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Responses of submerged plant *Vallisneria natans* growth and leaf biofilms to water contaminated with microplastics

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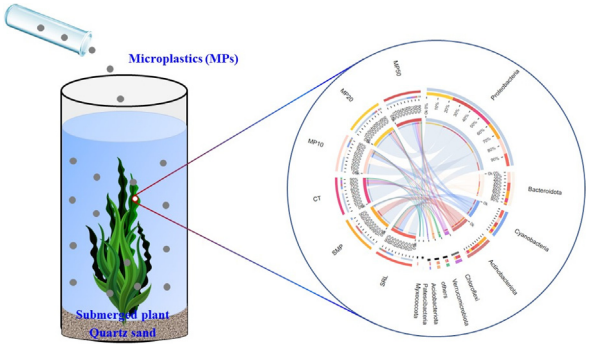
HIGHLIGHTS

- MPs can induce the oxidative stress responses of *V. natans*.
- *V. natans* microstructure was seriously damaged by MPs at 50 mg L^{-1}.
- MPs have enrichment effect on *Proteobacteria* and *Cyanobacteria* in leaf biofilms.

GRAPHICAL ABSTRACT



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ABSTRACT

Microplastics pose a serious threat to ecological processes and environmental health. To evaluate the toxic effects of the exposure of microplastics on submerged plants and biofilms, eel grass (*Vallisneria natans*) was exposed to different concentrations of microplastics (10–50 mg L^{-1}). The changes in microbial community on leaf biofilms were also tested. The results showed that the ratio of variable fluorescence to maximum fluorescence was largely unchanged, but the contents of chlorophyll *a* and b increased by 56.5% and 23.0% respectively. Different concentrations of exposure to microplastics effectively induced antioxidant responses, such as increasing the activities of superoxide dismutase, peroxidase and catalase, as well as increasing the activity of glutathione S-transferase and the contents of glutathione and malondialdehyde. In addition, the leaf flesh cells of *Vallisneria natans* showed some degree of organelle damage when examined by transmission electron microscopy. Moreover, a high-throughput sequencing analysis showed that the abundances and structure of the microbial community on the leaf biofilms were altered by exposure to microplastics. These results demonstrated that environmentally relevant concentrations of microplastics could disrupt homeostasis, induce effective defense mechanisms of *Vallisneria natans* and alter the biofilms in aquatic ecosystems.

1. Introduction

Microplastics (MPs) are particles that are composed of synthetic polymers <5 mm in size (Cole et al., 2015). The presence of MPs in the environment is now a global problem, since the particles pose a threat to the health of terrestrial and aquatic ecosystems worldwide

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(Corinaldesi et al., 2021). Studies have shown that MPs are common in aquatic environments (Niu et al., 2021), including polymers such as polyethylene, polypropylene, polystyrene, and polyamide (Nava & Leoni, 2021). MPs can be discharged into natural water in the form of personal care products, optoelectronic products and targeted drugs (Brewer et al., 2021). Studies have found that the main sources of MPs are effluent discharge from sewage treatment plants, rainwater runoff and atmospheric deposition (Jrlskog et al., 2020; Liu et al., 2019; Sang et al., 2021; Vcs et al., 2021). In a study of surface water of the Baram River Estuary, Choong et al. (2021) found that the abundance of MPs ranged from 0.55 ± 0.071 to 1.85 ± 1.48 mg L^{-1} or from 9.3 ± 1.27 to 18 ± 1.41 particles L^{-1}. Wezel et al. (2016) reported that the concentration of MPs in wastewater treatment plants in the Netherlands was as high as 66 μg L^{-1}. Baldwin et al. (2016) studied 29 tributaries of the Great Lakes in six states in the USA. The highest concentration of MPs was 32 particles m^{-3}, with a median of 1.9 particles m^{-3}. MPs can directly or indirectly enter the body of aquatic organisms (An et al., 2021; Zhang et al., 2021), and thus, be consumed by humans as food, causing potential harm to human health (Wang et al., 2020b; Zhu et al., 2019).

The pollution of water resources has had a significant impact on the environment (Hayes and Vanni, 2018) and can lead to a serious loss of the ecosystem services provided by aquatic environments (Taranu et al., 2017). As an important low trophic level producer, submerged aquatic plants not only maintain the stability of aquatic ecosystems but also provide valuable ecosystem services, such as the removal of nutrients and heavy metals from a water body (Keskinkan et al., 2003; Nigam and Gopal, 2013). A biofilm can be defined as a community of microorganisms attached to a surface (Davey and O'toole, 2000). Studies have shown that biofilms play an important role in the process of purifying polluted water by affecting primary production, food chain and the migration of pollutants in the sediment-water interface (Writer et al., 2011). Submerged plants can provide an adherent surface for the microbial community composed of bacteria or algae and produce adherent biofilms (Jones et al., 2000). Biofilms have synergistic and antagonistic effects on submerged plants to purify water pollutants. On the one hand, the growth of a large number of biofilms can hinder the absorption of nutrients and affect the gas exchange of plants, which has a negative impact on plants (Drake et al., 2003). Alternatively, biofilms can absorb relevant pollutants in water and have a synergistic effect with plants (Gong et al., 2018; Shih-Hsin et al., 2017).

In recent years, many researchers have studied the effects of MPs on microorganisms and aquatic plants. Wang et al. (2020a) exposed marine green microalgae (*Platymonas helgolandica*) to different concentrations of polystyrene nanoplastics for 6 days, and these results demonstrated that nanoplastics can reduce the vitality of microalgae by damaging the morphology of their cells and the functions of their organelles (Wang et al., 2020a). Ye et al. (2021) also found that 26 bacterial taxa that were members of Proteobacteria and Bacteroidetes introduced microbial dysbiosis and dysfunction in the surrounding seawater after exposure to 10- and 200-m of polystyrene MPs (Ye et al., 2021). Furthermore, Yu et al. (2020) found that high concentrations of MPs induce high ecotoxicity and oxidative damage to the aquatic plant common bladderwort (*Utricularia vulgaris*) (Yu et al., 2020). However, there have been few studies about the effect of MPs on submerged plant biofilms.

Vallisneria natans (*V. natans*) is good at purifying polluted water and exists widely in the aquatic environment. To address this lack of research, the effects of different concentrations of MPs on the physiology and biochemistry of *V. natans* were studied. In this study, the growth and water quality changes caused by *V. natans* following exposure to MPs were detected. The changes of antioxidant system and ultrastructure of *V. natans* under the stress of MPs were also tested. Furthermore, changes in the abundance and structure of microbial biofilm communities caused by the exposure to MPs toxins were also studied. The results will enhance the understanding of the ecological effects of MPs on submerged plants and the process of water purification.

2. Materials and methods

2.1. Plant materials

V. natans was selected for this study because it tolerates elevated nutrient levels and was readily available for research purposes. Mature plants were purchased from the Tiancun Horticultural Company (Shanghai, China) and then cultivated in a tank filled with a 1/10 Hoagland solution as the growing medium. To simulate natural growing conditions in a laboratory environment, the experiment was conducted under climate-controlled conditions with a temperature that was maintained at 25 ± 2 °C. Daily 12 h–12 h light-dark cycles were applied at a light flux density of 90 μmol quanta m^{-2} s^{-1}, measured as the photosynthetic photon flux density (PPFD) using a quantum meter (Spectrum Technology, Inc., USA). 10 g ± 1 g of *V. natans* was transferred to a cylindrical plexiglass container that was 398 mm high and 148 mm wide and had a wall with a diameter of 3 mm (ADA aqua soil, Aqua Design Amano Company, Japan) that contained water collected from Lake Ri on the Jiangwan campus of Fudan University (Shanghai, China, 31°20′14″N, 121°30′35″E) and 30 mm of quartz sand (silica sand, Aqua Design Amano Company, Japan). Prior to planting, the leaves of *V. natans* were wiped with deionized water to remove the original biofilm. The concentration of nitrogen and phosphorus in each experimental system was subsequently regulated to mimic eutrophic conditions in the natural environment.

