Application of a Partial Nitrogen Lab-Scale Sequencing Batch Reactor for the Treatment of Organic Wastewater and Its N$_2$O Production Pathways, and the Microbial Mechanism

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Abstract: Partial nitrification (PN) is a widely used wastewater treatment process. Here a lab-scale sequencing batch reactor for PN (PN-SBR) was constructed and run with artificial organic wastewater for 225 days. Results showed that the SBR reached a stable PN state after 174 days of operation and >98% of NH$_4^+$-N was removed and >60% was converted to NO$_2^-$-N with low effluent NO$_3^-$-N content. In a PN-SBR cycle at stage IV, the release of N$_2$O was accompanied by the production of hydroxylamine, occurring mainly in the conversion from anaerobic to aerobic phases, and the amount of N$_2$O produced was about 6.3% of the total nitrogen. The N$_2$O isotopic signature results suggested that hydroxylamine oxidation was the main pathway for N$_2$O production. Illumina MiSeq sequencing results showed that Proteobacteria and Bacteroidetes were the dominant phyla throughout the operation period. Many heterotrophic nitrifiers were significantly enriched, leading to ammonia removal and nitrite accumulation, including Acidovorax, Paracoccus, Propionibacteriaceae_unclassified, Shinella, Comamonas and Brevundimonas. Representative strains were isolated from the reactor and they were capable of efficiently producing nitrite from ammonia. These results provide a guide for the direct running of PN reactors for treating organic wastewater and help to understand the microbial processes and N$_2$O release pathways and the microbial mechanism of partial nitrification.

Keywords: partial nitrification; organic wastewater; N$_2$O emissions; microbial mechanisms; heterotrophic nitrifiers

1. Introduction

Partial nitrification (PN) is a controlled process for ammonia oxidation to the nitrite accumulation stage. Recently, PN has been adopted either for nitrite shunting or the intermediate nitrite generation step for anammox [1]. PN-Anammox, a newly developed process for biological nitrogen removal from wastewater, achieves a PN effluent with a NO$_2^-$:NH$_4^+$ molar ratio of 1.31 suitable for subsequent anammox [2], which consumes less energy and produces less sludge [3,4]. However, full PN is necessary as the first step for nitrite shunting. Studies have mainly focused on the PN of inorganic synthetic wastewater [5–7], but not that of organic wastewater. It is difficult to achieve PN of organic wastewater because of the inhibitory effect of organic matter on the activity of ammonia-oxidizing bacteria when the C/N ratio is >0.25 [8,9]. However, most wastewater, such as municipal and industrial, contains organic compounds with a C/N ratio higher than 5. In this case, the successful PN of organic wastewater is of significance for both PN-Anammox and nitrite shunting.

Further, despite many obvious advantages, PN promotes more nitrous oxide (N$_2$O) production than the traditional nitrification process, leading to secondary pollution [10]. N$_2$O is an important trace gas in the atmosphere, contributing to both the greenhouse effect and stratospheric ozone destruction [11]; sewage disposal is a significant contributor to N$_2$O generation and release [12]. Therefore, controlling the release of N$_2$O in PN has significance.
for minimizing N₂O emissions. Determining the pathways for N₂O production in PN remains a challenge. Recent studies have mainly focused on the release kinetics and the effect factors [13]; there are few studies focused on the N₂O generation pathways in reactors with different performances, and the contribution of different pathways is inconsistent. Other studies suggest that: heterotrophic denitrification is the main contributor to N₂O production in SBAR [14], hydroxylamine metabolism is a key pathway in autotrophic partial nitrification granules [15], and N-nitrosation hybrid N₂O production in a bioreactor is the dominant N₂O production mechanism [16].

Microorganisms play important roles in wastewater treatment. Conventional nitrification is a two-step reaction in which ammonium is first oxidized to nitrite by ammonia-oxidizing bacteria (AOB) and then to nitrate by nitrite-oxidizing bacteria (NOB). In PN, the reaction is controlled at the stage of continuously generating nitrite and nitrate would be directly used as an electron acceptor for denitrification, instead of being further oxidized to nitrate, which is performed by inhibiting the activity of NOB but maintaining the activity of AOB [17]. In early reports, both AOB and NOB are autotrophic nitrifiers. Most heterotrophic members affiliated with phyla as Chloroflexi, Proteobacteria, and Bacteroidetes are widely reported to co-exist with AOB [18,19]. Heterotrophic bacteria are widely prevalent in partial nitrification-based processes; some heterotrophic bacteria, such as the members of the genera Pseudomonas, Paracoccus, Alcaligenes, Acinetobacter, and Thauera, are reported to be able to simultaneously oxidize ammonia to nitrite/nitrate and reduce nitrite/nitrate to N₂, called heterotrophic nitrification and aerobic denitrification (HNAD) [20,21]. However, the ecological roles and related functional genes of these heterotrophic bacteria remain unclear. Few studies have examined ammonia monoxygenase (AMO) and hydroxylamine oxidase (HAO) in heterotrophic bacteria. Some enzymes associated with denitrification have been reported to be sensitive to O₂ and therefore unable to reduce nitrite to N₂. These facts challenge our understanding of the ammonia to N₂ process in these HNAD bacteria under aerobic conditions [22].

