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Characterization of a Microbial Community in an Anammox Process Using Stored Anammox Sludge

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Abstract: This study investigated a rapid start-up anaerobic ammonium oxidation (Anammox) process by inoculation with stored Anammox sludge and characterized the associated microbial communities. The Anammox process took only 43 days to start. A high nitrogen removal rate of 1.13 kg N m^{-3} d^{-1} and a nitrogen loading rate of 1.28 kg N m^{-3} d^{-1} were achieved. The ratio of ammonium removal to nitrite removal to nitrate production (1:1:0.2) was slightly lower than the theoretical value, which indicated nitrogen removal by denitrification in the reactor. Illumina high-throughput sequencing of sludge samples confirmed the co-existence of Anammox bacteria and denitrifying bacteria in the reactor and demonstrated that denitrifying bacteria play a role in nitrogen removal during the Anammox process. The dominant microbes in the reactor were *Proteobacteria*, *Chlorobi*, *Chloroflexi*, and *Planctomycetes*. However, only one species of Anammox bacteria, *Candidatus jettenia*, was identified and had an abundance of 4.92%. Our results illustrate the relationship between Anammox reactor performance and microbial community succession.

Keywords: Anammox; bacterial community; start-up; up-flow anaerobic sludge blanket

1. Introduction

Anaerobic ammonium oxidation (Anammox), which converts ammonia and nitrite to nitrogen gas under anaerobic conditions (Equation (1)) [1], was first discovered in a denitrifying fluidized bed reactor in the early 1990s [2]. Since then, the Anammox process has become a promising technology for the removal of nitrogen contaminants [3,4] and is a novel alternative to conventional biological processes for the treatment of wastewater. Stoichiometry of the Anammox process has been revised by a membrane bioreactor (MBR) with free Anammox bacteria and a negligible amount of non-Anammox bacteria [5]. The process is a highly efficient and cost-effective technology for treating effluents with low C/N and high ammonia content as it allows for oxygen and organic carbon source retention and has an excellent nitrogen removal capacity [6].

$$NH_4^+ + 1.32NO_2^- + 0.066HCO_3^- + 0.13H^+ \rightarrow 1.02N_2 + 0.26NO_3^- + 0.066CH_2O_{0.5}N_{0.15} + 2.03H_2O \quad (1)$$

A limitation to the widespread application of the Anammox process is the long start-up time due to the relatively long doubling time of Anammox bacteria (10–11 days), with a maximum specific growth rate of 0.0027 h^{-1} [1,7]. Therefore, methods for decreasing or shortening the start-up period of Anammox reactors, including seeding with different types of sludge, altering reactor types, and using different types of bacterial carriers, have been the subject of increasing interest. Sludge with a high abundance of active Anammox bacteria should be used to seed Anammox reactors in order to accelerate the initiation of the Anammox process. Several types of seed sludge have been tested

for Anammox bacteria enrichment, including conventional activated sludge, denitrifying sludge, and nitrifying sludge. However, the start-up period for reactors seeded with non-acclimatized sludge is still quite long [8] due to low numbers of Anammox bacteria present in the sludge [9], aerobic inhibition, or the presence of organic matter [10–12].

Many different habitats, including engineered systems [1,13] and natural environments [14], contain anaerobic ammonium-oxidizing bacteria. Several studies have investigated bacterial diversity in Anammox reactors using molecular techniques [15,16]. However, compared to conventional molecular biology techniques, high-throughput sequencing can provide more complete information about microbial community structures [17–19]. A full investigation of the bacterial community in the Anammox processes would provide a comprehensive understanding of the microorganisms responsible for the removal of nitrogen compounds and their interactions. This information could be used to engineer microorganisms to improve the Anammox process.

The objective of this study was to investigate a rapid start-up Anammox procedure using an up-flow anaerobic sludge blanket (UASB) reactor inoculated with stored Anammox sludge. Additionally, this study aimed to characterize the microbial community structure and dynamic changes that occur during the Anammox process using Illumina high-throughput sequencing technology based on 16S rRNA. These findings will improve Anammox process start-up and help to better understand nitrogen conversion during the Anammox process.

