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RESEARCH ARTICLE

Morphological and growth responses of two green algal strains to toxic *Microcystis* are dependent on the cultivation growth phase of filtrate and target strain

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Abstract – Allelopathic interactions amongst phytoplankton are considered an important factor contributing to species competition and succession in aquatic ecosystems, but their mechanisms in plankton dynamics are poorly described. In this study, whether toxic *Microcystis aeruginosa* could affect the growth of *Chlorella vulgaris* and *Kirchneriella* sp. was examined according to filtrate experiments at different cultivation phases. Results indicated that *M. aeruginosa* filtrate significantly influenced the growth and morphological characteristics of the two target green algae, which were dependent on the cultivation growth phase of filtrate and target strain. At the beginning of the experiment, the formation of a large *C. vulgaris* colony was induced by *M. aeruginosa* filtrate. The effects of filtrate in the stationary phase (SP) was more significant than that of the exponential phase (EP). Subsequently, the colonies gradually broke into small colonies or single cells. The growth rate of *C. vulgaris* was finally promoted in the filtrate treatment. For *Kirchneriella* sp., the colonies formed and remained in *M. aeruginosa* filtrate under EP until the end of the experiment. Smaller colonies were observed in *Kirchneriella* sp. by *M. aeruginosa* filtrate under SP than those in the control, and larger colonies were not detected. The growth rate of *Kirchneriella* sp. was inhibited in the filtrate of EP but was promoted in SP. This study provided new insights into the interaction between the morphological responses and growth effects of algae and proposed a new theoretical basis for algal succession in aquatic ecosystems.

Keywords: *Microcystis aeruginosa* / *Chlorella vulgaris* / *Kirchneriella* sp / allelopathy / colony formation

1 Introduction

Allelopathy, derived from the words ‘allelon’ and ‘pathos’, is a process by which specific biomolecules are secreted by one plant or bacterial species to possibly inhibit or benefit other plants or bacterial species. In aquatic ecosystems, allelopathic interaction also occurs between algae and plays an important role in species competition, succession except for other factors, such as hydrodynamics, nutrients and temperature (Suikkanen *et al.*, 2004; Dunker *et al.*, 2013; Rzymski *et al.*, 2014; Accoroni *et al.*, 2015; Wang *et al.*, 2017b). Many examples of allelochemical interactions exist amongst cyanobacteria and their competitors which may affect the seasonal dynamics of these algae (Legrand *et al.*, 2003). For example, allelochemicals secreted by *Cylindrospermopsis raciborskii* can inhibit the growth of its competitors, such as *Microcystis aeruginosa*, thereby contributing to the stable dominance of *C. raciborskii* in a tropical lake (Figueredo *et al.*, 2007; Mello *et al.*, 2012). In Lake Kinneret, high biomass of *Microcystis* are observed until the end of winter, thereby causing either missing or delayed *Peridinium* bloom (Sukenik and Kaplan, 2002; Vardi *et al.*, 2002). Sukenik and Kaplan (2002) and Vardi *et al.* (2002) further suggested that the filtrate of *Microcystis* monoculture can significantly inhibit the growth of the dinoflagellate *Peridinium gatunense*. In addition, *M. aeruginosa* elicits allelopathic effects on competitive *Scenedesmus quadricauda* (Zheng *et al.*, 2008) and *Quadrigula chodatii* (Zhang *et al.*, 2013). Cyanobacteria and green algae also demonstrated seasonal changes in eutrophic lakes, such as Lake Taihu (Cai *et al.*, 2012) and Lake Dianchi (Dong *et al.*, 2015) in China. Bar-Yosef *et al.* (2010) also demonstrated that the monoculture of *Aphanizomenon ovalisporum* can secrete allelochemicals on competitors. The underlying mechanism may involve the promotion of phosphorus (Pi) supply by cylindrospermopsin producers through the induction of alkaline phosphatase secretion in other phytoplankton, suggesting that their abundance increases despite the reduced (Pi) supply from watersheds (Bar-Yosef *et al.*, 2010).

Growth effects have been extensively investigated. In fact, the interaction between algae can induce growth effects but also have morphological influences (Mello *et al.*, 2012). These

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morphological influences might also be essential for algal competition and interaction. However, studies on morphological influences amongst phytoplankton have been rarely performed. During growth, which is related to the beginning and the full *Microcystis* dominance/bloom in nature, substances produced in different growth phases are diverse. Studies comparing the effects of extracellular metabolites from different growth phases are also limited.

