Application of an enrichment culture of the marine anammox bacterium “*Ca. Scalindua* sp. AMX11” for nitrogen removal under moderate salinity and in the presence of organic carbon

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Graphical Abstract

ABSTRACT

Seawater can be directly used for toilet flushing in coastal areas to reduce our dependence on desalination and freshwater resources. The presence of high-salt content in the generated wastewater from seawater toilet flushing could limit the performance of conventional biological nitrogen removal processes. Anaerobic ammonium oxidation (anammox) process is regarded as one of the most energy-efficient process for nitrogen removal from N-rich waste streams. In this study, we demonstrated the application of a novel marine anammox bacterium (*Candidatus Scalindua* sp. AMX11) in a membrane bioreactor (MBR) to treat moderate-saline (~1.2% salinity) and N-rich organic (2 mM acetate) solution, prepared using real seawater. The MBR showed stable performance with nitrogen removal rate of $0.3 \text{ kg-N m}^{-3} \text{ d}^{-1}$ at >90% N-removal efficiency. Furthermore, results of ^{15}N stable isotope experiments revealed that anammox bacteria was mainly responsible for respiratory ammonification through NO_3^- reduction to NH_4^+ via NO_2^- , and the by-products of respiratory ammonification were used as substrates by anammox bacteria. The dominant role of anammox bacteria in nitrogen removal under saline and organic conditions was further confirmed by genome-centric combined metagenomics and meta-transcriptomic approach. Taken together, these results highlight the potential application of marine anammox bacteria for treating saline wastewater generated from seawater toilet flushing practices.

Keywords: Biological nitrogen removal; Anammox; Seawater toilet flushing;

Candidatus Scalindua

1. Introduction

As the demand on freshwater resources for potable and non-potable uses increases due to the rapidly growing human population, the per capita availability of freshwater in many regions is likely to decrease (Wang et al., 2017b, 2018). Over 50% of the world's population resides within 60 km of the coast (Polunin, 2008), and seawater can be directly utilized for non-potable uses such as toilet flushing to reduce the demand on freshwater resources. The use of seawater for toilet flushing is already in practice in several coastal cities (Tang et al., 2007). These practices indicate that the salinity of the generated wastewater (i.e., used seawater and freshwater) ranges from 1 to 1.7 % (Yang et al., 2015).

It has been demonstrated that the best configuration for biological nitrogen removal to achieve energy-efficient wastewater treatment is the A-B configuration with anaerobic treatment of organic carbon (A-stage) followed by mainstream partial nitrification and anaerobic ammonium oxidation (anammox) treatment (B-stage) (Ali and Okabe, 2015; Gu et al., 2018; McCarty, 2018; Wan et al., 2016). Organic pollutants in saline wastewaters can be degraded or recovered as an energy source in anaerobic digestion (Aspé et al., 1997; Lefebvre and Moletta, 2006) or bio-electrochemical systems (Kim and Logan, 2013). The effect of saline wastewater is more pronounced on autotrophic microbial communities responsible for nitrogen removal in biological wastewater treatment processes (Wang et al., 2017b) due to their low diversity and functional redundancy compared to heterotrophs (Fuhrman, 2009; Loreau et al., 2001; Saikaly and Oerther, 2011). Further, saline wastewaters could produce hyperosmotic stress in anammox bacteria, which might lead to cell plasmolysis due to high osmotic pressure (Ali et al., 2014).

Anammox bacteria can be broadly classified into two physiological groups; 1) freshwater anammox (belonging to genera *Candidatus Brocadia*, *Ca. Kuenenia*, *Ca. Anammoxoglobus*, and *Ca. Jettenia*), and 2) marine anammox (belonging to genus *Ca. Scalindua*) (Sonthiphand

et al., 2014). Based on existing literature, there are two strategies to treat nitrogen-rich saline wastewater through anammox process; one is adaptation of freshwater anammox bacteria to saline wastewater and another is application of marine anammox bacteria to treat saline wastewater (Li et al., 2018b). Previous studies indicated that anammox bacteria derived from freshwater environments have the potential to treat nitrogen-rich saline wastewater by gradually acclimatizing anammox bacteria to increasing salt (NaCl or NaCl + KCl) concentration (up to 20–30 g L⁻¹) within 60–100 days (Dapena-mora et al., 2010; Kartal et al., 2006; Malovanyy et al., 2015; Yan et al., 2016 Wang et al., 2017a; Gonzalez-Silva et al., 2017). Further, studies showed that different freshwater anammox bacteria have different tolerance to salinity, with *Ca. Kuenenia* able to adapt to high salinity (15–30 g L⁻¹) (Kartal et al., 2006; Yan et al., 2016; Wang et al., 2017a; Gonzalez-Silva et al., 2017), whereas *Ca. Jettenia* and *Ca. Brocadia* are very sensitive to salinity higher than 4 g L⁻¹ (Ali et al., 2015; Gonzalez-Silva et al., 2017; Wang et al., 2017a).

Marine anammox bacteria are better suited for nitrogen-rich saline wastewater treatment compared to freshwater anammox bacteria due to their intrinsic tolerance to salinity, and they could maintain a stable nitrogen removal performance at a wide range of salt concentration (0–50 g L⁻¹) and in response to salinity shock (Wei et al., 2016; Li et al., 2018;). However, they are less studied compared to freshwater anammox bacteria for saline wastewater treatment (Kawagoshi et al., 2009; Kindaichi et al., 2011; Toro et al., 2017; van de Vossenberg et al., 2008). Also, these previous studies on marine anammox bacteria were conducted using inorganic artificial seawater medium with salinity of ~3%, and studies on the effect of different saline conditions (1–3% salinity) on nitrogen removal efficiency of marine anammox bacteria are lacking. It has been indicated that certain elements in inorganic artificial seawater medium might be essential for the growth and activity of marine anammox bacteria (Wei et al., 2016). It is therefore essential to use real seawater to demonstrate the potential application of marine

anammox bacteria for treating saline wastewaters generated from seawater toilet flushing practices. Further, if marine anammox bacteria are to be implemented as B-stage of an A-B process, the effect of residual organic carbon from stage A on their activity should be investigated. Recent studies demonstrated the application of freshwater anammox for the treatment of saline and N-rich organic wastewater (Li et al., 2018a; Rio et al., 2018). However, studies on the long-term impact of organic carbon on marine anammox process using real seawater are lacking (Table S1).

