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Comparison of the start-up and long-term operation characteristics of floc sludge and biofilm integrated anammox reactors.

Xia Q.*, Zheng X., Shang W., Zhang W., Wang Y., Sun Y., and Li P.
North China Municipal Engineering Design & Research Institute Co. Ltd, Tianjin, China
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*Corresponding author: Qiongqiong Xia: E-mail: xqqwater@126.com
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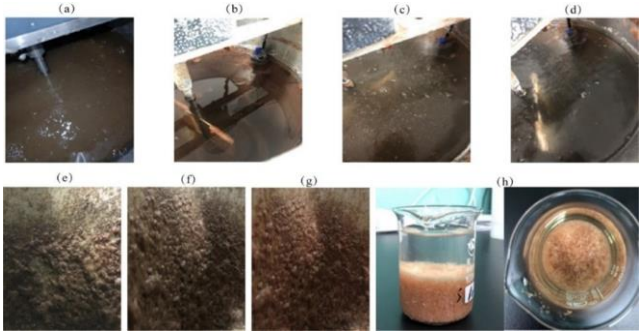
Graphical Abstract



Abstract

Integrated anammox process has competitive advantages of low energy and carbon consumption in nitrogen removal. Because of the coupling of partial nitrification and anammox process, complex physicochemical and biological interactions occur in integrated anammox reactors, and long-term operation might change the characteristics which is seldom reported in previous studies. In this study, an activated sludge bioreactor and a moving bed biofilm reactor (MBBR) reactor using integrated anammox process were investigated over 450 days and compared from the startup to stable operation period. The results showed that after long-term operation, the flocs in the sludge system firstly gathered to form biofilm, and the biofilm would further form granular sludge after being manually scrapped and detached. The average ammonia removal rate (ARR) and total nitrogen removal rate (TNRR) in the transformed granular sludge system were 98.4% and 82.3%, respectively. In the MBBR reactor, because of the mass transfer resistance, the ARR and TNRR were constrained by aeration, with average values of 87.5% and 74.2% respectively, and it would be difficult to inhibit Nitrite Oxidizing Bacteria (NOB) after long-term operation due to the protection of biofilm. The biofilm and granular sludge samples in the reactors were analyzed by high-throughput sequencing of 16S rDNA amplicon, and the results revealed that the abundance of ammonia oxidation bacteria (AOB), NOB and anaerobic ammonia oxidation bacteria (AnAOB) in the biofilm was 5.66%, 2.99% and 21.10%, respectively, while the three functional bacteria in granular sludge was 7.62%, 0.34% and 6.85%, respectively. This study provides an in-depth understanding the mechanism of anammox process and broaden the feasibility of its application.

Keywords: integrated anammox reactors, long-term operation, MBBR, floc sludge, granular sludge, biofilm detachment

1. Introduction

Integrated anammox is a new process which couples nitritation with anammox in the same reactor to achieve cooperative nitrogen removal without carbon source. In the practical engineering of global anammox application, integrated reactors are dominant and are mainly used for the nitrogen removal from wastewater with high ammonia nitrogen and low carbon/nitrogen ratio (e. g. reject of sludge anaerobic digestion and landfill leachate) (Lackner *et al.*, 2014; Scherson *et al.*, 2015). The main functional microorganisms of the integrated anammox process are Ammonia Oxidizing bacteria (AOB) and anammox bacteria (AnAOB), which can be enriched in both activated sludge and biofilm systems (Christensson *et al.*, 2011; Gilbert *et al.*, 2015; Guo *et al.*, 2016; Kowalski *et al.*, 2018; Lotfi *et al.*, 2019). Usually, activated sludge flocs are more likely to enrich AOB due to lower internal oxygen transfer resistance (Liu *et al.*, 2017; Zhang *et al.*, 2015; Zheng *et al.*, 2016) while biofilm (including granular sludge) is considered to be an effective way to enhance biomass retention of microorganisms, especially AnAOB biomass (Jia *et al.*, 2017).

