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Changes in Wastewater Treatment Performance and the Microbial Community during the Bioaugmentation of a Denitrifying *Pseudomonas* Strain in the Low Carbon–Nitrogen Ratio Sequencing Batch Reactor

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Abstract: The low carbon–nitrogen ratio (C/N) of influent wastewater results in the insufficient carbon source for the process of denitrification in urban wastewater treatment plants (WWTPs). A denitrifying bacterial strain, *Pseudomonas* sp. JMSTP, was isolated and demonstrated effective denitrification ability under a low C/N ratio of 1.5–4 (*w*/*w*) in anoxic conditions. Sequencing batch reactor (SBR) studies were conducted to test the bioaugmentation of JMSTP on total nitrogen (TN) removal under the influent COD/N ratio of 3/1. After the second bioaugmentation, the TN of effluent in the bioaugmented SBR was significantly lower than that in the control SBR. Redundancy analysis results showed that there was a positive correlation between *Pseudomonas* sp. abundance and TN removal in the bioaugmented SBR. Microbial community analysis showed that, especially after the second bioaugmentation, the abundance of *Pseudomonas* sp. decreased rapidly, but it was still much higher than that in the control SBR. Correlation network analysis showed that after the addition, *Pseudomonas* sp. had no significant co-occurrence relationship with other native bacteria, owing to the quick increase and decrease. Our results suggest that JMSTP shows the potential to enhance TN removal through bioaugmentation. Since the effect of bioaugmentation gradually diminishes, further research is still needed to investigate its long-lasting applications.

Keywords: bioaugmentation; denitrification; network analysis; *Pseudomonas*; low C/N ratio

Citation: Chen, T.; Yang, X.; Sun, Q.; Hu, A.; Qin, D.; Li, J.; Wang, Y.; Yu, C.-P. Changes in Wastewater Treatment Performance and the Microbial Community during the Bioaugmentation of a Denitrifying *Pseudomonas* Strain in the Low Carbon–Nitrogen Ratio Sequencing Batch Reactor. *Water* **2022**, *14*, 540. https://doi.org/10.3390/w14040540

Academic Editors: Dibyendu Sarkar, Rupali Datta, Prafulla Kumar Sahoo and Mohammad Mahmudur Rahman

Received: 29 December 2021
Accepted: 8 February 2022
Published: 11 February 2022

1. Introduction

With the rapid development of the economy and the continuous increase in urbanization, water pollution has been a severe problem in China. Therefore, the environmental standards implemented at existing wastewater treatment plants have become stricter [1]. For example, the effluent standard of total nitrogen (TN) discharged from wastewater treatment plants has been improved from 20 mg/L to 15 mg/L, or even 10 mg/L in certain areas, and therefore a more effective strategy for TN removal will be required. Bioaugmentation, which enhances the removal of pollutants in original systems by adding functional strains or populations [2–5], has shown the potential to improve nitrogen removal in wastewater treatment systems [6,7].

In the full-scale conventional nitrification–denitrification process, removing TN is the conversion of ammonia to nitrate via nitrite with oxygen, and the conversion of nitrate to

nitrogen gas or nitrous oxide via nitrite in the anoxic environment [8]. The whole biochemical reaction is catalyzed by several known enzymes, such as ammonia monooxygenase, nitrite oxidoreductase, nitrate reductase, nitrous oxide reductase, and the functional genes, including *amo*A, *nar*G, *nir*, *nor*B, *nos*Z, were characterized [9,10]. In general, heterotrophic denitrifying bacteria are involved in the conventional nitrification–denitrification process. As the carbon-to-nitrogen ratio in China's wastewater influents can be as low as about 3 or less (*w*/*w*), insufficient carbon limits denitrification in the entire nitrogen removal process [2,11]. Therefore, strains with denitrification functions at low C/N ratios have attracted increasing attention. The isolation and characterization of highly functional strains with high denitrification efficiencies at low C/N ratios are important. To date, *Paracoccus*, *Pseudomonas*, *Bacillus*, *Alcaligenes*, and *Rhodococcus* sps. have been reported to have a denitrification efficiency at low C/N [12–15]. However, limited studies have successfully inoculated selected bacteria into activated sludge systems by bioaugmentation to improve nitrogen removal efficiency. Therefore, further bioaugmentation studies are needed to evaluate denitrification performance and understand the mechanism based on the structure and function of microbial communities.

In natural environments, microorganisms do not exist individually, but they directly or indirectly form complex microbial interactions, such as mutualism, symbiosis, parasitism, predation, favoritism, and competition. Interaction has positive, negative, and neutral effects on microbial populations. Denitrification was boosted via inter-genus cooperation, competitive and symbiotic relationships between key taxa (*Azoarcus*, *Paracoccus*, *Thauera*, *Stappia*, and *Pseudomonas* sps.), and other heterotrophic bacteria in aeration reactors to construct the optimal niche contributing to the high TN removal efficiency [16]. Chen et al. used a mixture of heterotrophic nitrification–aerobic denitrification bacteria, including *Agrobacterium tumefaciens* sp. LAD9, *Comamonas testosteroni* sp. GAD3, and *Achromobacter xylosoxidans* sp. GAD4, to enhance the denitrification of a pilot-scale SBR [17]. The results show that when the COD/N ratio is 8, the effluent could meet the first class requirement of the National Municipal Wastewater Discharge Standards of China (TN $\leq$ 15 mg/L). Based on the community analysis results, they considered that although the inoculated mixed bacteria were present in low abundance, they might have a synergistic effect with other indigenous microorganisms in the reactor. Therefore, whether bioaugmented functional microorganisms can have benign interactions with the original microorganisms in the system is of particular concern. High-throughput sequencing data contains large amounts of population information and has the great advantage of exploring microbial interrelationships. The combination of network analysis and high-throughput sequencing provides a valuable research tool for discovering the microbial relationships needed for the construction and stable maintenance of microbial communities [18].

This study aims to improve nitrogen removal efficiency by bioaugmentation in the laboratory-scale bioreactor and understand the underlying mechanism through the changes of microbial communities. First, a denitrifying bacterial strain was isolated from activated sludge, and the denitrification characteristics of the strain were studied. The performance of nitrogen removal ability of the isolated strain was investigated in a laboratory-scale sequencing batch reactor (SBR) by bioaugmentation. The denitrification performance of the inoculated reactor is discussed with reference to the characteristics of the microbial community and the relative abundance of the bioaugmented strain. Furthermore, the shift of microbial communities is linked and discussed.

2. Materials and Methods

2.1. Media and Isolation Strategy

Three media for isolating facultative anaerobic denitrifying bacteria are as follows:

Enrichment medium (CH_3COONa = 2.84 g; KNO_3 = 2 g; KH_2PO_4 = 0.5 g; $MgSO_4 \cdot 7H_2O$ = 0.41 g; NaCl = 3.28 g; pH = 7.2–7.5; distilled water = 1000 mL; C/N = 3/1; COD/N = 8/1).

Isolation medium (CH_3COONa = 2.84 g; KNO_3 = 2 g; KH_2PO_4 = 1 g; K_2HPO_4 = 1 g; $FeCl_2 \cdot 6H_2O$ = 0.5 g; $CaCl_2 \cdot 7H_2O$ = 0.2 g; $MgSO_4 \cdot 7H_2O$ = 1 g; NaCl = 10 g; Agar = 20 g; distilled water = 1000 mL; bromothymol blue (BTB, 1% in ethanol) = 1 mL/L; pH = 7.2–7.5).