We hypothesised that morphological responses could be detected between algal interactions, in addition to growth effects. Furthermore, we assumed that the morphological and growth responses of other algae to *M. aeruginosa* were growth-phase dependent. To test this hypothesis, we chose a toxic cyanobacterium, namely, *M. aeruginosa*, and two common green algae, namely, *Chlorella vulgaris* and *Kirchneriella* sp., as target species. This study aimed to demonstrate (1) the growth responses of two green algal strains to *M. aeruginosa*; (2) whether *M. aeruginosa* influences the morphological characteristics of two green algal strains; (3) whether filtrates of *M. aeruginosa* from different cultivation phases have various influences on the growth and morphological characteristics of the target green algae. This study contributed new insights into the allelopathic interaction between algae and provided a new theoretical basis for algal succession in eutrophic lakes, rivers, reservoirs and aquaculture ponds.

2 Materials and methods

2.1 Algal culture

Axenic strains of toxic cyanobacteria *M. aeruginosa* (FACHB-905) and green alga *C. vulgaris* (FACHB-8) and *Kirchneriella* sp. (FACHB-1134) were all obtained from Freshwater Algal culture Collection of Institute of Hydrobiology, Chinese Academy of Sciences (deposited in Wuhan, China). *M. aeruginosa* (FACHB-905) was isolated from Dianchi Lake (Kunming, China), and was reported to produce microcystin-LR at approximately 0.61 μg per 10^7 viable cells (Sun *et al.*, 2012).

Prior to the experiment, all strains were cultured separately with sterile BG11 medium (Rippka *et al.*, 1979), at a climate-controlled temperature of 25 °C, under a cool-white fluorescent illumination of 25 μmol photons s^{-1} m^{-2} in a 12 h:12 h light–dark cycle. Both cultures were shaken three times per day to prevent sedimentation.

2.2 Experimental design

2.2.1 Preparation of cell-free filtrates associated with *M. aeruginosa*

The biomass of *M. aeruginosa* was determined by its optical density (OD) at 665 nm (blue-green algae) under ultraviolet/visible spectrophotometer, and the initial OD_{665} was 0.2 (with cell density 1.86×10^9 cells/L). The incubation conditions were the same as the maintenance of stock cultures, as mentioned above.

One part of the cell-free *M. aeruginosa* filtrate was obtained when *M. aeruginosa* grew to the exponential phase (EP) (cultured for 5 days, when $OD_{665} = 0.56$) and the other part was harvested in the stationary phase (SP) by cultivating for 30 days (when $OD_{665} = 1.56$) after inoculation (Wang *et al.*, 2017a). *M. aeruginosa* cultures of different growth phases were centrifuged with a speed of 8000 r m^{-1} for 10 min. Then, each supernatant was filtered through Whatman GF/C filters (Whatman International Ltd., Maidstone, England) for subsequent experimentation (Mello *et al.*, 2012).

2.2.2 Effects of cell-free filtrates associated with *M. aeruginosa* on *C. vulgaris* and *Kirchneriella* sp.

When the two green algae were in the exponential stages, the formal experiment started. The experiment was performed in 250 mL Erlenmeyer flasks. The experiment had two treatments (EP, SP), in addition to the control, which had no *Microcystis* filtrate (filtrated BG11 medium). The initial inoculation at OD_{680} (OD at 680 nm for green algae) of the tested green alga *C. vulgaris* and *Kirchneriella* sp. was 0.1 for both, with cell densities of 4.57×10^8 cells/L and 3.98×10^8 cells/L, respectively. Each treatment was repeated in triplicate; thus, 18 culture flasks were obtained in total. Cells grew in flasks containing 150 mL of solution (controls, filtrate BG11 medium; in cell-free filtrates in EP or SP, nutrients were added similar to that of BG11 medium to compensate for nutrient decrease during the preliminary cultivation period) (Mello *et al.*, 2012).

