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Evaluating the allelochemical effects of *Syzygium cumini* leaves extract on *Microcystis aeruginosa* by pulse-amplitude-modulated fluorometry and flow cytometry

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ABSTRACT

The allelochemical effects of *Syzygium cumini* leaves extract (SCLE) on the growth inhibition and physiological properties for *Microcystis aeruginosa* were evaluated in laboratory setting. The chlorophyll-a (Chl-a) concentration of the algae was 24.4 μg L^{-1} and the relative content of Chl-a was 0.9% at 2.0 g L^{-1} dose of SCLE treatment till the end of 15 d experiment. Results from fluorescein diacetate and propidium iodide showed SCLE inhibited esterase activity at early time, and affect cell integrity at later period. Inhibition of photosynthetic activity was reflected by relative electron transport rates (rETRs), the maximal electron transport rate ($rETR_{max}$) and quantum yield of photosystem II (F_v/F_m) of algal cell. The photosynthetic parameters inhibited by SCLE occurred more rapidly than cell growth suggested the causative relationship though the effectiveness is temporary or at early stage.

Keywords: Allelopathy; *Syzygium cumini*; Esterase activity; Photosystem; Flow cytometry; PAM fluorometry

1. Introduction

In recent decades, harmful algal blooms have received increasing attention worldwide because they can cause serious environmental problems [1,2]. Algal blooms in some countries or regions causes enormous economic losses. The negative influences include many aspects, such as colour, taste, oxygen level, unpleasant odour problem, aquatic animal survival in affected water bodies [3] and inefficient operation of filtration processes [4]. Bloom-forming cyanobacterial algal such as *Microcystis* species are highly toxic to various fish species, as well as other terrestrial animals including humans [5].

Allelopathy is a favourable method for cyanobacterial bloom control through the production and release of allelochemicals to interact with cyanobacterial species [6–8]. Allelochemicals are supposed to be easily degradable and environmentally safer than traditional herbicides [9]. Most allelochemicals are secondary metabolites and their bioactive substances are natural compounds and crude plant extracts [10]. The isolation of pure compounds from crude extracts is costly and labour intensive, while employing active ingredients from natural materials as is seems more economically viable [11]. Crude extracts have higher potential application for removing cyanobacteria in water bodies than purified active ingredient [12,13]. In addition, it seems practical to use extracts of terrestrial plants [10] than aquatic plants to control cyanobacteria [14,15].

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Syzygium cumini, which belongs to the Myrtaceae family, is commonly found in tropical and subtropical regions. *S. cumini* leaves have been tested for anti-inflammatory, antimicrobial and antioxidant activity [16–18], and have been attributed for their medicinal properties in the Indian folklore medicine system [19,20]. Phytochemical investigations indicate that there are rich phenolic acids in *S. cumini* leaves, such as tannins, acetyl oleanolic acid and ellagic acid [18,21], which are also common allelochemicals to inhibit the growth of algae [22,23]. However, the suppression mechanisms for algae of allelochemicals are partly known, mainly including cell integrity, esterase activity and photosynthetic capacity [10]. In addition, *S. cumini* is often found near lakes or ponds, usually located in central area of urban communities. Nevertheless, little literatures focus on the potential application of *S. cumini* leaves extract (SCLE) for alga blooms control and possible mechanisms.

In the study, we investigated the effects on *Microcystis aeruginosa* after medium-term SCLE exposure in laboratory settings. Pulse amplitude modulated (PAM) fluorometry was applied to evaluate the growth and photosynthetic performance of *M. aeruginosa*, and flow cytometry (FCM) was used to assess the membrane characteristics and metabolic status of algal cells [24].

2. Materials and methods

2.1. SCLE preparation and culture of M. aeruginosa

S. cumini leaves collected from Guangzhou, Guangdong Province, China, were rinsed with deionized water for three times, dried at 40°C for 5 d in oven. Dried leaves of *S. cumini* were powdered. Each gram of powered was soaked in 10 mL deionized water for 5 d at 26°C ± 1°C in the darkness. The mixture was then filtered using membrane (0.22 μm) and kept at 4°C until use.

