Role of Organic/Sulfide Ratios On DNRA Cultivation And The Microbial Community Structure of A Co-Driven Sequencing Biofilm Batch Reactor

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Abstract

Denitrification and dissimilatory nitrate reduction to ammonium (DNRA), are two competing pathways in nitrate reducing process. In this study, a series of C/S ratios from 8:1 to 2:4 was investigated in a sequencing biofilm batch reactor (SBBR) to determine the role of reducers (sulfide and acetate) on their competition. The results showed the proportion of DNRA increased in high electron system, either in organic rich or in sulfide rich system. The highest DNRA ratio increased to 16.7% at the C/S ratio of 2:3. Excess electron donors, particularly sulfide, were favorable for DNRA in a limited nitrate environment. Moreover, a higher reductive environment (ORP <-400 mV) can be used as an indicator for the occurrence of DNRA. 16s RNA analysis demonstrated that *Grobacter* was the main functional bacteria of DNRA in the organic rich system, while *Alphaproteobacteria* and *Desulfomicrobium* were dominant DNRA bacteria in the sulfide rich system. DNRA cultivation could enrich nitrogen conversion pathways in conventional denitrification systems. This provides the great insight into nitrogen removal in high nitrogen containing sewage with low C/N.

Highlights

1. Redundant electron donors, particularly sulfide facilitates the occurrence of DNRA.
2. Extent of DNRA did not keep increasing with the amount of electron donors in organic rich and sulfide-rich systems.
3. ORP lower than -400mV can indicate the occurrence of DNRA.
4. DNRA functional bacteria group varied with the types of electron donors.

Introduction

During the treatment of high nitrogen containing sewage without organic carbon (low C/N), the addition of an external electron donor is necessary to ensure a high nitrogen removal efficiency. Either sulfide / sulfur / thiosulfate or organics could be electron donor and they were effectively applied for low C/N wastewater treatment. In the mixed culture systems, sulfur components act as electron donor, and sulfur oxidizing bacteria (SOB) participate in sulfide-driven autotrophic denitrification by utilizing nitrate as electron acceptor (Xu et al. 2014). When organic components function as electron donors, denitrifiers participate in traditional heterotrophic denitrification. However, in recent years, many scholars proposed dissimilatory nitrate reduction to ammonium (DNRA), a new N transfer path which was a competing microbial nitrate-reduction processes with denitrification either in autotrophic or in heterotrophic situation (Giblin et al. 2013; Lu et al. 2013; Dolejs et al. 2015).

The presence of DNRA in municipal wastewater treatment plants (WWTPs) and its potential use in anammox process was highly evaluated. Wang et al. 2020 investigated samples from six WWTPs and found that DNRA bacteria were detected in all treatment units, though, the relative abundance of DNRA bacteria was less than that of denitrifying bacteria. Li et al. 2020 found that the nitrogen removal was enhanced in a anammox system by a Fe(II)-dependent DNRA-anammox process, in which DNRA was

performed by anammox bacteria, and provide NH_4^+ and NO_2^- to assist anammox process. Moreover, Li et al. 2020 found NH_4^+ was produced by DNRA process in a microbial fuel cell system, which was in favor of struvite formation. Since DNRA correlated with nitrogen conversion and could auxiliary enhancing nitrogen removal process, the conditions of its occurrence and competition with denitrification need to be further study.

