Enrichment and characterization of Anammox bacteria in a non-woven membrane reactor

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ABSTRACT

An upflow column reactor packed with nonwoven fabric carrier was used to enrich anaerobic ammonium oxidation (Anammox) sludge. After 101 days, the reactor Anammox sludge concentration increased from 470 to 3,118 mg·L⁻¹. In the stable operating stage, the average total nitrogen (TN) volume loading rate was 818.3 mg·L⁻¹, and the maximum removal efficiencies of NH₄⁺-N, NO₂⁻-N and TN were 65.9, 81.2 and 63.8%, respectively. Scanning electron microscopy (SEM) showed that the cultivated sludge was dominated by a mix of short rod-shaped and spherical bacteria, which accumulated to the typical cauliflower-like aggregates assumed to be the Anammox culture. Fluorescence in situ hybridization (FISH) analysis using 16S rRNA showed that the dominant population developed in the reactor when hybridized with both PLA46 and Amx820 gene probes. This indicates that the cultivated biomass may comprise Planctomycetes bacteria. The results of real-time quantitative PCR (qPCR) showed that these bacteria formed 45 to 60% of the total bacteria in the Anammox sludge. The study demonstrated successful detection and enrichment of Anammox bacteria in wastewater.

Key words: Anammox bacteria, enrichment, FISH, nonwoven fabric carrier, qPCR, SEM

HIGHLIGHTS

- Anammox bacteria was successfully enriched by a designed non-woven membrane reactor.
- The maximum TN removal rate was 63.8% in stable operation.
- The SEM and FISH analysis showed Anammox bacteria coexisted with the others as the dominant population.
- The qPCR results showed a 45% to 60% proportion of Planctomycetes in the culture.
1. INTRODUCTION

Removing the nitrogen components from wastewater is important since they can cause eutrophication in waters receiving them, giving rise, potentially, to aquatic ecosystem deterioration and/or human health issues (Moghaddam & Sargolzaei 2013; Tedengren 2021). Conventional processes for biological nitrogen removal involve two principal steps, nitrification and denitrification. However, these processes have several problems, including system complexity, large environmental footprints, and high operating costs. Recently, a promising cost-effective method for ammonium removal from wastewater, referred to as anaerobic ammonium oxidation (Anammox), has been developed (van de Graaf et al. 1996). Anammox includes a partial nitrification step, and requires only half of the ammonium to be nitrified to nitrite, while the remainder is converted subsequently into nitrogen. Anammox bacteria, which are related to five Planctomycetes genus and branched off as a monophyletic cluster, can oxidize ammonium under anoxic conditions, using nitrite as the electron acceptor, to produce nitrogen gas (Kartal et al. 2013; Yang et al. 2020). Recently, Zhao et al. (2019) confirmed that marine eutrophication could exacerbate Anammox bacteria growth.

The Anammox process is not generally considered suitable for practical applications due to its low growth rate (1 to 2 weeks) and biomass (Awata et al. 2013; Ali et al. 2015). Various bioreactors types have been used to enrich Anammox microorganisms, including the fluidized (or fixed) bed reactor, sequencing batch reactor (SBR), membrane bioreactor (MBR), up-flow anaerobic bioreactor (UAB), continuous stirred-tank reactor (CSTR) and others (Scaglione et al. 2015; Ji et al. 2019; Zhang & Okabe 2020). Non-woven carriers have been applied successfully to reactors both for starting up and the long-term operation of the Anammox process (Wang et al. 2016).

In this study, Anammox bacteria were enriched by connecting an external set of non-woven membrane modules with an anaerobic reactor. The primary objective was to develop a cost-effective reactor configuration capable of enriching Anammox bacteria for practical application. Fluorescence in situ hybridization (FISH) and scanning electron microscopy (SEM) were used to confirm successful Anammox enrichment.

2. MATERIALS AND METHODS

2.1. Reactor setup and Anammox enrichment

A 1.5 L glass Anammox up-flow column reactor, packed with polyester non-woven biomass carrier and sealed to maintain an anaerobic environment, was used for enrichment – see Figure 1. Reactor pH was maintained...
between 7.5 and 8.0 with NaHCO₃ solution. Several outlets were drilled to collect gas, sludge, and other samples. The Anammox biomass used for inoculation came from a column reactor. The main characteristics of the biomass were mixed liquor suspended solids (MLSS) 4 g·L⁻¹, mixed liquor volatile suspended solids (MLVSS) 3.2 g·L⁻¹, and MLVSS/MLSS 0.8.

