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First identification of marine anammox bacteria in Indonesia under tropical aquaculture conditions

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Abstract. *Zainuddin EN, Gumelar G, Ismail S, Rustama MM, Zulkarnaini Z. 2025. First identification of marine anammox in Indonesia under tropical aquaculture conditions. Biodiversitas 26: 6469-6479.* Marine anaerobic ammonium oxidation (anammox) bacteria offer a promising and sustainable pathway for nitrogen removal from aquatic environments, particularly in shrimp aquaculture, where nitrogen-rich waste can cause environmental problems. This study aimed to identify marine anammox bacteria (MAB) under tropical conditions in two filter bioreactors (FtBRs) operated at tropical ambient temperatures with seawater salinity of 32-33 ppt. FtBR 1 was inoculated with intensive shrimp pond sludge, while FtBR 2 received a mixture of sludge and freshwater anammox granules (*Candidatus* Brocadia fulgida). Microbial community profiling was performed using Illumina HiSeq sequencing targeting the V3-V4 regions of the 16S rRNA gene. Both reactors successfully enriched the marine anammox species *Candidatus* Scalindua wagneri, accounting for 7% (FtBR 1) and 21% (FtBR 2) of the phylum Planctomycetota. In contrast, *Candidatus* Brocadia fulgida failed to survive under high salinity. Notably, *Xanthomarina gelatinilytica* emerged as the dominant genus in both systems, suggesting its important role in the hydrolysis and mineralization of organic nitrogen originating from shrimp aquaculture sludge. MAB were successfully identified and enriched despite their low relative abundance, suggesting potential interactions with co-occurring denitrifying communities. This study represents the first documented identification of MAB in Indonesia. These findings represent the first documented presence of MAB in Indonesia from aquaculture sludge. This work provides a foundation for developing localized, low-energy nitrogen removal technologies for sustainable shrimp aquaculture in coastal regions.

Keywords: Anammox, *Candidatus*, filter bioreactor, shrimp aquaculture, tropical

INTRODUCTION

Shrimp aquaculture is a rapidly growing sector in Indonesia that supports coastal livelihoods and export markets, but intensive production generates nitrogen-rich effluent from uneaten feed and shrimp metabolism. This effluent contains ammonium, nitrite, nitrate, urea, and organic nitrogen, which can drive eutrophication, harmful algal blooms, oxygen depletion, and sediment degradation in receiving waters, ultimately reducing pond productivity. Maintaining water quality remains particularly challenging for small- and medium-scale farms with limited treatment infrastructure (Hukom et al. 2020). Although recirculating aquaculture systems and in-pond treatments are increasingly applied to limit effluent discharge, their effectiveness in saline environments is constrained by high energy demand, carbon supplementation requirements, and reduced efficiency of conventional nitrification-denitrification pathways. These limitations underscore the need for alternative, low-energy microbial nitrogen transformation processes, such as anaerobic ammonium oxidation (anammox).

Anammox offers an alternative and energy-efficient pathway for nitrogen removal by converting ammonium (NH_4^+) directly into nitrogen gas (N_2) using nitrite (NO_2^-) as an electron acceptor under anaerobic conditions, offering a highly efficient alternative. Anammox is estimated to contribute up to 50% of fixed nitrogen loss in marine ecosystems globally, underscoring its ecological significance in saline environments (Dalsgaard et al. 2005). Marine anammox bacteria (MAB), particularly *Candidatus* Scalindua, are considered obligate halophiles capable of thriving at salinities typical of seawater (Zhang and Okabe 2020). Their application in engineered systems, including marine RAS and brine treatment, has shown promising results in temperate regions (Micolucci et al. 2023; Zulkarnaini et al. 2024b).

Indonesia, with its extensive shrimp farming industry, provides an ideal setting for investigating the potential of anammox bacteria for nitrogen removal from aquaculture effluent (Gumelar et al. 2022). Despite Indonesia's position as one of the world's largest shrimp-farming nations, very few studies have examined the presence, cultivation, or functional role of MAB in tropical aquaculture systems.

Existing Indonesian research has primarily focused on freshwater anammox enrichment or nitrogen removal in non-saline wastewater (Zulkarnaini et al. 2024a, 2025). As a result, there remains a significant knowledge gap regarding the adaptation, enrichment dynamics, and microbial interactions of marine anammox species-particularly *Candidatus* Scalindua-under tropical environmental conditions and within shrimp aquaculture sludge. Consequently, the presence, identity, and enrichment potential of MAB under tropical aquaculture conditions remain largely unexplored. Anammox bacteria under tropical aquaculture conditions remain largely unexplored.

The cultivation and identification of MAB hold significant promise for sustainable nitrogen removal in aquatic environments, particularly in shrimp aquaculture, where nitrogen-rich waste poses severe environmental challenges (Ismail et al. 2022). The use of FtBR for cultivating anammox bacteria is particularly advantageous due to its high surface area for biofilm attachment, low operational costs, and suitability for various environmental conditions. This reactor type has been shown to support stable anammox activity, making it a viable option for treating shrimp farm wastewater (Gumelar et al. 2024; Zulkarnaini et al. 2024a).

Our previous study showed fast start-up of the anammox process using intensive shrimp solid waste in two FtBRs at tropical ambient temperature. Nitrogen removal performance was almost the same between the use of intensive shrimp solid waste inoculum and the addition of fresh anammox granule (*Candidatus* Brocadia fulgida) to the inoculum (Gumelar et al. 2024).

