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Bioreactor performance and functional gene analysis of microbial community in a limited-oxygen fed bioreactor for co-reduction of sulfate and nitrate with high organic input

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HIGHLIGHTS

- Co-removal of nitrate and sulfate from high organic-laden wastewater was achieved.
- Limited-oxygen fed enhanced sulfur recovery, up to 70%.
- Functional genes of microbial community were analyzed at limited-oxygen conditions.
- Limited oxygen hold strong impact on sulfide-oxidizing genes (fccA/B, sox).

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ABSTRACT

Limited-oxygen mediated synergistic relationships between sulfate-reducing bacteria (SRB), nitrate-reducing bacteria (NRB) and sulfide-oxidizing bacteria (SOB, including nitrate-reducing, sulfide-oxidizing bacteria NR-SOB) were predicted to simultaneously remove contaminants of nitrate, sulfate and high COD, and eliminate sulfide generation. A lab-scale experiment was conducted to examine the impact of limited oxygen on these oxy-anions degradation, sulfide oxidation and associated microbial functional responses. In all scenarios tested, the reduction of both nitrate and sulfate was almost complete. When limited-oxygen was fed into bioreactors, S^0 formation was significantly improved up to ~70%. GeoChip 4.0, a functional gene microarray, was used to determine the microbial gene diversity and functional potential for nitrate and sulfate reduction, and sulfide oxidation. The diversity of the microbial community in bioreactors was increased with the feeding of limited oxygen. Whereas the intensities of the functional genes involved in sulfate reduction did not show a significant difference, the abundance of the detected denitrification genes decreased in limited oxygen samples. More importantly, sulfide-oxidizing bacteria may alter their populations/genes in response to limited oxygen potentially to function more effectively in sulfide oxidation, especially to elemental sulfur. The genes fccA/fccB from nitrate-reducing, sulfide-oxidizing bacteria (NR-SOB), such as *Paracoccus denitrificans*, *Thiobacillus denitrificans*, *Beggiatoa sp.*, *Thiomicrospira sp.*, and *Thioalkalivibrio sp.*, were more abundant under limited-oxygen condition.

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1. Introduction

Nitrate (NO_3^-) contamination of surface and ground water is a relevant problem due to its health risk for methemoglobinemia in infants and spur eutrophication of water bodies. Agricultural runoff, wastewater discharges, and septic tanks are common sources of these contaminants [1]. The most common technology for nitrate removal from wastewater streams is microbial reduction, denitrification. Denitrification, the microbial reduction of NO_3^- to nitrite (NO_2^-) to form nitrogen gas (N_2), involves the stepwise reduction driven by a series of enzymes [2–4]:

$$NO_3^- \xrightarrow{NO_3^- reductase} NO_2^- \xrightarrow{NO_2^- reductase} NO \xrightarrow{NO\ reductase} N_2O \xrightarrow{N_2O\ reductase} N_2$$

Sulfate (SO_4^{2-}) is another respiratory electron acceptor commonly found in water and wastewater as a coexistent content of nitrate. The most common two-stage biological process for SO_4^{2-} removal is microbial SO_4^{2-} reduction to sulfide (S^{2-}) by sulfate-reducing bacteria (SRB) and S^{2-} oxidation to sulfur (S^0) by sulfide-oxidizing bacteria (SOB) or nitrate-reducing, sulfide-oxidizing bacteria (NR-SOB). Concomitant SO_4^{2-} reduction and biological S^{2-} oxidation with limited oxygen in a single reactor has proven to be a promising and cost-effective alternative for remediating water contaminated with the compound, and a low dissolved oxygen (DO) concentration has been demonstrated to play an important role in the coexistence of SRB and SOB [5–7].

In addition, during co-reduction of NO_3^- and SO_4^{2-}, NO_3^- availability also increases the potential that S^{2-} oxidation will occur because nitrate-reducing, sulfide-oxidizing bacteria (NR-SOB) such as *Thiomicrospira denitrificans* and some strains of *Thiomicrospira sp.*, *Thiobacillus sp.*, and *Acrobacter sp.* can oxidize S^{2-} with NO_3^- as electron acceptor [8–11]. The NR-SOB mediated biooxidation of S^{2-} (also termed denitrifying sulfide removal, DSR) has been studied extensively over a range of reactor operation and performance, reactor configurations, mechanism and modeling or microbial community [12]. However, banking on the fact that the majority of NR-SOB is chemolithotroph that uses sulfide as an electron donor and nitrate as an electron acceptor, heterotrophic nitrate-reducing bacteria (h-NRB) may out-compete NR-SOB for the common electron acceptor in the presence of high organic input [12]. Thus it is important to understand how to maintain the balance between the h-NRB and NR-SOB during the co-reduction of NO_3^- and SO_4^{2-} with high organic input to reduce sulfide generation as much as possible.

