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Effects of the submerged macrophyte *Ceratophyllum demersum* (Ceratophyllaceae) and the cladoceran *Moina micrura* (Cladocera: Moinidae) on microalgal interactions

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ABSTRACT

Introduction: Cyanobacterial blooms in tropical water bodies are increasingly common, because of eutrophication and rising temperatures. Consequently, many freshwater systems are affected, by reducing water quality, biodiversity, and ecosystem services. With the increased frequency of harmful algal blooms, the development of biological tools to improve water quality is an urgent issue.

Objective: To evaluate the effects of a submerged macrophyte and a cladoceran on the microcystin-producing cyanobacteria *Microcystis aeruginosa* (NPLJ-4) and the chlorophyte *Raphidocelis subcapitata* (BMIUFRPE-02) in mixed cultures.

Methods: Two parallel experiments were carried out for ten days to evaluate the effects of the submerged macrophyte *Ceratophyllum demersum* and the cladoceran *Moina micrura* on microalgal interactions. Microalgal strains were cultivated in the ASM1 culture medium, under controlled laboratory conditions. The first experiment presented four treatments: M (*C. demersum*), Z (*M. micrura*), MZ (*C. demersum* and *M. micrura*), and C (control). Meanwhile, the second experiment consisted of five treatments, in which the microalgae were cultivated together at different *Microcystis*:*Raphidocelis* ratios: 1:0, 3:1, 1:1, 1:3, and 0:1. Biomass and growth rates of the strains were evaluated every two days, which were statistically treated with three-way or two-way repeated-measures ANOVA.

Results: In the first experiment, *M. aeruginosa* was significantly inhibited in M and MZ treatments from the second day, and Z from the fourth, while *R. subcapitata* showed no reduction in its biomass in any treatment. On the other hand, *R. subcapitata* was stimulated from the eighth and tenth days in M treatment and only on the eighth day in Z treatment. In the second experiment, *M. aeruginosa* was significantly inhibited when cultivated with *R. subcapitata* in low ratios (*Microcystis*:*Raphidocelis* ratio of 1:3) throughout the experiment, while the chlorophyte was stimulated in that treatment.

Conclusions: The coexistence of a cyanobacterium with a green alga did not alter the main negative response of *M. aeruginosa* to the submerged macrophyte and zooplankton but stimulated the green alga. Accordingly, the introduction of submerged macrophytes and cladocerans already adapted to eutrophic conditions, both isolated and combined, proved to be a good method to control cyanobacterial blooms without negatively affecting other coexisting phytoplankton species.

Key words: microalgal blooms; allelopathy; biomanipulation; competition; grazing.

The occurrence of cyanobacterial blooms in freshwater environments, in response to anthropogenic eutrophication and climate change (Moura et al., 2018; O'Neil et al., 2012), has seriously damaged aquatic biota (Paerl & Otten, 2013), leading to the death of fish, mollusks, and crustaceans (Ibelings & Chorus, 2007; Zurawell et al., 2005). For the most part, blooms affect aquatic ecosystems through the production of toxic metabolites (e.g., Microcystins, Li et al., 2021) by certain cyanobacteria species (Cirés & Ballot, 2016; Harke et al., 2016). Moreover, algal blooms can represent a serious threat to freshwater biodiversity, reducing water quality and ecosystem functioning (Amorim & Moura, 2021).

Recurrent toxic blooms of these organisms seriously compromise the quality of water and make it unsuitable for human consumption, generating economic (Carmichael & Boyer, 2016) and health problems (Chen et al., 2009; Zurawell et al., 2005) and, in more serious cases, can be fatal (Azevedo et al., 2002). Among the main bloom-forming genera, *Microcystis* stands out with wide geographical distribution and several microcystin-producing morphospecies (Harke et al., 2016; O'Neil et al., 2012; Wiegand & Pflugmacher, 2005). To control algal blooms, laboratory and *in situ* studies using aquatic plants (e.g., Amorim & Moura, 2020; Amorim et al., 2019a;) and zooplankton organisms (e.g., Amorim & Moura, 2020; Amorim et al., 2019b; Diniz et al., 2019; Severiano et al., 2018; Severiano et al., 2021) have been carried out in tropical regions, specifically in the Northeastern region of Brazil. In that region, the biomanipulation of fish to control eutrophication and phytoplankton blooms can show negative (Menezes et al., 2010), positive or no effects (e.g., Dantas et al., 2019; Okun et al., 2008).