A comprehensive insight into the microbial ecology of anammox process for saline wastewater treatment is also needed to optimize system performance. Amplicon sequencing, although a powerful technique for alpha and beta diversity studies (Ali et al., 2019b), does not provide information on community function (Gonzalez-Silva et al., 2017). Recently, genome-resolved metagenomics and metatranscriptomics (Lawson et al., 2017) have been applied to investigate the expression of genes in anammox and heterotrophic bacteria in a laboratory-scale anammox bioreactor treating non-saline wastewater. It is pertinent to mention that previous studies using marine (Awata et al., 2013; Li et al., 2018b; van de Vossenberg et al., 2008) or freshwater anammox bacteria (Ali et al., 2015; Kartal et al., 2006; Oshiki et al., 2016) for the treatment of nitrogen-rich saline wastewater did not use genome-resolved metatranscriptomics approach to investigate the effect of different salinity and organics on anammox process. Thus, studies on genome-centric metatranscriptomics to investigate the effect of different salinity and organics on expression of genes in anammox bacteria are lacking.

The objective of this study was to evaluate the nitrogen removal performance of a novel marine anammox bacterium, *Ca. Scalindua* sp. AMX11 (Ali et al., 2019a), in a membrane bioreactor (MBR) operated at different saline conditions (1.2–3.5% salinity) and continuously fed with real seawater supplemented with ammonium, nitrite and acetate (0–4 mM) as the main source of organic carbon. The marine anammox bacterium was initially enriched and cultured

in an up-flow column bioreactor continuously fed with fresh Red Sea water supplemented with ammonium and nitrite. *Ca. Scalindua* sp. AMX11 has 84% nucleotide-level genomic similarity with the closest related anammox bacterium *Ca. Scalindua rubra*, which was detected in Red Sea ecosystem (Ali et al., 2019a). However, *Ca. Scalindua rubra* could not be enriched in a bioreactor (Speth et al., 2017).

MBR was used in this study to effectively retain the marine anammox biomass because their doubling time is longer (~14 days) (Awata et al., 2015, 2013; van de Vossenberg et al., 2008) relative to freshwater anammox bacteria (2–4 days) (Zhang et al., 2017b). The contribution by anammox, denitrification and respiratory ammonification (*i.e.* dissimilatory nitrate reduction to ammonium) pathways were determined by stable ^{15}N isotope batch incubation experiments. Combined metagenomics and metatranscriptomics approach was used to determine genome-resolved transcription activities under different operating conditions to underpin the microbial community responsible for nitrogen removal in the MBR.

2. Materials and methods

2.1. Membrane bioreactor construction and operation

Ca. Scalindua enrichment culture was harvested as a biofilm, attached to a non-woven sheet, from an up-flow column reactor continuously fed with fresh Red Sea water supplemented with ammonium and nitrite (Ali et al., 2019a). The anammox biofilm was detached from the non-woven sheet by gentle shaking and seeded into a 7.5-L (5 L effective volume) MBR equipped with a microfiltration (average pore size 0.1 μm) hollow fiber membrane module (Zena-membrane, Czech Republic) (Fig. S1). Subsequently, the MBR was operated in three different phases (Table S2) for 140 days at a nitrogen-loading rate (NLR) of 0.24–0.40 $\text{kg N}\cdot\text{m}^{-3}\text{d}^{-1}$. The salinity of the feed was gradually reduced in Phases 2 and 3 by mixing Red Sea water with deionized water (DIW) to mimic the salinity of wastewater generated from seawater

toilet flushing practice. The MBR was continuously fed with fresh Red Sea water (~3.5% salinity) supplemented with NH_4^+ (8–14 mM), NO_2^- (9–15 mM), sodium acetate (0–4 mM), CaCl_2 100 mg L^{-1} , MgSO_4 300 mg L^{-1} , KH_2PO_4 30 mg L^{-1} , KHCO_3 500 mg L^{-1} and trace element solutions (van de Graaf et al., 1995). The MBR was operated at $25 \pm 2^\circ\text{C}$ and pH 7.5–8.0 with mixing by a metal propeller at a stirring speed of 150 rpm. Anoxic conditions were maintained by continuous purging with argon: CO_2 (95:5) gas at a rate of 10 mL min^{-1} . The synthetic solution was fed continuously at a rate of $\sim 5 \text{ L d}^{-1}$ and hydraulic retention time of one day. The sludge retention time was kept at 33 days by constantly wasting sludge at a rate of 150 mL d^{-1} to maintain the biomass concentration at $\sim 0.5 \text{ mg-protein mL}^{-1}$. Biomass, influent and effluent samples were collected on a regular basis for measuring the concentrations of NH_4^+-N , NO_2^--N and NO_3^--N using HACH kits (HACH, CO, USA) following manufacturer's instructions. Concentrations were measured by a spectrophotometer (D5000, HACH, CO, USA).