In this study, we evaluated S^{2-} elimination and S^0 production under co-reduction of nitrate and sulfate conditions in a bioreactor fed with limited oxygen. We applied a fixed O_2 supply rate to each bioreactor which was selected based on our previous batch results with which S^0 formation was significantly improved [13]. We hypothesized that effectively synergistic communities among SRB, h-NRB, and (NR-)SOB could be developed by controlling the O_2 supply rates to suppress sulfide generation. The performance of the limited O_2-fed bioreactor was compared to that of a control, keeping anaerobic condition during the experiments. In addition, the mass balance for SO_4^{2-}, S^0, and S^{2-} in reactors was examined to evaluate the development of the internal sulfur cycle. Here, we evaluated the interplay among O_2 fed, SO_4^{2-} reduction, NO_3^- reduction and S^{2-} oxidation. We also focused on how these performances factors are linked to the structure of the microbial community.

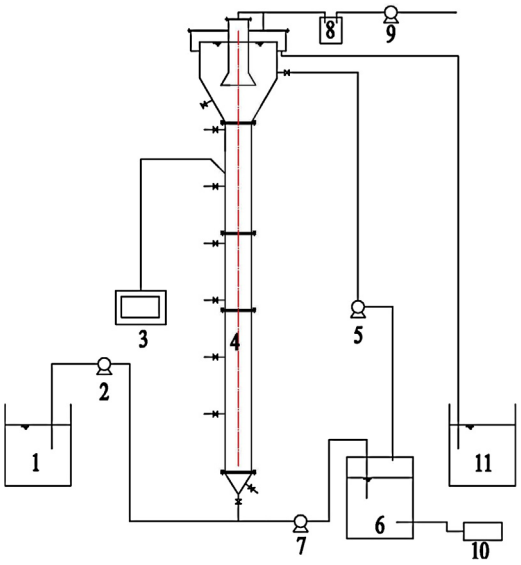

Fig. 1. Scheme of the expanded granule sludge bed (EGSB) reactor used in the experiments.

2. Materials and methods

2.1. Reactor setup and operational conditions

Experiments were conducted using expanded granular sludge bed (EGSB) reactors made of Perspex. The total volume was 4 L, with a working volume of 1 L. The reactors were insulated and the temperature was maintained at 30 °C via electric resistance heating. A gas-washing device collected the H_2S gas generated at the column top. Limited-oxygen condition was maintained using the regulated flow of air with a mass flow controller from an air cylinder, and air was injected into a separated aeration tank as previously described [7] (Fig. 1). Since gas–liquid mass transfer resistance exists, moderate stirring was required in the aeration tank to avoid air stripping as much as possible.

Two EGSB reactors were set up and inoculated with granule sludge from EGSB reactor operated by [14] for more than six months. The EGSB reactors were both operated in a continuous mode with an influent flow rate of 5.4 L/day and a recirculation rate of 54 L/day in each reactor for complete mixing of the liquid. For Reactor A, the substrates were sulfate and organic carbon (Table 1) and once the concentration of SO_4^{2-} and COD in the effluent reached a steady state (the variations of COD and SO_4^{2-} effluent concentrations were less than 10% over a minimum of three hydraulic retention times (HRT) and each steady state had a duration of a minimum of 20 days [1]), limited oxygen was fed into the reactor with a fixed O_2 supply rate of 0.5 ml min^{-1} $L_{reactor}{}^{-1}$. For Reactor B, all operating conditions were the same as Reactor A except for nitrate added to the influent and the oxygen was fed at 1.0 ml min^{-1} $L_{reactor}{}^{-1}$. In both reactors, organic carbon (lactate) was supplied in excess since relatively high organic input in wastewater. Recently, in certain environment nitrate has been shown to enhance sulfide bio-oxidation by NR-SOB [9]. Thus to clarify whether nitrate or limited-oxygen improved biological sulfide oxidation to S^0, in this study we also operated Reactor A.

The feed medium contained (g/L): Na_2SO_4 as S, 1.478; KNO_3 as N, 0.815; lactate as C, 5 ml/L; NH_4Cl, 0.575; $CaCl_2$, 0.070; $MgSO_4{\cdot}7H_2O$, 0.100; K_2HPO_4, 0.22; and 1 ml of trace solution [15]. The pH was adjusted to 8.0 ± 0.3 with bicarbonate. Before pumped into reactors, the feed medium was sparged with N_2 for 10 min to remove oxygen from aqueous phase.

Table 1
Experimental conditions for Reactor A and B.

Steady states	Reactor A			Reactor B			
	O_2 (ml $min^{-1}-L_{reactor}{}^{-1}$)	inf-$SO_4{}^{2-}$(mg/L)[a]	inf-COD (mg/L)[c]	O_2 (ml $min^{-1}L_{reactor}{}^{-1}$)	inf-$SO_4{}^{2-}$ (mg/L)[a]	inf-$NO_3{}^{-}$ (mg/L)[a]	inf-COD (mg/L)[c]
1	0	1000	3000	0	1000	500	3000
2	0.5	1000	3000	1.0	1000	500	3000

[a] Variations in the influent concentrations are presented in Fig. 1.
[b] O_2 supply rate was selected based on results described by [13].
[c] Excess organic carbon (lactate) was supplied in the study.

2.2. GeoChip

GeoChip 4.0 is a new generation of functional gene arrays, with ~80,000 probes covering approximately >280,000 gene sequences from >400 functional gene families involved in carbon, nitrogen, phosphorus and sulfur cycles, energy metabolism, antibiotic resistance, metal resistance and organic contaminant degradation [16]. Such a high-throughput tool is developed with an aim to study microbial community composition, structure and functional activity; and link microbial communities to ecosystem processes and functioning. Since its publication this tool has been extensively used to analyze various ecosystems [17].