The *M. aeruginosa* FACHB-915 strain was obtained Freshwater Algae Culture of Hydrobiology (China). Algae were grown for about 10 d in 250 mL Erlenmeyer flasks in BG-11 medium. The conditions were 12:12 h light/dark cycle under light of 30 μmol photons m^{-2} s^{-1} and temperature was set at 26°C ± 1°C [25]. 1 mL of each algal suspension was subcultured to 100 mL BG-11 medium. The extract of leaves was added to microalgae cultures at the final concentrations of 0.4, 0.8, 1.2, 1.6 and 2.0 g L^{-1}.

2.2. PAM fluorometry measurements

M. aeruginosa growth was determined by measuring Chl-a concentrations using PAM fluorometry (Phyto-PAM, Walz, Effeltrich, Germany) equipped with a special emitter-detector unit, and data were outputted with the software Phyto-Win v2.13. Calibration was performed with chlorophyll extracted with acetone. Four-millilitre algal samples were measured on 0, 1, 4, 7, 10 and 15 d after SCLE exposure.

The rapid photosynthesis versus irradiance curves technique was employed to determine the photosynthetic activity of *M. aeruginosa* treated with SCLE. The effective quantum yields measured, which were generated by 10 incremental actinic light intensities (photosynthetically available radiation, PAR) namely 16, 164, 264, 464, 664, 864, 1,064, 1,264, 1,464 and 1,664 μmol photons m^{-2} s^{-1}. The corresponding relative electron transport rates (rETRs) were calculated, and a complete rapid light curve (RLC) was constructed. The actinic light irradiance step was applied for 30 s. RLC curves were fitted using the model described [26]. Detailed procedures are described elsewhere [27].

The maximal electron transport rate ($rETR_{max}$) was estimated by using the Platt equation [28]. The maximum optical quantum yield of PS II (F_v/F_m) was calculated as $F_v/F_m = (F_m - F_0)/F_m$, where F_m and F_0 indicated the maximum and minimum fluorescence light yields in a saturating light pulse after dark adaptation for 5 min, respectively [29].

2.3. Flow cytometric measurements

The effects of SCLE on cell integrity and esterase activity of *M. aeruginosa* were investigated by FCM analysis. Accuri 6 FCM (Becton Dickinson, USA) equipped with FL2 (585/40 nm) and FL1 (533/30 nm) detectors to measure the changes on cell membrane integrity and esterase activity, respectively. Cells were stained with propidium iodide (65 mg L^{-1}, PI, Sigma, St. Louis, MO) and fluorescein diacetate (10 mg L^{-1}, FDA, Sigma, St. Louis, MO) for 15 min. Medium flow rate was applied and about 12,000 cells were counted by FCM for each sample. Data were collected and analysed using BD-Accuri 6 software. Detailed procedures are described in Xiao et al. [30,31].

2.4. Statistical analysis

All experiments were in triplicate. Data were presented as mean ± 1 standard deviation. The differences between control and treated samples were tested by ANOVA with the SPSS analytic package 16.0 (SPSS Inc., Chicago, IL, USA). $p < 0.05$ was considered statistically significant.

3. Results and discussion

3.1. Extract of SCLE on the growth of M. aeruginosa

The concentration of Chl-a was closely related to the growth of algae and photosynthetic activity. The effect of *S. cumini* leaf extract on the Chl-a concentration of *M. aeruginosa* is shown in Fig. 1. The Chl-a concentration remained at low level from day 0 to day 4 in all groups. The Chl-a concentrations increased slowly at the low dose extract of leaf treatment (0.4 and 0.8 g L^{-1}) until day 7, which were 1,095 and 194.8 μg L^{-1} on day 15, respectively. However, it was about 24.0 μg L^{-1} at the highest dose of extract (2.0 g L^{-1}), while it was 2,800 μg L^{-1} in the control at the end of experiment. The growth of algae was substantially inhibited within the 15-d period ($p < 0.01$, ANOVA). The EC_{50} values were only 0.12 g L^{-1} on day 7 and increased to 0.35 on day 15 (data not shown).

