



An efficient method of nitrate–nitrogen removal from wastewater based on pyrrhotite autotrophic denitrification

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ABSTRACT

Wastewater treatment plant is the primary source of nitrogen discharge. Some studies have shown excessive nitrate–nitrogen concentration as the main reason for excessive total nitrogen discharge in China. To solve this problem from the sewage treatment plant, autotrophic denitrifying bacteria were added to the simulation device equipped with pyrrhotite to remove nitrate–nitrogen from wastewater. The purified strains were inoculated into the simulated wastewater. When the ratio of pyrrhotite to quartz sand was 1:1 and the influent NO_3^- -N concentration was 30.0 mg/L, the removal effects of NO_3^- -N and total nitrogen were the best, and the removal rates were 95.9% and 71.9%, respectively. Meanwhile, sulfate in the system gradually accumulated, up to 233 mg/L. To further study the removal mechanism of nitrate–nitrogen in the autotrophic denitrification process, the evolution of microbial community in the purified strain was studied by 16S rDNA sequencing. The sequencing results showed that the dominant bacteria was the genus *Sulfurimonas*, with an abundance of 66.6%, which was increased by more than 40.0% compared with some studies. This method improves the removal rate of nitrate–nitrogen and makes the total nitrogen in effluent meet the sewage discharge standard (15.0 mg/L). The results showed that autotrophic denitrifying bacteria can be screened and purified with thiosulfate as the substrate, and most of the NO_3^- -N can be reduced to N_2 using pyrrhotite as an electron donor, while successfully converting nitrate–nitrogen into N_2 and realizing the complete removal of nitrogen from wastewater. Meanwhile, autotrophic denitrification does not need additional carbon sources, which greatly reduces operational energy consumption. This study provides an efficient and low-energy nitrate–nitrogen removal method and provides a theoretical basis for removing nitrate–nitrogen in wastewater lacking organic matter.

Keywords: Anaerobic sludge; Autotrophic denitrification; Nitrate–nitrogen; Pyrrhotite; Wastewater

1. Introduction

Total nitrogen is an important index affecting the stable operation of sewage treatment plants. Nitrogen removal

mainly depends on biological metabolism. Currently, biological treatment is performed by bacteria that convert nitrate into nitrogen. Heterotrophic denitrification is a process in which microorganisms use organic carbon as

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electron donor and energy to reduce nitrate. Autotrophic denitrification, in contrast to heterotrophic denitrification, reduces nitrate by oxidizing inorganic substances, and it does not require an additional carbon source. Common electron donors include S, H₂, Fe²⁺/Fe³⁺, and S²⁻. Among them, sulfur and reduced iron, when used as denitrification substrates, have the advantages of stable pH value, good NO₃⁻ removal effect, and minimal SO₄²⁻ formation [1]. Zhu et al. [2] compared the performance of sulfur-limestone autotrophic denitrification reactor with a sulfur-siderite autotrophic denitrification reactor, and found synergistic denitrification of sulfur and iron carbonate (FeCO₃) had significant advantages. The sulfur-siderite system had a higher denitrification rate, less intermediate products (NO₂ and N₂O) accumulation, and less sulfate output. From the perspective of cost-saving, some researchers use elemental sulfur and affordable pyrite (FeS₂ and FeS) as substrates for denitrification. The results showed that sulfur and ferrous sulfide co-matrix [3] and sulfur and pyrite co-matrix [4] can both remove nitrate and keep the pH value of the system stable.

Ferrous sulfide or pyrrhotite can be used as an electron donor to remove nitrogen from wastewater. Li et al. [5] removed part of phosphorus in the water while removing biological nitrogen using pyrrhotite. Fu et al. [6] used pyrite chemical sludge with ferrous sulfide as the substrate and microorganisms to remove nitrogen from coking wastewater with low chemical oxygen demand in the reactor, and the results showed that the main functional bacteria were autotrophic microorganisms. Trouve et al. [7] compared the rates of nitrate reduction by *Thiobacillus* denitrification with autotrophic denitrification using FeS, S₂O₃²⁻, FeS₂, and S as substrates, and the order of reaction rates was S₂O₃²⁻ > FeS > FeS₂ > S. Li et al. [5] used natural pyrrhotite as biological filter material to construct pyrrhotite autotrophic denitrification biofilter (PADB), integrated with anaerobic sludge to perform autotrophic denitrification for the removal of nitrogen and phosphorus in wastewater lacking organic matter. The experiments showed that PADB can effectively remove NO₃⁻ and total nitrogen (TN) in wastewater lacking organic matter.

Currently, there are the following problems in denitrification of the sewage treatment plants in China: (1) The traditional method only converts the ammonia nitrogen in the wastewater into nitrate-nitrogen and does not completely remove the nitrogen from the system; (2) total nitrogen discharge exceeding the standard; (3) low treatment efficiency and high energy consumption. To solve these problems, this study provides an efficient and low-energy nitrate-nitrogen removal method. It mainly includes: (1) screening and purification of autotrophic denitrifying bacteria; (2) explore the influencing factors of autotrophic denitrification (substrate, influent nitrate-nitrogen concentration, and explaining the reaction principle of the experiment through the reaction equation); (3) the evolution of microbiota during enrichment is investigated and analyzed by 16S rDNA segment sequencing, further explore the reaction mechanism of autotrophic denitrification, which provided a theoretical basis for removing nitrate-nitrogen and controlling total nitrogen in the sewage treatment plant.

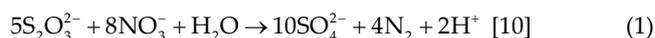
2. Materials and methods

2.1. Experimental materials

The natural pyrrhotite used in the experiment was purchased from Tongling City, Anhui Province. After crushing and sieving, pyrrhotite particles with a particle size of 0.500–1.00 mm were obtained. Soak the sieved particles in 10.0% HCl solution for 2 h to remove the oxides formed on the surface, and then wash them with deionized water 6–8 times until the pH of the washing solution is neutral. Seal and store them after drying. Anaerobic sludge is collected from the return sludge of waste treatment plant in Jinan, Shandong Province, China.

