Pamukkale Üniversitesi Mühendislik Bilimleri Dergisi
Pamukkale University Journal of Engineering Sciences

Changes in microbial structure in the anammox population at high-temperature

Yüksek sıcaklıkta anammox popülasyonunda mikrobiyal yapıdaki değişiklikler

Tuğba SARI[1*]

[1]Department of Bioengineering, Marmara University, Istanbul, Türkiye.
tgbasarii@gmail.com

Received/Geliş Tarihi: 27.08.2024 Revision/Düzeltme Tarihi: 24.01.2025 doi: 10.5505/pajes.2025.69449
Accepted/Kabul Tarihi: 15.02.2025 Research Article/Araştırma Makalesi

Abstract

Anammox bacteria are crucial in converting ammonia nitrogen into N_2 gas in anoxic conditions, offering sustainable alternatives to biological wastewater treatment. However, temperature fluctuations in wastewater treatment plants significantly impact the activity of microorganisms, including anammox bacteria. This underscores the importance of understanding the influence of temperature on microbial ecology. Although extensive research has been conducted on anammox activity across various temperatures, the effects of high temperatures on microbial diversity within anammox consortia remain largely unexplored. This study aims to investigate the effects of high temperatures on microbial performance and shifts in microbial diversity in anammox bioreactors. Two separate bioreactors were employed to validate the findings. After enriching anammox cultures under mesophilic conditions, the bioreactors were exposed to 65°C for 9 hours, followed by a recovery phase at 35°C. Thermal stress led to a sharp decline in nitrogen removal efficiency, with NH_4^+-N and NO_2^--N removal rates decreasing by up to 83.59% and 91.35%, respectively. Even after reducing influent concentrations, nitrogen removal performance remained impaired. Metagenomic analyses revealed substantial shifts in microbial composition at elevated temperatures. While phyla such as Chloroflexi and Bacteroidota diminished, Proteobacteria and Firmicutes proliferated. Notably, Candidatus Kuenenia (anammox genus) declined, indicating its sensitivity to thermal stress. These findings provide valuable insights into how high temperatures influence microbial diversity in anammox systems. Future research should focus on improving microbial resilience and stability to optimize anammox system performance in harsh environmental conditions.

Keywords: Anammox, Wastewater treatment, High temperature, Metagenomics, Microbial composition

Öz

Anammox bakterileri, anoksik koşullar altında amonyak azotunu N_2 gazına dönüştürmede kritik öneme sahiptir ve biyolojik atık su arıtımına sürdürülebilir alternatifler sunar. Ancak, atık su arıtma tesislerindeki sıcaklık dalgalanmaları, anammox bakterileri de dahil olmak üzere mikroorganizmaların aktivitelerini önemli ölçüde etkilemektedir. Bu durum, sıcaklığın mikrobiyal ekoloji üzerindeki etkisini anlamanın önemini ortaya koymaktadır. Çeşitli sıcaklıklarda anammox aktivitesi üzerine kapsamlı araştırmalar yürütülmüş olmasına rağmen, yüksek sıcaklıkların anammox konsorsiyumlarındaki mikrobiyal çeşitlilik üzerindeki etkileri büyük ölçüde keşfedilmemiştir. Bu çalışma, anammox biyoreaktörlerinde yüksek sıcaklıkların mikrobiyal performans üzerindeki etkilerini ve mikrobiyal çeşitlilikteki değişimleri araştırmayı amaçlamaktadır. Bulguları doğrulamak için iki ayrı biyoreaktör kullanılmıştır. Anammox kültürleri mezofilik koşullar altında zenginleştirildikten sonra, biyoreaktörler 9 saat boyunca 65 °C'ye maruz bırakılmış ve ardından 35 °C'de bir geri kazanım sürecine tabi tutulmuştur. Termal stres, azot giderim verimliliğinde keskin bir düşüşe neden olmuş; NH_4^+-N ve NO_2^--N giderim oranları sırasıyla %83,59 ve %91,35'e kadar azalmıştır. Giriş konsantrasyonlarının azaltılmasına rağmen, azot giderim performansı iyileşme göstermemiştir. Metagenomik analizler, yüksek sıcaklıklarda mikrobiyal bileşimde önemli değişiklikler olduğunu ortaya koymuştur. Chloroflexi ve Bacteroidota gibi filumlar azalırken, Proteobakteriler ve Firmicutes artmıştır. Özellikle, Candidatus Kuenenia (anammox türü) bir düşüş göstermiştir ve bu durum bu türün termal strese duyarlılığını ortaya koymaktadır. Bu bulgular, yüksek sıcaklıkların anammox sistemlerindeki mikrobiyal çeşitlilik üzerindeki etkilerine ilişkin değerli bilgiler sunmaktadır. Gelecekteki araştırmalar, zorlu çevre koşullarında anammox sistemlerinin performansını optimize etmek için mikrobiyal dayanıklılığı ve stabiliteyi artırmaya odaklanmalıdır.

Anahtar kelimeler: Anammox, Atık su arıtımı, Yüksek sıcaklık, Metagenomik, Mikrobiyal kompozisyon

1 Introduction

Anaerobic ammonium oxidation (anammox) is an important reaction in the global nitrogen cycle, where ammonia nitrogen (NH_4^+-N) is converted into N_2 gas in anoxic environments by anammox bacteria [1]. They have been identified as a significant contributor to nitrogen loss in various ecosystems, with reported contributions of 18-36% in groundwater, 4-37% in paddy soils, 9-13% in lakes, and 1-8% in estuaries [2]. Inspired by nature, anammox process has become a more sustainable and cost-effective alternative to traditional biological wastewater treatment methods [3]. Thus far, researchers have developed several anammox-based technologies, including Completely Autotrophic Nitrogen Removal Over Nitrite (CANON), a Single Reactor System for High-Rate Ammonium Removal Over Nitrite (SHARON)-Anammox, Partial Denitritation and Anammox (PD/A), and so on [4]. Over the past two decades, anammox-based processes have gained popularity due to their ability to reduce both initial investment costs and operational energy consumption in wastewater treatment plants [5]. These processes have been successfully applied to treat a wide range of wastewaters, including digested blackwater, sludge digester effluents, waste brine [4], and effluents from various industrial sectors such as

*Corresponding author/Yazışılan Yazar

photovoltaic manufacturing, pharmaceuticals, tanning, coking, monosodium glutamate production, and semiconductor and food processing [6],[7]. Many anammox-based plants are now in operation at different scales for the treatment of both sidestream [8]-[10] and mainstream wastewater [5],[11],[12]. This widespread adoption highlights the transformative potential of anammox technologies in modern wastewater management practices [13].

Temperature is one of the most critical environmental factors governing microbial life, significantly affecting both growth and metabolic rates [14]. In microbial ecosystems, temperature influences enzymatic activity, which regulates the transformation of substrates during biological reactions [15]. Optimal temperature varies across bacterial species, including anammox taxa, reflecting differences in their physiological characteristics [16]. Anammox bacteria exhibit a broad physiological temperature range between -5°C and 80°C, depending on their habitat [17]. While anammox bacteria existed in marine ecosystems typically thrive between 15°C and 25°C [18], those used in wastewater treatment generally perform optimally within the 30°C to 40°C range [19]. It has been reported that temperature also directly impacts key enzymatic functions in the central anammox metabolism, such as the activity of the hydrazine dehydrogenase enzyme, which is essential in electron transport [20]. At lower temperatures, nutrient transport is hindered, and cell membranes shift to a gel-like state. Consequently, microbial activity declines due to restricted nutrient flow [21]. Nevertheless, anammox bacteria demonstrate reversible inhibition at lower temperatures, with reports showing that adapted biomass maintains higher activity under these conditions compared to non-adapted biomass [22]. Conversely, higher temperatures, especially above 40°C, can cause cell lysis and cytochrome release, leading to a significant loss of anammox activity and potentially rendering the process ineffective [21]. For instance, temperatures reaching 45°C can cause biomass lysis, as evidenced by a color change in the liquid phase to orange owing to cytochrome release [23]. In a similar manner, Han [24] reported the complete loss of anammox activity above 40°C, while attempts to select for thermophilic anammox bacteria in activated sludge at temperatures of 55-60°C were unsuccessful [25]. Moreover, Isanta et al. [26] conducted a temperature shift from 35°C to 46°C, sustained over a period of 8 days, which led to a sharp decline in the nitrogen removal rate of the anammox bacteria. But interestingly, the anammox activity was recovered after the temperature was returned to its initial level (35°C), eventually reaching the pre-shock nitrogen removal levels [26]. In addition, understanding microbial community dynamics in extreme environments provides valuable insights into the resilience and adaptability of microorganisms under stress conditions. Recent studies have shown that microbial communities in extreme environments, such as deep-sea hydrothermal vents and polar regions, exhibit unique adaptations that enable them to survive and function under harsh conditions. For example, they have employed diverse strategies to cope with extreme temperatures, salinity, and pressure, including specialized membrane structures and efficient DNA repair mechanisms [27]. These adaptations are crucial for maintaining metabolic functions and community stability in fluctuating environments. Moreover, research indicates that microbial communities in extreme environments can undergo rapid evolutionary changes, allowing them to respond quickly to environmental stressors [28]. These progresses highlight the critical role of microbial diversity and adaptability in maintaining ecosystem functionality under extreme environmental conditions.

Apart from this, the operation of wastewater treatment plants is extensive and complex, necessitating adherence to specific discharge standards [29] and accommodating variable influent characteristics [30]. Temperature variations also pose significant challenges for wastewater treatment plants, as they directly influence the metabolic rates and community structures of microorganisms responsible for biological treatment processes [31]. Given the temperature sensitivity of microorganisms responsible for biological treatment [32], understanding the impact of temperature on microbial ecology is therefore critical. Recent studies have highlighted that temperature changes can alter the composition and activity of microbial communities, potentially leading to decreased treatment efficiency [33]. For instance, certain microbial populations may become dominant at specific temperatures, disrupting the ecological balance necessary for optimal wastewater treatment. Moreover, extreme temperatures can inhibit the growth of essential microorganisms or promote the proliferation of undesirable species, further complicating the treatment process [34]. These studies collectively emphasize the importance of understanding and managing temperature effects on microbial ecology to maintain effective performance of wastewater treatment plants.