2. Materials and Methods

2.1. UASB Configuration

A plexiglass-made UASB reactor with a working volume of 5.6 L, an inner diameter of 11.2 cm, and a height of 65 cm was used to carry out the experiment (Figure 1). Black sponge was used to completely cover the reactor to prevent light penetration. The temperature was maintained at 30 ± 1 °C with a water jacket. The concentration of dissolved oxygen (DO) in the influent was kept below 0.05 mg L^{-1} by flushing the synthetic wastewater with N_2. Influent pH was maintained at 7.4–7.6. Synthetic wastewater was pumped into the reactor from the bottom of the reactor.

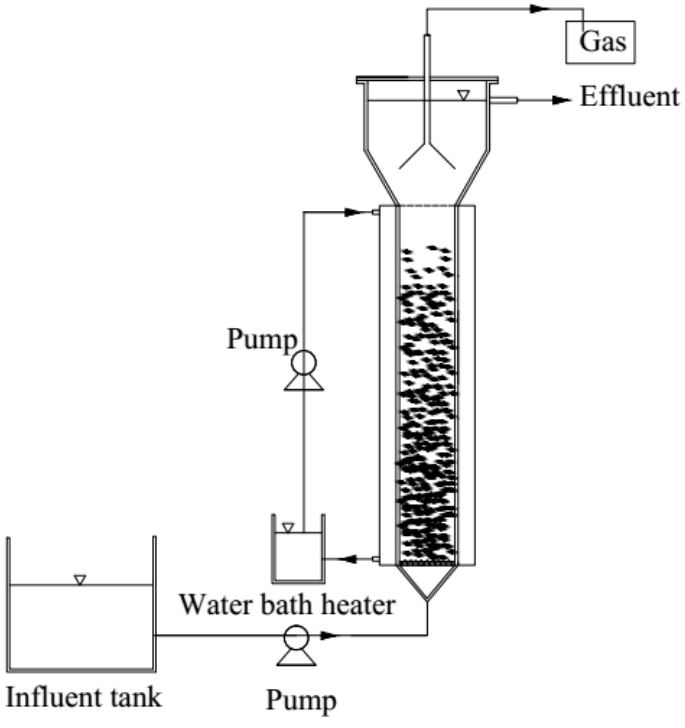

Figure 1. Schematic diagram of the Anammox-up-flow anaerobic sludge blanket (UASB) reactor.

2.2. Inoculation

The reactor was seeded with 300 mL of activated Anammox sludge taken from a lab-scale Anammox-UASB reactor in our laboratory and stored at 4 °C for 6 months. The characteristics of the seed sludge after 6 months storage are listed in Table 1.

Table 1. Characteristics of seed sludge.

Seed Sludge	SS (g L^{-1})	VSS (g L^{-1})	VSS/SS
Anammox activated sludge	23.24	17.24	0.74

2.3. Synthetic Wastewater

The UASB reactor was fed with synthetic wastewater. Ammonium (NH_4^+-N) and nitrite (NO_2^--N) were added to the mineral medium as required in the form of $(NH_4)_2SO_4$ and $NaNO_2$, at a molar ratio of 1. The mineral media was composed of 0.01 g L^{-1} KH_2PO_4, 0.00565 g L^{-1} $CaCl_2{\cdot}2H_2O$, 0.3 g L^{-1} $MgSO_4{\cdot}7H_2O$, 1.25 g L^{-1} $KHCO_3$, 0.00625 g L^{-1} $FeSO_4$, 0.00625 g L^{-1} EDTA, and 1.25 mL L^{-1} trace elements solution [20]. The trace element solution contained 15 g L^{-1} EDTA, 0.014 g L^{-1} H_3BO_4, 0.99 g L^{-1} $MnCl_2{\cdot}4H_2O$, 0.25 g L^{-1} $CuSO_4{\cdot}5H_2O$, 0.43 g L^{-1} $ZnSO_4{\cdot}7H_2O$, 0.19 g L^{-1} $NiCl_2{\cdot}6H_2O$, 0.21 g L^{-1} $NaSeO_4{\cdot}10H_2O$, 0.22 g L^{-1} $NaMoO_4{\cdot}2H_2O$, and 0.05 g L^{-1} $NaWO_4{\cdot}2H_2O$.

