Efficacy of Nitrogen and Phosphorus Removal and Microbial Characterization of Combined A²O-MBBR Constructed Wetlands

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Abstract: A combined anaerobic–anoxic–oxic moving bed biofilm reactor (A²O-MBBR) constructed wetlands process was used to treat low carbon-to-nitrogen (C/N) simulated sewage. The results showed that the removal rates of chemical oxygen demand (COD), ammonia nitrogen (NH₄⁺-N), total nitrogen (TN), and total phosphorus (TP) by this process were 94.06%, 94.40%, 67.11%, and 84.57%, respectively, and the concentrations of COD, NH₄⁺-N, TN, and TP in the effluent were lower than the Class I-A standard of GB18918-2002. In the anoxic zone, NH₄⁺-N had an inhibitory effect on phosphorus uptake via phosphorus-accumulating organisms (PAOs). The highest community diversity was observed in the anoxic zone sludge at 24 d. During the water-quality-shock loads stage, microbial community diversity decreased in a combined A²O-MBBR constructed wetlands reactor. At the phylum level, bacteria within the mature activated sludge were dominated by Proteobacteria, while Planctomycetes bacteria were the dominant species in the constructed wetlands. At the genus level, Tolumonas spp. were the dominant species in the 12 d and 24 d constructed wetlands and the anaerobic zone, with relative abundance percentages ranging from 20.24 to 33.91%. In the water-quality-shock loads stage, they were replaced by denitrifying bacteria such as Herbaspirillum spp. Unclassified_Burkholderiales was the dominant species in the constructed wetlands, with a relative abundance of 33.09%.

Keywords: A²O-MBBR; constructed wetland; low C/N; nitrogen and phosphorus removal; microbial community

1. Introduction

Most sewage treatment plant influents are characterized by low C/N [1]. With the deepening processes of modernization and urbanization, people’s living standards are improving, and sewage discharge standards are becoming more stringent, with 52.2% of sewage treatment plants having influent C/N less than 4.0 [2]. Because denitrification and phosphorus-removal processes require sufficient carbon sources, nitrogen and phosphorus removal is inefficient under low C/N conditions [3]. The traditional method of upgrading the nitrogen-removal capacity of sewage treatment plants is to put in external carbon sources, which leads to higher carbon emissions, contrary to the goal of “Carbon Peaking and Carbon Neutrality”, and to improve the effect of phosphorus removal, often used at the end of the process to put in phosphorus-removal chemicals, which increases the operating costs of sewage treatment plants. Therefore, enhancing the efficacy of nitrogen and phosphorus removal from sewage under low C/N conditions has become a research difficulty in the sewage treatment industry.

The anaerobic–oxic (A/O), anaerobic–anoxic–oxic (A²O), and oxidation ditch processes are mature domestic sewage treatment processes that occupy mainstream positions...
in municipal wastewater treatment plants, of which A²O is the most commonly used synchronous nitrogen- and phosphorus-removal process [4]. Moving bed biofilm reactor (MBBR) upgrading is a relatively inexpensive and efficient upgrade program for sustainably upgrading municipal sewage treatment plants, which saves land and construction costs and is suitable for upgrading small sewage treatment plants [5]. Embedding MBBR in the A²O process optimizes the distribution of carbon sources, increases the microbial structure’s stability, improves the reactor’s internal biomass, and effectively enhances the effectiveness of nitrogen removal and phosphorus removal [6]. Constructed wetlands are weak against shocks and often need to be combined with other water treatment processes to work together. The effluent quality of the combined process is stable and can meet the standard requirements. Compared with a single constructed wetlands treatment system, a combined A²O–MBBR constructed wetlands treatment system shows a preferable treatment effect [7].

A combined A²O-MBBR constructed wetland process reactor was designed and prepared, with MBBR suspended filler being injected into the constructed wetlands, on top of which Copperhead was planted. Using low C/N simulated sewage as the research object, we investigated the start-up and water-shock load resistance of the A²O-MBBR constructed wetlands reactor, combined with a correlation analysis of the operating conditions and water quality indexes to study the efficiency of the reactor at removing nitrogen and phosphorus. We examined the characteristics of the microbial community structure and the diversity in the spatial and temporal variations of the reactor operation via high-throughput sequencing technology [8]. The final validation of the efficacy of combined A²O-MBBR constructed wetlands for nitrogen and phosphorus removal from low C/N sewage under shock loads conditions and its effect on the microbial community structure occurred.

