Journal of Plant Biology (2021) 64:501–510
https://doi.org/10.1007/s12374-021-09322-5

RESEARCH ARTICLE

Allelopathic Inhibition Effects of *Myriophyllum spicatum* on Growths of Bloom-Forming Cyanobacteria and Other Phytoplankton Species in Coexistence Experiments

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Received: 6 April 2021 / Revised: 17 May 2021 / Accepted: 24 May 2021 / Published online: 21 June 2021

Abstract
Many studies have attempted to find measures for control of cyanobacterial harmful algal blooms (cyanoHABs) caused by *Microcystis*, *Anabaena*, and other bloom-forming plankton species. We have investigated allelopathic inhibition of the submerged macrophyte *Myriophyllum spicatum* on four phytoplankton species of two taxonomic groups: Chlorophyta *Selenastrum capricornutum*, *Scenedesmus obliquus*, and cyanobacteria *Microcystis aeruginosa* (different strains for toxic, non-toxic, the North Han River originated (NHR) and colonies) and *Anabaena circinalis*. Inhibitions of unicellular cyanobacteria *M. aeruginosa* were over 50% for three consecutive days from the 3rd to the 5th day of the coexistence. *M. spicatum* even inhibited *M. aeruginosa* at a high initial concentration (1.1 mg L^{-1} Chl-*a*). Moreover, *M. aeruginosa* in a mixture of four phytoplankton species (*S. capricornutum*, *S. obliquus*, *M. aeruginosa* and *A. circinalis*) was selectively inhibited by *M. spicatum*. The inhibition of toxic, non-toxic, and NHR of *Microcystis* by *M. spicatum* were not significantly different. Colonial cyanobacteria strains were mostly not inhibited by *M. spicatum*.

Keywords Allelopathy · *Myriophyllum spicatum* · *Microcystis aeruginosa* · Harmful algal bloom · Cyanobacteria · Phytoplankton

Introduction

Many freshwater ecosystems are becoming eutrophic due to rapid industrialization and urbanization, leading to severe ecological and economic damage (Huisman et al. 2018; Shin et al. 2018). Many studies have been performed to find measures for control of cyanobacterial harmful algal blooms (cyanoHABs) such as blooms caused by *Microcystis*, *Anabaena*, and other bloom-forming plankton species.

Due to ongoing need for development of species-specific or selective control methods to reduce damage on the environment, scientists are now attempting to use aquatic, especially submerged, vascular plants to control cyanoHABs (Hilt and Gross 2008; Kim et al. 2008; Jančula and Maršálek 2011; Kwon et al. 2012; Huisman et al. 2018). Inhibition of phytoplankton by macrophyte has been well known and various mechanisms have been suggested, such as shading, lower temperatures, nutrient competition, and allelopathy (Molisch 1937; van Donk et al. 2002; Gross 2003; Hilt and Lombardo 2010; Zhu et al. 2010; He et al. 2016).

Among submerged macrophytes, *Myriophyllum* have been widely investigated for their allelopathic interactions with algae and/or cyanobacteria (Planas et al. 1981; Gross and Süfeld 1994; Gross 1999, 2000; Nakai et al. 2000, 2005,2012; Körner and Nicklisch 2002; Leu et al. 2002; Nam et al. 2008; Bauer et al. 2009; Hilt and Lombardo 2010; Zhu et al. 2010; Švanys et al. 2014; He et al. 2016). In particular, it is known that *M. spicatum* produces several polyphenol compounds and fatty acids that inhibit growth of *M. aeruginosa* (Gross 2000; Nakai et al. 2000, 2005, 2012; Leu et al. 2002).

In a previous study, Joo et al. (2007) surveyed submerged macrophytes in 21 reservoirs in South Korea. Afterwards,

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M. spicatum and *Hydrilla verticillata* were identified as possible candidates for releasing allelochemical substances that diminish phytoplankton growth. Subsequently, coexistence experiments of Nam et al. (2008) have found that *M. spicatum* could significantly ($p < 0.05$) inhibit the growth of *M. aeruginosa*. Additionally, a study of Körner and Nicklisch (2002) showed inhibitory effects of various species of green algae, diatom, and cyanobacteria by coexistence experiments with *M. spicatum*, and He et al. (2016) demonstrated programmed cell death of *M. aeruginosa* influenced by co-culturing with *M. spicatum*.

Although various studies of submerged macrophyte allelopathy on phytoplankton have been conducted, it is inevitable to discuss fragmentary results since the experiments were conducted under different experimental conditions (Hilt 2006; Gross et al. 2007; Hilt and Gross 2008). Information about allelopathic mechanisms of macrophytes in freshwater ecosystem and the role of aquatic plants in water management is insufficient. To reveal the ecological relevance of submerged macrophytes with phytoplankton, it is necessary to attempt additional coexistence or mesocosm experiments and in situ research considering the dynamics of natural phytoplankton communities (Körner and Nicklisch 2002; Gross et al. 2007; Hilt and Gross 2008; Nam et al. 2008; Hilt and Lombardo 2010; Švanys et al. 2014).

In the present study, we compared allelopathic inhibition effects of various strains of green algae and bloom-forming cyanobacteria by conducting various sets of in vivo one-on-one coexistence experiments with *M. spicatum* L. The goal of this study was to determine selective inhibition effects on cyanobacterial strains by *M. spicatum*, and to elucidate the differences of inhibition effects depending on various forms (including wild colonies), concentrations, and strains of *M. aeruginosa*. The inhibition effects were quantified as changes in relative biomass (Chl-*a*).

