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Achieving fast start-up of anammox process by promoting the growth of anammox bacteria with FeS addition

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The effects of FeS on nitrogen removal performance and microbial community of anammox process were studied. During the start-up period, the removal efficiencies of nitrite and total nitrogen were significantly improved by FeS. The addition of FeS increased the content of iron ions in the reactor and promoted the synthesis of heme c, which was involved in the formation of various enzymes. Compared with the control, the abundance of anammox bacteria in the FeS reactor was increased by 29%, and the expression level of the *nirS* gene (encoding cd₁ type nitrite reductase containing heme) was nearly doubled. The content of nitrite reductase (ammonia-forming) in the community was increased by 26.4%. The difference in functional bacteria and enzyme contents in the microbial community resulted in a difference in nitrogen removal rate (NRR) between the two reactors. High-throughput results indicated that FeS increased the richness and diversity of microbial community and enhanced the metabolic function of the microbial community. The addition of FeS did not change the dominant position of *Ca. Kuenenia* in both reactors. But the relative abundance of heterotrophic denitrifying bacteria was reduced with FeS, which may be related to the inhibition effect of S²⁻ produced by FeS.

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INTRODUCTION

The increasing concentrations of inorganic nitrogen in surface water have had a serious impact on many aquatic organisms, leading to the deterioration of freshwater, estuaries and marine ecosystems. The discharge of wastewater containing nitrogen such as industrial wastewater and domestic sewage is primarily responsible for nitrogen pollution. The traditional biological nitrogen removal process was developed based on the action of nitrifying bacteria and denitrifying bacteria. Since the process is carried out under different conditions by various organisms, a longer hydraulic retention time (HRT) and high excess sludge production are required to achieve complete denitrification. In addition, nitrification requires oxygen and denitrification requires an adequate source of organic carbon, which increase the operating cost. As a new environmental-friendly biological nitrogen removal process, the anammox process has effectively solved the problems of low total nitrogen removal and high energy consumption in the traditional biological nitrogen removal process, and has been a research hotspot¹.

The anammox process is catalyzed by anammox bacteria belonging to the phylum of Planctomycetes, which oxidizes ammonium into nitrogen gas with nitrite as electron acceptor in the absence of oxygen and organic carbon (Eq. (1))². The activity and 16S rRNA gene sequence of anammox bacteria have been detected in various anaerobic environment¹, and there are 27 species classified as anammox bacteria so far³. However, the anammox process has not yet become the mainstream process

for treating domestic sewage due to extremely long doubling time and sensitivity to the environmental conditions of anammox bacteria⁴. Lauren et al. reported that when temperature dropped from 29 °C to 12.5 °C, the doubling time of anammox bacteria increased from 18 days to 79 days⁵. Therefore, more efforts should be devoted to shortening the startup time and improving the stability of anammox process.

Iron sulfide minerals distributed in water, shallow or tidal sands, are ubiquitous in nature. The Fe²⁺ can be released due to the acid solubility of FeS (Eq. (2))⁶. Several studies have demonstrated that anammox bacteria possessed large amounts of iron-rich “particles” inside the anammoxosome, and these iron-rich “particles” usually were Fe–S proteins and heme proteins with Fe²⁺ as a cofactor which partakes in electron transport including the transition of ammonium to dinitrogen gas^{7,8}. It has been reported that the start-up time of the anammox reactor was shortened by 20 days when the concentration of Fe²⁺ in influent was increased by a factor of three and heme c content increased significantly⁹. Several studies demonstrated that appropriate Fe²⁺ addition significantly enhanced anammox activity through improving the bacterial growth rate and accelerated the anaerobic granulation process^{9–12}. Guo et al. reported that the addition of ZVI could release iron ions, which was beneficial for promoting anammox bacteria¹³. Ma et al. documented that the heme c concentration was positively correlated with nitrogen removal rate, thus heme c could serve as an indicator to evaluate the anammox performance¹⁴. Iron element plays an important role in anammox metabolism. It is reasonable to hypothesize that the activity of anammox bacteria could be improved by the addition

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of FeS with suitable concentration.

Microbial oxidation of FeS, which links to the efficiency of denitrification, dissimilatory nitrate reduction to ammonium (DNRA) and anammox, plays an important role in the N cycle and S cycle¹⁵. Approximately one-third of the NO_3^- removal by microbial FeS oxidation in groundwater aquifer, freshwater wetland, and sediments had been reported¹⁶. FeS could function as an alternative electron donor for sulfur-dependent autotrophic denitrification. Nitrate reduction was achieved as described in Eq. (3) using pyrrhotite (Fe_{1-x}S , $0 < x < 0.125$) as the biofilter medium and autotrophic denitrifiers as the seed in lab¹⁷. And DNRA process could occur due to HS^- release (see Eq. (4))¹⁸. Moreover, according to previous reports, anammox usually integrated with denitrification in anammox reactor, forming a co-metabolic process¹⁹. The co-existence of anammox and sulfur-driven autotrophic denitrifying bacteria or sulfur-depend DNRA is also important due to possible inhibitory effect of sulfide in the water on anammox²⁰. The impact of FeS on denitrification had been studied extensively, while the roles of FeS in anammox reactor were still unknown.

