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Insights into a shift of microbial communities during the start-up of anammox reactor under mainstream and sidestream conditions

Ming Zeng[a], Tingting Du[a], Zimeng Wu[a], Nan Wu[b,*], Shiyu Xie[b], Hongli Li[a], Yuhe Tian[a]

[a]*College of Marine and Environmental Sciences, Tianjin University of Science & Technology, 300457 Tianjin, China, emails: ming.zeng@tust.edu.cn (M. Zeng), 1404093761@qq.com (T.T. Du), 1321331912@qq.com (Z.M. Wu), 1208383522@qq.com (H.L. Li), 1159336342@qq.com (Y.H. Tian)*

[b]*College of Engineering and Technology, Tianjin Agricultural University, Tianjin 300384, China, emails: nwu@tjau.edu.cn (N. Wu), 962503751@qq.com (S.Y. Xie)*

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ABSTRACT

Although anaerobic ammonium oxidation (anammox) owns energy-neutral advantages, the start-up of anammox faced the challenges of a long time and complex environmental factors. This study investigates the shift of microbial communities during the start-up of the anammox reactor under mainstream and sidestream conditions. Two microbial morphologies (biofilm and gel bead) were compared. Different from *Candidatus Brocadia*, only *Candidatus Kuenenia* was retained under sidestream conditions, whose relative abundance arrived at 15.40% and 2.17% in biofilm and gel bead, respectively. However, anammox bacteria were strongly against temperature (20°C ± 0.5°C) and pH (6.7–7.3) under mainstream conditions, instead of competition with other nitrogen removal microorganisms. Although gel beads could start up the bioreactor in a short time of 14 d, the abundance of anammox bacteria, *Nitrosomonas* and denitrifiers in gel beads was lower than those in the biofilm. Finally, the anammox bacteria were proved to own significant positive relations with temperature and pH. Overall, our findings give information to facilitate the start-up of an anammox-based bioreactor.

Keywords: Anammox; Start-up; Mainstream condition; Sidestream condition; High-throughput sequencing

1. Introduction

Anaerobic ammonium oxidation (anammox) is a novel biological nitrogen removal process, where ammonia and nitrite are transformed into nitrogen at the same time by anammox bacteria, with the production of a small amount of nitrate [1]. Compared with the traditional nitrification/denitrification process, the anammox process has the advantages of high efficiency, cost-effective, less sludge production and reduction of 50% oxygen demands and 100% organic carbon sources [2]. These advantages allow the anammox process to have broad application prospects. However, due to the slow growth of anammox bacteria and the long start-up time, it is difficult to start up an anammox-based process in full-scale wastewater treatment [3]. For instance, the start-up time of the laboratory-scale anammox reactors usually ranged from 91 to 1,000 d [4].

The start-up is of great importance for the operation of an anammox-based bioreactor. Considering the relatively slow growth rate of anammox bacteria, it is necessary to find a suitable source of inoculated sludge for the rapid start-up of the anammox process [5]. Furthermore, one of the most essential tasks during the start-up process is to activate and to amplify the functional bacteria in the inoculated sludge, in order to achieve the acceptable performance of anammox system [6].

* Corresponding author.

The morphology of anammox biomass during the start-up process may have certain impacts on the anammox performance and microbial communities of nitrogen removal. Actually, the suspended sludge is usually selected to be seeded microbial morphology for anammox-based nitrogen removal technique, which could be easy to maintain in a stable anammox process [7]. Sources of seeded sludge include sewage treatment plants, anaerobic digestion tanks and marine sediment [8]. Furthermore, some effective strategies were carried out to start up an anammox bioreactor, such as the optimization of its structure, the sludge scouring strategy and the use of magnetic and electrostatic fields [9]. The membrane bioreactor [10], moving-bed biofilm reactor [11] and sequencing batch reactor [12] are suggested to start up anammox bioreactors. Different anammox sludge sources include nitrifying sludge inoculation, denitrifying biofilm, common sludge inoculation and mixed inoculation [13].

Generally, the start-up of the anammox process can be divided into four stages according to the ammonium removal performance of the reactor, namely cell lysis stage, lag stage, active promotion stage and fixation stage [9]. The biofilm is suitable for the start-up of the anammox process, due to its long sludge residence time, good settling characteristics and strong tolerance of adverse conditions [7]. Specifically, the carrier to attach and grow biofilm plays an important role. For example, certain types of gel carriers can enhance the performance of the anammox process [14,15]. Besides the biofilm, the gel bead by microbial immobilization technique is another medium for anammox bacteria. Polyvinyl alcohol (PVA), sodium alginate (SA), polyethylene glycol and polyurethane are widely used as embedding agents. This technique has the advantages of long cell residence time, high cell density and easy separation of solid and liquid phases. In addition, research shows that a rapid start-up of the anammox process in the upflow reactors was successfully achieved by immobilizing a small amount of biomass in PVA-SA gel beads [16].

The main application objects of anammox are digestion liquids with high ammonia nitrogen (500–2,000 mg/L), low C/N ratio and high temperature (25°C–35°C) [17]. Recently, the domestic sewage with low ammonia nitrogen concentration (20–60 mg/L) and low/medium temperature (10°C–25°C) has attracted more attention [17]. The condition of the former digestion liquids is defined as the sidestream condition, while the latter domestic sewage is set to be the mainstream condition. Nowadays, the start-up of anammox under sidestream conditions is assumed to be easier than the mainstream conditions [18]. However, so far, no related study on the start-up of anammox under these two different conditions based on the same inoculated microorganism.

