

# Effect of dissolved oxygen on elemental sulfur generation in sulfide and nitrate removal process: characterization, pathway, and microbial community analysis

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**Abstract** Microaerobic bioreactor treatment for enriched sulfide and nitrate has been demonstrated as an effective strategy to improve the efficiencies of elemental sulfur ( $S^0$ ) generation, sulfide oxidation, and nitrate reduction. However, there is little detailed information for the effect and mechanism of dissolved oxygen (DO) on the variations of microbial community in sulfur generation, sulfide oxidation, and nitrate reduction systems. Polymerase chain reaction denaturing gradient gel electrophoresis (PCR-DGGE) was employed to evaluate the variations of microbial community structures in a sulfide oxidation and nitrate reduction reactor under different DO conditions ( $DO$  0–0.7  $mg \cdot L^{-1}$ ). Experimental results revealed that the activity of sulfide-oxidizing bacteria (SOB) and nitrate-reducing bacteria (NRB) could be greatly stimulated in 0.1–0.3  $mg \cdot DO \cdot L^{-1}$ . However, when the DO concentration was further elevated to more than 0.5  $mg \cdot L^{-1}$ , the abundance of NRB was markedly decreased, while the heterotrophic microorganisms, especially carbon degradation species, were enriched. The reaction pathways for sulfide and nitrate removal under microaerobic conditions were also deduced by combining batch experiments with functional species analysis. It was likely that the oxidation of sulfide to sulfur could be performed by both aerobic heterotrophic SOB and sulfur-based autotrophic denitrification bacteria with oxygen and

nitrate as terminal electron acceptor, respectively. The nitrate could be reduced to nitrite by both autotrophic and heterotrophic denitrification, and then the generated nitrite could be completely converted to nitrogen gas via heterotrophic denitrification. This study provides new insights into the impacts of microaerobic conditions on the microbial community functional structures of sulfide-oxidizing, nitrate-reducing, and sulfur-producing bioreactors, which revealing the potential linkage between functional microbial communities and reactor performance.

**Keywords** Sulfide-oxidizing · Nitrate-reducing · Dissolved oxygen · Elemental sulfur generation · Microbial community

## Introduction

$SO_2$  and  $NO_x$  released from the process of coal, fuel oils, and waste combustion result in serious environmental pollution and impact significantly on the ecosystem and human life. Utilizing microorganisms to treat  $SO_2$  and  $NO_x$  seems to be one of the most promising alternatives to conventional air pollution control techniques, because biological process is cost-effective with no secondary pollutant production (Ding et al. 2014; Wang et al. 2015).

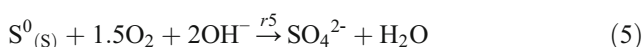
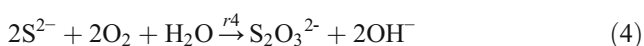
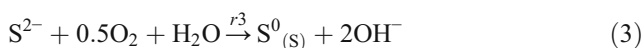
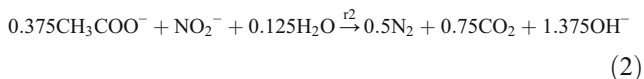
A novel method for simultaneous flue gas desulfurization and denitrification was investigated recently (Fig. S1). The novel method composed of two processes was demonstrated performing well with respect to sulfide and nitrate treatment in microaerobic conditions (Wang et al. 2015). When sulfide, nitrate, and carbon source were present simultaneously, the absence of oxygen makes the activities of heterotrophic denitrifiers significantly depressed by sulfide of high concentrations, resulting in the accumulation of abundant nitrite. And then the accumulated nitrite could inhibit the activities of

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autotrophic denitrifiers, which followed by the collapse of the denitrifying sulfide removal process (Chen et al. 2010a, c). Nevertheless, the addition of limited oxygen could greatly relieve the inhibition of sulfide to denitrification by oxidizing sulfide to sulfur or sulfate via the following oxidation-reduction reaction equation (Eqs. 1–5) (Chen et al. 2010a; Wang et al. 2016).



Furthermore, under microaerobic conditions, the glucose could be degraded by fermenting bacteria to small molecule organic acids (lactate, alanine, valine, acetate, and  $\alpha$ -ketoglutarate) (Huang et al. 1998), which were more easily utilized by microorganism (NRB) and could enhance the performance of bioreactor with respect to denitrification. Therefore, the nitrate removal efficiency in the simultaneous sulfide, nitrate, and carbon source removal system could be improved with limited oxygen (Chen et al. 2010a, c). However, the abundance of carbon degradation bacteria (fermenting bacteria) would continuously increase with the raising of DO levels (Xu et al. 2014b), and superfluous fermenting bacteria would compete with denitrification bacteria for carbon source (Bernat and Wojnowska-Baryła 2007). On the other hand, oxygen appeared to be as an alternate and energetically preferable electron acceptor for facultative NRB (Xu et al. 2014b). So, oxygen of excess may compete with nitrate for the same enzymes (or electron donor), resulting in a lower nitrate reduction rate (Bernat and Wojnowska-Baryła 2007; Knowles 1982). According to the above analysis, the DO is a key factor to determine the coexistence and performance of SOB, carbon degradation bacteria, and NRB and then influences the efficiencies of sulfide oxidation, nitrate reduction, and  $\text{S}^0$  generation (Chen et al. 2010a; Wang et al. 2015; Xu et al. 2014b).

So, understanding the relationship among SOB, carbon degradation bacteria and NRB under different DO in the bioreactor are meaningful to the improvement of sulfide and nitrate removal efficiencies. However, previous studies were focused on sulfide and nitrate removal efficiencies in microaerobic conditions (Wang et al. 2015) or the diversity of microbial communities in anoxic sulfide and nitrate

removal bioreactors (Chen et al. 2008; Huang et al. 2015a, b; Mora et al. 2015). The bacterial community structures of microaerobic bioreactors need to be explored urgently. The aims of this work are to investigate the changes of the diversity, structure, and abundances of functional species at different DO levels with the use of polymerase chain reaction denaturing gradient gel electrophoresis (PCR-DGGE) technique and to reveal the linkage between the abundances of functional species and the performance of reactor. The reaction pathways for sulfide and nitrate removal under microaerobic conditions were also deduced by combining batch experiments with functional species analysis to better understand the interaction between sulfide-oxidizing and denitrification, which was helpful for better reactor performance and control.

## Materials and methods

### Bioreactor design

A polymethyl methacrylate cylinder (diameter 100 mm and height 800 mm) with 3.7 L active volume was used for this research. The microaerobic activated sludge reactor was covered by a water jacket to keep the operational temperature at  $30.0 \pm 0.5$  °C (Fig. S2). Influent was introduced into the bottom of the bioreactor with a peristaltic pump. Air stream is introduced into the bioreactor by a sparger fixed at the bottom. DO was adjusted by changing gas flow rates ( $20$ – $600$  mL  $\cdot$  min $^{-1}$ ).

