

Article

Nitrogen Removal Characteristics and Comparison of the Microbial Community Structure in Different Anaerobic Ammonia Oxidation Reactors

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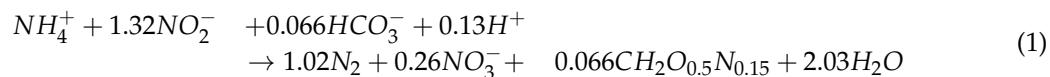


Abstract: Nitrogen removal characteristics and the comparison of the microbial community structure were investigated in different anaerobic ammonia oxidation (Anammox) reactors: an anaerobic sequencing batch reactor (ASBR) and a biofilter reactor. The Anammox systems were inoculated with sludge from the second settling tank of a wastewater treatment plant in Guangzhou, China. After successful start up of Anammox, the microbial community structure of different Anammox reactors were studied through high-throughput sequencing. The results showed that anaerobic ammonium oxidation in the ASBR reactor could successfully start up after 134 days, while Anammox in the biofilter could start up after 114 days. In both systems, total nitrogen removal was at 80% after more than 200 days of operation. The diversity of denitrifying microorganisms was high in both reactors, with Planctomycetes as the main taxa. Anammox bacteria belonging to the genera *Candidatus Anammoxoglobus* and *Kuenenia*, were dominant in the ASBR, while all three genera of *Candidatus*, *Anammoxoglobus*, *Kuenenia*, and *Brocadia*, could be detected in the biofilter reactor. Therefore, the biofilter starts up faster than the ASBR, and contains richer species, which makes it more suitable to domesticate Anammox bacteria.

Keywords: Anammox; ASBR; biofilter; high-throughput sequencing

1. Introduction

Anaerobic ammonium oxidation (Anammox) was discovered and named in 1995 by researchers of the Delft University of Technology, Netherlands [1]. It is an alternative nitrogen removal process oxidizing $\text{NH}_4^+ - \text{N}$ into nitrogen, using $\text{NO}_2^- - \text{N}$ as an electron acceptor. The reaction equation was shown as follows:



The bacteria involved in this process, Anammox bacteria, are anaerobic autotrophic bacteria, and therefore Anammox is an anaerobic process and does not require an additional carbon source [2]. In this sense, it has been recognized as the most cost effective biological nitrogen removal approach. However, Anammox bacteria have a long doubling time, resulting in long start up periods for batch reactors and difficulties in enrichment, which severely limits large-scale applications.

Therefore, the selection of a suitable reactor for anaerobic ammonia oxidation plays an important role in the enrichment of Anammox bacteria. At present, most studies on Anammox oxidation have

focused on three aspects: the rapid start up of Anammox reactors under different conditions [3–5], the factors potentially influencing Anammox bacteria [6,7], and the combined process of Anammox and other nitrogen removal processes [8–11]. All of these studies involved two types of reactors, an anaerobic sequencing batch reactor (ASBR) and a biofilter reactor [12]. They were assumed to be suitable reactors to enrich Anammox bacteria as ASBR could hold Anammox bacteria in its ideal sludge state [13] and the biofilter could prevent the loss of microorganisms due to its filter [14]. However, each study was only carried out using one type of reactor, and very few studies have been reported on the comparison of the two reactors during the start up of the Anammox process. Much attention has been given to the comparison of different seeding sludge sources [15,16]. Whether, with same seeding sludge source, the type of reactor influences results, and which type of reactor is more suitable for Anammox, are worth investigation.

At present, six genera of Anammox bacteria have been described, namely *Brocadia*, *Kuenenia*, *Jettenia*, *Anammoxoglobus*, *Scalindua*, and *Anammoximicrobium* [17]. Of these bacteria, *Scalindua* was found in seawater, while the other genera occur in various sewage systems [18]. In the early days, polymerase chain reaction (PCR) was used to detect Anammox bacteria [19]. Due to the limitations of conventional PCR, high-throughput sequencing has emerged with a number of advantages such as high accuracy, high speed and sensitivity, and it is highly automated [20]. It is, therefore, increasingly being used to detect bacterial species in various environments, especially in activated sludge systems, and it is also an effective tool to indicate the transformation of functional bacteria in the system, explaining the nitric–nitrogen effect in the system from the perspective of molecular biology.

