

Efficient feeding strategy to enhance the start-up of anaerobic ammonium oxidation process in an anaerobic up-flow biofilm column reactor

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ABSTRACT

Anaerobic ammonium oxidation (anammox) performed by bacteria that belong to the Planctomycetes phylum could potentially be applied to treat wastewater with high ammonium content. However, anammox application is hindered by the slow growth of bacteria and nitrite and free ammonia inhibition. Thus, this study aims to enrich anammox culture in a 1.0 L anaerobic up-flow biofilm column reactors equipped with a non-woven fabric material, under three different feeding modes: (i) batch, (ii) fed-batch and (iii) continuous, to enhance the start-up of anammox process. A 16S rDNA gene analysis targeting planctomycetes-anammox bacteria was performed on various sludge sources to screen the appropriate inoculum of anammox microorganisms prior to the enrichment study. A reactor with a continuous mode of feeding showed the most effective consumption of N-NH_4^+ and N-NO_2^- , with the highest nitrogen removal rate of $0.28 \text{ kg N m}^{-3} \text{ d}^{-1}$ at 0.6 d hydraulic retention time and 27°C , compared to the fed-batch and batch of 0.14 and $0.004 \text{ kg N m}^{-3} \text{ d}^{-1}$, respectively. The enriched anammox culture showed as close to the '*Candidatus* Kuenenia stuttgartiensis' genera based on 16S rDNA analysis. Fluorescence in-situ hybridization analysis targeting the 16S rDNA gene of anammox bacteria further confirmed the existence of an anammox population in the enriched culture.

Keywords: Anaerobic up-flow; Anammox; FISH; Deammonification; Nitric oxide removal

1. Introduction

Excessive nitrogen levels from untreated wastewater disposal promote the excessive growth of green algae and cyanobacteria. To protect our ecosystem, it is crucial to remove excess nitrogen from wastewater before it can be discharged into the environmental waters. During wastewater treatment, the biological nitrogen removal (BNR) process

known as sequential nitrification-denitrification is commonly used. Nitrification involves a two-step biological process that takes part under aerobic conditions, including (i) the oxidation of ammonium to nitrite and (ii) the oxidation of nitrite to nitrate aided by ammonia-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB). Denitrification is a nitrate reduction process that is facilitated by heterotrophic anaerobic bacteria to produce dinitrogen gas. Limitations for this process include the high cost of maintaining separate

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aerobic-anaerobic units, as well as the need for organic carbon sources as electron donors during the denitrification process [1].

The anaerobic ammonium oxidation (anammox) performed by bacteria that belong to the planctomycetes phylum was discovered in the early 21st century in Netherlands inside a BNR system [2,3]. It would undoubtedly affect the functional microorganisms and the biochemical reaction in the wastewater system, especially on the autotrophic biomass systems if no appropriate solution is deployed [4]. In the anammox process, ammonia and nitrite are simultaneously transformed into nitrogen gas by anaerobic ammonia-oxidizing bacteria, with a small amount of production of nitrate [5]. The anammox process promises lower operational costs and sludge production, no employment of organic carbon supplementation and a more effortless combination of the aerobic-anaerobic units. It is suitable for the treatment of high-strength ammonium wastewater, like leachate in a landfill, which is believed to inhibit the nitrification process [6]. Landfill leachate contains many organic and inorganic compounds, including a wide range of heavy metals [7]. As such, if the leachate is not well treated, it can pollute both the surface and groundwater resources.

Anammox has been used at the laboratory scale and in full-scale anammox reactors to treat various kind of wastewater, such as landfill leachate in a 20 L sequencing batch reactor (SBR) [8], digester liquor in a 22 L up-flow fixed-bed reactor filled with a polyester non-woven fabric carrier [9], pig manure effluents [10], turtle breeding wastewater [11] and pharmaceutical wastewater [12]. An important component of biofilm treatment technologies is the substratum that is used for the attachment and growth of the biofilm. A study by Zekker et al. [13] found that the biofilm concentration increased to 5.96 g/L on day 450 from an initial 2.47 g/L, with the anammox biomass developed in protected anaerobic zones under moderately aerobic conditions [13]. The most recent data indicate that there are more than 100 full-scale anammox reactors that have been successfully implemented worldwide as of early 2015 [14,15].

So far, the anammox process has been successfully applied to the treatment of ammonium-rich wastewaters, such as sludge digestion liquid (known as sidestream treatment), and now, anammox has been adopted for treating not only high-nitrogen wastewater in the sidestream but also the low-ammonia sewage in mainstream [16]. Despite the advantages of the anammox process, the long start-up time, due to the extremely slow growth rate of the bacteria (0.072 d^{-1} at 32°C), has contributed to the difficulty in implementing this process [17]. In addition, anammox bacteria are also sensitive to various environmental factors and can be negatively affected by light exposure and the presence of oxygen. Anammox growth could be inhibited by certain substrates, such as nitrite, phosphate, and hydrazine at high concentrations. An anammox system was reported to be irreversibly inhibited by the presence of nitrite at a concentration of $\geq 694.6 \text{ mg L}^{-1}$ [18]. Meanwhile, moderate inhibition of anammox activity was observed at hydrazine concentration $>0.96 \text{ mg L}^{-1}$ [18]. Additionally, 1.28 mg L^{-1} of dissolved oxygen can completely inhibit anammox activity [19]. However, the inhibition of anammox bacteria resulting from exposure to oxygen is reversible depending on the type

of anammox system used for the enrichment process [20,21]. Thus, a proper study in applying appropriate enrichment methods should be conducted to enrich the slow-growing bacteria. It is believed that the proper selection of inoculum, appropriate reactor configuration system and optimal operating conditions can enhance the anammox reaction, thus shortening the start-up time of anammox activity [21].

In this study, we investigated anammox enrichment in an anaerobic up-flow biofilm (UBF) reactor with non-woven fabric as a biomass carrier by applying three different feeding modes (batch, fed-batch, and continuous), to determine the efficacy of anammox process start-up. The identification of the enriched anammox bacteria was also performed to further confirm their presence using 16S rDNA gene analysis and a fluorescence in-situ hybridization (FISH) technique. This study provides important findings on the selection of suitable feeding strategies that can be utilized to enhance the start-up of anammox process. Moreover, the data presented may be also useful in widening its application in the BNR system of wastewater.

2. Materials and methods

2.1. Selection of inoculum

There were four different sources of wastewater sludge namely from sanitary landfill leachate, sewage, palm oil mill effluent sludge (POME), municipal solid waste (MSW) landfill leachate that was used to screen appropriate inoculum for anammox enrichment. Approximately 500 mL of sludge was collected from the bottom of the ponds since this zone is believed to provide an aerobic-anaerobic interface, which has a higher probability to contain anammox bacteria. The sludge samples were preserved at -20°C until further use and were subjected to physicochemical analyses of NH_4^+ , NO_3^- , NO_2^- , total suspended solids (TSS) and volatile suspended solids (VSS) [22], as well as molecular identification of anammox bacteria. The sludge sample that shows the highest similarity to anammox bacteria, based on basic local alignment search tool (BLAST) results of the retrieved sequences, was selected as the inoculum for the enrichment study. The sludge was washed several times with sterilized distilled water, followed by centrifugation at 4,000 rpm until the COD $< 20 \text{ mg/L}$ was achieved, prior to being used in the enrichment experiment.

2.2. Chemical analyses

The concentrations of NH_4^+ , NO_3^- and NO_2^- were determined with an ion chromatography system DIONEX ICS-1100 (Thermo Fisher Scientific Inc., US) equipped with an IonPac AS23 analytical $4 \times 250 \text{ mm}$ cation column. Samples were prepared by centrifugation at 2,500 rpm for 10 min before filtration, using $0.45 \mu\text{m}$ pore-sized membranes. TSS and VSS were determined according to the standard methods for the examination of water and wastewater [22]. The pH was measured using a pH meter PB10 (Sartorius, Göttingen, Germany).