This study identified MAB from tropical Indonesian shrimp aquaculture using FtBR operated at ambient tropical temperature and seawater (salinity 32-33 ppt). By characterizing bacterial community composition, anammox abundance, and associated microbial consortia, this work provides the first identification of MAB in Indonesia and offers critical insights for developing localized, energy-efficient nitrogen removal strategies suitable for tropical aquaculture systems. By identifying the specific anammox species and their optimal cultivation conditions, this study contributes to the broader goal of improving wastewater management practices in aquaculture, ultimately supporting environmental sustainability and economic viability in the industry. We hypothesized that shrimp aquaculture sludge, under tropical temperature and marine salinity conditions, can serve as an effective inoculum for enriching MAB-particularly *Candidatus* Scalindua wagneri-despite its low initial abundance and in the absence of pre-existing marine anammox enrichment systems in Indonesia.

MATERIALS AND METHODS

Sample collection and DNA extraction

Biofilm samples analyzed in this study were collected from two Filter Bioreactors (FtBR 1 and FtBR 2) previously operated by Gumelar et al. (2024) at the end of reactor operation (day 120). In that study, the reactors were run at a hydraulic retention time of 24 h using seawater with a salinity of 32-33 ppt under anaerobic conditions suitable for anammox enrichment. The present study did not involve reactor operation; instead, it focused solely on microbial community analysis to identify and assess the enrichment of MAB. Reactor performance and nitrogen removal data are reported elsewhere (Gumelar et al. 2024). Intermediate time-point sampling was not performed; therefore, the microbial data represent an end-point characterization of microbial community composition rather than temporal dynamics. The samples were extracted using the ZymoBIOMICS DNA Miniprep Kit according to the manufacturer's protocol.

Microbial community analysis

The bacterial community analysis utilized Illumina HiSeq 2500 PE250 (Novogen, Korea), focusing on the V3-V4 hypervariable regions of the 16S rRNA gene. Amplification was achieved with 341F/R806 primer sets, with sequences as follows: 341F 5′-CCT AYG GGR BGC ASC AG-3′ and 806R 5′-GG ACT ACN NGG GTA TCT AAT-3′. Polymerase Chain Reaction (PCR) reactions employed Phusion High-Fidelity PCR master mix (New England Biolabs), beginning with an initial denaturation at 98°C for 2 min, followed by 35 cycles of annealing from 65°C to 55°C for 15 sec, and extension at 68°C for 30 sec. Annealing temperature decreased by 1°C per cycle until reaching 55°C (Rahayu et al. 2024). The PCR products were quantified and assessed by mixing equal volumes of 1× loading buffer (containing SYBR green) with the PCR products. Subsequently, they were subjected to electrophoresis on a 2% agarose gel for visualization. Samples exhibiting a prominent band between 400 and 450 base pairs were selected for subsequent experiments.

High-throughput sequencing analysis

Sequencing libraries were constructed using the NEBNext® UltraTM DNA Library Prep Kit for Illumina, following the prescribed protocol, with index codes incorporated. Evaluation of library quality was conducted using a Qubit® 2.0 Fluorometer (Thermo Scientific) and an Agilent Bioanalyzer 2100 system. Subsequently, the library underwent sequencing on an Illumina platform, producing 250 bp paired-end reads.

Paired-end sequenced data were merged using the FLASH software (Magoč and Salzberg 2011), yielding raw tag data, which were subsequently filtered through QIIME (v1.7.0) to procure high-quality tags (Caporaso et al. 2010). The filtering process employed the UCHIME algorithm, setting a threshold of a quality score >20 and an error rate <0.01 (Q20) to obtain high-quality tags. These tags underwent comparison against the gold database using the UCHIME algorithm to identify and eliminate chimera sequences, resulting in effective tags. The effective tags, representing sequenced data, were further analyzed using UPARSE software (Edgar 2013). Sequences were clustered into operational taxonomic units (OTUs) at a 97% similarity threshold. Representative OTU sequences were taxonomically assigned using the SILVA 132 reference database with a confidence threshold of 0.8-1 (https://www.arb-silva.de/). An OTU-based approach was adopted to maintain consistency with previous marine anammox studies and available

reference datasets. Although Amplicon Sequence Variant (ASV)-based methods provide higher taxonomic resolution, the use of OTU clustering may limit discrimination among closely related taxa and is therefore acknowledged as a methodological limitation. An OTU-based approach was adopted to maintain consistency with previous marine anammox studies and available reference datasets. Although Amplicon Sequence Variant (ASV)-based methods provide higher taxonomic resolution, the use of OTU clustering may limit discrimination among closely related taxa and is therefore acknowledged as a methodological limitation.

RESULTS AND DISCUSSION

Sequencing output, OTUs, community richness, and diversity

High-throughput sequencing successfully generated 175,139 raw paired-end reads for FtBR 1 and 177,782 reads for FtBR 2 (Table 1). After merging, quality filtering, and chimera removal, the number of effective high-quality tags retained was 83,272 for FtBR 1 and 82,394 for FtBR 2, confirming strong dataset reliability. Clustering of effective tags at 97% similarity produced in average 2,578 OTUs in FtBR 1 and 2,429 OTUs in FtBR 2, indicating substantial taxonomic richness in both reactors.