Based on previous works, in the present study, the influence of FeS on anammox process was investigated with three main objectives: (1) evaluate the start-up time and performance of anammox process in a UASB reactor with/without the addition of FeS, (2) reveal the microbial community variations in anammox reactor driven by FeS, and (3) explore the influence mechanism of FeS.

RESULTS AND DISCUSSION

Effects of FeS on nitrogen removal

The start-up period could be divided into two phases based on the operating strategy of the reactor, as illustrated in Table 1. The first phase was characterized by high HRT and low substrate concentration (days 0–18), in which the HRT was 48 h and the concentrations of influent $\text{NH}_4^+\text{-N}$ and $\text{NO}_2^-\text{-N}$ were 50 and 60 mg L^{-1} , respectively. The second phase was characterized by low HRT and high substrate concentration (days 24–68), in which the HRT was 36 h and the theoretical concentrations of influent $\text{NH}_4^+\text{-N}$ and $\text{NO}_2^-\text{-N}$ were 100 and 120 mg L^{-1} , respectively.

The effluent ammonium concentration was significantly higher than that of influent at the beginning of the reactor operation shown in Fig. 1a. On the first day, the effluent $\text{NH}_4^+\text{-N}$

concentration of R1 and R2 reached 106.0 and 80.6 mg L^{-1} , respectively, nearly twice as high as the influent $\text{NH}_4^+\text{-N}$ concentration. This is mainly due to the fact that some microorganisms were unable to adapt to the new environmental conditions, inducing cellular lysis²¹. At the same time, effluent $\text{NO}_2^-\text{-N}$ concentration of R1 and R2 on the fourth day were 18.4 and 17.3 mg L^{-1} , respectively, with the removal efficiency of more than 70% (Fig. 1b); and $\text{NO}_3^-\text{-N}$ accumulated in the effluent. The high-throughput results showed that Nitrospirae, which contained massive nitrite-oxidizing bacteria (NOB), accounted for a higher proportion in the inoculation sludge (Supplementary Fig. 1)²². qPCR results also indicated that NOB abundance was higher in the inoculation sludge as shown in the section “Effect of FeS on functional bacteria abundance”. Therefore, the removal of $\text{NO}_2^-\text{-N}$ in the beginning might be attributed to the role of nitrification. Denitrification also might promote the decrease of $\text{NO}_2^-\text{-N}$ through using the organic matter which was released by decay of biomass²³. From day 7 to day 10, effluent $\text{NH}_4^+\text{-N}$ of R1 and R2 decreased rapidly from 38.1 and 49.4 mg L^{-1} to 6.8 and 6.8 mg L^{-1} , respectively, however the removal rate of $\text{NO}_2^-\text{-N}$ did not change much. From day 1 to day 18, the accumulation of $\text{NO}_3^-\text{-N}$ in R1 and R2 gradually decreased from 10 mg L^{-1} to 0 mg L^{-1} . These phenomena indicated that NOB was gradually eliminated in the low-oxygen environment and the activity of anammox bacteria was increasing. In addition, microbial metabolism and decay of biomass will release organic carbon, which can be used as carbon sources by denitrifying bacteria²³. From day 4 to day 18, the total nitrogen removal efficiency (TNRE) of R1 and R2 increased from 30.4% and 22.2% to 96.0% and 98.3%, respectively. On day 18, the values of removed $\text{NO}_2^-\text{-N}/\text{NH}_4^+\text{-N}$ and produced $\text{NO}_3^-\text{-N}/\text{removed NH}_4^+\text{-N}$ were 1.14 and 0 in R1 while these were 1.17 and 0 in R2, which was the result of the combined action of nitrifying bacteria, denitrifying bacteria and anammox bacteria.

