Fast start-up of expanded granular sludge bed (EGSB) reactor using stored Anammox sludge

Zhang Wenjie, Zhang Yuanyuan, Li Liang, Zhang Xuehong and Jin Yue

ABSTRACT

Stored Anammox sludge (SAS) was used in an expanded granular sludge bed (EGSB) reactor treating synthetic wastewater with the aim of evaluating its possible use as seed sludge. The SAS had been kept in a refrigerator (4°C) without any feed. After 2 years, only 1–2% Anammox bacteria could survive in the SAS. However, it soon prevailed in the EGSB reactor after loading. Accordingly, the start-up of the EGSB reactor was successfully completed in 34 days. The biomass turned to round reddish granular sludge from irregular brown floc at the end of this study. The results indicate that SAS could serve well as seed sludge. The required time for start-up of the Anammox reactor using SAS was thus demonstrated to be shorter than that of uncultivated sludge under experimental conditions.

INTRODUCTION

The traditional denitrification process consumes a great deal of energy and needs additional carbon sources to treat low carbon to nitrogen ratio (C/N) wastewater. Since the first discovery of anaerobic ammonium oxidation (Anammox) (Mulder et al. 1995), the Anammox process has been put forward as an innovative and promising way for denitrification owing to the characteristics of Anammox bacteria.

However, it was reported that Anammox bacteria grew slowly and the doubling time was approximately 11 days (Strous et al. 1998). As a result, the start-up period was usually very long. For instance, it took Van der Star et al. (2007) 3.5 years to start the first full-scale plant. Shortening the start-up period was a critical point in the application of the Anammox process. Research efforts have increasingly focused on the culture and enrichment of Anammox bacteria by different groups. Previous studies usually seeded the reactors with aerobic activated or anaerobic sludge (Araujo et al. 2001). Most of these strategies had to start the process at low loading rates and influent concentrations, and then prolong the start-up period (Suneethi & Joseph 2011). Until recent years it was considered that cultivated Anammox bacteria could be employed as inoculum, leading to a rapid start-up compared with other kinds of seed sludge (Vazquez-Padin et al. 2009). Nevertheless, taking out a good deal of sludge from a running Anammox reactor could affect the denitrification performance substantially and even cause collapse of the system. A better strategy, which could reduce the impact on the reactor, was recommended: to remove a smaller amount of sludge more frequently. Under these circumstances, the sludge had to be preserved at a low temperature in a method referred to as stored Anammox sludge (SAS), otherwise the Anammox bacteria would self-degrade rapidly. Consequently, evaluating SAS as seed sludge was a key point for application of the Anammox process.

In this study, SAS that had been kept in a refrigerator with a low temperature (4°C) was employed as seed sludge to start an expanded granular sludge bed (EGSB) reactor. Gene analysis on the 16S rDNA gene was applied to analyze the microbial population shift in the reactor.

MATERIALS AND METHODS

Anammox reactor and feed media

The reactor had an inner diameter of 14 cm with a total liquid volume of 10 L including a reaction section of 8 L and a settling zone of 2 L. The reactor was made of acrylic resin and was equipped with a water jacket for temperature control and was maintained at about 35 ± 1°C. Sampling
ports were located at heights of 3, 17, 20 and 25 cm above the bottom of the reactor. Part of the effluent was collected in one recycling vessel with mixer and heating equipment. The total volume of the recycling vessel was 5 L. The pH was controlled by one pH online controller (TPH/T-10, LongerPump, China) with 0.5 mol/L H₂SO₄. The reactor was enclosed in a black-vinyl sheet to inhibit the growth of photosynthetic bacteria.

The reactor was operated in up-flow mode, with influent introduced at the bottom by a peristaltic pump (BT100-2J, LongerPump, China). A recirculation pump (BT600-2J, LongerPump, China) was set to dilute the influent with the recycling water in the recycling vessel.

The reactor was fed with synthetic inorganic wastewater with a NO₂⁻-N to NH₄⁺-N molar ratio of 1.0–1.2. The Anammox nutrient medium consisted of 40–120 mg-N/L (NH₄)₂SO₄, 40–150 mg-N/L NaNO₂, 1,500 mg/L KHCO₃, 50 mg/L K₃PO₄, 200 mg/L MgSO₄·7H₂O, 226 mg/L CaCl₂·2H₂O, and 1 mL of trace element solution I and II (Zhang et al. 2010). The influent storage tank was flushed with nitrogen gas to maintain dissolved oxygen (DO) concentration under 0.5 mg/L, then Na₂SO₃ with the concentration of 40 mg/L (proven to be harmless to Ana-mox bacteria, data not shown) was induced to keep DO at nearly zero.