2. Materials and Methods

2.1. Experimental Setup and Operating Parameters

The combined A²O-MBBR constructed wetlands test setup is shown in Figure 1. The device’s main body was plexiglass, with a total adequate volume of 211 L. The ratio of the effective volumes of the anaerobic zone, anoxic zone, and oxic zone was 2:2:7. The effective volume of the constructed wetlands was 64 L, with 14 L in the upper substrate layer and 50 L in the lower MBBR fill layer. The anaerobic zone and anoxic zone were equipped with mechanical stirrers with a stirring speed of 20–70 r/min and a hydraulic retention time (HRT) of 5 h. The mechanical stirrers can be used in the anaerobic zone and anoxic zone. The oxic zone was equipped with constructed wetlands and an aeration system, and the dissolved oxygen (DO) concentration was maintained at 2.0–4.0 mg/L, with an HRT of 17.5 h and a sludge retention time (SRT) of 20 d. The oxic zone was designed with constructed wetlands and an aeration system. A 50 W plant growth light was provided above the constructed wetlands. All three ponds were dosed with MBBR fill, with a fill rate of 30% [9]. MBBR packing adopted white high-density polyethylene suspension carrier packing, model XFTL-A-900-25×10 (Henan Shuangxin, Zhengzhou, China) [10]. Constructed wetlands substrate layer was planted with Hydrocotyle vulgaris, a wetland aquatic plant effective at TN removal [11]. The volcanic substrate was used to immobilize Culex pipiens, adsorb NH₄⁺-N in the water body, and provide a better environment for denitrifying bacteria to survive and improve the TN removal rate [12].

COD, NH₄⁺-N, TN, TP, DO, and pH were monitored in the sewage tanks, anaerobic zones, anoxic zones, and oxic zones during the combined A²O-MBBR constructed wetlands process. Sludge was collected from the anaerobic zone, anoxic zone, oxic zone, and constructed wetlands and couriered to Sangon Biotech (Shanghai) Co., Ltd. (Shanghai, China) for high-throughput sequencing analysis to study the structure and function of microbial communities in the combined treatment process.
2.2. Operating Condition

Simulated sewage was prepared from glucose, NH₄Cl, KH₂PO₄ and KNO₃. The pH of the influent was adjusted to about 8.0 with Ca(OH)₂ and NaHCO₃. To maintain various trace elements required for microbial life activities, the concentration of the preparation was adjusted by adding 1 mL of trace element nutrient solution per 150 L of simulated sewage, concerning the formulation of trace element nutrient solution by Xinru Jiang et al. [13]. The inoculated activated sludge was taken from the secondary sedimentation tank of the Guilin Wulidian Sewage Treatment Plant (GWSTP), with MLSS of 1910 mg/L and MLVSS of 1080 mg/L. The mixed liquid reflux ratio in the system was 200%, and the sludge reflux ratio was 12.5%.

The test was divided into three phases; first, start-up stage (0–12 d) used rapid sludge discharge method to start the combined A²O-MBBR constructed wetlands process reactor. The design influent’s COD, NH₄⁺-N, TN, and TP were 300, 30, 40, and 2 mg/L, respectively [14]. During the water quality improvement stage (13–24 d), the influent’s COD, NH₄⁺-N, TN, and TP were designed to be 350, 40, 47.5, and 2 mg/L, respectively, and the inoculated sludge was domesticated to adapt to the higher concentration of low C/N domestic sewage; during the shock loads stage (25–36 d), the influent’s COD, NH₄⁺-N, TN and TP were designed to be 400, 50, 55 and 2 mg/L, respectively, to investigate the efficacy of A²O-MBBR combined with constructed wetlands in treating high-concentration and low-C/N domestic sewage for denitrification and removal of phosphorus.

2.3. Methods of Analysis

2.3.1. Methods for Analyzing Conventional Water Quality Indicators

The reference standards for analytical methods of conventional water quality indicators are shown in Table 1.

Table 1. Analysis methods and reference standards for traditional quality of water indicators.

