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Article

Combined Effects of C/N Ratio and NO_3^- -N Concentration on the Partial Denitrification (PD) Performance Under Lower Temperature: Substrate Variation, Nitrite Accumulation and Microbial Transformation

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Abstract

Using an intermittent SBR process with sodium acetate as additional carbon source, the combined effects of influent carbon-to-nitrogen ratio (C/N =0.8, 1.5, 2.5, 3.5, 4.5) and nitrate (NO_3^- -N) concentration (40 and 80 mg/L labeled R_{40} and R_{80}) on the nitrite (NO_2^- -N) accumulation during the partial denitrification (PD) process were investigated at low temperatures (4 - 12 °C), where the microbial diversity and functional bacteria evolution were also explored by high-throughput sequencing. The results showed that the 3.5- R_{40} and 2.5- R_{80} systems both presented the optimal NO_2^- -N accumulation at temperature of 10 °C, with the NO_2^- -N transformation rate (NTR) of 66.89% and 76.79%, respectively. In addition, as the temperature reduced from 10 °C to 5 °C, the NO_2^- -N accumulation performance was significantly suppressed, where the average effluent NO_2^- -N of 3.5- R_{40} (20.00 → 11.00 mg/L) and 2.5- R_{80} (43.00 → 18.90 mg/L) systems reduced by nearly half. It's worth noting that there was almost no NO_2^- -N accumulation at C/N of 0.8, although higher NO_3^- -N concentration promoted NTR under the same C/N ratio. The high-throughput sequencing showed that *Proteobacteria* and *Bacteroides* increased significantly from 35.31%, 18.34% to 51.69% - 60.35% and 18.08% - 35.21% as compared with the seeding sludge. *Thauera* and *Flavobacterium* as the main contributors to NO_2^- -N accumulation accounted for 31.83% and 20.30% at the C/N ratio of 2.5 under low temperature of 5 °C. The above discussion suggested that higher temperature (10 °C), lower C/N ratio (2.5 - 3.5), and higher NO_3^- -N concentration (80 mg/L) were more favorable for the stable PD formation.

Keywords: Partial denitrification; Temperature; C/N ratio; NO_3^- -N concentration; NO_2^- -N accumulation; Functional bacteria

1. Introduction

In recent years, environmental issues have garnered increasing attention from all sectors of society, among which water resources are closely related to human survival, as such, how to treat sewage productively and energy-efficiently has become the hot issues. Biological nitrogen removal (BNR) processes effectively degrades nitrogenous compounds from wastewater and facilitates compliance with the discharge standards outlined by national policy (Thakur & Medhi, 2019). Particularly, the anaerobic ammonia oxidation (Anammox) process reacts ammonia (NH_4^+ -N, electron donor) with nitrite (NO_2^- -N, electron acceptor) to generate nitrogen gas (N_2) and nitrate (NO_3^- -N) (Hu et al., 2024; Li et al., 2024), which manifests huge advantages such as reduced aeration, low sludge production and less carbon source addition (Wang & Yang, 2022). Consequently, partial

denitrification (PD, $\text{NO}_3\text{-N} \rightarrow \text{NO}_2\text{-N}$) has gained considerable attention due to its role in promoting stable nitrite accumulation, which is essential for enhancing the real application of Anammox process. Studies have shown that there are a number of factors that affect PD performance, including temperature, carbon-to-nitrogen (C/N) ratio, carbon source, dissolved oxygen (DO), and pH values (Di Capua et al., 2022; Lu et al., 2023; Wu et al., 2024), etc. In these studies, all the findings indicated that PD could be utilized to enhance the traditional BNR efficiency for the upgrading and reconstruction of wastewater treatment plants (WWTPs).

Regarding the effects of temperature on PD, most scholars have focused on ambient temperatures ranging from 15 to 30 °C, revealing that this range of temperature exerts a positive impact on the $\text{NO}_2\text{-N}$ accumulation. According to Li et al., extending the *hydraulic retention time* (HRT) can also improve the $\text{NO}_3\text{-N}$ removal although low temperatures may impede it (Li et al., 2020), which pointed out that the denitrification rate increased gradually with the elevation in temperature. In particular, lower temperatures (15 - 17 °C) could result in oversaturated DO conditions and suppress the microbial activity associated with denitrification rates, thereby restricting the occurrence of anoxic denitrification throughout the entire operational period (Yao et al., 2013). Extreme temperature variations can induce alterations in the structural integrity of cell membranes, including lipids and proteins (Strous et al., 2006). However, few scholars have explored the $\text{NO}_2\text{-N}$ accumulation characteristics of PD at lower temperatures (Hoover et al., 2016), highlighting the importance of enhancing nitrogen removal in winter. Additionally, many scholars have investigated the impact of C/N ratio on PD performance. It was shown that for a given nitrate concentration, the peak $\text{NO}_2\text{-N}$ productions increased with higher C/N ratios (Ge et al., 2012). Moreover, the experimental data suggested an increase in both the maximum $\text{NO}_2\text{-N}$ amount and the $\text{NO}_2\text{-N}$ accumulation rate with rising $\text{NO}_3\text{-N}$ concentrations (Mohan et al., 2016). In the combined partial denitrification-anammox (PD/A) process, the nitrate removal efficiency (NRE) maintained around 89% - 90% with a mean nitrate-to-nitrite transformation ratio (NTR) of 84.43% at a C/N ratio of 2.5 (Fan et al., 2024). Hence, the factors of temperature, C/N ratio, and influent $\text{NO}_3\text{-N}$ concentration are all critical for starting the PD process and maintaining stable operation, especially in cold regions.

This study established a sequencing batch reactor (SBR) process with intermittent aeration by utilizing sodium acetate as the external carbon source, and the combined effects of C/N ratio and $\text{NO}_3\text{-N}$ concentration on PD performance under low-temperature conditions were investigated by analyzing substrate variations during both long-term operation and typical cycles. The microbial community structure was assessed through high-throughput sequencing analysis to reveal the $\text{NO}_2\text{-N}$ accumulation characteristics under different conditions, with the aim of providing theoretical foundations for the popularization and application of the PD/A process.

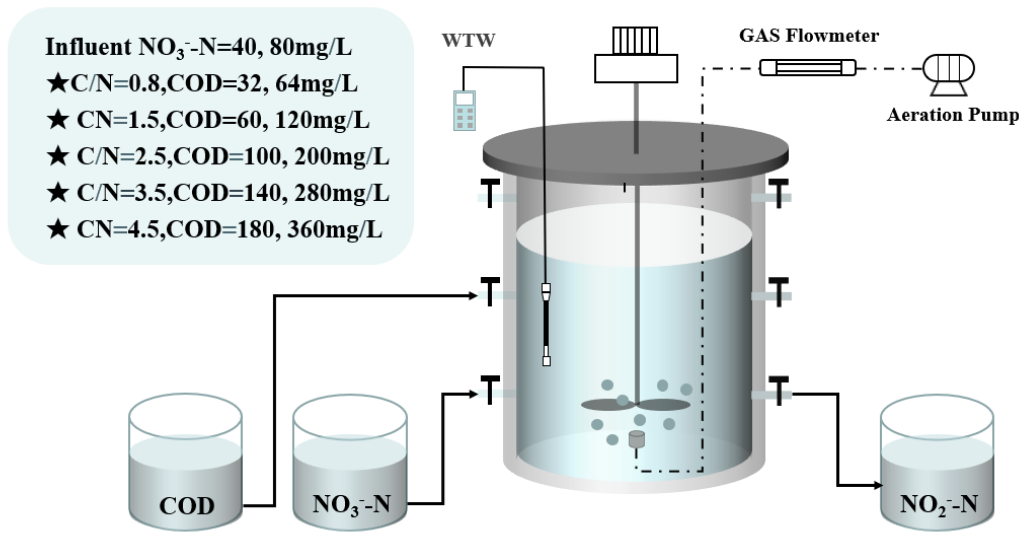
2. Materials and Methods

2.1. Experimental Device and Operational Mode

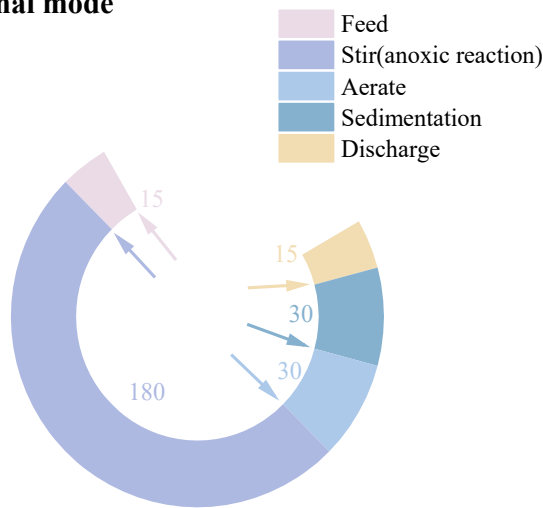
As shown in Figure 1a, the cylindrical PD - SBR reactor (working volume: 10 L) was operated at room temperature, and the running time stretched across November to December with the ambient temperature changing from $10 \pm 2^\circ\text{C}$ to $5 \pm 2^\circ\text{C}$. The reactor was fitted with a mechanical stirrer (IKA REO basic C type), while the microporous aeration was adopted at the bottom through a gas flow meter (LZB-4) combining with an air pump (ACO-6603). The experimental process, operated in an anoxic/aerobic alternating mode (Figure 1b), consisting of four cycles per day. Each operational cycle lasted 270 min, with the following duration for each stage: influent stage (15 min), anoxic stage (180 min), aerobic stage (30 min), sedimentation stage (30 min), and drainage stage (15 min). At the beginning of each cycle, 5 L of synthetic wastewater, along with appropriate amounts of $\text{NO}_3\text{-N}$ and COD were pumped into the reactor, followed by agitation under anoxic conditions for the production of $\text{NO}_2\text{-N}$ accumulation. To maintain better biological activity of sludge (Du et al., 2017a), a brief

aeration period of 30 min was conducted and approximately 50 mL sludge was discharged before sedimentation to ensure that the sludge retention time (SRT) sustained around 25 days (Table 1).

