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Combined toxic effects of anatoxin-a and microcystin-LR on submerged macrophytes and biofilms

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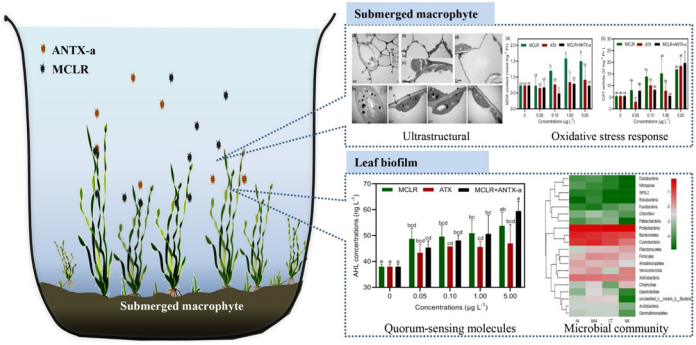
GRAPHICAL ABSTRACT



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ABSTRACT

Hazardous substances, such as anatoxin-a and microcystin-LR, are released into the aquatic environment during cyanobacterial blooms, causing significant ecological risk. To assess the toxic effects of anatoxin-a, microcystin-LR and their combined exposure on submerged macrophytes and biofilms, *Vallisneria natans* was exposed to solutions containing different concentrations of anatoxin-a and microcystin-LR (0.05–5.00 μg L^{-1}). Results showed that *Vallisneria natans* was sensitive to anatoxin-a of 0.05 μg L^{-1}, and antagonistic effects were induced at combined microcystin-LR and anatoxin-a exposure. Single and combined exposure effectively induced antioxidant responses such as promoted activities of superoxide dismutase, peroxidase and catalase, as well as increased glutathione S-transferase, glutathione and malondialdehyde content. In addition, anatoxin-a and microcystin-LR could also be absorbed by *Vallisneria natans* and trigger plant defense responses, generating increased concentrations of the phytohormones abscisic acid and strigolactones. Moreover, the abundances and structure of the microbial community in periphyton biofilms were altered by combined anatoxin-a and microcystin-LR exposure. The enhanced concentration of N-acylated-L-homoserine lactone indicated that the assessed cyanotoxins had a significant influence on quorum-sensing in biofilm microbial communities. These results demonstrated that anatoxin-a and microcystin-LR at environmentally relevant concentrations could disrupt homeostasis, induce effective defense mechanisms of *Vallisneria natans* and alter biofilms in aquatic ecosystems.

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1. Introduction

Eutrophication of lakes and reservoirs results in frequent and abundant outbreaks of cyanobacterial blooms (Carmichael, 2008), which present a high ecological risk to the aquatic environment. Cyanotoxins are secondary metabolites generated by different genera of bloom forming cyanobacteria, including cytotoxins, neurotoxins and dermatoxins (Buratti et al., 2017). Recently, microcystin-LR (MCLR) has been identified as the most common and the most toxic structural microcystin variant (Pérez and Aga, 2005). According to the World Health Organization, the guideline maximum concentration of MCLR in drinking water is 1.0 μg L^{-1}, with concentrations ranging from 0.1–10.0 μg L^{-1} in most lakes (Lahti et al., 1997). Anatoxin-a (ANTX-a) is a potent cyanobacterial neurotoxin, which presents a serious health risk to humans and can be lethal to aquatic organisms (Sabart et al., 2015). These cyanobacterial pollutants maybe have adverse effects on aquatic systems and human health even at low exposure levels (Liu et al., 2018a).

In aquatic ecosystems, submerged macrophytes play an important role in the purification of eutrophic water and plants are frequently exposed to cyanotoxins during blooms and subsequent cyanobacterial decay (Kosten et al., 2009; Wiegand and Pflugmacher, 2005). It has been reported that MCs released into waterbodies could cause inhibitory effects on the growth and photosynthesis of aquatic plants, which can induce oxidative stress by promoting the release of reactive oxygen species (ROS) in plants (Jose and LEFLAIVE, 2007). The effects of ANTX-a exposure on aquatic organism have also been explored, with previous studies investigating the generation, removal and degradation of ANTX-a in aquatic systems. However, the effects of ANTX-a on submerged macrophyte have seldom been reported. There has been an abundance of investigations on the detrimental influence of ANTX-a on animals, including muscle overstimulation, leading to paralysis and causing the inhibition of abdominal breathing (Osswald et al., 2007). Although this cyanotoxin is neurotoxic to animal, Dr. Mitrovic group found that ROS was formed in the plant cell with ANTX-a exposure (Osswald et al., 2007). Meanwhile, Dr. Kaminski group reported that ANTX-a can be phytoremediated by aquatic macrophytes such as *Lemna trisulca L.* in natural ecosystems (Kaminski et al., 2014). However, different kinds of cyanotoxins commonly co-exist in waterbodies and therefore, investigations of single toxin exposure to aquatic plants with cyanotoxins such as MCs and ANTX-a, are not representative of the real environment. This highlights that a comprehensive study of the combined toxic effects of MCLR and ANTX-a is necessary.

Furthermore, numerous reports have indicated that environment stress triggers many changes to the biochemical and physiological characteristics of plants, due to increased synthesis of phytohormones (Wang et al., 2003). For instance, heavy metals, drought, extreme temperatures and nutrient deficiency have been found to induce changes in gene expression, as shown with abscisic acid (ABA) synthesis in plant stress responses (Danquah et al., 2014). The plant hormone strigolactone (SL), can regulate a variety of growth and developmental processes in plants and has been investigated for its involvement in plant defense responses (Gomez-Roldan et al., 2008; Torres et al., 2014). Investigations into ABA and SLs are rapidly emerging, expanding our understanding of their relevance in plant physiology. However, no study has yet assessed the relationship of ABA and SLs with defense responses to MCLR and ANTX-a exposure in submerged macrophytes.