2.4. Anammox Operation Strategy

In this study, the experiment was conducted in three phases (Table 2). The start-up stage included the propagation phase (phase I) and the stationary phase (phase II). In phase I, Anammox activity increased gradually. During phase II, Anammox activity and performance remained steady. After successful initiation, the experiment entered the performance improvement stage (phase III), in which the hydraulic retention time (HRT) was shortened and the nitrogen concentration was improved to enhance Anammox-UASB performance. No sludge was removed from the reactor during the operation.

Table 2. Phases of experimental setup.

Phases	Days	Inlet NH_4^+-N and NO_2^--N (mg N L^{-1})	HRT (h)	NLR (kg N m^{-3} day^{-1})
I	1–12	20	24	0.04
	13–26	20	12	0.08
	27–43	40	12	0.16
II	44–54	40	5	0.38
III	55–64	40	3	0.64
	65–79	80	3	1.28

Notes: hydraulic retention time (HRT), nitrogen loading rate (NLR).

2.5. Analytical Methods

Filtered samples from the influent and the effluent were collected simultaneously and analyzed every day. Analytical measurements of ammonium (NH_3-N), nitrite (NO_2^--N), nitrate (NO_3^--N), suspended solids, and volatile suspended solids in the system were performed according to standard methods [21].

2.6. High-Throughput Sequencing

Biomass samples A1, A2 and A3 were collected from the Anammox reactor on day 1, 13 and 79 for high-throughput sequencing. Primers 515F (5′-GTGCCAGCMGCCGCGG-3′) and 907R

(5′-CCGTCAATTCMTTTRAGTTT-3′) [22] were used to amplify the V4–V5 region of the bacterial 16S rRNA gene. DNA amplicons were separated by electrophoresis on a 2% (*w*/*v*) agarose gel and recovered using an AxyPrep DNA Gel Extraction Kit (Axygen Ltd., Hangzhou City, China). A DNA library was then constructed and analyzed using the Miseq platform (Illumina Inc., San Diego, CA, USA) at Majorbio BioPharm Technology Co., Ltd. (Shanghai, China). Acquired reads with low-quality, such as those shorter than 150 bp or containing one or more ambiguous bases, were removed from the sequencing datasets using RDP tools (http://pyro.cme.msu.edu/). In addition, Chao and Shannon indices of alpha diversity were calculated using QIIME [23]. Operational taxonomic units were assigned using UCLUST at a 97% similarity level [24].

3. Results and Discussion

3.1. Rapid Start-Up of the Anammox Process (Phases I and II)

Variation in the concentrations of influent and effluent nitrogen compounds throughout the study are illustrated in Figure 2a. Influent nitrogen (ammonium and nitrite) concentrations were kept at 40 mg N L^{-1} during phase I with a HRT of 24 h. A continuous and sharp increase in ammonium and nitrite removal was observed on days 1–5. Nitrite and ammonium removal efficiencies on day 5 were 96% and 100%, respectively (Figure 2b), indicating good performance of the treatment process. These results confirm that Anammox bacteria can quickly adapt to new environments. Compared to previously reported start-up procedures, Anammox sludge stored at 4 °C for 6 months can quickly recover for inoculation of nitrifying sludge without an adaptation phase [25–27].

