Research Article

Diversity of Anammox Bacteria from Landfill Treatment Plant Sludge in Tropical Area

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Abstract
The anaerobic ammonium oxidation (anammox) process is known as the warm process. Tropical areas have an advantage due to their consistent temperature throughout the year. This study analyzed the diversity of anammox bacteria in the tropical area using leachate sludge from a landfill as an inoculum in a filter bioreactor (FtBR) and observed nitrogen removal performance. Ammonium and nitrite concentrations of 70, 150, and 200 mg-N/L were delivered into the reactor continuously with hydraulic retention time (HRT) of 24 h and 12 h and run for 131 days at ambient tropical temperature (25–28 °C). High performance achieved with nitrogen removal rate (NRR), nitrogen removal efficiency (NRE), and ammonium conversion efficiency (ACE) were 0.866 kg-N/m^3·d, 99.19%, and 98.90%, respectively. The cultivated leachate sludge could perform an anammox process with four anammox species, *Candidatus Brocadia fulgida*, *Candidatus Brocadia sapporoensis*, *Candidatus Brocadia* sp *uncultured*, *Candidatus Jettenia* sp with abundance 6.52%, 13.82%, 0.77%, and 0.69%, respectively. These findings contribute to the advancement of biotechnology in wastewater treatment, particularly in tropical countries, and highlight the potential for highly cost-effective technology.

Keywords: Anammox, Filter bioreactor, Landfill treatment, Leachate sludge, Nitrogen removal, Tropical area

1 Introduction

Microorganisms play specific roles in wastewater treatment processes, such as in nitrification and denitrification-based nitrogen removal processes. Such as nitrification and denitrification-based nitrogen removal processes [1]. Four distinct groups of autotrophic microorganisms oxidize ammonia: ammonia-oxidizing bacteria (AOB), ammonia-oxidizing archaea (AOA), comammox bacteria (complete oxidation of ammonia to nitrate), and anammox (anaerobic ammonium oxidation) bacteria [2]. Recently, researchers have focused on Anaerobic ammonium oxidation (anammox) bacteria, which have been found to offer significant advantages, including energy savings, sludge reduction, and greenhouse gas emissions (N_2O) [3], [4]. This bacteria converts ammonium (NH_4^+) to nitrogen gas (N_2) using nitrite (NO_2^-) as an electron acceptor [5]. This process is important for removing nitrogen from engineered and natural systems. Additionally, it offers a potential alternative to conventional wastewater treatment methods by efficiently eliminating fixed nitrogen compounds [6]. Anammox bacteria have been found in ecological and wastewater treatment, where their abundance is influenced by the simultaneous presence of ammonium and nitrite [7]. In many wastewater treatment plants, anammox is present, usually around 1% of the total population. Various sludge from the environment [8]–[11] and wastewater treatment plants, such as nitrifying, denitrifying, or methane sludge, can be used as an inoculum for initiating the enrichment of anammox cultures [12].

The selection of the operational setting and the organic content present in the influent may affect the enrichment of different species of anammox bacteria from a single inoculum source. Consequently, different species of anammox bacteria may be enriched from a single inoculum source. For instance, the successful enrichment of *Candidatus Kuenenia stuttgartiensis* relies on a completely inorganic mineral medium that supports autotrophic growth. On the other hand, an organic carbon supplement is necessary to enrich *Candidatus Anammoxoglobus propionicus* (utilizing propionate or acetate, respectively). Adding specific organic compounds facilitates the growth and enrichment of these species of anammox bacteria [13], [14].

Many types of reactors for the biological process could be used for anammox, such as Upflow Anaerobic Filter process (UAF), Anaerobic Fluidized Bed (AFB) Reactor, Upflow anaerobic Sludge Blanket (UASB), and facultative pond systems [15]. In application, anammox is used in the form of granules, suspended sludge, and biofilm. Biofilm is attached to the carrier, and the wastewater is treated and passes through the media [16]–[20].

The application of anammox in wastewater treatment has made significant progress. In particular, Isaka *et al.* pioneering work demonstrating the successful implementation of the anammox process in an anaerobic biofilter reactor (ABF) [21]. This reactor uses porous polyester non-woven supports as the immobilizer liner for Anammox bacteria. The study showed the ability to maintain stable nitrogen removal performance in the temperature range of 20–22 °C. Additionally, the research achieved an impressive nitrogen conversion rate of 8.1 kg-N/m^3·d, underscoring the feasibility of anammox technology for effective nitrogen reduction. On the other hand, Zulkarnaini *et al.* cultivated anammox in a string wound filter and achieved nitrogen removal efficiency up to 81% at start-up [22]. The present study explores the utilization of a PVC string wound filter as a carrier for anammox cultivation, aiming to enhance further the understanding of its performance and potential in wastewater treatment.

