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Purification of Micro-Polluted Lake Water by Biofortification of Vertical Subsurface Flow Constructed Wetlands in Low-Temperature Season

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Abstract: In this study, a novel lab-scale biofortification-combination system (BCS) of *Oenanthe javanica* and *Bacillus series* was developed to improve the treatment ability of vertical subsurface flow constructed wetlands (VSFCW) at low temperatures (0–10 °C). The results showed that BCS-VSFCW overcame the adverse effects of low temperature and achieved the deep removal of nutrients. In addition, the removal rates of chemical oxygen demand (COD), ammonia nitrogen (NH_4^+-N), total nitrogen (TN), and total phosphorus (TP) by BCS-VSFCW were 38.65%, 28.20%, 18.82%, and 14.57% higher than those of blank control, respectively. During the experiment, *Oenanthe javanica* and low temperature tolerant *Bacillus* complemented each other in terms of microbial activity and plant uptake. Therefore, VSFCW combined with *Oenanthe javanica* and low temperature tolerant *Bacillus* has a promising future in low temperature (<10 °C) areas of northern China.

Keywords: vertical subsurface flow constructed wetlands (VSFCW); low temperature season; *Oenanthe javanica*; microorganism; biofortification

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1. Introduction

Eutrophication in micro-polluted lake ecosystems has an increasing trend worldwide due to unreasonable anthropogenic activities [1]. Constructed wetlands (CWs) are an efficient, economical, and eco-friendly sewage purification technology, especially suitable for the treatment of micro-polluted lake and river water [2–4]. Among these types of wetlands, the vertical subsurface flow constructed wetlands (VSFCW) require less area and strong oxygen transfer capability and have been widely used in the treatment of micro-polluted lake water [5,6].

The existence and activity of plants and microorganisms are important for VSFCW [7,8], which can improve their effectiveness in the treatment of wastewater [9,10]. VSFCW are considered to be a physical, chemical and biological process to remove pollutants, including matrix adsorption and precipitation, plant absorption and microbial degradation. Studies have shown that plants play an important role in removing nitrogen, phosphorus, and heavy metals from sewage [11]. In addition, plants can improve the porosity of matrix, increase oxygen transfer, promote the growth of matrix microorganisms, and provide attachment sites for microorganisms [12,13]. Moreover, the pollutants in micro-polluted lake water were degraded by complex microbial community in wastewater through its assimilation and proliferation, which improved the overall treatment performance of the VSFCW [14]. However, in the low-temperature season, some aquatic plants have difficulty in adapting to low temperature, and microbial activity is reduced, leading to a reduced

removal efficiency of VSFCW [15,16]. Species such as *Phragmites australis* and *Typha orientalis* are commonly used in VSFCW for an improved performance in the warmer environment; however, in the low-temperature season, these species would deteriorate or wither [17]. The activities of most microorganisms in VSFCW are weakened under low-temperature conditions and only a few microorganisms are able to maintain normal life activities [18]. Consequently, the treatment ability of VSFCW in the low-temperature season is poor.

Biofortification is an effective method to improve the removal efficiency of VSFCW pollutants in the low-temperature season [19]. A type of biofortification seeks improvement of the removal of pollutants from CWs by planting cold-resistant aquatic plants or adding low-temperature resistant microorganisms with specific functions [20,21]. Currently, most studies on the treatment effect of bio-enhanced constructed wetlands in low-temperature season focus on the screening of wetland plants. These studies also confirm the feasibility of biofortification as a method to improve the treatment of wastewater in VSFCW at low temperatures [22–24]. Bioaugmentation through the addition of inoculated microbial is an excellent method [25]. The screening and application of low temperature tolerant microorganisms in VSFCW are less studied. Existing studies only discuss the effect of low temperature resistant microorganisms on the treatment efficiency of VSFCW [26].

Oenanthe javanica is an aquatic vegetable that can grow in low-temperature conditions and has edible value [27]. Wang et al. [28] planted *Oenanthe javanica* in constructed floating wetlands to improve the nitrogen removal performance at low temperature. The results demonstrated the positive role of *Oenanthe javanica* on nitrogen removal from wastewater during the cold season. However, studies on the synergistic effect of *Oenanthe javanica* and low temperature tolerant microorganisms on the purification of micro-polluted lakes in VSFCW during the low-temperature season have not been reported. The behavior of *Oenanthe javanica* and microorganisms in VSFCW is not fully understood.

In the present study, a laboratory-scale vertical subsurface flow constructed wetlands was established to plant cold-tolerant *Oenanthe javanica* and add high-efficiency composite microbial preparations in a low-temperature season (0 °C–10 °C). The main purpose of the study was to evaluate the treatment effect of *Oenanthe javanica* and other added microbial agents on VSFCW in low-temperature season, and to explore the effect of microbial addition on the microbial community structure of VSFCW. The results of this study are of significant implications for the continuous and stable operation of VSFCW in the low-temperature season.

