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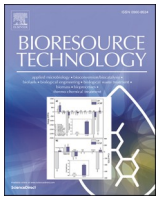

Low maintenance anammox enrichment and nitrogen removal with an anaerobic baffled reactor

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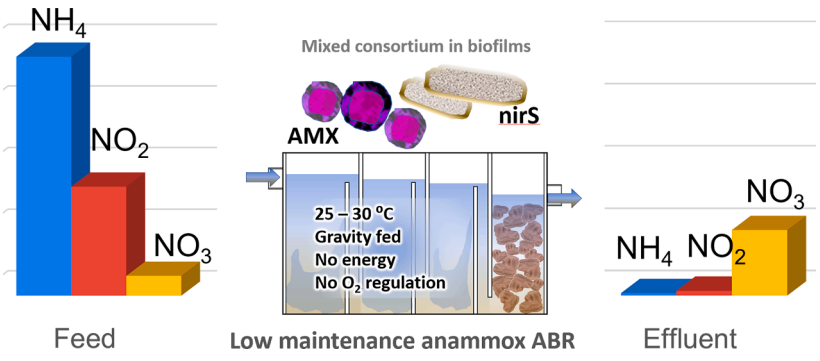
HIGHLIGHTS

- Low-maintenance anaerobic baffled reactor for anammox enrichment operated 335 days.
- Anammox ABR consistently removed > 90% of N despite oxygen fluctuations.
- Anammox communities confirmed with qPCR and anammox activity test.
- Denitrifier genes also present, indicating mixed consortium responsible for N removal.

GRAPHICAL ABSTRACT



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ABSTRACT

The stringent growth requirements of anammox bacteria may be a challenge for employing the anammox process for nutrient removal at household or decentralized scales, where low maintenance systems are more successful. Enrichment of anammox bacteria was achieved by 100 d using a lab-scale (32 L) anaerobic baffled reactor (ABR). Even though strict anaerobic conditions were not imposed, NH_4-N and NO_2-N removals of >90% were maintained after ~100 d, with greatest removals observed in the first two chambers of the four-chamber ABR. Batch anammox activity tests and results of qPCR analyses confirmed the presence of anammox bacteria in all four ABR chambers. Changes in fluorescent peaks and indices supported that intracellular compounds from reactor biomass evolved along the ABR. The presence of denitrifiers, confirmed by qPCR, and lower NO_2/NH_4 ratios than predicted by stoichiometry indicated that nitrification–denitrification processes also may have contributed to the high N removal in the anammox ABR.

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1. Introduction

Anaerobic ammonium oxidation (anammox), an autotrophic process in which ammonium (NH_4^+) and nitrite (NO_2^-) are metabolized to dinitrogen gas and nitrate (NO_3^-) (Strous et al., 1999, van de Graaf et al., 1996), has been widely shown to be an effective method for nitrogen (N) removal and has been applied at the field scale for side-stream wastewater treatment processes, such as treatment of centrate and other reject water (Lackner et al., 2014; Wang et al., 2020), which are rich in reduced N compounds and low in labile carbon (C). By contrast, the use of anammox for mainstream municipal wastewater treatment has rarely been demonstrated (Cao et al., 2018; Bunse et al., 2020), owing to the challenges with growing sufficient biomass and achieving partial nitritation under real world conditions (Wang et al., 2020). The challenges associated with growing anammox bacteria for large-scale, conventional centralized wastewater treatment applications include long startup times, maintenance of anaerobic conditions in reactors, sensitivity to temperature and pH, and inhibition by sulfide, nitrite, ammonia, phosphate, and other compounds found in different wastewater streams (Talan et al., 2021; Jin et al., 2012).

To enrich anammox bacteria, many studies adapted approaches from the seminal papers of van de Graaf et al. (1995) and Strous et al. (1999), which involve supplying trace elements and nutrients in a feed solution under strict anaerobic conditions. In all cases, argon or nitrogen gas are maintained in the headspace, sometimes mixing is required, and temperature and pH are typically maintained at optimal conditions (temperature of 30–40 °C and pH of 7 to 8; Strous et al., 1999; Strous et al., 1999; Egli et al., 2001; Jin et al., 2012). Reactors commonly used for anammox enrichment include sequencing batch reactors (SBRs), continuous feed upflow reactors, and upflow anaerobic sludge blanket (UASB) reactors, which result in the growth of anammox granules (Chen et al., 2017). To enrich anammox in biofilms, membrane bioreactors, moving bed biofilm reactors, and anaerobic baffled reactors (ABRs) also have been employed and are often augmented with biomass carrier media (eg., volcanic rock, plastic carriers, zeolites) for retaining anammox in the biofilm sorbed to media surfaces (Lu et al., 2018; Wang et al., 2019; Adams et al., 2020). In general, studies evaluating ABR systems are less common in the literature, with only three ABR-related studies, one reporting on an ABR with biomass carriers (Wang et al., 2019) and two others reporting on an ABR without biomass carriers (Chen et al., 2017; Chen et al., 2018), appearing in the recent literature. A gap in our understanding exists about the resilience of anammox growth and the importance of the sludge blanket as a medium for anammox biomass in ABR systems.

