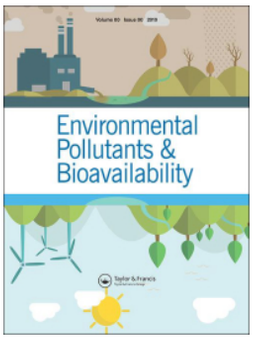

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Interaction of the concentration of S^{2-}, SO_4^{2-} and microbial community by controlling nitrogen stripping during sulfate-rich wastewater treatment

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ABSTRACT

Nitrogen stripping plays a vital role during the sulfate-rich wastewater treatment process by sulfate-reducing bacteria (SRB). However, the interaction between nitrogen stripping, sulfate reduction capacity, S^{2-} concentration, and microbial community structure is rarely studied. Here, two anaerobic systems were conducted to analyze these interactions simultaneously. Results showed that the toxic products (H_2S) were stripped by nitrogen at first. Then, the increased relative abundance of fermentative bacteria accelerated the carbon sources conversion, and *Desulfovibrio, Dethiosulfovibrio*, and *Sulfurospirillum* facilitated the sulfate reduction after nitrogen stripping. Finally, the produced S^{2-} concentration was increased by 12%, and the sulfate reduction rate was increased by 9.2% compared to that without nitrogen stripping.

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KEYWORDS
Nitrogen stripping; *Desulfovibrio*; coexistence; anaerobic system; sulfate-rich wastewater

1 Introduction

The annual production of mine wastewater in China is more than 1.1×10^{10} m^3, and the resource utilization rate is only 20%, with a large amount of mine wastewater is directly discharged [1,2]. Acid mine drainage (AMD) is continuously produced from abandoned mines and contains many high-concentration sulfate ions (SO_4^{2-}) and various heavy metals with low pH. It brings severe threats to mine equipment, the ecological environment, and human health [3]. There is an urgent need for an economical, practical, and resource-recyclable method to protect the sustainability of ecosystems and reduce resource waste. There are many methods for treating AMD, among which biological technology by SRB has attracted much attention due to their low cost and environmental friendliness [4,5].

S^{2-} plays an essential role in reducing sulfate by SRB [6]. On the one hand, S^{2-} transformed by excessive sulfate can bind with metals to precipitate as metal sulfides under the metabolism of SRB [7]. On the other hand, S^{2-} can be converted to elemental sulfur by sulfur-oxidizing bacteria. Unfortunately, current research predominantly focuses on growth characteristics, utilization of different substrates, and sulfate reduction ability of SRB [8–11]. However, little research has been done on S^{2-} under the metabolic reactions of SRB. Based on previous studies, only a tiny S^{2-} reacts with heavy metals, and a large amount of sulfur is not analyzed [12]. A study by Vasquez et al. reported that 833 mg/L of SO_4^{2-}-S in the influent and a total of 473 mg L^{-1} of S^{2-} in the effluent (including sulfate and generated metal sulfides), with approximately 43% of sulfur are not described in the effluent [13]. Therefore, it is necessary to pay attention to S^{2-} when SRB treats sulfate-rich wastewater.

The formation of S^{2-} is affected by many factors. On the one hand, diverse microorganisms have different metabolic profiles because of their inherently different functions. As well, there is a competition between SRB and other bacteria. Additionally, the coexisting hydrolytic bacteria and fermentative bacteria may affect the activity of SRB. The above reason may affect the activity of SRB, which has the most considerable influence on S^{2-} production [14]. Therefore, it is essential to understand the microbial community successions during the sulfate-rich wastewater reduction process. On the other hand, several types of sulfur-containing products (H_2S, HS^-, and S^{2-}) are found during the SRB metabolism process [15], and the undissociated H_2S can penetrate the cell wall and inhibit the activity of SRB, causing the change in S^{2-} production [16]. If toxic products (such as H_2S) can be removed, the activity of SRB will not be inhibited, and thus the sulfate reduction reaction will be accelerated. Yao compared the effect of nitrogen stripping on sulfide production when using SRB to treat AMD and found that the sulfide content in the nitrogen stripping group is 2.09 times higher than that in the group without nitrogen

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stripping [17]. Thusly, nitrogen stripping is a feasible method for removing toxic products in the sulfate-rich wastewater treatment by SRB. Furthermore, the existence of toxic products can influence the microbial community composition. As reported earlier, the added H_2S changed the microbial growth environment, which might cause the relative abundance of *Hydrogenophaga* sp, *Clostridium populeti, Bacteroides* sp, *Pseudomonas* sp, and *Dysgonomonas wimpennyi* to change [18]. Here, the formation of S^{2-} is affected by the microbial community and nitrogen stripping. Meanwhile, the succession of microbial communities is affected by nitrogen stripping in the sulfate-rich wastewater treatment by SRB. However, it is still unclear about the relationship of microbial community succession, nitrogen stripping, and S^{2-} formation during the sulfate-rich wastewater treatment by SRB.

