Start-up and Nitrogen Removal Performance of DAMO-anammox Inoculated with Anaerobic Granular Sludge in MBfR

Xiaoting Guo, Ying Shi, Xiaojing Yao
School of Resources and Safety Engineering, Central South University, Changsha, 410083, China
E-mail: 2316030969@qq.com

Abstract. This study investigated the rapid start-up of the DAMO-anammox strategy using inoculum of anaerobic granular sludge in MBfR. The results showed that the reactor containing DAMO microorganisms was rapidly and successfully started up after 73 days with only feeding nitrate. Anammox microorganisms were obtained after 1.5-month of ammonium addition. The nitrogen removal rate achieved 603.9 mg N/L/d in this study. Microbial analysis results suggested that the newly discovered DAMO archaea, DAMO bacteria and anammox bacteria were not detected in this enriched culture. The DAMO-anammox process may be carried out by other microbes. This deduction remains to be further established in the future.

1. Introduction
Nitrogen pollution resulting from excessive discharge of domestic and municipal wastewater to the water bodies has incurred a great concern of society, thus increasing demand for more effective and economical technology for nitrogen removal. Recently, more attention has been focused on a novel and cost-effective biological nitrogen removal process namely anaerobic ammonium oxidation (anammox) integrating with denitrifying anaerobic methane oxidation(DAMO)process[1-3]. With methane as the sole electron donor, DAMO archaea reduced the nitrate produced by anammox to the nitrite, the nitrite generated by DAMO archaea was utilized by anammox and DAMO bacteria under anoxic condition. As a result, NO$_3^-$/NO$_2^-$ were reduced to N$_2$ and CH$_4$ was oxidised to CO$_2$. This largely save the operational costs for wastewater treatment due to no requirement of aeration, additional carbon sources supply compared to conventional microbial nitrogen removal processes[4, 5]. To date, several cultures containing DAMO-anammox microorganisms were obtained from freshwater sediment[6], anammox granules[7], enriched culture of DAMO microorganisms or co-culture of DAMO-anammox microorganisms[1, 8], a mixture of methanogenic sludge and activated sludge[9]. However, the enrichment of DAMO-anammox consortia is a lengthy process(4.5-9months), which restricted its application to wastewater treatment. In this case, an alternative seed source should be used for shortening enrichment time of DAMO-anammox microorganisms. The previous work has showed that membrane biofilm reactor (MBfR) was a powerful way to enrich co-culture of DAMO-anammox microorganisms for its developing and retaining high concentrations of extremely slow growing microorganisms[1, 6]. The aim of this study was to hasten the start-up of DAMO-anammox process by inoculating anaerobic granular sludge in the MBfR.
2. Materials and methods

2.1. Reactor set-up
The MBfR system for enrichment of co-culture of DAMO and anammox microorganisms was set up (Fig. 1). The total volume of the membrane module was 60 mL with 1 mL of hollow fibres, 13 mL interior space for gas supply and 45 mL external space outside the fibres for liquid, respectively. Feeding gas (99.99%, CH₄) was supplied into the interior of hollow fibres. Liquid in the reactor was recirculated by a peristaltic pump and gas pressure was maintained by a pressure stabilizing valve.

![Figure 1. Schematic diagram of the DAMO-anammox reactor](image)

2.2. Inoculum and medium
Anaerobic granular sludge was sampled from a domestic wastewater treatment plant in Changsha, China. A synthetic medium contained the following compounds: NaHCO₃ (0.5 g L⁻¹), KH₂PO₄ (0.05 g L⁻¹), CaCl₂·2H₂O (0.3 g L⁻¹), MgSO₄·7 H₂O (0.3 g L⁻¹), an acidic trace element solution (1.0 mL/L) and an alkaline trace element solution (1.0 mL/L). The acidic trace element solution and the alkaline trace element solution were prepared as described in previous study[10].

