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# Elemental sulfur recovery and spatial distribution of functional bacteria and expressed genes under different carbon/nitrate/sulfide loadings in up-flow anaerobic sludge blanket reactors

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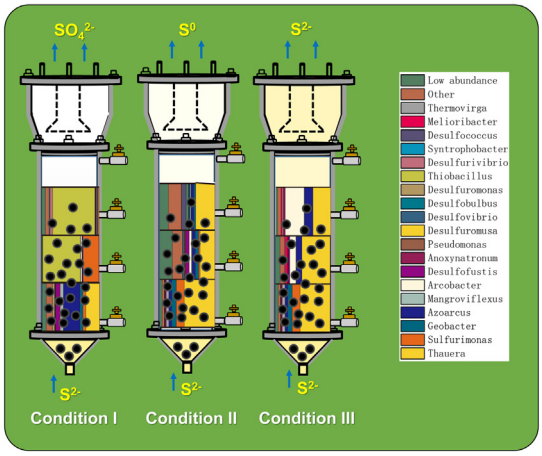
## HIGHLIGHTS

- The variations of influent loading altered $S^0$ recovery rate and bacterial structure.
- The optimized loads of acetate, nitrate and sulfide were 0.95, 0.79, and 0.34 kg $d^{-1}$ $m^{-3}$, respectively.
- The highest $S^0$ recovery rate was 77.9% when *Thauera* and *Sulfurimonas* were predominant.
- Insufficient loading caused a low $S^0$ yield and sulfide oxidation activity.
- Excess nutrient loading caused the over-oxidation of $S^0$ to sulfate.

## GRAPHICAL ABSTRACT

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## ABSTRACT

To characterize the impact of influent loading on elemental sulfur ($S^0$) recovery during the denitrifying and sulfide oxidation process, three identical, lab-scale UASB reactors (30 cm in length) were established in parallel under different influent acetate/nitrate/sulfide loadings, and the reactor performance and functional community structure were investigated. The highest $S^0$ recovery was achieved at 77.9% when the acetate/nitrate/sulfide loading was set to 1.9/1.6/0.7 kg $d^{-1}$ $m^{-3}$. Under this condition, the genera *Thauera*, *Sulfurimonas*, and *Azoarcus* were predominant at 0–30, 0–10 and 20–30 cm, respectively; meanwhile, the *sqr* gene was highly expressed at 0–30 cm. However, as the influent loading was halved and doubled, $S^0$ recovery was decreased to 27.9% and 45.1%, respectively. As the loading was halved, the bacterial distribution became heterogeneous, and certain autotrophic sulfide oxidation genera, such as *Thiobacillus*, dominated, especially at 20–30 cm. As the loading doubled, the bacterial distribution was relatively homogeneous with *Thauera* and *Azoarcus* being predominant, and the *nirK* and *sox* genes were highly expressed. The study verified the importance of influent loading to regulate $S^0$ recovery, which

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could be achieved as *Thauera* and *Sulfurimonas* dominated. An influent loading that was too low or too high gave rise to insufficient oxidation or over-oxidation of the sulfide and low $S^0$ recovery performance.


## 1. Introduction

A high amount of sulfate ($SO_4^{2-}$) is generated from manufacturing industries, such as pulp production and the pharmaceutical and petrochemical industries [1], which often lead to a high accumulation of sulfide ($HS^-$ and $S^{2-}$) in liquid phase by sulfate-reducing bacteria (hypothetical biochemical equation (1)) [2,3]. Sulfide is toxic and odorous, and it can corrode concrete and steel; thus, efficient removal technologies of sulfide from a liquid phase are requisite and urgently needed [4].

$$SO_4^{2-} + \text{organicmatter} \rightarrow HS^- + H_2O + HCO_3^- \quad (1)$$

When both $S^{2-}$ and $NO_3^-$ are present, sulfide-oxidizing bacteria (SOB) can transfer $S^{2-}$ to $S^0$ by utilizing $NO_3^-$ as an electron acceptor, called the denitrifying sulfide removal (DSR) process [5]. Previous studies have suggested that only autotrophic bacteria can perform the DSR process [6,7]; however, some heterotrophic bacteria have abnormally high DSR capability [8,9], which inspired a novel process for $S^0$ recovery and the simultaneous removal of nitrate, sulfide and organic carbon (hypothetical biochemical equation (2)). In many cases, the over-oxidation of sulfide has resulted in a high amount of sulfate, thiosulfate and sulfide remaining in effluent as the main products [10–14], largely restricting the reclamation of elemental sulfur ($S^0$) (hypothetical biochemical equation (3)).