(a) Experimental device



(b) Operational mode



Cycle time 270min

Figure 1. Experimental device and operational mode of the PD - SBR system.

2.2. Seeding Sludge and Synthetic Wastewater

The seeding sludge (SS) used in the test was obtained from the biological pond of Tangwang WWTP (China, Yangzhou), known for its effective denitrification performance. The initial mixed volatile suspended solids (VSS) of the PD - SBR reactor was kept at about 2500 mg/L (Zhang et al., 2022). The test water was artificially simulated with the carbon and nitrogen sources provided by sodium acetate (CH₃COONa) and sodium nitrate (NaNO₃), respectively. The reactors were categorized into two groups based on the initial NO₃⁻-N concentration: R₄₀ (NO₃⁻-N: ~ 40 mg/L) and

R₈₀ (NO₃-N: ~ 80 mg/L), and the COD concentration fluctuated within the range of 32 mg/L to 360 mg/L, giving the initial C/N ratios of 0.8, 1.5, 2.5, 3.5, and 4.5, respectively.

Table 1. Influent quality characteristics.

Systems	C/N	Influent NO ₃ -N	Influent COD	NTR ^A	NRE ^A	Other parameters
		(mg/L)	(mg/L)	(%)	(%)	
R ₄₀	0.8	40±1	32±8	0.50	8.63	Operation cycle: 270 min Cycle: 120 VSS: 2500±100 mg/L SRT: 25 d Temperature: 10 - 5 °C
	1.5	40±2	60±7	27.79	31.10	
	2.5	40±1	100±5	31.54	40.15	
	3.5	40±1	140±9	52.87	54.96	
	4.5	40±2	180±8	48.87	45.67	
R ₈₀	0.8	80±1	64±6	19.15	9.16	
	1.5	80±2	120±7	46.00	24.68	
	2.5	80±1	200±6	57.12	51.19	
	3.5	80±1	280±8	60.42	55.75	
	4.5	80±2	360±9	56.98	40.55	

A: The average values during the stable operation.

Moving on to the C/N ratio, R₄₀ and R₈₀ were further subdivided into five sorts: 0.8-R₄₀ and 0.8-R₈₀ (C/N=0.8), 1.5-R₄₀ and 1.5-R₈₀ (C/N=1.5), 2.5-R₄₀ and 2.5-R₈₀ (C/N=2.5), 3.5-R₄₀ and 3.5-R₈₀ (C/N=3.5), 4.5-R₄₀ and 4.5-R₈₀ (C/N=4.5), and each system was operated for 120 cycles (Table 1). Meanwhile, in order to maintain good microbial growth and metabolism, 1 mL trace element solution (per liter: 0.03 g CuSO₄·5H₂O, 0.06 g Na₂MoO₄·2H₂O, 0.12 g MnCl₂·4H₂O, 0.12 g ZnSO₄·7H₂O, 0.15 g H₃BO₃, 0.15 g CoCl₂·6H₂O, 0.18 g KI, 1.5 g FeCl₃·6H₂O, and 10 g ethylene diamine tetraacetic acid (EDTA)) (Zhang et al., 2022) was fed into the reactor during each cycle.

2.3. Analytical Methods

All wastewater samples were filtrated through 0.45 μm filters before analysis. COD was monitored by a COD quick-analysis apparatus (LH-3C, Lanzhou, China), NO₂-N was determined by N-(1-naphthyl)-ethylenediamine spectrophotometry, NO₃-N was analyzed by UV spectrophotometry (U-T6), and the above determinations were made by the standard methods (AWWA, 2005). The temperature was monitored by the WTW meters (WTW Multi 3420, Germany).

The removal efficiency of total nitrogen (Re_{TN}) was calculated:

$$Re_{TN} \text{ (}\%) = [1 - (NO_2\text{-}N_{eff} + NO_3\text{-}N_{eff}) / (NO_2\text{-}N_{inf} + NO_3\text{-}N_{inf})] \times 100\% \text{ (1)}$$

NTR and NRE were expressed by the following equations (Fan et al., 2024):

$$\text{NTR (\%)} = (\text{NO}_2\text{-N}_{\text{eff}} - \text{NO}_2\text{-N}_{\text{inf}}) / (\text{NO}_3\text{-N}_{\text{inf}} - \text{NO}_3\text{-N}_{\text{eff}}) \times 100\% \quad (2)$$

$$\text{NRE (\%)} = (\text{NO}_3\text{-N}_{\text{inf}} - \text{NO}_3\text{-N}_{\text{eff}}) / \text{NO}_3\text{-N}_{\text{inf}} \times 100\% \quad (3)$$

Where $\text{NO}_3\text{-N}_{\text{inf}}$, $\text{NO}_2\text{-N}_{\text{inf}}$, $\text{NO}_3\text{-N}_{\text{eff}}$ and $\text{NO}_2\text{-N}_{\text{eff}}$ were the influent and effluent concentrations of $\text{NO}_3\text{-N}$ and $\text{NO}_2\text{-N}$ in the PD - SBRs, respectively, mg/L.

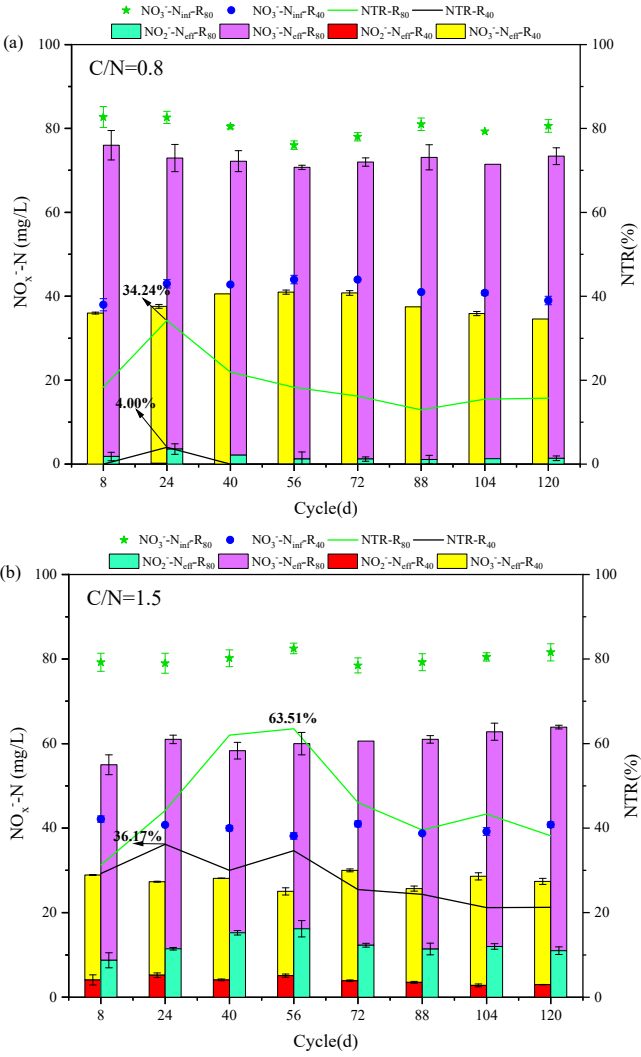
2.4. High-Throughput Sequencing

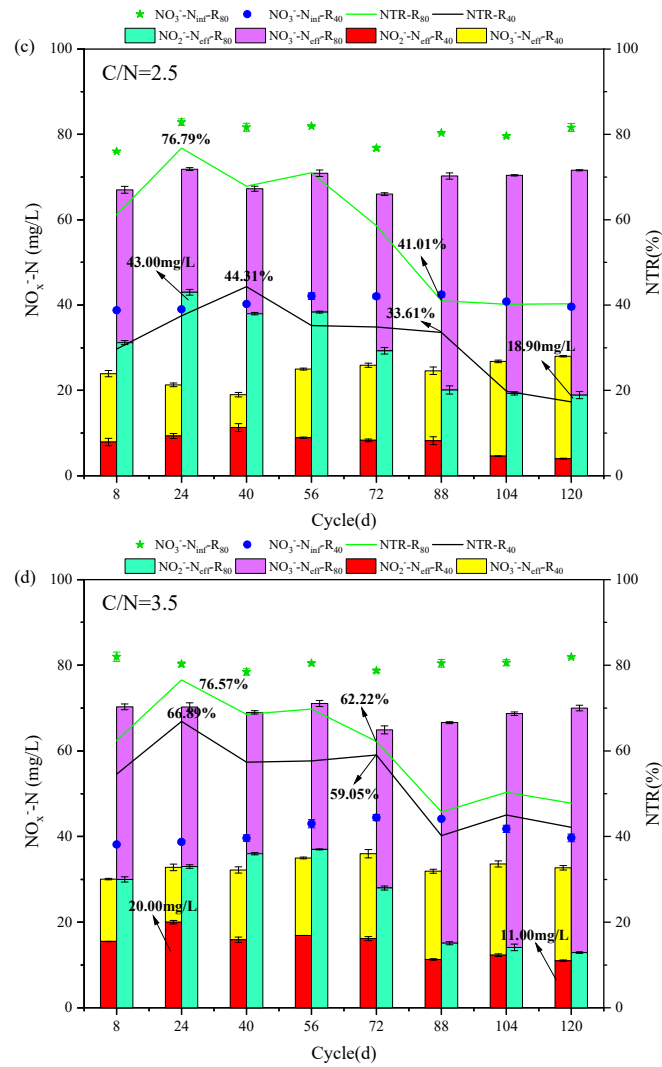
The SS and five PD - SBR samples of the R_{80} group after the steady operation were collected for high-throughput sequencing analysis. Following the manufacturer's instructions, DNA was extracted using the E.Z.N.A.® Soil DNA Kit (Omega Bio-tek, Norcross, GA, USA). The NanoDrop1000 spectrophotometer was used to determine the qualitative and quantitative analysis at optical densities (OD) of 260 and 280 nm. In a sequential manner, the polymerase chain reaction (PCR) amplification was performed on an ABI GeneAmp® 9700 PCR System using primers 338F (5'-ACTCCTACGGGAGGCAGCA-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3'). Thereafter, the PCR products were implemented on the Illumina MiSeq PE300 platform (Personalbio Biotechnology Co., Ltd., Shanghai, China) (Zhang et al., 2022), and all the raw data were provided by Shanghai Meiji BioCloud (SRP057140).