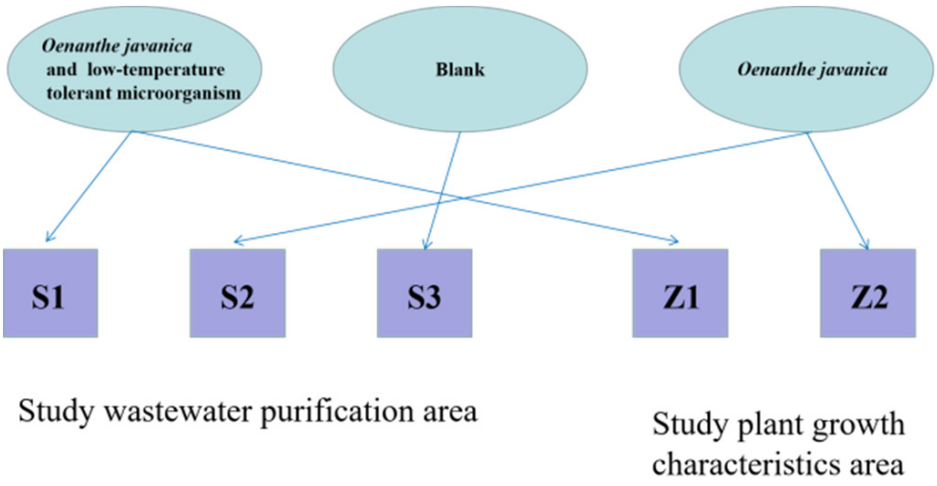
2. Materials and Methods

2.1. Experimental Wetland System

2.1.1. Design and Operation of Wetland

The experimental site was established at a transparent rain shelter at the Zhengzhou University, Northern China (112°42′–114°14′ E, 34°16′3–4°58′ N). The selected *Oenanthe javanica* seedlings were purchased from the Anqing Aquatic Vegetable Research Institute. The micro-wetland systems were made of plastic with a subsurface vertical flow style. The nutrient removal efficiency and plant growth characteristics of VSFCW were studied in two groups (Figure 1a). The first group (S1–S3) was used to study the effect of water treatment. The groups of S1, S2, and S3 were set up as combination of *Oenanthe javanica* and microbial agents, just the *Oenanthe javanica* without microbial agents, and just microbial agents without *Oenanthe javanica*, respectively. The second group (Z1–Z2) was used to study the growth characteristics. Group Z1 and Z2 were divided into *Oenanthe javanica* and microbial agents and *Oenanthe javanica* without microbial agents, respectively. The general layout of the experimental facility is shown in Figure 1b and the schematic cross section of the VSFCW unit is presented in Figure 2b. The VSFCW unit is 50 cm long, 36 cm wide, and 25 cm deep. The VSFCW unit uses gravel of 1.5–2.0 cm diameter as supporting medium and its depth is 4 cm to prevent blockage at the bottom of PVC pipe. These units were filled with a composite filler as a matrix to a depth of 20 cm. The composite fillers were composed of round ceramsite, blast furnace-granulated slag, soil, and sawdust with a

volume ratio of 3:3:2:1. The effective particle size (D10) of round ceramsite is 0.8–1.5 cm, and the particle size of the blast furnace slag is 2.0–3.0 mm. The soil was taken from the CWs of Xinmi City (Xinmi, China). To improve the accuracy of this study, three replicates were operated per microcosm from September to November for approximately 3 months. The theoretical hydraulic retention time was about 1.5 days, the inflow rate of VSFCW was 5.56 mL/min, and about 8 L of lake water was added every day. The micro-polluted lake water used in the experiment was taken from Xiliu lake in Zhengzhou (Zhengzhou, China), and micro-polluted lake water was pumped into VSFCW units from tanks at the same flow rate through peristaltic pumps.



(**a**)

(**b**)

Figure 1. The general layout of the experimental facility (**a**); VSFCW treatment system (**b**).

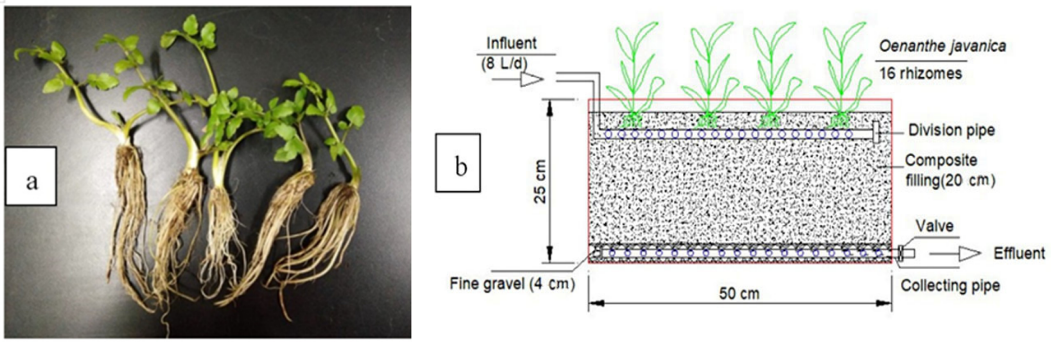


Figure 2. *Oenanthe javanica* plants (**a**); Schematic section of the VSFCW (**b**).

2.1.2. Plant Culture and Operation

In September, the whole plant of *Oenanthe javanica* (Figure 2a) with a basically similar biomass (the weight difference was less than 1 g, the height difference was less than 0.5 cm) was cultivated in a container with 10% Hoagland's solution after the roots had been washed to remove soil and dead plant tissue. After a month of acclimation, the plants were transplanted into VSFCW units (sixteen plants per unit).

2.1.3. Preparation of a Low-Temperature Resistant Microbial Agents

The added microorganisms are low-temperature resistant microorganisms isolated from the soil of the Xinmi City (Xinmi, China) CWs. In the preliminary experiment, they were screened and found to possess 99% similarity with *Bacillus* according to 16S rDNA sequencing and BLAST homology comparison. The low-temperature resistant microbial agents were composed of *Bacillus licheniformis*, *Bacillus cereus*, and *Bacillus amyloliquefaciens*, wherein the proportion of *Bacillus licheniformis*: *Bacillus cereus*: *Bacillus amyloliquefaciens* was 10:2:0.25.

The cultured microorganisms were expanded using a medium containing beef extract (1 g), glucose (5 g), NaCl (5 g), and yeast extract (10 g), added to distilled water, and the pH was adjusted to 7 (1 L). At the beginning of experiment, the surface of VSFCWs were sprayed with microbial liquid (800 mL). The concentration of microflora was 50×10^8 cfu/mL. The VSFCW treatment system is shown in Figure 2b.

2.2. Sample Collection and Analytical Methods

2.2.1. Water Sampling and Analysis

The actual micro-polluted lake water was continuously injected into the constructed wetlands by peristaltic pump, the average CODcr, NH_4^+-N, TN, and TP were 40 ± 6 mg/L, 2.8 ± 0.4 mg/L, 3.7 ± 0.5 mg/L, and 0.3 ± 0.09 mg/L, respectively. To check the efficiency of sewage treatment, the samples were collected from the inlet and outlet of the VSFCW from 9 a.m. to 11 a.m. during the day. All samples were analyzed for chemical oxygen demand (COD), total nitrogen (TN), ammonia-nitrogen (NH_3-N), and total phosphorus (TP), according to the Standard Methods for testing water and wastewater (APHA, 2005). Water temperature and pH were measured with a portable analyzer (HQ30d53LEDTM, HACH, Loveland, CO, USA) in the field [29].