**Stored Anammox sludge**

In this study, the SAS, which was provided by Kumamoto University (Zhang et al., 2010), had been kept in a refrigerator with a low temperature (4 °C) for nearly 2 years. Then, 2 L SAS was added into the EGSB reactor. The mixed liquor suspended solids (MLSS) and mixed liquor volatile suspended solids (MLVSS) in the reactor were 4.0 and 3.0 g/L, respectively. Before loading, sludge activity was measured in batch test mode.

**Analytical method**

NO₂⁻-N and TN were measured by a colorimetric method according to *Standard Methods* (APHA 1995). Mixed liquor suspended solids and suspended solids (SS) were measured in accordance with *Standard Methods* (APHA 1995). NH₄⁺-N was measured by the Nessler’s reagent spectrophotometry (water quality-determination of ammonia nitrogen-Nessler’s reagent spectrophotometry (HJ 535-2009)). NO₃⁻-N was calculated by total nitrogen (TN) and NO₂⁻-N. The pH and DO were measured using a pH meter (9010, Jenco, USA) and a DO meter (6010, Jenco, USA), respectively.

**Batch experiments for Anammox sludge activity measurement**

To estimate the activity of Anammox sludges, batch experiments were carried out in 250 mL conical flasks. The conical flasks were sealed with butyl-rubber stoppers and purged with N₂ gas to remove oxygen. Intensive magnetic stirring was adopted to reduce mass transfer limitation (Okabe et al. 2011). The Anammox nutrient medium consisted of 20 mg-N/L (NH₄)₂SO₄, 25 mg-N/L NaNO₂. The pH of the medium was adjusted at 7.3. The bottles were then incubated at 35 °C. Gas and medium samples were taken for chemical analyses at appropriate time intervals. Since nitrite might be utilized by denitrification bacteria, ammonium was preferred to evaluate the Anammox sludge activity.

**DNA extraction and high-throughput 16s rRNA gene pyrosequencing**

After 139 days of operation, the particle-based granules were taken out from the Anammox reactor. A granular sludge sample was first ground with a pestle under liquid nitrogen. Meta-genomic DNA was extracted using the E.Z.N.A. Soil DNA Kit (OMEGA Biotec. D5625-01, USA) according to the manufacturer’s instructions. Amplification of the 16S rRNA gene was performed using primers 27F (forward primer: 5'-AGAGTTTGATCCTGGCTCAG-3') and 533R (reverse primer: 5'-TTACCGCGGCTGCTGGCAC-3'). Polymerase chain reaction (PCR) was carried out according to the following thermocycling parameters: 120 s initial denaturation at 95 °C, 25 cycles of 30 s at 95 °C, 30 s at 55 °C, 30 s at 72 °C, 5 min final elongation at 72 °C, 10 °C until halted by user. Duplicate PCR products were pooled and purified using the AXYGEN gel extraction kit (Axogen, USA) (Feng et al. 2012).

Pyrosequencing was carried out using a 454 Life Sciences Genome Sequencer FLX Titanium instrument (Roche). Sequences were clustered into operational taxonomic units (OTUs) by setting a 0.03 distance limit (equivalent to 97% similarity) using the MOTHUR program.

**RESULTS AND DISCUSSION**

**Reactor performance**

Phase 1 (day 0 to day 18) in Figure 1 represents the treatment performances for the Anammox reactor during the recovering period. The initial concentration of influent
NH$_4$-N and NO$_2$-N were controlled at 40 mg/L (Figure 1). On day 1, the removal efficiencies of NH$_4$-N, NO$_2$-N and TN were 66.79, 92.00 and 73.83%, respectively. The conversion ratio of NO$_2$-N and NH$_4$-N was 1.65, which shows some variance with the expected stoichiometric ratio of 1.32 (Strous et al. 1998). A progressive decline in effluent NH$_4$-N and NO$_2$-N was observed during the first 5 days. On day 6, the concentrations of influent NH$_4$-N and NO$_2$-N were boosted to 70 and 75 mg/L. On day 14, the concentrations of influent NH$_4$-N and NO$_2$-N were gradually increased to 120 and 150 mg N/L, respectively, corresponding to an increase of nitrogen loading rate (NLR) from 1.00 to 1.08 g/L/d. Three days later, the removal efficiencies of NH$_4$-N and NO$_2$-N stabilized at 90.56 and 99.25%. In the meantime, the conversion ratio of NO$_2$-N and NH$_4$-N decreased to 1.30; it can be deduced that the Anammox bacteria had completely recovered and prevailed in the reactor.

During the steady phase, NH$_4$-N and NO$_2$-N concentrations were raised to 150 and 150 mg/L on day 18. The removal efficiencies of NH$_4$-N and NO$_2$-N were steadily enhanced and finally stabilized at above 93 and 99%. The average conversion ratio of NO$_2$-N and NH$_4$-N was about 1.33, which was very close to the theoretical ratio, indicating that the start-up of the EGSB reactor was achieved successfully. During this study, DO was controlled below 0.20 mg/L and no adverse impact was observed in the reactor.