On the 21st day, when influent $\text{NH}_4^+\text{-N}$ and $\text{NO}_2^-\text{-N}$ concentrations increased to 100.3 and 138.1 mg L^{-1} , effluent $\text{NH}_4^+\text{-N}$ and $\text{NO}_2^-\text{-N}$ concentrations of R1 increased to 6.5 and 24.2 mg L^{-1} , respectively; while those of R2 increased to 2.6 and 19.9 mg L^{-1} . On the 24th day, when HRT decreased from 48 h to 36 h, effluent $\text{NH}_4^+\text{-N}$ and $\text{NO}_2^-\text{-N}$ continued to increase. At this time, the abundance of anammox bacteria in the reactors was relatively low and had not played a dominant role. Meanwhile, the cell lysis phase was over and denitrifying bacteria activity began to decrease with the continuous consumption of organic substance²³. Therefore, the $\text{NH}_4^+\text{-N}$ and $\text{NO}_2^-\text{-N}$ removal efficiencies fluctuated widely when the nitrogen loading rate (NLR) increased. Moreover, the higher removal rate of $\text{NH}_4^+\text{-N}$ and $\text{NO}_2^-\text{-N}$ in R2 can be attributed to the promotion effect of FeS on anammox growth. On the 27th day, effluent $\text{NO}_2^-\text{-N}$ concentration of R1 and R2 reached the highest values (81.8 mg L^{-1} , 71.1 mg L^{-1}); the TNRE was the lowest, which were 52.8% and 61.0%, respectively. After this point, the $\text{NH}_4^+\text{-N}$ and $\text{NO}_2^-\text{-N}$ removal efficiencies of both R1 and R2 gradually increased and there were significant differences in total nitrogen removal capability between the two reactors. As shown in Fig. 1c, the TNRE of R2 on the 30th day increased to 73.3%; R1 achieved a TNRE of over 70% 12 days later, while the TNRE of R2 reached over 80% at this time. On the 45th day, the accumulation of nitrate appeared again in the effluent of the two reactors, meaning anammox was predominant. On the 51st day, the $\text{NH}_4^+\text{-N}$ and $\text{NO}_2^-\text{-N}$ removal in R2 reached more than 85% simultaneously, and the values of removed $\text{NO}_2^-\text{-N}/\text{NH}_4^+\text{-N}$ and produced $\text{NO}_3^-\text{-N}/\text{removed NH}_4^+\text{-N}$ were 1.12 and 0.17, respectively, closing to the theoretical stoichiometric ratio of anammox reaction, which marks that anammox reactor was started up successfully²¹. Based on Eq. (1) and the experimental data on day 51, an assumed transformation model was constructed to reflect the pathways of the nitrogen conversions in the system as shown in Supplementary Fig. 2. Due to the lack of oxygen and organic matter and the inhibition of denitrification by

Table 1. Operational conditions of R1 and R2 under different phases.

Period (day)	$\text{NH}_4^+\text{-N}$ (mg L^{-1})	$\text{NO}_2^-\text{-N}$ (mg L^{-1})	HRT (hour)
Star-up			
0–18	52 ± 3	65 ± 3	48
19–23	100 ± 10	120 ± 10	48
24–68	100 ± 10	120 ± 10	36
Stabilization			
69–84	150 ± 15	185 ± 15	36
85–97	200 ± 15	245 ± 15	36
98–120	200 ± 15	245 ± 15	24
123–150	200 ± 15	245 ± 15	12

Fig. 1 Nitrogen removal performances of R1 and R2. **a** Influent and effluent $\text{NH}_4^+\text{-N}$ concentration; **b** Influent and effluent $\text{NO}_2^-\text{-N}$ concentration; **c** Nitrogen loading rate (NLR), nitrogen removal rate (NRR), and total nitrogen removal efficiency (TNRE).

FeS, anammox played a dominant role. The same phenomenon occurred in R1 on day 56. Bi et al. studied the effect of Fe(II) concentration on the start-up of anammox process with a HRT of 12 h and found that the start-up time of anammox process could be shortened from 70 to 58 days when the concentration of Fe(II) ranged from 1.68 to 3.36 mg L^{-1} ²¹. Because the concentration of Fe(II) was relatively lower than previous study, the influence was relatively less but this method is more convenient. The heme c content at day 50 in R2 was higher than that in R1 as shown in the section “ Fe^{2+} release and Heme c content”, demonstrating that the activity of anammox bacteria in R2 was higher than that in R1. In summary, FeS effectively shortened the start-up time and improved the nitrogen removal performance.

On the 71st day, when influent $\text{NH}_4^+\text{-N}$ and $\text{NO}_2^-\text{-N}$ concentrations increased to 150 mg L^{-1} and 180 mg L^{-1} , respectively, the $\text{NH}_4^+\text{-N}$ and $\text{NO}_2^-\text{-N}$ removal rates in the two reactors decreased. On the 75th day, effluent $\text{NH}_4^+\text{-N}$ concentrations of R1 and R2 increased to 37.1 and 35.3 mg L^{-1} , meantime effluent $\text{NO}_2^-\text{-N}$ concentration increased to 93.3 and 84.8 mg L^{-1} . Although the nitrogen removal rate of the two reactors decreased obviously after the NLR was increased, it quickly recovered to the original level. As shown in Fig. 1a, b, on day 81, effluent $\text{NH}_4^+\text{-N}$ in R1 and R2 decreased to 11.1 and 7.1 mg L^{-1} and effluent $\text{NO}_2^-\text{-N}$ concentrations decreased to 16.5 and 6.2 mg L^{-1} . The TNRE increased to about 90%. This indicated that the reactors have a certain capacity in resistance to weak shock loading due to the enrichment of anammox bacteria. And, when influent $\text{NH}_4^+\text{-N}$ and $\text{NO}_2^-\text{-N}$ were further increased, effluent $\text{NH}_4^+\text{-N}$ and $\text{NO}_2^-\text{-N}$ concentrations of R2 were significantly lower than these of R1. Meantime, the responses caused by the unit intensity of shock (R) of R2 was substantially lower than these of R1 as shown in Supplementary Table 1, indicating that R2 had more resistance to shock loading rate. The same trend was observed when HRT were

further shortened to 36 h and 12 h, suggested that the stability of anammox reactors can be improved with the addition of FeS.

