Temperature-driven microbial dynamics and functional shifts in a pilot-scale partial nitrification/anammox reactor treating low-ammonium rare earth tailwater

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Abstract: This study fills a critical research gap by evaluating the year-round performance of a pilot-scale partial nitrification/anammox (PN/A) reactor treating low-ammonium rare earth tailwater under seasonal temperature fluctuations. Operated without thermal insulation, the system achieved a peak total nitrogen removal efficiency of 87.5% during warm periods, while experiencing a 58.4% decline in winter. Microbial analyses revealed that all core functional groups were affected by cold stress. Notably, the PN reactor maintained suppression of nitrite-oxidizing bacteria, preserving community structure integrity, with *Nitrosomonas* maintaining 11.46% relative abundance. In contrast, *Candidatus Brocadia* declined from 15.26% to 4.89%, while denitrifying bacteria exhibited stronger cold tolerance and maintained nitrogen removal via denitrification. Pathway and electron transport analyses confirmed a functional shift from Anammox to denitrification during low-temperature periods. The findings offer practical insights into improving PN/A stability under seasonal variation and provide guidance for advancing its engineering application in the treatment of low-ammonium wastewater.

Key words: partial nitrification/anammox; bacteria activity; metagenomic; temperature shock

1. Introduction

Rare earth elements, often referred to as the "vitamins of modern industry," are increasingly utilized in advanced materials and high-tech applications. In southern China, ammonium sulfate is commonly used in the extraction of ion-adsorption rare earth deposits, leading to the release of substantial ammonium into surface runoff, forming low ammonia nitrogen tailwater [1]. The ammonium concentration in rare earth tailwater (RET) ranges from 50-110 mg/L in spring and 90-160 mg/L in winter. The uncontrolled discharge of this untreated tailwater into surface waters threatens ecosystem stability and raises concerns about human exposure. The primary treatment approach for RET is the modified constructed rapid infiltration system [2]. However, its large land footprint, high operational costs, and inconsistent pollutant removal performance limit its practical application. Therefore, developing innovative nitrogen removal technologies is crucial to mitigating the environmental impact of RET.

Anaerobic ammonium oxidation (Anammox) is an advanced and highly efficient biological nitrogen removal technology. First discovered and named by Mulder in 1995 [3], Anammox has since become a focal point in both microbial and engineering research [4]. The metabolic pathway of Anammox has been extensively elucidated, and the process has been successfully implemented in biological wastewater treatment. The partial nitrification-Anammox (PN/A) process, centered around Anammox, is a cost-effective and energy-efficient nitrogen removal strategy [5]. Compared to conventional nitrification-denitrification (DN) processes, PN/A can reduce aeration energy consumption by up to 60%, eliminate the need for external organic carbon, and generate

significantly less sludge [6]. In recent years, research on PN/A has predominantly focused on optimizing laboratory-scale reactors, while the number of full-scale implementations remains limited. To date, only around 100 full-scale PN/A systems have been successfully operated worldwide, shifting research priorities toward engineering applications. For high-ammonium wastewater, such as landfill leachate [7] and liquid-ammonia mercerization wastewater [8], numerous successful pilot and full-scale applications have been reported. However, achieving stable PN/A performance under low-ammonium conditions remains challenging. Under low-ammonium conditions, AOB enrichment is inherently difficult, whereas nitrite-oxidizing bacteria (NOB) can thrive in low-substrate environments, leading to process instability[9]. Previous studies have demonstrated that zeolite can enhance local ammonium concentrations via ion exchange, suppressing excessive NOB proliferation and overcoming a critical bottleneck in PN/A [10-14]. Nevertheless, the long-term reliability of zeolite as a stable PN medium in low-ammonium wastewater requires further experimental and engineering validation. Additionally, at low ammonium concentrations and nitrogen loading rates, AnAOB exhibits slow growth, and improper operational control may lead to granule deterioration, ultimately compromising process performance [15]. Thus, despite its potential, the application of PN/A for low-ammonium wastewater treatment still faces significant technical hurdles, necessitating further investigation and optimization.

In the promotion and engineering application of the PN/A process, the impact of seasonal temperature variations cannot be overlooked. Due to the influence of

temperate climates, wastewater treatment systems often experience significant temperature fluctuations, which pose an additional challenge to the stable operation of PN/A [16]. Previous studies have shown that within a moderate temperature range (30-40°C), AnAOB and AOB exhibit high activity [17]. However, seasonal temperature variations can substantially affect process performance [18]. In particular, at temperatures below 20°C, the growth rate of NOB surpasses that of AOB, making it difficult to maintain a stable supply of nitrite as a substrate for AnAOB [19]. Furthermore, low temperatures inhibit the activity and growth rate of AnAOB, thereby weakening nitrogen removal efficiency [20, 21]. Studies have reported that for every 5°C decrease in temperature, the growth rate of AnAOB declines by 30%-40% [22], consequently limiting nitrogen removal performance and negatively impacting enzyme activity, reaction activation energy, and microbial growth. During winter, the activity of functional microorganisms is significantly suppressed, leading to a notable reduction in nitrogen removal efficiency. Although maintaining temperatures above 30°C in full-scale applications can help stabilize PN/A performance, this approach incurs substantial energy consumption, undermining the economic viability and sustainability of the process [23]. Therefore, future research should focus on evaluating the stability and engineering feasibility of PN/A under seasonal temperature fluctuations, assessing its performance under natural temperature conditions. Such investigations will facilitate process optimization and contribute to the broader implementation and scaling up of PN/A technology.

In this study, a pilot-scale PN/A reactor was constructed and operated over the

long term to investigate its performance and microbial community dynamics under natural conditions. The objectives of this study are as follows: (1) to evaluate the performance and nitrogen removal pathways of the PN/A process during long-term operation; (2) to assess in situ changes in the activity of functional microorganisms and nitrogen removal mechanisms;(3) to explore the shifts and interactions within the microbial community in response to seasonal temperature variations;(4) to elucidate the impact of low temperatures on the abundance of microbial functional genes. The findings of this study are expected to provide further insights into the long-term operational mechanisms of PN/A and offer valuable guidance for its engineering-scale implementation.

2. Material and methods

2.1 The pilot-scale PN/A process

The pilot-scale PN/A system was located in a rare earth mining area in southern Jiangxi Province, China (24°54′16.8″N, 114°47′59.2″E). The system primarily consisted of a two-stage PN reactor and an anammox reactor (Fig. 1). The PN reactor was a steel cylindrical tank with an inner diameter of 3 m and a height of 4.5 m, providing a working volume of 30 m^3. It was packed with 80-mesh zeolite, which had an ammonium adsorption capacity of 3.0 mg NH_4^+-N/g, with a total packing volume of 5 m^3. The reactor was aerated using a connected air blower. Similarly, the anammox reactor was a steel cylindrical tank with the same working volume of 30 m^3. It was inoculated with a small amount of mature anammox sludge and operated using a suspended sludge system rather than a biofilm-based process for nitrogen removal. The

treated effluent was discharged through a sedimentation tank, where hydraulic flow facilitated sludge recirculation back to the anammox reactor. Both reactors were equipped with online pH monitoring, liquid level sensors, and temperature control instruments. Alkalinity ($NaHCO_3$) required for the PN reactor was supplied via a metering pump from an alkalinity storage tank. The anammox reactor received carbon supplementation (sodium acetate) through a metering pump from a carbon source tank to maintain a suitable microbial environment and maintain a C/N ratio of 3.