3. Results and Discussion

3.1. Substrate Variation and Nitrite Accumulation in the Long-Term Operation

The evolutions of $\text{NO}_3\text{-N}_{\text{inf}}$, $\text{NO}_3\text{-N}_{\text{eff}}$ and $\text{NO}_2\text{-N}_{\text{eff}}$, NTR, and NRE at different temperatures in R_{40} and R_{80} groups were revealed and compared at given C/N ratios (0.5, 1.5, 2.5, 3.5, and 4.5) (Figure 2). All 10 PD-SBR reactors were operated for 120 cycles at room temperature, which can be categorized into two phases based on the temperature change trends: Phase I (1 - 56 cycles) with the temperature fluctuating around $10 \pm 2^\circ\text{C}$, and Phase II (57 - 120 cycles), with the temperature maintaining at approximately $5 \pm 2^\circ\text{C}$.





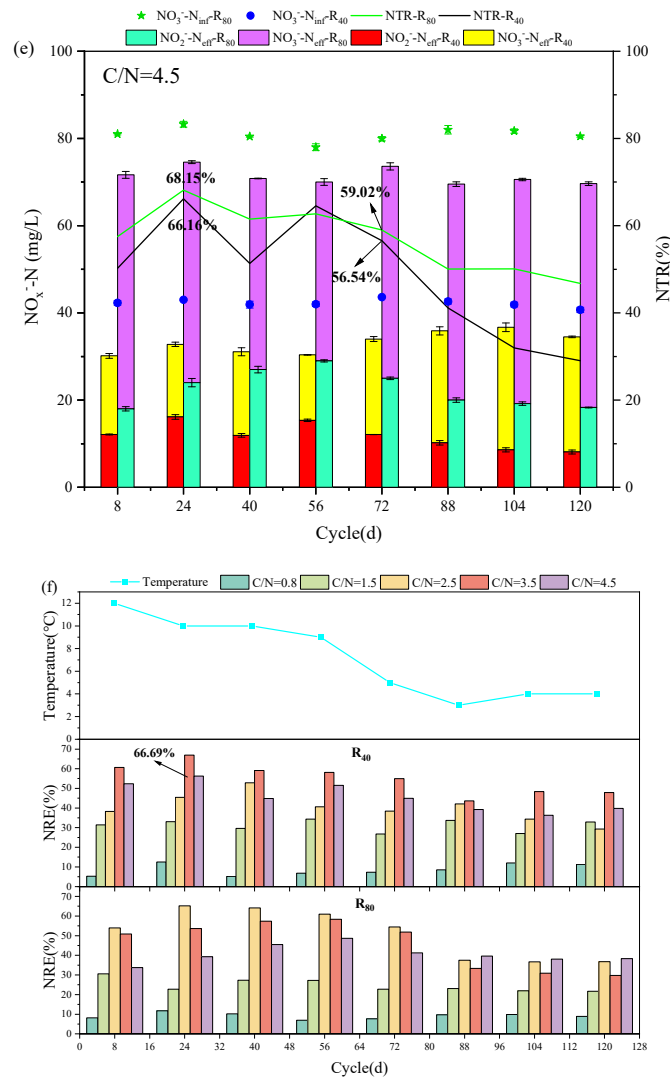


Figure 2. Variations of substrate concentration, NTR, and NRE in different reactors.

For the R_{40} systems, the average $\text{NO}_2\text{-N}_{\text{eff}}$ values were 0.056, 4.63, 9.35, 17.07, and 13.88 mg/L in Phase I, while the concentrations were reduced to 0, 3.30, 6.28, 12.70, and 9.75 mg/L in Phase II, respectively. Due to the sensitivity of denitrifying bacteria to temperature changes and the greater impact of temperature on PD (Du et al., 2016b), the average $\text{NO}_2\text{-N}_{\text{eff}}$ in Phase I was higher than that in Phase II of both R_{40} and R_{80} groups. Thereinto, the peak NTR occurred at the temperature of 9 - 10°C in the R_{80} systems under all C/N ratio conditions (0.8- R_{80} (34.24%), 1.5- R_{80} (63.51%), 2.5- R_{80} (76.79%), 3.5- R_{80} (75.57%), 4.5- R_{80} (68.15%)), presenting the same rules with the R_{40} systems (0.8- R_{40} (4.00%), 1.5- R_{40} (36.17%), 2.5- R_{40} (44.31%), 3.5- R_{40} (66.89%), 4.5- R_{40} (66.16%)). According to previous studies, lower temperatures (<10°C) inhibited microbial activity associated with denitrification rates, thereby disrupting anoxic environment and limiting $\text{NO}_2\text{-N}$ accumulation (Yao et al., 2013). Similarly, in this study, when the temperature was lowered from 10 °C to 5 °C, the NTR decreased significantly in the R_{40} (0.8- R_{40} (4.00% → 0.00%), 1.5- R_{40} (36.17% → 21.21%), 2.5- R_{40} (44.31% → 17.32%), 3.5- R_{40} (66.89% → 42.15%), 4.5- R_{40} (66.16% → 29.03%)) and R_{80} (0.8- R_{80} (34.24% → 12.94%), 1.5- R_{80} (63.51% → 38.19%), 2.5- R_{80} (76.79% → 40.21%), 3.5- R_{80} (76.57% → 45.76%), 4.5- R_{80} (68.15% → 38.07%)) groups (Figure 2). It was noteworthy that the average effluent $\text{NO}_2\text{-N}$ of the 3.5- R_{40} (20.00 → 11.00 mg/L) and 2.5- R_{80} (43.00 → 18.90 mg/L) systems was reduced by nearly half.

In addition, a positive correlation between NRE and NTR was observed, for example, in the 3.5- R_{40} system, NRE peaked to 66.69% at 10 °C but decreased to 54.99%, 47.86%, and 43.59% as the

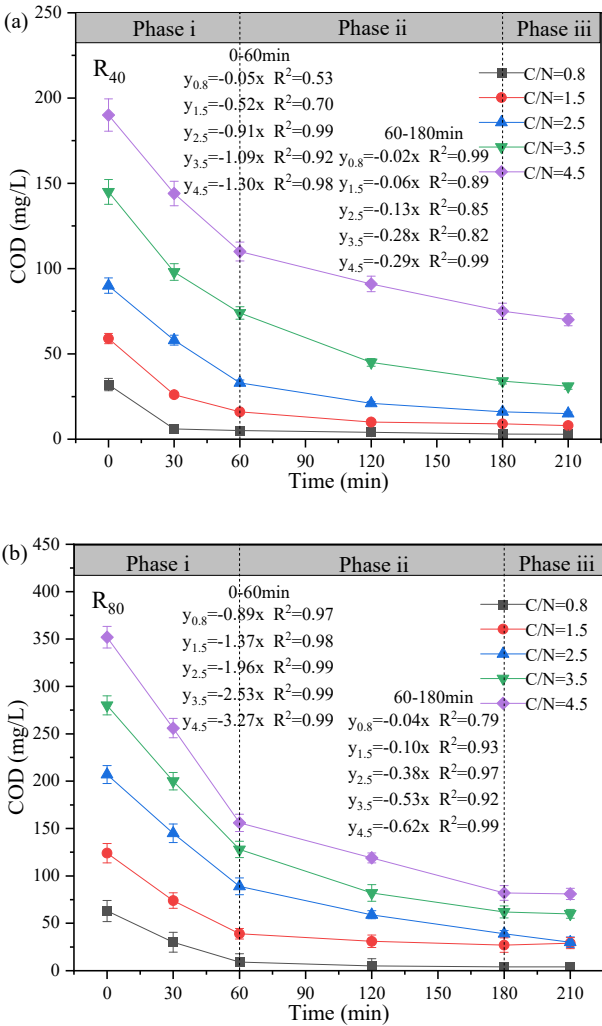
temperature dropped to 5°C, 4°C, and 3 °C, respectively (Figure 2f), showing that the nitrogen removal efficiency was depressed with decreasing ambient temperature (Wu et al., 2024). Actually, the rate of PD denitrification was related to enzyme activities, specifically nitrate reductase (*Nar*) and nitrite reductase (*Nir*). Studies have shown that *Nir* was more sensitive to temperature than *Nar*, and *Nir* inhibition under high temperatures led to more NO₂-N accumulation (Li et al., 2014). However, it was reported that lowering the temperature from 22°C to 14°C resulted in enhanced NO₂-N accumulation along with an increase in NRE from 72.2% to 77.2% (Li et al., 2023). It was also found that the nitrite accumulation was facilitated from 60% to 75% as temperature reducing from 30°C to 20°C (Liu et al., 2022), and the PD performance could be enhanced under low temperature of 15°C (Gao et al., 2022). Combined with the results of this experiment, it can be inferred that the PD performance was free from the negative influences of lower temperature at 10°C, but the deterioration of NO₂-N accumulation and NTR occurred by lowering temperature to a certain level (<5°C).

