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Isolation and characterization of nitrate-reducing bacteria which enhance souring and steel corrosion in crude oil well environment

Doctoral Thesis by

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Chapter 1. General introduction

1.1 Reservoir souring and Microbiologically Influenced Corrosion (MIC)

Most of the oil in the world is produced by waterflooding, a secondary oil recovery process that allows the extraction of up to 40-60% of oil from the reservoirs. Waterflooding operations are carried out through the injection of water (particularly seawater, if the well is located offshore) to re-pressurize the reservoir, displacing the oil from reservoir rock pores and pushing it the producer wells (Davidova et al. 2001). This secondary oil recovery process, which typically relies on water injection is relatively cheap to use and has a high yield in oil recovery. Fig 1.1 shows the common method of secondary oil recovery using water injection

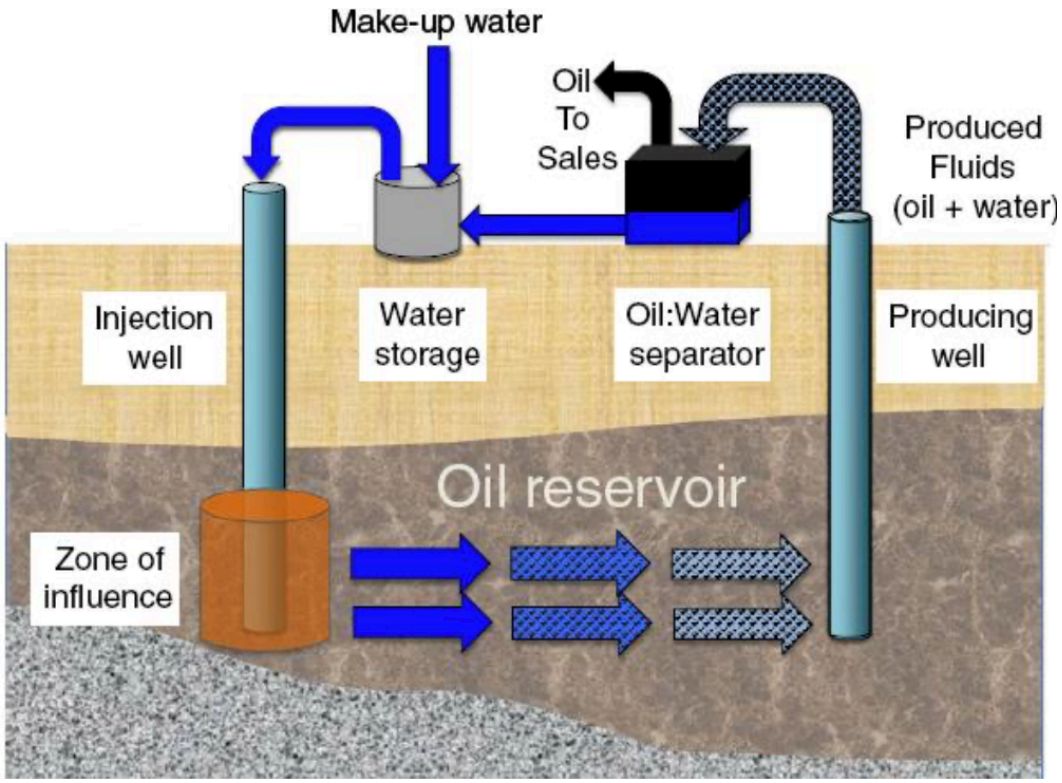

Figure 1.1 Schematics for water re-injection process (Gieg et al. 2011)

The presence of sulfate in the injection water and the presence of organic electron donors in the oil phase mix near to the injection wellbore region will stimulate the growth of sulfate reducing bacteria (SRB) (Kumaraswamy et al. 2011), which is the main cause of reservoir souring. These bacteria reduce the sulfate present in the injected water, especially the sea water and generate hydrogen sulfide (H_2S). The H_2S gas is poisonous and corrosive. The production of this gas can lead to serious environmental problems and economic loss such as reservoir souring, natural gas and oil contamination, metal surface corrosion, and the plugging of reservoirs due to metal sulfide accumulation and subsequent decrease in oil recovery (Hubert and Voordouw 2007; Lambo et al. 2009). In many cases, the reservoir at the primary production stage is a hostile environment to microbial activities. As a result of water injection, a more favorable environment develops. This could be caused by the suitable temperature distribution or availability of sulfate and nutrients. Substantial levels of fatty acids are available in many formation waters that can be used directly by SRB. In offshore reservoirs, seawater is injected and provides an extensive source of sulfate. There should be a good mixing of formation and injection water to have a suitable environment for SRB activity and growth. One common practice in water injection operations is to re-inject the produced water. In this case, the injection water holds fatty acids that normally exist in the formation water and provides all the required compounds for SRB activity. Therefore, bacteria grow in the area around the wellbore and later form an immobile microbial biofilm. In this situation, the reaction zone for production of H_2S is in the vicinity of injectors (Davidova et al. 2001). In addition, H_2S is commonly regarded as having harmful consequences as soluble sulfide or precipitated metal sulfide (e.g. FeS) are corrosive to metal pipes and equipment (Lambo et al. 2009). Biocorrosion is established as a severe problem in the oil and gas industry, also described as microbiologically influenced corrosion (MIC). SRB are the most widespread bacteria involved in anaerobic MIC due to the presence of sulfate in aqueous environments such as seawater, which is used in water

injection to increase oil reservoir pressure (Xu and Gu 2014). SRB are anaerobic bacteria that can produce hydrogen sulfide by using sulfate as the terminal electron acceptor (Zhang et al. 2015). In addition, MIC can happen under a variety of terminal electron accepting conditions. Currently, nitrate and nitrite are commonly used in the oil and gas industries to reduce SRB activity which often resulted in the corrosion of steel pipes and equipment and crude oil souring (Miller et al. 2018). MIC may happen under nitrate reducing conditions through several mechanisms. First, nitrate can sustain thickened biofilms, leading to localized cathodic and anodic regions on metal, resulting in pitting corrosion (Beech 2004). Second, nitrite, an intermediate in dissimilatory nitrate reduction, can be generated in fluids and oxidize Fe^0 in a uniform corrosion process. However, the surrounded accumulation of nitrite may cause in pitting (Lin et al. 2008). Nitrite is also often used as an corrosion agent (Hubert and Voordouw 2007). Lastly, nitrate can help in the direct microbial metabolism of Fe^0 in steel or cathodic H_2 intake, both resulting in the carbon steel Fe^0 oxidation and often leading to pitting (Miller et al. 2018; Windt et al. 2003).

1.2 Souring mechanism

Several mechanisms are suggested for reservoir souring including microbial and geochemical reactions such as thermochemical sulfate reduction and decomposition of organic sulfur (Herbert, 1987). However, sulfate reduction by SRB is believed to be the most dominant mechanism of reservoir souring during water flooding (Ligthelm et al. 1991). SRB utilize various organic compounds as electron donors for sulfate reduction as shown in Fig.1.2.

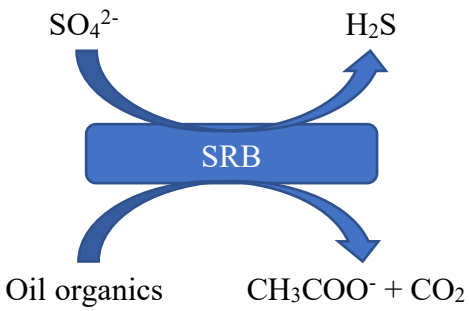

Figure 1.2. Common mechanism to H_2S generation in SRB

Studies show that SRB can use almost 100 compounds as substrates, most of them are abundant in the formation water (Barton, 1995). Therefore, with the supply of sulfate in reservoir, SRB can potentially generate H_2S using organic compounds from crude oil as the source of carbon and energy. During water flooding, the temperature around the injectors decreases and provides better conditions for SRB growth. The water backflow samples from injectors in an oil field in California showed that a region of high bacterial activity existed close to the injectors (Mckinley et al. 1988). The backflow from injectors in the Skejold field in Norway showed the existence of viable SRB population and H_2S concentrations as high as 60 mg/L (Larsen, 2002).

1.3 Microorganisms responsible for MIC

Anaerobic iron MIC is the outcome oxidation of iron and the reduction of electron acceptors, like those of sulfate nitrate and proton, which is a non-oxygen oxidant. Microbes related to MIC are omnipresent. Various bacteria may induce MIC attack along with iron oxidizing bacteria (IOB), sulfate reducing bacteria (SRB), and acid producing bacteria (APB). SRB are the corrosive bacteria most widely studied since sulfate is pervasive (Jia et al. 2017). Nitrate injection is used for promoting growth of NRB to inhibit SRB growth in order to

prevent reservoir souring due to biogenic H_2S production by SRB. However, NRB can also cause MIC (Jia et al. 2017).

1.3.1 Sulfate reducing bacteria (SRB)

SRB are most often assumed as a culprit for MIC. As an electron acceptor, sulfate is used for SRB respiration. Organic carbon is used as electron donors, such as volatile fatty acid. The molecular hydrogen is as an electron donor by the SRB. The oxidation-reduction reaction, such as the exchange of electron gives energy for metabolism of SRB (Barton et al. 2003). Starvation of organic carbon which frequently happened on the surface of a carbon steel with growing biofilm. Due to the scarce of organic carbon in the bulk-fluid phase, SRB can use Fe^0 as electron donor for generating energy, in order to overcome energy deficient condition due to carbon starvation (Xu et al. 2013). Xu and Gu (2011) revealed that *Desulfovibrio vulgaris* was desperate for organic carbon became more serious in starvation experiments associated with the biofilms of *D. vulgaris* originally developed in a pure culture medium under the same conditions on C1018 carbon steel coupons. Microorganisms need energy for maintenance even if they do not grow. Fe^0 is considered as an electron donor and is used by SRB for biocatalysis in the production of energy. Since the electrons generated from oxidation of iron unable to flow in an aqueous solution, they must be uptake directly by microbes. Therefore, on the surface of iron, there should be a biofilm for the electron transfer (Xu et al. 2013). Many SRB may also use element sulfur, nitrate, and fumarate as terminal electron acceptors (Barton et al. 2003). Nitrate is particularly noteworthy among these chemicals in the oil and gas industry since it can be used to inhibit reservoir souring by biogenic H_2S (Gieg et al. 2011).

1.3.2 Nitrate reducing bacteria (NRB)

Under minimal oxygen conditions, facultative NRB such as *P. aeruginosa* and *Bacillus licheniformis* can utilize nitrate as a terminal electron acceptor for their respiration (Mayer,

1994). Through denitrification, NRB can reduce nitrate to ammonium or nitrogen. There are two ways of reducing NO_3^- involving different reductions in denitrification metabolism pathways: assimilatory pathway ($NO_3^- \longrightarrow NO_2^- \longrightarrow NH_3^+$) and dissimilatory pathway ($NO_3^- \longrightarrow NO_2^- \longrightarrow NO \longrightarrow N_2O \longrightarrow N_2$) (Hassett et al. 2002; Yoon et al. 2002). *P. aeruginosa* has been documented to be able to grow in diesel and jet fuels and cause MIC (Striebich et al. 2014). MIC induced by aerobic *P. aeruginosa* biofilms has been extensively studied (Hamzah et al. 2013). Yuan's group (2008; 2007) researched MIC of the *Pseudomonas* NCIMB 2021 bacterium on 304-type stainless steel. Their results revealed that in the presence of aerobic *Pseudomonas* NCIMB 2021, the passivating film was damaged on the surface of stainless-steel. The researchers found that the corrosion rate and the pit depth improved over time. Although *P. aeruginosa* commonly grows aerobically, under restricted oxygen condition or strictly anaerobic condition, through denitrification using nitrate (NO_3^-) or nitrite (NO_2^-) as an appropriate electron acceptor in its respiratory chain, it can form biofilms on environmental surfaces (Eschbach et al. 2004). Products in gas phase, such as nitric oxide (NO), nitrous oxide (N_2O), and molecular nitrogen (N_2) can be generated during the denitrification process. Yoon et al. (2002) showed that *P. aeruginosa* can generate a more robust biofilm and experience a morphological change showing filamentous cells with anaerobic respiration (Yoon et al. 2011). Nitrate have been extensively introduced into reservoirs in the oil and gas industry to inhibit SRB growth for reservoir souring control (Gieg et al. 2011). Nitrate stimulates the growth of NRB and NRB may induce MIC (Xu et al. 2013). Their results indicated that under anaerobic condition, *B. licheniformis* was identified to corrode C1018 carbon steel as a corrosive nitrate reducer against. *P. aeruginosa* may be able to grow as NRB and induce MIC.