Each culture was cultivated in conditions similar to those described above and shaken three times daily to avoid sedimentation. The experiments lasted for 7 days, and each algal culture was sampled every day to measure growth, colony proportion and cell numbers per colony in *C. vulgaris* and *Kirchneriella* sp. The details were as follows: samples of 3 mL were used to determine the biomass of the target green algae *C. vulgaris* and *Kirchneriella* sp. by measuring its OD at 680 nm (OD_{680}) (ultraviolet/visible spectrophotometer), and samples of 2 mL were fixed with Lugol' solution and viewed under an inverted microscope within 24 h at 400× magnification to determine the cell density, colony proportion and number of cells per colony (Lürling and Van Donk, 1997). In the study, the colony was identified as a minimum of three cells; the colony proportion was determined using the ratio between the number of cells in colonies and the total number of algae counted in each treatment. The number of cells per colony was assessed by the ratio of the total number of algae counted and the number of colonies in each treatment.

2.3 Statistical analysis

The slope of the regression between ln-transformed OD_{680} and the experimental time (day) was used as an estimate of the population growth rate (Lürling, 2006; Mello *et al.*, 2012). The effect of the growth phase of *M. aeruginosa* on the growth rate of target algae was tested using a one-way ANOVA with the growth phase as an independent factor and subsequent separate *post hoc* tests to detect the differences between treatments ($\alpha = 0.05$) (Fisher LSD) (Wang *et al.*, 2017a).

The percentage of the cells in the colonies and the number of cells per colony of each treatment during the experiment were compared through a repeated-measure ANOVA ($\alpha = 0.05$). Both tests were followed by Tukey's *post hoc*

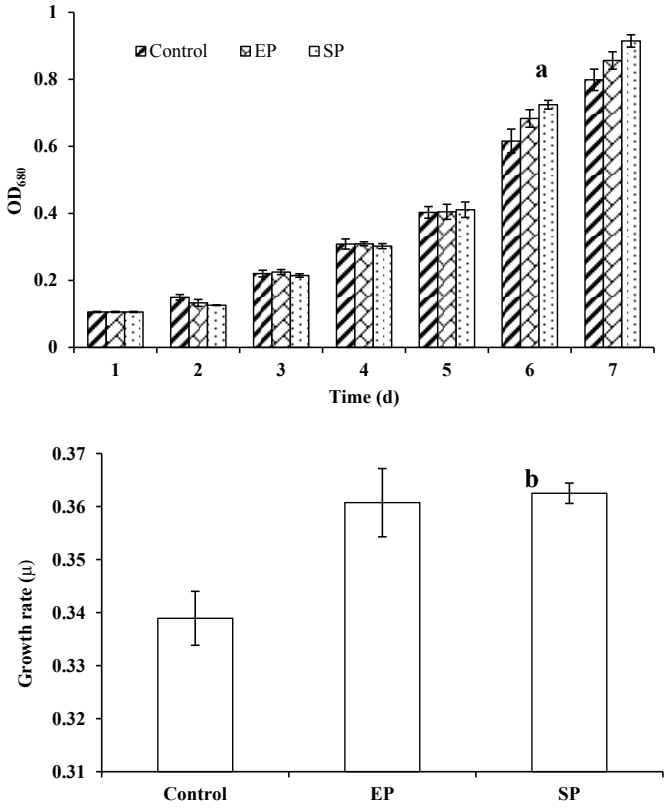

Fig. 1. OD_{680} (a) and growth rate (b) of *C. vulgaris* in the control and treatment with filtrate of *M. aeruginosa.*

