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Novel Stepped Combined Constructed Wetland For Surface Water Removal: Enhancing the Performance and Responses of Microbial Communities

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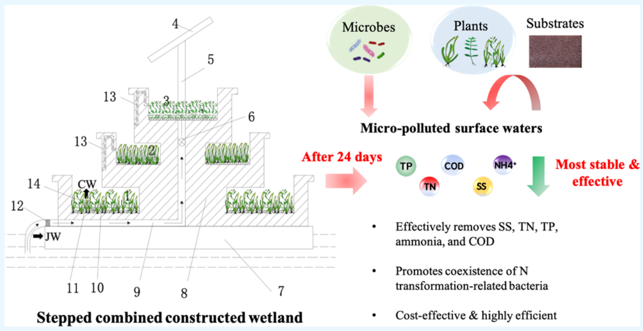
ABSTRACT: Micro-polluted surface waters (MPSWs) draw increased concern for environmental protection. However, traditional treatment methods such as activated sludge, ozone activated carbon, and membrane filtration suffer from high cost and susceptibility to secondary pollution and are rarely used to address MPSWs. Herein, a new stepped combined constructed wetland planted with *Eichhornia crassipes* without additional inputs was developed. In a 60-day experiment conducted in a drainage canal, we evaluated contaminant removal and bacterial communities using laboratory analysis and amplicon sequencing. Our results showed that the stepped combined constructed wetland achieved impressive removal rates for various contaminants. It was able to remove 70% of suspended solids, 51% of total nitrogen, 55% of total phosphorus, 70% of ammonia nitrogen, and 64% of the chemical oxygen demand. The dominant bacterial phyla found in stepped combined constructed wetland was Proteobacteria and Actinobacteria, with average relative abundances of 43.4 and 19.9%, respectively. We also observed clear differences in bacterial genera between the influent and effluent water samples. Specifically, we found that the stepped combined constructed wetland significantly reduced the abundance of bacteria such as *hgcl clade*, *Rhizorhapis*, and *Cyanobacteria*, while increasing the abundance of bacteria like *Flavobacterium*, *Limnohabitans*, *Alpinimonas*, *Erwinia*, and *Saccharibacteria*. The dominant bacterial community comprised nitrifying bacteria (*Azoarcus* and *Nitrospira*), denitrifying bacteria (e.g., *Mycobacterium*, *Paracoccus*, *Ralstonia*, *Rhodobacter*, *Escherichia Shigella*), and nitrogen-fixing bacteria (*Rhizobium*, *Bradyrhizobium*, *Azospirillum*). Notably, the abundance of nitrogen-fixing bacteria, nitrite-oxidizing bacteria, and denitrifying bacteria increased with the stepped combined constructed wetland presence. The stepped combined constructed wetland technology is highly cost-effective, with a total investment of only 259.83 USD. The majority of this investment is used for construction, with minimal expenditure required for operation and maintenance. Therefore, the stepped combined constructed wetland presents an economical and environmentally friendly solution for pollutant removal in slow flow and still water areas. It offers numerous benefits, including improved pollutant removal efficiency, low cost, ecological advantages, and extensive development potential, in various fields.



1. INTRODUCTION

A growing number of polluted surface waters were defined as micro-polluted surface waters (MPSWs) as a result of growing awareness and concern for environmental protection,[1] including rivers, lakes, reservoirs, streams, canals, and ponds. Compared to domestic wastewater and industrial wastewater, MPSWs contained relatively low levels of pollutants such as nitrogen, phosphorus, and organic matter, with most less than 10 mg/L of nitrogen, phosphorus, and organic matter.[2] A traditional treatment method for water, such as flocculation and sedimentation, activated sludge, ozone activated carbon, photocatalytic oxidation, and membrane filtration, had drawbacks such as high cost, membrane obstruction, and susceptibility to secondary pollution and was rarely used to address MPSWs. The constructed wetland was low-cost, had high efficiency and environmental friendliness, possessed an excellent *in situ* remediation capacity, as well as aesthetic and economic value. Currently, constructed wetland were widely applied in China,[3,4] United States,[5] Japan, The Netherlands,[6] and Australia.[7]

Island plants influenced the efficacy of nutrient assimilation and created favorable conditions for organic matter degradation. By taking nitrogen and phosphorus from the aquatic environment, plants produced their cellular components and energy, and roots and foam carriers facilitated colonization of

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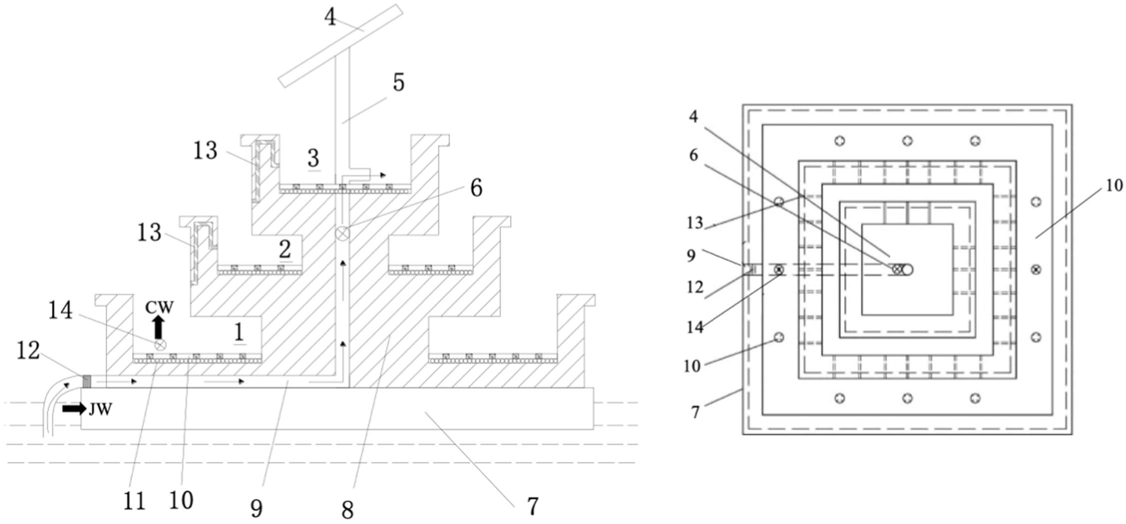

Figure 1. Design drawing of the stepped combined constructed wetland water purification system. (1) Bottom trough; (2) middle trough; (3) top trough; (4) solar power panel; (5) bracket; (6) pump; (7) light polystyrene foam board; (8) stepped combined constructed wetland; (9) water inlet port; (10) pearl foam mat; (11) ceramsite; (12) mesh screen filter; (13) siphon; and (14) a drainage device.