Alpha-diversity metrics revealed clear differences in microbial community structure. FtBR 1 exhibited higher richness and diversity, with 2,492 observed species, a Shannon Index of 8.567, and a Simpson Index of 0.990, demonstrating a more evenly distributed microbial community. FtBR 2 contained 2,335 observed species, with a Shannon Index of 7.888 and a Simpson Index of 0.983, suggesting slightly lower diversity. Chao1 and ACE richness estimators were similar between reactors, indicating comparable potential species richness despite differences in observed taxa. Good's coverage values of 0.999 (FtBR 1) and 0.995 (FtBR 2) confirmed that sequencing depth was sufficient to capture nearly all microbial taxa. FtBR 1 showed higher phylogenetic diversity (PD_whole-tree = 206.988) than FtBR 2 (190.922), indicating greater evolutionary breadth among its microbial members.

The higher richness and diversity observed in FtBR 1 likely reflect the complex microbial community inherent to shrimp pond sludge, which contains diverse heterotrophic and nitrogen-transforming bacteria adapted to organic-rich sediments. This broader community composition may enhance functional redundancy and ecological stability during early biofilm development.

FtBR 2, although slightly less diverse, demonstrated comparable potential richness based on Chao1 and ACE values. The lower evenness in FtBR 2 could be attributed to selective pressures from the additional freshwater anammox inoculum, allowing specific taxa-including *Candidatus* Scalindua wagneri-to become more dominant under saline anaerobic conditions. This selective community structure aligns with the observed enrichment behavior of marine anammox species.

The combined sequencing depth, OTU richness, and diversity profiles indicate that FtBR 1 maintained a richer and more phylogenetically diverse microbial assemblage, while FtBR 2 exhibited a more specialized community structure that may facilitate targeted enrichment of MAB.

Microbial community analysis

This study employed Illumina NT-250 for microbiological analysis to discern the diversity of microbial communities and the proportional contribution of each phylum to anammox species relative to total bacterial composition within the sample.

Figure 1 illustrate phylum-based microbial community abundance, with results cross-referenced against the SILVA138 reference database utilizing the UCHIME algorithm, and subsequent sequence analysis facilitated by the Uparse software to ensure accurate phylum-to-species detection. In FtBR 1, Pseudomonadota exhibited the highest dominance at 48.9%, followed by Desulfobacterota at 10.2% and Chloroflexi at 8.1%. Notably, Planctomycetota, housing anammox bacteria, was identified in reactor samples, albeit in modest quantities of 0.2%. Conversely, in FtBR 2, Pseudomonadota dominated at 53.7%, followed by Desulfobacterota (4.5%), Chloroflexi (4.6%), and Bacteriodota (7.8%). Planctomycetota was identified at 0.3%.

From the phylum abundance data, both reactors exhibited similar profiles, with Pseudomonadota dominating and Planctomycetota in minor abundance. Studies on MAB using seawater as a substrate have shown Pseudomonadota dominance throughout a 303-day study period. However, Planctomycetota exhibited a significant increase starting from day 206 (Yokota et al. 2018a). Microbial analysis of anammox reactors consistently reveals Pseudomonadota and Chloroflexi as dominant or highly abundant (Pereira et al. 2017). Intensively farmed shrimp pond sediment, sourced from sedimentation ponds, likely has a high organic content, favoring the dominance of Pseudomonadota. Denitrifying bacteria perform optimally in environments rich in organic carbon. In this study, reactor sediment was not removed for the first 120 days to minimize disturbances that could negatively impact growing anammox bacteria. Anammox bacteria have traditionally been considered chemolithotrophic, although some species can oxidize short-chain fatty acids. The addition of small amounts of organic acids minimally affects biomass yields and specific growth rates in some freshwater bacteria, but there is little literature on the effects of organic carbon on MAB (Zhang and Okabe 2020).

Figures 2-3 portrays bacterial abundance at the genus level. A clear difference was observed between the microbial communities in FtBR 1 and FtBR 2 (Table 2). Although Pseudomonadota dominated both reactors, FtBR 1 maintained a more evenly distributed and phylogenetically diverse community, consistent with its higher Shannon (8.567) and PD_whole_tree (206.988) indices. At the genus level, both reactors were dominated by taxa commonly associated with saline and coastal environments.

Table 1. Sequencing output, OTUs, and Alpha-Diversity Indices for FtBR 1 and FtBR 2

Parameter	Raw paired-end reads	Raw tags	Clean tags	Effective tags	Effective (%)	Average length (nt)	GC (%)	OTUs (97% similarity)	Observed species	Shannon Index	Simpson Index	Chao1 richness	ACE richness	Good's coverage	PD_whole_tree
FtBR 1	175,139	155,390	151,762	83,272	47.55%	418	54.39	68,416	2,492	8.567	0.99	2,493.84	2,512.82	0.999	206.988
FtBR 2	177,782	155,864	153,125	82,394	46.35%	416	53.98	69,092	2,335	7.888	0.983	2,490.20	2,539.04	0.995	190.922

