High-Efficiency Mixotrophic Denitrification for Nitrate Removal in High-Sulfate Wastewater Using UASB Reactor

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Abstract: The efficient removal of nitrate from industrial wastewater containing high concentrations of both sulfate and nitrate presents a major challenge in the field of water treatment. In this study, we investigated the use of an Upflow Anaerobic Sludge Blanket (UASB) reactor for the removal of nitrate from wastewater by gradually increasing the sulfate concentration (ranging from 1 g/L to 10 g/L) and the NO₃⁻-N concentration (ranging from 30 mg/L to 300 mg/L). Through this approach, the activated sludge was successfully acclimated to tolerate high-sulfate conditions. The results demonstrated a remarkable NO₃⁻-N removal capacity of 288 mg/L·d in wastewater with a high sulfate concentration of 10 g/L, leading to a nitrate removal efficiency exceeding 96.0%. The analysis of sulfate and sulfide concentrations, as well as the characterization of the microbial community, revealed the occurrence of autotrophic and heterotrophic denitrification processes in the reaction system. The autotrophic denitrifying bacteria found were Raoultella and Shinella, while the heterotrophic denitrifying bacteria included Klebsiella, Simplicispira, and Thauera. The organic carbon sources were found to be a critical factor influencing the denitrification performance of the system. Furthermore, the effects of different chemical oxygen demand (COD)/SO₄²⁻ ratios (0.3, 0.5, and 1) were examined in wastewater containing a sulfate concentration of 10 g/L and a NO₃⁻-N concentration of 300 mg/L. The results showed that increasing the COD/SO₄²⁻ ratio enhanced the removal rate of NO₃⁻-N, maintaining it above 98.0% when COD/SO₄²⁻ was 1. Additionally, the enhancement of the sulfate reduction reaction in the system was observed, and the enrichment of heterotrophic microorganisms such as Megasphaera, Lactobacillus, and Buttiauxella was observed.

Keywords: UASB process; high-sulfate wastewater; nitrate removal; mixotrophic denitrification; microbial community

1. Introduction

In various industries, such as thermal power plants, chemical industries, and printing and dyeing industries, the wastewater generated during production processes often contains high concentrations of sulfate and nitrate [1–3]. The discharge of untreated wastewater containing nitrate can have severe ecological consequences and pose a significant threat to human health [4,5]. In addition, stringent sewage discharge standards have been established to reduce the emission of nitrate and other nitrogen compounds [6]. However, due to the high salinity of sulfate in the wastewater and the potential formation of toxic by-products during the treatment process, the removal of nitrate from high-sulfate wastewater has remained a major challenge in this field.

Biological nitrogen removal has become the most widely used method for treating nitrogen-containing wastewater due to its economic feasibility, simplicity, and absence of secondary pollution compared to physical and chemical nitrogen removal methods [7]. Heterotrophic denitrification is the most common method for nitrate removal, where denitrifying bacteria utilizes organic matter as an electron donor during anaerobic respiration to...
reduce nitrate to nitrite and then further to nitrogen gas [8,9]. Currently, there are studies exploring autotrophic denitrification for nitrate removal using sulfide as an electron donor in wastewater [10,11]. However, these studies have been limited to wastewater that contains a certain amount of sulfide and cannot be applied to sulfide-free wastewater treatment.

Sulfate-reducing bacteria are a group of microorganisms that utilize sulfate as an electron acceptor to carry out metabolic activities using energy derived from the oxidation of organic matter [12]. These bacteria can be easily enriched when treating sulfate-containing wastewater. Sulfate-reducing bacteria reduce sulfates to sulfides, which create conditions favorable for autotrophic denitrification. Meanwhile, sulfides are toxic and corrosive, posing risks to microorganisms and the environment, and reducing sulfide concentrations in the reactor is beneficial for wastewater treatment. Hence, the combined use of autotrophic and heterotrophic denitrification methods may provide an effective approach for nitrate removal from sulfate-rich wastewater.

