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Cultivation of anammox bacteria from a tropical lake in Indonesia using a novel filter bioreactor to enhance nitrogen removal efficiency

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ABSTRACT

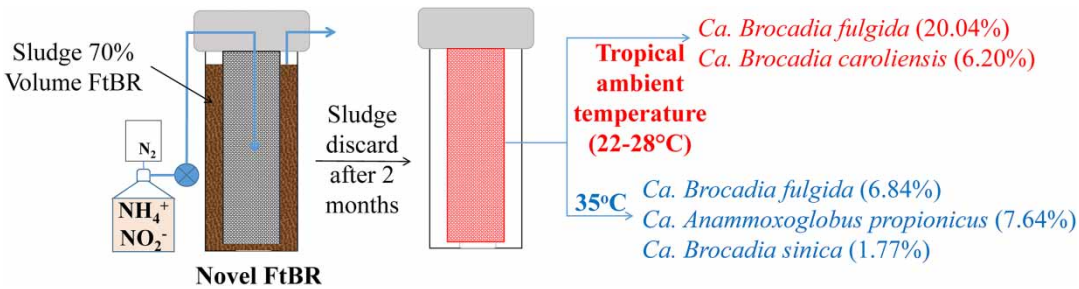
This study presents a novel strategy for cultivating anammox bacteria from tropical environments using a filter bioreactor (FtBR). Two bioreactors were inoculated with sediment sludge from an Indonesian lake and operated at different temperatures: tropical ambient (22–28 °C) in Reactor 1 and 35 °C in Reactor 2. After 106 days, Reactor 1 developed a red carmine anammox biofilm, while Reactor 2 remained similar to its initial state. Reactor 1 achieved a higher and more stable nitrogen removal rate (0.27 kg-N/m^3·d) compared with Reactor 2 (0.21 kg-N/m^3·d), indicating a 28.6% greater efficiency. The operational temperature significantly influenced the diversity and abundance of anammox bacteria. *Candidatus Brocadia caroliensis* (6.20%) was detected in Reactor 1, whereas *Candidatus Anammoxoglobus propionicus* (7.64%) and *Candidatus Brocadia sinica* (1.77%) were found only in Reactor 2. Additionally, *Candidatus Brocadia fulgida* was more abundant in Reactor 1 (20.04%) than in Reactor 2 (6.84%). These findings demonstrate that temperature plays a crucial role in starting the anammox process in FtBRs with a resident inoculum from tropical environments, significantly affecting bacterial growth and nitrogen removal efficiency.

Key words: anammox bacteria, filter bioreactor (FtBR), Lake Koto Baru, nitrogen removal, tropical ambient temperature, tropical environments

HIGHLIGHTS

- A novel technique for cultivating anammox bacteria from tropical environments using filter bioreactor (FtBR).
- High abundance and diversity of anammox species in tropical temperature operation compared to 35 °C.
- The first identified anammox bacteria, *Candidatus Brocadia fulgida*, *Candidatus Brocadia caroliniensis*, *Candidatus Brocadia sinica*, and *Candidatus Anammoxoglobus propionicus* in Indonesia from Lake Koto Baru.

GRAPHICAL ABSTRACT



1. INTRODUCTION

In the environment, anaerobic ammonium oxidation (anammox) bacteria convert ammonium into nitrogen gas under anaerobic conditions using nitrites as donor electrons (Van Kemenade *et al.* 2024). Anammox process is more efficient than the conventional nitrification–denitrification process, which can achieve a 60% reduction in oxygen demand, eliminate the need for organic carbon addition, and reduce land requirements. Additionally, the lower production of N_2O greenhouse gases (i.e., N_2O) and waste active sludge makes this technology more environmentally friendly (Ali & Okabe 2015). This process is widely utilized for nitrogen removal from wastewater, with over 114 large-scale anammox reactors currently in operation worldwide, and the number is steadily growing (Lackner *et al.* 2014). Furthermore, it fosters the exploration and enrichment of anammox bacteria across diverse ecosystems. Presently, 23 species spanning seven genera have been identified, including *Ca. Anammoxoglobus*, *Ca. Anammoximicrobium*, *Ca. Brasilis*, *Ca. Brocadia*, *Ca. Jettenia*, *Ca. Kuenenia*, and the marine anammox group within *Ca. Scalindua* (Narita *et al.* 2017; Miao *et al.* 2019). The distribution of known anammox species encompasses regions, such as Europe, China, Japan, Russia, and Brazil. However, they are predominantly located in sub-tropical zones (except for Brazil), rather than tropical areas.

Anammox bacteria represent a subset of slow-growing microorganisms, as indicated by their doubling time of 10–12 days (Van Der Star *et al.* 2007); although faster rates have been reported ranging from 2.1 to 3.9 days (Zhang *et al.* 2017). This characteristic poses challenges for their practical application, rendering conventional microbiological cultivation techniques ineffective (Fujii *et al.* 2002). Moreover, their low environmental abundance requires laboratory enrichment from environmental samples for identification purposes, yet achieving pure cultures remains elusive. Various enrichment strategies have been explored using different reactor configurations such as sequencing batch reactors (SBRs), fluidized bed reactors (FBRs), gas-lift reactors (GLRs), and membrane bioreactors (MBRs) (Nsenga Kumwimba *et al.* 2020). MBRs emerge as particularly promising due to their ability to enrich slow-growing bacteria, including anammox species. However, the widespread adoption of MBRs is hindered by their high procurement costs, limited market availability, and operational complexities associated with membrane fouling (Wu *et al.* 2023). Previous efforts by Zulkarnaini *et al.* (2018) demonstrated the use of a string wound filter for cultivating anammox biofilms with *Candidatus Brocadia sinica* granules at 35 °C and employed filter feeding strategies to grow anammox biofilm before start-up one-stage nitritation/anammox process (Zulkarnaini *et al.* 2024). Building upon this work, they introduced a novel reactor, termed the filter bioreactor (FtBR), in which the same string wound filter was installed as a supporting medium on cartridge housing filters. This innovative setup facilitated the cultivation of anammox bacteria using environmental sludge as an inoculum, thus extending the applicability of anammox biofilm cultivation techniques (Gumelar *et al.* 2024; Zulkarnaini *et al.* 2024).

In addition to the enrichment strategy, procuring anammox inoculants is important for developing a bioreactor-based anammox process. Anammox bacteria are omnipresent across diverse ecosystems and geographical regions due to their crucial role in aquatic and marine nitrogen removal (Ismail *et al.* 2022; Gumelar *et al.* 2024). Exploring and discovering anammox bacteria within local environments offers significant advantages over importing strains from external sources. However, the existing body of information and research pertaining to this subject remains relatively scarce, especially for the anammox inoculants originating from tropical countries (Nsenga Kumwimba *et al.* 2020). Given Indonesia's status as a tropical country, it represents a promising venue for exploring anammox bacteria within its indigenous environmental contexts.

Typically, anammox bacteria grow in nitrogen-polluted environments or those with elevated nitrogen concentrations, where they often play a crucial role in mitigating eutrophication in lakes, as demonstrated in Japan (Yoshinaga *et al.* 2011) and China (Zhu *et al.* 2013). In Indonesia, Lake Koto Baru has become a focal point for studying eutrophication, a consequence of nutrient accumulation from surrounding agricultural areas. The lake surface is predominantly covered by *Salvinia* with a lesser presence of *Eichhornia crassipes*, while the shallow lake bed is characterized by the accumulation of decaying plant matter over several years (Putra *et al.* 2020). Given these ecological conditions, Lake Koto Baru presents an ideal candidate location for investigating and characterizing anammox bacteria within tropical environments. This study aims to cultivate and identify anammox bacteria within this setting using a FtBR approach. Sediment samples were collected from Lake Koto Baru, Tanah Datar, Indonesia, and inoculated into the FtBR reactors. Two operational runs were conducted for 200 days: one at a range of tropical ambient temperatures (22–28 °C) in Reactor 1, and the other at a constant temperature of 35 °C in Reactor 2. Microbial abundance was assessed via Illumina MiSeq sequencing.

2. MATERIALS AND METHODS

2.1. Sample collection

Lake Koto Baru, situated in Tanah Datar, Indonesia, spans an area of 126,963 m^2. Approximately 85.3% of its surface has transitioned into marshland, encroached upon by vegetation. Originally boasting a depth of approximately 20 m, the lake has experienced a shallowing phenomenon, with depths ranging from 0.5 to 4 m, attributed to sedimentation and the accumulation of organic matter from plant debris. Surrounding agricultural activities have further exacerbated nutrient and soil deposition, including nitrogen compounds, in the lake ecosystem.

Sediment samples were collected in June 2020 from four designated points, as shown in Figure 1, utilizing a polyethylene cylinder-handled sampler with dimensions of 15 cm in height and 5.08 cm in diameter. Point I (0°23′23.075″, 100°24′5.400″) was situated proximal to the lake's inlet channel, whereas Point II (0°23′20.674″, 100°24′3.110″) was located in the northern sector of the lake, where surface vegetation coverage was prominent. Conversely, Point III (0°23′17.376″, 100°24′1.537″) resided in the northern region without any surface vegetation. Lastly, Point VI (0°23′17.682″, 100°23′59.766″) was positioned near the lake outlet channel. Sample depths varied across collection points, with 0.6 m at Point I, 0.5 m at Point II, 1.0 m at Point III, and 0.5 m at Point IV.

Dissolved oxygen (DO) and pH were measured onsite. Collected samples were immediately transported to the laboratory, where total solids (TS) and volatile suspended solids (VS) concentrations were measured. The filtered samples' ammonium, nitrate, and nitrite concentrations were also determined.