Recently, researchers have found that activated sludge flocs and biofilms often coexist in the same anammox system and that the morphology of microbial aggregation has been changing in these hybrid systems (Innerebner *et al.*, 2007; Vlaeminck *et al.*, 2010; Winkler *et al.*, 2012). In fact, AnAOB seems to be characterized by a tendency to self-aggregate and it is often observed that AnAOB flocs in hybrid systems gradually increase in size, with some

forming granular sludge (Jia *et al.*, 2017; Ali *et al.*, 2018; Wang *et al.*, 2020). Cheng *et al.* (2016) showed that in a system containing activated sludge flocs and granular sludge, the granular sludge increased from 300 μm to 500~600 μm in 118 d. YANG *et al.* (2019a) found that the particle size of sludge flocs gradually increased from 115.9 μm to 189.9 μm after 127 d of operation in an integrated fixed film activated sludge (IFAS) system. Song *et al.* (2021) found that granular sludge can be formed after the detachment of biofilms in an IFAS reactor operating for 220 d.

As is shown above, in hybrid systems, changes in the physical properties of floc sludge, granular sludge and biofilm would not be observed soon for any system, because the physicochemical and biological interactions of the anammox process were not likely triggered in a short-term process. On the other hand, studies on the aggregation mechanism of AnAOB have shown that the flocculation ability of anammox granular sludge is significantly higher than that of anammox sludge (Wang *et al.*, 2021b). Thus, it can be inferred that the time required to observe the changes in the morphology of microbial aggregation in a pure activated sludge anammox system is longer than that required in a hybrid system containing both granular sludge and flocs. In addition, in a pure biofilm system, such as MBBR, their operation is influenced by the biofilm thickness, but changes in biofilm thickness may only become apparent after a longer period of time. Therefore, understanding the long-term operational characteristics of the system is critical for the mechanistic and application studies of the integrated anammox process. However, fewer tests and studies have been conducted for long-term operation of anammox systems, with most studies lasting less than one year.

In this study, a sludge reactor and a parallel MBBR reactor were operated for more than 450 days, both of which applied the integrated anammox process to treat simulated wastewater containing high concentrations of ammonium nitrogen. The changes in the morphology of functional microbial aggregates from start-up to stable operation of the two reactors were monitored over time, and the microbial community characteristics of the two reactors were analyzed. The results of this study contribute to an in-depth understanding of the mechanism of the anammox process and promote its application in wastewater treatment.

2. Materials and methods

2.1. Configuration of reactor

Two same reactors were applied, and the experiment diagram was shown in Figure1. The effective volume of reactor was 32 L (internal diameter 30 cm, high 55 cm) with mechanical speed mixer, sand aerator and heating rod equipped inside. The aeration was controlled by an adjustable gas pump and a rotameter, and the influent flow was controlled by a peristaltic pump. Reactor 1 was a MBBR reactor, and reactor 2 was a granular sludge reactor. Reactor 1 was filled with suspended filler carrier (SPR-1 type) with a diameter of 25 mm, a height of 10 mm, a specific surface area of 450 m^2/m^3 and a filling rate of 30%.

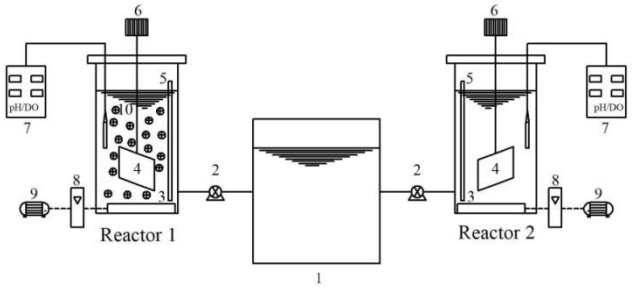


Figure 1. Schematic diagram of the treatment system. 1) Feed tank; 2) peristaltic pump; 3) sand aerator; 4) stirring paddle ; 5) heating rod; 6) stirring motor; 7) pH/DO (dissolved oxygen) measuring instrument; 8) rotameter; 9) air pump; 10) suspended carrier.

2.2. Raw Wastewater

The reactors were fed continuously by synthetic wastewater, and HRT was 48 h. The wastewater took $(NH_4)_2SO_4$ as nitrogen source, the ammonia concentration was 380~410 mg/L, which was in the middle range of concentration of reject water from anaerobically digested sewage sludge (200~1000 mg/L). $NaHCO_3$ was added to adjust alkalinity, and proportion of m (HCO_3^-): m (NH_4^+-N) was 1.0~1.5. Other chemicals included KH_2PO_4 (28 mg/L), $MgSO_4{\cdot}7H_2O$ (300 mg/L), $CaCl_2$ (136 mg/L) and two kinds of trace element nutrient solutions (1 mL/L). Solution Ⅰ had the following compositions: EDTA (5000 mg/L), $FeSO_4$ (5000 mg/L), and solution II had the following compositions: EDTA (15 000 mg/L), $ZnSO_4{\cdot}7H_2O$ (430 mg/L), $CoCl_2{\cdot}6H_2O$ (240 mg/L), $MnCl_2{\cdot}4H_2O$ (990 mg/L), $CuSO_4{\cdot}5H_2O$ (250 mg/L), $Na_2MoO_4{\cdot}2H_2O$ (220 mg/L), $NiCl_2{\cdot}6H_2O$ (190 mg/L), $Na_2SeO_4{\cdot}10H_2O$ (210 mg/L). The influent was kept near an anaerobic condition with the DO of 0.04~0.10mg/L by removing DO from the tap water with pure nitrogen.

