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Title

Impact of nitrate addition on the bacteria population dynamics of sulfate-reducing and nitrate-reducing bacteria in the oilfield-produced water

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Abstract

Nitrate injection has been widely used to minimize the production of biological hydrogen sulfide in oil and gas field industry. In environments exposed to seawater injection, nitrate is used to chemically and biologically control the growth of sulfate-reducing bacteria (SRB). The aim of this study was to investigate the changes in the bacterial community in response to nitrate addition used to control biological souring. Specifically, we investigated the effect of nitrate addition in an artificial souring experiment, using diluted crude oil as substrate and electron donor. *Desulfotignum* sp. was the predominant SRB under all conditions tested. Addition of nitrate at the beginning (N_0) repressed the growth of SRB, as revealed by chemical and bacterial community analysis, concomitant with significant growth of the nitrate-reducing bacteria (NRB) *Thalassospira* sp. Nitrate addition after SRB growth (at day 28, N_{28}) successfully remediated the sulfide produced by SRB, but no significant reduction in sulfate was observed subsequently; moreover, the bacterial communities before and after nitrate addition remained identical. The phenomenon in the N_{28} experiment might be the result of the role of *Arcobacter* sp., which are nitrate-reducing sulfide-oxidizing bacteria, and/or the ability of *Desulfotignum* sp. to

reduce nitrate and/or nitrite as a stress response. Thus, SRB might persist after nitrate addition, potentially causing subsequent SRB outbreaks.

Keywords

Arcobacter, *Desulfotignum*, nitrate injection, nitrate-reducing bacteria, souring, sulfate-reducing bacteria, secondary recovery, *Thalassospira*

1. Introduction

In order to increase the productivity of crude oil, recovery methods have been developed. Water injection serves as main oil recovery method to be used whenever the natural derive became inefficient, known as secondary recovery (Plankaert, 2005). In offshore oil exploration, seawater is commonly injected into the reservoir, although this may cause several problems, including biological souring. Seawater contains high concentrations of sulfate (up to 27 mM) that can enhance the growth of sulfate-reducing bacteria (SRB) in the reservoir. Biological souring is a serious problem in the oil and gas industry because it deteriorates the quality of crude oil and increases both the environmental threat and production cost (Gieg et al. 2011).

To date, several methods for preventing and treating biological souring have been developed based on physicochemical and biological approaches, most of which seek to mask the activity of SRB. Widely used physiochemical approaches include injection of biocides (Jayaraman et al. 1999; Nemati et al. 2001; Tang et al. 2009; Yin et al. 2016), SRB metabolic inhibitors such as nitrite and molybdate (Nemati et al. 2001; Tang et al. 2009), or air injection to prevent anaerobic condition (Ochi et al. 1998). An alternative approach is nitrate injection, which seeks to promote the growth of nitrate-reducing bacteria (NRB) as competitors of SRB for the electron donors in the reservoir, such as volatile fatty acids (Agrawal et al. 2012). NRB stimulated by nitrate injection serve not only as competitors for SRB, but also as consumers of biological sulfide produced by microbial souring (De Gusseme et al. 2009); furthermore, the resultant nitrite, produced as an intermediate metabolite, can repress the growth of SRB (Tang et al., 2009). NRB that can reduce nitrate autotrophically in presence of sulfide are known as nitrate-reducing, sulfide-oxidizing bacteria (NR-SOB) (De Gusseme et al. 2009). Thus, nitrate injection might be used to prevent and treat souring.

Nitrate injection is an attractive solution to souring because nitrate is cost-effective, relatively non-toxic, and can distribute evenly in the reservoir (Dunsmore et al. 2006; Gieg et al. 2011). Field-test of nitrate injection (65mg/L) in high-temperature oil reservoir showed that nitrate injection could reduce sulfate-reducing prokaryotes (SRP) activity and seemed to affect microbial community in the reservoir

(Gittel et al. 2009). However, nitrate injection sometimes fails to control biological souring (Kjellerup et al. 2005; Kaster et al. 2007; Gieg et al. 2011). Specifically, the usage of nitrate injection in a low-temperature reservoir was associated with an SRB outbreak in the deeper zone of the reservoir, resulting in sulfide production (Agrawal et al. 2011). Meanwhile, the methods for predicting the optimal nitrate concentration for such applications continue to be debated. Application of nitrate is thought to be necessary for overcoming microbial souring under high carbon concentrations (Myhr et al. 2002). Hence, the information about the bacterial community before and after nitrate injection under biological souring conditions is required in order to design suitable approaches for these applications. Accordingly, the objective of this study was to investigate the effect of nitrate injection as a prevention and treatment method on the bacterial community before and after biological souring.

2. Materials and methods

2.1 Artificial Souring Experiment

Oil field water (OFW) was taken from an oil field (Akita, Japan) in a 20 L poly-tank that was completely filled with a mixture of water/oil. The sample was kept at 4°C until use. This oil field has not previously been subjected to water flooding (the psychochemical characteristic of OFW was showed as supplementary information, Table SI-2). The inoculum used in this research was generated by condensing the OFW by centrifugation at 11,000 × *g* for 15 min in a 500 mL centrifuge tube (Hasegawa et al. 2014). The original oil field water total bacteria concentration was 10^9 copies/ mL. The inoculum used in this study was 100 times concentrated oil field water, with total bacteria concentration 10^{11} copies/mL. The oil layer was separated and further used as the crude oil source (the psychochemical characteristic of crude oil was showed as supplementary information, Table SI-1). The pellet was washed with phosphate-buffered saline (PBS pH 7.2; 237 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2mM K_2HPO_4) and diluted with 20 mL PBS for further use as the inoculum. Aliquots of 1 mL of OFW before and after condensation was subjected to 16S rRNA gene quantification to estimate total bacteria concentration.

