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RESEARCH ARTICLE

Growth and morphological responses of *Chlorella vulgaris* at different initial algal densities to treatment with *Ceratophyllum demersum* methanol extracts

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Abstract – The allelopathically inhibitory effects of submerged macrophytes on phytoplankton have been extensively studied and are utilised as an effective strategy for water restoration. However, this technique has been minimally implemented in natural in situ water bodies because of the unclear and complex interactions involved. Our study considered the uneven density distribution of algae in natural aquatic ecosystems and the biomass-dependent effects of submerged macrophytes on target algae. *Ceratophyllum demersum* methanol extracts of gradient concentration on *Chlorella vulgaris* with different initial algal density were conducted in the present laboratory study. Results indicated that methanol extracts of *C. demersum* could not only inhibit the growth of but also promote colony formation of *C. vulgaris*. *C. vulgaris* of a low density exerted increased inhibition and colony proportion responses to *C. demersum* extracts. By 0.42 g/mL *C. demersum* treatment, the inhibition rate on *C. vulgaris* under 0.02 IAD (Initial Alagal Density) and 0.05 IAD ($p < 0.05$) was 88.7%, 70.9%, respectively. We also suggested that the effects of *C. demersum* were biomass dependent, such that extracts with high concentration could produce increased inhibitory effects on *C. vulgaris*. According to GC-MS analysis, the study revealed five potential compounds (i.e. hexanoic, acetoacetic, azelaic, palmitic and stearic acid) in the *C. demersum* methanol extracts. However, the individual or combined effects of those compounds require further exploration. This study proposed certain theoretical basis for future water restoration by submerged macrophytes, that the biomass of the macrophytes and the density of the algae should both be taken into account.

Keywords: Submerged macrophytes / green algae / allelopathy / initial algal density / biomass

1 Introduction

In natural shallow freshwater ecosystems, phytoplankton and aquatic macrophytes are the primary producers and play important roles in the energy flow of the food chain. However, given that phytoplankton and aquatic macrophytes share similar demands for growth, they exhibit extreme competition. One of the strategies employed by submerged plants to resist competitors is the release of active compounds, such as allelochemicals. Allelochemicals possess algicidal effects – an effective strategy to control harmful nuisance blooms; furthermore, these compounds are easily degradable and environmentally safe (Narwal, 2006). Allelopathic interactions between macrophytes and algae are complex and unclear; moreover, such interaction is controlled by abiotic environmental factors, such as nutrients, light and temperature (Leflaive and Ten-Hage, 2009; Graneli and Salomon, 2010; Antunes *et al.*, 2012; Barreiro and Hairston, 2013) and is dependent on action modes (Gao *et al.*, 2017). Regarding natural water bodies, further research is required to support the broad utilisation of submerged macrophytes for freshwater restoration (Jiang *et al.*, 2015). Firstly, aside from inhibiting bloom-forming cyanobacteria, allelochemicals also come into contact with many other algae, resulting in species-specific and diversified growth effects in natural aquatic ecosystems (Chang *et al.*, 2012; Eigemann *et al.*, 2013; Santonja *et al.*, 2018). Thus, when these plants are applied for the inhibition of harmful algae, the responses of other phytoplankton, such as green algae, must also be considered because some green algae are favourable food for zooplankton and other economical animals. Besides, the density distribution of phytoplankton in natural shallow freshwater ecosystems varies across water regions and may be affected by wind (Jiang *et al.*, 2015). Therefore, the interactions between algae with varying densities and aquatic macrophytes must be studied to establish

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Table 1. Experimental design of the experiments.

Initial algal density	Blank (no treatment)	DMSO Control	Treatment I 0.25 g/mL	Treatment II 0.42 g/mL	Treatment III 1.25 g/mL
IAD = 0.02	Parallel 1 to 3	Parallel 4 to 6	Parallel 7 to 9	Parallel 10 to 12	Parallel 13 to 15
IAD = 0.05	Parallel 1 to 3	Parallel 4 to 6	Parallel 7 to 9	Parallel 10 to 12	Parallel 13 to 15

an effective water restoration method. Furthermore, allelochemical influences are suggested to be dependent on macrophyte biomass (Zheng *et al.*, 2013; Zuo *et al.*, 2015; Donadi *et al.*, 2019); Thus when submerged macrophytes are applied for water restoration, the appropriate biomass must be considered. According to the issues mentioned above, understanding the effects between gradient macrophytes and algae of different densities was necessary in water restoration by submerged macrohytes in the practical natural in situ water bodies. However, so far, relevant studies have been rarely conducted and the active substances that participate in this process have yet to be determined for the mechanisms discussed.

