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

Yuhua Lyu [a], Song Yan [a,*], Zheng Gong [b] and Shoutong Zhang [a]

[a] School of Environmental and Chemical Engineering, Dalian Jiaotong University, 794 Huanghe Road, Shahekou District, Dalian 116028, China
[b] School of Life Science, Liaoning Normal University, 850 Huanghe Road, Shahekou District, Dalian 116081, China
*Corresponding author. E-mail: yansong209@sohu.com

YL, 0000-0002-4742-8579

ABSTRACT

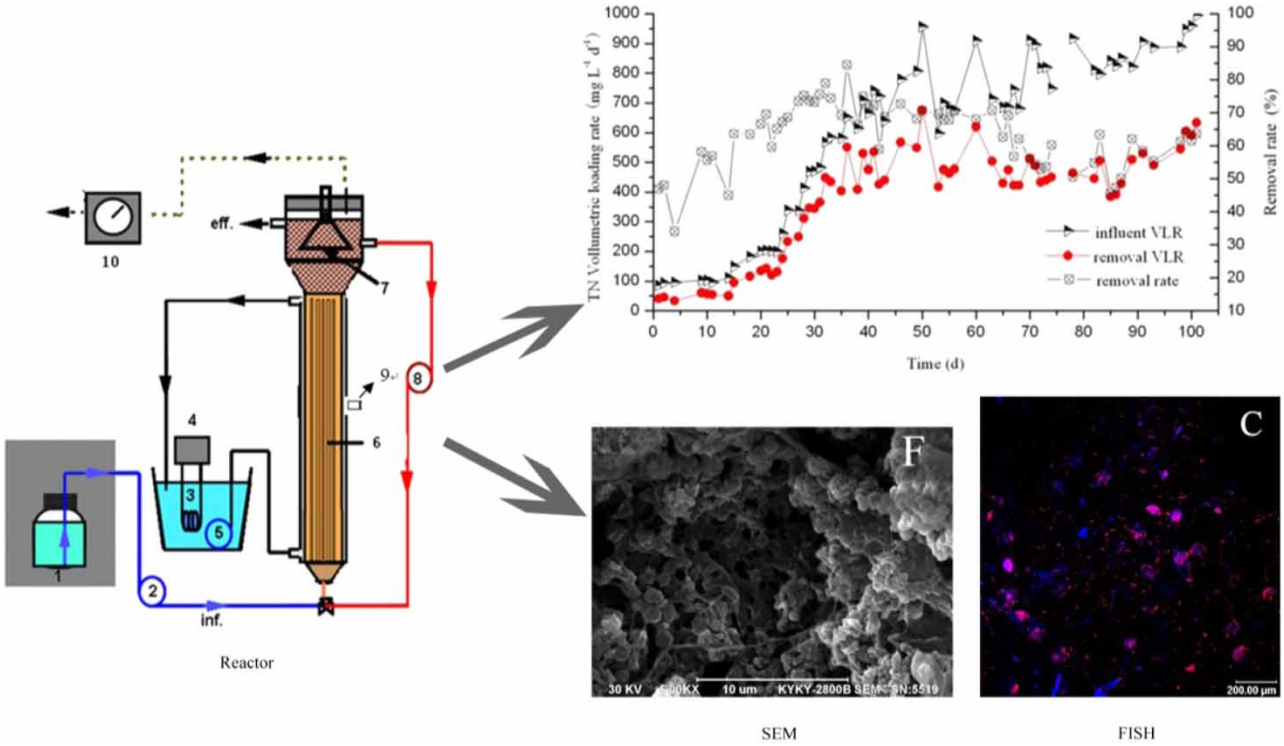
An upflow column reactor packed with nonwoven fabric carrier was used to enrich anaerobic ammonium oxidation (Anammox) sludge. After 101 days, the reactor Anammox sludge concentration increased from 470 to 3,118 mg·L^{-1}. In the stable operating stage, the average total nitrogen (TN) volume loading rate was 818.3 mg·L^{-1}, and the maximum removal efficiencies of NH_4^+-N, NO_2^--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.