The medium used in this study was filter-sterilized (0.22 μM, MillexGV, Millipore) natural seawater collected from Ogasawara (Tokyo, Japan) (the psychochemical characteristic of natural sea water was showed as supplementary information, Table SI-3). Seawater (50 mL) was overlaid with 5 mL of 10% crude oil diluted in the biologically inert branched-chain alkane 2,2,4,4,6,8,8-heptamethylnonane (HMN). The medium was prepared in 70 mL glass vials, closed with butyl rubber caps, and autoclaved. The headspace was filled with N_2 gas. One milliliter of condensed OFW was used as the bacterial inoculum. Four conditions were set up in this study: $N_{w/o}$ (without nitrate addition), N_0 (nitrate added at the beginning to prevent souring), N_{28} (nitrate added at day 28 to treat souring), and abiotic control. All conditions were conducted in triplicate. On days 0 and 28, a solution of 1 M sodium nitrate solution was added to yield a final concentration of 27 mM; this concentration was chosen because it is identical to the level of sulfate in seawater. All vials were incubated horizontally at 30°C in the dark on a shaking incubator (80 rpm)

Every week, a 600 μL sample of the water phase was taken. The sample was centrifuged at 6,500 × g for 10 min; the resultant supernatant was used for chemical analysis, and the pellet was used for bacterial analysis. The pellet was washed twice with sterile PBS. Genomic DNA was extracted by the bead-beating method (Tanji et al. 2014) followed by phenol–chloroform extraction. The bead-beating procedure was conducted at 6.0 ms^{-1} for 40 s on a FastPrep-24 Instrument (MP biomedical LLC, Santa Ana, CA). A NanoDrop 2000 spectrophotometer (Thermo Fischer Scientific, Wilmington, DE) was used to assay and measure DNA quality and concentration. An oil-phase sample for crude oil analysis was taken every two weeks.

2.2 Chemical analysis

Sulfide concentration was measured using a kit based on the methylene blue method (NANOCOLOR standard experiment sulfide; Machery-Nagel Corp; Germany) (Trueper & Schlegel, 1964). Anion concentration was measured using ion chromatography (with TSK Gel Super Anion AZ column; TOSOH Corporation, Tokyo, Japan) with a flow rate of 0.8 mL min^{-1}. Volatile fatty acid concentration was measured using a High-Performance Liquid Chromatography system (SCR102H

column; CSS-10A detector; Shimadzu, Tokyo, Japan). The crude oil sample was analyzed using a gas chromatography system (GC2014; Shimadzu) equipped with a flame ionization detector (325°C) using helium and hydrogen as the carrier gas. The column used in this experiment as HP-PONA (Agilent Technologies, Santa Clara, CA), 50 mm × 0.2 mm (0.5 μm film thickness). The GC conditions were as described previously (Hasegawa et al. 2014). The crude oil we used originally contained 38 mM toluene. Pristane, a persistent fraction in crude oil, was used as an internal standard. The relative abundance of each hydrocarbon fraction in crude oil was calculated as follows:

Relative abundance (%) = $((A/B)/(A_0/B_0)) \times 100$

Note:

A = Peak area of specific fraction at time = t

B = Peak area pristane at time = t

A_0 = Peak area of specific fraction at time = 0

B_0 = Peak area of specific pristane at time = 0

2.3 Bacterial analysis

Quantitative PCR (qPCR) was performed to measure the abundance of ribosomal and two functional genes respectively as follow: 16S rRNA (ribosomal gene, for estimating total number of bacteria (Nadkarni et al. 2002), *bssA* (Benzysuccinate synthase α-subunit, which catalyzes the addition of fumarate in toluene degradation (Funk et al. 2014); for estimating abundance of toluene-degrading bacteria), and *dsrA* (Dissimilatory sulfite-reductase α-subunit, which involved in reduction of sulfite (Widdel and Hanser, 1992); for estimating the abundance of SRB).

Standard genes for 16S rRNA and *dsrA* used in this study were isolated from *Desulfovibrio desulfuricans* (ATCC 13699), while *bssA* standard gene was isolated from *Desulfotignum* sp. (isolated in this lab). All the isolated genes were cloned using pGEM-T vector system (Promega Corp. Madison, WI) in *E. coli* JM109 competent cells as described previously (Yan et al. 2008; Tanji et al. 2014). PCR was performed in 20-μL volumes containing 10 μL Thunderbird™ SYBR® qPCR mix (TOYOBO Co., Ltd., Osaka, Japan), 0.4 μL of 10 μM forward and reverse primers (the primers set for each gene was

137 available in Table 1), 0.4 μL of 50× ROX reference dye, 6.8 μL of Bacterial free water and 2 μL of DNA
138 sample. The qPCR was performed on a Step One Real-Time PCR system (Applied Biosystem, Waltham,
139 MA) with the following conditions: initial denaturation at 95°C for 20 s; 40 cycles (95°C for 30 s for
140 denaturation and annealing temperature of 60°C for 30 s) and one additional melt-curve cycle (95°C for
141 15 s, 60°C for 60 s, and 95°C for 15 s). The standard curves produced in each measurement had R^2
142 values of 0.99±0.05 and efficiency value for 16S rRNA, *bssA*, and *dsrA*, respectively was: 98%, 90%,
143 and 93% (detail information was shown as supplementary information, table SI-4). The abundance of
144 these genes was analyzed statistically using Student's t-test (type 3) and population Pearson correlation
145 coefficient.

146 The bacterial community was examined using an Illumina MiSeq sequencing system. The 16S
147 rRNA metagenome sequencing library was generated by two-stage PCR with different primer sets, as
148 shown in table 1. The first-stage PCR was performed in 25-μL volumes containing 2.5 μl 10× ExTaq
149 buffer, 2 μl dNTP Mix, 0.4 μL of 10 μM of each primer, 0.625U ExTaq polymerase (Takara Bio Inc.,
150 Japan), and 1 μl of the DNA extract with PCR conditions were as follows: initial denaturation at 95°C
151 for 5 min; 25–30 cycles (95°C for 30 s, 55°C for 30 s and 72°C for 1 min) and final elongation at 72°C
152 for 5 min. PCR fragments was observed by electrophoresis using 2% agarose gel in 1X TBE (8.9 mM
153 Tris, 8.9 mM Boric acid, 2 mM EDTA) to confirmed the expected size of product (512 bp). The product
154 of first-stage PCR was purified and the DNA concentration was measured, for the second-stage PCR
155 the purified DNA concentration from first-PCR was adjusted to 2.5 ng/uL as DNA template.