Chlorella vulgaris is an asexual freshwater green alga, which can be found in freshwater bodies worldwide. *Ceratophyllum demersum* is also commonly found in eutrophic freshwater habitats (Zhao *et al.*, 2012). In the present work, the algal density and concentration gradients of macrophyte extracts were designed to further investigate how the submerged macrophyte *C. demersum* interacts with the green alga *C. vulgaris*. Finally, according to Gas Chromatography-Mass Spectrometry (GC–MS), we also aimed to discuss the potential active compounds isolated from *C. demersum*. This study aimed to provide further theoretical knowledge for the interaction between photosynthetic organism macrophytes and algae. Besides, according to discuss the responses of beneficial green algae, rather than cyanobacteria as in the previous reported studies, to the submerged macrohytes, our study would provide some basis for determining the practicability and feasibility in the future water restoration by macrophytes.

2 Materials and methods

2.1 Algal culture and macrophytes

An axenic culture of *C. vulgaris* (FACHB-8) was obtained from the Freshwater Algae Culture Collection of Institute of Hydrobiology, Chinese Academy of Sciences (Wuhan, China). Prior to experimentation, the stock culture was incubated in 500 mL Erlenmeyer flasks with 300 mL of BG_{11} mediumin, as reported by Rippka *et al.* (1979), at room temperature (25 °C) and under an irradiance of 25 μmol photons s^{-1} m^{-2} (12-h light:12-h dark cycle). The algae at exponential phase were utilised for the following experimentation.

The submerged macrophyte samples were collected from Tuan River, Nanyang, China (32°43′49″ N, 112°0′40″ E) and then acclimatised to laboratory for 1 month under cultivation conditions of *C. vulgaris* monoculture to simulate co-existence of the macrophytes and algae in natural water regions.

2.2 Preparation of *C. demersum* methanol extracts and algal cultivation

Fresh *C. demersum* was repeatedly washed with sterile water and prepared in 3, 5 and 15 g fresh weight (FW) as concentration gradients. The samples were ground, transferred to sterilised water, soaked and extracted for 24 h. The samples were centrifuged at 8000 r/min for 10 min, and the supernatants were filtered through a 0.22 μm membrane and concentrated using HLB extraction cartridges. The extracts were eluted with methanol, evaporated to dryness by using a nitrogen blower and diluted with 40 μL of dimethyl sulfoxide (DMSO, >98%).

C. vulgaris grown until the exponential phase was incubated in the medium with *C. demersum* methanol extracts, and the nutrients added were similar to those present in BG_{11} medium. In order to discuss the roles of algal density played in the responses of *C. vulgaris* to *C. demersum*, two initial cultivation optical densities (OD_{680}) of *C. vulgaris* were set at 0.02 and 0.05, that the values of which were determined by ultraviolet/visible spectrophotometer. The final concentrations of *C. demersum* extracts were 0.25, 0.42 and 1.25 g/mL. The solvent (DMSO) control for the exposure group and a blank containing the culture media without any treatment were prepared simultaneously. All treatments were performed in triplicate (Tab. 1), and the samples were cultivated in conditions similar to those of *C. vulgaris* monoculture. The entire experiment lasted for 7 d.

2.3 Parameter measurement

2.3.1 Photosynthetic pigments (Chl*a*) of *C. vulgaris*

The algal cells were centrifuged (12,000 r/min, 10 min, 4 °C) and extracted using 95% ethanol for 24 h at 4 °C in the dark. Absorbance was read at 665 and 470 nm (A_{665} and A_{649}) by using an ultraviolet/visible light spectrophotometer. Chl*a* concentrations were calculated according to Lichtenthaler and Buschmann (2001) as follows:

$$\text{Chl}a\ \text{concentration} = 13.95 \times A_{665} - 6.88 \times A_{649}.$$

2.3.2 Inhibition of *C. vulgaris* by *C. demersum* extracts

The inhibition rates (I) of *C. vulgaris* in the treatment groups compared with the DMSO control were measured as follows:

$$I(\%) = (C_t - C_c)/C_c \times 100\%,$$

where C_c and C_t represent the Chl*a* contents of *C. vulgaris* in the DMSO control and each treatment group, respectively.

2.3.3 Cell counting

Each replicate was examined under an inverted microscope at 400× magnification, and the morphological characteristics of the colonies were assessed using a minimum of three cells. Colony proportion was determined by calculating the ratio of the number of cells in the colonies to the total number of algae counted in each treatment (Dong *et al.*, 2018).