2.2. Microbial culture

To prepare a 1,000 mL liquid medium, we weighed 5.00 g Na₂S₂O₃·5H₂O, 2.00 g KH₂PO₄, 2.00 g KNO₃, 1.00 g NaHCO₃, 0.500 g NH₄Cl, 0.500 g MgCl₂·6H₂O, and 0.010 g FeSO₄·7H₂O; then dissolved and fixed the volume to 1,000 mL [5,8,9]. The medium was transferred into a 1,000 mL conical flask and we inoculated 50.0 mL of anaerobic sludge. The conical flask was sealed with a rubber plug with two glass tubes A and B. Tube A was used to flush N₂, and tube B was connected to another conical flask to collect the generated N₂. The conical flask was washed with pure N₂ for 10 min. To remove air, the tubes were sealed and cultured at 25°C. The success of the microbial culture was evaluated by the volume of gas generated (measured by the drainage gas gathering method). After four times of culturing, the gas volume of each culture was stable at about 240 mL, indicating that the microbial culture was successful. The theoretical volume of N₂ is 240 mL, which is calculated using the following formula:



2.3. Analysis method

2.3.1. Water sample analysis

Before anion analysis, the samples were filtered through a 0.22 μm syringe filter, the anion (nitrate and sulfate) was measured by ion chromatography [11], and the concentrations of total nitrogen [12] were determined by spectrophotometry.

2.3.2. Sequencing analysis of sludge

The seed sludge and cultured inoculum were stored at -80°C for follow-up experiments. The two samples were centrifuged, cleaned, and repeated 3–4 times. The supernatant was discarded, and the precipitate DNA was extracted with a soil DNA kit. The 16S rDNA gene of bacteria was amplified by polymerase chain reaction, which was then cloned and sequenced. The number of DNA samples sequenced by 16S rDNA was 2. Firstly, the total DNA of the sample was extracted and electrophoretically detected. The V3-V4 region of the gene was used as the target fragment for PCR amplification. The bacterial primer

sequences were 338F 5'-ACTCCTACGGGAGGCAGCA-3' and 806R 5'-GGACTACHVGGGTWTCTAAT-3'. The amplified products were purified, quantified, and homogenized to form a sequencing library. The constructed library was subject to library quality inspection first, and the qualified library was sequenced with Illumina HiSeq 2500 [13]. In the sequencing results, Chao and Ace's indexes simply reflect the number of species in the community, but they do not represent the abundance of each species in the community [14]. Shannon and Simpson's indexes are used to measure community diversity [14,15]. Affected by species abundance and species evenness in the sample community, Chao, and ACE indexes are used to measure community abundance. In the case of the same species abundance [13], the greater the uniformity of each species in the community, the greater the community's diversity; the larger the Chao, Ace, and Shannon indexes, the smaller the Simpson index, indicating a higher sample's species diversity.

2.3.3. Sequence composition, operational taxonomic units, and alpha diversity analysis

Autotrophic denitrifying bacteria were screened with thiosulfate as a substrate. To further study the removal mechanism of nitrate–nitrogen in the autotrophic denitrification process, the changes in sludge community structure after purification were analyzed by sequencing. Samples were collected from seeding sludge and the screened bacterial solution for microbial community structure analysis. The sequence composition, operational taxonomic unit (OTUs) number, and microbial community alpha diversity of the two samples are shown in Table 1, approximately 68,374–74,268 effective sequences were obtained, the original sequence contains primer sequences on both ends, and the optimized sequence number is reduced and filtered using cutadapt [16] (version 1.18). The corresponding number after sample clustering was in the range of 1,235–2,785. After four screenings, ACE index, Chao index, and Shannon index were lower than those of the seeding sludge, whereas Simpson index was higher than that of the seeding sludge, indicating that microbial diversity was decreasing. The changes in microbial sequence composition, OTUs number, and alpha diversity index showed that the main functional microorganisms in the screened strains were selectively enriched over time, and the microorganisms were highly selected in the autotrophic denitrification system.

2.4. Physical and chemical properties of wastewater

This study takes simulated wastewater as the treatment object. The concentration gradient of simulated wastewater was set regarding the wastewater shown in Table 2,

Table 1
Sequence composition, OTUs, and alpha diversity

Number	Sequences	OTUs	ACE index	Chao index	Shannon index	Simpson index
SS	74,268	2,785	3,412.275	3,301.835	4.943065	0.060723
TE	68,374	1,235	1,434.228	1,352.534	2.249427	0.417462

Note: SS in the table refers to seed sludge and TE refers to sludge after screening and purification.

which listed the basic physical and chemical properties of municipal wastewater.

3. Results and discussion

3.1. Influence factors of nitrogen removal in autotrophic denitrification system

3.1.1. Effect of substrate on removal rate of NO_3^- -N and TN

Fig. 1 shows the effect of substrate on the removal rate of nitrate–nitrogen and total nitrogen during autotrophic denitrification of pyrrhotite as an electron donor. It can be seen that from 0 to 144 h, the concentrations of nitrate–nitrogen and total nitrogen in three different substrate ratios gradually decreased. Among them, T₁ had the best effect, the concentration of NO_3^- -N decreased from 30.0 mg/L to 1.24 mg/L, the removal rate was 95.9%, and the concentration of TN decreased from 31.0 to 8.69 mg/L, the removal rate was 71.9%. The concentration of NO_3^- -N and TN changed relatively slowly in the first 24 h, which may be due to the growth and adaptation stage of microorganisms. After 24 h, the concentration of NO_3^- -N and TN changed faster.

