Influence of high-load shocks on achieving mainstream partial nitrification: Microbial community succession

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ABSTRACT

Driving microbial community succession through the regulation of operational strategies is crucial for achieving partial nitrification (PN) in municipal wastewater. However, at present, there is a decoupling between the strategic regulation of PN systems and the succession characteristics of the microbial community. This study examined the correlation between microbial community succession and PN performance under two high-load shocks (HLS1 and HLS2) treating actual sewage. During HLS1, the influent organic loading rate (OLR) and nitrogen loading rate (NLR) increased from 116.7 ± 37.7 to 219.7 ± 24.7 mg COD/(g VSS·d) and 0.21 ± 0.02 to 0.33 ± 0.02 kg N/m^3/d respectively, with the nitrite concentration and nitrite accumulation ratio only reaching 11.7 ± 2.7 mg/L and 49.3 ± 13.9 %, respectively. During HLS2, the influent OLR and NLR increased from 123.5 ± 17.2 to 300.3 ± 49.2 mg COD/(g VSS·d) and 0.19 ± 0.03 to 0.32 ± 0.03 kg N/m^3/d respectively, resulting in a nitrite accumulation ratio of 89.4 ± 10.7 %. The system achieved efficient PN performance and sustained for 124 days. High-throughput sequencing results showed that community diversity remained consistently high, and the community composition returned to its initial state following a minor succession during HLS1. During HLS2, the high-load shock reduced the richness and evenness of the microbial community. The community underwent succession in a new direction, leading to community composition and function changes. The results indicate that the realization, stabilization, and disruption of PN are influenced not only by operational parameters but also by microbial community structure.

1. Introduction

Enhancing pollutant removal efficiency while reducing energy consumption is an essential goal in wastewater treatment plant (WWTP) operation. As a novel biotechnology for nitrogen removal, partial nitrification (PN) shortens the nitrification process and results in nitrite accumulation (Guo et al., 2023), and the accumulated nitrite can subsequently be catalytically converted to nitrogen gas by denitrifying bacteria or anaerobic ammonia-oxidizing (anammox) bacteria (Qiao et al., 2022). This PN-coupled process offers economic benefits by reducing aeration energy consumption by 25 % during nitrification, conserving 40 % of organic carbon needed for subsequent denitrification, and eliminating the need for organic carbon in the anammox process (Kosgey et al., 2022).

The dynamic characteristics of real municipal wastewater with low ammonia concentrations (e.g., fluctuations in water quality, pH, temperature) render mainstream PN processes highly complex. In the dynamic competition between ammonium oxidizing bacteria (AOB) and nitrite oxidizing bacteria (NOB), AOB cannot maintain a definitive competitive advantage, making stable nitrite accumulation difficult to achieve. Extensive research has been focused on elucidating the mechanisms underlying the relationship between functional microorganisms and nitrogen transformation to improve nitrogen removal performance in WWTPs (Hu et al., 2021; Wang et al., 2021). Traditionally, AOB and NOB have been regarded as the two primary competing microorganisms in PN systems, with the key to success lying in the selective enrichment of AOB and suppression of NOB (Soliman and Eldyasti, 2018). However, current PN processes still struggle with limited predictability and controllability in response to dynamic changes (e.g., fluctuations in water quality, pH, temperature), hindering the achievement of stable nitrite accumulation (Zhao et al., 2022).

Recently, several studies have observed a "decoupling" between the taxonomic composition of microbial communities and their functional traits in response to environmental changes. This phenomenon has also

been observed in wastewater bioreactors, such as those used for nitrogen removal or methane production, where the taxonomic composition changes considerably over time, yet system performance remains stable (Louca et al., 2018). Therefore, many studies have attempted to characterize the microbial communities in wastewater treatment using molecular biology techniques. In a well-functioning PN, microorganisms can form a dynamic ecosystem at the micro-scale in response to external environmental changes (Kent et al., 2019). This ecosystem can further facilitate complex interactions between community microbes and correlate strongly with overall performance (Cerruti et al., 2021). The operational complexity of PN is largely due to the lack of effective microbial community regulation strategies. This highlights the fact that we have yet to fully understand the regulatory characteristics of microbial communities in PN. Therefore, revealing the ecological features that drive the realization of PN will enhance the understanding of the relationship between microbial community dynamics and system function

in wastewater treatment processes (Jian et al., 2024; Wang and He, 2019).