2.2. ^{15}N Tracer batch incubation experiments

Standard anaerobic techniques were adopted in the batch incubation experiments as described elsewhere (Ali et al., 2015). The biomass from the MBR was washed twice to remove residual NH_4^+ , NO_3^- , and NO_2^- and suspended in an inorganic medium with a composition similar to the composition of synthetic solution used in the MBR but without addition of NH_4^+ , NO_3^- , NO_2^- , and acetate. Also, the inorganic medium contained 2 mM of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, pH 7.8). Next, the biomass was dispensed into 10-mL glass serum vials (Chemglass Life Sciences, NJ, USA). The biomass concentration in the vials ranged from 0.1–0.6 $\text{mg-protein mL}^{-1}$. Biomass concentration was determined as protein concentration using DC Protein Assay Kit (Bio-Rad, Tokyo, Japan) with bovine serum albumin as the standard. To prevent unintentional contamination with ambient air during incubation and

gas sampling, the headspace in the vials was replaced with helium gas at a positive pressure (50–75 kPa). The vials with oxygen-free biomass suspensions were pre-incubated overnight at room temperature (~25°C) to eliminate residual substrates. The activity test was initiated by adding $^{15}\text{N-NO}_3^-$ and $^{13}\text{C-acetate}$ (2 mM each) using a gas-tight syringe (VICI; Baton Rouge, LA, USA). Some vials were also supplemented with $^{14}\text{N-NH}_4^+$ (2 mM). The vials were incubated in triplicates at 25°C in the dark for several hours.

The concentrations of $^{29}\text{N}_2$, $^{30}\text{N}_2$, and $^{13}\text{CO}_2$ gases in the headspace of the vials were determined by gas chromatography mass spectrometry (GC/MS) analysis (Ali et al., 2015; Yoshinaga et al., 2011). Fifty microliter of headspace gas was collected using a gas-tight syringe (VICI; Baton Rouge, LA, USA), and immediately injected into a GC (Agilent 7890A system equipped with a CP-7348 PoraBond Q column) combined with 5975C quadrupole inert MS (Agilent Technologies; Santa Clara, CA, USA), and $m/z = 29, 30$, and 45 were monitored. The gas samples were collected almost on an hourly basis depending upon the gas production. The amounts of $^{29}\text{N}_2$, $^{30}\text{N}_2$ and $^{13}\text{CO}_2$ gases were determined using a standard curve prepared with $^{30}\text{N}_2$ and $^{13}\text{CO}_2$ standard gas (>98% purity) (Sigma-Aldrich, USA). All batch incubation experiments (~8–12 h) were performed in triplicates.

Stable isotopic compositions of NH_4^+ and NO_2^- in the liquid samples were determined as previously described (Isobe et al., 2011). The liquid samples were collected at the end of incubation. The cell suspension was filtered through a 0.45 μm syringe filter (Advantec; Tokyo, Japan) and the filtrates were dispensed into sealable glass vials. NO_2^- was chemically reduced to N_2O gas using sodium azide, and the $^{15}\text{N}/^{14}\text{N}$ ratio of the N_2O gas ($m/z = 44, 45$, and 46, respectively) in the headspace was examined by GC/MS. NH_4^+ was converted to NO_2^- by hypobromide oxidation procedure as mentioned elsewhere (Lachouani et al., 2010). Similarly, NO_3^- in the liquid samples was converted to NO_2^- through vanadium chloride reduction protocol (Lachouani et al., 2010). Before converting NH_4^+ and NO_3^- to NO_2^- , samples were

treated with sulfamic acid to reduce residual NO_2^- to N_2 gas (Granger and Sigman, 2009). The $^{15}\text{N}/^{14}\text{N}$ ratios were quantified using standard curves prepared from NaNO_2^- solution containing the ^{15}N atom at known ratios; *i.e.*, 0.37, 5.31, 10.3, 24.4, 49.8, and 99.2 atom% ^{15}N .

2.3. Metagenomic analyses

Metagenomic analyses were performed to facilitate genome-resolved transcription analyses for profiling the gene expression across the recovered population genomes. Two independent biomass samples for metagenomics were collected from the MBR after one (Phase-1) and four months (Phase-3) of operation. The DNA in each sample was extracted using the Fast DNA spin kit (MP Biomedicals, Tokyo, Japan) according to the manufacturer's instructions. Metagenome library was prepared using the NEB Next Ultra II DNA library preparation kit (New England Biolabs, USA). The DNA library was paired-end sequenced (2 x 301bp) on a HiSeq 2500 system (Illumina, USA). Raw reads obtained in the FASTQ format were processed for quality filtering using Cutadapt package (v. 3.7.1) (Martin, 2011). Trimmed reads were assembled using SPAdes version 3.11.1 (Bankevich et al., 2012). The reads were mapped back to the assembly using Burrows-Wheeler Aligner (BWA, v. 0.7.15-r1142-dirty) (Li and Durbin, 2010) to generate coverage files for metagenomics binning. These files were converted to the sequence alignment/map (SAM) format using samtools (Li et al., 2009). The metagenome-assembled genomes (MAGs) were retrieved from assembled scaffolds by binning based on sequence composition, differential coverage and read-pair linkage by employing CONCOCT program available within anvi'o software (Eren et al., 2015). The MAGs were refined manually if needed by following the instructions provided on the anvi'o website (<http://merenlab.org/2016/06/22/anvio-tutorial-v2/>, retrieved on December 2, 2018). Completeness and contamination of MAGs were determined in anvi'o based on occurrence and a set of single-copy marker genes (Campbell et al., 2011). Subsequently, MAGs were annotated

using Prokka ([Seemann, 2014](#)). The nomenclature of the annotated genome of *Ca. Scalindua rubra* (MAYW01) was utilized for annotation of anammox MAG.