Furthermore, anammox process has proven its efficacy in nitrogen reduction across a range of temperatures from 15–40 °C [23], [24]. Anammox species, leading to shifts in dominance anammox process under ambient temperature conditions. This study aims to investigate the diversity of anammox bacteria in a tropical environment under such circumstances, shedding light on its feasibility and effectiveness for nitrogen removal.

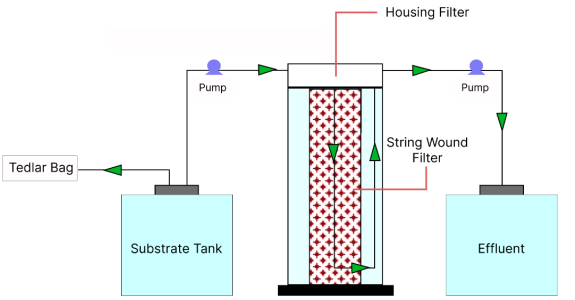

Figure 1: Configuration of filter bioreactor (FtBR).

The present study intends to build upon these findings by further exploring the potential of anammox for nitrogen removal under tropical temperature conditions. Through comprehensive analysis of the microbial community using Illumina MiSeq sequencing, valuable insights can be gained regarding the performance and suitability of anammox in tropical regions.

2 Materials and Methods

2.1 *Reactor setup and inoculum*

The inoculum utilized in this experiment was from a wetland within the leachate treatment plant located at Air Dingin Landfill, Padang City, West Sumatra, Indonesia. The specific coordinates for sample collection were recorded as 100°22'49" E and 0°49'26" S, precisely near the lagoon effluent. Volatile suspended solid (VSS) inoculum sludge was 2.20 g/L, and 375 mL of sludge was added to the FtBR for the experiment's start-up.

FtBR was a cylindrical column (effective volume of 1.5 L). The membrane module (PVC string wound filter, pore size of 0,5 µm) was set up within the reactor. The reactor was completely covered with aluminum foil to avoid light penetration and prevent heterotrophic bacteria growth. Figure 1 shows the configuration of FtBR for cultivation. Hydraulic Retention Time (HRT) was kept at 24 h to feed effective substrate for anammox. The reactor was connected to a tedlar bag containing gas N_2 to maintain an anoxic condition.

2.2 *Operational condition*

The FtBR was operated in a room with tropical ambient temperature (without temperature regulation). The synthetic wastewater was added with 70–200 mg-N/L $(NH_4)_2SO_4$ and $NaNO_2$ as food for anammox bacteria. During Stage I (days 1-65), NO_2^--N and NH_4^+-N concentrations were maintained at 70 mg-N/L and increased gradually. The substrate containing (per liter of tap water) 500 mg $KHCO_3$, 27.2 mg KH_2PO_4, 300 mg $MgSO_4.7H_2O$, 180 mg $CaCl_2.7H_2O$, 1 mL of trace element I, and trace element II [17].

The water quality of the influent and effluent of the reactor was collected and analyzed twice a week. Initially, the nitrogen concentration of the influent was 70 mg-N/L until day 65. After that, NO_2^--N and NH_4^+-N were raised to 150 mg-N/L up to day 74 for stage II. In the next stage, HRT was set for 12 h with the same nitrogen concentration until day 89. The concentration was increased to 200 mg-N/L until day 131.Water samples were analyzed using the standard methods. NH_4^+-N was measured by the Nessler method, NO_2-N by spectrophotometry, and NO_3^--N by ultraviolet screening spectrophotometry. pH and temperature were determined potentiometrically with a portable digital pH meter.

