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Enhanced nitrogen removal for low C/N wastewater via preventing futile carbon oxidation and augmenting anammox

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ABSTRACT

Efficient carbon use is crucial for biological nitrogen removal. Traditional aerobic processes can waste carbon sources, exacerbating carbon deficiency. This study explores an anaerobic/oxic/anoxic system with sludge double recirculation to improve nitrogen removal in low C/N wastewater. This system integrated aerobic nitrification after the carbon intracellular storage, separating carbon and nitrogen by denitrifying glycogen-accumulating organisms (DGAOs) with endogenous partial denitrification and Anammox within the anoxic units. A significant efficiency of 91.02±7.01% chemical oxygen demand (COD) was converted into intracellular carbon in anaerobic units, significantly reducing carbon futile oxidation in the aerobic units by effectively separating COD from ammonia. Intracellular storage of carbon sources and microbial adaptation to carbon scarcity prevent futile oxidation of COD in the aerobic units even with short-term high dissolved oxygen (DO), thereby enhancing nitrogen removal under anoxic conditions with sufficient intracellular carbon source. The microbial analysis identified *Candidatus Brocadia* as the dominant anammox bacteria, in combination with the activity of DGAOs and other related microbial communities, accounting for 37.0% of the TN removal. Consequently, the system demonstrated remarkable nitrogen removal efficiencies, achieving 81.3±3.3% for total nitrogen (TN) and 98.5±0.9% for ammonia nitrogen while maintaining an effluent COD concentration of 17.2±9.1 mg/L, treating the low C/N of 4.18 in the influent wastewater. The findings in this study provide a sustainable and energy-saving technique for conventional WWTPs to meet strict discharge standards by avoiding futile oxidation of COD and encouraging anammox contributions.

1. Introduction

Conventional biological wastewater treatment processes typically involve autotrophic microbes for nitrification and heterotrophic microbes for denitrification to effectively remove nitrogen from wastewater (Zeng et al., 2023). However, many wastewater treatment plants (WWTPs) in China struggle to efficiently remove total nitrogen (TN) due to a limited carbon source in influent, impeding denitrification (Smith et al., 2019). To achieve strict TN discharge standards, two strategies are used: optimizing carbon usage in influent for pre-denitrification by nitrate liquid recirculation in process units like A/A/O, or using Anammox, which is independent of organic carbon. Both strategies use nitrification or partial nitrification, which consumes carbon sources in aerobic units. Therefore, alternative approaches are needed to prevent aerobic carbon oxidation and improve denitrification. Recent biological nitrogen removal technologies include the anammox process, which directly converts ammonia nitrogen ($NH_4^+ - N$) and nitrite ($NO_2^- - N$) into nitrogen gas (N_2) without an organic electron donor or oxygen (Cao et al., 2019; Cui et al., 2021). However, since municipal wastewater typically contains NH_4^+–N without the required $NO_2^- - N$, PN or partial denitrification (PD) is necessary to produce $NO_2^- - N$. These processes lead to the consumption of organic matter, thus depleting carbon sources needed for later denitrification stages.

Compared to pre-denitrification, endogenous denitrification (ED) with denitrification glycogen-accumulating organisms (DGAOs) can achieve full denitrification with adequate carbon sources. (Winkler

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et al., 2011). These organisms convert carbon from wastewater into intracellular stores like polyhydroxyalkanoates (PHAs) or glycogen (Gly), which then serve as electron donors in anoxic units for denitrification (Wang et al., 2008). DGAOs also facilitate endogenous partial denitrification (EPD) by reducing nitrate (NO_3^- – N) to NO_2^- – N, providing NO_2^-–N for anammox processes and optimizing the use of limited carbon in low C/N influent (Rubio-Rincon et al., 2017). Pre-anaerobic treatment transforms influent organic matter into these intracellular carbon sources, enhancing cooperation between anammox bacteria and DGAOs for effective nitrogen removal (Ji et al., 2017).

Post-endogenous denitrification (PED) has recently been studied (Zhao et al., 2018a; Zhao et al., 2018b). In PED, DGAOs store external carbon sources in the anaerobic units, preventing oxidation in the subsequent aerobic units. Moreover, low DO conditions in the aerobic units saves aeration costs and preserves carbon sources for partial denitrification/anammox (PD/A) and ED processes.

Effective nitrogen removal from wastewater with low C/N remains a critical challenge, prompting innovations in treatment configurations such as anaerobic/oxic/anoxic (AOA) systems with sludge double recirculation (SDR-AOA). Previous studies (Gao et al., 2024; Zhang et al., 2018) have demonstrated the excellent nitrogen removal performance of SDR-AOA configurations. However, the efficient utilization and control of carbon sources within these systems have not been comprehensively studied. Our research addresses this gap by focusing on the separating of carbon and nitrogen by DGAOs and optimized utilization of carbon sources for nitrogen removal in SDR-AOA systems. Specifically, we investigate how carbon source management at different stages enhances denitrification efficiency and facilitates anammox. This study contributes to advancing our understanding of AOA-based nitrogen removal strategies, emphasizing the role of internal carbon storage in enhancing treatment efficiency.