Phylogenetic analysis was performed for the recovered MAGs and closely related genomes downloaded from the National Center for Biotechnology Information (NCBI) genome repository. The MAGs and closely related genomes were imported in anvi'o to create a set of concatenated ribosomal proteins adapted from [Campbell et al. \(2011\)](#). The anvi'o command “*anvi-get-sequences-for-hmm-hits*” was used with *-concatenate* flag to generate concatenated amino acid sequences. The phylogenetic tree was computed by MEGA7 using the concatenated amino acid sequences by applying neighbor-joining statistical method ([Kumar et al., 2016](#)). Branch node support values were calculated by performing 1000 bootstrap iterations and using the Poisson model.

2.4. Metatranscriptomic analyses

Duplicate biomass samples for metatranscriptomics were collected from the MBR during Phase-1 (day 30), Phase-2 (day 55) and Phase-3 (day 115) when the reactor performance was stable and stored in RNAlater™ Stabilization Solution (Invitrogen™, USA) until further processing. Total RNA in each sample was extracted using the PowerBiofilm RNA Isolation kit (QIAGEN, Germany) according to the manufacturer's instructions. After ribosomal RNA (rRNA) depletion using the Ribo-zero Magnetic kit (Illumina, USA) and DNase treatment using the DNase MAX kit (MoBio Laboratories Inc.), the samples were cleaned and concentrated using the RNeasy MinElute Cleanup kit (QIAGEN, Germany) and successful rRNA removal was confirmed using TapeStation HS RNA Screentapes (Agilent Technologies, USA). The samples were then prepared for sequencing using the TruSeq Stranded Total RNA kit (Illumina, USA) according to the manufacturer's instructions. The samples were pooled in equimolar concentrations and sequenced on HiSeq 2500 system (Illumina, USA) using a 1x50

bp Rapid Run (Illumina, USA). Raw sequence reads in FASTQ format were trimmed using USEARCH (v10.0.2132; [Edgar, 2010](#)) -fastq_filter with the settings -fastq_minlen 45 -fastq_truncqual 20. The reads were then mapped to the predicted protein coding genes generated from Prokka (v1.12) ([Seemann, 2014](#)) for each MAG. Reads with a sequence identity below 0.98 were discarded. The count tables were imported to R ([R Core Team, 2013](#)) and processed using the default DESeq2 workflow ([Love et al., 2014](#)), and visualized using ggplot2. Principle component analysis (PCA) and hierarchical clustering analysis (HCA) of overall sample similarity was done using DESeq2 normalized counts (log transformed), through the vegan package ([Oksanen et al., 2017](#)) in R platform.

2.5. Data availability

All sequencing data associated with this project are available at the National Center for Biotechnology Information (NCBI) under BioProject PRJNA482223. Annotated GenBank files for the MAGs described in this study can be found under accession numbers listed in [Table S5](#).

3. Results

3.1. Performance of the MBR during the three phases of operation

The MBR inoculated with the enriched marine anammox culture (*Ca. Scalindua* sp. AMX11) was operated for 30 days (phase-1) with synthetic inorganic solution prepared using Red Sea water (~3.5% salinity) ([Table S2](#)). Stable performance with nitrogen removal rate (NRR) of $0.35 \pm 0.02 \text{ kg-N m}^{-3} \text{ d}^{-1}$ and nitrogen removal efficiency of ~90% was observed in phase 1 ([Fig. 1](#)). In phase 2 (day 31–55), the salinity of the synthetic inorganic solution was gradually reduced by mixing Red Sea water with DIW to reach a salinity of 1.8% on day 55. The performance of the MBR in phase 2 was similar to phase 1 with NRR of $0.34 \pm 0.01 \text{ kg-N}$

$\text{m}^{-3} \text{d}^{-1}$ and nitrogen removal efficiency of ~90% (Fig. 1). The calculated stoichiometric ratios of removed NO_2^- to removed NH_4^+ ($\text{Rem-}\Delta\text{NO}_2^-/\text{Rem-}\Delta\text{NH}_4^+$) and produced NO_3^- to removed NH_4^+ ($\text{Pro-}\Delta\text{NO}_3^-/\text{Rem-}\Delta\text{NH}_4^+$) were in the range of 1.0–1.5 and 0.08–0.12, respectively. These ratios were close to the theoretical anammox stoichiometric ratios of 1.15 ($\text{Rem-}\Delta\text{NO}_2^-/\text{Rem-}\Delta\text{NH}_4^+$) and 0.10 ($\text{Pro-}\Delta\text{NO}_3^-/\text{Rem-}\Delta\text{NH}_4^+$) (Lotti et al., 2014), indicating anammox process was mainly responsible for nitrogen removal in the MBR during phase 1 and 2 of operation. This was further supported by metagenomics and metatranscriptomics (see section 3.3).

During phase 3 (56–140 day), the salinity was further reduced to 1.2% and acetate concentration was progressively increased from 0 to 4 mM. Typically, NO_2^- concentration was below the detection limit throughout the experimental period except at an acetate concentration of 4 mM, where the anammox activity was deteriorated with effluent NO_2^- concentration reaching 50 mg-N L^{-1} . However, anammox activity was recovered after reducing the acetate concentration to 2 mM corresponding to a $\text{COD}/\text{NH}_4^+-\text{N}$ ratio of 1.0.

Stable reactor performance was achieved at acetate concentration of 2 mM with NRR of $0.27 \pm 0.01 \text{ kg-N m}^{-3} \text{d}^{-1}$ and ~92% N-removal efficiency. The average effluent NO_3^- concentration was reduced from 17 mg-N L^{-1} to 3 mg-N L^{-1} after the addition of acetate (2 mM), which improved the treatment efficiency. However, the calculated stoichiometric ratios were different from the conventional anammox process, with relatively higher NO_2^- removal (average of 1.61) and lower NO_3^- production (average of 0.04) per mole of NH_4^+ removed. This suggests that other bioprocesses such as heterotrophic denitrification, anammox, respiratory ammonification, etc. might be contributing to nitrogen removal in the presence of organic carbon. Therefore, experiments were conducted using stable isotope labeled substrates to determine the possible bioprocesses responsible for nitrogen removal in the presence of organic carbon.