In the practical application process, compared with heterotrophic denitrification, after adding autotrophic denitrifying bacteria, pyrrhotite, and quartz sand, autotrophic denitrification does not need to add carbon source and produces less sludge, which greatly reduces the treatment cost. Studies have shown that the cost of heterotrophic denitrification electron donor (methanol) was 1.05 (\$/kg nitrate) and the sludge yield was 0.71–1.2 g (biomass production per 1 g of NO_3^- -N denitrified) [17]; the cost of heterotrophic denitrification (PHBV) was 6.9–7.6 (\$/kg nitrate) and the sludge yield was lower than 0.71–1.2 g/g-N [18]. The cost of autotrophic denitrification electron donor (sulfur) was 0.43 (\$/kg nitrate) and the sludge yield was 0.15–0.57 g/g-N, the cost of pyrite-based autotrophic denitrification was 0.57 (\$/kg nitrate) and the sludge yield was 0.186 g/g-N [19]. Li et al. [5] research showed that based on the pyrrhotite price of \$100/ton, the cost of pyrrhotite consumed in the PADB was estimated as 0.01 \$/m³. According to the above research, in autotrophic denitrification, only a small amount of electron donors are added, and the cost of electron donors and sludge

Table 2
Physical and chemical properties of municipal wastewater

Index (mg/L)	NO_3^- -N	TN	PO_4^{3-} -P	F ⁻	Cl ⁻
Municipal wastewater	40.2	44.4	7.52	0.340	107

yield are only 2/1 or less of that of heterotrophic denitrification, which will not become a new burden of sewage treatment.

3.1.2. The effect of influent NO_3^- -N concentration

Fig. 2 shows the effect of influent NO_3^- -N concentration on NO_3^- -N and TN removal by autotrophic denitrification. With the increase of NO_3^- -N concentration, the reduction

amount of NO_3^- -N gradually increased and the removal rate also increased. In the first 24 h, the removal rate was relatively low because the microorganism was in the growth adaptation period. After 24 h, the removal rate increased linearly and finally tended to be flat. When the influent NO_3^- -N was 30.0 mg/L, after 144 h autotrophic denitrification process, NO_3^- -N and TN decreased to 1.24 and 8.69 mg/L, respectively, and the removal rates were 95.9% and 71.9%, respectively.

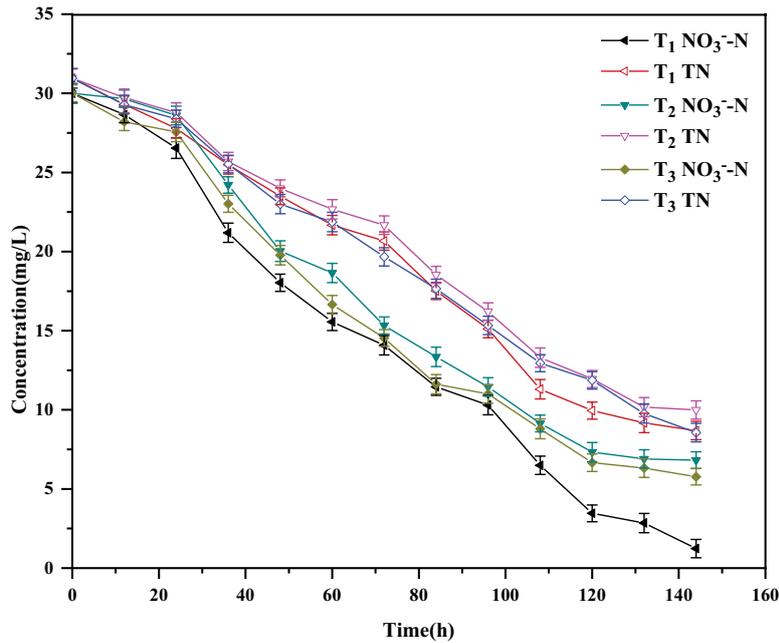


Fig. 1. Effect of substrate on the removal rate of NO_3^- -N and TN. Note: T₁: pyrrhotite:quartz sand = 1:1; T₂: quartz sand; T₃: pyrrhotite.

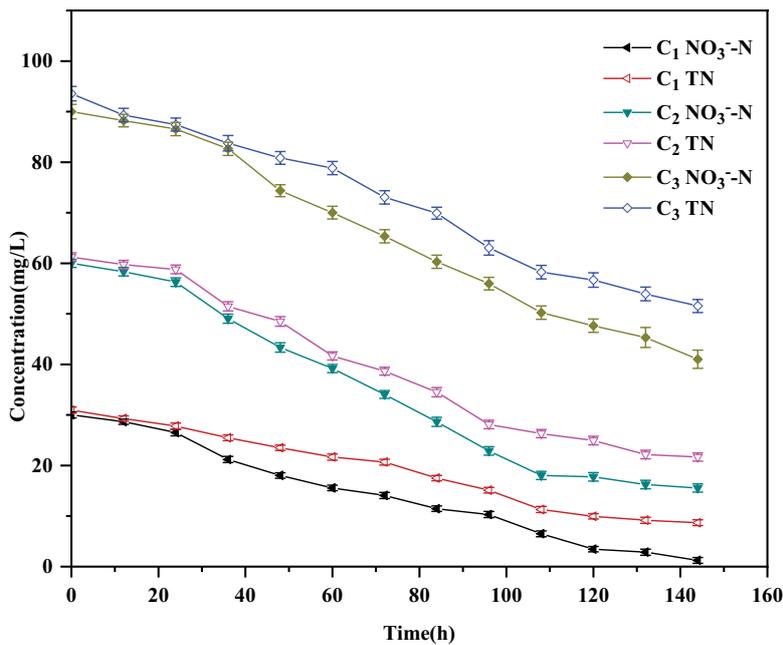


Fig. 2. Effect of influent NO_3^- -N concentration on NO_3^- -N and TN removal. Note: C₁: NO_3^- -N = 30.0 mg/L; C₂: NO_3^- -N = 60.0 mg/L; C₃: NO_3^- -N = 90.0 mg/L.

