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Original Research

Effects of Different Substrates on the Growth and Rhizosphere Microorganisms of *Vallisneria natans*

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Abstract

The modified material at the bottom of lakes plays a key role when restoring and reconstructing the ecology of waterbodies. However, the effects of different substrate types on the growth and development of submerged plants and rhizosphere microorganisms remain unclear. Therefore, this study analyzed the effects of five substrates (attapulgite, biochar, cinder, maifanite, and quartz sand) on the growth and rhizosphere microorganisms of *Vallisneria natans (Lour.) Hara* (*V. natans*). The results showed that during the culture period, the mean plant height of *V. natans* peaked in the maifanite group (26.75 cm), the mean leaf number peaked in the biochar group (12.13), and the mean root activity peaked in the cinder group (83.42 U g^{-1} min). There was no significant difference in the total chlorophyll content among the groups during culture ($P > 0.05$). The malondialdehyde content of *V. natans* leaves in all groups, except quartz sand group, peaked early in the culture period (10 days). Moreover, the superoxide dismutase activity of the five groups increased first and then decreased; however, the catalase activity in the maifanite group decreased significantly compared with the levels in the other groups ($P<0.05$). This indicates that maifanite supplementation can accelerate the adaptation of *V. natans* to changes in the environment. After the addition of substrate, the species of dominant bacteria in the rhizosphere remained nearly unchanged, but their relative abundance was different. Specifically, the relative abundance of *Desulfobacterota* increased in each group, peaking at 9.77% in the maifanite group. This study showed that different substrates had different growth-promoting

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effects on submerged plants, with maifanite exerting the greatest effect. The findings provide a technical reference for selecting the best substrate improver for *in situ* ecological restoration.

Keywords: substrate, submerged plants, rhizosphere microorganisms, growth index

Introduction

Rapid economic development is associated with the entry of large amounts of nutrients into waterbodies including lakes, making lake eutrophication one of the most common and challenging ecological problems [1, 2]. Most nutrients in lake ecosystems are stored in the sediment and can be recycled into the water column through various mechanisms [3]. At present, the main approaches for controlling lake eutrophication include sediment dredging [4], physicochemical adsorption [5], and ecological restoration [6]. Sediment dredging can reduce the pollution load of lakes on a large scale; however, sediment removal requires a large treatment area and is expensive [7]. Meanwhile, when adsorbent materials such as iron salt, aluminum, and lanthanum-amended clay are added to a lake, they can establish an absorbing layer between the sediment and the water environment, stabilize and increase the adsorption capacity of the sediment, and thus reduce the overall circulation of sediment nutrients in the water column [8, 9]. However, this simply reduces the flux of nutrients from the sediment to the water. Therefore, to successfully restore the ecology of lakes, research has increasingly focused on ecological restoration technology that is low in cost and has a lasting restorative effect.

Submerged macrophytes are widely used in the restoration of aquatic ecosystems and are often considered a key material in the restoration of eutrophic lakes [10]. In the process of restoring the ecology of lakes, submerged plants directly regulate the migration and transformation of nutrients in sediment and overlying water by absorbing nutrients in the sediment [11, 12]. Submerged macrophytes can also directly inhibit phytoplankton biomass through allelopathy, thus acting as a buffer against the harmful consequences of eutrophication in lake ecosystems [13]. However, the key factor for the recovery of submerged macrophytes is whether they can establish a good root system in the sediment, and the nutrient state of the sediment also affects the growth and distribution of submerged macrophyte communities and their species composition [14]. It was found that the eutrophication of sediment not only accelerated the deterioration of waterbodies but also limited the growth of submerged macrophytes [15, 16]. Therefore, to enable submerged macrophytes to properly take root and grow stably, it is particularly important to improve the quality of the sediment substrate.

Maifanite, cinder, and other materials are widely used as substrate improvement materials owing to their environmental friendliness and economic applicability [17, 18]. The addition of these materials to sediment not only deactivates the surface sediment but also provides a surface for microbial growth [19, 20]. It was found that substrate not only affected the growth rate of aquatic plants [21] but was also a key factor for the stable anchoring of aquatic plant via their roots [22]. However, the relationships of substrate with submerged macrophyte growth and rhizosphere microorganisms remain poorly understood.

This study aimed to investigate whether substrate affects physiological changes during the growth of submerged macrophytes and the effects of substrate on the structure of the microbial community in the rhizosphere of submerged macrophytes. The enzyme activities related to submerged macrophyte tissues and the community structure of rhizosphere microorganisms were also analyzed in this study.

