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Bio-augmentation with dissimilatory nitrate reduction to ammonium (DNRA) driven sulfide-oxidizing bacteria enhances the durability of nitrate-mediated souring control

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ABSTRACT

Biological souring (producing sulfide) is a global challenge facing anaerobic water bodies, especially the oil reservoir fluids. Nitrate injection has demonstrated great potential in souring control, and dissimilatory nitrate reduction to ammonium (DNRA) bacteria was proposed to play crucial roles in the process. How to durably control souring with nitrate amendment, however, remains undiscovered. Herein, *Gordonia* sp. TD-4, a DNRA-driven sulfide-oxidizing bacterium, was used to elucidate the effects of bio-augmentation with DNRA bacteria on the durability of nitrate-mediated souring control. The results revealed that nitrate amendment combined with bio-augmentation with TD-4 after souring could effectively control souring and enhance the durability of nitrate-mediated souring control, while nitrate amendment before souring failed to persistently control souring. Nitrate amendment before and after souring resulted in different evolution dynamics of nitrate-reducing bacteria. Denitrifying bacteria were enriched in reactors amended with nitrate before souring or in dissolved sulfide exhausted reactors amended with nitrate after souring. The heterotrophic denitrifying activity of denitrifying bacteria, however, decreased the durability of nitrate-mediated souring control. Comparative and functional genomics analysis identified potential niche adaptation mechanisms (autotrophic and heterotrophic nitrate/nitrite reduction, including DNRA and denitrification) of predominant SRB in nitrate-amended environments, which were responsible for the rapid resumption of sulfide accumulation after the depletion of nitrate and nitrite. Pulsed injection of nitrate combined with bio-augmentation with DNRA-driven sulfide-oxidizing bacteria was proposed as a potential method to enhance the durability of nitrate-mediated souring control. The findings were innovatively applied to simultaneous bio-demulsification and souring control of emulsified and sour produced water from the petroleum industry.

1. Introduction

Biological souring (producing sulfide) of oil reservoir fluids, including produced water (PW), constitutes a ubiquitous challenge facing the petroleum industry (Marietou et al., 2020; Okpala and Voordouw, 2018; Qi et al., 2021; Williamson et al., 2020). The activity of sulfate-reducing bacteria (SRB) results in souring (Gieg et al., 2011; Korte et al., 2015; Marietou et al., 2020; Williamson et al., 2020). Nitrate is an alternative to biocides for the control, mitigation, and prevention of bio-souring in oil reservoirs fluids (An et al., 2010; Gassara et al., 2015; Qi et al., 2021; Voordouw et al., 2009). Nitrate reshapes the microbial communities by stimulating nitrate-reducing bacteria (NRB) in oil reservoirs fluids (Dutta et al., 2020; Kamarisima et al., 2018; Marietou et al., 2020). The competition for electronic donors between NRB and SRB, the inhibition of nitrite on SRB, and the oxidation of sulfide by nitrate-reducing and sulfide-oxidizing bacteria (NR-SOB) constitute three key mechanisms of nitrate-mediated souring control (An et al., 2010; Dolfing and Hubert, 2017; Marietou et al., 2020). Nitrate-mediated souring control, however, continuously relies on the dosages of nitrate/nitrite, and exhaustion of nitrate/nitrite will produce a rapid resumption of sulfide accumulation (Agrawal et al., 2012; An et al., 2010; Jurelevicius et al., 2021; Prajapat et al., 2018; Voordouw et al., 2009). The long-term potency of nitrate-mediated souring control and the adaptation of SRB to nitrate-amended environments seem to

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Table 1
Experimental grouping and sampling times for high throughput sequencing.

Groups	3 d	5 d	7 d	9 d	13 d	14 d	21 d	Culture conditions
reactor_1	s13	s15/s15_1*	s17	s19	s113	–	s121	after souring: TD-4 + nitrate (since day 5)
reactor_2	s23	s25	s27	s29	–	s214	–	after souring: nitrate (since day 5)
reactor_3	s33	s35	s37	s39	s313	–	–	before souring: TD-4 + nitrate
reactor_4	s43	s45	–	s49	s413	–	–	before souring: nitrate
reactor_5	s53	s55	–	s59	s513	–	–	control: without TD-4 and nitrate

* Bio-augmentation with TD-4.

determine the success of nitrate-mediated souring control.

Considering the competitive roles of NRB, inoculation of competitive microbial strains (bio-augmentation) as an antagonist has been proposed for souring control (Fan et al., 2020; Hubert and Voordouw, 2007; Zhao et al., 2016). Among all the putative candidates of competitive NRB, NR-SOB should the best candidate (Hubert and Voordouw, 2007). Dissimilatory reduction of nitrate to ammonium (DNRA) (Hubert and Voordouw, 2007; Li et al., 2020; Song et al., 2013; Wang et al., 2021) and denitrification (Zhang et al., 2020, 2018) are two potential pathways of NR-SOB to reduce nitrate. The potential roles of DNRA, rather than denitrification, in nitrate-mediated souring control, were highlighted in recent literature (Dolfing and Hubert, 2017; Jahanbani Veshareh and Nick, 2019; Marietou et al., 2020). DNRA bacteria were widely reported to inhabit environments with high C/NO_3^- ratios and sulfide-rich environments (Murphy et al., 2020; van den Berg et al., 2015; Wang et al., 2021). Sour oil reservoirs seem to provide the requisite conditions for the enrichment of DNRA bacteria. The process of DNRA consumes more electron donors compared to complete denitrification when reducing the equivalent amounts of nitrate (Dolfing and Hubert, 2017; Kraft et al., 2014; Pandey et al., 2020), which implied that DNRA bacteria possess more competitiveness over SRB compared to heterotrophic denitrifying bacteria (DNB). Moreover, the lower reducing rates of nitrate and nitrite in DNRA compared to denitrification can prolong the retention of nitrate and nitrite during souring control (Kraft et al., 2014), which can enhance the long-term potency of nitrate-mediated souring control (Qi et al., 2021). However, the long generation time of DNRA bacteria compared to DNB weaken its advantage in souring control (Kraft et al., 2014). In theory, bio-augmentation with DNRA-driven SOB can augment the long-term potency of nitrate-mediated souring control. Whether bio-augmentation with DNRA-driven SOB is practical and effective to enhance the potency of nitrate-mediated souring control, however, remains to be investigated due to the complexity of interactions between NRB and SRB in co-culture systems (Agrawal et al., 2012; Han et al., 2020; Kraft et al., 2014; Wang et al., 2021). Furthermore, the key-driven factors that contribute to the short potency of nitrate-mediated souring control resulting from the interactions between SRB and NRB remain undiscovered.

