia similis* displayed a higher clearance rate (1.67-fold) than *D. laevis* when fed only with edible green algae *Selenastrum capricornutum* **(Fig. 2)**. However, when fed with cyanobacteria, *D. similis* significantly decreased its CR such that it achieved a filtration rate similar to the small-bodied *D. laevis*. Once filamentous CYRF-01 constituted 50% of the food, it was already possible to record a significant decrease in the *D. similis* CR **(Fig. 2)**. The same was seen in *M. aeruginosa* NPLJ-4 when it constituted 90% of the total food available (ANOVA, $F_{(2,18)}$= 19.22, p<0.0001). In contrast, *D. laevis* displayed an increase in clearance rate when exposed to mixed food with cyanobacteria as 50% of the total biomass **(Fig. 2)**.

On the other hand, *Daphnia* clones significantly inhibited the ingestion rate (IR) of the edible algae *S. capricornutum* when mixed with each single cyanobacteria (ANOVA, $F_{Microcystis(2,17)}$= 263.9, p<0.0001; $F_{Raphidiopsis(2,17)}$= 111.2, p<0.0001) and both cyanobacteria (ANOVA, $F_{mixed(2,15)}$= 98.75, p<0.0001) **(Suppl.)**. In addition, although the clearance rate in *D. similis* was higher when fed with edible food, *D. laevis* showed a significantly higher ingestion rate (1.43-fold) under the same condition **(Fig. 3)**.

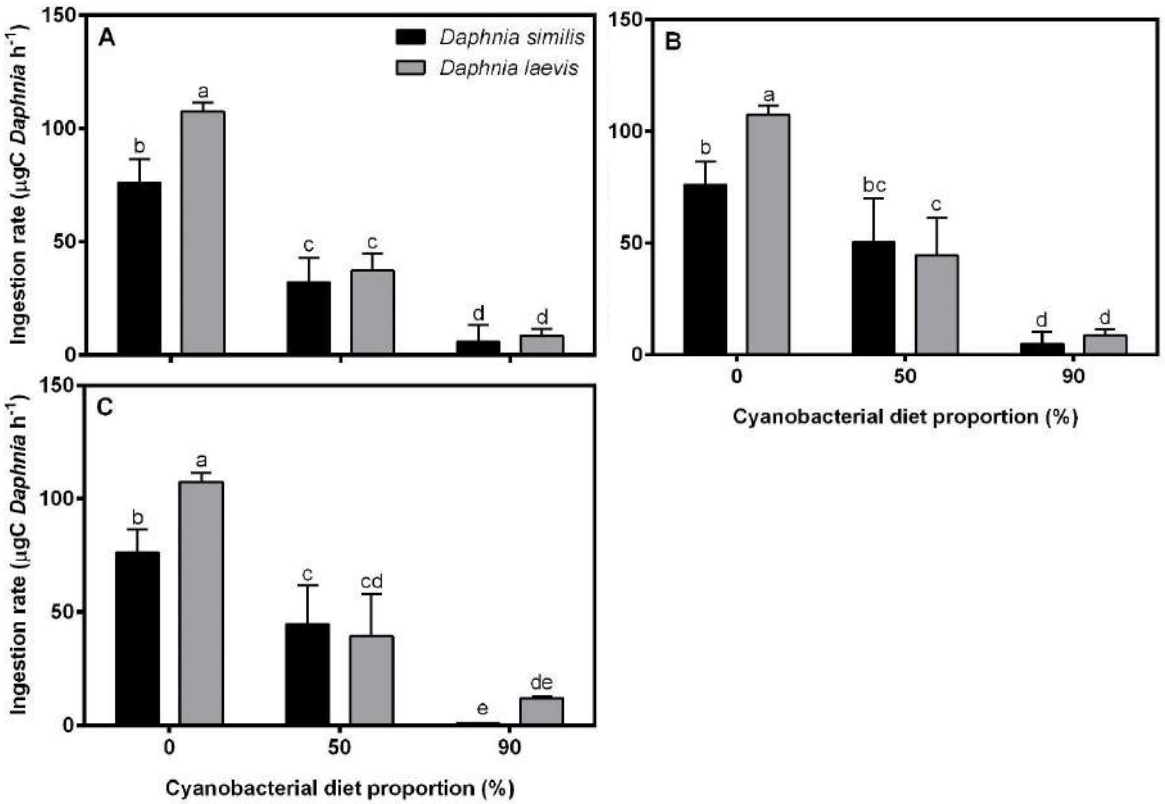

Figure 3. Ingestion rate of edible algae (*Selenastrum capricorutum*) biomass by (black bars) *Daphnia similis* and (grey bars) *Daphnia laevis* under different proportions of **(A)** *Microcystis aeruginosa* NPLJ-4, **(B)** *Raphidiopsis raciborskii* and **(C)** mixed cyanobacterial diets. Different letters mean statistical diferences (Tukey HSD, p < 0.05).

Zooplankton displayed no selective behavior when fed with both single and mixed toxic cyanobacteria at different proportions (Chesson's $\alpha = 0.5$ and $\alpha = 0.33$, respectively; $p>0.05$) **(Fig. 4).** Despite displaying some variations in feeding rate, a null Chesson's coefficient estimated for the *Daphnia* clones indicated a generalist feeding behavior when these zooplankters were fed with cyanobacteria.

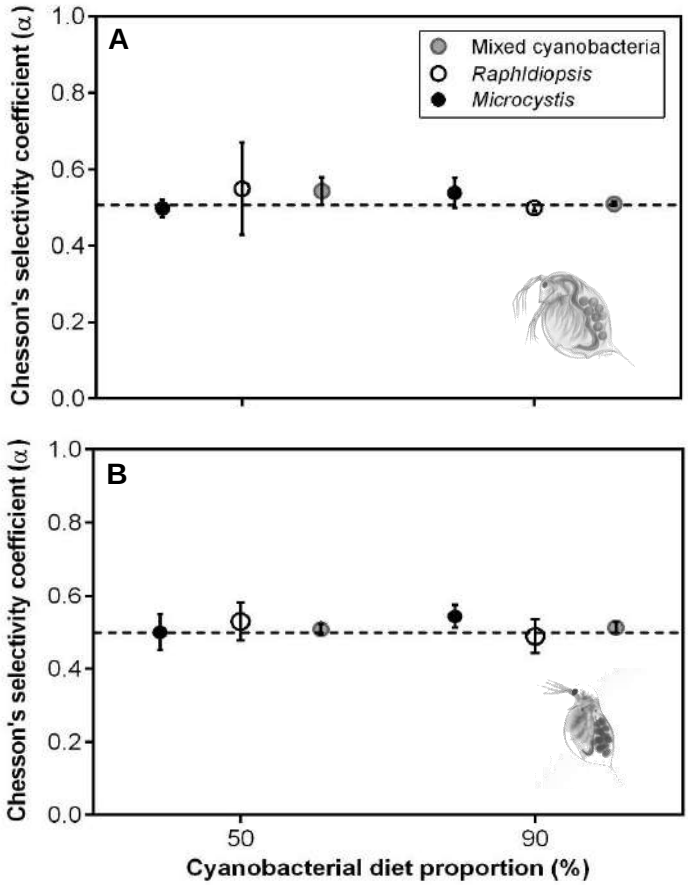

Figure 4. Chesson's selectivity to edible algae (*Selenastrum capricorutum*) of **(A)** *Daphnia similis* and **(B)** *Daphnia laevis* under different proportions of single and mixed cyanobacterial diets at 400 µgC L^{-1}. $\alpha > 0.3$ and 0.5 indicates selectivity under both mixed and single *(Raphidiopsis* or *Microcystis*) cyanobacterial diet, respectively.

Discussion

A significant effect of body-size was accounted to *Daphnia* grazing. Despite both *Daphnia* species had shown a generalist feeding behavior, only the large-bodied one displayed a negative impact in the filtration rate when submitted to toxic cyanobacteria diet. Therefore, our hypothesis was partially accepted.

In the present study, the *Daphnia* clearance rate measured by carbon biomass and chlorophyll-*a* concentration showed good agreement. Linear regressions from both CR_{carbon} and $CR_{Chl\text{-}a}$ under different diets had equal slopes (<1). Similar findings were also reported by Lürling and Verschoor (2003) investigating the feeding behavior of the rotifer *Brachionus rubens* fed with a combined diet of *Scenedesmus obliquus* and *M. aeruginosa.* The authors found a strong correlation between CR based on chlorophyll fluorescence and carbon biomass, signifying the PHYTO-PAM measurements to be a reliable tool for assessing zooplankton grazing experiments.

Microcystin- and saxitoxin-producing cyanobacteria provided at increasing proportions as both single and mixed food negatively affected the feeding behavior of large-bodied *Daphnia* but not small-bodied *Daphnia*. In addition to the toxicity, it is also important to regard the coupled effect of morphology exerted by the filamentous cyanobacterium *R. raciborskii* CYRF-01 (trichomes 30–211 µm) in our results. Once prey morphology affects zooplankton feeding efficiency, long-sized filaments and colonies may increase the prey's grazing resistance, mainly against large generalist cladocerans (Fulton III and Paerl 1987; Litchman and Klausmeier 2008).

Other findings have also evidenced the impacts of filamentous saxitoxin-producing cyanobacteria on the grazing performance of large *Daphnia* species such as in Panosso and Lürling (2010), who showed a decrease in CR of *D. magna* fed with *R. raciborskii* CYRF-01 at different trichome lengths (61±23.4–137.1±64.1 µm) and biomass proportions relative to *Tetradesmus* (*Scenedesmus*) *obliquus*. Fabre et al. (2017) showed a 10-fold decrease in *D. pulex* CR when fed with the toxic *R. raciborskii* CYRF-01 and MVCC19 compared with the edible green algae *Chlamydomonas chlorastera*. Additionally, Ferrão-Filho et al. (2017) showed a marked decrease in the ability of *D. similis* to filter nutritious food (*Raphidocelis subcapitata*) when mixed (1:1) with *R. raciborskii* CYRF-01.

Unlike the large *Daphnia*, *D. laevis* exhibited no inhibition in clearance rate when exposed to cyanobacteria. An apparent similarity in CR across cyanobacterial concentrations suggests that *D. laevis* was somehow capable of handling toxic *Microcystis* and *Raphidiopsis* in both single and mixed diets regardless of the increased particulate toxins and prey morphology. Some studies have demonstrated that small

zooplankton can break filamentous cyanobacteria in order to facilitate cell ingestion (Bouvy et al. 2001; Gulati et al. 2001; Kâ et al. 2012; Rangel et al. 2016; Sikora and Dawidowicz 2017), maintaining relatively constant grazing rate despite the phytoplankton biomass. Furthermore, due to the variation in hydrodynamics of water flow through the filtering combs, small-bodied species act more as paddles than sieves, reducing clogging of the filtering chamber (Abrusán 2004) and facilitating the expulsion of undesired particles.

In contrast, once small zooplankton survive the morphological resistance of prey and become able to feed on the smaller particles, the feeding behavior becomes ruled by food chemical composition (e.g., bioactive secondary metabolites). This assumption has been supported by studies which assess the impact of toxic cyanobacteria on the grazing behavior of small-bodied zooplankton which have shown that these animals are sensitive to both saxitoxin- and microcystin-producing strains (Soares et al. 2010; Rangel et al. 2016; Ferrão-Filho et al. 2017). Data on native cladocerans such as *Moina micrura* and *D. laevis* have shown selective behavior in choosing edible algae, especially when combined with filamentous cyanobacteria (Ferrão-Filho et al. 2017).

It is also important to regard the total carbon biomass concentration. Once above a small limiting food concentration, the grazing rate gradually decreases and feeding remains constant (Peters 1984). In our study, food was provided at a final concentration of 400 µgC L^{-1}; however, many studies have assessed *Daphnia* grazing performance under higher biomasses (e.g., >1000 µgC L^{-1}) such as Fabre et al. (2017) who showed, even when *D. pulex* was fed with edible green algae (*Chlamydomonas chlorastera*), that as the final biomass increased (500 – 5000 µgC L^{-1}) the CR displayed a ~3-fold decrease. The rotifer *Brachionus calyciflorus* also exhibited decreased grazing behavior when the edible *S. obliquus* was provided in a food suspension above 1000 µgC L^{-1} (Soares et al. 2010).

Nevertheless, in addition to an apparent effect of *Daphnia* body size on grazing performance, the predicted ingestion rate indicated a similar pattern of inhibition in both zooplankters when fed with toxic cyanobacteria as single and mixed food. A significant decrease was observed in the ingestion rate of edible algae. During foraging, cladocerans may create a stream of filtration by the rhythm of their thoracic appendages, bringing

particles into the filtration chamber. However, these organisms can also expel undesired particles with the aid of the post-abdomen. According to Peters (1984), the ingestion (or feeding) rate is the measure of biomass (or energy) flow into the zooplankton while the clearance (or grazing) rate is the volume of food suspension that the animal would need to remove that biomass over a given time interval to provide its measured ingestion. In addition, analyzing the gut transit time (*GTT*) for *D. magna* fed with single and mixed diets comprising the green algae *S. obliquus* and toxic and non-toxic *M. aeruginosa*, Chesney et al. (2019) found that despite the toxin content *M. aeruginosa* impaired zooplankter food ingestion. Therefore, these findings support our results that cyanobacteria reduce feeding rate in *Daphnia*.

In addition to the cyanobacterial impact on *Daphnia* grazing, in general large-bodied *D. similis* exhibited a filtering ability of edible food higher (~1.5-fold) than the smaller *D. laevis*. Sikora and Dawidowicz (2017) also reported differences in the filtering rate between the large *D. pulicaria* and the small *D. longispina* at a magnitude of approximately 4.27–6.33-fold. However, although the structure of zooplankton assemblages in tropical waterbodies comprises mostly small-sized crustaceans and rotifers, these species can occur at high densities (500–5000 individuals L^{-1}, see Pagano (2008)), which promote a similar role in top-down control of phytoplankton as that in temperate freshwater environments provided by large zooplankton.

D. similis displayed a marked reduction (~50%) in grazing when fed with *R. raciborskii.* Saxitoxins can exert acute effects on zooplankton such as paralyzing limbs, inhibiting thoracic appendage beating, and reducing the post-abdominal rejection of undesirable particles (Ferrão-Filho and Silva, 2020). Additionally, saxitoxin-producing cyanobacteria are usually reported as affecting the swimming behavior of freshwater zooplankton by decreasing mobility and activity parameters (time spent swimming and resting, distance traveled, and mean velocity) (Ferrão-Filho et al. 2014). Recently, Ferrão-Filho and Silva (2020) found that neurotoxic cyanobacteria can slow down heartbeats in *Daphnia*. Indeed, the authors' results are in line with saxitoxins' effects as these toxins also block calcium channels (Wiese et al. 2010), which are abundant in the cardiac muscle.

In our study, the saxitoxin-producing *R. raciborskii* CYRF-01 was provided as food to a *Daphnia* species ranging from 6×10^3 to 3×10^4 cells mL^{-1}. Restani and Fonseca (2014) estimated 10^4 cells mL^{-1} of CYRF-01 as the minimum concentration required to induce immobilization in *D. laevis* neonates. However, in our findings *D. laevis* adults did not show any acute effect of saxitoxin exposure after 3 hours of exposure. According to Ferrão-Filho and Kozlowsky-Suzuki (2011), the response of these animals depends on different cyanobacterial traits and exposure modes (intact cells, extracts from field and cultured strains, and purified toxins). Moreover, there are also differences in sensitivity along the life cycle.

On the other hand, no negative particle size effect was attributed to *M. aeruginosa* NPLJ-4 as it was provided as unicells (<5 µm). However, it is well known that microcystin, whether in cells or dissolved, can impact antioxidant enzymes in zooplankton (Ortiz-Rodriguez and Wiegand, 2010; Esterhuizen et al. 2016), reproduction and survivorship (Ferrão-Filho and Kozlowsky-Suzuki, 2011; Ferrão-Filho et al. 2017); however, despite analyzing the cell quota of microcystin in NPLJ-4, it is unclear whether inhibitory effects are regulated by these toxins or other bioactive metabolites. A previous investigation characterized *M. aeruginosa* NPLJ-4 as a producer of aeruginosin, a peptide inhibitor of serine proteases (Silva-Stenico et al. 2011). Once disrupted, these enzymes involved in protein catabolism might impair digestive process, thus allowing cyanobacterial cells to pass through zooplankton gut and be excreted intact (Porter, 1976). This supports the assumption that cyanobacteria constrain energy transfer into the aquatic food web.

So far, few studies have tested the combined action of cyanotoxins as dissolved or cell-bound toxins (Freitas et al. 2014; Esterhuizen-Londt et al. 2016; Ferrão-Filho et al. 2017). Studying the combined effects of cell extracts of the hepatotoxic *M. aeruginosa* NPLJ-4 and the neurotoxic *Sphaerospermopsis torques-reginae* (previously named as *Anabaena spiroides*) (ITEP-024), Freitas et al. (2014) showed synergistic effects on the survivorship and feeding rates of *Daphnia magna*, a non-native temperate cladoceran species. Esterhuizen-Londt et al. 2016 also investigated the impacts of both dissolved pure and bloom-derived cyanotoxins and reported severe synergistic effects on *D. pulex* oxidative enzymes. In our study, in addition to using living cells and a non-native cladoceran species (*D. similis*), we also used a native cladoceran species (*D. laevis*) naturally

occurring in tropical freshwaters. However, once the animals were exposed to living cells instead of pure toxins, the effects of compounds (e.g., cyanopeptides) other than the well-known cyanotoxins cannot be ruled out. Ferrão-Filho et al. (2017) also found that mixed diets comprising microcystin- and saxitoxin-producing cyanobacteria might disrupt not only physiological and population parameters but also feeding behavior in different cladoceran species, including native ones.

In conclusion, toxic cyanobacteria provided as both single and mixed food impaired *Daphnia* ability to feed on edible algae, but affected clearance rate differently on small- and large-bodied species. However, regarding zooplankton community, further studies with species representing different size classes are needed to provide robust evidence of the size effect on grazing. Overall, *D. similis* and *D. laevis* displayed nonselective behavior and reduced grazing as cyanobacteria increased in the food suspension. Factors such as manageability, mechanical interference and toxicity certainly play a role in cladocerans feeding behavior and are determinant of their occurrence under dense cyanobacterial blooms.

Acknowledgements

This work was supported by the National Council for Scientific and Technological Development – CNPq and by the Carlos Chagas Filho Foundation for the Support of Science in Rio de Janeiro Development – FAPERJ. M.C.P.V. was supported by the CNPq PhD scholarship.

Author contributions

M.C.P.V., A.S.F.F. and S.M.F.O.A. designed the research; M.C.P.V. and T.F.C.P.R., G.S.D.S performed research; M.C.P.V., A.S.F.F. and S.M.F.O.A. analyzed and reviewed data; and M.C.P.V. wrote the paper.

Declaration of interest

The authors declare no conflict of interest.

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Supplementary data

Table 1 – Results of the Two-way ANOVA for differences in clearance rates of *Daphnia* clones under single (*Microcystis* or *Raphidiopsis*) and mixed cyanobacterial diets. ns: no significant differences.

Clearance rate	**SS**	**Df**	**MS**	***F***	***P***
Microcystis diet	1.867	2	0.9335	$F_{(2,18)}$= 19.22	**<0.0001**
Daphnia clones	0.9536	1	0.9536	$F_{(1,18)}$= 19.63	**<0.001**
Interaction	0.6864	2	0.3432	$F_{(2,18)}$= 7.065	**<0.001**
Raphidiopsis diet	1.671	2	0.8354	$F_{(2,17)}$= 20.04	**<0.0001**
Daphnia clones	0.2749	1	0.2749	$F_{(1,17)}$= 6.595	**<0.05**
Interaction	0.7097	2	0.3549	$F_{(2,17)}$= 8.513	**<0.01**
Mixed diet	0.2937	2	0.1468	$F_{(2,17)}$= 1.516	ns
Daphnia clones	0.5227	1	0.5227	$F_{(1,17)}$= 5.397	**<0.05**
Interaction	0.4915	2	0.2457	$F_{(2,17)}$= 2.573	ns

Table 2 – Results of the Two-way ANOVA for differences in ingestion rates of *Daphnia* clones under single (*Microcystis* or *Raphidiopsis*) and mixed cyanobacterial diets. ns: no significant differences.

Ingestion rate	**SS**	**Df**	**MS**	***F***	***P***
Microcystis diet	29595	2	14797	$F_{(2,17)}$= 263.9	**<0.0001**
Daphnia clones	976.7	1	976.7	$F_{(1,17)}$= 17.42	**<0.001**
Interaction	1000	2	500	$F_{(2,17)}$= 8.917	**<0.01**
Raphidiopsis diet	28891	2	14445	$F_{(2,17)}$= 111.2	**<0.0001**
Daphnia clones	533.4	1	533.4	$F_{(1,17)}$= 4.107	ns
Interaction	1443	2	721.3	$F_{(2,17)}$= 5.553	**<0.05**
Mixed diet	25832	2	12916	$F_{(2,15)}$= 98.75	**<0.0001**
Daphnia clones	800.1	1	800.1	$F_{(1,15)}$= 6.117	**<0.05**
Interaction	1248	2	624	$F_{(2,15)}$= 4.77	**<0.05**

CAPÍTULO 2

Daphnia gessneri infochemicals enhance toxin production, but does not affect the fitness of the bloom-forming cyanobacterium *Microcystis aeruginosa* NPLJ-4

Manuscrito a ser submetido ao periódico *Hydrobiologia*

***Daphnia gessneri* infochemicals enhance toxin production, but does not affect the fitness of the bloom-forming cyanobacterium *Microcystis aeruginosa* NPLJ-4**

Mauro Cesar Palmeira Vilar[1]*, Thiago Ferreira da Costa Pena Rodrigues[1]* & Sandra Maria Feliciano de Oliveira e Azevedo[1]

* Both authors contributed equally for this study

[1]Laboratory of Ecophysiology and Toxicology of Cyanobacteria, Carlos Chagas Filho Institute of Biophysics, Federal University of Rio de Janeiro, Rio de Janeiro - 21949-902, Brazil.

Corresponding author: Mauro Vilar (maurovilar@gmail.com)
Carlos Chagas Filho Biophysics Institute, Federal University of Rio de Janeiro, Rio de Janeiro 21949-902, Brazil
Phone: +55 21 39386648

Abstract

Cyanobacteria are photosynthetic microorganisms that compose phytoplankton and therefore have a trophic relationship with zooplankton, which represent an important link for energy flux in aquatic food webs. Several species can form blooms and produce bioactive metabolites, that is, cyanotoxins. However, the ecological and adaptative role of these toxins have not been determined. One of the most studied hypotheses attributes to cyanotoxins the function of defense against herbivory once grazing pressure by zooplankton plays a role in phytoplankton top-down control. Thus, the present study evaluated the ecophysiological response of the cyanobacterial strain *Microcystis aeruginosa* NPLJ-4 to the cladoceran *Daphnia gessneri*. Exposure to predator infochemicals consisted of cultures established in ASM-1 medium prepared in a filtrate from a culture of adults of *D. gessneri* at an environmentally relevant density. *Daphnia* infochemicals promoted a significant increase in toxin production by *M. aeruginosa*. However, no differences in growth were observed, despite a significant increase in both maximum photosynthetic efficiency and electron transport rate in response to zooplankton. Additionally, there was no significant variation in the production of exopolysaccharides. Overall, although an induced defense response was demonstrated, there were no effects on *M. aeruginosa* fitness, which maintained its growth in the presence of predator cues.

Keywords: Microcystins, induced defenses, zooplankton, cyanobacteria

Introduction

Cyanobacterial blooms represent a water issue that is recurrent worldwide due to artificial eutrophication. Moreover, these phenomena have been boosted by climate change due to alterations in rain and drought regimes, as well as temperature increase (O'Neil et al., 2012; Huisman et al., 2018). Nevertheless, cyanobacteria are a natural group of the phytoplankton community, contributing to primary production and having a strict link with zooplankton in aquatic food webs. However, several cyanobacterial genera produce a range of bioactive secondary metabolites, which can impact zooplankton survivorship and reproduction (Ferrão-Filho and Kozlowsky-Suzuki, 2011, Esterhuizen-Londt et al., 2016, Nandini et al., 2017, Ferrão-Filho et al., 2019) and even swimming and feeding behavior (Ferrão-Filho et al., 2014, Bownik, 2016, 2017, Ferrão-Filho and Silva, 2020), thereby limiting its role in phytoplankton top-down control.

Among toxic cyanobacteria, *Microcystis* is a widely distributed genus that has been reported to form dense blooms and produce toxic peptides, such as microcystins (MCs) and other cyanopeptides (Harke et al., 2016, Huang and Zimba, 2019). Microcystins are chemically characterized as cyclic heptapeptides and classified according to their action mechanism as potent inhibitors of phosphatases protein 1 and 2A (Carmichael et al., 2001). These toxins have been associated with outbreaks with human (Azevedo et al., 2002) and animal intoxications (Masango et al., 2010).

There is a consensus about the environmental promoters of harmful algal blooms. However, little is known about the factors that regulate toxin production. MCs are among the most studied cyanotoxins (Merel et al., 2013; Omidi et al., 2018), and their production has been described as regulated by several abiotic factors, such as nutrient stoichiometry (Wagner et al., 2019), light (Wiedner et al., 2003, Chaffin et al., 2018) and temperature (Paerl and Huisman, 2008, Walls et al., 2018). Nevertheless, the role of biotic interactions as a trigger on MC production is still incipient, focusing primarily on phytoplankton allelopathy and zooplankton predation (Jang et al., 2003, Yang et al., 2014, Chia et al., 2018, Princicota et al., 2019). Although the ecological role of microcystin has not been determined, many of its effects are attributed to its defensive potential. Although cyanotoxin genes date back to before the reported existence of metazoans (Rantala et al., 2004), cyanometabolites might have evolved their role over time as an adaptative response against predators (Kaplan et al., 2012). This idea is supported by the finding that

toxic species (or strains) display a higher potential for avoiding predators compared to nontoxic ones (Rohrlack et al., 1999, Chislock et al., 2013, Holland and Kinnear, 2013).

In axenic cultures, toxic cyanobacterial strains generally maintain basal toxin production, which has raised questions about the possible self-regulatory and signaling functions of these secondary metabolites for the cell itself (see Omidi et al., 2018). However, cyanobacteria may experience an enhancement in toxin production in response to a predator, which has been considered an inducible defense. Jang et al. (2003, 2007, 2008) reported an increase in the production of microcystins by *M. aeruginosa* when exposed, either directly or indirectly (infochemicals), to *Daphnia*. These responses have also been observed at the molecular level as Pineda-Mendoza, Zúñiga and Martínez-Jerónimo (2014), which evidenced an increase in both microcystin production and MC biosynthesis marker (*mcy*A) expression by *M. aeruginosa* strains exposed to infochemicals released from *Daphnia magna*. Conversely, it is important to regard the intraspecific variation in defensive traits in the same species, once genotypes that display different responses can be found (Van Gremberghe et al., 2009). Therefore, regarding toxin production as a grazing defense, natural selection may act on cyanobacterial genotypes that display higher plasticity or those that are less plastic but naturally produce a higher amount of toxins.

Unlike toxin production, colonial phytoplankton species kept under laboratory conditions tend to grow in a single-celled morph after multiple generations, but they might display de novo colony formation after being exposed to predators (Verschoor et al., 2004, Wu et al., 2013). Such studies as Roccuzzo et al. (2016) and Yasumoto et al. (2005, 2008) have already shown that the ability to form colonies does not seem to be lost, being a phenotype that can be induced by the presence of zooplankton, which is signaled by infochemicals (kairomones), chemically characterized for *Daphnia* as cationic surfactants (aliphatic sulfates and sulfamates) that might interact with the lipid layer of cells. Additionally, Harke et al. (2017) evaluated the transcriptome of *M. aeruginosa* LE-3 exposed to *Daphnia* spp. and observed a significant increase in transcripts related to growth and the stress response and those encoding exopolymeric substances (EPSs) but no variations in cyanotoxin biosynthesis. These EPSs are also referred to as extracellular polysaccharides and have an important role in colony formation, being described for maintaining colonial morphology in Microcystis (Xiao et al., 2018). Thus, the aim of our study was to investigate changes in microcystin and

exopolysaccharide (EPS) production, as well as in the growth and photosynthesis of a *M. aeruginosa* strain in response to indirect (infochemical) exposure to the tropical zooplankton *D. gessneri*. Therefore, we tested the hypothesis that exposure to herbivorous zooplankton induces chemical defenses in cyanobacteria but does not affect their fitness.

Material and Methods

Phytoplankton and Zooplankton cultures

The cyanobacterial strain *Microcystis aeruginosa* NPLJ-4 was isolated from Jacarepaguá Lagoon-RJ and has been kept under laboratory conditions for more than 20 years. It was characterized as producer of bioactive peptides such as [D-leu^1]microcystin-LR variants, cyanopeptoline and aeruginosin (PP1/2A and serine endopeptidases inhibitors) (Ferreira et al. 2010; Silva-Stenico et al. 2011). Stock cultures were maintained in ASM-1 medium (Gorham et al. 1964) at 24±1°C, 50 µmol photons m^{-2} s^{-1} under a 12h photoperiod. Under these conditions, *Microcystis* occurred mainly as unicells and 2 cells coenobium with a mean cell diameter of 4.45±0.69 µm.

Zooplankton consisted of the neotropical species *Daphnia gessneri* isolated from Barra do Braúna Reservoir, in 2018 (Minas Gerais, Brazil). *D. gessneri* had been maintained in the laboratory for many generations before the study. The zooplankter was kept in RT medium (Tollrian 1993) enriched with commercial 0.1% (~2.25 mgC L^{-1}) humic extract (Microbe-lift® Amazon Black & Soft Water Conditioner, USA) at initial pH 7.6; 24±1 °C, 50 µmol photons m^{-2} s^{-1} and 12h:12h dark-light cycle. Animals were fed with *Selenastrum capricornutum* cell suspension at a final concentration of 400 µgC L^{-1} once every two days. Green algae population was dominated by unicells (length-width mean dimensions 13.80±1.56 x 3.18±0.66 µm).

Experimental design

Zooplankton infochemicals obtention

To obtain the filtrate with zooplankton infochemicals, 2L cultures of *D. gessneri* were established at a density of 60 individuals L^{-1}, fed with suspension of *S. capricornutum* at 1000 µgC L^{-1} and incubated for 72 hours. After the incubation time, the zooplankton was removed by pre-filtration in a glass fiber filter and subsequent membrane filtration (0.22 µm, Whatman®) to remove the remaining cells of microalgae, bacteria and other

particles. Control condition consisted of RT medium with food suspension without zooplankton. Both infochemicals-rich and control filtrates were used as reconstituted water on the preparation of the cyanobacterial culture media.

Cyanobacteria exposure to infochemicals

To evaluate cyanobacterial responses to the infochemicals released by zooplankton, a 10^5 cells mL^{-1} inoculum of *M. aeruginosa* NPLJ-4 were made in 1L erlenmeyers (n=3) filled with 600 mL of the filtrates (both treatment and control filtrate) enriched with stock solutions from the ASM-1 culture media, after appropriate stoichiometric adjustments regarding the standard concentrations of nutrient in ASM-1.

The experiment was run for 6 days and samples were taken every two days for growth measures, photosynthetic parameters, EPS and toxin analysis.

Growth and photosynthesis analysis

Harvested samples were preserved with 1% acetic lugol solution for cell counting in Fuchs-Rosenthal chamber under optical microscope expressed in cell mL^{-1}. Subsequently, cell density values were converted to biovolume (mm^3 L^{-1}) from the average cell volume (μm^3) (Hillebrand et al. 1998; Sun and Liu 2003).

Growth rate was calculated during exponential growth phase following the equation:

$$\mu = \frac{(lnN_2 - lnN_1)}{(t_2 - t_1)}$$

where, N_2=final biovolume; N_1=initial biovolume; t_2=final time; t_1=initial time.

Photosynthetic performance was analyzed using a PHYTO-PAM fluorometer (Heinz Walz GmbH, Germany) equipped with the PHYTO-EDF detection unit for measuring cyanobacterial fluorescence. Fluorescence data were converted to chlorophyll-a concentration (μg L^{-1}). Light curves were performed with dark acclimated culture samples (F_0) at an interval of 10 s between each pulse, whose luminous intensity varied from 16 to 764 µmol photons m^{-2} s^{-1}. From these curves, the values of photosynthetic yield (relative *Fv'/Fm'*), maximum electron transport rate (ETR_{max}), light harvesting efficiency (α) and the light saturation parameter (*Ik*) were obtained as a function of irradiance. Also, the maximum effective quantum efficiency of PSII was obtained as $\phi_m = [(F_m - F)/ F_m]$,

where F is fluorescence of the dark-adapted sample and F_m is the maximum dark-adapted fluorescence.

Exopolysaccharides (EPS) analysis

The potential for cells aggregation was assessed indirectly from the estimative of the concentration of dissolved and cell-bound polysaccharides according to the method described by Dubois et al. (1956) and adapted for phytoplankton organisms by Myklestad and Haug (1972).

10 ml culture samples were taken every two days, centrifuged and the supernatant collected, filtered in glass fiber filter, preserved with 0.1% sodium azide and stored at -80°C until dissolved carbohydrates quantification. Conversely, pellet (cellular biomass) samples were stored at -80°C and freeze-dried to subsequent cell-bound carbohydrates quantification. Cellular (cell-bound and intracellular) were extracted with 80% sulfuric acid solution and incubated during 20 h at room temperature until to be stopped with ultrapure water addition under ice bath.

1 mL aliquots of the extracts (cell extract and supernatant) were harvested and enriched with 0.25 mL of 5% phenol and 2.5 ml of standard sulfuric acid solution (95-97%) were added. After 30 minutes, the optical density was determined by spectrophotometry (Shimadzu – UV mini 1240) at 485 nm. Quantification of both cell-bound and dissolved carbohydrates was performed using a standard glucose curve (Sigma-Aldrich) and expressed as $pg \times cell^{-1}$ and $\mu g \times mL^{-1}$, respectively.

Microcystin analysis

Microcystins from freeze-dried cells were extracted with 50% acidified (5% acetic acid) methanol solution. The supernatant was collected, evaporated (up to 50% of the total volume) in a thermostatic bath with N_2 injection, pre-purified in a solid phase extraction C_{18} cartridge (SPE) and eluated in 100% methanol prior to analysis. For dissolved microcystins, culture samples were filtered (0.22 μm, Whatman®), freeze-dried and also resuspended in 100% methanol. Both samples followed to MCs analysis in a High-Performance Liquid Chromatography system coupled to a photodiode array UV detector (HPLC-PDA Shimadzu). Mobile phase consisted of 20 mM ammonium acetate buffer solution + acetronitrile (72:28 v/v, pH 5.0) as described in Sivonen et al. (1992) using a C18 column (5 μm – Phenomenex®) with detection at 238 nm.

Obtained peaks were analyzed and UV absorption spectra compared to standard microcystin-LR solution from the National Research Council (NRC) - Institute of Marine Biosciences (Canada). Cell and biovolume quota of microcystin were expressed as $pg \times cell^{-1}$ and $\mu g/mm^3$, respectively. Both total volumetric intracellular and extracellular microcystin were expressed in $\mu g \times L^{-1}$.

Application of first-order rate kinetics to assess total microcystin production

The specific cell division rate (μ_c) and the specific microcystin production rate (μ_{MC}) were calculated during the exponential growth phase according to simple first-order rate kinetics using either cell concentration (cells mL^{-1} of culture medium) or volumetric intracellular microcystin concentration data (ng mL^{-1}), respectively (both specific rates are reported in units per d^{-1}).

The ratio between μ_{MC} and μ_c was calculated to assess different patterns of toxin production coupled to the growth cycle and consequent changes in the toxin biovolume (or cell) quota (Q_{tox}) as described in Orr et al. (2018), such that:

Equation (i) describes the condition where the intracellular μ_{MC} between t_0 and t_n is slower than μ_c, resulting in a lower Q_{tox}

(i) $$0.5 < \frac{\mu_{stx}}{\mu_g} < 1$$

Equation (ii) describes the condition where the intracellular μ_{MC} between t_0 and t_n is a function of μ_c, resulting in a constant Q_{tox} (1:1 growth-toxin relationship).

(ii) $$\frac{\mu_{stx}}{\mu_g} = 1$$

Equation (ii) describes the condition where the intracellular μ_{MC} between t_0 and t_n is higher than μ_c, resulting in an increased Q_{tox}.

(iii) $$\frac{\mu_{stx}}{\mu_g} > 1$$

Data analysis

All data were checked for normality and homoscedasticity of variances. Growth rate data and toxin production–cell division ratios were analyzed by Student's t-test to verify any significant effect of zooplankton infochemicals. Variations in growth, microcystin cell quota, total microcystin pool size and carbohydrate content along culture time were

verified using repeated measure two-way ANOVA with a *post hoc* Bonferroni test. All analyses were performed using GraphPad Prism 6.0 software. Additionally, linear regressions between μ_c and μ_{MC} were performed using SigmaPlot 12.0.

Results

Infochemicals released by the cladoceran *D. gessneri* stimulated a significatively higher toxin production by *M. aeruginosa* NPLJ-4 but did not exert effects on cyanobacterium growth and photosynthesis that could conversely reduce its fitness.

M. aeruginosa NPLJ-4 displayed a similar growth curve by both cellular biovolume and chlorophyll-a concentration, and no significant differences in growth rate when treated with *D. gessneri* infochemicals ($\mu_{control}$ = 0.19±0.01 day^{-1}; $\mu_{treatment}$ = 0.20±0.005 day^{-1}; *T* test, p>0.05) **(Suppl. 1; Fig. 1)**.

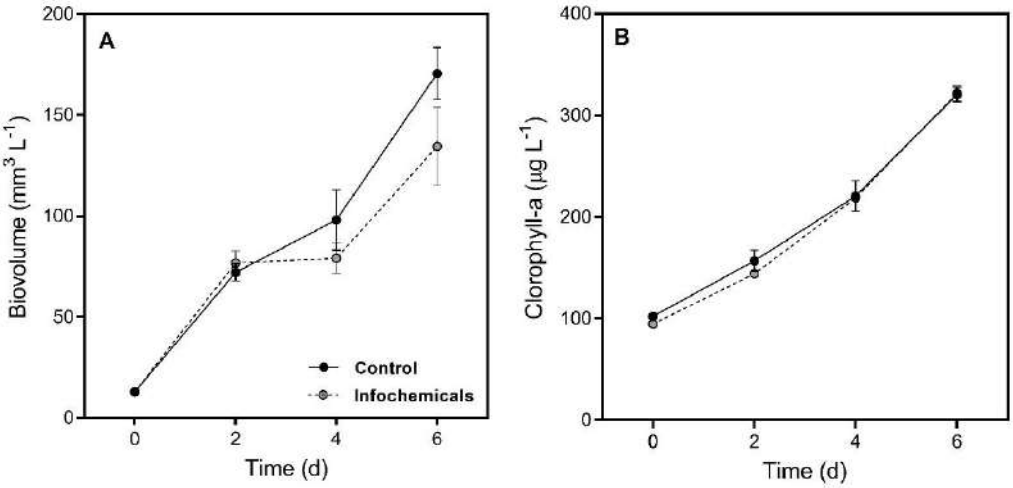

Figure 1. Growth curves in A) biovolume and B) chlorophyll-a concentration for *Microcystis aeruginosa* NPLJ-4 (dashed grey line) exposed and (black line) non-exposed to *Daphnia gessneri* infochemicals.

Additionaly, no significant effects of *Daphnia* infochemicals were found in the light harvesting efficiency (α), relative PSII quantum yield (*Fv*'/*Fm*') and light saturation parameter (*Ik*) **(Table 1).** The later increased significantly in both conditions over the experiment (Two-way RM-ANOVA, p<0.0001). The light saturation parameter defines the light threshold in which photosynthesis is inhibited.

Conversely, a significant increase in the maximum electron transport rate (ETRmax) was observed on the 6th day in response to the infochemicals (Bonferroni's test, p<0.05)

in addition to an increase in this photosynthetic parameter over the incubation time (two-way RM-ANOVA, $p<0.0001$) **(Table 1)**.

Additionally, the maximum PSII quantum yield reached by *M. aeruginosa* NPLJ-4 through irradiation curves evidenced an early enhancement in photosynthetic efficiency in response to predator cues (Bonferroni's test, $p<0.0001$) **(Suppl. 2; Fig. 2)**.

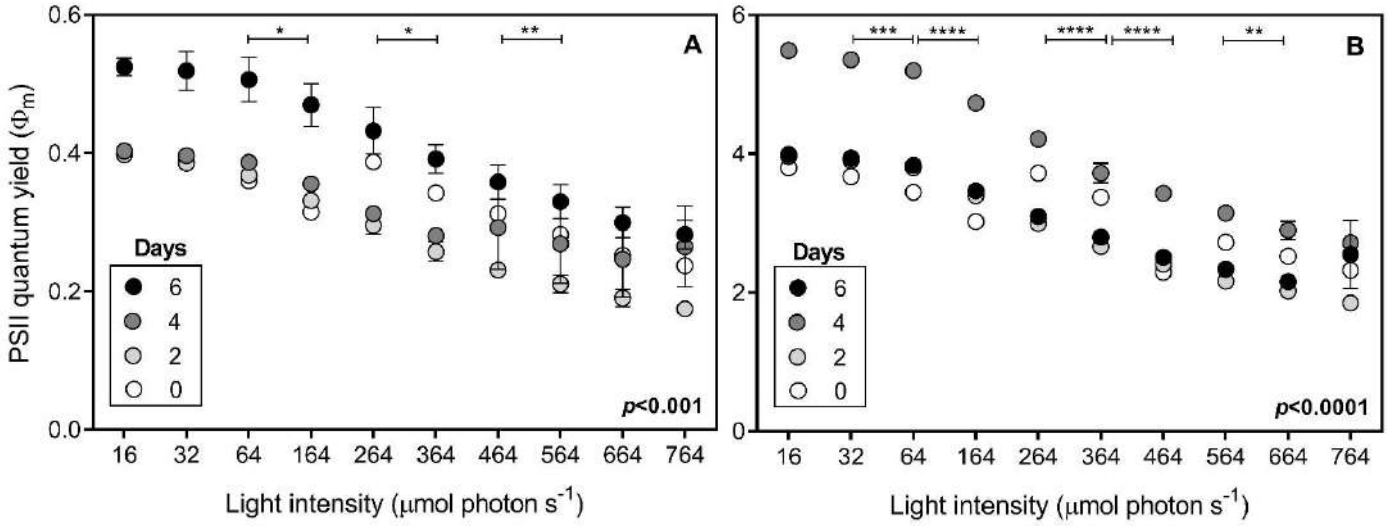

Figure 2. Maximum PSII quantum yield derived from photosynthesis irradiation curve of *Microcystis aeruginosa* NPLJ-4 (A) non-exposed and (B) exposed to *Daphnia gessneri* infochemicals along 6-days culture. Significative difference (***; ****) = Bonferroni's test, $p<0.001$; $p<0.0001$, respectively.

In addition, at this condition the cyanobacterium seemed to be more sensitive displaying a significative decrease in photosynthetic efficiency when exposed to light intensities above 32 µmol photon m^{-2} s^{-1} across increased irradiation pulses. Conversely, in the control it was observed an increase in maximum PSII quantum yield at the last day of incubation (Bonferroni's test, <0.001), but an overall decrease in photosynthetic efficiency only from 64 µmol photon m^{-2} s^{-1}, indicating a higher light tolerance **(Fig. 2)**. Under control conditions, an increase in the maximum PSII quantum yield was only observed on the last day of incubation (Bonferroni's test, $p<0.001$).

Table 1. Photosynthetic parameters of *Microcystis aeruginosa* NPLJ-4 (INFO) exposed and (CTRL) non-exposed to *Daphnia gessneri* infochemicals. A) Yield – Relative PSII quantum yield at a saturation pulse of 36 PAR (Photosynthetically active radiation), ETRmax – maximum electron transport rate, Ik – light saturation parameter and Alpha (α) – light harvesting efficiency. SD = standard deviation. Different letters mean statistical differences (Bonferroni's test, $p<0.05$).

Photosynthetic Parameters	**Yield** (Relative Fv'/Fm')		**ETR_{max}** (µmol e m^{-2} s^{-1})		**Ik** (µmol photon m^{-2} s^{-1})		**Alpha** (µmol photon m^{-2} s^{-1})	
Time (d)	**CTRL** (Mean±SD)	**INFO** (Mean±SD)	**CTRL** (Mean±SD)	**INFO** (Mean±SD)	**CTRL** (Mean±SD)	**INFO** (Mean±SD)	**CTRL** (Mean±SD)	**INFO** (mean±sd)
0	0.55±0.00[a]	0.54±0.00[a]	71.90±0.00[a]	76.10±0.00[a]	310±0.00[a]	343.10±0.00[a]	0.23±0.00[a]	0.22±0.00[a]
2	0.54±0.03[a]	0.52±0.01[a]	84.73±6.19[a]	88.83±4.16[a]	371.43±25.90[a]	386.83±25.99[a]	0.23±0.00[a]	0.23±0.00[a]
4	0.52±0.01[a]	0.52±0.01[a]	97.27±4.46[a]	94.60±3.93[a]	413.90±19.77[a]	409.17±21.01[a]	0.24±0.00[a]	0.23±0.00[a]
6	0.51±0.02[a]	0.54±0.00[a]	94.17±3.52[a]	102.63±0.57[b]	425.53±10.44[a]	441.13±4.24[a]	0.22±0.01[a]	0.23±0.00[a]

No significant differences were found in cellular carbohydrates produced by *M. aeruginosa* NPLJ-4 when exposed to predator infochemicals. However, an increase in released carbohydrates was observed at the late exponential growth phase (6th day) (Bonferroni's test, $p<0.01$) **(Fig. 3)**.

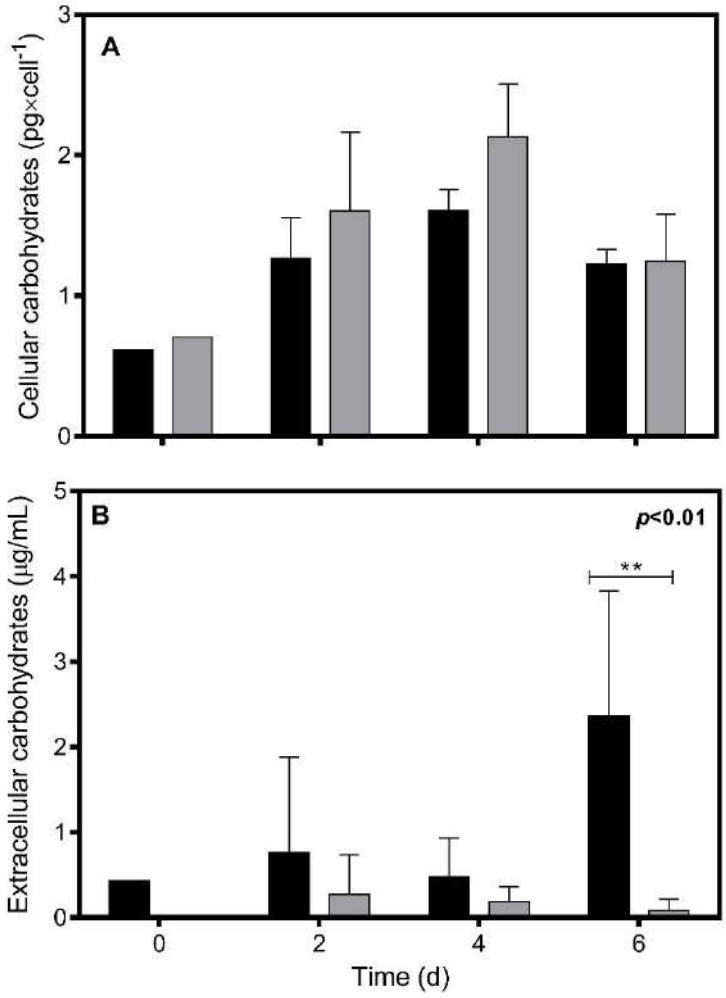

Figure 3. Cell-bound and extracellular carbohydrates as a proxy of exopolysaccharides produced *Microcystis aeruginosa* NPLJ-4 (black bars) non-exposed and (grey bars) exposed to *Daphnia gessneri* infochemicals. Significative difference (**) = Bonferroni test, $p<0.01$.

Daphnia infochemicals significantly enhanced microcystin production by *M. aeruginosa* NPLJ-4 (control= 0.023±0.007 pg $cell^{-1}$ and 0.509±0.160 µg mm^{-3}; zooplankton infochemicals= 0.045±0.008 pg $cell^{-1}$ and 0.974±0.178 µg mm^{-3}), evidencing a chemically induced defense response by the cyanobacterium (Bonferroni's

test, $p<0.01$) **(Suppl. 3 and 4; Fig. 4).** Moreover, despite the higher cell/biovolume microcystin quota at the beginning of the experiment **(Fig. 4)**, which represents the physiological state (late exponential phase) of the cells in the inoculum, the volumetric concentration of microcystin indicates that the total amount of toxin in the culture at this same time was lower in comparison to the subsequent days of incubation **(Fig. 5)**. Overall, both the cellular microcystin quota and volumetric concentration increased over incubation time (two-way RM-ANOVA, $p<0.01$) **(Fig. 4 and 5)**. The latter also represents the increase in cell concentration in both experimental conditions.

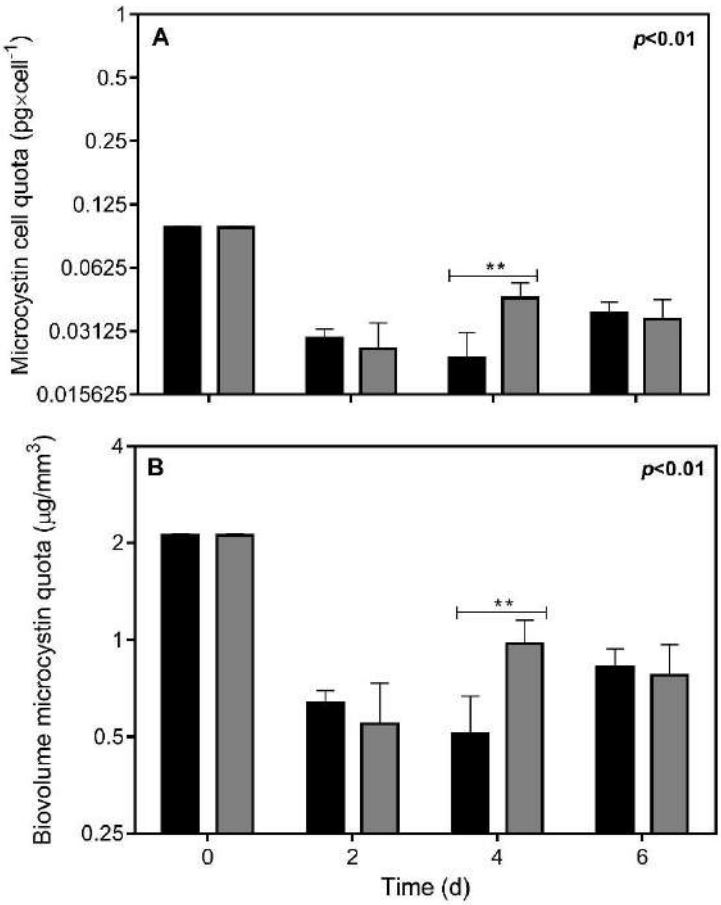

Figure 4. Microcystin A) cell and B) biovolume quota produced by *Microcystis aeruginosa* NPLJ-4 (black bars) non-exposed and (grey bars) exposed to *Daphnia gessneri* infochemicals. Significative difference (**) = Bonferroni test, $p<0.01$.

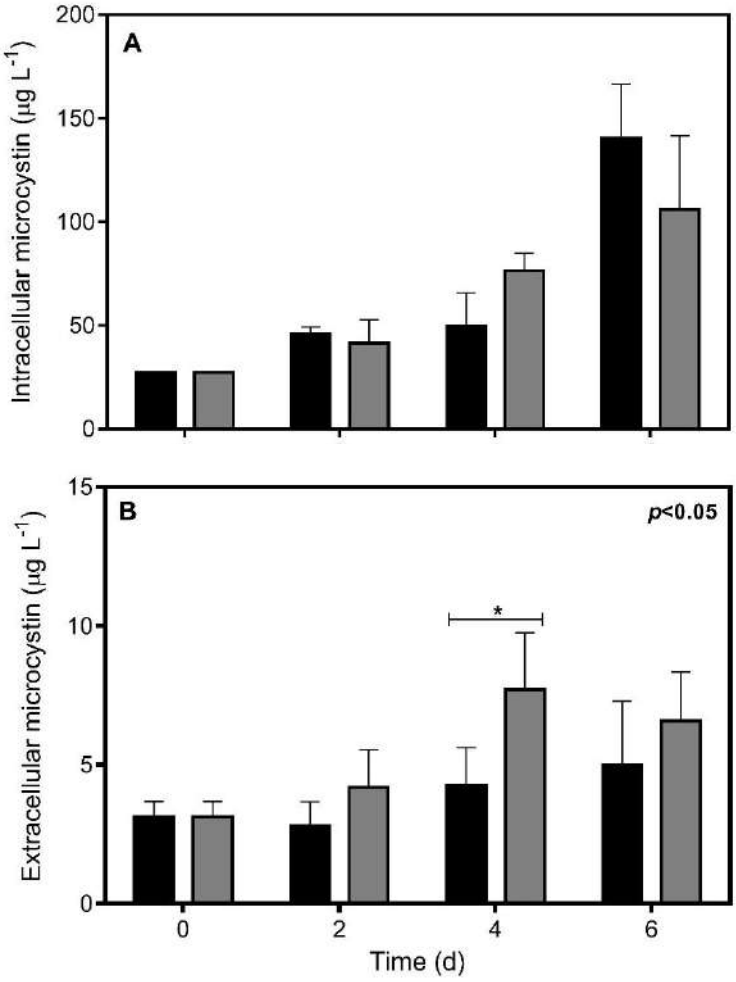

Figure 5. Total volumetric amount of (A) intracellular and (B) extracellular microcystin produced by *Microcystis aeruginosa* NPLJ-4 (black bars) non-exposed and (grey bars) exposed to *Daphnia gessneri* infochemicals. Significative difference (*) = Bonferroni test, $p<0.05$.

Regarding the extracellular amount of MC, there was also a significant peak in the dissolved fraction of toxin in response to zooplankton cues **(Fig. 5B)**. Predator-threat signal effects in *Microcystis* over the incubation time resulted in microcystin release ranging from 3.34–14,98% relative to the total microcystin produced, while in the control, it ranged from 2.05–8.56%.

First-order rate kinetics enabled us to identify different patterns in the relationship between toxin production/cell division in *M. aeruginosa* NPLJ-4. These data analyses enabled us to observe that without zooplankton infochemicals, microcystin production by the cyanobacterium was strictly related to the growth rate **(Fig. 6A)**. On the other hand, predator cues seemed to govern toxin production, as no linear relationship was found

between the cell division rate in the presence of infochemicals **(Fig. 6B)**. These findings are supported by the μ_{MC}/μ_c ratio, which displayed values significantly higher than 1 **(T=10.29, *p*<0.001; Fig. 7)**, indicating that the specific rate of microcystin production was faster than the specific cell division rate at the log phase, resulting in increased cell toxin accumulation, as shown in **Figure 4**.

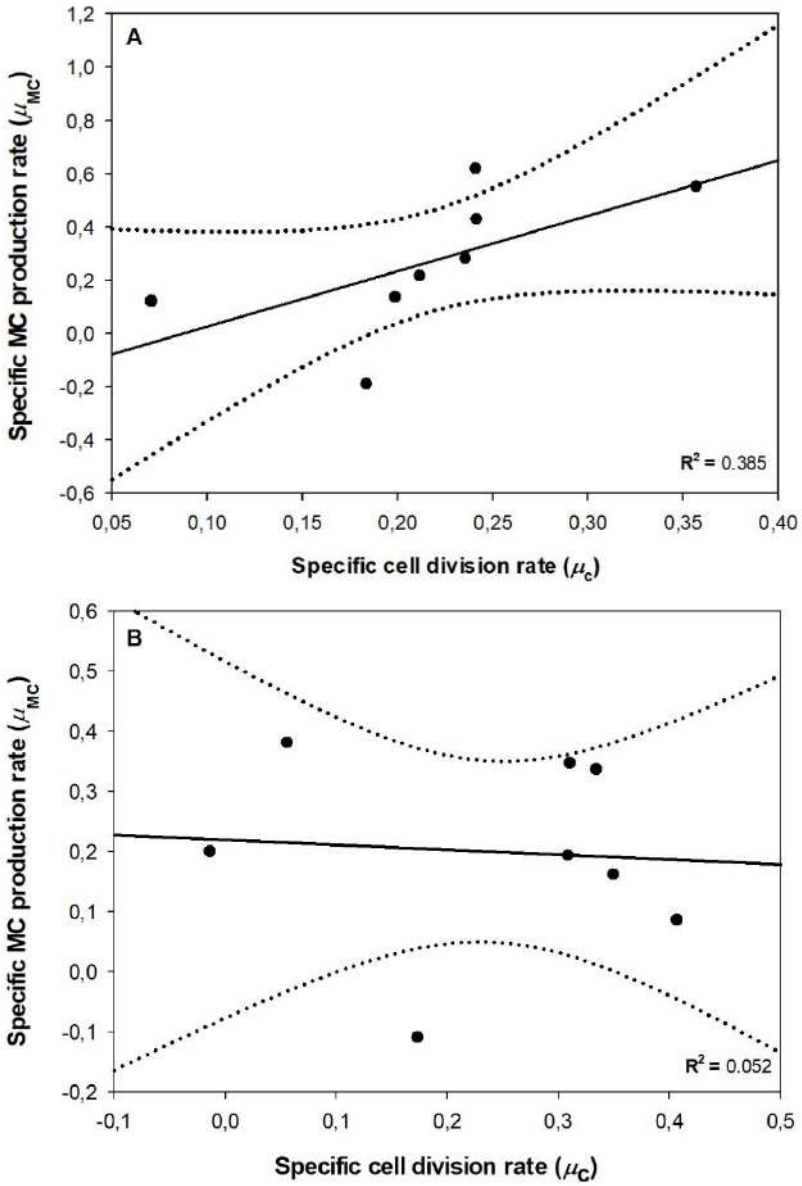

Fig.6. Linear regression of specific cell division rate (μ_c) plotted against specific (MC) microcystin production rate (μ_{MC}) for *Microcystis aeruginosa* NPLJ-4 grown (A) without and (B) with *Daphnia gessneri* infochemicals. Data point represents µc and µMC at each 2-days interval along growth cycle. Dotted lines mean 95% confidence interval.