2.3. Genomic DNA extraction and polymerase chain reaction (PCR) purification

Sludge samples were centrifuged at 5,000 rpm for 10–15 min and the pellets were washed several times with distilled

water. Genomic DNA from the sludge samples was extracted using a PowerSoil DNA Isolation kit (#12888-100 Mo Bio Laboratory Inc., USA) following the manufacturer’s instructions. Polymerase chain reaction (PCR) amplification of 16S rDNA gene sequences from the entire bacterial communities in the extracted genomic DNA samples was performed using the bacterial universal primers 27F and 1492R (Table 1) [21,23–27]. Partial 16S rDNA gene sequence of anammox bacteria was amplified using a planctomycetes specific primer (Pla46) in combination with an anammox specific primer (Amx386 or Amx820), as previously described [28]. PCR reactions contained 10 µL of 2X KAPA Taq ReadyMix with dye (#KM1002), 0.5 µL of each forward and reverse primer, 2 µL of genomic DNA sample and an adequate amount of sterile distilled water to bring the final volume to 20 µL. The PCR protocol consisted of an initial denaturation at 95°C for 4 min, followed by 35 cycles of denaturation at 95°C for 45 s, an annealing of 54°C–56°C for 45 s and extension at 70°C for 1 min, with a final extension at 72°C for 3 min. The annealing temperature was changed prior to optimization, according to the combination of primers used in PCR. PCR amplification was performed using a Bio-Rad MyCycler™ Thermal Cycler (Bio-Rad, US). PCR purification was performed using a Qiagen QIAquick® PCR Purification kit (Cat. 28104) (Venlo, Netherlands) following the manufacturer’s protocol. PCR products were electrophoresed on 1.0% w/v agarose gels and stained with ethidium bromide.

2.4. DNA sequencing

PCR reactions producing a visible band were sent to First BASE Laboratories Sdn Bhd., Malaysia for DNA sequencing. Retrieved sequences were aligned using the multiple sequence alignment program CLUSTALW in the Biology Workbench through the website (<http://workbench.sdsc.edu>). Sequence similarity searches of the aligned sequences were conducted using the BLAST network service of the GenBank database through the website (<http://www.ncbi.nlm.nih.gov>). The nearest relatives of bacterial species were identified based on the highest percent of similarity identified from the BLAST results.

2.5. Anaerobic UBF column reactor

Anammox enrichment was performed in an anaerobic UBF reactor consisting of three column reactors (Fig. 1a), applying three different feeding modes. Each column reactor has an internal diameter of 80 mm, a height of 200 mm and a working volume of 1 L. Non-woven fabric material (8 cm × 6 cm × 6 cm) with a surface area of 432 cm² (Fig. 1b) was inserted into each column reactor for biofilm attachment. All tubing and connectors used 6.0 mm (internal diameter) silicone tubing.

2.6. Enrichment conditions and feeding strategy

The conditions for anammox enrichment in the anaerobic UBF column reactor are summarised in Table 2. The reactor was covered with black cloth to avoid light interference, which may encourage the growth of light-dependent microorganisms. The mineral medium was fed into the reactor

Table 1
List of primers and oligonucleotides probes used for amplification of 16S rDNA genes and FISH analysis

Primer name/ oligonucleotides probes	Specificity (16S rDNA)	Sequence (5'-3')	Target site (bp)	Label	Fluorescence dye	Hybridization condition ^a		References
						% ^b	mM ^c	
27F	Universal bacteria	AGAGTTTGAATCMTGGCTCAG	8–27	Forward	-	-	-	[23]
1492R	Universal bacteria	CGGTTACCTTGTACCGACTT	1,492–1,507	Reverse	-	-	-	[23]
Pla46	Planctomycete	GGATTAGGCATGCAAGTC	46–63	Forward	-	-	-	[24]
Amx368	Anammox	CCTTTCGGGCCATTGGCGAA	368–385	Reverse	-	-	-	[21]
Amx820	Brocadia, Kuenenia	AAAACCCCTCTACTTAGTCCCC	820–841	Reverse	-	-	-	[21]
EUB338	Universal	GCTGCCTCCCGTAGGAGT	-	-	Rhodamine Green	30	180	[25]
EUB338II	Universal	GCAGCCACCCGTAGGTGT	-	-	Rhodamine Green	30	180	[26]
EUB338III	Universal	GCTGCCACCCGTAGGTGT	-	-	Rhodamine Green	30	180	[26]
Amx368	Anammox	CCTTTCGGGCCATTGGCGAA	-	-	Rhodamine Red	15	318	[27]

^aHybridization condition was performed at 46°C.

^b% of Formamide.

^cConcentration of NaCl.

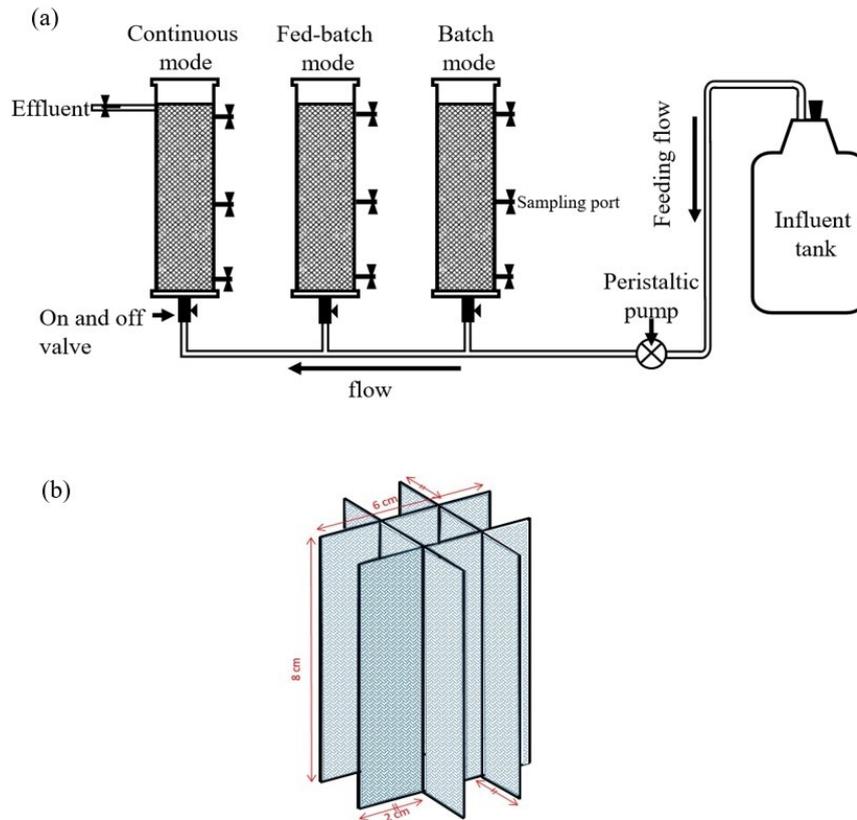


Fig. 1. Schematic diagram of anaerobic up-flow biofilm column reactor, S1, S2 and S3 are the reactor sampling ports (a) and non-woven fabric material with total surface area of 432 cm² as biomass supporter (b).

Table 2
Operational condition of anammox enrichment in anaerobic up-flow biofilm column reactor

Column reactor	1	2	3
Feeding mode	Batch	Fed-batch	Continuous
Volume of inoculum (mL)	250	250	250
Biomass carrier	Non-woven fabric	Non-woven fabric	Non-woven fabric
Reactor working volume (L)	1	1	1
pH	7.75 ± 0.25	7.75 ± 0.25	7.75 ± 0.25
Operating temperature (°C)	27°C ± 3°C	27°C ± 3°C	27°C ± 3°C
Dissolved oxygen (mg L ⁻¹)	<0.01 ± 0.002	<0.01 ± 0.002	<0.01 ± 0.002
NLR (kg N m ⁻³ d ⁻¹)	–	0.16 ± 0.01	0.14–0.38 ± 0.01

column from the bottom part of the reactor using a peristaltic pump (Masterplex L/s, Cole-Parmer, US). The medium contained (per L) 1.81 g NH₄HCO₃, 0.35 g NaNO₂, 0.06 g NaH₂PO₄·H₂O, 0.1 g MgSO₄·7H₂O, 0.2 g NaHCO₃, 1.05 g ethylenediamine-tetraacetic acid (EDTA) and 1.0 mL each of trace element solutions 1 and 2. Trace element solution 1 contained (per L) 5 g FeSO₄·7H₂O and 5 g EDTA. Trace element solution 2 contained (per L) 15 g EDTA, 0.43 g ZnSO₄·7H₂O, 0.24 g CoCl₂·6H₂O, 0.63 g MnCl₂, 0.25 g CuSO₄·5H₂O, 0.22 g Na₂MoO₄·2H₂O, 0.19 g NiCl₂·6H₂O, 0.21 g Na₂SeO₄·10H₂O, 0.01 g H₃BO₃ and 0.05 g NaWO₄·2H₂O [29]. The chemicals used were of analytical grade and were obtained from Fisher

Chemical, US. The medium was autoclaved and flushed with nitrogen gas for at least 20 min prior to use. The enrichment was carried out at room temperature of 27°C ± 3°C with an operating pH of 7.75 ± 0.25. Dissolved oxygen was measured using YSI 550A, USA dissolved oxygen meter. Nitrogen flushing for 30–40 min was carried out two times a week after sampling, until dissolved oxygen of <0.01 ± 0.002 mg/L was achieved, to maintain anaerobic conditions in the reactor [30].

A batch mode feeding was operated without any addition or removal of substances, except for sampling. The enrichment was conducted in repeated batch with first and second batch operated for 100 and 80 d, respectively.