The influent medium consisted of 200 mg NH₄⁺-N in the form of a solution of 1.89 g (NH₄)₂SO₄, 1.25 g KHCO₃, 0.025 g KH₂PO₄, 0.3 g CaCl₂·2H₂O, 0.2 g MgSO₄·7H₂O, 0.00625 g FeSO₄, 0.00625 g EDTA per liter, together with trace elements.

2.2. Chemical analysis of water quality

Water samples were analyzed using the standard methods for water and wastewater examination (APHA 2012). NH₄⁺-N and NO₂⁻-N were measured by different colorimetric methods, and NO₃⁻-N by ultraviolet spectrophotometry. Total nitrogen (TN) was measured using a TOC analyzer equipped with a total nitrogen-measuring unit (TOC-VCPR, Shimadzu). pH was determined potentiometrically with a portable digital pH meter, and DO with a portable digital DO meter (YSI, Model 55, USA).

Genomic DNA for PCR amplification was extracted using TIANamp Bacteria DNA Kit (Tiangen, China). The crude extract was further purified using an Agarose Gel DNA Purification Kit Ver.2.0 (TaKaRa, China), according to the manufacturer’s instructions.

The Anammox bacteria amplification was performed with Ana-F and revised Ana-R primers, and of eubacteria with 338F and 518R primers. PCR reactions were performed on 25-μL samples according to the instructions of SYBR Premix Ex TaqTM (TaKaRa, China). All PCR runs included control reactions without template DNA to test for possible non-specific amplification and all samples were run in triplicate. Standard curves for qPCR were generated via serial decimal dilutions of plasmid DNA containing specific target (Planctomycetes) gene inserts – Planctomycetes is a representative of Anammox-related bacteria – and the correlation coefficients (r²) of the standard curve were greater than 0.98.

2.3. FISH analysis and SEM observation

In situ hybridization was performed using the standard hybridization protocol (Nielsen 2009). The 16S rRNA-targeted oligonucleotide probes used are listed in Table 1. The cy3- and FITC-labeled derivatives used as probes came from TaKaRa (Dalian, China). Images were acquired with an epifluorescence microscope (Olympus BX51, Japan) together with the instrument’s standard software package (version 4.0).
The 16S rRNA-targeted oligonucleotide probes used for in situ detection of AOB and Anammox bacteria were: betaproteobacterial AOB-specific NSO190 (40% formamide length position: 190–208) labeled with Cy3, Planctomycetes-specific PLA46 (30% formamide; length position: 46–63) labeled with Cy5 and Anammox-specific AMX820 (40% formamide; length position: 820–841) labeled with Cy5. Domain-specific EUB338, EUB338-II and EUB338-III labeled with fluorescein isothiocyanate (FITC) were used to detect all bacteria in situ.

The biofilm’s morphology characteristics were observed using SEM (JEOL JSM-5600LV). The nonwoven fabric specimens for SEM were fixed with glutaraldehyde for 3 hours in paraformaldehyde solution and, subsequently, dehydrated through a graded series – 25, 50, 75, 90 and 100% – of ethanol solutions (three times for each concentration), before being gold-coated by sputtering.

3. RESULTS AND DISCUSSION

3.1. Enrichment of Anammox sludge

The reactor was operated for 101 days, which can be divided into three stages: unstable (days 0 to 14), transition (15 to 70), and effective and stable (71 to 101). All three were in accordance with previous studies (Yu et al. 2013).

During the unstable stage, NH$_4^+$-N and NO$_2^-$-N removal rates increased from 28 and 54.9% to 62.2 and 92.7%, respectively, and the TN removal rate was 58.1%. NH$_4^+$-N and NO$_2^-$-N were removed simultaneously, accompanied by the generation of NO$_3^-$-N and air bubbles, demonstrating that Anammox sludge activity was recovered in the reactor.

During the transition period, the influent nitrogen concentration was raised gradually, from 72.7 to 267.5 mg·L$^{-1}$ for NH$_4^+$-N and 75.2 to 254.5 mg·L$^{-1}$ for NO$_2^-$-N. At the same time, a corresponding improvement in volume load was also obtained, from 204.2 to 504.5 mg·TN·L$^{-1}$. The removal ratios also increased, from 54.8% to 83.7% for NH$_4^+$-N and 71 to 97% for NO$_2^-$-N, while that for TN rose from 59.6 to 84.5%. A large amount of deep red granular sludge adhered to the nonwoven fabrics, forming Anammox aggregates.