Materials and Methods

Materials

We collected *M. spicatum* from a wetland of Tando Lake (37° 16′ 45″ N, 126° 37′ 27″ E) and the Gongreung stream (37° 41′ 14″ N, 126° 52′ 23″ E) located in Gyeonggi-do, South Korea. *M. spicatum* used in the first experiment set was collected from Tando Lake. After a winter season, the *M. spicatum* community disappeared and other aquatic plants such as *Potamogeton crispus*, *Najas graminea*, and *Trapa japonica* became dominant. Therefore, we changed the sampling site to the Gongreung stream for other experiments. After sampling, the plants were washed at the site and stored in a sample box with the water of the habitat then transferred to the laboratory. At the laboratory, plants were washed carefully with tap water using a soft brush to remove deposits and then rinsed with distilled water. Only the apical shoot parts (5 cm for the first coexistence experiment 'One-on-one Trials of Various Phytoplankton Species With *M. spicatum*', 8 cm for remains) were used for experiments.

Selenastrum capricornutum (UTEX 1648, the University of Texas Algae Culture Collection) and *Scenedesmus obliquus* (UTEX 383) were used for Chlorophyta. *Microcystis aeruginosa* (UTEX LB 2385; toxic, UTEX LB 2386; non-toxic, and NHSB 150,821; the North Han River originated strain, obtained from Konkuk Univ.) and *Anabaena circinalis* (obtained from the Konkuk University) were used for Cyanobacteria. The University of Texas Algae Culture Collection provided information on the toxicity of two *Microcystis* strains, UTEX 2385 and UTEX 2386. In addition, we measured microcystin-LR and microcystin-RR in these two *Microcystis* strains using HPLC–ESI–MS (Yang and Park 2017). The UTEX 2385 contained only microcystin-LR, while microcystin-LR and microcystin-RR were not detected in the UTEX 2386. We used the same strains of *Microcystis* as those used in the study of Yang and Park (2017). Modified triple Nitrate Bold Basal Medium (3NBBM) (Bold 1949; Stanier et al. 1971; Starr, 1993) was used to cultivate *S. obliquus* and *M. aeruginosa*. We used modified 1NBBM for *S. capricornutum* and modified 6NBBM for *A. circinalis*. We collected colonial *M. aeruginosa* from Seoho reservoir in Suwon, South Korea (where *M. aeruginosa* blooming usually occurs every year) and cultivated them in modified 3NBBM after filtering through 368 μm mesh and identified them using an optical microscope (×400, ×1000) based on Desikachary (1959) and Jung (1993). All strains were cultured in 2-L flasks with 1 L of fresh medium suitable for each species to ensure adequate supply of nutrients. All cultures were placed in a temperature-controlled chamber at 25 ℃ under fluorescent light 40 μmol photons m^{-2} s^{-1} at a light period of 16 h d^{-1} and grown to their exponential growth phase. Experiments were performed using high-temperature and high-pressure sterilized test vials (transparent glass, without lids) and liquid medium to minimize external contamination.

Coexistence Experiments

One-on-one Trials of Various Phytoplankton Species With *M. spicatum*

M. spicatum and four phytoplankton species of two taxonomic groups [Chlorophyta: *S. capricornutum* (average initial biomass (Chl-*a*): 395 μg Chl L^{-1}, SE ± 32) and *S. obliquus* (128 μg Chl L^{-1}, ± 28), Cyanobacteria: *M. aeruginosa* (UTEX LB 2385, 471 μg Chl L^{-1}, ± 18) and *A. circinalis* (550 μg Chl L^{-1}, ± 34)] were used for 7-day coexistence experiments. Each strain was diluted based on optical

density (OD) of 0.1 at 800 nm and then determined for chlorophyll *a* concentration.

Coexistence experiments (keeping the same *M. spicatum*) were conducted repeatedly every day using daily refreshed algal cultures and growth media for consecutive days of experiment periods (Fig. 1). The experiment periods were set to confirm the inhibitory reaction of unicellular *M. aeruginosa* for three consecutive days from the first day that significant inhibition effects began to appear (Nam et al. 2008). Each test vial (the initial of *M. aeruginosa*; $n=3$, remains; $n=5$) received 100 mL of fresh phytoplankton with medium and a 5-cm apical shoot of *M. spicatum* (dry weight 0.42 g L^{-1}, ±0.08 at the end of the experiments). The control group contained only 100 mL of phytoplankton and medium without a plant.

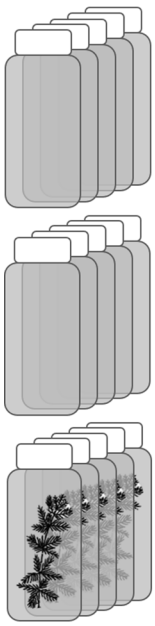
Aliquots of initial samples of each phytoplankton each day were kept for later Chl-*a* measurement. All phytoplankton strains (controls and the plant added groups) were refreshed daily (24 h) to minimize the impact of nutrient competition between *M. spicatum* and algal plankton. Coexistence experiments were conducted at 25 ℃ under fluorescent light with light intensity of 34 µmol photons m^{-2} s^{-1} at a 16 h d^{-1} light period. The test vials were gently shaken three times a day so that phytoplankton could be evenly distributed. To determine chlorophyll *a* concentrations, 50 mL of homogenized samples (*S. capricornutum*, *S. obliquus*, *M. aeruginosa*, and *A. circinalis*) was filtered through glass microfiber filters (1.2 µm pore size, Whatman GF/C). Afterwards, these filters were put into light blocking tubes (prerinsed with aqueous acetone) and stored at – 20 °C. Chlorophyll *a* content was then measured using a Turner Designs Trilogy Fluorometer according to EPA Method 445.0, without the acidification step (Welschmeyer 1994; Eaton et al. 2005). Chlorophyll *a* concentrations measured at the beginning (C_0) and after 24 h (C_n) with control (C_c) were used to calculate relative biomass (*RB*) (OECD Guidelines for the Testing of Chemicals 2011, #201), and inhibition (*I*) (Park et al. 2010; OECD 2011; Yuan et al. 2020) for each species each day using the following equations:

$$\mathrm{RB} = C_\mathrm{n}/C_0$$

$$I(\%) = \left(1 - C_\mathrm{n}/C_\mathrm{c}\right) \times 100$$

A Mixture of Cyanobacteria and Green Algae with *M. spicatum*

To determine the selective inhibition effects of *M. spicatum* on *M. aeruginosa*, a 4-day co-existence experiment was conducted using a mixture of *M. aeruginosa* (UTEX 2385), *A. circinalis*, *S. capricornutum** and *S. obliquus*. Each phytoplankton strain was prepared by diluting them to have an OD of 0.1 at 800 nm and then mixed in equal volume (25% for each). We used a modified method of coexistence experiment, which used 8 cm apical shoot of *M. spicatum* and 50 ml of the algal mixture for each test vial (0.28 g dw L^{-1}, ±0.02 at the end of the experiments). To



