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The presence of nitrate- and sulfate-reducing bacteria contributes to ineffectiveness souring control by nitrate injection

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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, by controlling the growth of sulfate-reducing bacteria (SRB) chemically and biologically. This study aimed to investigate the changes in the bacterial community in response to nitrate addition used to control biological souring. Specifically, we examined 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. Isolation of *Desulfotignum* YB01 (D. YB01) proved the resistance of this predominant SRB in high nitrate environment. Simultaneous reduction of sulfate and nitrate by D. YB01 was also observed in this study. Therefore, 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.

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 geological pressure 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 a high concentration of sulfate (up to 27 mM) that can enhance the growth of sulfate-reducing bacteria (SRB) in the reservoir. Biological souring is a severe 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 methods include injection of biocides (Jayaraman et al., 1999; Nemati et al., 2001; Tang et al., 2009), SRB metabolic inhibitors such as nitrite and molybdate (Nemati et al., 2001; Tang et al., 2009), and 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 (VFA) (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 the presence of sulfide is 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 in high-temperature (Gittel et al., 2009) as well as in low-temperature oil reservoir (Agrawal et al., 2012) resulting in inconsistencies results of its effect to inhibit SRB, as SRB outbreak was observed in the more in-depth zone of the reservoir on a low-temperature reservoir. Another failure of nitrate-mediated souring control was also

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reported elsewhere (Kjellerup et al., 2005; Kaster et al., 2007; Gieg et al., 2011), but the solid reasoning to explain those phenomena were still debatable. The changes of a microbial community before and after nitrate treatment might serve as critical information to evaluate the efficiency of this treatment. 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

Oilfield water (OFW) was taken from an oil field (Akita, Japan) in a 20 L poly-tank that was 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). Total bacteria of OFW is 10^9 16S rRNA copies/mL. The inoculum used in this study was 100 times concentrated OFW, 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, 2 mM 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 seawater 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 (N_0) and 28 (N_{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, and on a shaking incubator (80 rpm).

Every week, a 600 μL sample of the water phase was taken. The sample was centrifuged at 6500 × g for 10 min; the 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. All sample in N_{28}-day 28 was taken before nitrate addition, except sample for anion concentration analysis was taken at both times before and after nitrate addition.

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) (Truper and 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 (GC 2014; 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 of 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 pristine at time = t
A_0 = Peak area of specific fraction at time = 0
B_0 = Peak area of specific pristine at time = 0

2.3. Bacterial analysis

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

Standard genes for 16S rRNA and *dsrA* used in this study were isolated from *Desulfovibrio desulfuricans* (ATCC 13,699), 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 available in Table 1), 0.4 μL of 50 × ROX reference dye, 6.8 μL of Bacterial free water and 2 μL of DNA sample. The qPCR was performed on a Step One Real-Time PCR system (Applied Biosystem, Waltham, MA) with the following conditions: initial denaturation at 95 °C for 20 s; 40 cycles (95 °C for 30 s for denaturation and annealing temperature of 60 °C for 30 s) and one additional melt-curve cycle (95 °C for 15 s, 60 °C for 60 s, and 95 °C for 15 s). The standard curves produced in each measurement had R^2 values of 0.99 ± 0.05 and efficiency value for 16S rRNA, *bssA,* and *dsrA*, respectively was: 98%, 90%, and 93% (detail information was shown as supplementary information, table SI-4). The abundance of these genes was analyzed statistically using Student's t-test (type 3) and population Pearson correlation coefficient.

The bacterial community was examined using an Illumina MiSeq sequencing system. The 16S rRNA metagenome sequencing library was generated by two-stage PCR with different primer sets, as shown in Table 1. The first-stage PCR was performed in 25-μL volumes containing 2.5 μL 10 × ExTaq buffer, 2 μL dNTP Mix, 0.4 μL of 10 μM of each primer, 0.625U ExTaq polymerase (Takara Bio Inc. Japan), and 1 μL of the DNA extract with PCR conditions were as follows: initial denaturation at 95 °C 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 for 5 min. PCR fragments were observed by electrophoresis using 2% agarose gel in 1X TBE (8.9 mM Tris, 8.9 mM Boric acid, 2 mM EDTA) to confirmed the expected size of the product (512 bp). The product of first-stage PCR was purified, and the DNA concentration was measured, for the second-