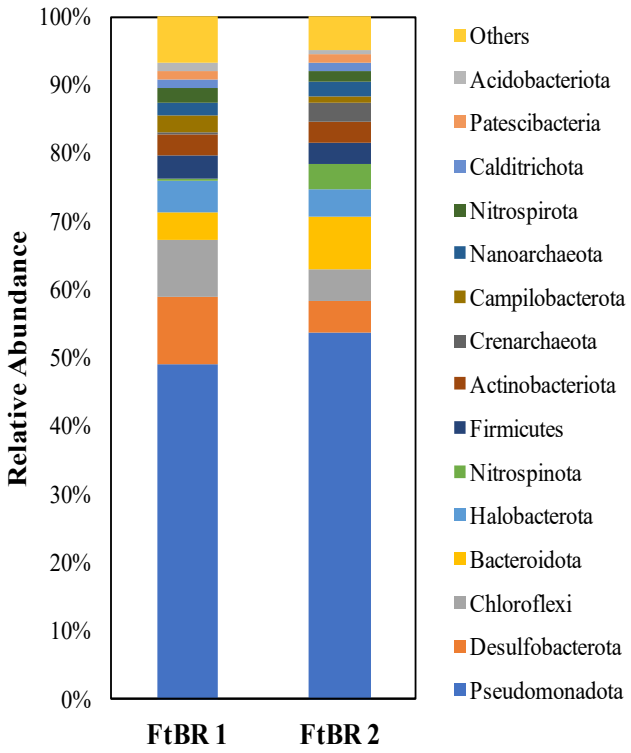

Figure 1. Microbial community relative abundance of FtBR 1 and FtBR 2 at the phylum level. Relative abundance below 1% is classified as others

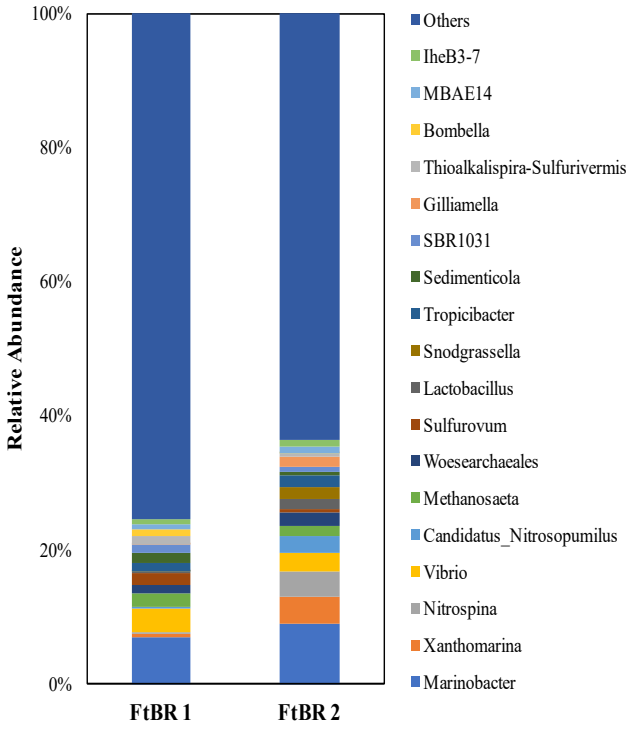

Figure 2. Microbial community relative abundance of FtBR 1 and FtBR 2 at the genus level. Relative abundance below 1% is classified as others

In FtBR1, *Marinobacter* was the most common genus (6.98%), followed by *Vibrio* (3.50%) and *Methanosaeta* (1.96%), with smaller portions of the community represented by *Nitrospina* (0.41%), *Xanthomarina* (0.39%), and *Candidatus* Nitrosopumilus (0.13%). *Marinobacter* also showed the highest proportion (8.89%) in FtBR2, while *Xanthomarina* (4.01%) and *Nitrospina* (3.76%) were more common in this reactor compared to FtBR1. *Vibrio* accounted for 2.80%, and *Candidatus* Nitrosopumilus and *Methanosaeta* made up 2.63% and 1.57%, respectively. *Candidatus* Scalindua wagneri was significantly more enriched in FtBR 2, accounting for approximately 21% of Planctomycetota, compared to 7% in FtBR 1. This indicates that despite its lower overall diversity, FtBR 2 provided more favorable conditions for marine anammox proliferation, likely due to the selective pressure created by the addition of freshwater anammox granules that failed to survive in marine salinity. Thus, FtBR 1 supported broader microbial richness, whereas FtBR 2 achieved stronger functional specialization and more efficient anammox enrichment, demonstrating a trade-off between diversity and targeted functional selection.

Discussion

Identification of MAB

The identification of *Candidatus* Scalindua wagneri in both reactors signifies its status as a relatively difficult-to-cultivate marine anammox bacterium, requiring a long time to reach stability. Other studies have also identified the same species in reactors inoculated with seawater discharge (Kawagoshi et al. 2010) and from Hiroshima Bay with a sequence similarity of 96% (Kindaichi et al. 2011). The low abundance observed in both reactors aligns with findings from UASB reactor studies, where *Candidatus* Scalindua was initially identified in small quantities. However, inoculation with marine anammox on day 119 resulted in a significant increase in relative abundance by day 323, with processing capability and growth equivalent to freshwater anammox bacteria used in large-scale reactors (Yokota et al. 2018a). The stability of anammox reactor performance is largely determined by the inoculum used. With minimal anammox bacteria present in the inoculum, the required time for cultivation will be longer. The use of inoculum from specialized marine reactors with high salinity levels demonstrates high dominance (Awata et al. 2015a).

At salinity levels above 30 ppt, *Candidatus* scalindua can grow optimally. However, at lower salinity levels, other anammox bacteria such as *Candidatus* Kuenenia, *Candidatus* Jettenia, and *Candidatus* Brocadia can thrive. According to Yokota et al. (2018a), bacteria are able to survive, but in very small abundance. Salinity is the most important differentiating factor in anammox bacterial abundance. The abundance of *Candidatus* Scalindua increases proportionally with salinity levels ranging from 20-35 ppt, and all marine anammox species are obligate halophiles (Zhang and Okabe 2020).

