Research Article

Microbial Community Structure and Nitrogen in a Saline Filter Bioreactor Inoculated with Shrimp Pond Sludge

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Abstract
Understanding the composition of microbial communities is essential for optimizing anammox-based nitrogen removal in saline wastewater environments. This study examined the microbial diversity and ecological structure within a filter bioreactor inoculated with shrimp pond sludge and operated under saline conditions (30.1–33.0 ppt) and fed with synthetic seawater containing 70 mg-N/L of ammonium and nitrite with hydraulic retention time 24 h. Over 175 days of operation, the reactor maintained stable nitrogen removal, with peak ammonium conversion and nitrogen removal efficiencies of 46.25% and 44.33%, respectively. High-throughput 16S rRNA gene sequencing illuminated a diverse microbial community, dominated by *Candidatus Brocadia* (8.07%), alongside significant representations of *Candidatus Jettenia* (0.88%). The microbial consortium also included key nitrifying bacteria such as *Nitrosomonas* and *Nitrospira*, indicating synergistic interactions in nitrogen transformation. These taxa play key functional roles in nitrogen transformation, biofilm stability, and adaptation to saline, anoxic environments. Phylogenetic analysis showed close affiliations between amplicon sequence variants and recognized anammox species, such as *Candidatus Brocadia sinica* and *Candidatus Jettenia asiatica*. The detection of freshwater-associated anammox genera in a saline system highlights their ecological adaptability and potential application in saline wastewater treatment. Overall, this study provides insights into microbial consortia that drive anammox processes in engineered saline environments and supports the development of biological nitrogen removal strategies for marine and coastal applications.

Keywords: Anammox, Filter bioreactor, Microbial community, Nitrogen removal, Shrimp pond sludge

1 Introduction

Nitrogen (N) pollution is a major environmental issue with wide–ranging implications for aquatic ecosystems, human health, and economic stability. This problem primarily arises from excessive inputs of reactive nitrogen compounds, such as ammonium, nitrite, and nitrate, into the environment due to human activities, including domestic wastewater discharge, industrial effluents, and agricultural runoff [1]. Although essential for biological functions, these compounds become harmful when present in excess. Eutrophication, oxygen depletion, and subsequent mortality of aquatic organisms are direct consequences of nitrogen over-enrichment in water bodies.

Conventional biological nitrogen removal technologies involving aerobic nitrification and anoxic denitrification are often energy–intensive and require additional carbon sources. In contrast, anaerobic ammonium oxidation (anammox) presents a more sustainable and cost-effective alternative. This process involves ammonium oxidation using nitrite as an electron acceptor to produce nitrogen gas. The anammox process is characterized by a lower energy demand and reduced sludge production compared to

traditional methods [2], [3]. Anammox bacteria have been isolated from various anoxic environments, including freshwater and marine ecosystems, with currently 24 species identified within seven known genera under the phylum *Planctomycetota* [4]. *Candidatus Scalindua* is the only genus confirmed to thrive in saline marine conditions [5].

Marine anammox bacteria (MAB), particularly those classified under *Candidatus Scalindua*, exhibit unique physiological traits that enable them to function effectively in saline conditions. These include halophilic properties, a high affinity for nitrite, and the ability to maintain metabolic activity across a wide temperature (10–30 °C) and salinity (1.5–4.0%) range [6]. Despite their ecological importance in marine nitrogen cycling, studies on MAB remain limited, and their cultivation under controlled reactor systems is still a developing field. The performance of anammox–based systems is closely linked to the microbial community structure within the bioreactor [7]. Recent innovations, such as the filter bioreactor (FtBR), provide promising platforms for anammox enrichment. FtBRs utilize filter media, such as string–wound filters, to support biofilm formation and microbial attachment, enhancing nitrogen removal efficiency [8]. Prior research has demonstrated nitrogen removal efficiencies of up to 72% using FtBRs inoculated with shrimp pond sludge, indicating the process's potential compatibility with marine microbial communities [9].

However, most studies to date have focused predominantly on freshwater anammox species. There remains a significant gap in understanding the microbial ecology of marine anammox systems, especially under FtBR operation. For instance, Yokota *et al*. successfully enriched *Candidatus Scalindua wagneri* in a upflow anaerobic sludge blanket (UASB) reactor [10], while Lulrahman cultivated multiple anammox species, including *Candidatus Anammoxoglobus propionicus* and *Candidatus Brocadia sinica,* using FtBR with seawater [11].

Few studies have investigated the cultivation of anammox in FtBR inoculated with shrimp pond sludge. Ismail *et al*. examined the development of an anammox reactor for aquaculture wastewater treatment and confirmed its feasibility for nitrogen removal under saline conditions [12]. Similarly, Guno Gumelar *et al.* demonstrated a rapid start–up of a marine anammox process using shrimp pond solid waste as inoculum, achieving high nitrogen removal performance [13]. However, the study primarily focused on reactor operation and stoichiometric confirmation, providing limited information on the underlying microbial community structure.

In this study, an FtBR inoculated with shrimp pond sludge was operated using seawater–based synthetic wastewater to examine both the performance and microbial ecology of the anammox process under high salinity conditions (30–33 ppt). High–throughput next–generation sequencing (NGS) was employed to identify and quantify the microbial taxa responsible for nitrogen transformation within the reactor. The objectives of this research were to evaluate the nitrogen removal efficiency and overall reactor performance under saline conditions, to determine the dominant anammox and associated microbial taxa forming the FtBR biofilm. The findings from this study provide a new understanding of saline FtBR operation and demonstrate its potential for application in nitrogen removal from marine and aquaculture wastewater treatment systems.