Considering all, the microbial changes within anammox cultures exposed to high temperatures remain relatively unexplored despite extensive research on anammox activity at various temperatures [17],[21],[35]. To address this gap, the current study aims to investigate the effects of high temperatures on microbial diversity within an anammox bioreactor. After enriching anammox cultures under mesophilic conditions, the cultures were subjected to 65°C, and their recovery performance was monitored through nitrogen (ammonia and nitrite) removal profiles. Concurrently, changes in microbial structure were analyzed using 16S rRNA amplicon-based next-generation sequencing, providing insights into how thermal stress affects microbial diversity in anammox bioreactors. Overall, this study represents a critical step in understanding the resilience of the anammox community under extreme thermal stress, which has direct implications for optimizing wastewater treatment technologies.

2 Materials and Methods

2.1 Synthetic wastewater composition

Synthetic wastewater is commonly utilized in laboratory-scale experiments, including anammox, owing to its advantages such as reproducibility and the ability to maintain controlled experimental conditions [36]. In contrast, the inherent complexity of real wastewater can result in varying outcomes during experiments and may exhibit synergistic effects within biological systems [37]. Therefore, the synthetic wastewater was preferred to use in this study and its composition was specifically formulated to support the growth and activity of anammox bacteria in bioreactors. $(NH_4)_2SO_4$ and $NaNO_2$ were used to provide NH_4^+-N and nitrite nitrogen (NO_2^--N) as substrates, respectively. The ratio of NH_4^+-N to NO_2^--N was maintained between 1:10 and 1:15. In addition, the composition, as previously tabulated by Can et al. [38], included the following components: 0.174 g/L K_2HPO_4, 0.073 g/L $CaCl_2$, 0.102 g/L $MgCl_2$, 1 mL trace element solution (TES) #1 containing 10 g/L $Na_2EDTA{\cdot}2H_2O$ and 5 g/L $FeSO_4$, and 1 mL TES #2 containing 10 g/L $Na_2EDTA{\cdot}2H_2O$, 0.43 g/L $ZnSO_4{\cdot}7H_2O$, 0.24 g/L $CoCl_2{\cdot}6H_2O$, 0.99 g/L $MnCl_2{\cdot}4H_2O$, 0.25 g/L

$CuSO_4 \cdot 5H_2O$, 0.19 g/L $NiCl_2 \cdot 6H_2O$, and 0.014 g/L H_3BO_4. Additionally, 1.04 g/L $NaHCO_3$ was added to the fresh feed solutions to both supply inorganic carbon for the anammox bacteria and buffer the pH of the environment. When the reactors were first put into operation, 50 mg/L $NaNO_3$ was added to the bioreactor, and then, for the rest of the operation periods, $NaNO_3$ was decreased to 25 mg/L. To eliminate dissolved oxygen (DO) and maintain oxygen-free environment conditions within the bioreactors, the freshly prepared synthetic wastewater solutions were always purged with N_2 gas before being fed into the bioreactors. This careful preparation ensured that the synthetic wastewater provided a consistent and controlled environment for anammox bacteria in the present study.

2.2 Experimental setup and inoculation of parent bioreactor

A lab-scale and side-armed parent bioreactor (R1) with an effective volume of 2 L was established to enrich anammox culture. The bioreactor was inoculated with seed sludge taken from a plexiglass-based up-flow continuous bioreactor, which had been operating for more than 13 years in the Sustainable Environmental Technologies Laboratory at the Department of Environmental Engineering, Marmara University. The bioreactor was operated in sequencing batch bioreactor (SBR) mode with a total cycle duration of 24 hours, including filling (5 minutes), reaction (23 hours), settling (30 minutes), and effluent withdrawal (25 minutes). The mixed gas of N_2/CO_2 (95/5%) was also introduced to the bioreactor to ensure anoxic conditions and supply the inorganic carbon as well. The temperature was kept constant at 35 ± 0.5°C, while hydraulic retention time was set at 2 days throughout the operation. The reactor was fed daily with freshly prepared synthetic wastewater, and the enrichment process was continued for 300 days. Once a steady state and efficient operation (≥ 95% removal efficiency for NH_4^+-N and NO_2^--N) was achieved, secondary seed sludges were harvested from the parent bioreactor. They were then used to inoculate experimental bioreactors designed to be exposed to high temperatures.

2.3 Operation strategies for experimental bioreactors

Two separate lab-scale bioreactors with different working volumes were established using the seed sludge taken from R1: a 500-mL bioreactor (R2) and a 1-L bioreactor (R3). These bioreactors were designed to replicate the experiment, with only minor differences in the duration of their start-up periods and their working volumes. The operation strategies, including exposure setup to high temperatures, subsequent recovery periods, and the composition of the synthetic wastewater, were kept the same across R2 and R3 to ensure reliable comparisons. Both bioreactors were operated similarly to R1 during their start-up periods. During these periods of both bioreactors, the influent concentrations of NH_4^+-N and NO_2^--N were also gradually increased to acclimatize the anammox bacteria to their new environment. Following the start-up periods and prior to the application of thermal stress, both reactors were fed as described in Section 2.2. Initially, the nitrogen treatment performance of both bioreactors was monitored to verify the stability and continuation of microbial activity. Once the nitrogen removal performance in the reactors reached 59.60 ± 9.61% (~15 hours), the incubator temperature was increased from 35 ± 0.5°C to 65 ± 1°C. Subsequently, the enriched anammox cultures were exposed to the elevated temperature (65°C) for 9 hours, considering the total cycle duration. After the high-temperature exposure, the temperature was quickly reduced back to 35 ± 0.5°C, as the optimal temperature range is 30-40°C for the anammox bacteria [35], and both bioreactors were immediately fed with fresh synthetic wastewater. The bioreactors continued to operate for further 64 days under these conditions, allowing the observation of the recovery capability of the enriched anammox cultures after being exposed to high temperatures.

2.4 16S rRNA amplicon-based next-generation sequencing and microbial diversity analyses

At the end of the enrichment period for R1 (day 300) and the final days of the operation periods for R2 (day 120) and R3 (day 110), sludge samples were harvested from the bioreactors. The genomic DNA of the biomass samples was extracted using the FastDNA SPIN Kit (MP Biomedicals LLC, France), following the manufacturer's instructions. For microbial community analysis, 16S rRNA amplicon-based next-generation sequencing was performed using the Illumina NovaSeq platform. This process, along with the bioinformatics analyses (from raw data processing to the determination of operational taxonomic units (OTUs)), was carried out by BMLabosis in Ankara, Türkiye. Details of metagenomic analysis methods were previously outlined by Sari et al. [39]. Primer7 software was employed to calculate alpha diversity indices, while Past 4.09 was used to determine beta diversity and similarity indices.

2.5 Analytical and statistical analyses

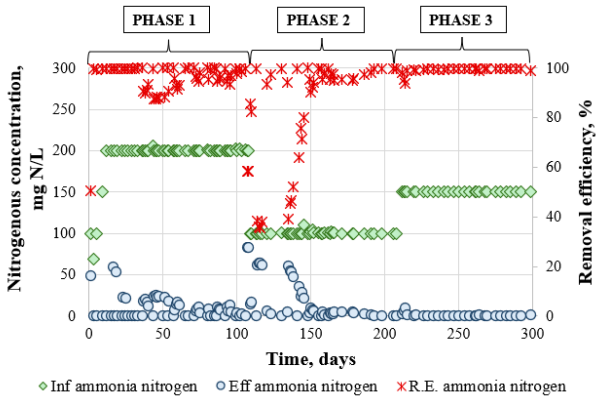
HQ40D digital portable multimeter kit (HACH, USA) were used to monitor DO and pH, while NO_2^--N, and NH_4^+-N were measured according to Standard Methods. Results are expressed as the mean ± standard deviation using Microsoft Excel. The Shapiro-Wilk test was applied to examine the normality of quantitative variables using GraphPad Prism (version 8.0.2). To compare the results obtained from different experimental groups, a paired t-test or Welch's t-test was conducted by Microsoft Excel according to sample size. In addition, Pearson's correlation was analyzed by Past4.09 based on the OTUs genus level. In all statistical analyses, the results were considered statistically significant when p was equal to < 0.05.

3 Results and discussion

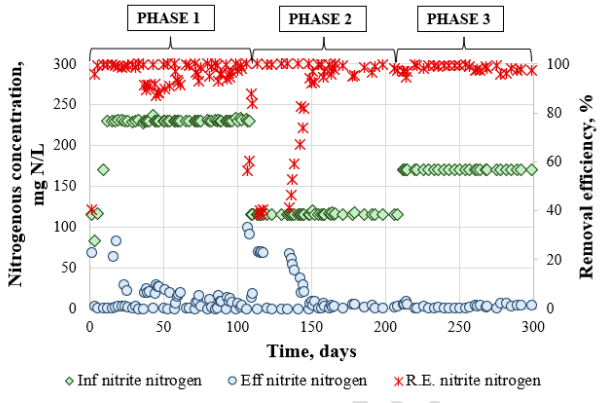
3.1 Enrichment period of anammox culture in parent bioreactor

The operation period of R1 was divided into three distinct phases called Phase I, Phase II, and Phase III based on the influent concentrations of NH_4^+-N and NO_2^--N, as illustrated in Figure 1. In Phase I (days 1-108), the initial substrate concentrations were adjusted to 100 mg/L for NH_4^+-N and 115 mg/L for NO_2^--N. As the nitrogen treatment capacity of the bioreactor was observed to be above 99%, the concentrations of NH_4^+-N:NO_2^--N were stepwise increased to 150 mg/L:170 mg/L, and subsequently to 200 mg/L:230 mg/L. Between days 12 and 106 in Phase I, the enriched anammox culture simultaneously treated NH_4^+-N and NO_2^--N with a removal efficiency of 96.56 ± 4.11% and 96.38 ± 3.89%, respectively (Figure 1a, 1b). Toward the end of the operation in Phase I, it was determined that the incubator did not distribute the hot air inside uniformly. Following this technical issue, the influent substrate concentrations were reduced to 100 mg/L for NH_4^+-N and 115 mg/L for NO_2^--N, named Phase II (days 109-211). In the subsequent period, the nitrogen removal performance dropped

significantly to 37.04 ± 1.18% and 39.52 ± 0.72% for NH_4^+-N and NO_2^--N, respectively. This reduced anammox performance persisted for approximately 50 days (days 107-148). Afterward, the nitrogen treatment capacity of the parent bioreactor began to recover, achieving ≥ 90% removal efficiency for both substrates by day 149. Nevertheless, this situation, caused by technical parameters, slowed down the enrichment process of the anammox bacteria. However, this also indicated that anammox bacteria exhibited a strong recovery capability after experiencing adverse and unexpected environments, despite the initial suppression of their activity [26],[40],[41].



a)



b)

Figure 1. Nitrogen treatment performance of the parent anammox bioreactor (R1). a) NH_4^+-N and b) NO_2^--N removal trends during the enrichment period. Inf: influent; Eff: effluent; R.E.: removal efficiency.