beneficial microorganisms by providing nutrients, oxygen, and habitat.[8] Floating island plants were classified as emergent plants, floating-leaved rooted plants, floating plants, and submerged plants.[9,10] In general, the capacity of plants to accumulate pollutants varied depending on the type of plant and pollutant.[11]*Eichhornia crassipes*was widely used in various environmental and climatic zones due to its robust roots and efficient pollutant sorption ability.[12,13]

A greater role was played by microorganisms in the reduction of nitrogen, phosphorus, and organic pollutants in the constructed wetland. As an example, microorganisms provided food for aquatic animals and plants and participated in the biogeochemical cycle such as C, N, P, and S, which effectively degraded organic pollutants.[14,15] Microbial communities were considered to be the primary pollutant removal mechanisms, but more research focused on constructed wetlands. Microbial communities in floating treatment wetlands in horizontal−vertical flow received relatively less attention, as most researchers were interested in plants and their effluent removal mechanisms. Colares[15] used VOSviewer to analyze 396 publications from floating treatment wetlands; the most common topics mentioned were macrophytes, growth, and nutrient removal. To better understand the ecological functions performed by microorganisms in a new horizontal−vertical flow constructed wetland, we used amplicon sequencing to examine the bacterial community composition in influent and effluent samples in the present invention.

We investigated the following aspects: (1) the efficacy of stepped combined constructed wetland in removing pollutants, (2) bacterial community characteristics in stepped combined constructed wetland influent and effluent samples, especially differences concerning nitrogen-transforming microorganisms, and (3) the economic benefits of stepped combined constructed wetland. Our results will be helpful for this newly constructed wetland device, which is widely used in waters such as lakes, reservoirs, ponds, and artificial landscapes.

2. MATERIALS AND METHODS

2.1. Stepped Combined Constructed Wetland Construction. This stepped combined constructed wetland was composed of three clean water troughs (Figure 1). These troughs were installed sequentially from the bottom to the top.[16] The diameters of the troughs from top to bottom are 49.5, 80, and 121 cm. A light polystyrene foam board was fixed under the troughs to provide sufficient buoyancy. A solar power panel was installed on the top of the constructed wetland to provide energy to the pump. The top trough and the middle trough were respectively equipped with 2−4 outlet holes, and the outlets were fitted with latex tubes to form a siphon pipe. The mesh screen filter was located at the inlet port, and the depth of the water in the bottom trough was controlled by a drainage device. 6.5 cm tall*E. crassipes*were selected for the floating island experiment and planted in a pearl foam mat floating in the trough. One seedling per hole was planted in order to prevent plant mortality caused by high density. Additionally, a layer of ceramsite with a particle size of 5−20 mm was positioned between the planting mat and the bottom surface of the trough.

2.2. Sampling Sites and Design. The study was conducted from May to June 2022, over a 60-day period, with water samples collected three times daily at 10:00 am in triplicate. The stepped combined constructed wetland was located near a drainage canal at the Xuancheng campus of Hefei University of Technology (Anhui, China). The main sources of the sewage were domestic and kitchen sewage, which were discharged into the drainage canal by a drainage pump. Background samples were collected from the drainage canal with the sampling point located approximately 1 m from the stepped combined constructed wetland. During the experiment, the water inlet of the stepped combined constructed wetland was continuously filled with sewage from this drainage canal, and the treated water was discharged through the stepped combined constructed wetland outlet. Considering the relative stability of the system during the middle and late stages of the experiment, the sampling interval was extended from 4 to 8 days. JW and CW represented

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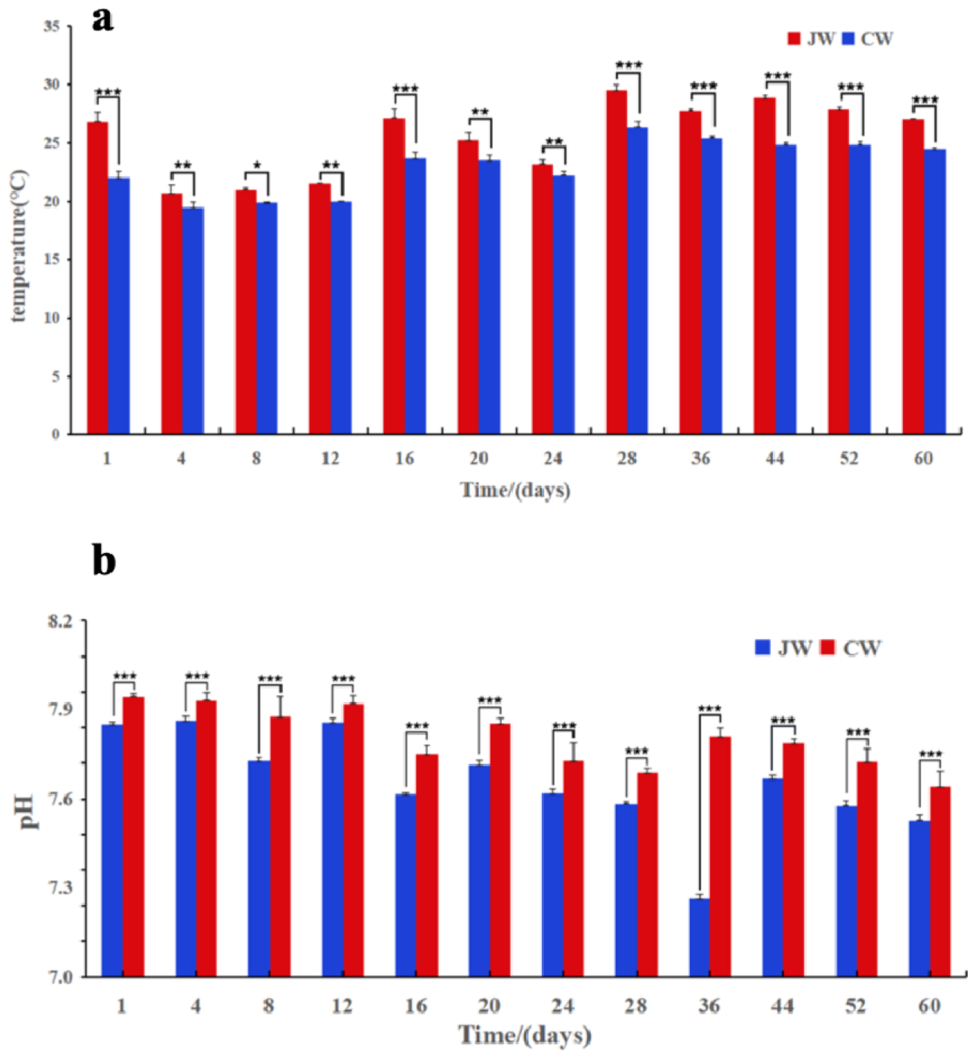

Figure 2. Temperature (a) and pH (b) of influent and effluent water of a stepped combined constructed wetland. JW represents the influent water, and CW represents the effluent water.

influent and effluent water, respectively, and the numbers indicated the sampling days (Figure 2).