The factors influencing DNRA have been identified in hypoxic or anaerobic environments (e.g. marine estuary sediments, and rice farmland), which includes microbial generation time, supply of nitrite relative to nitrate, the carbon/nitrogen ratio, pH, copper, presence of sulfide, supply of fermentation products, natural seawater and temperature (Roberts et al. 2014; Brett et al. 2015; Yin et al. 2017; Kessler et al. 2018; Zhu et al. 2018; Hoagland et al. 2019; Pandey et al. 2019; Pan et al. 2021). Nitrate limitation, a result of high COD:N ratio promotes nitrate conversion to ammonium, and DNRA increases with the elevation of COD:N ratio (4.9 to 7.7 mg COD/mg N) in a chemostat enrichment culture (Van den Berg et al. 2015). Change in COD:N ratio alters the oxidation state of the environment and the oxidation state or prevailing redox potential is the key factor to affect the competition (Matheson et al. 2002). In addition, Redox potential (ORP) influenced the competition between DNRA and denitrification, and the oxidation state can be influenced by the presence of reductants, such as sulfide, ferrous ion, manganese ion (Buresh and Patrick 1981). To distinguish these factors and to verify the critical ones, further studies in well-defined DNRA phenomenon should be conducted and the DNRA-functional bacteria should be identified in wastewater treatment systems.

Cultivation of DNRA from a traditional denitrification system and investigation of factors affecting its occurrence and competition with denitrification are of great significance. In this study, sodium acetate and sulfide were used as electron donors to determine the impacts of the C:S ratio on the N-conversion pathways under nitrate limitation conditions. The objectives of this study are: 1) to investigate the performance of the N-conversion process, with organics and sulfide hybrid functioning as electron donors and NO_3^- as electron acceptor in a sequencing biofilm batch reactor (SBBR). 2) to identify the interaction between denitrification and DARN in different C/S ratios. 3) to identify the main-functional bacteria of nitrogen paths in hybrid culture system.

Materials And Methods

2.1 Bioreactor set-up and operation

This experiment uses sequencing biofilm batch reactor (SBBR), which is a cylindrical container made of plexiglass with an inner diameter of 130 mm, a height of 165 mm and an effective volume of 2 L. The experimental inoculation sludge was taken from the Dengjiacun sewage treatment plant in Xi'an. The biofilm carrier was suspended in a cylindrical container which uses a hydroformyl fiber and a polyester fiber composite filler, and the cylindrical container was placed on a thermostatic magnetic stirrer to control 25°C water temperature and stirring speed of 400 r/min. The influent and effluent water were controlled by a peristaltic pump with an automatic control system. The dissolved oxygen (DO), pH and

Oxidation-Reduction potential (ORP) electrode probes were respectively placed in the reactor to monitor the operation of the system in real time.

The reactor was operated periodically, and the influent and effluent water were controlled by an automatic monitoring system. The influent and effluent water ratio was 1:1, the pH of influent water was controlled at 7.7 ± 0.1 , and the hydraulic retention time (HRT) was 12 hours. In order to minimize the interference of temperature and DO, the 25°C water was placed on a thermostat before each process line test, and it was blown off with nitrogen for 30 mins to make DO less than $0.3 \text{ mg}\cdot\text{L}^{-1}$. At the same time, the biofilm was washed 3 times with pure water to reduce the interference of the remaining substances in the previous cycle. After the preparation is completed, the freshly prepared influent water was injected into the reactor, and samples were collected at intervals of time for detection.

2.2 Materials

During the experiment, variable C:S ratios were obtained by varying the acetate concentration or sulfide concentration, act as dual electron donor in the influent with constant nitrate concentration, the single electron acceptor (Table 1). And other additions in each influent were NaHCO_3 $800 \text{ mg}\cdot\text{L}^{-1}$, KH_2PO_4 $350 \text{ mg}\cdot\text{L}^{-1}$, CaCl_2 $50 \text{ mg}\cdot\text{L}^{-1}$, Trace element 2 ml. The composition of trace element was H_3BO_3 $50 \text{ mg}\cdot\text{L}^{-1}$, CuCl_2 $30 \text{ mg}\cdot\text{L}^{-1}$, ZnCl_2 $50 \text{ mg}\cdot\text{L}^{-1}$, $(\text{NH}_4^+)_6\text{Mo}_7\text{O}_{21}\cdot 4\text{H}_2\text{O}$ $50 \text{ mg}\cdot\text{L}^{-1}$, $\text{MgSO}_4\cdot\text{H}_2\text{O}$ $500 \text{ mg}\cdot\text{L}^{-1}$, $\text{CoCl}_2\cdot 6\text{H}_2\text{O}$ $50 \text{ mg}\cdot\text{L}^{-1}$, AlCl_3 $50 \text{ mg}\cdot\text{L}^{-1}$, NiCl_2 $50 \text{ mg}\cdot\text{L}^{-1}$. In addition, HCL of $1 \text{ mL}\cdot\text{L}^{-1}$ was used to adjust pH of the liquor.