Submerged macrophytes can produce allelochemicals that are important tools for controlling cyanobacteria (Amorim & Moura, 2020; Zhu et al., 2010). The secondary metabolites released by plants into the water column can inhibit the activity of photosystem II and rupture the cell membrane, which kills the cyanobacteria (Mohamed, 2017). Macrophyte allelopathy is a biological alternative that can minimize impacts caused by harmful cyanobacteria and improve water quality (Chen et al., 2012; Hilt & Gross, 2008; Vanderstukken et al., 2014). However, unlike cyanobacteria, green algae are resistant to the inhibitory effect of allelochemicals from submerged macrophytes (Amorim et al., 2019a; Dong et al., 2014; Zhu et al., 2010). Nevertheless, it is believed that when in coexistence with a green alga, cyanobacteria can be stimulated by macrophyte allelochemicals instead of being inhibited (Chang et al., 2012).

Another way to reduce cyanobacterial blooms is through zooplankton since the herbivory pressure exerted by these organisms negatively affects the biomass of phytoplankton species (Amorim et al., 2019b; Diniz et al., 2019; Severiano et al., 2018). However, that method does not always negatively affect cyanobacteria, because they can produce large filaments and colonies, along with the production of toxic metabolites, which reduce the grazing of zooplankton on cyanobacteria (Ger et al., 2016). Cyanotoxins can negatively affect the quality of life for zooplankton, leading to death, retarding growth, and changing the ingestion rate (Bownik, 2016; Santos et al., 2021). Moreover, cyanotoxins can affect more seriously larger zooplankton than small organisms, however, the former ones can graze more efficiently on phytoplankton (Guo & Xie, 2006). With that, some zooplankton species can select palatable phytoplankton organisms, which can, in turn, increase cyanobacterial biomass (Leitão et al., 2018; Severiano et al., 2021).

Therefore, the present study aims to (1) verify the effect of the submerged macrophyte *Ceratophyllum demersum* L. and the cladoceran *Moina micrura* Kurz, 1874 on the interaction between the cyanobacteria *Microcystis aeruginosa* (Kützing) Kützing and the chlorophyte *Raphidocelis subcapitata* (Korshikov) Nygaard, Komárek, Kristiansen & Skulberg; and (2) understand the relationships between

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cyanobacteria and chlorophytes under different dominance scenarios for both groups.

MATERIALS AND METHODS

Phytoplankton organisms and culture conditions: For this study, two non-axenic strains of microalgae were selected: the cyanobacterium *M. aeruginosa*, and the chlorophyte *R. subcapitata*. The *M. aeruginosa* strain (NPLJ-4) (Cyanobacteria), which has been proven to produce four variants of microcystins (Amorim et al., 2017), was provided by the Laboratory of Ecophysiology and Toxicology of Cyanobacteria (LETC) at the Federal University of Rio de Janeiro (Rio de Janeiro, Brazil). The *R. subcapitata* strain (BMIU-FRPE-02) (Chlorophyceae) was obtained from the Microalgae Culture Collection at the Federal Rural University of Pernambuco - BMIU-FRPE, Recife (Pernambuco, Brazil).

Cyanobacteria and chlorophyte were grown in 1 000 ml Erlenmeyer flasks filled with 800 ml of ASM1 culture medium (Gorham et al., 1964), in a climatic chamber with controlled conditions of temperature (25 °C ± 1.5), light intensity (40 µmol m^{-2} s^{-1}), pH (7.5), and 12 h photoperiod. All cultures were homogenized three times a day to avoid agglomeration and sedimentation of cells. Microalgae were cultivated until biomass of 50 mg L^{-1} was obtained.

Collection and maintenance of submerged macrophyte *Ceratophyllum demersum*: The submerged macrophyte *C. demersum* was collected from the Carpina reservoir, Lagoa do Carro municipality (Pernambuco, Brazil). During the sampling, 20 cm apical branches from young plants were selected and transported to the laboratory, where they were washed with distilled water jets and a soft brush to remove epiphytic microalgae, small invertebrate animals, and adhered sediments. The macrophytes were cultivated in 8 l aquaria (20 cm^2) that were filled with filtered tap water and maintained under the same conditions described for microalgae, however, the aquaria were constantly aerated with aquarium aerators. Until the experiments were carried out, the water was renewed twice a week to prevent the proliferation of insects, small mollusks, and microorganisms.

Obtaining and culture of the cladoceran: The cladoceran *M. micrura* was collected from the Mundaú reservoir, Garanhuns municipality (Pernambuco, Brazil). This reservoir presents intense cyanobacteria blooms formed mainly by *Microcystis* spp. and *Raphidiopsis raciborskii* (Woloszynska) Aguilera, Berrendero Gómez, Kastovsky, Echenique & Salerno (Moura et al., 2015; Amorim et al., 2020). This cladoceran was collected by filtering 100 l of water with a 68 µm mesh plankton net and identified under an optical microscope using specialized bibliography. The individuals were selected and cultivated separately in 30 ml test tubes, filled with 20 ml of reconstituted water (70 % mineral water + humic acid and 30 % water from the environment that was filtered through a 20 µm mesh net), for subsequent selection of genetically identical clones from the same mother.