In this study, two different reactor types, namely an ASBR reactor and a biofilter reactor, were used to start up anaerobic ammonia oxidation. High-throughput sequencing technology was used to characterize the microbial community structure in the two systems. The overall aims of this study were: 1) to obtain a more suitable reactor to start up Anammox oxidation; 2) to reveal any potential shifts in the microbial community from seeding sludge to enriched sludge; and 3) to determine the dominant bacteria in different reactors.

2. Materials and Methods

2.1. Reactor Configuration

The experiments were conducted in an ASBR reactor and a biofilter made of plexiglass with a working volume of 4.0 L. Both reactors were operated at ambient temperature (the range was between 20 to 26 °C). The ASBR was equipped with a mechanical mixer operated constantly at 60 rpm for mixing, while the biofilter reactor was equipped with a pump for an upward flow to continuously supply the substrate. Both reactors were covered with a black plastic bag to block out the light [21].

2.2. Operational Strategies

The two reactors were initially seeded with sludge obtained from the second settling tank of a wastewater treatment plant in Guangzhou, China. The ASBR reactor was operated on a 12 h cycle, consisting of five phases: 3 min filling, 10 h of anaerobic reaction, 0.5 h settling, 3 min of discharging 2.0 L of effluent, and 1.5 h idle. Two cycles were conducted per day and the hydraulic retention time (HRT) of ASBR was 24 h. For the biofilter, the initial HRT was 24 h; in both reactors, the pH of the influent was 7.5 ± 0.1 .

The synthetic wastewater used consisted of NH₄Cl, NaNO₂, and medium solution; NH₄Cl and NaNO₂ were dosed in the feeding medium to provide NH₄⁺-N and NO₂⁻-N. The medium solution contained the following nutrients: NaHCO₃ at 0.2 g·L⁻¹, KH₂PO₄ at 0.068 g·L⁻¹, and 10 mL of each trace element solution I, II, and III. The compositions of trace element solution I, II, and III are shown in Table 1. The pH was maintained at 7.5 ± 0.1 at the beginning of each cycle by adding sulfuric acid solution (1 M) or sodium hydroxide solution (1 M).

Table 1. The compositions of trace element solution I, II, and III.

Trace element solution	Reagent	Concentration
I	FeSO ₄ ·7H ₂ O	45 g·L ⁻¹
	EDTA	25 g·L ⁻¹
II	KCl	3.5 g·L ⁻¹
	NaCl	2.5 g·L ⁻¹
	CaCl ₂ ·2H ₂ O	3.5 g·L ⁻¹
	MgSO ₄ ·7H ₂ O	2.5 g·L ⁻¹
III	EDTA	75 g·L ⁻¹
	ZnSO ₄ ·7H ₂ O	2.15 g·L ⁻¹
	CoCl ₂ ·6H ₂ O	1.2 g·L ⁻¹
	MnCl ₂ ·4H ₂ O	4.95 g·L ⁻¹
	CuSO ₄ ·5H ₂ O	1.25 g·L ⁻¹
	Na ₂ MoO ₄ ·2H ₂ O	1.1 g·L ⁻¹
	NiCl ₂ ·6H ₂ O	0.95 g·L ⁻¹
	Na ₂ SeO ₄ ·10H ₂ O	1.05 g·L ⁻¹
	H ₃ PO ₄	0.07 g·L ⁻¹

2.3. Sample Collection, DNA Extraction, and High-Throughput Sequencing

Biomass samples from the two reactors were collected after 240 days of operation to characterize the microbial community structure via high-throughput sequencing. The obtained mixed liquid sludge samples were filtered through filter paper (11 cm, 45 um) and weighed 0.20–0.25 g for DNA extraction. Genomic DNA was extracted by the Power Soil DNA Isolation Kit (Mobio, USA) and diluted to 10 ng/μL for subsequent analysis.