Sulfate-reducing bacteria are heterotrophic microorganisms that compete with heterotrophic denitrifying bacteria for substrates [13,14], which can affect nitrate removal efficiency. However, the enrichment of sulfate-reducing bacteria may enhance the autotrophic denitrification process by increasing sulfide concentrations. Thus, the influent COD/SO₄²⁻ ratio could be a crucial factor affecting nitrate removal from wastewater.

The treatment of saline wastewater has always been a hot topic in the field of wastewater treatment. This is because salinity will have adverse effects on microorganisms, such as resulting in the separation of microbial cell fluid in wastewater, thereby reducing and destroying enzyme activity [15]. Therefore, the high salinity of sulfate in wastewater can have adverse effects on the biological removal of nitrate. In addition, sudden increases in salinity in the influent wastewater can lead to the release of intracellular components, further affecting microbial activity. However, gradually increasing the salinity level of the influent can allow microorganisms to adapt to the high-salinity environment and mitigate the impact of high salinity on the reaction system [16]. While Zhang et al. [17] achieved a nitrate removal capacity of 134.6 mg/L·d for wastewater containing a sulfate concentration of 300 mg/L using a membrane bioreactor, there is currently a lack of research on the denitrification of wastewater with high sulfate concentrations, specifically at 10 g/L.

UASB process is a commonly used anaerobic treatment method. Liu et al. [18] successfully removed nitrate from water using sulfide as an electron donor in a UASB reactor. The UASB reactor, as an oxygen-free closed system, can capture and remove toxic gases such as hydrogen sulfide produced during the reaction process, reducing their impact on the environment and human health [19,20]. Therefore, it is suitable for the treatment of sulfate-rich wastewater in this study.

The objectives of this study were as follows: (1) to gradually acclimate the activated sludge to tolerate high sulfate conditions by increasing the sulfate and nitrate nitrogen concentrations in the influent, and assess the feasibility of this method for efficiently removing nitrate from sulfate wastewater; (2) to analyze the changes in sulfate and sulfide concentrations and characterize the microbial community structure to determine the occurrence of autotrophic and heterotrophic denitrification in the reaction system; and (3) to explore the influence of organic matter on nitrate removal efficiency and sulfate and sulfide concentrations in the reaction system by varying the influent COD/SO₄²⁻ ratio.

2. Materials and Methods

2.1. Reactor Design and Operation

As shown in Figure 1, the reactor used in this study was a methyl methacrylate vessel with a total effective volume of 9.568 L and a height of 200 cm. The reaction zone had a diameter of 6 cm, a height of 150 cm, and a volume of 4 L. The settling zone had a diameter of 15 cm, a height of 35 cm, and a volume of 5.568 L. The reactor was equipped with 8 sampling ports spaced 18 cm apart. The temperature was maintained at 35 °C using a heating rod and a temperature controller. The influent was pumped from the bottom of the reactor, and a return port near the three-phase separator facilitated internal
circulation. The reflux ratio was set at 30:1, the influent flow rate was 6.9 mL/min, and the hydraulic retention time (HRT) was 24 h. The flow and circulation of wastewater in the reaction system, as well as flow rate control, were achieved using a peristaltic pump (BT100-1L, Longer Pump). Gases produced in the reactor were absorbed using 2 mol/L sodium hydroxide solution.

![Figure 1. UASB reactor and process flow diagram.](image)

2.2. Properties of Synthetic Wastewater and Seed Sludge

The wastewater used in this study was flue gas desulfurization and denitrification wastewater obtained from a thermal power plant, containing 7.6–10 g/L of sulfate, 15–56 mg/L of COD, and 16.8–300 mg/L of nitrate and nitrite. Due to the significant fluctuations in pollutant concentrations in actual wastewater, synthetic wastewater was employed in this research to maintain stable pollutant levels and enhance reproducibility. In addition, the COD concentration in the actual wastewater is low, which is not enough to maintain heterotrophic nitrification, so a certain amount of carbon source is added in the experiment process. The synthetic wastewater consisted of anhydrous glucose as the carbon source, KNO₃ as the source of NO₃⁻-N, and anhydrous Na₂SO₄ as the source of sulfate.

