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Enhanced nitrogen removal through supernatant-assisted recovery in an ANAMMOX reactor after biomass loss

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Abstract. The ANAMMOX process offers energy-efficient nitrogen removal but remains vulnerable to biomass loss due to the slow growth of ANAMMOX bacteria. This study evaluated the potential for performance recovery after biomass washout by supplementing with supernatant from a stable ANAMMOX reactor. A 5L reactor experienced intentional Mixed Liquor Suspended Solid(MLVSS) loss and was operated under reduced nitrogen loading. Recovery was initiated by adding 4 L of filtered supernatant. Reactor performance improved rapidly, with MLVSS increasing from 340 mg/L to 590 mg/L and Nitrogen Removal Rate(NRR) from 0.076 to 0.1832 kg-N/m³/day within 8 days. Sponge-type carrier media also showed biomass accumulation. The results suggest that the supernatant contained components that stimulated ANAMMOX activity, enabling fast recovery. Supernatant supplementation may offer a practical strategy for restoring ANAMMOX performance after biomass loss.
Keywords: ANAMMOX; biomass loss; carrier media; MLVSS; restoration

1. Introduction

Microorganism based biological treatment has become a cornerstone of modern wastewater management, with growing emphasis on process monitoring, environmental adaptability, and ecological sustainability. Recent studies on wetland-based systems, watershed-scale pollutant dynamics, and sediment-associated contaminants have further underscored the importance of microbial stability and adaptive operation in achieving long-term treatment performance under variable environmental conditions (Kang and Gil 2023, Youn *et al.* 2024, Im and Gil 2023). Anaerobic ammonium oxidation (ANAMMOX) has emerged as a promising and sustainable alternative for nitrogen removal in wastewater treatment (Ibrahim *et al.* 2016). This process enables the direct conversion of ammonium (NH_4^+) and nitrite (NO_2^-) into dinitrogen gas (N_2) under anoxic conditions (Mulder *et al.* 1995), eliminating the need for organic carbon and significantly reducing energy consumption and operational costs (Wett *et al.* 2013, Agrawal *et al.* 2017). Compared to conventional nitrification- denitrification systems, ANAMMOX exhibits several advantages, including lower sludge production, reduced aeration demand, and minimal greenhouse gas emissions (Siegrest *et al.* 2008, Kartal *et al.* 2010). This characteristic is of particular significance in the current era, where climate change driven by global warming has emerged as a critical environmental challenge (Im and Gil 2024, Lee *et al.* 2024). As a result, it has been widely regarded as an innovative solution for meeting increasingly stringent nitrogen discharge regulations. Despite these benefits, the full-scale application of ANAMMOX processes remains limited by several operational challenges. One of the most critical issues is the vulnerability of ANAMMOX biomass to physical disturbances and hydraulic fluctuations, which can lead to the loss of microbe community from the reactor (Morales *et al.* 2015). Since the growth rate of ANAMMOX bacteria is inherently slow, the recovery from biomass washout or loss events is often prolonged and unpredictable (Strous *et al.* 1999, Dosta *et al.* 2008). Such instability not only compromises treatment performance but also raises concerns regarding long-term process reliability (Reino *et al.* 2018, Niederdorfer *et al.* 2021). To enhance the resilience of ANAMMOX systems, various strategies have been proposed, including biofilm-based reactor designs, carrier media, and the use of granular sludge (Egli *et al.* 2001, Vlaeminck *et al.* 2010, Adams *et al.* 2020). However, these approaches primarily serve as preventive measures against biomass loss and may have limited effectiveness in addressing significant biomass washout once it has occurred during reactor operation. Restoring the functionality of an ANAMMOX reactor after biomass washout can be attempted through bioaugmentation (Abdulsalam *et al.* 2011, Herrero and Stuckey 2015). This method involves the addition of concentrated ANAMMOX sludge; however, due to the inherently slow growth rate and strict cultivation requirements of ANAMMOX microorganisms, obtaining sufficient biomass at high concentrations can be challenging (Pimenov *et al.* 2022). Another possible strategy involves the artificial supplementation of quorum sensing (QS) compounds to stimulate microbial activity based on the QS mechanisms of ANAMMOX bacteria (Tang *et al.* 2015, Parin *et al.* 2021). Despite its theoretical potential, the high cost of commercially available QS compounds poses a

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significant limitation to the economic feasibility of this approach. Recent studies have advanced biomass acquisition strategies through enrichment and immobilization techniques, which improve the availability and retention of active ANAMMOX biomass (Liu *et al*. 2025, Fu *et al*. 2023). However, because these methods still rely on continuous biomass production and direct inoculation, they remain resource-intensive and may be insufficient for rapid, cost-effective recovery from sudden biomass loss in full-scale operations. Given these persistent limitations, alternative post-disturbance recovery strategies merit exploration. The supernatant from a well-functioning ANAMMOX reactor offers such an alternative. It is likely to contain biologically active constituents, including suspended or planktonic ANAMMOX cells that remain metabolically active despite not being retained within biomass aggregates (Oshiki *et al*. 2013, Kartal and Keltjens 2016, Okabe *et al*, 2023). Additionally, cell fragments and extracellular polymeric substances (EPS) in the supernatant can promote microbial aggregation or facilitate attachment (Anburajan *et al*. 2021, Wong *et al*. 2023). Signaling molecules such as QS compounds, known to regulate microbial communication and coordination (Valle *et al*. 2004, Waters and Bassler 2005), may also be present. Building on this rationale, this study hypothesizes that the supernatant obtained from a stable ANAMMOX reactor contains biologically active metabolites particularly quorum sensing related substances that can facilitate the reactivation of inhibited or biomass depleted ANAMMOX cultures. This approach offers a practical and cost-effective alternative to conventional bioaugmentation or the addition of commercially sourced QS compounds, with potential for non-invasive, resource-efficient, and scalable application in full-scale systems. The objectives of this study are to evaluate the feasibility of restoring ANAMMOX reactor performance after induced MLVSS loss through supernatant supplementation, and assess its potential to support the functional restoration of microbial activity as part of resilient operational frameworks for maintaining process stability under biomass-related perturbations.

2. Materials and Methods

2.1 Reactor setup and initial operation

A lab-scale anaerobic ammonium oxidation (ANAMMOX) reactor with an effective working volume of 5 L was employed in this study. The initial mixed liquor volatile suspended solids (MLVSS) concentration in the reactor was maintained at 1,800 mg/L. ANAMMOX granule sludge used for inoculation was enriched from activated sludge obtained from the J wastewater treatment facility. The reactor was operated under batch feeding mode with a hydraulic retention time (HRT) of 1.25 days. HRT control was achieved by allowing 20 minutes of quiescent settling, followed by withdrawal of 4 L of supernatant and replenishment with an equal volume of synthetic wastewater. The synthetic wastewater contained ammonium nitrogen (NH_4^+-N) at a concentration of 100 mg/L and nitrite nitrogen (NO_2^--N) at 125 mg/L. In addition, the following components were included to support microbial growth: $KHCO_3$ (1.25 mg/L), KH_2PO_4 (0.01 mg/L), $MgCl_2 \cdot 7H_2O$ (0.14 mg/L), $CaCl_2 \cdot 2H_2O$ (0.14 mg/L), trace element A (1 mL/L), and trace element B (1 mL/L) (van de Graaf *et al*. 1996).

2.2 Mid-term operation: Induction of biomass loss

Following an initial stabilization period of 10 days, a significant biomass loss event was simulated to assess the resilience and recovery potential of the ANAMMOX system. Under fully mixed conditions, 3.5 L of the reactor's internal volume was intentionally withdrawn to mimic accidental biomass washout. Subsequently, the reactor was operated for 7 days with synthetic influent containing 70 mg/L of NH_4^+-N and 90 mg/L of NO_2^--N, while maintaining the HRT at 1.25 days.

2.3 Late-stage operation: Supernatant supplementation and restoration

A restoration strategy was implemented at the end of the mid-term operation using supernatant obtained from a stable ANAMMOX reactor. After 20 minutes of settling, 4 L of supernatant was removed from the reactor and replaced with an equivalent volume of filtered supernatant collected from another stable ANAMMOX reactor. The supplemented supernatant was pre-filtered to remove residual solids, ensuring that only the soluble fraction, including potential quorum sensing (QS) molecules, was introduced. In addition, 35 pieces of porous sponge media made of polyvinyl alcohol (PVA) with a pale ivory color were added to the reactor to provide surface area for microbial attachment and growth during the recovery phase.

Following this supplementation, the reactor was operated with synthetic wastewater containing 70 mg/L of NH_4^+-N and 90 mg/L of NO_2^--N at an HRT of 1.25 days. As the restoration progressed, the influent nitrogen concentrations were gradually increased to evaluate the functional restoration of ANAMMOX activity. The detailed operational conditions for each stage, including influent composition, reactor configuration and specific remarks are summarized in Table 1.