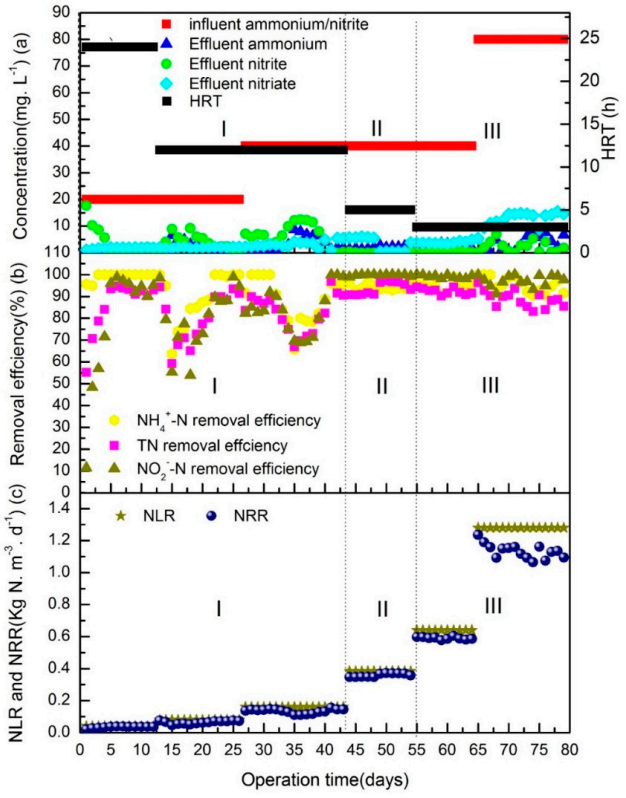

Figure 2. Performance of the Anammox-UASB reactor over 79 days of operation showing (**a**) concentrations of influent NH_4^+-N/NO_2^--N, effluent NH_4^+-N, effluent NO_2^--N, effluent NO_3^--N, and hydraulic retention time (HRT); (**b**) NH_4^--N removal efficiency, total nitrogen (TN) removal efficiency, and NO_2^--N removal efficiency; and (**c**) nitrogen loading rate (NLR) and nitrogen removal rate (NRR).

In order to further promote Anammox activity, the HRT was reduced from 24 h to 12 h on day 13 and the nitrogen loading rate (NLR) was increased to 0.08 kg N m^{-3} d^{-1}. On day 15, effluent ammonium and nitrite concentrations had increased and their removal efficiencies had decreased to 64% and 55%, respectively. After a further 7 days, the removal efficiencies of ammonium and nitrite increased to 88.48% and 81.99%, respectively. As HRT decreased, the removal efficiency of total nitrogen (TN; NH_4^+-N and NO_2^--N) decreased significantly. Similar results were observed when influent ammonium and nitrite were increased to 40 mg N L^{-1} on day 27. On day 35, effluent ammonium and nitrite concentrations reached 8.7 mg N L^{-1} and 12.17 mg N L^{-1} and their removal efficiencies decreased to 65.75% and 69.58%, respectively. After a further 5 days, the removal efficiencies of ammonium and nitrite increased to 82.18% and 80.07%, respectively. These results demonstrate that the Anammox bacteria gradually adapted to and continuously proliferated in a new environment with increasing NLR.

On day 44, during phase II, the HRT was reduced from 12 h to 5 h and the NLR was increased to 0.38 kg N m^{-3} day^{-1}. As shown in Figure 2, the UASB reactor operated stably for 11 days. During that time, influent ammonium and nitrite concentrations were maintained at 40 mg N L^{-1} (Figure 2a), with a nitrogen removal rate (NRR) of 0.36 kg N m^{-3} day^{-1}. The TN removal efficiency increased to 97%, while the effluent nitrite concentration remained below 0.1 mg N L^{-1}, indicating ideal nitrogen removal performance. These results demonstrate that steady-state conditions and efficient nitrite and ammonium removal were established in the reactor.

Successful initiation of the Anammox process is indicated by steady ammonium and nitrite removal. The time for starting up Anammox in this study is much shorter than previously reported [15,26,28–30]. Wang (2016) detected Anammox activity after 67 days with NLR 5.78 × 10^{-2} kg N m^{-3} d^{-1} and NRR 4.64 × 10^{-2} kg N m^{-3} d^{-1} [31], while we obtained Anammox activity after approximately 43 days of cultivation with NLR 16 × 10^{-2} kg N m^{-3} d^{-1} and NRR 15 × 10^{-2} kg N m^{-3} d^{-1}. This can be attributed to the use of stored Anammox sludge in our study.

