Pilot-scale enrichment of anammox biofilm using secondary effluent as source water
Runxian Tao, Xingcan Zheng, Xingfang Guo, Mai Li, Shifeng Shen, Min Yang, Yongli Sun and Fansong Wu

ABSTRACT

Enough biomass of anaerobic ammonium oxidation (anammox) bacteria is essential for maintaining a stable partial nitrification/anammox (PN/A) wastewater treatment system. Present enrichment procedures are mainly labor-intensive and inconvenient for up-scaling. A simplified procedure was developed for enrichment of anammox biofilm by using secondary effluent as source water with no supplement of mineral medium and unstrict control of influent dissolved oxygen (DO). Anammox biofilm was successfully enriched in two pilot-scale reactors (XQ-cul and BT-cul) within 250 and 120 days, respectively. The specific anammox activity increased rapidly during the last 2 months in both reactors and achieved 2.54 g N2-N/(m2·d) in XQ-cul and 1.61 g N2-N/(m2·d) in BT-cul. Similar microbial diversity and community structure were obtained in the two reactors despite different secondary effluent being applied from two wastewater treatment plants. Anaerobic ammonium oxidizing bacteria genera abundance reached up to 37.4% and 43.1% in XQ-cul and BT-cul biofilm, respectively. Candidatus Brocadia and Ca. Kuenenia dominated the enriched biofilm. A negligible adverse effect of residual organics and influent DO was observed by using secondary effluent as source water. This anammox biofilm enrichment procedure could facilitate the inoculation and/or bio-augmentation of large-scale mainstream PN/A reactors.

Key words | anammox, biofilm, enrichment procedure, large scale, microbial community, secondary effluent

HIGHLIGHTS

- Secondary effluent was used as source water to enrich anammox biofilm in pilot MBBR reactors.
- Residual organics and DO showed negligible adverse effect on the enrichment process.
- Specific anammox activity of 1.61 g N/(m2·d) was successfully gained within 4 months.
- The relative abundance of anammox genera reached 43% in enriched biofilm.
- The enrichment procedure is proposed for up-scaling application.
INTRODUCTION

Partial nitritation/anammox (PN/A) is an attractive autotrophic nitrogen removal technology for the merits of energy-saving and no need for carbon sources. The PN/A process has been successfully applied in the treatment of sludge liquor, landfill leachate, and other ammonium-rich wastewater (Van der Star et al. 2007; Joss et al. 2009; Lackner et al. 2014). In the case of municipal wastewater, full-scale implementation of the PN/A process has more inherent challenges including relatively lower and variable influent nitrogen concentration in mainstream systems, seasonal fluctuation of temperature especially in cold winter and strict effluent requirements (Xu et al. 2015; Cao et al. 2017). Considerable researches have been done in this field, which have deepened our knowledge of operational strategies, reactor configurations, and microbial community and interactions about mainstream PN/A systems (Agrawal et al. 2014; Nsenga Kumwimba et al. 2020). However, most of these studies were conducted on laboratory-scale and only a few pilot-scale experiments are reported to date (Gustavsson et al. 2014, 2020; Lemaire et al. 2014; Lotti et al. 2015; Hoekstra et al. 2018). To develop a mainstream PN/A system, a key point is effectively accumulating and holding sufficient anammox biomass in the reactor. Unfortunately, in-situ cultivation and enrichment of anammox bacteria in mainstream conditions is difficult and time consuming due to their sensitivity to operational conditions and slow growth rate with a typical doubling time of about 11 days (Strous et al. 1998; Van der Star et al. 2007). For this reason, most mainstream PN/A reactors were inoculated at the start-up period and/or bio-augmented during operation with enriched anammox biomass in the form of granular sludge or carriers-supported biofilm (Lotti et al. 2014; Gilbert et al. 2015; Wett et al. 2015). Especially, all the above-mentioned pilot-scale reactors with several cubic metre volumes were inoculated with a large amount of seed anammox biomass, which was usually obtained from pilot- or full-scale side-stream PN/A reactors treating sludge liquor. Therefore, it is a necessity to find a practicable enrichment procedure for accumulating enough anammox biomass to promote the establishment of an up-scaling mainstream PN/A system and reduce start-up time, especially if there is no available side-stream system for inoculation.