The PN/A process was operated for one year, with the operational periods of the PN reactor categorized by season as follows: cycles 1-21 corresponded to spring, cycles 22-72 to summer, cycles 73-134 to autumn, and cycles 135-195 to winter. The anammox reactor was initiated only after the PN reactor had been successfully started and stabilized to ensure the effective accumulation and conversion of nitrite within the system. Subsequently, the PN/A process was operated in a two-stage sequential mode. However, due to the relatively high nitrite concentration in the PN reactor effluent, potential inhibition of AnAOB could occur. To mitigate this, the effluent from the PN reactor was mixed with raw influent from the equalization tank before entering the anammox reactor, thereby reducing nitrite concentrations and optimizing the reaction conditions. The operational period of the anammox reactor was also classified by season: cycles 1-30 corresponded to summer, cycles 31-91 to autumn, and cycles 92-153 to winter.

2.2 Wastewater and seed sludge

The low-ammonium RET in this study was sourced from mining activities at a

rare earth mine in Ganzhou City, Jiangxi Province. The seed sludge for the PN reactor was obtained from the activated sludge of a wastewater treatment plant in Longnan, Ganzhou City, with mixed liquor volatile suspended solids (MLVSS) of approximately 3 g/L. The anammox seed sludge was collected from the Canon reactor in the laboratory, with MLVSS of approximately 0.3 g/L. The specific characteristics of the wastewater as shown in Tab. S1-S3 (Supplementary Materials). Other necessary elements were supplied according to the literature.

2.3 Analytical methods and calculations

The concentrations of nitrogen species and COD were detected according to the standard analysis method, all samples were filtered through 0.45 μm filter membrane before analysis. The temperature and dissolved oxygen (DO) were both measured by a digital DO meter (HQ30d, HACH, USA) and pH by a pH meter (PHS-3C, INESA Scientific Instrument Co. Ltd, China). Mixed liquor suspended solids (MLSS) and MLVSS were measured following the standard method [24]. The extraction and analysis of extracellular polymeric substances (EPS) was conducted according to the procedures outlined by Li [25], pre-treatment had removed any residual substances. The nitrite accumulation rate (NAR), nitrogen production rate (NPR), nitrogen loading rate (NLR), nitrogen removal rate (NRR), total nitrogen removal rate (TNRE), and free ammonia (FA) concentration were calculated using calculation method in Text S3 (Supplementary Materials).

2.4 PN/A functional bacterial activity

A series of ex situ batch tests were conducted to evaluate the specific activity of

ammonium-oxidizing bacteria (SAOA) and the specific anammox activity (SAA). The SAOA and SAA methods are described in Text S1-S2 (Supplementary Materials).

2.5 microbiological analysis

To investigate the differences in functional bacteria and microbial community in the PN/A system, high-throughput sequencing was employed to analyze the microbial community structure and changes in biofilm samples from different operational stages of the reactors. The sludge samples were collected from suspended sludge, cycle 0, cycle 20 and cycle 80 in the PN reactor. In the anammox treatment unit, the sludge samples were collected on cycle 0, on cycle 50, on cycle 110. Bacterial universal primers were used to amplify the 16S rRNA of the sludge sample. The analysis method is described in Text S4 (Supplementary Materials). Metagenomic was extracted from Anammox sludge samples for metagenomic analysis. For specific methods, see Table S5 (Supplementary Materials). The raw sequencing data generated in this study have been deposited in the NCBI database under accession number PRJNA1293347.

3. Results and discussion

3.1. Long term performance of the PN/A reactor

3.1.1 Impact of seasonal temperature on the stable supply of nitrite

Unlike conventional NOB inhibition strategies (e.g., intermittent aeration, high-temperature control, and low DO), this study employed zeolite as a filler in the PN reactor to facilitate a stable partial nitrification process. Zeolite has been widely reported as an effective ammonia storage medium due to its high cation exchange capacity and strong selectivity for ammonium ions [10]. The underlying mechanism

involves in situ enrichment of FA, elevating its concentration above the NOB inhibition threshold (0.1-1.0 mg/L), thereby effectively suppressing NOB growth and activity, ultimately leading to selective NOB washout. The PN reactor, filled with zeolite, was operated for a total of 196 cycles (Fig. 2a-c). During Phase I, the reactor temperature gradually increased (17-28°C), significantly enhancing AOB activity. By cycle 5, the oxidation rate of NH_4^+-N to NO_2^--N exceeded the formation rate of NO_3^--N for the first time, and NO_2^--N accumulation continued to increase thereafter, indicating the successful startup of partial nitrification. In comparison with the original sample, biofilm development was observed in the zeolite pore structure (Fig. S1). The rapid establishment of partial nitrification could be attributed to two key factors: (i) the introduction of zeolite promoted localized FA accumulation, inhibiting NOB growth while facilitating AOB enrichment, and (ii) the continuous temperature rise further accelerated AOB growth and metabolism, enhancing ammonia oxidation efficiency. During this phase, the hydraulic retention time (HRT) was reduced from 48h to 24h to increase the nitrogen loading rate. However, the NAR remained stable, demonstrating the effectiveness of the zeolite-based strategy in maintaining stable partial nitrification. During summer (Phase II), the operating temperature of the PN reactor ranged from 20°C to 33°C, with an average of 26°C. Influenced by the temperate climate, the region experienced high precipitation in summer, leading to significant seasonal fluctuations in the temperature and nitrogen content of the real RET entering the reactor. As a result, influent NH_4^+-N and NO_3^--N concentrations exhibited considerable variation, averaging 65.28 ± 20.83 mg/L and 36.32 ± 9.77 mg/L, respectively. In contrast, the

effluent NO_2^--N and NO_3^--N concentrations reached 120.27 ± 72.72 mg/L and 57.03 ± 19.80 mg/L, respectively. Notably, NO_3^--N accumulation was significantly lower than NO_2^--N oxidation, achieving a high NAR. This result indicates that NOB activity was effectively suppressed, ensuring an adequate NO_2^--N supply to the anammox reactor. As autumn (Phase III) commenced, ambient temperatures declined substantially (13-30°C). Temperature fluctuations influenced the performance of the PN reactor, particularly affecting AOB activity. The effluent NO_2^--N concentration decreased to 85.49 ± 78.12 mg/L, with an average NAR of approximately 85%, marking a decline from the summer phase (Phase 2). Previous studies have shown that when temperatures drop below 15°C, AOB growth rates decrease significantly, allowing NOB to gradually regain a competitive advantage. In winter (Phase IV), temperatures continued to decline (8-16°C). Despite the low temperatures, the PN reactor maintained effective NOB suppression, with an average NAR of 77.12%. This result suggests that the system retained partial nitrification capability under cold conditions, although overall reactor performance was negatively impacted, as evidenced by a substantial decrease in NO_2^--N accumulation (18.86 ± 8.03 mg/L). Previous studies have demonstrated that low temperatures generally exert a stronger inhibitory effect on AOB than on NOB [9, 26]. However, the limited decline in NAR observed in this study suggests that the zeolite-based strategy mitigated the adverse effects of low temperatures on PN. This could be attributed to the ability of zeolite to locally enrich NH_4^+-N via ion exchange, thereby maintaining FA concentrations at levels sufficient to inhibit NOB, even under cold conditions. Since the anammox process relies on a 1:1 molar ratio of NH_4^+-N to NO_2^--

N, the insufficient NO_2^--N supply during this phase led to an imbalance in the NO_2^--N/NH_4^+-N ratio, thereby affecting overall PN/A nitrogen removal performance. Without additional temperature control measures, the system may experience a decline in nitrogen removal efficiency during winter.