$$S^{2-} + 1.25CH_3COOH + 2.4NO_3^- + 2.4H^+ \rightarrow S^0 + 2.5CO_2 + 1.2N_2 + 2.7H_2O + 2OH^- \quad \Delta G^{0\prime} = -1463.1\,kJ/reaction \quad (2)$$

$$S^{2-} + 1.25CH_3COOH + 3.6NO_3^- + 1.6H^+ \rightarrow SO_4^{2-} + 2.5CO_2 + 1.8N_2 + 2.3H_2O + 2OH^- \quad \Delta G^{0\prime} = -2100.1\,kJ/reaction \quad (3)$$

To solve this problem, several studies have attempted to search for high $S^0$ recovery strategies through optimizing the different carbon/nitrate/sulfide ratios. Chen et al. [14] found that selective $S^0$ recovery was improved when the sulfide/nitrate molar ratio was set to 5/2 rather than 5/5 or 5/8 in an up-flow reactor. Cardoso et al. [15] reported that the $S^0$ generation rate gradually decreased with an increase of the sulfide/nitrate molar ratio under the fixed carbon/nitrate molar ratio of 1/1 in an upflow anaerobic sludge blanker (USAB) reactor.

The contaminant loading, which reflects the treatment capacity, is regarded as one of the most important parameters during the operating design and engineering application of the reactor [16,17]. Seeking for the effective strategy to regulate $S^0$ recovery and avoid the unwanted or unnecessary biochemical reactions, like $S^{2-}$ over oxidization or sulfide reduction, has become a tough issue urgently to be addressed [18]. The previous study demonstrated that both the microbial community and reactor performance varied significantly under different sulfide/nitrate ratios [18]; however, until now, the impact related to the spatial structure and function of the bacterial community and reactor performance caused by the variations in carbon/sulfide/nitrate loading is hardly understood.

Therefore, in this study, three lab-scale UASB reactors were established to test $S^0$ recovery and the removal efficiency of acetate, sulfide and nitrate as well as the spatial abundance and distribution of functional bacteria and genetic activities under different acetate/sulfide/nitrate loadings. The aim of the study was to evaluate the impact of the different influent loadings on $S^0$ recovery and DSR efficiencies and to provide a detailed investigation of the functional bacterial community and genetic activities in response to the shift in acetate/sulfide/nitrate loading.

## 2. Materials and methods

### 2.1. Experimental set-up

Three identical UASBs with an effective volume of 0.85 L was operated under the optimized acetate-C/nitrate-N mol ratio of 1/2 and sulfide-S/nitrate-N mol ratio of 5/6 [18]. The influent contained sulfide (200 mg $L^{-1}$), $NO_3^-$-N (127.5 mg $L^{-1}$), $Ac^-$-C (45 mg $L^{-1}$), $Ca^{2+}$ (25 mg $L^{-1}$), $Mg^{2+}$ (10 mg $L^{-1}$) and essential trace element. The influent loading was gradually increased as the hydraulic retention time (HRT) decreased from 8 h (condition I) to 4 h (condition II) and then 2 h (condition III). With the HRT of 8 h, influent concentration of acetate, nitrate and sulfide were 0.95, 0.79 and 0.34 kg $d^{-1}$ $m^{-3}$, respectively. With the HRT of 4 h, influent concentration of acetate, nitrate and sulfide were 1.84, 1.53 and 0.66 kg $d^{-1}$ $m^{-3}$, respectively. When the HRT reduced to 2 h, the influent concentration of acetate, nitrate and sulfide were 3.9, 3.26 and 1.4 kg $d^{-1}$ $m^{-3}$, respectively. The influent sulfide that ranged between 0.34–1.4 kg $d^{-1}$ $m^{-3}$, was referred from other studies [10,12]. The reactors were wrapped with electrothermal wire to maintain a consistent operating temperature of 30 ± 1 °C. Initially, the reactors were inoculated with an equal sludge (17 g TSS $L^{-1}$) from the anaerobic sludge thickener of the WenChang Wastewater Treatment Plant (Harbin, China). The micronutrients in 1 L of feed wastewater were as follows: 22 mg of $ZnSO_4$, 55 mg of $CaCl_2$, 50.6 mg of $MnCl_2{\cdot}4H2O$, 11 mg of $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$, 15.7 mg of $CuSO_4{\cdot}5H_2O$, and 16.1 mg of $CoCl_2{\cdot}6H_2O$. Bicarbonate (1 g $L^{-1}$) was employed to maintain the influent pH of 8.0 ± 0.3. The concentrations of acetate, nitrate and sulfide were determined at intervals until the treatment efficiency of the reactors achieved a steady state after more than 30 days.