2.2.1. DNA extraction, amplification, and labeling

For a full understanding of the microbial community structure evolution resulting from limited-oxygen, we respectively sampled the granule sludge from Reactor A (1# and 2# represent sample without and with limited oxygen fed respectively) and Reactor B (3# and 4# represent sample without and with limited oxygen fed) once the reactors displayed steady-state reduction of either $SO_4{}^{2-}$ only or $SO_4{}^{2-}$ and $NO_3{}^{-}$.

Approximately 100 ng of DNA that was previously extracted from the samples [18,19] was amplified using the Templiphi kit and labeled 1.5 μg DNA with Cy3 using random primers and Klenow. Labeled DNA was purified (QIAquick purification kit; Qiagen, Valencia, CA) and dried in a SpeedVac (45 °C, 45 min; ThermoSavant, Waltham, MA, USA) before hybridization.

2.2.2. Hybridization and data pre-processing

All hybridizations were carried out at 42 °C with 40% formamide for 16 h on a MAUI hybridization station (Biomicro, Salt Lake City, UT, USA). After hybridization, the arrays were scanned (NimbleGen MS200, Madison, WI, USA) at a laser power of 100%. Signal intensities were measured based on scanned images, and spots with signal-to-noise ratios lower than 2 were removed before statistical analysis as described previously [16,20]. Functional gene diversity was calculated using Shannon-Weaver index (H) via the freely available software (http://www2.biology.ualberta.ca/jbrzusto/krebswin.html).

2.3. Analytical procedures

Influent and effluent from the reactors were centrifuged using a HettichRotofix 32 centrifuge at 3000 × g for 10 min for further analysis. An ion chromatography (Dionex ICS-3000) measured the concentration of sulfate ($SO_4{}^{2-}$), thiosulfate ($S_2O_3{}^{2-}$) nitrate ($NO_3{}^{-}$), and nitrite ($NO_2{}^{-}$) in the collected liquor samples following 0.45-μm filtration. Sample separation and elution were performed using an IonPac AG4A AS4A-SC 4 mm analytic column with carbonate/bicarbonate eluent (1.8 mmol dm^{-3} Na_2CO_3/1.7 mmol l^{-1} $NaHCO_3$ at 1 cm^3 min^{-1}) and a sulfuric regeneration (H_2SO_4, 25 mmol l^{-1} at 5 cm^3 min^{-1}). Sulfide concentration (including H_2S, HS^- and S^{2-}) was determined according to the methylene blue method [21]. Gas chromatography (6890, Agilent, USA) was used to measure the compositions of gas (CO_2, CH_4). Both volatile suspended solids and suspended solids were measured according to Standard Methods [22]. Measurements for the concentrations of total organic carbon (TOC) and inorganic carbon (IC) were taken by the TOC analyzing instrument (TOC-VCPH, Japan) equipped with platinum catalyst quartz tube. The flow rate of oxygen gas was 130 ml/min and the furnace temperature was 680 °C. The dissolved oxygen in liquid samples was measured by DO meter (pH/Oxi 340i, WTW, Germany). A pH/ORP meter (pHS-25) determined the pH and oxidation-reduction potential (ORP) of liquid samples. Since some SRB are capable of utilizing $NO_3{}^{-}$ as an electron acceptor and reducing $NO_3{}^{-}$ to ammonium [23], $NH_4{}^{+}$ was analyzed according to Standard Methods [22].

S^0 generation could not be measured from effluent samples, because much of the S^0 remained in the EGSB attached to the sludge (data not shown). Therefore, S^0 production was calculated according to the following equation (eq. 1) [24]:

$$[S^0] = [Influent\ total\ S] - [effluent\ SO_4^{2-}] - 2 * [effluent\ S_2O_3^{2-}] - [effluent\ HS^-] \quad (1)$$

3. Results

3.1. Reactor performance

Fig. 2 and Tables S1 and S2 showed the average concentration of $NO_3{}^{-}$, $SO_4{}^{2-}$ and COD in the influent and effluent for Reactors A and B. For all scenarios tested, both the nitrate and sulfate were almost completely reduced and no $NO_2{}^{-}$ production was observed; and this was likely the case due to the unrestricted electron donor (organic carbon) availability. Nevertheless, S^0 formation differed greatly with/without limited oxygen fed. Whereas only 4.2% (Reactor A) and 4.0% (Reactor B) sulfur in influent were converted to S^0 when without limited oxygen fed into bioreactors, a peak of 71.8% and 72.6% S^0 formation was achieved in limited-oxygen fed bioreactors. These findings might be explained by an enhanced activity of SOB in granule sludge to carry out sulfide oxidation to elemental sulfur under limited-oxygen condition. We addressed this interpretation in the following section that presents the GeoChip results. $S_2O_3{}^{2-}$ accumulation when limited-oxygen fed was not observed for any Reactor A or B cases, indicating that the chemical sulfide oxidation was negligible [25]. In addition, ammonium production was not detected in our study (data not shown). These results indicated that limited-oxygen fed had little or no negative effect on $SO_4{}^{2-}$ and $NO_3{}^{-}$ reduction. Considerable differences were observed for S^0 formation and the formation achieved in the reactors with limited-oxygen fed was generally higher than those always operated under anaerobic conditions.

