WEP Warwick Evans Publishing

International Journal of Natural Resources and Environmental Studies

ISSN: 3006-2012 (Print), ISSN: 3006-0834 (Online) | Volume 8, Number 5, Year 2026
DOI: https://doi.org/10.62051/ijnres.v8n5.13
Journal homepage: https://wepub.org/index.php/IJNRES/index

Research on the Enhancement of an Anammox System Using Iron Derived from Excess Sludge

Yuhe Zhao*, Xinqiang Ning

College of Food and Brewing Engineering, Sichuan University of Science & Engineering, Yibin, China

*Corresponding Author: Yuhe Zhao

ABSTRACT

This study uses iron recovered from excess sludge to enhance an anammox system. Results show that sludge-derived iron significantly improves nitrogen removal efficiency, optimizes extracellular polymeric substances (EPS) composition, and increases heme c content to strengthen microbial ac tivity. Microbial analysis reveals that iron selectively enriches anammox functional bacteria. The re actor with K1 carrier performs best. This approach realizes sludge resource utilization and enhance s anammox process, achieving waste treatment by waste.

KEYWORDS

Anammox; Ferric ion; Excess sludge.

1. INTRODUCTION

With the acceleration of urbanization, the scale of wastewater treatment in China has continued to grow, leading to a substantial increase in excess sludge production. According to statistics, by the end of 2023, the annual production of excess sludge with 80% water content from municipal wastewater treatment plants in China had exceeded 60 million tons. The high cost of excess sludge treatment and disposal, which accounts for approximately 50% to 60% of the total operating costs of wastewater treatment plants, has become a major challenge facing the wastewater treatment industry. On the other hand, excess sludge is rich in iron, primarily derived from the addition of iron salt coagulants. If these iron resources are not effectively recovered and reused, it not only results in resource waste but also increases the burden of sludge treatment. Iron is an essential trace element for the growth and metabolism of anaerobic ammonium-oxidizing bacteria, known as anammox bacteria. It participates in the synthesis of key enzymes and electron transfer processes[1], playing a crucial regulatory role in enhancing bacterial activity and maintaining system stability. The addition of external iron has become a common approach to strengthen anammox systems[2]. Utilizing excess sludge as a low-cost iron source for anammox systems achieves the dual objectives of sludge resource utilization and process performance enhancement. This study uses excess sludge as an iron source, recovering iron from excess sludge and applying it to enhance anammox systems, thereby addressing the fate of iron in sludge while providing a low-cost iron source for the anammox process, achieving the goal of treating waste with waste.

In this study, excess sludge was used as the iron source. Iron was recovered from the sludge through a pyrolysis extraction method, and the resulting iron-based material was added to an anammox system. The effects of this sludge-derived iron source on anammox bacterial activity, nitrogen removal

performance, and microbial community were investigated to evaluate the feasibility of enhancing anammox systems using iron derived from excess sludge.

2. EXPERIMENTAL MATERIALS AND METHODS

2.1. Experimental Setup and Operating Procedure

Three identical ANR reactors were operated in parallel as shown in Figure 1-1. The reactor main body was made of plexiglass, with a total effective volume of 30 L and a height-to-diameter ratio of 4.25. The ANR reactor was operated in continuous flow mode. Influent was supplied steadily from the bottom of the reactor via a peristaltic pump, and aeration was controlled by a rotameter. Bubbles generated by bottom aeration carried the sludge-liquid mixture upward along the center, which then recirculated to the bottom along the outer side. Separation of sludge granules from the effluent was achieved by gravity sedimentation, and the treated effluent was discharged from the outlet through an overflow weir. In this study, K1 carrier was added to reactor R1, polyurethane sponge carrier was added to reactor R2, and a no-carrier blank control group was designated as CK. After the reactors had stabilized, excess sludge was added from day 103 to day 164. The nitrogen removal performance of the reactors and the biofilm formation on the carriers were monitored. The ANR reactors were operated at 35 ± 2°C, with dissolved oxygen maintained at 0.5 ± 0.2 mg/L, pH at 8 ± 0.2, and a hydraulic retention time of 15 hours.