### 2.2. Analytical methods

At steady state, influent and effluent samples (3–10 mL) were collected from the inlet and outlet of the reactors, and the concentrations of acetate, $NO_3^-$, $HS^-$, $SO_4^{2-}$, and $S_2O_3^{2-}$ were analysed. The pH and oxidation-reduction potential (ORP) of the liquid samples from inside the reactor were determined with a pH/ORP meter (FE20; Merrler Toledo, Switzerland). TSS was determined according to standard methods [19]. The concentrations of $H_2S$, $HS^{-1}$ and $S^{2-}$ were determined according to the methylene blue method [20]. The concentrations of sulfate, thiosulfate, nitrate, nitrite and acetate were measured by an ion chromatograph (ICS-90A; Dionex, USA) with a column (IonPac AG4A AS4A-SC 4 mm, Dionex, USA) after filtration through a millipore filter of 0.45 μm. The production of elemental sulfur in the effluent was calculated according to the following equation [21]: [$S^0$] = [Influent $HS^-$] + [Influent $SO_4^{2-}$] + [Influent $S_2O_3^{2-}$] – [Effluent $HS^-$] – [Effluent $SO_4^{2-}$] – [Effluent $S_2O_3^{2-}$]. All

of the results were calculated from the average of triplicate samples.

### 2.3. DNA extraction and 454 pyrosequencing

After a continuous run for approximately 30 days, samples (20 mL) in the UASB reactors at the heights of 10, 20, 30 cm were harvested and stored in 50 mL sterile plastic test tubes at −80 °C before DNA and RNA analysis. DNA was extracted using the PowerSoil DNA Isolation kit (MoBio Laboratories Inc, USA) according to the manufacturer's instructions. The concentration and purity of the extracted DNA were measured with a Nanophotometer (P-class, Implen, Germany). The bacterial V1–V3 region of the 16S rRNA gene was amplified using the forward primer 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and reverse primer 533R (5′-TTACCGCGGCTGCTGGCAC-3′). The PCR products were purified using the GeneJET™ PCR purification kit (Fermentas, USA) and then sequenced by the Illumina sequencing platform. The sequences obtained from Illumina sequencing were analysed following the pipelines of Quantitative Insights into Microbial Ecology (QIIME) software (www.microbio.me/qiime) as described by previous studies [22]. The taxonomic classification of each phylotype was determined using the SILVA rRNA database project with over 97% of sequence similarity. The 16S rRNA gene sequence data was deposited in the NCBI Sequence Read Archive under the accession number of SRP052221.

### 2.4. RNA isolation and quantitative reverse transcription PCR (qRT-PCR)

The total RNA was extracted using the RNA PowerSoil™ Total RNA Isolation Kit (MoBio Laboratories Inc, USA) in accordance with the manufacturer's instructions. The RNA concentration and purity was measured with a Nanophotometer (P-class, Implen, Germany). The total RNA of 2 μg was reverse transcribed using the PrimeScript™ II 1st Strand cDNA Synthesis Kit (TaKaRa, Japan) following the manufacturer's instructions. The concentration and purity of cDNA were measured with a Nanophotometer (P-class, Implen, Germany). The *q*RT-PCR was performed on an ABI 7500TM Real-Time PCR System (Applied Biosystems, CA, USA). Flavocytochrome *c* (FCC) and sulfide: quinone oxidoreductase (*SQR*) were two isolated cytochrome *c* oxidoreductase genes in charge of sulfide oxidization[18]. The gene encoding sulfide quinone reductase (*sqr*) during the sulfur oxidation process was amplified using the specific primer pair sqrF (GCTCGGCAGCCTCAATAC) and sqrR (GGTCGGACGGTGGTTACTG) [23]. *The nirK* gene encodes the copper-containing nitrite reductase during the nitrate reduction process. This enzyme played an important role when nitrate reduced into nitrite. The *nirK* gene was detected by the specific primer pair F1aCu (ATCATGGTSCTGCCGCG) and R3Cu (GCCTCGATCAGRTTGTGGTT) [24]. The *soxB* gene, encoding the SoxB subunit of the Sox enzyme system, was considered a fundamental and primordial molecular mechanism for sulfur oxidation, and it oxidizes sulfide, elemental sulfur and thiosulfate to sulfate. The specific primer sets for amplification of the *soxB* gene were 710F (ATCGGYCAGGCYTTYCCSTA)/1184R (MAVGTGCCGTTGAARTTGC) [25]. The *q*RT-PCR mixture (25 μL) comprised 1 × SYBR Green *q*PCR Mix (Tiangen, China), primer sets (200 nM for each) and approximately 3 ng of template cDNA. The amplification conditions included an initial denaturation step for 3 min at 95 °C followed by 35 cycles of denaturation for 5 s at 95 °C; annealing for 10 s at 56 °C (*sqr*), 55 °C (*soxB*), and 57 °C (*nirK*); and extension for 20 s at 72 °C. The detailed PCR procedures for the amplification of the *sqr*, *soxB* and *nirK* genes were described in detail in the previous studies [23–25]. Calibration curves (log DNA concentration versus an arbitrarily set cycle threshold value) for the *sqr*, *soxB* and *nirK* genes were constructed using serial dilutions of the amplicons of single colonies. The gene copy number of the amplicon was calculated by multiplying the molar concentration of the amplicon by Avogadro's constant. The efficiencies of the real-time PCR assays were over 98%, and the $r^2$ value was 0.99. All experiments were performed in triplicate.

