Effects of toxic cyanobacterium *Microcystis aeruginosa* on the morphology of green alga *Chlorella vulgaris*

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**Abstract** – In eutrophic freshwater lakes, cyanobacteria and green algae are in succession due to abiotic factors. Allelochemical interaction also exists. In this study, we indicated that filtrates of *Microcystis aeruginosa* can influence the growth rate and photosynthetic pigments of the target alga named *Chlorella vulgaris*. We also determined the morphological appearance and colony formation of green alga *C. vulgaris* that were induced by chemicals associated with a competitor cyanobacterium named *M. aeruginosa*. However, microcysts (MCs) were not the active substances in this study. The morphology changes and growth of *C. vulgaris* affected by *M. aeruginosa* filtrates were dependent on the initial cultivation density of the target algae and the cultivation phase of *M. aeruginosa*. We also assumed that the morphology changes were defensive strategies utilised by *C. vulgaris* to resist *M. aeruginosa*. The temporary stress of *M. aeruginosa* was favourable to the growth of *C. vulgaris*. By contrast, the continuous induction of colony formation by *M. aeruginosa* in the field inhibited the growth of *C. vulgaris*. The present results provided new insights into the interaction between algae and theoretical basis for algae succession in the field.

**Keywords:** *Microcystis aeruginosa* / *Chlorella vulgaris* / allelopathy / colony formation / initial cultivation density / cultivation phase / microcystins

1 Introduction

Given the eutrophication and climate warming, cyanobacterial blooms occurred frequently in freshwater lakes, rivers, reservoirs and ponds worldwide (Paerl and Huisman, 2008; Li et al., 2012). In these areas, *Microcystis* is the major contributor (Davis et al., 2009; Paerl et al., 2010; Zhang et al., 2010); for example, *Microcystis* sp. dominated Lake Taihu (Chen et al., 2003; Cai et al., 2012; Ma et al., 2015) and Dianchi in China (Dong et al., 2015). *Microcystis* blooms have also received increasing attention and posed considerable threats to the survival of aquatic organisms and humans (Chen et al., 2003; Dittmann and Wiegard, 2006; Dong et al., 2015).

The underlying mechanisms of this *Microcystis* dominance and severe reduction of phytoplankton diversity have been widely studied but still not completely understood. Several studies have been conducted to evaluate the role of abiotic factors (mainly hydrodynamics, water temperature, light and nutrients) on the dominance of certain algae. However, recent studies reported that biotic factors underlie the succession between *Microcystis novacekii* and *Scenedesmus quadricauda* or between *Skeletonema costatum* and *Prorocentrum dentatum* (Kuwata and Miyazaki, 2000). Therefore, many researchers proposed that allelopathy may also be an important mechanism for certain algae to obtain a competitive advantage in freshwater ecosystem (Gross, 2003; Jin and Dong, 2003; Leão et al., 2009; Rzymski et al., 2014; Yang et al., 2014). Allelopathy is the release of secondary metabolites, which influence the growth of other organisms. Allelopathic inhibition has been observed in various interactions between cyanobacteria and green algae, and intraspecies competition within cyanobacteria, and green algae (Tab. 1).

Cyanobacteria are one of the most important producers of allelochemicals and toxins in freshwaters (Pflugmacher, 2002; El-Sheekh et al., 2010; Ma et al., 2015). Therefore, cyanobacteria may influence the outcome of competition and dominance of algal communities in aquatic environments by selectively suppressing other growth (Legrand et al., 2003; Gantar et al., 2008; Graneli et al., 2008; Ma et al., 2015). Phytoplankton composition in freshwater ecosystems (especially for eutrophic water region) varies, and it often includes cyanobacteria and green microalgae as major components (Chen et al., 2003; Okello et al., 2010; Cai et al., 2012). In eutrophic lakes, such as Lake Taihu, cyanobacteria and green
alge demonstrate seasonal succession (Cai et al., 2012). Our previous study also suggested that seasonal dynamics occur between cyanobacteria and green algae in Lake Dianchi, China (Dong et al., 2015).