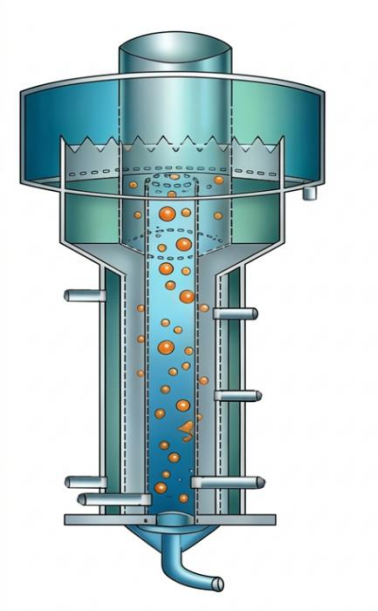

Figure 1. Schematic Diagram of the ANR Reactor

2.2. Experimental Wastewater and Seed Sludge

The experimental influent was taken from the anaerobic effluent of an IC reactor used for producing strong-flavor Baijiu in a distillery. The basic characteristics of the wastewater were as follows: COD of 550.23 ± 115 mg/L, TN of 600.11 ± 109 mg/L, and TP of 828.135 ± 7 mg/L. One milliliter of trace element solution was added per liter of wastewater. The anammox sludge used for inoculation, with a volatile suspended solids concentration of 3842 ± 152 mg/L, was obtained from a laboratory-scale upflow anaerobic sludge bed reactor.

Table 1. Economic Data Statistics

Composition	Concentration
FeNaEDTA	8.252
EDTA	15
H3BO3	0.014
ZnSO4·7H2O	0.43
CoCl·6H2O	0.24
NaSeO4·10H2O	0.21
MnCl·4H2O	0.99
CuSO4·5H2O	0.25
NiCl·6H2O	0.19
NaMoO4·2H2O	0.22
Na2WO4·2H2O	0.05
KH2PO4	10
CaCl·2H2O	5.6
MgSO4·7H2O	300

2.3. Preparation of Excess Sludge

The sludge concentration was adjusted by diluting the excess sludge to a total solid (TS) concentration of 50 g/L to ensure uniform reaction. Sodium hydroxide (NaOH) was added to adjust the pH to 10, thereby disrupting the cell wall and extracellular polymeric substance (EPS) structure of the excess sludge, which promoted organic matter degradation and the release of iron ions. The sludge was then heated in a water bath at 60°C for 30 minutes to decompose readily degradable organic compounds and avoid iron ion precipitation caused by high temperatures. Subsequently, hydrochloric acid (HCl) was added to readjust the pH to 7. The total iron concentration available in the pretreated liquid was 45.48 ± 7 mg/L, and the total amount of Fe^{2+} and Fe^{3+} in the system was maintained at 4.0 ± 1 mg/L.

2.4. Calculation Methods for Basic Parameters

(1) Removal efficiency

$$\text{Removal efficiency}(\%)=\frac{TN_{inf}-TN_{eff}}{TN_{inf}}\times 100\% \quad (1)$$

(2) ARE

$$ARE(\%)=\frac{NH_4^+-N_{inf}-NH_4^+-N_{eff}}{NH_4^+-N_{inf}}\times 100\% \quad (2)$$

2.5. Analytical Methods

2.5.1. Basic Water Quality Indicators

The influent and effluent of the reactor were centrifuged at 6000 r/min for 5 minutes and then passed through a 0.45 μm membrane. Monitoring was conducted in accordance with the Standard Methods for the Examination of Water and Wastewater.

2.5.2. Analytical Methods for Extracellular Polymeric Substances

Extracellular polymeric substances were divided into three layers: soluble EPS (S-EPS), loosely bound EPS (L-EPS), and tightly bound EPS (T-EPS). At the end of each cycle, 10 mL of biofilm sludge attached to the carrier was collected in a centrifuge tube and shaken for 30 minutes to obtain the sludge sample.