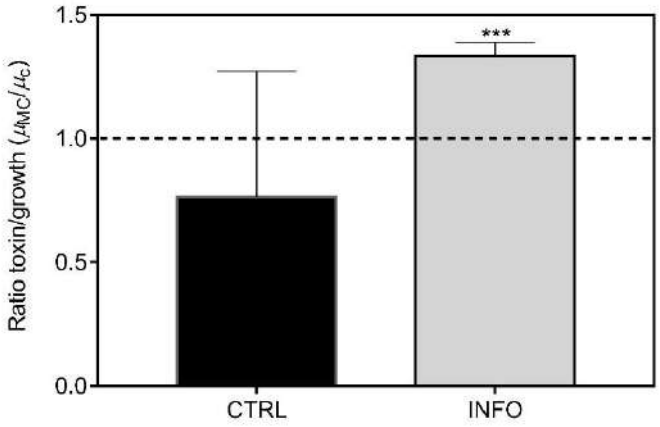

Fig.7. Ratio between specific microcystin production rate (μ_{MC}) and specific cell division (μ_c) obtained from first order rate kinetic (log phase) for *Microcystis aeruginosa* NPLJ-4 grown (black bar) without (CTRL) and (grey bar) with *Daphnia gessneri* infochemicals (INFO). Dashed line means 1:1 relationship between μ_{MC} and μ_c as described in Orr et al. (2018). (*) = Student T-test, $p<0.05$.

Discussion

Our findings support the hypothesis that exposure to herbivorous zooplankton enhances toxin production by *M. aeruginosa* NPLJ-4 but does not affect negatively its fitness.

Intracellular microcystin concentration presents a peak four days after indirect exposure of zooplankton, and then decreased subsequently as previously reported by Jang et al. (2003; 2007). The increase in microcystin can be related to an induced defense against the predator as reported for (Jang et al. 2003; 2007; 2008; Van Gremberghe et al. 2009; Akbar et al. 2017; Princiotta et al. 2019). Also, it is likely that microcystin decrease after exposure to infochemicals is a consequence of the degradation of zooplankton chemical signals (Jang et al. 2003).

However, it is also important to regard a compensatory effect due to an additional energy expenditure coupled to the early response on peaking cyanotoxin production. Enhancing cell content of cyanotoxins seems to be a rapid defense response directed to predation, once zooplankton is more sensitive when exposed by ingestion route.

Moreover, *M. aeruginosa* NPLJ-4 displayed a higher microcystin excretion when experienced predator threat-signals. An increase in dissolved content of microcystin is supposed to play a role on colony formation in *Microcystis* (see Gan et al. 2012). Either colony or coenobium are traits with a defensive potential as it can limit zooplankton

grazing pressure on phytoplankton. Therefore, it is likely that both chemical and morphological defenses responses in toxic *Microcystis* do not occur in parallel but subsequently as a reciprocal response mediated by microcystin production.

On the other hand, an increase in dissolved microcystin also has been suggested as an allelochemical response to competitors, since cyanotoxins have been reported to reduce microalgae growth (Sedmak and Elersek 2006; Kearns and Hunter 2000). Moreover, during a bloom microcystins are mostly intracellular until the bloom collapse in consequence of cell lysis (Rohrlack and Hyenstrand 2007). However, extracellular MCs (either dissolved in water or bound to other materials) typically make up less than 30% of the total MC concentration in raw water (Graham et al. 2010). In our study, the dissolved fraction comprised less than 15% of the total microcystin produced.

M. aeruginosa NPLJ-4 toxicity has been reported in the literature regarding its microcystin-LR analogues (Soares et al. 2004; Ferreira et al. 2010; Laughinghouse IV et al. 2012; Freitas et al. 2014; Amorim et al. 2017). However, the strain is also producer of others bioactive peptides such as the serine proteases-inhibitors cyanopeptolin and aeruginosin (Silva-Stenico et al. 2011) whose toxicity has been noticed, i.e., inhibition of trypsin and chymotrypsin activity (Rohrlack et al. 2005; von Elert et al. 2012; Janssen, 2019). Hence, it is likely that chemical induced defenses in cyanobacteria not only affect the production of well-known cyanotoxins, but other bioactive metabolites.

Besides toxin enhancement, *M. aeruginosa* NPLJ-4 did not display any reduction on its fitness that was evaluated by growth and photosynthetic parameters. Similar results were seen by Jang et al. (2007) investigating the responses of microcystin-producing strains of *M. aeruginosa* and *Planktothrix agardhii* which increased toxin production in response to *D. magna* infochemicals at different densities, but no changes in growth pattern. Some dinoflagelates also have shown a similar response as reported by Selander et al. (2006) which found a significative increase in gonyautoxins cell amount of *Alexandrium minutum,* but no changes in net growth rate.

On the other hand, some studies have reported a trade-off between growth and toxin production, such as Akbar et al. (2017) which evidenced a higher microcystin production by *M. aeruginosa* and *M. flos-aquae* when exposed to *Daphnia* infochemicals, followed by a decrease in the growth pattern. Filtrate from culture of the mixotrophic algae *Cryptomonas* has also promoted a trade-off between growth and microcystin production in *M. aeruginosa* (Princiotta et al. 2019). Conversely, contrasting findings were reported

by van Gremberghe et al. (2009), which found that different *M. aeruginosa* strains displayed a decrease pattern in growth but no changes in toxin production. A possible explanation may be that changes in *M. aeruginosa* toxins might occur in other bioactive compounds which are not well-known yet (Akbar et al. 2017). From other toxic phytoplanktonic species, Blossom et al. (2019), when investigating the costs of toxicity in saxitoxin-producing *Alexandrium* strains, did not find a growth reduction related to saxitoxins production, but with lytic activity which is associated to other metabolites poorly known. Thus, the energy storage of organisms is limited, therefore energy allocation for defense against grazers would impair growth and maintenance (Zhu et al. 2016).

In our study photosynthetic parameters were also used as traits to assess cyanobacterial fitness. Into the inducible defenses, a little is known on how photosynthesis in toxic cyanobacteria cope with predator direct or indirect exposure. Pioneer data are presented by Savic et al. (2019), which evaluated photosynthetic response of toxic and non-toxic strains of *M. aeruginosa* exposed to *D. magna* metabolites. Contrary to authors' expectations, the toxic strain decreased its photosynthetic activity (ETR_{max}<30) as well as chlorophyll-a content in response to *Daphnia*. Moreover, photosynthetic response to predator cues has already been evidenced through *Microcystis* transcripts' analysis (Harke et al. 2017).

Our study brings more detailed data on photosynthesis response once that has measured other parameters (i.e. *Ik*, α and ϕ_m) besides electron transport rate and chlorophyll-a concentration, in order to evaluate cell physiological state. In our findings, an early significative increase in the maximum quantum yield (ϕ_m) of *M. aeruginosa* NPLJ-4 parallel to microcystin peak under predator threat suggests a compensatory mechanism on energy loss by ramping up their generation of energy for cellular growth via photosynthesis. In addition, we also found a significative increase in ETR_{max} at the end of the experiment what is in agreement with Savic et al. (2019).

Carbohydrate analysis was used as a proxy of colony-forming exopolysaccharides production. According to Xiao et al. (2017) two mechanisms of colony formation in *Microcystis* are recognized: (i) cell division, where the cells remain attached by an mucilaginous envelope after binary fission, and (ii) cell adhesion, where single cells aggregates are formed by secreted adhesive EPSs. The authors found that colony formation has a positive linear relation (p<0.001, N=25) with increasing EPSs

concentration. Therefore, both mechanisms depend on either exopolysaccharide or other polymeric substances production to occur.

When exposed to predator cues *M. aeruginosa* NPLJ-4 displayed a maximum cell-bound carbohydrate production of 2.13±0.38 pg cell^{-1}, but no colonies were observed. Yang et al. (2008) observed an EPS content of 2.14 pg cell^{-1}, but unlike our findings, that EPS amount was coupled to *Microcystis* colony formation. Also, Li et al. (2013) observed the appearance of *Microcystis* colonies at lower EPS amounts ranging from 0.6–0.8 pg×cell^{-1}. An even lower value (0.34 pg×cell^{-1}) was reported by Wu and Song (2008) as associated to colony formation. These findings indicate that colony formation is coupled to exopolymeric substances other than carbohydrates. In fact, besides carbohydrates (and their derivatives products, i.e., uronic acids; neutral sugars), EPS also constitute a group of functional molecules such as amino acids and proteins (Hassler et al. 2011; Mancuso Nichols et al., 2005). Furthermore, the functional EPS amount associated to *Microcystis* colony formation varies among different experimental and analytical procedures (Xiao et al. 2018) as well along the culturing time.

In general, inducible defenses allow organisms to take advantage of the benefits of this mechanism, reducing the potential costs associated with the investment of defensive strategies, when not necessary (Agrawal et al. 1999). In terms of energy requirements, defenses that are induced only in the presence of predator are considered more efficient than constitutive defenses under variable grazing risk (Agrawal 1998). In this context, Van Donk et al. (2011) point out that under low cost of survival, this characteristic tends to remain undetectable, especially under optimal conditions, as observed in the laboratory. Moreover, toxigenic cyanobacterial strains are suggested to have a higher amount of proteins involved in cellular metabolism which is likely to lend the advantage on producing more energy to maintain the fitness, despite of the cost of toxin production is significant (Tonietto et al., 2012).

As pointed by Van Gremberghe et al. (2009), environmental conditions act as a co-factor and suggest further investigation in *Microcystis* responses to biotic interactions. Moreover, the authors state that the environment does not affect the ability of identifying variation on intraspecific responses. It lies in the fact that zooplankton-induced changes are strongly strain-specific. Hence, the later merit further investigation by examining different strains, which allows identifying more precisely the changes underlying induced defenses in a species. Thus, in our study toxin increase in *M. aeruginosa* NPLJ-4 when

exposed to *D. gessneri* infochemicals support the hypothesis of induced chemical defenses. Moreover, despite the energetic cost on producing toxins, no effects were counted to other physiological traits such as growth, biomass acquisition and photosynthetic performance of the cyanobacterium. Evaluating the reciprocal phenotypic responses of the cyanobacteria-zooplankton interaction provides information for understanding the adaptive pressure that such interactions exert on these organisms, which will invest in chemical and morphological defenses for the rapid evolution and maintenance of their populations in nature.

Acknowledgements

This work was supported by the National Council for Scientific and Technological Development – CNPq and by the Carlos Chagas Filho Foundation for the Support of Science in Rio de Janeiro Development – FAPERJ. M.C.P.V. and T.F.C.P.R. were supported by CNPq scholarships.

Author contributions

M.C.P.V. and S.M.F.O.A. designed the research; M.C.P.V. and T.F.C.P.R. performed research; M.C.P.V., T.F.C.P.R. and S.M.F.O.A. analyzed and reviewed data; and M.C.P.V. wrote the paper.

Declaration of interest

The authors declare no conflict of interest.

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Supplementary data

Table 1 – Results of the Two-way ANOVA for diferences in growth (biovolume and chl-a curves) of *Microcystis aeruginosa* NPLJ-4 exposed to *Daphnia gessneri* infochemicals in comparison to control condition along 6-days incubation. **ns**= no significant differences.

Biovolume	**SS**	**Df**	**MS**	***F***	***P***
Time	59120	3	19707	$F_{(3,12)}$= 244.6	<0.001
Infochemicals	949.2	1	949.2	$F_{(1,4)}$= 4.788	ns
Interaction	1571	3	523.6	$F_{(3,12)}$= 6.498	<0.001
Subjects (matching)	793	4	198.3	$F_{(4,12)}$= 2.46	ns
Residual	966.9	12	80.58		
Chlorophyll-a	**SS**	**Df**	**MS**	***F***	***P***
Time	166622	3	55541	$F_{(3,12)}$= 1018	<0.0001
Infochemicals	154.4	1	154.4	$F_{(1,4)}$= 2.459	ns
Interaction	174.6	3	58.21	$F_{(3,12)}$= 1.067	ns
Subjects (matching)	251.2	4	62.8	$F_{(4,12)}$= 1.151	ns
Residual	905.7	12	54.55		

Table 2 – Results of the Two-way ANOVA for diferences in maximum PSII yield obtained from irradiation curves of *Microcystis aeruginosa* NPLJ-4 exposed to *Daphnia gessneri* infochemicals in comparison to control condition along 6-days. **ns=** no significant diferences.

Control	**SS**	**Df**	**MS**	***F***	***P***
Time	0.2598	3	0.08661	$F_{(3,60)}$= 165.9	<0.0001
Increased irradiation	0.5474	9	0.06082	$F_{(9,20)}$= 107.5	<0.0001
Interaction	0.05403	27	0.00200	$F_{(27,60)}$= 3.833	<0.0001
Subjects (matching)	0.01132	20	0.00056	$F_{(20,60)}$= 1.084	ns
Residual	0.03132	60	0.00052		
Infochemicals	**SS**	**Df**	**MS**	***F***	***P***
Time	0.2608	3	0.08695	$F_{(3,60)}$= 920.9	<0.0001
Increased irradiation	0.6307	9	0.07008	$F_{(9,20)}$= 627	<0.0001
Interaction	0.07168	27	0.00265	$F_{(27,60)}$= 28.12	<0.0001
Subjects (matching)	0.00223	20	0.00011	$F_{(20,60)}$= 1.184	ns
Residual	0.0057	60	0.00009		

Table 3 – Results of the Two-way ANOVA for differences for microcystin cell and biovolume quota of *Microcystis aeruginosa* NPLJ-4 exposed (infochemicals) and non-exposed (control) to *Daphnia gessneri* infochemicals along 6-days incubation. **ns=** no significant diferences.

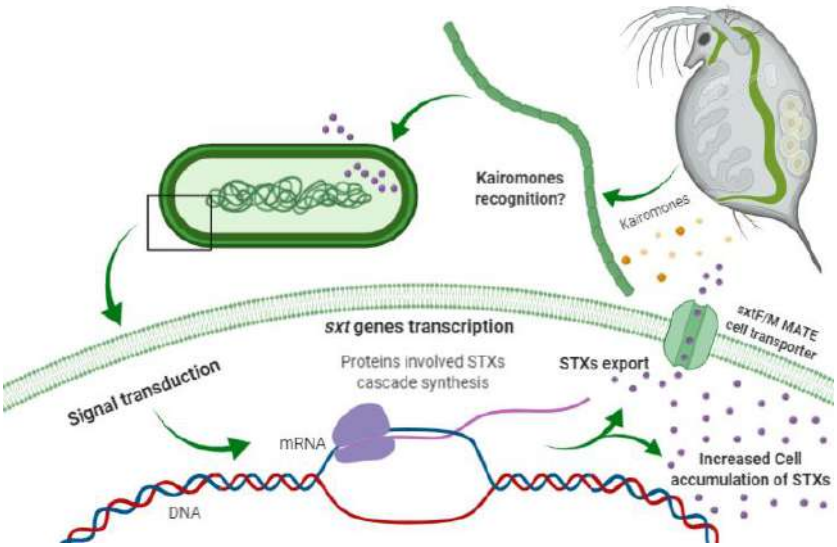
Microcystin cell quota	**SS**	**Df**	**MS**	***F***	***P***
Time	0.01904	3	0.00635	$F_{(3,12)}$= 241.4	<0.0001
Infochemicals	0.00009	1	0.00009	$F_{(1,4)}$= 1.481	ns
Interaction	0.00063	3	0.00021	$F_{(3,12)}$= 7.36	<0.01
Subjects (matching)	0.00026	4	0.0006	$F_{(4,12)}$= 2.262	ns
Residual	0.00034	12	0.00023		
Microcystin biovolume quota	**SS**	**Df**	**MS**	***F***	***P***
Time	8.987	3	2.996	$F_{(3,8)}$= 137.1	<0.0001
Infochemicals	0.04162	1	0.04162	$F_{(1,8)}$= 2.839	ns
Interaction	0.2976	3	0.09919	$F_{(3,8)}$= 6.765	<0.05
Subjects (matching)	0.1748	8	0.02185	$F_{(8,8)}$= 1.49	ns
Residual	0.1173	8	0.01466		

Table 4 – Results post-hoc Bonferroni's comparison test for differences for microcystin cell and biovolume quota of *Microcystis aeruginosa* NPLJ-4 (infochemicals) exposed and (control) non-exposed to *Daphnia gessneri* infochemicals along 6-days incubation. **ns=** no significant diferences. **CI=** confidence interval

CONTROL – INFOCHEMICALS (MC cell quota)	**Mean difference**	**95%CI of differences**	***P***
Time (d^{-1})			
0	0	-0.0141 – 0.0141	ns
2	0.003333	-0.0107 – 0.0174	ns
4	-0.02167	-0.0358 – -0.0076	<0.01
6	0.002333	-0.0118 – 0.0164	ns
CONTROL – INFOCHEMICALS (MC biovolume quota)	**Mean difference**	**95%CI of differences**	***P***
Time (d^{-1})			
0	0	-0.3103 – 0.3103	ns
2	0.08725	-0.2231 – 0.3976	<0.01
4	-0.4653	-0.7756 – -0.155	ns
6	0.0449	-0.2654 – 0.3552	ns

CAPÍTULO 3

Ecophysiological aspects and *sxt* genes expression underlying induced chemical defense in STX-producing *Raphidiopsis raciborskii* (Cyanobacteria) against *Daphnia*



Artigo a ser submetido ao periódico *Scientific Reports*

Ecophysiological aspects and *sxt* genes expression underlying induced chemical defense in STX-producing *Raphidiopsis raciborskii* (Cyanobacteria) against *Daphnia*

Mauro Cesar Palmeira Vilar[1], Thiago Ferreira da Costa Pena Rodrigues[1], Luan de Oliveira Silva[1], Ana Beatriz Furlanetto Pacheco[2] and Sandra Maria Feliciano de Oliveira e Azevedo[1]

[1]Laboratory Ecophysiology and Toxicology of Cyanobacteria, Carlos Chagas Filho Institute of Biophysics, Federal University of Rio de Janeiro, Rio de Janeiro – 21949-902, Brazil

[2] Laboratory Biologial Physics, Carlos Chagas Filho Institute of Biophysics, Federal University of Rio de Janeiro, Rio de Janeiro – 21949-902, Brazil

Corresponding author: Mauro Vilar (maurovilar@gmail.com)
Carlos Chagas Filho Biophysics Institute, Federal University of Rio de Janeiro, Rio de Janeiro 21949-902, Brazil
Phone: +55 21 39386648

Abstract

Cyanobacteria stand out in phytoplankton since it can form massive blooms and produce toxins. Regarding the fact that cyanotoxin genes date back from metazoans origin, the hypothesis that attributes to cyanotoxins the function of defense against herbivory is still under debate. Although their primary cellular function might be different, it is supposed that these metabolites also have evolved as a response against predators. Thus, the present study evaluated the ecophysiological and molecular response of a saxitoxin-producing strain *Raphidiopsis raciborskii* to infochemicals released by the cladoceran *Daphnia gessneri*. *R. raciborskii* was cultured in ASM-1 medium prepared with a filtrate obtained from a culture of *D. gessneri* at an environmentally relevant density. In these conditions, significant increases in saxitoxin production and in sxt gene transcript levels were observed, as compared to control. Concomitantly, cyanobacterial growth decreased, with no significant effects on photosynthesis or morphology. Overall, the induced defense response displayed a trade-off between toxin production and growth. These results shed light in the comprehension of the mechanisms underlying zooplankton-cyanobacteria interactions in aquatic food webs.

Keywords: Infochemicals, cyanotoxins, phytoplankton, saxitoxin, phenotypic plasticity

Introduction

Among ecological relationships, predator-prey interactions are forces that lead to evolving defensive adaptations (Vermeij et al. 1994). A series of traits that impair predation by zooplankton have been described in phytoplankton, i.g., formation of colonies, spines, and/or the production of harmful metabolites (see van Donk et al. 2011). In addition to the direct effect provided by active foraging, zooplankton also releases infochemicals which induce functional responses in phytoplankton species such as increased toxin production (Jang et al. 2003; Selander et al. 2006; Jang et al. 2007; 2008) and colony formation or trichome stretching which in turn may limit grazing pressure (Lürling, 2003; Lürling and Beekman, 2006; Wu et al. 2013; Wejnerowski et al. 2018). These infochemicals are also referred to as kairomones, i.e., chemical signals released by the predator which suggest a threat and commonly benefit the prey. In the cladocera *Daphnia* these chemicals were described as aliphatic sulphates and sulfamates (Yasumoto et al. 2005; 2008).

In many eutrophic water bodies cyanobacteria dominate the phytoplankton and represent one of the main sources of energy for omnivorous zooplankton in aquatic food webs. Several cyanobacterial species produce a range of bioactive metabolites considered as anti-grazer substances due to their well-described impacts on zooplankton survivorship, reproduction (Ferrão-Filho and Kozlowsky-Suzuki, 2011, Esterhuizen-Londt et al., 2016, Ferrão-Filho et al., 2019; Santos et al. 2020), swimming and foraging behavior (Bownik, 2016; Ferrão-Filho and Silva, 2020), thereby limiting its role in phytoplankton top-down control and ultimately impairing the energy flux in aquatic ecosystems.

Cyanobacteria are globally widespread and may form nuisance blooms, especially due to their potential of producing specialized secondary metabolites named cyanotoxins (Huisman et al. 2018), including microcystins (MCs), saxitoxins (STXs), cylindrospermopsins (CYNs) and anatoxins (ATXs). Although the chemical, pharmacological properties, and the biosynthesis pathways for these cyanotoxins have been well documented (Pearson et al. 2016), their roles on cyanobacterial ecology is not entirely clear (Kaplan et al. 2012; Holland and Kinnear 2013; Omidi et al. 2018).

Raphidiopsis raciborskii (formerly *Cylindrospermopsis raciborskii*, Aguilera et al. 2018) is one of the most common bloom-forming species in freshwater. It is a diazotrophic toxic cyanobacteria which has been first reported in tropical and sub-tropical

regions where it dominates (or co-dominates) phytoplanktonic communities. Nowadays, *R. raciborskii* is frequently recorded in temperate freshwater environments (Antunes et al. 2015). This recent expansion and invasive (or opportunistic) potential is ascribed to its phenotypic plasticity, noted as tolerance to low light, fluctuations in temperature and pH/CO_2, adaptations to acquire nutrients, higher competitive ability and grazing resistance due to morphological and metabolic versatility (Antunes et al. 2015; Burford et al. 2016; Jia et al. 2020; Vilar and Molica, 2020).

Additionally, some strains of *R. raciborskii* can produce toxic alkaloids with neurotoxic activity (saxitoxins; STXs) or cytotoxic activity (cylindrospermopsins; CYNs). Up to date, toxic South American strains produce STXs (Lagos et al. 1999; Molica et al. 2002; Piccini et al. 2013; Mesquita et al. 2019; Vilar and Molica, 2020) and despite some reports on the detection of CYN in environmental samples (Bittencourt-Oliveira et al. 2011; Lorenzi et al. 2018), no isolated strain has been characterized.

Saxitoxins are guanidinium-containing neurotoxic alkaloids that block ionic Na^+ and Ca^{2+}-channels, encompassing more than 50 analogues including non-sulphated (saxitoxin and neosaxitoxin), monosulphated (gonyautoxins), disulphated (C-toxins), and decarbamoyl variants and derivatives (Wiese et al., 2010). Moreover, these toxins distinguish from other cyanotoxins because the producing organisms are found in two life domains: Bacteria (Cyanobacteria) and Eukarya (marine dinoflagellates). In the later these toxins are referred to as paralytic shellfish toxins (Wang et al. 2016). Since STX analogues display different toxicities in animal cells depending on their functional groups, toxicity equivalent factors (TEFs) are addressed (Perez et al. 2011).

The biosynthesis of STXs depends on enzymes encoded in a gene cluster referred to as *sxt*, first described in *R. raciborskii* T3 (Kellmann et al. 2008), the same strain used in the present study. Saxitoxin biosynthesis depends on the expression of seven *sxt* genes, namely *sxtA*–*sxtD*, *sxtG*, *sxtS* and *sxtU*, which encode enzymes involved in the formation of the structural skeleton and other accessory *sxt* genes, encoding tailoring enzymes involved in chemical modifications, transport and regulation, and transposases (Jia et al. 2020).

STX-producing strains of *R. raciborskii* have demonstrated a variety of harmful effects on zooplankton organisms, especially on daphnids, such as decreased clearance rates (Panosso and Lürling 2010; Ferrão-Filho et al. 2017), mobility (Ferrão-Filho et al. 2010, 2014; Santos et al. 2020), growth, and reproduction (Soares et al. 2009; Costa et al. 2013).

The above-mentioned effects occur via direct contact through cells ingestion or handling impair due to trichome size mismatch. However, there is a lack of information on early adaptive responses in STX-producing cyanobacteria induced by indirect exposure, commonly associated to grazer's chemical signals, prior to damage caused by herbivory.

The evolution of inducible defenses appears to be favored over constitutive ones since grazing pressure by zooplankton varies both on temporal and spatial scales (Harke et al. 2017). In comparison with constitutive defenses, those induced by the presence or action of predators may be an effective way to minimize costs (Agrawal et al. 2001). If the presence of STXs can avoid predation, the optimal defense hypothesis (ODH) predicts that toxin cell content should increase in response to threat-cues in order to maintain population fitness (Selander et al. 2006). According to Pavia et al. (2002), ODH assumes that both chemical and morphological defenses are costly and therefore natural selection will act on those that optimize their benefit-cost ratio in terms of fitness.

The molecular basis for grazer induced defenses in phytoplankton is poorly understood. Therefore, monitoring the expression of target genes gives robustness to the observation of defensive phenotype, and also makes feasible to explore such responses as an adaptive trait. The conservation of *sxt* gene clusters in different cyanobacterial species indicates that STXs may play an adaptive function in producing cyanobacteria (Murray et al. 2010). To our knowledge, no study to date has investigated changes in the expression of genes related to STX synthesis upon cyanobacteria exposure to zooplankton or in the context of predator induced defenses. Thus, here we aimed to assess the effects of chemical cues produced by the neotropical daphnid *Daphnia gessneri* on morpho-physiological traits and *sxt* gene expression in the *Raphidiopsis raciborskii* strain T3. We tested the hypothesis that predator infochemicals promote a defense response (at molecular and physiological level) in the cyanobacterium but with an associated cost, as assumed by the optimal defense hypothesis (see McKey, 1974 and Van Donk et al. 1999).

Materials and Methods

Phytoplankton and zooplankton cultures

Raphidiopsis raciborskii T3 is a saxitoxin-producing strain isolated from the Billings reservoir (São Paulo, Brazil) (Lagos et al. 1999). Stock cultures were maintained in ASM-1 medium (Gorham et al. 1964) at 24±1°C, 50 µmol photons m^{-2} s^{-1} under a 12:12h light-

dark cycle. Under these conditions, T3 had an average trichome length of 74.91 ± 32.54 µm.

Zooplankton consisted of the neotropical species *Daphnia gessneri* isolated from Barra do Braúna Reservoir (Minas Gerais, Brazil) in 2018. Individuals were kept in RT medium (Tollrian 1993) enriched with commercial 0.1% (~2.25 mgC L^{-1}) humic extract (Microbe-lift® Amazon Black & Soft Water Conditioner, USA) at initial pH 7.6; 24±1 °C, 50 µmol photons m^{-2} s^{-1} and a 12/12h dark-light cycle. Animals were fed with a cell suspension of the green algae *Selenastrum capricornutum* at a final concentration of 500 µg C L^{-1} once every two days. Algae population was dominated by unicells (length-width mean dimensions 13.80±1.56 × 3.18±0.66 µm).

Experimental set-up

Zooplankton filtrate for kairomone obtention

Prior to experiments, *D. gessneri* clones were starved in fresh RT medium to empty their guts and to remove any superficial contamination. Cultures were established with adults at 60 ind L^{-1}, initially fed with a cell suspension of *S. capricornutum* at 500 µgC L^{-1} and incubated over 96h. Thereafter, animals were removed using a cup of plankton (60 µm mesh-size) and the zooplankton medium was sterilized by filtration through a 0.22-µm membrane. The obtained filtrate was used instead of water to prepare the ASM-1 culture medium, hereafter termed as infochemical-rich culture medium. As a control, *S. capricornutum* (500 µgC L^{-1}) was maintained in RT medium for 96h and a filtrate was obtained after filtration through a 0.22-µm membrane. The filtrate was used instead of water to prepare ASM-1.

Raphidiopsis raciborskii exposure to zooplankton conditioned medium

In this experiment zooplankton culture medium was used to evaluate whether *R. raciborskii* cells can detect and respond to predator infochemicals. An inoculum of *R. raciborskii* T3 with 20 mm^3 L^{-1} (~10^5 cells mL^{-1}) was prepared in 1-L Erlenmeyers filled with 600-mL of infochemical-rich and control culture medium (n = 3). The cultures were incubated over 6-days at 24±1 °C and 50 µmol photons m^{-2} s^{-1} under a 12/12h dark-light cycle. Samples were collected every two days to evaluate growth, morphological changes, photosynthetic activity, toxin content and expression of *sxt* genes.

Growth, morphology and photosynthetic measurements

Every two days, *R. raciborskii* culture samples were harvested and preserved with 1% acetic Lugol solution for trichome counting in a Fuchs-Rosenthal chamber under an optical microscope (Olympus BX51). Results are expressed in trichomes×mL^{-1}. Trichome density was converted to biovolume ($mm^3\ L^{-1}$) considering the mean trichome volume (μm^3) (Hillebrand et al. 1998; Sun and Liu 2003).

Growth rate was calculated during the exponential growth phase according to Fogg and Thake (1987) following the equation:

$$\mu = \frac{(lnN_2 - lnN_1)}{(t_2 - t_1)}$$

where N_2 = final biovolume; N_1 = initial biovolume; t_2 = final time; t_1 = initial time.

Additionally, trichome length and width (µm) at $T_{initial}$ and T_{final} in both conditions were measured to detect possible morphological variations induced by *Daphnia* conditioned medium. Samples were analyzed under an Olympus BX51 light microscope (250x and 400x magnification) coupled to an image-capture system (Canon t6i). Trichomes were measured using the Cell^B software package for image acquisition. Combining trichome length and mean cell number per trichome, we have estimated cell density (cell×mL^{-1}).

Fresh culture samples were obtained every two days to evaluate photosynthetic performance using a PHYTO-PAM fluorometer (Heinz Walz GmbH, Germany) equipped with the PHYTO-EDF detection unit for measuring cyanobacterial fluorescence. Saturation pulses (36 µmol photons m^{-2} s^{-1}) were applied and fluorescence data were converted to chlorophyll-a concentration (µg×L^{-1}) and photosynthetic yield (relative F_v'/F_m'). Light curves were performed with dark acclimated culture samples (F_0) with 10 s intervals between pulses. Light intensity varied from 16 to 764 µmol photons m^{-2} s^{-1}. From these curves, the values of maximum electron transport rate (ETR_{max}), light harvesting efficiency (α) and light saturation parameter (Ik) were obtained as a function of irradiance. Additionally, the maximum effective quantum efficiency of PSII was calculated as $\phi_m = [(F_m - F)/F_m]$, where F is the fluorescence of the dark-adapted sample and F_m is the maximum dark-adapted fluorescence.

Saxitoxins analyses and toxicity equivalency calculation

On days 0, 2, 4 and 6, aliquots of 50 mL were taken from *R. raciborskii* cultures and cells were harvested by centrifugation (7200 g, 10 min, 4 °C). The supernantant was filtered to measure the toxins in the dissolved fraction while the pellet was used to measure the intracellular toxin content. Samples were frozen at -20 °C, freeze-dried and the dry cellular biomass was combined with cells in the filters to extract saxitoxins. Toxin was extracted adding a 0.5 M acetic acid solution to the cells in 3 cycles of 1h each. At each cycle, the cell extract was centrifuged as previously mentioned, and the supernatants were sequentially combined. The volume was reduced to 1/5 through evaporation in a thermostatic bath (~80 °C) with compressed-air injection. For dissolved toxins analysis, after complete freeze-dry, samples were resuspended in a 0.5 M acetic acid solution. Both dissolved and cellular fractions were filtered in 0.22 µm syringe-filter prior to STXs analysis.

The analysis was performed on a Shimadzu Class VP liquid chromatography system with a fluorescence detector (RF-10A XL). A reversed phase C18 column (Lichrospher®; 150 mm x 4.6 mm; 5 µm - Merck) and a 20 µl loop injector was used. Chromatographic analyzes were performed according to the post-derivatization Oshima (1995) method using a mobile phase 2 mM sodium 1-heptanesulfonate in 30 mM ammonium phosphate and 5% acetonitrile at a flow rate of 0.8 mL min^{-1}. STXs were detected at 330 nm excitation wavelength and 400 nm emission wavelength.

For quantification of toxins, standard solutions of saxitoxin (STX) and neosaxitoxin (neoSTX) from the National Research Council (NRC) - Institute of Marine Biosciences (Canada) were used. Total saxitoxins content was expressed as toxin quota per cell ($fg \times cell^{-1}$) and per biovolume ($\mu g/mm^3$).

Total relative toxicity (saxitoxin equivalents, STX_{eq}) was expressed as cellular toxicity ($fg_{STXeq} \times cell^{-1}$) according to the toxicity equivalency factor (TEF) described for each saxitoxin analog (in this study, neoSTX and STX) in FAO/WHO (2016) and using the following equation:

$$STXeq = \sum_{i=1}^{n} (C_i x TEF_i)$$

in which C_i is the concentration of the individual toxin analog and its assigned TEF_i.

Application of first-order rate kinetics to assess total saxitoxin production

The specific growth rate (μ_g) and the specific total saxitoxin production rate (μ_{stx}) were calculated during the exponential growth phase according to simple first-order rate kinetics using either biovolume concentration (mm^3/L) and volumetric total intracellular saxitoxin data ($\mu g \times L^{-1}$), respectively (both specific rates are reported in units per d^{-1}).

The ratio between μ_{stx} and μ_g was calculated to assess different patterns of toxin production coupled to the growth cycle and consequent changes in the toxin biovolume (or cell) quota (Q_{tox}) as described in Orr et al. (2018), such that:

Equation (i) describes the condition where the intracellular μ_{stx} between t_0 and t_n is slower than μ_g, resulting in a lower Q_{tox}.

(i) $$0.5 < \frac{\mu_{stx}}{\mu_g} < 1$$

Equation (ii) describes the condition where the intracellular μ_{stx} between t_0 and t_n is a function of μ_g, resulting in a constant Q_{tox} (1:1 growth-toxin relationship).

(ii) $$\frac{\mu_{stx}}{\mu_g} = 1$$

Equation (ii) describes the condition where the intracellular μ_{stx} between t_0 and t_n is higher than μ_g, resulting in an increased Q_{tox}.

(iii) $$\frac{\mu_{stx}}{\mu_g} > 1$$

Expression of genes involved in STX biosynthesis

The level of transcripts of of *sxtI* and *sxtU* genes was measured to estimate the synthesis of enzymes involved in STX synthesis. The gene *sxtU* encodes a dehydrogenase which reduces the terminal aldehyde group of the saxitoxin (STX) precursor (Mihali et al. 2009). The gene *sxtI* encodes a carbamoyltrasferase which catalyses a carbamoyl transfer from carbamoylphosphate onto the free hydroxyl at C-13, forming STX. The reference gene used to calculate the relative expression of the target genes was *rpo*C1 (encodes the gamma subunit of the RNA polymerase) (Willis et al. 2019).

On days 0, 2, 4 and 6, 50 mL samples were taken from *R. raciborskii* cultures and cells were harvested by centrifugation (1500 g, 15 min, 4°C). TRIzol® (300 µL) was added to the pellets and the suspensions were immediately stored at -80°C. RNA

extraction was performed using the Direct-zol™ RNA Miniprep Plus (Zymo Research®) extraction kit according to the instructions provided by the manufacturer (including a DNAse digestion step). RNA samples were suspended in RNAse-free water and the nucleic acid quality and concentration were determined using the RNA HS Assay kit (Life Technologies) in a Qubit fluorometer (Thermo Fisher Scientific). To rule out DNA contamination, a PCR was carried out using 16S rDNA primers and the purified RNA samples as template. Synthesis of cDNA synthesis was performed with the GoScript Reverse Transcriptase enzyme (Promega) according to the manufacturer instructions. The cDNA products were quantified in Nanodrop, diluted 1:10 and tested for amplification with specific primers for *rpo*C1 (Willis et al., 2019).

The obtained cDNA was used in qPCR to quantify the relative abundance of *sxt*U and *sxt*I transcripts. For each sample, 3uL of a 1:10 cDNA dilution were mixed with 7.5 µL Sybr Green PCR MasterMix (Applied Biosystems), 0.6 µL of a 20 µM solution of each primer and nuclease free water to a final volume of 15 µL. Reactions were performed with the following primers: *sxt*U forward [5-ACTCCCAGAACATTCACATCG-3] – *sxt*U reverse [5-GGAATTGGTGTGTTTGGTGC-3] (Martinez De La Escaleira et al. 2014); *sxt*I forward [5-TGCAGTGGGAGCAGCTTTAG-3] – *sxt*I reverse [5-GATCGCCTGCTGTTGAAGTG-3] (Vico et al. 2016) and *rpo*C1 forward [5-GACATGGTTTTGGGAGCCTA-3] – *rpo*C1 reverse [5-CGTTATCCGGTTGTCCTGTT-3] (Willis et al. 2019). For each sample triplicate reactions were run on a QuantStudio 3 Real-Time PCR system (Thermo Fisher Scientific), with a pre incubation at 95 °C for 10 min and 40-cycles of amplification at 95 °C for 15 s; 60 °C for 1 min. A melting curve was obtained incubating at 95 °C for 15 s, 60 °C for 1 min and 95 °C for 15 s. Negative controls for each primer set were included in which nuclease free water was instead of cDNA template. The amplification efficiency of each primer set was calculated from standard curves using serial dilutions of cDNA, according to the equation: E = 10 (-1/slope) **(Suppl. Table 4 and Fig. 1)**. Transcript levels of the target *sxt* genes were normalized to the reference *rpo*C1 gene and the relative change in transcript levels was calculated using the ΔΔCT method of relative quantification (Livak and Schmittgen, 2001).

Statistical analysis

All data were checked for normality and homoscedasticity of variances. Specific growth rate data and ratio between specific toxin production and growth rate were analyzed by Student's t-test to verify any significant effect of zooplankton cues. Variations in growth, saxitoxins cell quota, cellular relative toxicity, total saxitoxin pool size and *sxt* genes expression over incubation time were verified using repeated measure two-way ANOVA with a post hoc Bonferroni's test. Morphological traits variation was analyzed using one-way ANOVA with a post hoc Dunnet's test using the morphological traits at the initial time as a control condition. The ΔΔCT data referent to gene expression was log transformed and two-way RM ANOVA used to compare the infochemical-rich samples with the control samples on each day. All analyses and graphs were performed using GraphPad Prism 6.0 software.

Results

Growth, morphology and photosynthetic response

Raphidiopsis raciborskii T3 displayed a significant decrease in biovolume (Two-way RM ANOVA, $p<0.001$) and in specific growth rate when exposed to *Daphnia* conditioned medium ($\mu_{control}$ = 0.47 ± 0.15 day^{-1}; $\mu_{kairomones}$ = 0.24 ± 0.04 day^{-1}; Student's T-test, $p<0.05$) as compared to control **(Fig.1A; Suppl. 1)**. No differences were observed for chlorophyll-a concentrations **(Fig. 1B)**.

Additionally, growth in the presence of predator infochemicals did not affect photosynthesis as estimated by the light harvesting efficiency (α), relative PSII quantum yield (*Fv*'/*Fm*'), light saturation parameter (Ik) and maximum electron transport rate (ETR_{max}) **(Table 1)**. However, a significant increase in the photosynthetic parameters ETR_{max} (two-way RM-ANOVA, $p<0.05$) and Ik (two-way RM-ANOVA, $p < 0.001$) was observed over time **(Table 1)**.

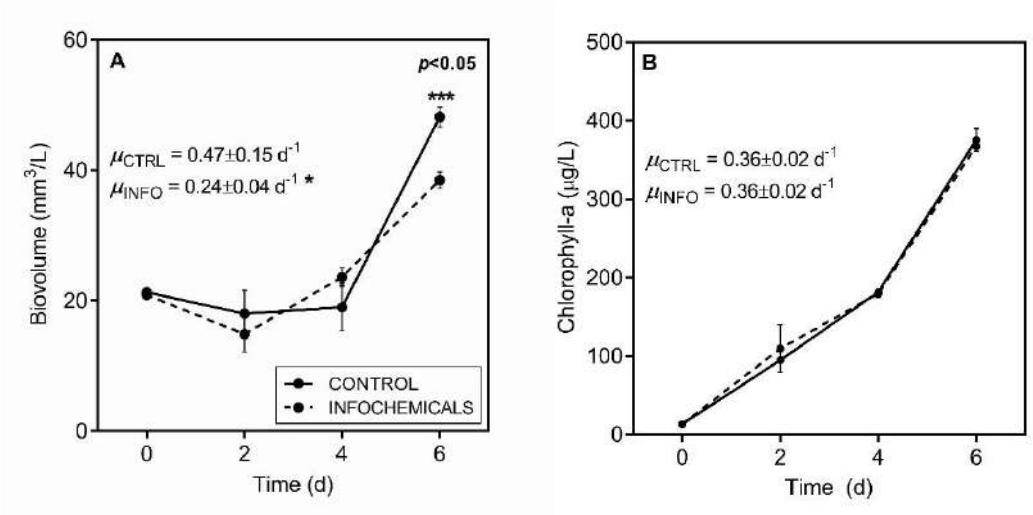


Figure 1. Growth curves and growth rates estimated by **A)** biovolume and **B)** chlorophyll-a concentration for *Raphidiopsis raciborskii* T3 exposed to *Daphnia gessneri* infochemical (dashed line) and in the control condition (solid line). Significant differences (*) = $p<0.05$; (***) = $p<0.001$, Bonferroni's Test.

Irradiation curves of *R. raciborskii* T3 (maximum PSII quantum yield) showed no effect of predator infochemicals **(Fig. 2)**. In both control and treatment conditions the cells were sensitive to light intensities above 64 µmol photons s^{-1} and upon increased light pulses *R. raciborskii* T3 displayed a significant decrease in photosynthetic efficiency (Two-way RM ANOVA, $p<0.0001$) **(Fig. 2)**.

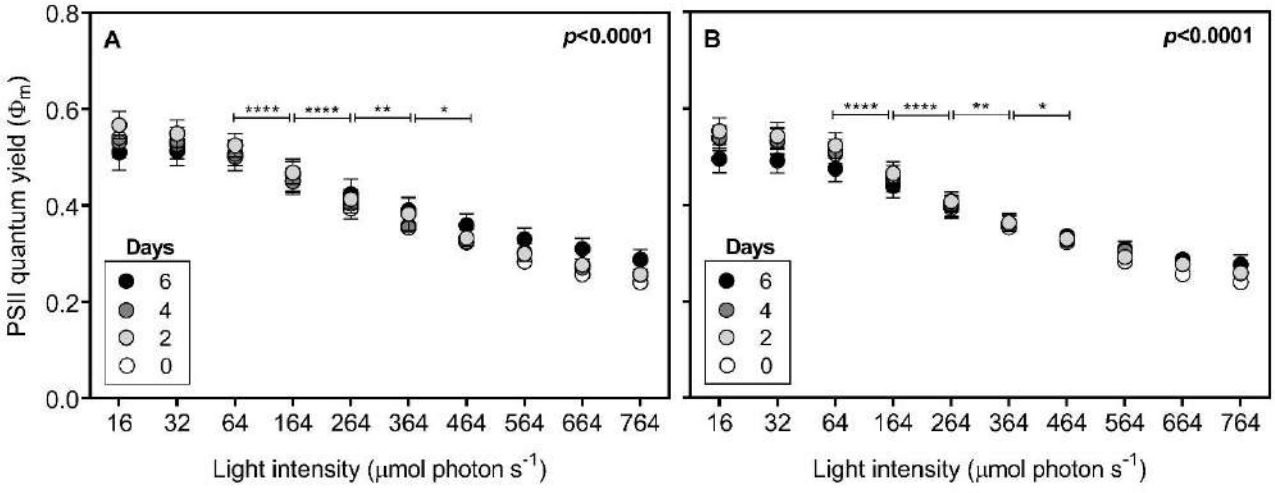


Figure 2. Maximum PSII quantum yield derived from photosynthesis irradiation curve of *Raphidiopsis raciborskii* T3 control (A) and exposed (B) to *Daphnia gessneri* infochemicals for 6 days. Significant differences **(*; **; ****)** = Bonferroni's test, $p<0.05$; $p<0.01$; $p<0.0001$, respectively.

Table 1. Photosynthetic parameters of *Raphidiopsis raciborskii* T3 exposed to *Daphnia gessneri* infochemicals **(INFO)** and in the control condition **(CTRL)**. Yield – Relative PSII quantum yield at a saturation pulse of 36 PAR (Photosynthetically active radiation), ETR_{max} – maximum electron transport rate, Ik **–** light saturation parameter and Alpha (α) **–** light harvesting efficiency. SD = standard deviation. Same letter indicates no statistical difference between experimental conditions (Bonferroni's test, $p<0.05$).

Photosynthetic Parameter Time (d)	**Yield** (Relative *Fv*'/*Fm*')		**ETR_{max}** (µmol e m^{-2} s^{-1})		**Ik** (µmol photon m^{-2} s^{-1})		**Alpha** (µmol photon m^{-2} s^{-1})	
	CTRL (Mean±SD)	**INFO** (Mean±SD)	**CTRL** (Mean±SD)	**INFO** (Mean±SD)	**CTRL** (Mean±SD)	**INFO** (Mean±SD)	**CTRL** (Mean±SD)	**INFO** (Mean±SD)
0	0.48±0.00[a]	0.48±0.00[a]	81.80±0.00[a]	81.80±0.00[a]	353.10±0.00[a]	353.10±0.00[a]	0.23±0.00[a]	0.22±0.00[a]
2	0.59±0.03[a]	0.48±0.13[a]	104.07±16.50[a]	77.70±31.40[a]	429.23±66.71[a]	359.10±69.31[a]	0.23±0.00[a]	0.23±0.00[a]
4	0.55±0.02[a]	0.53±0.01[a]	103.27±15.30[a]	102.47±8.08[a]	448.03±56.92[a]	440.23±36.25[a]	0.24±0.00[a]	0.23±0.00[a]
6	0.52±0.03[a]	0.51±0.01[a]	118.27±5.70[a]	109.77±6.73[a]	531.03±41.86[a]	514.60±41.86[a]	0.22±0.01[a]	0.23±0.01[a]

The morphology of *R. raciborskii* T3 did not change (Dunnet's test, $p > 0.05$) when exposed to *D. gessneri* infochemicals. Over 6 days in the presence of the conditioned medium trichomes ranged from an initial length of 74.91 ± 32.54 µm to 75.05 ± 40.44 µm. In the control condition trichome length ranged from 74.91 ± 32.54 µm to 79.69 ± 42.79 µm. Similarly, no changes were detected in trichome thickness, with a mean width of 2.63 ± 0.52 µm and 2.53 ± 0.43 µm in control and treatment conditions.

Saxitoxins production

D. gessneri infochemicals significantly enhanced saxitoxins quota in *R. raciborskii* T3 cultures. On the 6th day, in the treatment condition concentrations were 12.23±2.34 fg cell^{-1} and 0.63 ± 0.12 µg mm^{-3}, twice those measured in the control, 6.32±2.04 fg cell^{-1} and 0.31±0.10 µg mm^{-3} (Bonferroni's test, $p<0.001$), evidencing a chemically induced defense response by the cyanobacterium **(Fig. 3; Suppl. 2 and 3)**. In addition, neo-saxitoxin and saxitoxin contents increased significantly in response to the predator cues, resulting in a higher cellular relative toxicity (treatment = 22.24±4.02 fg_{STXeq} cell^{-1}; control = 11.41±3.89 fg_{STXeq} cell^{-1}; Bonferroni's test, $p<0.001$) **(Fig. 4)**.

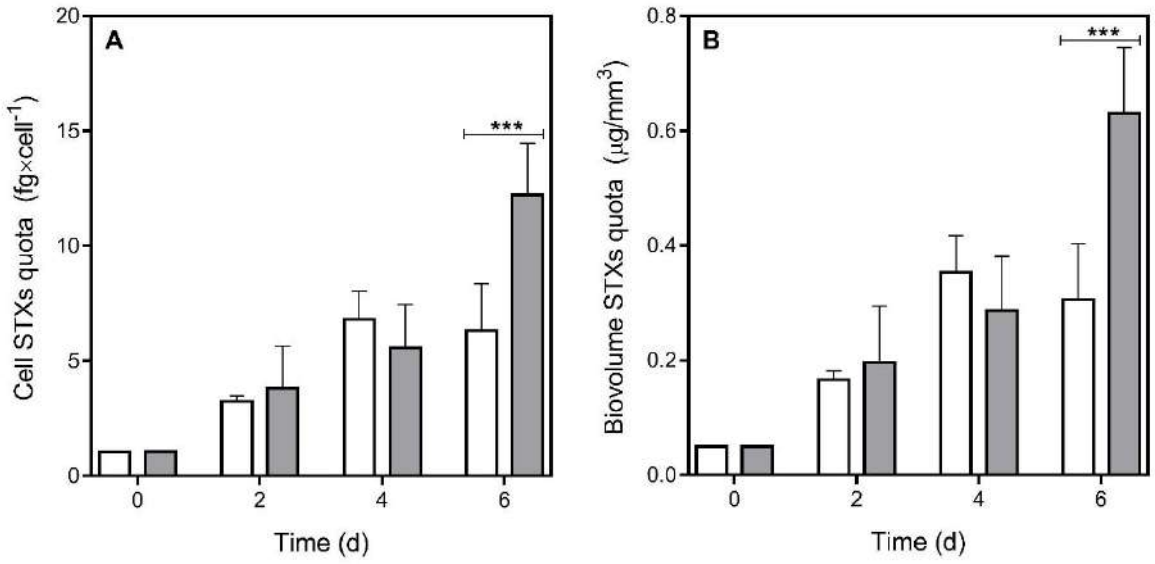


Figure 3. Saxitoxin quota by **A)** cells and **B)** biovolume of *Raphidiopsis raciborskii* T3 exposed to *Daphnia gessneri* infochemicals **(grey bars)** and in the control condition **(white bars)**. Significant differences (**; ***) = Bonferroni test, $p<0.01$; $p<0.001$.

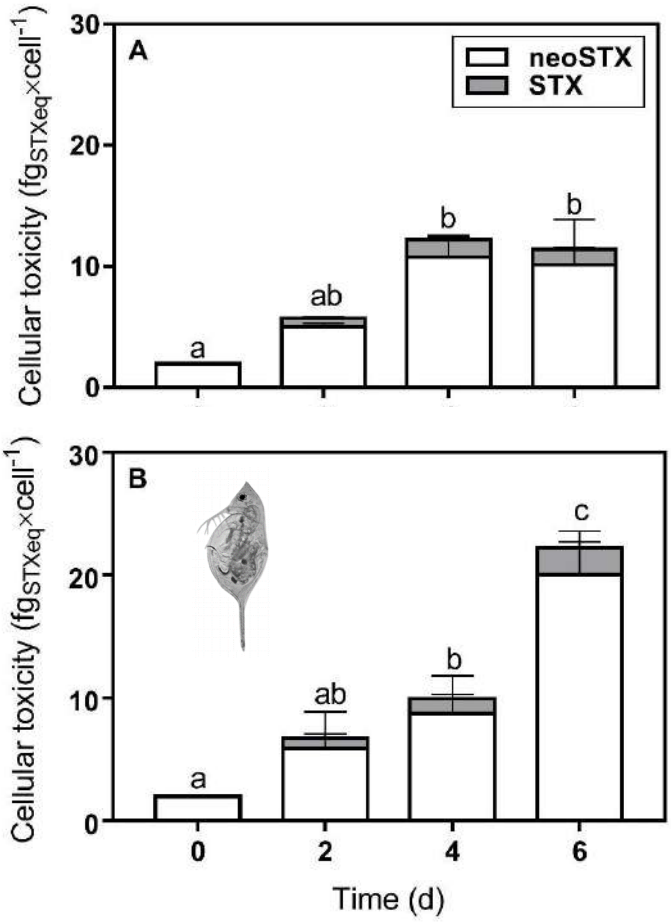


Figure 4. Cellular toxicity based on neo-saxitoxin **(white bars)** and saxitoxin **(grey bars)** contents of *Raphidiopsis raciborskii* T3 **(A)** in the control condition and **(B)** exposed to *Daphnia gessneri* infochemicals. Different letters mean significant differences (Bonferroni's test, $p<0.05$).

Regarding the extracellular amount of toxin, there was no significant effect of kairomones on the total pool of dissolved saxitoxins **(Figure 5)**. Moreover, predator cues resulted in a STXs release rate of 0.20 ± 0.04 µg L^{-1} d^{-1}, while in the control condition the release rate was 0.16 ± 0.03 µg L^{-1} d^{-1} (data not shown).

Overall, saxitoxin quota, cellular toxicity and volumetric STXs concentration increased over incubation time (two-way RM-ANOVA, $p<0.01$) **(Figs. 3, 4 and 5)**. The latter also represents the increase in cell concentration in both experimental conditions.

First-order rate kinetics showed different patterns in toxin production/growth in *R. raciborskii* T3. In the control condition saxitoxin production was coupled to growth **(**1:1 μ_{stx}/μ_g ratio) (**Fig. 6)**. In contrast, predator cues changed this ratio toward a μ_{stx}/μ_g mean value significantly higher than 1 (Student's T-test; T = 4.055, $p < 0.05$), indicating that

the specific rate of STXs production was faster than the specific growth rate, resulting in an increased cell toxin accumulation, as shown in **Figure 3**.

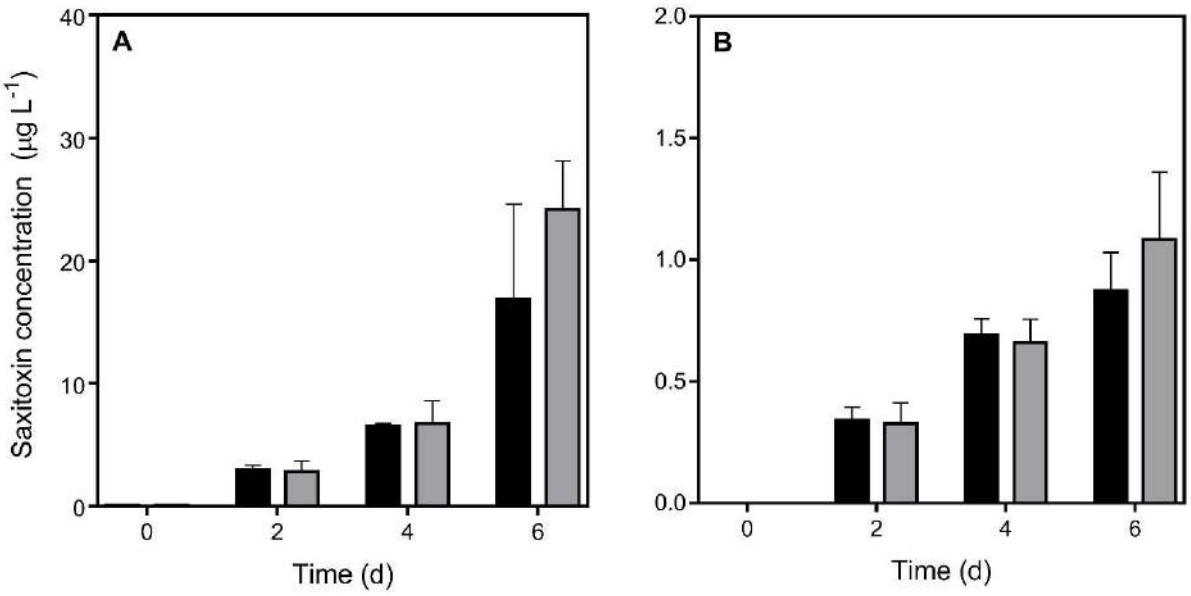


Figure 5. Concentrations of **(A)** intracellular and **(B)** extracellular saxitoxin in *Raphidiopsis raciborskii* T3 in the control condition **(black bars)** and exposed to *Daphnia gessneri* infochemicals **(grey bars)**. No significant differences were detected comparing the two conditions.

