Journal of Oceanology and Limnology
Vol. 40 No. 5, P. 1804-1818, 2022
https://doi.org/10.1007/s00343-022-1393-x

Competitiveness of alga *Microcystis aeruginosa* co-cultivated with cyanobacterium *Raphidiopsis raciborskii* confirms its dominating position*

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Received Nov. 18, 2021; accepted in principle Jan. 7, 2022; accepted for publication Mar. 7, 2022

Abstract *Microcystis aeruginosa* has always been regarded as the main culprit of cyanobacterial blooms in freshwater. However, in recent years, *Raphidiopsis raciborskii* has gradually replaced *M. aeruginosa* as the culprit of cyanobacterial blooms in some tropical and subtropical shallow lakes. To reveal which one plays a more dominant role, interactions between cylindrospermospin (CYN)-producing *R. raciborskii* and microcystins (MCs)-producing or non-MCs-producing *M. aeruginosa* strains were studied using bi-algal cultures at different initial ratios of biomasses of the two species at 25 °C. During the co-cultivation, the *M. aeruginosa* strains inhibited the growth and heterocyst formation of *R. raciborskii* filaments, and thus occupied a dominant position during the co-cultivation regardless of the initial biomass ratios in the cultures. In addition, the MCs-producing *M. aeruginosa* strain contributed to a higher portion of the total biomass and exerted a stronger inhibitory effect on *R. raciborskii* compared with the non-MCs-producing strain. However, the growth of both MCs-producing and non-MCs-producing *M. aeruginosa* strains was stimulated by *R. raciborskii* in the co-cultures compared with *M. aeruginosa* monoculture, indicating that *M. aeruginosa* could outcompete *R. raciborskii* if given enough time, enabling it to develop into the dominant species even in very low initial concentration. To our best knowledge, this is the first report on the loss of heterocyst formation by a species of cyanobacteria that resulted from interactions between two different species of cyanobacteria. These findings indicate that it is difficult for *R. raciborskii* to replace the dominant position of *M. aeruginosa* under the experimental environmental condition, and the allelopathic effects of *M. aeruginosa* on *R. raciborskii* could significantly contribute to the success of *M. aeruginosa*.

Keyword: competition; growth; heterocyst; *Microcystis aeruginosa*; morphology; *Raphidiopsis raciborskii*

1 INTRODUCTION

Cyanobacterial harmful algal blooms (CyanoHABs) are almost a ubiquitous phenomenon in stagnant water worldwide (Huisman et al., 2018; Paerl, 2018). These algal blooms have presented a great environmental challenge because of their severe ecological impacts and associated health threats not only to humans but also to the many aquatic animals that inhabit the water (Olokotum et al., 2020; Amorim and Moura, 2021; Plaas and Paerl, 2021).

* Supported by the National Key R&D Program of China (No. 2018YFE0103700), the National Natural Science Foundation of China (Nos. 41876124, 61871293, 42007372), and the Zhejiang Provincial Natural Science Foundation of China (Nos. LZ21C030001, LQ20C030008, LD21C030001, LQ18C030002)
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Microcystis aeruginosa, *Raphidiopsis raciborskii*, *Dolichospermum flos-aquae*, *Aphanizomenon flos-aquae*, and *Planktothrix* sp. are the most successful CyanoHAB-forming species in many freshwater ecosystems (Padisák and Reynolds, 1998; Nixdorf et al., 2003; Hoeger et al., 2004; Paerl et al., 2011). Among them, *M. aeruginosa* is the most important and the blooms it causes are the most frequent and widely distributed algal blooms as well as being the largest scale worldwide (Harke et al., 2016). In addition, during the proliferation of *M. aeruginosa*, the odor compounds and microcystins (MCs) that it produces can lead to serious environmental disaster (Dokulil and Teubner, 2000; Dittmann and Wiegand, 2006; Zhang et al., 2010; Kim et al., 2020).

In recent years, *R. raciborskii* has become the second most concerning species in terms of the causation of CyanoHABs. *Raphidiopsis raciborskii* has a particularly broad growth temperature range (Briand et al., 2004; Chonudomkul et al., 2004; Soares et al., 2013), well suited to different light habitats (Pierangelini et al., 2014a, 2015b; Kovács et al., 2016) and inorganic carbon centration (Pierangelini et al., 2014b, 2015a). In addition, it is more competitive when phosphorus and nitrogen availability is low and/or variable availability (Amaral et al., 2014; Burford et al., 2014, 2018; Willis et al., 2015). Therefore, *R. raciborskii* also causes algal blooms globally, and there seem to be signs of *R. raciborskii* replacing *M. aeruginosa* as the main culprit of algal blooms in some tropical and subtropical shallow lakes (Chislock et al., 2014; Burford et al., 2016; Lei et al., 2020; Xiao et al., 2020). Therefore, *R. raciborskii* and *M. aeruginosa* are of particular concern and these two species of cyanobacteria have received widespread attention (Nixdorf et al., 2003; Tomioka et al., 2011).

Currently, *R. raciborskii* is well regarded as an invasive cyanobacterial species in natural waters (Hamilton et al., 2005; Stüken et al., 2006; Moreira et al., 2015), and has gradually become the dominant species in some tropical and subtropical lakes where *M. aeruginosa* used to be the dominant species (Saker and Griffiths, 2001; Moisander et al., 2012). According to the results of field investigation, *R. raciborskii* and *M. aeruginosa* usually coexist in the same bodies of natural water as dominant species (Rzymski et al., 2014; Jia et al., 2020). However, to our knowledge, there has been a lack of studies focusing on the interactions between these two species using a bi-algal cultivation system in the lab, and only non-MCs-producing strains of *M. aeruginosa* were used in the previous studies that investigated the competition for dominance between *R. raciborskii* and *M. aeruginosa* (Rzymski et al., 2014; Bai et al., 2020; Jia et al., 2020). In addition, it has been reported that the competition between *R. raciborskii* and *M. aeruginosa* can be affected by environmental factors such as temperature, light, and nutrients (Marinho et al., 2013; Soares et al., 2013; da Silva Brito et al., 2018). Furthermore, the biological invasion is a dynamic process from low-density cell introduction to high-density cell replacement of the dominant species, and the biomass of *R. raciborskii* and that of *M. aeruginosa* are almost unequal in every natural body of water that has been tested (Mallon et al., 2015; Sukenik et al., 2015). Therefore, the competition for dominance between *R. raciborskii* and *M. aeruginosa* as initiated by different biomass ratios are more suitable for simulating their competition in natural waters.