Anaerobic ammonium oxidizing bacteria (AnAOB) responsible for the anammox reaction belong to the phylum Planctomycetes. Intense efforts have been made to research enrichment techniques of AnAOB since they were successfully developed in a fluidized bed reactor (FBR) fed with a mineral medium in 1996 (Van de Graaf et al. 1996). To start an AnAOB enrichment system, appropriate seed sludge, suitable reactor types, and operation/control procedure should be carefully considered. Inoculum was usually taken from conventional wastewater treatment systems (Third et al. 2005; Chamchoi & Nitisornvut 2007; Connnan et al. 2016), operating anammox reactors (Trigo et al. 2006; Ni et al. 2011), or marine sediment (Kindaichi et al. 2011; Rios-Del Toro et al. 2017). Diverse types of reactors were studied for AnAOB enrichment, including the sequencing batch reactor (SBR) (Jetten et al. 2005; Third et al. 2005).
2005; Chamchoi & Nitisoravut 2007; Ding et al. 2017; De Cocker et al. 2018), membrane bioreactor (Van der Star et al. 2008; Zhang & Okabe 2017), upflow anaerobic sludge blanket (UASB) reactor (Uyanik et al. 2011), FBR (Van de Graaf et al. 1996), closed sponge-bed trickling filter (Sánchez Guillén et al. 2015), and gas-lift reactor (Hendrickx et al. 2014). However, these reactors were mostly in laboratory-scale and the experiences of up-scaling AnAOB enrichment are rarely reported. For laboratory-scale enrichment, synthetic influent containing substances (ammonium and nitrite), mineral media and other trace elements were usually prepared in tap water or demineralized (demi) water to avoid organic contamination (Van de Graaf et al. 1996; Egli et al. 2001; Jetten et al. 2005; Third et al. 2005; Chamchoi & Nitisoravut 2007; Uyanik et al. 2011; Hendrickx et al. 2014; Sánchez Guillén et al. 2015; Connan et al. 2016; Choi et al. 2018). In addition, oxygen presence was carefully controlled by sparging the influent and flushing the reactors with N2, CO2, or Ar gas during experiments. These enrichment procedures are labor-intensive and inconvenient. Moreover, tap water or demi water may not be readily available for large-scale enrichment.

Secondary effluent from wastewater treatment plants (WWTPs) might be an alternative source water for AnAOB enrichment. It is convenient to obtain for a pilot-scale experiment usually performed in a WWTP. Organic matters could be effectively removed in WWTPs and the typical effluent chemical oxygen demand (COD) is below 30–50 mg/L. Moreover, the matrix of secondary effluent is more ‘sewage’-like than tap water, and that would help enriched AnAOB adapt to mainstream conditions. To the best of our knowledge, the present study is the first to use secondary effluent as source water for AnAOB enrichment. Some issues need a detailed investigation, which are possibly caused by the secondary effluent, including competition of heterotrophic bacteria, potential inhibition of residual dissolved oxygen, and what enrichment level could be expected.

This study aimed to develop a practicable procedure to enrich anammox biofilm for up-scaling mainstream PN/A systems. Secondary effluent from two different WWTPs was used as source water with adding of just ammonium and nitrite. Enrichment of carriers-supported anammox biofilm was attempted in two pilot moving bed biofilm bioreactors (MBBRs) which were operated by increasing nitrogen loads step by step. The enrichment process was described in terms of nitrogen removal rates and efficiencies. Anammox activity of the biofilm fixed on carriers was tracked during the process. Further, the microbial community structures were characterized and compared between the enriched biofilm cultures from the two reactors.

