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Allelopathic effects of harmful algal extracts and exudates on biofilms on leaves of *Vallisneria natans*

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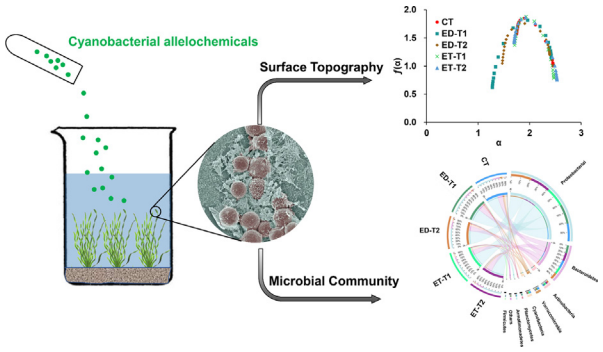
HIGHLIGHTS

- *M. aeruginosa* extract and exudate effects on *V. natans* leaf biofilm were tested.
- *M. aeruginosa* allelochemicals caused physiological stress on the leaf biofilm.
- *M. aeruginosa* allelochemicals altered the surface topography of *V. natans* leaf.
- Exudate led to greater changes to the microbial community in biofilm than extract.

GRAPHICAL ABSTRACT



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ABSTRACT

This study investigated the allelopathic effects of *Microcystis aeruginosa* (*M. aeruginosa*) extracts and exudates on the physiological responses, photosynthetic activity, and microbial structure of biofilms on leaves of *Vallisneria natans* (*V. natans*). By measuring physiological and photosynthetic indices, the results showed that *M. aeruginosa* allelochemicals inhibited photosynthesis, oxidative stress and antioxidant system stress response in the biofilms of *V. natans* leaves. Multifractal analysis found that the surface topography of *V. natans* leaves was altered due to the allelochemicals found in *M. aeruginosa*. Microbial diversity on the leaves was analyzed using high-throughput sequencing, and the results showed that *M. aeruginosa* exudates had a stronger effects on the microbial community structure of biofilms compared to extracts. These findings highlight how cyanobacterial allelochemicals induce negative effects on submerged macrophytes.

1. Introduction

Eutrophication of lakes often results in a complete loss of submerged macrophytes, and a subsequent regime shift towards the dominance of phytoplankton (Scheffer, 1990). In contrast, phytoplankton in hypereutrophic lakes is dominated by cyanobacteria (Sayer et al., 2010). The reduction of phosphorus and nitrogen in hypereutrophic lakes leads to a substantial challenge to policymakers due to its heavy load both from sediment and from external sources. Furthermore, nutrient reduction alone can have a limited effect or be unrealistic in the practice of controlling harmful algal blooms (HABs), as indicated in recent works (Janssen et al., 2017; Van Meter et al., 2018). Submerged macrophytes can suppress HAB formation, and this technique has

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been applied to remove cyanobacteria and restore the ecological environment in eutrophic water (Pan et al., 2011; Seto et al., 2013).

Previous studies mainly focused on the allelopathic effects of submerged macrophytes on cyanobacteria, and using the allelopathic effects to control cyanobacterial blooms (Eigemann, 2013; Gross et al., 2007; Pan et al., 2011; Zhu et al., 2010). Conversely, cyanobacterial allelochemicals can cause adverse effects on the growth, physiological and biochemical processes of submerged macrophytes (Ha and Pflugmacher, 2013; Mitrovic et al., 2004; Kang et al., 2015). However, it remains unclear how cyanobacterial allelochemicals affect submerged macrophytes.

Bloom-forming cyanobacteria can synthesize and release allelochemicals into the aquatic environment by exudation or lysing of cells. Decomposition of cyanobacterial cells can lead to the release of inhibiting compounds (Song et al., 2007), where extracts of cyanobacterial cells inhibit the growth and survival of submerged macrophytes. However, Zheng et al. (2013) indicated that cyanobacteria exudates might have stronger negative effects on submerged macrophytes than extracts of cyanobacteria. Xu et al. (2016) found that compounds exuded during cyanobacteria exponential growth phase was important for disrupting photosynthesis and inducing oxidative stress in submerged macrophytes. However, there were still arguments of the allelopathic interactions between cyanobacteria and submerged macrophytes.

The leaves of submerged macrophytes can provide a large attachment area for bacteria to settle, where the microbial community, including algae and other microbes, form biofilms within microbial communities (Michael et al., 2008). Submerged macrophyte biofilms play an important role in the allelopathic interactions between submerged macrophytes and algae or other phytoplankton (Drake et al., 2003; Hill and Dimick, 2002; Jones et al., 2002; Song et al., 2015). Thus, the submerged macrophyte biofilms can be regarded as main site of interaction between cyanobacteria and submerged macrophytes.

This study tested the effect of the extracts and exudates of cyanobacteria on the photosynthesis and bacterial community structure of the biofilms of submerged macrophytes. These interactions were studied using *Microcystis aeruginosa* (*M. aeruginosa*), one of the most harmful freshwater bloom-forming cyanobacteria (Black et al., 2011; Yamaguchi et al., 2015). Due to its survival and growth potential in eutrophic freshwater, *Vallisneria natans* (*V. natans*) has proven to be superior to other submerged macrophytes and was selected as the experimental subject (Qiu et al., 2001; Yan et al., 1995). This study aimed to test the following hypotheses: (1) *M. aeruginosa* can allelopathically damage the antioxidant defense system of leaves and inhibit photosynthesis in the leaves of *V. natans*; (2) allelochemicals change the bacterial communities and biofilm structure of the *V. natans* leaves; and (3) the allelopathic effects on the microbial community is higher in exudates than extracts.