In this study, a lab-scale sequencing batch reactor for PN (PN-SBR) was constructed and run with artificial organic wastewater to investigate performance for treating organic wastewater and the release patterns and production pathways of N₂O. The changes of effluents, the succession of microbial community structures, and related core bacteria involved in nitrogen metabolism were studied to reveal the microbial mechanisms of N₂O production in PN. The results are critical for understanding N₂O release pathways and the microbial mechanisms of PN for treating organic wastewater.

2. Materials and Methods

2.1. Start-Up and Operation of the PN-SBR

The reactor was made of plexiglass and designed to be airtight, with a gas collection bag connected from the gas sampling port at the top of the reactor. The working volume was 7.2 L, volumetric exchange rate was 25% and hydraulic retention time (HRT) was about 18 h and sludge retention time (SRT) was 6 days. The PN-SBR was initiated with artificial organic wastewater (NH₄Cl, 0.8 g; C₆H₁₂O₆ [glucose], 1.6 g; Na₂HPO₄·12H₂O, 0.2 g; tap water, 1000 mL; natural pH; the chemical oxygen demand (COD) content was ~1650 mg/L). The inlet load was maintained at an NH₄⁺-N concentration of 210 mg/L and C/N ratio ~3. Activated sludge used as the inoculum for the start-up was obtained from a SHARON-reactor, a lab-scale “UASB + SHARON + ANAMMOX” combined system built in our laboratory for the treatment of piggery wastewater [14]. 0.8 L of activated sludge was inoculated into the reactor with a concentration of suspended solids of 3.0 g/L. The PN-SBR was run in a sequencing batch manner at room temperature by increasing the NH₄⁺-N concentration of the influent and controlling the dissolved oxygen (DO) and pH. The operation parameters of the reactor were optimized continuously.
2.2. Analysis Methods for Water Quality Parameters and Gaseous and Dissolved N\textsubscript{2}O

Ammonium (NH\textsubscript{4}\textsuperscript{+}-N), nitrite (NO\textsubscript{3}\textsuperscript{-}-N), and nitrate (NO\textsubscript{2}\textsuperscript{-}-N) in the influent and effluent were measured by using the Aquakem 600 water quality analyzer (Thermo Scientific, Waltham, MA, USA); pH and DO were measured by a portable analyzer (Multi 3420). COD was measured using potassium permanganate method [23]. The gas flow rate into the reactor was regulated with an air pump and the volume of gas entering the reactor was measured with a digital gas flow meter. Exhaust gas was removed from the gas sampling port at the top of the reactor.

The dissolved N\textsubscript{2}O (D-N\textsubscript{2}O) concentration was determined using an N\textsubscript{2}O microelectrode system (N\textsubscript{2}O-R, Unisense monometer-9706, Aarhus, Denmark), in which ambient N\textsubscript{2}O is reduced on the surface of the metal cathode through the film at the top of the electrode, and the system converts the reduction current into a signal. Before measurement, the N\textsubscript{2}O microelectrode was calibrated according to the system manual and a standard curve was obtained. Gaseous samples were used for the measurement of gaseous N\textsubscript{2}O concentration and \textsuperscript{15}N-site preference (SP) value, and gaseous N\textsubscript{2}O at different aeration time points were collected from the sampling port using 1 L gas collection bags. Quantitative detection of gaseous N\textsubscript{2}O was performed using gas chromatography/mass spectrometry (model 7890A/5975C, Agilent) as previously described [24].

2.3. Analysis of Site Preference Values

Simultaneous determination of $\delta^{15}$N\textsubscript{bulk} and $\delta^{15}$N\textsubscript{α} of N\textsubscript{2}O molecules was performed by one injection using Isoprime100-Tracegas instruments. The N\textsubscript{2}O molecule has an asymmetrical linear structure with a molecular formula of N=N=O. According to the positions of the nitrogen and oxygen atoms in the center ($\alpha$) and the end ($\beta$), the value of the microbes’ preference for N in different positions in the N\textsubscript{2}O molecule is SP, SP value has no relationship with the $\delta^{15}$N isotopic composition of the N\textsubscript{2}O precursor. That is, SP is independent of the substrate $\delta^{15}$N during N\textsubscript{2}O production and can be an important tool to distinguish N\textsubscript{2}O sources from different environments, such as municipal wastewater treatment plants [25]. The formula of SP is as follows:

$$\delta^{15}N^i = \left(\delta^{15}R^i_{\text{sample}} - \delta^{15}R^i_{\text{standard}}\right)/\delta^{15}R^i_{\text{standard}}$$  \hspace{1cm} (1)

$$\delta^{15}N^\text{bulk} = \delta^{15}N^\alpha + \delta^{15}N^\beta / 2$$  \hspace{1cm} (2)

$$\delta^{15}N^\text{sample} = \delta^{15}N^\alpha - \delta^{15}N^\beta$$  \hspace{1cm} (3)

where $i = \alpha, \beta$, bulk, R = $^{15}N/^{14}N$, and $\delta^{15}N^\alpha$ and $\delta^{15}N^\beta$ denote isotopic values of the nitrogen atoms in the $\alpha$ and $\beta$ positions in the N\textsubscript{2}O molecule [16].

Since different pathways produce N\textsubscript{2}O with different SP values (NH\textsubscript{2}OH oxidation vs. NO\textsubscript{2}\textsuperscript{-} reduction), the analysis of the SP allows us to identify the pathway of N\textsubscript{2}O production. In specific pure cultures, the average SP values of N\textsubscript{2}O produced by NH\textsubscript{2}OH oxidation (SP\textsubscript{NIT}) and NO\textsubscript{2}\textsuperscript{-} reduction (nitrite denitrification and heterotrophic denitrification, SP\textsubscript{DNIT}) are 33 ± 4‰ and −1 ± 5.5‰, respectively [26,27]. If bacterial nitrification and denitrification were the causes of N\textsubscript{2}O production, and N\textsubscript{2}O has not been reduced, the contributions of NH\textsubscript{2}OH oxidation vs. NO\textsubscript{2}\textsuperscript{-} reduction were estimated using the following equation:

$$\text{Contribution of NH}_{2}OH\text{ oxidation} = \frac{\left[100 - \text{contribution of NO}_{2}^{-}\text{ reduction}\right]}{\left(\text{SP}_{\text{sample}} - \text{SP}_{\text{DNIT}}\right) / \left(\text{SP}_{\text{NIT}} - \text{SP}_{\text{DNIT}}\right)} \times 100$$  \hspace{1cm} (4)

2.4. Analysis of the Bacterial Community and Core Bacterial Genera

Sixteen activated sludge samples from different running stages of the PN-SBR were collected. Total DNA of the activated sludge sample was extracted using a QIAGEN DNeasy PowerSoil kit (12888-50), and the specific operation steps were carried out according to the kit instructions. The quality of extracted DNA was measured and checked with a NanoDrop.
spectrophotometer (Thermo Scientific, NanoDrop 2000, H455) for its 260/280 ratio and stored at −80 °C until further processing.

For bacterial community analysis, the primer set used was 338F/806R, which targets the variable region (V3–V4) of the bacterial 16S rRNA gene. All samples were conducted according to the formal experimental conditions. The PCR products were extracted using a Gel Extraction Kit and purified PCR products were adjusted to equimolar amounts and sequenced using Illumina Miseq PE300 platform by Majorbio Bio-pharm Technology Co. (Shanghai, China). The raw data were processed and chimeric sequences were filtered using QIIME toolkit, v. 1.8.0 [19] and the UCHIME program [28]. Ninety-seven percent of the sequence similarities were clustered into operational taxonomic units (OTUs) using UPARSE [29]. Classification assignment was performed using the Greengenes database [30]. Normalization was performed for all OTU-based analyses.

2.5. Isolation, Phylogenetic Analysis, and Characteristics of Culturable Nitrifying Bacteria

Two media, M1 and M2 (Table 1), were used to isolate bacteria from the reactor. Single colonies were picked from the solid plates and inoculated into the liquid media. After the strain was cultivated for 2 d, nitrite production was detected using the Griess reagent [31] and the positive strains were further studied. The 16S rRNA gene of the positive isolates was amplified and sequenced. Ammonia removal and the nitrite/nitrate production of the isolates belonging to the core genera in the bioreactor were further detected using the methods described above. To improve understanding of the structure and function of microbial communities in the SBR system, the relationship between Illumina MiSeq analysis and the representative isolated heterotrophic nitrifying bacteria were investigated using correlation network analysis. The relationship matrix was statistically analyzed. Spearman’s rank was calculated to reflect the correlation; genera with \( p < 0.05 \) were shown and the matrix was imported into Cytoscape for visualization. The phylogenetic tree was constructed using Mega 11 [32], including the core genera in the bioreactor and the representative, isolated, heterotrophic nitrifying bacteria.