2 Material and Methods

2.1 *Inoculum*

Sludge used as inoculum was collected during siphoning at a shrimp aquaculture pond in Katapiang Village, Batang Anai Sub–district, Padang Pariaman Regency, West Sumatra, Indonesia (0°45'24.8" S, 100°14'53.5" E). The sludge was taken from a depth of 0.5 to 1.0 meters below the water surface to ensure the collection of active sediment rich in microbial biomass. After collection, the sludge was stored in a refrigerator at -4 °C prior to its use in the reactors. The characteristic of the sludge was TSS 2,072 mg/L, VSS 1,476 mg/L.

2.2 *Reactor configuration*

The reactor utilized an inoculum sourced from shrimp pond sludge for start–up. This sludge, derived from feed residues and shrimp waste, contains a rich microbial community, including microorganisms known for their roles in nitrogen transformation and decomposition. The sludge originated from a high–salinity environment, suitable for marine anammox cultivation. A total of 1,500 mL of sludge was introduced into FtBR, corresponding to approximately 75% of the effective reactor volume. For optimal biofilm formation, according to Lotti *et al.*, approximately 30% of the reactor volume was filled with inoculum to support anammox bacterial attachment and initial growth [14].

The FtBR was equipped with a string–wound filter cartridge made of polypropylene with a pore size of 0.5 µm. This cartridge was positioned centrally within a 1,500 mL PVC filter housing (height: 10 inches) to facilitate the retention of biomass and prevent washout. The reactor was covered in aluminum foil to block light and inhibit photosynthetic bacterial growth. The substrate was rinsed with N_2 gas using a Tedlar bag to maintain anoxic conditions. A peristaltic pump delivered the influent at a flow rate of 1 mL/min. An outlet port with a gas valve at the top of the reactor allowed effluent sampling and the release of nitrogen gas, an end product of the anammox reaction. The biofilm development was visually monitored during the experiment, with the appearance of reddish biomass on the filter media as an indicator of anammox bacterial colonization [15]. The reactor was operated continuously for 175 days with a 24–h hydraulic retention time (HRT) under tropical ambient temperatures ranging from 25–28 °C. The FtBR configuration can be viewed in Figure 1.

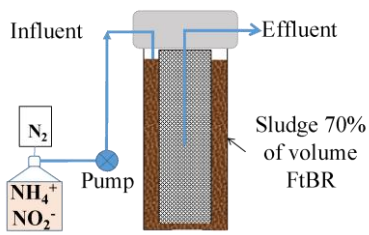

Figure 1: Configuration of FtBR.

2.3 *Synthetic wastewater and reactor operation*

Artificial wastewater was used as the substrate, prepared from seawater containing ammonium and nitrite at concentrations of 70 mg–N/L each, simulating a saline environment to support marine anammox bacteria. The substrate solution's composition details various chemical components and their concentrations in mg/L. The primary nitrogen sources are $(NH_4)_2SO_4$ and $NaNO_2$, both present at a concentration of 70 mg–N/L. The first group of trace elements, "Trace Element I," includes Na EDTA (II) at 6.370 mg/L and $FeSO_4$ at 5.000 mg/L. A second group, "Trace Element II," consists of Na EDTA (II) at 19.110 mg/L, $CoCl_2{\cdot}6H_2O$ at 240 mg/L, $MnCl_2{\cdot}4H_2O$ at 990 mg/L, $CuSO_4{\cdot}5H_2O$ at 250 mg/L, $NaMoO_4{\cdot}2H_2O$ at 220 mg/L, $NiCl_2{\cdot}6H_2O$ at 190 mg/L, $ZnSO_4$ at 0.241 mg/L, $NaSeO_4{\cdot}10H_2O$ at 240 mg/L, and H_3BO_4 at 140 mg/L. Trace element solutions I and II were added to the substrate to support microbial growth and optimize anammox activity. The salinity of the influent ranged from 30.1 to 33.0 ppt, replicating the natural habitat of halophilic anammox bacteria.

2.4 *Analytical methods*

The sample was collected 1–2 times a week. Influent and effluent sampling was carried out to measure concentrations of NH_4^+-N with Nessler spectrophotometric analysis, NO_2^-–N with spectrophotometric analysis, and NO_3^-–N with ultraviolet–screening spectrophotometric analysis based on standard methods [16].

2.5 *Calculation of nitrogen removal performance*

The performance of FtBR on nitrogen removal was calculated based on the nitrogen balance as shown in Equations (1)–(4) [8]:

$$ACE = \frac{[NH_4^+\text{-}N]_{in} - [NH_4^+\text{-}N]_{out}}{[NH_4^+\text{-}N]_{in}} \times 100\ \% \quad (1)$$

$$NRE = \frac{[NH_4^+\text{-}N]_{in} + [NO_2^-\text{-}N]_{in} - [NH_4^+\text{-}N]_{out} - [NO_2^-\text{-}N]_{out} - [NO_3^-\text{-}N]_{out}}{[NH_4^+\text{-}N]_{in} + [NO_2^-\text{-}N]_{in}} \times 100\ \% \quad (2)$$

$$NRR = \frac{[NH_4^+\text{-}N]_{in} + [NO_2^-\text{-}N]_{in} - [NH_4^+\text{-}N]_{out} - [NO_2^-\text{-}N]_{out} - [NO_3^-\text{-}N]_{out}}{HRT} \quad (3)$$

$$NLR = \frac{[NH_4^+\text{-}N]_{in} + [NO_2^-\text{-}N]_{in}}{HRT} \quad (4)$$

Where:

$[NH_4^+\text{-}N]_{in}$ = Influent ammonium concentration
$[NO_2^-\text{-}N]_{in}$ = Influent nitrite concentration
$[NH_4^+\text{-}N]_{out}$ = Effluent ammonium concentration
$[NO_2^-\text{-}N]_{out}$ = Effluent nitrite concentration
$[NO_3^-\text{-}N]_{out}$ = Effluent nitrate concentration
HRT = Hydraulic retention time