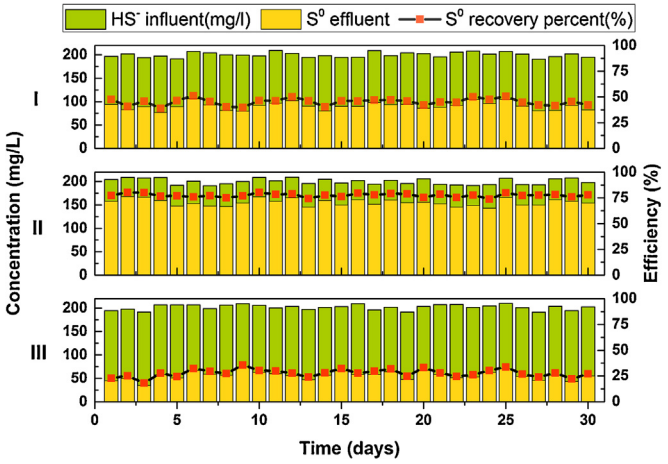


**Fig. 1.** Performance of $S^0$ recovery under different influent loadings of acetate/nitrate/sulfide in the UASB reactors during the continuous run for 30 days. Condition I: the loadings of acetate, nitrate and sulfide were 0.95 kg d$^{-1}$ m$^{-3}$, 0.79 kg d$^{-1}$ m$^{-3}$ and 0.34 kg d$^{-1}$ m$^{-3}$, respectively; Condition II: the loadings of acetate, nitrate and sulfide were 1.84 kg d$^{-1}$ m$^{-3}$, 1.53 kg d$^{-1}$ m$^{-3}$ and 0.66 kg d$^{-1}$ m$^{-3}$, respectively; Condition III: the loadings of acetate, nitrate and sulfide were 3.9 kg d$^{-1}$ m$^{-3}$, 3.26 kg d$^{-1}$ m$^{-3}$ and 1.4 kg d$^{-1}$ m$^{-3}$, respectively.

## 3. Results

### 3.1. UASB performances under the different acetate/nitrate/sulfide loadings in the influent

The steady running results of $S^0$ recovery and the removal efficiency of acetate/nitrate/sulfide are shown in Fig. 1 and Table 1. At the steady state of condition I (HRT = 8 h; acetate = 0.95 kg d$^{-1}$m$^{-3}$; nitrate = 0.79 kg d$^{-1}$ m$^{-3}$; sulfide = 0.34 kg d$^{-1}$ m$^{-3}$), the removal rate of acetate, nitrate and sulfide approached 100%, but the $S^0$ recovery rate was relatively low, achieving only 45.1%, and it was accompanied by a large amount of generated $SO_4^{2-}$ and $S_2O_3^{2-}$ (109.8 mg L$^{-1}$) (Fig. 1). As the loading ratio increased (condition II, HRT = 4 h; acetate = 1.84 kg d$^{-1}$m$^{-3}$; nitrate = 1.53 kg d$^{-1}$ m$^{-3}$; sulfide = 0.66 kg d$^{-1}$ m$^{-3}$), $S^0$ generation was substantially improved to 77.9% with a low accumulation of sulfate and thiosulfate (44.8 mg L$^{-1}$); meanwhile, the removal of acetate, nitrate and sulfide were stably maintained at 100% (Fig. 1; Table 1). However, as the loading of acetate/nitrate/sulfide further increased (condition III) to 3.9, 3.26 and 1.4 kg d$^{-1}$ m$^{-3}$ (HRT = 2 h), neither acetate nor nitrate could be completely removed. A large amount of sulfate and thiosulfate were generated (93.6 mg L$^{-1}$), and the $S^0$ generation rate decreased to 27.9% (Fig. 1; Table 1). The results indicated that sulfide partial-oxidation or over-oxidation occurred as the influent loading further decreased or increased.