Fermentation medium (peptone = 10 g/L; yeast extract = 5 g/L; NaCl = 10 g/L; pH = 7.2–7.5).

Denitrifying bacteria were isolated from activated sludge collected from a sewage treatment plant in Xiamen, China. The sludge was slightly treated with a tissue homogenizer. Nitrogen gas was blown into the enrichment medium for more than 15 min, sealed, and sterilized. Then, sludge was added for static culture. The medium was transferred when nitrate was consumed. During the transfer process, the C/N ratio and initial concentrations of NO_3^--N gradually decreased, eventually reaching by as low as 1.5 (*w*/*w*) and 200 mg/L, respectively. Bacteria in the logarithmic phase in the enrichment medium were diluted with gradients of 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5} and evenly spread on isolation medium plates. The light blue area indicated by bromothymol blue (BTB) was selected for further picking and streaking until pure bacteria were obtained [19].

2.2. Batch Experiment

The growth and denitrification capability of isolated strain JMSTP under different dissolved oxygen conditions were examined. The DO of anoxic environment was set to lower than 0.1 mg/L, and that of aerobic environment was set to higher than 8 mg/L. CH_3COONa and KNO_3 were used as the only carbon and nitrogen sources in batch experiments with different C/N ratios or different incubation temperatures, and the initial nitrogen concentration of the medium was 200 mg/L. The inoculation volume ratio was 5%. C/N of the batch experiment was set to 1, 1.5, 2, 3, and 4, respectively. The incubation temperature was set as 15 °C, 20 °C, 25 °C, and 30 °C, respectively.

The selected strain JMSTP was applied for the nitrate removal from actual wastewater in shake flasks. When the microorganisms were in the logarithmic growth phase in the fermentation medium at 30 °C, they were collected by centrifugation (8000 rpm and 10 min). A total of 0.39 g of the dry cell weight was added to 500 mL of the actual wastewater from the inlet of the sewage plant spiked with 30 mg/L NO_3–N. Control flasks were not inoculated with functional microorganism. They were placed in a shaker at 30 °C and 100 rpm, and duplicate flasks were conducted for each experimental setup. Samples were collected every 24 h for a total of 4 days, and pretreated by centrifugation (8000 rpm and 5 min) before further analysis.

2.3. Bioreactor Operation Strategy

Two 6 L SBRs were set up using artificial wastewater as the influent. One was for experimentation (JMSTP SBR), and the other was for control (control SBR). The mixed liquid suspended solid (MLSS) was set to 3000 mg/L using activated sludge collected from the sewage treatment plant in Xiamen, China. The SBR cycle time was 6 h, and the hydraulic retention time (HRT) was 12 h. The operational strategy was as follows: Fill (20 min) → Idle stage (60 min) → Aeration stage (120 min) → Anoxic/Anaerobic stage (90 min) → Settle stage (60 min) → Draw (10 min). The first inoculation of strain JMSTP was performed after 20 days when the two sets of reactors were stable for effluent TN in 18–22 mg/L. The inoculation dosage was 5% MLSS (*w*/*w*). This means adding strain JMSTP to achieve 150 mg/L in the JMSTP SBR, but no additional bacteria was added to the control SBR. Ten days later, the same inoculation dosage was conducted again for the second bioaugmentation. The effluent was collected once daily on the 1st–20th and 37th–50th days, and effluents from three different sequencing cycles per day were collected on 21–36 days after the inoculation. The composition of the artificial wastewater was as follows: $C_6H_{12}O_6$ (Glucose anhydrate) = 71.52 mg; $(NH_4)_2SO_4$ = 120 mg; $NaHCO_3$ = 220 mg; KH_2PO_4 = 24 mg; $MnCl_2$ = 0.19 mg; $CuCl_2$ = 0.022 mg; $MgSO_4$ = 5.6 mg; $FeCl_3 \cdot 6H_2O$ = 0.88 mg; $CaCl_2$ = 1.3 mg; $ZnCl_2$ = 0.0018 mg; distilled water to 1000 mL. The artificial wastewater contained COD of 76.53 mg/L and TN of 25.45 mg/L with COD/N = 3:1 (C/N = 9:8, *w*/*w*).

2.4. Analytical Methods

The cell optical density was measured at 600 nm (OD600) using a spectrophotometer (Metash, UV-5200). COD was detected by a COD analysis kit (5B-3C (V8), Lianhua Technology). NH_4^+–N, NO_3^-–N, and TP were detected using a flat-bottom 96-well plate method [20]. The MLSS was tested according to standard method procedures.

Polymerase chain reaction (PCR)-agarose gel electrophoresis was used to detect functional genes in the strain, including *nir*S, *nir*K, *cnor*B, *qnor*B, and *nos*Z, according to previous studies [21–23], and the details of primers are shown in Supplementary Materials Table S1. The DNA of strain JMSTP was extracted using FastDNA SPIN Kit for soils (Qbiogene-MP Biomedicals, Irvine, CA, U.S.A). A total of 10–50 ng of DNA was added to 25 μL of reaction solution containing 12.5 μL of TaqMan® Gene Expression Master Mix (2×) (Applied Biosystems, CA, U.S.A.), 1 μM of each primer, and sterile ddH_2O.

2.5. Microbial Community Analysis

DNA was extracted using FastDNA SPIN Kit for soils (Qbiogene-MP Biomedicals, Irvine, CA, U.S.A.). The V4-V5 region of 16S rRNA genes was amplified using a universal primer pair 515F (5′-GTG YCA GCM GCC GCG GTAA-3′) and 907R (5′-CCG YCA ATT YMT TTR AGT TT-3′). The samples were sequenced on an Illumina Miseq platform (Illumina Inc., San Diego, CA, USA) with the paired-end approach (2 × 300 bp) [24]. Amplicon sequencing data was processed using the LotuS pipeline, as described in the previous study [25]. The quality control settings were adjusted as follows: (1) average sequence quality > 27; (2) sequence length > 170 bp; (3) no ambiguous bases; (4) no barcode and primer mismatches; (5) homopolymer < 8 bp. The obtained qualified reads were chimera-checked and clustered into operational taxonomic units (OTUs) with a 97% identity cutoff using the UPARSE method [26]. Subsequently, the taxonomic assignment of each OTU was made using the RDP classifier in SILVA v132 as the reference database [27], with a bootstrap cutoff of 80% using the QIIME pipeline [28]. All samples were randomly subsampled to the smallest library size (105,000 reads) to standardize the uneven sequencing efforts. Data were uploaded to NCBI with numbers PRJNA706543. Twelve samples of each reactor from the 20th–23rd and 30th–37th days were analyzed (24 samples in total), with labels of B1 (1 day before the first bioaugmentation), D1_1~3 (1st–3rd days after the first bioaugmentation), and B2 (1 day before the second bioaugmentation), and D2_1~7 (1st–7th days after the second bioaugmentation), respectively.

Statistical analysis was performed using R × 64 4.0.2 (http://www.R-project.org/ access on 28 December 2021). The community diversity was calculated using R package vegan [29] and package picante [30]. Network analysis was conducted to evaluate the relationships between the species in the reactors using the R package igraph [31] and Hmisc [32]. The OTUs, with the occurrence of at least 75% in the samples, were used to build the network. The network was visualized using Gephi 0.9.2 (https://gephi.github.io/ access on 28 December 2021). Redundancy analysis (RDA) was analyzed using R package vegan [29] and drawn by Hellinger distance.