Our previous study isolated a nitrate-reducing bacterium, *Gordonia* sp. TD-4, to demulsify emulsified PW (Qi et al., 2021, 2022a). TD-4 can reduce nitrate only via the DNRA process (Fig. S1). The genome data also indicated that the genome of TD-4 contains a gene coding for sulfide:quinone oxidoreductase (*sqr*), which can oxidize sulfide to polysulfide chains (Fig. S2) (Shuman and Hanson, 2016). The addition of the viable bacterial cells of TD-4 into emulsified alkaline-surfactant-polymer (ASP) flooding PW resulted in the demulsification of emulsified ASP flooding PW (Qi et al., 2021, 2022a, 2022b). This procedure, i.e., bio-demulsification, transforms the microbial community structures and functions in PW through bio-augmentation (Herrero and Stuckey, 2015; Pandey et al., 2020). However, whether or not this procedure benefits nitrate-mediated souring control still needs to be determined.

Herein, we focused on the effects of bio-augmentation with DNRA-driven SOB, *Gordonia* sp. TD-4, combined with nitrate injection before and after souring on the durability of nitrate-mediated souring control. The concentration profiles of nitrate, nitrite, and sulfide were linked to the composition and functions of microbial communities to elucidate the key factors that determined the long-term potency of nitrate-mediate souring control. The adapting mechanisms of the predominant SRB, contributing to the resumption of accumulation of sulfide, in nitrate-amended environments were also investigated based on comparative and functional genomics analysis. The findings were innovatively applied to simultaneous bio-demulsification and souring control of emulsified and sour produced water from the petroleum industry. The results provide novel perspectives and revolutionary strategies for the treatment of anaerobic bio-sour fluids, especially those from the petroleum industry.

2. Methods and materials

2.1. Continuous experiments in co-culture conditions

The existence of sulfide may affect the competition between microbes, which further influences the souring control. Therefore, the effects of bio-augmentation with TD-4 on the nitrate-mediated souring control before and after souring were investigated, respectively, in five anaerobic reactors (Table 1).

Two anaerobic serum bottles (effective volume of 1 L, reactor_1 and reactor_2), containing 800 mL of Baar's medium (more information can be obtained in supporting information), were used to examine the effects of bio-augmentation of TD-4 on nitrate-mediated souring control after souring. The serum bottles were sterilized in an autoclave (115°C, 30 min). When the temperature decreased to approximately 90°C, the serum bottles were vacuumed immediately, and the headspace was filled with sterile N_2 to avoid direct contact with air. Subsequently, these two bottles were inoculated with 5 mL of SRB-contained microbial consortium (with OD600 = 1.0) and incubated at 170 rpm and 35°C for 5 d until souring was established. One of the bottles was bio-augmented with TD-4 and amended with nitrate (415 mg-N/L), while another bottle was amended with nitrate (415 mg-N/L) only. The volatile suspended solids (VSS) concentration of TD-4 after bio-augmentation was 0.17 ± 0.02 g-VSS/L. The bottles were continuously incubated at 170 rpm and 35°C for several days. The operating statuses of reactors were monitored closely by sampling without replacement. The samples were separately stored in two sterilized centrifugal tubes: one was stored at −80°C for high-throughput sequencing, while the other was immediately used for chemical analysis.

When investigating the effects of bio-augmentation of TD-4 on the nitrate-mediated souring control before souring, the experiments were also conducted with two anaerobic serum bottles (effective volume of 1 L, reactor_3 and reactor_4) containing 800 mL of nitrate (415 mg-N/L)-amended Baar's medium, respectively. Both TD-4 (5 mL of inoculant with OD_{600} = 1.0) and SRB-contained microbial consortium (5 mL of inoculant with OD_{600} = 1.0) were inoculated to reactor_3, while reactor_4 was only inoculated with SRB-contained microbial consortium (5 mL of inoculant with OD_{600} = 1.0). The serum bottles were sterilized in an autoclave (115°C, 30 min). When the temperature decreased to approximately 90°C, the serum bottles were vacuumed immediately, and the headspace was filled with sterile N_2. The reactors were stirred magnetically (170 rpm) in a heated water bath (35°C) and operated

continuously for several days. The operation statuses of reactors were monitored closely by sampling without replacement. The samples were separately stored in two sterilized centrifugal tubes: one was stored at −80°C for high-throughput sequencing, while the other was immediately used for chemical analysis.

Reactor_5 without amendment of nitrate and bio-augmentation was operated as control. These reactors were continuously operated for at least 13 days. When the accumulation of sulfide resumed after the exhaustion of nitrate and nitrite, the reactors were shut down. By considering the operating status of these reactors during the whole operation period, samples from specific periods were selected for time series high-throughput sequencing to determine the evolution of microbial communities. The details of grouping and sampling times for high-throughput sequencing were shown in Table 1.

2.2. High-throughput sequencing

Time series (Table 1) sludge samples from continuous operation of co-culture systems were used for high-throughput sequencing. DNA extraction and polymerase chain reaction (PCR) were performed as described elsewhere (Zhang et al., 2020). The V4 region of the 16S rRNA gene was amplified using primers 515F/806R. The PCR products were sequenced on the Illumina MiSeq platform. The raw sequences collected were deposited in NBCI under accession number PRJNA792215.

The raw sequences were analyzed using the QIIME2 platform (v2021.11) (Bolyen et al., 2019). The adapters in raw sequences were removed by the 'cutadapt' plug-in QIIME2. The sequence quality control was processed by the 'dada2' plug-in QIIME2, and high-quality feature (amplicon sequence variants, ASVs) sequences with a mean length of 270 bp were collected. The pre-trained Naive Bayes classifier (Silva 138 99% OTUs from 515F/806R region of sequences) provided by QIIME2 was used for taxonomic analyses with a confidence threshold of 70%. Top-20 dominant assigned genera were reported, and other genera with a relatively low abundance, unassigned genera, and uncultured genera were classified as 'others'.

2.3. Functional analysis of bacterial community

The putative functions of the bacterial community were predicted by PICRUSt2 (v2.4.1) based on the feature sequences and the feature table generated from the 'dada2' plugin in QIIME2 (Douglas et al., 2020). Specifically, the epa-ng method was used to place sequences into the reference tree. The output putative functions of the bacterial community were reported in the format of the Kyoto Encyclopedia of Genes and Genomes (KEGG) orthologs (KOs) and the KEGG pathway. The resultant KOs and pathway tables were rarefied using the R package vegan (v2.5–7). All hits of KOs associated with denitrification and DNRA were retained for functional composition and functional trait analyses.

Due to the limitation of PICRUSt2 (Douglas et al., 2020), comparative and functional genomics analyses were used to deduce the potential functions (KOs, Table S1) of the dominant genus of NR-SOB (Table S2) and SRB (Table S3). The genome data of a specific genus was retrieved in NCBI Genome Database that was available before December 1, 2021, and the protein-coding gene sequences of all the species were downloaded for KEGG assignment. Duplicate data were removed from the downloaded results based on the taxonomy ID. KOs and KEGG pathway annotations were based on the database of eggNOG 5.0 (Huerta-Cepas et al., 2019). Python library eggNOG-mapper (v2.1.3) was used to process the annotations, and seed orthologs were searched by diamond in sensitive mode. The dataset of the resultant annotations can be available on Mendeley Data (Qi, 2022). The resultant annotations were orderly retrieved to evaluate the gene functions associated with denitrification, DNRA, and sulfide oxidation. Briefly, the KOs numbers (shown in Table S1) were used to match the annotation results. The gene numbers hit during matching were reported.