2. Materials and methods

2.1. Cultivation of M. aeruginosa and V. natans

M. aeruginosa (FACHB-912), isolated from algal blooms in Lake Taihu, was obtained from the Freshwater Algae Culture Collection at the Institute of Hydrobiology at the Chinese Academy of Sciences (FACHB, Wuhan City, China). The cultures were grown in 1000-mL Erlenmeyer flasks with 250 mL of modified BG11 medium according to OECD standards (Table S1) (Rippka et al., 1979) under climate-controlled conditions at 28 ± 2 °C and 70–90% relative humidity under continuous light at 80 μmol quanta m^{-2} s^{-1}, which was measured as photosynthetic photon flux density (PPFD) by a quantum meter (Spectrum Technology, Inc., USA), with a 12:12 h light: dark cycle. Cell suspensions were homogenized using a magnetic stirrer for biomass and biochemical analyses.

Healthy *V. natans* plants were obtained from Pudong Tiancun Horticultural Company (Shanghai, China) and kept in container with 60 mm of sediments (ADA aqua soil, Aqua Design Amano Company, Japan) and aquaria containing tap water for 10 days to acclimate samples before the experiments. The plants were then cleaned with deionized water, and uniformly sized healthy and full plants were chosen for experiments.

2.2. Exudates and extracts of M. aeruginosa

Exudates and extracts were used in place of coexistence experiments to avoid the potential competition for living space between *M. aeruginosa* and *V. natans* (Xian et al., 2005; Wu et al., 2009). *M. aeruginosa* cultures were diluted into two different concentrations using deionized water, samples with *chlorophyll-a* (Chl-*a*) contents of 10 (T1) and 50 (T2) μg L^{-1}. These values were selected based on the two alert levels for HABs using the World Health Organization (WHO) guidelines (Chorus and Bartram, 1999).

To obtain exudates, 3 L of *M. aeruginosa* cultures of low and high Chl-*a* contents were centrifuged at 10,000 ×*g* for 10 min, and the supernatant was filtered through a 0.45 μm glass–fiber filter. The precipitate of *M. aeruginosa* cells was used to obtain extracts and was fixed with modified BG11 mediums to a final volume of 3 L. The samples were frozen at −80 °C for 8 h and thawed at room temperature. This freezing-thawing cycle was repeated three times to destroy all cell walls and membranes. After centrifugation at 3357 ×*g* for 10 min, the supernatant was filtered through a 0.45 μm glass–fiber filter. The color of the exudate medium was similar to the BG11 medium, while the extracts were closer to a blue color due to the phycocyanin content (Fig. S1).

2.3. Experimental design

After acclimation for 10 days, *V. natans* plants were divided into three groups: (1) grown in modified BG11 medium (CT); (2) treated with the exudates of *M. aeruginosa* (ED); and (3) treated with the extracts of *M. aeruginosa* (ET). ED and ET treatments were conducted with the two levels of *M. aeruginosa*, T1 and T2 (Treatment descriptions shown in Table 1). Twelve plants per treatment (three replicates each) with a fresh weight of 1.82 ± 0.28 g, shoot height of 14.22 ± 0.72 cm, and root length of 5.62 ± 1.21 cm were cultivated in a 5 L plexiglass container with 60 mm sediment (ADA aqua soil, Aqua Design Amano Company, Japan) and 3 L of modified BG11 medium. Plants were incubated at 28 ± 2 °C under continuous light of 80 μmol quanta m^{-2} s^{-1} (PPFD) with a 12:12 h light; dark cycle in a container. Leaf biofilm samples were collected on the 12th day of culture, in order to allow biofilm to form and stabilize.

2.4. Measurement of aquatic parameters and chlorophyll fluorescence

Aquatic parameters, including the pH and oxidation-reduction potential (ORP) of the media, were examined in water by a portable instrument (YSI, WEISS instrument, U. S.) every other day. The F_v/F_m ratios (maximum quantum yield of photosystem II (PSII)) of leaves were examined every two days by a portable PAM fluorometer (AquaPen-C, Photon Systems Instruments, Czech Republic) adopting the methods for submerged macrophytes from Hanelt and Roleda (2009).

Table 1
Description of the treatment conditions on *V. natans*.

Treatment	Culture of modified BG11 medium	Chlorophyll-*a* contents of *M. aeruginosa* for exudates addition	Chlorophyll-*a* contents of *M. aeruginosa* for extracts addition
CT	Yes	0	0
ED-T1	Yes	10 μg L^{-1}	0
ED-T2	Yes	50 μg L^{-1}	0
ET-T1	Yes	0	10 μg L^{-1}
ET-T2	Yes	0	50 μg L^{-1}

2.5. Measurement of total chlorophyll, malondialdehyde content, and enzyme activity

To determine total *chlorophyll* ($a + b$) levels, approximately 0.2 g fresh leaf samples with biofilm were collected in liquid nitrogen. Chlorophyll content was extracted using 90% hot ethanol, and total chlorophyll content was determined using a spectrophotometer at 649 and 665 nm. The contents of total *chlorophyll* ($a + b$) were calculated following the method of Wang et al. (2008).