Nested polymerase chain reaction, amplifying the V3–V4 region of the 16S rRNA gene, was conducted using the bacterial universal primers 341F (5'-CCCTACACGACGCTTCCGATCTG-3') and 805R (5'-GAATGGAGTTCCCTGGCACCCGAGAATTCCA-3'). For the amplification of each sample, we added 15 μL of 2× Taq master mix, 1 μL of Bar-PCR primer F (10 μM), 1 μL of primer R (10 μM), 10–20 ng of template DNA, and brought the solution up to 30 μL with ddH₂O. The PCR program consisted of an initial denaturation step at 94 °C for 3 min, followed by five cycles at 94 °C for 30 s, 45 °C for 20 s, and 65 °C for 30 s, followed by another 20 cycles at 94 °C for 20 s, 55 °C for 20 s, and 72 °C for 30 s, and a final extension step at 72 °C for 10 min. During the second amplification, we added 15 μL of 2× Taq master mix, 1 μL of Bar-PCR primer F (10 μM), 1 μL of Primer R (10 μM), 20 ng of the PCR product from the first step, and brought the solution up to 30 μL with ddH₂O. The PCR program consisted of an initial denaturation step at 95 °C for 3 min, followed by five cycles at 94 °C for 20 s, 55 °C for 20 s, and 72 °C for 30 s, and a final extension step at 72 °C for 5 min. The DNA-amplified samples were sequenced using an Illumina MiSeq sequencer (ID: PRJNA505712).

2.4. Analytical Methods

Effluent samples were collected daily into a 25 mL sampling cup and then filtered through 45 μm filters. Each sample was immediately stored at 4 °C and analyzed on the following day for ammonium nitrogen (NH₄⁺–N), nitrite nitrogen (NO₂[–]–N), and nitrate nitrogen (NO₃[–]–N), using Nessler's reagent spectrophotometry, N-(1-naphthyl)-ethylenediamine reagent spectrophotometry, and Thymol reagent spectrophotometry, respectively, with a UV spectrophotometer (UV-2550, SHIMADZU, Japan). The concentration of total nitrogen (TN) in the influent and the effluent equaled the sum of NH₄⁺–N, NO₂[–]–N, and NO₃[–]–N. Dissolved oxygen (DO) and pH were determined using oxygen and pH probes, respectively (WTW pH/Oxi 340i, WTW Company, Germany).

3. Results and Discussion

3.1. Nitrogen Removal at Different Stages

3.1.1. ASBR Reactor

The ASBR reactor was operated over 246 days, with a start up period of 134 days. After the start up period, influent TN was steadily increased. Microbial endogenous respiration leads to cell decay and lysis when substrate is depleted [22], therefore, increasing substrate concentration in influent over time is necessary. Based on the different conditions during the operational period, the process was divided into seven phases, as shown in Table 2. The concentration of effluent TN, TN removal efficiency, and the stoichiometric ratio in the ASBR over the whole operation time are shown in Figure 1. It should be noted that theoretical values of the nitrite removal/ammonium removal ratio and the nitrate accumulation/ammonium removal ratio were 1.32 and 0.26, respectively, based on equation 1 from the introduction.