Periphytic biofilms attached to submerged macrophyte surfaces, are microbial aggregates commonly observed in aquatic systems such as lakes and ponds, which also play a significant role in nutrient cycling in wastewater restoration (Zhao et al., 2018). Cyanobacterial blooms in eutrophical lakes induce adverse effects on biofilms and the natural competitors of palanktonic cyanobacteria in freshwaters are other photoautotrophs in the habitat - submerged macrophytes and periphyton (Trbojević et al., 2019). Recently, an increasing number of studies have attempted to utilize natural periphytic biofilms for eutrophic water restoration (Han et al., 2018). Previous studies have observed the ability of biofilms to absorb and biodegradate MCLR in water (Li et al., 2012). However, few studies have assessed the effects of ANTX-a in combination with different cyanotoxins on biofilms (Mitrovic et al., 2004). Moreover, quorum-sensing (QS) signal molecules produced by bacteria affect the formation and function of biofilms (Shrout and Nerenberg, 2012). As a QS signal molecule, N-Acyl-L-homoserine lactones (AHLs) could influence extracellular polymeric substances production, surface motility, interspecies competition, and growth regulation for specific bacterial species through the LuxR-homologue regulatory proteins initiate transcription of select genes (Engebrecht et al., 1983). But the variations of QS signals in biofilm under MCLR and ANTX-a stress have not yet been described.

Understanding of the way that cyanobacterial blooms and cyanotoxins affect macrophytes and periphyton is of crucial importance in maintaining and reestablishing aquatic ecosystem health. This work aimed to comprehensively investigate the combined toxic effects of ANTX-a and MCLR on submerged macrophytes and periphyton biofilms. Specifically, the present study investigated: (1) plant growth, oxidative responses and combined toxicity to ANTX-a and MCLR; (2) the uptake of ANTX-a and MCLR by *V. natans*; (3) changes in ABA and SL phytohormones in *V. natans*; and (4) microbial properties and AHLs in biofilms.

2. Materials and methods

2.1. Chemicals and plant materials

The freshwater perennial *V. natans* was selected as it is tolerant to eutrophic water conditions. Mature aquatic plants *V. natans* were purchased from the Tiancun Horticultural Company (Shanghai, China) and were grown in 1/10 Hoagland solution. In the plant growth chamber, light intensity was maintained at 80 μmol m^{-2} s^{-1} with 12 h light/ 12 h dark cycles at 25 °C. After 4–6 days of cultivation, similar plants were chosen and cleaned prior to subsequent experiments. MCLR (purity > 95.0 %) was obtained from Taiwan Algal Science Inc (Taiwan, China), while ANTX-a (purity > 98.0 %) was purchased from Enzo Life Sciences Inc (New York, USA).

2.2. Experimental design

5.0 g fresh weight (FW) plants were cultivated in a 5 L plexiglass container with 3 L Hoagland solutions and 50 mm of silica sand (ADA aqua soil, Aqua Design Amano Company, Japan). ANTX-a and MCLR were dissolved in distilled water, and the solutions were diluted to achieve final experimental concentrations of 0.05, 0.10, 1.00 and 5.00 μg L^{-1}. For the combined ANTX-a and MCLR treatments, concentrations of ANTX-a and MCLR maintained at equal levels of 0.05 + 0.05, 0.10 + 0.10, 1.00 + 1.00 and 5.00 + 5.00 μg L^{-1}. The control treatment utilized the same medium without the addition of toxins. Each treatment was performed in triplicate, with cultivation at 25 ± 2 °C and a light intensity of 70 − 75 μmol m^{-2} s^{-1} for 14 days, resulting in rapid biofilm growth and biofilm stability on leaves within 10 days. Distilled water was added daily to replenish evaporated water. At the end of the experimental period, plants and solution were collected for analysis.

2.3. Plant growth, enzyme activity and MDA determination

V. natans was rinsed and FW was measured to assess the biomass (g) of samples after 14 days of exposure. To establish the concentration and activity of plant enzymes, 1.0 *g* (FW) of whole plants material was rinsed and then frozen in liquid nitrogen, ground to a powder and combined with 10 mL 0.1 mol L^{-1} phosphate buffered saline (PBS) (pH 7.0). Following this, the extracts were centrifuged at 10,000 × g at 4 °C

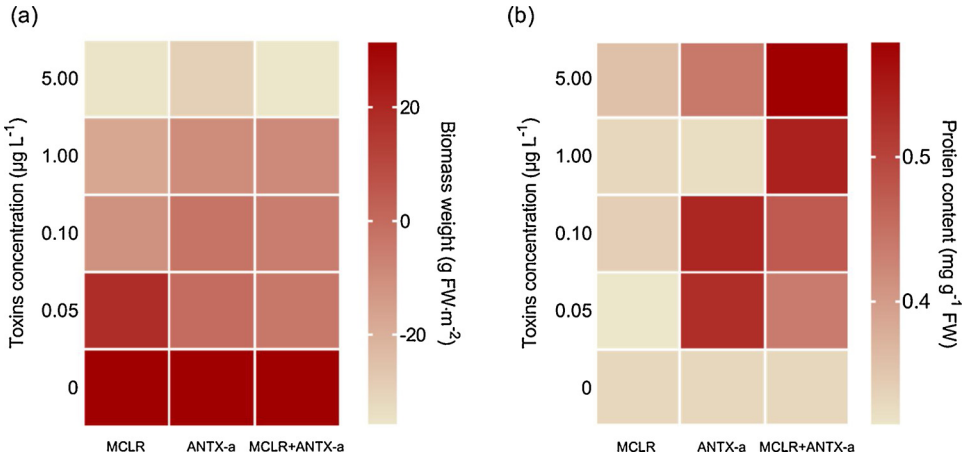

Fig. 1. Effects of MCLR, ANTX-a and their mixture on biomass (a) and protein (b) of *V. natans* leaves.

for 20 min to obtain supernatants. Superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), glutathione S-transferase (GST), glutathione (GSH) malondialdehyde (MDA) and the protein content were assessed in supernatants using a commercialized chemical assay kit: SOD assay kit, CAT assay kit, POD assay kit, GST assay kit, GSH and MDA assay kit (Nanjing Jiancheng Bioengineering Institute, China).