In the final stage, Anammox activity seemed stable and remarkable. The maximum removal efficiencies of NH$_4^+$-N, NO$_2^-$-N and TN were 65.9, 81.2 and 63.8%, respectively. After continuous cultivation for 101 days, the biofilm was reddish brown, and the reactor’s Anammox sludge concentration of had increased from 470 to 3,118 mg·L$^{-1}$.

The non-woven material’s specific surface and porosity were large, and the membrane module was designed to enhance microorganism retention and treatment efficiency. Non-woven material has been shown to be suitable for Anammox bacteria, which grow slowly, and is effective in maintaining high biomass retention with high effluent quality in Anammox treatment systems (Ren et al. 2018; Gu et al. 2020). During operation, the maximum TN removal rate achieved was 84.5%, almost the same level as in reports by others (Li et al. 2021).
3.2. SEM observation

Sludge samples were obtained from the reactor on day 101, and SEM used to observe how the biomass adhered to the nonwoven fabrics (Figure 3). The discontinuous floc was trapped between the fibers or attached to them. Various bacterial morphologies were observed, the main types being spherical and short rod-shaped. By eye, the sludge was reddish brown.

The SEM images of the granular sludge appeared to show many characteristics of Anammox enrichment cultures. All cells were about 1 μm in diameter and had craters on the cell wall. The accumulations showed a high degree of compactness and were cauliflower like (Wang et al. 2011; Xiong et al. 2013; Hu et al. 2018). Various other bacterial morphologies were also found in the sludge, indicating harmonious coexistence of Anammox culture with other organisms (Ren et al. 2018).

3.3. Fluorescence in situ hybridization analysis

The presence of Anammox bacteria in the enrichment culture was verified by FISH analysis. The analytical probes used are listed in Table 2. PLA46 and PLA886 target Planctomycetes bacteria, whereas AMX820 and
KST157 targets anaerobic ammonium-oxidizing bacteria (AOB), C. Brocadia anammoxidans and C. Kuenenia stuttgartiensis, while NSO190 targets AOB in the β-Proteobacteria. Most of the bacteria detected with AMX820 also hybridized with PLA46 (Figure 4), confirming the presence of Anammox bacteria and Anammox-related Planctomycetes. A few bacteria, presumably AOB, were detected with NSO190 (Figure 5). There were also other bacteria, besides Anammox bacteria and AOB, that hybridized with EUB338, although the signal was weak. This implied that Anammox and Anammox-related bacteria were dominant in the sludge’s microbial community on day 101, and coexisted with various other bacteria.

Hybridization signals found with the Amx820 probe were characteristic of Anammox cells (Liu et al. 2008). FISH analyses with Amx820 probes have been successful in detecting and observing Anammox bacteria in different environmental samples (Sánchez-Melsió et al. 2009). In the reactor, a dominant population developed and hybridized with both PLA46 and Amx820 probes, as observed Yasuhiro Date (Almstrand et al. 2014).

Figure 3 | Micrographs of the biofilm formed on the non-woven fabrics.

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Figure 3 | Micrographs of the biofilm formed on the non-woven fabrics.
Figure 4 | FISH analysis of *Planctomycetes* and Anammox bacteria in Anammox sludge. A Cy3-labelled probe AMX820; B Cy5-labelled probe PLA46; C Simultaneous staining of biomass with Cy3-labelled probe AMX820 and Cy5-labelled probe PLA46.
Figure 5 | FISH analysis of AOB in the Anammox sludge. A FITC-labelled probe NSO190; B Cy5-labelled probe EUB338 plus; C Simultaneous staining of biomass with FITC-labelled probe NSO190 and Cy5-labelled probe EUB338 plus.
3.4. Quantification of Anammox bacteria

The abundance of bacteria in the samples was estimated on the basis of 16S rRNA gene quantification using the qPCR method. The concentrations of eubacteria and Planctomycetes were $6.32 \times 10^4$ and $1.58 \times 10^4$ cells $\mu$L$^{-1}$, respectively. Assuming that the eubacteria contained 3.6 copies of 16S rRNA gene per cell genome, and Planctomycetes 1.5 to 2 copies, the Planctomycetes population was calculated as 45 to 60% of the total bacteria in the sludge. This is partially consistent with previous findings (Sobotka et al. 2017; Hu et al. 2018). The qPCR results were consistent with the FISH microscopy and confirmed Anammox bacteria as the dominant microorganisms in the reactor.