To determine malondialdehyde (MDA) content, approximately 0.5 g leaf samples were homogenized in 5 mL of 10% (w/v) trichloroacetic acid (TCA). The homogenate was centrifuged at 15,000 ×*g* for 10 min, and 4 mL of 20% TCA containing 0.65% (w/v) 2-thiobarbituric acid (TBA) was added to 1 mL of supernatant. The mixture was heated in a hot water bath at 95 °C for 25 min, and immediately placed in an ice bucket to stop the reaction. The samples were then centrifuged at 10,000 ×*g* for 5 min at 4 °C, and the absorbance of the supernatant was measured at 440, 532, and 600 nm. MDA equivalents were calculated following methods from Hodges et al. (1999).

To extract enzymes, 1 g of plant leaf samples was ground in liquid nitrogen and mixed with 10 mL of 50 mM potassium phosphate buffer (PBS, pH of 7.0) containing 1 mM EDTA and 1% polyvinylpyrrolidone (w/v). The mixture was centrifuged at 4 °C for 20 min at 15,000 × *g*, and the resulting supernatants were used to determine superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD) enzymatic activity using assay kits (XYbscience, China) following the instructions supplied by the manufacturer. The total chlorophyll, MDA content, and enzyme activities were measured on the last day (12 days).

2.6. Scanning electron microscopy (SEM) and multifractal analysis

SEM was used to visualize the distribution of bacteria and algae on the biofilm surface. For SEM analysis, *V. natans* leaves were carefully sampled from upper-middle part of the leaves, and cut into 0.8 × 0.8 cm squares and fixed in glutaraldehyde (2.5% in 50 mmol/L sodium cacodylate). After a double rinse with 0.1 M PBS (pH of 7.4), the leaf samples were dehydrated using a series of ethanol concentrations (20, 40, 60, 80 and 90%) for 15 min and with 100% ethanol twice for 15 min. Dried samples were visualized using SEM (Hitachi, Japan, S-3400 NII).

SEM images were used for subsequent multifractal analysis with software *FracLac for ImageJ* V.2.5 after binary conversion. In general, multifractal describes a measurement that is defined in a certain area or volume that can divide the defined domain into a sequence of subdomains in space. According to the singularity of the multifractal measurement, every subdomain constitutes a single fractal (Liang et al., 2013). Multifractals not only have fractal dimensions but also have singularity of every measurement, which constitutes the multifractal spectrum language (α)–$f(\alpha)$. Box counting is one of the methods in a multifractal analysis and involves counting the number of occupied boxes over a range of different box sized images. In this study, the box counting method was applied following similar approaches as previous studies (Liang et al., 2013; Wang et al., 2014). The width of the multifractal spectrum was $\Delta\alpha = \alpha_{max} - \alpha_{min}$, which quantitatively describes the degree of heterogeneity, and $\Delta f = f(\alpha_{max}) - f(\alpha_{min})$, which denotes the difference in the fractal dimensions between the maximum and the minimum probability subsets.

2.7. Analysis of total organic carbon (TOC) and microbial community of biofilms

TOC can be regarded as the most comprehensive measurement used to quantify organic matter content in biofilms attached to the leaf system (Leenheer and Croué, 2003). For the TOC analyses, approximately 1 g leaf samples were collected from healthy plants and transferred into a sterile 50 mL polyethylene bottle containing 20 mL of 10 mM PBS solution. After ultrasonication treatment for 1 min, mixtures were shaken for 5 min at 25 °C and exposed to ultrasonication for 1 min. The suspensions from the same sample were combined, and 100 μL suspension samples were analyzed using a total organic carbon analyzer (TOC-L CPH, Shimadzu, Japan) to measure the difference between the total carbon and inorganic carbon.

Parts of the suspension samples were used for DNA extraction after samples were passed through a sieve with a 50 μm mesh to remove plant debris, and subsequently centrifuged at 10,000 ×*g* for 5 min. Samples were preserved in plastic tubes at −80 °C. DNA extraction and high-throughput sequencing were carried out by Majorbio Bio-Pharm Technology Co. Ltd. (Shanghai, China). Briefly, microbial DNA was extracted from sediment samples using a E.Z.N.A.® Soil DNA Kit (Omega Biotek, Norcross, U. S.). The V3-V4 hypervariable region of 16S rRNA was amplified using a barcoded universal primer set, including 338F (ACTCCTACGGGAGGCAGCAG) and 806R (GGACTACHVGGGTWTCTAAT). High-throughput sequencing was conducted using the Illumina MiSeq platform and bioinformatic analysis were used to analyze the diversity of the microbial communities and to identify different species (Fadrosh et al., 2014).

2.8. Statistical analysis

One-way analysis of variances (ANOVA) was used to the test differences between the experimental groups (ED-T1, ED-T2, ET-T1 and ET-T2) and the control group (CT), where differences were considered statistically significant at $p < 0.05$. Data are presented as the means ± standard deviations of triplicate samples. An analysis of the structure and abundance of the bacterial community in the leaf biofilms of *V. natans* was performed using the free online platform Majorbio I-Sanger Cloud Platform (www.i-sanger.com).