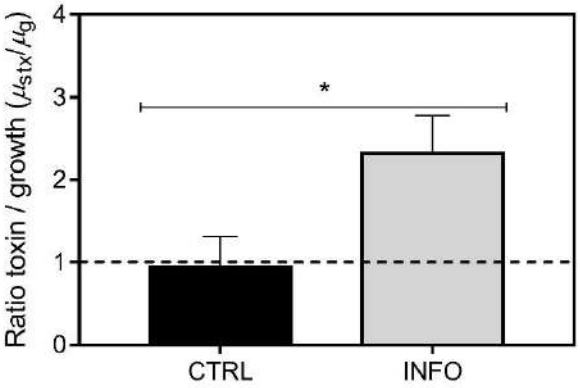


Figure 6. Ratio between specific total saxitoxin production rate (μ_{stx}) and specific growth rate (μ_g) obtained from first order rate kinetic (log phase) for *Raphidiopsis raciborskii* T3 grown in the control condition (CTRL) and with *Daphnia gessneri* infochemicals (INFO). Dashed line means 1:1 relationship between μ_{stx} and μ_g as described in Orr et al. (2018). (*) = *T*-test, $p<0.05$

Saxitoxin gene expression

A significant upregulation of the expression two saxitoxin related genes, *sxt*I (Two-way RM ANOVA, $F_{(1,4)} = 8.588$; $p < 0.05$) and *sxt*U (Two-way RM ANOVA, $F_{(1,4)} =$

153.2; $p < 0.001$) was detected in *R. raciborskii* T3 grown in the presence of zooplankton conditioned medium compared to control **(Fig. 7; Suppl. 4)**. In the presence of the predator infochemicals *sxt*I gene relative expression was significantly higher than the control at the sixth day of incubation (Bonferroni's test, $p < 0.0001$) (**Fig. 7A)**, concomitant to the significant increase in saxitoxin production **(see Fig. 3)**. Transcript levels increased over time ($F_{(3,12)} = 8.172$; $p < 0.01$) and also a significant interaction between both factors (*time* × *treatment*, $F_{(3,12)} = 10.13$; $p < 0.01$) occurred. For s*xt*U, transcript abundances were higher in the treatment as compared to control on the 2th and 4th days ($F_{(1,4)} = 153.2$; $p < 0.001$) (Bonferroni's test, $p < 0.0001$) **(Fig. 7B)**, four days before the detection of an increase in saxitoxin content. The transcript levels of this gene also displayed a significant interaction between both factors (*time* × *treatment*, $F_{(3,12)} = 85.65$; $p < 0.0001$). The abundance of *sxt*U transcripts decreased gradually over time and on the 6th day was similar in both conditions ($F_{(3,12)} = 137.7$; $p < 0.0001$).

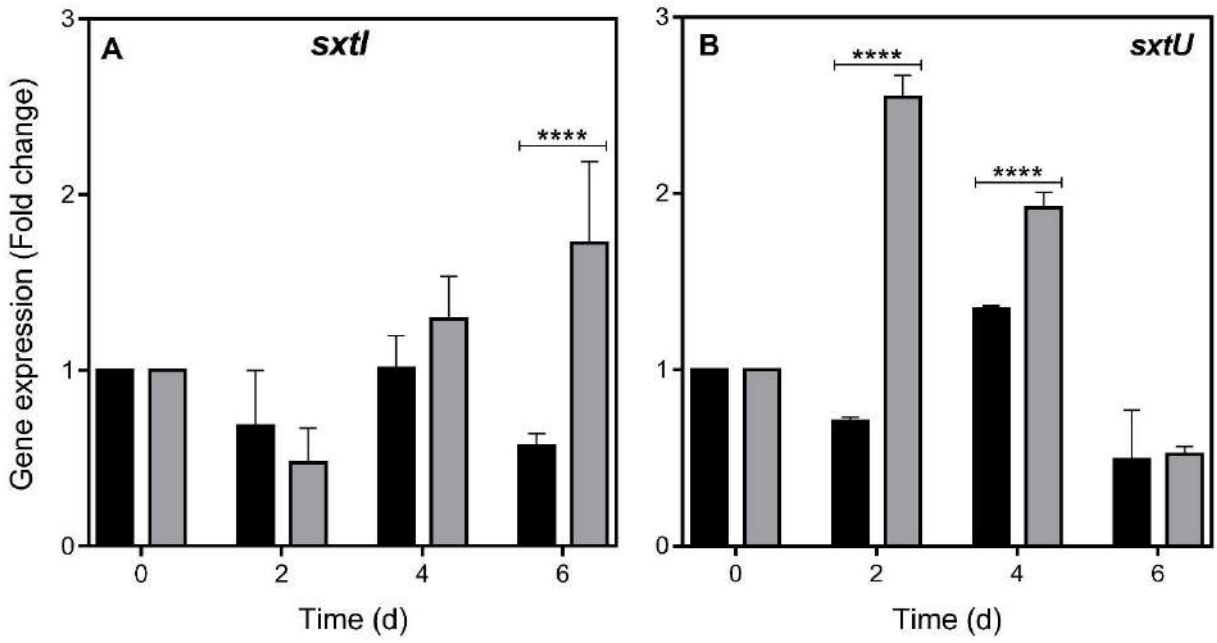


Figure 7. Relative expression of *sxt*I (A) and *sxt*U (B) genes in *Raphidiopsis raciboskii* T3 in the control condition (black column) and exposed to *Daphnia gessneri* infochemicals (grey columns), fold change calculated by the 2-ΔΔCT method. Significant differences comparing the two conditions are indicated by asterisks (Bonferroni's test, $p < 0.0001$).

Discussion

Infochemicals produced by *Daphnia gessneri* significantly affected growth, *sxt* gene expression and toxin content of *R. raciborskii* T3 during 6 days of incubation as we hypothesized. We have choosen to test the effect of infochemicals from a density of cladocerans compatible with those naturally occurring and we have observed a two-fold increase in cellular saxitoxin quotas as well an increase in *sxt* gene expression, evidencing an induced chemical defense response.

Many studies have characterized physiological responses underlying predator-induced chemical defenses in microcystin-producing cyanobacteria (Jang et al. 2007; Van Gremberghe et al., 2009, Akbar et al., 2017, Princiotta et al., 2019), including gene expression (Pineda-Mendoza et al. 2014; Harke et al. 2017). In contrast, an increase in saxitoxins production mediated by predator cues was only reported in marine dinoflagellates. For example, Selander et al. (2006) showed a more than 2-fold increase in saxitoxin production by *Alexandrium minutum* when exposed to kairomones of the copepod *Acartia tonsa*. Similar findings were evidenced for STX-producing *A. tamarense* and *A. fundyense* exposed to several predators (Selander et al. 2012; Senft-Batoh et al. 2015a; 2015b). Furthermore, despite the impact of C:N:P ratio on saxitoxins production, as suggested by the stoichiometric hypothesis (Van de Waal et al. 2014), it has been also shown that saxitoxin cellular content can increase in response to predator cues, regardless of nutrient condition (Griffin et al. 2019).

In our study, saxitoxin secretion was also assessed. *R. raciborskii* T3 has *sxtM*/*sxtF* genes which encode multi-drug and toxic compound extrusion (MATE) transport-proteins (Mihali et al. 2009). Yet, despite the increased STXs cellular quotas measured in *R. raciborskii* T3 after exposure to *D. gessneri* infochemicals, no significant effect on toxin secretion was evidenced. It is supposed that feeding is the most important route of exposure to toxins in aquatic systems (Ibelings and Havens 2008). Therefore, increasing toxin cellular amounts can be an effective defense against grazing pressure by zooplankton, instead of releasing these metabolites into the water. This assumption is supported by the observation of harmful acute effects and life history impairments on zooplankton when actively feeding on STXs-producing cyanobacteria (Freitas et al. 2014; Ferrão-Filho et al. 2014; 2019; Ferrão-Filho and Silva, 2020; Santos et al. 2020).

According to Yang et al. (2011), kairomone induced increase of STXs production is mediated by chemoreception without the involvement of mechanical damage. This

characterizes a specific defensive response that does not depend on active grazing. Furthermore, assessing inducible chemical defenses by exposing harmful algae and cyanobacteria to predator kairomones provide a more reliable response once direct exposure enables the occurrence of selective grazing on less toxic cells what might favor cells with a higher mean toxin content, and therefore may be indistinguishable from those whose induced toxin production have occurred (Selander et al. 2006).

In our study, two representative genes of the *sxt* cluster, *sxtI* and *sxtU,* showed increased transcript levels in *R. raciborskii* cells exposed to *D. gessneri* infochemicals. This response preceded the observed increase in saxitoxin cellular quotas. *sxtU* encodes a dehydrogenase which reduces the terminal aldehyde group of the saxitoxin precursor, whereas *sxtI* encodes a carbamoyl transferase which catalyses a carbamoyl transfer from carbamoylphosphate onto the free hydroxyl at C-13, forming STX (Mihali et al. 2009). These genes are reliable markers for assessing STX synthesis since they encode enzymes which participate in key steps of STX formation. Other studies have successfully used these *sxt* genes as reporters to assess saxitoxin biosynthesis in *R. raciborskii* (Beamud et al. 2016; Vico et al. 2016).

Yang et al. (2011) were the first to demonstrate a gene expression response of a STX-producing dinoflagellate to zooplankton cues, but no genes specific of the *sxt* cluster were identified as marker of toxin cell increase. Later, Wohlrab et al. (2017) assessed *sxtA* gene expression coupled to saxitoxin production in *A. fundyense* exposed to predator. However, despite a two-fold increase in STX cell quota, no significant changes were observed in gene transcription. Authors have suggested that *sxtA* might have been overexpressed early, at the onset of the dinoflagellate defense response, prior to the observed physiological changes. Here, we were able to evidence arise in *sxt* trancripts preceding the increase in saxitoxin cellular quota as part of the cyanobacterial response to predator cues.

Molecular events involved in induced chemical defenses in cyanobacteria are still poorly explored, especially regarding those saxitoxin-producing species. Although the presence of genes related to toxin synthesis precedes the existence of metazoans (Rantala et al., 2004), these toxins might have evolved to diversify their functions over time, acting also as an adaptive response against predators (Kaplan et al., 2012). Evidence of the adaptive potential of induced defense in STX-producing phytoplankton has been already demonstrated by Selander et al. (2006) which firstly reported a predator-mediated

induction of toxin production coupled to an increased resistance to grazers in *A. minutum*. Thus, it is likely that the potential adverse effects of STXs on zooplankton have undergone positive selection more recently, as suggested for microcystins by Rzymski et al. (2020).

We have also examined trichome length and thickness as proxy for induced morphological defense, but no significant changes were found. In freshwater, filamentous algae and cyanobacteria are expected to be more resistant to grazing and have life-history implications on herbivorous zooplankton. Cladocerans are key grazers which are forced to frequently clean their filtering apparatus from filaments by post-abdominal rejection movements, thereby also losing most edible food particles accumulated (Gliwicz and Siedlar 1980). On the other hand, several studies have shown the effects of predator kairomones on filamentous cyanobacterial morphology and most of them report increase in width (Cerbin et al. 2013; Wejnerowski et al. 2018) and subsequent impact on zooplankton life history (Wejnerowski et al. 2015). However, little is known about the impact of that interspecific interaction on cyanobacterial trichome length (Wejnerowski et al. 2018). Studies with other phytoplankton groups suggest that the filamentous morphology acts as a functional trait avoiding grazing by clogging the predator filtration apparatus, as seen in marine microalgae that can adjust chain length in response to predator cues. This is a known mechanism to minimize losses due to grazing (Selander et al. 2011; Bergkvist et al. 2012; Bjærke et al. 2015; Selander et al. 2019).

In parallel to the increased toxin production induced in response to predator, growth of *R. raciborskii* T3 decreased reaching low biovolume concentrations in this condition. Regarding toxic dinoflagellate species, Blossom et al. (2019), when investigating the costs of toxicity in saxitoxin-producing *Alexandrium* strains, did not find any growth reduction related to toxin production, but with lytic activity which is associated to other metabolites poorly known. Generally, when cellular growth is reduced in phytoplankton, a previous impact on photosynthesis is expected, since it consists of the energy-production machinery for biomass acquisition. Here, despite the negative effects on *R. raciborskii* growth, *D. gessneri* infochemicals did not affect photosynthetic parameters. Yet, a significant increase in the ETR_{max} and Ik parameters over incubation time has occurred, indicating enhanced light acquisition by *R. raciborskii*, consistent to self-shading caused by cell accumulation.

We examined photosynthetic parameters as traits to assess early changes in cyanobacterial physiology, considering that little is known about how predator-induced defenses in phytoplankton might affect photosynthesis. Savic et al. (2019) were the first to evaluate the photosynthetic response of toxic and nontoxic *Microcystis* strains exposed to *D. magna* kairomones. A decrease in photosynthetic activity and in chlorophyll-a content in the toxic strain were observed as a response to *Daphnia*. Later, the same group (Savic et al. 2020) reported that zooplankton cues did not affect *M. aeruginosa* photosynthetic activity under a similar experimental set, which probably occurred because only ETR_{max} was examined. In our study, the finding that zooplankton infochemicals had no significant effects on photosynthesis was based on a more detailed set of photosynthetic parameters (i.e., Ik, ETR_{max}, α and ϕ_m), besides the commonly used chlorophyll-a concentration and relative ETR to evaluate the physiological state of the cells. The photosynthetic response of *Microcystis* during exposure to zooplankton has also been assessed with a different approach through transcriptomic analysis (Harke et al., 2017). The authors identified a strong and significant differential expression of 20 genes associated with photosynthesis and gas vesicle production.

Overall, the imbalance between growth and saxitoxin production observed after exposure to zooplankton infochemicals indicate a trade-off. According to photosynthetic parameters, *R. raciborskii* cells were in a normal physiological state but still growth was reduced. Possibly, the threat represented by the infochemicals caused the cells to allocate energy toward increased toxin production instead cell division. In fact, studies addressing the effects of environmental stressors on *R. raciborskii* physiology suggest a trade-off between growth and toxin production (Vilar and Molica, 2020). Although different stress conditions can activate diverse metabolic pathways, they can share certain aspects on effects in cell physiology. Investigations with both saxitoxin and cylindrospermopsin producing *R. raciborskii* strains have evidenced this imbalance in response to abiotic stress (e.g. nutrient limitation, light and temperature stress) (Kenesi et al. 2009; Mesquita et al. 2019; Nor et al. 2019). However, our study is the first to report the response of a toxic *R. raciborskii* strain to a biotic stress. A trade-off response has been indicated by the first order kinetics rate calculated between saxitoxin production rate *versus* growth rate in the case of *R. raciborskii* exposed to zooplankton infochemicals, which resulted in a higher ratio (~2:1) as compared to control. The production of other cyanotoxins, such as microcystins, has been considered as a constitutive process directly coupled to cell

division (Orr and Jones, 1998, Orr et al. 2018). However, the total toxin pool size may not always be in a 1:1 proportion to cell concentration, since cells may achieve up to 3-fold variation in the toxin quota depending on their growth stage (Jähnichen et al., 2008). In addition, saxitoxin content depends not only on cell division rate, but can be also affected by environmental factors (Vilar and Molica, 2020).

Regarding energy requirements, defenses that are induced only under variable grazing threat/attack are considered more efficient than constitutive ones, since they enable organisms to exploit benefits reducing costs associated with the investment on constant defensive strategies (Agrawal, 1998; 1999). Numerous studies attempted to estimate the costs and benefits of defense mechanisms in phytoplankton, as reviewed by Pancic and Kiorboe (2018) which argue that the costs associated with induced defenses have largely been undetermined. These authors also assume that if there was no cost to predator-resistance traits expression, all species would evolve towards a state of equal defense, and the community would not be structured by predation. This claim agrees with the optimal defense hypothesis (ODH) which is based on the fundamental assumption that the benefits of defense outweigh the costs, especially at high predation pressure (McKey, 1974). Although ODH had been primarily proposed for plants defenses, this hypothesis also predicts defensive responses in single-celled organisms. This can occur in the phytoplankton, where phenotypically plastic organisms can express defensive traits whenever threatened. However, not all individuals can express defenses, since these responses might differ intraspecifically, what suggest that the associated costs are genotype-specific (Van Gremberghe et al. 2009). Therefore, regarding genotype-specific responses as well as the ability of a genotype express different phenotypes it is suggested that predator-prey interaction is submitted a selection pressure governed by both mendelian and non-mendelian evolving mechanisms.

In the present study *D. gessneri* infochemicals induced an upregulation of *sxt* genes and a subsequent increase in toxin content but no morphological changes in *R. raciborskii* T3. This suggest an induced chemical defense in response to the grazer. *R. raciborskii* T3 cells remained physiologically healthy but reduced growth. These results support the hypothesis that the defense of *R. raciborskii* to the presence of the predator may be costly. In general, *R. raciborskii* predominance in water bodies has been attributed to its phenotypic plasticity supporting several environmental filters as well as its dispersion capacity (Antunes et al. 2015; Burford et al. 2016). Assessing the potential of this species

to thrive also interaction filters (e.g. competitive strategies, predation and parasitism resistance) (Cadote and Tucker, 2017) can shed more light in the complex factors contributing to its adaptive success considering the plankton interaction network.

Acknowledgments

This work was supported by the National Council for Scientific and Technological Development – CNPq and by the Carlos Chagas Filho Foundation for the Support of Science in Rio de Janeiro Development – FAPERJ. M.C.P.V., T.F.C.P.R. and L.O.S. were supported by CNPq scholarships.

Author contributions

M.C.P.V. and S.M.F.O.A. designed the research; M.C.P.V., T.F.C.P.R., L.O.S. and A.B.F.P. performed research; M.C.P.V., A.B.F.P. and S.M.F.O.A. analyzed and reviewed data; and M.C.P.V. wrote the paper.

Declaration of interest

The authors declare no conflict of interest.

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Supplementary data

Table 1 – Results of the Two-way ANOVA for diferences in growth curves of *Raphidiopsis raciboskii* T3 in biovolume and chlorophyll-a (infochemicals) exposed and (control) non-exposed to *Daphnia gessneri* along 6-days incubation. **ns=** no significant diferences.

Control	**SS**	**Df**	**MS**	***F***	***P***

Time	0.2598	3	0.08661	$F_{(3,60)}$= 165.9	<0.0001
Infochemicals	0.5474	9	0.06082	$F_{(9,20)}$= 107.5	<0.0001
Interaction	0.05403	27	0.00200	$F_{(27,60)}$= 3.833	<0.0001
Subjects (matching)	0.01132	20	0.00056	$F_{(20,60)}$= 1.084	ns
Residual	0.03132	60	0.00052		
Infochemicals	**SS**	**Df**	**MS**	***F***	***P***
Time	0.2608	3	0.08695	$F_{(3,60)}$= 920.9	<0.0001
Infochemicals	0.6307	9	0.07008	$F_{(9,20)}$= 627	<0.0001
Interaction	0.07168	27	0.00265	$F_{(27,60)}$= 28.12	<0.0001
Subjects (matching)	0.00223	20	0.00011	$F_{(20,60)}$= 1.184	ns
Residual	0.0057	60	0.00009		

Table 2 – Results of the Two-way ANOVA for differences for total saxitoxin cell and biovolume quota of *Raphidiopsis raciborskii* T3 exposed (infochemicals) and non-exposed (control) to *Daphnia gessneri* along 6-days incubation. **ns=** no significant diferences.

STXs cell quota	**SS**	**Df**	**MS**	***F***	***P***
Time	224.9	3	74.95	$F_{(3,12)}$= 30.84	<0.0001
Infochemicals	10.48	1	10.84	$F_{(1,4)}$= 6.803	ns
Interaction	44.66	3	14.89	$F_{(3,12)}$= 6.124	<0.01
Subjects (matching)	6.162	4	1.541	$F_{(4,12)}$= 0.634	ns
Residual	29.17	12	2.431		
STXs biovolume quota	**SS**	**Df**	**MS**	***F***	***P***
Time	0.5828	3	0.1943	$F_{(3,12)}$= 31.02	<0.0001
Infochemicals	0.0308	1	0.0308	$F_{(1,4)}$= 7.524	ns
Interaction	0.134	3	0.0447	$F_{(3,12)}$= 7.133	<0.01
Subjects (matching)	0.0164	4	0.0041	$F_{(4,12)}$= 0.654	ns
Residual	0.0751	12	0.0063		

Table 3 – Results post-hoc Bonferroni's comparison test for differences in total saxitoxin cell and biovolume quota of *Raphidiopsis raciborskii* T3 exposed (infochemicals) and non-exposed (control) to *Daphnia gessneri* along 6-days incubation. **ns=** no significant diferences. **CI=** confidence interval

CONTROL – INFOCHEMICALS (STXs cell quota)	**Mean difference**	**95% CI of differences**	***P***
Time (d^{-1})			
0	-0.019	-3.432 – 3.394	ns
2	-0.5943	-4.007 – 2.819	ns
4	1.233	-2.18 – 4.646	ns
6	-5.906	-9.319 – -2.493	<0.001
CONTROL – INFOCHEMICALS (STXs biovolume quota)	**Mean difference**	**95% CI of differences**	***P***
Time (d^{-1})			
0	0	-0.1737 – 0.1737	ns
2	-0.03	-0.2037 – 0.1437	ns
4	-0.06667	-0.1071 – 0.2404	ns
6	-0.3233	-0.4971 – 0.1496	<0.001

Table 4 – Results of the Two-way ANOVA for differences for *sxtI* and *sxtU* relative expression by *Raphidiopsis raciborskii* T3 exposed (infochemicals) and non-exposed (control) to *Daphnia gessneri* along 6-days incubation. **ns=** no significant diferences.

sxtI	**SS**	**Df**	**MS**	***F***	***P***
Time	1.307	3	0.4356	$F_{(3,12)}$= 8.172	<0.01
Infochemicals	0.5646	1	0.5646	$F_{(1,4)}$= 8.588	<0.05
Interaction	1.621	3	0.5402	$F_{(3,12)}$= 10.13	<0.01
Subjects (matching)	0.263	4	0.0657	$F_{(4,12)}$= 1.233	ns
Residual	0.6396	12	0.0533		
sxtU	**SS**	**Df**	**MS**	***F***	***P***
Time	5.36	3	1.787	$F_{(3,12)}$= 137.7	<0.0001
Infochemicals	2.237	1	2.237	$F_{(1,4)}$= 153.2	<0.001
Interaction	3.335	3	1.112	$F_{(3,12)}$= 85.65	<0.0001
Subjects (matching)	0.0584	4	0.0146	$F_{(4,12)}$= 1.125	ns
Residual	0.1557	12	0.0130		

Table 5 – Efficiencies and standard curve parameters obtained by real-time qPCR analysis for the cyanobacterial *rpo*C1, *sxt*I and *sxt*U specific primers sets.

Target gene	Strain	Efficiency	Slope	*Y*-intercept	R^2	Melting temperature
***rpo*C1**	*R. Raciborskii* T3 genomic DNA	98.23	-3.365	13.49	0.9794	60 – 95 °C
***sxt*I**	*R. Raciborskii* T3 genomic DNA	107.18	-3.161	16.27	1.0000	60 – 95 °C
***sxt*U**	*R. Raciborskii* T3 genomic DNA	107.52	-3.154	15.43	0.9997	60 – 95 °C

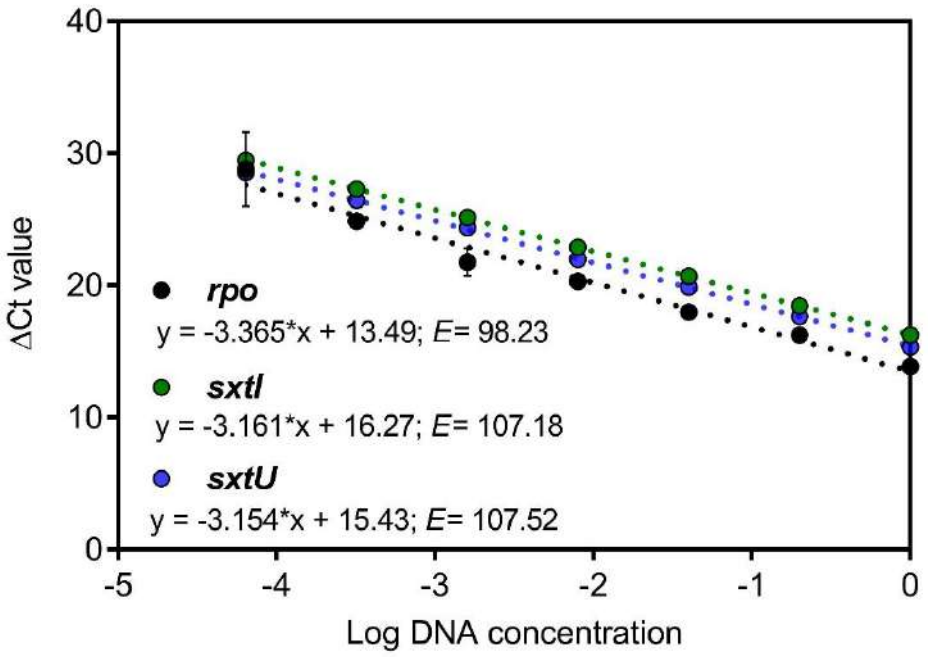

Figure 1 – Standard curve and its linear equation for cyanobacterial *rpo*C1, *sxt*I and *sxt*U specific primers.

CAPÍTULO 4

Diet quality overrides toxic Cyanobacteria impact on the tropical zooplankton *Daphnia gessneri*: insights from transgenerational response of antioxidant defenses

Manuscrito a ser submetido ao periódico *Journal of Plankton Research*

Short communication

Diet quality overrides toxic Cyanobacteria impact on the tropical zooplankton *Daphnia gessneri*: insights from transgenerational response of antioxidant defenses

Mauro Cesar Palmeira Vilar[1], Sabrina Loise de Morais Calado[2], Luan de Oliveira Silva[1], Helena Cristina Silva de Assis[2], Aloysio da Silva Ferrão-Filho[3] e Sandra Maria Feliciano de Oliveira e Azevedo[1]

[1]Laboratory of Ecophysiology and Toxicology of Cyanobacteria, Institute of Biophysics Carlos Chagas Filho, Federal University of Rio de Janeiro, Rio de Janeiro, RJ 21949-900; Brazil

[2]Laboratory of Environmental Toxicology, Department of Pharmacology, Federal University of Paraná, Curitiba, PR 21040–360, Brazil

[3]Laboratory of Evaluation and Promotion of Environmental Health, Instituto Oswaldo Cruz, FIOCRUZ, Rio de Janeiro, RJ 21040–360, Brazil

Corresponding author: Mauro Vilar (maurovilar@gmail.com)
Carlos Chagas Filho Biophysics Institute, Federal University of Rio de Janeiro, Rio de Janeiro 21949-902, Brazil
Phone: +55 21 39386648

Abstract

Cyanobacterial blooms are nuisance that constrain predators top-down control by both low nutritional value and the production of several toxins that affect zooplankton, particularly large ones such as *Daphnia* which is a key consumer in aquatic food webs. Conversely, an improved antioxidant and biotransformation system is hypothesized to underlie the physiological ability of zooplankton to resist cyanotoxins and to explain the transgenerational tolerance of *Daphnia* to toxic cyanobacteria. Thus, We conducted transgenerational experiments with *D. gessneri* by culturing the parental generation in variable food treatments (very-low, low, medium and high-quality diet) for 12-days and determined the impact of parental nutritional environment on offspring enzyme-mediated tolerance to a saxitoxin-producing cyanobacterium by assessing biotransformation and antioxidant biomarkers. The absence of PUFA-rich algae in food composition constrained the increase of glutathione-S-transferase and glutathione peroxidase activity over generations and buffered offspring tolerance to toxic cyanobacteria. Our results suggest that low quality food conditions in the maternal environment pose a direct constraint on offspring enzyme-mediated capacity to deal with toxic cyanobacteria.

Keywords: cladocerans, cyanotoxins, maternal effects, antioxidant biomarkers.

Introduction

Trophic interactions are the main forces which drive eco-evolutionary dynamics in populations. However, human interference such as artificial eutrophication of water bodies has impacted the energy transference in aquatic food webs, mainly by bottom-up mechanisms on promoting changes in phytoplankton structure toward cyanobacterial dominance (Ger et al. 2014; Huisman et al. 2018). Moreover, cyanobacteria have also been of concern due to the potential on producing a diverse array of secondary metabolites (commonly named cyanotoxins), and several of these are toxic and might act as neurotoxins, cytotoxins and phosphatases enzymes inhibitors (Carmichael et al. 2001; Buratti et al. 2017) to several aquatic organisms, including zooplankton.

In general, cyanobacteria are considered a poor food source for zooplankton by their low nutritional value, manageability, and harmful effects (Ferrão-Filho and Kozlowsky-Suzuki 2011; Ger et al. 2014). Therefore, despite achieving greater carbon biomass during their dominance, not only food quantity, but quality is of critical importance for zooplankton growth and reproduction (Pietrzak et al. 2010; Ferrão-Filho et al. 2019). Food quality is generally regarded in terms of elemental stoichiometry (i.e. C:N:P) (Sterner and Elser 2002), biochemical composition (Müller-Navarra et al. 2000; von Elert 2004), shape and size of food particles (van Donk et al. 1997; Fileto et al. 2007), and toxicity (Ferrão-Filho and Kozlowsky-Suzuki 2011; Ger et al. 2014; 2016).

Despite the potential toxicity and low nutritional value, cyanobacteria can represent an important alternative to the zooplankton diet, being at the base of the aquatic food chain and promoting the transfer of energy to subsequent links (Agrawal 1998). The most accepted hypothesis considers that such interaction is maintained by evolutionary pressures that define a balance between the production of toxic metabolites by cyanobacteria, as a chemical defense against predation, (Lampert 1981) and the acquisition of zooplankton tolerance to such a 'toxic diet' *via* transgenerational (or maternal) effect (Guo and Xie 2006). However, regarding that food quality can be highly variable within and among food webs in different natural environments, maternal effects should be included as an additional dimension into studies on how nutrition affects the physiology, ecology, and evolution of animal consumers (Frost et al. 2010).

Differently from other zooplankton groups (e.g. copepods and rotifers), cladocerans are generalists and therefore their dynamics in the environment is strongly affected by food availability and composition (i.e. phytoplankton succession). However, the persistence of some cladocerans during prolonged periods of cyanobacterial blooms has shown that these animals might dispose of strategies for maintaining their populations (Ger et al. 2014). Literature have shown that adaptive tolerance responses among those organisms are transferred to subsequent generations *via* "maternal effect" (Schwarzenberger and von Elert, 2013) and might differ interespecifically and between genotypes at the same species (Hairston et al. 2001; Guo and Xie 2006; Jiang et al. 2013).

Physiological resistance to toxic cyanobacteria in zooplankton is suggested as a higher efficiency of the detoxification system by enhancing constitutive defensive enzymes level in counterbalance to the intake of toxic cyanometabolites. The most accepted hypotheses indicate that tolerant cladocerans tend to have an overregulation of genes associated with the encoding of antioxidant enzymes (Wojtal-Frankiewicz et al. 2013; Wang et al. 2016) and to the mechanisms of cellular efflux, such as membrane transporters, multidrug exporters and permeases (Schwarzenberger et al. 2014). In addition, there is also an increase in the expression of genes encoding enzymes trypsin and chymotrypsin, in response to the presence of peptides inhibitors of digestive enzymes (Schwarzenberger and von Elert 2013). Thus, the potential tolerance to the metabolites, associated with the efficiency of a detoxification system, is expected to maximize the fitness of these organisms to deal with cyanobacterial constrains.

Agrawal et al. (1999) emphasize that the parental environment predicts the quality of the progeny's environment. Thus, mothers can transfer tolerance from maternal effects, endowing their offspring with phenotypes to thrive over potential threats such as predation and harmful substances, maintaining a stable fitness of the population. Pflugmacher et al. (1998) state that exposure to perennial blooms can cause the improvement of physiological tolerance to toxic cells, regarding more efficient detoxification mechanisms. Thereby, tolerance traits can be associated with an overregulation of enzymatic cascades to deal with diets of different composition, maintenance of cellular homeostasis, as well as the metabolism of harmful substances. In a recent investigation, Koussoroplis et al. (2017) reported a change in the level of expression and activity of lipases produced by *Daphnia pulex* depending on the type of

diet (cyanobacteria or green algae) provided. In addition to the genetic response, it is also important to consider that epigenetic information is of particular interest for the study of phenotypic plasticity, since previous studies indicate the change of the phenotype in response to environmental heterogeneity with the on-off state or the level of quantitative expression of genes, regulated by epigenetic factors (Marden 2008; Menzel et al. 2011).

Raphidiopsis raciborskii (previously *Cylindrospermopsis raciborskii*, Aguilera et al. 2018) is a bloom-forming potentially toxic cyanobacterial species which have been reported dominating in several regions. Up to date, isolated strains of *R. raciborskii* from South American waters have been reported to produce the neurotoxic saxitoxins (or paralytic shellfish toxins as referred to marine dinoflagellates), while in other regions that species produces the cytotoxic cylinderspermopsins (Antunes et al. 2015; Burford et al. 2016). In the present study we evaluated the effect of food quality underlying the transgenerational response of antioxidant defenses in *Daphnia gessneri* exposed to a saxitoxin-producing cyanobacterium.

Materials and Methods

Zooplankton cultures

Zooplankton consisted of the neotropical cladoceran species *Daphnia gessneri* isolated from Mucugê reservoir (Bahia, Brazil). The cladoceran was kept in RT medium (Tollrian 1993) enriched with commercial 0.1% (~2.25 $mgC \times L^{-1}$) humic extract (Microbe-lift® Amazon Black & Soft Water Conditioner, USA) at initial pH 7.6; 24±1 °C, 50 µmol photons m^{-2} s^{-1} and a 12/12h dark-light cycle. Animals were fed with the green algae *Chlamydomonas reinhartii* CHLRN-1 and *Ankistrodesmus stiptatus* ANRF-1 cells suspension at a final concentration of 500 $\mu gC \times L^{-1}$ once every three days.

Phytoplankton strains

The green algae strains *Ankistrodesmus stiptatus* ANRF-1 isolated from Funil Reservoir (Rio de Janeiro, Brazil) and the *Chlamydomonas reinhardtii* CHLRN-1 gently provided by the Petrobrás Research Center (CENPES/PETROBRÁS) are maintained on the Culture Collection of the Laboratory of Ecophysiology and Toxicology of Cyanobacteria (LETC/IBCCF-UFRJ) and were used to provide an edible and nutritious food. The microalgal strains ANRF-1 and CHLRN-1 grew as single cells and displayed

a mean cell size of 21.98 and 4.04 µm, respectively. Conversely, the saxitoxin-producing cyanobacterium *Raphidiopsis raciborskii* T3 isolated from *Billings* reservoir (São Paulo, Brazil) (Lagos et al. 1999) was used as non-edible low-quality food.

Stock cultures of all strains were kept in sterile liquid ASM-1 medium (Gorham et al. 1964) at 24±1°C, 50 µmol photons m^{-2} s^{-1} under a 12:12h light-dark cycle until the production of considerable biomass prior to variable food experiments. Under these conditions, T3 had an average trichome length of 74.91 ± 32.54 µm and a total saxitoxins cellular amount of 6.32±2.04 fg×$cell^{-1}$ which correspond to a total saxitoxins biomass amount of 0.31±0.10 µg/mm^3.

Experimental dietary treatments set-up

Diet quality set-up consisted of four strains mixtures where edible food was represented by *A. stiptatus* ANRF-1 and *C. reinhardtii* CHLRN-1, and non-edible when *R. raciborskii* T3 was in the mixture. These edible microalgal strains display a similar lipid cellular content but differential composition so that ANRF-1 was assumed as a highly nutritious food since its lipid composition consists mostly of highly unsaturated fatty acids (HUFAs), whereas CHLRN-1 was assumed as medium-quality food due to a higher relative amount of saturated fatty acids (Miranda et al. 2016). The toxic cyanobacterium was assumed as an unsuitable food for *Daphnia*. Food mixtures (treatments) were provided as diets varying in quality level relative to edible algae, but at the same final biomass 500 µgC×L^{-1} as the following proportions:

(i) High-quality diet (CHLRN-1 + ANRF-1; 1:1 biomass ratio)
(ii) Medium-quality diet (CHLRN-1)
(iii) Low-quality diet (CHLRN-1 + ANRF-1 + T3; 0.5:0.5:1 biomass ratio)
(iv) Very-low-quality diet (CHLRN-1 + T3; 1:1 biomass ratio).

For the transgenerational experiment third brood offspring was used. Initially the individuals were age-synchronized and obtained from a parental generation raised in a high-quality food environment to ensure a good physiological state. We set-up the neonates (<24h-old parental population = F_0) at a density 50 ind L^{-1} in 2 L beakers filled with 1.5 L of RT medium for each one of the four food treatments. A relatively high density was chosen to have enough animals for biochemical biomarkers analyses. In addition, since the animals had an apparently low clearance rate (~0.5 mL ind^{-1} h^{-1}, data not shown), the beakers received the different food mixtures once every three days and

the medium was refreshed at the same frequency, over a period of 12-days. That exposure duration was chosen because consists of the time interval where *D. gessneri* reached mean maturity and has released their third brood. Along the experiment, the first and second brood of the parental population (F_0) were discarded and the transgenerational effect of maternal diet quality was carried out with the third brood (offspring = F_1 population) during the same period under edible and non-edible food treatments. Therefore, F_1 population was exposed to the same nutritious and poor food environment experienced by their mothers.

Thereafter 10-individual (adults, 12-days old) groups were pooled in 1.5-mL microtube flasks (n=3). The excess of liquid medium was removed with a micropipette and the animals were stored at -80°C until the subsequent biochemical biomarkers analyses.

Biochemical biomarkers

To assess the antioxidant defense, we analyzed the activity of three key antioxidant biomarkers in whole-body homogenates of *Daphnia*: lipoperoxidation (LPO), glutathione S-transferase (GST) and glutathione peroxidase (GPx). Antioxidant system response has been described as good indicator of saxitoxin exposure, as well as for dietary stress (Steinberg et al. 2010; Melegari et al. 2012; Calado et al. 2020).

Pooled *Daphnia* (10 individuals per sample) were homogenized in phosphate buffer solution (0.1 M) at pH 7.0 in a proportion of 300 µL/sample. Homogenates were centrifuged at 15000 ×g for 30min at 4°C and the supernatants were collected and used to measure the glutathione peroxidase (GPx) and glutathione-S-transferase (GST) activities and lipoperoxidation levels (LPO). Protein concentration was measure prior to all biochemical analyses using bovine serum albumin as a standard (Bradford, 1976). A volume of 10 µL of supernatant (diluted 1:20) and 250 µL of Bradford reagent (Biorad®) were placed in a microplate and absorbance measured at 595 nm.

GPx activity was measured using the method of Paglia and Valentine (1967). A volume of 10 µL of supernatant and 130 µL of reaction medium (3.08 mM of sodium azide; 0.308 mM β-NADPH, reduced nicotinamide-adenine dinucleotide phosphate; 1.54 U/mL glutathione reductase and 3.08 mM reduced glutathione in 0.1 M sodium phosphate buffer, pH 7.0). After two minutes, 60 µL of 1.5 mM H_2O_2 was added. Absorbance was monitored at 340 nm and the activity was expressed as nmol/min/mg of protein.

GST activity was analyzed according to Keen et al. (1976) in which GST activity is measured using reduced glutathione (GSH) and 1-chloro-2.4-dinitrobenzene (CDNB) as substrates. Supernatant (20 µL) was placed in microplate, immediately followed by 180 mL of reaction medium (3 mM GSH, 3 mM CDNB, 0.1 M potassium phosphate buffer, pH 6.5). The absorbance increase was measured at 340 nm and the activity was expressed as nmol/min/mg of protein.

The analysis of LPO was carried out using the method proposed by Levine et al. (1994) at a wavelength of 530 nm (excitation) and 590 nm (emission). A volume of 100 µL of supernatant (resuspended in methanol 1:1 v/v) was mixed with 900 µL of reaction solution (0.1 mM xylenol orange, 25 mM H_2SO_4, 4.0 mM BHT (butylated hydroxytoluene) and 0.25 mM $FeSO_4NH_4$ (ammonium ferrous sulfate) added in this specific order in 90% grade methanol). After 30 min of reaction at room temperature, the absorbance was measured at 570 nm and the LPO was expressed as nmol hydroperoxides/mg of protein.

The biochemical analyses were carried out on a BioTek ELx800 Absorbance Microplate Reader (BioTek Instruments, Inc).

Statistical analysis

Data were evaluated for normality and homoscedasticity of variances and subsequently subjected to a two-way ANOVA to assess antioxidant enzymes levels in *D. gessneri* under different "food quality" and "across generations". Tukey HSD post hoc test was used to identify statistical differences. Graphs and analyzes were performed in the GraphPad prism 6.0.

Results and discussion

Changes in diet quality represented by different mixtures of green algae and toxic cyanobacteria at the same biomass amount significantly affected antioxidant defenses and lipid peroxidation in the zooplankton *D. gessneri* between food treatments and over generations **(Table 1)**.

Changes in diet quality had no impact on whole-body GST activity of *Daphnia* offspring from mothers fed with very-low, low and medium-quality diets so that the same basal GST level was maintained over generations **(Fig. 1)**. However, offspring from mothers kept under a high-quality diet displayed a significant increase in GST activity

when fed with 50% cyanobacteria (equivalent low-quality diet) **(Tukey HSD test, $p<0.01$; Fig. 1)**. Therefore, food treatments exerted a similar impact on GST activity in parental lineages, whereas only mothers fed with high-quality diet seemed to improve offspring enzyme response to toxic *R. raciborskii*.

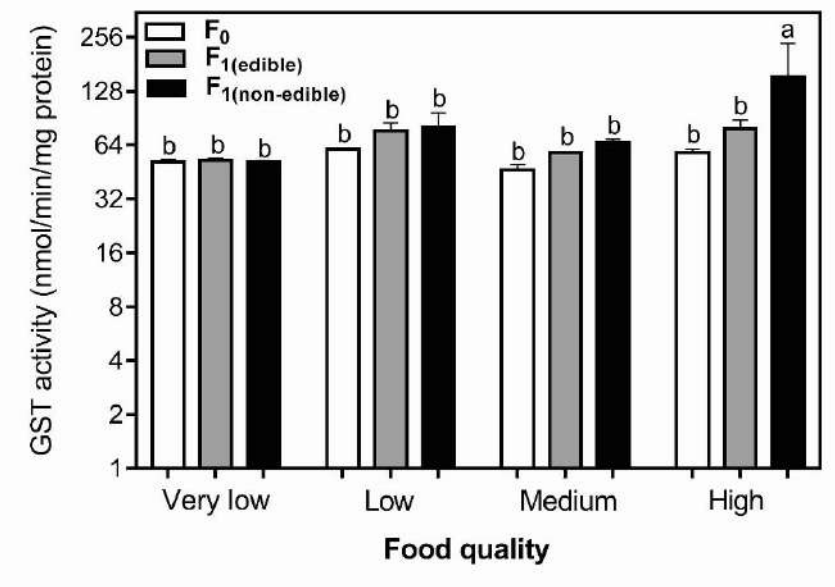

Figure 1 – Whole-body glutathione S-transferase (GST) activity over generations of *Daphnia gessneri* grown in different food treatments (very-low, low, medium and high-quality diets) after 12-days incubation. $\mathbf{F_0}$ = Mothers; $\mathbf{F_{1(edible)}}$ = offspring from mothers fed with edible food; $\mathbf{F_{1(non\text{-}edible)}}$ = offspring from mothers fed with cyanobacteria. Different letters mean statistical differences (Tukey HSD test; $p<0.05$).

GST plays a role on biotransformation process, thereby it is expected to increase its activity in the presence of toxicants, such as saxitoxins. Previous studies have shown GST increase in zooplankton as response to cyanotoxins (Chen et al. 2005; Kozlowsky-Suzuki et al. 2009; Ferrão-Filho et al. 2017). In our study, offspring from mothers kept under a highly-nutritious diet displayed a higher GST response to toxic cyanobacteria in diet. However, differently to our findings, we expected a higher GST activity in offspring from mothers exposed to cyanobacteria due to an inducible toxin tolerance by improving detoxification potential. It has been described by Ortiz-Rodriguez et al. (2012) which evidenced a transgenerational enhancement on antioxidant defenses of *D. magna* promoted through maternal cyanotoxin exposure. Nevertheless, *D. gessneri* has already shown to be tolerant to *R. raciborskii* in previous studies (Costa et al. 2013; Ferrão-Filho et al. 2014) and performed even better in a diet composed of *R. raciborskii* T3 (Costa et al. 2013). Thus, the mechanism of this tolerance is not yet well deciphered.

Table 1 – Results of the Two-way A NOVA for differences in antioxidant enzymes (GST and GPx) activity and lipid peroxidation (LPO) level over generations (transgenerational effect) of *Daphnia gessneri* grown in different food treatments (very-low, low, medium and high-quality diet) after 12-days incubation.

Two-way ANOVA	GST		GPx		LPO	
	F	***P***	***F***	***P***	***F***	***P***
Food quality	$F_{(3,24)}$= 6.14	<0.01	$F_{(3,24)}$= 90.6	<0.0001	$F_{(3,24)}$= 30.42	<0.001
Transgenerational effect	$F_{(2,24)}$= 5.72	<0.01	$F_{(2,24)}$= 75.38	<0.0001	$F_{(2,24)}$= 21.65	<0.0001
Interaction	$F_{(6,24)}$= 2.62	<0.05	$F_{(6,24)}$= 21.46	<0.0001	$F_{(6,24)}$= 6.99	<0.0001

Regarding GPx activity, there was a transgenerational increase depending on food source availability. Offspring from mothers grown in both low and high-quality diet environment displayed a significatively higher GPx activity when fed with diet partially composed of toxic cyanobacteria **(Fig. 2)**.

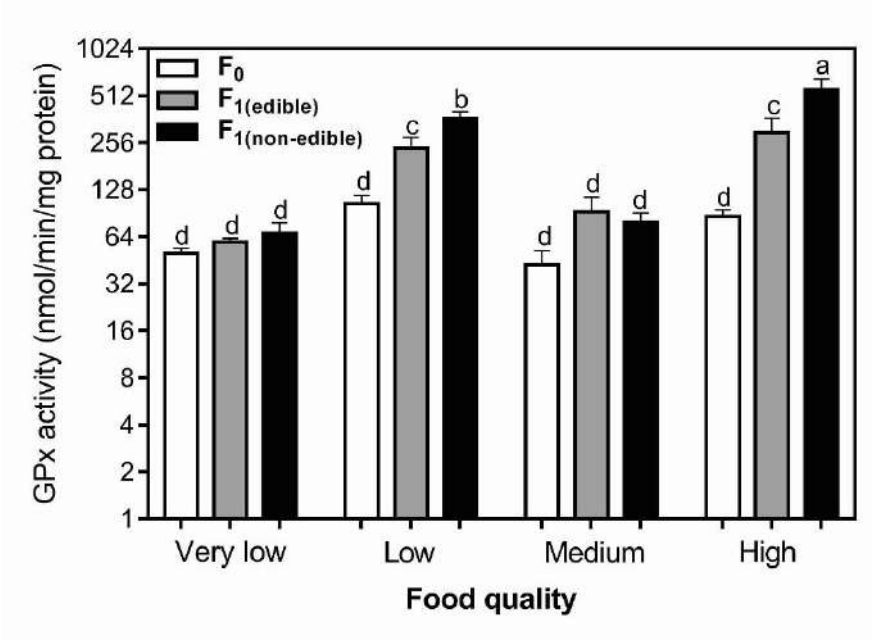

Figure 2 – Whole-body glutathione peroxidase (GPx) activity over generations of *Daphnia gessneri* grown in different food treatments (very-low, low, medium and high-quality diets) after 12-days incubation. $\mathbf{F_0}$ = Mothers; $\mathbf{F_{1(edible)}}$ = offspring from mothers fed with edible food; $\mathbf{F_{1(non\text{-}edible)}}$ = offspring from mothers fed with cyanobacteria. Different letters mean statistical differences (Tukey HSD test; p<0.05).

Conversely, no significant changes were found in GPx activity of F_1 population compared to its parental lineage raised in very-low and medium-quality diets. In general, GPx displayed a trend similar to GST. Both enzymes share the use of glutathione (GSH)

as a cofactor (Calado et al. 2019a), what might explain the similar pattern observed for their activity in our findings. A plastic increase in GPx was shown by Oexle et al. (2016) which have evidenced natural populations of *D. magna* to evolve an up-regulation of this enzyme induced by ultraviolet radiation exposure.

Furthermore, GPx also plays a role on protecting the integrity of cellular membranes (Van der Oost, 2003) and hence decreases lipoperoxidation caused by reactive species. In turn, LPO levels were in line with the results found to GPx, regarding the effects of maternal food environment on the transgenerational response of antioxidant system. Basal levels of lipid peroxidation in *Daphnia* were enhanced in offspring of mothers fed with high and low-quality diet, however offspring from the later only displayed a higher LPO level when fed with non-edible food **(Figure 3),** despite concomitant GPx increase.

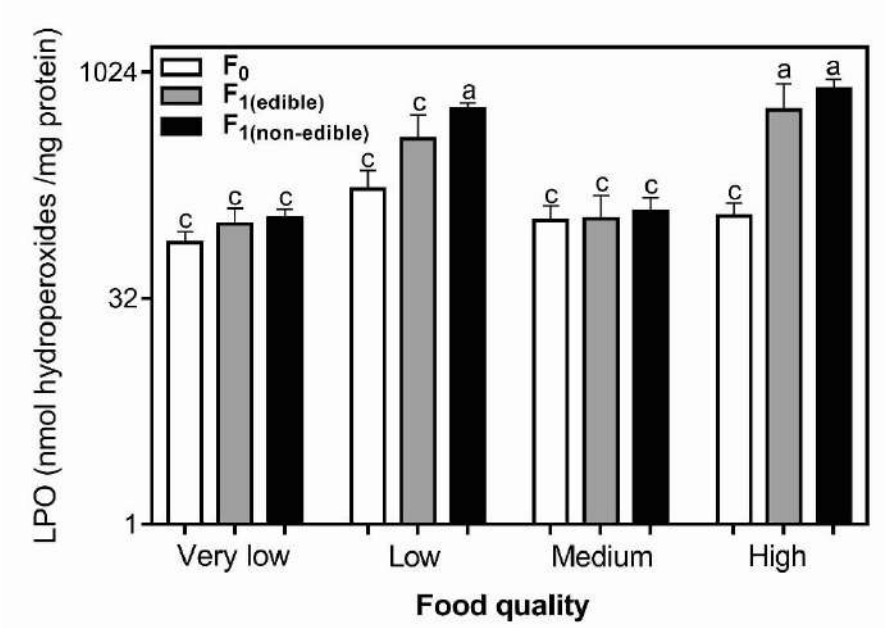

Figure 3 – Whole-body lipid peroxidation (LPO) level over generations of *Daphnia gessneri* grown in different food treatments (very-low, low, medium and high-quality diets) after 12-days incubation. $\mathbf{F_0}$ = Mothers; $\mathbf{F_{1(edible)}}$ = offspring from mothers fed with edible food; $\mathbf{F_{1(non\text{-}edible)}}$ = offspring from mothers fed with cyanobacteria. Different letters mean statistical differences (Tukey HSD test; $p<0.05$).

In our study, despite the increase in GPx in offspring when fed with non-edible food, there was also an increase in LPO, which may have resulted in a system failure in the degradation of reactive species resulting in oxidative stress. LPO is characterized by the process where reactive species attack polyunsaturated fatty acids of the cellular

membrane, inducing a chain reaction with lipid hydroperoxides as intermediate products (Halliwell and Gutteridge, 2007). Basically, peroxyl free radicals (ROO) can be rearranged through a cyclization reaction of endoperoxides and react with carbon double bonds in cell lipid layers, producing, among other final products of this process, malondialdehyde (MDA) (Melegari et al. 2012; Gagne, 2014). Considering that saxitoxins act blocking cellular ion channels, mainly constraining nervous impulse to occur, it is expected some level of membrane instability and therefore a greater susceptibility to the action of hydroperoxides. Accordingly, lipoperoxidation has been demonstrated as a biomarker to assess saxitoxins toxicity in studies *in vitro* with cells cultures (Melegari et al. 2012) and *in vivo* with aquatic organisms (Cao et al. 2018; Calado et al. 2020).

Overall, increased activities of the studied antioxidant and biotransformation enzymes under toxic cyanobacteria exposure have been observed before in zooplankton: GST in *D. longispina* (Vega and Pizarro, 2000) and in *D. commutata* (Balseiro et al., 2008); GST in copepods (Kozlowsky-Suzuki et al. 2009), including an cyanotoxin-induced increase in GST, CAT and LPO in *D. magna* (Ortiz-Rodriguez and Wiegand, 2010), GST and CAT in *D. laevis*, *D. similis* and *Moina micrura* (Ferrão-Filho et al. 2017). However, up to date, no study has evaluated the impact of *Daphnia*'s food environment on the antioxidant response to toxic cyanobacteria.

Previous studies have shown that exposure to toxic cyanobacteria induce zooplankton defenses that can be further transferred to offspring via maternal effects (Gustafsson et al. 2005, Ortiz-Rodríguez et al. 2012, Schwarzenberger and Von Elert 2013, Jiang et al. 2013). The present results expand the study of maternal effects to maternal food quality as a prerequisite to offspring enhanced detox response. Yet, differences among studies may also partly reflect temporal induction dynamics of antioxidant enzymes, although here we underline the plastic changes in basal enzyme production and its potential activity over generations.

As large generalists, daphnids are likely to have reduced ability on selecting the particles they ingest, thereby being exposed to fluctuating dietary lipid quantity and quality in their gut contents across various temporal scales (Koussoroplis et al. 2017). Thus, in our study, the switch to a cyanobacterial-rich diet implies both a decrease in highly-unsaturated fatty acids and sterols, which are essential for *Daphnia* good physiological state (see Müller-Navarra et al. 2000; von Elert, 2004; Ferrão-Filho et al.

2019). On the other hand, the green algal strains *Ankistrodesmus stiptatus* ANRF-1 and *Chlamydomonas reinhartii* CHLRN-1 provided as nutritious food items have been previously characterized as lipid-rich ones (Miranda et al. 2016). However, ANRF-1 seems to be more suitable for zooplankton nutrition regarding its lipid composition (as well as phosphate content) which consists mainly of unsaturated fatty acids (C18 chains such as alpha-linolenic acid, oleic acid and linoleic acid) compared to a higher amount of saturated fatty acids (C15 chains such as the pentadecanoic, heptadecanoic, arachidic and behenic acids) observed in CHLRN-1 (Miranda et al. 2016). Therefore, it is likely that the same maternal effect was not evidenced in mothers from medium-quality diet due the nutritional impairing caused by a relatively poor essential lipid composition provided by CHLRN-1 as the only edible food source.

In our study, nutritional limitation might have buffered the detox response to toxic cyanobacteria in the diet. In fact, increased biotransformation and antioxidant activity requires additional energy allocation (Ortiz-Rodriguez et al. 2012). The ability to predict the direction of evolution of physiological defense mechanisms against a certain stressor is further hampered by the fact that these often defend against a variety of stressors (as observed to antioxidant defenses), and these other stressors may also change across a temporal scale (Oexle et al. 2016). Another reason is that *Daphnia* offspring might rely more on behavioral mechanisms than its parthenogenetic mothers (see Garbutt and Little, 2014), making unnecessary a higher physiological antioxidant defense, even if toxic cyanobacteria increased in water. The findings suggest that maternal nutritional status is determinant on improving offspring defenses.

Zooplankton naturally experience food variation regarding fluctuations in phytoplankton. In this sense, cyanobacterial blooms dramatically impact on large *Daphnia* species which are shifted toward more selective microzooplankton (e.g. copepods, rotifers, small cladocerans) in the community (Ger et al., 2014; Jiang et al., 2014). In this study, somehow diet quality regarding the availability of edible (and nutritious) food items governed transgenerational antioxidant response to saxitoxin-producing cyanobacteria in *D. gessneri*. Thus, taking into account the insights from antioxidant defenses examined over generations, studies focusing on the phenotypic plasticity in *Daphnia* regarding its rapid evolution on tolerating toxic cyanobacteria must consider the availability of nutritious food items as abundant as non-edible ones (e.g. cyanobacteria) experienced by the parental lineage. In conclusion, we evidenced that food

environment, regarding the availability of high-quality food items, impacts maternal effects on zooplankton ability to respond to toxic cyanobacteria in diet.

Author contributions

M.C.P.V., A.S.F.F. and S.M.F.O.A. designed the research; M.C.P.V., L.O.S. and S.L.C. performed research; M.C.P.V., A.S.F.F., H.C.S.A and S.M.F.O.A. analyzed data; M.C.P.V. wrote the paper; A.S.F.F., H.C.S.A. and S.M.F.O.A. reviewed the paper.

Declaration of interest

The authors declare no conflict of interest.

Acknowledgments

This work was supported by the National Council for Scientific and Technological Development – CNPq and by the Carlos Chagas Filho Foundation for the Support of Science in Rio de Janeiro Development – FAPERJ. M.C.P.V. and L.O.S. were supported by CNPq scholarships.

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DISCUSSÃO GERAL

Os resultados apresentados na presente tese evidenciam respostas adaptativas na interação predador-presa usando como modelo organismos do zooplâncton e fitoplâncton; mais especificamente cladóceros e cianobactérias tóxicas. Neste estudo foram ressaltadas respostas dessa interação trófica no potencial de cianobactérias em dominar e persistir no fitoplâncton, bem como entender como *Daphnia*, um gênero representativo do zooplâncton onívoro, lida com a predominância desses microrganismos no ambiente. Assim, objetivou-se gerar subsídios ao conhecimento da dinâmica eco-evolutiva no plâncton dulcícola e adicionar mais uma dimensão ao conhecimento de fatores reguladores da produção de toxinas em cianobactérias.

