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Achieving Partial Nitrification via Intermittent Aeration in SBR and Short-Term Effects of Different C/N Ratios on Reactor Performance and Microbial Community Structure

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Abstract: A sequencing batch reactor (SBR) with an intermittent aeration mode was established to achieve partial nitrification (PN) and the short-term effects of C/N ratios were investigated. Stable nitrite accumulation was achieved after 107 cycles, about 56d, with the average ammonia nitrogen removal efficiency (ARE) and nitrite accumulation rate (NAR) of 96.92% and 82.49%, respectively. When the C/N ratios decreased from 4.64 to 3.87 and 2.32, ARE and NAR still kept a stable and high level. However, when the C/N ratio further decreased to 0.77, nitrite accumulation became fluctuation, and ARE, total nitrogen (TN), and chemical oxygen demand (COD) removal performance declined obviously. Except for four common phyla (*Proteobacteria*, *Bacteroidetes*, *Chloroflexi*, and *Actinobacteria*) in the wastewater treatment system, *Patescibacteria*, the newly defined superphylum, was found and became the most dominant phylum in the PN sludge for their ultra-small cell size. The only ammonia oxidation bacteria (AOB), *Nitrosomonas*, and nitrite oxidation bacteria (NOB), *Nitrospira*, were detected. The relative abundance of NOB was low at different C/N ratios, showing the stable and effective inhibition effects of intermittent aeration on NOB growth.

Keywords: partial nitrification; intermittent aeration; C/N ratio; high-throughput sequencing; microbial community

1. Introduction

Currently, in the trend of increasingly stringent nitrogen discharge standards, conventional biological nitrogen removal (BNR) technologies are gradually unable to meet demands due to the insufficient carbon sources and great energy consumption [1,2]. Compared with traditional processes, partial nitrification, as a novel BNR process, can reduce oxygen consumption during the aeration phase by 25% and save the organic carbon requirement by 40% during the denitrification phase [3]. However, PN only completes the process of converting ammonia nitrogen into nitrite nitrogen, the intermediate product. Other processes such as partial denitrification or anaerobic ammonia oxidation (anammox) are required to further remove nitrite nitrogen. In contrast with conventional BNR technologies, the novel combined processes can not only significantly save the carbon sources cost and energy consumption, but also reduce the sludge production. Both practicality and the economy can be realized, therefore, they became promising technologies in the wastewater treatment filed. Basically, the achievement of PN to accumulate nitrite is critical for those processes based on nitrite removal.

In order to attain stable PN, the key lies in repressing the activity of NOB and retaining AOB [4]. Various methods reported in many studies can be applied to achieve stable PN, such as adjusting the operational parameters (e.g., low dissolved oxygen (DO) [5], low temperature [6], high free ammonia

(FA) [7], and intermittent aeration [8]), adding some chemicals (e.g., hydroxylamine [9]), changing the reactor configuration (e.g., zeolite biological aerated filter [10]), or a combination of some control strategies [3,11]. Among the reported methods, widespread attention has been focused on intermittent aeration for its flexibility of operation in many kinds of reactors. In an intermittent aeration system, the alternating aerobic/anoxic condition can be built due to the repeated start and stop functions of the aeration system, inhibiting the activity of NOB while not affecting AOB [12]. Though the previous literature reported intermittent aeration as a cost-effective method to accumulate nitrite and its mechanism is understood to a certain degree, data are still scarce about the long-term stability and optimization of the aeration time. Further studies are necessary to improve the performance of this method.

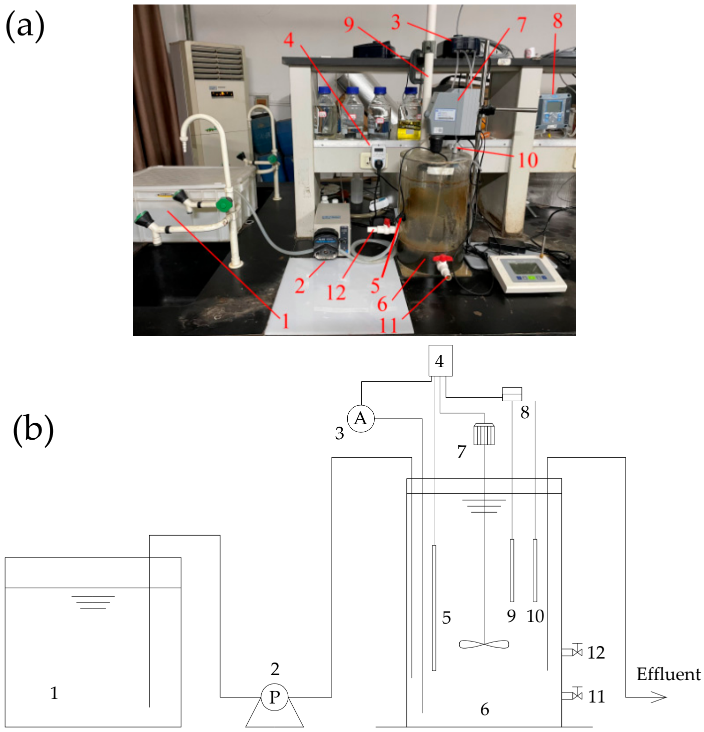
Generally, two main microbial guilds are involved in a PN system, the heterotrophic bacteria and autotrophic bacteria (AOB and NOB). As a substrate, the influent carbon source is a significant factor affecting nitritation. On the one hand, the existence of organic matter supported the growth of heterotrophic bacteria, resulting in competing DO with AOB [13]. In view of the lower growth rate of AOB, it is possible for AOB to be washed out from the reactor under a high COD concentration and nitrite nitrogen cannot accumulate successfully. It was reported that the stable PN was achieved at C/N ratios of 4–6 and heterotrophic bacteria possessed much growth advantage than AOB when C/N ratios were higher than 6 [14]. On the other hand, the sludge settleability is influenced by the C/N ratio, which is essential for the enrichment of AOB whose sludge retention time (SRT) is much higher than that of the heterotrophic microorganism. When the C/N ratios increased from 1.1 to 2.5, the sludge volume index (SVI) reduced from 111.7 to 83.9 mL/g and the granule sludge tended to increase, while the percentage of small floc sludge decreased, showing that the sludge settleability improved as the C/N ratio increased [15]. As the main constitution of the activated sludge matrix, extracellular polymeric substrates (EPS) played a role in binding microbial cells to form biomass flocculation and promoting sludge sedimentation [16,17]. The EPS content increased as the C/N ratio was raised, facilitating the formation of sludge floc with a large particle size and improving the sludge settleability [15]. Additionally, a too high or too low C/N ratio is also not conducive to forming a proper biomass concentration. The former will foster more heterotrophic growth and then lead to more sludge production [18], and the latter will cause less sludge production and even cause the decrease of biomass, both of which have a negative effect on keeping a certain SRT and retaining AOB. Hence, a suitable C/N ratio range is of great importance. However, information concerning the effects of the C/N ratio on the PN reactors performance and microbial community structure, especially in the intermittent aeration PN system, is still scarce.