2.2. Experimental design

MPs (<10 μm in size) easily attach to the surface of aquatic plants, thus, affecting the physiological and biochemical performance of the leaves (Kalíková et al., 2017; Weert et al., 2018). We used the results obtained by some researchers to guide the selection of particle sizes used in our study (Besseling et al., 2014; Yuan et al., 2018). We used polystyrene MPs with a diameter of two μm (2.5% w/v) in the experiments. The MPs were purchased from the Beisler Chromatography Technology Development Center (Tianjin, China). The experiment was divided into six treatment groups: (1) grown without MPs (Control, CT group), (2) microplastic concentration of 10 mg L^{-1} (MP10 group), (3) microplastic concentration of 20 mg L^{-1} (MP20 group), (4) microplastic concentration of 50 mg L^{-1} (MP50 group), (5) only Lake Ri water (SRL group), and (6) only Lake Ri water and 50 mg L^{-1} MPs (SMP group). The processing methods and numbers used in the experiments are shown in Table 1. There treatments were repeated in triplicate for each group, with the leaf biofilm samples collected at the end of the experiment (i.e., day 24), to ensure that the material available for study was abundant, and the microbial community composition had stabilized in the biofilm.

2.3. Determination of plant growth and chlorophyll

Plant fresh weight and root lengths were measured at the start and end of the experiment to quantify growth. The chlorophyll (Chl) content and primary light energy conversion efficiency (Fv/Fm) were used to quantify photosynthetic activity. The fresh weight and root length of plants were measured after wiping the surface liquid off with sterile paper. The Fv/Fm ratio was measured with a hand-held

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

Group	Microplastics in water (mg L^{-1})	With *V. natans*
CT	0	Yes
MP10	10	Yes
MP20	20	Yes
MP50	50	Yes
SRL	0	No
SMP	50	No

chlorophyll fluorescence meter. To calculate the content of chlorophyll *a* and b, a 0.2 g leaf sample was washed, dried, and then incubated in 10 mL of a 96% ethanol solution for 24 h to extract the chlorophyll. The chlorophyll content was determined using a spectrophotometer to record the absorbance at 649 nm and 665 nm. The content of chlorophyll was calculated using Eqs. (1) and (2) with the solvent (96% ethanol solution) used as a blank (Wellburn and Lichtenthaler, 1984).

$$\text{Chl a} = 12.7 \times D_{663} - 2.69 \times D_{645} \quad (1)$$

$$\text{Chl b} = 22.9 \times D_{645} - 2.69 \times D_{663} \quad (2)$$

2.4. Determination of water quality parameters

The concentrations of total organic carbon (TOC), ammonium-nitrogen (NH_4^+-N), total nitrogen (TN) and total phosphorus (TP) were used to characterize the water quality. During the 24-day experimental period, the water quality was tested every 4 days, with three replicates tested for each parameter on each occasion. The concentration of TOC and TN were determined using a TOC-L analyzer (Shimadzu, Japan). The TP content was determined by testing a needle filtered (MCE, 0.45 μm) water sample using molybdenum blue colorimetry and a Hach DR6000 at a wavelength of 700 nm. NH_4^+-N was determined at a wavelength of 420 nm using Nessler's reagent for the analysis.

2.5. Extraction and detection of total protein and related enzymes

To measure the activities of enzymes and content of total protein, we placed 1 g ± 0.1 g of a fresh sample of *V. natans* in a precooled agate mortar, added a small amount of liquid nitrogen and quickly homogenized the sample on an ice bath. A phosphate-buffered saline (PBS) solution with a pH of 7.0 ± 0.2 and a concentration of 0.1 mol L^{-1} was used as the buffer system. After the homogenate was centrifuged for 10 min at 10,000*g*, the supernatant was stored at −20 °C to determine the enzyme activities at a later timepoint. The supernatant was then removed from storage for the test, and the analysis was conducted at 0–4 °C. The concentrations of malondialdehyde (MDA) and total protein (TPro) and the activities of superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), reduced glutathione (GSH), glutathione sulfhydryl transferase (GST), nitrate reductase (NR), and alkaline phosphatase (AKP) were determined following the manufacturer's instructions (Nanjing Jiancheng Bioengineering Institute, China).

2.6. Microstructural analysis of plant cells

The biofilm of *V. natans* leaves was first fixed with a 2.5% glutaraldehyde solution, and then rinsed twice with 0.1 mol L^{-1} PBS buffer (pH 7.4). Finally, the samples were dehydrated in different concentrations of ethanol (20, 40, 10, 60, 80 and 90%). The sample was treated for 15 min and then immersed twice in 100% ethanol for 15 min. After the samples had been dried, a scanning electron microscope (S-3400 N; Hitachi, Tokyo, Japan) was used to study the morphological characteristics of the biofilm and identify the composition of the biological and extracellular polymers in the biofilm. An ultrathin section was used to observe the plant cells using transmission electron microscope (H-7650, Hitachi).

2.7. Confocal laser microscopy

A confocal laser scanning microscope (CLSM) and multiple fluorescence staining techniques were used to determine the spatial distribution of proteins and α-D-glucopyranose polysaccharides and β-D-glucopyranose polysaccharides in the biofilms and leaves. First, the biofilm of *V. natans* leaves was fixed with a 2.5% solution of glutaraldehyde. After approximately 24 h, the samples were stained with 50 μL of 1 g L^{-1} fluorescein-isothiocyanate (FITC) (Sigma-Aldrich, St. Louis, MO, USA), 100 μL of 250 mg L^{-1} concanavalin A (Con A) (Sigma-Aldrich), and 100 μL of 300 mg L^{-1} calcofluor white (CW) (Sigma-Aldrich). The sample was subsequently washed with 0.1 mol L^{-1} PBS buffer (pH 7.4) to remove the excess stain. Finally, the internal structure of the stained sample was observed using a CLSM (SP8; Leica, Wetzlar, Germany). The excitation/emission wavelengths of FITC, ConA and CW were 488 max 520 nm (green), 552 max 580 nm (red) and 405/435 nm (blue), respectively. Finally, the data were processed and displayed as 2D/3D images (Adav et al., 2010; Chen et al., 2007).

2.8. Analysis of the leaf biofilm microbial community

The leaf samples of *V. natans* were collected from three replicates and mixed. At the end of the 24-day experimental period, 0.5 ± 0.05 g of fresh full leaves were treated in 20 mL of a 0.1 mol L^{-1} PBS buffer and then exposed to 40 kHz ultrasound for 1 min. A culture oscillator (ZWY-240, Zhicheng, China) was then used to shake the sample for 5 min at a constant temperature of 4 °C, after which it was centrifuged for 5 min at 12,000*g* at 25 °C. The liquid was subsequently poured off, and the sediment was collected and stored at −80 °C. DNA extraction and high-throughput sequencing was conducted by the Majorbio Bio-Pharm Technology Co., Ltd. (Shanghai, China). After high-throughput sequencing on an Illumina PE300 (San Diego, CA, USA), the Illumina MiSeq platform was used to analyze the composition of the microbial community and conduct a difference analysis and other types of bioinformatics analyses. A bioinformatics analysis was used to describe the diversity of the microbial communities and verify the existence of microbial species (Li et al., 2020a).

