The Bioenhanced Effect of Biochar-Based Aerobic Denitrifying Bacteria on a Sequencing Batch Reactor under Different Stress Conditions

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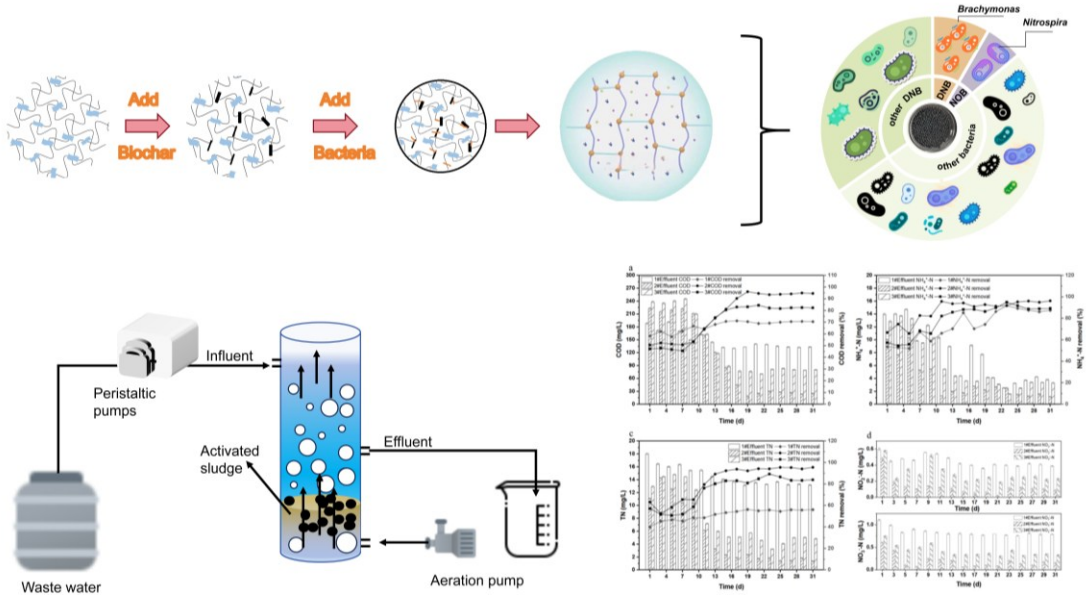
GRAPHICAL ABSTRACT



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Biochar-based aerobic denitrifying functional bacteria (BADB) have the advantages of excellent biochar adsorption performance and high nitrogen removal efficiency. In this study, a sequencing batch reactor (SBR) system was employed to explore the impacts of various stress conditions on the pollutant removal efficiency and metabolic pathways of BADB. Additionally, the adaptation mechanisms and response patterns of functional bacteria under different stress conditions were revealed. The structure of the microbial community was analyzed through high-throughput sequencing. The research results indicated that aerobic denitrification bacteria had good growth and nitrate removal performance under various conditions. Aeration rate exhibited positive relationship with denitrification efficiency, and the effect was enhanced when the ion concentration increased. Carbon source type significantly influenced the denitrification efficiency of functional bacteria, among which sodium acetate showed the best effect. Within the appropriate C/N ratio range, greater amounts of carbon led to higher denitrification efficacies of functional bacteria. Sequencing results revealed the key role of *Brachymonas* in organic degradation and denitrification process, with a percentage of 13.9% in the system. This study can provide a reference for the optimization and utilization of biochar-based aerobic denitrification technology, which is of great significance for improving the quality of water environment.

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Keywords: Biochar; Aerobic denitrification; Immobilization; Biological denitrification; Denitrification efficiency

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INTRODUCTION

With agricultural and industrial development, eutrophication of water bodies by nitrogenous pollutants has become the focus of global environmental management. The traditional biological method of nitrogen removal relies on "nitrification - denitrification", which needs to be carried out step by step in anoxic and aerobic environments, and there are problems such as complicated process flow, high energy consumption, and limited nitrogen removal efficiency (Qiu *et al.* 2025). Heterotrophic Nitrification-Aerobic

Denitrification (HN-AD) bacteria have transcended the conventional theoretical framework. Their distinct capacity for Simultaneous Nitrification and Denitrification (SND) offers a new direction for the innovation of wastewater denitrification technology (An *et al.* 2024). These strains can directly transform nitrite and nitrate into nitrogen gas under aerobic conditions. Not only does this shorten the denitrification pathway, but it also maintains the pH stability of the system through an alkalinity compensation mechanism, thereby significantly reducing the operational costs (Xiao *et al.* 2024).

HN-AD bacteria had demonstrated greater environmental adaptability in denitrification (Huang *et al.* 2020). To date, multiple HN-AD strains such as *Pseudomonas*, *Halomonas*, *Bacillus*, and *Acinetobacter* had been isolated from diverse environments (Song *et al.* 2021). These species mainly carry out the gradual reduction of nitrate to N_2 through a series of denitrification enzyme systems, such as Nitrate reductase (Nar), Nitrous oxide reductase (Nos), Nitrite reductase (Nir), Periplasmic nitrate reductase (Nap), and Nitric oxide reductase (Nor) (Zhu *et al.* 2020). N and C metabolism was found to be the foundation of all microbial cell metabolic processes involved in wastewater treatment through biological method (X. Yang *et al.* 2025). The biodegradabililty of the carbon source, its molecular weight, and its chemical structure could influence the activity of microbial enzymes, growth, metabolism, and N degradation (Lu *et al.* 2024). During nitrification, an appropriate carbon-nitrogen ratio (C/N) could markedly enhance the rate of nitrification (Zhao *et al.* 2017; Zhang *et al.* 2023). HN-AD bacteria exhibit a broad adaptability to varying C/N ratios (Bian *et al.* 2022; Hu *et al.* 2023). Dissolved oxygen (DO) is critical for both ammonia oxidation and denitrification. However, it may impede the growth and metabolic activities of anaerobic denitrifying bacteria (DNB) (Ji *et al.* 2023; Su *et al.* 2024). Notably, HN-AD bacteria could perform aerobic denitrification, with different strains requiring distinct optimal DO concentrations (Chen *et al.* 2019; Xia *et al.* 2020; Bian *et al.* 2022). Recent research has predominantly focused on individual purified strains, while studies on the denitrification effects and methods to enrich HN-AD bacteria within reactors remain scarce.

If the HN-AD bacteria were directly added to the biological system for better N removal, there will be problems such as easy loss, difficulty in enrichment, and the need for regular addition of functional strains. Additionally, multiple environmental stresses (*e.g.*, pH fluctuations, heavy metal impacts, carbon scarcity, *etc*.) in the actual wastewater treatment system could also inhibit the activity of free strains, thus limiting their practical applications (Liu *et al.* 2024; Lu *et al.* 2024; Sethi *et al.* 2025). Immobilizing HN-AD bacteria onto carriers not only prevents microbial loss but also enhances resistance to adverse conditions such as low temperatures, low C/N ratios, and heavy metals, while facilitating recycling (Li *et al.* 2025). Carrier materials play a pivotal role in microbial immobilization. Yang *et al.* (2021) used macro-genomic technology to analyze the metabolic pathways and community structure of microbes in the solid-opposed nitrification system. They found that the use of natural materials or artificial synthesis biodegradable materials as carbon sources and biofilm carriers not only solved the problem of the amount of the traditional carbon source injection, but also provided a safe and stable survival environment for the denitrifying bacterial flora. In an earlier study (Song *et al.* 2023), immobilization of HN-AD bacteria screened in the waste leachate environment with sodium alginate (SA) and polyvinyl alcohol (PVA) as the implanting carriers and *Artemisia argyi* stem biochar as an adsorption carrier led to better biofortification.