1.3.3 Acid producing bacteria (APB)

Under APB biofilms, there may be an acid environment due to secreted organic acids that may cause MIC (Xu et al. 2016). Iron oxidation along with the reduction of protons is

thermodynamically desirable. In contrast to sulfate or nitrate reduction, biocatalysis was not necessary in the reduction of proton (Gu, 2014). Organic acids formed in the bulk fluid by planktonic cells may support maintain a low pH environment. Underneath an APB biofilm, the pH can be much lower compared to the condition inside the bulk fluid. The reason is, in the APB biofilm, the amount of sessile cells is much greater than the amount of planktonic cell found in the bulk fluid (Gu, 2014). Organic acid attack at the same pH is much more corrosive than strong acids, because organic acids are weak acids.

1.4 Mechanisms of MIC caused by SRB and NRB

The study and understanding of SRB's contribution to corrosion is very important. Since 1934, researchers proposed different kinds of mechanisms. The first theory to describe MIC due to SRB using electrochemical reactions is the cathodic depolarization theory (CDT) (von Wolzogen Kuehr and von der Vlugt, 1934). In this theory, the atomic hydrogen deposited on the cathode was taken away using hydrogenase enzymes. Thus, the corrosion process is pushed forward. This principle is applicable only for SRB which is hydrogenase-positive.

The corrosion process through CDT can be explained in more details. A metal becomes polarized by losing positive metal ions (anodic reaction) when it contacts with water. In the absence of oxygen, the free electrons reduce water-derived protons (cathodic reaction). Next, hydrogen is produced and remained on the metal surface. This will result in a dynamic equilibrium. The hydrogen produced is expected to be consumed by SRB (according to reaction (4) indicated in Table 1.1. The consumption of hydrogen leads to oxidation of Fe. In this process, anodic metal dissolution is increased. As the result, corrosion products such as FeS and Fe $(OH)_2$ are produced.

Table 1.1 Cathodic depolarization mechanism of metal corrosion by SRB

Anodic reaction (1)	$4Fe \longrightarrow 4Fe^{2+} + 8e^-$
Water dissociation (2)	$8H_2O \longrightarrow 8H^+ + 8OH^-$
Cathodic reaction (3)	$8H^+ + 8e^- \longrightarrow 8H + 4H_2$
Hydrogen oxidation (4)	$SO_4^{2-} + 4H_2 \longrightarrow H_2S + 2H_2O + 2OH^-$
Corrosion product formation (5)	$Fe^{2+} + H_2S \longrightarrow FeS + 2H^+$
Corrosion product formation (6)	$3Fe^{2+} + 6OH^- \longrightarrow 3Fe(OH)_2$
Total reaction	$4Fe + SO_4^{2-} + 4H_2O \longrightarrow FeS + 3Fe(OH)_2 + 2OH^-$

Gu et al. (2009) proposed the concept of BCSR (biocatalytic cathodic sulfate reduction) based on electrochemical kinetics and bioenergetics. An SRB biofilm is produced on a steel surface according to the BCSR theory. The carbon source for SRB is required in the first stage in order to sustain metabolism. The biofilm becomes a mass transfer barrier in the next step, restricting the availability of carbon sources on the steel surface. When carbon sources for SRB respiration are reduced, SRB turn to elemental iron as an important source of sulfate in energy production. The reactions of oxidation/reduction are shown as Reactions (1) and (2) (Xu and Gu 2014):

Anodic: $4Fe \longrightarrow 4Fe^{2+} + 8e^-$ (Iron dissolution) $E°' = -447$ mV (1)

Cathodic: $SO_4^{2-} + 9H^+ + 8e^- \longrightarrow HS^- + 4H_2O$ (BCSR) $E°' = -217$ mV (2)

$E°$ is the reduction potential at 1 M solution (1 bar gas), 25°C, and pH 7. Table 1.1 shows the values of $E°$ for relative reactions in this work.

Table 1.2 The reduction potential of relative reactions at condition of 25°C, 1 M concentration (1 bar in gas), and pH 7 (Thauer et al. 2007)

Redox couple	n	$E^{\circ'}$ (mV)
Fe^{2+}/Fe^0	2	-447
CO_2 + acetate/lactate	4	-430
SO_4^{2-}/HS^-	8	-217
NO_3^-/NH_3	8	+360
$2NO_3^-/N_2$	10	+760

The reduction potential change, ΔE°, of the two oxidation/reduction reactions above is +230 mV. If the Gibbs free energy change, ΔG°, is negative for the combined redox reaction, the half reactions can occur spontaneously. The Gibbs free energy is calculated from $\Delta G^\circ = -nF\Delta E^\circ$, where n is the electron number and F is Faraday's constant. The ΔG° of Reactions (1) and (2) is negative, which means these reactions are spontaneous. Therefore, in the BCSR theory, it indicates the thermodynamic favorability of MIC by SRB. Sulfate is not known to cause MIC in the absence of SRB. This is because the reduction of sulfate is retarded by a high activation energy, which indicates that it is necessary for SRB to act as biocatalyst in order to force the reaction forward. In BCSR, the cathodic generally refer to the reaction whereby the reduction reaction is responsible for taking electrons donated from the anodic reaction (i.e., iron oxidation) on an iron surface. Some SRB species have different mechanisms for transferring electrons into the cytoplasm within the SRB cells from the surroundings of SRB cells for biocatalytic sulfate reduction (Thauer et al. 2007). In typical SRB MIC, sulfate is the terminal electron accepter for SRB respiration in anaerobic corrosion tests. On surface of coupon, Fe^0 acts as a fuel molecule for the respiration of SRB to generate energy when cells are locally starved of organic carbon in a biofilm (Xu and Gu. 2011). Fe^0, as an electron donor,

is in fact having higher energy than lactate, which is a common organic carbon for SRB because the equilibrium potential of Fe^{2+} /Fe^{0} is -447 mV, which is more pessimistic than the equilibrium potential of CO_2 + acetate/lactate (-430 mV) (Thauer et al. 2007), at 25°C, pH 7 and 1 M solutes (1 bar gasses). The reduction of sulfates happens within the cytoplasm of the SRB utilizing extracellular electrons provided by oxidation of iron with catalysis of enzyme in the sessile SRB cells that are located directly on the metal surface.

The extracellular electrons must be transferred to the SRB cytoplasm through various techniques of electron transfer. Fig.1.3 provides a schematic diagram for SRB's MIC mechanism via cross-cell wall electron transfer.

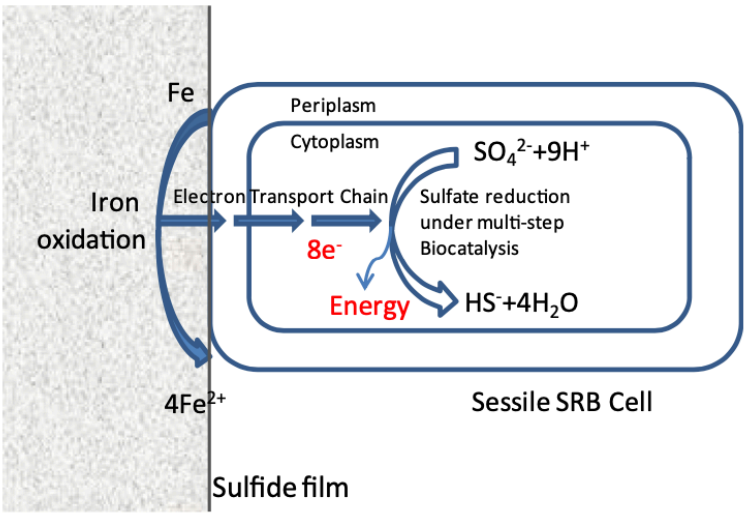

Figure 1.3 Schematic description of SRB MIC mechanism in which extracellular electrons are transported into the cytoplasm of the SRB (Xu and Gu 2014)

It is known that other types of bacteria, such as NRB, can also cause MIC. The theory of BCSR can be generalized to the analogous theory of BCNR (biocatalytic cathodic nitrate reduction) for MIC induced by NRB. By denitrification of oxidative bacteria or by fermentative bacteria, nitrate can be reduced to nitrogen (N_2) or to ammonium (NH_4^+) (Nijburg et al. 1998).

In BCNR, the oxidation reaction is identical to Reaction (1), page 11. It is followed by one of the following possible reduction reactions (Xu et al. 2013):

Cathodic reaction:

$$2NO_3^- + 10e^- + 12H^+ \longrightarrow N_2 + 6H_2O \text{ (BCNR) } E° = +760 \text{ mV} \quad (3)$$

$$NO_3^- + 8e^- + 10H^+ \longrightarrow NH_4^+ + 3H_2O \text{ (BCNR) } E° = +360 \text{ mV} \quad (4)$$

The $\Delta E°$ values of Reaction (1) coupled with Reactions (3) and (4) are +1207 mV and +807 mV, respectively. Thus, the $\Delta G°$ values of these two redox reactions are -582 kJ/mol nitrate and -623 kJ/mol nitrate, respectively. The values of $\Delta G°$ is negative indicates that these redox reactions are even more suitable thermodynamically favorable than those for SRB.

The MIC process induced by NRB should, therefore, be taken seriously and further examined. Fig.1.4 is indeed schematic diagram demonstrating that iron oxidation extracellular electrons are delivered to the cytoplasm of the NRB under biocatalysis for nitrate reduction contributing to MIC corrosion. In many other non-corrosion studies, Fe^0 has been found to be the sole energy source for NRB.

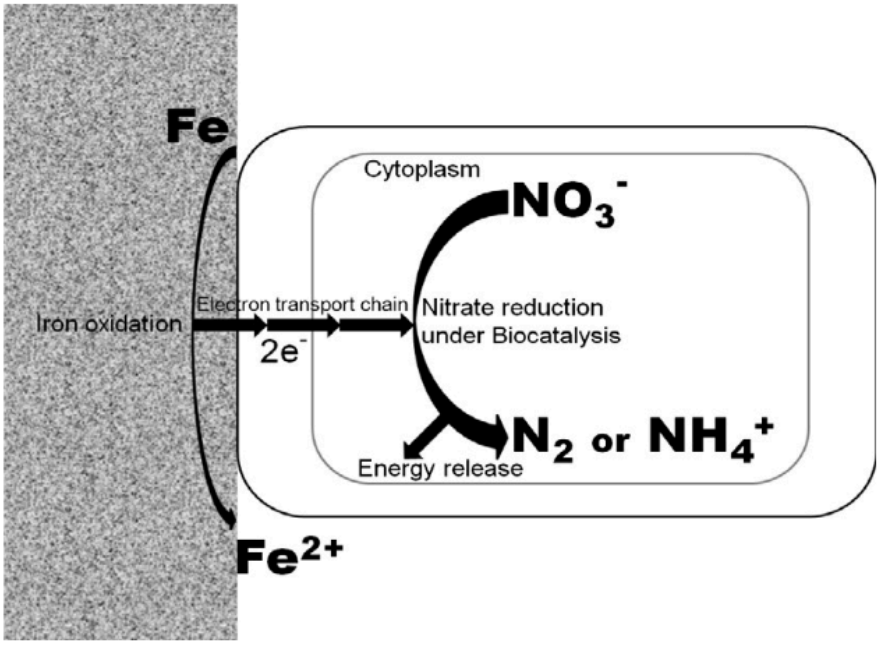

Figure 1.4 Illustration of the NRB MIC mechanism due to NRB use of extracellular electrons to reduce nitrate (Xu et al. 2013).

1.5 Mitigation of reservoir souring and MIC

One of the effective methods of reducing biofilm and MIC pigging (or scrubbing) is widely used in pipelines to minimize the activity of corrosion. It can move throughout the pipelines and clean up the biofilm and other deposits of corrosion physically (Gieg et al. 2011). In advanced smart pigs, it is equipped with sensors that has high resolution to detect the presence of pitting corrosion These sensors were utilize during the cleaning of the inner part of a pipeline too. Nevertheless, pigs cannot be used in some complicated designed pipelines. The application of pigging and biocidal treatment is high in cost, excluding the loss of revenue during the period of installation or treatment. Till now, a variety active and passive measures to mitigate reservoir souring have been suggested, such as reverse osmosis or membrane filtration to remove sulfide, sulfide scavenger addition (Gieg et al. 2011). The methods mentioned may involve efficiency or cost issues in offshore reservoir souring control. Biocides and nitrates are therefore widely used in practical application.