Nevertheless, despite the recent studies on the allelopathic interactions between cyanobacteria and green algae (Tab. 1), the effects of cyanobacteria, (1) allelopathic inhibition or stimulation on target algae and (2) the role of cultivation phase of cyanobacteria and initial biomass of target algae between these interactions, remain unknown. To date, related studies are rare. Thus, we need to improve our understanding of the inhibitory and stimulatory interactions amongst different cultivation biomass of the target algae to elucidate the role of allelopathy. In addition, previous studies mainly focused on the growth effects caused by allelopathic interaction. In recent years, some studies found that direct competition between phytoplankton can also influence the morphology of the target algae (Leflaive et al., 2008; Mello et al., 2012). However, the existing relative studies are limited, and many issues must be addressed. The ecological meaning of these morphological changes has also not been studied.

In the present study, we use toxic cyanobacterium Microcystis aeruginosa and green alga Chlorella vulgaris to determine (1) whether cell-free filtrates associated with M. aeruginosa can affect the growth, the underlying mechanism of this effect and the morphology of green alga C. vulgaris. We also ascertained (2) whether C. vulgaris with different initial inoculation biomass demonstrated different responses to cell-free filtrates associated with M. aeruginosa and (3) the ecological significance of morphological changes in C. vulgaris when it encountered M. aeruginosa. (4) Finally, we discussed whether microcysts (MCs) played roles in the effects of M. aeruginosa on C. vulgaris in our study. Previous studies suggested that MCs possibly act as allelopathic substances in interspecific phytoplankton interactions (Sedmak and Kosi, 1998; Kaebernick and Neilan, 2001).

2 Materials and methods

2.1 Strains and algal culture

A strain of cyanobacteria M. aeruginosa (FACHB-905), which was isolated from Dianchi Lake, China, and green alga C. vulgaris (FACHB-8) were provided by Freshwater Algae Culture Collection of Institute of Hydrobiology (FACHB), Chinese Academy of Sciences. Prior to experimentation, both of the strains were cultivated routinely using the sterile BG11 medium (Rippka et al., 1979) at 25°C, with continuous illumination of 25 μmol photons s⁻¹ m⁻² under a 12-h light: 12-h dark cycle. Both the cultures were shaken three times within a day to prevent algal sedimentation.

2.2 Experimental design

2.2.1 Experiment I: this experiment determined whether cell-free filtrates of M. aeruginosa can influence the growth and morphology of target green algae C. vulgaris and whether the growth phase of M. aeruginosa or the initial cultivation biomass of C. vulgaris plays any role

2.2.1.1 Preparation of cell-free filtrates associated with M. aeruginosa

The initial cultivation biomass of M. aeruginosa was determined by its optical density (OD) at 665 nm (blue-green alge) under ultraviolet/visible spectrophotometer, and the initial OD₆₆₅ was 0.2 (with cell density 1.86 × 10⁹ cells/L). The cell-free filtrate of M. aeruginosa was obtained when M. aeruginosa grew to the exponential stationary phase (EP) (cultured for 5 days, when OD₆₆₅ = 0.56) and stationary phase (SP) (cultivating for 30 days, when OD₆₆₅ = 1.56) (Wang et al., 2017). M. aeruginosa cultures at different growth phases were centrifuged at 8000 rpm for 10 min and filtered through Whatman GF/C filters (Whatman International Ltd., Maidstone, England) for subsequent experimentation (Mello et al., 2012).

2.2.1.2 Effects of cell-free filtrates associated with M. aeruginosa on C. vulgaris

All experiments were carried out in 250 mL Erlenmeyer flasks with 150 mL volume. The experimentation showed three gradients: control (filtrate BG11 medium) and EP and SP filtrates of M. aeruginosa (nutrients were added similar to that of BG11 medium to compensate for nutrient decrease during the preliminary cultivation period). Correspondingly, the initial inoculation OD values of tested green alga C. vulgaris were 0.05, 0.1 and 0.2, respectively (Tab. 2). All other

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**Table 1. Allelopathic interactions between algae.**