2.2.2. Plant Monitoring and Analyses

Every seven days, three plants of *Oenanthe javanica* in the VSFCW units Z1 and Z2 were measured in the laboratory. Plants removed from biomass testing sites were replaced by cultured plants, which were planted in other units and cultured under the same nutrient conditions. The extracted plants were washed with tap water to remove attached precipitates, then rinsed with distilled water and dried with absorbent paper. The maximum root length and plant height were determined. At the end of the experiment, the

changes in plant growth characteristics and biomass with net growth were calculated by subtracting the initial measurements from the measured value.

2.2.3. Soil Microbial DNA Extraction and High-Throughput Sequencing Analysis

Twenty days after the beginning of the experiment, the soil samples (5 cm deep) were collected from S1 and S2 units and labeled A1 and B1, respectively. With the end of wetland system, soil samples from units S1 and S2 were collected and labeled A2 and B2, respectively. Genomic DNA was extracted from soil subsamples using a Mobio Power Soil DNA isolation kit, and each sample was submitted to 1% agarose gel electrophoresis. PCR amplification was carried out with primers for the V4 region of the 16S rRNA gene. The PCR amplification was carried out in a 50 µL reaction system. The thermal cycle included initial denaturation at 95 °C for 5 min, followed by denaturation at 94 °C for 60 s, annealing at 57 °C for 45 s, and extension at 72 °C for 60 sec each of 34 cycles. Finally, it was kept at 72 °C for 10 min then stored at 10 °C. The amplification products of PCR were recovered and quantified by FTC-3000 TM real-time PCR [30]. MiSeq sequencing, sequence splicing, and classification to operational taxon units (OTUs) were performed by professional commercial sequencing companies (NoVogene, Beijing, China).

2.3. Data Analysis

Operational taxonomic units (OTUs) were characterized at 97% sequence identity using the Usearch software. Alpha-diversity indices including Chao, ACE, and Shannon were calculated by Mothur analysis at a 3% distance level. Results reported in triplicate ± standard error were displayed in tables and graphs. Statistical analysis was performed using the statistical program SPSS 22 (SPSS Inc., Chicago, IL, USA). The charts were produced with Origin 2017.

3. Results and Discussion

3.1. Plant Growth Characteristics and Biomass Changes

After the seedlings were transplanted, the original stems and leaves turned yellow and withered, and the new stems and leaves gradually grew out. In the subsequent experiments, plant growth tended to be stable. Two weeks after the plants were adapted to the environment, the physiological characteristics of the plants were determined on days 14, 21, 28, 35, 42, and 49. Plant growth characteristics are expressed by the trend of total length and net growth of plants at each stage, as shown in Figure 3a,b. *Oenanthe javanica* continued to grow slowly throughout the experimental period. The total length change and net growth of the plants in the Z1 unit were larger than those in the Z2 unit.

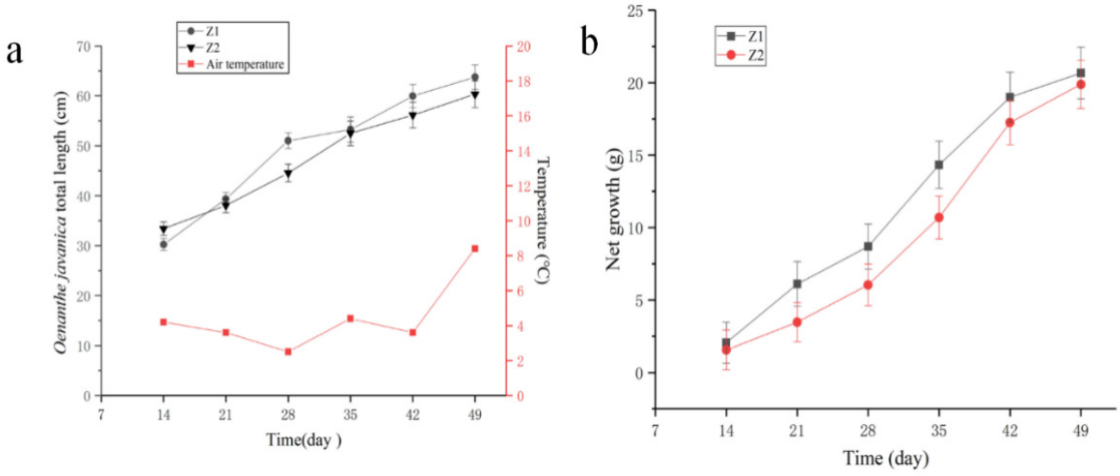


Figure 3. Variations of total length (**a**), net growth (**b**) of *Oenanthe javanica* in the different VSFCW units (Z1: added microorganisms unit; Z2: no added microorganisms unit).

At lower temperatures, *Oenanthe javanica* grows more slowly because the plant's growth is affected by temperature, light, and nutrients. In winter, the light duration is shorter, the temperature is lower, so the enzyme activity in plants decreases, and the metabolic ability of plants is decreased [31]. The optimum growth temperature for *Oenanthe javanica* is 20–25 °C [32], but during the experiment, the temperature was kept below 10 °C. Although *Oenanthe javanica* grows slowly in winter, it still has the advantage of resistance to cold compared to other plants commonly used in constructed wetlands. Consequently, *Oenanthe javanica* can be used in constructed wetlands in low-temperature areas. As shown in Figure 3a,b, plants in the Z1 unit grew better than those in Z2. This may be due to the promotion by the microbes added during the experiment. In the experimental operation, low-temperature resistant microorganisms screened from the soil were added to the Z1 unit. The soil of the constructed wetland system contains water, air, and various nutrients needed by microorganisms for growth and development, so that the microorganisms can maintain excellent assimilation and proliferation ability. The metabolites produced by microorganisms in soil can promote the growth and development of plants [33,34], and microorganisms in the soil can secrete some trace secondary metabolites, including plant hormones [35,36]. Phytohormones can improve the absorption of nutrient elements in plants to a large extent, thus promoting plant growth. Preliminary identification experiments showed that the microorganisms that were added in the experiment might be *Bacillus*. *Bacillus* has the ability to promote plant growth and to a certain extent, inhibit the reproduction of harmful bacteria and reduce the damage caused by pathogenic bacteria [37,38]. Therefore, the addition of appropriate microorganisms can effectively promote the growth of plants at low temperatures. In the current study on the use of microorganisms to enhance the low-temperature seasonal treatment effect of constructed wetlands, the purification performance of microorganisms on water quality under low-temperature conditions is important [15]. In future studies, consideration should be given to screening for low-temperature resistant microorganisms that support good water purification performance and promote plant growth.

