Microbial community structure and function in response to the shift of sulfide/nitrate loading ratio during the denitrifying sulfide removal process

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Optimized $S^{2-}/NO_3^-$ (S/N) molar ratio was 5/6 for $S^0$ recovery and nitrate removal.
Bacterial community and genetic activity remarkably modified as S/N ratio altered.
Desulfurization and denitrification genera was predominant at S/N ratio of 5/6.
Autotrophic $S^{2-}$ oxidation genera dominated and functioned under lower S/N ratios.
$NO_3^-$ reduction and $S^0$ over oxidation genera functioned with higher S/N ratios.

1. Introduction
Nitrate and sulfide compounds are frequently detected in sewage, refinery and pharmaceutical industry (Show et al., 2013; Yuan et al., 2014). They gave rise to the serious contamination in
many well-known forms based on their varied valent states – ammonia, nitrous acid, nitrate (NO$_3^-$), sulfate, sulfur dioxide, sulfide (S$^2-$), elemental sulfur (S$^0$) and etc. (Pikaa et al., 2014; Pokorna and Zabranska, 2015); among all of them, S$^2-$ and NO$_3^-$ are the most common forms as contaminants. When both S$^2-$ and NO$_3^-$ were present, sulfide oxidizing bacteria (SOB) was able to transfer S$^2-$ to S$^0$ by utilizing NO$_3^-$ as an electron acceptor, called the denitrifying sulfide removal (DSR) process (Wang et al., 2005); and the process supplies an effective means to recover S$^0$ and simultaneously remove of NO$_3^-$ and C/N molar ratio plays key roles in obtaining the high recovery and kinetics of DSR process based on eight SOB isolated strains.

Therefore, the extensive studies have conducted on the simultaneous removal of organic carbon, sulfite and nitrate (Chen et al., 2008a; Pokorna and Zabranska, 2015), focused on the high S$^0$ recovery rate under the different operating conditions and C/N/S ratios. Cai et al. (2008) reported that the S/N molar ratio controlled the S$^0$ recovery rate under the different Ac/NO$_3^-$ ratios with the intermittent mix at the rate of approximately 150 rpm. The trace element solution was continuously fed into the influent with a plunger pump (iPump2S, Baoding, China), with the detailed chemical composition described by Chen et al. (2008a). Bicarbonate (1 g L$^{-1}$) was employed to maintain the influent pH of 8.0 ± 0.3. All of the CSTR reactors were operated at a fixed HRT of 24 h. Concentration of Ac-C, NO$_3^-$ and S$^2-$ were determined at intervals until the reactors treatment efficiency achieved the steady state after more than 20 days.

2.2. Analytical methods

After achieved the steady state, influent and effluent samples (3–10 mL) were collected from inlet and outlet of the reactor and concentrations of acetate, NO$_3^-$, S$^2-$, SO$_3^{2-}$ and S$^0$ were continuously analyzed. The pH and oxidation–reduction potential (ORP) of liquid samples inside the reactor were determined with a pH/ORP meter (FE20; Merck Toledo, Switzerland). TSS was determined according to the standard methods (APHA, 1998). Concentrations of H$_2$S, HS$^-$ and S$^2-$ were determined according to the methylene blue method (Triuper and Schlegel, 1964). Concentrations of sulfate (SO$_4^{2-}$), thiosulfate (S$_2$O$_3^{2-}$), sulfite (SO$_3^{2-}$), nitrate (NO$_3^-$) and nitrite (NO$_2^-$) were measured by an ion chromatography (ICS-90A; Dionex, USA) after filtration with the Millipore filter of 0.45 μm. S$^0$ was analyzed according to the method described by Jiang et al. (2009). Briefly, elemental sulfur and sulfite were converted to thiosulfate at high pH, which was analyzed for S$_2$O$_3^{2-}$ by ion chromatograph. The final concentration of S$^0$ was calculated from the concentration of S$_2$O$_3^{2-}$ according to the reaction stoichiometry.