2.9. Statistical analysis

A *t*-test uses the *t*-distribution to infer whether the probability of difference between the average value of the theoretical distribution and the average of a sample is significant. The statistical software SPSS 18.0 (SPSS, Inc., Chicago, IL) was used to test the differences between multiple groups ($n \geq 3$) using a single-factor analysis of variance (ANOVA). The difference was regarded as statistically significant at $p < 0.05$.

3. Results and discussion

3.1. Analysis of plant growth

The effect of MPs on the fresh weight and root length of submerged plants is shown in Fig. 1(a–b). When the concentration of MPs was lower than 20 mg L^{-1}, the fresh weight and root length of *V. natans* increased, but when the concentration of MPs exceeded 20 mg L^{-1}, the inhibitory effect of the MPs started to become more apparent. At 50 mg L^{-1} group (MP50), the fresh weight and root length of *V. natans* decreased by 21.3% and 19.7%, respectively, compared with the control group (CT).

As shown in Fig. 1(c), at the beginning of the experiment, there was almost no difference in the Fv/Fm ratio between each group. At the end of the experiment, there was no significant difference in Fv/Fm between the experimental groups, and there was no significant change in the same group at two points in time (i.e., the beginning and the end of the experiment). This shows that different concentrations of MPs have no obvious effect on the primary light energy conversion efficiency of Photosystem II (PSII) in *V. natans* leaves.

The results presented in Fig. 1(d) show that the chlorophyll content in the leaves differed under different treatments. At the end of the experiment, the contents of chlorophyll *a* and chlorophyll *b* in the treatment groups MP10, MP20 and MP50 increased at first and then decreased as the concentration of MPs increased. When the concentration of MP was

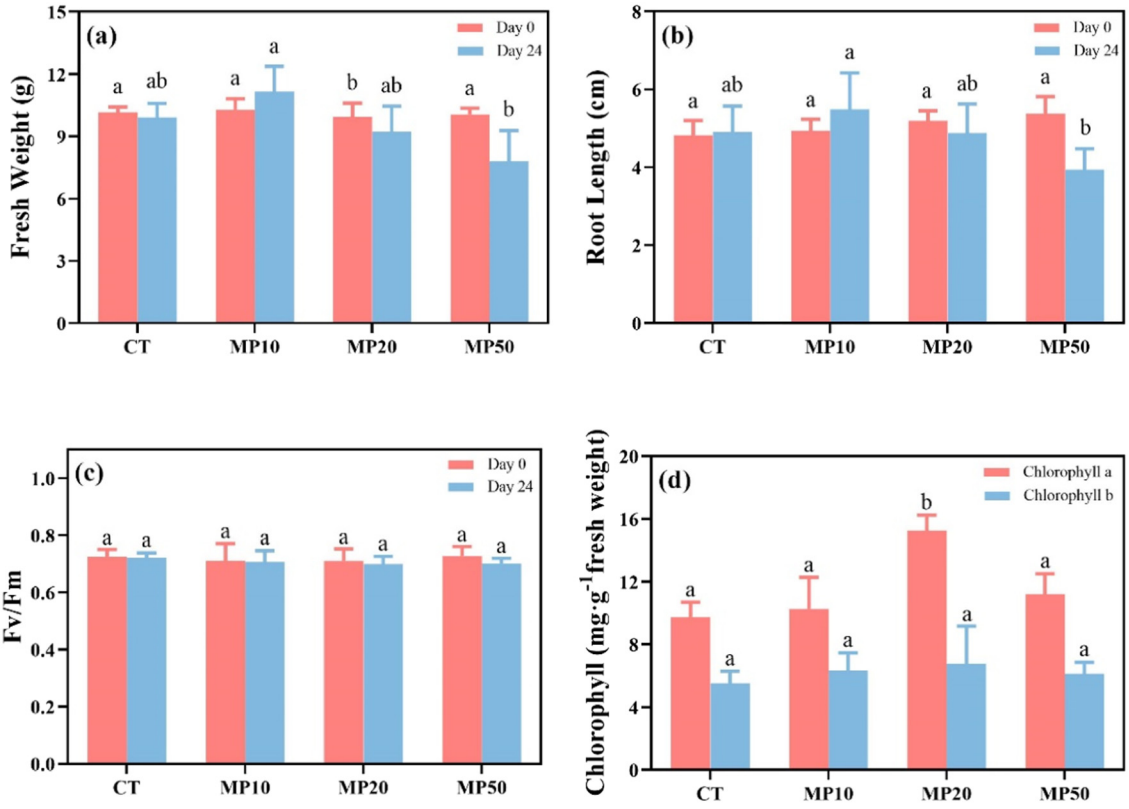

Fig. 1. Effects of microplastics on leaf fresh weight (a), root length (b), Fv/Fm (c), chlorophyll *a* and b (d) of *V. natans*. Values with different letters are significantly different ($p < 0.05$).

20 mg L^{-1}, the concentration of chlorophyll in the leaves increased significantly (56.5%) to a level higher than that of the control group (23.0%). When the MP concentration was 50 mg L^{-1}, the contents of chlorophyll *a* and chlorophyll *b* in leaves increased by only 14.9% and 11.1%, respectively, compared with the control group, with the content of chlorophyll *a* significantly different than that of the MP20 treatment group ($p < 0.05$). This showed that the concentrations of chlorophyll *a* and chlorophyll *b* would increase until an MP concentration threshold was exceeded, after which the rate of increase in the concentrations of chlorophyll *a* and b decreased. The concentrations of both chlorophyll types decreased significantly in the MP50 group. Nevertheless, these lower chlorophyll concentrations were still higher than that of the CT group.

The growth of biofilm has a negative effect on plant cells (Gong et al., 2018). For example, the increase in biofilm surface area could reduce the light intensity to which leaves are exposed, while cyanobacteria could release allelochemicals, such as microcystins, that inhibit photosynthesis in leaves (Pflugmacher, 2002). The presence of MPs could also reduce the amount of biofilm on the leaf surface and the proportion of cyanobacteria in the biofilm, and thus, decrease the competition for light energy. Consequently, the concentration of chlorophyll could increase and thus, enhance photosynthesis in the leaves. One could conclude that the reduction in the surface area of the biofilm and amount of cyanobacteria on the surface of *V. natans* leaves promoted the absorption of light energy.