2.6 *DNA Sequencing and bioinformatics*

High-throughput sequencing characterized the FtBR microbial community using 16S rRNA gene sequencing. DNA extraction utilized the QIAamp® PowerFecal® Pro DNA Kit to isolate microbial DNA from reactor biomass samples. About 250 mg of sample was added to a Power Bead Pro Tube with 800

μL of Solution CD1, followed by intense vortexing for effective cell lysis. After vortexing, centrifuging, and buffer addition, the DNA was purified with CD2, CD3, EA, and C5 solutions. The DNA was eluted with Solution C6 and stored at –20 °C for molecular analysis. Amplification of the V4 region of the 16S rRNA gene was performed via the first polymerase chain reaction (PCR) using primers 515F (5′-GTGCCAGCMGCCGCGGTAA-3′) and 806R (5′-GGACTACNNGGGTATCTAAT-3′), HotStar Taq Plus polymerase, dNTPs, and buffer in a UV–sterilized environment. PCR success was confirmed by agarose gel electrophoresis with Midori Green dye, showing clear DNA bands.

The DNA amplicons underwent purification using AMPurePX magnetic beads to remove excess primers, nucleotides, and enzymes. The second PCR added indexing primers for multiplexing using KAPA HiFi HotStar ReadyMix and specific primers. Samples were analyzed with an Agilent 2100 Bioanalyzer using a DNA 1000 chip to confirm amplicon sizes for Illumina sequencing. DNA libraries were prepared for sequencing on the Illumina MiSeq platform, which included dilutions, adding PhiX control libraries, and mixing reagents [17]. After sequencing, raw DNA reads were processed with the DADA2 pipeline for bioinformatics analysis, involving filtering, dereplication, error correction, chimera removal, and generation of Amplicon Sequence Variants (ASVs). This was followed by taxonomic classification, revealing detailed microbial diversity in the FtBR during anammox under saline conditions.

2.7 *Phylogenetic analysis*

Phylogenetic analysis of microbial communities in the FtBR employed a bioinformatics pipeline after high–throughput sequencing on the MiSeq Illumina platform. Raw paired–end reads were evaluated for quality, trimming low–quality bases and adapters. High-quality reads were retained through strict filtering, ensuring only reliable sequences were included. Error rates were modeled to enhance denoising accuracy. Identical reads were dereplicated, reducing computational load while preserving information.

The DADA2 algorithm in R Studio inferred exact amplicon sequence variants (ASVs), correcting sequencing errors without traditional clustering for single-nucleotide resolution of microbial variants. After denoising, paired–end reads were merged to reconstruct full-length sequences of the V4 region of the 16S rRNA gene, with chimeric sequences removed to avoid false positives in taxonomic identification. The ASV table was taxonomically classified using the SILVA ribosomal RNA gene database, specifically silva_nr99_v138.1_train_set and silva_species_assignment_v138.1, for species-level identification.

The ASV data and metadata were imported into the phyloseq package in R for ecological analysis, including alpha diversity (species richness) and beta diversity (community composition variations). This framework provided a comprehensive understanding of the FtBR reactor's microbial diversity and phylogenetic composition, enabling robust profiling of communities and identifying key functional bacteria involved in nitrogen removal, such as anammox and nitrifying bacteria.

Furthermore, the sequence of each ASV was analyzed using GenBank NCBI (National Center for Biotechnology Information). The data have been deposited with accession numbers PX526093 and PX526094. Subsequently, the Phylogenetic Tree was constructed utilizing ARB Software with the neighbor-joining method, and branch support was evaluated using 100 bootstrap replications [18]. The identification of phylogenetic neighbors and the calculation of pairwise 16S rRNA gene sequence similarity were accomplished using https://www.genome.jp/tools-bin/clustalw.

3 Results and Discussion

3.1 *Nitrogen removal performance*

Nitrogen removal performance in the FtBR was evaluated to determine the effectiveness of the anammox process throughout the experimental period. Key performance indicators included ammonium conversion efficiency (ACE), nitrogen removal efficiency (NRE), nitrogen removal rate (NRR), and nitrogen loading rate (NLR). The highest observed ACE and NRE were recorded on day 90, reaching 46.253% and 43.797%, respectively. Maximum nitrogen removal activity was attained on day 120 with an NRR of 0.084 kg–N/m³·d, while the peak NLR occurred on day 7, measured at 0.216 kg–N/m³·d. NRR reflects the quantity of nitrogen removed per unit volume and time, providing insight into the reactor's operational performance, whereas NLR represents the nitrogen loading input over time. Throughout the operation, NLR ranged from 0.166 to 0.216 kg–N/m³·d, while NRR fluctuated between 0.006 and 0.084 kg–N/m³·d. The increasing NRR trend, particularly the peak on day 120, indicated enhanced nitrogen elimination attributed to the stable

establishment and activity of anammox bacteria within the reactor system.

The influent concentrations of ammonium (NH_4^+–N) and nitrite (NO_2^-–N) were maintained at 70 mg-N/L. ACE, calculated as the percentage reduction in ammonium concentration from influent to effluent relative to the influent concentration, reflected the specific conversion of ammonium by anammox bacteria. NRE, representing the total nitrogen removal efficiency, was derived from the difference between total nitrogen concentrations in influent and effluent and normalized to the ammonium concentration. Initial ACE and NRE were low, recorded at 2.002% and 2.841%, respectively, reflecting the early adaptation phase of the microbial community. ACE reached its highest value on day 90, while NRE peaked at 44.331% on day 120, corresponding with optimal anammox activity. A subsequent decline in NRE to 40.88% was observed on day 139, suggesting competing processes, such as nitrification, which may have temporarily suppressed anammox efficiency. Nonetheless, by the end of the operational period, NRE recovered to 43.290%, indicating system resilience and reestablished anammox dominance. These findings confirm the FtBR effectiveness for nitrogen removal under saline conditions and highlight the importance of operational stability. A graph of the nitrogen removal performance during the study can be seen in Figure 2.