2. Results

2.1. Overall performance of the SDR-AOA system

The SDR-AOA system operated for 224 days, divided into four phases for analysis according to changes in operating parameters (Fig. 2). During Phase I, the average DO in the aerobic zone was 1.6±0.8 mg/L, with effluent TN and NH_4^+–N were 8.8±5.3 mg/L and 1.2±1.6 mg/L, respectively, The removal efficiencies were 82.30±11.33% for TN and 97.4±3.6% for NH_4^+ – N. While TN removal efficiency fluctuated (Fig. 2a), the NH_4^+–N removal remained consistently high (Fig. 2b). In Phase II, the DO was reduced to 0.6±0.2 mg/L (**Table S1**) to assess performance under low DO, resulting in effluent concentrations of NH_4^+–N and TN were 4.3±4.6 mg/L and 11.3±7.6 mg/L, respectively. This adjustment led to a decline in NH_4^+–N removal efficiency to 83.1 ±17.3% due to reduced nitrification. To enhance nitrogen removal, polyethylene carriers with a surface area of 800 m^2/m^3 (K4) were introduced into the system during Phase III, this change resulted in an increase of the effluent NH_4^+–N concentration to 3.3±3.5 mg/L. In Phase IV, with an influent C/N ratio of 4.18±0.43 under low DO, achieved NH_4^+–N and TN removal efficiencies of 98.5±0.9% and 81.4 ±3.3%, respectively. COD removal remained stable throughout the operation, with an average effluent COD concentration of 17.3±9.1 mg/L, meeting the Class IV surface water environmental quality standard of China for COD (<20 mg/L) (Administration, 2002).

2.2. Nitrogen removal performance

The nitrogen removal performance in different zones was analyzed in the enhanced system in Phase IV (Fig. 3). The effluent TN was 5.1±1.9 mg/L, with influent NH_4^+ – N, NO_3^- – N, TN and COD were 50.5±6.3 mg/L, 0.6±0.2 mg/L, 51.1±6.3 mg/L, and 202.0±32.4 mg/L.

During the Phase IV, nutrient variations were measured over three cycles (n=3), tracking changes in intracellular carbon sources (Fig. 3). In the anaerobic zone, the concentration of NH_4^+–N decreased from 50.5 ±6.3 mg/L to 24.2±2.5 mg/L, primarily due to the dilution effects of recirculated sludge, the concentration of NO_3^-–N remained stable. The anaerobic zone contributed 12.7% to TN removal (**Eq. S8**), because of heterotrophic denitrification of NO_2^-–N and NO_3^-–N from recirculated sludge. COD decreased from 202.0±32.4 mg/L to 18.3±9.2 mg/L not only due to the diluting but also because of a transformation to intracellular stores with a 92.1% COD_{intra} efficiency (**Eq. S1**). PHAs and Gly were measured at 2.2 mmol C/L and 15.3 mmol C/L, respectively, indicating the primary site for PHAs synthesis was the anaerobic zone.

In the second aerobic unit, the concentration of NH_4^+–N decreased from 24.2±2.5 mg/L to 4.2±1.1 mg/L, while NO_3^-–N increased to 15.6 ±3.2 mg/L, and the total nitrogen (TN) decreased to 20.0±3.4 mg/L. These suggests the occurrence of significant simultaneous nitrification

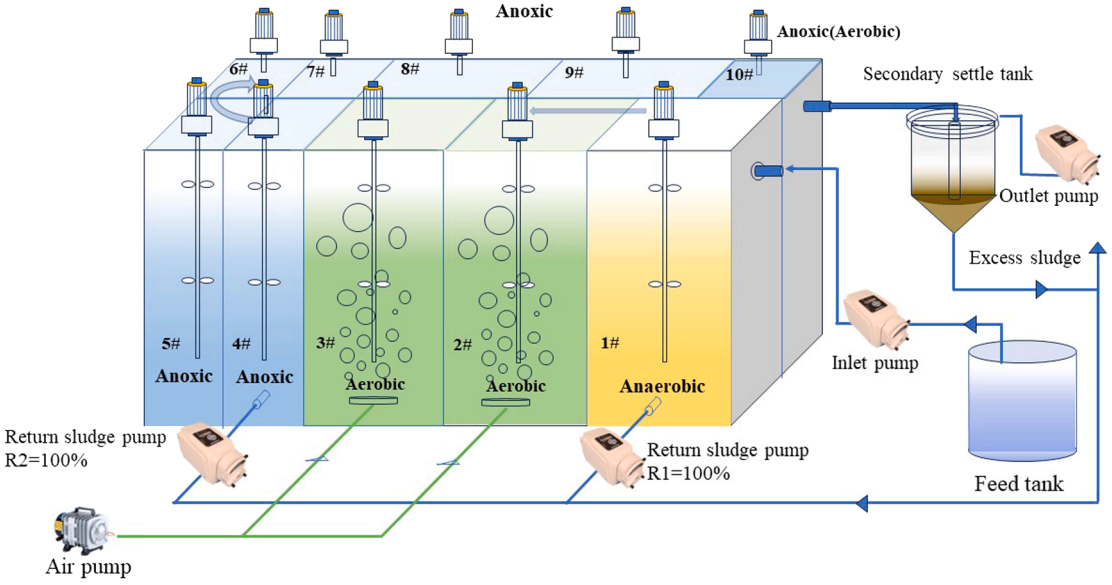

Fig. 1. Schematic diagram of the continuous flow reactor.