3.2. Effects of MPs on water quality parameters

The presence of *V. natans* can reduce the content of nutrients in water, while the existence of MPs will change the physiological state of *V. natans*, which, in turn, would have an impact on water quality. The changes in the concentrations of TOC, NH_4^+-N, TN and TP under different treatment conditions are shown in Fig. 2. During the 24-day experimental period, the concentrations of all of these parameters in the CT group decreased, which could be related to the growth of *V. natans*. However, in the groups treated with different concentrations of MPs, the concentrations of TP, TN and NH_4^+-N decreased greatly at the beginning of the experiment, which could be attributed to the adsorption and dispersion of MPs. The adsorption of nitrogen, phosphorus and other elements in the water by MPs could explain the significant improvement in water quality in a short time. On the 4th day of the experiment, the difference in the concentrations of MPs brought about notable changes in water quality. The results presented in Fig. 2 show that the concentration of TOC for the MP50 and SMP groups increased sharply after the addition of MPs but decreased slowly after reaching peaks at 35.65 mg L^{-1} and 21.54 mg L^{-1}, respectively, on the 4th day. This indicated that submerged plants or water microorganisms tended to release organic matter into the water at high concentrations of MP. For the rest of the experiment, the TOC concentrations for the MP50 and SMP groups gradually decreased until they reached similar concentrations to those in the other groups. This indicated that the MPs at this concentration had not reached the threshold for microbial or submerged plants, with the biota starting to absorb organic carbon after the eighth day following a period of adaptation. The trend of degradation for NH_4^+-N was similar to that displayed by TOC (Fig. 2(b)). NH_4^+-N for the MP50 and SMP groups peaked at 0.307 mg L^{-1} and 0.156 mg L^{-1}, respectively, on the 8th day and then decreased slowly. This pattern was similar to that for the other groups at the end of the experiment.

The results shown in Fig. 2(c) indicate that there was no significant difference in the efficiency of TN degradation for the MP10, MP20, MP50 and SMP groups at different concentrations of MPs. The efficiency of degradation for all of the aforementioned groups was >75%. These values (viz. >75%) were nearly 40% higher than those for the CT and SRL groups. This indicated that the existence of submerged plants (*V. natans*) has a significant effect on the degradation of TN. The results in Fig. 2(d) indicate that the degradation of TP in the MP10, MP20 and MP50 experimental groups was higher than that in the other experimental groups. The degree of degradation was also very similar, with degradation rates of 47.5%, 51.4% and 58.6%, respectively, which are

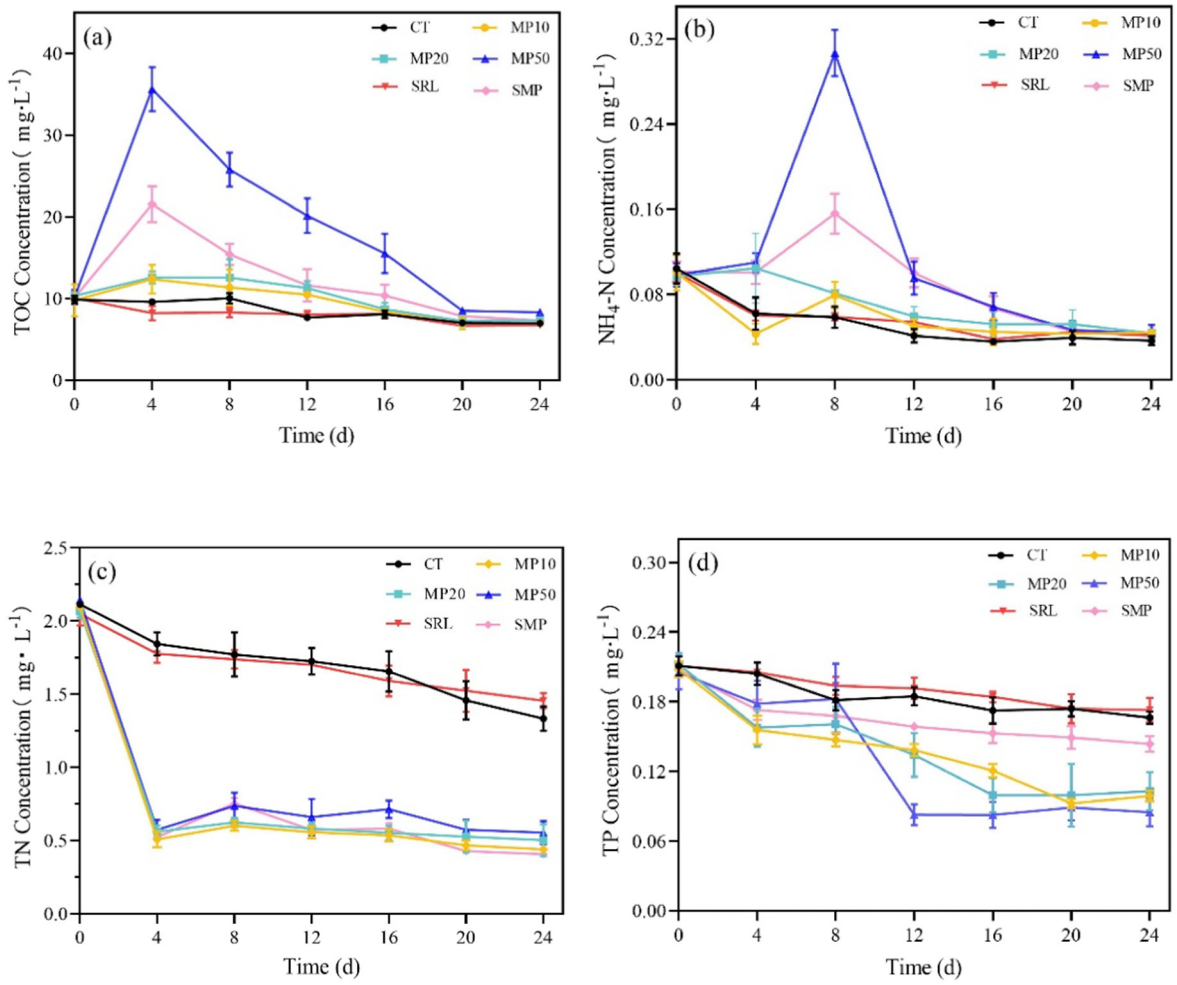

Fig. 2. Changes of water quality parameters during the culture of *V. natans*.

much higher than the 21.2% for the CT group. This shows that at some concentrations of MPs, the reduction in the concentration of TP by *V. natans* is higher than that for the non-MPs.

3.3. Effects of MPs on enzyme activity

The level of lipid peroxidation in plants can be determined by measuring the MDA content of leaves. The results in Fig. 3(a) show that the concentration of MDA in the MP10 group increased significantly to a level 133.6% higher than that for the CT group. There was no significant difference between the other experimental groups and CT. Therefore, *V. natans* appears to be more sensitive to MDA secretion after slight stimulation, although it would be unlikely for a plant to show a stress response until a threshold had been exceeded.

The plant antioxidant system can eliminate the reactive oxygen species (ROS) produced in the organism and play an enormous role in the protective defense response of the plants (Livingstone, 2001). The results in Fig. 3(b–d) show that the concentrations of CAT, POD and SOD in the antioxidant system of *V. natans* increased after being stressed by low concentrations of MPs. This indicated that plants could activate the antioxidant mechanism to protect themselves when stressed by low concentrations of MPs. When the concentration of MPs increased to 50 mg L^{-1}, the efficacy of the *V. natans* antioxidant system was compromised and could not completely eliminate the toxicity of the active ROS, such as the superoxide anion, by producing enzymes. In these conditions, the enzyme activity decreased significantly, particularly the activity of CAT, which decreased to 48.9% of that of the CT group. This indicated that the plant was about to go into apoptosis.

GSH and GST can help cells maintain a normal immune system function by acting as antioxidants, facilitating integration and mediating detoxification. The results in Fig. 3(e–f) show that at concentrations of MP10, MP20 and MP50, all the plants had higher activities of GSH and GST. These activities were significantly different from those of the control group. Some studies have shown that an increase of glutathione will increase the ratio of glutathione to oxidized glutathione and enhance the enable *V. natans* to detoxify toxic compounds in response to external stimuli (Li et al., 2021). However, the ability of the MP50 group of *V. natans* to resist external damage was significantly lower than that in the MP20 group owing the MPs exceeding the stress threshold for the plant, which led to the weakening of its ability to resist external damage.