### 3.2. Bacterial diversity and community composition under different acetate/nitrate/sulfide loadings in the influent

The Illumina high-throughput sequencing was adopted to determine the spatial abundance and diversity of bacterial composition at different heights of the UASB reactor (Fig. 2). More than 26 types of bacterial genera (relative abundance ≥ 1%) were obtained

**Table 1**
Average concentration of acetate, nitrate and sulphur ($SO_4^{2-}$, $S_2O_3^{2-}$, $S^{2-}$ and $S^0$) in effluent under different loadings in the UASB reactors at steady state.

| Running reactor | Acetate-C (mg $L^{-1}$) | Nitrate-N (mg $L^{-1}$) | $SO_4^{2-}$-S and $S_2O_3^{2-}$-S (mg $L^{-1}$) | $S^{2-}$ (mg $L^{-1}$) | $S^0$ (mg $L^{-1}$) | Recovery of $S^0$ (%) |
|---|---|---|---|---|---|---|
| I | 0 | 0 | 109.8 ± 5.4 | 0 | 90.4 ± 5.8 | 45.1 ± 6.0 |
| II | 0 | 0 | 44.8 ± 4.8 | 0 | 155.2 ± 6.3 | 77.9 ± 3.6 |
| III | 11.34 ± 4.1 | 5.25 ± 3.3 | 93.6 ± 8.5 | 51.7 ± 10.3 | 56.7 ± 3.9 | 27.9 ± 3.2 |

The data were the average results from triplicate samples with the standard deviation shown on the right side of "±".

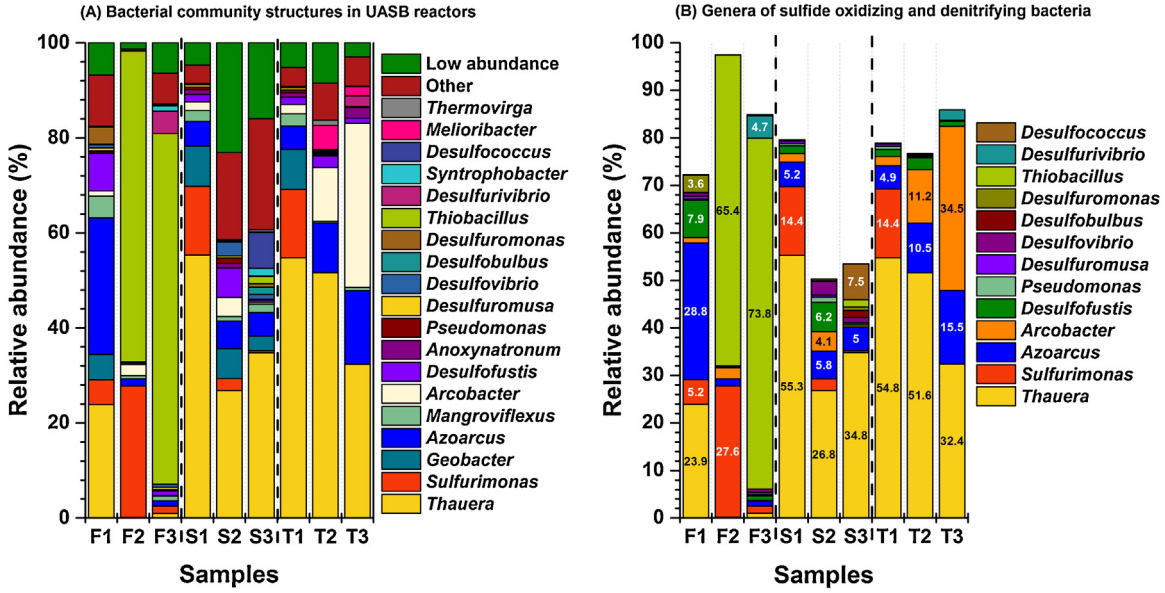


**Fig. 2.** Bacterial community structures by genus in the UASB reactors corresponding to different sample heights. F1, F2 and F3 indicate the heights of 10 cm, 20 cm and 30 cm in condition I of the UASB reactor; S1, S2 and S3 indicate the heights of 10 cm, 20 cm and 30 cm in condition II of the UASB reactor; and T1, T2 and T3 indicate the heights of 10 cm, 20 cm and 30 cm in condition III of the UASB reactor.

