The fresh macroalga, Gracilaria lemaneiformis significantly inhibit the photophysiological activities of red-tide causing microalga, Scrippsiella trochoidea

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Abstract. The thecate dinoflagellate bloom microalga, *Scrippsiella trochoidea,* was co-cultured with different grams of fresh macroalga *Gracilaria lemaneiformis* under laboratory conditions, to characterize the allelopathic inhibition effect of the seaweed on photophysiological activities of the microalga. Cell numbers was measured, and Chlorophyll a (Chl *a*) fluorescence transient O-J-I-P curves associated with its specific parameters were determined. Both concentration and time-dependent inhibition of *S. trochoidea* were observed when the fresh seaweed was added. The cell numbers and pigments contents were markedly decreased; the O-J-I-P curve coupled with its specific parameters was reduced. The inhibitory effects of the macroalga on the microalga, according to the JIP-test and pigments contents, include a decrease in the number of active reaction centers, the blocking-up of the electron transport chain. This study suggests that fresh *G. lemaneiformis* is effective in inhibiting photophysiological activity of *S. trochoidea*, and thus be a potential 'tool alga' for controlling *S. trochoidea* blooms.

Introduction

Nitrogen and phosphorus loading from industrial, agricultural and municipal sources accelerates eutrophication in coastal zones worldwide[1]. Eutrophication may cause explosive growth of phytoplankton including harmful algal blooms (HABs), which have significant detrimental effects on fishery resources, marine ecosystems and human health around the world[2, 3]. Climate induced changes can also act synergistically with anthropogenic nutrient enrichment to increase harmful algal bloom frequency and geographical extent[4,5]. A cosmopolitan species, *Scrippsiella trochoidea* (Stein) Loeblich III, is distributed mainly in neritic habitats from the tropical to cold-temperate seas[6,7,8]. Formerly deemed as a non-toxic species, *S. trochoidea* is a thecate dinoflagellate bloom microalga reported from Japan, Korea and China[9,10], and was reported recently also as a toxic species responsible for killing larvae of bivalve species[11]. In bloom conditions in the field, *S. trochoidea* was observed to produce smooth and calcareous or non-calcified cysts[8]. Wild and cultured fish and shellfish kills have been reported to be associated with blooms of *S. trochoidea* in Australia[12] and China[13].

Therefore, there is a need to develop management and mitigation strategies to respond to HABs. Various physical approaches, such as light-shading and solar ultraviolet radiation[14,15], and chemical strategies[16,17] have been developed. However, the large-scale application of these methods is limited by high cost and the potential for ecological secondary pollution[18,19]. In contrast, biological controls using macroalgae such as *Ulva pertusa* and *Gracilaria* species are found to mitigate HABs effectively. These species are indigenous to the marine environment, easy to collect, low cost and environmentally benign [20–22].

G. lemaneiformis, an edible red alga broadly distributed and intensively cultivated in the coastal areas of China, is an economically important alga for agar extraction [23] and extraction of other natural products with important bioactivity[24,25], and is also used as a food addictive in aquaculture [26]. It has been shown that *G. lemaneiformis* and other macroalgae have an inhibitory effect on the growth of some HAB species whether they are added as fresh thalli, culture filtrate, water-soluble extract or dry

powder[27–29]. These macroalgae can therefore mitigate the negative effects of HABs by varying the make up of the phytoplankton community, changing the dominant species and decreasing microalgal abundance[30].

Despite a number of studies on growth of HABs, the photosynthetic inhibitory mechanisms by which fresh seaweed may affect HABs remains unconfirmed. In this study, the inhibition of *S. trochoidea* photosynthesis by fresh *G. lemaneiformis* is characterized. As photosynthesis is the primitive driving force of physiological and biochemical processes in photoautotrophs, characterizing the photobiological profile provides useful information about the mechanisms by which fresh *G. lemaneiformi* acts as a potential algicide souse against blooms of *S. trochoidea* which cause red tide.