No primeiro capítulo foram abordados atributos comportamentais de duas espécies de *Daphnia* em espectros distintos de tamanho e representativas de diferentes latitudes. Buscou-se avaliar o impacto de florações mistas e uni-específicas de cianobactérias tóxicas no comportamento alimentar dessas espécies. Florações formadas de um único táxon são comuns, mas em muitos ambientes aquáticos tropicais e sub-tropicais a co-dominância de mais de um táxon como *Microcystis* spp. e *Raphidiopsis raciborskii* já tem sido reportada em mais de 20% das florações registradas (SOARES et al., 2013; MOURA et al., 2015). A ocorrência de florações mistas dessas espécies representa um risco elevado em potencial uma vez que em sua maioria estão associadas à presença de mais de uma cianotoxina, como microcistinas e saxitoxinas. O efeito sinérgico de hepatotoxinas e neurotoxinas produzidas por cianobactérias já tem sido reportado para organismos aquáticos (FREITAS et al., 2014; FERRÃO-FILHO et al., 2017).

Em nosso estudo a taxa de filtração mostrou-se proporcional ao tamanho do zooplâncton, de forma que *D. similis* apresentou uma maior taxa de filtração de alimento nutritivo em relação à *D. laevis*. No entanto, quando adicionadas cianobactérias à dieta, esses cladóceros responderam de forma similar, com uma redução significativa na ingestão de alimento. Florações de cianobactérias têm impactos diretos no zooplâncton pois em sua maioria são formadas por espécies potencialmente tóxicas e de atributos morfológicos (ex.: colônias, filamentos) que limitam a predação por esses animais. Assim, na medida em que essas características reduzem a ingestão de alimentos pelo zooplâncton, podem em consequência afetar o crescimento e a reprodução, promovendo impactos variáveis na dinâmica do plâncton e, dependendo da magnitude, também podem levar ao desacoplamento trófico (SOMMER et al., 2012).

Os capítulos 2 e 3 abordaram a produção de toxinas em *Microcystis aeruginosa* e *Raphidiopsis raciborskii* – duas espécies de cianobactérias potencialmente tóxicas e formadoras de florações – como uma defesa química induzida pelo predador. Concomitantemente avaliou-se o estado fisiológico das células a fim de monitorar os custos associados à resposta de defesa. Neste estudo, cairomônios de *Daphnia* induziram um aumento significativo na produção de cianotoxinas, no entanto este incremento na toxicidade não esteve associado a custos que afetassem o *fitness* das cianobactérias. Embora haja a evidência de custos metabólicos associados à produção de cianotoxinas (BRIAND et al., 2012), até então não foram detectados custos elevados associados às defesas induzidas (LÜRLING, 2020). É provável que esses custos não ocorram sob um 'ótimo fisiológico' como observado em cultivos de laboratório, mas em condições de limitação de recursos, ex.: redução de nutrientes (ver PANČIĆ; KIØRBOE, 2018).

Este é o primeiro estudo de defesas induzidas em cianobactérias para a América do Sul, sendo também o primeiro registro na literatura da evidência química e molecular da produção de toxinas em *R. raciborskii* induzida pelo predador. Esta cianobactéria é reportada como uma espécie invasora e oportunista, formando florações e ocorrendo no plâncton de corpos d'água em diferentes latitudes. *R. raciborskii* também tem sido descrita quanto à sua plasticidade fenotípica conferindo tolerância à limitação de recursos (luz, nutrientes, CO_2, etc), elevado potencial competitivo e resistência à predação (BURFORD et al., 2016; RANGEL et al., 2020). Portanto, estudos que investiguem a Biologia desta espécie fornecerão cada vez mais informações ao entendimento dos mecanismos pelos quais *R. raciborskii* compõe e muitas vezes domina o fitoplâncton em diversos ambientes aquáticos.

Variações morfológicas como a formação de colônias e estruturas celulares (ex.: espinhos, processos), e a fixação desses fenótipos em populações fitoplanctônicas já tem sido descrito como uma resposta adaptativa para lidar com a predação do zooplâncton (PANČIĆ; KIØRBOE, 2018; LÜRLING, 2020). Entretanto, o conhecimento sobre a indução da produção de toxinas em espécies nocivas de microalgas e cianobactérias como defesa química contra a predação ainda é pouco explorado. De forma geral, a produção de cianotoxinas como um traço funcional na defesa contra a herbivoria tem sido abordada como uma característica constitutiva (RANGEL et al., 2020). Portanto, considerando a diversidade intraespecífica relativa à produção celular desses metabólitos, podendo variar em unidade de fentograma a picograma por célula, o forrageamento seletivo em células

menos tóxicas pode agir como uma força que direciona a evolução à predominância de linhagens mais tóxicas. No entanto, a capacidade de uma linhagem em aumentar a quota celular de toxina como resposta ao predador permite que a seleção natural exercida pelo zooplâncton ocorra não apenas em um *pool* de genótipos distintos (seleção mendeliana), mas também sofra efeitos da plasticidade fenotípica (seleção não-mendeliana) que pode reduzir a diferença intraespecífica em traços funcionais de potencial defensivo expressos constitutivamente.

A predação pelo zooplâncton representa um dos mais importantes processos de perda populacional, portanto é considerada uma das maiores forças de seleção do fitoplâncton. A produção de metabólitos bioativos e/ou atributos morfológicos que limitem a predação suporta a hipótese da defesa e evidencia seu elevado potencial adaptativo. De acordo com Smetacek (2001) a evolução no plâncton é regida pela proteção e não pela competição. Assim, a interação trófica entre cianobactérias tóxicas e o zooplâncton herbívoro representa um modelo robusto para o estudo das respostas fenotípicas recíprocas que integram a dinâmica eco-evolutiva entre esses organismos em sistemas aquáticos.

No quarto capítulo a resposta transgeracional das defesas antioxidantes de *D. gessneri* foi avaliada sob diferentes dietas compostas de cianobactéria e algas verdes com perfis distintos de ácidos graxos. Foi possível observar uma influência da qualidade nutricional do alimento fornecido a fêmeas partenogenéticas no potencial de suas proles em responder à presença de *R. raciborskii* tóxica na dieta. No estudo, apesar de ser esperado um aprimoramento da resposta antioxidante nas proles proveniente de mães alimentadas com cianobactéria tóxica, este ambiente alimentar materno se mostrou relevante na resposta transgeracional sobretudo no que diz respeito à presença de uma dieta rica em ácidos graxos essenciais. Apesar de aqui não integrarmos informações do impacto da dieta no crescimento e reprodução de *D. gessneri*, dados do estresse oxidativo e enzima de biotransformação demonstram a resposta fisiológica desses animais, a qual antecede mudanças no *fitness* e determinarão quão resiliente esses organismos podem ser em manter a homeostase redox sob estresse variado. Ademais, o clone de *D. gessneri* usado no presente estudo já tem sido reportado por não sofrer variações significativas no *fitness* em dietas composta pela cepa *R. raciborskii* T3 – produtora de saxitoxinas. (Costa et al. 2013).

De acordo com Oexle et al. (2016) a capacidade de prever a direção da evolução dos mecanismos de defesa fisiológica contra um certo estressor é ainda mais dificultada pelo

fato de que estes costumam agir contra uma variedade de estressores (a exemplo das defesas antioxidantes e enzimas de biotransformação) e esses outros estressores também podem mudar em uma escala temporal. Mudanças essas que neste estudo são relacionadas às variações na abundância relativa de itens alimentares nutritivos e nocivos, experimentadas pelo zooplâncton herbívoro como flutuações temporais (ex.: ciclo sazonal) no fitoplâncton que levem à dominância de cianobactérias. Assim, as diferenças entre respostas do biomarcardores também podem refletir parcialmente a dinâmica de indução temporal de enzimas de defesas antioxidantes, embora aqui sejam ressaltadas as mudanças plásticas na produção basal e sua atividade potencial ao longo das gerações.

Essa tese de doutorado amplia o conhecimento da dinâmica eco-evolutiva no plâncton em ecossistemas eutrofizados onde florações de cianobactérias são frequentes. Entender da base molecular à comportamental, as respostas de resistência recíproca na interação *Daphnia*-cianobactéria, é fundamental uma vez que a predação e limitação nutricional estabelecidos nessa interação constituem o filtro biológico que regula a diversidade e substituição de espécies no fitoplâncton e zooplâncton, os quais por sua vez podem interferir no fluxo de energia em ecossistemas aquáticos.

CONCLUSÕES

i) – Ambas estimativas de biomassa através do biovolume (= carbono) e fluorescência da clorofila-a permitiram a mensuração dos efeitos de cianobactérias tóxicas no comportamento alimentar de *Daphnia* spp.

ii) – A manutenção de uma taxa de filtração relativamente constante associada a uma redução na ingestão total de carbono (referente ao alimento nutritivo) em diferentes concentrações simples e mistas de cianobactérias indicam um comportamento generalista dos cladóceros avaliados, o que foi evidenciado por um índice de seletividade nulo.

iii) – Infoquímicos do zooplâncton *D. gessneri* induziram um aumento na produção de cianotoxinas (microcistinas e saxitoxinas) em *Microcystis aeruginosa* NPLJ-4 e *Raphidiopsis raciborskii* T3, mas nenhuma variação morfológica, evidenciando uma resposta de defesa química induzida pelo predador.

iv) – Não foi registrado nenhum custo significativo na fotossíntese e crescimento associados à defesa química induzida nas cianobactérias, além de uma redução pontual no crescimento de *R. raciborskii* T3.

v) – O aumento na produção de saxitoxinas (STX + neoSTX) em *R. raciborskii* T3 foi acompanhado de uma sobrerregulação nos genes *sxt*U e *stx*I, sendo o primeiro relato de regulação gênica da produção de saxitoxinas em cianobactérias mediada *via* infoquímico do predador.

vi) – A qualidade do ambiente alimentar materno em *D. gessneri* no que se refere à disponibilidade de alimento nutritivo e cianobactéria tóxica, afetou a produção basal de defesas antioxidantes e enzima de biotransformação na prole.

vii) – A presente tese destaca aspectos comportamentais, morfo-fisiológicos e moleculares como respostas fenotípicas recíprocas que afetam a dinâmica eco-evolutiva do zooplâncton herbívoro e cianobactérias tóxicas.

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ANEXOS

Trabalhos publicados em anais de eventos (resumos e resumos expandidos)

- **VILAR, M.C.P.;** SILVA, L.O.; RODRIGUES, T.F.C.P.; FERRÃO-FILHO, A.S.; AZEVEDO, S.M.F.O. Feeding behavior in Cyanobacteria-tolerant and non-tolerant *Daphnia laevis* (Cladocera) populations. XVII Congresso Brasileiro de Limnologia & II Congresso Ibero-Americano de Limnologia, 2019, Florianópolis – Santa Catarina.

- RODRIGUES, T.F.C.P.; SILVA, L.O., **VILAR, M.C.P.;** AZEVEDO, S.M.F.O. Toxin production by *Microcystis aeruginosa* (Cyanobacteria) enhanced by predator infochemicals: is it a functional response? XVII Congresso Brasileiro de Limnologia & II Congresso Ibero-Americano de Limnologia, 2019, Florianópolis – Santa Catarina.

- **VILAR, M.C.P.;** RODRIGUES, T.F.C.P.; PARANHOS, R.R.; SILVA, L.O.; AZEVEDO, S.M.F.O. Toxin production by microcystin- and saxitoxin-producing Cyanobacteria in response to *Daphnia gessneri* infochemicals. XI International Conference on Toxic Cyanobacteria, 2019, Cracóvia – Polônia.

- MESQUITA, F. M.; PINTO, L. M. O.; **VILAR, M. C. P.**; OLIVEIRA, D. F.; NASCIMENTO, J. H. M. ; AZEVEDO, S. M. F. O. ; ZIN, W. A. Oral sublethal microcystin-LR does not affect pulmonary mechanics but compromises mitochondrial function. In: XI International Conference on Toxic Cyanobacteria, 2019, Cracóvia – Polônia.

- REIS, G.C.; **VILAR, M.C.P.;** FERRÃO-FILHO, A.S. Efeitos da cianobactéria *Cylindrospermopsis raciborskii* (CYLCAM-1) sobre parâmetros reprodutivos e comportamento alimentar de *Daphnia* spp. In: XV Congresso Brasileiro de Ecotoxicologia, 2018, Aracaju-SE.

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Environmental Science and Pollution Research
https://doi.org/10.1007/s11356-020-09737-6

RESEARCH ARTICLE

Can small-bodied *Daphnia* control *Raphidiopsis raciborskii* in eutrophic tropical lakes? A mesocosm experiment

Aloysio S. Ferrão-Filho[1] · Uanderson J. Pereira[2] · Mauro C.P. Vilar[3] · Leonardo de Magalhães[4] · Marcelo M. Marinho[4]

Received: 1 November 2019 / Accepted: 15 June 2020

Abstract

Raphidiopsis raciborskii is being considered an expanding, invasive species all over the world. It is a potentially toxin producer cyanobacterium and form blooms specially in (sub)tropical lakes, causing concern to public health. Thus, controlling such phenomena are of vital importance. To test the hypothesis that a tropical clone of *Daphnia laevis* is able to reduce the biomass of *R. raciborskii*, we performed a mesocosm experiment simulating a bloom of this cyanobacterium in field conditions and exposing it to ecologically relevant densities of daphniids. In addition, we tested the hypothesis that omnivorous fish would be able to exert a top-down effect on *Daphnia*, decreasing the effectiveness of this control. We used treatments with (10 and 20 *Daphnia* L^{-1}) or without *Daphnia* and fish (3 per mesocosm). *Daphnia* was able to significantly reduce the biomass of *R. raciborskii* only at the highest density tested. Fish had low effect on *Daphnia* biomass, but it is suggested that nutrient recycling by fish might have contributed to the higher *R. raciborskii* biomass in fish treatments. This is the first evidence of *Daphnia* control over saxitoxin-producing cyanobacteria in a tropical ecosystem.

Keywords Cyanobacteria · Zooplankton · Toxins · Top-down control · Biomanipulation

Introduction

Cyanobacterial blooms have been a matter of concern worldwide as they can cause harmful effects to animals and humans (Azevedo et al. 2002; Ferrão-Filho and Kozlowsky-Suzuki 2011). These organisms produce a series of metabolites with toxic properties, among the most known are hepatotoxins (microcystins and nodularin), neurotoxins (anatoxins, saxitoxins, and the amino acid β-methylamino-L-alanine—BMAA), cytotoxins (cylindrospermopsins), dermatotoxins (aplysiatoxins and lyngbyatoxins), and skin-irritant extra-cellular lipopolysaccharides (Chorus and Bartram 1999; Buratti

Responsible Editor: Vitor Manuel Oliveira Vasconcelos

Highlights Effects of *Daphnia laevis* and fish on cyanobacterial biomass were tested in mesocosms. *Daphnia* significantly reduced the biomass of *R. raciborskii* only at 20 individuals/L. Fish had low effect on *Daphnia* biomass and likely affected cyanobacteria by N/P. This is the first evidence of *Daphnia* control over saxitoxin-producing cyanobacteria.

✉ Aloysio S. Ferrão-Filho
aloysio@ioc.fiocruz.br

Uanderson J. Pereira
ujpereira@gmail.com

Mauro C.P. Vilar
maurovilar@gmail.com

Leonardo de Magalhães
ldemagalhaes@hotmail.com

Marcelo M. Marinho
manzi.uerj@gmail.com

[1] Laboratory of Evaluation and Promotion of Environmental Health, Instituto Oswaldo Cruz, FIOCRUZ, Rio de Janeiro, RJ 21040-360, Brazil

[2] Postgraduate Program in Biological Sciences, Department of Botany, Nacional Museum, Federal University of Rio de Janeiro, Rio de Janeiro, RJ 21940-590, Brazil

[3] Laboratory of Ecophysiology and Toxicology of Cyanobacteria, Institute of Biophysics Carlos Chagas Filho, Federal University of Rio de Janeiro, Rio de Janeiro, RJ 21949-900, Brazil

[4] Laboratory of Ecology and Physiology of Phytoplankton, Department of Plant Biology, Rio de Janeiro State University, Rio de Janeiro 20550-900, Brazil

Published online: 27 June 2020

et al. 2017). There has been a consensus among scientists that climate change, along with eutrophication, have boosted cyanobacterial development, increasing blooms frequency and intensity worldwide (Pearl et al. 2016). Thus, mitigating the global expansion of cyanobacterial blooms is a major challenge facing researchers and resource managers.

Toxic cyanobacteria can have a range of harmful effects on zooplankton such as feeding inhibition, decreased survivorship, and reproduction (DeMott et al. 1991; Ferrão-Filho et al. 2000; Nogueira et al. 2006). However, the effects depend on the type and concentration of toxin, as well as on the sensitivity of the zooplankton species/lineage (Ferrão-Filho and Kozlowsky-Suzuki 2011). Despite its toxic potential, cyanobacteria are also regarded a poor food for zooplankton by either its inherent low nutritional value, as low content of essential polyunsaturated fatty acids (PUFAs; DeMott and Müller-Navarra 1997), or to its difficult manageability, as they generally present colonial or filamentous forms (Porter and Orcutt 1980; De Bernardi and Giussani 1990; Bednarska et al. 2014). This is indeed the reason why the use of large cladocerans such as *Daphnia* has been considered of limited applicability on biomanipulation for restoration of lakes (Matveev et al. 1994; Chislock et al. 2013; Urrutia-Cordero et al. 2016).

The cyanobacterium *Raphidiopsis raciborskii* (Woloszynska) Aguilera, Berrendero Gómez, Kastovsky, Echenique & Salerno (basionym *Cylindrospermopsis raciborskii* (Woloszynska) Seenayya & Subba Raju) (Aguilera et al. 2019) has been considered an invasive (and opportunistic) species originally reported from the tropics, but in the last years, it has expanded to other latitudes and adapted to a wide range of temperature and light regimes (Antunes et al. 2015), what have been attributed to the increased global temperature (Burford et al. 2016). High physiological plasticity such as adaptation to low light, lower temperatures, and high affinity for nutrients, besides low susceptibility to predation, have provided this species a higher competitive advantage and has been also responsible for its expansion worldwide (Bonilla et al. 2012; Antunes et al. 2015; Burford et al. 2016). The strains isolated from different regions have shown that they can produce the neurotoxins of the saxitoxins (STXs) group (Lagos et al. 1999; Soto-Liebe et al. 2010; Vale et al. 2008) or the cytotoxic alkaloid cylindrospermopsin (CYN) (Li et al. 2001; Saker and Neilan 2001). Although there has been some report of the occurrence of CYN in field samples (Bittencourt-Oliveira et al. 2011; Lorenzi et al. 2018), South American strains isolated up to date are all reported to produce STXs (Lagos et al. 1999; Molica et al. 2002; Yunes et al. 2003; Piccini et al. 2013; Mesquita et al. 2019). Saxitoxin-producer strains of *R. raciborskii* have demonstrated a variety of harmful effects on zooplankton organisms, especially of the cladocerans group, such as decreased clearance rates (Panosso and Lürling 2010; Ferrão-Filho et al. 2017), mobility (Ferrão-Filho et al. 2010, 2014a), growth, and reproduction (Soares et al. 2009; Costa et al. 2013). However, some species are less affected and show good survivorship and reproduction despite of high proportion of *R. raciborskii* in the diet (Costa et al. 2013; Ferrão-Filho et al. 2014b; Ferrão-Filho et al. 2019).

In that context, biomanipulation appears as the management of the food chain with the purpose to improve water quality by reducing nutrients and undesirable phytoplankton biomass (Shapiro et al. 1975). It is based on the concept of trophic cascade (Brooks and Dodson 1965; Carpenter et al. 1985), in which top predator fish control smaller planktivorous fish populations, decreasing its predation on zooplankton, which, in turn, increases grazing pressure on phytoplankton. Therefore, manipulating higher trophic levels by adding piscivores, or removing planktivores, would theoretically increase the size spectra, abundance, and grazing pressure of herbivorous zooplankton and thus reduce algal abundance, promoting water clearance and improving water quality (Hansson et al. 1998; Chen et al. 2013; Chislock et al. 2013; Wilson and Chislock 2013). The efficacy of this technique relies on the dominance of large-bodied herbivore *Daphnia* in the zooplankton of temperate water bodies (Jeppesen et al. 2005; Lacerot et al. 2013). Biomanipulation has been extensively used in the restoration of temperate waterbodies (Søndergaard et al. 2007), but its effectiveness in subtropical and tropical lakes is not well evaluated (Jeppesen et al. 2007). Cladocerans of the genus *Daphnia* are scarce or absent in (sub)tropical aquatic ecosystems, whereas zooplankton communities are often dominated by rotifers, copepods, and small-sized cladocerans (Sarma et al. 2005).

Although *Daphnia* species are present in (sub)tropical regions, they are often smaller than their counterpart from temperate lakes, such as *D. magna* and *D. pulex* (Meerhoff et al. 2007; Lacerot et al. 2013). Therefore, top-down control by zooplankton is expected to be less effective in warmer lakes. There are also great differences in the structure and complexity of aquatic community between temperate and tropical lakes that weaken the link between primary consumers to higher trophic levels (Attayde and Hansson 1999; Meerhoff et al. 2007). In general, top-down control depends on the consumers' community structure regarding size spectra, relative abundance, and feeding behavior. Higher richness and dominance of omnivorous fish that breed throughout the whole year and are not controlled either by the availability of zooplankton or predation by piscivorous species are considered the main reasons why biomanipulation measures have failed in tropical lakes (Lazzaro 1997; Jeppesen et al. 2005, 2007). Consequently, tropical and subtropical communities may be strongly regulated by more complex, food web–type interactions in relation to the classical linear food chains of temperate lakes (Stein et al. 1995). Therefore, trophic cascades frequently documented in temperate lakes may be less common in the tropics. However, this hypothesis has not been adequately tested, and further biomanipulation experiments in tropical and subtropical regions are needed to examine the generality of this method as a tool to improve water quality in these systems.

Thus, the aim of this study was to evaluate if a small-bodied neotropical *Daphnia* clone can control filamentous cyanobacterial biomass in tropical systems. Therefore, the following hypotheses were tested: (1) small-bodied *Daphnia* can negatively affect the biomass of filamentous cyanobacteria by herbivory pressure, (2) *Daphnia* nutrient recycling (uptake and excretion of N and P) will not influence on nutrient availability for phytoplankton, (3) the presence of omnivorous fish weakens the top-down control exerted by *Daphnia*. We performed an outdoor mesocosm experiment (20-L transparent acrylic cylinders) containing an increased biomass of *R. raciborskii* in lake water and added the species *Daphnia laevis* as a grazer. We also tested the top-down effect on *R. raciborskii* biomass by adding omnivorous fish to control zooplankton abundance. With this design, we tried to test if general hypothesis of top-down control, applied to temperate lakes, would stand for tropical systems where toxic cyanobacterial blooms frequently occur.

Material and methods

Site of the experiment

Camorim reservoir (22° 55′ 54.44″ S/43° 28′ 22.44″ W) is an artificial lake built in 1908 for water supply and electric energy generation situated in the western part of the municipality of Rio de Janeiro (Brazil). It is a shallow (1.8 ± 0.8 m average depth) reservoir located at 436 m altitude in the Pedra Branca ridge, surrounded by Atlantic forest and varying from mesotrophic to eutrophic state according to total phosphorus (mean = 35.2 ± 18.4; min = 12; max = 82 μg TP L^{-1}) and chlorophyll (mean = 54.0 ± 26.0; min = 6,9; max = 128.5 μg L^{-1}) (Cunha et al. 2013). It has been used as a source of water for the neighborhood population, through a system of aqueducts going downhill and reaching a small water plant in the State Park of Pedra Branca (PEPB). Recurrent algal blooms of the diatom *Aulacoseira ambigua* (Grunow) Simonsen and of the potentially toxic cyanobacterium *R. raciborskii* occur in the Camorim reservoir, which has been a matter of concern for the water plant managers. Three saxitoxin-producing strains of *R. raciborskii* were isolated from the reservoir previously (Mesquita et al. 2019), and saxitoxin was also detected in seston samples from 1-year monitoring program (2012–2013) to study the limnological variables and phytoplankton and zooplankton communities (Ferrão-Filho et al. 2019). This system has been used for many years for water supply for the neighborhood, but its operation was interrupted since 2015 because of recurrent blooms of toxic cyanobacteria.

Cyanobacteria and Daphnia cultures

The STXs-producing *R. raciborskii* strain CYLCAM-2 was isolated from Camorim reservoir (Mesquita et al. 2019) and maintained in the Culture Collection of the Laboratory of Ecology and Physiology of Phytoplankton, University of Rio de Janeiro State (UERJ). Stock cultures prior to the experiments were established under the following conditions: WC medium, temperature of 25 °C, irradiance of 30 μmol photons m^{-2} s^{-1}, and photoperiod of 12/12 h light-dark cycle.

The species *D. laevis* Birge, 1878 was isolated from the eutrophic Ibirité reservoir (Minas Gerais State, Brazil), with previous record of *Microcystis* spp. blooms (Garcia et al. 2009). Stock cultures were established in filtered water from Camorim reservoir until the animals achieve adult phase (size 1.4–1.6 mm). The daphnids were fed a 2:1 mixture of two green algae *Ankistrodermus falcatus* (Braun) and *Pseudokirchneriella subcapitata* (Korshikov), respectively, at a total concentration of 400 μg C L^{-1}, in temperature of 24 °C, at dim light and photoperiod of 12/12 h light-dark cycle.

Experimental design and field procedures

A mesocosm experiment was performed between 13th and 29th of January 2016 in one of the decantation tanks of the water supply and wastewater company (CEDAE) situated in the basis of PEPB. These tanks have very similar physical-chemical characteristics to the reservoir water, but with lower turbidity and more stable conditions, thus perfect to test our specific hypotheses. The mesocosms consisted of 12 acrylic cylinders of 20 L volume, disposed in a 3 × 4 design, and hang by a fluctuating metallic structure through the use of PET bottles filled with air. The experimental design is described in Fig. 1.

At the beginning, all cylinders were filled with water from the tank containing the natural phytoplankton and zooplankton community and received also an addition of nitrate and phosphate, at a final concentration of 51.0 mg L^{-1} $NaNO_3$ (~ 37.20 mg L^{-1} NO_3^-) and 5.2 mg L^{-1} K_2HPO_4 (~ 2.83 mg L^{-1} PO_4^-), respectively. The treatments were (1) control, with tank water only; (2) cyanobacterium *R. raciborskii* (saxitoxin-producing strain CYLCAM-2; hereafter called CYL), in exponential growth phase was inoculated at t = 0 at a biomass concentration of 1.5 mg C L^{-1} (43 μg L^{-1} chlorophyll-*a*) in all cylinders except controls; (3) CYL + 200 *Daphnia* (10 *Daphnia* L^{-1}); and (4) CYL + 400 *Daphnia* (20 *Daphnia* L^{-1}). Ecologically relevant densities of *D. laevis* for (sub)tropical environments were based on the study of Brandão et al. (2012). In both 3 and 4 treatments, the *Daphnia* was added as adults (7–10 days old) on day 7. Treatment 4 received 3 fish per cylinder on day 13. Omnivorous fish of the species *Astyanax* sp. (Characidae) (Esteves 1996) with mean size of 28.2 (± 3.42) mm and weight of 0.44 (± 0.13) g were collected in the Camorim reservoir one day before its addition and maintained in 5 L of lake water without food. Treatments

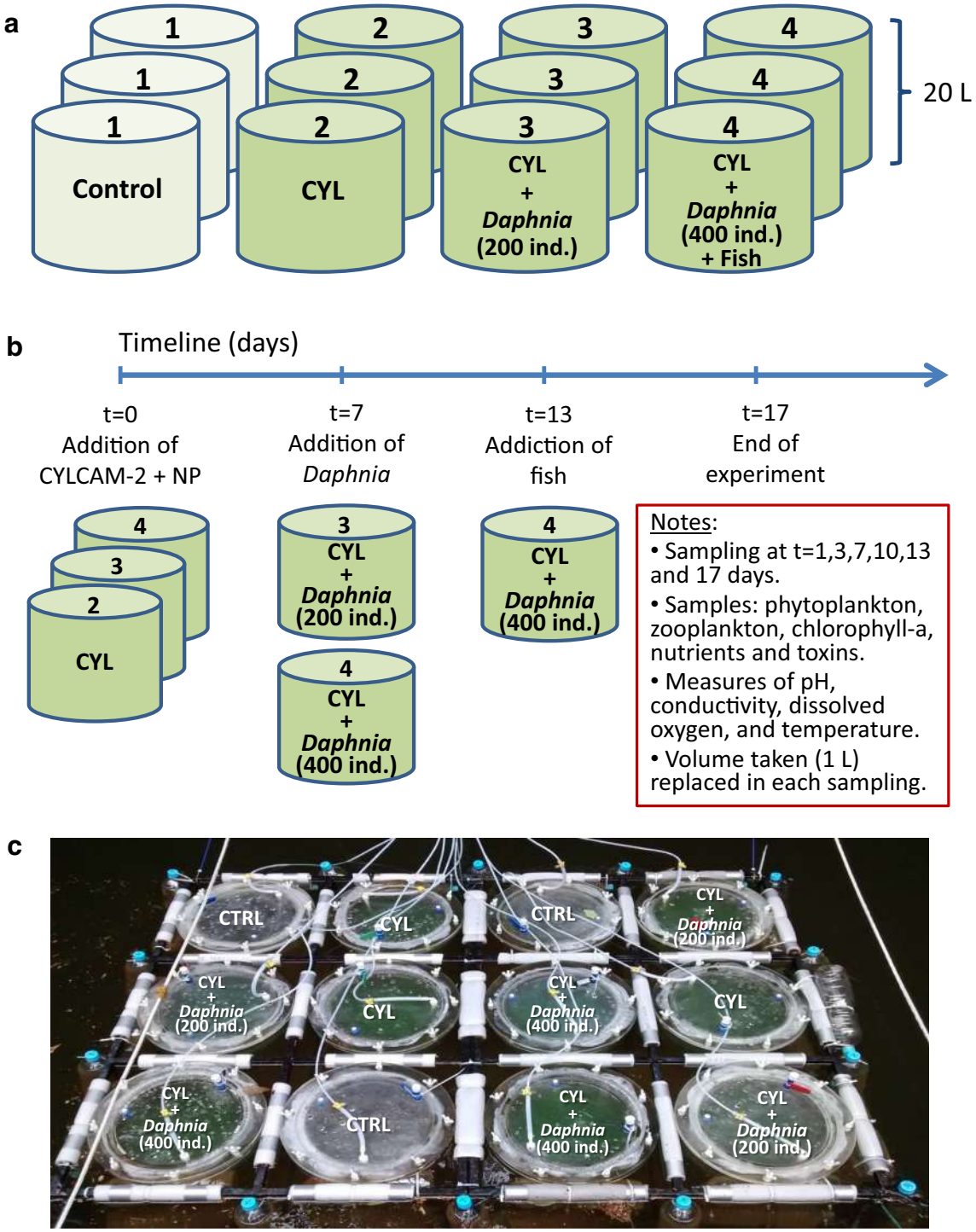

Fig. 1 Experimental design of the mesocosms. **a** Control and treatments; **b** timeline with the additions of CYLCAM-2 inoculum ($t = 0$), *Daphnia* ($t = 7$) in two densities, and fish ($t = 13$); **c** photo of the mesocosms showing the random disposition of the treatments

were randomly assigned to each cylinder to not create a gradient in physical conditions (i.e., light and temperature). The cylinders were hermetically sealed, having no exchange with the tank water, and had an aeration system with the use of an air pump to avoid sedimentation of phytoplankton cells in the cylinders.

Sampling and counting of phytoplankton and zooplankton

Samples were taken by silicone tubes inserted through a hole in the cylinder lids with the use of a manual vacuum pump. Samples of 100 mL were taken for chlorophyll-*a*, saxitoxins,

and phytoplankton analyses. Total and cyanobacterial chlorophyll-a concentrations were measured using phytoplankton analyzer PHYTO-PAM (Heinz Walz GmbH, Effeltrich, Germany). The PHYTO-PAM was calibrated against a spectrophotometric determination of Chl-a from the *R. raciborskii* strain, which was done with a 90% acetone extraction based on Ritchie (2006). PHYTO-PAM applies four excitation wavelengths allowing distinguish between the three different major pigment-based phytoplankton groups (Lürling et al. 2018). Chlorophyll-a concentrations determined in the blue channel is referred as cyanobacterial chlorophyll-a and the sum of the green and brown channel as eukaryote algae chlorophyll-a. Total chlorophyll-a is the sum of all three channels (Schreiber 1998). The phytoplankton sample was fixed with 1% acetic Lugol's solution, and cells were counted in the inverted microscope (Zeiss, Axiovert 10) (Utermöhl 1958), being enumerated in random fields (Uhelinger 1964). The average phytoplankton biovolume ($mm^3\ L^{-1}$) was estimated by the product of the densities of each species (ind. mL^{-1}) by the mean volume of its cells, considering the average size of about 25 individuals (Hillebrand et al. 1999). The carbon content of phytoplankton (μg C L^{-1}) was estimated for each species from biovolume, according to Rocha and Duncan (1985). Samples for zooplankton community were taken by filtering 1 L of water from cylinders in a 50-μm mesh size net and concentrating to the volume of 100 mL and fixed with 4% formalin solution. Zooplankton organisms were counted and measured in a dissecting microscope (Olympus SZ6145STR). The density of zooplankton was quantified by counting individuals in 6 × 6 cm gridded (10 × 10 fields) acrylic counting chamber; the biomass of each taxon was calculated using equations of regression length-weight reported in Dumont et al. (1975) and Castilho-Noll and Arcifa (2007).

Samples (100 mL) for nutrient analysis included nitrite (NO_2^-), nitrate (NO_3^-), ammonium (NH_4^+), and soluble reactive phosphorus (SRP) and were analyzed according to Wetzel and Likens 1990). At every sampling date, measures of temperature, pH, conductivity and dissolved oxygen were made with portable multiparameter probe.

Saxitoxins analyses

To determine saxitoxin (STX) concentration in seston, 100 mL samples were filtered in borosilicate filters (45 mm diameter, Sartorius 13400, Germany) and stored at − 35 °C. Toxins extraction were performed with 0.5 M acetic acid and subsequently analyzed according to Oshima et al. (1995) using a Shimadzu HPLC system with a silica-base reversed phase C18 column. The toxin was detected using a fluorescence detector at a wavelength excitation at 330 nm and an emission at 390 nm. Chromatograms were compared with those obtained from saxitoxin and neo-saxitoxin standards purchased from the Institute of Marine Bioscience, National Research Council of Canada (Halifax, NS, Canada). Data were expressed as total volumetric concentration of saxitoxins (μg STXs L^{-1}).

Statistical analyses

Two-way repeated measures ANOVA (RM ANOVA) was performed to test the effects of time (t) treatment (Z) and the interaction of these factors ($t \times Z$) on the physical, chemical, and biological parameters. Bonferroni posttests were performed to compare differences between treatments at an $\alpha = 0.05$. The statistical tests were performed in the program GraphPad Prism 5.0.

Results

Physical and chemical variables

Temperature, dissolved oxygen, conductivity, and pH were similar among the treatments and exhibited relatively small variation during the experiment. According to the RM ANOVA, those variables experienced significant variations along the experiment (time (t); Table 1), but only conductivity showed a significant effect of treatment (Z), with the control showing significantly lower values than the treatments throughout the experiment ($F = 14.347$; $P < 0.05$). Temperature varied about 5 °C with a drop in the middle of the experiment due to a cold front that resulted in lot of rain in the region. Dissolved oxygen showed a slight increase towards the middle of the experiment reaching values near to saturation (~ 100%), especially in the treatments with CYL, which reflects the high primary productivity in those treatments. As expected, pH showed a steady increase from the beginning of the experiment to the 10th day, which reflects the CO_2 consumption during the phytoplankton development in the mesocosms and decreased slightly to the end of the experiment.

Except for the SRP, which showed no effect of treatment (Z) or an interaction between time and treatment ($t \times Z$), all other nutrients showed significant variations with time (t), treatment (Z), and an interaction between them (Table 1; Fig. 2). During the experiment, the concentrations of nitrate ($N\text{-}NO_3^-$) and soluble reactive phosphorus (SRP) decreased steadily in control and in all treatments, but faster for $N\text{-}NO_3^-$ in the treatments with CYL, reflecting nutrient consumption by the phytoplankton biomass development. Despite the high input of nutrients in the beginning of the experiment, the concentrations of $N\text{-}NO_3^-$ became significantly lower ($P < 0.05$) in the treatments compared with control on the 10th day and maintained this pattern on 13th and 17th days, reaching values close to zero in CYL treatments by the end of the experiment (Fig. 2a). SRP concentrations declined from day 1 to day 13 of

Table 1 Minimum, maximum, mean, standard deviation, and results of the two-way repeated measures ANOVA of physical and chemical variables in the mesocosms. Significant *P* values ($P < 0.05$) marked in italics

Parameters	Min.	Max.	Mean	SD	Time (*t*)		Treatment (*Z*)		Interaction (*t* × *Z*)	
					F	*P*	*F*	*P*	*F*	*P*
Temperature (°C)	14.9	20.1	17.7	1.6	1277.51	*< 0.001*	3.838	0.076	0.920	0.553
Dissolved oxygen (mg L^{-1})	7.5	12.7	9.1	1.2	73.66	*< 0.001*	5.963	0.051	2.744	0.090
pH	7.1	10.4	8.5	0.9	145.57	*< 0.001*	2.480	0.158	10.774	*< 0.001*
Conductivity (μS cm^{-1})	100.5	156.1	127.7	14.1	141.71	*< 0.001*	14.347	*0.004*	1.849	0.074
N-NO_3^- (mg L^{-1})	0.03	54.4	19.6	17.4	959.95	*< 0.001*	37.434	*< 0.001*	9.479	*< 0.001*
N-NO_2^- (mg L^{-1})	0.003	1.8	0.38	0.5	33.54	*< 0.001*	6.053	*0.030*	4.593	*< 0.001*
N-NH_4^+ (mg L^{-1})	0.17	5.76	2.42	1.7	11.48	*< 0.001*	13.645	*0.004*	3.305	*0.003*
DIN (mg L^{-1})	0.48	40.2	17.9	12.0	897.80	*< 0.001*	9.187	*0.012*	7.467	*< 0.001*
SRP (mg L^{-1})	0.09	5.21	2.31	1.7	443.28	*< 0.001*	0.716	0.577	1.960	0.057
DIN:SRP	3.22	140.1	26.7	27.9	7.36	*0.004*	1.946	0.224	3.308	*0.003*

the experiment and increased slightly at the end of the experiment (except for the control that only decreased) (Fig. 2b). On the other hand, ammonium ion (N-NH_4^+) showed a steady increase through the experiment in all treatments that received CYL, with the control showing an opposed trend from the 7th day, with significant lower values than treatments with CYL (Fig. 2c). Nevertheless, there were no significant differences ($P > 0.05$) in N-NH_4^+ concentration between treatments with CYL. The DIN:SRP atomic ratio had a significant effect of time (*t*) and time vs. treatment interaction (*t* × *Z*; Table 1; Fig. 2d) and oscillated slightly between the threshold values of 13 and 50 from the beginning to the 10th day of the experiment, in the control and in all the treatments, showing neither N or P limitation (Kosten et al. 2009). On the 13th day DIN:SRP ratio increased in control and even more in the treatments with CYL showing a higher consumption of P by the cyanobacterium, although by the still high concentration of SRP, there was no P-limitation (Davidson et al. 2012). However, neither significant differences were found between DIN:SRP ratio of the control and treatments nor between treatments with CYL in any period of the experiment ($P > 0.05$).

Phytoplankton community

As high concentrations of nutrients (such as N and P) were added to all mesocosms at the beginning of the experiment, phytoplankton biomass increased exponentially (Fig. 3), while nutrient levels declined (Fig. 2). Total phytoplankton

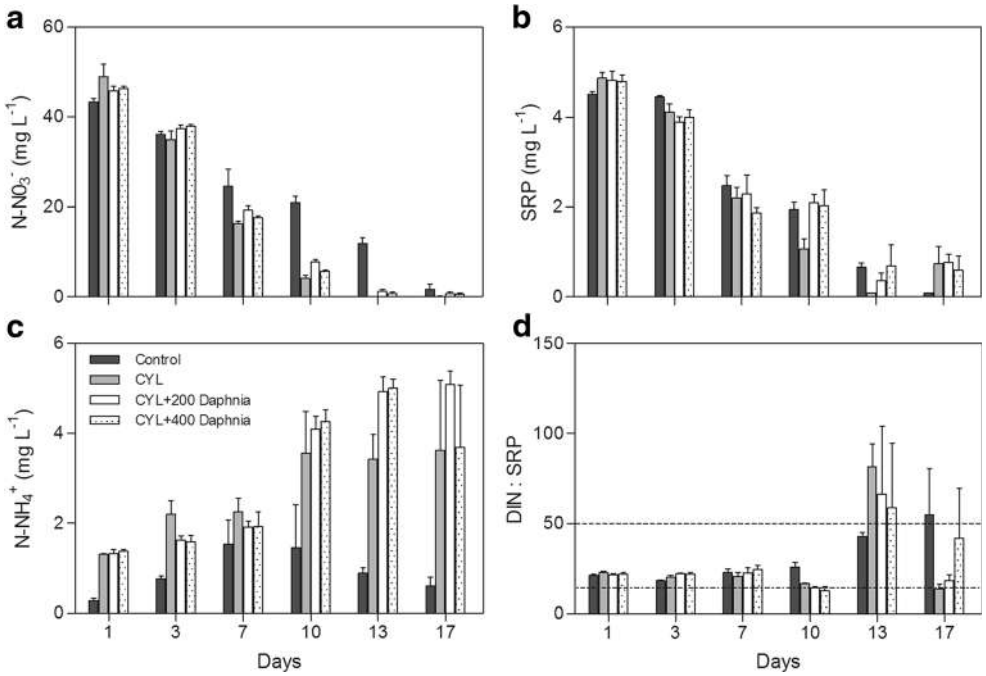

Fig. 2 Mean (± standard error) concentration (mg L^{-1}) of **a** nitrate, N-NO_3^-; **b** soluble reactive phosphorus, SRP; **c** ammonium ion, N-NH_4^+; and **d** DIN:SRP atomic ratio in control and treatments during the experiment. Inferior dotted line indicates lower limit for N limitation of phytoplankton growth and superior dashed line indicates upper limit for limitation by P (according to Kosten et al. 2009)

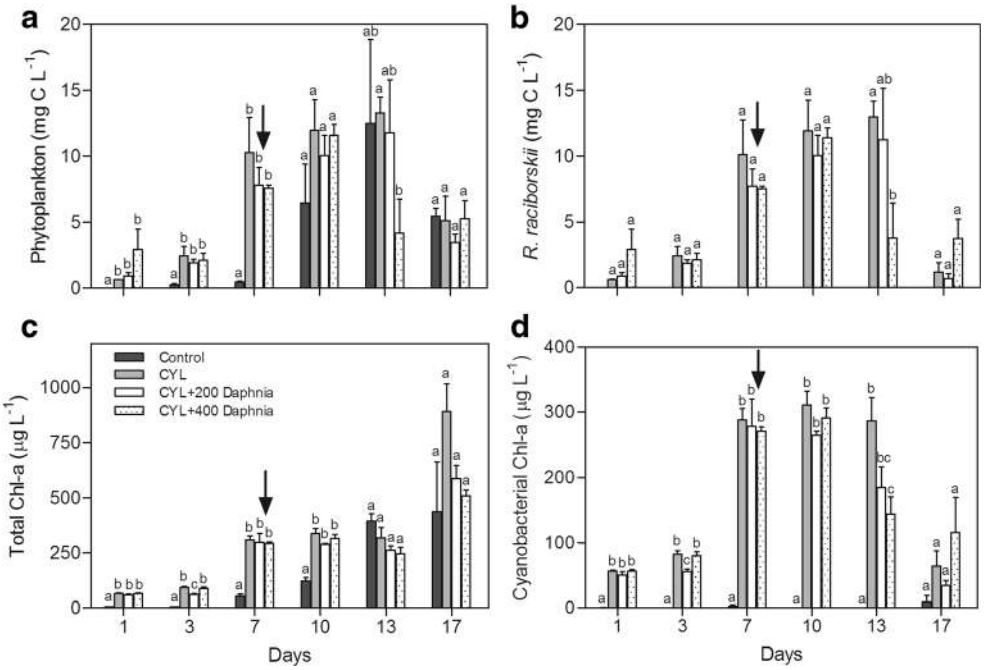

Fig. 3 Mean values (± standard error) of the phytoplankton biomass: **a** total phytoplankton carbon (µg C L^{-1}), **b** *Raphidiopsis raciborskii* carbon (µg C L^{-1}), **c** total chlorophyll-a (µg L^{-1}), and **d** cyanobacterial chlorophyll-a (µg L^{-1}) in control and treatments during the experiment. Different letters indicate significant differences. Setae indicate the day of the *Daphnia* addition

biomass was negatively correlated to nutrient concentrations (SRP: $r = -0.68$, $P < 0.001$; N-NO_3^-: $r = -0.69$, $P < 0.001$) suggesting that the nutrient uptake was coupled to primary production.

Phytoplankton community was markedly different in the control mesocosms compared with those ones that received CYL across the experiment. In the controls (no CYL added), the maximum phytoplankton biomass was reached in the 13th day of the experiment, and the species that contributed mostly were the diatoms *Achnanthidium* sp. and *Aulacoseira* spp. and the chlorophytes *Coelastrum proboscideum*, *Monoraphidium nanum*, *Nephrocytium* sp., *Nephrochlamys willeana*, and *Scenedesmus* sp. In the CYL treatments, besides *R. raciborskii* comprised over 90% of the phytoplankton biomass in all treatments, it exhibited a substantial increase until the 13th day. At the end of the experiment, there was a great reduction of the total phytoplankton biomass, including *R. raciborskii*, being the community dominated by chlorophytes, except for the treatment that received fish, in which the biomass of the cyanobacterium showed no variation from 13th to 17th day (Fig. 3).

According to the RM ANOVA, there was a significant variation in phytoplanktonic biomass with time (*t*), among treatments (*Z*) as well as an interaction between these factors (*t* × *Z*; Table 2). Pairwise multiple comparison procedures showed that there were significant differences (Bonferroni posttest, $P < 0.05$) between control and treatments until the 7th day of experiment ($P < 0.05$), while there were no significant differences between treatments until the 10th day of experiment. On day 10, no significant differences were detected between control and treatments or between treatments with or without *Daphnia*. On the 13th day, however, the total phytoplankton biomass in CYL + 400 *Daphnia* was drastically reduced and was significantly lower ($P < 0.002$) than those recorded in CYL (~ 32% of the treatment without *Daphnia*). The reduction in biomass in this treatment on day 13 was about 64% from that of the previous date (day 10). On the last day of the experiment, no significant differences were detected in the total phytoplankton biomass between control and treatments or between treatments with or without *Daphnia* (Fig. 3a).

With the exception of Cryptophyceae and Euglenophyceae, other groups of the phytoplankton had a significant variation with time (*t*), among treatments (*Z*) or time vs. treatment interaction (*t* × *Z*; Table 2).

Biomass of the cyanobacterium *R. raciborskii* significantly varied with time (*t*), among treatments (*Z*), and an interaction between these factors also was found (*t* × *Z*; Table 2). Until the 10th day of the experiment, there were no significant differences between treatments with or without *Daphnia*. On day 13, however, the biomass of *R. raciborskii* was significantly lower ($P < 0.05$) in CYL + 400 *Daphnia* compared with CYL (~ 29% of the treatment without *Daphnia*), but did not differ significantly from CYL + 200 *Daphnia*. On the last day of the experiment (17th day), there were no significant differences on biomass of *R. raciborskii* between control and treatments or between treatments with or without *Daphnia* and fish (Fig. 3b).

Chlorophyll-a analysis showed that the total amount of Chl-*a* had a significant variation with time (*t*), among treatments (*Z*) and an interaction between these factors (*t* × *Z*; Table 2). Total Chl-*a* increased steadily until the end of the experiment in all treatments, reaching its highest value in CYL treatments on day 17 (Fig. 3c), mainly due to the dominance

Table 2 Results of two-way repeated measure ANOVA for the biomass of the plankton community in the mesocosms. Significant P values ($P < 0.05$) marked in italics

Parameters	Time (t)		Treatment (Z)		Interaction ($t \times Z$)	
	F	P	F	P	F	P
Cyanophyceae	12.37	*< 0.001*	34.38	*< 0.001*	5.95	*< 0.001*
Cryptophyceae	0.89	0.524	0.86	0.513	1.52	0.160
Chrysophyceae	1.98	0.168	8.38	*0.014*	2.14	*0.037*
Bacillariophyceae	8.34	*0.002*	18.94	*0.002*	8.44	*< 0.001*
Chlorophyceae	3.70	*0.037*	3.45	0.092	1.46	0.184
Zygnematophycae	13.57	*< 0.001*	13.98	*0.004*	4.15	*< 0.001*
Euglenophyceae	0.76	0.597	1.17	0.396	0.98	0.501
R. raciborskii	12.40	*< 0.001*	34.79	*< 0.001*	5.98	*< 0.001*
Total phytoplankton	40.99	*< 0.001*	27.99	*< 0.001*	2.97	*< 0.001*
Chlorophyll-a	252.06	*< 0.001*	128.34	*< 0.001*	16.04	*< 0.001*
STXs	650.80	*< 0.001*	277.30	*< 0.001*	75.24	*< 0.001*
Daphnia	43.71	*< 0.001*	42.32	*< 0.001*	9.78	*< 0.001*
Rotifers	74.80	*< 0.001*	10.32	*0.009*	9.45	*< 0.001*
Total zooplankton	78.04	*< 0.001*	38.75	*0.044*	11.03	0.710

of chlorophytes. Control mesocosm differed from CYL treatments until day 10, and after that, there were no significant differences between control and treatments as well as between treatments. Cyanobacterial chlorophyll-a concentration showed a similar growth pattern to the cell biomass of *R. raciborskii* (Fig. 3d). In spite of the absence of *R. raciborskii* in the controls, other cyanobacteria (mainly *Pseudanabaena catenata*) were present but with a significantly lower Chl-a concentration than the treatments with CYL. Except for day 3, there were no significant differences in cyanobacterial biomass among treatments with CYL until day 10. On day 13, however, there was a significant reduction (~ 50%) in the CYL + 400 *Daphnia* (20 ind. L^{-1}) treatment relative to CYL treatment without *Daphnia*. At the end of the experiment, cyanobacterial Chl-*a* had a drastic decrease in all treatments, except in CYL + 400 *Daphnia*, which maintained about the same level as the previous date, although there were no differences between treatments.

Only saxitoxin (STX) variant was found in the phytoplankton samples. During the experiment, a steady increase of saxitoxin (STX) was observed in the treatments that received CYL until day 10, having a drastic decrease on days 13 and 17 of the experiment (Fig. 4). There was an effect of time (t), treatment (Z), and time vs. treatment ($t \times Z$; Table 2). Control had undetectable STX, as expected, once there was no *R. raciborskii* (CYL). Also, there was a significant increase in STX from day 3 in the treatment with CYL + 400 *Daphnia* (Fig. 3). After ten days, all treatments reached the highest STX concentrations, and subsequently, there was a significant decay of toxins in all treatments, reflecting the reduction of cyanobacterial biomass (Fig. 3b).

Zooplankton community

The zooplankton community was exclusively dominated by rotifers in the control and in CYL treatments throughout the whole period of the experiment and until the addition of *Daphnia* in the other treatments (CYL + 200 *Daphnia* and CYL + 400 *Daphnia*) (Fig. 5). Cladocerans were only registered after introduction of *D. laevis* to the treatments CYL + 200 *Daphnia* and CYL + 400 *Daphnia* on the 7th day. There were no records of copepods in the mesocosms during the period of the experiment. After the addition of *Daphnia*, the zooplankton biomass on days 7 and 10 was composed mostly by *Daphnia* (~ 93 and 58%, respectively), being overpassed by rotifers biomass after that (~ 72 and 92%, at days 13 and 17, respectively). The species of rotifers that contributed

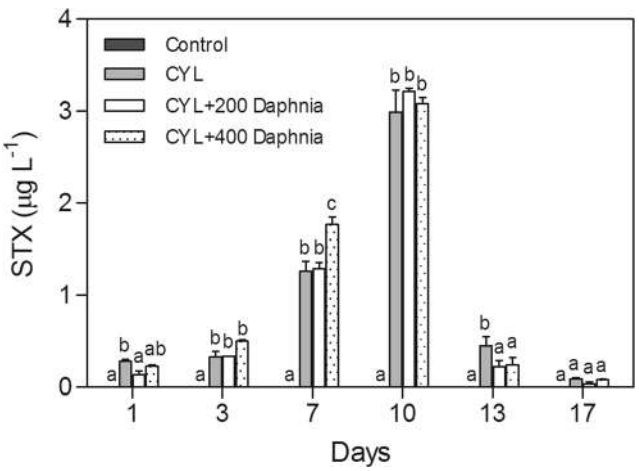

Fig. 4 Mean values (± standard error) of cell-bound saxitoxin (STX) concentration in control and treatments during the experiment

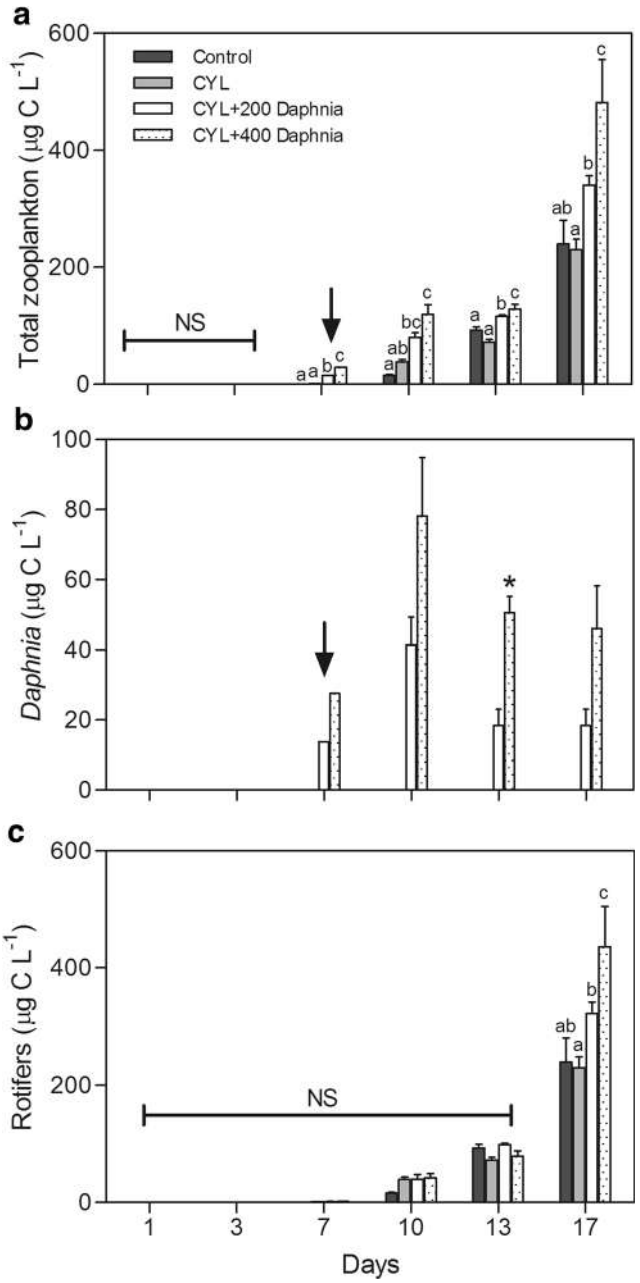

Fig. 5 Mean values (± standard error) of the zooplankton biomass (µg C L^{-1}): **a** total zooplankton, **b** *Daphnia*, and **c** rotifers in control and treatments during the period of the experiment. NS nonsignificant. Different letters and asterisk indicate significant differences. Setae indicate the day of the *Daphnia* addition

mostly to the total biomass (relative contribution > 50% on at least one of the experiment days) were: *Bdelloidea* sp., *Brachionus angularis*, and *Trichocerca* sp.

Total zooplankton biomass, as well as that of rotifers and *Daphnia*, varied significantly with time (t) and treatments (Z) and had a time vs. treatment interaction ($t \times Z$; Table 2). From de first day to the 7th day of the experiment (before *Daphnia* addition), zooplankton abundance were extremely low in the mesocosms (barely detectable), with rotifers being the only ones found, with densities ranging from 0 to 80 individuals L^{-1} (data not shown). There were no significant differences in the total zooplankton biomass between control and treatments, as well as between treatments on the first and third day of experiment. From the 7th day of the experiment, there was a considerable increase of total zooplankton biomass in the control and treatments (Fig. 5a), especially in the treatments that received *Daphnia*, which not only differed between each other but also presented significantly higher total zooplankton biomass ($P < 0.05$) than those recorded in CYL and in control. No significant differences were observed between control and CYL.

From the 7th to the 13rd day, *Daphnia* biomass increased and them decreased, staying relatively constant from day 13 to day 17 (Fig. 5b). Although average values were higher in the CYL + 400 *Daphnia* than in the CYL + 200 *Daphnia*, the only significant difference was found on the 13th day.