2.3.4 Gas chromatography–mass spectrometry (GC–MS) analysis

At the start of the experiment, fresh plants (15 g FW) were selected, rinsed with distilled water, carefully checked under a microscope to remove adherent zooplankton (Lürling *et al.*, 2006), homogenised by grinding and extracted in 300 mL of sterile water for 24 h. The supernatants were allowed to pass through 0.22 μm filters to exclude the influence of adherent bacteria (Fisher *et al.*, 2016), and the filtrates were stored for subsequent use. The filtrates were concentrated in the extraction column of HLB extraction cartridges, washed with ultrapure water and vacuum dried for further experimentation. The concentrates were washed with acetone and bis-trimethylsilyl fluoroacetamide (Sigma Aldrich, USA) silanisated before GC–MS analysis was conducted.

GC–MS was performed using an Agilent 6890N GC-mass spectrometer and 5973I mass spectrometer. The column of the instrument was an HP-5MS (0.25 mm × 30 m × 0.25 μm). The carrier gas used was helium, and the flow rate was 1.0 mL/min. Column heating was conducted as follows: temperature was initially set to 50 °C for 1 min, ramped to 280 °C at 10 °C/min and maintained for 5 min. Injection was performed in splitless mode at a volume of 1 μL.

2.4 Data analysis

Mean values and standard deviations were calculated for the different replicates ($n = 3$). One-way ANOVA was conducted to test for significant differences in Chl*a* content, inhibition rate and colony proportion among different treatments by using SPSS 13.0 for Windows (Wang *et al.*, 2017). A value of $p < 0.05$ was considered statistically significant in all analyses.

3 Results

The inhibition of *C. vulgaris* by *C. demersum* methanol extracts was dependent on the initial algal density (IAD) of *C. vulgaris* and on the concentration of *C. demersum* extracts. As shown in Figure 1B, the highest inhibition occurred on the third day. Meanwhile, by 0.42 g/mL *C. demersum* methanol extract treatment, the inhibition rate on *C. vulgaris* under 0.02 IAD was greater than that under 0.05 IAD ($p < 0.05$), that was 88.7%, 70.9%, respectively. However, when we treated *C. vulgaris* at low (0.25 g/mL) or high (1.25 g/mL) *C. demersum* methanol extract concentrations, no remarkable differences were detected between the two IADs (Fig. 1A and C).

The inhibition of *C. vulgaris* was also dependent on *C. demersum* extract concentration (Fig. 1). The highest inhibition occurred on the third day of cultivation, showing inhibition rates of 47%, 88% and 99% for extract concentrations of 0.25, 0.42 and 1.25 g/mL, respectively (Fig. 1). The addition of extracts once at the start of the experiment led to inhibitory effects that gradually decreased by the third day (Fig. 1A and B). However, the inhibitory effects of 1.25 g/mL extract remained high at the end of the experiment (Fig. 1C).

In the control, the colony proportion of *C. vulgaris* was approximately 2–5% (data not shown). However, *C. demersum* methanol extracts remarkably induced *C. vulgaris* colony