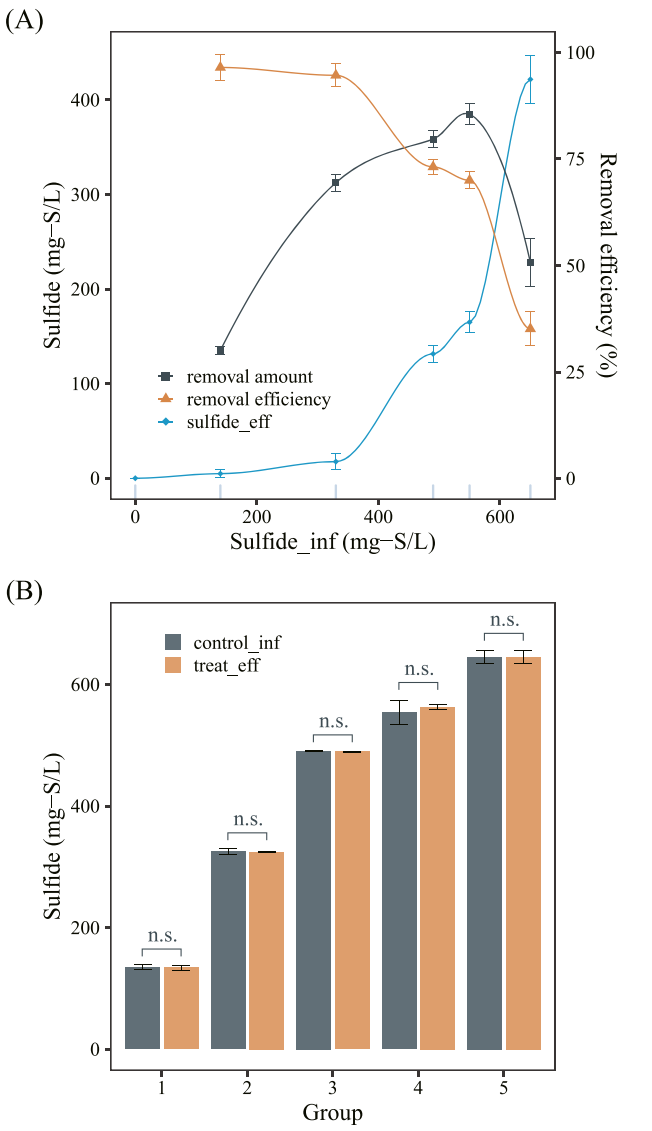

Fig. 1. Effects of different dosages of sulfide on TD-4 under conditions amended with (A) nitrate or (B) ammonia (no significant, n.s.).

2.4. Other analytical procedures and methods

Batch experiments investigating the effects of dissolved sulfide and undissolved sulfide on TD-4 were detailed in supporting information (SI). Analytical procedures of chemical compounds including the concentration of dissolved sulfide, undissolved sulfide, nitrate, nitrite, and crude oil concentration, were also stated in SI. One-way analysis of variance (ANOVA) analysis and student's *t*-test were performed in R.

3. Results and discussion

3.1. Feasibility of bio-augmentation with TD-4 on nitrate-mediated souring control

3.1.1. Effects of sulfide on TD-4 under pure culture conditions

Nitrate and ammonia nitrogen were utilized as sole nitrogen sources, respectively, to simulate nitrate-reducing conditions and non-nitrate-reducing conditions and to investigate the effects of dissolved sulfide on TD-4 in pure culture conditions. The batch experiments showed that TD-4 could effectively remove sulfide under anaerobic nitrate-reducing conditions (Fig. 1A). When the influent concentration of sulfide increased to 650 mg-S/L, the removal efficiency was still higher than