Therefore, this study conducted two anaerobic systems to simultaneously analyze the interaction of microbial community succession, nitrogen stripping, S^{2-}, and sulfate reduction ability. The SRB inoculum was enriched, and the sulfate reduction ability, the concentration of S^{2-} by controlling nitrogen stripping during sulfate-rich wastewater treatment by SRB were considered. Additionally, the response to microbial communities was also investigated.

2 Materials and methods

2.1 SRB inoculum

The microorganisms were derived from anaerobic sludge at the Nibu Bay Wastewater Treatment Plant (Qingdao, Shandong Province, China). Postgate'S medium C was used for the culture of SRB [19]. This medium was composed of 0.65 g L^{-1} KH_2PO_4, 0.06 g L^{-1} $MgCl_2 \cdot 6H_2O$, 1.0 g L^{-1} Na_2SO_4, 0.04 g L^{-1} $CaCl_2 \cdot 2H_2O$, 1.0 g L^{-1} NH_4Cl, 1.0 g L^{-1} yeast extract, and 2.0 mL L^{-1} sodium lactate. The pH of the media was adjusted to 7 and then sterilized at 121°C for 20 min. After cooling, 1.2 g L^{-1} of $Fe(NH_4)_2{\cdot}(SO_4)_2 \cdot 6H_2O$ and 0.5 g L^{-1} of L-cysteine sterilized by ultraviolet (UV) radiation for 30 min were added, and the samples were cultured at 28°C and 70 rpm min^{-1} in a shaker. After two days, when the solution turned ink-colored and the bottle smelled like rotten eggs (detected by using lead acetate test paper), 10% (v/v) of the above culture solution was transferred to fresh Postgate'S medium C, and the same culture conditions were repeated three times to enrich the SRB. Efficient SRBs were obtained and identified by Shanghai Majorbio Bio-pharm Technology Co., Ltd.

Subsequently, the morphology and the growth process of the SRB inoculum were evaluated. The pH and redox potential (ORP) during growth were tested together to investigate the growth characteristics of the SRB inoculum. SRB inoculum (10% [v/v]) were added to the fresh Postgate'S medium C (without the addition of $Fe(NH_4)_2{\cdot}(SO_4)_2 \cdot 6H_2O$, the medium that was not inoculated was used as a blank control, and the OD_{600}, pH, and ORP in the medium were measured every two hours. All experiments were performed three times.

2.2 Design, set up, and operation of anaerobic system SRB reaction device

Considering the influence of initial sulfate concentration and nitrogen stripping on sulfate reduction by SRB, two set anaerobic systems of experimental devices were designed (Figure 1). Device (a) consisted of bottle A only, filled with sulfate-rich wastewater and SRB. Device (b) was composed of bottles A, B, and nitrogen bottles. Bottle B is filled with $CuCl_2$ solution, absorbing H_2S by nitrogen stripped. The practical volumes of

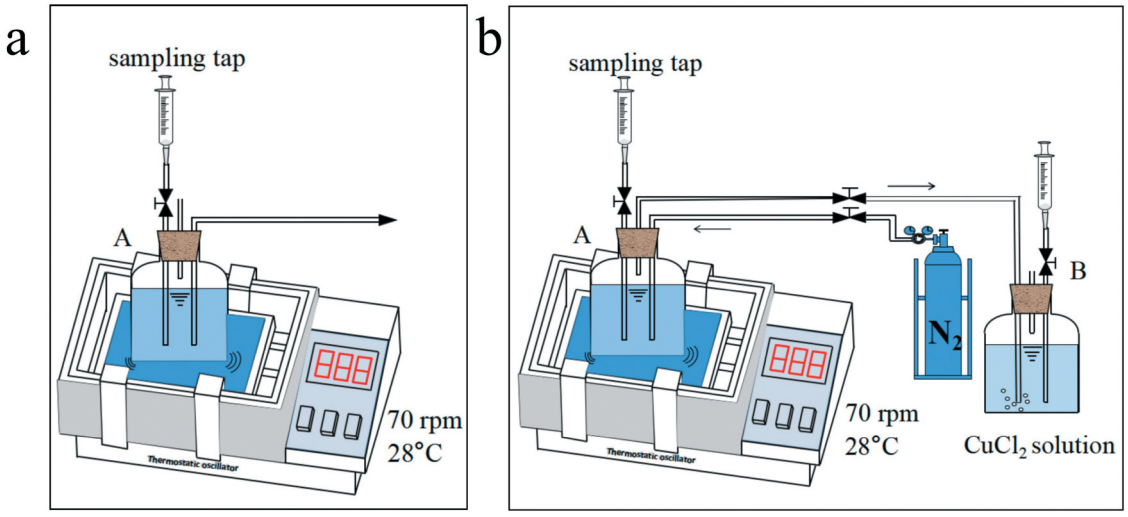

Figure 1. Experimental reaction device. (a): bottle A was filled with sulfate-rich wastewater and SRB; (b): bottle A, bottle B filled with $CuCl_2$ solution and nitrogen stripping.