3.1.2 Long term operation performance of Anammox reactor

The Anammox reactor served as the primary nitrogen removal unit. Following the successful initiation of PN in the PN reactor, the Anammox reactor was operated in series with the PN reactor, forming an efficient PN/A synergistic nitrogen removal system. However, previous studies have demonstrated that AnAOB are highly sensitive to environmental fluctuations, with sudden changes in dissolved oxygen (DO) levels, substrate concentrations, and temperature potentially inhibiting their activity [8]. In this study, the Anammox reactor was initiated in May (Fig. 2d-f). Given that the experimental site is located in a temperate monsoon climate zone, summer temperatures were not a limiting factor for Anammox startup. In contrast, significant rainfall runoff during the rainy season led to considerable fluctuations in influent water quality, particularly affecting the NO_2^--N/NH_4^+-N ratio. To ensure stable AnAOB growth, two key measures were implemented: (i) blending the effluent from the PN reactor with RET before entering the Anammox reactor to buffer substrate concentration fluctuations; and (ii) inoculating the system with mature Anammox sludge to accelerate AnAOB enrichment and stabilize system operation. During Phase I, as the AnAOB community gradually adapted and enriched, the TNRE of the Anammox reactor continuously increased, eventually stabilizing above 50%, indicating successful reactor

startup and achieving the expected nitrogen removal performance. Upon completion of startup, the system entered the loading enhancement phase. To improve treatment capacity, the HRT was adjusted. In Phase II, reactor performance further improved, with the maximum TNRE increasing to 87.5%, while the NLR and NRR reached 0.131 kg N/m³/d and 0.107 kg N/m³/d, respectively. Additionally, due to the strong self-aggregating properties of AnAOB, a large amount of deep-red Anammox sludge was observed in the reactor, further confirming the activity and efficient enrichment of AnAOB. In Phase III, as winter temperatures gradually decreased, the performance of the Anammox reactor was significantly affected. At this stage, the ambient temperature dropped to an average of 12°C, leading to a substantial reduction in NO_2^--N production within the PN reactor, which disrupted the NO_2^--N/NH_4^+-N ratio in the Anammox reactor influent. When NO_2^--N supply was insufficient, NH_4^+-N could no longer be removed via the Anammox pathway, resulting in a sharp decline in TNRE from a peak of 86.3% to a minimum of 36.4%. Consequently, the primary nitrogen removal pathway shifted from Anammox to denitrification (Fig. S2). However, due to the imbalance in influent nitrogen composition, the overall nitrogen removal performance of the system could not recover to the levels observed in Phase II. Further analysis indicated that temperature fluctuations directly influenced the dominant nitrogen removal pathway. During the warmer summer and autumn seasons, Anammox played a dominant role in nitrogen removal. However, as temperatures decreased, the system's nitrogen removal pathway gradually transitioned from Anammox to DN. When the temperature remained below 15°C for an extended period, DN contributed more to nitrogen removal than

Anammox, highlighting the higher sensitivity of AnAOB to low temperatures compared to denitrifying bacteria (DNB). The ΔNO_3^--N /ΔTN ratio further confirmed the predominance of DN during winter.

This study monitored the long-term stability of the pilot-scale PN/A system and demonstrated the feasibility of using PN/A technology for treating RET. As the first-stage unit in the two-stage PN/A process, the PN reactor achieved stable partial nitritation over the long term, while the Anammox reactor was only affected when temperatures dropped below 12°C. Considering the local climate characteristics, the reaction temperature remained above 15°C for most of the year, with a lower temperature risk occurring only between November and December. The Anammox reactor thus transitioned from energy-efficient autotrophic to less efficient heterotrophic DN-a key trade-off in PN/A system, ensuring nitrogen removal continuity even under suboptimal conditions.

3.2. Sludge characteristics in the PN/A reactor

Sludge samples were collected at different operational phases to evaluate reactor performance through sludge characterization. MLVSS and mixed liquor suspended solids (MLSS) were used to quantify the sludge biomass and its active fraction (Fig. 3a). By the 90th operational cycle, both MLVSS and MLSS remained at high levels, with the MLVSS/MLSS ratio approaching 0.8, indicating a high proportion of active biomass and sustained sludge activity in the reactor. However, after 120 cycles, a temperature decline led to a decrease in the MLVSS/MLSS ratio. The activity of AOB was particularly affected, and after 160 cycles, a significant reduction in sludge biomass

was observed, with the MLVSS/MLSS ratio dropping to 0.6. AOB activity is known to be governed by enzymatic processes that are highly temperature-dependent. This shift directly compromised nitrite production and overall PN reactor performance.

For the Anammox reactor, MLVSS and MLSS exhibited a distinct trend of initial increase followed by a subsequent decline over the operational period (Fig. 3b). By the 110th cycle, MLSS and MLVSS had significantly increased, suggesting that as the reactor stabilized after startup, gradual acclimation led to biomass accumulation, improving sludge quantity and active fraction. However, prolonged exposure to temperatures below 12°C resulted in a decline in active biomass due to sustained low-temperature stress. The correlation between temperature fluctuations, MLVSS, MLSS, and nitrogen removal efficiency highlighted temperature as a critical factor in nitrogen removal capacity deterioration. Moreover, low-temperature stress destabilized EPS, weakening sludge structure and promoting disintegration. When the MLVSS/MLSS ratio dropped below 0.7, sludge settleability deteriorated, and floating sludge was observed on-site, likely due to microbial death and sludge disintegration under prolonged low-temperature conditions. These results highlight that temperature stress not only reduces microbial activity but also disrupts floc integrity, further impairing reactor function.