Fig. 1 illustrates the configuration of the experimental system and the sequential operational stages deigned for this study. The initial operation involved starting up a 5 L ANAMMOX reactor using enriched sludge collected from a 60L suspended growth ANAMMOX reactor. In the mid-term operation, accidental biomass loss was simulated by withdrawing 3.5 L of suspended sludge from the reactor while maintaining agitation. During the late-stage operation, the reactor was refilled with 4 L of supernatant obtained from the same 60 L seed reactor to evaluate the recovery potential of ANAMMOX activity through supernatant dosing. This figure thus provides as integrated overview of the experimental sequence and the conceptual framework underlying the recovery assessment.

2.4 Analytical methods

Ammonium nitrogen (NH_4^+-N), nitrite nitrogen (NO_2^--N),

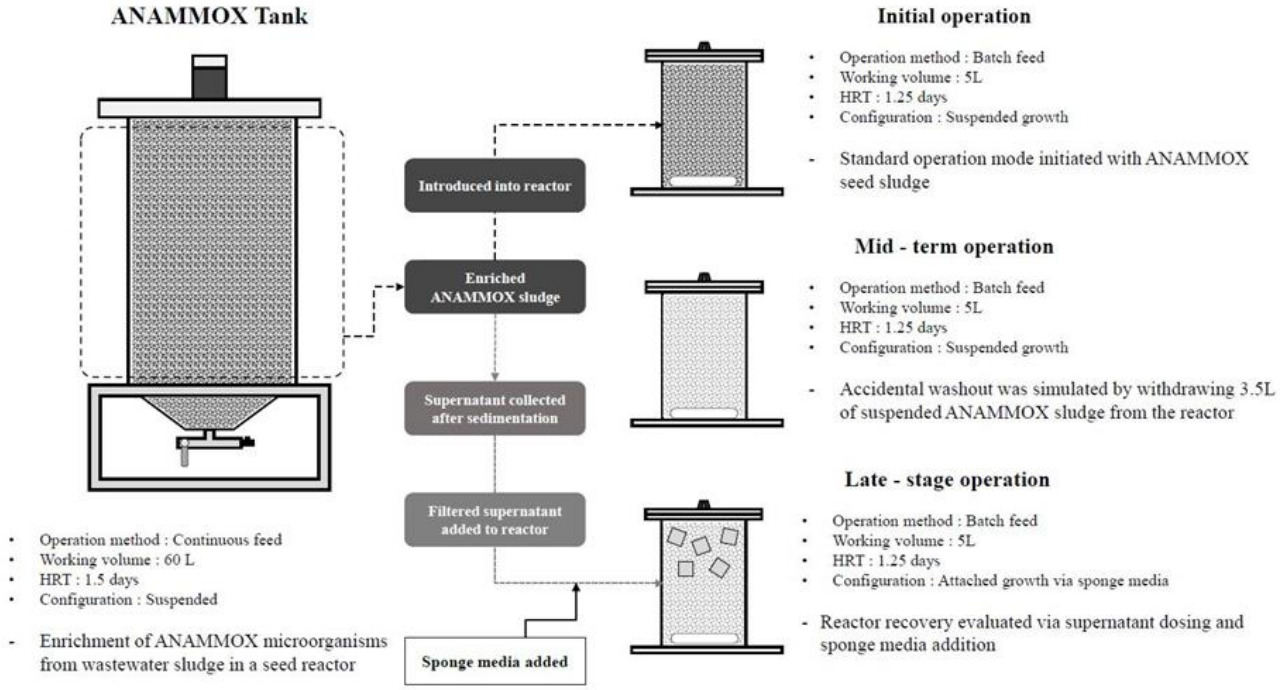

Fig. 1 Visual summary of biomass loss and restoration via supernatant and sponge media addition in ANAMMOX system

Table 1 Summary of operational conditions for each experimental stage

Operational stage	Period (Day)	Influent Ammonium	Influent Nitrite	Remarks
Initial operation	1 – 10	100 mg/L	125 mg/L	Standard operation with ANAMMOX granules
Mid-term operation	11 - 17	70 mg/L	90 mg/L	Biomass loss simulated by withdrawing 3.5L of sludge
Late-stage operation	18 - 33	70 mg/L – 110 mg/L	90 mg/L – 130 mg/L	Supernatant added from stable ANAMMOX reactor

and MLVSS were measured following standard analytical protocols outlined in the Standard Methods for the Examination of Water and Wastewater (APHA *et al*. 2012). The NH_4^+-N and NO_2^--N concentrations were determined using a UV-Vis spectrophotometer (DR3900, Hach, USA) equipped with pre-programmed methods for colorimetric analysis. The instrument utilizes specific wavelength detection for each nitrogen species, enabling accurate quantification in both influent and effluent samples.

Quantification of MLVSS attached to the porous PVA sponge media was conducted by measuring the weight of volatile substances detached from the carrier surface. The measured volatile fraction was considered representative of the MLVSS associated with the biofilm attached to the sponge media (Noor *et al*. 2023). A representative sponge sample was immersed in deionized water and subjected to ultrasonic treatment in an ultrasonic bath for 30 minutes. Manual agitation and scrapping were then applied to ensure thorough removal of the attached biomass (Fonseca *et al*. 2019). The detached suspension was filtered to remove sponge fragments, and the collected solids were subjected to volatile solids analysis at 550 °C using a muffle furnace. The analytical procedure partially followed the Standard Methods for the Examination of Water and Wastewater (APHA *et al*. 2012), with adaptations made to accommodate the characteristics of the sponge material.

3. Results and discussion

3.1 Origin and characterization of inoculum ANAMMOX granules

The ANAMMOX granules used in this study were sourced from a laboratory-scale reactor that had been operated for 427 days and developed through a stepwise enrichment process. The start-up of this seed reactor was initiated using return sludge, which had previously been identified as the most suitable inoculum type for ANAMMOX enrichment. The start-up of this seed reactor was initiated using return sludge collected from the J municipal wastewater treatment plant. The use of return sludge was guided by previous observations by Lee and Gil (2022), which suggested its potential suitability for facilitating ANAMMOX enrichment during the start-up process. The operation was divided into three distinct phases based on nitrogen removal patterns and microbial adaptation. The performance trend of the 60 L ANAMMOX

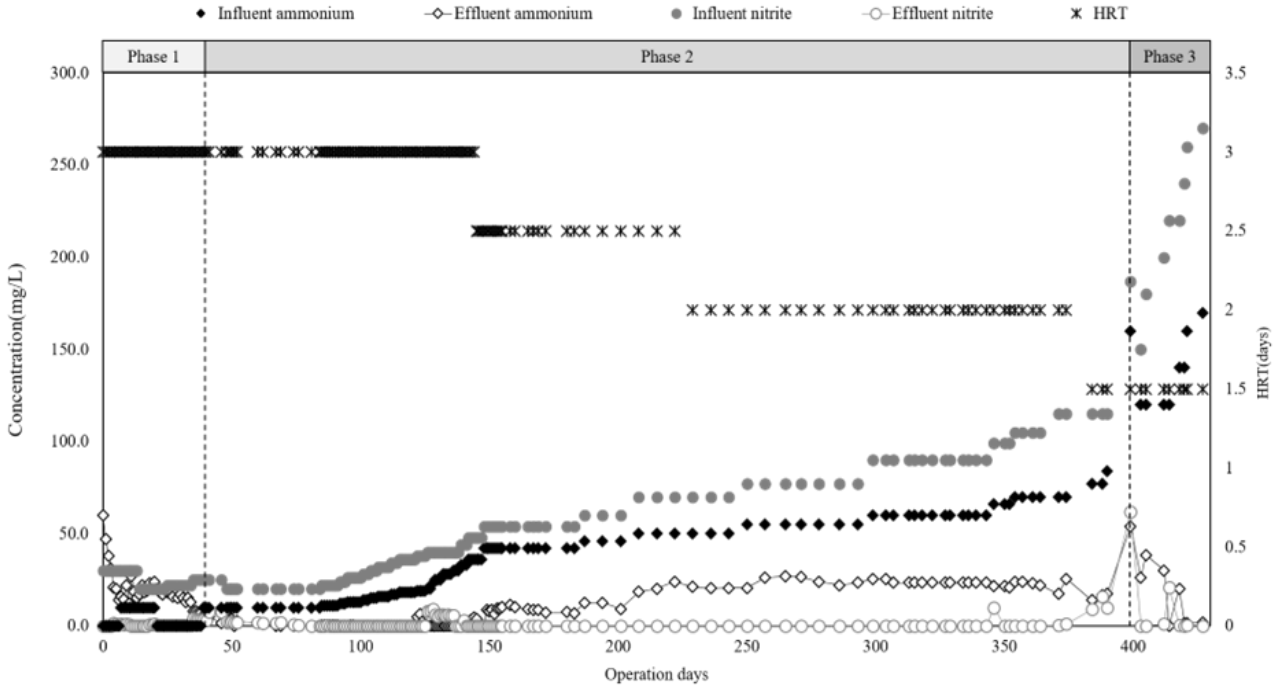

Fig. 2 Operation chart of ANAMMOX granule cultivation reactor

reactor used for inoculum preparation is presented in Fig. 2, reflecting the stable nitrogen removal achieved throughout its long-term enrichment phase.

