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Effects of Distinct Revegetation Methods on Growth and Microbial Properties of *Vallisneria natans*

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Abstract: This study investigated the effects of the mud-sinking (MS) method, agar gel-sinking (AS) method and agar gel-sinking with artificial aquatic mat (ASA) method on the growth, physiological characteristics, water purification capacity, and associated microbial community of the different organs of *Vallisneria natans* (*V. natans*). Results showed that the growth of agar-based growth (group AS and ASA) were more effective than the mud-wrapped method (group MS), exhibiting longer length, higher fresh weight and biomass of agar-based *V. natans* with the artificial aquatic mat (group ASA) being higher than those of other groups. MS caused a stress response in the oxidative system, which then inhibited photosynthesis. Results of water quality measurements showed that the three planting methods positively affected water purification without significant differences ($p > 0.05$). Besides, there was no significant difference ($p > 0.05$) between the microbial communities in terms of the roots and those found in rhizosphere soils in the MS group with high throughput sequencing. Meanwhile, the addition of agar in the AS and ASA groups increased the diversity of rhizosphere soil microbial communities and reduced the diversity of root microbial communities. Microbial community compositions in the rhizosphere soil and root differed significantly ($p < 0.05$). High throughput sequencing and scanning electron microscopy (SEM) also revealed that the biofilm on the surfaces were different, with Proteobacteria and Cyanophyta consistently dominating. This study provides new insights on the more effective revegetation methods of *V. natans*, researched the environmental impact of the addition of agar, and provides some theoretical support for the revegetation of submerged macrophytes under ecological restoration.

Keywords: revegetation methods; submerged macrophyte; microbial properties; *Vallisneria natans*

1. Introduction

For eutrophic lakes, pollution from external inputs affected the normal succession of plant communities, severely affecting the vegetation and causing degradation of the lake's ecosystem. In most cases, gradually transformation is observed from grass-dominated to grass-algae dominated communities, further shifting to algae-dominated environments [1,2], primarily due to eutrophication. Nutrient accumulation in the lake stimulates phytoplankton blooms, the biomass of which significantly reduces light penetration, decreasing dissolved oxygen concentration, which then limit the growth of the submerged plants. Mortality and subsequent decomposition of the submerged plants will then cause secondary pollution and further aggravate the deterioration of the water quality. As a result, the biodiversity of lakes decreases rapidly, and the community structure becomes a single-species

dominated community [3–5], which has very poor stability. Studies have shown that for lakes with less pollution, and aquatic plants not seriously degraded, artificial intervention to remove external stress showed that the macrophyte communities can be restored to some extent [6,7]. However, changes in external conditions (e.g., temperature, dissolved oxygen content), seem to again stimulate phytoplankton comeback, with a large number of outbreaks re-emerging as the dominant community in eutrophic lakes [8–10]. Therefore, ecological restoration should not only rely on pollution control, but would also need to artificially restore and rebuild the biological community particularly the submerged plants. However, we should develop better restoration methods for macrophytes in general. Submerged plants have been gaining interest since they grow fast and can absorb large amounts of nutrients from the sediment and overlying water, causing the dissolved oxygen to increase and the water quality to improve. Their secretion of allelochemicals can also effectively inhibit the growth of algae. Meanwhile, it can provide habitat and food for benthic animals, increasing species richness of the aquatic environment [11,12]. Thus, it is extremely important to develop submerged plant revegetation method with a high survival rate suitable for poor water quality environments.

Nowadays, although there had been much research done on plant growth and stresses in extreme or adverse environments (e.g., sediment fertilization, water depth, benthonic animal and etc.), studies on revegetation methods of submerged plants remain rare [13–16]. The research on current revegetation methods of submerged plants have been limited to sowing, cuttings and sinking. Traditional sinking method refers to covering the root with mud, wrapping and bundling the root with cloth, throwing the plants into the water, and using gravity to sink it to the bottom. Since the 1990s, successful restoration of submerged vegetation in shallow eutrophic lakes have already been done, but fluctuations in water levels, low transparency, and feeding by aquatic animals have become major obstacles to these efforts [17–19]. Studies showed that the survival rate of the cutting method was the highest, followed by the sinking method, and finally the sowing method, and there was no significant difference in the biomass of individual plants [20]. However, the cutting method is difficult to operate and requires a lot of manpower and material resources, while seed germination rate of the sowing method is low and time-consuming. Traditional sinking method on the other hand causes damage to plants, with the loss of mud reducing the survival rate of plants.

Thus, in this study, agar was used to lessen such disadvantages, and it can provide rich resources, such as nutrients. The introduction of agar will also greatly affect the structure of the associated microbial communities. In aquatic ecosystems, microorganisms drive most of the biogeochemical cycling and nutrient transfers. In ecological restoration, changes in bacterial abundance, community structure, and diversity can be used as important indicators in assessing the effect of the methods being employed [21,22]. Past studies on the restoration of submerged plants mainly compared the effects of common methods on plant biomass and water quality [20,23,24], but not the microbial communities. This study explored and segmented current understanding of the responses of microbial communities during restoration.

Vallisneria natans (*V. natans*) is a common perennial submerged plant with wide adaptability and high tolerance to pollution. It is regularly used as a pioneer species in vegetation restoration efforts in eutrophic waters [25].

This study then adopted *V. natans* as the target research species. By examining the growth of *V. natans* when transplanted using mud-sinking (MS) method, agar gel-sinking (AS) method, and agar gel-sinking with artificial aquatic mat (ASA) method, the effects of different planting methods on plant damage and photosynthesis were determined. The impact of different planting methods of *V. natans* on purification of water bodies and bacterial communities on the root system, rhizosphere soil, and plant leaf surfaces were then evaluated.