3.2. Wastewater Purification Performance of Wetland System

The water temperature during the study was below 10 °C, and the effluent water temperature of each unit was similar to the influent water temperature and air temperature (Figure 4a). The influent pH of the VSFCW unit was 7.6 ± 0.2, and the effluent pH values of the S1, S2, and S3 units were similar, 8.1 ± 0.2 (Figure 4b). This may be caused by the hydrolysis of metal ions in slag and ceramsite in the VSFCW filler, a similar phenomenon appears in the relevant literature [39,40].

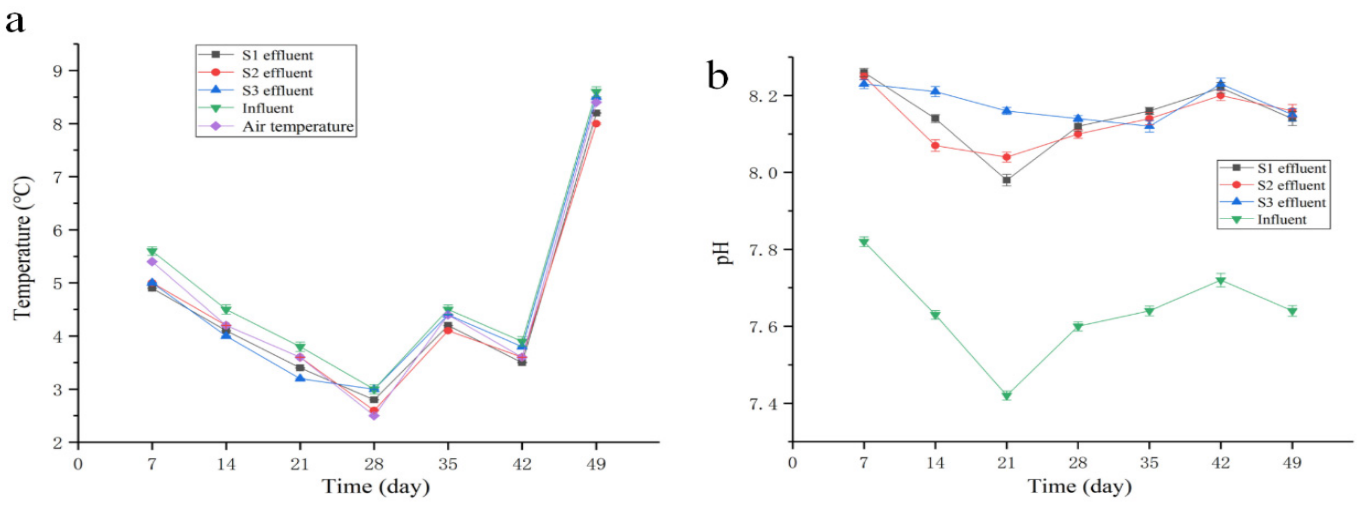


Figure 4. *Cont.*

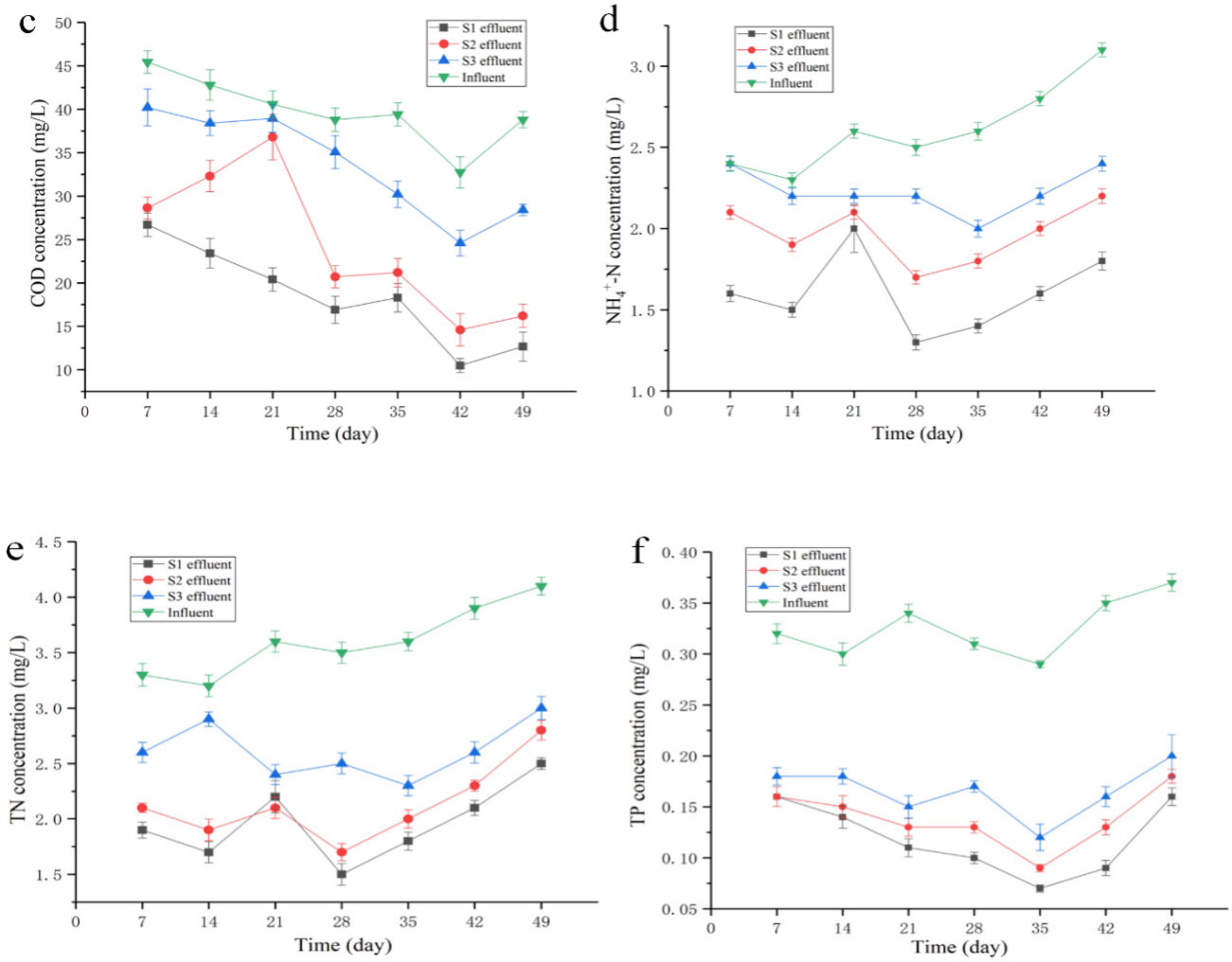


Figure 4. Influent and effluent temperature in S1–S3 (**a**); influent and effluent pH in S1–S3 (**b**); influent and effluent COD concentrations in S1–S3 (**c**); influent and effluent NH_4^+-N concentrations in S1–S3 (**d**); influent and effluent TN concentrations in S1–S3 (**e**); influent and effluent TP concentrations in S1–S3 (**f**).

The influent and effluent concentration of COD in the VSFCW during the monitoring period are shown in Figure 4c. The COD concentration of the influent fluctuated slightly during the study period, about 39.43 mg/L. The average removal efficiencies of COD for the S1, S2 and S3 were 54.17%, 39.20%, and 15.52%, respectively, and the average COD effluent concentration were 18.41 mg/L, 23.34 mg/L, and 33.31 mg/L, respectively, which can comply with the Grade III (20 mg/L), IV (30 mg/L), and V (40 mg/L) of *Environmental Quality Standards for Surface Water* (GB3838-2002) in China. The results show that there was a significant difference in the removal ability of COD between plants and microorganisms in the VSFCW. This is because the removal of COD in constructed wetlands mainly depends on plants and microorganisms than the substrates [29,41]. In addition, comparing the COD removal effects of S2 and S3 units, it was revealed that planting *Oenanthe javanica* can effectively improve the COD treatment ability of VSFCW. This may be due to the absorption and transformation of organic matter by *Oenanthe javanica*, which promotes the life activities of substrate microorganisms [42]. Microbial inoculant was added to the S1 unit during the experiment, and the effluent COD of S1 unit and S2 unit were compared; it was found that adding microorganisms promoted the COD removal effect of the VSFCW. This is because the addition of microbial agent promotes the growth of the *Oenanthe javanica*, improving the treatment ability of the COD. Although the laboratory is operated under low-

temperature conditions, the COD purification effect of the VSFCW with *Oenanthe javanica* and the microorganisms was better than that of some constructed wetlands treated in warm season reported previously [29,43], and similar reports also appeared in this literature [44].