Phase 1 was characterized by effluent ammonium concentrations exceeding those in the influent. This occurred due to the decomposition of biomass that was not acclimated to the ANAMMOX environment, leading to the release of ammonium and organic matter. The increased organic load temporarily promoted heterotrophic denitrification (Date *et al*. 2009, Tao *et al*. 2013). Phase 2 marked the beginning of simultaneous ammonium and nitrite removal, indicating that the surviving microbial community had adapted to the anaerobic conditions, enabling the gradual proliferation of ANAMMOX bacteria (Chen *et al*. 2012). Phase 3 corresponded to the exponential growth phase of ANAMMOX microorganisms, with a rapid increase in nitrogen removal activity (Deore *et al*. 2022). The seed sludge collected at the end of Phase 3, specifically on Day 427, exhibited a nitrogen removal rate (NRR) of 0.292 kg-N/m³/day and was used as the inoculum for the experimental reactor in this study.

Prior to the operation of the ANAMMOX reactor, a series of preliminary batch tests were performed to quantitatively evaluate the nitrogen removal activity of the inoculated ANAMMOX granule sludge. To ensure the reproducibility and reliability of the obtained results, three independent batch tests, designated as Sample No. 1, Sample No. 2, and Sample No. 3, were conducted under identical operating conditions. Although the start-up phase of the seed reactor exhibited stable enrichment of ANAMMOX biomass, additional batch-scale verification was required to confirm its intrinsic nitrogen conversion capability. Each test was carried out for approximately 11 hours, during which water samples were collected at 2-hour intervals, with an additional measurement taken at the 7-hour mark to capture short-term concentration variations. The summarized outcomes of these reproducibility tests are presented in Fig. 3.

The Sample No.1 (Fig. 3 (a)) experiment began with a mixed liquor volatile suspended solids MLVSS concentration of 925 mg/L. The feed solution included 101 mg/L of NH_4^+-N and 115 mg/L of NO_2^--N. The reactor showed a noticeable reduction in substrate concentrations after 6 hours of operation, with NH_4^+-N and NO_2^--N decreasing to 50 mg/L and 58 mg/L, respectively. Gas bubbles appeared prominently at the 7-hour mark, which coincided with further decreases in NH_4^+-N to 12 mg/L and NO_2^--N to 16 mg/L. The NH_4^+-N concentration dropped to 5.4 mg/L by the 9 hour, while NO_2^--N was completely depleted. Both nitrogen compounds were fully removed by 11 hours, and NH_4^+-N reached 0 mg/L. The Sample No. 2 (Fig. 3(b)) experiment started with an MLVSS concentration of 1,010 mg/L and initial nitrogen concentrations of 100 mg/L for NH_4^+-N and 120 mg/L for NO_2^--N. Within 6 hours, NH_4^+-N and NO_2^--N levels were reduced to 53 mg/L and 60 mg/L, respectively. Unlike Sample No.1, no further removal was observed at the 7-hour mark. However, both nitrogen species were entirely removed by 11 hours. In the Sample No. 3(Fig. 3(c)) test, which began with an MLVSS of 990 mg/L, initial NH_4^+-N and NO_2^--N concentrations were 105 mg/L and 120 mg/L, respectively. At 6 hours, both concentrations were reduced by approximately half, reaching 56 mg/L and 62 mg/L. Similar to Sample No.1, intensive gas production was detected at 7 hours, during which NH_4^+-N and NO_2^--N were further reduced to 12.4 mg/L and 26 mg/L, respectively. Complete removal of both nitrogen compounds was again achieved by 11 hours. All three tests consistently demonstrated rapid nitrogen removal

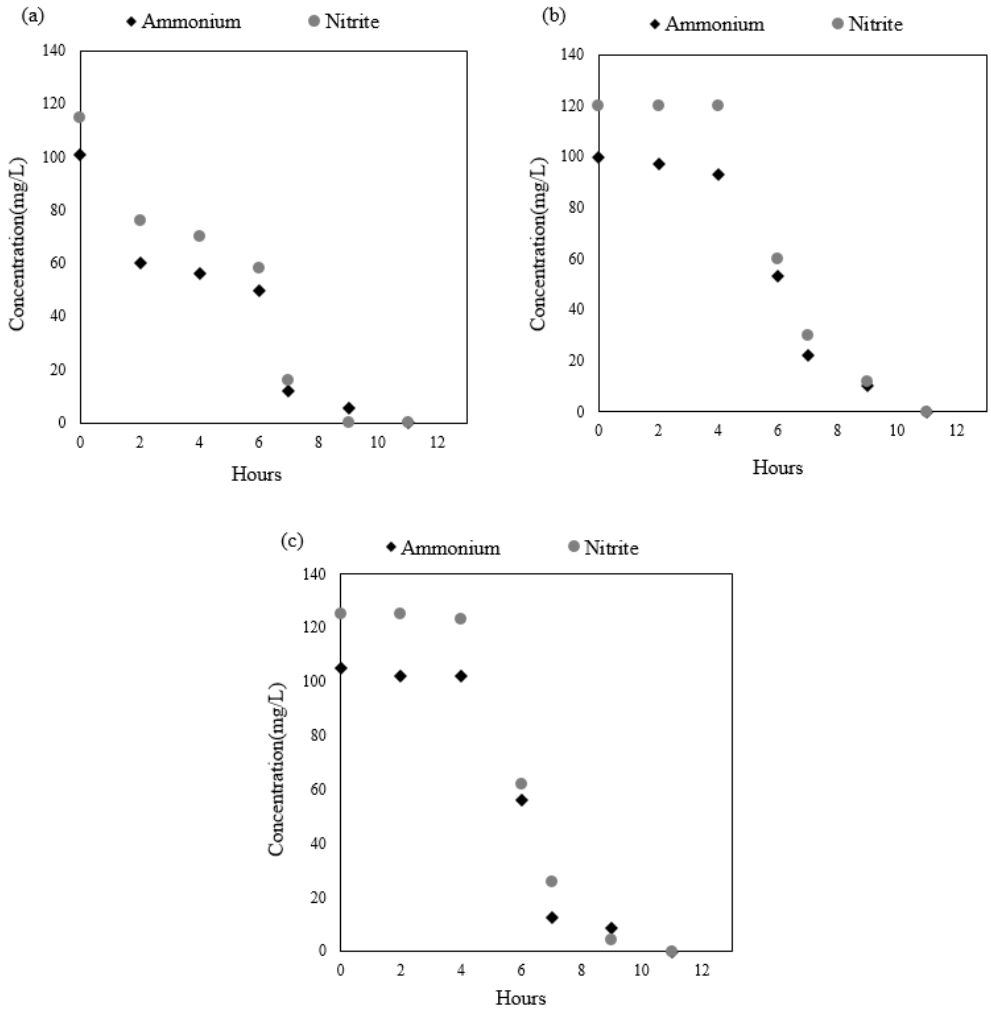

Fig. 3 Preliminary batch test result about ANAMMOX granule sludge (a) Sample No.1, (b) Sample No.2, (c) Sample No.3