The experiment was divided into two stages: the salt-tolerant acclimation stage and the COD/SO₄²⁻-promotion stage. The salt-tolerant acclimation stage was divided into five phases: from days 0 to 14, the influent sulfate and NO₃⁻-N concentrations were 1 g/L and 30 mg/L; from days 14 to 28, the influent sulfate and NO₃⁻-N concentrations were 2 g/L and 100 mg/L; from days 28 to 42, the influent sulfate and NO₃⁻-N concentrations were 3 g/L and 200 mg/L; from days 42 to 56, the influent sulfate and NO₃⁻-N concentrations were 5 g/L and 300 mg/L; and from days 56 to 84, the influent sulfate and NO₃⁻-N concentrations were 10 g/L and 300 mg/L. The COD/SO₄²⁻-promotion stage was divided into three phases: from days 0 to 14, the influent COD concentration was 3000 mg/L with a COD/SO₄²⁻ ratio of 0.3; from days 14 to 28, the influent COD concentration was 5000 mg/L with a COD/SO₄²⁻ ratio of 0.5; and from days 28 to 42, the influent COD concentration was 10,000 mg/L with a COD/SO₄²⁻ ratio of 1. The main pollutant concentrations in synthetic wastewater at each stage are shown in Table 1. To meet the growth and metabolic requirements of microorganisms, 65.81 mg KH₂PO₄ and 1 mL of trace element solution were added to every 1 L of synthetic wastewater. Per liter trace elements consisted of 25 g MgSO₄·7H₂O, 5.5 g MnCl₂·4H₂O, 0.5 g CaCl₂, 0.68 g ZnCl₂, 1.2 g CoCl₂·6H₂O, 1.2 g NiCl₂·6H₂O, 4 g FeCl₂, 1.2 g H₃BO₃, and 1.2 g (NH₄)₆Mo₇O₂₄·4H₂O. NaHCO₃ was added...
into the water to adjust the alkalinity and pH was stabilized at around 7.6 in the influent. The reactor maintained a dissolved oxygen (DO) level of 0–0.2 mg/L, a pH of 7.6 ± 0.2, and a temperature of 35 °C throughout the operation.

Table 1. Main pollutant concentrations in synthetic wastewater at each stage.

<table>
<thead>
<tr>
<th></th>
<th>0–14 d</th>
<th>Salt-Tolerant Acclimation Stage</th>
<th>COD/SO\textsubscript{4}\textsuperscript{2−} Promotion Stage</th>
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<tr>
<td></td>
<td>14–28 d</td>
<td>28–42 d</td>
<td>56–84 d</td>
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<td>Sulfate (g/L)</td>
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<td>NO\textsubscript{3}−-N (mg/L)</td>
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<td>100</td>
<td>300</td>
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<td>COD (mg/L)</td>
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The activated sludge used as seed sludge in the reactor was obtained from the methane-producing granular sludge at the Jiangdong Sewage Treatment Plant in Ningbo, China. The obtained sludge was initially washed with distilled water to remove inorganic particles and fine flocs, followed by inoculation into the reactor and cultivation using synthetic wastewater without salt. The initial mixed liquor volatile suspended solids (MLVSS) concentration was 3.436 g/L.

2.3. Analytical Methods

Water samples were collected every 2 days to analyze various water quality indicators. The influent and effluent were filtered with a 0.22 μm filter membrane to determine the concentrations of NO\textsubscript{3}−-N, NO\textsubscript{2}−-N, COD, S\textsuperscript{2−} and SO\textsubscript{4}\textsuperscript{2−} according to the standard methods [21]. Specifically, a spectrophotometer (756S, Shanghai Lengguang Technology Company) was used to measure the concentrations of NO\textsubscript{3}−-N, NO\textsubscript{2}−-N, S\textsuperscript{2−}, and SO\textsubscript{4}\textsuperscript{2−}, while the COD concentration was determined using the potassium dichromate method. Total nitrogen (TN) was calculated as the sum of NO\textsubscript{3}−-N and NO\textsubscript{2}−-N.

2.4. Microbial Community Analysis

Sludge samples were collected from the reactor at the end of the salt-tolerant acclimation stage and the end of the COD/SO\textsubscript{4}\textsuperscript{2−} promotion stage. The samples were pretreated and subjected to high-throughput sequencing by Shanghai Bioengineering Company. The whole process consists of the following steps: (1) Extraction of DNA. (2) The extracted DNA used primers 515F (5′-GTGCCAGCMGCCGCGGTAA-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) to amplify the V4 region of the 16S rRNA gene. (3) High-throughput sequencing was performed on Illumina MiSeq platform.