Biocide seems to be most common and most accurate technique to treat biofilm. Sessile cells are stated to be much more tenacious in a biofilm than planktonic cells in bulk fluids. A ten times higher dose of biocides is always needed in order to eradicate biofilm compared to the dosage required to eradicate planktonic cells (Videla, 1996). Repeated biocide treatment is usually required to control biofilm (Vance and Thrasher, 2005) because it is impossible to keep a pipeline system sterile. Glutaraldehyde and Tetrakis (hydroxymethyl) phosphonium sulfate (THPS) are the currently and most widely used biocides in the oil and gas industry, which are both biologically degradable. Nevertheless, high dosages of biocides are not appropriate because more stringent environmental regulations and large-scale biocide technologies are becoming more expensive in oil and gas pipelines. Efficient dosing of biocides is required. Compared to the application of nitrate, chemical components in the reservoir may utilize biocides through chemical reaction and may limit the application of biocides (Nemati et

al. 2001). Furthermore, biocide treatments are costly and difficult to deliver to the effective souring zone at adequate concentration as the huge surface area of reservoir rock provides ample sites for biocide sorption (Ezeuko et al. 2013). Nitrate and nitrite in subsurface environments, on the other hand, are very portable chemicals and do not adsorb to most porous materials. Therefore, the transport of nitrate/nitrite should not be a limiting factor. Previous studies demonstrate the addition of nitrate/nitrite may be more efficient than biocides in achieving longer periods of souring inhibition (Gieg et al. 2011; Reinsel et al. 1996). Nitrate is regarded as a highly cost-effective addition to oil fields to monitor souring and less hazardous substitute to treatment with biocide, especially in offshore oil fields where the concentration of sulfate in injection water is high, as well as in oil fields (Reinsel et al. 1996; Telang et al. 1997). Nitrate addition, in practice, promotes NRB growth and inhibits the growth and activity of SRB. It has proposed several steps, including: (Fig.1.5a) electron donors in reservoirs would be outcompeted by heterotrophic nitrate-reducing bacteria (hNRB) from the SRB, which in turn reduces the production of sulfide (Gittel et al. 2009; Hubert and Voordouw 2007); (Fig.1.5b) the addition of nitrate leads to certain SRB to change their metabolism from reducing sulfate to reducing nitrate (Seitz and Cypionka 1986); (Fig.1.5c) nitrate-reducing sulfide- oxidizing bacteria (NR-SOB) conduct nitrate-dependent sulfide oxidation to sulfate and thus reducing available concentrations of sulfide (Davidova et al. 2001). In addition, nitrite generates and increases the local redox potential that inhibits SRB (Greene et al. 2003). Also, the main SRB enzyme (dissimilatory sulfite reductase; DsrAB), which catalyzes sulfite reduction to sulfide is inhibited by nitrite and this would prevents the production of sulfide (Greene et al. 2003).

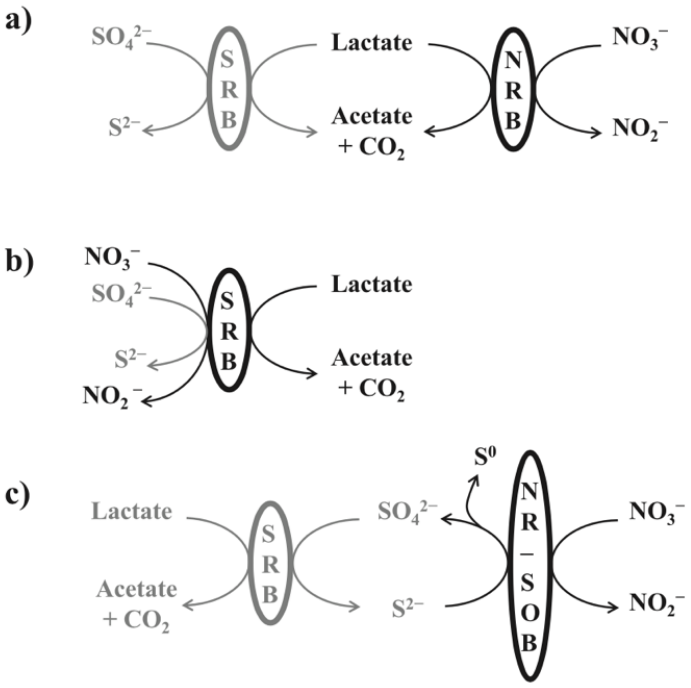

Figure 1.5 Effect of nitrate on the sulfur cycle: (a) SRB-NRB competition for the same organic electron donor (lactate) resulting in inhibition of sulfate reduction to sulfide; (b) nitrate is the favored electron acceptor for SRB, and therefore sulfate is not reduced; (c) oxidation of sulfide to sulfate and sulfur by nitrate-reducing, sulfide-oxidizing bacteria (NR-SOB) (Etique et al. 2018)

In oil fields with high temperature, nitrite is more beneficial than nitrate because it reacts directly with sulfide and provides inhibition of sulfate reduction for long period. Reinsel et al. (1996) noticed that glutaraldehyde (a type of biocide) had no long-term inhibitory effects, but rather reduced the SRB population. SRB possibly expanded at its original growth rate after glutaraldehyde removal and simultaneously produced H_2S. On the other hand, the concentration of H_2S started to increase six days after the removal of nitrite from the column. This indicates that after nitrite removal, SRB was not killed by nitrite, but rather was still inhibited; the original number of cells was still available and they were able to generate H_2S after removal of the inhibitory effect (Reinsel et al. 1996).

1.6 Aims of this research

The effect of nitrate addition on the souring caused by SRB, which was screened from oil field water (OFW) of Japan and was named YB01, has been reported (Kamarisima et al. 2018). It revealed that the nitrate addition to the seawater at the beginning of incubation could control souring. Based on the analysis by using 16S rRNA gene, *Thalassospira* sp., one of the NRB, became dominant instead of SRB, such as *Desulfotignum* sp., from the beginning. On the other hand, in conditions without addition of nitrate or with addition of nitrate to treat souring at day 28, souring occurred and *Desulfotignum* sp. became dominant. Therefore, to clarify the relationship between SRB and NRB growth with and without nitrate addition to the seawater, Isolate NRB from this environment was conducted. The objective of this research was therefore to screen NRB strains from OFW. Through the application of the roll-tube method (Hungate 1969) in the nitrate rich medium, one of the NRB was isolated and identified. Although *Thalassospira* sp. was dominant after addition of nitrate based on the results of microbial consortia analysis, isolated culturable strain was identified, *Marinobacter* sp. and was named YB03. *Marinobacter* sp. is also well known as a ubiquitous NRB in the environment, especially in seawater (Bowman et al. 2015; Singer et al. 2011). The effect of nitrate on reservoir souring and corrosion behavior of carbon steel by *Marinobacter* sp. are still not well understood and further research is needed. Thus, another objective was to investigate its souring activity and its corrosion behavior of carbon steel.

1.7 Structure of the thesis

The structure of this study is shown in Fig.1.6 below.

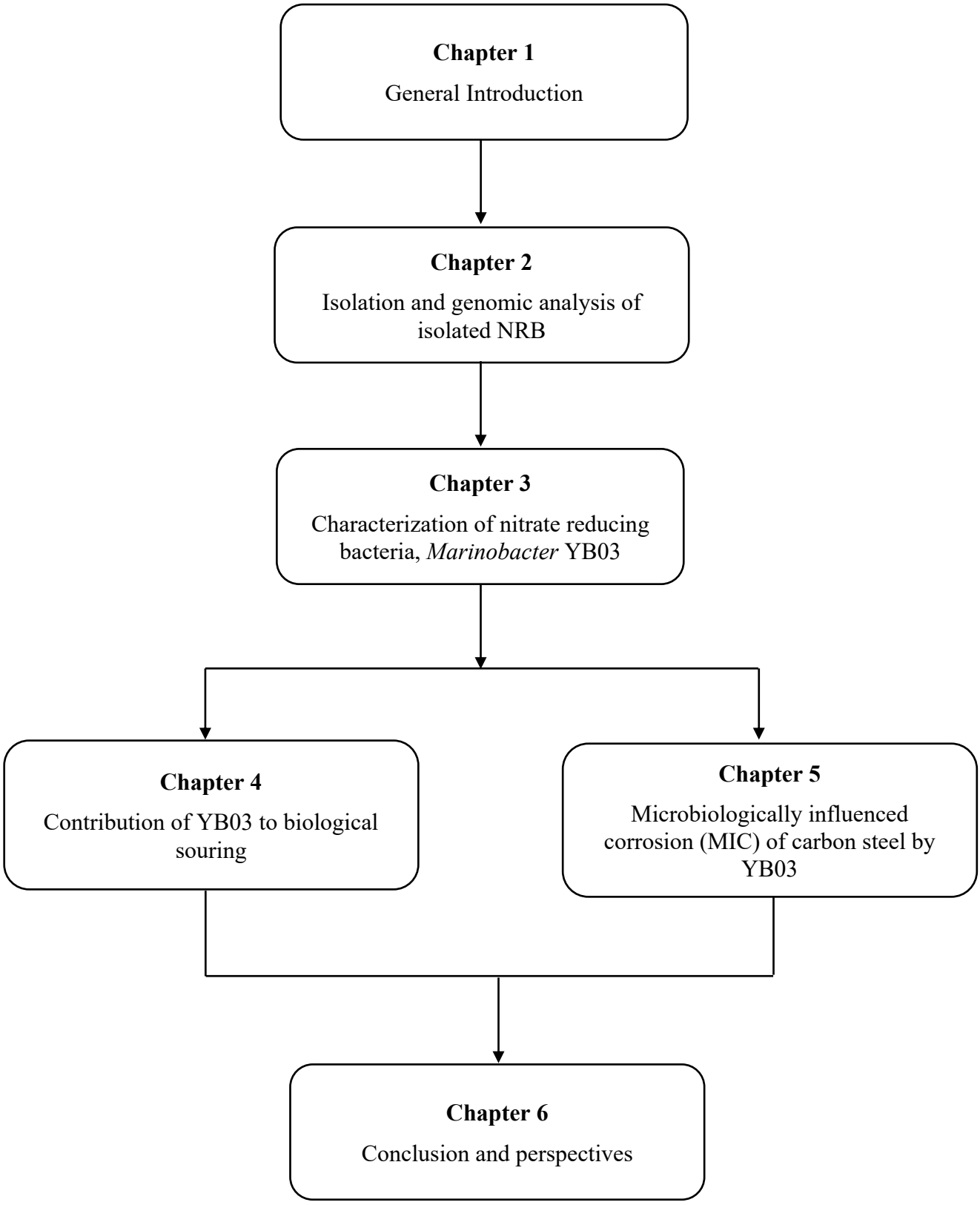

Figure 1.6. Thesis structure. The arrows indicate relationship between each chapter.

Chapter 2. Isolation and genomic analysis of isolated NRB

2.1 Introduction

In the process of sea water flooding as a secondary oil-recovery process, the increase of sulfide concentration in the crude oil, which is well-known as souring could induces the deterioration of oil quality and the corrosion of pipelines. It is thought that the souring of crude oil is due to the biological reaction of sulfate-reducing bacteria (SRB) which reduces the sulfate in the seawater into sulfide in the environment. To prevent souring in the oil reservoir, there are some countermeasures, such as the addition of biocides, filtration of injection water, addition of nitrate (Dopffel et al. 2018; Grigoryan et al. 2008; Hubert et al. 2003; Hubert and Voordouw 2007; Kjellerup et al. 2005; Myhr et al. 2002) and so on. Considering the economic cost, environmental burdens and general versatility, nitrate injection is widely applied (Eckford and Fedorak 2004; Rizk 2015; Schwermer et al. 2008; Telang et al. 1997). This is because the oxidation reduction potential of nitrate is much higher than that of sulfate, it is thought that the added nitrate can be preferentially reduced by nitrate-reducing bacteria (NRB) associated with the oxidation of the electron donor (Li et al. 2018). As a result, nitrate addition is expected to suppress the activity of SRB and control souring in a crude oil reservoir. The effect of nitrate addition on the souring caused by SRB, which was screened from oil field water (OFW) of Japan and was named YB01, has been reported (Kamarisima et al. 2018). The bacterial community of OFW was dominated by *Arcobacter* sp., followed by *Thalassospira* sp., *Desulfotignum* sp., and *Fusibacter* sp. in all conditions Based on 16S rRNA NGS analysis (Fig.2.1). The bacteria communities in $N_{w/o}$ (without nitrate addition) and N_{28} (nitrate added at day 28) were similar. Under these conditions, *Desulfotignum* sp. which is an SRB was more abundant. Unlike $N_{w/o}$ and N_{28} in which *Desulfotignum* sp. were significantly enriched, *Thalassospira* sp. was enriched only in N_0 (nitrate added at the beginning). *Thalassospira* sp., is classified as a member of the *Alphaproteobacteria* and is belonged to NRB (Kodama et al.

2008). Bacterial community analysis proved that the addition of nitrate at the beginning could promote the growth of NRB and repress the growth of SRB. However, the SRB still could not be eliminated from the community.

Therefore, to clarify the relationship between SRB and NRB's growth under the conditions with and without nitrate addition to the seawater, it is necessary to isolate NRB from the environment. The objective of this chapter was therefore to screen NRB strains from OFW.