2. Materials and Methods

2.1. Materials

V. natans plants were obtained from an aquatic plant company (Pudong Tiancun Horticultural Company, Shanghai, China) and cultured for 7 days in aquaria containing tap water before the experiment. The plants were then cleaned with deionized water and only healthy plants of similar size were chosen for subsequent experiments. Quartz sand (particle size 4–7 mm, Huilong Environmental Company, Jiangsu, China), fishnet (mesh side length 4 cm, Boxing Net Industry Company, Jiangsu, China) and artificial aquatic mat (Shanghai Fan-resistant Aquarium Products Co., Ltd., Shanghai, China) were first treated with deionized water and UV sterilization before use. Agar powder was purchased from Sinopharm Chemical Reagent Co., Ltd.

The overlying water was obtained from Moon Lake, a typical shallow landscape lake in Jiangwan Campus, Fudan University. Water samples were uniformly mixed. The sediment was sampled from the surface layer of Zhushan Bay in Taihu Lake by a grab dredger.

2.2. Experimental Design

After culturing for 7 days, the *V. natans* individuals were divided into three groups, and evenly separated into five small bundles. Each group had a fresh weight of 20.30 ± 0.42 g, a dry weight of 4.06 ± 0.41 g, a shoot height of 16.83 ± 1.83 cm and a root height of 9.20 ± 2.20 cm. These were then cultivated into three Plexiglas buckets (inner diameter 600 mm, height 600 mm) at 18 ± 2 °C under 80 μmol quanta m^{-2} s^{-1} (PPFD) light and a 16:8 h light-dark cycle. In the Plexiglas buckets, the sediment depth was set at 10 cm, and the overlying water depth at 40 cm to simulate typical contaminated shallow lakes (Figures 1 and 2). In the first group (hereafter referred to as mud-sinking method, MS), the root of the *V. natans* plants was wrapped with sediment from Taihu Lake to form a cone shape, and the sediment was tied by fishnet. The wrapped *V. natans* plants were put into the water then sank to the bottom. Meanwhile, in the second group (hereafter referred to as agar gel-sinking method, AS), the treatments of the root were modified. A 3% agar solution was prepared, heated to a boiling point, and cooled down to 42 °C; then, *V. natans* plants were placed in the center of small crucibles with a diameter of 5 cm and approximately 2 g quartz sand were added. After that, agar solution was poured quickly into the crucibles immersed in a cold-water bath. When the agar solidified, the content of the crucible was taken out, which included the agar base plant integrated grass seedling. In the third group (hereafter referred to as agar gel-sinking with artificial aquatic mat method, ASA), the *V. natans* plants were cultivated into the bucket in the same way as the second group but 20.00 ± 0.08 g string artificial aquatic mat were evenly added into the bucket to increase the microbial adhesion area [26].

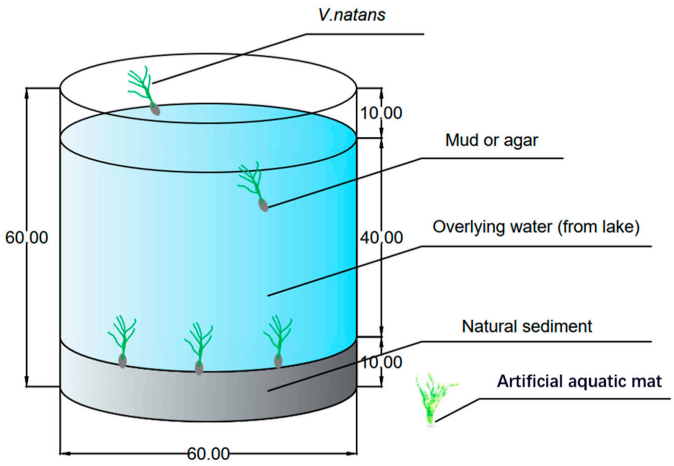
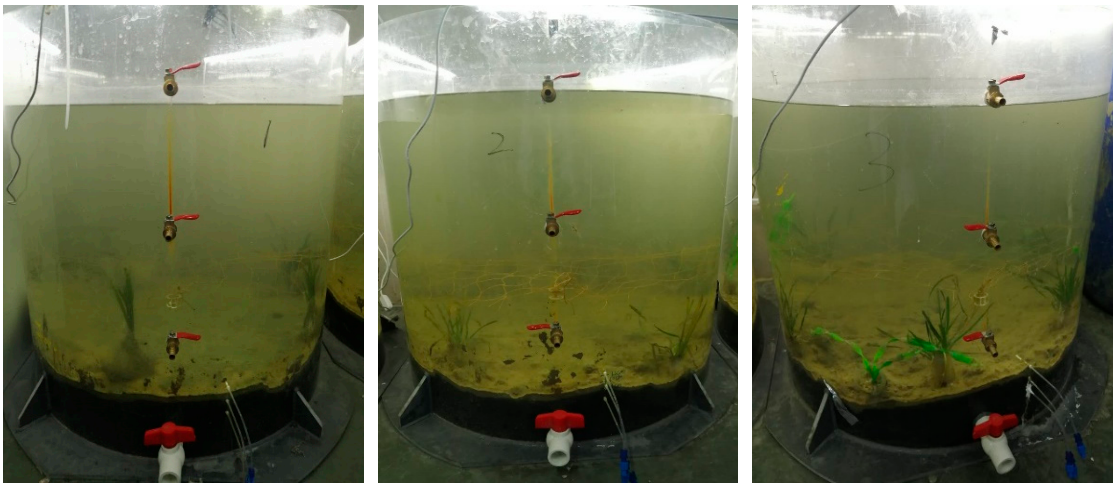


Figure 1. Schematic diagram of the experimental unit (cm).



(**a**) (**b**) (**c**)

Figure 2. Experimental units for *V. natans* plants transplanted. Notes: (**a**) is mud-sinking method (MS); (**b**) is agar gel-sinking method (AS); (**c**) is agar gel-sinking with artificial aquatic mat method (ASA).