3.2. Performance Improvement (Phase III)

After successful Anammox-UASB initiation, the NLR was gradually increased to improve Anammox performance. On day 55, the HRT was reduced from 5 to 3 h. On day 70, ammonium and nitrite concentrations were increased from 40 to 80 mg N L^{-1} in order to gradually raise the NLR to 1.28 kg N m^{-3} day^{-1}. Nitrogen removal performance was stable during this period as depicted in Figure 2. The removal efficiencies of ammonium and nitrite were 94.97% and 98.49%, respectively. The process stabilized after the HRT was reduced and the influent nitrogen concentration was increased. Nitrogen removal performance was ideal with average removal efficiencies of 91.28% for TN, 94.97% for NH_4^+-N, and 98.49% for NO_2^--N (Figure 2b). NLR was 1.28 kg N m^{-3} day^{-1} and an average NRR of 1.13 kg N m^{-3} day^{-1} was achieved (Figure 2c). TSS and VSS were 3075 mg L^{-1} and 2214 mg L^{-1} on day 79, although no sludge was removed from the reactor during the operation.

3.3. Stoichiometry

Anammox bacteria were not free in the activated sludge during the operation. So, the theoretical stoichiometric ratios [1] of NO_2 to NH_4^+ ($R_{NO2^-/NH4^+}$) 1.32 and NO_3^- to NH_4^+ ($R_{NO3^-/NH4^+}$) 0.26 were used to analyze the Anammox process. However, after accounting for differences in operating conditions, seed sludge, substrates, and reactor configurations in the various Anammox reactors, the ratio of nitrite consumption to ammonium consumption was in the range of 0.5–4 [32].

During initiation of the Anammox process, the stoichiometric ratios of $R_{NO2^-/NH4^+}$ and $R_{NO3^-/NH4^+}$ experienced large fluctuations and were much lower than the theoretical ratios with average values of 0.96 and 0.10, respectively (Figure 3). This was mainly due to the influence of denitrification [1,33]. As the HRT was reduced to 5 h during phase II, the average $R_{NO2^-/NH4^+}$ and $R_{NO3^-/NH4^+}$ values approached the theoretical ratio at 1 and 0.16, respectively (Figure 3). In phase III, $R_{NO2^-/NH4^+}$ and $R_{NO3^-/NH4^+}$ remained stable with average values of 1.03 and 0.2, respectively.

As NLR increased, $R_{NO2^-/NH4^+}$ and $R_{NO3^-/NH4^+}$ reached 1.32 and 0.26, respectively. These results indicate that the Anammox reaction became the predominant reaction in the Anammox reactor [1]. However, $R_{NO2^-/NH4^+}$ and $R_{NO3^-/NH4^+}$ were always lower than the theoretical values during the Anammox process. This may be explained by denitrification occurring in the Anammox-UASB reactor.