156 The second-stage PCR used primers containing overhanging adapter sequences for the target
157 locus using Nextera® XT Index Kit v2 Set D (Illumina Inc., CA, USA) (Table 1). The second-stage
158 PCR was performed in 25-μL volumes containing 2.5 μl 10× ExTaq buffer, 2 μl dNTP Mix, 5 μL of of
159 indexed primer, 0.625U ExTaq polymerase (Takara Bio Inc., Japan), and 1 μl of purified DNA from 1st
160 PCR with PCR conditions as follows: initial denaturation at 95°C for 5 min; 8 cycles (95°C for 30 s,
161 55°C for 30 s and 72°C for 1 min) and final elongation at 72°C for 5 min. The PCR products from each
162 stage were purified using the QIAquick® PCR Purification Kit (QIAGEN, Hilden, Germany). The final

product was then analyzed quantitatively and qualitatively using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE). The Illumina MiSeq sequencing was performed by Hokkaido System Science. All the results of Illumina MiSeq sequencing reads was deposited in NCBI BioProject (accession number PRJNA360017). All 16S rRNA MiSeq sequencing reads analyzed using QIIME version 1.9.1 (Caporaso et al. 2010).

3. **Results**

3.1 Souring and volatile fatty acid production

Chemical analysis was performed on four conditions (Fig. 1). In the abiotic control, we observed no changes of measured anion concentration or production of volatile fatty acid (data not shown). Under the control condition ($N_{w/o}$), we observed a decrease in sulfate and an increase in sulfide. After the 70-day incubation, sulfate decreased by 10 mM and sulfide increased by 6 mM. The addition of nitrate at day 28 was done since on day 28 the SRB already reached the active state, it was confirmed by the continuous production of hydrogen sulfide along with reduction of sulfate. Thus, condition was ideal to introduce nitrate in order to see the effect of its to SRB population (Fig. 1, N_{28}). Furthermore, a drastic reduction in sulfide and increase in sulfate were observed 7 days after nitrate addition (Fig. 1, N_{28}), indicating that addition of nitrate could treat souring. At the end of the experiment, the sulfide produced in N_{28} had completely disappeared. The decrease in sulfide concentration in N_{28} resulted in an increase in the sulfate concentration (Fig. 1, N_{28}); i.e., the sulfide reduction and sulfate production were not equal. Meanwhile, after addition of nitrate on day 28, we observed a reduction in nitrate (to 5.7 mM) and production of less than 1 mM nitrite. Thus, addition of nitrate when SRB are active can mask souring activity.

Moreover, in the N_0 condition, we did not observe a major decrease in sulfate nor production of sulfide (both signs of bacterial souring) in comparison with $N_{w/o}$. Thus, the addition of nitrate at the beginning totally masked biological souring. Meanwhile, in N_0, nitrate continuously decreased to 7.4 mM on day 70, and less than 1 mM nitrite was produced. The reduction of nitrate and production of nitrite were unequal, possibly because nitrite, the intermediate product, can be further reduced to

ammonia and nitrogen gas. Thus, NRB were present in N_0. Based on VFA production (Fig. 2), acetate production was observed only in conditions $N_{w/o}$ and N_{28}; the concentration reached its peak with the value of 260 μM after 14 days of incubation, and then started to decline. When nitrate added to condition N_{28}, the production of acetate was completely abolished. Production of other VFAs was undetected in all conditions.

3.2 Substrate consumption

Based on GC analysis, in the aromatic fraction, only toluene was efficiently consumed during the study (Fig. 3a, mostly in $N_{w/o}$ and N_{28}; detailed chromatogram was shown as supplementary information, Fig S1-1). In the $N_{w/o}$ condition, the toluene concentration continuously decreased and was fully consumed by day 42. The addition of nitrate in the N_{28} condition decreased the consumption of toluene relative to $N_{w/o}$, in which toluene was fully consumed after a 70-day incubation. By contrast, toluene depletion in N_0 was slower than under the other conditions: at the end of the experiment, less than 50% of toluene in N_0 had been consumed. In addition to toluene consumption, consumption of benzene was also observed under all three conditions. As shown in Fig. 3b, consumption of benzene started to accelerate after day 42 in N_0, but after day 56 in other conditions. Thus, benzene degradation was affected by the presence of toluene and contribute as alternative carbon source after toluene.

3.3 Analysis of bacterial dynamics

The bacterial dynamics was analyzed using two type of approach, next-generation sequencing (Illumina Miseq) and quantification of ribosomal and functional genes. The next-generation sequencing was aimed to reveal bacterial consortia based on 16S rRNA gene diversity, while the gene quantifications was aimed to quantify particular gene which reflect the abundance of particular group of bacteria such as SRB (*dsrA*) and toluene-degrading bacteria (*bssA*). The quantification of ribosomal and functional genes in all conditions was shown in Fig. 4.

In general, the quantification of the 16S rRNA gene under all conditions revealed that the number of total bacteria decreased over time. There was no significant difference of 16S rRNA abundance among all conditions ($P > 0.5$), except for abiotic control which the 16S rRNA abundance keep stable at

$10^2 – 10^3$ copies/mL (data not shown). By contrast, the copy numbers of the other two genes, which targeted specific bacteria, exhibited different trends. Quantification of *dsrA* gene, which reflect the abundance of SRB, increased in all condition except N_0. The abundance of *dsrA* after 27mM nitrate addition at the beginning was remained stable around 10^4 copy number/mL until day 28 and decreased by one log on the following day, indicate the SRB growth was suppressed in this condition. While the abundance of *dsrA* on $N_{w/o}$ and N_{28} was stable around 10^7 copy number/mL, three log higher than the initial condition (t=0). Quantification of *bssA* revealed that the abundance of toluene-degrading bacteria increased under all conditions, especially in $N_{w/o}$ and N_{28}; the gene was least abundant under the N_0 condition. Therefore, the addition of nitrate at the beginning of the experiment might influence the abundance of toluene-degrading bacteria and SRB, as the abundance of *dsrA* and *bssA* in N_0 against $N_{w/o}$ and N_{28} was significantly different ($P< 0.05$). Furthermore, based on the data shown in Fig. 4, the abundance of *bssA* corresponded to the abundance of *dsrA*, even though the absolute abundances differed. Thus was supported by the population Pearson Correlation Coefficient, which the abundance of *dsrA* and *bssA* in $N_{w/o}$ and N_{28} has strong correlation ($\rho > 0.85$), while in N_0 the abundance of these functional gene has moderate correlation ($0.80 >\rho> 0.50$).