The mean sludge activity at different operational phases was assessed. The SAOA and SAA followed trends similar to MLVSS (Fig.3d-e). As the temperature decreased from 30°C to 10°C, SAOA declined from a peak of 4.54 mg N/g VSS·d to 0.91 mg N/g VSS·d, representing nearly a fivefold reduction, underscoring the enzymatic and

membrane transport limitations experienced by AOB under cold stress. The decline in SAA was even more pronounced. When the temperature dropped to 10°C, the metabolic rate of AnAOB significantly decreased, demonstrating partial inactivation or washout of AnAOB. Furthermore, the growth rate of AnAOB was constrained by low nitrogen loading rates. During the winter dry season, reduced surface runoff led to lower nitrogen concentrations in the influent, resulting in decreased NLR. The coupling of low NLR with low temperature created a dual constraint-both energetic and physiological-on AnAOB growth, explaining the marked drop in activity and sludge biomass observed during winter.

EPS are microbial secretions that influence sludge physicochemical properties and pollutant removal efficiency [27]. EPS synthesis is dynamically regulated in response to environmental signals, particularly temperature, which modulates both microbial stress responses and metabolic allocation. EPS samples were collected from the Anammox reactor throughout the operational period (Fig. 3c). By the 90th cycle, total EPS concentration exhibited a continuous upward trend, increasing from 22.44 mg/g VSS to 69.12 mg/g VSS, indicating high microbial metabolic activity. Under warm conditions, elevated metabolic rates allowed microbes to allocate more energy toward protein. Protein components strengthened the bioaggregate matrix, improving mechanical resilience and promoting sludge granulation. The increasing protein/polysaccharide ratio suggested that EPS functioned as an adaptive tool for biofilm fortification under optimal temperatures.

Following a temperature decline to 15°C at the 110th cycle, EPS further increased

to 72.67 mg/g VSS. This transient spike in EPS reflects an immediate microbial stress response that trigger overproduction of EPS to insulate cells and stabilize extracellular microenvironments. However, as the temperature continued to decrease, prolonged low-temperature stress led to a reduction in EPS production. A relative increase in polysaccharide content was observed, indicating that under environmental stress, polysaccharides may play a protective role. Notably, the reduction in EPS concentration lagged behind the decline in SAA, indicating that while AnAOB metabolism was severely inhibited, heterotrophic populations-such as DNB-remained metabolically active and continued contributing to EPS synthesis. This functional redundancy suggests that EPS dynamics are governed by the collective microbial consortia, not solely AnAOB. The sustained EPS presence may have helped maintain reactor structure despite loss of AnAOB-driven autotrophic activity. The concurrent decline in MLVSS and shift in EPS composition points to microbial lysis, particularly of sensitive AnAOB, under prolonged low-temperature stress. This lysis released soluble microbial products, which likely served as secondary carbon sources fueling DNB metabolism [28]. This cross-feeding interaction-where lysed autotrophic cells support heterotrophic activity-represents a stress-adaptive survival mechanism within the microbial consortium. This is further confirmed by metagenomic data (section 3.4), which show DNB enrichment during winter and support the hypothesis of temperature-driven pathway shifts from Anammox to DN.

3.3. Microbial community population succession

To investigate the temporal evolution of microbial communities in PN and

anammox reactors, sludge samples were collected at multiple operational stages and subjected to 16S rRNA high-throughput sequencing (Fig. 4). For the PN reactor, samples were taken at cycles 0, 20, 80, 120, and 190, and designated PN1-PN5. Anammox reactor samples were collected at cycles 0, 50, 110, and 150, labeled AMX1-AMX4. The dominant phyla in the PN reactor included *Proteobacteria*, *Firmicutes*, *Actinobacteria*, and *Chloroflexi*. The relative abundance of *Proteobacteria* increased from 31.7% at startup to a peak of 66.6%, followed by a slight decline to 58.9% as temperatures dropped, indicating limited sensitivity to cold stress. Given the metabolic diversity within *Proteobacteria*, it is plausible that certain subgroups exhibit enhanced metabolic plasticity under low-temperature stress, enabling them to rapidly adapt and maintain viability. In contrast, the decline in *Firmicutes* suggests limited suitability for survival in the RET matrix, likely due to insufficient organic substrates to support their fermentative and acidogenic metabolism. *Actinobacteria*, primarily aerobic heterotrophs involved in organic matter degradation, also exhibited sensitivity to both nutrient availability and temperature fluctuations [29]. *Chloroflexi*, however, showed no significant decline in relative abundance under cold conditions. Their ecological competitiveness under low-temperature stress may be attributed to their K-strategy growth traits and robust EPS production, which supports biofilm formation and environmental resistance. Within *Proteobacteria*, AOB exhibited temperature-dependent dynamics. *Nitrosomonas* increased in relative abundance during reactor start-up, reaching 13.42%, and further rose to 14.23% during the autumn operational phase. However, its abundance declined to 11.46% under winter conditions. Neither

391 *Nitrobacter* or *Nitrospira* were detected, indicating effective suppression of NOB, and
392 confirming that partial nitrification was maintained despite low-temperature stress.
393 Notably, other denitrifying genera were also present. *Limnobacter* and *Thermomonas*,
394 both capable of nitrate reduction, were detected, with *Thermomonas* increasing from
395 1.8% to 3.47%. This suggests the formation of aerobic–anoxic microenvironments
396 within the sludge granules. The proliferation of *Thermomonas* underscores the
397 resilience of certain denitrifiers under environmental stress [30]. Overall, the functional
398 taxonomic composition of the PN reactor remained stable, indicating that low
399 temperatures primarily affected intracellular diffusion rates and enzyme activity, rather
400 than altering community structure. This functional resilience suggests that the reactor's
401 performance could recover with the return of favorable temperatures.

402 In the anammox reactor, phylum-level composition similarly remained stable
403 throughout operation. Dominant phyla included *Patescibacteria*, *Proteobacteria*,
404 *Planctomycetes*, *Actinobacteria*, and *Chloroflexi*. The relative abundance of
405 *Patescibacteria* remained high despite temperature decline. This phylum, widely
406 distributed across diverse environments, is frequently involved in DN and may function
407 as a syntrophic partner of AnAOB, utilizing their metabolic by-products. Over time,
408 *Patescibacteria* appeared to occupy ecological niches formerly dominated by
409 *Proteobacteria*, which typically require richer nitrogen and carbon sources-conditions
410 less prevalent in RET. However, *Patescibacteria* abundance declined slightly in winter,
411 potentially due to their streamlined genomes and limited metabolic capacity under low
412 temperatures. In contrast, certain psychrophilic *Proteobacteria* subgroups exhibited

