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Use of *Myriophyllum aquaticum* to inhibit *Microcystis aeruginosa* growth and remove microcystin-LR

Uso de *Myriophyllum aquaticum* para inibir o crescimento de Microcystis aeruginosa e remover microcistina-LR

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ABSTRACT

Harmful algal blooms are one of the greatest challenges when preserving water sources, especially when involving cyanobacteria such as *Microcystis aeruginosa*. Finding remediation possibilities is needed, and one of them has been the use of macrophytes such as the species *Myriophyllum*, which have presented allelopathic mechanisms of phytoplankton control. Thus, this work aimed to evaluate the inhibition of *M. aeruginosa* cell growth in a co-exposure with *Myriophyllum aquaticum* and the influence on microcystin-LR concentration. The experiments were carried out using a culture of *M. aeruginosa* ($1x10^6$ cells mL^{-1}) in a co-exposure with *M. aquaticum* for seven days. The inhibitory effects were investigated by counting the cells; the effects on photosynthetic pigments were measured and microcystin-LR was quantified in the culture medium on the last experimental day. To evaluate the possible effects of competition for nutrients and space, the concentration of total orthophosphate was quantified and treatment with plastic plants was used. The experiments with *Myriophyllum aquaticum* achieved the total inhibition of *M. aeruginosa* growth and a significant reduction of the photosynthetic pigments (> 98%). Additionally, we observed a reduction of microcystin-LR concentration (79%) in the tests with macrophytes when compared to the control. Competition for space and nutrients was not observed, demonstrating that the effects on *M. aeruginosa* were caused by aquatic macrophyte presence. These results may indicate that *M. aquaticum* causes inhibitory effects on cyanobacteria growth by allelopathic effects and removes microcystin-LR.

Keywords: allelopathy; cyanobacteria; cyanotoxins; submerged aquatic macrophytes; nature-based solutions.

RESUMO

Florações de cianoabactérias são consideradas um dos maiores desafios na preservação de fontes hídricas, especialmente quando estão presentes espécies como *Microcystis aeruginosa.* A descoberta de alternativas de remediação faz-se necessária, e uma delas é o uso de macrófitas aquáticas, como as espécies do gênero *Myriophyllum*, que apresentam atividades alelopáticas para o controle fitoplanctônico. Diante disso, este trabalho teve como objetivos avaliar a inibição do crescimento de células de *M. aeruginosa* em uma coexposição com *Myriophyllum aquaticum* e avaliar a remoção de microcistina-LR. Os experimentos foram conduzidos com cultivos de *M. aeruginosa* ($1x10^6$ células mL^{-1}) em coexposição com *M. aquaticum* por sete dias. Os efeitos inibitórios foram investigados por contagem celular. Os efeitos nos pigmentos fotossintéticos foram mensurados, além da quantificação de microcistina-LR no último dia experimental. Para avaliar possíveis efeitos de competição por nutrientes e espaço, realizou-se a quantificação da concentração de ortofosfato total e utilizou-se um tratamento com planta de plástico. Os experimentos com *M. aquaticum* apresentaram inibição total do crescimento de *M. aeruginosa* e redução significativa na concentração dos pigmentos fotossintéticos (> 98%). Além disso, foi constatada redução na concentração de microcistina-LR (79%) nos testes com macrófitas quando comparadas ao grupo controle. Não foi observada competição por espaço e nutrientes, o que demonstra que os efeitos sobre *M. aeruginosa* foram causados pela presença da macrófita aquática. Dessa forma, estes resultados podem demonstrar que *M. aquaticum* gerou a inibição no crescimento de cianobactérias por efeitos alelopáticos, além de remover a microcistina-LR das águas.

Palavras-chave: alelopatia; cianobactérias; cianotoxinas; macrófitas aquáticas submersas; soluções baseadas na natureza.

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Introduction

Anthropic activities such as agriculture and industry have been responsible for negative impacts on aquatic ecosystems (Ramya et al., 2020; Kakade et al., 2021). Increased atmospheric carbon dioxide, the incorrect use and disposal of fertilizers, and the dumping of domestic sewage have significantly contributed to the eutrophication of water bodies (Ramya et al., 2020; Kakade et al., 2021). As a result, increased harmful algal blooms, mainly of cyanobacteria, have been noted, becoming an emergent environmental concern (Touzet et al., 2016; Kakade et al., 2021).