The relative content of Chl-a affected by different doses of SCLE were measured to study the growth inhibition effect on *M. aeruginosa*. Our results show that a range of 0.4–2.0 g L^{-1} *S. cumini* leaf extract effectively inhibited the growth of *M. aeruginosa* (Fig. 2). *M. aeruginosa* growth was slightly promoted by the extract between day 0 and day 4. It is worth noting that the contents of Chl-a were higher at SCLE dosage

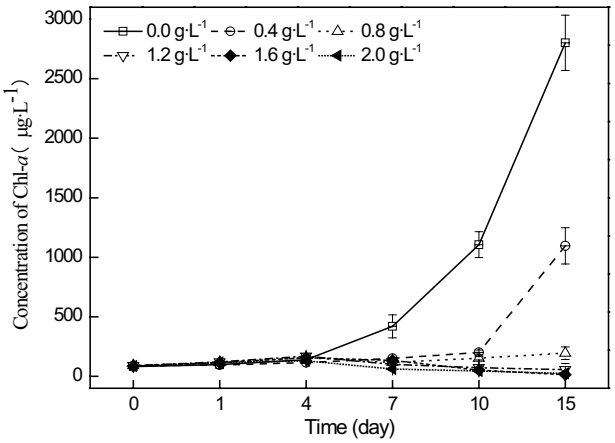

Fig. 1. Chl-a concentration in *M. aeruginosa* culture after 0, 1, 4, 7, 10 and 15 d of exposure to SCLE. Data are presented as mean ± standard deviation (n = 3).

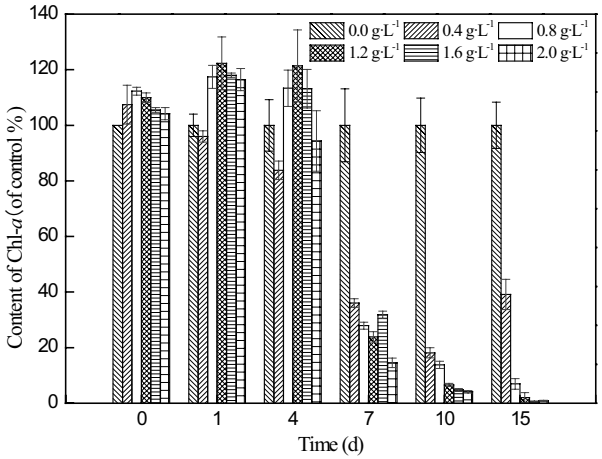

Fig. 2. Inhibition effect on growth of *M. aeruginosa* cultures after SCLE exposure. Data are presented as mean ± standard deviation (n = 3).

of 0.8–1.6 g L^{-1} than the dosages of 0.4 g L^{-1} and control before day 4. The relative content of Chl-a reached from about 110% to 120% compared with the control when the extract dose was 0.8–1.6 g L^{-1} on day 4. However, the extract began to exert strong inhibition activity after 4-d exposure. On 7-d, the relative content of Chl-a concentration decreased to 14.5% at highest concentration (2.0 g L^{-1}) of extract applied. This further decreased to 4.5% (95.5% inhibition) on day 10 and 0.8% on day 15 (99.2% inhibition) (Fig. 2).