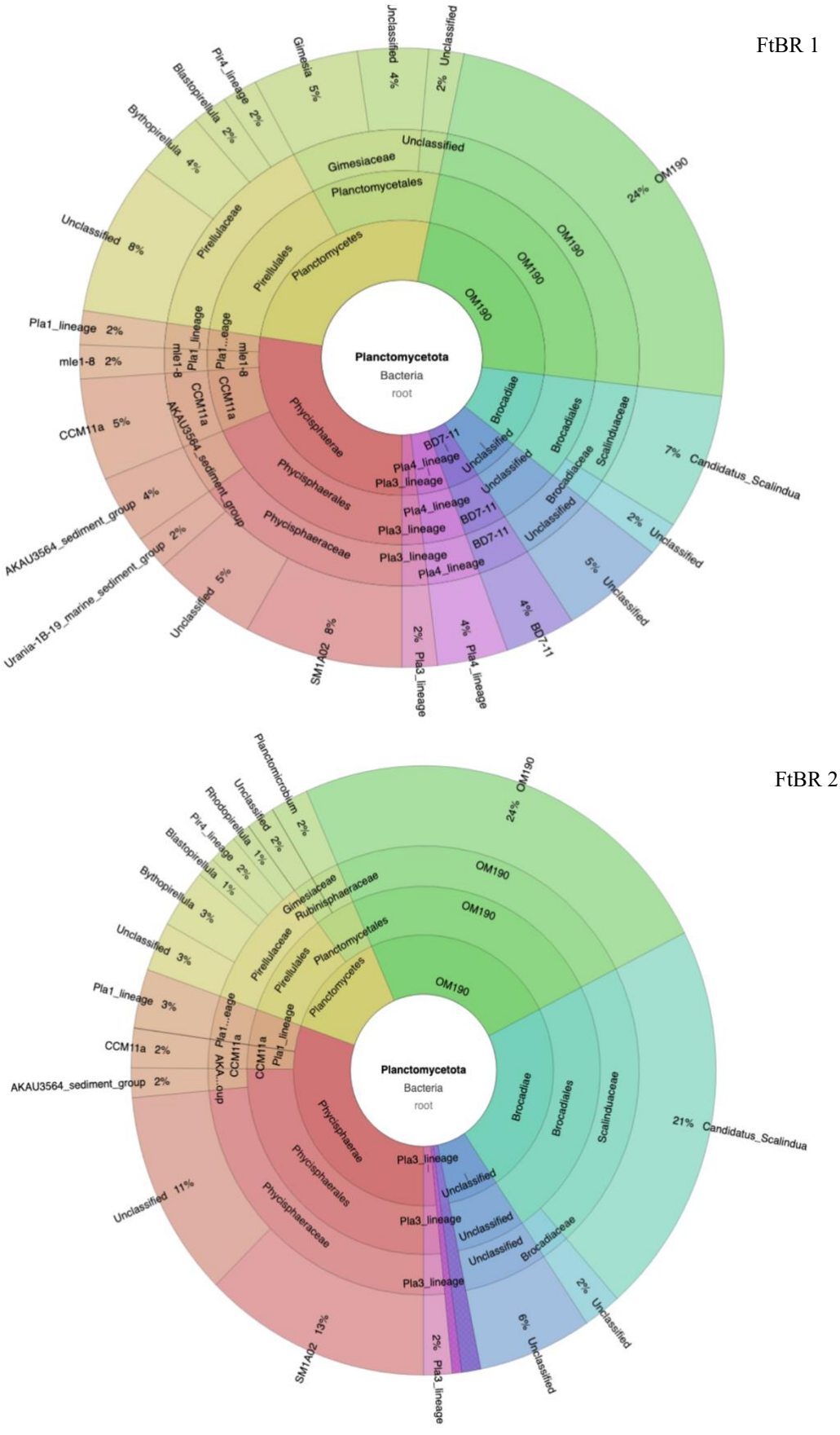

Figure 3. Krona microbial community abundance at the genus level belongs to Planctomycetota. The percentage of marine anammox (*Candidatus* Scalindua) was relative to Planctomycetota. Unclassified indicates that a portion of the sequences could not be assigned to known genera based on current reference databases

Table 2. Comparison of microbial composition and anammox abundance between FtBR 1 and FtBR 2

Reactor	FtBR 1	FtBR 2	Comparison Summary
Dominant phyla	Pseudomonadota (48.9%), Desulfobacterota (10.2%), Chloroflexi (8.1%), Bacteroidota (4.21%)	Pseudomonadota (53.7%), Bacteroidota (7.89%), Chloroflexi (4.62%), Desulfobacterota (4.51%)	Similar dominant groups, but FtBR 2 shows higher Pseudomonadota and Bacteroidota
Planctomycetota (anammox phylum)	0.20%	0.30%	Slightly higher presence in FtBR 2
Dominant genera	*Marinobacter* (6.98%), *Vibrio* (3.50%), *Methanosaeta* (1.96%)	*Marinobacter* (8.89%), *Xanthomarina* (4.01%), *Nitrospina* (3.76%)	FtBR 1 shows broader functional distribution, FtBR 2 shows selective enrichment
Marine anammox bacterium (*Candidatus* Scalindua wagneri)	~7% of Planctomycetota	~21% of Planctomycetota	FtBR 2 has a 3× higher Scalindua abundance
Freshwater anammox (*Candidatus* Brocadia fulgida)	Not detected (unable to grow at high salinity)	Not detected (washed out early)	Both reactors confirmed the inability of freshwater anammox to survive 32-33 ppt
Community diversity (Shannon)	8.567	7.888	FtBR 1 is more diverse and even
Phylogenetic diversity	206.988	190.922	FtBR 1 contains more evolutionarily diverse taxa
Functional implication	Higher richness and functional redundancy; slower selective enrichment	Lower richness but stronger functional specialization; faster Scalindua enrichment	FtBR 1: broad capability, FtBR 2: targeted anammox cultivation