Table 1
Nitrate, sulfide and acetate concentrations of influent in different experimental periods.

Periods	Operation (d)	C/S /(mol)	COD /(mg·L ⁻¹)	S ²⁻ -S /(mg·L ⁻¹)	NO ₃ ⁻ -N /(mg·L ⁻¹)
a	0–28	8:1	260	32	28
b	29–47	4:1	130	32	28
c	48–66	2:1	65	32	28
d	67–90	2:2	65	64	28
e	91–117	2:3	65	96	28
f	118–140	2:4	65	128	28

2.3 Analytical procedures

Samples from different reactors were filtered by $0.45 \mu\text{m}$ microfiltration membrane. The concentration of $\text{NH}_4^+\text{-N}$, $\text{NO}_2^-\text{-N}$, and $\text{NO}_3^-\text{-N}$ was measured according to the standard methods (APHA 2005). Sulfide concentration (including H_2S , HS^- and S^{2-}) was determined by the methylene blue method (Truper and Schlegel 1964); $\text{SO}_4^{2-}\text{-S}$ was adopted reagent turbidity method of USEPA1 SulfaVer4; COD was analyzed

using a COD quick-analysis apparatus (American Hach Company). The redox potential (ORP) was measured using the PHSJ-3F portable intelligent meter (Chengdu Century Ark Technology Co., Ltd.).

2.4 Microbiological analysis

Samples collected from each steady stage in this experiment were retained for microbial community analysis. The 16S rRNA gene was amplified using a primer set (338F/806R) targeting the V3 and V4 hypervariable regions of both the bacteria and archaea domains (Florentino et al. 2015). The Illumina MiSeq sequencing service (Illumina Inc., San Diego, CA) was provided by Shanghai Majorbio Bio-pharm Technology Co., Ltd. And the data were analyzed on the online platform of Majorbio Cloud Platform. Evolutionary analyses were conducted in MEGA5.

In this experiment, the sequence with the highest abundance was selected as the representative sequence, and the OTU number change and sample hierarchy clustering relationship were plotted. According to the plot, an optimal clustering similarity value could be determined, and on this basis, OTU analysis and microbial taxonomy analysis were conducted. The relevant analysis of this experiment was carried out at a similar level approaching 100%. After the effluent substance remained stable at each stage of the experiment, samples were taken for the composition of the microbial components. Besides, we carried out the evolutionary relationships of taxa. The evolutionary history was inferred using the Neighbor-Joining method in this experiment with the bootstrap consensus tree speculated from 1000 replicates. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The corrected sequences were compared with those stored in GenBank using the Basic Local Alignment Search Tool algorithm (<http://www.ncbi.nlm.nih.gov/blast>). The sequences have been deposited in the GenBank under accession number SRR10518522.