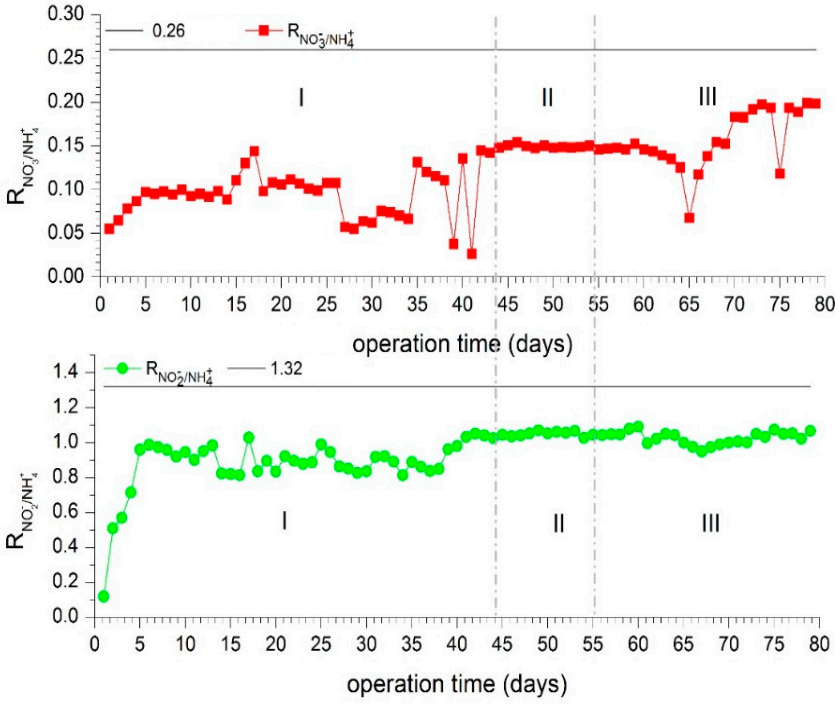

Figure 3. Ratios of nitrite consumption to ammonium consumption ($R_{NO3^-/NH4^+}$) and nitrate production to ammonium consumption ($R_{NO3^-/NH4^+}$) during the Anammox process.

3.4. Microbial Community Structures of Anammox Sludge

Microbial community structure and dynamic changes during the Anammox process were evaluated by Illumina high-throughput sequencing. Biomass samples were collected on days 1 (A1), 13 (A2), and 79 (A3). As shown in Table 3, 83,855 sequences were obtained with an average sequence length of 396 bp. The 16S rRNA sequence similarity was above 99%, indicating a high diversity of bacteria in the samples. Chao indices indicated that the species richness decreased over the course of the experiment. The Shannon index, which is commonly used to characterize microbial community diversity [34,35], revealed that diversity increased over the course of the experiment. In this study, the Anammox sludge was stored for 6 months, which led to the death of most microbes. When re-cultured, new species gradually appeared, resulting in a more diverse microbial community structure over time.

Table 3. Anammox sludge bacterial richness and diversity indices.

Samples	Reads	0.97					
		OTU	Ace	Chao	Coverage	Shannon	Simpson
A1	24189	240	261	264	0.9985	3.16	0.0983
A2	22456	247	259	262	0.9988	3.36	0.0757
A3	26310	237	248	250	0.9991	3.52	0.0624

Notes: operational taxonomic units (OTU).

The classification of bacteria from the three samples at the phylum level is shown in Figure 4. Similar to a previous study that treated synthetic wastewater with the Anammox process [36], *Proteobacteria*, *Chlorobi*, *Chloroflexi*, *Acidobacteria*, *Armatimonadetes*, *Planctomycetes*, and *Bacteroidetes* were the major phyla detected in each sample.

As shown in Figure 4, *Proteobacteria* was the major phyla although the relative abundance decreased over time from 46.69% at A1 to 36.17% at A3. *Proteobacteria* is the most common phyla in soil and wastewater treatment plants and includes all ammonium oxidizing bacteria and most nitrite oxidizing bacteria [37]. The abundance of *Chloroflexi* increased gradually over time from 7.69% at A1 to 18.04% at A3. It has been reported that *Chloroflexi* degrades and uses cellular compounds derived from dead microbes and their metabolites [38]. Chu et al. [19] also found that the abundance of *Chloroflexi* (28%) was high in a one-stage nitritation-Anammox batch reactor. In addition, the abundance of *Bacteroidetes* in our study decreased over time from 4.52% at A1 to 3.78% at A3. *Bacteroidetes* co-exist with Anammox bacteria and were reported to have an abundance of 11% in an Anammox reactor operated for 500 days [39]. *Bacteroidetes* are also thought to degrade high molecular weight compounds [40]. The main functional Anammox bacteria, *Plantomycetes* [41], was found by Cao et al. (2016) to have an abundance of 8.39% in an Anammox-UASB reactor. In our study, there was a significant increase in *Planctomycetes* from 3.82% at A1 to 8.56% at A3. Based on our analysis, a stable Anammox microbial community is made up of *Proteobacteria*, *Chloroflexi*, and *Bacteroidetes* acting synergistically and competing for substrate, DO, and metabolic products.