Activated sludge obtained from the secondary sedimentation tank of a municipal wastewater treatment plant in Chunliu Wastewater Treatment Plant, Dalian, China was incubated into the bioreactor. SOB and NRB cultures were enriched by sulfide and nitrate-laden medium. Table S1 shows the reactor startup stages operation parameters. The sulfide and nitrate-laden synthetic wastewater were prepared according to the following composition: first with  $800 \pm 30$  mg  $\cdot$  L $^{-1}$   $\text{S}^{2-}$  ( $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$ ),  $220 \pm 10$  mg  $\cdot$  L $^{-1}$  ( $\text{NaNO}_3$ ),  $2200 \pm 105$  mg  $\cdot$  L $^{-1}$  (glucose),  $500 \pm 20$  mg  $\cdot$  L $^{-1}$  yeast extract,  $2000 \pm 98$  mg  $\cdot$  L $^{-1}$   $\text{NaHCO}_3$ ,  $46 \pm 2$  mg  $\cdot$  L $^{-1}$  ( $\text{KH}_2\text{PO}_4$ ), and other trace metals at hydraulic retention time (HRT) of 8.8 h, pH  $8.0 \pm 0.2$ . After the domestication of activated sludge in microaerobic system for sulfide and nitrate removal was finished, the loading rate increased stepwise in 67 days to  $8.72 \pm 0.04$  (S),  $2.49 \pm 0.04$  (N), and  $23.52 \pm 0.55$  (COD) kg  $\cdot$  m $^{-3}$  day $^{-1}$ , respectively, at HRT of 2.2 h.

### Effects of DO on $\text{S}^0$ generation efficiency and sulfide and nitrate removal efficiencies

When the performance of the bioreactor was stable, the DO was stepwise changed in order to investigate the effects of DO on  $\text{S}^0$  generation efficiency and sulfide and nitrate removal

efficiencies. The degradation period consisted of four stages, I ( $DO < 0.1 \text{ mg} \cdot \text{L}^{-1}$ ), II ( $0.1 < DO < 0.3 \text{ mg} \cdot \text{L}^{-1}$ ), III ( $0.3 < DO < 0.5 \text{ mg} \cdot \text{L}^{-1}$ ), and IV ( $0.5 < DO < 0.7 \text{ mg} \cdot \text{L}^{-1}$ ). During each changing period, influents and effluents were collected every day to measure concentrations of sulfide, sulfate, thiosulfate, nitrate, nitrite, and COD.

### Batch cultures

Batch tests were conducted in order to explore the substrate consumption mechanism. Two media used in this study were prepared as follows: organic carbon source (S+N+C) fed medium, containing  $263.8 \pm 4.1$  ( $8.2 \pm 0.1 \text{ mM}$ )  $\text{mg} \cdot \text{S}^{2-} \cdot \text{L}^{-1}$  ( $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$ ),  $144.0 \pm 1.7$  ( $10.3 \pm 0.1 \text{ mM}$ )  $\text{mg} \cdot \text{NO}_3^- \cdot \text{N} \cdot \text{L}^{-1}$  ( $\text{NaNO}_3$ ),  $500 \pm 20 \text{ mg} \cdot \text{L}^{-1}$  yeast extract,  $1500 \pm 83 \text{ mg} \cdot \text{COD} \cdot \text{L}^{-1}$  (glucose) ( $8.06 \pm 0.11 \text{ mM}$ ) and other trace metals, and organic-free medium (S+N), which was identical to the one above, except that it contained 0 mM organic carbon. Mature sulfide and nitrate removal granules were formed after cultivation for 129 days in microaerobic conditions. Activated sludge was collected by centrifugation at 10,000 rpm for 5 min, washed twice with required medium, and resuspended in the same medium to achieve initial VSS of  $4000 \pm 196 \text{ mg} \cdot \text{L}^{-1}$ . One hundred milliliter aliquots of required culture were transferred into 125-mL serum bottles. Serum bottles were kept open and were not subjected to helium gas stripping. The bottles then were inoculated at  $30 \pm 1.0 \text{ }^\circ\text{C}$  with no shaking (Chen et al. 2010a), while the pH was adjusted to  $8.0 \pm 0.2$ . In each batch tests experimental condition, three serum bottles with identical feed were prepared to confirm data reproducibility, and an abiotic control was also performed. Liquid samples were periodically taken with sterile needle and syringe.

### Analysis procedures

An ion chromatography (Dionex ICS-1100, USA) was employed to monitor the concentrations of sulfate, thiosulfate, nitrate, and nitrite in the collected liquor samples which had been filtrated through  $0.45\text{-}\mu\text{m}$  filters. Sample separation and elution were performed in an IonPac AG22 AS22 4-mm analytical column with carbonate/bicarbonate as eluent (Chen et al. 2009). The dissolved sulfide concentration was measured by methylene blue method (Trüper and Schlegel 1964). A pH/ORP meter (pHS-30, China) was employed to determine the pH and oxidation-reduction potential (ORP) of liquid samples. Concentrations of DO were measured by a DO meter (YSI 550A, USA). Quantities of COD, SS, and VSS were determined according to the standard methods (APHA 2005). Elemental sulfur production was calculated by theoretic equation according to (Eq. 6) (de Graaff et al. 2012):

$$[\text{S}^0] = [\text{InfluentS}] - [\text{SO}_4^{2-}] - 2 * [\text{S}_2\text{O}_3^{2-}] - [\text{HS}^-] \quad (6)$$

### DNA extraction, 16S rDNA amplification, and DGGE analysis

Microaerobic sludge samples were collected from stages I ( $DO < 0.1 \text{ mg} \cdot \text{L}^{-1}$ ), II ( $0.1 < DO < 0.3 \text{ mg} \cdot \text{L}^{-1}$ ), III ( $0.3 < DO < 0.5 \text{ mg} \cdot \text{L}^{-1}$ ), and IV ( $0.5 < DO < 0.7 \text{ mg} \cdot \text{L}^{-1}$ ) to analyze the bacterial community. In each period, once the sulfide and nitrate removal efficiencies were stable, activated sludge was sampled from the bioreactors for 3 days and the samples were fully mixed. All of the samples were stored at  $-80 \text{ }^\circ\text{C}$  before the extraction of total DNA.