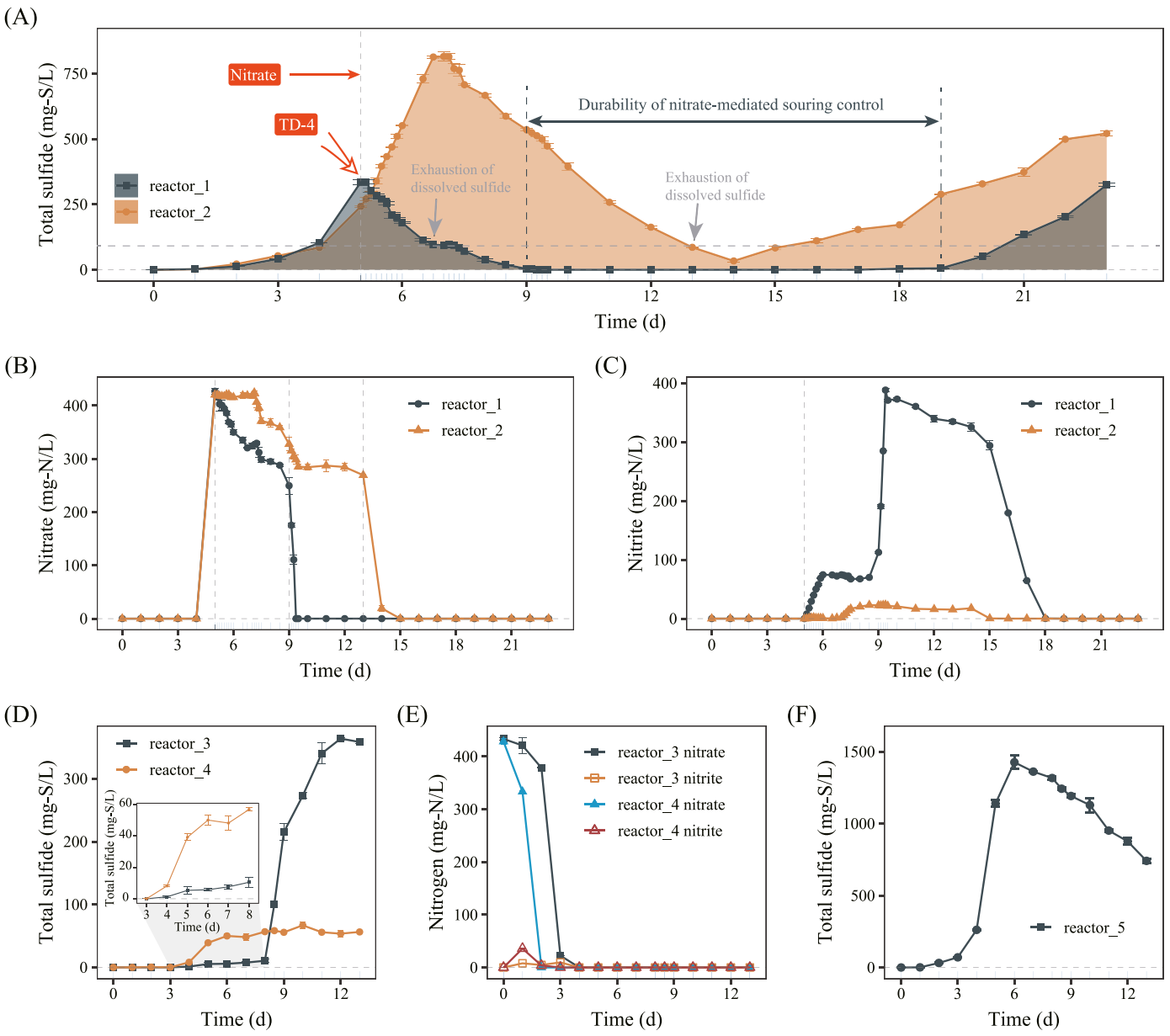

Fig. 2. Effects of bio-augmentation with TD-4 after (A, B, C) and before (D, E) souring on nitrate-mediated souring control. Time-dependent profiles of (A) sulfide concentrations, (B) nitrate concentrations, and (C) nitrite concentrations; Time-dependent profiles of (D) sulfide concentrations and (E) nitrogen concentrations; (F) Control experiments without nitrate amendment and bio-augmentation.

35% (Fig. 1A). However, when ammonia was utilized as a sole nitrogen source, sulfide could not be removed (Fig. 1B). These results suggested that bio-demulsifying bacterium TD-4 was a DNRA-driven sulfide-oxidizing bacterium. The high tolerance of TD-4 to sulfide concentrations was consistent with reports that sulfide favor SOB and DNRA bacteria over heterotrophic DNB (Dolfing and Hubert, 2017; Murphy et al., 2020). An interesting phenomenon was that the culture medium turned black in the following hours after the injection of sulfide, indicating existed Fe^{3+} was reduced to Fe^{2+}, and black undissolved sulfide precipitations (FeS) were formed. The formed sulfide precipitations could also be oxidized by TD-4 since the black color of the medium ultimately faded. Further experiments demonstrated that the oxidation of sulfide by TD-4 could be a carbon sources-independent process, and the oxidizing rate of dissolved sulfide (10.13 mg-S/(L•h)) was approximately two times higher than undissolved sulfide (4.94 mg-S/(L•h)) (Fig. S3). TD-4 preferred sulfide as electronic donors, and the heterotrophic nitrate reduction of TD-4 did not occur when sulfide existed (Fig. S4). Similar results were also reported, in which heterotrophic reduction of nitrate occurred only after the depletion of sulfide (An et al., 2010; Lambo et al., 2008).

3.1.2. Feasibility of bio-augmentation under co-culture conditions before and after souring

Bio-augmentation with pure cultures often fails to perform as expected due to the interactions between microbes ascribed to the complexity of microbial communities (Herrero and Stuckey, 2015). To further investigate the feasibility of bio-augmentation with TD-4 on nitrate-mediated souring control, bio-augmentation combined with nitrate amendment (Table 1) was performed in co-culture conditions before and after souring, respectively. The results revealed that nitrate amendment after souring could control souring (Fig. 2A), while nitrate amendment before souring failed to persistently control souring (Fig. 2D). Time-dependent concentration profiles of sulfide showed that bio-augmentation with TD-4 combined with nitrate amendment after

Fig. 3. Effects of bio-augmentation with TD-4 on (A) the community structures of nitrate-mediated souring control and (B) predominant SRB at the genus level.

souring (reactor_1) resulted in durability in nitrate-mediated souring control, which was manifested by the immediate cessation of sulfide accumulation and long-term exhaustion of sulfide (Fig. 2A). In contrast, the control group (reactor_2), only amended with nitrate after souring, responded slowly to nitrate amendment, and sulfide was continuously accumulated for 2 d (Fig. 2A). In addition, the sulfide was not exhausted during the whole period (Fig. 2A). Continuous accumulation of sulfide after nitrate amendment might be attributable to the low abundance and activity of NRB (Murphy et al., 2020; Tugtas and Pavlostathis, 2007). In reactor_1, sulfide concentrations decreased linearly during the initial 1.5 d (5.0 d - 6.5 d) after nitrate injection (Fig. 2A). The removal rate of sulfide was as high as 10.16 mg-S/(L•h), which was consistent with the oxidation rate of dissolved sulfide of TD-4 (Fig. S3A). The specific sulfide removal rate reached 60.60 ± 10.08 mg-S/(g-VSS•h). During the following days (from 6.5 d to 9.0 d), the sulfide removal rate was diminished, and the highest sulfide removal rate was only approximately 2.14 mg-S/(L•h) (Fig. 2A). The oxidation of undissolved sulfide (FeS) might account for the retarded oxidizing rate of sulfide (Fig. S3B). When undissolved sulfide (FeS) was exhausted, the color of reactor_1 turned orange-yellow (Fig. S5), indicating that polysulfide was formed (Nanda et al., 2021). Bio-augmentation with TD-4 combined with nitrate amendment after souring exhibited durability in nitrate-mediated souring control since the accumulation of sulfide did not recover during the next 9.5 d (from 9.5 d to 19.0 d) (Fig. 2A). In reactor_2, sulfide concentrations began to steadily decrease after 2.25 d of nitrate amendment (Fig. 2A). The sulfide concentration decreased to 33.70 mg-S/L on day 14.0 (Fig. 2A), and then gradually recovered after the depletion of nitrate and nitrite on day 15.0 (Fig. 2B and C). Reactor_3 and reactor_4 that were amended with nitrate before souring failed to control souring persistently, and sulfide accumulated since day 4 (Fig. 2D). Sulfide in reactor_3 sharply accumulated since day 8, while sulfide in reactor_4 did not unrestrained accumulate but reached a plateau period (Fig. 2D). A dynamic balance of generation and consumption of sulfide seemed to be achieved, even though nitrate and nitrite have been depleted. More interestingly, the reactor_5 without nitrate amendment and bio-augmentation showed a decrease in sulfide concentrations after a peak (Fig. 2F). Complex microbial electrosynthetic processes might be responsible for nitrate-independent sulfide oxidation (Blázquez et al., 2016; Luo et al., 2019; Marshall et al., 2017). Besides, trace amounts of oxygen might be utilized by SOB to oxidize sulfide (Fang et al., 2020).