After at least 20 individuals grew in each tube, one clonal lineage was selected and the cladocerans were transferred to 250 ml Erlenmeyer flasks filled with 200 ml of reconstituted water and constant aeration. Thirty days before the experiments, the cladocerans were cultivated in 1 000 ml Erlenmeyer flasks at 27 °C, 40 µmol m^{-2} s^{-1} light intensity, constant aeration, and 12 h photoperiod; and were fed with the chlorophyte *R. subcapitata* every day.

Experimental design: The experiments were carried out in an aseptic room with the same conditions described for the cultivation of the microalgae strains. Twenty-four 1 000 ml Erlenmeyer flasks were filled with 500 ml of ASM1 nutrient medium containing cyanobacteria and chlorophyte inoculum. Two parallel experiments were carried out for ten days to observe the allelopathic effects of *C. demersum* and the grazing pressure of the cladoceran *M. micrura*, both isolated and together, on the interaction between *M. aeruginosa* and

R. subcapitata; as well as to observe possible allelopathic interactions between cyanobacteria and green algae at different dominance ratios.

The experimental design consisted of eight treatments with three replicates that were divided between the two parallel experiments (Table 1). For both experiments, all microalgal treatments were grown in coexistence or isolated with initial biomass of approximately 35 mg l^{-1}. In the first experiment, the proportion of microalgae biomass was 1:1 in all treatments. In the second experiment, the interaction treatments consisted of different concentrations of *Microcystis* and *Raphidocelis*, while the controls consisted of the isolated cultures of the cyanobacterium and the chlorophyte. Thus, there was a gradient in the *Microcystis* and *Raphidocelis* ratios of M:R = 1:0; 3:1; 1:1; 1:3; and 0:1 (Table 1).

To determine the exact ratio between *Microcystis* and *Raphidocelis* cultures for the experiments, the biomasses of the stock cultures were analyzed. The proportions were achieved using the ASMI medium dilution method. Three days before the experiments began, young and apical branches of *C. demersum* were selected, washed several times with distilled water, cut to obtain an 8 gFW l^{-1} biomass (g of fresh weight), then grown in ASM1 medium for acclimatization. Similarly, three days before the experiment, visibly healthy (actively swimming) cladocerans of the same age were selected (120 ind l^{-1}) and transferred to 250 ml Erlenmeyer flasks containing ASM1 medium for acclimatization.

At the beginning of the experiment, one macrophyte branch was added to each experimental unity in the M and MZ treatments. Similarly, 50 *M. micrura* individuals were transferred to each experimental unit in the Z and MZ treatments. During the transfer of cladocerans to microalgae cultures, about 5 ml of ASM1 medium was also transferred, thus, the same amount of medium from the cladoceran cultures was added to all treatments. Aliquots of 1 ml were collected every two days for 10 days (0, 2, 4, 6, 8, and 10) from all experimental units to determine cell density and biovolume. Microalgal density was determined by counting cells in a Fuchs-Rosenthal chamber (hemocytometer) under an optical microscope. Also, the length and width of the cells were measured to obtain the biovolume as proposed by Hillebrand et al. (1999), which was multiplied by density for conversion into biomass. In the second experiment, growth rates were determined for each species in all treatments, following the formula described by Wood et al. (2005).

Statistical analyses: A three-way repeated-measures ANOVA was used to compare the biomass of microalgae in the first experiment, based on the factors Macrophyte, Zooplankton,

TABLE 1
Descriptions of the treatments used in the allelopathy and grazing experiment (Exp. 1) and microalgae interaction experiment (Exp. 2) with different proportions of microalgae

Experiment / Treatment	*Microcystis* Percentage (%)	*Raphidocelis* Percentage (%)	*Ceratophyllum demersum*	*Moina micrura*
Exp.1 / M	50	50	X	-
Exp.1 / Z	50	50	-	X
Exp.1 / MZ	50	50	X	X
Exp.2 / 1:0	100	0	-	-
Exp.2 / 3:1	75	25	-	-
Exp.1 and 2 / C and 1:1	50	50	-	-
Exp.2 / 1:3	25	75	-	-
Exp.2 / 0:1	0	100	-	-

X: presence; -: absence. The 1:1 treatment of the second experiment was also used as a control for the first since they were developed in parallel.