2.2. Enrichment reactor and operational conditions

The mud samples were stored at 4 °C in the dark, and an equal volume of each sample was mixed before the enrichment experiment using a novel filter reactor (FtBR) shown in Figure 2.

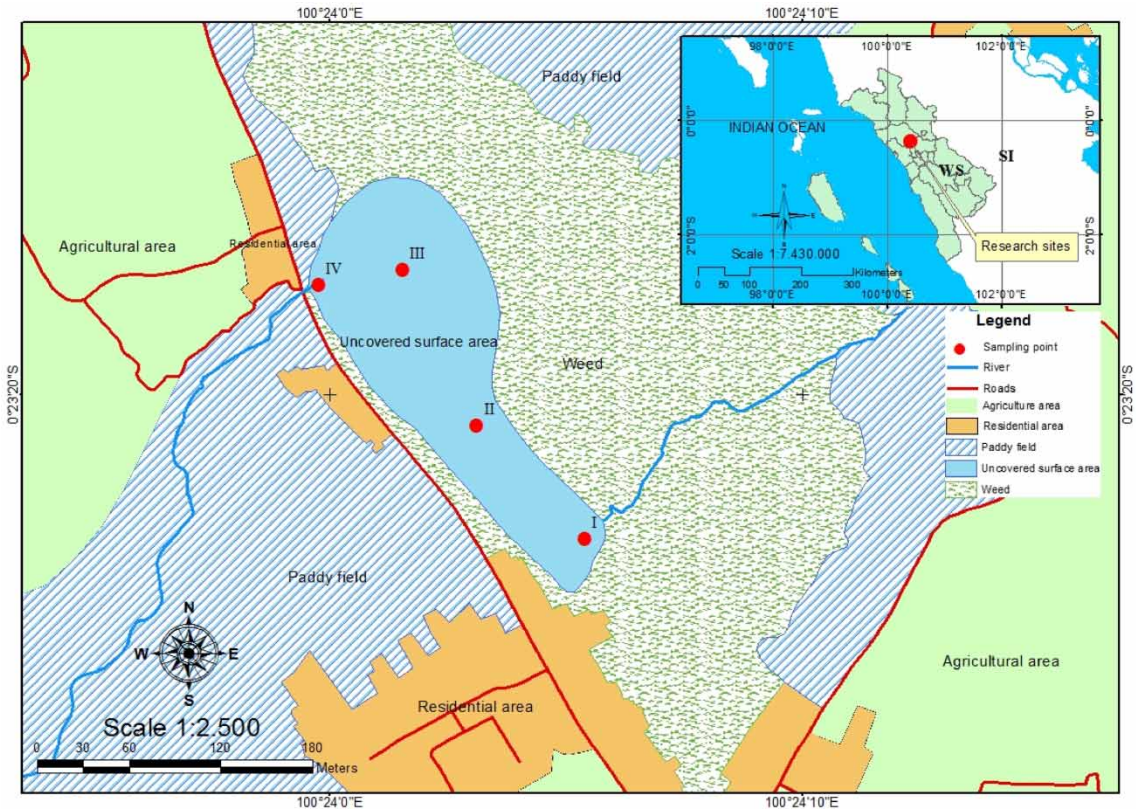


Figure 1 | Map of sampling points location. The sampling points were representative of the lake's condition. I, inlet; II, plant surface covered area; III, surface-free area; IV, outlet channel of the lake; WS, West Sumatra Province, Indonesia; SI, Sumatra Island.

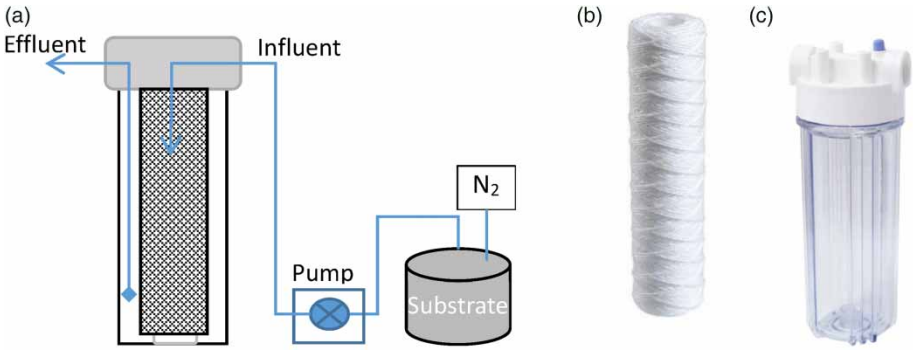


Figure 2 | Configuration of FtBR for cultivation (a), string wound filter (b), and housing filter (c).

FtBR was made of a housing filter (10 inches, Nanotec, China), and a string wound filter (0.5 μm pore size; PURETREX, USA) was mounted in the center of the reactor as a biofilm supporting media. The reactor was covered with aluminum foil to prevent the growth of photosynthetic bacteria. The sediment mud mixture filled around 70% of the reactors. The substrate is fed into the reactor through the hollow inner part of the filter using a peristaltic pump. The flow rate was controlled for 24-h hydraulic retention time (HRT). The substrate container was equipped with a Tedlar bag containing nitrogen gas to maintain anoxic conditions.

Two FtBRs were operated at different temperatures, i.e., tropical ambient temperature (22–28 °C) in Reactor 1 and 35 °C in Reactor 2. Table 1 shows the operational conditions of FtBRs. At the beginning of the reactor's operation (Period 1), 70 mg-N/L $(NH_4)_2SO_4$ and $NaNO_2$ were added to the mineral medium (per liter of distillate water) containing 500 mg $KHCO_3$; 27.2 mg KH_2PO_4; 300 mg $MgSO_4{\cdot}7H_2O$; 180 mg $CaCl_2{\cdot}7H_2O$; and 1 mL trace elements I and II (Zulkarnaini *et al.* 2021). At the end of Period 1, all suspended solids derived from inoculum were withdrawn from the reactor since biofilm was attached to the filter. Ammonium and nitrite concentrations were gradually increased from 70 to 150 mg-N/L in Periods 2–4.

Temperature and pH were monitored every day. Influent and effluent were sampled twice weekly, and the concentrations of ammonium, nitrite, and nitrate were analyzed. Observations of the anammox process were analyzed based on calculations using the ammonium conversion efficiency (ACE, %), nitrogen removal efficiency (NRE, %), and nitrogen removal rate (NRR, kg-N/m^3·d).

2.3. Analytical methods

DO was measured using a DO meter (DO-5510, Lutron, Taiwan). Temperature and pH were measured using a thermo-hygrometer (Corona gl-89, China) and pH meter (STARTER300, Ohaus, USA), respectively. Chemical oxygen demand (COD), TS, and VS concentrations were analyzed according to APHA (2017). Total phosphorus (TP) and total organic carbon (TOC) concentrations were analyzed using a perchloric acid method and a TOC analyzer (TOC-L, Shimadzu, Japan). The concentrations of ammonia and nitrite were analyzed by the Nessler and spectrophotometric method, while nitrate was measured by an ultraviolet spectrophotometric method with a UV–Vis spectrophotometer (Shimadzu 1800, Kyoto, Japan), according to APHA (2017).

Table 1 | Comparison of operational conditions between Reactor 1 (22–28 °C) and Reactor 2 (35 °C)

		Concentration (mg-N/L)			Temperature (°C)		
Period	Time (d)	NH_4^+–N	NO_2^-–N	HRT (h)	Reactor 1	Reactor 2	Note
1	0–62	70	70	24	22–28	35	
2	63–91	70	70	24	22–28	35	Seeding sludge removed at day 63
3	92–105	100	100	24	22–28	35	
4	106–200	150	150	24	22–28	35	

2.4. Microbial community analysis

At the end of Period 3, the biofilm was sampled and the microbial community was analyzed. The total DNA from biofilm samples was extracted using a PowerSoil DNA Isolation Kit (QIAGEN, Germany) following the manufacturer's protocol. The V3–V4 region of 16S *rRNA gene* was amplified from the extracted DNA using universal forward primer 515F (5′-ACTCC-TACGGGAGGCAGCAG-3′) and reverse primer 806R 5′-GGACTACHVGGGTWTCTAAT-3′) for bacterial community (Caporaso *et al.* 2012) and 340F (5′-CCTACGGGNGGCWGCAG-3′) and 806Rb (5′-GGACTACHVGGGTWTCTAAT-3′) primers for archaeal community (Gantner *et al.* 2011; Apprill *et al.* 2015). The PCR products were sequenced using the Illumina MiSeq sequencing (Illumina, USA). The UPARSE (Edgar 2013) and the QIIME2 platform (Caporaso *et al.* 2010) were used to process and analyze the sequences as described by Mawarda *et al.* (2022). Phylogenetic tree was constructed using ARB software.

3. RESULTS

3.1. Characteristics of collected sediment mud

Table 2 shows the characteristics of the collected mud samples. TS and VS concentrations were 40.2–42 and 25.7–27.6 g/L, respectively. There were no significant differences in mud sample characteristics across the sampling points. The VS/TS ratios ranged from 64 to 65%, indicating a high organic content in the mud. Whereas the dissolved oxygen carbon (DOC) concentrations were approximately 40 mg/L, with ammonium and nitrate being present in the supernatant, suggesting that the lake was eutrophic. The TP concentration, ranging from 1.56 to 1.82 mg-P/L, exceeded the water quality standard criteria in the Indonesian Government Regulation. These characteristics are comparable to those of lakes in Indonesia, where eutrophication is a common phenomenon (Soeprobowati *et al.* 2021; Izzati *et al.* 2022; Ruthena *et al.* 2022; Komala *et al.* 2024).