Compared with the non-MCs-producing *M. aeruginosa* strain, the MCs-producing strain exerts a higher inhibitory effect on other co-existing microalgae because of its MCs-producing ability, and therefore, it usually acquires a greater advantage in such competition for dominance (Li and Li, 2012; Lei et al., 2015; Ma et al., 2015). MCs are well-known toxicants that are primarily responsible for the inhibitory effects of *M. aeruginosa* strains, which can induce oxidative stress and eventually inhibit the photosynthesis and growth of their competitors (Campos et al., 2013; Yang et al., 2014; Kaur et al., 2019). Besides MCs, other compounds released by *M. aeruginosa* strains are found to be inhibitory to its competitors, and together with MCs, they can exert a synergistic effect (Campos et al., 2013; Yang et al., 2014). A few studies have demonstrated that *R. raciborskii* strains with the ability to synthesize cylindrospermopsin (CYN), and can rely on its contribution to enhance their competitiveness and extend their population size (Bar-Yosef et al., 2010; Sukenik et al., 2012). In addition, the CYN pool size of *R. raciborskii* is constitutive and not affected by light and CO_2 conditions, thus toxicity of blooms formed by *R. raciborskii* is determined by the absolute abundance of *R. raciborskii* cells within the water column and the relative abundance of toxic and nontoxic strains (Pierangelini et al., 2015b), although different CYN-producing strains possess different constituent CYN cell quotas (Willis et al., 2015). As a hepatotoxic alkaloid, the main targets of CYN included green algae (Bar-Yosef et al., 2010; Campos et al., 2013, Pinheiro et al., 2013) and cyanobacterial species (Rzymski and Poniedzialek, 2014; Rzymski et

al., 2014). For example, it was reported that 10- and 50-μg/L CYN (both of which are environmentally-relevant concentrations) can induce growth inhibition and cell necrosis of *M. aeruginosa*, respectively (Rzymski et al., 2014). Both MCs and CYN can exert similar effects on the cyanobacteria with respect to the process of their specific competition for dominance, but the effects of direct cell-to-cell contact between CYN-producing *R. raciborskii* and MCs-producing *M. aeruginosa* remain unclarified.

The consequences of competition between two different species depend on the niche difference and relative fitness difference between the two species, as well as the spatial and resource constraints (MacDougall et al., 2009; Narwani et al., 2013). These consequences can be the replacement of one of the species by the other, the failure of one of the species to maintain a steady population growth, or the coexistence of both species in a more balanced stage. Coexistence is defined as the ability of each of the two species to invade an established population of the other species from rarity (Chesson, 2000). Both *R. raciborskii* and *M. aeruginosa* are CyanoHABs-forming species with similar environmental adaptation strategies, such as high growth rate, high genetic diversity, and the possession of gas vesicles to attain buoyancy, indicating that they may occupy the same niche (Gugger et al., 2005; Duan et al., 2009). However, as two distinct species, they also have their own biological characteristics besides the obvious differences in morphology. For example, *R. raciborskii* can fix nitrogen with its heterocysts to alleviate nitrogen deficiency (Plominsky et al., 2013; Yang et al., 2018b), whereas *M. aeruginosa* has no heterocyst but can form colonies to gain greater buoyancy and withstand higher tolerance to a stressful environment (Mantzouki et al., 2016; Xiao et al., 2018). These biological similarities and differences determine their different competitive potentials for dominance in the same water body.

Based on the above-mentioned field results and the biological similarities and differences, it is reasonable to speculate that *R. raciborskii* can coexist with *M. aeruginosa*, but the consequences of such competition would depend on whether the *M. aeruginosa* strain is capable or incapable of producing MCs. To verify the hypothesis, a CYN-producing strain of *R. raciborskii* was co-cultivated with either an MCs-producing or non-MCs-producing strain of *M. aeruginosa* at different initial biomass ratios, and the contribution of biomass from each species to the total algal biomass in the culture and the morphological changes of the two species were determined to assess their competitive potentials as well as to predict the success of *Raphidiopsis* and *Microcystis* blooms in natural waters.

2 MATERIAL AND METHOD

2.1 Strain and culture condition

Pure cultures of CYN-producing *R. raciborskii* FACHB-3438, MCs-producing *M. aeruginosa* FACHB-905, and non-MCs-producing *M. aeruginosa* FACHB-526 were obtained from the Institute of Hydrobiology, Chinese Academy of Sciences located in Wuhan City of China. These species were grown in 250-mL Erlenmeyer flasks, each containing 150 mL of sterilized CT liquid medium (Watanabe and Ichimura, 1977). The cultures were incubated at 25±0.5 °C and exposed to 40 μmol photons/(m^2·s) in a 12-h∶12-h light-dark cycle, as it somewhat replicates the environmental conditions of night and day.

2.2 Co-cultivation experiments

To explore the competitive potentials of *R. raciborskii* and the two *M. aeruginosa* strains under conditions of unequal biomass densities, different initial biomass ratios of *R. raciborskii* to either *M. aeruginosa* strain were used, and these were as follows: (1) 100% *R. raciborskii* (100R); (2) 95% *R. raciborskii* and 5% *M. aeruginosa* (95R∶5M); (3) 75% *R. raciborskii* and 25% *M. aeruginosa* (75R∶25M); (4) 50% *R. raciborskii* and 50% *M. aeruginosa* (50R∶50M); (5) 25% *R. raciborskii* and 75% *M. aeruginosa* (25R∶75M); (6) 5% *R. raciborskii* and 95% *M. aeruginosa* (5R∶95M); and (7) 100% *M. aeruginosa* (100M).

The cultures containing the 95R∶5M and 5R∶95M biomass ratios were regarded as the invasive groups, based on the 95∶5 resident-to-invader ratios (Narwani et al., 2013; Gallego et al., 2019). *M. aeruginosa* was the invader and *R. raciborskii* was the defender in the culture with 95R∶5M ratio, while the roles were reversed in the culture with the 5R∶95M ratio. To obtain the above-mentioned co-cultures, all the strains at the logarithmic growth phase were harvested by centrifugation at 10 000×*g* and resuspended in fresh medium to acquire the designated biomass ratios. Both monocultures and co-cultures were prepared to have an equal initial total biomass density of 0.016 mg/mL, which correspond to 2×10^8, 7×10^8, and 6×10^8 cells/mL, respectively, in the monoculture of CYN-producing *R. raciborskii*, MCs-producing

and non-MCs-producing *M. aeruginosa*. To get the biomass densities of the three microalgae used in this study, the cell concentrations were determined by cell counting with an inverted microscope (ECLIPSE Ts2, Nikon, Tokyo, Japan) and their morphologies were recorded using a digital camera system (Nikon DS-Ri2, Tokyo, Japan). The biovolume calculation was performed according the method of Hillebrand et al. (1999) and their biomasses were estimated by assuming an average density for phytoplankton was 1 g/mL. All the cultures were prepared in 250-mL flasks, each containing 150-mL cell suspensions. Triplicate samples were prepared for each treatment and all the cultures were grown under the same temperature and light intensity as stated above. During the cultivation period, all flasks were shaken three times per day and their positions in the incubator were randomly adjusted after shaking to eliminate the possible uneven irradiance levels caused by the arrangement of their positions in the incubator. The experiment lasted for 24 days, and samples of the cultures were taken every three days.