2.3. Operations
The operation of the reactor consisted of four stages: stage 1 (Day 0–193), stage 2 (Day 194–292), stage 3 (Day 293–340), and stage 4 (Day 341–603). During stage 1, no medium was exchanged into the system to make the biomass attached to the surface of membrane biofilms for initial two weeks. Subsequently, the MBfR was operated under SBR and only nitrate feeding was added to the medium for enrichment of DAMO microorganisms. During stage 3, the medium of the influent contained 200 mg N/L nitrate and gradually increased to 300 mg N/L nitrate. During stage 3, 200 mg N/L ammonium and 200 mg N/L nitrate was added to reactor for enrichment of anammox bacteria. During stage 4, the MBfR was operated as SBR with cycle time of 3.5d (Day 341–576) and 2.5d (Day 577–603). The temperature was maintained at 25ºC. pH value was controlled at 7.0–7.5 by adding 1M HCL and 1M NaOH. To prevent the air into the system, gas (99.99% N₂) was used to sparge the reactor regularly. The partial pressure of CH₄ maintained at approximately 20 KPa.

2.4. Analytical methods
The concentrations of NH₄⁺, NO₃⁻, NO₂⁻ were measured with ammonium molybdate tetrahydrate spectrophotometry (UV1800, shimadzu, Japan). The consumption rates (rNH₄⁺, rNO₃⁻) were determined through linear regression of their concentration profiles.
2.5. Microbial analysis

Fast DNA SPIN Kit for Soil was used to extract total DNA. Universal primers (341b4F-806R) was used for the PCR amplifications with high-throughput Illumina MiSeq PE 300. The methods were described in previous study [11].

3. Results

3.1. Fast start-up of DAMO process

As is shown in Fig. 2, during the stage 1, nitrate was only supplied into the reactor. The nitrate removal rate closed to zero for initial two weeks. For the next one month, nitrate concentration was fluctuated, and nitrate removal rate increased gradually. From Day 50-72, the nitrate concentration stepwise decreased and nitrate removal rate reached 42.9 mg N/L/d. Ondwards Day 73, obvious removal rate of nitrate was obtained. During stage 2, the reactor was operated as fed-batch mode at an HRT of 3.5d with the influent only containing nitrate. Stable performance was achieved after Day 208. On Day 245, the influent nitrate concentration increased to 300 mg N/L/d. The nitrate remained in the range of 53.6-71.3 mg N/L/d, giving rise to increasing improvement of nitrate removal rate. Nitrite concentration was undetectable level during the operation.

To verify the occurrence of DAMO process, a batch test on Day 227 to Day 230 without methane feeding was performed with the results shown in Fig. 3. Without CH4 supply, the nitrate reduction rate was about 2.7 mg N/L/d compared to 36.3 mg N/L/d with CH4 supply, suggesting that the DAMO microorganisms were responsible for nitrate reduction.

3.2. Enrichment of co-culture DAMO-anammox microorganisms

As is shown in Fig. 4, during stage 3, ammonium and nitrate were supplied into the reactor for enrichment of anammox bacteria with a constant HRT of 3.5d. The effluent ammonium concentration was fluctuated and even higher than the influent ammonium concentration for initial 10 days. The impossible reasons may be attributed to cell lysis and breakdown of organic nitrogen to ammonium, causing high effluent concentration of ammonium. After Day 333, the ammonium concentration in the effluent decreased slowly to 181.3 mg N/L/d with ammonium removal rate of 54.2 mg N/L/d at the end of stage 3, which indicated the realization of anammox. At the same time, the nitrate concentration in the effluent increased to 23 mg N/L on Day 336, giving rising to the nitrate removal efficiency of 85%. From Day 343, nitrate concentration of the influent increased to 300 mg N/L, and
the nitrate concentration in the effluent rapidly increased to 179.8 mg N/L/d, which resulted in nitrate removal ratio decreasing by ~45%. During the last stage 3, removal rate of nitrate arrived at 256.3 mg N/L/d.