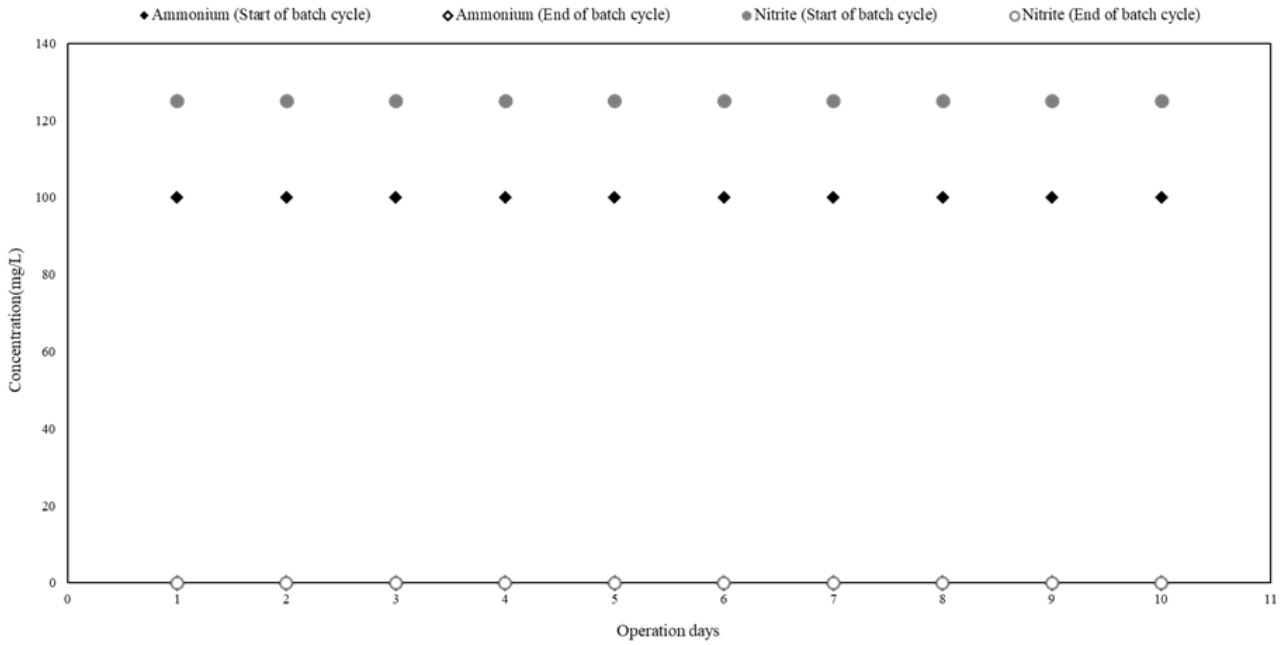

Fig. 4 Comparison of ammonium and nitrite concentrations at the start and end of batch operation during the initial stage of the 5 L ANAMMOX reactor

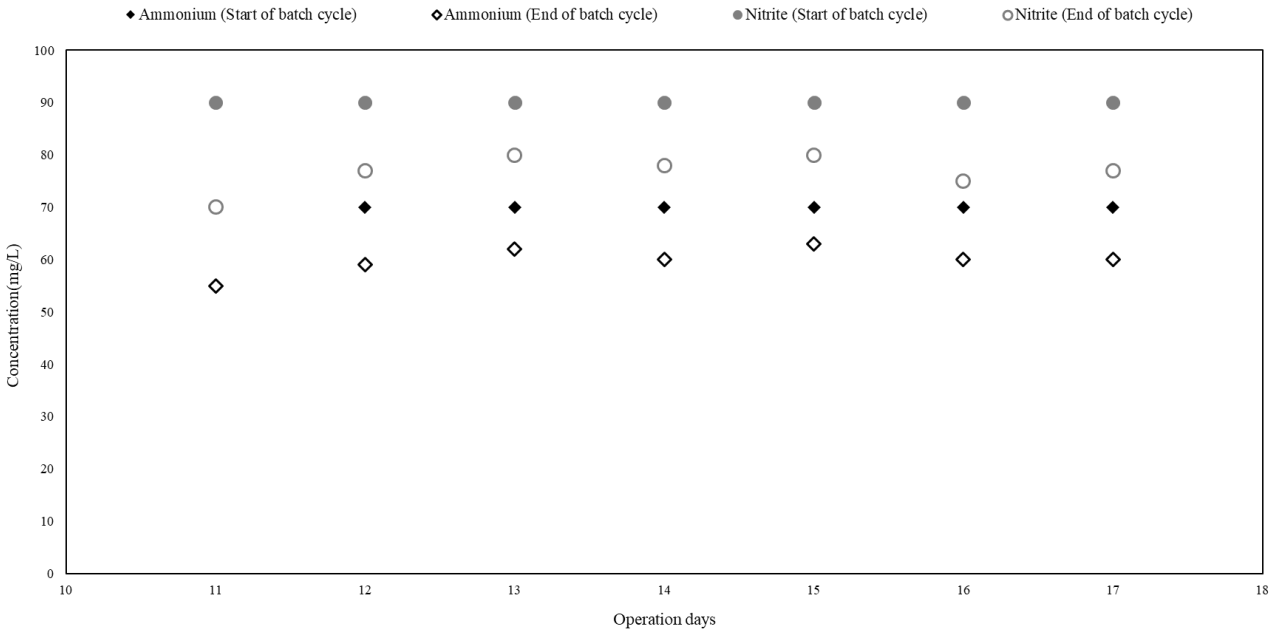

Fig. 5 Variation of ammonium and nitrite concentrations at the start and end of batch operation during the mid-term stage following biomass withdrawal

commencing at 6 hours, with full depletion of NH_4^+-N and NO_2^--N observed at 11 hours. The calculated NRR were 0.471 kg-N/m³/day for Sample No.1, 0.480 kg-N/m³/day for Sample No.2, and 0.501 kg-N/m³/day for Sample No.3. These results validated the high ANAMMOX activity of the inoculated sludge and supported its application in subsequent continuous reactor operation.

Fig. 4 presents the performance of a 5L ANAMMOX reactor operated for 10 days at an HRT of 1.25 days, using the same inoculated sludge as that employed in the preliminary batch tests. The reactor was initially seeded with biomass at an MLVSS concentration of 1,800 mg/L. During the initial 10-day operation period, the reactor exhibited stable nitrogen removal performance under the applied operational conditions. With an influent concentration of 100 mg/L for NH_4^+-N and 125 mg/L for NO_2^--N, effluent concentrations of NH_4^+-N and NO_2^--N were consistently maintained 0 mg/L respectively, indicating active ANAMMOX microbial activity. The MLVSS concentration remained stable at around 1800 mg/L, and no significant fluctuations were observed throughout the period. These results confirm that the ANAMMOX biomass had successfully acclimated to the reactor environment and the synthetic feed composition.

3.2 Reactor operation under biomass loss conditions

The reactor was subjected to a simulated biomass loss event by removing 3.5 L of mixed liquor while maintaining complete mixing. This intervention was followed by the introduction of synthetic influent adjusted to achieve target concentrations of 70 mg/L for NH_4^+-N and 90 mg/L for NO_2^--N. At the beginning of this operational phase, the MLVSS concentration in the reactor was measured at 320 mg/L as MLVSS. A 7-day operation under these conditions was conducted with a constant HRT of 1.25 days. The performance of the ANAMMOX reactor during this period is illustrated in Fig. 5.

Effluent concentrations of NH_4^+-N and NO_2^--N were initially measured at 55 mg/L and 70 mg/L, respectively. As the operation progressed, both nitrogen species in the effluent gradually increased. By day 17, NH_4^+-N had reached 60 mg/L, while NO_2^--N rose to 77 mg/L. This trend indicates a progressive decline in nitrogen removal efficiency, likely attributable to the significant loss of ANAMMOX biomass. The reduced microbial activity within the reactor, resulting from the biomass washout, appears to have impaired the system's ability to effectively remove NH_4^+-N and NO_2^--N.

3.3 Recovery operation using supernatant addition

The restoration phase began after a 18 day period of impaired reactor performance. The strategy involved replacing 4 L of the reactor content, following a 20 minutes settling period, with an equal volume of filtered supernatant obtained from a separate, stably operated ANAMMOX reactor. The supernatant was pre-filtered to remove solids, thereby introducing only the soluble fraction, which was expected to contain planktonic ANAMMOX cells, extracellular substances, and potentially QS molecules. The operational results following the addition of the supernatant are presented in Fig. 6.

Synthetic influent was subsequently reintroduced at concentrations of 70 mg/L for NH_4^+-N and 90 mg/L for NO_2^--N, maintaining the same HRT of 1.25 days. At the beginning of the recovery operation following supernatant addition, the effluent concentrations of NH_4^+-N and NO_2^--N were measured at 30 mg/L and 35 mg/L, respectively. These values represented approximately a two-fold improvement