4. CONCLUSIONS

The study's purpose was to enrich Anammox bacteria in an upflow column reactor packed with a nonwoven fabric carrier, and confirm the enrichment culture using SEM and FISH analysis. After 101 days of continuous cultivation, the maximum TN removal rate of 84.5% was achieved, and a stable enrichment culture was established in the reactor. The reactor exhibited high biomass retention capability based on the nonwoven material, which provided a good environment for Anammox bacteria.

SEM observation and FISH analysis of the cultivated sludge on day 101 showed that Anammox bacteria were the dominant population, and coexisted with other bacteria within the biofilm.

Planctomycetes-related bacteria accounted for 45 to 60% of the total bacterial population after Anammox enrichment. Anammox bacteria were detected and enriched successfully.

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DATA AVAILABILITY STATEMENT

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

REFERENCES

Ali, M., Oshiki, M., Awata, T., Isobe, K., Kimura, Z., Yoshikawa, H., Hira, D., Kindaichi, T., Satoh, H., Fujii, T. & Okabe, S. 2015 Physiological characterization of anaerobic ammonium oxidizing bacterium Candidatus Jettenia caeni. Environmental Microbiology 17(6), 2172–2189.

Almstrand, R., Persson, F., Daims, H., Ekenberg, M., Christensson, M., Wilén, B.-M., Sörensson, F. & Hermansson, M. 2014 Three-dimensional stratification of bacterial biofilm populations in a moving bed biofilm reactor for nitritation-anammox. International Journal of Molecular Sciences 15(2), 2191–2206.

American Public Health Association (APHA) 2012 Standard Methods for the Examination of Water and Wastewater, 22nd edn. American Public Health Association/American Water Works Association/Water Environment Federation, Washington DC, USA.

Awata, T., Oshiki, M., Kindaichi, T., Ozaki, N., Ohashi, A. & Okabe, S. 2013 Physiological characterization of an anaerobic ammonium-oxidizing bacteria belonging to the ‘Candidatus scalindua’ group. Applied and Environmental Microbiology 79(13), 4145–4148.

Gong, Z., Liu, S., Yang, F., Bao, H. & Furukawa, K. 2008 Characterization of functional microbial community in a membrane-aerated biofilm reactor operated for completely autotrophic nitrogen removal. Bioresource Technology 99(8), 2749–2756.

Gu, W., Wang, L., Liu, Y., Liang, P., Zhang, X., Li, Y. & Huang, X. 2020 Anammox bacteria enrichment and denitrification in moving bed biofilm reactors packed with different buoyant carriers: performances and mechanisms. Science of The Total Environment 719, 137277.

Hu, Q.-Y., Kang, D., Wang, R., Ding, A.-Q., Abbas, G., Zhang, M., Qiu, L., Lu, H.-F., Lu, H.-J. & Zheng, P. 2018 Characterization of oligotrophic an AOB culture: morphological, physiological, and ecological features. Applied Microbiology and Biotechnology 102(2), 995–1003.

Ji, X., Wu, Z., Sung, S. & Lee, P.-H. 2019 Metagenomics and metatranscriptomics analyses reveal oxygen detoxification and mixotrophic potentials of an enriched anammox culture in a continuous stirred-tank reactor. Water Research 166, 115039.

Kartal, B., de Almeida, N. M., Maalcke, W. J., Op den Camp, H. J. M., Jetten, M. S. M. & Keltjens, J. T. 2013 How to make a living from anaerobic ammonium oxidation. FEMS Microbiology Reviews 37(3), 428–461.

Li, Y., Huang, X. & Li, X. 2021 Use of a packed-bed biofilm reactor to achieve rapid formation of anammox biofilms for high-rate nitrogen removal. Journal of Cleaner Production 321, 128999.

Liu, S., Yang, F., Xue, Y., Gong, Z., Chen, H., Wang, T. & Su, Z. 2008 Evaluation of oxygen adaptation and identification of functional bacteria composition for anammox consortium in non-woven biological rotating contactor. Bioresource Technology 99(17), 8273–8279.
Moghaddam, A. & Sargolzaei, J. 2013. A review over diverse methods used in nitrogen removal from wastewater. *Recent Patents on Chemical Engineering* 6(2), 133–139.