For S-EPS: The sample was centrifuged at 5900 r/min for 15 minutes, and the supernatant was filtered through a 0.45 μm membrane.

For L-EPS: After the first centrifugation step, phosphate buffer solution was added to the sludge to bring the sample volume back to 5 mL. The centrifuge speed was adjusted to 9000 r/min. After 20 minutes of centrifugation, the supernatant was collected and filtered to obtain the loosely bound layer.

For T-EPS: The sludge remaining after the second step was disrupted by ultrasonic treatment. The sludge sample was resuspended to 5 mL and centrifuged for 25 minutes at 16000 r/min. The supernatant was collected and filtered through a 0.45 μm membrane to obtain the tightly bound layer.

The contents of polysaccharides and proteins were determined using the anthrone-sulfuric acid method and the Folin-phenol reagent method, respectively.

2.5.3. Microbial Community Structure Analysis

The V3-V4 variable region of the prokaryotic 16S rRNA gene was selected as the amplification target, and the target fragment was amplified by PCR using specific primers. The forward primer was 338F (5′→3′: ACTCCTACGGGAGGCAGCAG), and the reverse primer was 806R (5′→3′: GGACTACHVGGGTWTCTAAT). After genomic DNA extraction and PCR amplification, the amplified products were sequenced and analyzed using the Illumina MiSeq sequencing platform. The sequencing work was completed by Shanghai Majorbio Bio-pharm Technology Co., Ltd. To construct an effective sequence dataset for each sample, all raw sequencing reads were subjected to quality trimming to remove random sequencing errors and low-quality sequences, ensuring the reliability of subsequent analyses. Preprocessing and analysis of the sequencing data were performed based on the Majorbio Cloud Platform. Using amplicon sequence variants as the analytical unit, alpha diversity indices including Shannon and Chao were calculated. On this basis, a systematic analysis of the microbial community structure was carried out.

3. RESULTS AND DISCUSSION

3.1. Effect of Iron-Rich Excess Sludge on Reactor Nitrogen Removal Performance

A 61-day experimental operation was conducted using excess sludge as the iron source. The nitrogen removal performance of all three reactor groups for various nitrogenous pollutants was significantly enhanced, with prominent differences observed among the groups. After the carrier acclimation was completed, the treatment performance became stable. Following the addition of iron ions, the trends of various water quality indicators decreased steadily, indicating that the system could quickly adapt to the iron-rich environment. Among the three reactors, R1 exhibited the best ammonium nitrogen removal performance. Its effluent ammonium nitrogen concentration decreased from 42.58 mg/L to 10.14 mg/L, and the removal efficiency increased to 97.55%. This carrier promoted sufficient contact between trace elements and microorganisms, effectively avoiding mass transfer limitations[3].

The effluent nitrite nitrogen concentration in all three reactors continuously decreased without significant accumulation. Iron ions significantly enhanced the ability of anammox bacteria to convert and utilize nitrite nitrogen. Only R2 experienced a brief fluctuation due to a lag in microbial community adaptation, after which it quickly returned to stability. Regarding nitrate nitrogen, only the concentration in R2 decreased steadily from 17.03 mg/L to 6.62 mg/L, while the other two groups showed a slight increase. The polyurethane sponge carrier combined with iron ions optimized the anaerobic environment, enhanced the activity of denitrifying bacteria, and accelerated nitrate nitrogen consumption.

