

FEASIBILITY OF SUSTAINABLE NITROGEN REMOVAL: INTEGRATION OF PARTIAL
NITRITATION-ANAMMOX WITH MEMBRANE AERATED BIOFILM REACTOR (MABR)

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Abstract

The presence of nutrients, such as nitrogenous compounds, in wastewater can pose serious environmental concerns to water systems leading to reduced water quality and potential risks to the public health. Nutrient removal in conventional wastewater treatment systems is becoming increasingly more costly due to the extensive energy requirements and high aeration costs. Anaerobic ammonium oxidation (Anammox) is an alternative method for nutrient removal which can reduce overall treatment costs due to less aeration requirements and less sludge production. Anammox process can be implemented with other innovative technologies, such as membrane aerated biofilm reactors (MABR) to achieve effective and sustainable nutrient removal. A major challenge associated with Anammox process is effective control of nitrite oxidizing bacteria (NOB). High temperature in wastewater treatment systems can promote Anammox bacterial growth and inhibit NOB activity. This research aims to investigate the feasibility of integrating Anammox processes with MABR technologies and to examine the effects of high temperature aeration supplied to MABR systems on Anammox bacterial growth and NOB suppression. The nitrogen removal by Anammox bacteria in a lab-scaled MABR is examined to determine the impact of aeration temperature on inhibition of NOB.

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List of Abbreviations

AOA: Ammonia Oxidizing Archaea
AOB: Ammonia Oxidizing Bacteria
Anammox: Anaerobic Ammonium Oxidation
COD: Chemical Oxygen Demand
DO: Dissolved Oxygen
EPS: Extracellular Polymeric Substances
MABR: Membrane Aerated Biofilm Reactor
N: Nitrogen
NH₄⁺: Ammonium
NO: Nitric Oxide
NO₂⁻: Nitrite
NO₃⁻: Nitrate
N₂O: Nitrous Oxide
NOB: Nitrite Oxidation Bacteria
O₂: Oxygen
PBS: Phosphate Buffer System
PCR: Polymerase Chain Reaction
PD-A: Partial Denitrification-Anammox
PN-A: Partial Nitritation-Anammox
qPCR: Quantitative Polymerase Chain Reaction
sCOD: soluble chemical oxygen demand
SND: Simultaneous Nitrification and Denitrification
SS: Suspended Solids

1. Introduction

1.1 Overview

Wastewater contains nitrogenous nutrients which can cause environmental concerns such as eutrophication and public health risks when discharged into aquatic systems (Hu et al., 2012; Rahimi, 2020). Nitrogen removal in wastewater treatment is often associated with extensive energy consumption and high treatment costs (Rahimi, 2020; Shourjeh et al., 2021; Walker, 2021). Conventional wastewater treatment typically includes nitrification and denitrification process which is associated with high oxygen requirements, long hydraulic retention times, and large footprint of land. These factors all contribute to the high treatment costs in conventional wastewater treatment systems. Additionally, the sludge that is produced needs to be managed for disposal and can comprise of up to 40% of the overall wastewater treatment management costs (Cho et al., 2020; Shourjeh et al., 2021; Walker, 2021). Anaerobic ammonium oxidation (Anammox) is an emerging technology first discovered in the 1990's that can convert ammonia (NH_4) and nitrite (NO_2) into nitrogen gas under anoxic conditions (Mulder et al., 1995). Anammox process can be operated with less aeration and a reduced footprint while producing less sludge, which can reduce overall wastewater treatment costs by providing more sustainable and efficient nutrient removal (Cho et al., 2020; Shourjeh et al., 2021). Membrane aerated biofilm reactors (MABR) can be integrated with Anammox process to provide an ideal environment for simultaneous biological reactions. Development of Anammox and MABR integration is an innovative technology that has the potential to be utilized as an alternative method for wastewater treatment.

1.2 Biological Reactions in Anammox Process with MABR

Anammox bacteria cultivation must be coupled with other bacterial groups due to its dependence on nitrite substrates which is not commonly found in natural waters (Ren et al., 2022). As shown in Figure 1.1, other bacterial groups that contribute to nitrogen removal include ammonia oxidizing bacteria (AOB), nitrite oxidizing bacteria (NOB), and denitrifying bacteria. Anammox can obtain nitrite substrate from AOB, which is known as partial nitrification (PN) process, or from denitrifying bacteria, which is known as partial denitrification (PD) process (Ren et al., 2022; Zhang et al., 2019).

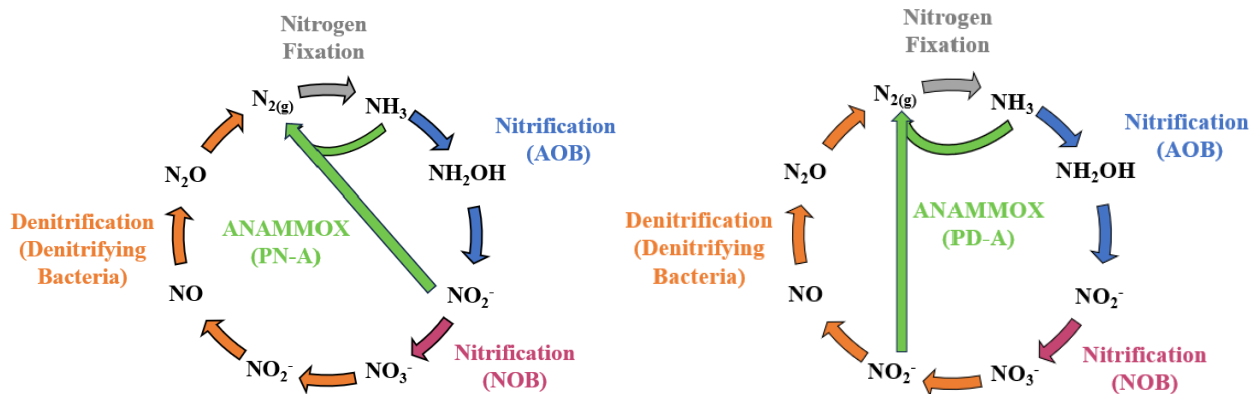
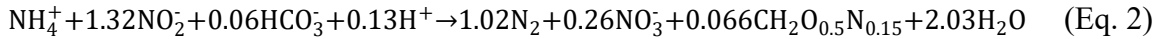


Figure 1.1. Schematic diagram of the key components in the nitrogen cycle for anammox processes.

In conventional wastewater treatment processes, nitrification processes are achieved by AOB and NOB, and denitrification processes are achieved by denitrifying bacteria. Nitrification is a two-step process involving both AOB and NOB (Soliman and Eldyasti, 2018). In the first step of nitrification, AOB oxidizes ammonia to hydroxylamine (NH_2OH), and then oxidizes hydroxylamine to nitrite. In the second step of nitrification, NOB oxidizes nitrite to nitrate. For denitrification, nitrate is converted to nitrite, nitric oxide (NO), nitrous dioxide (N_2O), and finally to nitrogen gas (N_2) by denitrifying bacteria (Trinh et al., 2021). The biological reactions for AOB, Anammox bacteria, NOB, and denitrifying bacteria can be described by Eq. 1-4, respectively (Soliman and Eldyasti, 2018; Trinh et al., 2021).



For the Anammox-MABR system to thrive, the four groups of bacteria (AOB, Anammox bacteria, NOB, and denitrifying bacteria) must coexist. One of the main advantages of MABR technologies is its capability to support simultaneous nitrification and denitrification (SND) reactions (Landes et al., 2021; Matsumoto et al., 2007; Zhang et al., 2020). As shown in Figure 1.2, the biofilm consists of an inner aerobic layer to support AOB and NOB growth and an outer anoxic layer to support Anammox and denitrifying bacteria growth (Landes et al., 2021). The nitrogen gas produced by both Anammox bacteria and denitrifying bacteria helps to maintain low dissolved oxygen (DO) concentration or anoxic conditions in the bulk solution of the Anammox-MABR system (Landes et al., 2021). A major challenge for successful integration of Anammox and MABR technologies is the effective control of NOB while accomplishing sufficient treatment (Bunse et al., 2020; Ma et al., 2015; Pathak et al., 2022).

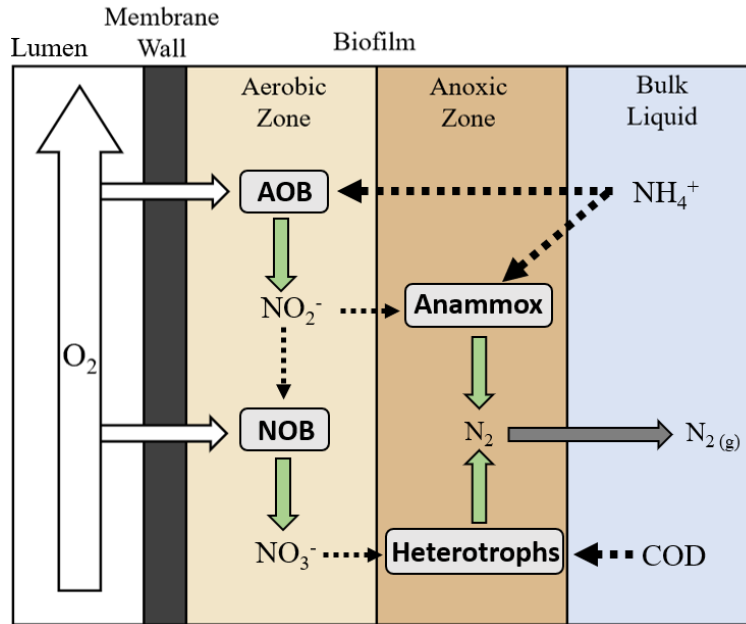


Figure 1.2. Schematic diagram of the expected biological reactions in the biofilm profile for Anammox-MABR systems.

1.3 Temperature Effect on NOB Suppression

The operational temperature of wastewater treatment processes can impact the metabolism of the bacteria in the system (Alisawi, 2020). Operation under mainstream processes between 10°C to 20°C have minimal differences in growth rate between AOB and NOB (Laureni et al., 2019). In contrast, a higher maximum growth rate for AOB compared to NOB is observed at higher temperatures greater than 20°C (Laureni et al., 2019). Due to the dominant growth rate of AOB over NOB at higher temperatures, operation of bioreactors under temperatures higher than 30°C was reported to be feasible for NOB elimination in nitrification processes (Chen et al., 2020; Laureni et al., 2019). A recent study demonstrated that operation under high temperatures (36°C) could maintain stable nitrite accumulation with low nitrate concentrations (Chen et al., 2020). However, high temperature operation is still unreliable since large energy requirements are associated with maintaining high water temperatures leading to increased operational costs (Chen et al., 2020).