This study aims to investigate the shift of microbial communities during the start-up of the anammox reactor under mainstream and sidestream conditions. Specifically, seeded anammox sludge was used to prepare two microbial systems (biofilm and gel bead) for the start-up of an anammox bioreactor. These two microbial systems were tested under mainstream and sidestream conditions at the same time. A high throughput sequencing technique was conducted to analyze the change of microbial communities during the start-up process under mainstream and sidestream conditions. The interaction between microbial communities and environmental factors was also investigated.

2. Materials and methods

2.1. Inoculation process

Seeded anammox sludge was sampled in an up-flow anaerobic sludge blanket fed with artificial wastewater (influent N loading rate of 0.4 g/(L d), pH of 7.8, the temperature of 31°C). In this study, the inoculated anammox sludge was mixed with activated suspended sludge. The activated sludge was sampled from a wastewater treatment plant in Tianjin. Meanwhile, two mixing patterns of inoculated anammox and activated suspended sludge were compared: one was the biofilm formed by these two types of sludge and the other one was the entrapment of these two types of sludge in the gel beads. The formation of biofilm was conducted by mixing these two types of sludge in an anaerobic bioreactor with the addition of carriers Kaldnes K1®, and the formation of gel beads was detailed in section 2.2. After two weeks of stabilizing biofilm and gel beads in the above artificial wastewater, these two microbial systems were tested in a start-up experiments under mainstream and sidestream conditions, respectively.

2.2. Entrapment technique

The gel beads to entrap microorganisms were prepared by mixing and melting PVA (7 w/v%) and SA (2 w/v%) under 90°C in a water bath to form the gel solution. When the gel solution was cooled down to 35°C, concentrated anammox sludge and activated suspended sludge (centrifuged at 3,500 rpm in 10 min) were mixed with the gel solution at 2 and 4 w/v%, respectively. Then, the gel solution was dropped into the solidifying solution (2 w/v% $CaCl_2$ and 50 w/v% $NaNO_3$) to produce gel beads (diameters approximately 3 mm). Furthermore, the gel beads were kept at 4°C for 24 h in the solidifying solution and washed with distilled water before the start-up experiments.

2.3. Start-up process

The biofilm and gel bead systems were tested to evaluate their start-up efficiency under mainstream and sidestream conditions, respectively. The whole experimental process is illustrated in Fig. 1. The prepared biofilm and gel bead were separately cultured in the 2 L cylinder bioreactors (diameter of 12 cm, the height of 23 cm) by continuous feeding mode. The feeding solutions were parallel pumped into the bioreactors that were controlled under mainstream and sidestream conditions in a water flow rate of 0.1 L/h with a hydraulic retention time of 26 h.

As shown in Table 1, the parameters of sidestream conditions included a temperature of 32°C ± 0.5°C and pH ranging between 8.0 and 8.4, while the parameters of mainstream conditions included a temperature of 20°C ± 0.5°C and pH ranging between 6.7 and 7.3. The temperature was precisely controlled by a cooler and a water bath cover. In terms of chemical compositions of artificial feeding solution, the chemical oxygen demand (COD) and NH_4^+–N

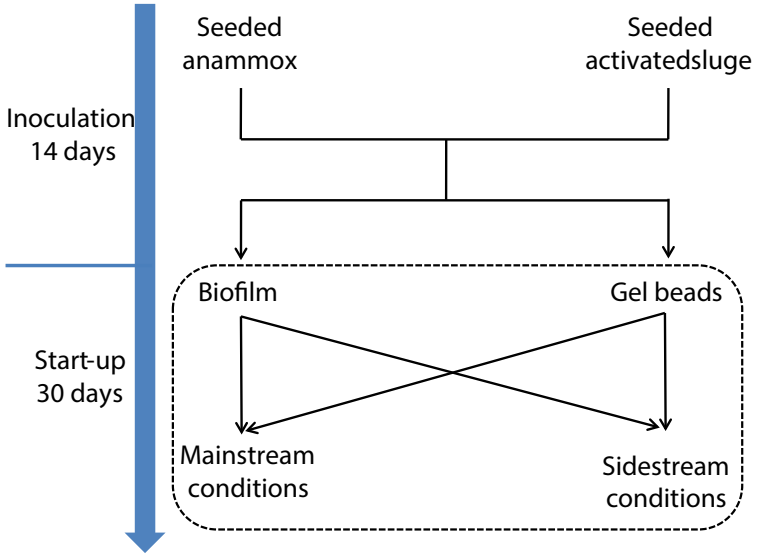

Fig. 1. Experimental procedure of inoculation and start-up periods.

Table 1
Operational parameters of the start-up process under mainstream and sidestream conditions

	Mainstream conditions	Sidestream conditions
Influent COD (mg/L)	80 ± 4	0
Influent TN (mg/L)	60 ± 6	600 ± 24
pH	7.0 ± 0.3	8.2 ± 0.2
Temperature (°C)	20 ± 0.5	32 ± 0.5
Dissolved oxygen	0.8 ± 0.3	0.8 ± 0.3
Biomass morphology	Biofilm and gel beads	Biofilm and gel beads

concentrations for the sidestream conditions were set to be 0 and 600 mg/L, respectively. While the COD and NH_4^+–N concentrations for the mainstream conditions were set to be 80 and 60 mg/L, respectively. Other common chemical components included KH_2PO_4 of 27 mg/L, $NaHCO_3$ of 500 mg/L, $CaCl_2{\cdot}2H_2O$ of 180 mg/L, and $MgSO_4{\cdot}7H_2O$ of 300 mg/L. The trace elements (1 mL/L) were added into the artificial wastewater, whose concentration can be referred to our previous study [7]. The dissolved oxygen concentration was controlled to be 0.8 ± 0.3 mg/L under both two conditions. The continuous feeding operation lasted for one month as the start-up process, and the biofilm and gel bead were sampled to analyze their microbial communities at the end of tests.