<table>
<thead>
<tr>
<th>Serial Number</th>
<th>Indicators</th>
<th>Analytical Methods or Monitoring Instruments</th>
<th>Reference Standard</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>NH₄⁺-N</td>
<td>Nessler’s reagent spectrophotometry</td>
<td>HJ 535-2009 [16]</td>
</tr>
<tr>
<td>3</td>
<td>TN</td>
<td>Alkaline potassium persulfate digestion UV spectrophotometric method</td>
<td>HJ 636-2012 [17]</td>
</tr>
<tr>
<td>4</td>
<td>TP</td>
<td>Ammonium molybdate spectrophotometric method</td>
<td>GB 11893-89 [18]</td>
</tr>
<tr>
<td>5</td>
<td>DO</td>
<td>HACH HQ30d</td>
<td>HJ 925-2017 [19]</td>
</tr>
<tr>
<td>6</td>
<td>pH</td>
<td>Leici PHSJ-6L</td>
<td>GB 6920-86 [20]</td>
</tr>
</tbody>
</table>
2.3.2. Analysis of Microbial Communities

Microorganisms in the anaerobic zone, anoxic zone, oxic zone, and constructed wetlands in the combined \( \text{A}^2\text{O-MBBR} \) constructed wetlands experimental setup were collected at 12 d, 24 d, and 36 d, respectively, and were correspondingly numbered as 1, Y, 1_Q, 1_H, 1_C, 2, Y, 2_Q, 2_H, 2_C, 3, Y, 3_Q, 3_H, and 3_C. Volcanic substrates were collected on d 18 and d 36 using the five-point sampling method, sealed in polyethylene sample bags, and correspondingly numbered 2–1 and 3–1.

The amount of DNA that should be added for polymerase chain reaction (PCR) was determined after the first round of amplification using the Qubit 3.0 DNA Detection Kit (Thermo Scientific, Waltham, MA, USA) for accurate quantification of genomic DNA, and the primers used for PCR were the 16SV3-V4 primers of the sequencing platform. Illumina bridge PCR-compatible primers were introduced for the second round of amplification. The library size was detected by 2% agarose gel electrophoresis, and the library concentration was determined using Qubit 3.0 fluorescence quantification. Sequencing was performed for high-throughput analysis after the library quality control was qualified.

Paired-end reads obtained from second-generation sequencing were first spliced according to the overlap relationship. After distinguishing the samples, the sequence quality was quality-controlled and filtered, followed by operational taxonomic unit (OTU) clustering analysis and species taxonomic analysis. Based on these analyses, a series of in-depth statistical and visual studies, such as statistical analyses of community structure, alpha diversity analyses, and functional predictions, were performed at each taxonomic level.

3. Results and Discussion

3.1. Operating Conditions of Combined \( \text{A}^2\text{O-MBBR} \) Constructed Wetlands

3.1.1. Volumetric Loading and Environmental Factors of Combined \( \text{A}^2\text{O-MBBR} \) Constructed Wetlands

The variation in volumetric loading (Nv), pH, and DO in the combined \( \text{A}^2\text{O-MBBR} \) constructed wetlands reactor, with the operating time, is shown in Figure 2. As can be seen in Figure 2, the Nv of the anaerobic zone in the combined \( \text{A}^2\text{O-MBBR} \) constructed wetlands reactor continued to increase with the increasing influent COD concentration, from an average of 1.30 kg COD/(m\(^3\)·d) in the start-up stage to 1.95 kg COD/(m\(^3\)·d) in the shock loads stage. The anoxic zone Nv averaged 0.95 kg COD/(m\(^3\)·d) and 1.03 kg COD/(m\(^3\)·d) for the start-up stage and water quality improvement stage, respectively, and 1.32 kg COD/(m\(^3\)·d) for the shock loads stage. The oxic zone Nv was consistently maintained at about 0.05 kg COD/(m\(^3\)·d). This shows that the combined \( \text{A}^2\text{O-MBBR} \) constructed wetlands process can carry a high Nv, resist shock loads, and have a stable treatment effect.

The DO of the oxic zone increased from 2 mg/L to 4 mg/L during the start-up stage and was depleted to 0.93 mg/L on d 6 due to damage to the aeration system. The DO of the oxic zone was controlled at 3~4 mg/L during the water quality improvement stage and shock loads stage [21]. During reactor operation, DO ranged from 1.5 to 0.5 mg/L in the anoxic zone and was less than 0.5 mg/L in the anaerobic zone. Low DO is one of the conditions for simultaneous nitrification–denitrification. Still, the carbon source can be a
limiting factor, so maintaining low DO for treating low C/N sewage may reduce nitrogen removal efficiency.