In this study, a SBR was built to achieve PN via the intermittent aeration treating synthetic wastewater with low ammonia nitrogen. On this basis, the aims of the present study were to: (1) Investigate the stability of this system; (2) evaluate the short-term effects of C/N ratios, which decreased gradually from 4.64 to 0.77 on the reactor performance after achieving PN; and (3) analyze how the microbial population changed under different C/N ratios through high-throughput sequencing.

2. Materials and Methods

2.1. The SBR Setup and Operation Protocol

The SBR, which had a height of 40 cm and an internal diameter of 24 cm, was composed of plexiglass with a 17 L working volume, as shown in Figure 1. The reactor was fed with 11 L of synthetic wastewater in the feeding tank using a peristaltic pump (BT100M, Chuangrui, Baoding, China). In addition, a mechanical stirrer (OS20-Pro, DLAB, Beijing, China) at 250 rpm ensured a completely mixed condition during the feeding and intermittent aeration phases. After feeding, the pH value of the mixed liquid was adjusted immediately at 7.7–8.4, which was suitable for the AOB growth [19,20] by adding 1 mol/L NaOH and the pH value was not controlled in the remaining reaction time. The temperature (20–29 °C) of the reactor was not controlled until it dropped below 20 °C due to the seasonal changes. In order to not reduce the AOB activity, a heating rod was used to maintain the temperature above 20 °C. Air was supplied from the bottom of the reactor through four porous stones connected to an air pump (SB-948, SeBo, Zhongshan, China) and the airflow was adjusted according to DO values monitored by a DO probe connected to a controller. All the equipment except for the pH meter was connected to a time controller to achieve automation.



1.Feeding tank 2.Feeding pump 3.Air pump 4.Time controller 5.Heating rod 6.SBR
7.Agitator 8.DO screen 9.DO probe 10.pH probe 11.Sludge outlet 12. Sampling outlet

Figure 1. The schematic diagram (**a**) and the photo (**b**) of the sequencing batch reactor (SBR).

The experiment was divided into two stages. In stage one (cycles 1–165), the purpose was to achieve stable PN via intermittent aeration at a C/N ratio of 4.64. Each cycle consisted of feeding (26 min), intermittent aeration (365–420 min, based on DO values and the ammonia nitrogen removal performance), settling (30–180 min, based on the sludge settleability), and decanting (6 min). The time controller turned on and off the air pump alternatively to create an intermittent aeration condition with the aerobic and anoxic periods of 20 min. DO was below 1.0 mg/L during the aeration phase and below 0.1 mg/L during the anoxic phase. Stage two (cycles 166–281) aimed to assess the short-term effects of different C/N ratios on the reactor performance. When achieving stable PN, the C/N ratios decreased gradually to 3.87 (cycles 166–197), 2.32 (cycles 198–240), and 0.77 (cycles 241–281), respectively. Different from stage one, DO changed drastically in stage two and the total time of intermittent aeration was adjusted to 340–420 min at the C/N ratio of 3.87 and 340 min at the C/N ratios of 2.32 and 0.77, respectively. During the entire experiment, the 100–200 mL sludge was discharged from the reactor at the end of the decanting phase irregularly and the mixed liquor suspended solids (MLSS) were 2283–4531 mg/L. The SRT was not controlled for this experiment.

2.2. Synthetic Wastewater and Seed Sludge

Synthetic wastewater was prepared from tap water and the composition of the wastewater was as follows (per liter): Glucose; NH_4Cl: 0.2635 g; KH_2PO_4: 0.0020 g; $NaHCO_3$: 0.4000 g; $MgSO_4$: 0.0200 g; $CaCl_2$: 0.0100 g; and 1.0 mL trace element solution I and II made from deionized water. Trace element solution I contained (per liter): EDTA: 2.5000 g; $FeSO_4$: 2.5000 g; and trace element solution II contained (per liter): EDTA: 2.5000 g; $CoCl_2 \cdot 6H_2O$: 0.1200 g; $CuSO_4 \cdot 5H_2O$: 0.1250 g; $MnCl_2 \cdot 4H_2O$: 0.4950 g; $NaMoO_4 \cdot 2H_2O$: 0.1100 g; $NiCl_2 \cdot 6H_2O$: 0.0950 g; $ZnSO_4 \cdot 7H_2O$: 0.2150 g. The glucose concentrations used at different C/N ratios were 0.3, 0.25, 0.15, and 0.05 g/L corresponding to COD concentrations of 320.1, 266.8, 160.1, and 53.35 mg/L, respectively. In addition, the concentrations of KH_2PO_4 and $CaCl_2$ increased to 0.0606 and 0.1000 g/L at the 170th and 186th cycles, respectively, to attempt to improve the sludge settleability due to the sludge bulking.

The inoculated sludge was collected from an aerobic tank in the Erlangmiao municipal wastewater treatment plant (Wuhan, Hubei, China), with a MLSS concentration of 6812 mg/L and SVI of 34 mL/g, respectively. The sludge was cultured in the SBR for 25 days to acclimatize the lab-scale conditions before starting this study, after which the MLSS concentration changed to 4181 mg/L and the SVI increased to 81 mL/g.

2.3. Batch Tests

Batch tests were conducted to assess the maximum activities of AOB and NOB at different C/N ratios. For both of the tests, 200 mL of mixed liquor were taken from the reactor and the supernatant was decanted after settling. The sludge was washed four times by deionized water to remove the remaining substrates, and then transferred into a 1 L conical flask, diluting with synthetic wastewater to 500 mL of which the characteristics were the same as SBR except for the nitrogen source. An amount of 40 mg/L NH_4^+-N (NH_4Cl) and 40 mg/L NO_2^--N ($NaNO_2$) were added into the wastewater for AOB and NOB activity tests, respectively. The magnetic stirrer ensured the mix of liquor. Both of the systems operated for 2 h and the liquid samples were taken every 30 min for the analysis of the concentrations of NH_4^+-N and NO_2^--N. The initial pH was 7.5–8.5 and the temperature was above 20 °C. Oxygen was supplied via an air pump, controlling the DO at 6–9 mg/L. The MLSS was kept at about 2000 mg/L for the whole operation.

The activities of AOB and NOB were determined by specific ammonia oxidation rate (SAOR) and specific nitrite oxidation rate (SNOR), respectively, which were calculated from the decreasing ammonia and decreasing nitrite curves [4]. The slope of the fitted line was divided by MLSS and the value was the SAOR or SNOR.