2.4. DNA extraction and high throughput sequencing

The samples should be pretreated for the high throughput sequencing analysis. Regarding the biofilm system, microorganisms on the surface of carriers were separated by centrifuging at 6,000 rpm in 10 min (TG16-WS Centrifuge, Cence, China). Concerning the gel bead system, gel beads were treated by a homogenater (FA25-D, Fluko, Germany) to allow the biomass to be exposed outside. The pretreated biofilm and gel beads were ready to extract DNA in the next step.

The pretreated samples were firstly immersed in PBS solution (Sangon Biotech, Shanghai, China) and then were stored at –20°C before the microbial analysis. The microbial DNA was extracted via the PowerSoil DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA). The 0.8% agarose gels were adopted to check the purity and quality of the DNA. The V3-V4 hypervariable region of the bacterial 16S rRNA gene was amplified by primers for 338F and 806R. The deep sequencing was performed on the Miseq platform at Allwegene Company (Beijing, China).

First, the raw data were filtered and low-quality sequences were removed. A sample-specific barcode sequence was separated for qualified reading and trimmed with IIIumina Analysis Pipeline Version 2.6. Then we can use QIIME to analyze the dataset. Finally, the sequences were clustered into operational taxonomic units (OTUs) with a similarity of 97%.

The significance level was presented by p values that were analyzed by Spearman correlation analysis. Spearman correlation analysis was conducted using SPSS software (version 19.0). Heatmaps and correlation plots were performed in R 3.5.3. Microsoft Excel 2010 was used for the generation of other plots.

3. Results

3.1. Bacterial community diversity analysis

The 40,976–72,531 high-quality sequencing tags were obtained for five samples (Table 2). The clustering analysis generated 227–664 OTUs. The Shannon index of seeded sludge (6.04) was greater than other samples (4.23–5.29), indicating a higher microbial community diversity in the seeded sludge. But the Chao1 index of seeded sludge was relatively lower than other samples. Regarding the microbial morphology, the Chao1 richness indices in gel bead and biofilm were generally comparable under the mainstream conditions, while this index in the gel bead was clearly higher than that in the biofilm under the sidestream conditions. However, the Shannon index shows a different phenomenon that the biofilm always owned a higher value than the gel bead.

3.2. Shift of microbial communities under mainstream conditions

There were 19 main phyla identified across all the samples (Fig. 2). *Proteobacteria* was the most abundant phylum in different samples, with an abundance of 49.8%–68.9%. A considerable amount of *Planctomycetes* (10.4%) were found in the seeded sludge but basically disappeared (<0.3% in the gel and biofilm) after the cultivation under mainstream conditions. On the contrary, *Bacteroidetes* (5.0% in the seeded sludge) were considerably enriched (8.1% in the gel and 24.6% in the biofilm) under mainstream conditions. In addition, compared to the seeded sludge, the abundance of *Armatimonadetes* and *Acidobacteria* increased under mainstream conditions. While the abundance of *Gemmatimonadetes* and *Chloroflexi* declined after the cultivation under mainstream conditions.

At the genus level (Fig. 3), the abundance of *Nitrosomonas*, one kind of ammonia-oxidizing bacterium (AOB), was reduced under mainstream conditions (decreasing from 10.2% in the seeded sludge to 1.4%–4.3% in the gel bead and biofilm). In the seeded sludge, two types of anammox bacteria were detected, namely *Candidatus Brocadia* (5.1%) and *Candidatus Kuenenia* (1.9%). After the cultivation under mainstream conditions, both these two kinds of anammox bacteria almost disappeared in gel bead and biofilm. Moreover, the dominant species of denitrifying bacteria significantly changed under the mainstream condition. For example, compared with the seeded sludge, *Hyphomicrobium* and *Comamonas* were substantially reduced in gel bead and biofilm (0.1%–0.5%). While *Denitratisoma* was considerably enriched in biofilm (9.27%) and *Thauera* was also enriched in gel bead (3.72%) compared with the seeded sludge.

3.3. Shift of microbial communities under sidestream conditions

Among the 22 main phyla identified in three samples (Fig. 4), *Proteobacteria* was the most abundant phylum (24.4%–49.9%), which is similar to the mainstream conditions. However, different from the mainstream conditions (Fig. 2), there were still abundant *Planctomycetes* after the cultivation of sidestream conditions. Specifically, compared with the seeded sludge, the abundance of *Planctomycetes* reduced to 2.4% in gel bead but increased to 18.2% in biofilm. The proportions of *Bacteroides* increased under sidestream conditions, reaching 53.3% and 8.1% in the gel bead and biofilm, respectively. Compared with the seeded sludge *Armatimonadetes* enriched significantly in the biofilm, but not in the gel bead. Moreover, the proportion of *Gemmatimonadetes* decreased under sidestream conditions. There was little change in the abundance of *Chloroflexi* in three samples.