Therefore, this study explored the correlation between microbial community succession and system performance. Under extreme operating conditions, such as high-load perturbations and forced sludge discharge, the microbial community is significantly disturbed, leading to succession in response to these changes thereby initiating PN (Li et al., 2021; Wang et al., 2024b). An ecological perspective suggests that regulating original microbial community is essential for the realization and stability of PN. Microbial community succession is bound to occur after the high-load shocks, but it is still unknown whether the pattern of microbial community succession is repetitive. Therefore, this study implemented two different intensities of high-load shocks to investigate the varying effects of shock load intensity on the composition and succession of the microbial community within the sludge system. The results potentially offer a new perspective for understanding and applying
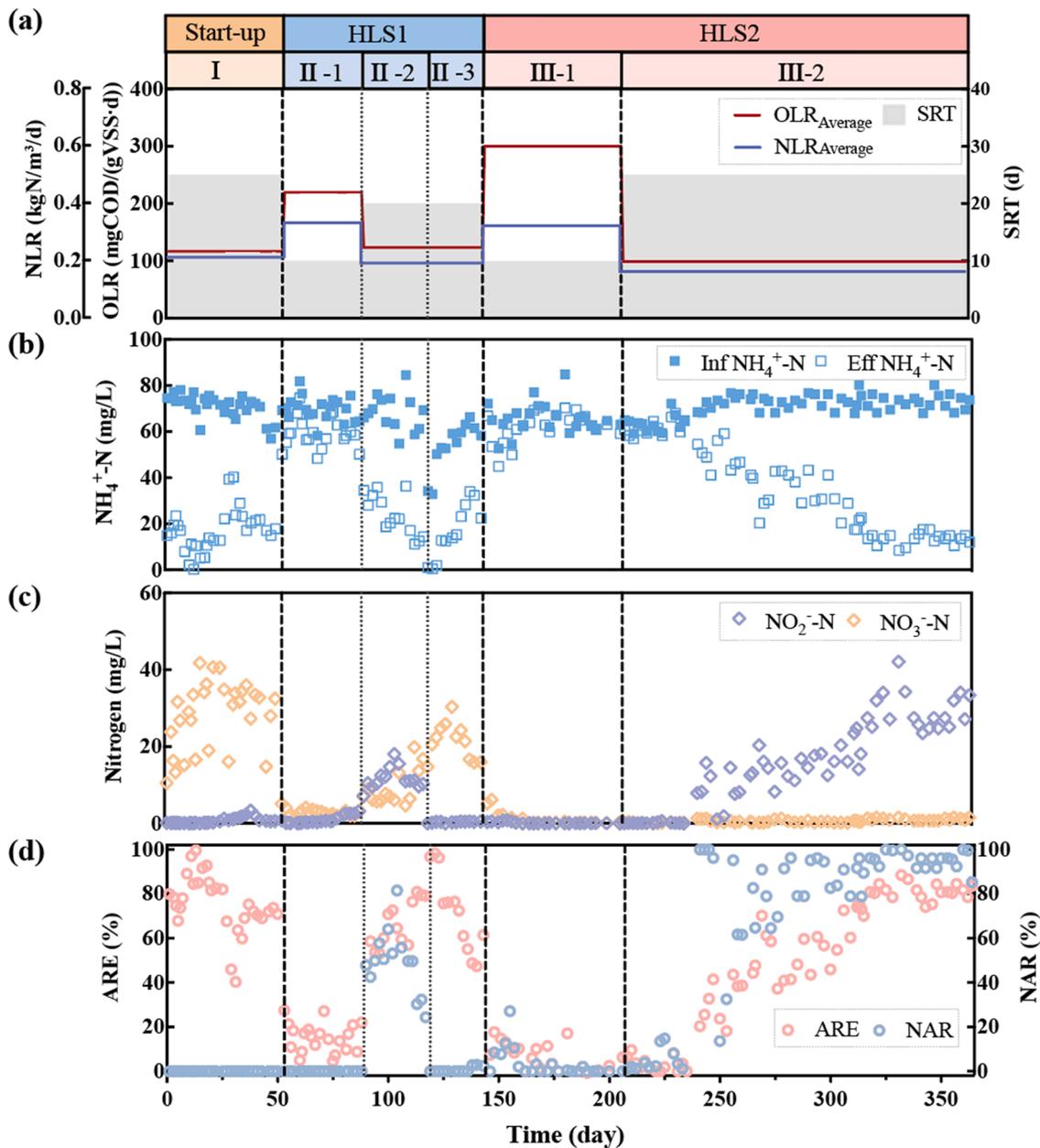
Fig. 1. Temporal changes in system performance. (a) Average organic loading rate (OLR), Average nitrogen loading rate (NLR) and sludge retention time (SRT), (b) ammonium concentration (NH_4^+-N) in the influent and at the end of the aerobic stage, (c) nitrite concentration (NO_2^--N) and nitrate concentration (NO_3^--N), (d) the ammonium removal efficiency (ARE) and nitrite accumulation ratio (NAR).

PN in wastewater treatment.

2. Results and discussion

2.1. Long-term system performance

The operational performance fluctuations during the three phases are depicted in Fig. 1. During the set-up stage (Days 1–53), the concentrations of NO_2^--N and NO_3^--N at the end of the aerobic stage were 0.7 $\pm$ 0.8 and 27.4 $\pm$ 9.5 mg/L, respectively, indicating that the system achieved complete nitrification.

During II-1 of HLS1 (Days 54–89), the HRT was adjusted to 5 h, and the aerobic time was shortened to 1.5 h. This disturbance, which lasted for 36 days, significantly caused the system to enter a nitrification-free state. In contrast, during II-2 (Days 90–119), the nitrification performance was enhanced by extending the aerobic time. The system successfully accumulated nitrite, with a NO_2^--N concentration of 11.7 $\pm$ 2.7 mg/L and a nitrite accumulation ratio (NAR) of 49.3 $\pm$ 13.9 %. Simultaneously, the NO_3^--N concentration was 9.3 $\pm$ 4.5 mg/L. The PN process was disrupted and transitioned into complete nitrification after 120 days (during II-3).