Materials and Methods

Sediment Collection

Jinyin Lake (114º07′N, 30º38′E) is a shallow lake (average depth 2.5 m, surface area 0.77 km^2) in the middle reaches of the Yangtze River, located in Wuhan, China. In this study, the center of Jinyin Lake was selected as the sampling point. In July 2022, the YSI EXO2 Multi-Parameter Water Quality Analyzer (YSI Inc., Yellow Springs, OH) was initially used to determine the pH and dissolved oxygen (DO) level of the overlying water at the sampling point. A sample of surface sediment with a thickness of approximately 15 cm was then obtained using a Peterson sampler. The collected samples were transported to the laboratory for preservation. Part of each sediment sample was used for determining the physical and chemical properties, while the rest was filtered using a 15-mm-diameter screen to remove impurities such as gravel and animal and plant residues, after which it was used for planting experiments. The pH and DO level of the overlying water of Jinyin Lake were 8.37 and 8.6 mg L^{-1}, respectively. The pH of the sediment was 7.76, the content of organic matter was 71.8 g kg^{-1}, and the contents of total nitrogen and total phosphorus were 3.26 g kg^{-1} and 0.65 g kg^{-1}, respectively.

Experimental Design

Vallisneria natans (Lour.) Hara (V. natans) is a dominant submerged macrophyte in the middle reaches of the Yangtze River, which is characterized by rapid growth, developed roots, and ease of planting. Therefore, *V. natans* pot experiments were conducted.

V. natans was cultured at 25ºC under a light intensity of 3500 lx for 1 week. The substrates employed in the experiment were attapulgite (AE), biochar (BR), cinder (CR), maifanite (ME), and quartz sand (QS) with a particle size of 3-5 mm. These substrates were obtained from Henan Province, China. The biochar was produced by carbonizing coconut shells at a temperature of 600ºC for a duration of 6 h. The cinder, on the other hand, was the residual waste generated from coal combustion in boiler equipment. In accordance with the method outlined by Wang et al. [23], the cinder underwent pretreatment to reduce the concentration of heavy metals. All substrates were thoroughly cleaned with distilled water before use.

The planting experiment was conducted with a polyethylene column (diameter, 15 cm; height, 50 cm). A 20-cm-thick layer of sediment was first placed in the planting column, after which a 1-cm-thick layer of substrate was spread on its surface. Healthy, equally sized seedlings (plant height, 10 cm) were selected to be planted in the cylinders, with five seedlings in each. Finally, distilled water was added and the water depth was maintained at 24 cm. The experiment began in August 2022 and samples were obtained every 10 days for 40 days. For each group of substrate materials, experiments were performed in triplicate.

Experimental Method

One *V. natans* sample was randomly selected at each sampling time, and its growth, physiological, and biochemical indicators were determined after cleaning the sample. The growth indicators included plant height, biomass, leaf number, and root length, while the physiological and biochemical ones included leaf chlorophyll content, malondialdehyde (MDA) content, superoxide dismutase (SOD) activity, catalase (CAT) activity, and root activity. Chlorophyll and MDA contents were determined using the Lichtenthaler–Arnon and thiobarbituric acid methods, respectively. SOD and CAT activities were determined using the guaiacol method. Root activity was determined using the triphenyltetrazolium chloride method [24].

Microcosm Experiments

Initial (blank control, CK) and final sediment samples were obtained and stored at -80°C for DNA extraction. Total genomic DNA was extracted from a 0.4-g sediment sample using the Fast DNA® Spin for Soil kit (MPBIO, USA) according to the manufacturer's protocol. DNA quality was determined by agarose gel electrophoresis with a mass concentration of 2%. The extracted DNA solution was stored in a freezer at -20ºC until later use. The V3–V4 regions of the 16S rRNA gene were amplified by PCR using the primers 341F (5′-CCTACGGGNGGCWGCAG-3′) and 805R (5′-GACTACHVGGGTATCTAATCC-3′) [25]. The PCR-amplified reaction system (20 μL) contained 4 μL of 5× FastPfu Buffer, 2 μL of dNTPs (2.5 mmol L^{-1}), 0.4 μL of FastPfu Polymerase, 0.8 μL of each primer (5 μmol L^{-1}), 0.2 μL of bovine serum albumin, 1 μL of template DNA, and 10.8 μL of ddH_2O. PCR was performed by initial denaturation at 95ºC for 3 min, followed by 30 cycles of denaturation at 95ºC for 30 s, annealing at 53ºC for 30 s, and extension at 72ºC for 45 s. PCR products were purified using the AxyPrep DNA (AXYGEN, USA) gel recovery kit and detected by 2% agarose gel electrophoresis. Then, the QuantiFluoruant™ blue fluorescence quantitative system was used to detect and quantify the PCR products and mix purified amplicons with the equimolar amount, followed by sequencing using HiSeq2500 PE250 on an Illumina HiSeq platform.