413 better cold adaptation. *Planctomycetes*, which thrive under moderate temperatures,
414 showed a substantial decline under winter conditions (from 15.69% during stable
415 operation to 5.25%), consistent with the suppression of anammox activity at
416 temperatures below 15 °C. *Actinobacteria*, known for their cold tolerance and
417 production of low-temperature-active cellulases and proteases, increased in abundance,
418 indicating metabolic resilience under cold stress. *Chloroflexi*, through the secretion of
419 hydrophobic EPS, helped maintain sludge structure and mitigated biomass washout,
420 thereby preserving anammox reactor stability [31]. At the genus level, *Candidatus*
421 *Brocadia* was the primary AnAOB. Its relative abundance rose from 1.25% at startup
422 to 15.26%, but fell to 4.89% under winter conditions. This decline likely reflects
423 reduced hydrazine dehydrogenase activity, which limited growth under low
424 temperatures. Meanwhile, *Saccharimonadales*-a genus within *Patescibacteria*-
425 maintained stable abundance (36.78–56.55%) despite cold stress [32]. Prior studies
426 suggest that *Saccharimonadales* utilize polysaccharides secreted by AnAOB as amino
427 acid sources, and in turn secrete secondary metabolites that support AnAOB growth,
428 forming a "metabolic dependency–protection" symbiotic relationship [32]. This
429 relatively novel taxon is highly resilient and may support nitrogen removal via DN
430 when anammox activity is suppressed by cold stress. Additionally, the relative
431 abundance of *Thermomonas* and *Comamonas* increased during winter. These genera
432 possess nitrate reductases with low-temperature tolerance, supporting the hypothesis
433 that nitrogen removal pathways shifted toward DN under cold conditions. Cell lysis
434 under cold stress may have released soluble organic matter, further fueling DN. Taken

together, phylum and genus-level analyses reveal that long-term low-temperature stress impaired the activity and growth of key functional microorganisms, significantly reducing the performance of the PN/A process during winter. These seasonal performance losses pose serious challenges for the full-scale application of PN/A technology. Addressing temperature-associated constraints is thus of critical engineering significance for advancing the practical implementation of PN/A process.

3.4. Temperature-driven shifts in energy and nitrogen metabolism

To further elucidate the functional shifts in microbial communities within the anammox reactor, metagenomic sequencing was performed on sludge samples collected at four key operational time points (0, 50, 110, and 150 cycle), designated AMX1 through AMX4 (Fig. 5). Functional gene annotation was conducted using KEGG Orthology, and the relative abundance of each gene category was quantified. In anammox, CO_2 fixation primarily occurs via the reductive acetyl-CoA pathway, with the Wood–Ljungdahl route serving as the dominant metabolic pathway. Several key enzymes involved in this process-including ferredoxin (Fd^{2-}, EC 1.2.1.74) and two NADPH-dependent enzymes (EC 1.17.1.10 and EC 1.5.1.5)-exhibited marked temperature sensitivity. These enzymes showed the highest relative abundances under summer conditions, but their expression levels declined significantly under low-temperature stress. Notably, in the AMX4 sample, the relative abundance of these enzymes decreased to 75% of that observed in AMX2, suggesting that cooler temperatures may impair NADPH biosynthesis and thereby hinder carbon fixation in AnAOB. In contrast, the tricarboxylic acid (TCA) cycle, a central pathway for energy

metabolism, showed minimal correlation with temperature fluctuations, indicating resilience to cold stress. Specifically, enzymes involved in NADH production, such as those catalyzing 3-hydroxypropyl-ThP metabolism (EC 2.3.1.61) and isocitrate dehydrogenase (EC 1.1.1.42), did not display notable temperature-dependent trends, implying that TCA-mediated electron transfer remains stable under low-temperature conditions. Beyond inorganic carbon metabolism, several organic carbon pathways-predominantly driven by DNB-also demonstrated temperature-specific behaviors. Among these, glycolysis and the pentose phosphate pathway generated substantial amounts of NADH and acetyl-CoA. While the intermediate glyceraldehyde-3-phosphate showed large fluctuations across samples, the terminal product of the pentose phosphate pathway, ribose-5-phosphate, remained unaffected by temperature. Its abundance in AMX4 reached 130% of that in AMX2, suggesting that this pathway may sustain high levels of electron transfer at low temperatures, thereby providing a sufficient NADH supply to support robust DNB growth.

Key functional genes associated with the anammox process include hzs and hdh [33]. Their initial expression levels were 3.62 and 4.90 RPKM, respectively, rising to 79.10 RPKM and 102.60 RPKM as the reactor matured. However, under low-temperature conditions, their expression in AMX4 declined to 41.10 RPKM and 46.11 RPKM, respectively, highlighting the substantial inhibitory effect of cold on hydrazine synthesis and oxidation, and consequently on nitrogen removal efficiency. While hao has long been recognized for catalyzing hydroxylamine oxidation to nitrite, recent studies also implicate it in nitric oxide production, underscoring its pivotal role in the

anammox process. At its peak in AMX2, hao expression reached 227.15 RPKM but progressively declined in AMX3 and AMX4. The concurrent downregulation of hzs, hdh, and hao under cold stress further confirms the high sensitivity of core anammox genes to temperature fluctuations. In the DN pathway, NO_3^--N is reduced to NO_2^--N via genes such as napA/B, nasB, and narG/H/I, then to nitric oxide by nirS/K, and finally to dinitrogen gas through norB/C/Z. Among these, the expression of the narG/H/I subunits remained stable across temperature regimes. Additionally, the nxr gene complex (nxrA/B), which catalyzes the oxidation of NO_2^--N to NO_3^--N, provides both reducing equivalents for autotrophic nitrifiers and compensates for electron loss in AnAOB. The expression of nxrA ranged from 367.35 to 416.78 RPKM and was unaffected by seasonal temperature changes, suggesting that while anammox activity may decline under cold conditions, it retains the potential for recovery upon temperature rebound. Further analysis of DN-related gene expression revealed only modest reductions in nirK and norB/Z under low-temperature conditions, indicating a greater resilience of DNB compared to AnAOB. The sustained activity of key nitrate reductases also implies that DN remains functionally robust at low temperatures, supporting the notion that DNB may exhibit superior adaptability and maintain effective nitrate reduction under cold stress [34].

3.5. Towards sustainable nitrogen removal from RET via PN/A

Although the application of the PN/A process has been promoted for many years, research on the treatment of RET under seasonal temperature fluctuations and low-strength ammonia conditions remains scarce. In this study, a pilot-scale PN/A process

was investigated to assess the effects of seasonal temperature variations on process performance and microbial community composition during the treatment of RET. The results indicate that under the relatively high temperature conditions in summer and autumn, the nitrogen removal performance steadily improved, with NLR and TNRE reaching peak values of 0.131 kg $N/m^3/d$ and 87.5%, respectively. This coincided with enhanced SAA and biomass enrichment, suggesting that high temperatures facilitated autotrophic nitrogen removal via the canonical PN/A pathway. In contrast, under winter low-temperature conditions, the short-term performance of the anammox process was not significantly affected, with AnAOB exhibiting strong low-temperature adaptability. However, long-term operation at low temperatures failed to achieve optimal PN/A performance, posing an engineering challenge-especially when temperatures fell below 12°C, at which the performance was only 36.4%. Encouragingly, the PN reactor consistently maintained a high NAR under cold conditions, effectively resolving the challenge of NOB suppression, largely due to the sustained inhibitory effect of zeolite on endogenous FA concentrations. Analysis of both process data and microbial profiles revealed that the contribution to nitrogen removal in the anammox reactor was mainly derived from the anammox reaction during summer, whereas in winter, DN became the dominant pathway. Moreover, heterotrophic DN in the reactor not only involved the reaction induced by a small addition of exogenous carbon but also likely utilized the slowly biodegradable organic matter released from biomass decay and fragmentation to accomplish endogenous DN-findings that are consistent with other's study [35]. To ensure overall stable nitrogen removal performance during winter, this study

recommends replacing anammox with DN for nitrogen removal during low-temperature periods, while selectively enriching and reinforcing AnAOB based on the annual temperature profile. This approach aligns pathway selection with microbial kinetic optima, enhancing overall resilience. Given that in Ganzhou the reaction temperature remains above 15°C for most of the year and drops only in November and December. The scalable application of the PN/A process can be achieved by operating DN during low-temperature periods and, upon temperature recovery, by adding exogenous hydrazine to selectively enrich AnAOB [35]. At the hardware level, strategies such as steam-assisted heating or enhanced insulation can be implemented to mitigate temperature-induced performance losses and improve nitrogen removal efficiency of the PN/A process. Although low temperatures affect the activity and abundance of functional bacteria, the PN/A nitrogen removal pathway remains a system advantage, and overcoming the constraints imposed by temperature is of significant engineering importance.