3. Results and discussion

3.1. Effect of M. aeruginosa allelochemicals on growth of V. natans

Both the extracts and the high level of exudates of *M. aeruginosa* had significantly increased the fresh weight of *V. natans* ($p < 0.01$, Fig. 1a). However, allelochemicals, especially in the extracts, had obvious adverse effects on shoot length growth ($p < 0.01$, Fig. 1b). Previous studies have reported that extracts or extracellular substances of cyanobacteria may have either growth-promoting or growth-inhibiting effects on different species under different environmental conditions (Safonova and Reisser, 2010; Shinohara et al., 2014). The results suggest that that algal organic matters (AOMs) or degraded algae may be utilized as nutrients by *V. natans* for growth of biomass, while the allelochemicals of cyanobacteria negatively affect shoot growth and length.

3.2. Effect of M. aeruginosa allelochemicals on photosynthesis of V. natans leaves

The F_v/F_m ratio of *V. natans* leaves was significantly lower in samples treated with extract and exudate compared to untreated controls. In the late stage of the experiment, the F_v/F_m ratio in the exudate groups (ED-T1 and ED-T2) was lower than that of the extract groups (ET-T1 and ET-T2) (Fig. 2). Zheng et al. (2013) reported similar results, finding that high concentrations of exudates and extracts of *M. aeruginosa* allelopathically inhibit the photosynthesis of submerged macrophytes, and the exudates had stronger effects. Cyanobacterial allelochemicals can decrease the F_v/F_m ratio in the leaves of submerged macrophytes significantly, and the reduction in photosystem II activity can underlie the allelopathic effects of *M. aeruginosa* on submerged macrophytes (Zheng et al., 2013). *M. aeruginosa* extracts and exudates resulted in adverse effects on *V. natans*, leading to increased reactive oxygen species (ROS) content and higher oxidative damage in the *V. natans* leaves.

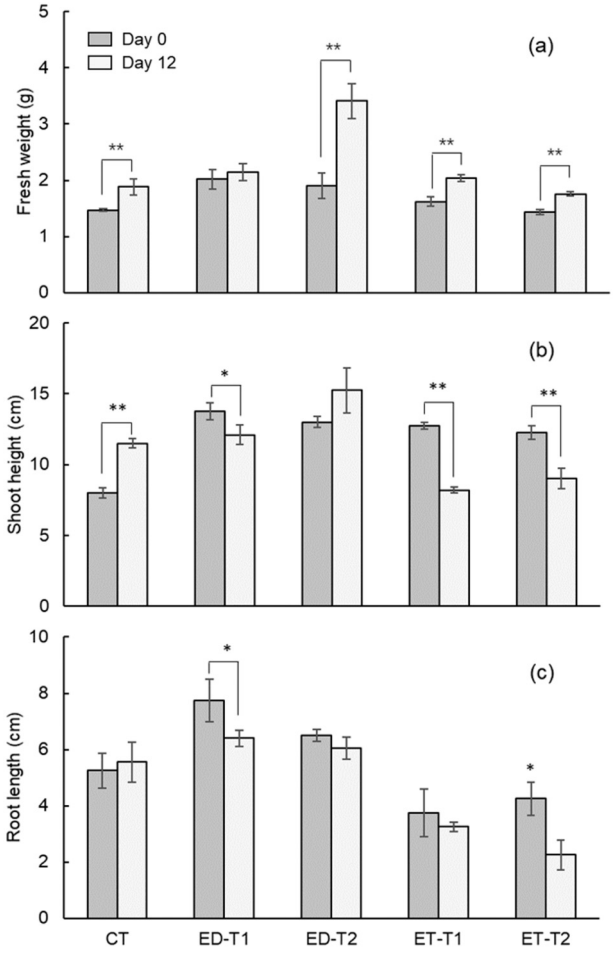

Fig. 1. Effects of *M. aeruginosa* exudates and extracts obtained from cultures of different *chlorophyll-a* contents on *Vallisneria natans*. * Indicates significant differences at $p < 0.05$, and ** indicates significant differences at $p < 0.01$.

ROS is considered to be one of the main reasons for photosynthesis inhibition (Richter et al., 1990).

3.3. Effect of M. aeruginosa allelochemicals on membrane lipid peroxidation and the antioxidant defense system of V. natans leaves

The degrees of oxidative damage in *V. natans* leaves were examined by determining the level of lipid peroxidation, which was estimated from the concentration of malondialdehyde (MDA) in the leaves. MDA content was higher in the exudate groups (ED-T1 and ED-T2) and the extract groups (ET-T1 and ET-T2) compared to the CT group (Fig. 3a), which could indicate hydroxyl radicals attacking the lipids and proteins and altering their structure. In general, the activities of SOD, POD, and CAT were higher in *V. natans* leaves in samples treated with extract and exudate compared to the untreated control (Fig. 3b, c, d). The increase in CAT, SOD and POD activities is likely related to the increased

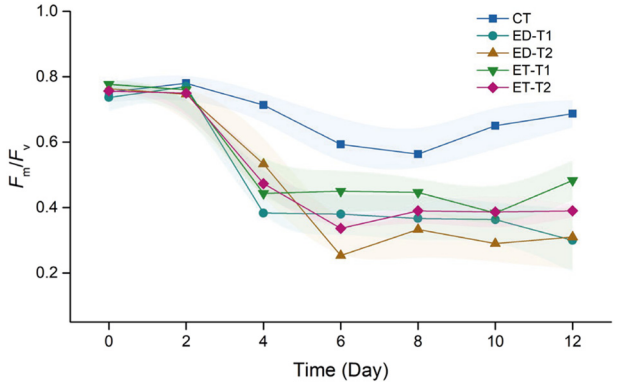

Fig. 2. Effects of *M. aeruginosa* exudates and extracts obtained from cultures of different *chlorophyll-a* contents on F_v/F_m of *Vallisneria natans*.