A fed-batch was performed at a fixed nitrogen loading rate (NLR) of $0.16 \text{ kg N m}^{-3} \text{ d}^{-1}$ (Eq. (1)), with the addition of medium in stages for lengths of 29, 11, 33, 22, 37, 25, and 23 d, respectively, for each stage. Meanwhile, the continuous mode was performed by continuous feeding of the NLR with $0.14\text{--}0.38 \pm 0.01 \text{ kg N m}^{-3} \text{ d}^{-1}$, with a hydraulic retention time (HRT) of $0.6\text{--}1.36 \pm 0.04 \text{ d}$. NLR was elevated according to the performance of anammox activity in the reactor. The samples collected were subjected to N-NH_4^+ , N-NO_3^- and N-NO_2^- analyses every 3 d. The specific N-NH_4^+ removal (Eq. (2)), percentage of N-NH_4^+ removal (Eq. (3)), percentage of N-NO_2^- removal (Eq. (4)) and nitrogen removal rate (Eq. (5)) were determined.

$$\text{NLR} = \frac{[\text{N-NH}_4^+ + \text{N-NO}_2^-]_{\text{influent}} \times \text{flow rate}}{\text{Reactor volume}} \quad (1)$$

$$\text{Specific N-NH}_4^+ \text{ removal} = \frac{[\text{N-NH}_4^+]_{\text{influent}} - [\text{N-NH}_4^+]_{\text{effluent}}}{\text{VSS} \times \text{HRT}} \quad (2)$$

$$\text{Percentage of N-NH}_4^+ \text{ removal} = \frac{[\text{N-NH}_4^+]_{\text{influent}} - [\text{N-NH}_4^+]_{\text{effluent}}}{[\text{N-NH}_4^+]_{\text{influent}}} \times 100\% \quad (3)$$

$$\text{Percentage of N-NO}_2^- \text{ removal} = \frac{[\text{N-NO}_2^-]_{\text{influent}} - [\text{N-NO}_2^-]_{\text{effluent}}}{[\text{N-NO}_2^-]_{\text{influent}}} \times 100\% \quad (4)$$

$$\text{Nitrogen removal rate} = \frac{[\text{N-NH}_4^+ + \text{N-NO}_2^-]_{\text{influent}} - [\text{N-NH}_4^+ + \text{N-NO}_2^-]_{\text{effluent}} \times \text{flow rate}}{\text{Reactor volume}} \quad (5)$$

2.7. FISH analysis

The sludge samples used for FISH analysis were obtained from the non-woven material of each reactor at the end of the enrichment period. Samples were immediately fixed with 4% paraformaldehyde (Sigma, USA), at 4°C for 3 h, before being washed with phosphate-buffered saline (PBS) (Bio-rad), and they were maintained in PBS-ethanol (1:1) at -20°C until further use. The hybridization of the samples was performed according to the FISH protocol described by Yusof et al. [7], with the inclusion of a pre-hybridization step using 10% of a blocking reagent (Boehringer, Germany) to reduce nonspecific binding from background solids. Specific oligonucleotide probes targeting the 16S rDNA gene were purchased from 1st Base Laboratory Sdn. Bhd., Malaysia for in-situ detection of anammox bacteria (Amx368) and total bacteria (EUB338, EUB338II, and EUB338III). The target sequences and hybridization conditions for FISH analyses used in this study are summarised in Table 1. An *E. coli* strain was used as a positive control for optimization

of FISH hybridization since the anammox bacteria were uncultured [31]. Fluorescence images were viewed under an inverted fluorescence microscope (Nikon Eclipse TE 2000-U, Japan, software: NIS-Elements BR3.0), using G-2A (excitation 510–560 nm) and B-2A (excitation 450–490 nm) for rhodamine red and rhodamine green filters, respectively.

2.8. Statistical analysis

One-way ANOVA statistical analysis was performed by using SPSS 16.0 software to compare the N-NH_4^+ and N-NO_2^- removal efficiency for three different modes of feeding strategy. The independent variables were the mode of feeding with three levels (batch, fed-batch and continuous). The dependent variable was the removal efficiency of the substances. The Tukey's HSD post hoc test was applied to identify where the significance lies. The *p*-value shows the significance of the *F*-ratio with a confidence level α of 0.05.

3. Results and discussion

3.1. Selection of inoculum for the enrichment of anammox bacteria

Various types of wastewater sludge were investigated for the presence of anammox bacteria prior to the selection of appropriate anammox inoculum. Table 3 shows the physicochemical characteristics of the sludge samples and the sequencing results obtained from the four different sources of wastewater sludge from treatment plants. The amplification of a 16S rDNA gene fragment targeting anammox organisms was performed using two sets of primers (Pla46-Amx368 and Pla46-Amx820) targeting planctomycetes-anammox bacteria. The data indicates, from the 14 sludge samples tested, that 7 samples show the presence of an anammox population due to a high number of similarities obtained from 16S rDNA analysis (Table 3). This could be explained by non-specific DNA binding, or the DNA concentration of the anammox bacteria was very low in the extracted genomic DNA. A PCR analysis of the partial 16S rDNA gene was unable to detect anammox bacteria from seed sludge with a very low-density anammox bacteria before the enrichment process was carried out. However, the PCR technique with specific primer targeting the 16S rDNA gene of anammox bacteria and DNA sequencing revealed the existence of an anammox bacterial population from the seed sludge. Activated sludge from an SBR of sanitary landfill leachate (1c), which had the highest similarity related to anammox bacteria, was selected as the inoculum for the subsequent anammox enrichment experiment. The selection of appropriate sludge samples is an important step to obtain the best inoculum for anammox enrichment [24].

3.2. Performance of anammox enrichment

3.2.1. Batch fermentation process

Anammox enrichment using a reactor with a batch feeding mode was operated for about 180 d. The reactor was inoculated with a high concentration of biomass ($3.8 \times 10^2 \text{ mg L}^{-1} \text{ VSS}$). Medium fed into the reactor contained NH_4^+ and NO_2^- at concentrations of $312 \pm 5.30 \text{ mg L}^{-1}$ and $72 \pm 2.94 \text{ mg L}^{-1}$, respectively. A strict deoxygenated condition

Table 3
Physicochemical characteristics of sludge samples and sequencing results obtained from 16 sRNA analysis

Wastewater	Type of sludge	Sample	Parameter					BLAST results		
			NH ₄ ⁺ (mg L ⁻¹)	NO ₂ ⁻ (mg L ⁻¹)	NO ₃ ⁻ (mg L ⁻¹)	VSS (mg L ⁻¹)	TSS (mg L ⁻¹)	pH	Similarity (%)	Accession
Sanitary landfill leachate	Untreated/Raw (Pond A)	1a	520.15 ± 11.2	^a ND	ND	4.0 × 10 ³ ± 301	8.8 × 10 ³ ± 389	7.8 ± 0.4	88%	JX465179
	Untreated/Raw (Pond B)	1b	529.94 ± 12.3	ND	ND	6.8 × 10 ³ ± 352	1.94 × 10 ⁴ ± 654	7.8 ± 0.4	88%	FN391170
	Activated (SBR 1 C)	1c	170.05 ± 4.7	ND	4.67 ± 1.2	3.66 × 10 ³ ± 233	1.55 × 10 ³ ± 118	7.9 ± 0.3	93%	FN391173
	Activated (SBR 1 D)	1d	196.99 ± 5.1	ND	2.51 ± 0.7	3.68 × 10 ³ ± 230	1.73 × 10 ³ ± 128	7.9 ± 0.3	92%	JX465194
Sewage	Untreated (Pond 1)	2a	215.12 ± 2.9	1.78 ± 0.06	2.29 ± 0.7	6.8 × 10 ³ ± 325	2.04 × 10 ⁴ ± 688	7.3 ± 0.4	0%	^b NA
	Untreated (Pond 2)	2b	158.21 ± 2.5	2.05 ± 0.03	2.08 ± 0.5	1.36 × 10 ³ ± 126	3.98 × 10 ⁴ ± 764	7.3 ± 0.3	0%	NA
	Untreated (Pond 3)	3a	363.17 ± 6.2	2.02 ± 0.03	2.66 ± 0.7	4.6 × 10 ³ ± 270	7.8 × 10 ³ ± 378	6.9 ± 0.2	0%	NA
	Untreated (Drainage)	3b	239.00 ± 1.9	1.82 ± 0.02	2.09 ± 0.5	3.8 × 10 ³ ± 230	3.8 × 10 ³ ± 268	7.0 ± 0.2	0%	NA
	Treated (Pond 1)	4a	93.51 ± 1.9	ND	56.89 ± 3.4	2.4 × 10 ² ± 187	8.14 × 10 ⁴ ± 875	7.7 ± 0.4	0%	NA
	Treated (Pond 2)	4b	83.33 ± 2.1	ND	60.39 ± 3.7	2.58 × 10 ² ± 220	4.02 × 10 ⁴ ± 701	7.8 ± 0.4	84%	JX465179
Palm oil mill effluent (POME)	Treated (Pond 3)	4c	87.17 ± 3.4	ND	ND	2.26 × 10 ² ± 220	7.84 × 10 ⁴ ± 866	7.8 ± 0.4	0%	NA
	Treated (Pond 4)	4d	86.50 ± 2.7	ND	3.68 ± 0.3	2.92 × 10 ² ± 246	4.32 × 10 ⁴ ± 698	7.8 ± 0.3	0%	NA
	Untreated/Raw (Pond 1)	5a	507.63 ± 9.7	ND	2.33 ± 0.3	3.9 × 10 ³ ± 247	1.53 × 10 ³ ± 120	7.8 ± 0.4	91%	JX465175
	Untreated/Raw (Pond 2)	5b	525.27 ± 8.8	ND	2.89 ± 0.7	3.18 × 10 ² ± 230	1.48 × 10 ³ ± 120	7.9 ± 0.3	91%	JX465179