In this experiment, we tested the effectiveness of the crude extract of *S. cumini* leaf on controlling the growth of *M. aeruginosa*. The significant decreases of Chl-a concentrations following addition of the SCLE might indicate that the extract had anti-algal activity, especially at early time (after day 4). However, the Chl-a contents were higher at medium dosage of SCLE (0.8–1.6 g L^{-1}) than other treatment at early time (before day 4), which might indicate that the extract also possessed stimulating activity and the growth of *M. aeruginosa* was slightly promoted. Other reports showed that allelochemicals and nutrients (such as N and P) coexisted in plant extract, which might exert both inhibition and stimulation effect on algal growth. This result is in accordance with other plant extracts, such as barely straw [32], rice straw [33,34] as well as some terrestrial plants [35,36], which could decrease growth of cyanobacteria *M. aeruginosa*. However, previous studies required high extract concentrations [10] and nutrients added might aggravate eutrophication [10,37] which make applications not feasible. In current study, lower concentration (2.0 g L^{-1}) the leaf extract of *S. cumini* was able to produce significant suppressing effect compared with crops straw (about 8.0 g L^{-1}) [31,38]. Nevertheless, our data also suggested that 4-d decomposition of powdered *S. cumini* leaves was also effective in inhibiting the growth of *M. aeruginosa*, which aligned with our previous study [36].

3.2. Effects on membrane integrity and esterase activity

PI-staining was utilized to assess the membrane integrity. The percentages of viable cells after SCLE exposure are shown in Fig. 3. Membrane integrity of *M. aeruginosa* cells was significantly compromised ($p < 0.05$, one way ANOVA) when SCLE concentrations were greater than 1.2 g L^{-1} after 5 d exposure, which showed adverse effects occurred in a dose-dependent manner (>1.2 g L^{-1}, $p < 0.05$) on day 5. The percentage of viable cells was reduced to 76.5% and 75.9% (1.2 and 2.0 g L^{-1}) on day 15, respectively.

Esterase, as an indicator of metabolic activity was commonly assessed by FDA fluorescence measurement [11]. Changes in esterase activity of *M. aeruginosa* at different treatment were observed (Fig. 4). On day 1, approximately 83.4% of cells shifted into the lower esterase activity state (S1) (5.2% in the control), 0.8% of cells shifted into the higher activity state (S3) (6.6% in the control) when *M. aeruginosa* was exposed to 2.0 g L^{-1} of SCLE (Fig. 4(a)). However, the esterase status of exposed cells was lowered on day 5 at higher dosages (2.0 g L^{-1}) compared with day 1 culture; 40.4% of cells shifted into the lower activity state (S1) (5.7% in the control, 83.4% on day 1), while the higher esterase activity state of cells was up to 18.1% (S3) (3.9% in the control, 0.8% on day 1) (Fig. 4(b)). After 15 d of exposure, approximately 44.7% of cells shifted into the lower esterase activity state and the higher activity state (S3) was 25.4% at dosages 2.0 g L^{-1} of SCLE (Fig. 4(c)).

The membrane integrity can be assessed using PI and FDA as a marker for membrane integrity [39]. To distinguish the causing the inhibition of FDA fluorescence, both membrane integrity and intercellular esterase experiment were conducted. Results of PI fluorescence clearly showed that membrane integrity was not severely affected even at high concentration (1.2 and 2.0 g L^{-1}), which discorded with the high inhibition and of FDA fluorescence even at the same SCLE concentration at early time. At the end of experiment, the inhibitory of esterase activity for algal cells was 43.3% and 44.7%, whereas the membrane integrity was 76.5% and 75.9%. In addition, the treatment group of 2.0 g L^{-1} showed no difference compared with the group of 1.2 g L^{-1} on days 5 and 15. This implied that *M. aeruginosa* was vulnerable during the initial period of exposure, but the surviving cells could physiologically adapt to the SCLE.

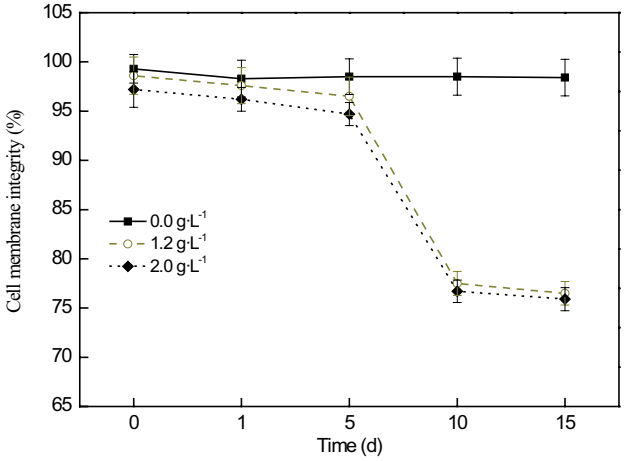

Fig. 3. Cell membrane integrity of *M. aeruginosa* treated with different dosages of SCLE. Data are presented as mean ± standard deviation (n = 3).