There were no significant differences ($P > 0.05$) in rotifer biomass between control and treatments and between treatments from the beginning to the 13th day of experiment (Fig. 5c). However, on the 17th day of the experiment, it was verified that the treatments with addition of *Daphnia* (CYL + 200 *Daphnia* and CYL + 400 *Daphnia*) differed significantly not only between each other but also from CYL and control ($P < 0.05$), by the largest rotifer biomass observed in those treatments.

Discussion

According to our first hypothesis, we predicted that if *Daphnia* can negatively affect cyanobacteria by herbivory pressure, there will be a significant decrease of *R. raciborskii* biomass in the treatments with *Daphnia* addition. Results on day 13 showed that the addition of *Daphnia* at a density of 20 individuals L^{-1} (CYL + 400 *Daphnia*) resulted in a drastic and significant reduction in *R. raciborskii* biomass (~ 71%) compared with the treatment without *Daphnia*. The second hypothesis predicted that nutrient availability would not differ between treatments with or without *Daphnia*. No significant differences were observed in the concentrations of NO_3^- and SRP between treatments with or without *Daphnia* during the whole period of the experiment. In addition, DIN:SRP ratios showed small variation until the 10th day and an elevation on day 13, although concentrations of NO_3^- and SRP indicated no signs of N or P limitation (Davidson et al. 2012). This is in agreement with our predictions and suggests a possible control of *R. raciborskii* biomass by higher densities of *D. laevis*. The effect of *Daphnia* herbivory on *R. raciborskii*, however, took about 1 week to show up since its introduction. On day 10, 3 days after its introduction, there was an increase in *Daphnia* biomass (more than double); however, it was not sufficient to cause a significant effect on *R. raciborskii* biomass. This delay is probably

related to high growth rate of the cyanobacterium, which was likely higher than the clearance rate of *Daphnia*. Likewise, the treatment of 10 *Daphnia* L^{-1} (CYL + 200 *Daphnia*) was not able to significantly reduce the cyanobacterial biomass.

Cladocerans of the genus *Daphnia* are considered key grazers in freshwater ecosystems, preying on phytoplankton populations and contributing to the recycling of nutrients (Bruce et al. 2006; Lacerot et al. 2013). They have high demand for P and must regulate P uptake from the diet by a stoichiometric mechanism in which the excess of P is excreted in order to maintain elemental (C, N, and P) homeostasis (DeMott 1998; Anderson et al. 2005). On the other hand, under P-limitation, animals must both maximize P uptake and excrete the excess C or to reduce C assimilation (Darchambeau et al. 2003; Anderson et al. 2005). Some studies have also shown that nutrients supplied by fish and zooplankton may affect the structure and dynamics of phytoplankton communities (Vanni 2002; Lacerot et al. 2013; Silva et al. 2014). As differences in nutrient supply rates and ratios can strongly affect the structure of phytoplankton communities, it has been hypothesized that zooplankton and fish might affect phytoplankton community structure by recycling nutrients at different rates and ratios (Danger et al. 2009; Sardans et al. 2012). Attayde and Hansson (1999), for instance, showed that nutrient supply by both fish and *Daphnia* reduced species richness and diversity of phytoplankton communities and increased algal biomass and dominance. However, nutrient recycling by fish supported a more diverse phytoplankton community than nutrient recycling by *Daphnia*. Both predation by planktivorous fish and zooplankton and N and P recycling might play an important role in phytoplankton structure. However, the morphology and ability of producing toxins, along with a high growth rate under nutrient-rich conditions, are traits that support cyanobacterial capacity of thriving grazing pressure and its dominance in phytoplankton community, respectively. Consequently, it causes a loss of diversity due to an increased cyanobacterial relative abundance. In our study, however, no significant differences were observed in the concentrations of NO_3^- and SRP between CYL treatments with or without *Daphnia* during the whole period of the experiment. Also, there were no significant differences in DIN:SRP ratio between treatments with or without *Daphnia*, indicating that P reduction was solely a result of the increase in phytoplanktonic biomass, not an effect of *Daphnia* of fish recycling. This fact suggests that it is unlikely that nutrient recycling by *Daphnia* or fish has played an important role in the mesocosms.

Planktivorous fish can exert an important predation pressure on large zooplankton such as *Daphnia*, altering the structure of plankton communities (Attayde and Hansson 1999; Attayde and Menezes 2008; Lacerot et al. 2013). In the presence of zooplanktivorous fish, top-down cascading effects are expected on phytoplankton communities, as an increase in phytoplankton biomass (Carpenter et al. 1985; McQueen 1998). The fish *Astyanax* sp. added in the CYL + 400 *Daphnia* treatment is abundant in Camorim reservoir. Although analysis of stomach contents of *Astyanax* sp. specimens in Camorim reservoir indicated that their diet is basically composed of periphyton, insect larvae, and detritus (unpublished data), Arcifa et al. (1991) have verified that two species of *Astyanax* (*A. fasciatus* and *A. bimaculatus*) can also feed on zooplankton, especially on cladocerans (*D. gessneri* and *Moina micrura*). Therefore, in the mesocosms that received fish, it is likely that *Daphnia* was preyed upon. However, on day 17 (the only day that the effect of fish predation could be analyzed), *Daphnia* biomass did not differ significantly in fish and fishless mesocosms, being also about the same as in the previous sampling date (day 13), which indicates that fish predation on *Daphnia* was not effective along these 4 days. As these fish are visual predators, turbidity in the mesocosms may have not allowed them to find the daphnids. Also, at the end of the experiment, two treatments that received *R. raciborskii* (CYL and CYL + 200 *Daphnia*), but did not received fish, suffered a great change in phytoplankton composition, being dominated by chlorophytes, while the treatment that received fish (CYL + 400 *Daphnia*) still had a high biomass of the cyanobacterium.

The reduction of cyanobacterial biomass (μg Chl-*a* L^{-1}) (especially *R. raciborskii*) in all treatments on the 17th day, together with the greenish yellow color of the water and the flocculation of algal biomass in all mesocosms, suggests that the senescence of the bloom is probably related to nutrient depletion in phytoplankton. The great reduction of NO_3^- and SRP in all mesocosms, indicated by the strong negative correlation between nutrients concentration and phytoplankton biomass, and also the higher DIN:SRP at the end of the experiment, suggested P-limitation (Kosten et al. 2009). For this reason, the late addition of fish on day 13 was of limited utility and may be interpreted with caution. Fish added was supposed to feed on *Daphnia* and reduce its grazing pressure, leading to increased algal biomass. However, although there was still high biomass of *R. raciborskii* in the fish treatment (CYL + 400 *Daphnia*), it was not significantly different from fishless treatment (CYL + 200 *Daphnia*).

Our experimental design was limited to test the effects of different densities of *Daphnia* in the biomass of *R. raciborskii*; therefore, the mechanism by which *Daphnia* acted on this cyanobacterium remains unclear. The most obvious possibility is that *Daphnia* increased *R. raciborskii* mortality rates directly by herbivory. Although previous studies reported low intake of filamentous cyanobacteria by differently sized *Daphnia* such as *D. galeata mendotae* (size 0.6–1.3 mm, Schoenberg and Carlson 1984), *D. cucullata* (size 1.2 mm), *D. hyalina* (size 1.9 mm), and *D. pulicaria* (size 2.3 mm; Gliwicz and Lampert 1990), more recent ones indicated relatively high ingestion/clearance rates by daphnids and other

cladocerans such as *Ceriodaphnia cornuta* and *Moina micrura* (small and medium size, respectively; Kâ et al. 2012), *D. laevis* (size 1.6 mm), *D. similis* (size 2.5 mm; Ferrão-Filho et al. 2017), *D. longispina* (size 1.1 mm), and *D. pulicaria* (size 1.4 mm; Sikora and Dawidowicz 2017). Thus, it is possible that *Daphnia* reduced filamentous cyanobacteria by direct ingestion. In addition, when cyanobacterial filaments are abundant, daphnids increase the frequency of rejection movements by their post-abdominal claws (Gliwicz and Siedlar 1980), and this action may increase breakage and/or sedimentation rates of algal filaments that are caught but not ingested, or even facilitate their ingestion by small zooplankton (Dawidowicz 1990; Bouvy et al. 2001). Fabbro and Duivenvoorden (1996) observed herbivory of small filaments of *R. raciborskii* by two rotifers (*Brachionus calyciflorus* and *B. angularis*) after the larger filaments were broken by *Daphnia lumholtzi*. Fernández and Alcocer (2018) showed also that large zooplankton such as the ostracod *Heterocypris incongruens* can facilitate the ingestion of colonial cyanobacteria (*Microcystis* cf. *panniformis*) by the smaller cladoceran *Moina macrocopa*. Therefore, the consumption of cyanobacteria by small zooplankton may be a result of facilitation by larger organisms capable of breaking, ingesting, and digesting cyanobacterial colonies and filaments. In fact, in all mesocosms, rotifer community increased exponentially, surpassing *Daphnia* biomass along the experiments. Thus, it is plausible to infer that a decrease in cyanobacterial density may have been caused directly or indirectly by *D. laevis* either by its herbivory pressure or facilitation to other zooplankters such as rotifers, which lead to further reductions in the biomass of phytoplankton in the mesocosms with *Daphnia*.

Cyanobacteria may reduce zooplankton fitness either by production of toxic metabolites (Ferrão-Filho and Kozlowsky-Suzuki 2011) or by its low nutritional quality related to low manageability and digestibility (De Bernardi and Giussani 1990; Bednarska et al. 2014) and to the lack of essential polyunsaturated fatty acids (PUFAs), such as eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3), and low levels of linoleic acid (LIN, C18:2n-6) and α-linolenic acid (ALA, C18:3n-3), which make cyanobacteria a poor resource to support growth and reproduction of *Daphnia* and other cladocerans (Brett and Müller-Navarra 1997; DeMott and Müller-Navarra 1997; Wacker and Martin-Creuzburg 2007). Also, several cyanobacteria strains are reported to produce a series of secondary metabolites that are toxic or inhibitory to zooplankton, decreasing survivorship, growth, and reproduction of several zooplankton species (Ferrão-Filho and Kozlowsky-Suzuki 2011; Schwarzenberger et al. 2013). Thus, both toxicity and low nutritional value are considered the main reasons of the poor control of cyanobacteria by zooplankton, as cyanobacteria-dominated lakes are generally considered unable to support production by higher trophic levels (Müller-Navarra et al. 2000, 2004).

Feeding seems to be the most important rout of exposure to toxins in aquatic systems (Ibelings and Havens 2008). Therefore, it is likely that aquatic organisms are exposed to low levels of dissolved toxins in the natural environment, and thus direct uptake of dissolved toxins from the medium does not represent the typical and ecologically relevant exposure route of aquatic organisms (Ferrão-Filho and Kozlowsky-Suzuki 2011). Dissolved toxins released from the lysed cells could, however, also exert effects on zooplankton (Bownik 2010). Several strains of *R. raciborskii* have been reported to produce either saxitoxins or cylindrospermopsin (Piccini et al. 2013). From the toxin-producer strains isolated in Brazil up to date, all of them are saxitoxins (STXs) producers (Lagos et al. 1999; Molica et al. 2002; Ferrão-Filho et al. 2010; Mesquita et al. 2019). Brazilian STXs-producing (STX+) strains of *R. raciborskii* have been shown to cause negative effects on cladoceran species, such as temporary inhibition of the swimming movements (Ferrão-Filho et al. 2009, 2010, 2014a) and reductions in clearance rates and fitness (Soares et al. 2009; Costa et al. 2013; Ferrão-Filho et al. 2014b, 2017). As the strain CYLCAM-2 is able to produce STXs, this may have contributed to the absence of control of *R. raciborskii* in the treatment with low *Daphnia* density (10 ind. L^{-1}) and even to the late control in the treatment with high *Daphnia* density (20 ind. L^{-1}).

Biomanipulation attempts reported positive, negative, and null correlation between cyanobacteria and zooplankton abundance (Søndergaard et al. 2007). Matveev et al. (1994), however, reported that *Daphnia carinata* was successfully used in small mesocosm (4 L) experiments in Australian lakes and resulted in a decrease in colonial cyanobacteria (*Microcystis* sp.) and filamentous diatom (*Melosira granulata* = *Aulacoseira granulata*). In a series of incubation experiments (250 mL) in Lake Agawam (NY, USA), Gobler et al. (2007) showed that *D. pulex* was able to reduce the biomass of *Microcystis*, but only when the microcystin synthetase gene (*mcyE*) was not being expressed, during fall, suggesting that the ability of such zooplankton to actively graze upon *Microcystis* may be strongly influenced by active toxin synthesis. In larger mesocosm experiments (4200 L) in Michigan and Alabama lakes using *D. pulicaria* clones that showed to be resistant to toxic *Microcystis*, Chislock et al. (2013) found that daphnids (*D. pulicaria*) were able to suppress phytoplankton biomass by over 70–80% and reduce microcystin relative to controls with no *Daphnia*, indicating that the toxic *Microcystis* does not prevent strong control of phytoplankton biomass by *Daphnia* genotypes that are adapted to environments with abundant cyanobacteria and associated cyanotoxins. In grazing experiments (10 L) with water from the eutrophic Lake Ringsjön (Sweden), Urrutia-Cordero et al. (2016) showed that the large-bodied *Daphnia magna* were capable of suppressing the abundance of filamentous

cyanobacteria such as *Aphanizomenon*, *Dolichospermum*, and *Planktothrix*, besides colonial *Microcystis*. However, in July, when the community was dominated by the spiral filament-forming *Dolichospermum crassum*, *Daphnia* was not able to control the cyanobacterial biomass. Those authors showed also a good correlation with field data before and after biomanipulation in Lake Ringsjön (since 2005), as 19 years of lake monitoring data (1996–2014) revealed that reducing fish predation increased the mean abundance (50%) and body size (20%) of *Daphnia*, as well as decreased the total amount of nutrients and the growth of the dominant cyanobacterial taxa, *Microcystis* and *Planktothrix*. All those previous studies were carried out in temperate lakes, with large-bodied *Daphnia*.

Tropical aquatic ecosystems are, however, dominated by small cladocerans such as *Bosmina*, *Diaphanosoma*, *Ceriodaphnia,* and *Moina* (Sarma et al. 2005), as well as small *Daphnia* (e.g. *D. laevis*), cyclopoid, and calanoid copepods (Lazzaro 1997; Bouvy et al. 2001; Jeppesen et al. 2007). Using *in situ* mesocosms (50 L), Severiano et al. (2018) showed that phytoplankton populations of a tropical reservoir exposed to treatments having 1 (control), 2, 3, and 4 times the biomass of local zooplankton (mainly rotifers and small cladocerans and copepods) had no significant effect on filamentous cyanobacteria (*Planktothrix agardhii* and *Raphidiopsis raciborskii*). In contrast, the treatments with 3 and 4 times zooplankton biomass negatively affected other cyanobacteria such as *Aphanocapsa* sp., *Chroococcus* sp., *Dolichospermum* sp., *Merismopedia tenuissima*, *Microcystis aeruginosa*, and *Pseudanabaena* sp. Total microcystin concentration both increased and decreased at different times depending on zooplankton treatment, while saxitoxin level was not significantly different between the treatments and control. In our study, STXs concentrations have dropped in all treatments on day 13, irrespective if they received *Daphnia* or not, but it was significantly lower in the treatments with *Daphnia* and corresponding to the drastic reduction of *R. raciborskii* in the treatment with CYL + 400 *Daphnia*. Some of the decrease in STXs in the treatment without *Daphnia* could, however, be explained by the senescence of the bloom and cell lysis. Probably, nutrient limitation has imposed restrictions to STX production in the cells and was metabolized. Although we did not measure extracellular STX, it is well-known that this family of toxins undergoes chemical and biological transformations that depend on pH, with toxicity being more stable at acidic conditions (Vale et al. 2008; Roggatz et al. 2019). The pH in the CYL treatments varied between 8.4 and 10.0 from 10th to 17th day. Thus, it is likely that most of the STX released from cells were degraded or either converted to less toxic variants in the water.

Contrary to predictions for tropical systems (Lazzaro et al. 2003; Jeppesen et al. 2005, 2007; Lacerot et al. 2013), our study showed that ecologically relevant densities of *D. laevis* (20 individuals L^{-1}) were able to reduce drastically (~ 71%) the biomass of a saxitoxin producing *R. raciborskii* strain. This reduction might be due to both mechanisms, direct predation on cyanobacterial filaments by *D. laevis* and facilitation to other zooplankton such as rotifers. This is the first experimental evidence that a tropical clone of a small-bodied *Daphnia* may be able to control neurotoxic filamentous cyanobacteria in the field. In fact, laboratory studies already showed that this *D. laevis* clone is able to feed on *R. raciborskii* and to maintain positive population growth rates even on a diet of 75–100% cyanobacteria (Ferrão-Filho et al. 2017, 2019). This clone has been isolated from the eutrophic lake Ibirité (MG, Brazil), which is dominated by *Microcystis* spp. (Garcia et al. 2009). Probably, previous exposure of native populations to toxic cyanobacteria may have provided this clone more tolerance after some generations. Therefore, our results together with previous studies show that biomanipulation procedures such as fish addition/removal as well as stocking of larger planktonic herbivores such as *Daphnia* in eutrophic lakes may be effective, but not enough to control cyanobacterial blooms.

Conclusions

Although biomanipulation of the food chain has gained much attention in temperate region, little is known on the effectiveness of this practice in tropical ecosystems. This study provided some evidence that cyanobacterial control by small-bodied *Daphnia* is possible in the tropics, even neurotoxic ones such as *R. raciborskii*. However, even though our results could not demonstrate, lakes with high densities of planktivorous fish might have decreased effectiveness of *Daphnia* control. Thus, other measures such as selective fishing and *Daphnia* stocking, as well as programs for nutrients (N and P) removal from the watershed, are needed to improve water quality.

Acknowledgments We would like to thank the State water supply and wastewater company (CEDAE) by allowing us to carry out the experiment in their decantation tanks and for all logistic and analysis of nutrients. We thank also Prof. Heitor Evangelista from the State University of Rio de Janeiro for providing the mesocosms and Fabio Q. Abreu and Diego A. C. da Silva for the help with mounting and demounting of the mesocosms.

Funding information This study was supported by Oswaldo Cruz Foundation (PAEF no. IOC-008-FIO-04).

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Harmful Algae 97 (2020) 101870

Contents lists available at ScienceDirect

Harmful Algae

journal homepage: www.elsevier.com/locate/hal

Changes in pH and dissolved inorganic carbon in water affect the growth, saxitoxins production and toxicity of the cyanobacterium *Raphidiopsis raciborskii* ITEP-A1

Mauro Cesar Palmeira Vilar[a,1], Renato José Reis Molica[b,*]

[a] Graduate Program in Ecology, Biology Department, Rural Federal University of Pernambuco, Recife, PE, Brazil
[b] Academic Unit of Garanhuns, Rural Federal University of Pernambuco, Bom Pastor Avenue, Garanhuns, PE, 55292-270, Brazil

ARTICLE INFO

Keywords:
Carbon concentrating mechanism
Phytoplankton
Cyanotoxins

ABSTRACT

Raphidiopsis raciborskii is a widely distributed, potentially toxic cyanobacterium described as a tropical-subtropical species. However, its occurrence in temperate regions has been expanding. Understanding the environmental factors underlying the expansion and colonization success of *Raphidiopsis* has been the object of numerous studies. However, less is known regarding its responses to pH and inorganic carbon in water. Thus, the aim of the present study was to investigate the effects of changes in pH and dissolved inorganic carbon on growth and saxitoxins production in the strain *R. raciborskii* ITEP-A1. We incubated batch cultures with different unbuffered and buffered pH (neutral-acid and alkaline) and inorganic carbon availability (CO_2-rich air bubbling and the addition of $NaHCO_3$) to assess the effect of these factors on the growth, toxin production as well as saxitoxins composition of the cyanobacterium. The carbon concentrating mechanism (CCM) system of ITEP-A1 was also characterized by an *in silico* analysis of its previously sequenced genome. The growth and saxitoxins production of *R. raciborskii* were affected. The addition of sodium bicarbonate and air bubbling enhanced the growth of the cyanobacterium in alkaline pH. In contrast, saxitoxins production and relative toxicity were decreased. Moreover, significant changes in the cellular composition of saxitoxins were strongly related to pH changes. ITEP-A1 potentially expresses the low-flux bicarbonate transporter BicA, an efficient CCM which uptakes most of its carbon from HCO_3^-. Hence, increasing the diffusion of CO_2 in alkaline eutrophic lakes is likely to increase *R. raciborskii* dominance, but produce less toxic blooms.

1. Introduction

Eutrophication has promoted cyanobacterial blooms in waterbodies worldwide and recently these phenomena have been boosted by climate changes – intensified by greenhouse gas emissions (O'Neil et al., 2012; Huisman et al., 2018). According to the literature, atmospheric pCO_2 levels were 272 µatm in the pre-industrial age and reached 346 µatm in the 1980s (Hall, 1989). Moreover, carbon dioxide concentrations are expected to be threefold greater by the end of the 21st century than the current 409.95 µatm (Holland et al., 2012; IPCC, 2017; NOAA, 2019). Together with land use and increased eutrophication, such conditions promote the alkalization of lakes, altering the dynamics of pH and dissolved inorganic carbon (DIC) and changing relative proportions of CO_2, HCO_3^- and CO_3^{2-} in water, which is believed to play a role in the proliferation of cyanobacterial blooms (Shapiro, 1997; Verspagen et al., 2014; Kaushal et al., 2017; Raven et al., 2020).

Besides dense biomass accumulation, some cyanobacterial species are potential producers of bioactive secondary metabolites denominated cyanotoxins, the most potent of which are paralytic shellfish toxins (PSTs) or saxitoxins (STXs). STXs are a group of 57 guanidinium-containing neurotoxic alkaloids that block voltage-gated sodium and calcium channels and modulate the gating behavior of potassium channels in mammals (Cusick and Sayler, 2013; Wiese et al., 2010). These toxins are chemically divided into non-sulfated (e.g., saxitoxin), mono-sulfated (e.g., gonyautoxins) and di-sulfated (C-toxins). Moreover, each of these may be missing the carbamoyl moiety (dc-toxins). The level of toxicity of STXs depends on the degree of sulfation, with non-sulfated analogs being the most toxic (Wiese et al., 2010). Hence, since STX analogues display different toxicities depending on their functional groups, toxicity equivalent factors (TEFs) are addressed

* Corresponding author.
E-mail address: renato.molica@ufrpe.br (R.J.R. Molica).
[1] Current Address: Laboratory of Ecophysiology and Toxicology of Cyanobacteria, Carlos Chagas Filho Institute of Biophysics, Federal University of Rio de Janeiro, Rio de Janeiro, RJ 21949–902, 52171–900, Brazil. E-mail: maurovilar@gmail.com

https://doi.org/10.1016/j.hal.2020.101870
Received 21 November 2019; Received in revised form 17 June 2020; Accepted 30 June 2020

(Perez et al., 2011).

STXs are distinct from other cyanotoxins, as the producing organisms are found in two domains of life: eukaryotic dinoflagellates, in which are referred as paralytic shellfish toxins, and prokaryotic cyanobacteria (Wang et al., 2016). Although much is known regarding their pharmacology and chemistry, these toxins have rarely been studied in terms of metabolism or the physiology of STXs-producing cyanobacteria (Negri et al. 1997; Pomati et al., 2004), despite the diversity of freshwater toxic mat-forming and bloom-forming species (Humpage et al., 1994; Lagos et al., 1999; Foss et al., 2012; Soto-Liebe et al., 2012; Borges et al., 2015; Cirés and Ballot, 2016; Wood et al., 2020).

Among STX-producing cyanobacteria, *Raphidiopsis raciborskii* (Woloszynska) Aguilera, Berrendero Gómez, Kastovsky, Echenique & Salerno [basionym *Cylindrospermopsis raciborskii* (Woloszynska) Seenayya & Subba Raju] (Aguilera et al., 2018) is one of the most widespread due to its invasive (or opportunistic) nature. The occurrence of this cyanobacterium was initially reported for tropical and subtropical regions (Padisák, 1997), but its current distribution includes temperate regions, as various ecotypes are experiencing warmer and longer seasons as a consequence of climate change (Piccini et al., 2011; O'Neil et al., 2012). *R. raciborskii* is also of particular concern due to its potential for producing both neurotoxic and cytotoxic alkaloids (Dittmann et al., 2013; Burford et al., 2016). To date, however, STX-producing strains have only been reported for South America (Piccini et al., 2011; Antunes et al., 2015).

A remaining question is whether this diversity represents an adaptation process by *R. raciborskii* to environmental changes experienced in the recent past or the evolution of different ecotypes due to geographical separation, as it continues to adapt to changing climatic conditions (Vico et al., 2020; Ribeiro et al., 2020). Thus, efforts have been made to investigate cyanobacterial responses to temperature and nutrients (Paerl and Huisman 2008; Walls et al., 2018; Wagner et al., 2019). However, these factors can affect the pH/CO_2 equilibrium in water, the consequences of which on growth and cyanotoxin production are still incipient.

Investigations regarding the influence of the impact of pH and CO_2 on phytoplankton began with King's (1970) hypothesis [subsequently tested by Shapiro (1984, 1990, 1997)], which assumes that under an alkaline pH and low CO_2 cyanobacteria may dominate the phytoplankton. Subsequent studies showed that, besides species fluctuations driven by water chemistry, cyanobacteria may 'construct' its niche through the establishment of a bloom by decreasing dissolved CO_2 and increasing the pH (Ibelings and Maberly, 1998; Vespagen et al., 2014), shifting DIC speciation towards carbon sources suitable to the functioning of the carbon concentrating mechanism (CCM), which is a possible driving agent of species succession (Lines and Beardall, 2018).

Different CCMs are identified among species and strains of phytoplankton (Badger and Price, 2003; Sandrini et al., 2013), which evolved as a system to make ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCo) more efficient by elevating the level of CO_2 near the active site of the enzyme to provide a balance between the processes of respiration and photosynthesis (Tomar et al., 2017). According to Beardall and Raven (2017), freshwater cyanobacteria usually have a RuBisCo with high half-saturation-concentrations for CO_2 ($K_{1/2}$ [CO_2]) and maximum uptake rate (V_{max} [CO_2]), and low relative specificity of Rubisco activity for CO_2/O_2 (S_{rel}), which means that its kinetic properties involve a low affinity for CO_2, operating below maximum capacity when internal CO_2 is in equilibrium with or lower than external CO_2.

Five different inorganic carbon uptake systems have been identified in cyanobacteria: two for CO_2 uptake ($NDH1_3$ and $NDH1_4$) and three for bicarbonate uptake (BCT1, BicA and SbtA) (Price et al., 2008). The latter two bicarbonate uptake systems (BicA and SbtA) are sodium-dependent symporters that respectively have a low affinity for bicarbonate and high flux rate and a high affinity for bicarbonate and low flux rate (Sandrini et al., 2016). Already in the 1980s, it was assumed that sodium was an important ion for cyanobacterial photosynthetic performance and, consequently, growth (Miller et al., 1984; Espie et al., 1988). This was subsequently related to bicarbonate sodium-dependent transporters (Price et al., 2008). Different cyanobacterial strains can express these carbon uptake systems in different ways to ensure carbon fixation rates in the occurrence of environmental changes in the availability of inorganic carbon (Price et al., 2008; Sandrini et al., 2016).

Most studies have focused on a single environmental variable. However, as variables affected by climate changes occur simultaneously with others, it is important to consider how their interactions drive harmful cyanobacteria and toxin production in aquatic ecosystems (O'Neil et al., 2012). Although pH is directly linked to the speciation of dissolved inorganic carbon, little is known regarding how the balance between pH and CO_2 in water impacts toxic cyanobacteria and the occurrence of blooms (Raven et al., 2020). Thus, we investigated the eco-physiological responses of a toxic cyanobacterium incubated in different pH/CO_2 scenarios. The aim of this study was to detect early responses to changes in pH and DIC on a saxitoxins-producing strain of *R. raciborskii*. Thus, we tested the hypothesis that *R. raciborskii* is a bicarbonate user able to produce considerable biomass at a high pH associated to a high HCO_3^- environment. Moreover, we hypothesized that pH/DIC variations directly affect saxitoxins production as well as STX analogues composition so that under low pH the cyanobacterium increases its toxicity.

2. Material and methods

2.1. Culture conditions and experimental design

The STX-producing ITEP-A1 strain of *Raphidiopsis raciborskii* was isolated from the Riacho dos Paus reservoir in the state of Pernambuco (Northeastern Brazil). *R. raciborskii* ITEP-A1 produces neosaxitoxin, decarbamoyl-saxitoxin and saxitoxin (neoSTX, dcSTX and STX, respectively). To date, no gonyautoxins have been detected in this strain (data not shown). Cultures were grown in 2-liter Erlenmeyer flasks in sterilized liquid ASM-1 medium (Gorham et al., 1964) at 24 ± 1 °C with 80 μmol photons (PAR) $m^{-2} s^{-1}$ and a 12-h light/dark cycle.

We used a factorial (3 × 2 × 2) experimental design with twelve treatments consisting of different combinations of pH and sources of DIC: unbuffered (initial pH 7.5) and buffered medium (pH 6.8 and 8.2) maintained with a buffer solution of 40.14 mM 2-[4-(2-hydroxyethyl) piperazin-1-yl] ethanesulfonic acid (HEPES, Sigma-Aldrich®) and adjusted with the addition of either HCl 1 M or NaOH 1 M combined with filtered air bubbling (~0.041% CO_2, air level) and without air bubbling. The aeration system consisted of a glass pipette immersed in the culture medium which passed through the erlenmeyer's hydrophobic cotton plug connected to a silicone hose, which in turn was attached to a 5 mL syringe filled with hydrophobic cotton, through which the compressed air passed. The aeration system was autoclaved assembled in the 2-L Erlenmeyer's. Also, there were treatments with the addition of sodium bicarbonate (0.2 mM $NaHCO_3$). Cultures without air bubbling were manually shaken two or three times daily during the 12 days of incubation. All experimental treatments were conducted in triplicate.

2.2. Biovolume and growth variables

Culture samples were preserved with 1% acetic Lugol's solution and trichome density (trichomes L^{-1}) was measured every two days using a Fuchs-Rosenthal chamber under optic microscope (AxioScope A1 and AxioVision 4.7.2 software, Zeiss). As cells were difficult to visualize in ITEP-A1, mean trichome volume was calculated according to its respective geometric form and together with density used to estimate biovolume ($mm^3 L^{-1}$) (Hillebrand et al. 1999; Sun and Liu, 2003). The net growth rate (μ) was calculated as treatment-specific over the time

interval in which the biovolume doubled (log-phase), according to the following equation: $\mu = (\ln B_{t2} - \ln B_{t1})/t_2\text{-}t_1$, in which Bt_2 and Bt_1 are the biovolume at times t_2 and t_1, respectively (Fogg and Thake, 1987).

2.3. Water chemistry measurements

pH and total alkalinity (mEq L^{-1}) were measured in unbuffered cultures using a pH meter (model pH 200, Marconi) and the titration method [Talling (1973) adapted by Gran (1952)], respectively. This consists of titrant volumes of standardized acid (H_2SO_4, sulfuric acid 0.1 N) required to reduce the pH of the medium to the range of 4.0 to 3.7. Alkalinity and pH were used as parameters to assess the carbonate system dynamics ($CO_{2(free)} \rightarrow HCO_3^- \rightarrow CO_3^{2-}$) as well as cation-anion balance in water. Water conductivity (μS cm^{-1}) and temperature (°C) were measured using a conductometer (Model CD-4303, Lutron).

2.4. Saxitoxins analysis and relative toxicity – high-performance liquid chromatography with fluorescence detection (HPLC-FLD)

Culture samples were withdrawn at the end of the experiments (early stationary growth phase), freeze-dried and subsequently submitted to saxitoxins extraction using 0.5 M acetic acid. After centrifugation (22,000x *g*), the supernatant was collected and filtered through a 0.22-μm Millex filter (Millipore) prior to injection. Chromatographic analyses were performed with a Shimadzu HPLC system using a silica-base reverse phase column (125 mm x 4.0 mm, 5 μm; C18 Phenomenex). Separations were performed in specific mobile phases for non-sulfated saxitoxins (STX, neoSTX and dcSTX). Post-column oxidation was performed as described in Oshima (1995). Fluorescent saxitoxins derivates were detected using a Shimadzu RF-10Axl fluorometric detector with excitation at 330 nm and emission at 390 nm. Toxins were identified and quantified by comparison with known retention times and integrated areas of standards, respectively. Saxitoxins standards were purchased from the Institute for Marine Bioscience, National Research Council of Canada (Halifax, Canada).

The data were grouped into total amount of saxitoxins (neoSTX + dcSTX + STX) and expressed as total saxitoxin content per biovolume unit ($ngSTX_{total}/mm^3$). Total relative toxicity (saxitoxin toxicity equivalence, STX_{eq}) was expressed as $ngSTX_{eq}/mm^3$ according to the toxicity equivalency factor (TEF) described for each saxitoxin analog in FAO/WHO (2016) and using the following equation:

$$STXeq = \sum_{i=1}^{n} (C_i \; x \; TEF_i)$$

in which C_i is the concentration of the individual toxin analog and its assigned TEF_i.

2.5. Bioinformatic analysis of CCM genes

The *R. raciborskii* ITEP-A1 genome shotgun sequence (Lorenzi et al., 2016) available in GenBank was investigated for the identification of carbon concentrating mechanism genes. Sequence similarities were checked for the predicted *BicA, Sbt1, BCT1,* carbonic anhydrases, *ccmM* and *NDH1* genes. To investigate the presence of these genes in ITEP-A1, we retrieved and analyzed raw genomic contigs through BLASTN (nucleotide sequence against nucleotide database) searches using *R. raciborskii* ITEP-A1 in the NCBI genome database, which is deposited in the DDBJ/ENA/GenBank under accession number LUBZ00000000. To confirm the identity of the proteins, a TBLASTN (amino acid sequence against nucleotide database) search was performed, with comparisons to the Uniprot database.

2.6. Statistical analysis

$Log_{(x+1)}$ transformed data on growth rate, toxin production and toxicity were tested for normality and equality of variances. The data were grouped into condition (air bubbling vs. no air bubbling) x treatment (bicarbonate vs. no bicarbonate) x pH level (unbuffered vs. buffered cultures) and submitted to parametric three-way ANOVA with either Tukey's HSD test or the Holm-Sidak post-hoc test. Growth curves and pH/inorganic carbon dynamics were assessed using one-way repeated-measures ANOVA with Tukey's HSD test. The statistical analyses were performed using the SigmaPlot 12.0 and graphs were created in GraphPad Prism 6.

3. Results

3.1. R. raciborskii growth, CCM genes and toxicity

Significant differences in the growth of *R. raciborskii* ITEP-A1 occurred throughout the twelve days of incubation under different pH and DIC conditions (RM ANOVA, $p < 0.001$). Air bubbling promoted a greater acquisition of biomass. However, from the 6th day onwards, non-aerated bicarbonate-rich cultures kept in unbuffered pH grew faster and reached a significantly greater biovolume than the other bicarbonate-rich and non-enriched treatments (air bubbling, RM ANOVA, $F_{(30,72)} = 16.17$, $p < 0.0001$) (**Fig. 1**). Moreover, even without air bubbling, bicarbonate-rich pH 8.2 buffered-cultures also exhibited significant growth (no air bubbling, RM ANOVA, $F_{(30,72)} = 5.629$, $p < 0.0001$) (**Fig. 1**).

The net growth rate of *R. raciborskii* was significantly improved with air bubbling (ANOVA, $F_{(1,24)} = 44.215$, $p < 0.001$). However, with the exception of aerated unbuffered medium, the addition of bicarbonate had no significant effect on the interaction with the other pH conditions in both air bubbling treatments (**Fig. 2**; **Table 1**). Under the different

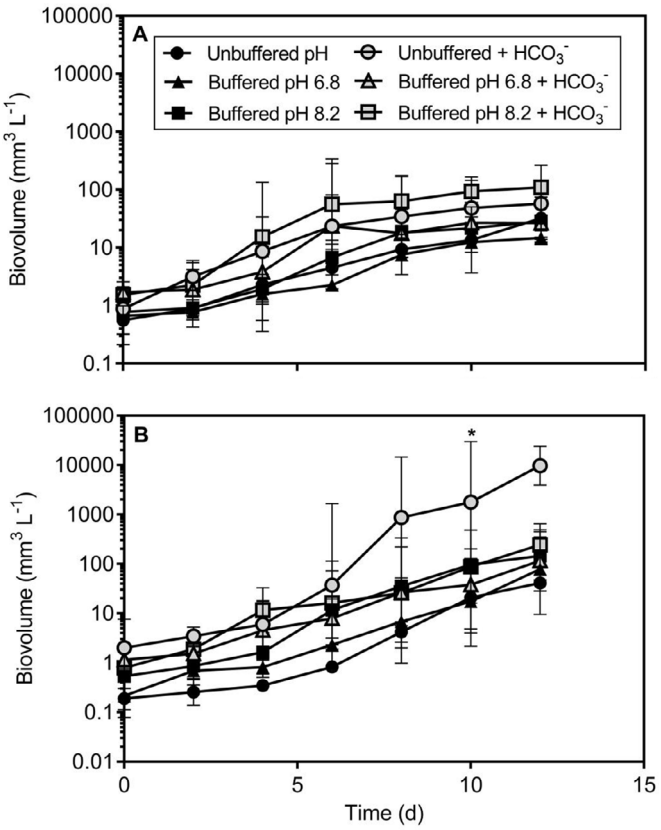

Fig. 1. *Raphidiopsis raciborskii* ITEP-A1 growth curves without **(A)** and with **(B)** air bubbling **(0.041% CO_2-rich air bubbling)** under different pH **(unbuffered; buffered 6.8 and 8.2)** and sodium bicarbonate supplementation **(HCO_3)**. (*) = $p < 0.05$; Tukey HDS Test.

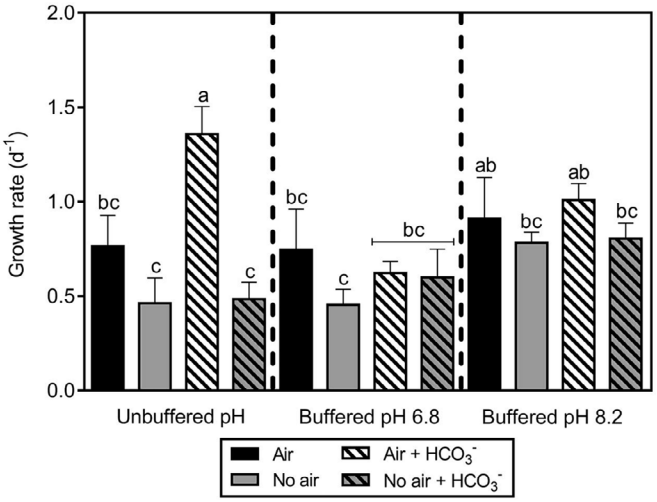

Fig. 2. Net growth rate of *Raphidiopsis raciborskii* ITEP-A1 culture grew with **(hatched column)** and without **(non-hatched column)** bicarbonate **($NaHCO_3$)** supplementation under different pH **(unbuffered; buffered 6.8 and 8.2)** and with/without air bubbling **(0.041% CO_2-rich air bubbling)**. Different letters mean significant diferences (Holm Sidak's multiple comparisons Test; $p < 0.001$).

Table 1
Results of the Three-way ANOVA for differences in growth rate of *R. raciborskii* ITEP-A1 under different pH/DIC conditions. ns: No significant differences.

Growth rate	SS	Df	MS	F	*P*
Air bubbling	0.0491	1	0.0491	44.215	<**0.001**
pH	0.0283	2	0.0141	12.735	<**0.001**
Bicarbonate ($NaHCO_3$)	0.00739	1	0.00739	6.661	<**0.05**
Air bubbling x pH	0.0205	2	0.0103	9.251	<**0.01**
Air bubbling x Bicarbonate	0.00111	1	0.00111	0.997	ns
pH x Bicarbonate	0.00663	2	0.00331	2.984	ns
Air bubbling x Bicarbonate x pH	0.0135	2	0.00677	6.100	<**0.01**
Residual	0.0266	24	0.00111		
Total	0.153	35	0.00438		

culture conditions, the growth rate ranged from 0.45 ± 0.08 d^{-1} to 1.22 ± 0.27 d^{-1}. Conversely, despite of bicarbonate supplementation and air bubbling, when kept under a buffered pH 6.8 the cyanobacterium was not able to enhance its growth, yet **(Fig. 2)**.

The CCM-gene survey and protein identification in the whole genome shotgun sequence of *R. raciborskii* ITEP-A1 revealed high identity and similarity with the genes *BicA, βCA, ccmM*, $NDH1_3$ and $NDH1_4$ **(Table 2)**. BLAST searches against the NCBI database with the contig 2 NZ_LUBZ01000002.1 yielded *Synechococcus* sp. *BicA* with 64% identity and 86% similarity (*E*-value = 0), confirming the sequence aligned in NZ_LUBZ01000002.1 as the low-affinity/high-flux bicarbonate uptake system BicA in *R. raciborskii* ITEP-A1. The same was seen with contig 1, which yielded *Mycobacterium tuberculosis* ATCC 25,618 βCA (beta carbonic anhydrase), with 43% identity and 88% similarity (*E*-value = 0), and contig 8, which yielded *Synechococcus elongatus* PCC 7942 ccmM (carbon dioxide concentrating mechanism protein), with 45% identity and 98% similarity (*E*-value = 0), confirming these proteins in *R. raciborskii* ITEP-A1 and supporting its performance under bicarbonate-rich conditions. Moreover, *S. elongatus* PCC 7942 NDH1 protein subunits (NdhF3/NdhD3/chpY and NdhF4/NdhD4/chpX protein complexes) were identified in the ITEP-A1 genome, with a similarity of >80% **(Table 2)**.

Total volumetric saxitoxins (STX_{total}) content in the *R. raciborskii* cultures incubated under different pH/CO_2 conditions ranged widely from 0.051 ± 0.01 to 151.98 ± 30.20 μg L^{-1} (data not shown). Changes in saxitoxins production were assessed in terms of total intracellular toxin content by normalizing it as the biovolume content ($ngSTX_{total}/mm^3$). Air bubbling (ANOVA, $F_{(2,18)} = 2.09$, $p < 0.05$) and sodium bicarbonate (ANOVA, $F_{(1,18)} = 214.07$, $p < 0.001$) exerted significant effects on total saxitoxins production **(Fig. 3, Table 3)**. The addition of bicarbonate promoted a significant decrease in the STX_{total} content, especially under air bubbling conditions, which showed a relative reduction of 99.94 ± 0.01% and 98.77 ± 0.27% in the unbuffered and buffered pH 6.8 treatments, respectively. Without air bubbling, the reduction was only significant in cultures kept at pH 6.8 (82.50 ± 0.01%), whereas no significant changes in toxin production or relative toxicity were found in the treatments with pH 8.2 **(Fig. 3)**.

The cellular composition of saxitoxins changed significantly in response to pH and air bubbling in both bicarbonate-rich ($F_{(5,36)} = 21.85$; $p < 0.01$) and non-enriched ($F_{(5,36)} = 14.52$; $p < 0.01$) cultures. Besides the differences in the total amount of saxitoxins, a proportional increase in saxitoxins analogues was found under unbuffered and buffered 6.8 pH **(Fig. 4)**, which resulted in a significative increase in toxicity ($F_{(5,24)} = 18.25$; $p < 0.0001$) **(Fig. 3B)**. In contrast, at pH 8.2, the relative contribution of dc-STX increased significantly in the absence of sodium bicarbonate (air bubbling = 72.08 ± 7.58%; no air bubbling = 69.55 ± 22.81%) but decreased about 28% when bicarbonate was added to the medium **(Fig. 4)**. With buffered alkaline pH, the relative toxicity of *R. raciborskii* ITEP-A1 decreased due to the increase in the relative amount of the less toxic analogue dcSTX (39–87%) **(Fig. 3 and 4)**.

3.2. Water chemistry

During the experiments, increases in pH ($F_{(21,56)} = 5.91$, $p < 0.01$) and alkalinity ($F_{(18,48)} = 21.86$; $p < 0.01$) were found in the unbuffered pH treatments, especially in the bicarbonate-rich medium with air bubbling **(Fig. 5)**, which promoted an increase near two-fold in alkalinity (~1.7 mEq L^{-1}) in comparison with the aerated. As expected, no changes in pH was found in the buffered culture media (pH 6.8 and 8.2). Moreover, water conductivity in our study has ranged from 0.0406 - 0.1547 S m^{-1} **(Supplementary Table 2)**.

4. Discussion

Growth, carbon concentrating mechanism and saxitoxins production

Overall, the cyanobacterium grew in the different conditions displaying no signals of inhibition, therefore, comparations were based on the maximum growth achieved in the experimental set-up. In this study, changes in the pCO_2 level of water represented indirectly by pH and directly by air bubbling (air level CO_2) and the addition of sodium bicarbonate, influenced growth and toxin production in *R. raciborskii* ITEP-A1 (hereafter ITEP-A1).

The net growth rate of ITEP-A1 was significantly enhanced in unbuffered bicarbonate-rich medium with aeration, followed by those cultures kept under a buffered pH 8.2. Indeed, bicarbonate promoted a mean increase of 42.36 ± 18.01% in ITEP-A1 net growth rate. These findings reinforce the study of Brito et al. (2018) who found greater biomass yields and net growth rates to this cyanobacterium promoted by both air bubbling and bicarbonate supplementation, even after successive re-inoculum. Bicarbonate promoted a higher pCO_2 environment and thereby an additional DIC source available for photosynthetic carbon metabolism. Moreover, besides improving CO_2 diffusion into the culture medium, aeration also enabled the cyanobacterium to broaden its surface of contact with nutrients and light by reducing self-shading. Other bloom-forming species, such as *Microcystis aeruginosa*, have also been shown to perform better with air bubbling, exhibiting an increase in both inorganic carbon acquisition and growth rate (Qiu and Gao, 2002). In addition, buffered medium (pH 8.2) displayed an increased conductivity **(Supplementary Table 2)** which also has been pointed to benefit ITEP-A1 growth (see Lima et al., 2020).

Table 2
Identity and similarity percentage BLASTN of BicA; SbtA; BCT1; α, β and γ carbonic anhydrase and carbon dioxide concentrating mechanism protein genes available in database with signficants alignments to *Raphidiopsis raciborskii* ITEP-A1 whole shotgun genome. Similarity <40% means a low possibility of the gene presence in genome. Highlighted (bold) values represent a significative match among sequences alignment. NdhF3/NdhD3/cupA and NdhF4/NdhD4/cupB protein complexes are referred to NDH-1_3 and NDH-1_4, respectively.

Alignments	Gene	Indentity	Query Cover (similatity)	Location in whole genome shotgun sequence
Synecochoccus sp. PCC 7002 with ITEP-A1	BicA	**64%**	**86%**	contig 2
Microcystis aeruginosa HUB524 with ITEP-A1	SbtA	47%	8%	contig 1
M. aeruginosa UvA V145 with ITEP-A1	SbtA	47%	8%	contig 1
Synechococcus sp. PCC 7002 with ITEP-A1	SbtA	30%	13%	contig 91
M. aeruginosa UTEX LB 2388 with ITEP-A1	SbtA	47%	8%	contig 1
Arthrospira platensis AGB-AP02 with ITEP-A1	SbtA	28%	20%	contig 5
Synechococcus sp. PCC 6301 with ITEP-A1	SbtA	29%	33%	contig 12
Aureococcus anophagefferens with ITEP-A1	BCT1	28%	21%	contig 1
Nostoc sp. PCC 7120 with ITEP-A1	α CA	42%	13%	contig 1
Mycobacterium tuberculosis ATCC 25,618 with ITEP-A1	β CA	43%	**88%**	contig 1
Arabidopsis thaliana with ITEP-A1	γ CA3	38%	66%	contig 7
Synechococcus elongatus PCC 7942 with ITEP-A1	ccmM	45%	**98%**	contig 8
S. elongatus PCC 7942 with ITEP-A1	ndhF3	38%	**95%**	contig 8
S.elongatus PCC 7942 with ITEP-A1	ndhD3	38%	**83%**	contig 8
S. elongatus PCC 7942 with ITEP-A1	cupA	40%	**93%**	contig 8
S. elongatus PCC 7942 with ITEP-A1	ndhF4	**53%**	**95%**	contig 8
S. elongatus PCC 7942 with ITEP-A1	ndhD4	**54%**	**95%**	contig 8
S. elongatus PCC 7942 with ITEP-A1	cupB	**62%**	**91%**	contig 8

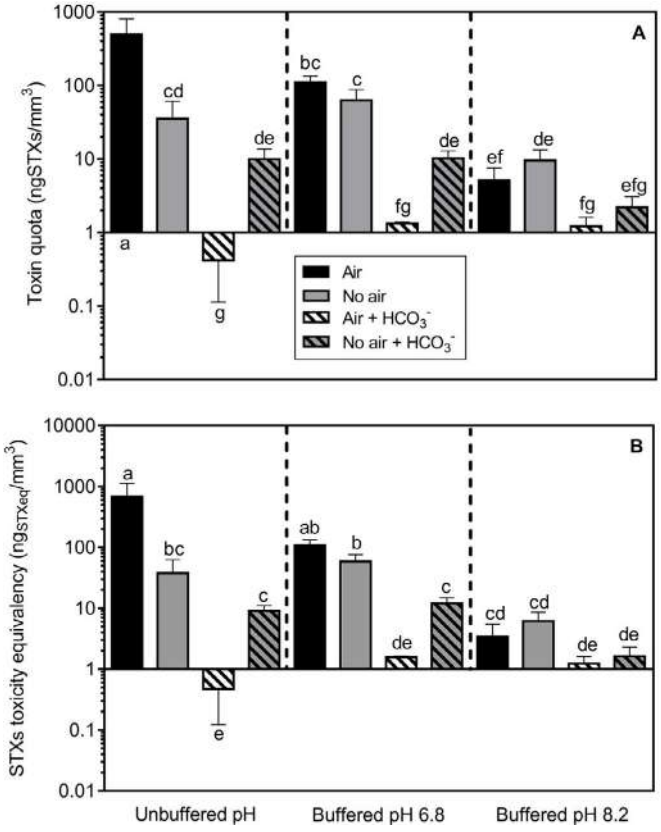

Fig. 3. Total saxitoxins quota per biovolume **(A)** and saxitoxins toxicity equivalency **(B)** with **(hatched column)** and without **(non-hatched column)** bicarbonate **($NaHCO_3$)** supplementation under different pH **(unbuffered; buffered 6.8 and 8.2)** and with/without air bubbling **(0.041% CO_2-rich air bubbling)**. Different letters mean significant diferences (Holm Sidak's multiple comparisons Test; $p < 0.001$).

On the other hand, under buffered pH 6.8 ITEP-A1 did not achieve a higher growth. There is an interdependence between the pH and DIC so that at pH 5 – 7 free-CO_2 has a higher proportion relative to bicarbonate which becomes only the significant DIC species at about pH 8.3 (Boyd, 2000). It is likely that somehow the cyanobacterium could

Table 3
Results of the Three-way ANOVA for differences in saxitoxins$_{total}$ quota/biovolume of *R. raciborskii* ITEP-A1 under different pH/DIC conditions. ns: No significant differences.

Saxitoxins quota per biovolume (ngSTX$_{total}$/mm^3)	SS	Df	MS	F	*p*
Air bubbling	0.222	2	0.111	2.090	<**0.05**
Bicarbonate	11.392	1	11.392	214.066	<**0.001**
pH	2.781	2	1.390	26.127	<**0.001**
Air bubbling x Bicarbonate	1.876	2	0.938	17.630	<**0.001**
Air bubbling x pH	0.290	4	0.0726	1.364	ns
Bicarbonate x pH	2.276	2	1.138	21.380	<**0.001**
Air bubbling x Bicarbonate x pH	1.997	4	0.499	9.381	<**0.001**
Residual	0.958	18	0.0532		
Total	20.257	35	0.579		

overcome carbonate chemistry changes occurred under a buffered (neutral-acid pH) higher-CO_2 culture medium, and thereby did not perform better at increased free CO_2. Pierangelini et al. (2014) found that high-CO_2 exert an impact on photosynthetic performance in *R. raciborskii* CS506 by down-regulating CCM activity and altering the use of light energy harvested by cells, consequently affecting photosynthetic yield. The authors state that little information is currently available on the photosynthetic responses of *R. raciborskii* which are needed to demonstrate changes in CCM activity and cell energetic requirements relative to the dynamics of the CO_2 supply. Moreover, our findings suggest that not only a high pCO_2 level, but a constant associated neutral-acid pH seems to represent a C-limiting condition for *R. raciborskii* which has been previously described as a bicarbonate-using cyanobacteria (Holland et al., 2012).

Furthermore, decreasing the pH may establish a stress condition where is expected an effect in membrane integrity once constituent amino acids tend to stabilize pH by gaining protons which in turn affects the hydrogen bonding formed between the molecules and impairs membrane properties such as selective permeability (Qian et al., 2014). Also, Van Bodegom (2007) lists some of the costs that exert an influence on growth, such as changes in metabolic pathways, energy-dissipating reactions, storage products, osmoregulation, extracellular losses, DNA and protein turnover and concentration of the substrate. Regarding the latter, e.g., pH affects the relative amount of inorganic carbon species in water which in turn are substrate for phytoplankton growth. Hence, depending on CCM affinity, changes on the proportion of carbon species

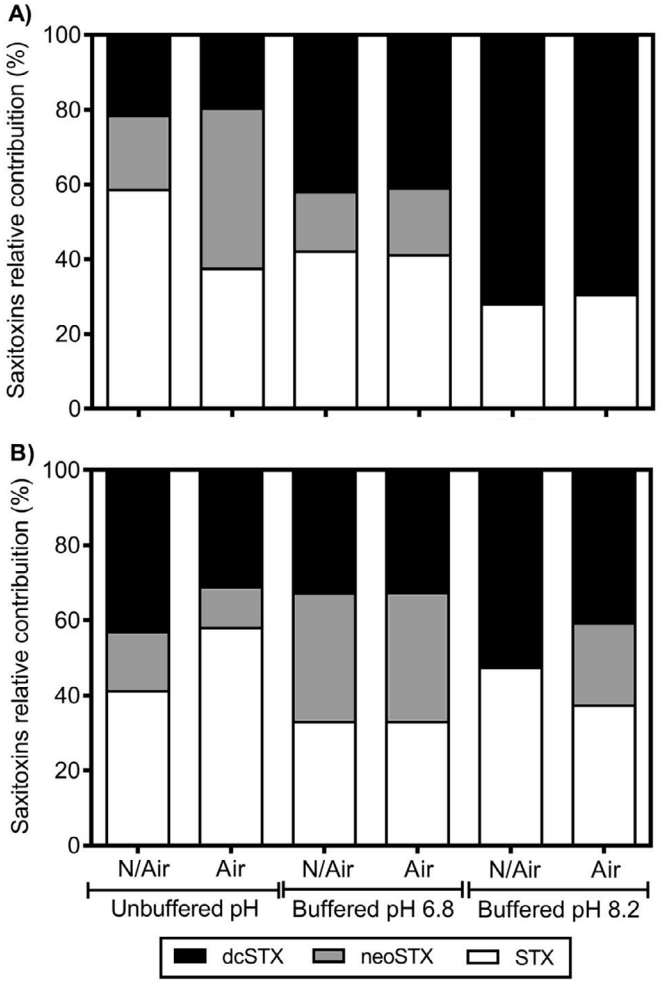

Fig. 4. Saxitoxins analogues contribution (%) relative to the total STXs produced by *R. raciborskii* ITEP-A1 kept under non-supplemented **(A)** and bicarbonate-supplemented **(B)** cultures at different pH **(unbuffered; buffered 8.2 and 6.8),** 0.041% CO_2-rich air bubbling condition **(Air)** and no air bubbling **(N/Air)**. Saxitoxin analogues: saxitoxin – STX **(white)**, neosaxitoxin – neoSTX **(gray)** and decarbamoyl-saxitoxin – dcSTX **(black)**.

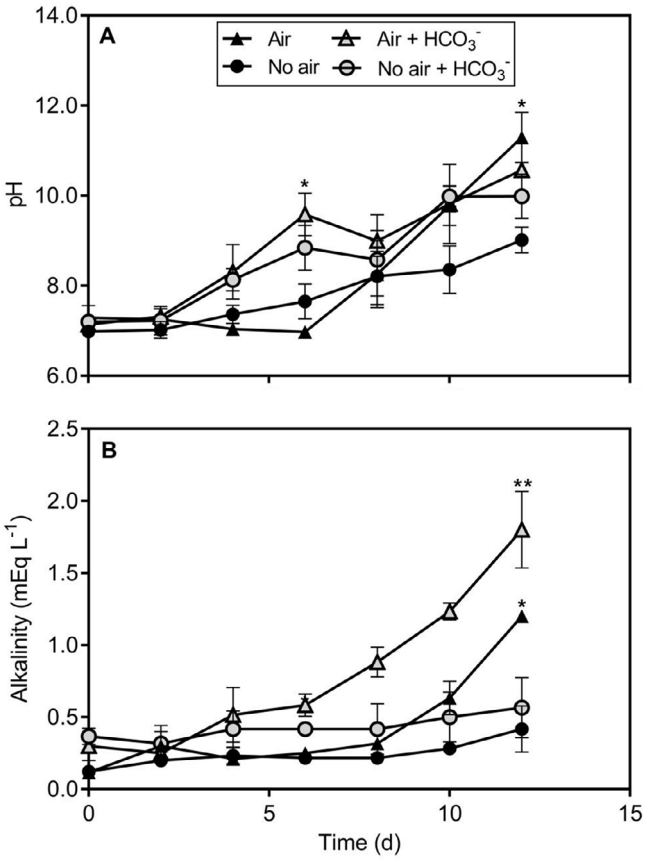

Fig. 5. pH **(A)** and alkalinity **(B)** dynamics in unbuffered culture with **(triangle)** and without **(circle)** 0.041% CO_2-rich air bubbling and supplemented with **(gray)** and without **(black)** bicarbonate ($NaHCO_3$). (*) = $p < 0.05$; (**) = $p < 0.01$, Tukey HDS Test.

might impair biomass acquisition. Thus, low pH is likely to have caused different effects on ITEP-A1 growth by affecting either carbonate system or ionic exchanges between cells and the surround environment.