2.3. Monitoring Plant Growth and Enzyme Activity

Plant growth was evaluated by measuring changes in fresh weight, dry weight, root length, shoot length, and photosynthetic efficiency including total chlorophyll (Chl (a + b)) and maximum quantum yield of PSII (F_v/F_m ratios). F_v/F_m ratios are optimal/maximal photochemical efficiency of PSII in the dark. Chl (a + b) was measured at the beginning and end of the experiment, which were extracted using 90% hot ethanol, and the absorbance measured at 649 nm and 665 nm, respectively. Chl contents were then calculated using the methods described by [27]. Maximum quantum yield of PSII was determined at the same frequency as the water quality parameter with a PAM 2100 fluorometer (AquaPen-C, Photon Systems Instruments, Czech Republic) [28]. The above indicators were measured in triplicate each sample at each time point.

Peroxidase (POD) and catalase (CAT) were detected at the beginning and end of the experiment. CAT is widely found in plant tissues—it has obvious ecological effects on a variety of adverse environmental factors (NO_2, SO_2, NH_3), which can decompose H_2O_2 produced by cell metabolism and prevent H_2O_2 from harming the plant. As one of the important protective enzymes in plants, POD can effectively prevent the accumulation of reactive oxygen species in plants, the increase and decrease of POD activity reflects the protective stress response of plants under the action of adversity factors. Around 1.00 ± 0.01 g of leaves were first flash frozen in liquid nitrogen, and then crushed with 10 mL 0.1 mol/L PBS (pH 7.0, containing 1 mmol EDTA and 1% polyvinylpyrrolidone (w/v)). The homogenate was centrifuged at 10,000× *g* to obtain supernatants at 4 °C for 20 min. The activities of POD, CAT and the total protein content were assessed by a commercial chemical kit (Nanjing Jiancheng Bioengineering Institute, China)) following the manufacturer's instructions. The above indicators were measured in triplicate each sample at each time point.

2.4. Water Quality Parameters

Total organic carbon (TOC) and total nitrogen (TN) were analyzed using a total organic carbon analyzer (TOC-L CPH, Shimadzu, Kyoto, Japan), and pH by a portable pH meter (PHB-4, Shanghai INESA Scientific Instrument Co., Ltd., Shanghai, China). Turbidity was measured by a portable turbidimeter (2100Q, HACH Water Analysis Instrument Co., Ltd., Shanghai, China). Then, NH_3-N was determined by standard methods [29]. The experiment lasted for 40 days, with the physiochemical indicators being observed every 3 days in the early period (0 d to 12 d) to track rapid changes, then every 5 days between 12 d to 32 d, and then terminated on 40 d.

2.5. Scanning Electron Microscopy (SEM) and Microbial Properties Analysis

SEM was used to visualize the surface morphology of plant leaves, which allowed visualization of the microbial distribution on leaf surface. On the 40th day, random samples were collected from the middle part of the *V. natans* leaves, cut into squares (0.8 × 0.8 cm), and fixed with glutaraldehyde (2.5% in 50 mmol/L sodium cacodylate) immediately. After a double rinse with 0.1 M PBS (pH of 7.4), the leaf samples were dehydrated using a series of ethanol concentrations (20, 40, 60, 80 and 90%) for 15 min, and with 100% ethanol twice for 15 min. Dried samples were visualized using SEM (Hitachi, Tokyo, Japan, S-3400 NII) [30].

Around 1 g of samples (*V. natans* leaves, artificial aquatic mat leaves and roots with biofilms) from every group was collected at the end of the experiment. Samples were then added with 20 mL 0.1 mol/L sterile PBS solution, and the mixture was incubated at 20 °C and centrifuged at 180 RPM for 20 min. Plant tissues were removed, and 20 mL 0.1 mol/L sterile PBS solution was added one more time before centrifugation at 180 rpm for about 20 min. The plant tissues were taken out again, and added with 20 mL 0.1 mol/L sterile PBS solution, then washed by ultrasonic for 10 min (parameters: 160 W, 30 s). Finally, PBS was removed, and the plant tissues were stored in the refrigerator at −80 °C for later use. All wash solution was collected and passed through 0.2 μm filter membrane (or 12,000 g centrifugation for 10 min to collect the precipitate). Collected filter membranes were stored at −80 °C for preservation [31]. Around 10 g rhizosphere soil was gathered at the end of the experiment from every group. Samples were quickly frozen in liquid nitrogen, and stored at −80 °C for later use [32]. Samples for microbiological testing were from random sampling in the reactor and mixing before testing. Each leaf, root and rhizosphere soil samples were collected in triplicate and mixed [33].

Microbial DNA of the leaves, roots and rhizosphere soil samples were extracted from by using the E.Z.N.A.®soil DNA Kit (Omega Bio-tek, Norcross, GA, USA) according to manufacturer's protocols. The final DNA concentration and purification were determined by NanoDrop 2000 UV-vis spectrophotometer (Thermo Scientific, Wilmington, NC, USA), and DNA quality was checked by 1% agarose gel electrophoresis. The V3-V4 hypervariable regions of the bacteria 16S rRNA gene were amplified with primers 338F (5′-ACTCCTACGGGAGGCAGCAG-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) by thermocycler PCR system (GeneAmp 9700, ABI, Wilmington, NC, USA). The PCR reactions were conducted using the following program: 3 min of denaturation at 95 °C, 27 cycles of 30 s at 95 °C, 30s for annealing at 55 °C, and 45 s for elongation at 72 °C, and a final extension at 72 °C for 10 min. PCR reactions were performed in a triplicate 20-μL mixture containing 4 μL of 5 × FastPfu Buffer, 2 μL of 2.5 mM dNTPs, 0.8 μL of each primer (5 μM), 0.4 μL of FastPfu Polymerase and 10 ng of template DNA. The resulted PCR products were extracted from a 2% agarose gel and further purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA) and quantified using QuantiFluor ™ -ST (Promega, Madison, WI, USA) according to the manufacturer's protocol. And purified amplicons were pooled in equimolar and paired-end sequenced (2 × 300) on an Illumina MiSeq platform (Illumina, San Diego, CA, USA) according to the standard protocols by Majorbio Bio-Pharm Technology Co., Ltd. (Shanghai, China).