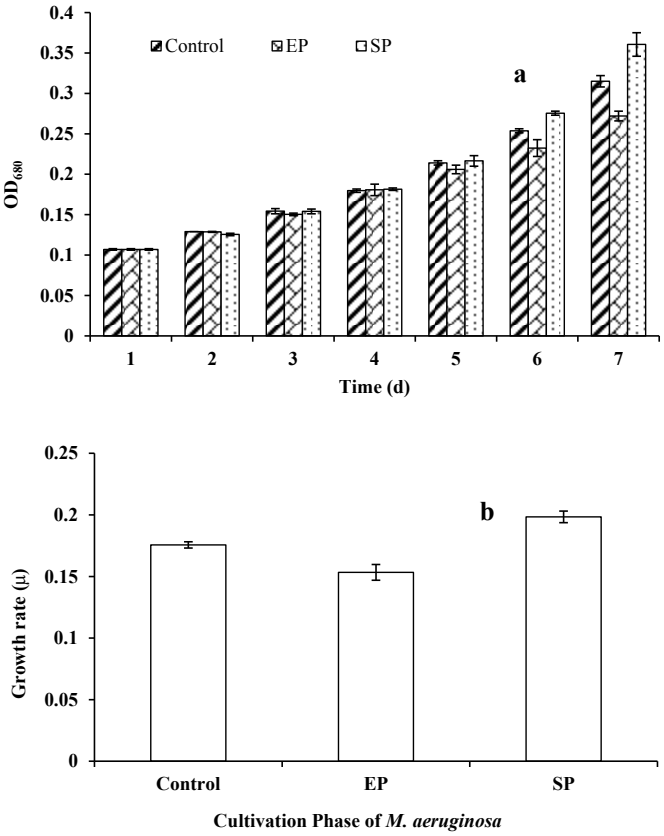

Fig. 2. OD_{680} (a) and growth rate (b) of *Kirchneriella* sp. in the control and treatment with filtrate of *M. aeruginosa*.

tests to detect the differences between treatments ($\alpha = 0.05$). $P < 0.05$ was considered significant in all of the analyses. Mauchly's sphericity was examined, and data were log-transformed when necessary. These tests were performed in SPSS 13.0 for Windows.

3 Results

3.1 Growth effects of *M. aeruginosa* filtrates on *C. vulgaris* and *Kirchneriella* sp.

The growth of *C. vulgaris* was slightly reduced on day 2, compared with that of the control (Fig. 1a). At the end of the experiment, we determined the growth rate of *C. vulgaris* and found that *M. aeruginosa* filtrate in EP and SP significantly increased the growth of *C. vulgaris* ($F = 21.856$, $P = 0.002$); however, further *post hoc* tests indicated that no significant differences were detected in growth rates of *C. vulgaris* in the treatment with *M. aeruginosa* filtrate in EP and SP ($P = 0.672$) (Fig. 1b).

The growth rates of *Kirchneriella* sp. in the treatment with *M. aeruginosa* filtrate in EP and SP were also significantly influenced compared with those of the control ($F = 65.805$, $P = 0.000$). Furthermore, *post hoc* tests showed that the growth of *Kirchneriella* sp. was significantly decreased when it was treated with *M. aeruginosa* filtrates under EP ($P = 0.001$), but was increased significantly when it was treated with *M. aeruginosa* filtrates under SP ($P = 0.001$) (Fig. 2).

3.2 Effects of *M. aeruginosa* filtrates on the morphological characteristics of two green algae

In addition to growth effects, the colony formation of *C. vulgaris* could be stimulated and its colony proportion could be significantly increased by *M. aeruginosa* filtrates (Fig. 3). The largest colony formed on day 2, and the number of cells per colony gradually decreased and reached values similar to that of the control at the end of the experiment.

According to repeated-measure ANOVA, the morphological response of *C. vulgaris* to *M. aeruginosa* filtrates was significantly influenced by the phase of *M. aeruginosa* filtrate treatment. The day and interaction between day and phase also significantly affected the number of cells per colony (day effects: $F = 161.728$, $P = 0.000$; treatment effects: $F = 3138.241$, $P = 0.000$; day × treatment effects: $F = 72.352$, $P = 0.000$) and colony proportion of *C. vulgaris* (day effects: $F = 29.213$, $P = 0.000$; treatment effects: $F = 1094.324$, $P = 0.000$; day × treatment effects: $F = 9.713$, $P = 0.000$). Tukey's *post hoc* test indicated that *M. aeruginosa* filtrates in EP and SP significantly increased the number of cells per colony in *C. vulgaris* ($P = 0.000$) compared with those in the control. The effects of *M. aeruginosa* filtrate in SP on the increase in the number of cells per colony of *C. vulgaris* were stronger than those in EP ($P = 0.000$). According to Tukey's *post hoc* test, the colony proportion of *C. vulgaris* in the control w *C. vulgaris* as significantly lower than that of *M. aeruginosa* filtrate treatment in EP and SP ($P = 0.000$). The colony