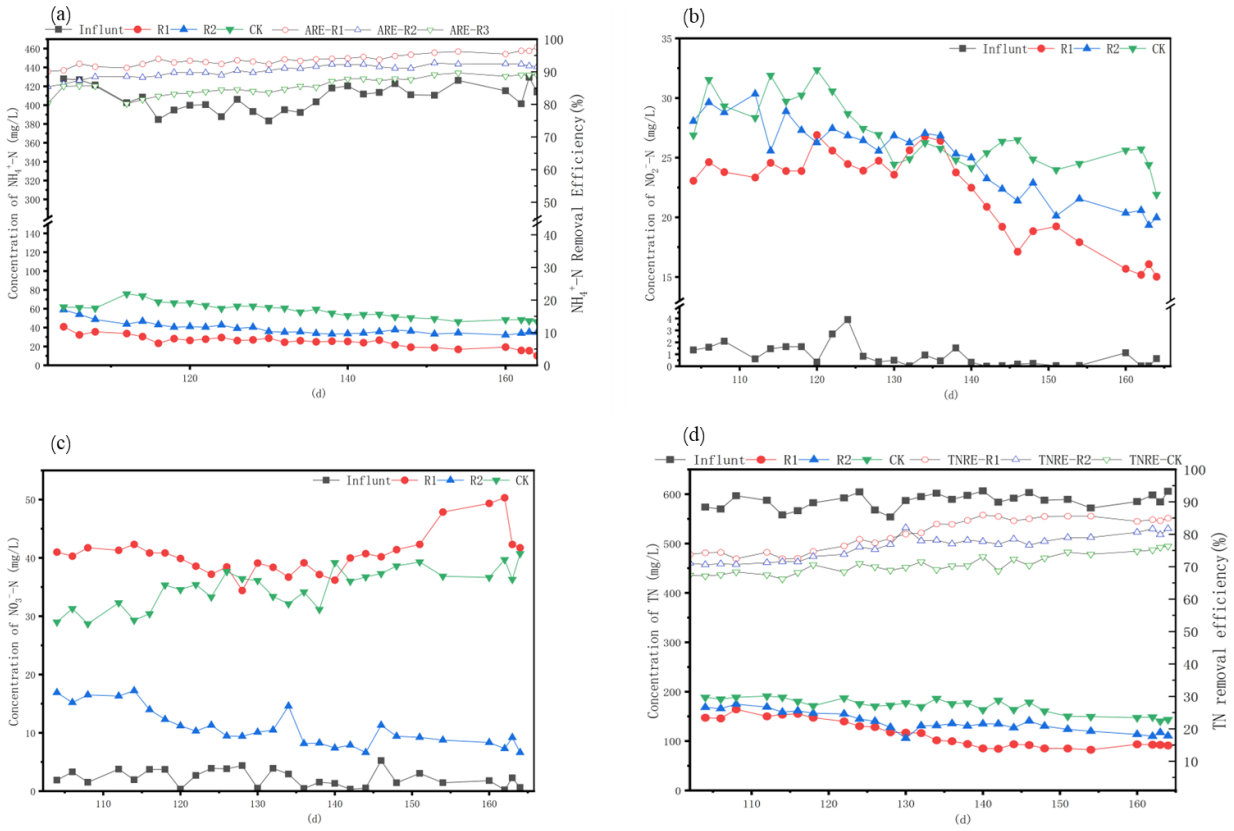

Figure 2 Nitrogen Concentration Variations in Different Reactors During Operation

The total nitrogen removal performance of all three reactors improved simultaneously. The total nitrogen removal efficiency of R1 increased from 73.80% to 84.98%, representing the most significant improvement. The overall system operated stably without obvious fluctuations, fully demonstrating that using excess sludge as an iron source added to the anammox system can effectively enhance the overall nitrogen removal performance of the system.

3.2. Effect of Iron-Rich Excess Sludge on EPS of Biofilm Sludge

Proteins and polysaccharides are important substances that maintain sludge structural stability and biological activity. Changes in their content can directly reflect the microbial metabolic level and environmental adaptability. On day 164 of the experiment, the extracellular polymeric substances of the sludge in the three reactors were analyzed. It was found that the combined effect of iron ions and different carriers synergistically regulated the anabolism and spatial distribution of EPS[4]. The total EPS content in the three reactors was highest in R1, followed by R2, and lowest in the control group CK. Compared with day 102, the EPS content increased in all groups, with R1 showing the most significant increase.