During the last 60 days of Phase II (days 155-211), the bioreactor operation stabilized with removal efficiencies of 97.76 ± 2.00% for NH_4^+-N and 97.91 ± 1.82% for NO_2^--N. As for Phase III (days 212-300), the substrate concentrations were increased again to 150 mg/L for NH_4^+-N and 170 mg/L for NO_2^--N. During the last phase, the parent bioreactor was stably operated with a removal efficiency of 99.22 ± 1.38% and 98.17 ± 1.37% for NH_4^+-N and NO_2^--N, respectively, until the end of the operation period (day 300). Influent and effluent pH were recorded at 7.81 ± 0.08 and 7.54 ± 0.17, respectively, throughout the entire operation period. During the experiments, the detailed monitoring and phase-wise adjustment of the substrate concentrations [41],[42] were critical in successfully enriching the anammox culture in R1 over the 300-day operation period.

3.2 Long-term operation of experimental bioreactors and temperature shock

To validate the experimental results regarding the effects of high temperatures on anammox performance and microbial community structure, two separate bioreactors, R2 and R3, were established. The total operation period lasted 120 days for R2 and 110 days for R3. Influent and effluent pH were measured as 7.89 ± 0.12 and 7.74 ± 0.23, respectively, in R2, while in R3, influent and effluent pH were determined to be 7.88 ± 0.12 and 7.56 ± 0.23, respectively. During the last five days of the start-up for R2, NH_4^+-N and NO_2^--N removal efficiencies were equal to 97.42 ± 2.44% and 98.42 ± 0.75%, respectively. As for R3, nitrogen treatment efficiencies corresponded to 93.45 ± 3.58% for NH_4^+-N and 98.96 ± 1.58% for NO_2^--N.

On days 56 for R2 and 46 for R3, both bioreactors were exposed to a high temperature of 65°C for 9 hours to assess the impact of thermal stress on the enriched anammox cultures. After the 9-hours exposure, the incubator temperature was reduced to the physiological range of anammox bacteria (35 ± 0.5°C). For the following 64 days, the nitrogen removal performance of both R2 and R3 was monitored, assessing the resilience of the anammox bacteria and their ability to maintain performance under physiological conditions following thermal stress. The exposure of the anammox cultures to 65°C significantly reduce their activity in both R2 and R3. This thermal stress led to a significant decrease in the treatment capacities for NH_4^+-N and NO_2^--N. The NH_4^+-N removal efficiency of R2 and R3 sharply decreased by 83.59% and 60.73%, respectively (Figure 2a, 3a). As for the NO_2^--N treatment capacity, it also declined by 89.69% and 91.35% for R2 and R3, respectively (Figure 2b and 3b). Compared to the 5-day pre- and post-temperature shock operations in both bioreactors, the decrease in nitrogen removal efficiency for NH_4^+-N and NO_2^--N was also statistically significant by a two-tailed paired t-test ($\alpha = 0.05$, $p = 2.3E\text{-}06 < 0.05$ for $\mu1 = \mu2$ for NH_4^+-N; $\alpha = 0.05$, $p = 6.1E\text{-}08 < 0.05$ for $\mu1 = \mu2$ for NO_2^--N).

In the following periods (days 57-120 for R2 and days 47-110 for R3), the influent concentrations of NH_4^+-N were reduced to 50 mg/L and NO_2^--N to 57.50 mg/L. Despite these changes, anammox performance remained suppressed, particularly with respect to NO_2^--N treatment in both reactors. This might be related to deterioration of not only anammox activity but also other genera responsible for nitrite oxidation, such as the Nitrospirota phylum, in the bioreactor. Due to the high temperature, the taxa from this phylum disappeared in both bioreactors, indicating their high temperature sensitivity. Although some nitrification bacteria have been reported to be successfully enriched at high temperatures (up to 65°C), their activity has been shown to decrease above 35°C [43]. In contrast, the NH_4^+-N removal performance in R2 showed a gradual recovery, reaching 86.80 ± 17.71% in the final week of the operation. This can be attributed to the growth of ammonia-oxidizing bacteria, primarily associated with Beta- and Gammaproteobacteria [44]. Although Betaproteobacteria were not detected, the abundances of Alpha- and Gammaproteobacteria were stimulated during the operation period, as detailed in 3.3.2 in detail. Additionally, Proteobacteria are generally known as aerobes; however, they can thrive in different environments with differing DO levels [45]. Therefore, the members of this phylum could be aerobic or anaerobic (strict or facultative) [46]. Considering the anoxic conditions in this study, it might be concluded that its facultative members were possibly present in anammox populations. In comparison

to NH_4^+-N treatment performance, the NO_2^--N removal efficiency continued to decline throughout the operation, indicating a more persistent inhibition in this pathway (Figure 2).

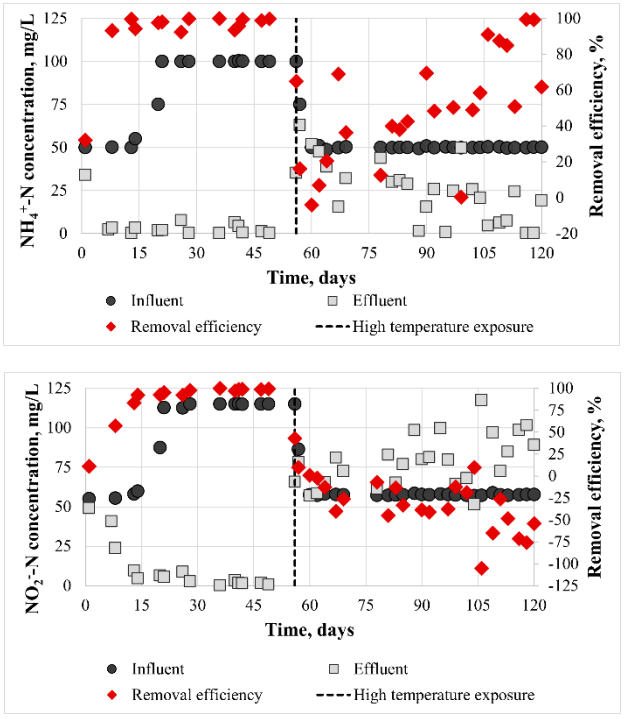


Figure 2. Nitrogen treatment performance of R2. a) NH_4^+-N and b) NO_2^--N removal trends throughout the operation.

Similar results were obtained for R3; however, the bioreactor exhibited a more stable performance in both NH_4^+-N and NO_2^--N removal compared to R2. Immediately after the post-exposure period (days 50-59), the NH_4^+-N treatment performance in R3 was determined to be -2.29 ± 2.54%. Afterward, NH_4^+-N removal capacity was slightly improved in the recovery period and reached 10.36 ± 10.16% between days 68 and 110, though microbial activity remained severely deteriorated. During this period in R3, the NO_2^--N removal efficiency declined to -10.10 ± 12.58% from 10.81 ± 13.51% on days 50-59 (Figure 3). Overall, results indicated that anammox bacteria were vulnerable at high temperatures, consistent with previous studies [21],[23]. Although the temperature was returned to its initial level (35°C) for a further 64 days, anammox activity could not be recovered in either R2 or R3 during this period (Figure 2, 3). The recovery period was also compared to the corresponding steady-state start-up period for each bioreactor, and the differences in nitrogen treatment performances were found to be statistically significant. A two-tailed Welch's t-test revealed the following results: $\alpha = 0.05$, p = 2.4E-07 < 0.05 for $\mu1 = \mu2$ for NH_4^+-N and p = 4.8E-17 < 0.05 for $\mu1 = \mu2$ for NO_2^--N in R2; $\alpha = 0.05$, p = 6.4E-24 < 0.05 for $\mu1 = \mu2$ for NH_4^+-N and p = 7.4E-21 < 0.05 for $\mu1 = \mu2$ for NO_2^--N in R3.

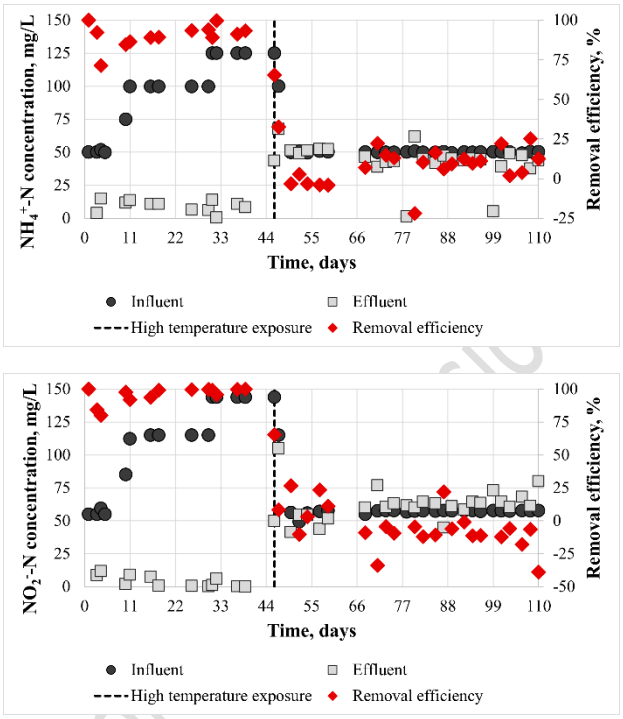


Figure 3. Nitrogen treatment performance of R3. a) NH_4^+-N and b) NO_2^--N removal trends throughout the operation.

3.3 Microbial structure changes in anammox consortia exposed to high temperatures

3.3.1 Alpha and beta diversity analyses

Good's coverage index was > 0.97% in each sample in this study, representing a high degree of sequencing analysis. In microbial ecology, alpha diversity analysis is a crucial step in understanding the complexity of microbial communities within different environments. This analysis provides insights into two key aspects of a community's structure, including richness and evenness. Richness refers to the number of different taxonomic groups, such as species, genera, or OTUs, present in a community, while evenness describes how uniformly individuals are distributed among the different taxonomic groups [47]. Alpha diversity metrics often combine richness and evenness to provide a holistic view of community structure. Common metrics include the Shannon Index, Simpson's Index, and Chao1, among others [48]. In the current study, compared to R1, exposure of the anammox culture to elevated temperatures led to an increase in the number of OTUs, suggesting an enrichment of the microbial community (Table 1). This is also consistent with the microbial community richness values. Specifically, Sobs, Chao1, and Chao2 indices showed an increase after high-temperature exposure in R2 and R3, indicating a rise in community richness (Table 1). Moreover, Shannon-Weaver and Simpson indices increased, representing a rise in microbial biodiversity in the bioreactors (Table 1).