2.3. Analysis of Physicochemical Characteristics. Samples were collected by using a 500 mL water sampler (WB-PM, Beijing Pulite Instrument Co. Ltd., China). 300 mL of water was used to measure water temperature (WT), pH, suspended solids (SS), total nitrogen (TN), total phosphorus (TP), ammonia nitrogen (NH_4^+-N), and chemical oxygen demand (COD). Water temperature was measured *in situ* with a glass-stem thermometer (Sycif, Shanghai, China). pH was measured by the glass electrode method; SS, TN, TP, NH_4^+-N, and COD were determined according to the standard methods of the State National Environmental Protection Administration of China.[17] Standard substances, certified reagents, and laboratory reagents were purchased from Sinopharm (Pharmaceutical Group Chemical Reagent Co. Ltd., China).

2.4. DNA Extraction, PCR Amplification, and Amplicon Sequencing. Approximately 200 mL samples were taken and filtered using a 0.22 μm membrane. DNA extraction was performed using the E.Z.N.A Water DNA kit D5525-01 (Omega Biotek, Norcross, Georgia). An amplification of DNA from 18 samples was amplified by primer set 338F (5′-ACTCCTACGGGAGGCAGCAG-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) to amplify the V3−V4 region of the 16S rRNA gene.[18] Using 1% agarose gel electrophoresis, band sizes of the samples were measured and stored at −20 °C. PCR products were purified using a DNA purification kit (BioFlux, Tokyo, Japan), and their concentrations were determined by spectroscopy with QuantiFluo-ST (Promega, Madison, Wisconsin). In Majorbio Biomedical Technologies' Illumina MiSeq platform (Illumina, San Diego, CA), amplicons were pooled at equimolar concentrations, and paired-end sequencing was performed following standard protocols.

2.5. Bioinformatics and Data Analysis. QIIME (version 1.9.1) was used to analyze the raw HiSeq sequencing data. Chimeric sequences were identified and removed using UCHIME. Using the RDP classifier algorithm (version 2.2 http://sourceforge.net/projects/rdp-classifier/), 16S rRNA sequences were analyzed against the Silva 16S rRNA database in order to cluster operational taxonomic units (OTUs) based on a 97% similarity threshold. Using MiSeq, we sequenced 18 samples of 16S rRNA genes from bacteria, resulting in 685,656 high-quality reads. Additionally, we selected 100 articles related to nitrogen-metabolizing microorganisms and screened for nitrogen-metabolizing bacteria therein as the basis for analyzing the abundance of nitrogen-metabolizing bacteria in the stepped combined constructed wetland.

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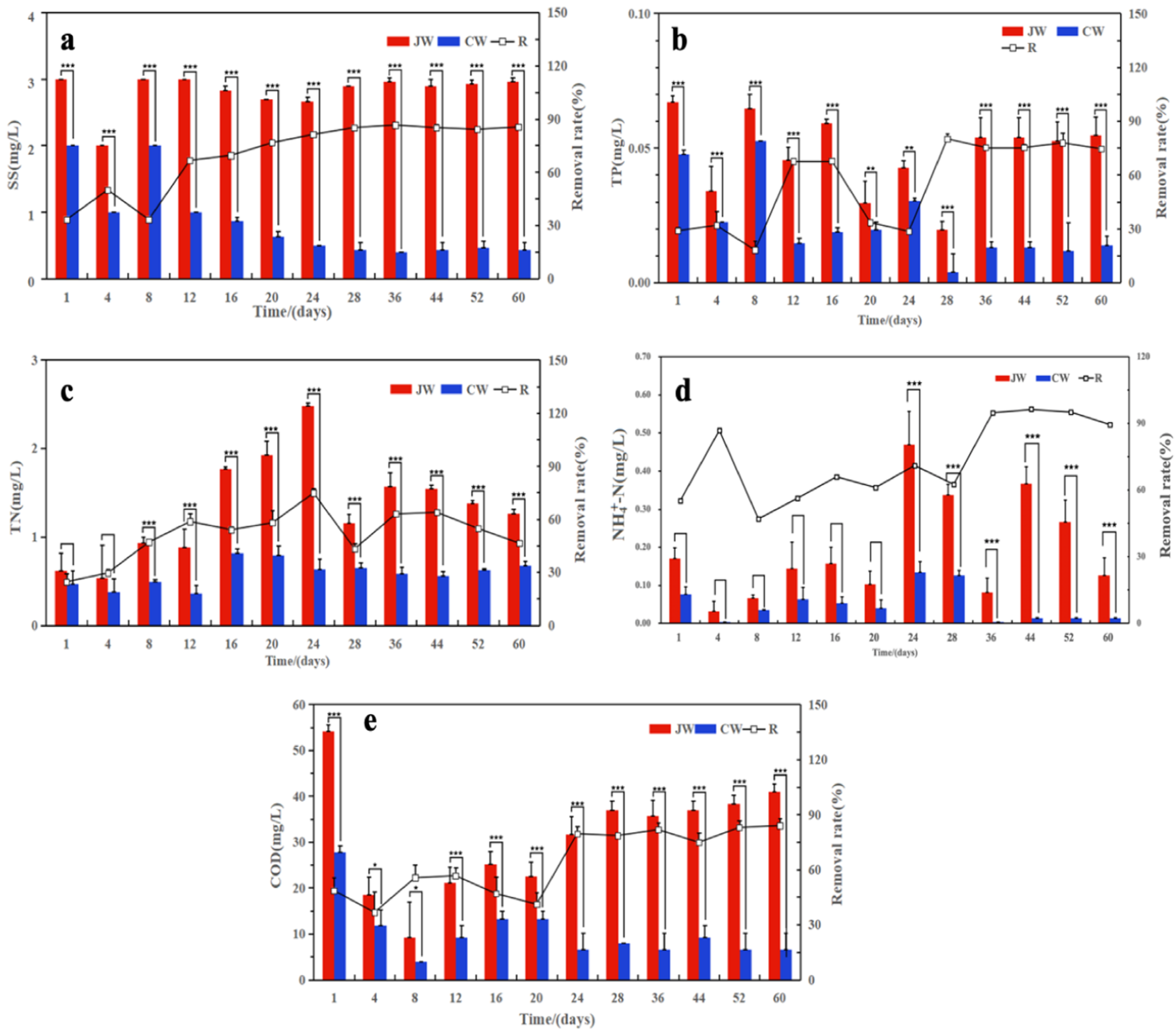

Figure 3. Suspended solids (a), total phosphorus (b), total nitrogen (c), ammonia nitrogen (d), and chemical oxygen demand (e) in influent and effluent water of a stepped combined constructed wetland. JW represents the influent water, CW represents the effluent water, and R represents the removal rate.