Table 1. Media used for isolation of bacterial strains from the reactor.

| Components (g/L) | Medium  |
|-----------------|---------|
|                 | M1      | M2  |
| \((\text{NH}_4\)\text{SO}_4\) | 0.66    | 0.33 |
| Sodium succinate | 4.72    | 1.0  |
| \(\text{KH}_2\text{PO}_4\)     | 0.5     | 0.5  |
| \(\text{Na}_2\text{HPO}_4\)    | 0.5     | 0.5  |
| MgSO\(_4\)        | 0.1     | 0.1  |
| Glucose           | 0       | 0.5  |
| Sodium pyruvate   | 0       | 0.3  |
| Vitamin complex solution | 0 | 1 mL |
| Amino acids complex solution | 0 | 5 mL |
| Trace element solution | 2 mL | 1 mL |

2.6. Metagenomic Analysis of Functional Genes in the PN-SBR

For the metagenomic analysis of microbial functional genes, activated sludge samples were collected at stage IV when the PN-SBR reached a stable PN state. The genome DNA was extracted as described above, and the PE library was constructed using a NEXTFLEX™ Rapid DNA-Seq Kit. Paired-end sequencing was performed on an Illumina Novaseq PE150 platform at Majorbio Bio-Pharm Technology Co (Beijing, China). The optimized sequences were then used for splicing assembly and gene prediction, and the gene sets were annotated by NR, eggNOG, and KEGG databases and the clusters of orthologous groups of proteins (COG).
3. Results and Discussion

3.1. Successful Running of the PN-SBR with Synthetic Organic Wastewater

The contents of NH$_4^+$, NO$_2^-$, and NO$_3^-$ in the effluent of the PN-SBR run with synthetic organic wastewater for the entire experimental period are shown in Figure 1. According to the variation of these parameters in the effluent, the operation period of the PN-SBR could be divided into four stages. Days 1 to 40 (Stage I) were the start-up stage; the influent NH$_4^+$-N was ~210 mg/L on day 5 to ~130 mg/L on day 35, and the removal of ammonia gradually increased from ~0 on day 5 to ~39% on day 35. Nitrite was accumulated gradually from ~25 mg/L on day 5 to ~75 mg/L on day 35, suggesting a successful start-up of the PN-SBR. From days 41 to 122 (Stage II), the outlet NH$_4^+$-N was further reduced to ~100 mg/L, accompanied by the accumulation of nitrite which increased gradually to ~100 mg/L. From days 123 to 173 (Stage III), the outlet NH$_4^+$-N decreased continuously to ~2 mg/L on day 173, and the effluent nitrite was maintained at 80–110 mg/L. From days 174 to 225 (Stage IV), the PN-SBR reached a stable PN state, with >98% removal of ammonia; >60% of ammonia was converted to nitrite, whose content in the effluent remained at ~140 mg/L on days 223–225 with low effluent NO$_3^-$-N, indicating robust PN performance of the PN-SBR with organic wastewater, with COD removal ~99% and effluent COD ~20 mg/L. This is significant for simplifying the treatment of organic wastewater in both PN-anammox and nitrite shunting since the anaerobic digestion process in which the organic matter is removed could be bypassed. This further decreases the running cost since the remaining organic matter can also be used as the carbon source for the denitrification process.

![Figure 1](image-url)  
*Figure 1.* The effluent concentration of NH$_4^+$, NO$_2^-$, and NO$_3^-$ in the PN-SBR over 225 days.

3.2. Performance of the PN-SBR in a Typical Cycle and D-N$_2$O Releasing Patterns

From days 174 to 225 (Stage IV), the PN-SBR reached a stable PN state. In this stage, the NH$_4^+$-N in the influent was almost completely consumed (~98%) and largely (~60%) converted to NO$_2^-$-N (Figure 1). Each running cycle lasted for 270 min, consisting of the aerobic feeding phase (20 min), aeration phase (220 min, aeration was started at 0 min), sludge settling phase (40 min), and discharging phase (245 s). The HRT was set to be 24 h and the air gas flow rate was controlled at 0.5 L/min. Figure 2a shows the trends of NH$_4^+$-N, NO$_2^-$-N, and NO$_3^-$-N during a typical cycle. The concentration of NH$_4^+$-N increased rapidly during the feeding phase, reaching the highest value (~48 mg/L) at the
end of the phase, and then gradually decreased to near zero at the end of the aeration phase. However, the trend of NO$_2^-$-N was opposite to that of NH$_4^+$-N. It was calculated that about 60% of NH$_4^+$-N was transformed to NO$_2^-$-N. During the sludge settling phase, the contents of NH$_4^+$-N and NO$_2^-$-N did not change much. The content of NO$_3^-$-N remained low throughout the running cycle. As shown in Figure 2b, the pH curve showed almost the same trend as that of NH$_4^+$-N. The dissolved oxygen (DO) level decreased rapidly from ~7.0 mg/L at the beginning of the aerobic feeding phase, and remained <0.65 mg/L for 60 min, then gradually increased to ~7.0 mg/L with the depletion of NH$_4^+$-N.