Deeper investigation of ABR systems for anaerobic N removal is of particular interest because anaerobic treatment systems (eg., ABRs, anaerobic filters, UASB reactors) are widely used as the main biological treatment process in decentralized wastewater treatment systems (DEWATS) installed by non-governmental organizations in thousands of low-income households, institutions, and neighborhoods in Asia, Africa, and Latin America (BORDA, 2017). Anammox may have immense potential for mainstream N removal in DEWATS, because the treated effluents already maintain reducing conditions and relatively small anammox reactors would be needed to polish the lower wastewater volumes, which could result in faster reactor startup. Low maintenance approaches for starting up anammox reactors will be particularly desirable, given that the reliance is often on low energy and maintenance requirements, gravity-fed flows, and low operation and maintenance costs in those settings where DEWATS are employed (Reynaud and Buckley, 2015; Singh et al., 2019). Therefore, more research on long-term operation under low maintenance conditions, applicable to decentralized wastewater treatment in low-income communities, is warranted. In this setting, there is a need to improve access to technologies for removing harmful levels of nutrients from wastewater before it enters water bodies, posing risks to aquatic life and human health.

The main objectives of this study were to evaluate the overall N removal efficiency of a low-maintenance ABR for enriching anammox bacteria over long term (335-d) operation and assess whether sludge blanket biofilms are able to support anammox under low-maintenance conditions relevant to the decentralized settings described above. Nutrients and trace elements were supplied at realistic wastewater concentrations and conditions, allowing for natural microbial consortia to develop (after inoculation) under air-exposed conditions and alternating continuous flow and static conditions. Evidence from tests of anammox activity, measurements of C and *N*-species concentrations and stoichiometric relationships, and analyses of qPCR products and fluorescence excitation emission matrix (EEM) peaks and indices were used in concert to evaluate: 1) the removal of NH_4-N and NO_2-N in the ABR, 2) transformations that occurred in each chamber of the ABR during anammox enrichment, and 3) the presence and activity of anammox bacteria.

2. Materials and methods

2.1. Reactor setup

An anaerobic baffled reactor (ABR, ~32 L capacity), which had previously been treating primarily domestic wastewater acquired from the influent of a water reclamation facility in San Diego, CA, USA and synthetic wastewater in the 6 months prior to the experimental period, was employed for this study. Chambers 1–4 comprised the following volumes, respectively: 12.3 L, 8.5 L, 5.6 L, and 5.8 L. Synthetic wastewater feed was discontinued, and, a new nutrient-rich feed and trace element solution (Table 1) was fed to the ABR for approximately 14 d. The new, low DOC (dissolved organic carbon) (~2.7 mg/L) solution, resulted in a shock to the ABR, with black discoloration in all chambers indicating senescence of the biological sludge blanket. During this period, approximately 50%–75% of the sludge blanket of each chamber was removed to expedite the conversion away from heterotrophic biological processes. Lava rock was added to chamber 4 to promote biofilm growth and minimize potential washout. Anammox biomass (scrapings and granules from a pilot-scale anammox enrichment SBR at Los Angeles County Sanitation District (LACSD)) was added evenly (by pipette) to chambers 2 – 4 to represent ~4% of the volume of each chamber.

Reactor feeding followed a semi-batch mode, with continuous pumping at a rate of 0.6725 L/h for 18 h followed by 6 h of static conditions. These conditions are representative of decentralized wastewater treatment systems treating domestic wastewater, where flow generally ceases overnight (Mladenov et al., 2018). High dissolved oxygen (DO) tap water was used in the preparation of the nutrient and synthetic wastewater solution (Table 2) and no anaerobic conditions

Table 1
Chemical composition of synthetic wastewater and trace elements solution.

Compound	Day 1	Day 30	Day 97
Nutrients and synthetic wastewater			
NH_4Cl	0.5 g/L	0.05 g/L	0.05 g/L
$NaNO_2$	0.5 g/L	0.05 g/L	0.05 g/L
$KHCO_3$	1.25 g/L	1.25 g/L	0.33 g/L
NaH_2PO_4	0.05 g/L	–	–
$CaCl_2$ 2H_2O	0.3 g/L	–	–
$MgSO_4$7H2O	0.2 g/L	–	–
$FeSO_4$7H2O	0.0118 g/L	–	–
Trace element solution	1.25 mL/L	–	–
Trace elements solution			
EDTA	150.0 mL	–	–
$ZnCl_2$	0.204 g/L	–	–
$CoCl_2$6H_2O	0.24 g/L	–	–
$MnSO_4$H2O	0.85 g/L	–	–
$CuSO_4$5H2O	0.25 g/L	–	–
$Na_2MoO_4$2H_2O	0.22 g/L	–	–
$NiCl_2$	0.10 g/L	–	–
H_3BO_3	0.01 g/L	–	–

Table 2
Reactor conditions and chemical composition of synthetic wastewater and trace elements solution.