![Figure 2. Variation in Nv, pH, and DO in A²O-MBBR combined with constructed wetland reactor over time.](image)

### 3.1.2. Plant Growth in Combined A²O-MBBR Constructed Wetlands

Figure 3 shows the growth status of *Hydrocotyle vulgaris* at different stages of the combined A²O-MBBR constructed wetlands. As seen in Figure 3a,d, on d 0, the leaf blades of *Hydrocotyle vulgaris* were small and numerous, with disorganized growth direction, generally yellowish leaf blades, petiole lengths of about 20 cm, and leaf blade diameters ranging from 2.5 to 3.5 cm. As can be seen in Figure 3b,e, on d 18, the growth direction of *Hydrocotyle vulgaris* was more uniform and growing vertically, and the higher the leaf blade nearer to the center light source was, the leaf blade appeared darker green, and the length of petiole was 25–30 cm. The diameter of the leaf blade was in the range of 4.0–5.1 cm. This indicates that the nitrogen, phosphorus, and trace elements were sufficient and that *Hydrocotyle vulgaris* absorbed and consumed the nitrogen in the sewage. As shown in Figure 3c,f, on d 36, the leaf blades of *Hydrocotyle vulgaris* were thinner than before. The dark green color of the leaf blades appeared slightly lighter than on d 18, but the diameter and height significantly changed, with petiole lengths ranging from 35 to 40 cm and blade diameters in the range of 6.0–7.2 cm. With the operation of the combined A²O-MBBR constructed wetlands, *Hydrocotyle vulgaris* significantly increased the petiole length and leaf blade diameter but decreased the number of split plants [22].

### 3.2. Efficacy of Combined A²O-MBBR Constructed Wetlands for Treatment of Low C/N Simulated Sewage

#### 3.2.1. COD Removal Effect of the System under Different Water Quality Conditions

The effect of the combined A²O-MBBR constructed wetlands reactor on COD removal is shown in Figure 4. As can be seen in Figure 4, during the start-up phase, the highest COD removal was 98.21% (5 d), the lowest was 67.59% (6 d), and the average removal was 87.42%, which is an unstable COD removal rate. This may be due to the environmental acclimatization period of the newly inoculated aerobic activated sludge. The lowest COD removal on d 6 of reactor operation was due to damage to the aeration system, resulting in a more inadequate system DO concentration. During the water quality improvement stage, the highest COD removal rate was 93.45% (d 24), the lowest removal rate was 87.22% (d 13), and the average removal rate was 90.9%. The COD removal rate showed a gradual upward trend, which indicated that the reactor operation status was gradually stabilized. On d 23 and d 24, the COD concentration in the anoxic zone abnormally increased, probably...
due to the low degree of mixing inside the anoxic zone due to the dislodgement of the agitator rotor head of the anoxic zone after collision with the MBBR filler. The sampling point was close to the anaerobic zone, and the COD concentration was back to normal on d 25 after repairing the anoxic zone on d 23. The influent COD concentration further increased during the shock loads stage, with a maximum influent COD of 450 mg/L (35 d), which exceeds the maximum design influent COD concentration (400 mg/L). The highest removal of influent COD was 97.38% (30 d), the lowest was 90.6% (33 d), and the average removal was 94.06%. The abnormally high COD concentration in the anoxic zone on d 32 was due to the significant increase in COD concentration in the anaerobic zone and the over-aeration of the aeration system in the oxic zone (DO = 4.19 mg/L), which led to high DO in the anoxic zone (DO = 1.56 mg/L), destroying the microbial environment in the anoxic zone, thus weakening the ability of the anoxic zone to degrade COD.

Figure 3. The effect of operation time on the growth of Hydrocotyle vulgaris. Where (a–c) are actual pictures of Hydrocotyle vulgaris with growth times of d 0, d 18, d 36 and (d–f) are schematic diagrams.