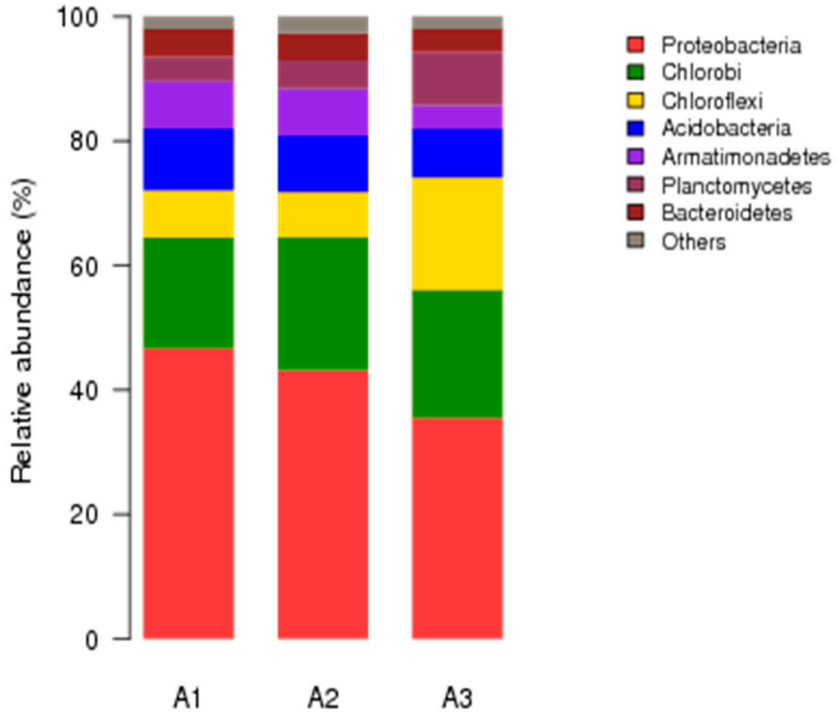

Figure 4. Relative abundance of major phyla in samples collected on day 1 (**A1**), day 13 (**A2**), and day 79 (**A3**).

To better understand the microbial community structure during the Anammox process, bacterial communities were identified at the genus level. As shown in Figure 5, abundances of the major genera in the three samples were minimally altered during the Anammox-UASB process. *Anaerolineaceae* (belonging to *Chloroflexi*) was the prominent genus in the Anammox-UASB. *Anaerolineaceae* abundance increased from 6.11% at A1 to 15.91% at A3, indicating that the anaerobic environment of influent water promotes *Anaerolineaceae* growth. Differences in abundances of genera

with abundances <1% were evident in the three samples, further demonstrating that the microbial community structure was altered throughout the Anammox process.