Conditions	Day 1	Day 80	Day 97
Temperature	Ambient	Adjusted with heat blanket	(heat blanket maintained throughout study period)
Maintenance of anaerobic condition	None	None	None
pH	Not adjusted	Not adjusted	Adjusted by decreasing $KHCO_3$ concentration

(eg., flushing with N_2 or argon gas) were imposed on the feed solution or the ABR headspace. The hydraulic residence time of the ABR system was 36 h and the upflow velocity was maintained at < 2.0 m/h. On day 80, a heat blanket was attached to the outside of the ABR, which increased water temperature inside the ABR by ~7 °C. This heat adjustment was maintained throughout the study to bring the ABR to conditions more typical of tropical and subtropical climates. On day 97, the pH was adjusted to fall within the preferred range for anammox (between 7.0 and 8.0, Strous et al., 1999); this was done by reducing the $KHCO_3$ concentration in the feed solution from 1.25 g/L to 0.33 g/L.

2.2. Water quality analysis

Water quality of the feed and effluent, were measured weekly. DO and temperature were measured using a YSI ProODO Optical DO meter, and pH and electrical conductivity were measured using a Fisherbrand Accumet AP85 portable waterproof meter. These water quality parameters were measured weekly in Chambers 1–3, but could not be measured in Chamber 4 because of tight spacing between the rock media. Dissolved organic carbon and total dissolved nitrogen (TDN) were determined on a Shimadzu TOC-L Total Organic Carbon and Total Nitrogen Analyzer using high temperature oxidation. Weekly sampling was also conducted for analyses of NH_4-N, NO_2-N, and NO_3-N in feed and effluent by spectrophotometry (APHA, 2017) with a HACH DR3900 spectrophotometer. Additionally, nitrogen species were measured in all four ABR chambers after the startup period (on days 165, 177, 198, 282, 311, 324, 325). Total suspended solids were determined gravimetrically (APHA, 2017).

2.3. Auto-fluorescence analysis

Autofluorescence of the endogenous organic compounds (e.g., from bacteria, biofilm, and microbial consortia, since no organic carbon was added to the reactor) in each chamber were analyzed using simultaneous UV–vis absorbance and fluorescence acquisition with the Horiba Aqualog. Three dimensional excitation-emission matrix spectra were acquired using a Horiba Scientific Aqualog Fluorometer using a 1 cm path length quartz cuvette as in Mendoza et al. (2020). Spectra were acquired at excitation wavelengths from 240 nm to 450 nm with an increment of 4.66 nm, emission wavelengths from 240 nm to 800 nm with an increment of 4.66 nm, and at an integration time of 0.25 s. Raw data from the Aqualog software were corrected for inner filter effects and Rayleigh masking in Matlab (ver 2015). EEM (excitation-emission matrix) data were normalized to the Raman area at 350 nm to provide intensities in Raman units (RU), and blank EEMs (using ultrapure water) were subtracted from sample EEMs. The fluorescence index (FI) was calculated as the ratio of fluorescence intensities at emission wavelengths of 470 nm and 520 nm and excitation wavelength of 370 nm. Lower values of FI (~1.3 to 1.4) are often associated with dissolved organic matter (DOM) of terrestrial sources, while higher values (~1.7 to 1.9) indicate microbially-derived DOM (McKnight et al., 2001). The humification index (HIX) was calculated as the ratio of peak area under the emission spectra at 435–480 nm to peak area from 300 to 345 nm obtained at an excitation wavelength of 254 nm (Zsolnay, 2003). Nomenclature and peak intensity locations reported for ubiquitous peaks A, T, and C follows Coble (1996).

2.4. Anammox activity test

Free-floating microbes and biofilm from sludge were sampled from each chamber on day 290, and were introduced to N_2-flushed serum bottles containing 40 mL of solution prepared with 100 mg/L NH_4–N and 123 mg/L NO_2-N. Bottles were incubated at 36 °C with continuous stirring at 150 RPM for 24 h. Ammonium, nitrate, and nitrite were analyzed as described above for samples and control (without biomass) to evaluate the presence and performance of anammox bacteria.