^aND means not detected and ^bNA means not available.

was maintained in the reactor by nitrogen flushing, as low dissolved oxygen concentration (0.04 mg L^{-1}) could negatively affect anammox activity [19].

The concentrations of N-NH_4^+ , N-NO_2^- and N-NO_3^- during anammox bacteria enrichment for batch feeding mode are shown in Fig. 2a. During the first 10 d of enrichment, the removal of N-NH_4^+ and N-NO_2^- was relatively slow. It took 10 d to remove 40.47 mg L^{-1} of N-NH_4^+ and 16.58 mg L^{-1} of N-NO_2^- from the culture. During the early stage of enrichment, anammox bacteria were still in the adaptation phase and had to compete with AOB and NOB, which were possible contributors to nitrogen removal in the reactor. It is believed that the removal of N-NH_4^+ and

N-NO_2^- during the earlier phase is associated with the ammonification and nitrification-denitrification process [29]. Moreover, in this study, a slight increase in N-NO_2^- was detected after the sludge inoculation, demonstrating a possible ammonium oxidation process by AOB in the reactor. To suppress nitrification, maintaining an anaerobic condition in the reactor was important, especially at the earlier stage of the enrichment process. Therefore, the deoxygenation process was strictly performed during the earlier phase of the enrichment process. Reducing the non-anammox organisms by providing a favorable condition for anammox growth (anaerobic conditions) results in a bacterial population that is enriched for anammox [32].

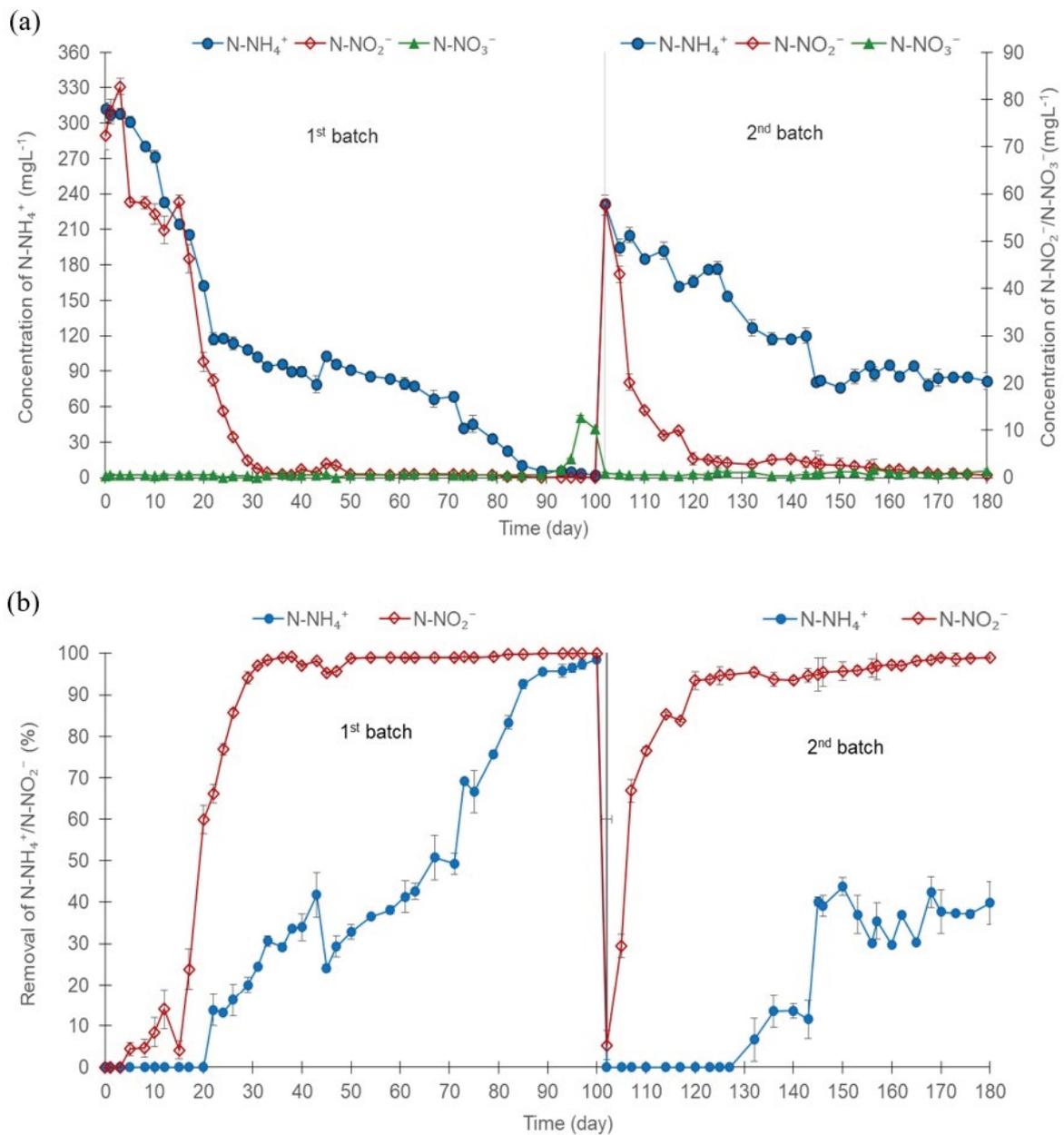


Fig. 2. Changes of (a) N-NH_4^+ , N-NO_2^- and N-NO_3^- concentration and (b) nitrogen removal percentage throughout anammox bacteria enrichment using a reactor with a batch feeding mode.

A simultaneous decrease of N-NH_4^+ and N-NO_2^- from the reactor was detected, starting at day 26, when the two substrates were consumed rapidly. The concurrent decrease in N-NH_4^+ and N-NO_2^- in a strict anaerobic environment showed that anammox activity started to occur in the system and became the dominant contributor to the nitrogen removal process. Although AOB can be inhibited under a low dissolved oxygen concentration of $0.15\text{--}0.55\text{ mg L}^{-1}$ [33], it also has been reported that complete nitrification can be achieved under the microaerobic condition of $0.5 \pm 0.3\text{ mg L}^{-1}$ with low aeration [34]. Nevertheless, the concentration of N-NO_2^- and N-NO_3^- were observed to be depleted throughout the enrichment process, suggesting that there was still a possible denitrification process occurring under the oxygen-limited conditions [35]. The denitrification process can occur simultaneously with the anammox process. Although organic carbon was not supplied, it can be derived from the decay of organic substances in the inoculum.

The reactor system took 89 d for N-NO_2^- to be completely removed from the system, while N-NH_4^+ remained present at a small concentration until day 100. The slow nitrogen removal might also due to the free ammonia (FA) concentration inhibition to the anammox bacteria in the system. The FA concentration calculated according to Rikmann et al. [4] at the beginning of the start-up process was 13.74 mg L^{-1} . The second batch of the enrichment process started occurring at day 101, at which time $57.66 \pm 2.12\text{ mg L}^{-1}$ of NO_2^- and $231.82 \pm 1.30\text{ mg L}^{-1}$ of NH_4^+ in enrichment medium was added into the reactor. The batch enrichment was necessary to supply the culture with sufficient NO_2^- and NH_4^+ to avoid substrate limitation, which may negatively affect the anammox activity. The culture experienced N-NO_2^- depletion for 12 d (day 89 to 100).