High temperature reactor operation can be reliably incorporated in MABRs by supplying high temperature aeration through the lumen of the membranes. In comparison to high water temperatures, maintaining aeration temperature supplied to MABR is more feasible and requires less energy consumption. Since MABRs supports an inner aerobic layer, the high temperature aeration supplied could inhibit NOB activity by providing more favourable growth conditions for AOB allowing AOB to outcompete NOB. Operation of Anammox-MABR systems with high temperature aeration can effectively control and suppress NOB while reducing the competition for nitrite substrate between Anammox bacteria and NOB.

1.4 Research Objectives

The research objectives presented in this thesis are:

- To demonstrate nitrogen removal by Anammox bacteria in a lab-scale MABR.
- To identify the limiting factors for PN/A in MABR.
- To examine the effects of high temperature air supplied in MABR systems on Anammox bacteria growth and NOB inhibition.

2. Literature Review: Factors for Optimal Growth Conditions

2.1 Overview

Anammox processes often relies on other bacteria to provide suitable substrate for utilization. For PN-A processes, AOB converts ammonia into nitrite to provide a supply of nitrite substrate for the Anammox bacteria. The optimal growth conditions for Anammox bacteria and AOB must be satisfied for these two bacteria to coexist for effective nitrogen removal. Additionally, since NOB and Anammox bacteria both compete for nitrite substrate, inhibition of NOB growth is desirable, however, controlling NOB growth in PN-A reactors have proved to be difficult for low strength wastewater (Ma et al., 2015).

2.2. Dissolved Oxygen

AOB are aerobic bacteria and thrive best in oxygen rich systems, however, even in limited DO concentrations, AOB was observed to still be capable of growth under anoxic conditions (Xie et al., 2017). This suggests that AOB can adapt to variable DO concentrations (Geets et al., 2006; Xie et al., 2017). In contrast, since Anammox bacteria prefer low DO or anoxic conditions, Anammox reactors are typically operated at DO concentrations less than 0.5 mgO₂/L (Cho et al., 2020; Shourjeh et al., 2021) or even less than 0.01 mgO₂/L (Cho et al., 2020). Since NOB is also dependent on AOB to provide nitrite, if there is low DO concentration or anoxic conditions in the reactor system and nitrite has accumulated, Anammox bacteria are more likely to consume the nitrite instead of NOB (Ma et al., 2015). To implement this, as nitrite accumulates, aeration would be turned off (Ma et al., 2015). By providing intermittent aeration, the reactor system can alternate between low DO conditions for AOB growth for a steady supply of nitrite, and anoxic conditions for Anammox bacteria growth for nutrient removal while suppressing the activity of NOB in the

system. Furthermore, during low DO conditions, as AOB oxidizes ammonia to nitrite, DO is also consumed which enhances the Anammox process by protecting Anammox bacteria from oxygen inhibition (Cho et al. 2020)

2.3. Temperature

Many studies have shown that AOB and Anammox bacteria are majorly inhibited under low temperature conditions with significantly reduced activities and abundance (Fernandes et al., 2018; Le et al., 2022; Lotti et al., 2015; Xie et al., 2017). Furthermore, temperature changes in a reactor system can impact the efficiency of nitrogen removal and the microbial community structure. At temperatures greater than 24°C, the growth rate of NOB was observed to be lower than AOB, however, at low temperatures, NOB are often dominant over AOB (Soliman and Eldyasti, 2018). For conventional wastewater treatment supporting AOB and NOB, the operational temperature is typically between 25°C to 35°C (Krzeminski et al., 2012). From previous studies, the growth rate of AOB and Anammox at 20°C to 30°C was 0.7-0.9d⁻¹ and 0.05-0.09d⁻¹, respectively (Cho et al., 2020). Anammox bacteria have a substantially slower growth rate compared to AOB which makes them more sensitive to changes in operating temperature (Cho et al., 2020). The optimal temperature for Anammox bacteria growth has been reported to be 35°C to 40°C (Chen and Jin, 2017; Cho et al., 2020; Shourjeh et al., 2021). Anammox processes at temperatures higher than 30°C are capable of producing high anammox activity and efficient nitrogen removal (Cho et al., 2020).

There has been an increasing interest in low temperature operations for wastewater treatment technologies due to drastic differences in raw influent into treatment facilities in regions with highly variable climates (Cho et al., 2020; Krzeminski et al., 2012). However, studies have shown that Anammox processes operated in low temperature conditions (<15°C) were not

sustainable and resulted in low nitrogen removal rates (Cho et al. 2020; Dosta et al., 2008). Anammox bacteria adaptability to temperatures from 10°C to 30°C has been observed, however, their activity was lower than at temperatures from 30°C to 35°C in comparison (Cho et al., 2020).

2.4. pH

For wastewater treatment systems, a neutral pH ranging between 6.5-9.0 is typically maintained. In presence of ammonia and nitrite, changes in pH can influence the concentration of free ammonia and free nitrous acid which is known to have inhibitory effect on both AOB and NOB (Shourjeh et al., 2021; Wang et al., 2023). Studies suggests that the optimal pH range for supporting AOB and Anammox growth are 7.0-8.0 (Shourjeh et al., 2021) and 6.7-8.3 (Chen and Jin, 2017; Xie et al., 2017), respectively. Moreover, favourable pH range for PN systems was found to be 7.5-7.8 (Shourjeh et al., 2021). A pH range of 7.5-8.5 have been suggested for effective NOB inhibition and nitrite accumulation (Shourjeh et al., 2021).

2.5. Carbon Sources

Municipal wastewater typically contains high concentrations of COD with COD/N ratio ranging from 10-14 (Zhang et al., 2019). In the presence of organic carbon sources, Anammox bacteria must compete with heterotrophic denitrifiers for nitrite substrates (Zhang et al., 2019). Studies have shown that the activity of Anammox bacteria can not be sustained in MABRs and there was a decrease in nitrite accumulation when the COD/N ratio is higher than 2 (Cho et al., 2020, Jin et al., 2012). Pretreatment of the raw influent should be considered to lower the COD in wastewater for effective Anammox processes while producing valuable end products such as biogas (Zhang et al., 2019). For a COD/N ratio less than 1, some studies implementing Anammox process and SNAD processes did not observe any adverse effects for either AOB or Anammox

bacteria (Anjali and Sabumon, 2017). For PN-A, the suggested optimal COD/N ratio is less than 0.5 (Miao et al., 2018).

3. Materials and Methods

3.1 Effluent Air Linear Velocity and Residence Time

The membranes used in the Anammox-MABR system are hollow fibre membranes from the ZeeLung module (SUEZ, Veolia Water Technologies & Solutions, Canada). The ZeeLung module contains 40 air permeable membrane fibres with 70 μ inner diameter and 95 μ m outer diameter. The air flow rate can be used to estimate the average effluent air linear velocity through the membrane can be described by Eq. 5 to determine if sufficient aeration was provided. Furthermore, the residence time of the air can be determined from Eq. 6.

$$\text{Average Linear Effluent Linear Velocity} = \frac{\text{Air Flow Rate}}{\text{Cross Sectional Area of the Flow}} \quad (\text{Eq. 5})$$

$$\text{Residence Time } (\tau_{\text{air}}) = \frac{\text{Average Linear Effluent Linear Velocity}}{\text{Length of Membrane}} \quad (\text{Eq. 6})$$

A preliminary MABR system was assembled consisting of 6 different membranes, each 50cm long, with three different temperatures of air supplied through the membranes (20°C, 40°C, 60°C). To improve the representation of the research objectives, the main findings from the preliminary MABR system indicated that the desired temperature of air was not retained for the entire residence time of air through the membrane. More details for the results from the preliminary MABR system can be found in Appendix A.

3.2 Anammox-MABR Configuration

The Anammox-MABR was constructed using 4 membrane fibers, each 7.5cm long. The preferred length of the membrane in the Anammox-MABR was determined to be less than 10cm from the results of a preliminary MABR system. Two different temperature air was supplied through the membranes to the Anammox-MABR system: 20°C and 60°C. The air was maintained

at the desired temperature by directing air flow through copper tubing in a constant temperature heated circulating bath. The temperature of air pumped into the Anammox-MABR was measured (AstroAI RMS 6000 Digital Multimeter), however, due to the heat loss along the length of the tubing, the inlet air temperature could not be accurately determined. The air temperature for each membrane will be represented based on their initial proposed aeration temperature condition. A schematic diagram and a picture of the Anammox-MABR system used in this study is shown in Figure 2. The ports 1 and 3 corresponds to the 20°C aeration temperature condition, while the ports 2 and 4 corresponds to the 60°C aeration temperature condition.