bottles A and B were 1 L, and rubber stoppers and polytetrafluoroethylene tapes sealed all the entrances of the bottles to ensure an anaerobic environment.

In each group of experiments, the initial sulfate concentrations of wastewater were 600 and 1000 mg L^{-1}, and 30% (v/v) SRB inoculum was added to sulfate-rich wastewater (Stripe nitrogen for 10 min before use to remove oxygen). The concentration of copper ions in bottle B used for absorption was 500 mg L^{-1}. The sulfate concentration, S^{2-} concentration, pH, and ORP during the reaction were measured at 0, 3, 6, 9, 12, and 15 h. All experiments were conducted and analyzed three times.

2.3 Analytical procedures

The morphology of the SRB inoculum was tested through scanning electron microscopy (FEI Quanta 250 FEG), and sample processing steps were based on Shi's study [20]. A pH meter (PHS-3C) and automatic potentiometric titrator (ZD-2) measured pH and ORP. Sulfate concentration was tested following the 'Water Quality-Determination of sulfate–barium chromate spectrophotometry' (HJ/T 342–2007). The S^{2-} concentration was determined using a sulfur ion concentration meter (Banter 931-S). The concentration of Cu^{2+} remaining in bottle B was measured to account for the H_2S produced in bottle A using a flame atomic absorption spectrometer (TAS-986 F).

2.4 Microbial community analysis

The samples (0 h, 15 h-without N_2, and 15 h-with N_2) at 600 mg L^{-1} initial sulfate concentration were filtered by a 0.22 μm aqueous filter membrane were collected first for high-throughput sequencing analysis. DNA extraction and polymerase chain reaction (PCR) amplification were performed. According to the manufacturer's instructions, the samples were using a FastDNA® Spin Kit for Soil (MP Biomedicals, U.S.). The DNA concentration and purity were determined with a NanoDrop 2000 UV–vis spectrophotometer, and DNA extract was checked on 1% agarose gel. PCR amplification was run by two primers of 338 F (ACTCCTACGGGAGGCAGCAG) and 806 R (GGACTACHVGGGTWTCTAAT) for each DNA sample, and the region was V3–V4 hypervariable region. PCR reactions were performed in triplicate. According to the manufacturer's instructions, the PCR product was extracted from 2% agarose gel, purified using an AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, USA), and quantified using a Quantus™ Fluorometer (Promega, USA). The third step was Illumina MiSeq sequencing. Purified amplicons were pooled in equimolar and paired-end sequenced on an Illumina MiSeq PE300 platform (Illumina, USA) according to the standard protocols by Majorbio Bio-Pharm Technology Co. Ltd. (Shanghai, China). The final step was the processing of sequencing data. Operational taxonomic units (OTUs) with 97% similarity cutoff were clustered, and chimeric sequences were identified and removed.

3 Results

3.1 Characteristics of SRB inoculum

In order to further analyze the succession of microbial community structure during the sulfate reduction process by SRB, we first studied the characteristics of SRB inoculum. The morphology of microorganisms is shown in Figure 2(a). The SRB was arc and rod-shaped, and their length was approximately 0.5 to 2 μm. As shown in Figures 2(b–c), the microorganisms were constituted by *Escherichia-Shigella, Desulfovibrio, Citrobacter, Rhodobacteraceae, Dethiosulfovibrio*, and others at the genus level. Since the first discovery of SRB by Beijerinck in 1895, more than 40 genera of SRB, such as *Desulfovibrio, Desulfotomaculum, Desulfonema, Desulfomonas, Desulfobacter*, and *Desulfococcus*, have been isolated successively [21].