2.3 *High-throughput sequencing analysis*

To analyze the microbial community, 5 mL biomass from day 131 was collected from 3 points (top, middle, and bottom) of the string wound filter. 40 mL of 50% ethanol was added and kept at –20 °C. Before DNA extraction, the sample was pelleted and washed with phosphate-buffered saline (8,000 × g, 2 min, 4 °C). Therefore, 0.20 g of the washed sample was DNA extracted using FastDNA SPIN Kit for Soil (MP Biomedicals, USA) following the manufacturer's instructions. Table 1 shows the thermocycling protocols and primer sequences. The DNA sample was amplified with the 515F and 806R primer set (Eurofins Genomics, Japan) for prokaryotes' 16S rRNA V4 region. PCR reactions were performed using 0.5 units of HotStarTaq DNA Polymerase (Qiagen, Germany) and 1.5 µL of DNA template at 95 °C for 5 min followed by 25 cycles of 94 °C (30 s), 55 °C (30 s), and 72 °C (60 s), and a final extension step at 72 °C for 10 min (First PCR). The second PCR (40 µL) was performed with 2 × KAPA HiFi HotStart ReadyMix (Roche, Switzerland) at 95 °C for 3 min followed by 11 cycles of 98 °C (20 s), 60 °C (15 s) and 72 °C (60 s), and a final extension step at 72 °C for 1 min. All PCR was performed in a T100 Thermal Cycler. Furthermore, amplified samples were sequenced using a Miseq system [25].

2.4 *Phylogenetic analysis*

The data was processed with Rstudio with the DADA2 package to identify the species present in the sample. Moreover, bacterial sequences were taken from silva_nr99_v138.1_train_set and silva_species_assignment_v138.1 data. The resulting data was the number of read sequences and bacterial categories from species to kingdom. Furthermore, the ASV was exported from R studio. ASV sequences belonging to the anammox genus were entered into the script for processing Phylogenetic Tree Data.

Additionally, the sequence of each ASV was searched at GenBank NCBI (National Center for Biotechnology Information). Finally, the Phylogenetic Tree is processed with the MEGA V.11 (Molecular Evolutionary Genetics Analysis). Identification of phylogenetic neighbors and calculation of pairwise 16S rRNA gene sequence similarity was achieved by using https://www.genome.jp/tools-bin/clustalw.

Table 1: Preparation of high-throughput sequencing analysis

Gene Target	Primer Name	Sequence (5′ - 3′)	Product Length (bp)	Thermal Profiles
Universal 16S rRNA	515F [26]	GTGCCAGCMGCCGCGGTAA	291	5 min at 95 °C, followed by 25 cycles of 30 s at 94 °C, 30s at 55 °C, 60s at 72 °C. Then final extension at 72 °C 10 min.
	805R [27]	GACTACHVGGGTATCTAATCC		
Illumina adapter sequence Forward sequence, 5′ -TCGTCGGCAGCGTCAGATGTGTATAAGAGAAG-3′ Reverse sequence, 5′ -GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-3′				

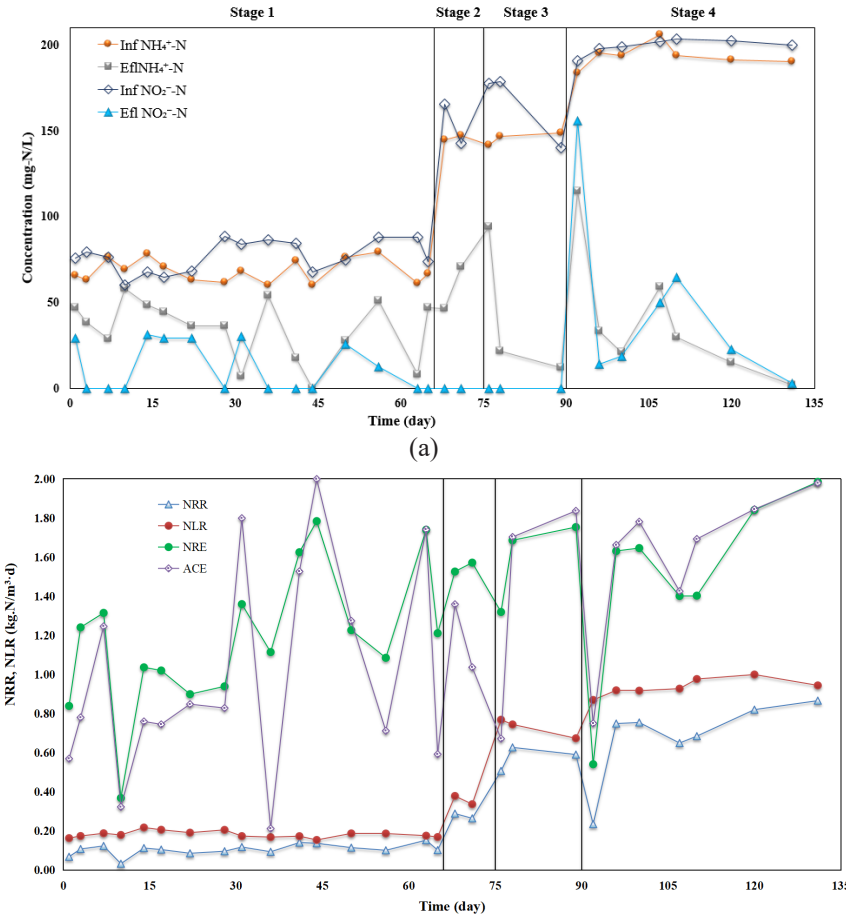

Figure 2: (a) Profile of nitrogen concentration on filter bioreactor (FtBR) (b) Nitrogen removal performance.