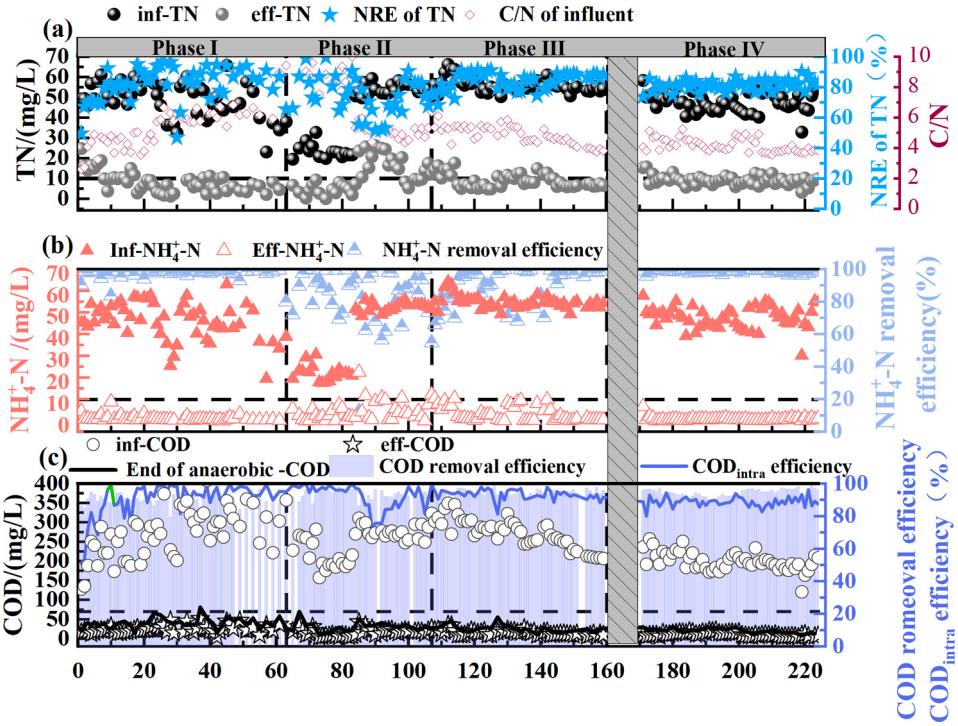

Fig. 2. Nutrient removal performance of the reactor: (a) Influent(inf.) TN, effluent (eff.) TN, the removal efficiency(R.E.) of TN and the C/N ratio of influent. (b) influent $NH_4^+ - N$, effluent $NH_4^+ - N$, and the removal efficiency of $NH_4^+ - N$, (c) influent COD, effluent COD, the removal efficiency of COD, and the COD_{intra}.

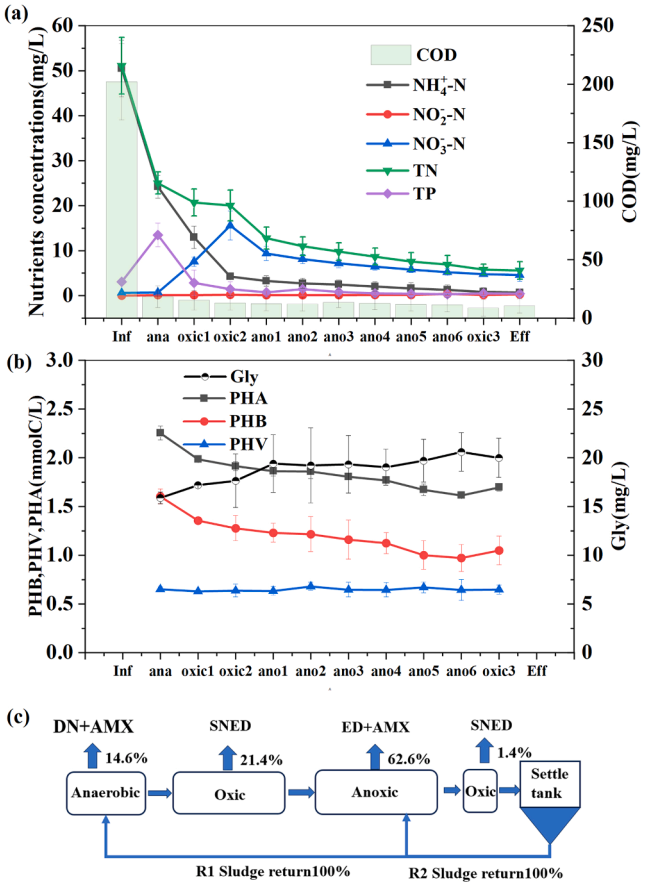

Fig. 3. (a)Typical cycle along the reactor in Phase IV(n=3): variations of COD, and (b)Nitrogen and intracellular carbon sources (PHA, PHB, PHV, and Glycogen).(c) Nitrogen removal contribution of each stage

and endogenous denitrification (SNED) processes in the aerobic zone. The PHAs content decreased to 1.9 mmol C/L, while the glycogen content increased to 17.6 mmol C/L by the end of the aerobic zone. The decrease in PHAs and the increase in glycogen, combined with the stability of COD concentration from the anaerobic to the aerobic zone, can both supporting SNED in the aerobic zone. SNED, which is common in AOA systems, plays a crucial role in nitrogen removal (Zhao et al., 2018b). Material balance analysis revealed that SNED occurred in aerobic units #1 and #2, contributing to 21.4±7.5% of TN removal (**Eq. S2, S3 and S9**). The effectiveness of SNED varied among the different operational phases of the system (Fig. 4). During Phase I, SNED accounted for 15.5±12.2% (**Eq. S2**). However, the reduction in DO since Phase II has led to an increase in SNED to 28.5 ± 16.0. In Phase IV, the