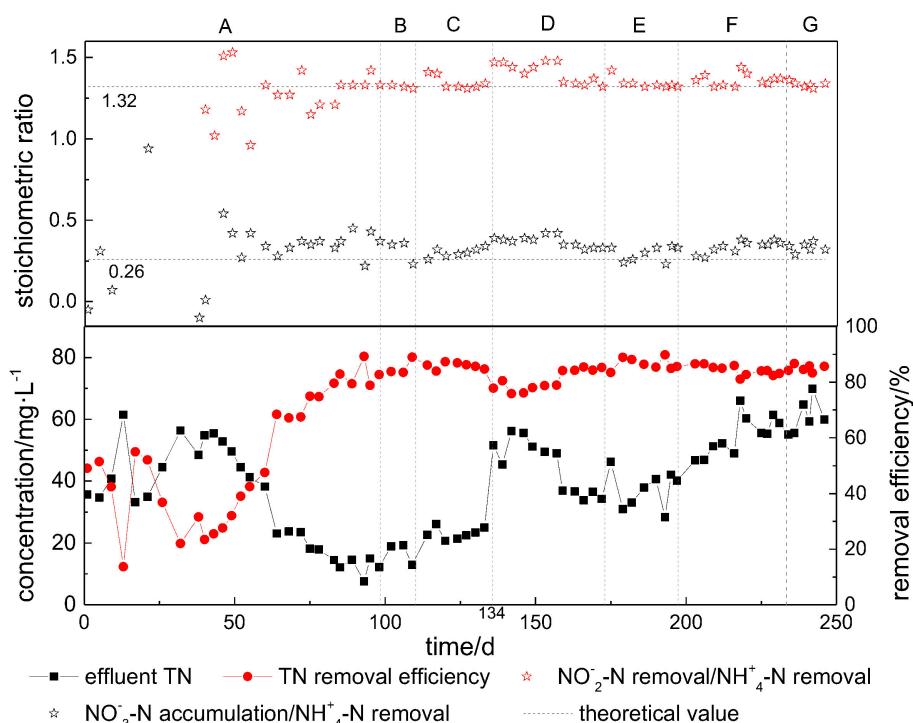


Figure 1. Total nitrogen (TN) concentration of effluent, TN removal efficiency, and stoichiometric ratios in an anaerobic sequencing batch reactor (ASBR) over the whole operation time.

Table 2. Total nitrogen concentration of the ASBR at different stages (phase A to G).

	A	B	C	D	E	F	G
Time/d	1–98	99–109	110–134	135–172	173–197	198–234	235–246
TN/mg·L⁻¹	72.28	116.96	163.36	232.96	279.36	348.96	418.56

During phase A (days 1–98), the average influent TN stabilized at $72.28 \text{ mg}\cdot\text{L}^{-1}$. Over time, with microbial endogenous respiration, the concentration of TN in the effluent gradually decreased, and TN removal efficiency increased and reached 89.25%. Because of the presence of Anammox bacteria in the system, the concentration of TN in the effluent did not reach zero. As the nitrite removal/ammonium removal ratio was close to the theoretical value, it was assumed that Anammox bacteria were present, but were not the dominant bacteria. To increase the activity of Anammox and avoid the Anammox microbial endogenous respiration caused by long periods of anoxic stirring after

substrate depletion, substrate concentration in influent was gradually increased from 116.96 mg L^{-1} to 418.56 mg L^{-1} . At the same time, the nitrate accumulation/ammonium removal ratio was getting closer to the theoretical value. The ratio between average ammonium removal, nitrite removal, and nitrate accumulation was 1:1.36:0.33. After the start up period, influent TN was steadily increased to improve microbial activity. The ASBR reactor could successfully start and maintain Anammox, with a TN removal efficiency above 80% after more than 200 days of operation. TN removal efficiency would decrease a short time after TN concentration increased at the start of each stage, and after operating for some time, it would recover to as high of an efficiency as before. During the whole start up the stoichiometric ratio was higher than the theoretical value, which means there were a variety of types of bacteria, and that they worked together to remove TN from the ASBR system.

3.1.2. Biofilter Reactor

The biofilter reactor was operated for 246 days, with a start up period of 114 days. Figure 2 shows the concentration of TN, TN removal efficiency, and the stoichiometric ratio in the biofilter reactor during the operating time. Based on the different operational conditions, the process was divided into 11 phases.