Figure 4. The removal efficiency of COD by A²O-MBBR combined with constructed wetlands reactor at different stages.
Under low C/N conditions, the oxic zone is in a COD-deficient state, so whenever problems with the operation of the anoxic zone result in elevated COD concentrations, effluent COD concentrations are barely affected. During the shock loads stage, the average COD removal of the water samples from the combined A2O-MBBR constructed wetlands reactor was slightly higher than the COD concentration of the A2O-MBBR reactor under the conventional C/N condition (93 ± 1.3%), which indicates that the combined A2O-MBBR constructed wetlands reactor has a preferable ability to resist the shock loads [23]. The average COD removal in the anoxic zone water samples was 56.31%, indicating that most COD acts as an electron donor for the denitrification reaction in the anoxic zone.

3.2.2. NH4+-N Removal Effect of the System under Different Water Quality Conditions

The effect of the combined A2O-MBBR constructed wetlands reactor on NH4+-N removal is shown in Figure 5. As shown in Figure 5, in the start-up stage, the NH4+-N removal rate of water samples is low and wildly fluctuates, and the NH4+-N removal rate of the water samples ranges from 27.59 to 78.51%. This may be due to the uneven aeration of the oxic zone during the start-up phase, which has a low DO concentration at the bottom (DO = 0.21 mg/L), presenting a hypoxic or even anaerobic condition, and the nitrification reaction lacks sufficient oxygen. Therefore, the aeration system was modified on d 12: multiple aeration heads were placed at the bottom of the oxic zone. During the water quality improvement stage, the average NH4+-N removal rate of the water samples was 95.69%. On d 23, d 24, and d 32, the NH4+-N concentration of the anoxic zone water sample was abnormally high, which was consistent with the trend of COD concentration, probably because the drastic change in environmental factors affects the activity of microorganisms inside the anoxic zone, leading to the decrease in the treatment efficiency of the anoxic zone. The influence of elevating the influent COD concentration on the NH4+-N removal rate during the shock loads stage was small, with the NH4+-N removal rate ranging from 88.77 to 99.24% and the effluent concentration ranging from 0.21 to 4.85 mg/L, which meets the GB18918-2002 [24] Class I-A discharge standard. When the system was shocked by a higher NH4+-N concentration, the impact on the nitrification reaction is small, and the combined A2O-MBBR constructed wetlands reactor has a preferable ability to remove ammonia nitrogen [25].

![Figure 5. The Removal efficiency of NH4+-N by A2O-MBBR combined with constructed wetlands reactor.](image_url)
3.2.3. TN Removal Effect of the System under Different Water Quality Conditions

The effect of the combined A\textsuperscript{2}O-MBBR constructed wetlands reactor on TN removal is shown in Figure 6. As seen in Figure 6, during the start-up stage, the TN concentration of effluent water samples ranged from 10.26 to 20.09 mg/L, and the TN removal rate went from 16.21 to 62.15%, with a significant fluctuation in the removal rate. There are two reasons for this situation: First, the necessary conditions for denitrification reaction are sufficient electron donors and anaerobic or anoxic zone conditions, while the average values of DO concentration in anaerobic and anoxic zones during the start-up stage were 0.43 mg/L and 0.84 mg/L, respectively, so the conditions for denitrification reaction were slightly worse. Second, the TN removal trend was similar to the trend of NH\textsubscript{4}\textsuperscript{+}-N removal but different from that of COD removal. This resulted in low TN removal due to lacking a sufficient carbon source for the denitrification reaction. During the water quality improvement stage, TN removal gradually increased due to the improved aeration system, with the highest TN removal rate of 74.79% (d 18), the lowest rate of 59.45% (d 15), and an average removal rate of 65.36%. This showed that the combined A\textsuperscript{2}O-MBBR constructed wetlands reactor is in stable operation. During the shock loads stage, the maximum influent TN concentration was 53.55 mg/L (d 32), the maximum TN removal was 74.85% (d 36), the minimum removal was 61.69% (d 29), and the average removal rate was 67.11%. On d 32, the TN concentration in the anaerobic zone was higher than the influent TN concentration because the impact of the high influent TN concentration and the low influent pH (pH = 6.98) combined to cause the pH of the anaerobic zone to decrease to 6.3, and the rate of the nitrification reaction was more significant than that of the denitrification reaction, which resulted in the accumulation of NO\textsubscript{3}\textsuperscript{-} production in the anaerobic zone. Overall, the total nitrogen removal rate in the shock loads stage gradually increased, indicating that the combined A\textsuperscript{2}O-MBBR constructed wetlands system has satisfactory nitrogen removal efficiency and strong impact load resistance.