Harmful algal blooms cause negative impacts on environments, and they negatively affect human health and activities such as aquaculture (Zohdi and Abbaspour, 2019; Tanvir et al., 2021). Cyanobacteria bloom causes problems in aquatic ecosystems, including alteration of the trophic chain structure and local functionality due to the deoxygenation of the water and disturbance in odor and color (Lu et al., 2017). Also, the release of cyanotoxins during the bloom may decrease ecosystem biodiversity because they can cause toxicity to non-target organisms (Ramya et al., 2020; Munoz et al., 2021). Similarly, cyanotoxins are also toxic to humans, and their occurrence in water may become a public health problem (Ramya et al., 2020; Munoz et al., 2021).

Microcystis aeruginosa is one of the most common cyanobacteria that establish blooms in reservoirs and other aquatic environments, degrading them mainly by producing microcystins (Li et al., 2016). These chemicals are hepatotoxic cyanotoxins that impact human and animal health (Ramya et al., 2020), and they are associated with cancer, hepatic dysfunction, and acute episodes of intoxication (Kudela et al., 2015).

The ecological, aesthetic, and economic problems generated by cyanobacteria are commonly treated by applying conventional algaecides, oxidative chemical compounds such as potassium permanganate, hydrogen peroxide, and anti-algae flocculants (Wang et al., 2017b; Torres et al., 2020; Munoz et al., 2021). However, many of these compounds present environmental toxicity and the potential to generate secondary pollutants (Meng et al., 2015; Tazart et al., 2021). Therefore, the development of economic and environmentally friendly technologies to control cyanobacteria blooms is needed, and the use of allelopathic strategies could be a possibility (Chen and Guo, 2014; Tazart et al., 2021; Zhu et al., 2021).

Allelopathy consists of the positive or negative effect that an organism exerts on other organisms by releasing compounds (named allelochemicals) into the environment (Li et al., 2021; Zhu et al., 2021) Studies have shown that these substances may be a feasible alternative to combat cyanobacteria blooms because they are biodegradable and, depending on the concentration used, they do not present toxicity to the environment (Meng et al., 2015; Li et al., 2021). By releasing allelochemicals, it was observed that some submerged macrophytes inhibited the growth of cyanobacteria by allelopathy (Huang et al., 2020; Tazart et al., 2021; Zhu et al., 2021). Moreover, these plant species are important to maintain the quality of lakes since they compete with phytoplankton for light and nutrients, reduce suspended sediments, and contribute to water purification (Li et al., 2021). So, these techniques can be considered an efficient nature-based solution (NBS) that can corroborate to mitigate the impact of harmful algae blooms (Kitamura et al., 2021; Zhu et al., 2021).

Myriophyllum aquaticum (Vell.) Verdc is a submerged macrophyte that presents fast growth in a eutrophic environment, phytoremediator potential, and can regulate ecosystems by allelopathic effects (Cheng et al., 2008). *M. aquaticum* was reported as a promising species to control cyanobacteria (Cheng et al., 2008; Wang et al., 2017a), mainly by inhibiting cell growth. Thus, this work aimed to evaluate the inhibitory activity of *M. aquaticum* on *Microcystis aeruginosa* through co-exposure experiments, as well as to investigate the effects on photosynthetic pigments and the impact on microcystin-LR concentration. The present work hypothesizes that *M. aquaticum* can have effects on cyanobacteria cell growth, mainly by affecting photosynthetic pigments and the capacity to reclaim microcystin-LR from the medium. In addition, we hypothesized that inhibition processes are caused by allelopathic mechanisms of macrophytes and these effects are independent of other competition processes (space and nutrients, as orthophosphate).