3. Results and Discussion

3.1. Salt-Tolerant Acclimation Stage

3.1.1. Changes in Nitrogen Concentration

Figure 2a shows the changes in NO\textsubscript{3}−-N concentration and the removal rate in the reactor during the salt-tolerant acclimation stage. From days 0 to 14, with an influent NO\textsubscript{3}−-N concentration of 30 mg/L and a sulfate concentration of 1 g/L, the removal rate of NO\textsubscript{3}−-N exceeded 93.3% after day 4, indicating the excellent nitrate removal performance of the reaction system. From days 14 to 28, NO\textsubscript{3}−-N concentration in influent was 100 mg/L and sulfate concentration was 2 g/L. Initially, as the concentrations of both NO\textsubscript{3}−-N and sulfate increased (days 14 to 18), the NO\textsubscript{3}−-N concentration in effluent showed an upward trend, leading to a decrease in the NO\textsubscript{3}−-N removal rate to 76.6%. However, as the reaction progressed, the NO\textsubscript{3}−-N concentration in the effluent decreased to 5.5 mg/L, resulting in an increase in the NO\textsubscript{3}−-N removal rate to above 89.3%. These findings suggest that the initial increase in NO\textsubscript{3}−-N and sulfate concentrations had a negative impact on nitrate removal, but as the reaction progressed, the activated sludge adapted to the new environment,
leading to improved nitrate removal performance. This is the same as the study of Xu et al. [22]. From days 28 to 42, with an influent NO$_3^-$-N concentration of 200 mg/L and a sulfate concentration of 3 g/L, the NO$_3^-$-N removal rate initially decreased to a minimum of 73.1%. However, with time, the removal rate increased again and stabilized above 81.2% after day 38. From days 42 to 56, with an influent NO$_3^-$-N concentration of 300 mg/L and a sulfate concentration of 5 g/L, the NO$_3^-$-N concentration in the effluent rapidly increased, reaching a maximum of 133.4 mg/L, and the removal rate dropped to a minimum of 53.6%. However, as the reaction progressed, the removal rate increased again and reached 85.9% on day 56. From days 56 to 84, with an influent NO$_3^-$-N concentration of 300 mg/L and a sulfate concentration of 10 g/L, the removal rate initially decreased and then increased. At this time, activated sludge in the reaction system basically adapted to the environment with high NO$_3^-$-N concentration and high sulfate concentration. After 76 days, the NO$_3^-$-N removal rate remained above 96.0%, and the NO$_3^-$-N concentration in the effluent was lower than 11.7 mg/L. These results demonstrate the successful acclimation of activated sludge to effectively remove nitrate from high-sulfate wastewater.

![Figure 2](image)

**Figure 2.** Changes in NO$_3^-$-N (a), NO$_2^-$-N (b), and TN (c) concentrations in the reactor during the salt-tolerant acclimation stage.