3 Results and Discussion

3.1 *Nitrogen removal performance*

Figure 2 shows nitrogen concentration and the removal in the whole experiment. During the initial 65 days of Stage I, the influent concentration of NH_4^+-N and NO_2^--N remained at approximately 70 mg-N/L, HRT of 24 h. The reactor's performance during this period exhibited fluctuations, with the highest recorded removal efficiencies of 99.91% for ammonium and 100% for nitrite. However, the lowest observed efficiencies were 10.62% for ammonium and 13.93% for nitrite. Correspondingly, the nitrogen removal rate (NRR) reached an average of 0.107 kg-N/m^3·d.

On day 66, the influent NH_4^+-N and NO_2^--N concentrations were progressively increased to 150 mg-N/L and maintained at this level for ten days during Stage II. During this stage, the effluent ammonium concentration demonstrated a notable decrease, achieving a maximum ACE of 91.79%. The average NRR during this period was 53.37%. On day 75, the HRT was decreased to 12 h. At this stage, the ACE was 70.16% on average. At the last stage, from day 90 to 131, the influent was 200 mg/L and HRT 12 h, ACE stabilized at about 83.08% with the highest at 98.90%. In the last stage, NRR achieved maximum of 0.866 kg-N/m^3·d.

Entirely, nitrogen removal fluctuations were observed despite consistent influent composition and hydraulic load. These occurred due to variations in operational conditions and environmental stressors,

including temperature, pH, dissolved oxygen levels, and organic content. Notably, in real-world applications, anammox bacteria may exhibit susceptibility to extraneous compounds such as sulfides, toxic metals, alcohols, phenols, and antibiotics, which could serve as potential inhibitors due to the intricate nature of the wastewater matrix [28].

3.2 *Effect of water temperature on nitrogen removal*

Temperature plays an important role in the process and growth of anammox, affecting nitrogen removal efficiency and microbial community structure. Temperature fluctuations can affect the physical response of anammox, leading to inhibitory and destabilizing effects in low-nitrogen wastewater [29]–[31]. The optimum temperature range for most anammox species in wastewater treatment is between 25–40 °C [32]. Moreover, Anammox bacteria's growth is significantly influenced by temperature. For instance, within the 20–30 °C temperature range, anammox bacteria displayed growth rates ranging from 0.05 to 0.09/day [29]. In a study conducted by Park *et al.*, a start-up of lab-scale Anammox reactors seeded with activated sludge at an ambient temperature of 20 °C. However, the maximum NRR achieved was 0.64 kg-N/m^3·d [33]. In comparison, this study achieved stability at 0.934 kg-N/m^3·d after day 90.

The water temperature in the experimental system ranged between 25 °C–28 °C. Interestingly, despite the absence of temperature control, the variations in water temperature did not significantly impact the performance of the reactor in terms of nitrogen removal efficiency. Temperature in the tropical environment is highly advantageous for the cultivation of anammox bacteria. The stable temperature conditions in tropical regions offer several benefits, including reducing costs associated with temperature-controlled equipment making the process more cost-efficient.

3.3 *Microbial community analysis*

Microbial community analysis was conducted from anammox biomass on day 131 of the experiment. The taxonomic assignment analysis revealed the presence of 25 phylum-level lineages belonging to both Bacteria and Archaea domains. Figure 3 shows microbial community abundance at the phylum level.

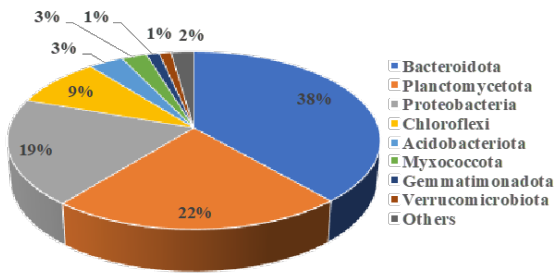

Figure 3: Microbial community abundance at phylum level. The relative abundance <1% is classified as others.