Nielsen, P. H. 2009. *FISH handbook for biological wastewater treatment.* *Water Intelligence Online* 8.

Ren, L.-F., Lv, L., Kang, Q., Gao, B., Ni, S.-Q., Chen, Y.-H. & Xu, S. 2018. Microbial dynamics of biofilm and suspended flocs in anammox membrane bioreactor: the effect of non-woven fabric membrane. *Bioresource Technology* 247, 259–266.

Sánchez-Melsió, A., Cáliz, J., Balaguer, M. D., Colprim, J. & Vila, X. 2009. Development of batch-culture enrichment coupled to molecular detection for screening of natural and man-made environments in search of anammox bacteria for N-removal bioreactors systems. *Chemosphere* 75(2), 169–179.

Scaglione, D., Ficara, E., Corbellini, V., Tornotti, G., Teli, A., Canziani, R. & Malpei, F. 2015. Autotrophic nitrogen removal by a two-step SBR process applied to mixed agro-digestate. *Bioresource Technology* 176, 98–105.

Schmid, M. C., Maas, B., Dapena, A., van de Pas-Schoonen, K., van de Vossenberg, J., Kartal, B., van Niftrik, L., Schmidt, I., Cirpus, I., Kuenen, J. G., Wagner, M., Sinninghe Damsté, J. S., Revsbech, N. P., Mendez, R., Jetten, M. S. M. & Strous, M. 2005. Biomarkers for in situ detection of anaerobic ammonium-oxidizing (Anammox) bacteria. *Applied and Environmental Microbiology* 71(4), 1677–1684.

Sobotka, D., Tusznyska, A., Kowal, P., Ciesielski, S., Czerwionka, K. & Makinia, J. 2017. Long-term performance and microbial characteristics of the anammox-enriched granular sludge cultivated in a bench-scale sequencing batch reactor. *Biochemical Engineering Journal* 120, 125–135.

Tedengren, M. 2021. Eutrophication and the disrupted nitrogen cycle: this article belongs to Ambio’s 50th anniversary collection. *Ambio* 50(4), 733–738.

van de Graaf, A. A., de Bruijn, P., Robertson, L. A., Jetten, M. S. M. & Kuenen, J. G. 1996. Autotrophic growth of anaerobic ammonium-oxidizing micro-organisms in a fluidized bed reactor. *Microbiology* 142(8), 2187–2196.

Wang, T., Zhang, H., Gao, D., Yang, F., Yang, S., Jiang, T. & Zhang, G. 2011. Enrichment of Anammox bacteria in seed sludges from different wastewater treating processes and start-up of Anammox process. *Desalination* 271(1–3), 193–198.

Wang, T., Zhang, H. & Yang, F. 2016. Performance of Anammox process and low-oxygen adaptability of Anammox biofilms in a FBR with small ring non-woven carriers. *Ecological Engineering* 86, 126–134.

Xiong, L., Wang, Y.-Y., Tang, C.-J., Chai, L.-Y., Xu, K.-Q., Song, Y.-X., Ali, M. & Zheng, P. 2013. Start-Up characteristics of a granule-based anammox UASB reactor seeded with anaerobic granular sludge. *BioMed Research International* 2013, 1–9.

Yang, Y., Li, M., Li, H., Li, X.-Y., Lin, J.-G., Denecke, M. & Gu, J.-D. 2020. Specific and effective detection of anammox bacteria using PCR primers targeting the 16S rRNA gene and functional genes. *Science of The Total Environment* 754, 139387.

Yu, Y.-C., Gao, D.-W. & Tao, Y. 2013. Anammox start-up in sequencing batch biofilm reactors using different inoculating sludge. *Applied Microbiology and Biotechnology* 97(13), 6057–6064.

Zhang, L. & Okabe, S. 2020. Ecological niche differentiation among anammox bacteria. *Water Research* 171, 115468.

Zhao, Z., Cao, Y., Fan, Y., Yang, H., Feng, X., Li, L., Zhang, H., Xing, L. & Zhao, M. 2019. Ladderane records over the last century in the East China Sea: proxies for anammox and eutrophication changes. *Water Research* 156, 297–304.

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