2.3. Experiment scheme

A nitritation reactor was constructed first to provide necessary nitrite substrate for the growth of AnAOB. Reactor 1 was initiated by first cultivating normal nitrification biofilm, followed by acclimate to a nitritation biofilm, and then to an integrated anammox biofilm. The microbes in reactor 1 grow primarily within the biofilms of suspended carriers. Reactor 2 was directly inoculated with activated sludge with good nitrification performance to start, and NOB inhibition was gradually achieved by controlling dissolved oxygen, and finally AnAOB was cultivated in the same reactor.

2.4. Analytical methods

DO was measured with a Hach HQ40d multi DO probe, and pH was recorded by pH detector (PHS-3C, China). NH_4^+-N, NO_2^--N and NO_3^--N were measured according to the standard methods by the UV/visible spectrophotometer (DR6000 HACH, USA). All samples were filtrated through a 0.45 μm filter prior to analyses.

2.5. Metagenomic sequencing

Total genomic DNA was extracted from carrier samples using the E.Z.N.A.® DNA Kit (Omega Bio-tek, Norcross, GA, U.S.). Concentration and purity of extracted DNA was determined with TBS-380 and NanoDrop2000, respectively. DNA extract quality was checked on 1% agarose gel. DNA extract was fragmented to an average size of about 400 bp using Covaris M220 (Gene Company Limited, China) for paired-end library construction. Paired-end library was constructed using NEXTFLEX Rapid DNA-Seq (Bioo Scientific, Austin, TX, USA). Adapters containing the full complement of sequencing primer hybridization sites were ligated to the blunt end of fragments. Paired-end sequencing was performed on Illumina Hiseq Xten (Illumina Inc., San Diego, CA, USA) at Majorbio Bio-Pharm Technology Co., Ltd. (Shanghai, China) using HiSeq X Reagent Kits.

3. Results and discussion

3.1. The start-up of reactors

During the start-up stage, DO in both reactor 1 and reactor 2 were controlled to 0.5~1.0 mg/L. As shown in Figure 2, the initial NAR (nitrite accumulation rate) in reactor 1 was close to zero and then increased to over 85.2% within 35 d. While the NAR of reactor 2 was 74.4% on the first day after startup and rose to 89.7% within 7 d, significantly higher than that of reactor 1. Therefore, nitritation can be achieved faster by suspended activated sludge than biofilm, which is consistent with that reported by Sun *et al.* (Sun *et al.*, 2017).

As is known, high concentrations of ammonia can inhibit NOB by forming free ammonia (FA) under high pH. In biofilm structure, AOB lives in the appearance of the biofilm, and NOB is mainly located in the middle to inner layer of the membrane, so it is protected by the biofilm and FA has less effect on it. However, NOB in activated sludge is completely exposed to FA. Therefore, this study suggests that the protection of NOB by the biofilm is the main reason for the slower progress of nitritation in reactor 1.

This phenomenon was also observed by Wang *et al.* (2015), Piculell *et al.* (2016) and Kental *et al.* (2019). They suggested that the effectiveness of FA on NOB inhibition was related to the particle/biofilm size, and that thick biofilm or big granular sludge had difficulty in maintaining NOB inhibition due to the limitations of FA mass transfer. In contrast, Zhu *et al.* (2018), Morales *et al.* (2016). and Pérez *et al.* (2020) concluded that NOB inhibition was more successful in thicker biofilms. This was mainly due to the fact that in the above studies, the range of influent ammonia was 50~75 mg/L. Under this influent condition FA was less likely to inhibit NOB and DO was the only condition to inhibit NOB. When the biofilm was thicker, the concentration of DO diffusing into the interior of the biofilm was lower, which was unfavorable to the growth of NOB.