After 0.25 g (wet weight) mixed sludge of each stage was weighed, the total DNA extraction was performed according to the manufacturer's instructions with a PowerSoil DNA Isolation Kit (MO BIO, USA). The extracted DNA samples were stored in a  $-20 \text{ }^\circ\text{C}$  freezer before use. For DGGE analysis, the bacterial 16S ribosomal RNA (rRNA) gene was amplified by bacterial primer sets GC-341F ( $5'\text{-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GCC TAC GGG AGG CAG CAG-3}'$ ) and 907R ( $5'\text{-CCT ACG GGA GGC AGC AG-3}'$ ). Polymerase chain reaction (PCR) amplification was conducted by a thermal cycler (Veriti 96-Well Fast Thermal Cycler, Applied Biosystems, USA) with a touchdown PCR method as following program: initial reaction mixtures were held at  $95 \text{ }^\circ\text{C}$  for 5 min, followed by 30 cycles of  $94 \text{ }^\circ\text{C}$  for 30 sec,  $50 \text{ }^\circ\text{C}$  for 1 min, and  $72 \text{ }^\circ\text{C}$  for 2 min per cycle. Finally, an extension step of 10 min at  $72 \text{ }^\circ\text{C}$  was performed. PCR products of the proper size were confirmed by electrophoresis through a 1 % agarose gel stained with GoldView (Sangon Biotech Co., Ltd., Shanghai, China) in  $0.5 \times \text{TAE}$  buffer, and DGGE was performed at  $60 \text{ }^\circ\text{C}$  with a D-CODE System Universal Mutation (BioRad Co., Ltd., USA), which was conducted according to the manufacturer's instruction. The PCR products were applied directly on a DGGE gel of 8 % polyacrylamide with a linear denaturing gradient ranging from 30 to 70 % (100 % denaturant was a mixture of 7 mM urea and 40 % [v/v] formamide). Electrophoresis was run at a constant voltage of 180 V for 6 h in  $1 \times \text{TAE}$  buffer. Subsequently, the gels were stained with GoldView (Sangon Biotech Co., Ltd., Shanghai, China) in  $1 \times \text{TAE}$  buffer for 40 min, and gel digital images were obtained by the Gel Doc 2000 System (BioRad Co., Ltd., USA).

Each gel slice containing an obvious electrophoretic band was excised and placed in a 1.5-mL Eppendorf tube incubated with TE buffer. Then, the Eppendorf tubes were stored at  $4 \text{ }^\circ\text{C}$  for 12 h. DGGE profiles were analyzed using the Quantity One software. The Shannon-Wiener index was calculated using the Shannon method. The richness (S) of the microbial population was examined using the number of bands in each lane. Selected DGGE bands were excised and re-amplified by PCR using the primers described above without the GC clamp. The PCR products were sequenced at Sangon

Biotech Co., Ltd. (Shanghai, China). The sequenced 16S rDNA gene was analyzed using the NCBI search tool to identify the closest matching sequence. The sequences were compared with the GenBank databases using the Basic Local Alignment Search Tool algorithm to determine the approximate phylogenetic affiliations. Representatives from the different clone types were submitted to the ribosome database (<http://rdp.cme.msu.edu/index.jsp>).

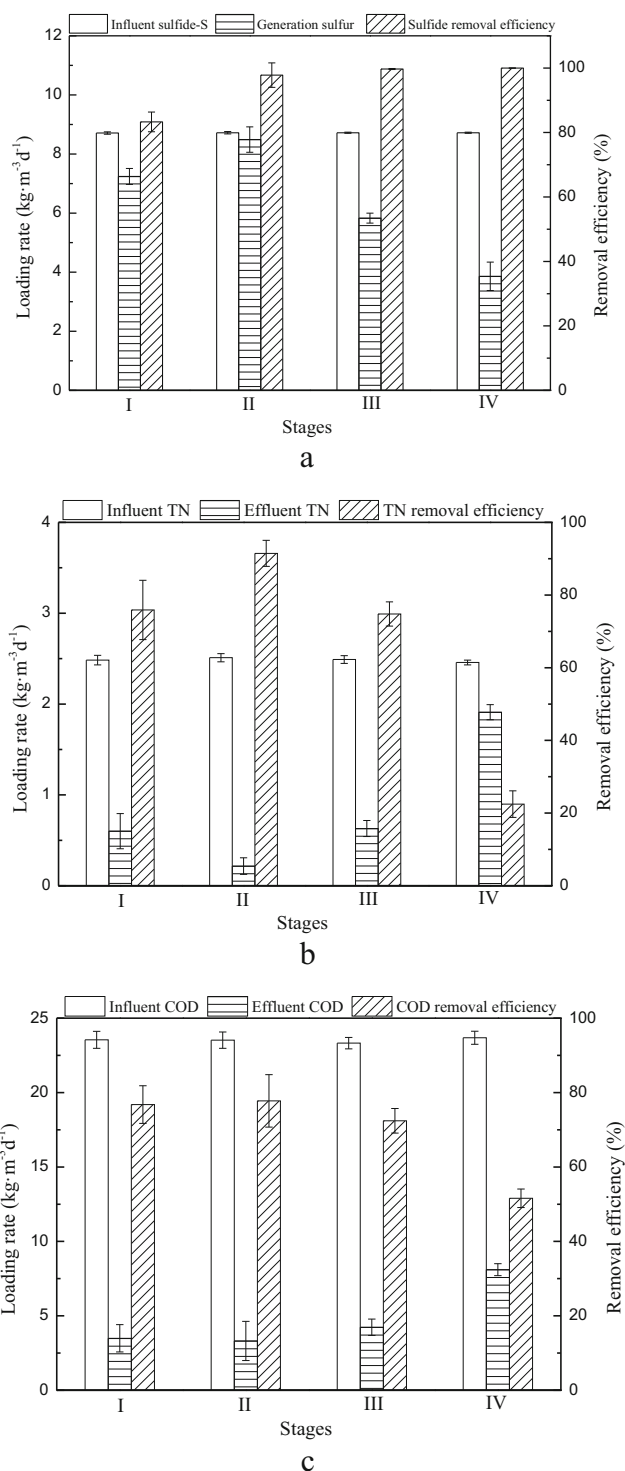
The sequences obtained were aligned in the GenBank DNA database with the BLAST algorithm (<http://www.ncbi.nlm.nih.gov/genbank/>). Sequences obtained from band 1 to 11 were deposited in the GenBank with the accession number of KT819574, KT825876~KT825885.