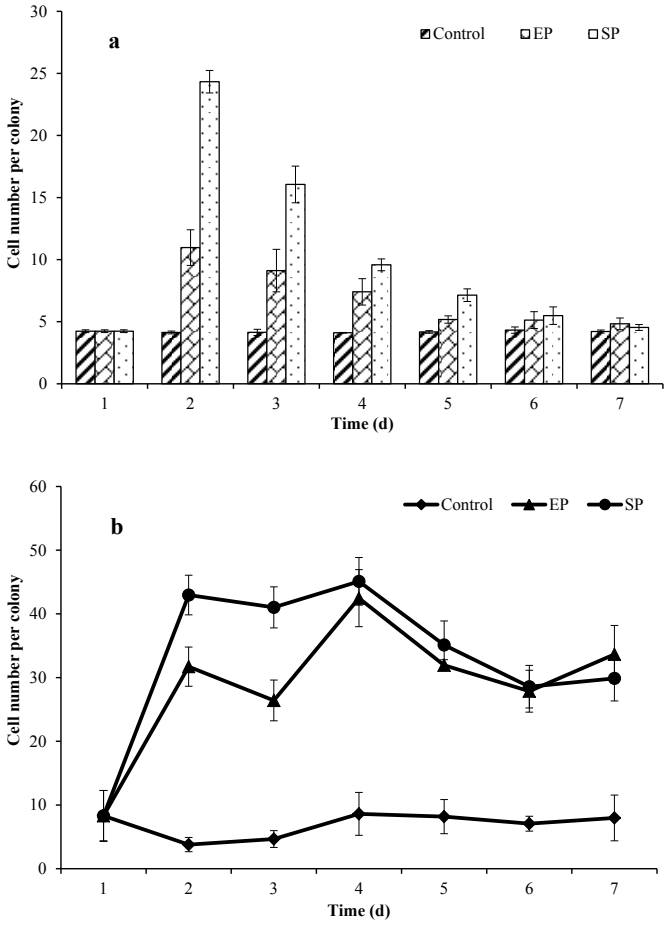

Fig. 3. Cell number per colony (a) and colony proportion of *C. vulgaris* (b), in the control and treatment with filtrate of *M. aeruginosa*.

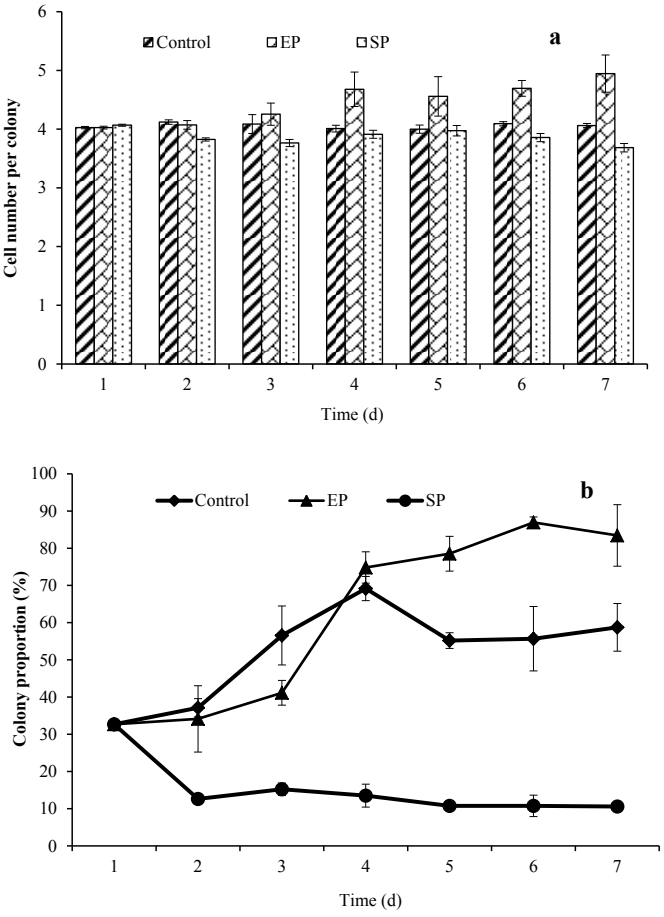

Fig. 4. Cell number per colony and colony proportion of *Kirchneriella* sp. (b) in the control and *M. aeruginosa* filtrate.

proportion of *C. vulgaris* in SP was much higher than that of *C. vulgaris* in EP ($P = 0.003$).