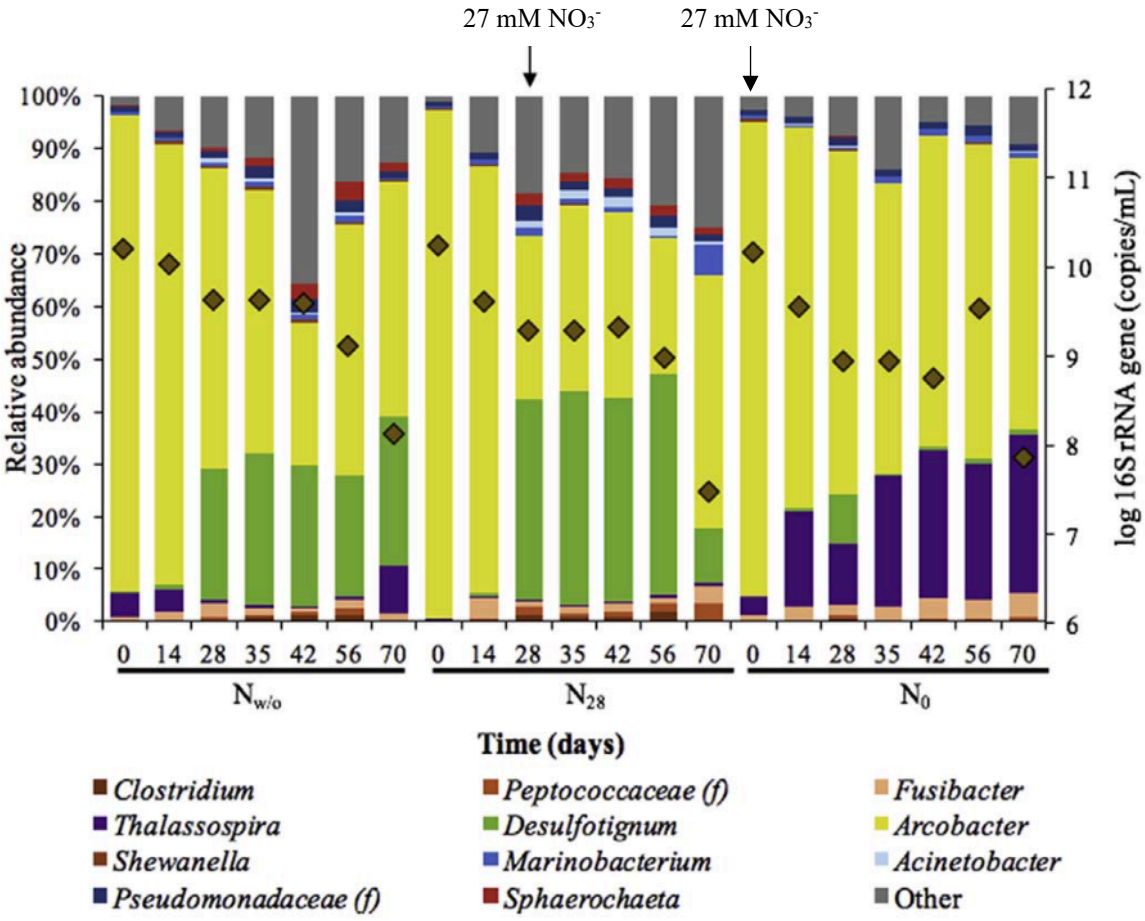

Figure 2.1 Bacterial community profile in 70 days of incubation, as determined by 16S rRNA gene quantification (diamond). Arrow indicates time of nitrate addition (Kamarisima et al. 2018).

2.2 Materials and methods

2.2.1 Isolation of Nitrate-reducing bacteria (NRB)

From an oil field located in Akita, Japan, OFW was collected in a 20-liter poly-tank. OFW is a mixture of water and oil. Up to use, the sample was kept at 4°C. The selected oil field has never been treated by water flooding. The physicochemical properties of the OFW and inoculum have been described previously (Kamarisima et al. 2018).

NRB was isolated from OFW. First, enrichment culture of NRB was performed by inoculating 1mL of condensed OFW (10^{11} copies/mL) in freshwater medium. Added to that was 5 mL of 10% diluted crude oil, used as a carbon source. The biologically inert branched-chain alkane 2,2,4,4,6,8,8- heptamethylnonane (HMN) was used to dilute the crude oil. The incubation condition was anaerobic, 30°C, in the dark for 130 days. The freshwater medium was composed of 0.50 g of $MgCl_2 \cdot 6H_2O$, 0.10 g of $CaCl_2 \cdot 2H_2O$, 0.30 g of NH_4Cl, 0.20 g of KH_2PO_4, 0.50 g of KCl, 0.05 g of Na_2SO_4 and 1000 mL of distilled water. After autoclaving, 1 mL of trace element A (Supplementary Table S1), 1 mL of trace element B (Supplementary Table S2), 30 mL of $NaHCO_3$ 1 M (84 g of $NaHCO_3$ in 1000 mL in distilled water), 1 mL of vitamin mixture (Supplementary Table S3), 1mL of thiamine solution (10 mg of thiamine Cl· HCl in 100 mL of sodium phosphate buffer, 10 mM, pH 3.4), and 1 mL of vitamin B12 (5 mg of cyanocobalamin in 100 mL of distilled water) was added to the medium. The enrichment culture was then reinoculated in the same medium and incubated for about 30 days before isolation by roll-tube technique. The freshwater medium was mixed with agar 3%. A single colony was selected, inoculated in a new freshwater medium supplemented with 10% crude oil and enriched. To identify NRB, 16S rRNA gene was amplified by PCR using universal primers (27F/1492R). Primer sequences were 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGCTACCTTGTTACGACTT-3'). After purification of PCR product, it was sequenced by DNA analyzer (3730xl, Applied Biosystems). Subsequently, based on the 16S

rRNA gene sequence, the strain was identified by Basic Local Alignment Search Tool (BLAST). The strain was named YB03. According to the result of BLAST analysis, the closest genes to YB03 strain was *Marinobacter*. A phylogenetic tree was constructed based on 16S rRNA gene alignment was according by neighbor-joining method on MEGA X software (https://www.megasoftware.net/) after the multiple alignment with 16S rRNA gene sequences of relative *Marinobacter* species by Clustal W. A *Deslufotignum* sp. strain YB01 (MG554646) (Kamarisima et al. 2018) was used as an out group of the tree.

2.2.2 Whole genome analysis

For determination of the whole genome sequence, after cultivation of YB03 in the freshwater medium supplemented with 5 mM of volatile fatty acid mixture (acetate, formate, lactate and propionate) for 10 days, the genome of YB03 was extracted and purified by DNA isolation kit QIAGEN blood and tissue.

DNA extraction using the QIAGEN protocol, briefly for Gram-negative strains, the pellet was resuspended in the 180 µL of STL buffer, add 20 µL of RNase A solution, mix, and incubate for 2 min at room temperature. After incubation, add 20 µL of the proteinase K (20 mg/mL stock solution) to the sample. Mix and incubate for 30 min at 55°C. Add 200 µL of lysis solution C, vortex thoroughly and incubate at 55°C at 10 min. For column preparation, add 500 µL of column preparation solution to each binding column, add centrifuge at 12000×g for 1 min. Discard the eluate. Add 200 µL of ethanol (95-100%) to the lysate and mix thoroughly by vortexing for 5-10 seconds. The mixture was transferred into the binding column, followed by centrifugation for 1 min at 6500×g. Discard the collection tube containing the eluate and place the column in a new 2 mL collection tube. The column was then washed with 500 µL of wash solution 1 and then 500 µL of wash solution concentration, centrifuging each time for 1 min and 3 min at 6500×g and 12000×g. The column was placed in a sterile

Eppendorf tube, 100 µL of elution solution added and centrifuge for 1 min at 6500×g to elute purified bacterial DNA.

The whole-genome sequence analysis (100bp paired end, data size 1 GB) was conducted by next generation sequencer (NGS) (Illumina HiSeq4000) of BGI (Shenzhen, China). The clean reads were used for de novo assembling by velvet 1.2.10 (https://www.ebi.ac.uk/~zerbino/velvet/) under the condition of k-mer size 85. Then, to construct the draft whole genome, all contigs formed were applied to Multi-Draft based Scaffolder (MEDUSA) web server (http://combo.dbe.unifi.it/medusa) using *Marinobacter hydrocarbonoclasticus* ATCC49840 genome (NC_017067) as a comparison genome. The draft genome was annotated by Rapid Annotation using Subsystem Technology (RAST) server (http://rast.theseed.org/FIG/rast.cgi).

2.3 Results

2.3.1 Isolation of NRB

NRB were isolated from oil field water (OFW) with 50 mL of seawater or freshwater as medium, supplemented with 5 mL of 10% crude oil as the substrate. The enrichment of NRB was conducted in the liquid medium for 30 days. After that, the enriched culture was inoculated into same medium with 3% agar for isolation and purification of single colony. After 2 months incubation, 5 colonies from seawater medium and 9 colonies from freshwater medium were obtained. Further analysis revealed that the 5 colonies from seawater medium were not NRB from the viewpoint of their nitrate reducing activity. Among 9 colonies from freshwater medium, 3 colonies were identified as NRB. However, based on 16S rRNA gene identification, all the 3 colonies were identical. Therefore, only one colony was selected for further analysis.

Enrichment of NRB was conducted in the liquid culture consisting of water and oil (HMN) phases. Transfer of substrate in the crude oil from the HMN phase to water phase is

depending on the equilibrium coefficient of each component in the crude oil between two phases. In contrast, in the screening condition, the substrate in the crude oil vaporize from the HMN-layer to the gas phase depending on the partial pressure of each component of the crude oil. These gases will then dissolve into the agar. Differences of substrate supply to the microbes between the enrichment and screening medium made it difficult to screen *Thalassopira sp*. from the enrichment culture.

According to 16S rRNA gene identification, NRB isolate belonged to genus *Marinobacter* and was named *Marinobacter* YB03. The genome sequences of *Marinobacter* YB03 were deposited in the DDBJ/EMBL/GenBank under the accession number AP019537.

The phylogenetic position of YB03 was determined based on 16S rRNA gene sequence. The phylogenetic tree constructed by neighbor-joining and maximum-likelihood algorithms at MEGA X software is shown in Fig.2.2. The 16S rRNA gene sequence (1402 bp) of *Marinobacter* YB03 was found to have the highest sequence similarity to the 16S rRNA gene of *Marinobacter hydrocarbonoclasticus* (99.57%).

Moreover, only two complete whole genome data of *Marinobacter hydrocarbonoclasticus* species are currently available in the database. They are strain ATCC49840 (NC_017067) and strain VT8 (NC_008740). According to the result of whole genome alignment using BLASTn against ATCC49840 and VT8, YB03 shared 94.30% and 97.30% identity with query coverage of 59% and 61%, respectively. The identity of the 16S rRNA gene of YB03 compared with strain ATCC49840 and strain VT8 were 96.50% and 98.20%, respectively. From this result, we suggested that YB03 shares a high similarity with these species.

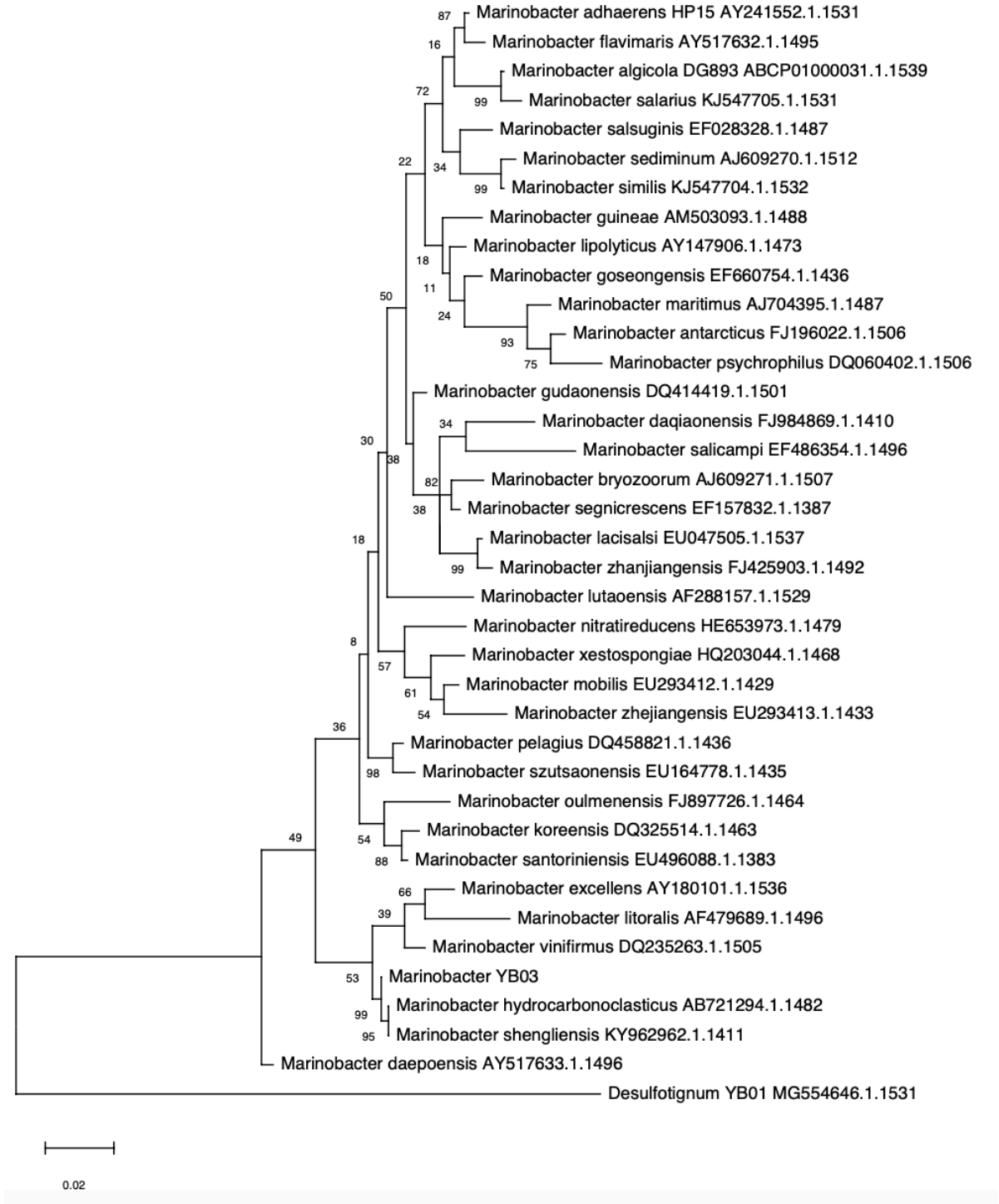

Figure 2.2 Phylogenetic tree based on 16S rRNA gene sequences, showing the relationship of *Marinobacter* YB03 to the type strains of other *Marinobacter* species. *Desulfotignum* YB01 MG554646 is used as an outgroup. Bar, 0.02 substitutions per nucleotide position.