<table>
<thead>
<tr>
<th>Taxa</th>
<th>Influencing algae</th>
<th>Target algae</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Green algae</td>
<td>Chlorella vulgaris</td>
<td>Pseudokirchneriella subcapitata</td>
<td>Fergola et al., 2007</td>
</tr>
<tr>
<td></td>
<td>Chlamydomonas reinhardtii</td>
<td>Haematocrucus pluvialis</td>
<td>McCracken et al., 1980</td>
</tr>
<tr>
<td></td>
<td>Olisthodiscus luteus</td>
<td>Skeletonema costatum</td>
<td>Pratt, 1966</td>
</tr>
<tr>
<td>Cyanobacteria-green algae</td>
<td>Microcystis aeruginosa</td>
<td>Scenedesmus obliquus</td>
<td>Zheng et al., 2008</td>
</tr>
<tr>
<td></td>
<td>Quadrigula chodatii</td>
<td>Microcystis aeruginosa</td>
<td>Zhang et al., 2013</td>
</tr>
<tr>
<td></td>
<td>Chlamydomonas reinhardtii</td>
<td>Anaabaena flos-aquae</td>
<td>Kearns and Hunter, 2002</td>
</tr>
<tr>
<td></td>
<td>Microcystis aeruginosa</td>
<td>Oocystis marsonii</td>
<td>Dunker et al., 2013</td>
</tr>
<tr>
<td>Cyanobacteria-Cyanobacteria</td>
<td>Microcystis sp.</td>
<td>Nostoc sp.</td>
<td>Valdor and Aboal, 2007</td>
</tr>
<tr>
<td></td>
<td>Cylindropermopsis raciborskii</td>
<td>Microcystis aeruginosa</td>
<td>Mello et al., 2012</td>
</tr>
<tr>
<td></td>
<td>Microcystis aeruginosa</td>
<td>Microcystis wesenbergii</td>
<td>Yang et al., 2014</td>
</tr>
</tbody>
</table>
Table 2. Experimental design of the present study.

<table>
<thead>
<tr>
<th>Initial cultivation biomass (OD)</th>
<th>Control</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Filtrate of BG11 medium</td>
<td>Filtrate of EP</td>
</tr>
<tr>
<td>OD = 0.05</td>
<td>Parallel 1–3</td>
<td>Parallel 4–6</td>
</tr>
<tr>
<td>OD = 0.1</td>
<td>Parallel 1–3</td>
<td>Parallel 4–6</td>
</tr>
<tr>
<td>OD = 0.2</td>
<td>Parallel 1–3</td>
<td>Parallel 4–6</td>
</tr>
</tbody>
</table>

cultivation conditions were the same as described above. The experiments lasted for 7 days.

2.2.1.3 Parameter measurement

To evaluate the effects of *M. aeruginosa* on the morphology of *C. vulgaris*, samples (2 mL) of *C. vulgaris* were viewed under an inverted microscope at 400× magnification. The cell density was determined using Utermöhl method (Lund et al., 1958; Paxinos and Mitchell, 2000). The number of cells per colony and the colony proportion (minimum of three cells) were calculated according to the method of Lürling and Van Donk (1997). Inhibition (I) or enhancement (E) of colony formation to the control was calculated on the basis of the number per colony and colony proportion as follows (Wang et al., 2017):

\[
I/E = \frac{T}{C} - 1,
\]

where \(C\) represents the control data and \(T\) represents the filtrate treatment data.

Furthermore, to evaluate the allelopathic effects of *M. aeruginosa* on the growth of *C. vulgaris*, the chlorophyll \(a\) (Chla) of target green alga *C. vulgaris* was measured and calculated according to the method of Lichtenhaler and Buschmann (2001). The slope of the regression between transformed Chla and time (the lasting experimental period) was used as an estimate of the population growth rate (Lürling, 2006; Mello et al., 2012).

2.2.2 Experiment II: role of MCs in the interaction between *M. aeruginosa* and *C. vulgaris*

In the present study, we selected strain *M. aeruginosa* (FACHB-905), which was isolated from Dianchi Lake (Kunming, China) for study. This strain produces MC-LR of approximately 0.61 μg per 10^7 viable cells (Sun et al., 2012). Thus, to detect whether MC-LR played roles in effects on *C. vulgaris*, we also sorted to purified MC-LR (Taiwan Algal Science Inc., Taiwan R.O.C.) experiments. Four MC-LR concentrations (0.25, 1, 10, 100 μg L^-1), the nutrition was added the same as that of BG11) (Gan et al., 2012) were used to investigate the influences of MC-LR on *C. vulgaris*. The control was prepared without MCs. During the whole experiments, the growth and morphology changes of *C. vulgaris* were monitored every day. The experiments lasted for 7 days. The Chla content, growth rate, colony proportion and cell number per colony of *C. vulgaris* were calculated as that in Section 2.2.1.