3. Results

3.1. Isolation of Nitrogen Removal Bacteria

After several months of enrichment with KNO_3 as the only nitrogen source, one of thirteen isolated strains was selected due to its better nitrogen removal efficiency. This strain belongs to the genus *Pseudomonas*, with high similarity to *Pseudomonas songnenensis* sp. NEAU-ST5-5(T) (99.51%, Figure 1a). Therefore, it is named *Pseudomonas* sp. JMSTP. Based on the scanning electron microscope imaging technique, strain JMSTP has a rod-like shape (Figure 1b). Its growth and denitrification ability was tested under both aerobic and anaerobic conditions (Figure 2). The strain showed exponential growth and reached a stable stage after 12 h of inoculation under aerobic conditions. The denitrification capacity achieved a NO_3^-–N removal efficiency of 20.4%. Under anaerobic conditions, the strain grew relatively slowly and reached a stable stage within 30 h, while its denitrification

capacity reached 100% within 24 h, with a brief accumulation of nitrite. Denitrification using nitrite was verified using CH_3COONa and $NaNO_2$ as the sources of carbon and nitrogen at the initial concentrations of 300 and 100 mg/L, respectively. The results show that JMSTP could remove more than 97% of nitrite within 12 h (Table S2). As shown in Figure S1, the strain is associated with positive PCR reactions for genes *nir*S, *cnor*B, and *nos*Z. The sequencing of the PCR products suggested that the strain harbors the genes of interest and were deposited in NCBI with accession numbers OL442066, OL442065 and OL456216, respectively.

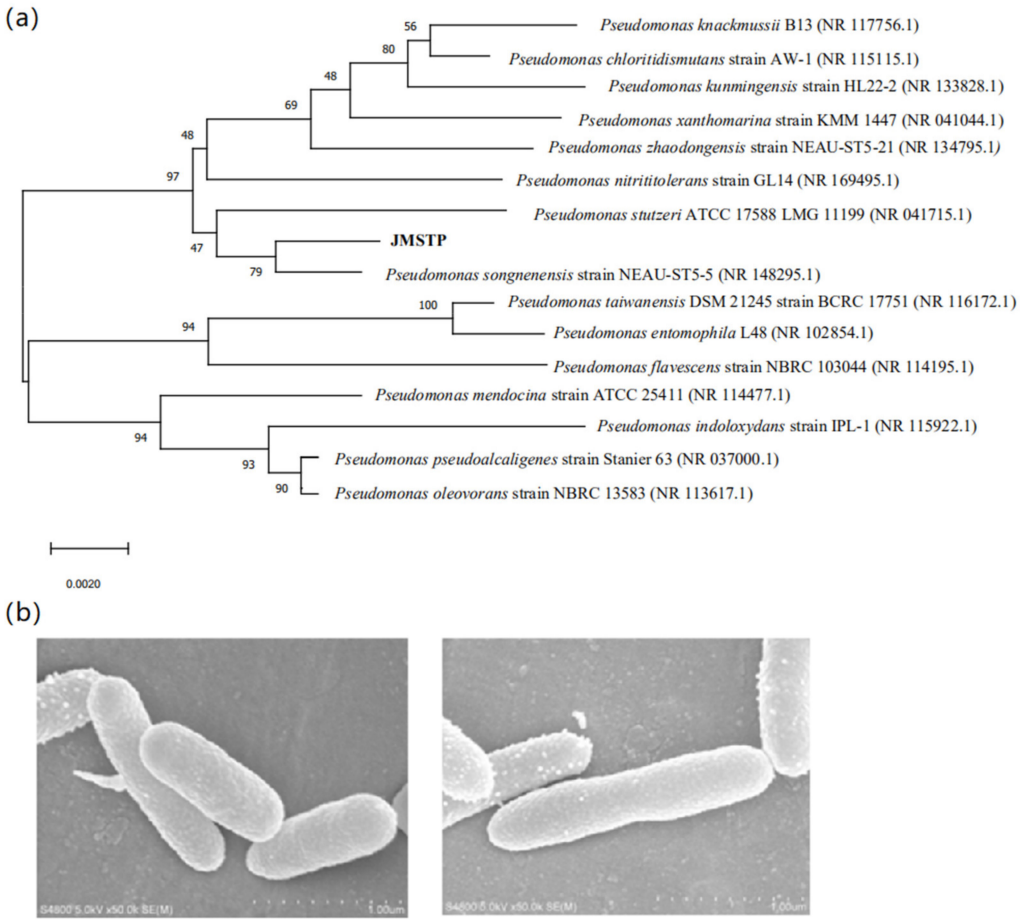

Figure 1. Phylogenetic tree derived from 16S rRNA gene sequence of strain JMSTP (**a**) and scanning electron microscope of strain JMSTP (**b**).

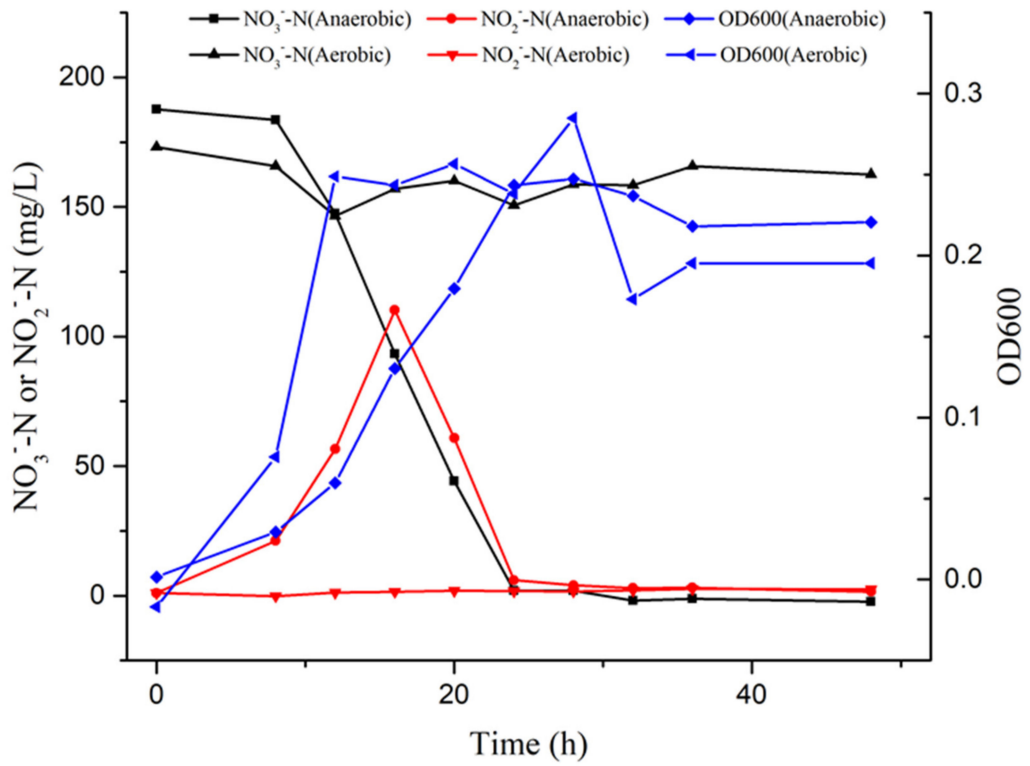

Figure 2. Growth and denitrification of strain JMSTP under different dissolved oxygen conditions (25 °C, C/N = 1.5, the DO of anaerobic < 0.1 mg/L, aerobic > 8 mg/L).