2.3. DNA extraction and 454 pyrosequencing

After continuous running for about 50 days, samples (3–10 mL) were harvested from the middle of the five reactors with a sterilized sample spoon and stored in a 50 mL sterile plastic test tubes at −80 °C before went for DNA and RNA analysis. DNA was extracted using the PowerSoil DNA Isolation kit (MoBio Laboratories Inc, USA) according to the manufacturer’s instructions. Concentration and purity of the extracted DNA were measured with Nanophotometer (P-class, Implen, Germany). Bacterial V1-V3 region of 16S rRNA gene was amplified using the forward primer 5′-AGAGTTTGATCCTGGCTCAG-3′ and reverse primer 533R (5′-TACCCTGCGTCACTCC-3′). PCR products were purified using GeneJET™ PCR purification kit (Fermentas, USA) and then went for pyrosequencing on the 454 Genome Sequencer FLX platform. The sequences obtained from 454 pyrosequencing were analyzed following the pipelines of Quantitative Insights into Microbial Ecology (QIME) software (www.microbio.me/qime) as described by the previous studies (Caporaso et al., 2010; Loudon et al., 2014). Taxonomic classification of each phylotype was determined.
using the SILVA rRNA database project with over 97% of sequence similarity, as suggested by Wang et al. (2007). The 16S rRNA gene sequence data were deposited in NCBI Sequence Read Archive under the accession number of SRR2136643.

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

The total RNA was extracted using RNA PowerSoil™ Total RNA Isolation Kit (MoBio Laboratories Inc, USA) in accordance with the manufacturer’s instructions. RNA concentration and purity was measured with a Nanophotometer (P-class, Implen, Germany). The total RNA of 2 μg was reversely transcribed using PrimeScript™ II 1st Strand cDNA Synthesis Kit (TaKaRa, Japan) following manufacturer’s instruction. Concentration and purity of the cDNA were measured with Nanophotometer (P-class, Implen, Germany). The qRT-PCR was performed on an ABI 7500TM Real-Time PCR System (Applied Biosystems, CA, USA). Gene encoding sulfide quinone reductase (sqr) during sulfur oxidation process was amplified using the specific primer pairs of sqrF (GGTCGGACGGTGGTTACTG) and sqrR (GGTCGGACGGTGGTTACTG) (Yin et al., 2014). nirK gene encodes the copper-containing nitrite reductase during nitrate reduction process. This enzyme played an important role when nitrate reduced into nitrite. NirK gene was detected by specific primer pairs of F1aCu (ATCATGGTSCTGCCGCG) and R3Cu (GCCTCGAT-CAGRTTGTTGTT) (Throbäck et al., 2004). SoxB gene, encoding SoxB subunit of the Sox enzyme system, considered as a fundamental and primordial molecular mechanism for sulfur oxidation, which oxidize sulfide, elemental sulfur and thiosulfate to sulfate. The specific primer sets for amplification of soxB gene were 710F (ATCGGACCCTCCTCGCG) and 1184R (MAVGTGCCGTTGAARTTG) (Tourna et al., 2014). The qRT-PCR mixture (25 μL) consisted of 1 × SYBR Green qPCR Mix (Tiangen, China), primer sets (200 nM for each) and about 3 ng of template cDNA. The PCR procedures for amplification of sqr, soxB and nirK genes were described in detail in the previous studies (Yin et al., 2014; Throbäck et al., 2004; Tourna et al., 2014). Calibration curves (log DNA concentration versus an arbitrarily set cycle threshold value) for sqr, soxB and nirK genes were constructed using serial dilutions of amplicons from single colonies. Gene copy number of the amplicon was calculated by multiplying the molar concentration of the amplicon by Avogadro’s constant. Efficiencies of real-time PCR assays were over 95% and r² were 0.99. All experiments were performed in triplicates.

### 3. Results and discussion

#### 3.1. CSTR performances with the different S²⁻/NO₃⁻ ratios

In this study, Ac-C/NO₃⁻ ratio was fixed at 1/2 to insure the complete transformation of NO₃⁻ to N₂ gas (Lee and Wong, 2014), and while, S²⁻/NO₃⁻ ratios were altered to determine the optimized condition for S₀ recovery. In C-I (S²⁻/NO₃⁻ ratio = 5/2; S²⁻/Ac-C ratio = 5/1), acetate, NO₃⁻ and S²⁻ were completely removed, but the recovery rate of S₀ only achieved at 24.0%, accompanied with a large amount of SO₄²⁻ generated (153.5 mg L⁻¹) (Fig. 1). As the loading ratios of acetate and nitrate increased (C-II and C-III), S₀ generation rate was gradually improved and correspondingly, the produced SO₄²⁻ was decreased, which was probably caused by the increased electron acceptor (NO₃⁻) supply for sulfide oxidation (Fig. 1 and Table 2). The highest yield of S₀ (84.4%) was observed at C-III. Here, the produced sulfate was the lowest (31.6 mg L⁻¹) and while, the removal rates of acetate, NO₃⁻ and S²⁻ were maintained at 100% (Fig. 1 and Table 2), indicating an optimal running condition with the balanced molar concentration of acetate, S²⁻ and NO₃⁻. However, as the loading ratios of acetate and NO₃⁻ further increased (C-IV and C-V), neither acetate nor NO₃ could be completely consumed (Table 2). Meanwhile, a large amount of SO₄²⁻ was generated and the S₀ generation rate was quickly dropped to 12.3% and 2.9% at C-IV and C-V, respectively. The above results gave information on the optimized S²⁻/NO₃⁻ ratio of 5/6 when Ac-C/NO₃⁻ ratio was fixed at 1/2, and in contrast, S²⁻ over oxidation was occurred as the S²⁻/NO₃⁻ ratio decreased or increased. Previously, Cai et al. (2008) evaluated the optimized S²⁻/NO₃⁻ ratio as 5/2 for selectively generation of S₀ and N₂ gas, and however, the Ac-C/NO₃⁻ ratio was not clearly elaborated. Cardoso et al. (2006) discovered the depressed S₀ generation rate with the increase of S/N molar in a UASB reactor by fixing at C/N molar ratio of 1/1, but the removal rates of NO₃⁻ was not mentioned.