During the start-up period, the $\text{NO}_3^-\text{-N}$ concentration in R2 was substantially higher than that in R1 as shown in Supplementary Fig. 3, which might be attributed to the inhibition of denitrification process in R2 by FeS^{24,25}. However, in the stabilization period, the $\text{NO}_3^-\text{-N}$ concentration in R2 was substantially lower than that in R1. This was due to the lack of organic matter in R1 which inactivated denitrifying bacteria. Meantime, the presence of FeS in R2 might promote sulfur autotrophic denitrification and DNRA to reduce nitrate. The KEGG function prediction result as shown in the section “Effect of FeS on microbial community” verified this inference.

FeS structure change

The appearance of FeS with dark brown color, particle size between 1 and 5 mm and compact texture before being added to the reactor was observed (Supplementary Fig. 4). After 180 days of reactor operation, the FeS materials remaining in R2 were found to be covered with a layer of sludge. And the appearance displayed clear differences: most of the color changed from dark brown to khaki and the texture was sparse, which may be caused by the oxidation of FeS. Moreover, the red anammox granule sludge as shown in Supplementary Fig. 4 was observed in R2. Touching these red anammox granule sludge felt that the interior was relatively hard, which was made of FeS particles. FeS may promote the formation of anammox granular sludge.

To further understand the structure change, the morphology of FeS before and after reaction were observed by SEM at different magnifications. As shown in Fig. 2c, d, there were many honeycomb style holes on the surface and inside of the FeS particles after the reaction. The voids formed on the surface may facilitate the attachment of microorganisms, which acted like microbial carriers. Therefore, anammox granular sludge containing

Fig. 2 SEM of FeS. Before (a, b) and after (c–f) reaction.

FeS as inert cores formed in R2. In addition, $\text{Fe}^{2+}/\text{Fe}^{3+}$ produced by oxidation and ionization of FeS could weaken the electrostatic repulsion among negatively charged anammox cells and promote the aggregation of anammox bacteria into zoogloea by the effect of salt bridge²⁶. Thus, the addition of FeS could promote the formation of anammox granular sludge, then improve the stability of the reactor. Figure 2e, f showed that many plate-shaped secondary minerals were produced after the reaction of FeS. In the presence of dissolved oxygen (DO), O_2 can diffuse into the FeS surface and oxidize Fe^{2+} to Fe^{3+} (Eq. (5))⁶. The formation of these secondary minerals may hinder the release of iron ions from FeS^{27} .

Effect of FeS on functional bacteria abundance

The abundance of anammox bacteria in the two reactors were monitored during the period of their operation. As shown in Fig. 3a, the copy numbers of anammox 16S rRNA gene in the inoculation sludge was 3.31×10^6 copies per ng DNA. After 150 days of cultivation, the copy numbers of anammox 16S rRNA gene in R1 and R2 (1.21×10^7 , 1.42×10^7 copies per ng DNA) were significantly higher than that in the inoculation sludge. The data

demonstrate that although the content of anammox in the inoculation sludge was low, anammox bacteria can be rapidly enriched and the reactor could be properly started-up as long as the cultural conditions for anammox bacteria growth were suitable. The anammox 16S rRNA gene copy numbers of R1 and R2 were 5.68×10^6 and 7.04×10^6 copies per ng DNA on day 70, respectively. Compared with R1, the abundance of anammox bacteria in R2 was increased by 29%. The contrast in cell quantities between R1 and R2 indicated that the addition of FeS with this concentration promoted the growth of anammox bacteria. Combined with the water quality results, the faster growth rate of anammox bacteria in R2 was responsible for the higher removal efficiencies of $\text{NH}_4^+\text{-N}$ and $\text{NO}_2^-\text{-N}$ and shorter start-up time of reactor.

In addition to anammox, the contents of ammonia-oxidizing bacteria (AOB), NOB and denitrifying bacteria also affect the start-up time and nitrogen removal capacity of anammox reactor. Compared with the inoculation sludge, the expression levels of *amoA* ($\text{NH}_4^+ \rightarrow \text{NO}_2^-$) and *nirS* ($\text{NO}_3^- \rightarrow \text{NO}_2^-$) genes in both R1 and R2 were increased, while the expression levels of *Nitrospira spp.* 16S rRNA genes ($\text{NO}_2^- \rightarrow \text{NO}_3^-$) and *nirK* ($\text{NO}_3^- \rightarrow \text{NO}_2^-$) genes were decreased (Fig. 3b). The expression levels of *Nitrospira*

Fig. 3 The qPCR results of sludge samples. **a** Anammox 16S rRNA gene copy number in different period; **b** other functional genes copy number on day 70. Data indicate average, and error bars represent standard deviation of the results from three independent sampling, each tested in triplicate.