In Fig. 5, the structure of bacterial community in all conditions through the time was plot in histogram and tandem with the abundance of 16S rRNA gene quantification by qPCR to reflect the total number of bacteria in all sample (dot graph- second axis). In average around 90% of total read could be classified at the genus level, and genera with relative abundances below 1% were grouped as 'other'. Based on NGS analysis, the bacterial community of OFW was dominated by *Arcobacter* sp., followed by *Thalassospira* sp., *Desulfotignum* sp., and *Fusibacter* sp. (Fig. 5, t_0 in all conditions). *Arcobacter* sp. is considered an NR-SOB because it can oxidize sulfide chemoautotrophically by reducing nitrate as an electron acceptor (De Gusseme et al. 2009; Tang et al. 2012).

In general, the bacterial communities in $N_{w/o}$ and N_{28} were similar. In these conditions, *Desulfotignum*, a member of the Deltaproteobacteria class, was more abundant. *Desulfotignum* is an SRB (Drzyga et al., 1993). Thus, the enrichment of these particular bacteria provides evidence of souring

under the $N_{w/o}$ and N_{28} conditions (before nitrate addition) (Fig. 1). After nitrate addition, the community remained similar until the end of experiment. Unlike $N_{w/o}$ and N_{28}, in which *Desulfotignum* were greatly enriched, *Thalassospira* was enriched in N_0. *Thalassospira*, a member of the Alphaproteobacteria, is an NRB (Kodama et al. 2008). However, the presence of *Desulfotignum* was still detected under this condition, especially at day 28, when its abundance was obviously increased (Fig 5, N_0). Relative to Fig. 4-N_0, the ratio between copy number of *dsrA* and 16S rRNA gene increased significantly from day 14 to day 28, providing further support for the presence of *Desulfotignum* in N_0. Bacterial community analysis proved that addition of nitrate at the beginning could stimulate the growth of NRB and repress the growth of SRB, even though it could still not fully eliminate SRB from the community.

Other genera present at relatively low abundance in all conditions could be distinguished into two main groups: (1) fermentative bacteria, which can use hydrocarbon as their carbon source and produce organic acid with/without utilize sulfate or nitrate as electron acceptor (Sherry et al. 2013), consisting of *Acinetobacter* sp., *Clostridium* sp., *Shewanella* sp., and *Sphaerocheata* sp.; and (2) heterotrophic NRB (hNRB), which used hydrocarbon and/or organic acid as electron donor while reduce nitrate, consisting of members of the *Pseudomonaceae, Peptococcaceae, Marinobacterium* sp. (Feng et al. 2011; Ruan et al., 2016).

4. Discussion

In this study, we evaluated the effects of nitrate addition on biological souring and its impact on the bacterial community. The bacterial community could be divided into four groups (Fig. 6): (1) fermentative bacteria, (2) hNRB, (3) NR-SOB, and (4) SRB. Each group was thought to play special role in biological souring under each condition.

SRB and hNRB might share similar sources of electron donors, such as the hydrocarbon fraction in crude oil and/or VFA (Grigoryan et al. 2008). In this study, the most favorable electron donors used by SRB and hNRB were different. Based on Fig. 2 and 3, in $N_{w/o}$ (which was dominated by SRB) toluene acted as the main electron donor as well as the carbon source. The dominant SRB in $N_{w/o}$ and N_{28} were identified as *Desulfotignum* sp., suspected to be the main degrader of toluene under all conditions. These

bacteria have been reported as potential threat to cause souring by reducing sulfate while using toluene and VFA as an electron donor as well as the main carbon source (Hasegawa et al. 2014; Tanji et al. 2014, Li et al. 2016). By contrast, in N_0 no VFA was detected from the beginning of the experiment, and the degradation of the hydrocarbon fraction was slow in comparison with the other conditions. Thus, based on this study the bacteria in N_0, which were dominated by hNRB, tended to use VFA as their main electron donor (Agrawal et al. 2012). The dominant hNRB in N_0 was identified as *Thalassospira* sp., which can degrade various type of aromatic hydrocarbons, including poly-aromatic hydrocarbon (PAH) (Liu et al. 2007). The ability of *Thalassospira* sp. to degrade toluene under anaerobic condition was still unclear. In addition, all the less dominant genera showed in this study (such as *Acinetobacter* sp., *Clostridium* sp., *Marinobacterium* sp., and *Pseudomonaceae*) was known to have ability to degrade aromatic hydrocarbon (Zylstra et al. 1997, Feng et al. 2011, and Ruan et al. 2016), which also responsible for degradation of aromatic fraction in conditions with or without nitrate addition (Fig. 3). The absence of VFA in general during this study may because of the VFA that has been produced by fermentative bacteria and SRB was readily consumed by hNRB as most favorable electron donor (Fig.6). As discussed in Gittel et al (2009), nitrate reduction is thermodynamically more favorable than sulfate reduction, it results higher biomass yields by oxidation of electron donor that can be utilized by both sulfate and nitrate reducers, e.g. volatile fatty acids

Furthermore, after addition of nitrate in N_{28}, the sulfide produced by SRB was fully oxidized and no additional sulfide was produced, although the bacterial community did not change. The dominant bacteria in N_{28} were classified as NR-SOB and SRB. NR-SOB are chemoautotrophic bacteria that can oxidize sulfide coupled to reduction of nitrate. Oxidation of sulfide under denitrifying condition could lead to formation of sulphur or sulfate based on the following reaction (Cardoso et al. 2006; Tang et al. 2009):

$$1.6\ NO_3^- + S^{2-} + 1.6\ H^+ \rightarrow SO_4^{2-} + 0.8\ N_2 + 0.8\ H_2O \quad \text{(a)}$$

$$0.4\ NO_3^- + S^{2-} + 2.4\ H^+ \rightarrow S^o + 0.2\ N_2 + 1.2\ H_2O \quad \text{(b)}$$

Meanwhile, hNRB and SRB reduce nitrate and sulfate, respectively, according to the following equations (Coombe et al. 2010; Sunde and Torsvik, 2005):

$$C_7H_8 + 7.2\ NO_3^- + 0.2\ H^+ \rightarrow 7\ HCO_3^- + 3.6\ N_2 + 0.6\ H_2O \quad (c)$$

$$C_7H_8 + 4.5\ SO_4^{2-} + 3H_2O \rightarrow 7\ HCO_3^- + 4.5\ HS^- + 2.5\ H^+ \quad (d)$$

Based on the equation (a) and (b), sulfide could be completely oxidized into sulfate or partially oxidized into sulfur. The detail metabolism of sulfide oxidation under denitrifying condition of NR-SOB in the community was still unclear and need further studied. Based on equation (c) and (d), toluene will oxidize faster when coupling with sulfate reduction compare to nitrate, which explain the phenomena on Fig 3.

