UNIVERSIDADE FEDERAL RURAL DE PERNAMBUCO
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CIHELIO ALVES AMORIM

# EFEITOS ALELOPÁTICOS MÚTUOS ENTRE A MACRÓFITA AQUÁTICA *Egeria densa* PLANCH. E A CIANOBACTÉRIA FORMADORA DE FLORAÇÕES *Microcystis*

RECIFE, PE
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Dissertação apresentado ao Curso de Mestrado do Programa de Pós-Graduação em Botânica da Universidade Federal Rural de Pernambuco como requisito para obtenção do título de Mestre em Botânica.

Orientadora: Ariadne do Nascimento Moura
Coorientadores: Cláudia Ulisses e Ênio Wocyli Dantas

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Dissertação defendida e aprovada pela banca examinadora em 16 de agosto de 2017.

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Dedico à Vida!

Dedico à vida da forma como ela é,
da forma como todos veem e como
querem que seja!

Dedico ao dom da criação, do
surgimento das novas vidas e da
variabilidade das formas viventes!

Novamente dedico aos amigos,
pois são os melhores presentes que
a vida me deu!

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# LISTA DE FIGURAS E TABELAS

## REVISÃO BIBLIOGRÁFICA



## PRIMEIRO MANUSCRITO

**SEGUNDO MANUSCRITO**

Amorim, Cihelio Alves; M.Sc.; Universidade Federal Rural de Pernambuco; agosto de 2017; Efeitos alelopáticos mútuos entre a macrófita aquática *Egeria densa* Planch. e a cianobactérias formadora de florações *Microcystis*. Ariadne do Nascimento Moura (Orientadora), Cláudia Ulisses e Ênio Wocyli Dantas (Coorientadores).

## RESUMO

As macrófitas aquáticas são importantes para a manutenção dos estados alternativos claros dos ambientes aquáticos, pois podem liberar substâncias alelopáticas capazes de inibir o crescimento das algas planctônicas. Por outro lado, em ambientes túrbidos, resultantes das florações de cianobactérias, a presença de cianotoxinas pode afetar negativamente as macrófitas, causando estresse oxidativo e reduzindo o seu crescimento. Com o objetivo de elucidar as relações alelopáticas mútuas entre cianobactérias formadoras de florações e macrófitas submersas, nós testamos duas hipóteses através de estudos laboratoriais: (1) a liberação de aleloquímicos por *Egeria densa* reduz as taxas de crescimento de cepas tóxicas e não tóxicas de *Microcystis*; e (2) cepas tóxicas de *Microcystis* inibem o crescimento e biomassa de *E. densa*, além de ocasionar estresse oxidativo a essas plantas. Com base nisso, foram elaborados dois artigos: (1) Macrófitas submersas podem inibir cepas tóxicas e não tóxicas de cianobactérias igualmente? Efeitos alelopáticos de *Egeria densa* Planch. sobre *Microcystis* spp.; e (2) Respostas biométricas e fisiológicas de *Egeria densa* Planch. cultivada com cepas tóxica e não tóxica de *Microcystis*. No primeiro artigo, verificamos a influência da coexistência de *E. densa* e a aplicação de extratos desta planta (com os aleloquímicos dissolvidos) sobre a biomassa e taxas de crescimento de cepas tóxica de *M. aeruginosa* (MC+) e não tóxica de *M. panniformis* (MC-). No segundo, avaliamos as respostas fisiológicas e de crescimento de *E. densa* cultivadas com as cepas MC+ e MC-, verificando-se peroxidação lipídica, produção de peróxido de hidrogênio, alteração no conteúdo de pigmentos fotossintéticos e atividade das enzimas antioxidativas superóxido dismutase (SOD), catalase (CAT) e ascorbato peroxidase (APX). A cepa MC+ foi significativamente inibida quando cultivada com *E. densa*, enquanto que MC- exibiu uma resposta contrária, havendo estímulo do crescimento. Ao serem cultivadas sob influência do extrato de *E. densa*, as cepas MC+ e MC- foram inibidas, porém mais significativamente em MC-. A macrófita apresentou um pequeno incremento na produção de compostos fenólicos totais quando em coexistência com as cianobactérias, principalmente com a cepa tóxica. O crescimento e biomassa de *E. densa* foram inibidos quando em cultivo com a cepa MC+, com poucas alterações quando cultivadas com MC-. Além disso, a cepa MC+ inibiu a emergência de brotos e raízes nas plantas. Ambas as cepas mostraram uma redução nos teores de clorofilas totais, clorofila *a* e *b*, bem como um incremento dos carotenóides totais. As plantas cultivadas com MC+ apresentaram altas taxas de peroxidação lipídica e atividade enzimática, especialmente da APX. Nossos resultados suportam a hipótese de que existem interações alelopáticas mútuas entre *Microcystis* e *E. densa*. No entanto, *E. densa* necessita de um estímulo para liberar os aleloquímicos, como por exemplo o estresse ocasionado pelas microcistinas. A planta testada possui mecanismos de defesa enzimáticos que lhes permite coexistir com as microcistinas a curto prazo (cerca de oito dias), entretanto, a exposição prolongada (cinco semanas) às cepas tóxicas de *Microcystis* causa uma redução no crescimento da planta.

**Palavras chave:** alelopatia; cianobactérias formadoras de florações; estados alternativos dos lagos; estresse oxidativo; macrófitas submersas; microcistinas.

Amorim, Cihelio Alves; M.Sc.; Universidade Federal Rural de Pernambuco; August 2017; Mutual allelopathic effects of the aquatic macrophyte *Egeria densa* Planch. and the bloom-forming cyanobacterium *Microcystis*. Ariadne do Nascimento Moura (Adviser), Cláudia Ulisses and Ênio Wocyli Dantas (Co-advisers).

## ABSTRACT

Aquatic macrophytes are important for the maintenance of clear alternative states of aquatic environments, because they can release allelopathic substances capable of inhibiting the growth of planktonic algae. On the other hand, in turbid environments, resulting from cyanobacterial blooms, the presence of cyanotoxins can negatively affect the macrophytes, causing oxidative stress and reducing their growth. In order to elucidate the mutual allelopathic relationships between bloom-forming cyanobacteria and submerged macrophytes, we tested two hypotheses through laboratory studies: (1) the release of allelochemicals by *Egeria densa* reduces the growth rates of toxic and non-toxic strains of *Microcystis*; and (2) toxic strains of *Microcystis* inhibit the growth and biomass of *E. densa*, and cause oxidative stress on these plants. Based on this, two articles were written: (1) Why do aquatic macrophytes inhibit cyanobacteria? Allelopathic effects of *Egeria densa* Planch. on toxic and non-toxic strains of *Microcystis*; and (2) Can submerged macrophytes inhibit toxic and non-toxic strains of cyanobacteria equally? Allelopathic effects of *Egeria densa* Planch. on *Microcystis* spp. In the first article, we verified the influence of the coexistence of *E. densa* and the application of extracts of this plant (with dissolved allelochemicals) on the biomass and growth rates of toxic strain of *M. aeruginosa* (MC+) and non-toxic strain of *M. panniformis* (MC-). In the second, we evaluated the physiological and growth responses of *E. densa* cultivated with the MC+ and MC- strains, verifying the lipid peroxidation, hydrogen peroxide production, changes in photosynthetic pigment content and activity of the antioxidant enzymes superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (APX). The MC+ strain was significantly inhibited when cultivated with *E. densa*, while MC- exhibited a contrary response, with stimulating growth. When cultivated under the influence of *E. densa* extract, MC+ and MC- strains were inhibited, but more significantly in MC-. The macrophyte presented a small increment in the total phenolic compounds production when in coexistence with the cyanobacteria, mainly with the toxic strain. The growth and biomass of *E. densa* were inhibited when in co-culture with the MC+ strain, showing few changes when cultivated with the MC- strain. In addition, the MC+ strain inhibited the emergence of shoots and roots in plants. Both strains showed a reduction in total chlorophyll levels, chlorophyll *a* and *b*, as well as an increase in the total carotenoids content. The plants cultivated with MC+ strain presented high lipid peroxidation rates and enzymatic activity, especially for the APX. Our results support the hypothesis that there are mutual allelopathic interactions between *Microcystis* and *E. densa*. However, *E. densa* needs a stimulant to release the allelochemicals, such as the stress caused by microcystins. The tested plant has enzymatic defense mechanisms that allow them to coexist with microcystins during short-time exposure (about eight days), however, prolonged exposure (five weeks) to toxic strains of *Microcystis* causes a reduction in plant growth.

**Keywords**: allelopathy; alternative states of lakes; bloom-forming cyanobacteria; microcystins; oxidative stress; submerged macrophytes.

# SUMÁRIO

## 1 INTRODUÇÃO GERAL

O processo de eutrofização dos corpos hídricos, além do incremento da temperatura pelas mudanças climáticas, tem resultado em uma maior ocorrência de florações de cianobactérias tóxicas, gerando sérios problemas ecológicos (PAERL; HUISMAN, 2009; KOSTEN et al., 2012; O'NEIL et al., 2012; PAERL et al., 2016). Essa demasiada proliferação ocasiona graves consequências na utilização dos recursos hídricos, além de afetar as demais comunidades aquáticas, especialmente as macrófitas aquáticas (PAERL; OTTEN, 2013). Além disso, causa sérios prejuízos à saúde humana e animal, devido à liberação de cianotoxinas (CHORUS; BARTRAM, 1999). Deste modo, a redução das florações de cianobactérias tóxicas é prioritária no manejo das águas superficiais (HONG et al., 2009).

Geralmente, ambientes com florações de algas ou cianobactérias possuem uma baixa contribuição das macrófitas submersas, sendo a reintrodução dessa vegetação essencial na restauração destes ecossistemas (DONG et al., 2014). Uma vez que, a exsudação de aleloquímicos, compostos químicos liberados pelas plantas, é capaz de interferir no crescimento dos demais produtores primários aquáticos, como o fitoplâncton ou epifíton, fornecendo às macrófitas uma vantagem competitiva por luz e nutrientes (GROSS, 2003; HILT; GROSS, 2008; EIGEMANN; HILT; SCHMITT-JANSEN, 2013).

Rice (1984) definiu alelopatia como "qualquer efeito tóxico direto ou indireto de uma planta (incluindo microrganismos) em outra, através da produção de compostos químicos que escapam no ambiente". As reações aleloquímicas constituem a adaptação mais significativa das espécies e organização das comunidades, pois os aleloquímicos podem afetar a fisiologia, crescimento e desenvolvimento de outros organismos (REIGOSA et al., 1999; GROSS, 2009). Deste modo, as substâncias alelopáticas produzidas pelas macrófitas aquáticas atuam na inibição do crescimento fitoplanctônico, especialmente no controle de cianobactérias (VANDERSTUKKEN et al., 2014; ŠVANYS; PAŠKAUSKAS; HILT, 2014; ZUO et al., 2015). O modo de atuação destes aleloquímicos na redução das taxas de crescimento das cianobactérias é principalmente através da redução da atividade fotossintética (LEU et al., 2002; DZIGA et al., 2007; ZHU et al., 2010) ou causando estresse oxidativo (SHAO et al., 2009; ZHANG et al., 2010; CHENG et al., 2017).

Os aleloquímicos presentes nas macrófitas aquáticas apresentam diferentes formas de atuação em cepas tóxicas e não tóxicas de *Microcystis*, onde linhagens não tóxicas são mais sensíveis (ŠVANYS et al., 2016). Isso é devido principalmente a um mecanismo protetor das microcistinas contra o estresse oxidativo causado pelos aleloquímicos (DZIALLAS; GROSSART, 2011; ZILLIGES et al., 2011). Entretanto, pesquisas demonstram que este

mecanismo está relacionado à síntese e expressão gênica das microcistinas e não necessariamente às próprias toxinas (ŠVANYS et al., 2016). Porém, pesquisas envolvendo os efeitos alelopáticos de macrófitas sobre cianobactérias tóxicas e não tóxicas ainda não foram publicadas.

Macrófitas aquáticas e cianobactérias apresentam efeitos alelopáticos mútuos, através da liberação de aleloquímicos por ambas as partes, como compostos fenólicos pelas macrófitas e cianotoxinas pelas cianobactérias (MOHAMED, 2017). O principal motivo desta liberação é a inibição de seus competidores. Em ambientes aquáticos rasos, existe uma alternância na predominância de macrófitas e cianobactérias com consequências na dinâmica local. O estado alternativo claro é dominado por macrófitas, as quais inibem o fitoplâncton pela liberação de aleloquímicos e fornecimento de refúgio ao zooplâncton, enquanto que o estado túrbido é dominado por algas e cianobactérias, afetando as macrófitas através da síntese de cianotoxinas e sombreamento (SCHEFFER et al., 1993; 2001).

As macrófitas aquáticas são intensamente afetadas na presença de cianobactérias ou cianotoxinas, principalmente através da redução do crescimento e pigmentos fotossintéticos e estresse oxidativo (ZHENG et al., 2013; XU et al., 2016). Dentre estas cianotoxinas, as microcistinas, são as mais tóxicas e frequentes nos ambientes aquáticos (SVIRČEV et al., 2017), capazes de causar efeitos adversos a diversas plantas aquáticas (SAQRANE et al., 2007; ROMERO-OLIVA; CONTARDO-JARA; PFLUGMACHER, 2015a). Entretanto, as plantas possuem dois mecanismos fisiológicos capazes de diminuir os efeitos das cianotoxinas, a biotransformação e a defesa antioxidante (BABICA; BLÁHA; MARŠALEK, 2006).

Pesquisas que envolvem o efeito alelopático das macrófitas submersas sobre as cianobactérias são relevantes, pois buscam a elaboração de métodos eficientes no controle do crescimento destes organismos e auxiliam no melhor gerenciamento dos recursos hídricos, possibilitando melhorias na qualidade da água e diminuição de florações tóxicas. No entanto, o crescimento excessivo das cianobactérias, bem como a produção de toxinas, pode causar efeitos negativos sobre a vegetação aquática, como estresse oxidativo e bioacumulação de toxinas. Portanto, com o presente trabalho, nós verificamos os efeitos alelopáticos mútuos entre cianobactérias formadoras de florações do gênero *Microcystis*, com cepas tóxicas e não tóxicas, e a macrófita aquática invasora tropical, *Egeria densa* Planch.

O presente trabalho foi dividido em dois manuscritos, nos quais foram testadas duas hipóteses: (i) a liberação de aleloquímicos pela macrófita *E. densa* interfere negativamente no crescimento de cepas tóxicas e não tóxicas de *Microcystis*; e (ii) cepas tóxicas de *Microcystis* podem exercer um efeito negativo no desenvolvimento de *Egeria densa*, causando uma redução no crescimento e provocando estresse oxidativo nesta macrófita aquática.

# 2 REVISÃO BIBLIOGRÁFICA

## 2.1 Florações de cianobactérias e cianotoxinas

A eutrofização e a subsequente formação de florações tóxicas consistem nos principais problemas que afetam os ambientes aquáticos atualmente, os quais trazem sérios prejuízos ecológicos e de saúde humana (ZHANG et al., 2011; 2014; ZUO et al., 2012; ZHOU et al., 2014). Inúmeros fatores favorecem o aparecimento de florações de cianobactérias em ecossistemas de água doce, com destaque para a eutrofização e a elevação da temperatura (PAERL; HUISMAN, 2008; PAERL; OTTEN, 2013; MARIANI et al., 2015; PAERL et al., 2016). Especialmente pelos efeitos das mudanças climáticas, as cianobactérias têm se tornado cada vez mais invasoras em ambientes de água doce ao redor do mundo (MOOIJ et al., 2007; DAVIS et al., 2009; ELLIOTT, 2012; PAERL; PAUL, 2012; MARIANI et al., 2015; LÜRLING et al., 2017).