## Results

### Effects of DO on $S^0$ generation and sulfide and nitrate removal

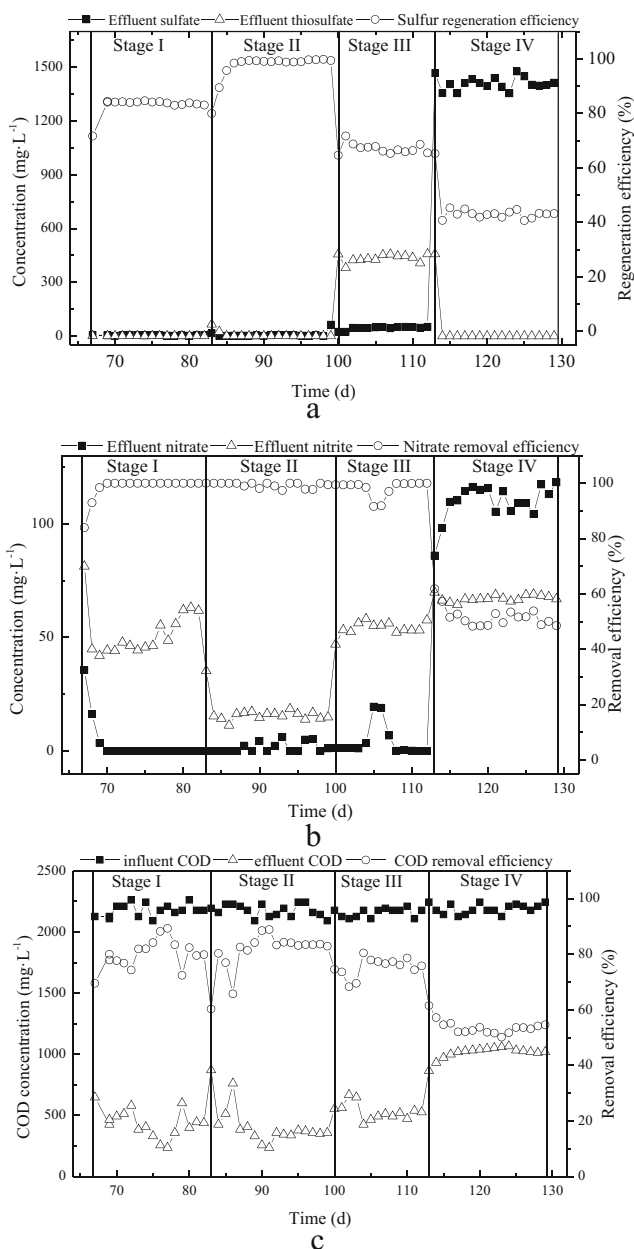
After start-up (66 days), the operation consisting of four stages continued for 63 days, totaling running for 129 days (Table S1). Figures 1 and 2 show the time course of  $S^{2-}$  loading rate,  $S^0$  loading rate, TN loading rate, COD loading rate, effluent thiosulfate, effluent sulfate, effluent nitrite, effluent nitrate, influent and effluent COD,  $S^{2-}$  removal ( $S^{2-}_{Re}$ ),  $S^0$  generation ( $S^0_{Ge}$ ), TN removal ( $TN_{Re}$ ), nitrate removal ( $N_{Re}$ ), and COD removal ( $COD_{Re}$ ) at four different DO levels during 63 days of continuous operation of the lab-scale system. As DO levels were shifted stepwise from  $<0.1 \text{ mg}\cdot\text{L}^{-1}$  to  $0.1 \text{ mg}\cdot\text{L}^{-1} < \text{DO} < 0.3 \text{ mg}\cdot\text{L}^{-1}$ ,  $0.3 \text{ mg}\cdot\text{L}^{-1} < \text{DO} < 0.5 \text{ mg}\cdot\text{L}^{-1}$ , and then to  $0.5 \text{ mg}\cdot\text{L}^{-1} < \text{DO} < 0.7 \text{ mg}\cdot\text{L}^{-1}$ , there were significant increases and subsequent decreases in the  $S^0_{Ge}$  (from  $83.1 \pm 3.1$  to  $98.3 \pm 2.6$  %,  $67.1 \pm 1.9$  and then to  $42.9 \pm 1.2$  %),  $TN_{Re}$  (from  $75.9 \pm 8.2$  to  $91.5 \pm 3.6$  %,  $74.8 \pm 3.3$  and then to  $22.5 \pm 3.7$  %),  $COD_{Re}$  (from  $79.9 \pm 5.3$  to  $81.0 \pm 7.3$  %,  $75.4 \pm 3.4$  and then to  $53.7 \pm 2.6$  %), while  $S^{2-}_{Re}$  increased significantly with DO concentration improved to  $0.1$ – $0.3 \text{ mg}\cdot\text{L}^{-1}$  and then kept stable with the further increase of DO levels (from  $83.3 \pm 3.1$  to  $97.8 \pm 3.8$  %,  $99.7 \pm 0.7$  and then to  $100 \pm 0.1$  %). Overall, the results showed that  $S^0$  generation and sulfide, nitrate, and COD removal performance was significantly affected by DO concentrations.

As shown in Figs. 1 and 2, the sulfide, nitrate, and COD removal simultaneously reached the highest efficiencies in the  $0.1$ – $0.3 \text{ mg}\cdot\text{DO}\cdot\text{L}^{-1}$ , and at same time, the  $S^0$  generation efficiency also reached a peak. A similar behavior was also observed in microaerobic bioreactor (Wang et al. 2015). It was suggested by previous research that the optimal DO concentration for nitrogen removal in CANON reactor was  $0.2 \text{ mg}\cdot\text{DO}\cdot\text{L}^{-1}$ , in which 75.4 % total nitrogen was removed (Varas et al. 2015).  $S^0$  generation efficiency decreased with DO increasing to more than  $0.3 \text{ mg}\cdot\text{L}^{-1}$ , because partial



**Fig. 1** Effects of DO on loading rates and removal efficiency. I ( $\text{DO} < 0.1 \text{ mg}\cdot\text{L}^{-1}$ ), II ( $0.1 < \text{DO} < 0.3 \text{ mg}\cdot\text{L}^{-1}$ ), III ( $0.3 < \text{DO} < 0.5 \text{ mg}\cdot\text{L}^{-1}$ ), IV ( $0.5 < \text{DO} < 0.7 \text{ mg}\cdot\text{L}^{-1}$ ). **a** Effects of DO on sulfide removal efficiency and  $S^0$  generation loading rate. **b** The effects of DO on TN influent and effluent loading rate, TN removal efficiency. **c** The effects of DO on COD influent and effluent loading rate, COD removal efficiency

sulfide was further oxidized into thiosulfate and sulfate by excessive DO. The values of oxidized sulfide (sulfate or



