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ENHANCED NITROGEN REMOVAL AND SLUDGE CHARACTERISTIC IN AEROBIC SBR BY SIMULTANEOUS NITRIFICATION AND DENITRIFICATION BACTERIA

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Highlights:

- activated sludge characteristic and nitrogen removal performance of the aerobic SBR were enhanced;
- *Saccharibacteria* abundance increased to 15.34% (vs 8.48% in control), directly contributing to 18% higher TN removal efficiency;
- first demonstration of HN-AD bacteria-driven granular sludge formation at C/N = 3.

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Abstract. Four efficient heterotrophic nitrification and aerobic denitrification (HN-AD) strains were applied in sequencing batch reactors (SBRs) via two bioaugmentation strategies to enhance the nitrogen removal and sludge characteristic. Synthetic domestic wastewater with NH_4-N concentrations of 30~50 mg L^{-1}, was treated in three SBRs, with DO maintained at 4±0.5 mg L^{-1}. Compared with the control (crude activated sludge), bioaugmentation improved TN removal by 7% averagely, increased nitration rate by 0.54 mg g^{-1} h^{-1}, and reduced sludge volume index at 30 min (SVI_{30}) by 3.5~14.7 mL g^{-1}. The maximum TN removal efficiency reached 50.37% with effluent TN concentration of 14.64 mg L^{-1}, meeting China's Class 1A discharge standard (TN ≤ 15 mg L^{-1}). SBR started by bacterial suspension without activated sludge exhibited high adaptability to low carbon/nitrogen ratio, achieving 35% TN removal at C/N = 3 (vs <10% in control), with <5% MLSS fluctuation versus 30% decline in control. Microbial community analysis revealed *Saccharibacteria* dominance (15.34% vs control's 8.48%) coupled with 7.6% reduction in filamentous *Saprospiraceae* (12.78% to 5.18%), collectively explaining the enhanced nitrogen removal and sludge settleability. This study provides the first evidence of granular sludge formation via HN-AD bacterial coaggregation under low C/N conditions, offering a novel strategy for energy-efficient wastewater treatment.

Keywords: SBR, bioaugmentation, simultaneous nitrification and denitrification, sludge characteristic, microbial community.

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1. Introduction

Ammonia nitrogen has become a key pollutant of the surface water in China while the emissions are still rising, leading to more aggravated eutrophication (Shi et al., 2019; Wang et al., 2024). Physical, chemical, biological, and combined treatment methods have been applied to mitigate the pollution trend to satisfy the increasingly stricter legislation limits. In particular, biological treatment is efficient and economical in removing nitrogen (Marchant et al., 2017). Due to the requirement differences in organic loading, sludge age, and dissolved oxygen between the nitrifying and denitrifying processes, it is difficult to achieve complete nitrogen removal by simple treatment. Multiple reactors or stages and step-feeding processes are always essential.

As a single sludge system, SBR has the advantages of flexible operation mode and low cost. However, Conventional nitrogen removal consists of two steps: nitrification by autotrophs under aerobic conditions and denitrification by heterotrophs under anaerobic conditions. Intermittent aeration process is a common strategy for SBR to removal nitrogen (Haddaji et al., 2023; Zhou et al., 2022a). Some studies explored to couple SBR with anammox system (Choi et al., 2019; Zhou et al., 2024), or introduce exogenous nitrate sewage to develop denitratation-anammox over nitrite process (Cao et al., 2024). Operations of these methods are relatively complex.

Simultaneous nitrification and denitrification (SND) processes in a single reactor under aerobic conditions can greatly simplify the construction and operation of wastewater treatment plants. In the early years, investigations on SND focused on the acclimation of activated sludge. The achievement of SND in a single tank mainly resulted from the aerobic-anaerobic micro-environment by biofilm or granule sludge (Hibiya et al., 2003). The stratified layers within granule sludge or biofilm can offer a distinct micro-environment for the anoxic and aerobic conditions required by different functional microorganisms in SBR (Chen et al., 2020). DO levels (4±0.5 mg L^{-1}) were optimized to balance nitrification (aerobic) and denitrification (microaerobic zones within flocs), a critical factor for SND efficiency.

Heterotrophic nitrification and aerobic denitrification (HN-AD) bacteria, or SND bacteria were discovered in the 1980s (Robertson & Kuenen, 1983). They were different from conventional nitrogen removal microorganisms, and capable of heterotrophic nitrification and aerobic denitrification. Thus, the vision that nitrification and denitrification process could be realized simultaneously in a single reactor in aerobic conditions relying on the action of a single type of microorganism come true. In the past few decades, a large number of HN-AD bacteria have been isolated from drinking water reservoir sediment (Zhang et al., 2018), domestic sewerage (Padhi et al., 2017), and activated sludge (Zhao et al., 2018). Due to their high growth rate and ability to remove multiple nitrogen aerobically, HN-AD bacteria have a competitive advantage over conventional autotrophic and anaerobic nitrifiers. Bioaugmentation with HN-AD bacteria has been studied by researchers in recent years. Chen et al. greatly enhanced the treatment of municipal wastewater in SBR using HN-AD bacteria, with dissolved oxygen (DO) maintained at 2~3 mg L^{-1} and TN concentration of effluent stabilized at 14.1 mg L^{-1}, and the importance of introduced HN-AD bacteria in facilitating nitrogen removal was confirmed (Chen et al., 2015).