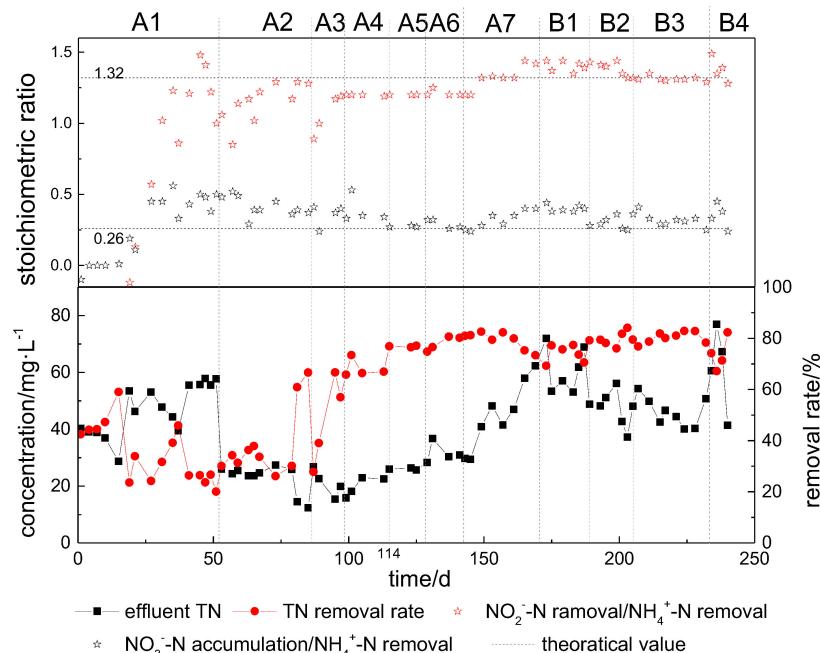


Figure 2. Total nitrogen (TN) concentration of effluent, TN removal efficiency, and stoichiometric ratios in the biofilter over the whole operation time.

The TN concentrations of influent and HRT values of the different phases are shown in Table 3. The values of TN gradually increased from A1 to A7; because of the gradual adaptation of the bacteria to the operational conditions and the increased biomass activity, the HRT decreased from 24 h to 8 h during phases B1 to B4.

Table 3. Influent TN concentration and hydraulic retention time (HRT) of each stage of the biological filter reactor.

	A1	A2	A3	A4	A5	A6	A7	B1	B2	B3	B4
Time/d	1–51	52–89	90–99	100–114	115–129	130–145	150–169	170–187	188–205	206–228	229–240
TN/mg·L ⁻¹	70	37.08	46.28	70	112.28	156.28	234.28	234.28	234.28	234.28	234.28
HRT/h	24	24	24	24	24	24	24	20	16	12	8

Note: The error of total nitrogen concentration in influent water at each stage was controlled within ± 1.00 .

The start up phase of the biofilter reactor lasted for 114 days, from phase A1 to phase A4. In phase A1, the nitrite removal/ammonium removal ratio was lower than the theoretical value which was below 1.32, while the nitrate accumulation/ammonium removal ratio was higher than 0.26, and even reached 0.59. The results also showed that the removal efficiency of NO_2^- -N was higher than that of NH_4^+ -N together with a high accumulation of NO_3^- -N, which resulted in a gradual increase in the concentration of effluent TN. As seen in Figure 2, at this phase, the TN in the effluent had accumulated significantly, up to $59.06 \text{ mg}\cdot\text{L}^{-1}$, and the TN removal efficiency was below 42.3%. These results also indicated that Anammox bacteria were not the dominant bacteria in the system and that nitrite-oxidizing bacteria (NOB) and denitrifying bacteria were more concentrated than the Anammox bacteria at that time. Previous studies have shown that this type of reactor could be operated stably when the substrate ratio was less than 1:1.62 [23]. Therefore, the concentration ratio of influent NO_2^- -N and NH_4^+ -N was reduced to 1.2 in order to induce growth of Anammox bacteria into the dominant taxa. At phase A2, the nitrite removal/ammonium removal ratio increased gradually from 0.94 to 1.31, while the nitrate accumulation/ammonium removal ratio decreased gradually from 0.51 to 0.31. The maximum TN removal efficiency was 60%. After 94 days of operation (at the end of phase A6), the concentration ratio of influent NO_2^- -N and NH_4^+ -N was recovered to 1.32 with high TN removal efficiency.

It should be noted that after more than 100 days of operation, at the end of phase A4, the TN removal efficiency was stable, and the stoichiometric ratio tended to be close to the theoretical value (due to the adjustment of the ratio of influent, the ratio of NO_2^- -N removal/ NH_4^+ -N removal tends towards 1.2 rather than 1.32). This showed that Anammox bacteria gradually became the dominant species in the system, indicating a successful start up phase.

Subsequently, to further increase the activity of Anammox bacteria, the influent TN was gradually increased to $234.28 \pm 1.00 \text{ mg}\cdot\text{L}^{-1}$. After 169 days of operation, the removal efficiency of TN reached 82.56%. An efficient way to increase TN load was shortening the HRT [24]. Considering that the HRT of the biofilter reactor could be controlled by adjusting the flow rate, it was shortened from 24 h to 8 h from phase B1 to B4. The nitrite removal/ammonium removal ratio fluctuated close to the theoretical value, while the nitrate accumulation/ammonium removal ratio was higher than the theoretical value. The ratio between average ammonium removal, nitrite removal, and nitrate accumulation was 1:1.33:0.32. Therefore, it was assumed that other denitrifying bacteria were still present in the system. The nitrite removal/ammonium removal ratio largely deviated after 230 days, and after adjusting the influent pH in the later stage, the system gradually returned to normal.