After the addition of the 2nd batch of medium, which started at day 101, N-NO_2^- showed a rapid decreasing pattern compared to N-NH_4^+ . This demonstrated that the denitrification activity was still taking place together with the anammox process in the system. There was no difference in the decreasing pattern of the N-NH_4^+ and N-NO_2^- concentration between the 1st and 2nd batch of enrichment, indicating that there was no impairment of anammox activity after N-NO_2^- starvation for 12 d. Considering that N-NO_3^- is the product of the anammox process, a low concentration of N-NO_3^- throughout the enrichment process strengthens the fact that there was still a simultaneous denitrification process occurring together with the anammox process. A complete deoxygenation step was required to completely remove heterotrophic denitrifiers from the reactor system, which was impossible to be achieved without deliberate flushing of nitrogen gas into the reactor throughout the enrichment process.

The nitrogen removal performances of the anammox enrichment in the batch reactor are shown in Fig. 2b. The batch anaerobic UBF column reactor took 26 d for the start-up of anammox activity. The continuous removal of N-NO_2^- and N-NH_4^+ could be detected starting from day 26, and the nitrogen removal percentage increased with time until all of the substrates were completely removed. The highest removal efficiency of anammox activity for this batch enrichment study was achieved on day 89, with N-NH_4^+ , N-NO_2^- and total nitrogen removal percentages of 95.66%, 100%, and 98%, respectively. There was a significant difference for N-NH_4^+

and N-NO_2^- removal between the three types of feeding strategy used: ($df = (3, 185)$, $F = 5.748$, $p = 0.004$) and ($df = (3, 185)$, $F = 23.144$, $p = 0.000$), respectively. The N-NH_4^+ and N-NO_2^- removal between batch and fed-batch ($p = 0.028$) and batch and continuous reactor ($p = 0.000$), respectively, were significantly different. The nitrogen removal rate achieved was $0.004\text{ kg N m}^{-3}\text{ d}^{-1}$, lower than the fed-batch and continuous reactors. The ratio of ammonium consumption to nitrite consumption to nitrate production at day 97 for the highest N-removal efficiency was 1:1.177:0.33. This experimental ratio was similar to the stoichiometric ratio of anammox (1:1.146:0.161) reported by Lotti et al. [36].

3.2.2. Fed-batch mode of feeding

The changes in the concentrations of N-NH_4^+ , N-NO_2^- and N-NO_3^- during anammox bacteria enrichment for fed-batch feeding mode are shown in Fig. 3a. In this operation, substrates are intermittently fed to the system, while the effluent is removed discontinuously. The fed-batch mode is one of the strategies to reduce the effect of substrate inhibition catabolite repression [37], or in this case, to minimize the shock NLR to the system. The removal of nitrogen during the initial phase of enrichment (days 1–10) was negligible since no anammox activity was expected during the initial period of enrichment [29,38]. The denitrification activity was the dominant process, and there was no possible anammox activity during the initial period of anammox bacterial enrichment. The N-NH_4^+ concentration increased slightly on day 12. A continuous decrease in the concentration of N-NO_2^- during the early phase (days 1–10) of the anammox enrichment was primarily caused by the denitrification process. The denitrification process during the initial phase in the anammox reactor occurs since it makes use of the remaining organic carbon in the culture. An increase in N-NH_4^+ during days 8–12 was due to the degradation of the dead organic matter present in the inoculum. The mineralization of organic nitrogen as a result of biomass decay contributes to the increase of N-NH_4^+ during the initial phase of the enrichment process [29].

A stable decrease in N-NH_4^+ and N-NO_2^- in the reactor was observed starting at day 30 of the enrichment. The medium was added once the N-NH_4^+ and N-NO_2^- was consumed by the culture. The N-NH_4^+ and N-NO_2^- concentrations were increased in almost every sample after the medium was added (days 40, 73 and 132), then began to show a decreasing pattern as the two substrates were utilized in the anammox process. This might have been due to the acclimatization of anammox bacteria to the changes in substrate concentrations.

It was observed that N-NO_2^- was rapidly consumed compared to N-NH_4^+ , although the ammonium and nitrite supplied in the influent was performed according to the stoichiometric ratio of the anammox reaction [36]. Low production of N-NO_3^- was detected throughout the enrichment process, indicating that a substantial denitrification process was occurring in the reactor. Taking into consideration the anaerobic environment present in the reactor and the depletion of the organic carbon source for the denitrification process as the enrichment time increased, the anammox activity was still the favored process responsible for the removal of N-NH_4^+ and N-NO_2^- in the reactor.

As shown in Fig. 3b, the removal of $N-NH_4^+$ and $N-NO_2^-$ increased starting at day 30, indicating the improvement of nitrogen removal by the anammox process as the enrichment time increased. A slight decrease in the nitrogen removal efficiency was observed when the medium was added, due

to the acclimatization of anammox bacteria to the changes in substrate concentrations. The concentrations of $N-NH_4^+$ ($135.7 \pm 5.72 \text{ mg L}^{-1}$) and $N-NO_2^-$ ($60.87 \pm 1.40 \text{ mg L}^{-1}$) introduced into the culture were considered relatively high. However, no inhibition of anammox activity resulting from

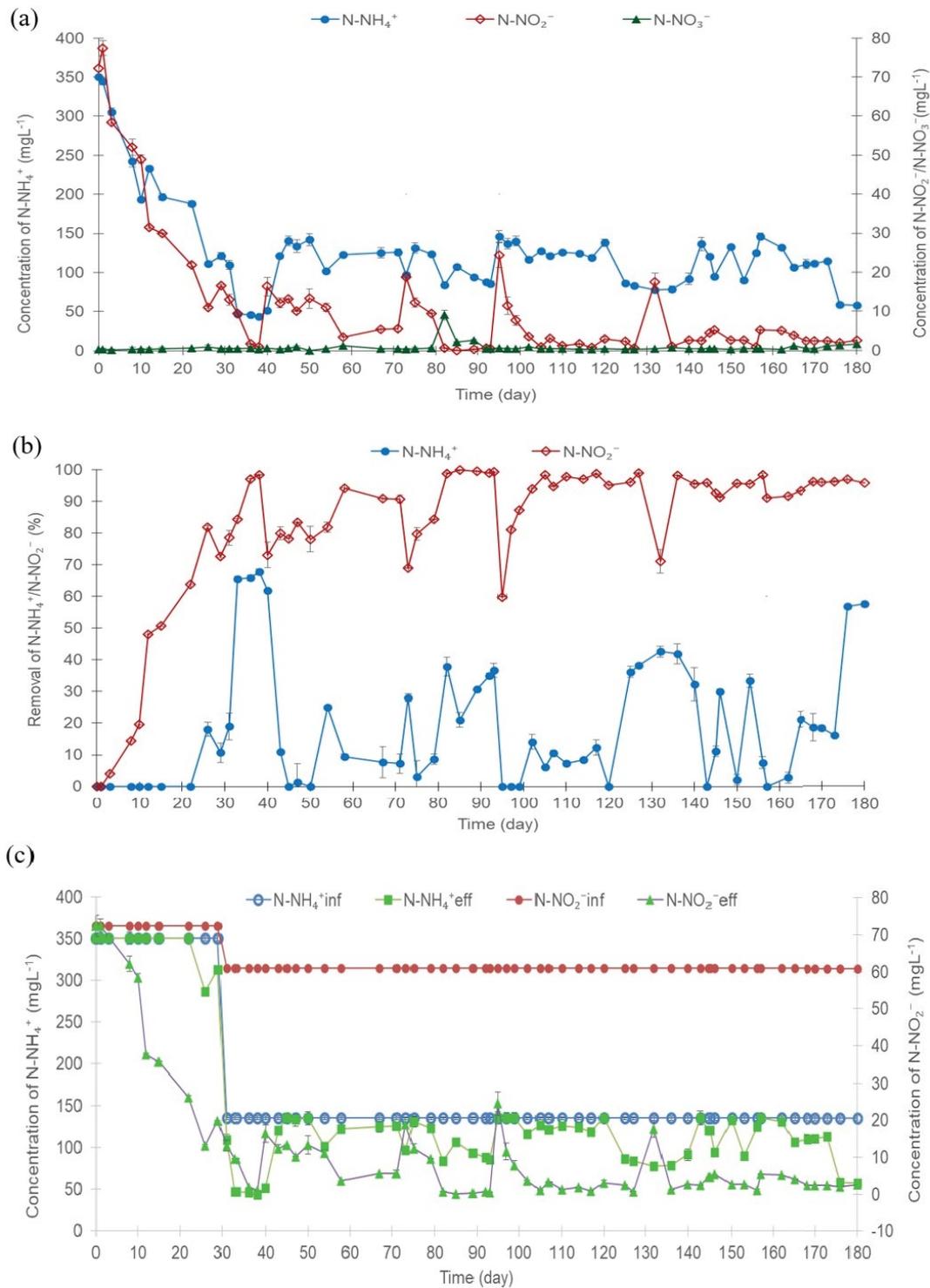


Fig. 3. Changes of (a) $N-NH_4^+$, $N-NO_2^-$ and $N-NO_3^-$ concentration, (b) nitrogen removal percentage and (c) $N-NH_4^+$, $N-NO_2^-$ influent and effluent concentration throughout anammox bacteria enrichment using a reactor with a fed-batch feeding mode.