GRAPHICAL ABSTRACT



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 bioreactor 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

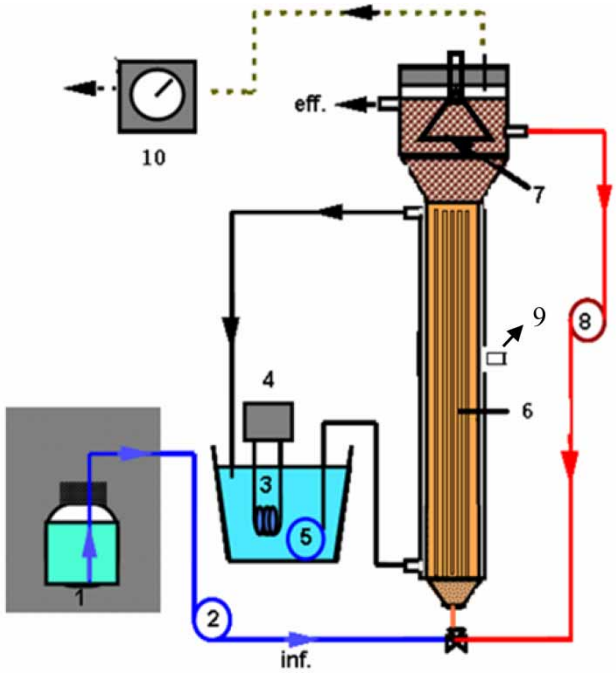


Figure 1 | Schematic of the Anammox enrichment reactor: (1) feed tank, (2) influent pump, (3) heater tank, (4) temperature controller, (5) hot water circulation pump, (6) non-woven membrane module, (7) three-phase separator, (8) circulation pump, (9) sample collection point, and (10) gas meter.

between 7.5 and 8.0 with $NaHCO_3$ 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 $g \cdot L^{-1}$, mixed liquor volatile suspended solids (MLVSS) 3.2 $g \cdot L^{-1}$, and MLVSS/MLSS 0.8.

The influent medium consisted of 200 mg NH_4^+–N in the form of a solution of 1.89 g $(NH_4)_2SO_4$, 1.25 g $KHCO_3$, 0.025 g KH_2PO_4, 0.3 g $CaCl_2 \cdot 2H_2O$, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.00625 g $FeSO_4$, 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). NH_4^+-N and NO_2^--N were measured by different colorimetric methods, and NO_3^--N by ultraviolet spectrophotometry. Total nitrogen (TN) was measured using a TOC analyzer equipped with a total nitrogen-measuring unit (TOC-VCPH, 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 (r^2) 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).

Table 1 | 16S rRNA-targeted oligonucleotide probes used in the study

Probe	Specificity	Target position (*E.coli*)[a]	%FA formamide[b]	NaCl (mM)[c]	Label	Reference
NSO190	AOB in the *β-Proteobacteria*	190–208	40	25	FITC (green)	Gong *et al.* (2008)
PLA46	*Planctomycetes*	46–63	30	159	Cy3 (red) or Cy5 (blue)	Schmid *et al.* (2005)
PLA 886	*Isosphaera*, *Gemmata*, *Pirellula*, *Plantomyces*	886–904	35	80	FITC (green)	Schmid *et al.* (2005)
AMX820	*Cand. 'Brocadia anammoxidans'* *Cand. 'Kuenenia stuttgartiensis'*	820–841	40	56	Cy3 (red) or Cy5 (blue)	Almstrand *et al.* (2014)
Kst157	*Cand. 'Kuenenia stuttgartiensis'*	157–175	25	159	FITC (green)	Schmid *et al.* (2005)
EUB338 plus	Eubacteria	338–355	20	170	Cy5 (blue)	Almstrand *et al.* (2014)

[a]16S rRNA position according to *Escherichia coli* numbering.
[b]Percentage formamide in the hybridization buffer.
[c]Millimolar concentration of sodium chloride in the washing buffer.

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 DICUSSION

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 to70), and effective and stable (71 to101). All three were in accordance with previous studies (Yu *et al.* 2013). The volumetric loading rate, removal volumetric loading rate and removal rate during these 101 days operating is represented in Figure 2.

During the unstable stage, NH_4^+-N and NO_2^--N (50 mg·L^{-1} each) were fed to the reactor. The 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 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).