Some HN-AD bacteria could excrete extracellular polymeric substance (EPS) and promote auto-aggregation and coaggregation among various bacteria (Hong et al., 2021). EPS played an important role in formation of sludge aggregation flocs that sludge settleability could be enhanced by improving the properties of EPS (Hu et al., 2022). Hong et al. bioaugmented a sequencing batch biofilm reactor with HN-AD bacteria with DO maintained at 4.5~5 mg L^{-1} and both biofilm formation and TN removal efficiency were enhanced (Hong et al., 2020). Currently, the investigations of auto-aggregation and promotive aggregation of HN-AD bacteria are mainly conducted in sequencing batch biofilm reactors (SBBRs) (Lu et al., 2023). The improvement of activated sludge characteristics in SBR by HN-AD bacteria has rarely been reported. Therefore, effects of HN-AD bacteria on the settleability of activated sludge in SBR should be studied.

Although HN-AD bacteria have been applied in SBR systems, two critical gaps remain: (1) most studies focus on single-strain inoculation, while consortia performance under low C/N conditions is poorly understood; (2) the comparative effectiveness of direct bioaugmentation (without activated sludge) versus mixed inoculation remains unquantified. This study innovatively addresses the gaps by: (1) Proposing two novel bioaugmentation strategies: (a) building sludge microbiome de novo using HN-AD consortia (S1), versus (b) enhancing existing sludge with HN-AD bacteria (S2); (2) Systematically evaluating their efficacy under C/N = 3–7, a range covering typical municipal to industrial wastewater.

2. Materials and methods

2.1. Substrate and inoculum

The activated sludge used as inoculum was collected from the aerobic tank of an anaerobic/anoxic/aerobic process in Liaozhong Ecological Sewage Treatment Plant (Shenyang, China). The mixed liquid suspended solid (MLSS) was about 5000 mg L^{-1}.

Synthetic wastewater was prepared as influent, containing KH_2PO_4, 0.01 g L^{-1}; trace element solution, 5 mL L^{-1}; sodium citrate, sodium succinate, and glucose as carbon sources; $(NH_4)_2SO_4$ as nitrogen source at desired concentrations. The composition of the trace element solution was (per liter): $MgSO_4{\cdot}7H_2O$, 2.5 g; $FeCl_2{\cdot}4H_2O$, 0.5 g; $MnSO_4{\cdot}H_2O$, 0.5 g; $CaCl_2$, 0.5 g.

The carbon sources were selected for their high biodegradability, yielding a BOD_5/COD ratio of 0.7–0.8, which indicates the predominance of readily oxidizable organic matter—a key factor for efficient microbial activity and nitrogen removal. The near-equivalence of BOD_5 and COD confirms the absence of refractory organics, consistent with typical domestic wastewater. The composition of carbon sources and nitrogen sources in the influent in different periods is given in Table 1.

Table 1. Operating condition at different phases

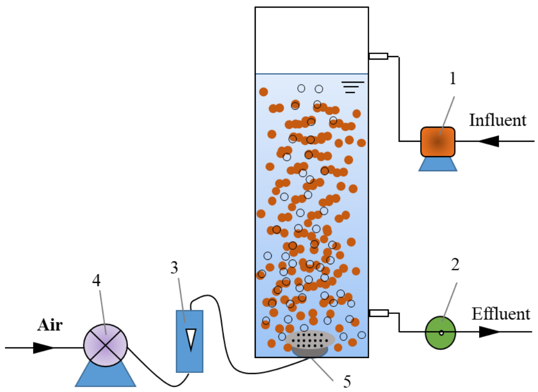
	Phase 0	Phase 1	Phase 2	Phase 3	Phase 4
Sodium citrate concentration (g L^{-1})	0.1022	0.1022	0.1431	0.1022	0.1022
Sodium succinate concentration (g L^{-1})	0.1206	0.1206	0.1688	0.1206	0.1206
Glucose concentration (g L^{-1})	0.0516	0.0516	0.0722	0.0516	0.0516
COD (mg L^{-1})	150±5	150±5	210±5	150±5	150±5
$(NH_4)_2SO_4$ concentration (g L^{-1})	0.1446	0.1446	0.1446	0.1446	0.2417
NH_4-N (mg L^{-1})	30±2	30±2	30±2	30±2	50±2
C/N	5	5	7	5	3
HRT (h)	8	6	8	8	8
Time (d)	0~21	22~31	32~41	42~51	52~61

Preparation of bacterial suspension: *Acinetobacter venetianus* PYI1, *Flavobacterium sasangense* PTHG, *Massilia neuiana* PTW21 (Zhao et al., 2017), and *Pseudomonas chengduensis* ZPQ2 were isolated in the lab and showed an excellent ability of HN-AD. *Massilia neuiana* PTW21 and

Pseudomonas chengduensis ZPQ2 also showed the ability of auto-aggregation. Strains of PYI1, PTHG, PTW21, and ZPQ2 were preserved in the Environmental Molecular Ecology Laboratory of Northeastern University, China. The strains were inoculated into 100 mL Luria-Bertani medium (LB) and incubated at 32 °C and 120 rpm for 24 h. The composition of LB was tryptone 10 g, yeast extract 5 g, and NaCl 10 g (per liter). When the pre-cultured bacterial suspensions were mixed in a proportion of 1:1:1:1 as inoculum, it presented high ammonia nitrogen (NH_4-N) removal efficiency of 68.3% in 24 hours with 50 mg L^{-1} ammonium-N used as the sole N source in pre-experiment. Based on this result, bacterial suspension of the selected strains was mixed in a proportion of 1:1:1:1 as inoculum into the SBRs.