![Figure 6. The removal efficiency of TN by A\textsuperscript{2}O-MBBR combined with constructed wetlands reactor.](image)

3.2.4. TP Removal Effect of the System under Different Water Quality Conditions

The effect of the combined A\textsuperscript{2}O-MBBR constructed wetlands reactor on TP removal is shown in Figure 7. As can be seen in Figure 7, during the start-up stage, the influent TP concentrations ranged from 1.41 to 3.77 mg/L, the anaerobic zone water sample TP concentrations ranged from 1.99 to 5.58 mg/L, the anoxic zone water sample TP concentrations ranged from 2.52 to 6.72 mg/L, and the effluent TP concentrations ranged from 0.70 to 2.49 mg/L, with the anaerobic zone and anoxic zone sample TP concentrations...
being more significant than the influent TP concentrations. This is because the inoculated sludge is phosphorus-rich, and the sludge is anaerobic until it is removed and shipped to the laboratory, so the reactor’s anaerobic and anoxic zones have high phosphorus concentrations prior to rapid sludge draining and membrane hanging. Therefore, the influent TP concentration was controlled to be within 2.0~3.0 mg/L in the water quality improvement stage, and the shock loads stage and a large amount of sludge discharge were also carried out. During the water quality improvement phase, the highest TP removal was 92.55% (20 d), and the lowest removal was 75.66% (13 d), with an average removal of 82.19%. The difference between the highest removal and the most insufficient TP removal was too significant because of the start-up phase and the high endogenous TP content, resulting in less significant pre-sludge removal. In the shock loads stage, the average removal rate was 84.57%, and the average effluent concentration was 0.22 mg/L, less than 0.5 mg/L, which reached the GB18918-2002 Class I-A discharge standard.

**Figure 7.** The removal efficiency of TP by A²O-MBBR combined with constructed wetlands reactor.

3.3. Correlation Analysis of Water Quality Indicators in Combined A²O-MBBR Constructed Wetlands

The correlation analysis of conventional water quality indicators and environmental factors in the anaerobic zone, anoxic zone, and oxic zone of the combined A²O-MBBR constructed wetlands was carried out using the R language version 3.6.0, and the results of the analysis are shown in Figure 8. As can be seen from Figure 8A, the anaerobic zone showed a highly significant strong positive correlation between NH₄⁺-N and TN, a highly significant moderate positive correlation between COD and NH₄⁺-N as well as COD and TN, and a highly significant moderate negative correlation between pH and TP. As can be seen in Figure 8B, the anoxic zone showed a highly significant moderate positive correlation between NH₄⁺-N and TP and a highly significant moderate negative correlation between pH and TP. As shown in Figure 8C, the oxic zone showed a highly significant positive correlation between pH and TP. As shown in Figure 8C, the oxic zone showed a highly significant positive correlation between NH₄⁺-N and TN as well as NH₄⁺-N and TP, and a high significant negative correlation between TP and pH as well as TP and DO. The results showed that the COD concentration in the anaerobic zone affects the nitrogen removal efficiency, the NH₄⁺-N and TN concentrations decrease with the decrease in COD concentration, and the combined A²O-MBBR constructed wetlands are suitable for the treatment of low C/N sewage. The inhibitory effect of NH₄⁺-N in the anoxic zone occurs on the phosphorus uptake rate of phosphorus accumulating organisms (PAOs) under anoxic conditions [26].
3.4. Analysis of Microbial Community Structure and Diversity in Combined A2O-MBBR Constructed Wetlands

High-throughput sequencing of the V3–V4 region of the bacterial 16S rRNA gene in the samples was performed using the Illumina platform (Illumina MiSeq, San Diego, CA, USA), and the OTUs at a 97% similarity level were taxonomically identified, analyzed for alpha diversity, and the samples were analyzed for clustering tree and relative abundance. The results of the alpha diversity analysis are shown in Table 2. As can be seen from Table 2, the coverage is between 99.34% and 99.99%, indicating that the analyzed results can cover the vast majority of the flora in the samples, which can reflect the actual information of the samples and is representative.