As shown in Fig. 5 similar to the mainstream condition, *Nitrosomonas* decreased (from 10.2% to <3.3%) and *Candidatus Brocadia* disappeared under the sidestream condition. Conversely, *Candidatus Kuenenia* turned out to be 2.2% and 15.4% in gel bead and biofilm, respectively. Compared with the mainstream condition, diversity and abundance of denitrifying bacteria generally decreased under sidestream conditions. For example, *Hyphomicrobium* disappeared in the gel bead and biofilm. Compared with the seeded sludge, *Comamonas* and *Denitratisoma* in the gel bead did not change significantly. But their abundance increased in the biofilm, especially for *Denitratisoma* that increased up to 6.23%.

3.4. Relation between microorganisms and environmental factors

As illustrated in Fig. 6, Spearman correlation analysis reveals that the anammox bacteria *Candidatus Kuenenia* had a strong positive relationship ($r = 0.95$, $P < 0.05$) with temperature (20°C ± 0.5°C in mainstream conditions; 32°C ± 0.5°C in sidestream conditions) and pH (7.0 ± 0.3 in mainstream

Table 2
Bacterial community diversity indices of five samples

Samples	Observed species	Shannon	Chao1	Goods coverage	PD whole tree
Seed[a]	349	6.04	351.4	0.999	38.6
Main_Gel[b]	604	4.93	649.6	0.999	60.8
Main_Biofilm[c]	575	5.13	817.0	0.998	52.8
Side_Gel[d]	664	4.23	924.3	0.999	58.6
Side_Biofilm[e]	227	5.29	227.0	0.999	25.5

[a]represents the seeded sludge;
[b,c]represent the gel bead and biofilm under mainstream conditions;
[d,e]represent the gel bead and biofilm under sidestream conditions.

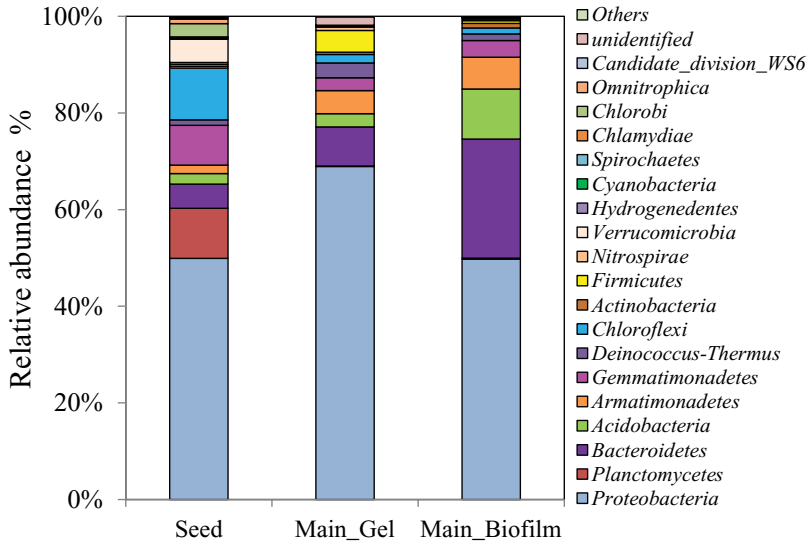

Fig. 2. Microbial community composition at phylum level in seeded sludge (Seed), gel bead (Main_Gel) and biofilm (Main_Biofilm) under mainstream conditions (relative abundance less than 0.1% is included as others).

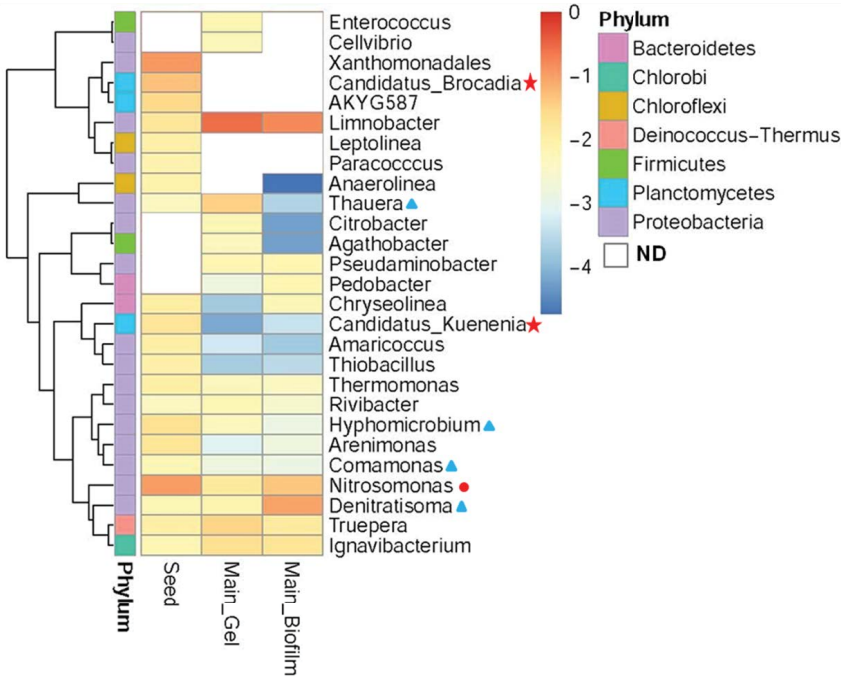

Fig. 3. Heat map of genera in seeded sludge (Seed), gel bead (Main_Gel) and biofilm (Main_Biofilm) cultured under mainstream conditions. Values plotted are the relative abundance after log10 transformed. Anammox bacteria: red ★; Ammonia-oxidizing bacteria: red ●; Denitrifier: blue ▲.

conditions; 8.2 ± 0.2 in sidestream conditions). Genera I-8 showed a similar variation in response to temperature and pH. Genera AKYG587 exhibited a negative relationship (r = –0.89, P < 0.05) with COD and significant positive relationship with total nitrogen (TN) (r = 0.97, P < 0.01). While the abundance of *Limnobacter* was negatively related to TN. Additionally, *Nitrosomonas* had a strong relationship with *Cellvibrio* (r = 0.97, P < 0.01).