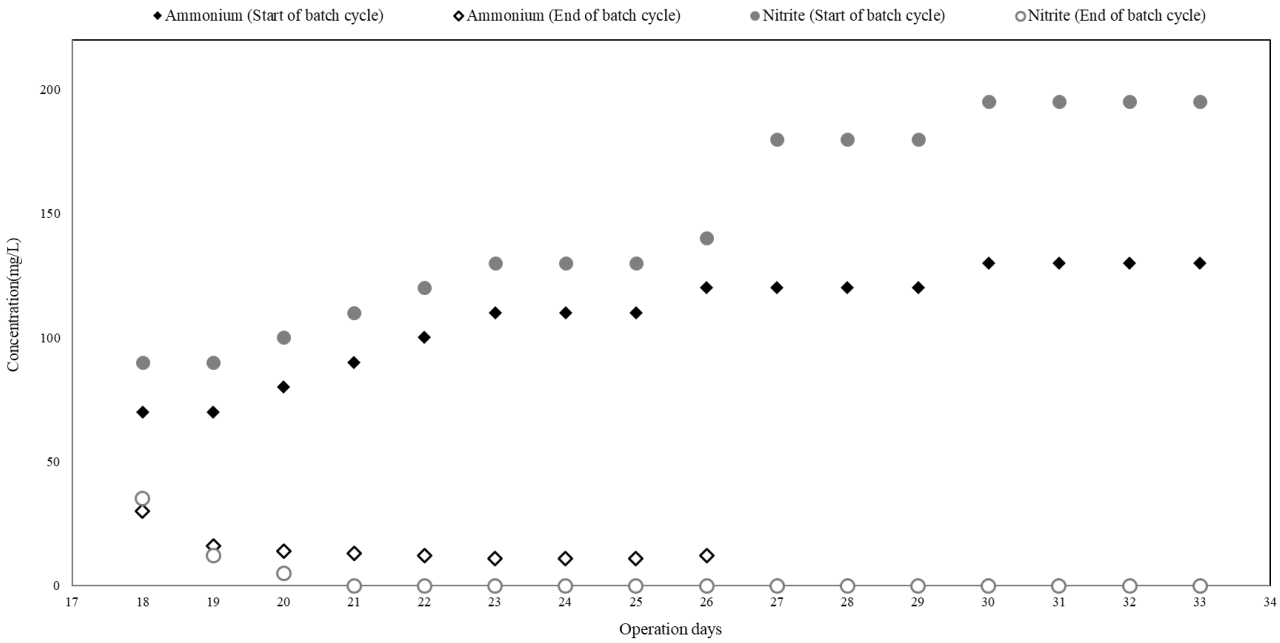

Fig. 6 Variation of ammonium and nitrite concentrations at the start and end of batch operation during the late-stage restoration with supernatant supplementation

compared to those observed during the biomass loss phase, suggesting a notable enhancement in nitrogen removal performance. On day 19, the effluent NH_4^+-N concentration further decreased to 16 mg/L, while the NO_2^--N concentration remained at 35 mg/L, indicating continued improvement in reactor performance. On day 20, the influent nitrogen concentrations were increased to 80 mg/L for NH_4^+-N and 100 mg/L for NO_2^--N. Under these conditions, the effluent NH_4^+-N and NO_2^--N concentrations were measured at 14 mg/L and 5 mg/L, respectively. Despite the increased substrate loading, both nitrogen species in the effluent decreased, confirming the active involvement of ANAMMOX bacteria in nitrogen removal. On days 21 and 22, the influent NH_4^+-N concentrations were further raised to 90 mg/L and 100 mg/L, respectively, while NO_2^--N concentrations were adjusted to 110 mg/L and 120 mg/L. As a result, the effluent NH_4^+-N concentration dropped to 12 mg/L, and NO_2^--N was completely removed from the system. These findings clearly demonstrate a recovery of ANAMMOX activity and its capacity to handle increased nitrogen loading. The reactor was operated for an additional three days with increased influent concentrations of 110 mg/L for NH_4^+-N and 130 mg/L for NO_2^--N. Under these conditions, the effluent NH_4^+-N concentration was measured at 11 mg/L, while NO_2^--N was completely removed, reaching 0 mg/L. These results indicate that approximately 90% of the influent NH_4^+-N was successfully removed, despite the previous biomass loss. High removal efficiencies for nitrogenous substrates were observed however, measurable concentrations of residual ammonium remained in the effluent. The fact that complete removal of ammonium and nitrite was achieved during the initial operation period prior to biomass loss indicates that the ANAMMOX microbial activity had not been fully restored following the recovery process. This result suggests that, although the system regained substantial nitrogen removal capacity, the functional performance of the microbial community did not entirely return to its pre-disturbance state. In response, the influent nitrite concentration was increased from 140 mg/L to 180 mg/L on Date 27, thereby adjusting the ammonium- to-nitrite ratio in the reactor to approximately 1:1.5. Following this adjustment, both ammonium and nitrite were completely removed from the system, indicating a substantial improvement in ANAMMOX performance. Sustained complete removal of nitrogenous compounds was observed even after the influent concentrations were increased while maintaining the ammonium-to-nitrite ratio at 1:1.5 following Day 28, when complete nitrogen removal was initially achieved. This continued performance indicates that the ANAMMOX process remained functionally stable under elevated substrate loading conditions, provided that the stoichiometric balance was maintained. Such a high removal efficiency suggests that the supernatant introduced during the recovery phase may have contained substances capable of stimulating ANAMMOX microbial activity. It is presumed that these substances, possibly including soluble microbial products or QS molecules, contributed to the reactivation and functional recovery of ANAMMOX bacteria following the biomass washout event. Complete removal of ammonium during the recovery phase of ANAMMOX activity appeared to require nitrite concentrations approximately 1.5 times higher than those of ammonium. This ratio exceeds the commonly reported stoichiometric range of 1:1 to 1:1.32 for ammonium to nitrite in conventional ANAMMOX processes (Verma *et al*. 2021), indicating a substantially increased nitrite demand (Liu *et al*. 2019). For reference, the canonical ANAMMOX stoichiometry is represented as:

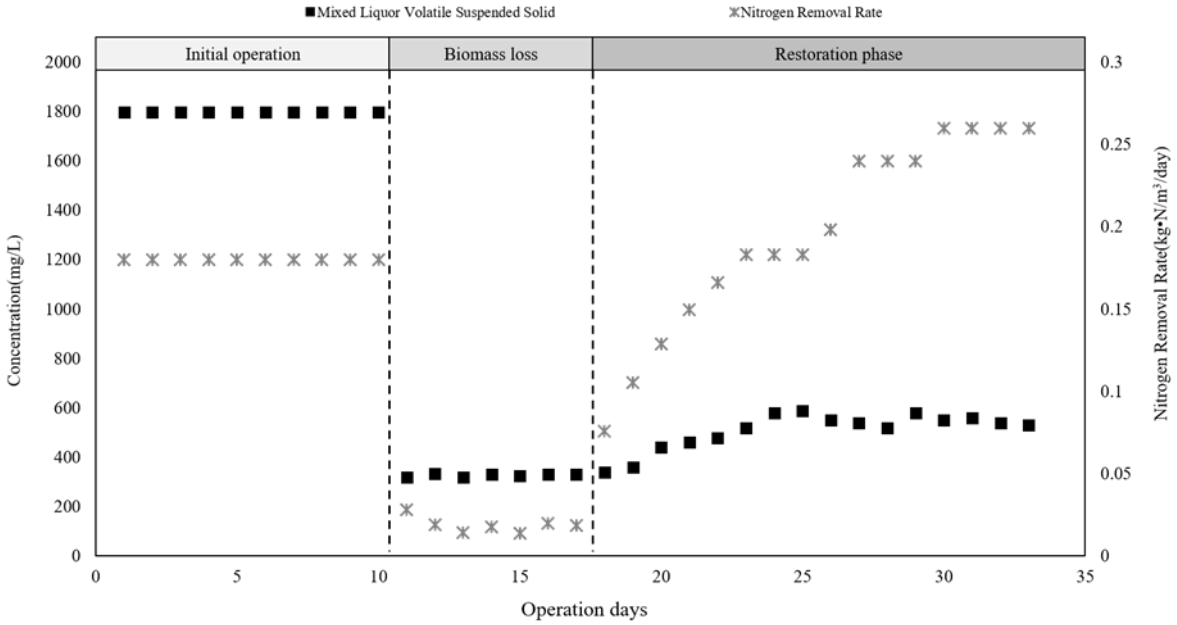

Fig. 7 Temporal trends of MLVSS concentration and nitrogen removal rate during the operation of the ANAMMOX reactor

$$NH_4^+ + 1.32\ NO_2^- \rightarrow 1.02\ N_2 + 0.26\ NO_3^- + 0.13\ H^+ + 0.0666\ Biomass + 2.03\ H_2O \quad (1)$$

where "Biomass" corresponds to the empirical formula $CH_2O_{0.5}N_{0.15}$. This expanded form accounts for proton production, water formation, and biomass synthesis, which is relevant for interpreting both nitrogen transformations and alkalinity changes in the reactor (Strous *et al*. 1999).

The elevated requirement is presumed to result from the concurrent activation of denitrifying bacteria within the sludge community, which may have been stimulated alongside ANAMMOX microorganisms through the addition of supernatant. Certain compounds that enhance ANAMMOX activity are also likely to promote the metabolic activity of denitrifiers. This effect is potentially attributable to the fact that both ANAMMOX bacteria and typical denitrifying species—such as *Micrococcus denitrificans*, *Comamonas nitrativorans*, and *Pseudomonas aeruginosa*—are classified as Gram-negative organisms and are presumed to share common QS compounds (Etchebehere *et al*. 2001, Pokorna *et al*. 2015). While this interpretation aligns with established knowledge of microbial interactions, it is important to recognize that the present study did not include direct measurements of EPS or QS molecules in the supernatant. Accordingly, the proposed mechanism should be regarded as a preliminary interpretation based on indirect evidence, and future investigations could benefit from detailed chemical and molecular analyses to elucidate the specific roles of these constituents in facilitating process recovery.