Em ecossistemas aquáticos, as florações de cianobactérias podem ser dominadas por cepas tóxicas ou não tóxicas (CHORUS; BARTRAM, 1999; SVIRČEV et al., 2017), sendo as cepas não tóxicas de *Microcystis* consideradas melhores competidoras por luz, destacando-se nos ambientes em que ocorrem (KARDINÄAL et al., 2007). Porém, em condições de elevadas temperaturas e nutrientes, cepas tóxicas podem ser predominantes em relação às não tóxicas, com maior produção de microcistinas (DAVIS et al., 2009). Portanto, o controle das florações de cianobactérias é fundamental para minimizar os riscos de contaminação dos organismos aquáticos e do homem em áreas tropicais (HONG et al., 2009; ZHANG et al., 2014). Uma vez que as florações de cianobactérias podem se tornar mais intensas devido ao cenário de eutrofização e mudanças climáticas.

Os principais gêneros formadores de florações em áreas tropicais são *Microcystis*, *Planktothrix*, *Cylindrospermopsis* e *Anabaena* (MOWE et al., 2015), destacando-se *Microcystis aeruginosa* Kütz. como a principal espécie formadora de florações em ambientes de água doce (HARKE et al., 2016). Estes gêneros são produtores de microcistinas, cilindrospermopsinas, saxitoxinas e anatoxina-a (WIEGAND; PFLUGMACHER, 2005; PAERL; HUISMAN, 2009; PAERL; OTTEN, 2013; OTTEN; PAERL, 2015). A região Nordeste apresenta maior ocorrência de florações com registros tóxicos no Brasil. Especialmente no estado de Pernambuco, diversas cianobactérias foram registradas em florações tóxicas, destacando-se *Microcystis aeruginosa*, *Planktothrix agardhii* (Gomont) Anagn. & Komárek e *Cylindrospermopsis raciborskii* (Wolosz.) Seenayya & Subba Raju, tendo sido registrada a ocorrência de diversas cianotoxinas em reservatórios de abastecimento, como microcistinas,

cilindrospermopsina, saxitoxinas e anatoxina-a(s) (MOLICA et al., 2005; BITTENCOURT-OLIVEIRA; SANTOS; MOURA, 2010; BITTENCOURT-OLIVEIRA et al., 2014; LORENZI et al., 2015; MOURA; ARAGÃO-TAVARES; AMORIM, submetido). Portanto, estratégias de controle das cianobactérias podem promover uma melhoria na qualidade da água para abastecimento, aumentando a oferta de água potável.

As microcistinas são as cianotoxinas mais amplamente distribuídas em ambientes aquáticos continentais e apresentam efeitos deletérios significativos nos organismos que entram em contato com variantes desta toxina (WIEGAND; PFLUGMACHER, 2005; MARTINS; VASCONCELOS, 2009; PAERL; HUISMAN, 2009). Mais de 240 variantes de microcistinas já foram descritas para os ambientes aquáticos de todo o mundo (SVIRČEV et al., 2017; SPOOF; CATHERINE, 2017), dentre estas, a microcistina-LR é a mais comumente registrada e atua principalmente inibindo as proteínas fosfatases 1 e 2A em plantas e animais (MacKINTOSH et al., 1990; DAWSON, 1998; CHORUS; BARTRAM, 1999).

As microcistinas são conhecidas por afetar diversos organismos que entram em contato com estas cianotoxinas, como por exemplo, hortaliças (CORDEIRO-ARAÚJO et al., 2015, 2016; BITTENCOURT-OLIVEIRA et al., 2016; DOBRAC et al., 2017; MACHADO et al., 2017), macrófitas aquáticas (MITROVIC et al., 2005; SAQRANE et al., 2007; ROMERO-OLIVA; CONTARDO-JARA; PFLUGMACHER, 2015a, 2015b), zooplâncton (LIANG et al., 2017a, 2017b), peixes (BOARU et al., 2006; FERREIRA et al., 2010) e mamíferos, incluindo o homem (CARMICHAEL et al., 2001; ZEGURA et al., 2003). O episódio mais conhecido de contaminação humana por microcistinas ocorreu em 1996 em Caruraru, Pernambuco, onde a presença destas toxinas na água que abastecia uma clínica de hemodiálise causou a morte de 76 pacientes (AZEVEDO et al., 2002).

## 2.2 Macrófitas aquáticas e seu potencial alelopático *in situ* e *in vitro*

Existem dois estados alternativos nos ambientes aquáticos, um claro, quando o ambiente é dominado por macrófitas aquáticas, e outro túrbido, devido à elevada biomassa fitoplanctônica (SCHEFFER, 1990; SCHEFFER et al., 1993; 2001). As plantas submersas são capazes de manter a qualidade da água em sistemas rasos (ZHANG et al., 2014), proporcionando uma maior transparência devido à liberação de aleloquímicos que atuam contra o crescimento fitoplanctônico (HILT; GROSS, 2008; BLINDOW; HARGEBY; HILT, 2014). A manutenção da claridade da água se deve também à capacidade das macrófitas de reduzir a resuspensão do sedimento e fornecer refúgio para peixes e zooplâncton, que atuam na herbivoria do fitoplâncton. No entanto, a elevada turbidez da água, proporcionada pelo

crescimento fitoplanctônico, reduz o desenvolvimento das macrófitas devido à menor disponibilidade de luz na coluna d'água (TAKAMURA et al., 2003) (Figura 1).

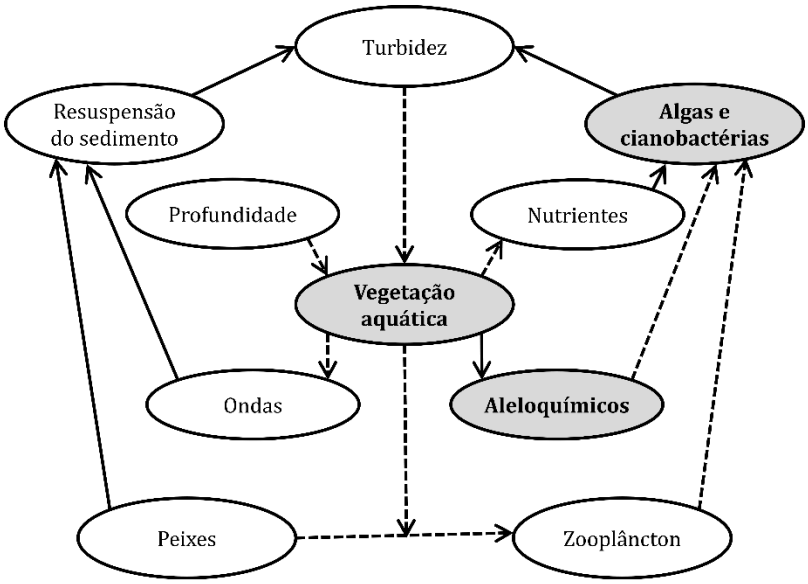


**Figura 1.** Interações ocorrentes nos ambientes aquáticos que influenciam na alternância dos estados alternativos claro e túrbido, com destaque para as relações alelopáticas entre macrófitas e fitoplâncton. Relações positivas são representadas por linhas contínuas e negativas por linhas tracejadas. Redesenhado de Scheffer et al. (1993).

A alelopatia pode ser observada tanto em plantas terrestres quanto aquáticas. Nos ecossistemas aquáticos, plantas submersas e flutuantes, juntamente com as algas, desempenham uma função primordial na dinâmica local (PFLUGMACHER, 2002). Nesses ambientes, a alelopatia ocorre em todos os grupos de macrófitas e do fitoplâncton, incluindo as cianobactérias; seus efeitos geralmente são negativos para outros organismos vivos, comumente inibindo o crescimento e fotossíntese (ZAK et al., 2012). No entanto, Li et al. (2016) ressaltam que, além da alelopatia, outros fatores, como competição por luz e nutrientes, podem dar às macrófitas uma maior vantagem em relação às cianobactérias. Macrófitas que apresentam um potencial para a alelopatia podem ser utilizadas no processo de restauração de ambientes aquáticos eutróficos, uma vez que estas plantas podem controlar o crescimento algal, especialmente de cianobactérias (GROSS; MEYER; SCHILLING, 1996; GHOBRIAL; NASSR; KAMIL, 2015).

A alelopatia consiste na interação bioquímica entre dois organismos, principalmente plantas, os quais secretam substâncias que interferem no desenvolvimento dos demais seres vivos (PFLUGMACHER, 2002). Esses aleloquímicos, principalmente polifenóis, compostos sulfúricos, poliacetilenos e ácidos graxos (GROSS, 1999), são secretados diretamente na água, podendo agir mais eficientemente, minimizando as chances de ocorrência das florações de cianobactérias (CHEN et al., 2012), uma vez que estes organismos são mais sensíveis aos

aleloquímicos das macrófitas aquáticas que as clorófitas (KÖRNER; NICKLISCH, 2002; ERHARD; GROSS, 2006; ZHU et al., 2010).

Dentre os estudos realizados com macrófitas aquáticas e seus aleloquímicos no controle de microalgas e cianobactérias tóxicas, destacam-se os de Zuo et al. (2012) que analisaram os efeitos algicidas de *Alternanthera philoxeroides* (Mart.) Griseb. sobre o crescimento do fitoplâncton, os quais foram mais eficientes contra *Microcystis aeruginosa* e *Chlorella pyrenoidosa* H.Chick. Chen et al. (2012) verificaram que *Nymphaea tetragona* Georgi, *Typha orientalis* Presl., *Nelumbo nucifera* Gaertn. e *Iris wilsonii* Wright inibiram o crescimento de *Microcystis aeruginosa*. Zhu et al. (2014) constataram um rápido crescimento de *M. aeruginosa* no início do co-cultivo com *Cyperus alternifolius* L. seguido de um crescente decréscimo, demonstrando efeitos inibitórios.

Zhu et al. (2010) verificaram que os polifenóis isolados de *Myriophillum spicatum* L. causaram uma redução significativa do fotossistema II e de toda a cadeia transportadora de elétrons de *M. aeruginosa*. Chang, Eigemann e Hilt (2012) revelaram que *Myriophyllum verticillatum* L. e o ácido tânico por ela liberado inibiram o crescimento de *M. aeruginosa* em culturas puras, sendo estimulado quando co-cultivada com a clorofícea *Desmodesmus armatus* (Chodat) E.Hegewald. Zhang et al. (2014) registraram uma inibição significativa das cianobactérias tóxicas *Microcystis aeruginosa* e *Anabaena flos-aquae* Brébisson e da clorofícea *Scenedesmus obliquus* (Turpin) Kütz. pelos efeitos dos exsudatos de *Najas minor* All. Zuo et al. (2015) verificaram que as macrófitas aquáticas inibiram o crescimento de *M. aeruginosa* e *C. pyrenoidosa* (clorofícea) através de compostos alelopáticos de *Potamogeton crispus* L., *Nymphoides peltatum* (Gmel.) O.Kuntze, *Ranunculus sceleratus* L. e *Alternanthera philoxeroides*.

Fortes efeitos inibitórios de *Myriophillum aquaticum* (Vell.) Verdc. em coexistência com *Anabaena flos-aquae* e *Microcystis aeruginosa*, acompanhados pela inibição da enzima antioxidante superóxido dismutase, foram verificados por Wang et al. (2017). Diversas macrófitas mostraram efeitos alelopáticos contra cianobactérias no estudo de Grutters et al. (2017), os autores verificaram que a quantidade de compostos fenólicos, e consequentemente a atividade alelopática contra *Dolichospermum flos-aquae*, não diferiu entre plantas nativas e invasoras. A variabilidade no potencial alelopático das macrófitas foi explicada principalmente pela filogenia, estratégia de crescimento das espécies e relação carbono:fósforo, com eucotiledôneas emergentes possuindo maior potencial alelopático.

Li et al. (2016) mostraram que extratos aquosos do tubérculo de *Sagittaria trifolia* L. inibiram significativamente o crescimento de *Microcystis aeruginosa* de uma forma dependente da concentração, com uma taxa de inibição de até 90,4% com extratos a uma concentração de

100% (v/v). Os autores também observaram fortes danos oxidativos na cianobactéria, causados pelos aleloquímicos da macrófita. Gao et al. (2017) mostraram que *Microcystis aeruginosa* foi inibida com a adição de N-fenil-1-naftalamina isolado das raízes de *E. crassipes*. A inibição do crescimento foi acompanhada pela redução da atividade do fotossistema II, entretanto, em elevadas concentrações, *Microcystis aeruginosa* foi estimulada.

Estudos que visam avaliar os efeitos alelopáticos dos aleloquímicos sobre cepas tóxicas e não tóxicas mostram que as linhagens não tóxicas são mais sensíveis. Como verificado por Švanys et al. (2016), linhagens não tóxicas de *Microcystis aeruginosa* são mais sensíveis ao ácido tânico, mostrando que algo relacionado à síntese das microcistinas confere uma maior tolerância aos aleloquímicos para as cepas tóxicas. Entretanto, Mulderij et al. (2005) mostraram que uma cepa tóxica de *Microcystis aeruginosa* foi mais sensível aos exsudatos de *Stratioides aloides* L. que uma linhagem não tóxica.

Poucos estudos relatam os efeitos alelopáticos de macrófitas sobre o fitoplâncton em ambientes naturais, devido principalmente a atuação de outros fatores, como limitação por luz e nutrientes, competição e herbivoria. O primeiro estudo a comprovar a presença de respostas alelopáticas de macrófitas aquáticas sobre o crescimento fitoplanctônico foi o de Hilt, Ghobrial e Gross (2006), mostrando que *Myriophyllum verticillatum* inibiu o fitoplâncton, principalmente as cianobactérias. Os efeitos puderam ser atribuídos à alelopatia devido não haver diferença entre os outros parâmetros ambientais nos tratamentos controle e de coexistência com a macrófita. Mulderij et al. (2006) revelaram que a taxa de crescimento da comunidade fitoplanctônica foi significativamente inibida quando em contato com *Stratioides aloides*.

O estudo de Dong et al. (2014) mostrou que a introdução de *Ceratophyllum demersum* L. em um lago eutrófico inibiu o crescimento de *M. aeruginosa*, beneficiando na restauração do lago. Švanys, Paškauskas e Hilt (2014) verificaram os efeitos (limitação de nutrientes e alelopatia) de *Myriophyllum spicatum* sobre o fitoplâncton e afirmaram que a macrófita é capaz de controlar mais eficientemente as populações de cianobactérias, sendo que cepas tóxicas e não tóxicas de *M. aeruginosa* foram igualmente inibidas, causando uma maior taxa de produção de microcistinas. Efeitos negativos sobre o fitoplâncton foram observados para as macrófitas *Egeria densa*, *Potamogeton illinoensis* Morong e *Elodea nuttallii* (Planch.) St. John por Vanderstukken et al. (2011; 2014). Essas plantas inibiram o fitoplâncton pela competição por nutrientes, porém outros experimentos mostraram que mecanismos biológicos, como alelopatia, podem também ter sido responsáveis pela redução do crescimento fitoplanctônico.

## 2.3 Efeitos nocivos das cianobactérias e cianotoxinas sobre as macrófitas aquáticas

As macrófitas aquáticas, quando na presença de cianotoxinas, podem apresentar uma redução em seu crescimento acompanhado pela diminuição dos pigmentos fotossintéticos, além de estresse oxidativo devido a maior produção de espécies reativas de oxigênio (PFLUGMACHER, 2002; 2004; SAQRANE et al., 2007). No entanto, estas plantas possuem dois mecanismos de defesa que lhes permitem coexistir com estas cianotoxinas: a biotransformação e o sistema de defesa antioxidante (PFLUGMACHER; STEINBERG, 1997; BABICA; BLÁHA; MARŠALEK, 2006). Estes dois mecanismos são os mais eficazes no combate aos efeitos tóxicos gerados pela exposição às cianotoxinas (ROMERO-OLIVA; CONTARDO-JARA; PFLUGMACHER, 2015b).