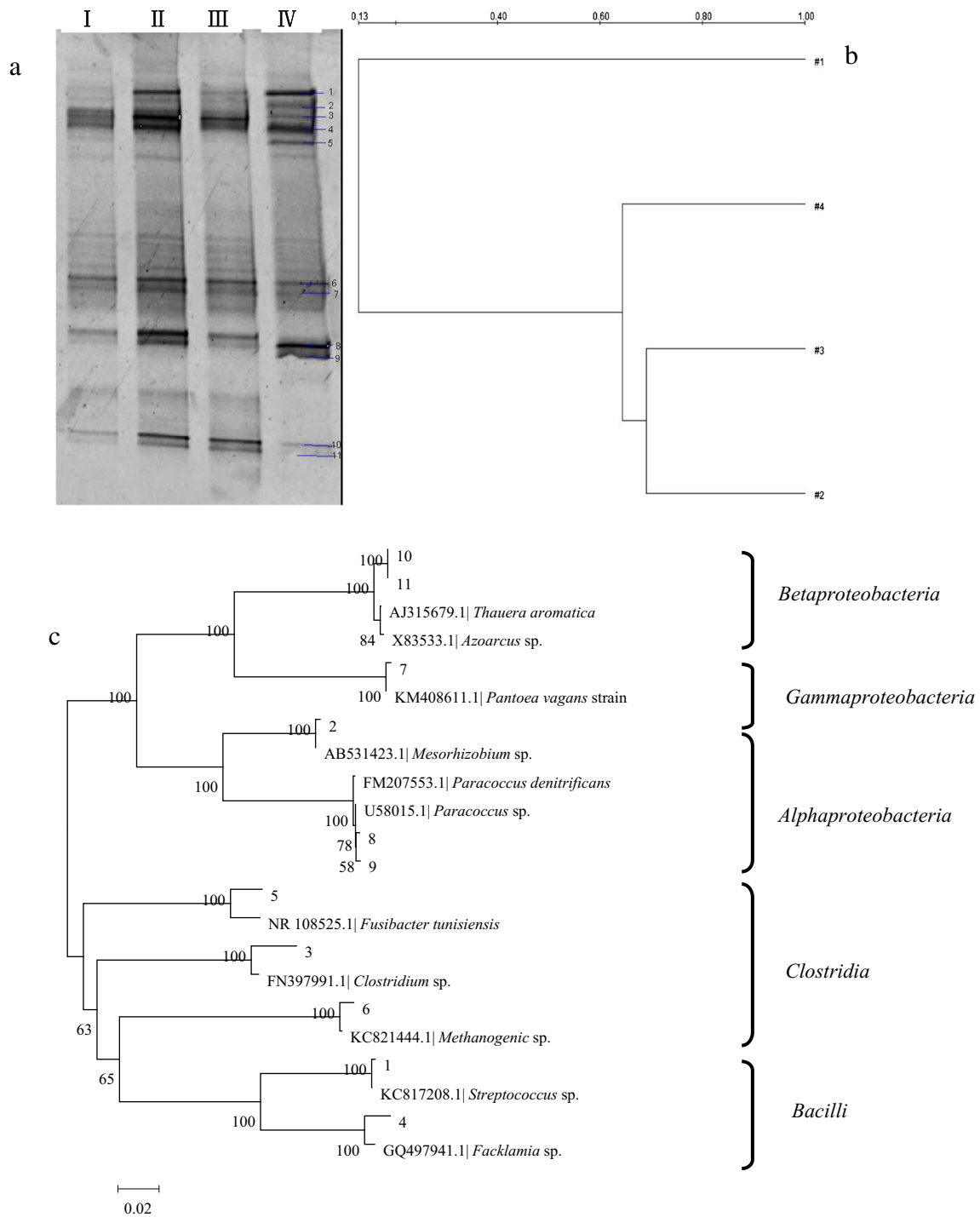
**Fig. 2** The effects of DO on conversion efficiency and effluent concentrations. *I* ( $DO < 0.1 \text{ mg} \cdot \text{L}^{-1}$ ), *II* ( $0.1 < DO < 0.3 \text{ mg} \cdot \text{L}^{-1}$ ), *III* ( $0.3 < DO < 0.5 \text{ mg} \cdot \text{L}^{-1}$ ), *IV* ( $0.5 < DO < 0.7 \text{ mg} \cdot \text{L}^{-1}$ ). **a** Effects of DO on  $S^0$  generation efficiency and effluent concentrations of sulfate and thiosulfate. **b** Effects of DO on TN removal efficiency and effluent concentrations of nitrate and nitrite. **c** Effects of DO on COD removal efficiency and concentrations of influent and effluent

thiosulfate) in effluent were indeed improved in higher DO levels when the influent sulfide concentration remained unchanged (Fig. 2a). A similar phenomenon was also observed in previous report that the increase of DO resulted in significant increases in  $S^0$  generation efficiency and subsequent decreases with the presence of a certain amount of sulfide (Roosta et al. 2011). During the degradation period (I–II), the consumed sulfide was mainly oxidized to elemental sulfur

with the sulfur generation efficiency gradually increased from  $83.1 \pm 3.1$  to  $98.3 \pm 2.6$  % (Figs. 1 and 2). The results of the present work were in agreement with those reported earlier, which implied that under limited oxygen conditions, almost all of sulfide was oxidized to elemental sulfur instead of sulfate in fluidized loop reactor (Krishnakumar et al. 2005).

### Effect of DO concentration on microbial community

Relative abundances (%) of bacterial species based on DGGE profile and Shannon diversity analysis were shown in Fig. 3 and Table 1. While the most similar sequences of predominant DGGE bands were listed in Table 2. Through phylogenetic analyses, the dominant populations in the reactors were mainly divided into five groups, including *Bacilli*, *Alphaproteobacteria*, *Betaproteobacteria*, *Gammaproteobacteria*, and *Clostridia* (Fig. 3c). According to DGGE profile and phylogenetic analysis, the microbial population structure and the microbial population diversity of microaerobic reactor were both varied among different stages of the sulfide and nitrate removal reactor. In the first stage of the operation ( $DO < 0.1 \text{ mg} \cdot \text{L}^{-1}$ ), the stripe intensity of sulfide and nitrate removal reactor was weaker, while microbial species (e.g., bands 3, 4, 6, 8, and 10) similar to species *Clostridium* sp., *Facklamia* sp., *Methanogenic prokaryote*, *Paracoccus denitrificans*, *Thauera aromatica*, respectively, were detected. In the second stage of the operation ( $0.1 \text{ mg} \cdot \text{L}^{-1} < DO < 0.3 \text{ mg} \cdot \text{L}^{-1}$ ), the variation in the number of DGGE bands and the enhanced stripe intensity indicated that the microbial population structure of sulfide and nitrate removal reactor was greatly altered with a sharp increase in microbial populations. Some new species appeared in the sulfide and nitrate removal reactor with stronger brightness, such as bands 1, 7, 9, and 11, the most similar species of which were *Streptococcus* sp., *Pantoea vagans*, *Paracoccus* sp., and *Azoarcus* sp., respectively. In the third stage of the continuous operation ( $0.3 \text{ mg} \cdot \text{L}^{-1} < DO < 0.5 \text{ mg} \cdot \text{L}^{-1}$ ), the microbial population diversity in the sulfide and nitrate removal reactor decreased significantly, and the stripe intensity became weaker. Some species disappeared in the sulfide and nitrate removal reactor, such as bands 1, 7, and 9, whose maximum similarity bacteria were *Streptococcus* sp., *Pantoea vagans*, and *Paracoccus* sp., respectively. In the fourth stage of the continuous operation ( $0.5 \text{ mg} \cdot \text{L}^{-1} < DO < 0.7 \text{ mg} \cdot \text{L}^{-1}$ ), the microbial community was altered distinctly. The carbon degradation bacteria (*Bacilli* and *Clostridia*) was obviously increased, while the NRB (*Azoarcus* and *Thauera*) was decreased dramatically. Some new species appeared in the sulfide and nitrate removal reactor, such as band 5, whose maximum similar species was *Fusibacter tunisiensis*. On the other hand, some species disappeared in the sulfide and nitrate removal reactor, such as bands 7 and 11, whose maximum similar species were *Pantoea vagans* and *Azoarcus* sp. According to the changes of



**Fig. 3** DGGE profile (a) and UPGMA dendrogram (b) neighbor-joining phylogenetic tree for the partial SO-NR sequences from 11 OTUs. The tree was constructed by the neighbor-joining in MEGA 4, and the scale bar represents 0.02 differences in nucleotide sequence (c)

microbial populations in sulfide and nitrate removal reactor, it could be concluded that the diversity and the number of microbial populations of sulfide and nitrate removal reactor had a closed relationship to DO concentration.