Material and Methods

Culture of cyanobacteria and plants

The *Microcystis aeruginosa* strain (code BB005, isolated from harmful bloom and provided by the Botany Department of Universidade Federal de São Carlos, Brazil) was cultivated in the ASM-1 medium (Gorham et al., 1964 with adaptations of Almeida et al., 2016) with inoculum at an initial concentration of $1.6x10^5$ cell mL^{-1}. The cultures were maintained at 25 ± 3°C under luminosity of 36.81 ± 2.58 µmol of photons.s^{-1}.$m^{2\ -1}$and photoperiod of 12:12 h (light: dark).

The *Myriophyllum aquaticum* (12 ± 2 cm of height) plants were purchased from specialized suppliers and maintained for two months in an aquarium of 20 L (approximately 15 plants per aquarium) containing dechlorinated water and coarse gravel sediment. The plants were disinfected and grown at 25°C under luminosity of 36.81 ± 2.58 µmol of photons.s^{-1}.$m^{2\ -1}$ and photoperiod of 12:12 (light: dark).

Experimental conditions

The experiments were performed on flasks (1,000 mL) containing 1 L of ASM-1 sterile medium. Three types of treatments were defined: *M. aquaticum* + *M. aeruginosa* (MMa), *M. aeruginosa* (Control), and artificial plants + *M. aeruginosa* (AMa).

In each flask, the MMa and AMa treatments, two *M. aquaticum* with 40 ± 2 cm in length of the submerged part and plastic plants, respectively, were used. The length of natural and artificial plants was standardized. In addition, the choice of artificial plants was based on the morphology that was most similar to *M. aquaticum*. All the treatments were inoculated with *M. aeruginosa* inoculum, corresponding to an initial density of $1x10^6$ cells mL^{-1}. The systems were kept under

luminosity and photoperiod at the same culture conditions described early, for seven days. For each treatment, three replicates were used.

Cyanobacteria growth

M. aeruginosa growth was evaluated by cellular counting in a Neubauer chamber, withdrawing aliquots (10 µL) every 24 hours. The inhibition rate (IR) was calculated according to Equation 1 (Cheng et al., 2008).

$$IR = [1 - N_0 \times N^{-1}) \times 100 \quad (1)$$

Where:
N = cell density in the MMa or AMa flask;
N_o = cell density in Control.

Orthophosphate analysis

The influence of this limiting nutrient on *M. aeruginosa* growth was evaluated by the quantification of total orthophosphate by the method of digestion using potassium persulfate (Valderrama, 1981). A calibration curve was prepared from monobasic potassium phosphate solutions at concentrations of 0.025; 0.05; 0.1; 0.15; 0.2; 0.25; 0.5; 1; 2; 4, and 8 mg mL^{-1} (linear regression: y = 0.0506x + 0.2213; r = 0.9819). To compare the values of orthophosphate in a non-consumable system and establish the influence of macrophyte alone, the analyses were also carried out in flaks with an artificial plant (AP) and with *M. aquaticum* only, without *M. aeruginosa* (M).

Photosynthetic pigments

Chlorophyll-α and phycobiliprotein concentrations were evaluated after the period of exposure. For chlorophyll-a analysis, aliquots (6 mL) were withdrawn from the samples, and they were centrifuged at 10,500 rpm (Hitachi) for 4 minutes. The supernatant was discarded, and 2 mL of acetone 80% (v/v) was added to the cellular pellet, homogenized, and refrigerated in the dark for 24 hours to extract the pigments. The liquid was homogenized and centrifuged again before spectrophotometric analysis (UV-1800, Shimadzu) at 646 and 663 nm. The chlorophyll-α concentration was calculated by Equation 2 (Lichtenthaler and Wellburn, 1983).

$$\text{Chlorophyll (mg } L^{-1}) = 12.21(A_{663}) - 2.81(A_{646}) \quad (2)$$

Where:
A_{663} = absorbance at 633 nm;
A_{646} = absorbance at 646 nm.