3.2. Nitrogen removal in FtBR

Figures 3 and 4 show the course of nitrogen concentrations and nitrogen removal performances. During Period 1 (0–62 days), when the bioreactors were fed with 70 mg-N/L of ammonium and nitrite at an HRT of 24 h, the ammonium concentration in the effluent was higher than in the influent. This is a typical phenomenon phase in the anammox process using sludge for start-up, called sluggish (Chen *et al.* 2012). The sluggish period occurs due to the breakdown of organic nitrogen in seeding mud into ammonium by bacteria. Furthermore, the increase in ammonium generated from dead aerobic and heterotrophic bacteria is attributed to environmental changes, where the cell lysis and the breakdown of organic nitrogen release ammonia (Wang *et al.* 2013).

The catabolism-produced ammonium was more rapid in Reactor 2 than in Reactor 1 due to higher temperature operation (Figure 3). In contrast, the nitrite concentration in effluent decreased rapidly in both reactors. This result is consistent with the

Table 2 | Characteristics of the mud samples collected from Lake Koto Baru

Sampling points		I	II	III	IV
Depth	m	0.6	0.5	1.0	0.5
pH		6.6	7.1	7.4	7.3
Temperature	°C	25.2	25.6	25.5	25.2
DO	mg/L	4.2	3.1	3.4	7.4
TS	g/L	40.2	42.2	41.0	40.6
VS	g/L	25.7	27.6	26.2	26.1
NH_4^+–N	mg-N/L	5.6	5.2	5.2	4.7
NO_2^-–N	mg-N/L	0.3	0.3	0.3	0.3
NO_3^-–N	mg-N/L	2.7	2.1	2.2	2.1
DOC	mg-C/L	37.3	41.0	39.5	38.6
TP	mg-P/L	1.82	1.62	1.58	1.56
COD sediment	mg/L	2,688[a]			

[a]Composite samples as inoculum.

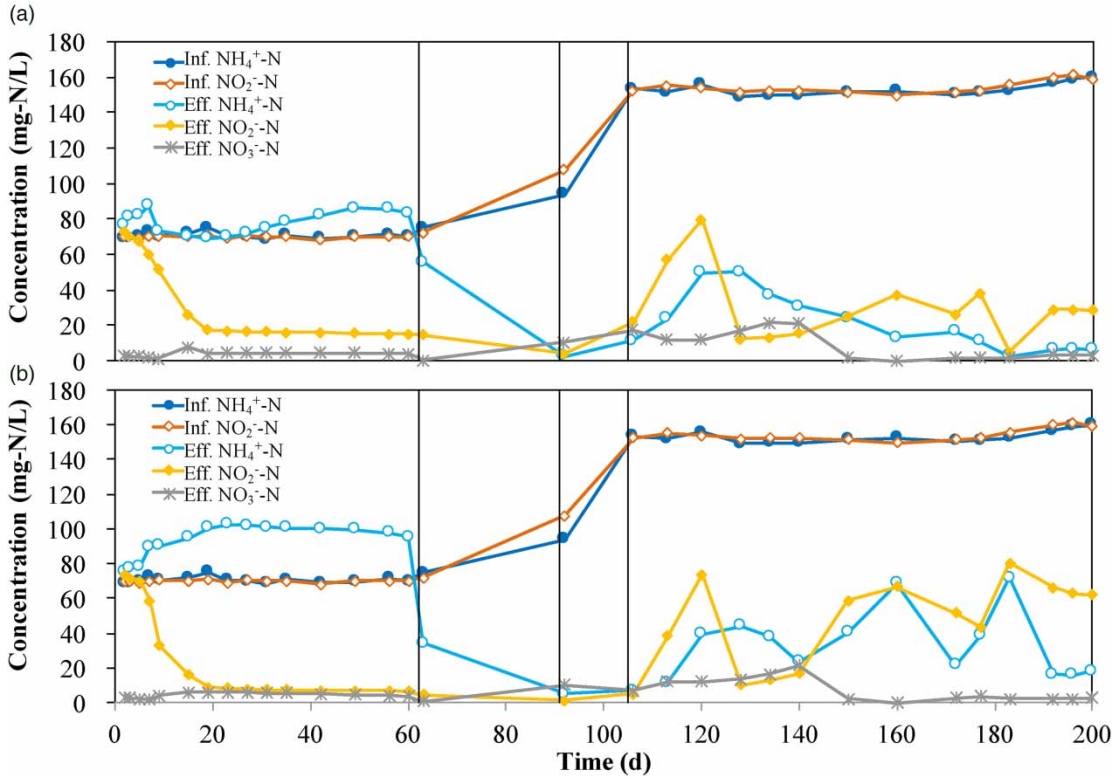


Figure 3 | Profile of nitrogen conversion during cultivation in Reactor 1 (a) and Reactor 2 (b).

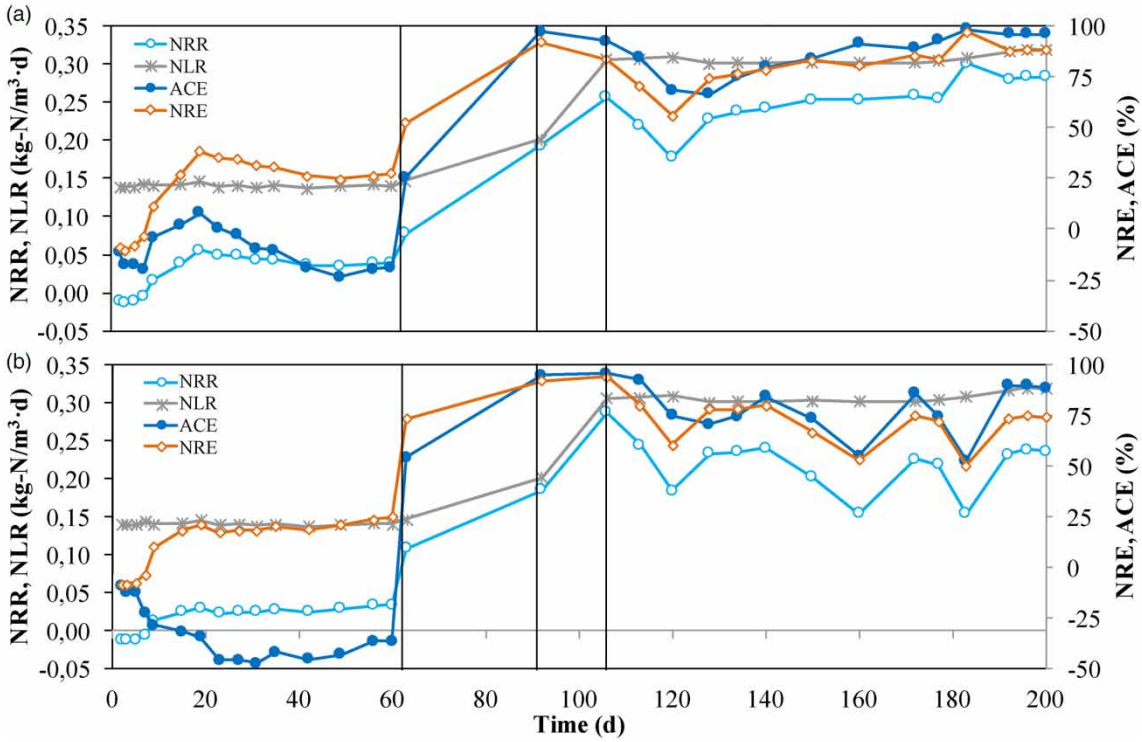


Figure 4 | Performance of nitrogen removal during the cultivation in Reactor 1 (a) and Reactor 2 (b).

previous reports, which reported a rapid reduction in nitrite concentration during the start-up period when using a mixture of aerobic activated sludge and nitrifying sludge (Wang *et al.* 2013) or sludge from an anaerobic digester of wastewater treatment plants (Chen *et al.* 2012). The rapid decline in nitrite concentration was likely associated with the activity of denitrifying bacteria, which potentially utilized organic compounds in the seeding sludge (Wang *et al.* 2013). The anammox activity appeared after 1 month of cultivation in the above experiment.

In contrast, the sluggish period continued over 2 months in this study. Since the lake was eutrophic, the sediment was estimated to contain a high concentration of biodegradable organic matter (COD 2,688 mg/L). In addition, the heterotrophic denitrification activity was likely high.

The maximum nitrogen removal performance during the first period in Reactor 1 based on ACE, NRE, and NRR values was 25.6%, 53.3%, and 0.085 kg-N/m^3·d, respectively. The results in Reactor 2 were higher than in Reactor 1, where ACE, NRE, and NRR were 54.4%, 74.9%, and 0.107 kg-N/m^3·d, respectively. Meanwhile, on day 63 (Period 2), the sludge was discharged from both reactors to reduce the effect of organic carbon, while the attached biofilm was preserved on the filter. On day 92 (Period 3), the ammonium and nitrite concentrations in the substrate were increased to 100 mg-N/L. Both effluent concentrations of ammonium and nitrite decreased significantly. Almost all nitrogen was removed from the reactor. In this third period, ACE, NRE, and NRR in Reactor 1 were 97.1%, 91.9%, and 0.19 kg-N/m^3·d, respectively, whereas in Reactor 2, they were 94.7%, 92.0%, and 0.16 kg-N/m^3·d, respectively.