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TABLE 2
Results of the three-way repeated-measures ANOVA comparing the effects of *Ceratophyllum demersum* (macrophyte), *Moina micrura* (zooplankton), and time on the biomass of *Microcystis aeruginosa* and *Raphidocelis subcapitata* in the first experiment

Factors	df	F	p
Microcystis aeruginosa			
Macrophyte	1	6681.14	< 0.001
Zooplankton	1	1129.62	< 0.001
Time	5	120.38	< 0.001
Macrophyte:Zooplankton	1	726.46	< 0.001
Macrophyte:Time	5	206.70	< 0.001
Zooplankton:Time	5	182.68	< 0.001
Macrophyte:Zooplankton:Time	5	194.49	< 0.001
Raphidocelis subcapitata			
Macrophyte	1	25.65	0.037
Zooplankton	1	23.12	0.041
Time	5	174.30	< 0.001
Macrophyte:Zooplankton	1	73.75	0.013
Macrophyte:Time	5	8.27	0.003
Zooplankton:Time	5	13.26	< 0.001
Macrophyte:Zooplankton:Time	5	20.39	< 0.001

Time, and their interactions. Likewise, a two-way repeated-measures ANOVA was used to compare the growth rates of the cultures in the second experiment, based on the factors Ratios, Time, and their interactions. Before the analysis of variances, the data were tested for normality with the Kolmogorov-Smirnov test and homoscedasticity with Bartlett's test. For statistical analyses, the R program was used, with a significance level of $P < 0.05$ (R Core Team, 2021).

RESULTS

Experiment 1: The cyanobacterium and green alga strains responded differently to the treatments and time (Table 2). *Ceratophyllum demersum* significantly reduced the biomass of *M. aeruginosa* from the second day of the experiment in treatment M ($P < 0.05$) (Fig. 1A). However, *R. subcapitata* was not significantly affected in treatment M when compared to the control ($P > 0.05$) until the sixth day. After that, *R. subcapitata* showed significantly higher biomass in the treatment M, compared to the control ($P < 0.05$) (Fig. 1B).

With the addition of cladocerans, there was a significant reduction in the biomass of *M. aeruginosa* ($P < 0.05$) from the fourth day (Fig. 1A). Regarding the green alga, *M. micrura* stimulated the growth of *R. subcapitata* on the eighth day, with significant differences when compared to the control ($P < 0.05$) and showing no significant grazing by cladocerans (Fig. 1B).

The coexistence of aquatic macrophytes with the cladoceran in the MZ treatment significantly reduced the biomass of *M. aeruginosa* from the second day of the experiment compared to the control ($P < 0.05$) but did not show significant differences to the M treatment ($P > 0.05$) (Fig. 1A). For green alga, the combined treatment of the plant and microcrustacean (MZ) did not alter the biomass of *R. subcapitata* ($P > 0.05$) (Fig. 1B). *Raphidocelis subcapitata* also did not show any significant differences between the treatments M, Z, and MZ ($P > 0.05$). During the experiment, *R. subcapitata* tended to form small to large colonies

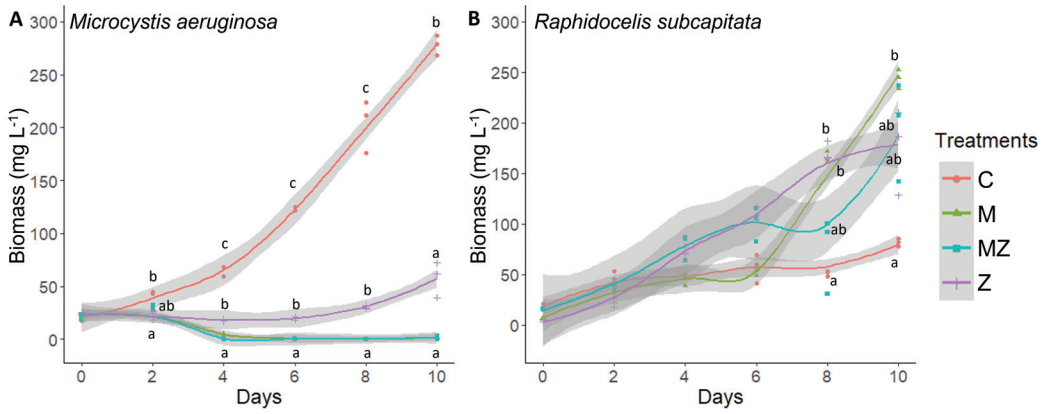

Fig. 1. Isolated effects of *Ceratophyllum demersum* (M) and *Moina micrura* (Z), besides the combined addition of *C. demersum* and *M. micrura* (MZ), and control (C) on **A.** *Microcystis aeruginosa* and **B.** *Raphidocelis subcapitata* in mixed cultures for ten days. The different letters indicate significant differences between treatments for each day ($P < 0.05$). Lines and shaded areas represent the mean and 95 % confidence interval, respectively.

in all treatments with the macrophyte and the cladoceran (data not shown).