Statistical Analysis

One-way ANOVA and Tukey's test ($P<0.05$) were performed using SPSS 20.0 software to analyze the significance of differences in the physiological and biochemical properties of the *V. natans* samples. The correlations among different substrate and physiological characteristics of *V. natans* were determined by redundancy analysis (RDA) using CANOCO 4.5, in which the lengths of the gradient were shorter than 3.0 by detrended correspondence analysis.

Results

Growth Indicators of *V. natans* on Different Substrates

Fig. 1 shows the changes in growth of *V. natans* during the culture period. There was no significant difference in plant height among the different substrates ($P>0.05$; Fig. 1a). During the entire culture period, the mean plant height of the ME group was the largest, at 26.75 cm, followed by that in the CR group (25.25 cm). In each sampling period, the increment of plant height since the last measurement was greater in the BR group than those in the AE and QS groups. During the same treatment group among the different culture period, the number of leaves in the ME and BR groups was significantly different ($P<0.05$) (Fig. 1b), and the maximum average number of leaves in the BR group was 12.13.

During the same treatment group among the entire culture period, the mean root lengths in the groups with the five substrates (AE, BR, CR, ME, QS) were 11.69, 13.03, 13.44, 13.63, and 12 cm, respectively, while the change in root length of the ME group was significantly different compared with other groups ($P<0.05$) (Fig. 1c). *V. natans* grew faster during the culture period, and the biomass of almost all groups peaked at 20-30 days of culture, apart from in the QS group (Fig. 1d).