No processo de biotransformação, ocorre a degradação das substâncias poluentes, dentre elas as cianotoxinas, e ocorre em três etapas. Na fase I ocorre a degradação e transformação das substâncias nocivas em compostos menos tóxicos. Na fase II estes compostos são conjugados à glutationa, os quais são metabolizados através da enzima glutationa S-transferase, deixando o composto com características mais hidrossolúveis. Na fase III, fase final da biotransformação, ocorre a compartimentalização das substâncias geradas na fase I, podendo ocorrer de duas maneiras diferentes, seja pela excreção (em animais), ou estocagem na parede celular ou em vacúolos (em plantas) (PFLUGMACHER; STEINBERG, 1997; DIXON; SKIPSEY; EDWARDS, 2010).

O sistema de defesa antioxidante das plantas aquáticas atua como uma resposta ao estresse biótico ou abiótico do meio. O principal modo de ação deste sistema é através da eliminação das espécies reativas de oxigênio, que podem causar danos às proteínas, lipídios e ao DNA (GILL; TUTEJA, 2010). O controle do estresse oxidativo nas plantas pode ocorrer através de mecanismos enzimáticos, através da atividade da catalase, superóxido dismutase, ascorbato peroxidase e glutationa peroxidase (MITTLER et al., 2004), ou não enzimáticos, com a atuação de carotenoides, compostos fenólicos, e tocoferóis (GILL; TUTEJA, 2010).

Efeitos nocivos das cianotoxinas sobre o desenvolvimento de macrófitas aquáticas foram verificados nos trabalhos de Ha e Pflugmacher (2013a; 2013b) e Ha, Contardo-Jara e Pflugmacher (2014), nos quais os autores mostraram que a macrófita *Ceratophyllum demersum* foi capaz de absorver uma grande quantidade de anatoxina-a nas primeiras 24 h do experimento, porém a planta reduziu seu crescimento após exposição à toxina, o que causou um efeito alelopático negativo, induzindo o estresse oxidativo na macrófita. O estudo de Rojo et al. (2013) mostrou que pequenas doses de microcistina-LR são capazes de afetar negativamente a

germinação e crescimento de algumas espécies de carofíceas e da angiosperma *Myriophyllum spicatum*.

Flores-Rojas, Esterhuizen-Londt e Pflugmacher (2015) mostraram que a exposição de *Lemna minor* L. à cilindrospermopsina causou um estresse oxidativo, porém, esta planta possui mecanismos de defesa que controlam os níveis de toxinas. Romero-Oliva et al. (2014) e Romero-Oliva, Contardo-Jara e Pflugmacher (2015a; 2015b) constataram que as macrófitas aquáticas *Ceratophyllum demersum*, *E. densa* e *Hydrilla verticillata* Royle apresentaram alterações fisiológicas e redução dos pigmentos fotossintéticos, quando expostas às microcistinas LR, RR e YR, porém, essas plantas acumularam e degradaram essas cianotoxinas. No entanto, os autores utilizaram extratos de florações, nos quais poderiam estar presentes outros compostos tóxicos diferentes das microcistinas, devido à presença de variadas espécies fitoplanctônicas. Ao exporem a macrófita *Ceratophyllum demersum* à neurotoxina β-N-metilamino-L-alanina, Downing, Esterhuizen-Londt e Downing (2015) registraram que esta planta foi capaz de remover rapidamente a cianotoxina do meio, além de absorver e modificar covalentemente a β-N-metilamino-L-alanina no interior de suas células, excretando a toxina em uma forma não tóxica, não detectada durante a depuração do meio.

Zhang et al. (2014), verificaram que *Microcystis aeruginosa*, quando em elevadas densidades ($10^7$ cél $mL^{-1}$), pôde inibir o crescimento de *Najas minor*, relacionando-se com a presença de cianotoxinas. Zheng et al. (2013) observaram que a germinação e biomassa de *Potamogeton malaianus* Miq. foi significativamente inibida pelos exsudatos de *M. aeruginosa* em altas concentrações, no entanto, extratos da cianobactéria não demonstraram efeitos na macrófita. Exsudatos e extratos de *Microcystis aeruginosa* também inibiram a germinação de *Ottelia acuminata* (Gagnep.) Dandy no estudo de Xu et al. (2015), os autores concluíram que plantas adultas promoveram o crescimento da cianobactéria testada. Xu et al. (2016) também perceberam um efeito negativo dos exsudatos de *Microcystis aeruginosa* sobre *Potamogeton crispus*, interrompendo a fotossíntese e causando estresse oxidativo desta macrófita.

## 2.4 Interações alelopáticas mútuas entre macrófitas e cianobactérias

Esses estudos (apresentados nos tópicos *2.2* e *2.3*) mostram que as cianobactérias e macrófitas aquáticas apresentam interações alelopáticas mútuas, com a liberação de substâncias que inibem o crescimento dos competidores, como os polifenóis (macrófitas) e as cianotoxinas (cianobactérias). Ambos os organismos liberam estas substâncias em situações de estresse, seja nas cianobactérias, ocasionado pelos polifenóis, ou nas macrófitas, provocado pelas cianotoxinas. Porém, ainda existe uma lacuna sobre como se dão estas relações entre macrófitas

e cepas tóxicas e não tóxicas de cianobactérias, especialmente sobre qual o papel das microcistinas no estímulo da liberação de aleloquímicos pelas plantas aquáticas. Além disso, ainda não se conhecem os efeitos de linhagens não tóxicas de cianobactérias sobre as macrófitas aquáticas.

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## 3 PRIMEIRO MANUSCRITO

# Can submerged macrophytes inhibit toxic and non-toxic strains of cyanobacteria equally? Allelopathic effects of *Egeria densa* Planch. on *Microcystis* spp.

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**Abstract**

Shallow aquatic environments have two alternative states, one clear, dominated by aquatic macrophytes, and the other turbid, with significant phytoplankton growth. However, little is known about which factors affect the allelopathic response of macrophytes on toxic and non-toxic strains of cyanobacteria, especially what causes the release of allelopathic compounds. Therefore, we tested the hypothesis that allelochemicals released by *Egeria densa* negatively interfere in the growth of toxic and non-toxic strains of *Microcystis*. We investigated the effects of this macrophyte on biomass and growth and inhibition rates of a microcystin-producing (MC+) strain of *Microcystis aeruginosa*, and another non-microcystin-producing (MC-) strain of *Microcystis panniformis*, through coexistence experiments and with application of the aqueous extract of *E. densa*. This aquatic macrophyte has allelopathic compounds that can act to inhibit cyanobacteria, such as polyphenols and flavonoids, with a total concentration of phenolic compounds of 15.78 mg eTA $gDW^{-1}$ (mg equivalents of tannic acid per g dry weight). The MC+ strain was significantly inhibited when in coexistence with the macrophyte, whereas MC- was stimulated. With the application of the aqueous extract, both strains were inhibited, but the non-toxic strain was more sensitive to allelochemicals. These results demonstrate that *E. densa* has allelopathic compounds capable of inhibiting the growth of *Microcystis*, but are only released under stress conditions, such as that caused by the presence of microcystins in coexistence with the toxic strain. These results are important for future biomanipulation strategies of aquatic macrophytes, in which the presence and proportion of toxic and non-toxic strains of cyanobacteria should also be considered.

**Keywords**: Allelochemicals; control of *Microcystis*; cyanobacterial blooms; invasive aquatic plants; polyphenols.

## Introduction

The control of toxic cyanobacterial blooms is the main problem for managing continental aquatic environments. These blooms are increasingly common and influenced by several factors, such as eutrophication and climate change (Paerl and Huisman 2008, Smith and Schindler 2009, Paerl and Otten 2013). However, this accelerated growth has several negative impacts on aquatic biota and human populations, especially due to the release of cyanotoxins (Carmichael and Boyer 2016). Cyanobacterial blooms can be formed by toxic and non-toxic strains (Svirčev et al. 2017), however, with the increased eutrophication and higher temperatures, toxic *Microcystis* blooms may become more significant, resulting in a higher production of microcystins (Davis et al. 2009). Therefore, controlling cyanobacteria is fundamental in reducing contamination for aquatic organisms and, consequently, humans.

Shallow aquatic environments have two alternative states, a clear state dominated by aquatic vegetation, and a turbid state with high phytoplankton biomass (Scheffer et al. 1993, 2001). Macrophytes play an important role in maintaining the clear state, by providing shelter for zooplankton, the main consumers of phytoplankton (Jeppesen et al. 1997), or by releasing allelopathic substances that inhibit phytoplankton growth (Hilt and Gross 2008). Several studies have demonstrated the potential of aquatic macrophytes in inhibiting cyanobacteria growth, in particular *Microcystis aeruginosa* Kütz. (e.g. Chang et al. 2012, Švanys et al., 2014), which is considered one of the most toxic and most commonly encountered cyanobacteria in aquatic environments worldwide (Harke et al. 2016).

Several species of aquatic macrophytes are considered invasive, as they can colonize new areas and develop in a disorderly way, which can damage the dynamics and structure of native species (Havel et al. 2015). One theory that explains the success of these plants is the "new weapons hypothesis" (Callaway and Ridenour 2004), which predicts that some exotic plants possess biochemical weapons, such as the production of allelochemicals, that they use at new colonization sites and gives them a competitive advantage by increasing their invading

potential. The macrophyte *Egeria densa* Planch. Has caused great economic and ecological losses and is currently one of the most harmful aquatic species, additionally, it has been estimated that there will be an increase in the distribution of this macrophytes throughout the world until 2070 (Gillard et al. 2017). The success of its wide distribution is associated with its phenotypic plasticity, competition (whether by light or nutrients), propagule production, mutualism, and invasional meltdown (Fleming and Dibble 2015). Moreover, *E. densa* can suppress phytoplankton growth through competition for nutrients and release of allelochemicals, which increases its colonization success (Vanderstukken et al. 2011). However, when coexisting with toxic strains of *Microcystis*, this macrophyte is intensely affected (Amorim et al. 2017).

The allelopathy between aquatic macrophytes and phytoplankton consists of biochemical interactions between these organisms, which can inhibit or stimulate competitors (Gross et al. 2007). The aquatic macrophytes can synthesize a wide variety of allelochemicals, especially polyphenols, sulfur compounds, polyacetylenes, and fatty acids, that control phytoplankton growth (Gross 1999). The synthesis and release of these compounds are influenced by environmental variables, biological aspects of the species, or by biotic interactions with other organisms (Bauer et al. 2009). Polyphenols are the most commonly released allelochemicals and act differently on cyanobacteria, either by inhibiting or retarding growth, oxidative stress, or reducing photosynthesis (Laue et al. 2014). In *M. aeruginosa* the main action site for the allelochemicals is the photosystem II, through damage caused in the electron transport chain (Zhu et al. 2010).

Despite this, little is known about how allelochemicals of aquatic macrophytes affect toxic and non-toxic strains of *Microcystis*. Results among previous studies vary, with distinct responses for toxic and non-toxic strains. Furthermore, it has been demonstrated that toxic strains of *Microcystis* are more sensitive to exudates of *Stratiotes aloides* L. (Mulderij et al. 2005). In addition, in a natural ecosystem, the macrophyte *Myriophyllum spicatum* L. was

also able to inhibit toxic and non-toxic populations of *M. aeruginosa* (Švanys et al. 2014). Recently, non-toxic strains of *M. aeruginosa* were found to be more sensitive to allelochemicals of aquatic macrophytes, such as tannic acid (Švanys et al. 2016). However, studies on the allelopathic effects of submerged aquatic macrophytes on toxic and non-toxic strains of cyanobacteria in coexistence have not yet been published. Therefore, knowledge about how cyanotoxins stimulate the release of allelochemicals by aquatic macrophytes in coexistence is scarce.

The objective of this study was to analyze the influence of allelopathic substances emitted by the aquatic macrophyte *E. densa* on the growth of blooming cyanobacteria of the genus *Microcystis*. Thus, we tested the hypothesis that the release of allelochemicals by *E. densa* negatively impacts the growth rates of toxic and non-toxic strains of *Microcystis*. Therefore, we evaluated the growth of cyanobacteria in coexistence with *E. densa*, to verify if the macrophyte inhibits toxic and non-toxic cyanobacteria equally; and through the application of the aqueous extract of the plant to analyze if the plant has compounds that inhibit the growth of toxic and non-toxic strains, which eventually are not released in coexistence.

## Material and Methods

*Organisms and culture conditions*

Two strains of *Microcystis* were used in our experiments, one microcystin-producer (MC+) and one non-producer (MC-). The toxic strain of *M. aeruginosa* (NPLJ-4) was obtained from the Collection of Cyanobacteria of the Federal Rural University of Pernambuco. This strain presents [D-Leu$^{-1}$] microcystin-LR as the most representative toxin, with about 90% of the total microcystins, in addition to three other unidentified microcystin variants (Amorim et al. 2017). The non-toxic strain was *M. panniformis* (BCCUSP29) obtained from the Brazilian Cyanobacteria Collection of the University of São Paulo. This

129 strain belongs to the *M. aeruginosa* complex, due to a great genetic similarity between these
130 species (Bittencourt-Oliveira et al. 2001), and does not produce microcystins (Bittencourt-
131 Oliveira 2003). The cultures of the cyanobacteria were kept in ASM1 nutrient medium
132 (Gorham et al. 1964) in a climatic chamber with controlled conditions of temperature (25°C ±
133 1.5), light intensity (40 µmol photons $m^{-2}$ $s^{-1}$), pH (7.5), photoperiod (12 h), and homogenates
134 three times a day. Cultures were maintained until a density of 1.5 $\times 10^6$ cel $mL^{-1}$ of the MC+
135 strain and 2.5 $\times 10^6$ cel $mL^{-1}$ of the MC- strain was obtained during the end of the exponential
136 growth phase. This difference was due to the lower cellular volume of *M. panniformis*,
137 resulting in a biomass of 100 mg $L^{-1}$ for both strains (Amorim et al. 2017).
138 The submerged aquatic macrophyte *E. densa* was selected because of its potential
139 inhibitory effect on phytoplankton and cyanobacteria shown in previous studies of
140 coexistence in a subtropical reservoir (Vanderstukken et al. 2011). Branches of *E. densa*
141 (about 25 cm) were collected from the Tabocas Reservoir, in Belo Jardim, Pernambuco,
142 Brazil (8°14'32" S; 36°22'35") in September 2016, one month before the experiments started.
143 This reservoir presents conditions of mesotrophy without cyanobacterial blooms (Amorim et
144 al. 2017). In the laboratory, the plants were washed several times with running water and
145 ultrapure water to remove debris and periphytic organisms, they were then kept in 8L tanks
146 containing tap water under the same conditions for cyanobacteria cultures and constant
147 aeration. Apical branches of young plants (early stages of development) were used because
148 the meristematic growth tissues have a greater amount of active allelochemicals (Gross 2003)
149 and adult plants can stimulate the growth of cyanobacteria (Xu et al. 2015).
150
151 *Preparation of aqueous extract of E. densa*
152 Young *E. densa* branches were cut into small pieces, frozen at -80 ° C, and freeze-
153 dried. Then, the dried material was macerated in a mortar and immersed in 50% (v/v)
154 methanol for two hours at a ratio of 100 mL to 1 g dry weight (DW) of the macrophyte to

155 extract allelopathic compounds (Erhard and Gross 2006). This solution was rotoevaporated
156 and filtered on glass fiber filters with 0.45 μm porosity. The aqueous extract was prepared
157 three days prior to the experiments, diluted in ASM1 medium with twice the nutrient
158 concentration and stored at 4 °C in the dark to avoid photodecomposition of the
159 allelochemicals.
160
161 *Phytochemical analysis and quantification of total phenolic compounds in E. densa*
162 Phytochemical analysis of *E. densa* was performed using analytical thin layer
163 chromatography. We used the extract of the plant extracted in 50% methanol prepared as
164 mentioned above. The extract was then separated into aqueous and chloroform phases to
165 distinguish hydrophobic and hydrophilic compounds. The presence of polyphenols,
166 flavonoids, coumarins, saponins, tannins, and triterpenes, the eluents present in the mobile
167 phase, and the revealing substances for each metabolite are described in Table 1. Metabolites
168 were detected through the application of specific revealing substances and visualization in
169 ultraviolet light (between 254 and 365 nm). The presence of saponins was determined by
170 shaking the extract on a vortex for 5 minutes.
171 To determine the total phenolic compounds (TPC), 10 mg of freeze dried *E. densa*
172 material was extracted in 5 mL of 80% ethanol for 10 minutes at 80°C and subsequently
173 centrifuged at 5000 rpm for 10 minutes to remove the debris. Then, 2 mL of 1% sodium
174 dodecyl sulfate and 2 mL of 0.01 M $FeCl_3$ solution in 0.01 N HCl were added to 1 mL of *E.*
175 *densa* extract according to the methodology of Hagerman and Butler (1978). After 30
176 minutes, the reduction of $Fe^{3+}$ to $Fe^{2}$ through the activity of the phenolic compounds was
177 checked spectrophotometrically at 510 nm and the amounts of the compounds determined by
178 the regression formula (Absorbance = (0.1109 × TPC) - 0.0035, $R^2 = 0.9999$, $p < 0.05$)
179 obtained with the tannic acid calibration curve at concentrations ranging from 25 to 350 μg

180 $mL^{-1}$. The results were expressed in mg equivalents of tannic acid per g dry weight (mg eTA
181 $gDW^{-1}$).