As illustrated in Figure 4d, when the influent NH_4^+-N concentration ranged from 2.4 mg/L to 3.2 mg/L, the average efficiency of S3 was 10.39%, while that of S1 and S2 were 38.59% and 24.21%, respectively. The average effluent NH_4^+-N concentration of S1, S2, S3 were 1.45 mg/L, 1.97 mg/L, and 2.31 mg/L. Most of S1 and S2 complied with the Grade IV (1.5 mg/L) and V (2 mg/L) of the *Environmental Quality Standards for Surface Water* (GB3838-2002) in China, respectively. The main mechanism of NH_4^+-N removal in constructed wetlands might be related to the absorption of plants, substrates, and the action of microorganisms. However, the life activities of microorganisms are affected by temperature, and the temperature above 15 °C is beneficial to the NH_4^+-N removal [41,45].

The TN removal capacity of the VSFCW was shown in Figure 4e. The influent TN concentration was stabilized in the range of 3.4–4.0 mg/L, and the average TN concentrations in the effluent of S1, S2, and S3 were 1.96 mg/L, 2.13 mg/L, and 2.61 mg/L, with average removal efficiencies of 45.79%, 41.04%, and 26.97%, respectively. The results show that the removal effect of TN by planting *Oenanthe javanica* and adding microorganism agent was much better than that of the blank control group. TN removal rates were consistent with the reported results [16,29]. Nitrification and denitrification are the main processes of nitrogen removal. Studies have confirmed that anammox is also one of the mechanisms of nitrogen removal in constructed wetlands [41,46]. In addition, changes in NH_4^+-N and TN, as well as changes in microbial communities in S1, S2, and S3, indicated that the addition of *Oenanthe javanica* and microorganism agent could improve nitrification significantly. Ammonia is oxidized to NO_3^-, it is difficult to convert to N2 due to the VSFCW not being able to provide a suitable oxygen environment, while aquatic plants can better compensate for this defect by adsorption NO_3^- [29,41].

The TP removal performance of the VSFCW is illustrated in Figure 4f, the average influent TP concentration was 0.33 mg/L; the average effluent TP concentrations were 0.10 mg/L (S1), 0.14 mg/L (S2), and 0.17 mg/L (S3), which complied with the Grade II (0.1 mg/L) and III (0.2 mg/L) of *Environmental Quality Standards for Surface Water* (GB3838-2002). The average removal efficiencies of TP for the S1, S2, and S3 were 63.66%, 57.77%, and 49.09%, respectively. The results show that *Oenanthe javanica* and low-temperature resistant *Bacillus* to TP removal efficiency in VSFCW is limited under low-temperature conditions. These results confirmed that the main pathway of TP removal in VSFCW is physicochemical action of the matrix, including adsorption, coprecipitation, ion exchange, and ligand exchange [6,47,48]. This phenomenon is also reflected in similar literature [16]. Furthermore, the growth of *Oenanthe javanica* was maintained during the experiment, which enhanced the removal of TP in VSFCW. At the same time, since the life activities of microorganisms require a certain phosphorus level, the removal efficiency of TP in VSFCW was also slightly improved after the addition of low-temperature tolerant microorganisms.