Overall, sulfide oxidation accompanied nitrate reduction and nitrite accumulation, and the resumption of sulfide accumulation was found after the exhaustion of nitrate/nitrite (Fig. 2B and C). Dramatic decrease in nitrate concentrations were identified in four nitrate-amended reactors (Fig. 2B, E). The sharp decline of nitrate in reactor_1 resulted in a marked accumulation of nitrite from 70.05 mg-N/L to 388.25 mg-N/L, while the sharp decline of nitrate in reactor_2 did not result in the

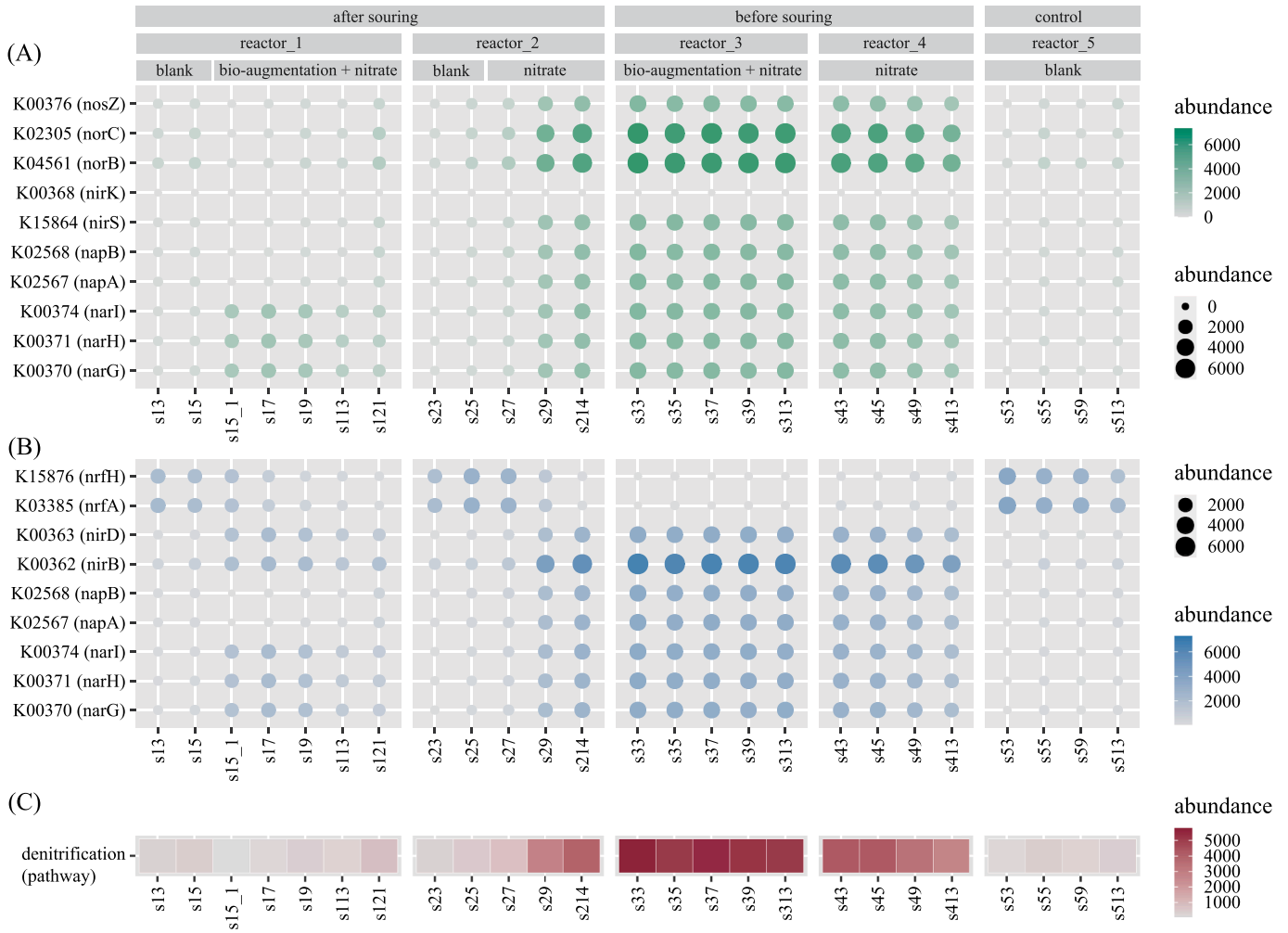

Fig. 4. The relative abundance of (A) denitrification-associated KOs, (B) DNRA-associated KOs, and (C) denitrification pathway.

accumulation of nitrite (Fig. 2C). The sharp decline of nitrate in reactor_1 was found after the exhaustion of sulfide on day 9; whereas the sharp decline of nitrate in reactor_2 was identified after the exhaustion of dissolved sulfide on day 13 (Fig. 2A). This might be ascribed to the resumption of heterotrophic nitrate-reducing activity of NRB after the depletion of dissolved sulfide (An et al., 2010; Lambo et al., 2008). Accumulated nitrite in reactor_1 was gradually reduced since day 9.5 and dramatically reduced from 325.5 mg-N/L to 0 since day 14 (Fig. 2C). The activity of heterotrophic DNRA of TD-4 might account for the gradual reduction of nitrite; whereas, the sharp reduction of nitrite was attributed to the activity of heterotrophic DNB (Fig. 3A) (Kraft et al., 2014). Nitrate in reactors amended with nitrate before souring was consumed quickly within 3 days, during which transient accumulation of nitrite was recorded (Fig. 2E). The accumulation of sulfide was unsurprisingly restored when nitrate and nitrite were depleted. These results indicated the long-term potency of nitrate-mediated souring control was associated with the persistent existence of nitrate and nitrite, and bio-augmentation combined with nitrate amendment after souring could enhance the long-term potency in nitrate-mediated souring control.

3.2. Effects of bio-augmentation with DNRA bacteria on the shaping of microbial community structures

3.2.1. Effects of bio-augmentation with DNRA bacteria after souring

Prior to nitrate amendment, the microbial communities were dominated by typical fermentation genera, such as *Bacteroides* (18.0%–28.0%), *Romboutsia* (12.0%–32.0%), and *Clostridium sensu stricto* (4.1%–14.2%) (Fig. 3) (Zeybek et al., 2020). The dominant SRB genera, before nitrate amendment, were *Desulfomicrobium* (0.6%–1.8%), *Desulfobulbus* (0.1% - 0.9%), and *Desulfovibrio* (0.5%–0.9%). When souring was established on day 5, bio-augmentation with *Gordonia* sp. TD-4 changed the microbial structures, which manifested as an increase in the relative abundance of TD-4 from 0 to 25.5% (Fig. 3). TD-4 became the dominant genus, but this competitive advantage over *Thioclava* lasted less than 8 d (Fig. 3). The relative abundance of TD-4 showed a decreased trend with the exhaustion of sulfide since day 9, and the abundance of TD-4 was only 8.6% on day 21. Bio-augmentation with TD-4 resulted in the enrichment of *Thioclava* since day 7, especially after the exhaustion of sulfide on day 9. The abundance of *Thioclava* increased to 13.3% on day 9, and then to 49.6% on day 21. In contrast, the control reactor, which was only amended with nitrate, resulted in the enrichment of *Pseudomonas* (Fig. 3). *Thioclava* was also enriched to 8.7% on day 14 (when the sulfide concentration was lowest) in reactor_2. The enrichment of *Pseudomonas* was responsible for sulfide oxidation in reactor_2 (Zhang et al., 2020, 2018). The prevalence of *Thioclava* and *Pseudomonas* after the exhaustion of sulfide showed a high correlation with the rapid consumption of nitrate and nitrite (Fig. 2B and C).