2.6. Statistical Analysis

Statistical analysis was performed by Version 17.0 of SPSS for Windows (SPSS, Chicago, IL, USA). One-way analysis of variance (ANOVA) and a *t*-test were used to test the difference between of experiment groups, and followed by the least significant difference at $p < 0.05$. In this paper, the error bars on the bar plots indicate standard deviation. The microbial properties of *V. natans* were conducted by a web-based platform Majorbio I-Sanger Cloud Platform (www.i-sanger.com). PCoA (Principal Component Analysis) is a non-binding method of data downscaling that can be used to study similarities or differences in the composition of sample communities, and it was performed by Majorbio I-Sanger Cloud Platform (www.i-sanger.com). Raw FASTQ files were quality-filtered by Trimmomatic and merged by FLASH with the following criteria: (i) The readings were truncated at any site receiving an average quality score <20 over a 50 bp sliding window. (ii) Sequences

whose overlap being longer than 10 bp were merged according to their overlap with mismatch no more than 2 bp. (iii) Sequences of each sample were separated according to barcodes (exactly matching) and Primers (allowing 2 nucleotide mismatching), and reads containing ambiguous bases were removed. Operational taxonomic units (OTUs) were clustered with 97% similarity cutoff using UPARSE (version 7.1 http://drive5.com/uparse/) with a novel 'greedy' algorithm that performs chimera filtering and OTU clustering simultaneously. The taxonomy of each 16S rRNA gene sequence was analyzed by RDP Classifier algorithm (http://rdp.cme.msu.edu/) against the Silva (SSU123) 16S rRNA database using confidence threshold of 70%.

3. Results and Discussion

3.1. Effects of Distinct Revegetation Methods on Growth, Physiological Parameters, and Enzyme Activity of V. natans

3.1.1. Growth of *V. natans*

Figure 3 showed that the fresh weight and shoot height of *V. natans* in groups AS and ASA were significantly higher than that in group MS ($p < 0.05$). Further, ASA fresh weight and biomass increased more significantly than AS ($p < 0.05$). However, AS and ASA were not as good as MS in root growth ($p < 0.05$), which could be due to the agar encapsulation hindering the growth of the root system. Previous studies showed that agar penetration resistance is higher than soil, and an increase in penetration resistance will reduce root elongation [34]. The results showed that when comparing 0 d and 40 d, the growth rates of roots in the experimental group were MS (35.71%), AS (14.58%) and ASA (9.09%), respectively. The growth rate of the leaf length is MS (8.82%), AS (20.00%) and ASA (18.92%), respectively.

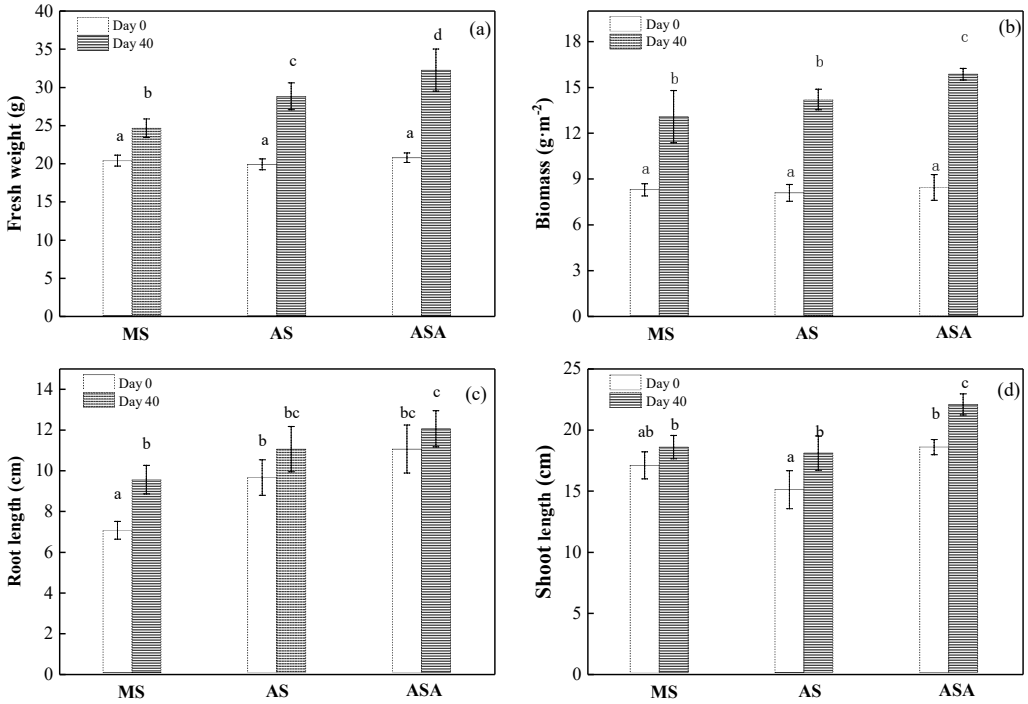


Figure 3. Effects of the different revegetation methods on the growth of *V. natans*.

3.1.2. Physiological Parameters and Enzyme Activity of *V. natans*

The F_v/F_m ratios and total chlorophyll are shown in Figures 4 and 5. The F_v/F_m ratios showed no significant ($p > 0.05$) changes during the experiment, indicating that different revegetation methods had no influence in the potential maximum photosynthetic capacity of *V. natans*. Meanwhile, the total

chlorophyll of AS and ASA were higher ($p < 0.05$) than that of MS, indicating that cultivation with agar did not diminish the plant's photosynthetic capacity.