3.2. Denitrification in the Batch Study

Since the low C/N ratio limits denitrification in wastewater treatment systems, denitrification efficiencies were tested by strain JMSTP at the C/N ratios of 1, 1.5, 2, 3, and 4. As shown in Figure 3a, strain JMSTP has a rapid denitrification ability in the C/N range of 1–4. Removal efficiencies were 100% within 38 h for C/N ratios 3 and 4, but 77.62, 96.45, and 90.43% for C/N ratios 1, 1.5, and 2, respectively. In addition, the denitrification ability of strain JMSTP was tested at 15, 20, 25, and 30 °C with a C/N ratio of 3. As shown in Figure 3b, complete denitrification is achieved in the temperature range of 20–30 °C.

Attempts were made to see if strain JMSTP can be adapted to real urban sewage. As shown in Figure 4, fast denitrification is achieved in the JMSTP group with NO_3^-–N concentrations reduced to 1 mg/L within 24 h, but to 12 mg/L in the control group. Nitrate concentrations increased slightly from 24 to 96 h, and this may be due to aerobic nitrification or the release of nitrate due to the death and decomposition of sludge microorganisms [33,34]. The removal efficiency of NO_3^-–N was 87.20% at 96 h with strain JMSTP, which was higher than 71.15% in the control group.

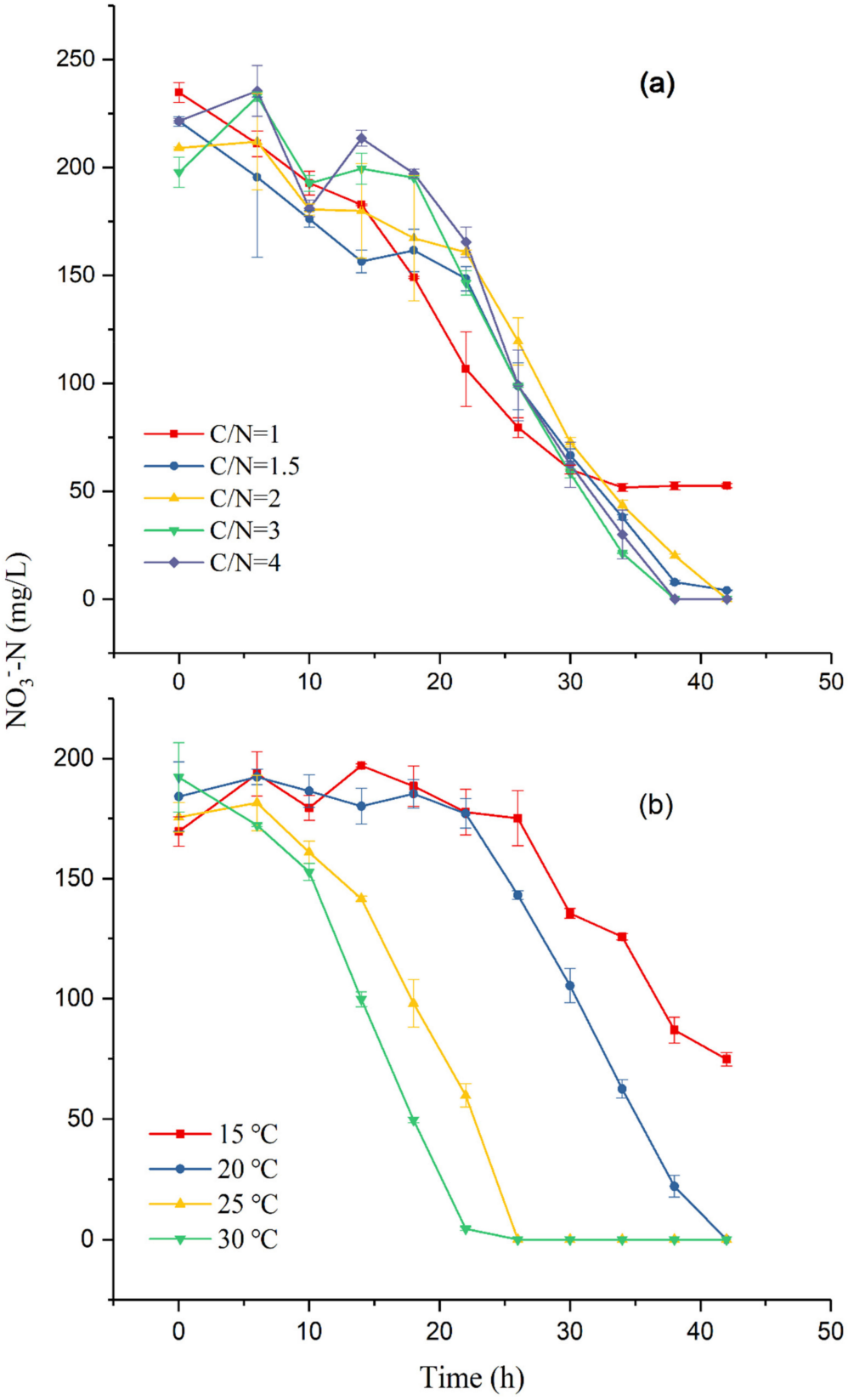

Figure 3. Removal of nitrate concentrations by strain JMSTP under different C/N ratios (**a**) and temperatures (**b**).

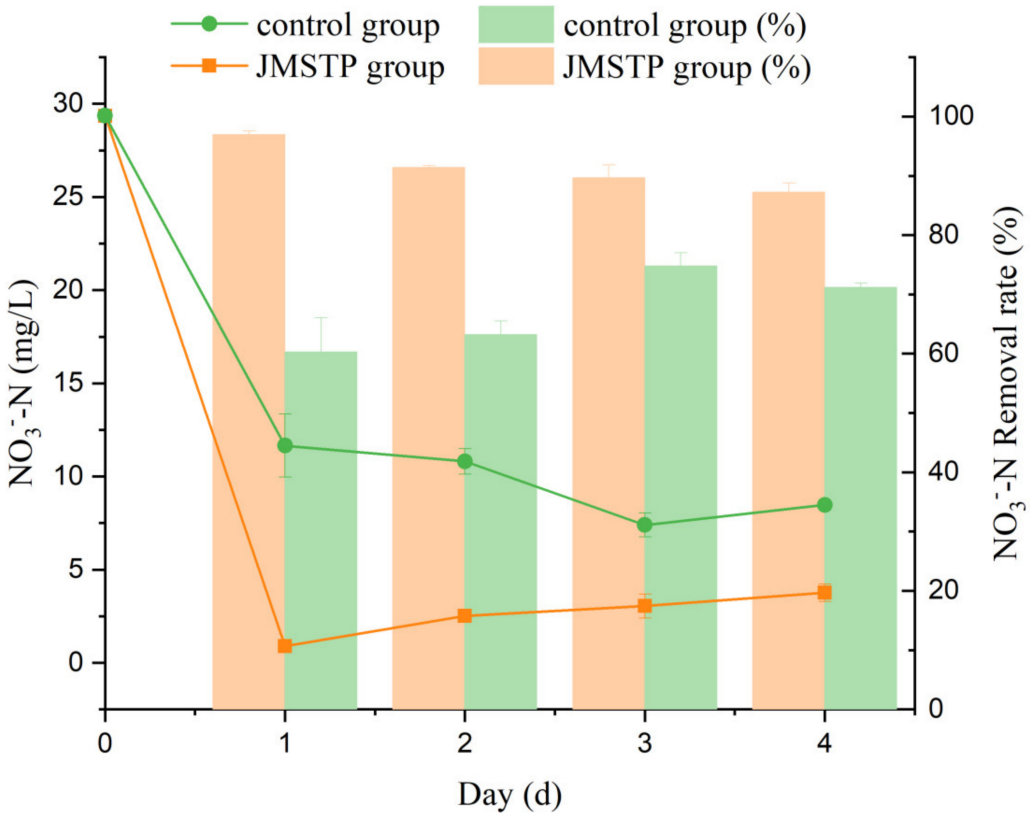

Figure 4. Removal of nitrate concentrations by strain JMSTP in real wastewater.