Experiment 2: The mixed cultures, with different ratios of *M. aeruginosa* and *R. subcapitata*, showed distinct responses to the treatments and time (Table 3). In the controls, *M. aeruginosa* (M:R 1:0) maintained a constant growth (Fig. 2A), while *R. subcapitata* (M:R 0:1) decreased its growth during the experiment (Fig. 2B). For the treatment with 75 % *Microcystis* and 25 % *Raphidocelis* (M:R 3:1), both cyanobacterium and green alga did not differ from the controls throughout the experiment ($P > 0.05$) (Fig. 2A, Fig. 2B). The opposite effect was observed for the treatment with 25 % *Microcystis* and 75 % *Raphidocelis* (M:R 1:3), when the green alga strain significantly inhibited the growth of *Microcystis* from the second day of the experiment ($P < 0.05$) (Fig. 2A). Also, *Raphidocelis* presented higher growth rates in treatment M:R 1:3 than in M:R 1:1 on the fourth and eighth days. In equal proportions of microalgal biomass, i.e. 50 % of both strains (M:R 1:1), neither strain differed significantly from the controls ($P > 0.05$) (Fig. 2).

Regarding the ratio between the biomass of *M. aeruginosa* and *R. subcapitata* in the treatments with the dominance of *M. aeruginosa*

TABLE 3
Results of the two-way repeated-measures ANOVA comparing the effects of different ratios between *Microcystis* and *Raphidocelis* and time on the growth rate of *Microcystis aeruginosa* and *Raphidocelis subcapitata* in the second experiment

Factors	df	F	p
Microcystis aeruginosa - Growth rate			
Ratios	3	1049.02	< 0.001
Time	4	16.27	< 0.001
Ratios:Time	12	22.26	< 0.001
Raphidocelis subcapitata - Growth rate			
Ratios	3	5.74	0.034
Time	4	152.95	< 0.001
Ratios:Time	12	2.75	0.017

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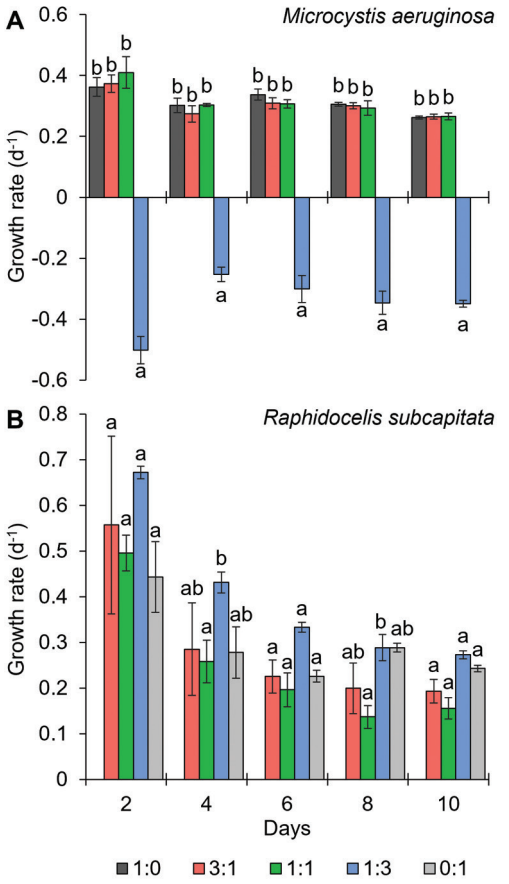

Fig. 2. Growth rate of **A.** *Microcystis aeruginosa* and **B.** *Raphidocelis subcapitata* in treatments with different M:R (*Microcystis*:*Raphidocelis*) ratios: 1:0, 3:1, 1:1, 1:3, and 0:1 during ten days of the experiment. The different letters indicate significant differences between treatments for each day ($P < 0.05$). Bars and error bars represent the mean and standard deviation, respectively.

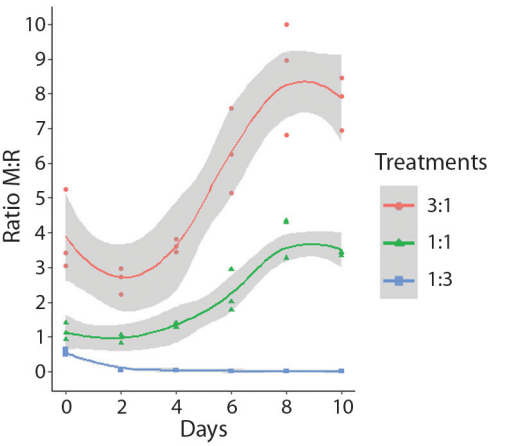

Fig. 3. The ratio between the biomass of *M. aeruginosa* and *R. subcapitata* in treatments with 75 % *Microcystis* and 25 % *Raphidocelis* (3:1), 50 % *Microcystis* and 50 % *Raphidocelis* (1:1), and 25 % *Microcystis* and 75 % *Raphidocelis* (1:3) during 10 days of the experiment. Lines and shaded areas represent the mean and 95 % confidence interval, respectively.