The results showed that biofilter reactors could also be used to acclimate and enrich Anammox bacteria. Anammox bacteria could be enriched more rapidly in biofilter reactors than in an ASBR. In addition, the TN load could be increased indirectly by shortening the HRT in a biofilter. After more than 200 days of operation, TN removal efficiency in the biofilter stabilized at more than 80%.

3.2. Microbial Community Analysis in Different Systems

3.2.1. Microbial Community Richness and Alpha Diversity

Three 16S rRNA gene libraries were constructed via Illumina MiSeq sequencing of the seed sludge (sample a), the ASBR (sample b), and the biofilter (sample c) community, with 41,708, 52,976, and 63,983 high-quality reads, respectively. All indexes are shown in Table 4.

Table 4. Microbial community richness and alpha diversity in seed sludge (a), domesticated sludge of the ASBR reactor (b), and domesticated sludge of the biofilter (c).

Estimator index	a	b	c
ACE index	107,031.0	267,200.6	420,401.1
Chao index	47,726.38	88,476.66	138,852.20
Shannon index	6.75	5.10	5.18
Simpson index	6.3×10^{-3} .	0.04	0.06
Coverage index	0.86	0.86	0.84

The value of the Shannon diversity index indicates the abundance of species within a community; larger values indicate a higher phyla diversity. Among the three investigated samples, seed sludge had the highest diversity (Shannon index = 6.75), while the ASBR and the biofilter reactor had values of 5.10 and 5.18, respectively.

The results showed that the seed sludge from the secondary sedimentation tank of the wastewater treatment plant contained more bacterial species, and after cultivation and acclimation to a special environment, the activated sludge in the reactor was gradually being replaced and enriched, resulting in a decreased diversity. Based on this, it could be inferred that the microbial community structures were affected by the different reactor features, and different bacterial groups were enriched in both the ASBR and the biofilter reactor.

3.2.2. Changes in Microbial Community Structure During Operation

The similarities and differences among the three systems were identified using hierarchical cluster analysis shown in Figure 3. Some strains in the seed sludge clearly differed from those in the ASBR and the biofilter, for example the color of *Planctomycetes* in the ASBR and the biofilter seemed relatively red, which means that after long-term domestication, *Planctomycetes* gradually become the dominant group and was successfully enriched in both reactors.

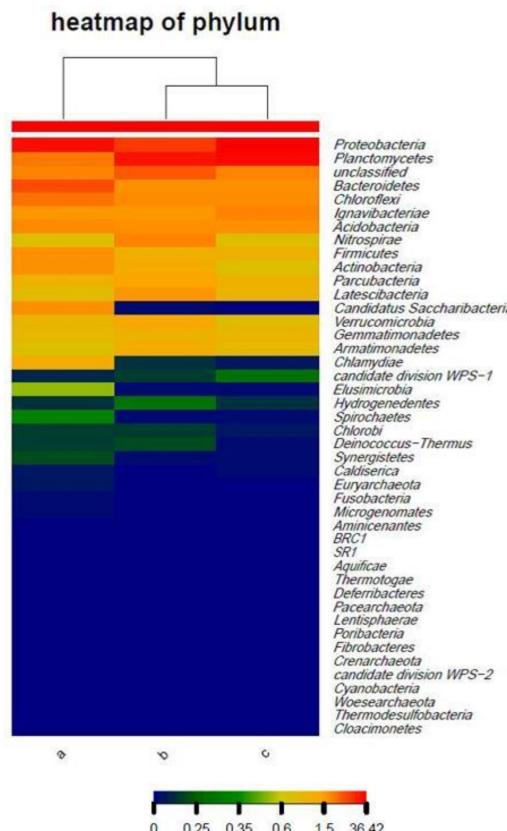


Figure 3. Heatmap of 16S rRNA metagenomics of the microbial populations of a, b, and c samples. Samples a, b, and c were for seed sludge, ASBR sludge, and biofilter reactor sludge, respectively. Color scale from blue (lowest abundance) to red (highest abundance).