2.3 Determination of specific growth rate

The sampled cell suspensions were fixed with 1% Lugol's iodine solution and the numbers of cells in the samples were then counted under a microscope (ECLIPSE Ts2, Nikon, Tokyo, Japan). The biovolume of each species was calculated by multiplying its abundance with its measured cell size (Hillebrand et al., 1999). The specific growth rate (μ) of the species was calculated using the following formula (Orr and Jones, 1998):

$$\mu=(\ln C_2-\ln C_1)/(t_2-t_1), \quad (1)$$

where C_1 and C_2 represent the biomass concentrations (mg/mL) of CYN-producing *R. raciborskii* plus MCs-producing or non-MCs-producing *M. aeruginosa* at time t_1 and t_2.

2.4 Morphological examination

To explore the final differences in morphologies of *R. raciborskii* filaments in the co-cultivation, the photographs of the filaments were recorded with a digital microscope camera system (Nikon DS-Ri2), and the widths of 100 filaments were randomly measured with the Nikon software NIS Element Ar 3.0 (Nikon, Tokyo, Japan) at the end of cultivation. The width of the vegetative cell was equal to that of the filament. Furthermore, the ratios of the number of filaments containing heterocysts to the total number of filaments in different treatments were determined as we accidentally discovered that the heterocysts in the filaments were disappearing during the co-cultivation process. In addition, at the end of cultivation, only a small quantity of *R. raciborskii* filaments was observed when the initial biomass of *R. raciborskii* relative to *M. aeruginosa* was less than 50%. Therefore, the ratios of *R. raciborskii* filaments containing the heterocysts to the total filaments were only determined for co-cultures where the initial biomass of *R. raciborskii* was more than 50%. About 1 000 filaments in each culture were examined for this purpose.

2.5 Determination of total dissolved nitrogen (TN) and phosphorus (TP) in the cultures

To address the changes of the extracellular microenvironment during the cultivation, the concentrations of total dissolved nitrogen (TN) and total dissolved phosphorus (TP) were measured every three days. After sampling, the cell suspension was immediately filtered through a 0.45-μm cellulose acetate film, and the filtrate was used for determination of the concentrations of TN and TP by potassium persulfate oxidation and Mo-Sb antispectrophotometry. For each culture, the determination of TN and TP was carried out for three replicates.

2.6 Statistical analysis

Data were presented as mean ± standard deviation for each set of measurements. Shapiro-Wilk and Levene tests were used to assess the normality and homogeneity of variance. Differences between groups were analyzed by *t*-test or one-way ANOVA followed by Tukey's multiple comparison test, and statistical significances were considered at the $P<0.05$ level. Statistical analysis was performed with SPSS V.16.0 (SPSS Inc., USA), and all figures were generated using Origin 9.0 (OriginLab, USA).

3 RESULT

3.1 Effects of biomass ratios and incubation time on the growth of *R. raciborskii* and *M. aeruginosa* in the co-cultures

In the monocultures (Control, 100R), the biomass densities of *R. raciborskii* increased with prolonged incubation (Fig.1a–b). However, when *R. raciborskii* was co-cultivated with *M. aeruginosa*, its density significantly decreased at all tested initial *R. raciborskii* to *M. aeruginosa* biomass ratios. In addition, the inhibition of *R. raciborskii* growth was stronger when it was co-cultivated with the MCs-

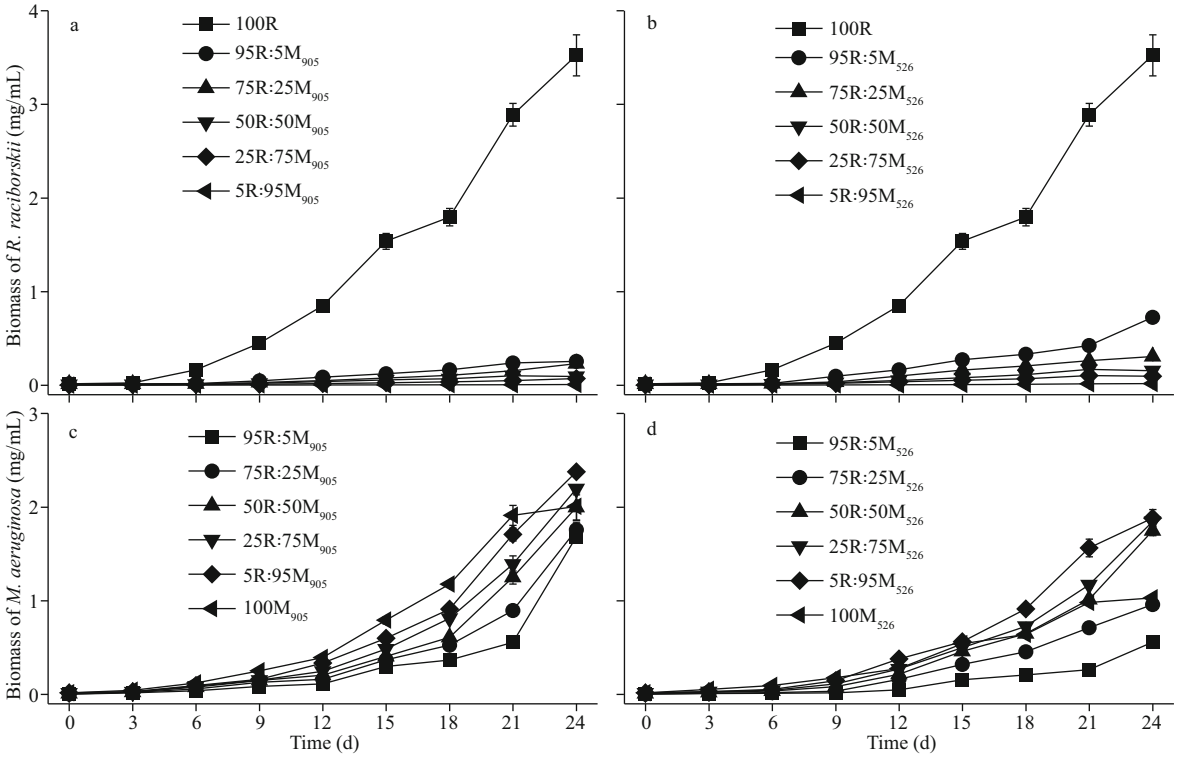

Fig.1 Changes in algal biomass for cultures with different initial *R. raciborskii* to *M. aeruginosa* biomass ratios

Biomass of *R. raciborskii* co-cultured with MCs-producing (a) and non-MCs-producing (b) *M. aeruginosa* strains. Biomass of MCs-producing (c) and non-MCs-producing (d) *M. aeruginosa* strains co-cultured with *R. raciborskii*. Data are the means±standard errors from triplicate determinations.

producing *M. aeruginosa* strain compared with the non-MCs-producing *M. aeruginosa* strain. At the end of the cultivation, the densities of *R. raciborskii* were 0.25, 0.23, 0.09, 0.07, and 0.01 mg/mL in the co-cultures where the initial biomass ratios of *R. raciborskii* to the MCs-producing *M. aeruginosa* strain were 95R:5M, 75R:25M, 50R:50M, 25R:75M, and 5R:95M, respectively (Fig.1a). The corresponding densities of *R. raciborskii* in the cases of the co-cultures containing the non-MCs-producing *M. aeruginosa* strain at the same initial biomass ratios were 0.73, 0.31, 0.16, 0.10, and 0.02 mg/mL (Fig.1b).