During III-1 of HLS2 (Days 145–207), the aerobic time was reduced to 1.5 h, and the HRT was adjusted to 5 h for 63 days. This led to a decline in nitrification performance, causing the system to enter a nitrification-free state once again. During III-2 (Days 208–364), the HRT was extended to 8 h, with an aerobic time of 3 h. However, the system did not exhibit nitrification performance during the first 27 days (Days 208–234). However, when the HRT was further adjusted to 10–13 h during 235–364 days, the aerobic stage was gradually extended to 4–5.5 h, gradually enhancing nitrification performance. This led to a significant improvement in nitrite accumulation, with the concentrations of NO_2^--N and NO_3^--N were 19.8 $\pm$ 9.1 and 0.8 $\pm$ 0.4 mg/L, respectively. Notably, the NAR reached to 89.4 $\pm$ 10.7 %, indicating a stable PN performance.

In the initial stages of both transitions from the high-load shock phase to the low-load phase, both ARE and NAR increased concurrently. However, in II-2, as ARE gradually increased, NAR progressively decreased, indicating that while the nitrification performance of the system improved, a shift toward a complete nitrification state was gradually emerging. In contrast, in III-2, both ARE and NAR increased in tandem, stabilizing at approximately 80 % and 95 %, respectively. This suggests that a more stable and efficient short-range nitrification performance was achieved in HLS2.

2.2. Functional bacteria analysis

The effect of high-load shocks on nitrifying bacteria was investigated by analyzing changes in their maximum activity and abundance. As illustrated in Fig. 2, a strong correlation was observed between the changes in maximum activity and abundance of nitrifying bacteria and the variations of load during HLS1. The AOB activity decreased from 7.41 to 2.42 mg-O_2/g-VSS/h. The relative abundance increased from $(4.1 \pm 0.57) \times 10^7$ to $(9.6 \pm 0.44) \times 10^7$ copies/g-VSS, nearly doubling. Additionally, the dominant AOB underwent a significant shift, changing from the initial ASV115, ASV189, ASV424, and ASV11741 to ASV2850 and ASV4177. It was hypothesized that the high-load perturbation accelerated community succession and migration (Wang et al., 2024a), with strains that adapted more quickly to the altered conditions becoming dominant. The NOB activity decreased from 4.54 to 0.36 mg-O_2/g-VSS/h, which was also accompanied by a reduction in their relative abundance. During the subsequent low-load phase of HLS1, the AOB activity gradually increased from 2.42 to 10.33 mg-O_2/g-VSS/h, and the absolute abundance decreased to $(3.9 \pm 0.23) \times 10^7$ copies/g-VSS. Whereas, the absolute abundance of NOB remained relatively stable. However, their activity increased from 0.36 to 4.57 mg-O_2/g-VSS/h, corresponding with the observed change in nitrification performance.

During III−1 (HLS2), high organic loading rate (OLR), nitrogen loading rate (NLR) and short SRT (10 d) simultaneously inhibited nitrifying bacteria. AOB and NOB were eliminated due to their slow growth and inability to adapt to the non-steady-state environment, resulting in their relative abundance falling below the detection limit (Li et al., 2021; Sun et al., 2021b). The absolute abundance of AOB and NOB decreased from $(3.9 \pm 0.23) \times 10^7$ copies/g-VSS to $(1.1 \pm 0.11) \times 10^6$ copies/g-VSS and from $(4.4 \pm 0.28) \times 10^8$ copies /g-VSS to $(6.3 \pm 0.68) \times 10^7$ copies/g-VSS, about 97 % and 89 % reduction, respectively. The activities of AOB and NOB also decreased to 0.1 and 0 mg-O_2/g-VSS/h, respectively. In summary, the results indicated that maintaining stable nitrification became challenging when the OLR and NLR increased to 300.3 $\pm$ 49.2 mg COD/(g VSS·d) and 0.32$\pm$0.03 kg N/m^3/d, respectively.

During III−2 (HLS2), the AOB activity increased to 4.63 mg-O_2/g-
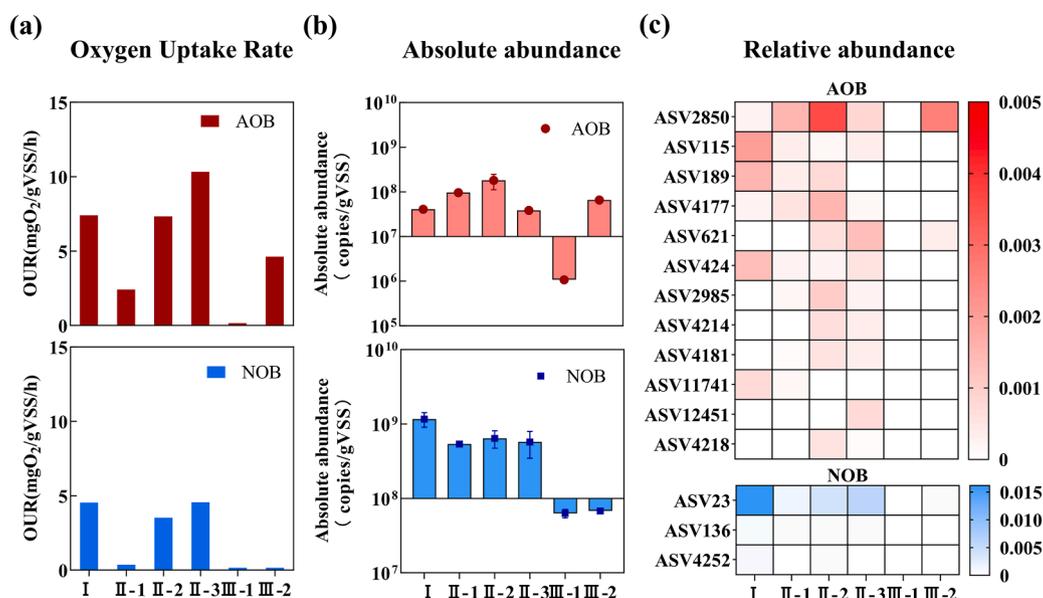
Fig. 2. Correlation analysis of nitrifying bacteria. (a) Maximum activities determined by maximum oxygen-consuming respiration rate (OUR), (b) absolute abundance of AOB and NOB determined by qPCR, and (c) relative abundance of AOB and NOB at the ASV level determined by high-throughput sequencing.