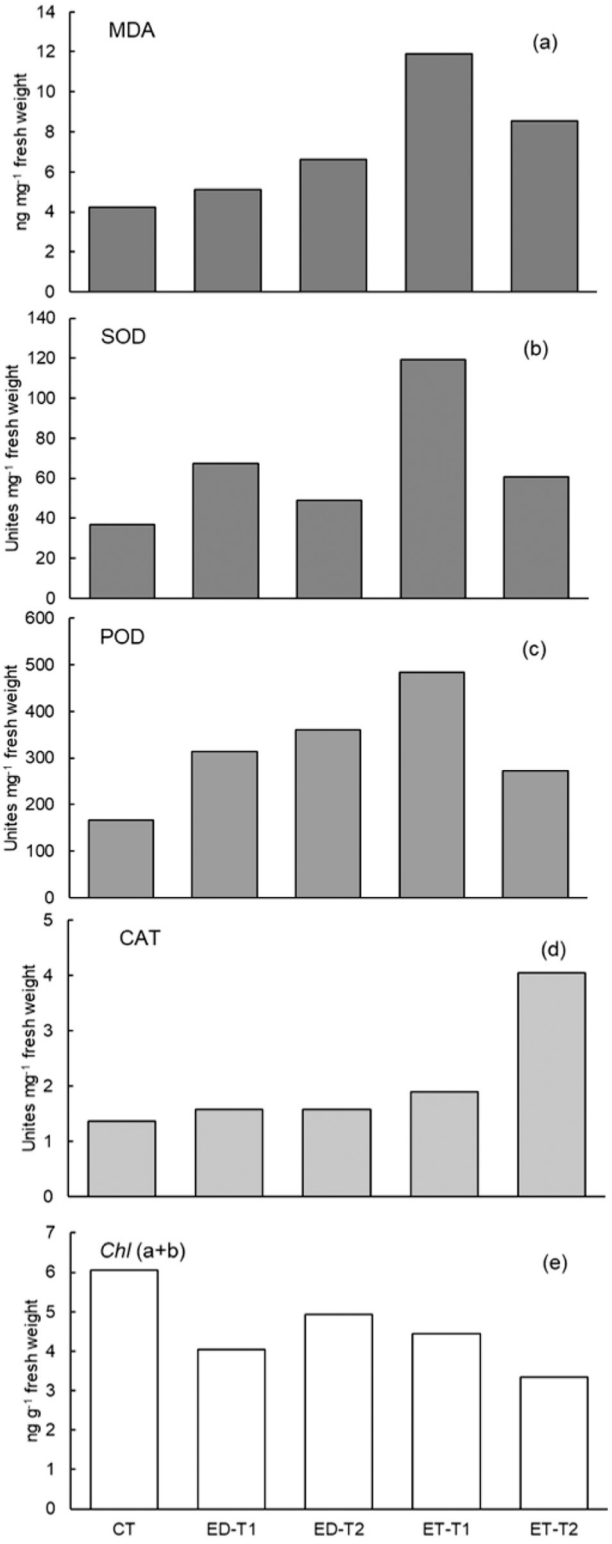

Fig. 3. Effects of *M. aeruginosa* exudates and extracts obtained from cultures of different *chlorophyll-a* contents on antioxidant system of *Vallisneria natans*.