In terms of bacterial size, NOB are smaller than AOB and AnAOB (Vlaeminck *et al.*, 2010; Trinh *et al.*, 2021; Yang *et al.*, 2019b), so this paper considered that NOB was more easily washed out in sludge reactors comparing to MBBR reactors where NOB was located in the inner layer of AOB. The stronger FA inhibition and easy wash-out feature of NOB in reactor 2 allowed nitrite nitrogen to accumulate more rapidly.

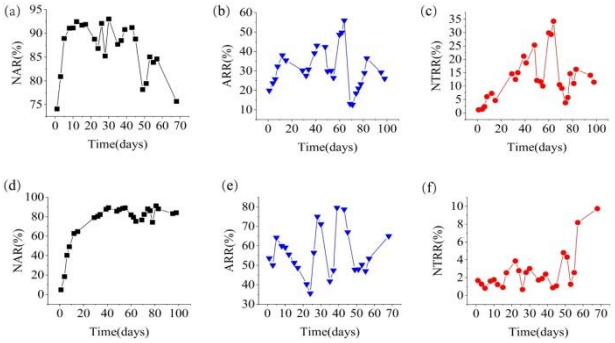


Figure 2. The performance of reactors during the start-up stage. (a), (b) and (c) are for reactor 1; (d) , (e) and (f) are for reactor 2

At 0~48 d, the ammonia nitrogen removal rate (ARR) of reactor 1 ranged from 19.9% to 43.0% (a mean of 31.4%), and the total nitrogen removal rate (TNRR) ranged from 1.1% to 21.2% (a mean of 9.5%). After the completion of start-up (48~98 d), when the biofilm had begun to grow to a certain thickness and the aeration was not adjusted, the ARR was lower, ranging from 12.3% to 56.1% (a mean of 30.8%), similar to the level of the previous stage, and the TNRR was 4.4%~34.2% (a mean of 15.7%).

For reactor 2, the ARR was 36.2%~80.3% (a mean of 56.4%), and TNRR was 1.2%~8.7% (a mean of 3.3%) after start-up (at 1~72 d). It can be seen that the ARR of the sludge reactor was higher than that of the biofilm reactor, while the TNRR was lower. The reason was that DO could penetrate inside the flocs in the activated sludge, affecting the growth of AnAOB and the removal of total nitrogen, while the anaerobic environment inside the biofilm provided better conditions for the growth of AnAOB.

3.2. Changes from suspended sludge to granular sludge

The concentration of DO was reduced to 0.3~0.5 mg/L at 72~105 d in reactor 2 due to the failure of aerator, and during this stage it was observed that the sludge in reactor 2 aggregated together and attached to the inner wall (Figure 3-e), the bottom aeration disc and mixer paddles, forming a biofilm, and the effluent was more transparent (Figure 3-b) compared to the startup stage (Figure 3-a). The main reason was that the lower DO resulted in less turbulence in the system and the water flow had less rushing force on the inner wall of the reactor, so the biofilm was not easy to be detached. In addition, no external carbon source was added to the system, in which the sludge contained mainly autotrophic bacteria, and the dense structure of the autotrophic bacteria promoted the secretion of more extracellular polymers (EPS), which provided sticking and winding for the bacteria, allowing microbial aggregates to bind together even under hydraulic shear force (Li *et al.*, 2010; Xia *et al.*, 2015). Zhao *et al.* (2019) and Fan *et al.* (2016)

also found in their studies that AOB had superior settleability and are more likely to cluster and adhere to each other at low organic concentrations. As is shown in Figure 4, during this stage, the ARR was 18.6%~37.3% (a mean of 27.9%) and the TNRR was 4.2%~10.6% (a mean of 7.3%) in reactor 2. The ARR was significantly lower than that of at the startup, mainly due to its relative higher DO diffusion resistance decreasing the activity of AOB.

At 106~291 d, to improve the system removal efficiency, the DO concentration in reactor 2 was raised to 1.0~1.5 mg/L. The results showed that the ARR was 29.1%~70.4% (a mean of 51.2%), and the TNRR increased to 22.4%~57.1% (a mean of 39.5%), indicating that the system had changed into integrated anammox system. The biofilm at this stage was darker and redder in color (Figure 3-f), showing that some AnAOB had grown in the system.