2.2. Bioreactor operation

Three parallel bench-scale reactors (Figure 1) with a 4 L working volume were employed. Each operational cycle comprised: (1) 20-min influent feeding, (2) 5- or 7-h aeration (DO: 4±0.5 mg/L), (3) 20-min settling, (4) 10-min effluent withdrawal, and (5) 10-min idle phase (total cycle time: 6–8 h). The volumetric exchange ratio was 60% and DO was maintained at 4±0.5 mg L^{-1} by aeration apparatus.



Note: 1 – submersible pump; 2 – magnetic valve; 3 – rotameter; 4 – aeration pump; 5 – bubble diffuser.

Figure 1. Schematic diagram of the SBR

The inoculum of reactors was as follows: 4 L of pre-cultured mixed bacterial suspension in SBR1 (S1 for short); 2 L of pre-cultured mixed bacterial suspension and 2 L of activated sludge in SBR2 (S2 for short); 2 L of activated sludge and 2 L of synthetic wastewater in SBR3 (S3 for short) as control group.

The experimental period can be divided into 6 phases with different HRT or C/N. Operating parameters in different phases are given in Table 1. During the start-up phase, mixed bacterial suspension was inoculated into S1 and S2 at 10% (v:v) additionally on day 7 and day 14 to strengthen the bioaugmentation.

2.3. Microbial community analysis

The activated sludge was collected respectively on day 10, day 20, day 30, day 40, day 50, and day 60 to analyze the succession of microbial community structure. The samples were suspended in 0.9% (w/v) sodium chloride solution and centrifuged at 4000 rpm for biomass collection. Then stored at –20 °C until DNA extraction.

DNA was first extracted using a PowerSoil^{TM} DNA Isolation Sample Kit (MoBio, USA), followed by the instructions given by the manufacturers. Then the V3-V4 regions of the 16S rRNA genes were amplified with 341F (5′-CCT ACG GGN GGC WGC AG-3′) and 806R (5′-GGA CTA CHV GGG TWT CTA AT-3′). The PCR amplification was done in GeneAmp 9700 Thermo Cycler (ABI, USA) and the process was as follows: 95 °C preheating for 5 min, 94 °C for 30 s (denaturation), 55 °C for 30 s (annealing), 72 °C for 1.5 min (extension) for 30 cycles, and 72 °C for 7 min (final extension). The amplicon was analyzed on an Illumina MiSeq platform (Majorbio, China). Mothur 1.30.1 was used for computational analysis with the average length of contigs of 500 bp, and referenced to the bacterial database SILVA.

2.4. Analytical methods

Culture samples were centrifuged at 6000 rpm using a high-speed tabletop centrifuge (LG16-A, Leiboer, China) and liquid supernatant was used for chemical analysis. NH_4-N, NO_3^--N, NO_2^--N, TN, and COD were determined according to standard methods (American Public Health Association [APHA], 2012). DO and pH were measured using an oxygen electrode (HQ30d, HACH) and pH electrode (HI98183, HANNA), respectively. MLSS and SVI_{30} were measured according to the standard methods for the Examination of Water and Wastewater (APHA, 2012). The analysis repeated twice, and each concentration in figures represents the average concentration.

3. Results and discussion

3.1. Nitrogen removal performance

As demonstrated in Figure 2, the removal efficiency of NH_4-N in S1 was poor at the start-up stage due to the low biomass. However, it increased significantly later and reached about 85% at the late start-up period, coincident with the TN removal efficiency trend. SND bacteria achieved simultaneous nitrification-denitrification through enzymatic synergy and oxygen gradient regulation (Jin et al., 2019). Combined with the rapid growth of biomass, activated sludge with high efficiency of nitrogen removal could be cultured successfully within 15~20 days only with HN-AD bacterial suspension. TN removal efficiency in S2 surpassed S3 on day 8 and stabilized at 46% at the end of the start-up phase, 7% higher than that in S3.

Under conditions of HRT as 8 or 6 hours and C/N as 7 or 5 (day 22~51), NH_4-N removal efficiencies in all the reactors remained stable at almost 100%, except for S1 during day 22~24. As for TN removal, S2 performed better, showing a 2.3%~3.2% advantage over S3. The maximum TN removal efficiency in S2 is 50.37%, with the effluent TN concentration of 14.64 mg L^{-1}, NH_4-N concentration