3.2 Effect of different conditions on sulfate reduction ability by microorganisms

Different initial sulfate concentrations affect microorganisms' growth and reduction ability due to the different osmotic pressures. As observed in Figure 3, the sulfate reduction rate was 40.82% when the initial sulfate concentration was 600 mg L^{-1}, and it was 32.40% when the initial sulfate concentration was 1000 mg L^{-1}. Regardless of the initial sulfate concentration, the pH during the reactions was maintained in the range of 7.02–7.11, and the ORP values decreased more obviously at the initial sulfate concentration of 600 mg L^{-1} than that of the initial sulfate concentration was 1000 mg L^{-1}. With nitrogen stripping, the sulfate reduction rate was improved by 9.25% compared with that without nitrogen stripping at 600 mg L^{-1} initial sulfate concentration, and there was an approximately 7.48% increase at 1000 mg L^{-1} initial sulfate concentration. These demonstrated that nitrogen stripping is effective for sulfate-rich wastewater treatment by SRB. In addition, a visible elevation of alkalinity was observed in the SRB treatment of sulfate-rich wastewater after nitrogen stripping.

3.3 Effect of different conditions on S^{2-} production during sulfate reduction process by microorganisms

S^{2-} as the target product of SRB metabolism and can form stable metal sulfide precipitation with heavy metal ions in wastewater, the S^{2-} concentration with different nitrogen stripping conditions was studied, and the results were

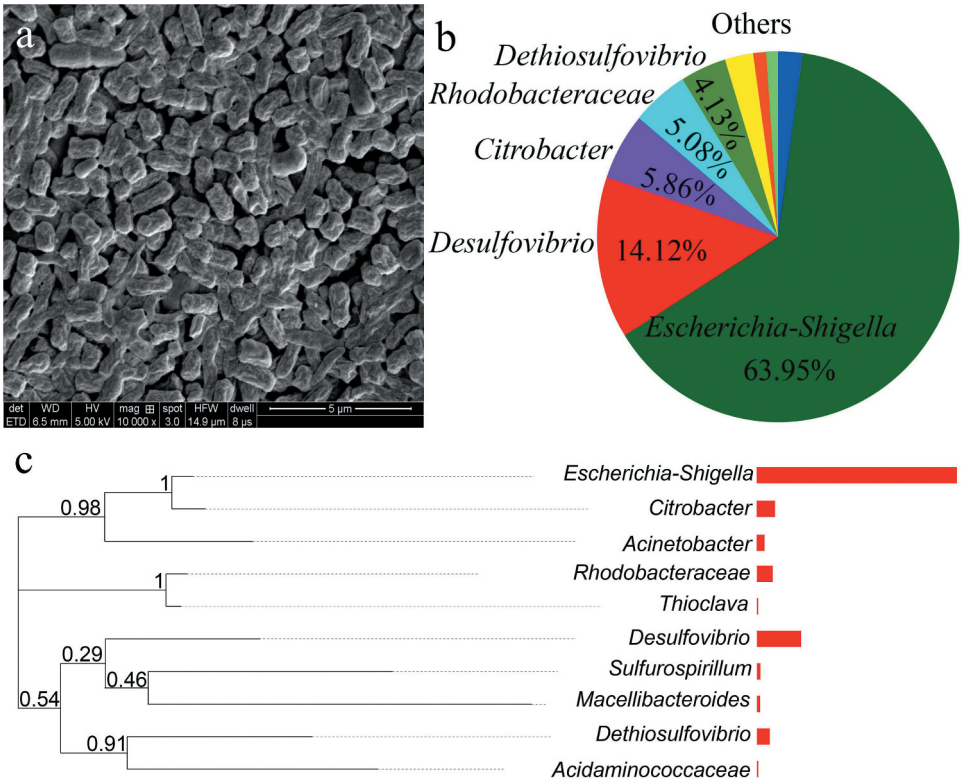

Figure 2. Screening and identification of microorganisms. (a) the morphology of microorganisms; (b) the composition of SRB inoculum; (c) the phylogenetic tree on genus level of microorganisms.

shown in Figure 4. The S^{2-} concentration increased from 97 mg L^{-1} to 103 mg L^{-1} with 600 mg L^{-1} initial sulfate concentration, and it grew from 98 mg L^{-1} to 106 mg L^{-1} with 1000 mg L^{-1} initial sulfate concentration. After nitrogen stripping, the concentration of S^{2-}increased from 97 mg L^{-1} to 115 mg L^{-1} and 120 mg L^{-1} when the initial sulfate concentration increased from 600 mg L^{-1} to 1000 mg L^{-1}, which was approximately 12% higher than the final S^{2-} concentrations in the system without nitrogen stripping. After 15 h bioreaction, the concentration of H_2S (as sulfur) blown off was 85.14 and 109.99 mg L^{-1} with an initial sulfate concentration of 600 and 1000 mg L^{-1}, respectively.