2.4. Analytical Methods

The liquid samples were taken every two cycles and were filtered by a 0.45 μm filter membrane immediately before the analysis of NH_4^+-N, NO_2^--N, and NO_3^--N. The COD was measured using a rapid measuring analyzer (5B-3A, Lianhua Tech Co., Ltd., Beijing, China) twice a week. MLSS, SVI, and nitrogen species were measured according to the Chinese water and wastewater monitoring methods, fourth edition. TN was determined by the sum of NH_4^+-N, NO_2^--N, and NO_3^--N. DO and pH were monitored by a DO detector (SC200, Hach, Loveland, CO, USA) and a pH meter (FE 28, Mettler-Toledo, Greifensee, Switzerland), respectively.

The actual COD was supposed to deduct part of the oxidation of nitrite from the measured values and was calculated by Equation (1) [21], where $COD_{measured}$ (mg/L) was the measured value, 1.143 was the oxygen demand (g) by oxidizing 1 g NO_2^--N, and NO_2^--N (mg/L) was the measured value. NAR was calculated by Equation (2), where NO_2^--N_{eff} (mg/L) and NO_3^--N_{eff} (mg/L) were the concentrations of NO_2^--N and NO_3^--N in effluent, respectively, and NO_2^--N_{inf} (mg/L) and NO_3^--N_{inf} (mg/L) were those in influent. The FA concentration was estimated using Equation (3) [22], where NH_4^+-N (mg/L) was the concentration of NH_4^+-N in the reactor and T (°C) was the temperature of the mixed liquor.

$$COD = COD_{measured} - 1.143NO_2^{-}{-}N \tag{1}$$

$$NAR(\%) = \frac{NO_2^{-}{-}N_{eff} - NO_2^{-}{-}N_{inf}}{(NO_2^{-}{-}N_{eff} - NO_2^{-}{-}N_{inf}) + (NO_3^{-}{-}N_{eff} - NO_3^{-}{-}N_{inf})} \times 100\% \tag{2}$$

$$\mathrm{FA} = \frac{17}{14} \times \frac{\mathrm{NH_4}^{+}\!-\!\mathrm{N}\cdot 10^{\mathrm{pH}}}{10^{\mathrm{pH}} + \exp\left(\frac{6344}{273+\mathrm{T}}\right)} \tag{3}$$

2.5. DNA Extraction, PCR Amplification, and High-Throughput Sequencing Analysis

The sludge samples were collected at the end of 0, 110, 195, 237, and 270 cycles for Illumina MiSeq sequencing to analyze the shift of microbial community, respectively. The samples were sent to Majorbio Bio-pham Technology Co., Ltd. (Shanghai, China) to extract genomic DNA and detect the concentrations, purity, and integrity. The primer set used in the PCR amplification was 338F (5′-ACTCCTACGGGAGGCAGCAG-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) for the V3 and V4 region [23]. The PCR assay used the TransStart Fastpfu DNA Polymerase reaction system with a total volume of 20 μL, the component of which contained 4 μL 5 × FastPfu Buffer, 2 μL 2.5 mM dNTPs, 0.8 μL 5 μM forward primer, 0.8 μL 5 μM reverse primer, 0.4 μL FastPfu Polymerase, 0.2 μL BSA, and 10 ng of template DNA. PCR programs included a cycle of initial denaturation at 95 °C for 3 min, followed by 27 cycles of denaturing at 95 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 45 s, followed by a cycle of final extension at 72 °C for 10 min. Each sample was amplified in triplicate experiments and the obtained PCR products were detected by 2% agarose gel electrophoresis, as well as qualified using the QuantiFluor$^{\mathrm{TM}}$-ST Fluorometer (Promega, Madison, WI, USA).

A mixture of PCR products with an appropriate ratio were sequenced on the Illumina MiSeq PE300 platform (Majorbio Bio-pham Technology Co., Ltd., Shanghai, China). The paired-end reads from the raw data were merged using the FLASH software (version 1.2.11) and then the sequences were processed by the QIIME software (version 1.9.1) for controlling the quality. The sequences with a similarity at 97% or more were classified into the same operational taxonomic units (OTUs) by the Usearch software (version 7). Taxonomy analysis was conducted using the RDP classifier and Silva database.

3. Results and Discussion

3.1. Achievement of Partial Nitrification via Intermittent Aeration

The reactor performance in stage one was presented in Figure 2. Due to the change of aeration mode and low DO (<1.0 mg/L), the sludge could not acclimate the new environment rapidly, resulting in the decrease of ARE from 99.86% at cycle 1 to 51.47% at cycle 37 (Figure 2a). Despite the decrease of ARE, the effluent NO_2^--N concentrations were on the increase, while NO_3^--N reduced daily and NAR increased to 64.50% (Figure 2b), indicating that the intermittent aeration promoted a rapid start of PN. Considering the activity, even in the initial period (cycle 29), the activity of AOB (5.25 mg N/(g MLSS·h)) was higher than that of NOB (3.48 mg N/(g MLSS·h)) (Table 1), which also accelerated the realization of PN. In cycles 38 to 107, the reactor was in an unstable period, in which ARE and NAR ranged from 50.18 to 93.01% and 52.45 to 73.39%, respectively (Figure 2b). After operating 107 cycles, approximately 54d, the system achieved stability, with the average effluent NH_4^+-N, NO_2^--N, and NO_3^--N of 1.26, 12.42, and 2.78 mg/L (Figure 2a), respectively. The average ARE and NAR reached 96.92% and 82.49% (Figure 2b), respectively. Though the activity of AOB decreased slightly, it was still retained at a high level (3.89 mg N/(g MLSS·h) at cycle 113 and 4.29 mg N/(g MLSS·h) at cycle 160) (Table 1). However, the activity of NOB reduced about 3–4 times with 0.96 mg N/(g MLSS·h) and 0.83 mg N/(g MLSS·h) at cycles 113 and 160, respectively (Table 1). The variation in the activities of two microorganisms confirmed that an intermittent aeration PN system was beneficial to the AOB growth, while stably inhibiting NOB. As shown in Figure 2c, the effluent TN was almost stable and maintained at 12.35–29.32 mg/L, corresponding to the average TN removal efficiency (TNRE) of 55.43%, suggesting that the activity of denitrifying bacteria was steady during stage one The rapid achievement of PN and the stable operation over 50 cycles were mainly due to three aspects. First, the alternating aerobic/anoxic condition could be provided by the intermittent aeration, which was the most significant factor. Compared with AOB, the growth rate of NOB decreased for the deactivation of a critical enzyme (nitrite oxidoreductase) during the transition from anoxic to aerobic conditions [12], inhibiting the further oxidation of NO_2^--N. Furthermore, during the anoxic environment, the decay rate of AOB was lower than that of NOB [24,25]. The two physiological traits of AOB above ensured that it was dominant in the nitrifier guild. Second, it was reported that the concentration of FA to inhibit NOB was 0.1–1.0 mg/L, whereas that of AOB was 10–150 mg/L [22]. In this study, FA kept at 1.96–4.92 mg/L during stage one and the growth of NOB, therefore, could be inhibited while not affecting AOB, giving rise to the rapid accumulation of NO_2^--N. Finally, AOB possessed a lower oxygen half saturation constant (0.2–1.5 mg/L) than NOB (1.2–1.5 mg/L) [26]. The constant refers to the corresponding DO concentration when the growth rate of AOB/NOB reaches the half of the maximum growth rate. It reflects the oxygen affinity of AOB/NOB. The smaller it is, the larger the affinity obtained. The fitted Monod equation can be reached through measuring the specific nitrite/nitrate oxidation rates under different DO concentrations. Then, the oxygen half saturation constant can be acquired according to the equation. Therefore, the oxygen affinity of AOB was higher than that of NOB. DO was controlled below 1.0 mg/L during stage one, consequently DO was mainly utilized by AOB rather than NOB. Therefore, due to the insufficient DO, NO_2^--N produced by AOB could not be further oxidized to NO_3^--N. A stable PN could be achieved rapidly through combining the intermittent aeration with controlling FA and DO, simultaneously.