2.3 Statistical analysis

Mean values and standard deviations were calculated for different replicates (\(n = 3\)). The effect of different inoculation biomass of *C. vulgaris* and the growth phase of *M. aeruginosa* on the growth rate of *C. vulgaris* were assessed using a two-way ANOVA with biomass and growth phase as independent factors and subsequent separate post hoc tests (Fisher LSD) (Wang et al., 2017).

The photosynthetic pigment, enhancement or inhibition of percentage of cells in colonies and the number of cells per colony of each treatment during the experiment were compared using a repeated measure ANOVA (\(\alpha = 0.05\)). Both tests were followed with Tukey's post hoc tests to detect the differences between treatments (\(\alpha = 0.05\)). A value of \(P < 0.05\) was considered significant in all analyses. Mauchly's sphericity was evaluated. Data were log-transformed when necessary. All these tests were performed in SPSS 13.0 for windows (Mello et al., 2012).

3 Results

3.1 Effects of *M. aeruginosa* filtrates on the cell number per colony and colony proportion of *C. vulgaris*

*M. aeruginosa* filtrates can influence the morphology of *C. vulgaris*. During the cultivation, particles were significantly observed in the treatment with *M. aeruginosa* filtrate (Fig. 1). According to microscopic observation and data analysis, it was also suggested that the largest colony formed on the second day under *M. aeruginosa* filtrate treatment and the cell number per colony gradually reduced, and, till the end of the experiment, no significant differences were detected between the control and the treatment (Figs. 2 and 3).

According to repeated measure ANOVA analysis, the morphology response of *C. vulgaris* to *M. aeruginosa* filtrates was significantly influenced by its initial cultivation density and phase of *M. aeruginosa* filtrate treatment. The day and the interaction amongst density, day and phase also exerted significant effects on the cell number per colony and colony proportion of *C. vulgaris* (Tab. 3). Further Tukey's post hoc test indicated that significant differences were detected in cell number per colony amongst the three initial target algal cultivation densities (\(P = 0.000\)). The SP and EP *M. aeruginosa* filtrates both exerted significant influences on the cell number per colony of *C. vulgaris* (\(P = 0.000\)) compared with those of control. Significant differences were detected in the cell number per colony of *C. vulgaris* between SP and EP *M. aeruginosa* filtrate treatments (\(P = 0.000\)).

The Tukey's post hoc test results also suggested that significant differences existed in the colony proportion of *C. vulgaris* between SP and EP *M. aeruginosa* filtrate treatments (\(P = 0.000\)). The different initial cultivation ODs of the target algae also played significant roles in the response of *C. vulgaris* colony proportion (\(P = 0.000\)).

3.2 Responses of photosynthetic pigments (Chla) of *C. vulgaris* to *M. aeruginosa* filtrates

There is no difference in the Chla contents of *C. vulgaris* between control and experiments (*M. aeruginosa* filtrates
under the SP and EP) during the first 3 days. However, on the fourth day, the photosynthetic pigments of \textit{C. vulgaris} were significantly higher in SP and EP \textit{M. aeruginosa} filtrate treatments than those of the control (Fig. 4).

According to repeated measure ANOVA analysis, the Chl contents in \textit{C. vulgaris} were significantly influenced with its initial cultivation biomass and the phase of \textit{M. aeruginosa} filtrate treatment. The day and the interaction amongst density, day and phase also exerted significant effects on the Chl contents of \textit{C. vulgaris} (Tab. 2). Tukey’s post hoc test indicated that SP and EP \textit{M. aeruginosa} filtrates exhibited significant influences on Chl contents in \textit{C. vulgaris} compared with those of control (\(P = 0.000\)). Significant differences were also detected in Chl contents of \textit{C. vulgaris} between SP and EP \textit{M. aeruginosa} filtrate treatments (\(P = 0.002\)).