Thereby, we could confirm that the reduction of FDA fluorescence was not because of the algal cells disruption induced by SCLE exposure completely. It could be explained that algal cell esterase is one of the targets for allelopathy to *M. aeruginosa* at early time [40]. The esterase activity responded more rapidly than membrane integrity determined by FCM after SCLE exposure. Similarly, this finding aligned with other reports such as tannic acid and ferulic acid allelochemicals [23] and other arbor leaves extract [36]. In contrast, membrane integrity was recognized as a slower and less sensitive endpoint than esterase activity [11]. Contrary, the esterase activity was regarded as the most sensitive and rapid response to allelochemicals compared with other physiological effects [40]. Both the esterase activity and membrane integrity of algal cells might influence the growth of *M. aeruginosa*. The former parameter played relative important role at early time of treatment, and the membrane integrity demonstrated at later stage.

3.3. Effects on photosynthetic activity

Measuring PS II activity in algae by PAM fluorometry allows better understand algal physiological mechanisms [41–43]. The rETR/E curves of *M. aeruginosa* treated with SCLE measured by PAM fluorometry is shown in Fig. 5. The SCLE treatment group showed a similar pattern of rETR in response to PAR, which was generated by PAM fluorometry. It showed significant difference of the rETR in response to PAR between the treatment and control on day 1 ($p < 0.05$). The rETR values significantly decreased ($p < 0.05$), even at low concentration of extract (0.4 g L^{-1}) on day 7. rETR level increased gradually corresponding to PAR and SCLE concentrations and rebounded even at the highest dose of extract (2.0 g L^{-1}). Interestingly, we also observed that the values of rETR were higher at dosage of 2.0 g L–1 than 1.6 g L^{-1} after 7 d SCLE exposure.

The changes of F_v/F_m and $rETR_{max}$ of *M. aeruginosa* induced by SCLE treatment were shown in Fig. 6. It demonstrated the inhibition of $rETR_{max}$ and F_v/F_m of *SCLE* exposed *M. aeruginosa*