3.4 Microbial community differences on sulfate-rich wastewater treatment by controlling nitrogen stripping

3.4.1 Alpha diversity analysis of different samples

16S rRNA genes were amplified and sequenced through high-throughput sequencing, and all OTUs were clustered at a setting cut off 97% similarity. A total of 32,048 sequences per sample were collected after subsampling to determine microbial diversity. Community richness and diversity in each sample are estimated, and the results are shown in Figure 5. As shown in Figures 5(a–c), the microbial community richness has a slight downward trend after nitrogen stripping. As for microbial community diversity, an increasing tendency was found under the sulfate-rich wastewater treatment process of SRB (Figures 5(d–f)). Moreover, the results indicated that nitrogen stripping negatively influenced microbial community diversity.

3.4.2 Microbial community composition analysis of different samples

The distribution of microorganisms was examined to reveal the functional microbial community among the three samples. The phylum with the highest representation was *Proteobacteria* representing 77.86%, 68.91%, and 59.96% among the samples of 0 h, 15 h-without N_2, and 15 h-with N_2, respectively, followed by *Desulfobacterota* (14.12%, 15.58%, and 16.63%), *Bacteroidota, Synergistota*, and *Firmicutes* (Figure 6(a)).

At the class level, the relative abundance of nine bacterial phyla was identified with over 1%. The most abundant phyla were *Gammaproteobacteria*, as the main part of *Proteobacteria* representing 72.32%, 58.36%, and 54.61% among the samples of 0 h, 15 h-without N_2, and 15 h-with N_2, respectively, followed by *Desulfovibrionia, Baceroidia, Alphaproteobacteria, Synergistia, Campylobacteria, Clostridia, Actinobacteria*, and *Negativicutes*. *Alphaproteobacteria* were classified as *Proteobacteria*, accounting for 5.54%, 10.55%, and 5.37% among the samples (Figure 6(b)).

As shown in Figure 6(c), the bacterial community of samples altered after sulfate reduction (only the top 10 genera). The distribution of some specific functional

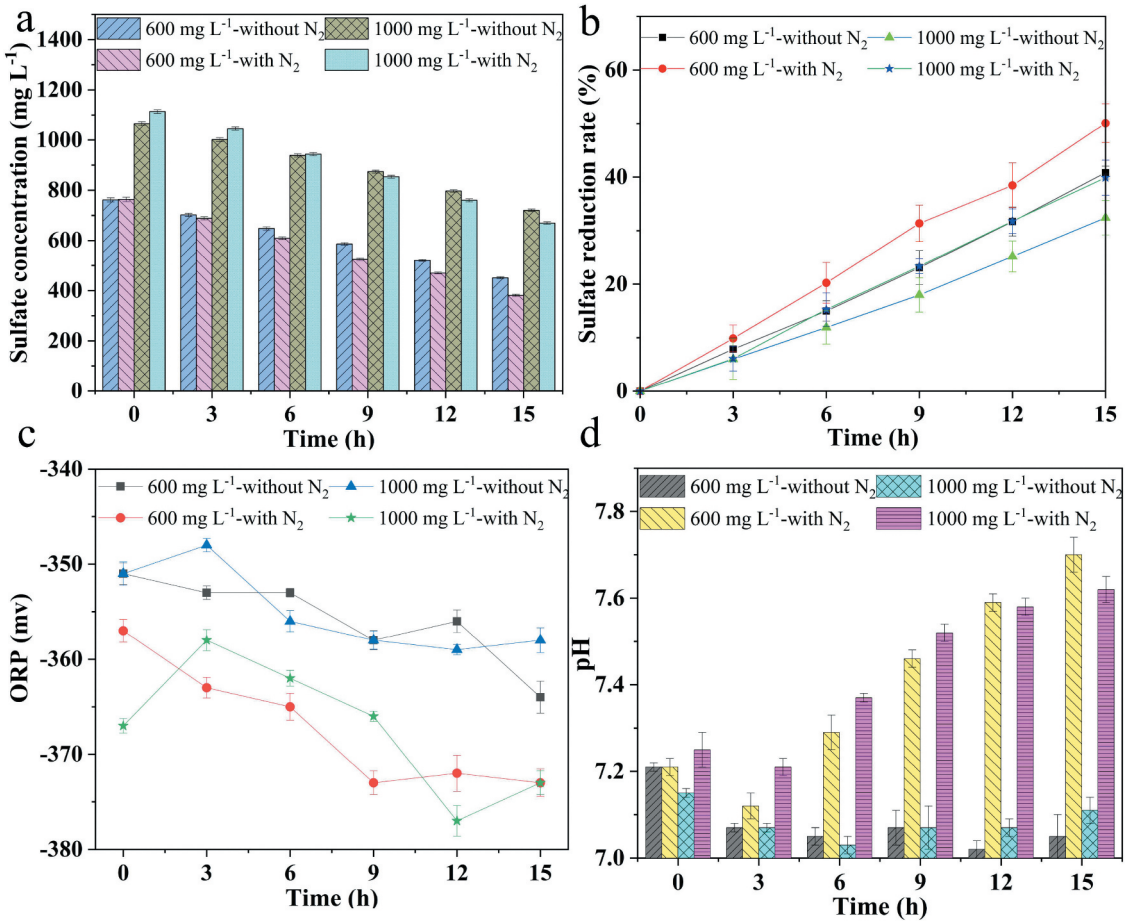

Figure 3. The characteristics of different conditions on sulfate reduction by microorganisms. (a) sulfate concentration; (b) sulfate reduction rate; (c) ORP; (d) pH.