The dominant phyla included Bacteroidota (38.49%), Planctomycetota (22.43%), Proteobacteria (19.39%), Choloroflexi (9.00%), and others phyla (10.70%). Archaea were found in relatively smaller proportions, represented by two genera comprising less than 1% of the microbial community abundance. As a member of the Planctomycetota phylum, Anammox emerged as a significant contributor, ranking second in dominance within the bioreactor. This observation is consistent with the findings of Begmatov *et al.*, where the microbial community in a wastewater treatment process was largely governed by Proteobacteria (averaging 27.8%) and Bacteroidota (15.7%) [34]

3.4 *Diversity anammox bacteria*

Figure 4 shows microbial community abundance at the genus level. The result shows the dominant was PHOS-HE36 unclassified at 14.97%, followed by two genera *Candidatus Brocadia*, with abundance at 13.82% and 6.52%. Furthermore, the other two anammox species were also detected with low abundance (<1%). PHOS-HE36 is reported as denitrification bacteria and coexists with anammox sludge that converts produced nitrate by anammox and organic sludge from inoculum to nitrogen gas [35]. Phylogenetic analysis of the 16S rRNA gene (Figure 5) assigned four ASVs to the genus *Candidatus Brocadia* (16S ASV2, 16S ASV16, 16S ASV135, 16S ASV152). Based on sequence similarity in Table 2, four anammox species were grown in FtBR. They were *Candidatus Brocadia fulgida*, *Candidatus Brocadia sapporoensis*, *Candidatus Brocadia*, *Candidatus Jettenia* sp. The high diversity of cultivated anammox bacteria using sludge from tropical environments could prove that tropical environments

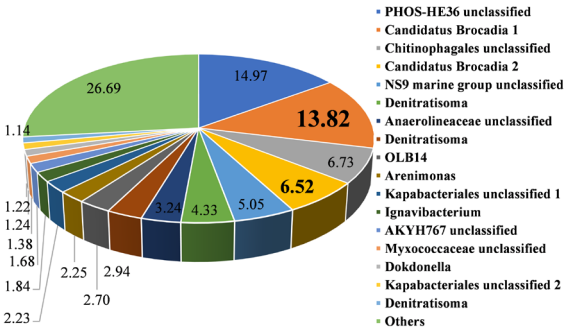

Figure 4: Microbial community at the genus level. The relative abundance <1% is classified as others, the number after the genus name to distinguish different species.

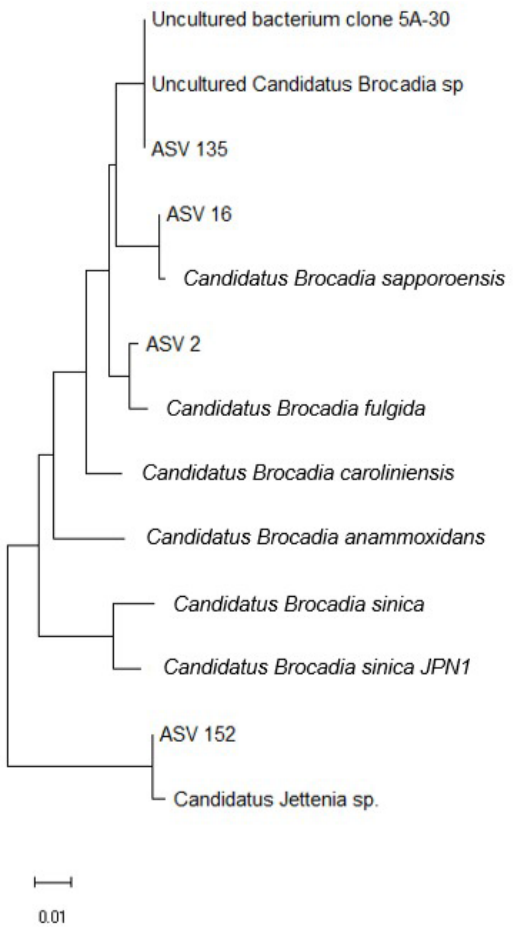

Figure 5: Phylogenetic tree representing the affiliation of 16S rRNA clone sequences of anammox bacteria in the reactor.

serve many anammox bacteria communities. Even anammox abundance was not the dominant species for 131 days operation FtBR due to slow-growth bacteria; the abundance could be increased over time.