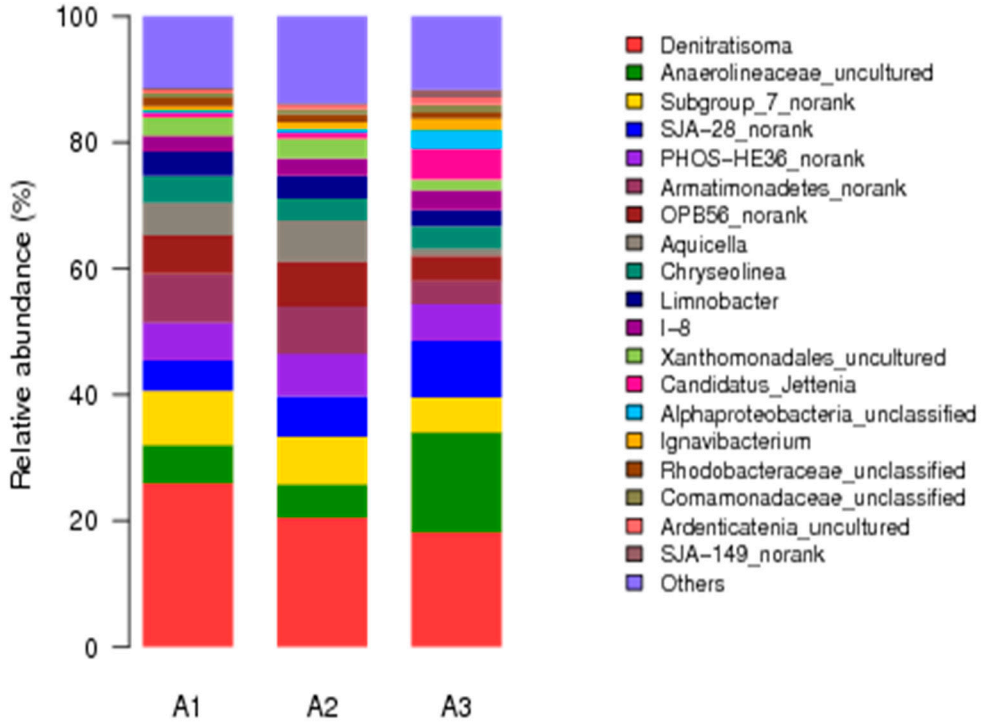

Figure 5. Top 19 genera in samples collected on day 1 (**A1**), day 13 (**A2**), and day 79 (**A3**).

Heterotrophic, denitrifying *Denitratisoma* (belonging to *Proteobacteria*) was the prominent genus during the Anammox process, although its abundance decreased from 25.92% at A1 to 18.11% at A3. This may have affected TN removal in the Anammox reactor. *Denitratisoma* can denitrify through the degradation and utilization of cellular compounds and metabolites of dead microbes. A previous study also found that *Denitratisoma* was the dominant genus, with an abundance of 23.6%, in an Anammox reactor treating synthetic waste water [36].

In our study, the abundance and diversity of Anammox bacteria during the Anammox process was very low. Only one species of Anammox bacteria, *Candidatus jettenia*, was identified and increased in abundance from 0.65% at A1 to 4.92% at A3. Other researchers have also reported that, despite accounting for only a small proportion of the total bacteria, Anammox bacteria are highly efficient in ammonium and nitrite removal in Anammox reactors [42]. Li et al. [39] reported successful operation of an Anammox reactor for more than 500 days with an abundance of Anammox bacteria of only 16%. *Candidatus brocadia* and *Candidatus kuenenia*, which were previously reported to be the predominant Anammox bacteria [43], were not detected in our Anammox-UASB reactor. Different sludge types and operating conditions can lead to shifts in the abundances of the three genera of Anammox bacteria. Indeed, the factors influencing the abundance of the different Anammox bacteria are still unclear.

Results of Illumina high-throughput sequencing showed that denitrifying bacteria and Anammox bacteria can co-exist in the same reactor. In particular, during the start-up stage, *Denitratisoma* contributed to high efficiency nitrogen removal by the Anammox-UASB.

4. Conclusions

In this study, the Anammox process was successfully initiated in 43 days in an UASB reactor using stored Anammox sludge. The average removal efficiency of TN was 95% with an NLR of

1.28 kg N m^{-3} d^{-1} and an NRR of 1.16 kg N m^{-3} d^{-1}. The ratio of the removal of ammonium to the removal of nitrite to the production of nitrate (1:1:0.2) was slightly lower than the theoretical value (1:1.31:0.26), indicating that nitrogen removal by denitrification occurred throughout the Anammox process. Illumina high-throughput sequencing indicated that the dominant microbes in the reactor were *Proteobacteria*, *Chlorobi*, *Chloroflexi*, and *Planctomycetes*. *Denitratisoma* was also highly abundant and contributed to high efficiency nitrogen removal. The only species of Anammox bacteria identified was *C. jettenia*, which had an abundance of 4.92% in the reactor. The co-existence of Anammox bacteria and denitrifying bacteria confirmed that denitrifying bacteria play a role in nitrogen removal during the Anammox process.

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Conflicts of Interest: The authors declare no conflict of interest in any aspect of the data collection, analysis or the preparation of this paper.

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