182 To evaluate the production of total phenolic compounds in *E. densa* in response to
183 cultures with toxic and non-toxic strains of *Microcystis*, we carried out a parallel experiment
184 with the same plant and cyanobacteria biomass and the same conditions as the other
185 experiments (subsection 2.4). Three treatments were used to evaluate the producing of total
186 phenolic compounds, in coexistence of *E. densa* with the MC+ strain (1), coexistence of the
187 plant with the MC- strain (2), and control with the cultivation of the plants without
188 *Microcystis* strains (3). We checked the amounts of total phenolic compounds at the
189 beginning (day 0) and end (day 14) of the experiment. The analyses were performed
190 according to Hagerman and Butler (1978), however, 100 mg of fresh weight (FW) were
191 macerated and the results expressed in mg equivalents of tannic acid per g of fresh plant
192 weight (mg eTA $gFW^{-1}$).

193

194 *Experimental design*

195 Two experiments were performed in parallel to verify the effects of *E. densa* on toxic
196 and non-toxic strains of *Microcystis*. Both started with the same cyanobacteria densities for
197 each strain, $1.6 \times 10^6$ cel $mL^{-1}$ (± 0.29) for MC+ and $2.55 \times 10^6$ cel $mL^{-1}$ (± 0.29) for MC-,
198 corresponding to the biomasses of 110.04 mg $L^{-1}$ (± 19.7) and 105.29 mg $L^{-1}$ (± 11.9),
199 respectively. The experiments were conducted in a climatized aseptic room with the same
200 conditions previously described for the cultivation of cyanobacteria. We used Erlenmeyer
201 flasks with 1000 mL capacity containing 850 mL of ASM1 culture medium, inoculated with
202 MC+ and MC- cyanobacteria cultures.

203 In the first experiment, we evaluated the effects of *E. densa* culture on the coexistence
204 with cyanobacterial strains. Three days prior, the plants were washed five times with ultrapure
205 water to remove algae and adhered animals, and were kept in ASM1 medium for acclimation.

When cyanobacterial cultures reached the predicted biomass, two macrophytes branches were added, totaling a biomass of 8.2 gFW $L^{-1}$ in the coexistence treatments with the toxic (MMC+) and non-toxic (MMC-) strain, while the control treatments consisted of toxic (CMC+) and non-toxic (CMC-) cyanobacteria cultures without plants or extract.

In the second experiment, cyanobacteria responses to the application of *E. densa* extract with a concentration of 1 gDW $L^{-1}$ in both the toxic (EMC+) and non-toxic (EMC-) strains were verified. The control treatments also consisted in the culture of cyanobacteria without the aquatic macrophyte or its aqueous extract, like the previous experiment. The same amount of ASM1 medium, saturated in nutrients, was added to the control and coexistence treatments, which corresponded to the extract volume applied in the EMC+ and EMC- treatments. The addition of nutrients in all treatments was to avoid nutrient limitation for cyanobacteria cultures during the experiments. At the end of the experiment, the cultures submitted to the aqueous extract were re-inoculated in a new ASM1 culture medium to verify the viability of the cells present.

The two experiments were performed in three replicates per treatment for 14 days, and the effects of coexistence and macrophyte extracts on cyanobacteria were analyzed every two days. On sampling days, 1 mL of the cyanobacteria cultures were collected to determine the density of the MC+ and MC- strains in all treatments, which was verified by counting the cells in the Fuchs-Rosenthal chamber. The biomass of the strains was determined through the biovolume calculations proposed by Hillebrand et al. (1999) considering the cellular dimensions. Then, the mean biovolume of the strains was multiplied by the density to obtain the biomass.

*Determination of growth and inhibition rates*

The growth rates (μ) of the MC+ and MC- strains submitted to the different treatments were calculated based on the biomass values between the sampling days and the initial day of

the experiments. We used the formula proposed by Wood et al. (2005): $\mu$ ($d^{-1}$) = (ln(*Nt*) – ln($Nt_0$)) / ($t - t_0$), where N represents the cyanobacterial biomass on the different days of experiments (*t*) and the initial time ($t_0$). The inhibition rate (IR) of the cyanobacteria was expressed as a percentage and evaluated through the differences between the treatments for the two strains, calculated as follows: IR = ((*Nt* – *Nc*) / *Nc*) × (-100%); where *Nt* represents the biomass in the treatments of coexistence and application of the extract and *Nc* is the biomass in the control treatments. Thus, an inhibition rate with positive values represent stimulus, and negative values represent inhibition of the strains.

*Statistical analysis*

To verify the differences between total phenolic compounds between treatments at the beginning and end of the experiment, we used a factorial ANOVA a × b, which was also used to verify differences in cyanobacteria biomasses based on time factors, treatments, and in the interaction between them. These analyses were followed by a one-way ANOVA to verify the differences between treatments for each sampling days. Cyanobacteria biomass was compared between treatments and days of sampling and not between strains, because they had different growth patterns. To test the differences between the treatments for the average cell biovolume of the strains, we performed a one-way ANOVA between the treatments. The values of growth and inhibition rates were compared using the one-way ANOVA test for each sampling day. Before performing the analyses of variance, the data was tested for normality using the Kolmogorov-Smirnov test and homoscedasticity using the Bartlett test. When we found significant differences, the analyses were followed by the Tukey a posteriori test. Statistical analysis was performed using the R program with significance level set at $p < 0.05$ (R Core Team 2017).

## Results

*Total phenolic compounds in E. densa*

In the foliar tissues of *E. densa*, polyphenols, flavonoids, triterpenes, and saponins were registered. In addition, this macrophyte had 15.78 mg eTA $gDW^{-1}$ of total phenolic compounds, we used this amount in the aqueous extract during the experiment. We observed a small increase in the production of total phenolic compounds in *E. densa* when submitted to MC+ and MC- strains during the experiment, accompanied by a reduction in production for the control treatment, however, no significant differences were observed between the treatments ($F = 1.253$, $p > 0.05$) or sampling days ($F = 0.1215$, $p > 0.05$) (Fig. 1).

*Effects of E. densa on biomass of Microcystis strains*

The cyanobacteria tested showed different responses to allelochemicals of *E. densa*, either in coexistence or with the application of the extract, with significant differences between treatments (MC+: $F = 242.78$, $p < 0.001$; MC-: $F = 331.643$, $p < 0.001$) and sampling days (MC+: $F = 119.87$, $p < 0.001$; MC-: $F = 4.458$, $p < 0.001$).

The presence of *E. densa* in the coexistence experiment significantly delayed the growth of the MC+ strain, and generated an opposite response for the MC- strain by stimulating growth, which was twice as high as in the control (Fig. 2). Inhibition of the MC+ strain started on the second day, when the MMC+ densities were significantly lower than CMC+, becoming more pronounced throughout the experiment (Fig. 2A). On the eighth day of the experiment, the MC-strain, in coexistence with *E. densa*, presented higher growth when compared to the control (Fig. 2B).

The aqueous extract of *E. densa* significantly inhibited the growth of the two *Microcystis* strains evaluated, being more expressive in the MC- strain. Initially, there was a stimulation of the MC+ strain until the fourth day, however, from the eighth day, this line presented a decrease, reaching a biomass lower than at the beginning of the experiment (Fig.

2A). The MC- strain was more sensitive to the allelochemicals present in the extract, being inhibited significantly on the fourth day, with biomass close to zero at the end of the second experiment (Fig. 2B). When re-inoculated the cultures of the MC- strain submitted to the extract did not present growth due to the cellular unviability caused by the allelochemicals, however, there was growth of the MC+ strain, demonstrating its lower sensitivity to the extract.

We verified an increase in the biovolume of the cyanobacteria treated with the aqueous extract of *E. densa*. While in the treatment of coexistence and in control there was a reduction of the mean biovolume of *Microcystis* for both strains (Fig. 3). Cellular biovolume of cyanobacteria presented significant differences between treatments of MC+ ($F = 44.9$, $p < 0.001$) and MC- ($F = 12.06$, $p < 0.001$) strains. The values of the coexistence treatment did not differ from the control for both strains ($p > 0.05$), while the cyanobacteria MC+ and MC-, treated with the aqueous extract of *E. densa* were significantly higher in relation to the treatments of coexistence (MC+: $p < 0.001$, MC-: $p < 0.05$) and control treatment (MC+: $p < 0.001$, MC-: $p < 0.001$).

*Effects of E. densa on the growth and inhibition rates of Microcystis strains*

The toxic and non-toxic strains of *Microcystis* presented different growth rates in response to the different treatments. For the MC+ strain, cyanobacteria grown with *E. densa* was inhibited on the second day of experiment. The growth rate of EMC+ was higher than that of the control until the fourth day, then decreased, with growth rates lower than control starting on day 10 (Fig. 4A). From the beginning of the experiment, the MC- strain showed negative growth rates in the EMC- treatment, demonstrating the high sensitivity of this cyanobacterium to the aqueous extract of *E. densa*. Unlike the microcystin-producing strain, the MC- strain had higher growth rates when grown with *E. densa* than the control (Fig. 4B).

Looking at Fig. 5, the different responses of the *Microcystis* strains to the *E. densa* allelochemicals become more evident. The inhibition rate was significantly different between treatments ($F = 21.12$, $p < 0.001$), mainly in the coexistence treatment, in which inhibition of the MC+ strain became a stimulus in the MC- strain ($p < 0.001$). For the treatment with aqueous extract of *E. densa*, both strains of *Microcystis* were significantly inhibited, showing no significant differences between the two ($p > 0.05$), and the MC- strain showing major reduction in growth.

**Discussion**

Our results show that *E. densa* has allelochemicals that are able to significantly inhibit different strains of *Microcystis* (Experiment 2), however when in coexistence, inhibition rates for toxic and non-toxic strains showed different behaviors (Experiment 1). In coexistence, *E. densa* significantly inhibited the growth of the microcystin-producing lineage, whereas there was a stimulation of the non-producing strain. These results suggest that the presence of microcystins in the culture medium may stimulate this macrophyte to release a greater amount of allelochemicals that are toxic to *Microcystis*.

More than 40 aquatic macrophytes have the potential to inhibit cyanobacteria, with submerged species being the most efficiently allelopathic (Mohamed 2017). Many studies involving the allelopathic activity of macrophytes were developed using plant extracts or the allelochemicals dissolved in the medium at high concentrations, which do not reflect conditions found in natural environments. In addition, the concentration of allelochemicals released by macrophytes in lakes is much lower than the average lethal concentration required for inhibition of cyanobacteria (Nakai et al. 2000). However, studies have shown that a submerged macrophyte was able to inhibit phytoplankton growth in a shallow ecosystem over long periods (Vanderstukken et al. 2014). Therefore, coexistence experiments, as the present study, best reflect the conditions found in aquatic environments and can evaluate other factors

that affect cyanobacterial growth in addition to allelopathy, as competition for light and nutrients, or mechanical effects of the plant. In our study, the effects of *E. densa* on the strains of *Microcystis* could be attributed to allelopathy due to an increase in the biomass of the non-toxic lineage in coexistence with the plant, as well as the inhibition of both strains with the application of the aqueous extract, demonstrating that there was no competition for resources and no interference of plant morphology on the growth of cyanobacteria.

The submerged macrophyte *E. densa* is known to be an invasive species capable to develop abundantly in aquatic environments (Riis et al. 2010). One of the factors that guarantees the success of its colonization could be related to the production of a greater quantity of phenolic allelochemicals that act to inhibit phytoplankton growth and other plants, which could give this macrophyte a competitive advantage. However, contrary to this, research suggests that invasive and non-invasive macrophytes do not present relevant differences in total phenolic compound contents (Harrison et al. 2017). Moreover, these plants have similar allelopathic potential against cyanobacteria, regardless of the location of plant origin, and the variability in allelopathic potential of the macrophytes is mainly explained by phylogeny of the species, growth strategy, and the ratio of carbon and nutrients in the plant (Grutters et al. 2017).

As verified in our study, *E. densa* possesses efficient allelopathic compounds in the control of cyanobacteria, demonstrated by the presence of total phenolic compounds. Another possible explanation for its success as an invasive, would be the presence of different allelochemicals in this plant compared to those of the other plants in the introduced environments. In these ecosystems, the other species do not present resistance to new allelochemicals, and are more affected in the presence of *E. densa*, as predicted by the "new weapons hypothesis" (Callaway and Aschehoug 2000, Bais et al. 2003, Callaway and Ridenour 2004).

Many factors influence the allelopathic potential of macrophytes, as nutrient limitation, luminosity (Gross 2003), pH (Bährs et al. 2013), bacterial adherence (Bauer et al. 2012), seasonality (Bauer et al. 2009), and stage of plant development (Xu et al. 2015). In our experiments, the growth stimulus of the non-toxic strain proved the absence of competition in coexistence with the plant, in addition, before the experiments we added an extra amount of nutrients to avoid resource limitation. Another important factor that influences the allelopathic potential is the mixing of more than one allelochemical, which increases its inhibitory capacity (Zhu et al. 2010). The highest inhibition of the strains observed when treated with the aqueous extract of *E. densa* may have been due to the action of several substances present in the extract, which may have synergistic or additive effects on *Microcystis* growth.

Previous studies have demonstrated a variability in the inhibition of toxic and non-toxic strains of *Microcystis* caused by the allelochemicals of aquatic macrophytes, with non-microcystin-producing strains being more sensitive (Švanys et al. 2016). One of the main forms of action of the allelochemicals is the promotion of oxidative stress in cyanobacteria (Zhang et al. 2010, Cheng et al. 2017). However, toxic strains of *Microcystis* are less affected by allelochemicals due to the action of microcystins on the protective system against oxidative stress (Dziallas and Grossart 2011, Zilliges et al. 2011). This fact justifies the lower sensitivity of the microcystin-producing strain to the aqueous extract of *E. densa*. However, this protective mechanism is linked to some process related to the synthesis of microcystins and not necessarily to the toxins per se, since a deficient strain for the production of microcystins was also tolerant to the allelochemical tannic acid (Švanys et al. 2016). Moreover, exposure of *M. aeruginosa* to allelochemicals causes an increase in the expression of genes related to the synthesis of microcystins (Shao et al. 2009), which increases their protective capacity.