MATERIALS AND METHODS

Reactor setup

Two pilot-scale reactors with the same configuration (Figure 1) were installed respectively in Xiqu WWTP and Beitang WWTP (Tianjin, China). Both reactors consist of three main parts including a substrate tank for dissolving NH4Cl and NaNO2, an influent tank for preparing the experiment influent and a well-mixed MBBR for enriching the anammox biofilm. The reactor in Xiqu WWTP (labeled as XQ-cul) has a working volume of 0.7 m3, and that of the reactor in Beitang WWTP (labeled as BT-cul) is 1.8 m3. Plastic biofilm carriers (600 m2/m3, Spring, China) were filled into both reactors, with a volume of 300 L in XQ-cul and 750 L in BT-cul. Both reactors were automatically controlled by the PLC program. Secondary effluent was pumped from the secondary clarifier of each WWTP into the influent tank when the liquid level reached the low-value setting. Meanwhile, NH4-N and NO2-N solution in the substrate tank was pumped into the influent tank. It took 5–10 min to prepare the influent. When the liquid level reached the high value, the raw water pump stopped, and the influent pump started to feed the biofilm reactor. The running program of the two reactors included: stirring continuously, feeding for about 6 and 12 h for BT-cul and XQ-cul respectively (due to their different flow rates and influent tank volumes), feeding paused for 5–10 min to prepare influent, and no settling or decanting steps. The hydraulic retention time of XQ-cul and BT-cul was 16.1 h and 16.7 h, respectively. During the whole experiment, the temperature in the reactors was kept at 30 ± 2 °C by the water-bath system. The effluent of the reactors was drained into the sewer and back to the WWTP influent chamber.

Firstly, XQ-cul was operated from October 2018 to June 2019 and inoculated with 10 L of carriers which were pre-cultured in a 25 L laboratory anammox reactor previously. Then, 60 L of carriers was taken from XQ-cul after 6 months of enrichment and used as inoculum for BT-cul. After that, 60 L of new carriers was supplemented in XQ-cul. The inoculum carriers and another 690 L of new carriers were added into BT-cul in April 2019, and the experiment in BT-cul ended in July 2019.

Enrichment procedure

The initial concentration of NH4+-N and NO2-N was set at 20–30 mg/L in the influent tank by dissolving an
appropriate amount of NH₄Cl and NaNO₂ reagent (technical grade) in the substrate tank and adjusting the operation time of the dosing pump. Tap water was used to dissolve NH₄Cl and NaNO₂ with no addition of extra mineral materials nor trace elements.

The nitrogen concentration in the feed was increased stepwise, thus increasing the nitrogen load rate (NLR) by prolonging the dosing time of substrates when total nitrogen (TN) removal kept stable or effluent NO₂⁻/C⁰⁻-N was below 10 mg/L. During the experiment, the molar ratio of NH₄⁺-N/NO₂⁻-N was kept around 1.0 in the feed. To avoid nitrite inhibition, the dosing time would be reduced to the value of the previous step to lower the effluent concentration once effluent NO₂⁻-N exceeded 50 mg/L.

The dissolved oxygen (DO) in XQ-cul influent was about 5.0–7.5 mg/L, and that in BT-cul influent was about 3.0 mg/L. The higher DO content in XQ-cul influent was attributed to a design flaw of the secondary clarifier of Xiqu WWTP with a large drop in water level in its outlet well where secondary effluent was pumped for XQ-cul. No treatment was done to control DO in the influent tank during the experiment.

Source water quality

Secondary effluent from Xiqu WWTP and Beitang WWTP was used as the source water for XQ-cul and BT-cul, respectively. Table 1 shows the main characteristics of the influent and secondary effluent of these two WWTPs. The Xiqu WWTP is located in an integrated industrial park involving companies of automobile, manufacturing, electronic and