Beta diversity indices are often employed to measure the overall biodiversity changes in microbial communities from one habitat to another [49]. Thus, their use is very useful for comparing different biomass samples across environmental parameters. To achieve this, several global beta diversity measures are generally conducted, such as Whittaker, Cody, Routledge, Wilson-Shmida, Mourelle, and others [50]. Among them, three indexes with the highest scores were selected for

this study: Whittaker, Cody, and Wilson-Shmida. Following the exposure of the anammox population to 65°C, these indices escalated (Table 2), indicating changes in microbial community composition within the experimental bioreactors. On the other hand, when comparing R2 and R3, beta diversity indices decreased (Table 2), suggesting that their compositions were more similar to each other than to R1. Beta similarity indices, derived from beta diversity indices, convey what percentage of biological diversity is similar or common among the habitats. In the present study, Dice (Sorensen) and Jaccard based on presence/absence data and Bray-Curtis and Horn based on abundance data similarity indices were conducted (Table 2). Results pointed out that similarity between samples decreased as a consequence of the temperature exposure, consistent with findings from the beta diversity indices.

3.3.2 Variations in the anammox populations

3.3.2.1 Microbial community variation at phylum level

The anammox culture is not limited to typical anammox bacteria. Instead, a diverse array of microorganisms coexists in the bioreactors, supporting the anammox process. These microorganisms can participate in complementary metabolic activities, such as degrading organic matter or producing intermediate compounds that anammox bacteria can utilize. Thus, they contribute to creating and maintaining a highly conducive environment for anaerobic ammonia oxidation [51]. The microbial community within the anammox bioreactors underwent notable shifts at the phylum level following exposure to high temperatures. These shifts also reflect the adaptive responses of communities to changing environmental conditions throughout the operation period. In the present study, Bacteroidota, Planctomycetota, and Chloroflexi are the major contributors to microbial composition in R1, accounting for 93% of the total population (Figure 4). Herein, anammox bacteria are affiliated with the Brocadiae class, a branch of Planctomycetota phylum [52].

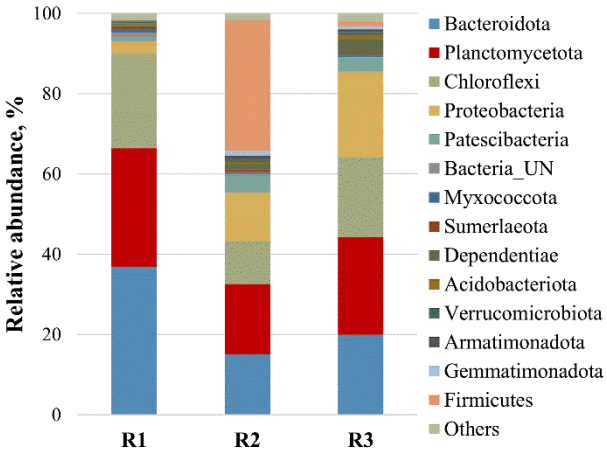


Figure 4. Microbial community structures at phylum level throughout the operation periods. The graph represents phyla with a relative abundance of at least 0.5% in any sample.

Table 1. Alpha diversity indices of biomass samples.

Sample	Reads	OTUs number	Gini Simpson's	Shannon	Sobs	Chao1	Chao2
R1	108969	118	0.874	2.594	137.33 ± 15.21	137.54 ± 0.59	137.33 ± 0.59
R2	46893	144	0.945	3.568	192.67 ± 4.03	192.79 ± 0.44	271.29 ± 19.42
R3	87220	150	0.929	3.371	230 ± 0.00	230.07 ± 0.30	346.15 ± 29.12

Table 2. Beta diversity and similarity indices of biomass samples.

Comparison between samples	Beta diversity			Beta similarity			
	Whittaker	Cody	Wilson-Shmida	Dice	Jaccard	Bray-Curtis	Horn
R1-R1	0	0	0	1	1	1	1
R1-R2	0.473	62	0.473	0.870	0.769	0.300	0.565
R1-R3	0.403	54	0.403	0.872	0.773	0.573	0.808
R2-R3	0.340	50	0.340	0.844	0.731	0.356	0.694

In anammox bioreactors, there is a syntrophic relationship between anammox bacteria and the Chloroflexi phylum that benefits from the organic matter provided by the anammox bacteria [53]. Moreover, the Chloroflexi phylum has been previously suggested to play a crucial role in facilitating sludge granulation, flocculation processes and biofilm formation due to their filamentous growth [54]. A recent study [55] also proposed that surface (S-) layer proteins detected in the extracellular polymeric substances (EPS) of anammox biofilms fuse with Chloroflexi network junctions to create a biofilm scaffold. Chloroflexi cells then perform glucogenesis and develop into a cross-linked network. Afterward, glucose is fermented into CO_2 to be fixed by anammox bacteria [55]. Some novel Chloroflexi species have also been identified as putative nitrite-oxidizing bacteria at high temperatures (~70 °C) [56]. Moreover, Bacteroidota species are known for their ability to degrade complex organic materials, including EPS. Similar to the Chloroflexi phylum, species belonging to the Bacteroidota phylum contribute to the anammox population by playing a crucial role as scavengers of EPS and in the degradation of cell debris [57]. In the present study, Pearson's analysis also demonstrated a strong and positive correlation between the phyla of Chloroflexi and Bacteroidota and the Planctomycetes phylum, based on their OTUs levels ($r = 0.999$, $p = 0.02 < 0.05$ for Chloroflexi; $r = 0.92$, $p = 0.26 > 0.05$ for Bacteroidota). Additionally, the high temperature decreased the relative quantities of both Chloroflexi and Bacteroidota. However, the Bacteroidota phylum was more vulnerable to high-temperature exposure. This is most likely due to the fact that lower temperatures are more favorable for the proliferation of Bacteroidota taxa [58] compared to Chloroflexi [59]. Proteobacteria is another phylum that is often detected in anammox bioreactors, as most denitrifiers in the microbial population are affiliated with the Proteobacteria phylum [60]. Its proportion in the microbial population was determined to be relatively small in R1 (3%); however, its relative abundance increased by at least 9.3% in R2 and R3 following the high-temperature exposure of anammox culture (Figure 4). A recent

study reported that Chloroflexi and Proteobacteria alternately play important roles in microbial networks [61]. Few studies have also reported that the relative content of Proteobacteria, Bacteroidetes, and Chloroflexi was distributed in the stable interval, indicating their irreplaceable role in anammox cultures [62]. A similar situation was also observed in this study. While the relative abundances of Chloroflexi and Bacteroidota decreased, the Proteobacteria displayed an opposite trend in the biomass samples (Figure 4). Especially in R1 and R3, their total distribution was close to each other (at 60%). In R2, the Firmicutes phylum was also involved in the core microbial consortium, being remarkably stimulated and reaching 32.4% (Figure 4). Some studies have indicated that the dominant phylum may change from Proteobacteria to Firmicutes with changing environmental conditions (e.g., organic content) [63]. The existence and dominance of both Proteobacteria and Firmicutes phyla in the microbial consortia have been previously verified in thermal regions between 52.3°C and 90.8°C [64], consistent with the current study. Taxa within the Firmicutes phylum are also significant contributors to N_2O consumption in microbial ecosystems, alongside classical denitrifiers typically found within the Proteobacteria phylum [65]. It has been suggested that Firmicutes species possess genes related to denitrification and ammonification genes. Therefore, these bacteria are well-equipped to thrive in nutrient-rich, anaerobic conditions by participating in nitrogen transformations that contribute to overall nitrogen removal in bioreactors [63].

In the present study, Patescibacteria and Dependentiae also represented temperature-adaptive phyla in the microbial population. Towards the end of the operation periods of R2 and R3, the relative abundances of Patescibacteria and Dependentiae were stimulated by 2.64 ± 0.25% and 1.94 ± 1.06%, respectively (Figure 4). To date, Patescibacteria has been identified in a variety of environments, including groundwater sediment, lakes, and activated sludge, as well as in anammox enrichment cultures that are supplied solely with ammonia as the energy source [66]. Although they do not participate directly in the nitrogen or sulfur cycles in microbial communities [67], Patescibacteria likely play significant ecological roles, such as producing short-chain fatty acids and degrading chitin-related compounds [66]. They likely depend on symbiotic relationships with other microorganisms in the reactor to obtain essential nutrients [68]. Therefore, in the current study, Patescibacteria might have adapted to the reactor environment by consuming organic compounds derived from other microorganisms, facilitating their growth or proliferation. As for Dependentiae, taxa in this phylum, recognized for its parasitic or symbiotic lifestyle, exhibits notable acid tolerance and contributes significantly to organic metabolism within microbial communities [69]. This metabolic role may have helped them adapt to the environment, even at high temperatures in this study. The relative quantities of the phyla Armatimonadota and Gemmatimonadota were slightly increased by at most 1.2% as a result of temperature exposure. Although they are not predominant phyla, they are frequently detected taxa in anammox consortia [70],[71].