Different strains of the same phytoplankton species may exhibit different sensitivities to environmental stressors. This characteristic has been demonstrated for several strains of

386 *Scenedesmus subspicatus* Chodat, which showed quite different responses to concentrations
387 of herbicide atrazine, suggesting that genetic alterations may provide different physiological
388 responses to aquatic xenobiotics (Behra et al. 1999). These adaptations are well-considered
389 for epiphytic algae in response to allelochemicals of macrophytes, which co-evolve with host
390 plants, providing them resistance to allelochemicals (Hilt 2006). For phytoplankton, this
391 relationship is not very clear, since several *Pediastrum duplex* Meyen strains, even with a
392 great variation in sensitivity to *M. spicatum*, did not show differences in the sensitivity to
393 strains isolated from lakes dominated by *Myriophyllum* or lakes without this macrophyte,
394 suggesting that there was no adaptation to the allelochemicals (Eigemann et al. 2013).
395 In addition, it is known that non-toxic strains are better competitors for light
396 (Kardinäal et al. 2007), whereas toxic strains have higher growth rates under conditions of
397 high nutrient concentrations (Vézie et al. 2002) and temperature (Dziallas and Grossart 2011).
398 However, under laboratory conditions, *M. aeruginosa* presents a reduction in the production
399 of microcystins with the increasing temperature (Celeste et al. 2017). In response to
400 allelochemicals, non-toxic strains are more sensitive (Švanys et al. 2016), as demonstrated in
401 our study, in which the MC- strain showed lower growth rates in response to the
402 allelochemicals present in the aqueous extract of *E. densa*, and did not grow when re-
403 inoculated in a new culture medium. In addition, the microcystin-producing strain was
404 significantly inhibited by the plant, whereas the non-producing strain, when coexisting with
405 *E. densa*, grew more than double in relation to the control.
406 The production of allelochemicals in plants is intensified under stress conditions,
407 either biotic or abiotic, and the synthesis of these compounds is regulated by stress intensity
408 (Reigosa et al. 1999, Gross 2003). Additionally, the combination of different stressors may
409 increase the production of phenolic compounds by plants and consequently alter the effects on
410 the target organisms of the allelochemicals (Einhellig 1996). Therefore, the presence of
411 microcystins in the cultivation of *E. densa* in coexistence with the toxic strain of *Microcystis*

412 acted as a stress factor to the plant, causing oxidative stress and leading the plant to produce a
413 greater amount of polyphenols, especially flavonoids, efficient non-enzymatic antioxidants in
414 plants (Pietta 2000, Gill and Tuteja 2010), as demonstrated by the increase in the production
415 of total phenolic compounds when in coexistence with the microcystin-producing strain.
416 Recent studies have demonstrated that *E. densa* is intensely affected when coexisting with
417 microcystin-producing strains of *Microcystis*, with reduced growth, photosynthetic pigment
418 alteration, and oxidative stress (Amorim et al. 2017). In addition to the protective mechanism
419 of oxidative stress, phenolic compounds may also have acted as powerful allelochemical
420 inhibitors of the toxic strain of *Microcystis*, while the non-toxic strain showed an increase in
421 its growth, since the absence of microcystins did not cause the release of allelochemicals by
422 the plant in the medium, even having them in its tissues.

423 Few studies have shown the efficiency of macrophytes in the control of
424 phytoplankton from natural environments, especially cyanobacteria, through allelopathic
425 mechanisms (Hilt et al. 2006, Švanys et al. 2014, Vanderstukken et al. 2014). In the eutrophic
426 environments there is an alternation in the dominance of the aquatic vegetation, with
427 submerged macrophytes being negatively affected under conditions of high nutrient
428 concentrations, whereas floating macrophytes are favored (Scheffer et al. 2003). In addition,
429 submerged macrophytes produce less phenolic compounds under low luminosity conditions,
430 such as in turbid eutrophic environments dominated by phytoplankton (Cronin and Lodge
431 2003), making it difficult to control cyanobacteria by the allelopathic compounds of aquatic
432 vegetation.

433

434 **Conclusions**

435 This work demonstrates the allelopathic effects of a globally occurring invasive
436 aquatic macrophyte on toxic and non-toxic cyanobacteria lineages in coexistence, important
437 for understanding how cyanobacteria react to allelochemicals produced by aquatic vegetation.

Our hypothesis was partially confirmed, because only the toxic strain was inhibited in coexistence with *E. densa*, suggesting that the microcystins acted as a stress factor to the plants, which caused a higher production of allelochemicals. Although the non-producing strain was stimulated in coexistence, *E. densa* demonstrated to have allelochemicals capable of inhibiting both strains with the application of the aqueous extract, especially for the non-toxic strain. Therefore, the allelopathic response of this macrophyte is dependent on stress, such as that caused by cyanotoxins, to release significant amounts of allelochemicals capable of inhibiting the growth of cyanobacteria. This information is relevant in bioremediation strategies of cyanobacterial blooms using aquatic macrophytes, since the proportion of toxic and non-toxic strains may influence the allelopathic response of macrophytes.

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**Authors contribution**

CAA and ANM designed the study. CAA conducted the experiments and analyzed the samples. CAA, CU and ANM performed the phytochemical analysis of *E. densa*. CAA, EWD and ANM performed the statistical analysis. CAA and ANM interpreted the data and wrote the manuscript with a critical revision of all authors.

**Conflicts of interest**

The authors declare no conflicts of interest.

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618

619 **Figures legends**

620 **Fig. 1.** Total phenolic compounds (TPC) in *E. densa* submitted to the culture with
621 microcystin-producing (MC+) and non-producing (MC-) strains of *Microcystis*, compared to
622 control (CT) without the *Microcystis* strains, at the start (Day 0) and the end (Day 14) of the
623 coexistence experiment. Same letters represent no significant differences between treatments
624 or days (Tukey: $p > 0.05$).

625 **Fig. 2.** Evaluation of the growth (biomass) of the toxic *M. aeruginosa* - NPLJ-4 (MC+) (A)
626 and non-toxic *M. panniformis* - BCCUSP29 (MC-) (B) strains, submitted to the treatments of
627 coexistence (MMC+ and MMC-) and aqueous extract (EMC+ and EMC-) of *E. densa*, related
628 to controls treatments (CMC+ and CMC-). Different letters represent significant differences
629 between the treatments (Tukey: $p < 0.05$).

630 **Fig. 3.** Mean cellular biovolume of *M. aeruginosa* - NPLJ-4 (MC+) (A-C) and *M.*
631 *panniformis* - BCCUSP29 (MC-) (D-E) strains, submitted to the treatments of coexistence
632 (MMC+ and MMC-) and aqueous extract (EMC+ and EMC-) of *E. densa*, related to controls
633 treatments (CMC+ and CMC-).

634 **Fig. 4.** Growth rate of *M. aeruginosa* - NPLJ-4 (MC+) (A) and *M. panniformis* - BCCUSP29
635 (MC-) (B) strains, in coexistence with *E. densa* (MMC+ and MMC-) and with the aqueous
636 extract of this macrophyte (EMC+ and EMC-), compared to controls (CMC+ and CMC-).
637 Different letters represent significant differences between the treatments (Tukey: $p < 0.05$).

638 **Fig. 5.** Inhibition rate of the microcystin-producing (MC+) (A) and non-producing (MC-) (B)
639 strains of *Microcystis*, cultivated in coexistence with *E. densa* (MMC+ and MMC-) and its
640 aqueous extract (EMC+ and EMC-).

641 **Table 1.** Mobile phase eluting system used for phytochemical analysis of the aqueous extract
642 of *E. densa*.

| Metabolites | Eluting system | Revealed with | Reference |
|---|---|---|---|
| Polyphenols | Toluene + $HCO_2H$ + EtOH (40:50:25 v/v) | Sulfuric vanillin 1% | Harbone (1984) |
| Flavonoids | AcOEt + $HCO_2C$ + AcOH + $H_2O$ (100:11:11:26 v/v) | Sulfuric vanillin 1% | Harbone (1984) |
| Coumarins | Ether + Toluene + AcOH 10% (50:50:50 v/v) | KOH + EtOH 10% | Harbone (1984) |
| Tannins | $CHCl_3$ + $CH_3OH$ + $H_2O$ (65:30:5 v/v) | $FeCl_3$ 1% | Harbone (1984) |
| Triterpenes | Toluene + $HCO_2H$ + EtOH (40:40:10 v/v) | Heating to 100 °C by 5 minutes | Wagner and Bladt (1996) |

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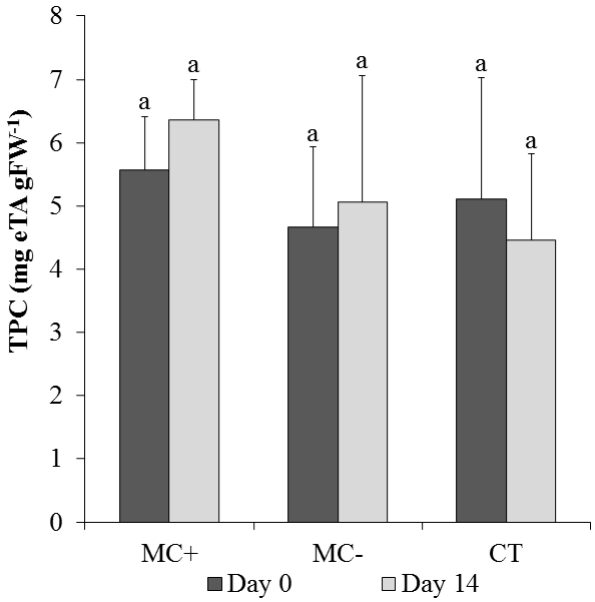


646

647 **Fig. 1**

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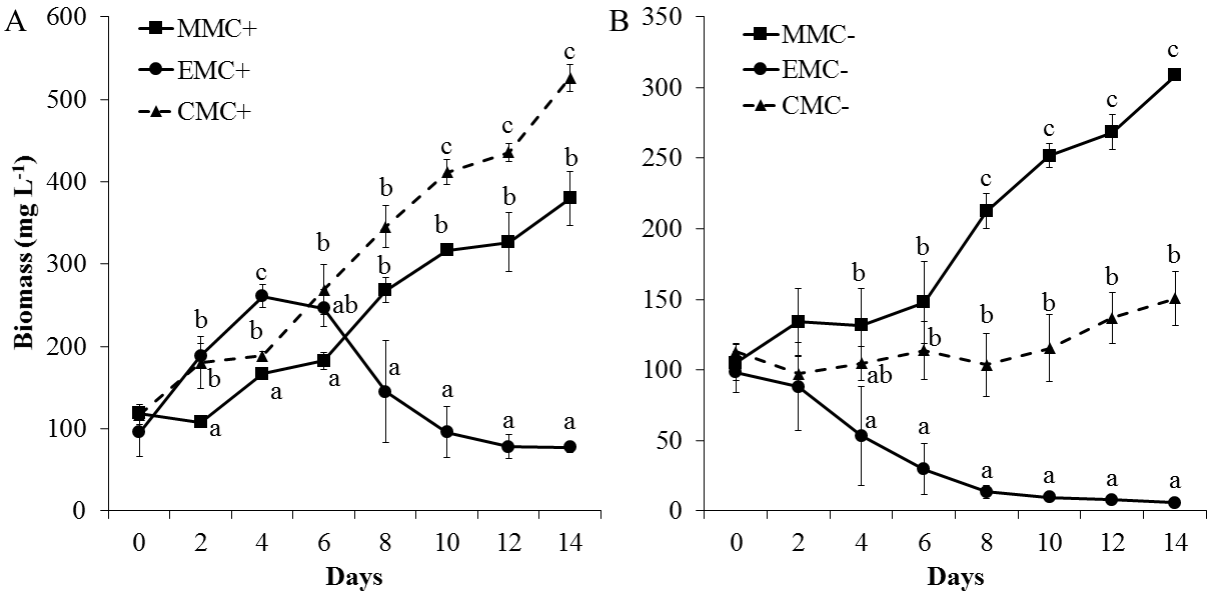


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650 **Fig. 2**

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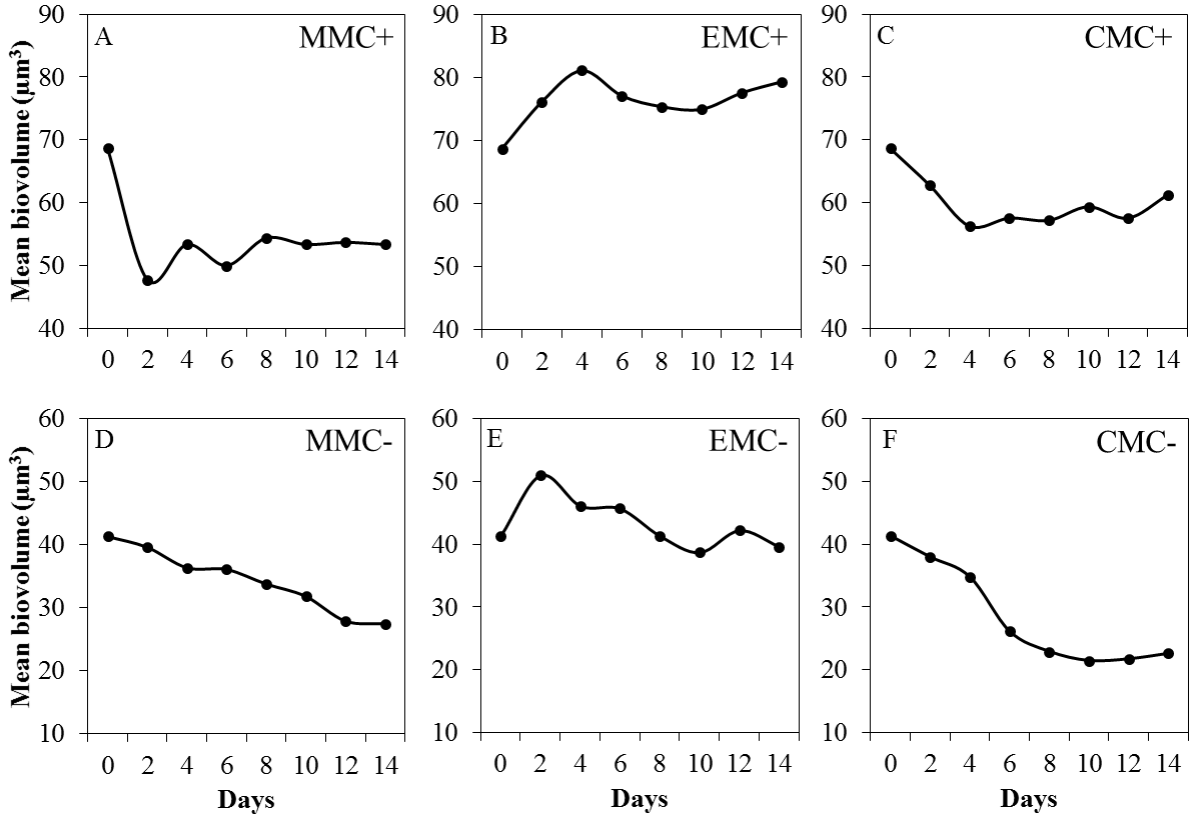