| Parameters | Xiqu WWTP | Beitang WWTP |
|------------|-----------|--------------|
| COD        | 127 ± 55  | 342 ± 100    |
| sCOD       | 51 ± 17   | 129 ± 37     |
| NH₄⁺-N     | 12.65 ± 7.93 | 31.94 ± 4.66 |
| TN         | 28.08 ± 7.93 | 46.18 ± 6.99 |
| Total P    | 2.14 ± 1.18  | 4.49 ± 1.49  |
| Chloride   | 500-700    | 650-1,100    |
information, and biopharmaceutical industries. It receives mainly treated industrial wastewater (50,000 m³/d). Due to the strict discharge standards in China, the influent of Xiqu WWTP has been pre-treated well before entering the sewer system. The treatment train of Xiqu WWTP consists of grit chambers, HYBAS biological units (with adding of polyethylene carriers in aerobic tanks), secondary clarifiers, post-denitrification filters, Fenton oxidation tanks, pulse clarifiers, and a dissolved air flotation tank. Beitang WWTP receives mainly municipal wastewater with a capacity of 150,000 m³/d. The treatment train includes aerated grit chambers, modified Bardenpho units, secondary clarifiers, magnetic sedimentation tanks, ozonation tanks, and deep-bed filters. The ratio of domestic wastewater in the influent of Xiqu WWTP is much lower than that of Beitang WWTP. It caused a significant difference in the raw water quality between the two WWTPs. However, the main parameters of the two secondary effluents in Table 1 were relatively similar except for chlorides.

**Analytical methods**

Grab samples were collected from the influent tanks and the effluent of the enrichment reactors at least once or twice a week. Analyses of concentrations of NH₄-N, NO₂-N, NO₃-N, TN, and COD were performed according to standard methods (APHA 2017).

**Activities measurement**

The manometric method was applied for evaluating anammox activities of the biofilm during the enrichment process as described elsewhere (Scaglione et al. 2009; Gilbert et al. 2014; Van Loosdrecht et al. 2016) and modified in this study. OxiToP Control AN-6 (WTW, Germany) was chosen to monitor the change of head-space pressure caused by nitrogen gas produced from anammox bioreaction. In each ex-situ batch test, 6–10 carriers were taken from the enrichment reactors and rinsed carefully with tap water three times to remove particulates and residual substrates. The carriers were then put in a glass bottle in which 200 mL tap water, stripped with high-purity nitrogen gas for 10 min beforehand, was added. The bottle was sealed with a sensor cap and flushed for an additional 30 min with nitrogen gas to achieve anoxic conditions. To stabilize the temperature and pressure, it was put in a thermostat cabinet (WTW, Germany) at a set temperature (15, 20, and 30 °C) and mixed with a magnetic stirrer for 60 min. After that, substrate solutions (NH₄Cl and NaNO₃) were spiked in through the septum with a syringe and needle to provide 10 mg/L NH₄-N and 10 mg/L NO₂-N. The change of the head-space pressure was read by the OxiToP Control OC 110 (WTW, Germany), and then the produced N₂ was calculated via stoichiometry and ideal gas law. The curve of produced N₂ vs. time was drawn and its maximum slope was obtained. From this, the maximum specific anammox activity (SAA, gN₂-N/(m² carriers·d)) was assessed. All tests were conducted in triplicate and the average values of SAAs were used in analysis. Only new added carriers were chosen for the SAA measurement in BT-cul.

**DNA extraction and high-throughput sequencing**

Ten carriers were harvested from XQ-cul (operation day 245) and BT-cul (operation day 108) respectively. Activated sludge was collected on the same day from the oxic tanks of XQ WWTP and BT WWTP for comparison. In total four homogenized biomass samples were used to extract genomic DNA.

Bacterial 16S rRNA gene V4 fragments covering bacteria and archaea were amplified with universal primers 515F (GTGYCAGCMGCCGCGGTAA) and 806R (GGACTACNVGGGTWTCTAAT). Polymerase chain reaction (PCR) was performed in 20 μL reactions using TransStart Fastpfu DNA Polymerase (TransGen Biotech, Beijing, China) under the following conditions: 95 °C for 3 min, 27 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 45 s, and 72 °C for 10 min. The purified fragments were sequenced by the Illumina Miseq PE300 platform (Majorbio Biopharm Technology Co., Ltd., Shanghai, China). In total, 261,209 high-quality sequences were obtained for four samples with an average length of 253 bp after quality control and screening. The sequences were clustered into operational taxonomic units (OTUs) with a 97% identity threshold. Taxonomic classification of OTUs was performed using the RDP Classifier algorithm and bacterial SILVA 132 database. Alpha diversity indexes were calculated by Mothur software (v. 1.30.2). All the data analysis was performed on the free online Majorbio Cloud Platform (www.majorbio.com).