2.5 Modification and presentation

$\text{NH}_4^+\text{-N}$ determination was interfered by S^{2-} , While in this experiment, S^{2-} was used as one of electron donors, a great amount of S^{2-} was added. Therefore, in order to eliminate the interference of S^{2-} , this experiment drew a fitting curve based on the influence data of different S^{2-} concentrations of 30 groups in a single state (Fig S1). NH_4^+ in the effluent was corrected by the total detected value minus the interference value (Eq. (1)).

$$\text{C}(\text{NH}_4^+\text{-N})_e = \text{C}(\text{NH}_4^+\text{-N})_t - \text{C}(\text{NH}_4^+\text{-N})_i \quad \text{Eq. (1)}$$

e-effluent, t-total, i-interference

As emissions of nitric and nitrous oxide were not detected, the nitrogen not accounted for in ammonium, nitrate, nitrite or biomass was assumed to be converted to N_2 . When the proportion of biomass assimilation was neglected, the nitrate conversion pathways in this experiment were mainly included denitrification and DNRA. Ammonium production was attributed to nitrate reduction by DNRA. Hence, the

proportion of denitrification and DNRA was calculated using Eq. (2). However, it should be clarified that the denitrification rate calculated here contains the proportion of nitrate to nitrite.

$$\text{DNRA rate} = \frac{c(\text{NH}_4^+ - \text{N})_{\text{eff}}}{c(\text{NO}_3^- - \text{N})_{\text{inf}}} \times 100\% \quad \text{Denitrification rate} = 1 - \text{DNRA rate} \quad \text{Eq(2)}$$

The reductive S^{2-} participates is further converted into substances such as S^0 , $\text{S}_2\text{O}_3^{2-}$, SO_3^{2-} , SO_4^{2-} in the process of sulfur autotrophic denitrification (Xu et al. 2013). In this experiment, we measured S^{2-} and SO_4^{2-} , and the intermediate substance of S (S^0 , SO_3^{2-} , $\text{S}_2\text{O}_3^{2-}$, S_n^{2-}) was calculated as the minus of S^{2-} and SO_4^{2-} -S. In addition, sulfide always interferes with organic measurement in terms of COD, therefore, COD indicated the amount of both organic and sulfide.

Results And Discussion

3.1 Effect of C/S on nitrogen conversion

Heterotrophic denitrification occurs with enough organic as electron donor, while autotrophic denitrification occurs with sulfide as electron donor. Similarly, the DNRA process can also be classified into fermentative DNRA and respiratory DNRA, based on the type of electron donors. The C/S ratio altered the nitrogen conversion pathways. With the increase of the C/S ratio from 2:1 to 8:1, the organic concentration increased from $65 \text{ mg} \cdot \text{L}^{-1}$ to $260 \text{ mg} \cdot \text{L}^{-1}$, while the sulfide concentration was maintained at a relatively low level ($32 \text{ mg} \cdot \text{L}^{-1}$). Thus, two electron donors coexisted in this system. The reaction rates of nitrate gradually increase from 12.07, 14.98 to $20.00 \text{ mg} \cdot (\text{L} \cdot \text{h})^{-1}$ with the C/S ratio being 2:1, 4:1, and 8:1, respectively. Nitrite accumulated during the whole process at the C/S ratio of 2:1 and the electron donors were completely consumed (Fig. 1), indicating that electron donors might be insufficient, and partial denitrification was performed. With more organic matters, nitrite firstly accumulated and was then quickly converted. At the C/S ratio of 8:1, there was nearly no nitrite residual, revealing that complete heterotrophic denitrification performed. Meanwhile, ammonia was detected at the conditions of the C/S ratio of 4:1 and 8:1 (1.75 and $0.17 \text{ mg} \cdot \text{L}^{-1}$), with limited electron acceptor of nitrate and excess of electron donor (with more organic than sulfide), indicating the presence of fermentative DNRA, which was consistent with previous study (Van den Berg, et al. 2015).

Sulfide outweighed organic matters at the C/S ratio of 2:2, 2:3, 2:4 with limited nitrate and sufficient electron donors, and the reaction rate of nitrate was 14.53, 13.9, $14.23 \text{ mg} \cdot (\text{L} \cdot \text{h})^{-1}$, respectively, which were higher than that of the C/S of 2:1, while lower than that of the C/S of 4:1 and 8:1, indicating the autotrophic N conversion rate was slower than that of heterotrophic process. In addition, there was a slight nitrite accumulation, and NH_4^+ was detected as the concentration of 3.09, 4.9 and $2.5 \text{ mg} \cdot \text{L}^{-1}$ in the effluent at the C/S of 2:2, 2:3 to 2:4, respectively, manifesting that respiratory DNRA process was enhanced in autotrophic processes.