In both nitrate-amended reactors after souring, the predominant SRB (Fig. 3B) (*Desulfobacterota* phylum (Fig. S6), including *Desulfomicrobium, Desulfobulbus*, and *Desulfovibrio*) maintained a high fraction, indicating that nitrate-mediated souring control after souring did not possess lethal effects on SRB. Unique adaptation mechanisms of SRB in nitrate-amended environments might be responsible for the responsive

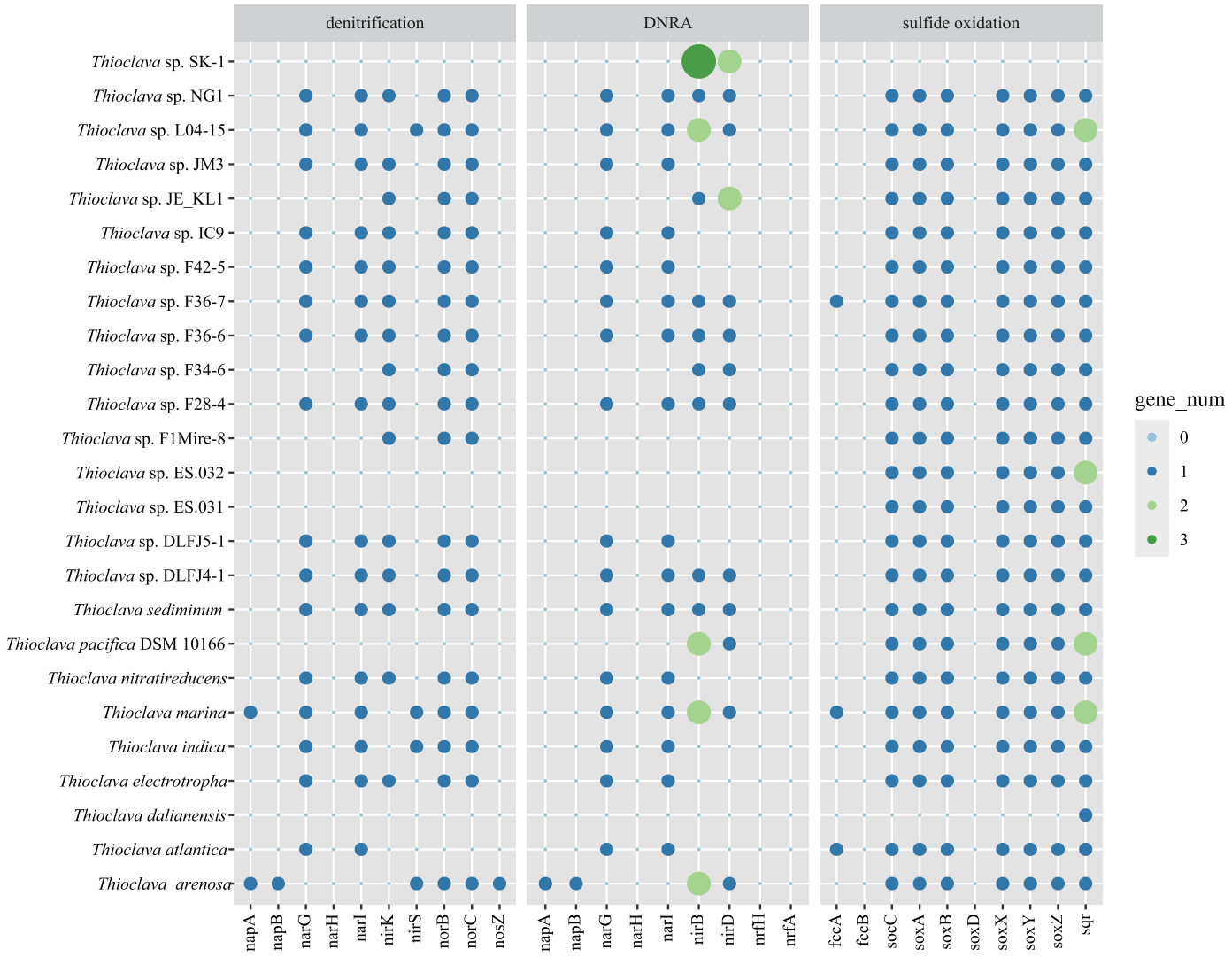

Fig. 5. Nitrate reduction pathway and sulfide oxidation pathway of *Thioclava*.

resumption of sulfide accumulation after the exhaustion of nitrate/nitrite (Gieg et al., 2011; Jurelevicius et al., 2021; Kamarisima et al., 2018; Voordouw et al., 2009). This result also highlighted the crucial role of the persistent existence of nitrate and nitrite on the durability of nitrate-mediated souring control (An et al., 2010; Fan et al., 2020; Qi et al., 2021).

3.2.2. *Effects of bio-augmentation with DNRA bacteria before souring*

Nitrate amendment before souring resulted in a different evolution dynamic of microbial communities (Fig. 3A). On the one hand, bio-augmentation with TD-4 before souring did not perform as expected. TD-4 failed to be the dominant bacteria, and its relative abundance was less than 0.2% during the whole operating period (Fig. 3A). On the other hand, *Pseudomonas* constituted the dominant genus in both reactors (Fig. 3A). The complex interactions between microbes might be responsible for this phenomenon (Herrero and Stuckey, 2015). *Pseudomonas* was a typical genus of heterotrophic DNB (Zhang et al., 2020). Without the inhibition of sulfide on heterotrophic DNB (Murphy et al., 2020; Tugtas and Pavlostathis, 2007), the competition for nitrate and nitrite between heterotrophic DNB and DNRA bacteria and the long generation time of DNRA bacteria might account for the failure of the prevalence of TD-4 (Kraft et al., 2014). The high heterotrophic denitrifying activity of *Pseudomonas* was responsible for the dramatic consumption of nitrate and nitrite, which resulted in the transiency of nitrate-mediated souring control (Fig. 2D, E).

Although without the amendment of nitrate, the relative abundance of so-called SOB, including *Thioclava* (Luo et al., 2019) and *Sulfurospirillum* (Berg et al., 2019), exhibited a growing tendency over time in reactor_4 and reactor_5, respectively (Fig. 3A). The relative abundance of *Thioclava* increased from 3.0% on day 5 to 4.3% on day 14 in reactor_4, while the relative abundance of *Sulfurospirillum* was even as high as 7.2% in reactor_5 on day 13. *Sulfurospirillum* can use many kinds of electronic acceptors, including Fe^{3+}(Berg et al., 2019), CO_2 (Marshall et al., 2017), and trace amounts of O_2 (Marshall et al., 2017), to oxidize sulfide. The existence of potential electroactive microbes, including *Trichococcus* (Saheb-Alam et al., 2019), *Sulfurospirillum* (Blázquez et al., 2016; Marshall et al., 2017), *Thioclava* (Luo et al., 2019), indicated that nitrate-independent sulfide oxidation might be due to the microbial electrosynthetic processes. More details should be investigated in the future.