3.2. Overall functional gene diversity of microbial communities

To understand whether limited-oxygen affected community composition and structure, the granular sludge communities in the reactors were analyzed at the steady state of the experiments

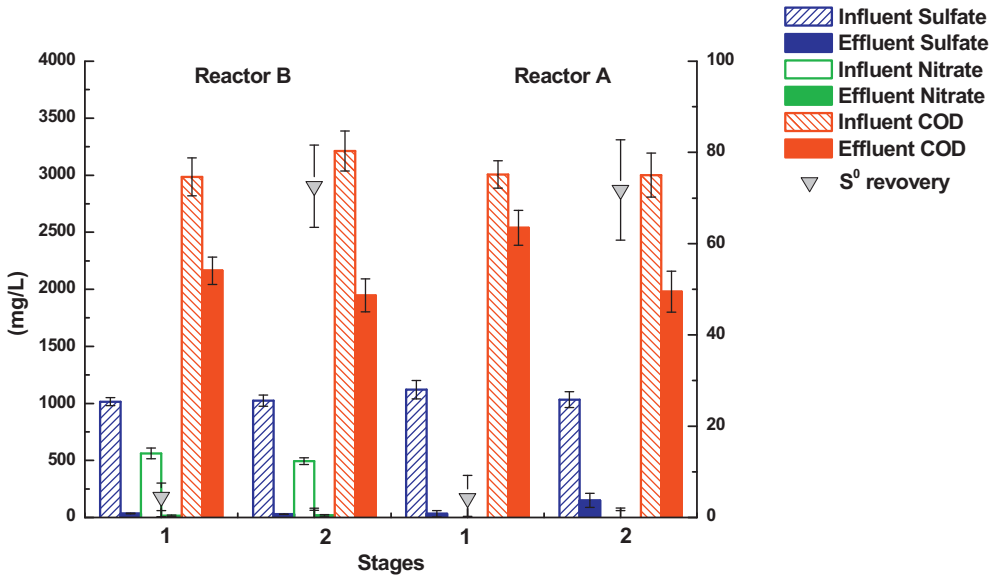

Fig. 2. Reactor performance for the operating conditions shown in Table 1. (a) reactor operating with sulfate and lactate as substrate; (b) reactor operating with sulfate, nitrate and lactate as substrate.

using GeoChip 4.0. A total of 24124 genes (approximately 24% of all designed probes) displayed significant hybridization signals with at least one of the four samples. Overall, the gene numbers and signal intensities detected varied significantly across these samples, ranging from 15708 to 20853 genes (Table 2). The diversity of these communities varied considerably based on the Simpson's and Shannon-Weaver indices. In general, the samples from limited-oxygen fed bioreactors contained higher gene numbers and signal intensities than those from anaerobic ones. Furthermore, the overall genetic diversity detected in samples from each reactor under limited-oxygen condition and anaerobic condition suggested that limited-oxygen condition had strong effects on the microbial communities. An average of 58.3% genes (range of 48.4–63.1%) was shared among the samples (Table 2). The percentage of unique genes varied from 4 to 18%, which is relatively high considering that the inocula used for reactors were all originated from [14].

To determine whether limited-oxygen fed affected the overall patterns of microbial community functional structure, PCA (Principal Component Analysis) was performed based on all detected functional genes (data not shown). The analysis revealed that the samples from Reactor B were clustered together and were well separated from those samples from Reactor A; while the samples 1# and 2# were clustered separated. These results indicate that the limited-oxygen condition had a more clear effect on the community functional structure in systems with a lack of nitrate substrate.

3.3. Changes in functional genes involved in carbon degradation, sulfate/nitrate reduction and sulfur oxidation

Genes involved in carbon degradation were examined in this study to provide a better understanding of microbial diversity in these communities. A variety of carbon degradation genes were detected among these reactors (Fig. 3), including amylase, xylanase, and endochitinase. The detected genes involved in carbon degradation were highly diverse and the relative abundance of these genes varied considerably among these reactors. Sample 4# had the highest abundance of carbon degradation genes indicating the potential for removal of a variety of types of organic carbon in wastewater.

Genes involved in denitrification were also examined in this study. A variety of denitrification genes were detected among these samples (Fig. 4), including narG, nirK, nirS, norB and nosZ. The genes involved in denitrification had a high diversity and the detected gene numbers in sample 4# were lower than that in sample 3#. This suggests that limited-oxygen has an inhibitory effect on denitrification. The inhibition of denitrification by oxygen observed in this study is in agreement with the findings of other studies. And we further addressed the interpretation in the discussion section.

Sulfate reduction genes and sulfur oxidation genes were specifically analyzed as they are important for understanding bacteria related to the process of S^0 formation. A variety of sulfate-reduction genes, including AprA, APS_AprA, APS_AprB, CysJ, dsrA, dsrB and

Table 2
Gene overlap (unshaded number and percentages), gene uniqueness (shaded gene number and percentages), and diversity indices for each sample.