Table 2: Anammox abundance in FtBR

Species	ASV	Sequence Similarity (%)
Ca. Brocadia fulgida	ASV_2	98%
Ca. Brocadia sapporoensis	ASV_16	98%
Ca. Brocadia	ASV_135	98%
Ca. Jettenia sp.	ASV 152	98%

On the other hand, the bacteria that responsible for the oxidation of ammonium and nitrite were found in a small amount with approximately 0.733% of the total population. Nitrite and ammonium oxidizing bacteria were identified as *Nitrospira*, *Candidatus nitrotoga*, and *Nitrosomonas*. This finding shows anammox's significant presence and contribution in the reactor process, and nitrite-oxidizing bacteria was relatively minor.

In a previous study by Hsu *et al.* Anammox enrichment was performed using sludge obtained from an anaerobic landfill leachate treatment system [36]. Initially, the predominant anammox microorganism in the sludge was *Candidatus Brocadia* sp. However, significant changes appeared after the installation of the artificial environment. The ammonia and nitrite removal rates increased from 16–92% and 22–99.9%, respectively. It is plausible that *Candidatus Anammoxoglobus propionicus* was already present in the original leachate sludge but in such limited quantities that it remained undetectable. The exact cause of this transition in the Anammox community remains unclear. Nevertheless, with the introduction of the artificial medium and reactor operation under controlled conditions, *Candidatus Anammoxoglobus propionicus* outcompeted other frequently encountered anammox microorganisms, ultimately becoming the dominant species [36].

Yang *et al.*, introduced pellets enriched with active anammox bacteria into landfill leachate treating WWTPs, demonstrating successful outcomes [37]. Following the pellet inoculation, the anammox process was effectively established in the aeration tanks of four full-scale WWTPs, where the anammox

communities were predominantly led by *Brocadia*, accompanied by minor members such as *Kuenenia* and *Anammoxoglobus*. Introducing pellets enriched with active anammox bacteria into landfill leachate treating WWTPs has demonstrated successful outcomes [37].

Moreover, the substantial ammonium influx from the leachate influent exhibited a positive correlation with the enhancement of Nitrosomonas, likely due to the increased availability of free ammonia, supporting rapid growth. Concurrently, alongside ammonia oxidation by AOB, the provision of nitrite facilitated the growth of NOB. The genera Nitrospira, Nitrobacter, and Nitrotoga, displaying various niche adaptations, serve as canonical NOB in wastewater treatment systems [37].

3.5 *The prospect of anammox cultivation in a tropical area*

The tropical region offers significant advantages for wastewater treatment utilizing biotechnology due to its stable temperature conditions, which are crucial for the optimal performance of microorganisms involved in the process. In this experimental study, the stable temperature range of 25–28 °C was maintained throughout the operation, enabling the successful removal of nitrogen compounds from the wastewater. Notably, a high nitrogen removal rate of 0.934 kg-N/m^3·d was achieved, demonstrating the efficiency of the anammox process in the treatment system.

To further evaluate the applicability of anammox, a scale-up experiment is required. Considering various operational factors and potential challenges, it is important to see Anammox's capability in real conditions. Scale-up studies provide valuable insights into the scalability and practicality of anammox technology, allowing for optimizing process parameters and developing cost-effective strategies for nitrogen removal in wastewater treatment plants.

4 Conclusions

FtBR operated to cultivate anammox bacteria in tropical ambient temperatures (25–28 °C). This reactor demonstrated highly successful with ACE, NRE, and NRR 98.90%, 99.19%, and 0.866 kg-N/m^3·d, respectively. This experiment successfully cultivated four anammox species belonging to *Candidatus Brocadia* in a tropical environment without requiring temperature control. It enhances cost-efficiency by eliminating temperature regulation expenses. These results underscore anammox technology's potential as a sustainable and cost-effective solution for nitrogen removal in tropical regions, expanding its feasibility and offering a promising approach for wastewater treatment with reduced operational costs. Furthermore, to fully realize the potential and scalability of this innovative approach, Full-scale research and pilot studies are warranted.

Acknowledgments

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Author Contributions

Z.Z.: Planned this study and finalized the paper; N.A.: Writing - original draft, conducting metagenomic and metatranscriptomic experiments, and analyzing data. H.C.A.: Performed nitrogen analysis, collected the samples; P.S.K.: Oversaw nitrogen analysis and contributed to the synthesis writing. S.S.: contributed to the synthesis drafting. NM: oversaw metagenomic and metatranscriptomic analysis experiments. The paper was approved by all authors.

Conflicts of Interest

The authors declare no conflict of interest.

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