3.3.1 Biomass recovery in reactor after supernatant addition

Improved removal of NH_4^+-N and NO_2^--N was observed following the addition of supernatant to the reactor, which had previously experienced biomass loss. To further investigate the underlying mechanisms of this recovery, the concentration of microorganisms in the reactor was assessed by measuring the MLVSS. Fig. 7. illustrates the temporal variation in MLVSS concentration during the recovery operation after supernatant supplementation.

The initial phase showed stable MLVSS at 1800 mg/L and NRR at 0.18 kg-N/m³/day, indicating consistent activity of the inoculated ANAMMOX granules. Biomass was intentionally removed on Day 11, leading to a sharp decline in MLVSS to 320 mg/L and a reduction in NRR to 0.028 kg-N/m³/day, which further decreased to 0.0136 kg-N/m³/day by Day 15. The introduction of supernatant resulted in a gradual increase in MLVSS, reaching 590 mg/L on Day 25 and stabilizing at 530 mg/L by Day 33. In parallel, NRR increased continuously from 0.076 kg-N/m³/day to a final value of 0.26 kg-N/m³/day, surpassing the initial performance level. Nitrogen removal rates recorded during the later stage of operation were higher than those in the initial phase. This increase aligned with elevated concentrations of nitrogenous compounds in the influent. Fig. 6 shows that nitrogen removal during this period involved both ANAMMOX microorganisms and denitrifying bacteria. A correlation MLVSS and NRR was identified from the initial operation phase through the biomass loss and into the early restoration phase. Increased MLVSS concentrations were accompanied by proportional increases in NRR, indicating that nitrogen removal efficiency was dependent on the amount of ANAMMOX biomass present in the reactor. MLVSS levels remained stable between Day 25 and Day 33 of operation, with an average concentration of 551 mg/L, a standard deviation of 22.6 mg/L, a coefficient of variation of 4.10%, and a range of 520–590 mg/L, indicating minimal fluctuation in suspended biomass. Over the same period, NRR continued to increase, suggesting that the improvement

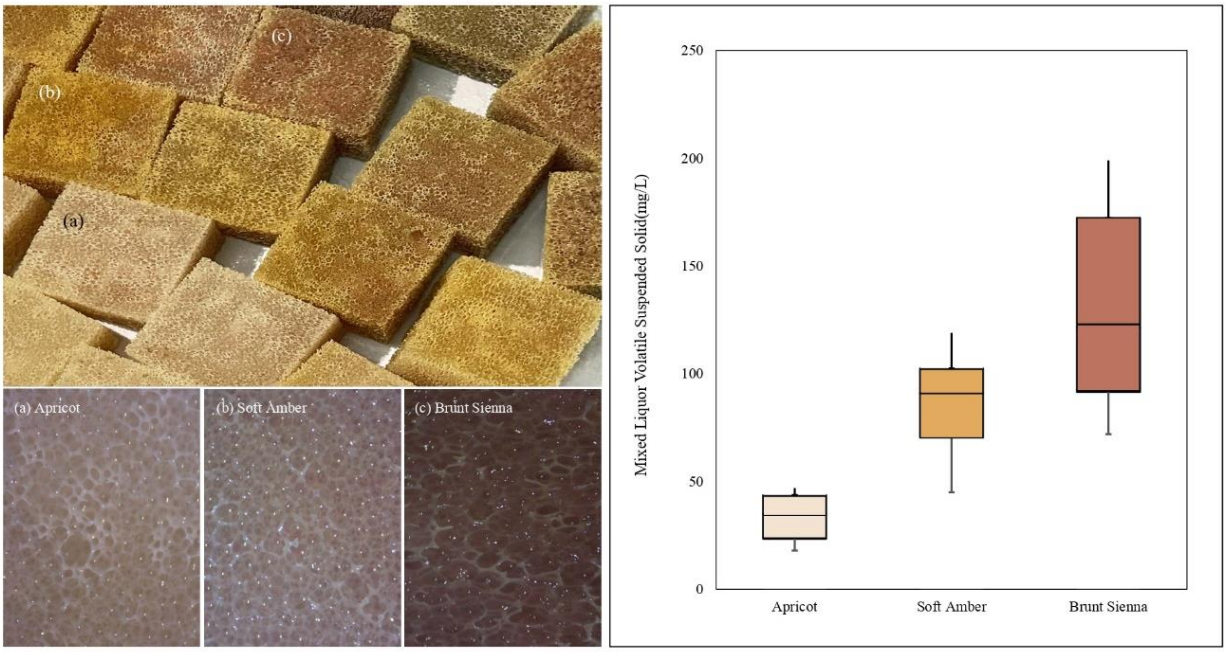

Fig. 8 Variation in MLVSS concentration on sponge media by color category

in nitrogen removal performance was driven by microbial growth on sponge carriers and the associated development of attached biofilms, rather than by an increase in suspended-phase biomass.

3.3.2 Evaluation of ANAMMOX biomass accumulation on sponge media

A rapid increase in NRR was observed during the restoration phase, whereas MLVSS showed a relatively moderate recovery. Along with these quantitative trends, visual changes were also evident within the reactor. The turbidity of the bulk liquid decreased progressively, resulting in a clearer appearance, and the sponge media exhibited noticeable color changes. These observations indicate that microorganisms present in the reactor attached to the surface of the sponge media during mixing and subsequently grew on the carriers. Sponge media with visible microbial accumulation formed a floating layer in the upper region of the reactor. Numerous air pockets were observed surrounding the media, likely caused by nitrogen gas produced through ANAMMOX activity on the biofilm-attached surfaces. These visual changes support the assumption of active microbial colonization and gas production on the sponge carriers. MLVSS distribution within the sponge media was investigated by measuring MLVSS in the internal sections of the carriers. The results are presented in Fig. 8.

On Day 33 of operation, sponge media in the reactor were classified into three distinct color categories based on visual appearance: Apricot, Soft Amber, and Burnt Sienna. Visual inspection revealed a clear trend in which darker and redder coloration corresponded to greater microbial colonization, suggesting progressive biomass accumulation on the media. To quantitatively verify this observation, MLVSS measurements were conducted for sponge media from each color category. A total of 35 sponge media were identified, with 8 categorized as Apricot, 10 as Soft Amber, and 17 as Burnt Sienna. Eight sponge media were selected from each color group for MLVSS analysis. The Apricot-colored media showed a minimum MLVSS value of 18 mg/L, a maximum of 47 mg/L, and an average of 35 mg/L. The Soft Amber group presented values ranging from 45 to 119 mg/L, with an average of 91 mg/L. The Burnt Sienna group exhibited the highest biomass levels, with MLVSS ranging from 72 to 199 mg/L and an average of 121.5 mg/L. The original color of the sponge media was pale ivory. A clear trend was observed in which darker and redder coloration correlated with higher MLVSS concentrations, suggesting progressive microbial colonization. Based on the average MLVSS values and the number of sponges in each color group, the total amount of biomass attached to the sponge media was estimated to be 3255.5 mg. This corresponds to an equivalent MLVSS concentration of 651.1 mg/L in the reactor, representing biomass attached to the sponge media, and is approximately 1.22 times higher than the suspended-phase MLVSS value of 530 mg/L observed on Day 33 (Fig. 7). The distinction between these two measurements indicates that the continuous improvement in NRR during the restoration phase was primarily attributable to microbial growth on the carriers and the associated development of attached biofilms, rather than an increase in suspended biomass.

3.4 Summary of operational stages

The following synthesis summarizes the key observations from each operational stage, highlighting the evolution of MLVSS levels, attached biomass accumulation, nitrogen removal rates, and major performance changes. Table 2 compiles these results to facilitate direct comparison of

Table 2 Summary of operational conditions for each experimental stage

Operational stage	MLVSS (mg/L)	Attached biomass (eq. MLVSS)	NRR (kg·N/m^3/day)	Key observations
Initial operation	1800	-	0.18	Stable nitrogen removal
Mid-term operation	320 - 330	-	0.0136	Significant performance decline due to biomass loss
Late-stage operation	340 - 590	651	0.26	Recovery of performance; biofilm formation on sponge media

system behavior during the initial operation, mid-term biomass loss, and late-stage recovery phases.