In FtBR 2, inoculated with fresh anammox, *Candidatus* Brocada fulgida, did not yield discernible differences in profile or nitrogen reduction performance compared to non-inoculated conditions. This can be attributed to the inability of *Candidatus* Brocadia fulgida, a primary species within the inoculum, to adapt to high salinity levels. The salinity levels utilized in this study ranged between 32-33 ppt. Sudden acclimatization processes rendered this bacterium incapable of survival, as salinity directly affects the performance and population dynamics of anammox bacteria (Gonzalez-Silva et al. 2017). While certain freshwater anammox bacteria can tolerate elevated salinity levels, their metabolic activity and population density may decline under such conditions (Gonzalez-Martinez et al. 2018). In addition to freshwater environments, anammox bacteria have been identified in shrimp pond sediment, suggesting their potential role in nitrogen cycling within subtropical aquaculture ecosystems (Amano et al. 2011).

The use of *Candidatus* Brocadia fulgida as the inoculum for this study was due to the absence of MAB in Indonesia (Zulkarnaini et al. 2025). Some studies indicate that freshwater anammox species may possess tolerance and adaptability to saline environments. Okabe et al. (2024) demonstrate that freshwater anammox species such as Kuenenia stuttgartiensis can survive in salinity levels of up to 6% salinity, while *Candidatus* Jettenia and *Candidatus* Brocadia sinica decreased activity at 1-2% salinity.

The inability of *Candidatus* Brocadia fulgida to survive in FtBR 1 can be attributed to the inherent metabolic and osmotic limitations of freshwater anammox bacteria when exposed to marine salinity. Brocadia species are adapted to freshwater or low-salinity environments and lack the osmoprotective mechanisms necessary to maintain cellular homeostasis at 32-33 ppt. Elevated salinity imposes strong osmotic pressure that disrupts membrane integrity, destabilizes key metabolic enzymes, and impairs the proton motive force required for anammox metabolism. Okabe et al. (2024) reported that the activity of Brocadia and Jettenia sharply declines at salinity levels as low as 1-2%, while only halophilic MAB such as *Candidatus* Scalindua possess compatible solute accumulation, modified membrane lipids, and salt-tolerant enzymatic systems enabling growth under high salinity (Okabe et al. 2024). Osmotic stress also increases ATP demand for cellular maintenance, diverting energy away from ammonium oxidation and nitrite reduction, further reducing the competitiveness of Brocadia in saline environments. As a result, freshwater Brocadia fulgida was unable to adapt and was rapidly washed out, whereas *Candidatus* Scalindua wagneri successfully established and enriched under the saline, anoxic conditions of the reactors.

Cultivating MAB is a time-consuming process, and not all efforts lead to successful identification due to the challenges in cultivating these bacteria. In the sediment cultivation of Hiroshima Bay, MAB were not identified in the first reactor for 200 days (Kindaichi et al. 2011). In a cultivation with inoculum from Gullmar Fjord sediment, no significant anammox activity was observed during the first five months. Identification results indicated minimal presence of anammox bacteria (<2%), but after nine months of culture, their abundance increased, stabilizing dominance after 14 months (van de Vossenberg et al. 2008). In reactors inoculated with coastal mud sediment, no anammox bacteria were identified initially. However, inoculum sourced from nitrogen-rich coastal areas exhibited satisfactory performance after 330 days (Kawagoshi et al. 2009). A similar scenario was observed in a study using sediment from aquaculture ponds, where no anammox activity was observed in the first 174 days, with anammox bacteria accounting for only 0.3% of the community. However, after 277 days, the reactor

demonstrated robust performance, with anammox bacteria comprising 55% of the population (Li et al. 2019). Anammox bacteria were also found in low abundance (1.74%) during 194 days in a partial reactor (Li et al. 2019). Denitrifying and anammox bacteria can collaborate within the same ecosystem to process nitrogen, making this consortium applicable in waste treatment with high organic content (Tal et al. 2006).

In comparison with previous studies, not all reactors can identify MAB within a short period (200 days) (Kindaichi et al. 2011). *Candidatus* Scalindua wagneri was the primary marine anammox bacterium found in all successful anammox reactors. The success of anammox reactors in increasing the abundance of anammox bacteria takes a relatively long time (over 300 days) (Kawagoshi et al. 2009). Both reactors successfully enabled the identification of *Candidatus* Scalindua wagneri at low relative abundance within a 120-day period, representing an initial stage of marine anammox enrichment.