For *M. aeruginosa*, the biomass density increased with prolonged cultivation, and when higher initial biomass relative to *R. raciborskii* was used at the time of inoculation, higher biomass was also achieved at the end of the co-cultivation (Fig.1c–d). Furthermore, at the same *R. raciborskii* to *M. aeruginosa* biomass ratios, the MCs-producing *M. aeruginosa* strain grew better than the non-MCs-producing one (Fig.1c–d). At the end of the cultivation, the densities of the MCs-producing *M. aeruginosa* strain co-cultivated with *R. raciborskii* at initial biomass ratios of 95R:5M, 75R:25M, 50R:50M, 25R:75M, and 5R:95M increased to 1.68, 1.75, 2.00, 2.20 and 2.38 mg/mL, respectively, from an initial density of 0.016 mg/mL (Fig.1c). For the same *R. raciborskii* to *M. aeruginosa* biomass ratios, the corresponding densities of the non-MCs-producing *M. aeruginosa* strain reached 0.56, 0.96, 1.75, 1.84, and 1.88 mg/mL at the end of the co-cultivation (Fig.1d). Thus, regardless of the initial biomass ratios of *R. raciborskii* to *M. aeruginosa* (both MCs-producing and non-MCs-producing strains), the biomass of *M. aeruginosa* relative to that of *R. raciborskii* exhibited an increasing trend at the end of the co-cultivation. This suggested the ability of *M. aeruginosa* to proliferate out-competed *R. raciborskii* in the co-culture system.

The average specific growth rate of *R. raciborskii* in the co-cultures was suppressed by both strains of *M. aeruginosa* (Fig.2a–b). The inhibition of *R. raciborskii* growth exerted by the MCs-producing *M. aeruginosa* strain was stronger than that exerted by the non-MCs-producing strain at all initial biomass ratios tested (Fig.2a). At the end of the cultivation, the average specific growth rate of *R. raciborskii* in the monoculture was 0.22/d, but the specific growth rates of *R. raciborskii* in the co-cultures with initial *R. raciborskii* to MCs-producing *M. aeruginosa* biomass ratios of 95R:5M, 75R:25M, 50R:50M, 25R:75M and 5R:95M decreased to 0.12, 0.12, 0.10, 0.12, and 0.10/d, respectively (Fig.2a). Similarly, a

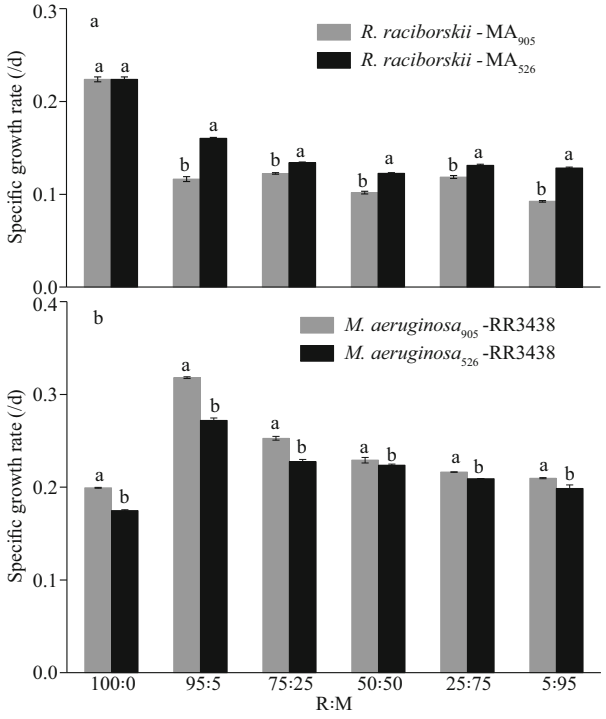

Fig.2 Specific growth rates of *R. raciborskii* and *M. aeruginosa* co-cultivated at different initial biomass ratios

a. specific growth rates of *R. raciborskii* co-cultivated with MCs-producing or non-MCs-producing *M. aeruginosa* at different initial biomass ratios; b. specific growth rates of MCs-producing and non-MCs-producing *M. aeruginosa* co-cultivated with *R. raciborskii* at different initial biomass ratios. Data are the means±standard errors from triplicate determinations. The lower case letters a and b indicate significant differences at the $P<0.01$ level. MA_{905}, MA_{526}, and RR3438 stand for *M. aeruginosa*$_{905}$, *M. aeruginosa*$_{526}$, and *R. raciborskii* 3438.

decrease in the specific growth rate of *R. raciborskii* was also evident when it was co-cultured with the non-MCs-producing *M. aeruginosa* strain, reaching 0.16, 0.13, 0.12, 0.13, and 0.13/d, respectively when the biomass ratios were 95R:5M, 75R:25M, 50R:50M, 25R:75M, and 5R:95M (Fig.2a). However, the average specific growth rates of the MCs-producing and non-MCs-producing *M. aeruginosa* strains in the co-cultures were significantly ($P<0.01$) higher than those in the monocultures (Fig.2b). The specific growth rates of *M. aeruginosa* strain at initial biomass ratios of 95R:5M, 75R:25M, 50R:50M, 25R:75M and 5R:95M were 0.32, 0.25, 0.23, 0.22, and 0.21/d, respectively, in the case of the MCs-producing strain, and were 0.27, 0.23, 0.22, 0.21, and 0.20/d, respectively, for the non-MCs-producing strain (Fig.2b).