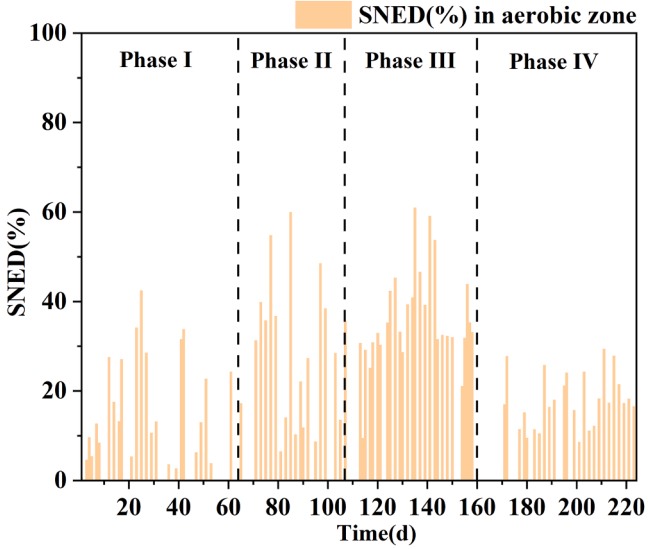

Fig. 4. The ratio of SNED in aerobic zone.

proportion of SNED decreases to 17.9±6.1% due to the lower C/N.

In the post-anoxic zone, COD remained low and unchanged, while NO_3^-–N reduced significantly to 4.6±1.4 mg/L (Fig. 3a), indicating effective ED. Meanwhile, PHAs concentration decreased to 1.6 mmol C/L, while glycogen increased to 20.0 mmol C/L, suggesting that PHAs provided the energy for denitrification (Fig. 3b). Material balance analysis shows that ED accounted for 55.7±12.4% of nitrogen removal in this zone (**Eq. S10**), highlighting the crucial role of post-anoxic in overall nitrogen removal performance.

Alao, the observed significant reduction of NH_4^+–N (**Eq. S4-S5**) in anoxic zones suggests the occurrence of anammox (Fig. 3a). Furthermore, the absence of significant nitrite accumulation in these zones, coupled with stable COD levels and reduced internal carbon sources (Fig. 3b), further indicates that the required nitrite electron acceptors were likely obtained through ED bacteria utilizing internal carbon sources to reduce nitrate nitrogen. Previous studies have shown that nitrite does not accumulate in some integrated PDA processes. Some of the nitrite produced by PD is used by Anammox and the other part is used by DGAOs to reduce nitrite to nitrogen (Li et al., 2020, Ji et al., 2020). Overall, the high-efficiency performance of the system was ensured by the coordinated action of SNED in the aerobic zone and ED in the anoxic zone. The co-contribution of each reaction zone to nitrogen removal is shown in Fig. 3c.

2.3. Verification of anammox activity in batch experiments

In the anoxic units of the AOA system, a decrease in NH_4^+–N suggested the enrichment of anaerobic ammonium-oxidizing bacteria (AnAOB), aligning with findings from previous studies (Cao et al., 2019; Gong et al., 2013). This reduction was linked to Anammox activity, confirmed by three batch experiments conducted between days 108 and 224 under anoxic conditions with DO below 0.1 mg/L. The results showed that NH_4^+-N degradation occurred in both Group A and Group B, where nitrate and nitrite were present, respectively, while there was no apparent decrease in NH_4^+-N in Group C, where only NH_4^+-N was available. This suggests that the reactions in Group A were caused by the interaction between NO_2^- and NH_4^+, while those in Group B could be attributed to PD (NO_3^- to NO_2^-) and simultaneous nitrite-ammonia reactions (Fig. 6). These results support the effectiveness of AnAOB in the system, aligning with the nitrite-ammonia reaction.

The contribution of Anammox to nitrogen removal was evaluated using **Eq. S7**. Group A and Group B demonstrated NH_4^+-N removal rates of 0.15±0.02 mg N/(gSS·h) and 0.14±0.01 mg N/(gSS·h), respectively, and TIN removal ratios of 38.8% and 85%. These findings highlight the critical role of Anammox in nitrogen removal within the AOA system, especially when paired with PD in Group B. Meanwhile, the lack of NH_4^+-N reduction in Group C further validates the role of Anammox in the system and underscores the importance of nitrate/nitrite in the reaction.