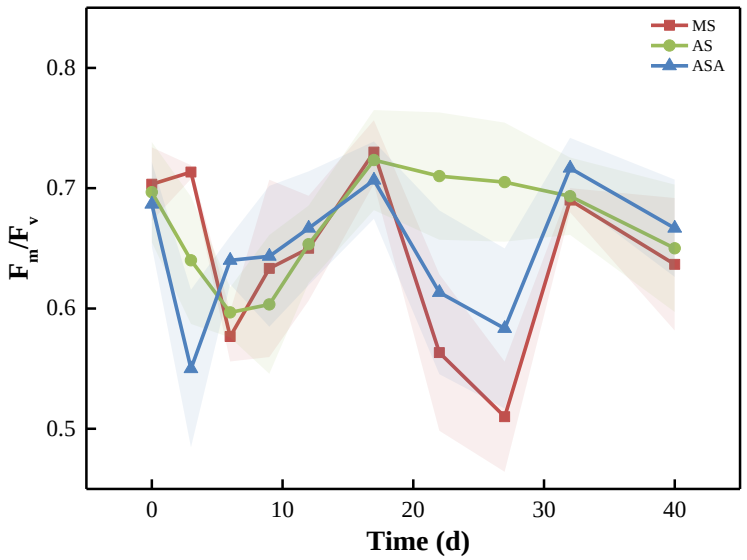


Figure 4. Change in *V. natans* F_v/F_m ratios in MS, AS and ASA groups. Notes: The shaded area indicates error range.

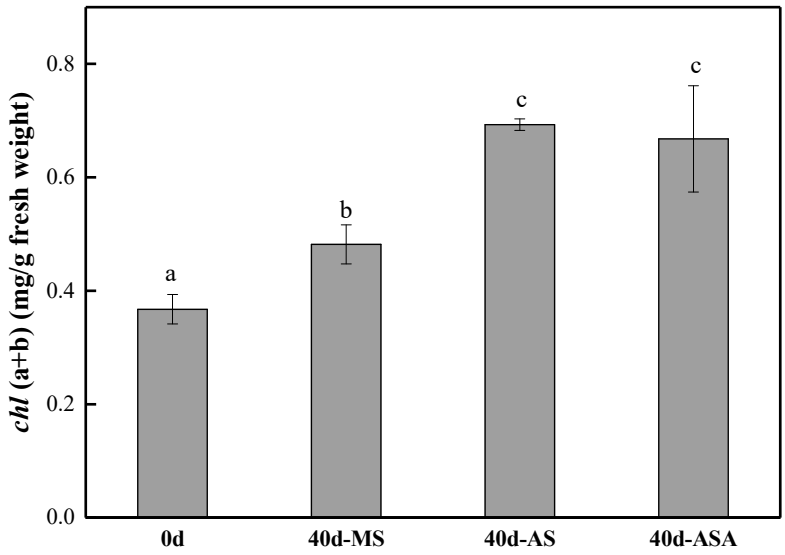


Figure 5. Total chlorophyll content of *V. natans* in MS, AS and ASA groups.

To investigate the impact of stress effect of the three revegetation methods on the plant, the antioxidant activity was monitored (Figure 6). Compared with the initial state, the enzyme activity of all the plants have been increased under different planting method, probably because the plants had some mechanical damage during the planting process. In MS, the concentrations of POD and CAT were significantly ($p < 0.05$) higher than that of AS and ASA. POD and CAT indicate plant regulation of its own protective system to eliminate harmful free radicals, which were also related to the reactive oxygen species (ROS) [35]. The mud in MS group was came from the sediment of Taihu lake, which had high nitrogen and phosphorus contents [36]. Studies have shown that increased nitrogen and phosphorus concentrations could lead to eutrophic stress, causing the enzyme activity to increase [37,38]. In addition, previous studies also showed that POD levels increased significantly after plants were mechanically damaged [39]. This study demonstrates that the damage to the anti-oxidation

defense system caused by MS was greater than that of AS or ASA on *V. natans*, further confirming the superiority of agar cover on the growth of *V. natans*.

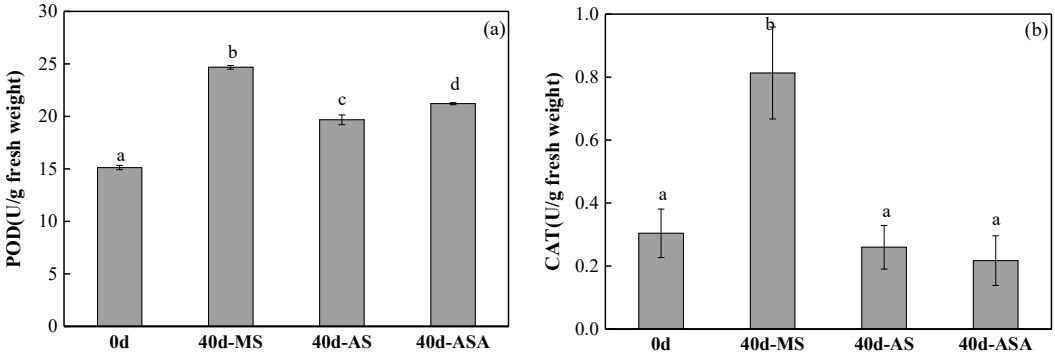


Figure 6. Enzyme activity of *V. natans* in MS, AS and ASA groups.

3.2. Effects of Distinct Revegetation Methods on the Results of Water Purification

Changes in water quality parameters including TOC, TN, NH_3-N, turbidity and pH are shown in Figure 7. Previous studies have shown that submerged plants significantly influence water purification processes [40–42]. This study revealed that the *V. natans* under the three planting methods has a significant ($p < 0.05$) purification effect on the water body, but there is no significant difference ($p < 0.05$) between the three planting methods thought t-test, indicating that the potential purification capacity of agar-based and mud-wrapped *V. natans* were similar. TOC (Figure 7b) concentration decreased through the removal by *V. natans* on water pollutants and promotion of organic matter released from sediment to overlying water. The purification effects of *V. natans* became more evident before 32 d, and in the late stage, growth was slower, causing much of the change in TOC. The tendency of TN (Figure 7a) was similar to TOC, but differed in the peak. Specifically, NH_3-N (Figure 7c) increased in the first 3 d, and then continuously declined towards the end of the experiment since it is the form of nitrogen easiest to utilize [43,44].

pH was basically stable, only ranging from 7.74–8.18 (Figure 7e). The effects of *V. natans* on the water body were evident, particularly on turbidity (Figure 7d). At the initial stage of operation, the system was always disturbed by water intake, causing the sediment to be resuspended and the turbidity to reach 47.64 NTU followed by sharp decrease to 2 NTU within 12 d, and then stabilizing at about 2 NTU by the end of the experiment.