NR is the rate-limiting enzyme for nitrogen assimilation in higher plants. It can regulate nitrogen metabolism by acting as a catalyst for nitrate reduction, thereby affecting plant photosynthesis and carbon metabolism (Kaiser and Huber, 2001). The results in Fig. 3(g) show that the degree of stress associated with the MP10, MP20 and MP50 groups was much higher than that for the CT group. This indicates that *V. natans* promotes nitrogen assimilation by increasing the activity of NR and that the metabolism of organic carbon is more noticeable, resulting in an enhancement of photosynthesis. This provides a possible explanation for the decrease in the degree of eutrophication of water treatments after the fourth day of the experiment.

The AKP concentration in leaves can change in response to changes in nutrient concentrations in water. When the concentration of inorganic phosphorus was insufficient, the activity of AKP increased accordingly. The results in Fig. 3(h) show that the concentration of AKP in the

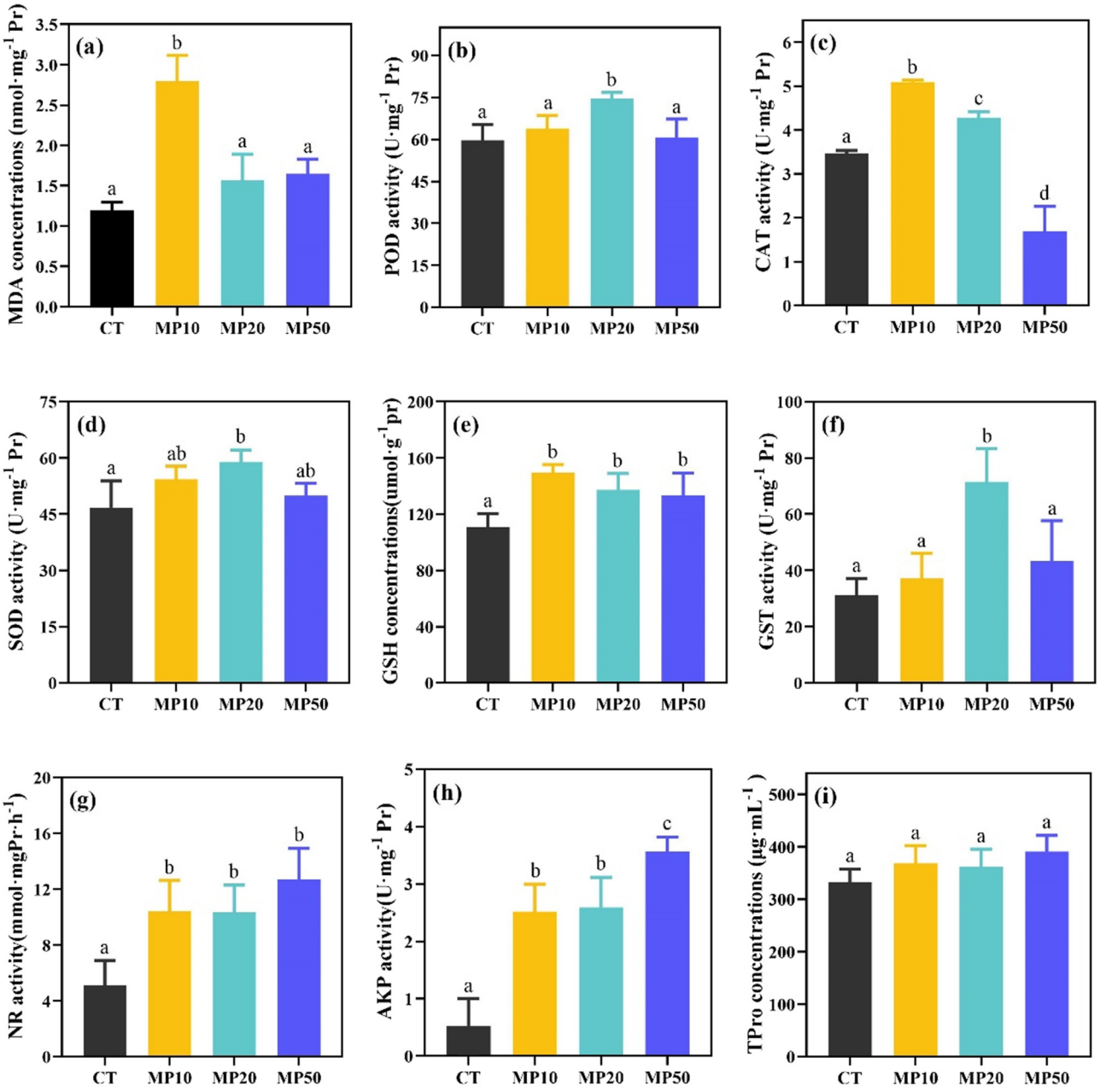

Fig. 3. Effects of different concentrations of microplastics on antioxidant system and other enzyme activities of *V. natans*.

experimental group increased in response to different MP concentrations, which was significantly different from that in the CT group. This indicated that MPs could stimulate the secretion of AKP by *V. natans* in response to changes in the external environment. Therefore, one could conclude that MPs indirectly affect the phosphorus concentration in water, which then indirectly affects the activity of AKP in the cells of *V. natans*. The results displayed in Fig. 3(i) also show that the TPro concentration increased gradually in conjunction with an increase in the concentration of MPs. Thus, there is a feedback relationship between *V. natans* and external stimulation with the stress response proportional to the intensity of stimulation within a certain range.

3.4. TEM analysis of leaf flesh cells

The leaf sections of *V. natans* were photographed using transmission electron microscopy (TEM). The results shown in Fig. 4(a–b) indicate that the chloroplasts of the control leaf cell were a regular fusiform organelle; the cell membrane was clear, and the matrix was dense with the thylakoid sheets neatly arranged in the chloroplast. The mitochondria were oval and the stroma dense. The nuclear membrane is intact; the nucleolus is clear; the nucleoplasm is evenly distributed, and the number of starch granules and osmiophilic granules are small. In contrast, the results shown in Fig. 4(c–d) show that at 10 mg L^{-1} (MP10) concentrations of MPs, the number of osmiophilic granules in the mesophyll cells of *V. natans* increased significantly, and the starch granules became large and full. Related studies have shown that a large number of osmiophilic granules produced in chloroplasts indicate plant senescence and pathological changes (Chen et al., 1992). In addition, the accumulation of starch has been considered a plant protection strategy to increase the likelihood of survival when subjected to environmental stress (Singh et al., 2013).

