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# Application of Reeds as Carbon Source for Enhancing Denitrification of Low C/N Micro-Polluted Water in Vertical-Flow Constructed Wetland

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**Abstract:** Constructed wetlands have been applied to micro-polluted rivers and lakes. However, they often show poor nitrogen removal efficiency due to insufficient carbon sources for complete denitrification in the waters. In this study, a vertical-flow wetland system was built, in which reeds as a carbon source were added in the middle layer of the substrate. Thereby, the effect of the reed carbon source on denitrification of micro-polluted rivers and lakes with a low C/N ratio in the wetland and the denitrification mechanism were studied. The results showed that the concentrations of $NH_4^+$-N, $NO_3^-$-N and $NO_2^-$-N in the effluent of the constructed wetland were reduced to 0.17–0.35, 0.20–0.49 and 0.01–0.02 mg/L after adding the reed carbon source, and the removal efficiencies of the system for $NH_4^+$-N and $NO_3^-$-N reached 93.84% and 84.69%, respectively. The abundances of *nirK*, *nirS*, *hzo* and *nrfA* genes in the wetland substrate increased by 95.51%, 54.96%, 52.89% and 731.95%, respectively, which was considered to be related to the enhanced denitrification, anammox and dissimilatory nitrate reduction to ammonium of the wetland system. Reed planting promoted the increased abundances of *amoA* and *nxrB* genes, which might play a positive role in enhancing nitrification in wetland systems. The result of this study may provide a theoretical basis for the ecological restoration of low C/N micro-polluted water bodies.

**Keywords:** constructed wetland; low C/N micro-polluted water; reed carbon source; denitrification



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## 1. Introduction

At present, a large amount of low C/N effluents are discharged into surface rivers and lakes, causing serious eutrophication of water bodies. Therefore, nitrogen removal has become an urgent problem to be solved in surface water restoration. As a composite ecosystem, constructed wetlands can efficiently remove organic matter and nutrients such as nitrogen and phosphorus in wastewater through the diverse synergistic effects of substrates, plants and microorganisms. In addition, it has been widely used in the wastewater of urban sewage treatment plants, agricultural wastewater and industrial wastewater for deep processing [1,2], as well as storm-water control and treatment [3]. Studies have shown that the main pathways of nitrogen removal in constructed wetlands include nitrification, denitrification, anammox and dissimilatory nitrate reduction to ammonium (DNRA) driven by microbial metabolic activities [4]. The organic carbon source acts as an electron donor and energy source for microbial metabolism in the denitrification process, which is one of the main limiting factors of denitrification [5]. Micro-polluted rivers and lakes with low C/N levels often lack organic carbon sources, resulting in poor microbial denitrification in constructed wetlands. Therefore, researchers usually use an external carbon source to solve this problem.

Various carbon sources have been used to improve the denitrification performance of constructed wetlands. Conventional liquid low-molecular organic carbon sources such

as methanol, ethanol and acetate have problems such as rapid consumption, difficult storage, high price and difficult dosage control, and do not meet the criteria of sustainable development [6]. However, new carbon sources such as biodegradable polymers have complex manufacturing processes and high operating costs, so that large-scale applications cannot be achieved in the short term [7]. Therefore, finding a new carbon source with low price, high efficiency and environmental protection has become an urgent necessity for the research and development of constructed wetlands. As a high-carbon solid carbon source, the plant carbon source has the advantages of sufficient source and low cost. It can continuously degrade and release organic substances under the action of microbial enzymes, and has the characteristics of long-term release and convenient administration after one addition, and has gradually become a research hotspot [8].

At present, most municipal sewage treatment plants in China have a high removal efficiency for organic matters in wastewater, while the removal efficiency for TN is low. China's most stringent wastewater discharge standard (GB 18918-2002, China) for municipal sewage treatment plants allows total nitrogen levels (TN 15 mg/L) that are significantly higher than the Class IV standard (TN 1.5 mg/L) requirements for surface water quality (GB 3838-2002, China). During the dry season or near the outlets of the sewage treatment plants, the C/N value of the river usually reaches 2–3. While in the rainy season or far from the outflows of the sewage treatment plants, the C/N content of the river increases slightly after the dilution of the river water [9]. Jia et al. [10] showed that the wetland system denitrification process is significantly improved by using agricultural biomass materials such as wheat straw, apricot kernels and walnut shells as additional carbon sources in the treatment of nitrogen-rich wastewater with a TN concentration of 41.79 to 44.24 mg/L in constructed wetlands. Li et al. [11] used alkali-treated corn stalks as an additional carbon source to treat low C/N agricultural effluents with a TN concentration of 12 mg/L. The results showed that with a hydraulic residence time of 3 days, the TN removal rate of the constructed wetland increased by 37.2%. Zhang et al. [12] used *Platanus acerifolia* leaf litter as an additional carbon source to treat low C/N wastewater with a $NO_3^-$-N concentration of 20 mg/L in a laboratory-scale vertical-flow wetland. The results showed that the wetland system's TN removal rate increased by 36.04%. The above research results showed that plant carbon sources can effectively improve the denitrification effect of constructed wetlands. Reeds are one of the most widespread wetland plants in the world. If not harvested after ripening, it will rot in the wetland, increasing the risk of pollution and clogging. The reeds are high in carbon and low in nitrogen. A large amount of organic carbon is released during its decomposition to promote microbial denitrification, and reeds have good potential as a solid carbon source. It is therefore imperative to recycle and reuse reed waste. However, there are still few studies on reed carbon sources that promote denitrification in constructed wetlands. In addition, existing research mainly focuses on plant carbon sources to improve denitrification of heavily polluted water bodies with high TN and high $NO_3^-$-N concentrations, but little research has been conducted on denitrification of low C/N micro-polluted rivers and lakes with low TN concentration.