spp. 16S rRNA genes could reflect the content of NOB in anammox reactor²⁸. As anammox was cultured in an anaerobic environment, which was not conducive to the growth of NOB, the content of NOB was gradually decreased with the increase of culture time. And the expression level of *Nitrospira spp.* 16S rRNA genes in the inoculated sludge was 2.14×10^6 copies per ng DNA, which was consistent with the higher nitrite removal efficiency initially. On day 70, the expression levels of *amoA* gene in R1 and R2 were 1.34×10^4 and 2.07×10^3 copies per ng DNA, while anammox 16S rRNA gene expression level was 5.68×10^6 and 7.04×10^6 copies per ng DNA. It was clear that the content of anammox was two or three orders of magnitude higher than AOB. The qPCR results also demonstrated that the anammox bacteria were dominant after 70 days of operation, at which time the removal of ammonium nitrogen was mainly from anammox. In addition, the expression level of *amoA* gene in R2 was much lower than that of R1, and the NOB content of both reactors was higher than AOB content on day 70 (Fig. 3b). FeS could react with dissolved oxygen (DO) in the reactor, leading to an inhibitory effect on the growth of AOB⁶. But *Nitrospira-like* NOB has higher hypoxia tolerance ability than AOB. Liu et al. reported that when the reactor was operated under low oxygen conditions ($0.16 \text{ mg DO L}^{-1}$) for a long time, some of *Nitrospira-like* NOB can adapt to anaerobic environment and maintain activity²⁹. Both *nirS* and *nirK* are functional genes of denitrifying bacteria. The expression level of *nirS* gene in R2 (2.05×10^6 copies per ng DNA) was higher than that of R1 (1.11×10^6 copies per ng DNA), while the expression of *nirK* gene in R2 (3.27×10^6 copies per ng DNA) was slightly lower than that of R1 (3.65×10^6 copies per ng DNA). According to previous reports, the *nirK* gene encodes copper-containing nitrite reductase and the *nirS* gene encodes heme-containing *cd*₁ nitrite reductase which contains heme *d* as its catalytic center³⁰. And iron ions are involved in the synthesis of various types of heme. It is reasonable to speculate that the synthesis of *cd*₁ nitrite reductase in microorganisms was promoted after adding FeS into the reactor.

Fe²⁺ release and Heme c content

The effluent Fe²⁺ and intracellular heme *c* concentrations were determined and illustrated in Fig. 4. Initially, the Fe²⁺ content in the effluent of R1 and R2 was similar because FeS particles with compact texture had a smaller specific surface area (Fig. 2a, b) and released less iron ions (Fig. 4a). After the reactor was operated for a period, the effluent Fe²⁺ concentration of R2 was significantly higher than that of R1. On the 30th day, the effluent Fe²⁺ concentration of R1 and R2 were 0.132 and 1.762 mg L⁻¹, respectively. The results on days 45 and 60 also showed that

there was a significant difference in effluent Fe²⁺ concentration between R1 and R2. During this period, massive holes were corroded on the surface and inside of FeS particles as shown in Fig. 2, the specific surface area of FeS increased and the activity of FeS was higher, contributing to more release of iron ions. On day 70, the content of heme *c* in R1 and R2 was 7.2 and 11.8 μmol per g-protein, respectively (Fig. 4b). It has been reported that Fe²⁺ was involved in the formation of heme *c*, which was the active center of many enzyme proteins³¹. In many biochemical reactions, the transformation of substrate and intermediate is accomplished by the catalysis and electron transfer of c-type heme protein^{32,33}. Anammox cells contain a large amount of multi-heme cytochromes, for example one subunit of hydroxylamine oxidoreductase enzyme (HAO) binds 8 heme *c*³⁴. In this experiment, the positive correlation between Fe²⁺ and heme *c* confirmed that the concentration of Fe²⁺ in the reactor could be increased with the addition of FeS, then promoting the synthesis of heme *c*. On the 75th and 90th days, the Fe²⁺ content in the effluent of both reactors became lower, probably because the abundance of anammox bacteria increased gradually, corresponding to an increased consumption of iron ions. At the same time, the results showed that the content of Fe²⁺ in R2 effluent did not differ much from that in R1 effluent. On one hand, as the reaction progress, secondary minerals and biofilm were formed on the surface of FeS (Fig. 2), which led to a decrease in FeS activity. On the other hand, the abundance of anammox bacteria in R2 was higher than that in R1 (Fig. 3), thus more iron ions would be consumed.

Effect of FeS on microbial community

Through clustering analysis of OTU, the number of OTUs shared among samples and the number of OTUs unique to each sample can be intuitively observed. The number of OTUs shared by the R1 and R2 samples was 816, which accounted for 71.8% and 69.9% of the total OTUs, respectively; the number of OTU unique to R1 was 321 and that for R2 was 352 (Supplementary Fig. 5). The addition of FeS led to different species composition of the two communities. The shared OTUs number of R1 and R2 samples with inoculated sludge was 168, accounting for 14.8% and 14.4% of the total OTUs of R1 and R2 samples, respectively. Obviously, after domestication, the R1 and R2 samples were less similar to the inoculated sludge.