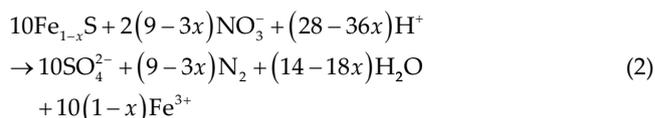
When the influent NO_3^- -N concentration was 60.0 and 90.0 mg/L, after 144 h autotrophic denitrification process, the removal rates of NO_3^- -N were 74.1% and 54.4%, respectively, and the removal rates of TN were 64.6% and 46.6%, respectively. It can be seen that with the increase of nitrate–nitrogen influent concentration, the removal rates of nitrate–nitrogen and total nitrogen showed a decreasing trend. A high concentration of nitrate–nitrogen will affect the autotrophic denitrification process, which further showed that the removal effect of experimental cultured strains on a low concentration of nitrate–nitrogen is more obvious.

The above data show that autotrophic denitrifying bacteria can be screened and purified with thiosulfate, and 95.9% NO_3^- -N can be reduced to N_2 with pyrrhotite as the electron donor. Zhang [20] found that when the initial NO_3^- -N concentration was 25.0 mg/L, the NO_3^- -N concentration continued to decrease after 12 h, from 24.9 ± 0.420 mg/L to 11.7 ± 1.08 mg/L at the end of the experiment. At this time, the NO_3^- -N removal rate was only 53.0%. Li et al. [5] found that the optimal reaction hydraulic retention time of the reactor with pyrite/dolomite as substrate was 4 d, and the removal rate of NO_3^- -N was 67.2%. Compared with them, this experiment can achieve a higher removal rate in a short time.

3.2. Sulfate accumulation during the reaction process of autotrophic denitrification

The accumulation of sulfate during autotrophic denitrification is shown in Fig. 3. The concentration of sulfate first showed a gradually increasing trend, and finally tended to be flat. In the early stage, the sulfate concentration rises rapidly, because pyrrhotite can be oxidized by oxygen to generate SO_4^{2-} , and the system started to mix with oxygen and react with the substrate to form oxide. In addition, the

substrate added to the system can react with oxygen, at the same time, the strains in the system began to consume nitrate nitrogen and produce sulfate, resulting in a high sulfate concentration of water outlet in the early stages [21,22], and the system operation tended to be stable in the later stage, with a flat sulfate production. The reaction equation of autotrophic denitrification of pyrrhotite [10] is:



The removal of nitrate–nitrogen and the production of sulfate both explain the smooth progress of autotrophic denitrification in the system, and the occurrence of the reaction [Eq. (2)] was proved. With the gradual accumulation of sulfate, the reaction accelerated gradually until the reaction was completed in about 100 h (Fig. 3), and the sulfate concentration reached the highest and remain stable. At 144 h, sulfate reached the highest level, and the sulfate concentrations in the six systems were 193 (C_1), 236 (C_2), 222 (C_3), 193 (T_1), 183 (T_2), and 183 (T_3) mg/L respectively. The maximum sulfate concentration reached 236 mg/L. In the two systems (C_1 and T_1) with the best denitrification effect, the sulfate concentration was 193 mg/L, which is lower than the standard limit of sulfate (calculated as SO_4^{2-}) 250 mg/L specified in the supplementary standard limit of the surface water source of centralized domestic and drinking water in the environmental quality standards for surface water (GB 3838-2002 replaces GB 3838-88, GHZB 1-1999), which will not pose a threat to the quality of sewage in sewage treatment.

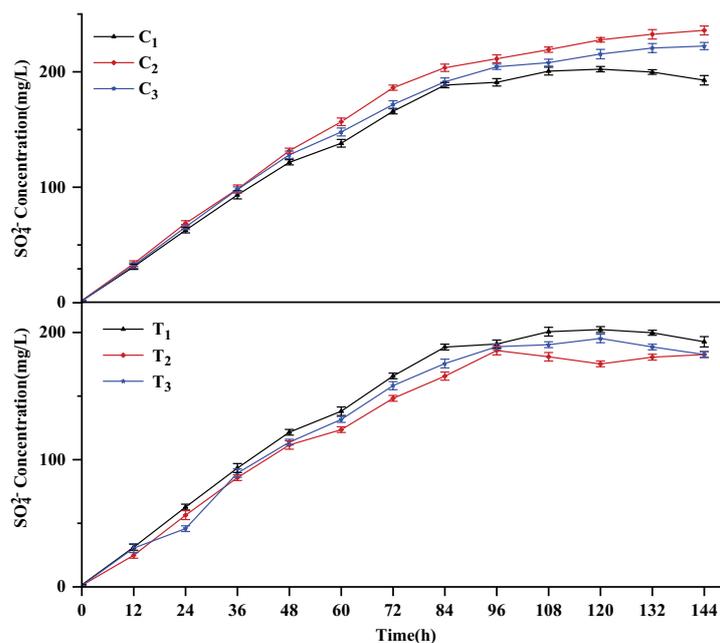


Fig. 3. The accumulation of sulfate during autotrophic denitrification. Note: T_1 : pyrrhotite:quartz sand = 1:1; T_2 : quartz sand; T_3 : pyrrhotite C_1 : NO_3^- -N = 30.0 mg/L; C_2 : NO_3^- -N = 60.0 mg/L; C_3 : NO_3^- -N = 90.0 mg/L.

3.3. Microbial community composition and removal mechanism of nitrate–nitrogen

3.3.1. Analysis of microbial community structure

We sequenced and analyzed the sludge samples before and after culture, observed the community structure of the samples at different classification levels, drew a histogram, used the histogram to show the species composition and abundance of different samples [23], and the two samples were classified and analyzed at the phylum and genus levels.