Figure 2b shows the changes in NO$_2^-$-N concentration in the reactor during the salt-tolerant acclimation stage. During the first 32 days, there was almost no production of NO$_2^-$-N. However, at day 34, when the influent sulfate concentration was 3 g/L and the influent NO$_3^-$-N concentration was 200 mg/L, NO$_2^-$-N started to accumulate and continued to increase with increasing influent NO$_3^-$-N concentration. The maximum concentrations of NO$_2^-$-N at each stage were 44.5 mg/L, 111.4 mg/L, and 245.5 mg/L, respectively. The accumulation of NO$_2^-$-N in the effluent may be attributed to the insufficient availability of carbon sources. However, considering that the influent organic matter concentration was sufficient in this study, the rapid increase in influent NO$_3^-$-N concentration may have affected the denitrifying bacteria. The reduction rate of NO$_3^-$-N to NO$_2^-$-N is higher than the reduction rate of NO$_2^-$-N to N$_2$ [23,24]. As the denitrifiers in the activated sludge gradually adapted to the high NO$_3^-$-N concentration environment, there was almost no accumulation of NO$_2^-$-N in the effluent after day 68.
Figure 2c shows the changes in TN concentration in the reactor during the salt-tolerant acclimation stage. TN concentration was represented by the sum of NO$_3^-$-N and NO$_2^-$-N because there was no NH$_4^+$-N in the influent and effluent. From days 0 to 28, the TN removal rate remained above 88.0%, and the TN concentration in the effluent was lower than 6.5 mg/L. However, from days 28 to 64, with increasing NO$_3^-$-N concentration in the effluent. However, from days 28 to 64, with increasing NO$_3^-$-N and sulfate concentrations, NO$_2^-$-N began to accumulate, and the removal of NO$_3^-$-N was also affected. The TN concentration in the effluent increased to a maximum of 297.1 mg/L, and the TN removal rate dropped to only 2.0%. However, as the denitrification bacteria in the activated sludge gradually adapted to the high NO$_3^-$-N and sulfate environment, the nitrate removal performance improved, and there was almost no accumulation of NO$_2^-$-N in the wastewater. After day 76, the TN removal rate remained above 95.9%, and the TN concentration in the effluent was lower than 11.9 mg/L.

3.1.2. Changes in Sulfur Concentration

Figure 3a shows the changes in sulfate concentration in the reactor during the salt-tolerant acclimation stage. The sulfate concentration in effluent decreased as the reaction progressed, indicating the reduction of sulfate by sulfate-reducing bacteria. With increasing sulfate concentration, the reduction of sulfate also increased, reaching a maximum of 3644.8 mg/L on day 60. Figure 3b shows the changes in S$^{2-}$-concentration in the reactor during the salt-tolerant acclimation stage. Sulfide was detected in the effluent from days 0 to 32, and the highest S$^{2-}$ concentration reached 59.1 mg/L on day 14. However, as the reaction progressed, the S$^{2-}$ concentration in the effluent rapidly decreased after day 22, and after day 34, almost no sulfide formation was observed in the effluent. Yongsiri et al. [25] showed that when pH = 7–8, the proportion of hydrogen sulfide gas produced in the total sulfide in water was only 5–45%, while the pH in the reactor in this study was maintained at 7.6 ± 0.2, indicating that autotrophic denitrification may have occurred in the reactor after day 34. Autotrophic denitrifying bacteria utilize S$^{2-}$ as an electron donor for nitrate removal, resulting in a decrease in the S$^{2-}$ concentration.

![Figure 3](image-url)

Figure 3. Changes in sulfate concentration (a) and S$^{2-}$ concentration (b) in the reactor during the salt-tolerant acclimation stage.

3.1.3. Changes in COD Concentration

Figure 4 shows the changes in COD concentration in the reactor during the salt-tolerant acclimation stage. As shown in the figure, it can be observed that the COD removal efficiency remained relatively stable throughout the entire salt-tolerant acclimation stage. The influent COD concentration ranged from 2932.2 to 3104.6 mg/L, while the effluent COD concentration ranged from 2002 to 2197 mg/L. The maximum COD removal rate achieved was 34.7%, and the minimum was 30.5%.
with the addition of organic carbon sources, leading to a higher reduction of sulfates.

promotion stage. As the COD/SO$_4^{2-}$ ratio increased, the average reduction of sulfate concentration in the effluent increased continuously, with values of 619.3 mg/L, 855.8 mg/L, and 1108.0 mg/L, respectively. This was consistent with the study by Li et al. [26]. Due to the fact that sulfate-reducing bacteria are heterotrophic organisms, their activity increases with the addition of organic carbon sources, leading to a higher reduction of sulfates.

Figure 4. Changes in COD concentration in the reactor during the salt-tolerant acclimation stage.