The ACE, Chao1, Simpson and Shannon listed in Table 2 are the alpha diversity indexes that reflect the richness and diversity of the community. The ACE and Chao 1 indexes are mainly used to indicate the richness of the community. In general, the larger the two index values are, the higher the richness of the community is.

Fig. 4 Effluent Fe^{2+} concentration and the content of Heme c. **a** effluent Fe^{2+} concentration; **b** the content of Heme c. Data indicate average, and error bars represent standard deviation of the results from three independent sampling, each tested in triplicate.

Table 2. The OTU numbers and bacterial diversity indices of sludge samples.

Sample	OTU numbers	ACE	Chao 1	Simpson	Shannon
SS	953	1075	1040	0.967	6.49
R1	1136	1137	1137	0.975	7.23
R2	1167	1168	1168	0.978	7.33

SS seed sludge.

Comparing the ACE and Chao1 index values of R1 and R2 samples, the richness of R2 community was higher than that of R1. The Simpson and Shannon indexes account for the richness and evenness of the community. The larger the two index values are, the higher the diversity of the community is. As shown in Table 2, the two index values of R2 samples were higher than these of R1, so the diversity of R2 community was higher. In summary, the community of R2 sample had higher richness and diversity. During the cultivation and acclimation process, some species in the seed sludge couldn't adapt to the new environmental conditions and were gradually washed out from the system. The addition of FeS reduced the tendency of some species to disappear under its role in facilitating the formation of granular sludge.

It can be seen from the results of microbial diversity analysis that the addition of FeS had a certain influence on the number of species of R1 and R2. The differences in microbial community composition at different classification levels with or without the presence of FeS were shown in Fig. 5.

The microbial community composition of R1 and R2 was similar at phylum classification level (Fig. 5a). The dominant phylum in two reactors was Proteobacteria, accounting for 40.1% and 29.6%, respectively, followed by Chloroflexi (12.5% and 14.1%). Other reports also showed there were higher contents of Proteobacteria and Chloroflexi in anammox reactor^{35,36}. The relative abundance of Planctomycetes which anammox belonged to in R1 and R2 was 9.1% and 9.9%, respectively. The values were not very high, mainly due to the small proportion of Planctomycetes in the inoculated sludge (Supplementary Fig. 1) and the slower growth rate of the anammox bacteria. The proportion of Acidobacteria in R1 and R2 showed obvious difference, with relative abundances of 7.0% and 11.9%, respectively. Several publications demonstrated that some microorganisms belonged to Acidobacteria have the ability to dissimilate iron reduction with various simple organic acids

such as acetate as alternative electron donors under anaerobic conditions^{37–39}. In addition, the relative abundance of Nitrospirae which *Nitrospira* belonged to in R1 and R2 was extremely low compared with the inoculated sludge, which was reduced from 16.58% to 0.45% and 0.15%, respectively (Supplementary Fig. 1). This result was consistent with the water quality.

Figure 5b showed the genus of the top 9 abundance in the microbial community of R1 and R2. The most abundant genus in R1 was *Halomonas*, accounting for 9.7%. Most parts of the microbes belonged to *Halomonas* were denitrifying bacteria, which could reduce NO_3^- -N to NO_2^- -N⁴⁰. *Denitratisoma* with a high relative abundance (7.3%) in R1 is also one type of denitrifying bacteria⁴¹. The proportions of *Halomonas* and *Denitratisoma* in R2 was 6.5% and 4.3%, respectively, significantly lower than these in R1. The relative abundance of *Thiobacillus*, which was the major autotrophic denitrifier detected in most sulfur-based autotrophic denitrification reactors, increased from 0.012% in R1 to 0.041% in R2 with the addition of FeS^{42,43}. The most abundant genus in R2 was *Clone_Anammox_20*, accounting for 9.0%. *Clone_Anammox_20* and *Clone_Anammox_2* are a class of microorganisms with anammox function. The most abundant anammox genus obtained in both reactors was “*Ca. Kuenenia*” and the proportion was relatively close. In order to further explore the effect of FeS on the distribution of anammox bacteria, the composition of R1 and R2 samples on *Brocadia* family classification level was analyzed. The *Brocadia* family in R1 consisted of three anammox genus, “*Ca. Kuenenia*”, “*Ca. Brocadia*” and “*Ca. Jettenia*”, accounting for 99%, 0.9%, and 0.1%, while the *Brocadia* family in R2 consisted of two anammox genus, “*Ca. Kuenenia*” and “*Ca. Brocadia*”, accounting for 98% and 2%, respectively (Fig. 5c). The dominant anammox bacteria in R1 and R2 was “*Ca. Kuenenia*”, and the proportion of “*Ca. Brocadia*” in R2 was higher than in R1. Other works have found that some of the anammox bacteria under the genus “*Ca. Kuenenia*” and “*Ca. Brocadia*” could oxidize Fe^{2+} with NO_3^- -N while anammox process occurred⁴⁴. Thus, FeS might affect the distribution of species and relative abundance of anammox genus but did not change the dominant status of the anammox bacteria in the community.