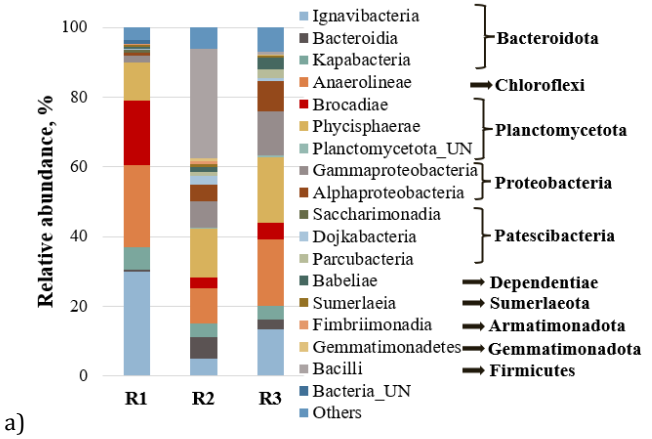
3.3.2.2 Microbial community variation in the Planctomycetota phylum

Figures 5a and 5b illustrate the predominant classes and genera in the anammox population, respectively. Within the phylum Planctomycetota, two primary classes have been identified in anammox systems: Phycisphaerae and Brocadiae (Figure 5a). Notably, all known anammox genera are affiliated with Brocadiae (Figure 5a). Since they have no pure cultures, all anammox taxa have been named "Candidatus" (Ca.) [72]. So far, six anammox genera have been explored: *Jettenia* [73], *Anammoxoglobus* [74], *Scalindua* [75], *Kuenenia* [76], *Brocadia* [77], and *Loosdrechtia aerotolerans* [78]. In addition, the presence of anammox bacteria has been documented in many different anoxic environments, including freshwater, slightly saline water, marine and terrestrial ecosystems, hydrothermal vents, hypersaline basins, sea ice, permafrost soils, and wastewater treatment systems [79]. Different anammox species may inhabit in different habitats. For instance, Ca. *Kuenenia* has been mainly observed in wastewater treatment systems, especially under high-strength conditions [60]. In this study, where synthetic wastewater was utilized, the only detected anammox genus was Ca. *Kuenenia* (Figure 5b). Exposure of the enriched anammox cultures to high temperatures caused a considerable reduction in the relative quantity of Ca. *Kuenenia* by 14.47 ± 0.82%, indicating the sensitivity of anammox bacteria. This could be also attributed to the severe deterioration in simultaneous nitrogenous treatment performance during the reactor operations (Figures 2, 3).

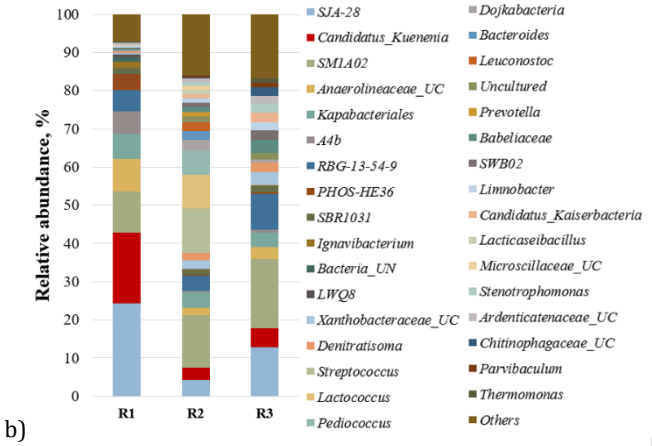
SM1A02 is a species belonging to the class Phycisphaerae within Planctomycetota. It is also the only species from this class identified in the present study (Figure 5b). The presence of *SM1A02* may play a critical role in maintaining system stability by protecting anammox species from environmental changes [80]. While the exact role and function of *SM1A02* remain unclear [81], its coexistence with anammox species has been documented in previous studies. Some research has suggested that *SM1A02* may contribute to denitrification [82] or exhibit nitrification properties [83], and there is also speculation about its potential anammox capabilities [84]. The previous studies also pointed out its great adaptation to extreme environmental conditions, with its abundances being remarkably stimulated [39],[85]. In the current study, its relative quantity also increased by at least 2.98%, exhibiting high tolerance to harsh environmental conditions (Figure 5b). However, a statistically significant correlation was not found between its abundance and the anammox reactions throughout the operation period by Pearson's analysis ($r = -0.73$, $p = 0.48 > 0.05$ for NH_4^+-N; $r = -0.27$, $p = 0.92 > 0.05$ for NO_2^--N), which is also consistent with the literature [39].

3.3.2.3 Microbial community variation at class and genus levels, except for anammox bacteria

Ignavibacteria, Bacteroidia, and Kapabacteria classes are the main contributors to the Bacteroidota phylum, while Anaerolineae form the major class of the Chloroflexi phylum. These classes, except for Bacteroidia, were vulnerable to high temperatures, especially the Ignavibacteria and Anaerolineae classes (Figure 5a). Among Bacteroidota species, those belonging to the Ignavibacteria group are facultative anaerobic heterotrophs [57]. In this study, *SJA-28*, *PHOS-HE36*, and *Ignavibacterium* were detected under the Ignavibacteria class (Figure 5b). Their presence has been previously reported in autotrophic nitrogen removal reactors and heterotrophic denitrification systems [41],[86]-[89]. *Ignavibacterium album* possesses nitrite reductase [90], a key enzyme in the dissimilatory reduction of nitrate to ammonium or nitrite [91]. Like *Ignavibacterium*, *SJA-28* has also been reported to be involved in the NO_3^--N reduction of NO_2^--N, helping to improve the nitrogen removal efficiency in the bioreactors [86].



a)



b)

Figure 5. Microbial community structures at class level (a) and genus level (b) throughout the operation periods. In the graphs, each taxonomic data represents at least 0.7% and 1% relative abundance in any sample at the class and genus level, respectively. UC: uncultured.

Due to the versatile metabolism of *Ignavibacterium*, it is considered mixotrophic [86], capable of directly assimilating carbon from acetate and indirectly from metabolites in the system, where EPS, other organic products, and HCO_3^- serve as carbon sources [90]. Additionally, *PHOS-HE36* likely has the same metabolic lifestyle using alternative carbon sources, similar to *Ignavibacterium* [92]. In the current study, in R1, *SJA-28* was the most dominant genus with a relative quantity of 24.27%, while *PHOS-HE36* had a 4.13% abundance in the community. The abundances of both genera decreased during the experiments due to the high-temperature exposure. Moreover, although *Ignavibacterium* had a relatively small abundance (1.54%) following the enrichment period of the bioreactors, it also showed susceptibility to high temperatures and almost disappeared from the microbial consortia (Figure 5b). The *Kapabacteriales* genus, which belongs to Kapabacteria, is another type of heterotrophic microorganism detected in autotrophic systems [93]. However, research on them is limited. A recent genomic analysis has indicated that *Kapabacteriales* metabolizes acetate via the glyoxylate pathway [94]. This genus was slightly and adversely affected by high temperatures. Throughout the operation periods, its abundance was detected to be 4.82 ± 1.23% (Figure 5b). As for the genera belonging to Bacteroidia, they have potential as a N_2O sink in the microbial population [70]. *Bacteroides*, *Prevotella*, and uncultured *Chitinophagaceae* were identified in the Bacteroidia class in this study with relatively low abundances but higher than other genera in Bacteroidia, and their quantity was stimulated by elevated temperatures (Figure 5b).

Moreover, Anaerolineae class consists of obligately anaerobic bacteria [57]. They metabolize various organic compounds, including sugars, producing short-chain fatty acids and hydrogen gas [95]. Additionally, Anaerolineae has been revealed to potentially play a crucial role as N_2O reducers in anammox systems, similar to Ignavibacteria [70]. In this study, the most identified taxa in the Anaerolineae class were *A4b*, *RBG-13-54-9*, *SBR1031*, and uncultured Anaerolineaceae (Figure 5b), together accounting for 21.74% in R1. Although *A4b* and uncultured Anaerolineaceae were sensitive to high temperatures, it seems that *RBG-13-54-9* and *SBR1031* adapted to changing environmental conditions, especially in R3 (Figure 5b). As these microorganisms need to utilize organic matter, the decayed biomass in the bioreactor might have facilitated their proliferation during the operation. Recent metagenomic studies have also provided deeper insights into the roles of Chloroflexi and Bacteroidota phyla in anammox bioreactors. Members of these phyla may participate in nitrogen removal in anammox bioreactors through interactions via NO_x^- in the community [54],[57],[96],[97]. In this study, a strong and positive correlation was also determined between NO_2^--N removal and the several predominant genera belonging to either the Chloroflexi or Bacteroidota phylum by Pearson's correlation (Table 3).

Table 3. Pearson's correlation between OTUs of predominant genera and NO_2^--N removal performance in bioreactors.

Phylum	Genus	*r* value
Bacteroidota	*SJA-28*	0.88
	Kapabacteriales	0.90
	PHOS-HE36	1.00*
Chloroflexi	*A4b*	1.00**
	Anaerolineaceae_UC	0.97
Planctomycetota	Ca. *Kuenenia*	0.99

*$p < 0.05$; **$p < 0.01$

Alphaproteobacteria and Gammaproteobacteria were the only identified classes of the Proteobacteria phylum, and their quantities were notably boosted in R2 and R3 (Figure 5a). In the microbial cultures exposed to high temperatures, the Alphaproteobacteria class mainly consisted of *SWB02*, *Parvibaculum*, and uncultured Xanthobacteraceae, whereas the Gammaproteobacteria class comprised the genera *Denitratisoma*, *Limnobacter*, *Stenotrophomonas*, and *Thermomonas*, which are denitrifiers in the community (Figure 5b). *Parvibaculum* is a genus known for its role in the degradation of complex and refractory organic compounds, making it a significant player in the remediation of contaminated environments. This genus is often detected in soil and wastewater treatment systems [98]. Denitrifiers (e.g. *Limnobacter*) also cooperate with other functional microorganisms in the microbial community. For instance, in anammox bioreactors, heterotrophic microorganisms utilize NO_3^--N and soluble microbial products produced by anammox bacteria as substrates for their metabolic processes [99]. Therefore, they may have supported the anammox community against harsh environmental conditions, as seen in the present study.

In addition to these, *LWQ8*, *Dojkabacteria*, and Candidatus *Kaiserbacteria* were detected in deep branches of

Patescibacteria, while *Babeliaceae* was the only genus identified within the Dependentiae phylum. Among them, *LWQ8* [100] and *Dojkabacteria* [101] have been reported to live in organic-rich environments. In the present study, *LWQ8* had a relatively small abundance (< 0.1%) throughout the operation, while the *Dojkabacteria* genus was stimulated by 1.42 ± 0.90% in R2 and R3 (Figure 5b). As for Candidatus *Kaiserbacteria*, its coexistence with ammonium-oxidizing bacteria, nitrite-oxidizing bacteria, and anaerobic methane-oxidizing bacteria has been previously reported [102],[103]. However, articles on Candidatus *Kaiserbacteria* are limited, and therefore, information on this genus is scarce. Although it has been reported as a nitrite-reducing bacteria [104], its function could not be analyzed in several studies [103]. The current study revealed that this genus appeared at high temperatures (in both R2 and R3), even though it was not detected in R1 (Figure 5b). *Streptococcus*, *Lactococcus*, *Pediococcus*, *Leuconostoc*, and *Lacticaseibacillus*, which are affiliated with the Firmicutes phylum, together accounted for 30.21% in R2 after the high-temperature exposure of the anammox bioreactor. Among them, *Lactococcus*, a lactic acid bacteria, was previously found in an anaerobic hydrolysis denitrification reactor [105].