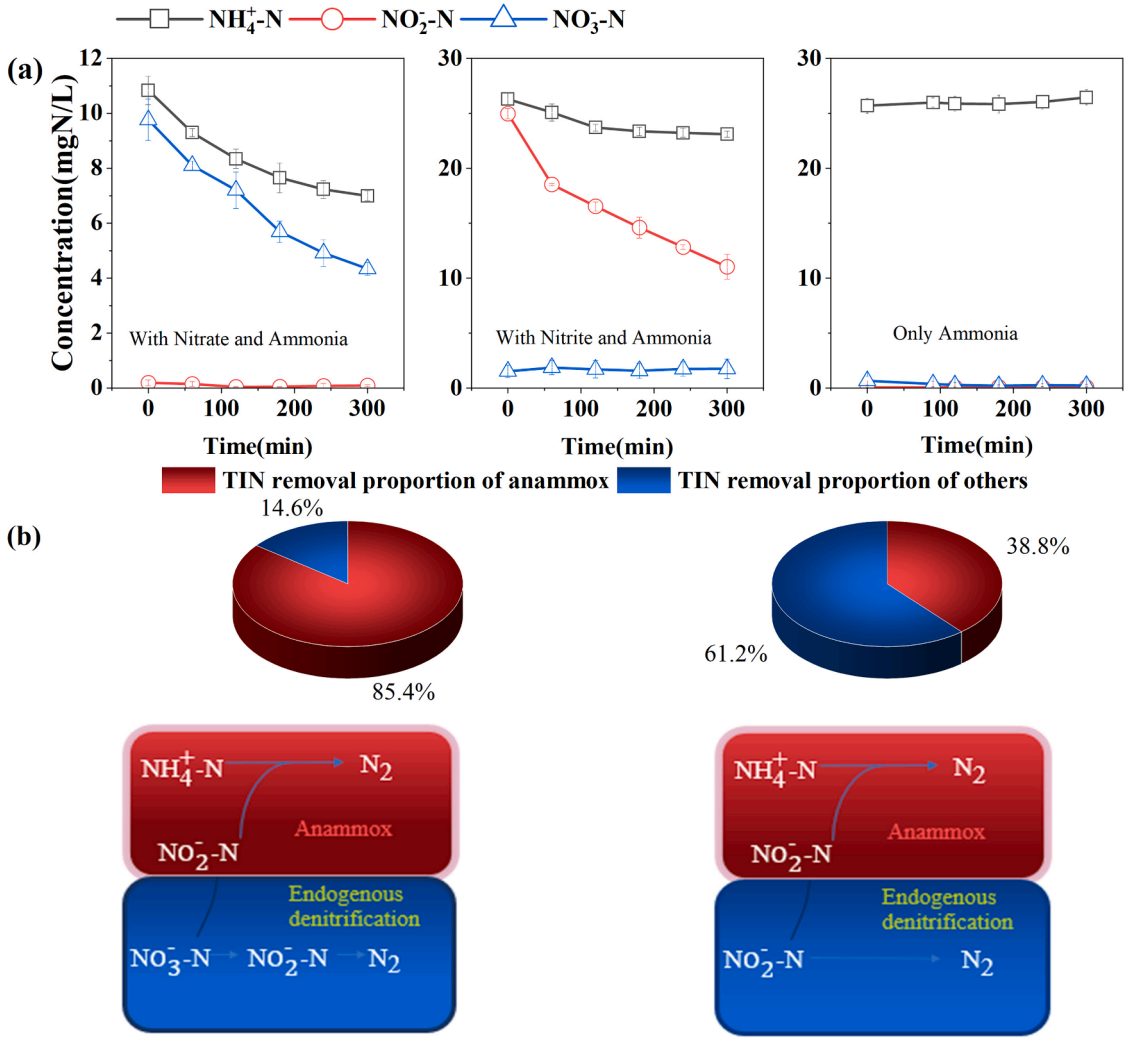

Fig. 5. (a) The results of the anaerobic ammonium oxidation activity verification experiment in groups A, B, and C, (b) The mechanisms of nitrogen removal and the contribution of anaerobic ammonium oxidation in group A and group B.