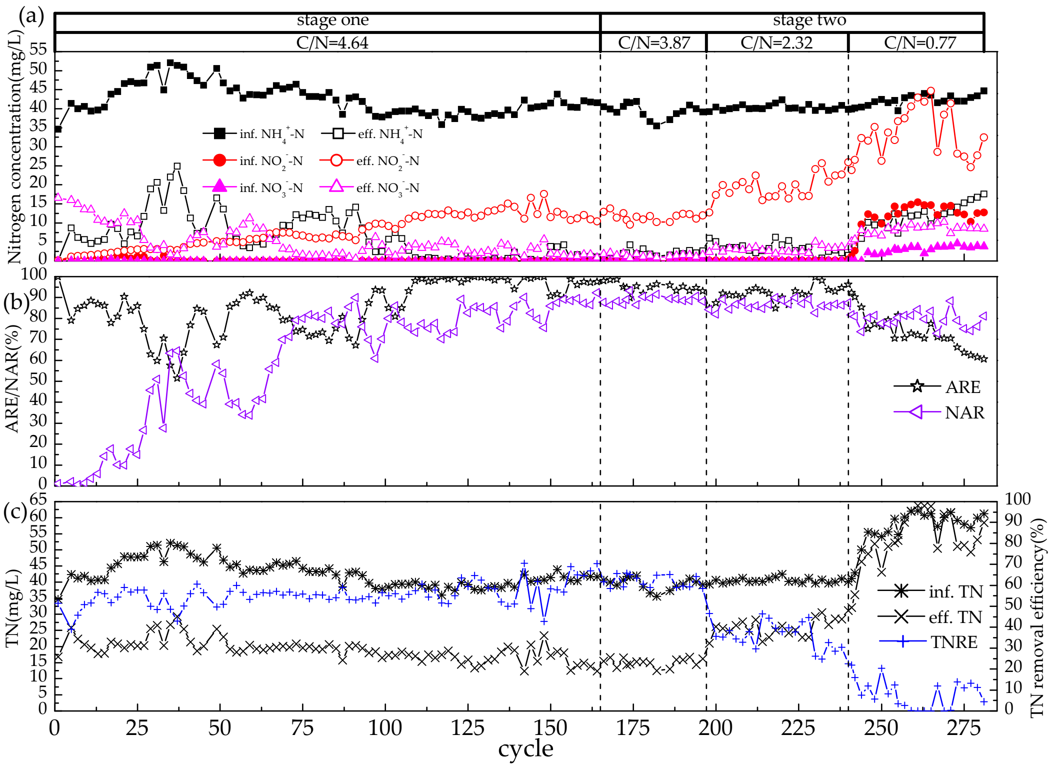


Figure 2. The reactor performance during the whole experiment: (**a**) The influent and effluent concentrations variation in NH_4^+-N, NO_2^--N, and NO_3^--N; (**b**) ammonia nitrogen removal efficiency (ARE) and nitrite accumulation rate (NAR); (**c**) the influent and effluent concentrations variation in total nitrogen (TN) and total nitrogen removal efficiency (TNRE).

Table 1. The maximum activities of ammonia oxidation bacteria (AOB) and nitrite oxidation bacteria (NOB) at different C/N ratios.

C/N	Cycle	SAOB (mg N/(g MLSS·h))	SNOB (mg N/(g MLSS·h))
4.64	29	5.25	3.48
	113	3.89	0.96
	160	4.29	0.83
3.87	196	5.51	1.01
2.32	238	6.29	1.04
0.77	281	2.04	Almost zero

To further understand the nitrogen conversion process, a typical cycle (cycle 162) was investigated and the results were shown in Figure 3. The removal of NH_4^+-N and the accumulation of NO_2^--N were observed in every aerobic phase (Figure 3a) and the effluent NH_4^+-N and NO_2^--N were 1.20 and 10.91 mg/L, respectively. When aeration ceased, DO dropped fast below 0.1 mg/L (Figure 3b) and AOB, hence, could not continuously oxidize NH_4^+-N in the absence of DO. The NO_2^--N concentrations reduced during every anoxic phase mainly due to denitrification. Moreover, the NO_3^--N concentrations were always at a low level with almost zero in the first 160 min and only increased to 1.70 mg/L at the end of the reaction (Figure 3a), which implied that NOB was at a low activity state for the whole cycle. Additionally, TN concentrations kept a decreased tendency, even at the aerobic phase (Figure 3a), inferring that simultaneous nitrification and denitrification (SND) might occur. The uneven distribution of DO in SBR created the possibility for SND, contributing to the TN removal. After reacting for 360 min, DO increased fast above 1.0 mg/L (Figure 3b), indicating that the substrates, NH_4^+-N, including the carbon source, were almost depleted and the reaction reached the end. The pH value increased slightly in the first 6 min and then decreased as the NH_4^+-N concentrations reduced (Figure 3b). The decline in the pH value was ascribed to nitrification of ammonia, which was a process consuming alkalinity (Equation (4)) [27]. The pH profile gradually stabilized in the last few minutes, which also suggested that PN terminated.