Co-existence Experiments		Used Strains	Experiment Period (day)
2.2.1	Various phytoplankton species with *M. spicatum*	*Selenastrum capricornutum* *Scenedesmus obliquus* *Microcystis aeruginosa* *Anabaena circinalis*	7
2.2.2	A mixture of green algae and cyanobacteria with *M. spicatum*	*S. capricornutum* + *S. obliquus* + *M. aeruginosa* + *A. circinalis*	4
2.2.3	Various strains of *M. aeruginosa* with *M. spicatum*	Toxic *M. aeruginosa* (UTEX 2385) Non-Toxic *M. aeruginosa* (UTEX 2386) NHR *M. aeruginosa* (Origin: the North Han River)	8
	Various forms of *M. aeruginosa* with *M. spicatum*	Unicells Colonies Cleaved-Colonies	5
2.2.4	Various concentrations of *M. aeruginosa* with *M. spicatum*	x0.1, x0.5, x1, x2	5
Supp. Fig. 1	Extended from *2.2.1*	*Parachlorella* sp. *Synechocystis* sp.	5 7

Fig. 1 Design of the co-existence experiments. Both unialgal cultures and medium used for all experiments were refreshed daily (24 h) measuring the initial and final chlorophyll *a* concentrations

determine chlorophyll *a* concentrations, 30 mL of homogenized algal samples was filtered using Whatman GF/C, and filtered volume was calibrated to calculate Chl-*a* content.

The next process of Chl-*a* analysis was the same as described in Sect. *2.2.1*. An extra step for cell densities was done in this chapter. An aliquot of the algal mixture from each vial was quenched everyday with Lugol's solution right before the algal mixture had been changed with a fresh one. The aliquot samples were then counted for cell densities to determine the inhibition effect of *M. spicatum* on each species.

One-on-one Trials of Various Stains and Forms of *M. aeruginosa* with *M. spicatum*

In this chapter, three different strains and three different forms of *M. aeruginosa* were used to see the differences on inhibitory reactions in coexistence experiments with *M. spicatum*.

The first experiment used UTEX LB 2385 (toxic), UTEX LB 2386 (non-toxic) and NHR strain (NHSB 150,821 originated from the North Han River) to compare inhibition effects of *M. spicatum* on toxic, non-toxic and a field originated (the North Han River in South Korea) strains of *M. aeruginosa*. In this experiment, we used 8 cm apical shoot of *M. spicatum* and 50 ml of unialgal cultures for each test vial (0.86 g dw $L^{-1}, \pm 0.03$ at the end of the experiments).

Next, unicellular, colonial and cleaved-colonial *Microcystis aeruginosa* strains were used for the experiment to elucidate the effects of gelatinous sheath of *Microcystis* when this blue-green algae coexisted with *M. spicatum*. Cleaved-colonial *M. aeruginosa* was prepared by sonication for 5 min with a Bioruptor UCD-200 (Diagenode). Cleaved colonies were each composed of three to five cells per colony. We used 8 cm apical shoot of *M. spicatum* and 50 ml of unialgal cultures for each test vial (0.47 g dw $L^{-1}, \pm 0.03$ at the end of the experiments).

To determine chlorophyll *a* concentrations, 30 mL of homogenized algal samples were filtered through Whatman GF/C, and the volume were calibrated later for Chl-*a* content calculation. Following steps of the Chl-*a* analysis were the same as those described in Sect. 2.2.1.

Various Concentration Gradients of *M. aeruginosa* with *M. spicatum*

A series of coexistence experiments were conducted using four different OD levels of *M. aeruginosa* to see its inhibitory effects on cyanobacteria at various concentrations. *Microcystis aeruginosa* (UTEX 2385) was diluted to 0.2 OD (1081 µg Chl $L^{-1}, \pm 13.8$), 0.1 OD (592 µg Chl $L^{-1}, \pm 7.1$), 0.05 OD (330 µg Chl $L^{-1}, \pm 4.1$) and 0.01 OD (71 µg Chl $L^{-1}, \pm 1.6$) at 800 nm. With the reference concentration set as 0.1 OD, OD values shown above were described as 2 times ($\times 2$), $\times 1$, $\times 0.5$, and $\times 0.1$ of the reference concentration, respectively. We used 8 cm apical shoot of *M. spicatum* and 50 ml of each diluted culture for a test vial (0.86 g dw $L^{-1}, \pm 0.03$ at the end of the experiments). To determine chlorophyll *a* concentrations, 30 mL of homogenized algal samples was filtered using Whatman GF/C, and filtered volume was calibrated later to calculate Chl-*a* content. Chlorophyll *a* analysis was performed by the same workflow shown in Sect. *2.2.1*.

Statistical Analysis

All results were expressed as mean values with ± standard error (SE). Data were assessed for normality (Shapiro–Wilk test) and homogeneity of variance (Bartlett test and *F* test) at the 0.05 significance level. Significant differences among experimental results were determined by one-way analysis of variance (ANOVA) with subsequent post hoc analysis by Tukey's HSD tests and non-parametric Kruskal–Wallis tests with subsequent Bonferroni tests at $p < 0.05$. To compare the control groups (without *M. spicatum*) and the treated groups (with *M. spicatum*), Student *t* test was applied for parametric data at 0.05 significance level. When non-homogeneity of variance was found, Welch's *t* tests were applied, and Wilcoxon–Mann–Whitney tests were applied for non-parametric data at 0.05 significance level. All statistical analysis were processed using R program (4.0.3).