in total, with 16, 16 and 21 types in conditions I, II and III, respectively (Fig. 2A). Under the low influent loading (condition I), the bacterial composition was more diverse at 10 cm than at 20 and 30 cm. Among them, the predominant denitrifying and sulfide-oxidizing bacteria which converted sulfide to $S^0$ and $NO_3^-$ to $N_2$ were *Azoarcus* (28.8%) and *Thauera* (23.9%) at the first 10 cm; meanwhile, *Thiobacillus* was dominant at both 20 cm (65.4%) and 30 cm (73.8%) (Fig. 2B) [26,27]. Compared with condition I, the bacterial distribution was relatively homogeneous along with the reactor in conditions II and III (Fig. 2A). In condition II, *Thauera* was the most abundant genus which was highly distributed at 10 cm (55.3%) and then gradually decreased with increasing height (26.8% at 20 cm; 34.8% at 30 cm). In addition, *Sulfurimonas* sp., one of the denitrifying and sulfide-oxidizing bacteria that transfer sulfide to thiosulfate or sulfate with nitrate as an electron acceptor, was also dominant at 10 cm (14.4%) [28]. However, the occupied genera related to sulfide-oxidizing bacteria were less abundant as the height increased to 20 and 30 cm. In condition III, *Thauera* (54.8%, 10 cm; 51.6%, 20 cm; 32.4%, 30 cm) was still the most dominant genus, and in addition, *Arcobacter* (2.0%, 10 cm; 11.2%, 20 cm; 34.4%, 30 cm) and *Azoarcus* (4.9%, 10 cm; 10.5%, 20 cm; 15.5%, 30 cm) were selectively predominant at the different reactor heights. Among them, *Arcobacter* was able to produce filamentous sulfur as the end product of the sulfur cycle [29]. This indicated that the diversity of autotrophic/heterotrophic denitrifying and sulfide-oxidizing bacteria at the heights of 20 and 30 cm responded to the different nutrient supplementation of the lower (condition I) and higher (condition II and III) influent loading. The results of the spatial bacterial diversity and structure composition decided the removal efficiency and $S^0$ recovery rate.

### 3.3. Quantitative expression of the genes related to denitrification and sulfide oxidation under different acetate/nitrate/sulfide loadings in the influent

The *q*RT-PCR was conducted to estimate the expression of functional genes, including *nirK*, *sqr* and *soxB*, during the denitrification and sulfide oxidation processes from the different reactor heights and acetate/nitrate/sulfide loadings (Fig. 3). The efficiency values were 0.97, 0.98 and 0.98 for the *nirK*, *sqr* and *soxB* genes, respectively, with an $R^2$ value of 0.99. From 10 to 30 cm in condition I, the expressed *nirK* gene was gradually decreased with the log values ranging from 4.0 to 1.5. When influent loading was further increased (condition II and III), the expressed *nirK* was gradually increased, manifesting the enhanced denitrification activity. In addition, the spatial distribution of *nirK* was rather homogenous in conditions II and III, demonstrating the extended denitrification activity from the lower to the higher part of the reactor.

Flavocytochrome *c* (FCC) and quinone oxidoreductase (SQR) were two isolated cytochrome *c* oxidoreductase genes responsible for sulfide oxidation; however, FCC does not often occur in a variety of sulfur-oxidizing bacteria [30]. Thus, the gene activity representing the process of sulfide oxidation to $S^0$ was indicated by the *sqr* gene in this study. In condition I, the expressed *sqr* gene was relatively higher in 20 cm (log value 2.1) compared with 10 cm (log value 1.6) and 30 cm (log value 0.9), suggesting that high $S^0$ generation activity occurred at the height of 20 cm. As the influent loading increased in condition II, the expressed *sqr* gene was improved with the log value ranging from 3.4 to 4.2 (Fig. 3), reflecting the high sulfide oxidation activity. The result was consistent with the high $S^0$ recovery rate as shown in Fig. 1. In comparison, the expressed *sqr* gene was much lower with the log values ranging from 1.6 to 1.2 in