$$NH_4^+ + 1.5O_2 \rightarrow 2H^+ + H_2O + NO_2^- \quad (4)$$

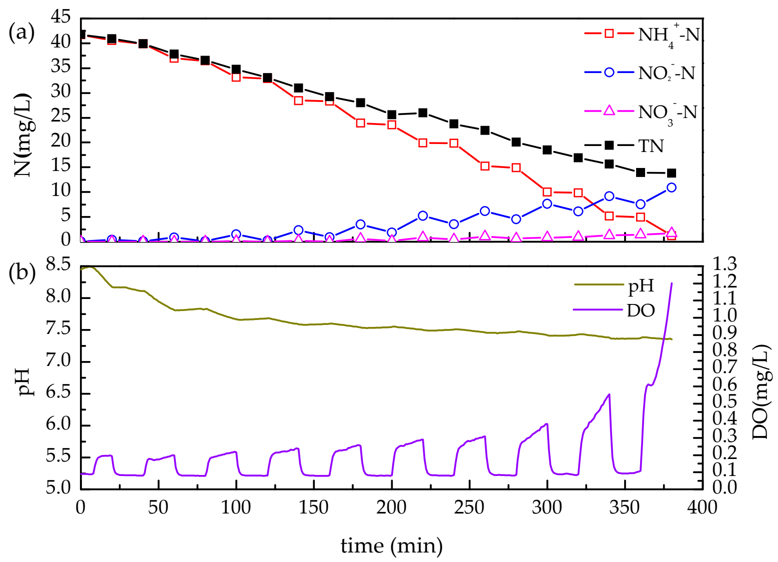


Figure 3. Variation in nitrogen species concentrations, pH, and dissolved oxygen (DO) in a typical cycle (cycle 162): (**a**) NH_4^+-N, NO_2^--N, NO_3^--N, and TN; (**b**) pH and DO.

3.2. Short-Term Effects of Different C/N Ratios on the Reactor Performance

Figure 2 presented the reactor performance at different C/N ratios during stage two. When influent C/N ratios decreased from 4.64 to 3.87 and 2.32, the average effluent NH_4^+-N was 2.34 and 3.26 mg/L, respectively, corresponding to the average ARE of 94.02 and 91.71%, respectively (Figure 2a). Despite a slight decrease of ARE, the stable removal of NH_4^+-N was still maintained. At a C/N ratio of 3.87, no obvious change of the effluent NO_2^--N was observed, with the average of 11.27 mg/L, whereas the effluent NO_2^--N rose up at a C/N ratio of 2.32 (Figure 2b). The effluent NO_3^--N still kept a low level, with the averages of 1.42 and 3.05 mg/L at the C/N ratios of 3.87 and 2.32, respectively (Figure 2a). The average NAR (88.85% and 86.48% at the C/N ratios of 3.87 and 2.32, respectively) was higher than that at the C/N ratio of 4.64 (82.49%) (Figure 2b). This result was in agreement with a previous study [28], which found that the nitrite accumulation increased with the decreasing C/N ratio in moving bed biofilm reactors. The higher stability of the nitrite accumulation performance was also observed when the C/N ratio decreased, indicating that the nitrite accumulation could be stabilized as the C/N ratio decreased. Due to the decrease of the C/N ratio, the available carbon source for heterotrophic bacteria was not enough, resulting in a decline of the activity, and the effluent COD increased from 24.20–49.28 mg/L at a C/N ratio of 4.64 to 72.18–80.10 mg/L at a C/N ratio of 3.87, and reached the maximum at cycle 224 at a C/N ratio of 2.32 (Figure 4). Consequently, the autotrophic bacteria could out-compete heterotrophic bacteria for substrates. Moreover, the activity of AOB increased as the C/N ratio decreased and reached 5.51 and 6.29 mg N/(g MLSS·h), respectively, which were much higher than those of NOB with 1.01 and 1.04 mg N/(g MLSS·h), respectively (Table 1). AOB was still dominant in the nitrifier guild, while NOB was inhibited effectively. However, when the C/N ratio reduced to 0.77, the nitrite accumulation became fluctuation but was still maintained at a high level with NAR of 72.94–88.34%. This was likely due to the DO, which needed more time to drop below 0.1 mg/L when aeration ceased and the corresponding anoxic time was shortened. As the inhibited performance to NOB was proportional to the anoxic duration [12], the growth rate of NOB recovered to some extent, which led to further oxidation of NO_2^--N and the unstable nitrite accumulation. Though NAR kept over 70%, ARE dropped from 92.21% at cycle 241 to 60.55% at cycle 281 (Figure 2a). Due to the lack of carbon source at the C/N ratio of 0.77, excess NO_2^--N and NO_3^--N in the last cycle could not be completely denitrified using the influent carbon source during the feeding process, resulting in high concentrations of nitrogen after feeding. It seems that these nitrogen concentrations could exert an inhibited effect on AOB and NOB (SAOB and SNOB decreased to 2.04 mg N/(g MLSS·h) and almost zero, respectively (Table 1)), leading to the worsened ARE. Further studies were needed to interpret this phenomenon.

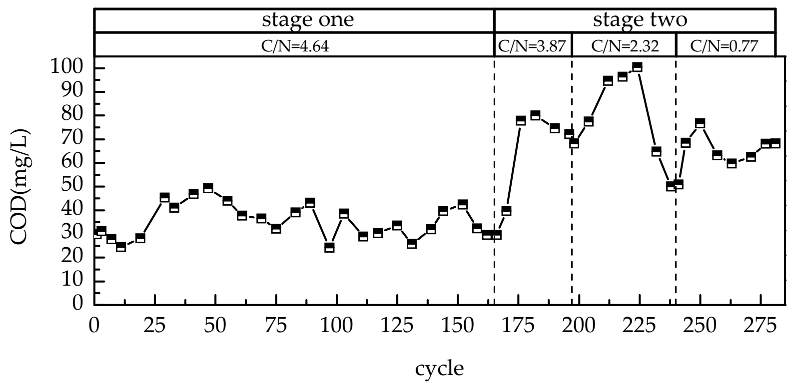


Figure 4. Chemical oxygen demand (COD) concentrations in effluent.

The TN removal performance did not exhibit an obvious variation when the C/N ratio decreased to 3.87 and the effluent TN and the average TNRE were 12.51–16.70 mg/L and 61.80%, respectively, but the TN removal began to deteriorate at a lower C/N ratio of 2.32 with only 22.60% at cycle 240

(Figure 2c). A further decrease to 0.77 caused little TN conversion and the TN concentration in effluent even exceeded that in influent at some cycles (Figure 2c). It was also reflected from Figure 4, where the COD concentration in effluent was close to that in influent. Since the anammox bacteria were not detected in this study, denitrification and partial denitrification by the heterotrophic denitrifying bacteria were the main TN removal way. As the C/N ratio decreased, on the one hand, DO rose up rapidly during the aerobic phase and decreased slowly when aeration stopped, resulting in a shorter anoxic time. On the other hand, the substrate, carbon source, was insufficient for denitrifying bacteria. It was observed that the effluent COD at stage two was notably more than that at stage one. The decrease of the heterotrophic bacteria activity was one of the causes, and also the variation in EPS could not be overlooked. Under low organic carbon availability, the EPS secreted by microbes were ready to hydrolyze leading to the formation of soluble microbial products (SMP) [14], another EPS which was in a dissolved form [29]. Therefore, SMP concentrations in effluent increased as the C/N ratio decreased. As a result, effluent COD rose up and even exceeded influent COD at the C/N ratio of 0.77 (Figure 4). Though PN was considered to be beneficial to treat wastewater with a low C/N ratio, it seems that the lower limit of C/N ratio was supposed to exist with respect to ARE, NAR, and TNRE. In this study, NH_4^+-N and TN removal performance started to worsen at a C/N ratio of 0.77, the minimum range of the C/N ratio needed more investigations.