Results

One-on-one Trials of Various Phytoplankton Species with *M. spicatum*

Growths (relative biomass) of four different phytoplankton species of two taxonomic groups (Chlorophyta: *S. capricornutum* and *S. obliquus*, Cyanobacteria: *M. aeruginosa* (UTEX LB 2385) and *A. circinalis*) coexisted with *M. Spicatum* for 7 days are shown in Fig. 2. Since the results are relative to the initial concentration (Chl-*a*) of each phytoplankton species (per 1 day), only *M. aeruginosa* (the white-colored bars) appeared to be strongly suppressed by coexisting with *M. spicatum*. Inhibition effects of *M. spicatum* on *M. aeruginosa* began on the 4th day of the coexistence (one-way ANOVA and Tukey's HSD, $F = 42.3$, $p < 0.001$) and the inhibition appeared consecutively until the end of the experiment (day 4 to day 7). Two species in green algae, *S. capricornutum* and *S. obliquus*, and a cyanobacterial species *A. circinalis* maintained the general growth phase with slight changes. Additional results of subsequent coexistence experiments using two different algal species with *M. spicatum* showed that green algae *Parachlorella* sp. was not strongly influenced by allelopathic effects

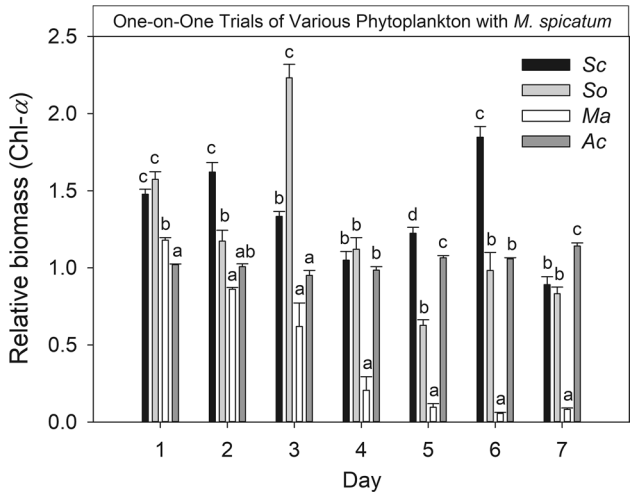


Fig. 2 Chlorophyll *a* based relative biomass of two phytoplankton species (*Selenastrum capricornutum* (*Sc*) and *Scenedesmus obliquus* (*So*)) and two cyanobacteria species (*Microcystis aeruginosa* (*Ma*) and *Anabaena circinalis* (*Ac*)) from individual coexistence experiments with *M. spicatum* (*Ms*). Error bars show standard errors ($n=5$). Different small letters indicate significant differences among the four strains assessed by one-way analysis of variance (ANOVA) with subsequent post-hoc analysis (Tukey's HSD, $p<0.05$)

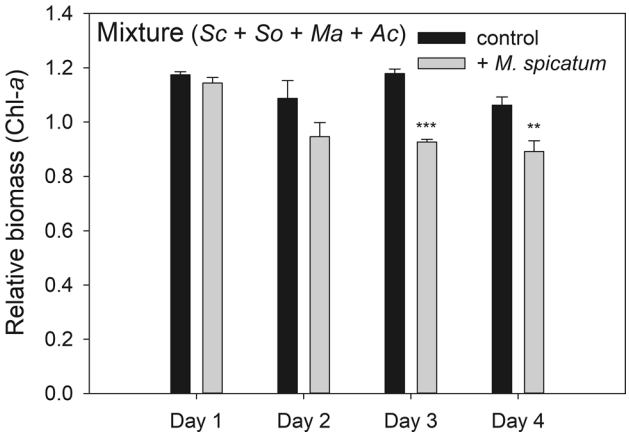


Fig. 3 Relative biomasses (chlorophyll-*a*) of a mixture with four algal species (*S. capricornutum*, *S. obliquus*, *M. aeruginosa*, and *A. circinalis*) from the coexistence experiment with *M. spicatum*. Error bars show standard errors ($n=5$). Student *t* tests, Welch's *t* tests (when non-homogeneity of variance was found) and Wilcoxon-Mann–Whitney tests (for non-parametric data) were performed at 0.05 significance level, and differences between control (without *M. spicatum*) and *M. spicatum* coexisted group (+*M. spicatum*) for each day are indicated by asterisks

while cyanobacteria *Synechocystis* sp. was significantly inhibited for 3 consecutive days (Supplementary Fig. 1).

A Mixture of Cyanobacteria and Green Algae with *M. spicatum*

Relative biomass of an algal mixture of *S. capricornutum*, *S. obliquus*, *M. aeruginosa*, and *A. circinalis* during a 4-day coexistence experiment with *M. spicatum* are displayed in Fig. 3. Student *t* test and Welch's *t* test (for data with non-homogeneous variance) were done to compare relative biomass between controls (without *M. spicatum*) and the *M. spicatum* added groups on each day. All data were assessed for normality and homogeneity of variance, and Wilcoxon–Mann–Whitney tests were done for non-parametric data at 0.05 significance level. Differences in relative biomass between the controls and the *M. spicatum* added groups on day 1 and day 2 were insignificant ($p>0.05$) while there were significant differences on day 3 ($^{***}p<0.001$) and day 4 ($^{**}p<0.01$). Additionally, *M. aeruginosa* was indeed selectively inhibited (from day 2 to day 4) by *M. spicatum*, which did not appear to influence the other three plankton taxa in the mixture (Fig. 4).