Samples[a]	1#	2#	3#	4#
1#	594(3.8%)	14144(63.1%)	12066(62.1%)	10096(55.5%)
2#		3774(18.1%)	13712(59.8%)	10903(48.4%)
3#			730(4.6%)	10727(60.8%)
4#				724(3.9%)
No. of genes detected	15708	20853	15793	18567
Simpson's (1/D)	15624.5	20703.1	15711.5	18503.4
Shannon-Weaver (*H'*)	9.6	9.9	9.7	9.8
Sulfur Recovery[b] (%)	4.2	71.8	4.5	72.6

[a] These samples were extracted from Reactor A and B, respectively. Different samples represented different operating conditions: 1#, sulfate and organic carbon (lactate) as substrates without limited-oxygen fed; 2#, sulfate and organic carbon (lactate) as substrates with limited-oxygen fed; 3#, sulfate, nitrate and organic carbon (lactate) as substrates without limited-oxygen fed; 4#, sulfate, nitrate and organic carbon (lactate) as substrates with limited-oxygen fed. The diversity data was obtained with the granular sludge communities using GeoChip 4.0 at the steady state of reactors.

[b] Sulfur recovery was obtained according to Eq. (1).

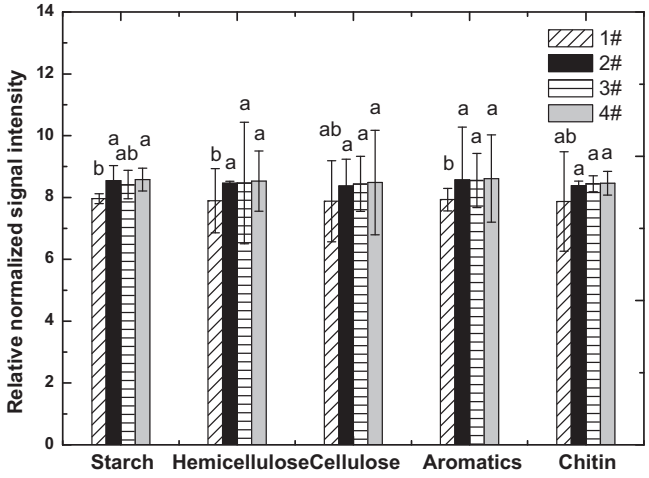

Fig. 3. Relative abundance of the genes involved in carbon degradation among the samples. Abscissa Starch, Hemicellulose, Cellulose, Aromatics and Chitin represent genes involved in corresponding carbon degradation, and they are arranged from labile carbon to recalcitrant carbon.

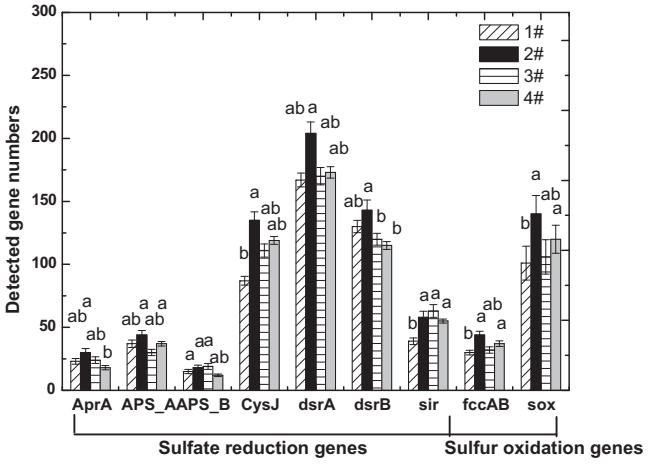

Fig. 5. Relative abundance of the genes involved in sulfate reduction and sulfide oxidation among the samples, including AprA, APS_AprA, APS_AprB, CysJ, dsrA, dsrB and sir; fccA/B and sox.

sir, and sulfur-oxidation genes, including fccA, fccB and sox, were detected among the study samples (Figs. 5 and 6 and S1). The genes involved in sulfate reduction were varied in a small range among samples, and in all of the samples, ~85% sulfate reduction was achieved. GeoChip 4.0 contains probes from several sulfur oxidation bacteria. A total of 213 sulfur-oxidation genes were detected in these samples, and the sulfur oxidation genes fccA, fccB and sox, which are key factors associated with enzyme synthesis, have been shown to be involved in sulfur oxidation and S^0 formation in the reactors [26]. fccA/fccB, which are involved in sulfide oxidation to sulfur, could be detected from bacteria, such as *Thermus sp.* and *Methylobacillus sp*. In addition, *Allochromatium vinosum* and *Rhodobacter sphaero* were highly abundant in all samples. The fccA/fccB genes from *Chlorobium* species were the next abundant genera in reactors. The genes fccA/fccB from nitrate-reducing, sulfide-oxidizing bacteria such as *Paracoccus denitrificans*, *Thiobacillus denitrificans* and *Beggiatoa sp.* were also detected in the samples, but their abundance varied significantly among these four samples. Furthermore, the gene sox, which act as an oxidase during sulfide oxidization to sulfur/thiosulfate/sulfate, could be detected from bacteria such as *Bradyrhizobium sp.* and *Sulfitobacter sp. Roseobacter sp.* and *Roseovarius sp.* were highly abundant in all samples related to the sox gene. Similar to the fccA/fccB genes, the sox genes from these sulfur-oxidizing bacteria, *Chlorobium limicola*, *P. denitrificans*, *T. denitrificans* and *Beggiatoa sp.*, were also detected in the samples, but varied abundance was observed in the four samples. The distribution of the fccA/fccB and sox genes varied substantially among different samples, with sample 2# and 4# (limited-oxygen fed) having more abundant sulfur-oxidation genes. Interestingly, some NR-SOB, such as *Beggiatoa sp., Thiobacillus denitrificans, P. denitrificans*, some strains of *Thiomicrospira sp.*, and *Thioalkalivibrio sp*. in samples 2# and 4#, were observed more than that in sample 1# and 3#. In particular, the abundance of *T. denitrificans* in limited-oxygen fed bioreactor was approximately three to five-fold times higher than that in the non-limited-oxygen bioreactor (Fig. 7).