The protein content in the EPS of each group was much higher than that of polysaccharides, making protein the main component. The protein content in R1 reached 151.63 mg/g VSS, an increase of 32.7% compared to the early experimental period. R2 had a protein content of 140.34 mg/g VSS, an increase of 37.6%, and the CK group had 109.99 mg/g VSS, an increase of 47.1%. Iron ions effectively promoted microbial protein synthesis, with a more pronounced promoting effect on suspended microorganisms. The polysaccharide contents in the three groups were 95.05 mg/g VSS, 90.82 mg/g VSS, and 71.53 mg/g VSS, respectively, representing increases of 29.3%, 39.6%, and 60.8%. The increase in polysaccharide content stabilized the sludge floc structure, and together with proteins, improved sludge flocculation performance and shock resistance.

After the addition of excess sludge, the proportion of tightly bound EPS increased in all three groups. The proportion in R1 rose to 67.9%, in R2 to 66.9%, and in the CK group to 66.8%. Excess sludge promoted the conversion of EPS to the tightly bound form, compacted the internal structure of the sludge, and reduced the loss of soluble EPS. This effect was more prominent under the carrier systems. The K1 carrier facilitated full contact between microorganisms and iron ions, accelerating the accumulation of tightly bound EPS. Overall, the two reactor groups equipped with carriers had higher contents of various EPS components than the blank group, and the K1 carrier was more suitable for the iron-rich environment than the polyurethane sponge carrier, being more conducive to microbial attachment, metabolism, and EPS accumulation optimization.

Iron ions participated in microbial electron transfer, enhanced bacterial activity, and promoted EPS synthesis. They also complexed with proteins and polysaccharides to enhance their stability and flocculation performance. The carriers immobilized microorganisms[5], strengthened synergistic effects among microbial communities, and amplified the regulatory effect of iron ions. Together, they optimized the physicochemical properties of the sludge and effectively improved the overall operational performance of the anammox system.

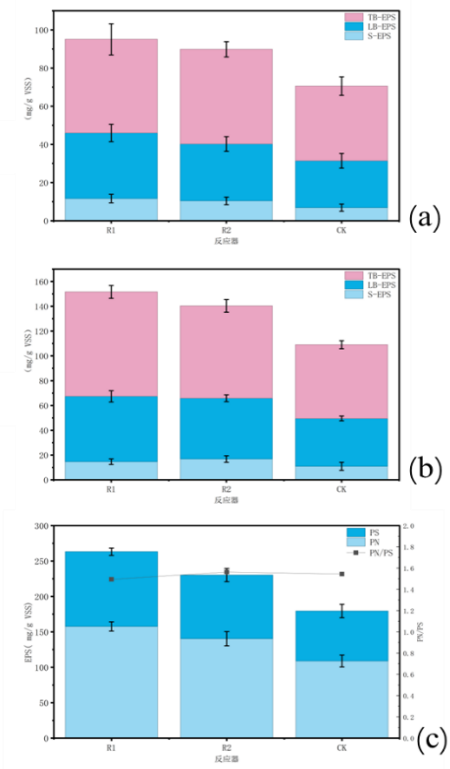

Figure 3 Protein and Polysaccharide Concentrations in Different Reactors

3.3. Differential Analysis of Heme Content as Influenced by Iron-Rich Excess Sludge

On day 164 of the experiment, the heme c content in the sludge of the three reactor groups was measured. It was found that the concentration in each group had increased compared to that before the addition of excess sludge[6]. Iron ions participated in heme synthesis and also enhanced the activity of the microbial respiratory chain, promoting the synthesis and expression of nitrogen removal enzymes. The heme c content in the CK group increased from 0.915 μmol/g VSS to 1.12 μmol/g VSS, representing an increase of 22.4%, confirming that iron enhanced the nitrogen removal activity of anammox bacteria. The content in the R2 group increased to 1.56 μmol/g VSS, an increase of 14.1%. Although the concentration was higher than that of the control group, the rate of increase was relatively low. This was attributed to the tendency of the polyurethane sponge carrier to