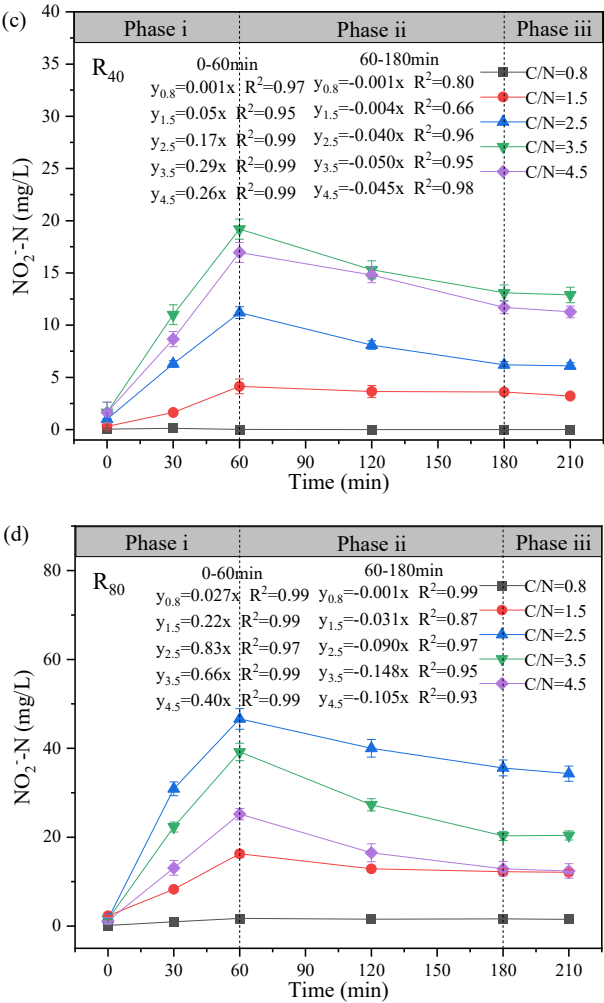
Specifically, the average NTR values of the R₄₀ group at the five conditions (C/N=0.5, 1.5, 2.5, 3.5, and 4.5) were much lower than those of the R₈₀ group. Under the same influent C/N of 0.8 (Figure 2a), the average NTR and NRE were only 0.5% and 8.63% in the R₄₀ systems, while the corresponding values reached to 19.5% and 9.16% in the R₈₀ systems, respectively. The peak NTR was 44.31% in the 2.5-R₄₀ system and 76.79% in the 2.5-R₈₀ system, which was consistent with previous findings that higher NO₃-N concentrations promoted nitrite accumulation at the same C/N ratio (e.g., 0.8-R₄₀: 0.5 %; 0.8-R₈₀: 19.16 % (Zhang et al., 2023d)). Similarly, NTR increased from 87.76% to 89.31% when NO₃-N concentration climbed from 20 mg/L to 150 mg/L (Du et al., 2016b), and the possible reason has been speculated to the inhibited NO₂-N reduction triggered by the unbalanced activities between *Nar* and *Nir* under higher NO₃-N environment (Zhang et al., 2022). Because more NO₃-N concentration will significantly increase the *Nar* activity and accelerate the reduction of NO₃-N to NO₂-N.

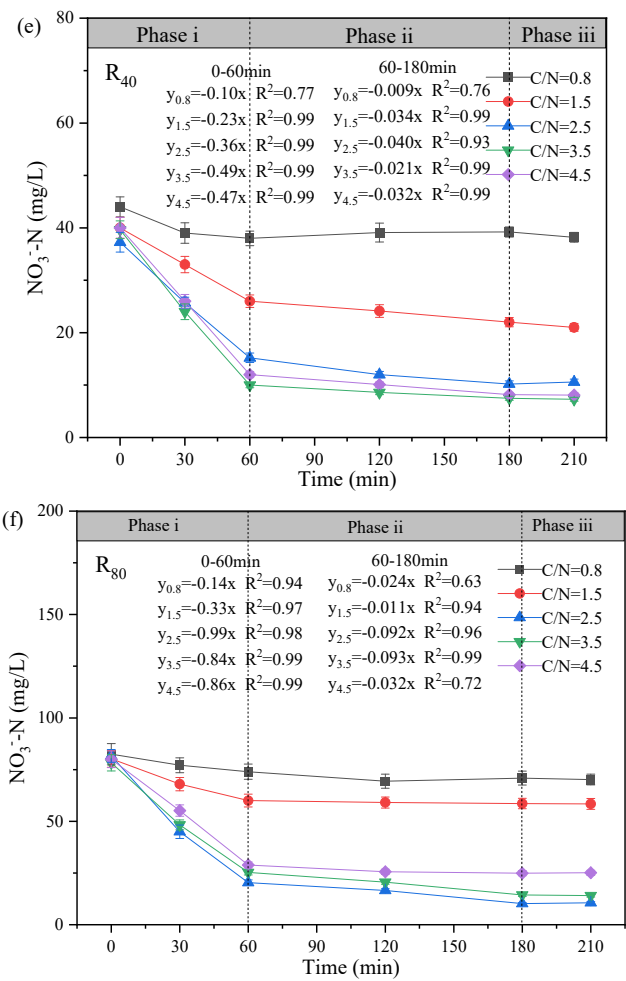
Moving on to the influence of C/N ratio, the mean NTR increased from 46% to 60.42% in the R₈₀ systems, while NRE fluctuated from 24.68% to 55.75% as the C/N ratio increased from 1.5 to 3.5 (Figure 2b-d). However, when the C/N ratio continued to soar up to 4.5 (Figure 2e), the mean values of NTR and NRE exhibited a decreasing trend, measuring 56.98% and 40.55%, respectively. The above trends of NTR and NRE changed aligned with those in the R₄₀ systems, displaying an optimal C/N range around 2.5 - 3.5. The optimal C/N ratio range of 1.5 - 3.0 was also previously proposed (Zhang et al., 2021b), and numerous scholars have shown similarly NO₂-N accumulation and NTR in excess of 80% when the C/N ratio was set between 2.5 and 3.0 using sodium acetate as carbon source (Cao et al., 2016). The optimal NTR value of 45.28% - 47.50% at C/N ratios of 2.5 - 3 was also reported with a significant decline in NTR when C/N exceeded 3.5 (Li et al., 2014). These findings indicated that both excessively high and low C/N ratios were unfavorable for NTR and NRE. However, different from these conclusions, it was worth noting that it had been argued that the key factor affecting denitrification was the residual NO₃-N concentration rather than C/N ratio (Le et al., 2019), which implied that stable NO₂-N accumulation was virtually independent of the C/N ratio (Du et al., 2016b). In fact, except NO₃-N concentration, both temperature and C/N ratio were the essential factors influencing NO₂-N accumulation in PD systems. On the one hand, the low temperatures resulted in decreased microbial activity and lower reaction rates, so NO₃-N and COD concentrations were more demanded than high-temperature environment (Park et al., 2017). On the other hand, better NO₂-N accumulation also achieved at a relatively low temperature with higher C/N ratios of surpassing 4 (Shen et al., 2020), because the low temperature prompted an increase in the optimal C/N ratio.

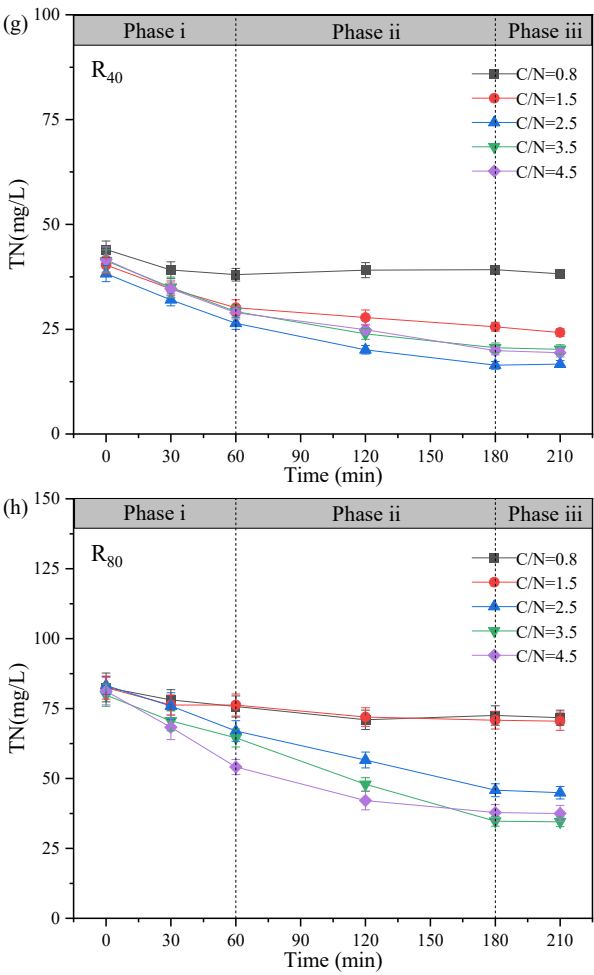
3.2. Substrate Variation and Nitrite Accumulation in the Typical Cycle

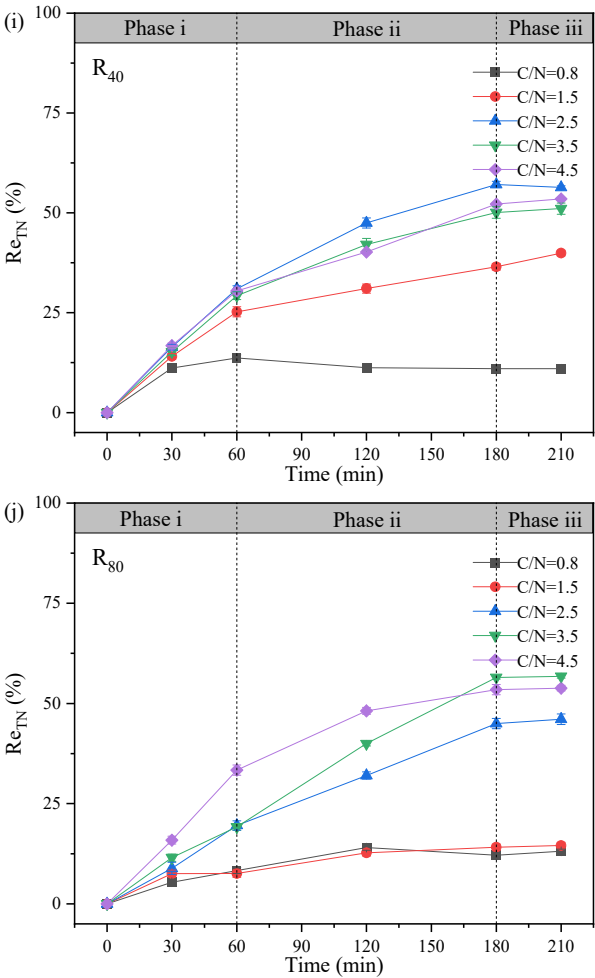
The variation profiles of NO₃-N, NO₂-N and COD in the typical cycle at the test temperature of 10 ± 2°C was exhibited, and the mechanism of NO₂-N accumulation during the stable operation was further elaborated (Figure 3). Based on the observed changes in substrate concentration over reaction time, the denitrification process can be divided into three distinct phases: Phase i (0 - 60 min), Phase ii (60 - 180 min), and Phase iii (180 - 210 min).