4. Conclusions

This study constructed a pilot-scale PN/A reactor to treat low-ammonium RET, and systematically investigated the long-term effects of seasonal temperature fluctuations on an uninsulated system. During warmer periods, the PN/A process achieved a maximum TNRE of 87.5%. However, low temperature resulted in a 58.4% reduction. All core functional microbial groups were negatively impacted by cold stress. Encouragingly, the PN reactor maintained effective suppression of NOB under low temperature, preserving ecological niche integrity, with Nitrosomonas retaining a

relative abundance of 11.46%. In contrast, Candidatus Brocadia-the dominant Anammox bacterium-declined from a peak of 15.26% to 4.89%, whereas DNB demonstrated greater cold tolerance. Analyses of both nitrogen transformation pathways and electron transport further confirmed the superior low-temperature adaptability of DNB compared to AnAOB. These findings suggest that PN/A can enhance denitrification maintains reactor performance during low temperature periods. As temperatures increased, targeted operational strategies enabled the selective enrichment of AnAOB, thereby overcoming the temperature limitations that hinder the broader engineering application of the PN/A process.

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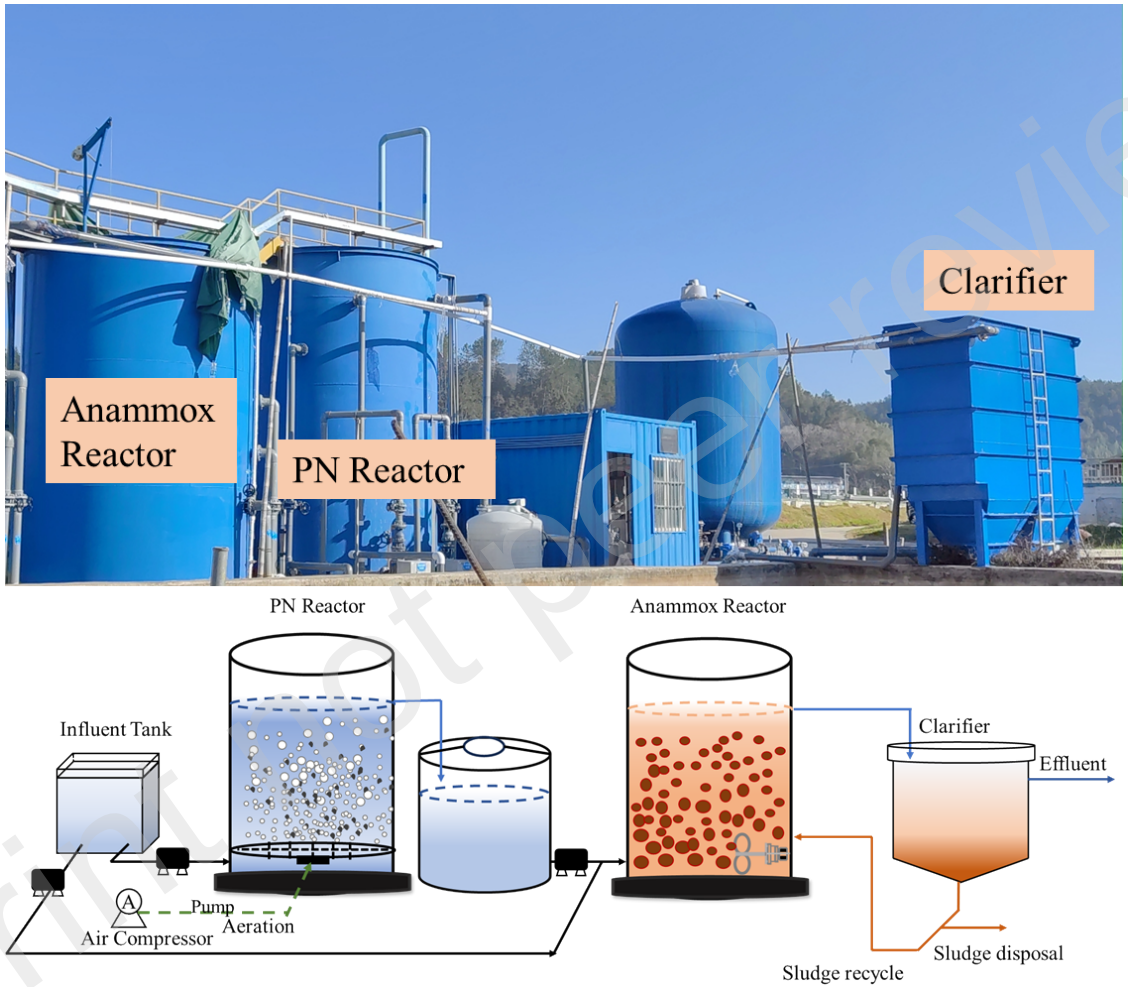

Fig. 1. Schematic of the PN/A reactor.