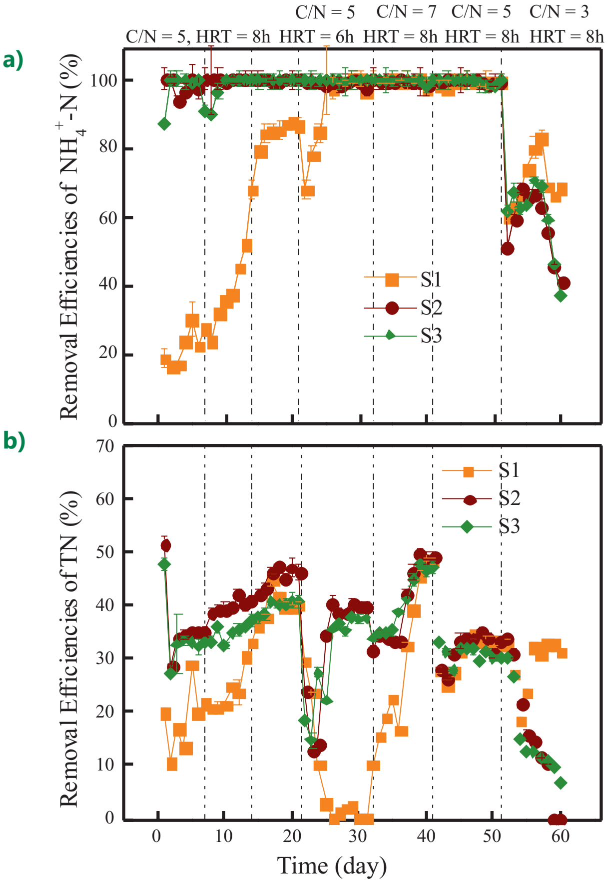


Figure 2. Removal efficiencies of: a) NH_4-N; b) TN

of 0.07 mg L^{-1}, NO_3-N concentration of 14.58 mg L^{-1} and NO_2-N concentration of 0 mg L^{-1}. Stable DO ensured simultaneous nitrification-denitrification, with DO of 4–4.5 mg L^{-1}. Compared with similar studies, a maximum TN removal efficiency of 66% in the column-type aerobic SBR was achieved with C/N of 20 and HRT of 6~24 h (Khan et al., 2024). And the average TN removal efficiency is 61% treating olive oil mill wastewater in SBR with C/N > 60 and HRT of 24 h (17 h aeration, 4 h anoxia, 2 h anaerobic and 1 h others) (Rifi et al., 2022). TN removal efficiency in S2 was not very competitive due to the low C/N condition and relatively short HRT. But the concentration of TN and NH_4-N has met the first-class requirement of the National Municipal Wastewater Discharge Standards of China (NH_4-N≤5 mg L^{-1}, TN≤15 mg L^{-1}). S1 also performed better than S3 under conditions of HRT at 8 hours and C/N at 7 or 5. The results indicated an obvious advantage of both bioaugmentation strategies in nitrogen removal under general circumstances. However, it was a hard hit for S1 as HRT was adjusted from 8 hours to 6 hours, with TN removal efficiency down to 0%, presumably because HRT shortening increased the resulting volumetric loading rate. It has been demonstrated that an ecosystem with high biodiversity has a better capacity to resist environmental stress (Ou et al., 2016). On the contrary, S1, a system composed of only several species, would be very weak to the shock loading of pollutants.

When C/N was further decreased to 3 (day 52~61), TN and NH_4-N removal efficiency decreased sharply in all the reactors. But the downtrend was curbed in S1 after a short adaptation time of 3 days. NH_4-N and TN removal efficiencies ultimately stabilized at about 65% and 35%. Combined with the previous researches, with continuous operation of the reactor and an increasing population of microorganisms, the optimal C/N required would gradually reduce in the SND process (Lang et al., 2020). So the activated sludge in S1 might be easier to adapt to C/N lowering, on account of a higher proportion of HN-AD bacteria. The concentration of NH_4-N and TN in the effluent from S1 were 19 and 35 mg L^{-1} respectively, exceeding the first-class requirement of the National Municipal Wastewater Discharge Standards of China, indicating that an additional carbon source is necessary under low C/N to meet the nitrogen removal requirement.

During most of the operation phases, loading rate of COD (Figure 3a), TN (Figure 3b) and NH_4-N (Figure 3c) was as follows: S1 > S2 > S3. TN loading rate of S1 was higher than S2 and S3 on day 60 by 1.04 mg g^{-1} h^{-1} and 0.15 mg g^{-1} h^{-1} respectively. S2 performed average loading improvement of 0.54 mgNH_4-N g^{-1} h^{-1}, 0.15 mgTN g^{-1} h^{-1} and 1.94 mgCOD g^{-1} h^{-1}, compared with S3.