Overall, the performance of anammox systems can be severely impacted by temperature fluctuations, particularly elevated temperatures that frequently occur in wastewater treatment plants. In high-temperature environments, the decrease in the relative abundance of certain genera, such as Ca. *Kuenenia* and *Ignavibacterium*, poses a risk of system inefficiency since they are vital for nitrogen removal processes. Consequently, future optimization strategies could focus on maintaining a balanced microbial community capable of withstanding thermal fluctuations, such as by introducing or selecting less temperature-sensitive anammox genera. Insights derived from this study may also pave the way for further research into the co-evolution and functional integration of anammox bacteria with other microbial populations under stress conditions. Understanding these interactions at a mechanistic level could inform the development of predictive models for system behavior, enabling more precise management strategies. As global wastewater treatment demands continue to rise, the ability to design adaptable and efficient anammox-based systems will be crucial for meeting environmental regulations while reducing energy and resource consumption. Furthermore, these findings could guide the development of better temperature monitoring and control systems for real-world applications, ensuring that microbial communities remain functional despite rising or fluctuating temperatures.

4 Conclusions

This study primarily investigated the impacts of high temperatures on anammox performance and microbial community structure in bioreactors. Exposure to 65°C for 9 hours significantly impaired nitrogen removal efficiency, with reductions of up to 83.59% for NH_4^+-N and 91.35% for NO_2^--N. Despite returning the reactor operation to optimal conditions (35°C), anammox activity did not recover in either bioreactor within the subsequent 64 days, indicating the high vulnerability of anammox bacteria to thermal stress. In addition, exposure to high temperatures of anammox consortia revealed significant shifts in microbial composition. Phyla such as Bacteroidota and Chloroflexi, responsible for organic matter degradation and biofilm formation, were adversely affected by elevated temperatures, with Bacteroidota being particularly sensitive. In contrast, Proteobacteria and Firmicutes increased in abundance, reflecting their resilience and potential role in supporting nitrogen transformations under thermal stress. Within the phylum Planctomycetota, two main classes were identified: Phycisphaerae and Brocadiae. The class Brocadiae represented by Ca. *Kuenenia*, the primary anammox genus identified, notably decreased by 14.47% due to thermal stress, highlighting their vulnerability. On the contrary, the class Phycisphaerae, *SM1A02* genus, showed increased resilience, potentially contributing to system stability. Overall, these findings illuminate the effects of high-temperature stress on microbial diversity in anammox bioreactors. Future research should focus on strategies tp enhance microbial resilience and stability to optimize the performance of anammox systems under harsh environmental conditions.

5 Acknowledgment

The author expresses her gratitude to Marmara University, Molecular Ecology Laboratory, and Sustainable Environmental Technologies Laboratory for the infrastructure to perform the experiments.

6 Author contribution statement

In this manuscript, xxxx contributed conceptualization, methodology, experimental study, data collection, results analysis, literature investigation, and original draft writing.

7 Ethics committee approval and conflict of interest statement

"There is no need to obtain an ethics committee approval for this manuscript".

"There is no conflict of interest with any person/institution in the manuscript".

8 References

[1] [1] Jetten MSM, Strous M, van de Pas-Schoonen KT, Schalk J, van Dongen UGJM, van de Graaf AA, Logemann S, Muyzer G, van Loosdrecht MCM, Kuenen JG. "The anaerobic oxidation of ammonium". *FEMS Microbiology Reviews*, 22(5), 421-437, 1998.

[2] [2] Sonthiphand P, Hall MW, Neufeld JD. "Biogeography of anaerobic ammonia-oxidizing (anammox) bacteria". *Frontiers in Microbiology*, 5, 2014.

[3] [3] Wang P, Lu B, Liu X, Chai X. "Accelerating the granulation of anammox sludge in wastewater treatment with the drive of "micro-nuclei": A review". *Science of the Total Environment*, 860, 160238, 2023.

[4] [4] Adams M, Issaka E, Chen C. "Anammox-based technologies: A review of recent advances, mechanism, and bottlenecks". *Journal of Environmental Sciences*, 148, 151-173, 2025.

[5] [5] Trinh HP, Lee S-H, Jeong G, Yoon H, Park H-D. "Recent developments of the mainstream anammox processes: Challenges and opportunities". *Journal of Environmental Chemical Engineering*, 9(4), 105583, 2021.

[6] [6] Ren Z-Q, Wang H, Zhang L-G, Du X-N, Huang B-C, Jin R-C. "A review of anammox-based nitrogen removal technology: From microbial diversity to engineering applications". *Bioresource Technology*, 363, 127896, 2022.

[7] [7] Wu P, Chen J, Garlapati VK, Zhang X, Wani Victor Jenario F, Li X, Liu W, Chen C, Aminabhavi TM, Zhang X. "Novel insights into anammox-based processes: A critical review". *Chemical Engineering Journal*, 444, 136534, 2022.
[8] [8] Wang Q, Tan G-YA, Azari M, Huang X, Denecke M, Men Y, Jung J-Y, Okabe S, Ali M, Huang Y-T, Wu Z, Lo W-h, Gu J-D, Lin J-G, Lee P-H. "Insights into the roles of anammox bacteria in post-treatment of anaerobically-treated sewage". *Critical Reviews in Environmental Science and Technology*, 48(6), 655-684, 2018.
[9] [9] Lackner S, Gilbert EM, Vlaeminck SE, Joss A, Horn H, van Loosdrecht MCM. "Full-scale partial nitritation/anammox experiences – an application survey". *Water Research*, 55, 292-303, 2014.
[10] [10] Zuo F, Yue W, Gui S, Sui Q, Wei Y. "Resilience of anammox application from sidestream to mainstream: A combined system coupling denitrification, partial nitritation and partial denitrification with anammox". *Bioresource Technology*, 374, 128783, 2023.
[11] [11] Huang X, Mi W, Chan YH, Singh S, Zhuang H, Leu S-Y, Li X-z, Li X, Lee P-H. "C-n-s synergy in a pilot-scale mainstream anammox fluidized-bed membrane bioreactor for treating chemically enhanced primary treatment saline sewage". *Water Research*, 229, 119475, 2023.
[12] [12] Cao S, Koch K, Du R, Wells GF, Ye L, Drewes JE. "'Toward mainstream anammox by integrating sidestream treatment". *Environmental Science & Technology*, 56(15), 10553-10556, 2022.
[13] [13] Shaw DR, Tobon Gonzalez J, Bibiano Guadarrama C, Saikaly PE. "Emerging biotechnological applications of anaerobic ammonium oxidation". *Trends in Biotechnology*, 42(9), 1128-1143, 2024.
[14] [14] Gonzalez JM, Aranda B. "Microbial growth under limiting conditions-future perspectives". *Microorganisms*, 11(7), 2023.
[15] [15] Byrne N, Strous M, Crépeau V, Kartal B, Birrien J-L, Schmid M, Lesongeur F, Schouten S, Jaeschke A, Jetten M, Prieur D, Godfroy A. "Presence and activity of anaerobic ammonium-oxidizing bacteria at deep-sea hydrothermal vents". *The ISME Journal*, 3(1), 117-123, 2008.
[16] [16] Kouba V, Bachmannová C, Podzimek T, Lipovová P, van Loosdrecht MCM. "Physiology of anammox adaptation to low temperatures and promising biomarkers: A review". *Bioresource Technology*, 349, 126847, 2022.
[17] [17] Tomaszewski M, Cema G, Ziembinska-Buczynska A. "Influence of temperature and ph on the anammox process: A review and meta-analysis". *Chemosphere*, 182, 203-214, 2017.
[18] [18] Zhang L, Okabe S. "Ecological niche differentiation among anammox bacteria". *Water Research*, 171, 115468, 2020.
[19] [19] Strous M, Kuenen JG, Jetten MSM. "Key physiology of anaerobic ammonium oxidation". *Applied and Environmental Microbiology*, 65(7), 3248-3250, 1999.
[20] [20] Le L-T, Jeon J, Dang B-T, Bui X-T, Jahng D. "Influence of temperature on anammox reaction and microbial diversity in a bio-carriers reactor under mainstream conditions". *Environmental Technology & Innovation*, 25, 102178, 2022.
[21] [21] Chen X, Liu L, Bi Y, Meng F, Wang D, Qiu C, Yu J, Wang S. "A review of anammox metabolic response to environmental factors: Characteristics and mechanisms". *Environmental Research*, 223, 115464, 2023.
[22] [22] Lotti T, Kleerebezem R, van Loosdrecht MCM. "Effect of temperature change on anammox activity". *Biotechnology and Bioengineering*, 112(1), 98-103, 2015.
[23] [23] Dosta J, Fernández I, Vázquez-Padín JR, Mosquera-Corral A, Campos JL, Mata-Álvarez J, Méndez R. "Short- and long-term effects of temperature on the anammox process". *Journal of Hazardous Materials*, 154(1), 688-693, 2008.
[24] [24] Han YM. "Study on temperature influence on nitrogen removal properties in anammox". *Advanced Materials Research*, 955-959, 484-487, 2014.
[25] [25] Toh SK, Webb RI, Ashbolt NJ. "Enrichment of autotrophic anaerobic ammonium-oxidizing consortia from various wastewaters". *Microbial Ecology*, 43(1), 154-167, 2002.
[26] [26] Isanta E, Bezerra T, Fernández I, Suárez-Ojeda ME, Pérez J, Carrera J. "Microbial community shifts on an anammox reactor after a temperature shock using 454-pyrosequencing analysis". *Bioresource Technology*, 181, 207-213, 2015.
[27] [27] Shu W-S, Huang L-N. "Microbial diversity in extreme environments". *Nature Reviews Microbiology*, 20(4), 219-235, 2022.
[28] [28] Li S-J, Hua Z-S, Huang L-N, Li J, Shi S-H, Chen L-X, Kuang J-L, Liu J, Hu M, Shu W-S. "Microbial communities evolve faster in extreme environments". *Scientific Reports*, 4(1), 6205, 2014.
[29] [29] Güner ED. "Environmental risk assessment for biological wastewater treatment plant". *Pamukkale Universitesi Muhendislik Bilimleri Dergisi*, 24(3), 476-480, 2018.
[30] [30] Newhart KB, Holloway RW, Hering AS, Cath TY. "Data-driven performance analyses of wastewater treatment plants: A review". *Water Research*, 157, 498-513, 2019.
[31] [31] Alisawi HAO. "Performance of wastewater treatment during variable temperature". *Applied Water Science*, 10(4), 89, 2020.
[32] [32] Gude VG. "Energy and water autarky of wastewater treatment and power generation systems". *Renewable and Sustainable Energy Reviews*, 45, 52-68, 2015.
[33] [33] Cui D, Li A, Zhang S, Pang C, Yang J, Guo J, Ma F, Wang J, Ren N. "Microbial community analysis of three municipal wastewater treatment plants in winter and spring using culture-dependent and culture-independent methods". *World Journal of Microbiology and Biotechnology*, 28(6), 2341-2353, 2012.
[34] [34] Ai C, Yan Z, Zhou H, Hou S, Chai L, Qiu G, Zeng W. "Metagenomic insights into the effects of seasonal temperature variation on the activities of activated sludge". *Microorganisms*, 7(12), 713, 2019.
[35] [35] Sobotka D, Zhai J, Makinia J. "Generalized temperature dependence model for anammox process kinetics". *Science of the Total Environment*, 775, 145760, 2021.
[36] [36] O'Flaherty E, Gray NF. "A comparative analysis of the characteristics of a range of real and synthetic wastewaters". *Environmental Science and Pollution Research*, 20(12), 8813-8830, 2013.
[37] [37] Madeira CL, de Araújo JC. "Inhibition of anammox activity by municipal and industrial wastewater pollutants: A review". *Science of the Total Environment*, 799, 149449, 2021.
[38] [38] Can S, Sari T, Akgul D. "Recovery profile of anaerobic ammonium oxidation (anammox) bacteria inhibited by