3.3. Denitrification in SBR

Laboratory-scale SBRs were performed to test nitrogen removal using activated sludge with the bioaugmentation of strain JMSTP. Particular emphasis was placed on denitrification efficiencies under harsh conditions with a carbon to nitrogen ratio of 9:8 (*w*/*w*). Figure 5 shows the results of TN in effluent. A total of 30 cycles were analyzed within the 10 days of the first inoculation, of which 21 cycles of JMSTP SBR had lower TN in effluent than control SBR. However, there was no significant difference in TN of effluent between the two reactors (*t*-test, $p > 0.05$). The effluent of the two cycles after our inoculation was visually turbid. The COD of the effluent in JMSTP SBR can be observed to increase sharply in the cycle after inoculation (Figure 5) and reaches 110 mg/L. However, when the effluent sample was filtered through a 0.45 μm syringe filter, the soluble COD dropped to about 40 mg/L. After the second inoculation, although COD still appeared as a sharp rise after inoculation, the TN concentration in JMSTP SBR was consistently lower than that in the control SBR (*t*-test, $p < 0.05$). This indicates the improvement of nitrogen removal by strain JMSTP bioaugmentation. TN decreased to 10.53–15.54 mg/L from days 4 to 7 after inoculation in the JMSTP SBR, while it was 18.08–22.07 mg/L in the control SBR, under the influent TN of 25.45 mg/L. The TN residue in the effluent, which was lower than 16 mgN/L, and met the criteria of the Grade I standard A (TN $\leq$ 20 mgN/L) in China's wastewater discharge regulation.

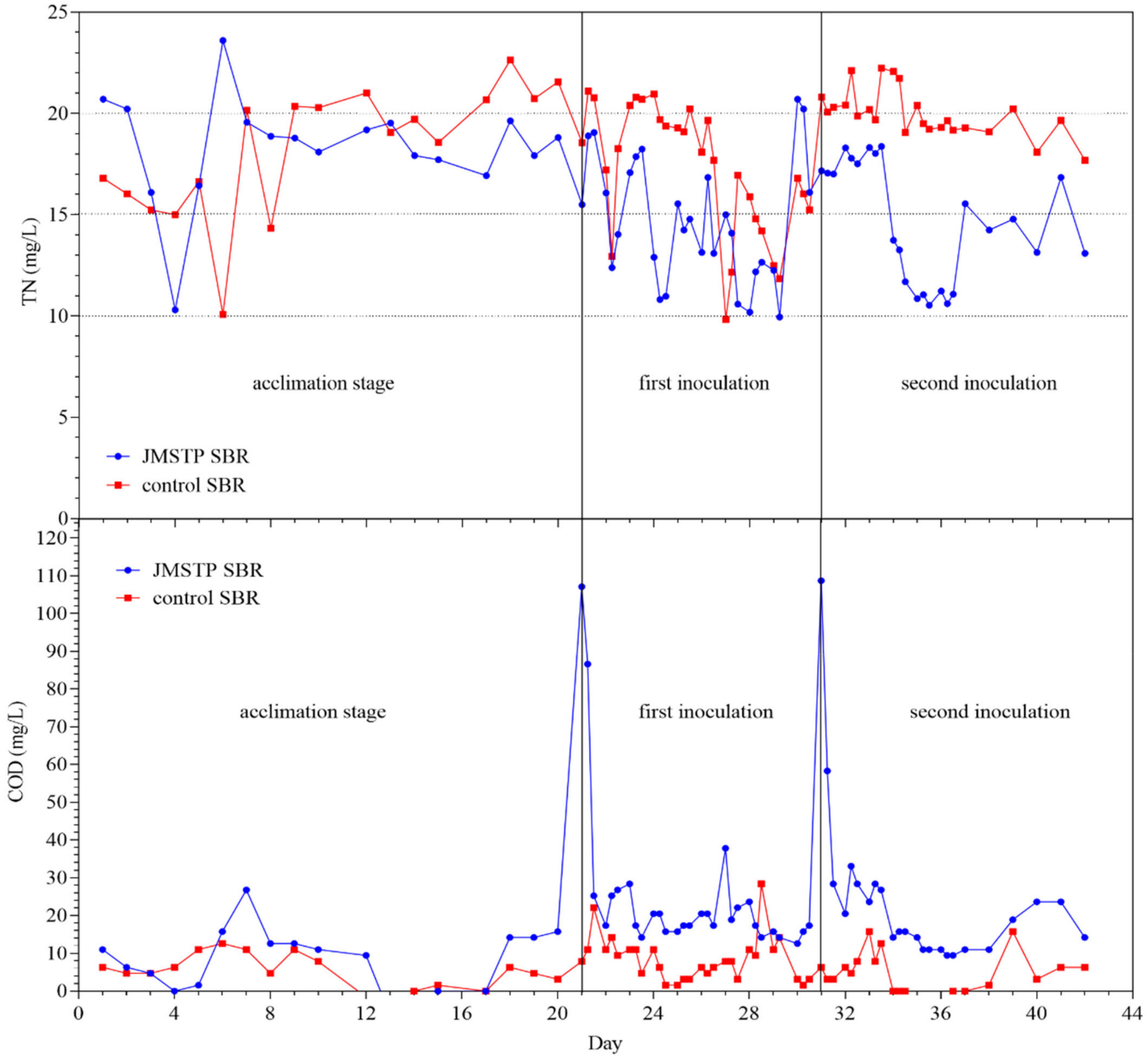

Figure 5. Variations of TN and COD concentrations in the effluent of the JMSTP SBR and control SBR using artificial wastewater.

3.4. Microbial Community Analysis in Lab-Scale SBR

The diversity of the microbial community and the correlation of different microorganisms in the experimental and control SBRs were analyzed using high-throughput sequencing techniques. Alpha diversity is a comprehensive indicator of richness (e.g., Chao1; Ace), evenness (e.g., Pielou), and the results of microbial diversity, e.g., the Shannon index and Simpson index, are shown in Table S3. The Goods coverage was over 99%, indicating that the sequencing depth basically covered the majority of the species in the samples. These indices of diversity generally showed similar trends between the two reactors. However, the indices after the day of each bioaugmentation in the JMSTP SBR decreased abruptly, but they recovered quickly from the second day. This means that the addition of strain JMSTP could only temporarily affect the diversity of the microbial community within the reactor. Chen et al. operated a bioaugmentation reactor and a control

reactor, and they found that the Shannon–Weaver indices of two reactors were similar under long-term operation [17], which is in agreement with our findings. The proportion of OTUs shared between the two SBRs fluctuate between 41.1% and 48.5% within 12 sampling days (shown in Table S4). However, the microbial communities of the two reactors had a similar percentage of shared OTUs before the bioaugmentation of strain JMSTP, implying bioaugmentation did not largely change the OTUs in the reactor [35].