Therefore, in this study, by constructing a vertical-flow wetland system and adding a crushed reed carbon source in the middle layer of the substrate, the effect of the reed carbon source on the denitrification effect of low C/N micro-polluted water in wetlands was investigated, and real-time quantitative PCR was performed to determine the abundance of denitrification function genes in the substrate and study the reed carbon source mechanism for enhanced denitrification in constructed wetlands to provide theoretical foundations and technical support for the ecological restoration of micro-polluted rivers and lakes with low C/N levels in constructed wetlands.

## 2. Materials and Methods

### 2.1. Experimental Materials

The substrate used in this experiment is river sand with a particle size range of 0.5 to 2.0 mm. The reed biochar was purchased from Jinfeng Environmental Protection Company

in Zhengzhou, China, with a carbonization temperature of 400 °C and a particle size range of 1 to 2 mm. The reed carbon source was washed and dried in an oven at 70 °C and then ground into particles with a particle size range of 1–2 mm using a 150-mill. The fine gravel was ordinary building gravel with a particle size range of 5 to 10 mm. The activated sludge used for inoculation was taken from the anoxic zone of a sewage treatment plant in Nanjing. The water used in the test was low C/N micro-polluted water with excessive nitrogen nutrient content in simulated surface rivers and lakes [13]. It was made by adding $C_6H_{12}O_6$, $NH_4Cl$, $KNO_3$ and $KH_2PO_4$ to tap water. The water quality indicators are shown in Table 1. All chemicals mentioned above are analytical grade.

**Table 1.** Experimental water quality.

| Parameters | COD | TN | $NH_4^+$-N | $NO_3^-$-N | TP | pH |
|---|---|---|---|---|---|---|
| concentration mg/L | 30.00 | 6.00 | 4.00 | 2.00 | 0.30 | 7.00 |

### 2.2. Experimental Devices and Operation

The structure of the vertical-flow wetland device is shown in Figure 1. The main body of the device is a column structure with a diameter of 15 cm, a net height of 60 cm, and a water outlet at the bottom. From bottom to top, the wetland was divided into a 5 cm gravel layer, a 40 cm substrate layer and a 15 cm aquifer layer, and substrate sampling ports were established at the top, middle and bottom of the substrate layer. According to the different wetland substrate fillers, a total of 3 groups of treatment systems are set up with 2 parallel groups in each group: (1) The substrate consisted of evenly mixed reed biochar + river sand (mass ratio 1:9), the mass of the mixture was 9.0 kg, 16.7 g/kg of reed carbon source was added to the substrate, and the reed carbon source and substrate filler were mixed evenly in the middle layer of the wetland and then reeds were planted, recorded as a reed carbon source wetland (RW); (2) reed carbon source was added to the wetland matrix, recorded as the control wetland (CW); (3) The substrate composition was exactly the same as that of RW, but no reeds were planted, which was recorded as no-plant wetland (N-RW). Five reeds at the same growth stage and the same fresh weight were transplanted in both RW and CW, and the plant roots were placed 10 cm above the substrate. The average pore volume of the filler in the wetland device was measured to be 2.00 L.

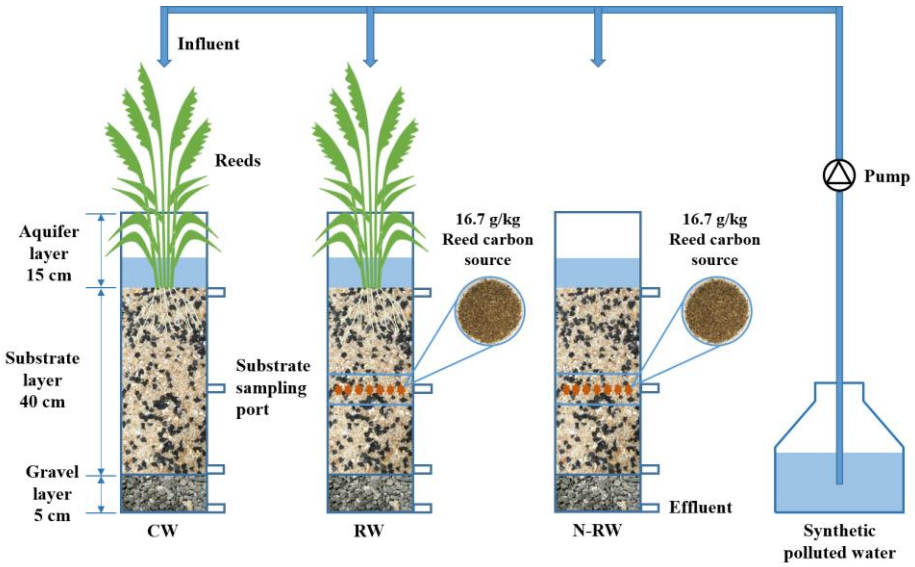


**Figure 1.** Schematic diagram of vertical-flow constructed wetland devices. Note: g/kg—the mass of reed carbon source per kilogram of substrate filler.