At 292~381 d, the DO concentration in reactor 2 increased to 1.8~2.2 mg/L, and the ARR was 73.1%~87.8% (a mean of 80.4%), the TNRR was 63.2%~76.3% (a mean of 68.7%). Although the DO concentration was often above 2 mg/L during this stage, the biofilm remained attached and was not washed away. Compared to reactor 1 at the stage of steady operation (379-415 d), reactor 2 had a higher DO but lower ARR and NTRR at 292~381 d, indicating that the biofilm in reactor 2 was too thick, reducing the diffusion of DO. It can also be seen from Figure 3-g that the biofilms at this stage was very thick.

At 382~426 d, the inner wall of reactor 2 was scrapped manually by a hard brush and the biofilm was found to be detached and suspended in water (Figure 3-c). The suspended biofilm was denser than normal activated sludge flocs and the system HRT was up to 48 h. Therefore, most of the detached biofilm was remained in the reactor even under continuous flow conditions. Compared to the previous stage (292~381 d), the DO concentration at 382~426 d decreased significantly although the aeration rate kept the same. The ARR was above 95% (a mean of 98.4%, Figure 4-b), and TNRR was 71.2%~85.8% (a mean of 82.3%, Figure 4-c). The removal rates were higher, and more stable compared to reactor 1. In addition, the nitrate concentration produced by the system at this stage was at normal levels, with△nitrate/△ammonia being about 10.3%~13.2% (a mean of 12.4%, Figure 4-d), indicating great inhibition of NOB by the system.

From 453 d, the ARR and TNRR changed less in reactor 1 and the effluent was getting more transparent (Figure 3-d). The presence of small red granular sludge with a compact structure and clear shape at the bottom of the reactor (see Figure 3-h) verified that biofilm detachment can form granular sludge. Currently, the formation of granular sludge from biofilm has been identified mainly in studies on IFAS reactors where sludge and biofilms coexist. All views in these studies that granular sludge originated from biofilm detachment processes were speculative and the detachment was a spontaneous process. While in this study, microorganisms mainly accumulated in the biofilm and there were few flocs in the system, which suggested that biofilm detachment was the real main factor leading to granular sludge formation. Furthermore, the detachment process could be controlled manually, which was essential to shorten the formation time of granular sludge in practical engineering.

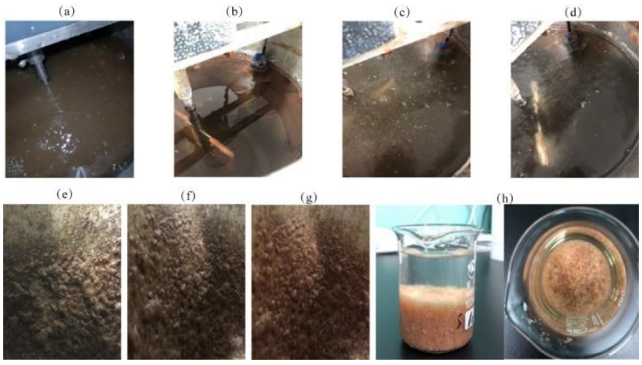


Figure 3. Photos of mixture, biofilm and granular sludge morphology in reactor 2. (a)The muddy mixture at start-up stage; (b) The clear effluent after the formation of biofilm; (c) The effluent containing detached biofilm; (d) The effluent after the formation of granular sludge; (e) The inner wall of reactor2 at 95d; (f) The inner wall of reactor2 at 221d; (g) The inner wall of reactor2 at 346d; (h) granular sludge morphology in reactor 2 (sampling at 417d).