One-on-one Trials of Various Stains and Forms of *M. aeruginosa* with *M. spicatum*

Inhibition effects of *M. spicatum* on three different strains of *Microcystis aeruginosa*, a toxin strain (UTEX 2385), a non-toxin strain (UTEX 2386), and the North Han River originated strain (NHR) are shown in Fig. 5. Relative biomass of the controls and the plant added groups were compared by Student *t* test and Welch's *t* test (for data with non-homogeneous variance). Toxic and non-toxic strains were significantly inhibited by allelopathic effects of *M. spicatum* beginning on the 6th and the 5th day of the experiments, respectively, and the inhibition effects were appeared consecutively until the last day of the experiment (day 8). While NHR strain showed significant difference only on day 7 (Fig. 5), results of one-way ANOVA with Tukey's post-hoc tests and non-parametric Kruskal–Wallis tests with Bonferroni tests comparing inhibitions (*I*s) of the three strains (day 6 to day 8, the days shown consecutive inhibition effects) showed no significant difference (Supplementary Fig. 2).

Differences in relative biomass of *M. aeruginosa* according to its various forms are displayed in Fig. 6. To see the differences between the controls and the *M. spicatum* added groups, Student *t* test and Welch's *t* test (non-homogeneity of variance) were applied after the assessment for normality and homogeneity of variance. Wilcoxon–Mann–Whitney tests were done for non-parametric data at 0.05 significance level. Unicellular *M. aeruginosa* strain (UTEX 2385) was significantly inhibited by *M. spicatum* beginning on the 3rd day and the inhibition effects appeared consecutively for 3 days (until day 5). *M. spicatum* did not influence colonial and cleaved-colonial strains (Fig. 6).

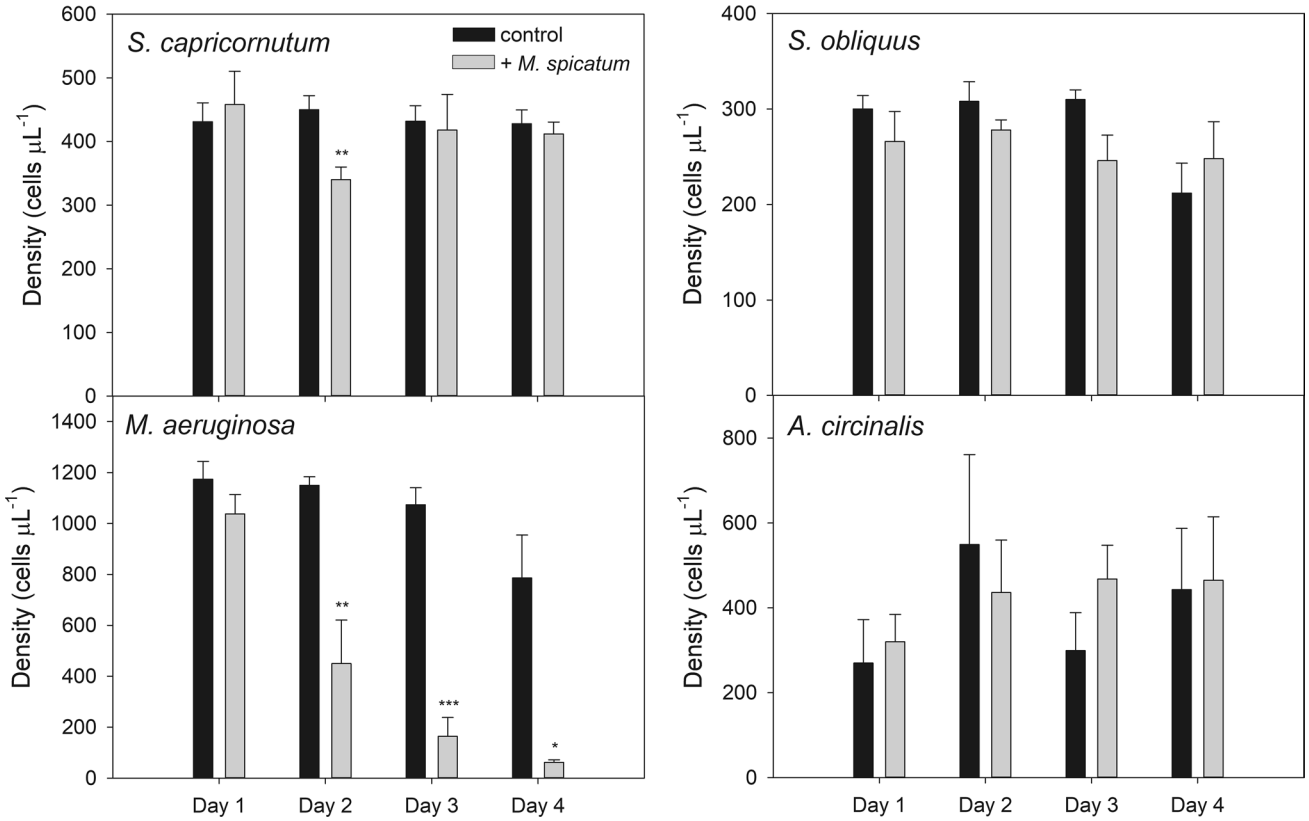


Fig. 4 Cell densities of the four algal species (*S. capricornutum*, *S. obliquus*, *M. aeruginosa*, and *A. circinalis*). Error bars show standard errors ($n=5$). Student t tests, Welch's t tests (when non-homogeneity of variance was found) and Wilcoxon-Mann–Whitney tests (for non-parametric data) were performed at 0.05 significance level, and differences between the control and the treated group on each day are indicated by asterisks