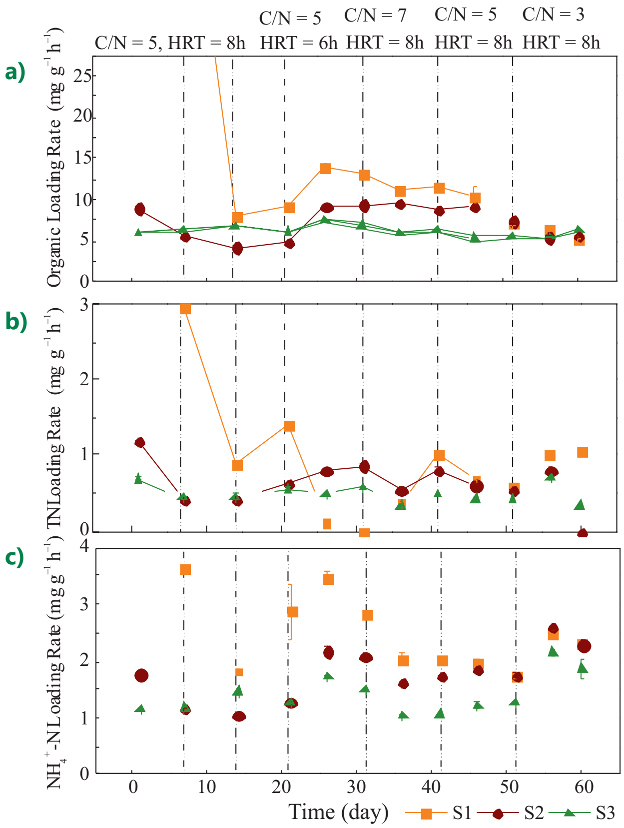


Figure 3. Pollutant loading rates under different operational conditions: a) organic; b) TN; c) NH_4-N

3.2. Nitrogen removal characteristics in different phase

As demonstrated in Figure 4a, changing trends of nitrogen differed greatly in different reactors in the same operation cycle on day 20. An obvious accumulation of nitrite up to 16.16 mg L^{-1} was found in S1 during the early two hours, which was in accordance with a previous study that nitrification and denitrification are not concurrent at the beginning of the nitrogen removal reaction (Jin et al., 2019). And the nitrite concentration of the effluent from S1 was 13.41 mg L^{-1}, which might be attributed to that the biomass was not enough to support sufficient denitrification. In the early two hours, TN concentration decreased by 10 mg L^{-1} and NH_4-N removal efficiency was up to 92% in S2. While in S3, TN concentration decreased by 0.8 mg L^{-1} and NH_4-N removal efficiency was 68%. Inoculation of HN-AD bacteria into activated sludge was conducive to accelerating the nitrogen removal rate of SBR. A termination of nitrification and denitrification appeared at the 5th hour, because of the insufficiency of carbon sources.

On day 30, TN concentration decreased at the beginning, but increased after 3.75 h in S1 and S2 (Figure 4b), presumably because of the enhancement of the adsorption capacity of the activated sludge. Ammonium absorption resulted in the decrease of TN, and then the release of denitrification products NO^{3-}-N and NO^{2-}-N increased TN. Early studies have shown that up to 20–25% dissolved