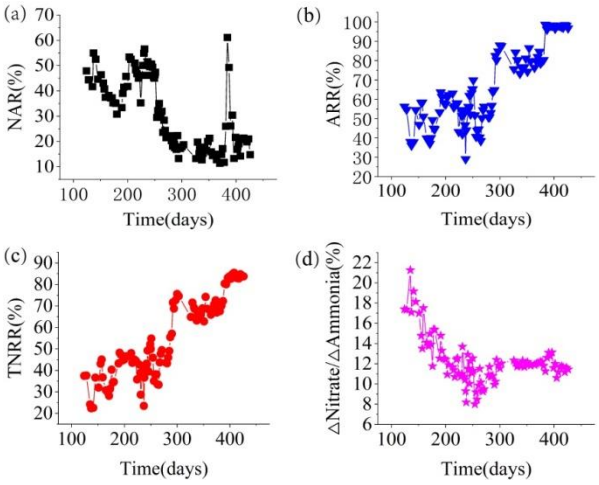


Figure 4. The performance of reactor 2 during the period of long-term operation

3.3. Long-term operation of MBBR integrated anammox process

After the start-up was completed, the DO concentration in reactor 1 increased to 1.0~1.5mg/L. As shown in Figure5, the ARR increased to 50.1%~76.8% (a mean of 64%) at 106~187 d. The increase in DO decreased the internal AnAOB activity, resulting in a reduction in the TNRR to only 1.3%-21.1% (a mean of 13.5%), which was comparable to the initial value during start-up stage. At 188~243 d, the TNRR of reactor 1 rapidly increased from 19.2% to 68.4%, and the ARR fluctuated between 51.1%~79.6%, and during this stage, the system had been substantially converted from nitritation to integrated anammox process. At 244~415 d, the ARR was

62.3%~98.4% (a mean of 87.5%), and the TNRR was 53.2%~90.1% (a mean of 74.2%). Overall, the removal rate of reactor 1 was lower than that of reactor 2 after stable operation, and Wang *et al.* (2012) also found in their study that the granular sludge anammox reactor performed better than the biofilm reactor. This may be due to the existence of an internal gap in the granular sludge, which serves as a channel for substrate and gas transport and improves the nitrogen removal efficiency (Chen *et al.*, 2010; Lu *et al.*, 2012; Wang *et al.*, 2021c).

Another obvious phenomenon observed in the long-term operation of reactor 1 was that once NOB had grown in the system then it would be difficult to fully inhibit it. For example, at 310~320 d, excessive aeration occurred in the reactor and the DO was reduced to 0.5~0.8 mg/L to ensure a continued inhibition of NOB, but the Δnitrate/Δammonia still increased to 16.5% to 18.1% after adjusting the DO. At 380 d, the nitrate concentration of effluent increased by approximately 50% than that before excessive aeration, and △nitrate/△ammonia was about 13.4%~16.8% (a mean of 15.5%), indicating that NOB was not fully inhibited even at the later stage of operation. In global projects using ammonia process to treat industrial wastewater, 30% of the systems have experienced an increase in nitrate nitrogen concentration (Li *et al.*, 2018).

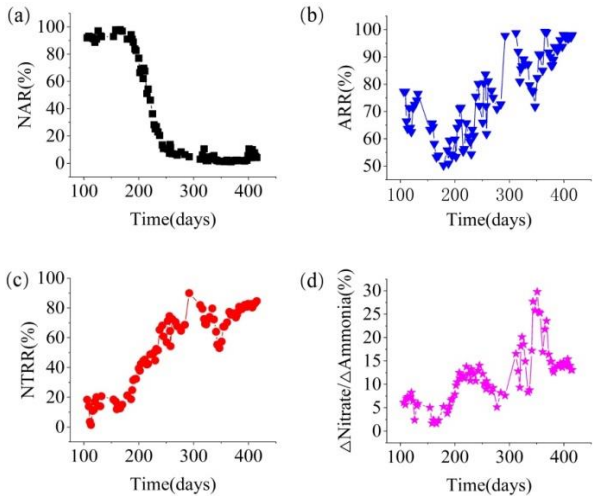


Figure 5. The performance of reactor 1 during the period of long-term operation

3.4. The relationship between the ARRs and the TNRRs

The ARRs and NTRRs of reactor 1 sometimes showed a linear relationship, which became more significant with the increase of biofilm thickness (Figure 6-1). From the start-up stage (1~105 d), the linear relationship was evident as the carriers used had previously had a thin biofilm grown on it, and the correlation coefficient was 0.627 3. At 106~187 d, a higher aeration rate was adopted, and the biofilm thickness remained thin, so the TNRRs increased slower than the ARRs, and the correlation coefficient decreased to 0.000 2. At 188~374 d, the linear relationship between ARRs and NTRRs gradually increased, with the correlation coefficient increasing to over 0.6. At the later operation stage (375~415 d), as the biofilm thickness increased, the DO diffusion resistance increased and total nitrogen removal was mainly restricted by ammonia removal, with the correlation coefficient as high as 0.914 9.