The phycobiliprotein analysis was performed using aliquots (10 mL) of the liquid sample that were withdrawn, and two cycles of freezing/thawing were carried out for cell lysis and release of pigments in the medium. After that, the samples were centrifuged, and the supernatant liquid was spectrophotometrically analyzed at 565 nm for phycoerythrin (PE), at 620 nm for phycocyanin (PC), and at 650 nm foro allophycocyanin (APC). The quantification was performed according to Equations 3 to 6 (Chapman and Kremer, 1988).

$$PC \text{ (mg } mL^{-1}) = [A_{620} - (0.72 \times A_{650})] / 6.29 \quad (3)$$

$$APC \text{ (mg } mL^{-1}) = [A_{650} - (0.191 \times A_{620})] 5.79 \quad (4)$$

$$PE \text{ (mg } mL^{-1}) = \{A_{565} - [(2.41 \times PC) - (1.41 \times APC)]\}/13.02 \quad (5)$$

$$\text{Total Phycobiliproteins, TP (mg } mL^{-1}) = PC + APC + PE \quad (6)$$

Where:
A_{620} = absorbance value at 620 nm;
A_{650} = absorbance value at 650 nm;
A_{665} = absorbance value at 665 nm.

Microcystin-LR analysis

The analyses were performed in a liquid chromatographic system (Prominence, Shimadzu) equipped with a quaternary pump (LC-2AT), degasser unit (DGU-20A), oven (CTO-20A), diode array detector (SPD-M20A), and a controller unit (CBM-20A) operated by the LC Solutions software. The separation was obtained on an analytical column with a polymeric octadecyl stationary phase (Xterra, Waters, 150 x 3 mm d.i., 3.4 µm of particle size). The quantification of microcystin-LR was conducted at 238 nm by linear regression (y = 322.481 x – 32832; r = 0.9998) of a validated method developed in our research group (Torres et al., 2020).

The samples (50 mL) were withdrawn on the last experimental day and submitted to three cycles of freezing-thawing for the cellular disruption and release of their content in the liquid medium. Then, the samples (40 mL) were centrifuged (8,000 rpm for 20 minutes at 10°C) to remove cell debris, and the supernatant was used in the pre-concentration procedure by solid-phase extraction (SPE). The C18 cartridges (1,000 mg, Applied Separations) were conditioned with 10 mL of methanol (HPLC grade, J.T. Baker) and 10 mL of ultrapure water (Mega UP, Megapurity), followed by 10 mL of the sample. The cartridge was dried for one minute. The cleanup was carried out by applying 10 mL of water and the elution with 10 mL of methanol. The eluate was completely evaporated and dissolved in 1 mL of water.

To evaluate whether the presence of *M. aquaticum* reduced the concentration of microcystin-LR by inhibiting growth or it directly affected the production of cyanotoxin, the concentration of microcystin-LR per cell was calculated. For this, the Equation 7 was used:

$$MCLR\ per\ cells = \frac{MCLR}{Cell\ No.} \quad (7)$$

Where:
MCLR = the concentration of microcystin-LR on the last experimental day;
cell No. = the cell concentration obtained on the last experimental day.

Data analysis

Data were expressed as the mean of three independent replicates. Data were submitted to Shapiro-Wilk normality, Levene's test for homogeneity, and evaluated by one-way ANOVA. The means were then compared by the Tukey test ($P < 0.05$). The factors which contribute the most to the inhibition rate of *M. aeruginosa* were analyzed by the Pearson correlation analysis with photosynthetic pigments and microcystin-LR. The analyses were carried out with software R, version 3.5.0 (R Core Team, 2018), and a significant level $p < 0.05$ was considered.

Results and Discussion

Inhibitory effects of *Myriophyllum aquaticum* on *Microcystis aeruginosa* growth

The presence of *M. aquaticum* significantly interfered with *M. aeruginosa* cellular growth when compared to the Control during the entire exposure duration ($F_{(2,6)} = 1225.9$; $p < 0.001$, Figure 1A; Figure 2) with a marked decline in cellular concentration from the third day. There was no observed significant difference between the Control and the treatments using artificial plants (AMa), demonstrating that there was no influence of space competition in the observed effect. Expressing these results in percent inhibition values, the rates obtained reached 100% on the seventh experimental day for MMa ($F_{(2,6)} = 551.3404$, $p < 0.001$, Figure 1B). On the other hand, when considering AMa, no inhibition of *M. aeruginosa* was observed. Qian et al. (2018) observed inhibitory effects on *Microcystis aeruginosa* growth in a co-culture with a submerged macrophyte (*Pontederia cordata*), as observed in our work, in which we reached 100% inhibitory effects. These results can corroborate the influence of submerged macrophytes to inhibit cyanobacteria cell growth.