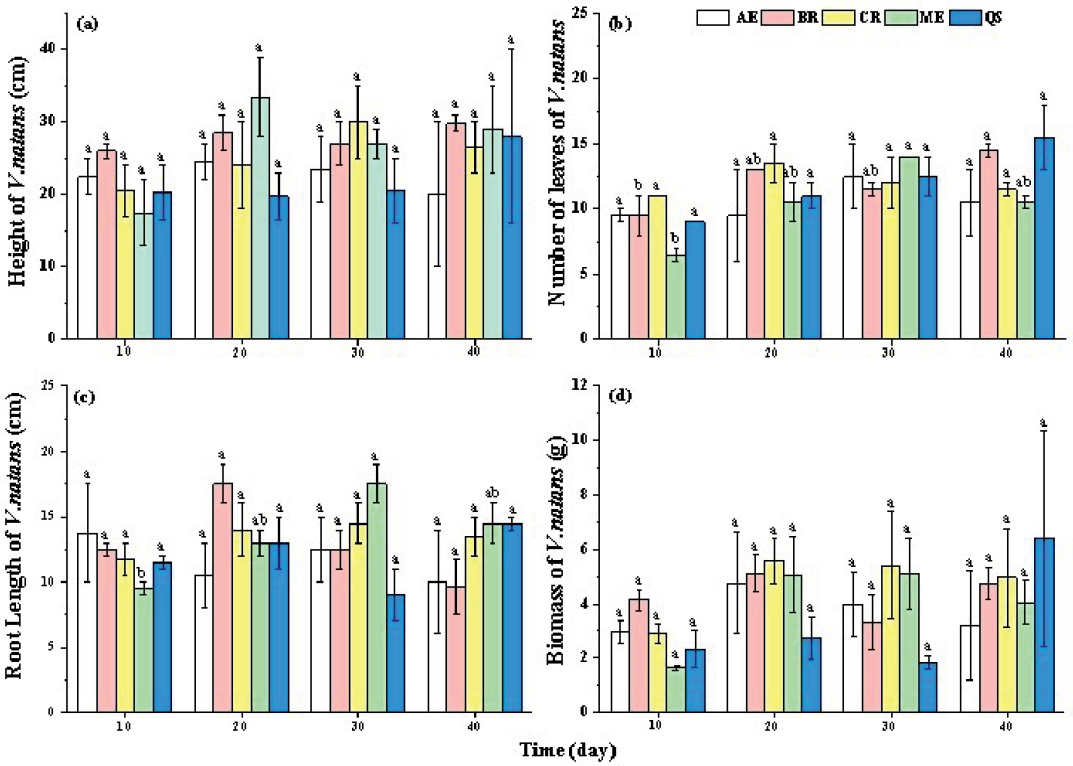

Fig. 1. Effects of different substrates on the growth of *V. natans*. Different lower-case letters in the bar chart indicate significant differences at the P<0.05 (Tukey's test) level in the same treatment group among the different times. Error bars indicate the standard deviation.

Ecophysiological Index Responses of *V. natans* on Different Substrates

The content of Chl-a in the leaves of *V. natans* in the AE and BR groups first increased and then decreased with increasing culture time, while the opposite trend was shown in the ME and QS groups (Fig. 2a). Apart from in the AE and QS groups, the content of Chl-b in the other groups was significantly different during the same treatment group among the whole experiment (P<0.05; Fig. 2b). The trend of variation of Chl-b content in leaves of *V. natans* in the AE and BR groups was similar to that of Chl-a. The content of Chl-b in the CR group peaked on the 20th day of culture at 0.62 mg g^{-1}. During the entire experiment, the maximum total chlorophyll content in the CR group was 1.15 mg g^{-1}, but there was no significant difference in the total chlorophyll content among all groups (P>0.05; Fig. 2c).

During the entire experimental period, there were similar trends of variation of CAT activity in the leaves of *V. natans* in the AE and CR groups (Fig. 3a), both of which showed a trend of first increasing and then decreasing. Meanwhile, the CAT activity in the ME group was decreased, and the difference was significant (P<0.05) during the entire culture period of the same treatment group. MDA content in the leaves of *V. natans* in all groups, except the QS group, peaked the first sampling time; however, in BR and ME groups, it gradually decreased, albeit not significantly (P>0.05; Fig. 3b). Meanwhile, the SOD activity of each group peaked at 20–30 days of culture and then gradually decreased. There were significant differences in SOD activity and root activity of *V. natans* in the QS group (P<0.05; Fig. 3c and 3d). Finally, during the entire culture period, the root activity of the AE, BR, CR, and ME groups first increased and then decreased, while the mean value of root activity was highest in the CR group (83.42 U g^{-1} min).

Redundancy Analysis

The first two RDA axes explain 79.5% and 18.6% of the variance, respectively (Fig. 4). The results show that root vitality and SOD activity were positively correlated with biomass, Chl-a, Chl-b, and Chl-a+Chl-b of *V. natans*. CAT activity was positively correlated with root vitality and MDA. BR was closest to the region of the plot representing QS, indicating that the substrates BR and QS had similar effects on plants.

Effects of Different Substrates on the Rhizosphere Microbial Community Structure

At the phylum level (Fig. 5), the amounts of bacteria in *Proteobacteria*, *Chloroflexi*, *Desulfobacterota*, *Acidobacteriota*, and *Nitrospirota* were the highest, accounting for 60% of the total in the order of relative

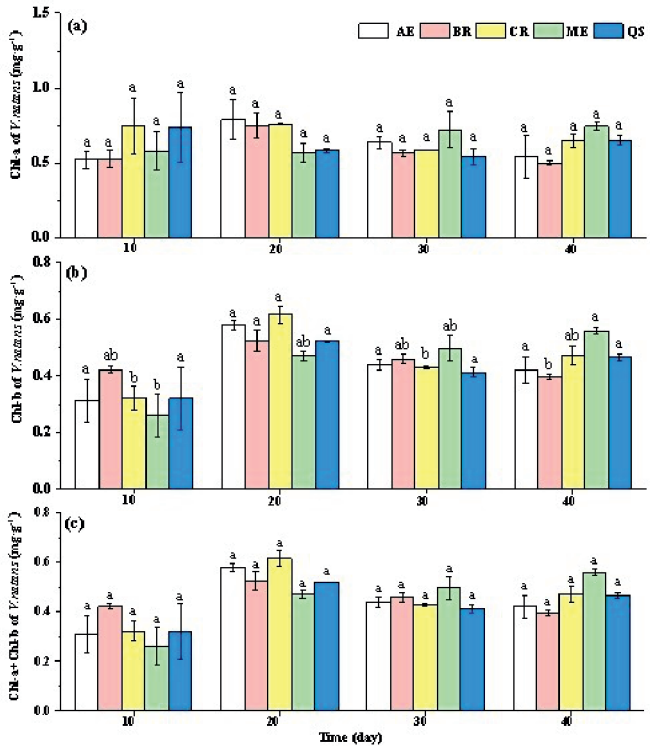

Fig. 2. Effect of different substrates on the chlorophyll content of *V. natans*. Different lower-case letters in the bar chart indicate significant differences at the P<0.05 (Tukey's test) level in the same treatment group among the different times. Error bars indicate the standard deviation.