Figure 6-2 shows the relationship between ARR and NTRR for reactor 2. At the start-up (1~72 d) and early operation stage (73~105 d), microbes in the reactor clustered as flocs which could be penetrated easily by dissolved oxygen. Therefore, the activity of AnAOB was low, TNRRs were mainly restricted by DO, and has a weak correlation with ARRs. At 106~291 d, the microbes were enriched predominantly in a biofilm and a clear linear relationship between the TNRRs and the ARRs was shown, with the correlation coefficient increasing to 0.737 6. During this stage, the structure of the biofilm remained relatively loose and the resistance of DO to penetration inside the biofilm was low. At 292~381 d, the correlation coefficient increased to 0.978 3 as the biofilm became more dense, and the stronger correlation between ARRs and NTRRs indicated that the diffusion of DO was the main factor influencing the removal of total nitrogen at this stage. At 382~426 d, the biofilm was detached, the system was filled with suspended biofilm fragments, and the correlation coefficient decreased rapidly to 0.020 9. However, the permeate transfer resistance within the biofilm fragments was slightly higher than that of the flocs, so the correlation coefficient was higher at this stage compared to the start-up stage (1~72 d) and the early operation stage (73~105 d).

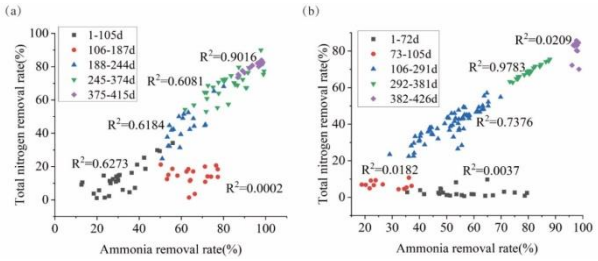


Figure 6. Correlation of ARRs and TNRRs for reactor 1 and reactor 2. (a) is for reactor 1; (b) is for reactor 2.

3.5. Structural characteristics of microbial communities and gene analyze of Nitrogen metabolism

At 460 d, carriers in reactor 1 (sample 1) and granular sludge in reactor 2 (sample 2) were taken out to analyze the microbial abundance by the method of metagenomic sequencing. The results showed that at the phylum level, Proteobacteria, planctomycetes and Chloroflexi had high abundance in both samples and were the main phylum in the integrated anammox system (Figure 7-a). Planctomycetes is the main phylum to which AnAOB belongs. It accounted for the highest proportion in sample 1, higher than that in sample 2. The main reason was that sample 2 was mainly composed of newly formed small granular sludge particles with relatively loose structure, while the biofilm in sample 1 was relatively dense and the internal DO is low, which provided favorable conditions for the growth and reproduction of AnAOB. The Chloroflexi is a common phylum in anammox systems, which is able to degrade metabolites of AnAOB and form

symbiotic relationships with other bacteria (Wu *et al.*, 2019; Keren *et al.*, 2020). The phylum proteobacteria plays a very important role in biological nitrogen removal process and is the dominant in most anammox systems (Tallec *et al.*, 2008; Ma *et al.*, 2015; Itoi *et al.*, 2007). To further investigate the community structure of nitrogen removal bacteria in the system, proteobacteria were subdivided. Proteobacteria can usually be classified into five classes, and four classes, *α*, *β*, *γ* and *δ*, were detected in this experiment, with the highest abundance of the *β-Proteobacteria* class, which covers almost all type of AOB (Liang *et al.*, 2017; Li *et al.*, 2014; Varas *et al.*, 2015). The *β-proteobacteria* abundance in samples 1 and 2 was 12.17% and 18.43%, respectively. Therefore, it can be concluded that the proportion of AOB in the reactor 2 was relatively high.

The AOB and NOB bacteria were analyzed at the genus level then and the results were presented in Table 1. The main members of AOB belong to the genus *Nitrosomonas*, *Nitrosolobus*, *Nitrosospira*, *Nitrosovibrio* and *Nitrosococcus* (Staley *et al.*, 2001). Four major genus other than *Nitrosolobus* were detected, and *Nitrosomonas* had the highest abundance, with 5.36% and 7.55% in sample 1 and sample 2, respectively, indicating that there was a higher proportion of AOB in sample 2. The main members of NOB belong to the genus *Nitrobacter*, *Nitrococcus*, *Nitrospira* and *Nitrospina*, of which the first three genus were detected in this study and the abundance of *Nitrospira* was the highest, with 2.95% and 0.34% in sample 1 and sample 2, respectively, indicating that the NOB was inhibited to a greater extent in sample 2.