Materials and methods

Culture of the seaweed

Fresh thalli of *Gracilaria lemaneiformis* were collected in April 2011 from the Nanao Island Cultivation Zone (116.6°E, 23.3°N), Shantou, Guangdong, China. Thalli were transported in 500 mL sterile bottles filled with sterile seawater (SSW) to the laboratory where they were rinsed thoroughly with 100mL SSW and treated with a mixture of penicillin, chloramphenicol, polymixin and neomycin at non-inhibiting concentrations after Nakai[31] and Jeong[32]. The medium used for algal cultivation was prepared according to the method of Jin[20]. The pH and salinity were adjusted to 8.0 and 30‰, respectively. Treated thalli were placed in sterile bottles containing SSW and were allowed to adapt to the laboratory environment for 5 d before use in experiments. Nutrients were enriched in the culture medium weekly by adding 100 μmol L^{-1} of $NaNO_3$ and 7μmol L^{-1} of NaH_2PO_4. Nutrient enrichment was stopped 1 week before the experiments. The temperature was kept at 20 ± 0.1 ºC on a 12:12 light:dark cycle. Illumination was provided by cool-white fluorescent lamps at 70 μmol photons m^{-2} s^{-1}. *G. lemaneiformis* fresh thalli were always maintained axenically under laboratory conditions and the effect of the environmental bacteria was considered negligible.

Culture of Microalga

S. trochoidea was cultured in modified f/2 medium[48] at 20°C, 70 μmol m^{-2} s^{-1} under a 12h:12h LD cycle. The initial pH and salinity of the culture medium were adjusted to 8.0 ± 0.02 and 30‰, respectively. The flask containing microalga was shaken manually twice daily, and grown to exponential phase for use in the experiments. Cells were inoculated into 500 mL Erlenmeyer flasks containing fresh f/2-enriched seawater until the total volume was 300 mL. The initial cell density was 1.0×10^4 cells mL^{-1}. The microalgal culture (monoculture) was used as a control throughout the experiment.

Experiments with microalga and fresh G. lemaneiformis

Fresh thalli of *G. lemaneiformis* was cut into 0.3 cm x 0.3 cm fragments to prevent the possible light shading and acclimatized in sterile seawater 48 h before use. Exponentially growing microalgae was inoculated with 1 and 2g/L fresh *G. lemaneiformis* at the same time in the culture medium. Monoculture with only *S. trochoidea* was served as the control. Three replicates were prepared for each experiment, and experiments were monitored for 9 days.

Measurement of chlorophyll fluorescence

Every other day, Chl *a* fluorescence transients of *S. trochoidea* were measured at room temperature using a plant efficiency analyser (PEA, Hansatech Instruments, Norfolk, England) with an actinic light of 3000 μmol photon m^{-2} s^{-1} [33]. Illumination was provided by an array of six high-intensity light-emitting diodes (with a peak wavelength of 650 nm), which were focused on the sample surface to provide homogeneous illumination over an area of 4 mm in diameter. All samples were dark-adapted for 15 min before measurement. The whole experiment lasted for 9 days.

The fluorescence intensities at 50 μs, 300μs (K-step), 2 ms (J-step) and 30 ms (I-step) were denoted as F_0, F_{300}μs, F_J and F_I, respectively, and F_m was assumed as the maximum fluorescence intensity[33]. The specific parameters were calculated according to the JIP-test [34]. To carry out the JIP test, several extracted and technical fluorescence parameters calculated from the measurements of the polyphasic

fluorescence transients are needed. They are: (1) the minimal fluorescence yield, F_0 (the fluorescence intensities when all reaction centers are open), (2) the maximal fluorescence yield, F_m (the excitation intensity is high enough to ensure the closure of all reaction centers), (3) the initial slope at the beginning of the variable fluorescence transients theoretically at time zero), dV/dt_0 [$=4(F_{300\mu s}-F_0)/(F_m-F_0)$]; and (4) the relative variable fluorescence at phase J, V_J [$=(F_J-F_0)/(F_m-F_0)$]. According to the JIP test, the energy flux for absorption (ABS), energy flux for trapping (TR) and enegry flux for electron transport (ET) per photosystem II (PSII) reaction center (RC) are given by equations 1-3, respectively. The concentration of the PSII reaction centers (RC/CS, indicating the density of active reaction center, i.e. photosynthetic units) are is given by equation 4 or 9 and 10.