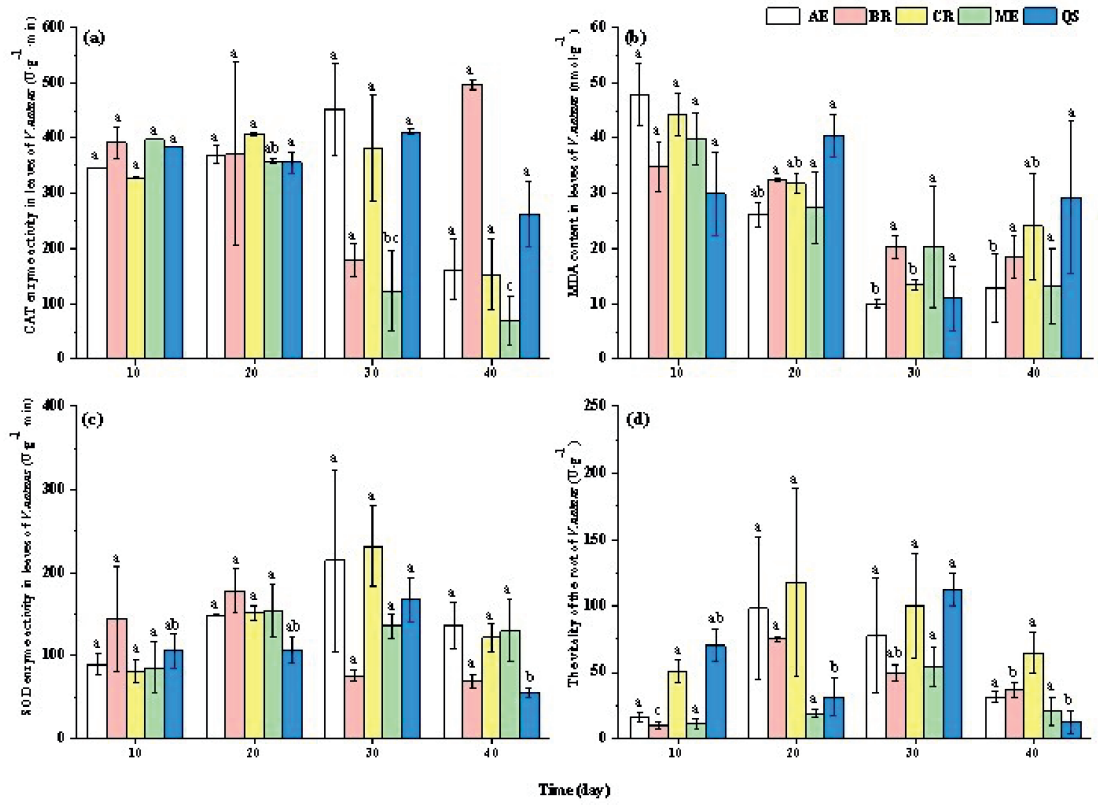

Fig. 3. Effect of different substrates on the ecophysiological indexes of *V. natans*. Different lower-case letters on the bar chart indicate significant differences at the P<0.05 (Tukey's test) level in the same treatment group among the different times. Error bars indicate the standard deviation.