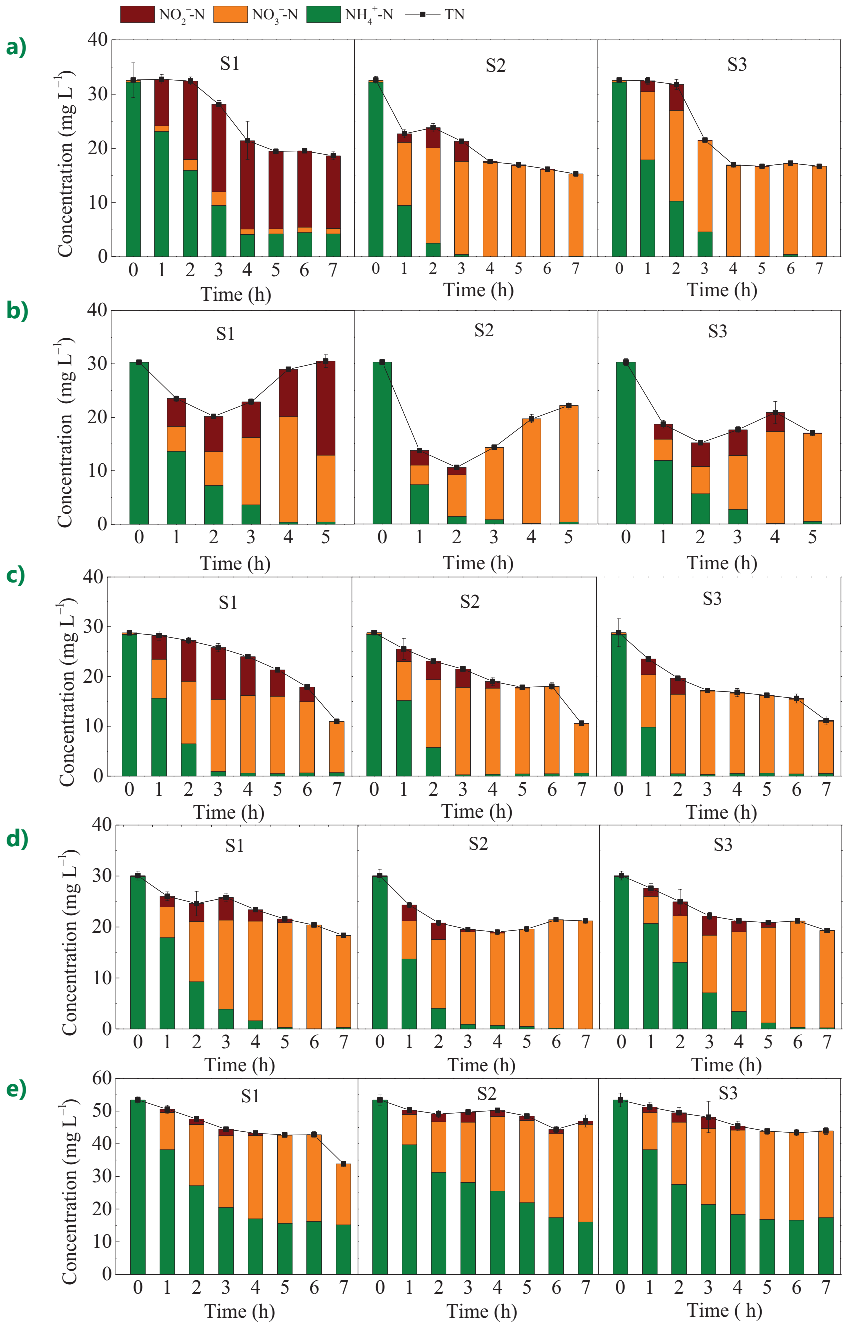


Figure 4. Changes of nitrogen in one cycle of SBR on: a) day 20; b) day 30; c) day 40; d) day 50; e) day 60

ammonium can be adsorbed to the activated sludge flocs under some conditions (Nielsen, 1996). In particular, aerobic granular sludge, with the maximum adsorption constant of 0.9~1.7 mgNH_4-N g^{-1}, exhibited an order of magnitude higher adsorption capacity, compared to activated sludge and anammox granules, whose maximum adsorption constants were 0.16~0.18 mgNH_4-N g^{-1} and 0.20 mgNH_4-N g^{-1} (Bassin et al., 2011). The nitrite concentration in S1during the first 4 hours stabilized at around 6.5 mg L^{-1}, but an obvious accumulation of nitrite up to 17.61 mg L^{-1} was found in the effluent. Perhaps because some ammonia absorbed by activated sludge during early time was released into the water, converted to nitrite in final stage of the cycle and discharged before denitrification could be fully carried out.

Nitrite accumulation in S1 was reduced on day 40 and could be completely degraded in the end (Figure 4c). With C/N adjusted from 7 to 5 (Figure 4d), the time it took to finish thorough ammoxidation increased greatly from 3, 3, and 1.5 hours on day 40 to 6, 4.5 and 3 hours on day 50. But TN could be greatly reduced during the early 1.5 hours in S1. As C/N was further adjusted to 3 (Figure 4e), nitrogen changing trends became remarkably similar in the reactors, except that TN and NH_4-N removal rates in S1 were higher than in the other reactors.

3.3. Characteristics of activated sludge

As demonstrated in Figure 5a, MLSS grew rapidly during the start-up phase in S1 but stabilized at a much lower concentration from day 14 than the general biological treatment system. MLSS of S2 was significantly lower than S3 most of the time by about 30%, indicating that the addition of SND bacteria may reduce the remaining sludge while maintaining the treatment effect. An unexpected phenomenon was found that granular structures could be observed in the sludge in S1 and S2 from about day 25 to day 35. The volumetric organic loading rate has been proven to favor aerobic granular sludge formation and stability (Carucci et al., 2019). It indicated that the formation of granular structure is possibly a result of HRT shortening, resulting in organic loading increase. However,

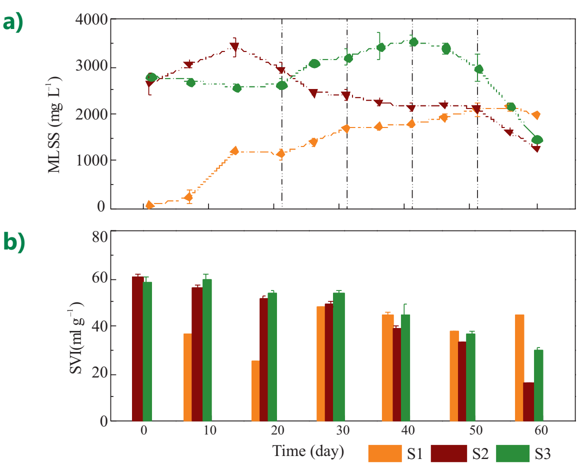


Figure 5. In the reactors: a) MLSS; b) SVI

no granular structure was found in S3 through the entire operation process. The formation of small aggregates and granular sludge is profit for sludge settleability and sludge bed compactness, as indicated by the SVI change trend in S2, compared to S3 (Figure 5b).

During day 42~51, C/N decreasing from 7 to 5, an obvious MLSS decline was found in S3, but not found in S1 and S2. A possible reason might be that bioaugmentation increased system tolerance to nitrogen load. With C/N further decreased 3, irreversible sludge particle disintegration and loss took place in S2 and S3. Bucci et al. reported that low COD/N ratio (3.3–5.0) would result in low growth rate of the biomass and decrease of some EPS-producing bacteria (Bucci et al., 2022). Thus the structure of activated sludge would be destroyed. Large quantities of fine-grained sludge were washed out with effluent, leading to a continuous decline of MLSS. And the sharp decrease of SVI to below 40 mL g^{-1} in S2 and S3 indicated that the activity of the sludge has dropped. While in S1, the indexes of activated sludge changed slightly, indicating strong ability to withstand nitrogen impact load.

During the entire experimental process, except for the last phase, SVI in S3 is higher than the other reactors, which might be interpreted as the settleability of sludge in S3 is poorer.