Table 2. Sequencing results and alpha diversity analysis of different samples.

<table>
<thead>
<tr>
<th>Sampling Location</th>
<th>Time</th>
<th>Coverage</th>
<th>Shannon</th>
<th>Chao</th>
<th>Ace</th>
<th>Simpson</th>
<th>Shannon Even</th>
</tr>
</thead>
<tbody>
<tr>
<td>anaerobic zone</td>
<td>12 d</td>
<td>0.996</td>
<td>5.406</td>
<td>2168</td>
<td>2201</td>
<td>0.033</td>
<td>0.711</td>
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<tr>
<td>anoxic zone</td>
<td>12 d</td>
<td>0.996</td>
<td>2.809</td>
<td>1185</td>
<td>1245</td>
<td>0.190</td>
<td>0.405</td>
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<td>constructed wetlands</td>
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<td>2152</td>
<td>0.029</td>
<td>0.715</td>
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<tr>
<td>oxic zone</td>
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<td>0.995</td>
<td>2.795</td>
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<tr>
<td>anaerobic zone</td>
<td>24 d</td>
<td>0.996</td>
<td>5.470</td>
<td>2057</td>
<td>2095</td>
<td>0.017</td>
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<tr>
<td>anoxic zone</td>
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<td>1.000</td>
<td>3.090</td>
<td>448</td>
<td>446</td>
<td>0.096</td>
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<tr>
<td>constructed wetlands</td>
<td>24 d</td>
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<td>5.865</td>
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<td>oxic zone</td>
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<td>3.228</td>
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<td>anoxic zone</td>
<td>36 d</td>
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<tr>
<td>constructed wetlands</td>
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<td>1.000</td>
<td>2.449</td>
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<td>243</td>
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<td>0.446</td>
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<tr>
<td>oxic zone</td>
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<td>5.462</td>
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<td>2067</td>
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<td>0.726</td>
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<td>volcanic substrate</td>
<td>18 d</td>
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<td>volcanic substrate</td>
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<td>1361</td>
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</tbody>
</table>

The alpha diversity analysis used the Shannon, Chao, Ace, Simpson, and Shannon evenness indices. The Shannon index of the anaerobic zone was the smallest on d 36, and...
community diversity was the lowest. The Shannon index of the anoxic zone was the largest on d 24, and the community diversity was the highest. The constructed wetlands had the smallest Chao index and the lowest community diversity on d 36. The anoxic zone had the most extensive Chao index and the highest community diversity on d 24. The constructed wetlands had the smallest Ace index and the lowest community diversity on d 36. The anoxic zone had the smallest Simpson index and the most elevated community diversity on d 24. The anoxic zone had the most extensive Ace index and the highest community diversity on d 24. The constructed wetlands had the smallest Simpson index and the most elevated community diversity on d 24. The anoxic zone had the most extensive Shannon evenness index and the highest community diversity on d 24. The Simpson index was the most significant, and community diversity was the lowest in the constructed wetlands on d 12. The Shannon evenness index was the most significant, and community diversity was the lowest in the anaerobic zones on d 12. The Shannon evenness index was the most significant, and community diversity was the highest in the anoxic zones on d 24. The community diversity was the highest in the anoxic zones on d 24. The results showed that sludge from the anoxic zone on d 24 of the sample was the highest in community distribution abundance, diversity, and evenness.

The results of the microflora clustering tree and relative abundance analysis are shown in Figure 9. The bacterial samples collected on d 12, d 24, and d 36 are named Group 1, Group 2, and Group 3, and the volcanic substrate is named Group 4, as shown in Figure 9B,D.