In addition, according to Tukey’s post hoc test, the initial cultivation density of \textit{C. vulgaris} also played important roles in the target algal response to \textit{M. aeruginosa} filtrates. The significant differences detected in the Chl contents of \textit{C. vulgaris} between the initial cultivation OD were 0.05 and 0.1 (\(P = 0.000\)), 0.05 and 0.2 (\(P = 0.000\)) and 0.1 and 0.2 (\(P = 0.004\)).

### 3.3 Effects of \textit{M. aeruginosa} filtrates on the growth rate of \textit{C. vulgaris}

The growth rate of \textit{C. vulgaris} was significantly affected with its initial cultivation density (\(df = 2; \bar{F} = 3650.708; P = 0.000\)). The Tukey’s post hoc test revealed significance in three groups. The growth rate of \textit{C. vulgaris} with low cultivation density (OD = 0.05) was significantly higher than those with OD of 0.1 and 0.2 (Fig. 5).

The growth rate of \textit{C. vulgaris} also demonstrated significant differences amongst control and treatment with different phases of \textit{M. aeruginosa} filtrates (\(df = 2; \bar{F} = 140.263; P = 0.000\)). Further, Tukey’s post hoc test showed that the growth rate of \textit{C. vulgaris} in control was significantly lower than those in SP and EP \textit{M. aeruginosa} treatments (\(P = 0.000\)). Significant difference was also observed in the growth rate of \textit{C. vulgaris} between \textit{M. aeruginosa} filtrate treatments in SP and EP (\(P = 0.000\)).

### 3.4 Effects of MC-LR on \textit{C. vulgaris}

Different from our expectations, the MC-LR with three concentrations exerted no significant influences on the growth and morphology of \textit{C. vulgaris} compared with that of control (data are not shown).

### 4 Discussion

Allelopathic inhibition is more frequently reported between algal interactions than that of promotion effects.
Cyanobacterin is a biochemical between algal interactions. The cyanobacterin secreted by Scytonema hofmannii can inhibit the growth of cyanobacteria (Gleason and Paulson, 1984), eukaryotic algae (Gleason and Baxa, 1986) and higher plants (Gleason and Case, 1986). Moreover, a bloom-forming cyanobacterium Anabaena flos-aquae can significantly inhibit the growth of Chlamydomonas reinhardtii (Kearns and Hunter, 2001). The exudates from Microcystis also inhibit the growth of Peridium gatunse (Sukenik et al., 2002). Considerable studies indicated that MCs or other allelochemicals associated with cyanobacteria are crucial in inhibiting competitive phytoplankton (Suikkanen et al., 2004; B-Béres et al., 2015; Cirés and Ballot, 2016; Harke et al., 2016) or macrophytes (Zheng et al., 2013; Xu et al., 2015; Xu et al., 2016). The most common reported modes of action of allelochemicals towards the target algae include the inhibition of photosynthesis by affecting photosystem II activity (Bagchi et al., 1990; Bagchi, 1995; Srivastava et al., 1998, 2001; Bagchi and Ray, 2001), inhibiting RNA synthesis of other organisms (Doan et al., 2000), interfering with its internal carbonic anhydrase activity (Sukenik et al., 2002), influencing the hormone concentration or disrupting the amino acid metabolism (Weir et al., 2004). The present study proposed that M. aeruginosa may influence the growth by affecting the morphology of target algae.

The present study proved the hypothesis that the cell-free filtrate of toxic M. aeruginosa can affect the morphology of target algae by inducing large colonies in C. vulgaris. The study also provided new insights in allelopathically induced colony formation between algae. To our knowledge, to date, only two previous studies reported about this induced morphological change in alga caused by another alga. Leflaive et al. (2008) demonstrated that the allelochemicals released by the filamentous alga Uronema confervicolor triggered the colony formation in Desmodesmus quadrispina. The other study of Mello et al. (2012) indicated that allelochemicals secreted by Cylindrospermopsis raciborskii can induce the colony formation of M. aeruginosa.