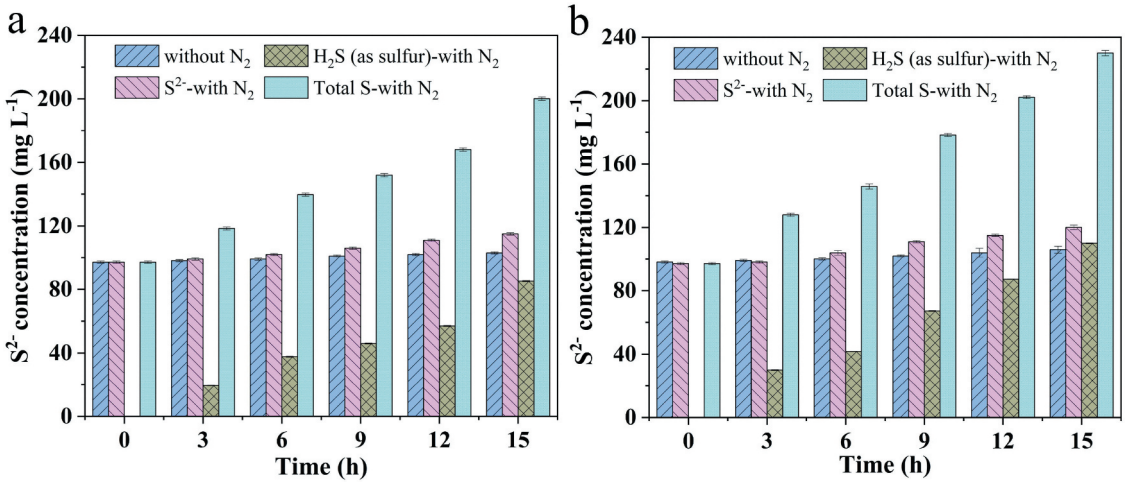

Figure 4. Effect of S^{2-} production concentration by microorganisms controlling nitrogen stripping during sulfate reduction. (a) initial sulfate concentration was 600 mg L^{-1}; (b) initial sulfate concentration was 1000 mg L^{-1}.

genera among the three samples is illustrated in Figure 6(d). The principal genera responsible for sulfate reduction were *Desulfovibrio*, accounting for 14.12%, 15.58%, and 16.63% among the samples of 0 h, 15 h-without N_2, and 15 h-with N_2, respectively, which performed an increasing tendency after the bioreaction. At the same time, *Dethiosulfovibrio* accounted for 4.13%, 2.06%, and 8.70%, respectively. *Thioclava* accounted for 0.44%, 1.02%, and 0.35%, which played an essential role in the biogeochemical sulfur cycle and sulfide and sodium thiosulfate oxidization [22]. Here, the relative abundance of *Desulfovibrio, Macellibacteroides, Dethiosulfovibrio, Citrobacter*, and *Sulfurospirillum* increased, while the relative abundance of *Escherichia-Shigella, Rhodobacteraceae*, and *Bacteroides* decreased after nitrogen stripping.