MAB has garnered attention due to its demonstrated efficacy in nitrogen removal from saline environments, especially those contaminated by aquaculture and marine–based effluents. Duc *et al.* and Kawagoshi *et al.* have successfully enriched MAB from marine sediments, with predominant affiliations to *Candidatus Scalindua spp.* These species have achieved nitrogen removal efficiencies as high as 88% under loading rates of 1.0 kg–N/m³·d, highlighting their robustness and scalability [19], [20]. Furthermore, Ali *et al.*, reported that MAB could efficiently treat moderately saline (~1.2% salinity) and nitrogen-rich organic waste streams, maintaining over 90% removal efficiency at 0.3 kg–N/m³·d [21].

Interestingly, this study observed the dominance of *Candidatus Brocadia*, a genus typically associated with freshwater or low–salinity systems, within a saline environment (up to 33 ppt). This finding diverges from the prevailing assumption that *Candidatus Scalindua* dominates marine systems, suggesting that *Candidatus Brocadia* possesses a previously underappreciated degree of halotolerance and ecological plasticity. These results align with observations by Duc *et al.*, who reported the co-existence of freshwater–origin anammox species such as *Candidatus Brocadia TKU–4* and *Candidatus Jettenia asiatica* in marine environments [19].

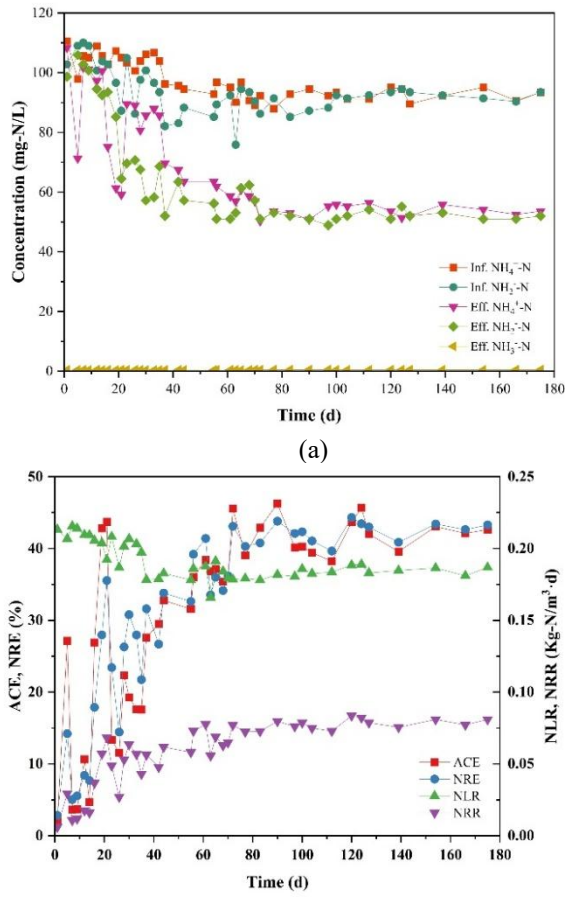

Figure 2: (a) Profile of nitrogen concentration (mgN/L), (b) Nitrogen removal performance (ACE and NRE, %; NLR and NRR, kg-N/m³·d) on FtBR.

The performance of anammox systems is often linked to temperature, where Kawagoshi *et al.*, reported optimal activity at 25 °C, a condition mirrored in the current FtBR setup [22]. Furthermore, the co–occurrence of other functional microbes, such as sulfur–oxidizing denitrifiers, has been noted in marine sediments and may suggest similar synergistic interactions within this FtBR system. Such microbial interactions could enhance the performance of system resilience and nitrogen removal under fluctuating environmental conditions.

The enrichment of *Candidatus Brocadia* under saline conditions expands the potential for applying

freshwater–origin anammox species in saline wastewater treatment. FtBR appears particularly suitable for supporting diverse consortia, including halotolerant species, enhancing adaptability across salinity ranges.

3.2 *Microbial community analysis*

Microbial community analysis was conducted from the reactor biomass on inoculum (0 days) and on day 132 of the experiment. The taxonomic classification at the phylum level (Figure 3) revealed that the dominant bacterial groups in the FtBR were *Pseudomonadota* (27.14%), *Chloroflexi* (17.59%), and *Planctomycetota* (14.75%) [23]. These phyla are frequently associated with nitrogen cycling in anammox systems. *Pseudomonadota*, often comprising ammonia–oxidizing bacteria (AOB), thrive under aerobic conditions, while *Planctomycetota* include obligate anaerobic anammox bacteria such as *Candidatus Brocadia*. *Chloroflexi* are implicated in the degradation of extracellular polymeric substances and decayed biomass, contributing to the stability of biofilms. The presence of these phyla aligns with findings from previous studies conducted in similar saline or ambient–temperature environments, confirming the adaptability of these microbial groups to FtBR systems.

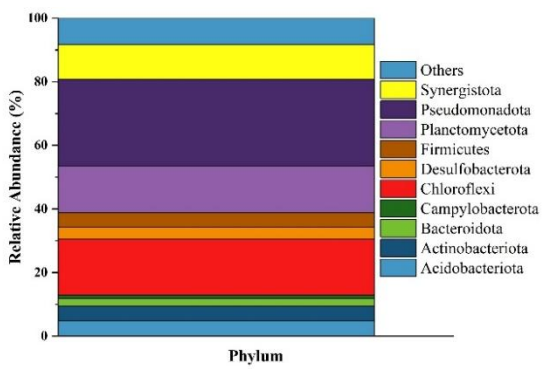

Figure 3: Microbial community abundance at the phylum level. The relative abundance <1% is classified as other.