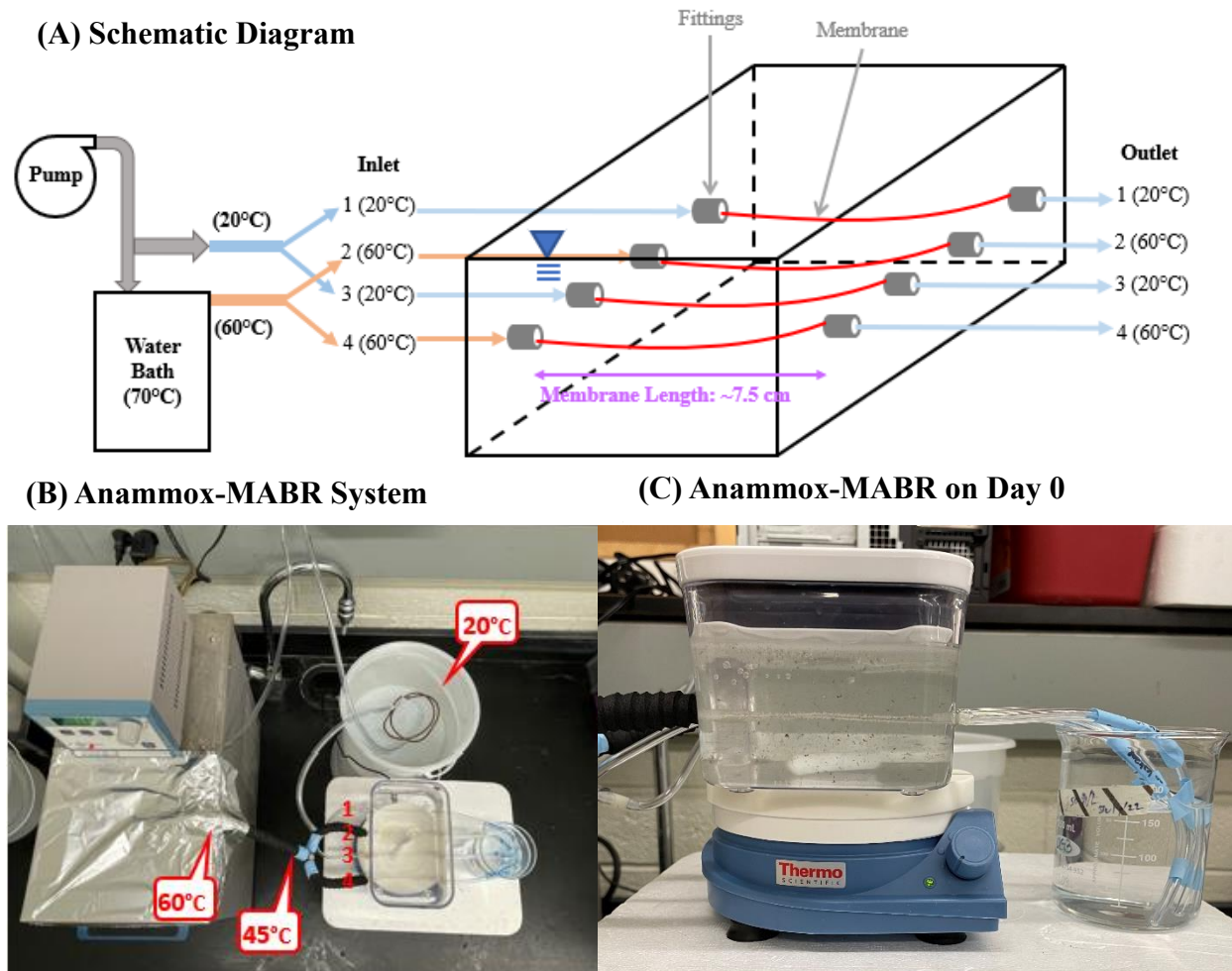


Figure 1. Schematic diagram of anammox-MABR configuration with 20°C (Port 1 & 3) and 60°C (Port 2 & 4) air, (B) Picture of anammox-MABR used in this study, and (C) the Anammox-MABR on Day 0.

The Anammox-MABR was enriched with a seed inoculum from a PN-A pilot MABR operated under 20°C from SUEZ, Veolia Water Technologies & Solutions, Canada. The total reactor volume was 600mL and the enrichment solution provided to the Anammox-MABR system includes: 38 mgN/L NH₄Cl, 50 mgN/L NaNO₂, 500 mg/L NaHCO₃, 50mM PBS (i.e. 8.66 g/L Na₂HPO₄·7H₂O, 2.14 g/L NaH₂PO₄, 0.13 g/L KCl), 0.1mL of 100X Vitamins, and 2.5mL of 10X Minerals. The composition of the vitamins and minerals can be found according to (Daniele et al., 1996; Maiti et al., 2005).

3.3 Experimental Methods

Samples from the bulk solution were collected from a port at the top of the Anammox-MABR system and analyzed. The pH (SevenMulti, Mettler-Toledo International Inc., USA), DO concentration (OX 4100H VWR, USA), and temperature were monitored daily. The samples were analyzed daily for ammonia (NH₄-N TNT832 HACH, USA), nitrite (NO₂-N TNT840 HACH, USA), nitrate (NO₃-N TNT835 HACH, USA), and chemical oxygen demand (COD Digestion Vials HACH, USA). The total suspended solids (TSS) and volatile suspended solids (VSS) were analyzed weekly.

The Anammox-MABR system was operated with a hydraulic retention time (HRT) of 14 days with an exchange ratio of 50%. The influent enrichment solution was purged with nitrogen (N₂) gas before addition into the Anammox-MABR system.

3.4 Nitrogen Removal Mass Balance Equations

The theoretical biological reactions and molar ratios as described in Eq. 1-4 were used to derive the mass balance equations, Eq. 7-10, to determine nitrogen removal and contribution from each target bacterial groups. Eq. 11-14 can be determined by solving the Eq. 7-10 for x_1, x_2, x_3, x_4 .

The estimated daily nitrogen removal (mg N/L/d) from AOB, Anammox bacteria, NOB, and denitrifying bacteria can be described as x_1, x_2, x_3, x_4 , respectively.

$$\Delta NH_4 = -x_1 - x_2 \quad (\text{Eq. 7})$$

$$\Delta NO_2 = x_1 - 1.32x_2 - x_3 \quad (\text{Eq. 8})$$

$$\Delta NO_3 = 0.26x_2 + x_3 - x_4 \quad (\text{Eq. 9})$$

$$\Delta sCOD \left(\frac{4 \text{ mol } NO_3^-}{5 \text{ mol } O_2} \right) \left(\frac{1 \text{ mol } N}{1 \text{ mol } NO_3^-} \right) \left(\frac{MW_N}{MW_{O_2}} \right) = -x_4 \quad (\text{Eq. 10})$$

$$x_1 = \left[\frac{-1.06\Delta NH_4 + \Delta NO_2 + \Delta NO_3 - 0.35\Delta sCOD}{2.06} \right] \quad (\text{Eq. 11})$$

$$x_2 = \frac{-\Delta NH_4 - \Delta NO_2 - \Delta NO_3 + 0.35\Delta sCOD}{2.06} \quad (\text{Eq. 12})$$

$$x_3 = \Delta NO_3 - 0.35\Delta sCOD - 0.26 \left[\frac{-\Delta NH_4 - \Delta NO_2 - \Delta NO_3 + 0.35\Delta sCOD}{2.06} \right] \quad (\text{Eq. 13})$$

$$x_4 = -0.35\Delta sCOD \quad (\text{Eq. 14})$$

3.5 16S Amplicon Sequencing Methods

The genomic DNA from the biofilm membrane samples was extracted using the DNA isolation method as described in (Stearns et al., 2015). The samples were further processed for purification using the MagMAX Express 96-Deep Well Magnetic Particle Processor with the MagMAX DNA Multi-Sample Kit (Applied Biosystems, USA). The purified DNA was used to amplify the V4 region of the 16S rRNA gene by conducting PCR using adapted primers as described by (Bartram et al., 2011). The primers were modified to 515f (GTG YCA GCM GCC GCG GTAA) and 806r (GGA CTA CNV GGG TWT CTA AT). The PCR protocol for the 515f/806r primer pair with the denaturing, annealing, and extension times and temperature can be described

in Table 1. The PCR components include 50 ng of DNA templates with 1U of Taq, 1x buffer, 1.5 mM MgCl₂, 0.4 mg/mL BSA, 0.2 mM dNTPs, and 5 pmol of each primer. The PCR products were checked by gel electrophoresis and the positive amplicons were sent for sequencing. All the amplicons were visually normalized based on the band intensity on the gel and pooled. The final pool was cleaned using the Promega ProNex Size-Selective Purification System (Promega, USA).

Table 1. PCR protocol for 16S amplicon sequencing used in this study.

Primer	Hold	Denaturation	Annealing	Extension*	Hold
515f	94°C	94°C	50°C	72°C	72°C
806r	5min	30s	30s	30s	10min

*After extension, repeat denaturation-annealing-extension steps 35X.

DNA sequence reads were filtered and trimmed based on the quality of the reads using Cutadapt with a minimum quality score of 30 and a minimum read length of 100bp (Martin, 2011), and then the amplicon sequence variants were resolved from the trimmed raw reads by DADA2 (Callahan et al., 2016). Finally, bimeras were removed and taxonomy was assigned using the RDP classifier against the SILVA database version 1.3.8.

The genomic DNA extraction, PCR amplification, and sequencing with the Illumina MiSeq platform (paired-end reads, 2 × 300 base pairs [bp]) was conducted at the Farncombe Institute (McMaster University, Hamilton, ON, Canada).

16S amplicon sequencing analysis was conducted for the biofilm samples from each membrane for the two temperature conditions (20°C and 60°C) and for the bulk solution in the Anammox-MABR system. The microbial species were then assigned to a target bacterial group of interest: AOB, Anammox bacteria, NOB, denitrifying bacteria, and others (i.e., nitrogen/carbon fixation bacteria, sulfur reducing bacteria, other heterotrophic bacteria, fermenters, hydrolysis, etc.).

3.6 qPCR Methods

The DNA from the biofilm membrane samples were extracted using the Soil DNA Isolation Kit (Norgen Biotek Corp., Canada). Each biofilm membrane sample was 2cm in length and was weighed prior to DNA extraction. The specific targets of interest are the 16S rRNA genes for general bacteria, AOB, Anammox bacteria, and NOB as shown in Table 2.

Table 2. Specific target amplicons for qPCR and primers with associated sequences used in this study.

Target	Primer	Sequence	Base Pair Length
General Bacteria	16s rRNA 1055f	5' – ATG GCT GTC GTC AGCT – 3'	1392-1055+1= 338 bp
	16s rRNA 1392r	5' – ACG GGC GGT GTG TAC – 3'	
AOB	amoA-1f	5' – GGG GTT TCT ACT GGT GGT – 3'	822-349+1= 474 bp
	amoA-2r	5' – CCC CTC KGS AAA GCC TTC TTC – 3'	
Anammox Bacteria	Amx 809f	GCC GTA AAC GAT GGG CACT	1066-809+1= 258 bp
	Amx 1066r	AAC GTG TCA CGA CAC GAG CTG	
	Amx 368f Amx 820r	5' – TTC GCA ATG CCC GAA AGG – 3' 5' – AAA ACC CCT CTA CTT AGT GCCC – 3'	820-368+1= 453 bp
NOB	NSR 113f	5' – CCT GCT TTC AGT TGC TAC CG – 3'	1264-1113+1= 152 bp
	NSR 1264r	5' – GTT TGC AGC GCT TTG TAC CG – 3'	

The PCR protocol for each primer pair, the denaturing, annealing, and extension times and temperature can be described in Table 3. The PCR components include 12.5 µL iTaq Universal SYBR Green Supermix (BioRad, USA), 9.5 µL DI Water, 1 µL forward primer, 1 µL reverse primer, and 1 µL DNA Template.