After the 3 groups of constructed wetlands were erected, the test water in Table 1 and the activated sludge filtered through two layers of gauze were fully mixed in a volume ratio of 7:3, and the mixed solution was added to each wetland and immersed substrate to make a hanging of the film on the substrate and the hydraulic retention time was 24 h. After 21 days of domestication, the $NO_3^-$-N concentration in the wetland effluent tended to be stable, and the wetland systems were commissioned as successful. The constructed wetland entered the continuous operation phase, and the simulated micro-polluted water was pumped into each wetland; the hydraulic residence time was 24 h, and the continuous operation was carried out at room temperature (15–25 °C) for 3 months.

### *2.3. Experimental Sampling and Analysis*

#### 2.3.1. Analytical Techniques

COD was measured by a 6B-200C COD rapid tester by rapid digestion spectrophotometry, with a range of 5–500 mg/L. $NH_4^+$-N was measured by Nessler's reagent spectrophotometry, with a range of 0.025–2 mg/L. $NO_3^-$-N was measured by ultraviolet and visible spectrophotometry, with a range of 0.08–4 mg/L. $NO_2^-$-N was measured by N-(1-naphthyl)-ethylenediamine dihydrochloride spectrophotometry, with a range of 0.003–0.20 mg/L. $NH_4^+$-N, $NO_3^-$-N and $NO_2^-$-N were all measured by the UV-5500 spectrophotometer; see *Monitoring and Analysis Methods of Water and Wastewater* for specific operation methods [14]. When the concentration exceeds the range, the water sample should be diluted accordingly.

#### 2.3.2. Water Sampling and Analysis

During the continuous operation of the constructed wetland, the quality of the influent and effluent of the wetland was monitored every 3 days and the COD, $NH_4^+$-N, $NO_3^-$-N, $NO_2^-$-N and pH were measured in the water samples. The determination methods of COD, $NH_4^+$-N, $NO_3^-$-N and $NO_2^-$-N are shown in Section 2.3.1. The pH was measured with a 400-type pH meter.

#### 2.3.3. Denitrogenation Functional Gene Abundance Sampling and Analysis

After the wetland operation, the upper, middle and lower substrates were sampled from the substrate sampling ports of the three groups of wetlands. The upper, middle and lower substrates of each group of wetlands were weighed with the same quality and mixed evenly, and sent to the Zhengzhou Research Center of the Cotton Research Institute of the Chinese Academy of Agricultural Sciences. Enzymes encoded by genes for nitrification, anammox, denitrification and dissimilatory nitrate reduction to ammonium (DNRA) and their specific catalytic functions are shown in Table 2. Real-time quantitative PCR was used to determine the abundances of denitrogenation function genes (*amoA*, *nxrB*, *hzo*, *nirK*, *nirS*, *nrfA*) in the wetland substrate of each group. The primer sequences of related genes were shown in Table 3, and the specific experimental steps referred to the method of Liang et al. [15].

**Table 2.** Enzymes encoded by genes for nitrification, anammox, denitrification and DNRA and their specific catalytic functions.

| Nitrogen Cycle Step | Gene | Enzyme | Catalytic Function |
|---|---|---|---|
| Nitrification | *amoA* | Ammonia monooxygenase | $NH_4^+ \rightarrow NO_2^-$ |
| Nitrification | *nxrB* | Nitrite oxidoreductase | $NO_2^- \rightarrow NO_3^-$ |
| Anammox | *hzo* | Hydrazine oxidase | $N_2H_2 \rightarrow N_2$ |
| Denitrification | *nirK* and *nirS* | Nitrite reductase | $NO_2^- \rightarrow NO$ |
| DNRA | *nrfA* | Nitrite reductase | $NO_2^- \rightarrow NH_4^+$ |

**Table 3.** List of the primers for qPCR.

| Targeting Gene | Primer | Sequence (5′-3′) | Reference |
|---|---|---|---|
| *amoA* | amoA-1F<br>amoA-2R | GGGGTTTCTACTGGTGGT<br>CCCCTCKGSAAAGCCTTCTTC | Rotthauwe et al., 1997 [16] |
| *nxrB* | nxrB-169F<br>nxrB-638R | TACATGTGGTGGAACA<br>CGGTTCTGGTCRATCA | Pester et al., 2014 [17] |
| *hzo* | hzocl1F1<br>hzocl1R2 | TGYAAGACYTGYCAYTGG<br>ACTCCAGATRTGCTGACC | Schmid et al., 2008 [18] |
| *nirK* | nirK-1aCuf<br>nirK-3Cur | ATCATGCTSCTGCCGCG<br>GCCTCGATCAGRTTGTGGTT | Henry et al., 2004 [19] |
| *nirS* | cd3af<br>r3cd | GTSAACGTSAAGGARACSGG<br>GASTTCGGRTGSGTCTTGA | Liu et al., 2010 [20] |
| *nrfA* | nrfAF2aw<br>nrfAR1 | CARTGYCAYGTBGARTA<br>TWNGGCATRTGRCARTC | Welsh et al., 2014 [21] |

### *2.4. Statistical Analysis*

All statistical analyses were performed using SPSS 20.0 (SPSS Inc., Chicago, IL, USA) and data were subjected to a one-way ANOVA and a multi-sample Kruskal–Wallis ANOVA, an independent nonparametric test. For all tests, differences and correlations were considered statistically significant if $p < 0.05$.