The treatment with *M. aeruginosa* filtrate at different cultivation phases also significantly influenced the cell number per colony (repeated ANOVA, day effects: $F = 13.242$, $P = 0.000$; treatment effects: $F = 211.252$, $P = 0.000$; day × treatment effects: $F = 6.589$, $P = 0.000$) and colony proportion (repeated ANOVA, day effects: $F = 92.966$, $P = 0.000$; treatment effects: $F = 590.400$, $P = 0.000$; day × treatment effects: $F = 23.268$, $P = 0.000$) of *Kirchneriella* sp (Fig. 4) . Furthermore, Tukey's *post hoc* test indicated that significant differences were detected in the number of cells per colony of *Kirchneriella* sp. between the control and *M. aeruginosa* filtrates in EP and SP ($P = 0.000$). According to Tukey's *post hoc* test, the colony proportion of *Kirchneriella* sp. in the control was significantly lower than that in *M. aeruginosa* filtrate treatment in EP ($P = 0.001$). The colony proportion of *Kirchneriella* sp. in SP was much lower than that in *M. aeruginosa* filtrate in EP ($P = 0.000$).

4 Discussion

Culture filtrate method is a classic approach to determine allelopathy between phytoplankton species (Wang *et al.*, 2017b). Considering our hypothesis, we demonstrated the remarkable allelopathic effects of toxic *M. aeruginosa* on *C. vulgaris* and *Kirchneriella* sp. Our results also suggested that *M. aeruginosa* filtrate could cause growth effects (growth rate) and influence the morphological characteristics of the tested green algae. To the best of our knowledge, this study is the first to report the cyanobacterium-induced colony formation of green algal strains. Moreover, our studies suggested that these effects were dependent on the growth stages of *M. aeruginosa* and the strain of the target algae.

Allelopathy functions through the release of allelochemicals into the surrounding water which can negatively affect other competitive organisms. However, allelopathic stimulations have been also reported in the interaction between algae (Pratt, 1966; DellaGreca *et al.*, 2010; Campos *et al.*, 2013). Our results also indicated that *M. aeruginosa* filtrates could promote the growth rate of *C. vulgaris*. This consistency might be attributed to the dependence of the effects of algae on their concentration (Campos *et al.*, 2013; Pinheiro *et al.*, 2013; Rzymski *et al.*, 2014; B-Béres *et al.*, 2015). Pratt (1966) suggested that high concentrations of *Olisthodiscus luteus* can inhibit the growth of *Skeletonema costatum*, whereas low concentrations stimulate the growth of *S. costatum*. DellaGreca *et al.* (2010) further reported that the growth of *Pseudokirchneriella subcapitata* is stimulated by *C. vulgaris* of low densities but is inhibited by *C. vulgaris* of high densities. Low

concentrations of cyanobacteria or crude extracts can promote the growth of *C. vulgaris* (Campos *et al.*, 2013), *Chlamydomonas reinhardtii* and *Nannochloropsis* sp. (Pinheiro *et al.*, 2013). Song *et al.* (2013) also indicated that the growth of *Botryococcus braunii* is induced by filtrates derived from low or moderate *C. vulgaris* density cultures but is prevented in the filtrate derived from a high *C. vulgaris* density culture. The present study also discussed the influences of the cultivation phase of *M. aeruginosa* filtrate on the target algae. Some studies reported that the inhibitory effects of filtrates in SP are stronger than those of other growth phases (Arzul *et al.*, 1999; Suikkanen *et al.*, 2004; Wang *et al.*, 2017b). Conversely, other studies have indicated that the effects of filtrates obtained from cultures of SP on target species are weaker than those derived from the same monoculture in EP (Suikkanen et al., 2004; Zhang *et al.*, 2013; Wang et al., 2017a). The present study revealed that the effects of different cultivation phases of *M. aeruginosa* on target algae were also species dependent (Leflaive and Ten-Hage, 2007). For *C. vulgaris*, no significant differences were detected in the growth effects caused by *M. aeruginosa* filtrate in EP and SP. However, *M. aeruginosa* filtrate in EP inhibited the growth of *Kirchneriella* sp., whereas *M. aeruginosa* filtrate in SP significantly promoted its growth.