2.5. Microbial community analysis

DNA from duplicate samples of the sludge blanket in each chamber of the ABR were extracted on day 317 and analyzed by real-time quantitative PCR (qPCR) for anammox (16S), denitrifiers (nirS), and eubacteria (for normalization purposes) by the LACSD Microbiology Laboratory (certified by Environmental Laboratory Accreditation Program). In brief, DNA extraction was conducted using the Applied bioSystems PowerUp SYBR Green Master Mix using a method derived from Tsushima et al. (2007). The anammox assay (AMX) utilizes primers amx809f-amx1066r and generates an expected 285 bp product. The sequence used for creating the standard is *Candidatus Kuenenia stuttgartiensis* isolate kuenenia_mbr1_ru-nijmegen genome assembly, chromosome: Kuenenia_stuttgartiensis_MBR1 (GenBank: LT934425.1). The nirS assay utilized NirS F1-LACSD and NirS R1-LACSD = 58.2oC). All bacteria were quantified by a quantitative PCR system (LightCycler® 480) using manufacturer software. Salmon sperm DNA (Invitrogen cat# 15632-011 at 10 mg/mL, lot number: 2353841) was used as a sample processing control (SPC) in the DNA extractions to identify inhibition and deficiencies during DNA extraction. There were no qPCR inhibitions based on the salmon sperm control. The AMX and nirS cell densities were normalized to eubacteria cell densities to give a percentage value. Normalization of AnAOB qPCR results against the EUB qPCR results has been used in a number of studies over the past decade for investigating the enrichment levels of anammox bacteria (eg., Wang et al., 2019; Davery et al., 2013). In our study, normalization was performed to compensate for variations in sampling to eliminate erroneous impact of the sampling difference. In addition, PCR products from the samples submitted were also analyzed by gel electrophoresis (Supplemental Fig. 1) to verify product size.

3. Results and discussion

3.1. Nitrogen removal performance

Water quality parameters (pH, temperature, and DO) in the chambers of the ABR (Fig. 1a–c) shifted in response to changes in reactor conditions (Table 2). The Phase 1 (startup) period of the ABR was considered to be ~108 days (shaded region in Fig. 1). During Phase 1, NH_4-N removal was variable, ranging from 15% to >90% (Fig. 1d). Measurements of NH_4-N and NO_2-N in the feed tank during Phase 1 (Fig. 1d and e) reflected the reduction from a starting feed concentration of ~200 mg/L for each to a final feed concentration of ~50 mg/L NH_4-N, which is a value more typically encountered in raw wastewater (Metcalf and Eddy, 2014), and ~20 mg/L for NO_2-N. Although NO_3-N was not added in the feed solution, nitrate did form at low concentrations under the ambient, air exposed conditions of the feed tank (Fig. 1f).

During Phase 2, measurements of NH_4-N in the feed tank ranged from 20 to 50 mg/L NH_4-N and NO_2-N remained ~20 mg/L (Fig. 1d). Despite variability in the feed concentrations of ammonium, NH_4-N removals stabilized at > 90% (mean 98.8% ± 2.0%) over the entirety of Phase 2, from day 108 to day 335. With the exception of one sampling date, NO_2-N removals were also > 90% (mean 95.6% ± 4.7%) over the