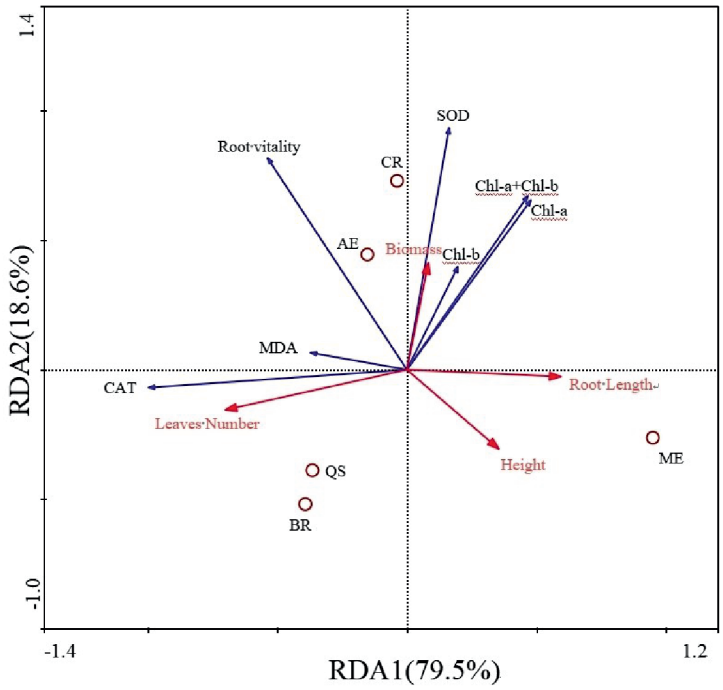

Fig. 4. RDA results of the impact of different substrates on *V. natans* ecophysiological indexes and main growth indicators.

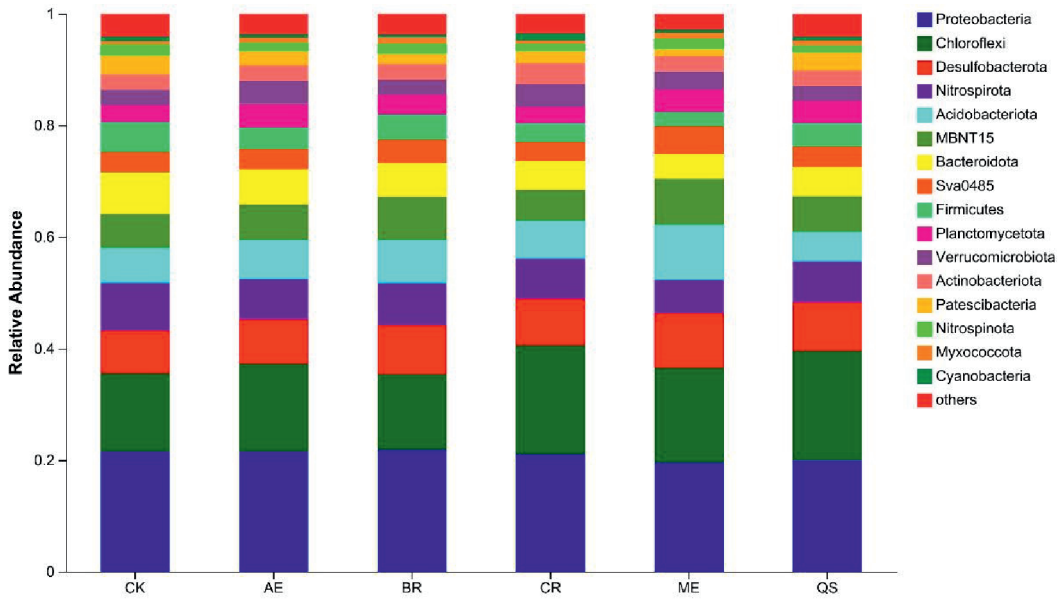

Fig. 5. Microbial community structure for each sample at the phylum level (CK, sediment sample before substrate addition).

abundance in the five substrate groups. Although the microbial communities were similar among the five substrate groups, the relative abundance of *Proteobacteria* in each group was slightly different from that in the CK group before culture. However, we still found significant interspecific differences in the remaining four phyla. The relative abundance of *Chloroflexi* in the other three groups was lower than that in the CK group, except for the CR and QS groups. The relative abundance of *Desulfobacterota* in all substrate groups was higher than that in the CK group (7.65%). Moreover, the relative abundances of *Desulfobacterota* and *Acidobacteriota* were highest in the ME group, at 9.77% and 9.96%, respectively. The relative abundance of *Nitrospirota* in the substrate group was lower than that in the CK group (8.53%).