3.2. Isotope labeling experiments revealed anammox as the dominant process for nitrogen conversion in the presence of organic carbon

Batch incubation experiments with $^{15}\text{N-NO}_3^-$ and $^{13}\text{CH}_3\text{COOH}$ (2 mM each) were conducted to investigate the nitrogen conversion pathways in the presence of organic carbon. In addition, $^{14}\text{N-NH}_4^+$ was added to some of the batch incubations (Fig. 2 and Fig. S2), which would preferentially be utilized and promote the accumulation of $^{15}\text{NH}_4^+$ (Brunner et al., 2013). In the presence of $^{14}\text{N-NH}_4^+$, the nitrogen gas produced was mainly $^{29}\text{N}_2$ (as a product of $^{14}\text{N-NH}_4^+$ and $^{15}\text{N-NO}_2^-$ which is generated from reduction of $^{15}\text{N-NO}_3^-$), whereas in the absence of $^{14}\text{N-NH}_4^+$ the main nitrogen gas produced was $^{30}\text{N}_2$ due to reduction of $^{15}\text{N-NO}_3^-$ to $^{14}\text{N-NH}_4^+$ via $^{15}\text{N-NO}_2^-$ through respiratory ammonification by anammox bacteria (Fig. 2 and Fig. S2). To determine if heterotrophic denitrification is responsible for dissimilatory nitrate reduction to ammonium (DNRA), Penicillin G (0.5 mM), a compound that is not active against anammox but inhibits specifically the activity of most heterotrophs (Hu et al., 2013), was added to the vials. Interestingly, there was no significant difference between the incubations with and without Penicillin G, indicating that heterotrophic denitrifiers were not responsible for nitrate reduction. In both incubations (i.e., with and without Penicillin G), approximately ~60% of the $^{15}\text{NO}_3^-$ was converted to $^{15}\text{NO}_2^-$, $^{15}\text{NH}_4^+$, $^{29}\text{N}_2$, and $^{30}\text{N}_2$ gas. In contrast, only ~5% of the $^{15}\text{NO}_3^-$ was transformed to N-related products in batch incubations with acetylene (0.1 mM), a compound that selectively inhibits anammox bacteria, further supporting the lack of contribution of heterotrophic denitrification. Taken together, these results confirmed that anammox bacteria were mainly responsible for reducing $^{15}\text{NO}_3^-$ with the oxidation of $^{13}\text{CH}_3\text{COOH}$ to $^{13}\text{CO}_2$ at an oxidation rate of $0.94 \pm 0.02 \text{ nmol} \cdot \text{mg-protein}^{-1} \cdot \text{min}^{-1}$. The NO_3^- reduction rate by marine anammox bacteria was $2.1 \pm 0.7 \text{ nmol-N} \cdot \text{mg-protein}^{-1} \cdot \text{min}^{-1}$.

To further confirm that anammox bacteria were responsible for nitrogen conversion in the MBR, combined metagenomics and metatranscriptomics approaches were used to establish genome-resolved transcription activities under different conditions.

3.3. High transcriptional activity of anammox bacterium “*Ca. Scalindua* sp. AMX11”

The total DNA extracted from the biomass samples were sequenced to obtain a system-wide view of the microbial community (Table S3 & S4) in the MBR. The combined metagenome assembly led to the extraction of 17 near-complete (completeness > 75%; redundancy < 4%) draft MAGs from the metagenome samples (Table S5). The recovered 17 population genomes accounted for 82% (Phase–1) and 71% (Phase–3) of the quality filtered sequence reads, indicating that the sequencing depth was sufficient to obtain a comprehensive insight of the microbial community structure in the MBR. Hereafter, we will refer to these 17 organisms by their identifiers mentioned in Table S5.

A phylogenetic tree was constructed from the recovered population genomes and the reference genomes based on a set of concatenated ribosomal protein sequences (Fig. 3). The MAGs recovered from the MBR represent organisms belonging to the phyla Armatimonadetes (1), Bacteroidetes (3), Calditrichaeota (5), Chloroflexi (2), Proteobacteria (4), and Planctomycetes (2, including the novel anammox bacterium *Ca. Scalindua* sp. AMX11, MAG ID: AMX). Phylogenomics analysis showed a clear phylogenetic distance between our species (AMX) and previously reported marine anammox bacterial species, suggesting it is an entirely new species (Fig. 3). As expected, *Ca. Scalindua* sp. AMX11 was the most abundant organism in the MBR (Fig. 4a). However, their relative abundance decreased during reactor operation (34% in phase III compared to 53% in phase I). Nevertheless, when comparing different phases based on activity (i.e., RNA), AMX had the highest (93–96%) gene expression among the microbial community in the MBR during the three phases of operation (Fig. 4b). Interestingly,

the other members in the community that displayed moderate relative abundance (<12%) in the MBR showed very low (<1%) overall gene expression. Since the majority of transcription reads (93–96% of transcription per million) belonged to *Ca. Scalindua* sp. AMX11 (Table S4), subsequent analysis was only performed for AMX-related transcription reads.