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653 **Fig. 3**

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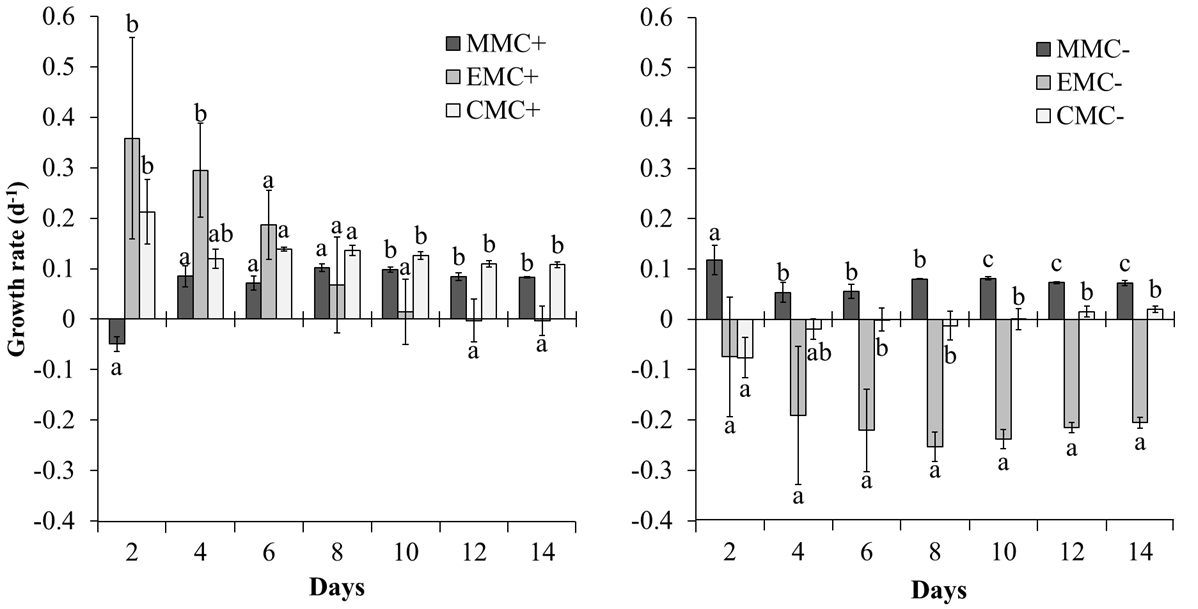
Growth rate (d-1)
MMC+
EMC+
CMC+
MMC-
EMC-
CMC-
Days
Days

Fig. 4

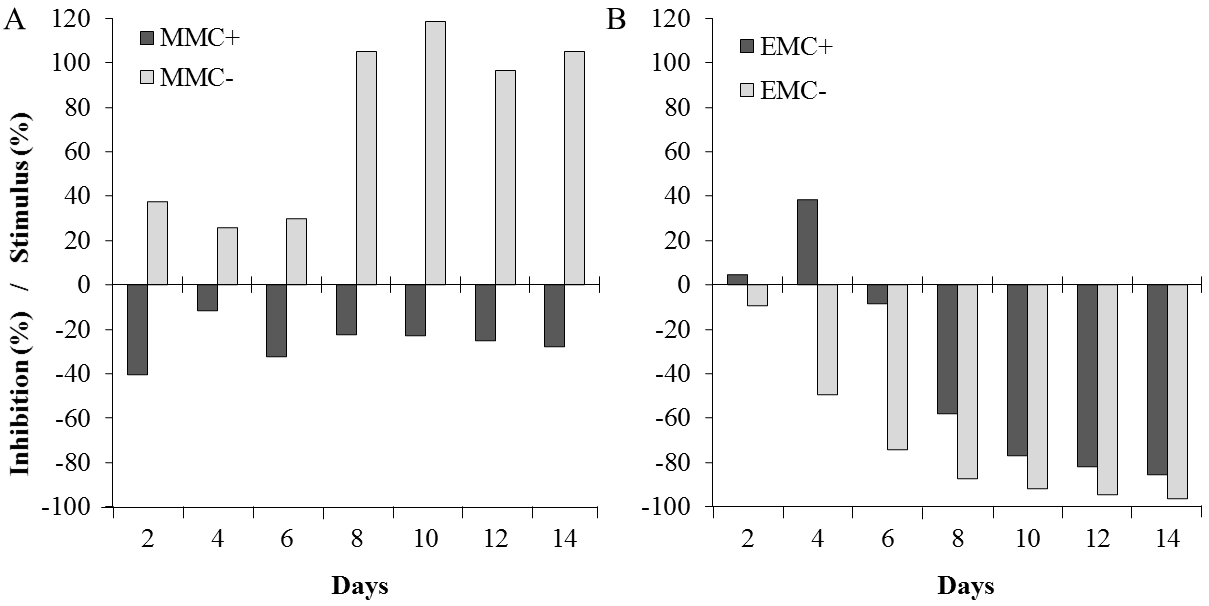
A
B
Inhibition (%) / Stimulus (%)
MMC+
MMC-
EMC+
EMC-
Days
Days

Fig. 5

## 4 SEGUNDO MANUSCRITO

# Biometric and physiological responses of *Egeria densa* Planch. cultivated with toxic and non-toxic strains of *Microcystis*

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### Highlights

Microcystin producing strain inhibited *Egeria densa* growth, shoot and root emission

Toxic strain of *Microcystis* caused significant lipid peroxidation in *E. densa*

*Egeria densa* increased the anti-oxidative system against toxic *Microcystis*

*Microcystis* caused an increase and then a decrease in enzymatic activity of *E. densa*

Non-toxic strain of *Microcystis* did not cause severe damage to the *E. densa*

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# Biometric and physiological responses of *Egeria densa* Planch. cultivated with toxic and non-toxic strains of *Microcystis*

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ABSTRACT

Cyanobacterial blooms are becoming increasingly common in aquatic environments around the world, mainly due to eutrophication and climate change. Cyanotoxin-producing strains (e.g., microcystins (MC) producers) may be present in these blooms, affecting the growth of other aquatic organisms, such as aquatic macrophytes. In this study, we evaluated the morphometric and physiological responses of the aquatic macrophyte *Egeria densa* to the exposure to a toxic strain of *Microcystis aeruginosa* (MCs producer) and a non-toxic *Microcystis panniformis* (non-MC producer). The effects of *Microcystis* strains on *E. densa* growth and biomass were verified for five weeks (Experiment 1) and physiological responses were evaluated for 14 days (Experiment 2). Prolonged exposure of *E. densa* to the MC producing strain reduced growth, accompanied by the inhibition of shoot and root emission. Both *Microcystis* strains caused a decrease in the content of photosynthetic pigments, like total chlorophyll and chlorophyll *a* and *b*, accompanied by an increase of carotenoids. At the beginning of the MC-producing strain exposure, *E. densa* showed an increase in the activity of the anti-oxidative enzymes superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (APX), accompanied by an increase in the levels of malondialdehyde, indicating lipid peroxidation. During the 14th day of exposure, the activity of antioxidant enzymes remained similar to the control, suggesting that *E. densa* has an efficient anti-oxidative system to control the reactive oxygen species produced in response to the stress caused by microcystins. However, when prolonged exposure occurred, possible damage to proteins may have affected the growth and development of *E. densa*. No changes were observed in the enzymatic activity of the plants exposed to the non-MC producing strain, suggesting that this cyanobacterial strain do not cause significant damage to the development of *E. densa*. These results are important for understanding the anti-oxidative defense mechanisms of aquatic macrophytes when coexisting with an MC producing strain.

## 1. Introduction

Aquatic macrophytes are important for maintaining a clear state of shallow lakes (Scheffer et al., 1993), because they present mechanisms that reduce the amount of phytoplankton biomass, either indirectly, by providing shelter for zooplankton (Jeppesen et al., 1997), or directly, by releasing chemical compounds that inhibit phytoplankton growth (Hilt and Gross, 2008; Eigemann et al., 2013). However, eutrophication and global temperature rise, as effects of climate change, favor the persistence of harmful cyanobacterial blooms (CyanoHABs) (Mariani et al., 2015; Paerl et al., 2016; Lürling et al., 2017). These blooms may pose contamination risk to man and aquatic organisms, mainly due to the release of cyanotoxins (Paerl and Otten, 2013; Carmichael and Boyer, 2016). The synthesis of these toxins in blooming events favors cyanobacteria growth, as it inhibits the growth of competing species through allelopathy and kills the predators of cyanobacteria (Granéli et al., 2008). Submerged macrophytes are strongly affected by these cyanotoxins because they are in direct contact with these metabolites in the water (Pflugmacher, 2004).

Among the bloom-forming cyanobacteria, *Microcystis aeruginosa* Kütz. stands out as the most commonly recorded species in continental aquatic environments around the world (Harke et al., 2016). This species is able to synthesize several variants of microcystins (MCs), which are the most harmful and widely distributed hepatotoxins (Martins and Vasconcelos, 2009; Paerl and Huisman, 2009). To date, more than 240 variants of MCs are known (Svirčev et al., 2017; Spoof and Catherine, 2017), among them MC-LR is the most frequent in aquatic ecosystems and mainly acts in the inhibition of protein phosphatases 1 and 2A (PP1 and PP2A, respectively) (MacKintosh et al., 1990; Dawson, 1998). The toxicity of MCs has been demonstrated in

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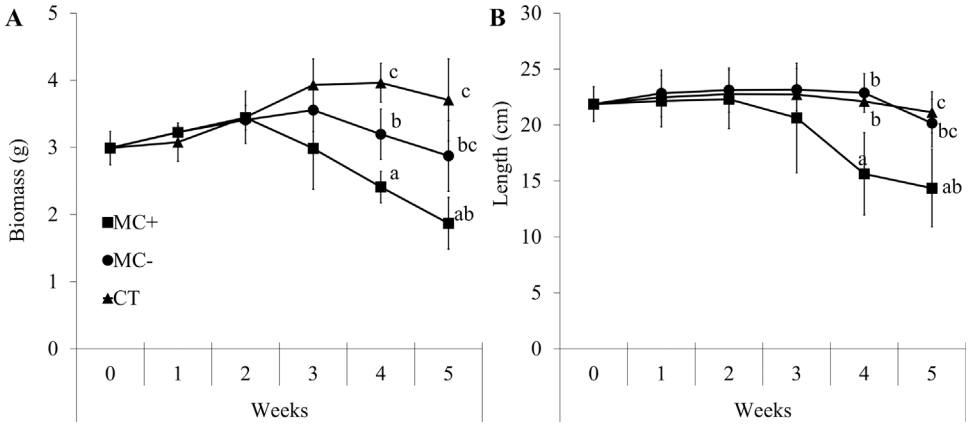


**Fig. 1.** Evaluation of the biomass (A) and length (B) of *E. densa* submitted to the culture with toxic (MC+) and non-toxic (MC−) strains of *Microcystis* and in the control (CT). Different letters represent significant differences between treatments (Tukey: $p < 0.05$).


**Fig. 2.** Development of *E. densa* after five weeks (35 days) of cultivation in coexistence with toxic (MC+) and non-toxic (MC−) strains of *Microcystis* and in the control (CT), showing the emission of shoots (S) and roots (R).

cultured plants (Bittencourt-Oliveira et al., 2016), aquatic macrophytes (Romero-Oliva et al., 2015a), zooplankton (Liang et al., 2017), fish (Boaru et al., 2006), and mammals, including man (Carmichael et al., 2001; Žegura et al., 2003).

In aquatic plants, MCs can cause a reduction in growth, alteration in photosynthetic pigments or oxidative stress through increased production of reactive oxygen species (ROS) (Saqrane et al., 2007). However, these plants have two defense mechanisms that allow them to coexist with these cyanotoxins, biotransformation and anti-oxidative defense (Babica et al., 2006). These strategies are the most efficient against the harmful effects caused by MC exposure (Romero-Oliva et al., 2015b). Biotransformation is the process of degradation of the polluting substances, among them the MCs, and occurs in three phases. In stage I the harmful substances are transformed into less toxic compounds; in phase II these compounds are conjugated to glutathione (GSH) to be metabolized by glutathione S-transferase (GST); in phase III these substances are compartmentalized in the cell wall or vacuoles (Dixon et al., 2010). The anti-oxidative system acts as a response to biotic or abiotic stress and the main mode of action is through the elimination of ROS (e.g., $O_2\cdot^-$, $H_2O_2$, OH· e $^1O_2$; Mittler, 2017), which can cause damage to proteins, lipids, and DNA (Gill and Tuteja, 2010). The control of oxidative stress in plants can occur through enzymatic mechanisms, as catalase (CAT), superoxide dismutase (SOD), ascorbate peroxidase (APX) and glutathione peroxidase activity (Mittler et al., 2004), or non-enzymatic activity, through the performance of carotenoids, phenolic compounds, and tocopherols (Gill and Tuteja, 2010).

Thus, we investigated the physiological and growth responses of *Egeria densa* Planch. to the exposure of toxic and non-toxic strains of *Microcystis*. The submerged aquatic macrophyte *E. densa* occurs abundantly in tropical regions, and can form large stands. Considering this, we verified whether this macrophyte supports coexistence with a MC-producing *Microcystis* strain, especially [D-Leu$^1$]MC-LR, as well as compared the performance of plants growing with a non-MC producing strain. With this, we aimed to verify how the submerged aquatic macrophytes were affected when cultivated with MCs producing cyanobacteria and what are their defense mechanisms against these toxins.

## 2. Materials and methods

### 2.1. Culture of aquatic macrophyte and cyanobacteria

The aquatic macrophyte *E. densa* was collected from the Tabocas Reservoir, a mesotrophic reservoir without cyanobacterial bloom events, which is located in Belo Jardim, Pernambuco, Brazil (8°14'32 "S, 36°22'35"). The plants were identified with help of taxonomic keys and specialized literature (Cook and Urmi-König, 1984). Branches 25 cm in length were washed several times with running water, followed by Milli-Q water to completely remove debris and organisms adhered to the plants. The macrophytes were cultivated in 8-L aquaria containing tap water, at a temperature of 25 °C, constant aeration, photoperiod of 12 h and light intensity of 40 μmol photons $m^{-2}\,s^{-1}$, which was measured with a photometer (LI-COR model LI-250).

The *E. densa* macrophyte was exposed to two strains of *Microcystis*. The *M. aeruginosa* – NPLJ-4 strain (MC + ) produces 4 MC variants, mainly [D-Leu$^1$]MC-LR with almost 90% of the total MC content, the other variants were not identified. This strain was obtained from the Coleção de Cultivo de Cianobactérias from Unidade Acadêmica de Garanhuns at the Universidade Federal Rural de Pernambuco. The *M. panniformis* – BCCUSP29 strain (MC-), non-MC producing (Bittencourt-Oliveira, 2003), was obtained from the Brazilian Cyanobacteria

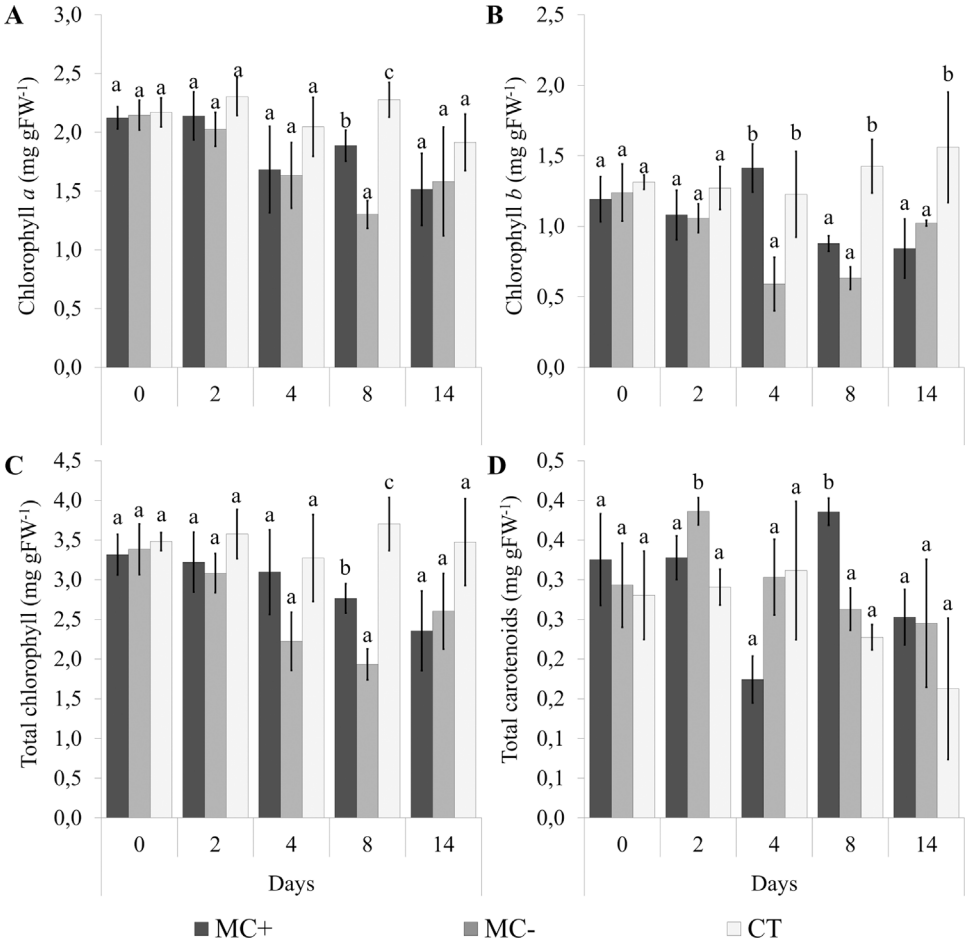


**Fig. 3.** Photosynthetic pigment content in *E. densa* submitted to the culture with toxic (MC+) and non-toxic (MC−) strains of *Microcystis* and in the control (CT). A: chlorophyll *a*; B: chlorophyll *b*; C: total chlorophyll; D: total carotenoids. Different letters show significant differences between treatments (Tukey: $p < 0.05$).