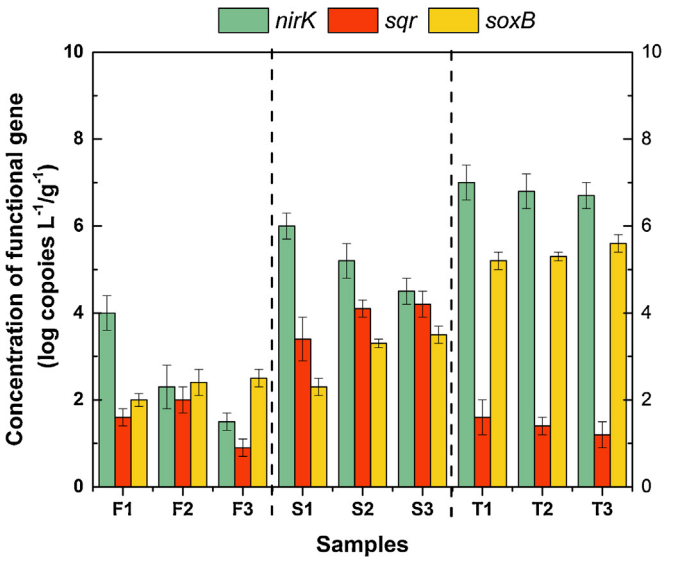


**Fig. 3.** Quantitative expression of genes related to denitrification and sulfide oxidation, including *nirK*, *sqr* and *soxB* under different loadings of acetate/nitrate/sulfide in the UASB reactors. F1, F2 and F3 indicate the heights of 10 cm, 20 cm and 30 cm in condition I of the UASB reactor; S1, S2 and S3 indicate the heights of 10 cm, 20 cm and 30 cm in condition II of the UASB reactor; and T1, T2 and T3 indicate the heights of 10 cm, 20 cm and 30 cm in condition III of the UASB reactor.

condition III, which manifested low sulfide oxidation activity. The *SoxB* gene was utilized as an indicator of sulfide oxidation, $S^0$ and $S_2O_3^{2-}$ to $SO_4^{2-}$. As the acetate/nitrate/sulfide loading increased, the expressed *soxB* gene was increased stepwise from 2.0 to 5.6 (log value), manifesting the lifting activity of $SO_4^{2-}$ generation (Fig. 3).

## 4. Discussion

In this study, the impact of influent loading (condition I, II and III) on both the reactor performance and bacterial community and function were thoroughly investigated under the optimized acetate/nitrate/sulfide ratio of 3:6:5 [18]. The study provided suggestions on how influent loading regulated reactor performance and impacted the bacterial community, spatial distribution and function. The study gave suggestions to regulate the $S^0$ recovery through optimization of the influent loading.

Three influent loading parameters were set up in this study: deficient nutrients as condition I, sufficient nutrients as condition II, and excess nutrients in condition III. In condition I, although all acetate, nitrate and sulfide could be removed, the $S^0$ recovery rate was much lower (45.1%). When both acetate and nitrate were insufficient, $S^0$ was regarded as an energy storage polymer in prokaryotes. $S^{2-}$ was converted to $S^0$ and then further oxidized to $SO_4^{2-}$ under autotrophic conditions, which was conducted by the most abundant genera, *Azoarcus* and *Thiobacillus*. At the height of 30 cm, the expressed *sqr* was much lower compared with at 10 cm and 20 cm, reflecting the low sulfide oxidation activity. The estimated bioprocesses of the functional bacteria under condition I (nutrient insufficient) were found to be as follows: sulfide was oxidized to $S^0$, and then $S^0$ was further oxidized to sulfate/thiosulfate, as verified by the abundance of chemolithoautotroph sulfide-oxidizing bacteria, such as *Thiobacillus* and *Sulfurimonas* [28,29]. The hypothetical transformation equations were listed as follows: $x\,S^{2-} + y\,CO_2 + z\,H^+ \rightarrow x\,S^0 + y$ Organic carbon $+ z/2\,H_2O$; $x\,S^0 + y\,CO_2 + z\,H_2O \rightarrow x\,SO_4^{2-} + y$ organic carbon $+ 2z\,H^+$ [29].