Based on this, the present study employed SA and PVA as immobilization matrices, with *Artemisia argyi* stem biochar serving as the adsorbent support. These composite

particles were used to immobilize the HN-AD bacteria. Their addition to the SBR reactor had the effects of constructing a biofortification system of BADB. The research focused on the mechanisms governing the influence of different stress conditions, such as aeration rate, C/N ratio, carbon source, *etc*. on the efficiency of their denitrification. A further goal was to make clear the critical value of the functional bacterial group's stress tolerance, as well as to provide theoretical support for the biological fortification technology under the complex water quality conditions. The results of the study will promote the transition of aerobic denitrification technology from laboratory research to engineering application. The results also can have an important practical value for the deep treatment of high ammonia-nitrogen industrial waste waters.

EXPERIMENTAL

Preparation of Seed Sludge and Immobilized Functional Microbial Gel Spheres

This experiment used sludge from a sewage treatment plant sludge in Nanyang. Its sludge settling ratio, initial concentration, volatile sludge concentration, and other indexes were detected by using the procedures described in the Urban Construction Industry Standard of China (CJ/T 221-2005). After the sludge was recovered and left for 24 hours, the supernatant was excluded as the sludge used in this test reactor, and its sludge settling proportion at 30 min (SV30) was about 28.4%. Other sludge characteristics were as follows: mixed liquor suspended solids (MLSS): 4370 mg/L; Mixed liquor volatile suspended solids (MLVSS): 3550 mg/L; MLVSS/MLSS ratio: 0.81; and sludge volume index at 30 min (SVI): 65.0.

The detailed preparation method of the immobilized spheres with loaded microorganisms was shown in the earlier paper published by the group (J. Song *et al.* 2023), and the embedded bacterial strains were the bacteria screened in the activated sludge of the waste leachate biological treatment system by the previous personnel of this group.

Reactor Construction for Different Systems

Three identical SBR reactors were set up with specifications of 120 mm internal diameter, 400 mm height, 3.7 L effective volume, and 3.3 height-to-diameter (H/D) ratio. The reactors were numbered from left to right as 1#, 2#, and 3#, where 1# reactor was filled with activated sludge only, without gel spheres, 2# was filled with activated sludge and immobilized functional microorganisms gel spheres, and 3# was filled with activated sludge and unloaded functional microorganisms gel spheres. Activated sludge and unloaded functional microorganisms were added into the reactor.

The test used artificial water simulation of domestic sewage as the source of water intake. To ensure stable water quality, on-site preparation was undertaken. The chemical oxygen demand (COD) of prepared water was provided separately by anhydrous sodium acetate (NaAc), total phosphorus (TP) was provided separately by potassium dihydrogen phosphate (KH_2PO_4), and ammonia nitrogen (NH_4^+-N) was provided separately by ammonium chloride (NH_4Cl) alone. Influent pH was adjusted to the neutral level. The pH was no longer controlled during the operating phase of the reactor, and the water temperature was room temperature. At the same time, a solution of trace elements (1 mL/L) was added during the preparation of artificially simulated wastewater to provide necessary trace elements for microbial reproduction and growth. The composition of the wastewater

used for artificial preparation and the added trace elements were as shown in Table 1.

At the beginning of the experiment, about 400 mL of thickened activated sludge was injected into each reactor, and 66 g of immobilized functional microorganisms and unloaded functional bacteria were injected into reactors 2# and 3#, respectively. Reactor operation was conducted in the same A/O/A mode, with each cycle set at 8 h and a hydraulic retention time (HRT) of about 16 h. Three cycles were operated per day, with the following cycle composition: influent time of 5 min, anaerobic phase of 155 min, aerobic phase of 160 min, anoxic phase of 125 min, settling time of 30 min, and anaerobic phase of 30 min. In the anaerobic stage, no stirring and aeration were provided to control the corresponding DO below 0.20 mg/L. In the aerobic stage, the aeration was carried out by the air pump, and the mechanical stirrer was also started to work to keep DO values above 2.0 mg/L. In the anoxic stage, aeration stopped, and the stirring kept DO concentrations within 0.20~0.50 mg/L. The system was regularly drained during operation, and the water temperature was indoor temperature; the actual water temperature was about 20±2 °C.

Table 1. Artificial Water Quality and Trace Element Composition of Water Distribution

Water Quality Indicators	Concentration (mg/L)	Composition of Trace Element Reserve	Concentration (mg/L)
COD	450	H_3BO_3	500
NH_4^+-N	30	$MnSO_4·4H_2O$	400
TP	6	$ZnSO_4·7H_2O$	400
Trace Element Reserve	1mL/L	$Na_2MoO_4·4H_2O$	200
		$CuSO_4·5H_2O$	100
/	/	$CoCl_2$	100
/	/	KI	100
/	/	$NiCl_2$	100

Reactor Construction for Different Conditions of Operation

Influence of aeration rate on the decontamination performance of reaction system

To explore the impacts of varying DO levels on the decontamination effect of immobilized functional microbial gel spheres, a control group 4# and a reactor 5# with changed aeration rate were set up with the same influent as in Table 1. The test was carried out with the sludge that had been matured and operated stably for more than 15 d in 2#. At the beginning of the test, 1 L of sludge and 90 g of immobilized functional microorganism gel spheres were added to each reactor. After the system was stabilized, the operation mode was kept unchanged, and the aeration rate of reactor 5 was set to 3 L/min, and that of reactor 4 was kept at 2 L/min.

Impact of C/N on decontamination in the reaction system

An SBR reactor identical to 2.2 was utilized, noted as 6#. The previous operation mode and water distribution were kept the same as 4#. For the purpose of researching the impact different C/N on the decontamination effect of immobilized functional microbial gel spheres, after the system operation was stabilized, the COD of the water distribution in the 6# reactor was changed to 240 mg/L, so that the C/N of the system was 8:1, and the rest of the conditions were kept unchanged, and the subsequent decontamination effect was observed.

Influence of different sources of carbon on the decontamination performance of reaction system

An SBR reactor identical to that of 2.2, noted as 7#, was utilized. To study the impact of C source on the decontamination of immobilized functional microbial gel spheres, all other conditions were kept constant, and only the carbon source was changed to glucose in reactor 7#.

Measurement and Analysis

According to the standard method for determination of COD, TN, NH_4^+-N, NO_3^--N, NO_2^--N, MLVSS, MLSS, and SVI_{30} (Apha, 2012). The pH was monitored through a pH meter (pHS-25, Shanghai Leici Instrument Factory, China).