The fourth period started on the 106th day when the concentration of ammonium and nitrite increased to 150 mg-N/L. At the beginning of this stage, nitrogen removal performance declined because of the shock loading; then, the ammonium and nitrite concentrations gradually decreased in Reactor 1. During this period, the maximum ACE, NRE, and NRR values in Reactor 1 were 98.2%, 93.0%, and 0.303 kg-N/m^3·d, respectively. In contrast, ammonium and nitrite concentrations were unstable in the effluent from Reactor 2. Meanwhile, the maximum values of ACE, NRE, and NRR were 88.6%, 68.9%, and 0.214 kg-N/m^3·d, respectively. This indicates that the reactor performances in Reactor 1 were better than those in Reactor 2 in this period (Figure 4). The results showed that temperature influenced the performance of nitrogen removal where sudden changes in temperature (temperature shock) may affect the activity and viability of indigenous microorganisms on Reactor 2. The water temperature in Lake Koto Baru at the sampling date (31 June 2020) was 25.8 °C, whereas the experimental temperature in Reactor 1 was 22–28 °C (23.9 °C on average). Enrichment temperature near the origin environment showed better performances.

3.3. The growth of anammox biofilm

In addition to nitrogen removal performance, biomass color change was observed in FtBRs, as shown in Figure 5. In the initial stage (0–10 days), the reactor contained a brownish-black inoculum. Significant changes occurred on day 106 in Reactor 1, where reddish-brown biofilm was formed. The biofilm development in the FtBR took longer than reported by Tsushima *et al.* (2007), who observed a color change by day 40. This discrepancy may be attributed to differences in the optimal conditions preferred by the inoculum, as the microbial communities utilized in this study were derived from a different geographical location and environmental condition compared with those employed in the referenced study. Indeed, previous studies showed even within the same species, different strains of anammox bacteria can have different genomic architecture and metabolic capacity (Bielski & Islam 2024; Wang *et al.* 2024).

Nonetheless, this indicates that the biomass in the FtBR exhibited the characteristics of anammox bacterial biomass, consistent with the reactor's anaerobic nitrogen removal performance. At the end of the experiment, the red carmine biofilm became thicker and covered the filter. This observation was similar to the study by Zhang *et al.* (2015), who reported that the enrichment of anammox bacteria was successful if the sludge color changed from brown to reddish and bright red. According to Ma *et al.* (2011), the red color is also typical of anammox bacteria enrichment. The biofilm attached to the filter and housing of FtBR. On the other hand, there was no difference in the biofilm's color of inoculum in Reactor 2, in which NRR was lower than in Reactor 1. The microbial community analysis below explains the above phenomenon.

3.4. Microbial community abundance

Biofilm was collected after 200 days for microbial community analysis using Illumina Miseq sequencing. The 27,493 sequences from FtBR operated at ambient tropical temperature (Reactor 1) and 29,732 sequences from FtBR operated at 35 °C (Reactor 2) were used for taxonomic analysis. The relative abundance reads of each operational taxonomic unit (OTU) was calculated from the total OTU reads in the sample. The results presented in Figure 6 showed the predominant

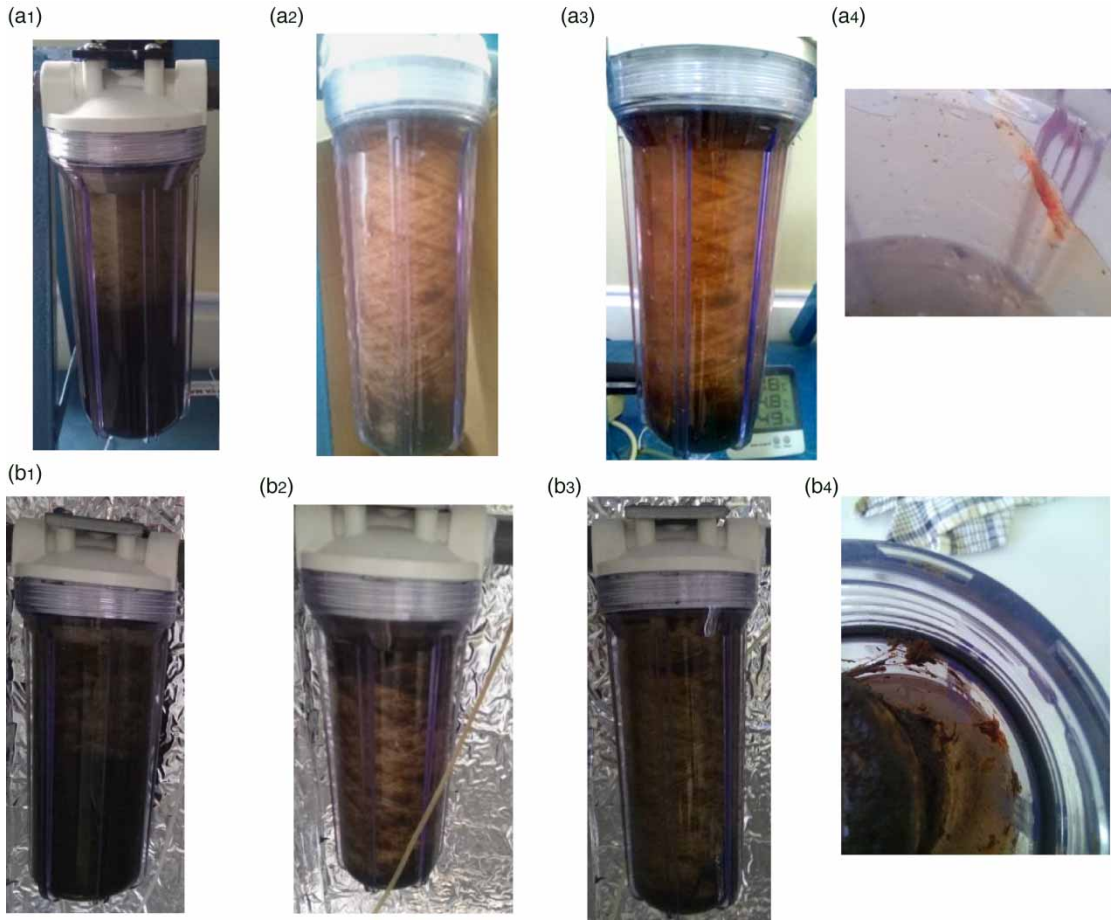


Figure 5 | The change of biomass's color in FtBR: (a1) Reactor 1 at day 0, (a2) Reactor 1 at day 106, (a3) Reactor 1 at day 180, (a4) biofilm on Reactor 1 wall; (b1) Reactor 2 at day 0, (b2) Reactor 2 at day 106, (b3) Reactor 2 at day 180, and (b4) biofilm on Reactor 2 wall.

phyla detected at levels of over 1%. In both experimental runs, the three most predominant phyla were Proteobacteria (31.6% in Reactor 1, 21.3% in Reactor 2), Planctomycetes (27.5% in Reactor 1, 18.5% in Reactor 2), and Bacteroidetes (24.1% in Reactor 1, 17.8% in Reactor 2). The proportions of Chloroflexi (6.49% in Reactor 1, 9.2% in Reactor 2), Ignavibacteriae (2.2% in Reactor 1, 8.1% in Reactor 2), and Acidobacteria (1.05% in Reactor 1, 14.5% in Reactor 2) were observed. Meanwhile, BRC1 (1.43%) and Verrucomicrobia (1.03%) were detected exclusively in Reactor 1 at relatively low abundance, whereas a minor presence of RBG-1 and Nitrospirae was observed in Reactor 2.

At the genus level, 412 genera were detected in Reactor 1 and 408 in Reactor 2. Figure 7 shows the relative abundance at the genius level of over 1%. There are big differences in the community among enrichment temperatures. In Reactor 1, the top three genera were *Candidatus Brocadia_1* (20.04%), *Saprospiraceae uncultured* (11.65%), and *Candidatus Brocadia_2* (6.20%). *Candidatus Brocadia* is a known anammox bacteria. It is reported that the Saprospiraceae family can hydrolyze and utilize complex carbon sources and play an essential role in the breakdown of complex organic compounds in the environment (Xia *et al.* 2008; McIlroy & Nielsen 2014).

The low abundance of the *Anaerolineaceae* family was observed, where these microorganisms live mutually with anammox bacteria by utilizing organic material from dead cells or organic material from seeding sludge (Kindaichi *et al.* 2012). In contrast, Reactor 2 had five genera with an abundance higher than 5%, which are *Mycobacterium* (14.24%), *PHOS-HE51* (11.00%), *Candidatus Anammoxoglobus* (7.64%), *Ignavibacterium* (7.08%), and *Candidatus Brocadia_1* (6.84%). Another anammox bacteria identified in FtBR operated at 35 °C (Reactor 2) was *Candidatus Brocadia_3* (1.77%). It was reported that *Ca. Anammoxoglobus* outcompeted in the presence of an organic substance such as propionate (Kartal *et al.* 2007) or sulfate (Liu *et al.* 2008) and operated in a controlled temperature of 33–35 °C. These results indicate that anammox