2.4. ANTX-a and MCLR determination and analyses

To determine the ANTX-a and MCLR concentration in plants, 1.0 *g* (FW) plant material was prewashed and frozen, crushed and combined with 10 mL 0.1 mol L^{-1} PBS (pH 7.0). Then, the homogenate was centrifuged (4000 × g for 20 min) with the supernatant collected and filtered through a glass-fiber filter (0.45 μm) (Mitrovic et al., 2004). The concentrations of ANTX-a and MCLR were analyzed using a MCLR enzyme-linked immunosorbent assay (ELISA) kit and ANTX-a ELISA kit (Mlbio Company, China). All processing operations were performed at 4 °C and each sample was analyzed in triplicate.

2.5. ABA, SL determination and microscopic analysis

To determine the ABA and SL contents in plants, 0.5 g (FW) frozen plant root material was ground to powder with liquid nitrogen and combined with 5 mL 0.1 mol L^{-1} PBS (pH 7.0). Extraction and purification of ABA and SL in root material was carried out after 14 days, using the method reported by Dr. Bacaicoa group (Bacaicoa et al., 2009). Quantification of ABA and SL was performed using the ABA ELISA kit and SL ELISA kit (Mlbio Company, China), respectively.

Plant leaves were fixed with 2.5 % glutaraldehyde for 24 h and then flushed twice with 0.1 mol L^{-1} PBS (pH 7.4). Fixed leaves were then dehydrated with different concentrations of ethanol (20, 40, 60, 80 and 90 %) for 15 min and then immersed twice in 100 % ethanol for 15 min. A transmission electron microscope (H-7650, Hitachi, Japan) was used to observe ultra-thin sections of dried plant cells.

2.6. Microbe and AHL signal molecule examination

Biofilms on leaves were collected after 14 days of treatment, with the methods of biofilm microbe collection from leaves described in a previous study (Li et al., 2019). An E.Z.N.A. Soil DNA Kit (Omega, D5625-01, USA) was used for microbial DNA extraction and high-throughput sequencing of DNA was provided by Majorbio Bio-Pharm Technology Co., Ltd. (Shanghai, China) using an Illumina MiSeq platform. The bacterial community structure and abundance of leaf biofilms were analyzed using the online platform Majorbio I-Sanger Cloud Platform (www.i-sanger.com).

To obtain the AHL signal molecules in biofilms, leaves covered by biofilms were frozen, ground to powder and mixed with 9 mL 0.01 mol L^{-1} PBS (pH 7.0). The homogenate was centrifuged at 4000 × g for 20 min to collect the supernatant. The AHLs content in the supernatant was analyzed using an AHL ELISA kit (Mlbio, China) according to the method reported by Dr. Chen group (Chen et al., 2010).

2.7. Statistical analysis

Combined toxic effects of MCLR and ANTX-a were calculated using Abott' s formula. The expected inhibitions of the mixture were expressed as percent (Cexp) and the ratio of inhibition (RI) for the mixture of toxicants was calculated according to the research of Dr. Wang (Wang et al., 2017).

All data was processed using GraphPad Prism. Homogeneity of variance among groups was analyzed by performing Levene' s test. When necessary, data were transformed and normalized to reduce the heterogeneity of variance. Comparisons between different treatments were performed by two-way analysis of variance (ANOVA), followed by Fishers least significant difference (LSD)-t tests. Each experiment was performed in triplicate and statistical significance was $p < 0.05$.

3. Results and discussion

3.1. Plant growth and combined toxicity assessment

The effects of ANTX-a, MCLR and their combined effects on the growth of *V. natans* were observed (Fig. 1), with their combined toxicity shown in Table 1. All the algae toxins showed negative effects on the biomass of *V. natans* (Fig. 1a). Higher toxin concentrations significantly decreased the biomass of plants. Meanwhile, low ANTX-a concentrations (0.05 μg L^{-1}) induced more toxicity in plants than the equivalent MCLR concentration. The mixed solution of MCLR and ANTX-a (0.05 + 0.05 μg L^{-1}) also induced a notable decrease in plant biomass compared with single MCLR and ANTX-a exposure. In previous studies, biomass of the aquatic plant *Lemna minor* was reduced when treated with ANTX-a of 5 μg mL^{-1}, while *Medicago sativa* seedling germination was inhibited by exposure to ANTX-a of 5 ng mL^{-1} (Kaminski et al., 2014; Pflugmacher et al., 2010). Exposure to different concentrations of ANTX-a induced various effects on each assessed plant. The results of the present study indicated that *V. natans* was sensitive to low concentrations of ANTX-a, which suggested *V. natans* could be applied as an indicator of harmful algal blooms. Furthermore, Dr. Jiang group confirmed that low MC—LR concentrations (1.0 μg L^{-1}) induced damage to aquatic plants (Jiang et al., 2011). In addition, when exposed to MCLR and ANTX-a mixtures at higher concentrations (5.00 μg L^{-1}), the final protein concentration in plants was approximately two-fold more than that of the control plants (Fig. 1b). The previous study also

Table 1
Observed biomass and protein contents inhibition of plants exposed to MCLR, ANTX-a and their mixture.

Indicators	MCLR (μg L^{-1})	A	ANTX-a (μg L^{-1})	B	MCLR + ANTX-a (μg L^{-1})	Observed Inhibition (%)	C_{exp} (%)	*RI*
Biomass	0.05	−11.40	0.05	0.20	0.05 + 0.05	2.40	−11.18	−0.21
	0.10	7.20	0.10	1.60	0.10 + 0.10	3.40	8.68	0.39
	1.00	1.00	1.00	6.20	1.00 + 1.00	5.60	7.14	0.78
	5.00	22.40	5.00	18.60	5.00 + 5.00	22.60	36.83	0.61
Protein	0.05	−1.33	0.05	−38.74	0.05 + 0.05	−40.69	−40.59	1.00
	0.10	−9.90	0.10	−55.11	0.10 + 0.10	−53.16	−70.47	0.75
	1.00	−10.98	1.00	−66.80	1.00 + 1.00	−74.60	−85.12	0.88
	5.00	−14.97	5.00	−72.56	5.00 + 5.00	−87.07	−98.39	0.88

Expected inhibition (Cexp) and ration of inhibition (RI) were calculated with Abott's formula.