All plots were drawn by OriginPro version 8.5.1. A *t*-test was performed using PASW Statistics 19.0.0 to evaluate differences in attributes between JW and CW. A one-way analysis of variance (ANOVA) was used to evaluate the important comparisons between indicated groups. A significance level of $P < 0.05$ was determined, and the mean data results were expressed as mean ± standard deviation (Mean ± SD). Bar plots, hierarchical clustering analysis (HCA), and heatmap analysis were performed using R. 3.3.1. HCA was a useful tool for analyzing water quality parameters in terms of their origin and influencing factors. Heatmap analysis accounted for all consensus genera within the top 50 relative abundances. The stepped combined constructed wetland design drawing was accomplished using computer-aided design software.

3. RESULTS

3.1. Effect on Pollutant Removal of the Ecological Floating Island. Reference samples were collected near a sewage outlet to investigate the background water quality of campus sewage. The average water temperature was 23.1 °C. Both the COD and TN concentrations were relatively high. In the campus drainage canal, organic pollutants were present in high concentrations, and water quality improvements were necessary.

The water quality was significantly improved after stepped combined constructed wetland treatment ($P < 0.05$). A significant stratification of water temperature and pH was observed on the first day of the experiment (Figure 2), with the mean water temperature of the effluent samples being 25.5 ± 3.2 °C, 2.5 °C ($P = 0.05$) lower than the influent water temperature (23.0 ± 2.3 °C). In contrast, the influent pH was 7.65 ± 0.17, and the effluent pH was 7.80 ± 0.10. After 60 days, the effluent pH increased slightly, and the pH difference remained at 0.15.

SS, TN, TP, NH_4^+-N, and COD were removed at an average rate of 70, 51, 55, 70, and 64% (Figure 3). Compared to the

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Table 1. K-Means Clustering Analysis of the Removal Rates of All Samples (from the First Day to the 60th Day) Based on Seven Water Quality Indexes

reference index	initial unstable stage (1−8 days)	better effective stage (2−20 days)	most-stable and effective stage (24−36 days)
temperature	0.097	0.056	0.105
pH	−0.013	−0.016	−0.024
SS	0.389	0.789	0.803
TP	0.263	0.310	0.741
TN	0.337	0.663	0.548
NH_4^+-N	0.634	0.675	0.800
COD	0.469	0.603	0.722
number of clusters	3	2	7

effluent samples (0.85 ± 0.58 mg/L), the SS values (2.82 ± 0.28 mg/L) in the influent samples are relatively high. During the middle and late stages of the experiment (28 days), the removal rate of SS remained at a high and stable sate (about 85%). TP concentration averaged 0.05 ± 0.01 mg/L in the influent and 0.02 ± 0.01 mg/L in the effluent. By the end of the experiment, the average TN removal rate was 51%, and the highest removal rate (75%) occurred after 24 days. The TN effluent concentration was stabilized at 0.59 ± 0.04 mg/L. COD concentrations of the influent and effluent were 30.972 ± 11.991 and 10.251 ± 6.389 mg/L. During the middle stages of the experiment (24 days), the removal rate of COD remained at a stable state (about 79%).

To gauge the consistency of the stepped combined constructed wetland system's pollutant removal performance, we undertook a meticulous examination of how removal rates evolved over time. We used a K-means clustering method, which grouped our data based on temperature, pH, SS, TN, NH_4^+-N, TP, and COD. This approach unveiled a clear three-stage pattern (Table 1). These stages were categorized as the initial unstable stage, the more effective stage, and the most-stable and effective stage.

The first, labeled as the "unstable cluster," included the initial three sampling days (days 1, 4, and 8). The second phase, in the "stable cluster," covered days 12, 16, and 20. The third phase, labeled as the "most-stable cluster," encompassed the later days (24, 28, 36, 44, 52, and 60) (Figure 4a). Just as the K-means analysis revealed, it was clear that the stepped combined constructed wetland's effectiveness was at its peak during the period we have referred to as the "most-stable cluster." Interestingly, during this time frame, the system not only performed well but also maintained its high efficiency. The three stages of effective purification corresponded to the three water quality clusters. The bacterial community of the influent water was clustered on one branch, while the bacterial community of the effluent water was clustered on each of the three branches (Figure 4b and Supporting Figure S1). A variety of factors, such as the stage of plant growth and the quality of influent water, contributed to the difference in bacterial communities.

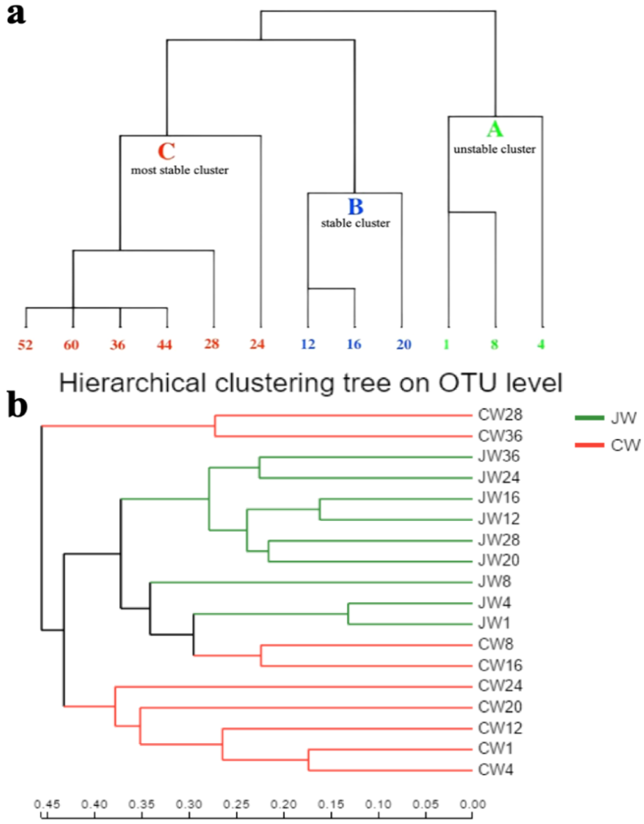

Figure 4. Hierarchical clustering analysis of the removal rates (a) and bacterial communities (b) of all samples. Sequences with over 97% similarity (species level) are defined as a single operational taxonomic unit (OTU).