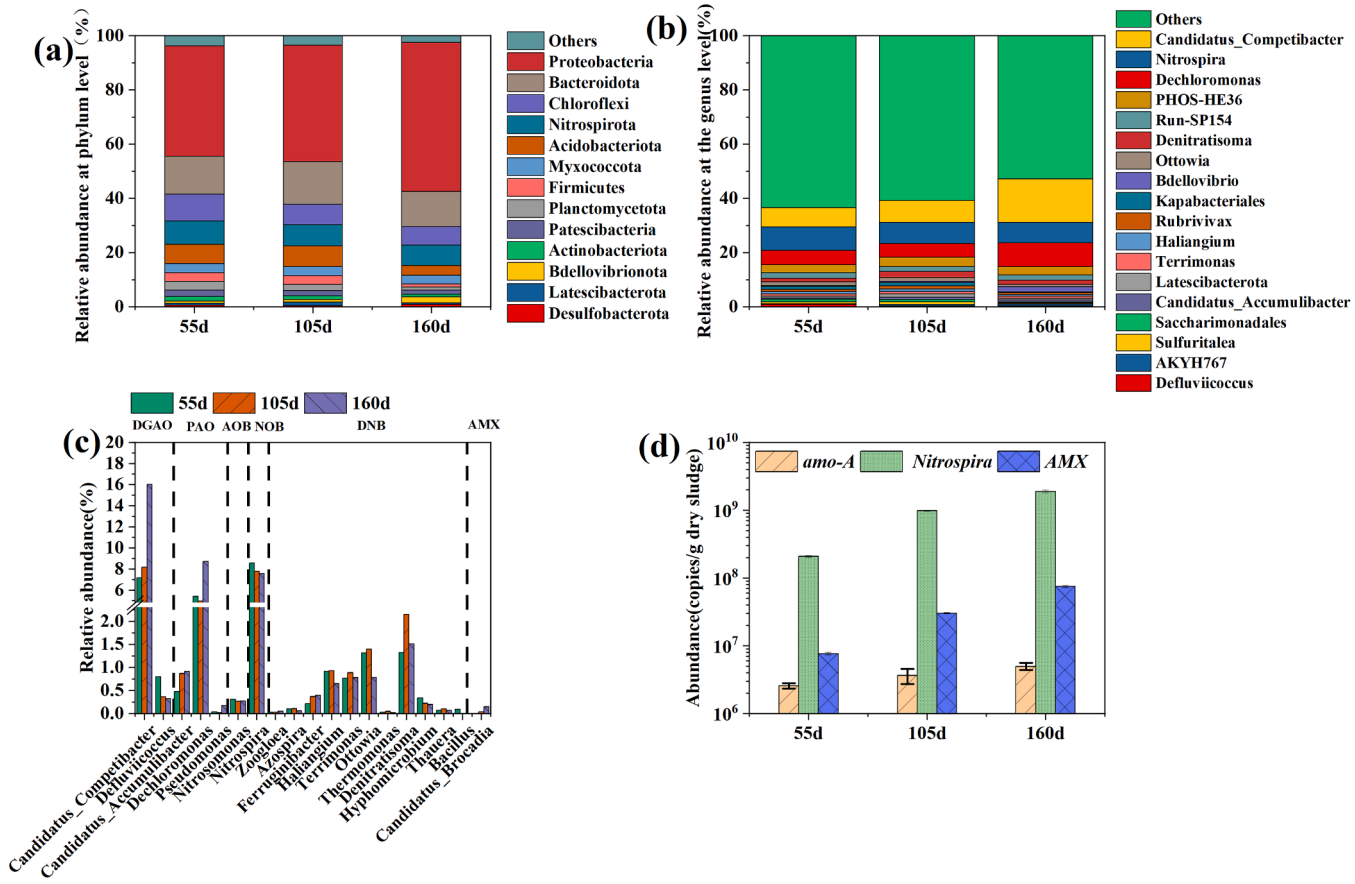

Fig. 6. Microbial community structure analysis and the qPCR of the key functional gene on 53 d, 105 d, and 160 d: (a) relative abundance at the phylum level (%); (b) relative abundance at the genus level (%); (c) the relative abundance of key nutrient removal microorganisms in SDR-AOA system; (d) the qPCR results of functional genes.

2.4. Successful coupling of endogenous denitrification and Anammox

The SDR-AOA system effectively integrated the anammox process with ED by using carriers and maintaining low residual NH_4^+-N levels through controlled DO (**Fig. S1 and S2**). The system also demonstrated significant intracellular carbon storage in the anaerobic zone (Fig. 2). This study emphasizes the sustained performance of the Anammox process as crucial for the stability and effectiveness of nitrogen removal in the system. Anammox was identified as the primary nitrogen removal pathway, accounting for 37.0% of the TN removal (**Eq. S6**).

The qPCR results indicate a rapid increase of AnAOB within the system, with functional genes of AnAOB rised from $7.70\pm0.32\times10^6$ copies/g dry sludge to 7.50×10^7 copies/g dry sludge (Fig. 6d). The Anammox genus *Candidatus Brocadia* was the only one identified This aligns with prior studies, which noted successful AnAOB enrichment in the biofilms of mobile carriers in A^2/O municipal wastewater treatments (Li et al., 2019). The low organic content in the anoxic zone was also beneficial for AnAOB (Li et al., 2020b).

2.5. Microbial structure and primary functional microorganism analysis

Microbial community analysis of suspended sludge on days 55, 105, and 160 revealed that *Proteobacteria, Bacteroidota, Chloroflexi*, and *Nitrospirota* were the dominant phyla (Fig. 6a). *Proteobacteria*, essential for nitrogen metabolism, increased from 40.6% to 55.0%, while *Nitrospirota* decreased from 8.55% to 7.57% (Li et al., 2020a; Ni et al., 2022).

At the genus level, *Candidatus_Competibacter*, a key ED bacterium, rose from 7.2% to 16.2%, indicating the system's support for endogenous denitrifying microorganisms, which likely contributed to the excellent nitrogen removal performance under low aerobic energy conditions (Ma et al., 2023; Zhang et al., 2024). The system environment facilitated the growth of endogenous denitrifying microorganisms, which likely enhanced the SDR-AOA system's nitrogen removal efficiency under low C/N and low aerobic energy conditions. Additionally, microorganisms such as *Denitratisoma* and *Candidatus_Accumulibacter* played key roles in the phosphorus cycle (Li et al., 2020a; Zhao et al., 2018b). And nitrifying bacteria such as *Nitrosomonas* (AOB) and *Nitrospira* (NOB), along with the anammox bacterium *Candidatus_Brocadia*, were also present, indicating a diverse nitrification and denitrification capability within the system (Fig. 6b and c).

2.6. Quantitative analysis of N metabolism function genes by qPCR

qPCR analysis on days 50, 105, and 160 showed a gradual increase in amoA, *Nitrospira*, and *AMX* gene copies (Fig. 6d). *Nitrospira* gene abundance, indicating high NOB activity, rose from $2.08\pm0.06\times10^8$ copies/g dry sludge to $1.89\pm0.82\times10^9$ copies/g dry sludge. preventing nitrite accumulation (**Fig. S3**). The *amoA* gene, representing AOB activity, increased from $2.57\pm0.23\times10^6$ copies/g dry sludge on day 55 to an abundance of $4.99\pm0.62\times10^6$ copies/g dry sludge by day 155. The significant rise in *AMX* gene copies by day 160, along with the presence of *Candidatus Brocadia* (Fig. 6b), confirmed the anammox pathway's role in nitrogen removal, consistent with studies showing NH_4^+-N conversion to N_2 under anaerobic conditions without nitrite accumulation (Fig. 2, Shi et al., 2023; Zhang et al., 2020).

In this study, no nitrite accumulation was found during the whole operation cycle of the reactor (Fig. 2). Moreover, the high relative abundance of *Nitrospira* could result in the rapid conversion of nitrite to

nitrate by NOB. The anammox microorganisms relied on nitrite produced by denitrification to convert NH_4^+-N into N_2 (Wang et al., 2023). Simultaneously, anammox microorganisms also competed with denitrifying bacteria for nitrite substrates. Therefore, it was a reasonable guess that the synergistic and competitive mechanisms of anammox and denitrifying microorganisms coexist in the SDR-AOA system. This synergy and competition mechanism together contributed to the high TN removal performance of the system.