experience pore clogging, which hindered mass transfer and only allowed for stable enrichment of bacterial species. The heme c concentration in the R1 group reached 1.76 μmol/g VSS, with an increase of 22.5%, the highest among the three groups. This indicated that the K1 carrier, having low mass transfer resistance, facilitated the penetration of iron ions into the deeper layers of the biofilm for utilization by the microbial community, forming a synergistic interaction with iron ions and maximizing heme synthesis.

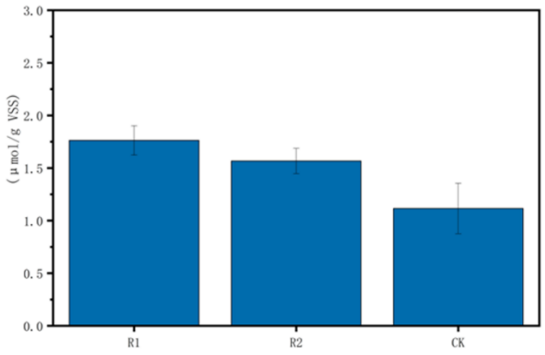

Figure 4 Concentrations of Heme c in Different Reactors

3.4. Microbial Community Structure Analysis

Table 2 Sludge microbial alpha diversity index

Samples	Sobs	ACE	Chao	Shannon	Simpson	Coverage
R1	581	581	581	4.261581	0.043971	1
R2	516	516	516	4.261996	0.038093	1
CK	727	727	727	4.735577	0.028757	1

On day 164, microbial sequencing of sludge from the three reactor groups was performed. As shown in Table 2, the Coverage index for each group was close to 1[7], indicating that the sequencing results fully reflected the actual community structure and that the analysis was highly reliable. To compare the effect of carrier type on microbial community diversity, the alpha diversity data from day 102 were also included in the analysis. The results showed that compared with the period before the addition of excess sludge as the iron source, the Sobs, ACE, and Chao indices of all reactor groups decreased. This indicated that the introduction of iron ions exerted selective pressure on the microbial community, reducing some non-dominant bacterial species, simplifying the community structure, and shifting the system from high diversity to high functionality. Previous studies have confirmed that the enrichment of specific functional microbial groups in biological treatment systems is more conducive to enhanced pollutant removal than high diversity alone.

Horizontal comparison of the data from each group after iron ion addition revealed that the species richness indices of the CK group remained higher than those of R1 and R2, and the Shannon index was also higher. However, the CK group exhibited the weakest nitrogen removal performance. This was because, although the CK group had a complex community structure, the functional bacteria were widely dispersed, lacking effective enrichment of dominant nitrogen-removing microbial groups. Moreover, the Simpson index indicated high community evenness without prominent dominant bacteria, making it difficult to form an efficient functional metabolic system, which limited nitrogen removal efficiency.

After the addition of the polyurethane sponge carrier to the R2 reactor, the alpha diversity indices were lower than those of CK but higher than those of R1, indicating that some functional bacteria were enriched to a certain extent. The adsorption capacity and porous structure of the sponge carrier provided attachment sites for microorganisms and created localized hypoxic or anaerobic microenvironments favorable for the growth of denitrifying bacteria, thus achieving better nitrogen

removal performance than CK. However, limited by structural stability and mass transfer conditions, the degree of functional bacterial enrichment in R2 was less than that in R1, resulting in moderate overall treatment performance.

The K1 carrier in the R1 reactor, owing to its large specific surface area and favorable hydraulic characteristics, formed a stable and distinctly stratified biofilm structure, creating a gradient of aerobic, hypoxic, and anaerobic microenvironments that facilitated the synergistic action of nitrifying and denitrifying bacteria. Additionally, iron ions further promoted electron transfer and enhanced denitrification efficiency. Therefore, although the species richness of R1 was lower than that of CK, its Shannon index remained at a relatively high level while the Simpson index was low. The community maintained a certain degree of diversity while enriching distinct dominant functional bacteria, ultimately achieving the best nitrogen removal performance.