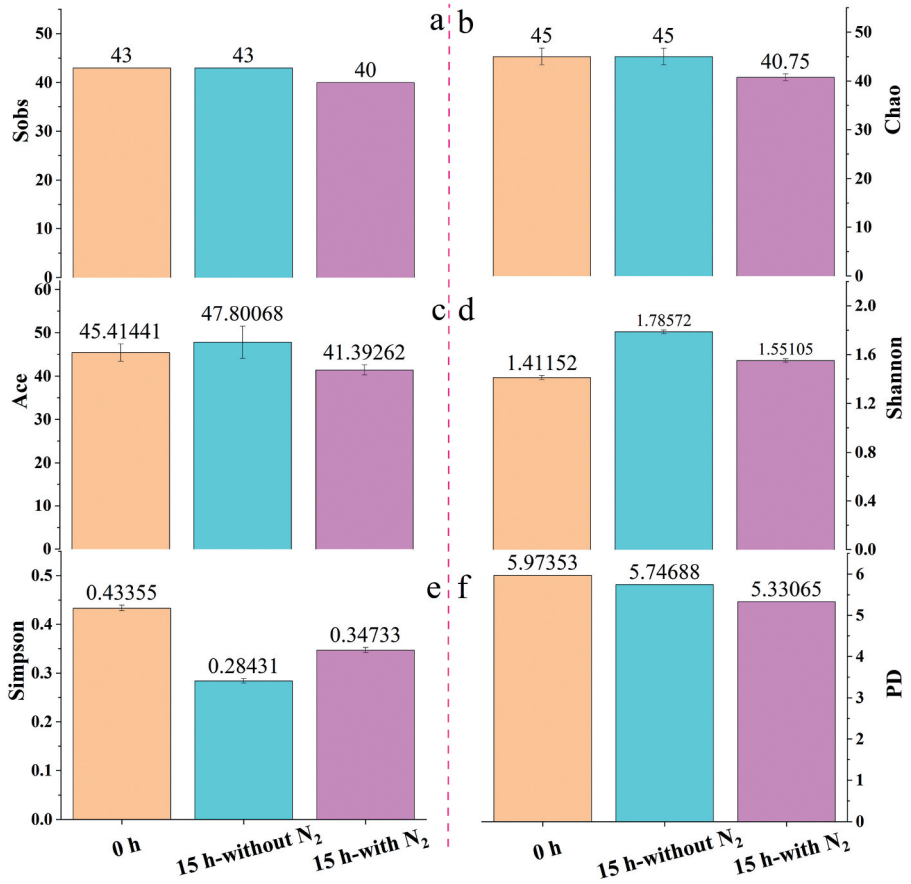

Figure 5. Microbial community richness and diversity among three samples. (a-c) the indexes of microbial community richness; (d-f) the indexes of microbial community diversity.

4 Discussion

This study comprehensively investigated the sulfate reduction rate, S^{2-} concentration, and microbial community by controlling nitrogen stripping during sulfate-rich wastewater treatment by SRB. In the sulfate reduction process, SRB generated several reduction products, including H_2S, HS^-, and S^{2-}, especially when H_2S caused the most toxicity to the growth and performance of SRB [23]. Consequently, nitrogen gas can strip the H_2S generated during the sulfate reduction by SRB to decrease the toxicity. Other studies suggested that when the concentration of H_2S reached 40–50 mg L^{-1}, SRB was inhibited, and SRB activity was irreversibly destroyed when the concentration of H_2S exceeded the toxicity level for 3–6 h [24]. Different SRB possessed different tolerances to H_2S, and the toxicity of H_2S produced during sulfate reduction to SRB may be direct and reversible [25,26].

In this paper, the concentration of H_2S (as sulfur) blown off by nitrogen was 85.14 mg L^{-1} after 15 h bioreaction with an initial sulfate concentration of 1000 mg L^{-1}, respectively. At the same time, the S^{2-} production concentration was increased from 103 to 120 mg L^{-1} compared to that without nitrogen stripping. Moreover, the sulfate reduction rate was increased by 9.2% to 50.08%. In other words, toxicity products were blown by nitrogen, and SRB activity was restored, thereby enhancing the sulfate reduction ability.

Furthermore, the H_2S was blown off by nitrogen stripping, with the evolution of microbial community structure. Specifically, the relative abundance of *Desulfovibrio, Macellibacteroides, Dethiosulfovibrio*, and *Sulfurospirillum* increased. *Desulfovibrio* has an essential role in anaerobic systems [27], and it was a typical hydrogenotrophic SRB, indicating that these SRB can use hydrogen to reduce sulfate [28]. Moreover, *Dethiosulfovibrio* reduced thiosulfate and elemental sulfur but not sulfate to hydrogen sulfide [29]. *Macellibacteroides*, a type of fermentative bacteria, produced hydrogen for hydrogenotrophic and enhanced the activity of *Desulfovibrio* [30]. *Sulfurospirillum*, a type of sulfite-reducing bacteria [31] and Nogueira et al listed *Sulfurospirillum* as SRB genera [5]. In addition, the species of *Bacteroides* has a relatively high abundance in 15 h-with N_2 sample. *Bacteroides* was a hydrolytic bacterium that could participate in protein degradation and convert amino acids to acetate and had the function of being tolerant to high concentrations of metals and metalloids [32]. It is inferred from these results that the increased fermentative bacteria (*Macellibacteroides*) accelerated the conversion of carbon sources. The sulfate was transformed to sulfite by