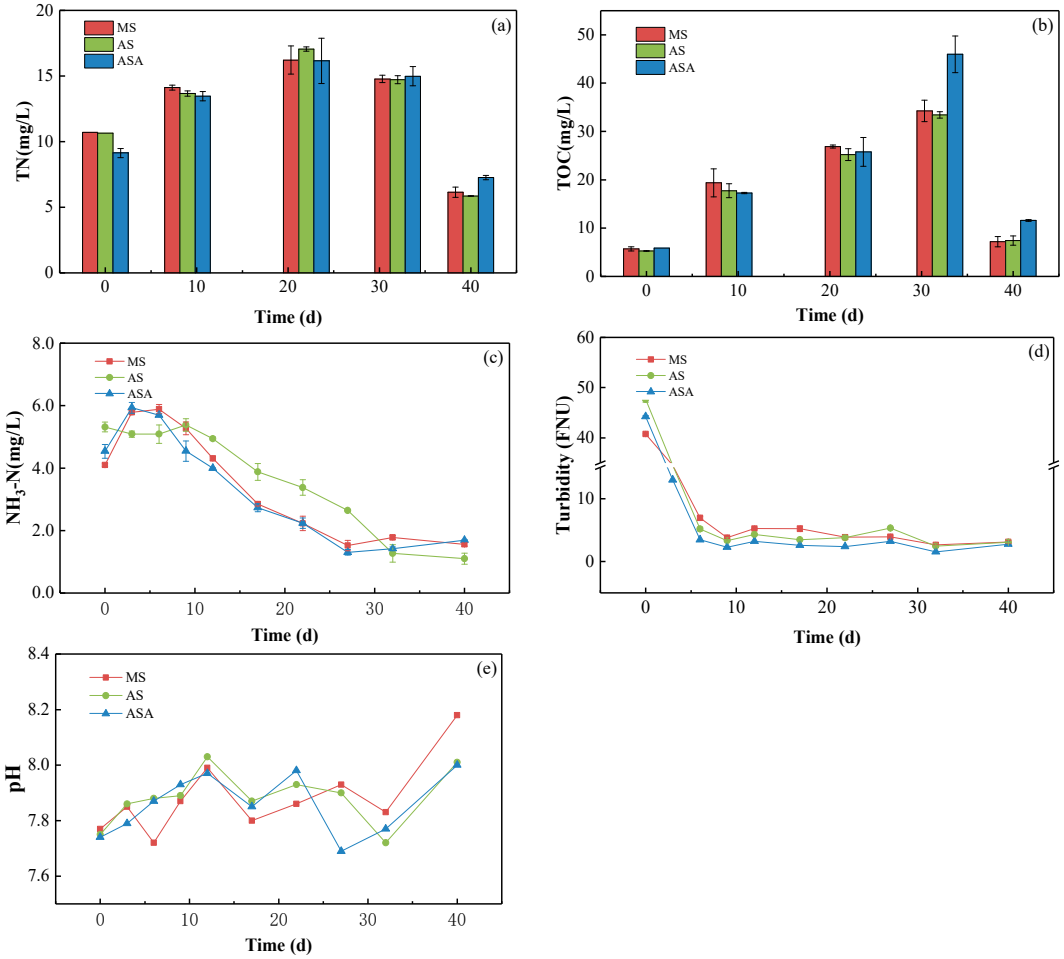


Figure 7. Changes in water quality parameters in MS, AS and ASA groups.

3.3. Effects of Distinct Revegetation Methods on the Microbial Properties of V. natans Root and Rhizosphere Soil

The structure and abundance of microbiota in the root and rhizosphere soil were studied using high throughput sequencing of 16S rRNA. At 97% similarity to cluster operational taxonomic units (OTU), a total of 34,1967 high-quality sequences were obtained, with the average length of the sequence at 420.74. All samples had high Good's coverage indices, indicating that the sequencing results were reliable and representatives. Compared with the MS group, the AS and ASA groups had lower bacterial richness and diversity in their roots, and higher richness and diversity in their rhizosphere soils (Table 1). These results indicate that agar changed the local environmental in the soil but increased the microbial diversity of rhizosphere soil by inhibiting the exchange between the root system and the surrounding soil.

The bar plot (Figure 8) also clearly showed that the agar-based roots in group AS and ASA were dominated by Bacteroidetes (58.38–60.86%), while the root wrapped by mud had almost the same community as the rhizosphere soil, with Proteobacteria as the dominant taxa (49.59–51.48%). Previous results [45,46] showed that Proteobacteria and Chloroflexi both dominated in the sediment of eutrophic waters. In samples 40-s-AS and 40-s-ASA, microbial compositions were highly similar. The content of Chloroflexi (30.00–32.88%) and Proteobacteria (35.05–37.53%) in samples 40-s-AS and 40-s-ASA were more approximative than that in sample 40-s-MS, indicating that the addition of agar was more suitable for the survival of Chloroflexi [47,48]. In the agar-based group, Bacteroidetes was the dominant group. Studies have shown that the growth rate of Bacteroidetes on agar medium is faster, indicating that agar is more conducive for its growth [49].

Table 1. Summary of the indices of community richness and diversity of bacterial taxa in MS, AS and ASA groups.

Samples	Ace	Chao	Coverage	Shannon	Simpson	Sobs
40d-s-MS	3168.89	3083.75	0.97	6.08	0.011	2400.00
40d-s-AS	3341.26	3252.70	0.98	6.24	0.007	2572.00
40d-s-ASA	3351.64	3326.66	0.97	6.24	0.007	2564.00
40d-r-MS	3747.64	3750.71	0.97	6.54	0.005	2912.00
40d-r-AS	934.22	781.22	0.99	2.63	0.313	576.00
40d-r-ASA	1648.57	1457.83	0.99	3.51	0.176	1037.00

Notes: 40d-s-AS is rhizosphere soil of the last day in group AS, 40d-s-ASA is rhizosphere soil of the last day in group ASA, 40d-s-MS is rhizosphere soil of the last day in group MS, 40d-r-MS is root of the last day in group MS, 40d-r-AS is root of the last day in group AS, 40d-r-ASA is root of the last day in group ASA.