At the phylum level, the dominant phyla in the control reactor are *Bacteroidetes*, *Actinobacteria*, *Proteobacteria*, *Chloroflexi*, *Nitrospirae*, and *Planctomycetes* sps. The JMSTP SBR has one additional dominant phylum, which is *Synergistetes* sp. (relative abundance > 0.01). The types of core microorganisms at the phylum level are generally similar between the two reactors. A bar graph of relative abundance at the genus level is shown in Figure 6. *Pseudomonas* sp. JMSTP showed a significant decrease on the second day after each inoculation, which was consistent with the COD results. However, the relative abundance of *Pseudomonas* sp. JMSTP was much higher than that of the control SBR at the range of 20.98–150.73% from days 4 to 7, after the second inoculation, resulting in the apparent decrease in effluent TN in JMSTP SBR. RDA was used to explore the relationship between environmental parameters and microbial community distribution in the JMSTP SBR. As shown in Figure 7, there is a negative correlation between *Pseudomonas* sp. JMSTP and TN of effluent; in other words, with the increase in JMSTP abundance in the reactor, the TN of effluent decreases.

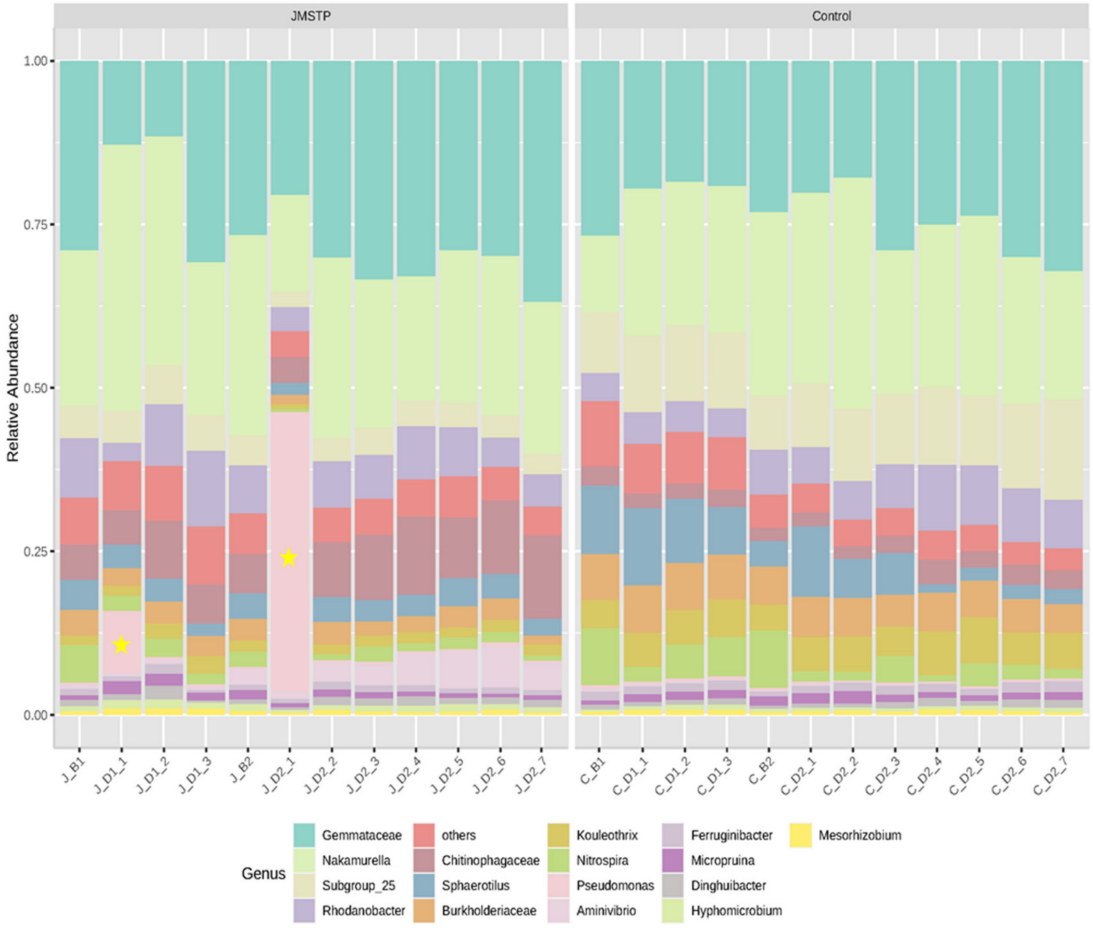

Figure 6. Relative abundance of the bacterial genera of the microbial community from reactors. C stands for control SBR, and J stands for JMSTP SBR.

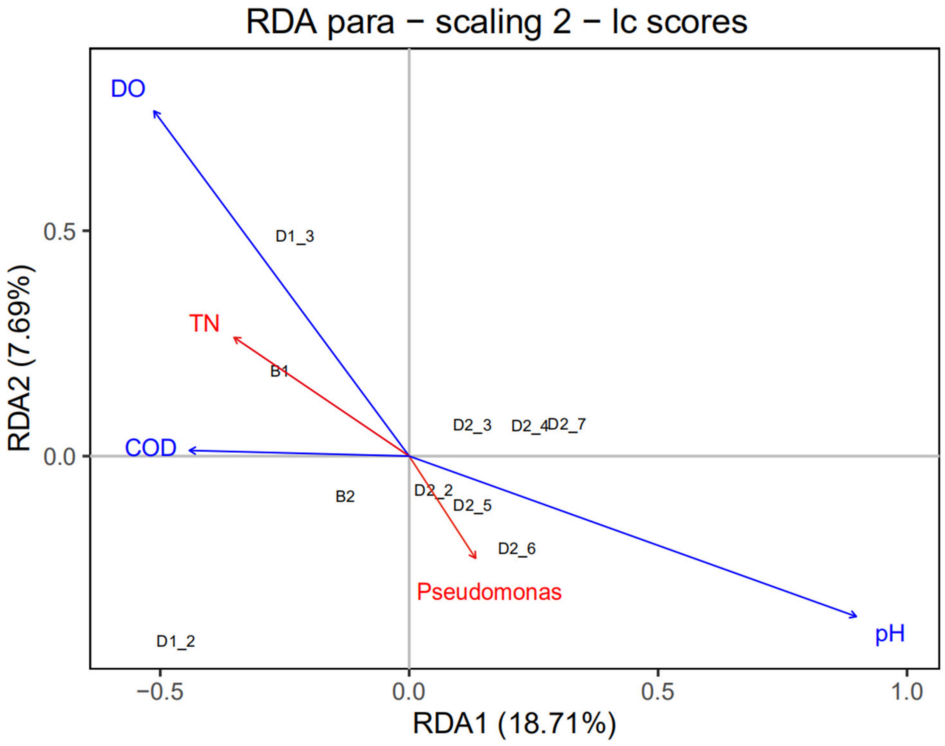

Figure 7. RDA between environmental parameters and microorganisms.