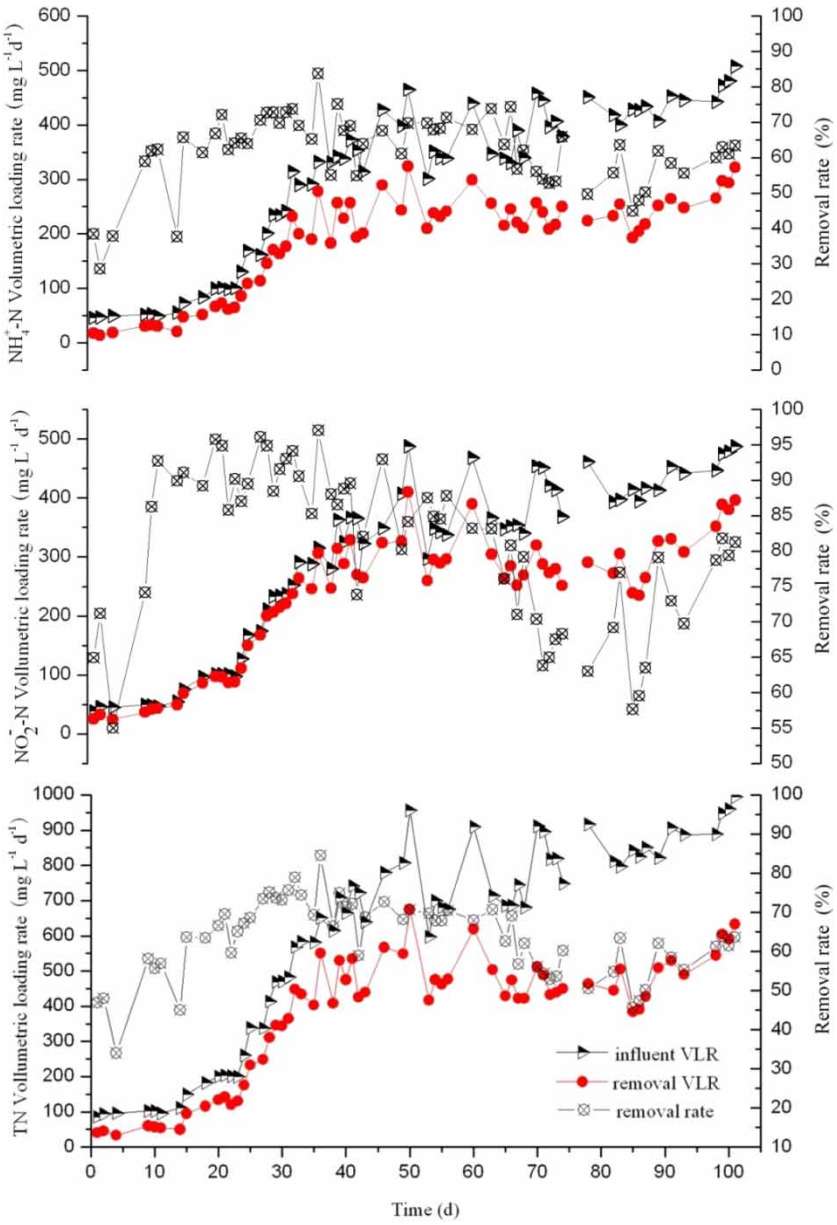


Figure 2 | Profiles of volumetric loading, removal volumetric loading, and removal rates during operation.

3.2. SEM observation

Sludge samples were obtained from the reactor on day 101, and SEM was 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 1. PLA46 and PLA886 target *Planctomycetes* bacteria, whereas AMX820 and