(M:R 3:1) or in equal proportions (M:R 1:1), there was an increase in the relative participation of cyanobacteria compared to green algae from the fourth day until the end of the experiment. On the other hand, under the dominance of *R. subcapitata* (M:R 1:3), *M. aeruginosa* showed a reduction in relative participation from the second day (Fig. 3).

DISCUSSION

Competition for nutrients (Zhang et al., 2013), light (Marinho et al., 2013), and allelopathy (Bittencourt-Oliveira et al., 2015; Harel et al., 2013) are variables that strongly regulate the development of phytoplankton species. The availability of nitrogen and phosphorus contributes to the growth and helps to maintain the metabolism of microalgae, increasing the biomass of species that better absorb inorganic compounds (Carey et al., 2012; Markou et al., 2014). Nevertheless, in our study, we excluded the effect of nutrient competition by using ASM1 culture medium in optimum quantities for the development of both strains.

The presence of morphological structures that facilitate fluctuation, as well as the presence of accessory pigments that protect cells from excessive light, are adaptive advantages that cyanobacteria species present (Carey et al., 2012). *Microcystis aeruginosa* is a strong competitor for light, as verified by Marinho et al. (2013). However, we discarded this type of competition through the uniform and random light supply between treatments, as well as by manually agitating the experimental units three times a day to reduce cell sedimentation.

Finally, by excluding other possible forms of competition, we suggest that allelopathy was the main mechanism of action among photosynthesizing organisms, especially the macrophyte *Ceratophyllum demersum*.

In treatments with *C. demersum*, cyanobacterial biomass was reduced too much after the second day of coexistence. Previously, Amorim et al. (2019a) reported that the biomass of *M. aeruginosa* was inhibited by the presence of *C. demersum* in unialgal cultures. Therefore, the macrophyte effect could be attributed to allelopathy herein, as also verified by Nakai et al. (1999), Dong et al. (2014), and Amorim et al. (2019a). Furthermore, in a field experiment, Amorim and Moura (2020) showed a significant reduction in cyanobacterial blooms composed mainly of *Microcystis* spp. (reduction of 85 % in the total biomass and 99 % in the biomass of filamentous morphotypes) by *C. demersum* in a tropical reservoir in Northeast Brazil.

Studies highlight that *M. aeruginosa* is sensitive to chemical compounds released by several macrophyte species (Nakai et al., 1999; Zhu et al., 2010). In this case, *Ceratophyllum demersum*, a free-living submerged macrophyte, can produce secondary metabolites (Hilt & Gross, 2008) which inhibit competitors' photosystem II, compromising the photosynthetic activities of the target cell (Körner & Nicklisch, 2002). Sulfur or lipophilic labile sulfur compounds are the major allelopathic substances released by *C. demersum* (Wium-Andersen et al., 1983). Also, some volatile compounds from *C. demersum*, including fatty compounds, terpenoids, phenolic compounds, and phthalates, can show a strong inhibitory effect on *M. aeruginosa* (Xian et al., 2006). Further compounds present in *C. demersum* tissues, such as hexanoic acid, phthalic acid, octanedioic acid, butenoic acid, azelaic acid, palmitic acid, alpha-linolenic acid, and pentanedioic acid, can also inhibit the growth and induce colony formation in green algae species (Dong et al., 2019). Therefore, allelopathy can act as one of the main adaptative strategies of submerged macrophytes, especially *C. demersum*, in their competition with phytoplankton (Gross et al., 2003), as well as to maintain the clear state of shallow lakes (Hilt & Gross, 2008). So, this macrophyte can support the eutrophic and cyanobacterial blooms conditions, as it has potent antioxidant and biotransformation mechanisms to alleviate the effects of cyanotoxins on its physiology, besides removing cyanotoxins from the water (Pflugmacher, 2004).