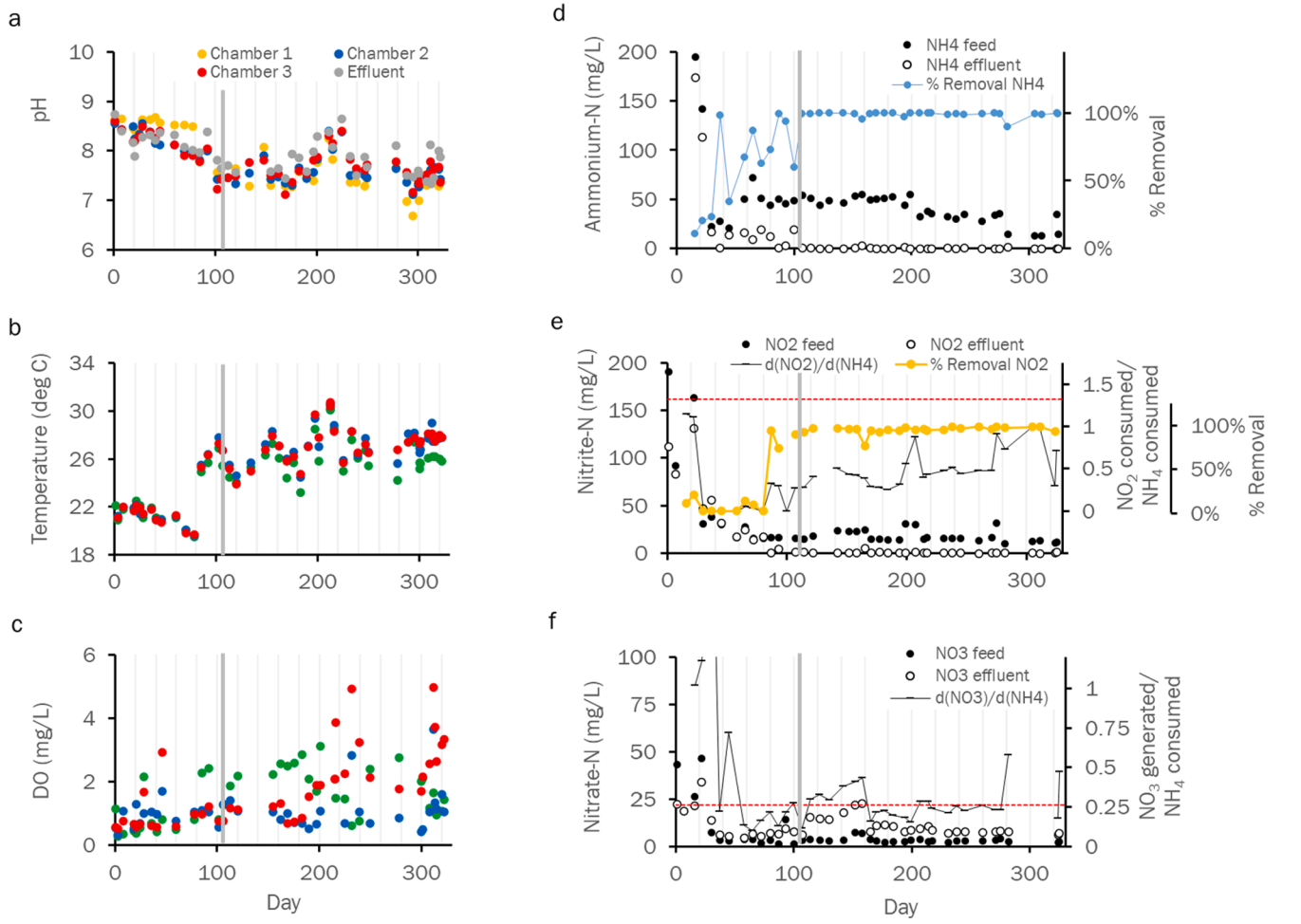


Fig. 1. Evolution of water quality parameters, a) pH, b) temperature, and c) dissolved oxygen, in in each ABR chamber and nitrogen species, d) ammonium-N, e) nitrite-N, and f) nitrate-N, in the feed and effluent of the anammox enrichment ABR. Percent removals of NH_4-N and NO_2-N and ratios of NO_2-N removed per NH_4-N removed and NO_3-N produced per NH_4-N removed are also shown as well as ratios expected based on stoichiometry (red dashed lines). Phase 1 (startup) is denoted by the vertical line at day 108. Temperature and DO were not measured in chamber 4 due to inaccessibility due to rock media. pH values also shown for ABR effluent. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

entirety of Phase 2. During this phase, pinkish coloration was occasionally observed on biofilm inside the ABR, but the color could be generally classified as brownish-gray.

There was an increase in overall DO concentrations and in the fluctuation of DO over time, especially after day 200, which may be due to the slow but gradual transition from highly reducing conditions, which were present during the ABR's previous anaerobic treatment of wastewater, to a greater occurrence of DO concentrations > 1.0 mg/L due to the higher DO feed solution. Also, DO measurements were taken at the water surface and during pumping conditions, which may have resulted in more elevated values. High and persistent DO concentrations are known to inhibit anammox growth (Strous et al., 1999) and support the growth of nitrite oxidizing bacteria (NOB). However, reviews of one-stage anammox processes with intermittent aeration (Li et al., 2018) and partial nitritation/anammox (PN/A) (Qiu et al., 2021) report the general consensus that intermittent aeration can successfully inhibit NOB and lead to high N removals because anammox bacteria recover from inhibition faster than NOB and can consume nitrite produced during aeration phases. Although intermittent aeration was not employed in the ABR of this study, the plug-flow nature of the ABR combined with intermittent feeding schedule may have resulted in a more resilient anammox biomass within the biofilms present in the reactor.

Changes in environmental conditions, which were imposed to achieve more optimal temperature and pH during the enrichment period, had variable effects on the removal of NH_4-N and concentrations of other N species. The increase in temperature from ambient temperature (between 19 °C and 22 °C) to an average of 27 °C ± 1.5 °C after day 80 did not result in immediate changes to N removal (Fig. 1b). However, the decrease in the feed concentration of $KHCO_3$ from 1.25 mg/L to 0.33 mg/L ~two weeks later, on day 97, which resulted in a pH decrease from 8.17 ± 0.40 to 7.55 ± 0.27 (Fig. 1c), did coincide with a shift to high NH_4-N removal rates that were consistently > 90%.

Based on the full stoichiometry reported for anammox metabolism in a SBR by Strous et al. (1998), the stoichiometric ratio between nitrite and ammonium uptake (NO_2/NH_4 ratio) and the ratio between nitrate production and ammonium uptake (NO_3/NH_4 ratio), should be 1.23 and 0.26, respectively. Under high purity conditions achieved in a membrane bioreactor, Lotti et al. (2014) determined slightly different stoichiometric ratios: a NO_2/NH_4 ratio of 1.146 and a NO_3/NH_4 ratio of 0.161. Although the NO_2-N removal rates were observed to be > 90% in the current study, our NO_2/NH_4 ratio was only 0.50 ± 0.23 due to the lower NO_2-N in the influent. The NO_3/NH_4 ratio of 0.27 ± 0.11 was in the range suggested by both Strous et al. (1998) and Lotti et al. (2014). The low average NO_2/NH_4 ratio shows that NH_4-N and NO_2-N removals were met in the ABR reactor without the NO_2-N addition (of 1.23 times that of NH4-N) that is recommended from stoichiometric ratios of reactants. The high removals were, however, likely due to a microbial

consortia existing in the ABR that also may have contained nitrifying and denitrifying organisms that could aid in N removal.