3. Discussion

Multiple techniques, including physical separation of COD and NH_4^+−N like external nitrification, have been developed to enhance carbon utilization in denitrification. (Chen et al., 2023; Mahmoud et al., 2022). These methods often require additional infrastructure and complexity (Hu et al., 2001; Kapagiannidis et al., 2011). In contrast, the SDR-AOA configuration used in this study achieves efficient COD and NH_4^+−N separation within a single unit, simplifying the process and enhancing integration.

Our study highlights the role of DGAOs, particularly *Candidatus Competibacter* and *Defluviicoccus*, in converting organics into PHAs or Gly under anaerobic conditions (Fig. 3b). With a two-hour anaerobic hydraulic retention time (HRT), 92.1% of COD was stored (**Table S1**, Phase IV), reducing carbon oxidation during aerobic nitrification to just 5.10% (**Eq. S1**). This conservation of carbon and energy ensured intracellular carbon adequacy for later heterotrophic processes, optimizing metabolism and reducing external carbon needs.

Compared to the conventional SDR-AOA process that controlled high DO (Gao et al., 2023b), the low DO strategy, combined with the use of carriers in the SDR-AOA system, promoted SNED in the aerobic zone (Figs. 3 and 4b). The integration of SNED lightened the denitrification load in subsequent anoxic phases, thereby improving the TN removal effectiveness (Fig. 3b). In addition, the formation of a micro-anoxic environment within the floc was also crucial for the SNED (Fig 4b). Therefore, the futile oxidation of COD can be prevented by strategically storing carbon sources and managing DO levels.

Low DO levels can inhibit complete nitrification, leaving residual NH_4^+-N that supports anammox activity (Gao et al., 2023a). This environment favors anammox bacteria by giving them a competitive edge (Cao et al., 2017; Li et al., 2020b). Slowly degradable carbon sources like PHA and Gly are used by ED bacteria, improving access to nitrite nitrogen for anammox bacteria (Xu et al., 2021). Additionally, secondary sludge recirculation enhances denitrification (Gao et al., 2020; Zhang et al., 2018). In this study, the high COD_{intra} efficiency and extended anoxic duration led to partial microbial hydrolysis, increasing PHA and Gly content in the recirculated sludge and further strengthening ED and EPD/A in the anoxic zones (Zhang et al., 2018) (Fig. 5).

4. Conclusions

In traditional wastewater treatment, biological nitrogen removal involves a process of aerobic nitrification or partial nitrification that turns NH_4^+−N into NO_3^-−N or NO_2^- − N. To avoid the futile oxidation of COD during this aerobic unit, which could result in an inefficient utilization of carbon source and energy wastage, it is critical to keep COD and NH_4^+−N separate before entering the aerobic units. The configuration of SDR-AOA effectively stored carbon sources. It emphasized the conversion of external COD to internal carbon sources (PHAs and Gly), thus conserving COD throughout the aerobic units, which allows for optimized utilization of these stored carbon sources. By controlling DO and enhancing ED, the SNED processes were strengthened in aerobic units, and the microbial community was optimized to efficiently utilize carbon for comprehensive nitrogen removal by ED and anammox in anoxic units. This approach significantly enhances the role of anammox in nitrogen removal, protecting the carbon source from being oxidized fruitlessly in aerobic units, even with short-term elevated DO. The AnAOB enrichment and anammox activity in the AOA system were enhanced due to the restricted levels of DO and separation of carbon, along with the cooperative effects of endogenous denitrification. This approach effectively removes nitrogen from low C/N wastewater, introducing new methods for preserving and using carbon in the biological nitrogen removal process.

5. Materials and methods

5.1. Setup and operation of the reactor

A reactor made of polymethyl methacrylate with a working volume of 366 L was established (Fig. 1). This system also included a secondary settle tank with a working volume of 122 L. The reactor was divided into ten zones. The first zone has a volume of 45 L, the second and third zones have volumes of 48 L each, the fourth to seventh zones have volumes of 21 L each, the eighth and ninth zones have volumes of 58.5 L each, and the tenth compartment has a volume of 24 L.

In this study, the influent water was pumped into the system by the peristaltic pump from a water tank. Double sludge recirculation was applied, in which the first sludge recirculation and the second sludge recirculation from the settling tank were pumped into an anaerobic zone and first anoxic zone with recirculation ratios of R1 of 100% and R2 of 100%. The activated sludge collected from a pilot-scale continuous plug-flow A/O/A reactor with a flow rate of 100 m^3/d, which performed nitrification and ED, was inoculated in the system, and the SRT was controlled at 40–90 days. The total operating time of the system was 224 days. The operation of the system was divided into four phases according to different operating conditions. In Phase I (1-63 d), the DO concentration in the aerobic zone was maintained at 1.6±0.8 mg/L to investigate the performance of the reactor in treating real sewage. In Phase II (64-107 d), to evaluate the performance of nitrogen removal under the condition of low aeration energy by SNED, the DO level in the aerobic zone was decreased from 1.6±0.8 mg/L to 0.6±0.2 mg/L. In Phase III (108-160 d), to enhance the SNED and anammox performance carriers (K4) made of polyethylene with a specific surface area of 800 m^2/m^3 were filled on day 108, which accounts for a filling ratio of 15% of the specific zone. To avoid clogging the water holes and simulate the conditions in a real sewage plant, we only added carriers in the larger compartments (2#, 3#, 8#, and 9# zones in Fig. 1). The system operated stably as the modified SDR-AOA mode in Phase III. In Phase IV (161-224 d), the influent C/N ratio was reduced to evaluate the performance of the modified SDR-AOA system on nutrient removal with a low C/N ratio. During the entire operating period, the temperature was maintained at around 25°C. The hydraulic retention time (HRT) was 16 h by maintaining the influent flow rate at 549.0 L/d during the whole operation. The various operational parameters through different operational phases of the reactor are shown in **Table S1**.