In our study, *M. aeruginosa* was significantly inhibited in coexistence with an allelochemical-producing macrophyte and green algae, differing from the results observed by Chang et al. (2012), who recorded the stimulus of *M. aeruginosa* in coexistence with a submerged macrophyte and the chlorophyte *Desmodesmus armatus* (R. Chodat) E. Hegewald. Furthermore, *R. subcapitata* was not inhibited in coexistence with *C. demersum* (M and MZ treatments), corroborating the results of Amorim et al. (2019a), who found that biomass from *R. subcapitata* was not significantly affected by *C. demersum* throughout the experiment. This response could be attributed to the low sensitivity of chlorophytes to the inhibitory metabolites released by macrophytes in comparison to cyanobacteria, as verified by Zhu et al. (2010). Moreover, Körner and Nicklisch (2002) suggest that phytoplankton species can physiologically adapt to allelochemicals. One important evolutionary adaptation of green algae to coexist with allelopathically active macrophytes is colony formation (Dong et al., 2018), as also observed in our study for *R. subcapitata.*

In tests with the addition of cladocerans, we observed that the cyanobacterial biomass was significantly reduced from the fourth day and the green alga was stimulated on the eighth day, differing statistically from the control. This result differs from previous findings in the literature, where zooplankton are assumed to select the palatable food source in co-cultures, and thus stimulate cyanobacterial growth (Leitão et al., 2018; Severiano et al., 2021). However, in experimental studies, Guo and Xie (2006) found that populations of *M. micrura* pre-exposed to toxic strains *M. aeruginosa* may become more resistant to cyanobacteria

metabolites compared to other large cladocerans, enabling the predation of cyanobacteria. Herein, the cladoceran *M. micrura* proved to be resistant to toxins from the *Microcystis* strain, which may have favored its predation on cyanobacteria, considering that the cladoceran was isolated from a reservoir with a history of *Microcystis* blooms (Moura et al., 2015; Amorim et al., 2020). Similarly, Santos et al. (2021) showed that cladocerans isolated from lakes with cyanobacterial blooms are less affected by cyanotoxins through the diet or the absorption of dissolved toxins.

Although being considered a palatable food source for zooplankton, *R. subcapitata* was stimulated in treatment Z, instead of being grazed. This can be attributed to the colony formation in the presence of predators or competitors. Both the presence of macrophytes and zooplankton grazers can induce colony formation in green algae species, with a strong effect in the presence of zooplankton cues, which can act as an important anti-grazer defense (Zhu et al., 2021). The stimulus of *Raphidocelis* in cladoceran treatments can also be attributed to a reduction in *Microcystis* biomass. Furthermore, zooplankton contributes to nutrient cycling in the water column, favoring the growth of phytoplankton species (Attayde & Hansson, 1999), which may justify the significant stimulus of *R. subcapitata* in Z treatment.

As for the interaction between microalgae, different results between *Microcystis* and *Raphidocelis* were observed. *Raphidocelis subcapitata* inhibited *M. aeruginosa* in the treatment with low concentrations of cyanobacteria (M:R ratio 1:3). Li and Li (2012), in co-cultivation experiments with *M. aeruginosa* and *Anabaena*, found that the species with higher proportions at the beginning of cultivation (1:9 and 9:1) remained dominant throughout the experiment. In our study, although *M. aeruginosa* did not inhibit *R. subcapitata* growth when dominant (M:R 3:1), the ratio between *Microcystis* and *Raphidocelis* increased throughout the experiment, showing that *M. aeruginosa* remained dominant, corroborating with Li and Li (2012). Another factor that may be strictly related to the inhibition of *M. aeruginosa* is allelopathy. Some chlorophyte species can release chemical compounds that inhibit cyanobacteria, as verified by Harel et al. (2013), who demonstrated that *Scenedesmus* sp. inhibited the growth of *Microcystis* sp. by producing secondary metabolites that disrupted the cell membranes of cyanobacteria.

Bittencourt-Oliveira et al. (2015) showed that the density of *M. aeruginosa* decreased in mixed cultures with 1:1 ratios of cyanobacteria and chlorophytes *Monoraphidium convolutum* (Corda) Komárková-Legnerová and *Scenedesmus acuminatus* (Lagerheim) Chodat, with more significant effects when coexisting with the latter species. However, in our experiment, the growth rates of *M. aeruginosa* and *R. subcapitata* did not differ statistically from controls in the treatment with proportions of M:R 1:1. Li and Li (2012) found that *M. aeruginosa* and *Anabaena*, in 1:1 ratios, maintained similar growth for 15 days, reinforcing that the dominance of a given strain is related to the values of inoculated biomass on the first day. This result also reinforces the main effects of the submerged macrophyte and the cladoceran in the first experiment, proving that the microalgae species do not affect each other under the same proportion of biomass.