3.2. Changes in the Abundance of the Bacterial Community. *Proteobacteria* were one of the most abundant phyla, accounting for 40.7 and 46.1% of the total sequences in the influent and effluent samples, respectively (Figure 5a). However, the bar graph of the community structure showed that the abundance of *Proteobacteria* had decreased by 5−7% in the stable and most stable stage of the stepped combined constructed wetland (Figure 5b). Other dominant phyla in the influent and effluent samples, respectively, were *Actinobacteria* (20.6 and 19.2%), *Bacteroidetes* (11.2 and 18.5%), *Cyanobacteria* (16.0 and 3.9%), *Verrucomicrobia* (4.1 and 1.4%), *Saccharibacteria* (0.6 and 4.3%), *Firmicutes* (2.7 and 1.3%), and *Chloroflexi* (1.0 and 0.3%). Moreover, *Chlamydiae*, *Parcubacteria*, *TM6*, *Acidobacteria*, and others comprised less than 0.5% of the total bacterial sequences. In the most-stable operation of the stepped combined constructed wetland, the relative abundances of *Verrucomicrobia*, *Saccharibacteria*, and *Firmicutes* increased by 3.4, 4.1, and 0.5 times, respectively.

Heatmap analysis was used to identify the dominant bacterial genera and their relative abundances (Figure 6). As an example, 13, 12, and 7% of the rRNA sequences in JW (influent samples) were attributed to *hgcl clade*, *Cyanobacteria*, and *Rhizorhapis*, respectively (Figure 6a). Their relative abundance decreased to 5, 3, and 0% after treatment. A similar trend was observed for *Sporichthyaceae*, *FamilyI*, *Verrucomicrobiaceae*, *Synechococcus*, *Zymomonas*, and *Lactobacillus* as well.

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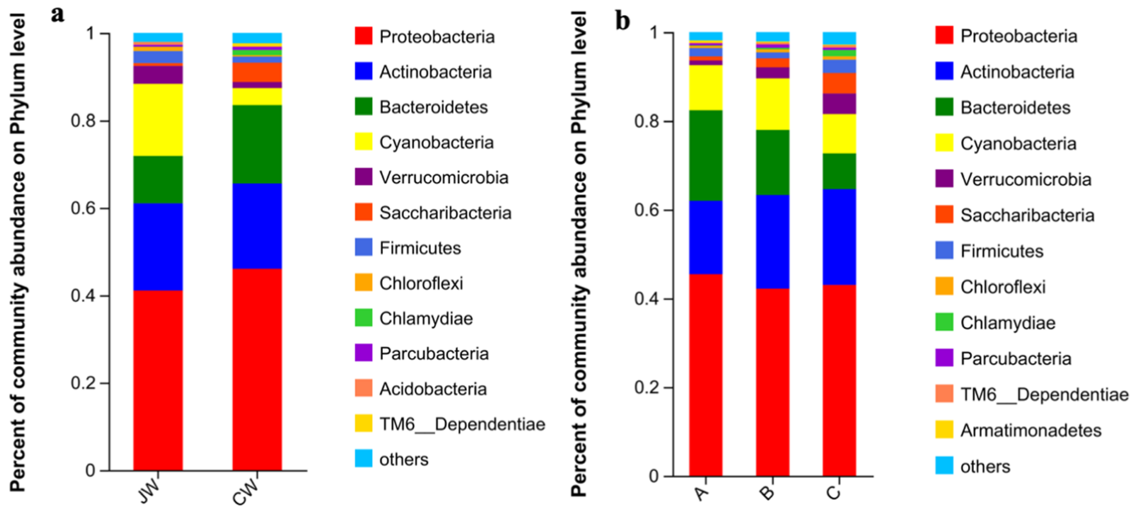

Figure 5. Bacterial community compositions at the phylum level, as revealed by pyrosequencing. (a) Divided according to the influent and effluent samples and (b) divided according to the three distinct stages during the operation.

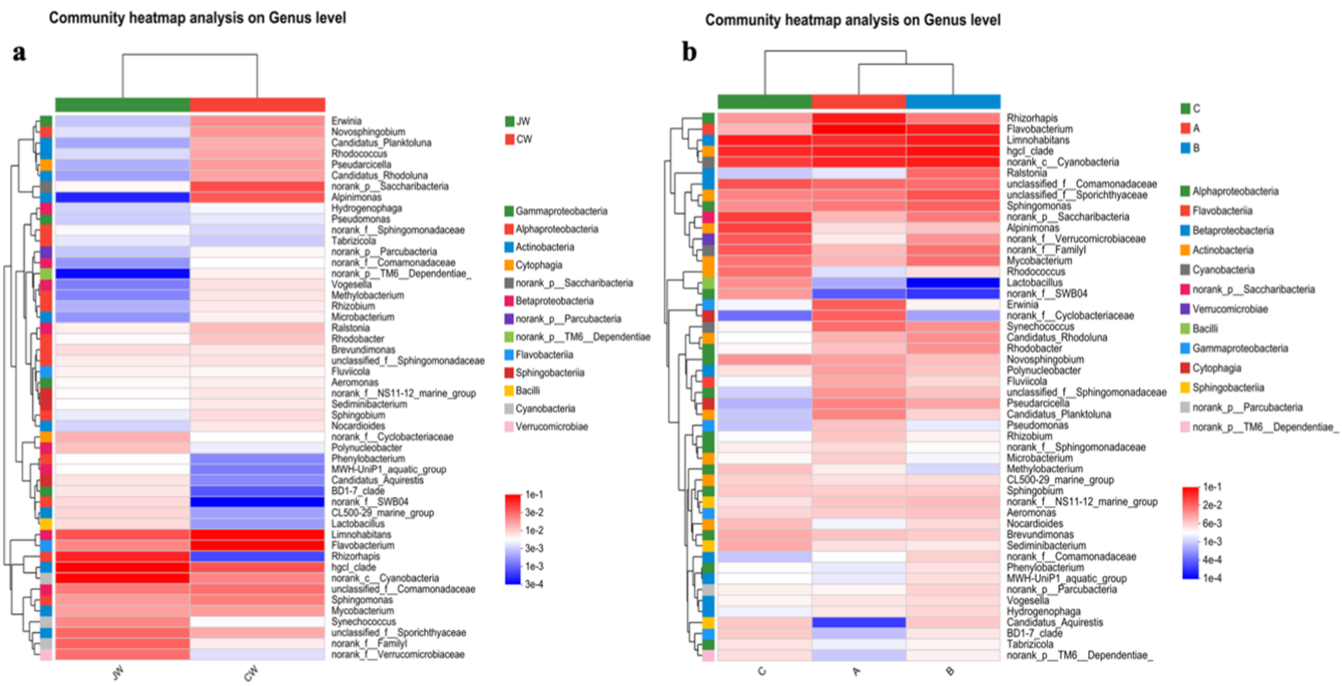

Figure 6. Heatmap of the top 50 bacterial genera in influent and effluent waters of a stepped combined constructed wetland. The color intensity shows the abundance of genera; the color key is indicated at the bottom right. (a) Divided according to the influent and effluent samples and (b) divided according to the three distinct stages during the operation.