At the genus level (Figure 4), the most abundant taxa in the FtBR were *Candidatus Brocadia* (8.07%), *Marinobacter* (3.18%), and *EBM-39* (2.92%). *Candidatus Brocadia* is a well–established anammox genus known for its metabolic versatility and dominance in freshwater and engineered systems. Its presence in this study highlights the establishment of an active anammox consortium under saline conditions. *Marinobacter*, although not a core nitrogen-removing bacterium, plays an indirect role in nitrogen elimination through partial denitrification and hydrocarbon degradation. Its halotolerant nature makes it highly adaptable to saline environments, thus supporting microbial synergy in the FtBR. *EBM–39*, a less-characterized genus, has also been frequently observed in nitrogen–rich and anoxic environments. While its functional role remains uncertain, it is hypothesized to assist in nitrate/nitrite turnover or organic matter transformation, contributing to the ecological balance within the reactor. The consistent enrichment of these genera, particularly *Candidatus Brocadia*, *Marinobacter*, and *EBM–39*, reflects a microbial community optimized for nitrogen removal under saline and anoxic conditions. Their interactions may form a robust metabolic network that enhances the overall performance and resilience of the anammox process in the FtBR system.

Salinity plays a critical role in shaping the activity and composition of microbial communities in anammox–based systems. Recent studies have shown that with proper acclimatization, anammox bacteria can tolerate salinities up to 30 g NaCl/L, maintaining stable nitrogen removal performance [24]. In a study by Wang *et al.* stepwise salinity increments from 0.61% to 3.10% in a partial nitritation/anammox (PN/A) reactor treating landfill leachate achieved nitrogen removal efficiencies up to 85.7%, demonstrating the robustness of adapted microbial consortia under gradual saline stress [24].

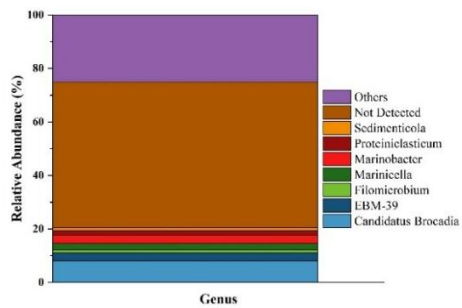

Figure 4: Microbial community abundance at the genus level. The relative abundance <1% is classified as other.

However, salinity fluctuations can impose osmotic shock, negatively impacting microbial activity and biomass stability. Dsane *et al.*, observed that sudden exposure to high–salinity conditions can significantly suppress specific anammox activity (SAA) and lead to a temporary collapse of nitrogen removal efficiency [25]. These stressors also altered

microbial community dynamics, often resulting in reduced relative abundance of salt–sensitive genera such as *Candidatus Brocadia* and *Candidatus Jettenia*.

Different anammox genera display varying salinity tolerances. For example, *Candidatus Kuenenia* has demonstrated greater resilience under elevated salinity compared to *Candidatus Brocadia* and *Jettenia* [24], [26]. This variation is likely linked to physiological adaptations, including adjustments in extracellular polymeric substances (EPS) production, intracellular ATP concentrations, and membrane transport systems, which modulate osmotic balance and protect enzymatic functions under saline stress [26].

The current study's observation of *Candidatus Brocadia* as the dominant anammox genus under salinity levels of 30.1–33.0 ppt suggests that the acclimatization process and biofilm–based FtBR configuration may have provided a favorable niche for its survival and activity. Biofilm environments are known to buffer environmental fluctuations and enhance microbial resistance to salinity by facilitating microbial aggregation and EPS secretion. Nonetheless, the relatively moderate nitrogen removal efficiency observed may reflect a transitional phase in microbial adaptation, with potential inhibition from nitrite accumulation or competition from other nitrogen–transforming organisms.

These findings support the idea that the operational strategy, including gradual salinity adjustment and stable environmental parameters, is crucial for maintaining an active anammox community in saline systems. Future research should explore long-term shifts in microbial composition under variable salinity regimes and evaluate strategies to promote the enrichment of salt–tolerant anammox species, such as *Candidatus Scalindua* or *Kuenenia*, for optimized nitrogen removal in high-salinity wastewaters.

3.3 *The role of nitrogen–related bacteria*

The microbial community in the FtBR encompassed various bacteria involved in nitrification, including anammox bacteria, ammonia–oxidizing bacteria (AOB), and nitrite–oxidizing bacteria (NOB). Anammox bacteria dominated the community at 8.95%, indicating their crucial role in nitrogen removal. The AOB and NOB populations mainly consist of *Nitrosomonas* and *Nitrospira* species. The presence of AOB in anoxic anammox reactors has been reported in previous studies. It is suggested that certain AOB can persist under limited oxygen conditions due to their ability to convert nitrite into N_2O or NO gases, despite the lack of aerobic ammonium oxidation activity. Highlighting their roles and interactions within the reactor. Inhibition within the reactor is likely caused by nitrite (NO_2^-) accumulation due to insufficient ammonium (NH_4^+) available for the theoretical anammox reaction. Elevated nitrite concentrations can be toxic to some anammox bacteria, reducing their population density. This results in a shift in the microbial community, with a decline in the relative abundance of anammox bacteria. When anammox bacteria are inhibited and their capacity for nitrite reduction diminishes, it creates an ecological niche for other microorganisms, particularly NOB and AOB, to proliferate. These bacteria utilize nitrite and ammonia as their substrates, respectively, and with reduced competition from anammox bacteria, their abundance increases.