As mentioned earlier the addition of nitrate not only enrich the NRB as the competitor for SRB, but also create toxic environment for SRB by producing nitrite as intermediate metabolite. The presence of nitrate and nitrite in the environment may pose a specific stress to SRB; consistent with this, these ions suppress sulfate reduction ability *in situ* (Jenneman et al, 1989; Davidova et al, 2001; Qiang et al, 2010). Therefore, some SRB have developed defense mechanisms by acquiring nitrate/nitrite reducing enzymes (Greene et al. 2003; Preira et al. 2000; Haveman et al. 2005). In such cases, SRB may also act as NRB in the presence of nitrate and therefore could maintain SRB community under nitrate-rich environment. In addition, NR-SOB could engage in synergetic interactions with SRB, which can reduce the produced sulfide concomitant with nitrate reduction. Then, resulting condition which less toxic to other bacteria ((1) and (2), Fig. 6) to survive, as sulfide was toxic to the most of bacteria. In this context, chemical analysis monitoring (as pictured in Fig 1, N_{28} after nitrate addition) could yield false-negative results about the activity of SRB. If such interaction were to happen over a long period, it could cause an SRB outbreak after nitrate was fully reduced. Therefore, complete elimination of SRB from the community remains challenging.

5. Conclusion

In conclusion, nitrate injection represents a promising tool for preventing and treating souring in oil exploration, especially offshore. Application of nitrate injection at the places where souring already happened, might be less effective compare with the one where have not. Therefore, the process itself

should be monitored closely to prevent SRB outbreaks, which could cause larger problems. Moreover, the use of lower nitrate concentrations is recommended, given that in this study we observed a nitrate reduction of less than 10 mM of nitrate during a 70-day experiment.

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Figure

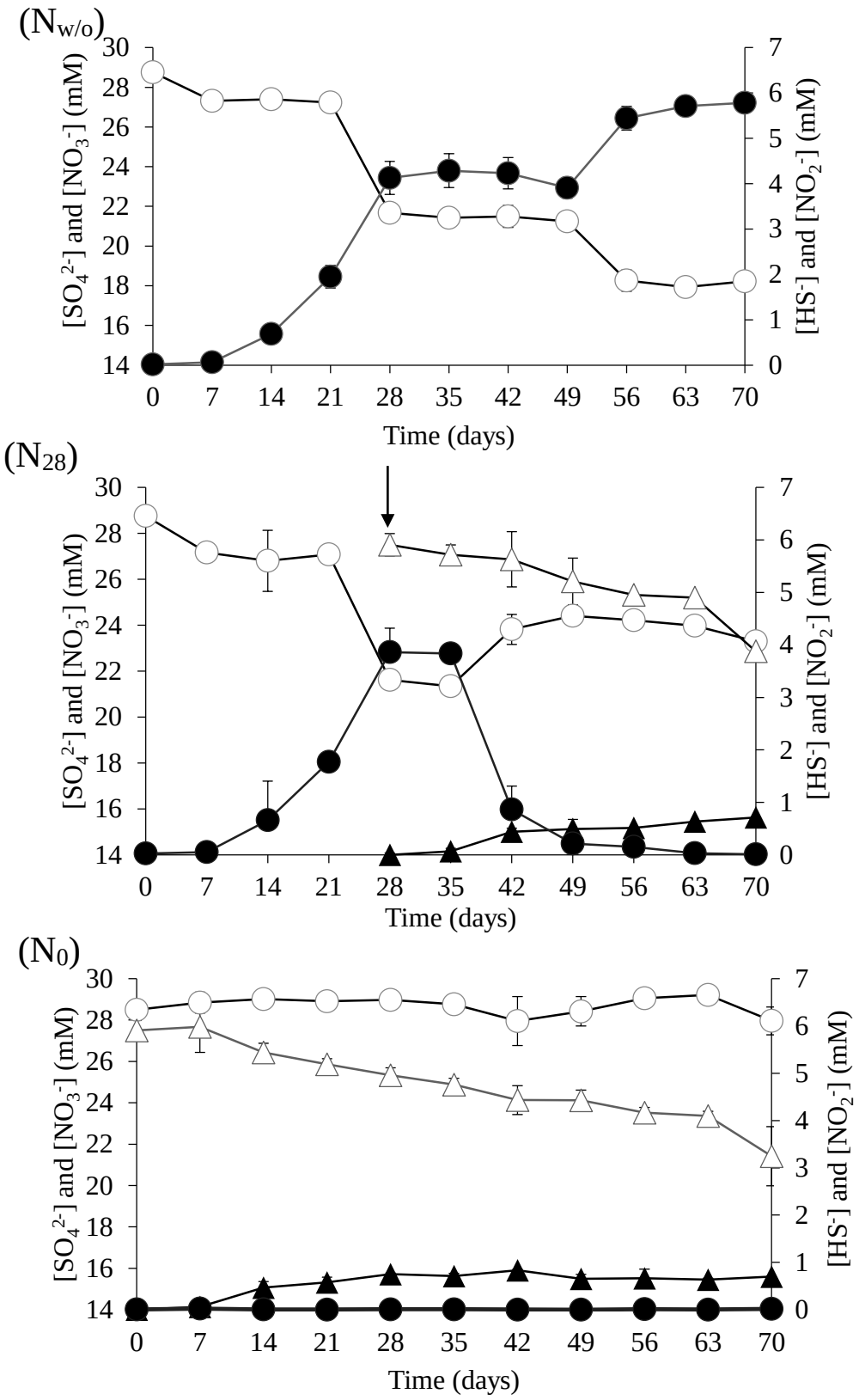


Fig. 1 Chemical analysis of sulfate (open circle), sulfide (closed circle), nitrate (open triangle), and nitrite (closed triangle)**.** Arrow indicates time of nitrate injection (n=3)