Table 3. PCR Protocols for the primers used in this study.

Target	Primer	Hold	Denaturation	Annealing	Extension*	Hold
General Bacteria	16s rRNA 1055f	95°C	95°C	55°C	72°C	72°C
	16s rRNA 1392r	5min	45s	30s	30s	5min
AOB	amoA-1f	95°C	95°C	55°C	72°C	72°C
	amoA-2r	5min	45s	45s	1min	5min
Anammox Bacteria	Amx 809f	95°C	94°C	55°C	72°C	72°C
	Amx 1066r	5min	20s	20s	20s	5min
	Amx 368f	95°C	95°C	59°C	72°C	72°C
	Amx 820r	5min	45s	45s	45s	5min
NOB	NSR 113f	95°C	94°C	65°C	72°C	72°C
	NSR 1264r	5min	30s	30s	30s	5min

*After extension, repeat denaturation-annealing-extension steps 40X.

The PCR protocol was validated using gel electrophoresis as shown in Figure 3.1 and compared to the reference band to match the bp length of each primer from Table 2.

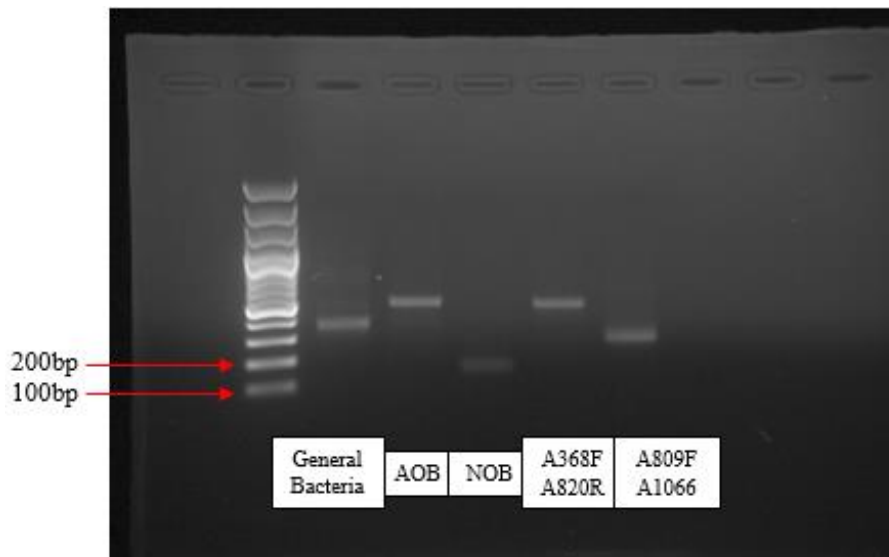


Figure 3.1. Picture of the gel electrophoresis results for primer validation.

The PCR products are purified using the PCR Kleen Spin Columns (BioRad, USA) and then analyzed using Nanodrop Spectrophotometer (Thermo Fisher Scientific, USA). The DNA concentration of purified PCR product is quantified using Qubit dsDNA Quantification Assays (Thermo Fisher Scientific, USA).

Standard curves can be constructed by conducting qPCR for 10-fold serial dilution of the purified PCR product. The qPCR protocol for each primer pair, the denaturing, annealing, and extension times and temperature can be described in Table 4. The qPCR components include 10 μL iTaq Universal SYBR Green Supermix (BioRad, USA), 6 μL DI Water, 1 μL forward primer, 1 μL reverse primer, and 2 μL DNA Template.

Table 4. qPCR Protocols for the primers used in this study.

Target	Primer	Hold	Denaturation	Annealing	Extension*
General	16s rRNA 1055f	95°C	98°C	55°C	72°C
Bacteria	16s rRNA 1392r	2min	5s	30s	30s
AOB	amoA-1f	95°C	95°C	55°C	72°C
	amoA-2r	3min	30s	30s	45s
Anammox	Amx 809f	94°C	94°C	55°C	72°C
	Amx 1066r	30s	10s	15s	15s
Bacteria	Amx 368f	98°C	98°C	59°C	72°C
	Amx 820r	2min	5s	30s	30s
NOB	NSR 113f	95°C	95°C	53°C	72°C
	NSR 1264r	3min	30s	30s	30s

*After extension, repeat denaturation-annealing-extension steps 40X. Following PCR, melt curves were generated between 65°C to 95°C in 0.5°C increments to ensure PCR specificity.

The gene copy number can be calculated from the DNA concentration and the bp length of the primer used as shown in Eq. 15. The efficiency of each standard curve constructed can also be calculated using Eq. 16.

$$\frac{\text{copies}}{\mu\text{L}} = \frac{N_A \times 10^{-9}}{(\text{length in bp}) \times 660} \times (\text{ng}/\mu\text{L}) = 9.26 \times 10^{11} \times \left(\frac{\text{ng}/\mu\text{L}}{\text{bp}} \right) \quad (\text{Eq. 15})$$

$$\text{Efficiency (\%)} = \left(10^{-\frac{1}{\text{Slope}}} - 1 \right) * 100\% \quad (\text{Eq. 16})$$

The standard curves for each primer are shown in Figure 3.2 along with their respective efficiencies. The relative abundance of the sample can then be determined by conducting qPCR analysis as described in Table 4 on the extracted genomic DNA and calculating the gene copy number from Figure 3.2 for each respective target amplicon of interest.

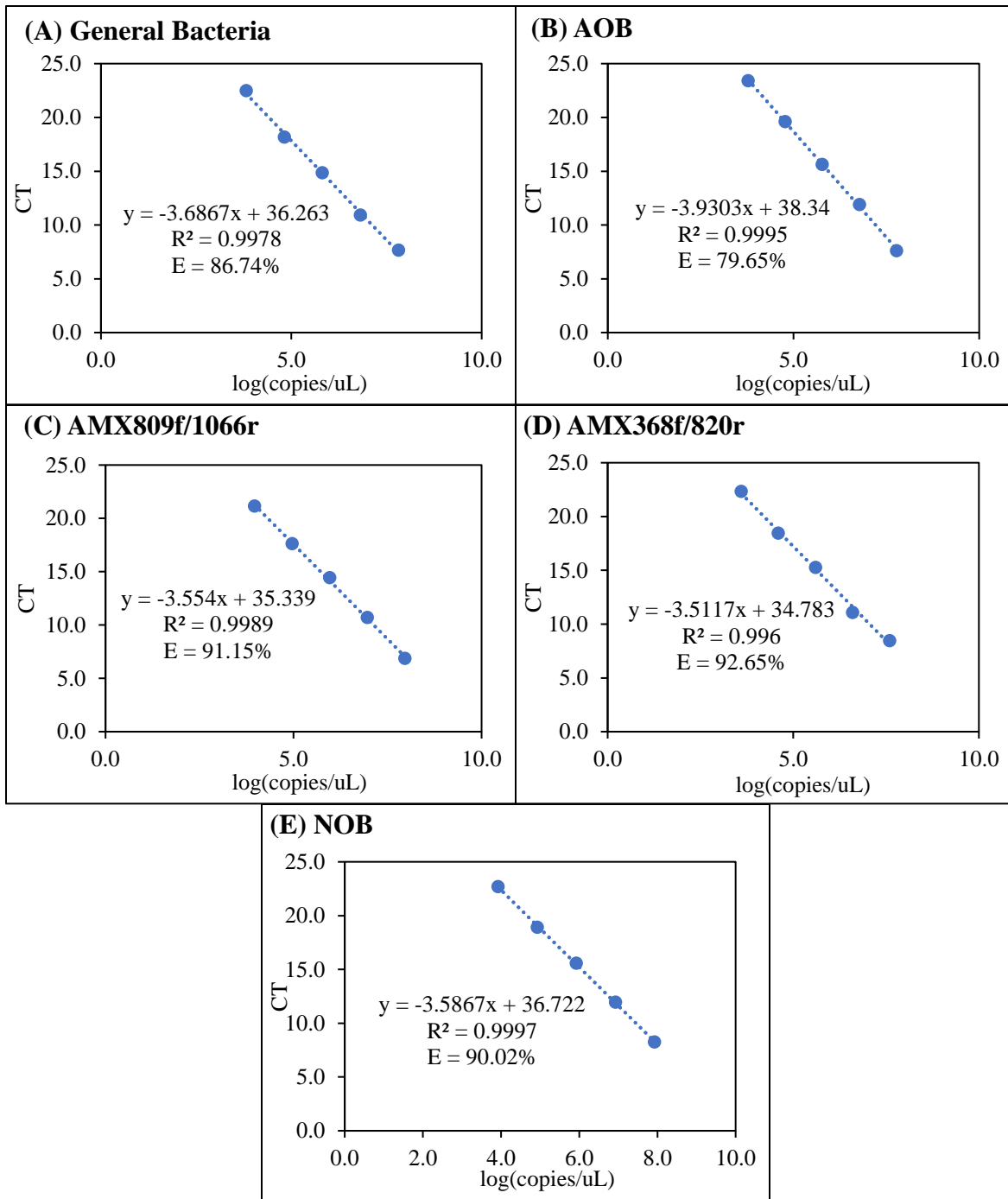


Figure 3.2 Standard curves constructed from qPCR methods for each primer pair for (A) General Bacteria, (B) AOB, (C) Anammox bacteria (AMX809f/1066r), (D) Anammox bacteria (AMX368f/820r), (E) NOB.