3.2 Contribution of each algal biomass to the total biomass during co-cultivation

To visually show the dominating competitiveness

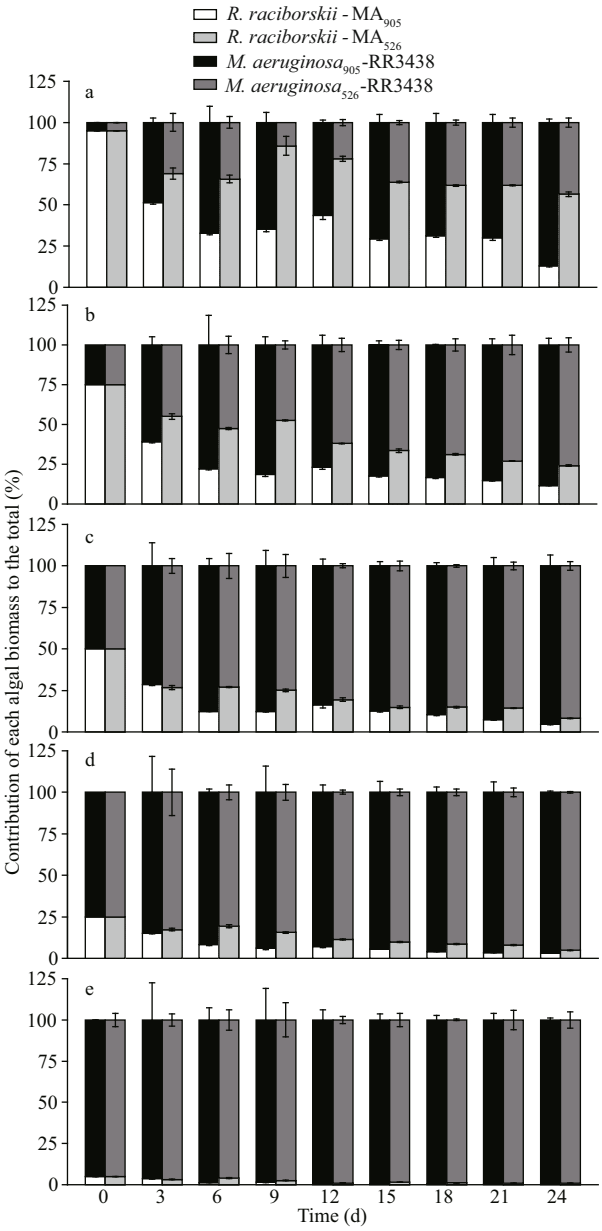

Fig.3 Contribution of *R. raciborskii* and the two *M. aeruginosa* strains to the total biomass in the co-cultures at different initial biomass ratios and incubation times

a. 95R:5M; b. 75R:25M; c. 50R:50M; d. 25R:75M; e. 5R:95M. Data are the means ± standard errors from triplicate determinations.

of the microalgae during the co-cultivation, the contribution of each algal biomass to the total biomass during the entire co-cultivation period was determined. Overall, the contribution of *R. raciborskii* to the total biomass was positively correlated with its initial biomass in the culture, but such contribution decreased with prolonged cultivation time, with the decrease occurring at a faster rate when the co-cultivated *M. aeruginosa* strain was the MCs-producing strain (Fig.3a–e). Correspondingly, the

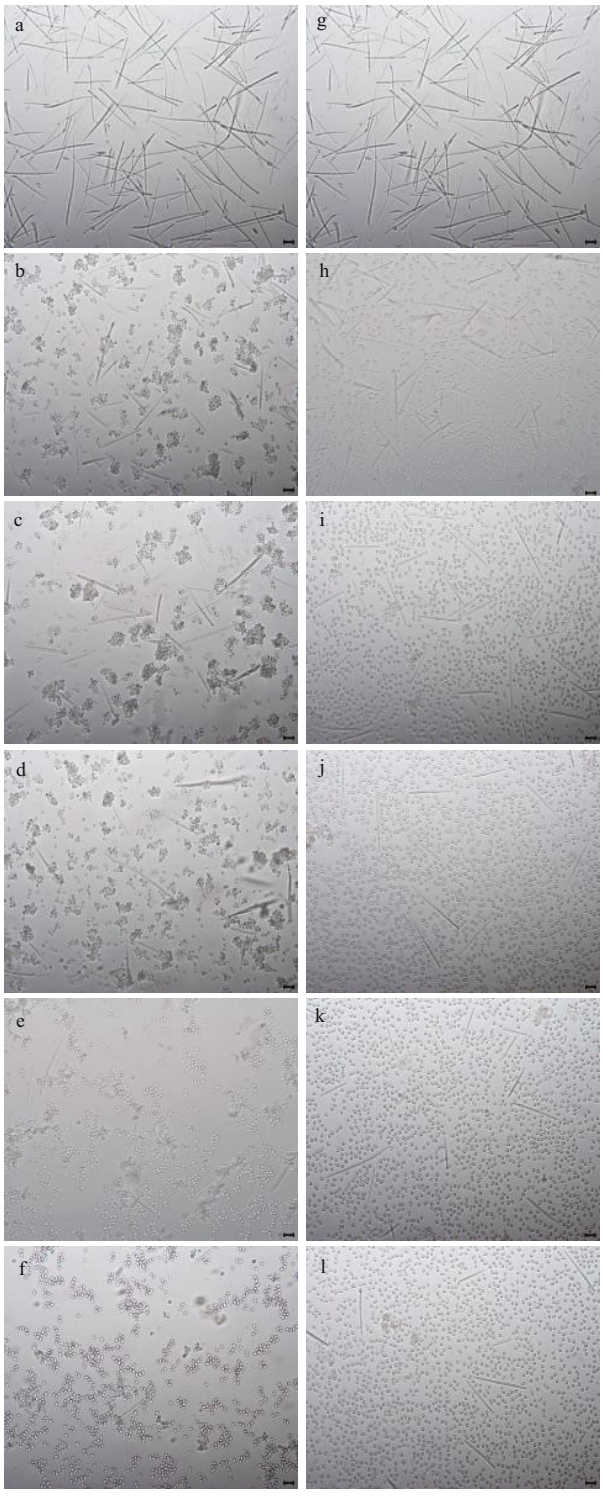

Fig.4 Morphological characteristics of the filaments of *R. raciborskii* co-cultivated with *M. aeruginosa*

Raphidiopsis raciborskii was co-cultivated with MCs-producing (a–f) and non-MCs-producing (g–l) *M. aeruginosa* strains for 24 days at different initial biomass ratios. a and g: monocultured *R. raciborskii* filaments; b–f: *R. raciborskii* filaments co-cultivated with MCs-producing *M. aeruginosa* strain at initial biomass ratios of 95R:5M (b), 75R:25M (c), 50R:50M (d), 25R:75M (e), and 5R:95M (f); h–l: *R. raciborskii* filaments co-cultivated with non-MCs-producing *M. aeruginosa* strain at the corresponding biomass density ratios as in b–f. Scale bars: 10 μm.

contribution of *M. aeruginosa* to the total biomass also increased with prolonged cultivation time and the contribution of the MCs-producing strain exceeded that of the non-MCs-producing strain. At the end of the cultivation, the biomass contribution of either *M. aeruginosa* strain was significantly higher (P<0.01) than that of *R. raciborskii*, except in the culture where the initial biomass of *R. raciborskii* was 95% of the total initial biomass and that *R. raciborskii* was co-cultured with the non-MCs-producing strain of *M. aeruginosa* (Fig.3a). For the co-cultures with initial biomass ratios of 95R:5M, 75R:25M, 50R:50M, 25R:75M, and 5R:95M, the biomass contribution of *R. raciborskii* decreased by 84.94%, 88.36%, 95.49%, 96.90%, and 99.97%, respectively, when MCs-producing *M. aeruginosa* was the co-cultured strain, or by 43.39%, 75.76%, 91.85%, 95.09%, and 99.91%, respectively, when non-MCs-producing *M. aeruginosa* was the co-cultured strain (Fig.3a–e). The results suggested that loss in biomass contribution by *R. raciborskii* was less severe when it was co-cultured with the non-MCs-producing *M. aeruginosa* strain, and in cultures with higher initial *R. raciboskii* biomass than those with higher initial *M. aeruginosa* biomass.