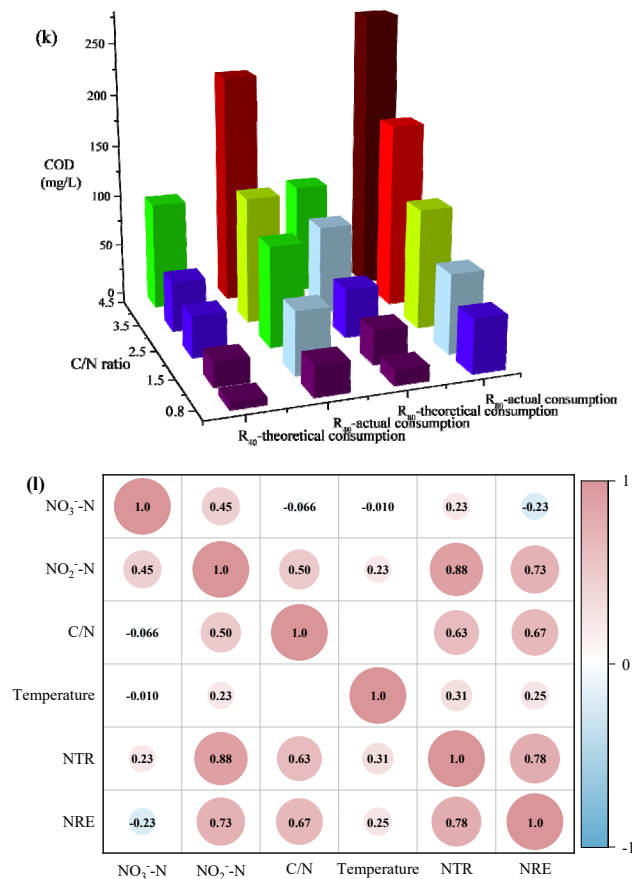


Figure 3. Variations of COD (a-b), NO_2^- -N (c-d), NO_3^- -N (e-f), TN (g-h), Re_{TN} (i-j) linked with carbon source consumption (k) and correlation analysis (l) in the typical cycles.

On the one hand, in the R_{80} group, the COD utilization amounts increased from 54 mg/L to 196 mg/L when the C/N ratio varied from 0.8 to 4.5, which were much higher than the COD consumption in the R_{40} systems under the same C/N condition (Fig. 3a-b). This implied that the high influent NO_3^- -N could promote the high-efficient utilization of carbon sources. Furthermore, the COD degradation rate was positively correlated with the C/N ratio in all systems ($y_{4.5}(1.30) > y_{3.5}(1.09) > y_{2.5}(0.91) > y_{1.5}(0.52) > y_{0.8}(0.05)$ in the R_{40} group (Figure 3a); $y_{4.5}(3.27) > y_{3.5}(2.53) > y_{2.5}(1.96) > y_{1.5}(1.37) > y_{0.8}(0.89)$ in the R_{80} group (Figure 3b)). It was worth noting that 4.5- R_{40} (70.00 mg/L), 3.5- R_{80} (60.00 mg/L) and 4.5- R_{80} (81.00 mg/L) showed higher COD residuals, probably due to the “carbon breakthrough” phenomenon (Ge et al., 2012), implying that the denitrifying bacteria no longer conducted the excess COD in the denitrification stage, consequently, not all COD was utilized for NO_3^- -N removal. As well, the theoretical and actual COD consumptions were further calculated based on the operating data (Figure 3k), in which 1.14 and 1.72 mg COD was utilized to reduce 1 mg of NO_3^- -N to NO_2^- -N and 1 mg of NO_2^- -N to N_2 (Zhang et al., 2023c). It was found that the actual COD consumption was generally higher than the theoretical values for all C/N ratios, especially for 4.5- R_{40} and 4.5- R_{80} . It was speculated that the low temperature led to inhibited enzyme activity and reduced electron transfer, and higher C/N ratio facilitated the replenishment of energy loss for microorganisms to maintain cellular activity through endogenous respiration (Zhang et al., 2023d). However, under the lower C/N ratios (e.g., 0.8- R_{80} and 1.5- R_{80} systems), the effluent COD at 180 min (5 - 27 mg/L) was much lower with continued degradation of NO_3^- -N, presumably owing to the possibility of sludge adsorption (Ji et al., 2025; Kotrba et al., 2001). Hence, the COD concentration exhibited a slight increasing trend (5 → 8 mg/L; 27 → 32 mg/L) after aeration (180 - 210 min) due to the secondary release of carbon adsorbed on the sludge flocs (Figure 3b).

On the other hand, obvious $\text{NO}_2\text{-N}$ accumulation (Figure 3c-d) was observed as well as significant $\text{NO}_3\text{-N}$ reduction (Figure 3e-f). During Phase i, with the addition of carbon and nitrogen sources, $\text{NO}_3\text{-N}$ concentration decreased rapidly (slopes: 0.10 - 0.47 in the R_{40} group; 0.14 - 0.99 in the R_{80} group), accompanied by the rapid $\text{NO}_2\text{-N}$ generation. The peaks of $\text{NO}_2\text{-N}$ accumulation appeared at 60 min ($19.20 \text{ mg/L (3.5-R}_{40}) > 16.96 \text{ mg/L (4.5-R}_{40}) > 11.20 \text{ mg/L (2.5-R}_{40}) > 4.14 \text{ mg/L (1.5-R}_{40})$; $46.6 \text{ mg/L (2.5-R}_{80}) > 39.20 \text{ mg/L (3.5-R}_{80}) > 25.20 \text{ mg/L (4.5-R}_{80}) > 17.71 \text{ mg/L (1.5-R}_{80})$) in addition to 0.8- R_{40} . Correspondingly, the highest $\text{NO}_2\text{-N}$ accumulation rates of the two groups were also found at C/N ratios between 2.5 ($y_{2.5} = 0.17x$; $y_{2.5} = 0.83x$) and 3.5 ($y_{3.5} = 0.29x$; $y_{3.5} = 0.66x$), respectively. The results were consistent with previous studies that C/N ratios of 2.5 - 3.5 favored nitrite accumulation, while increasing C/N to 3.5 or higher may result in suppressive $\text{NO}_2\text{-N}$ transformation (Yuan et al., 2013). It suggested that limited carbon sources can facilitate greater enrichment of PD denitrifying bacteria (Takeda et al., 2024; Zhang et al., 2021a) although extreme low C/N ratio (e.g., 0.8) may inhibit microbial growth or colonization (Peng et al., 2021). At Phase ii, the two groups were accompanied by a significant decrease in $\text{NO}_2\text{-N}$ concentration with the slopes changing around 0.001 - 0.045 (R_{40}) and 0.001 - 0.148 (R_{80}), indicating that the excessive $\text{NO}_2\text{-N}$ was gradually denitrified to N_2 as an electron acceptor (Park et al., 2017). Obviously, $\text{NO}_2\text{-N}$ was almost non-existent at the C/N ratio of 0.8, and studies have shown that insufficient carbon source led to bacterial lysis and death, which deteriorated the PD performance (Fu et al., 2022). As for Phase iii, the concentrations of $\text{NO}_3\text{-N}$ and $\text{NO}_2\text{-N}$ in all reactors were basically unchanged, confirming that the nitrifying bacteria (especially $\text{NO}_2\text{-N}$ -oxidizing bacteria, NOB) had been swept out during the short aeration (Zhang et al., 2022).

From the perspectives of TN (Figure 3g-h) and Re_{TN} variations (Figure 3i-j), the denitrification patterns of the two groups were comparable, and TN removal mainly concentrated in Phases i and ii (0 - 180 min). In Phase iii (180 - 210 min), the TN concentrations were overall unchanged. For the R_{80} systems, the effluent TN concentrations were 71.73, 70.50, 44.90, 34.50, and 37.50 mg/L, corresponding to Re_{TN} of 13.1%, 14.6%, 46.1%, 56.8%, and 53.8% with the comparisons of R_{40} systems (e.g., 13.3% (C/N=0.8), 39.9% (C/N=1.5), 56.3% (C/N=2.5), 51.1% (C/N=3.5), and 53.5% (C/N=4.5)). Generally, the denitrification performance was greatly enhanced when the influent C/N ratio increased from 0.8 to 3.5 - 4.5. It was supposed that the influent carbon source was relatively insufficient at C/N ratios of 1.5 - 2.5 especially in the R_{80} systems, contributing to lower Re_{TN} but higher nitrite accumulation (Zhang et al., 2022). Conversely, under higher C/N ratios of 2.5 - 3.5, the overall performance was relatively remarkable, despite of slight differences in $\text{NO}_2\text{-N}$ accumulation and Re_{TN} , which demonstrated that the effective denitrification system could be carried out only when the carbon source was sufficient (Xu et al., 2018). Besides, the correlation analysis (Figure 3l) revealed that the C/N ratio was positively with $\text{NO}_2\text{-N}$ concentration ($r=0.50$, $p < 0.01$), NTR ($r=0.63$, $p < 0.01$) and NRE ($r=0.67$, $p < 0.01$), conforming to the superior PD performance in the 3.5- R_{40} and 2.5- R_{80} systems (Figure 2 and Figure 3). Notably, temperature showed an un conspicuous correlation with NTR ($r=0.31$, $p < 0.01$) and NRE ($r=0.25$, $p < 0.01$), suggesting a strong shock resistance ability to environmental factors.