VSS/h, and their absolute abundance increased from $(1.1 \pm 0.11) \times 10^6$ to $(6.6 \pm 0.45) \times 10^7$ copies/g-VSS. The relative abundance of AOB increased from below the detection limit to 0.29 %, with ASV2850 remaining the dominant strain. In contrast, the activities and relative abundance of NOB remained below the detection limit, indicating that AOB was more dominant than NOB during the low-load phase.

2.3. Analysis of community diversity

As shown in Fig. 3, significant changes in microbial community composition resulted from perturbations. Principal coordinate analysis (PCoA) based on Bray-Curtis distance revealed four distinct microbial assemblages across the six stages, defining the succession into four
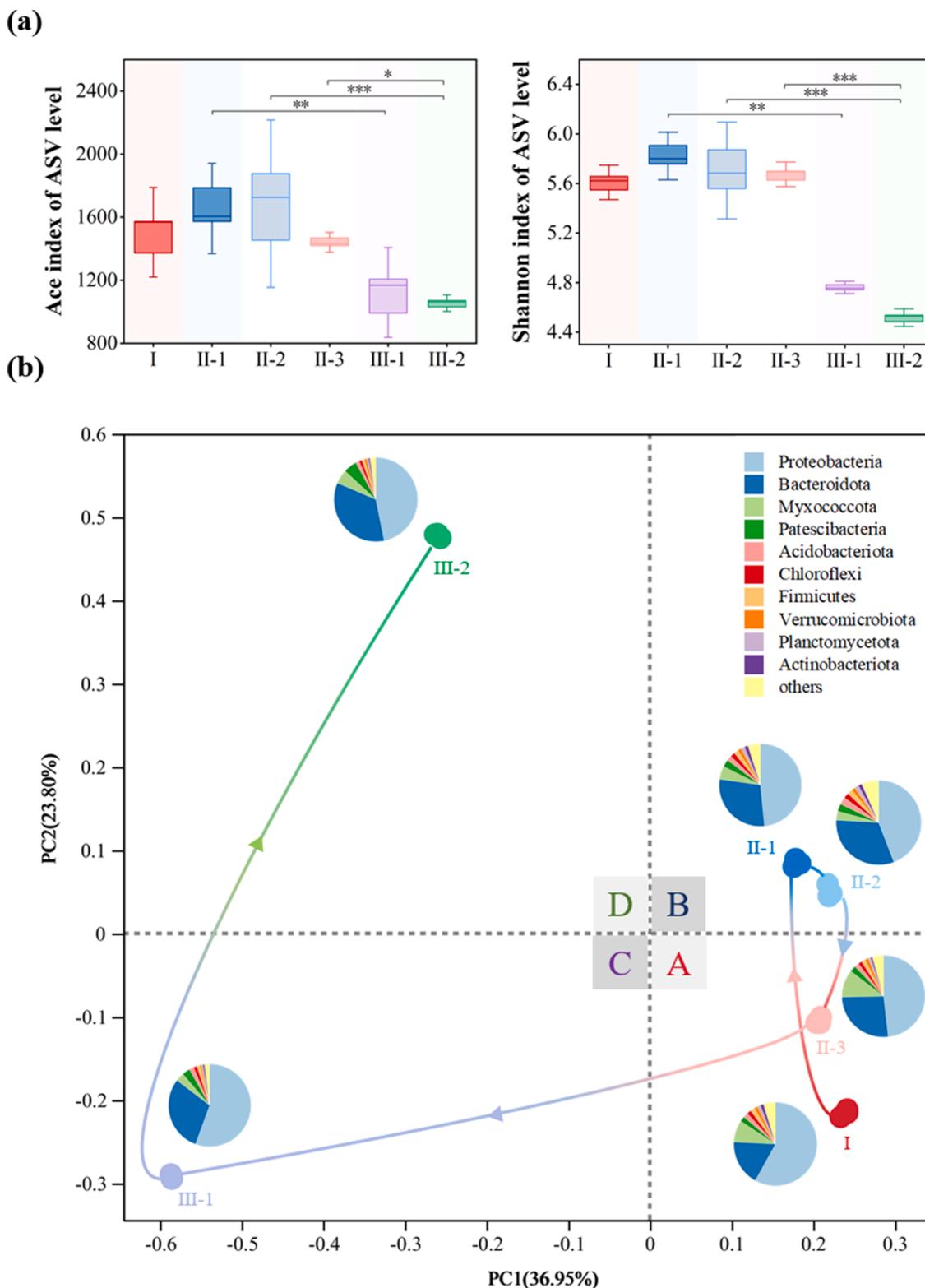
Fig. 3. Microbial community diversity. (a) Alpha diversity, measured using the ACE and Shannon indices; (b) Microbial community succession dynamics, depicted through pie charts showing the distribution of high-abundance phyla analyzed at the ASV level.

distinct regions: A, B, C, and D. These phases collectively represent the complete succession trajectory of microbial communities in an activated sludge system under load perturbations (Fig. 3b). Additionally, microbial diversity is an important factor in understanding community dynamics (Sun et al., 2021a). The application of ACE and Shannon indices provided a quantitative assessment of changes in microbial community richness and evenness, respectively (Fig. 3a).