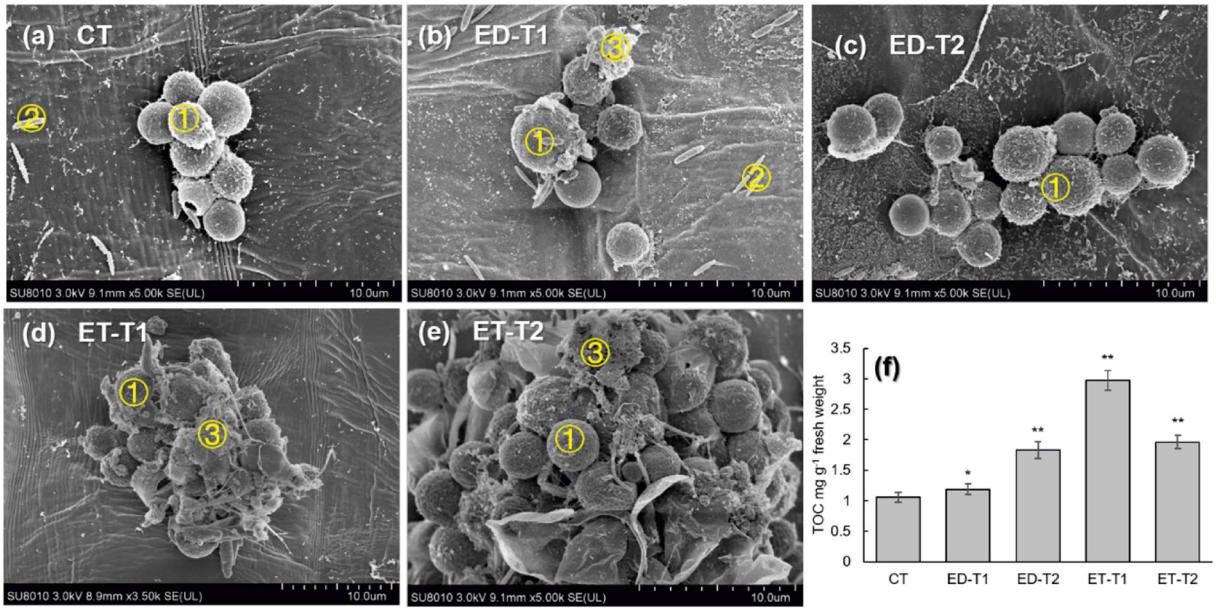

Fig. 4. SEM images (a–e) and TOC changes (f) of biofilms attached to leaves of *Vallisneria natans* of CT, ED, and ET groups. ①, cocci aggregates; ②, bacillus aggregates; ③, organic matter or extracellular polymers. * indicates significant differences at $p < 0.05$, and ** indicates significant differences at $p < 0.01$.

production of ROS, which will be detoxified as a result of the antioxidant reactions. The activities of the antioxidases were noticeably higher in the extract groups (ET-T1 and ET-T2) than in the exudate groups (ED-T1 and ED-T2) (Fig. 3b, c, d), suggesting that the extracts of *M. aeruginosa* caused more damage to the antioxidant defense system of *V. natans* leaves than the exudates. In addition, the total chlorophyll content of *V. natans* leaves in both the extract and exudate groups was significantly lower than the control, and this was likely caused by the cellular damage of leaves (Fig. 2e).

Exudates at low and high concentrations affected the antioxidant response of leaves in a similar manner, which the low concentration of extracts caused more damage on antioxidant defense system than the high concentration extracts, based on the activity of MDA, SOD, and POD enzymes. Samples were placed under experimental condition for 12 days, as the formation of biofilms on the leaf surface requires at least 10 days of culture. However, 12 days may be too long to assess the oxidative stress and antioxidant response of the leaves, as previous studies measuring the effects of external factors on oxidative stress of *V. natans* measured these parameters in <7 days (Ge et al., 2012; Wang et al., 2011). In addition, the high extract concentration may have led to the unusual results in extracts groups. It is speculated that there may be a saturation point for allelochemicals to affect antioxidant responses in leaves, and when the allelochemicals concentration is higher than the saturation value, increasing allelochemicals concentrations will not affect antioxidant response in leaves. However, more studies are needed to test this hypothesis.

3.4. Effect of *M. aeruginosa* allelochemicals on the biofilm community of *V. natans* leaves

3.4.1. SEM and multifractal analysis of microbes in leaf biofilms of *V. natans*

To investigate the morphology of biofilms attached to the leaf surfaces of *V. natans*, SEM images of the leaf surface were obtained (Fig. 4). A large number of particles were observed on the surface of *V. natans* leaves, including cocci, bacillus, and other forms of bacteria. The biofilms of the ET-T1 and ET-T2 groups visually had more microbes and derived organic matters than the other three groups (Fig. 4d and e). The results of the TOC analysis also supported the results of the SEM images (Fig. 4f). The TOC contents in the ET-T1 and ET-T2 groups were significantly higher than the CT, ED-T1, and ED-T2 groups ($p < 0.01$). Both extracts and exudates of cyanobacteria can be utilized as a nutrient source for the growth of bacteria and phytoplankton, and thus increase the TOC content. Organic matters in cyanobacterial extracts are about five times higher than exudates (Gao et al., 2010), and extracts of cyanobacteria can supply nutrients for the growth of bacteria and phytoplankton compared to exudates; this may have led to the higher TOC

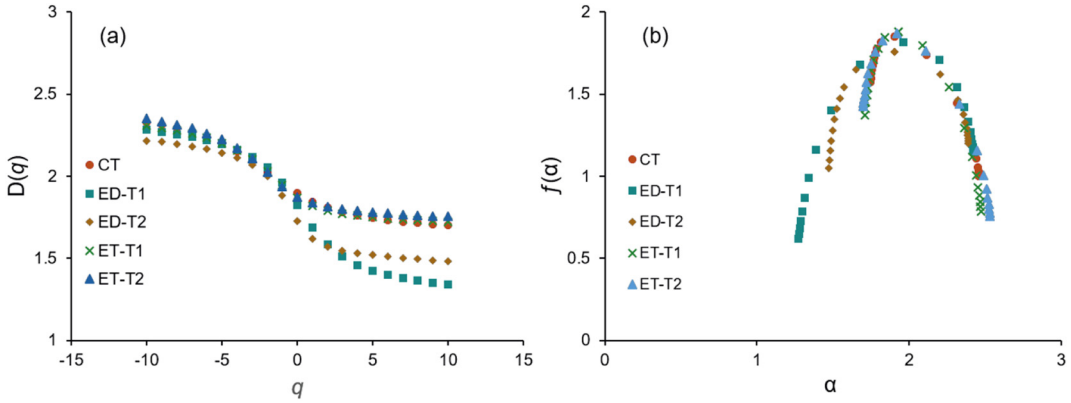

Fig. 5. (a), Generalized dimension spectra q-D(q) and (b), Multifractal spectra $f(\alpha)$ of leaf-biofilm of *Vallisneria natans*.