3.3. Species Diversity

The smoothest SS curve implied the highest microbial diversity (Figure 4a), while the steepest curve at the C/N ratio of 2.5 represented the lowest biodiversity (Zhang et al., 2022). Microbial diversity suggested that the sludge diversity in the PD - SBR reactors had been changed through the cultivation with different influent C/N ratios. The clustering analysis (Figure 4b) demonstrated that at a 0.97 similarity threshold (Zhang et al., 2021b), the species composition for C/N ratio of 2.5 - 3.5 was most similar, with similarity distance coefficients (SDC) of 0.22, equating to the similarity of 78%. In particular, data from the typical cycle (Figure 3) showed that $\text{NO}_2\text{-N}$ accumulation under these conditions were closely aligned. In contrast, when the C/N ratios were 0.8 and 1.5, the sludge samples exhibited the most similarity with SS species composition, with a distance coefficient of 0.43, corresponding to 57% similarity. The operational taxonomic units (OTUs) in the overlapping portions of the Venn diagrams (Figure 4c) represented the species number that were common to the different samples, while non-overlapping portions symbolized the species number that were unique to the

corresponding samples (Ji et al., 2025; Zhang et al., 2024). The common number of OTUs for the five samples was 514, which accounted for 27.35% (SS), 37.35% (C/N=0.8), 38.36 (C/N=1.5), 55.15% (C/N=2.5), 47.46% (C/N=3.5), and 53.65% (C/N=4.5) in the respective samples. However, the individual number of OTUs being unique to the samples, were 1275 (SS), 862 (C/N=0.8), 826 (C/N=1.5), 418 (C/N=2.5), 569 (C/N=3.5), and 444 (C/N=4.5), implying that the continuous enrichment of unique genera with higher microbial aggregation especially at C/N=2.5.

Combined with the microbial diversity index presented in Table 2, the sequence range of all samples fell between 44032 and 60472, with 932 - 1,879 OTUs obtained by clustering valid sequences at 97% similarity. Although the coverage of each sample exceeded 0.99, the abundance and diversity of sludge samples varied significantly compared to SS. Overall, the microbial diversity decreased across the five reactors, as confirmed by different diversity indices. Specifically, Chao and ACE for community richness declined from 2084 and 2052 to ranges of 1296 - 1752 and 1276 - 1727, respectively. Additionally, Shannon and Simpson for community diversity (Du et al., 2019) also shifted considerably, with the minimum Shannon value of 3.81 and the maximum Simpson value of 0.095 at C/N = 2.5, proving that the microbial richness was at its lowest. The above results also coincided with the changes of OTUs illustrated in Figure 4.

Table 2. Comparisons of microbial diversity index.

Samples	Sequence	OTUs	ACE	Chao	Shannon	Simpson	Coverage
SS	50347	1879	2084	2052	6.09	0.006	0.993
C/N=0.8	60472	1376	1752	1727	4.88	0.040	0.991
C/N=1.5	49551	1340	1693	1666	4.87	0.038	0.992
C/N=2.5	46142	932	1296	1276	3.81	0.095	0.992
C/N=3.5	50825	1083	1469	1445	4.29	0.057	0.992
C/N=4.5	44032	958	1564	1370	3.84	0.076	0.992

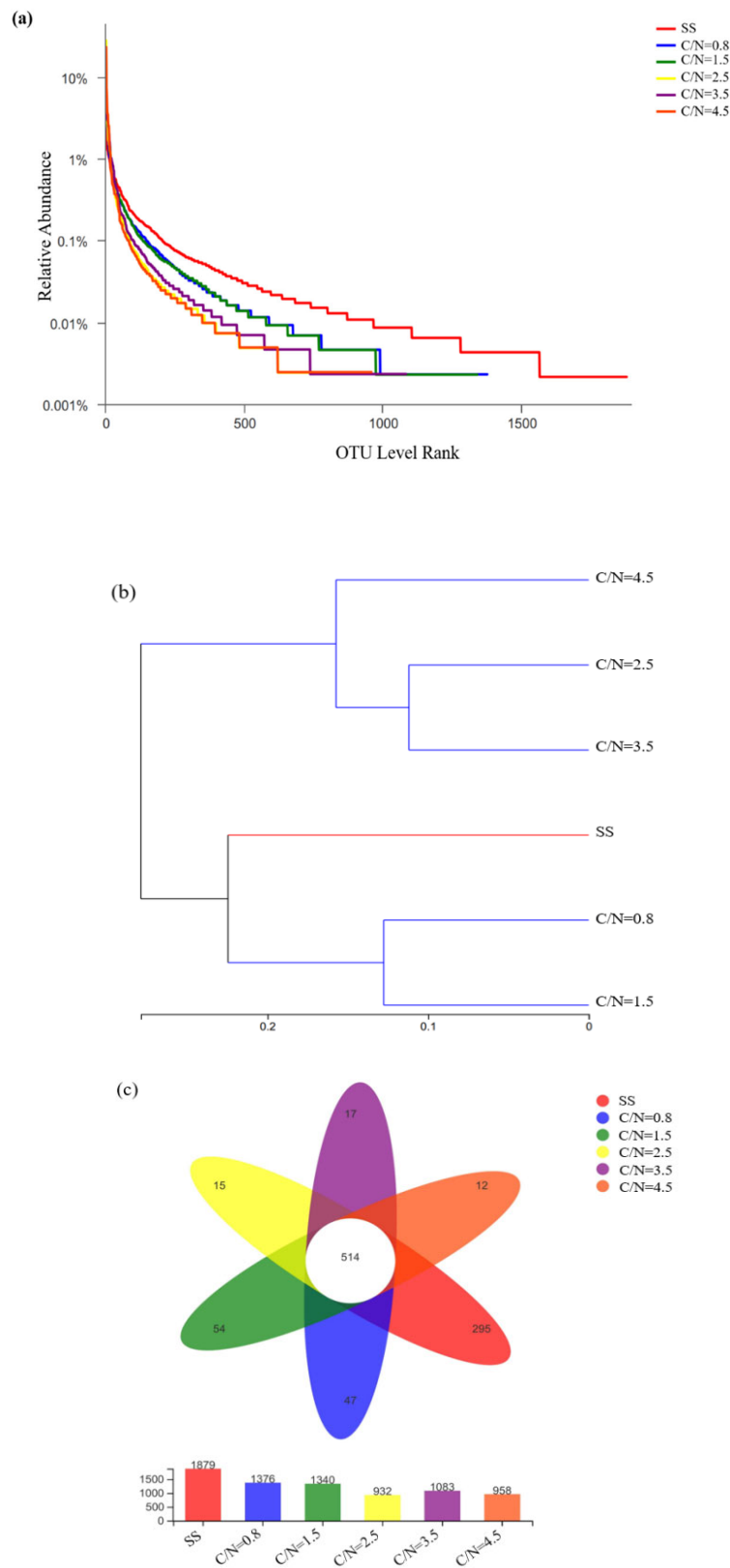
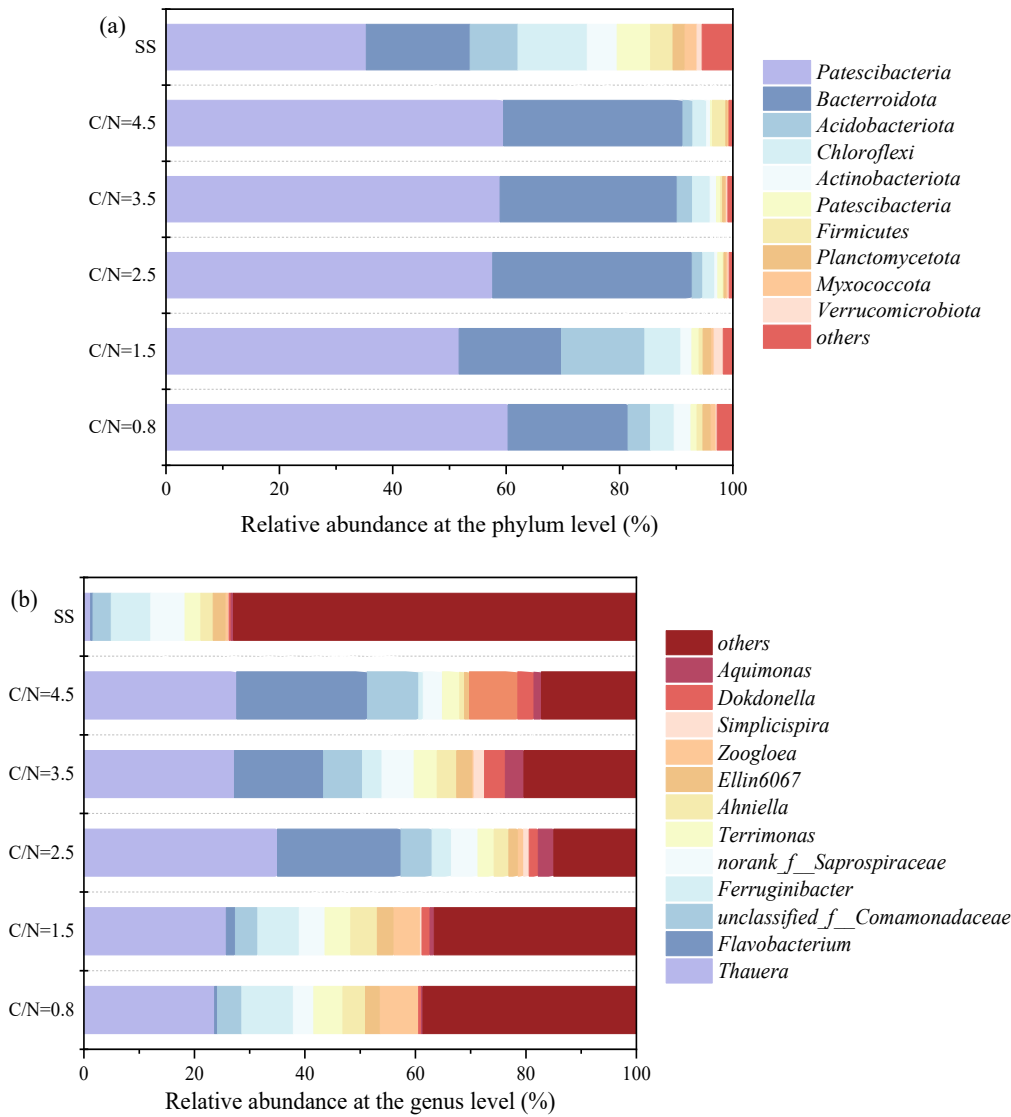


Figure 4. Microbial diversity analysis based on OTUs (a: Rank-Abundance curves; b: Hierarchical clustering tree; c: Venn diagram).