In silico analysis enabled the identification of the low-affinity sodium-dependent bicarbonate uptake system BicA in the genome of *R. raciborskii* ITEP-A1. Together with growth experiments, this offers support to evidence shown by Sandrini et al. (2013) that the BicA transporter system can exert an impact on cyanobacterial growth and biomass acquisition. Our findings corroborate those found by Willis et al. (2019) after examining the core genome of two toxic *R. raciborskii* strains for the carbon concentration mechanism system. The authors found the bicarbonate uptake gene (*bicA*), a carbonic anhydrase (*β-CA*), genes encoding the NAD(P)H-quinone oxidoreductase (*ndhD4*) and CCM proteins.

The CO_2 uptake NDH-1 *ndhD3/ndhF3/cupA*-type and *ndhD4/ndhF4/*cupB-type genes were also identified. As shown by Shibatta et al. (2001; 2002) studying CO_2-uptake systems in *Synechocystis* 6803, NdhD3/NdhF3/cupA proteins (NDH-1_3 complex) are induced under low pCO_2 conditions, whereas NdhD4/NdhF4/cupB proteins (NDH-1_4 complex) have 3.0-fold lower affinity for CO_2 but constitutive functioning. Moreover, cupA-cupB and NdhD3/F3-NdhD4/F4 are CO_2-uptakers and H^+-antiporters (Battchikova et al., 2011) that collectively enable light-dependent CO_2 uptake and hydration (HCO_3^- formation), respectively. Thus, NDH-1_3 and NDH-1_4 complexes may be involved in the conversion of CO_2 to HCO_3^-, indicating the bicarbonate affinity of ITEP-A1. Reinforcing these findings, Holland et al. (2012) and Lines and Beardall (2018) found higher growth efficiency and a CO_2 concentrating mechanism in *R. raciborskii*, which has been labeled a bicarbonate-user species, what justify ITEP-A1 best performance under a bicarbonate-enriched environment.

In the present study changes in pH as well as dissolved inorganic carbon affected STXs production and composition. Bicarbonate supplementation in culture medium clearly downshifted saxitoxins concentration, so that a mean decrease of 99.78 ± 0.21% was found **(see Fig. 3)**. Besides many studies have investigated *R. raciborskii* performance in response to CO_2 (Holland et al., 2012; Pierangelini et al., 2014; Brito et al., 2018; Lines and Beardall, 2018; Willis et al., 2019), an experimental design which evaluate toxins production by this species in response to that factor is still incipient. On this subject, most of the studies are focused on *Microcystis* (Liu et al., 2016) and marine microalgae (Fu et al., 2010; Van de Waal et al. 2014a). Accordingly, Van de Waal et al. (2014a) reported a reduction of 20–33% in STXs production by the dinoflagellate *Alexandrium tamarense* in response to a high pCO_2 scenario. Conversely, the highest saxitoxins cellular amount achieved in our experiments was detected under a lower growth, e.g., in unbuffered cultures and with air bubbling the cyanobacterium reached a relatively lower net growth rate, but the highest toxin content; about 100-times higher than the obtained without air bubbling. It is likely that aeration has played a role on enhancing saxitoxins production by ITEP-A1.

It is also important to consider the slight increase in sodium concentration experienced by the cyanobacterium due to bicarbonate

($NaHCO_3$) enrichment and the adjustment of the pH by the addition of the NaOH solution. Combined effects of DIC and sodium on STXs-producing *R. raciborskii* were also described by Carneiro et al. (2013), who found a decrease in neosaxitoxin and saxitoxin production in response to carbonate salts (5 mM $MgCO_3$ and Na_2CO_3), which reduced toxin production up to 3.0-fold from the exponential to stationary growth phase. Proteogenomic studies on *Anabaena circinalis* (=*Dolichospermum circinale*) have shown that increasing the sodium chloride concentration promotes a decrease in total STXs and in the expression of the sxtC protein (D'Agostino et al., 2016). In contrast, some investigations have shown an increase in STXs production in response to ionic changes. Soto-Liebe et al. (2012) suggest that high concentrations of Na^+ or an ion gradient loss may positively affect enzymes involved in the synthesis of STXs in *R. brookii* D9. Pomati et al. (2003, 2004) also studied the effects of sodium in the cyanobacterial strain *R. raciborskii* T3, in which increased Na^+ assimilation was related to the cellular accumulation of saxitoxins. However, when compared to most studies (Pomatti et al. 2003, 2004; Soto-Liebe et al., 2012; Ongley et al., 2016), the sodium concentration in the present experimental set-up was far below the concentration range (>10 mM) in which saxitoxins enhancement is reported, but within the range of the functioning of bicarbonate transporters, resulting in remarkable biomass production.

There was an impact of pH on STXs cell amount. Unbuffered cultures experienced a wide range of pH (7.5–11.0) what resulted in differential toxin production among CO_2 conditions. Unlike, buffered cultures displayed a pattern where under constant alkaline pH 8.2 ITEP-A1 has shown about 90% less toxins relative to neutral-acid pH 6.8. These results are in line with Ongley et al. (2016) which found a saxitoxin cellular reduction of 18 and 64% in *R. raciborskii* T3 and *A. circinalis* AWQC131C when cultured under buffered pH 9.0, respectively. Additionally, the authors found an increase in STXs transcripts (e.g. *sxtM*) which encodes a toxin efflux cell membrane transporter, what is suggested to have a function on cellular ion balance. On the other hand, a dramatic drop in pH (< 5.0) also has been reported to increase saxitoxins excretion, previously pointed by Qian et al. (2014) as a stress caused by the cation-anion balance on the functioning of many membrane-associated proteins. Accordingly, in our study pH did not reach critical values that could induce a toxin stress in the cyanobacterium.

A high pCO_2 environment provided by bicarbonate promoted an increased carbon acquisition (as biomass growth) which in turn was likely to have affected saxitoxins production. Van de Waal et al. (2013) evidenced lower saxitoxins cell content related to both higher C:N and C:P ratio, suggesting that the synthesis of N-rich toxins such as STXs might be affected under conditions of enhanced cellular C acquisition. Similar findings were reported by Liu et al. (2016) and Fu et al. (2010) for microcystin and karlotoxin production, respectively. According to assumptions put forth by Van de Waal et al. (2014b), an input of inorganic carbon is suggested to elevate the C:N:P ratio such that the relative excess of energy and synthesized organic C would be used for cell growth rather than being shunted into N- or P-rich molecules, such as bioactive alkaloids.

Stoichiometric hypothesis suggests that the production of cyanotoxins usually follow a synthesis dynamic that at least partially depends on the interplay between their own stoichiometric composition and the relative availability of key nutrients (Van de Waal et al. 2014b) as means to provide a cellular internal elemental equilibrium. Saxitoxins are nitrogen-rich alkaloids whose cellular content tends to increase under conditions of phosphorus limitation (N:*P* >10; Brandenburg et al., 2020), e.g., Van de Waal et al. (2014b, 2015) found that high N:P ratios influence the synthesis of STXs, which can increase by as much as 28% under low phosphate conditions. Moreover, amongst cyanotoxins, saxitoxins have the lower C:N ratio (1.5), thus an increase in internal nitrogen drives their production to maintain the stoichiometric regulation of these nutrients (Brandenburg et al., 2020). In contrast, Vico et al. (2016) have not observed any effects of nitrogen (*N*+ and N-) on STXs biosynthesis as *sxtI/sxtU* transcripts and cell toxin quota. However, the authors only assessed STXs production after 6-days incubation, and at this time STXs transcripts in *N*+ displayed an increase trend what is likely to suggest an upward toxin response.

CO_2 and pH also affected the STX analogues profile in *R. raciborskii* ITEP-A1. Unbuffered and buffered neutral-acid pH increased the amount of total saxitoxins, in which the composition consisted mainly of STX and neoSTX. Higher neoSTX and STX amounts indicate an increase in the relative cellular toxicity of ITEP-A1 once these are the most toxic analogues (Perez et al., 2011; Munday et al., 2013). The saxitoxins analogues level of toxicity depends on the degree of sulfation, with non-sulfated analogs being the most toxic based on hydroxyl and carbamoyl radicals (neoSTX > STX > dcSTX) (Wiese et al., 2010). However, sulfated GTXs 1–4 and 2–3 have already shown to be as toxic as non-sulfated derivatives (Munday et al., 2013) what brings attention to other moieties besides sulfate group. Yet, to date, no GTXs were detected in *R. raciborskii* ITEP-A1.

In contrast, when kept under pH 8.2 the cyanobacterium downshifted its relative toxicity due to a greater production of dcSTX (39–87%). Van de Waal et al. (2013) indicate that under limited conditions, hydrolase-like enzymes may be affected, enabling synthesized STX to be hydroxylated towards neoSTX or dcSTX. Thus, surrounded pH may affect the cellular environment promoting chemical transformations in these toxins, such as the addition of sulfur or hydroxyl, which are respectively provided by sulfotransferase and cephalosporin hydroxylase enzymes. Moreover, constant alkaline pH seemed to establish a condition which has affected saxitoxin production toward a decarbamoylated analogue what suggest an effect on O-carbamoyltransferase enzymes.

Under maintaining conditions (non-aerated unbuffered medium) saxitoxins produced by ITEP-A1 displayed the following composition: dcSTX (21.64 ± 8.89%), neoSTX (19.87 ± 2.88%) and STX (58.48 ± 8.04%). However, in the same conditions, but at a four-times lower light regime, STXs profile in the ITEP-A1 strain was as follow: dcSTX (62.58%), neoSTX (32.50%) and STX (4.90%) (Bittencourt-Oliveira et al., 2016). Therefore, reinforcing that saxitoxin analogues synthesis is governed by different environmental factors. The production of saxitoxins (more than 50 STX analogues and derivatives have been reported) is known to be affected by more than one environmental condition and there may be changes in content and composition throughout growth. Moreover, a little is known regarding their chemical transformations as well as total toxin production and the role they play in cell metabolism within the producing organism (Wiese et al., 2010) what rises questions on varying ecophysiological functions, as their production is strongly related to diverse environmental factors.

Investigations addressing the impact of the environmental stress on the ecophysiology of toxic *R. raciborskii* generally indicate a trade-off between growth and toxin production. Mesquita et al. (2019) found that the highest STXs production was accompanied by a lower growth rate in different strains of *R. raciborskii* when assessing the combined effect of light intensity and temperature. Moreover, cylindrospermopsin-producing strains of *R. raciborskii* have exhibited an imbalance between growth and toxin production in response to nitrogen-limiting conditions (Saker and Neilan, 2001; Kenesi et al., 2009). Saker and Griffiths (2000) and Nor et al. (2019) reported a similar pattern in which cylindrospermopsin ranged from high concentrations at 20–25 °C to low concentrations or even below the detection limit at >35 °C, which is an optimal temperature range for growth. Conversely, despite some studies suggest toxin production is coupled to growth rate, we also found significantly different saxitoxins quotas achieved under similar growth rates **(Figs 2 and 3)**. Therefore, saxitoxins production seems to depend not only on cell division rate, but also on environmental variables which must be examined multidimensionally as synergistic factors. Accordingly, here we attempted to put apart the effects of carbonate chemistry and pH to determine whether the observed changes in STXs content are mainly due to shifts in pH or by changes in CO_2 availability.

Water chemistry

The addition of sodium bicarbonate and air bubbling in unbuffered cultures promoted significant increases in pH and alkalinity **(see Fig. 5)**. There is a positive inter-relation between pH and alkalinity so that the relative proportion of HCO_3^- and CO_2^{3-} ions is pH-dependent. Also, it is likely that CO_2 input provided by air bubbling into the cultures might have contributed to carbonate system. An increase in these variables throughout the cellular growth of ITEP-A1 suggests inorganic carbon uptake by this cyanobacterium (e.g. Zhang et al., 2014; Farias-Silva et al., 2016). In addition, the maximum alkalinity registered in our study (2 mEq L^{-1}) is on the range (0.12 – 5.57 mEq L^{-1}) of those reported for tropical and subtropical lakes in which *Raphidiopsis* has predominated (see Bouvy et al., 1999, 2000; Brentano et al., 2016). Also, cellular uptake of nutrients by algae may cause alkalinity to rise in culture medium (Brewer and Goldman, 1976; Zhang et al., 2014) due to the release of ions such as NH_3, HPO_4^-, $H_2BO_4^-$, $[R]COO^-$. Overall, photosynthesis enhances pH along culture incubation; hence it is suggested that alkalinity could also be changed as well (Zhang et al., 2014).

Furthermore, at the end of incubation, the pH in all unbuffered cultures was higher than 9.0. This is a feature of a water with a low calcium concentration where bicarbonate and carbonate are associated with sodium or potassium, carbonate does not precipitate, and pH rises reaching values above 11 (Boyd, 2000). A lower pCO_2 level is expected under such conditions, with a gradual shift in relative inorganic carbon amount from HCO_3^- to carbonate (CO_3^{2-}). In many lakes pH increase may occur during algal blooms which form hydroxide as algae consumes all carbonate alkalinity (Gao et al., 2012). This supports the notion that these microorganisms may modify their environment in such a way that gives them a competitive advantage over other phytoplankton species. However, Ji et al. (2017) demonstrated that this assumption does not capture the ability among and within phytoplankton taxa to use different dissolved inorganic carbon species.

In the present study we also found a possible effect of sodium in the treatments enriched with $NaHCO_3$, as this ion was increased approximately 26-fold (a total Na^+ upshift from 0.002 mM to 0.057 mM relative to the standard ASM-1 culture media). This concentration of Na^+ induces maximum or near-maximum activity of the sodium-dependent bicarbonate transporters BicA and SbtA, considering the half saturation constant of 1–2 mM exhibited by these transporters (Shibata et al., 2002). Moreover, *R. raciborskii* growth seems to be supported even under ionic concentrations above the half-saturation range of the transporters, as reported by Carneiro et al. (2013), who found that T3 strain exhibited growth under 5 mM NaCl ($\mu = 0.197 \pm 0.02\ d^{-1}$), with a significant decrease only beginning at 10 mM ($\mu = 0.104 \pm 0.01\ d^{-1}$). Also, Duvall et al. (2018) found growth inhibition in different strains of *Raphidiopsis* and *Dolichospermum* only at concentrations ≥ 85.55 mM of sodium chloride (NaCl salinity, ≥ 5 g L^{-1}). As suggested by Pomati et al. (2004), sodium stress may result from an imbalance in the activity of Na^+/H^+ antiporters, which ensure regular intracellular concentrations of this ion. Thus, considering the range of salinity in which *R. raciborskii* ITEP-A1 was maintained in our experimental set-up, a possible stress response can be discarded. Therefore, the effect of changes in pH and inorganic carbon are likely the main factors that affected the growth and toxin production in the cyanobacterium.

5. Conclusion

Here, we provide further information on the physiological responses of the STXs-producing strain *R. raciborskii* ITEP-A1 exposed to different pH/CO_2 conditions. Despite of thriving under different pH and carbonate chemistry, ITEP-A1 reached a better growth under bicarbonate supplementation. Conversely, a clear negative influence of a higher pCO_2 level established by sodium bicarbonate and aeration on saxitoxins production was found. To our knowledge, the influence of CO_2 on *R. raciborskii* saxitoxins production has not previously been reported. However, despite bicarbonate enrichment, pH played a role on growth and toxin production, suggesting that pH-depending changes in carbonate chemistry drive these physiological responses. In addition, besides temperature and nutrients, deciphering how changes in pH and DIC affect growth and total STXs production may shed light on the success of *R. raciborskii* genotypes with different CCM systems in terms of dominating waterbodies, and provide insights that enable predicting blooms in a CO_2-saturated scenario. Our findings reinforce that, although a high-CO_2 environment intensify the occurrence of cyanobacterial blooms, these blooms will probably be less toxic.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

We thank the anonymous reviewers for their valuable comments, which helped us to improve the quality of this paper. This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Finance Code 001.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.hal.2020.101870.

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Ecotoxicol. Environ. Contam., v. 15, n. 1, 2020, 1-8
doi: 10.5132/eec.2020.01.01

EEC

Acute toxicity of neurotoxin-producing *Raphidiopsis* (*Cylindrospermopsis*) *raciborskii* ITEP-A1 (Cyanobacteria) on the neotropical cladoceran *Macrothrix spinosa*

Santos, G.S.D., Vilar, M.C.P. & Azevedo, S.M.F.O.

Laboratory of Ecophysiology and Toxicology of Cyanobacteria, Carlos Chagas Filho Institute of Biophysics, Federal University of Rio de Janeiro, Rio de Janeiro, 21949-902, Brazil.

Received December 12, 2019; Accept February 12, 2020

Abstract

Cyanobacterial blooms are recurrent phenomena in several water bodies, mostly eutrophic. They are considered a public health problem, especially considering harmful species like *Raphidiopsis raciborskii*, a bloom-forming cyanobacteria recorded as a producer of neurotoxic and cytotoxic alkaloids. The present study aimed to assess the acute toxicity of a saxitoxin-producing strain *Raphidiopsis raciborskii* ITEP-A1 on the zooplankter *Macrothrix spinosa*. Cladoceran clones isolated from an eutrophic system with cyanobacterial blooms records and an oligotrophic one with no bloom record; both from Pernambuco (Northeastern Brazil), were tested for *R. raciborskii* ITEP-A1 toxicity. Acute toxicity assays were carried out with newborns (<24h, n=10) and the animals were exposed to three concentrations of cyanobacterial cell biomass (μgC L^{-1}). Quantification of saxitoxins (STX and neoSTX) was done by high-performance liquid chromatography coupled with fluorescence detector (HPLC-RF). The EC_{50}(48h) for the Duas Unas and Prata clones were 359 and 189 μgC L^{-1}, respectively. Obtained results showed that *M. spinosa* Prata was more sensitive to *R. raciborskii* than Duas Unas, reinforcing the importance of analyzing the previous life history of the test organisms regarding their native environments. However, both clones demonstrated high sensitivity to *R. raciborskii*, which enhances their potential for biomonitoring toxic cyanobacterial blooms in tropical reservoirs.

Keywords: Cyanotoxin, tropical, zooplankton

INTRODUCTION

Widespread pollution from human activity followed by the eutrophication of water bodies has continuously increased the occurrence of cyanobacterial blooms, a worldwide phenomenon and environmental issue. Aquatic environments that are rich in nutrients, particularly nitrogen and phosphorus, are directly connected to the prevalence of blooms, however, it is not the only factor influencing the predominance of cyanobacteria. Rising CO_2 levels and global warming are also believed to favor harmful cyanobacterial blooms (Paerl & Huisman, 2009), increasing their frequency, intensity and duration in many ecosystems all over the world (Huisman *et al*., 2018).

Some of the most recorded bloom- or mats-forming cyanobacterial genera are *Planktothrix*, *Microseira* (syn. *Lyngbya* and *Plectonema*)*, Dolichospermum*, *Microcystis* and *Raphidiopsis* (formely *Cylindrospermopsis*) (Mowe *et al*., 2015). The latter is commonly found in East and Southeast Asia, Australia and South America, with difference in the type of toxin reported by their isolated strains – for instance, while *Raphidiopsis* in Australia, East and Southeast Asia produces cylindrospermopsins (CYNs), in South America isolated strains from that species are reported to produce saxitoxins (STXs) (Antunes *et al.*, 2015; Burford *et al.*, 2016; Mowe *et al.*, 2015). The later are a group of neurotoxic carbamate alkaloids which act as ionic channel blockers and affect the nervous system, leading to paralysis and death (Wiese *et al.*, 2010). These toxins are also produced by some genus of eukaryotic marine dinoflagellates such as *Alexandrium* which concerns due to forming red tides and compromising seafood in marine and coastal ecosystems (Anderson *et al.*, 2012).

*Corresponding author: Mauro Cesar Palmeira Vilar; e-mail: maurovilar@gmail.com

In freshwater systems saxitoxins are usually associated to *Raphidiopsis raciborskii* (Woloszynska) Aguilera, Berrendero Gómez, Kastovsky, Echenique & Salerno (basionym *Cylindrospermopsis raciborskii* (Woloszynska) Seenayya & Subba Raju) (Aguilera *et al.*, 2018) which is an important bloom-forming and toxin-producing filamentous cyanobacteria, predominant in Brazilian aquatic environments (Bouvy *et al.*, 2001; Soares *et al.*, 2013; Guedes *et al.*, 2018). Its presence in water bodies poses risks to endemic organisms by ingestion of cells or absorption of toxin dissolved in water (Costa *et al.*, 2013), as well as might impact public health by compromising water supply and fish.

Some studies have shown that cyanobacteria exert a variety of effects on zooplankon, such as decrease in survivorship, growth and reproduction (Costa *et al.*, 2013; Ferrão-Filho *et al.*, 2011; Lürling, 2003; Vilar *et al.*, 2014). These effects, however, vary according to the zooplankton species. Some have developed, throughout life history, the ability to coexist with certain species of cyanobacteria. On the other hand, reports suggest that cyanobacteria might have resistance to predation by zooplankton; specifically when there is dominance of *R. raciborskii* in a water body, there is inhibition of the growth of zooplankton as they have difficulty using the large filaments and colonies of cyanobacteria as food (Leonard & Pearl, 2005; Havens & East 2006).

Although saxitoxins are potent paralyzing alkaloids and feeding inhibitors, studies suggest they present low lethality effects in invertebrates (Ferrão-Filho *et al.*, 2013). The immobilizing effect of the toxin affects the movements of cladocerans by paralyzing their swimming appendages, but once they are still able to filter-feed even with immobilized limbs they do not suffer from starvation nor respiratory arrest. Thus, as observed by Costa *et al.* (2013) and Ferrão-Filho *et al.* (2010), the continuous filtration of food particles whilst paralyzed allows the organisms to survive for long periods. Nevertheless, there is still a lack of research on the effects of STXs on tropical freshwater zooplankton.

Daphnia spp. are among the most commonly used organisms in freshwater quality monitoring programs (ABNT, 2016; USEPA, 2002). Nowadays, most ecotoxicological research in tropical environments are conducted with acclimated exotic cladoceran species from temperate regions, such as *Daphnia similis* Claus, 1876 and *Daphnia magna* Straus, 1820. In the other hand, very few studies are carried out with native ones (Freitas & Rocha, 2011), therefore, there is a growing preference for using native species on ecotoxicological assessments, as they are more representative of the environment and local-species sensitivity.

On that context, *Macrothrix* appears to be a potential organism to be used in ecotoxicological assays, although studies on cladocerans from the family Macrothricidae are still incipient. Available data on sensitivity are on the species *Macrothrix elegans* Sars, 1901 and *Macrothrix flabelligera* Smirnov, 1992 that have shown potential as suitable test-organisms for a variety toxicity tests (Araújo *et al.*, 2008; Moreira *et al.*, 2014; Moreira *et al.*, 2017). These animals' adaptability, short life cycle and easy maintenance in laboratory facilitate their choice as potential biomonitors for tropical aquatic systems. Species in this genus are described as having an oval body in lateral view, a dilated antennule being identified by the serrations along the dorsal part of its valve (Fuentes-Reinés *et al.*, 2015; Fuentes-Reinés *et al.*, 2018).

Although there is an apparent lack of in-depth studies regarding the tropical species, *Macrothrix spinosa* King, 1853 is reportedly well-distributed in South American water bodies (Fuentes-Reinés *et al.*, 2018; Sousa *et al.*, 2018). *M. spinosa* is often presented as synonym of *Macrothrix squamosa* Sars, 1901. This synonymy is based on superficial characteristics, such as the aspect of the carapace, antennule, and post abdomen (Elmoor-Loureiro, 2007). In contrast, recent studies suggest that *Macrothrix* spp. tend to have continental or regional distribution, which indicates that *M. spinosa* – originally described from Australia, and its congener *M. squamosa* might be different species (Elmoor-Loureiro, 2007; Fuentes-Reinés *et al.*, 2015; Fuentes-Reinés *et al.*, 2018). Regardless, further revision is important to know the diversity of potentially sensitive species on water biomonitoring. Thus, the present study aimed to evaluate the toxicity of a neurotoxin-producing *Raphidiopsis raciborskii* on the cladoceran *Macrothrix spinosa.* Moreover, this is the first report of the effects of a saxitoxin-producing cyanobacteria on that zooplankton species. Therefore, the findings may contribute to the knowledge on the potential of a (sub)tropical zooplankton as a tool on the biomonitoring of freshwaters.

MATERIALS AND METHODS

Cultivation and maintenance of organisms

The cyanobacterial strain used in this study was a saxitoxin-producing *Raphidiopsis raciborskii* ITEP-A1 which was isolated from Riacho dos Paus reservoir located in Arcoverde, countryside of Pernambuco state, Brazil. The culture were maintained in ASM-1 (Gorham *et al.*, 1964) medium under initial pH 8.0, controlled temperature (24 ºC ± 1) and 12h dark:light cycle under light intensity (50 µmol m^{-2} s^{-1}) . Culture samples were collected for acute toxicity tests and toxin analysis during exponential growth phase (about 8 days).

Two clones of the cladoceran *M. spinosa* isolated from Prata and Duas Unas reservoirs (Pernambuco, Brazil) were used in this study. Duas Unas is known as a eutrophic water body with previous report of cyanobacterial blooms (Bittencourt-Oliveira *et al.*, 2014; Lorenzi *et al.*, 2018). On the other hand, Prata reservoir is an oligotrophic water body with no previous cyanobacterial bloom report (Almeida *et al.*, 2012).

Zooplankton samples were collected with plankton net (60 µm mesh-size) in the littoral zone of the waterbodies. Animals were anaesthetized with carbonated water (1:20 v/v), sorted and disposed in Petri dish where parthenogenetic females of *M.*

spinosa were isolated under stereoscope and kept in laboratory during several generations. Adults of *M. spinosa* ranged from 0.943–0.961 mm. Animals were cultured in artificial RT culture medium (Tollrian, 1993) with 20% mineral water enriched with commercial humic extract Microbe Lift® 0.1% (approximately 2.25 mg L^{-1} dissolved carbon content). They were kept under a controlled temperature of 24 ±1 ºC, 12h dark:light cycle and fed by mixed cell suspension of green algae *Selenastrum capricornutum* Printz and *Ankistrodesmus stipitatus* Komárková-Legnerová at a final biomass 1 mgC L^{-1}.

Cyanobacterial cell biomass measurements

R. raciborskii ITEP-A1 biomass (µgC L^{-1}) was estimated according to Rocha & Duncan (1985) using the mean trichome volume (µm^3) (Hillebrand *et al.*, 1999) after measuring 50 units, and estimated using trichome density from Fuchs-Rosenthal chamber counting.

Acute toxicity test

To evaluate *R. raciborskii* ITEP-A1 impact on cladocerans' mobility both clones of *M. spinosa* were exposed to different biomass concentrations, with maximum concentration defined after preliminary acute toxicity test.

Tests were carried out with 10 newborns (<24h) of each *M. spinosa* clone in triplicates. The neonates were put in test tubes containing 25 mL of different concentrations of *R. raciborskii* ITEP-A1 biomass: 150, 300 and 500 µgC L^{-1}, and a control which consisted of cyanobacteria-free culture media. Test organisms were exposed to cyanobacterium for 48h. After incubation period, the number of immobile individuals in each concentration as well as in the control was recorded for estimative of half maximal effective concentration (EC_{50} 48h) using the Trimmed Spearman-Karber method (Hamilton *et al.*, 1977).

Bibliographic survey of cladoceran sensitivity to saxitoxin-producing cyanobacteria

It was performed a bibliographic survey about published articles on acute toxicity assay experiments testing the effects of saxitoxin-producing cyanobacteria on cladocerans. Previous toxicity studies, published between 1990 and September 2019, were consulted in the database Web of Science using the following keywords and combinations: *bioassay and cyanobacteria* (456 articles); *cyano* and toxicity and Cladocera* (16 articles); *neurotoxicity and cyanobacteria* (73 articles); *saxitoxin and cyanobacteria and Cladocera* (3 articles); *Macrothrix and toxicity* (6 articles).

Aiming to compare our results to the available literature on cladoceran sensitivity to saxitoxin-producing cyanobacteria, the publications were filtered based on the following criteria: 1) the study assessed the acute toxicity of saxitoxin-producing *R. raciborskii* on Cladocera; 2) animals were exposed to intact cyanobacterial cell (or fresh biomass); 3) half maximal effective concentration values in cell density (cell mL^{1}) or biomass (µgC L^{-1}).

Saxitoxins extraction and analysis by HPLC-RF

Saxitoxins extraction from cell biomass were performed using 0.5 M acetic acid. After centrifuging (22,000x g), supernatant was collected and filtered through 0.22 µm Millex (Millipore) prior to injection. Chromatographic analyses were performed on a Shimadzu HPLC using a silica-based reverse phase column and separations were carried out under specifics mobile phases for non-sulfated saxitoxins (saxitoxin and neosaxitoxin) (hereafter STX and neoSTX) coupled to post-column oxidation as described in (Oshima, 1995). Fluorescent saxitoxins derivates were detected using a Shimadzu RF-10Axl fluorometric detector with excitation at 330 nm and emission at 390 nm. Toxins were identified and quantified by comparison with known retention times and integrated areas of standards, respectively. The standards of saxitoxins were purchased from Institute for Marine Bioscience, National Research Council of Canada (Halifax, Canada). Data were shown as total saxitoxin (neo-saxitoxin and saxitoxin) quota per biomass unit ($ngSTX_{total}$/µgC).

Statistical analysis

To identify any significant difference in the mean number of affected individuals, the one-way analysis of variance (ANOVA) was used with *post hoc* Dunnet test in order to ascertain the lowest-observed-adverse-effect level. All statistical analyses were performed using the software SigmaPlot 11.0 and attending the basic premises of the above-mentioned tests.

RESULTS

Cyanobacterial measurements and toxin content

The average length and width of filament obtained was, respectively, 78.38 (±38.91) µm and 2.99 (±0.58) µm resulting in an average trichome volume of 550.35 µm^3 and biomass value of 108,988.601 µgC L^{-1}. The Total saxitoxin concentration normalized by trichome biomass was 0.2 $ngSTX_{total}$ $µgC^{-1}$.

Acute toxicity of R. raciborskii on M. spinosa

In the acute bioassays, the effects of *R. raciborskii* ITEP-A1 characterized by paralysis of swimming appendages was noticed in both clones. After 24h of exposure to the cyanobacterium, all replicates already presented immobile organisms in all concentrations of biomass (data not shown). After 48h of exposure that number grew, as the effects of the cyanobacteria affected in more individuals. The immobilization effect was higher with the higher concentrations of cyanobacterial biomass (500 µgC L^{-1}) (Figure 1).

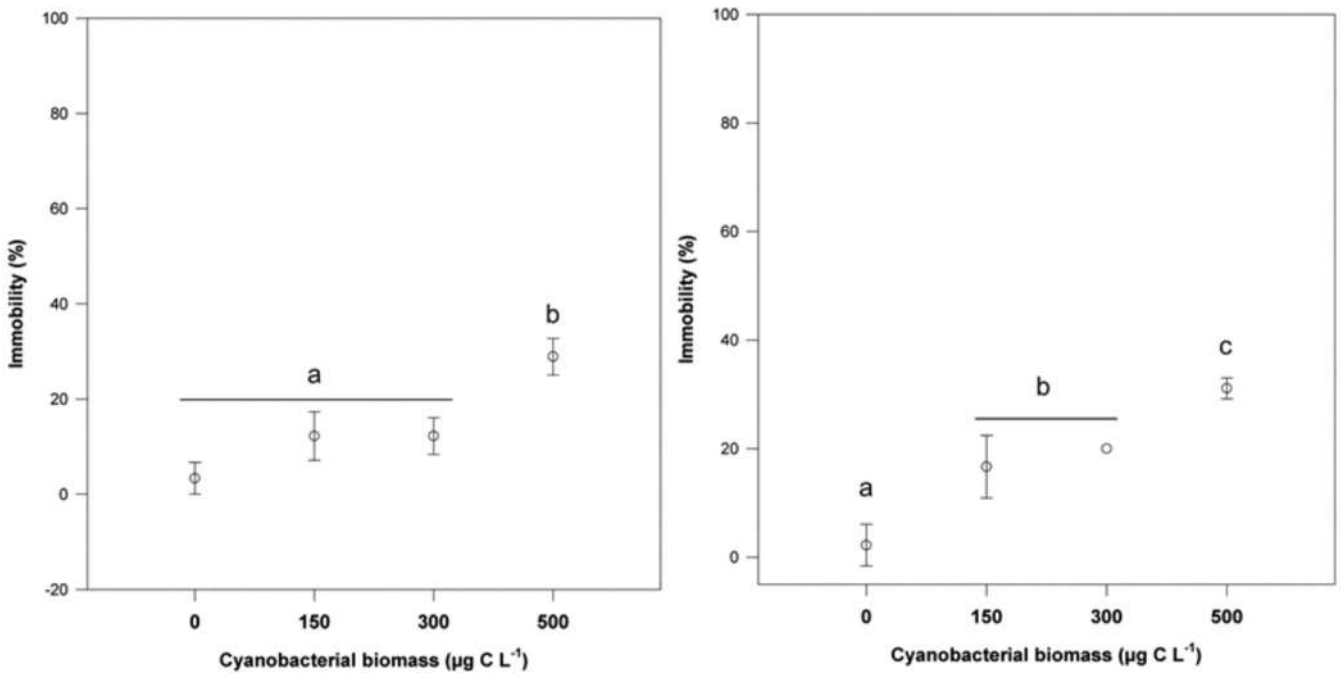

Figure 1. Dose-response of *Macrothrix spinosa*, clones (A) Duas Unas and (B) Prata under acute exposition to toxic *Raphidiopsis raciborskii* ITEP-A1. Letters = Significant difference compared to the control (Dunnett, p <0.05), representing the lowest-observed-adverse-effect.

The lowest-observed-adverse-effect concentrations were equal to or above 150 µgC L^{-1} for Prata and 300 µgC L^{-1} for Duas Unas *M. spinosa* clones (Dunnett, p <0.05). As showed in Table 1, EC_{50} (48h) for Duas Unas was 359 µgC L^{-1}, approximately two times higher than the EC_{50} (48h) for Prata, evidencing that individuals originated from Prata reservoir show more sensitivity to *R. raciborskii* ITEP-A1 than the organisms from Duas Unas reservoir.

Bibliographic survey

When analyzed the bibliographic survey it was found 456 articles when used the terms *bioassay and cyanobacteria*, and 73 articles to *neurotoxicity and cyanobacteria.* The most specific the keywords, less articles were found: *cyano* and toxicity and cladocera* (16 articles); *saxitoxin and cyanobacteria and cladocera* (3 articles); *Macrothrix and toxicity* (6 articles). After screening, only 3 papers out of >400 publications reported data that fitted the established criteria and presented EC_{50} values that were then compared to those of the present study, as summarized in the Table 2.

DISCUSSION

In the present study a tropical zooplankton species *M. spinosa* showed to be sensitive to ecologically relevant concentrations of saxitoxin-producing cyanobacteria, regarding its potential on biomonitoring cyanobacterial blooms in tropical water bodies.

When comparing to previous toxicity studies of STX-producing *R. raciborskii* strains, the EC_{50} (48h) values to *M. spinosa* clones Duas Unas 359 µgC L^{-1} (~3.930 cell mL^{1}) and Prata 189 µgC L^{-1} (~2.607 cell mL^{1}) were both similar or dissonant to those with standard cladocerans, such as *D. similis*. Also, although *M. spinosa* has not yet been standardized regarding acute toxicity tests, the species presents similarities with standard test-organisms. For instance, data presented by Zagatto *et al.* (2012) demonstrated different sensitivities in relation to our findings – *D. similis* exposed to *R. raciborskii* T2 strain showed less sensitivity when compared to our data, while for the T3 strain the daphnid showed more sensitivity. The same was reported by Ferrão-Filho *et al.* (2014), where EC_{50} values for *D. similis* exposed to the strain *R. raciborskii* CYRF-01 evidenced more sensitivity than our findings. In contrast, when analyzed the data showed by Ferrão-Filho *et al.* (2010), the EC_{50} values for *Daphnia pulex* and *Moina micrura* Kurz, 1875 exposed to CYRF-01 were not so far off from our data, with *D. pulex* exhibiting near equal sensibility to CYRF-01 as *M. spinosa* Prata did to ITEP-A1. Therefore, these data showed us that sensitivity of different cladoceran species or clones to STX-producing cyanobacteria can vary, either by intrinsic differences on tolerance or because different strains may display differences in their toxin profile and total amount of metabolites, which affects the relative toxicity. Also, we cannot disregard the potential

Table 1. Half maximal effective concentration values (EC_{50} – 48h) and 95% confidence intervals (CI) estimated for both clones of *M. spinosa.*

Test organism	EC_{50} – 48h (95% CI) µgC L^{-1}
Macrothrix spinosa (Duas Unas)	359.27 (323.37 – 399.16)
Macrothrix spinosa (Prata)	188.99 (73.83 – 483.75)

Table 2. Half maximal effective concentration values (EC_{50}) obtained from the literature of cladocerans exposed to saxitoxin-producing strains of *Raphidiopsis raciborskii*.

Test organism	Parameters	EC_{50} (µgC L^{-1})	EC_{50} (cell mL^{-1})	Reference
Macrothrix spinosa	Immobilization - 48 h	359.27 (323.37 – 399.16)	3,930	Present study
Macrothrix spinosa	Immobilization - 48 h	188.99 (73.83 – 483.75)	2,607	Present study
Daphnia similis	Immobilization - 48 h	-	302.56 x10^3	Zagatto *et al.* (2012)
Daphnia similis	Immobilization - 48 h	-	0.218 x10^3	Zagatto *et al.* (2012)
Daphnia similis	Immobilization - 2 h	31.5 (13.8 – 48.0)	-	Ferrão-Filho *et al.* (2014b)
Moina micrura	Immobilization - 2–3 h	-	8,905	Ferrão-Filho *et al.* (2010)
Daphnia pulex	Immobilization - 2–3 h	-	2,133	Ferrão-Filho *et al.* (2010)

variation among experimental designs. Those differences, even small, allow some variation in sensitivity.

Saxitoxin-producing cyanobacteria are usually reported as affecting swimming behavior of freshwater zooplankton by decreasing mobility and activity parameters (time spent swimming and resting, distance traveled, and mean velocity) (Ferrão-Filho *et al.*, 2008; 2014b). Saxitoxins can exert acute effects on zooplankton such as paralyze limbs, inhibit thoracic appendages beating, besides reducing the post-abdominal rejection of undesirable particles (Ferrão-Filho *et al.*, 2008; 2014b). Most recently Ferrão-Filho & Silva (2020) evidenced that neurotoxic cyanobacteria can reduce *Daphnia*'s heartbeat. Indeed, that result is in line with saxitoxins effects once these toxins also block calcium channels (Wiese *et al.*, 2010) which are abundant in the cardiac muscle.

Bibliographic survey allowed to evidence that most of data produced on STX-producing cyanobacteria impact on zooplankton focused on chronic effects such as growth, survivorship and reproduction (Soares *et al.*, 2009; Costa *et al.*, 2013; Ferrão-Filho *et al.*, 2014a; Ferrão-Filho *et al.*, 2019). Therefore, the lack of investigations regarding the acute toxicity of STX-producing cyanobacteria to freshwater zooplankton, especially those of the Macrothricidae family, limits a more in-depth, extensive comparison in our study. Moreover, once STX-producing *R. raciborskii* is up to date reported to South America, most of the studies concentrates in this area, although still incipient. However, considering that *R. raciborskii* is an opportunistic and potentially-toxic species which has dominated in waterbodies from different regions and more recently spread to temperate zones, it is important to produce data on its early impacts on aquatic biota in order to predict future environmental outbreaks as well as subsidize monitoring programs on water quality.

Besides zooplankton sensitivity, cyanobacterial toxicity also depends on the composition of secondary metabolites, such as known cyanotoxins and other non-characterized molecules, as well as their specific cell content. Several of these secondary metabolites are shown to be toxic to plants, invertebrates and vertebrates (Huisman *et al.*, 2018) and, considering the same strain can produce more than one toxic metabolite, it becomes difficult to link the adverse effects to one single known toxin (Ferrão-Filho *et al.*, 2011).

There are substantial differences between the sensibilities of different bioindicator species (Ferrão-Filho *et al.*, 2017). Body length (surface area/volume ratio), having a more selective diet and metabolic differences are some of the characteristics interfering on the effects of toxicants in the organism, and can be limiting factors when choosing a bioindicator species for water monitoring assessments (Costa *et al.*, 2013; Ferrão-Filho *et al.*, 2011; 2014; Gustafsson *et al.*, 2004). However, geographical differences also play a part in a species' sensitivity (Araújo *et al.*, 2008). In some studies, autochthonous tropical species have demonstrated higher sensitivity to certain compounds than their temperate counterparts (Moreira *et al.*, 2014; Moreira *et al.*, 2017), which leads to an increasing interest in the use of native species in ecotoxicological assessments, as the results tend to be more representative of local impacts (Araújo *et al.*, 2008). Despite this, water quality assessments in tropical regions are still mostly based on methods developed in temperate regions, which may diminish the ecological relevance of results (Araújo *et al.*, 2008; Moreira *et al.*, 2014).

The obtained results also revealed that the two clones of *M. spinosa* isolated from different locations were differently affected by *R. raciborskii* metabolites (Table 1). Curiously, the clone isolated from the eutrophic lake with previous occurrence of cyanobacterial blooms seems to be more tolerant to *R. raciborskii*. It is suggested that *M. spinosa* Duas Unas which has previously experienced cyanobacteria, may have evolved tolerance along generations. Chislock *et al.* (2019) evidenced that clones of *Daphnia pulicaria* from eutrophic lakes tend to have higher growth rates and survival when

fed toxic cyanobacteria than populations from oligotrophic environments where cyanobacteria are less abundant. Besides species-specific traits, intraspecific (clonal) particularities should also be taken into consideration. In our study we used two clones of the same species, however the differences in tolerance to toxic dietary cyanobacteria could be linked to their dietary history in their respective sampling locations as hypothesized by Hairston *et al.* (2001).

In addition, small-size zooplankton generally represents the size spectra predominant under cyanobacterial dominance. Studies show that different body length among *Daphnia* spp. clones, as well as predation rate, promote different responses to filamentous cyanobacteria (Gilbert *et al.*, 1990; Spitze, 1992). However, when considering the changes in zooplankton structure along bloom establishment, clonal differences are probably as large as those among species, and therefore as important for community structure (Hietala *et al.*, 1995).

According to Ortiz-Rodríguez *et al.* (2012), exposure of the parental generation to certain substances results in more tolerance from future generations. Gustafsson *et al.* (2004), for instance, revealed that parts of *D. magna* populations that had been pre-exposed to toxic cyanobacteria *Microcystis aeruginosa* had a higher tolerance manifested as higher survivorship even under cyanobacterial food than those populations that were not pre-exposed. Results from Sarnelle *et al.* (2005) also indicated that *D. pulicaria* clones isolated from eutrophic lakes were less inhibited by toxic *Microcystis* than *D. pulicaria* isolated from oligotrophic lakes.

This phenomenon is suggested as a result of natural selection driving adaptation to cyanobacteria producing toxic or inhibitory compounds (Sarnelle *et al.*, 2005). When facing water bodies with recurring toxic cyanobacterial blooms, selection may act on zooplanktonic grazers and favor those that evolve resistance to the compounds and are best able to survive, grow, and reproduce (Ferrão-Filho *et al.*, 2011; Hairston *et al.*, 2001; Sarnelle *et al.*, 2005).

In *Daphnia*, many adaptive responses, such as responses to dietary changes in the environment, are maternally transferred to the next generation (Schwarzenberger *et al.*, 2013). Hairston *et al.* (1999) revealed these short-term evolutionary adaptations in *Daphnia* to an hepatotoxin-producing cyanobacterial strain, but no studies have reported this effect stimulated by saxitoxin-producing cyanobacteria, hence the need of future studies to thoroughly analyze these transgenerational effects on the latter.

Nevertheless, it is likely that a disparity in sensibility between Duas Unas and Prata clones of *M. spinosa* comes from the fact that Duas Unas Reservoir is a eutrophic environment, with recorded *R. raciborskii* blooms occurrence, while Prata lake does not show any recorded bloom. It is presumed that previous exposition to cyanobacterium and its metabolites resulted in transgenerational adaptations, justifying the neonates from Prata lake being two times more sensible than those from Duas Unas.

The obtained results reinforce the importance of analyzing whether the test organisms had previous contact with toxicants in their natural environments, seeing that a correlation can be made between tolerance of toxic cyanobacteria by *M. spinosa* and prevalence of cyanobacteria in their habitat. Difference in sensitivity between species and clones of the species is definitely important to factor in when choosing test-organisms in ecotoxicological assays, once transgenerational heritage (rapid evolutionary events) seemingly has great influence in bioindicators' responses to toxicants. Besides, the use of ecologically-relevant test-subjects benefits tropical ecotoxicology, as it prospects tropical standardization and the responses are more relevant to the regions. Furthermore, despite of the difference among clones, native Macrothricidae have shown to be sensitive model organisms and have the potential for biomonitoring toxic cyanobacterial blooms in tropical reservoirs.

ACKNOWLEDGEMENTS

The authors would like to acknowledge the National Research Council (CNPq) for the PhD scholarship and research grant provided to M.C.P.V and S.M.F.O.A., respectively, and the anonymous reviewers for the valuable suggestions on improving this work.

We also thank Dr. Renato Molica (Federal Rural University of Pernambuco) for kindly provide the cyanobacterial strain used in this study and Alane S. A. Santos (Laboratory of Phycology, Federal Rural University of Pernambuco) for kindly provide *M. spinosa* clones used in this study.

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Harmful Algae 95 (2020) 101793

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Morphology and molecular phylogeny of a new PST-producing dinoflagellate species: *Alexandrium fragae* sp. nov. (Gonyaulacales, dinophyceae)

Suema Branco[a,*], Mair M.M. Oliveira[b], Fabiano Salgueiro[c], Mauro C.P. Vilar[d], Sandra M.F.O. Azevedo[d], Mariângela Menezes[a]

[a] Laboratório de Ficologia, Departamento de Botânica, Museu Nacional / Universidade Federal do Rio de Janeiro, Quinta da Boa Vista, s/no, São Cristóvão, 20940-040 Rio de Janeiro, Brazil
[b] Laboratório de Biomineralização, Instituto de Ciências Biomédicas, Universidade Federal do Rio de Janeiro, Cidade Universitária, 21941-590 Rio de Janeiro, Brazil
[c] Laboratório de Biodiversidade e Evolução Molecular, Universidade Federal do Estado do Rio de Janeiro, Av. Pasteur 458, sala 512, Urca, 22290-240 Rio de Janeiro, Brazil
[d] Laboratório de Ecofisiologia e Toxicologia de Cianobactérias, Instituto de Biofísica Carlos Chagas Filho, Universidade Federal do Rio de Janeiro, 21949-902 Rio de Janeiro, Brazil

ARTICLE INFO

Keywords:
Alexandrium fragae sp. nov.
ITS
LSU
rRNA
South Atlantic coastal waters
SSU

ABSTRACT

The genus *Alexandrium* comprises some of the most potentially toxic marine algae. A new toxic species of *Alexandrium, A. fragae* sp. nov., was found in Guanabara Bay, Rio de Janeiro, southern Brazil. The new species produces GTX2&3 and STX. The cell morphology of *A. fragae* resembles *A. minutum* in many characters, including the small size; the rounded-elliptical shape; and the shapes of the apical pore complex (APC), first apical plate (1′), sixth precingular plate (6″), and anterior and posterior sulcal plates (s.a. and s.p.). The main diagnostic characters of *A. fragae* are the ornamentation pattern, smooth epitheca and reticulated hypotheca, all of which were present in both natural populations and cultures. Phylogenies inferred from the ITS, LSU, and SSU rDNA of *A. fragae* showed that *A. fragae* clustered in a well-supported clade, distinct from other *Alexandrium* species. Morphology and molecular analyses based on ITS and LSU rDNA indicated that *A. fragae* strains and *Alexandrium* sp. from Japan (D163C5, D164C6) are a single species. Our findings suggest that the *Alexandrium* morphotype with a smooth epitheca and reticulated hypotheca, previously identified as *A. minutum* in different geographic regions, may corresponds to *A. fragae*.

1. Introduction

The members of the dinoflagellate genus *Alexandrium* Halim, which occurs worldwide in coastal waters, have been studied intensively in recent decades. This is largely because several of the species form blooms and produce potent toxins that are responsible for losses of wild and cultured shellfish, and also cause human illness from consumption of contaminated shellfish or bony fish (Anderson et al., 2012; Lim et al., 2007). Species of *Alexandrium* are identified from morphological characteristics such as the cell size and shape, and the arrangement of the plate tabulation (Balech, 1995). However, delimitation of *Alexandrium* species based on morphological criteria has been problematic because of the morphological plasticity of certain characters, e.g., the presence or absence of the ventral pore and the position of the anterior attachment pore (Leaw et al., 2005; Touzet et al., 2008; Varela et al., 2012; John et al., 2014), often leading to misidentification. Incorporation of molecular techniques into taxonomic and phylogenetic studies of *Alexandrium* has helped to improve species identification and the circumscription of the genus (Anderson et al., 2012; John et al., 2014). Approaches applying molecular markers have also revealed a high degree of cryptic diversity in *Alexandrium* (Genovesi et al., 2011; John et al., 2014; Laporte et al., 2014), as well as phenotypic and genetic diversity (McCauley et al., 2009; Casabianca et al., 2012; Toebe et al., 2013; Dia et al., 2014).

The genus encompasses about 30 currently accepted taxa (Anderson et al., 2012). Although some species of *Alexandrium* have been documented in South America (Balech, 1995, 2002; Montoya et al., 2010; Almandoz et al., 2014), the local diversity of this genus continues to be underestimated because of the small number of studies of material from coastal samples. Until now, nine species of *Alexandrium* have been

* Corresponding author.
E-mail address: branco.suema@mn.ufrj.br (S. Branco).

https://doi.org/10.1016/j.hal.2020.101793
Received 21 October 2019; Received in revised form 27 February 2020; Accepted 1 March 2020

recorded in South America: *A. affine* (Inoue & Fukuyo) Balech, *A. catenella* (Whedon & Kofoid) Balech, *A. fraterculus* (Balech) Balech, *A. gaarderae* Nguyen-Ngoc & Larsen [= *A. concavum* (Gaarder) Balech (Larsen and Nguyen-Ngoc, 2004)], *A. kutnerae* (Balech) Balech, *A. monilatum* (Howell) Balech, *A. ostenfeldii* (Paulsen) Balech & Tangen [= *A. peruvianum* (Balech & Mendiola) Balech & Tangen (Kremp et al., 2014)], *A. tamiyavanichii* Balech, and *A. tamutum* Montresor, Beran & John (Balech, 1995; Vera et al., 1999, 2002; Montoya et al., 2010; Varela et al., 2012; Almandoz et al., 2014; Menezes et al., 2018). Of these, *A. fraterculus, A. gardnerae, A. kutnerae, A. catenella, A. tamiyavanichii* and *A. tamutum* have been recorded from Brazil (Balech, 1979; Menezes et al., 2018). Also, cysts of *Alexandrium* cf. *minutum* and populations of *A. minutum* Halim were reported from Rio de Janeiro State by Juliano and Garcia (2006) and Menezes et al. (2007) respectively. The latter formed an extensive toxic bloom along the beaches of Rio de Janeiro city on 23 April 2007 (Menezes et al., 2007).

Here, we describe the species *A. fragae* sp. nov., based on morphological investigations and phylogenetic analyses of ITS, LSU, SSU, and rDNA sequences. We hypothesize that *A. fragae* is probably the bloom-forming species identified as *A. minutum* by Menezes et al. (2007) from Rio de Janeiro beaches. This new taxon is compared with the morphotypes of *A. minutum* with pronounced reticulation on the hypotheca that were found in the Gulf of Naples (Montresor et al., 1990; Balech, 1995), Japan (Yuki, 1994; Kaga et al., 2006), Jamaica (Ranston et al., 2007), and Hong Kong (Law and Lee, 2013).

2. Methods

2.1. Field samples

Phytoplankton samples containing cells of *Alexandrium* were collected from subsurface waters in Guanabara Bay, Rio de Janeiro, Brazil (22.7902°S, 43.1057°W) (Fig. 1) in February 2014. These samples were used to establish cultures (by cell isolation) and for morphological analyses. A fixed sample from a bloom of *Alexandrium* collected on the Rio de Janeiro coast in April 2007 (Menezes et al., 2007) was also examined but was used only in the morphological analyses.

2.2. Isolation and culture conditions

Single cells were isolated from Guanabara Bay water samples, with a micropipette, and placed in 96-well plates containing 200 µL of K medium (Keller et al., 1987). The strains were maintained in K medium, salinity 30, at a temperature of 20–23 °C, photon flux density of 70 µE $m^{-2}.s^{-1}$, and 12:12 h light: dark photocycle.

2.3. Morphological analysis

For cell characterization and measurements, live cells from natural populations and cultures were examined under an Olympus BX51 microscope (Olympus, Tokyo, Japan) with a QCapture Suite image system, version 2.68 (QImaging, Bethesda, MD, USA). The thecal plates were stained with 1% calcofluor white (Fritz and Triemer, 1985) and observed with the same microscope, equipped with a UV mercury lamp and an Olympus UV filter set.

Ultrastructural details were examined by means of scanning electron microscopy. The cells were fixed with 2% paraformaldehyde for 2–24 h and adhered to a glass plate coated with poly L-lysine. They were washed with 0.1 M sodium cacodylate buffer and then dehydrated through an ethanol series. The dehydrated cells were dried in a critical-point dryer, sputter-coated with gold, and observed with JEOL/EO 1.0 JSM-6390/6510, 15/30 kV, WD 12/10 mm (Peabody, MA, USA) and FEI Quanta 250, 20–25 kV, WD 9.6–10.2 mm (Brno, Czech Republic) scanning electron microscopes. The dried holotype material of this strain was deposited in the Museu Nacional, Universidade Federal do Rio de Janeiro, Brazil.

2.4. PCR amplification and DNA sequencing

The algal DNA was extracted from 5 mL of exponentially growing culture, using a NucleoSpin® Plant II extraction kit (Macherey-Nagel GmbH & Co. KG, Duren, Germany) according to the manufacturer's protocol. The SSU, LSU (D1–D3) and ITS (including 5.8S rDNA gene) regions of rDNA were amplified using primers 18ScomF1 (5′-GCTTGT CTCAAAGATTAAGCCATGC-3′) and 18ScomR1 (5′-CACCTACGGAAAC CTTGTTACGAC-3′) (Zang et al., 2005); D3F (5′-ACGAAGGATTTGCAC GTCAG-3′) and D1R (5′-ACCCGCTGAATTTAAGCATA-3′) (Scholin et al., 1994; Litaker et al., 2003); and ITSA (5′-GTAACAAGGTHTCCG-TAGGT-3′) and ITSB (5′-AKATGCTTAARTTCAGCRGG-3′) (Sato et al., 2011), respectively. The PCR mixture of 25 µL contained 1 U GoTaq® DNA polymerase (Promega, Madison, WI, USA), 1 × GoTaq® Flexi buffer (Promega), 1.25 mM $MgCl_2$ solution (Promega), 0.16 mM dNTPs (Thermo Scientific, Inc., USA), 8 pmol of each primer, 0.2 µg of Bovine Serum Albumin (BSA) (New England Biolabs, Inc.) and ca. 15 ng genomic DNA. The polymerase chain reaction (PCR) protocol was as follows: initial denaturation for 5 min at 94 °C, followed by 35 cycles of

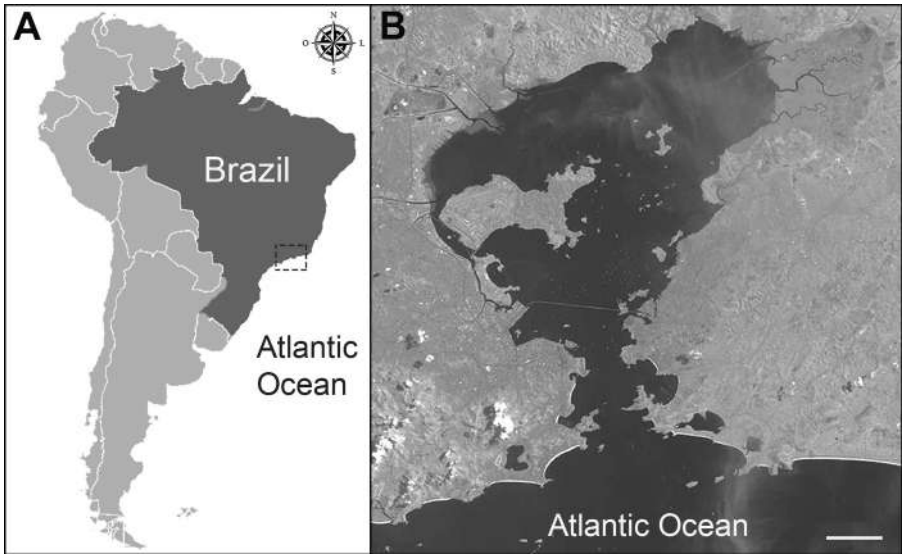

Fig. 1. A) Map of South America showing Brazil with the study area (dotted area). B) The study area in Guanabara Bay. Scale bar: 7 km.