3.3. Microbial Community Analysis

3.3.1. Changes of Microbial Community after Achieving Partial Nitrification

Two samples were collected at the beginning of the experiment (cycle 0) and during the PN stable period (cycle 110) to investigate how the microbial community changed from nitrification to PN. The coverage indices of S0 and S1 were more than 99.5% (Table 2), indicating that the two samples covered most of the bacteria community and could present the real state of the microbe. A total of 913 and 627 OTUs were identified in S0 and S1, respectively (Table 2), suggesting that the community richness decreased after achieving PN. This result could also be confirmed by chao and ace indices. Due to the fact that the compositions of the synthetic wastewater were less complex than those of the real municipal wastewater, some bacteria might be washed out from the system, resulting in the lower richness of the PN sludge. Another index, Shannon index, reflected the community diversity, and the larger it was, the higher the diversity obtained [30]. S0 had a higher Shannon index (5.25) than S1 (3.73) (Table 2), thus the diversity of the PN sludge reduced compared to the seed sludge. The Simpson index, which had the opposite trend (the smaller it was, the higher the diversity acquired) [30], also demonstrated this result (Table 2). In a word, both community richness and diversity decreased after realizing PN.

Table 2. Microbial alpha diversity of five samples (seed sludge, S0; C/N = 4.64, S1; C/N = 3.87, S2; C/N = 2.32, S3; C/N = 0.77, S4).

Samples	Sobs	Shannon	Simpson	Ace	Chao	Coverage
S0	913	5.25	0.020	967.5	968.3	0.9968
S1	627	3.73	0.091	754.9	742.9	0.9953
S2	481	3.62	0.055	639.7	626.0	0.9955
S3	462	3.72	0.067	595.2	605.2	0.9961
S4	416	3.69	0.069	508.2	506.0	0.9969

Proteobacteria, *Bacteroidetes*, *Chloroflexi,* and *Actinobacteria* were four ubiquitous phyla in the activated sludge. In this study, most bacteria also belonged to the four phyla in the two samples (Figure 5a). *Proteobacteria* (23.5%), *Bacteroidetes* (29.0%), and *Chloroflexi* (16.3%) were more dominant in S0 than those in S1 (14.4%, 16.7%, and 3.4%, respectively). *Bacteroidetes* played a role in degrading high molecular weight compounds, which was the important part in the carbon cycle [31]. *Chloroflexi* also participated in organic carbon degradation and they could utilize carbohydrates [32].

Apart from removing carbon, *Bacteroidetes* and *Chloroflexi* also contributed to nitrogen removal by denitrification [33,34]. Different from the three phyla above, the relative abundance of *Actinobacteria* increased from 11.2% in S0 to 15.0% in S1. Except for the four common phyla, another phylum, *Patescibacteria* which was little reported in the PN sludge in previous studies, was found to be another main phylum in two samples. This phylum had the least relative abundance (10.9%) among the five main phyla in S0, and then became the most dominant phylum in S1 (48.0%). *Patescibacteria* was the newly defined superphylum, which was found to be prevalent in aquifer environments, for example, groundwater with less nutrient and low DO [35]. The reason why this phylum dominated in S1 might lie in their ultra-small cell size, making them possess the advantage to maximize the use of the nutrient as the high surface-to-volume ratio [36,37].

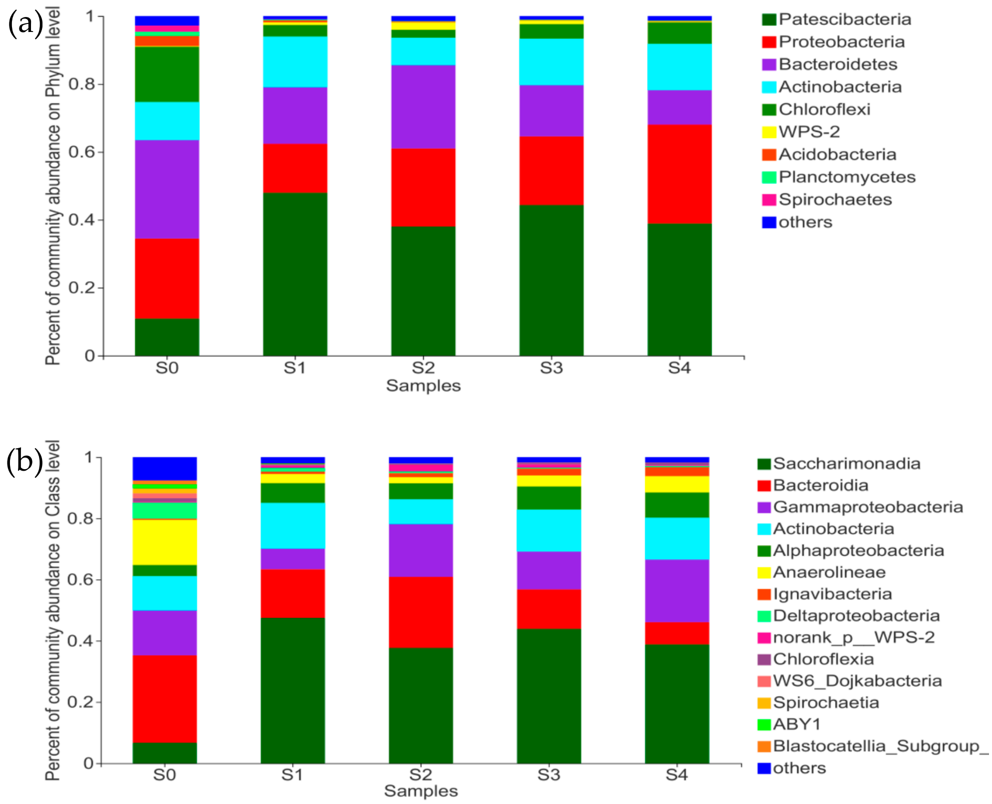


Figure 5. Microbial community structures of five samples (seed sludge, S0; C/N = 4.64, S1; C/N = 3.87, S2; C/N = 2.32, S3; C/N = 0.77, S4) at the phylum (**a**) and class (**b**) level (bacteria whose relative abundance were less than 1% were merged into others).