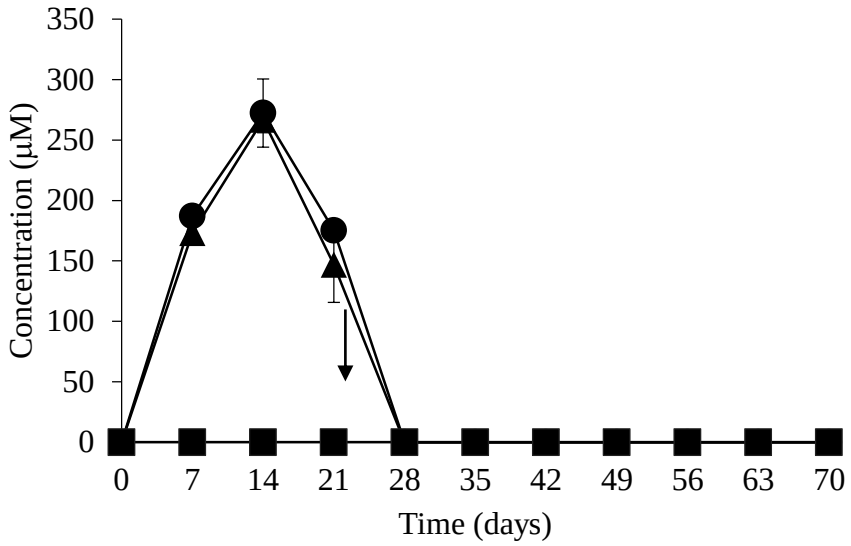


Fig. 2 Acetate production in abiotic control (square), $N_{w/o}$ (triangle), N_{28} (circle) over 70 days of incubation. Arrow indicates time of nitrate injection (n=3). In N_0 condition the production of acetate was undetected

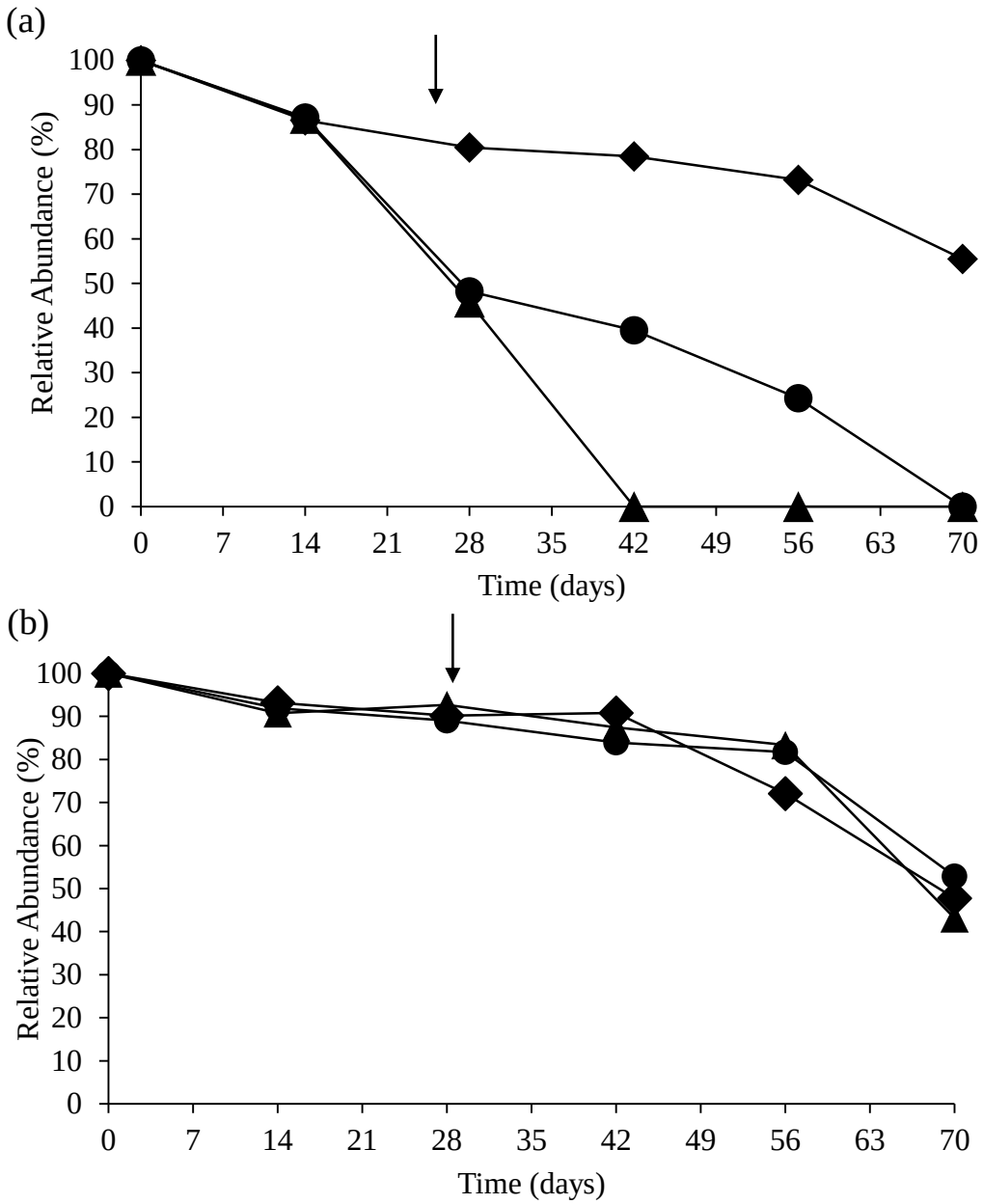


Fig. 3 Substrate consumption (a) toluene and (b) benzene in $N_{w/o}$ (triangle), N_{28} (circle), and N_0 (diamond) over 70 days of incubation. Arrow indicates time of nitrate injection (n=1).