The results presented in Fig. 4(e–f) reflect the physiological status of mesophyll cells for the MP20 group with a number of features evident, including separation of the plasma wall, increased distortion of the cell

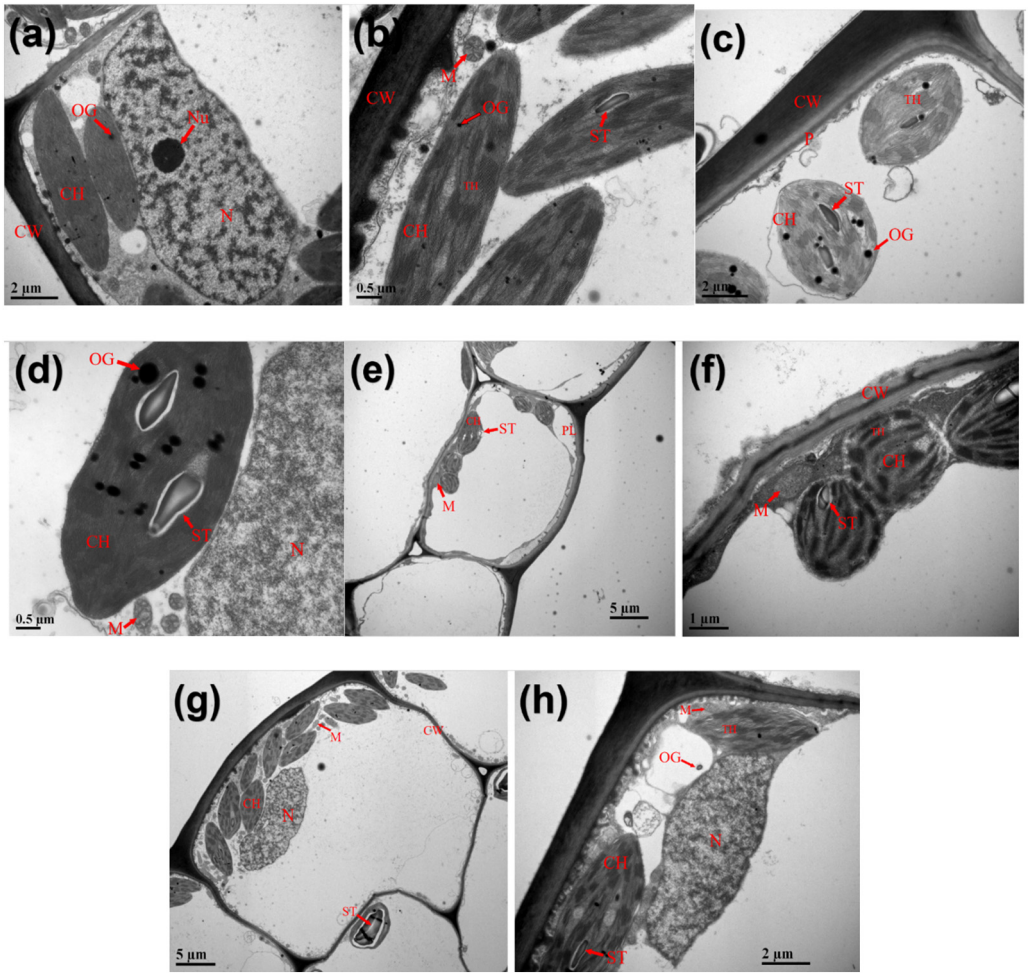

Fig. 4. TEM images of the leaf surface of *V. natans* (a and b): CT group; (c and d): MP10 group; (e and f): MP20 group; (g and h): MP50 group. Note the intact organelles and membranes. Abbreviations: CH, chloroplast; CW, cell wall; ST, starch; M, mitochondria; N, nucleus; Nu, nucleoli; OG, osmophilic granules; P, plasma membrane; PL, plasmolysis; TH, thylakoid.

wall, a change in chloroplast morphology, a blurred double-layer membrane structure, and twisted and disordered thylakoid lamellae. Simultaneously, both the number of ridges (i.e., swellings of the mitochondrial lining) in mitochondria and the degree of intimal folding decreased. When the concentration of MPs was 50 mg L^{-1}, as shown in Fig. 4(g–h), the starch granules were further enlarged; the mitochondria vacuolated and disintegrated; part of the chloroplast envelope ruptured and disintegrated; the thylakoid lamella dissolved or disappeared; part of the thylakoid dispersed in the cytoplasm; the nucleus gradually contracted; the nucleolus disappeared, and the chromatin condensed in the nucleus. This showed that the mesophyll cells of *V. natans* produce a stress response to protect themselves under certain concentrations. In contrast, at higher concentrations, the organelles in the mesophyll cells of *V. natans* would be seriously damaged. Studies have found that some microplastic particles can inhibit cell growth by their absorption to the plant surface. Such potential damage can occur owing to the shade that blocks algal pores or gas exchange, and embedding into microalgal cells (Fu et al., 2019; C. Zhang et al., 2016). This could be the direct cause of organelle damage.

3.5. Effects of MPs on the spatial distribution of extracellular polymeric substances (EPS) in the biofilm of leaves

The results presented in Fig. 5 show that when the leaves of *V. natans* were treated with different concentrations of MPs, the spatial distributions of the biofilm EPS differed. Fluorescein-isothiocyanate (FITC) can bind various antibodies and has strong green fluorescence in an alkaline solution (Powell et al., 2015). Concanavalin A (Con A) can bind to α-D-glucopyranose on a biofilm in red, and Calcofluor White (CW) can be combined with β-D-glucopyranose to produce a blue color (Li et al., 2020b). In the CT group, β-D-glucopyranose was dominant and was accompanied by a small amount of α-D-glucopyranose. The CT group also had the lowest concentration of protein of all the groups studied. This indicated that the surface of *V. natans* leaves would not secrete extracellular protein without external stress. Simultaneously, the amount of extracellular protein present in the leaf surface biofilm increased in response to successively larger concentrations of MPs associated with the groups MP10, MP20 and MP50. This showed that within a certain range of external MP related stress, the cells of *V. natans* would trigger the secretion of EPS as a protective mechanism. Some studies have shown that the increase of protein content in the biofilm may have a positive effect on the physicochemical and physiological characteristics of microorganisms (Xue and Seo, 2013). However, when biofilms are stressed by exogenous substances, they can oxidize polysaccharides on the surface of their EPS (Han et al., 2017). This could be the reason for the reduction of α-D-glucopyranose and β-D-glucopyranose on the EPS surfaces of MP50. In addition, α-D-glucopyranose polysaccharides and β-D-glucopyranose polysaccharides are unevenly distributed on the biofilm, which could be owing to the fact that polymers play an important role in maintaining the structural integrity of the biofilm (Lux et al., 2004). Therefore, the changes in the functional characteristics and spatial structure of biofilms are important indicators of the reaction of biofilms to MPs.