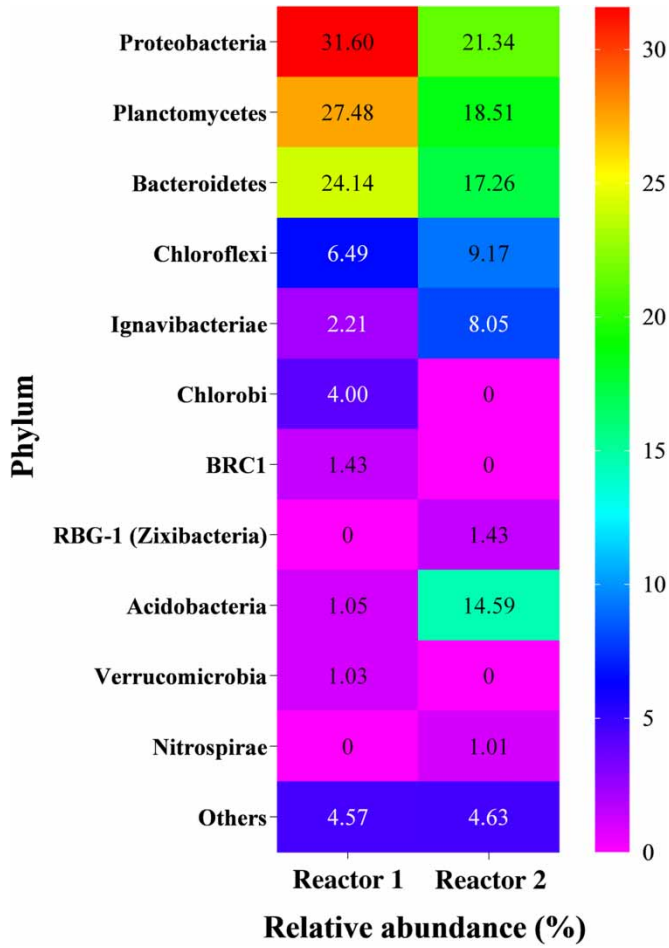


Figure 6 | Microbial community abundance at the phylum level. The total abundance lower than 1% classified as other.

bacteria were successfully enriched in tropical ambient temperature and 35 °C operation (both reactors), though the dominant anammox consortium was different.

3.5. Phylogenetic analysis

Figure 8 shows the phylogenetic analysis of anammox bacteria. Three kinds of OTU were clustered in *Ca. Brocadia* (OTU 15, 7191, and 14). The relative abundance of each OTU was 20.04, 6.20, and 0.01% in Reactor 1 and 6.84, 0.08, and 1.77% in Reactor 2 for OTU 15, 7191, and 14, respectively. Most abundant *Ca. Brocadia*, OTU 15 has 99% sequence similarity to *Ca. Brocadia fulgida*. Meanwhile, OTU7191 and OTU14 were related to *Ca. Brocadia caroliniensis* (98% sequence similarity) and *Ca. Brocadia sinica* (98% sequence similarity), respectively. In contrast, OTU33 has a 100% similarity with C*a. Anammoxoglobus propionicus*. Kartal *et al.* (2007) reported that *Ca. Anammoxoglobus propionicus* could outcompete other anammox bacteria and heterotrophic denitrifiers in the presence of propionate and ammonium when operated at 33 °C using an sequencing batch reactor (SBR) reactor. It is important to mention that high temperature in Reactor 2 might lead to the growth of *Ca. Anammoxoglobus propionicus*.

In this study, a high-rate anammox reaction was successfully established within 90 days, resulting in the enrichment of anammox bacteria by over 25% under tropical ambient temperatures, using the newly developed reactor and procedure. These findings suggest the potential applicability of the reactor for developing anammox-based wastewater treatment under tropical ambient conditions. Additionally, the dominant anammox bacterial species identified showed close phylogenetic relationships with *Ca. Brocadia fulgida*.

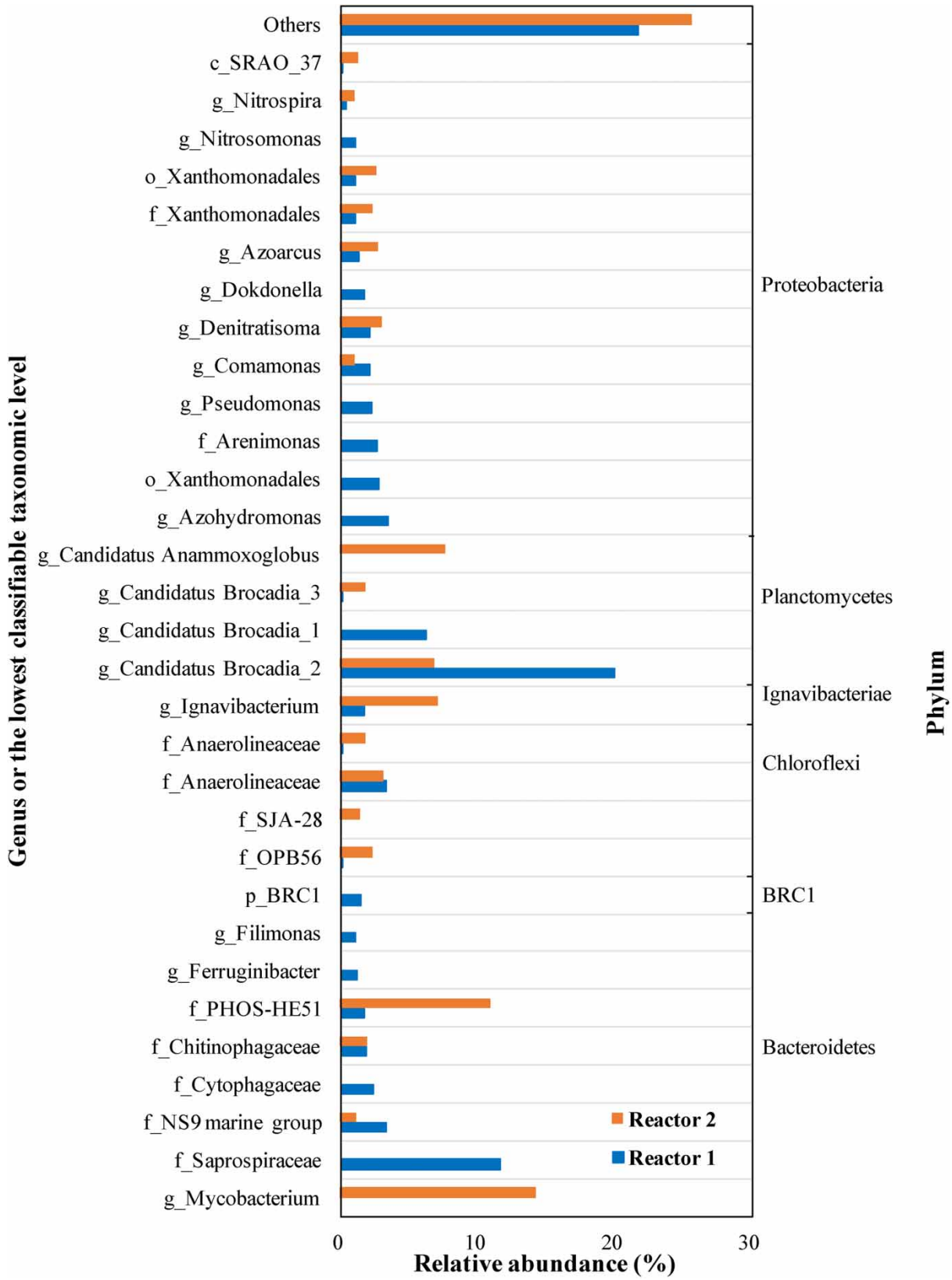


Figure 7 | Microbial community abundance at the genus or lowest classifiable level taken from Reactor 1 (blue) and Reactor 2 (orange). The relative abundance of bacteria below 1% is classified as others.

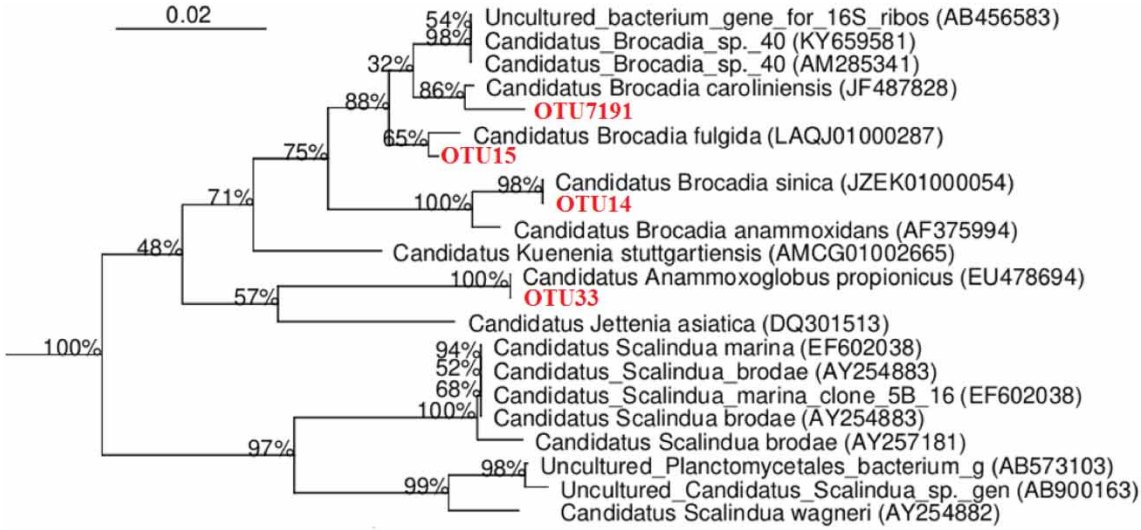


Figure 8 | Phylogenetic tree constructed by Neighbor-Joining (NJ) methods using ARB software based on 16S rRNA gene sequences of known anammox bacteria. The scale represents the number of nucleotide changes per sequence position. The nodes are supported by bootstrap values from 1,000 replicates.