**RESULTS AND DISCUSSION**

**Nitrogen removal performance**

Figure 2(a) shows the nitrogen removal performance in XQ-cul during 250 days of enrichment. In the first 130 days, the
enrichment process was interrupted by two upsets on day 56 and day 102. The first upset on day 56 (in winter) was caused by freezing of the secondary effluent supply and the influent pipelines. It took several days to clear away and reconnect the pipelines and the reactor stopped running, but temperature control was kept during this period. When sampling after a week, TN removal efficiency decreased dramatically from 37.5% (day 54) to 7.2% (day 65). As a result, tiny bubbles produced from anammox biofilm stopped appearing in the reactor. After a month of operation, TN removal was recovered to 38.6%. However, the secondary clarifier in Xiqu WWTP, from which the source water was pumped, was stopped for maintenance and repair at day 102. After that, the stirrer and heater in XQ-cul broke down in succession. These mechanical failures caused the stoppage of XQ-cul for over 20 days. In this period, secondary effluent was replaced by tap water to prepare the influent for XQ-cul. The reactor was fed in batch mode once a day. When restarting and sampling on day 130, only 5.7% of TN was removed. In the succeeding stage, the enrichment in XQ-cul kept stable and the influent nitrogen concentration was increased gradually resulting in an NLR of up to 1.49 g N/(m²·d) at the end of the experiment (Figure 2(b)). The nitrogen removal rate (NRR) kept rising simultaneously and reached 1.20 g N/(m²·d) on day 250. More than 80% TN removal efficiency was stably achieved after day 190.

Compared to XQ-cul, the NLR was increased more progressively in BT-cul (Figure 2(d)) to promote the rapid enrichment of anammox biofilm. After 4 months of operation, the NLR reached 1.90 g N/(m²·d) and a higher NRR was achieved of up to 1.42 g N/(m²·d) in BT-cul. As a result, BT-cul had a lower N removal performance than XQ-cul (Figure 2(c)). During their last 60 days of operation,
the average TN removal efficiencies were 71.2% and 81.2% for BT-cul and XQ-cul, respectively. As shown in Figure 3, the effluent \( \text{NH}_4^+\cdot \text{N} \) and \( \text{NO}_2^-\cdot \text{N} \) concentrations of BT-cul were obviously higher than those of XQ-cul in the last 60 days, but never above the setting \( \text{NO}_2^-\cdot \text{N} \) threshold concentration of 50 mg/L to avoid the inhibition of nitrite.

To validate the role of anammox route on N removal in the two reactors, the ratio of consumed \( \text{NO}_2^-\cdot \text{N} \) to \( \text{NH}_4^+\cdot \text{N} \) (\( \Delta \text{NO}_2^-\cdot \text{N}/\Delta \text{NH}_4^+\cdot \text{N} \)) and the ratio of produced \( \text{NO}_3^-\cdot \text{N} \) to consumed \( \text{NH}_4^+\cdot \text{N} \) (\( \Delta \text{NO}_3^-\cdot \text{N}/\Delta \text{NH}_4^+\cdot \text{N} \)) were calculated. The value was \( 1.28 \pm 0.10 \) and \( 0.32 \pm 0.06 \) for XQ-cul and \( 1.27 \pm 0.18 \) and \( 0.31 \pm 0.07 \) for BT-cul during their last 60 days. The two ratios were close to the anammox stoichiometrical value of 1.32 and 0.26, respectively (Strous et al. 1998). It indicated the removal of N species was mainly carried out by anammox bacteria, and side-reactions like nitrification and denitrification were negligible.