3.2. COD/SO$_4^{2-}$ Promotion Stage

3.2.1. Changes in Nitrogen Concentration

Figure 5a shows the changes in NO$_3^-$ and NO$_2^-$ concentrations in the reactor during the COD/SO$_4^{2-}$ promotion stage. From days 0 to 14, with a COD/SO$_4^{2-}$ ratio of 0.3, apart from day 2, where the effluent NO$_3^-$ concentration was 50.5 mg/L with a removal rate of only 82.5%, the NO$_3^-$ removal rate fluctuated around 94% for the rest of the study period. With increasing COD/SO$_4^{2-}$ ratio, the removal rate of NO$_3^-$ gradually increased and stabilized above 98.0%, indicating that an increased organic carbon source favored nitrate removal. No significant accumulation of NO$_2^-$ was observed throughout the COD/SO$_4^{2-}$ phase. Figure 5b shows the changes in TN concentration in the reactor during the COD/SO$_4^{2-}$ promotion stage. Similar to the NO$_3^-$ trend, the removal rate of TN increased gradually with increasing COD/SO$_4^{2-}$ ratio, reaching a stable level of 97.9%, and TN concentration in the effluent was below 6.5 mg/L.

Figure 5. Changes in NO$_3^-$ and NO$_2^-$ concentrations (a) and TN concentrations (b) in the reactor during the COD/SO$_4^{2-}$ promotion stage.

3.2.2. Changes in Sulfur Concentration

Figure 6a shows the changes in sulfate concentration in the reactor during the COD/SO$_4^{2-}$ promotion stage. As the COD/SO$_4^{2-}$ ratio increased, the average reduction of sulfate concentration in the effluent increased continuously, with values of 619.3 mg/L, 855.8 mg/L, and 1108.0 mg/L, respectively. This was consistent with the study by Li et al. [26]. Due to the fact that sulfate-reducing bacteria are heterotrophic organisms, their activity increases with the addition of organic carbon sources, leading to a higher reduction of sulfates.
Figure 6b shows the changes in $S^{2-}$ concentration in the reactor during the COD/SO$_4^{2-}$ promotion stage. There was almost no sulfide accumulation in the effluent during the first 14 days. However, from days 14 to 20, with a COD/SO$_4^{2-}$ ratio of 0.5, the highest $S^{2-}$ concentration in the effluent reached 18.5 mg/L. This indicated that the increased organic carbon source promoted the production of sulfide. From days 28 to 42, with a COD/SO$_4^{2-}$ ratio of 1, the $S^{2-}$ concentration in the effluent decreased, the highest concentration of $S^{2-}$ in the effluent was only 2.85 mg/L, and there was almost no accumulation of sulfide in the effluent after day 34, while the amount of sulfate reduced was increased. It is plausible to suggest that the increased COD concentration during this phase might have stimulated the proliferation of heterotrophic non-denitrifying bacteria in the reactor, which led to a decrease in the proportion of heterotrophic denitrifying bacteria and an enhancement in autotrophic nitrification. As a result, the concentration of $S^{2-}$ in the effluent experienced a noticeable decline.

![Figure 6](image_url)  
**Figure 6.** Changes in sulfate concentration (a) and $S^{2-}$ concentration (b) in the reactor during the COD/SO$_4^{2-}$ promotion stage.

### 3.2.3. Changes in COD Concentration

Figure 7 shows the changes in COD concentration in the reactor during the COD/SO$_4^{2-}$ promotion stage. As observed in the figure, from days 0 to 14, with an influent COD concentration of 3000 mg/L and a COD/SO$_4^{2-}$ ratio of 0.3, the effluent COD concentration ranged from 2107.3 to 2174.6 mg/L, achieving the highest removal rate of 30.5%. From days 14 to 28, with the influent COD concentration increased to 5000 mg/L and a COD/SO$_4^{2-}$ ratio of 0.5, and the effluent COD concentration ranged from 2116.7 to 2936.6 mg/L, resulting in the highest removal rate of 49.2%. Finally, during days 28 to 42, with the influent COD concentration further raised to 10,000 mg/L and a COD/SO$_4^{2-}$ ratio of 1, the effluent COD concentration ranged from 6894.7 to 7320.3 mg/L, and the removal rate reached its peak at 31.4%. Notably, it can be observed that the optimal COD/SO$_4^{2-}$ ratio of 0.5 yielded the highest removal rate of COD in the reaction system.
were also present. Notably, all of these genera have denitrification capabilities. This shift in relative abundances suggests that the presence of Firmicutes increased after the end of salt-tolerance acclimation stage and sample JD2 from the end of COD/SO$_4^{2-}$ promotion stage, while the proportion of Proteobacteria decreased.