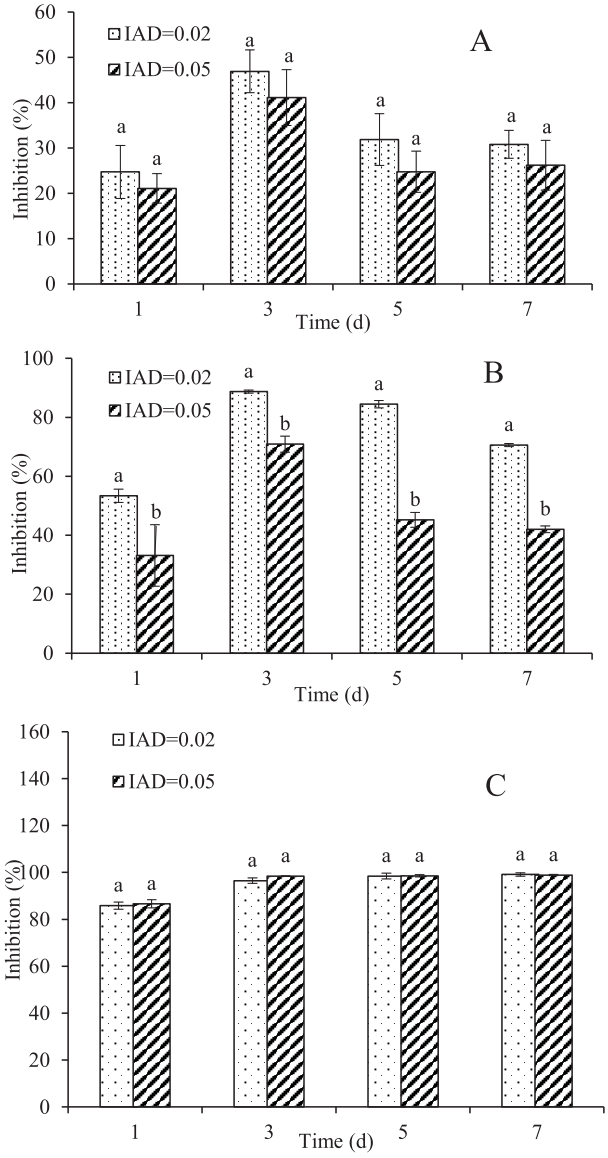

Fig. 1. Inhibition of *C. vulgaris* under different initial algal densities (IAD) by 0.25 g/mL (A), 0.42 g/mL (B) and 1.25 g/mL (C) of *C. demersum* methanol extracts.

formation, and the highest colony proportion was detected on the third day (Fig. 2). Treatment with different extract concentrations showed considerable differences, and the highest colony proportion of *C. vulgaris* treated with of 1.25 g/mL extract concentration was 21%, whereas the colony proportion was 66% and 62% after treatment with 0.25 and 0.42 g/mL extracts, respectively. Regarding the duration of the experiment, colony proportion gradually decreased since the third day.

The initial algal density could also affect the responses of *C. vulgaris* to macrophytes. As shown in Figure 2, *C. vulgaris* with low algal density was more sensitive to treatment with low extract concentration and formed more colonies compared with that with high algal density. Conversely, in high extract concentration, no differences were detected between the two density groups.

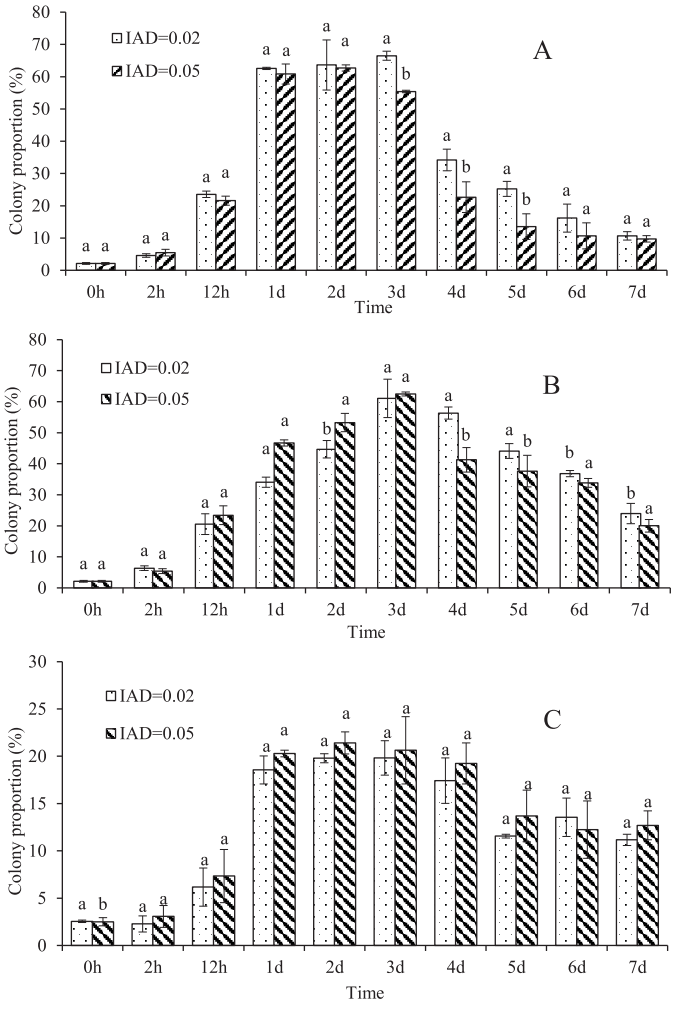

Fig. 2. Colony proportion of *C. vulgaris* under different initial algal densities (IAD) in the treatment with 0.25 (A), 0.42 (B) and 1.25 g/mL (C) *C. demersum* methanol extracts.