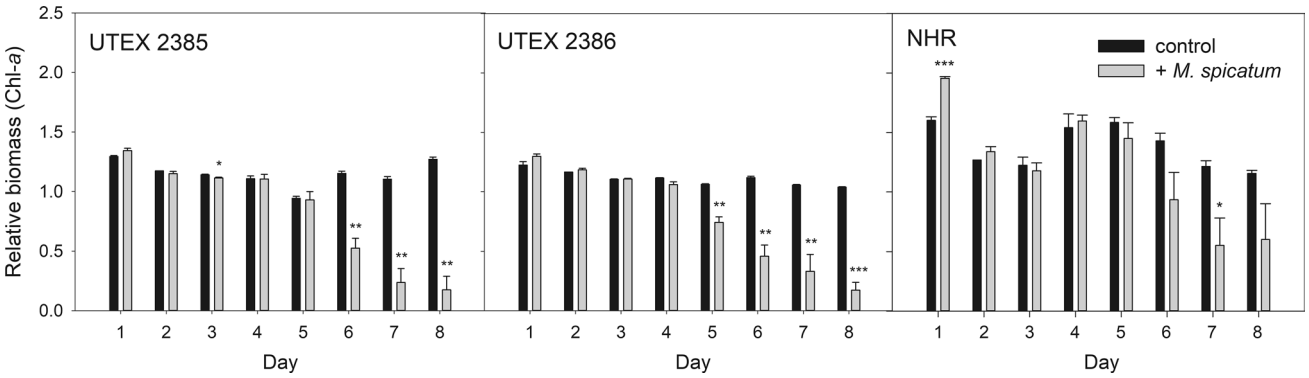


Fig. 5 Chlorophyll *a* based relative biomass of three different strains of *Microcystis aeruginosa* (toxin strain: UTEX 2385, non-toxin: UTEX 2386, the North Han River originated: NHR) from coexistence experiments with *M. spicatum*. Error bars show standard errors ($n=5$). Student t tests, Welch's t tests (when non-homogeneity of variance was found) and Wilcoxon-Mann–Whitney tests (for non-parametric data) were performed at 0.05 significance level, and differences between control (without *M. spicatum*) and treated group (with *M. spicatum*) on each day are indicated by asterisks

Various Concentration Gradients of *M. aeruginosa* with *M. spicatum*

Average relative biomass of each four different concentration levels of *M. aeruginosa* (UTEX 2385) coexisted with *M. spicatum* were compared with controls (without the plant) (Fig. 7). On the first day, only the lowest level concentration group (×0.1) began to be suppressed (Welch's t test,

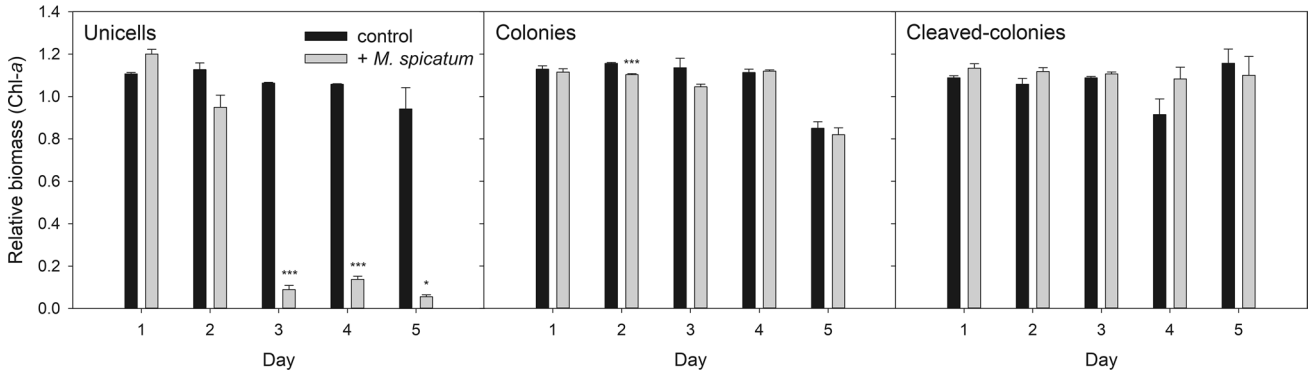


Fig. 6 Chlorophyll *a* based relative biomass of three different forms of *Microcystis aeruginosa* (unicells, colonies, cleaved-colonies) from coexistence experiment with *M. spicatum*. Error bars show standard errors (unicells: $n=3$, others: $n=5$). Student *t* tests, Welch's *t* tests (when non-homogeneity of variance was found) and Wilcoxon-Mann–Whitney tests (for non-parametric data) were performed at 0.05 significance level, and differences between the control and the treated group on each day are indicated by asterisks

$p=0.02$) and from the 2nd day, average relative biomass of all four different groups ($\times 0.1, \times 0.5, \times 1$ and $\times 2$) were lower than their controls. From the 3rd day to the last day (day 5), concentration groups of all levels showed significant differences consecutively for 3 days (Student *t* test, Welch's *t* test and Wilcoxon–Mann–Whitney test at 0.05 significance level). In the case of the $\times 1$ group, the average relative biomass was 0.02 on day 5.