In the three samples, 44 phyla were detected, of which eight were present in all three samples; the phyla involved in nitrogen removal were *Proteobacteria*, *Planctomycetes*, and *Nitrospirae*. The proportions of *Proteobacteria*, *Planctomycetes*, and *Nitrospirae* in the different samples are summarized in Table 5. After enrichment in the reactors, the *Planctomycetes* increased in both reactors to become 31.08% of all bacteria in the ASBR and 33.79% in the biofilter.

Table 5. The percentages of *Proteobacteria*, *Planctomycetes*, and *Nitrospirae* in seed sludge (a), domesticated sludge of the ASBR reactor (b), and domesticated sludge of the biofilter (c).

	Sample a	Sample b	Sample c
<i>Proteobacteria</i>	33.46%	23.72%	36.42%
<i>Planctomycetes</i>	9.7%	31.08%	33.79%
<i>Nitrospirae</i>	0.37%	6.91%	0.39%

Numerous bacterial populations are involved in nitrification, denitrification, and Anammox processes. Figure 4 illustrates the phyla abundance of bacterial communities in the seed sludge, the ASBR sludge, and the biofilter sludge. Most bacteria belonged to the *Proteobacteria* and the *Planctomycetes*, which accounted for over 50% of all bacteria in the ASBR and biofilter samples. According to these results, prolonged sludge domestication could make the target species gradually become the dominant one.

Ammonia oxidizing bacteria (AOB) and some NOB belong to the group *Proteobacteria*, while Anammox bacteria belong to *Planctomycetes* and other NOB belong to *Nitrospirae*. In the seed sludge, *Planctomycetes* only accounted for 9.7% of the population, but after long-term domestication, both reactors contained more *Planctomycetes*, indicating that Anammox bacteria were successfully enriched. The seed sludge contained a large amount of AOB, and after a relatively long domestication period, AOB were still abundant in the two reactors. So ammonium in the reactors could be removed through the cooperation of AOB and Anammox bacteria. Unlike the biofilter reactor, the ASBR contained a certain amount of *Nitrospirae* bacteria, which can convert NO_2^- -N to NO_3^- -N, and therefore, the concentration of NO_3^- -N in the effluent of the ASBR reactor was slightly higher than that in the biofilter reactor.

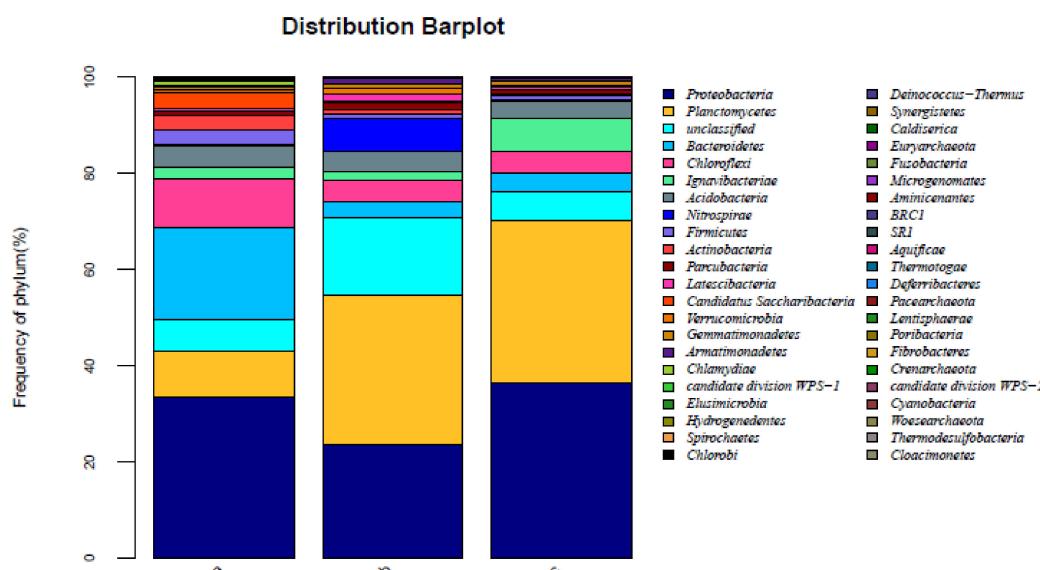


Figure 4. The phyla abundance of bacterial communities in a, b, and c samples. Samples a, b, and c were for seed sludge, ASBR sludge, and biofilter reactor sludge, respectively.