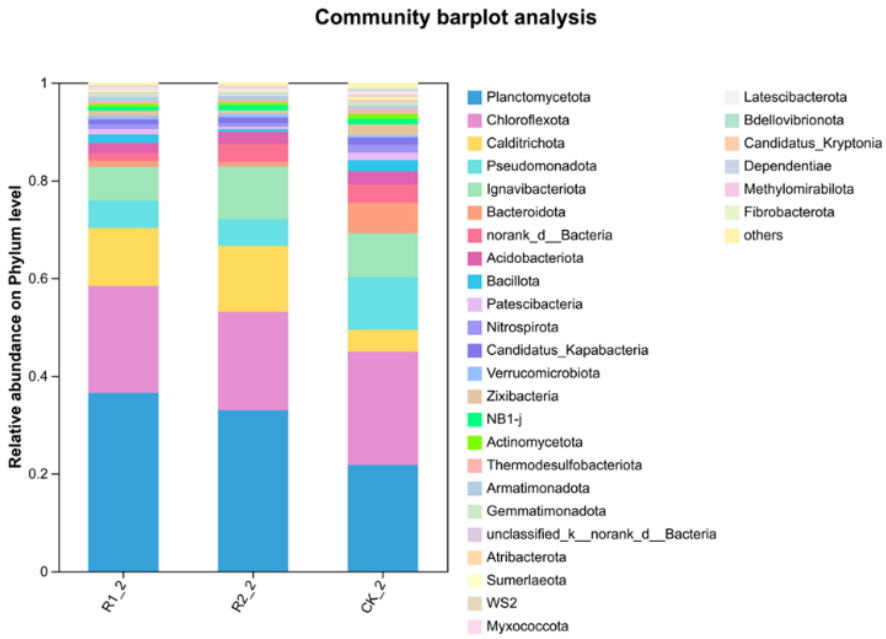

Figure 5 Microbial community structure at the phylum level in different reactors

As shown in Figure 5, the microbial community analysis at the phylum level revealed that before the addition of iron ions, Planctomycetota and Chloroflexota were the dominant phyla[8]. After the addition of iron ions, the abundance of Planctomycetota increased overall and remained dominant, with abundances of 36.53% in R1, 32.99% in R2, and 21.73% in CK. Iron ions, acting as a functional enzyme cofactor, promoted the growth of anammox bacteria. The abundance of Chloroflexota decreased slightly but remained relatively stable in R1, possibly due to substrate competition caused by the enrichment of Pseudomonadota and environmental inhibition mediated by iron ions[8]. The abundance of Calditrichota decreased, indicating reduced competitiveness. In contrast, the abundance of Pseudomonadota, which includes denitrifying bacteria and ammonia-oxidizing bacteria, increased, directly contributing to enhanced nitrogen removal performance. Acidobacteriota and Bacteroidota, which are involved in organic matter degradation and iron cycling, emerged as new dominant phyla, and the response of microbial communities to iron ions varied depending on the type of carrier used[9].