The DESeq2 workflow was applied to normalize the read counts (Love et al., 2014). Both PCA and HCA (Fig. 5) using DESeq2 normalized counts were performed to establish the congruency among technical replicates. Both analyses showed a high reproducibility among replicates, whereas the comparisons between different conditions indicated the up- or down-regulation of genes under specific conditions. The differentially expressed genes were identified statistically for phase 2 (89 out of 3886 genes, p-value < 0.01, Table S6) and phase 3 (1370 genes out of 3886 genes, p-value < 0.01, Table S7), while considering phase 1 as a control. It is pertinent to mention that the N-cycle related genes (Fig. S3) were not significantly up or down-regulated under moderate saline conditions (salinity ~ 1.8%; Table S6) in Phase 2. Similarly, N-cycle related genes were not significantly up or down-regulated after the addition of organics in Phase 3 (salinity ~ 1.2%; Table S7 and Fig. S3) except for the upregulation of the copper-containing nitrite reductase (*nirK*) gene responsible for the reduction of NO₂⁻ to nitric oxide, and subsequently to hydrazine N₂H₄ (Table S7 and Fig. S3).

Our results showed that the decrease in salinity did not influence the transcription activity, and this was also evident with the similar performance of the MBR in phase I and II (Fig. 1). However, the addition of acetate in phase 3 induced a significant change in the transcription activity which was also reflected in the MBR stoichiometry during phase 3. This was further supported by PCA (Fig. 5) where most of the variation (93%) was explained in principle component 1 (PC1) suggesting the critical role of acetate in stimulating the change in the transcription activity of *Ca. Scalindua* sp. AMX11. Particularly, we noticed NO₃⁻ transporters gene (Gene ID: FDANJJPB_03011) was up-regulated (p-value < 0.05) (Table S7) under the

presence of organic carbon suggesting that *Ca. Scalindua* sp. AMX11 actively utilized NO_3^- for respiratory ammonification. This was also reflected in the improved N removal performance of the MBR because of NO_3^- reduction under organic addition (Fig. 1). Further, our metatranscriptomics results support the isotopic experiments (Fig. 2) where anammox process was mainly responsible for respiratory ammonification through NO_3^- reduction to NH_4^+ via NO_2^- .

4. Discussion

It is anticipated that 75% of the world's population will reside within 60 km of the coast by 2020 (Yang et al., 2015), and more coastal cities may implement seawater for toilet flushing, which represents ~30% of the total domestic water demand, to reduce our dependence on freshwater resources and desalination – an energy intensive process (2.5–9 kWh energy required per m^3 of produced water) (Wakil et al., 2017). Therefore, demonstrating an energy-efficient biological process to treat saline wastewater generated from toilet flushing is urgently needed. Here, we demonstrated using complementary approaches including reactor performance, stable isotope experiments, metagenomics, and metatranscriptomics that *Ca. Scalindua* enrichment culture efficiently treated N-rich real seawater under organic conditions. *Ca. Scalindua* enrichment culture adapted quickly (~35 days) to salinity (~1.2%) and organics (acetate 2mM) and showed stable operation with >90% N-removal efficiency and NRR of 0.3 $\text{kg-N m}^{-3} \text{ d}^{-1}$, which was comparable to previously reported (0.07 to 10 $\text{kg-N m}^{-3} \text{ d}^{-1}$) NRRs (Kawagoshi et al., 2009; Kindaichi et al., 2011; Toro et al., 2017; van de Vossenberg et al., 2008; Yokota et al., 2018). It should be noted that these previous studies used inorganic synthetic seawater prepared using artificial salt compared to real seawater supplemented with organics in the current study. Also, in the current study the startup period to adapt with moderate salinity and organics was shorter (about one month) compared to previous studies

(more than two months) on freshwater and marine anammox bacteria (Li et al., 2018b). Gonzalez-Silva et al. (2017) reported that anammox biomass (enriched with genus *Ca. Brocadia*) needed longer period (~6 months) to adapt to low (0.3%) salinity conditions and suggested that this genus could not tolerate high salt concentration. However, once *Ca. Kuenenia* dominated the community, stable anammox performance was achieved at high salinity (3%) in a relatively short period (~1.5 months) using artificial (NaCl and KCl) seawater. Thus, the choice of inoculum is essential to reduce the startup period for saline wastewater treatment. Although, *Ca. Kuenenia* could gradually adapt to high salinity (15-30 g L⁻¹) using artificial salt (*i.e.* NaCl and KCl) (Gonzalez-Silva et al., 2017; Kartal et al., 2006; Wang et al., 2017a; Yan et al., 2016), previous studies using sea salt or real seawater always enriched for *Ca. Scalindua* species (Kawagoshi et al., 2009; Kindaichi et al., 2011; Nakajima et al., 2008; Toro et al., 2017; van de Vossenberg et al., 2008; Yokota et al., 2018). This could be due to the presence of essential micronutrients in sea salt and real seawater that provide a competitive advantage for the growth of *Ca. Scalindua* over freshwater anammox bacteria (Wei et al., 2016). Further, *Ca. Scalindua* has high-affinity to ammonium (3 µM) and nitrite (0.45 µM) as compared to other anammox species (Oshiki et al., 2016). A previous study reported that *Ca. Scalindua* has high gene expression for ammonium transport (*amtB*) and formate/nitrite transport (*focA*) proteins as compared to other anammox species (van de Vossenberg et al., 2013). In addition, the optimal growth temperature of *Ca. Scalindua* is close to ambient temperature (~25°C) (Awata et al., 2013; van de Vossenberg et al., 2008), and much lower than the optimal growth temperatures of other anammox species (*i.e.* 30–37°C) (Oshiki et al., 2016). High-affinity to ammonium and nitrite, and optimal growth at ambient temperature make *Ca. Scalindua* ideal for mainstream B-stage application where ammonium and nitrite concentrations are lower than sidestream wastewater and temperature is generally close to ambient temperature.