To further explore the influence mechanism of FeS on nitrogen transportation, PICRUSTs was used in this experiment to predict the contents of enzymes related to NO_2^- -N conversion based on KEGG database. As shown in Fig. 6a, nitrite can be reduced to nitrogen (NO_2^- -N $\rightarrow$ N_2) through denitrification and ammonia nitrogen (NO_2^- -N $\rightarrow$ NH_4^+ -N) through dissimilatory nitrate reduction to ammonium (DNRA), in addition to being removed by

Fig. 5 The microbial community of sludge samples at different levels on day 180. **a** Phylum level; **b** top 9 abundant genera at genus level; **c** the microbial community of *Brocadiaceae*.

Fig. 6 Prediction of community functions based on KEGG. **a** Nitrogen invertase content; **b** metabolism functions.

anammox. The nitrite reductase (ammonia-forming) content of R2 was significantly higher than that of R1, while nitrite reductase (NO-forming) and nitric oxide reductase content of R2 was lower than that of R1. It had been reported that some DNRA bacteria can conduct DNRA process with sulfide (S^{2-}) or elemental sulfur (S^0) as electron donors⁴⁵. And sulfide had an inhibitory effect on nitrous oxide reductase and nitric oxide reductase, which can inhibit the denitrification reaction, have an inhibitory effect on nitrite reductase (NO-forming) due to the accumulation of NO and promote the nitrite reduction reaction by the DNRA process^{24,25,46}. In addition, heme was involved in the formation of nitrite

reductase (ammonia-forming)⁴⁷. Robertson et al. found that the addition of Fe^{2+} improved DNRA and inhibited denitrification^{48,49}. It is postulated that the iron ions and sulfur ions released by FeS encouraged the occurrence of DNRA process and somehow decreased the denitrification reaction. Therefore, the removal rates of NO_2^- -N in the two reactors were significantly different, and the removal rates of NH_4^+ -N were similar. This may also account for the relatively low abundance of denitrifying bacteria in R2. Moreover, Fig. 6b showed the predicted metabolism function of the two reactors' communities, and the results indicated that the metabolic function of R2 was slightly higher

than that of R1. It can be seen that the addition of FeS to the anammox reactor can increase microbial metabolism.

Engineering significance

As a new type of environmentally-friendly biological nitrogen removal process, the anammox process has been a research hotspot, but it still encounters some issues to limit its wider application. Anammox bacteria are slow-growing microorganisms, and are sensitive to environmental conditions, such as salinity and organic carbon⁵⁰. Another challenge of the anammox process system is the maintenance of effluent quality since about 10% nitrate would be produced in the anammox reaction, failing to meet discharge standards⁵¹.

In this study, the start-up time of the anammox reactor was shortened and the nitrogen removal rate was significantly increased with the addition of FeS. There were mainly two reasons: On one hand, FeS promoted the formation of anammox granular sludge and increased the abundance of anammox bacteria; on the other hand, FeS stimulated the synthesis of the heme *c*, which participated in the synthesis of a variety of enzymes. In addition, FeS can promote the DNRA process by inhibiting denitrification. Microbial oxidation of FeS, which links to the efficiency of denitrification, DNRA and anammox, plays an important role in the N cycle and S cycle¹⁵. According to previous report, FeS could function as an alternative electron donor for sulfur-dependent autotrophic denitrification⁵². Nitrate reduction was achieved by using pyrrhotite as the biofilter medium and autotrophic denitrifiers as seed in lab¹⁷. And DNRA process could occur due to HS[−] release¹⁸. This study found that FeS could promote the start-up of anammox process and promote the nitrite reduction reaction by the DNRA process through inhibiting denitrification. Therefore, it is possible to couple anammox with sulfur-autotrophic DNRA or sulfur-autotrophic denitrification in full-scale application by adding FeS to improve the total nitrogen removal efficiency.

METHODS

Reactor operation condition

Two reactors, R1 and R2 were set up in this experiment. R1 was the control group; R2 was the experimental group, which operating conditions except the addition of FeS were consistent with R1. FeS was only added once during the whole experiment with a concentration of 3 g L^{−1}. The FeS was purchased from Aladdin with a purity of 99.99%.

The UASB reactor with an effective volume of 5 L was used to culture anammox bacteria (Supplementary Fig. 6). In order to distribute water uniformly, a layer of glass beads with a particle size of 4 mm was laid on the bottom of the reactor. Meanwhile, a certain number of carriers were added to the reactor to reduce the loss of the activated sludge. As shown in Supplementary Fig. 6, the unique design of the reactor allowed the carriers to be suspended in the middle of the reactor, which can effectively achieve the function of retaining sludge. In addition, the UASB reactor was equipped with an effluent recycling system, the reflux rate was maintained at 135 L d^{−1} and the up-flow velocity was maintained at about 1.12 m h^{−1}. With the progress of the experiment, the speed of the influent peristaltic pump was gradually increased to shorten the HRT as shown in Table 1. The outside of the reactor was covered with a layer of tin foil paper for protection from light. The influent was purged for 5–10 min per day using nitrogen gas with a purity of 99.9% to maintain anaerobic condition. The DO in the reactor was maintained lower than 0.1 mg L^{−1}. The reactor temperature was maintained at 32 ± 1 °C by a water bath.