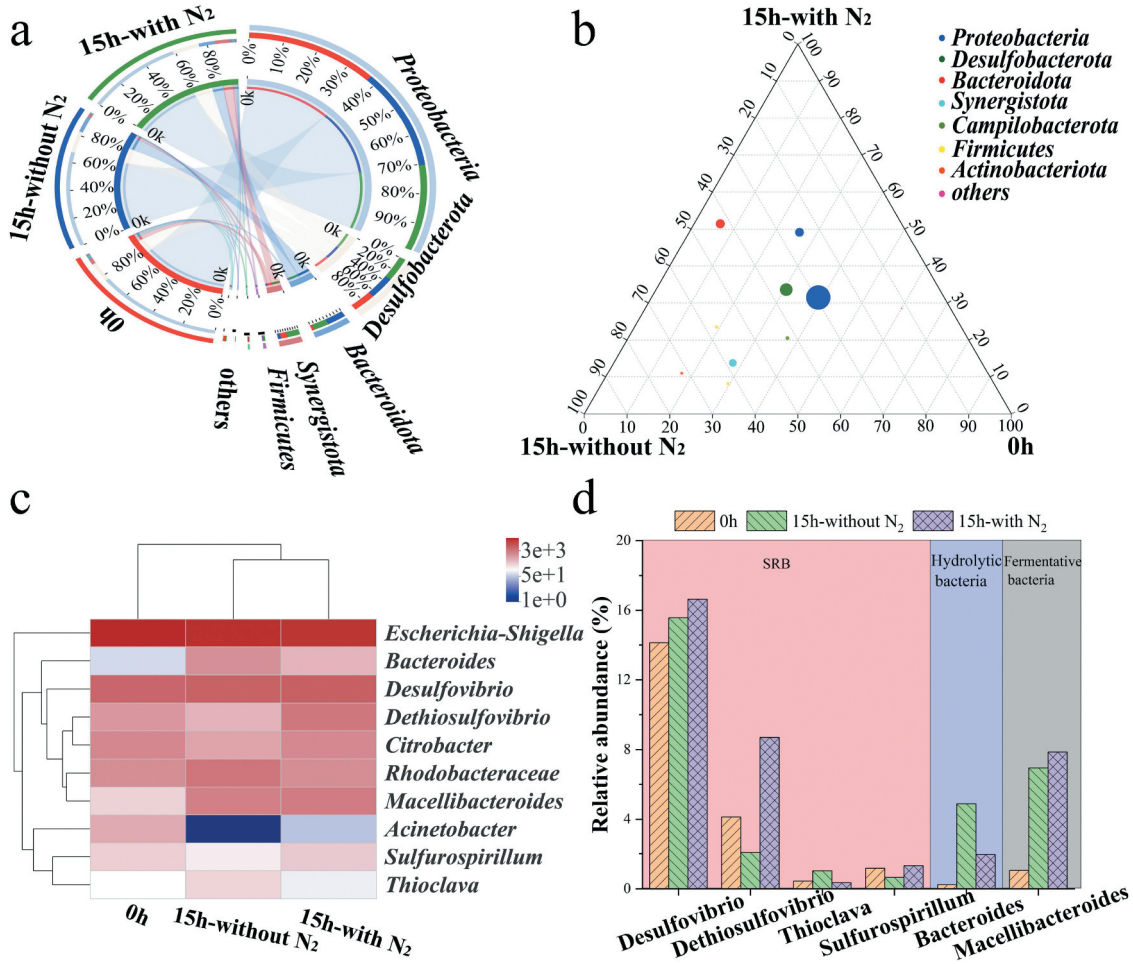

Figure 6. Species composition analysis at different levels among three samples. (a) the sample-species relationship circle diagram at the phylum level; (b) the ternary analysis at the class level; (c) the heatmap at the genus level; (d) the distribution of specific functional genera among three samples.

Desulfovibrio, and the bacteria of *Dethiosulfovibrio* and *Sulfurospirillum* converted sulfite to S^{2-}. Thus, the produced S^{2-} concentration increased, and sulfate reduction ability improved.

5 Conclusions

In this study, the contributions of nitrogen stripping to sulfate reduction by SRB were comprehensively investigated. Stripped H_2S, favorable S^{2-} production, and considerable sulfate reduction ability implied a vital contribution of nitrogen stripping in the sulfate-rich wastewater. The hydrolytic and fermentative microorganisms coexistent with the SRB, and the increased fermentative bacteria accelerated the conversion of carbon sources. The relative abundance of *Desulfovibrio, Dethiosulfovibrio*, and *Sulfurospirillum* increased after nitrogen stripping, thereby facilitating the sulfate reduction. These findings provide advanced incisive insights into the effect of nitrogen stripping on sulfate-rich wastewater treatment by SRB from S^{2-}, sulfate reduction ability, and microbial community successions.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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