All statistical analyses were performed using SPSS version 15.0. Pair-sample t-tests were used to assess whether the water quality was significantly different between samples based on p-values. A p-value of <0.05 was considered significant.

High-throughput Sequencing

Appropriate amounts of sludge were extracted as test samples at the end of each operation cycle. The sludge samples obtained from 1#, 2#, 3#, 4#, 5#, 6#, and 7# were designated as S1, S2, S3, S4, S5, S6, and S7, respectively. After sampling, they were immediately frozen for preservation and entrusted to Sangon Biotech Co., Ltd. (Shanghai, China) for Illumina MiSeq sequencing. Using the genomic DNA of the above samples as templates, PCR amplification the V3-V4 sections of 16S rRNA genes of bacteria were amplified with primers B341F and B785R. Quality control of high-throughput sequencing sequences was conducted using Cutadapt (v1.9.1), Prinseq (v0.20.4), and Flash (1.2.3) software. The 97% similar sequences were collected into operational taxonomic units (OTUs). Using the Silva database and Mothur (1.30.1) software, the composition of microbial population in each sample was statistically analyzed at the species level. In addition, coverage and alpha diversity indices of microbes in the individual samples were calculated, namely, the Simpson, Shannon, ACE, and Chao1 indices.

RESULTS AND DISCUSSION

Effluent Decontamination by Different System Reactors

Previous studies had confirmed the specific role of biochar as a carrier (J. Song *et al.* 2023). Figure 1 illustrates the pollutant removal performance of different reactor systems ($p < 0.05$). The COD removal performance of Reactor 2# remained at approximately 94% after stabilization ($p < 0.05$). In contrast to the experimental data for Reactor 1# and 3#, the degradation of organic matter in Reactor 2# was maintained at a relatively elevated level. It was concluded that the loaded microorganisms played a significant role in the reaction process. The functional bacteria acclimated to the sewage environment during this period, and their activity increased rapidly. The removal performance was enhanced and remained stable during operation (Fig. 1a). Based on the experimental data from Reactor 1# and 3#, although the activated sludge possessed a certain COD removal capacity, it was unstable and susceptible to environmental factors. In contrast, the immobilized functional microorganism enhancement system demonstrated relatively robust resistance to impact loading. This was attributed to the highly porous structure of the biochar, which provided excellent growth sites for microorganisms. Thus,

the biochar exhibited a favorable adsorption effect on COD, and microorganisms accelerated its degradation. Additionally, during the operation of Reactor 2# and 3#, the effluent COD initially increased slightly, and then it gradually decreased. The reason for the initial increase was likely that the gel spheres, which were initially tested in a shaker, were subjected to their first operational trial in the reactor. Their mechanical strength was insufficient to withstand the shear force generated by the reactor's mixing device, resulting in the fracture and subsequent washout of a portion of the gel spheres. This disrupted the system within the reactor. The subsequent decline in COD might have been due to the remaining intact gel spheres continuing degradation after the fractured ones were discharged. After approximately four weeks of operation, COD removal in the reactor stabilized essentially. The COD removal effect of Reactor 2# was optimal, indicating that the immobilized functional microbial gel spheres could effectively eliminate COD.

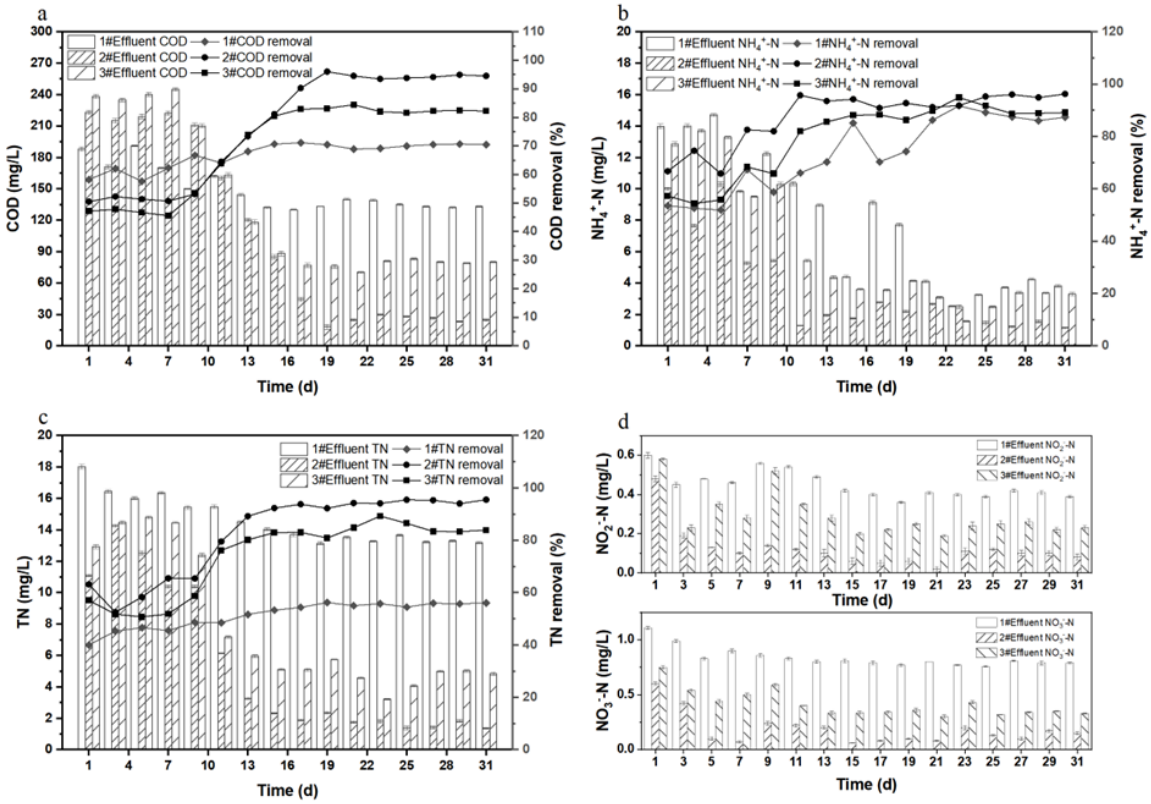

Fig. 1. Variation of pollutant removal in reaction systems 1#, 2#, and 3# with operating time: (a) COD removal; (b) NH_4^+-N removal; (c) TN removal; (d) NO_3^--N and NO_2^--N changes. The three systems were: 1# (activated sludge only), 2# (activated sludge with immobilized functional microorganism gel spheres), and 3# (activated sludge with unloaded gel spheres).