3.5. Performance of nutrient removal

Due to the superior abundance of nitrogen removal microorganisms in the biofilm system, its nitrogen removal performance was shown in Fig. 7. NH_4^+–N was reduced under both conditions. NH_4^+–N was reduced from 430 to 390 mg/L under sidestream conditions, and from 70 to 20 mg/L under mainstream conditions. However, only a small amount of

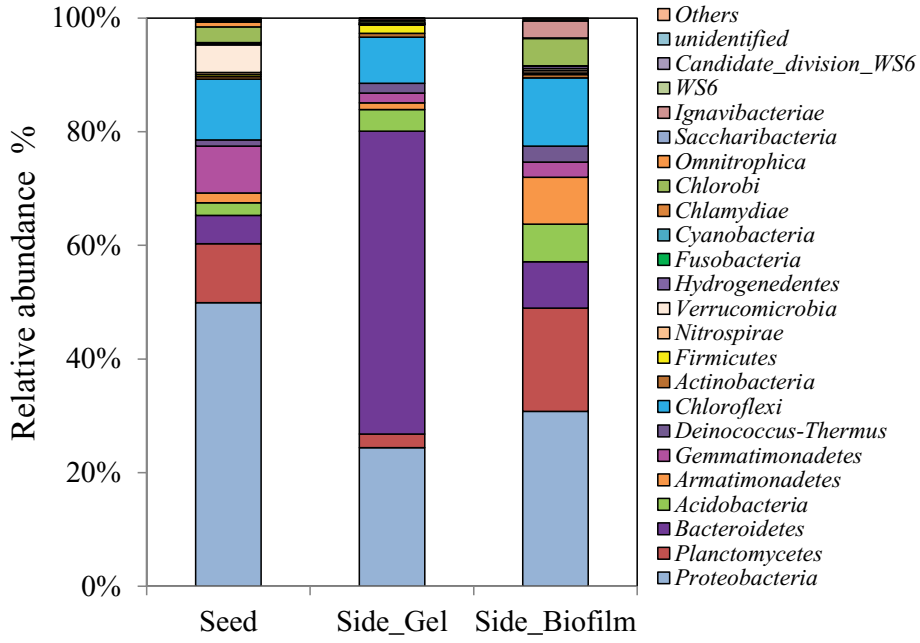

Fig. 4. Microbial community composition at phylum level in seeded sludge (Seed), gel bead (Side_Gel) and biofilm (Side_Biofilm) under sidestream conditions (relative abundance less than 0.1% is included as others).

NO_2^-–N was produced under sidestream conditions, while a large amount of NO_2^-–N was produced under mainstream conditions. NO_3^-–N has not increased much. The above shows that the PN/A process occurred in sidestream conditions, while the mainstream was only the PN process. This was consistent with microbiological results.

Due to the addition of $NaHCO_3$, there was no great change of pH value that was 7.0 ± 0.3 and 8.2 ± 0.2 in mainstream and sidestream conditions, respectively. According to the other study, high pH value was easy to arouse the free ammonia (FA) accumulation. Specifically, the pH value between 7.5 and 8.5 was usually conducive to the accumulation of nitrite, and the optimal pH value of anammox activity was about 8. In addition, the high pH value of anammox can also cause the changes of FA and free nitrous acid (FNA) concentration, which play a key role in the inhibition of nitrite-oxidizing bacteria (NOB) and AOB and the enrichment of anammox bacteria [19].

4. Discussion

4.1. Change of anammox bacteria during start-up

Two common species of anammox bacteria, namely *Candidatus Kuenenia* and *Candidatus Brocadia*, were observed in samples after the start-up process. They have frequently detected members within the anammox group [7,20]. Compared with the mainstream condition, the sidestream condition appeared to be more suitable for the growth of *Candidatus Kuenenia*. However, *Candidatus Brocadia* could survive in neither mainstream nor sidestream conditions. This could be attributed to the different characteristics of these two species that have been reported by other studies. For example, *Candidatus Kuenenia* had a higher affinity with nitrite compared with *Candidatus Brocadia*, and was able to remove nitrate from wastewater via the dissimilatory nitrate reduction process [21]. *Candidatus Kuenenia*-like species were found to dominate the anammox bacteria in a biofilm reactor [22]. In short, *Candidatus Kuenenia* was likely to become the dominant anammox member during the start-up process, while *Candidatus Brocadia* was eliminated through selection.

It is also interesting to observe the different phenomenon between mainstream and sidestream conditions. The anammox bacteria were only retained after the start-up under sidestream conditions. Neither *Candidatus Brocadia* nor *Candidatus Kuenenia* was observed under mainstream conditions. This is in accordance with the general cognition that the mainstream anammox faces great challenges. Hence, a two-stage partial nitrification-anammox system was usually taken to realize the start-up and stable operation under mainstream conditions. For instance, a novel microbial carrier was adopted with anammox bacteria immobilized on the volcanic carriers [23]. A control strategy was also proposed to optimize the operation [24]. However, a single-stage system was tested in the present study. Unfortunately, the anammox bacteria were eliminated under mainstream conditions, which were probably attributed to the inhibition of environmental factors or the competition among different nitrogen removal microorganisms, that is, anammox bacteria and denitrifiers. According to the result of the relation between microorganisms and environmental factors, the inhibition of environmental factors seems to be the main reason. The application of mainstream anammox is affected by seasonal temperature change [13]. Further efforts need to be made to regionalize different nitrogen removal microorganisms and

the gel immobilization technique is likely to be a suitable alternative.