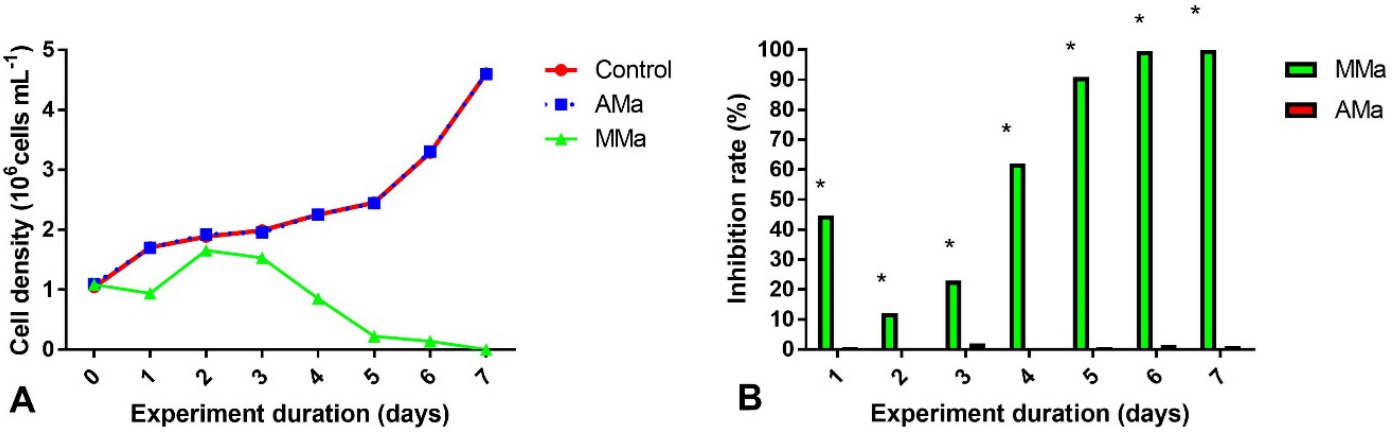
The interference of light and space with the inhibition effects was evaluated using artificial plants in independent flasks with the same experimental conditions and no effects on cyanobacteria growth were observed (Figures 1A and 2). The concentration of orthophosphate was measured to verify the influence of nutrients on the observed effect. Ferreira et al. (2018) showed that orthophosphate is an essential and limiting nutrient to cyanobacteria growth. However, we do not see the nutritional limitation of plants on cyanobacteria growth since they were not different between the Control group and *M. aquaticum* (MMa) in orthophosphate concentrations (Figure 3). Moreover, there was no correlation between total orthophosphate and the rate of cell density growth ($r = 0.3239$; $p = 0.3967$).

Effects on photosynthetic pigments of *Microcistys aeruginosa*

Chlorophyll-*a* (Figure 4) and the accessory pigments (phycoerythrin, phycocyanin, and allophycocyanin) were significantly reduced ($p < 0.0001$, Table 1) in *M. aeruginosa* grown in the presence of macrophytes. A strong correlation was observed between cellular growth and pigments ($r = 0.999$; $p < 0.0001$), with decreasing cell growth being reflected in reduced pigment concentrations. No significant effects ($p > 0.05$) of artificial plants were observed on *M. aeruginosa* pigments.

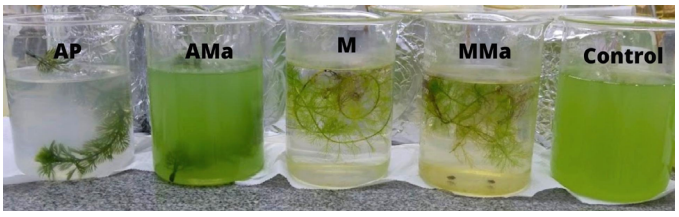
Wang et al. (2017a) observed that growth inhibition of *M. aeruginosa* and *Anabaena flos-aquae* strains was associated with allelochemicals being released by *M. aquaticum*. Similarly, Cheng et al. (2008) reported that *M. aquaticum* could secrete some allelochemicals in the medium, which reduces *M. aeruginosa* growth. The principal effects of allelochemicals secreted by *M. aeruginosa* were the inhibition of superoxide dismutase enzyme activity (leading to oxidative stress), in addition to chlorophyll-α reduction, as also observed in our study (Figure 3).