zno nanoparticles". *Water Science and Technology*, 85(1), 342-353, 2021.
[39] [39] Sari T, Akgul D, Mertoglu B. "Long-term response of anammox process to hydrazine under different exposure strategies". *Journal of Environmental Chemical Engineering*, 12(5), 113600, 2024.
[40] [40] Kaewyai J, Noophan P, Wantawin C, Munakata-Marr J. "Recovery of enriched anammox biofilm cultures after storage at cold and room temperatures for 164 days". *International Biodeterioration & Biodegradation*, 137, 1-7, 2019.
[41] [41] Wan K, Yu Y, Hu J, Liu X, Deng X, Yu J, Chi R, Xiao C. "Recovery of anammox process performance after substrate inhibition: Reactor performance, sludge morphology, and microbial community". *Bioresource Technology*, 357, 127351, 2022.
[42] [42] Chen X, Liu L, Bi Y, Meng F, Wang D, Qiu C, Yu J, Wang S. "The recovery of a sequencing biofilm batch reactor—anammox system: Performance, metabolic characteristics, and microbial community analysis". *Sustainability*, 15(13), 10454, 2023.
[43] [43] Mehrani M-J, Sobotka D, Kowal P, Ciesielski S, Makinia J. "The occurrence and role of nitrospira in nitrogen removal systems". *Bioresource Technology*, 303, 122936, 2020.
[44] [44] Braker G, Conrad R. *Chapter 2 - diversity, structure, and size of n2o-producing microbial communities in soils—what matters for their functioning?* Editors: Laskin AI, Sariaslani S, Gadd GM. Advances in applied microbiology, 33-70, Academic Press, 2011.
[45] [45] Cevallos MA, Degli Esposti M. "New alphaproteobacteria thrive in the depths of the ocean with oxygen gradient". *Microorganisms*, 10(2), 2022.
[46] [46] Kersters K, De Vos P, Gillis M, Swings J, Vandamme P, Stackebrandt E. *Introduction to the proteobacteria*. Editors: Dworkin M, Falkow S, Rosenberg E, Schleifer K-H, Stackebrandt E. The prokaryotes: Volume 5: Proteobacteria: Alpha and beta subclasses, 3-37, New York, Springer New York, 2006.
[47] [47] Willis AD. "Rarefaction, alpha diversity, and statistics". *Frontiers in Microbiology*, 10, 2019.
[48] [48] Feranchuk S, Belkova N, Potapova U, Kuzmin D, Belikov S. "Evaluating the use of diversity indices to distinguish between microbial communities with different traits". *Research in Microbiology*, 169(4), 254-261, 2018.
[49] [49] Diserud OH, Odegaard F. "A multiple-site similarity measure". *Biology Letters*, 3(1), 20-22, 2007.
[50] [50] Gülsoy S, Özkan K. "Tür çeşitliliğinin ekolojik açıdan önemi ve kullanılan bazı indisler". *Turkish Journal of Forestry*, 9(1), 168-178, 2009.
[51] [51] Stegenta-Dąbrowska S, Sobieraj K, Rosik J, Sidełko R, Valentin M, Białowiec A. "The development of anammox and chloroflexi bacteria during the composting of sewage sludge". *Sustainability*, 14(16), 10248, 2022.
[52] [52] Jetten MS, Op den Camp HJ, Kuenen JG, Strous M. ""Candidatus brocadiaceae" fam. Nov". *Bergey's manual of systematics of Archaea and Bacteria*, 1-10, 2015.
[53] [53] Kindaichi T, Yuri S, Ozaki N, Ohashi A. "Ecophysiological role and function of uncultured chloroflexi in an anammox reactor". *Water Science and Technology*, 66(12), 2556-2561, 2012.
[54] [54] Bovio-Winkler P, Guerrero LD, Erijman L, Oyarzúa P, Suárez-Ojeda ME, Cabezas A, Etchebehere C. "Genome-centric metagenomic insights into the role of chloroflexi in anammox, activated sludge and methanogenic reactors". *BMC Microbiology*, 23(1), 45, 2023.
[55] [55] Wong LL, Lu Y, Ho JCS, Mugunthan S, Law Y, Conway P, Kjelleberg S, Seviour T. "Surface-layer protein is a public-good matrix exopolymer for microbial community organisation in environmental anammox biofilms". *Isme j*, 17(6), 803-812, 2023.
[56] [56] Spieck E, Spohn M, Wendt K, Bock E, Shively J, Frank J, Indenbirken D, Alawi M, Lücker S, Hüpeden J. "Extremophilic nitrite-oxidizing chloroflexi from yellowstone hot springs". *The ISME Journal*, 14(2), 364-379, 2020.
[57] [57] Oshiki M, Takaki Y, Hirai M, Nunoura T, Kamigaito A, Okabe S. "Metagenomic analysis of five phylogenetically distant anammox bacterial enrichment cultures". *Microbes and Environments*, 37(3), 2022.
[58] [58] Su Z, Zhao J, Lu Z, Wang M, Guo C, Song X, Guo X, Cai M, Wu Z. "The effects of different temperature conditions on sludge characteristics and microbial communities of nitritation denitrification". *Journal of Water Process Engineering*, 50, 103283, 2022.
[59] [59] Thiel V, Fukushima S-I, Kanno N, Hanada S. *Chloroflexi*. Editor: Schmidt TM. Encyclopedia of microbiology. 4th ed, 651-662, Oxford, Academic Press, 2019.
[60] [60] Zhang Q, Zhang J, Zhao L, Liu W, Chen L, Cai T, Ji X-M. "Microbial dynamics reveal the adaptation strategies of ecological niche in distinct anammox consortia under mainstream conditions". *Environmental Research*, 215, 114318, 2022.
[61] [61] Ya T, Huang Y, Wang K, Wang J, Liu J, Hai R, Zhang T, Wang X. "Functional stability correlates with dynamic microbial networks in anammox process". *Bioresource Technology*, 370, 128557, 2023.
[62] [62] Zhang Q, Zheng J, Zhao L, Liu W, Chen L, Cai T, Ji X-M. "Succession of microbial communities reveals the inevitability of anammox core in the development of anammox processes". *Bioresource Technology*, 371, 128645, 2023.
[63] [63] Pekyavas G, Yangin-Gomec C. "Response of anammox bacteria to elevated nitrogen and organic matter in pre-digested chicken waste at a long-term operated uasb reactor initially seeded by methanogenic granules". *Bioresource Technology Reports*, 7, 100222, 2019.
[64] [64] Li H, Yang Q, Li J, Gao H, Li P, Zhou H. "The impact of temperature on microbial diversity and aoa activity in the tengchong geothermal field, china". *Scientific Reports*, 5(1), 17056, 2015.
[65] [65] Pitombo LM, do Carmo JB, de Hollander M, Rossetto R, López MV, Cantarella H, Kuramae EE. "Exploring soil microbial 16s rrna sequence data to increase carbon yield and nitrogen efficiency of a bioenergy crop". *GCB Bioenergy*, 8(5), 867-879, 2016.
[66] [66] Hosokawa S, Kuroda K, Narihiro T, Aoi Y, Ozaki N, Ohashi A, Kindaichi T. "Cometabolism of the superphylum patescibacteria with anammox bacteria in a long-term freshwater anammox column reactor". *Water*, 13(2), 208, 2021.
[67] [67] Zhu W, Zhang X, Yuan S, Sang W, Tang X, Zhang S. "Effects of different aeration strategies on removal of organics, nitrogen and phosphorus in sequencing batch biofilm reactor (sbbr): Performance, microbial community and nitrogen cycling pathways". *Journal of Water Process Engineering*, 49, 103118, 2022.