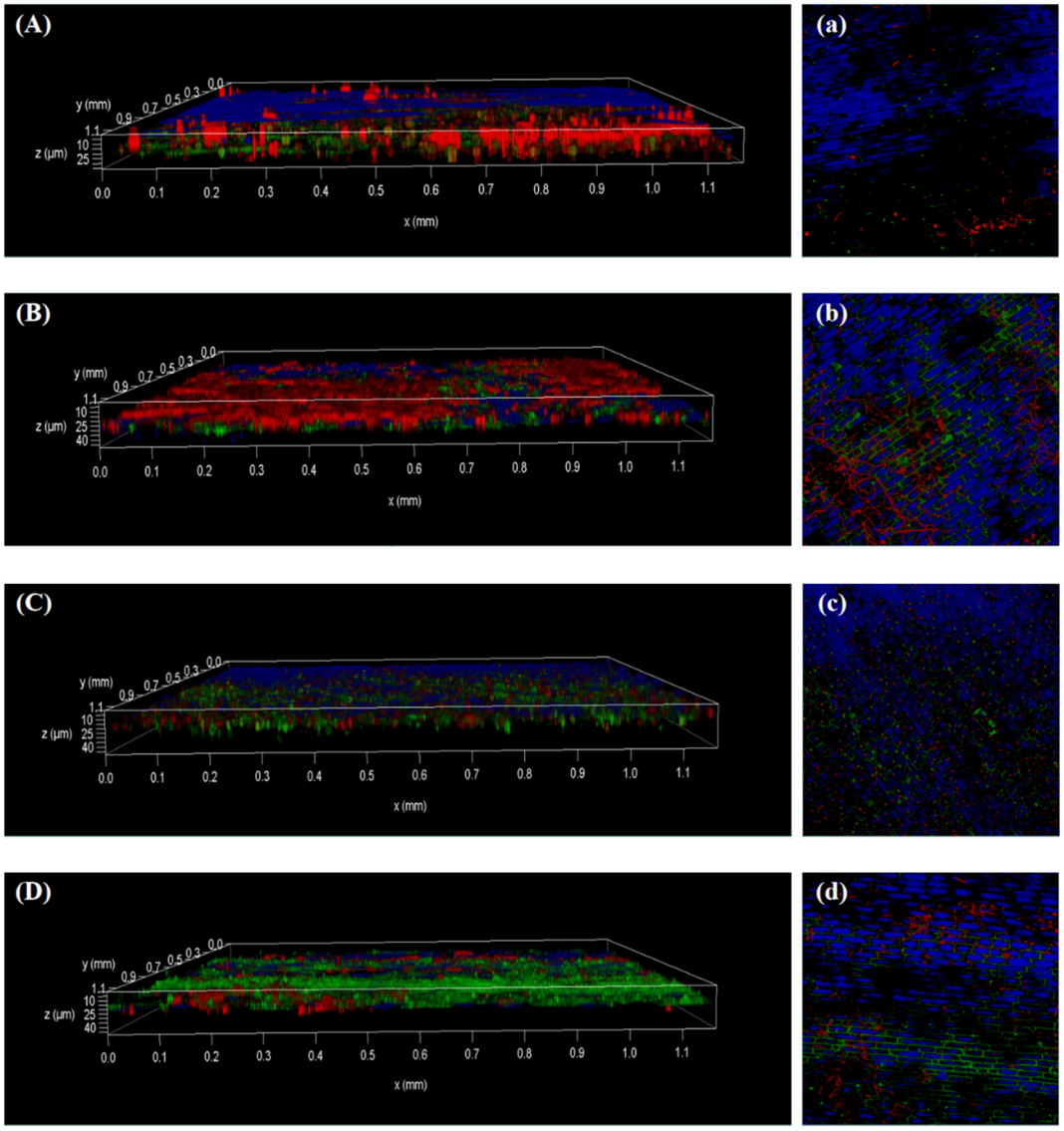

Fig. 5. Effects of different concentrations of microplastics on the spatial distribution of biofilm EPS in leaves of *V. natans*. The left picture shows the three-dimensional superimposed stereoscopic projection, and the picture on the right shows the two-dimensional images of the brightest layer in the corresponding group of samples (A and a: CT; B and b: MP10; C and c: MP20; D and d: MP50). The biofilm on the surface of *V. natans* was stained with FITC (green for protein), ConA (red for α-D-glucopyranose polysaccharide) and CW (blue for β-D-glucopyranose polysaccharide). The image scale on the right is 100 μm.

3.6. Analysis of microbial diversity

The microorganisms in the biofilm were affected by environmental parameters and leaf distribution (S. Zhang et al., 2016). To study the richness and diversity of the microbial community in the biofilm under MP stress, analyses of alpha diversity and 16S rRNA sequences were conducted on a single sample of biofilm.

3.6.1. Analysis of microbial diversity

Table A.$_1$ in the Appendix shows the microbial diversity index for the different treatment groups. In this study, the high coverage index (coverage) was above 0.99. This showed that the results of the measurements were a good representation of the real situation for the microbial community. The Sobs, ACE and Chao 1 diversity indices showed that the microbial community richness for the CT, MP10, MP20 and MP50 groups was higher than those of the SRL and SMP groups. This indicated that submerged plants had a positive effect on microbial enrichment in the system. However, the richness of the microbial community associated with the CT group with *V. natans* and the SRL group without bitter grass decreased significantly after the addition of different concentrations of MPs. The degree of decrease increased with an increase in the concentrations of MP. This indicated that the presence of MPs would affect microbial diversity. There was a negative exponential relationship between the Simpson index and microbial community diversity, and a positive correlation between the Shannon index and microbial community diversity. Based on the data in the table, the microbial community diversity of the CT group was the highest. This could be attributed to exposure to low levels of external stress. One could conclude that the existence of submerged plants was associated with an expansion of the diversity and abundance of the microbial population. In contrast, the microbial population diversity of other experimental groups decreased in the presence of MPs (MP10, MP20, MP50, and SMP) and when submerged plants (SMP) were absent.

3.6.2. Taxonomic distribution of microorganisms at the phylum and genus levels

To study the effects of MPs on microorganisms, we evaluated the hypervariable zone of V3–V4 by establishing the biofilm community structure and diversity on the leaves of submerged plants as shown in Fig. A$_1$.

The Venn diagram shows the distribution of the number of operational taxonomic units (OTUs) in the biofilm and provides a visualization of the number of common and unique OTUs for different samples to show the compositional similarity and overlap of different samples (Huang et al., 2014). As shown in Fig. S1(a) of the Appendix, the number of single OTUs in different treatments was 158 (CT), 42 (MP10), 35 (MP20), 58 (MP50), 83 (SRL) and 29 (SMP). The results showed that the addition of MPs had a significant effect on the species diversity of microbes on the biofilm. The results of hierarchical clustering also showed that the existence of MPs changed the microbial community structure of biofilm on the *V. natans* leaf surfaces. As shown in Fig. S1(b), the distance between MP10, MP20, MP50 and the CT groups increased with an increase in the concentration MPs. This indicated that the microbial community structure changed in response to the concentration of MPs present. The differences in the microbial communities associated with the SRL, SMP and CT groups were particularly significant. This indicated that the existence of submerged plants had a substantial impact on microbial community composition.

The results in Fig. 6(a–b) show the distribution of the nine dominant phyla in all the samples and the proportional representation of dominant