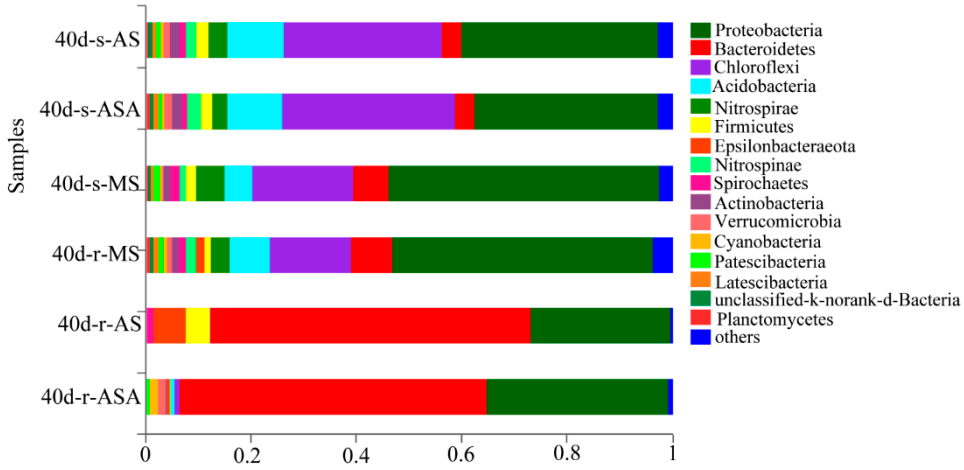


Figure 8. Bar plot of the bacterial community in the rhizosphere soil and root at the phylum level. (Notes: 40d-s-AS is rhizosphere soil of the last day in group AS, 40d-s-ASA is rhizosphere soil of the last day in group ASA, 40d-s-MS is rhizosphere soil of the last day in group MS, 40d-r-MS is root of the last day in group MS, 40d-r-AS is root of the last day in group AS, 40d-r-ASA is root of the last day in group ASA.).

3.4. Biofilm Attached to the Leaves of V. natans

High throughput sequencing of 16S rRNA and scanning SEM were used to investigate the biofilm attached to the leaves of *V. natans* under three planting methods. Results showed that bacteria-like particles were distributed on the surface of the leaves such as cocci, bacilli and other forms of bacteria and organic matter. Biofilms were unevenly distributed on the leaf surface of MS, AS, ASA and artificial aquatic mat in ASA (ASA-AL) (Figure 9). The microbial distributions of AS and ASA were more similar. Cocci bacteria and organic matter were more commonly found in the MS group, while other samples in group AS and ASA were more found with bacilli. High-throughput sequencing results showed that the dominant microbes on the leaf surface were *Proteobacteria*, *Cyanobacteria*, and *Bacteroides*, which was consistent with previous studies [23]. Principal coordinates analysis (PCoA) and community bar plot analysis (Figures 10 and 11) showed that the addition of agar certainly affected the surface microbes, with the most significant difference observed in the distribution of Proteobacteria and Cyanophyta on the surface of artificial aquatic mat and *V. natans*. This may due to the symbiotic and competitive relationships between the leaves of *V. natans* and the microbial community on the leaves. For example, the leaves provide the nutrients needed for the growth of *Cyanophyta*, and photosynthetic *Cyanophyta* can provide oxygen for *V. natans*. *Proteobacteria* are mostly anaerobic or facultative anaerobic bacteria, which grow more favorably in inorganic surfaces [50,51].