## 3. Results and Discussion

### *3.1. Nitrogen Removal Effect of Reed Carbon Source Constructed Wetland*

#### 3.1.1. Removal Effect of $NH_4^+$-N

As shown in Figure 2, the $NH_4^+$-N concentration of the influent from RW, CW and N-RW was 3.95–4.90 mg/L and the $NH_4^+$-N concentration of the effluent was 0.17–0.35, 0.14–0.39 and 0.30–0.52 mg/L, respectively. The corresponding average removal rates of $NH_4^+$-N were 93.84 ± 1.22%, 94.01 ± 1.57% and 90.70 ± 1.38%, respectively. It was seen that the concentration of $NH_4^+$-N in the effluent of the three groups of wetlands was low, and the removing effect of $NH_4^+$-N was good and stable. The comparison between RW and CW showed that the addition of the reed carbon source had no significant impact on $NH_4^+$-N removal in constructed wetlands ($p > 0.05$). Wetland nitrification mainly occurred in the top layer of the substrate and reeds were planted in both RW and CW, which increased the dissolved oxygen content in the top layer of the substrate and promoted the nitrification reaction [22]. The reed carbon source in RW was added to the middle layer of the substrate, which had little impact on the nitrification of the top layer of the substrate, so the $NH_4^+$-N removal rates of RW and CW were almost the same.

The comparison between RW and N-RW showed that the $NH_4^+$-N removal rate of RW was 3.14% higher than that of N-RW wetlands, and reed planting could improve the $NH_4^+$-N removal effect of constructed wetlands. The oxygen secretion function of reed roots could create an alternating aerobic/anoxic environment in the wetland, increase the number and activity of denitrifying microorganisms, and help synchronize the occurrence of nitrification and denitrification. Some rhizosphere microorganisms in the wetland could promote the growth of reeds and improve the absorption of nitrogen in the sewage by reeds. At the same time, plant absorption could also restore adsorption capacity of wetland substrates and promote adsorption of $NH_4^+$-N in wastewater by wetland substrates. The three synergistically enhanced the removal of $NH_4^+$-N from wetlands [23]. However, the removal rate of 3.14% is comparatively lower, but the phenomenon still works for constructed wetlands. Perhaps the plants would not have grown enough to ensure greater uptake of $NH_4^+$-N in addition to nitrification. Similar behavior was reported by Albuquerque et al. where plant growth is one of the main issues with constructed wetland startup [24].

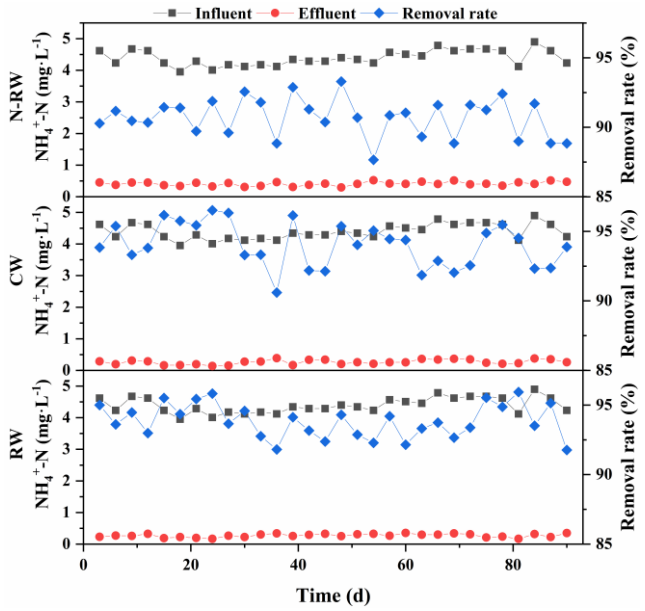


**Figure 2.** Changes of $NH_4^+$-N concentration and corresponding removal rate in influent and effluent of three groups of wetland systems.

### 3.1.2. Removal Effect of $NO_3^-$-N

From Figure 3, it can be seen that the influent $NO_3^-$-N concentration of RW, CW and N-RW was 2.06 to 2.71 mg/L and the $NO_3^-$-N concentration of the effluent was 0.20 to 0.49, 2.72 to 3.55 and 0.25 to 0.59 mg/L. The corresponding average removal rates of $NO_3^-$-N were 84.69 ± 3.35%, −31.29 ± 12.63% and 83.76 ± 3.22%, respectively. The comparison between RW and CW showed that the $NO_3^-$-N concentration in the RW effluent was low and stable, and the $NO_3^-$-N removal rate was increased by 115.98% compared to CW. The addition of a reed carbon source could significantly improve the $NO_3^-$-N removal effect of constructed wetlands ($p < 0.05$). The decomposition of the reed carbon source in RW created an anoxic/anaerobic environment with a high concentration of organic matter in the middle and lower layers of the substrate, which greatly enhanced the denitrification process in the system. At the same time, the abundant pore structure on the surface of the reed carbon source could also be used as a carrier for the growth and attachment of denitrifying bacteria, which together promoted the removal of $NO_3^-$-N from wetlands [25]. The $NO_3^-$-N concentration in the CW effluent was higher than in the influent mainly because the denitrification rate was low due to the lack of organic carbon. In addition, the high nitrification rate in CW promoted the continuous conversion of $NH_4^+$-N into $NO_3^-$-N in the influent, which accumulated a large amount of $NO_3^-$-N in CW.

The comparison between RW and N-RW showed that the $NO_3^-$-N removal rate of RW was only 0.93% higher than that of N-RW, and reed planting had no significant impact on $NO_3^-$-N removal in constructed wetlands ($p > 0.05$). This is because only five reeds were planted in RW and the state of reed development does not allow a higher role for nitrogen uptake and nitrogen removal. The removal of nitrogen forms in the wetland were mainly associated to nitrification, denitrification and some non-conventional pathways, as strongly observed by Albuquerque et al. [26].