Specific fluxes or specific activities of photosystem:

$$ABS/RC = [(dV/dt_0)/V_J]/[1-(F_0/F_m)] \quad (1)$$

$$TR_0/RC = (dV/dt_0)/V_J \quad (2)$$

$$ET_0/RC = [(dV/dt_0)/V_J]\cdot(1-V_J) \quad (3)$$

Density of reaction centrescenters:

$$RC/CS = [V_J/(dV/dt_0)]\cdot[1-(F_0/F_m)]\cdot F_0 \quad (4)$$

Quantum efficiencies or flux ratios:

$$\varphi_{Po} = TR_0/ABS = 1-(F_0/F_m) = F_v/F_m \quad (5)$$

$$\psi_0 = ET_0/TR_0 = (1-V_J) \quad (6)$$

Phenomenological fluxes or phenomenological activities:

$$ABS/CS_0 \approx F_0 \quad (7)$$

$$RC/CS_0 = \varphi_{Po}\cdot(V_J/F_0)\cdot(ABS/CS_0) \quad (8)$$

$$TR_0/CS_0 = \varphi_{Po}\cdot(ABS/CS_0) \quad (9)$$

$$ET_0/CS_0 = \varphi E_o\cdot(ABS/CS_0) \quad (10)$$

Where ABS/CS_0 represents absorption flux per excited cross-section of sample (at t=0), indicating the quantity of antenna chlorophyll; RC/CS_0, the amount of active PSII reaction centers per excited cross-section (at $t = t_{F0}$). TR_0/CS_0, the trapping flux per excited cross sectionat at t = 0; ET_0/CS_0, the electron transport flux per excited cross sectionat at t = 0.

Determination of Chl a and carotenoid

Every other day, the sample of *S. trochoidea* was collected in a 15ml tube and centrifuged for 10 min at 5000 rpm at 20 °C in the high speed freezing centrifuge (5810R, Eppendorf, Germany). The sediment was extracted in 4ml absolute methanol for 24 h at 4°C in the dark. This extract was centrifuged at 5000 rpm for 10 min and analyzed for Chl *a* and carotenoid content with a scanning spectrophotometer (UV 530, Beckman Coulter, USA). The Chl *a* and carotenoid concentration was calculated according to Wellburn [35].

Data statistical analysis

Data were analyzed by two-way ANOVA followed by a multiple comparison using the least significance difference (LSD) test. Calculations and statistical analyses were performed with SPSS 13.0 for Windows. *P*-values of 0.05 were considered as a significant.

Results and Discussion

Results

Compared with the first day, the cell mumbers in each treatment was significantly higher ($P<0.05$) on the third day, and the inhibitory effect between 1 and 2 g/L fresh seaweed treatment was not very obviously, while all of which were significantly lower than the control (Fig. 1). On the day 5, 7, 9, the

addition of fresh *G. lemaneiformis* lowered cell numbers of *S. trochoidea*, with a positive correlation between the amount of fresh biomass added and the amount of inhibition (Fig. 1).

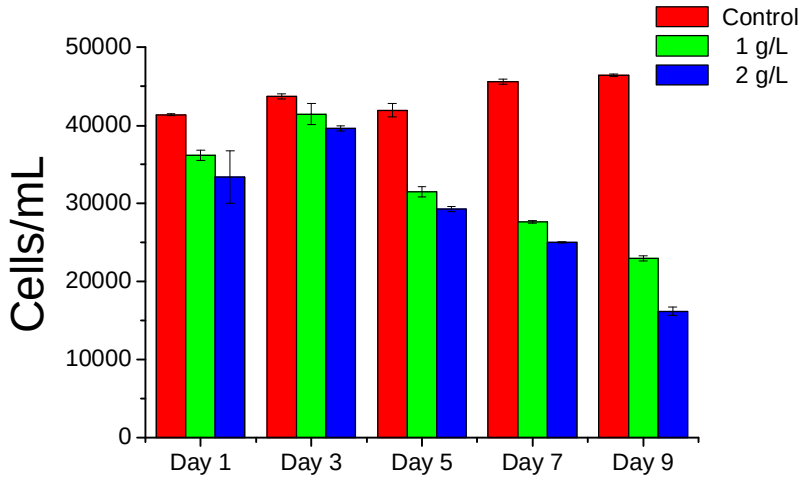

Fig.1. The cell concentration of *S. trochoidea* co-cultured with different quantities of fresh macroalga *G. lemaneiformis* on different days.