3.3 Morphological changes of *R. raciborskii* co-cultured with *M. aeruginosa*

The morphological changes in *R. raciborskii* and *M. aeruginosa* that have been co-cultivated for 24 days are shown in Fig.4. Changes in the morphological parameters of *M. aeruginosa* were ignored in the present study because no visible morphological changes were found. For the monoculture of *R. raciborskii*, the filaments contained heterocysts (Fig.4a & g), but an obvious loss of heterocysts in the filaments occurred when the strain was co-cultured with the MCs-producing strain of *M. aeruginosa* (Fig.4b–f), and the percent of heterocyst-containing filaments also decreased with increased initial biomass ratios of MCs-producing *M. aeruginosa* to *R. raciborskii* (Fig.5a). The loss of heterocyst in *R. raciborskii* filaments also occurred when the strain was co-cultured with the non-MCs-producing *M. aeruginos*a strain (Fig.4h–l), but the loss occurred at a lower rate (Fig.5b). For the mono-cultured *R. raciborskii*, the heterocyst-containing filaments accounted for 97.36% of the total number of filaments. However, when *R. raciborskii* was co-cultivated with the MCs-producing *M. aeruginosa* strain at initial biomass ratios of 95R:5M, 75R:25M, and 50R:50M, the percentage

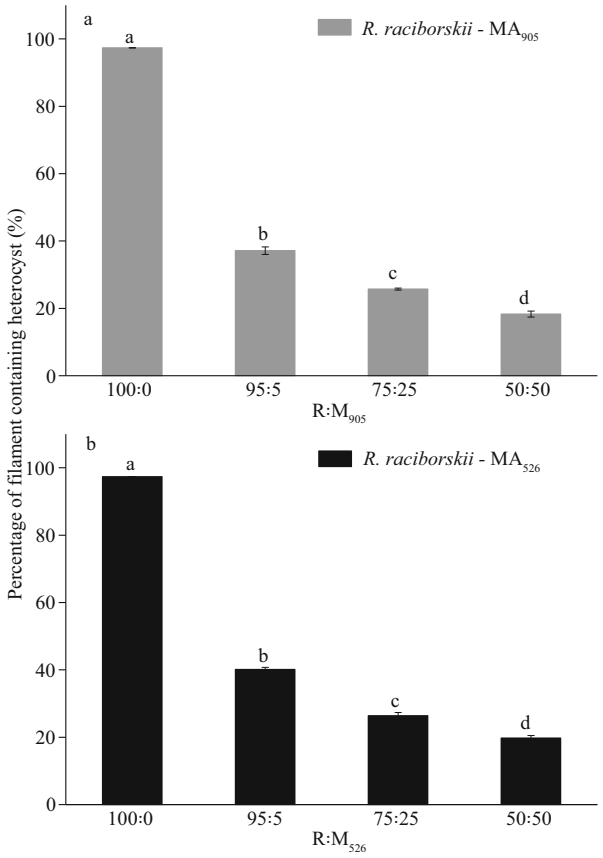

Fig.5 Percentage of heterocyst-containing *R. raciborskii* filaments co-cultivated with *M. aeruginosa*

Raphidiopsis raciborskii was co-cultivated with MCs-producing (a) and non-MCs-producing (b) *M. aeruginosa* strains at different initial biomass ratios for 24 days. Data are the means±standard errors based on 1 000 randomly chosen filaments. The lower case letters indicate significant differences at the $P<0.05$ level.

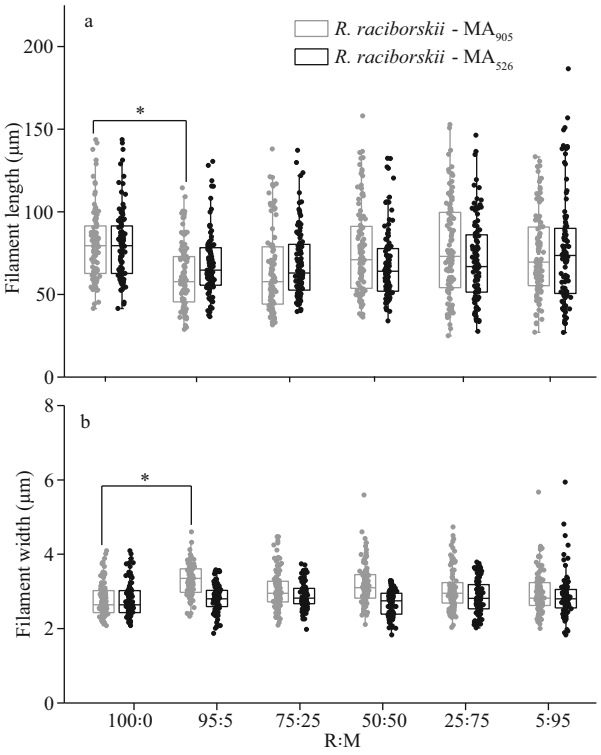

Fig.6 Changes in filament length (a) and width (b) of *R. raciborskii* co-cultivated with *M. aeruginosa* at different initial biomass ratios for 24 days

Data are the means±standard errors based on 1 000 randomly chosen filaments. Asterisk indicates a significant difference at the $P<0.05$ level.

of heterocyst-containing filaments decreased to 37.13%, 25.75% and 18.30%, respectively (Fig.5a). As for *R. raciborskii* co-cultivated with the non-MCs-producing *M. aeruginosa*, this percentage decreased to 40.17%, 26.40%, and 19.77%, respectively (Fig.5b).

In addition to the changes in the heterocysts of the filaments, the average length and width of the filaments of *R. raciborskii* co-cultivated with the MCs-producing *M. aeruginosa* strain also decreased significantly ($P<0.05$) when the initial biomass ratio was 95R:5M (Fig.6a–b). In contrast, no significant difference in the average length and width of the filaments were detected when *R. raciborskii* was co-cultivated with the non-MCs-producing *M. aeruginosa* strain regardless of the initial biomass ratios of the cultures and incubation time.

3.4 Changes in TN and TP during the cultivation period

Changes in TN and TP in the media of both *R. raciborskii* monoculture and *R. raciborskii*-*M. aeruginosa* co-culture showed a gradually decreasing trend with prolonged incubation (Fig.7), but the rate of decrease for TN was slower in the case of the monoculture (Fig.7a–b). In addition, the rates of decrease for TN were similar in the co-cultures containing either the MCs-producing or non-MCs-producing *M. aeruginosa* strain with no significant difference between the two cultures regardless of incubation time. As for TP, its concentration in both the monoculture and co-culture decreased sharply from the point of inoculation to Day 9, followed by a more gradual decrease that also fluctuated slightly among the different set of cultures (Fig.7c–d). Unlike the change in TN, the TP concentration in both *R. raciborskii* monoculture and *R. raciborskii*-*M. aeruginosa* co-culture displayed almost identical trends, with no significant difference between the two cultures regardless of incubation time and the initial biomass ratios in the case of the co-culture (Fig.7c–d).