### 3.1.3. Removal Effect of $NO_2^-$-N

In the process of microbial denitrification, $NO_3^-$-N must go through the reduction process of $NO_3^-N \rightarrow NO_2^-N \rightarrow NO \rightarrow N_2O \rightarrow N_2$ and is finally converted into gaseous nitrogen, such as $N_2O$ or $N_2$, and discharged. However, if the organic carbon source in the wetland is insufficient, the nitrite reductase will be at a disadvantage in competing with the nitrate reductase for electron donors, so the reaction stops at the $NO_2^-$-N generation stage, resulting in the accumulation of $NO_2^-$-N [27]. Therefore, the $NO_2^-$-N concentration

in wetland effluent is one of the important indicators for evaluating the denitrification stability of constructed wetlands.

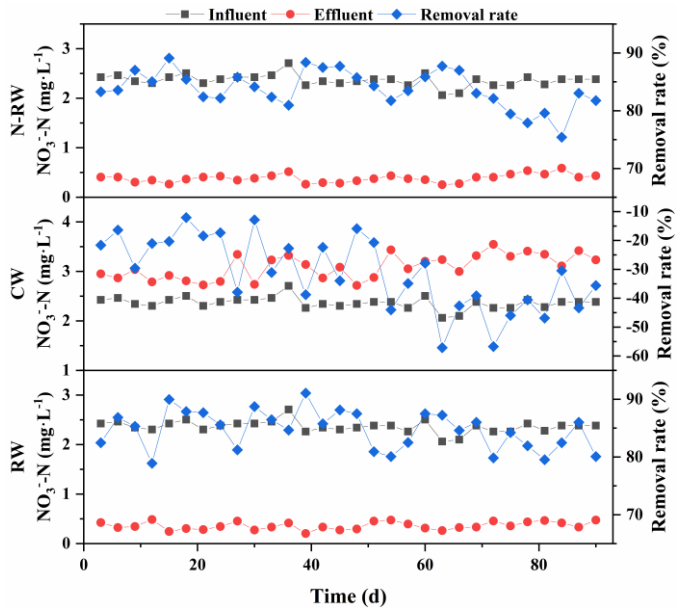


**Figure 3.** Changes of $NO_3^-$-N concentration and corresponding removal rate in influent and effluent of three groups of wetland systems.

As shown in Figure 4, the influent water of RW, CW and N-RW did not contain $NO_2^-$-N; the effluent $NO_2^-$-N concentration was 0.01–0.02, 0.03–0.04 and 0.01–0.02 mg/L, and the corresponding mean values were 0.02 ± 0.00, 0.03 ± 0.00 and 0.02 ± 0.00 mg/L. It was found that the $NO_2^-$-N concentration in the effluent of the three groups of constructed wetlands was at a low level. The reed carbon source in RW could provide a sufficient organic carbon source for denitrifying bacteria. The denitrification reaction in RW was relatively thorough, and there was no accumulation of $NO_3^-$-N and $NO_2^-$-N, indicating that RW had high denitrification stability. Although the $NO_2^-$-N concentration in the CW effluent was only slightly higher than that of RW, the reduction rate of $NO_3^-$-N in the process of microbial denitrification was reduced due to the lack of organic carbon sources in the CW, resulting in the accumulation of a large amount of $NO_3^-$-N in the wetland [27]. Therefore, the TN-removing effect of CW was poor.

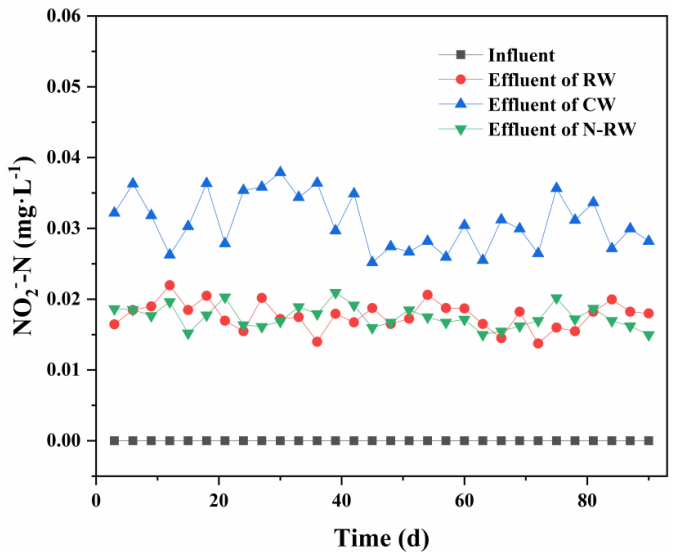


**Figure 4.** Changes of $NO_2^-$-N concentration in influent and effluent of three groups of wetland systems.

The comparison between RW and N-RW showed that reed planting had no significant effect on $NO_2^-$-N removal in constructed wetlands ($p > 0.05$). This may be due to the small number of reeds planted in RW in this experiment, which did not have a significant impact on $NO_2^-$-N removal. The main limiting factor in the wetland denitrification process was still whether the organic carbon source was sufficient [28].