The increases in nitrite and ammonium concentrations were synchronous with the decrease in nitrate concentration (Fig. 1), suggesting that they were likely generated via nitrate transformation. Proportions of different NO_3^- -N transfer pathways were established in Table 2. Sufficient electron donors (organics) with limited NO_3^- -N favored the occurrence of DNRA (Van den Berg et al. 2015). The highest DNRA rate took place at the C/S ratio of 4:1 in organic rich process (5.8 %) and the C/S ratio of 2:3 in sulfide rich process (16.7 %). This finding confirms that denitrification always remained dominant when competing with DNRA for nitrate during the entire operation. Notably, when electron donors were redundant, DNRA rate adversely declined either in heterotrophic dominated or in autotrophic dominated process. This phenomenon was also observed in previous study (Van den Berg et al. 2017) that the highest NH_4^+ conversion rate (0.43 mmol/h) occurred at the Ac/N ratio of 1.5:1 mol/mol, but not under the Ac/N of 1.87:1 mol/mol.

Table 2
Proportions of different N-conversion pathways

	C/S = 8:1	C/S = 4:1	C/S = 2:1	C/S = 2:2	C/S = 2:3	C/S = 2:4
Denitrification(%)	99.4	94.2	100	89.7	83.7	91.6
DNRA(%)	0.6	5.8	0	10.3	16.7	8.4

In the electron donor transfer process, the complete removal of 1 mol C (CH_3COO^-) requires 4 mol of electrons, and the complete oxidation of 1 mol S^{2-} requires 8 mol of electrons. Therefore, the total amount of electron donor in the C/S of 8:1 and 2:4 is equal to 40 mmol/L, similarly, in the C/S of 4:1 and 2:2 system total amount of electron donor is equal to 24 mmol/L. As a result, the fraction of DNRA in autotrophic dominated system (the C/S of 2:4 and 2:2) was apparently higher than that in heterotrophic dominated system (the C/S of 8:1 and 4:1) (Table 2). This phenomenon may be attributed to the fact that sulfide exhibits a much stronger electron-feeding capability over organic, as a result, the respiratory DNRA may be more easily cultivated in autotrophic environment.

3.2 The role and behavior of sulfide and organics in N-conversion pathways

The COD/N ratio is inextricably linked to the oxidation state, and the oxidation state can also be influenced by the presence of reductants (Van den Berg et al. 2015). Other competition affecting factors such as pH, sulfide concentration, or the type and complexity of electron donors may have a decisive effect on the dominant nitrate reducing pathway (Beate et al. 2014). In this experiment, sulfide was introduced in the biofilm system as a mixed electron donor with organics. Sulfide in all conditions was quickly degraded, with synchronously increase of SO_4^{2-} and other sulfur intermediates (SO_3^{2-} , S^0 , $\text{S}_2\text{O}_3^{2-}$, S_n^{2-}) (Qian et al. 2019; Zhang et al. 2018). Even at the C/N of 8:1 and 2:2, 2:3, 2:4, sulfide was regenerated after NO_3^- was consumed. This phenomenon might happen in rich electron donor systems, either organics or sulfide was dominant. A possible reason was that sulfide was firstly used as the

electron donor to reduce nitrate, generating SO_4^{2-} as well as sulfur intermediates, then these intermediates acted as electron acceptors and were further reduced by microorganisms. It was reported that sulfur reducers can use elemental sulfur as the electron acceptor to oxidize many organic matters such as formate, acetate, lactate, pyruvate and sugars (Hedderich et al. 1999). In this study, the organics used as the electron donor in this reverse process were either from influent substrate or from innercellular carbon source. Specifically, in organic rich system (the C/S of 8:1), the carbon source might come from the substrate (CH_3COO^-) (Fig. 1), while in sulfide rich systems (the C/S of 2:2, 2:3, 2:4), it might come from the innercellular carbon.