The main bioactive substances reported in the *Myriophyllum* genus are phenolic compounds (Zhu et al., 2010; Zhu et al., 2021). These macrophytes can produce allelochemicals as (+)-Catechin, caffeic acid, ellagic acid, gallic acid, nonanoic acid, pyrogallol, tellimagrandin II,



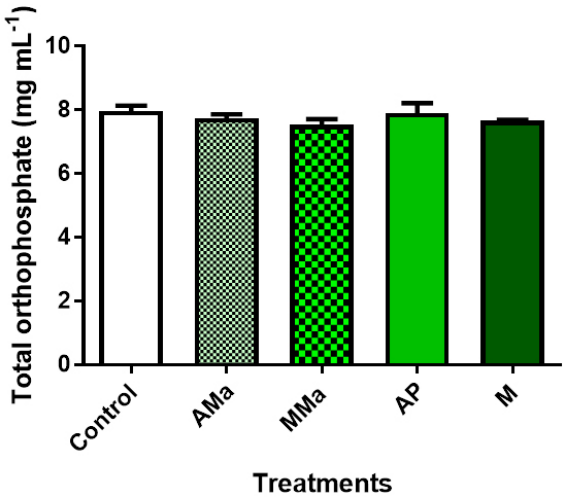
Control: only *M. aeruginosa*; AMa: artificial plants + *M. aeruginosa*; MMa: *M. aquaticum* + *M. aeruginosa*. The bars represent means ± SD of three replicates; *a significant difference considering $p < 0.05$ by ANOVA followed by the Tukey test.

Figure 1 – Inhibition effect on *Microcystis aeruginosa* cellular growth during exposure to *Myriophyllum aquaticum*. (A): Effect on cell density; (B): percentual inhibition.



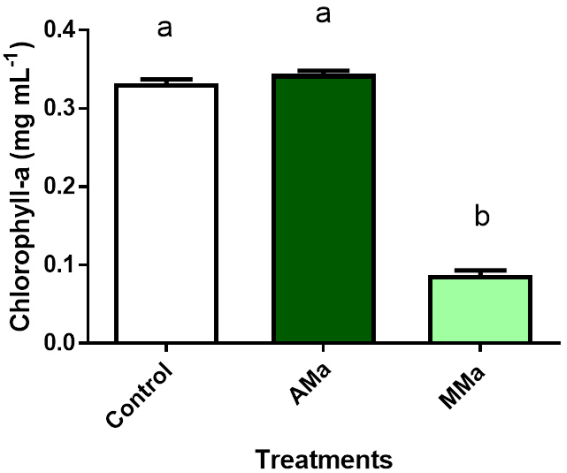
AMa: artificial plants + *M. aeruginosa*; MMa: *M. aquaticum* + *M. aeruginosa*; AP: artificial plants; M: *M. aquaticum*.

Figure 2 – Representative image of the bioassay on the last experimental day.



AMa: artificial plants + *M. aeruginosa*; MMa: *M. aquaticum* + *M. aeruginosa*; AP: artificial plants; M: *M. aquaticum*. The bars represent the means ± SD of three replicates.

Figure 3 – Orthophosphate concentration in the culture medium on the last experimental day.



Control: only *M. aeruginosa*; AMa: artificial plants + *M. aeruginosa*; MMa: macrophytes + *M. aeruginosa*. The bars represent the means ± SD of three replicates. Different letters indicated a significant difference considering $p < 0.05$ by the ANOVA test followed by the Tukey test.

Figure 4 – Chlorophyll-α concentration after the exposure duration (7 days).