4. DISCUSSION

4.1. The influence of temperature on the abundance and species variation of anammox

Different bioreactor treatments in this study involved variations in operational temperature: Reactor 1 operated at tropical ambient temperature (22–28 °C) and Reactor 2 at 35 °C. These differences resulted in the variation of relative abundance and the diversity of the dominant species of the growing anammox. This indicates that temperature is indeed a crucial factor in cultivating anammox bacteria. The optimal temperature for the activity of many anammox species ranges between 30 and 40 °C (Tomaszewski *et al.* 2017). Interestingly, this study revealed that the optimal temperature, indicated by the highest relative abundance of the anammox bacteria, falls within the tropical ambient temperatures, ranging from 22 to 28 °C.

Furthermore, the highest variation of anammox bacteria species diversity was observed at the operational temperature of 35 °C. Careful temperature control during the anammox process is essential, as temperatures exceeding 45 °C could permanently reduce anammox bacterial activity (Fernández *et al.* 2012). Yet, Vandekerckhove *et al.* (2020) reported the successful adaptation of thermophilic anammox bacteria by gradually increasing the temperature of a mesophilic inoculum to 50 °C. Additionally, it is well-established that the tropical temperature range is conducive to the anammox process for nitrogen removal, with suitable temperatures ranging from 25 to 38 °C (Trigo *et al.* 2006), 20–30 °C (Isaka *et al.* 2007), and approximately 20 °C (Ma *et al.* 2013). This substantiates that the anammox process can be operated at various temperatures; however, selecting the appropriate species of anammox bacteria is critical due to variations in anammox activity among different species.

The species *Ca. Brocadia sinica* had the smallest abundance within the biomass of Reactor 1. This is because *Ca. Brocadia sinica* cannot thrive well at tropical ambient temperatures, since the bacterial optimum growth is at 45 °C (Oshiki *et al.* 2011). However, at 35 °C, *Ca. Brocadia sinica* exhibits improved growth rates, as shown by an increase in abundance to 1.77%. Meanwhile, the identification of the species *Ca. Anammoxoglobus propionicus* can be considered rare among anammox researchers (Hsu *et al.* 2014). This species was initially discovered through laboratory-scale reactor studies involving the addition of the chemical compound propionate to the substrate (Kartal *et al.* 2007), although research conducted by Hsu *et al.* (2014) successfully obtained the species *Ca. Anammoxoglobus propionicus* without the addition of propionate. Interestingly, *Ca. Anammoxoglobus propionicus* was not found in Reactor 1, indicating that this species cannot survive and adapt to tropical ambient temperatures in tropical climates.

The species *Ca. Brocadia fulgida* emerges as the most dominant species in terms of abundance percentage in both reactors, accounting for 20.04% in Reactor 1 and 6.85% in Reactor 2. In Reactor 1 specifically, *Ca. Brocadia fulgida* was the most dominant species. This indicates that the tropical ambient temperature maintained in this study, within the range of

22–28 °C, falls within the optimum temperature range for the activity of this species. Yet, despite its non-dominant status in Reactor 2, *Ca. Brocadia fulgida* continues to grow together with the dominant species *Ca. Anammoxoglobus propionicus*, with a difference in abundance of <2%. The relative abundance difference of the *Ca. Brocadia fulgida* between Reactor 1 and Reactor 2 indicates that the temperature of 35 °C lies outside the optimum condition for this bacterium, possibly leading to its decreased activity. However, given that the temperature variance falls within approximately ±5 °C of the optimum range, this temperature remains conducive to the growth, activity, and competitive coexistence of *Ca. Brocadia fulgida* alongside other species of anammox bacteria.

Ca. Brocadia fulgida was first identified in a study on cultivating anammox bacteria with organic acetic acid (Kartal *et al.* 2008). One of the beneficial features of *Ca. Brocadia fulgida* is its ability to oxidize acetate, consequently establishing dominance over other anammox bacterial species within the reactor in the presence of acetate (Jenni *et al.* 2014). Additionally, *Ca. Brocadia fulgida* can effectively coexist with *Nitrosomonas* in forming biofilm thickness (Liu *et al.* 2017). Therefore, the opportunity to carry out a one-stage anammox process becomes more feasible with a combination of *Nitrosomonas* AOB (ammonia-oxidizing bacteria) and *Ca. Brocadia fulgida* as the anammox bacteria. Furthermore, the most beneficial advantage of the anammox process using *Ca. Brocadia fulgida* is a substantial reduction in nitrate production by 40–68% compared with other bacterial species (Winkler *et al.* 2012).

4.2. Eutrophic ecosystem as a potential anammox niche

Candidatus Brocadia and *Candidatus Kuenenia* are frequently found in wastewater treatment facilities (Kuenen 2008). Despite ongoing efforts, the precise ecological niche of anammox bacteria remains rudimentary, with elevated nitrogen concentrations serving as a principal indicator of their environmental presence. Ammonium serves as a primary substrate for anammox bacteria and other microorganisms; thus, AOB in the environment supplies nitrite for nitrogen removal. At the time of sampling, the water of Lake Koto Baru, which had experienced eutrophication, contains ammonium, nitrite, and nitrate. Remineralization processes, such as anaerobic degradation of organic matter from decomposing vegetation at the lake bottom, constitute a potential ammonium source for anammox, AOB, and ammonia-oxidizing archaea (AOA), functioning as crucial participants in the lake's nitrogen cycle. Additionally, ammonium inputs from residual fertilizers in the surrounding agricultural areas, dissolved and accumulated in the lake, further contribute to the nitrogen load. AOB-mediated ammonium oxidation at a depth of 1–1.5 m in the lake surface produces nitrites, which can subsequently be converted into nitrogen gas by anammox bacteria.

The discovery of anammox bacteria in tropical regions underscores their pivotal role in nitrogen removal processes. Anammox bacteria exhibit adaptability to diverse environmental conditions, with their presence being contingent upon the availability of ammonium and nitrite substrates. These findings serve as a valuable reference for investigating anammox bacteria across various ecosystems in tropical regions. Indigenous anammox bacteria from the local environment are expected to outperform exogenous strains in nitrogen removal, owing to the former's established adaptation to local environmental conditions, thereby ensuring enhanced operational efficiency. Agustina *et al.* (2017) reported high anammox-nitrogen removal, a maximum NRR of 1.05 kg-N/m^3·d, at tropical temperatures using a non-woven and UASB reactor with the KSU-1 strain of anammox bacteria from Osaka University. Similar results were reported by Zulkarnaini *et al.* (2020) with various carriers, including plastic and palm fiber (organic), using *Ca. Brocadia sinica* from Hiroshima University for low nitrogen concentration. However, the color of anammox biomass changed from red carmine to brown because it was operated below the optimum temperature. This could be an obstacle to future application of the anammox process due to the high cost of temperature control. Consequently, future implementations of the anammox process for nitrogen removal in wastewater treatment or environmental contexts can be globally applicable by utilizing enriched anammox bacteria sourced from the local area. This approach might offer cost efficiencies during the initiation phase of anammox reactors, as it obviates the need for transporting biomass from distant regions.

4.3. Implication for implementing anammox technology in a tropical country

This study, for the first time, attempted to cultivate native anammox bacteria from a tropical lake in Indonesia, through a novel FtBR. This research has the potential to advance future efforts in cultivating anammox bacteria in Indonesia and other tropical countries. The identification of four anammox bacteria (*Candidatus Brocadia caroliensis*, *Candidatus Anammoxoglobus propionicus*, *Candidatus Brocadia sinica*, and *Candidatus Brocadia fulgida*) provides a crucial perspective on the potential discovery of further genera and species within this bacterial group, capable of growing in tropical ambient

temperatures, prevalent in tropical climates. This research sheds light on the presence of anammox bacteria within tropical ecosystems and lays the groundwork for the development of comprehensive, large-scale anammox reactors. However, we acknowledge that there are several limitations in our study. First, multiple reactors are needed to confirm the reproducibility and repeatability of the results, providing a more robust conclusion for statistical analyses. Second, future research must aim for multiple sampling points to monitor the dynamic of anammox bacteria diversity and growth. This way we would be able to track the abundance of these bacteria across different operational stages.

Nevertheless, this research serves as the first step for more advanced application of anammox bacteria in the treatment of domestic and industrial wastewater. The effluents discharged from municipal wastewater treatment plants consistently fall short of meeting Indonesian effluent quality standards, resulting in widespread eutrophication issues in the country's aquatic environments. Eutrophication, predominantly leads to oxygen depletion, fish mortality, unpleasant odors, and toxicity concerns, exemplified by the eutrophication incident in Lake Koto Baru. While initiatives have been undertaken to leverage the anammox process to address these challenges (Wijaya & Soedjono 2018), the limited research on anammox in Indonesia poses a barrier to its implementation, despite the process being discovered over 29 years ago and having been successfully applied in full-scale reactors worldwide.

Therefore, there are significant opportunities to integrate anammox processes into wastewater treatment systems, aiming to mitigate eutrophication concerns in the tropical environment by utilizing indigenous anammox bacteria. Simultaneously, the development of large-scale anammox reactors could serve to supplement or replace existing processes, such as the wetland system employed in the fertilizer industry. This application holds promise in establishing a national standard for effluent quality, thereby addressing the eutrophication issue in Indonesia.

5. CONCLUSIONS

The successful cultivation of the anammox culture from tropical environmental sediment highlights the remarkable adaptability and resilience of these bacteria in such conditions. Temperature plays a crucial role in their activity, with optimal performance observed within the tropical ambient range of 22–28 °C, outperforming operations at 35 °C. The pioneering discovery of anammox bacteria from Indonesia, facilitated by a novel FtBR, paves the way for future anammox research in tropical regions. The identification of two distinct anammox species, *Ca. Brocadia fulgida* and *Ca. Brocadia caroliensis*, which were dominant in this temperature range, offers valuable insights for the potential development of large-scale anammox reactors under similar conditions. Despite challenges in meeting effluent standards, leveraging indigenous anammox bacteria presents a promising alternative approach for wastewater treatment and combating eutrophication in tropical water bodies. This finding not only advances the understanding of anammox processes in tropical climates but also underscores the potential for sustainable and efficient wastewater management in these regions.