Previous studies showed that freshwater anammox bacteria can perform respiratory ammonification and mediate NO_3^- reduction process while using organic carbon as a source of electrons, subsequently, utilizing the produced NO_2^- and NH_4^+ in conventional anammox process (Ali et al., 2015; Kartal et al., 2007). *Ca. Scalindua* has a relatively versatile metabolism (Oshiki et al., 2017). It was previously reported that *Ca. Scalindua* can use NO_2^- , NO_3^- , and metal oxides as alternative electron acceptors (van de Vossenberg et al., 2013, 2008) and can perform DNRA process in the presence of organic (*i.e.*, propionate, acetate, formate) in oceanic oxygen minimum zones (Jensen et al., 2011). Our results from isotopic experiments (Fig. 2), indicated that in presence of organics, anammox bacteria dominated in the recycling of NO_3^- through respiratory ammonification of NO_3^- via NO_2^- . The oxidation rate of acetate ($0.94 \pm 0.02 \text{ nmol} \cdot \text{mg-protein}^{-1} \cdot \text{min}^{-1}$) was comparable to a previous study performed with enriched *Ca. Scalindua* using synthetic (prepared with sea salt) seawater (van de Vossenberg et al., 2008). Also, the NO_3^- reduction rate ($2.1 \pm 0.7 \text{ nmol-N} \cdot \text{mg-protein}^{-1} \cdot \text{min}^{-1}$) by marine anammox bacteria in the current study was an order of magnitude lower than conventional anammox activity, yet comparable with previously reported NO_3^- reduction rates (Oshiki et al., 2016). As NO_3^- concentration (0.10 mole NO_3^- produced per mole of NH_4^+ removed) (Lotti et al., 2014) in the effluent is still an issue in the application of anammox process for wastewater treatment (Ali and Okabe, 2015), the dissimilatory reduction of NO_3^- to NO_2^- and NH_4^+ with oxidation of acetate is an important trait when implementing marine anammox process for the treatment of saline wastewater generated through seawater toilet flushing practices. In the current study, the average effluent NO_3^- concentration from the MBR was reduced after the addition of acetate, resulting in an increase in the total nitrogen removal efficiency.

A combined metagenomics and metatranscriptomics approach were used in this study to determine genome-resolved abundance and transcription activities of the microbial community in the MBR operated under different conditions. Though, there was a slight change in the

neighboring microbial community, anammox bacterium (AMX) was the most abundant member of the microbial community despite changes in salinity and in the presence of organics. Previous studies on freshwater anammox showed succession in the microbial community in response to salinity change (Awata et al., 2012; Huifeng et al., 2019; Kartal et al., 2006). It should be noted that MAGs belonging to Proteobacteria (*i.e.* PRO1, PRO2, PRO3 and PRO4) in the current study increased in relative abundance during reactor operation. A recent genome-based study on a full-scale RO system using the same intake source from the Red Sea reported Proteobacteria-affiliated bacteria as dominant members (64%) in the seawater population (Belila et al., 2016; Rehman et al., 2018). Since the MBR was equipped with a tight microfiltration membrane, which can effectively retain planktonic bacterial cells coming into the bioreactor with seawater feed, it was likely that these Proteobacteria-affiliated bacteria naturally occurring in the Red Sea immigrated into the bioreactor with the seawater feed and increased in relative abundance during reactor operation. This was further supported by the fact that MAGs belonging to Proteobacteria showed low relative gene expression (Fig. 4), suggesting that their functional or ecological role was not significant in the MBR.

The ability of neighboring microbial community to recycle NO_3^- back to NO_2^- for supporting anammox bacterial growth and bioprocess efficiency was reported by previous studies (Lawson et al., 2017; Speth et al., 2016). For example, in a lab-scale non-saline anammox bioreactor, the neighboring Chlorobi-affiliated bacteria could be active protein degraders, catabolizing extracellular peptides while recycling NO_3^- to NO_2^- as ~30% of the gene expression originated from Chlorobi-affiliated bacteria (Lawson et al., 2017). In the current study, Chlorobi-affiliated bacteria were not recovered from the metagenome.

Our transcription activity results (Fig. 4b) confirmed isotopic experiments, where anammox bacterium (AMX) gene expression accounted for more than 93% of the total gene expression of the microbial community in the MBR under organics addition, and NO_3^-

transporters gene was significantly up-regulated suggesting that in the presence of organics, NO_3^- was actively taken up and transported via NO_3^- transporters. The MBR was operated under NO_2^- limiting condition (Zhang et al., 2017a) and NO_2^- concentration in the effluent was below the detection limit. The presence of acetate stimulated DNRA process which supplemented additional NO_2^- via NO_3^- reduction. The recycled NO_2^- produced via DNRA process served anammox bacteria under NO_2^- -limited environment. This could partially explain the upregulation of *nirK* in Phase 3 (Table S7 and Fig. S3).

Many of the existing wastewater treatment plants are configured as A-B process and PN/A can be easily implemented in existing B-stage for achieving energy-efficient treatment of wastewater (McCarty, 2018). Currently, PN/A is mainly applied for sidestream wastewater treatment (Lackner et al., 2014). Some of the issues that need to be resolved for full-scale implementation of mainstream PN/A are operation under low temperature and low ammonium concentrations (Ali and Okabe, 2015). Nevertheless, research on mainstream PN/A is rapidly expanding with several lab- (Lotti et al., 2014) and pilot-scale (Han et al., 2016; Hoekstra et al., 2018; Lotti et al., 2015; Wett et al., 2013; Yang et al., 2016) studies using real wastewater. These studies clearly demonstrated the biological feasibility of PN/A for mainstream wastewater treatment. In particular, Hoekstra et al., (2018) demonstrated the long-term stable operation of pilot-scale mainstream PN/A process (the pilot reactor was operated for almost a year and half). Also, as previously mentioned, marine anammox bacteria might be better suited for addressing these challenges because of their high affinity to ammonium and nitrite with optimum growth close to ambient temperature.