To obtain more mechanistic insights into the effect of increased DO concentrations on functional species, several key species involved in sulfur, nitrate, and carbon cycling were

analyzed. The detected species involved in sulfide oxidation, denitrification, and carbon degradation were highly diverse, and the relative abundance of these genes varied considerably among the four stages (Fig. 3 and Table 1).

Carbon degradation bacteria including *Streptococcus* sp. (band 1) (Mickelson 1972), *Clostridium* sp. (band 3) (Monot et al. 1982), *Facklamia* sp. (band 4) (Di Giacomo et al. 2007),

**Table 1** Relative abundance (%) of bacterial species based on DGGE profile analysis

DGGE band	I DO<0.1 mg·L <sup>-1</sup>	II 0.1<DO<0.3 mg·L <sup>-1</sup>	III 0.3<DO<0.5 mg·L <sup>-1</sup>	IV 0.5<DO<0.7 mg·L <sup>-1</sup>
1 <i>Streptococcus</i> sp.	5.45	8.76	6.83	11.01
2 <i>Mesorhizobium</i> sp.	4.55	4.25	4.27	9.30
3 <i>Clostridium</i> sp.	15.46	5.81	13.67	6.31
4 <i>Facklamia</i> sp.	18.03	14.99	20.03	16.02
5 <i>Fusibacter tunisiensis</i>	2.90	1.48	2.05	11.06
6 <i>Methanogenic</i> sp.	4.55	11.45	11.60	7.86
7 <i>Pantoea vagans</i>	20.34	11.34	11.89	8.30
8 <i>Paracoccus denitrificans</i>	13.95	14.93	9.36	15.67
9 <i>Paracoccus</i> sp.	4.48	12.61	5.13	8.61
10 <i>Thauera</i> sp.	6.83	8.81	10.49	3.39
11 <i>Azoarcus</i> sp.	3.46	5.57	4.68	2.47

*Methanogenic prokaryote* (band 6) (Ito et al. 2012), and *Fusibacter tunisiensis* (band 5) (Ben Hania et al. 2012) were detected in the reactor. The DGGE identified five denitrifiers in the bioreactor, namely *Mesorhizobium* sp. (band 2) (Costa et al. 2000), *Pantoea vagans* (band 7) (Feng et al. 2012), *Paracoccus denitrificans* (band 8) (Shi et al. 2013), *Paracoccus* sp. (band 9) (Shi et al. 2013), *Thauera aromatica* (band 10) (Zhao et al. 2013), and *Azoarcus* sp. (band 11) (Lee et al. 2014) (Fig. 3). Additionally, the sulfide-oxidizing bacteria in the microaerobic reactor were noted to be *Paracoccus* (bands 8 and 9) (Quentmeier et al. 2003).

*Mesorhizobium* sp. was reported to have the capability for denitrification with methane as external carbon source in oxygen-limited conditions (Costa et al. 2000). *Pantoea vagans* was associated with denitrification and refractory organic degradation under low DO concentration (Feng et al.

2012). *Paracoccus* sp. was confirmed to perform denitrification reactions no matter under anaerobic (Vackova et al. 2011) or aerobic conditions (Shi et al. 2013). *Azoarcus* sp. could reduce nitrate through autotrophic pathways (Lee et al. 2014). In detail, nitrate could be reduced by the reducing equivalent formed in the process of sulfide oxidation (Lee and Wong 2014). Meanwhile, *Azoarcus* sp. was also reported to have the capability for heterotrophic nitrate-reducing in both anaerobic and aerobic conditions (Rhee et al. 1997). *Thauera aromatica* was reported to be able to reduce nitrate with organics as substrate and was also demonstrated that CO<sub>2</sub> and N<sub>2</sub> were the end products in both anaerobic (Hubert and Voordouw 2007) and aerobic conditions (Luo et al. 2014; Zhao et al. 2013). *Paracoccus* sp. is an SOB that can oxidized sulfide with O<sub>2</sub> as electron acceptors whether in lithotrophic or mixotrophic conditions (Quentmeier et al. 2003), and it also

**Table 2** Identity of dominant DGGE bands

DGGE band	Phylogenetic affiliation	Genbank accession number	Isolation source
1	<i>Streptococcus mitis</i> strain V24-181 16S rRNA gene, partial sequence	KT819574	Fermentation process
2	<i>Mesorhizobium</i> sp. IV-19 gene for 16S rRNA, partial sequence	KT825876	Solid media
3	<i>Clostridium</i> sp. AN-D partial 16S rRNA gene, isolate AN-D	KT825877	Anaerobic process
4	<i>Facklamia</i> sp. KO_PS21 16S rRNA gene, partial sequence	KT825885	Cultivable microbial
5	<i>Fusibacter tunisiensis</i> strain BELH1 16S rRNA gene, partial sequence	KT825878	Anaerobic reactor treating olive-mill wastewaters
6	<i>Methanogenic prokaryote</i> enrichment culture B31_4_153 16S rRNA gene, partial sequence	KT825879	Hypothermia methanogenic condition
7	<i>Pantoea vagans</i> strain LJ16 16S rRNA gene, partial sequence	KT825880	Fermentation process
8	<i>Paracoccus denitrificans</i> partial 16S rRNA gene, isolate E4	KT825881	Sulfur mustard gas
9	<i>Paracoccus</i> sp. KS1 16S rRNA gene, complete sequence	KT825882	Sulfur-based autotrophic denitrification
10	<i>Thauera aromatica</i> 16S rRNA gene, strain SP	KT825883	Denitrifying conditions
11	<i>Azoarcus</i> sp. mXyN1 partial 16S rRNA gene, strain mXyN1	KT825884	Anaerobic denitrifying conditions

has been proven to have the sulfide-oxidizing ability under denitrifying conditions (Chen et al. 2010b).