A comparative analysis of samples from region A (I and II-3) exhibited that the ACE indices were 1505±93 (I) and 1441±21 (II-3), respectively, while the Shannon indices were 5.609±0.046 (I) and 5.674±0.033 (II-3), respectively, with a statistically significant difference (P < 0.05). This period was marked by high microbial species diversity and various metabolic pathways, which contributed to maintain stability within a defined range of disturbances. This highlights the system's inherent resistance and resilience to external perturbations (Fang et al., 2022).

A comparative analysis of samples from region B (II-1 and II-2 of HLS1) exhibited ACE indices of 1655±94 (II-1) and 1686±174 (II-2), and Shannon indices of 5.822±0.063 (II-1) and 5.705±0.129 (II-2), respectively. Both indices exhibited statistically significant differences (P < 0.05). However, the indices were significantly higher than those of the samples from region A (ACE: 1505±93, 1441±21; Shannon: 5.609 ±0.046, 5.674±0.033). Furthermore, PCoA signified a clear distinction between the microbial communities of region B and region A along the primary axis. This separation indicates that the load disturbance not only improved microbial community succession but also increased diversity, providing strong support for community stability under the load disturbance.

In region C (III-1 of HLS2), the ACE and Shannon indices were significantly lower, at 1123±94 and 4.760±0.016, respectively (P < 0.05), compared to the corresponding indices in region B (ACE: 1655 ±94, 1686±174; Shannon: 5.822±0.063, 5.705±0.129). This decrease in alpha diversity indices suggests a significant loss of microbial community richness and evenness. The process was marked by the dominance of opportunistic microorganisms that rapidly exploited available resources, adapted to fluctuating conditions, and proliferated, thereby driving the community's successional trajectory (Reznick et al., 2002; Vuono et al., 2015).

Region D (III-2) exhibited a further decline in microbial community diversity, with significantly reduced ACE and Shannon indices of 1055 ±17 and 4.517±0.024, respectively (P < 0.05). This suggests that community diversity did not recover in conjunction with system performance during the low-load phase following the high-load disturbance. PCoA analysis showed that the microbial communities in III-2 shifted in a new direction. At this stage, unchecked proliferation and nutrient depletion among heterotrophic bacteria led to the decline of microorganisms that had previously thrived by rapidly exploiting resources and adapting to unstable conditions (Vuono et al., 2015). Conversely, stable, slower-growing microorganisms reestablished dominance (Neuenschwander et al., 2018).

A stable ecosystem can either remain in its original state in response to perturbation or move to another stable state. That is, by changing its structure to maintain the necessary functions to adapt to the perturbation, defining as resistance and resilience (Cabrol and Malhautier, 2011; Herold et al., 2020). In regions A and B (HLS1), these communities demonstrate resilience and recover from such perturbations. In contrast, the successions in regions C and D (HLS2) indicate system destabilization caused by high-intensity, high-load disturbances. This phase is characterized by a directional shift in community composition along an uncertain trajectory, which may result in a reconfiguration, either reaching a new steady state or reverting to the previous cycle. The resulting stable PN process likely represents a novel equilibrium established by the restructured microbial community as the system recovers from the high-load disturbance, reflecting adaptive reorganization in response to environmental challenges.

2.4. Correlation between disturbance intensity and microbial community succession

Achieving PN is a complex, dynamic evolutionary process, in which the magnitude of disturbances and the management of the recovery phase are critical to maintaining PN stability. In this study, the microbial community demonstrated relative stability during periods of low-intensity disturbance (Dueholm et al., 2022). Although minor shifts in community structure were observed alongside changes in system performance, the community tended to return to its initial state as system performance recovered. This reversion may be due to the high inherent diversity and complexity (Fig. 3).

The transient nitrite accumulation during the low-intensity perturbation-recovery stage reflects the challenges associated with achieving and maintaining PN, which are closely related to the spontaneous recovery capabilities of more diverse microbial communities. In contrast, high-intensity disturbances led to secondary succession within the microbial community, characterized by changes in both composition and diversity (Bagchi et al., 2014). This suggests that the disturbance intensity exceeded the community's resilience threshold, causing a directional shift in community dynamics (Lu et al., 2018; Nguyen et al., 2018).

Even after the load intensity was returned to its original level, the community's diversity remained reduced, which diminished the microbial community's resilience. These shifts in community composition and functionality consequently influenced system performance. However, the community may eventually stabilize into a new equilibrium through the implementation of appropriate operational strategies (Cerruti et al., 2021). This transition is macroscopically reflected in the stable accumulation of nitrite within the activated sludge system. In practical engineering applications, the prolonged PN process observed during the long-term operation of high ammonium wastewater systems can be attributed to high-intensity perturbations combined with strategic operational controls (Cao et al., 2022; Chen et al., 2023; Jiang et al., 2021). This approach highlights the importance of understanding and managing the interplay between microbial community dynamics and environmental stressors to achieve and maintain effective wastewater treatment effects.