**Sludge characteristics**

It is reported that the red color of mature Anammox bacteria is attributed to the red pigment involved (Chen et al. 2011; Tang et al. 2011). The red color could be deemed as the indicator of Anammox accumulation. In this study, the biomass turned to round reddish granular sludge on day 34 from irregular brown floc on day 1. The evolution of sludge appearance in this study was exactly similar with previous studies (Li et al. 2012).

To determine the Anammox sludge activity, batch experiments were carried out by using (1) before stored, (2) after preserved, and (3) after 33-day start-up Anammox sludges (Figure 2). The results showed that Anammox sludge activity was only 1–2% of the original value (390 mg NH$_4$-N/g MLVSS/d on average) after 2 years preservation. With the after 33-day start-up, the activity could quickly recover from 3.8 to 40 mg NH$_4$-N/g MLVSS/d on average.

**Bacterial diversity**

As shown in Figure 3, bacterial diversity was investigated on day 1 and on day 34, respectively. The pie graph indicates that, at the beginning of the start-up, other kinds of bacteria and no rank bacteria comprised 30.8 and 38.4% of the community population, respectively. Along with the process of start-up, the values dropped to 12.34 and 8.01%. In this study, the seed sludge used in the Anammox reactor was inoculated from a 50-L reactor with a non-woven biocarrier (Zhang et al. 2010), which was known to be clustered with...
KSU-1 and KU-2 strains. After 45 days of cultivation with synthetic wastewater, *Candidatus Kuenenia stuttgartiensis* appeared and dominated in the reactor, while the initial KSU-1 and KU-2 strains were rarely detected. The phylogenetic analysis showed that about 59.24% in genus level was identified as *Paenisporosarcina*. It could be concluded that *Paenisporosarcina* might also contain a strain (known as *Paenisporosarcina* uncultured bacterium), which is similar to the Anammox bacteria.

*Zhang et al.* (2010) studied the Anammox community shift using synthetic wastewater and digester liquor. A shift occurred in the Anammox bacteria between the KSU-1 strains and the new species (*kumadai-1*) during the real wastewater period. It seems that the bacteria species was based on the cultural condition. In this study, tap water was used for making synthetic wastewater, while groundwater had been used in earlier studies. Because of this variation, some trace components in the tap water used in

Figure 3 | Bacterial diversity on day 1 (a) and day 34 (b).
this study might give Candidatus Kuenenia stuttgartiensis a competitive advantage over KSU-1 and KU-2. In addition, an EGSB reactor with pH adjuster was used in this study, while the reactor used in former studies was operated without recirculation and pH control. Microbial community shift is a really complicated process, and it requires further research to identify the exact mechanism. Furthermore the shift of the Anammox community indicates that Candidatus Kuenenia stuttgartiensis has great potential to adapt the cultural condition in this study.

The comparison of the start-up performance of different Anammox processes was summarized in Table 1. In most cases, a lengthy hydraulic retention time (HRT) was adopted in order to retain enough sludge in the Anammox reactors. In this study, HRT was shortened at 6 h. Compared with other seed sludge, seeding with SAS could decrease the start-up time of the Anammox process greatly (from 200–360 days to one month). Furthermore, only a little floating sludge was observed during the study period, while its occurrence was more general in many other studies (Araujo et al. 2011). It might be contributed by SAS, which was employed as seed sludge, and then the settling ability of biomass could be enhanced.

In this study, a doubling time of 7–13 days could be estimated, which coincided with the reported doubling time of 11 days (Strous et al. 1998). As shown in Table 1, compared with previous studies, the doubling time of SAS was relatively short (36–68% of the normal value). This means that the increasing of Anammox sludge might be accomplished in a reduced time. It could be concluded that SAS could be used as seed sludge of for the Anammox reactor, thus accelerating the start-up period.

CONCLUSIONS

The Anammox process was successfully started-up in an EGSB reactor using SAS as seed sludge within 34 days. The influent concentrations of NH₄-N and NO₂-N were gradually increased from 40 mg N/L to 150 and 150 mg N/L, respectively, corresponding to an increase of NLR from 0.32 to 1.12 g/L/d. The maximum removal efficiency of TN of 87.93% was obtained on day 32 with NLR of 1.12 g/L/d. In conclusion, employing SAS as seed sludge could successfully start the Anammox reactor and largely shorten the start-up period.

ACKNOWLEDGEMENTS

This research was supported by the National Natural Science Foundation of China (No. 51108108), Guangxi Natural Science Foundation (2013GXNSFCA019018), Guangxi Science and Technology Development Project (1298014-14).

REFERENCES


First received 15 September 2013; accepted in revised form 7 January 2014. Available online 25 January 2014