The NH_4^+-N decontamination performances of the system were stable at 87% (1#), 96% (2#), and 89% (3#) throughout the whole reaction stage ($p < 0.05$). This initially indicated that the immobilized gel spheres loaded with the dominant bacterial strains improved NH_4^+-N removal in the system to a degree, which can be attributed to nitrification. The differences in NO_3^--N and NO_2^--N contents are presented in Fig. 1d. Combined with Fig. 1b, it could be observed that nitrification was stronger in reactor 2# than in 1# and 3#, and the NH_4^+-N removal in reactor 3# was also stronger than that in 1#. It was hypothesized that the reason for this might be that the biochar pores supported microbial growth and enhanced NH_4^+-N removal (Zhou *et al.* 2017). Moreover, it had been

pointed out that the carbon-rich biochar may also serve as a C source to promote denitrification (Zhou *et al.* 2017). The nitrite and nitrate contents in the effluent from reactor start-up to the system stabilization were low. In combination with TN removal (Fig. 1c), these results showed that the TN removal performance was unstable in the initial stage due to the lack of microbial adaptation to the environment. The removal performance increased significantly in the middle and late stages of the experiment, which might be mainly due to alterations in the structure of microbial population and the evolution of nitrifying bacterial community structure (D. Chen *et al.* 2023). After completion of reaction, nitrite and nitrate levels decreased under denitrification. TN removal stabilized at approximately 84% (1#), 94% (2#), and 87% (3#), respectively ($p < 0.05$). Despite the fluctuations in the influent TN concentration, the TN removal rate generally presented an upward trend. Effluent TN slightly increased at the outset. It was noteworthy that the main cause of TN fluctuation was related to NO_3^--N and NH_4^+-N, which may be because of the reductions in C/N and DO (Huang *et al.* 2022). However, as the operation time increased, effluent TN level gradually declined, and the TN removal performance in 1# and 3# at the end of the reaction could reach more than 85%. TN removal in the 2# reactor with the immobilized gel ball of loaded bacteria was more than 95% ($p < 0.05$). The results demonstrated that, compared with the activated sludge method alone, the combined immobilized functional microbial gel ball possessed a more efficient nitrogen removal capacity.

Effect of Different Aeration Rates on Biological Systems Loaded with Microbially Immobilized Spheres

To investigate the influence of aeration rate on the decontamination efficacy of biogel balls, the other conditions were kept the same and the aeration rate was varied. TN and NH_4^+-N removal performances are given in Fig. 2(b, c). When the C/N of influent was 15:1, NH_4^+-N removal efficacy (NRE) of system reached about 90%, with a maximum removal of 99.2% at an aeration rate of 3 L/min ($p < 0.05$). The results showed that the appropriate increase of the aeration in different environments could improve the NRE of immobilized gel spheres. NRE comparison of different experimental groups showed significantly positive effects of aeration on nitration process, DO concentration, and nitrification (Jin *et al.* 2019). There was adequate DO in the 5# reactor to promote both nitrification and denitrification. The supply of external oxygen supplementation (particularly at high C/N levels) boosted NRE because, at greater ratios of C/N in influent, organic matter contended with autotrophic nitrifying microbes for DO, while heterotrophic microbial reproduction restrained the growth of autotrophic nitrifying microorganisms (Peng *et al.* 2025). This might be the cause of the decline in the NH_4^+-N removal rate under a high carbon-to-nitrogen ratio. In HN-AD, NH_4^+ first by ammonia monooxygenase (AMO), ammonia nitrogen was oxidized to NH_2OH. Hydroxylamine oxidoreductase (HAO) catalyzed the transformation of hydroxylamine into NO_2^-, which was then converted to NO_3^- by nitrite oxidoreductase. Reduction of nitrite and nitrate is crucial for denitrification. During the denitrification process, both NO_3^- and O_2 could serve as the ultimate electron acceptors, and O_2 was consumed when NO_3^- was eliminated. This suggested that HN-AD bacteria may have relatively high demands for DO. The generation of NO_3^--N and NO_2^--N is presented in Fig. 2d. Compared with 4#, nitrification and denitrification in 5# reactor with a higher aeration rate were more rapid.

It was also observed that 5# with the same higher aeration rate was more effective in COD removal (Fig. 2a), presumably because the more environmentally adapted HN-AD

bacteria proliferated faster, thus contributing to the more significant COD removal in reactor 5#. Another main reason is that, at high aeration rate, the DO in the reactor was sufficient to supply enough oxygen for organic matter oxidation, while there was still abundant oxygen for oxidation of NH_4^+-N. Therefore, COD and NH_4^+-N removal efficiencies were more significant at high aeration rate under this condition. These results indicated the selectivity of heterotrophic bacteria associated with organic matter removal and nitrification-related AOB for high DO. At both aeration rates, the system showed COD and nitrogen removal advantages for different DO environments, especially at slightly higher DO.

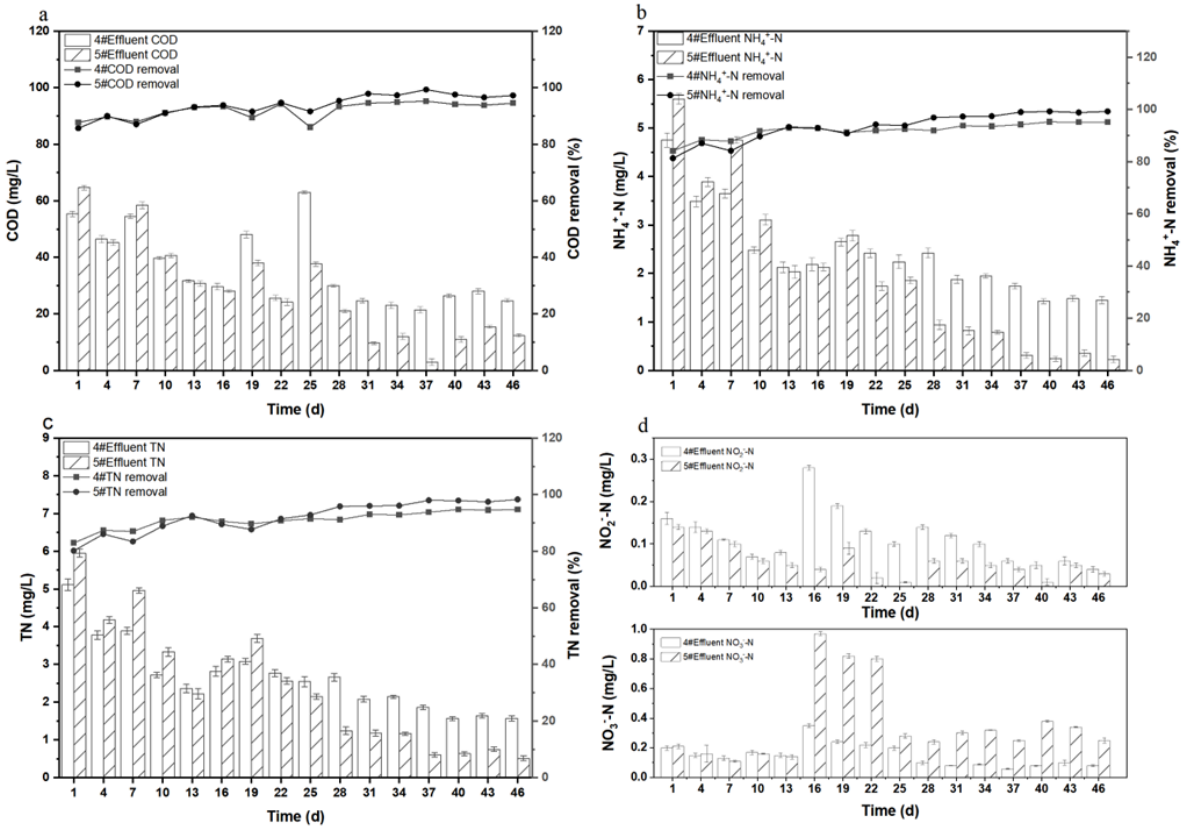

Fig. 2. Variation of pollutant removal in reaction system 4# and 5# with operating time: (a) COD removal; (b) NH_4^+-N removal; (c) TN removal; (d) NO_3^--N and NO_2^--N change