4 DISCUSSION

Co-cultivation is considered to be a good method

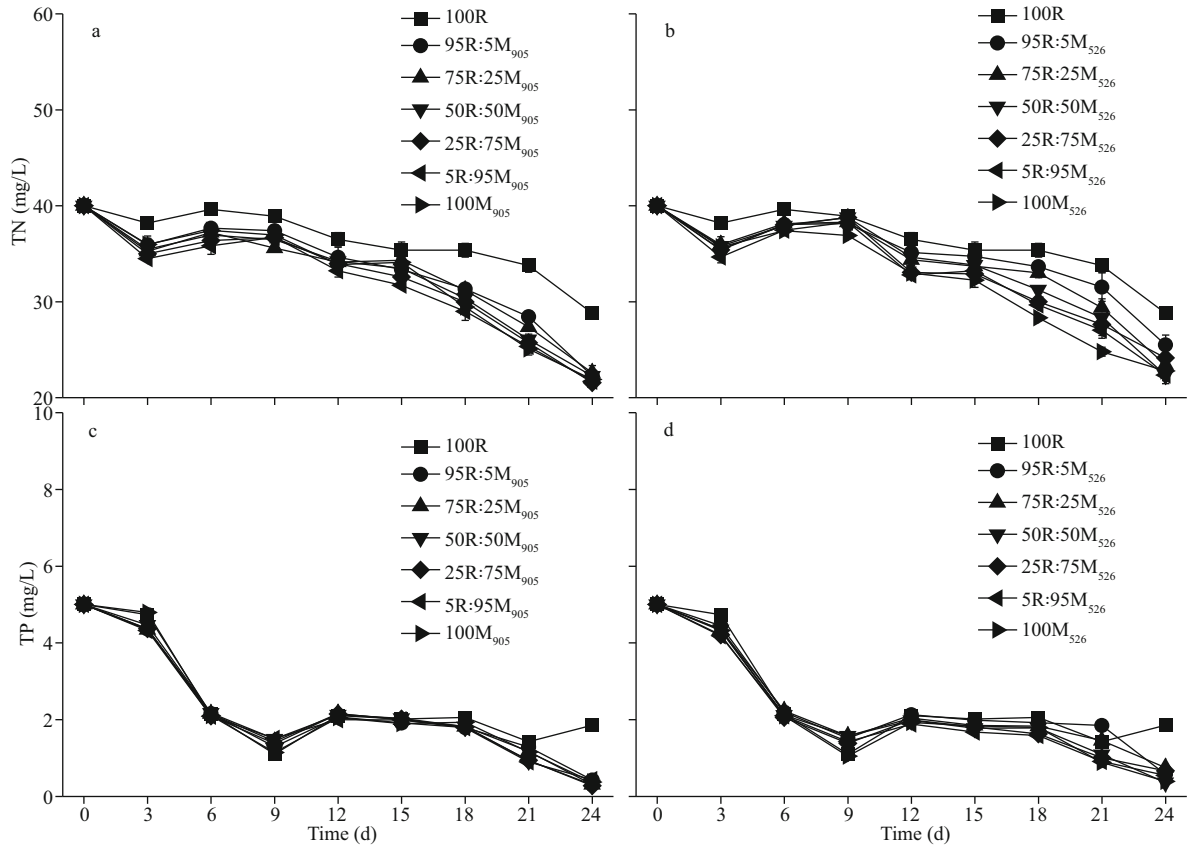

Fig.7 Changes in total soluble nitrogen (TN) and total soluble phosphorus (TP) concentrations in *R. raciborskii*-*M. aeruginosa* co-cultures at different initial biomass ratios and incubation times

a and c: *R. raciborskii* co-cultured with MCs-producing *M. aeruginosa* strain; b and d: *R. raciborskii* co-cultivated with non-MCs-producing *M. aeruginosa* strain. Data are the means±standard errors from triplicate determinations.

for studying the allelopathic interaction between two different phytoplankton species because direct evidence for allelopathic interactions under natural aquatic conditions is difficult to provide owing to the interferences from other processes (Sukenik et al., 2002; Legrand et al., 2003; Ma et al., 2015; Dunker et al., 2017; Chia et al., 2018). Through co-cultivation, we have shown in the present study that the growth rates of *R. raciborskii* could be inhibited by both MCs-producing and non-MCs-producing *M. aeruginosa* strains (Figs.1a–b & 2a). In addition, the inhibition of *R. raciborskii* growth exerted by the MCs-producing *M. aeruginosa* strain was greater than that exerted by the non-MCs-producing one (Figs.1a, b, & 2a). This is in accordance with the reported allelopathic effects of *M. aeruginosa* on several other species of green algae and dinoflagellates (Sukenik et al., 2002; Bittencourt-Oliveira et al., 2015; Ma et al., 2015; Yang et al., 2018a; Omidi et al., 2019). MCs-producing *M. aeruginosa* strains can suppress the growth and photosynthesis of their competitors by producing MCs as well as other odor substances (Pietsch et al., 2001; Singh et al., 2001; Pflugmacher, 2002; Hu et al., 2004; Babica et al., 2007; Wang et al., 2017), which give them an advantage in such competition (Ma et al., 2015; Wang et al., 2017).

MCs are allelochemicals that can induce suppression of the growth of cyanobacteria and eukaryotic algae (Sukenik et al., 2002; Qian et al., 2009; Tan et al., 2019). The mechanism of growth suppression is rather diverse, and it includes reducing carbon dioxide fixation and nitrogen fixation activities (Singh et al., 2001), obstructing electron transport by specifically binding to relevant sites in photosystem II (PSII) (Keating, 1999), hindering adenosine triphosphate (ATP) synthesis (Zhu et al., 2010), abolishing intracellular carbonic anhydrase activity (Sukenik et al., 2002), and causing oxidative damage (Qian et al., 2009). Besides the negative allelopathy, a recent study has found that *M. aeruginosa* can inhibit the growth of *Chlorella vulgaris* via the release of linoleic acid (Song et al., 2017).