Collection at the Universidade de São Paulo. The cultures of these cyanobacteria were carried out in Erlenmeyer flasks (1-L capacity) containing 850 mL of ASM1 medium (Gorham et al., 1964), in a climatic chamber with a temperature of 25 °C, light intensity of 40 µmol photons $m^{-2} s^{-1}$, photoperiod of 12 h, pH 7.5, and were manually homogenized three times a day.

### 2.2. Identification of MCs in M. aeruginosa – NPLJ-4 strain

The cultures of *M. aeruginosa* at the end of exponential growth phase were lyophilized, added to 75% methanol and then the solution was stirred for 1 h for extraction of microcystins (MCs). The sample was centrifuged (11,180 × *g* for 10 min at 4 °C) and the supernatant was re-extracted twice for 30 min (Fastner et al., 1998). The solution was dried under warm air, resuspended in 2 mL of 20% methanol (HPLC-grade) and then frozen at −18 °C until the high performance liquid chromatography (HPLC) analysis.

The analysis was carried out in a HPLC (LC20A model, Shimadzu Prominence, Japan) equipped with a C18 column (5 µm diameter particles, 250 × 4.6 mm), UV–vis detector with diode array, oven at 40 °C and the mobile phase was water and acetonitrile both containing 0.05% trifluoroacetic acid (Lawton et al., 1994). The flow rate was 1 mL $min^{-1}$ and the injection volume was 40 µL. The composition gradient was 30% of acetonitrile for 10 min, 35% until 30 min and then 70% after 30 min. The chromatograms were monitored at 238 nm and the absorption spectra of the peaks observed between 200 and 300 nm. The MCs produced by *M. aeruginosa* − NPLJ-4 were identified by comparisons of their absorption spectra with a MC-LR standard.

### 2.3. Exposure of E. densa to toxic and non-toxic Microcystis strains

Two experiments were carried out in parallel, in which the *E. densa* branches were exposed to the cultures of the producing (MC+) and non-producing (MC−) strains of MCs. The experiments were carried out in 1-L Erlenmeyer flasks containing 850 mL of culture medium and the cyanobacteria with a biomass of 100 mg $L^{-1}$ at the end of exponential growth phase. The control treatment contained only ASM1 medium to verify the independent effects of the strains. The experiments were conducted with three replicates for each treatment in a climatized room with the same plant and cyanobacteria culture conditions. Three days before the experiments the plants were cut and grown in ASM1 nutrient medium for acclimatization.

In the first experiment, 3 g of *E. densa* were exposed to the MC+ and MC− cultures to determine the growth (length) and biomass (fresh weight) of the macrophytes main branches, weekly for five weeks. In the second experiment 4 g of the plant were exposed to the strains for physiological analysis. Samples were collected on days 0, 2, 4, 8, and 14. For that, 0.7 g of fresh weight (FW) were removed by a cutting from the plants in each replicate, washed with Milli-Q water to remove adhered *Microcystis* cells and immediately frozen in liquid nitrogen and stored at −20 °C for further analyzes.

### 2.4. Content of photosynthetic pigments

The photosynthetic pigments were extracted with 80% acetone (v/v) according to Lichtenthaler (1987). Plant tissue samples (0.1 gFW) was macerated in 80% acetone with mortar and pestle, then the extract was preserved in the dark at 4 °C for 24 h. The extract was centrifuged at 11,180 × *g* for 10 min to remove tissue debris and the absorbance of

**Table 1**
Results of factorial ANOVA a × b comparing chlorophyll *a* and *b*, total chlorophyll, total carotenoids, soluble proteins, hydrogen peroxide, malondialdehyde, and activities of enzymes superoxide dismutase, catalase and ascorbate peroxidase in *E. densa* based on the time and treatments factors, and the interaction of these factors, submitted to cultures with toxic and non-toxic strains of *Microcystis*.

| Variables | Factors | Df | F | *p* |
|---|---|---|---|---|
| Chlorophyll *a* | Time | 4 | 8.044 | < 0.001 |
| | Treatments | 2 | 11.615 | < 0.001 |
| | Interaction Time × Treatments | 8 | 1.981 | > 0.050 |
| Chlorophyll *b* | Time | 4 | 2.439 | > 0.050 |
| | Treatments | 2 | 21.729 | < 0.001 |
| | Interaction Time × Treatments | 8 | 5.224 | < 0.001 |
| Total chlorophyll | Time | 4 | 5.139 | < 0.010 |
| | Treatments | 2 | 19.575 | < 0.001 |
| | Interaction Time × Treatments | 8 | 2.995 | < 0.050 |
| Total carotenoids | Time | 4 | 6.477 | < 0.001 |
| | Treatments | 2 | 3.334 | < 0.050 |
| | Interaction Time × Treatments | 8 | 4.484 | < 0.010 |
| Soluble proteins | Time | 4 | 2.046 | > 0.050 |
| | Treatments | 2 | 1.445 | > 0.050 |
| | Interaction Time × Treatments | 8 | 2.04 | > 0.050 |
| Hydrogen peroxide | Time | 4 | 4.768 | < 0.010 |
| | Treatments | 2 | 5.258 | < 0.050 |
| | Interaction Time × Treatments | 8 | 3.781 | < 0.010 |
| Malondialdehyde | Time | 4 | 33.174 | < 0.001 |
| | Treatments | 2 | 21.491 | < 0.001 |
| | Interaction Time × Treatments | 8 | 5.026 | < 0.001 |
| Superoxide dismutase | Time | 4 | 19.393 | < 0.001 |
| | Treatments | 2 | 9.324 | < 0.001 |
| | Interaction Time × Treatments | 8 | 3.827 | < 0.010 |
| Catalase | Time | 4 | 12.439 | < 0.001 |
| | Treatments | 2 | 3.043 | > 0.050 |
| | Interaction Time × Treatments | 8 | 9.774 | < 0.001 |
| Ascorbate peroxidase | Time | 4 | 13.330 | < 0.001 |
| | Treatments | 2 | 53.100 | < 0.001 |
| | Interaction Time × Treatments | 8 | 14.570 | < 0.001 |

supernatant determined spectrophotometrically at 647 and 663 nm for total chlorophyll and chlorophyll *a* and *b*. Total carotenoids was determined at 470 nm.

### 2.5. Hydrogen peroxide ($H_2O_2$) and lipid peroxidation in E. densa

To verify the quantities of $H_2O_2$ and malondialdehyde (MDA), *E. densa* samples were macerated in liquid nitrogen and 0.1% trichloroacetic acid (TCA) and 20% polyvinylpolypyrrolidone (PVPP) in the proportion of 1 g to 10 mL. Then the extract was centrifuged at 11,180 × g for 10 min at 4 °C. The levels of $H_2O_2$ in the plants were determined according to the methodology of Alexieva et al. (2001). To this end, 200 µL of the supernatant were mixed in 200 µL of 100 mM potassium phosphate buffer (pH 7.5) and 800 µL of potassium iodide. This was then incubated for 1 h in the dark in an ice bath followed by 20 min at room temperature and the absorbance reading was at 390 nm. Lipid peroxidation was determined by the amount of MDA in plants according to Heath and Packer (1968). An aliquot of 250 µL of the extract supernatant was added to 1000 µL of 20% TCA and 0.5% tiubarbituric acid (TBA). The samples were then incubated at 95 °C for 30 min and transferred to ice bath for 10 min to stop the reaction. The solution was centrifuged at 11,180 × *g* for 10 min and absorbances read at 535 and 600 nm. The results of $H_2O_2$ and MDA were expressed in µmol $gFW^{-1}$.

### 2.6. Enzymatic analysis

The activity of the enzymes SOD (EC 1.15.1.1), CAT (EC 1.11.1.6) and APX (EC 1.11.1.11) was determined based on spectrophotometric readings from the same extract. In which a branch of *E. densa* was macerated in liquid nitrogen and homogenized with 100 mM potassium phosphate buffer (pH 7.5), 1 mM ethylenediaminetetraacetic acid (EDTA) and 4% PVPP in the ratio of 1 g to 10 mL. The enzyme extract was centrifuged at 11,180 × *g* for 20 min and the supernatant stored at −20 °C for further analysis.

The activity of SOD was verified after the reaction between 50 µL of the enzyme extract and 2950 µL of solution containing 50 mM of sodium phosphate buffer (pH 7.8), 13 mM methionine, 75 µM tetrazolium nitroblue, 1 mM EDTA, and 5 µM riboflavin. The solution was kept in the light for 5 min for the reaction to occur. The activity was then measured at 560 nm (Giannopolitis and Ries, 1977). The activity of CAT was measured by the reaction between 50 µL of the extract and 2950 µL of 50 mM potassium phosphate buffer (pH 7.0) containing 20 mM $H_2O_2$, where the decrease in $H_2O_2$ concentrations were verified at 240 nm during one minute at 30 °C (Havir and McHale, 1987). APX was determined by ascorbate decay (AsA), demonstrating its degradation. The reaction started after mixing 100 µL of the extract, 2700 µL of 50 mM potassium phosphate buffer (pH 6.0) containing 0.5 mM AsA and 200 µL of 30 mM $H_2O_2$. The decrease in AsA was observed at 290 nm for one minute (Nakano and Asada, 1981). The results of enzyme activities are expressed in katal per mg of protein (nkat or µkat $mgPT^{-1}$), which represents the conversion rate of one mol of substrate per second. The content of plant-soluble proteins was determined by the Bradford (1976) method using bovine albumen serum to calibrate the standard curve.

### 2.7. Data analysis

A factorial ANOVA a × b was used to verify the statistical differences between biomass values and growth of aquatic macrophytes, in addition to the pigment content, soluble proteins, $H_2O_2$, MDA, and activity anti-oxidative enzymes between treatments and sample collection days. Before ANOVA, the data were tested for normality and homoscedasticity using the Kolmogorov-Smirnov and Bartlett tests, respectively. When significant differences were verified, the analyses were followed by the Tukey test. Software R 3.4.0 was used with significance level of $p < 0.05$ (R Core Team, 2016).

## 3. Results

### 3.1. Effects of toxic and non-toxic strains of Microcystis on the growth of E. densa

In long term exposures, macrophytes exposed to the MC+ strain showed an evident inhibition of biomass (fresh weight) (Fig. 1A) and consequently growth (length) at the end of the experiments (Fig. 1B). The biomass reduction of the *E. densa* branches started after the fourth week of experiment when exposed to the MC+ strain, showing a fresh weight 50% lower than in control plants at the end of the experiments. After four weeks, the branches exposed to MC− also showed a decrease in their fresh weight, demonstrating that competition for resources, like nutrients and light, or other metabolites, like lipopolysaccharides, may have contributed to the reduction of plant growth. The experiment was finished in the fifth week due to a small decrease in fresh weight also in the control treatment, during which time we also observed the growth of periphytic algae on the plants and inside the Erlenmeyer flasks. Macrophyte biomass differed significantly between treatments ($F = 12.319$, $p < 0.001$), with values in MC+ lower than in MC- and control. Regarding the length of the plants, we verified the same pattern in plants exposed to the MC+ strain, which decreased significantly in relation to the control from the fourth week, showing a reduction of 32% at the end of experiment. Throughout the experiment, the MC- strain did not cause significant changes in the growth of the plants, remaining close to the control. During the fifth week, there was a slight reduction in the length of the plants exposed to MC− and in the control, also due to the colonization of the periphyton. The treatments presented differences between them ($F = 9.577$, $p < 0.001$), with MC+ causing a greater inhibition in the length of macrophytes.

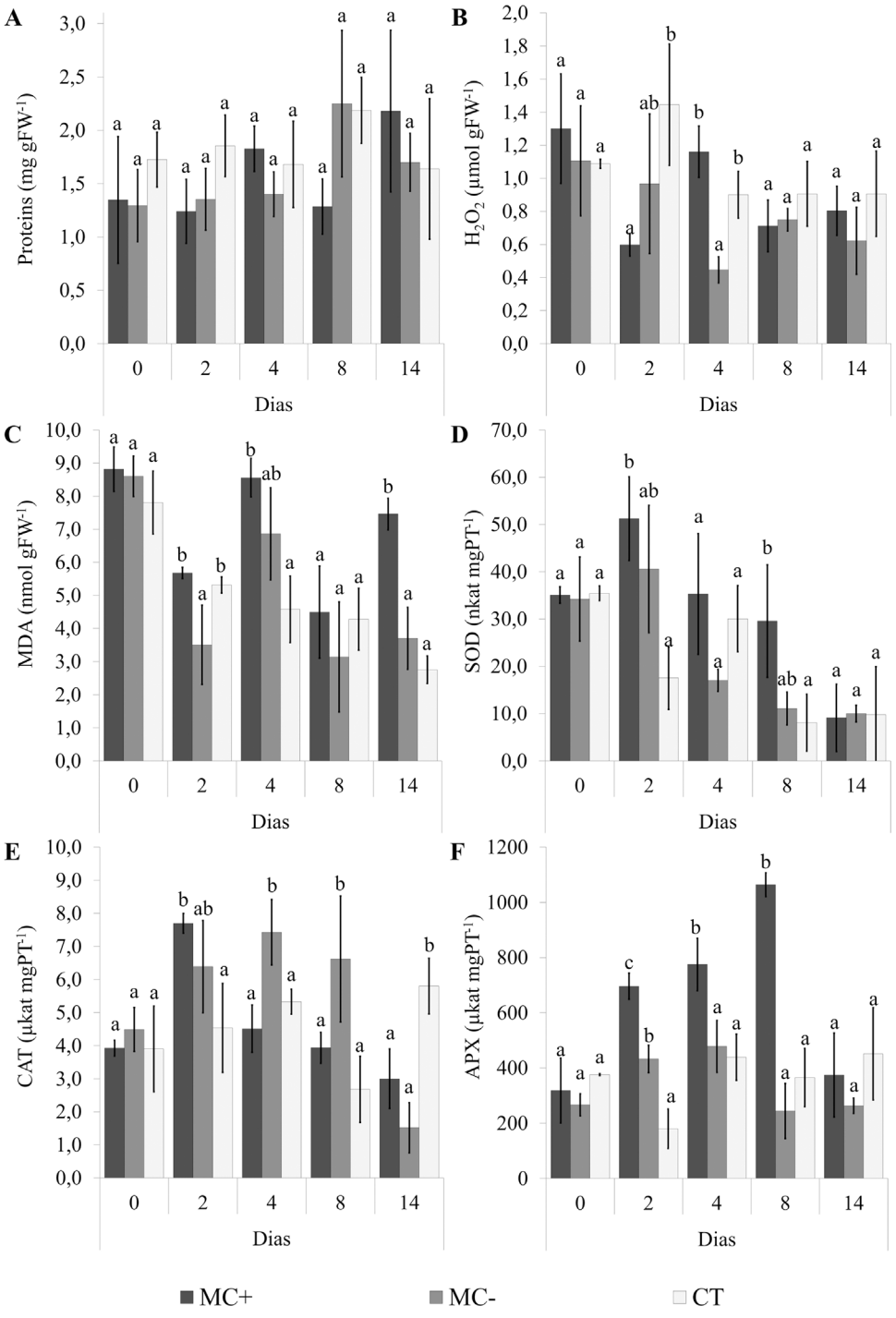


**Fig. 4.** Analysis of oxidative stress in *E. densa* submitted to cultivation with toxic (MC+) and non-toxic (MC−) strains of *Microcystis* and in the control (CT). A: proteins; B: hydrogen peroxide ($H_2O_2$); C: malondialdehyde (MDA); D: superoxide dismutase (SOD); E: catalase (CAT); F: ascorbate peroxidase (APX). Different letters show significant differences between treatments (Tukey: $p < 0.05$).