Influence of Different C/N on the Biological System of Immobilized Spheres Loaded with Microorganisms

Carbon to nitrogen ratio as a key parameter that directly affects the efficiency and effectiveness of the denitrification process, where microorganisms require a C source as a donor of electrons for denitrification, thus reducing nitrate to nitrogen. Most studies have shown that a proper carbon to nitrogen ratio promotes the TN and ammonia removal and increases the rate of nitrification. Too high or too low a carbon to nitrogen ratio could reduce TN and ammonia removal, and when the C/N is low, it usually exhibits poor TN removal and denitrification capabilities (Hill and Khan 2008; Sha *et al.* 2024). However, when the C/N exceeds the ideal level, it results in slightly lower capacities of TN removal and ammonia oxidation (Hu *et al.* 2023). This experiment explored the treatment of wastewater by the reaction system at C/N of 15 and 8.

The TN and NH_4^+-N removal effects, as well as nitrite and nitrate contents in the effluent are shown in Fig. 3 NH_4^+-N removal was greatly affected by C/N, and the two

reactors showed different NRE in the reaction stage. In the last stage, NRE at C/N 15 and 8 were 95.2% and 88.3% ($p < 0.05$), respectively. The extent of TN removal in the 4# reactor remained at a relatively high and stable level of about 94% ($p < 0.05$). Nitrate was non-accumulative, and denitrification was more thorough. TN removal in 6# system with low carbon and nitrogen ratio was lower than that of the 4# reaction system with high carbon and nitrogen ratio. After two weeks of operation, nitrate accumulation was more obvious, and the rate of TN removal fluctuated. Shi *et al*. (2024) showed that TN and NH_4^+-N removal improved significantly with increasing carbon to nitrogen ratio, and that carbon supplementation enhanced both nitrification and denitrification. Functional pellet-loaded HN-AD bacteria could adapt to an extensive C/N range, while normal HN-AD bacteria need greater C/N levels than the autotrophic nitrifying bacteria (ANB) and had shown optimal carbon to nitrogen ratios of 8 to 15 in several studies. It was thus clear that high carbon to nitrogen ratios were more favorable to functional pellet-loaded systems. They were more conducive to denitrifying microbial proliferation, as well as to activity It could be seen that a high carbon to nitrogen ratio was more favorable to the functional pellet loading system and the proliferation and activity of denitrifying microorganisms.

In terms of COD removal (Fig. 3a), 4# and 6# were roughly close to each other, both reaching more than 90% ($p < 0.05$).

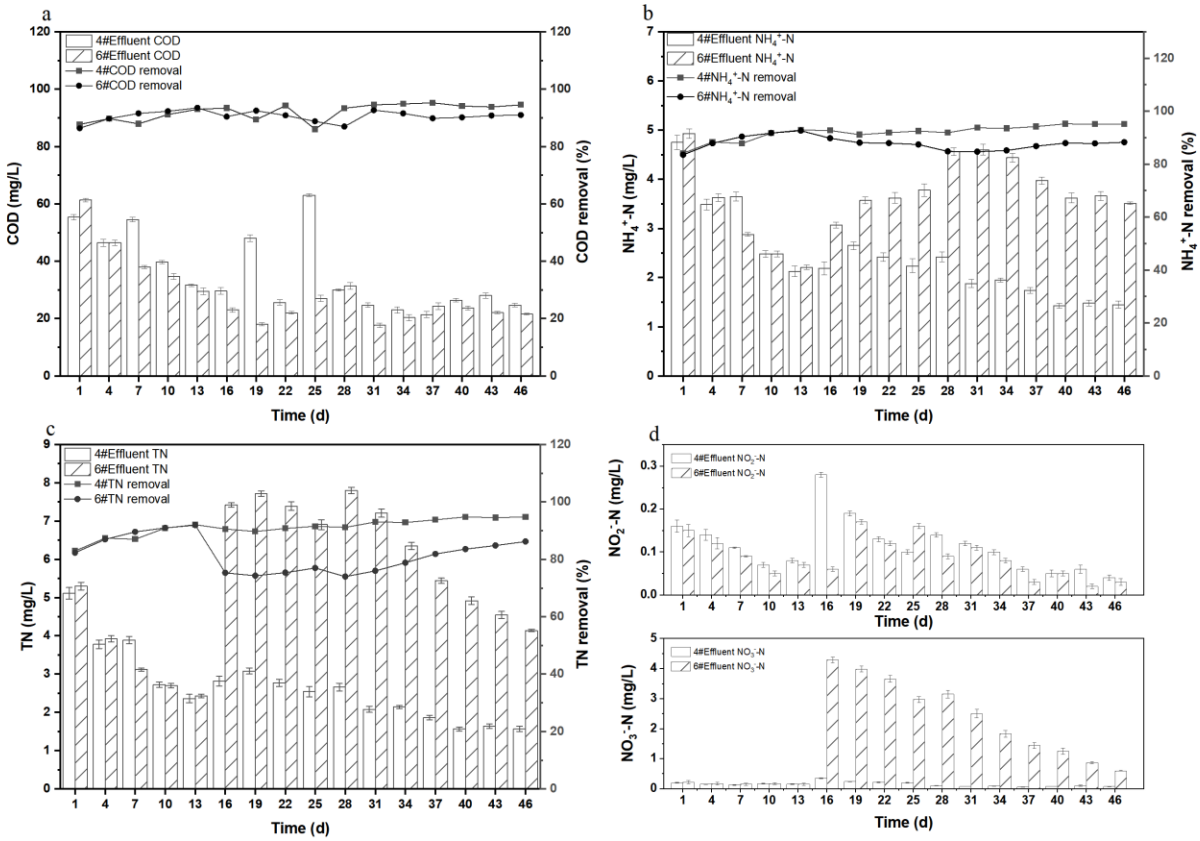

Fig. 3. Variation of pollutant removal in reaction system 4# and 6# with operating time: (a) COD removal; (b) NH_4^+-N removal; (c) TN removal; (d) NO_3^--N and NO_2^--N change

Influence of C Sources on Immobilized Spheres with Microorganisms

To understand the influence of C source on decontamination of immobilized materials, the effluent indicators were measured during the reactor operation in the presence of different C sources. Carbon source has been found to be crucial for denitrifying bacteria as a source of electron and energy (M. Chen *et al.* 2023), and denitrifying bacteria are known to utilize organic carbon sources while denitrifying. The process of nitrification denitrification is a redox reaction, wherein the carbon source provides the required electrons and energy for microbial growth. The extent of bacterial growth varies with the type of C source, which affects the degree of nitrate reduction and accumulation of intermediates, and therefore can have a great influence on denitrification/nitrification rates (Lu *et al.* 2024). The glucose used as a carbon source easily caused the system to acidify, which lowered the local pH and impaired the activity of microorganisms involved in nitrogen metabolism, leading to a slower denitrification rate. In addition, NO_2^- accumulation was greater in presence of glucose as a C source. As shown in Fig. 4, COD was found to be rapidly utilized in the reactor in the presence of exogenous C sources ($p < 0.05$), indicating that the acetate and glucose were readily biodegradable (Sun and Li 2024). However, the reactors showed significant differences in denitrification performance, which was closely connected to the C metabolic pathway of the microbes. When the source of C was glucose, COD removal efficiency declined substantially and the pH value in the system gradually decreased from the initial 7.2 to 6.3 ± 0.2. This may be due to the acidic intermediates such as short-chain fatty acids (*i.e.*, propionic acid, acetic acid) generated by glucose during microbial metabolism, which led to the acidification of the liquid phase (Huang *et al.* 2024).