[68] [68] Ya T, Liu J, Zhang M, Wang Y, Huang Y, Hai R, Zhang T, Wang X. "Metagenomic insights into the symbiotic relationship in anammox consortia at reduced temperature". *Water Research*, 225, 119184, 2022.
[69] [69] Zhang H, Du B, Li D, Peng Z, An Z. "Enhancing pollutant removal and desalination in microbial desalination cells using a rotating algal biofilm cathode". *Desalination*, 586, 117832, 2024.
[70] [70] Oba K, Suenaga T, Yasuda S, Kuroiwa M, Hori T, Lackner S, Terada A. "Quest for nitrous oxide-reducing bacteria present in an anammox biofilm fed with nitrous oxide". *Microbes Environ*, 39(1), 2024.
[71] [71] Pimenov NV, Nikolaev YA, Dorofeev AG, Grachev VA, Kallistova AY, Mironov VV, Vanteeva AV, Grigor'eva NV, Berestovskaya YY, Gruzdev EV, Begmatov SA, Ravin NV, Mardanov AV. "Bioaugmentation of anammox activated sludge with a nitrifying bacterial community as a way to increase the nitrogen removal efficiency". *Microbiology*, 91(2), 133-142, 2022.
[72] [72] Peeters SH, van Niftrik L. "Trending topics and open questions in anaerobic ammonium oxidation". *Current Opinion in Chemical Biology*, 49, 45-52, 2019.
[73] [73] Quan ZX, Rhee SK, Zuo JE, Yang Y, Bae JW, Park JR, Lee ST, Park YH. "Diversity of ammonium-oxidizing bacteria in a granular sludge anaerobic ammonium-oxidizing (anammox) reactor". *Environmental Microbiology*, 10(11), 3130-3139, 2008.
[74] [74] Kartal B, Rattray J, van Niftrik LA, van de Vossenberg J, Schmid MC, Webb RI, Schouten S, Fuerst JA, Damsté JS, Jetten MSM, Strous M. "Candidatus "anammoxoglobus propionicus" a new propionate oxidizing species of anaerobic ammonium oxidizing bacteria". *Systematic and Applied Microbiology*, 30(1), 39-49, 2007.
[75] [75] Schmid M, Walsh K, Webb R, Rijpstra WI, van de Pas-Schoonen K, Verbruggen MJ, Hill T, Moffett B, Fuerst J, Schouten S, Sinninghe Damsté JS, Harris J, Shaw P, Jetten M, Strous M. "Candidatus "scalindua brodae", sp. Nov., candidatus "scalindua wagneri", sp. Nov., two new species of anaerobic ammonium oxidizing bacteria". *Systematic and Applied Microbiology*, 26(4), 529-538, 2003.
[76] [76] Schmid M, Twachtmann U, Klein M, Strous M, Juretschko S, Jetten M, Metzger JW, Schleifer K-H, Wagner M. "Molecular evidence for genus level diversity of bacteria capable of catalyzing anaerobic ammonium oxidation". *Systematic and Applied Microbiology*, 23(1), 93-106, 2000.
[77] [77] Kartal B, van Niftrik L, Rattray J, van de Vossenberg JLCM, Schmid MC, Sinninghe Damsté J, Jetten MSM, Strous M. "Candidatus 'brocadia fulgida': An autofluorescent anaerobic ammonium oxidizing bacterium". *FEMS Microbiology Ecology*, 63(1), 46-55, 2008.
[78] [78] Yang Y, Lu Z, Azari M, Kartal B, Du H, Cai M, Herbold CW, Ding X, Denecke M, Li X, Li M, Gu J-D. "Discovery of a new genus of anaerobic ammonium oxidizing bacteria with a mechanism for oxygen tolerance". *Water Research*, 226, 119165, 2022.
[79] [79] Oshiki M, Satoh H, Okabe S. "Ecology and physiology of anaerobic ammonium oxidizing bacteria". *Environmental Microbiology*, 18(9), 2784-2796, 2016.
[80] [80] Fu J-J, Wang Y, Yang J-H, Huang D-Q, Zhang Q, Huang Y, Chen J-R, Fan N-S, Jin R-C. "Mitigating the detrimental effects of salt stress on anammox process: A comparison between glycine betaine and mannitol". *Science of the Total Environment*, 851, 158221, 2022.
[81] [81] Xu L-Z-J, Zhang Q, Fu J-J, Zhang J-T, Zhao Y-H, Jin L-Y, Fan N-S, Huang B-C, Jin R-C. "Deciphering the microbial community and functional genes response of anammox sludge to sulfide stress". *Bioresource Technology*, 302, 122885, 2020.
[82] [82] Wang Y, Wang X, Niu J. "Implemented impediment of extracellular electron transfer–dependent anammox process :Unstable nitrogen removal efficiency and decreased abundance of anammox bacteria". *Chemosphere*, 337, 139415, 2023.
[83] [83] Tian S, Tian Z, Yang H, Yang M, Zhang Y. "Detection of viable bacteria during sludge ozonation by the combination of atp assay with pma-miseq sequencing". *Water*, 9(3), 2017.
[84] [84] Yang R, Mao W, Wang X, Zhang Z, Wu J, Chen S. "Response and adaptation of microbial community in a canon reactor exposed to an extreme alkaline shock". *Archaea*, 2020, 8888615, 2020.
[85] [85] Sari T, Akgul D, Mertoglu B. "Enhancement of hydrazine accumulation in anammox bioreactors". *Chemosphere*, 359, 142293, 2024.
[86] [86] Ya T, Du S, Li Z, Liu S, Zhu M, Liu X, Jing Z, Hai R, Wang X. "Successional dynamics of molecular ecological network of anammox microbial communities under elevated salinity". *Water Research*, 188, 116540, 2021.
[87] [87] Allegue T, Arias A, Fernandez-Gonzalez N, Omil F, Garrido JM. "Enrichment of nitrite-dependent anaerobic methane oxidizing bacteria in a membrane bioreactor". *Chemical Engineering Journal*, 347, 721-730, 2018.
[88] [88] Hu L, Cheng X, Qi G, Zheng M, Dang Y, Li J, Xu K. "Achieving ammonium removal through anammox-derived feammox with low demand of fe(iii)". *Frontiers in Microbiology*, 13, 918634, 2022.
[89] [89] Yang N, Zhan G, Li D, He X, Zhang Y, Jiang Q, Liu H, Wang C. "Performance and microbial community of a novel non-aeration-based up-flow bioelectrochemical filter (ubef) treating real domestic wastewater". *Chemical Engineering Journal*, 348, 271-280, 2018.
[90] [90] Zhang S, Zhang Z, Xia S, Ding N, Liao X, Yang R, Chen M, Chen S. "The potential contributions to organic carbon utilization in a stable acetate-fed anammox process under low nitrogen-loading rates". *Science of the Total Environment*, 784, 147150, 2021.
[91] [91] Ahmad HA, Guo B, Zhuang X, Zhao Y, Ahmad S, Lee T, Zhu J, Dong Y, Ni S-Q. "A twilight for the complete nitrogen removal via synergistic partial-denitrification, anammox, and dnra process". *npj Clean Water*, 4(1), 31, 2021.
[92] [92] Ribeiro H, Wijaya IMW, Soares-Santos V, Soedjono ES, Slamet A, Teixeira C, Bordalo AA. "Microbial community composition, dynamics, and biogeochemistry during the start-up of a partial nitritation-anammox pathway in an upflow reactor". *Sustainable Environment Research*, 32(1), 18, 2022.
[93] [93] Zhang J-F, Lai C-Y, Cao X-X, Hartmann EM, Zhao H-P. "High ammonia loading rate and biofilm reattachment initiated partial nitrification and anammox in a membrane aerated biofilm reactor". *Journal of Water Process Engineering*, 58, 104829, 2024.
[94] [94] Ding J, Qin F, Li C, Tang M. "Long-term effect of acetate and biochar addition on enrichment and activity of denitrifying anaerobic methane oxidation microbes". *Chemosphere*, 338, 139642, 2023.
[95] [95] Sun L, Toyonaga M, Ohashi A, Matsuura N, Tourlousse DM, Meng XY, Tamaki H, Hanada S, Cruz R, Yamaguchi T, Sekiguchi Y. "Isolation and characterization of flexilinea

flocculi gen. Nov., sp. Nov., a filamentous, anaerobic bacterium belonging to the class anaerolineae in the phylum chloroflexi". *International Journal of Systematic and Evolutionary Microbiology*, 66(2), 988-996, 2016.

[96] [96] Lawson CE, Wu S, Bhattacharjee AS, Hamilton JJ, McMahon KD, Goel R, Noguera DR. "Metabolic network analysis reveals microbial community interactions in anammox granules". *Nature Communications*, 8(1), 15416, 2017.

[97] [97] Speth DR, in 't Zandt MH, Guerrero-Cruz S, Dutilh BE, Jetten MSM. "Genome-based microbial ecology of anammox granules in a full-scale wastewater treatment system". *Nature Communications*, 7(1), 11172, 2016.

[98] [98] Qiu J, Li T, Lü F, Huang Y, Li C, Zhang H, Shao L, He P. "Molecular behavior and interactions with microbes during anaerobic degradation of bio-derived dom in waste leachate". *Journal of Environmental Sciences*, 126, 174-183, 2023.

[99] [99] Cheng L, Yang W, Liang H, Nabi M, Li Y, Wang H, Hu J, Chen T, Gao D. "Nitrogen removal from mature landfill leachate through enhanced partial nitrification-anammox process in an innovative multi-stage fixed biofilm reactor". *Science of the Total Environment*, 877, 162959, 2023.

[100] [100] Luo Y, Yi K, Zhang X, Li B, Cao R, Pang Y, Li M, Hou C, Lv J, Li X, Li D. "Simultaneous partial nitrification, denitrification, and phosphorus removal in sequencing batch reactors via controlled reduced aeration and short-term sludge retention time decrease". *Journal of Environmental Management*, 344, 118598, 2023.

[101] [101] Li J, Chen X, Yang Z, Liu Z, Chen Y, Wang Y-e, Xie H. "Denitrification performance and mechanism of sequencing batch reactor with a novel iron-polyurethane foam composite carrier". *Biochemical Engineering Journal*, 176, 108209, 2021.

[102] [102] Li S, Li J, Yang S, Zhang Q, Li X, Zhang L, Peng Y. "Rapid achieving partial nitrification in domestic wastewater: Controlling aeration time to selectively enrich ammonium oxidizing bacteria (aob) after simultaneously eliminating aob and nitrite oxidizing bacteria (nob)". *Bioresource Technology*, 328, 124810, 2021.

[103] [103] Li Y, Wang J, Hua M, Yao X, Zhao Y, Hu J, Xi C, Hu B. "Strategy for denitrifying anaerobic methane-oxidizing bacteria growing under the oxygen-present condition". *Science of the Total Environment*, 742, 140476, 2020.

[104] [104] Toumi M, Abbaszade G, Sbaoui Y, Farkas R, Ács É, Jurecska L, Tóth E. "Cultivation and molecular studies to reveal the microbial communities of groundwaters discharge located in hungary". *Water*, 13(11), 1533, 2021.

[105] [105] Wang C, Yu G, Yang F, Wang J. "Formation of anaerobic granules and microbial community structure analysis in anaerobic hydrolysis denitrification reactor". *Science of the Total Environment*, 737, 139734, 2020.