![Figure 2. During a typical cycle of the PN-SBR, the change in the contents of: NH$_4^+$, NO$_2^-$, and NO$_3^-$ (a); DO and pH (b); dissolved N$_2$O and hydroxylamine (inset) (c); integrated area of N$_2$O amount (d); $\delta^{18}$O, $\delta^{15}$N$_{bulk}$, SP value, and the contribution (%) of gaseous N$_2$O during the aeration phase (e).](image)

For sewage-treatment plants that use nitrification and nitrification technology, there are three kinds of bacteria in the sludge: ammonia-oxidizing bacteria (AOB, NH$_4^+$ $\rightarrow$ NO$_2^-$), nitrite-oxidizing bacteria (NOB, NO$_2^-$ $\rightarrow$ NO$_3^-$), and aerobic heterotrophic bacteria (organics $\rightarrow$ CO$_2$) [33]. Establishing a stable PN reaction requires limiting the growth of NOB, making AOB and/or heterotrophic nitrifiers dominant flora, and increasing the nitrite accumulation. To do so, there are many factors that need to be controlled, including DO, pH, and aeration time. The physiological characteristics of AOB and NOB differ in the following ways: AOB has a greater affinity for oxygen than NOB; low dissolved oxygen (0.5–1 mg/L) has a higher inhibitory effect on NOB than on AOB [34]; the optimal pH for AOB is 7.0–8.5 and for NOB is 6.5–7.5, so pH should be controlled at 7.5–8.5 [35]. In addition, the nitrosation reaction consumes alkalinity, so it is necessary to supplement alkalinity while adjusting the pH. The substrate of AOB is NH$_4^+$-N, and NO$_2^-$-N as a reaction product is the substrate of NOB. Therefore, the appropriate aeration time was explored in the early
stage of reactor start-up, and the aeration was stopped after the nitrite was accumulated such that NOB could not react with nitrite substrate and grow by itself. In this PN-SBR, low levels of DO were maintained most of the time, indicating that the microorganisms in the reactor could use DO efficiently and that the oxidation of ammonium had been implemented. This utilized dissolved oxygen efficiently, suggesting ammonium oxidation was proceeding. At the end of the aeration phase, microbial growth and metabolism were inhibited due to the lack of nutrients, which led to the DO increasing again.

Figure 2c displays the changes of D-N2O content during a typical running cycle. D-N2O content increased rapidly from ~0.2 mg/L at the beginning of the feeding phase to its peak (3.5 mg/L) after feeding for 10 min, and then dropped to ~0.4 mg/L at the end of the feeding phase and maintained a low level afterward. This release pattern of D-N2O was accompanied by that of hydroxylamine production (Figure 2c), suggesting that the release of D-N2O was from the oxidation of hydroxylamine. Similar to our results, Chandran et al. found that N2O emissions assigned to nitrification were generally much higher than those of heterotrophic denitrification-related emissions, especially with recovery from anoxic conditions to aerobic conditions, rather than imposing anoxic conditions [36]. There were two main forms of N2O in the reactor: gaseous state and dissolved state. N2O has a high solubility in water (Henry’s coefficient of N2O is 0.024 M/atm whereas that of oxygen is only 0.0013 M/atm [37]), and some of the N2O would be present in the dissolved form, which contributed to the release of dissolved N2O in the gaseous form during the aeration phase. In addition, as we observed in the PN-SBR, the effect of high pH on N2O emission is also apparent, which is consistent with the results of an AOB enrichment nitrification system at slight acid or neutral pH [38].

Figure 2d,e exhibits the gaseous N2O amount and SP values for different sampling times during the aeration period, respectively. Since the content of N2O was lower than 50 ppm after 100 min, it is difficult to make a correct quantitative calculation. Here, the N2O concentration and SP values within the first 100 min of one typical cycle were plotted. After several periodic replications, it was found that the trend of the gaseous N2O concentration was similar to that of the D-N2O. Gaseous N2O content increased rapidly from ~200 ppm at the beginning of the aeration period to ~1180 ppm (its peak) after aeration for 10 min, dropped to 900 ppm at the end of the feeding phase (aeration for 20 min), and then gradually declined to ~30 ppm at the end of the aeration period. The mathematically integrated area of N2O within 100 min was calculated, and the total emission of N2O accounted for about 6.3% of total nitrogen load, based on the gas flow rate, HRT, and the effective volume of the reactor.

The isotope signatures of δ15Nα, δ15Nβ, δ15Nbulk and δ18O-N2O were obtained and the SP values of gaseous N2O were calculated according to the formula described above. Results showed that the SP values in this PN-SBR ranged from 19–23‰ during the first 100 min of aeration (Figure 2e). The SP values remained relatively constant within N2O molecules although their concentration changed over the running time. Based on SP values, the approximate contribution of NH2OH oxidation and N2O reduction to N2O emission during the aeration period can be calculated by assuming that each point was linearly proportional to the SP values. The contribution of the NH2OH oxidation pathway accounted for >59% of the total N2O production. The results of δ18O and δ15Nbulk were also used for N2O source partitioning, which elucidates the trends in the sources of N2O production.