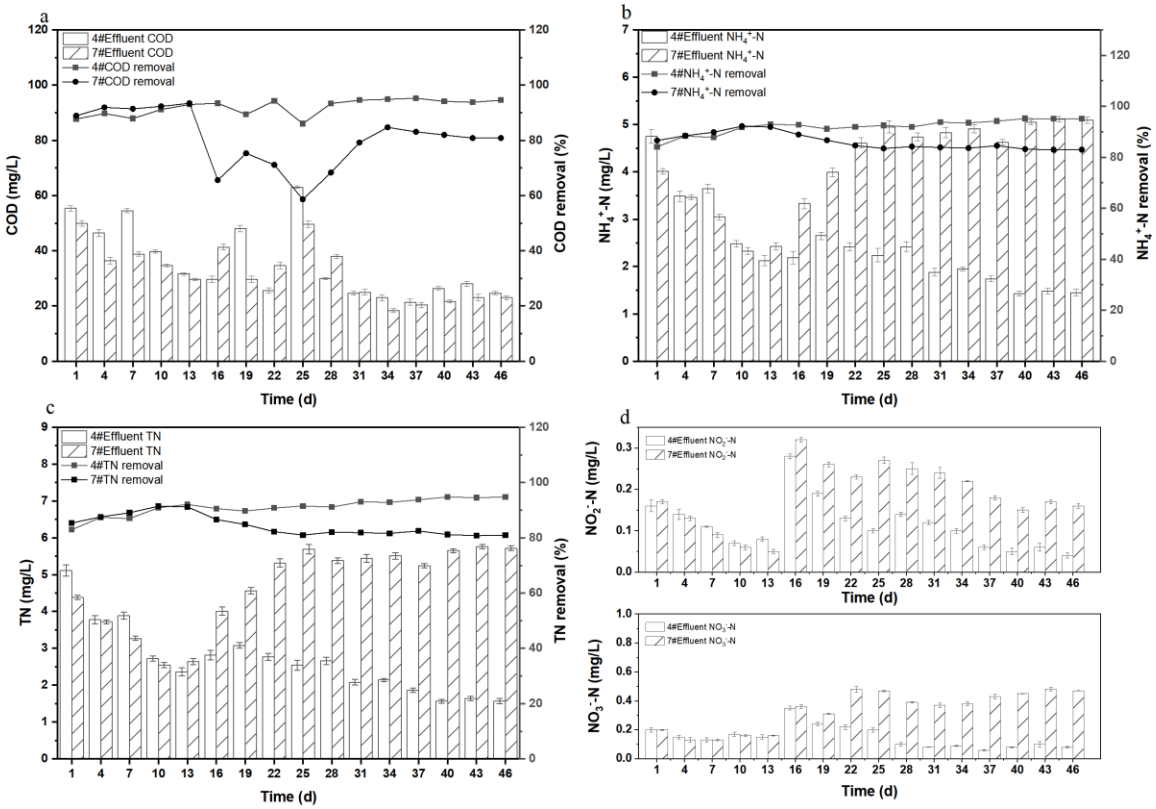

Fig. 4. Variation of pollutant removal in reaction system 4# and 7# with operating time: (a) COD removal; (b) NH_4^+-N removal; (c) TN removal; (d) NO_3^--N and NO_2^--N change

Analysis of the Dynamic Structure of Microbial Populations

Results of sequencing and alpha diversity analysis

The sequence data of sludge samples were analyzed and screened for quality. The partial results are shown in Table 3. The numbers of valid sequences in S1, S2, S3, S4, S5, S6, and S7 samples were 26284, 24519, 27325, 23147, 22392, 19055 and 22618, and the OTUs obtained at the 97% similarity level were 737, 729, 745, 709, 660, 649 and 600, respectively. The Coverage index was more than 0.99 for all samples. Simpson and Shannon indices represent the statistical homogeneity and diversity of microbial population, and Chao and ACE represent the richness of the microbial community structure.

Table 2. Sequencing Results and Diversity Indices of Species Diversity and Richness Index Analysis

Operating conditions	Sample	Number	OTUs	Ace	Chao	Shannon	Simpson	Coverage
Initiation phase	S1	26284	737	787.762	791.451	5.137	0.014133	0.996
	S2	24519	729	763.235	772.596	5.382	0.010244	0.997
	S3	27325	745	785.402	800.781	5.273	0.010763	0.996
Different working conditions	S4	23147	709	750.034	747.616	4.649	0.04636	0.996
	S5	22392	660	748.679	748.483	4.849	0.018675	0.994
	S6	19055	649	760.124	764.068	4.743	0.024078	0.992
	S6	22618	600	699.105	691.733	4.335	0.037696	0.994

Table 2 shows that the diversity of microorganisms in the three reactor samples (1#, 2#, and 3#) set up for the evaluation study of the treatment effect of different systems was higher than that of the reactor samples for the subsequent evaluation experiments of the different influencing factors. This may be due to the more homogeneous composition of the artificially dispensed water, and the microbial diversity of the 4#, 5#, 6#, and 7# reactors, which were used with the sludge of the reactor #2 as the initial sludge for the evaluation of the subsequent operation, was reduced. The diversity of microbes in the reactors with carriers was slightly greater compared to the control system, and the increase in aeration further improved the diversity of microbes within the system.

Structure of microbial populations

Relative abundance greater than 1% was considered as dominant phylum, and on this basis there were 11 dominant phylum in the three reactors in the research experiments of different systems. Among them the absolute dominant phylum was *Proteobacteria* with relative abundances of 27.7%, 45.0%, and 51.9%, respectively (Fig. 5A). *Proteobacteria* had been consistently found to be the most abundant phylum in the bioreactors because of its P and N removal potential (Liao *et al.* 2021). The average relative abundance of *Bacteroidetes* was greater than 10%. In addition, *Firmicutes*, *Chloroflexi*, *Nitrospirae*, *Planctomycetes* and *Armatimonadetes* were detected.