3.3 The indicator of ORP in N-conversion pathways

Redox potential (ORP) had been found to affect the competition between denitrification and DNRA. ORP was controlled in sediment suspensions by sparging with different N_2/O_2 gas mixtures and measured a higher DNRA activity at lower potentials (Patrick et al. 1973). In addition, the increase of ORP might inhibit growth of the DNRA bacteria, resulting in the domination of denitrifiers (Buresh and Patrick 1981). The change in COD:N ratio alters the oxidation state of the environment and the oxidation state or prevailing ORP is the key factor affecting the competition (Matheson et al. 2002). Therefore, the presence of reductants such as sulfide other than COD/N is linked to the oxidation state of the environment. ORP was monitored in this study to verify its importance for DNRA (Fig. 2).

At the C/S ratio of 8:1, ORP presents a gradual decline. At C/S of 2:1, ORP gradually increased with excessive nitrate remaining, indicating that the lack of electron donor resulted in low denitrification efficiency. At the C/S of 2:3, there was a sharp drop followed by a gradual decrease, and at the C/S of 2:4, a sharp decrease of ORP was followed by an insignificant variation. ORP was always below -400mV throughout the entire process at these two C/S ratios, indicating that the electron donor was excessive and the denitrification rate was relatively fast. At the C/S of 2:3, the indicator points of NO_3^- -N reduction occurred on the 10th minute. When the same amount of electron donors was added, sulfide showed a lower ORP. When the ORP was below -400mV , sulfide releases took place in the reactor at the C/S ratios of 8:1, 2:2, 2:3 and 2:4. All the lines below the red dotted line indicated the DNRA process (Fig. 2), which produced ammonia nitrogen. Previous study reported that ammonia nitrogen was produced when ORP was below -250mV (Van den Berg et al. 2016). Hence, ORP can act as an indicator of the occurrence of DNRA.

3.4 Bacteria communities in two systems

To understand the dynamics of the microbial community and N-conversion pathways in different C/S ratios biofilm reactor, sludge samples from different stages were analyzed. The number of OTUs in the respective sludge samples (C/S of 8:1, 4:1, 2:2, 2:3, 2:4) were 210, 262, 229, 136 and 161, respectively. Accordingly, shannon indexes and rarefaction curves were showed in Fig S2, indicating that the sequences were sufficient to represent the diversity of microbial communities. To further analyze the core communities and the main functional bacteria, bacterial taxonomic identification was performed (Table S1). The compositions of the microbial community and their correlation with operation conditions were

displayed in Fig S3 and Fig. 3. By 16s rRNA, Neighbor-joining phylogenetic tree was developed in the SBBR system with C/S adjustment (Fig. 4).

The composition of the microbial community analysis showed that Proteobacteria dominated in both organic rich and sulfide rich systems. In organic rich system, it was followed by Bacteroidetes, Patescibacteria. While in sulfide rich system, it was followed by Bacteroidetes, Firmicutes, Synergistetes, Acidobacteria (Fig S3). All of the above species were reported to have the function of nitrogen removal (Wu et al. 2018; Zhai et al. 2018). Proteobacteria are rich in microbial species, including Gammaproteobacteria, Deltaproteobacteria, as well as Alphaproteobacteria in our systems, and some microorganisms have different functional genes that can express the denitrification process and sulfur metabolism, and are one of the important bacteria to remove organic matter, carbon, and sulfur compounds in wastewater (Robinson et al. 2018).