3.3. *Potential mechanisms responsible for the durability of nitrate-mediated souring control*

3.3.1. *Heterotrophic denitrification determined the durability of nitrate-mediated souring control*

The success of nitrate-mediated souring control relied on the persistent existence of nitrate and nitrite. The nitrate-reducing activity

after the depletion of dissolved sulfide seemed to determine the persistent existence of nitrate and nitrite. Since the sulfide-driven autotrophic nitrate reduction could not quickly reduce nitrate and nitrite (Fig. 2), and the heterotrophic nitrate reduction did not occur before the depletion of sulfide (Fig. S4B) (An et al., 2010; Lambo et al., 2008). The heterotrophic nitrate reduction after the depletion of sulfide might contribute to the rapid consumption of nitrate and nitrite. The predicted function of microbial communities showed that enormous amounts of denitrification-associated genes were enriched in reactors amended with nitrate before souring (Fig. 4A), and the denitrification pathway was also enriched correspondingly (Fig. 4C). This result suggested that nitrate amendment before souring resulted in the enrichment of heterotrophic DNB, which in turn quickly consumed nitrate and nitrite (Fig. 2E) and decreased the potency of nitrate-mediated souring control. Indeed, heterotrophic denitrifying bacteria, represented by *Pseudomonas* (Zhang et al., 2020; Zhao et al., 2016), thrived and became the predominant genera in these reactors (Fig. 3A). A similar result was also found in reactor_2 since day 9 (Fig. 4A). It should be noted that reactor_2, reactor_3, and reactor_4 also enriched DNRA-associated KOs (Fig. 4B). This might be because the genome of *Pseudomonas* usually contains two sets of nitrate reduction pathways (DNRA and denitrification). DNRA activity was mainly performed during the existence of sulfide, while denitrifying activity was mainly performed after the depletion of sulfide (Murphy et al., 2020; Pandey et al., 2020; Tugtas and Pavlostathis, 2007).

A rapid decline in nitrate was found in four reactors, respectively (Fig. 2B, E). The results of PICRUSt2, however, did not show enrichment of a high abundance of denitrification-associated genes in reactor_1 (Fig. 4A). The prosperity of *Thioclava* after the exhaustion of dissolved sulfide was proposed to be responsible for the dramatic declines of nitrate in reactor_1 (Fig. 3A). Indeed, the comparative and functional genomics analysis of all published genome data of *Thioclava* (Table S2) confirmed this assumption (Fig. 5). The results showed that denitrification-specific genes, e.g., nitrite reductase (*nirKS*), and nitric oxide reductase (*norBC*), widely harbored in the genome of *Thioclava* (Fig. 5). Without the inhibition of sulfide on *norBC* (Murphy et al., 2020; Tugtas and Pavlostathis, 2007), the shorter generation times as well as the higher rates of nitrate and nitrite reduction of DNB, compared to DNRA bacteria (Kraft et al., 2014), constituted the main reasons for the gradual decrease in the relative abundance of TD-4 and the increase in the relative abundance of *Thioclava* after the exhaustion of dissolved sulfide (Fig. 3A). These results not only formally indicated that heterotrophic denitrification decreased the durability of nitrate-mediated souring control, but also further suggested that continuous injection of a high dosage of nitrate was less beneficial for long-term souring control (Callbeck et al., 2011; Gassara et al., 2015; Grigoryan et al., 2009; Prajapat et al., 2018; Voordouw et al., 2009). Continuous injection of nitrate likely resulted in the prevalence of denitrifying bacteria, especially when dissolved sulfide was exhausted. High activity of heterotrophic denitrification resulted in the failure in nitrate breakthrough, which might constitute the main reasons why some oil reservoirs failed in nitrate-mediated souring control (Callbeck et al., 2011; Grigoryan et al., 2009; Voordouw et al., 2009).

Competition for electronic donors between heterotrophic NRB and SRB was proposed as a basic mechanism in nitrate-mediated souring control (Dolfing and Hubert, 2017; Gassara et al., 2015; Gieg et al., 2011). Heterotrophic nitrate reduction, however, only occurred after the depletion of sulfide (Fig. S4B) (An et al., 2010; Lambo et al., 2008). Theoretically, the richness of available carbon sources in oil reservoir fluids could provide abundant organic electronic donors for microbes, including heterotrophic NRB and SRB (Agrawal et al., 2012; Nowak et al., 2018; Okpala and Voordouw, 2018). Therefore, souring control by the competition of heterotrophic NRB with SRB for available carbon sources may have limited applicability, especially in oil reservoirs that are rich in carbon sources available for microbes (Agrawal et al., 2012; Lambo et al., 2008). Long-term souring control was expected to be achieved by prolonging the retention of nitrate and nitrite in oil reservoir fluids (An et al., 2010; Fan et al., 2020; Gieg et al., 2011; Qi et al., 2021). The persistence of nitrate and nitrite, to a great extent, relied on the interactions between denitrification and DNRA. DNRA possesses a lower reducing rate of nitrate and nitrite compared to denitrification (Kraft et al., 2014), which can improve the durability of nitrate-mediated souring control (Qi et al., 2021). Bio-augmentation with DNRA will compensate for the disadvantages of the long generation time of DNRA bacteria (Kraft et al., 2014) and further enhance the durability of nitrate-mediated souring control. A continuous dose of nitrate, however, will promote the abundance and the heterotrophic denitrifying activity of heterotrophic DNB (Fig. 4), especially after the exhaustion of sulfide, which in turn results in a decrease in the durability of nitrate-mediated souring control. Pulsed injection of low concentrations of nitrate, therefore, is an ideal method in souring control (Callbeck et al., 2011; Voordouw et al., 2009), since it can keep a relatively low concentration of sulfide in the reservoir fluid, and a low concentration of sulfide maintains the competitive advantage of DNRA bacteria over heterotrophic DNB.

3.3.2. Autotrophic and heterotrophic nitrate/nitrite-reducing ability endowed SRB with unique niche adaptation mechanisms

The relative abundance of predominant SRB (Fig. 3B) (*Desulfobacterota* phylum, Fig. S6), including *Desulfomicrobium, Desulfovibrio*, and *Desulfobulbus*, maintained a high fraction during nitrate amendment post-souring, which constituted a key reason why sulfide accumulation resumed rapidly after the exhaustion of nitrite (Fig. 2A) (Kamarisima et al., 2018; Voordouw et al., 2009). The reduction of sulfur and polysulfide might be another key reason responsible for the quick resumption of sulfide accumulation. Comparative genome analysis was then used to assess the potential response of the predominant SRB genus (Table S3) to nitrate and nitrite. Complete DNRA pathway (including genes of *narI, nirB, nrfH*, and *nrfA*) was identified in all six species of *Desulfomicrobium*, seven species (a total of nine) of *Desulfobulbus*, and 59 species (a total of 67) of *Desulfovibrio* (Fig. S7). The genome of some species of *Desulfovibrio* also contained denitrification-specific genes, e.g., *nirK* and *norB*. Interestingly, numerous species of *Desulfovibrio* possess the homologous gene of *sqr* (Fig. S7), which can catalyze the oxidation of sulfide to polysulfide chain (Shuman and Hanson, 2016). These results illuminated the uncharacterized metabolic abilities of SRB, in which autotrophic, as well as heterotrophic, nitrate and nitrite reduction, including DNRA and denitrification, are broadly distributed in the predominant genus of SRB. These potential adaptation mechanisms allow the niche expansion of SRB in nitrate-amended environments (Kamarisima et al., 2018; Korte et al., 2015), which prompts us to make updated decisions toward nitrate-mediated souring control. Considering suitable amounts of sulfide benefit for heterotrophic DNB inhibition, controlling the concentration of sulfide at a relatively low level should be the optimal strategy for nitrate-mediated souring control. Therefore, dynamically regulating, rather than obliterating, the activity of sulfate reduction is more beneficial and realistic, which in turn further underscores the importance of long-term potency of nitrate-mediated souring control. By pulsed injection of nitrate combined with bio-augmentation with DNRA-driven SOB, not only can the activity of sulfate reduction and heterotrophic denitrification be regulated, but also the potency of nitrate-mediated souring control can be extended.