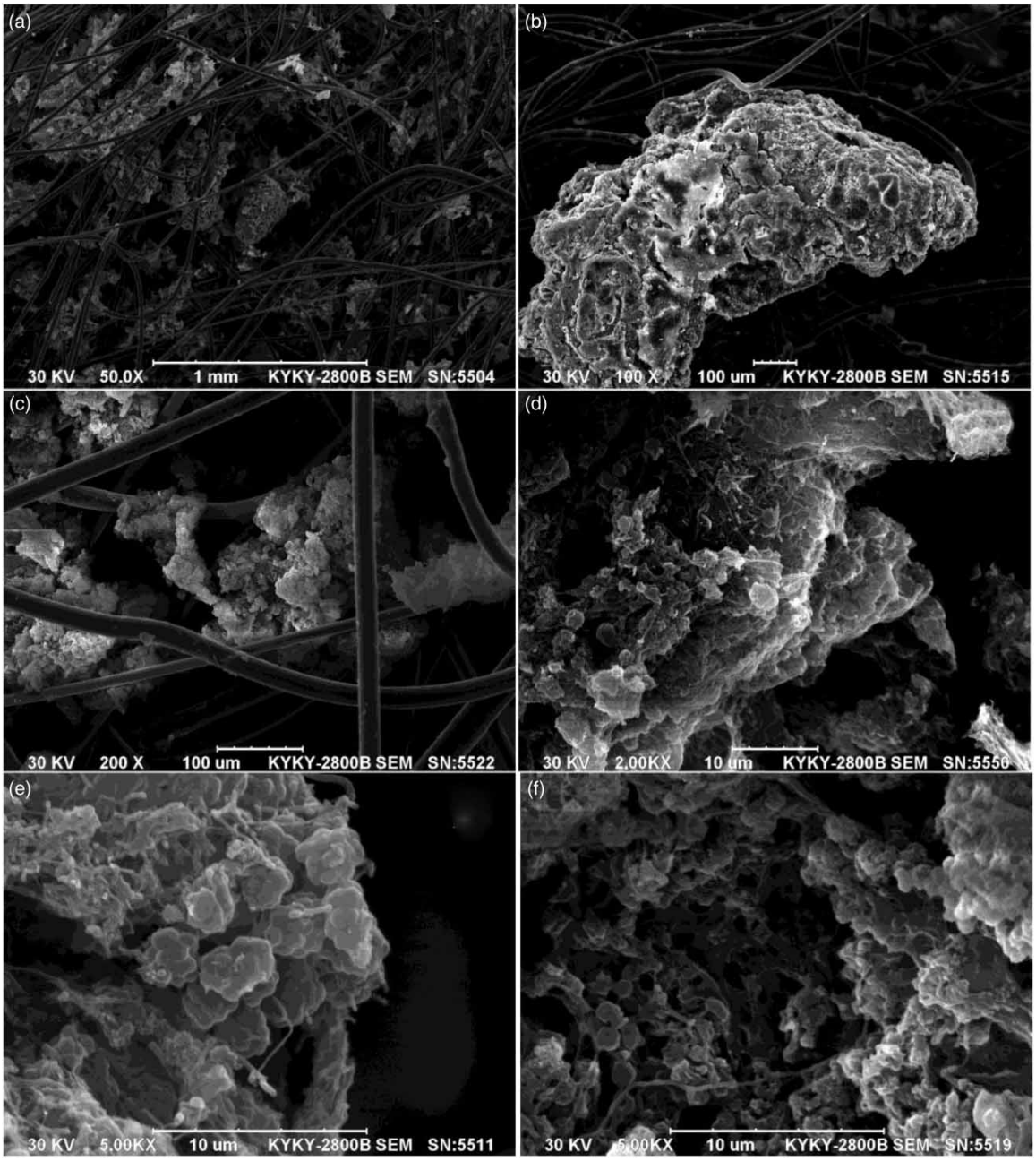


Figure 3 | Micrographs of the biofilm formed on the non-woven fabrics.

KST157 target 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 (Sànchez-Melsió *et al.* 2009). In the reactor, a dominant population developed and hybridized with both PLA46 and Amx820 probes, as observed by Yasuhiro Date (Almstrand *et al.* 2014).