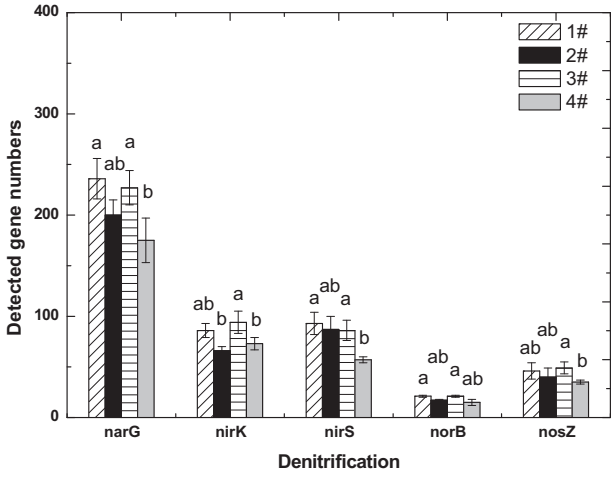

Fig. 4. Relative abundance of the genes involved in denitrification among the samples, including narG, nirK, nirS, norB and nosZ.

4. Discussion

Limited-oxygen mediated synergistic relationships between SRB, NRB and SOB with accompanying improvement the conversion of toxic and corrosive sulfide to insoluble S^0 is a promising strategy for the potential co-reduction of NO_3^- and SO_4^{2-} with relatively high organic input in wastewater. For the operating conditions tested in the study, high efficiency removal of sulfate and nitrate was achieved and a peak S^0 formation of ~70% was achieved in samples 2# and 4#. This was the first demonstration that under limited-oxygen conditions, sulfate, nitrate and organic carbon can be successfully removed simultaneously and meanwhile S^0 formation reached a relatively high level. Based on the bioreactor performance, it was hypothesized that limited oxygen conditions affect bioreactor indigenous microbial communities and their functions. To test this, we used community DNA, rather than mRNA, to measure the metabolic potential of microbial communities by the abundance change of key functional genes and their associated populations as the detection of functional activity with mRNA currently presents a number of challenges, such as low abundance, rapid turnover, and instability [16].

It is important to establish the mechanistic linkages between microbial community structure and bioreactor performance [27]. Since lactate was sole carbon source in the study, a relatively high diversity of carbon-degradation genes detected by GeoChip 4.0 could be due to utilization of excreted compounds by microorganisms as well as degradation of dead biomass grown on lactate [28]. Having a diverse community such as in these systems may be important in pollutant removal by maintaining environments