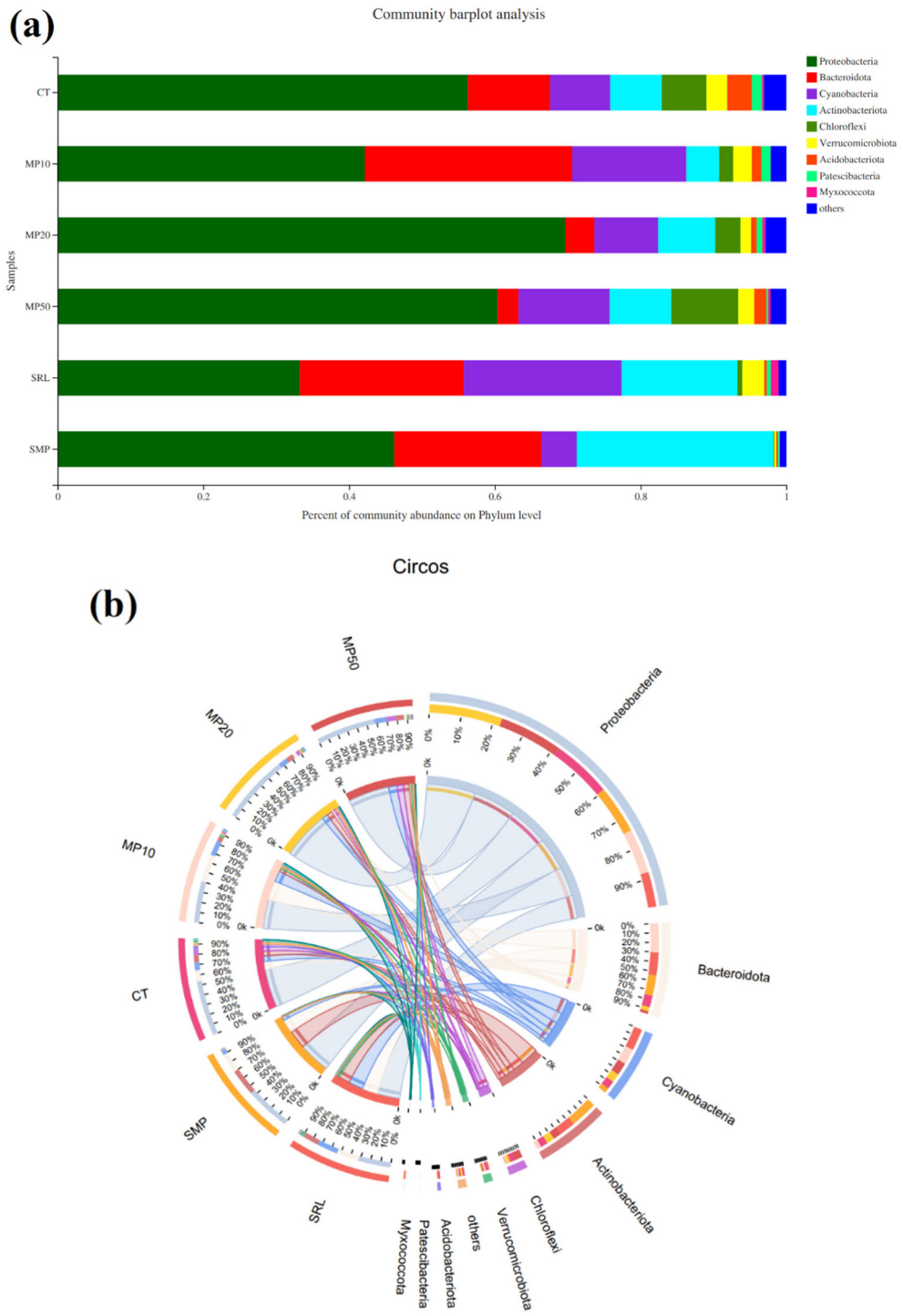

Fig. 6. Microbial community analysis: (a) community bar diagram; (b) sample and species relationship Circos diagram.

species in each group of samples, as well as the proportional representation of dominant species in different samples. The dominant bacteria in biofilm samples included Proteobacteria (33.26%–69.66%), Bacteroidetes (2.89%–28.43%), Cyanobacteria (4.87%–21.65%), Actinobacteria (4.48%–26.99%), Chloroflexi (0.10%–9.17%) and Verrucomicrobiota (0.25%–3.02%). The presence of these microphyla confirmed the results reported by other studies for submerged plant biofilms (Jiang et al., 2019). In addition, there are other microphyla that occupy a smaller proportion (<3.0%) of the microbial communities. The stability of a biofilm community is determined by the dominant microorganisms on the biofilm (Yi et al., 2014). This study showed that Proteobacteria were the most common phyla of all the groups. This may be owing to the ability of Proteobacteria to degrade organic macromolecules (Zhao et al., 2020). Compared with the control group, the addition of different concentrations of MPs did not change the species composition of microbial communities but resulted in significant increases in the relative abundance of different microphyla. The results may be owing to the effect of surface ionic charge of MPs on their toxicity. Weathering and degradation processes, such as photo-oxidation, which can lead to carbonyl group formation, can change the ionic charge of the microplastic surface (Bellingeri et al., 2019). Bacteria are sensitive to differences in electrical charges. The abundances of Proteobacteria, Actinobacteria and Chloroflexi decreased in group MP10, while the relative abundances of Proteobacteria, Actinobacteria and Chloroflexi increased with an increase in the concentration of MPs present in groups MP20, MP50 and SMP. The abundances of Bacteroidetes and Cyanobacteria showed a different trend with the populations increasing significantly in group MP10 but decreasing slightly in groups MP20, MP50 and SMP. The population size of Verrucomicrobiota varied little between the different treatment groups and control groups. It has been reported that some bacteria have preferred concentrations of MPs, particularly Proteobacteria, Bacteroidetes and Cyanobacteria (Guariento et al., 2009). In the earlier results for TEM and CLSM analyses, we concluded that the surface biofilm and internal organelles of submerged plant leaves were damaged and changed in response to stress induced by the presence of MPs. Bacteria may respond to these changes by altering their abundance to stably exist in the presence of MPs. In general, the existence of MPs changes the composition and distribution of biofilms.

The composition of the microbial community was further analyzed at the genus level, as shown in Fig. S2. Heat maps were established for the 50 most abundant genera, with red representing high abundance and blue representing low abundance. The abundances of *unclassified_f__Comamonadaceae* (2.04%–25.29%) and *unclassified_f__Comamonadaceae* (2.04%–25.29%) were the highest in the samples. The dominant species were *norank_f__Rhizobiales_Incertae_Sedis* (1.09%–5.40%), *Gemmobacter* (0.74%–7.65%) and *Mycobacterium* (1.55%–5.51%). The abundances of *Candidatus_Aquirestis*, *Limnohabitans* and *norank_f__Sporichthyaceae* were elevated in the group without submerged plants (SRL and SMP), but they tended to be absent in the groups with submerged plants (CT, MP10, MP20 and MP50). The opposite is true for *Phreatobacter* and *Mesorhizobium*. The sample clustering tree showed that the six experimental groups could be assigned to two categories, depending on whether they contain submerged plants or not. This showed that the existence of submerged plants would result in changes at the microbial community level. This could be used to explain why the microbial genera present in different water bodies show obvious differences. The abundances of *Chryseobacterium*, *JGI_0001001-H03*, *Methylophilus* and *Brevundimonas* decreased gradually with an increase in the concentration of MPs present with the genera abundant in the CT and MP10 groups but decreased to less than 0.02% in the MP50 group. This could be caused by the synergistic effect of *V. natans* releasing organic matter after being stressed. This showed that the difference in the concentration of MPs would also bring significant changes to the abundance of microorganisms. This analysis shows that the existence of submerged plants and the concentration of MPs are important factors that affect the species composition and abundance of microorganisms.

4. Conclusions

This experiment comprehensively investigated the toxic effects of MPs on submerged macrophytes and periphyton biofilms. The results showed that MPs altered the growth and chlorophyll content of *V. natans*. Moreover, an antioxidant response was induced that promoted the activities of CAT, SOD, POD and GST, along with increasing the concentrations of GSH and MDA. Furthermore, the extracellular protein content of *V. natans* increased significantly when stimulated by MPs. Moreover, exposure to the MPs toxin induced stronger changes in the abundances and structure of the biofilm microbial community. This information is potentially useful to identify possible interventions to reduce the impacts of MPs on these taxa and the identification of methods for the ecological restoration and reconstruction of habitats for the submerged plants.

CRediT authorship contribution statement

Jiawei Zhang: Conducting experiments, Data analysis, Investigation, Writing - Original draft preparation.
Deying Huang: Methodology, Data analysis, Data curation, Investigation.
Hong Deng: Resources, Writing - Reviewing and editing.
Jibiao Zhang: Conceptualization, Resources, 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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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2021.151750.

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