Correlation network analysis is used to explore the possible relationships between the microorganisms in two reactors. The R package was used to calculate the Spearman's correlation coefficient. The significance (*p*-value) and correlation (R-value) are controlled at 0.05 and 0.6, respectively. The genus-level visualization results are shown in Figure 8, and each node represents a genus category. The color of the node represents a different phylum. As shown in Figure 8a, the phylum categories that appear on the nodes are *Proteobacteria* (41.18%), *Planctomycetes* (19.61%), *Chloroflexi* (13.73%), *Acidobacteria* (9.8%), *Actinobacteria* (5.88%), and *Bacteroidetes* sps. (3.92%). The intergeneric correlation shown in Figure 8a is mainly positive, with two negative correlations between *Terrabacter* and *Aminivibrio* sps., and *Pseudoxanthomonas* and *Hydrogenophaga* sps. Figure 8b shows the results of network analysis for the control SBR. It is worth noting that *Pseudomonas* sp. JMSTP demonstrated a co-occurrence pattern with other native bacteria in the reactor. In addition, the control SBR showed more genus nodes and relationships than JMSTP SBR.

(**a**)

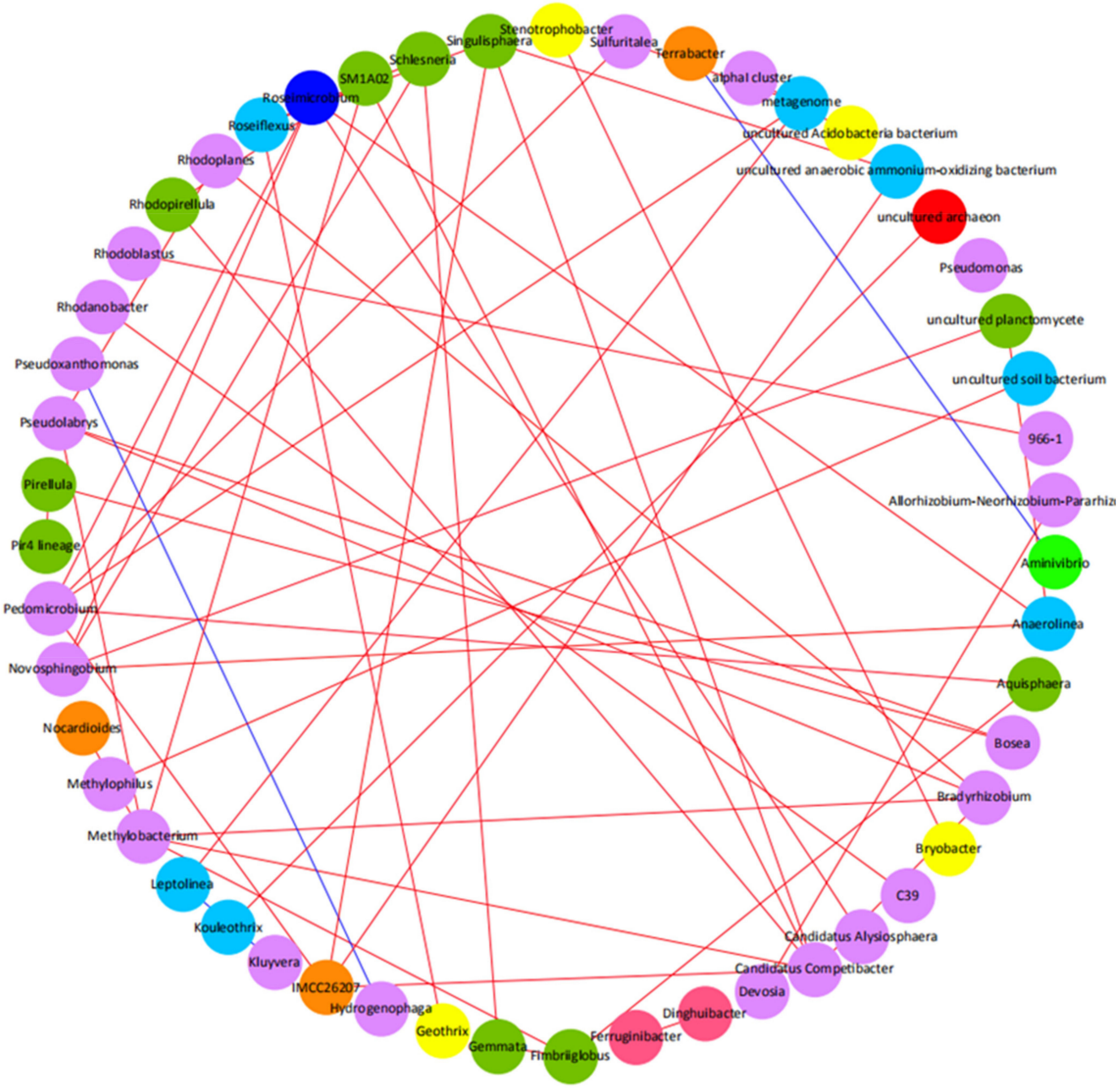

Figure 8. *Cont.*

(**b**)