3.3. Microbial Community Characteristics

A 16S rDNA gene sequencing process was carried out to assess the effects of added microorganisms and low-temperature on microorganisms in VSFCW, and to gain insight into the microbial community structure changes in VSFCW.

According to 97% similarity, OTUs, ACE, Chao, and Shannon of four samples were obtained by high-throughput sequencing (Table 1). A total number of 111,713, 105,164, 105,501 and 116,209 sequences were obtained for A1, A2, B1, and B2 respectively. The highest operational taxonomic units (OTU) number of 3753 was observed in the A2 unit, while the lowest found was 3389 in the B2 unit. The OTUs in A1 and B1 were 3648 and 3389, respectively. The number of OTUs can reflect the diversity of sample microbial communities. Thus, the diversity of microbial communities in the S1 group of VSFCW was improved under low-temperature conditions. In the S2 group, the microbial community diversity was reduced by the influence of the low temperature. The ACE and Chao indices

are commonly used to assess the abundance of species in a microbial community. The two indices of original soil (A1, B1) were not much different. However, after the operation of the VSFCW with microbial addition in A2, the two indicators were higher than in A1; the artificial wetland without the added microbial group operated for a period of time (B2), and both indices were lower than in B1. The Shannon Diversity Index not only reflects the richness of species but also how the abundance of species is distributed. The Shannon indices of A1, A2, B1, and B2 were 9.50, 9.79, 9.57, and 9.06, respectively. The ACE, Chao, and Shannon index all showed that the order of richness and diversity of four samples is A2 > B1 > A1 > B2.

Table 1. Bacterial diversity of sample A1, A2, B1 and B2.

Sample ID	Sequences	OTUs	ACE	Chao	Shannon
A1	111,713	3648	3672.31	3651.90	9.50
A2	105,146	3753	4988.66	4988.66	9.79
B1	105,501	3604	3569.52	3569.52	9.50
B2	116,209	3389	3354.47	3354.47	9.06

The rarefaction curve shows whether the total microbial population in the sample can be fully reflected in high-throughput sequencing. If the end of the scarcity curve becomes more gradual, the total biopopulation measured will be close to the actual sample biomass [49]. The end of the curve obtained by the experimental sequencing approached a gradual trend, indicating that the total number of populations in this sequencing is close to the actual sample biomass (Figure 5). According to the rarefaction curve, it can be concluded that the biodiversity of sample A2 is higher than in the other three groups. Under low-temperature in winter, the life activities of microorganisms are affected, so the richness and diversity of B2 are lower than that of B1. However, due to the application of low-temperature resistant microorganisms to the S1 unit, the abundance and diversity of the A2 sample is higher than that of the A1. Therefore, addition of low-temperature resistant microorganisms can increase the richness and diversity of microbial communities in constructed wetlands.

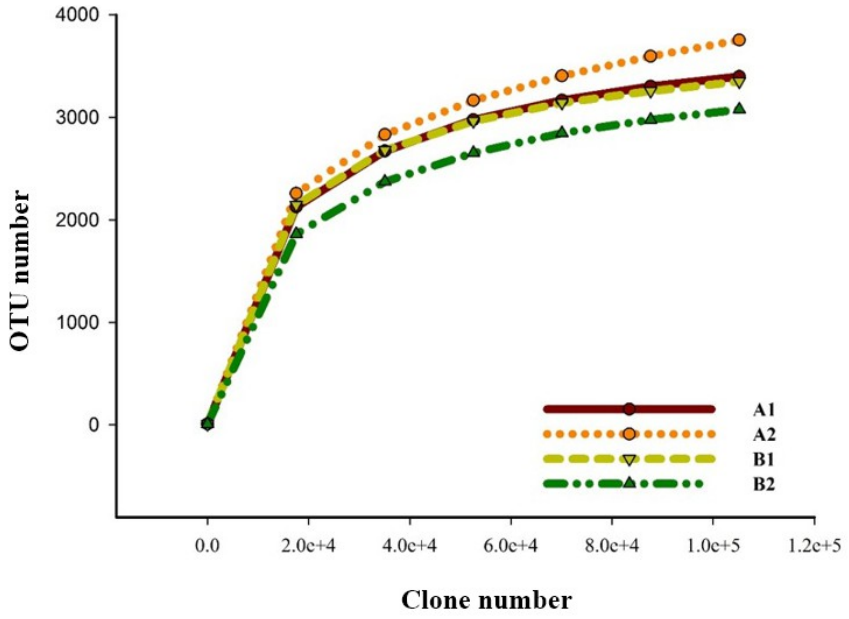


Figure 5. Rarefaction analysis of OTUs (operational taxonomic units).

A phylogenetic tree was constructed with four samples based on 97% homology (Figure 6), it can be seen that the A2 and B1 samples have the highest homology, while other

three samples have the lowest homology with B2. Since the microorganisms added during the experiment were obtained by sieving from the wetland soil, the homology of A2 sample was closer to A1 and B1. B2 is a sample of wetland that has been running continuously for a long time under low-temperature conditions, and the activity of microorganisms is reduced under low-temperature conditions, so the homology of B2 samples is quite different from the other three samples.

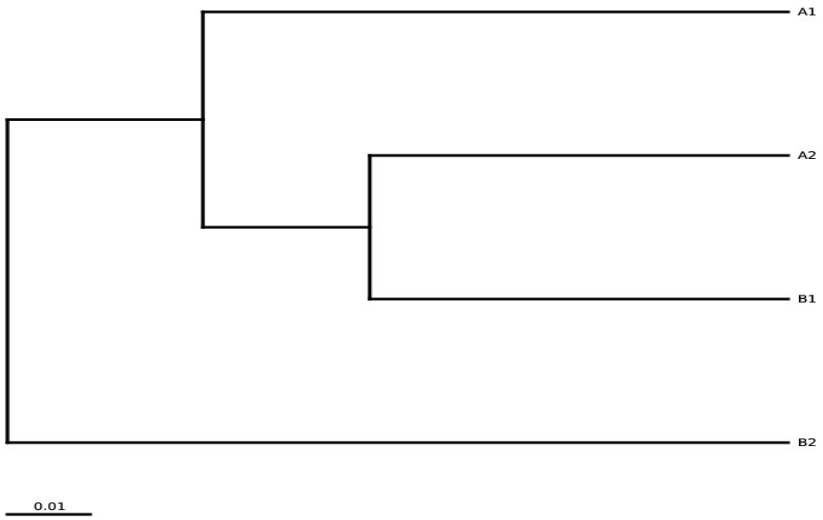


Figure 6. A taxonomic tree showing the bacteria isolation from soil samples.