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DECLARATION OF GENERATIVE AI AND AI-ASSISTED TECHNOLOGIES IN THE WRITING PROCESS

During the preparation of this work, the authors used ChatGPT in order to improve the language and readability. After using this tool/service, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

CONFLICT OF INTEREST

The authors declare there is no conflict.

REFERENCES

Agustina, T. E., Novia, N., Diansyah, G., Ike, M. & Soda, S. (2017) Nitrogen removal by anammox biofilm column reactor at moderately low temperature, *Indonesian Journal of Fundamental and Applied Chemistry*, **2**, 78–82. https://doi.org/10.24845/ijfac.v2.i4.78.

Ali, M. & Okabe, S. (2015) Anammox-based technologies for nitrogen removal: advances in process start-up and remaining issues, *Chemosphere*, **141**, 144–153. https://doi.org/10.1016/j.chemosphere.2015.06.094.

APHA (2017) *Standard Methods for the Examination of Water and Wastewater*, 23rd edn. Washington, DC, USA: American Public Health Association.

Apprill, A., Mcnally, S., Parsons, R. & Weber, L. (2015) Minor revision to V4 region SSU rRNA 806r gene primer greatly increases detection of SAR11 bacterioplankton, *Aquatic Microbial Ecology*, **75**, 129–137. https://doi.org/10.3354/ame01753.

Bielski, R. G. & Islam, M. A. (2024) Pangenome-scale mathematical modelling of ANAMMOX bacteria metabolism, *SynBio*, **2** (1), 70–84. https://doi.org/10.3390/synbio2010005.

Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., Fierer, N., Peña, A. G., Goodrich, J. K., Gordon, J. I., Huttley, G. A., Kelley, S. T., Knights, D., Koenig, J. E., Ley, R. E., Lozupone, C. A., Mcdonald, D., Muegge, B. D., Pirrung, M., Reeder, J., Sevinsky, J. R., Turnbaugh, P. J., Walters, W. A., Widmann, J., Yatsunenko, T., Zaneveld, J. & Knight, R. (2010) Correspondence QIIME allows analysis of high-throughput community sequencing data intensity normalization improves color calling in SOLiD sequencing, *Nature Publishing Group*, **7**, 335–336. https://doi.org/10.1038/nmeth0510-335.

Caporaso, J. G., Lauber, C. L., Walters, W. A., Berg-Lyons, D., Huntley, J., Fierer, N., Owens, S. M., Betley, J., Fraser, L., Bauer, M., Gormley, N., Gilbert, J. A., Smith, G. & Knight, R. (2012) Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms, *ISME Journal*, **6**, 1621–1624. https://doi.org/10.1038/ismej.2012.8.

Chen, C. j., Huang, X. x., Lei, C. x., Zhu, W. j., Chen, Y. x. & Wu, W. x. (2012) Improving anammox start-up with bamboo charcoal, *Chemosphere*, **89**, 1224–1229. https://doi.org/10.1016/j.chemosphere.2012.07.045.

Edgar, R. C. (2013) UPARSE: highly accurate OTU sequences from microbial amplicon reads, *Nature Methods*, **10**, 996–998. https://doi.org/10.1038/nmeth.2604.

Fernández, I., Dosta, J., Fajardo, C., Campos, J. L., Mosquera-Corral, A. & Méndez, R. (2012) Short- and long-term effects of ammonium and nitrite on the Anammox process, *Journal of Environmental Management*, **95**, S170–S174. https://doi.org/10.1016/j.jenvman.2010.10.044.

Fujii, T., Sugino, H., Rouse, J. D. & Furukawa, K. (2002) Characterization of the microbial community in an anaerobic ammonium-oxidizing biofilm cultured on a nonwoven biomass carrier, *Journal of Bioscience and Bioengineering*, **94**, 412–418. https://doi.org/10.1016/S1389-1723(02)80218-3.

Gantner, S., Andersson, A. F., Alonso-Sáez, L. & Bertilsson, S. (2011) Novel primers for 16S rRNA-based archaeal community analyses in environmental samples, *Journal of Microbiological Methods*, **84**, 12–18. https://doi.org/10.1016/j.mimet.2010.10.001.

Gumelar, G., Zulkarnaini, Z. & Zainuddin, E. (2024) Nitrogen removal performance in bioreactor filters using intensive shrimp pond solid waste and anammox bacterial inoculum, *Journal of Sustainable Science and Management*, **19**, 55–62. https://doi.org/10.46754/jssm.2024.01.005.

Hsu, S.-C., Lai, Y.-C., Hsieh, P.-H., Cheng, P.-J., Wong, S.-S. & Hung, C.-H. (2014) Successful enrichment of rarely found *Candidatus Anammoxoglobus propionicus* from leachate sludge, *Journal of Microbiology and Biotechnology*, **24**, 879–887. https://doi.org/10.4014/jmb.1401.01016.

Isaka, K., Sumino, T. & Tsuneda, S. (2007) High nitrogen removal performance at moderately low temperature utilizing anaerobic ammonium oxidation reactions, *Journal of Bioscience and Bioengineering*, **103**, 486–490. https://doi.org/10.1263/jbb.103.486.

Ismail, I. N., Taufik, M., Umor, N. A., Norulhuda, M. R., Zulkarnaini, Z. & Ismail, S. (2022) Anammox process for aquaculture wastewater treatment: operational condition, mechanism, and future prospective, *Water Science and Technology*, **86**, 3093–3112. https://doi.org/10.2166/wst.2022.403.

Izzati, M., Soeprobowati, T. R. & Prasetyo, S. (2022) Characterization of three selected macrophytes – an ecological engineering approach for effective rehabilitation of Rawapening Lake, *Journal of Ecological Engineering*, **23**, 277–287. https://doi.org/10.12911/22998993/152047.

Jenni, S., Vlaeminck, S. E., Morgenroth, E. & Udert, K. M. (2014) Successful application of nitritation/anammox to wastewater with elevated organic carbon to ammonia ratios, *Water Research*, **49**, 316–326. https://doi.org/10.1016/j.watres.2013.10.073.

Kartal, B., Rattray, J., van Niftrik, L. A., van de Vossenberg, J., Schmid, M. C., Webb, R. I., Schouten, S., Fuerst, J. A., Damsté, J. S., Jetten, M. S. M. & Strous, M. (2007) *Candidatus* '*Anammoxoglobus propionicus*' a new propionate oxidizing species of anaerobic ammonium oxidizing bacteria, *Systematic and Applied Microbiology*, **30**, 39–49. https://doi.org/10.1016/j.syapm.2006.03.004.

Kartal, B., Van Niftrik, L., Rattray, J., Van De Vossenberg, J. L. C. M., Schmid, M. C., Sinninghe Damsté, J., Jetten, M. S. M. & Strous, M. (2008) *Candidatus* '*Brocadia fulgida*': an autofluorescent anaerobic ammonium oxidizing bacterium, *FEMS Microbiology Ecology*, **63**, 46–55. https://doi.org/10.1111/j.1574-6941.2007.00408.x.

Kindaichi, T., Yuri, S., Ozaki, N. & Ohashi, A. (2012) Ecophysiological role and function of uncultured *Chloroflexi* in an anammox reactor, *Water Science and Technology*, **66**, 2556–2561. https://doi.org/10.2166/wst.2012.479.

Komala, P. S., Zulkarnaini, Z., Kurniati, R. I. & Fauzi, M. (2024) The effect of anthropogenic activities on the spatial distribution of total nitrogen and total phosphate in Lake Maninjau, *Journal of Water and Land Development*, **60**, 71–78. https://doi.org/10.24425/jwld.2024.149109.

Kuenen, J. G. (2008) Anammox bacteria: from discovery to application, *Nature Reviews Microbiology*, **6**, 320–326. https://doi.org/10.1038/nrmicro1857.

Lackner, S., Gilbert, E. M., Vlaeminck, S. E., Joss, A., Horn, H. & van Loosdrecht, M. C. M. (2014) Full-scale partial nitritation/anammox experiences – an application survey, *Water Research*, **55**, 292–303. https://doi.org/10.1016/j.watres.2014.02.032.

Liu, S., Yang, F., Gong, Z., Meng, F., Chen, H., Xue, Y. & Furukawa, K. (2008) Application of anaerobic ammonium-oxidizing consortium to achieve completely autotrophic ammonium and sulfate removal, *Bioresource Technology*, **99**, 6817–6825. https://doi.org/10.1016/j.biortech.2008.01.054.

Liu, T., Quan, X. & Li, D. (2017) Evaluations of biofilm thickness and dissolved oxygen on single stage anammox process in an up-flow biological aerated filter, *Biochemical Engineering Journal*, **119**, 20–26. https://doi.org/10.1016/j.bej.2016.12.006.

Ma, B., Zhang, S., Zhang, L., Yi, P., Wang, J., Wang, S. & Peng, Y. (2011) The feasibility of using a two-stage autotrophic nitrogen removal process to treat sewage, *Bioresource Technology*, **102**, 8331–8334. https://doi.org/10.1016/j.biortech.2011.06.017.

Ma, B., Peng, Y., Zhang, S., Wang, J., Gan, Y., Chang, J., Wang, S. S. S., Wang, S. S. S. & Zhu, G. (2013) Performance of anammox UASB reactor treating low strength wastewater under moderate and low temperatures, *Bioresource Technology*, **129**, 606–611. https://doi.org/10.1016/j.biortech.2012.11.025.