We also indicated that the cell-free filtrates of M. aeruginosa in EP and SP treatments significantly reduced the growth of C. vulgaris at the first 3 days during the cultivation periods. Such reduction was also accompanied with the colony formation of C. vulgaris. This was in accordance with that of Zhu et al. (2016) who reported that colony formation requires excessive energy investment, which inhibits the algal growth. Our previous study suggested that
the colony formation in *C. vulgaris* is due to the accumulation of intracellular carbohydrate (Dong et al., 2018) or carbohydrate secretion. For the target algae, this colony formation may be a defensive strategy to counter adverse conditions (such as competitor toxic *Microcystis*). Mello et al. (2012) also suggested *M. aeruginosa* forms colonies, which is potentially a defensive mechanism against growth-inhibiting allelochemicals produced by *C. raciborskii*. This hypothesis was supported by Park et al. (2009) who also showed that, when exposed to an allelopathic compound isolated from rice (*Oryza sativa*), the growth inhibition in *M. aeruginosa* is stronger in single cells than in colonies. In the field, the large colonies prevent zooplankton from eating them (Van Donk et al., 2011), which is also accompanied with disadvantageous effects, such as increased sedimentation (Dong et al., 2013), and reduced growth rate. Thus, if the *Microcystis* stress is temporary, then the preliminary carbohydrates stored in colonies may provide sufficient energy for them to grow and develop (Dong et al., 2018). This hypothesis was in agreement with our results. In the present study, the fresh cell-free filtrate of *M. aeruginosa* was only added once at the beginning of the experiment (the concentration was maximal). It was suggested that the growth of *C. vulgaris* was reduced, which was accompanied with colony formation at the beginning of the experiment (Figs. 2 and 3). Afterwards, with the degradation of active compounds in the filtrate with the increased experimental time (Wang et al., 2017; Dong et al., 2018), the colonies gradually broke into small colonies and single cells. Until the end of the experiment (seventh day), no differences were detected between the control and filtrate treatments. The final growth rate of *C. vulgaris* in the filtrate treatment was significantly enhanced (Fig. 5). Thus, we concluded that in laboratory experiment, adding filtrates only once was advantageous for the growth and development of *C. vulgaris*. This result was in agreement with that of Zak et al. (2012) who demonstrated the stimulated growth of *C. vulgaris* induced by cyanobacteria *Nodularia spumigena*. In the field, the continuous secretion of allelochemicals from *Microcystis* will inhibit the growth of these target green algae, where morphology changes may be crucial.

In the present study, two key issues were also addressed. The first issue is whether filtrates from different culture periods of *M. aeruginosa* exerted different effects on *C. vulgaris*. Previously, limited number of studies have investigated and compared the effects of extracellular metabolites from different growth stages of algae. However, considerable inconsistency exists amongst research results regarding the question of which growth phase of cyanobacteria exerts the strongest allelopathic effects. Arzul et al. (1999) demonstrated a stronger allelopathic influence of three *Alexandrium* species in SP than that in EP. Suikkanen et al. (2004) indicated that the toxicity of *N. spumigena* in SP is higher than that in EP. Nevertheless, other authors suggested a completely opposite effect: filtrates obtained from cultures of the SP exert less effect on target species than that of filtrates obtained from the same monoculture in the EP (Suikkanen et al., 2004; Wang et al., 2017). The present study proved the hypothesis that the effects of *M. aeruginosa* on target alga *C. vulgaris* depended on cyanobacterial growth phase. The *M. aeruginosa* filtrate treatment of SP exerted more significant influences on the morphology and growth of *C. vulgaris* than that under EP treatment.

Table 3. Repeated measures analysis for Chla contents in *C. vulgaris* and cell number per colony and colony proportion of *C. vulgaris* in control versus *M. aeruginosa* filtrates treatment, and amongst three initial cultivation densities during days 1–7.