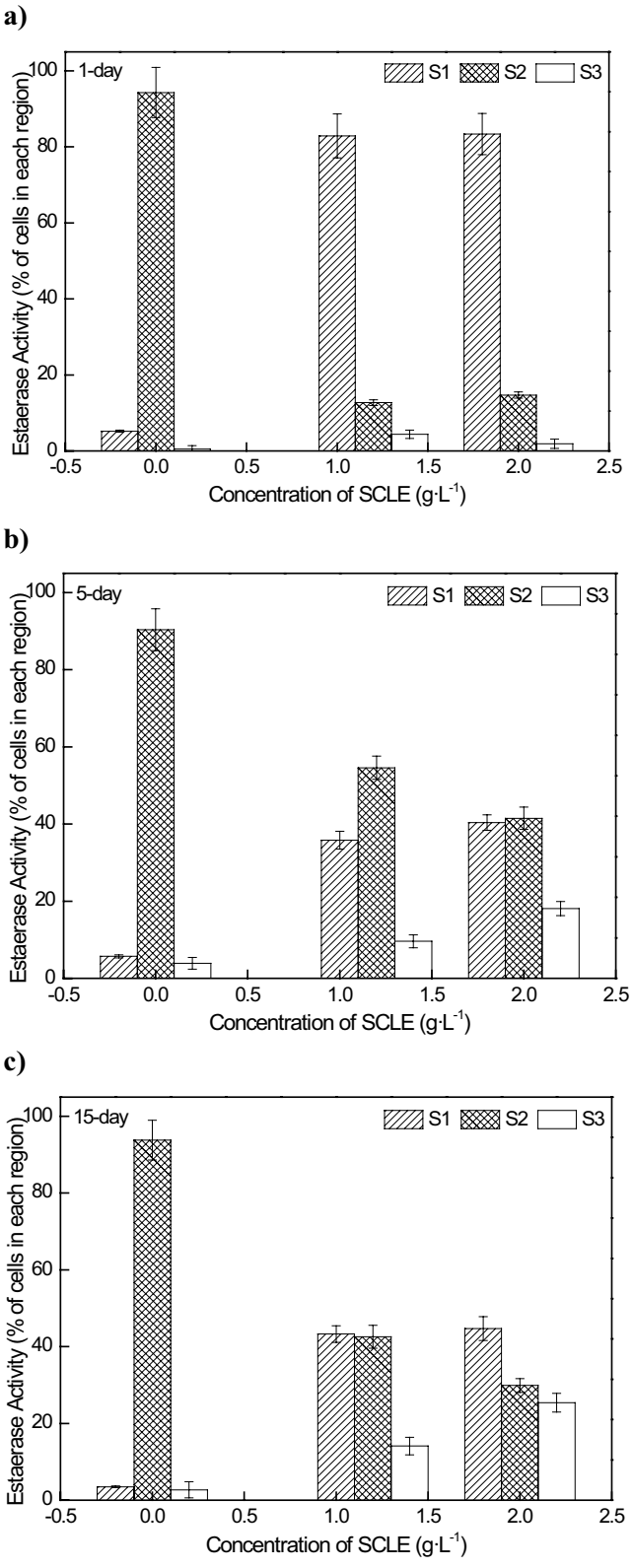

Fig. 4. Esterase activity determined by FCM in *M. aeruginosa* after SCLE exposure ((a) 1 d; (b) 5 d and (c) 15 d). S2 indicates esterase activity in the control cells; S1 represents a decrease in esterase activity and S3 represents an increase in esterase activity. Data are presented as mean ± standard deviation (n = 3).