#### 3.2. Bacterial diversity and community composition with the different S²⁻/NO₃⁻ ratios

The 454 pyrosequencing was adopted to determine the abundance and diversity of bacterial populations in these reactors. Over 10,000 qualified sequences were produced with an average length of 450 bps for each bacterial community (Supplementary Table 1).
information Fig. S1). More than 35 types of bacterial genus were recovered altogether, and among all of them, bacterial communities in C-I and C-V have a relative higher diversity with Shannon indices of 3.31 and 4.04, respectively, compared with C-II, C-III and C-IV with Shannon indices varying from 2.02 to 2.73 (Table 3).

The obvious different bacterial composition was observed between C-I to C-V based on the different loading ratios of acetate and nitrite (Fig. 2). C-I sample was dominated with *Arcobacter* \(20.8\%\), *Desulfobulbus* \(14.9\%\) and *Thermovirga* \(10.7\%\). Among of them, *Arcobacter* was able to oxidize sulfide autotrophically into filamentous sulfur and simultaneously fix carbon dioxide to organic compounds (Wirsen et al., 2002). *Thermovirga* was anaerobic sulfur reducing bacteria that utilizing organic acid as carbon source and electron donor (Göker et al., 2012), and *Desulfobulbus* was able to reduce both sulfate and sulfite (Laanbroek et al., 1984). Microbial community structures were similar in C-II to C-III, that predominated with *Thiobacillus* \(31.5–31.6\%\), *Enterobacter* \(27.2–4.8\%\), *Stappia* \(13.4–26.7\%\), *Rhizobium* \(4.0–7.0\%\), and *Thauera* \(2.6–10.2\%\), respectively. Among of them, sulfide oxidation and denitrification bacteria which converted sulfide to \(S_0\) and \(NO_3^-/C_0\) to \(N_2\) were predominant, including genera of *Thauera*, *Enterobacter*, *Thiobacillus* and *Stappia* (Liu et al., 2006, 2015; Meyer et al., 2007; Schedel and Trüper, 1980). Bacterial community structures of C-IV and C-V were much different from the others, that C-IV was occupied with genera of *Ochrobactrum* \(54.1\%\), *Stenotrophomonas* \(16.7\%\) and *Flavobacterium* \(10.4\%\), while, C-V was predominant with genera of *Exiguobacterium* \(61.8\%\), *Anaerolineaceae* \(8.5\%\) and *Leptolinea* \(3.9\%\). Of which, *Leptolinea*, *Exiguobacterium*, *Flavobacterium*, and *Ochrobactrum* were in charge of denitrification or sulfate reduction under heterotrophic condition (Frühling et al., 2002; Kitamikado et al., 1981; Mahmood et al., 2009; Yamada et al., 2006). These clear differences indicated
the variation of acetate and nitrate loading ratio markedly influenced the bacterial diversity and community composition.