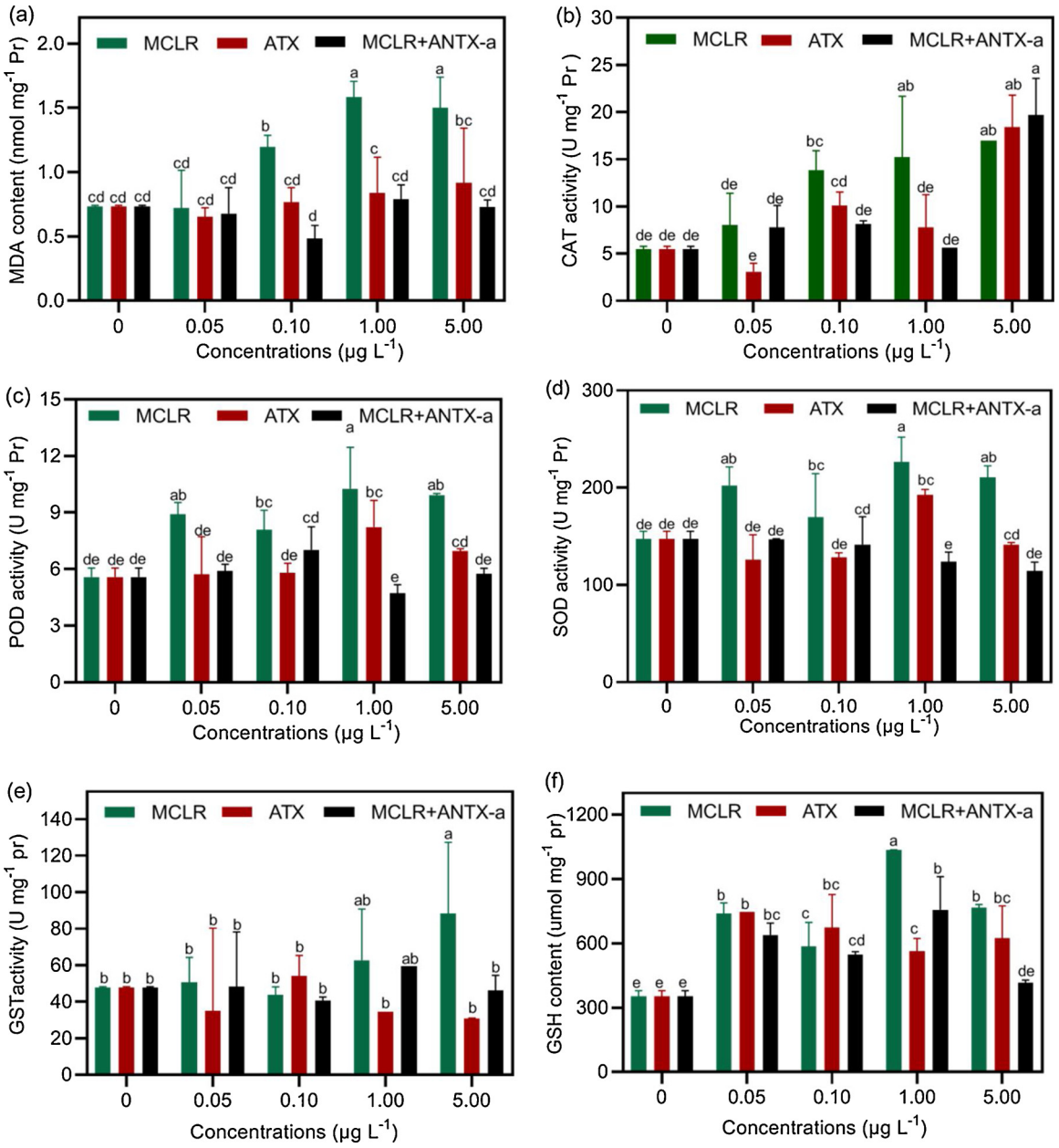

Fig. 2. Effects of MCLR, ANTX-a and their mixture on antioxidant system (a–f) of *V. natans* leaves. Data are means ± standard deviation analyzed from three replicates. Different characters indicate significant difference ($P < 0.05$).

confirmed that more soluble protein generated as the plant defense response, which caused by the harmful effects of reactive oxygen (Qifang, 2008; Qing et al., 2018). These results indicated that submerged macrophytes increased their protein content when they were exposed to higher toxin concentrations and toxin mixtures.

The toxic effects of combined exposure were assessed, as shown in Table 1. When using biomass as an indicator, all of the ratio of inhibition (*RI*) values were significantly lower than 1, indicating that