4. Results and Discussion

4.1 Anammox-MABR Performance

The overall performance of the Anammox-MABR system was determined by monitoring key operational parameters throughout the 111 days operational period such as nitrogen concentrations in the bulk solution of the Anammox-MABR system. The bacterial groups expected to contribute to nitrogen removal include AOB, Anammox, NOB, and denitrifying bacteria. For all four bacterial groups of interest to coexist, there will be competition for substrate within the Anammox-MABR system. In PN-A systems, the Anammox bacteria will compete with AOB for ammonia, and NOB for nitrite (Zhang et al., 2019). As shown in Figure 4.1, there is a rapid consumption of ammonia throughout the entire operational period. Due to the rapid ammonia consumption, NH_4Cl is periodically injected into the Anammox-MABR system to maintain sufficient ammonia substrate. This consumption can mainly be attributed to AOB converting ammonia to nitrite, and it can be considered the rate limiting reaction due to their low yield and nitrite dependence from Anammox bacteria. From Day 0 to Day 20, there is an indication of a low presence of NOB due to the increase in nitrite and stable nitrate concentrations in the system. However, after Day 20, nitrite decreased while nitrate increased drastically, which implies high NOB activity. Following Day 40, the nitrite concentration was maintained consistently at $\sim 45\text{mgN/L}$ despite the rapid increase in nitrate which demonstrates some degree of NOB were controlled and suppressed in the Anammox-MABR system.

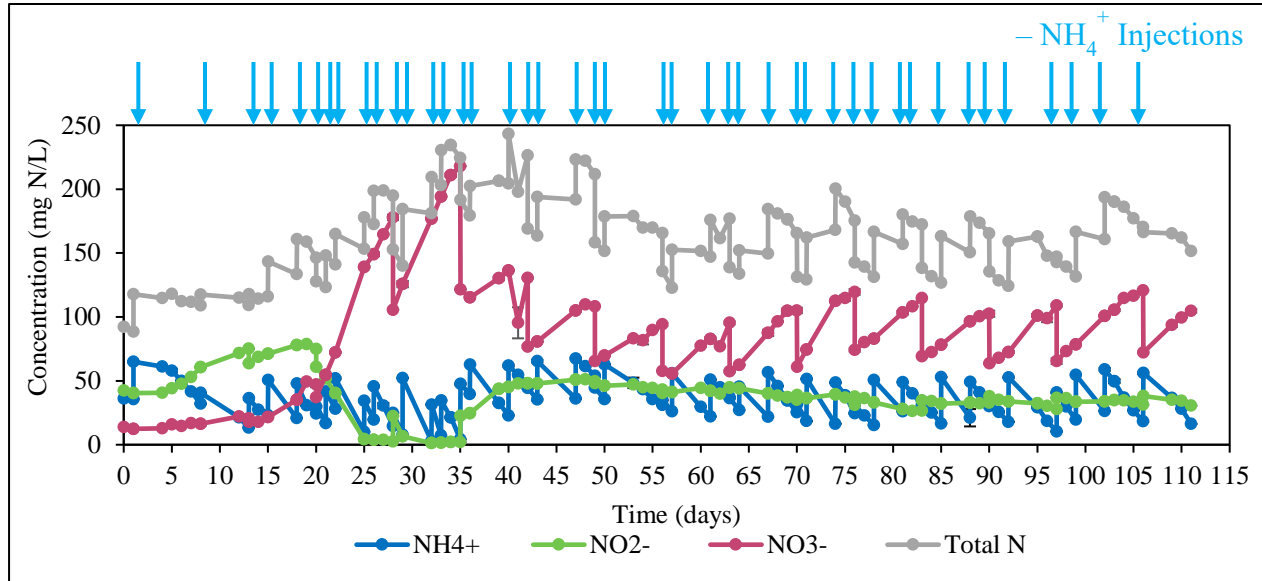


Figure 4.1. Nitrogen concentrations for Anammox-MABR performance throughout 111 days operational period with blue arrows indicating NH_4Cl injections.

Optimal growth of Anammox bacteria is dependant on the ratio of the substrate available for utilization, as a result, the nitrite to ammonia molar ratio is a crucial parameter to monitor for Anammox systems. From the Anammox reaction for anoxic conditions in Eq. 2, the optimal nitrite to ammonia molar ratio is 1.32 (Soliman and Eldyasti, 2018; Trinh et al., 2021). As shown in Figure 4.2, the nitrite to ammonia molar ratio during Day 0 to Day 40 was unstable indicating that the growth of Anammox bacteria may not be ideal, however, from Day 40 onwards, the nitrite to ammonia molar ratio was stable and only fluctuates due to addition of NH_4Cl to maintain sufficient ammonia substrate. Overall, the average nitrite to ammonia molar ratio for the Anammox-MABR system was 1.20 with a 9.5% difference in comparison to the optimal ratio.

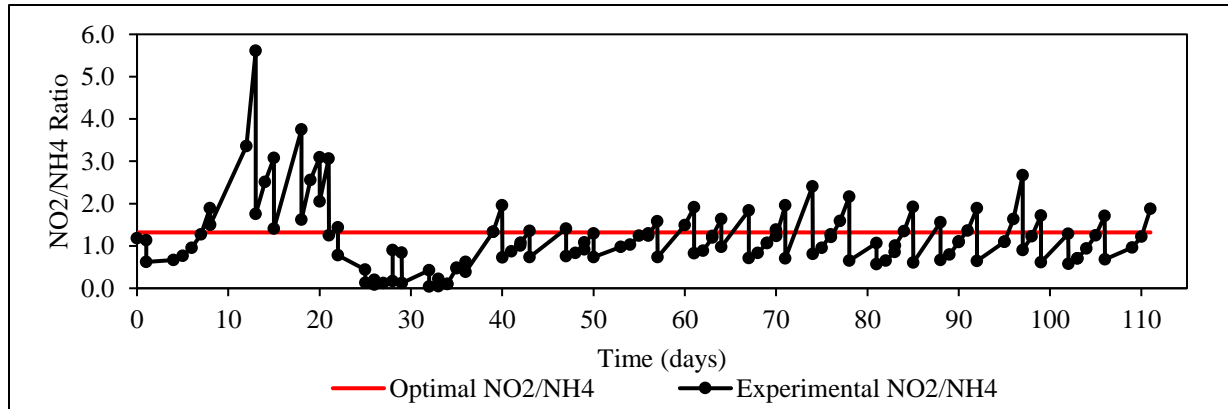


Figure 4.2. Comparison of optimal and experimental nitrite to ammonia molar ratio for Anammox-MABR performance throughout 111 days operational period.

Other operational parameters such as pH, reactor temperature, and DO were also monitored for the Anammox-MABR system as shown in Figure 4.3. The optimal pH range for AOB and anammox bacteria growth are 7.0-8.0 (Shourjeh et al., 2021) and 6.7-8.3, respectively (Chen and Jin, 2017; Xie et al., 2017). To satisfy growth conditions for all four bacterial groups, the pH in the Anammox-MABR system was maintained at a range of 7.0-7.5 with periodic NaOH injections. Additionally, the reactor temperature was kept consistently at $24.4 \pm 1.0^\circ\text{C}$. Despite Anammox bacteria requiring anoxic conditions, the Anammox-MABR system could not achieve low DO conditions until Day 40. The average DO concentration from Day 0 to Day 40 was 1.78 ± 1.62 mgO_2/L , and then from Day 40 onwards, the average DO concentration was maintained at 0.36 ± 0.14 mgO_2/L . While low DO conditions were achieved for the Anammox-MABR system, the lowest DO concentration attainable was still not comparable to the DO concentrations of <0.1 mgO_2/L in typical Anammox processes (Cho et al. 2020). The variation in DO concentrations in the Anammox-MABR system will be discussed further in Section 4.2.

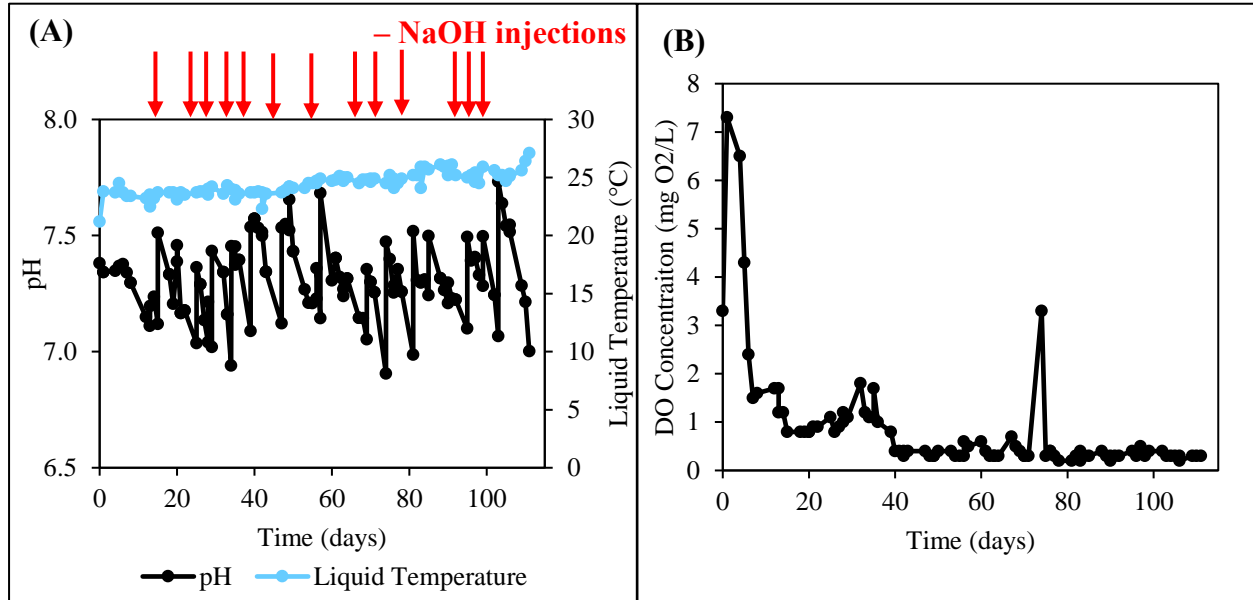


Figure 4.3. (A) pH and reactor temperature, and (B) DO concentrations for Anammox-MABR performance throughout 111 days operational period with red arrows indicating NaOH injections.