Bacterial populations of S0 and S1 at the class level were depicted in Figure 5b. Sixty two bacterial classes were detected in the two samples. The main classes were *Saccharimonadia*, *Bacteroidia*, *Actinobacteria*, *Anaerolineae*, *Alphaproteobacteria*, *Gammaproteobacteria*, and *Deltaproteobacteria*. *Gammaproteobacteria* and *Deltaproteobacteria* accounted for 14.7% and 5.2% in S0, respectively, and decreased to 6.7% and 1.2% in S1, respectively, while *Alphaproteobacteria* increased from 3.6% in S0 to 6.5% in S1. *Gammaproteobacteria* and *Alphaproteobacteria* classes contained many hydrogen-oxidizing denitrifiers such as *Aeromonas* sp. and *Pseudomonas* sp. [38]. The three classes, *Actinobacteria*, *Bacteroidia*, and *Anaerolineae* showed the same trend as the phyla they belong to (*Actinobacteria*, *Bacteroidetes*, and *Chloroflexi*, respectively) and changed from 11.2%, 28.6%, and 14.7% to 15.0%, 15.9%, and 3.0%, respectively. *Saccharimonadia*, one of the subdivisions of *Patescibacteria* phylum, became the most dominant class in S1 and accounted for 47.5%, which was almost seven times more than that in S0 (6.7%).

In the two samples, only one AOB and NOB genus, *Nitrosomonas* and *Nitrospira*, were detected and they were the common nitrifying bacteria in many wastewater treatment plants. *Nitrosomonas* and

Nitrospira accounted for 0.09% and 0.25% in S0, respectively, but the former increased to 0.28% and the latter decreased to 0.05% in S1 after achieving PN, indicating that intermittent aeration was an effective and rapid method to accumulate AOB and to wash out NOB. It was noteworthy that *Nitrospira* could not be eliminated completely in the system, which was confirmed by the existence of NO_3^--N in effluent.

3.3.2. Shift of Microbial Community under Different C/N Ratios

To understand the shift of microbial community, samples were taken during the stable period at different C/N ratios (cycle 195, 237, and 270). The coverage indices of S2-S4 exceeded 99.5%, presenting satisfactory sequencing results (Table 2). The OTUs detected were decreased as the C/N ratio reduced, with 481, 462, and 416 OTUs at the C/N ratios of 3.87, 2.32, and 0.77, respectively. Both ace and chao indices decreased as the C/N ratio reduced, implying that the community richness would show a decline tendency when the C/N ratio reduced. Different from richness, the community diversity did not show a regular variation and the values were similar, inferring that the change in the C/N ratio had no obvious effect on community diversity.

Venn diagrams were used to evaluate the similarities and differences of microbial community at different C/N ratios [39], and the results were depicted in Figure 6. There were 14 shared phyla in S1–S4 and only S1 had one unique phylum. The most abundant shared phylum was *Patescibacteria*, accounting for 42.4%, and the others were *Proteobacteria* (21.7%), *Bacteroidetes* (16.6%), *Actinobacteria* (12.7%), *Chloroflexi* (4.1%), and WPS-2 (1.1%). In general, S1–S4 shared most phyla, indicating that the change in the C/N ratio had no significant influence on the number of phyla in this system. The microbial community structures in S2-S4 were shown in Figure 5a. *Patescibacteria* was still the dominant phylum in the three samples, accounting for 38.0%, 44.4%, and 38.9%, respectively. This result was probably a consequence of the trait of this phylum as mentioned in Section 3.3.1 in the insufficient nutrient environment at a lower C/N ratio. Compared with S1, the relative abundance of *Proteobacteria* increased to 23.0%, 20.2%, and 29.1% in S2-S4, respectively. *Proteobacteria*, as the common phylum in many wastewater treatment systems, contained most nitrogen removal related microbes, such as AOB, NOB, and denitrifying bacteria [40]. *Bacteroidetes* increased to 24.5% in S2 in contrast with S1 and decreased to 15.1% and 10.2% when the C/N ratios further decreased from 3.87 to 2.32 and 0.77, respectively. On the contrary, *Chloroflexi* first decreased to 2.3% at a C/N ratio of 3.87 and then increased to 4.3% and 6.3% at the C/N ratios of 2.32 and 0.77, respectively. This was likely due to the fact that *Chloroflexi* could adapt to the decreasing carbon source compared with *Bacteroidetes*. Another main phylum, *Actinobacteria*, accounted for 8.1%, 13.8%, and 13.7% in S2, S3, and S4, respectively.

At the class level, the four samples had 31 shared classes, where *Saccharimonadia* belonging to *Patescibacteria* was in the majority with the relative abundance of 42.1%. The other main classes were *Bacteroidia* (14.8%), *Gammaproteobacteria* (14.2%), *Actinobacteria* (12.7%), *Alphaproteobacteria* (6.9%), *Anaerolineae* (3.5%), *Ignavibacteria* (1.8%), and norank_Wps-2 (1.1%) (Figure 6b). Except for S4, unique classes were detected in the other three samples with 4, 3, and 1 classes in S1, S2, and S3, respectively. Shared classes were still the main part of the class level microbial community. Decreasing in the C/N ratio did not affect the predominance of *Saccharimonadia*, the relative abundance of which were 37.7%, 44.0%, and 38.9% at the C/N ratios of 3.87, 2.32, and 0.77, respectively. *Bacteroidia* was the second dominant class at a C/N ratio of 3.87 (S2), reaching 23.2%, and continuously decreased in the C/N ratio (12.8% for S3 and 7.2% for S4) (Figure 5b). *Actinobacteria* first decreased from 15.0% in S1 to 8.1% in S2, while it increased to 13.8% in S3 and kept at the same level in S4 (13.7%) (Figure 5b). The two classes belonging to *Proteobacteria*, *Gammaproteobacteria*, and *Alphaproteobacteria* showed an increasing trend. *Alphaproteobacteria* reduced to 5.2% slightly in S2 compared with S1 and then increased to 7.5% in S3 and 8.2% in S4. *Gammaproteobacteria* accounted for 17.2%, 12.4%, and 20.5% in S2, S3, and S4, respectively, becoming the second dominant class in S4 eventually. However, another class *Deltaproteobacteria* belonging to *Proteobacteria* decreased to a very low level, with the relative abundance of only 0.54%, 0.31%, and 0.39% in S2, S3, and S4, respectively, indicating that this class possibly could not adapt to decreasing in the C/N ratio and could be washed out gradually from the system. The other main classes included *Ignavibacteria* and *Anaerolineae* and their abundance increased as the C/N ratio decreased. *Ignavibacteria* increased to 1.3%, 2.2%, and 2.9% in S2, S3, and S4, respectively. Some species of *Anaerolineae* were able to degrade carbohydrates [41]. In S2–S4, *Anaerolineae* accounted for 2.0%, 3.6%, and 5.3%, respectively.