Compared with the mainstream conditions, sidestream conditions were confirmed to favor the growth of anammox bacteria during the start-up process. For example, the abundance of anammox bacteria in the mainstream PN/A reactor was reported to decrease from 6.6×10^{11} to 3.2×10^{11} copies/L [17]. In the sidestream treatment of the anaerobic digester filtrate process, *Kuenenia stuttgartiensis* was found to dominate the enriched anammox community [25]. High temperature is a key characteristic of sidestream conditions, which might serve as one of the most important parameters for the start-up of anammox bioreactor. A temperature of 35°C for the growth and metabolism of anammox bacteria was suggested to maintain anammox activity for the acceleration of the reactivation process after the storage [26].

Considering the anammox bacteria in biofilm and gel bead under sidestream conditions, the substantial enrichment of *Candidatus Kuenenia* only occurred in the biofilm rather than in the gel (Fig. 5), suggesting that biofilm was more favorable to the growth of anammox bacteria compared to the gel bead. This is in accordance with previous studies that anammox bacteria tend to grow in the form of biofilm [27]. The reason might be attributed to the mass transfer resistance of nutrients inside the gel matrix. Thus, the activity of anammox bacteria inside the gel bead was lower than the biofilm on the surface of carriers. Nevertheless, gel immobilization technique is still demonstrated to be an efficient strategy to initiate anammox reactors with the minimal quantity of anammox biomass [16].

4.2. Effects of environmental factors on anammox

Anammox process is greatly affected by various environmental factors such as pH, temperature and organic matter. Temperature is a key parameter of microbial metabolism and growth, which is directly related to the performance of an anammox reactor. As we all know, medium temperature from 30°C to 37°C is considered as the most suitable condition for anammox [28]. Zekker et al [29] found that a predominance of *Candidatus Brocadia* persisted when the temperature was decreased from 20°C to 15°C and even an increase in the abundance of its copy numbers was observed. Besides, the same observation was made in a study of switching the influent streams (cold mainstream wastewater vs. warm sidestream wastewater) in an anammox biofilm reactor [30]. The other important parameter for anammox growth is pH. The optimum pH for the growth of anammox bacteria has been reported between 6 and 9 [31]. Strong acid and strong base can inhibit the activity of anammox bacterial process [32].

Recent studies show that the proper influent organic content can improve the nitrogen removal effect and the operation stability of PN/A. It has been reported that the C/N ratio for the superior anammox process was in the range of 0.3–1.0. The optimum C/N ratio of stable PN/A was generally 0.5. A high C/N ratio inhibited autotrophic denitrification. Relatively speaking, a lower C/N ratio was conducive to the stability of PN/A, but the nitrogen removal efficiency was usually low due to the production of nitrate [33]. Besides, a related study showed that the interaction between pH and temperature was also significant.

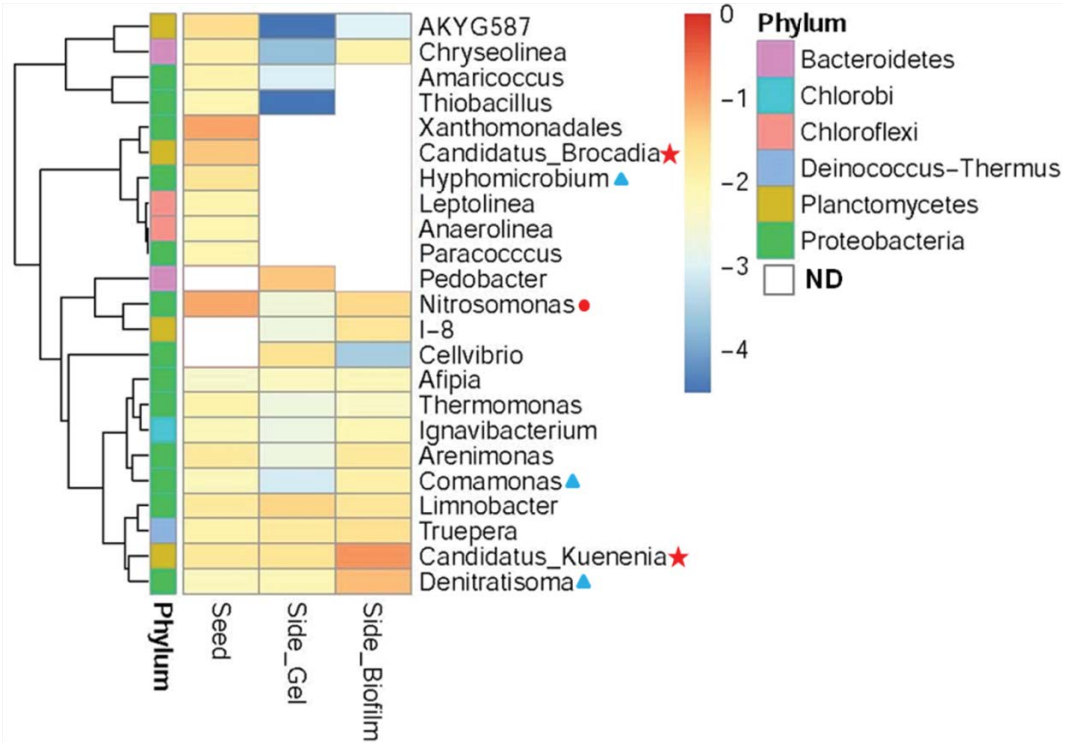

Fig. 5. Heat map of genera in seeded sludge (Seed), gel bead (Side_Gel) and biofilm (Side_Biofilm) under sidestream conditions. Values plotted are the relative abundance after log10 transformed Anammox bacteria: red ★; Ammonia-oxidizing bacteria: red ●; Denitrifier: blue ▲.