5.2. Wastewater characteristic

Municipal wastewater used in this study was pumped from the grit chamber of a wastewater treatment plant located in Shenzhen. The variation range of influent NH_4^+−N and COD concentrations were 17.5-64.1 mg/L and 97.0-352.0 mg/L, respectively. The detailed influent wastewater and effluent characteristics of the A/O/A reactor at different operating phases are listed in **Table S4**. After 135 days of stable operation of the SDR-AOA reactor, the influent changes to synthetic wastewater due to the maintenance of the sewage plant. The characteristics of synthetic sewage were consistent with those of actual sewage. The compositions of synthetic wastewater and trace element solution were prepared according to (Xu et al., 2023). The details are listed in **Table S2**.

5.3. Anaerobic ammonium oxidation activity verification experiment

Three sludge samples from the reactor were washed thrice with deionized water and had MLSS levels of 4000±500 mg/L. These samples were placed into three anoxic batch reactors labeled A, B, and C. Group A contained NH_4^+–N and NO_3^- – N. Group B was given NH_4^+–N and $NaNO_2$, while Group C was given NH_4Cl only. Initial concentrations of NH_4^+ – N, NO_3^- – N, and NO_2^-–N were set at 25 mg/L for Group B and C. For Group A, the initial NH_4^+-N and nitrate nitrogen concentrations were 10 mg/L. Group A and B were performed in 1000 mL sealed glass vessels at 30°C. Anoxic conditions were ensured by halting dinitrogen flow until DO drop below 0.03 mg/L.

5.4. Analytical methods

Mixed liquor samples were collected 3-5 times per week, and all the samples were filtered with filter paper (0.45 μm) before analysis. COD was measured by a COD quick analysis apparatus (HACH Company, USA). The parameters, including NH_4^+ – N, total nitrogen (TN), COD, NO_2^- – N, NO_3^- – N, MLSS, and mixed liquor volatile suspended solids (MLVSS), were determined according to the standard methods (APHA, 2005). The DO and temperature were monitored using the HACH HQ40d flexi portable meter (HACH Company, USA). The freeze-dried biomass was taken to measure PHA and Gly. PHA was determined by the sum of poly-b-hydroxybutyrate (PHB) and poly-b-hydroxyvalerate (PHV), which were monitored by a gas chromatography-mass spectrometry (Agilent 7890N/5970B) with an Agilent HP-5MS column. Gly was analyzed using the anthrone method (Oehmen et al., 2005). The internal carbon source storage ratio (COD_{intra}, %) in the anaerobic section, the SNED efficiency (%) in the aerobic zone, the contribution rate of SNED (%), the contribution of anammox in the anoxic zone (AMX, %), and the nitrogen removal ratio in different functional areas were calculated (see supplementary materials).

5.5. Bacterial 16s rRNA genes sequence determination

To amplify the bacterial 16s rRNA genes, the activated sludge samples were collected on day 60 in phase I, day 116 in phase II, and 208 in phase VI, respectively. The obtained activated sludge samples were stored at -80 °C. The genomic DNA of activated sludge samples was extracted with a water DNA kit (Omega, USA). Nanodrop 2000 (Thermo Fisher Scientific, USA) was further used to determine the concentration of DNA and the value of 260/280 (Ren et al., 2024). Afterward, the bacterial 16s rRNA genes were amplified with universal primers (**Table S3**) and analyzed using the lllumina MiSeq platform of The Beijing Genomics Institute (BGI). The raw sequencing data were processed by QIIME (v1.9.1) with sequence grouping according to operational taxonomic units (OTUs) and 97% identity thresholds using UPARSE (7.0.1090) (Zhen et al., 2022) and classified using the SILVA bacteria database (Bremen, Germany).

5.6. Quantification of functional genes related to nitrogen metabolism

qPCR was performed to quantify the functional genes related to nitrogen metabolism using the specific primers (**Table S3**). The freeze-dried biomass was collected on days 1, 63, and 166, and repeated three times. The genetic material was then extracted using the Fast DNA Spin kits for soil (Bio 101, USA) following the manufacturer's protocol. After extraction, DNA samples were determined by a Nanodrop 2000 (Thermo Fisher Scientific, USA). qPCR was performed using the ABI7300 qPCR system (Applied Biosystems, USA). The operation methods of qPCR were carried out according to the literature (Zheng et al., 2020).

CRediT authorship contribution statement

Song Chen: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Qing-Pei Zhang:** Writing – review & editing, Resources, Methodology, Investigation, Conceptualization. **Jin-Song Zhang:** Supervision, Resources, Funding acquisition, Conceptualization. **Na An:** Writing – review & editing, Resources. **Hai-Yang Yu:** Writing – review & editing, Data curation, Conceptualization. **Xiang Fu:** Writing – review & editing, Data curation. **Zhi-Hua Li:** Writing – review & editing, Writing – original draft, Supervision, Funding acquisition, Conceptualization.

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.

Data availability

Data will be made available on request

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.wroa.2024.100253.

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