However, for *M. aeruginosa* strains, co-cultivation with *R. raciborskii* stimulated their growth rates and the stimulating effects decreased with increased proportions of initial biomass of *R. raciborskii* in the

co-cultures (Figs.1c–d & 2b). In addition, under such co-cultivation, the toxic *M. aeruginosa* strain achieved a higher growth rate than the non-toxic one (Fig.2b). This is also supported by other previous experimental studies. For example, environmentally relevant concentrations of CYN were found to have no effect on the growth of the cyanobacteria *Nannochloropsis* sp. and *Chlamydomonas reinhardti* (Pinheiro et al., 2013), whereas CYN was found to stimulate the growth of the green alga *C. vulgaris* despite its relatively high concentration, up to 179 μg/L (Campos et al., 2013). Taken together, these results may suggest that *M. aeruginosa*, similar to other microalgae, can cope with CYN-induced oxidative stress through increasing the activity of antioxidant enzymes (Campos et al., 2013). Furthermore, the biomass contribution of the MCs-producing or non-MCs-producing *M. aeruginosa* strain to the total biomass increased regardless of the initial *R. raciborskii* to *M. aeruginosa* biomass ratios, and a ratio of 5R:95M resulted in the complete elimination of *R. raciborskii* from the co-culture on the 9th day and 21th day for the MCs-producing and non-MCs-producing *M. aeruginosa* strains, respectively (Fig.3e). According to the trend of biomass contribution by each algal strain to the total biomass during the entire co-cultivation period (Fig.3a–e), it could be inferred that with further incubation time, both the MCs-producing and non-MCs-producing *M. aeruginosa* strains could eventually exclude *R. raciborskii* completely regardless of the initial biomass ratios, but the non-MCs-producing strain would need a longer incubation to completely replace *R. raciborskii*.

Raphidiopsis species have been reported to exert stronger inhibitory effects than pure CYN on the growth of green algae and cyanobacteria (Campos et al., 2013; Rzymski et al., 2014). *Raphidiopsis raciborskii* FACHB-3438 is a CYN-producing species (Jiang et al., 2014). However, the higher growth rates of both MCs-producing and non-MCs-producing *M. aeruginosa* strains observed when they were co-cultivated with *R. raciborskii* FACHB-3438 (Fig.2b) suggested that CYN could stimulate the growth of *M. aeruginosa*. Furthermore, it could also mean that the MCs-producing species are more likely to gain a competitive advantage over their competitors than the CYN-producing species.

Under natural conditions, most of the *R. raciborskii* filaments contain heterocysts needed for nitrogen fixation, a function that gives this alga an advantage in the competition with non-nitrogen-fixing algae, especially in N-deficient environments (Moisander et al., 2012; Yang et al., 2017). In the present study, the formation of heterocysts in *R. raciborskii* filaments was inhibited by *M. aeruginosa* (Figs.4–5), and the inhibitory effect increased with an increased proportion of *M. aeruginosa* biomass in the co-culture. In addition, co-cultivation with the MCs-producing *M. aeruginosa* strain led to a greater loss of heterocysts than co-cultivation with the non-MCs-producing one (Fig.5a–b). To our knowledge, this is the first report to mention the disappearance of heterocysts in cyanobacterial filaments caused by allelopathy although the physiological mechanism is unclear. A previous study has reported that *M. aeruginosa* can exert an inhibitory effect on the differentiation of heterocysts in the filamentous cyanobacterium *Trichormus variabilis* through an unknown mechanism (Bártová et al., 2011). In addition, co-culture with *M. aeruginosa* can also result in the growth reduction of *Planktothrix* (Briand et al., 2019). In either case, the molecular mechanism responsible for the effect has not been clarified. However, the disappearance of heterocysts in the *R. raciborskii* filaments and the morphological changes observed for the filaments could be an adaptation feature resulting from the pressure brought by the presence of *M. aeruginosa* in the culture. This could increase the biotic stress for *R. raciborskii*, although the direct effects on the competition for dominance between *R. raciborskii* and *M. aeruginosa* remain unclear. Therefore, a further study involving the use of multiple environmental factors and multiple technical methods should be carried out to reveal the precise effects of the extracellular microenvironment on the morphological changes of *R. raciborskii* filaments and the underlying molecular mechanisms.

The competition for dominance between the two algae is affected by many factors, such as temperature (Ma et al., 2015; Yang et al., 2018a), nutrient levels (Marinho et al., 2013; Chia et al., 2018), algae species even strains of the competitors (Sukenik et al., 2002; Ma et al., 2015; Wang et al., 2017). For example, in the co-cultivation of *Scendesmus obliquus* with *M. aeruginosa*, *S. obliquus* is more competitive at 15 °C, the two species are equally competitive at 20–30 °C, but *M. aeruginosa* is more competitive at 35 °C (Yang et al., 2018a). Furthermore, studies have shown that *R. raciborskii* is able to utilize a range of nitrogen sources such as ammonium, nitrate, and urea with a clear preference for ammonium (Burford et al., 2006; Stucken et al., 2014), thus appears to

gain competitive advantage under low and fluctuating dissolved nitrogen concentrations, in line with the term "facultative diazotroph" coined by Moisander et al. (2012). In addition, *R. raciborskii* has high dissolved inorganic phosphorus uptake rates and storage capacity relative to a range of other cyanobacterial species (Isvánovics et al., 2000; Prentice et al., 2015). Consistent with this, *R. raciborskii* can dominate in a range of phosphorus levels (Chislock et al., 2014). In this study, the changes in the concentrations of TN and TP in the co-cultures displayed a similar trend without any significant differences among the different sets of cultures regardless of the initial biomass ratios and incubation time (Fig.7a–d). This may be due to the demand for nitrogen and phosphorus by the two algae being completely satisfied throughout the incubation period. Therefore, the different effects that the MCs-producing and non-MCs-producing *M. aeruginosa* strains had on *R. raciborskii* as shown in the present study, were unlikely to be caused by variations in nutrients in co-cultivations.

5 CONCLUSION

In the co-cultivation of *R. raciborskii* with *M. aeruginosa* at different biomass ratios, the growth of *R. raciborskii* was inhibited by both MCs-producing and non-MCs-producing *M. aeruginosa* strains, with the former exerting a stronger inhibition than the latter. On the other hand, co-cultivation of *M. aeruginosa* with *R. raciborskii* stimulated the growth of the *M. aeruginosa*. Therefore, in the co-cultivation, *R. raciborskii* could be completely eliminated by *M. aeruginosa* even when *R. raciborskii* was present in dominating proportion at the start of the cultivation (R∶M=95∶5 in biomass ratio), and during the cultivation, the MCs-producing *M. aeruginosa* strain took less time to replace *R. raciborskii* than the non-MCs-producing strain. Furthermore, the co-cultivation of *R. raciborskii* with *M. aeruginosa* led to the disappearance of heterocysts containing in filaments of *R. raciborskii*. Compared with the non-MCs-producing *M. aeruginosa* strain, the MCs-producing strain exerted a more potent inhibitory effect on *R. raciborskii*, thereby, enabling it to acquire stronger competitiveness for dominance These results indicated that it is difficult for *R. raciborskii* to replace the dominant position of *M. aeruginosa* in the same natural water if based purely on the allelopathy between the two species. This study has provided important evidence pertaining to the competition for dominance between *R. raciborskii* and *M. aeruginosa*, and essentially ruled out the possibility that *R. raciborskii* could replace the dominant position of *M. aeruginosa* in natural waters.

6 DATA AVAILABILITY STATEMENT

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

7 ACKNOWLEDGMENT

The authors highly appreciate Dr Alan K. Chang (Wenzhou University) for his effort in the revision of the manuscript's language, and the anonymous reviewers for their very useful and insightful comments.

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