3.4. Dominant Microbial Community

The microbial distribution of functional bacteria in each sludge sample at temperature of 3 - 12 °C was compared at both phylum and genus levels (Figure 5). Compared to SS, the proportions of *Proteobacteria* and *Bacteroidetes*, the two dominant phyla, increased from 35.31%, 18.34% to 51.69% - 60.35% and 18.08% - 35.21%, respectively. This shift resulted in an enhancement of sludge denitrification performance and improved the conversion rates of NO₃-N to NO₂-N, while *Chloroflexi* capable of oxidizing NO₂-N to NO₃-N reduced from 8.42% to 1.47% - 3.95% (Figure 5a). Another phylum, *Myxococcota*, which is unfavorable for NO₂-N accumulation (Zhang et al., 2023a), also decreased significantly with the increasing C/N ratio, dropping from 2.10% (SS) to 0.17% (C/N=4.5). Additionally, *Patescibacteria* (1.27% - 0.38%), *Actinobacteria* (2.95% - 0.53%), *Firmicutes* (3.97% - 0.15%), and *Acidobacteria* (14.70% - 1.74%) all of which play vital roles in degrading carbohydrates during heterotrophic metabolism (He et al., 2018), exhibited a significant decreasing trend under all working conditions compared to SS.



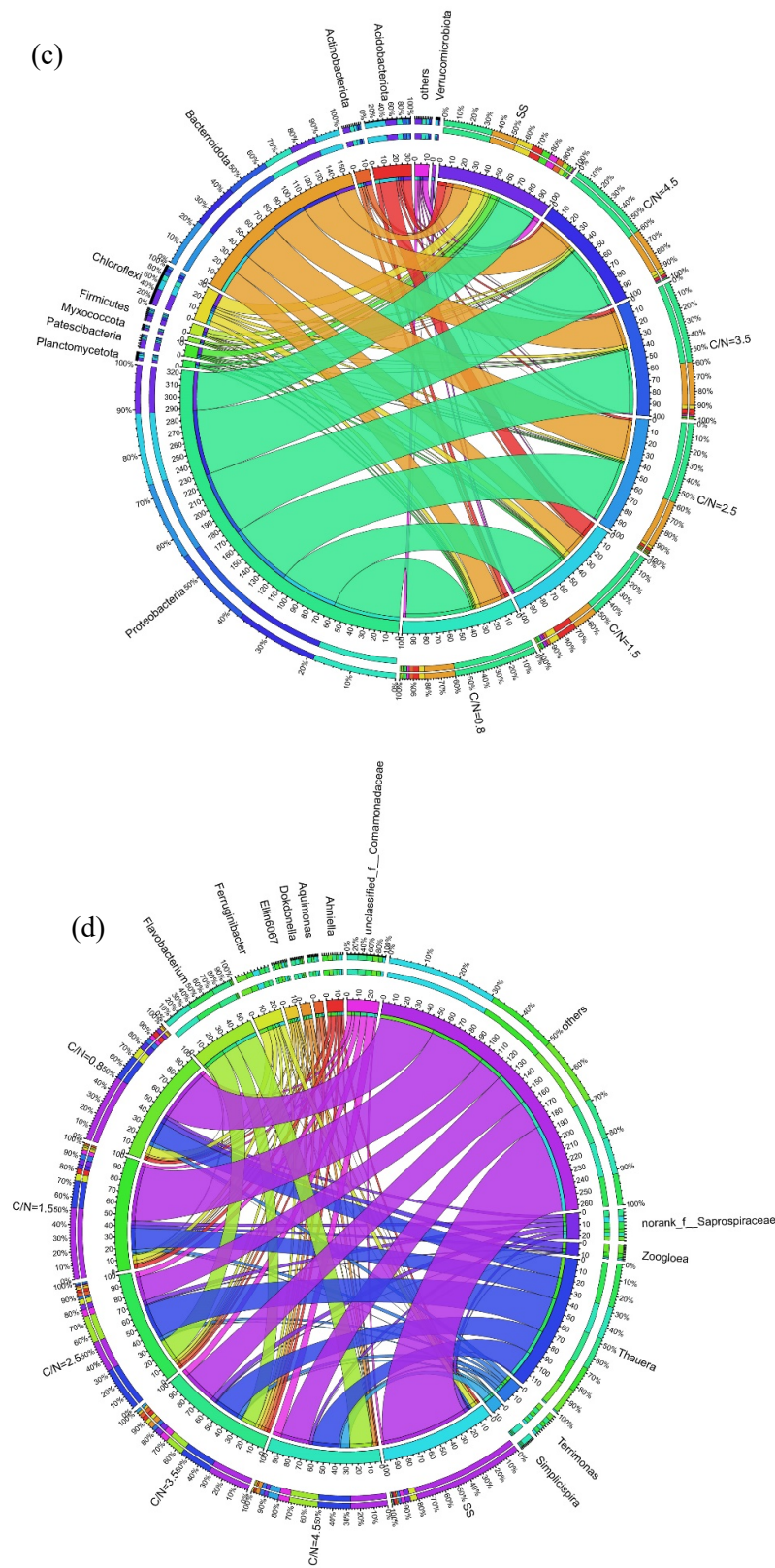


Figure 5. Microbial taxonomic compositions at the phylum (a, c) and genus level (b, d).

Significant differences were observed at genus level across the six samples (Figure 5b and Figure 5d), where *Thauera* known as denitrifying bacteria that stably maintain NO_2^- -N accumulation (Du et

al., 2017a), accounted for a relatively high percentage of 19.31% - 31.83% (C/N=0.8 - 4.5) than SS (0.87%). *Thauera* enrichment was associated with the inhibition of NO₂-N reduction (Du et al., 2016b) due to denitrifying enzyme synthesis at different electron acceptors (NO₃-N and NO₂-N). The macro-genomic analyses showed that the abundance of NO₃-N reductase (*Nar*: NO₃-N → NO₂-N) was higher than that of NO₂-N reductase (*Nir*: NO₂-N → NO) in *Thauera* (Qian et al., 2019). Moreover, *Nar* was more competitive than *Nir* for electron donors during denitrification, highlighting the strong influence of C/N ratio on the activities of *Nar* and *Nir* (Zhang et al., 2024). At lower C/N ratios, *Nar* preferentially utilized the carbon source to reduce NO₃-N, which inhibited *Nir* due to insufficient carbon access and resulted in NO₂-N accumulation. Conversely, at higher C/N ratios, where carbon sources were sufficient, *Nir* could also access adequate carbon capacity, reducing the likelihood of NO₂-N accumulation. *Flavobacterium* exhibited similar denitrifying properties to *Thauera* (Chen et al., 2020; Králová, 2017; Zhang et al., 2024) and also proliferated in the reactors, increasing from 0.31% (SS) to 0.42% - 20.85% (C/N=0.8 - 4.5). In particular, at C/N ratio of 2.5, the two genera accounted for 31.83% and 20.30%, respectively, confirming the earlier findings that better NO₂-N accumulation was achieved (Figure 2 and Figure 3).

It was noteworthy that *Flavobacterium* showed better denitrification ability and strong adaptability to cold climates (Králová, 2017), and its abundance reached to 20.30% and 20.85% at C/N ratios of 2.5 and 4.5, respectively, which significantly higher than the other reactors (ranged from 0.31% to 13.94%). It was also found that *Flavobacterium* was more effective to generate NO₂-N in cold conditions, and its abundance increased to 16.6% when the temperature reduced from 32 °C to 5 °C (Chen et al., 2020). Considering the ambient temperature changes in this experiment, it was hypothesized that the low-temperature environment (5 - 10°C) accelerated the enrichment of *Flavobacterium*, which was the key factor in the enhanced PD performance observed at C/N ratio of 2.5 (Figure 2 and Figure 3). Besides, *Saprospiraceae*, *Comamonadaceae* and *Terrimonas*, which play key roles in the denitrification process, accounted for 3.03% - 5.01%, 3.07% - 8.20%, and 2.66% - 3.62%, respectively. The genera *Ferruginibacter* and *Simplicispira*, primarily involved in the degradation of organic matter (Chen et al., 2020), changed from 0.73%, 0.02% to 5.64% and 7.73%. *Zoogloea*, which is vital for bioconcentration and sludge granulation (Zhang et al., 2020), also showed better enrichment at C/N ratios of 0.8 and 1.5 with the abundances achieving at 5.66% and 3.66%, implying the COD release triggered by sludge adsorption (Figure 3b). Meanwhile, the results indicated that the genus of *Zoogloea* could also maintain a good biological activity at low C/N ratios.

3.5. Application Feasibility of PD Related Processes

Currently, PD is the most common method to achieve NO₂-N accumulation in wastewater treatment, offering many advantages over conventional BNR processes. As shown in Figure 6a, PD often combined with the Anammox process, creating a new application prospect with outstanding advantages of higher economic value (e.g., stable NO₂-N production, high NO₃-N removal, low carbon consumption) and environmental benefit (e.g., reduced greenhouse gas, low sludge production) (Fan et al., 2024; Le et al., 2019). However, it still faces many technical challenges in terms of long start-up cycle, microbial activity inhibition, unstable NO₂-N production, and low treatment efficiency. Typically, the transcription and translation levels were suppressed with reduced metabolism-related enzyme activities at low temperatures (Zhen et al., 2021).

In practical engineering, *napA* and *narG* genes were important factors for NO₂-N accumulation in the PD process, and the relative abundance of *narG* gene decreased with increasing reaction temperature from 10 - 52 °C (Liao et al., 2018). Hence, at lower temperatures, denitrification was less competitive for NO₂-N generation (Wang et al., 2018) and the *nirS*, *nirK* genes that further converting NO₂-N to NO were hindered, except a substantial increase in functional genes of *narG* and *napA* (Peng et al., 2023). Taken together, it can be concluded that the essential reasons for the higher NTR and NO₂-N accumulation at higher temperature were the stimulant functional genes of *narG* and *napA* accompanied by restrained abundances of *nirS* and *nirK*. However, it was also found that the repression degree of *narG*, *nirS* and *nirK* was unequal with the temperature decreasing from 30°C to

5°C although more severe inhibition of *nirS* and *nirK* promoted better NTR (Peng et al., 2023), which was not consistent with the observations of present experiments. Meanwhile, it has also been recently found that *narG* abundance decreased and *napA* abundance increased when the temperature decreased from 25°C to 15 °C, suggesting that *napA* genes gradually prevailed in low-temperature environments (Gong et al., 2025). However, the potential mechanism of temperature effect on transcribed gene needs to be further investigated.