### *3.2. Effects of Reed Carbon Source on COD and pH of Constructed Wetland Effluent*

#### 3.2.1. Changes in COD of Wetland Effluent

It can be seen from Figure 5 that the COD concentration of the influent from RW, CW and N-RW was 29.15–36.45 mg/L and the COD concentration of the effluent was 26.06–29.75, 12.49–19.99 and 38.34–49.60 mg/L, respectively. The corresponding average removal rates of COD were 14.65 ± 4.35%, 48.29 ± 7.67% and −28.17 ± 9.79%, respectively. The comparison between RW and CW showed that the addition of a reed carbon source could increase the COD concentration of wetland effluents to a certain extent, and the COD removal rate was 33.64% lower than that of CW, but the COD concentration of the effluent from RW was stable and less than 30 mg/L, which met the corresponding surface water standard, i.e., Class IV (*Environmental Standards for Surface Water Quality* GB 3838-2002, China); COD, TN, $NH_4^+$-N and TP should be below 30, 1.5, 1.5 and 0.3 mg/L, respectively [29]. This was because the reed carbon source in RW could release organic matter continuously and stably, which significantly increased the organic matter content in the wetland. Most of the organic matter was removed by substrate adsorption, microbial aerobic degradation and denitrification consumption, and the remaining organic matter would flow out of the wetland system with the effluent [30].

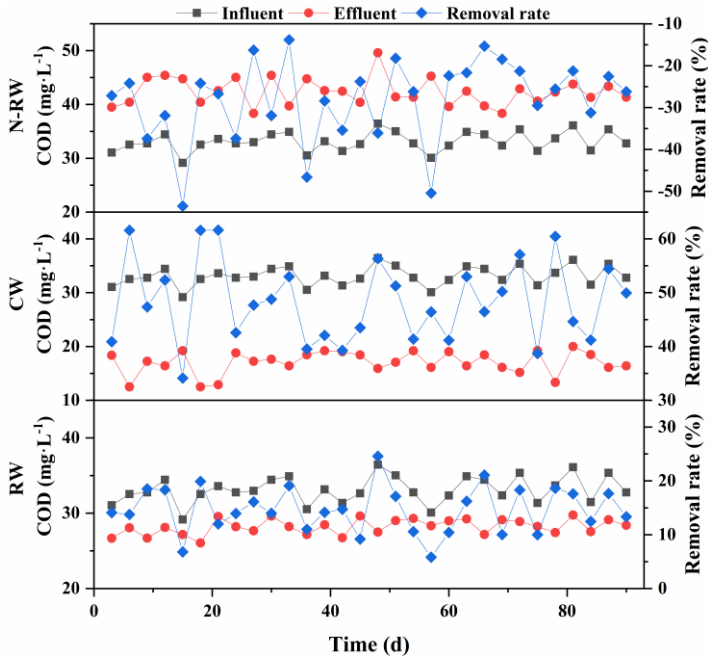


**Figure 5.** Changes of COD concentration and corresponding removal rate in influent and effluent of three groups of wetland systems.

The comparison between RW and N-RW showed that the COD removal rate of RW was 42.82% higher than that of N-RW. Planting reeds could significantly improve the COD removal effect of constructed wetlands ($p < 0.05$). The entwined reed roots played a role in loosening the wetland substrate and carrying oxygen to the plant roots, improving the dissolved oxygen environment in the substrate. At the same time, the reed roots could provide an attachment surface for the growth and reproduction of microorganisms and promote the degradation of organic matter in the wetland by aerobic microorganisms [31].

3.2.2. Changes in pH of Wetland Effluent

As shown in Figure 6, the influent pH of RW, CW and N-RW was 6.96–7.18; the effluent pH of wetland was 7.51–7.72, 7.86–8.08 and 7.16–7.41, respectively, and the corresponding mean values were 7.60 ± 0.04, 7.94 ± 0.05 and 7.27 ± 0.07, respectively. It was evident that the pH of the effluent from the three groups of constructed wetlands was higher than that of the influent. This was mainly because the conversion of $NO_3^-$-N in the wetland into $N_2$ under the action of denitrifying bacteria generated alkalinity at the same time. The reed char in the substrate contained mineral elements like Ca and Mg which would also make the water basic after hydrolysis [32].

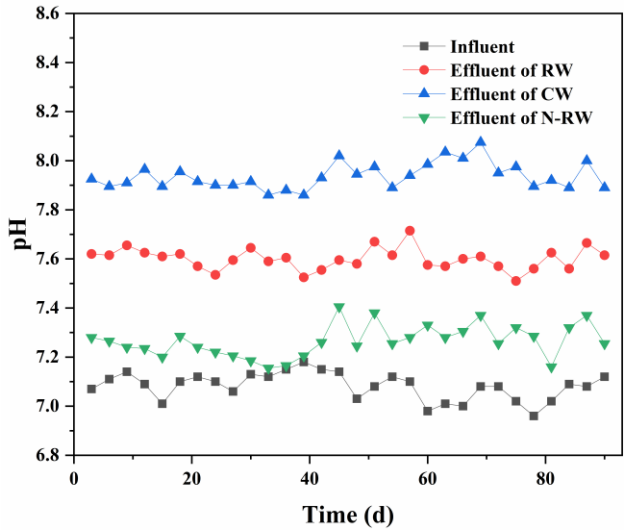


**Figure 6.** Changes of pH in influent and effluent of three groups of wetland systems.

Comparison between RW and CW showed that the addition a of reed carbon source could significantly decrease the pH of effluent from constructed wetlands ($p < 0.05$). Hydrolysis of the reed carbon source in RW could liberate some organic acids. As the concentration of organic matter in the system increased, anaerobic fermentation became the main way to remove organic matter, and acidic metabolites would be produced, leading to a decrease in pH of wetland effluents [33]. However, the pH of the RW effluent was still in the range of 7.51 to 7.72, which was suitable for metabolic reproduction of denitrifying microorganisms.