Comparison of the sequences clustered into OTUs from each library was performed to determine the proportions of the bacterial populations that are shared among the A1, A2, B1, and B2 sites. A Venn diagram of the microbial community were drawn according to the OTUs of each of the four samples (Figure 7). The Venn diagram can visually represent the similarity of species between different samples [50]. The 2173 OTUs represented were shared among all the sites, accounting for 48% of the total number of OTUs (4494). The same OTUs of A1 and A2, and B1 and B2 were 2977 and 2555, respectively. Thus, after running for a period of time under low-temperature conditions, the similarity of the biomes in the S1 group was higher than that in the S2 group and adding low-temperature resistant *Bacillus* may promote the tolerance of wetland microorganisms in low temperatures.

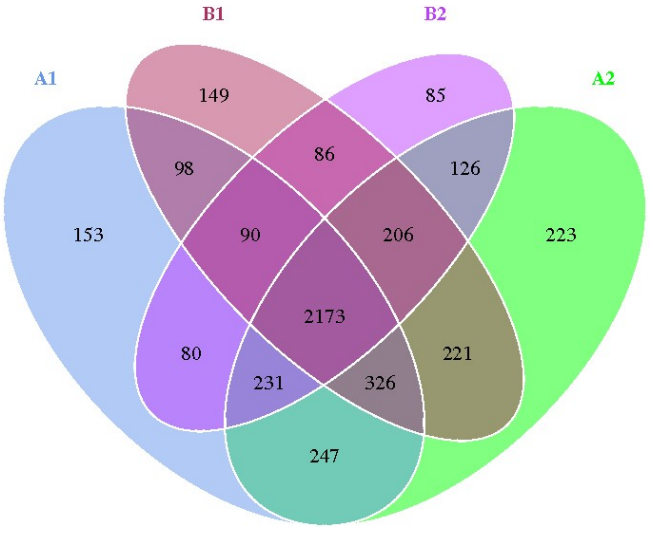
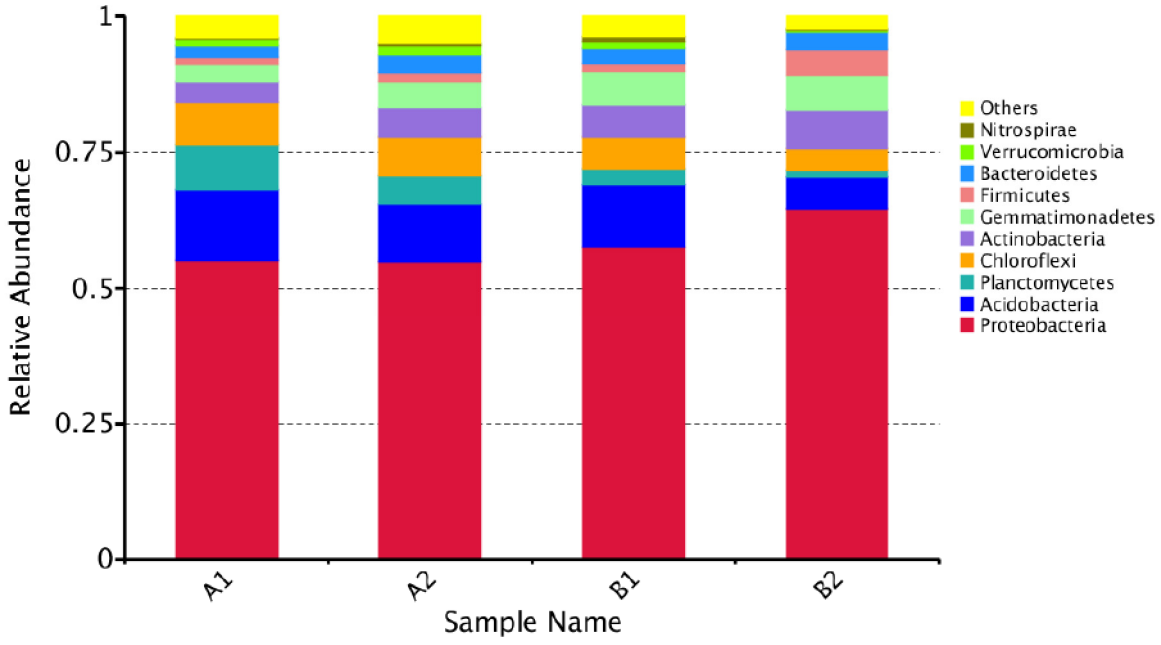
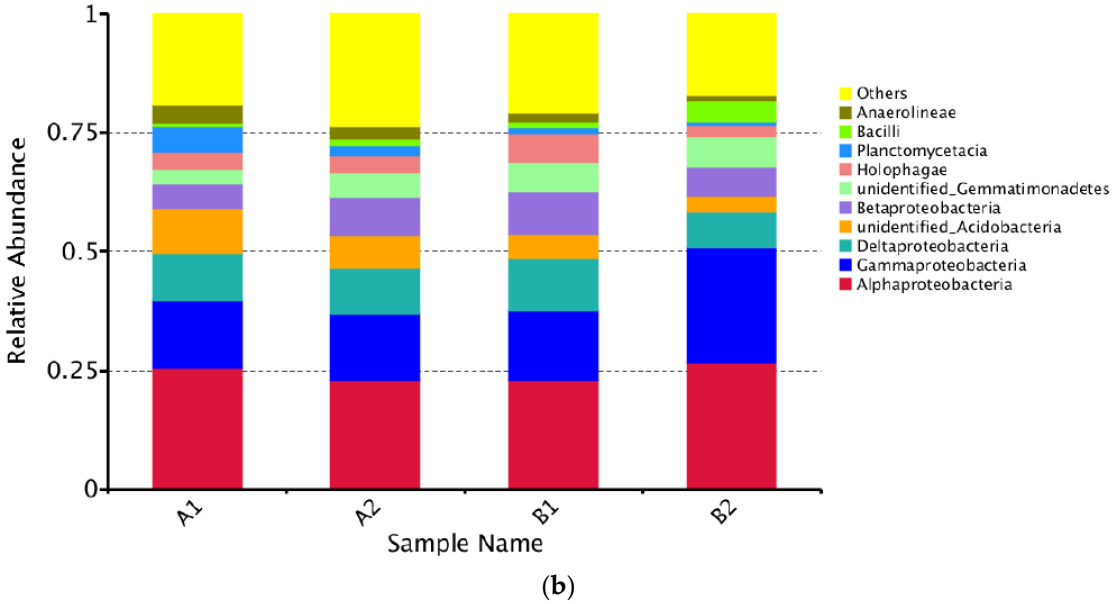