The denitrifying bacteria activity was expected to be minimal since no organic source was included in the enrichment solution. However, heterotrophic denitrify bacteria can still utilize available carbon sources from degradation of dead cells present (Lew et al., 2012). The sCOD concentration was monitored throughout the entire operational period as shown in Figure 4.4 to determine utilization of organic carbon sources by denitrifying bacteria. An important factor to consider for sCOD measurements in Anammox systems is the presence of nitrite which can overestimate sCOD due to interference (Ferraz and Yuan, 2017). The theoretical oxygen demand from nitrite oxidation was accounted for in evaluating the nitrite interference for the sCOD measurements in Figure 4.4. Furthermore, the suspended solids within the reactor were monitored to determine the biomass concentration within the Anammox-MABR system.

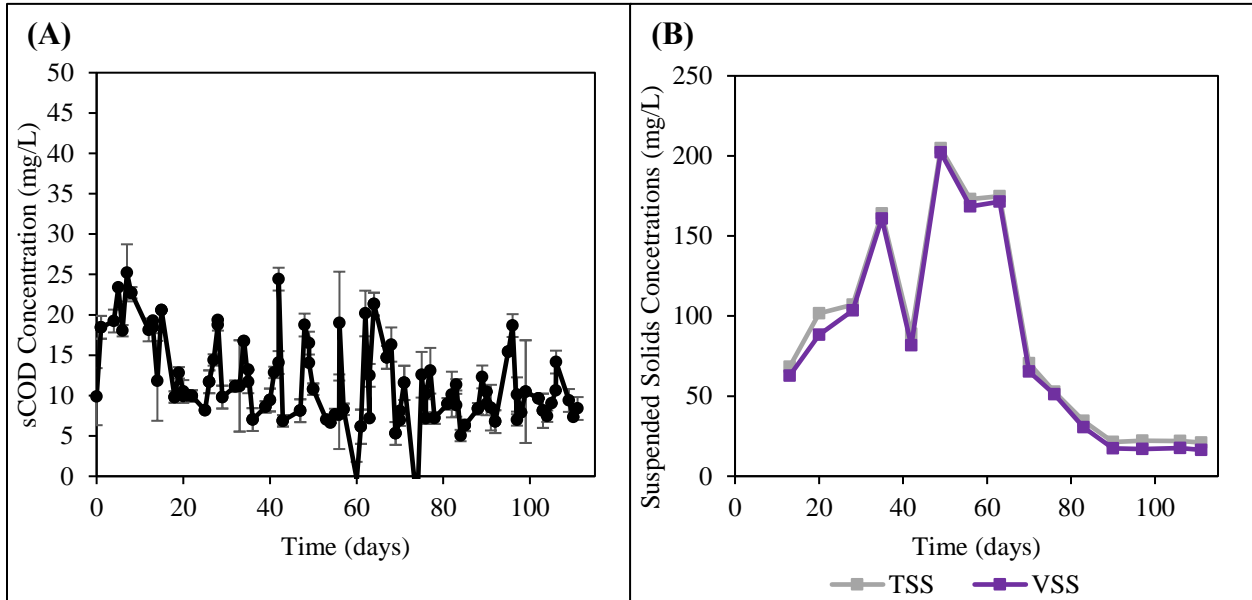


Figure 4.4. (A) sCOD and (B) Suspended Solids concentrations for Anammox-MABR performance throughout 111 days operational period.

4.2 Rate of Oxygen Transfer through Membranes

In the Anammox-MABR system, the biological reactions that occur can be impacted by the dissolved oxygen concentration present in the bulk solution. While it has been shown in studies that Anammox bacteria can coexist with AOA and AOB in DO concentrations over 2 mgO₂/L, high concentrations of DO can inhibit the activity of Anammox bacteria (Cho et al., 2020). However, as shown in Figure 4.3 (B), for the first 5 days of operation, the DO concentration was much greater than 2 mgO₂/L and continues to remain above 1 mgO₂/L until Day 40. Despite the high DO concentration in the Anammox-MABR system, the performance was not compromised until after Day 20. A simple test was conducted on Day 35 with clean membranes and no aeration through the membranes. As shown in Figure 5, the DO concentration in the bulk solution was initially more than 2 mgO₂/L and gradually decreased to below 0.5 mgO₂/L within 120 mins when no aeration was provided. This suggests that the membranes in the Anammox-MABR system have a high rate of oxygen transfer which can also be associated with a high membrane air permeability.

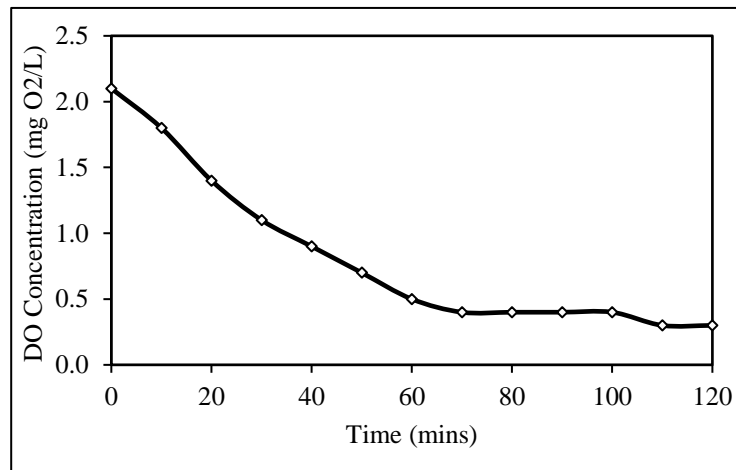


Figure 5. Dissolved oxygen concentrations for MABR system with no aeration conducted on Day 35 for 120 mins.

To control the rate of oxygen transfer in the Anammox-MABR system, a substantially lower air flow rate was supplied to lower the DO concentration in the bulk solution. The average effluent air linear velocity and residence time of air for the Anammox-MABR can be calculated

according to Eq. 5 and 6, respectively, and can be described in Table 5 for each temperature condition before and after Day 35. After reducing the air flow rate supplied to the Anammox-MABR system on Day 35, the DO concentration as shown in Figure 4.3 (B) decreased and was maintained below 0.5 mgO₂/L which allowed the system to quickly recover within a few days. By Day 40, the Anammox-MABR system had stabilized despite the increased accumulated nitrate due to higher NOB activity. The average DO concentration in the Anammox-MABR system was 0.4 mgO₂/L, however, it was still higher than typical Anammox systems with DO concentration <0.1 mgO₂/L (Cho et al., 2020).

Table 5. Effluent air linear velocity and residence time (τ_{air}) for the membranes in Anammox-MABR system throughout the 111 days operational period for 20°C and 60°C air temperature conditions.

Condition	Day 0 to Day 35		Day 35 to Day 111	
	Effluent Linear Velocity (cm/s)	Residence Time, τ_{air} (sec)	Effluent Linear Velocity (cm/s)	Residence Time, τ_{air} (sec)
20°C – A	32.25±4.81	0.23±1.56	8.24±2.04	0.91±3.68
20°C – B	17.30±3.78	0.43±1.98	4.94±1.35	2.12±18.02
60°C – A	4.71±2.66	1.59±2.81	3.53±0.42	1.52±5.54
60°C – B	5.06±4.78	1.48±1.57	6.24±1.69	1.20±4.44

Since air is being heated before it is pumped through the membrane, the estimated effluent linear velocity can be used as an indication for if the air supplied through the membranes will remain sufficient heat. Even at reduced air flow rate from Day 35 onwards, the residence time of the air through the membranes for both temperature conditions as shown in Table 5 were approximately 1-2 seconds implying that the air supplied experiences negligible heat loss through the Anammox-MABR system.

4.3 Nitrogen Removal by Specific Bacterial Group

The daily nitrogen removal rate by each specific bacterial group (AOB, Anammox bacteria, NOB, and denitrifying bacteria) can be estimated based on electron and mass balance from the Eq. 1-4. An assumption associated with the equations constructed to estimate the nitrogen removal for each specific bacterial group from the mass balance equations is that only denitrifying bacteria utilize organic carbon source, whereas AOB, Anammox bacteria, and NOB utilize inorganic carbon sources such as CO_2 (Soliman and Eldyasti, 2018; Trinh et al., 2021). Since there were no organic substrate provided to the enrichment solution, the only source of organic substrate in the Anammox-MABR system is from degradation of dead bacteria cells, implying that a reduction in sCOD would only be attributed in denitrifying bacteria activity. This suggests that there is nitrate consumption from denitrifying bacteria based on mass balance with respect to the reduction of sCOD. The estimated nitrogen removal from mass balance can be determined using the Eq. 11-14.

The Anammox-MABR system is designed to operate as PN-A process to remove both ammonia and nitrite. The removal for each substrate is shown in Figure 6 with the specific bacterial group contribution for the respective removal. For ammonia removal, AOB and Anammox bacteria had an average daily removal rate of 7.59 mgN/L/d (77.0%) and 2.06 mgN/L/d (23.0%), respectively. While AOB was predominantly responsible for ammonia removal in the Anammox-MABR system, there seems to be minor or no competition between AOB and Anammox bacteria as both groups of bacteria are associated with a slow growth rate (Cho et al., 2020). Since the ammonia concentrations in the Anammox-MABR system was never completely depleted and additional NH_4Cl was added periodically as shown in Figure 4.1, the ammonia substrate was sufficient for both AOB and Anammox bacteria.