Studies have shown that N2O emissions may be promoted in a PN bioreactor due to the high ammonia loading and nitrite accumulation, as well as a large amount of aeration and other conditions. It has been reported that N2O emissions increased rapidly at the beginning of a cycle and then slowed after 30 min [39], and this result was consistent with ours. We also found that during the inception phase of aeration, the high NH4+ N influent water entered the reactor and was converted to NH3OH, and an imbalance with the rate of further oxidation to nitrite caused the NH3OH accumulation to function as the source for N2O emission [40]. Terada et al. reported that N2O emissions in a PN process were mainly derived from hybrid N-nitrosation and NH2OH pathways [7]. Compared with complete
nitrification, PN culture produced more N\textsubscript{2}O, and the hydroxylamine pathway was the dominant pathway [41]. The pathway of N\textsubscript{2}O production is complex and involves the actions of various microorganisms, including autotrophic AOB, heterotrophic nitrifiers, and nitrite-oxidizing bacteria (NOB), in addition to the chemical decomposition of intermediate metabolites [42]. Early studies suggested that denitrification by both AOB and heterotrophic nitrifiers was the main source of N\textsubscript{2}O production in PN [43]; however, it has been found that the hydroxylamine oxidation pathway and nitrite-mediated hydroxylamine oxidation also contribute significantly to N\textsubscript{2}O release in the PN process [6,7,42]. Moreover, the abiotic reaction of hydroxylamine with nitrite might happen in biological systems with nitrite and hydroxylamine accumulation [44].

3.3. Succession of Microbial Community Structure in Different Stages of the PN-SBR

Illumina sequencing was conducted for the 16 samples during the whole running period. The raw sequence reads ranged from 44,819 to 69,935 and were standardized to 44,819 for further analysis. The taxonomic structure of bacterial communities resulted in the classification of 19 phyla, 32 classes, 79 orders, 143 families, 228 genera, and 371 OTUs based on a 0.97 threshold across all samples. The Good’s coverage for all 16 samples was >99.8%, indicating that the sequencing depth was sufficient to cover the majority of microorganisms in the samples. The alpha diversity analysis results of different stages indicated that the Shannon and Chao indexes were higher in stages I and II, and decreased in stages III and IV; by contrast, the Simpson index decreased in stages I and II and increased in stages III and IV. These results suggested that the community richness and community diversity of the PN-SBR increased in the early days and decreased thereafter (Table 2).

### Table 2. Alpha diversity index for samples from different running stages of the PN-SBR.

| Index   | Stage I       | Stage II      | Stage III      | Stage IV      |
|---------|---------------|---------------|---------------|---------------|
| Shannon | 2.60 ± 0.08   | 3.09 ± 0.09   | 2.55 ± 0.19   | 1.95 ± 0.14   |
| Simpson | 0.13 ± 0.01   | 0.09 ± 0.01   | 0.16 ± 0.04   | 0.35 ± 0.07   |
| Chao    | 206.48 ± 19.96| 254.73 ± 10.92| 216.16 ± 23.92| 170.84 ± 17.07|

Proteobacteria and Bacteroidetes were the dominant phyla throughout the whole operation period, accounting for an average relative abundance of 55–78% and 13–35%, respectively, in different stages (Figure 3a). The changes in bacterial community composition at the class level are shown in Figure 3b. The $\gamma$-proteobacteria accounted for more than 70% of the total during stage I, decreased significantly in stage II, and then increased from 13.8% on day 127 to 73.60% on day 222 (stages III and IV). The decrease of $\gamma$-proteobacteria was accompanied by the increase of $\alpha$-proteobacteria (41.14% on day 127). Figure 3c shows the results of hierarchically clustered heatmap analysis at the genus level. The top 50 genera were clustered hierarchically. Many bacteria involved in nitrogen metabolism were significantly more abundant after the PN-SBR process, including the members of the HNAD genera Acidovorax, Brevundimonas, Comamonas, Paracoccus, an unclassified genus of the family Chitinophagaceae, and an unclassified genus of the family Propionibacteriaceae. The total relative abundance of these six genera increased with the running of the PN-SBR (16.21% in stage I, 30.67% in stage II, 41.71% in stage III, and 55.47% in stage IV), much higher than that in other PN reactors for synthetic inorganic wastewater [7]. The relative abundance of Nitrosomonas, the only AOB genus detected in the PN-SBR, increased from ~0.4% in the early days to ~0.9% during stage IV, indicating that AOB was enriched to some degree. The AOB relative abundance was lower than that in similar reactors for inorganic wastewater, such as in a SHARON reactor; the relative abundance of the genus Nitrosomonas ranged from 1.0–4.4% [33], much lower than that (up to 16.1%) in a different PN reactor for synthetic inorganic wastewater [7]. The reason for the low abundance of AOB may be that their growth was inhibited by the organic compounds presented. These results indicate that there were other ammonia-oxidizing microbes, such as HNAD bacteria,
in this PN-SBR, except for autotrophic AOB in regard to the high ammonia removal and transformation ability of the PN-SBR.