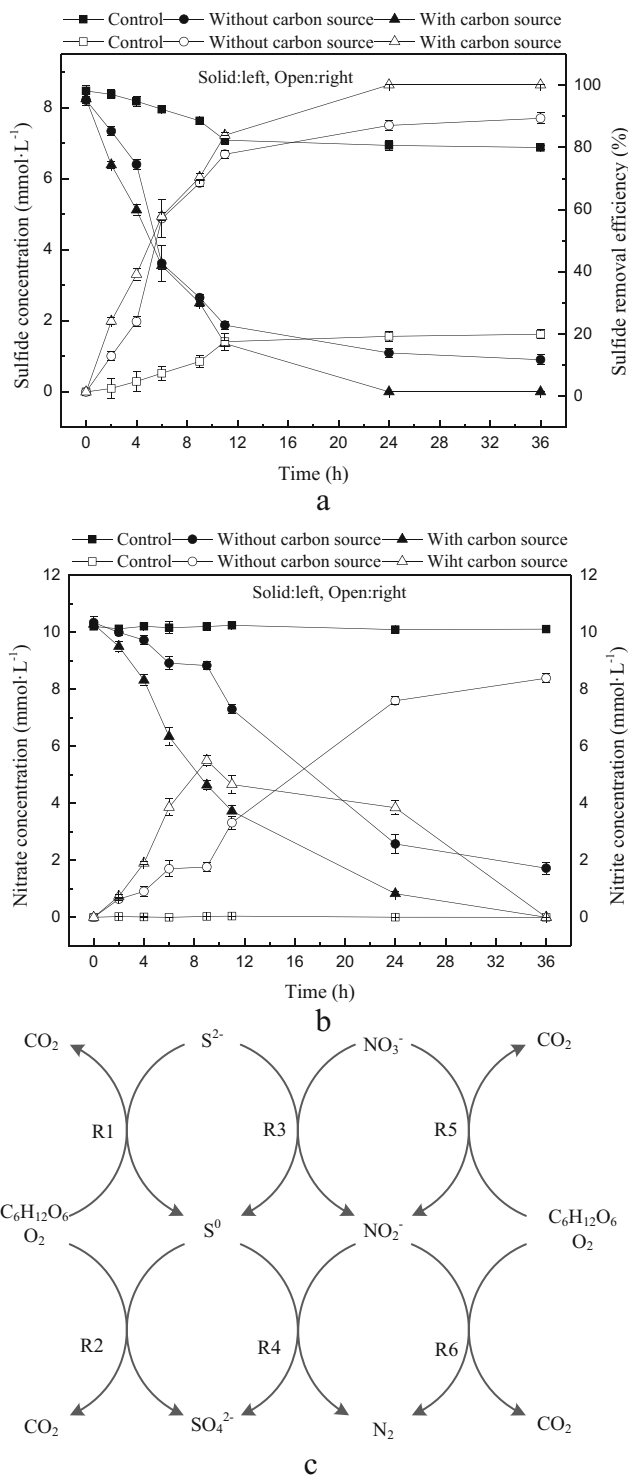
**Batch cultures**

Batch cultures were conducted to further investigate the mechanism of the sulfide and nitrate removal process under microaerobic conditions. The removal performances of batch tests are presented in Fig. 4. It could be observed that the sulfide and nitrate removal sludge in S+N+C medium oxidized sulfide and reduced nitrate at higher rates than that in S+N medium (Fig. 4).

During the first 4 h, 3.1 mM sulfide as well as 2.0 mM nitrate (nitrate to nitrite) was removed in the presence of organic carbon source. Meanwhile, only 1.8 mM sulfide and 0.6 mM nitrate were eliminated in the absence of substrate. Between 4 and 9 h, 2.6 mM sulfide was oxidized, and 3.6 mM nitrate (nitrate to nitrite) was reduced in S+N+C medium. Correspondingly, 3.8 mM sulfide and 0.9 mM nitrate (nitrate to nitrite) were removed in S+N medium. In the presence of organic carbon source, nitrite began to decline at 9 h, accompanied by continuous decline of nitrate. It only needed 24 h for the sulfide (8.2 mM) being completely removed and 36 h for full TN removal (10.3 mM) with sludge incubated in S+N+C medium. However, when the organic carbon source was absent, only 7.3 mM sulfide and 8.6 mM nitrate (nitrate to nitrite) were removed even in roughly 36 h.

**Discussion**

Microaerobic mediated synergistic relationships between SOB and NRB, accompanying the improvement of the efficiency of sulfide conversion to  $S^0$ , are a promising strategy for the potential co-reduction of enriched sulfide and nitrate. For the operating conditions tested in the study, both high removal efficiencies of sulfide and nitrate and peak  $S^0$  formation efficiency ( $98.3 \pm 2.6\%$ ) were achieved in stage II. Compared with anoxic reactor, sulfide removal loading rate ( $8.72 \pm 0.04 \text{ kg-S} \cdot \text{m}^{-3} \cdot \text{d}^{-1}$ ) in microaerobic bioreactor was higher, and the performance of simultaneous sulfide and nitrate removal was also enhanced (Beristain-Cardoso et al. 2008; Chen et al. 2008, 2009; Reyes-Avila et al. 2004). Limited oxygen supply could not only improve the overall  $S^0$  conversion efficiency significantly but also substantially increased the sulfide and nitrate removal efficiencies. However, the efficiencies of  $S^0$  conversion and nitrate removal were decreased with the continuous increase of DO level. The variation of the performance of the microaerobic sulfide and nitrate removal bioreactor might be related to the evolution of the microbial functional communities with the changing DO concentration.



**Fig. 4** Effects of carbon source on sulfide, nitrate removal in microaerobic conditions, and the suggested pathway involved in the degradation process. **a** Effects of carbon source on sulfide removal in microaerobic conditions. **b**. Effects of carbon source on nitrate removal in microaerobic conditions. **c**. Suggested denitrification, sulfide and organic carbon source oxidation pathway under microaerobic conditions

This study comprehensively characterized the diversity, structure, and metabolic potential of the microbial



communities and investigated the shift of functional species in response to elevated DO levels. PCR-DGGE data analysis showed that the overall diversity, structure, and composition of the microbial communities had a closed relationship to DO levels.