### Anammox activity evolution during enrichment

Anammox activity was tracked throughout the enrichment process to assess the development of anammox biofilm on carriers. After the first 130 d of operation with two upsets (see section ‘Nitrogen removal performance’), the carriers in XQ-cul had been fixed with a thin layer of biofilm with brown color. However, an extremely low SAA of only \( 0.09 \) g \( \text{N}_2\cdot \text{N}/(\text{m}^2\cdot \text{d}) \) was detected on day 136, indicating the running problems had ruined anammox activity of the biofilm. In BT-cul, no visible biofilm was found on the carriers within the first month, supported by the result of non-detectable SAA on day 23 (Figure 4(a)). The N removal in BT-cul during this period was probably attributed to the seed biomass. Thus, the SAA results of XQ-cul after day 130 were compared with BT-cul for their close starting SAA and within the same time interval of 4 months (Figure 4(a)). The evolution of anammox activity showed similar trends with two distinct stages in both reactors. In the first 2 months, the SAAs increased slowly, then boomed
rapidly in the next 2 months. Nevertheless, the final SAA in XQ-cul was much higher and reached 2.54 g N₂/N/(m²·d). It could be explained by two assumptions. (1) Some AnAOB might have been enriched on the carriers during the first 130 d. Though the SAA (day 136) was low after the upsets occurred, the anammox activities recovered when the reactor was running properly. (2) The ‘rough’ surface of the carriers with pre-developed biofilm is more suitable for fixation of AnAOB than are the fresh carriers in BT-cul. The thicker biofilm on XQ-cul carriers also proved that (Figure 5). On the other hand, anammox biofilm with high activity up to 1.61 g N₂/N/(m²·d) could be successfully enriched in 4 months using secondary effluent as source water, as done in BT-cul.

Temperature effect on anammox activity was tested at 15, 20, and 30 °C, with the carriers taken from XQ-cul on day 180 and 250 (Figure 4(b)). The SAAs detected at 30 °C represented the maximum anammox activities of the biofilm. With the temperature decreasing from 30 °C to 20 °C, the SAAs reduced by 68 and 49% for the sample of day 180 and day 250, respectively. When further decreasing to 15 °C, the typical temperature in winter of many WWTPs, only 16 and 25% of the maximum SAAs remained, respectively. A more severe effect of declined temperature suffered by the sample of day 180 was probably attributed to different enrichment levels of AnAOB at different sampling time. Based on the above results, enough anammox biomass with overcapacity activity is assumed to be needed in a mainstream PN/A system in winter for counterbalancing the effect of declined temperature on anammox activity. As proposed by other researchers (Wett et al. 2013; Cao et al. 2017), anammox biomass bio-augmentation would be critical to maintaining a stable mainstream PN/A system, especially during low-temperature stage. An enrichment reactor, as we used in the present study, is therefore a good choice for bio-augmentation when a side-stream system is absent.

**Microbial communities of enriched biofilm**

High-throughput amplicon sequencing was used to analyze two biofilm samples from XQ-cul and BT-cul and two samples (XQ-s and BT-s) from the corresponding WWTPs. The clustered OTUs ranged from 1,021 to 1,664 per sample. A satisfactory sequencing depth was obtained with all the coverage indexes above 99%. Table 2 shows the alpha diversity parameters including richness indices (Sobs, Chao and ACE) and diversity indices (Shannon and Simpson). The BT-s showed much higher species richness and more evenness of microbial community than XQ-s as revealed by their significant difference in the estimated indicators. The remarkable distinction between the two activated sludges might be attributed to different raw water quality (see Table 1) between the two WWTPs. On the other hand, the BT-cul biofilm had a slightly higher bacteria richness and lower diversity compared with the XQ-cul biofilm. The difference between the two biofilm samples was less obvious, though the carriers-supported biofilm was cultivated with different secondary effluent as source water.