During stage 4, the ammonium concentration of the effluent gradually decreased to 119 mg N/L, which resulted in the removal rate of ammonium increasing to 126 mg N/L/d on Day 413. The nitrate concentration maintained relatively stable at about 166 mg N/L after one-month adaption of change of nitrate concentration and then decreased stepwise from 173.6 to 103 mg N/L. From Day 434-513, nitrate and ammonium concentration in the effluent fluctuated in the range of 230-247 mg N/L and 104.1-137.63 mg N/L with 300 mg N/L ammonium and 300 mg N/L nitrate in the influent, respectively. To improve the quality of the effluent, the influent containing nitrate and ammonium decreased to 150 mg N/L. The effluent ammonium concentration gradually decreased to 74.6 mg N/L on Day 571. Meanwhile, the effluent ammonium concentration further dropped to 0.2 mg N/L on Day 566. To further increase nitrogen loading rate, the HRT was decreased to 2.5d. Following the decrease of HRT, the effluent nitrate and ammonium concentration increased from 0.2 to 69.6 mg N/L and 47.6 to 107.2 mg N/L temporarily. But after two-week adaption, the effluent nitrate and ammonium concentration kept relatively stable at approximately 51.5 mg N/L and 81.5 mg N/L. The ammonium and nitrate removal rates in the end of this phase were 244.7 and 301mg N/L/d, respectively.

3.3. Nitrogen performance of DAMO-anammox

Fig.5 showed nitrate and ammonium removal rates from Day 520 to 603 with 150 mg N/L nitrate and ammonium in the influent at HRT of 2.5d. The nitrate and ammonium removal rates gradually increased and arrived at 318.9 mg N/L/d and 285 mg N/L/d, accounting for 603.9 mg N/L/d of nitrogen removal rate in the end of experiment.

3.4. Microbial analysis

As was shown in Fig.6. After 12-month enrichment, archaea and bacteria accounted for 5.51% and 94.49%. In the phylum level, the dominant microbials in the reactor were Proteobacteria(60.74%), Bacteroidetes(8.8%), Planctomycetes(7.6%), Euryarchaeota(4.23%), Actinobacteria(4.1%), Gemmatimonadetes(3.61%), Chlamydiae(3.6%), Choroflexi(3.1%). In the order level, the dominant microbes in the reactor contained Rhizobiales, Xanthomonadales, Phycisphaerales with the relative abundance of 34.79%, 9.19% and 5.76%, respectively. However, the currently known DAMO archaea, DAMO bacteria and anammox bacteria were not found in this enrichment culture. But Methanosarcinales with the relative abundance of 1.94% which have been enriched in several DAMO culture under feeding.
nitrate and methane[12], were detected in this biofilm. Furthermore, the Phycisphaerae class containing Phycisphaerales order have been found in the anammox enrichment and several DAMO cultures[2].

![Figure 6](image-url)

Figure 6. Microbial distributions of the enriched culture at the Phylum level

![Figure 7](image-url)

Figure 7. Microbial distributions of the enriched culture at the Order level

4. Discussion
The DAMO process occurred after the enrichment of 73 days with nitrate removal rate of 42.9 mg N/L/d. The enrichment time of DAMO microorganisms was relatively short compared to the previous studies(3-18month). This indicated that the MBfR was a good system to enrich DAMO microorganisms. After 1.5 months of feeding ammonium, the decrease of ammonium concentration was obviously observed. This suggested that the formation of DAMO microorganisms could promote the development of anammox bacteria. In this study, simultaneous decrease of the nitrate and ammonium concentrations and undetectable level of nitrite in the effluent showed that the partial nitrite generated from nitrate reduction by DAMO archaea was consumed by anammox bacteria. The high-throughout sequencing analysis showed that the currently known DAMO archaea, DAMO bacteria and anammox bacteria were not found in this enrichment culture. But the Methanosarcinales, which have been enriched in several DAMO culture under feeding nitrate and methane[12], were detected in this reactor. Furthermore, the Phycisphaerae Class containing Phycisphaerales Order were found in the anammox enrichment and several DAMO cultures. Based on the experimental data and high-throghout sequencing results, we deduced that the DAMO-anammox process may be accomplished by other microbes. This deduction remains to be further elucidated in the future.
5. Conclusion
The MBfR inoculated with anaerobic granular sludge showed an excellent performance for its fast start-up DAMO-anammox process and obtaining high TN removal rate. The DAMO process occurred only after 73 days. By adding ammonium, anammox microorganisms were obtained after 1.5-month. The nitrogen removal rate achieved 603.9 mg N/L/d in this study. Microbial analysis suggested that the discovered DAMO archaea, DAMO bacteria and anammox bacteria were not detected in this enriched culture. The DAMO-anammox process may be carried out by other microbes. This deduction remains to be further established in the future.