Fig. 4 shows the species' relative abundance heat map of sludge samples at the phylum level. The dominant flora of seed sludge was the phylum *Proteobacteria*, with an abundance of 55.2%, followed by the phylum *Bacteroidetes*, *Acidobacteria*, and *Actinobacteria*, with an abundance of 18.6%, 5.27%, and 3.96%, respectively. The four dominant floras accounted for 83.0% of the total community. In the screened and purified sludge, the main phylum was the phylum *Epsilonbacteraeota*, with an abundance of 67.2%, followed by the phylum *Proteobacteria*, *Bacteroidetes*, and *Acidobacteria* with an abundance of 26.8%, 2.35%, and

0.98%, respectively. The proportion of four dominant bacteria in the total community was 97.3%. Although the seed sludge and the dominant bacteria after screening and purification contain the phylum *Proteobacteria*, *Bacteroidetes*, and *Acidobacteria*, their relative abundances were different. The proportion of the screened and purified sludge in the total community was higher than that of the seed sludge, indicating that the bacteria were selectively enriched and screened under the conditions studied, and the community structure changed as well. The research results of Fu et al. [6] showed that the dominant bacterium was the phylum *Proteobacteria*, with an abundance of 44.4%. Zhang [20] found that the phylum *Betaproteobacteria* and the phylum *Epsilonproteobacteria* were the dominant floras in the system, and the corresponding relative abundances were 49.4%, 13.7%, respectively. Ma et al. [24] found that the phylum *Proteobacteria* (42.2%–51.5%) was the dominant bacteria in its operating system. In contrast, among the strains screened in this experiment, the abundance of the phylum *Epsilonbacteraeota* was the highest (67.2%), followed by the phylum *Proteobacteria* (26.8%), and the proportion of dominant bacteria was also at a higher level.

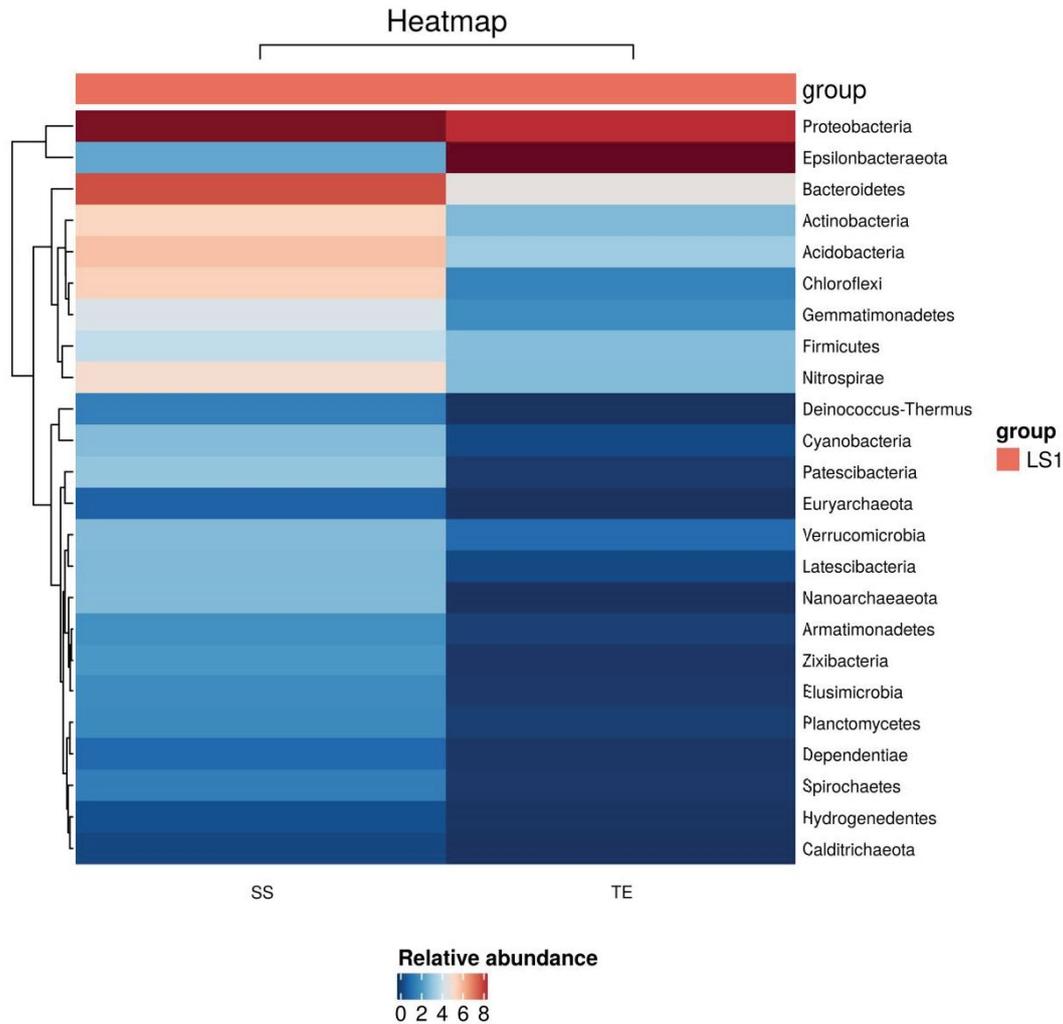


Fig. 4. Heat map of the relative abundance of sludge samples at the phylum level (SS: seeding sludge, TE: screening purified sludge).

The microbial composition of the two samples at the genus level is shown in Fig. 5. Fig. 5 shows that the genus *Thauera* was the dominant flora of seeding sludge, with an abundance of 26.8%. In addition, the genus *Truepera* (8.79%), *Nitrosomonas* (3.75%), and *Limnobacter* (2.18%) were also the main flora in the seeding sludge sample, accounting for 41.5% of the total community. Compared with seeding sludge, the genus *Sulfurimonas* was the dominant flora in the screened inoculated sludge, with an abundance of 66.6%, followed by the genus *Thiobacillus*, *Halothiobacillus*, and *Thiomonas*, accounting for 9.43%, 3.92%, and 1.89%, respectively, which is more than 80.0% of the total community. The relative abundance of seeding sludge was significantly different from that of screened sludge (41.5% vs. 81.5%), and the main groups of seeding sludge were diverse at the genus level.