3.2. Chamber-by-chamber analysis

The characteristics and removal of N species in each chamber differed due to the plug-flow nature of the ABR. Removal of NH_4-N was typically greatest in Chamber 1, which had 58% removal on average, followed by Chamber 2, which had an additional 31% removal on average (Table 3). By the time water reached Chambers 3 and 4, influent NH_4-N concentrations were already reduced by 90%. The removal of NO_2-N was also highest in the first two chambers, but Chambers 3 and 4 also had substantial removals (Table 3). On the two occasions when samples were collected for DOC and TDN analyses, DOC concentrations were low in the influent and in all chambers, ranging from 2.5 to 2.9 mg/L. DOC was not added in the feed; therefore, additional sources of organic compounds must be autochthonous, derived from the reactor biomass. TDN concentrations decreased most notably between Chambers 1 and 2. These results further support that the fourth chamber of the ABR provides limited N removal function and may be redundant, as most of the N removal (96% of ammonium and 76% of nitrite) occurred in Chambers 1 – 3.

Anaerobic batch tests conducted on day 290 containing biomass, ammonia, and nitrite, had specific anammox activity (SAA) ranging from 0.28 to 0.48 g N/g VSS·d, which is within the range that has been reported for other reactors maintained at different temperatures (0.03 g N/g VSS·d at 10 °C to 0.86 g N/g VSS·d at 40 °C; Tomaciewski et al., 2017). Over the 24-h incubation period, removals ranged from 14% of NH_4-N to 25% of NO_2-N in Chambers 1 and 3. The greatest removal, 56% of NH_4-N and 23% of NO_2-N, and highest SAA (0.48 g N/g VSS/d) occurred using biomass from Chamber 4 (Table 4), which, incidentally, was the only chamber containing lava rock media. These results support other studies (Adams et al., 2020) showing that the inclusion of biomass carrier media is beneficial for growth of active anammox communities and may even shorten the start-up time of the anammox process (Lu et al., 2017). Nitrite and ammonium could not be measured in Chamber 2 due to initially high readings that exceeded the detection limits of the nutrient analysis kits; nitrate concentrations of Chamber 2 were similar to those of Chambers 1 and 3.

3.3. Microbial activity traced with fluorescence

Fluorescence spectroscopy has been used to track microbial sources and activity in natural and engineered systems. Although the use of EEM fluorescence as a tool to evaluate anammox growth and the performance of anammox reactors is relatively new (last decade), a large number of studies have now documented changes in fluorescent peaks and components in anammox reactors (e.g., Lu et al., 2017; Hou et al., 2017; Ruscalleda et al., 2014; Yang et al., 2018; Xia et al., 2019; Zhang et al., 2019; Wang et al., 2020). Most studies have found that fluorescent peaks increased over long-term anammox enrichment, with some studies indicating greater increases in humic-like peaks (eg., Ruscalleda et al., 2014) and other observing greater increases in protein-like peaks (eg., Hou et al., 2017). In the present study, we did not track changes in fluorescence over the duration of anammox enrichment. Nevertheless, from our snapshot analysis, we did find that fluorophores associated with tryptophan (Peak T; ex/em of 275/340 nm) were often present at intensities as high or higher than humic and fulvic acids (Peaks A and C; ex/em of 240/450 nm and 350/500 nm, respectively) (Fig. 2).

In all chambers, a fluorescence shoulder was present at 420 nm excitation and an emission between 460 nm and 490 nm (shown as a "+" in Fig. 2), resulting in what gives the impression of a "stretching" of the peak C region toward higher excitation wavelengths. The association of the 420 nm peak with anammox activity was first reported by Ruscalleda et al. (2014), who ran an upflow biofilm anammox reactor and found their parallel factor analysis (PARAFAC)-derived component 2, containing the 420 nm peak, increased both over long-term (~120 d) and batch experiments. Later Hou et al. (2017) also observed this peak in an anammox enrichment reactor and determined that it was due to the increased presence of intracellular compounds. Previous studies only associated this peak with Coenzyme 420, a compound produced by methanogens (Song et al., 2019). Hou et al. (2017) further showed that the fluorescence at 420 nm excitation correlated significantly with nitrogen removal rate in their anammox membrane bioreactor. Here we show that the increase in fluorescence at the excitation/emission of ~420/470 nm affects the ratio of emission intensities at 470 nm to 520 nm, which are used to calculate the FI (Mcknight et al., 2001), thereby resulting in an increase in the FI to values > 2.0. Therefore, the high FI values (from 1.91 to 2.25) in the anammox ABR may be due to the fluorescence of the intracellular compounds responsible for this peak. The only peak intensity that increased from chamber 1 to chamber 4 was that of humic-like Peak C. The increase in the HIX in later chambers also may reflect preferential degradation of organic compounds, presumably derived from sludge in the ABR. Chamber-by-chamber increases in the HIX have been reported previously in a decentralized facility in South Africa that utilized an ABR to treat domestic wastewater (Mladenov et al., 2018).