### 3.3. Microbial Community Analysis

Figure 8a shows the relative abundance of phylum in two samples. After the salt-tolerance acclimation stage, the dominant phylum in the reactor was Proteobacteria (97.73%), followed by Firmicutes (0.56%) and Bacteroidetes (0.55%). However, after the COD/SO$_4^{2-}$ promotion stage, the relative abundance of phylum in the reactor was Proteobacteria (53.18%), Firmicutes (36.79%), Actinobacteria (6.79%), and Bacteroidetes (2.48%). This shift in relative abundances suggests that the presence of Firmicutes increased after the COD/SO$_4^{2-}$ promotion, while the proportion of Proteobacteria decreased.

Figure 8b shows the relative abundance of genus in two samples. After the salt-tolerant domestication stage, the major genera in the reactor were Unclassified (56.21%), Raoultella (11.71%), Simplicispira (11.36%), and Klebsiella (10.51%). Thauera (7.88%) and Shinella (2.33%) were also present. Notably, all of these genera have denitrification capabilities. Raoultella is an autotrophic denitrifying bacterium [27,28], Klebsiella can perform heterotrophic denitrification under anaerobic conditions [29], Simplicispira is a heterotrophic denitrifying bacterium that utilizes biopolymers as a carbon source [30], Thauera is a common heterotrophic denitrifying bacteria [31], and Shinella is an autotrophic denitrifying bacterium that utilizes sulfide as an electron donor [32]. After the COD/SO$_4^{2-}$ promotion stage, the relative abundance of each genus in the reactor shifted to Megasphaera (17.16%), Lactobacillus (16.92%), Raoultella (16.94%), and Klebsiella (13.52%). Unclassified (5.63%), Buttiauxella (11%), Bifidobacterium (3.41%), and Prevotella (3.06%) were also present. A comparison with samples JD1 revealed an enrichment of Megasphaera, Lactobacillus, and Buttiauxella. Megasphaera and Buttiauxella are both crucial fermenting and hydrogen-producing bacterial genera [33,34], while Lactobacillus can transform glucose and other sugars into lactic acid [35].

In conclusion, the microbial community analysis indicated the presence of both autotrophic and heterotrophic denitrifying bacteria in the reaction system. Therefore, this
study demonstrated the occurrence of mixed trophic denitrification in the removal of nitrate from sulfate wastewater.

4. Conclusions
In this study, a high-sulfate wastewater treatment process using the UASB reactor was investigated for efficient nitrate removal. The results showed that the activated sludge could be acclimated to tolerate high-sulfate conditions and achieve a nitrate removal efficiency exceeding 96.0%. Autotrophic and heterotrophic denitrification processes were found to occur in the reaction system. The enrichment of autotrophic denitrifying bacteria, including *Raoultella* and *Shinella*, and heterotrophic denitrifying bacteria, including *Klebsiella*, *Simplicispira*, and *Thauera*, was observed. The availability of organic carbon sources was a critical factor influencing the denitrification performance of the system. Increasing the COD/\(\text{SO}_4^{2-}\) ratio enhanced nitrate removal efficiency and the sulfate reduction reaction in the system. The enrichment of heterotrophic microorganisms, such as *Megasphaera*, *Lactobacillus*, and *Buttiauxella*, was observed at a high COD/\(\text{SO}_4^{2-}\) ratio. These findings provide valuable insights into the development of high-efficiency mixotrophic denitrification processes for nitrate removal from sulfate-rich wastewater.

**Author Contributions:** Conceptualization, H.Z. and Y.W.; methodology, Y.W. and M.J.; validation, H.Z.; formal analysis, Y.W.; investigation, Y.W. and M.J.; resources, H.Z. and Y.W.; data curation, Y.W. and M.J.; writing—original draft preparation, Y.W.; writing—review and editing, H.Z., J.Y. and M.X.; visualization, Y.W.; supervision, H.Z.; project administration, H.Z.; funding acquisition, H.Z. All authors have read and agreed to the published version of the manuscript.

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