The color intensity of the heatmap demonstrated an increase in *Flavobacterium* (10%), *Limnohabitans* (7%), *Alpinimonas* (4%), *Saccharibacteria* (4%), and *Erwinia* (3%) following the stepped combined constructed wetland treatment. Additionally, *Rhodococcus*, *Planktoluna*, *Pseudarcicella*, *Rhodoluna*, *Comamonadaceae*, *Sphingomonas*, *Novosphingobium*, *Rhizobium*, and *Rhodobacter* were also enriched in the effluent samples. At the same time, the relative abundances of *Cyanobacteria* and *Rhizorhapis* had decreased by 28.2 and 81.7%, respectively (Figure 6b). In the most stable stage of operation, *Saccharibacteria* increased by 4.10 times, *Alpinimonas* by 7.24 times, *Verrucomicrobiaceae* by 6.02 times, and *Rhodococcus* by 9.86 times. Although the abundance of *Flavobacteirum* increased at the effluent, it decreased by 87.53% over the most stable stage.

Hydrochemical parameters accounted for 43.04% of the variance in the bacterial community (Supporting Figure S2). Interestingly, COD, TP, SS, TN, and ammonia nitrogen, which distinguished JW samples from others, significantly influenced the composition of the bacterial community (Supporting Figure S2a). However, there was no distinct separation observed during the three stages (Supporting Figure S2b). SS (r^2 = 0.67) and TP (r^2 = 0.48) were identified as the primary factors determining the composition of the bacterial community in all samples. Removing SS and TP from influent water resulted in some differences in microbial community

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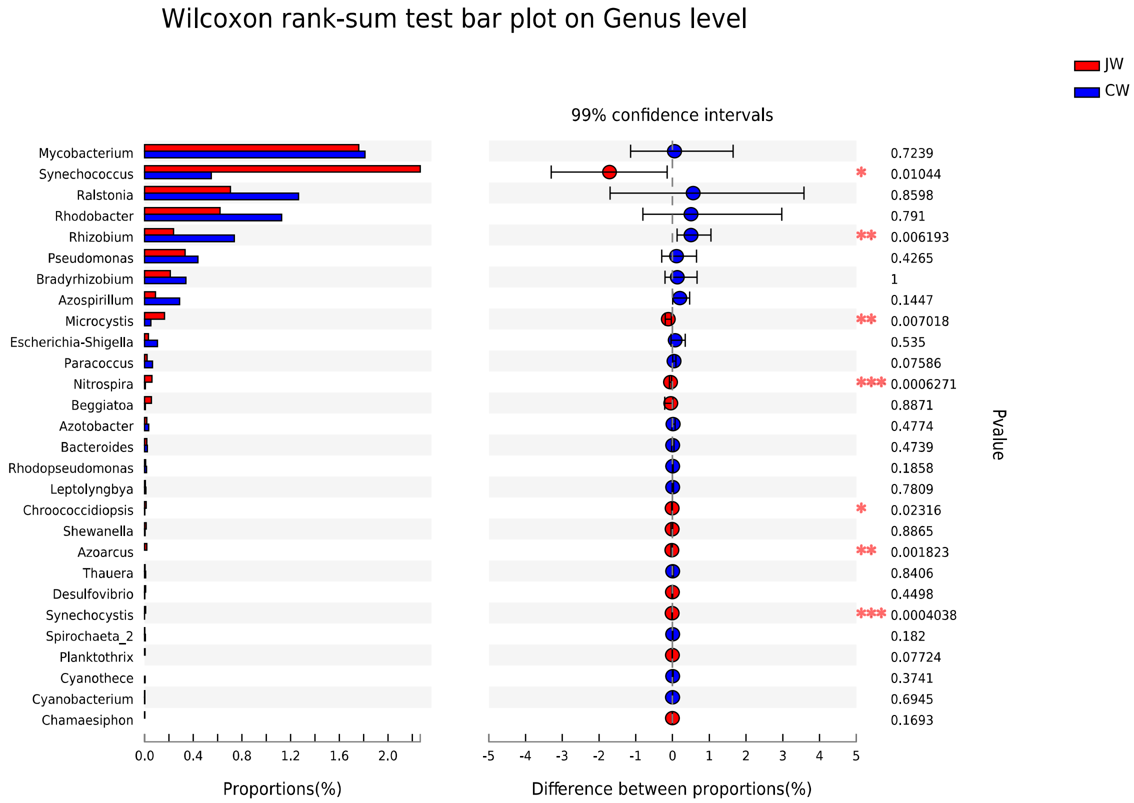

Figure 7. Relative abundance of significantly different N transformation-related bacteria in influent and effluent waters of a stepped combined constructed wetland. The one-way ANOVA test was used to evaluate the importance of comparisons between indicated groups. $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

composition caused by a stepped combined constructed wetland.

3.3. Changes in the Abundance of Bacteria Involved in Nitrogen Transformation. Nitrogen transformation requires the collaboration of various microorganisms, and most of them are unable to complete all of the steps. Bacteria in the stepped combined constructed wetland were involved in nitrogen fixation, nitrification, denitrification, anammox, assimilation, and ammonification. As a result, nitrogen-transforming bacteria in the stepped combined constructed wetland predominantly transformed and degraded nitrogenous substances in water via nitrification, denitrification, and nitrogen fixation (Figure 7).

Pseudomonas was involved in both nitrite oxidation and nitric oxide reduction, and its abundance decreased by 80.84% in the most stable stage (Supporting Figure S3), whereas in the effluent of stepped combined constructed wetland, it increased by 32.04% (Figure 7). *Rhizobium*, *Bradyrhizobium*, *Azospirillum*, and *Cyanophyta* were the primary nitrogen-fixing bacteria. *Rhizobium* and *Bradyrhizobium* exhibited similar trends in abundance during the most stable stage (Supporting Figure 3S), with a significant increase in abundance observed in the effluent. *Cyanophyta* such as *Synechococcus*, *Microcystis*, *Chroococcidiopsis*, and *Synechocystis* were present in lower numbers in the effluent water. Although the abundance of *Microcystis* decreased in the effluent, it increased during the most stable stage (Supporting Figure S3), primarily influenced by rising temperatures. Nitrate reduction to nitrite could have been mediated by *Paracoccus* and *Mycobacterium*, while nitrite oxide reduction to nitrous oxide or dinitrogen gas may have been mediated by *Ralestonia*, *Rhodobacter*, *Escherichia*, and *Shigella*. The above genera were likely involved in denitrification together, and the abundance of nitrite-reducing bacteria significantly increased after both stepped combined constructed wetland treatment and the most stable stage (Figure 7 and Supporting Figure S3).