The comparison between RW and N-RW showed that reed planting could significantly improve the pH of the wastewater in constructed wetlands ($p < 0.05$). The oxygen secretion function of reed roots could improve the dissolved oxygen content and redox potential in the wetland substrate, which promoted the degradation of organic matter in the system by aerobic microorganisms and reduced the production of organic acids. At the same time, the exudates from the reed roots could be used as organic carbon sources to promote microbial denitrification, increasing the alkalinity generated by the denitrification reaction [34]. Therefore, the pH of the effluent from RW is higher than that from N-RW.

*3.3. Effects of Reed Carbon Source on Denitrification Function Genes*

3.3.1. Changes in Abundances of *amoA*, *nxrB* and *hzo* Genes

Constructed wetlands mainly remove $NH_4^+$-N from polluted waters mainly through microbial nitrification and anammox. The nitrification process of ammonia nitrogen consists of two steps. First, ammonia-oxidizing bacteria (AOB) oxidizes ammonia to nitrite, and nitrite is further oxidized to nitrate by nitrite-oxidizing bacteria (NOB) [35]. The first step is the rate-limiting step of nitrification, in which ammonia monooxygenase is encoded by the *amoA* gene, and the nitrite oxidoreductase in the second step is encoded by the *nxrB* gene [36]. As shown in Figure 7a,b, the mean abundances of *amoA* and *nxrB* genes in RW, CW and N-RW were 2.55, 2.74 and 1.90 copies/μg and 1.04, 1.17 and 0.73 copies/μg,

respectively. Compared with CW, the abundances of *amoA* and *nxrB* genes in RW were only reduced by 7.12% and 11.46%. The addition of the reed carbon source in the middle layer of the wetland substrate had no significant effect on the abundances of *amoA* and *nxrB* genes, and would not inhibit the nitrification reaction in the system. The comparison of RW and N-RW showed that reed planting had a significant effect on the abundances of *amoA* and *nxrB* genes. The abundances of *amoA* and *nxrB* genes in RW increased by 34.29% and 41.24%, respectively, compared with N-RW. This suggested that reed planting could increase the abundance of nitrification genes in substrates and thereby improve nitrification in wetland systems.

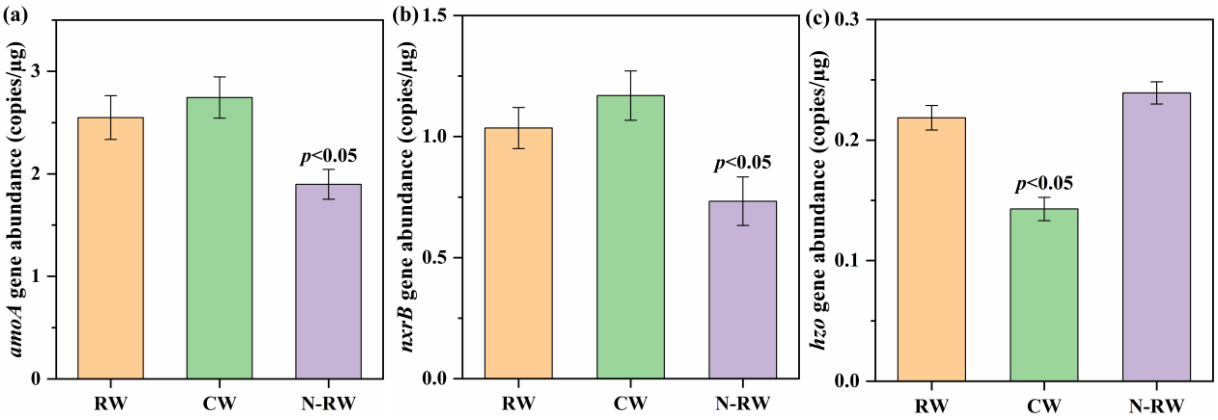


**Figure 7.** (**a**) *amoA* gene abundance; (**b**) *nxrB* gene abundance; (**c**) *hzo* gene abundance in three groups of wetland systems. Note: $p < 0.05$ compared to RW.

Anammox is ubiquitous in aquatic environments. The anammox bacteria use $NO_2^-$-N in the wastewater as an electron acceptor and $NH_4^+$-N as an electron donor to oxidize $NH_4^+$-N to $N_2$. The *hzo* gene is essential for anammox, and controls the conversion of the intermediate hydrazine ($N_2H_4$) to the final product $N_2$ [37]. As shown in Figure 7c, the mean abundance of *hzo* gene in RW, CW and N-RW was 0.22, 0.14 and 0.24 copies/μg, respectively. Comparison of RW and CW showed that addition of the reed carbon source had a significant effect on *hzo* gene abundance. The abundance of the *hzo* gene in RW increased by 52.89% compared to CW. This suggested that the addition of a reed carbon source could increase the abundance of the *hzo* gene in substrates and thereby improve anammox in wetland systems. Comparing RW and N-RW showed that reed planting had no significant effect on *hzo* gene abundance. The abundance of the *hzo* gene in RW was only 8.63% lower than in N-RW. Comparing Figure 7a–c, it was seen that the abundance of the *hzo* gene was lower than that of the *amoA* and *nxrB* genes, indicating that the contribution of anammox was lower than that of nitrification.