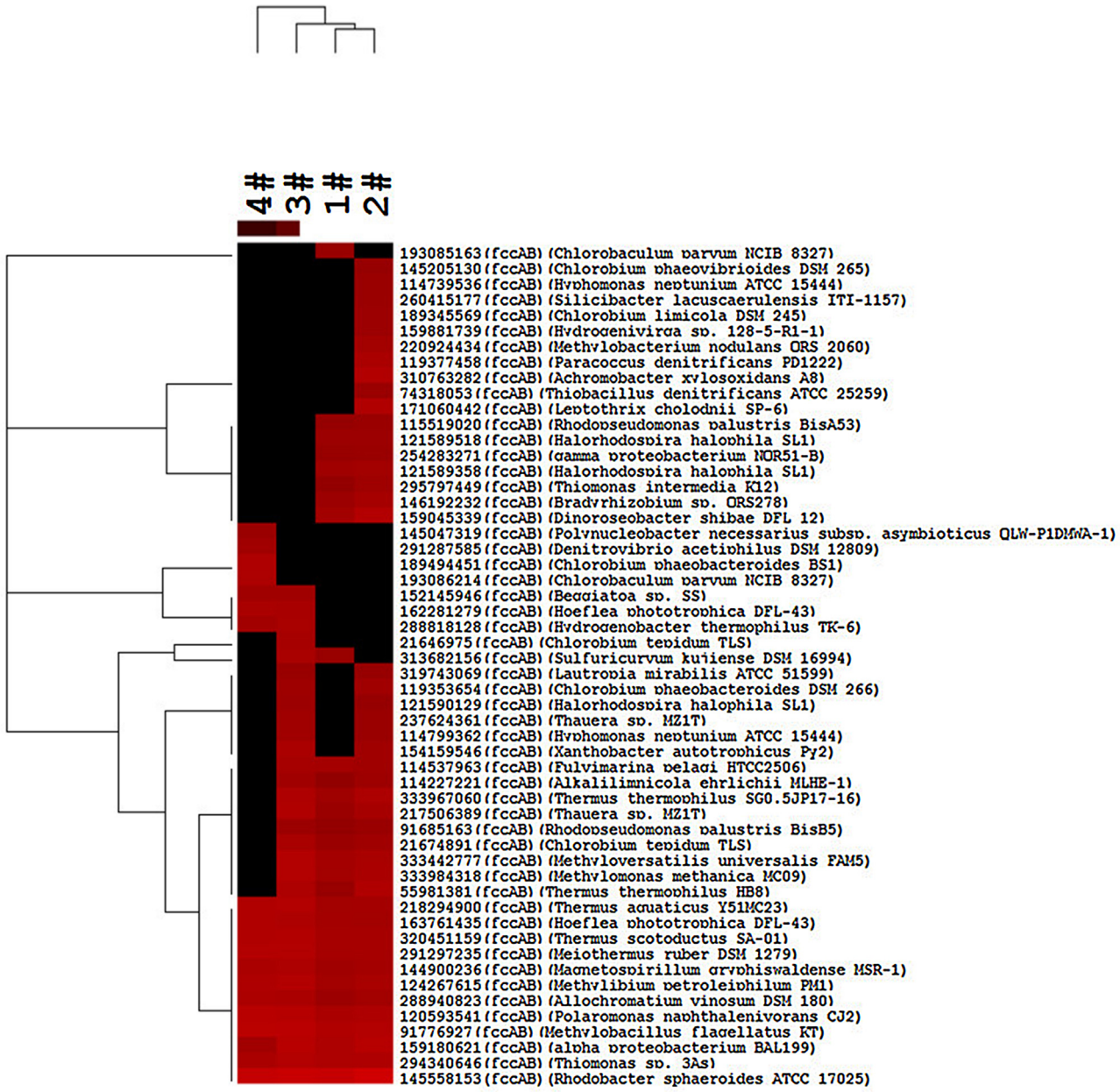

Fig. 6. Hierarchical cluster analysis of fccA/B genes based on hybridization signals. The figure was generated using CLUSTER and visualized with TREEVIEW. Black represents no hybridization above background level and red represents positive hybridization. The color intensity indicates differences in hybridization patters.

favoring S^0 formation. Further studies on the relationships among substrate, biomass, reactor performance and microbial community structure and function are needed. Despite limited-oxygen was fed to the reactors, GeoChip analysis revealed a relatively high functional and phylogenetic diversity of sulfate- and nitrate-reducing bacteria and limited oxygen showed no negative effect on sulfate reduction bacteria in our system. The diversity of the microbial community may be maintained by keeping a low oxygen level in the bioreactor via sulfide-oxidizing bacteria consumption of oxygen [29], or O_2 respiration by some sulfate-reducing bacteria [30–32]. The inhibition of nitrate reduction by oxygen in this study has also been reported by many other researchers [33–37]. Studying the factors that influence the deterioration in open anoxic reactor, Plosz et al. [35] demonstrated that oxygen entering an anoxic reactor through the surface may not just affect denitrification metabolically, but also kinetically, due to increased dissolved oxygen (DO) concentration exerting an inhibitory effect on the denitrification rate. In the work reported by Oh and Silverstein [34] it was shown that mixed liquor DO as low as 0.09 mg/L was found to significantly inhibit denitrification, resulting in a rate decrease of 35%. While some researchers have reported that pure strains of denitrifying bacteria grow simultaneously using both oxygen and nitrate electron acceptors [38–41], oxygen appears to be available as an alternate and energetically preferable electron acceptor for facultative denitrifying bacteria, and regulate synthesis of nitrate reductase enzyme and inhibits denitrification in pure cultures of facultative denitrifying bacteria so that substrate electrons flow to oxygen cytochromes [36,42]. Therefore, oxygen may compete with nitrate for the same enzymes resulting in a lower nitrate reduction rate (data not shown here).

As distinct microbial communities, especially sulfide-oxidizing bacteria, were observed between the reactors with limited-oxygen fed or not, it was expected that some microbial communities related to S^0 formation were stimulated or enriched in the

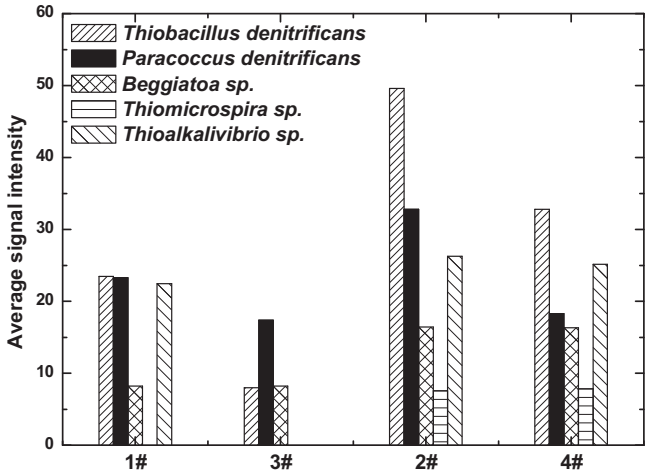

Fig. 7. The relative abundance of fccA/B and sox genes detected from some NR-SOB species, including *Thiobacillus denitrificans, Paracoccus denitrificans, Beggiatoa sp., Thiomicrospira sp., Thioalkalivibrio sp.* The relative abundance of genes was calculated from different samples based on the average signal intensity of each microorganism.