Table 2. Alpha diversity index for samples from different running stages of the PN-SBR.

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|--------|-------------|-------------|-------------|-------------|
| Shannon| 2.60 ± 0.08 | 3.09 ± 0.09 | 2.55 ± 0.19 | 1.95 ± 0.14 |
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| Chao   | 206.48 ± 19.96 | 254.73 ± 10.92 | 216.16 ± 23.92 | 170.84 ± 17.07 |

Figure 3. Cont.
Figure 3. Microbial community compositions at the phylum level (a), class level (b), and hierarchically clustered heatmap analysis results at the genus level (c).

3.4. Correlation Network Analysis and Characteristics of Core Bacteria from the PN-SBR

The relationships between core bacterial genera, identified from Illumina sequencing, and those heterotrophic nitrifying bacteria isolated were investigated using correlation network analysis. In all samples, the genera with a sum of relative abundance greater than 1% in all four stages were considered as “core” genera. The top 20 genera in terms of total abundance at the taxonomic level were selected and the microbial network was analyzed. During the whole operation of the reactor, the relative abundance of *Acidovorax*, *Chitinophagaceae_unclassified*, *Propionibacteriaceae_unclassified*, *Paracoccus*, *Burkholderiaceae_norank*, *Thauera*, and *Comamonas* was the highest. In the correlation network (correlation value $r > 0.5$), the highest betweenness centrality (BC) values in the network were found for the genera *Comamonas*, *Burkholderiaceae_norank*, *Comamonas*, *Thermomonas*, *Shinella*, and *Para-
cococcus, which show that they were the core bacterial genera of the PN-SBR (Figure 4a). More than 50 heterotrophic nitrifying bacterial strains were isolated, and 25 of them belonged to the core bacteria classified into six groups. Among them, six representative strains were characterized for their nitrifying performance (Table 3). Results (Table 3) showed that all the strains were efficient in nitrification, with NH$_4^+$-N removal >40% and NO$_2^-$-N formation (4.98–18.89 mg/L).

| Strain      | Taxonomy               | NH$_4^+$-N Removal (%) | NO$_2^-$-N Formed (mg/L) | NO$_3^-$-N Formed (mg/L) |
|-------------|------------------------|------------------------|---------------------------|--------------------------|
| SBR-Zi-1    | Paracoccus             | 96.3 ± 3.9             | 18.885 ± 3.183            | 1.718 ± 0.032            |
| SBR-SF13    | Comamonas             | 61.7 ± 4.1             | 5.371 ± 0.989             | 0.921 ± 0.135            |
| SBR-SF4     | Acidovorax            | 46.4 ± 2.8             | 6.543 ± 1.033             | 1.132 ± 0.019            |
| SBR-SC2     | Shinella               | 50.3 ± 1.5             | 4.982 ± 0.686             | 0.964 ± 0.074            |
| SBR-S-SH9-1 | Propionibacteriaceae   | 65.1 ± 5.7             | 13.786 ± 2.977            | 3.260 ± 0.145            |
| SBR-SFI-2   | Brevundimonas         | 48.3 ± 2.4             | 7.317 ± 0.683             | 0.311 ± 0.009            |

Figure 4. (a) The core microbial network in the reactor showed the interaction of different genera. The size of a node indicates the abundance of the genus. The color of the node corresponds to the taxonomy classification. The edge color represents the correlation of positive (green) or negative (red). (b) The phylogenetic tree of core bacteria and isolated nitrifying bacteria in the reactor.

Table 3. Nitrifying performance of six representative strains after cultivation for 48 h.

Phylogenetic analyses based on the 16S rRNA gene sequences of the strains and those of the high throughput database showed that all the six strains could match their representative OTUs with 100% sequence similarity (Figure 4b). As shown in Figure 4b, these pairs were Acidovorax sp. SBR-SF4 and OTU 291, Comamonas sp. SBR-SF13 and OTU 3, Propionibacteriaceae strain SBR-S-SH9-1 and OTU 327, Paracoccus sp. SBR-Zi-1 and OTU 129, Shinella sp. SBR-SC2 and OTU 384, and Brevundimonas sp. SBR-SFI-2 and OTU 60. As mentioned above, the relative abundance of these OTUs increased with the progress of the PN-SBR and their represented strains were efficient in nitrification (Table 3). It can be concluded that these core genera, together with other core genera, contributed
3.5. Metagenomic Analysis of Microbial Functional Genes in the PN-SBR