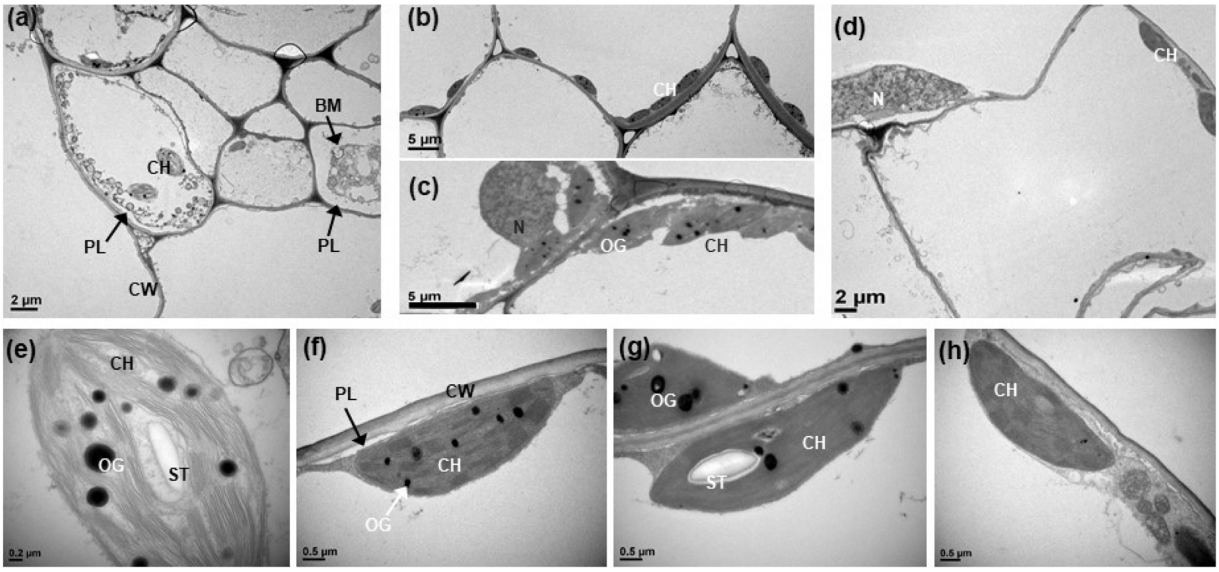

Fig. 3. Ultrastructural changes of *V. natans* exposed to 5.00 μg L^{-1} MCLR (a, e), 5.00 μg L^{-1} ANTX-a (b, f) and their mixture (c, g), control group (CT) (d, h) for 14 days. BM: broken plasma membrane; CH, chloroplast; OG, osmiophilic granules; PL, plasmolysis; ST, starch; N, nucleolus; P, plasma membrane; TH, thylakoids; CW, cell wall.

(a) Part of broken plasma membrane and plasmolysis observed in a mesophyll cell exposed to MCLR treatment. (b) and (f) Small osmiophilic granules were generated in chloroplasts in ANTX-a group. (c) Chromatin condensation and much osmiophilic granules observed when exposed to mixture treatment. (d) Intact organelles and (h) Thylakoid lamellae arranged orderliness in chloroplast in CT group. (e) Small starch and bigger osmiophilic granules were generated in chloroplasts exposed to MCLR treatment. (g) Note the starch accumulated in mixture MCLR and ANTX-a solution.

their interactive effects were antagonistic. Meanwhile, when MCLR and ANTX-a concentrations of 0.05 + 0.05 μg L^{-1} were applied, the *RI* value was 1 when protein was used as an indicator, suggesting that the interactive effects were additive. However, higher combined concentrations of MCLR and ANTX-a exhibited lower *RI* values (*RI* < 1). The occurrence of cyanotoxins could exert a long-term impact on organisms co-existing in biocrust communities (Chrapusta et al., 2015). Previous study also found that the combined effects of Cu and MCLR exposure were antagonistic when their concentrations were higher (Wang et al., 2017), and the Cu^{2+} and Cu^{+} could catalyze the production of ROS as the same with ANTX-a. Therefore, this study observed that the interactive effects of MCLR and ANTX-a on submerged macrophytes were antagonistic.

3.2. Oxidative response and structural characteristics of leaves

Membrane lipid peroxidation of plant tissues is shown in Fig. 2a. The concentrations of MDA were significantly increased with exposure to higher concentrations of MCLR, while it was not notably increased with ANTX-a exposure. The MDA level in plant tissues exposed to a mixture of toxins was notably lower than that with exposure to single toxins at higher concentrations (5.00 μg L^{-1}). Similar results have also been observed in previous study, with higher MCLR and Cu mixture concentrations inducing lower MDA levels when compared with single toxin exposures (Wang et al., 2017). The results of the present study indicated that MCLR caused most injury to the membrane system of plant cells. Similar trends were also observed in CAT, POD and SOD activities in plants exposed to single MCLR and ANTX-a treatments (Fig. 2b, c, d). The enhanced activities of antioxidant enzymes confirmed that toxin exposure promoted detoxification processes in plants. In antioxidant reactions, H_2O_2 was generated from superoxide by SOD dismutase, and then reduced by POD and CAT (Jiang et al., 2011; Sarvajeet Singh and Narendra, 2010). Meanwhile, combined toxin exposure also resulted in lower POD and SOD activities compared with single toxin exposure ($p < 0.05$), which also confirmed that the interactive effects on plants were antagonistic. Overall, exposure to ANTX-a, MCLR and a combination of the two induced CAT enzyme activities, with MCLR contributing the strongest inhibition of the assessed toxins.

As shown in Fig. 2e and f, the activity of GST was gradually increased with exposure to enhanced MCLR concentrations, with the highest value observed at 5.00 μg L^{-1}. Meanwhile, significant differences in GST activity and GSH concentrations were observed between exposure to MCLR and ANTX-a alone, at higher concentrations (> 0.1 μg L^{-1}), with no obvious changes in GST activity observed with exposure to low concentrations of ANTX-a (< 0.1 μg L^{-1}) alone. Forming a MCLR-GSH conjugate has been reported as the important step in MCLR detoxification in aquatic organisms (Liu et al., 2018b). The increase of GSH content was probably because of synthesis of new GSH triggered by exposure to low concentrations of MC−LR which was also confirmed by Dr. Jiang group (Jiang et al., 2011). Previous studies have confirmed that MCLR could be transformed to a glutathione conjugate by GST and that GST had the ability to detoxify metabolites of lipid peroxidation (Stephan et al., 2007; Stüven and Pflugmacher, 2007). In the present study, the notable increase in GST activity with exposure to higher MCLR concentrations ($p < 0.05$), confirmed that a conjugation reaction occurred, while there was no significant difference following ANTX-a exposure. In addition, the GSH content was higher in toxin exposed plants compared with the control group, which could be considered as an adaptive mechanism of antioxidants (Valavanidis et al., 2006).