Table 2
Multifractal parameters of leaf surface from *V. natans*.

Samples	α_{min}	α_{max}	$f(\alpha_{min})$	$f(\alpha_{max})$	$\Delta\alpha$	$\Delta f(\alpha)$
CT	1.75	2.46	1.57	1.00	0.71	0.57
ED-T1	1.27	2.42	0.62	1.14	1.15	−0.53
ED-T2	1.47	2.39	1.05	1.20	0.92	−0.15
ET-T1	1.71	2.47	1.37	0.79	0.76	0.59
ET-T2	1.70	2.53	1.43	0.76	0.83	0.67

contents in samples treated with extracts compared to exudates. AOM in cyanobacterial extracts may also directly lead to an increase in TOC content (Granéli et al., 1999; Zhang et al., 2010).

To understand how extract and exudate treatments affected biofilm distribution, a multifractal analysis using the SEM images (Fig. S3) was employed to analyze the topographic characters of biofilms attached to the *V. natans* leaves. The generalized dimension spectrum series D (q) of values of different orders in each group are in descending order instead of being constant (Fig. 5a; Table 2); thus, these series comprise obvious multifractal characteristics (Liu et al., 2016). The multifractal singular spectra were all asymmetrical upper convex curves, and the exudate groups (ED-T1 and ED-T2) had typical left deviation multifractals, while the CT and extract groups (ET-T1 and ET-T2) had right deviation multifractals, which indicated the patches of biofilm were small in the CT and extract groups and large in the exudate groups. The order of width of the multifractal spectrum $\Delta\alpha = \alpha_{max} - \alpha_{min}$ was ED-T1 > ED-T2 > ET-T1 > ET-T2 > CT (Table 2), suggesting that the exudate group was more heterogeneous than the extract and CT groups. These results suggest that the extracts and exudates of *M. aeruginosa* can damage the leaf surface and increase the roughness of the leaf surface. $\Delta f = f(\alpha_{min}) - f(\alpha_{max})$ represents the difference in the fractal dimensions between the minimum probability ($\alpha = \alpha_{min}$) and the maximum probability ($\alpha = \alpha_{max}$), where $\Delta f < 0$ denotes small probability subsets predominate and $\Delta f > 0$ denotes large probability subsets predominate in a given sample. The Δf values in the CT and extract groups were larger than zero, and Δf was below zero in the exudate groups, following the order ET-T2 > ET-T1 > CT > ED-T2 > ED-T1 (Table 2). These results indicate that the microbes in the probability subset of the maximum biofilm distribution are larger than in the minimum subset in the exudate groups, while the opposite occurs in the CT and extract groups.