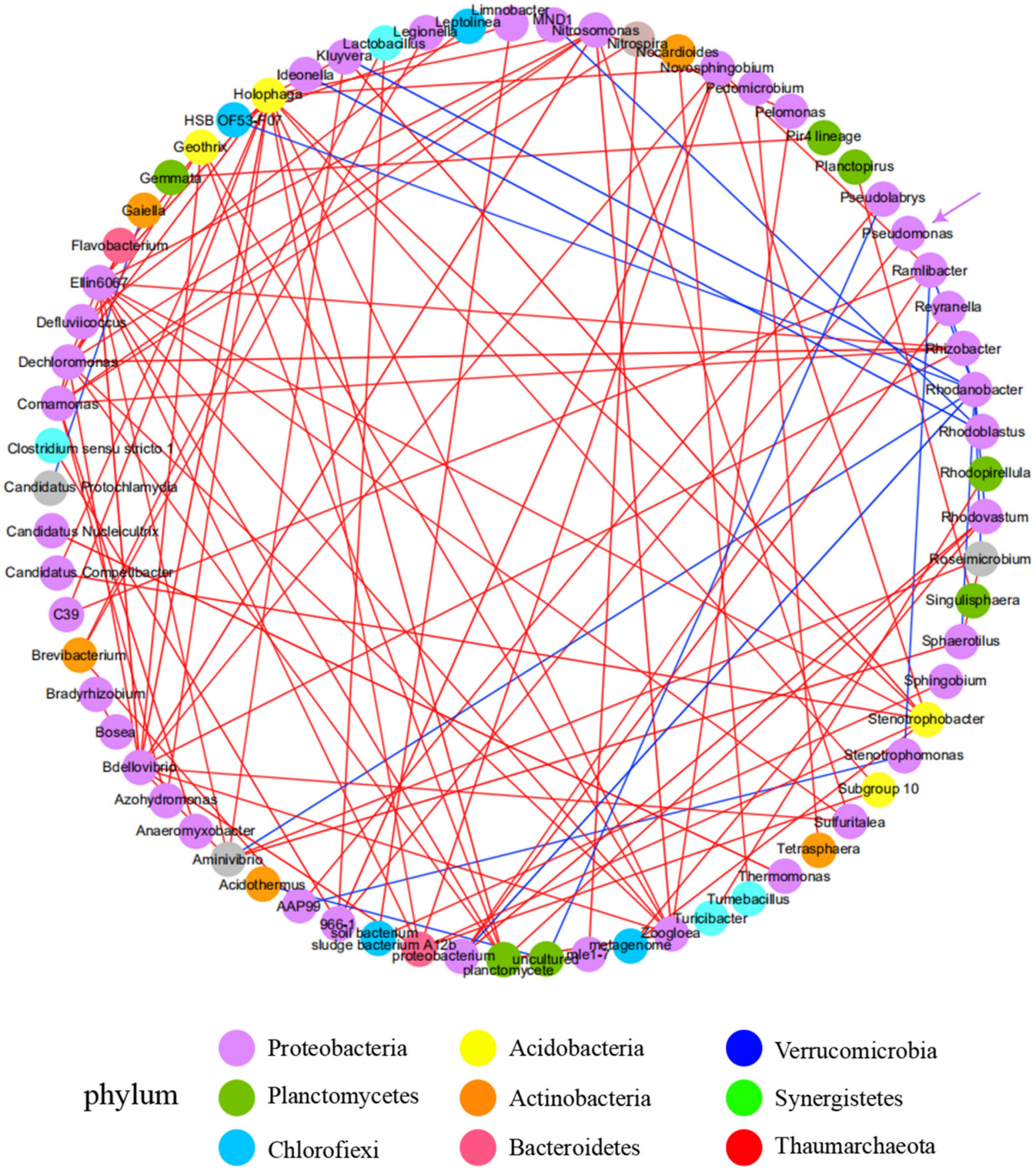

Figure 8. The bacterial genus network of microbial communities in JMSTP (**a**) and control (**b**) reactors. Each node represents a genus. The colors of the nodes indicate different major phylum. Red and blue edges indicate the positive and negative correlations, respectively.

4. Discussion

In this research, a strain, JMSTP, which can highly denitrify at low C/N, was obtained through laboratory screening. PCR agarose gel electrophoresis and gene sequencing confirm that the strain harbors the functional genes. The *nir*S-encoded cytochrome cd1 enzyme can catalyze the reduction of NO_2^- to NO, which is essential to denitrification [22]. Nitric oxide reductase encoded by *cnor*B is the next functional gene that converts NO to N_2O [21]. The reduction in N_2O is the final step in denitrification and is catalyzed by nitrous oxide reductase encoded by the *nos*Z gene [23]. Gene sequencing results show that strain JMSTP can perform nitrate removal via conventional denitrification pathways through nitrite to nitric oxide, and then via nitric oxide to nitrous oxide and further nitrogen gas. *Pseudomonas stutzeri* GF2 was also found to carry three functional genes and to work in low C/N conditions [36]. High denitrification efficiency at relatively low C/N ratios and sufficient denitrification efficiency under 20–30 °C suggest that strain JMSTP has the potential to improve the denitrification of urban sewage treatment through bioaugmentation. In experiments with actual wastewater, although the concentration of nitrate fluctuated due to the death of microorganisms [33,34], the removal efficiency of the experimental group was still relatively good (*t*-test, $p < 0.01$). These results indicate that strain JMSTP can improve nitrate removal in actual sewage with a complex organic composition [37,38].

In laboratory-scale SBRs with a C/N of 9:8 (*w*/*w*), which is a harsh environment for microorganisms, JMSTP did not perform very well at the beginning. The result was similar to the first inoculation of two bioaugmentation experiments by Bouchez et al. [37,39]. The insignificant results may be due to the lack of adaptation of the inoculated microorganisms, insufficient substrate, competition between the introduced species and native biomass, and the grazing by protozoa [40]. Bouchez et al. considered that their results were more likely due to the grazing by protozoa. However, through filtration and COD detection, the reason may be that JMSTP did not successfully form flocs with the activated sludge within 6 h and was rapidly washed out from the reactor. After the second inoculation, there was a decrease in JMSTP SBR. The TN residue in the effluent, which was lower than 16 mgN/L, and met the criteria of the Grade I standard A (TN $\leq$ 20 mgN/L) in China's wastewater discharge regulation. The present study suggested that the bioaugmentation of strain JMSTP in the activated sludge could enhance nitrogen removal, even at low carbon sources. The TN in the effluent increased gradually from the 7th day after the second inoculation in the JMSTP SBR, even though it was still lower than that of the control SBR, implying that the effects of bioaugmentation on denitrification gradually reduced. Therefore, it was suggested that the continuous bioaugmentation by cultivating strain JMSTP could further enhance the denitrification effect [38,39]. We have to investigate how functional microorganisms can better use their advantages in the real environmental conditions, and which techniques can facilitate the performance.

The result of the diversity of the microbial community indicates that the sudden introduction in a single microorganism into the activated sludge system does not have a huge impact [40]. Although the relative abundance of *Pseudomonas* sp. decreased significantly after being added, but the decrease in the total nitrogen, which was observed in the reactor, was also related to the addition of JMSTP. In the network analysis diagram, there is no statistical correlation between *Pseudomonas* sp. and other genera in the bioaugmented reactor. This may be due to the large dosage of inoculation and a sharp decrease in relative abundance after inoculation. Namely, no other genus was found to have such a substantial increase and decrease as *Pseudomonas* sp. Previous studies have suggested that although the added bacteria were not detected, the bacteria they added were still believed to have synergistic effects with the native microorganisms [41,42]. Therefore, it could be speculated that strain JMSTP was likely overdosed and it could synergize with native microorganisms after long-term operation. The control SBR showed more genus nodes and relationships than JMSTP SBR. For an ecological network, more nodes and edges are related to the stability of the network structure. The result suggests that the large dosage of strain JMSTP

might cause some interference to the stability of the network of the microbial community in JMSTP SBR. The microbial ecology needs a period of stabilization, which might correspond to the variation of effluent TN in the first few days after the bioaugmentation.

5. Conclusions

A denitrifying bacterium, *Pseudomonas* sp. JMSTP, was isolated and showed a denitrification ability under low carbon–nitrogen ratios. Under the influent COD/N = 3, the TN of effluent in the 6 L SBR could be less than 15 mgN/L after the bioaugmentation of *Pseudomonas* sp. JMSTP, which was significantly lower than that in the control SBR without bioaugmentation. RDA shows a negative correlation between *Pseudomonas* sp. abundance and TN concentrations in the bioaugmented SBR. The addition of *Pseudomonas* sp. JMSTP in activated sludge temporarily affected the diversity of the microbial community. The relative abundance of *Pseudomonas* sp. could achieve an amount 150% higher than that in the control SBR, after the second bioaugmentation of *Pseudomonas* sp. JMSTP. Correlation network analysis of the bioaugmented SBR found no clear co-occurrence pattern between *Pseudomonas* sp. and other native bacteria, due to the dramatic increase and decrease in *Pseudomonas* sp. abundance. This study suggests that bioaugmentation can be an effective approach for improving TN removal in urban sewage treatment plants. However, since the one-time bioaugmentation of the functional strain can only last for a few days, specific bioaugmentation strategies are needed to be considered further.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/w14040540/s1. Table S1. Primers for functional genes. Table S2. Nitrite removal capacity of JMSTP. Figure S1. The result of PCR sequencing analysis of *nir*S, *nor*B, and *nos*Z. Table S3. Community richness and diversity indices of two reactors. Table S4. OTUs of two reactors at each sampling day during experiment. Text S1. SEM operation process.

Author Contributions: Conceptualization, C.-P.Y. and Q.S.; methodology, X.Y.; software, T.C.; validation, C.-P.Y., Q.S. and A.H.; formal analysis, Q.S.; investigation, Y.W.; data curation, T.C.; writing—original draft preparation, T.C.; writing—review and editing, C.-P.Y.; visualization, T.C.; supervision, D.Q.; project administration, J.L.; funding acquisition, C.-P.Y. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by National Nature Science Foundation of China (41807411), the FJIRSM&IUE Joint Research Fund (RHZX-2019-005), and the STS Project of Fujian-CAS (2016T3004, 2021T3059, 2021T3014).

Conflicts of Interest: The authors declare no competing financial interest.

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