Ultrastructural changes in *V. natans* exposed to cyanotoxins, are shown on Fig. 3. The plasma membrane was broken and plasmolysis was observed in the cells of plants exposed to MCLR (Fig. 3a). Meanwhile, bigger size and more abundant osmiophilic granules were observed in chloroplasts (Fig. 3e) following MCLR exposure, as compared with ANTX-a exposure (Fig. 3f). Previous studies have reported that the generation of osmiophilic granules in plants is related to senescence and pathological changes (Chen et al., 1992). Chromatin condensation was also found in plant cells exposed to combined toxin treatments (Fig. 3c). These results indicated that MCLR toxin treatment induced more damage to *V. natans* plant cells than ANTX-a. Bigger starch granules were also generated in chloroplasts exposed to MCLR or a mixture of toxins (Fig. 3e, g), which indicates that these plants suffered more

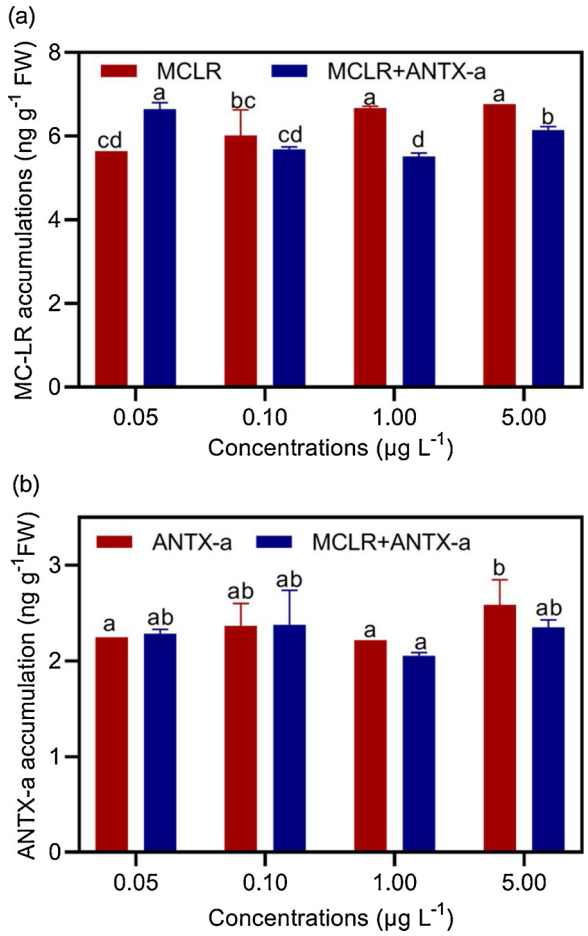

Fig. 4. MCLR (a), ANTX-a (b) accumulation in leaves of *V. natans* exposed to single and mixture solution for 14 days. Data are means ± standard deviation analyzed from three replicates. Different characters indicate significant differences ($P < 0.05$).

environmental stress (Singh et al., 2013).

3.3. Uptake of ANTX-a and MCLR by V. natans

The accumulation of ANTX-a and MCLR in leaves is shown in Fig. 4. The maximum uptake of MCLR was 6.77 ng g^{-1} FW when exposed to 5.00 μg L^{-1} MCLR alone, which increased with enhanced toxin concentrations (Fig. 4a). Previous studies have confirmed that the uptake of MCLR reached a plateau at MCLR concentrations of 1.0 μg L^{-1} (Jiang et al., 2011). The results of the present study indicated the uptake plateau occurred at the same concentration. Meanwhile, the accumulation of MCLR was significantly enhanced when the exposure concentration was higher than 1.0 μg L^{-1}. When *V. natans* was exposed to low concentrations of the mixed ANTX-a and MCLR solution (0.05 + 0.05 μg L^{-1}), the level of bioaccumulation was greater than that of mixed solution at higher concentrations. These findings indicated that lower concentrations of ANTX-a promoted the accumulation of MCLR in plants.

The highest bioaccumulation of ANTX-a in *V. natans* was 2.59 ng g^{-1} FW, which was observed with ANTX-a exposure at 5.00 μg L^{-1} (Fig. 4b), with no significant differences being observed in others treatment. Previous studies have indicated that the level of accumulation of ANTX-a was associated with initial toxin concentrations and the accumulation of ANTX-a in plants reached 19.3 μg g^{-1} FW with exposure to ANTX-a at 25 mg L^{-1} after 14 days (Kaminski et al., 2014). However, concentrations of ANTX-a rarely reached such a high concentration (25 mg L^{-1}) in the natural environment (Bumke-Vogt et al., 1999). Besides, the accumulation of ANTX-a also showed no significant difference in the mixed ANTX-a and MCLR solution. Overall, these results indicated that ANTX-a and MCLR could be taken up by plants at environmentally relevant concentrations, allowing transfer through the aquatic food wed as a food source for fish and other animals, finally affecting human health (Tessier et al., 2008).

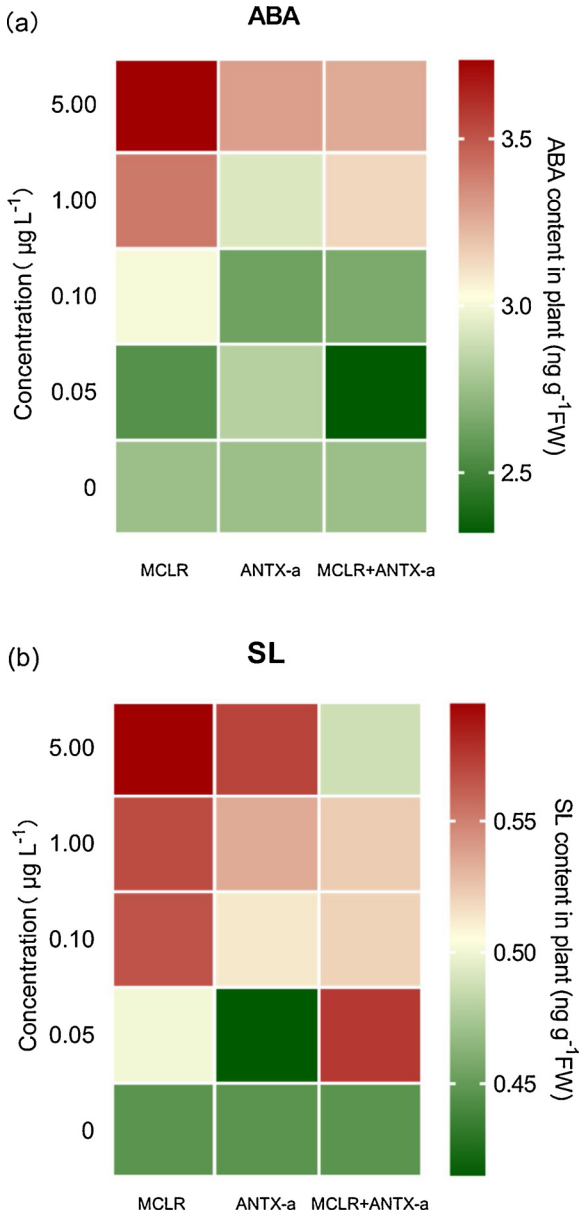

Fig. 5. the content of ABA (a) and SL (b) generated in leaves of *V. natans* exposed to single and mixture solution for 14 days. Data are means analyzed from three replicates.