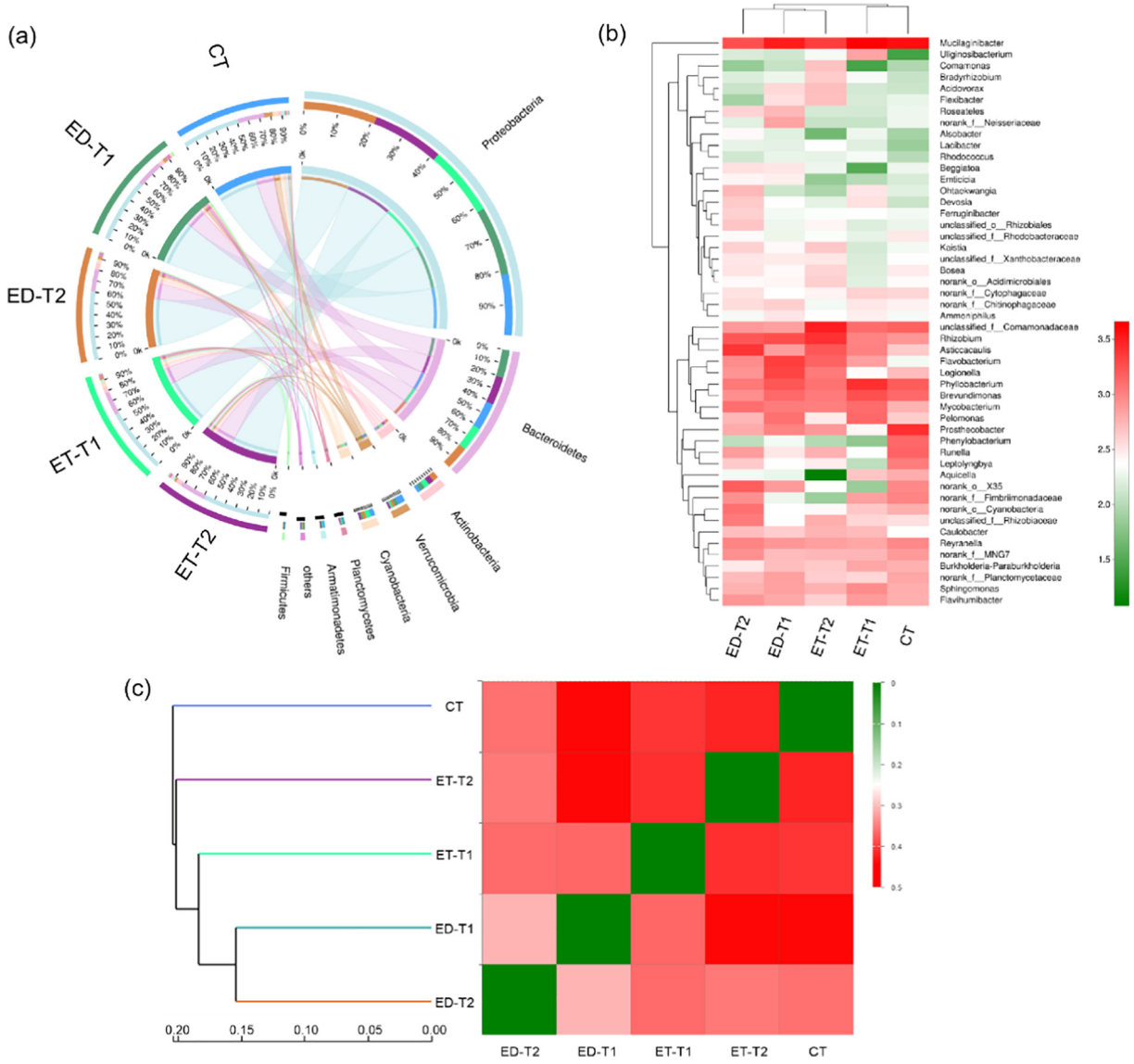

Fig. 6. Distribution of microbial community for each sample. (a), Circos plot, the width of the bars from each phylum indicate the relative abundance of that phylum in the sample; (b), Heat map of different samples at genus level and (c), Hierarchical clustering tree and distance heatmap on OTU level.

Specifically, the exudates of *M. aeruginosa* may increase single colony-like biofilms, and extracts increase multiple colony-like biofilms.

3.4.2. Structure and abundance of the bacterial community in the leaf biofilms of V. natans

The 16S rRNA sequence was used to investigate the structure and abundance of the microbial community in the biofilms of *V. natans* leaves. Sequences were classified into a total of 667 operational taxonomic units (OTUs) at a 97% similarity threshold. The width of the curves increased with increasing OTU, indicating that the microbial community was diverse on the *V. natans* leaves (Fig. S4). In addition, the evenness in the microbial composition and the microbial diversity were similar among the five treatment groups. Using 184,161 sequences, four microbial α diversity indices were observed: Shannon, Simpson, Chao1, and Good's coverage (Table S2). The Good's coverage indices were high in all groups, indicating that the sequencing results represent the real condition of the samples. The Shannon indices of the five groups were very similar, indicating that the richness and evenness of the microbial community were very similar across treatments. The Simpson value of the CT group (0.027) was higher than the experimental groups, except for ET-T2 (0.031), suggesting that the cyanobacterial allelochemicals decrease the microbial abundance in most treatments.

All samples had similar predominant bacterial species (Fig. 6a). *Proteobacteria*, *Bacteroidetes*, *Actinobacteria*, *Verrucomicrobia*, and *Cyanobacteria* were the five most dominant phyla in the leaf biofilm samples of *V. natans*. Similarly, Gong et al. (2018) also reported that these five phyla are dominant in leaf biofilms of submersed macrophytes. *Proteobacteria* was the most abundant phylum across treatment conditions: CT (53%), ED-T1 (59%), ED-T2 (63%), ET-T1 (60%), and ET-T2 (61%). *Bacteroidetes* was the second-most dominant phylum, with an abundance of 24% in the CT group, 24% in the ED-T1 group, 21% in the ED-T2 group, 21% in the ET-T1 group, and 24% in the ET-T2 group. The *Cyanobacteria* content was relatively low, ranging from 1.8% to 4.9%, which was in accordance with the SEM results (Fig. 4, Fig. S3). The microbial composition analysis was performed at the genus level (Fig. 6b) and the red color represented the genera with the highest abundance in the corresponding sample. But, the green color represented the genera with the lowest abundance. *Mucilaginibacter* was the most abundant among the five groups. *Phenylobacterium* and *Aquicella*, genera were abundant in the CT group and they decreased significantly in the experimental groups. So, the cyanobacterial allelochemicals affected the microbial community structure.

The results of hierarchical clustering analysis also supported the finding that the allelochemicals of *M. aeruginosa* altered the structure of the microbial community of biofilms on *V. natans* leaves (Fig. 6c). A distance heatmap showed that exudates and extracts resulted in different effects on microbial communities. The exudates of *M. aeruginosa* led to greater changes in the structure of the biofilm microbial community compared to the extracts of *M. aeruginosa* (Fig. 6c), which was in agreement to the conclusions of Zheng et al. (2013).

4. Conclusion

The allelopathic effects of *M. aeruginosa* extracts and exudates on the biofilms of leaves of *V. natans* were investigated. The results indicated that *M. aeruginosa* allelochemicals could inhibit photosynthesis, oxidative stress and antioxidant system stress response of the biofilms of *V. natans* leaves. The allelochemicals of *M. aeruginosa* damaged the leaf surface and increased the roughness of the leaf surface. This study also showed that the exudates of *M. aeruginosa* may increase single colony-like biofilms, while extracts have the opposite effects. The exudates of *M. aeruginosa* caused greater changes in the microbial structure of biofilms in *V. natans* leaves compared to extracts. The study provides information on how cyanobacterial allelochemicals induce adverse effects in submerged macrophytes.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2018.11.296.

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