*Figure 6(a)* presents the major phyla (read abundance >1%) of the sludge and biofilm samples. Due to the different influent characteristics of the two WWTPs, XQ-s and BT-s showed distinctive microbial community structures. *Proteobacteria* dominated the two sludge samples with comparable relative abundance (34.3% in XQ-s and 27.0% in BT-s). Other main phyla in BT-s are were distributed comparatively evenly including *Bacteroidetes*,

| Table 2 | Alpha diversity values for enriched biofilm samples from XQ-cul and BT-cul compared with that of the activated sludge samples from corresponding WWTPs |
|---------|
| Sample  | Sobs  | Chao  | ACE     | Shannon | Simpson | Coverage |
| Sludge  | XQ-s  | 1,135 | 1,536.65 | 1,578.72 | 3.96    | 0.1063   | 0.9902   |
|         | BT-s  | 1,664 | 1,974.03 | 1,972.50 | 6.08    | 0.0049   | 0.9906   |
| Biofilm | XQ-cul| 1,021 | 1,444.62 | 1,365.88 | 4.22    | 0.0650   | 0.9918   |
|         | BT-cul| 1,105 | 1,598.59 | 1,503.26 | 3.91    | 0.1104   | 0.9906   |

*Figure 5* The photos of enriched carriers-supported anammox biofilm. BT-cul: biofilm in BT-cul after 120 d; XQ-cul: biofilm in XQ-cul after 250 d.
Chloroflexi, Planctomycetes, and Acidobacteria, while in XQ-s, Armatimonadetes accounted for a large abundance of 30.8%. As for enriched biofilm samples, however, the total composition at the phyla level was quite similar. Planctomycetes, which related to anammox bacteria, was the dominant phylum with relative abundance of 43.6% and 47.8% in biofilm of XQ-cul and BT-cul, respectively. Other subdominant phyla in the two biofilm samples were in turn Proteobacteria (accounting for 24.2% in XQ-cul vs. 26.2% in BT-cul), Chloroflexi (15.3% vs. 8.2%), Bacteroidetes (5.5% vs. 6.9%), and Acidobacteria (5.6% vs. 2.7%). In this study, the community structure of the anammox biofilm enriched with secondary effluent as source water showed barely noticeable difference with other researches using tap water (Connan et al. 2016; Rios-Del Toro et al. 2017).

AnAOB became the most abundant species after enrichment in both reactors as revealed by analysis at genus level. Three frequently observed anammox genera were detected including Candidatus Brocadia, Ca. Kuenenia, and Ca. Jettenia in both enriched biofilm samples (Figure 6(b)). In total, AnAOB genera accounted for 37.4 and 43.1% in XQ-cul and BT-cul biofilm, respectively. Ca. Brocadia was the most abundant population in BT-cul biofilm, while Ca. Kuenenia accounted for a higher abundance in XQ-cul biofilm. Ca. Brocadia and Ca. Kuenenia are thought to be growth rate strategist and affinity strategist, respectively (Van der Star et al. 2008). It might explain the abundance difference between the two genera in XQ-cul and BT-cul samples. As shown in Figure 3, BT-cul biofilm was in a eutrophic condition with relatively higher substrates concentration during the last 60 days. This situation is more favorable for Ca. Brocadia with a higher specific growth rate. In addition, Denitratisoma was found in both samples, which is a typical group of heterotrophic denitrifying bacteria and supposedly utilizes the bacteria lysates (Cao et al. 2016). Other genera involved in nitrogen removal including Nitrosomonas (AOB) and Nitrospira (NOB) were detected at extremely low abundance in both biofilm samples.