Among them, Gammaproteobacteria including *Thauera*, *Thiobacillus*, *Hydrogenophaga*, *Pseudoxanthomonas*, *Sulfuritalea*, has the highest relative abundance in both types of systems. Some organisms of Gammaproteobacteria contain nirK, nirS genes for S oxidation and NO_x reduction (Brett et al. 2015; Freitag and Prosser 2003; Jones et al. 2017). And some microorganisms of Gammaproteobacteria have the nrfA gene and are functionally related to DNRA (Behrendt et al. 2014; Yin et al. 2017). Therefore, Gammaproteobacteria plays a major role in the removal of nitrates and sulfides.

Thauera is an important functional bacterium that degrades complex organic matters under denitrification conditions. It contains the nirS gene and can reduce nitrate and nitrite, which is closely related to heterotrophic denitrification (Behrendt et al. 2014; Liu et al. 2000). In organic rich system, this bacterium dominated, which was 40.89% at C/S of 8:1 and 37.85% at C/S of 4:1. In addition, some scholars have also found that this bacterium is a heterotrophic denitrifying sulfur oxidizing bacterium in the presence of oxidized sulfide under hypoxic conditions, indicating that it also has a desulfurizing function (Cytryn et al. 2005). Correspondingly, in sulfide rich system, its abundance was 10.04% (C/S of 2:2), 17.70% (C/S of 2:3), and 18.80% (C/S of 2:4), which was relatively lower compared with that in organic rich system. Moreover, *Thiobacillus*, prefers chemolithoautotroph. When both acetate and nitrate were insufficient, S⁰ was regarded as an energy storage polymer in prokaryotes. While under autotrophic conditions, S²⁻ was converted to S⁰ and then further oxidized to SO₄²⁻. Accordingly, in our systems, when sodium acetate was sufficient (C/S of 8:1), the abundance was 13%. When C/S was decreased to 4:1, *Thiobacillus* increased to 16%. Notably, in sulfide rich system, this bacterium dominated, its proportions were 37%, 47%, and 30%, respectively under C/S ratio of 2:2, 2:3 and 2:4, under which *Thiobacillus* plays an important role in sulfur-oxidizing.

While, *Hydrogenophaga*, a hydrogen-oxidizing bacterium, which accounted for 10.22% under C/S of 8:1 and 5.6% under C/S of 4:1, was absent in sulfide rich systems. *Pseudoxanthomonas* is heterotrophic bacterium, mainly relying on the attenuation of microbial cells (Wu, et al. 2018). When C/S ratio decreased, the bacterium gradually disappeared. *Sulfuritalea* is a lithoautotrophs, one species of

denitrifier might contain nirs-type gene, related with autotrophic denitrification. *Sulfuritalea* only existed in sulfide rich systems, the highest abundance was achieved at C/S ratio of 2:4 (18.71%) (Fig. 3).

3.5 DNRA functional bacteria in two systems

Deltaproteobacteria contains most of the sulfate-reducing bacteria, and which can regulate the fixation of nitrogen. The addition of carbon sources can stimulate its heterotrophic nitrogen fixation (Robinson et al. 2018). The bacterium reduces sulfate with organic matter in freshwater sediment oxidation (Wu et al. 2018; Zhu et al. 2018). It was present in sulfate-rich areas and drove sulfate reduction (Brett et al. 2015). In organic rich system (C/N of 4:1 and 8:1), *Grobacter* in Deltaproteobacteria class accounted for a certain proportion (2.3% and 4.2%), where it was absent in sulfide rich system. It is reported that high COD condition is more beneficial to the growth of this bacterium, which can convert nitrate to ammonia (Van den Berg et al. 2015; Li et al. 2020). Therefore, the *Grobacter* was considered as the main functional bacterium of DNRA in organic rich system.