<table>
<thead>
<tr>
<th>Variable</th>
<th>RM test</th>
<th>df</th>
<th>F-value</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chla contents in <em>C. vulgaris</em></td>
<td>Phase of <em>M. aeruginosa</em> filtrates (phase)</td>
<td>2</td>
<td>143.400</td>
<td>0.000</td>
</tr>
<tr>
<td></td>
<td>Initial cultivation density of target algae (density)</td>
<td>2</td>
<td>1605.443</td>
<td>0.000</td>
</tr>
<tr>
<td></td>
<td>Time</td>
<td>2.742</td>
<td>7535.784</td>
<td>0.000</td>
</tr>
<tr>
<td></td>
<td>Time × phase</td>
<td>5.484</td>
<td>38.117</td>
<td>0.000</td>
</tr>
<tr>
<td></td>
<td>Time × density</td>
<td>5.484</td>
<td>255.406</td>
<td>0.000</td>
</tr>
<tr>
<td></td>
<td>Phase × density</td>
<td>4</td>
<td>17.255</td>
<td>0.000</td>
</tr>
<tr>
<td></td>
<td>Time × phase × density</td>
<td>10.968</td>
<td>3.311</td>
<td>0.002</td>
</tr>
<tr>
<td></td>
<td>Phase of <em>M. aeruginosa</em> filtrates (phase)</td>
<td>1</td>
<td>189.605</td>
<td>0.000</td>
</tr>
<tr>
<td></td>
<td>Initial cultivation density of target algae (density)</td>
<td>2</td>
<td>544.047</td>
<td>0.000</td>
</tr>
<tr>
<td></td>
<td>Time</td>
<td>2.491</td>
<td>735.450</td>
<td>0.000</td>
</tr>
<tr>
<td>Cell number per colony (-fold)</td>
<td>Time × phase</td>
<td>2.491</td>
<td>61.130</td>
<td>0.000</td>
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<td></td>
<td>Time × density</td>
<td>4.981</td>
<td>139.765</td>
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<tr>
<td></td>
<td>Phase × density</td>
<td>2</td>
<td>59.308</td>
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<td>Time × phase × density</td>
<td>5.641</td>
<td>32.443</td>
<td>0.000</td>
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<tr>
<td></td>
<td>Phase of <em>M. aeruginosa</em> filtrates (phase)</td>
<td>1</td>
<td>231.100</td>
<td>0.000</td>
</tr>
<tr>
<td></td>
<td>Initial cultivation density of target algae (density)</td>
<td>1</td>
<td>515.789</td>
<td>0.000</td>
</tr>
<tr>
<td></td>
<td>Time</td>
<td>1.765</td>
<td>772.467</td>
<td>0.000</td>
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<tr>
<td>Colony proportion (-fold)</td>
<td>Time × phase</td>
<td>1.765</td>
<td>33.352</td>
<td>0.000</td>
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<td>Time × density</td>
<td>3.529</td>
<td>75.843</td>
<td>0.000</td>
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<tr>
<td></td>
<td>Phase × density</td>
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<td>0.091</td>
<td>0.913</td>
</tr>
<tr>
<td></td>
<td>Time × phase × density</td>
<td>3.529</td>
<td>0.542</td>
<td>0.686</td>
</tr>
</tbody>
</table>

The second question is whether *C. vulgaris* with different initial inoculation biomass demonstrated different responses to cell-free filtrates associated with *M. aeruginosa*. During the cultivation, the final growth rate of *C. vulgaris* with low initial
cultivation biomass was significantly higher than that of higher cultivation density. In addition, more large colonies were detected in initial cultivation of *C. vulgaris* (OD = 0.05) than that of OD of 0.05 and 0.2. The present result was consistent with that of Jiang et al. (2015) who indicated that the allelopathic effects of *Hydrilla verticillata* on phytoplankton depended on phytoplankton density. Therefore, the high initial cultivation density of the target algae may resist remarkably against adverse conditions.

Finally, the present study also showed that the biochemicals associated with *M. aeruginosa* may play vital roles in affecting the growth and inducing colony formation of green-algae *C. vulgaris*, because no significant differences were observed in final nutrition content between the control and treatment groups. Bittencourt-Oliveira et al. (2016) speculated that the biochemical MCs may play roles in the colony formation of *C. vulgaris*. Although Sedmak and Elersèk (2005) and Gan et al. (2012) suggested that *M. aeruginosa* can exhibit large cell volumes when exposed to MCs, our simulated study suggested that MC-LR was not the key factor that influenced the morphology of *C. vulgaris*. This result was in agreement with that of Perreault et al. (2011) who reported that saxitoxins (released by *C. raciborskii*) induce no toxic effects on algal cells (*C. reinhardtii*). Thus, further separation and identification experiments should be conducted to analyse the active substances in the cell-free filtrates of *M. aeruginosa*, which influences the morphology and growth of *C. vulgaris* in our study.

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