limited-oxygen fed reactors. Indeed, it was observed that nitrate-reducing, sulfide-oxidizing bacteria (NR-SOB), including *Beggiatoa sp, T. denitrificans, P. denitrificans*, and some strains of *Thiomicrospira sp.*, and *Thioalkalivibrio sp*. were enriched in limited-oxygen fed reactors (Figs. 6 and 7 and Fig. S1). *Beggiatoa* species are sulfide-oxidizing bacteria that play an important role in the benthic sulfur cycle [43], and the species can oxidize sulfide to S^0 and subsequently to SO_4^{2-} with nitrate or oxygen as electron acceptor [44]. When oxygen is used as electron acceptor, the oxidation of sulfide to S^0 is pH neutral, whereas the oxidation of S^0 to sulfate is acidogenic [45,46]. In our study, the pH of the influent and effluent in sample 2# and 4# is 8.0 ± 0.3 and 7.7 ± 0.2, respectively which could have resulted from the oxidation of sulfide to S^0 with oxygen. The abundance of *Beggiatoa* species in limited-oxygen reactor was relatively higher than that in non-limited-oxygen fed reactor. Sulfide oxidation was carried out at a low level due to inadequate nitrate available as high rate of heterotrophic denitrification in non-limited-oxygen reactor; however, when limited oxygen was fed into the bioreactor, supplemented with electron acceptor like nitrate, the sulfide oxidation by *Beggiato*a species could reach a high level.

Similarly, the abundance of *T. denitrificans*, *P. denitrificans*, *Thiomicrospira sp.* and *Thioalkalivibrio sp*. in limited-oxygen reactor was relatively higher than that in non-limited-oxygen fed reactor (Fig. 7). Recently, many researchers reported that addition of nitrate to a sulfate-rich environment had been shown to enhance the biological oxidation of sulfide by NR-SOB and diminish sulfide generation [8–11,47]. However, in our system, although nitrate was added to sample 3# (without limited oxygen fed), no obvious enhancement for biological oxidation of sulfide by nitrate-reducing, sulfide-oxidizing bacteria (NR-SOB) was observed and S^0 formation was still maintained at a low level compared with sample 1#. These results indicated that indigenous NR-SOB was not stimulated by addition of nitrate and this phenomenon might be explained by the fact that organic carbon-driven heterotrophic denitrification outcompeted the sulfide-driven autotrophic denitrification and both the denitrifiers would compete for nitrate. However, the limited-oxygen fed scheme changed the status. Under limited-oxygen condition, the autotrophic NR-SOB species were enriched and these bacteria could oxidize sulfide with oxygen as electron acceptor whereas heterotrophic denitrifiers still utilized organic carbon to proceed in denitrification. Furthermore, Chen et al. [48] also indicated that the sulfide-oxidizing rate could be increased higher than 1.5 times than that under anaerobic condition in denitrifying sulfide removal process and the sustainable maximum sulfide threshold was also increased from 200 to 300 mg/L under limited-oxygen condition (DO = 0.2–0.5 mg/L). Under the limited-oxygen condition, the low efficiency of NR-SOB in the uptake of nitrate to drive denitrification was reversed, and the competitive relations between heterotrophic denitrifiers and NR-SOB were relieved. Thus, the biological sulfide oxidation by NR-SOB was enhanced leading to a high S^0 formation.

In addition, *Chlorobium tepidum* and *Chlorobium limicola*, which can grow in low oxygen and vanishingly low light conditions [49], were detected frequently in limited-oxygen samples by GeoChip (Fig. 6). And the high proportion of *Methylobacterium sp.* genes detected might be due to oxidation of methane generated in the reactors by methanogens [50]. Herein it was not possible to clearly tell what role these bacteria served in sulfur formation. Perhaps they could be important in our system by maintaining environments favoring sulfur generation. During our experiments with limited-oxygen fed, methane production was observed and thus methanogenic bacteria should be responsible for the formation of methane. The survival of these extremely oxygen-sensitive organisms could be due to O_2 removal from their biotopes by non-enzymatic reduction of O_2 by H_2S formed by the SRB; this might have contributed to the extensive distribution of the methanogens in nature [51].

5. Conclusions

The study investigated the performance of a limited-oxygen fed bioreactor for co-reduction of nitrate and sulfate with high organic input. With limited-oxygen fed, both the nitrate and sulfate were completely removed and ~70% of sulfur in influent was converted to elemental sulfur. The microbial community analysis by GeoChip revealed that the functional genes involved in sulfide oxidation (fccA/B, sox) were expressed at significantly higher levels in limited oxygen samples than that in anaerobic samples. The successful co-removal of nitrate, sulfate and high organics in the limited-oxygen fed bioreactor provided a promising technology for practical wastewater treatments.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jhazmat.2014.06.006.

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