Alphaproteobacteria and *Desulfomicrobium* belong to Proteobacteria. In sulfide rich system (with C/S of 2:2, 2:3, 2:4), these two bacteria accounted for 0.64%, 0.31%, 0.29% and 0.1%, 0.7%, 1.2%, respectively. They are metabolic pluripotent organism with multiple functional genes, which can reduce organics, nitrates, sulfates and phosphates, and some species may be related to DNRA (Wu et al. 2018). *Clostridia*, a fermentative bacteria, can reduce nitrate to nitrite and then decompose to ammonium (Rehr and Klemme 1989). This bacterium may play a role in the DNRA process. Interestingly, it only has a relatively small proportion in sulfide rich system. Therefore, the above bacteria, though in a small proportion, are closely related with respiratory DNRA in sulfide rich system in our experiment.

3.6 Bacteria related with sulfide reproduction

Desulfomicrobium belongs to *Proteobacteria*. It is involved in the removal of sulfides and the reduction of unstable sulfur compounds. *Desulfurella* strain TR1 can use glycerol, methanol, acetate as the electron donors to reduce sulfur to sulfide (Florentino et al. 2015). Only a few sulfur reducing bacteria (SRB) can grow with element sulfur, and *Desulfomicrobium* can biologically reduce sulfur to sulfide using organic carbons as electron donors (Rabus et al. 2006; Qiu et al. 2017). With C/S ratio increased from 2:2 to 2:4, the abundance of *Desulfomicrobium* was increased, resulting in the oxidation of sulfide in systems. Furthermore, it is reported that *Clostridium* in Firmicutes and *Geobacter* in Proteobacteria can use elemental sulfur as the electron acceptor to oxidize many organic matters (Hedderich et al. 1999). *Clostridium* was abundant in sulfide rich system, while *Geobacter* was abundant in organic rich system (Fig. 3), which might play the function of sulfide reproduction in each system.

Overall, the phylogenetic compositions of the microbial communities demonstrated that the heterotrophic aerobic communities tend to be replaced by the autotrophic anaerobic communities in sulfide rich system. And the microbial composition in the sulfide rich system showed high potential for the occurrence of chemolithotrophic nitrate reduction, coupled with sulfur oxidation, as well as the presence of DNRA pathway.

Conclusion

The effects of the C/S ratios on the occurrence of DNRA and the effect of electron donor types on N-conversion pathways were extensively studied in a SBBR. Sulfide, the alternative electron donor is more likely to be used for the reduction of nitrate in sulfide rich system and is of significance in DNRA cultivation in traditional denitrification systems. The DNRA proportion was the highest at the C/S ratio of 2:3, since higher reductive environment was performed and it was favorable for respiratory DNRA. However, the proportion of DNRA did not increase as the amount of electron donor was increased in organic rich and sulfide rich systems. Different DNRA functional bacteria dominated in these two systems, though the microbial diversity and abundance were lower than that of denitrifier group.

Declarations

Author contributions Xiaoling Li performed data analysis and drafted the work; Jianqiang Zhao contributed to the study, concept and design; Yuhao Zhang performed the investigation and assisted material preparation; Jiaojie He and Kaili Ma helped to analyzed the data and revised the work. Chunshuang Liu provided useful suggestions on manuscript draft.

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Availability of data and materials All data generated or analyzed during this study are included in this published article (and its supplementary information files).

Ethics approval and consent to participate Not applicable.

Consent for publication Not applicable.

Competing interests The authors declare no competing interests.

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Figures

Figure 1

Variations of N, S with time at different C/S ratios, including: C/S=8:1, C/S=4:1, C/S=2:1, C/S=2:2, C/S=2:3, C/S=2:4.

Figure 2

Variations of ORP with time at different C/S ratios.

Figure 3

The relationship between the microbial species and C/S ratios

Figure 4

Neighbor-joining phylogenetic tree present in the SBBR system with C/S ratio adjustment. Closely related sequences, with the respective Genbank accession numbers in the parentheses, are shown as references. Bootstrap values are shown in percentages of 1000 replicates, when higher than 50%. The scale bar represents 0.02 substitutions per nucleotide position. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site.

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