### 3.3.2. Changes in Abundances of *nirK*, *nirS* and *nrfA* Genes

The addition of the reed carbon source to the constructed wetland substrate created external conditions for microbial denitrification and dissimilatory nitrate reduction to ammonium (DNRA). The process of converting $NO_3^-$-N to $N_2$ by denitrification involves four steps: $NO_3^-N \rightarrow NO_2^-$-N, $NO_2^-N \rightarrow NO$, $NO \rightarrow N_2O$ and $N_2O \rightarrow N_2$ [38]. The process of reducing $NO_2^-$-N to NO is an important rate-limiting step in denitrification. Nitrite reductase is the rate-limiting enzyme that performs this step and the corresponding coding genes are *nirK* and *nirS* [15]. As shown in Figure 8a,b, the mean abundances of the *nirK* and *nirS* genes in RW, CW and N-RW were 1.26, 0.64 and 1.37 copies/μg and 228.72, 147.60 and 243.12 copies/μg, respectively. It was seen that the abundance of the *nirS* gene was much higher than that of the *nirK* gene, indicating that the nitrite reductase in the three wetland groups was mainly encoded by the *nirS* gene. Comparison of RW and CW showed that the addition of the reed carbon source had a significant effect on the abundances of *nirK* and *nirS* genes. Compared to CW, the abundances of *nirK* and

*nirS* genes in RW increased by 95.51% and 54.96%, respectively. This suggested that the addition of a reed carbon source could increase the abundance of denitrification genes in substrates, thereby improving denitrification in wetland systems. Comparison of RW and N-RW showed that reed planting had no significant impact on the abundance of *nirK* and *nirS* genes. The abundances of *nirK* and *nirS* genes in RW were only 8.01% and 5.92% lower than that in N-RW, respectively.

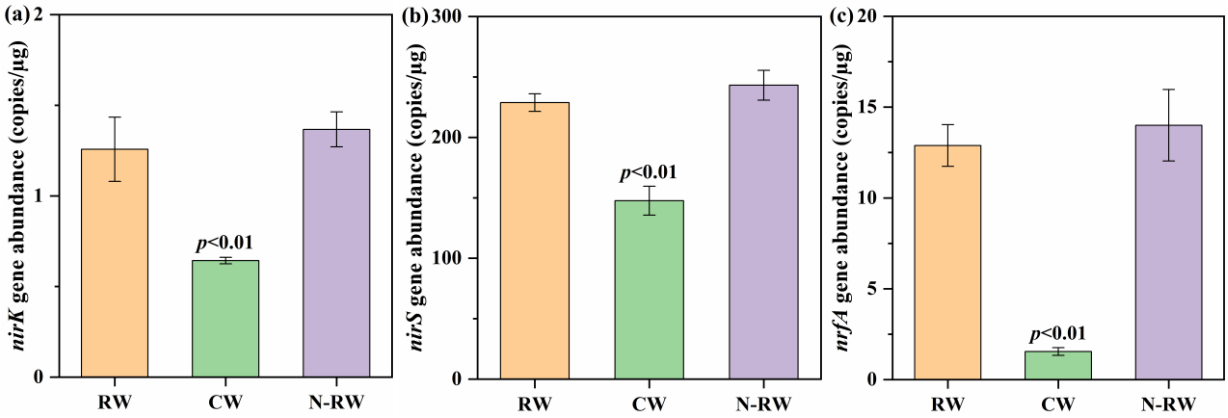


**Figure 8.** (**a**) *nirK* gene abundance; (**b**) *nirS* gene abundance; (**c**) *nrfA* gene abundance in three groups of wetland systems. Note: $p < 0.01$ compared to RW.

DNRA can reduce $NO_3^-$-N to $NH_4^+$-N, and then $NH_4^+$-N is further removed by microbial nitrification, denitrification and anammox. It can be seen that DNRA is an important linkage process in the nitrogen cycle and plays an important role in the nitrogen cycle of constructed wetlands. The reduction of nitrite to ammonium by nitrite reductase is a key step in DNRA, and its encoding gene is *nrfA* [39]. It can be seen from Figure 8c that the mean abundance of the *nrfA* gene in RW, CW and N-RW were 12.89, 1.55 and 14.00 copies/μg, respectively. Comparison of RW and CW showed that the addition of the reed carbon source had a significant effect on the abundance of the *nrfA* gene. The abundance of the *nrfA* gene in RW increased by 731.95% compared to CW. This suggested that the addition of a reed carbon source could increase the abundance of the *nrfA* gene in substrates and thereby improve DNRA in wetland systems. Comparing RW and N-RW showed that reed planting had no significant effect on the abundance of the *nrfA* gene. The abundance of the *nrfA* gene in RW was only 7.96% lower than in N-RW.

## 4. Conclusions

When treating low C/N micro-polluted water with constructed wetlands, adding a reed carbon source could greatly increase the organic matter content in the wetland and greatly improve the removal effect of $NO_3^-$-N and $NO_2^-$-N by the wetland. The concentrations of $NH_4^+$-N, $NO_3^-$-N and $NO_2^-$-N in wetland effluent were reduced to 0.17–0.35, 0.20–0.49 and 0.01–0.02 mg/L, respectively, and the removal rates of $NH_4^+$-N and $NO_3^-$-N reached 93.84% and 84.69%. The addition of a reed carbon source increased the abundances of *nirK*, *nirS*, *hzo*, and *nrfA* genes in the substrate by 95.51%, 54.96%, 52.89% and 731.95%, respectively, thereby improving the denitrification, anammox and DNRA in the wetland system. Wetland reeds increased the abundances of *amoA* and *nxrB* genes by 34.29% and 41.24%, respectively, improving the nitrification in the wetland system.

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