2.5. New possibilities for the analysis of PN initiation strategies

The importance of microbial communities in ecosystem function is well established. Although researchers have made efforts to manipulate microbial communities in engineered ecosystems, the rules governing community assembly have not been clearly elucidated (Ning et al., 2020). Since microbial communities in engineered ecosystems are managed, it has traditionally been assumed that community assembly is primarily driven by deterministic processes (Jia et al., 2018). Deterministic processes are those where biotic factors, such as species characteristics and interspecies interactions, along with abiotic factors, such as environmental conditions, drive the outcomes. Microorganisms typically inhabit environments that are suitable for specific ecological niches (Liu et al., 2019; Xia et al., 2018). Therefore, targeted succession of microbial communities can be achieved by regulating operational strategies, allowing engineered ecosystems to selectively perform specific functions (e.g., nitrogen removal, carbon removal, hydrogen production). However, the application of regulation theory in the PN has not yet been able to give a clear analysis between the regulation of the operating strategy and the change of the function, and this strategy for the same parameter has extensive variability in the startup and operation of different PN (Bolsan et al., 2024). The regulation strategy does not guarantee the certainty of the startup and the stability of the operation of PN (Mo et al., 2023).

Inlet loading was identified as a key factor influencing the role of deterministic processes in community assembly (Dueholm et al., 2022). In this study, a high-load shock regulation strategy was applied twice to

drive directed microbial community succession (HLS1 and HLS2). However, the response of the microbial community differed between the two shocks. In HLS1, the microbial community consistently maintained high biodiversity, demonstrating biological resistance and resilience to external perturbations. Although microbial community succession initially progressed towards achieving PN, the community spontaneously recovered to its original composition over time, leading to the disruption of PN during long-term operation. In contrast, in HLS2, the ultra-high-load shocks significantly reduced microbial community diversity, diminished community resistance, and resilience, and drove the community succession towards a new state. The experiments exhibited that stochastic processes were predominant in the community succession triggered by the two high-load shocks (Dueholm et al., 2022; Kinnunen et al., 2017). The stochastic process assumes that species are ecologically equivalent, regardless of differences in their competitive abilities, and that microbes inhabit environments with the same or overlapping ecological niches (Ning et al., 2019). The PN process emerged as an unstable intermediate mediator during the transition from a stable microbial community subjected to shocks to a new steady state. In essence, stable PN reflects the nitrogen removal performance associated with a specific microbial community structure.

The difficulty and stability of PN are closely linked to the initial state and succession of microbial community structure (Balmonte et al., 2018; Yuan et al., 2021). Specifically, it primarily relates to two factors in the shock-driven microbial community succession process: the initial characteristics of the microbial community (such as community composition and diversity) and the perturbation parameters (including intensity and type of disturbance) (Fig. 4). Therefore, we can induce the microbial community to shift in a specific direction by regulating operational strategies, thereby constructing the desired functional microbial communities (e.g., PN, hydrogen production, methane production, etc.). The study by Xuemei Liu et al. suggests that high nitrogen loading gives nitrifying bacteria a competitive advantage over other strains, ultimately dominating the succession of the microbial community, which leads to the continuous accumulation of nitrite (Liu et al., 2024). This provides a new perspective for the regulation of PN.