Discussion of the impacts of secondary effluent

Generally, high content of organic matters is thought to be harmful to AnAOB because of the competition for nitrite between AnAOB and heterotrophic bacteria (Chamchoi et al. 2008; Jin et al. 2012). This is one of the reasons for tap water or demi water being usually employed in lab-scale enrichment of AnAOB. However, some researchers reported anammox bacteria have a more versatile metabolism and could utilize organics such as acetate or propionate as the supplementary electron donors (Kartal et al. 2007; Kartal et al. 2008). Organic matters in low concentration did not have a significant negative impact on AnAOB. In the present study, residual organics in secondary effluent exhibited negligible inhibition impact on AnAOB. The soluble COD (sCOD) was below 50 mg/L in both
secondary effluents (Table 1). Most importantly, organics in secondary effluent are mainly non-biodegradable, consisting of humic and fulvic acids, soluble microbial products, and recalcitrant synthetic chemicals (Shon \textit{et al.} 2006). Negligible removal of COD could be observed in the two enrichment reactors (data not shown for the sake of brevity). The residual organics would not promote the booming of heterotrophic bacteria in enrichment reactors. It could be confirmed by the sequencing analysis results that AnAOB genera dominated in the biofilm and were not outcompeted by heterotrophic bacteria. On the other hand, the matrix of secondary effluent is more complex than tap water or demi water. We hypothesize the inorganic constituents are enough for the growth of AnAOB and no mineral salts were added except ammonium and nitrite. The results showed that it is non-problematic for either enrichment speed or enrichment level with secondary effluents from two distinct WWTPs. From this point of view, secondary effluent could be a good choice of source water for large-scale enrichment of anammox biomass.

DO should be strictly controlled because high DO could inhibit AnAOB seriously (Jin \textit{et al.} 2012). It was reported that AnAOB showed no activity at low DO exposure of 0.02 mg/L (Seuntjens \textit{et al.} 2018). Inactive gases such as nitrogen, CO$_2$ or Ar were usually used to strip oxygen from the influent and reactors. In the present study, the DO concentration was about 3–7 mg/L in the influent of the pilot reactors, which was brought with the secondary effluent. No control was carried out to eliminate DO in both influents for simplifying the enrichment operation. An adverse effect of influent DO was not observed during the experiment. It could be explained as follows: (1) the ratio of DO to ammonium/nitrite was extremely low in the influents (e.g., 7 mg O$_2$:120 mg NH$_4^+$-N); both enrichment reactors were completely mixed and oxygen in the flow was rapidly dispersed when entering the reactors and could be consumed with a relatively little amount of ammonium or nitrite; (2) nitrogen gas produced from anammox reaction played a role of gas-stripping as fine bubbles were always observed in both reactors during normal running stage; (3) attached AnAOB were protected by the biofilm structure because of the mass transfer resistance from the liquid body to carrier surface. Certainly, DO in the enrichment reactors need to be closely monitored and too high a DO should be avoided. During the whole experiment, the DO concentration in both reactors was maintained below 0.1 mg/L. Except for the dispersion and consumption of oxygen as discussed above, the high temperature in the reactors might be another factor for the low DO, since oxygen solubility drops at high temperatures. Nitrogen removal mainly occurring in anammox route was testified by the ratios of N species (ie. $\Delta$NO$_2^-$-N/ $\Delta$NH$_4^+$-N and $\Delta$NO$_3^-$-N/$\Delta$NH$_4^+$-N, see section ‘Nitrogen removal performance’) and rare detection of aerobic bacteria of AOB and NOB (see section ‘Microbial communities of enriched biofilm’). The results suggest that it seemed to be unnecessary to carefully control DO in anammox enrichment influent.

**CONCLUSIONS**

The enrichment procedure of anammox biofilm was simplified in this study by using secondary effluent as readily accessible source water with no supplement of mineral medium and unstrict control of influent DO. Anammox biofilm with SAA up to 1.61 g N$_2$-N/(m$^2$·d) was successfully enriched within 4 months. A high enrichment level of AnAOB genera was achieved. Residual organics and DO in secondary effluent showed a negligible adverse effect on the growth of AnAOB. The simplified enrichment procedure could be easily implemented under automatic operation on large scale for inoculation and/or bio-augmentation for pilot or prototype mainstream PN/A reactors.

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**DATA AVAILABILITY STATEMENT**

All relevant data are included in the paper or its Supplementary Information.

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