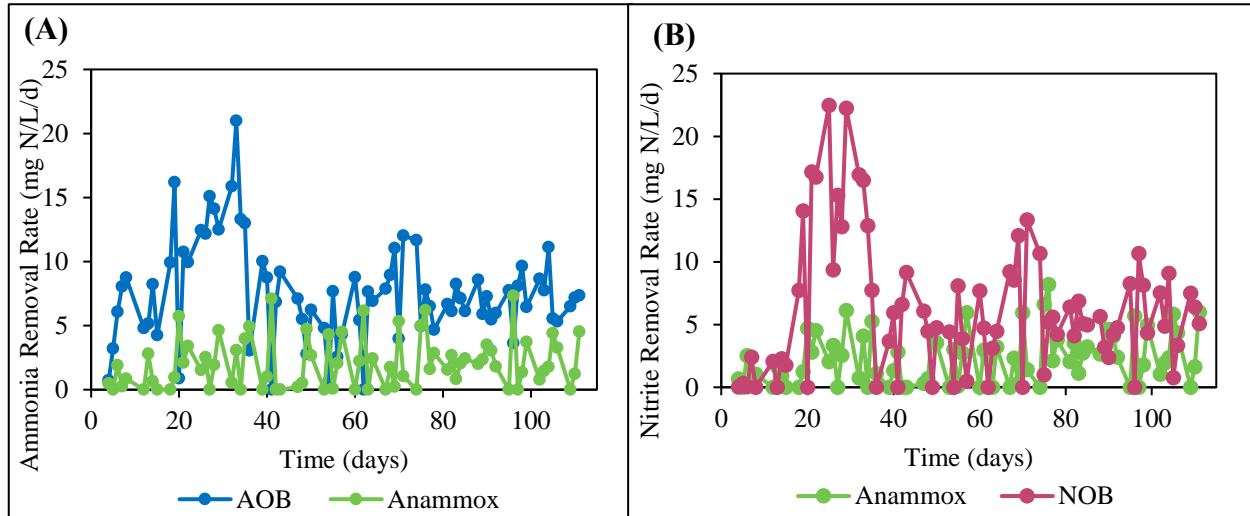


Figure 6. Estimated daily removal rate for (A) ammonia removal and (B) nitrite removal contributions for target bacterial groups based on electron and mass balance in the Anammox-MABR throughout the 111 days operational period.

Likewise, for nitrite removal, Anammox bacteria and NOB had an average daily removal rate of 2.29mgN/L/d (35.6%) and 6.20mgN/L/d (64.4%), respectively. The total nitrogen removal rate from Anammox by combining the removal of ammonia and nitrite was 4.35 mgN/L/d. In contrast, the nitrite concentrations from Day 25 to Day 35 were completely depleted as shown in Figure 4.1 and the nitrite removal rate for NOB from Day 20 to Day 35 was substantially higher compared to Anammox bacteria. During this period, Anammox bacteria could not compete with NOB for nitrite substrate due to the presence of high DO in the system. Subsequently, after Day 40, the nitrite concentrations were not completely depleted and maintained approximately 45 mgN/L as shown in Figure 4.1, this suggests that the active competition between Anammox bacteria and NOB can be observed despite the higher nitrite removal rate from NOB compared to Anammox bacteria. Although the nitrogen removal from NOB does not contribute to the total removal substantially, the presence of NOB in the Anammox-MABR system can still impact the overall performance and influence the growth of Anammox bacteria due to the increased competition for available nitrite substrate.

4.4 Microbial Communities

The relative abundance of each specific bacteria group can also be analyzed from 16S amplicon sequencing and qPCR to determine the overall performance of the Anammox-MABR system. 16S amplicon sequencing was conducted on the biofilms of each membrane for the two aeration temperature conditions (20°C and 60°C) and for the bulk solution in the Anammox-MABR system. The microbial species were assigned to a target bacterial group of interest: AOB, Anammox bacteria, NOB, denitrifying bacteria, and others (i.e., nitrogen/carbon fixation bacteria, sulfur reducing bacteria, other heterotrophic bacteria, fermenters, hydrolysis, etc.). As shown in Figure 7, the most abundant bacterial group was AOB with a relative abundance for 20°C and 60°C aeration temperature conditions increasing from 3.9% to 47.3±10.9% and 40.4±23.1%, respectively from Day 0 to Day 111. However, for Anammox bacteria, the relative abundance for both 20°C and 60°C aeration temperature conditions decreased from 8.5% to less than 0.001% from Day 0 to Day 111. The low detection for Anammox bacteria from 16S amplicon sequencing analysis could potentially be due to poor amplification of the 16S rRNA gene with the universal bacterial primers (Li and Gu, 2011). For NOB, the relative abundance for the 20°C and 60°C aeration temperature conditions increased from 0.2% to 15.7±8.9% and 19.81±15.08%, respectively from Day 0 to Day 111. In contrast, despite the overall increase in the relative abundance of NOB for both temperature conditions, it remained below 20% indicating that NOB were not necessarily predominant over Anammox bacteria. Furthermore, between the 20°C and 60°C temperature conditions, there was only a 5.8% difference, however since the 60°C air temperature condition was more variable compared to 20°C, this suggests that the increase in relative abundance of NOB over the entire operational period could potentially be even greater for higher temperature aeration condition.

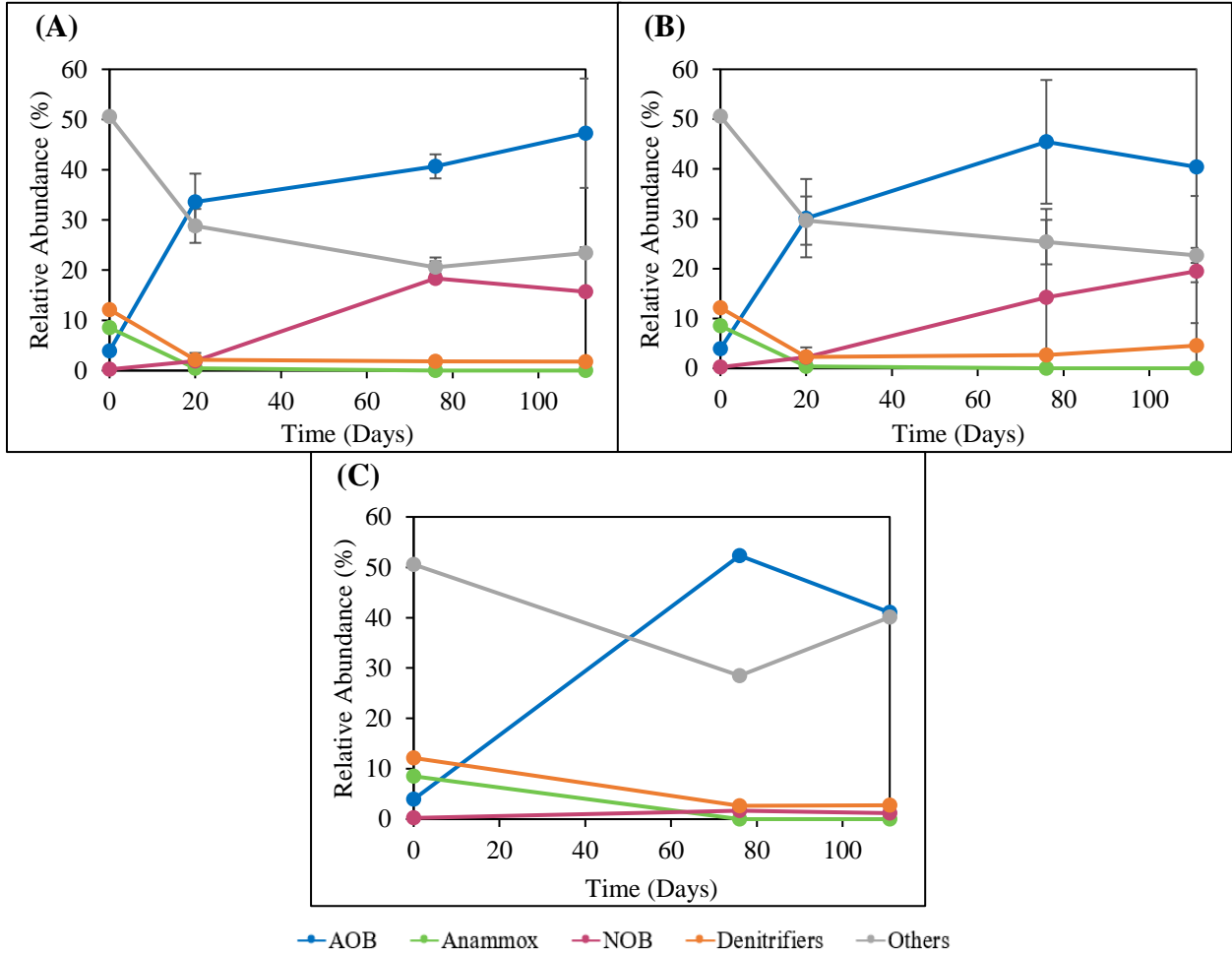


Figure 7. Relative abundance of microbial communities from 16S amplicon sequencing analysis for the V4 region of 16S rRNA gene in Anammox-MABR for (A) T=20°C, (B) T=60°C, and (C) Bulk Solution.

In comparison to both the temperature aeration conditions, the relative abundance of AOB in the bulk solution was also higher than 40% indicating that AOB could support both attached and suspended growth condition with negligible repercussions. However, the relative abundance of NOB in the bulk solution was less than 2% indicating majority of NOB prefer to attach to the membrane which supports a higher DO concentration in the aerobic biofilm. This suggests that the higher DO concentration at the membrane surface, which is significantly impacted by the membrane’s air permeability characteristics, will also influence NOB growth.

Since abundance for Anammox bacteria yielded low detection from 16S amplicon sequencing, other methods such as qPCR can be conducted to determine more accurate detection and representation of the microbial communities present in the Anammox-MABR system. Using the standard curves from Figure 3.2, the abundance of each target bacterial group and temperature condition for can be reported as copies/ng DNA/cm² membrane as shown in Table 6. The relative abundance for each bacterial group of interest was determined by normalizing with the general bacteria as shown in Table 7. The copy numbers are normalized by the DNA concentration and the effective total surface area of each membrane sample. Each target bacterial group of interest for qPCR analysis can be described from 16S rRNA with single copy genes, therefore the abundance can be directly compared (Speth et al. 2016; Zhao et al. 2023). The qPCR analysis was only conducted on Day 111 for the Anammox-MABR system. From the two Anammox bacteria primers used, AMX809f/1066r was able to detect Anammox bacteria in the Anammox-MABR more effectively compared to AMX368f/820r.

Table 6. Abundance of target bacterial groups in Anammox-MABR for 20°C and 60°C air temperature conditions quantified by qPCR assays on Day 111.

Condition	General	AOB	Anammox (AMX809f/1066r)	Anammox (AMX368f/820r)	NOB
	Copies/ng DNA/cm ² membrane				
20°C	2837799	720616	23344	38	1196
60°C	1909612	647375	25293	62	3123

Table 7. Relative abundance of target bacterial groups normalized by general bacteria in Anammox-MABR for 20°C and 60°C air temperature conditions quantified by qPCR assays on Day 111.