After the third week of experiment, leaf loss was observed in the plants exposed to the MC+ strain, as well as a degradation of the basal and mainly apical regions of the stem. These characteristics contributed to the reduction in growth and fresh weight of the plants evaluated in the following weeks. In addition, the plants had a pale chlorotic appearance, especially in the basal region. For the branches exposed to the MC- strain, these characteristics were less evident, with few morphological and color changes and similar behavior as the control. The toxic strain of *Microcystis* also inhibited the emission of shoots from the lateral meristems and the emergence of roots in all replicates, which may reflect the microcystins' performance on the growth of the tested plants. The MC- strain had the same effect, but with the emission of lateral shoots in one of the replicates. In the control treatment two to four shoots and three to four roots were emitted (Fig. 2).

### 3.2. Effects of toxic and non-toxic strains of Microcystis on pigment content of E. densa

The plants exposed to the MC+ and MC- strains presented a reduction in chlorophyll *a* content on 8th day, with values lower than the control, especially in the plants exposed to MC- strain with values 43% lower than the control plants (MC+: $p < 0.05$; MC-: $p < 0.001$) (Fig. 3A). Chlorophyll *b* content reduced from the 4th and 8th days for plants exposed to MC- ($p < 0.05$) and MC+ ($p < 0.01$) strains, respectively, with significant differences to the control up to the 14th day (Fig. 3B). The reduction in photosynthetic pigments content,

chlorophyll *a* and *b*, also caused a reduction in the total chlorophyll, which when exposed to MC+ ($p < 0.01$) and MC- ($p < 0.001$) presented values lower than the control on the 8th day of exposure (Fig. 3C). The plants showed a higher production of carotenoids when exposed to MC+ only on the 8th day ($p < 0.001$), whereas the MC- strain caused an increase in carotenoid content in plants only on the 2nd day ($p < 0.01$) (Fig. 3D). Chlorophyll *a*, total chlorophyll and carotenoids showed significant differences among the treatments and the sampling days, whereas chlorophyll *b* differed only in response to the treatments (Table 1).

### 3.3. Evaluation of oxidative stress in E. densa cultivated with toxic and non-toxic strains of Microcystis

Plant exposure to the toxic and non-toxic strains of *Microcystis* did not cause significant changes to the amounts of proteins in plant tissues ($p > 0.05$) (Fig. 4A, Table 1). There was a reduction in $H_2O_2$ production in plants exposed to MC+, whereas in the control there was an increase on the second day followed by a decrease. The amount of $H_2O_2$ only differed significantly in the plants exposed to MC+ on the 2nd day ($p < 0.05$) and MC- on the 4th day ($p < 0.05$), with lower values in relation to the control (Fig. 4B, Table 1). Lipid peroxidation was a strong indicator of oxidative stress in the tested plants, where the levels of MDA in the branches exposed to the MC+ strain were significantly higher in relation to MC- on the 2nd ($p < 0.05$) and 14th ($p < 0.001$) days and the control on the 4th ($p < 0.01$) and 14th ($p < 0.001$) days. On the 2nd and 8th days, the MDA values in the plants exposed to the MC+ strain decreased with respect to the beginning of the experiment (Fig. 4C, Table 1).

The response of the SOD, CAT and APX enzyme activity of the plants exposed to the MCs producing strain was treatment and time dependent, with a significant increase in the activity of these enzymes followed by a decrease. Plants exposed to MC+ increased SOD activity by 1.46 times on the 2nd day in relation to the beginning and 2.93 times in relation to the control ($p < 0.05$), followed by a decrease, however, the values continued higher than in the control until the 8th day ($p < 0.05$), reducing to values similar to the control on the 14th day ($p > 0.05$). While the plants exposed to the MC- strain did not differ in relation to the control ($p > 0.05$), decreasing over the days (Fig. 4D, Table 1). CAT activity of the plants exposed to MC+ showed a significant increase on the second day (1.96 times in relation to the beginning of the experiment) with values 41% higher than the control ($p < 0.05$), followed by a decrease on the following days. For MC- the plants responded with an increase in CAT activity up to the 8th day. Plants exposed to MC+ and MC- showed a reduction in CAT activity on the 14th day, with values lower than the control (Fig. 4E, Table 1). The APX enzyme showed a significant increase in its activity on the 2nd day (2.19 times), reaching 3.34 times on the 8th day of culture in relation to the beginning of the experiment and returning to values close to the control on the 14th day ($p > 0.05$). APX was significantly higher in plants treated with MC+ than in MC- ($p < 0.001$) and control plants ($p < 0.001$) until the 8th day. Plants submitted to MC- increased APX activity followed by a decrease on 8th and 14th days, and differ from the control only on the 2nd day ($p < 0.01$) (Fig. 4F, Table 1).

## 4. Discussion

The *E. densa* was significantly affected when coexisting with the toxic strain, presenting a reduction in biomass and consequently in growth. Also, the exposure to the toxic strain caused oxidative stress in plants due to the amount of MDA in plant tissues, which is an expressive indicator of lipid peroxidation and causes degradation of the membranes. However, the plants present enzymatic defense mechanisms capable of reducing the effects caused by the MC-producing strain, with the APX enzyme being the one that responded better to exposure to the MC+ strain.

For aquatic macrophytes, the main cause of growth inhibition is cyanotoxins, such as anatoxin-a (Ha and Pflugmacher, 2013), cylindrospermopsin (Flores-Rojas et al., 2015) and microcystins (Jiang et al., 2011). Since MCs are potent inhibitors of protein phosphatases 1 and 2A, important for various biochemical and physiological processes (MacKintosh et al., 1990), these toxins also negatively affect plant growth. Inhibition of protein phosphatases may trigger several problems for the plant, affecting mitosis, development of apical and floral meristems, and root growth and development (Smith and Walker, 1996; Luan, 1998). This agrees with the results observed herein, in which a possible inhibition of the protein phosphatases may have reduced the growth of the plants exposed to the MC+ strain, as well as inhibited apical, lateral and root meristems growth. Furthermore, the possible presence of other harmful compounds, like lipopolysaccharides, may have contributed for the biomass reduction in plants exposed to both strains.

Morphological changes in *E. densa* were only observed in the fourth week of exposure to the MC+ strain, which may be due to damages in the antioxidative system or biotransformation mechanisms of toxic compounds present in MC+ strain, such as the MCs. At the beginning of the experiments, both strains were at the end of exponential growth phase, which means that the *Microcystis* cells was in senescent stage with an increased releasing of MCs in the toxic strain. In addition, cyanobacteria may have released a greater amount of cyanotoxins in response to allelochemicals of *E. densa*, causing cellular lysis and consequent release of cyanotoxins, provoking such changes, as well as biomass and branch length reduction.

The plants exposed to MC+ and MC- strains presented a degradation of the photosynthetic pigments in short exposure, which was evidenced by the chlorosis of the branches in MC+ in long-term exposure, which was not observed in the plants exposed to the MC- strain and in the control. Similarly, many studies demonstrated the effects of MCs on photosynthetic parameters and alteration of pigments in algae (García-Espín et al., 2017), agricultural plants (Machado et al., 2017), and aquatic macrophytes (Rojo et al., 2013). MCs stimulate the production of ROS and consequently MDA in aquatic macrophytes, with several biochemical and physiological effects, such as changes in photosynthetic pigment content and oxidative stress (Corbel et al., 2014). However, the plants responded with a small increase of carotenoids content on the 8th day in plants exposed to MC+ strain. Carotenoids can act as powerful non-enzymatic anti-oxidative defense (Gill and Tuteja, 2010). Plants exposed to MC- strain also showed a reduction in total chlorophyll, chlorophyll *a* and *b* content, which may be due to the light limitation occasioned by the growth of cyanobacteria or the actuation of other toxic compounds, like lipopolysaccharides.

In the case of oxidative stress, the high amount of ROS triggers a series of reactions that cause lipid peroxidation, mainly forming MDA (Esterbauer et al., 1991). During lipid peroxidation phospholipids, the main component of the cell membranes, are degraded and fragmented, where reactive aldehydes, such as MDA, are released, causing damage to cellular components, DNA, and proteins (Mueller, 2004). The presence of $H_2O_2$ in plants can also play a beneficial role, acting on cell proliferation, stress acclimatization, defense against pathogens, signal transduction, and regulation of metabolism (Mittler, 2017). Therefore, the balance between benefits and losses of $H_2O_2$ is determined by the concentration of this ROS in the organelles and the activity of the antioxidative enzymes (Shigeoka et al., 2002). Plants exposed to MC+ and MC- strains showed a decrease in MDA and $H_2O_2$ content on the 2nd day, possibly due to the short acclimatization time prior to the experiments.

ROS are naturally produced, but under unfavorable conditions such as water scarcity, high temperatures, or the presence of pollutants, such as cyanotoxins, the accumulation of these metabolites is intensified (Mittler, 2002). Another way of producing ROS is through the biotransformation reactions of xenobiotics in plants through the glutathione cycle (Polle, 2001), where with the activity of the glutathione

S-transferase and glutathione reductase enzymes the production of $H_2O_2$ in plants exposed to MCs is increased (Pflugmacher, 2004). ROS is highly toxic to plant tissues and organelles, causing damage to proteins, lipids, carbohydrates, and DNA, and can lead to cell death when at high levels (Bernstein et al., 2010). However, enzymatic and non-enzymatic anti-oxidative mechanisms can eliminate or convert ROS to less toxic components (Chen et al., 2004).

Under oxidative stress, the first enzyme that acts to control ROS is the SOD, which convert $O_2\cdot^-$ to $H_2O_2$ and $O_2$ (Mittler, 2002). Then the $H_2O_2$ released by SOD reactions is catalyzed and removed by CAT and APX enzymes (Asada, 1992). The maintenance of adequate levels of ROS in cells depends on the balance between the activity of the SOD, CAT and APX enzymes and changes or damages to certain enzymes are compensated by a greater activity of the others. Thus, when CAT activity is reduced, APX activity is potentiated (Apel and Hirt, 2004). This fact justifies the high APX activity in plants exposed to the MC+ strain, when CAT reduced its activity, contributing to the maintenance of $H_2O_2$ levels close to those of the control. In addition, APX has a higher affinity for $H_2O_2$ than CAT, as it is the main enzyme that acts on oxidative stress control (Gill and Tuteja, 2010), further contributing to the maintenance of tolerable levels of $H_2O_2$ in plants exposed to MC+.

The APX enzyme has been shown to be more efficient in the control of oxidative stress caused by MC+ strain, while for the MC- strain there was an increase in the values of CAT activity, demonstrating that other compounds, such as lipopolysaccharides, or competition for light and nutrients may have caused oxidative stress in plants. The enzymatic activity of SOD, CAT and APX enzymes remained close to the control in plants exposed to the MC+ and MC- *Microcystis* strains on the 14th day of exposure, possibly in response to protein damage. This result was also verified in *E. densa* exposed to a crude extract from a cyanobacterial bloom containing three MC congeners, where the enzymatic activity was kept lower than the control after 14 days of exposure (Romero-Oliva et al., 2015a). The presence of cyanotoxins, like β-N-methylamino-L-alanine, also may reduce the enzymatic anti-oxidant activity by the inhibition of antioxidant enzymes (Esterhuizen-Londt et al., 2011).

## 5. Conclusions

The anti-oxidative mechanisms of *E. densa* exposed to the toxic and non-toxic strains of *Microcystis* were efficient in controlling the oxidative stress of the plants during a period of three weeks. After this, intense modifications were observed in the plants cultivated with the toxic strain. These characteristics suggest that prolonged exposure to MCs can cause serious damage to aquatic plants, damaging the pigment content and anti-oxidative system of *E. densa*. This macrophyte was less affected morphometrically or physiologically when grown with non-MC-producing strain of *Microcystis*. This work demonstrates for the first time the effects of exposure of aquatic macrophytes to toxic and non-toxic strains in coexistence, important for understanding how these plants relate to different strains of cyanobacteria in natural ecosystems.

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## 5 CONSIDERAÇÕES FINAIS

Neste estudo, nós demonstramos pela primeira vez os efeitos alelopáticos mútuos da coexistência entre *Egeria densa* e linhagens tóxica e não tóxica de cianobactérias do gênero *Microcystis*. Os resultados apresentados são úteis para o entendimento da teoria de estados alternativos, a qual prediz que os ambientes aquáticos rasos possuem dois estados de dominância de macrófitas ou do fitoplâncton. Como observado no presente trabalho, as relações alelopáticas mútuas existentes entre macrófitas submersas e cianobactérias pode influenciar na dominância destes organismos, através da liberação de substâncias que inibem o crescimento de seus competidores.

A macrófita submersa *Egeria densa* é capaz de inibir significativamente o crescimento de linhagens produtoras de microcistinas, *Microcystis aeruginosa*. Por outro lado, linhagens não produtoras são estimuladas quando em coexistência com esta macrófita. Tais resultados sugerem que *Egeria densa* libera seus aleloquímicos no ambiente somente em situações de estresse, como o ocasionado pelas microcistinas nos experimentos de coexistência com a cepa tóxica. Por outro lado, *Egeria densa* apresentou significativas alterações fisiológicas e morfológicas ocasionadas pelo cultivo com a cepa de *Microcystis* produtora de microcistinas. A planta apresentou uma redução do crescimento (biomassa e comprimento dos ramos), acompanhada de alterações morfométricas, tais como alteração na coloração dos ramos, bem como inibição da emissão de brotos, raízes e degradação da região apical das plantas. A cepa não produtora de microcistinas não causou alterações severas nas plantas testadas.

Estes resultados, apesar de serem uma abordagem experimental em laboratório, trazem importantes indicações de como podem ocorrer as interações alelopáticas entre macrófitas aquáticas e cianobactérias nos ecossistemas de água doce, uma vez que os experimentos de coexistência são os que melhor refletem as condições ambientais. No entanto mais estudos precisam ser feitos especialmente em regiões tropicais, por serem as regiões mais afetadas pelas florações de cianobactérias, que muitas vezes são perenes e com a presença de várias espécies de cianobactérias. Além disso, a presença e proporção de linhagens tóxicas e não tóxicas deve ser levada em consideração em estratégias de biomanipulação com o uso de macrófitas aquáticas submersas.

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