The AnAOB were also analyzed at the genus level (Table 1). The present studies identified five genus of AnAOB in wastewater, *Candidatus Brocadia*, *Candidatus Kuenenia*, *Candidatus Scalindua*, *Candidatus Anammoxoglobus* and *Candidatus Jettenia*, which have different physiological properties (Kartal *et al.*, 2013; Oshiki *et al.*, 2016) and only one genus can usually compete to be the dominant species in AnAOB-enriched cultures (Bhattacharjee *et al.*). The results showed that, both samples contained *Candidatus Brocadia* and *Candidatus Kuenenia*, and *Candidatus Kuenenia* was the dominant genus, verifying that *Candidatus Kuenenia* is competitive at high ammonia nitrogen concentrations. The abundance of *Candidatus Kuenenia* was 20.51% and 4.55% in sample 1 and sample 2, respectively, and the abundance of *Candidatus Brocadia* was 0.59% and 2.30% in sample 1 and sample 2, respectively, indicating that *Candidatus Kuenenia* was more abundant in biofilm and *Candidatus Brocadia* was more abundant in granular sludge with relatively loose structures. In this paper, we suggest that biofilm is more tolerant to high concentrations of ammonia and therefore can enrich *Candidatus Kuenenia* more easily, which is consistent with the findings of Wang (Wang *et al.*, 2021a).

The results of the nitrogen cycle process analyze (Figure 7-b) showed that the *narG*, *narZ*, *narH*, and *narY* involved in the dissimilatory nitrate reduction process were the most abundant, and the abundance of *hao* involved in the nitrification process was the second. Dissimilatory nitrate reduction is a process of reducing nitrate to nitrite, which is not only the first step in the denitrification process, but also provides nitrite for anammox process. The *hao* gene is mainly produced by AOB, and its function is oxidizing hydroxylamine to nitrite. The results showed that the difference in the abundance of n*arG, narZ, narH,* and *narY* in sludge and carrier was small, while the abundance of *hao* in granular sludge was 1.9 times in carriers. Not only in the nitrification processes, but in the whole nitrogen cycle, the gene abundance in the granular sludge sample was higher than that in the carrier sample, especially the gene involved in denitrification, nitrification and anammox process, which was also the main reason for the better nitrogen removal effect of the granular sludge reactor than the biofilm reactor.

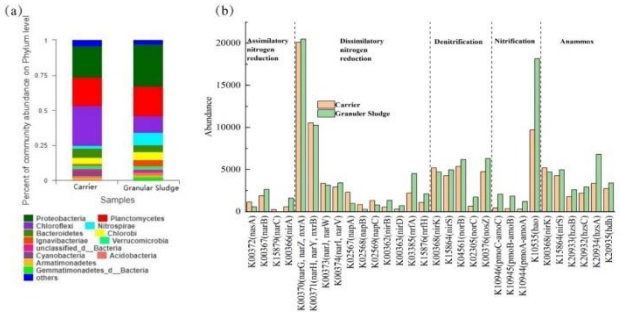


Figure 7. Metagenomic sequencing analysis results for carrier in reactor 1 and granular sludge in reactor 2. (a) Microbial characteristics at phylum level; (b) Nitrogen metabolism gene abundance.

Table 1. The abundance of nitrogen removal bacteria on genus level.

Type	Genus	Sample 1(%)	Sample 2(%)
AOB	*Nitrosomonas*	5.36	7.55
	Nitrosospira	0.23	0.04
	Nitrosovibrio	2.98E-05	9.55E-03
	Nitrosococcus	0.07	0.03
	Sum	5.66	7.62
NOB	*Nitrococcus*	0.02	3.76E-03
	Nitrobacter	0.02	6.72E-03
	Nitrospira	2.95	0.33
	Sum	2.99	0.34
Anammox	*Candidatus_Kuenenia*	20.51	4.55
	Candidatus_Brocadia	0.59	2.30
	Sum	21.10	6.85

4. Conclusions

This study investigated a floc sludge bioreactor and a MBBR reactor for more than 450 days and compared them from the start-up to stable operation period. The results showed that in the floc sludge bioreactor, flocs aggregated into biofilms first and granulated after being detached artificially in the sludge integrated anammox system, which provide the first evidence that the sludge granulation can be achieved by operational control in a continuous flow reactor. The study also found that, compared to the sludge bioreactor, it was more difficult to

achieve NOB inhibition inside the MBBR reactor due to the protection of the biofilm, and the total nitrogen removal was restricted by aeration. This study provides an in-depth understanding the mechanism of anammox process and broaden the feasibility of anammox applications.

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