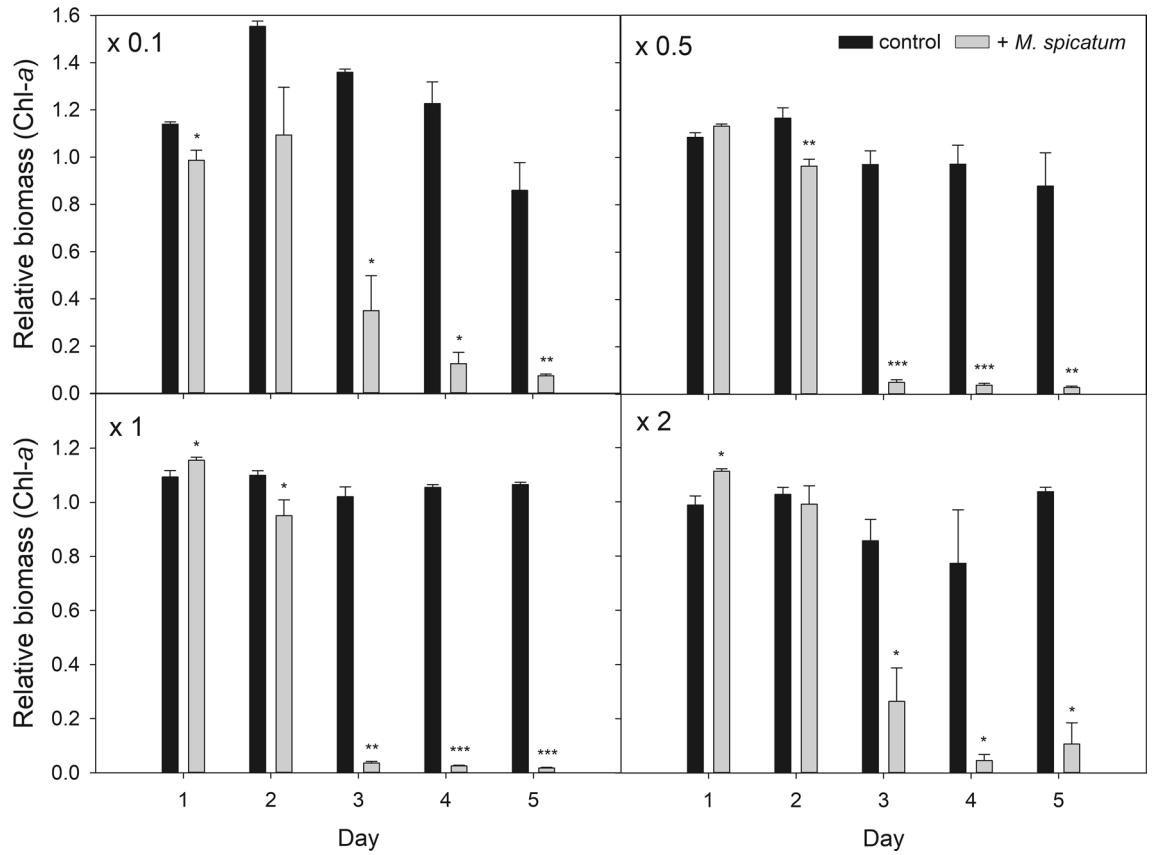


Fig. 7 Chlorophyll *a* based relative biomass of four different concentrations of *Microcystis aeruginosa* ($\times 0.1, \times 0.5, \times 1$ and $\times 2$) from co-existence experiment with *M. spicatum*. Error bars show standard errors ($n=5$). Student *t* tests, Welch's *t* tests (when non-homogeneity of variance was found) and Wilcoxon-Mann–Whitney tests (for non-parametric data) were performed at 0.05 significance level, and differences between control (without *M. spicatum*) and treated group (with *M. spicatum*) on each day are indicated by asterisks

Discussion

The present study indicates that *M. spicatum* has allelopathic inhibition effects on unicellular cyanobacteria, especially *M. aeruginosa* even when its initial concentration is very high (1081 μg Chl L^{-1}, ± 14), which is higher than those in previous studies (Nakai et al. 2000; Nam et al. 2008; Zhu et al. 2010; Chang et al. 2012; Švanys et al. 2014; He et al. 2016).

In our various coexistence experiments, *M. spicatum* showed high inhibition effects only on unicellular cyanobacteria beginning on the 3rd to 5th day of coexistence (Figs. 2, 6 and Supplementary Fig. 1). In addition, *M. aeruginosa* was selectively inhibited in a mixture of two green algal species and two cyanobacteria species (Fig. 4). Our results support many previous studies reporting that green algae and epiphytes were relatively insensitive to *M. spicatum* while *M. aeruginosa* and other cyanobacterial species were selectively suppressed (Jasser 1995; Nakai et al. 2000; Körner and Nicklisch 2002; Leu et al. 2002; Gross 2003; Hilt and Gross 2008; Nam et al. 2008; Zhu et al. 2010; Švanys et al. 2014; He et al. 2016). However, in a study of various proportions of mixture using green algae *Desmodesmus armatus* and cyanobacteria *M. aeruginosa* that coexisted with *M. verticillatum* (Chang et al. 2012), the results varied depending on the factors. In the present study, mixture experiment was unique in 4-day-coexistence-experiment using daily refreshed mixture of the four agal species (two Chlorophyta *S. capricornutum* and *S. obliquus*, two cyanobacteria *M. aeruginosa* and *A. circinalis*). We designed experiments to exclude nutrient competition and to confirm that the inhibitory effect appeared for at least 3 consecutive days (applied to all experiments in the present study). Since the inhibitory effects on unicellular cyanobacteria generally begins to appear on the 3rd to 6th day, it is considered that a sufficient experimental period is important to confirm allelopathic effects.

Inhibitory effects of *M. spicatum* on toxic and non-toxic *M. aeruginosa* in this study were congruent with a mesocosm study on *M. spicatum* (Švanys et al. 2014) except that colonial cyanobacteria were not inhibited by *M. spicatum* in present study while Švanys et al. (2014) detected that *M. spicatum* constantly suppressed growth of cyanobacteria in natural phytoplankton community. Such discrepancy might be due to a relatively very low initial concentration of cyanobacteria (27 μg Chl L^{-1}) used in their study compared to our colonial *M. aeruginosa* concentration (average initial Chl-*a* = 715 μg L^{-1}, ± 12) (Fig. 6). Likewise, *Anabaena circinalis* was not affected by the allelopathic inhibition effect of *M. spicatum*. *Anabaena* species are also known as mucilaginous filamentous cyanobacteria (Prasanna et al. 2006), supporting our results regarding *A. circinalis* (Fig. 2). Our interpretation is that allelochemicals produced by *M. spicatum* could not penetrate the mucilage of colonial *M. aeruginosa* and *A. circinalis*. Considering that cyanobacteria generally form colonies in a natural environment (Canter-Lund and Lund 1995; Latour et al. 2004; Yang and Kong 2012), further studies are necessary using various strains of harmful phytoplankton species and their outdoor conditions to investigate the ecological relevance of macrophyte allelopathy.