Table 2 provides a consolidated summary of the operational progression of the ANAMMOX reactor, capturing key parameters such as suspended MLVSS concentrations, attached biomass levels, and nitrogen removal performance across three distinct phases: initial operation, mid-term biomass loss, and late-stage recovery. During the initial operation, high suspended MLVSS levels supported stable nitrogen removal activity, establishing a baseline for reactor performance. The mid-term phase, characterized by a simulated biomass loss event, resulted in a sharp decline in suspended MLVSS and a corresponding reduction in NRR, highlighting the system's vulnerability to biomass washout. In the late-stage recovery phase, although suspended MLVSS showed only moderate improvement, a substantial increase in attached biomass on sponge media was observed, with concentrations exceeding those of the suspended phase. This accumulation coincided with a marked recovery and eventual enhancement of NRR, indicating that biofilm formation on the carriers played a pivotal role in restoring system functionality and resilience.

4. Conclusions

This study demonstrated the potential for recovering ANAMMOX reactor performance following biomass loss by utilizing supernatant obtained from a stable ANAMMOX system and the following conclusions were obtained.

1. The removal of 3.5 L of mixed liquor from the ANAMMOX reactor resulted in a sharp decrease in biomass concentration, with MLVSS dropping from 1800 mg/L to 320 mg/L. This reduction led to a significant decline in ANAMMOX activity, as reflected by a drop in NRR from 0.18 to 0.028 kg-N/m³/day, and further down to 0.0136 kg-N/m³/day by Day 15. These findings confirm the high sensitivity of the ANAMMOX process to biomass loss and its strong dependence on maintaining adequate microbial concentration within the system.

2. Throughout the recovery phase, MVLSS in the reactor increased from 340 mg/L to 590 mg/L within 8 days, accompanied by a corresponding rise in NRR from 0.076 to 0.1832 kg-N/m³/day. These results indicate that the supernatant not only supported microbial retention but also likely provided biochemical or signaling components that enhanced ANAMMOX bacterial activity.

3. The concentration of MLVSS attached to the porous PVA-based sponge media on Day 33 of reactor operation was 651.1mg/L which is 1.22 times higher than the suspended phase MLVSS concentration measured at the same time. This suggests that the combined use of supernatant and support media may enhance the robustness and resilience of ANAMMOX systems under stress conditions.

In conclusion, supernatant supplementation offers a cost-effective and resource-efficient approach for restoring ANAMMOX reactor function following biomass loss. Beyond laboratory scale validation, this strategy demonstrates strong scalability and seamless compatibility with full-scale wastewater treatment operations, making it particularly valuable as a contingency measure in practical field applications. Its minimal infrastructure requirements, adaptability to existing reactor configurations, and avoidance of direct biomass inoculation or costly QS compound dosing underscore its practicality for rapid, on-site deployment. By enabling prompt recovery and maintaining stable nitrogen removal performance, this method has the potential to significantly reduce operational downtime and enhance process resilience under variable field conditions. Future research should focus on comprehensive chemical and molecular characterization of ANAMMOX reactor supernatant to identify active constituents such as EPS and QS molecules and to elucidate their individual contributions to process recovery. These insights will facilitate the development of targeted, mechanism-based recovery protocols that strengthen the robustness of ANAMMOX systems in large-scale wastewater treatment facilities.

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References

Abdulsalam, S., Bugaje, I., Adefila, S. and Ibrahim, S. (2011), "Comparison of biostimulation and bioaugmentation for remediation of soil contaminated with spent motor oil", *Int. J. Environ. Sci. Technol.*, **8**, 187-194. https://doi.org/10.1007/BF03326208

Adams, M., Xie, J., Xie, J. Chang, Y., Guo, M., Chen, C. and Zhang, T.C. (2020), "The effect of carrier addition on Anammox start-up and microbial community: A review", *Rev. Environ. Sci. Biotechnol.*, **19**, 355-368. https://doi.org/10.1007/s11157-020-09530-4

Agrawal, S., Karst, S.M., Gilbert, E.M., Horn, H., Nielsen, P.H. and Lackner, S. (2017), "The role of inoculum and reactor

configuration for microbial community composition and dynamics in mainstream partial nitritation anammox reactors", *MicrobiologyOpen*, **6**(4), e00456. https://doi.org/10.1002/mbo3.456

Anburajan, P., Kim, Y., Rice, S.A. and Oh, H.S. (2021), "Bacterial signaling and signal responses as key factors in water and wastewater treatment", *J. Water Proc. Eng*., **44**, 102434. https://doi.org/10.1016/j.jwpe.2021.102434

APHA, Awwa, WEF. (2012), "Standard methods for Examination of Water and Wastewater", 22nd ed. American Public Health Association, Washington, DC, USA.

Chen, C.J., Huang, X.X., Lei, C.X., Zhu, W.J., Chen, Y.X. and Wu, W.X. (2012), "Improving Anammox start-up with bamboo charcoal", *Chemosphere*, **89**(10), 1224-1229. https://doi.org/10.1016/j.chemosphere.2012.07.045

Date, Y., Isaka, K., Ikuta, H., Sumino, T., Kaneko, N., Yoshie, S., Tsuneda, S. and Inamori, Y. (2009), "Microbial diversity of anammox bacteria enriched from different types of seed sludge in an anaerobic continuous-feeding cultivation reactor", *J. Biosci. Bioeng*., **107**(3), 281-286. https://doi.org/10.1016/j.jbiosc.2008.11.015

Deore, R., Kumar, R., Mirza, M.W. and Khan, A.A. (2022), "Selecting suitable seed sludge for anammox enrichment: Role of influent characteristics and reactor operational conditions", *Bioresour. Technol*., **347**, 126719. https://doi.org/10.1016/j.biortech.2022.126719

Dosta, J., Fernández, I., Vázquez-Padín, J.R., Mosquera-Corral, A., Campos, J.L., Mata-Alvarez, J. and Méndez, R. (2008), "Short- and long-term effects of temperature on the Anammox process", *J. Hazard. Mater*., **154**(1-3), 688-693. http://doi.org/10.1016/j.jhazmat.2007.10.082

Egli, K., Fanger, U., Alvarez, P.J.J., Siegrist, H., Van der Meer, J.R. and Zehnder, A.J.B. (2001), "Enrichment and characterization of an anammox bacterium from a rotating biological contactor treating ammonium-rich leachate", *Arch. Microbiol*., **175**, 198-207. https://doi.org/10.1007/s002030100255

Etchebehere, C., Errazquin, M.I., Dabert, P., Moletta. R. and Muxí, L. (2001), "Comamonas nitrativorans sp. nov., a novel denitrifier isolated from a denitrifying reactor treating landfill leachate", *Int. J. Syst. Evol. Microbiol*., **51**(3), 977-983. https://doi.org/10.1099/00207713-51-3-977

Fonseca, D.L. and Bassin, J.P. (2019), "Investigating the most appropriate methods for attached solids determination in moving-bed biofilm reactors", *Bioprocess Biosyst. Eng*., **42**, 1867-1878. https://doi.org/10.1007/s00449-019-02182-x

Fu, Y., Wen, X., Huang, J., Sun, D., Jin, L. (2023). "Advances in the efficient enrichment of anammox bacteria", *Water*, **15**(14), 2556. https://doi.org/10.3390/215142556

Herrero, M. and Stuckey, D.C. (2015), "Bioaugmentation and its application in wastewater treatment: a review", *Chemosphere*, **140**, 119-128. https://doi.org/10.1016/j.chemosphere.2014.10.033

Ibrahim, M., Yusof, N., Mohd Yusoff, M.Z. and Hassan, M.A. (2016), "Enrichment of anaerobic ammonium oxidation (anammox) bacteria for short start-up of the anammox process: A review", *Desalin. Water Treat*., **57**(30), 13958-13978. https://doi.org/10.1080/19443994.2015.1063009

Im, J. and Gil, K. (2023), "Characteristics of micro-plastics in stormwater sediment basin: Case study of J wetland", *Membr. Water Treat*., **14**(4), 147-153. https://doi.org/10.12989/mwt.2023.14.4.147

Im, J. and Gil, K. (2024), "Research on effects of reducing temperature and CO_2 emissions by green wall: Case study of G city", *Ecol. Eng*., **208**, 107382. https://doi.org/10.1016/j.ecoleng.2024.107382

Kang, C. and Gil, K. (2023), "Constructing an Internet of things wetland monitoring device and a real-time wetland monitoring system", *Membr. Water Treat*., **14**(4), 155-162. https://doi.org/10.12989/mwt.2023.14.4.155

Kartal, B. and Keltjens, J.T. (2016), "Anammox biochemistry: A tale of heme c proteins", *Trends Biochem. Sci*., **41**(12), 998-1011. https://doi.org/10.1016/j.tibs.2016.08.015

Kartal, B., Kuenen, J.G. and van Loosdrecht, M.C.M. (2010), "Sewage treatment with anammox", *Science*, **328**(5979), 702-703. http://doi.org/10.1126/science.1185941