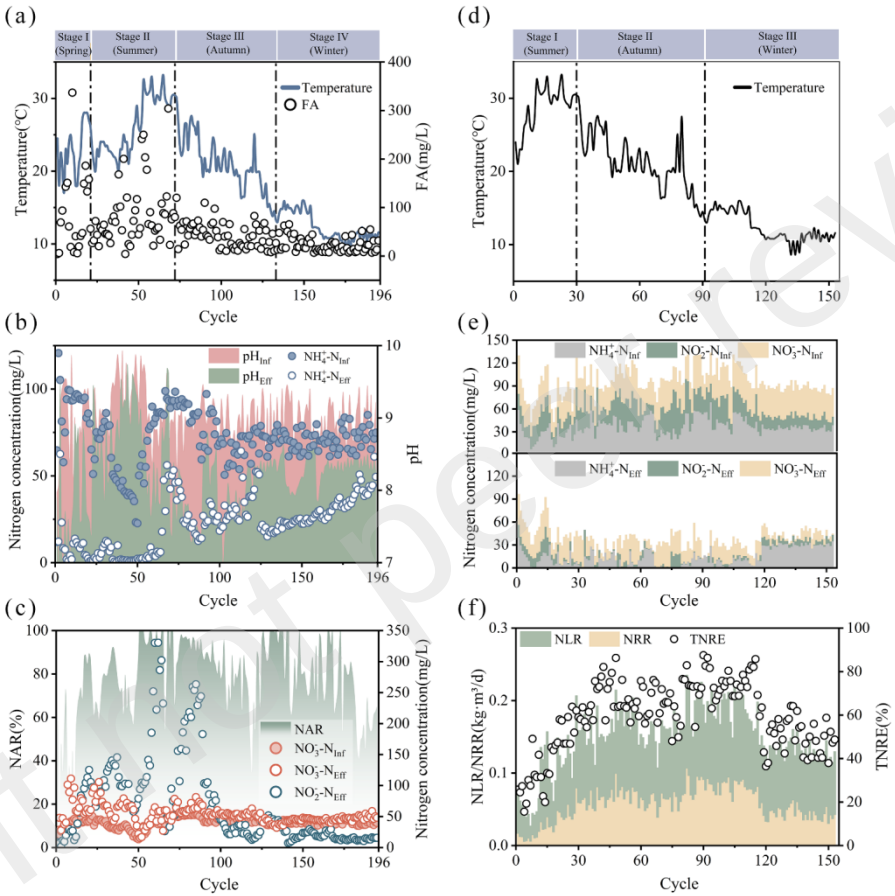

Fig. 2. Performance in the PN/A reactor: (a) FA concentration and temperature of PN reactor; (b) influent and effluent NH_4^+-N, pH of PN reactor; (c) influent and effluent NO_3^--N, effluent NO_2^--N, NAR of PN reactor; (d) temperature of Anammox reactor; (e) influent and effluent nitrogen of Anammox reactor; (f) NLR、NRR and TNRE of Anammox reactor.

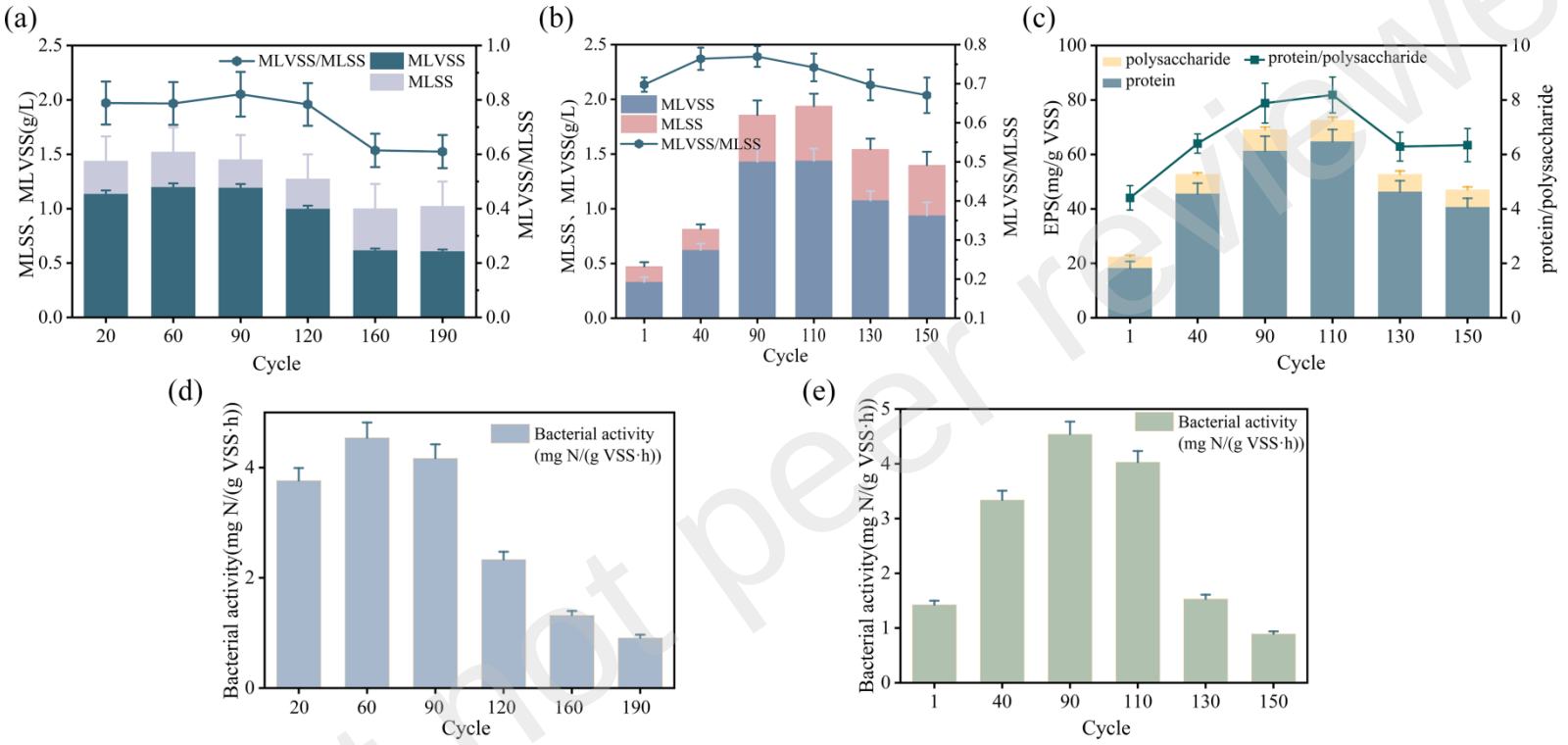

Fig. 3. Characteristics of sludge in PN/A reactor: (a) MLVSS、MLSS and MLVSS/ MLSS of PN reactor; (b) MLVSS、MLSS and MLVSS/ MLSS of Anammox reactor; (c) extracellular polymeric substance of Anammox reactor; (d) SAOA of PN reactor; (e) SAA of Anammox reactor.