Under the sufficient nutrient condition (condition II), the $S^0$ yield was improved to 77.9%; meanwhile, the complete removal of acetate, nitrate and sulfide was stably maintained. Within the bacterial community, *Thauera* and *Sulfurimonas* became the dominant genera instead of *Azoarcus* and *Thiobacillus*. The *sqr* gene was highly expressed through the reactor, and the high quantity of the expressed *sqr* gene also confirmed the high activity of sulfide oxidation to $S^0$ compared with the other two conditions. In comparison, the expressed *soxB* gene was lower; however, it improved as the influent loading increased compared with condition I. The log *sqr*/*soxB* value was 1.48, 1.24 and 1.24 for the heights of 10 cm, 20 cm and 30 cm, respectively, indicating the higher sulfide oxidation activity to $S^0$ than to sulfate/thiosulfate. The estimated bioprocesses by the functional bacteria under condition II (nutrient sufficient) were found to be as follows: first of all, most of the dominate genera (*Thauera*, *Sulfurimonas* and so on) were capable of oxidizing $S^{2-}$ to $S^0$ when $NO_3^-$ was applied as the electron acceptor ($S^{2-} + 0.4NO_3^- + 1.2H_2O \rightarrow S^0 + 0.2N_2 + 2.4OH^-$) [22]. Meanwhile, nitrate may participate in acetate degradation ($NO_3^- + 0.63CH_3COO^- + 0.37CO_2 \rightarrow 0.5N_2 + 0.13H_2O + 1.63HCO_3^-$) as confirmed by the abundance of the nitrate-reducing genus *Azoarcus* and highly expressed *nirK* (Figs. 2 and 3). Under the optimized influent loading (condition II), the spatial distribution of bacterial community and functional genes were rather uniform throughout the reactor (Figs. 2 and 3), indicated the fine running status of the reactor.

As the influent loading further increased (excess nutrients, condition III), the presence of sulfide-oxidizing and denitrifying bacterial genera (*Thauera* and *Azoarcus*) was higher than condition II (Fig. 2B); however, acetate, nitrate and sulfide could not be completely removed; meanwhile, the $S^0$ yield in the effluent decreased to 27.9%. This result was probably because the high loading exceeded the treatment capacity of the UASB reactor, the high residue of nitrate led to nitrate reduction, and the over-oxidation of sulfide to $S^0$ occurred as the major bioprocess. The highly expressed *nirK* and *soxB* genes and the less expressed *sqr* genes compared with condition II also verified the lifting activities of nitrate reduction and $SO_4^{2-}$ generation and the decreased activity of sulfide oxidation to $S^0$. The estimated bioprocesses by the functional bacteria under condition III (excess nutrient) were found to be as follows: $S^{2-} + 1.6NO_3^- + 1.6H^+ \rightarrow SO_4^{2-} + 0.8N_2 + 0.8H_2O$; $S^0 + 1.2NO_3^- + 0.4H_2O \rightarrow SO_4^{2-} + 0.6N_2 + 0.8H^+$ [22]. In addition, a probable explanation is that the rate of electron production from the TCA cycle is slower than sulfide oxidation. The sulfur cycle could be a supplement electron donor for denitrification because complete sulfide oxidation could supply more electrons for denitrification (i.e. $S^{2-} \rightarrow S^0$, 2e; $S^{2-} \rightarrow SO_4^{2-}$, 8e). The results proved that in the absence of nutrients the predominant microorganism was affiliated with autotrophic bacteria, whereas the abundance of heterotrophic bacteria was higher when supplemented with excess nutrients. Therefore, it is inferred that the influent loading played an important role in the UASB reactor because it largely altered both the reactor performance and the spatial bacterial community and functional activity. The optimization of influent loading to a sufficient nutrient condition by avoiding the deficient and excess nutrient conditions would largely enhance the $S^{2-}$ recovery efficiency and maximize the abundance and activities of denitrifying and sulfide-oxidizing bacteria.

Optimization of influent loading to nutrient sufficient condition by avoiding the nutrient deficient and over sufficient conditions would largely enhance the $S^{2-}$ recovery efficiency and maximize the abundance and activities of denitrifying sulfide-oxidizing bacteria.

## 5. Conclusions

The change in influent loading largely impacted both $S^0$ recovery and the removal efficiencies of acetate, sulfide and nitrate; the change also significantly altered the bacterial community structure and genetic activity in the UASB reactor. The highest $S^0$ recovery

was achieved at 77.9% under the acetate, nitrate and sulfide loading of 0.95, 0.79, and 0.34 kg $d^{-1}$ $m^{-3}$, respectively, corresponding with the predominance of *Thauera*, *Sulfurimonas*, and *Azoarcus* at 0–30, 0–10 and 20–30 cm, respectively, and the highly expressed *sqr* gene throughout the reactor height. The dominant bacterial community switched into an autotrophic sulfide oxidation mode or over-oxidation mode as evidenced by the domination of *Thiobacillus* (20–30 cm) or *Thauera* and *Azoarcus* (0–30 cm), as the influent loading was halved or doubled, respectively. The processes were accompanied by either low *sqr* genetic activity or highly expressed *nirK* and *sox* genes. The study provided suggestions for the effective control of the DSR process and $S^0$ recovery activity through regulation of the influent acetate/nitrate/sulfide loading.

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