1 min denaturation at 94 °C, 1 min annealing at 50, 55 or 58 °C (ITS, LSU or SSU, respectively) and 1 min extension at 72 °C, plus a final extension of 5 min at 72 °C. The PCR products were purified and sequenced by Macrogen (Seoul, Korea) in both directions, using the PCR primers. The sequences obtained were deposited in GenBank.

2.5. Phylogenetic analysis

Phylogenetic analyses were performed for each rDNA region (ITS, LSU and SSU) individually. *Alexandrium* sequences obtained from this study and from GenBank (S1–3) were aligned using the online package MAFFT version 7 (https://mafft.cbrc.jp/alignment/server/), followed by manual editing. The Maximum Likelihood (ML) analyses were conducted using the software MEGA 7.0 (Kumar et al., 2016). The evolutionary model used was the Tamura-Nei model with gamma distributed sites (TN93 + G), which was the best available for the tree of the rDNA region data (ITS, LSU and SSU), using MEGA 7.0 (Kumar et al., 2016). The robustness of the inferred topology was tested by bootstrap resampling (1000 replicates). Bayesian inferences (BI) were obtained with the software MrBayes 3.2 (Ronquist et al., 2012). Rather than selecting a nucleotide substitution model by a priori model selection, we used the "lset nst = mixed" Markov Chain Monte Carlo procedure, consisting of two independent trials with four chains. The chains were run for 5000,000 generations and sampled every 100th cycle. Posterior probability (PP) values for the resulting 50% majority-rule consensus tree were estimated after discarding the first 20% of the trees as burn-in. All trees were rooted using *Alexandrium affine, A. fraterculus* and *A. tamiyavanichii* sequences as outgroup.

Average evolutionary genetic distances (*p*-distance) between sequences were estimated using the software MEGA 7.0 (Kumar et al., 2016). The analyses were conducted using the Maximum Composite Likelihood model (Tamura et al., 2004). All positions containing gaps and missing data were eliminated. The analysis involved 33 SSU sequences, with a total of 1584 positions in the final dataset; 87 LSU sequences, with a total of 477 positions in the final dataset; and 68 ITS sequences, with a total of 382 positions in the final dataset.

2.6. Paralytic shellfish toxin extraction and analysis

To determine the production of paralytic shellfish toxins (PSTs, also known as saxitoxins) by *A. fragae* strains, batch cultures harvested in the late exponential phase were filtered on borosilicate filters (45-mm diameter) to concentrate the cell biomass and then extract the toxins using 0.5 M acetic acid. After centrifuging (20,000 × g), the supernatant was collected and filtered through 0.22 µm Millex (Millipore) prior to injection. High-performance liquid-chromatography analyses were performed on a Shimadzu HPLC, using a silica-base reverse-phase column (125 mm × 4.0 mm, 5 µm; C18 Phenomenex) and separations were carried out under specific mobile phases for non-sulfated PSTs (saxitoxin, neosaxitoxin and decarbamoyl-saxitoxin, hereafter STX, neoSTX and dcSTX respectively) and sulfated PSTs (Gonyautoxins 1&4, 5 and 2&3, hereafter GTX 1&4, GTX 5 and GTX 2&3, respectively) coupled to post-column oxidation, as described by Oshima (1995). Fluorescent saxitoxin derivates were detected using a Shimadzu RF-10Axl fluorometric detector with excitation at 330 nm and emission at 390 nm. Toxins were identified by comparison with known retention times and integrated areas of standards, respectively. The standards of all toxins were purchased from the Institute for Marine Biosciences, National Research Council of Canada (Halifax, Canada).

Also, in order to detect saxitoxin potentially produced in amounts below the limit of detection (< 2.24 µg L^{-1}) in HPLC, the STX content in the cell extract was quantified using the enzyme-linked immunosorbent assay (ELISA) commercial kit (Beacon Analytical Systems Inc., USA) in a HIDEX sense microplate reader (HIDEX, Finland) at absorbance 450 nm.

3. Results

3.1. Morphological analyses

Alexandrium fragae sp. nov. S. Branco and M. Menezes (Fig. 3B).

Diagnosis. Single cell; elliptical, ovate to spherical, often with a slightly pentagonal outline; 18.5–31.8 µm long, 16.8–25.6 µm wide. Cingular small lists along the sutures between cingular plates and both epithecal and hypothecal plates; sulcal lists along the sutures between sucal and hypothecal plates. Theca surface with irregularly distributed pores; epitheca smooth, hypotheca reticulated. Plate formula Po, 4′, 6″, 6c, 8 s, 5‴ and 2⁗. Apical pore complex obovate to elliptical; apical pore comma-shaped; anterior attachment pore not observed. Plate 1′ irregularly rhomboidal, with connection to apical pore complex. Ventral pore located on either anterior or posterior half of anterior right margin of plate 1′. Plate 6″ longer than wide or as long as wide. Plate s.a. longer than wide or as long as wide. Plate s.p. wider than long or as long as wide; posterior attachment pore not observed. Phototrophic. The species is defined by the nucleotide sequences of the holotype strain UFRJ-MN01: SSU (GenBank: KX097019), LSU (GenBank KX097015), ITS (GenBank KX097011).

Etymology: The species epithet is dedicated to dr. Santiago Fraga, for his significant contributions to the taxonomy of harmful marine dinoflagellates.

Holotype here designated: Fig. 3B from strain UFRJ-MN01 conserved on an SEM stub (designation R 232655), deposited at the herbarium of the Museu Nacional, Universidade Federal do Rio de Janeiro, Brazil.

Isotype here designated: Strain UFRJ-MN02 conserved on an SEM stub (designation R 232,654), deposited at the herbarium of the Museu Nacional, Universidade Federal do Rio de Janeiro, Brazil.

Type locality: Guanabara Bay, Rio de Janeiro, Brazil.

Reference Strains: Strains UFRJ-MN01 and UFRJ-MN02, deposited in the Phycological Culture Collection of the Museu Nacional, Universidade Federal do Rio de Janeiro.

Cells of both cultures (UFRJ-MN01 and UFRJ-MN02) have shapes ranging from elliptical or ovate to spherical, often with a slightly pentagonal outline (Figs. 2A; 3A–C; 4A–B; 5A and D). The epitheca is hemispherical or conical, with the sides convex or almost straight (Fig. 3A); the epitheca and hypotheca are usually equal in size, and sometimes the epitheca is slightly larger than the hypotheca. Cell size ranged from 18.5 to 31.8 µm in length and from 16.8 to 25.6 µm in width, L:W ratio 1.1– 1.3 ($n = 100$ cells). The cingulum is markedly excavated, with the right end displaced one cingular width toward the posterior pole of the cell (Fig. 3B). The margins of the cingulum and hypothecal sulcus generally have lists (Fig. 3B).

The plate pattern is APC, 4′, 6″, 6c, 8 s, 5‴ and 2⁗ (Fig. 4). The first apical plate (1′) has four or five sides, with a direct or indirect connection to the APC, and the posterior margin is generally straight, sometimes slightly concave or convex (Figs. 3B and D; 5A–D). The ventral pore is generally located on the anterior or posterior half of the margin suture between plates 1′ and 4′ (Figs. 3B and D; 5A–C) or, rarely, on the suture between plates 1′ and 6″ (Fig. 5D). The sixth precingular (6″) plate is generally pentagonal, longer than wide, or sometimes as long as wide (Figs. 3B and D; 5A–D). The apical pore complex (APC) shape is oboval to elliptical, with the apical pore comma-shaped on the center, and small pores on the plate margin (Fig. 5G). An anterior attachment pore in the apical pore complex was not observed. The anterior sulcal plate (s.a.) is as long as wide or longer than wide, with the anterior margin straight or convex and the posterior margin concave, sometimes with a more-developed projection to the right (Figs. 3B and D; 5C and D). The posterior sulcal plate (s.p.) is wider than long or as long as wide (Figs. 3D; 5H). Posterior and anterior attachment pores were not observed. All sulcal plates were easily observed in light microscopy after the cells were stained with calcofluor (Fig. 3D). The variations found in the shape of the main *A. fragae* plates

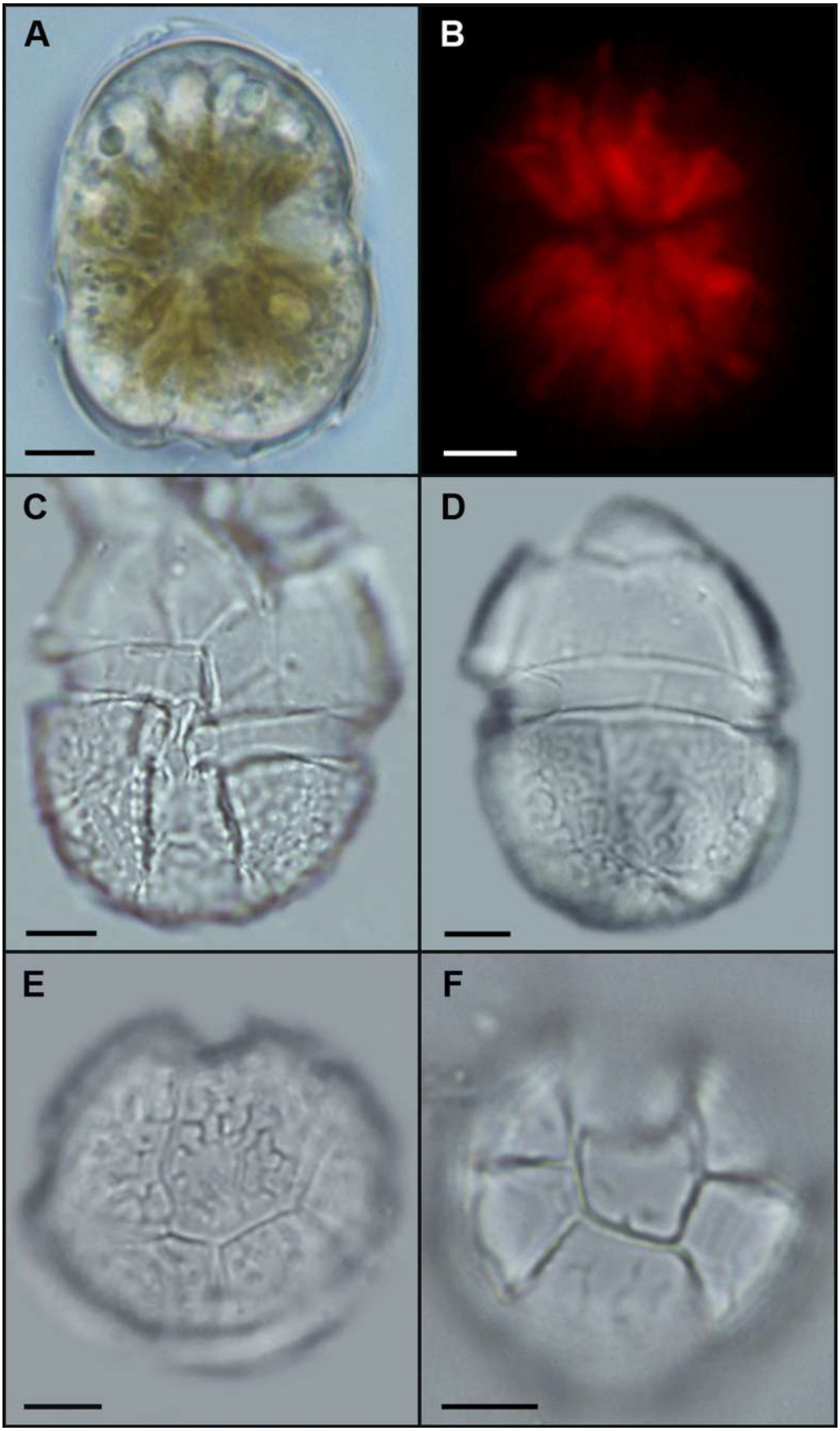

Fig. 2. Light and epifluorescence micrographs of *Alexandrium fragae* sp. nov. in exponential growth phase (strain UFRJ-MN02). A) Live cell in ventral view. B) Live cell with chloroplast autofluorescence. C-F) Thecal plates showing the ornamentation pattern, with epitheca smooth and hypotheca reticulated. C) Ventral view. D) Dorsal view. E) Antapical view showing strong reticulation. F) Antapical view showing slight reticulation. Scale bars: 5 μm.

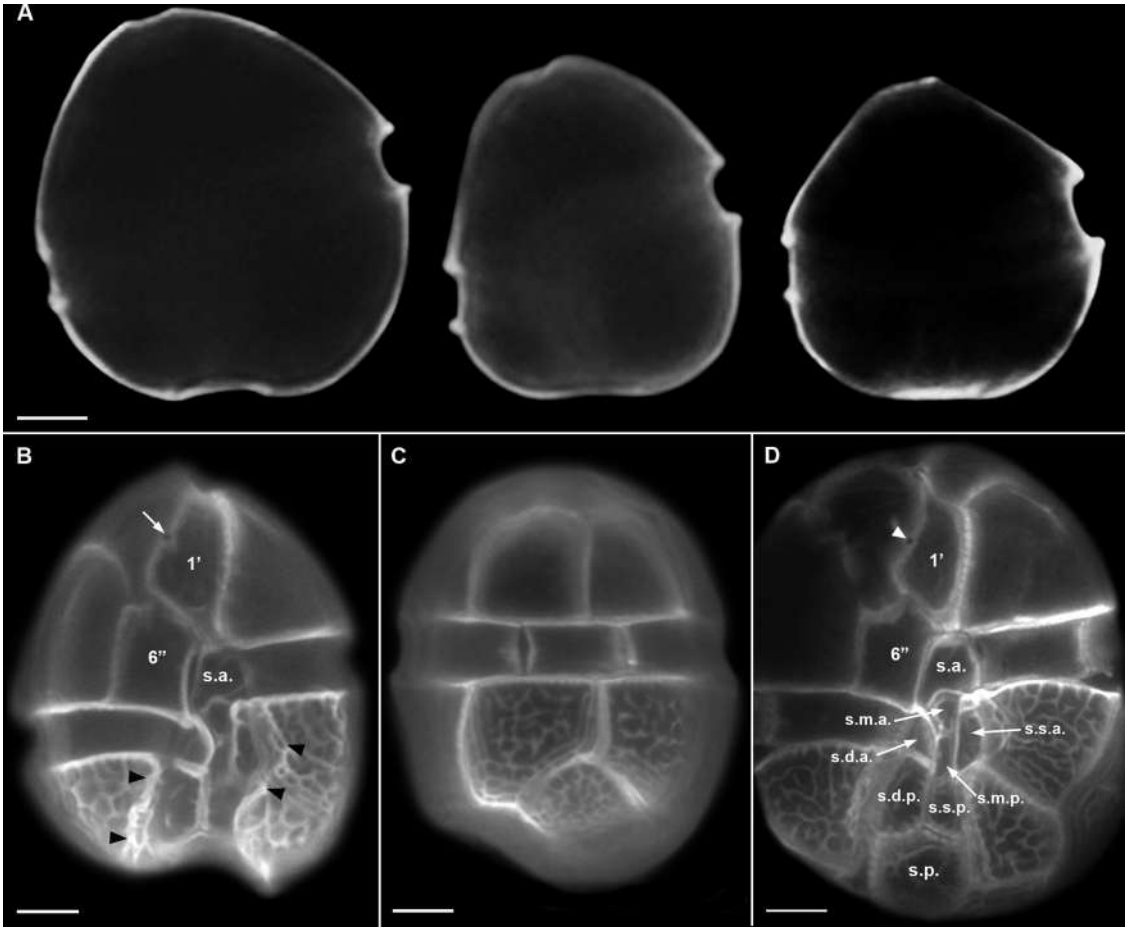

Fig. 3. Epifluorescence micrographs of *Alexandrium fragae* sp. nov. in exponential growth phase (A, strain UFRJ-MN02; B-D, strain UFRJ-MN01). A) Cell shape variations. B) Ventral view showing the first apical plate (1′) with a ventral pore (white arrow), the sixth precingular plate (6″), and the sulcus with well-developed lists (black arrowheads). C) Dorsal view. D) Ventral view showing the first plate (1′), the sixth precingular plate (6″), and eight sulcal plates. Sulcal plate abbreviations: s.a. = anterior sulcal plate; s.p. = posterior sulcal plate; s.s.a. = left anterior sulcal plate; s.s.p. = left posterior sulcal plate; s.d.p. = right posterior sulcal plate; s.d.a. = right anterior sulcal plate; s.m.a. = anterior median sulcal plate; s.m.p. = posterior median sulcal plate. Scale bars: 5 μm.

(Po, 1′, 6″, Sa, Sp) used in taxonomic identification are shown in Supplementary material S4.

The theca shows a distinct ornamentation pattern, easily observed in light and electron microscopy. The epitheca has a smooth surface and the hypotheca has reticulation (Figs. 2C–F; 3B–D; 5). Both the epitheca and hypotheca have small and irregularly distributed pores; a pore row is present on the cingulum margins (Fig. 5I). The cingular plates appear to have no reticulation (Fig. 3B–D). The sulcal plates often show strong reticulation, except the anterior sulcal plate (s.a.) (Fig. 3D).

The pattern of ornamentation varied gradually throughout the

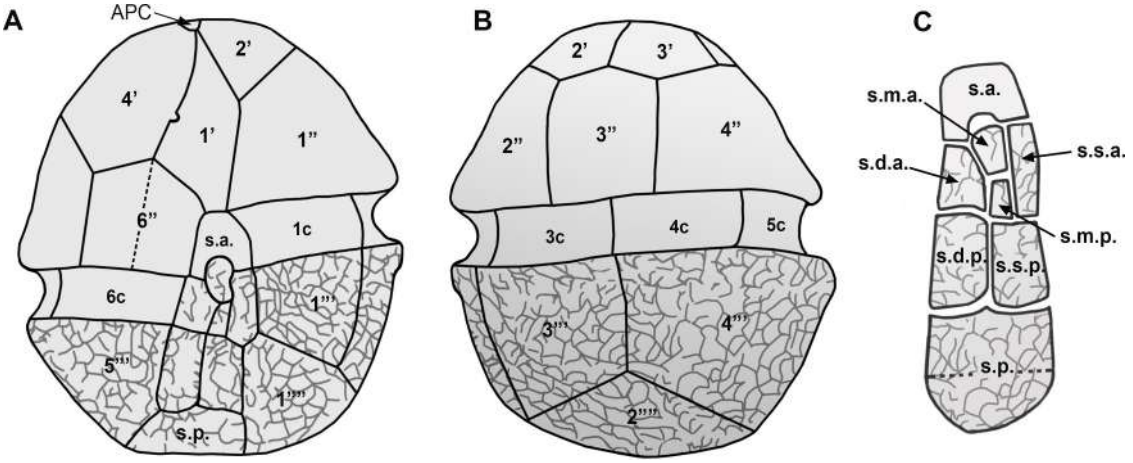

Fig. 4. Drawings of *Alexandrium fragae* sp. nov. A) Ventral view; the dashed line on the middle of the sixth precingular plate (6″) indicates that this plate can vary from as long as wide to longer than wide. B) Dorsal view. C) Sulcal plates; the dashed line on the middle of posterior sulcal plate (s.p.) indicates that this plate can vary from as long as wide to wider than long.

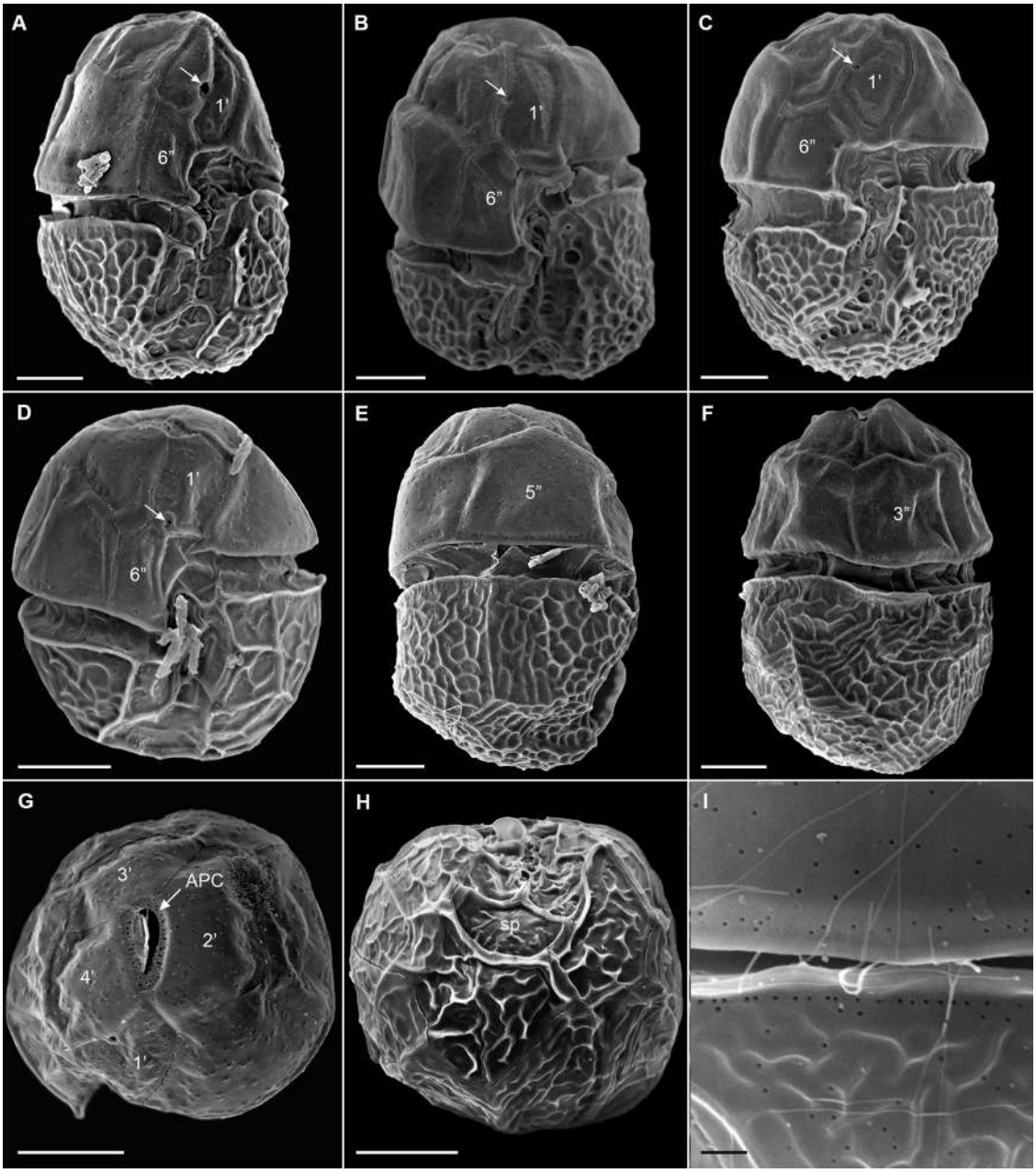

Fig. 5. Scanning electron micrographs of *Alexandrium fragae* sp. nov. in exponential growth phase (strain UFRJ-MN02). A-D) Ventral view showing the first apical plate (1′) with a ventral pore (arrow) and the narrow (A, B and D) and wide (C) sixth precingular plate (6″). E) Right lateral view. F) Dorsal lateral view. G) Apical view showing the apical pore complex (APC) connected to the first apical plate (1′). H) Antapical view showing the posterior sulcal plate (s.p.). I) Small and irregularly distributed pores on the epitheca and hypotheca and a pore row on the cingulum margin. Cell ornamentation pattern with epitheca smooth and hypotheca reticulated, both with pores irregularly distributed and pore row on the cingulum margins. Scale bars: 5 μm (A-H) and 1 μm (I).

development of the theca. During the initial exponential growth phase, the following ornamentation patterns have been found: i) few young cells with smooth hypothecal plates (n = 3 cells, Fig. 6A), ii) some cells with weak discontinuous ridges irregularly distributed on the hypothecal plates (n = 20 cells, Fig. 6B–E), and iii) many cells with slight reticulation with shallow polygonal areolae on the hypothecal plates (n = 97 cells, Fig. 6F and G). At the end of the exponential growth phase, the hypothecal plates exhibit strong reticulation with deep polygonal areolae (n = 200 cells, Fig. 6H). At the end of the exponential growth phase and stationary growth phase, hypothecal plates with different degrees of reticulation have been found in the same cell (Figs. 6I; S5).

Planozygotes with two longitudinal flagella (S5), pellicle cysts (S5), and couplets of recently divided cells (data not shown) were often observed in growing cultures. Sometimes, armored cell pairs, unequal in size and attached through the sulcal regions, were observed (S5).

Light- and electron-microscopy analyses of a wild population of *Alexandrium*, sampled from the bloom at Rio de Janeiro in 2007, showed cells with shapes and dimensions within the range of variation observed for cultured material: ovate, irregularly elliptic to almost spherical, 17.1 to 32.3 μm in length and 15.2 to 32.3 μm in width, and L:W ratio 0.9– 1.3 (n = 100 cells) (Fig. 7). Also, there were no differences in the plate pattern of the theca, in the lack of decoration on the epitheca surface, or in the presence and variation of the degree of ornamentation on the hypotheca surface (Fig. 7C–H). Couplets of recently divided cells (Fig. 7A) and pellicle cysts (Fig. 7B) were numerous.

The morphological features of *A. fragae* and morphologically closely related species of *Alexandrium* (*A. minutum, A. tamutum, A. insuetum* and

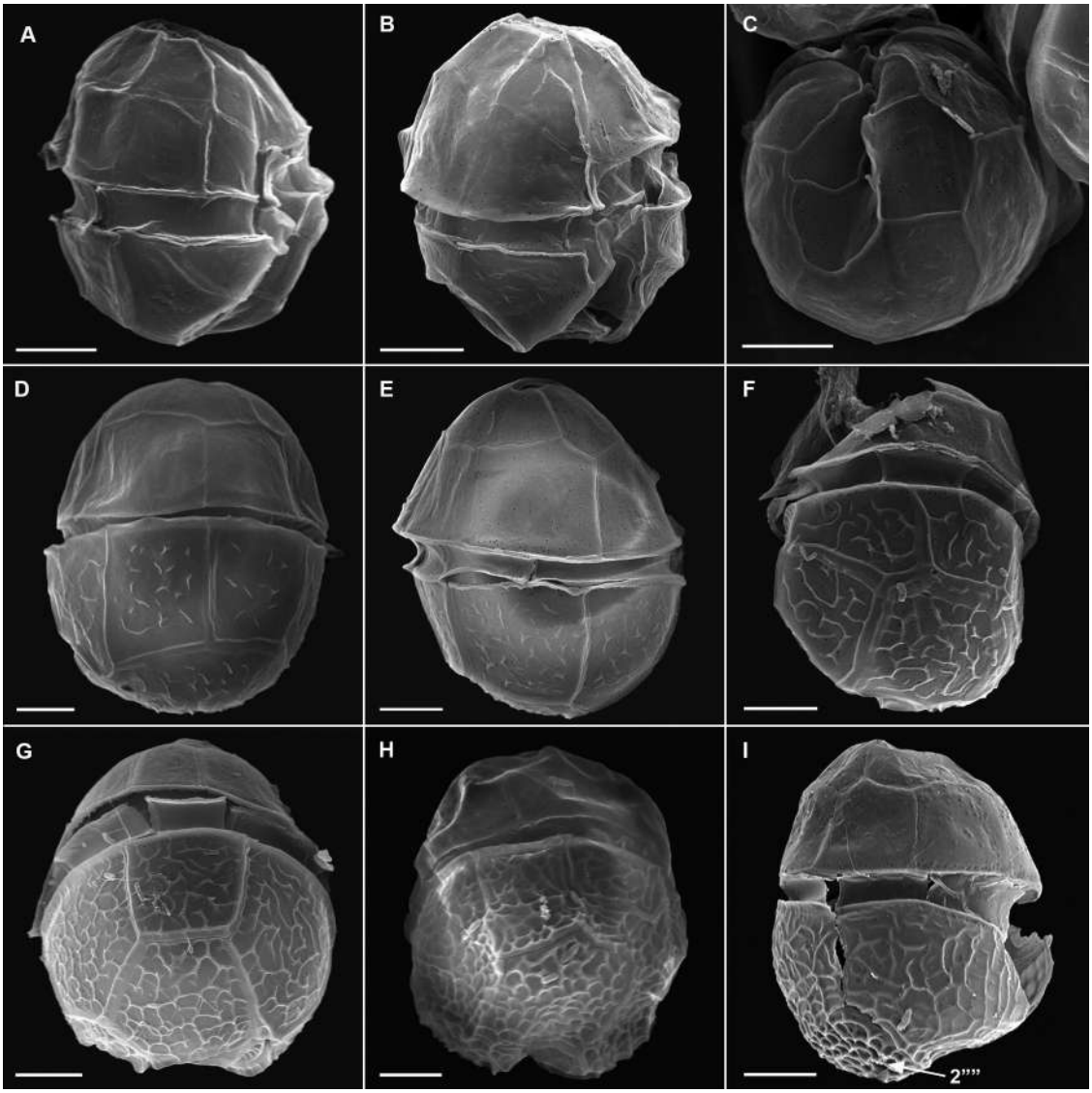

Fig. 6. Scanning electron micrographs of *Alexandrium fragae* sp. nov. (strain UFRJ-MN02) in the initial (A-G) and final exponential growth phase (H and I). A-H) Cells with different degrees of reticulation on the hypotheca. I) Cell with unequal ornamentation on the hypotheca, showing the second antapical plate (2‴) with more-developed reticulation. Scale bars: 5 μm.

A. balechii) are compared in Table 1 and Fig. 8.

3.2. Phylogenetic analyses

Genetic data for SSU, LSU and ITS rDNA were successfully obtained from *A. fragae* strains UFRJ-MN01 and UFRJ-MN02. In sum, 1634 and 1621 phylogenetically informative bp of the sequence were obtained from sequencing the SSU region, 848 and 845 bp for the LSU, and 565 and 518 bp for the ITS/5.8S rDNA of strains UFRJ-MN01 and UFRJ-MN02, respectively.

All phylogenetic trees based on SSU, LSU and ITS rDNA recovered the same clades of *Alexandrium* species (Figs. 9–11, respectively). The relationships among *Alexandrium* species clades were most noticeable in the SSU rDNA tree (Fig. 9). The BI and ML trees based on SSU rDNA sequences showed two main groups: i) a group composed of *A. minutum, A. fragae* and *A. insuetum* (PP = 0.54; BS = 52%), and ii) a group composed of *A. tamutum, A. ostenfeldii* and *A. andersonii* (PP = 0.96; BS < 50%). *Alexandrium fragae* sequences formed a highly supported clade (PP = 1; BS = 100), which grouped close to the *A. minutum* clade with moderate BI support (PP = 0.85).

The BI and ML trees based on the LSU and ITS rDNA sequences (Figs. 10 and 11, respectively) showed that sequences of *A. fragae* (strains UFRJ-MN01 and UFRJ-MN02) grouped together with sequences of two strains of *Alexandrium* sp. from Japan (strains D163C5 and D164C6 in Lilly et al., 2005). The clade composed of the Brazilian sequences (strains UFRJ-MN01 and UFRJ-MN02) and strains from Japan (D163C5 and D164C6) had a high support value (PP = 1.0; BS = 99%), forming a monophyletic group separate from other recognized *Alexandrium* species. The ML and BI trees based on LSU rDNA sequences showed the *A. fragae* strains (UFRJ-MN01 and UFRJ-MN02) and strains from Japan (D163C5 and D164C6) close to the *A. andersonii* clade, with low support (PP = 0.61; BS = 59%).

The average genetic distances (*p*-distance) between sequences of *Alexandrium* species used in the phylogenetic analyses were 0.006–0.080 for SSU, 0.030–0.270 for LSU and 0.047–0.396 for ITS rDNA. The average genetic distances between *A. fragae* and other *Alexandrium* species were 0.028–0.080 for SSU, 0.092–0.206 for LSU and 0.142–0.374 for ITS rDNA. The average genetic distances between *A. fragae* sequences from Brazil (strains UFRJ-MN01 and UFRJ-MN02) and *Alexandrium* sp. sequences from Japan (strains D163C5 and

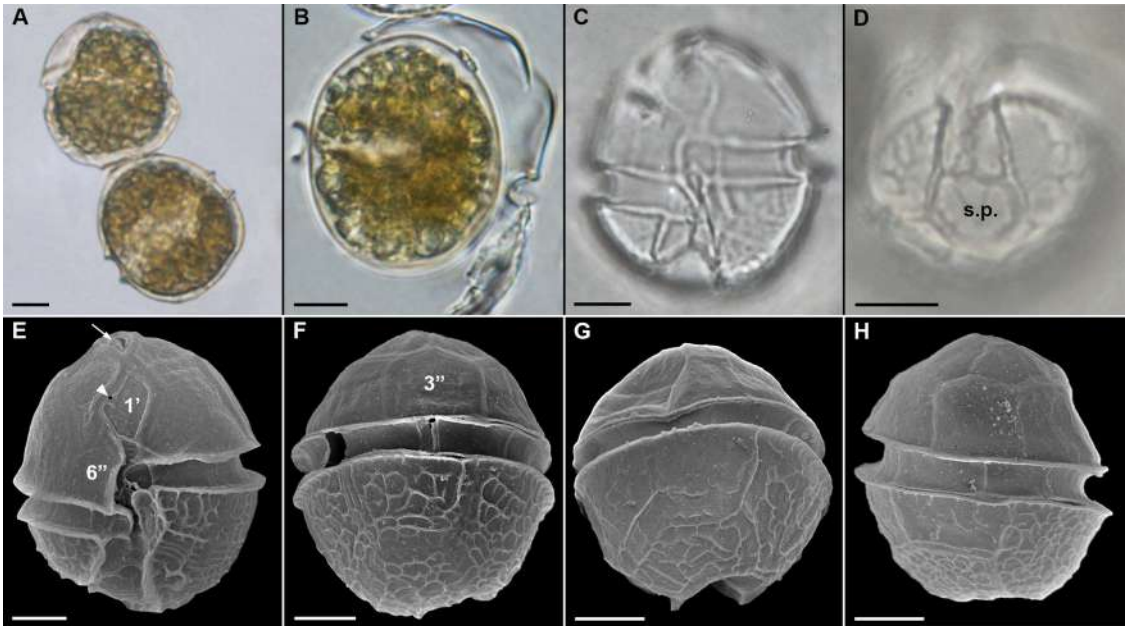

Fig. 7. Light and scanning electron micrographs of bloom-forming *Alexandrium* sp. (natural population from bloom in 2007). A) A couple of recently divided cells. B) Pellicle cyst. C) Ventral view. D) Antapical view showing reticulation on the hypotheca. E) Ventral view showing the first apical plate (1′) with a ventral pore (arrowhead), the sixth precingular plate (6″), and the apical pore complex (arrow). F-H) Dorsal view showing strong (F) and unequal (G and H) reticulation on the hypotheca. Scale bars: 5 μm.

D164C6 from GenBank) were low, 0.013 for LSU and 0.029 for ITS rDNA, compared to the average distance between the species of the genus. All average genetic distances obtained are listed in Supplementary material S6–8.

3.3. Profile of paralytic shellfish toxins

Analysis of PSTs of two *A. fragae* isolates by HPLC-RF revealed that only strain UFRJ-MN01 produced gonyautoxin (GTX 2&3) (Supplementary material S9 and 10). However, ELISA analysis was able to show saxitoxin production in both UFRJ-MN01 and UFRJ-MN02. A total amount of 0.036 ng_{GTXs} mL^{-1} was obtained from UFRJ-MN01 cell extract, with a total cellular toxin concentration of 2.82 fg_{GTXs} $cell^{-1}$, which indicated a relative production of 51.42 and 48.58% of GTX-2 and −3, respectively. Moreover, both *A. fragae* UFRJ-MN01 and UFRJ-MN02 produced average saxitoxin amounts of 0.0012 and 0.0010 ng_{STX} mL^{-1}, corresponding to 0.094 fg_{STX} $cell^{-1}$ and 0.144 fg_{STX} $cell^{-1}$, respectively. According to the toxin analysis, *A. fragae* strains displayed a total cell toxin quota of 2.914 and 0.144 fg_{PSTs} $cell^{-1}$. Furthermore, despite the differences in the cellular toxin content, embryo-larval toxicity tests with sea urchins and the brine shrimp *Artemia salina* demonstrated harmful effects of both *A. fragae* strains (data not shown).

4. Discussion

4.1. Morphology

Alexandrium fragae shares several morphological characters with *A. minutum*, such as the small cell size and the morphology of the first plate (1′), apical pore complex (APC), anterior sulcal plate (s.a.), and posterior sulcal plate (s.p.). The two species also have the ventral pore, when present, on the suture between the 1′ and 4′ plates, and the absence of an attachment pore in the apical pore complex (APC) and in the posterior sulcal plate (s.p.). Furthermore, the strains of *A. fragae* show the same variation in the aspect ratio of plate 6″, which can vary from as long as wide to longer than wide, as recorded for strains of *A. minutum* by Lilly et al. (2005, Table 2 in Lilly et al., 2005) and Lim et al. (2007, Fig. 3 and Table 3 in Lim et al., 2007). However, the ornamentation pattern of the theca differs between the two species. Cells of *A. fragae* in both the cultures and the natural populations showed a smooth epitheca and reticulated hypotheca. *Alexandrium minutum* was described originally by Halim (1960) with smooth plates, and later redescribed by Balech (1989) with a thin thecal wall and a very faint, irregular and incomplete reticulum often present on some plates, especially on the s.p. and 1′ plates. Other authors have identified natural populations and strains as *A. minutum,* with plates entirely smooth or with very fine ornamentation consisting of an areolated theca and primitive reticulation on some plates (Balech, 1995; Hansen et al., 2003; Lilly et al., 2005).

Some natural populations and strains identified as *A. minutum* have also been described with strong reticulation on the hypotheca. These reticulated morphotypes of *A. minutum* were found in the Gulf of Naples (Montresor et al., 1990; Balech, 1995), Japan (Yuki, 1994; Kaga et al., 2006), Jamaica (Ranston et al., 2007), Hong Kong (Law and Lee, 2013) and Brazil (Menezes et al., 2007). This last population was found forming a bloom along some beaches of Rio de Janeiro city, very near the type locality of *A. fragae.* Paralytic shellfish-poisoning toxin profiles have been confirmed in this population (Menezes et al., 2007). Unfortunately, to our knowledge no molecular data for a reticulated *Alexandrium* morphotype from the Gulf of Naples, Jamaica or Hong Kong are available. Nor do we have molecular data for the *A. minutum* morphotype identified from Rio de Janeiro city. However, Lilly et al. (2005) provided a phylogenetic analysis of *A. minutum* morphotypes with a reticulated hypotheca isolated from Japan (strains D163C5 and D164C6); their results grouped the two strains (with 100% similarity) as a separate group from the *A. minutum* clade. The reticulated morphotypes of *Alexandrium* isolates from Japan (strains D163C5 and D164C6) showed a similar morphology to the Brazilian isolates of *A. fragae,* leading us to consider them as a single species, which was corroborated by the molecular analysis (see below). However, there are no accurate studies of the morphology of these isolates from Japan (strains D163C5 and D164C6).

The lack of comparative data on the presence of reticulation on the hypotheca in natural populations associated with cultures of morphotypes identified as *A. minutum* has generated uncertainty about the stability of this character in the taxonomy of the group (Montresor

Table 1.
Comparison of morphological features of *Alexandrium fragae* sp. nov. and morphologically similar species.

Character	Alexandrium fragae sp. nov.	Alexandrium minutum[a, b, c]	Alexandrium tamutum[d]	Alexandrium insuetum[e]	Alexandrium balechii[e]
Cell shape	Asymmetric, oval to spherical	Oval to elliptical[b]	Round to elliptical	Oval	Epitheca long and conical, hypotheca rounded or somewhat irregular to trapezoidal
Cell dimensions ($l \times w$, μm)	18.5- 31.8 × 16.8- 25.6	16.0–29.0 × 13.0–24.0[a, b]	19.0–34.0 × 19.0–30.2	26.5–28.5 × 24.0–26.5	25.0–42.0 × 24.0–39.5
Apical pore complex (APC)	Oval to elliptical or comma-shaped	Oval to elliptical[b]	Slightly convex on left margin and straight on right margin	Oval to almost triangular	Oval
Apical attachment pore	Not observed	Not observed [a, b, c]	Not observed	Not observed (?)	Not observed (?)
First apical plate (1′)	Irregularly rhomboidal; straight or concave anterior right margin, and straight anterior and posterior left margin	Rhomboidal; long anterior right margin and posterior left margin[b]	Irregularly rhomboidal; straight anterior right margin	"Goniodoma-type"; right margin almost straight and left margin longer than other sides and convex	Irregularly pentagonal
Contact between APC and 1′	Present (direct or indirect)	Present (direct or indirect)[b, c]	Present	Absent	Absent
Ventral pore	Present	Present[b,c] or absent[c]	Present	Present	Absent
Sixth precingular plate (6″)	Pentagonal; narrow ($l > w$) or wide (l ≅ w)	Pentagonal; narrow ($l > w$)[a, b, c] or wide (l ≅ w)[c]	Pentagonal; wide (l ≅ w)	Pentagonal; $l > w$	Triangular
Anterior sulcal plate (s.a.)	$l > w$ or l ≅ w	$l \geq w$[b]	l ≅ w	$l > w$	Wide, short and oblique
Posterior sulcal plate (s.p.)	$w > l$ or l ≅ w	$w > l$[b]	$w > l$	$w > l$	Narrow, long, almost arrowhead-shaped
Posterior attachment pore	Not observed	Not observed	Not observed	Not observed (?)	?
Plate ornamentation	Epitheca smooth and hypotheca reticulated, both with pores	Smooth or faint, irregular, and incomplete reticulum often on some plates, especially on s.p. and 1′ [b, c]	Smooth with small pores	Epitheca and hypotheca reticulated, both with pores	Epitheca smooth or with some small crests or reticulations, and hypotheca with crests or reticulations

l, length; w, width.
[a] Halim (1960).
[b] Balech (1989).
[c] Lilly et al. (2005).
[d] Montresor et al. (2004).
[e] Balech (1995).

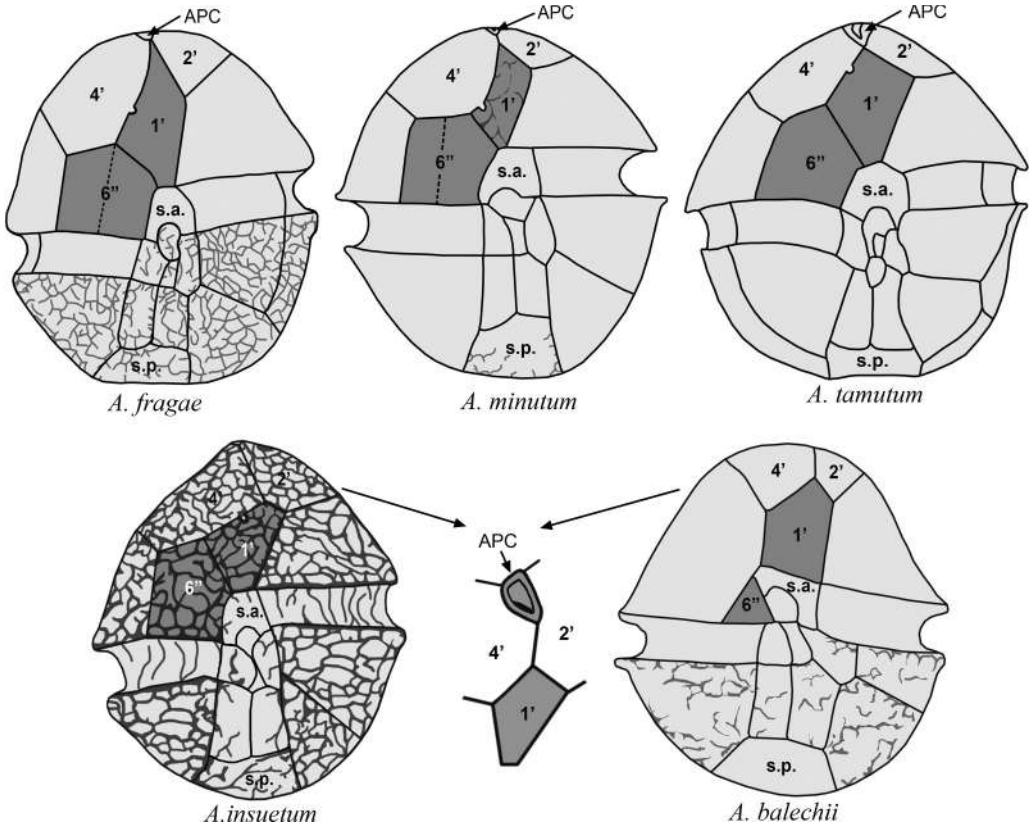

Fig. 8. Drawings of *Alexandrium fragae* sp. nov. and morphologically closely related species of the genus *Alexandrium*. Abbreviations: first apical plate = 1′, sixth precingular plate = 6″, apical pore complex = APC, s.a. = anterior sulcal plate, s.p. = posterior sulcal plate. The dashed line on the middle of the sixth precingular plate of *A. minutum* and *A. fragae* indicates that this plate can vary from as long as wide to longer than wide. Drawing of *A. minutum* was based on Balech (1989) and Lily et al. (2005), *A. tamutum* was based on Montresor et al. (2004), and *A. insuetum* and *A. balechii* were based on Balech (1995).

et al., 2004). However, cells of *A. fragae* always showed the same ornamentation pattern (smooth epitheca and reticulated hypotheca) in both natural populations and culture conditions. This leads us to assume that the presence of reticulation on the hypotheca in *A. fragae* is a stable character and that it distinguishes this new species from *A. minutum*.

Therefore, we hypothesize that the morphotype collected in Rio de Janeiro and identified as *A. minutum* by Menezes et al. (2007) corresponds to *A. fragae*. Menezes et al. (2007) analyzed cells, sampled from a bloom, that showed a densely reticulated hypotheca pattern under the light microscope. Our morphological analysis of the specimens from this same fixed sample examined by SEM corroborated the findings of Menezes et al. (2007); both results concord well with those observed for *A. fragae*, including the pattern and variation of hypotheca ornamentation.

Several morphological characters used in the description of *Alexandrium* species have been identified as unstable, and therefore not useful characters to define species boundaries. However, the majority of studies have concentrated on the shape and size of the thecal plates, ventral pore position, and capacity to form chains, among other characters (Leaw et al., 2005; Lim et al., 2007; John et al., 2014). The pattern of ornamental thecal plates is also included in the description of *Alexandrium* species, but little is known about the degree of variability in either natural or culture populations.

Although we did not observe the complete life cycle of the strains of *A. fragae*, cell pairs of unequal in size attached through the sulcal regions possibly correspond to conjugating anisogametes. Moreover, the couplets of recently divided cells were often observed in the cultures and in the natural population from a bloom. The occurrence of hypothecal plates with strong and very weak ornamentation in the same cell, observed in cells from both the culture and the bloom, seem to indicate the desmoschitic division process. The cysts observed in the cultured and bloom populations were surrounded by a thin cell wall, which suggests they were pellicle cysts because resting cysts are usually surrounded by a thicker wall (Bravo and Figueroa, 2014). These pellicle cysts may have originated young cells with a smooth theca by ecdysis. The theca maturation process may have originated the variation in the degree of ornamentation of the hypotheca.

Based on our results, it is also probable that the other morphotypes with a reticulated hypotheca identified as *A. minutum* found in the Gulf of Naples (Montresor et al., 1990), Japan (Yuki, 1994; Kaga et al., 2006), Jamaica (Ranston et al., 2007), and Hong Kong (Law and Lee, 2013) are in reality *A. fragae*.

Two *Alexandrium* species, *A. insuetum* Balech and *A. balechii* (Steidinger) Balech, also have thecal plates with reticulation similar to that of *A. fragae*. However, *A. insuetum* has the 1′ plate not connected to the APC and the reticulation covering the entire theca (Balech, 1995; Lilly et al., 2005), while *A. balechii* has the epitheca conical; the 1′ plate disconnected from the APC; and irregular crests or ridges, sometimes forming strong reticulation, on the hypotheca (Balech, 1995). *Alexandrium fragae* is similar to *A. tamutum* in the morphology of the first plate (1′), APC, anterior sulcal plate, and posterior sulcal plate. However, *A. tamutum* has the surface of the theca smooth, while in *A. fragae* the hypotheca is reticulated.

4.2. Phylogeny

The general topology of the phylogenetic trees (BI and ML) obtained in the present study was congruent, especially for the analyses based on SSU and LSU rDNA sequences. These topologies are also similar to the trees based on LSU and SSU rDNA sequences reported by other authors (Hansen et al., 2003; Kim et al., 2005; Lilly et al., 2005). In our phylogeny based on SSU rDNA sequences, two main clades were formed, the first composed of *A. minutum, A. fragae* and *A. insuetum* (PP = 0.54; BS = 52%) and the second composed of *A. tamutum, A. ostenfeldii* and *A. andersonii* (PP = 0.96; BS < 50%). These two clades together correspond to “Clade 2″ *sensu* Hansen et al. (2003, Fig. 6 in Hansen et al., 2003) and to “Group 2″ *sensu* Kim et al. (2005, Fig. 20 in Kim et al.,

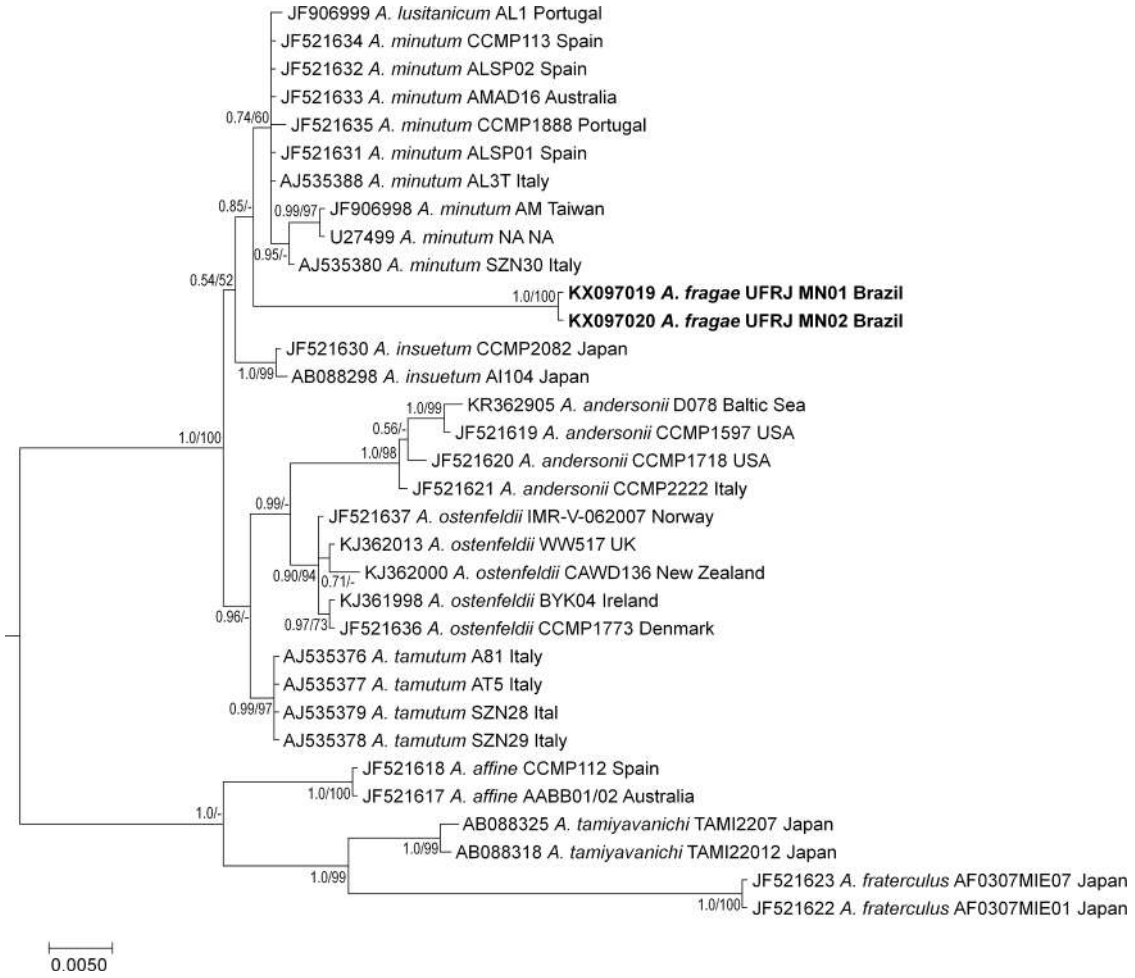

Fig. 9. Bayesian Inference tree based on SSU rDNA sequences. New sequences of *Alexandrium fragae* sp. nov. in this study are shown in bold. Branch support values are shown as BI/ML. Only values > 50% (ML) and 0.50 (BI) are shown. Hyphens indicate support values < 50% (ML) or < 0.50 (BI).

2005).

The trees based on SSU rDNA showed *A. fragae* positioned as a sister group of *A. minutum* (PP = 0.85; BS < 50%). *Alexandrium insuetum* was positioned near the *A. minutum* and *A. fragae* clade. These three species together composed a different clade (PP = 0.54; BS = 52%) from other *Alexandrium* species. On the other hand, the analyses based on LSU rDNA also showed *A. fragae* as a sister group of *A. andersonii* in the BI and ML trees (PP = 0.61; BS = 59%). Lilly et al. (2005), who developed an ML tree based on LSU rDNA sequences, showed the *A. minutum* clade near *Alexandrium* sp. strains from Japan (D163C5 and D164C6), and these two clades near *A. insuetum*, although in their tree the position of these clades was unsupported (Fig. 6 in Lilly et al., 2005). Our analyses based on LSU and ITS rDNA sequences showed the *A. fragae* strains from Brazil (UFRJ-MN01 and UFRJ-MN02) and *Alexandrium* sp. isolates from Japan (D163C5 and D164C6) as a monophyletic clade that was highly supported (PP = 1.0; BS = 99%). The average genetic distances (*p*-distance) between *A. fragae* (UFRJ-MN01 and UFRJ-MN02) and *Alexandrium* sp. (D163C5 and D164C6) were low compared to the values found among *Alexandrium* species that are currently accepted taxonomically. The genetic and morphological similarity between *A. fragae* (UFRJ-MN01 and UFRJ-MN02) and the *Alexandrium* sp. strains from Japan (D163C5 and D164C6, see above) indicates that they have the same taxonomic identity. Lilly et al. (2005) previously suggested, based on LSU rDNA sequences, that these isolates from Japan could correspond to a new species within the *A. minutum* clade. The results of our phylogenetic analysis based on LSU and ITS rDNA agree with the suggestion of Lilly et al. (2005) that the two isolates from Japan are a new species. The high bootstrap (ML) / posterior probability (BI) support of the *A. fragae* clade and the genetic distance (*p*-distance) between *A. fragae* and other species of *Alexandrium* support the proposal of a new species.

4.3. Toxin profile

Alexandrium is a potent PST-producing genus with several reported toxin-producing species, such as *A. diversaporum* (Murray et al., 2014), *A. minutum* (Hold et al., 2001; Comeau et al., 2019), *A. catenella* (Murray et al., 2012), *A. ostenfeldii* (Suikkanen et al., 2013; Van de Waal et al., 2015), and *A. tamiyavanichii* (Liow et al., 2019). Also, PTSs have been detected in bloom samples with *Alexandrium* complexes (Lefebvre et al., 2008), which provides evidence for the co-occurrence of species forming blooms.