To investigate which compounds play roles in the macrophyte–alga interaction, GC–MS analysis were performed. The following five compounds were found in the *C. demersum* methanol extracts (Fig. 3 and Tab. 2): hexanoic acid, acetoacetic acid, azelaic acid, palmitic acid and stearic acid, which are belonging to fatty acids.

4 Discussion

Allelopathic interactions between macrophytes and phytoplanktons have been extensively studied (Gross, 2003; Mulderij *et al.*, 2007; Hilt and Gross, 2008; Fleming and Dibble, 2015; Öterler, 2017). Given their environment-friendly effects to aquatic ecosystems, submerged macrophytes have been utilised as an effective strategy for water restoration (Wang *et al.*, 2015, 2017; Zeng *et al.*, 2017; Kim *et al.*, 2018). However, the use of submerged macrophytes for the restoration of a natural water region remains limited because of the numerous factors that must be considered for their application in this type of environment. One factor is the season. Allelochemicals secreted by macrophytes are secondary metabolites, the secretion of which is influenced by temperature and other abiotic factors. Santonja *et al.* (2018) detected significant differences among seasonal allelopathic effects of aqueous *Ludwigia hexapetala* leaf extracts. The species-specific sensitivity of phytoplankton to macrophyte allelochemicals (Gross, 2003; Hilt and Gross, 2008) is another concern. Hilt and Gross (2008) suggested that diatoms and cyanobacteria are more sensitive to allelochemicals than chlorophytes, but the inhibitory effects of submerged macrophytes on green algae are also observed (Dong *et al.*, 2013, 2018). Thus, the response of green algae, especially the favourable food for ecological consumers, must also be considered.

In the present study, we suggested that different initial algal densities of *C. vulgaris* also demonstrated different

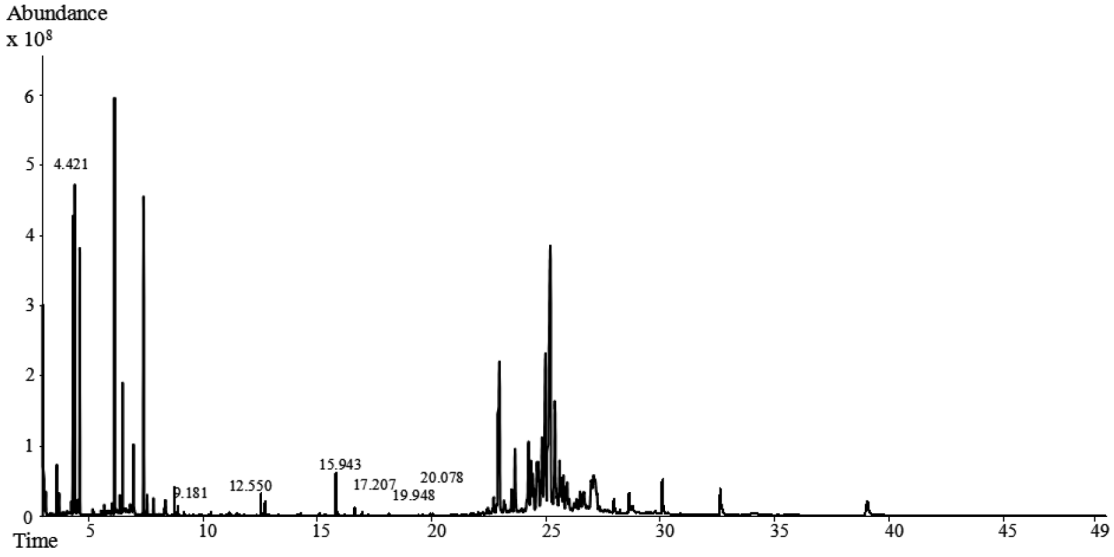

Fig. 3. GC–MS analysis of methanol extracts of *C. demersum*.

Table 2. Main active compounds obtained from *C. demersum* methanol extracts and identified through GC–MS.