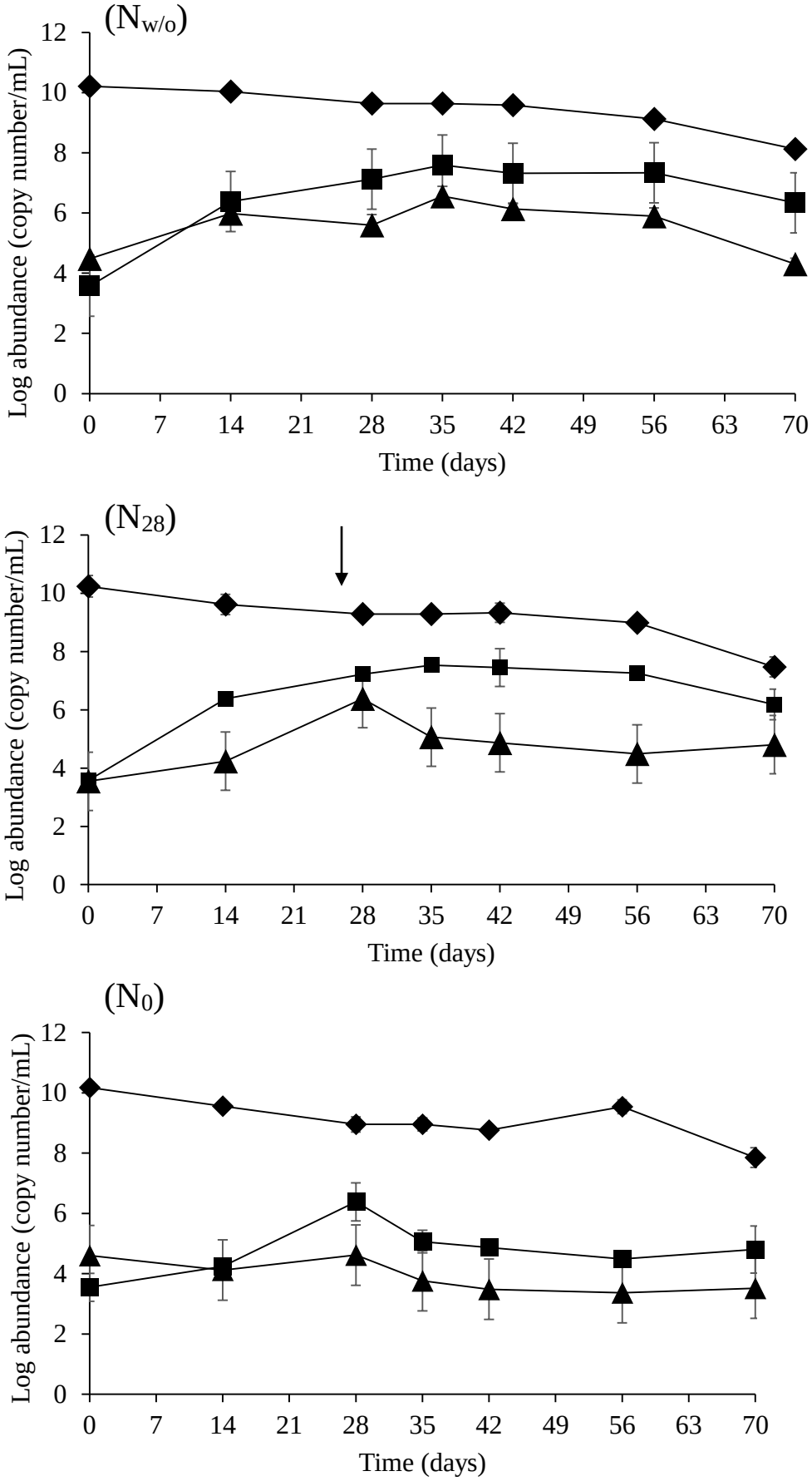


Fig. 4 Abundance of 16S rRNA (diamond), *dsrA* (triangle), and *bssA* (square) genes in $N_{w/o}$, N_{28}, and N_0 over 70 days of incubation. Arrow indicates time of nitrate injection (n=3). Presence of 16S rRNA gene in abiotic control was constantly detected as $\pm 10^3$ copy number/mL