The submerged macrophyte *C. demersum* considerably inhibited *M. aeruginosa* in coexistence with the green alga, controlling the cyanobacteria biomass, differing from previous research (e.g., Chang et al., 2012). However, this macrophyte stimulated *R. subcapitata* growth since the chlorophytes present physiological mechanisms that protect them against the allelopathic effects of *C. demersum*. The cladoceran *M. micrura* also reduced the biomass of *M. aeruginosa* and favored the growth of *R. subcapitata,* but in a less remarkable way than the macrophyte *C. demersum*, also differing from previous findings where zooplankton can graze on palatable food and stimulate cyanobacterial growth (Leitão et al., 2018). As we used a cladoceran isolated from a hypereutrophic reservoir, already adapted to cyanobacterial blooms, it could efficiently graze on

cyanobacteria. In small ratios, *M. aeruginosa* was severely inhibited by *R. subcapitata* in comparison to the competitor. However, in equal and dominant proportions, no cyanobacteria inhibition was observed. Unlike *M. aeruginosa*, *R. subcapitata* was not inhibited in low proportions but maintained constant growth when compared to the control. On the other hand, in cultures with lower proportions of *M. aeruginosa*, chlorophyte was stimulated.

These results reinforce the applicability of submerged macrophytes and cladocerans, both isolated and combined, to control cyanobacterial blooms. The coexistence with other microalgal species does not reduce the allelopathic effect of the submerged macrophytes or grazing efficiency of the cladoceran on cyanobacteria but can increase the inhibitory effect when the proportion of cyanobacteria is low. However, it is important to use organisms already adapted to the cyanobacterial blooms and eutrophic conditions. Besides that, the reduction of external sources of nutrients and fish biomanipulation should be considered to allow the development of macrophytes and zooplankton.

Ethical statement: the authors declare that they all agree with this publication and made significant contributions; that there is no conflict of interest of any kind; and that we followed all pertinent ethical and legal procedures and requirements. All financial sources are fully and clearly stated in the acknowledgements section. A signed document has been filed in the journal archives.

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RESUMEN

Efectos de un macrófito sumergido *Ceratophyllum demersum* (Ceratophyllaceae) y un cladócero *Moina micrura* (Cladocera: Moinidae) sobre las interacciones de microalgas

Introducción: Las proliferaciones de cianobacterias en los cuerpos de agua tropicales son cada vez más comunes, debido a la eutrofización y al aumento de las temperaturas. En consecuencia, muchos sistemas de agua dulce se ven afectados por la reducción de la calidad del agua, la biodiversidad y los servicios de los ecosistemas. Con el aumento de la frecuencia de la proliferación de algas nocivas, el desarrollo de herramientas biológicas para mejorar la calidad del agua es urgente.

Objetivo: Evaluar los efectos de una macrófita sumergida y un cladócero sobre la cianobacteria productora de microcistina llamada *Microcystis aeruginosa* (NPLJ-4) y la clorofita *Raphidocelis subcapitata* (BMIUFRPE-02) en cultivos mixtos.

Métodos: Se realizaron dos experimentos paralelos durante diez días para evaluar los efectos de la macrófita sumergida *Ceratophyllum demersum* y el cladócero *Moina micrura* sobre las interacciones microalgales. Se cultivaron cepas de microalgas en el medio de cultivo ASM1, en condiciones controladas de laboratorio. El primer experimento presentó cuatro tratamientos: M (*C. demersum*), Z (*M. micrura*), MZ (*C. demersum* y *M. micrura*) y C (control). El segundo experimento consistió en cinco tratamientos, en el que las microalgas se cultivaron juntas en diferentes proporciones de *Microcystis*:*Raphidocelis*: 1:0, 3:1, 1:1, 1:3 y 0:1. La biomasa y las tasas de crecimiento de las cepas se evaluaron cada dos días, y se trataron estadísticamente con ANOVA de medidas repetidas de dos o tres factores.

Resultados: En el primer experimento, *M. aeruginosa* se inhibió significativamente en los tratamientos M y MZ a partir del segundo día, y en Z a partir del cuarto, mientras que *R. subcapitata* no mostró reducción de su biomasa en ningún tratamiento. Por otro lado, *R. subcapitata* fue estimulada a partir del octavo y décimo día en el tratamiento M y solo en el octavo día en el tratamiento Z. En el segundo experimento, *M. aeruginosa* se inhibió significativamente cuando se cultivó con *R. subcapitata* en proporciones bajas (proporción de *Microcystis*:*Raphidocelis* de 1:3) durante todo el experimento, mientras que la clorófita se estimuló en ese tratamiento.

Conclusiones: La coexistencia de una cianobacteria con un alga verde no alteró la principal respuesta negativa de *M. aeruginosa* a la macrófita sumergida y al zooplancton, sino que estimuló al alga verde. En consecuencia, la introducción de macrófitos y cladóceros sumergidos ya adaptados a las condiciones eutróficas, tanto aislados como combinados, resultó ser un buen método para controlar las proliferaciones de cianobacterias sin afectar negativamente a otras especies de fitoplancton coexistentes.

Palabras clave: proliferaciones de microalgas; alelopatía; biomanipulación; competencia; pastoreo.

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