Figure 7. Venn diagram of OTU numbers of different soil samples.

The microbial community structure was analyzed at the level of phylum taxonomy, and the top 10 levels of samples were selected for analysis (Figure 8a). The abundance of Proteobacteria in the four samples A1, A2, B1, and B2 was the highest at 55%, 55%, 58%, and 65%, respectively, followed by the Acidobacteria with the abundance was 13%, 11%, 11%, and 6% respectively. The microbial community structure was analyzed at the class taxonomy level, and samples of the top 10 levels were selected for analysis (Figure 8b). The abundance of *Alphaproteobacteria* was the highest in the four samples A1, A2, B1, and B2, which were 26%, 23%, 23%, and 27%, respectively, followed by *Gammaproteobacteria*, which were 13%, 11%, 11%, and 6%, respectively.



(**a**)



(**b**)

Figure 8. Relative abundance at phylum level (**a**); relative abundance at class level (**b**).

Proteobacteria is the most common of the Phylum bacteria, including many nitrogen-fixing microorganisms and some pathogenic bacteria [51]. The relative abundance of

Proteobacteria in constructed wetlands was the highest, which was consistent with the experimental results [52–54]. The Proteobacteria are important bacterial flora which promote contaminant removal in water treatment [55]. In the experiment, the proportion of Proteobacteria in constructed wetlands without microorganisms increased gradually after a period of operation, which may be caused by the low-temperature resistance of Proteobacteria. However, the proportion of Proteobacteria did not change after a period of operation in constructed wetlands with low-temperature resistant microorganisms, which may be due to the influence of *Bacillus*, and Yavuztürk Gül et al. [56] also believed that *Bacillus* had a greater effect on the relative abundance of Proteobacteria. Acidobacteria is found widely in the soil and is abundant and may be an important contributor to the ecosystem (Eichorst et al. 2007). After running for a period of time, the decrease rate of *Acidbacillus* ratio in the constructed wetlands without microorganism addition was higher than that in the constructed wetlands with microorganism addition. This may be due to the influence of *Bacillus* on the life activities of Acidobacteria under low temperatures, thus changing the relative abundance of Acidobacteria.

Alphaproteobacteria play an important ecological function in VSFCW [55]. The relative abundance of *Alphaproteobacteria* in S1 and S2 did not change significantly. According to Figure 8b, the changing trend of *Gammaproteobacteria* in two groups of constructed wetlands further proves that *Bacillus* has a certain influence on the change of the microbial community in VSFCW. The relative abundance of *Gammaproteobacteria* increased with the stable operation of the system in S2, but the group of S1 remained unchanged. The *Gammaproteobacteria* include several medically and scientifically important groups of bacteria, such as the *Enterobacteriaceae*, *Vibrionaceae*, and *Pseudomonadaceae* [57]. Many important pathogens belong to this class, and include *Salmonella* spp., *Yersinia pestis*, *Vibrio cholerae*, and *Escherichia coli*. Plant pathogens such as *Xanthomonas axonopodis* pv. and *Pseudomonas syringae* pv. *Actinidiae* [58] are also *Gammaproteobacteria*. Therefore, the inhibition of *Bacillus* against harmful pathogens reduced the relative abundance of *Gammaproteobacteria* in the system.

According to Figure 8a,b, after the addition of *Bacillus*, the low temperature resistant *Bacillus* did not become the dominant species, but the purification effect of the constructed wetlands in the micro-polluted lake water was improved, and the microbial community structure of the constructed wetlands changed greatly. This indicates that the added microorganisms do not directly remove the pollutants through their own growth and reproduction but enhance the purification effect of the constructed wetlands by affecting the life activities of other microorganisms. The life activity of microorganisms is a complex process, and the purification effect of microorganisms should not be considered only for the purpose of adding microorganisms to improve the low-temperature purification effect of constructed wetlands. The addition of low-temperature-resistant *Bacillus* sp. in the experiment may promote the growth of microorganisms beneficial to sewage treatment and inhibit the life activities of pathogenic bacteria, thereby improving the purification effect of constructed wetlands.

4. Conclusions

Oenanthe javanica and low-temperature tolerant microbial agents in VSFCW have a high contribution rate to the treatment of micro-polluted lake water in low-temperature conditions (0–10 °C). Under the combined enhancement of plants and microorganisms, the removal rates of COD, NH_3-N, TN, and TP in the BCS-VSFCW were 38.65%, 28.2%, 18.82%, and 14.57% higher than those of the blank control, respectively. Screening suitable low temperature tolerant microorganisms can increase the diversity and abundance of microbial communities in VSFCW and inhibit the pathogenic bacteria to a certain extent. The synergistic effect of *Oenanthe javanica* and low-temperature tolerant microorganisms on the removal of pollutants in VSFCW was excellent compared to that in the control group. Therefore, the combination of *Oenanthe javanica* and low temperature tolerant *Bacillus* has a good prospect to enhance the low temperature treatment effect of VSFCW in the lake micro-polluted lake water.

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