It is worth noting that the pattern of variation in this bacterial community was intriguing and the potential scenarios were quite complex. The process of nitrate reduction to nitrite and the abundance of related N metabolism genes in the water was still needed for validation.

3.4. Evaluation of Economic Benefits of Planted Floating Islands. A variety of commercial products were used to achieve buoyancy or construct a framework, including polystyrene foam, acrylic sheets, clear hoses, and foam pads, all of which were readily available, inexpensive, and durable. Acrylic sheets cost less than 14.10 USD/kg, while polystyrene foams and pearl cottons cost only 3.10 USD/kg. We constructed a hybrid horizontal−vertical subsurface flow wetland using siphons and solar pumps to drive the water cycle with no additional energy costs. Maintenance was minimal, requiring only the removal and replacement of a few necrotic*E. crassipes*. The total cost of the stepped combined constructed wetland was 259.83 USD, including plants, pumps, accumulators, acrylic sheets, hoses, glue, wires, ropes, and labor (Table 2).*E. crassipe* occupied 95% of the water surface during the middle and end stages of the experiment, improving water quality in the drainage canal and providing a safe habitat for aquatic animals. Additionally, *E. crassipes*, being an ornamental plant, added an aesthetic value during growth and flowering.

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Table 2. Economic Benefit of Plant Floating Island

items	cost (USD)	total cost (USD)
Eichhornia crassipes	14.10	259.83
solar energy pump	36.66	
accumulator	33.55	
acrylic sheets	62.03	
transparent hoses	4.23	
PP, PVC, ABS, 502 glues	11.98	
iron wires	1.41	
EPE foam mats	7.05	
polystyrene foam	14.10	
ropes	4.23	
expenses of labor	70.49	
treatment capacity	4800 L/d	

4. DISCUSSION

Stepped combined constructed wetland is simple and easy to design, operate, and maintain, with a variety of floating island plants, fillers, and application scenarios, and provides a higher degree of removal efficiency, including nitrogen, phosphorus, and organic matter. Our device enhanced pollutant adsorption in water through the use of filler materials and plants. The vertical flow characteristic, achieved via water lifting and siphoning, transferred sewage longitudinally into the troughs. It was inferred that vertical flow increased DO in water through atmospheric diffusion and rhizome transfer.[19] However, further experimentation was required to validate this hypothesis. Consequently, the stepped combined constructed wetland developed independently and successfully patented in our study can organically integrate both surface flow artificial wetland and submerged flow artificial wetland within a small area, which not only improves the efficiency of pollutant removal but also has the advantage of small land areas and ecological landscapes.

4.1. Influence of Stepped Combined Constructed Wetland on Water Physicochemical Parameters. Interestingly, the treated water temperature in our study was almost 2.5 °C colder than that of untreated water. White and Cousins[5] demonstrated that effluent temperature decreased after flowing through areas with high vegetation cover. The stepped combined constructed wetland provided a natural habitat that reduced heat absorption and reflected most of the heat. Rezania S[20] demonstrated that after *E. crassipes* purification, the effluent pH increased from 6.95 to 7.10. Furthermore, it was suggested that effluent pH decreased after wetland treatment due to the combination of acid secreted by roots and aerobic microbial degradation.[21] In our study, the pH increased by 0.10 after treatment. While the growth of cyanobacteria could have altered the pH, this was ruled out, as the relative abundance of algae had decreased significantly. The nitrogen removal rate was the highest when the pH was between 7.0 and 7.5,[22] thus enhancing the pollutant treatment efficiency of the stepped constructed wetland.

The removal rates of SS, TN, TP, NH_4^+-N, and COD by stepped combined constructed wetland were 70, 51, 55, 70, and 64%, respectively (Figure 3). As a result of different redox conditions prevalent in different zones, the removal rates of NH_4^+-N and TN were high. COD levels were correlated with oxygen transfer rate,[21] and vertical flow greatly enhanced the removal rate of COD. The removal of phosphorus was accomplished by sedimentation, adsorption of fillers, and plant uptake. For example, *E. crassipes* also absorbed and assimilated phosphorus released from water and sediment,[23] as well as assimilated nitrogen and promoted nitrification−denitrification. The assimilation process also absorbed NH_4^+-N when *E. crassipes* continued to grow, and plants themselves starved.

Additionally, after 24 days of operating the stepped combined constructed wetland, the effluent quality stabilized, showing satisfactory removal of SS, TN, TP, and COD (Figures 3 and 4). This indicated the formation of a stable biofilm, highlighting the significant role of microorganisms in breaking down pollutants. Root development provided a larger surface area for the growth of biofilms,[15] thereby attracting more bacterial communities to inhabit, which promoted nitrogen transformation and organic matter degradation. As previous studies have shown,[15,19,21] the horizontal−vertical stepped combined constructed wetland brought oxygen into flooded areas and promoted nitrification, ammonia oxidation, and denitrification aerobically.

The removal of inorganic nitrogen by stepped combined constructed wetland primarily depended on filler adsorption and plant uptake, and it was transformed via microbially mediated processes. Higher temperatures resulted in a greater removal efficiency. Floating treatment wetlands exhibited an average hydraulic retention time (HRT) of 17 days, according to Colares.[15] Given the size of the stepped combined constructed wetland and the amount of water flowing into it, this study estimated an HRT of around 1.76 h. In contrast, most research results typically fell within a range of 1 to 2 weeks.[15] However, there were also domestic discharges from university campuses and nearby residential areas, with an HRT of 2.1 h.[24] Kusin[24] reported successful removal of 77% of the chemical oxygen demand (COD) and 21.7% of ammonia nitrogen from the water.

Hydraulic retention time (HRT) significantly influences the removal of N, P, and SS in constructed wetlands.[25] With an extended contact time between wastewater and microbial communities, nitrogen removal is typically enhanced, promoting nitrification and denitrification processes. Longer HRT also allows for increased settling of suspended solids and chemical precipitation and provides more time for plants and microbes to biologically absorb phosphorus. Moreover, a longer HRT tends to lead to improved rates of chemical oxygen demand (COD) removal. The effectiveness of COD removal is intricately connected to the efficient introduction of oxygen into the water.