It was worth noting that the phylum *Chloroflexi* was present in each sample, and in previous studies it had often been observed in autotrophic nitrogen removal systems as it could effectively degrade and use dead biomass and prevent the accumulation of organic waste in the system [25].

The phylum involved with nitrogen removal was selected for further analysis at the genus level. The results are shown in Figure 5.

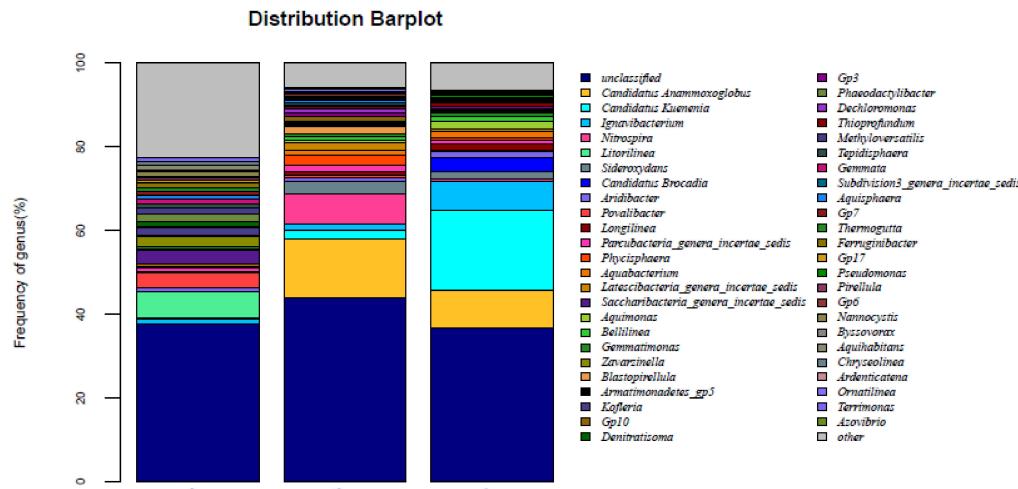


Figure 5. The genera abundance of bacterial communities in a, b, and c samples. Samples a, b, and c were for seed sludge, ASBR sludge, and biofilter reactor sludge, respectively.

Based on Figure 5, *Candidatus Anammoxoglobus* and *Candidatus Kuenenia* were mainly responsible for anaerobic ammonia oxidation in both the ASBR and the biofilter reactor. *Candidatus Brocadia* was of some importance in the biofilter reactor. In addition, *Denitamisoma* accounted for 0.27% of the bacteria in the biofilter reactor, and contributed to denitrification together with Anammox bacteria.

4. Conclusions

In this study, both the ASBR and the biofilter reactor could be used to start up Anammox processes. Stable nitrogen removal in both reactors was observed for more than 200 days, while high TN removal efficiencies were achieved (>80%). The start up period of the biofilter (114 d) was shorter than that of the ASBR (134 d). After enrichment in the reactors, the main organisms contributing to anaerobic ammonia oxidation were *Candidatus Anammoxoglobus* and *Candidatus Kuenenia* existing in both reactors. In addition to the above two species, the biofilter reactor also contained a small amount of *Candidatus Brocadia*, indicating a higher biological diversity in this reactor. The ratio of nitrite removal/ammonium removal and nitrate accumulation/ammonium removal were all higher than the theoretical values, indicating that not only Anammox bacteria, but also AOB, NOB, and denitrifying microorganisms were in both reactor types. The biofilter not only started up faster than the ASBR, but contained richer species as well, which showed it is more suitable for the domestication of Anammox bacteria.

Author Contributions: L.Z. and W.L. conceived and designed the experiments; W.L. and H.Y. performed the experiments; W.L. and Z.G. analyzed the data; L.Z. and S.L. contributed reagents/materials/analysis tools; W.L., L.Z. and S.L. wrote the paper; L.Z. and S.L. revised the paper.

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