Compared with the study of Yang et al. [25], the dominant bacterial group of sludge after screening was the genus *Sulfurimonas*, whereas in the study of Yang et al. [25], the dominant bacterial group was the genus *Thiomonas*, with an

abundance of 55.0%, and no trace of the genus *Sulfurimonas*. Among the strains cultured by Fu [26], the dominant strain was the genus *Thiomonas*, with an abundance of 51.3%, and the abundance of the genus *Sulfurimonas* was very low. Compared with them, we successfully screened the genus *Sulfurimonas* with a higher abundance and a higher proportion of bacteria. The genus *Sulfomonas* and genus *Thiobacillus* are sulfur-oxidizing bacteria used to reduce NO_3^- -N. They are typical autotrophic denitrifying bacteria that reduce nitrate while oxidizing sulfur or sulfide, and their denitrification performance is superior to that of conventional denitrifying bacteria [27]. Traditional denitrifying bacteria require an additional carbon source to the nitrogenous wastewater with low organic matter for denitrification reaction, and the sludge yield is high [28]. Compared with conventional denitrifying bacteria, autotrophic denitrifying bacteria can reduce nitrate by oxidizing inorganic matter in wastewater lacking organic matter without adding carbon source, and the sludge yield is low, which greatly reduces the cost of sewage treatment.

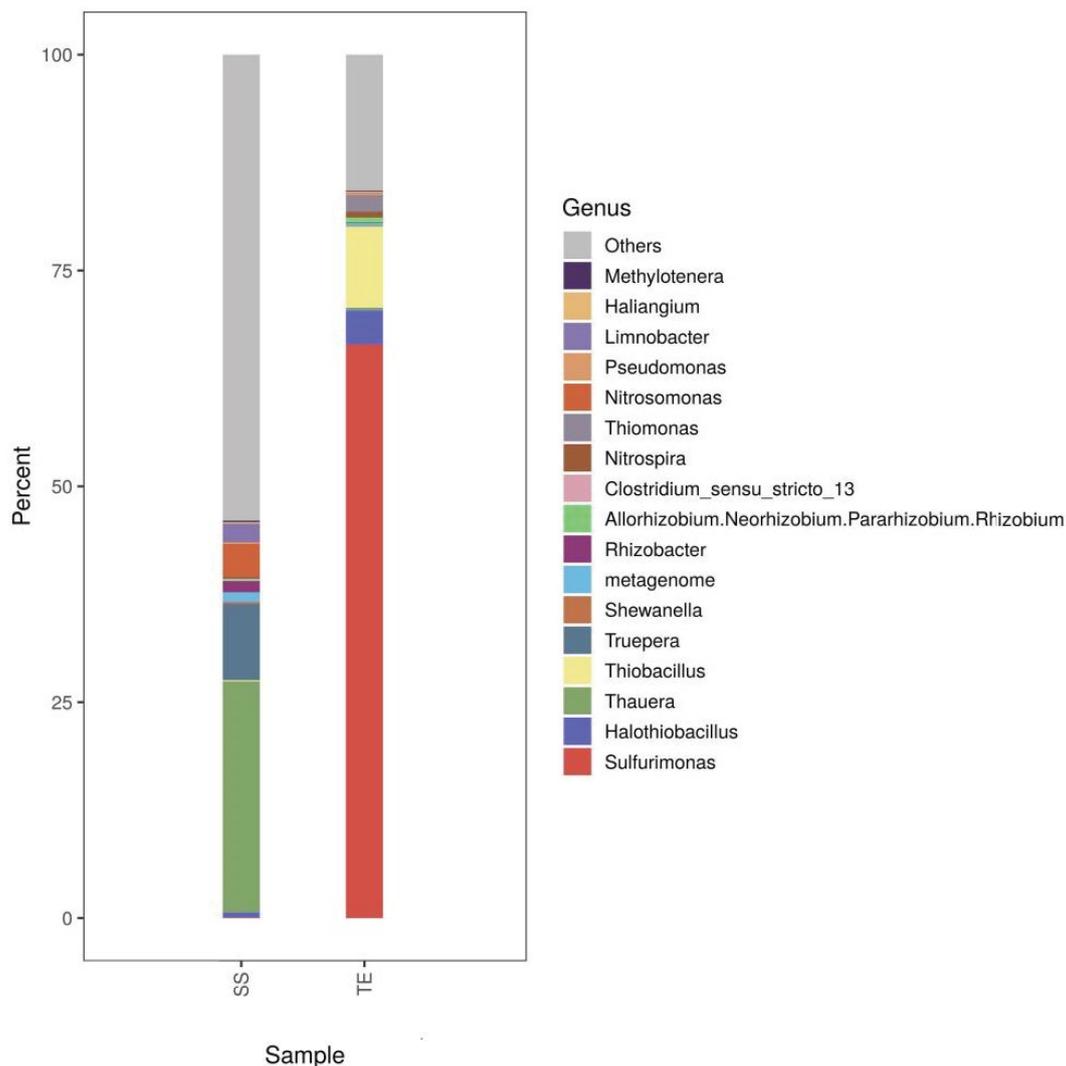


Fig. 5. Community structure at the genus level (SS: seeding sludge, TE: screening purified sludge).

Fig. 6 shows that the abundance of the genus *Sulfurimonas* and the genus *Thiobacillus* was the highest in the screened sludge, indicating that microorganisms have been growing in the medium containing thiosulfate for a long time, and their community structure changed significantly, indicating that the abundance of bacteria was induced via pollutants or nutrients, and transformed into the bacteria we need. The change in the microbial living environment made the flora in the system compete with each other. The change in environment promoted the continuous growth and reproduction of microorganisms suitable for the environment while squeezing the living space of other species. These bacteria gradually became the dominant flora in the system, and the unsuitable flora was gradually eliminated. The change of environment promoted the development of dominant flora in sludge, and community succession occurred [26].