Table 3. List of Key Functional Bacteria Related to N Removal

Key functional groups		Initiation phase			Different working conditions			
		S1	S2	S3	S4	S5	S6	S7
NOB	*Nitrospira*	3.703	0.940	0.838	0.709	0.210	1.287	2.212
DNB	*Brachymonas*－	0.697	6.795	4.911	10.020	13.920	1.499	5.202
	Saccharibacteria_genera_incertae_sedis	4.070	9.127	9.050	7.120	7.120	2.001	2.920
	Azonexus	0.131	0.441	0.282	15.072	8.218	1.875	2.186
	Acinetobacter＋	0.355	7.548	5.047	7.925	7.474	1.026	4.514
	Paracoccus＋	0.098	2.063	1.656	1.220	2.208	0.512	0.823
	Gemmobacter＋	0.848	3.371	2.181	1.456	0.764	0.672	0.651
	Delftia＋	0.012	0.148	0.282	0.099	3.144	3.095	1.527
	Pseudomonas＋	0.065	2.157	2.042	2.076	3.246	1.256	1.388
	Aeromonas＋	0.012	1.250	0.560	1.056	0.478	0.959	1.010
	Terrimonas	1.415	1.278	1.940	0.972	2.974	0.646	1.107
	Thermomonas	1.162	1.164	1.632	0.661	0.500	0.199	0.446
	Hyphomicrobium	0.644	1.168	1.014	1.119	0.201	0.159	0.215
	Aridibacter	4.629	1.587	2.075	1.417	2.215	0.287	1.666
	Dechloromonas＋	0.224	0.498	0.534	0.285	2.063	4.364	3.847
	Proteocatella	1.607	1.256	0.253	5.573	0.255	0.305	0.136
	Thiothrix	0.012	0.757	0.296	0.389	0.473	3.873	1.233
	Thauera＋	0.118	0.730	0.421	0.272	2.429	1.733	2.018
	Defluviimonas	0.420	2.195	1.369	0.786	0.353	0.230	0.247
	Ottowia＋	0.848	1.431	1.665	0.566	0.357	0.261	0.273
	Hydrogenophaga＋	0.008	0.156	0.172	0.104	0.263	3.090	1.275
	Arcobacter	0.053	0.403	0.549	2.147	0.027	0.270	0.047
	Novosphingobium	1.130	0.403	0.512	0.747	0.183	0.221	0.231
	Total number of DNBs	18.55	45.925	38.443	61.081	58.864	28.533	32.962

Most denitrifying bacteria were classified as *Ascomycota*. These bacteria have been found to be crucial for denitrification (Karanasios *et al.* 2010), while the phylum *Porphyromonas* may also perform denitrifying functions in intermediate stages (Kartal *et al.* 2007). The phylum *Nitrospirae* is a group of Gram-negative bacteria, in which *Nitrospirae* spp. function as nitrifying bacteria to oxidize nitrite into nitrate. *Nitrospirae* abundance levels in control system S1 and carrier-only system S3 were 3.76% and 1.84%, respectively, while *Nitrospirae* were reduced to non-dominant in the S2 system loaded with synchronous nitrifying and denitrifying functional bacteria, which may be due to the fact that the functional bacteria loaded in S2 system had the ability to perform SND, and they partially replaced the function of *Nitrospirae* in terms of ecological niche, leading to the decrease in *Nitrospirae*'s abundance.

At the level of class, 18 dominant classes were found in different systems, as shown in Fig. 5B. Many classes were prevalent in wastewater treatment systems, including *Alpha*, *Delta*-, and *Betaproteobacteria* as well as *Clostridia*, *Bacteroidia*, and *Sphingobacteria*. In comparison to S1, the abundance of *Betaproteobacteria*, *Gammaproteobacteria*, *Clostridia*, and *norank_Candidatus_Saccharibacteri*a doubled in S2 and S3 systems with additional vectors and functional bacteria. In contrast, the relative abundance of the four orders *Anaerolineae*, *Planctomycetia*, *Acidobacteria*, and *Nitrospira* decreased by a factor of two.

Figure 5C shows the 25 dominant genera in different reactors. Among them, 10 genera of microorganisms, namely *Zoogloea*, *Brachymonas*, *Acinetobacter, Clostridium_sensu_stricto*, *Gemmobacter*, *Paracoccus*, *Pseudomonas*, *Defluviimonas*, *Hydrogenophaga*, and *Ottowia*, all presented themselves as non-dominant bacteria in the blank control system S1, and growing into dominant genera in the S2\S3 system, with a growth rate of about 2-30 times. A review of the literature revealed that eight of these ten genera, except *Zoogloea* and *Clostridium_sensu_stricto*, were highly efficient denitrifying bacteria, and *Acinetobacter*, *Gemmobacter*, *Paracoccus*, *Pseudomonas* and *Hydrogenophaga* were typical of the *Acinetobacter*, *Paracoccus*, *Pseudomonas*, and *Hydrogenophaga* were typical aerobic denitrifying bacteria. *Acinetobacter*, *Paracoccus* and *Pseudomonas* were the strains used for loaded microbial immobilized spheres. Consequently, these genera were more abundant in the S2 system, as compared to S3 system, and the gel spheres carrier used in the present study was conducive to enhancement of denitrogenation.

A total of 9, 10, 5, and 9 phyla were identified within samples S4 to S7 (Fig. 5A), respectively. There were fewer phyla in the systems with insufficient carbon sources. The *Proteobacteria* phylum was dominant in all four reactors, with a relative abundance of more than 50% in all of them, including as high as 73.0% in reactor S6. The *Bacteroidetes* phylum was also dominant, with an average relative abundance of more than 18%. Three phyla, *Firmicutes*, *Chloroflexi*, and *Candidatus_Saccharibacteria*, had significant organic matter degradation, nitrogen treatment in wastewater treatment, with higher relative abundance in control system S4 than in other conditioned reactors. *Nitrospirae* remained non-dominant in other systems loaded with functional bacterial pellets except S5, which may be due to the increased aeration, prompting the function of *Nitrospirae*.

At the class level, compared with the other three reactors, the S7 reactor had the least number of dominant classes, which was 12 (Fig. 5B). *Betaproteobacteria* was its absolute dominant class, with a relative abundance as high as 43.7%. *Nitrospira* remained a non-dominant bacterium in the control system S4 and the well-aerated system S3, but its

relative abundance increased in the reactor with a low carbon-nitrogen ratio and glucose as the carbon source, transforming it into a dominant bacterium.

Figure 5C shows the 32 dominant genera that were observed in the three reactors. The relative abundance of aerobic DNB *Acinetobacter*, *Gemmobacter*, *Paracoccus*, *Pseudomonas*, *Delftia*, and *Hydrogenophaga* was found to be higher in the well aerated S5 system and the S4 control system than in the S6 and S7 systems, and it was hypothesized that the low C/N and inorganic C source may not favor the aerobic denitrifying bacteria. Glucose could negatively affect the growth of these bacteria. Synergistic denitrification between *Gemmobacter* and *Hydrogenophaga* and *Pseudomonas* had also been reported in the literature. The dominant genera with relative abundance more than 10% were *Brachymonas* and *Azonexus* in S4,*Brachymonas* in S5 system, and *Zoogloea* in S6 and S7 systems. *Brachymonas*, as a major genus of functional denitrifying bacteria in the short-range nitrification-denitrification process, was experimentally shown to specific conditions could increase the total nitrogen removal rate substantially (C. Yang *et al.* 2022).