AOB played a fundamental role in wastewater treatment systems, especially where partial nitrification and anammox processes are combined. AOB are responsible for the partial oxidation of ammonia to nitrite, which is then utilized by anammox bacteria. Even in reactors operating without oxygen, genera such as *Nitrosomonas* and *Nitrosospira* have been detected, though their aerobic ammonia oxidation activity may be inactive. For efficient ammonium removal, achieving partial conversion of ammonium to nitrite by AOB is essential, while inhibiting nitrite–oxidizing bacteria, such as *Nitrospira,* is crucial to reduce competition for nitrite. Therefore, low oxygen concentrations are maintained because NOB have a lower affinity for oxygen than AOB [27]. Overall, the microbial community composition changes reflect complex interactions among various nitrifying bacteria responding to environmental changes within the reactor. The suppression of anammox bacteria opens niches for other nitrogen–converting microorganisms to flourish, leading to shifts in their relative abundance and allowing the reactor to sustain nitrogen removal efficiency despite the reduced prevalence of anammox bacteria.

3.4 *Diversity of anammox bacteria*

The presence and relative abundance of anammox bacteria serve as critical indicators of nitrogen removal efficiency within the FtBR system, as these microorganisms directly convert ammonium (NH_4^+) and nitrite (NO_2^-) into nitrogen gas (N_2) under anoxic conditions. The composition and population density of the anammox genera were detected in the reactor and analyzed through molecular techniques such as 16S rRNA gene sequencing. Two anammox genera were

identified: *Candidatus Brocadia* (8.07%) and *Candidatus Jettenia* (0.88%). *Candidatus Brocadia* is one of the most extensively studied and widely recognized anammox genera. It plays a pivotal role in the global nitrogen cycle by directly converting ammonium and nitrite into dinitrogen gas, contributing significantly to nitrogen removal from wastewater without requiring organic carbon input.

Candidatus Brocadia is often enriched in FtBR due to its relatively fast growth rate and adaptability to varying environmental conditions, including moderate temperatures and fluctuating substrate concentrations. Morphologically, *Candidatus Brocadia* features unique intracellular compartments known as anammoxosomes, where the anammox process occurs. These cells also possess ladderane lipids in their membranes, which support their anaerobic metabolic activity. Genomic analysis has revealed that *Candidatus Brocadia* carries essential functional genes such as hzsA (hydrazine synthase) and hzo (hydrazine oxidoreductase), key enzymes in the anammox pathway. Due to its efficiency and robustness, *Candidatus Brocadia* is a cornerstone genus in developing low–energy, sustainable wastewater treatment systems. The second detected genus, *Candidatus Jettenia*, contributes to the biological nitrogen removal process by converting ammonium and nitrite to nitrogen gas in anaerobic environments. Although typically less abundant than *Candidatus Brocadia*, *Candidatus Jettenia* has demonstrated tolerance to low temperatures and varying salinity levels, making it a valuable candidate for applications in diverse wastewater treatment settings, including municipal and saline systems. Similar to *Candidatus Brocadia*, genomic studies have confirmed the presence of hzsA and hzo genes in *Candidatus Jettenia*, further confirming its functional role in the anammox process. However, its slower growth rate may result in its co-existence with other anammox species, contributing to the overall stability and resilience of the microbial community under suboptimal conditions.

Another essential genus of anammox bacteria typically found in marine or high-salinity environments is *Candidatus Scalindua*. Research by Ali *et. al.*, demonstrated the presence of *Candidatus Scalindua* in seawater with a salinity of 1.2%, where it achieved nitrogen removal efficiencies above 90% [21]. Further studies by Van Duc *et al.*, reported the dominance of *Candidatus Scalindua wagneri*, *Candidatus Jettenia asiatica*, and *Candidatus Brocadia TKU–4* in environments with a salinity of 2.7%. Van Duc *et al.*, indicated that freshwater–origin anammox species such as *Candidatus Jettenia asiatica* and *Candidatus Brocadia TKU–4* could still thrive in 2.7% salinity environments despite the prevailing presence of marine anammox species [19]. In contrast to previous studies, the present study revealed a dominant presence of *Candidatus Brocadia* in the FtBR, even under saline conditions. Specifically, *Candidatus Brocadia* accounted for 20.6% of the microbial community in an environment with a salinity of 10 g/L. This finding suggests *Candidatus Brocadia*'s broader environmental adaptability than previously assumed, emphasizing its potential for application in saline wastewater treatment systems.

Following genus–level classification, a phylogenetic analysis was conducted to evaluate the species-level similarity of the sequence results obtained in this study (Figure 5). Table 1 shows these similarity percentages more clearly.

Table 1: Anammox abundance in FtBR.

Sequence	Reference Species	Similarity
ASV_28 (2.94%)	*Candidatus Brocadia sp. UTAMX1*	99%
ASV_6 (4.86%)	*Candidatus Brocadia sinica (near)*	94%
ASV_112 (0.88%)	*Candidatus Jettenia asiatica*	98%

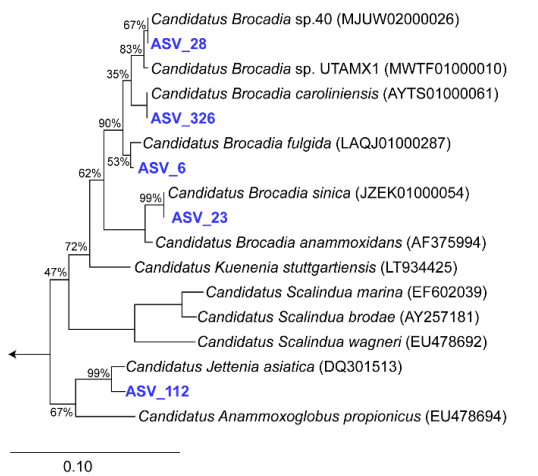

Figure 5: Phylogenetic tree of anammox bacteria on FtBR inferred by the neighbor–joining method with bootstrap support (n=100).