3.3. LefSe analysis of bacterial community structures with the different $S^2/\text{NO}_3$ ratios

LefSe was adopted to obtain the more insights of the differentiation and internal interactions of the determined bacterial affiliation in samples C-I to C-V, with the taxonomic tree generated as shown in Fig. 3. The cladogram showed taxa with LDA values was higher than 4.0 for clarity (Fig. S2). Phylum Proteobacteria was predominated in C-II, C-III, C-IV and C-V, and while, Bacteroidetes, Thermodontae and Spirochetes were enriched in C-III, C-IV and C-V. In contrast, at the fine taxonomy levels, C-I were consisted of Firmicutes and Chloroflex. Although the communities in C-II and C-III were similar (Fig. 2), their bacterial lineages were different. In C-II, 6 fine lineages had an LDA value of 4 or higher in phylum of Proteobacteria, and family Enterobacteriales was also enriched. In comparison, sample C-III was consisted of families of Hydrogenophilaeeae, Rhodocyclaceae, Rhodobacteraceae, Rhodobactaceae, and Porphyromonadaceae. LefSe highlighted the remarkable differences of bacterial community membership between samples C-I to C-V.

3.4. CCA diagram analysis

CCA diagram was applied to describe the correlations between the dominant bacterial genera and applied factors of acetate, $\text{NO}_3$ and $S^2$ in samples C-I to C-V, as shown in Fig. 4. Dominated genera in C-I (including Arcobacter, Desulphobulbus and Thermoviga) located on negative direction of horizontal axis were negatively correlated with acetate and $\text{NO}_3$. On the contrary, C-IV and C-V affiliated genera, such as Leptolinea, Exiguobacterium, Flavobacterium, Odoribacterium, and Parabacteroides, were distributed in the direction lines (or the extension lines) of acetate and nitrate, indicating their involvement of the removals of acetate and $\text{NO}_3$ in C-II and C-III affiliated genera, including Thauera, Enterobacter, Thiolellacillus and Stappia, were distributed either along with or closed to sulfide line. The positive correlation of $S^2$ and the abundant genera, explains the improved $S^0$ yield rates at C-II and C-III samples.

3.5. Quantitative expression of denitrification and sulfide oxidation related genes with the different $S^2/\text{NO}_3$ ratios

The qRT-PCR was conducted to estimate the expressed functional genes, including nirK, sqr and soxB, during the denitrification and sulfide oxidation processes with samples C-I to C-V (Fig. 5). Efficiency values were 0.98, 0.97 and 0.98 for nirK, sqr and soxB genes, respectively, with $R^2$ value of 0.99. From C-I to C-V, the expressed nirK gene was gradually increased from log values of 3.1 to 11.8, indicated the enhanced denitrification activities based on the high loading ratios of $\text{NO}_3$ and acetate. Sulfite oxidized to $S^0$ was targeted specifically using sqr gene. Expressed sqr gene was relatively higher in C-II (log value 5.1) and C-III (log value 6.9) compared with samples of C-I, C-IV and C-V, suggesting the high $S^3$ oxidation activity, which was coincident with the high recovery rate of $S^0$ in these two conditions (Fig. 1). In contrast, the expressed soxB gene in C-IV and C-V were much lower, with the log values of 1.4 and 0.9, reflecting the low $S^2$ oxidation activity. Presence of soxB gene was utilized as an indicator of oxidation of $S^2$, $S^0$ and $S_2\text{O}_3^-$ to $\text{SO}_4^2$. The gradual increase of the expressed soxB gene stepwise from 3.6 to 6.4 (log value) from C-I to C-V manifested the gradual lifting activity of $\text{SO}_4^2$ generation as the loading ratios of electron acceptor ($\text{NO}_3$) and organic carbon (Ac-C) increased. QRT-PCR analysis further confirmed the high $S^0$ generation activity under C-II and C-III and the high denitrification and sulfate generation activities under C-IV and C-V.

3.6. Discussion

The DSR process has shown a great potential in wastewater treatment application, since it transfers $S^2$ and $\text{NO}_3$ to completely water-insoluble $S^0$ and $\text{N}_2$ gas, and generates no secondary contaminant to water body (Chen et al., 2008a; Wang et al., 2005).
Fig. 3. Taxonomic tree generated using the LEfSe online software highlighting the biomarkers that statistically differentiated the samples under the different S\(^2\)/NO\(_3\) ratios. I: S/N ratio = 5/2, II: S/N ratio = 5/4, III: S/N ratio = 5/6, IV: S/N ratio = 5/8, V: S/N ratio = 5/9. The Ac-C(NO\(_3\))-N ratio was kept at 1/2 under all the conditions.