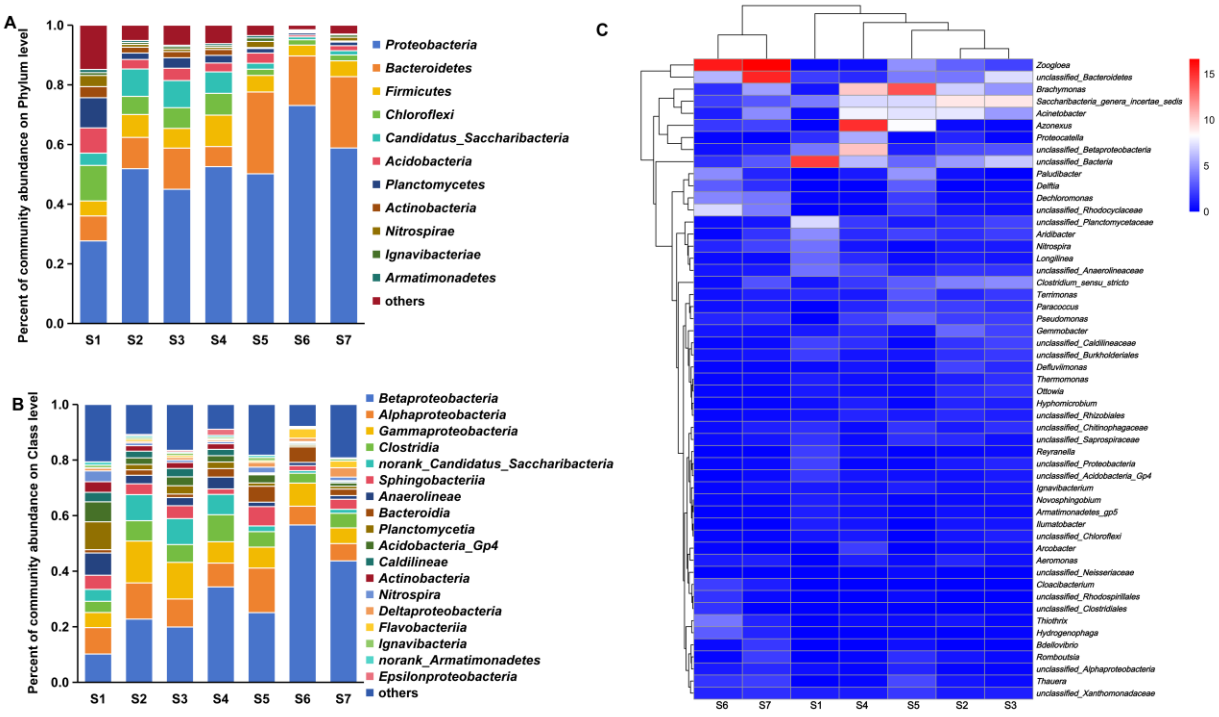

Fig. 5. Bacterial compositions of communities: microbial compositions distributions at phylum A, class B, and genus C level

Key functional species

To clarify the relationship between microorganisms and removal performances, studies on key functional species associated with carbon, nitrogen and Extracellular Polymeric Substances (EPS) production were particularly necessary. It is well known that traditional biological denitrification involves two main microbial processes, nitrification and denitrification, and only *Nitrospira*, which were reported as nitrite-oxidizing bacteria (NOB), were also observed in this study. The growth of *Nitrospira* was inhibited and its relative abundance became lower in the system loaded with synchronized nitrifying denitrifying functional bacteria. There were a large number of denitrifying bacteria (DNB)

to ensure the effective and reliable water nitrogen denitrification process, and most of the DNB carried out heterotrophic denitrification, thus consuming carbon sources during denitrification (Table 3).

A total of 22 species of DNB were detected in the three samples in the different system experiments, including aerobic denitrifying bacteria *Brachymonas*, *Acinetobacter*, *Paracoccus*, *Delftia*, *Pseudomonas*, *Gemmobacter*, *Thauera*, and *Aeromonas*. As can be seen from Table 3, the relative abundance of most aerobic DNB in the reactors of different systems showed a more significant increase under the effect of immobilization, which explains the higher nitrogen removal capacity of reactor #2, compared to reactor #1.

Under different working conditions, DNB growth was more pronounced in the S4 and S5 systems, with a combined relative abundance of more than 50%. At present, the denitrification pathway of aerobic denitrifying bacteria was relatively clear, *i.e.*, NO, N_2O, NO, N_2, NO_2^--N, NO_3^--N, and *Azonexus* had the highest abundance of 15.1% in the S4 reactor, and it was found that *Azonexus* could efficiently remove denitrification intermediates (NO_2^-) during denitrification, with slight accumulation of NO_2^- in this reactor, which was speculated to be the reason for the massive growth and reproduction of *Azonexus*. As can be seen from Table 3, the low carbon to nitrogen ratio (S6) and glucose carbon source (S7) were unfavorable factors for the growth of DNB. The DNB consumed a huge amount of C source to perform nitrification, which well explains the previous results about organic matter degradation, which aligned with the COD removal performance.

CONCLUSIONS

1. The reactor amended with BADB (Reactor 2#) demonstrated superior and stable pollutant removal compared with the control with only activated sludge (Reactor 1#) and the carrier-control (Reactor 3#). This confirms a synergistic effect in which the biochar-based gel spheres do not merely act as a physical support. Rather, they create an optimized micro-environment that enhances microbial activity, protects functional bacteria, and prevents washout, leading to a more efficient and robust system.
2. An elevated aeration rate (3 L/min) was found to be beneficial, significantly boosting the removal performances of COD, NH_4^+-N, and TN. This system can also maintain high processing efficiency under aerobic conditions.
3. The type and quantity of carbon source were found to be critical. Sodium acetate was a superior carbon source compared with glucose, which led to system acidification and performance deterioration. Maintaining a C/N ratio within an appropriate range was found to be essential for achieving high nitrogen removal efficacy. This study showed a broad range of carbon-nitrogen ratios (8 to 15).
4. The significant enrichment of key aerobic denitrifying genera, such as *Brachymonas*, *Acinetobacter*, *Paracoccus*, and *Pseudomonas*, directly explained the enhanced nitrogen removal capacity. The observed functional redundancy and community restructuring in response to different operational conditions provided biological resilience, ensuring stable performance under fluctuating environments.
5. *Brachymonas* was identified as a pivotal aerobic denitrifying bacterium in the system, with its relative abundance soaring under optimal conditions (up to 13.9%). Its prominent role underscores its importance in the short-range nitrification-

denitrification process and its potential as a biomarker for a healthy, high-performance BADB system.

6. This work provides operational parameters for optimizing BADB technology. The combination of a protective biochar-hydrogel carrier with a diversified and adaptable microbial community presents a highly promising strategy for the advanced treatment of high-ammonia nitrogen wastewater, facilitating the transition of aerobic denitrification from laboratory research to practical engineering application.

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Conflict of Interest

The authors declare no conflict of interest.

Use of Generative AI

The authors declare that no AI was used.

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