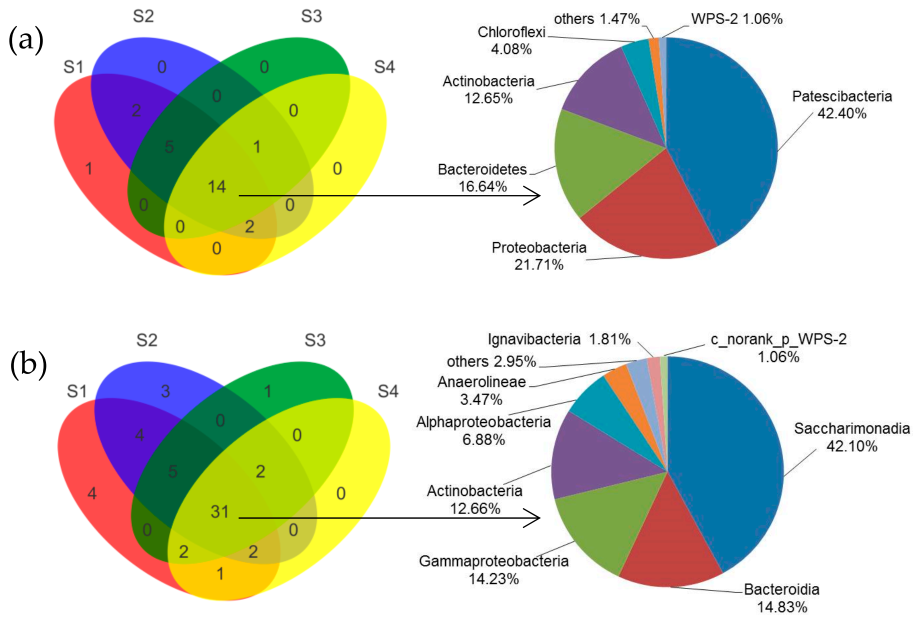


Figure 6. Venn of microbial communities from S1–S4 at the phylum (**a**) and class (**b**) level.

As the C/N ratio decreased, no more new AOB and NOB were found. The relative abundance of *Nitrosomonas* was still maintained at a higher level in contrast with NOB, with 1.2%, 0.9%, and 1.2% at C/N ratios of 3.87, 2.32, and 0.77, respectively, while *Nitrospira* was always in an inhibited state and accounted for only 0.04%, 0.06%, and 0.02%, respectively. Compared with the seed sludge, AOB accumulated about ten times. The dominance of *Nitrosomonas* could guarantee the good performance of PN at different C/N ratios. Approximately 10 denitrifying bacteria were found in this study (Table 3) [39,42]. *Candidatus_Competibacter*, *Dechloromonas*, *Rhodobacter,* and *Zoogloea* were the four main denitrifiers. *Dechloromonas* was the most dominant in S2, accounting for 10.3%. *Dechloromonas* could decompose benzene using nitrate as the electron acceptor [43]. *Candidatus_Competibacter* showed a decrease tendency as the C/N ratio reduced and was only 0.3% in S4, about half of that in S1, inferring that the low C/N ratio had a negative effect on it. *Candidatus_Competibacter* was capable of consuming the molecule organic matter and was a kind of glycogen accumulating organism, the competitor of the phosphate accumulating organism in the phosphorus removal process [44,45]. Apart from being involved in denitrification, *Zooglea* was also an important bacterium which could form sludge flocs [42]. The other commonly reported denitrifying bacteria, *Denitratisoma*, *Thauera,* and *Pseudomonas*, were found little in this study (less than 0.5% in each sample), implying that they contributed to denitrification slightly. Taken together, S2 had the most abundant denitrifying bacteria with the total relative abundance of 12.9% and had a good denitrification performance. However, further decreasing in the C/N ratio led to the elimination of the denitrifier. Finally, there was 5.5% of the denitrifier in S4, and only half of that in S2. The decrease in the denitrifier numbers and insufficient carbon source, together with the shortened anoxic time, resulted in an unsatisfactory denitrification performance at a lower C/N ratio.

Table 3. The relative abundance of denitrifying bacteria at different C/N ratios (S1–S4).

Denitrifying Bacteria	S1	S2	S3	S4
Candidatus_Competibacter	0.8%	0.5%	0.7%	0.3%
Dechloromonas	1.0%	10.3%	2.7%	0.9%
Denitratisoma	0.0%	0.0%	0.2%	0.3%
Dokdonella	0.0%	0.0%	0.0%	0.2%
Hyphomicrobium	0.2%	0.4%	0.4%	0.6%
Pseudomonas	0.0%	0.0%	0.0%	0.0%
Rhodobacter	0.3%	1.3%	2.9%	3.1%
Terrimonas	0.1%	0.0%	0.1%	0.1%
Thauera	0.1%	0.0%	0.0%	0.0%
Zoogloea	1.8%	0.3%	0.1%	0.0%
total	4.3%	12.9%	7.0%	5.5%

4. Conclusions

After 107 cycles and about 54d, a stable PN was realized, with an average ARE and NAR of 96.92% and 82.49%, respectively, and the stable performance was maintained over 50 cycles. When the C/N ratios decreased to 3.87 and 2.32, NAR increased slightly and nitrite accumulation became stabler. Moreover, ARE was kept at a high and stable level. However, when the C/N ratio further decreased to 0.77, NAR became fluctuation, and ARE, TN, and COD removal performance deteriorated. Therefore, the lower limit of the C/N ratio of wastewater was supposed to exist for a PN system. Both the microbial community richness and diversity of the PN sludge decreased compared with the seed sludge and *Proteobacteria*, *Bacteroidetes*, *Chloroflexi*, *Actinobacteria*, and *Patescibacteria* were the five main phyla. *Patescibacteria* had an ultra-small cell size and this trait made them possess the advantage of maximizing the use of the nutrient as a high surface-to-volume ratio. Therefore, it could become the most dominant phylum in the PN sludge. As the C/N ratio decreased, the community richness showed a decline tendency, with 627, 481, 462, and 416 OTUs at C/N ratios of 4.64, 3.87, 2.32, and 0.77, respectively. However, no obvious variation in diversity had been found. In addition, the main phyla did not change. During the whole experiment, the only AOB, *Nitrosomonas*, and NOB, *Nitrospira*, were detected. The relative abundance of AOB was much higher than that of NOB, indicating that the alternate aeration/anoxic environments were more beneficial for the accumulation of AOB.

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