From the above results and analysis, it can be seen that the dominant bacteria in the screened sludge were the genus *Sulfurimonas* and *Thiobacillus*, which played a major role in the denitrification system. Among them, the genus *Sulfurimonas* and *Thiobacillus* were gram-negative bacteria. These two microorganisms were typical sulfur-oxidizing nitrate-reducing bacteria, which belong to

chemoautotrophic microorganisms. They reduce nitrate while oxidizing sulfur or sulfide. They are currently widely used sulfur-oxidizing bacteria for reducing NO_3^- -N, which were used for sulfur autotrophic denitrification to treat NO_3^- -N in municipal sewage and groundwater [29–31]. The genus *Sulfurimonas* were globally distributed and especially predominant in deep-sea hydrothermal environments, the elemental sulfur reduction is quite common in different species of genus *Sulfurimonas*, their optimum growth temperature and pH were 4.0°C–45°C and 4.5–9.0, respectively [32]. The genus *Thiobacillus* belongs to the *Gammaproteobacteria* class. It is a typical chemoautotrophic denitrifying bacteria. It can use pyrrhotite as an electron donor for denitrification under anaerobic conditions [33], and it can use Fe (II) for autotrophic denitrification in addition to sulfide [33]. When the pH is 2.0–8.0 and the temperature is 20°C–50°C, it is most favorable for its growth. The genus *Halothiobacillus* is gram-negative chemoautotrophic bacteria, which appears alone or in pairs, can obtain energy from redox inorganic sulfur compounds, and is a halophilic microorganism. Its optimum growth temperature is 28°C–40°C, and its optimum pH is 6.0–8.0 [34,35]. The genus *Thiomonas* can grow heterotrophically

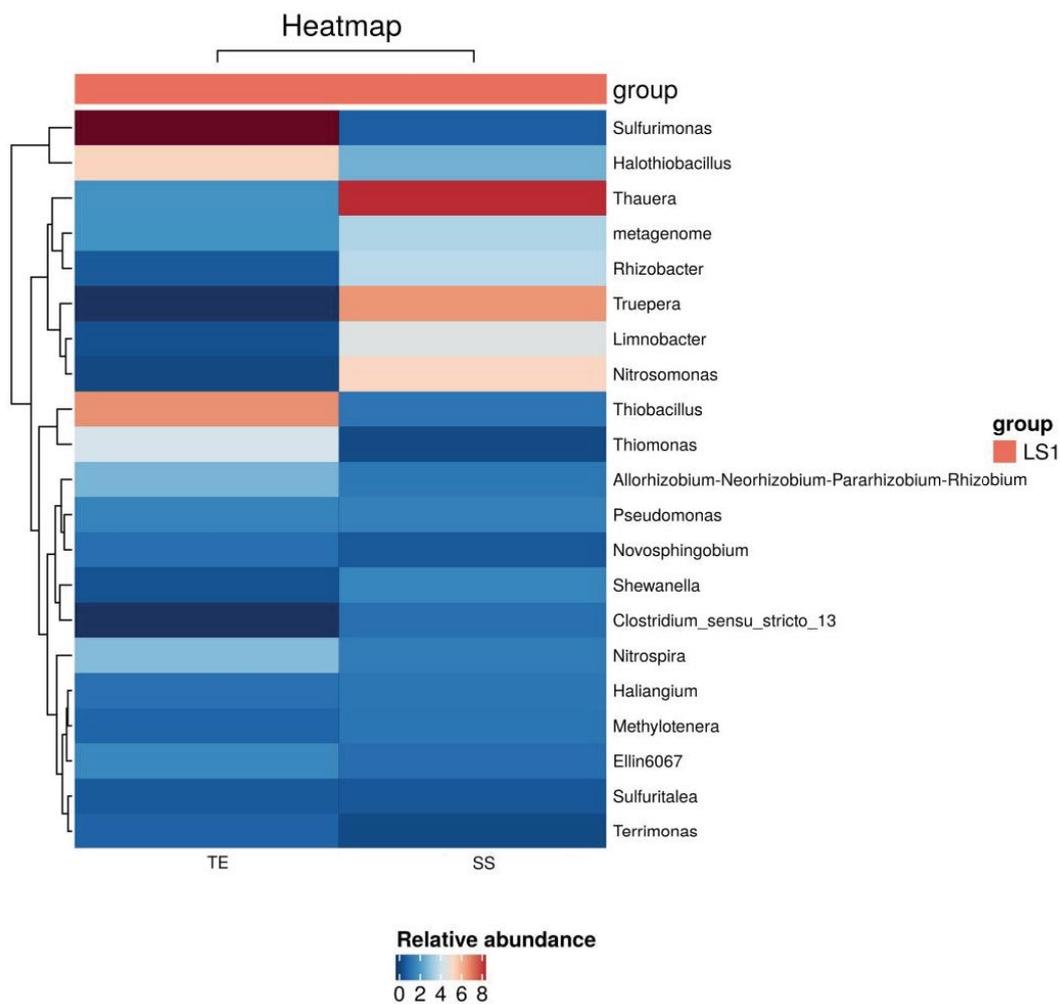


Fig. 6. Relative thermal abundance of sludge samples (SS: seeding sludge, TE: screening purified sludge).

and autotrophically, as well as in mixed cultures. It can grow autotrophically when pH is in the range of 2.3–9.0, optimum growth occurred at pH 7.5–8.0 and 30°C–37°C [36,37]. In this study, autotrophic denitrifying bacteria were cultured under neutral conditions at room temperature, which met the growth conditions of the above dominant bacteria.

3.3.2. Microbial communities shifts before and after autotrophic denitrification

Fig. 7 shows shifts of the main microbial communities before and after autotrophic denitrification. With the progress of autotrophic denitrification, the relative abundance of the genus *Sulfurimonas* in each system decreased significantly from 66.6% to 31.1%, 30.7%, 32.2%, 31.1%, 29.7%, and 33.9% respectively. The relative abundance of the genus *Thiobacillus* showed an increasing trend, increasing from 9.43% to 15.6%, 16.8%, 33.4%, 15.6%, 31.8%, and 20.5% respectively, this indicated that the genus *Sulfurimonas* can adapt to various environments, and the genus *Thiobacillus* was more acclimated to higher concentrations of $\text{NO}_3\text{-N}$ in the presence of pyrrhotite. Beller et al. [38] also founded that the genus *Thiobacillus* has strong catalytic ability under anaerobic conditions, and can reduce nitrate–nitrogen by oxidizing minerals and Fe(II) as electron donors.