Although inhibitions were high for both unicellular *M. aeruginosa* and *Synechocystis* sp. (Figs. 2, 4 and Supplementary Fig. 1), subsequent experiments were conducted (Figs. 5, 6, 7) only for *M. aeruginosa*, a world-wide harmful cyanobacterium (Huisman et al. 2018). Reference concentration ranges of *M. aeruginosa* used in this study (OD 0.1 at 800 nm) were about 411–632 μg L^{-1} in terms of chlorophyll *a*. These concentrations were high enough to be judged as a 'major outbreak and serious stage' of South Korea's harmful algal warning system (Ministry of Environment Republic of Korea 2019). In addition, according to 'WHO 2003 Recreational Guidance/Action Levels for Cyanobacteria, chlorophyll *a*, and Microcystin' (EPA 2019), they were in the range of 'High' level among levels of Low, Moderate, High, and Very High. Even the lowest level of *M. aeruginosa* (approximately 71 μg Chl L^{-1}, ± 1.6) was in the level of 'High' according to the WHO 2003 Guidance for Cyanobacteria (EPA 2019).

Myriophyllum spicatum collection site was changed once due to the disappearance of the Eurasian watermilfoil cluster. However, in this study, the difference in collection season seems more important than the difference in collection site. *M. spicatum* used in the experiment for comparing various *Microcystis* strains (Fig. 5) and the experiment using *Synechocystis* sp. and *Parachlorella* sp. (Supplementary Fig. 1) were collected in May, which was the first season of fresh growth of *M. spicatum* after a winter period. Inhibition effects of *M. spicatum* collected in May began on the 5th to 6th day of coexistence experiments. However, *M. spicatum* used for other experiments were collected in June to September, and their inhibition effects were shown within 3–4 days of coexistence experiments. We speculate that reaction rate of *M. spicatum* to inhibit cyanobacteria would be slow when they are freshly grown in spring. Adams and McCracken (1974) have compared Eurasian watermilfoil (*M. spicatum*) by season and found that phosphorus component is high while biomass is low in spring after ice has melted. Spencer and Ksander (1999) indicated seasonal differences in plant quality of Eurasian watermilfoil. Specifically, total phenolics and tissue carbon levels are relatively low in December–May. Studies of seasonal dynamics of allelochemicals in *M. spicatum* (Goss 2000) and *M. verticillatum* (Bauer et al. 2009) also showed the seasonal differences in inhibition

effects. Lombardo et al. (2013) used both laboratory and field approaches to investigate macrophyte–phytoplankton interactions considering various factors including seasonal changes, and results in the study provided various implications. Santonja et al. (2018) have shown that concentrations of major chemical compounds in leaf extracts of aquatic plants are significantly low in spring ($p < 0.0001$). These previous studies suggest that we should consider seasonal variabilities in allelopathic interactions between aquatic macrophytes and phytoplankton. Extended long-term experiments in both laboratory and in situ are necessary to clarify factors influencing seasonal changes of allelochemicals, especially whether seasonal *Microcystis* concentration might act as an environmental factor.

There are many previous studies that have discussed nutrient competition and the shading effect in allelopathy of submerged macrophytes (Gross et al. 2007; Lombardo et al. 2013). We excluded the nutrient competition since the coexistence experiments were designed to eliminate nutrient limitation using algal cultures and growth media that were daily refreshed. However, there was a possibility that *M. spicatum* might have been affected by shading from concentrated phytoplankton during the coexistence experiment (Fig. 7). Nevertheless, we are unable to explain how much light intensity contributed to the current results. Additionally, in a similar vein to Lombardo et al. (2013), we considered that the intensity of allelopathic inhibition effects of the submerged macrophyte appear to be somewhat dependent on plant health condition (discolored leaves and/or losing leaves). Although the algal inhibition according to the biomass (dry weight) of *M. spicatum* was not clearly seen in our results, the growth rate and biomass change of macrophyte still need to be considered as an important factor in the study of allelopathic effects (Körner and Nicklisch 2002; Gross et al. 2007; He et al. 2016). We consider that these factors are a part to be studied further along with seasonal variability of allelopathic effects.

Several studies have suggested that *M. spicatum* might possess potential allelochemicals such as polyphenols and fatty acids (Gross et al. 1996; Nakai et al. 2000, 2012; Leu et al. 2002; Gross et al. 2007; Zhu et al. 2010). Based on this, extended research of the present study is needed on the identification of allelopathically active compounds and factors influencing their production from the macrophyte.

In conclusion, our research indicated that (1) growth of unicellular cyanobacteria (*M. aeruginosa* and *Synechocystis* sp.) was inhibited by submerged macrophyte *M. spicatum* in coexistence experiments using daily refreshed target algal cultures. Inhibitions of unicellular *M. aeruginosa* was over 50% for 3 consecutive days from the 3rd to the 6th day of the coexistence. (2) *M. aeruginosa* was selectively inhibited among the algal mixture of four different species (*S. capricornutum*, *S. obliquus*, *M. aeruginosa* and *A. circinalis*). (3) The inhibition effects on toxic, non-toxic and NHR strains of *M. aeruginosa* were not significantly different. However, mucilaginous *M. aeruginosa* and filamentous *Anabaena circinalis* were not influenced by *M. spicatum*. (4) *Microcystis aeruginosa* at high initial concentration level (1.1 mg/L Chl-*a*) was also suppressed by *M. spicatum*.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s12374-021-09322-5.

Acknowledgements This work was supported by the National Research Foundation of Korea [2016R1A2B4015235].

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