It was found that maintaining a high pH value could overcome the negative effect of low temperature on anammox activity [31].

4.3. Change of nitrifying bacteria during start-up

In terms of nitrifiers, *Nitrosomonas* species were observed to be the dominant AOB under both mainstream and sidestream conditions, which can be verified by many other studies. In the two-stage PN/A system, the microbial ecology analysis for nitritation reactor before the anammox bioreactor showed the dominance of *Nitrosomonas* [25]. Besides, in a single-stage nitrogen removal system, it is also confirmed that the dominant genera of AOB were *Nitrosomonas* throughout the operation when the bioreactor changed from sidestream to mainstream [17].

The pH value between 7.5 and 8.5 is usually conducive to the accumulation of nitrite, and the optimal pH value of anammox activity is about 8. Actually, AOB is the tolerant microbial species to the FA [34]. FA is effective to inhibit NOB and enriches AOB, realizing rapid start-up and stability of partial nitrification [35]. High FA content inhibits not only NOB but also AOB [36].

4.4. Change of denitrifying bacteria during start-up

The common denitrifiers observed under mainstream and sidestream conditions include *Hyphomicrobium* (0.49% and 0.14% for gel bead and biofilm, respectively in mainstream conditions), *Denitratisoma* (0.78% and 9.27% for gel bead and biofilm, respectively in mainstream conditions; 0.79% and 6.23% for gel bead and biofilm, respectively in sidestream conditions) and *Thauera* (3.72% and 0.02% for gel bead and biofilm, respectively in mainstream conditions; 1.71% and 2.78% for gel bead and biofilm, respectively in sidestream conditions). Among them, *Denitratisoma* was capable of complete denitrification, which was observed in different nitrogen removal bioreactors [37]. *Thauera* as one denitrifying bacteria occurred commonly in aerobic–anaerobic coupled process for wastewater treatment [38]. All these denitrifications were detected in microbial communities under mainstream conditions, but there was a lack of *Thauera* in communities under sidestream conditions. Besides, the abundance of denitrifiers under mainstream conditions was higher than those under sidestream conditions. The reason might be the low temperature and relatively high organic matter concentration of mainstream conditions.