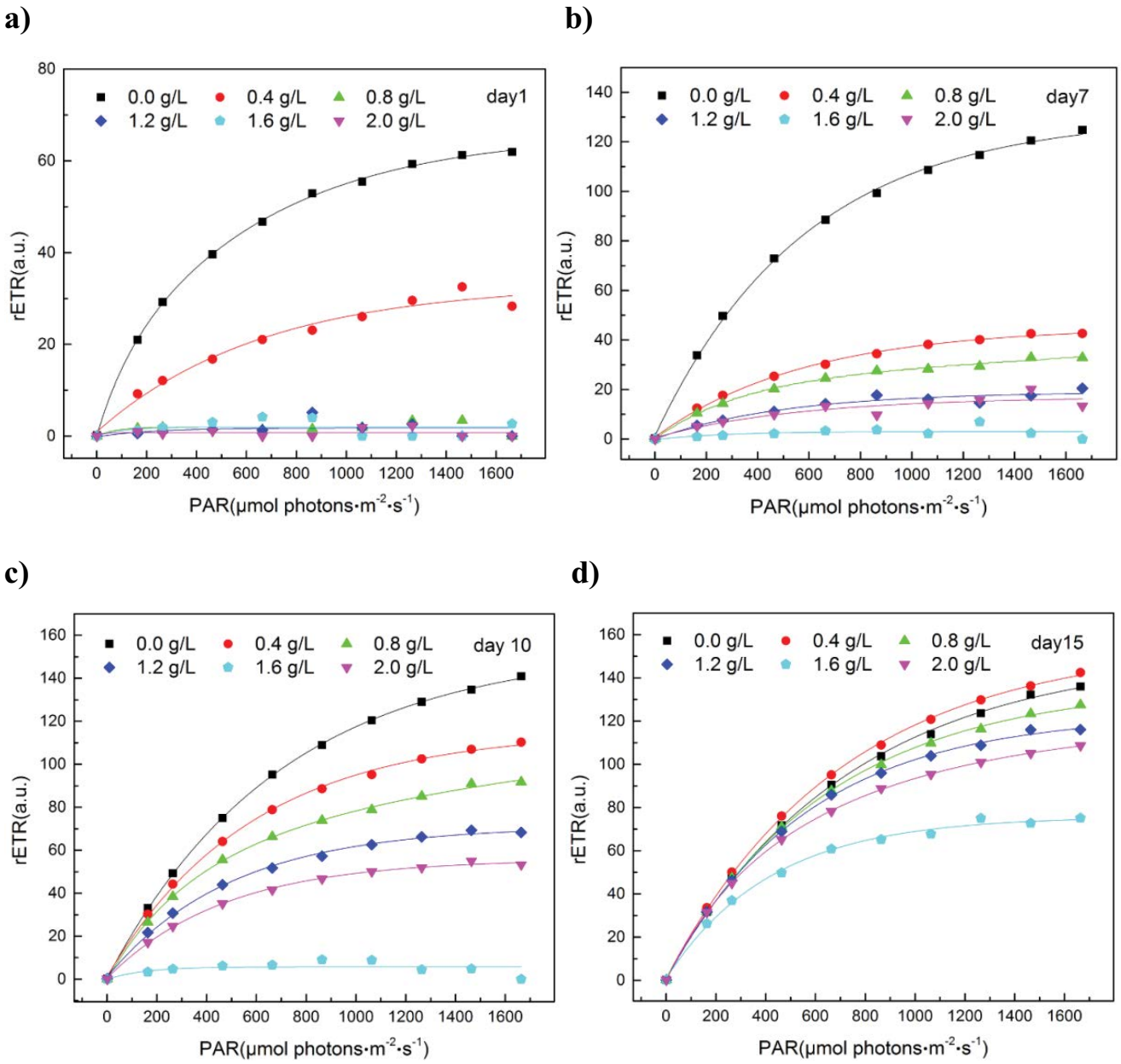

Fig. 5. Photosynthesis versus irradiance (rETR/E) curves for *M. aeruginosa* treated with *S. cumini* leaves extract. (a) 1 d; (b) 7 d; (c) 10 d and (d) 15 d. Each point represents the mean of three replicates.

cells during the incubation period. A significant inhibition ($p < 0.05$) of $rETR_{max}$ was observed at all doses of extract on day 0. After 7 d and 15 d of treatment, 2.0 g L^{-1} SCLE reduced $rETR_{max}$ from 119.8 to 19.2 μmol electrons m^{-2} s^{-1}, and from 142.1 to 106.5 μmol·electrons m^{-2} s^{-1}, respectively ($p < 0.05$) (Fig. 6(a)). Similar trend of F_v/F_m inhibition was. The F_v/F_m reduced from 0.55 to 0.05 after 10 d ($p < 0.01$), and increased to 0.30 after 15 d at 2.0 g L^{-1} dose of extract ($p > 0.05$) (Fig. 6(b)). In addition, the two parameters' values were also higher at dosage of 2.0 g L^{-1} than 1.6 g L^{-1} after 7 d SCLE exposure (Figs. 5 and 6).

Although the algicide action on phytoplankton is not fully understood, impairing the photosynthetic system may be one of the mechanisms [44,45]. Low electron transport chain could limit electron transport rate, Calvin cycle activities [44,46], adenosine-triphosphate (ATP) formation and carbon dioxide fixation [47]. In this study, photosynthetic activity demonstrated with the changes in rETR/E, $rETR_{max}$, and F_v/F_m was compromised by SCLE significantly, especially at early stage of treatment. The association between photosynthetic parameters and the SCLE treatment implied photosynthetic system might be the target. The decrease of rETR/E and $rETR_{max}$ indicated inhibition of electron transport rate led to lower photosynthetic capacity. Other studies also showed that the interruption of the electron transport might lead to the failure of photosynthesis [25]. In addition,