In addition to the C_3 system, the abundance of the genus *Halothiobacillus* has increased to a certain extent in other systems, among which T_1 (23.3%), T_2 (22.1%), and C_1 (23.3%) have the fastest growth. The abundance of the genus *Halothiobacillus* had been greatly improved, indicating that the genus *Halothiobacillus* is more adaptive to pyrrhotite than to $\text{Na}_2\text{S}_2\text{O}_3$. Like the genus *Sulfurimonas*, the genus *Thiomonas* showed a decreasing trend, but the abundance of the genus *Thiomonas* was very low before and after the reaction. The above data showed that in the autotrophic denitrification system, the genus *Sulfurimonas*, *Thiobacillus*, and *Halothiobacillus* played a major role in denitrification. It was in agreement that the genus *Thiobacillus* was the key player in the PAD based on synthetic pyrrhotite [39], and this was consistent with the fact that the genus *Sulfurimonas* was the main autotrophic denitrifying bacteria in the process of pyrite autotrophic denitrification [10,22].

4. Conclusions

Autotrophic denitrifying bacteria were screened and purified with thiosulfate as the substrate. The purified strains were inoculated into the simulated wastewater. When the ratio of pyrrhotite to quartz sand was 1:1 and the influent $\text{NO}_3\text{-N}$ concentration was 30.0 mg/L, the removal effects of $\text{NO}_3\text{-N}$ and TN were the best, and the removal rates were

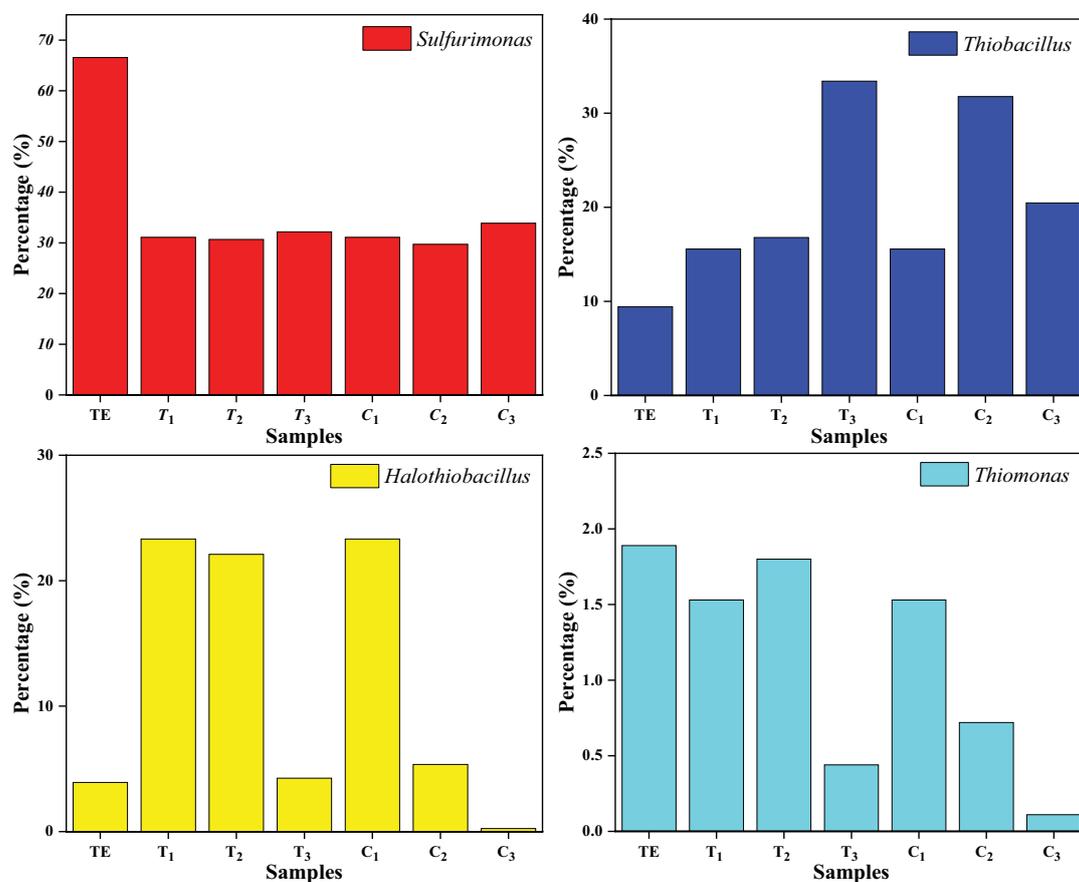


Fig. 7. Shifts of main bacterial groups on genus levels. Note: TE: inoculated sludge sample, T₁, T₂, T₃, C₁, C₂, C₃: sludge sample after T₁, T₂, T₃, C₁, C₂, C₃ system reaction.

95.9% and 71.9%, respectively. Simultaneously, In the process of autotrophic denitrification, sulfate in the system gradually accumulated, up to 233 mg/L. In the screened and purified sludge, the main bacteria were the phylum *Epsilonbacteraeota* and *Proteobacteria*, with abundances of 67.2% and 26.8%, respectively. The genus *Sulfurimonas* and *Thiobacillus* were the dominant bacteria in the final enrichment products, with abundances of 66.6% and 9.43%, respectively.

The influencing factors of autotrophic denitrifying bacteria during denitrification can be further investigated, and more materials that can be used as electron donors can be found to compare their properties and screen out the best combination. The next step is to investigate the possibility of applying autotrophic denitrifying bacteria in engineering practice.

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Contributions

T L (Master student) conducted all the experiments and wrote the manuscript. CS Z (Ph.D.) Methodology, Revised the manuscript, Funding acquisition. QF C (Ph.D.) Supervision, Funding acquisition. LZ L (Master student) Investigation, Analysis of the manuscript. L L (Master student) Data curation, Investigation. GR S (Master student) Data curation. BB G (Master student) Analysis of the manuscript.

Availability of data and materials

The datasets and materials used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare no competing interests.

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