Condition	AOB	Anammox (AMX809f/1066r)	Anammox (AMX368f/820r)	NOB
20°C	25.393%	0.823%	0.001%	0.042%
60°C	33.901%	1.325%	0.003%	0.164%

Comparing the two temperature conditions, the abundance of Anammox bacteria and NOB were higher under 60°C air conditions compared to 20°C, while the opposite is the case for AOB. The ratio between AOB and Anammox (AOB:Anammox) for the 20°C and 60°C aeration temperature conditions was 30.9 and 25.6, respectively, indicating that operating MABR with lower temperature air supplied promotes more AOB growth. Conversely, the ratio between NOB and Anammox (NOB:Anammox) for the 20°C and 60°C temperature conditions was 0.05 and 0.12, respectively. This suggests that supplying higher temperature aeration in the MABR may promote more NOB growth. The abundance for Anammox bacteria in the 60°C aeration temperature condition was higher compared to the 20°C air condition, however, the difference is only 8.0%, which this trade-off may not be worthwhile since the system would be compromised with increased NOB activity.

3.5 Nitrogen Removal and Bacterial Abundance Comparison

The estimated average daily nitrogen removal from can be compared to the relative abundance from Illumina sequencing to verify the removal contributions from each target bacterial group. The comparison between the estimated removal contributions and the relative abundance can provide an indication of the activity of the specific bacteria, however, it may not always be proportionally related. As shown in Table 8, there were not considerable differences between the estimated removal contributions and the relative abundance for each target bacterial group (all within 15%) with the exception for Anammox bacteria. As mentioned previously, the relative abundance of Anammox bacteria can not be compared using Illumina sequencing analysis due to low detection and must be supplemented with other alternative methods of quantification.

Table 8. Comparison of average daily nitrogen removal and relative abundance from 16S amplicon sequencing for each target bacterial group (AOB, Anammox bacteria, NOB, and denitrifying bacteria).

	AOB	Anammox Bacteria	NOB	Denitrifying Bacteria
Average Daily Nitrogen Removal Contribution (%)	41.11	26.30	28.79	3.80
16S Amplicon Sequencing Relative Abundance for 20°C Condition (%)	47.27	<0.001	15.72	1.78
16S Amplicon Sequencing Relative Abundance for 60°C Condition (%)	39.51	<0.001	19.81	4.78
qPCR Relative Abundance for 20°C Condition (%)	25.39	0.82	0.04	N/A
qPCR Relative Abundance for 60°C Condition (%)	33.90	1.33	0.16	N/A

The relative abundance for 16S amplicon sequencing and qPCR were not consistent with each other. However, since the amplification of primers used during qPCR analysis were validated with gel electrophoresis, this indicates that qPCR is more reliable in comparison to 16S amplicon sequencing. Furthermore, the sample collection for 16S amplicon sequencing and qPCR disturbs the biofilm on each membrane, the growth of Anammox bacteria could be potentially affected, therefore these analyses can not be conducted as frequently. This makes the estimated average daily nitrogen removal rate and contributions a good predictor of the activity of each bacterial group of interest.

5. Conclusions/Recommendations

The major challenges with integrating Anammox process with MABR technologies remain largely associated with controlling the DO concentration in the system's bulk solution. Since Anammox bacteria are considerably inhibited by presence of oxygen, it is desirable to achieve low DO concentrations. However, the membranes used in this study has characteristically high air permeability which made controlling the DO concentration in the bulk solution difficult. The average DO concentration after substantially reducing air flow rate was 0.4 mgO₂/L which is higher in comparison to typical Anammox process systems (<0.1 mgO₂/L). Other materials with a lower air permeability can be considered for membranes with Anammox process integration in the future.

By providing high temperature air for aeration supply into the MABR system, the relative abundance of Anammox bacteria increases slightly, however the relative abundance of NOB also increased which may be undesirable and must be taken into consideration. It can not be concluded that one temperature condition performed better than the other since the DO concentration in the bulk solution of the Anammox-MABR system was quite high and had significant impact on the overall performance of the system and on Anammox bacterial growth. An alternative method of decreasing DO concentration in the bulk solution is to periodically purge the MABR system with argon (Ar) gas to help achieve and maintain low DO concentrations.

The estimated daily nitrogen removal rate and contributions is an adequate predictor of the activity for each bacterial group of interest. The total contribution from AOB and Anammox bacteria (ideal PN-A process) was estimated to have a daily removal rate of 11.94 mg N/L/d (67.41%); where for AOB and Anammox was 7.59 mgN/L (41.11%) and 4.35 mgN/L (26.30%), respectively. The nitrogen removal rate and contributions from each target bacterial group were

comparable to the relative abundance within 15% except for Anammox bacteria due to poor amplification with universal primers for Illumina sequencing analysis which significantly impacted the relative abundance. While qPCR was proposed as an alternative method for quantifying the relative abundance of Anammox bacteria, only the general Anammox gene was targeted. The functional genes for each bacterial group of interest should be considered for future investigations to improve accurate representation of the microbial communities in the system.

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Appendix A: Preliminary MABR System Results

The preliminary Anammox-MABR system consist of 6 membranes, each 50cm long. Three different temperature aeration conditions were monitored: 20°C, 40°C, 60°C. The raw results for the nitrogen concentrations and DO concentrations are shown in Figures A1 and A2, respectively.

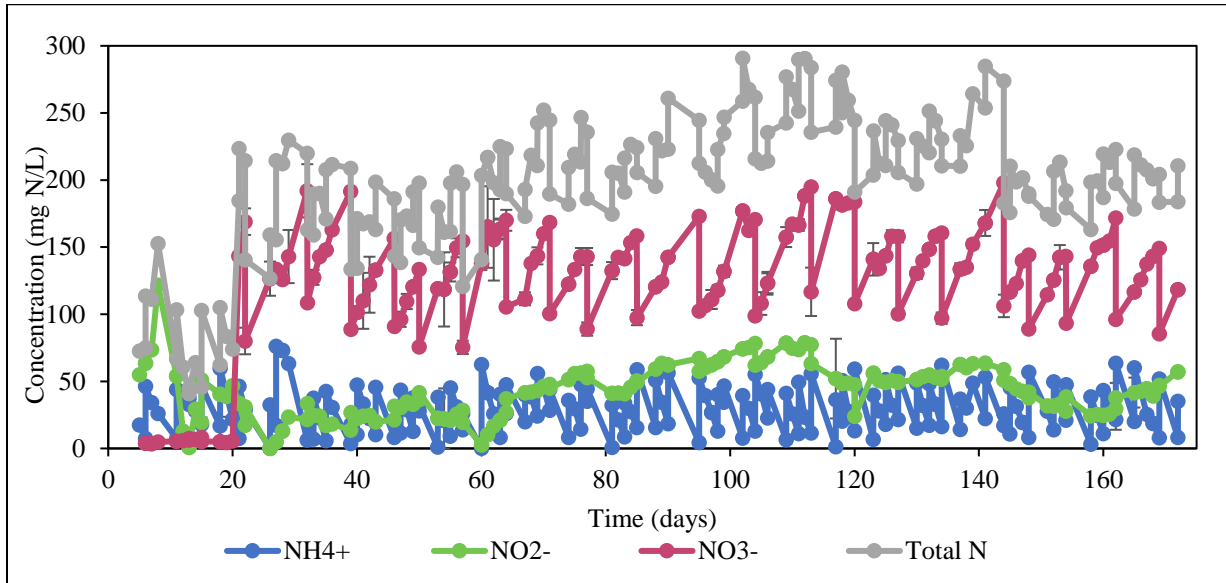


Figure A1. Nitrogen concentrations for the preliminary Anammox-MABR.

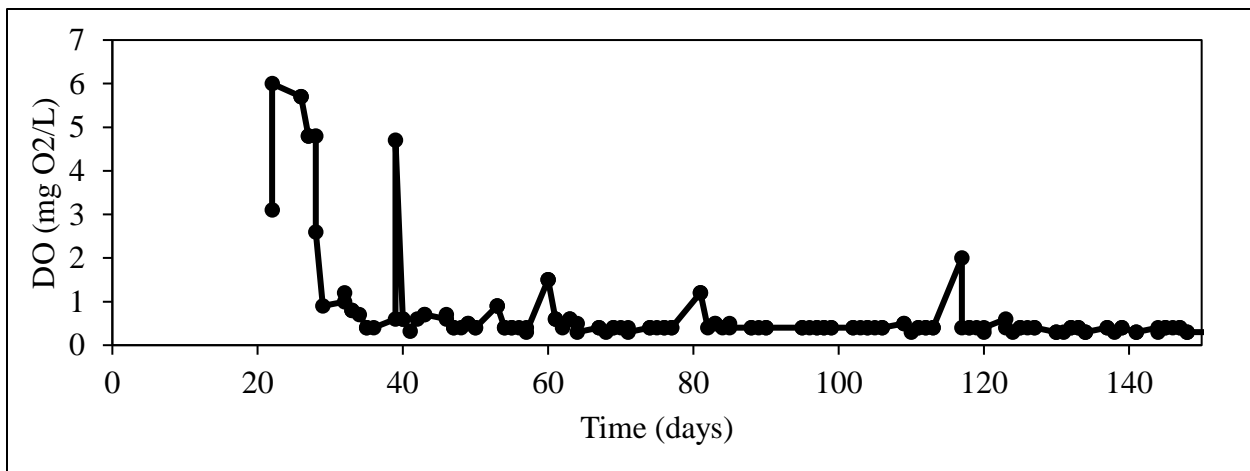


Figure A2. Dissolved oxygen concentration for the preliminary Anammox-MABR.

The linear velocity and residence time of the air through the membrane can be shown in Table A1. From these results, it was concluded that the residence time of the air through the membrane was too short and may significantly contribute to the high dissolved oxygen in the bulk solution as shown in Figure A2 which should be taken into consideration for future reactors.

Table A1. Effluent air linear velocity and residence time (τ_{air}) for the membranes in the preliminary Anammox-MABR.

Condition	Effluent Linear Velocity (cm/s)	Residence Time, τ_{air} (sec)
20A	7.04	0.14
20B	2.17	0.04
40A	3.79	0.08
40B	8.66	0.17
60A	8.66	0.17
60B	1.62	0.03