the high influent concentration was observed, since the removal of N-NH_4^+ and N-NO_2^- increased within days after the feeding. The highest N-NH_4^+ , N-NO_2^- and total nitrogen removal efficiencies (68%, 98%, and 89%, respectively) were recorded on day 38. It was found that the N-NH_4^+ removal between fed-batch and batch ($p = 0.028$) and fed-batch and continuous ($p = 0.004$) were significantly different. Meanwhile, the N-NO_2^- removal was significantly different only with the continuous reactor ($p = 0.000$). The nitrogen removal rate was $0.14 \text{ kg N m}^{-3} \text{ d}^{-1}$, which was higher than the batch reactor and lower compared to the continuous reactor. The removal of N-NO_2^- was higher than N-NH_4^+ throughout the enrichment period, which could be explained by the simultaneous denitrification process which reduces nitrite and nitrate into dinitrogen gas. The ratio of ammonium consumption to nitrite consumption to nitrate production for day 38 with the highest N-removal efficiency

was 1:1.664:0.015. A higher percentage of ammonium was removed compared to nitrite, and very low production of nitrate observed in the reactor might have been due to the denitrification activity in the reactor.

3.2.3. Continuous mode of feeding

The changes in the concentrations of N-NH_4^+ , N-NO_2^- and N-NO_3^- during anammox bacteria enrichment for continuous feeding mode are shown in Fig. 4a. Initially, the reactor was fed with $296 \pm 7.31 \text{ mg L}^{-1} \text{ N-NH}_4^+$ and $95 \pm 3.1 \text{ mg L}^{-1} \text{ N-NO}_2^-$ at an HRT of 1.36 d. A rapid decrease in N-NH_4^+ and N-NO_2^- was detected during days 1–22 of the enrichment. A total of $147.88 \pm 1.93 \text{ mg L}^{-1}$ of N-NH_4^+ and $25.78 \pm 0.18 \text{ mg L}^{-1}$ of N-NO_2^- was removed within 14 d of the start of the enrichment. Regardless of the observed decrease in N-NH_4^+ and N-NO_2^- , the concentrations of N-NH_4^+ and

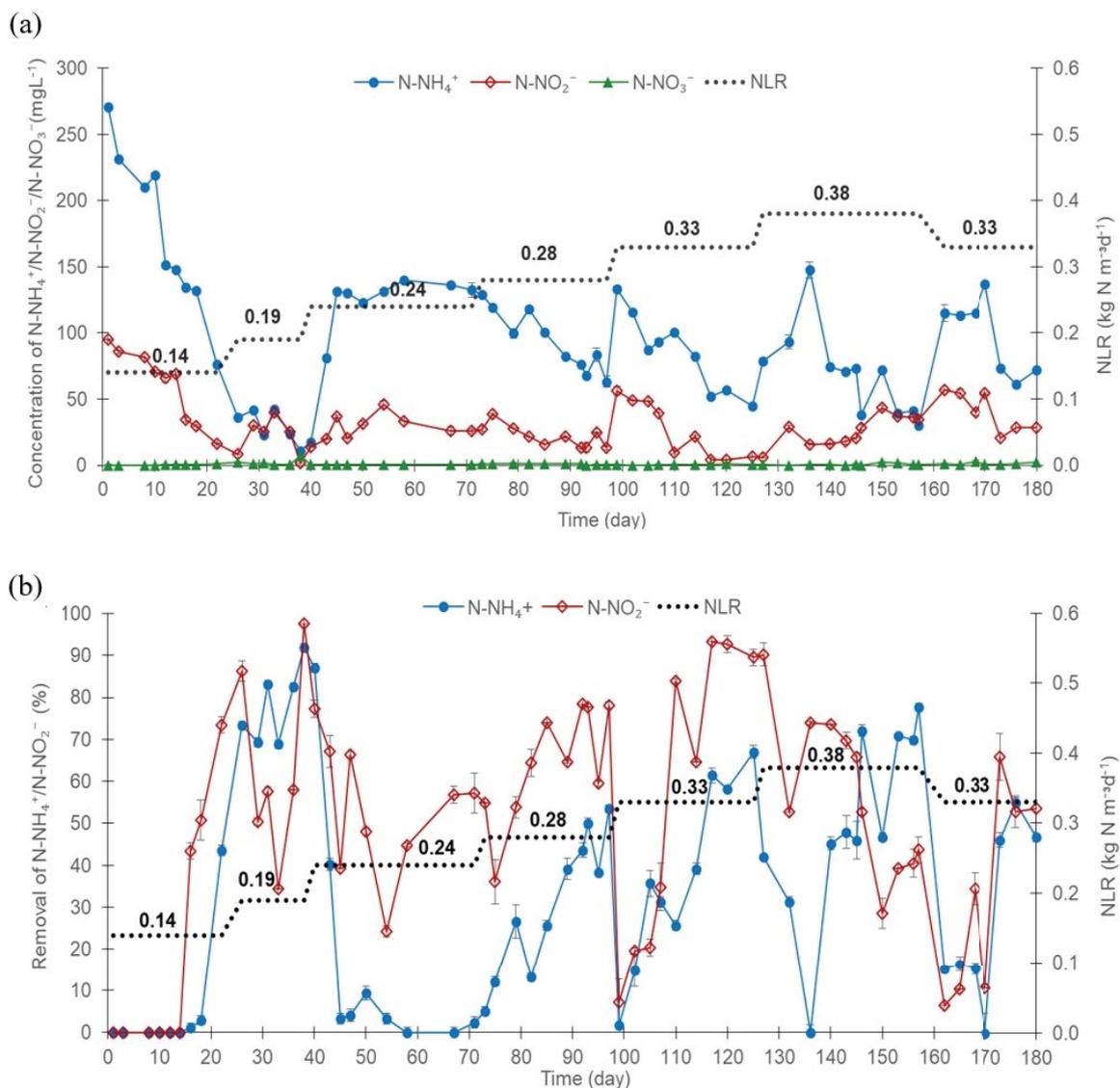


Fig. 4. Changes of (a) N-NH_4^+ , N-NO_2^- and N-NO_3^- concentration and (b) nitrogen removal percentage throughout anammox bacteria enrichment using a reactor with a continuous feeding mode.

N-NO_2^- in the effluent were still high for the first two weeks. The removal of N-NH_4^+ and N-NO_2^- associated with anammox activity was accounted for, starting at day 20, since days 1–10 of the enrichment were considered as the initial start-up period of anammox activity, in which no anammox activity was expected [39].

The NLR first increased from 0.14 to $0.19 \pm 0.01 \text{ kg N m}^{-3} \text{ d}^{-1}$ starting at day 23 by reducing the HRT to 1.04 d. A fluctuating trend in the concentrations of N-NH_4^+ and N-NO_2^- were observed, indicating an unstable performance of anammox activity in the reactor. When the NLR was further elevated from 0.19 to $0.24 \pm 0.01 \text{ kg N m}^{-3} \text{ d}^{-1}$, the concentrations of N-NH_4^+ and N-NO_2^- in the culture were observed to increase. The anammox performance became stable again within 3 d, as the concentrations of N-NH_4^+ and N-NO_2^- were observed to decrease as the substrates were consumed after the culture had adapted to the new conditions. An increase in the concentrations of N-NH_4^+ and N-NO_2^- , due to the acclimatization process, was obvious each time the NLR was elevated. The NLR was continuously increased until an impairment of the anammox process was observed at an NLR of $0.38 \pm 0.01 \text{ kg N m}^{-3} \text{ d}^{-1}$. Starting from day 150, the N-NO_2^- concentration increased and showed no decreasing pattern for 10 d. Afterward, the NLR was reduced to $0.33 \pm 0.01 \text{ kg N m}^{-3} \text{ d}^{-1}$, after which the N-NO_2^- concentration was observed to decrease again.