3.4. Application in simultaneous bio-demulsification and souring control

TD-4 was originally isolated to demulsify the emulsified ASP flooding PW from the petroleum industry (Qi et al., 2021). The procedure of bio-demulsification (Huang et al., 2014; Qi et al., 2021, 2022a) transforms the microbial communities and functions through bio-augmentation (Herrero and Stuckey, 2015). Consequently, during the process of bio-demulsification, sulfide oxidation driven by TD-4 should be logically functional. The bio-demulsifying mechanisms of

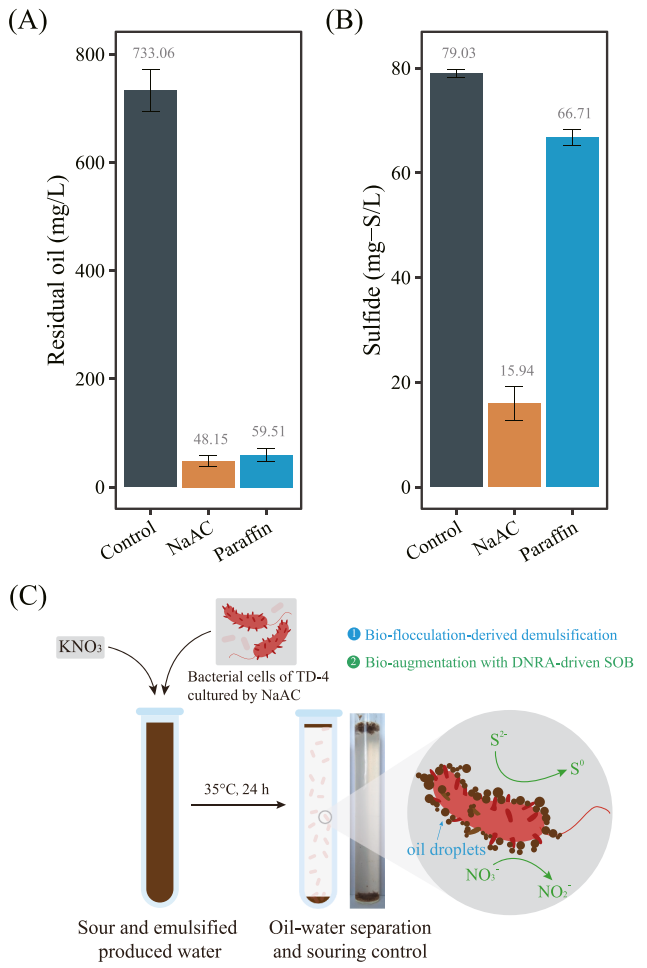

Fig. 6. Effects of carbon sources of bio-demulsifying bacteria on simultaneous bio-demulsification and nitrate-mediated souring control of ASP flooding PW. The concentration of (A) residual crude oil and (B) sulfide after 24 h of carbon sources-mediated bio-demulsification; (C) procedure and diagram of simultaneous bio-demulsification and nitrate-mediated souring control.

TD-4 and the distribution of bacterial cells of TD-4 in ASP flooding PW are mediated by carbon sources (water-soluble and oil-soluble carbon sources) (Qi et al., 2021, 2022b). We tested the effects of two bio-demulsifying mechanisms-involved demulsifying procedures that mediated by sodium acetate (NaAC) and paraffin oil on the nitrate-mediated souring control. The results demonstrated that simultaneous bio-demulsification and souring control could be achieved (Fig. 6). Both two kinds of carbon sources-mediated bio-demulsifying procedures could effectively separate the emulsified crude oil from ASP flooding PW (Fig. 6A), and the crude oil removal efficiency could reach 93.43%. Only the NaAC-mediated bio-demulsifying procedure (Fig. 6C), however, could effectively control souring (oxidizing sulfide) (Fig. 6B). The mean concentration of total sulfide post-demulsification (24 h) decreased from 79.03 mg-S/L to 15.94 mg-S/L, and the sulfide removal efficiency was as high as 79.83%. In contrast, the paraffin-mediated bio-demulsifying procedure only resulted in a slight decrease in sulfide concentration, and the sulfide removal efficiency was only 15.59%. This discrepancy was due to the distribution of bio-demulsifying bacteria in ASP flooding PW during demulsification (Qi et al., 2021, 2022b).

The development of the multifunctionality of bio-demulsifiers provided a new decision-making strategy to reduce the production costs of bio-demulsifiers (Qi et al., 2022a, 2022b), which was expected to speed up the application of bio-demulsification. The simultaneous bio-demulsification and souring control of emulsified and sour PW (Fig. 6C), proposed in this study for the first time, showed good compatibility with the sequencing batch settlement process which is widely used in oilfields for oil-water separation (Deng et al., 2005). Without altering or increasing the constructions of the oil-water separation process, bio-augmentation with DNRA-driven SOB, possessing bio-demulsifying ability, combined with pulsed injection of nitrate can not only improve the efficiency of oil-water separation, but also enhance the long-term potency of nitrate-mediated souring control.

4. Conclusions

- Nitrate amendment combined with bio-augmentation with DNRA-driven SOB after souring could effectively control souring and enhance the durability of nitrate-mediated souring control.
- Continuous injection of a high concentration of nitrate may be less beneficial for souring control, since denitrifying bacteria will be enriched, especially after the depletion of sulfide, and heterotrophic denitrifying activity will, in turn, decrease the long-term potency of nitrate-mediated souring control.
- Autotrophic and heterotrophic nitrate/nitrite reduction endowed SRB with unique niche adaptation mechanisms in nitrate-amended environments. Dynamically regulating, rather than obliterating, the activity of sulfate reduction seemed to be more beneficial and realistic.
- Pulsed injection of nitrate combined with bio-augmentation with DNRA-driven SOB was proposed to be a potential method to regulate the interactions between SRB and NRB and increase the durability of nitrate-mediated souring control.
- These findings were innovatively applied to simultaneous bio-demulsification and souring control of produced water from the petroleum industry, which has good adaptability to the current process and is expected to achieve a balance between cost and benefit.

Declaration of Competing Interest

None.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.watres.2022.118556.

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