Table 1 – Concentration of accessory photosynthetic pigments of *Microcystis aeruginosa* after a co-culture experiment (7 days) with *Myriophyllum aquaticum* (n = 3)*.

Treatments	Parameters		ANOVA		
	Phycocyanin	% of reduction	Df	F	p
Control (a)	0.0251 ± 0.0014 [c]	-	2	1,304.8	< 0.0001
AMa (b)	0.0252 ± 0.0016 [c]	-0.43			
MMa (c)	0.0004 ± 0.00001 [a,b]	98.05			
	Allophycocyanin	% of reduction	Df	F	p
Control (a)	0.0341 ± 0.0017 [c]	-	2	1,609.3	< 0.0001
AMa (b)	0.0342 ± 0.0016 [c]	-0.39			
MMa (c)	0.0005 ± 0.00000 [a,b]	98.04			
	Phycoerythrin	% of reduction	df	F	p
Control (a)	0.0139 ± 0.0007 [c]	-	2	1,690.7	< 0.0001
AMa (b)	0.0139 ± 0.0006 [c]	-0.38			
MMa (c)	0.0002 ± 0.00000 [a,b]	98.48			
	Total phycobiliproteins	% of reduction	df	F	p
Control (a)	0.0731 + 0.0035 [c]	-	2	873.77	< 0.0001
AMa (b)	0.0734 + 0.0035 [c]	-0.40			
MMa (c)	0.0012 + 0.00004 [a,b]	98.30			

*Different letters indicate a significant difference ($p < 0.05$) by the ANOVA test followed by the Tukey test; Control: only *M. aeruginosa*; AMa: artificial plants + *M. aeruginosa*; MMa: *M. aquaticum* + *M. aeruginosa*.

phenylpropanoid glucosides, and hydrolyzable tannins (Zhu et al., 2021). Nakai et al. (2012) observed that *M. spicatum* can release five polyphenols (catechin, eugeniin, gallic, pyrogallic, and ellagic acids) and three fatty acids in culture media and these allelochemicals were responsible for the inhibition of *M. aeruginosa* cell growth. The interactive effects of polyphenols and fatty acids can contribute to the effective allelopathic effects of this plant and increase the efficiency of cyanobacteria control (Gross et al., 1996). Leu et al. (2002) and Gross (2003) also demonstrated that tellimagrandin II produced by *Myriophyllum spicatum* has deleterious effects on cell growth and interferes with the photosynthetic apparatus — which can be associated with decreased photosynthetic pigments as observed in the present study (Table 1).

In cyanobacteria, the phycobilisomes concentrate the reactions that convert solar energy into high-energy electrons in photosystem II. They are composed of accessory pigments, mainly phycoerythrin, phycocyanin, and allophycocyanin, as well as chlorophyll-α (McColl 2018). Interferences in these pigments can promote reduced photosynthetic performance of cyanobacteria, which highlights the importance of these analyses. Moreover, there is another piece of evidence of the effects caused by the allelopathic substances released by macrophytes.

Cheng et al. (2008) used only the liquid media in which *M. aquaticum* had been developed and observed similar effects of reduced photosynthetic pigment concentrations.

Phenolic substances isolated from *Myriophyllum spicatum,* such as tellimagrandin II, affected photosystem II when in contact with cyanobacteria, degrading accessory pigments and causing cell death by inhibiting enzymes such as alkaline phosphatase (Leu et al., 2002). Zhu et al. (2010), when applying catechins, gallic, and ellagic acids extracted from *M. spicatum*, verified the effect on photosystem II of *M. aeruginosa,* reducing electron transduction. These phenolic compounds can cause isolated and synergic effects, mainly pyrogallic and gallic acid. The synergic effects can explain the macrophytes' efficiency in inhibiting cyanobacteria growth, reducing photosynthetic concentrations of the pigment, and affecting mainly non-photochemical quenching. These parameters efficiently screen the effects of allelochemicals to inhibit cyanobacteria isolated or in combined modes (Huang et al., 2020).

Allelopathic application is a promising strategy for cyanobacteria control, especially because this technique is inspired by natural phenomena. The use of plants and isolated allelochemicals is recommended and studies show the effects as interfering with photosynthesis, generating oxidative stress, and the possibility of causing other disturbances (Zhu et al., 2021).