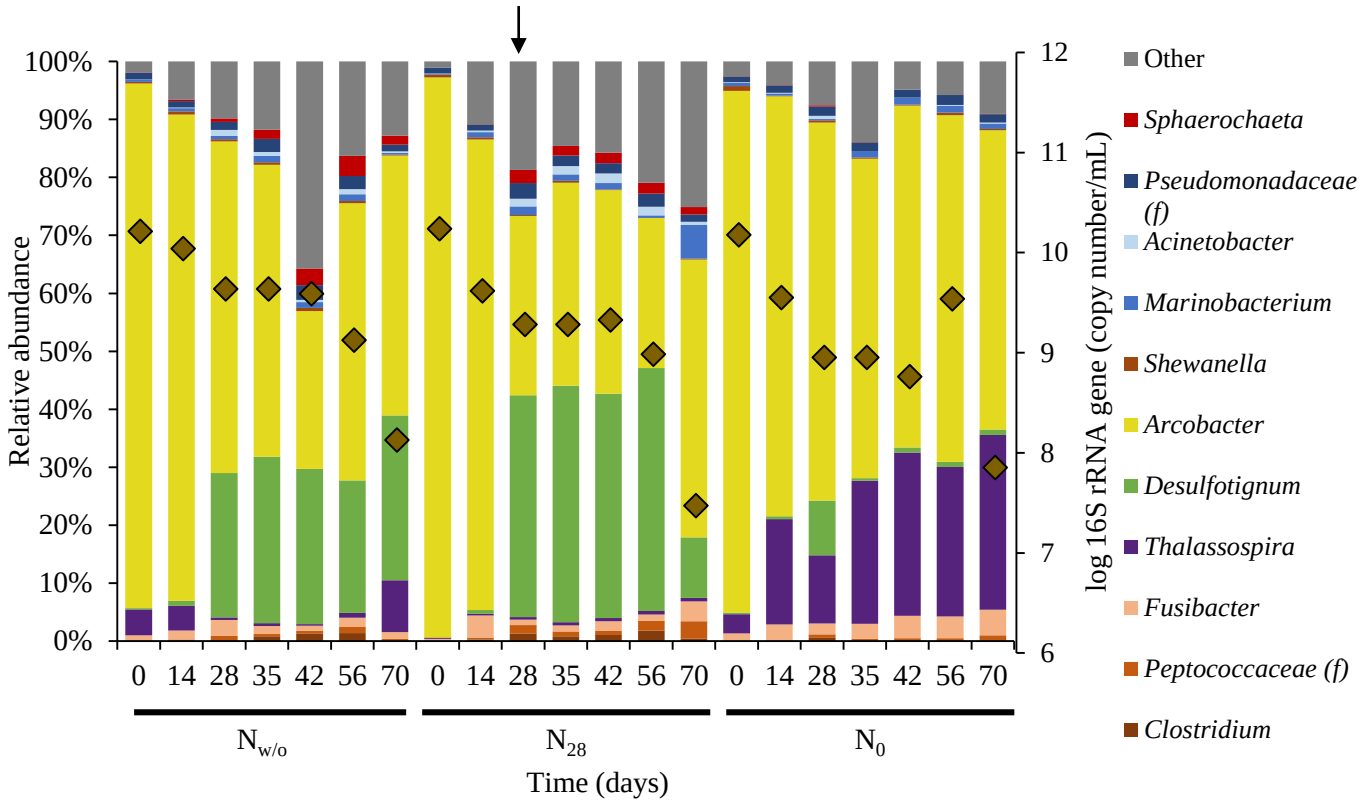


Fig.5 Bacterial community profile in 70 days of incubation, as determined by 16S rRNA gene quantification (diamond). Arrow indicates time of nitrate addition.

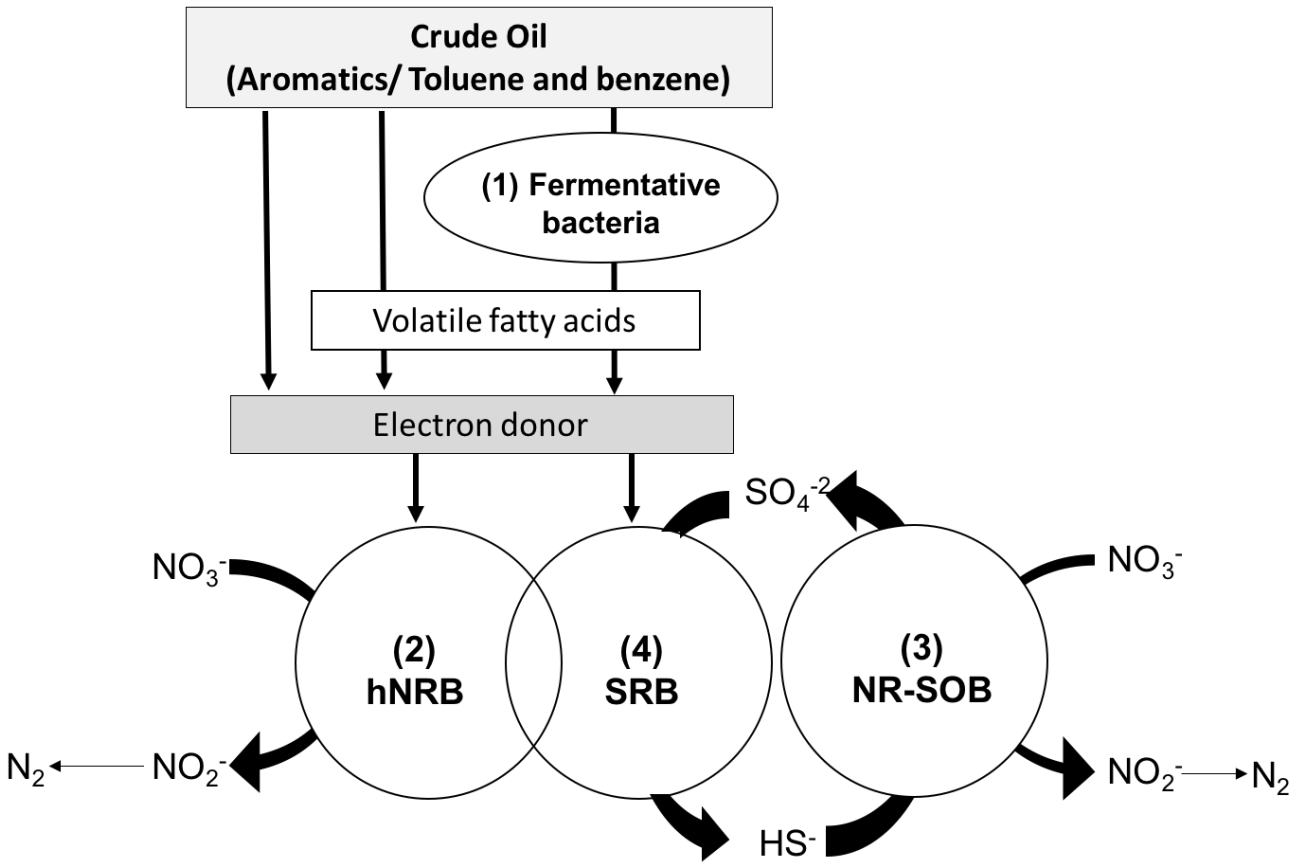


Fig. 6 Possible microbial interactions under microbial souring following nitrate injection

Table 1. List of primer set

Target Gene	Sequence (5' → 3')	Amplicon size	Reference
16S rRNA (qPCR)			
341F	CCTACGGGAGGCAGCAG	206 bp	[16,41,42]
519R	ATTACCGCGGCTGCTGG		
(Illumina 1st PCR)			[43]
16S-misseq F	TCGTCGGCAGCGTCAGATGTGTATAAGAGA CAGCCTACGGGNGGCWGCAG	512 bp	
16S-misseq R	GTCTCGTGGGCTCGGAGATGTGTATAAGAG ACAGGACTACHVGGGTATCTAATCC		
dsrA			
DSR1F-deg	ACSCAYTGGAARCACG	200 bp	[44,45]
RH3-dsr-R	GGTGGAGCCGTGCATGTT		
bssA			
bssA-q-F	TTCAGCAAGGAAGGGAAGGGAACGG	118 bp	This study
bssA-q-R	TTCAGCAAGGAAGGGAAGGGAACGG		