3.4. Microbial community structure succession

Through a high-throughput sequencing technique, the microbial community structure of sludge in SBR was investigated. As a metric for bacteria species richness and evenness, the observed numbers of operational taxonomic units (OTUs) and Shannon index of S1 changed slightly after the start-up phase, as shown in Table 2. While OTUs and Shannon index of S2 and S3 significantly decreased, compared with the initial activated sludge. Especially when the C/N was turned from 5 to 3 in the last phase, the Shannon index decreased from 4.30 and 4.66 to 3.65 and 3.90 in S2 and S3 respectively. These results possibly explained why S1 could better adapt to the shock loading of NH_4-N in phase 4. According to early research, high biodiversity is helpful for the maintenance and flexibility of the system to changing conditions (Wu et al., 2018).

Table 2. Biodiversity estimation in the activated sludge

Index	Rector	0d	10d	20d	30d	40d	50d	60d
OTUs	S1	–	315	604	572	494	506	558
	S2	1228	1259	1182	1093	700	731	491
	S3	1250	1296	1187	1145	690	761	634
Shannon	S1	0.39	3.58	4.16	3.92	3.84	3.80	4.01
	S2	5.48	5.70	4.94	4.50	4.09	4.30	3.65
	S3	5.56	5.58	4.96	4.99	4.39	4.66	3.90

Community structures were also found varying greatly in different reactors and stages. The 30 most abundant genera in each sample were selected for further analysis of the bacterial communities at the genus level in Figure 6.

Two of the most abundant genera accounted for almost 20% of original sludge samples, *Pseudomonas* (6.93%) and *Saprospiraceae* (12.78%). *Pseudomonas* is a group of heterotrophic aerobic bacteria that grows rapidly and distributes widely in soil, freshwater, seawater, and organisms. They play significant roles in nitrogen and phosphorus removal, as well as degrading a variety of simple or complex organic compounds. *Saprospiraceae* is a family of common filamentous bacteria that can metabolize glucose, galactose, and acetate (Xia et al., 2008), and weaken the settleability of sludge (Xu et al., 2018).

The introduced genera *Massilia*, *Acinetobacter*, *Flavobacterium,* and *Pseudomonas* occupied the dominant status in S1 during the start-up phase. The total relative abundance of the introduced species in S2 increased significantly from 10.65% on day 1 to 45.29% on day 20, but declined by over 30% (from 45.29% to 12.88%) during day 20~30, as well as in S1 (from 44.84% to 12.96%), consistent with the sharp decrease in TN removal efficiency. During days 30~40, it still decreased slightly, from 12.96%, 12.88%, and 4.46% to 7.58%, 7.27% and 0. During the last phase, the relative abundance of the introduced species in S1 increased from 9.82% to 14.78%, while it further decreased in S2, from 10.48% to 5.18%, corresponding to the sharp decline of NH_4-N and TN removal efficiency, suggesting that the increase of introduced species could indeed promote nitrogen removal performance of the activated sludge.

Saccharibacteria occupied the dominance in all the reactors during days 20~60, compared with the proportion of 2.44% in initial activated sludge. And the relative abundance of *Saccharibacteria* in S1 (15.34%) and S2 (12.62%) were higher than that in S3 (8.48%). It has been reported as the predominant taxon in some aerobic nitrifying SBRs with (Li et al., 2023; Liu et al., 2020; Zhang et al., 2020) or without (Hanada et al., 2014) carbon source, and in a membrane bioreactor with high nitrogen removal efficiencies treating landfill leachate (Remmas et al., 2017). Combined with previous results (Eo & Park, 2016), an increase in the relative abundance of *Saccharibacteria* was found by increasing N content in soil, suggesting a preference of *Saccharibacteria* in ammonia-rich environments. Although the abundance of the introduced species was not dominant in the system, a proper niche could be created to further enrich *Saccharibacteria* at suitable conditions (Zhou et al., 2022b), promoting nitrogen removal of the system.

The filamentous *Saprospiraceae* decreased significantly throughout the operation period, accompanied by a