Seed sludge and synthetic medium

The anaerobic granular sludge taken from a wastewater treatment plant was used as inoculation sludge and limited anammox bacteria were detected in the seed sludge. Before the sludge was transferred to the reactor, it was first washed with tap water, and then was added into phosphate buffer (pH = 7.2, 0.2 M) and sealed for overnight. About 800 mL

of inoculation sludge was added to the two reactors, resulting in an initial MLSS of 5500 ± 50 mg L^{−1} in each reactor.

The influent was synthetic wastewater composed of (NH₄)₂SO₄, NaNO₂, KHCO₃, CaCl₂, MgSO₄, KH₂PO₄ and trace element according to the literature⁵³. At the beginning of the experiment, the influent NH₄⁺-N and NO₂[−]-N concentrations were 50 mg L^{−1} and 60 mg L^{−1}, respectively, and then the concentrations were gradually increased as shown in Table 1.

Analyses of liquid samples and SEM

The effluents in R1 and R2 were sampled at certain intervals to determinate NH₄⁺-N, NO₂[−]-N, NO₃[−]-N and Fe²⁺ concentrations according to the standard methods⁵⁴.

The response caused by the unit intensity of the shock load (*R*) can be applied to characterize the impact of operational conditions on the performance of a reactor⁵⁵. The *R* was obtained from Eq. (6).

$$R = \frac{O_m - O_n}{I_i} \quad (6)$$

where *O_m* is the maximum substrate level in the effluent during the shock phase, *O_n* is the mean substrate level in the effluent under normal operation, and *I_i* is the shock concentration.

The used FeS particles were collected on day 180 from R2, then washed by absolute alcohol prior to observation using scanning electron microscopy (SEM, Hitachi S4800).

Heme *c*

Two gram of sludge samples taken from the reactors were washed with PBS (pH = 7.2, 20 mM) and transferred to the centrifuge tube, followed by the addition of PBS to 25 mL for freeze-thaw. Then, ultrasonic decomposition of samples was conducted for 15 min using an ultrasonic cell disruptor under the conditions of an output power of 500 W, an operating time of 4 s and an intermittent time of 6 s. The sludge samples after ultrasonic decomposition were centrifuged at 12000×g at 4 °C for 20 min, and the supernatant was retained as a crude enzyme extract. The content of heme *c* in the crude enzyme extract was measured from spectrophotometry of pyridine hemochrome according to the reported methods^{14,56}. Meanwhile, the protein concentration in the crude enzyme was determined by the modified Lowry method to evaluate heme *c* concentration quantitatively⁵⁷.

Microbial community analysis

Sludge samples were collected at the end of start-up period and stabilization period for quantitative PCR (qPCR) analysis. 0.5 g of sludge sample was weighed into PowerBead Tubes supplied by the PowerSoilTM DNA Isolation Kit (MO BIO Laboratories), in accordance with the manufacturer's instructions to extract the DNA in the samples. The extracted DNA was tested by microspectrophotometer to determine whether its concentration and quality met the requirements of subsequent experiments. The qPCR experiment was performed using a 20 μL reaction system consisting of 1 μL extracted DNA sample, 0.4 μL forward primer, 0.4 μL reverse primer, 10 μL SYBR Premix ExTap and 8.2 μL RNase free water. The analysis was repeated 3 times per sample for accuracy. The primers and amplification procedures for each gene were described in Supplementary Table 2.

Sludge samples were collected from R1 and R2 on day 180 for high-throughput sequencing, and the methods was consistent with our previous study⁵⁸.

Statistical analysis

The results of the experiments were demonstrated as the mean ± standard deviation using Microsoft Excel.

DATA AVAILABILITY

The data that support the findings of this study are available from the corresponding author upon reasonable request. The 16S rRNA gene sequences obtained in this study were submitted to the NCBI Sequence Read Archive (SRA) under accession numbers SAMN15871869–SAMN15871871.

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AUTHOR CONTRIBUTIONS

S.Q.N. conceived the concept, C.Z. and B.G. designed the study and methods. B.G. completed the start-up experiments. C.Z. and B.G. completed the laboratory analyses. S.Q.N., X.Z., L.R., S.A., Z.Q., Z.C., and J.H. completed the data analysis. C.Z. and B.G. composed the paper drafts and S.Q.N., X.Z., L.R., S.A., Z.Q., Z.C., and J.H. provided

comments and revisions to the drafts. C.Z. and B.G. contributed equally to this work and were co-first author.

COMPETING INTERESTS

The authors declare no competing interests.

ADDITIONAL INFORMATION

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