2.3.2 General features of YB03 genome and comparative genomics analysis

The genome sequence was submitted to the RAST server (Aziz et al. 2008). The total sequencing length of the genome of *Marinobacter* YB03 contains 4,300,401 bp with 57.50% GC content. This draft genome contains 3983 genes. The number of RNA genes was 58, including 4 rRNAs (3 16Sr RNA gene and 1 5S rRNA gene), and 54 transfer RNAs (tRNAs). However, 23S rRNA gene was not found in the draft genome of YB03.

A comparison of YB03 with other nitrate reducing bacteria within RAST server, *Marinobacter hydrocarbonoclasticus* VT8 (Genome ID 351345.5), as its closest neighbor with a score of 500, followed by *Hahella cheiuensis* KCTC 2396 (Genome ID 349521.5) with a score of 423, *P. putida* KT 2440 (Genome ID 160488.1) with a score of 214, and *P. aeruginosa* PA01 (Genome ID 208964.1) with a score 153. In RAST, closest neighbors are allocated automatically with the use of a neighbor score. The definition of neighbor score is the number of times where the neighbor genome is shown as the top BLAST hit against a candidate gene from the set of unique genes within the query genome. The taxonomy of the query genome is not necessarily indicated by the closet neighbor. However, it could imply the level of similarities in assigned functions between the query genome and the closet neighbor (Hauptmann et al. 2017).

The genomes of strain YB03 and other genomes were compared. Their general genomic characteristics are summarized in Table 2.1. The genomic features of *Hahella cheiuensis* KCTC 2396, such as genome size, number of coding sequences, were higher than that of other strains. However, the GC content of strain KCTC 2396 was lower than that of other stains. With respect to size and other genomic features, *Marinobacter* YB03 is generally similar to the *Marinobacter hydrocarbonoclasticus* VT8.

Table 2.1 General features of the *Marinobacter* YB03 and comparison with other genomes of NRB

Genomes	Feature			
	Size (bp)	GC (%)	Number of coding sequences	Number of RNAs
Marinobacter. YB03	4,300,401	57.50	3983	58
M. hydrocarbonoclasticus VT8	4,326,849	57.30	3994	60
Hahella cheiuensis KCTC 2396	7,215,267	53.90	6426	82
P. aeruginosa PA01	6,264,404	66.60	5727	76

Multiple genome aliments using Mauve. Alignment of the five genomes (YB03, VT8, PA01, and KCTC 2396) was performed using Mauve (Darling et al. 2010) (Fig.2.3). The upper graph corresponds to YB03; below that, VT8; and the last three graphs are PA01, KT2440 and KCTC 2396, respectively.

Blocks of different colors represent LCBs (Local Collinear Blocks). White areas are used to show the degree of similarity. When the similarity is high, the colored area is higher. In comparison, larger white portions define areas of low similarity. Completely white areas within a colored block (LCB) represent fragments that are not aligned or the absence of sequence elements specific to a genome. Sequences outside of colored blocks do not have homologues. As it can be observed in Fig.2.3, the genome of YB03 was aligned with that of VT8. This alignment showed a high homology between YB03 and VT8 because there are not many white spaces which indicate sequences not in homology blocks. This suggests that both species share many their genome sequences. As the result from the multiple genome alignment, there are some white spaces and white area within a LCB in the genome of PA01 and KCTC 2396.

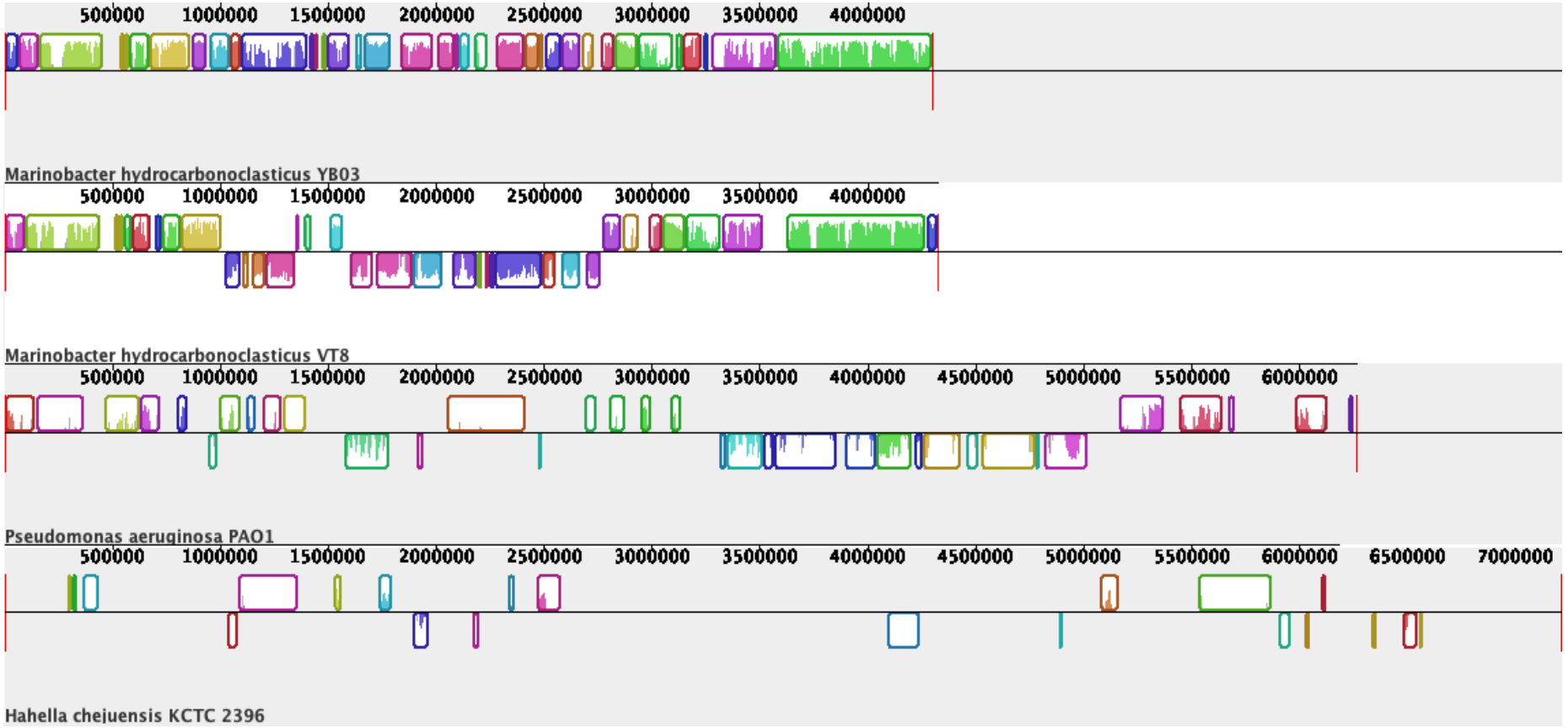

Figure 2.3 Multiple aliment of YB03, VT8, PA01 and KCTC 2396 genomes using Mauve. Inside each box a sequence identity similarity profile is shown. Boxes with the same colors are local collinear blocks (LCBs), suggesting homologous DNA regions in each genome. LCBs shown below the horizontal black line are reverse complements of the reference LCB. Red vertical bar demarcates chromosome boundaries.

2.3.3 Metabolic prediction of YB03

Genes related to dissimilatroy nitrate reduction, denitrification and assimilatory sulfate reduction were encountered in YB03. Pathways obtained from KEGG (https://www.genome.jp/kegg/pathway.html). For nitrate metabolism of YB03, based on genome analysis, the gene set encoding for dissimilatory nitrate reduction was found on the draft genome of YB03, as described in Table 2.2. The dissimilatory nitrate reduction pathway consisted of two main steps, nitrate reduction and ammonifying nitrite reduction. The YB03 has the gene set for both steps. The gene set encoding for nitrate reduction in YB03 was identified to form NarGHI complex (respiratory nitrate reductase). The second step in dissimilatory nitrate reduction is ammonifying nitrite reduction. The NarGHI complex facilitated the reduction of nitrate to nitrite by using formic acid as an electron donor (Simon et al. 2013). Based on genome analysis, the YB03 has the *nirBD* gene coding for nitrite reductase.

Denitrification can be represented in a redox reaction:

$$2NO_3^- + 10e^- + 12H^+ \longrightarrow N_2 + 6H_2O \quad (1)$$

This reaction, is carried out in four enzymatic steps:

$$NO_3^- + 2e^- + 2H^+ \longrightarrow NO_2^- + H_2O \quad (2)$$

$$NO_2^- + e^- + 2H^+ \longrightarrow NO + H_2O \quad (3)$$

$$2NO + 2e^- + 2H^+ \longrightarrow N_2O + H_2O \quad (4)$$

$$N_2O + 2e^- + 2H^+ \longrightarrow N_2 + H_2O \quad (5)$$

Respiratory nitrate reductase (NarGHI) catalyzes the first step Eq (2), whereby it reduces nitrate to nitrite. Nitrite in the second step Eq (3) which can be catalyzed by

cythochrome cd_1 (NirS) is further reduced to NO. Two nitric oxide molecules are conjugated in a third step Eq (4) to form nitrous oxide and water. This is accomplished by a nitric oxide reductase (NorBC). Finally, a nitrous oxide reductase is responsible for the fourth and final step (Eq.5) of reducing nitrous oxide to dinitrogen. For monitoring the process of denitrification in the environments, these key enzymes are often used (Braker et al. 2001; Santoro et al. 2008; Oakley et al. 2007; Smith et al. 2007; Tamegai et al. 2007; Nunoura et al. 2013). Denitrification is generally associated with anoxic conditions because oxygen could hinder the a activity of denitrifying enzyme by represses synthesis of new denitrifying enzyme as well (Bazylinski and Blakemore, 1983).

Nitrate reductase. The reduction of nitrate to nitrite is catalyzed by nitrate reductases in accordance with Eq (2). Organisms reduce nitrate for generating cellular function energy (respiration, denitrification) and eliminating excess energy from the metabolism of cells (dissimilatory ammonification). The pathway of the respiratory nitrate reduction requires the membrane-bound nitrate reductase (Nar). It has been isolated from a variety of organisms (Ballard et al. 1988). Nars are involved in generating a proton motive force across the membranes (Jormakka et al. 2003). NarG contains the enzyme's active site, while the rest of the subunits possess electron transfer centers. The NarGH complex is found in the cytoplasm but remains anchored by the transmembrane NarI subunit to cytoplasmic membrane at the inner surface. YB03 has NapAB (Periplasmic nitrate reductase) as the activity of nitrate reductase. Under anaerobic conditions, this enzyme is expressed the final electron acceptor is nitrate, as in the case of Nar from denitrifiers.

Nitrite reductase. In the dissimilatory denitrification chain, Nitrite reductase (Nir) is a key enzyme that catalyzes the reduction of NO_2^- to NO. Cytochrome cd_1 is encoded by the *nirS* gene. Cytochrome cd_1 nitrite reductase are homodimetric protein that contain one c-type heme and one d1-type heme in every subunit.