3.4. Changes in plant phytohormones

The concentrations of ABA and SL following treatment are shown in Fig. 5. Notable differences were observed for ABA concentrations in plants exposed to higher toxin concentrations (> 1.00 μg L^{-1}) ($p < 0.05$). The highest value of ABA in plants was 3.74 ng g^{-1} FW, following exposure to MCLR concentration of 5.00 μg L^{-1}. Meanwhile, higher concentrations of ABA were generated in plants exposed to MCLR, as compared with exposure to ANTX-a or the mixed toxins solution (Fig. 5a). Previous studies have indicated that the vital phytohormone ABA is a central regulator of plant growth and protects plants

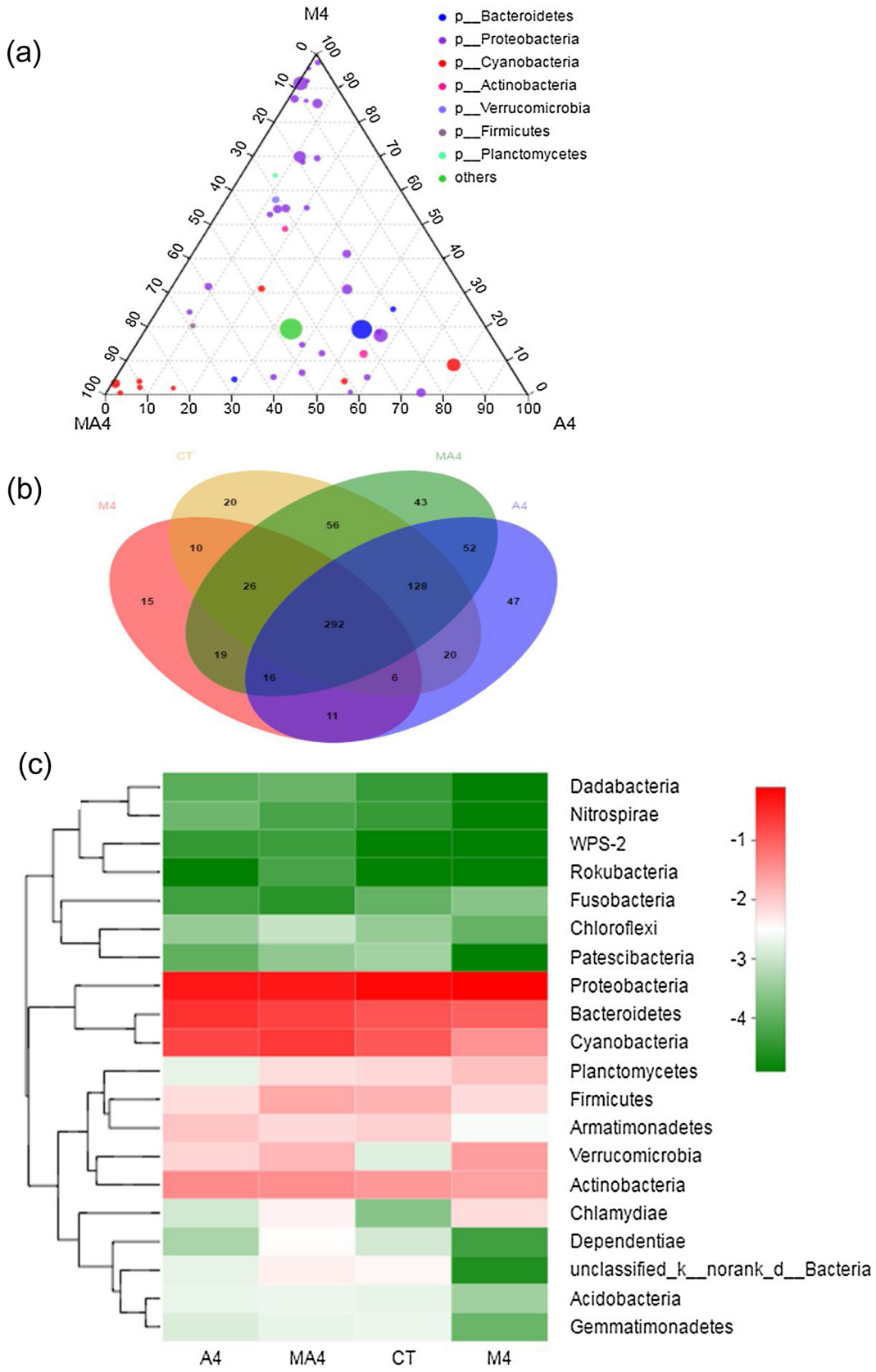

Fig. 6. Microbial community analysis: (a) Ternary Plot, (b) Venn diagram of distribution of OTUs and (c) heat-map of the different samples at the genus level in different samples. a, the color was classified by phylum level.M4, biofilms in MCLR with concentration of 5.00 μg L^{-1}; A4, biofilms in ANTX-a with concentration of 5.00 μg L^{-1}; MA4, biofilms in MCLR and ANTX-a mixture with concentration of 5.00 μg L^{-1} respectively; CT, control group.

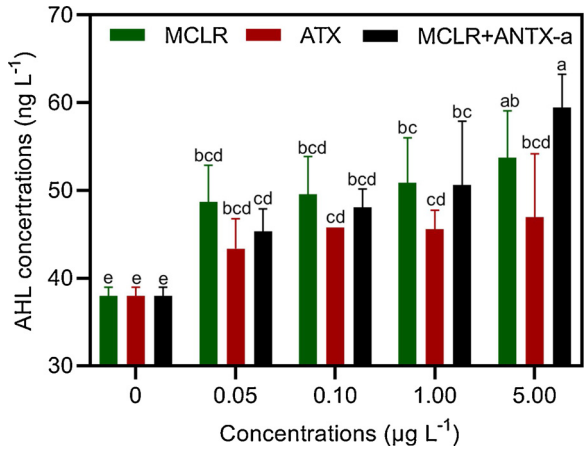

Fig. 7. Effects of MCLR and ANTX-a on AHL signals molecule content in water.

against environment stress (Fen et al., 2012). ABA also played a major role in cell signaling networks, adjusting plant defense responses (Jurriaan et al., 2009). However, no studies have observed that ABA is generated from submerged macrophytes following exposure to MCLR and ANTX-a. The results of the present study showed the promotion of ABA generation in plants exposed to MCLR and ANTX-a toxin stress, with individual and combined exposures.