This study hypothesized that shrimp aquaculture sludge, under tropical temperature and marine salinity conditions, can serve as an effective inoculum for the enrichment of MAB, particularly *Candidatus* Scalindua, despite their low initial abundance and in the absence of pre-existing marine anammox enrichment systems in Indonesia. The detection and enrichment of *Candidatus* Scalindua wagneri in both filter bioreactors after 120 days directly support this hypothesis. Although Scalindua remained a minor fraction of the total microbial community, its consistent presence across reactors operated at 32-33 ppt salinity and ambient tropical temperature confirms that shrimp aquaculture sludge contains viable marine anammox populations capable of establishment under appropriate selective conditions.

The absence of freshwater anammox species, including *Candidatus* Brocadia fulgida, further reinforces the salinity-driven selection proposed in the hypothesis and highlights the ecological specificity of MAB. These findings demonstrate that tropical shrimp aquaculture sludge can function as a natural reservoir for MAB and that filter bioreactors provide suitable conditions for their initial enrichment. However, the relatively low abundance observed indicates that longer operational periods was required for further enrichment, consistent with reports from other marine anammox cultivation studies.

Microbial nitrogen removal in an aquaculture system

The success of nitrogen removal in marine environments, particularly in aquaculture systems, is not solely dependent on the presence of anammox bacteria but also relies heavily on synergistic interactions within the broader microbial community. In this study, despite the low relative abundance of *Candidatus* Scalindua wagneri, 7% and 21% of the phylum Planctomycetota in FtBR 1 and FtBR 2, respectively, the reactors exhibited promising nitrogen removal, indicating that even low-abundance MAB can be functionally significant when supported by complementary microbial taxa.

In both reactors, non-anammox bacteria—especially *Xanthomarina*, *Marinobacter*, *Halomonas*, *Pseudomonas*, and *Nitratireductor*—played important ecological roles in supporting nitrogen cycling. *Xanthomarina*, a key degrader of complex organic matter, likely accelerated ammonification and increased ammonium availability, while *Marinobacter*, *Halomonas*, and *Nitratireductor* likely contributed to nitrite and nitrate transformations that may have supplied substrates for anammox activity. These interactions help explain stable nitrogen removal even with low *Candidatus* Scalindua abundance. Compared with prior studies, our results align with Awata et al. (2015), who showed that salinity strongly promotes marine anammox species and inhibits freshwater *Candidatus* Brocadia. However, the extent of *Candidatus* Scalindua enrichment in our reactors was lower, likely due to shorter operational periods, higher organic loading, and differences in reactor configuration (Awata et al. 2015; Yokota et al. 2018b). Our findings indicate that enriched marine anammox communities in tropical systems require longer operation and more controlled conditions to approach the dominance levels reported in previous studies. Anammox communities in tropical systems require longer operation and more controlled conditions to approach the dominance levels reported in previous studies.

Interestingly, the genus *Xanthomarina*, specifically *Xanthomarina gelatinilytica*, emerged as the most abundant genus in both reactors. Isolated from seawater, this genus, belonging to the Flavobacteriaceae family, is typically associated with the degradation of complex organic compounds, particularly polysaccharides and proteins, and the cycling of nutrients (Vaidya et al. 2015). In the context of shrimp aquaculture effluent, which is rich in organic nitrogen compounds such as amino acids, proteins, and urea, *Xanthomarina* likely plays a central role in the hydrolysis and mineralization of organic nitrogen into ammonium (NH_4^+) from sediment or solid waste in wastewater treatment facilities.

Instead of anammox, other bacteria in tiny abundance contribute indirectly to nitrogen cycling, including *Marinobacter* (Alqahtani et al. 2019), *Pseudomonas* (Yi et al. 2023), *Halomonas* (Wang et al. 2017), and *Nitratireductor* (Labbé et al. 2004). Many of these genera are known denitrifiers, indicating the presence of multiple nitrogen transformation pathways within the system; for instance, *Nitratireductor* participates in nitrate-to-nitrite conversion, supplying nitrite for the anammox process. Huang et al. (2020) reported *Cobetia*, *Marinomonas*, *Marinobacterium*, and *Halomonas* are dominant in nitrogen removal in mariculture environments, utilizing various pathways including assimilation and denitrification.

This microbial consortium, characterized by a high abundance of facultative anaerobes and halophiles, likely maintains suitable anaerobic conditions and supplies necessary intermediates, thereby facilitating the anammox process. Such findings support the notion that functional resilience and nitrogen removal efficiency in marine bioreactors depend on the entire microbial network, not just the abundance of anammox bacteria alone.

Although *Candidatus* Scalindua wagneri was present at low relative abundance (7% in FtBR 1 and 21% in FtBR 2 within the phylum Planctomycetota), both reactors demonstrated promising nitrogen removal performance. This indicates that even minimal populations of MAB can contribute significantly to nitrogen transformation, provided that the surrounding microbial community supports their activity.

Proposed mechanism of conversion of organic nitrogen to nitrogen gas

The nitrogen conversion pathway proposed in Figure 4 was a conceptual model derived from the microbial community composition observed in this study and established pathways reported in the literature. This schematic was intended to illustrate potential nitrogen transformations and microbial interactions rather than to represent empirically verified process rates or pathways within the reactors. The nitrogen removal pathway in the shrimp wastewater treatment system involves multiple microbial processes and nitrogen compound conversions. First, organic nitrogen compounds (proteins, amino acids from feed and waste) are hydrolyzed by heterotrophic bacteria such as *Xanthomarina*, *Tropicibacter*, and *Marinobacter* into ammonium (NH_4^+), initiating the nitrogen cycle (Harwati et al. 2009; Vaidya et al. 2015; Pan et al. 2024). This is followed by partial nitrification, where *Nitrosomonas* (AOB) oxidizes NH_4^+ into NO_2^-, and *Nitrospina* (NOB) may further oxidize NO_2^- into NO_3^-, although high salinity and limited oxygen could restrict full nitrification (Foesel et al. 2008; Lücker et al. 2013). In anaerobic zones, *Candidatus* Scalindua wagneri facilitates the anammox process, converting NH_4^+ and NO_2^- into N_2 gas and water, making it a key pathway for autotrophic nitrogen removal (Awata et al. 2015).