The c-type heme act as an electron entry site, whereby it receives electrons from c-type cytochromes, before d1-type heme uses those electrons to reduce nitrite. Heme d_1 is the site of nitrite binding and reduction (Yamanaka and Okunuki 1963).

Nitric oxide reductase. One of the key enzymes in the denitrification pathway is Nitric oxide reductase (Nor), which is catalyzed by specific enzymes. Nor reduces nitric oxide radical (NO) to nitrous oxide (N_2O) in a two-electron/proton reaction. The nitric oxide reductase which are commonly found in denitrifying bacteria is a membrane-bound dimer of subunits encoded by the *norB* and *norC* genes (Hendriks et al. 1998). The NorC subunit responsible for the electron transfer from the physiological electron donor to the catalytic subunit, the NorB subunit (Hendriks et al. 1998).

Nitrous oxide reductase. The reduction of nitrous oxide is the final step in the denitrification pathway. It is catalyzed by the nitrous oxide reductase enzyme. The gene encoding nitrous oxide reductase (nosZ) is essentially unique to denitrifying bacteria and has commonly been used in environmental samples to detect denitrifier specific DNA (Zumft and Kroneck 2007).

The amino acid sequence comparison of NarGHI, NirBD, NapA, NirS, NorB, and NosZ is shown in Table 2.2. The amino acid identity of the enzymes responsible for denitrification and dissimilatory nitrate reduction in YB03 shared high similarity with *M. hydrocarbonoclasticus* VT8 (93.60-99.00%). However, the gene coding NapA (periplasmic nitrate reductase) was missing in the genome of *M. hydrocarbonoclasticus* VT8. Furthermore, the identity of NirD between YB03 and *M. hydrocarbonoclasticus* VT8 was relatively low (77.20%). In the genome of *Hahella chejuensis* KCTC 2396 and *P. aeruginosa* PAO1, the gene coding NirD was missing. Only NirB was found in all species.

Having a complete set of the genes will allow YB03 to produce energy needed for its metabolism by using electron donor available in the environment, thereby, limiting its

dependence to other organisms. We selected *Pseudomonas aeruginosa* PAO1 (NC_002516) and *Hahella chejuensis* KCTC 2396 (NC_007645) because those bacteria were likely undergoing similar nitrate metabolism according to the RAST server and Kyoto Encyclopedia of Genes and Genome (KEGG). Moreover, whole genome sequencing of *Marinobacter* sp. is limited in the database, whereby only two strains of *Marinobacter hydrocarbonoclasticus* were sequenced. The genes related to denitrification and dissimilatory nitrate reductions are identical between those two strains, therefore we only selected VT8 as a representative.

Table 2.2 Similarity of amino acid alignment of the enzymes responsible for denitrification and dissimilatory nitrate reduction. ND: not detected.

[Unit: %]

Bacteria	Denitrification				Dissimilatory nitrate reduction				
	NapA	NirS	NorB	NosZ	NarG	NarH	NarI	NirB	NirD
M. hydrocarbonoclasticus VT8	ND	93.60	97.50	94.80	98.90	99.00	93.80	98.00	77.20
Hahella chejuensis KCTC 2396	84.10	81.50	87.70	81.50	84.00	85.70	68.70	49.10	ND
P. aeruginosa PAO1	83.40	82.50	85.40	84.10	83.50	88.40	70.70	52.20	ND

Pathways of ammonia assimilation

There are two pathways for ammonia assimilation: the GDH (glutamate dehydrogenase) and GS-GOGAT (glutamine synthetase, GS; glutamate synthase or glutamine 2-oxoglutarate aminotransferase, GOGAT) pathways. The GDH pathway contains a single enzyme, called GDH. The cyclic GS-GOGAT pathway consists of two enzymes, called GS and GOGAT.

The ammonia ion, NH_4^+ will be integrated through glutamine synthase after uptake by bacterial cell. It could be integrated via glutamine 2-oxoglutarate aminotransferase or

glutamate dehydrogenase. These enzymes integrate NH_4^+ into glutamine and glutamate which are amino donor in the generation of other amino acids.

Pathways of sulfate assimilation

For sulfur metabolism of YB03, the gene set encoding for the assimilatory pathway of sulfate reduction was identified in the genome of YB03. The assimilatory sulfate reduction, APS (adenosine 5'-phosphosulfate) is turned into PAPS (3'-phoshoadenosine-5'-phosphosulfate) by the adenylyl sulfate kinase (CysC). Furthermore, PAPS is reduced to sulfite by PAPS reductase (CysH), and sulfite is gradually reduced to sulfide by an assimilatory sulfite reductase (CysI) (Grein et al. 2013; Tsang 1981).

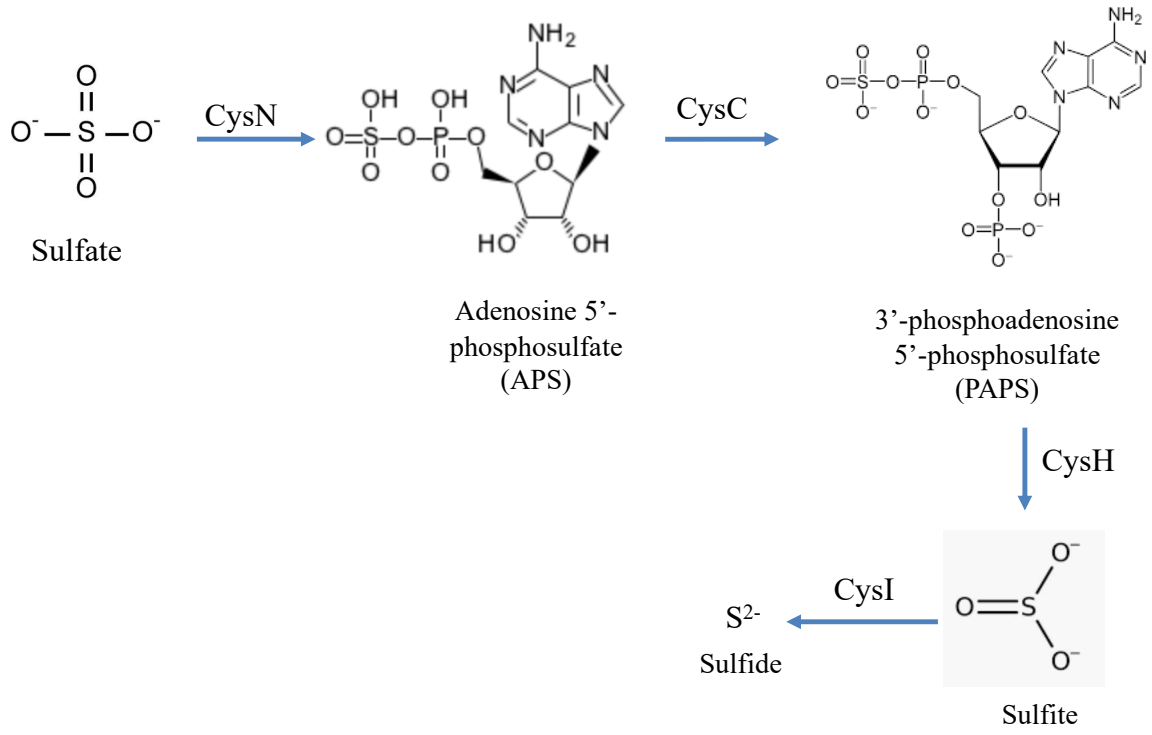

Figure 2.4. The assimilatory pathway of sulfate reduction. The chemical name is indicated below the structure. The enzyme name is indicated above the arrow.

The amino acid sequence comparison of gene coding for assimilatory sulfate reduction is shown in Table 2.3. The set of the genes for assimilatory sulfate reduction was found in all species. YB03 shared high similarity with *M. hydrocarbonoclasticus* VT8 (95.90-99.30%) for the amino acid identity of the enzyme responsible for assimilatory sulfate reduction.

Table 2.3 Similarity of amino acid alignment of the enzymes responsible for assimilatory sulfate reduction.

[Unit: %]

Bacteria	CysN	CysD	CysH	CysI
M. hydrocarbonoclasticus VT8	96.70	99.30	95.90	97.10
Hahella chejuensis KCTC 2396	76.70	94.40	82.10	85.30
P. aeruginosa PAO1	65.80	87.00	73.60	82.90

Chapter 3. Characterization of nitrate reducing bacteria, *Marinobacter* YB03

3.1 Introduction

The genus *Marinobacter,* member of the family *Alteromonadaceae*, in *Proteobacteria*, was first reported by Gauthier et al. (1992). The representative species is *Marinobacter hydrocarbonoclasticus*, which comprises of Gram negative, aerobic, flagellated, halophilic, hydrocarbonic and rod-shaped bacterium (Gauthier et al. 1992). In their metabolism, *Marinobacter* strains use oxygen or nitrate for reducing specific amino acids and organic acids into energy and carbon. Many *Marinobacter* strains, including only species members called *M. hydrocarbonoclasticus* and *M. aquaeolei*. They were isolated from environments contaminated with petroleum hydrocarbon. Branched and unbranched alkanes, cycloalkanes, and monoaromatic and polyaromatic hydrocarbons are the primary constituents of petroleum hydrocarbon (Alain et al. 2012). The strain *M. hydrocarbonoclasticus* ATCC 49280T, was found in the Mediterranean Sea from hydrocarbon contaminated sediments based on the ability of this bacterium to metabolize crude oil as a carbon source. Several crude oil aliphatic components including hexadecane and the aromatic compound phenanhrene have been shown to be degraded (Gauthier et al. 1992). *M. hydrocarbonoclasticus* can use either aromatic hydrocarbons or aliphatic as sole carbon sources. In freshwater environments, degradation of aromatic hydrocarbons and alkanes under anaerobic condition by utilizing nitrate as terminal electron acceptor, was previously noted and extensively studied. Almost all the nitrate-reducing strains isolated are so far originated from terrestrial and freshwater environments. They were classified to the class *Betaproteobacteria*, and more specifically to the genera *Thauera, Azoarcus* and *Georgfuchsia* (Dolfing et al. 1990; Fries et al. 1994; Hess et al. 1997; Rabus and Widdel, 1995; Ehrenreich et al. 2000). Two of the few exceptions until now are the hydrocarbon-degrading denitrifiers, which belongs to the class *Gammaproteobacteria.* They

have been isolated from river sediment (genus *Dechloromonas*) (Chakraborty et al. 2005) and ditch sediment (strain HdN1) (Ehrenreich et al. 2000).

Aliphatic and aromatic hydrocarbons have also been confirmed to degrade by *M. aquaeolei*, which isolated from an offshore oil well in Vietnam. However, the specific compounds it degraded were not identified (Huu et al. 1999). The distribution of aromatic hydrocarbon degradation ability within the *Marinobacter* genus is uncertain. Furthermore, the degradation ability of molecules of polycyclic aromatic hydrocarbon (PAH) has not been examined for *Marinobacter* strains.

Similarly, aromatic degradation mechanisms by *Marinobacter* strains were never explored. In this study, the hydrocarbon preferences of isolate *Marinobacter* strain is demonstrated.

3.2 Materials and methods

3.2.1 Substrate range of YB03

Freshwater medium was used in this study. The substrate sources were varied as follows: crude oil 10%, BTEX (Benzene, Toluene, Ethylbenzene, o-,m-,p-xylene), Alkane (C_6-C_{10}), and Alkane (C_{11}-C_{16}). The mixture of BTEX, Alkane (C_6-C_{10}) and Alkane (C_{11}-C_{16}) were prepared from 2% stock solution. All substrates were diluted in the HMN. 5 mL of YB03 culture was transferred into 50 mL of freshwater medium. Nitrogen gas was filled into the headspace. The freshwater medium was sterilized by autoclaving. Three replicates were done for each condition. All vials were incubated horizontally in the dark at 30^{o}C and on a shaking incubator (100 rpm).