The NRB are generally found abundant in nitrate laden wastewater treatment processes. It had been commonly accepted that NRB were strict anaerobes. However, in recent years, a growing number of studies have demonstrated that some NRB could utilize both oxygen and nitrate as electron acceptors (Bergaust et al. 2012; Luo et al. 2014; Shi et al. 2013; Vackova et al. 2011; Zhao et al. 2013). Higher abundances of NRB functional species were detected in stage II than that in stage I, indicating that limited oxygen had promoted the activity of NRB in the bioreactor. The same behavior was also observed in other researches that the abundance of NRB in limited oxygen fed bioreactor was approximately three to fivefold higher than that in anoxic bioreactor (Xu et al. 2014b). Nevertheless, a higher level of DO ( $0.3\text{--}0.5\text{ mg}\cdot\text{L}^{-1}$ ) was detrimental to both the growth rate and viability of NRB. It was suggested that oxygen appeared to be energetically preferable for facultative NRB. Oxygen could regulate the synthesis of nitrate reductase enzyme and inhibit the denitrification of facultative NRB, resulting in substrate electron flow to oxygen cytochromes (Wu et al. 1994). So, the lower nitrate reduction rate in higher DO concentration might be attributed to the competition between nitrate and oxygen for the same enzymes of transferred electron (Xu et al. 2014a). In early studies, it was also demonstrated that once the concentration of oxygen was lowered or removed, reduced *cytochrome b* might resume its transfer of electrons to *cytochrome c*, ultimately resulting in the reduction of nitrate (John 1977). DGGE data also showed the inhibition effect of high concentrations of DO ( $0.5\text{ mg}\cdot\text{L}^{-1}$ ) on NRB, as lower numbers and significant lower abundances of NRB functional species were detected in stage VI than that in stage I, stage II, and stage III. The reduction of NRB species might be due to the suppression of the thriving fermentation microorganisms which could strongly compete for carbon resources with NRB. The low nitrate removal efficiency in stage VI might be mainly caused by the low abundance of NRB instead of the competition between the excessive oxygen and nitrate or the inhibition of the excessive oxygen to the synthesis and activity of nitrate reductase.

The SOB species were reported to be able to use oxygen as electron acceptor, and it was also demonstrated that the heterotrophic growth of SOB could increase the rate of  $\text{S}^0$  oxidation (Hassan et al. 2013). The abundances of SOB species significantly increased in stage II ( $0.1\text{ mg}\cdot\text{L}^{-1} < \text{DO} < 0.3\text{ mg}\cdot\text{L}^{-1}$ ) (Fig. 3), suggesting that the sulfide oxidation process might be stimulated at stage II. This inference was supported by the great increase in the  $\text{S}^0$  conversion efficiency during stage II. In addition, the rapid sulfide degradation

caused by the thriving SOB mitigated the inhibition of sulfide for denitrifier, which partially contributed to the slight increase of the nitrate removal efficiency in stage II (Chen et al. 2009).

Interestingly, the abundance of carbon degradation species increased significantly in stage II when compared to stage I. And then the increasing DO concentration sustained a continued growth in the abundance of carbon degradation species. Stage IV had the highest abundance of carbon degradation species indicating the sludge potential capacity for removing a variety of types of organic carbon in bioreactor. It has been reported that the presence of organic carbon source could increase both the  $\text{S}^0$  oxidation rate and the nitrate removal rate (Chen et al. 2010a). So, the increased abundance of carbon degradation species might be due to the growing organic carbon requirement caused by the thriving of heterotrophic SOB and NRB. Additionally, although the abundance of SOB species had no obvious increase with the continuous increase of DO levels ( $\text{DO } 0.3\text{ mg}\cdot\text{L}^{-1}$ , Fig. 3), the decrease of  $\text{S}^0$  conversion efficiencies appeared (Figs. 1 and 2). It has been demonstrated that with excessive oxygen supply, sulfide was oxidized into  $\text{S}^0$  in the first place and then  $\text{S}^0$  continued to be oxidized into thiosulfate or sulfate (Jensen et al. 2011).

The bioreactor performance and functional species analysis of microbial community were joined with the specific removal rates of sulfide, nitrate, and carbon source in the batch tests to infer a possible explanation for the sulfide oxidation and denitrification metabolic pathway in continuous culture (Fig. 4c). It was observed that nitrate reduction proceeded in two steps: nitrate was first reduced to nitrite, and then nitrite was further reduced to nitrogen in the second step. The reduction rate of nitrate to nitrite (R3) with the oxidation of sulfide to sulfur is far lower than that with the oxidation of glucose (R5) (Fig. 4b). Meanwhile, the reduction rate of nitrite to  $\text{N}_2$  with the participation of sulfur oxidation (R4) is also significantly lower than that related to the oxidation of glucose (R6). In terms of the removal of sulfide, the batch culture results showed that the rate of sulfide oxidation to sulfur in the presence of oxygen (R1) is much higher than that caused by the reduction of nitrate to nitrite (R3) (Fig. 4a). In general, compared with the performance without organic carbon source medium, the specific consumption rates of sulfide with organic carbon source showed an increase of 1.72 times (highest), while denitrification rates with organic carbon source showed an increase of 2.98 times (highest). The behavior was also observed with the addition of acetate, in which both sulfide oxidation and denitrification rate were increased by roughly 55 % (Reyes-Avila et al. 2004). Those results demonstrated that mixotrophic denitrification rate was higher than the sulfur-based autotrophic denitrification rate. What is more, the production of sulfate (R4)

was significantly depressed by the mixotrophic denitrification (R6) process (Sahinkaya and Dursun 2012). The key species analysis in the microaerobic bioreactor also confirmed the sulfide oxidation and denitrification metabolic pathway. As shown in Figs. 3 and 4, Tables 1 and 2, the functional species in stage II included *Streptococcus* sp., *Clostridium* sp., *Facklamia* sp., *Methanogenic prokaryote*, *Pantoea vagans*, *Paracoccus* sp., *Thauera aromatica*, and *Azoarcus* sp. It was likely that the metabolic pathway of the oxidation of sulfide to sulfur at stage II was based on the reactions R1 and R3, while nitrate was reduced by the reactions R3, R5, and R6.

Overall, the results obtained in this study demonstrated that the microbial community functional compositions and structures changed dramatically in different dissolved oxygen concentrations. The abundances of NRB species involved in nitrate reduction processes increased with DO levels gradually elevated from <0.1 to 0.1–0.3 mg·L<sup>-1</sup>. Nevertheless, when the DO levels were further increased, the abundances of NRB species were observed to decrease slightly, which became significant with DO concentration more than 0.5 mg·L<sup>-1</sup>. The abundances of SOB species participating in sulfur/sulfide oxidation processes significantly increased with DO concentration growing within the range from 0.1 to 0.3 mg·L<sup>-1</sup> and remained stable with a further increase in DO concentration. Interestingly, the abundance of carbon degradation species continued to increase with the raising of DO levels. The variations of the abundances coincided with the performance of the microaerobic bioreactor.

Therefore, in the process of sulfide and nitrate removal, it is important to maintain an appropriate DO concentration to achieve the highest nitrate and sulfide removal efficiencies as well as the highest sulfur generation efficiency. Plenty of valuable information was provided by the PCR-DGGE analysis of functional microbial communities, which was meaningful for optimizing the performance of the microaerobic sulfide and nitrate removal reactor. Results obtained in the present research can be used as a design tool for removing enriched sulfide and nitrate efficiently in simultaneous flue gas desulfurization and denitrification process.

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#### Compliance with ethical standards

**Ethical approval** This article does not contain any studies with human participants or animals performed by any of the authors.

**Conflict of interest** The authors declare that they have no competing interests.

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