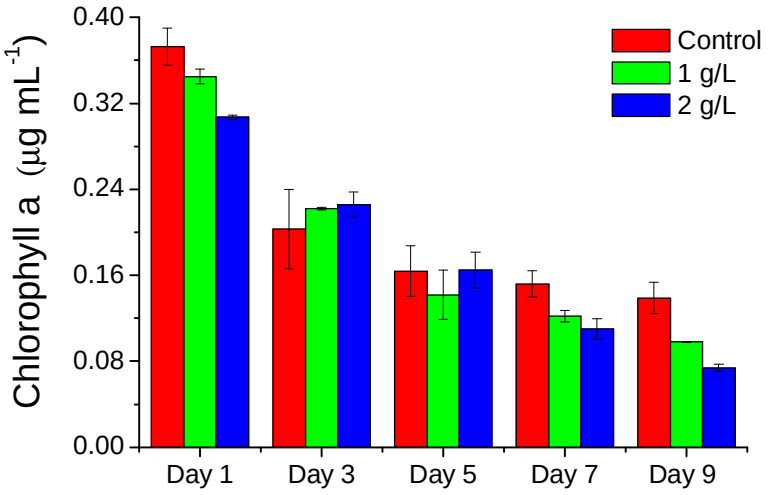

Fig.2. The chlorophyll a concentration of *S. trochoidea* co-cultured with different quantities of fresh macroalga *G. lemaneiformis* on different days.

The chlorophyll a concentration of *S. trochoidea* co-cultured with different quantities of fresh macroalga *Gracilaria lemaneiformis* on different days was declined, which was same as in the cell concentration (Fig. 2).

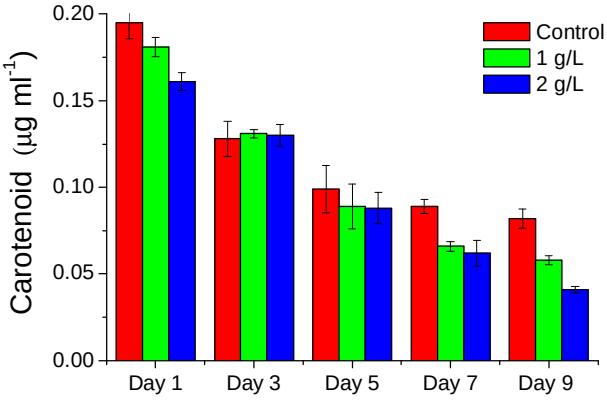

Fig.3. The carotenoid concentration of *S. trochoidea* co-cultured with different quantities of fresh macroalga *G. lemaneiformis* on different days.

The same pattern was followed by the carotenoid concentration of *S. trochoidea* co-cultured with different quantities of fresh macroalga *G. lemaneiformis* on different days (Fig. 3).

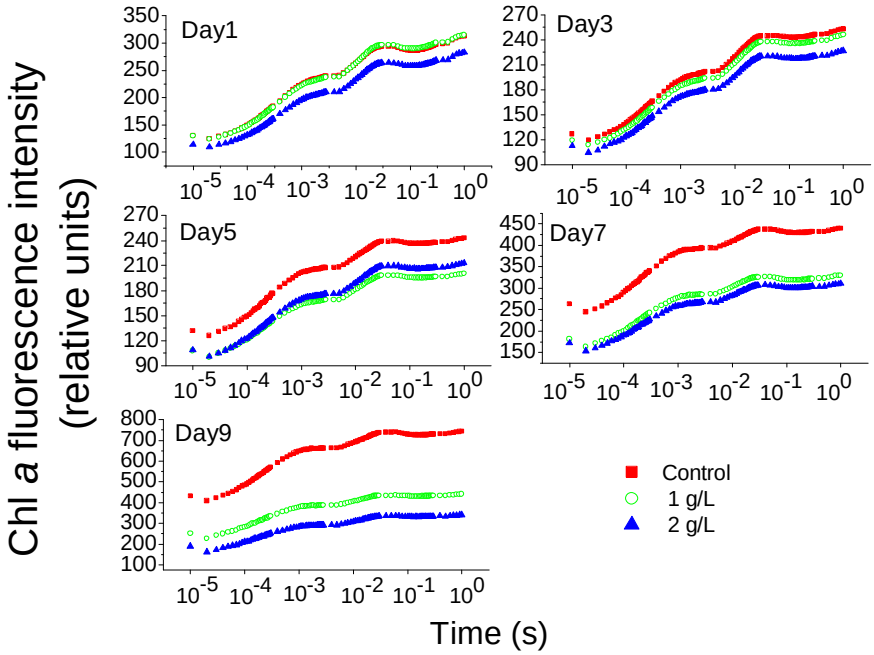

Fig.4. The O-J-I-P curves of *S. trochoidea* co-cultured with different quantities of fresh macroalga *G. lemaneiformis* on different days.

Figure 4 shows fluorescence induction curves for dark-adapted samples. The addition of fresh *G. lemaneiformis* lowered the polyphasic chlorophyll fluorescence transients (OJIP) intensities of *S. trochoidea* during the whole experiment period, with a positive correlation between the amount of fresh biomass added and the amount of inhibition (Fig. 4).