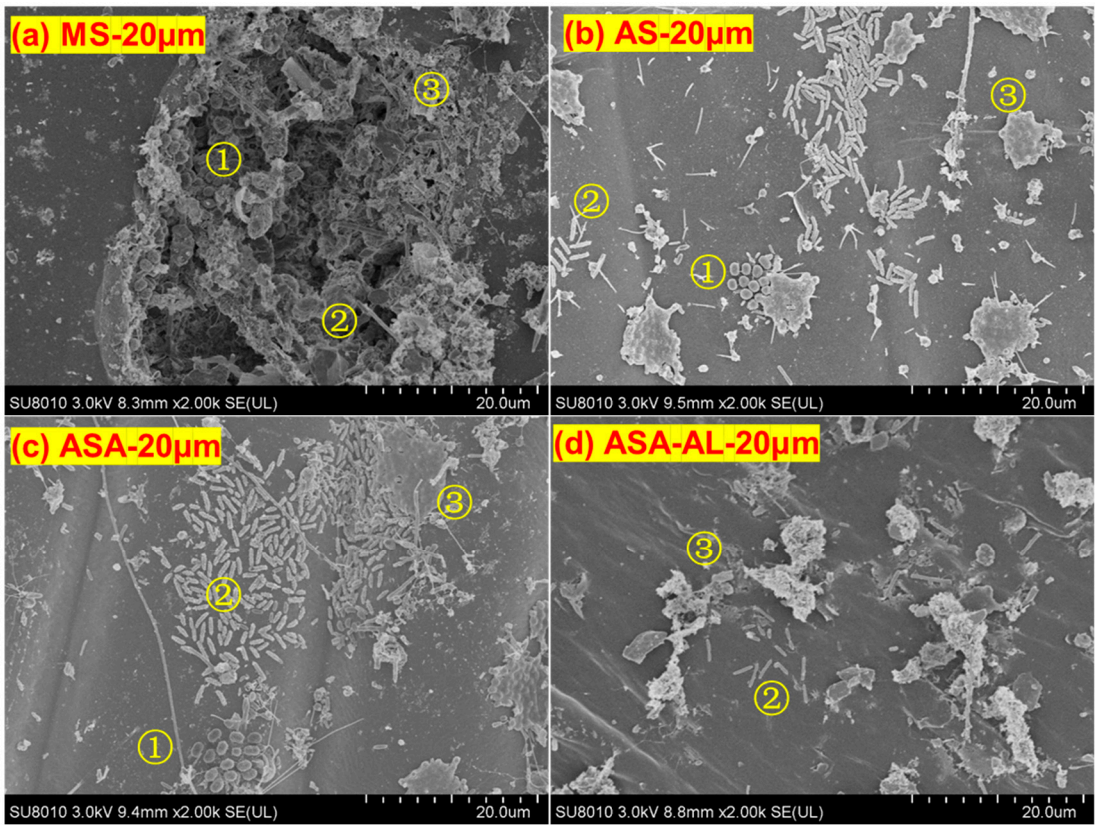


Figure 9. Scanning Electron Microscopy (SEM) images (**a**–**d**) of biofilms attached to *V. natans* leaves. (Notes: ①, cocci aggregates; ②, bacillus aggregates; ③, organic matter or extracellular polymers.).

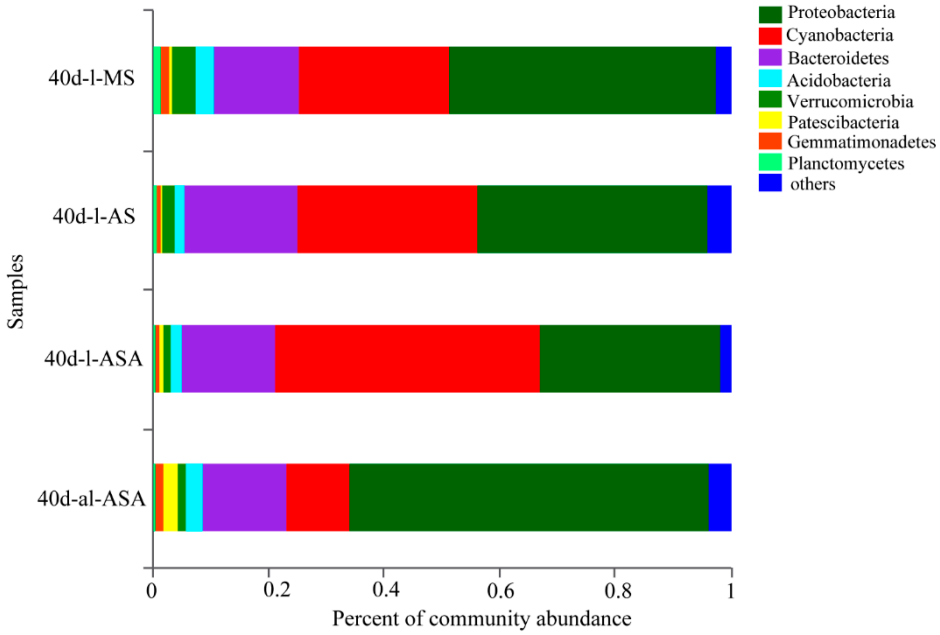


Figure 10. Community bar plot analysis of leaves on phylum level. (Notes: 40d-l-MS is leaf of the last day in group MS, 40d-l-AS is leaf of the last day in group AS, 40d-l-ASA is leaf of the last day in group ASA, 40d-al-ASA is artificial aquatic mat leaf of the last day in group ASA.).

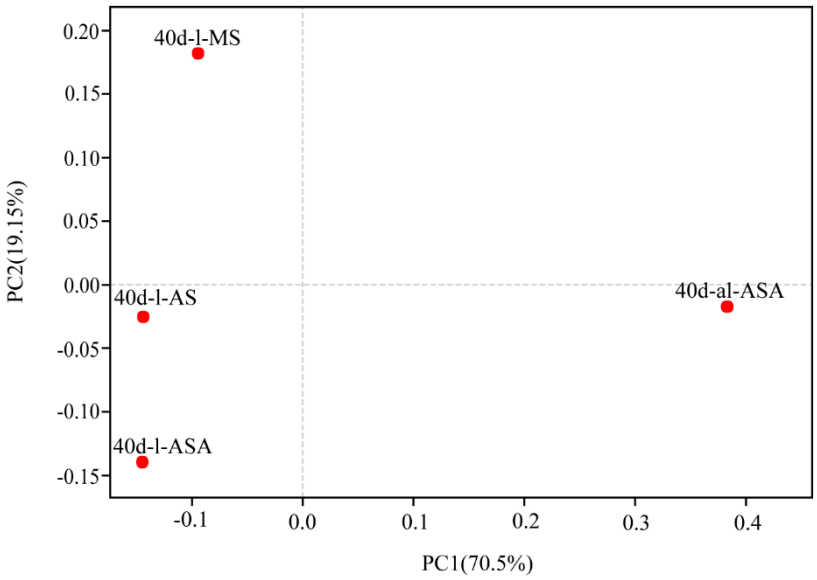


Figure 11. PCoA plot of bacterial communities on the leaves of *V. natans* on phylum level. (Notes: 40d-l-MS is leaf of the last day in group MS, 40d-l-AS is leaf of the last day in group AS, 40d-l-ASA is leaf of the last day in group ASA, 40d-al-ASA is artificial aquatic mat leaf of the last day in group ASA.).

4. Conclusions

In this paper, the effects of three revegetation methods on the growth of *V. natans*, water purification effects, and changes in associated microbial communities were studied. Results showed that compared with the mud-wrapped method, the agar-based method effectively promoted plant growth and photosynthesis, and reduced physical damage to plants, with the antioxidant system stress response much lighter. In terms of water purification effects, the three planting methods differed little. Adding agar did not reduce water quality based on the variables we measured, but it did cause significant changes in the structure of microbial communities in the root, rhizosphere soil, and leaf surfaces. This study indicates that using agar to sink aquatic plants may improve plant performance over existing mud-sinking approaches, which may enhance restoration efforts in degraded aquatic ecosystems.

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