Upon examination of the specific findings, ASV_6 emerged as the most prevalent sequence, accounting for 4.86% of the bacterial community and displaying a 94% similarity to a species closely related to *Candidatus Brocadia sinica*. Another noteworthy sequence, ASV_28, constituted 2.94% of the sample

and demonstrated 99% similarity to *Candidatus Brocadia sp. UTAMX 1*. Other sequences were present in lower abundances; for instance, ASV_112 comprised 0.88% of the community and shared a 98% similarity with *Candidatus Jettenia asiatica*. The "*Candidatus*" prefix for the reference species indicates that these are provisionally named organisms identified through molecular methods but not yet cultured and formally described according to the bacteriological code. The high similarity scores for most ASVs to known anammox–related genera such as *Brocadia* and *Jettenia* imply their likely functional roles within the FtBR system, which are often associated with nitrogen removal processes in wastewater treatment.

3.5 *Biofilm matrix and microbial resilience under saline conditions*

The biofilm matrix (extracellular polymeric substances/EPS) plays a critical protective role in buffering salinity stress by creating a sophisticated molecular shield that helps microorganisms survive high–salt environments. EPS consists mainly of polysaccharides, proteins, nucleic acids, and lipids that together form a hydrated, viscoelastic matrix embedding microbial cells. This matrix establishes microenvironments that moderate physicochemical fluctuations, particularly those caused by osmotic stress, thereby enabling stable microbial activity under saline conditions.

Experimental evidence demonstrates significant protective effects of EPS against salinity stress. Steele *et al.* showed that diatoms encapsulated within an EPS matrix maintained photosynthetic capacity within 4% of baseline levels during salinity shock, while unprotected cells experienced up to a 64% decline [28]. Similarly, Banerjee *et al.*, reported that EPS reduced sodium ion availability and enhanced bacterial colonization in salt-stressed environments, confirming its importance in microbial resilience [29]. Alhoqail *et al.*, further revealed that EPS–producing bacteria can restrict sodium absorption, improve beneficial ion intake (K^+, Ca^{2+}, Mg^{2+}), and elevate antioxidant levels, highlighting the multifaceted protective mechanisms conferred by EPS under osmotic stress [30]. The overall protective mechanisms include restricting sodium absorption, improving the uptake of beneficial ions, forming protective coatings around cell surfaces, maintaining intracellular metabolic stability, and reducing oxidative damage. Moreover, studies across multiple bacterial species consistently demonstrate EPS's ability to enhance cell survival, indicating that it serves as a robust and generalizable stress-buffering strategy.

Recent work by Zhou *et al.*, provides additional insight into EPS–mediated salinity tolerance in anammox systems. Their study demonstrated that conventional biological treatment methods are often less efficient for treating anaerobic digestion liquor from food waste due to high salinity and elevated ammonia–nitrogen content. However, anammox bacteria could effectively treat high-salinity wastewater after adequate acclimation. In a salt-tolerant anammox granular sludge (AGS) system operated across varying salinity levels (0–25 g/L NaCl), Zhou *et al.*, observed stable nitrogen removal (> 86 %) at 15 g/L following adaptation, with peak specific anammox activity (SAA) of 6.73 mg–N/h·g VSS at 5 g/L and inhibition only at 25 g/L NaCl [31].

Notably, increases in EPS provided enhanced structural protection and supported microbial aggregation. Enrichment of salt–tolerant anammox bacteria (*Candidatus Brocadia*, *Candidatus Kuenenia*) and heterotrophic denitrifiers, along with dynamic regulation of nitrogen-cycling genes (hzo, hzs, narG, nirS) and enzyme activities (Hao, Nar, Nir, Amo), was critical to adaptation under saline conditions. These findings confirm that elevated EPS production and compositional shifts play a pivotal role in sustaining anammox activity under high salinity, consistent with the protective mechanisms proposed for the FtBR system in this study.

In the present FtBR system, EPS likely played a pivotal role in enabling *Candidatus Brocadia* and other microorganisms to tolerate salinity levels of 30.1–33.0 ppt. The hydrated EPS layer would have slowed ion diffusion and reduced the osmotic gradient across cell membranes, while the biochemical composition of the matrix (polysaccharide and humic fractions) helped retain water and maintain ionic equilibrium. Additionally, EPS provided structural support for biofilm attachment on the filter media, improving biomass retention and reactor stability. The combination of physical shielding, ionic buffering, and antioxidant protection afforded by EPS thus offers a plausible explanation for the sustained nitrogen removal performance and microbial resilience observed in this study.

Future research should focus on quantifying EPS fractions (loosely bound, tightly bound, and total EPS), assessing polysaccharide–to–protein ratios, and analyzing functional groups using FTIR or NMR spectroscopy. Correlating these data with nitrogen removal performance and microbial community

composition would help elucidate the precise contribution of EPS to salinity tolerance and FtBR robustness.

3.6 *Nitrogen removal trends in marine anammox systems*

Nitrogen removal performance among anammox-based systems varies widely depending on inoculum source, reactor type, and salinity (Table 2). In this study, the FtBR inoculated with shrimp–pond sludge at 30.1–33.0 ppt showed relatively low performance (43.8% NRE; 0.084 kg–N/m³·d), likely due to the dominance of *Ca. Brocadia*, a genus known to be less tolerant of full-strength seawater conditions. High salinity can disrupt osmotic balance and inhibit enzyme activity in non-marine anammox species.

Systems dominated by *Ca. Scalindua*, which is naturally adapted to marine and brackish environments, consistently demonstrated higher removal rates at similar or lower salinities. Both the UASB reactor with marine sediment at 3% salinity and the shrimp–aquaculture column reactor at 2.7% achieved 88% NRE, with NRR far exceeding those of FtBR systems [10]. This reflects the strong halotolerance and metabolic efficiency of *Scalindua* lineages.

Performance differences among FtBRs also highlight the influence of inoculum and acclimation. Estuary sludge under 29.7–32.4 ppt resulted in very low activity [11], whereas shrimp-pond sludge at 20–25 ppt supported high removal (82.49% NRE) [12]. Likewise, anammox granules exposed to 5–25 g/L NaCl maintained stable activity (>86% NRE) due to their protective biofilm structure [31].