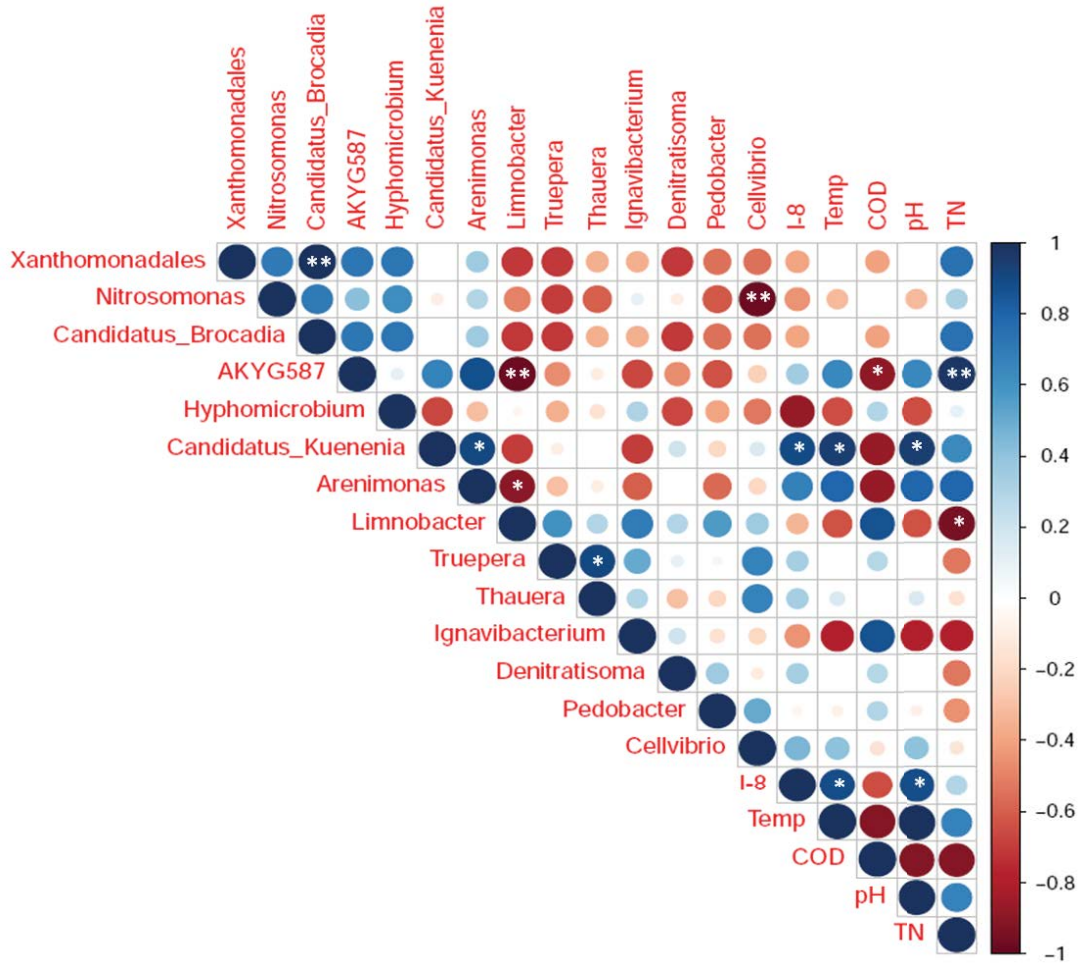

Fig. 6. Spearman correlation analysis between the abundance of main genera and physicochemical properties in all samples. Blue and red colors represent positive and negative correlations.*P< 0.05, ** P < 0.01.

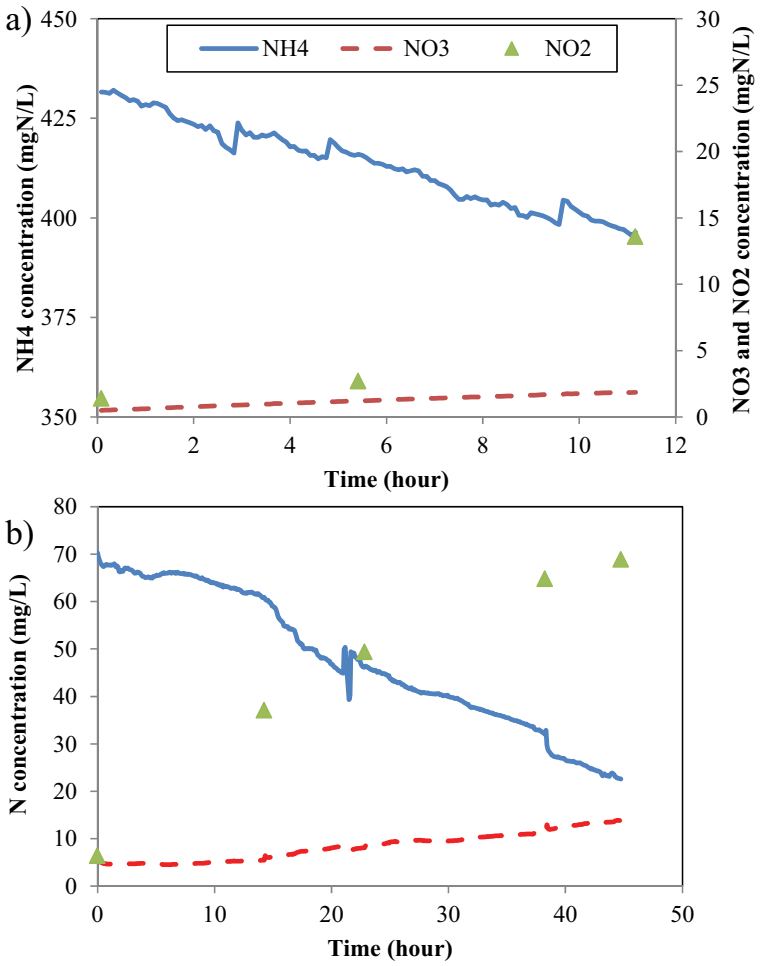

Fig. 7. Change of NH_4^+–N, NO_3^-–N and NO_2^-–N concentrations of biofilm system under sidestream and mainstream conditions.

Regarding the microbial communities for nitrogen removal, it seems that no significant relationship was observed among *Nitrosomonas*, denitrifiers and anammox bacteria. This means that the completion among different nitrogen removal microorganisms was negligible during the start-up process in both the biofilm and gel systems. Moreover, relationships between nitrogen removal microorganisms and environmental factors (temperature, pH, influent COD and TN) were investigated. The *Nitrosomonas* and denitrifiers had no significant relationships with environmental factors. However, the anammox bacteria was proved to own significant positive relations with the temperature and pH. The anammox bacteria also exhibited a negative relation with the influent COD concentration although this relationship was not significant. Similarly, it was indicated that the acidic (pH ≤ 6.5) and alkaline (pH ≥ 8.5) conditions inhibited the activity of the anammox bacteria process, based on nitrogen removal performance with different pH [32]. According to the study of Chen et al. [39], the coexistence of denitrification and anammox could exist when the influent COD concentration was lower than 99.7 mg/L, but elevated COD could further deteriorate the anammox activity, indicating that the COD concentration was the most important factor regulating the bacterial community structure.

5. Conclusion

The shift of microbial communities during the start-up of the anammox reactor under mainstream and sidestream conditions has been well investigated.

- Sidestream conditions with high temperatures were favorable for the enhancement of anammox bacteria during the start-up process.
- Regarding the mainstream conditions, the single-stage nitrogen removal process in this study seemed to lack advantages compared with the two-stage nitrogen removal process.
- Moreover, *Candidatus Kuenenia* instead of *Candidatus Brocadia* was easy to dominate in the microbial communities. The biofilm morphology was preferential to be selected rather than gel bead under sidestream conditions.
- *Nitrosomonas* and denitrifiers had no significant relationships with environmental factors, while the anammox bacteria was proved to own significant positive relations with the temperature and pH.

Overall, our findings give information about the succession of nitrogen removal microorganisms under mainstream

and sidestream conditions to facilitate the start-up of anammox-based bioreactor.

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