3. Conclusion

This study successfully achieved efficient PN by high load shocks to treat actual sewage. Additionally, the impact of load perturbation intensity on the microbial community within the activated sludge was examined. During HLS1, the nitrite reached 11.7 ± 2.7 mg/L, accompanied by NAR of 49.3 ± 13.9 %. Notably, the microbial community maintained high diversity throughout and, after a minor temporary shift, returned to its initial composition. During HLS2, the NAR reached 89.4 ± 10.7 %, and the system demonstrated stable operation for 124 days. However, the high-load shock induced a simultaneous decrease in microbial community abundance and evenness, resulting in a significant alteration of community structure and a non-directional shift in succession direction. The results suggest that stable PN is indicative of denitrification performance associated with a specific microbial community structure and offer valuable insights into the stable operation of PN processes from an ecological perspective.
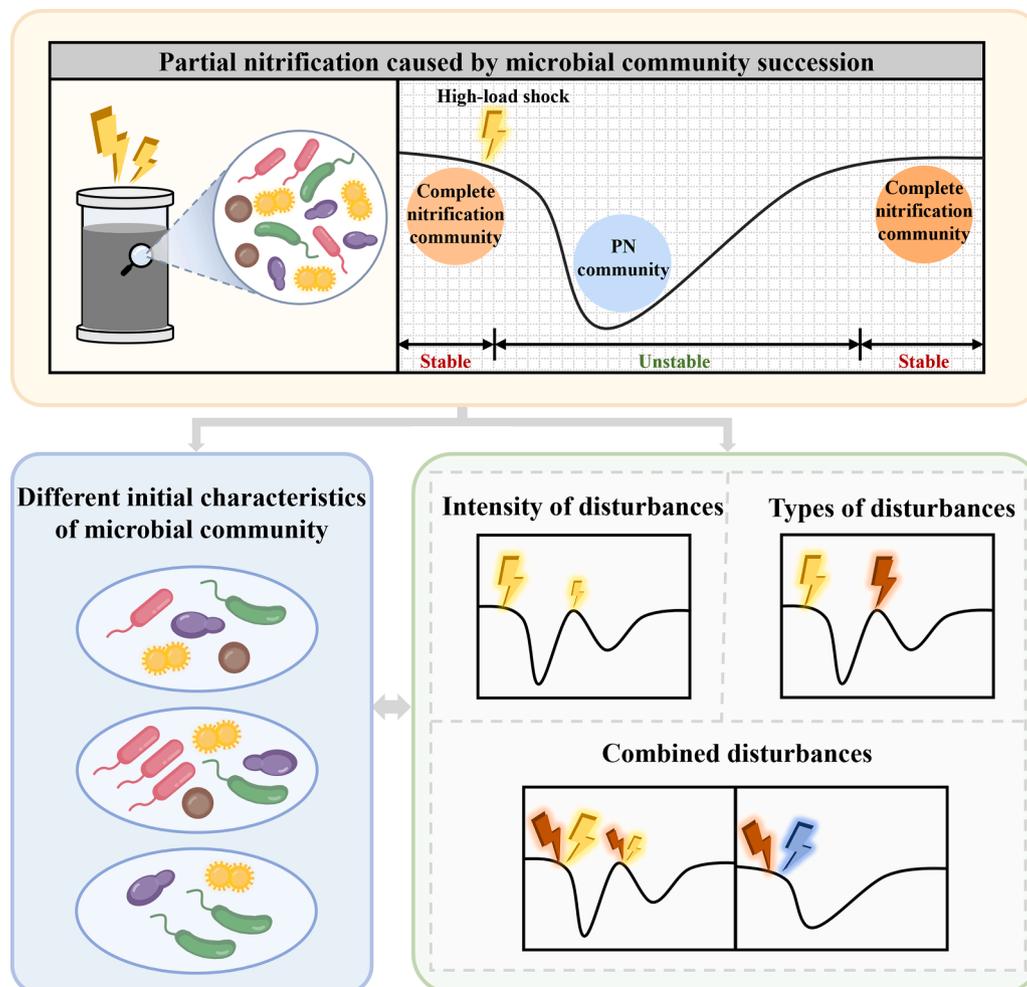
Fig. 4. Schematic diagram of the partial nitrification caused by microbial community succession.

4. Materials and methods

4.1. Experimental design

A single-stage SBR was used to treat natural domestic wastewater, with a working volume of 10 liters and a discharge ratio of 50 %. The SBR operated under an anoxic/oxic (A/O) mode, completing 3 to 4 cycles per day. Each cycle consisted of a 15-min influent stage, a 45-min anaerobic stage, and an aerobic stage lasting 90 to 300 min, depending on the specific operational phase. This was followed by a 30-min sedimentation stage, with drainage and idle stages ranging from 30 to 180 min. The parameter settings for each phase can be found in Table 1.

Aeration was regulated with a gas flow meter installed between the aeration pump and the air compressor. To maintain a consistent liquid temperature of $25 \pm 1\ °C$, heating devices were used in both the influent tank and the SBR. Sludge retention times (SRTs) were managed at 10, 20, and 25 days by adjusting the quantity of activated sludge discharged at the end of the aerobic stage. Additionally, a mechanical stirrer operating at 110 rpm was used to ensure thorough mixing of sludge and wastewater.

The experiment was divided into three phases by reducing the hydraulic retention time (HRT) to increase the influent OLR and NLR: Set-up (I), HLS1 (II-1, II-2, and II-3), and HLS2 (III−1 and III−2). During the set-up stage (1–53 days), the system operated with a long SRT of 25 days, and the gas flow rate was maintained at 150 mL/min. The first perturbation of HLS1 II−1 (54–89 days) involved increasing the discharge of activated sludge at the end of the aerobic stage, shortening the aerobic time, which reduced the SRT and increased the influent load. In HLS1 II−2 and HLS1 II−3 (days 90–144), the SRT was extended to 20 days to decrease the OLR and NLR, and the aerobic time was increased to 3 h. In HLS2 III−1 (days 145–207), the system underwent a second perturbation by shortening the aerobic time to 1.5 h, which increased the OLR and NLR. In HLS2 III−2 (days 208–354), the aerobic time was extended to 3–5.5 h, and the OLR and NLR were reduced to investigate the system's long-term response. These adjustments were implemented to assess the performance and stability across a range of operational conditions, enabling a comprehensive evaluation of its robustness and adaptability.

4.2. Inoculation sludge and wastewater

The inoculated sludge used in the experiments was sourced from the Gaobeidian WWTP. In Beijing, China, which utilizes an Anaerobic/Anoxic/Oxic (A^2O) process for domestic wastewater treatment. The sludge mixture had an average mixed liquor-suspended solids (MLSS) of 4740 mg/L.

The experiments utilized domestic wastewater pre-treated in septic tanks from a Beijing residential area, which is notable for its typically low C/N ratio. The specific pollutant characteristics of this wastewater are summarized in Table 2.

4.3. Analytical methods

The water samples of influent, end of the anoxic stage and end of the oxic stage were collected, filtered through 0.45 μm filters. The concen-

Table 1
The operating conditions in different phases.

Phase	Time (d)	Cycles (per d)	HRT (h)	SRT (d)
I	1–53	4	8	25
II−1	54–89	4	5	10
II−2	90–119	4	8	20
II−3	120–144	4	8	20
III−1	145–207	4	5	10
III−2	208–364	3	8–13	25

Table 2
Domestic wastewater quality.