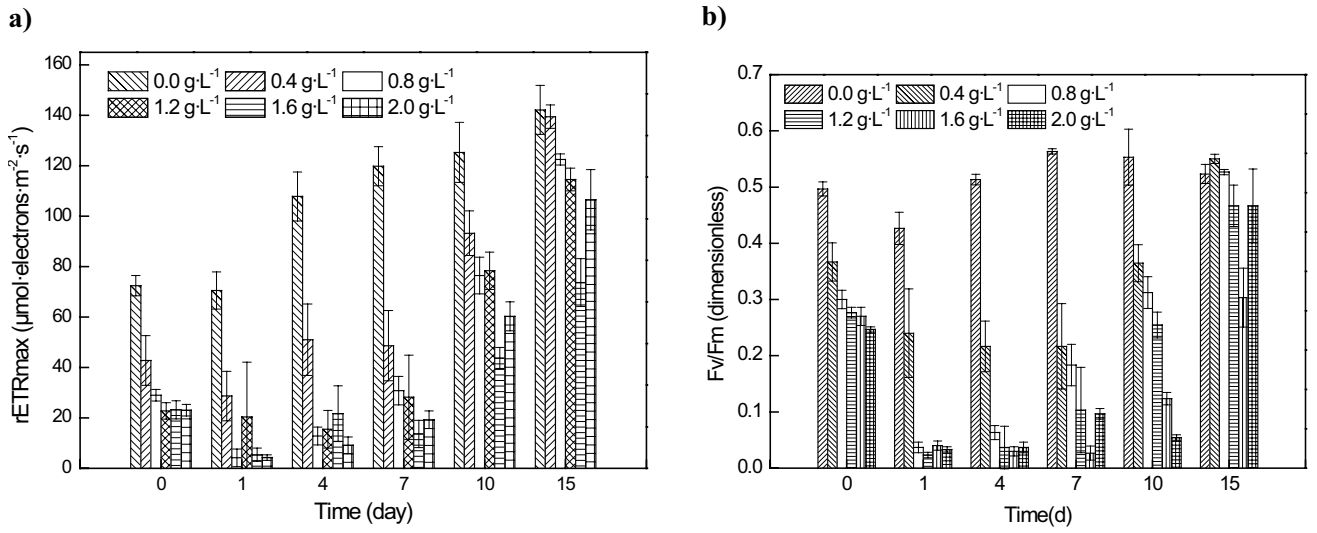

Fig. 6. The photosynthetic parameters of $rETR_{max}$ and F_v/F_m for *M. aeruginosa* determined by PAM fluorometry after SCLE exposure. (a) rETRmax; (b) F_v/F_m. Data are presented as mean ± standard deviation.

all photosynthetic parameters responded more rapidly than the growth and integrity for *M. aeruginosa* (Figs. 1, 3, 5 and 6). The photosynthetic activity slowly resumed suggested that the impairment was temporary and reversible. In the study, the rETR/E and $rETR_{max}$ parameters remained at low levels at early stage especially with high dose of SCLE. However, algistatic agent and nutrients such as nitrogen and/or phosphorus from decomposing *S. cumini* leaves were brought into algal cultures at the same time, both inhibiting and stimulating effects were present. As a result, photosynthetic parameters mentioned earlier were higher even at higher dosage after SCLE exposure at later period. These findings also suggested that photosynthetic parameters are more indicative on the status of *M. aeruginosa* than biomass.

4. Conclusions

In conclude, the Chl-a concentration of *M. aeruginosa* decreased significantly following addition of the SCLEs and kept low till the end of 15 d experiments. The results of PI and FDA fluorescence experiment showed the inhibition of esterase activity is one of the targets for allelopathy to *M. aeruginosa*. As for the results of rETR/E, $rERT_{max}$ and F_v/F_m, which indicated that photosynthesis was another important target for SCLE on *M. aeruginosa*. The electron transport rate of algal cells may be hindered by SCLE at early period, then started to repair themselves slowly. The results also showed that both inhibition and stimulation effectiveness are existence because of algistatic agent and nutrients in SCLE at the same time. Further study should test SCLE as natural agent(s) to manage cyanobacteria blooms at the simulating natural conditions.

Acknowledgements

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