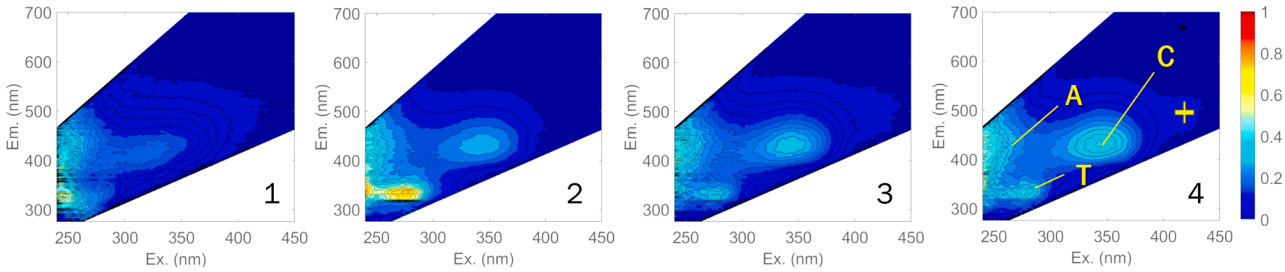
Table 3
Mean concentrations and standard deviations (in parentheses) of N species, DOC, and TDN in water column of feed tank, effluent tank, and four chambers of the ABR.

Constituent			Chambers				
	n	Feed	1	2	3	4	Effluent
NH_4-N (mg/L)	7	33 (19)	14 (14)	3.7 (5.7)	1.4 (2.6)	0.50 (0.70)	0.30 (0.52)
NH_4-N removal	–	–	58%	31%	7%	3%	0%
NO_2-N (mg/L)	7	16 (7.8)	12 (12)	6.1 (9.7)	4.0 (8.1)	1.0 (1.3)	1.1 (1.6)
NO_2-N removal	–	–	25%	37%	13 %	18%	−1%
Nitrate-N (mg/L)	7	2.6 (0.70)	4.7 (0.89)	6.1 (1.3)	7.8 (1.5)	8.1 (2.2)	8.0 (1.9)
DOC (mg/L)	2	2.7 (0.28)	2.8 (0.05)	2.7 (0.06)	2.8 (0.18)	2.51 (0.06)	2.92 (0.06)
TDN (mg/L)	2	28 (6.5)	20 (4.1)	8.5 (0.40)	6.8 (0.90)	5.7 (2.2)	5.5 (2.1)

Table 4
Mean concentrations with standard deviations (in parentheses) and percent removal of N species during 24-h anammox activity test on day 290.

			Chambers			
Constituent	*n*	Control	1	2*	3	4
Ammonium-N (mg/L)	2	110 (2.3)	85 (5.7)	R	90 (1.1)	46 (1.9)
Nitrite-N (mg/L)	2	120 (2.6)	89	R	95 (1.6)	92 (4.0)
Nitrate-N (mg/L)	2	11 (0.64)	27 (2.0)	26	27 (2.1)	29 (0.32)
NH_4-N removal	2	n/a	20%	n/a	14%	56%
NO_2-N removal	2	n/a	25%	n/a	20%	23%
Specific anammox activity, SAA (g N/g VSS/d)	1	n/a	0.28	n/c	0.40	0.48

* R = concentrations exceeded the range of the analysis kits, and sample preservation was compromised before dilution and re-analysis could be performed, therefore, NH_4-N and NO_2-N in chamber 2 could not be reported; n/a = not applicable; n/c = specific activity could not be calculated because of missing N concentrations for this chamber.



Index/Peak	Chamber 1	Chamber 2	Chamber 3	Chamber 4
FI	1.91	2.17	2.14	2.25
HIX	0.73	0.81	1.47	1.38
Peak C	0.16	0.29	0.31	0.38
Peak T	0.19	0.67	0.32	0.36

Fig. 2. EEMs of water samples from each chamber of the anammox ABR, analyzed on day 201 and corresponding FI and HIX values and Peak C and T intensities.

3.4. Microbial community composition

The qPCR analysis showed positive detection of qPCR targets for anammox (16S) (of the species of *Candidatus Kuenenia stuttgartiensis*) in all four chambers of the ABR (Fig. 3). There was notable variability in anammox bacteria concentrations (ranging from 6-log to 8-log gc/mL), even within the same chamber. Substantial variability was also observed when anammox concentrations were normalized to eubacteria (which was carried out to compensate for sampling effects), with percentages ranging from 26% to 69%, indicating that all chambers were enriched in anammox bacteria (Fig. 3). Our values are in the range reported by Wang et al. (2019) for the last chamber of an anaerobic baffled biofilm reactor after 163 d of operation, but lower than those reported for a SBR with pure anammox bacterium in granular sludge (Bae et al., 2016).