The water phase was sampled weekly. Next, the taken 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 bead-beating method was used for Genomic DNA extraction,

followed by phenol-chloroform extraction (Tanji et al. 2014). The bead-beating procedure was performed using FastPrep-24 instrument (MP biomedical LLC, Santa Ana, CA) at 6.0 ms^{-1} for 40 s. The quality and concentration of DNA was measured by using NanoDrop 2000 spectrophotometer (Thermo Fischer Scientific, Wilmington, DE). An oil-phase sample was taken every two weeks for sample analysis

3.2.2 Chemical analysis

An ion chromatography (with TSK gel super anion AZ column; TOSOH Corporation, Tokyo, Japan) was used to measure the concentration of nitrate and nitrite with the setting of flow rate at 0.80 mL/min. Volatile fatty acid concentration was measured using a High-Performance Liquid Chromatography system (SCR 102H column; CSS-10A detector; Shimadzu, Tokyo, Japan). The oil-phase samples were analyzed using a gas chromatography system (GC2014; Shimadzu) equipped with a flame ionization detector (325^{o}C), using helium and hydrogen as the carrier gas. The column used was HP-PONA (Agilent Technologies, Santa Clara, CA), 50 mm × 0.20 mm (0.50 μm film thickness). GC conditions have been previously described (Tanji et al. 2014). Pristine was used as an internal standard. I measured the relative abundance of each hydrocarbon as follows (Kamarisima et al. 2018):

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

note:

A : Peak area of a 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

3.2.3 Bacterial analysis

A quantitative real time PCR (qPCR) was carried out to assess the presence and the relative quantity of microbial DNA. 16S rRNA gene copy number was used for estimating the total number of bacteria (Nadkarni et al. 2002). Universal bacterial primers (341F and 519R) was used to amplify a region of 206 bp as described previously (Bru et al. 2008; Tanji et al. 2014). Primer sequences were 341F (5'-CCTACGGGAGGCAGCAG-3') and 1492R (5'-ATTACCGCGGCTGCTGG - 3'). Each PCR reaction was performed in a 20 µL final volume containing 10 µL Thunderbird™ SYBR® qPCR mix (TOYOBO Co., Ltd., Osaka, Japan), 0.40 µL of 10 µM forward and reverse primers, 0.40 µL of 50×ROX reference dye, 6.80 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). The reaction condition was set as follow: initial denaturation at 95ºC for 20 s; 40 cycles (95ºC for 30 s for denaturation and annealing temperature of 60ºC for 30 s) and one additional melt-cure curve (95ºC for 15 s, 60ºC for 60 s, and 95ºC for 15 s).

3.3 Results and discussion

3.3.1 Cell growth and nitrate reduction by YB03

The total number of bacteria was observed by quantification of 16S rRNA gene as shown in Fig.3.1. The abundance of 16S rRNA gene in the condition supplemented with crude oil and BTEX increased up to two logs from the initial abundance. Meanwhile, in the condition supplemented with alkane mixture, the abundance of 16S rRNA gene only increased by one log.

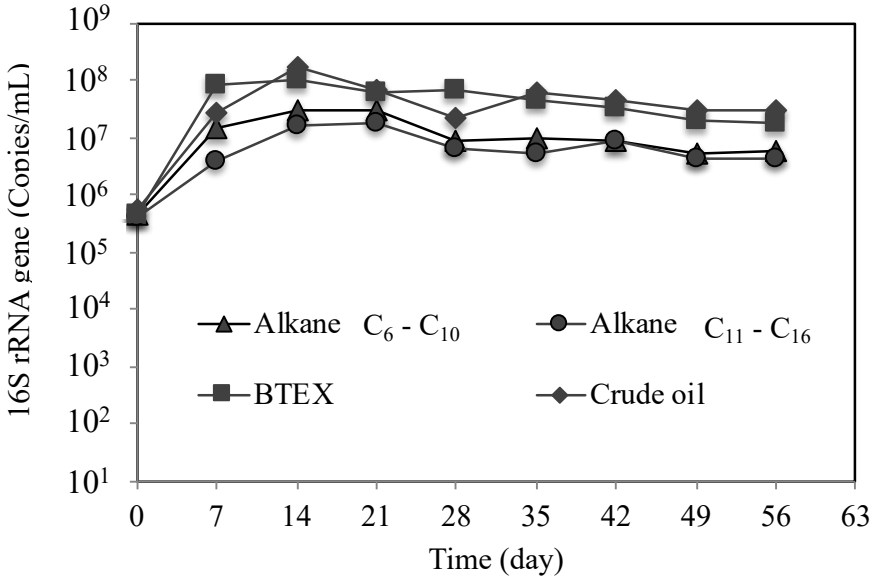

Figure 3.1 The abundance of 16S rRNA gene represent total number of bacteria in the condition supplemented with alkane mixture, BTEX and crude oil

The nitrate reduction and nitrite production concentration in the condition supplemented with alkane mixture, BTEX and crude oil as substrate were shown in Fig 3.2. In the condition with BTEX mixture and crude oil, the productions of nitrite were observed at 1.05 mM and 0.94 mM, while nitrate were reduced by 17.10 mM and 16.10 mM, respectively. In the condition supplemented with alkane (C_6-C_{10}) and alkane (C_{11}-C_{16}), the productions of nitrite were 0.48 mM and 0.39 mM, while reductions of nitrate were 20.59 mM and 21.67 mM, respectively. The nitrate reduction and nitrite production were not equal, because nitrite was the intermediate product, can be in turn being reduced to nitrogen gas and ammonia. Thus, among petroleum hydrocarbon, YB03 preferred to utilize aromatic hydrocarbon as a substrate for nitrate respiration better than saturated hydrocarbon.

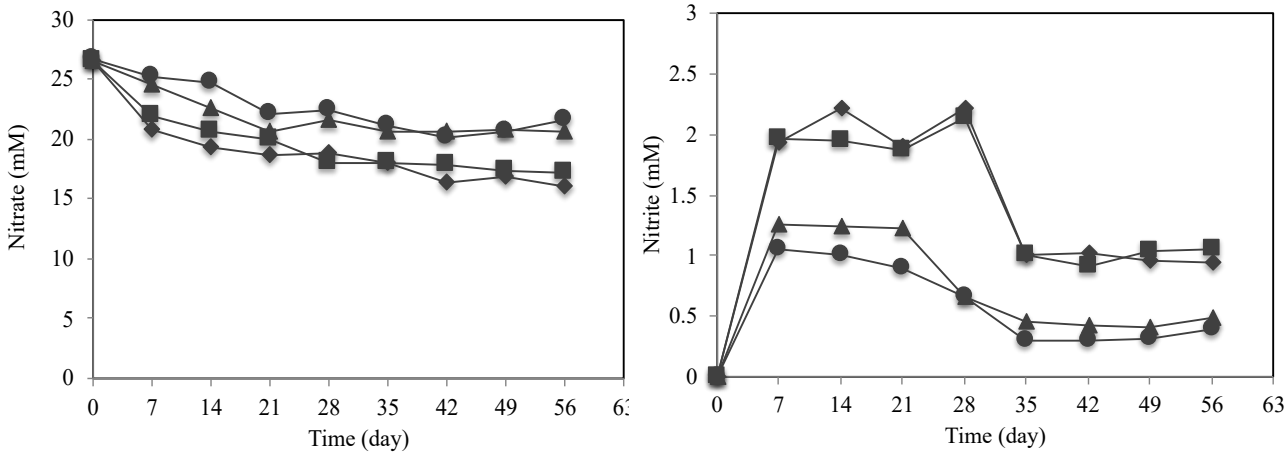

Figure 3.2 Nitrate and nitrite concentration in the condition supplemented with alkane mixture, BTEX and crude oil as substrate. (▲) Alkane C_6-C_{10}; (●) Alkane C_{11}-C_{16}; (■) BTEX; (◆) Crude oil.

3.3.2 Hydrocarbon consumption and production of volatile fatty acid by YB03

The substrate range by utilization of BTEX, alkane (C_6-C_{10}) and alkane (C_{11}-C_{16}) is shown in Fig. 3.3 (A-C). The hydrocarbon profile in the crude oil was shown in Fig 3.4. The hydrocarbon profile in the BTEX is shown in Fig. 3.3 (A). Only toluene and m,p-xylene were consumed efficiently in the aromatic fraction based on GC analysis. The first fraction consumed by YB03 was toluene, followed by m,p-xylene. The toluene and m,p-xylene continuously decreased and were finished by day 35 and 42, respectively. The consumption of hydrocarbon was observed in the BTEX condition was the same in crude oil condition (Fig.3.4). Other than toluene and m,p-xylene, there were no other fractions entirely consumed by YB03 during the study. The alkane fraction was the least favorable substrate for YB03, and longer chain alkane became the most unfavorable. Toluene showed the highest rate of degradation. Besides, it was found to be the most favorable BTEX hydrocarbon for anaerobic degradation by the enriched culture, which was consistent with other report comparing the biodegradation of various BTEX compounds (Lovley 1997; Heider et al. 1998; Dou et al. 2008).

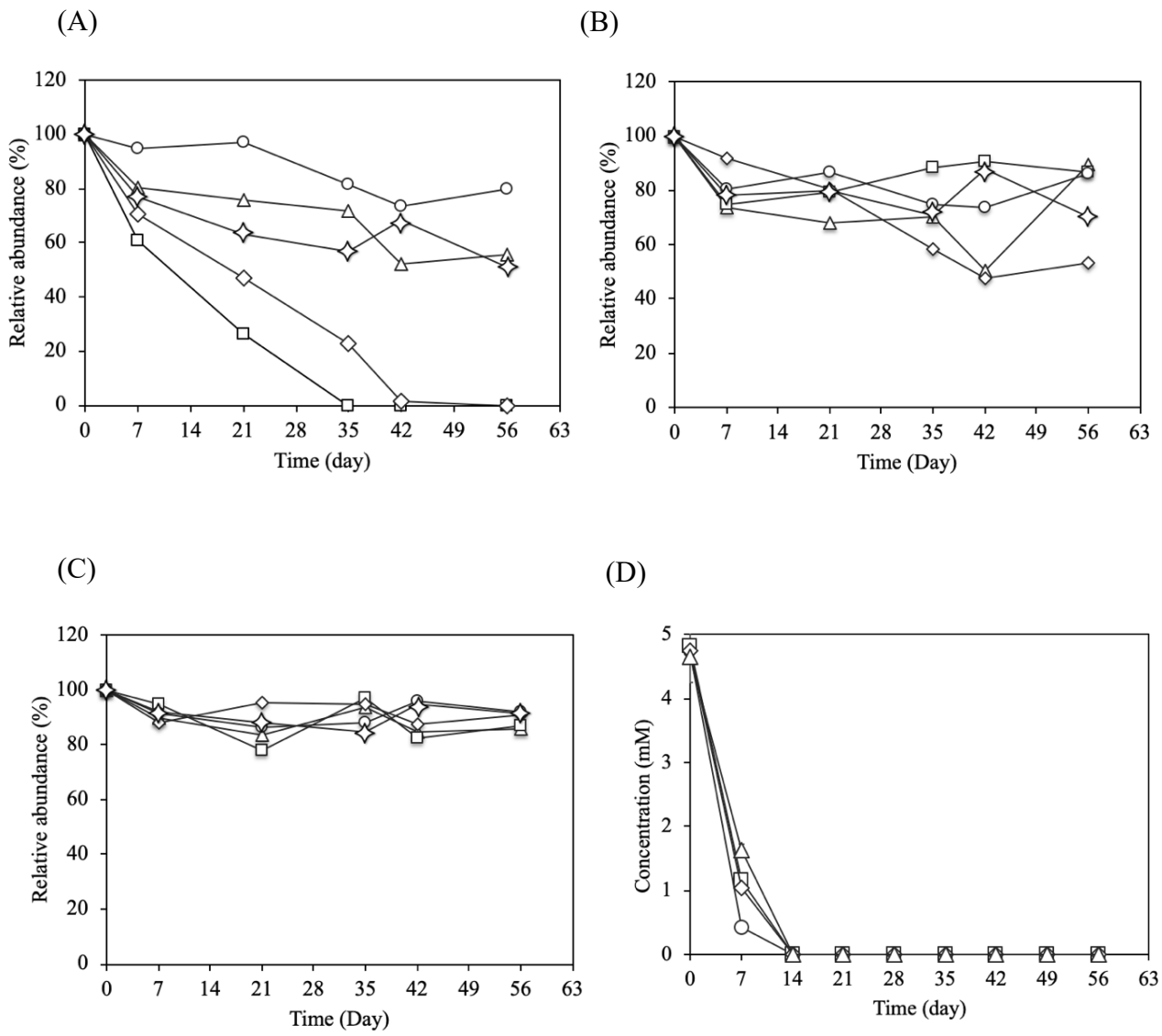

Figure 3.3 Consumption of various substrates of (A) BTEX: benzene (△), toluene (☐), ethylbenzene (○), m,p-xylene (◇), o-xylene (✧). (B) Alkane C_6 – C_{10}: C_6 (△), C_7 (☐), C_8 (○), C_9 (◇), C_{10} (✧). (C) Alkane C_{11} – C_{16}: C_{11} (△), C_{13} (☐), C_{14} (○), C_{15} (◇), C_{16} (✧). (D) volatile fatty acids: acetate (○), foramte (☐), lactate (◇), and propionate (△)

Therefore, the hydrocarbon preferences as substrate for YB03 were toluene and m,p-xylene overlaid by BTEX mixture and crude oil. The consumption of VFAs is shown in Fig. 3.3 (D). The consumption of VFAs was observed when VFA mixtures were used as a substrate.

Among the four types of VFAs, YB03 was observed to consumed acetate better. The 5 mM of VFAs was entirely consumed by day 14.