Mawarda, P. C., Le Roux, X., Acosta, M. U., van Elsas, J. D. & Salles, J. F. (2022) The impact of protozoa addition on the survivability of *Bacillus* inoculants and soil microbiome dynamics, *ISME Communications*, **2**, 1–9. https://doi.org/10.1038/s43705-022-00166-9.

McIlroy, S. J., Nielsen, P. H., (2014) The family Saprospiraceae. In: Rosenberg, E., DeLong, E. F., Lory, S., Stackebrandt, E. & Thompson, F. (eds.) *The Prokaryotes: Other Major Lineages of Bacteria and the Archaea*. Berlin, Heidelberg: Springer Berlin Heidelberg, pp. 863–889.

Miao, L., Yang, G., Tao, T. & Peng, Y. (2019) Recent advances in nitrogen removal from landfill leachate using biological treatments – a review, *Journal of Environmental Management*, **235**, 178–185. https://doi.org/10.1016/j.jenvman.2019.01.057.

Narita, Y., Zhang, L., Kimura, Z. i., Ali, M., Fujii, T. & Okabe, S. (2017) Enrichment and physiological characterization of an anaerobic ammonium-oxidizing bacterium '*Candidatus Brocadia sapporoensis*', *Systematic and Applied Microbiology*, **40**, 448–457. https://doi.org/10.1016/j.syapm.2017.07.004.

Nsenga Kumwimba, M., Lotti, T., Şenel, E., Li, X. & Suanon, F. (2020) Anammox-based processes: how far have we come and what work remains? A review by bibliometric analysis, *Chemosphere*, **238**, 1–21. https://doi.org/10.1016/j.chemosphere.2019.124627.

Oshiki, M., Shimokawa, M., Fujii, N., Satoh, H. & Okabe, S. (2011) Physiological characteristics of the anaerobic ammonium-oxidizing bacterium '*Candidatus Brocadia sinica*', *Microbiology (N Y)*, **157**, 1706–1713. https://doi.org/10.1099/mic.0.048595-0.

Putra, R. P., Zulkarnaini, Z. & Komala, P. S. (2020) Start-up proses anammox menggunakan lumpur telaga Koto Baru sebagai inokulum, *Jurnal Teknologi Lingkungan*, **21**, 9. https://doi.org/10.29122/jtl.v21i2.4155.

Ruthena, Y., Anggoro, S. & Soeprobowati, T. R. (2022) Phytoplankton community structure in Lutan Lake, Palangka Raya, Central Kalimantan, Indonesia, *AACL Bioflux*, **15**, 2052–2060.

Soeprobowati, T. R., Jumari, J., Saraswati, T. R., Suhry, H. C. & Gell, P. (2021) Land-use changes concerning the riparian vegetation in Galela Lake, North Maluku, Indonesia, *Ecological Engineering*, **170**, 106368. https://doi.org/10.1016/j.ecoleng.2021.106368.

Tomaszewski, M., Cema, G., Buczyńska, A. Z. & Ziembińska-Buczyńska, A. (2017) Influence of temperature and pH on the anammox process: a review and meta-analysis, *Chemosphere*, **182**, 203–214. https://doi.org/10.1016/j.chemosphere.2017.05.003.

Trigo, C., Campos, J. L., Garrido, J. M. & Méndez, R. (2006) Start-up of the anammox process in a membrane bioreactor, *Journal of Biotechnology*, **126**, 475–487. https://doi.org/10.1016/j.jbiotec.2006.05.008.

Tsushima, I., Ogasawara, Y., Kindaichi, T., Satoh, H. & Okabe, S. (2007) Development of high-rate anaerobic ammonium-oxidizing (anammox) biofilm reactors, *Water Research*, **41**, 1623–1634. https://doi.org/10.1016/j.watres.2007.01.050.

Vandekerckhove, T. G. L., Props, R., Carvajal-Arroyo, J. M., Boon, N. & Vlaeminck, S. E. (2020) Adaptation and characterization of thermophilic anammox in bioreactors, *Water Research*, **172**, 1–10. https://doi.org/10.1016/j.watres.2019.115462.

Van Der Star, W. R. L. L., Abma, W. R., Blommers, D., Mulder, J.-W. W., Tokutomi, T., Strous, M., Picioreanu, C., van Loosdrecht, M. C. M. M., Loosdrecht Van, M. C. M. & van Loosdrecht, M. C. M. M. (2007) Startup of reactors for anoxic ammonium oxidation: experiences from the first full-scale anammox reactor in Rotterdam, *Water Research*, **41**, 4149–4163. https://doi.org/10.1016/j.watres.2007.03.044.

van Kemenade, Z. R., Erdem, Z., Hopmans, E. C., Sinninghe Damsté, J. S. & Rush, D. (2024) Loss of nitrogen via anaerobic ammonium oxidation (anammox) in the California Current system during the late Quaternary, *Biogeosciences*, **21** (6), 1517–1532. https://doi.org/10.5194/bg-21-1517-2024.

Wang, T., Zhang, H., Yang, F., Li, Y. & Zhang, G. (2013) Start-up and long-term operation of the anammox process in a fixed bed reactor (FBR) filled with novel non-woven ring carriers, *Chemosphere*, **91**, 669–675. https://doi.org/10.1016/j.chemosphere.2013.01.026.

Wang, D., Meng, Y., Huang, L. N., Zhang, X. X., Luo, X. & Meng, F. (2024) A comprehensive catalog encompassing 1376 species-level genomes reveals the core community and functional diversity of anammox microbiota, *Water Research*, **266**, 122356. https://doi.org/10.1016/j.watres.2024.122356.

Wijaya, I. M. W. & Soedjono, E. S. (2018) Domestic wastewater in Indonesia: challenge in the future related to nitrogen content, *International Journal of GEOMATE*, **15**, 32–41. https://doi.org/10.21660/2018.47.06582.

Winkler, M. K. H., Yang, J., Kleerebezem, R., Plaza, E., Trela, J., Hultman, B. & van Loosdrecht, M. C. M. (2012) Nitrate reduction by organotrophic Anammox bacteria in a nitritation/anammox granular sludge and a moving bed biofilm reactor, *Bioresource Technology*, **114**, 217–223. https://doi.org/10.1016/j.biortech.2012.03.070.

Wu, M., Zhang, M., Shen, L., Wang, X., Ying, D., Lin, H., Li, R., Xu, Y. & Hong, H. (2023) High propensity of membrane fouling and the underlying mechanisms in a membrane bioreactor during occurrence of sludge bulking, *Water Research*, **229**, 119456. https://doi.org/10.1016/j.watres.2022.119456.

Xia, Y., Kong, Y., Thomsen, T. R. & Nielsen, P. H. (2008) Identification and ecophysiological characterization of epiphytic protein-hydrolyzing Saprospiraceae ('*Candidatus epiflobacter*' spp.) in activated sludge, *Applied and Environmental Microbiology*, **74**, 2229–2238. https://doi.org/10.1128/AEM.02502-07.

Yoshinaga, I., Amano, T., Yamagishi, T., Okada, K., Ueda, S., Sako, Y. & Suwa, Y. (2011) Distribution and diversity of anaerobic ammonium oxidation (anammox) bacteria in the sediment of a eutrophic freshwater lake, Lake Kitaura, Japan, *Microbes Environment*, **26**, 189–197. https://doi.org/10.1264/jsme2.ME10184.

Zhang, L., Liu, M., Zhang, S., Yang, Y. & Peng, Y. (2015) Integrated fixed-biofilm activated sludge reactor as a powerful tool to enrich anammox biofilm and granular sludge, *Chemosphere*, **140**, 114–118. https://doi.org/10.1016/j.chemosphere.2015.02.001.

Zhang, L., Narita, Y., Gao, L., Ali, M., Oshiki, M. & Okabe, S. (2017) Maximum specific growth rate of anammox bacteria revisited, *Water Research*, **116**, 296–303. https://doi.org/10.1016/j.watres.2017.03.027.

Zhu, G., Wang, S., Wang, W., Wang, Y., Zhou, L., Jiang, B., Op Den Camp, H. J. M., Risgaard-Petersen, N., Schwark, L., Peng, Y., Hefting, M. M., Jetten, M. S. M. & Yin, C. (2013) Hotspots of anaerobic ammonium oxidation at land-freshwater interfaces, *Nature Geoscience*, **6**, 103–107. https://doi.org/10.1038/ngeo1683.

Zulkarnaini, Z., Yujie, Q., Yamamoto-Ikemoto, R. & Matsuura, N. (2018) One-stage nitritation/anammox process using a biofilm reactor with two-inflow, *Journal of Water and Environment Technology*, **16**, 106–114. https://doi.org/10.2965/jwet.17-050.

Zulkarnaini, Z., Afrianita, R. & Putra, I. H. (2020) Application of anammox process in nitrogen removal using up-flow anaerobic sludge blanket reactor, *Jurnal Teknologi Lingkungan*, **21**, 31–39.

Zulkarnaini, Z., Komala, P. S. & Almi, A. (2021) Anammox biofilm process using sugarcane bagasse as an organic carrier, *Indonesian Journal of Biotechnology*, **26**, 25. https://doi.org/10.22146/IJBIOTECH.58554.

Zulkarnaini, Z., Matsuura, N., Kanazawa, S., Honda, R. & Yamamoto-Ikemoto, R. (2024) Optimizing start-up strategies for the two-inflow nitritation/anammox process: influence on biofilm microbial community composition, *Water Science & Technology*, **89**, 1583–1594. https://doi.org/10.2166/wst.2024.065.

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