The trend of the percent removal of N-NH_4^+ and N-NO_2^- throughout the enrichment process is illustrated in Fig. 4b. The percentage of nitrogen removed increased starting in the third week, with an NLR of $0.19 \text{ kg N m}^{-3} \text{ d}^{-1}$. The highest N-NH_4^+ and N-NO_2^- removal achieved was on day 38 with 91.8% and 97.5%, respectively, with the attained nitrogen removal rate at $0.18 \text{ kg N m}^{-3} \text{ d}^{-1}$. The percent of N-NH_4^+ and N-NO_2^- removed were observed to continuously decrease after the NLR was elevated due to the acclimatization process of anammox bacteria towards the changes in the environment and the substrate concentrations in the reactor. The highest operating NLR tested in the reactor was $0.38 \pm 0.01 \text{ kg N m}^{-3} \text{ d}^{-1}$. The anammox activity was most effective between day 100 and day 125, with an operating NLR of $0.33 \pm 0.01 \text{ kg N m}^{-3} \text{ d}^{-1}$. An efficient anammox performance was observed at day 125, with N-NH_4^+ , N-NO_2^- and total nitrogen removal of 67%, 89%, and 85%, respectively, and a high specific anammox activity of $0.34 \text{ g N-NH}_4^+ \text{ g-VSS}^{-1} \text{ d}^{-1}$. The N-NH_4^+ removal was significantly different from the fed-batch reactor ($p = 0.004$). Meanwhile, there were significant differences for N-NO_2^- removal between this reactor with batch ($p = 0.000$) and fed-batch ($p = 0.000$). The nitrogen removal rate achieved was $0.28 \text{ kg N m}^{-3} \text{ d}^{-1}$, which was higher than the batch and fed-batch reactors. The ratio of ammonium consumption to nitrite consumption to nitrate production for the day with the highest nitrogen removal efficiency was 1:1.530:0.016. The presence of heterotrophs and nitrifiers in an anammox system is an advantage since they can make use of any remaining oxygen in the system and leave the reactor in anaerobic condition that favors the growth of anammox bacteria [40].

This study was compared to previous studies on anammox enrichment in different bioreactor systems (Table 4) [32,40–45]. It was observed that 295–350 $\text{mg L}^{-1} \text{ N-NH}_4^+$ and

72–95 $\text{mg L}^{-1} \text{ N-NO}_2^-$ of influent used for anammox enrichment in this study was higher than the influent concentrations used for anammox enrichment processes in the previous studies. It is likely that the use of non-woven fabric material with a high surface area of 432 cm^2 is capable of retaining higher biomass for the anammox process to occur and speeds up the enrichment process. It is also suggested that the substrate concentration across the cross-section of the non-woven material might be reduced. Thus, the anammox bacteria existed in the inner part of the material are less exposed to substrate inhibition. During the 180 d enrichment process, no inhibition effect of the high nitrite and ammonium concentration was observed in the three reactors in this study, suggesting that about 400 mg L^{-1} of N-NO_2^- and N-NH_4^+ in the influent was acceptable for the enrichment of anammox bacteria in a 1.0 L up-flow anaerobic biofilm reactor. The anammox enrichment using a batch feeding mode is feasible, particularly for a simple and uncomplicated anammox batch process compared to fed-batch and continuous feeding mode reactors. Although the batch experiment recorded the highest removal efficiency for ammonium, it was not considered to be the most effective technique among the three feeding modes. The batch feeding mode reactor does not support a high loading capacity of the substrate. The concentration of ammonium and nitrite in the influent was kept low to prevent substrate inhibition.

The enrichment of anammox in a fed-batch mode is not a common culturing practice for anammox. However, this study discovered that it is possible to enrich anammox bacteria in a fed-batch reactor with a promising nitrogen removal efficiency, with percentages of N-NH_4^+ and N-NO_2^- removal of 68% and 98%, respectively. A fed-batch system allowed for the adaptation of anammox biomass to the new conditions, as the influent was introduced to the reactor in stages according to the nitrogen removal performance. The fed-batch mode of feeding avoids inhibitory effects of the substrate on the bacteria since the concentration of the ammonium and nitrite in the system are diluted [7].

In this study, a reactor with a continuous feeding mode was the most convenient and efficient operating system, as it allowed for the manipulation of the influent NLR according to the performance of anammox activity to provide the culture favorable conditions for the growth of anammox bacteria. Throughout the enrichment period, the anammox activity performance was observed to be most stable at an NLR of $0.33 \pm 0.01 \text{ kg N m}^{-3} \text{ d}^{-1}$, with an efficient nitrogen removal rate of $0.28 \text{ kg N m}^{-3} \text{ d}^{-1}$, suggesting that the value used as the maximum nitrogen loading capacity allowed for the reactor to operate efficiently. Anammox enrichment in bioreactor systems previously took longer than 200 d to achieve a stable and high performance of anammox activity [40,42,44]. However, a high nitrogen removal efficiency of anammox activity was achieved in this study within 180 d and is considered comparable to the most efficient anammox enrichment practices.

3.3. Identification and detection of the anammox bacteria

3.3.1. Partial 16S rDNA analysis

Sludge samples and biofilms on a non-woven carrier from each reactor on day 179 of enrichment were subjected

Table 4
Performance of different bioreactor systems for anammox enrichment

Reactor type	Feeding mode	Working volume (L)	Supporting material	Enrichment period (d)	Influent concentration (mg L ⁻¹)	HRT (d)	Highest N-NH ₄ ⁺ removal efficiency (%)	References
UBF	Batch	1	Non-woven	180	72 ± 2.94 (N-NO ₂ ⁻) 312 ± 5.30 (N-NH ₄ ⁺)	–	98.55 ± 0.29	This study
UBF	Fed-batch	1	Non-woven	180	72 ± 2.90 (N-NO ₂ ⁻) 350 ± 6.29 (N-NH ₄ ⁺)	–	67.75 ± 0.88	This study
UBF	Continuous	1	Non-woven	180	95 ± 3.10 (N-NO ₂ ⁻) 296 ± 7.31 (N-NH ₄ ⁺)	0.6	91.85 ± 0.97	This study
SBR	Continuous	7	–	150	50–70 (NO ₂ ⁻) 40–60 (N-NH ₄ ⁺)	–	80	[32]
Gas lift	Continuous	7	–	200	70 (NO ₂ ⁻) 70 (N-NH ₄ ⁺)	1	88	[40]
SBR	Continuous	1	–	–	375 (NO ₂ ⁻) 375 (N-NH ₄ ⁺)	0.625	78	
RBC	Continuous	1.7	–	100	350 (NO ₂ ⁻) 350 (N-NH ₄ ⁺)	0.167	99	[41]
UASB	Continuous	6	–	325	90 (TIN)	3.5	60 (TIN)	[42]
MBR	Continuous	4.8	Membrane	60	50 (NO ₂ ⁻) 50 (N-NH ₄ ⁺)	2	90	[43]
MBR	Continuous	8	Membrane	>250	552 (NO ₂ ⁻) 552 (N-NH ₄ ⁺)	2	–	[44]
MSBR	Continuous	5	Membrane	375	390 (NO ₂ ⁻) 390 (N-NH ₄ ⁺)	1	90	[45]

UBF: Up-flow biofilm, SBR: Sequencing batch reactor, RBC: Rotating biological contactor, UASB: Up-flow anaerobic sludge blanket, MBR: Membrane bioreactor, MSBR: Membrane sequencing batch reactor, and TIN: Total inorganic nitrogen.

to genomic DNA extraction, PCR amplification, and DNA sequencing. The primers Pla46-AMX368 were used to amplify a 16S rDNA fragment from the planctomycetes-anammox organisms. An additional primer pair (Pla46-AMX820) was used to amplify the 16S rDNA gene of *Candidatus Brocadia* sp. and *Candidatus Kuenenia* sp. Genomic DNA of anammox cultures and the biofilm from the non-woven carrier in column reactors 1, 2 and 3 formed a band with a product size of approximately 10 kb after being electrophoresed in a 1.0% w/v agarose gel and stained with ethidium bromide. PCR amplification using the primer combination of Pla46F-AMX368 formed a single band with a size of approximately 350 bp, while the primer pair Pla46F-AMX820 yielded a PCR band with a product size of approximately 900 bp. A sequence similarity search showed a 97%–99% similarity of the amplified 16S rDNA gene in the enrichment culture to the anammox organisms in NCBI GenBank. The reactor with the batch feeding mode was associated with anaerobic ammonium-oxidizing planctomycetes and '*Candidatus Kuenenia stuttgartiensis*', while the fed-batch and continuous reactors show a possible presence of '*Candidatus Kuenenia stuttgartiensis*' (Fig. 5), with a similarity level in the range of 97%–99%. This finding confirmed that anammox bacteria were present in the enrichment culture and contributed to the removal of ammonium and nitrite in the reactors.