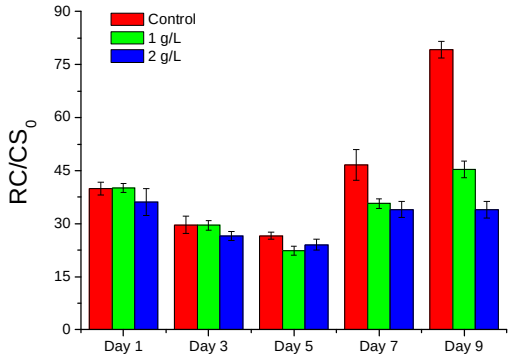

Fig.5. The active reaction centers per excited cross section at t = 0 (RC/CS_0) in *S. trochoidea* co-cultured with different quantities of fresh macroalga *G. lemaneiformis* on different days.

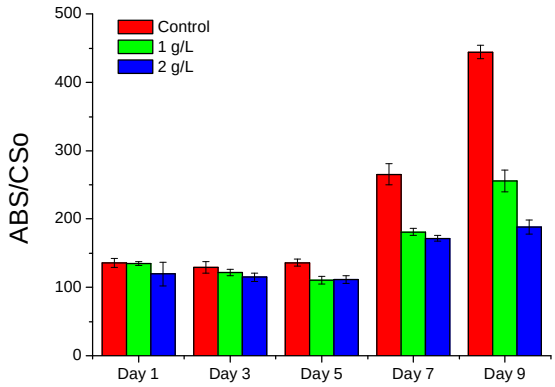

Fig.6. The absorption flux per excited cross section at t = 0 (ABS/CS_0) in *S. trochoidea* co-cultured with different quantities of fresh macroalga *G. lemaneiformis* on different days.

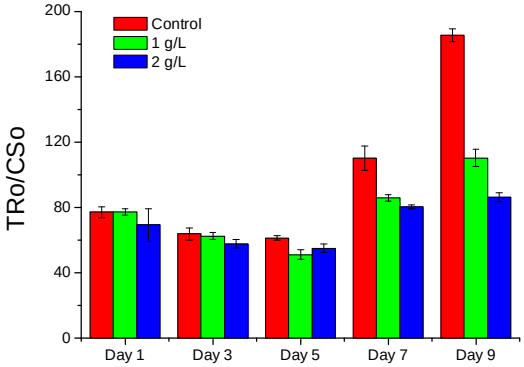

Fig.7. The trapping flux per excited cross section at t = 0 (TR_0/CS_0) in *S. trochoidea* co-cultured with different quantities of fresh macroalga *G. lemaneiformis* on different days.

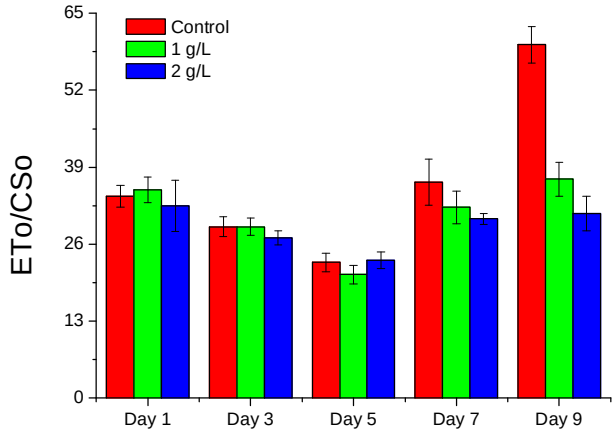

Fig.8. The electron transport flux per excited cross section at t = 0 (ET_0/CS_0) in *S. trochoidea* co-cultured with different quantities of fresh macroalga *G. lemaneiformis* on different days.

The same concentration-dependent inhibition patterns were found in RC/CS_0, ABS/CS_0, TR_0/CS_0 and ET_0/CS_0 on the first day (Figs. 5, 6, 7 and 8). On the third day, the difference between 1 g and 2 g L-1 fresh biomass was not very obviously, while the difference was still significant ($P<0.01$) when compared with control (Figs. 5, 6, 7 and 8).

Discussion

Allelopathy is the biochemical interaction, both stimulatory and inhibitory, between primary producers or between primary producers and microorganisms[36]. When *S. trochoidea* was cultured with different concentration of fresh *G. lemaneiformis*, fresh thalli of *G. lemaneiformis* was cut into 0.3 cm x 0.3 cm fragments to prevent the possible light shading, leading to the assumption that allelochemicals inhibited the microalga in this study.