Our study describes a new toxin-producing *Alexandrium* species, although the cellular content of PSTs in *A. fragae* isolates was below (< 0.002 pg_{PSTs} $cell^{-1}$) those reported for other species in the genus. A low concentration of PSTs was also found in a bloom of *Alexandrium*, identified as *A. minutum* by Menezes et al. (2007), in Rio de Janeiro in April 2007. Hold et al. (2001) found PST production in *A. minutum* (reported as *A. lusitanicum*) and *A. tamarense* strains ranging from 0.675–11 pg_{PSTs} $cell^{-1}$ between axenic and non-axenic cultures. Also, Lefebvre et al. (2008) reported cell toxin contents from 2.5–14 pg_{PSTs} $cell^{-1}$ for *Alexandrium* spp. isolates. However, Anderson et al. (2012)

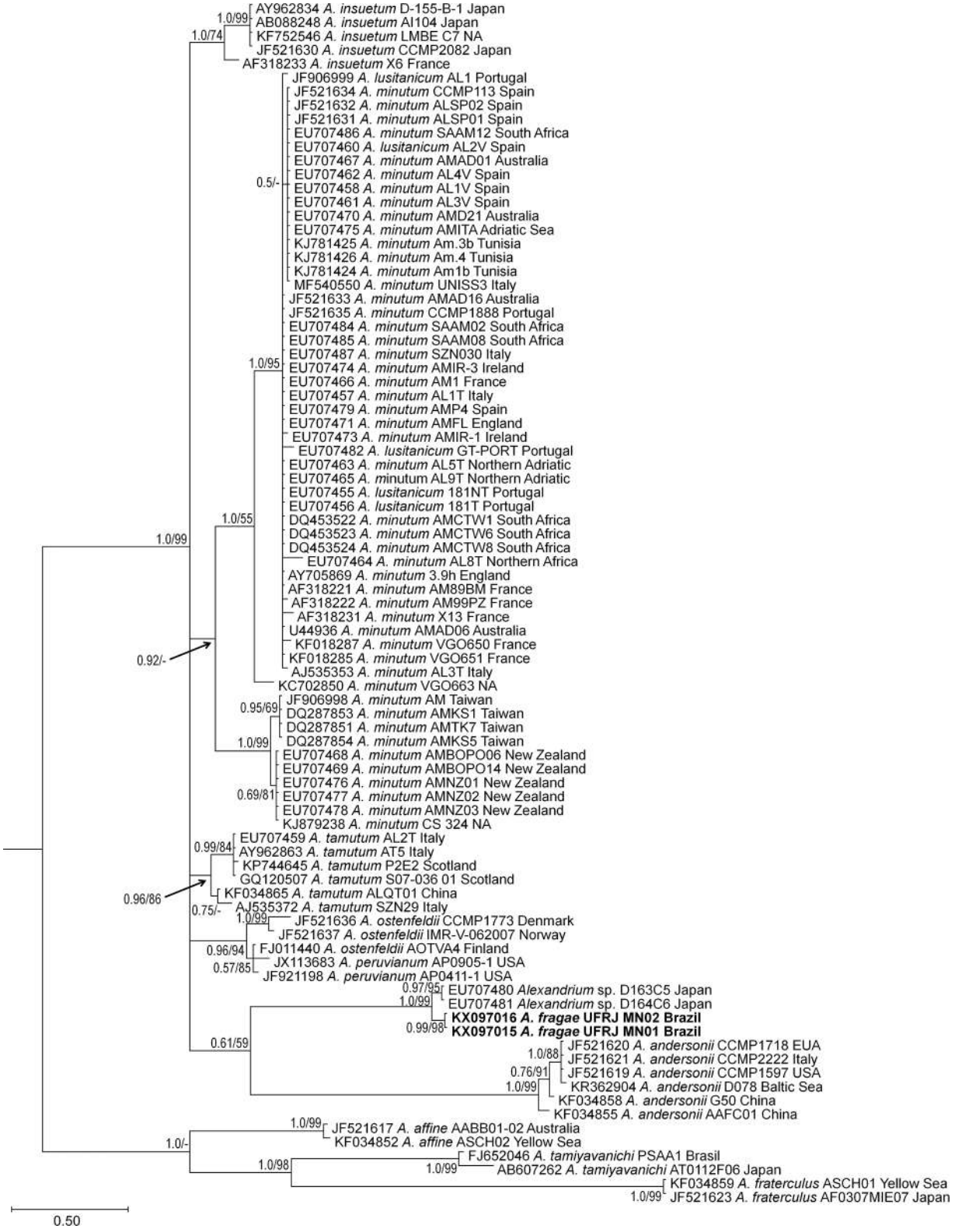

Fig. 10. Bayesian Inference tree based on LSU rDNA sequences. New sequences of *Alexandrium fragae* described in this study are shown in bold. Branch support values are shown as BI/ML. Only values > 50% (ML) and 0.50 (BI) are shown. Hyphens indicate support values < 50% (ML) or < 0.50 (BI).

pointed out that within *Alexandrium* species, strain-specific cell toxin quotas can vary from undetectable to > 100 fmol cell^{-1} during growth phases or in different environmental conditions, even in strains isolated from the same location. Van de Waal et al. (2015) found different amounts of PSTs in 20 clones of *A. ostenfeldii,* which varied from 9.5 to 50 pg$_{PSTs}$ cell^{-1}. Suikkanen et al. (2013) found that *A. ostenfeldii* strains varied in PST production along a salinity gradient (6 up to 35) as well as in different growth phases, ranging from 17.6 pg$_{PSTs}$ cell^{-1} to non-detectable concentrations. It is likely that in our study, the optimal culture conditions were not favorable for toxin production in *A. fragae*.

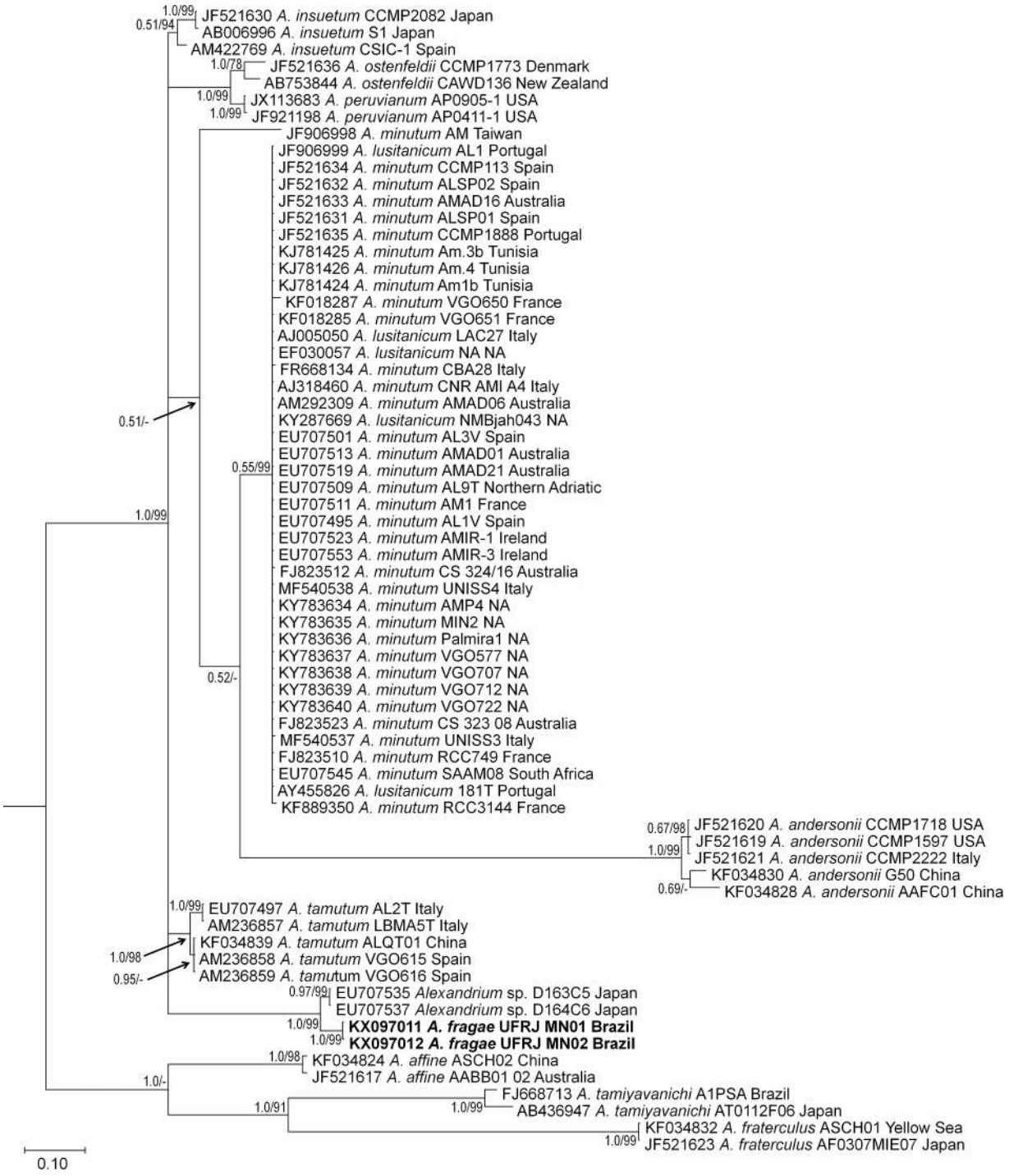

Fig. 11. Bayesian Inference tree based on ITS rDNA sequences. New sequences of *Alexandrium fragae* sp. nov. described in this study are shown in bold. Branch support values are shown as BI/ML. Only values > 50% (ML) and 0.50 (BI) are shown. Hyphens indicate support values < 50% (ML) or < 0.50 (BI).

According to previous findings, saxitoxin production seems to increase under stress conditions. Therefore, further investigations on the physiological performance of *A. fragae* under different culture conditions, as performed with other species by Hold et al. (2001) and Suikkanen et al. (2013), will provide more reliable information on its range of toxin production. These findings support the statement that the production of paralytic shellfish toxins in *Alexandrium* species might vary widely depending on the physiological state and biotic interactions of these dinoflagellates (e.g., Hold et al., 2001; Selander et al., 2006) as well as on environmental factors.

5. Conclusions

Our study provides a description of a new species of *Alexandrium, A. fragae*, based on morphology and sequences from the ITS, LSU and SSU regions of rDNA. The new species shows overlapping morphological characters with *A. minutum*; however, the reticulation on the hypotheca of *A. fragae* proved to be a good character in discriminating this species. The reticulation is a stable character in *A. fragae*: it is present in natural populations and is maintained under culture conditions. The molecular analysis indicated that *A. fragae* and *Alexandrium* sp. isolates from Japan (D163C5 and D164C6) are the same species. *Alexandrium fragae*

produces GTX2&3 and STX. Although *A. fragae* produced relatively small amounts of toxins, other metabolites (e.g., spirolides and gymnodimine; see Suikkanen et al. (2013) and Van de Waal et al. (2015)) besides PSTs might determine *Alexandrium* toxicity. Thus, *A. fragae* represents a potential risk for bloom occurrence and toxin production in coastal marine ecosystems.

Declaration of Competing Interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

This research was funded by FAPERJ (Carlos Chagas Filho Foundation for Research Support of Rio de Janeiro State, Brazil, Edital Biota RJ 2013 to M.M.) and CNPq (National Council for Scientific and Technological Development, Brazil, Edital Universal 2013 to M.M.). We are grateful to Dr. Inácio D. da Silva Neto and technician Marcelo Henrique de Oliveira at UFRJ, Rio de Janeiro, for assistance with SEM. We are also grateful to Dr. Marcos Farina for making available the facilities of the Laboratory of Biomineralization, UFRJ, for the preparation of materials for SEM. We appreciate the help of Dr. Janet W. Reid in improving the English text.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.hal.2020.101793.

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Harmful Algae 86 (2019) 96–105

Contents lists available at ScienceDirect

Harmful Algae

journal homepage: www.elsevier.com/locate/hal

Intraspecific variability in response to phosphorus depleted conditions in the cyanobacteria *Microcystis aeruginosa* and *Raphidiopsis raciborskii*

Iame Alves Guedes[a,1], Ana Beatriz F. Pacheco[a,1], Mauro C.P. Vilar[a], Mariana M. Mello[b], Marcelo Manzi Marinho[c], Miquel Lurling[d,e], Sandra M.F.O. Azevedo[a,*]

[a] Carlos Chagas Filho Biophysics Institute, Federal University of Rio de Janeiro, Rio de Janeiro 21949-902, Brazil
[b] Federal University of Juiz de Fora, Juiz de Fora, MG 36036-900, Brazil
[c] Laboratory of Ecology and Physiology of Phytoplankton, Department of Plant Biology, University of Rio de Janeiro State, Rua São Francisco Xavier 524—PHLC Sala 511a, 20550-900, Rio de Janeiro, Brazil
[d] Aquatic Ecology & Water Quality Management Group, Department of Environmental Sciences, Wageningen University, P.O. Box 47, 6700 AA, Wageningen, the Netherlands
[e] Department of Aquatic Ecology, Netherlands Institute of Ecology (NIOO-KNAW), P.O. Box 50, 6700 AB, Wageningen, the Netherlands

ARTICLE INFO

Keywords:
Ecotypes
Phosphorus uptake
Strain variability
Cylindrospermopsis

ABSTRACT

Phosphorus loading plays an important role in the occurrence of cyanobacterial blooms and understanding how this nutrient affects the physiology of cyanobacteria is imperative to manage these phenomena. *Microcystis aeruginosa* and *Raphidiopsis raciborskii* are cyanobacterial species that form potentially toxic blooms in freshwater ecosystems worldwide. Blooms comprise numerous strains with high trait variability, which can contribute to the widespread distribution of these species. Here, we explored the intraspecific variability in response to phosphorus depleted conditions (P-) testing five strains of each species. Strains could be differentiated by cell volume or genetic profiles except for those of the same species, sampling location and date, though these presented differences in their response to (P-). Although differently affected by (P-) over 10 days, all strains were able to grow and maintain photosynthetic activity. For most *M. aeruginosa* and *R. raciborskii* strains growth rates were not significantly different comparing (P+) and (P-) conditions. After ten days in (P-), only one *M. aeruginosa* strain and two *R. raciborskii* strains showed reduction in biovolume yield as compared to (P+) but in most strains chlorophyll-*a* concentrations were lower in (P-) than in (P+). Reduced photosystem II efficiency was found for only one *R. raciborskii* strain while all *M. aeruginosa* strains were affected. Only two *M. aeruginosa* and one *R. raciborskii* strain increased alkaline phosphatase activity under (P-) as compared to (P+). Variation in P-uptake was also observed but comparison among strains yielded homogeneous groups comprised of representatives of both species. Comparing the response of each species as a whole, the (P-) condition affected growth rate, biovolume yield and chlorophyll yield. However, these parameters revealed variation among strains of the same species to the extent that differences between *M. aeruginosa* and *R. raciborskii* were not significant. Taken together, these results do not support the idea that *R. raciborskii*, as a species, can withstand phosphorus limitation better than *M. aeruginosa* and also point that the level of intraspecific variation may preclude generalizations based on studies that use only one or few strains.

1. Introduction

Eutrophication is a major water quality issue worldwide and phosphorus (P) enrichment of lakes is one of the key factors that trigger and maintain cyanobacterial blooms (Smith and Schindler, 2009; O'Neil et al., 2012; Rangel et al., 2012). During progressive P-loading of lakes 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 species, among which *Raphidiopsis raciborskii* (Woloszynska) Aguilera, Berrendero Gómez, Kastovsky, Echenique & Salerno (basionym *Cylindrospermopsis raciborskii* (Woloszynska) Seenayya & Subba Raju) (Aguilera et al., 2018) and *Microcystis aeruginosa* are both toxic and common (Soares et al., 2013; Antunes et al., 2015; Burford et al., 2016;

* Corresponding author.
E-mail address: sazevedo@biof.ufrj.br (S.M.F.O. Azevedo).
[1] Both authors contributed equally for this study.

https://doi.org/10.1016/j.hal.2019.03.006
Received 7 December 2018; Received in revised form 18 February 2019; Accepted 14 March 2019

Harke et al., 2016). Insights in competitive interactions, as well as responses to environmental conditions, are crucial to improve our understanding of cyanobacterial succession and dominance. In the early stages of bloom formation, depending on relative biomass and local loading, dissolved nutrient pools may be depleted (Catherine et al., 2008; Rangel et al., 2012). In this scenario, P-loading in freshwater ecosystems can enhance the occurrence of cyanobacterial blooms (Harke et al., 2016). Thus, understanding how this nutrient affects the physiology of different species of cyanobacteria is imperative to understand and manage blooms.

M. aeruginosa and *R. raciborskii* are cyanobacterial species that form potentially toxic blooms in freshwater ecosystems worldwide (Antunes et al., 2015; Harke et al., 2016). The broad geographic distribution of these two species may be explained by different ecologic strategies. *R. raciborskii* is a filamentous diazotrophic cyanobacterium, initially isolated from a tropical site, but then reported worldwide, being considered an invasive species (Antunes et al., 2015; Burford et al., 2016). This expansion is potentially harmful since strains are described as producers of cylindrospermopsin or saxitoxins (Ohtani et al., 1992; Lagos et al., 1999; Molica et al., 2002). Phylogenetic analyses including representative strains collected in different continents 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). Genomic comparisons with strains from diverse locations indicated a core genome covering 62% of the genes (Abreu et al., 2018), while comparing strains isolated from the same environment the core genome corresponded to 86% (Willis et al., 2018). Both analyses indicate a minor flexible set of genes that can be related to specific adaptations to local conditions (Willis et al., 2018). The ecological success of *R. raciborskii* results from the combination of genetic variability and great phenotypic diversity. The latter is reflected by the existence of diverse ecotypes (Briand et al., 2004; Chonudomkul et al., 2004; Haande et al., 2008; Piccini et al., 2011; Bonilla et al., 2012; Xiao et al., 2017).

M. aeruginosa is a species with the ability to form highly-buoyant colonies, and one of the most common bloom-forming cyanobacteria in freshwater, with potential to produce the heptapeptide microcystin (O'Neil et al., 2012; Dittmann et al., 2013; Harke et al., 2016). Evaluation of its global biogeography revealed that this species represents a homogenous taxon (van Gremberghe et al., 2011; Harke et al., 2016). Comparative genome approaches indicated that the high adaptive capacity of *M. aeruginosa* is based on a high genomic plasticity with a core genome including only half of that and a vast flexible genome, which can account for the ecological success of diverse strains in various environments (Humbert et al., 2013).

Some studies have described the ability of high phosphorus uptake and storage of *R. raciborskii* (Wu et al., 2009; Bai et al., 2014; Isvánovics et al., 2000) and *M. aeruginosa* (Olsen, 1989; Marinho and Azevedo, 2007; Harke et al., 2012). 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 et al. (2013) found that although *R. 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 et al. (2009) observed that *R. raciborskii* more effectively uptakes and assimilates phosphate than *M. aeruginosa*. These findings led to the idea that *R. raciborskii* is an opportunistic species regarding the use of this nutrient (Antunes et al., 2015; Burford et al., 2016). 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 (Shen and Song, 2007; Marinho et al., 2013; Amaral et al., 2014; Willis et al., 2016; Bolius et al., 2017; Xiao et al., 2017; Willis et al., 2019) and most of these studies were done with no more than two strains. Comparisons based on individual or very few strains overlook the possible magnitude of strain variation within and between species, and limit our understanding on ecologically relevant traits as already pointed out by some studies (Rocap et al., 2003; Alexova et al., 2011; Aryal et al., 2014; Sandrini et al., 2015; Willis et al., 2016; Bolius et al., 2017; Xiao et al., 2017).

To better understand intraspecific and interspecific variability in physiological traits related to limiting P conditions, we examined growth, photosynthetic efficiency, alkaline phosphatase activity and P uptake rate of five *R. raciborskii* and *M. aeruginosa* strains under conditions of phosphorus limitation. Thus, we examined the hypothesis that *R. raciborskii* can withstand P restriction better than *M. aeruginosa*. In both species, a considerable plasticity was observed, reflected in high intraspecific variability in traits, which precludes generalizations on the performance on the species level.

2. Material and methods

2.1. Strains and maintenance conditions

The experiments were performed with five *R. raciborskii* and five *M. aeruginosa* strains isolated from different Brazilian freshwater environments. For each species the set included both strains from the same reservoir and strains from different geographical origins. Strains were obtained from culture collections of Brazilian laboratories (Supplementary Table 1). As typical, *M. aeruginosa* strains grew as single cells in culture, not in colonies. Cultures were maintained in 100 mL of WC medium (modified according to Lürling and Beekman, 2006) in 250 mL Erlenmeyer flasks kept in incubators (Sanyo Gallenkamp Orbital Incubator, Loughborough, United Kingdom) 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 h. Cultures were not grown axenically, but regular microscopic inspection revealed that biomass of heterotrophic bacteria remained low. To decrease bacterial contamination, cultures were rinsed weekly for at least one month prior to the start of the experiments. For such, cultures were centrifuged (4,000 × *g*, 10 min) in 50-mL centrifuge tubes and cells were resuspended in sterile WC medium. This procedure was performed three times and after that, cells were resuspended in fresh WC medium and returned to culture under the maintenance conditions.

2.2. Characterization of the strains

For the characterization of the strains of both species, they were cultivated under the maintenance conditions described above. Morphometric characterization was performed on cells sampled during 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 (*R. raciborskii* strains) were measured in at least 70 cells of each strain with a Zeiss Axio Imager microscope (Carl Zeiss, Göttingen) at magnifications 400× and 1000×. The cell volume was calculated according to Hillebrand et al. (1999).

2.3. Phycocynin intergenic region (cpcBA) amplification and sequencing

DNA was extracted from cells collected from the exponential growth phase using the Wizard Genomic DNA Purification kit (Promega) and used as a template to amplify the *cpcBA* locus according to Neilan et al. (1995). The amplified region includes part of the coding regions for two phycobilisome subunits (*cpcB* and *cpcA*) and a highly variable intergenic spacer region that is useful in differentiating cyanobacterial strains. 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′ CCAG TACCACCAGCAACTAA 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). The GenBank accession numbers for the sequences reported here are MG029614- MG029623.

2.4. Cyanobacterial repeated-sequence PCR

R. 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.

2.5. Phosphorus depletion experiments

Experiments were performed with the strains of both cyanobacterial species subjected to phosphate depleted (P-) and replete/control (P+) conditions. The inoculum was obtained from maintenance cultures, with cells in the exponential growth phase. To ensure that these cultures were not phosphorus depleted at the start of the experiments, four days before the inoculation cultures were centrifuged (4000 × *g*, 10 min) in 50-mL centrifuge tubes, washed with sterile WC medium twice, resuspended in fresh WC medium and returned to culture under the maintenance conditions. To initiate phosphorus limitation experiments cells were centrifuged (4000 × *g*, 10 min) in 50-mL centrifuge tubes, washed with sterile (P-) WC medium twice and resuspended in 100 mL of (P-) WC medium or (P+) WC medium for an initial cell concentration of 1×10^6 cells mL^{-1}. P depleted WC medium was WC medium in which K_2HPO_4 was omitted and instead KCl was supplemented to prevent K deficiency and (P+) WC medium was the standard medium (modified according to Lurling and Beekman, 2006). The experiment was performed in 250 mL Kitasato flasks, in triplicates for 10 days, with sampling each two days. Samples were taken for the determination of chlorophyll-*a* concentration, photosynthetic efficiency, biovolume and alkaline phosphatase activity.

2.6. Photosynthetic and growth parameters

Chlorophyll-a fluorescence and the photosynthetic efficiency (Photosystem II quantum yield) measurements were performed with the pulse-amplitude modulated photosynthesis yield phytoplankton analyzer (PHYTO-PAM, Heinz Walz GmbH, Effeltrich, Germany). The maximum effective quantum efficiency of PSII is represented by $\phi_m = [(F_m - F)/ F_m]$, where F is fluorescence of the dark-adapted sample and F_m is the maximum dark-adapted fluorescence after applying a single saturation pulse (3000 µmol m^{-2} s^{-1} for 0.2 s).

Biovolume was measured with an automated cell counter (Casy Cell Counter, Schaerfe System GmbH, Reutlingen, Germany) with a 120-µm capillary, directly after sampling. Biovolume yield (final biovolume - initial biovolume) was calculated to estimate biomass increase after 10 days in (P+) and (P-) conditions. The specific growth rate (µ) was calculated during the exponential phase (growth interval where $R2 \geq 0.95$) based on chlorophyll-*a* concentrations according to the following equation: $\mu = (\ln \text{Chl-a}_{t2} - \ln \text{Chl-a}_{t1})/t_2\text{-}t_1$ where Chl-at_2 and Chl-at_1 are chlorophyll-a concentration at times t_2 and t_1, respectively (Fogg and Thake, 1987).

2.7. Alkaline phosphatase activity

The alkaline phosphatase (AP) 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). Briefly, 2 mL of culture was centrifuged (3,000xg, 5 min) and the pellet resuspended in (P-) WC (pH 8.0) with 5 nM p-nitrophenyl phosphate (Sigma) in a final volume of 1.5 mL. After incubation in the same culture maintenance conditions for one hour, reaction was terminated by the addition of 1:10 volume of 4 M NaOH. The samples were centrifuged (3,000xg, 5 min) and the supernatant was used to determine the AP activity. Five nM pNPP in (P-) WC was used as the control. Absorbance was determined at 405 nm in a spectrophotometer and compared to the standard absorbance for pNPP in (P-) WC. The enzyme activity was normalized by biovolume concentration.

2.8. Phosphate uptake rates

To determine the phosphate uptake rate, the strains were maintained for ten days in culture on (P-) WC (Kitasato flasks of 500 mL containing 250 mL of culture). To that, a pulse of phosphate (10 µmol L^{-1} K_2HPO_4) was added and the inorganic phosphorus concentrations were measured every 10 min over a 4 h period. Samples for the monitoring of dissolved P concentrations were taken in triplicate after filtration with GF/C membranes and stored frozen at −20 °C until analysis. Concentrations of dissolved phosphate were determined with a continuous flow analyzer (CFA, Skalar Analytical BV). The initial biovolume was measured with an automated cell counter (Casy Cell Counter, 216 Schaerfe System GmbH, Reutlingen, Germany). Phosphate uptake rates were determined according to Marinho et al. (2013), as the initial linear slope of the curve of phosphate concentrations vs. time.

2.9. Data analysis and statistics

Cell volume and maximum uptake rates among strains of *R. raciborskii* and *M. aeruginosa* in control conditions were evaluated statistically running one-way ANOVAs in the tool pack SigmaPlot version 12.3 (Systat Software, Inc., San Jose, CA, USA). The chlorophyll and biovolume yield of *R. raciborskii* and *M. aeruginosa* under (P+) and (P-) conditions were statistically evaluated by a two-way ANOVA. The chlorophyll-*a* concentration and photosynthetic efficiency through time were evaluated by running a two-way repeated measure ANOVA, while comparison of the responses of each strain separately under (P-) and (P+) conditions were evaluated by running two-way repeated measures ANOVA. In addition, growth rates of each strain under the experimental condition were analyzed by two-way ANOVA. Growth rate and chlorophyll/biovolume yield in *M. aeruginosa* and *R. raciborskii* under (P+) and (P-) were evaluated by paired *t*-test using integrated data of all strains as replicates of the studied species. The comparison of AP activity among the five strains of *M. aeruginosa* and *R. raciborskii* submitted to phosphorus limitation was performed using two-way repeated measures ANOVA after data normalization relative to cyanobacterial biovolume in cultures. The ANOVAs were followed by pairwise multiple comparison procedures (Tukey HSD test) to distinguish means that were significantly different ($p < 0.05$). All analysis and graphs were performed on GraphPad Prism 6 software or SigmaPlot version 12.3.

3. Results

3.1. Characterization of R. raciborskii and M. aeruginosa strains

To assess the intraspecific variability of *R. 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 detailed morphometrics, genetic profiles obtained by PCR for highly iterated palindromic repeats (HIP1),

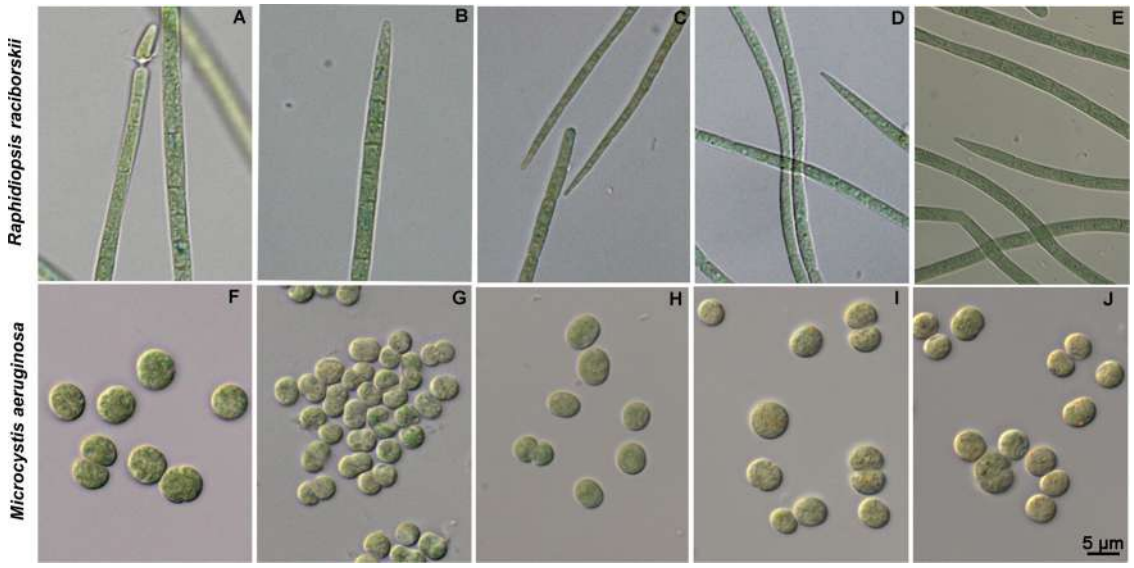

Fig. 1. Microscopic image of *R. raciborskii* (up) and *M. aeruginosa* (bottom) strains. (A) CYL 1, (B) CYL 2, (C) T3, (D) CYLP, (E) CYRF T3 (F) MIRF, (F) MIRS, (G) LEA4, (H) LEA12, (I) LEA13. Scale bar = 5 µm.

and by sequencing of the phycocyanin intergenic region (*cpcBA*).

The morphometric characterization of the strains revealed significant differences in cell volume among *M. aeruginosa* strains ($F_{4,486}$ = 183.7; $p <$ 0.001) and *R. raciborskii* strains ($F_{4,297}$ = 19.8; $p <$ 0.001; Fig. 1, Supplementary Table 2). Three strains of *M. aeruginosa* showed similar mean cell volumes (MIRF, LEA12 and LEA13), while the other two (LEA4 and MIRS) had a significantly smaller cell volume. Among *R. raciborskii* strains, CYRF had a significant higher cell volume than all the others.

The *cpcBA* intergenic region sequences confirmed the morphological identification of the strains of each species, with more than 99% of similarity with *M. aeruginosa* or *R. 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 (CYLP = CYL1 = CYL2 = T3; LEA12 = LEA13). HIP1 PCR revealed three profiles among the five tested *R. raciborskii* strains and four profiles among the five tested *M. aeruginosa* strains, so still some strains could not be differentiated (CYL1 = CYL2 = T3; LEA12 = LEA13) (Fig. 2). *R. raciborskii* and *M. aeruginosa* strains isolated from the same location at the same moment (CYL1, CYL2 and LEA12, LEA13), showed similar profiles in the HIP1 PCR and also identical *cpcBA* sequences and cell volumes.

3.2. Physiological response of R. raciborskii and M. aeruginosa strains to phosphorus depletion

All tested strains displayed a significant increase in chlorophyll-*a* concentrations over time despite phosphorus depletion ($F_{4,8}$ = 1073; $p <$ 0.0001 for *R. raciborskii,* and $F_{4,8}$ = 540.3; $p <$ 0.0001 for *M. aeruginosa*) (Fig. 3, Table 1). Growth rates derived from chlorophyll concentration during exponential phase showed a slightly but generally non-significant difference comparing (P+) and (P-) conditions for most *M. aeruginosa* and *R. raciborskii* strains (Fig. 4). However, chlorophyll-*a* concentrations were significantly higher in (P+) than in (P-) ($F_{9,18}$ = 145.2; $p <$ 0.0001 for *R. raciborskii,* and $F_{9,18}$ = 260.2; $p <$ 0.0001 for *M. aeruginosa*), which was apparent from day 6 onward in most strains (Fig. 3). As a result, the final chlorophyll yield was reduced after 10 days for all strains, except for one *R. raciborskii* strain (CYRF) (Fig. 5A, Supplementary Table 3).

P depletion affected differently the efficiency of photosystem II in

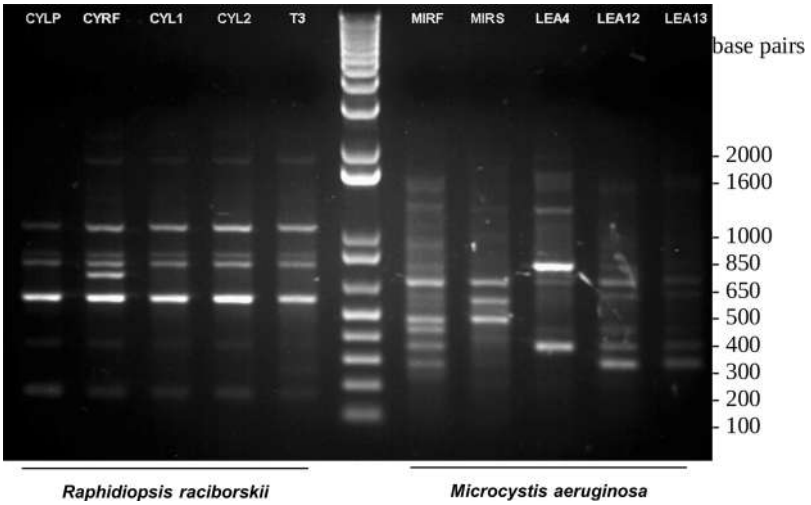

Fig. 2. HIP1 PCR profile of *R. raciborskii* (left) and *M. aeruginosa* (right) strains. HIP1 PCR revealed three profiles among the five tested *R. raciborskii* strains and four profiles among the five tested *M. aeruginosa* strains.

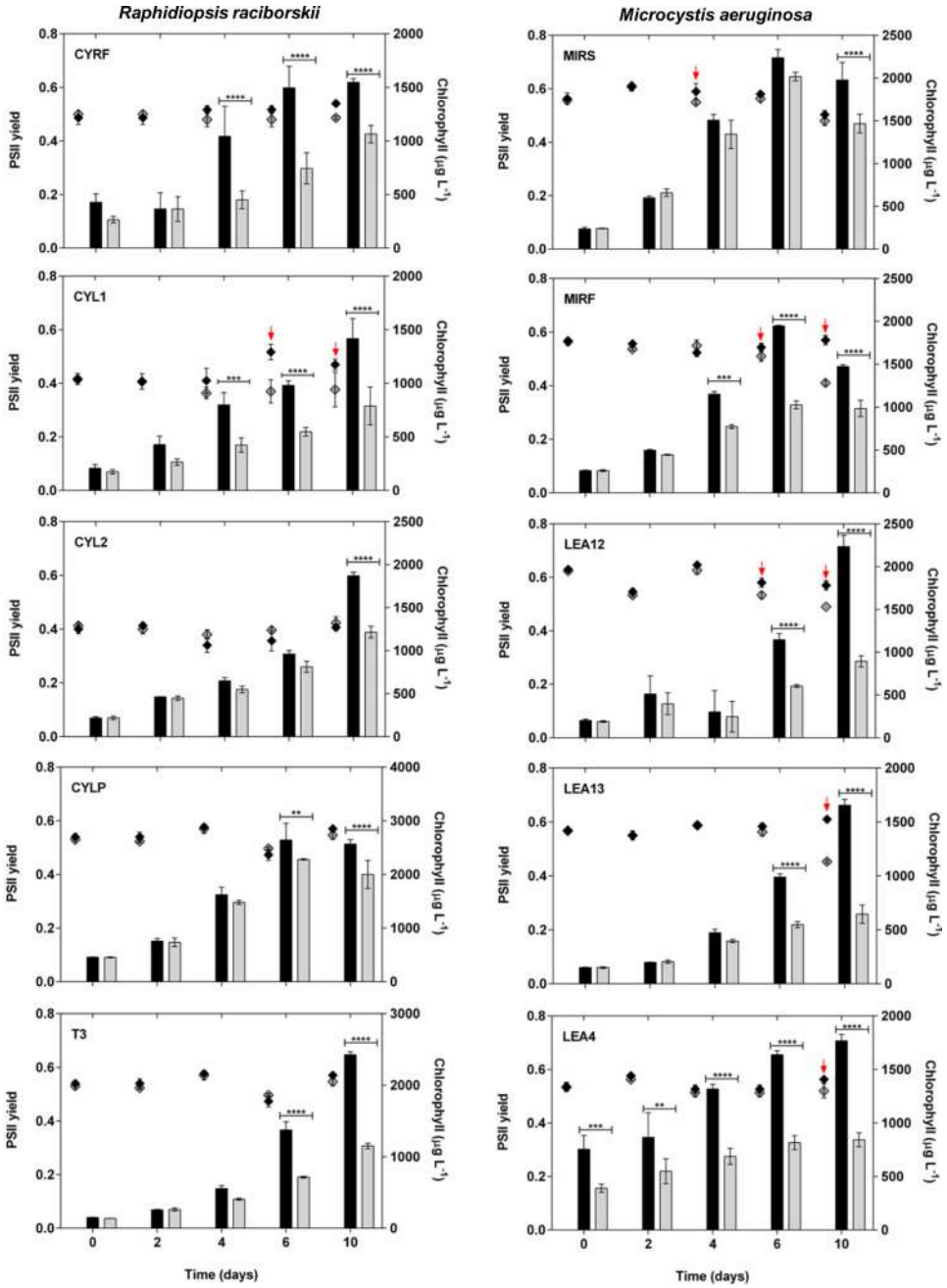

Fig. 3. Chlorophyll-a concentration (bars) and efficiency of photosystem II (diamonds) of *R. raciborskii* and *M. aeruginosa* strains after 10 days of culture under phosphate availability (black) or limitation (grey). Asterisks indicate significant differences. Tukey, (**) = $p < 0.01$; (***) = $p < 0.001$; (****) = $p < 0.0001$. Arrows indicate significant differences between the values of photosystem II efficiency.

these two species (Fig. 3, Table 1). Significantly reduced photosystem II efficiency was found for only one *R. raciborskii* strain (CYL1) while for *M. aeruginosa* all strains were affected, and in most strains it occurred from day 6 onward.

Considering biovolume yield, after ten days in the absence of phosphorus, only one *M. aeruginosa* strain showed a significant reduction ($F_{1,20} = 382.3$; $p < 0.01$) as compared to (P+). In contrast, this condition promoted a significant decrease in the biovolume yield for two *R. raciborskii* strains (CYRF and CYLP) (Fig. 5B and Supplementary Table 3).

When all strains were grouped and we compared the response of each species as a whole, it could be observed that P depletion significantly affected the growth rate ($T = 4.141$, df = 14, $p < 0.001$ for *M. aeruginosa* and $T = 3.634$, df = 14, $p < 0.01$ for *R. raciborskii*), biovolume yield ($T = 4.254$, df = 14, $p < 0.001$ for *M. aeruginosa* and $T = 3.529$, df = 14, $p < 0.01$ for *R. raciborskii*), and chlorophyll yield ($T = 8.507$, df = 14, $p < 0.0001$ for *M. aeruginosa* and $T = 7.090$, df = 14, $p < 0.0001$ for *R. raciborskii*) (Fig. 6). According to these parameters, the response of *M. aeruginosa* and *R. raciborskii* to P deprivation could not be distinguished (Supplementary Fig. 1).

AP activity was measured on the 2nd and 6th days of culture under both (P+) and (P-) conditions (Fig. 7 and Supplementary Table 4). Two *M. aeruginosa* strains increased AP activity under (P-) as compared to (P+), but this occurred in different intensities and sampling times. Only one *R. raciborskii* strain (CYL1) showed a significant increase in AP activity under P depletion.

Variation in V_{max} values among strains was also observed (Fig. 8). A one-way ANOVA on log transformed V_{max} values (to fulfill ANOVA

Table 1
Results of the Two-way repeated measure ANOVA for differences in quantum yield of Photosystem II (PSII-Yield) and Chlorophyll-*a* concentration (Chl-*a*) in *M. aeruginosa* and *R. raciborskii* strains under phosphorus limitation.

Microcystis aeruginosa	SS	Df	MS	F	*p*
Time (Chl-a)	2.836×10^7	4	7.90×10^6	540.3	< **0.0001**
Phosphorus-strain interaction (Chl-a)	1.451×10^7	9	1.613×10^6	260.2	< **0.0001**
Time-Strain-Phosphorus interaction (Chl-a)	1.137×10^7	36	315721	37.22	< **0.0001**
Residual	610669	72	8482		
Time (PSII-Yield)	0.06073	4	0.01518	80.47	< **0.0001**
Phosphorus-strain interaction (PSII-Yield)	0.07496	9	0.008329	23.10	< **0.0001**
Time-Strain-Phosphorus interaction (PSII-Yield)	0.1507	36	0.004186	33.25	< **0.0001**
Residual	0.009064	72	0.0001259		
Raphidiopsis raciborskii	SS	Df	MS	F	*p*
Time (Chl-a)	3.724×10^7	4	9.31×10^6	1073	< **0.0001**
Phosphorus-strain interaction (Chl-a)	1.919×10^7	9	2.132×10^6	145.2	< **0.0001**
Time-Strain-Phosphorus interaction (Chl-a)	1.036×10^7	36	287432	29.77	< **0.0001**
Residual	695162	72	9655		
Time (PSII-Yield)	0.02249	4	0.005623	51.74	< **0.0001**
Phosphorus-strain interaction (PSII-Yield)	0.4558	9	0.05065	61.89	< **0.0001**
Time-Strain-Phosphorus interaction (PSII-Yield)	0.1473	36	0.004090	10.55	< **0.0001**
Residual	0.02790	72	0.0003876		

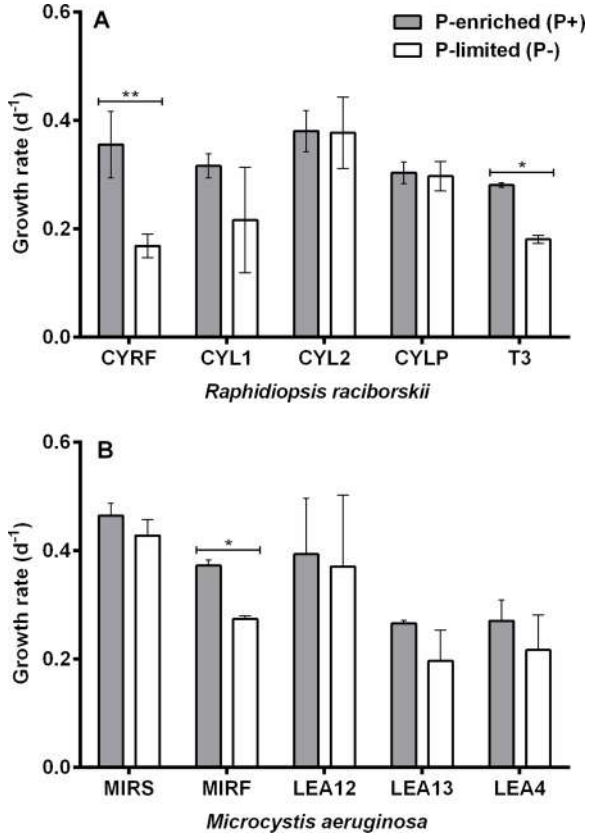

Fig. 4. Growth rates of (**A**) *R. raciborskii* and (**B**) *M. aeruginosa* strains under phosphate limitation (white) or availability (grey). Asterisks indicate significant differences. Tukey, (*) = $p < 0.05$; (**) = $p < 0.01$.

requirements) indicated significant differences ($F_{9,29} = 104.9$; $p < 0.001$) and a post hoc comparison yielded homogeneous groups comprised of representatives of both species (Fig. 8).

4. Discussion

In this study we have explored the intraspecific variability of *R. raciborskii* and *M. aeruginosa* to perceive more comprehensively the response of these species to phosphorous availability. The results do not support the hypothesis that *R. raciborskii*, as a species, can thrive better in P restriction than *M. aeruginosa*.

Most *R. raciborskii* and *M. aeruginosa* strains could be differentiated by morphology, HIP1 PCR profiles or *cpcBA* sequences. In the case of strains of the same species isolated from the same sampling location and date, these traits could not distinguish them, although they presented differences in some physiological traits in response to low P. *R. raciborskii* strains presented similar or identical HIP1PCR profiles, indicating a low variability in this trait as already noted (Saker and Neilan, 2001). This was also observed by Willis et al. (2016) who found identical HIP1PCR profiles among 24 *R. 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 (Wilson et al., 2005; Bittencourt-Oliveira et al., 2007).

The differences among strains in the cell volume were also more pronounced in *M. aeruginosa* than in *R. raciborskii*. Xiao et al. (2017) observed the opposite pattern when comparing eight *R. raciborskii* strains (including straight and coiled morphologies) and four *M. aeruginosa* strains. These different results may be due to the use of different cultivation time and conditions (different light intensities and temperatures) or to the set of strains included in each study.

To further explore this inter- and intraspecific variability we assessed the physiological responses of *R. raciborskii* and *M. aeruginosa* strains to P depletion. All strains were able to grow under phosphorus deficiency for ten days in spite of the reduction in growth rate, biovolume yield or chlorophyll yield. This is in agreement with other studies on these species that also compared growth in P replete and P depleted conditions (Wu et al., 2012; Harke and Gobler, 2013; Bai et al., 2014; Willis et al., 2019). The response of cyanobacteria to low P availability includes an increase in P uptake, the utilization of organic compounds such as phosphomonoesters and phosphonates, the induction of phosphatases, the use of phosphorus storage compounds, such as polyphosphates and changes in lipid composition (Schwarz and Forchhammer, 2005; Vahetera et al. 2007; Harke et al., 2012; Bai et al., 2014; Harke and Gobler, 2013; Wang et al., 2018). In our experiments, the most likely explanation of sustained growth for 10 days, even when cells were placed in (P-) medium, is initial luxury consumption and subsequent reuse of internal storage of phosphorus, once cyanobacterial cells were not P depleted. 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 *R.*

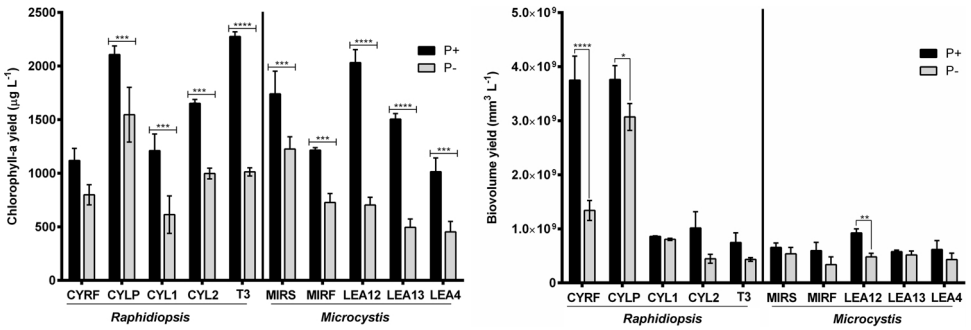

Fig. 5. Chlorophyll (A) and biovolume yield (B) of *R. raciborskii* and *M. aeruginosa* strains after 10 days of culture under phosphate availability (black) or limitation (grey). Asterisks indicate significant differences. Tukey, (*) = $p < 0.05$; (**) = $p < 0.01$; (***) = $p < 0.001$; (****) = $p < 0.0001$.

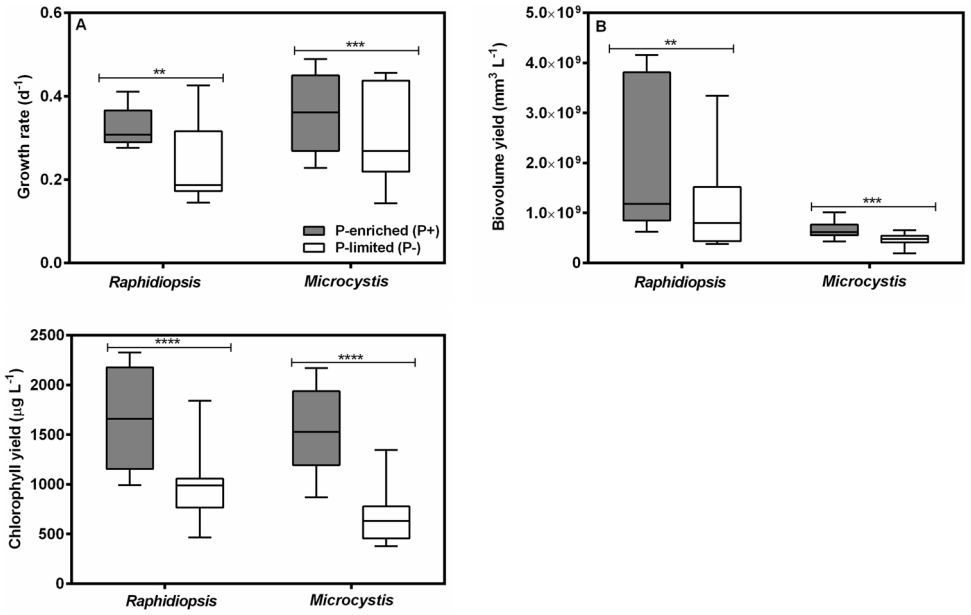

Fig. 6. Box plots comparing growth rates (A), biovolume yield (B) and chlorophyll yield (C) of *M. aeruginosa* and *R. raciborskii* under phosphate enriched (grey) and depleted (white). Bars represent minimum and maximum values. *T*-test, (**) = $p < 0.01$; (***) = $p < 0.001$; (****) = $p < 0.0001$.

raciborskii (Noyma et al., 2015). This storage could theoretically sustain 3–4 generations of growth in (P-) conditions (Droop, 1973; Morel, 1987), and probably is involved in the sustained growth observed even after 10 days in a (P-) medium.

Although the growth response to low P or P depletion have been already studied for both cyanobacterial species, 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; Amaral et al., 2014; Willis et al., 2019). Using four unicellular *M. aeruginosa* strains, Shen and Song (2007) observed that growth rates were inhibited by 51–79% in relatively low initial P concentrations (200 µg L^{-1} P). Comparing two ecotypes of *R. raciborskii*, Piccini et al. (2011) showed that under low initial P concentrations (10 µM K_2HPO_4) their growth rate differed more than two-fold. Similarly, the addition of P, either as repeated pulses or as a single dose, to P depleted cultures resulted in different growth rates for two *R. raciborskii* strains (Amaral et al., 2014). In contrast, Willis et al. (2015, 2019) tested three or two *R. raciborskii* ecotypes, respectively, and observed similar growth rates under low P (0-25 µM K_2HPO_4). Here, we clearly observed strain variability in both species, the growth rate of some strains was affected by P depletion while that of others was not. Considering the growth response to low P as measured by a reduction of biomass production, we observed a wider variation among *R. raciborskii* strains than among *M. aeruginosa* strains, which responded in a similar way to the (P-)

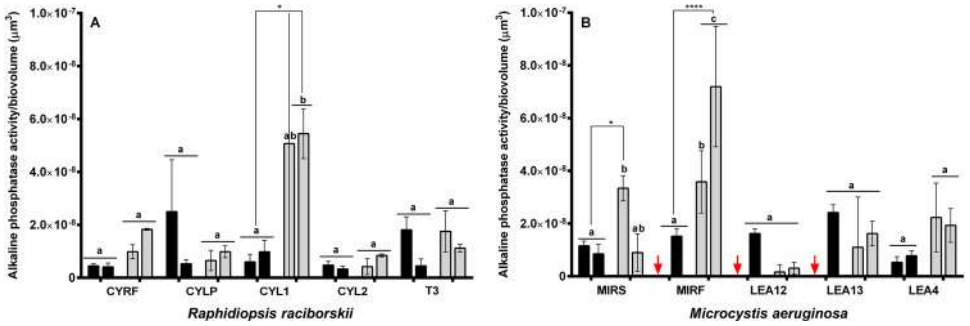

Fig. 7. Alkaline phosphatase activity relative to biovolume of **(A)** *R. raciborskii* and **(B)** *M. aeruginosa* strains under phosphate enriched (black) or depleted (grey) in the 2nd and 6th day of culture. Red arrows indicate undetectable activity. Different letters indicate significant differences. Tukey, (*) = $p < 0.05$; (**) = $p < 0.01$; (****) = $p < 0.0001$.

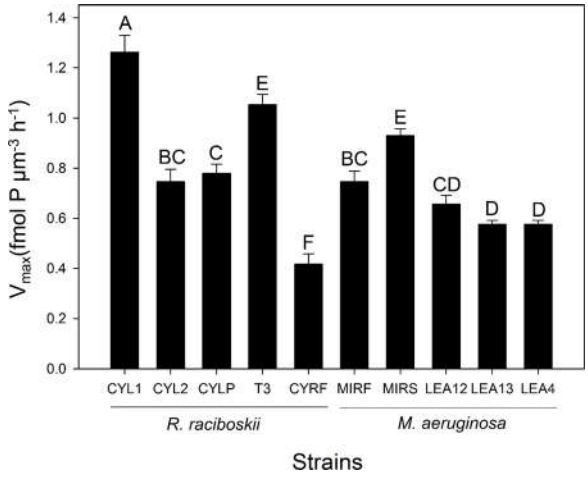

Fig. 8. Maximum uptake rate of phosphorus (*Vmax*) of *R. raciborskii* and *M. aeruginosa* strains after addition of a pulse of KH_2PO_4 to starved cells. Letters indicate homogeneous groups according to the Tukey test ($p < 0.05$).

condition.

A much general and evident response to (P-) was apparent comparing chlorophyll yields. A decrease in chlorophyll concentration was observed for all but one strain and was significant and similar for both species. This is in agreement with previous studies testing the effect of P depletion on *R. raciborskii* (Wu et al., 2012; Bai et al., 2014) and unicellular *Microcystis* strains (Shen and Song, 2007). Despite this change, the strains of both species were able to maintain growth and the photochemical efficiency of the PSII reaction centers. In (P-) a 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 and Sun, 2016). Only in one *R. raciborskii* strain and all *M. aeruginosa* strains photosystem II efficiency was reduced in the (P-) condition, but in most cases reductions were not high and were apparent only on later times. Consequently, the transfer of the cells into a (P-) environment did not impose such a strong P stress over the 10 day incubation period that photosystem II damage occurred. As already noted, intracellular P stocks probably accounted for the tolerance to external P depletion. In agreement with our results, Bai et al. (2014), testing one strain of *R. raciborskii*, found that P depletion did not affect photosynthesis activity, at least for a restricted time of P depletion. 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. In our case, all *M. aeruginosa* strains were unicellular and had this activity affected, but in spite of this, they were able to grow, indicating that *M. aeruginosa* was also able to thrive in this condition.

AP activity may be ecologically relevant to both species to overcome P limitation and acquire P from organic compounds. In this work, during six days of culture in a P limited condition, three *M. aeruginosa* strains increased AP activity with varied profiles, while only one *R. raciborskii* strain did so. Possibly, the low external P levels during this period were not sufficient to fully increase the enzyme activity as the cells could still rely on internal P storage, since the inocula were not P starved before the experiment. A diverse pattern of AP activity levels for *M. aeruginosa* strains, as observed here, has also been pointed out in a previous study comparing eight strains of *M. aeruginosa* (Shen and 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 AP activity can vary. For *R. raciborskii*, although some studies have tested AP activity in varying P sources and concentrations (Wu et al., 2012; Bai et al., 2104), only a recent work has compared two strains and showed different AP activity per cell for them (Willis et al., 2019). In that work, no increase in AP activity was observed for the strains up to ten days in P depleted conditions, as compared to P replete medium, similarly to what we have observed here for *R. raciborskii* strains. Different levels of AP activity can result from distinct sensitivities for phosphorus starvation for each strain and can affect their fitness.

Variation in V_{max} values was observed among strains of the two species. In general, *R. raciborskii* showed higher variation in maximum uptake rate than *M. aeruginosa*. Other studies also compared the maximum uptakes rates of these two species with a few strains, with contradictory findings. Using two strains of each species, Marinho et al. (2013) found higher V_{max} values for *M. aeruginosa*. In contrast, Wu et al. (2009), using one strain of each species observed that *R. raciborskii* had a higher phosphate uptake rate than *M. aeruginosa*. These findings, together with our data, do not support the general idea that *R. raciborskii* has greater P uptake ability than *M. aeruginosa* as mentioned in some previous studies (Isvánovics et al., 2000; Antunes et al., 2015).

Many freshwater ecosystems are frequently limited in inorganic phosphorus and during blooms most of this element is incorporated into the biomass. Our results, as well as others found in the literature (Shen and Song, 2007; Wu et al., 2009; Harke et al., 2012; Wu et al., 2012; Harke and Gobler, 2013; Bai et al., 2014; Willis et al., 2015), show that *M. aeruginosa* and *R. raciborskii* are able to withstand P depletion, supporting growth and photosynthetic activity, and this relies on luxury uptake of P, efficient storage of polyphosphate and AP activity. Thus, management strategies based on phosphorous depletion must take into consideration that these organisms can tolerate P limitation, and that the populations can be sustained for some time, at least until internal P storage run out. Consequently, if these organisms remain in the water column after a P reduction intervention, for instance with a P adsorbent e.g, 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 may be 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 been established.

The intraspecific variability of *Microcystis* and *R. raciborskii* strains in the environment has been evidenced in previous studies (Cai et al., 2012; Pobel et al., 2012; Guedes et al., 2014; Willis et al., 2016, 2018) and this characteristic could provide a better resilience to a population under nutrient limitation. Thus, the persistence of these species can be attributed in part 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 illustrating this strain variability. Of note is the fact that working with five strains and a single parameter provided data to evidence the variability of these strains and precluded the conclusion that *R. raciborskii* is better adapted to phosphorus limitation than *M. aeruginosa*. Further studies working with a larger number of strains and more than a single nutrient may reveal an even greater variability. The physiological diversity of many freshwater cyanobacteria is underscored in laboratory-based studies, however future efforts using an extended number of strains are imperative for a better understanding of their ecophysiology in an environmental change scenario.

Acknowledgements

This work was conducted under the auspices of the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Brazil/NUFFIC, The Netherlands (project 045/12). This work was supported by National Council for Scientific and Technological Development (CNPq) (project 446216) and by the Carlos Chagas Filho Foundation for the Support of Science in Rio de Janeiro Development (FAPERJ) (project E-26/201.146/2014). I.A.G was supported by a CAPES fellowship (project 10001/14-0). [CG]

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.hal.2019.03.006.

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