Effects on the concentration of intracellular microcystin-LR

After seven days of exposure, intracellular microcystin-LR concentration was significantly decreased in the growth media by the presence of *M. aquaticum* ($F_{(2,6)} = 38.14$; $p = 0.0002$, Table 2). On the other hand, it was not affected by the presence of artificial plants ($F_{(2,6)} = 38.19$; $p > 0.05$, Table 2). The same effects were observed for the concentration of microcystin-LR produced per cell in the presence of *M. aquaticum* ($F_{(2,6)} = 50.27$; $p < 0.0001$, Table 2).

Table 2 – Total intracellular microcystin-LR and microcystin-LR produced per cell concentration after the exposure duration (7 days; n = 3) of *M. aeruginosa* to *M. aquaticum.**

Treatments	Parameters		ANOVA		
	Microcystin-LR	% of reduction	Df	F	P
Total Microcystin-LR (µg.L^{-1})					
Control (a)	0.073 ± 0.001^{c}	-	2	38.14	0.002
AMa (b)	0.071 ± 0.01^{c}	3.329%			
MMa (c)	$0.015 \pm 0.011^{a,b}$	78.990%			
Microcystin-LR per cell (ng.L^{-1})					
Control (a)	16.034 ± 0.552^{c}	-	2	50.27	< 0.001
AMa (b)	15.541 ± 2.808^{c}	2.710%			
MMa (c)	$0.015 \pm 0.011^{a,b}$	99.904%			

Different letters indicate a significant difference ($p < 0.05$) by the ANOVA test followed by the Tukey test.

Besides inhibiting *M. aeruginosa* growth, total intracellular microcystin-LR removal (79%) was observed, as well as removal of the microcystin-LR produced per cell (> 99%). In a review of the interactions among compounds with allelopathic properties produced by macrophytes and cyanobacteria, Mohamed (2017) explained that in addition to controlling cell density, the macrophytes had the potential for phytoaccumulation and phytotransformation of cyanotoxins with potential metabolization and reduced toxicity, indicating that these species can be used to mitigate the impacts of harmful algal blooms. Besides, it is known that methanolic extracts of *M. aquaticum* corroborate to reduce microcystin-LR concentration and *M. aeruginosa* cell growth (Kitamura et al., 2021). In addition to the possible capacity of phytoremediation, the present results of microcystin-LR produced per cell demonstrated that allelopathic mechanisms could interfere in the production of cyanotoxins by cyanobacteria and more studies should be conducted to understand the possible mechanisms involved.

Pflugmacher et al. (2015) described the performance of a combination of live submerged macrophytes *Ceratophyllum demersum*, *Elodea canadenses*, and *Myriophyllum spicatum* and they showed that microcystins (LR, RR, and YR) were reduced from 67 to 85%. Calado et al. (2019) observed that an experiment using *Egeria densa, Ceratophyllum demersum,* and *Myriophyllum aquaticum* removed 100% of microcystin-LR after three days. These studies contributed to the understanding that microcystin-LR can be effectively removed and corroborated the hypothesis that phytoremediation can occur in addition to allelopathic effects, which makes the use of aquatic macrophytes in the control and remediation of harmful algal blooms even more beneficial.

Finally, the present work indicated the allelopathic effects of *Myriophyllum aquaticum* in inhibiting *M. aeruginosa* growth, causing effects on the photosynthetic apparatus. It is efficient to reduce microcystin-LR concentrations. These results can encourage the use of macrophytes in water remediation treatments for harmful *Microcystis* blooms.

Conclusions

The inhibitory effect of *Myriophyllum aquaticum* on the cellular growth of *Microcystis aeruginosa* was observed without interferences on nutrients and space restriction, demonstrating that the observed inhibition did not occur by competition but by allelopathy mechanisms. Concerning the reduction of photosynthetic pigments and microcystin-LR (total and produced per cell), the results demonstrated that the application of submerged macrophytes species is a powerful and promising tool to remediate harmful algal blooms and can be considered an efficient nature-based solution.

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