Lee, B., Kang, C. and Gil, K. (2024), "Effects of upstream pollution patterns on the water quality of Paldang Lake", *Membr. Water Treat.*, **15**(4), 185-192. https://doi.org/10.12989/mwt.2024.15.4.185

Lee, D. and Gil, K. (2022), "Influence of sludge type, MLSS, and substrate ratio on stable implementation of ANAMMOX". *Ecol. Eng*., **178**, 106564. https://doi.org/10.1016/j.ecoleng.2022.106564

Liu, T., Hu, S., Yuan, Z. and Guo, J. (2019), "High-level nitrogen removal by simultaneous partial nitritation, anammox and nitrite/nitrate-dependent anaerobic methane oxidation", *Water Res*., **166**, 115057. https://doi.org/10.1016/j.watres.2019.115057

Liu W., Hou, L., Huang F., Pan, Z., Li, J., Tang, P., Zhu, Y., Li, D. and Yao, X. (2025). "Advancements in entrapment immobilization technology for enhancing Anammox process: Material effects, preparation strategy and application", *Bioresour. Tech*., **433**, 132741. https://doi.org/10.1016/j.biortech.2025.1327

Morales, N., Val del Río, Á., Vázquez-Padín, J.R., Méndez, R., Mosquera-Corral, A. and Campos, J.L. (2015), "Integration of the Anammox process to the rejection water and main stream lines of WWTPs", *Chemosphere*, **140**, 99-105. http://doi.org/10.1016/j.chemosphere.2015.03.058

Mulder, A., van de Graaf, A.A., Robertson, L.A. and Kuenen, J.G. (1995), "Anaerobic ammonium oxidation discovered in a denitrifying fluidized bed reactor", *FEMS Microbiol. Ecol*., **16**(4), 177-184. https://doi.org/10.1016/0168-6496(94)00081-7

Niederdorfer, R., Fragner, L., Yuan, L., Hausherr, D., Wei, J., Magyar, P., Joss, A., Lehmann, M.F., Ju, F. and Burgmann, H. (2021), "Distinct growth stages controlled by the interplay of deterministic and stochastic processes in functional anammox biofilms", *Water Res*., **200**, 117225. https://doi.org/10.1016/j.watres.2021.117225

Noor, A., Mohamed Kutty, S.R., Baloo, L., Affam, A.C., Jagaba, A.H., Saeed Ghaleb, A.A., Yahya Almahbashi, N.M., Ahmad, T., Nawab, M.S. and Birniwa, A.H. (2023), "Parametric optimization of additive manufactured biocarrier submerged in sequencing batch reactor for domestic wastewater treatment", *Heliyon*, **9**(4), e14840. https://doi.org/10.1016/j.heliyon.2023.e14840

Okabe, S., Ye, S., Lan, X. Nukada, K., Zhang, H., Kobayashi, K. and Oshiki, M. (2023), "Oxygen tolerance and detoxification mechanisms of highly enriched planktonic anaerobic ammonium-oxidizing (anammox) bacteria", *ISME Commun*., **3**(45). https://doi.org/10.1038/s43705-023-00251-7

Oshiki M, Awata T, Kindaichi T, Satoh H. and Okabe S. (2013), "Cultivation of planktonic anaerobic ammonium oxidation (anammox) bacteria using membrane bioreactor", *Microbes Environ*., **28**(4), 436-443. https://doi.org/10.1264/jsme2.ME13077

Parin, I., Parnian, I. and Ahmed, E. (2021), "Holistic insights into extracellular polymeric substance (EPS) in anammosx bacterial matrix and the potential sustainable biopolymer recovery: A review", *Chemosphere*, **274**, 129703. https://doi.org/10.1016/j.chemosphere.2021.129703

Pimenov, N.V., Nikolaev, Y.A., Dorofeev, A.G. Grachev, V.A., Kallistova, A. Yu., Mironov, V.V., Vanteeva, A.V., Grigor'eva, N.V., Berestovskaya, Y.Y., Gruzdev, E.V., Begmatov, S.A., Ravin, N.V. and Mardanov, A.V. (2022), "Bioaugmentation of anammox activated sludge with a nitrifying bacterial community as a way to increase the nitrogen removal efficiency",

Microbiology, **91**, 133-142. https://doi.org/10.1134/S0026261722020102

Pokorna, D. and Zabranska, J. (2015), "Sulfur-oxidizing bacteria in environmental technology", *Biotech. Adv.*, **33**(6) part 2, 1246-1259. https://doi.org/10.1016/j.biotechadv.2015.02.007

Reino, C., Suarez-Ojeda, M.E., Perez, J. and Carrera, J. (2018), "Stable long-term operation of an upflow anammox sludge bed reactor at mainstream conditions", *Water Res.*, **128**, 331-340. https://doi.org/10.1016/j.watres.2017.10.058

Siegrest, H., Salzgeber, D., Eugster, J. and Joss, A. (2008), "Anammox brings WWTP closer to energy autarky due to increased biogas production and reduced aeration energy for N-removal", *Water Sci. Technol.*, **57**(3), 383-388. https://doi.org/10.2166/wst.2008.048

Strous, M., Kuenen, J.G. and Jetten, M.S.M. (1999), "Key physiology of anaerobic ammonium oxidation key physiology of anaerobic ammonium oxidation", *Appl. Environ. Microbiol.*, **65**(7), 3248-3250. https://doi.org/10.1128/AEM.65.7.3248-3250.1999

Tang, X., Liu, S., Zhang, Z. and Zhuang, G. (2015), "Identification of the release and effects of AHLs in anammox culture for bacteria communication", *Chem. Eng. J.*, **273**, 184-191. https://doi.org/10.1016/j.cej.2015.03.045

Tao, Y., Gao, D.W., Wang, H.Y., de Kreuk, M. and Ren, N.Q. (2013), "Ecological characteristics of seeding sludge triggering a prompt start-up of anammox", *Bioresour. Technol.*, **133**, 475-481. https://doi.org/10.1016/j.biortech.2013.01.147

Valle, A., Bailey, M.J., Whiteley, A.S. and Manefield, M. (2004), "N-acyl-L-homoserine lactones (AHLs) affect microbial community composition and function in activated sludge", *Environ. Microbiol.*, **6**(4), 424-433. https://doi.org/10.1111/j.1462-2920.2004.00581.x

van de Graaf, A.A., Debruijin, P., Robertson, L.A., Jetten, M.S.M. and Kuenen, J.G. (1996), "Autotrophic growth of anaerobic ammonium-oxidizing micro- organisms in a fluidized bed reactor", *Microbiology*, **142**, 2187-2196. http://doi.org/10.1099/13500872-142-8-2187

Verma, S., Daverey, A. and Lin, J.G. (2021), "Successful start-up of anammox process from activated sludge and anaerobic sludge in a sequencing batch reactor using an unconventional strategy, *Int. Biodeterior. Biodegr.*, **156**, 105132 https://doi.org/10.1016/j.ibiod.2020.105132

Vlaeminck, S.E., Terada, A., Smets, B.F., De Clippeleir, H., Schaubroeck, T., Bolca, S., Demeestere, L., Mast, J., Boon, N., Carballa, M. and Verstraete, W. (2010), "Aggregate size and architecture determine microbial activity balance for one-stage partial nitritation and anammox", *Appl. Environ. Microbiol.*, **76**. https://doi.org/10.1128/AEM.02337-09

Waters, C.M. and Bassler, B.L. (2005), "Quorum sensing: cell-to-cell communication in bacteria", *Annu. Rev. Cell Dev. Biol.*, **21**, 319-346. https://doi.org/10.1146/annurev.cellbio.21.012704.131001

Wett, B., Omari, A., Podmirseg, S.M., Han, M., Akintayo, O., Gomez Brandon, M., Murthy, S., Bott, C., Hell, M., Takacs, I., Nyhuis, G. and O'Shaughnessy, M. (2013), "Going for mainstream deammonification from bench to full scale for maximized resource efficiency", *Water Sci. Technol.*, **68**(2), 283-289. https://doi.org/10.2166/wst.2013.150

Wong, L.L., Lu, Y., Ho, J.C.S. Mugunthan, S., Law, Y., Conway, P., Kjelleberg, S. and Seviour, T. (2023), "Surface-layer protein is a public-good matrix exopolymer for microbial community organisation in environmental anammox biofilms", *The ISME J.*, **17**, 803-812. https://doi.org/10.1038/s41396-023-01388-y

Youn. H., Kang, C. and Gil, K. (2024), "Water quality management strategy based on organic matter characteristics of stream and lakes in the Namhan River Watershed", *Membr. Water Treat.*, **15**(3), 99-106. https://doi.org/10.12989/mwt.2024.15.3.000

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