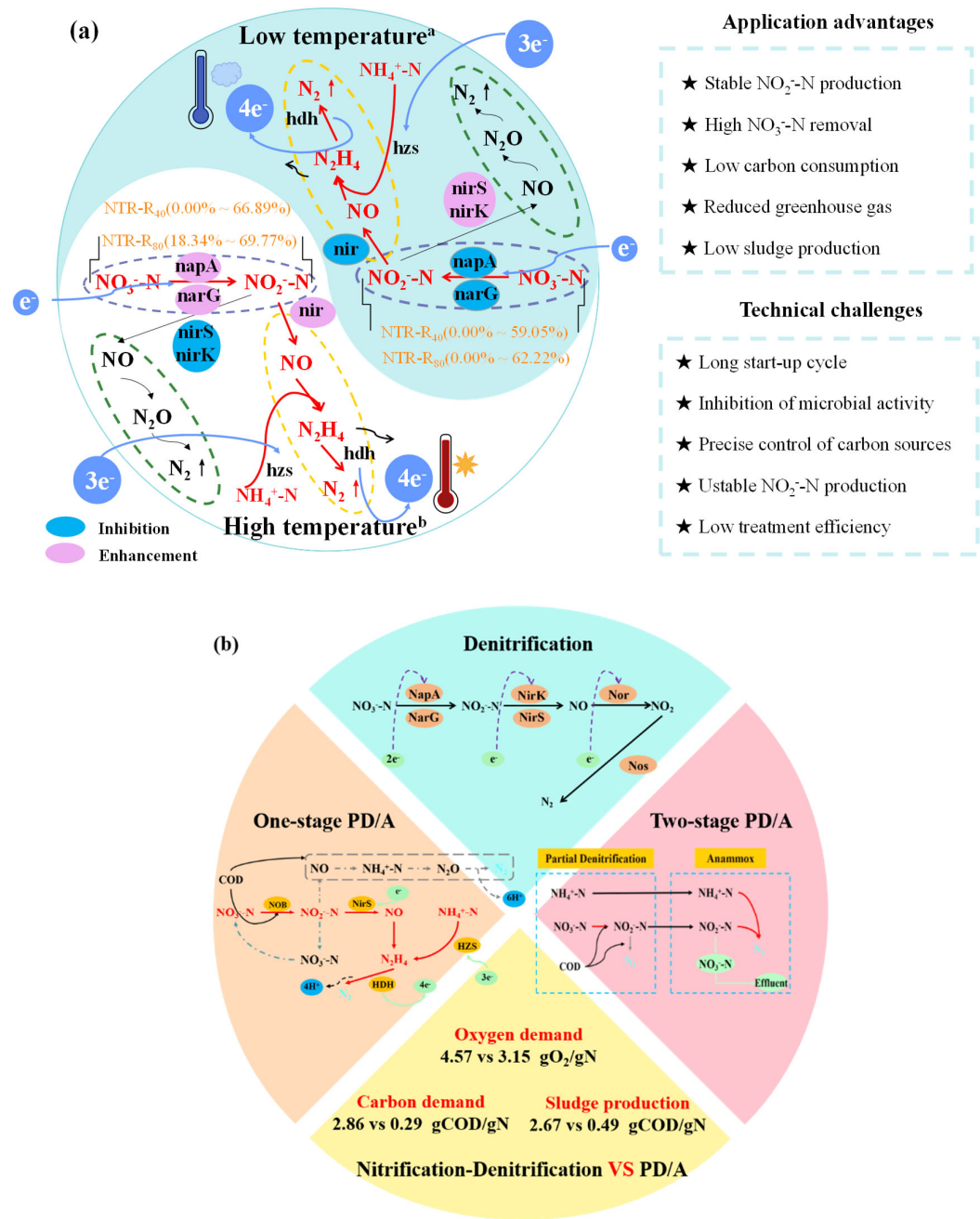


Figure 6. Mechanism analysis linked with temperature (a) and potential application of PD/A process (b).

Furthermore, a summary of how temperature, C/N ratio and NO_3^- -N concentration affecting PD performance in relation to NO_2^- -N buildup was provided (Table 3). It was found that NTR reached to 71.7% at a temperature of about 22 °C under the C/N ratio of 2.5 (Gong et al., 2013), and the value rose to 83.3% when the temperature was increased to 23.6 - 28.8 °C (Du et al., 2017b). As a contrast, at the same C/N ratio (2.5), NTR at lower temperatures (3 - 12°C) was 76.79% in the present study (R_{80}

system) but it changed to 68.15% - 75.57% as the C/N ration rose to 3.5 - 4.5. Consisting with the results of our study, the PD process presented a high NTR of 80% at the C/N ratio of 3.0 under temperatures between 16 - 28°C (Du et al., 2016b), however, NTR declined to 57.5% when the temperature dropped to 10.6 - 18.3°C (Du et al., 2016a). In addition to high temperature, suitable carbon source, lower C/N ratio and higher NO₃-N concentration were also the key factors to obtain stable NO₂-N production (Zhang et al., 2023d), and many studies have reached similar conclusions that NTR was around 80% when the C/N ratio was set between 2.5 and 3.0 using acetate as a carbon source (Du et al., 2017b; Du et al., 2016b; Gong et al., 2013). In the case of glycerol, the peak NTR of 72.5% was attained at a C/N ratio of 2.6 (Matthew et al., 2017), showing the importance of carbon source type (Zhang et al., 2024). Even so, conventional denitrification processes required a higher C/N of 4.0 - 6.0 (Nancharaiah & Venugopalan, 2011), which significantly increased the carbon demand and operating cost of low C/N ratio wastewater treatment. Moreover, the industrial products often generated significant amounts of high-strength NO₃-N wastewater (Mohan et al., 2016), which typically relied on traditional BNR technologies that were costly with limited applicability. As shown in Figure 6b, one-stage and two-stage PD/A systems were regarded as the two important approaches to treat NO₃-N rich and domestic wastewater simultaneously (Du et al., 2017a; Gao et al., 2022), where the oxygen demand, carbon source requirement and sludge production could be reduced by 31.07%, 89.86%, and 81.65% when compared with the traditional nitrification-denitrification (Zhang et al., 2023b). For real application, the two-stage PD/A process often showed prominent advantages under low-temperature operation (Du et al., 2016a), since the separate regulation of two reactors was more suitable to resist changes in environmental factors for low C/N ratio wastewater treatment.

Table 3. Comparisons of PD performance under different C/N ratios, NO₃-N concentrations and temperatures.

Reactors	Working volume (L)	Carbon source	C/N ratio	Influent NO ₃ -N (mg/L)	Temperature (°C)	NTR (%)	References
SBR	10	Acetate	2.5	41.6	22±2	71.7	(Gong et al., 2013)
SBR	5	Acetate	2.5	30 - 400	23.6 - 28.8	83.3	(Du et al., 2017b)
SBR	5	Acetate	3.0	25	16 - 28	~80	(Du et al., 2016b)
SBR	5	Acetate	3.0	50	10.6 - 18.3	57.5	(Du et al., 2016a)
SBR	12	Glycerol	2.5 - 2.8	100	20 - 23	72.5, C/N=2.6 76.79, C/N=2.5	(Matthew et al., 2017)
SBR	10	Acetate	2.5 - 4.5	80	3 - 12	75.57, C/N=3.5 68.15, C/N=4.5	This study

In addition, systems treating wastewater with elevated influent NO₃-N concentrations (30 - 400 mg/L) (Du et al., 2017b) exhibited significantly superior NTR compared to those with lower NO₃-N

levels. In this study, the PD performance of the R₈₀ group also outperformed that of the R₄₀ group (Figure 2-3). Previous research corroborated that when influent NO₃-N concentration was increased from 29.5 mg/L to 64.9 mg/L, the optimal NO₂-N accumulation correspondingly rose from 18.9 mg/L to 37.8 mg/L (Sun et al., 2009). The reason may be attributed to the fact that the higher NO₃-N concentration provided more sufficient electron acceptors (Zhang et al., 2023d) to promote the metabolic activity of PD denitrifying bacteria by inhibiting the degradation rate of NO₃-N. To sum up, these results indicated that moderately elevating influent NO₃-N concentration or nitrate loading rates (NLR) enhanced the stability of PD systems (Cao et al., 2019), which could effectively alleviate the inhibition effect of low temperature, providing a new operation strategy of PD processes for low temperature conditions. Even so, there is a pressing need to explore additional effective control strategies of PD/A process, as well as to investigate the impacts of heavy metals, antibiotics, and other substances on microbial activity.

4. Conclusions

The NO₂-N accumulation and NTR performance were markedly suppressed as the temperature decreased from 10 °C to 5 °C, which may be due to the suppression of *narG* gene transcription at low temperatures. The 3.5-R₄₀ and 2.5-R₈₀ systems demonstrated the optimal PD efficiency, and elevated influent NO₃-N concentration effectively alleviated the inhibition effect of low temperature. Notably, the PD performance of the R₈₀ group surpassed that of the R₄₀ group in both long-term operation and typical cycles, which was attributed to the preferential utilization of abundant electron acceptors by *Nar*, thereby accelerating the reduction of NO₃-N to NO₂-N. The optimal C/N ratio range of 2.5 - 3.5 promoted the enrichment of distinct bacterial taxa with higher aggregation levels (932 - 1083 OTUs), predominantly within the phyla of *Proteobacteria* and *Bacteroidetes*. Specifically, the enrichment of *Thauera* and *Flavobacterium* played a pivotal role in NO₂-N accumulation, contributing to the enhanced PD performance especially under low-temperature conditions.

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