Acknowledgement
This work was sponsored by Project of Natural Science Foundation of China (No. 51608536).

References
[1] Shi Y, Hu S, Lou J, Lu P, Keller J and Yuan Z 2013 Nitrogen removal from wastewater by coupling anammox and methane-dependent denitrification in a membrane biofilm reactor Environ. Sci. Technol. 47 11577-82.
[2] Xie G-J, Cai C, Hu S and Yuan Z 2017 Complete nitrogen removal from synthetic anaerobic sludge digestion liquor through integrating anammox and denitrifying anaerobic methane oxidation in a membrane biofilm reactor Environ. Sci. Technol. 51 819-27.
[3] Xie G-J, Liu T, Cai C, Hu S and Yuan Z 2018 Achieving high-level nitrogen removal in mainstream by coupling anammox with denitrifying anaerobic methane oxidation in a membrane biofilm reactor Water. Res. 131 196-204.
[4] van Kessel M A H J, Stultiens K, Slegers M F W, Guerrero Cruz S, Jetten M S M, Kartal B and Op den Camp H J M 2018 Current perspectives on the application of N-damo and anammox in wastewater treatment Curr. Opin. Biotechnol. 50 222-27.
[5] Bagchi S, Biswas R and Nandy T 2012 Autotrophic Ammonia Removal Processes: Ecology to Technology Critical Reviews in Environmental Science and Technology 42 1353-418.
[6] Ding Z W, Lu Y Z, Fu L, Ding J and Zeng R J 2017 Simultaneous enrichment of denitrifying anaerobic methane-oxidizing microorganisms and anammox bacteria in a hollow-fiber membrane biofilm reactor Appl. Microbiol. Biotechnol. 101 437-46.
[7] Zhu B, Sánchez J, van Alen T A, Sanabria J, Jetten M, Ettwig K F and Kartal B 2011 Combined anaerobic ammonium and methane oxidation for nitrogen and methane removal Biochem. Soc. Trans. 39 1822.
[8] Luesken F A, Sanchez J, van Alen T A, Sanabria J, Op den Camp H J M, Jetten M S M and Kartal B 2011 Simultaneous nitrite-dependent anaerobic methane and ammonium oxidation Appl. Environ. Microbiol. 77 6802-07.
[9] Ding Z-W, Ding J, Fu L, Zhang F and Zeng R 2014 Simultaneous enrichment of denitrifying methanotrophs and anammox bacteria Appl. Microbiol. Biotechnol. 98 10211-21.
[10] Ettwig K F, van Alen T, van de Pas-Schoonen K T, Jetten M S M and Strous M 2009 Enrichment and molecular detection of Denitrifying methanotrophic bacteria of the NC10 phylum Appl. Environ. Microbiol. 75 3656-62.
[11] Lu Y-Z, Ding Z-W, Ding J, Fu L and Zeng R J 2015 Design and evaluation of universal 16S rRNA gene primers for high-throughput sequencing to simultaneously detect DAMO microbes and anammox bacteria Water. Res. 87 385-94.
[12] Wang S, Wu Q, Lei T, Peng L and Xia H 2015 Enrichment of denitrifying methanotrophic bacteria from Taihu sediments by a membrane biofilm bioreactor at ambient temperature Environ. Sci. Pollut. Res. Int. 23 5627-34.