Compared with an FtBR operated at 32–33 ppt in a previous study showing >82% NRE [9], the lower performance here suggests differences in microbial composition and salinity acclimation strategy. Overall, the data indicate that halotolerant taxa such as *Ca. Scalindua* are critical for high NRR under marine conditions, while *Brocadia*-dominated communities require moderate salinity or gradual adaptation to maintain activity.

3.7 *Limitations and recommendations*

Despite demonstrating the successful enrichment of anammox bacteria in a saline Filter Bioreactor (FtBR), several limitations were observed in this study. The nitrogen removal efficiency (NRE) peaked at 43.797%, which is modest compared to previous studies reporting efficiencies exceeding 70% in optimized systems [9], [33]. This suggests the need for further operational refinement. Additionally, the study explored a limited salinity range (30.1–33.0 ppt), thus not fully capturing the performance and tolerance thresholds of halophilic anammox species such as *Candidatus Scalindua*, which are known to thrive in broader marine salinity gradients [5], [21]. Another limitation lies in the low temporal resolution of microbial analysis, with sampling conducted only at the end of the experiment. This may have overlooked key microbial transitions or adaptations during reactor startup and stabilization. Moreover, while 16S rRNA gene sequencing offered valuable taxonomic insight, it did not reveal the functional genetic traits driving nitrogen transformations. Functional genomic or transcriptomic analyses would be beneficial to confirm the metabolic activity and ecological roles of the identified taxa [4]. Operational conditions such as HRT, influent composition, and trace element dosing were held constant throughout the experiment, limiting the ability to evaluate performance under variable environmental stresses.

Table 2: Comparison of nitrogen removal performance in various anammox-based systems.

Reactor	Inoculum	Salinity (ppt)	NRE (%)	NRR (kg-N/m·d)	Anammox bacteria	Ref.
FtBR	Shrimp pond sludge	30.1–33.0	43.8	0.084	*Ca. Brocadia*	This study
UASB	Marine sediment	3%	88	10.7	*Ca. Scalindua wagneri*	[10]
FtBR	Estuary sludge	29,7–32,4	16.87	0.026	-	[11]
Column	Shrimp–aquaculture pond	2.7%	88	0.83	*Ca. Scalindua spp.*	[32]
FtBR	Shrimp pond sludge	20–25	82.49	0.125	-	[12]
UASB	Anammox granular sludge	5–25 g/L NaCl	>86	1.05	*Ca. Brocadia, Ca. Kuenenia*	[31]
FtBR	Shrimp pond sludge	32–33	82.48, 83.06	0.12, 0.10	-	[9]

Despite these constraints, the study contributes novel insights into the potential of *Candidatus Brocadia*, a genus typically associated with freshwater environments, to dominate under saline conditions. This finding challenges the prevailing understanding that *Candidatus Scalindua* primarily occupies marine systems and suggests a broader ecological plasticity of *Brocadia* than previously documented [19]. Phylogenetic

analysis confirmed strong affiliations between dominant ASVs and *Candidatus Brocadia sinica*, indicating a functional role for this genus in saline nitrogen removal. These results are particularly significant given the growing interest in deploying low–energy, anammox–based technologies in saline wastewater treatment applications.

Future studies should investigate performance across a wider salinity range, utilize higher–resolution temporal monitoring, and incorporate metagenomic techniques to map the functional potential of microbial consortia. Genomic and transcriptomic studies have confirmed that key functional genes such as hydrazine synthase (hzsA) and hydrazine oxidoreductase (hzo) are essential markers for anammox metabolism, catalyzing the conversion of ammonium and nitrite into dinitrogen gas. Expression of these genes is closely associated with nitrogen removal performance and microbial adaptation under environmental stress, including salinity fluctuations. Integrating functional gene-based analyses in future studies will provide mechanistic insights into metabolic regulation and help elucidate the resilience of halophilic anammox species in saline environments.

Pilot–scale trials and alternative inoculum comparisons are also recommended to validate the scalability and robustness of FtBR systems in real–world marine wastewater treatment scenarios. The direct application to wastewater can employ a PN/A system due to the limited nitrite concentration typically found in wastewater. Nitrogen removal performance can be further enhanced by using MAB as inoculum in the reactor, since these bacteria exhibit superior activity at high salinity compared to freshwater anammox bacteria (FAB).

4 Conclusions

This study successfully demonstrated the enrichment of anammox bacteria in a saline FtBR system operated with shrimp pond sludge. The reactor achieved stable nitrogen removal, with a maximum efficiency of 43.797%, despite fluctuations in nitrogen loading and microbial adaptation phases. Microbial community analysis identified *Candidatus Brocadia* as the dominant anammox genus, instead of a small amount of *Candidatus Jettenia*. Nitrifying bacteria such as *Nitrosomonas* and *Nitrospira* further reflect a complex microbial ecosystem supporting nitrogen transformation under anoxic, saline conditions. The ability of *Candidatus Brocadia* to persist and thrive under high salinity suggests its potential in treatment systems for saline environments. These findings advance understanding of saline anammox processes and support the application of FtBRs in marine nitrogen management. MABs should be identified and enriched for application in aquaculture wastewater treatment, as they are more compatible with saline environmental conditions for future research.

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Author Contributions

Z.Z.: Planned this study and finalized the paper; M.V.A.: Writing original draft, conducting microbial community experiment, and analyzing data; Z.I.: Performed nitrogen analysis and contributed to synthesis writing; N.M.: oversaw microbial community analysis experiment. All authors have read and agreed to the published version of the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

Declaration of generative AI and AI-assisted technologies in the writing process

The authors utilized the ChatGPT tool to enhance the language and readability of the manuscript.

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