Concurrently, denitrification by facultative anaerobes such as *Sedimenticola*, *Sulfurovum*, and *Thioalkalispira* reduces NO_3^- and NO_2^- to N_2 gas under anoxic conditions, using organic carbon or reduced sulfur as electron donors (Brettar et al. 2006; Zhang et al. 2009; Russ et al. 2014). Additionally, *Methanosaeta*, a methanogen present in both reactors, can convert acetate to methane under anaerobic conditions, potentially competing with denitrifiers for organic substrates (Smith and Ingram-Smith 2007). This integrated system of hydrolysis, nitrification, anammox, and denitrification ensures robust nitrogen removal through both autotrophic and heterotrophic pathways. The enhanced microbial diversity and abundance in FtBR 2 suggest it offers better reactor performance for marine anammox cultivation, especially under tropical shrimp farming conditions. *Methanosaeta*, a methanogen present in both reactors, can convert acetate to methane under anaerobic conditions, potentially competing with denitrifiers for organic substrate. This integrated system of hydrolysis, nitrification, anammox, and denitrification ensures robust nitrogen removal through both autotrophic and heterotrophic pathways. The enhanced microbial diversity and abundance in FtBR 2 suggest it offers better reactor performance for marine anammox cultivation, especially under tropical shrimp farming conditions.

This study illustrates the synergy between heterotrophs, nitrifiers, denitrifiers, and anammox bacteria in transforming nitrogen-rich shrimp aquaculture waste into environmentally benign nitrogen gas. Such cooperation helps overcome the limitations of low-abundance anammox bacteria, as other functional groups compensate through complementary roles in nitrogen cycling. The overall pathway thus integrates multiple biogeochemical processes to achieve effective nitrogen removal.

Limitations and future research direction

This study presents the first successful identification and short-term cultivation of a marine anammox bacterium, *Candidatus* Scalindua wagneri, from tropical Indonesian aquaculture sludge using filter bioreactors. However, the relatively low abundance and slow enrichment of *Candidatus* Scalindua indicate that the results reflect initial establishment rather than mature or stable enrichment, limiting conclusions about scalability. The use of only two bioreactors and end-point microbial analysis at day 120 further restricts interpretation of enrichment dynamics, stability, and reproducibility. In addition, the absence of testing under variable nitrogen loadings or salinity conditions typical of aquaculture systems limits the applicability of the observed enrichment to real-world operations.

Future research should implement multi-time-point sampling to track microbial succession and anammox enrichment trajectories, conduct longer operational periods to promote higher *Candidatus* Scalindua dominance, and integrate quantitative PCR or metatranscriptomic analyses (e.g., *hzsA*, *hdh*, *nirS*) to verify active nitrogen-transforming pathways. Future research should explore longer operational periods, microbial community dynamics, and different inoculum sources to optimize enrichment efficiency. Additionally, investigating functional gene expression and reactor performance under varying loading rates will help elucidate the resilience and adaptability of marine anammox communities in dynamic aquaculture systems. Such efforts will accelerate the development of robust, localized anammox-based treatments for sustainable nitrogen removal in tropical aquaculture systems.

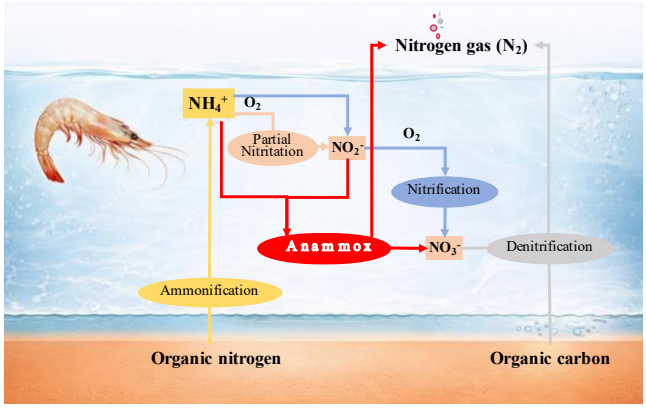

Figure 4. Nitrogen removal in aquaculture from organic nitrogen to nitrogen gas

In conclusion, this study provides the first confirmed identification and short-term enrichment of MAB from Indonesian aquaculture sludge. Using two filter bioreactors operated at ambient tropical temperature and seawater (salinity 32-33 ppt), we successfully detected and enriched *Candidatus* Scalindua wagneri at relative abundances of ~7% (FtBR 1) and ~21% (FtBR 2) within the phylum Planctomycetota after 120 days of operation. In contrast, the freshwater anammox species *Candidatus* Brocadia fulgida failed to survive at marine salinity, confirming its osmotic limitations. These findings demonstrate the feasibility of identifying and initiating enrichment of MAB from tropical aquaculture environments and provide a foundational microbiological reference for future functional and process-based investigations.

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