Generally, municipal wastewater has high COD/ NH_4^+ -N ratio (7–15) (Henze et al., 2008). This high ratio allows higher fraction of heterotrophic bacteria in the sludge which reduces the abundance and activity of anammox bacteria (Cao et al., 2017). However, the COD/ NH_4^+ -N ratio is much lower when PN/A is implemented as B-stage of A-B process, where the influent

COD/NH₄⁺-N ratio to mainstream PN/A (B-stage) is 0.5–2 after carbon removal (A-stage) (Hoekstra et al., 2018; Katuri et al., 2019). Red Sea water supplemented with ammonium, nitrite and acetate was used in the current study as a proof-of-concept to demonstrate the application of enriched Red Sea marine anammox bacteria to treat moderate saline wastewater. Relatively higher N concentration was used in the current study than what is typical of mainstream municipal wastewater to maintain a COD/NH₄⁺-N ratio between 0.5–2, which is typical of B-stage COD/NH₄⁺-N ratio. Future studies will be tested using Red Sea water supplemented with real municipal wastewater to better mimic wastewater generated from toilet flushing practices. A stable performance was achieved in the current study at a COD/NH₄⁺-N ratio of 1, however, anammox activity was deteriorated at a higher ratio (> 1.5). Recent studies reported stable activities in non-saline anammox reactors at higher COD/NH₄⁺-N ratio of 2 (Leal et al., 2016; Wang et al., 2019). In the current study, we did not provide enough time for the marine anammox bacterium to acclimatize to higher COD/NH₄⁺-N ratio and future studies should test the effect of gradual acclimatization of marine anammox bacteria to higher COD/NH₄⁺-N ratio (> 1.0). Also, future studies should demonstrate the application of marine anammox bacteria in granular or attached biofilm systems for mainstream PN/A treatment (Stage B) of saline wastewater.

5. Conclusions

The following conclusions can be drawn from the study:

- *Ca. Scalindua* enrichment culture can treat moderate-saline (~1.2% salinity) and organic (2 mM acetate) solution and showed stable operation with >90% N-removal efficiency and NRR of 0.3 kg-N m⁻³ d⁻¹.
- *Ca. Scalindua* sp. AMX11 exhibited consistent dominance in relative abundance and ecological function in the MBR under moderate-saline and organic conditions.

- Isotopic incubation experiments revealed that the novel anammox bacterium played a dominant role in respiratory ammonification of nitrate via nitrite under the presence of organics. Genome-resolved transcriptomics support isotopic experiments where anammox was mainly responsible for respiratory ammonification through nitrate reduction to ammonium via nitrite.
- Decrease in salinity did not influence the transcription activity of *Ca. Scalindua* sp. AMX11. However, the addition of organic induced a significant change in the transcription activity, and NO_3^- transporters gene was up-regulated possibly for NO_3^- uptake for respiratory ammonification.

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Fig. 1. Performance of the MBR during the different phases of operation. Phase 1 (0-30 days): MBR was operated with fresh Red Sea water containing NH_4^+ and NO_2^- ; Phase 2 (31-55 days): Red Sea water (~3.5% salinity) was mixed with deionized water at different ratios resulting in different salinities; Phase 3 (56-140 days): MBR was operated with addition of acetate. Dashed lines represent the separation of different phases. Black arrows represent biomass sampling events for metagenomics (Phases 1 & 3) and metatranscriptomics (Phases 1, 2 & 3). NLR: nitrogen loading rate; NRR: nitrogen removal rate.

Fig. 2. NO_3^- reduction to NH_4^+ via NO_2^- by respiratory ammonification: 3.5-mL of cell suspension was added into 10-mL glass vials and incubated for ~8-12 h after adding $^{15}\text{NO}_3^-$ and $^{13}\text{CH}_3\text{COOH}$. Some vials were also supplemented with $^{14}\text{NH}_4^+$, which would preferentially be utilized and promote the accumulation of $^{15}\text{NH}_4^+$. As a control, the same incubation was repeated in the absence of $^{14}\text{NH}_4^+$, which is designated as ‘w/o’. NO_2^- was not supplemented to any of the incubation vials. The heights of the bars represent the average values of triplicate batch tests. These incubations were repeated with specific inhibitors Penicillin G and Acetylene to selectively inhibit heterotrophic denitrification and anammox processes, respectively. The grey dashed line represents the amount of $^{15}\text{NO}_3^-$ added to an incubation vial.

Fig. 3. Neighbor-joining phylogenetic tree showing the evolutionary relationship between all recovered MAGs and closely related genomes downloaded from the NCBI genome repository. The tree includes MAGs (shown in bold colored text) recovered from the MBR. The GenBank accession numbers for each genome is provided in parentheses. Branch node numbers represent bootstrap support values and the bootstrap consensus inferred from 1000 iterations.

Fig. 4. Relative abundance and gene expression of microorganisms represented by the MAGs recovered from the anammox MBR. a) Relative abundance and b) gene expression based on transcription per million (TPM) values of metagenomics and metatranscriptomics reads that mapped to each MAG (Table S3). Seventeen MAGs were recovered from metagenomics, and these MAGs were presented here by their identifiers representing different phylum (Table S4).

Fig. 5. a) PCA plot displaying the six samples (duplicate samples in each phase) performed using DESeq2 rlog-normalized RNA-seq data. b) HCA of the six samples (duplicate samples in each phase). The heatmap was built using the DESeq2 package. Samples were clustered using HCA performed with DESeq2 rlog-normalized RNA-seq data, and the dendrogram represents the clustering results. The heatmap illustrates the pairwise distances between the different samples, with higher similarity indicated by higher intensity of color.