Fig. 4. Canonical correspondence analysis (CCA) comparing the correlations of bacterial community dynamics and applied acetate, NO\(_3\) and S\(^2\) applied in the under five operational conditions. The eigenvalues of horizontal and vertical axes equal to CCA1 of 35.66% and CCA2 of 29.21%. Each sample is represented by blue point; each genus is represented by a colored point (green for C-I, yellow for C-II and C-III, purple for C-IV, and brown for C-V), accompanied by the genus name. Environmental variable are indicated by red lines with variable names (in red) at the end. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
However, on account of the alterable valent states and multiple redox potentials of both S and N, exploration of the effective solution to regulate the DSR process and avoid the unwanted or unnecessary biochemical reactions, like $S^{2-}$ over oxidation or sulfite reduction, has become a tough issue urgently to be addressed. The previous studies majorly stressed on the physiological and kinetic behavior during nitrate removal and $S^0$ recovery (Chen et al., 2008a; Huang et al., 2015; Krishnakumar et al., 2005; Reyes-Avila et al., 2004), and however, functional microbes and genes in associated with $S^{2-}$ and NO$_3^-$ conversion processes were rarely concerned. For the first time, the study elaborated the bacterial community and genetic activity in response to the shift of Ac-C/NO$_3^-$/$S^{2-}$ ratio. The analysis of bacterial abundance and diversity and functional genes confirmed that the shift of $S^{2-}$/NO$_3^-$ ratio led to the obvious alteration of microbial community structure (Figs. 2–4) and genetic activity (Fig. 5) and gave hints on how the bacterial communities regulate the DSR process (Fig. 1 and Table 2).

$S^0$ yield rate was gradually improved as $S^{2-}$/NO$_3^-$ ratio increased in C-II and achieved the highest at C-III. Meanwhile, removal rates of NO$_3^-$ and acetate approached to 100% under these two conditions, suggested an optimized running condition with $S^{2-}$/NO$_3^-$ ratio of 5/6 (Fig. 1 and Table 2) in this study. Here, the estimated bioprocesses and the potential function of these dominant genera were revealed as follows: first of all, most of dominate genera (Thiobacillus, Enterobacter, Stappia, and Thauera) in C-II and C-III (Figs. 2 and 4) were capable of oxidizing $S^{2-}$ to $S^0$ as electron acceptor ($S^{2-}$ + yCO$_2$ + 2H$^+$ → $S^0$ + yCO$_2$ + 2H$^+$; $S^{2-}$ + H$_2$O → $xSO_4^{2-}$ + y organic carbon + 2H$^+$ (Wirsen et al., 2002). The expressed nirK was the lowest in C-I (Fig. 5), confirmed the low nitrate reducing activity. Since Desulfoviblbus were also dominant, it is estimated sulfate reduction was also possibly occurred ($SO_4^{2-}$ + CH$_3$COO$^-$ + H$^+$ → $S^{2-}$ + 2CO$_2$ + H$_2$O) (Laanbroek et al., 1984). The above results were supported by the positive expression of sox gene (Fig. 4).

As the loading ratios of acetate and NO$_3^-$ were in excess (C-IV and C-V), genera of Leptolinae, Exiguobacterium, Flavobacterium and Octobactrum were the most abundant, indicated the predominate bioprocesses that nitrate reduction and the over oxidation of $S^0$ to $S^{2-}$ under heterotrophic conditions were the ($S^{2-}$ + 1.6NO$_3^-$ + 1.6H$^+$ → SO$_4^{2-}$ + 0.8N$_2$ + 0.8H$_2$O; $S^{2-}$ + 1.2NO$_3^-$ + 0.4H$_2$O → SO$_4^{2-}$ + 0.6N$_2$ + 0.8H$^+$) (Huang et al., 2015). Meanwhile, the expressed nirK and sox genes achieved the highest value in C-IV and C-V, further verified the lifting activities of nitrate reduction and SO$_4^{2-}$ generation. A probable explanation is the rate of electron production from TCA cycle is slower than sulfide oxidation. The sulfur cycle could be a supplement electron donor for denitrification, since the complete sulfide oxidation could supply more electrons for denitrification (i.e., $S^{2-}$ → $S^0$, 2e; $S^{2-}$ → SO$_4^{2-}$, 8e).

### 4. Conclusion

The shift of $S^{2-}$/NO$_3^-$-N molar ratio impacted the $S^0$ recovery rate and the removal rates of NO$_3^-$ and acetate, and also significantly altered both the bacterial community structure and genetic activity. The optimized condition for $S^0$ recovery was determined with the SO$_4^{2-}$/NO$_3^-$ ratio of 5/6 and the acetate-C/NO$_3^-$-N ratio of 1/2, where the desulfurization and denitrification genera were dominant and sqr gene was highly expressed. The study gave suggestions for the effective control of the DSR process through the regulation of the bacterial communities.

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Appendix A. Supplementary data

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References