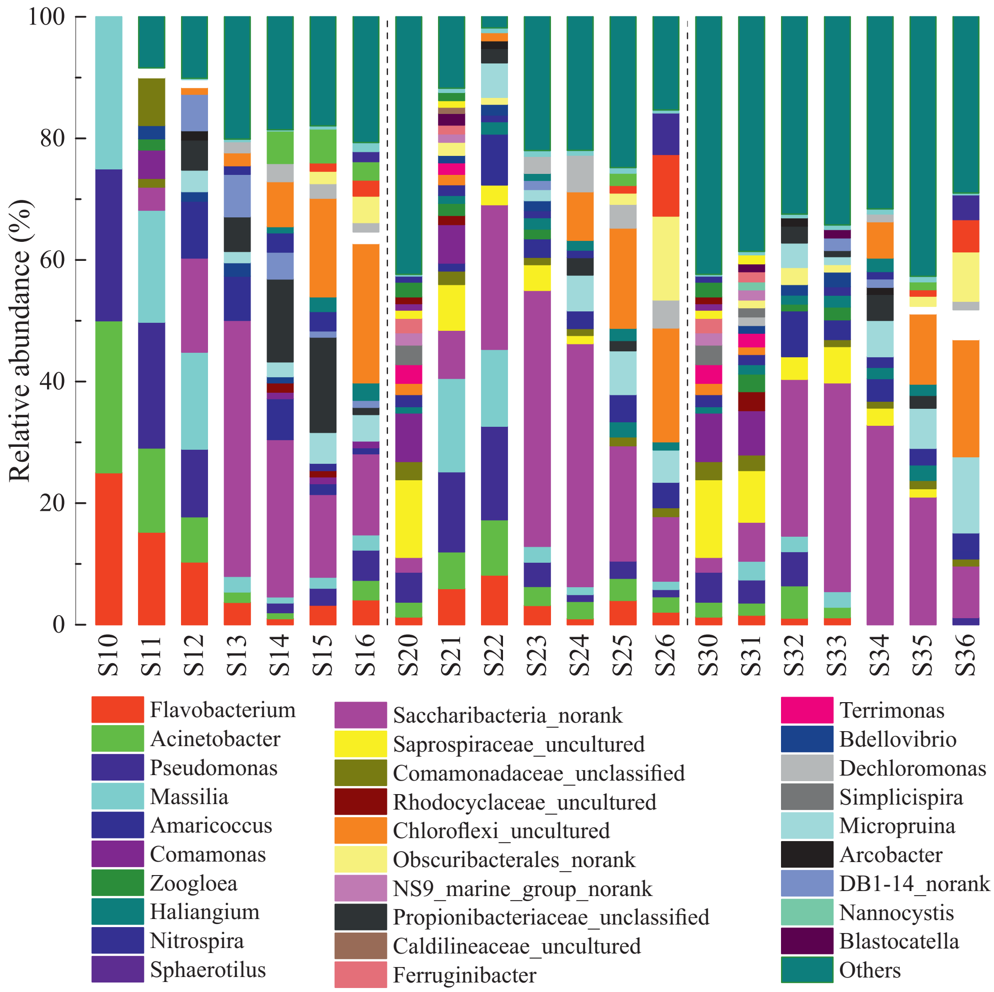


Figure 6. Analysis of microbial diversities: the top 30 genera were listed

decreased trend of SVI. It concurred with the conclusion of a previous study that the relative abundances of *Saprospiraceae* in bulking sludge samples were higher than those in normal samples (Xu et al., 2018). Xia et al. found that *Saprospiraceae* preferred alternating anaerobic-aerobic environments (Xia et al., 2008). Continuous aerobic conditions could contribute to the decrease of *Saprospiraceae*. Chi et al. reported that high organic content in wastewater would lead to the dominance of *Saprospiraceae* (Chi et al., 2018). Due to the efficient substrate utilization of HN-AD bacteria, the nutritional condition in S2 could also become more detrimental to *Saprospiraceae*. Thus compared with that in S3, the settleability performance of the activated sludge in S2 improved more, as SVI was 3.5~14.7 mL g^{-1} lower and the relative abundance of *Saprospiraceae* was 0.5%~1.5% lower. In addition, several members in *Pseudomonas* capable of producing EPS have always been reported, such as *Pseudomonas stutzeri* XL-2 (Xuesong et al., 2019) and *Pseudomonas mendocina* IHB602 (Hong et al., 2020). Previous studies have shown that EPS could allow cells of the microcolonies to adhere to each other and promote the formation of sludge aggregation flocs and maintain the stability of the polymer structures (Flemming & Wingender, 2010). Interaction between community structure and environment could promote bacterial community succession (Bouchez et al., 2000) and benefit the settleability of activated sludge. A similar phenomenon was observed in nitrogen treatment bioaugmentation biofilm reactors before the addition of some HN-AD bacteria (mainly Pseudomonas (Hong et al., 2020, 2021; Lu et al., 2023), and also some Alcaligenes (Jinxiang et al., 2018), Methylobacterium (Hong et al., 2024) and Zobellella (Xiang et al., 2023)) could significantly promote the secretion of EPS and biofilm formation.

4. Conclusions

The study evaluated two bioaugmentation strategies to promote nitrogen removal in SBR. Bioaugmentation with HN-AD bacteria improved the performance of SBR, achieving maximum TN removal efficiency of 50.37% with effluent TN concentration of 14.64 mg L^{-1}, meeting China's Class 1A discharge standard. Under conditions of HRT at 8 hours and C/N at 7 or 5, nitrogen removal and activated sludge settleability were greatly improved by inoculating microbes into activated sludge, reducing SVI30 by 3.5–14.7 mL g^{-1}. Activated sludge could be cultured successfully by the HN-AD bacterial suspension in 15~20 days. And it exhibited high adaptability to low C/N. Microbial analysis revealed that the succession of microbial community was greatly affected by bioaugmentation, with *Saccharibacteria* increasing to 15.34% and *Saprospiraceae* decreasing by 7.6%. Nitrogen removal improvement mainly resulted from the introduced bacteria and community succession they drove. *Saprospiraceae* played a crucial role in the decrease of activated sludge SVI. Importantly, this study provides the first report of granular sludge formation driven by HN-AD bacteria co-aggregation under low C/N conditions.

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Disclosure statement

The authors declare that they have no conflict of interest.

Author contributions

Y. P. (Lecturer) conducted the experiments and wrote the original draft. Z. S. (Assistant Professor) provided guidance on the overall research activities. J. L. (Assistant Lab Master) prepared the experimental materials and sites. W. Y. (Ph.D.) revised writing the initial version of the manuscript. X. Z. (Assistant Professor) conducted the work of sequencing and edited the manuscript.

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