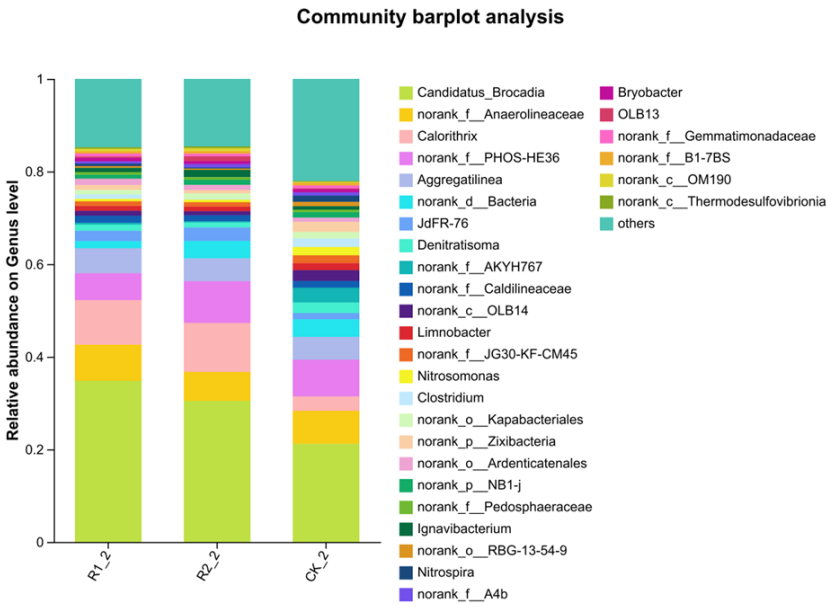

Figure 6 Microbial community structure at the genus level in different reactors

As shown in Figure 6, the results of the microbial community structure at the genus level indicated that after the addition of iron ions, the abundance of the core anammox bacterial genus increased in all three reactor groups[10]. The relative abundance of this genus was highest in R1 at 35.13%, followed by R2 at 30.48%, and lowest in CK at 21.21%. This trend in abundance was consistent with the actual nitrogen removal performance of the system. The K1 carrier created a suitable anaerobic attachment environment, and its combination with iron ions accelerated electron transfer, facilitating the substantial proliferation of functional bacteria. Related genera within the phylum Chloroflexota accounted for a relatively high proportion in each group, and as important associated bacteria, they helped maintain the structural stability of the biofilm and sludge[11]. The abundance of denitrifying bacteria in the blank group reached 2.29%, indicating that a carrier-free environment was more favorable for the growth of this group of bacteria. Additionally, two characteristic microbial groups were more abundant in R1, effectively promoting biofilm formation and the degradation of organic pollutants[12].

4. SUMMARY

(1) During the 62-day experiment in which excess sludge was added as an iron source, the K1 carrier reactor achieved the best nitrogen removal performance. The effluent ammonium nitrogen concentration remained stably below 10 mg/L, and the total nitrogen removal efficiency reached 84.98%, representing a significant improvement. The nitrogen removal performance of the other two reactors decreased successively. After the addition of iron ions, the stoichiometric ratios of all reactor groups approached the theoretical values, with the K1 carrier reactor exhibiting the most dominant anammox reaction.

(2) Iron ions effectively increased the rates of ammoniation and anammox reactions within the reactors, optimized microbial metabolic activity, and inhibited the activity of harmful microbial groups. Iron ions also increased the protein content in sludge extracellular polymeric substances, promoted the conversion of EPS to the tightly bound form, and stabilized sludge structure. The morphological characteristics of biofilms on different carrier surfaces changed in a differentiated manner. The biofilm structure on the K1 carrier was more compact, making it suitable for long-term high-load operation. Furthermore, iron ions significantly increased the heme c content in the sludge, with the most pronounced effect observed in the K1 carrier reactor, effectively accelerating electron transfer among the microbial community and enhancing microbial metabolic capacity.

(3) Exogenous iron ions reduced the overall microbial community diversity of the system while selectively enriching nitrogen-removing functional microbial groups. Iron ions effectively increased the abundance of dominant phyla and core functional genera associated with anammox. The enrichment effect of functional bacteria was most pronounced under the K1 carrier system. Iron derived from excess sludge served as an important cofactor, effectively promoting the growth and proliferation of anammox functional bacteria.

CONFLICTS OF INTEREST

The authors declare that they have no conflict of interest.

ACKNOWLEDGEMENTS

The support from the Graduate Innovation Fund of Sichuan University of Science & Engineering (Grant No. Y2024223) is gratefully acknowledged.

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