Number	Residence time	Compound
1	4.44	Hexanoic acid
2	5,99	Acetoacetic acid
3	12.55	Azelaic acid
4	16.64	Palmitic acid
5	19.95	Stearic acid

responses to *C. demersum* methanol extracts. Low-density target algae were more sensitive to inhibition than their high-density counterpart. The result was concordant with that of Hong *et al.* (2008), who also demonstrated that the growth of freshwater green alga *Selenastrum capricornutum* was inhibited by allelochemicals at low IAD. Our results also suggested that the uneven distribution of algal density in the natural water body due to the influences of the wind must be considered during restoration (Jiang *et al.*, 2015). The biomass of submerged macrophytes utilised in water restoration is another concern (Donadi *et al.*, 2019). Dense (vs. sparse) vegetation may considerably influence water quality, affecting phytoplankton or facilitating invertebrate grazers (Krause-Jensen *et al.*, 2008). However, in a natural water body, too dense vegetation may reduce the individual performances of phytoplankton and invertebrate grazers because of shading (Antonovics and Levin 1980). Besides, the inconsistent effects (stimulatory, inhibitory or no effects) observed among previous studies on submerged macrophytes and phytoplankton may be due to differences in the macrophyte biomass utilised in experimentations (Chen, 1999; Körner and Nicklisch, 2002; Wu *et al.*, 2007, 2011; Dong *et al.*, 2013). In the current work, we suggested that the biomass of macrophytes could interact with the density of the target algae in terms of macrophytes influencing algal effects. As illustrated in Figure 1, in an intermediate biomass of submerged macrophytes, the effects were considerably different between two algal densities (Fig. 1). Conversely, low and high concentrations showed no remarkable differences between the two algal densities. The results supported the suggestion of considering both the biomass and algal density of macrophytes during natural water restoration.

The present study suggested that the methanol extracts could not only affect the growth of but also the morphology of *C. vulgaris*. Concordant with the growth effects, the morphology of *C. vulgaris* with low density was more sensitive to the *C. demersum* extracts. The colony proportion of *C. vulgaris* with low IAD was higher than with high algal density after treatment with 0.25 and 0.42 g/mL *C. demersum* extracts (Fig. 2). However, given the remarkable inhibitory effects of the extracts on the growth of *C. vulgaris*, the colony proportion of *C. vulgaris* treated with 1.25 g/mL *C. demersum* extracts was considerably lower than that treated with the two other concentrations. This result also illustrated that aside from the biomass reduction of algae, the morphological responses to macrophytes during water restoration should also be considered. As suggested in previous studies (Van Donk *et al.*, 2011; Fisher *et al.*, 2016), the morphology of algae can affect predation by invertebrate animals or fish, thereby influencing the energy flow of the food chain in a freshwater ecosystem.

Our present short-time extract experiments successfully excluded the nutrient/light competition between macrophytes and target algae, which somehow suggested the allelochemical effects of macrophyte *Ceratophyllum demersum* on target algae *Chlorella vulgaris*. And according to GC-MS analysis, five effective peaks were analysed (Fig. 3 and Tab. 2), i.e. hexanoic acid, acetoacetic acid, azelaic acid, palmitic acid and stearic acid. All potential substances were fatty acids. The result was in agreement with Gao *et al.* (2011) who isolated and identified the allelochemicals from *Elodea nuttallii*, *Hydrilla verticillata*, *Vallisneria spiralis* also included azelaic, palmitic and hexanoic acid. Besides, Gao *et al.* (2011) identified 9 and 17 types of fatty acids from *Elodea nuttallii* cultured at 10 and 50 g FW/L, respectively. Such outcomes suggested that the extract secretions are also dependent of macrophyte biomass. Our few kind of substances identified might be due to the few biomass macrophytes utilization in the simulation experiments. What's more, so far, little was reported of which compounds played roles in the colony formation of *C. vulgaris* by *C. demersum*. Our identified fatty acids might be the active substances (Yasumoto *et al.*, 2005, 2008). Zuo *et al.* (2016) detected the synergistic and antagonistic interactions that occur among five allelochemicals on *Microcystis aeruginosa*. However, in this study, we did not examine the individual and combined effects of those active compounds in the interaction of *C. demersum* and *C. vulgaris*. The combined effects (antagonistic or synergistic) of those compounds detected in the present study on the growth and morphology of *C. vulgaris* must also be considered in future research. Whether the compounds that play roles in the growth effects also influence the morphology of the target algae must also be explored. Previous studies have suggested that it might be due to the excessive investment of energy in colony formation that imposed restrictions on the growth of green algae (Zhu *et al.*, 2016; Dong *et al.*, 2018). The potential mechanisms should be further investigated.

5 Conclusions

To conclude, this study suggested that beneficial green alga *C. vulgaris* also demonstrated growth inhibition and morphological responses to submerged macrophyte *C. demersum*, the process of which was target algal density and macrophyte biomass dependent. The results proposed that the utilization of submerged macrophytes in the eutrophic water region restoration should be cautious, which might influence the growth and morphology of green algae, thus affecting the energy flow of the food web.

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