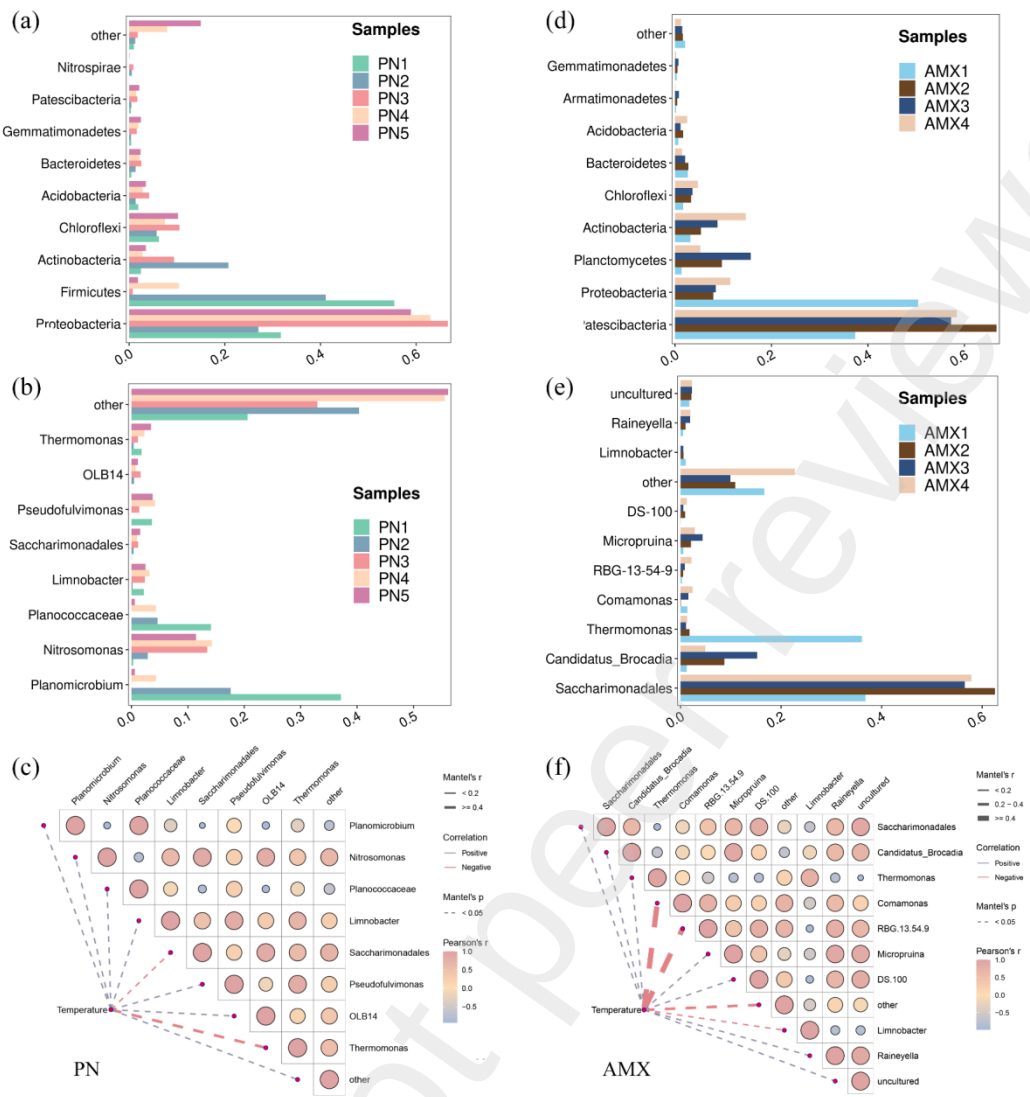

Fig. 4. Taxonomy classification of bacterial community composition of the PN/A reactor (a) phylum level in PN reactor; (b) genus level in PN reactor； (c) correlation between temperature and genus in PN reactor; (d) phylum level in Anammox reactor; (e) genus level in Anammox reactor; (f) correlation between temperature and genus in Anammox reactor.

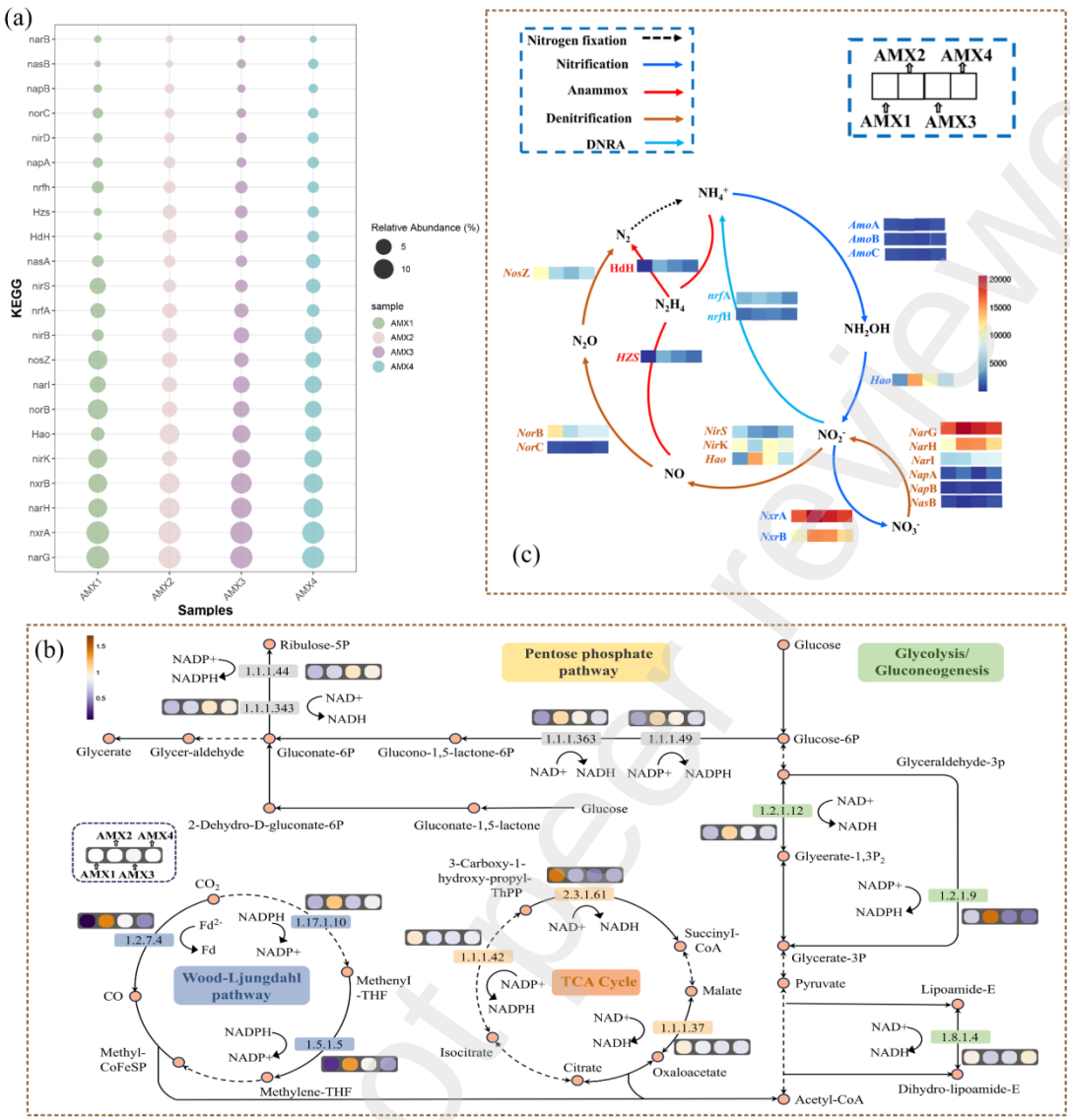

Fig. 5. Metagenomic of the anammox reactor: (a) relative abundance of nitrogen cycle functional genes; (b) nitrogen cycle functional gene mapping; (c) metabolic pathway energy transfer.