Given the fluctuating DO concentrations in the ABR and the robust biofilm formation along the senescent sludge blanket and along chamber sides and in the rock media of Chamber 4, other microbial communities were also likely to be co-existing in the ABR. The qPCR analysis showed that denitrifiers (nirS) were also present in all four chambers of the ABR. Denitrifier concentrations normalized to eubacteria represented between 18% and 32% (Fig. 3) and were, therefore, also important for N removal in the ABR. Dissolved organic carbon concentration of the feed solution was low (~2.7 mg/L; Table 3); therefore, the carbon source for denitrifiers must be autochthonous carbon, derived from cellular compounds of the microorganisms in the ABR sludge and biofilm.

Melt curve analysis revealed that signals from the first two chambers were a closer match to signals from the original anammox biomass used for reactor inoculation, whereas signals from Chambers 3 and 4 had slightly higher melting temperature, which may point to different mutants/variants from the original inocula. PCR products from the samples submitted were also analyzed by gel electrophoresis to verify product size (Supplemental Fig. S1). These images indicate that samples from all four ABR chambers were similar to the LACSD anammox strain in the inoculum originally used to start up the ABR.

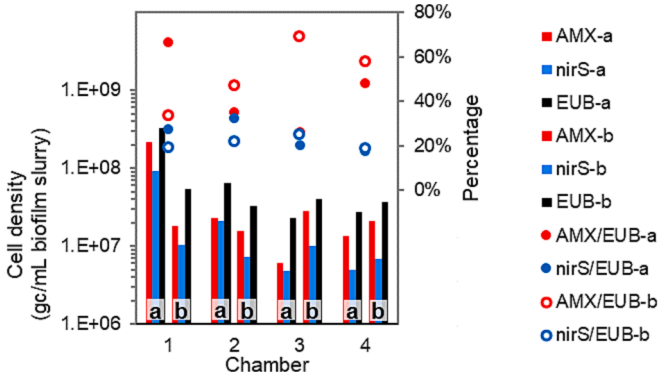


Fig. 3. Concentrations of anammox, denitrifiers, and eubacteria and normalized values of anammox/eubacteria (red markers) and denitrifiers/eubacteria (blue markers) for replicates "a" and "b" sampled from each chamber of the ABR. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Although nitrifying bacteria were not analyzed as part of this study, the fluctuating DO concentrations and increase in nitrate within the ABR chambers supports the idea that nitrifiers were also present. Indeed, in commercialized/full-scale operating deammonification reactors (e.g., Kruger ANITA^{TM}Mox, World Water Works DEMON) where the systems are aerated either continuously or intermittently, the systems still maintain anammox activity, and anammox are able to coexist with nitrogen oxidizing bacteria (Liu et al., 2014). Although DO is known to inhibit anammox activity, there are two important factors that may also be underway in the ABR of this study: (a) the inhibition is not irreversible; and (b) in biofilm systems, heterotrophic bacteria and nitrifiers can grow on the outer biofilm consuming the bulk liquid DO and shielding anammox and other anaerobic bacteria from oxygen exposure (Lemaire et al., 2013). Nevertheless, future work using amplicon sequencing and hybridization methods to evaluate microbial diversity specifically in the sludge blanket of ABR systems is recommended.

4. Conclusions

In the present study, >90% ammonium and nitrite removals were accomplished in a ~32 L anaerobic baffled reactor after ~100 d under low maintenance, intermittently-flowing conditions, at temperatures that were below the optimal range for anammox growth. Both sludge blanket biofilm (Chambers 1–3) and rock media biofilms (Chamber 4) harbored anammox bacteria, as confirmed by qPCR analysis and batch anammox activity tests in all four chambers. Denitrifier genes were also detected in the ABR chambers, reflecting the important contribution to high N removal by both anammox and nitrification–denitrification processes. Given the lower contribution to total N removal of the fourth ABR chamber but the potential importance of carrier media for maintaining biomass in biofilms and preventing washout of biomass, a three-chamber ABR with biomass carrier media in one or more chambers would be recommended for full-scale application. These results are important for the startup of anammox reactors for decentralized wastewater treatment.

CRediT authorship contribution statement

Elisa Rivera: Data curation, Formal analysis, Investigation,

Supervision. **Natalie Mladenov:** Conceptualization, Formal analysis, Investigation, Supervision, Funding acquisition. **Lilith Astete Vasquez:** Data curation, Formal analysis, Investigation, Supervision. **Grace McKenzie:** Data curation, Formal analysis, Investigation, Supervision. **Vanessa Gonzalez:** Data curation, Investigation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biortech.2022.128047.

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