In the present study, cell numbers of *S. trochoidea* was strongly suppressed by fresh *G. lemaneiformis*, and there was a clear concentration-dependent pattern, which was followed by chlorophyll a and carotenoid concentration, indicating the pigment complex in *S. trochoidea* was partial deactivation by the seaweed.

All oxygenic photosynthetic organisms investigated so far using show a polyphasic rise of of fluorescence transients during the first second of illumination, which are labelled as O-J-I-P[37]. O-J-I-P is a useful, non-invasive tool for the study of the photosynthetic apparatus, and more specifically the behavior of photosystem II [37, 38]. This O-J-I-P polyphasic transient has been found to change its shape according to changes in environmental conditions[39, 40]. Quantitative analysis of recorded O-J-I-P Chl a fluorescence transients, by means of the JIP-test, result in the calculation of several biophysical parameters, which contain information about the fluxes of photons, excitons, electrons and further metabolic events at a given physiological state, and which can be used to quantify PSII function to different stressors (refer to [37] for a review).

In our experiment, the O-J-I-P Chl a fluorescence transients, coupled with several biophysical parameters in *S. trochoidea* was depressed by the addition of fresh *G. lemaneiformis.* ABS/CS_0, absorption flux per excited cross-section of sample (at t=0) was declined, indicating the quantity of antenna chlorophyll had decreased, so that not enough light was harvested to support the subsequent photochemical process. The concentration of the PSII reaction centers (RC/CS) decreased, indicating the density of active reaction center, i.e. photosynthetic units decreased, which reduced the photochemical transferring of harvested and excited photons. ET_0/CS_0, the electron transport flux per

excited cross section (at t=0) was reduced, suggesting that photosynthetic electron transport was blocked, indicating that photochemical flux of photons, excitons, electrons and further metabolic events in the photosystem of *S. trochoidea* were depressed, and finally the decline of growth in microalga (expressed as cell conentration).

In this study, the main photosynthetic inhibition targets by *G. lemaneiformis* on *S. trochoidea* were a decrease in the quantity and sieze of antenna chlorophyll (reflected by the decrease of, chlorophyll a, carotenoid concentration and ABS/ CS_0), the number and trapping activity of active reaction centers (RC/CS_0, TR_0/CS_0); the blocking of the electron transport chain (a decrease of ET_0/CS_0).

Tang and Gobler[21] showed that the dried *Ulva lactuca* was equally or more potent than the fresh material, an observation consistent with the hypothesis that polyunsaturated fatty acids or organosulfur compounds are active allelopathic agents[41], which were also observed in dried seaweed of *G. lemaneiformis* and *G. tenuistipitat* in our previous experiments[42–44]. Dithiolane and trithiane compounds isolated and identified from *Characean* species were also found to have allelopathic effects on epiphytic diatoms and other phytoplankton[47]. Lu[45] isolated some secondary metabolites from *G. lemaneiformis*, such as glycolipid compounds, fatty acid compounds, amides, phenolic compounds and terpenoids. And by screening of allelochemicals, they found that the strongest allelopathy on the growth of the red tide alga, *Skeletonema costatum* was linoleic acid.

Our results show that fresh *G. lemaneiformis* strongly suppressed the photosynthesis of *S. trochoidea* culture, and that there was a clear concentration-dependent relationship reflected in a decreased cell number, pigment concentrations and O-J-I-P curve along with its specific parameters, such as the, RC/CS_0, ABS/ CS_0, TR_0/CS_0 and ET_0/CS_0, revealing that the oxygen evolution complex, reaction centre and electron transport were damaged and/or inhibited, which finally resulted in the decrease of photosynthesis and growth in *S. trochoidea*. Zhu[46] also found that allelopathic polyphenols, pyrogallic acid and gallic acid, isolated from submerged macrophyte Myriophyllum spicatum were the main factors responsible for the inhibition of PSII (from the oxygen-evolving complex to plastoquinone) and whole chain (from oxygen-evolving complex to the photooxidized chlorophyll) activities of *Microcystis aeruginosa*, a similar response in photosynthetic parameters was observed in our experiments.

Conclusions

The results of this study suggest that fresh *G. lemaneiformis* had negative allelopathic effects on the growth and photosynthesis of *S. trochoidea* and could thus be a potential algicide to control and mitigate *S. trochoidea* blooms.

Acknowledgements

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