4.2. Influence of Stepped Combined Constructed Wetland on Bacteria Communities and Nitrogen-Transforming Microorganisms. In previous constructed wetland studies, Proteobacteria, Actinobacteria, Bacteroidetes, Firmicutes, and Cyanobacteria were the dominant phyla of bacterial communities.[19,22] *Chloroflexi*, *Acidobacteria*, *Verrucomicrobia*, *Planctomycetes*, *Nitrospirae*, *Saccharibacteria*, and *Gemmatimonadetes* were also involved in the treatment of wastewater at constructed wetland.[26,27] Interestingly, the dominant phyla were similar to previous studies, and the effluent contained fewer *Cyanobacteria*, *α-Proteobacteria*, *Actinobacteria*, and *Verrucomicrobia*, while *Actinobacteria*, *β-Proteobacteria*, *Saccharibacteria*, and *γ-Proteobacteria* increased. *α-Proteobacteria* and *Actinobacteria* were primarily involved in nitrite reduction or NO reduction processes, while *β-Proteobacteria* and *γ-Proteobacteria* function as both nitrifying bacteria and nitrite-reducing bacteria.[14,28] The bacteria described above were jointly involved in nitrification and denitrification.

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Saccharibacteria were phylogenetically diverse and played a role in the degradation of various organic compounds, as well as sugars under aerobic, nitrate-reducing, and anaerobic conditions.[29] *Alphapinimonas*, isolated from river water, was capable of removing dissolved organic matter.[30] *Flavobacterium* was presented in many biological wastewater treatment systems and was involved in contaminant degradation and nutrient removal.[31] All of the mentioned bacteria significantly increased in the treated water, which was effective for degraded pollutants. The abundance of algae in the effluent decreased significantly following stepped combined constructed wetland treatment (Figures 6 and 7), including *Synechococcus*, *Microcystis*, *Chroococcidiopsis*, *Synechocystis*, and *Cyanobacteria*. Roots lowered water flow and turbulence, enhanced algae, and suspended sedimentation.

A large number of nitrifying bacteria (*Azoarcus* and *Nitrospira*) coexisted with denitrifying bacteria (*Mycobacterium*, *Paracoccus*, *Ralstonia*, *Rhodobacter*, *Escherichia Shigella*) and nitrogen-fixing bacteria (*Rhizobium*, *Bradyrhizobium*, *Azospirillum*) in stepped combined constructed wetland. A higher percentage of the nitrogen-fixing bacteria, α-Proteobacteria (such as *Rhizobium, Bradyrhizobium*, and *Azospirillum*) sequences were found after ecological floating island treatment.[32] The abundance of nitrogen-fixing bacteria, nitrite-oxidizing bacteria, and denitrifying bacteria was significantly increased, with the exception of cyanobacteria (Figure 7). The composition pattern of this bacterial community was quite intriguing, with potential scenarios being rather complex. According to the study by Samal K,[33] denitrification required more anaerobic conditions, and when vegetation cover approached 100%, it affected gas exchange and solar radiation, thereby promoting denitrification. Therefore, a plant coverage of 95% was considered to be a possible cause for the high abundance of denitrifying bacteria. However, further measurements of DO and the abundance of denitrification genes are still needed to validate this.

Numerous scientists[9,15,34] summarized the design, operation, plants, management, and application of constructed wetland. Vymazal J and Kropfelova L[34] concluded that earthwork, gravel, liners, plants, pipes, control structures, and miscellaneous items accounted for 7% of the total cost (27.4%, 27 to 53%, 13 to 33%, 2 to 12%, 6 to 12%, 3.1 to 5.7%, 1.8 to 12%, respectively). Earthworks and crushed rocks constituted the majority of the costs in previously constructed wetlands. The operation and maintenance costs of a stepped combined constructed wetland were minimal and much lower than those for concrete and steel technologies. Our invention reduced capital, operation, and maintenance costs by about one-fifth compared to the Zhang L L's[3] study.

A large majority of studies on floating islands did not include a cost analysis, and the estimated cost of constructed wetlands in China ranged from a few hundred to several hundred USD/m^2.[10] The new invention of stepped combined constructed wetland was equipped with solar panels and batteries that converted solar energy into electrical energy and stored it, thus raising the water flow without the need for additional energy sources. Additional aeration devices were planned to be installed in the future using electrical energy. Nevertheless, our invention had limited capabilities to adjust to changes in the water level and was better suited for a more stable water level.

5. CONCLUSIONS

The independently developed and patented stepped combined constructed wetland proved effective in removing pollutants from slow flow and still water areas by integrating horizontally and vertically constructed wetlands. After approximately 24 days of operation, the effluent quality stabilized. It demonstrated substantial removal rates for suspended solids (SS), total nitrogen (TN), total phosphorus (TP), ammonia nitrogen (NH_4^+-N), and chemical oxygen demand (COD), with average removal rates of 70% for SS, 51% for TN, 55% for TP, 70% for $NH4^+$-N, and 64% for COD. The constructed wetland fostered the coexistence of various bacteria involved in nitrogen cycling, including nitrifying bacteria like *Azoarcus* and *Nitrospira*, denitrifying bacteria like *Mycobacterium*, *Paracoccus*, *Ralstonia*, *Rhodobacter*, and *Escherichia Shigella*, and nitrogen-fixing bacteria like *Rhizobium*, *Bradyrhizobium*, and *Azospirillum*. The abundance of these bacteria in the effluent increased significantly except for *Cyanobacteria*. Microbial community analysis revealed that the constructed wetland created an environment simultaneously supporting aerobic, anoxic, and anaerobic conditions, enabling processes such as denitrification, nitrification, and anaerobic ammonia oxidation to collectively facilitate nitrogen transformation and removal. In conclusion, this technology offers a comprehensive and efficient solution for pollutant removal in slow flow and still water areas. Its ability to host multiple bacterial communities and facilitate nitrogen transformation make it an environmentally friendly and low cost-effective option for water treatment. Its patent, independent development, and successful implementation suggest significant potential for widespread application in various fields.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.4c05077.

> Hierarchical clustering analysis of the bacterial communities of all samples (from the first day to the 36th day) based on the genus (Supporting Figure S1); redundancy analysis (RDA) reveal hydrochemical parameters that influenced bacterial community composition (Supporting Figure S2); and relative abundance of significantly different of N transformation-related bacteria in the unstable and most stable stage during the operation (Supporting Figure S3) (PDF)

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Z.S.: Conceptualization, investigation, data curation, formal analysis, and writing—original draft. **Z.Y.**: Methodology and formal analysis. **P.G.**: Investigation. **K.Z.**: Investigation. **Y.L.**: Investigation. **Y.W.**: Investigation. **Q.L.**: Figures. **Q.L.**: Supervision and funding acquisition.

Notes

The authors declare no competing financial interest.

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