Norm	NH_4^+-N (mg/L)	NO_2^--N (mg/L)	NO_3^--N (mg/L)	COD (mg/L)	PO_4^{3-}-P (mg/L)
Maximum	84.8	0.6	1.6	335.8	0.6
Minimum	32.9	0	0	62.7	9.4
Average	68.3	0	0.4	160.9	5.8

trations of NH_4^+-N, NO_2^--N, NO_3^--N, COD, MLSS and MLVSS were measured manually according to standard methods (Federation and Association, 2005). Real-time monitoring of temperature, dissolved oxygen (DO), and pH was performed using a WTW-Multi 3420 portable meter (Germany). During operation, the DO concentration was maintained below 1 mg/L. The NAR (%) was defined as the ratio of nitrite generated to ammonia consumed during the oxic stage. The NAR (%) and ARE (%) were determined as follows:

$$NAR = \frac{NO_{2,oxic}^- - NO_{2,anoxic}^-}{NH_{4,\ anoxic}^+ - NH_{4,oxic}^+} \tag{1}$$

$$ARE = \frac{NH_{4,\ influent}^+ - NH_{4,\ oxic}^+}{NH_{4,\ influent}^+} \tag{2}$$

where $NH_{4,\ influent}^+$ (mg/L) is the concentration of the influent; $NH_{4,\ anoxic}^+$ (mg/L) and $NO_{2,anoxic}^-$ (mg/L) are the concentrations at the end of anoxic stage; $NH_{4,\ oxic}^+$ (mg/L) and $NO_{2,oxic}^-$ (mg/L) are the concentrations at the end of oxic stage.

4.4. Sample collection

Activated sludge samples were collected in triplicate during the set-up stage (48 days), HLS1 (89, 102, and 144 days), and HLS2 (200 and 256 days) to represent the microbial community at each operational stage. A total of 150 mL of mixed liquor samples was collected. The combined samples were then allowed to settle naturally, after which the excess supernatant was removed. The processed samples were dispensed into cryogenic storage tubes and stored at −80 °C until further analysis via high-throughput sequencing.

4.5. Maximum bacterial bioactivities

The oxygen uptake rate (OUR) was measured using a single substrate to assess the aerobic respiration rate of aerobic bacteria. The sludge was collected from the end of the aerobic stage. The mixed liquor was washed and resuspended with a washing solution of tap water, mineral substances (0.2 mM $MgSO_4 \cdot 7H_2O$, 2 mM $NaCO_3$, 0.2 mM KH_2PO_4, and $CaCl_2 \cdot 2H_2O$), and trace elements. The cleaning process was repeated until all remaining substrate traces were removed. Washed mixed liquor was diluted with washing solution to maintain the original volume. Endogenous respiration (OUR_1) was measured without substrate addition, and only $NaNO_2$ was added as substrate (20 mg N/L) to determine OUR_2. The OUR_3 was measured with the addition of $NaNO_2$ (20 mg N/L) and $NH_4\ HCO_3$ (20 mg N/L). The activities of nitrifying bacteria, NOB and AOB, were determined using the following equations (Li et al., 2021):

$$OUR_{NOB} = OUR_2 - OUR_1 \tag{3}$$

$$OUR_{AOB} = OUR_3 - OUR_2 \tag{4}$$

4.6. DNA extraction and quantitative pcr

DNA from the sludge samples was extracted using the Fast DNA Spin kit for Soil (MP Biomedicals, OH, USA), following the manufacturer's instructions. The concentration of the extracted DNA was measured

using an ND-1000 spectrophotometer (Thermo Scientific, USA), and DNA purity was evaluated based on OD260/280 values, with theoretical values ranging from 1.8 to 2.0. Following extraction, the DNA samples were apportioned into equivalent fractions. To ensure detection accuracy, parallel samples were prepared for PCR amplification of each sludge sample, with a 10-fold dilution performed prior to amplification. Absolute quantification was achieved using a standard curve, enabling the determination of the initial copy number in each sample. The results were expressed as the number of gene copies of each functional bacterium per g of VSS.

4.7. High-throughput sequencing analysis

Microbial community structure and diversity at various stages were analyzed using Illumina Miseq high-throughput sequencing technology by the Shanghai Majorbio Bio-pharm Biotechnology Limited Company (Shanghai, China). The V3-V4 region of the bacterial 16S rRNA gene was amplified using primers 338F (ACTCCTACGGGGAGGCAGCAG) and 806R (GGACTACHVGGGGTWTCTAAT) (Li et al., 2023). Sequence processing was conducted using QIIME2 software, which clustered sequences into amplicon sequence variants (ASV) with a 100 % similarity threshold. Taxonomic classification of ASVs was achieved using the Silva database via the Ribosome Database Project (RDP). Data analysis was performed on the Majorbio cloud platform (www.majorbio.com). The valid sequence data were deposited in the National Center for Biotechnology Information of the Sequence Read Archive database under accession numbers SUB14702274.

CRediT authorship contribution statement

Chenjie Jia: Writing – original draft, Methodology. **Jialin Li:** Writing – review & editing, Supervision, Project administration, Conceptualization. **Zhaoyang Li:** Writing – review & editing. **Liang Zhang:** Writing – review & editing, Supervision, Project administration.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.wroa.2025.100304.

Data availability

Data will be made available on request

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