To obtain a deeper understanding of the similarities and differences in the microbial community composition of each sample, a heat map containing the clustering relationship tree for each sample was constructed (Fig. 6). After clustering analysis, the microbial community was divided into four branches: CR and QS

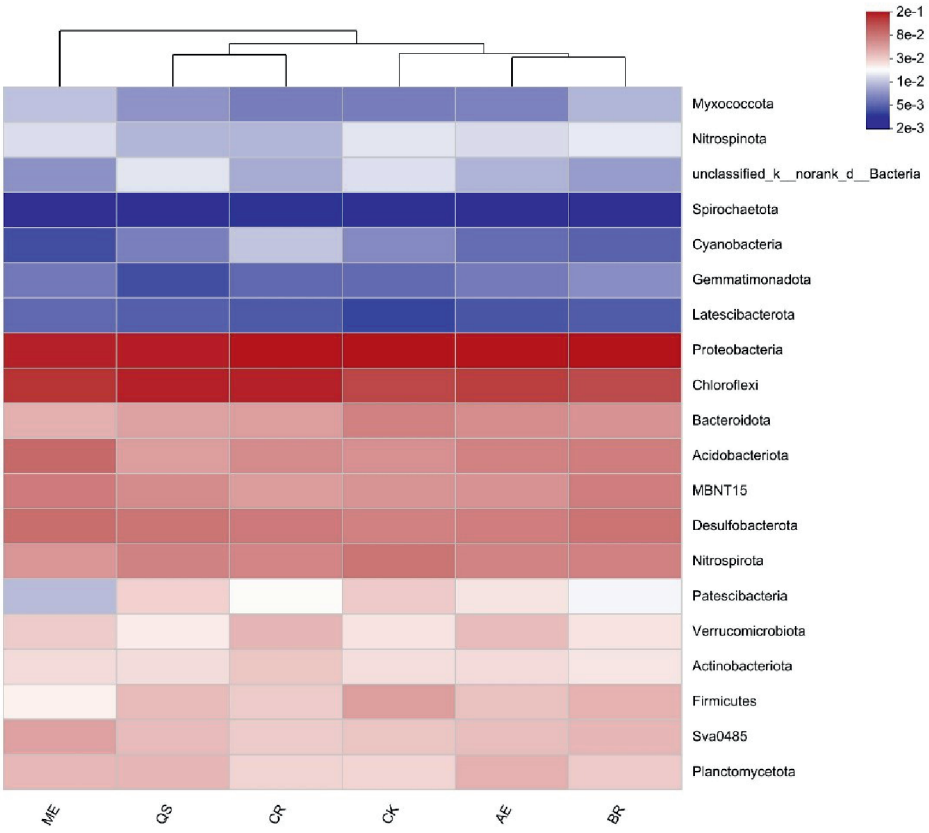

Fig. 6. Clustering heat map of microbial communities in each sample.

formed one branch, AE and BR formed another, while CK and ME formed a separate branch each. In addition, from the evolutionary relationships among the microbial community, samples of the ME group were shown to be distant from samples of the CK, AE, BR, CR, and QS groups.

Discussion

Studies have shown that the growth of submerged macrophytes in lakes can be affected by the addition of a substrate [26, 27]. For example, Bai et al. [17] found that vermiculite could significantly promote the plant height and biomass of *Vallisneria spiralis* (*V. spiralis*), while bentonite could increase the number of roots on *V. spiralis* by 130.77% [28]. These results are similar to those of the present study. Here, during the entire experimental period, the growth indexes of *V. natans* in each group revealed different increases, among which the plant height of *V. natans* had the largest increase in the ME group, while the mean leaf number of *V. natans* showed the largest increase in the BR group. This may be because the substrate contained abundant K^+, Ca^{2+}, Na^+, and other major and trace elements, which play important roles in the growth and propagation of submerged macrophytes [22, 28]. In addition, the roots of submerged macrophytes were slender, and substrate retention was conducive to the extension of plant roots. The stable environment brought about by the substrate has also been reported to promote the absorption of nutrients by plants [29].

When plants face external environmental stress, reactive oxygen species (ROS) accumulating in them cause oxidative damage to lipids and proteins in plant cells, potentially leading to cell death [30, 31]. In the presence of excessive ROS, plants activate their own antioxidant protection system to mitigate the damage caused by these ROS [32, 33]. The antioxidant enzyme SOD can catalyze the disproportionation of O_2^- in plants to produce H_2O_2 and O_2, while CAT can decompose H_2O_2 into H_2O and O_2 [34]. During the entire experiment reported here, SOD activity in each group generally initially increased and then decreased, indicating that *V. natans* protected itself by increasing SOD activity upon sensing the oxidative stress experienced under the new substrate environment. During the first 20 days of culture, there was no significant difference in CAT activity among all groups, indicating that *V. natans* of all groups produced a large amount of CAT to decompose H_2O_2, so as to reduce the H_2O_2-related toxicity. However, later in the experiment (40 days), CAT activity in the ME group was significantly reduced, which indicated that the addition of maifanite would enable *V. natans* to quickly adapt to changes in the environment.