Activated sludge samples were collected on days 222 and 223 (stage IV) for metagenomic analysis; 152,095,990 raw reads were obtained. After quality control, 150,193,718 clean reads were obtained, assembled to 211,184 contigs; 393,112 genes were annotated by MetaGene. According to the KEGG database (http://www.genome.jp/kegg/ (accessed on 17 June 2021)), the genes involved in nitrification (\( \text{NH}_4^+ \rightarrow \text{NH}_3\text{OH} \rightarrow \text{NO}_2^- \rightarrow \text{NO}_3^- \)) and denitrification (\( \text{NO}_3^- \rightarrow \text{NO}_2^- \rightarrow \text{NO} \rightarrow \text{N}_2 \)) were all detected (Figure 5), and the abundance of these genes is shown in Figure 6. Ammonia monooxygenase (Amo, EC 1.14.99.39) is the first enzyme for ammonia oxidation, which catalyzes the conversion of ammonia to hydroxylamine. Results (Figure 6a) showed that the abundance of amo was 0.053‰, and functional bacteria containing amo detected in this PN-SBR were all members of the genus *Nitrosomonas* (Figure 6b). The relative abundance of hydroxylamine dehydrogenase (HAO, EC 1.7.2.6), catalyzing the conversion of hydroxylamine to nitrite, was 0.22‰. Functional bacteria containing hao detected in the PN-SBR were mainly members of the genus *Nitrosomonas* (77.8%), followed by the genus *Comamonas* (5.47%). All the genes involved in denitrification were detected, including *nir* (nitrate reductase, EC 1.7.5.1, 3.23‰), *naph* (cytochrome nitrate reductase, EC 1.9.6.1, 0.12‰), *nirK* (nitrite reductase, EC 1.7.2.1, 1.43‰), *nrfA* (cytochrome nitrite reductase, EC 1.7.2.2, 0.31‰), *nirB* (NADH-dependent nitrite reductase, EC 1.7.1.15, 0.14‰), *nosZ* (nitrous oxide reductase, EC 1.7.2.4, 0.17‰), and *norB* (cytochrome c nitric oxide reductase, EC 1.7.2.5, 0.12‰). Functional bacteria containing these denitrification genes detected in this PN-SBR were mainly *Acidovorax*, followed by *Comamonas*, *Simplicispira*, *Paracoccus*, *Shinella*, and *Thauera*. The coexistence of these genes indicated that bacteria in the PN-SBR could carry out a complete denitrification pathway [45].

![Figure 5. KEGG pathway of nitrogen metabolism. A red box means the enzyme which was detected in the PN-SBR metagenomic database.](image-url)
Although the PN-SBR showed high nitrification activity, the abundance of nitrification genes (amo and hao) was much lower than that of denitrification ones (nirK, nar, norB, and nosZ). The results were consistent with those described above for microbial community structure (Figure 3). The possible reasons were the presence of a carbon source in the artificial wastewater, the high organic load (C/N ≈ 3), and that the collection of autotrophic bacteria was somewhat inhibited. The high nitrifying ability of this PN-SBR might result from the functional combination of AOB and HNAD bacteria which could also oxidize ammonia as discussed above. In addition, several heterotrophic nitrifying bacteria were isolated from the reactor using a culturable method, and the heterotrophic nitrification properties of six representative strains were further verified. Therefore, it is hypothesized that the nitrification role of heterotrophic bacteria was underestimated. It had been reported that the expression rate of amo in SBR is higher than that in denitrification [46], which may be another reason for the high nitrifying activity of this PN-SBR. The high nitrifying activity resulted in the transient accumulation of hydroxylamine and the same trends of D-N₂O and gaseous N₂O in the early feeding time of a typical cycle. It was also notable that the relative abundance of hao was quite high and so the activity of HAO could also oxidize hydroxylamine to form N₂O as a by-product [47,48]. In combination with SP values, it can be concluded that hydroxylamine oxidation contributed mainly to N₂O production in the PN-SBR, as summarized in Figure 7.
4. Conclusions

In this study, a PN-SBR was constructed for treating artificial organic wastewater. The results indicated that this PN-SBR successfully achieved robust PN performance with >98% ammonia removal and >60% transformation to nitrite, due to the significant increase in the abundance of heterotrophic nitrifiers with the progress of the reactor. About 6.5% of consumed ammonia was released as \( \text{N}_2\text{O} \) emissions, which were produced mainly (>59%) from hydroxylamine oxidation as indicated by the SP values of the \( \text{N}_2\text{O} \) molecules. These results provide a guide for the direct utilization of PN reactors for treating organic wastewater and are helpful to understand the microbial process and \( \text{N}_2\text{O} \) release pathways of partial nitrification.

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