As shown in Fig. 5b, the SL content increased in the presence of MCLR, ANTX-a and the combined toxin treatment. More SL generated with exposure to increased MCLR concentrations, with the highest value being observed under 5.00 μg L^{-1} MCLR exposure. In addition, the same results were also observed with exposure to ANTX-a, with a significant increase shown with exposure to 5.00 μg L^{-1} ANTX-a ($p < 0.05$). In contrast, the mixed solutions induced opposite results. Mixed solutions of low concentrations generated more SL in plants, indicating an antagonistic effect from ANTX-a and MCLR. As phytohormones, SLs are signaling molecules in the rhizospheric environment, regulating underground plant architecture by affecting root development (Kapulnik et al., 2011). Previous study have also confirmed that SL played a pivotal role in plant defense responses (Torres et al., 2014). This result indicated that ANTX-a and MCLR triggered plant defense responses by generating the phytohormones ABA and SL. Moreover, cross-talk between SL and ABA signaling pathways played a vital role in plant defense responses (Kohlen et al., 2011). Therefore, this study confirmed the generation of phytohormones such as ABA and SL, had an important effects on plant defense responses to ANTX-a and MCLR exposure.

3.5. Microbial properties and AHL signals in biofilms

The microbial community in biofilms were analyzed as shown on Fig. 6. Bacteroidetes, Proteobacteria, Cyanobacteria and Planctomycetes were the dominant phyla in biofilms, as shown by ternary plot analysis (Fig. 6a), with Proteobacteria being the main species existing in the MCLR exposed solution. Actinobacteria, Cyanobacteria and Planctomycetes were commonly observed in ANTX-a treatments and mixed toxin solution treatments. The compositional similarity and overlap of biofilm microbial communities in different treatments were established using Venn diagrams (Schloss et al., 2013). As shown in Fig. 6b, the unique operational taxonomic unit (OTU) numbers were 15 (M4), 20 (CT), 43 (MA4) and 47 (A4), indicating that ANTX-a and MCLR altered the microbial diversity of biofilms. As a chemical signal molecule of quorum-sensing, AHL was highly related to the bacterial diversity and density (Shrout and Nerenberg, 2012). In our previous study, the cyanobacterial (releasing MCLR) changed the abundances and structures of the biofilm microbial community and AHL content increased (Li et al., 2019). Thus, ANTX-a and the combination might have the same effects on microbial diversity of biofilms in the present study. In addition, distance heatmap analysis showed that different toxins had different effects on microbial communities (Fig. 6c). Proteobacteria and Bacteroidetes were the dominant microbes in biofilms at the genus level, with varying abundances in different toxin treatments. Previous studies have also confirmed the same microbial phyla were commonly observed in wastewater environments (Wang et al., 2016), maintaining the function and stability of biofilms (Lv et al., 2014). This result indicated that exposure to ANTX-a and the mixed toxin solution induced stronger changes in the abundances and structure of the biofilm microbial community.

The concentration of AHL signal molecules in biofilms were investigated, as shown in Fig. 7. AHL concentrations increased with enhanced toxin concentrations. The highest values were observed with exposure to the combined ANTX-a and MCLR solution (5.00 μgL^{-1}), with AHL levels being significantly higher than that of the other mixture treatments ($p < 0.05$). In addition, the AHL levels in all toxin treatments were significantly higher than that of the control group ($p < 0.05$). Previous studies have reported that QS systems played a vital role in biofilms formation and biofilm development (Moons et al., 2009). AHL signal molecules also participated in primary QS signaling pathways (Shrout and Nerenberg, 2012). Furthermore, AHL was highly related to the bacterial diversity and density and it was generated by microbial and altered in response to the environment stress (Cornforth and Mcnally, 2014). Therefore, enhanced AHL signaling in biofilms with ANTX-a and MCLR exposure, indicated that those toxins had an important influence on the biofilm structure. In addition, biofilms could cooperate with submerged macrophytes to modulate aquatic ecological functions, and play the vital and positive roles in the food chain, in modulating nutrient cycles, and in improving water quality (Yang et al., 2018). The present study confirmed that ANTX-a and MCLR caused the influence on biofilms structure and function, which might alter the aquatic ecological functions.

4. Conclusions

This experiment investigated comprehensively the combined toxic effects of ANTX-a and MCLR on submerged macrophytes and periphyton biofilms. Results demonstrated that *V. natans* was sensitive to low concentrations of ANTX-a, with antagonistic interactive effects of MCLR and ANTX-a on plants. Meanwhile, an antioxidant response was induced with promoted activities of CAT, SOD, POD and GST, along with increased GSH and MDA concentrations. Furthermore, ANTX-a and MCLR uptake by plants occurred at environmentally relevant concentrations. Additionally, this study confirmed the generation of the phytohormones ABA and SL, which had important effects on plant defense responses following ANTX-a and MCLR exposure. Moreover, ANTX-a and combined toxin exposure induced stronger changes in the abundances and structure of the biofilm microbial community. The enhanced AHL signal indicated that exposure to these toxins had a significant influence on biofilms. These results provided valuable information on the ecological effects of combined and individual exposure to ANTX-a and MCLR on submerged macrophytes and periphyton biofilms.

Author contribution statement

Qi Li: Conducting experiments, Data Analysis, Writing- Original draft preparation.

Peng Gu: Investigation, Resources.

Chen Zhang: Data Analysis.

Xin Luo: Data curation, Writing- Reviewing and Editing.

Hao Zhang: Writing- Reviewing and Editing.

Jibiao Zhang: Conceptualization, Methodology.

Zheng Zheng: Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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