As a product of membrane lipid peroxidation, MDA content reflects the degree of lipid peroxidation in plant

cell membranes and the adaptability of plants to changes in the external environment [35, 36]. Studies have shown that MDA accumulation can inactivate enzymes related to photosynthesis and respiration in plants [37]. In this study, MDA content in each group was higher at the early stage (0-20 days) than at the late stage of culture (20-40 days), which might have been because *V. natans* was introduced to a new living environment and thus suffered stress, thereby increasing MDA content. Moreover, the addition of a substrate activated the antioxidant protection system in the plants and removed ROS, thus inhibiting the production of MDA [38] and accelerating the adaptation of *V. natans* to the new environment.

There was no significant change in the dominant phyla among rhizosphere microorganisms between before and after culture in our research. Although the relative abundance of *Proteobacteria* was different in the different substrate environments, it remained the dominant phylum. Salta et al. [39] found the global biogeochemical cycle advantage of the microorganisms in *Proteobacteria*, which play a dominant role in various environments. This may be because *Proteobacteria* contains various bacteria involved in the carbon and nitrogen cycles, and these microorganisms were important contributors to biogeochemical processes [40]. Substrates were also shown to be critical to the growth of rhizosphere microbial communities. In this study, the relative abundance of *Desulfobacterota* in rhizosphere sediment increased with the addition of substrate, which is similar to the findings reported by Liu et al. [28]. The microorganisms in the phylum *Desulfobacterota* play an important role in the conversion of sulfur compounds in sediment as key species in the sulfur cycle [41]. A porous substrate not only provides stable breeding conditions for rhizosphere microorganisms and nutrients needed for their growth [42], but the amount of trace elements in the substrate is also directly involved in electron transfer and hormone synthesis in the form of inorganic salts [43]. This in turn contributes to the growth of microorganisms in the *Desulfobacterota* phylum and the conversion of sulfide in sediment.

In this study, the influence of maifanite on plants and microorganisms differed significantly from the influences of the other substrates (Fig. 4 and 6). Studies have shown that adding maifanite to sediment can not only promote plant growth but also significantly improve the diversity of rhizosphere microorganisms [42, 44]. This may be because only maifanite is rich in Se, Mn, and other trace elements, which play significant roles in promoting the growth and germination of plants [45]. In addition, maifanite contains a large number of active anion groups ($[\sim SiO]^{-}$), these $[\sim SiO]^{-}$ can adsorb microorganisms by multiple combinations of large cations ($-N^{+}$) in bacterial proteins [46]. The aggregation of these rhizosphere microorganisms can further promote the growth of plant roots [47].

Conclusions

In this study, five substrates were added to the surface of sediments in laboratory experiments, after which *V. natans* was planted to study the effects of the different substrates on its growth and physiological conditions as well as on rhizosphere microorganisms. The results showed that the substrates had different growth-promoting effects on *V. natans*. Maifanite was associated with the largest increase in the plant height of *V. natans*, while biochar was associated with the greatest number of leaves. Moreover, during the different culture periods, the change in root length in the maifanite group was significant. When faced with changes in the environment during growth, the SOD activity of each group exhibited the same trend of change, but late in the experiment (40 days), the CAT activity of the maifanite group was significantly reduced, indicating that the addition of maifanite accelerated the adaptability of *V. natans* to the new environment. RDA showed that biomass was positively correlated with root vitality, SOD activity, and chlorophyll content. While the predominant species of rhizosphere bacteria in each substrate group did not change significantly between before and after culture, the relative abundance of *Desulfobacterota* increased, with the greatest increase occurring in the maifanite group, indicating that the addition of substrate is conducive to the proliferation of sulfur-transforming microorganisms in the sediment. These results indicate that substrates (especially maifanite) play an important role in determining the growth of submerged macrophytes and the rhizosphere microbial community structure. This study thus provides a technical reference and theoretical basis for selecting the best substrate improver for in situ ecological restoration.

Acknowledgments

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Conflict of Interest

The authors declare no conflict of interest.

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