Many studies about phytoplankton phenotypic plasticity induced by physio-chemical variables, zooplankton, submerged plants or other algae have been published (Mulderij *et al.*, 2005; Leflaive *et al.*, 2008; Van Donk *et al.*, 2011; Mello *et al.*, 2012; Dong *et al.*, 2013, 2018). In addition to growth effects, the morphological responses of the two green algae to *M. aeruginosa* filtrate were described, and such responses were dependent on cultivation phase. For *C. vulgaris*, *M. aeruginosa* filtrate treatment promoted algal cells (*C. vulgaris*) to aggregate into more colonies compared with that of the control, and the effects of the SP filtrate were stronger than those of the EP filtrate. In our study, despite the slight inhibition of the growth of *C. vulgaris* at the beginning of the experiments, the final growth rate of *C. vulgaris* was enhanced. One explanation for this might be that the initial growth inhibition was related to the colony formation, that the growth inhibition was similar to the peak of colony formation of *C. vulgaris*. In the present study, the fresh cell-free *M. aeruginosa* filtrate was only added once at the beginning of the experiment (the concentration was maximal) and showed strong effects on colony formation of the target algae in the beginning. Thereafter, the colonies gradually broke into small colonies and single cells; until the end of the experiment (the seventh day), no differences were detected between control and filtrate treatment. This might be due to the degradation of the allelochemicals with the increasing experimental time. At the beginning of the experiment, the high amount of allelochemicals promoted the colony formation of the target algae, the process of which needed excessive investment of energy to accumulate more intracellular carbohydrate, which inhibited the growth of target algae *Scenedesmus* (Zhu *et al.*, 2016). Whereas, Dong *et al.* (2018) suggested that with the degradation of active compounds of the filtrate, the carbohydrates stored in colonies might provide sufficient energy for target algae *C. vulgaris* to grow and develop. Thus, in a laboratory experiment, the one-time addition of filtrates might be advantageous for the growth of *C. vulgaris*.

The response of growth and morphology of *Kirchneriella* sp. was different from that of *C. vulgaris*. The present results suggested that filtrates of *M. aeruginosa* in EP significantly promoted big and numerous colonies in *Kirchneriella* sp. These colonies still existed until the end of the experiments. Filtrates of *M. aeruginosa* in SP were accompanied with a small cell colony of *Kirchneriella* sp. This result was also considered to provide a theoretical basis for the different responses of *Kirchneriella* sp. to filtrates of *M. aeruginosa* in various cultivation phases. Colony formation required high amounts of energy, thus inhibiting growth, whereas single cells were preferable for algal growth (Zhu *et al.*, 2016). The different morphological responses of *Kirchneriella* sp. to filtrates of *M. aeruginosa* in EP and SP suggested that the two phases might possess various active ingredients which should be further identified and analysed (Pakdel *et al.*, 2013). Besides, the different responses of *C. vulgaris* and *Kirchneriella* sp. also suggested that the allelochemicals effects were species-dependent.

Finally, we discussed the substances implicated in the interaction between algae. Different allelochemical substances, including amino acids, enzymes, lipids, vitamins and toxins, released during algal growth might affect the growth of other algae (Leflaive and Ten-Hage, 2007). The secretion of exopolysaccharide (EPS) promotes the colony formation of target algae (Yang *et al.*, 2009; Yang and Kong, 2012). In our tests involving *M. aeruginosa* filtrates, a high amount of EPS was detected compared with that of the control (data not shown here). Thus, EPS might influence our study on algal colony formation, and other substances were implicated in this process. A large colony was detected in *Kirchneriella* sp. under EP *M. aeruginosa* filtrate treatment, whereas a small colony was observed in SP treatment compared with those of the control. For *Microcystis*, microcystin (MC) was considered a key factor affecting other algae (Valdor and Aboal, 2007; Yang *et al.*, 2014), and several cases involving MCs as allelopathic compounds have been reported (Pflugmacher, 2002; Yang *et al.*, 2014; Bittencourt-Oliveira *et al.*, 2015). The allelochemical properties of MC have been debated (Babica *et al.*, 2006), and recent reports have revealed that a non-toxic *Microcystis* strain can affect the growth of *C. vulgaris* (Ma *et al.*, 2015). Thus, other allelochemicals should be discussed in future studies.

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