Figure 9. Microbial community cluster tree and relative abundance analysis in A²O-MBBR combined with constructed wetlands reactor. (A) Combined analysis of sample clustering trees and histograms for bacterial samples at the phylum level; (B) Relative abundance plots for samples from different groups at the phylum level; (C) Combined analysis of sample clustering trees and histograms for bacterial samples at the gene level; (D) Relative abundance plots for samples from different groups at the gene level.
At the phylum level (Figure 9A,B), the microflora of the samples was dominated by *Proteobacteria*, whose relative abundance was 67.02%, 63.99%, 72.39%, and 71.66% in Group 1–4, respectively [27]. Similar to the results of the dilution curves, it may be that the more homogeneous the relative abundance of the different phyla in the sample colony is, the higher the alpha diversity of that colony is. The relative abundance of *Proteobacteria* decreased and then increased with the change in operation time and was higher in Group 3 than in Group 1, indicating that *Proteobacteria* dominated the mature activated sludge throughout the reactor. Significant differences in the relative abundance of *Planctomycetes* were found between the sludge and volcanic rock samples. Most bacteria in the phylum *Planctomycetes* are exclusively aerobic but include anaerobic ammonia-oxidizing bacteria, among others. This shows that the constructed wetlands have a suitable environment for the survival of *Planctomycetes* bacteria, strengthening the aerobic treatment capacity of the constructed wetlands.

At the genus level (Figure 9C,D), the relative abundance of *Tolumonas* in the constructed wetlands and anaerobic zones was significantly higher than other samples at d 12 versus d 24, with 33.91%, 28.91%, 20.24%, and 28.35% of the samples, respectively. The relative abundance of *Tolumonas* showed an accelerated decreasing trend during the run. It was replaced by *Herbaspirillum* and others, where the relative abundance of *Tolumonas* was 19.28% during the start-up stage, 13.12% during the water quality improvement stage, and only 0.08% during the shock loads stage, while the relative abundance of *Herbaspirillum* was 13.36%. The results showed that with the reactor’s operation, *Herbaspirillum* became the main denitrifying bacteria in the system, strengthening the reactor’s nitrogen removal efficacy. The dominant species in the volcanic substrate was *unclassified_Burkholderiales*, with a relative abundance percentage of 33.09%, which was widely present in the inter-root of *Hydrocotyle vulgaris*. *Burkholderia* belongs to the order *Burkholderiales* and is a plant-growth-promoting bacteria [28]. Thus, *unclassified_Burkholderiales* may have a growth-promoting effect on *Hydrocotyle vulgaris* [29]. In constructed wetlands, *unclassified_Burkholderiales* may enhance the uptake and utilization of nutrients such as nitrogen and phosphorus by promoting the growth of *Hydrocotyle vulgaris*, preventing the invasion of pathogens into *Hydrocotyle vulgaris*, improving the efficiency of nitrogen and phosphorus removal, and forming a healthy inter-root microbial community structure [30].

Microbial community similarities among structures and constructed wetlands at different stages at the gate level and genus level were explored by cluster analysis. It was found that anaerobic zones and constructed wetlands clustered together, oxic zones and aerobic zones clustered together, volcanic substrate was preferred, and anaerobic zones clustered with constructed wetlands.

4. Conclusions

(1) The removal rates of COD, NH$_4^+$-N, TN, and TP in low C/N simulated sewage by the combined $\text{A}^2\text{O}$-MBBR constructed wetlands process at the shock loads stage were 94.06%, 94.40%, 67.11%, and 84.57%, respectively, which were lower than the values stipulated in the Class I-A discharge standard of GB18918-2002.

(2) The root system of *Hydrocotyle vulgaris* and its attached microorganisms and the volcanic substrate in the combined $\text{A}^2\text{O}$-MBBR constructed wetlands enhanced the shock loading resistance of the scenario through adsorption and interception. It increased the nitrogen- and phosphorus-removal capacity of the reactor.

(3) A highly significant moderate positive correlation was observed between NH$_4^+$-N and TP in the anoxic zones, and NH$_4^+$-N inhibited phosphorus uptake by PAOs under anoxic conditions.

(4) The highest community diversity was found in the anoxic zone on d 24. The shock loads stage of water quality reduced the diversity of microbial communities in the combined $\text{A}^2\text{O}$-MBBR constructed wetlands reactor, with *Herbaspirillum* replacing *Tolumonas* as the dominant bacterial species.
(5) The mature activated sludge in the combined A\(^2\)O-MBBR constructed wetlands reactor was mainly *Proteobacteria*. *Planctomycetes* were the dominant species in the constructed wetlands, strengthening the aerobic treatment capacity of the constructed wetlands. *Unclassified Burkholderiales* enhanced the efficiency of nitrogen and phosphorus removal in the constructed wetlands by promoting the growth of *Hydrocotyle vulgaris* and providing a preferable environment for microorganisms to attach and immobilize.

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**References**


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