3.3.2. Fluorescence in-situ hybridisation

Anammox biomass from sludge and non-woven fabric material was fixed and hybridized using rhodamine red and green-labeled probes targeting the 16S rDNA gene of anammox bacteria (AMX368) and were viewed under a fluorescence microscope. FISH results of the anammox culture at the end of the enrichment period (day 178) for the three reactors are shown in Fig. 6. Both fluorescence signals of eubacterial-targeted probes and an anammox-targeted probe were detected as a result of simultaneous hybridization using EUB338, EUB338II and EUB338III and AMX368 oligonucleotide probes, suggesting that anammox bacteria dominated the bacterial population in the enrichment culture. A large number of AMX368-hybridised cells were detected in the sludge samples on day 178 for the continuous feeding mode reactor. Anammox cells in the enriched biomass of the reactor with a continuous feeding mode could be observed as microcolonies and produced the brightest fluorescence compared to the other two reactors. Thus, the enrichment conditions using the continuous feeding mode were the best for anammox enrichment in this study. The anammox detection proved that the substantial removal of nitrogen in the enrichment culture was promoted by the anammox process.

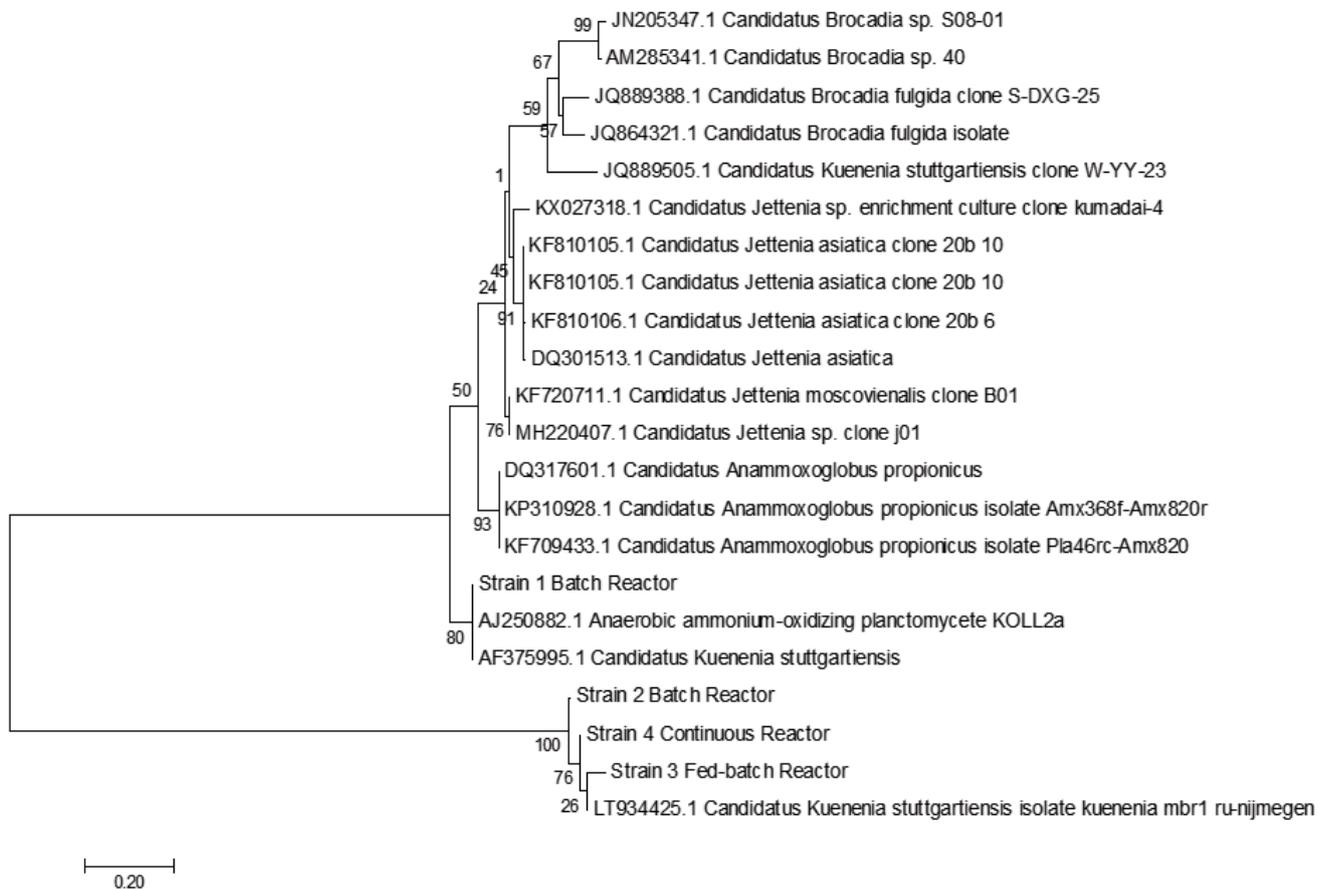


Fig. 5. Phylogenetic tree based on 16S rRNA sequences comparison of anammox bacteria obtained from all reactors. Numbers at the nodes show percentages of bootstrap values. Branch lengths corresponding to sequence differences are indicated by the scale bar.

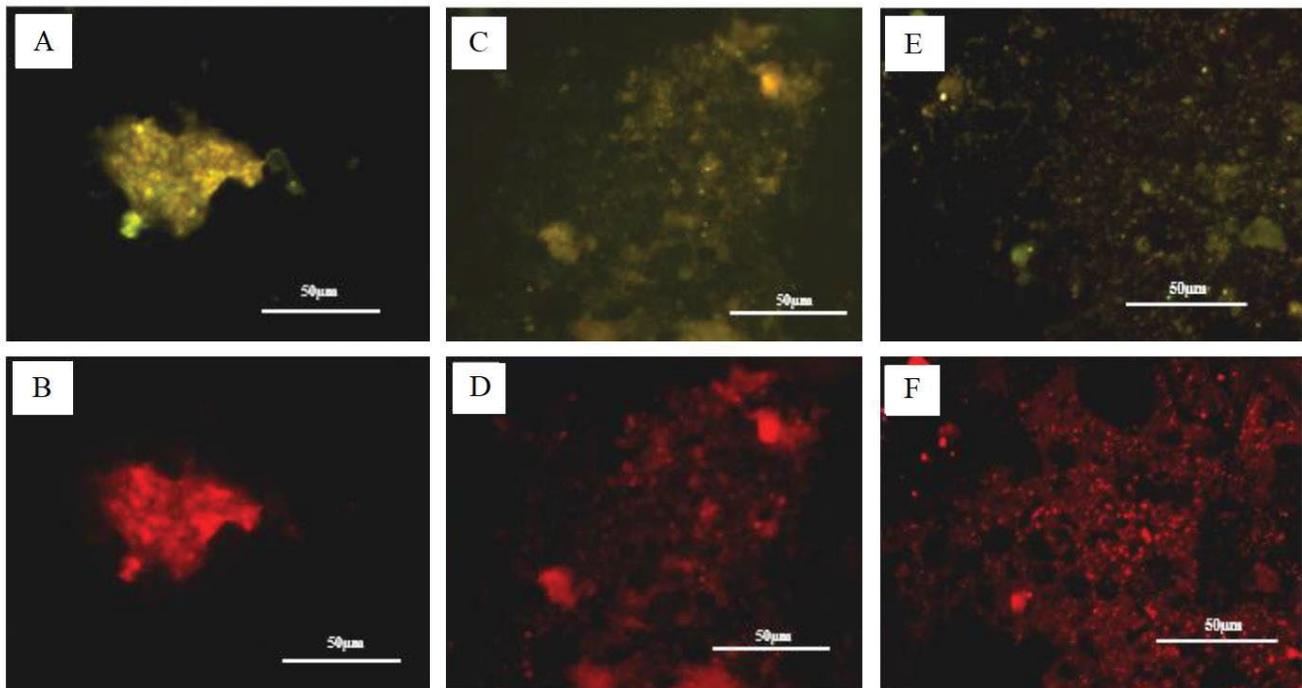


Fig. 6. Fluorescence in-situ hybridization of anammox culture on day 178 of enrichment using a reactor with a batch (a and b), fed batch (c and d), and continuous (e and f) feeding mode. Simultaneous hybridization with rhodamine-green labelled mixture of EUB338, EUB338II and EUB338III (green) and rhodamine red-labelled AMX368 (red).

4. Conclusions

In this study, a reactor with a continuous feeding mode was observed to be the most efficient strategy for anammox start-up in a UBF column reactor to produce a stable anammox population compared to the batch and fed-batch feeding modes. The reactor attained a high nitrogen removal rate and high specific anammox activity of $0.28 \text{ kg N}^{-1} \text{ m}^{-3} \text{ d}^{-1}$ and $0.34 \text{ g N-NH}_4^+ \text{ g-VSS}^{-1} \text{ d}^{-1}$, respectively. Microorganisms related to the anammox genera, '*Candidatus* Kuenenia stuttgartiensis' were detected in the reactors, revealing the presence of the anammox organisms in the culture. FISH analysis targeting the 16S rDNA gene of anammox bacteria further confirmed that the enriched microorganisms in the reactors were anammox bacteria, which appeared in the form of microcolonies, particularly for the reactor with a continuous mode of feeding.

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