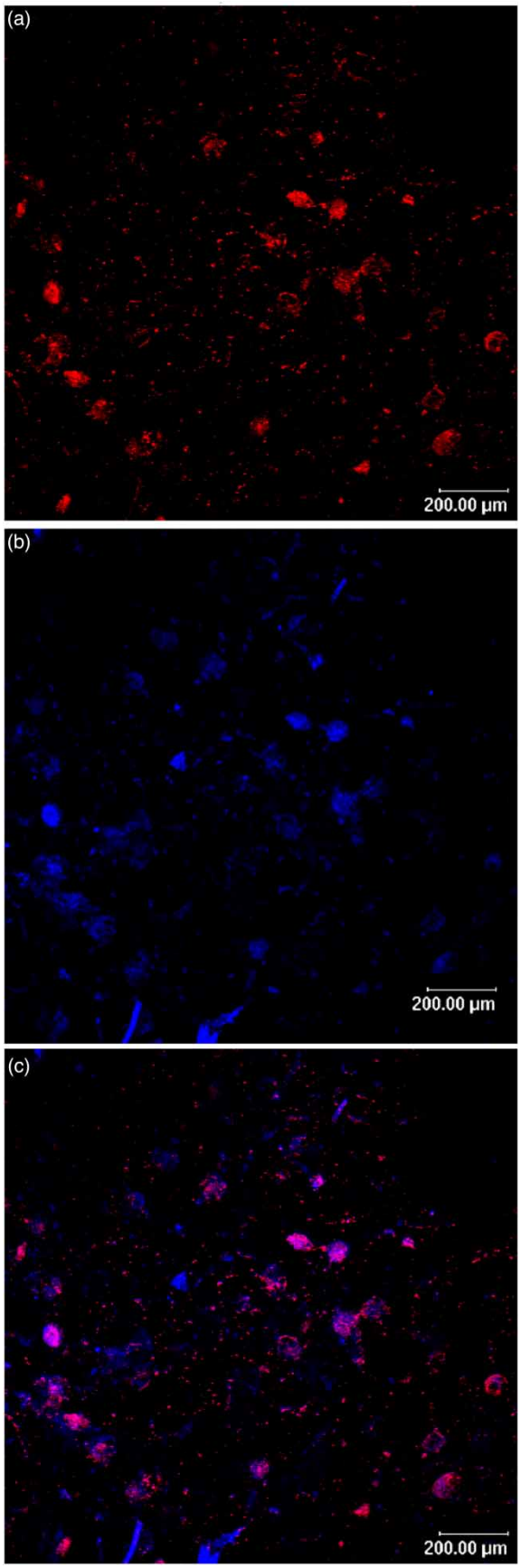


Figure 4 | FISH analysis of *Planctomycetes* and Anammox bacteria in Anammox sludge. A Cy3-labelled probeAMX820; B Cy5-labelled probe PLA46; C Simultaneous staining of biomass with Cy3-labelled probeAMX820 and Cy5-labelled probe PLA46.

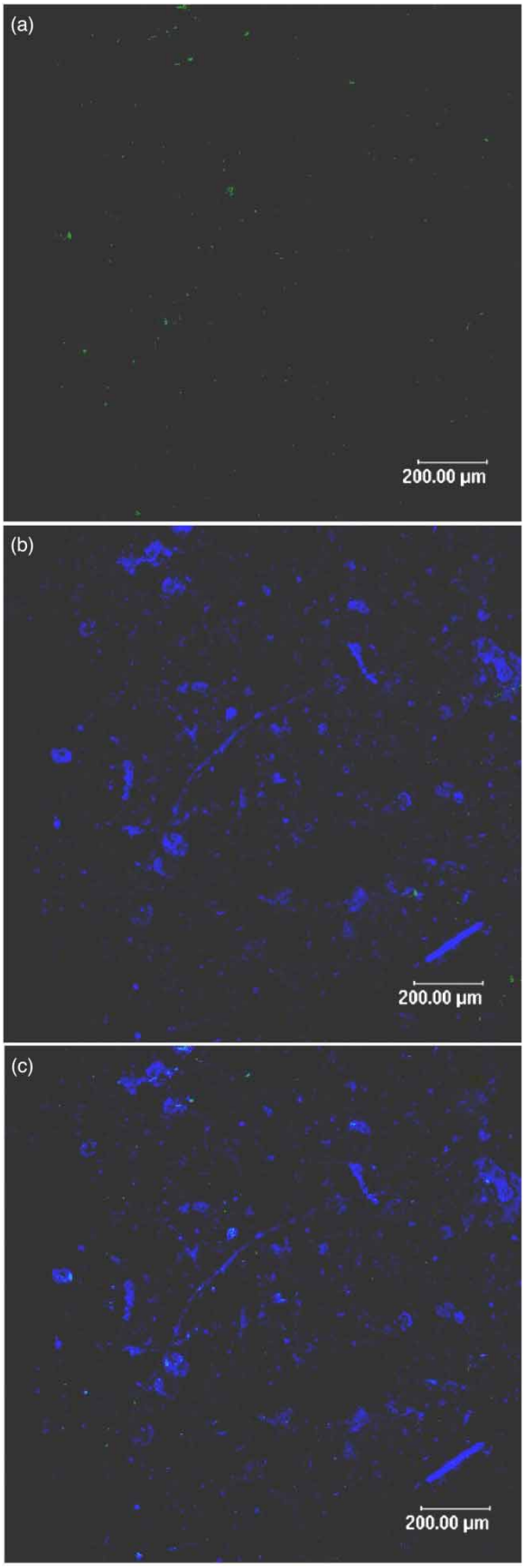


Figure 5 | FISH analysis of AOB in the Anammox sludge. A FITC-labelled probe NSO190; B Cy5-labelled probe EUB338 plus; C Simultaneous staining of biomass with FITC-labelled probe NSO190 and Cy5-labelled probe EUB338 plus.

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 6.32×10^4 and 1.58×10^4 cells μL^{-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.

ACKNOWLEDGEMENTS

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DATA AVAILABILITY STATEMENT

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

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