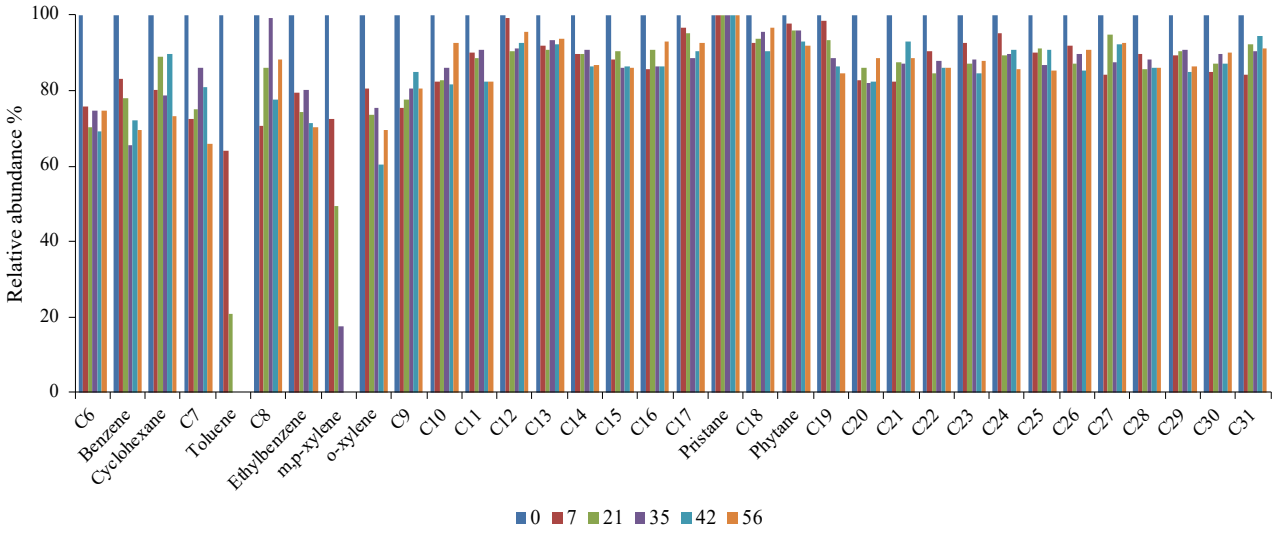

Figure 3.4 Crude oil hydrocarbon fraction consumption for 56 days. Pristine was used as an internal standard.

Some studies (Rabus and Widdel 1996; Rabus et al. 1999) demonstrated that strains of denitrifying bacteria which had been originally enriched and isolated by using the defined substrates made up of toluene, ethylbenzene, and m-xylene were able to grow on crude oil too by through the biodegradation of alkylbenzenes. Early reports of anaerobic xylene degradation under nitrate reducing conditions indicated that both m- and p- (but not o-) xylene were degraded (Kuhn et al. 1985). Some of the denitrifiers enriched and isolated with toluene (*Azoarcus sp.* strain T and *Azoarcus tolulyticus* Td15) were shown to grow also with m-xylene (Dolfing et al. 1990; Fries et al. 1994). Other denitrifying strains (mXyN1, M3) were directly isolated from enrichment cultures with m-xylene (Rabus and Widdel 1995; Hess et al. 1997). In some cases, the presence of m-xylene inhibited concomitant o- and p-xylene degradation (Mecken-stock et al. 2004; Morasch et al. 2004; Jahn et al. 2005). Although p-xylene is reported to exist for an extended period under anoxic conditions (Rabus and Widdel 1995), it

was observed to be degraded by enrichment cultures under denitrifying condition (Haner et al. 1995; Rotaru et al. 2010). However, among all the BTEX, benzene was difficult to be biodegraded, for example, some studies demonstrated that benzene was recalcitrant during the reduction of nitrate (Barbaro et al. 1992; Kao and Borden 1997).

The production of VFAs in the crude oil, BTEX, alkane (C_6-C_{10}) and alkane (C_{11}-C_{16}) was shown in Fig 3.5. Among all types of VFAs, only acetate was detected. Crude oil and BTEX conditions contribute to the highest production of acetate among all conditions. The production was detected under 0.07 mM. Acetate was produced until day 28 in BTEX and crude oil conditions, and further oxidized after that. Production of other VFAs was not detected in all conditions. Based on the possible pathway of crude oil component degradation under anaerobic condition proposed by Hasegawa et al. (2014), the pathway was divided into two steps. First, the reduction of crude oil component to VFAs as intermediate products. Second is further oxidation of VFA to carbon dioxide coupled with nitrate reduction.

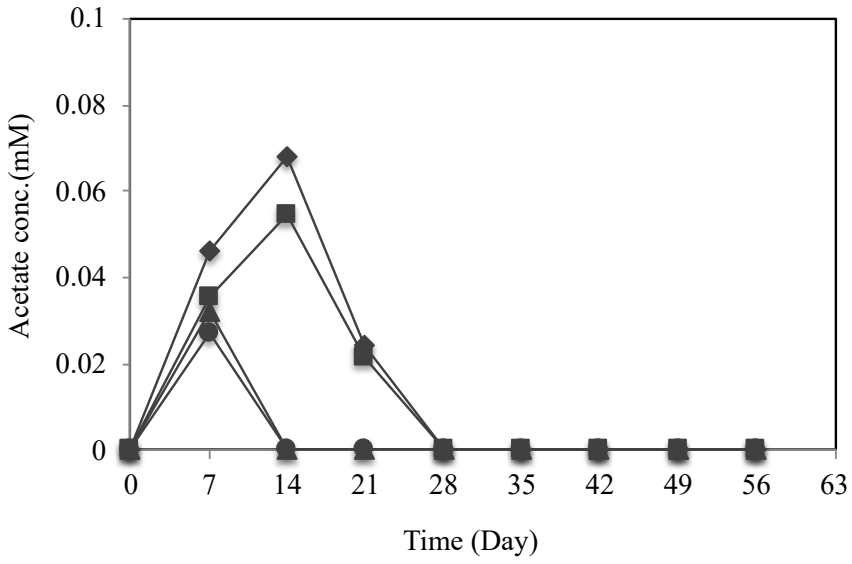

Figure 3.5 The production of acetate in the condition supplemented with crude oil, BTEX, alkane (C_6-C_{10}) and alkane (C_{11}-C_{16}) as substrate. (▲) Alkane C_6-C_{10}; (●) Alkane C_{11}-C_{16}; (■) BTEX; (◆) Crude oil.

3.4 Summary

The isolated strain YB03 was phylogenetically close to genus *Marinobacter*. The findings served insight information about the possibility of NRB that could also act as SRB with *Marinobacter* genus. The carbon sources and electron donor preferences of YB03 were toluene, m-,p-xylene and VFAs. The YB03 was able to utilize toluene and m-,p-xylene with minor production of acetate as an intermediate product. Moreover, the most favorable for VFA utilization as substrate by YB03 was acetate.

Chapter 4. Contribution of YB03 to biological souring

4.1 Introduction

Souring, unwanted production of hydrogen sulfide (H_2S) output by SRB in oil reservoirs, is a common concern during secondary oil recovery when water is injected to process the remaining oil. SRB converts sulfate to sulfide in the injection water and oxidizes organic electron donors present in the oil reservoir. Sulfide removal is required in terms of health and safety issues and to reduce the risk of corrosion of the pipeline and other side effects (Hubert and Voordouw 2007). Nitrate application may be very efficient in controlling reservoir souring by facilitating NRB growth, consuming nutrients that SRB need to grow, and thus inhibiting SRB activity (Gieg et al. 2011). Nitrate reduction may not only inhibit the production of sulfide, but NRB activates also eliminate existing sulfide from OFW. For example, nitrate can also be used as an electron acceptor for the re-oxidation of sulfide to sulfate or elemental sulfur by nitrate-reducing, sulfide-oxidizing bacteria. The latter reaction is summarized by:

$$5HS^- + 2NO_3^- + 7H^+ \longrightarrow 5S^o + N_2 + 6H_2O \qquad (1)$$

Furthermore, nitrite formed by NRB can also react with dissolved sulfide to the following formula to obtain elemental sulfur (Jenneman et al. 1996):

$$3HS^- + 2NO_2^- + 5H^+ \longrightarrow 3S^o + N_2 + 4H_2O \qquad (2)$$

Nevertheless, there also have been cases when addition of nitrate did not hinder the formation of sulfide (Kjellerup et al. 2005). Although nitrate-mediated souring control has been studied in the laboratory and in the field, much is still unknow about the details of microbial mechanisms involved in NRB-SRB interactions during nitrate addition for reservoir souring mitigation.

In chapter 2, one isolate (*Marinobacter* genus) named YB03, was identified as NRB

with sulfate reducing ability. Based on the whole genome analysis, the YB03 has the gene sets encoding for assimilatory sulfate reduction. Therefore, the purpose of this chapter is to investigate the souring activity by YB03.

4.2 Materials and methods

4.2.1 Souring experiment

Natural seawater harvested from Ogasawara (Tokyo, Japan) was filter-sterilized (0.22 μm, MillexGV, Millipore) and used as medium in this study. The physicochemical characteristics of natural seawater have been described previously (Kamarisima et al. 2018). 50 mL of seawater was covered with 5 mL of 2% toluene diluted in the HMN. The medium was made in 70 mL glass vials, closed with butyl rubber caps, and autoclaved. The N_2 gas was loaded into the headspace. 5 ml of enrichment culture were used for the bacterial inoculum. In this study, six conditions were performed: YB01 (without nitrate), YB01 + NO_3^- (with nitrate), YB03 (without nitrate), YB03 + NO_3^- (with nitrate), YB01 + YB03 (co-culture without nitrate), and YB01 + YB03 + NO_3^- (co-culture with nitrate). All conditions were performed in triplicate. A solution of 1M sodium nitrate was applied to get a final concentration of 27 mM; this amount was chosen because it is the same as the level of sulfate in seawater. All vials were horizontally incubated at 30°C, in the dark, and on a shaking incubator (100 rpm).

A 600 μL water phase sample was taken every week. The sample was centrifuged for 10 min at 6,500 × *g*; the supernatant was carefully transfer to new Eppendorf Tube® and was used for chemical analysis. The pellet was used for DNA extraction. The bead-beating method was used for Genomic DNA extraction, followed by phenol-chloroform extraction (Tanji et al. 2014). The bead-beating procedure was performed on a FastPrep-24 instrument (MP biomedical LLC, Santa Ana, CA) at 6.0 ms^{-1} for 40 s. The quality and concentration of DNA was measured using A NanoDrop 2000 spectrophotometer (Thermo Fischer Scientific, Wilmington, DE). An oil-phase sample was taken every two weeks for the crude oil analysis.

4.2.2 Chemical analysis

The concentration of sulfide was determined with a kit based on the methylene blue method (NANOCOLOR standard experiment sulfide; Macherey-Nagel Corp; Germany). Nitrate and nitrite concentration were measured using ion chromatography (with TSK gel super anion AZ column; TOSOH Corporation, Tokyo, Japan) with a flow rate of 0.80 mL/min. Volatile fatty acid concentration was measured using a High-Performance Liquid Chromatography system (SCR 102H column; CSS-10A detector; Shimadzu, Tokyo, Japan).

4.2.3 Bacterial analysis

Bacterial analysis was performed as described in section 3.2.3.

4.3 Results and discussion

4.3.1. The effect of nitrate addition to control souring

The souring activity of YB01, YB03 and the effect of nitrate addition were overserved by monitoring the changes in sulfate, sulfide, nitrate, and nitrite concentration for 63 days under the six conditions as shown in the Fig.4.1. Under the YB01 condition, an increase in sulfide (Fig. 4.1B) and a decrease in sulfate (Fig. 4.1A) were observed. After 63 days of incubation, sulfate decreased by 8.44 mM, and sulfide increased by 2.99 mM. The YB01 has the *dsrAB* gene coding for sulfite reductase as shown in Fig. 4.2. Compared with the (YB01+NO_3^-) condition, no significant decrease in sulfate and sulfide production was observed. The addition of nitrate in the YB01 condition was able to repress biological souring caused by YB01, as the decreasing sulfate in the YB01 and (YB01+NO_3^-) condition was significantly different ($P<0.05$). Moreover, the production of sulfide and reduction of sulfate were confirmed by YB01. This bacterium also has *narG* gene encoding for enzyme involves in reduction of nitrate to nitrite and further to ammonia (*nrfAH*/*nirB*). Nitrate reduction is thermodynamically more favorable than sulfate reduction (Gittel et al. 2009). Thus, in the presence of nitrate, YB01 preferentially used nitrate as electron acceptor and oxidized sulfide further to sulfate. There

was no production of sulfide, and the reduction of nitrate was observed. This phenomenon was confirmed by dissimilatory sulfate reduction pathway (Fig. 4.3). Thus, the addition of nitrate to YB01 culture completely masked the biological souring. After the 63-day incubation, concentration of nitrate decreased up to 6.18 mM (Fig. 4.1C) was observed and no production of nitrite could be detected.

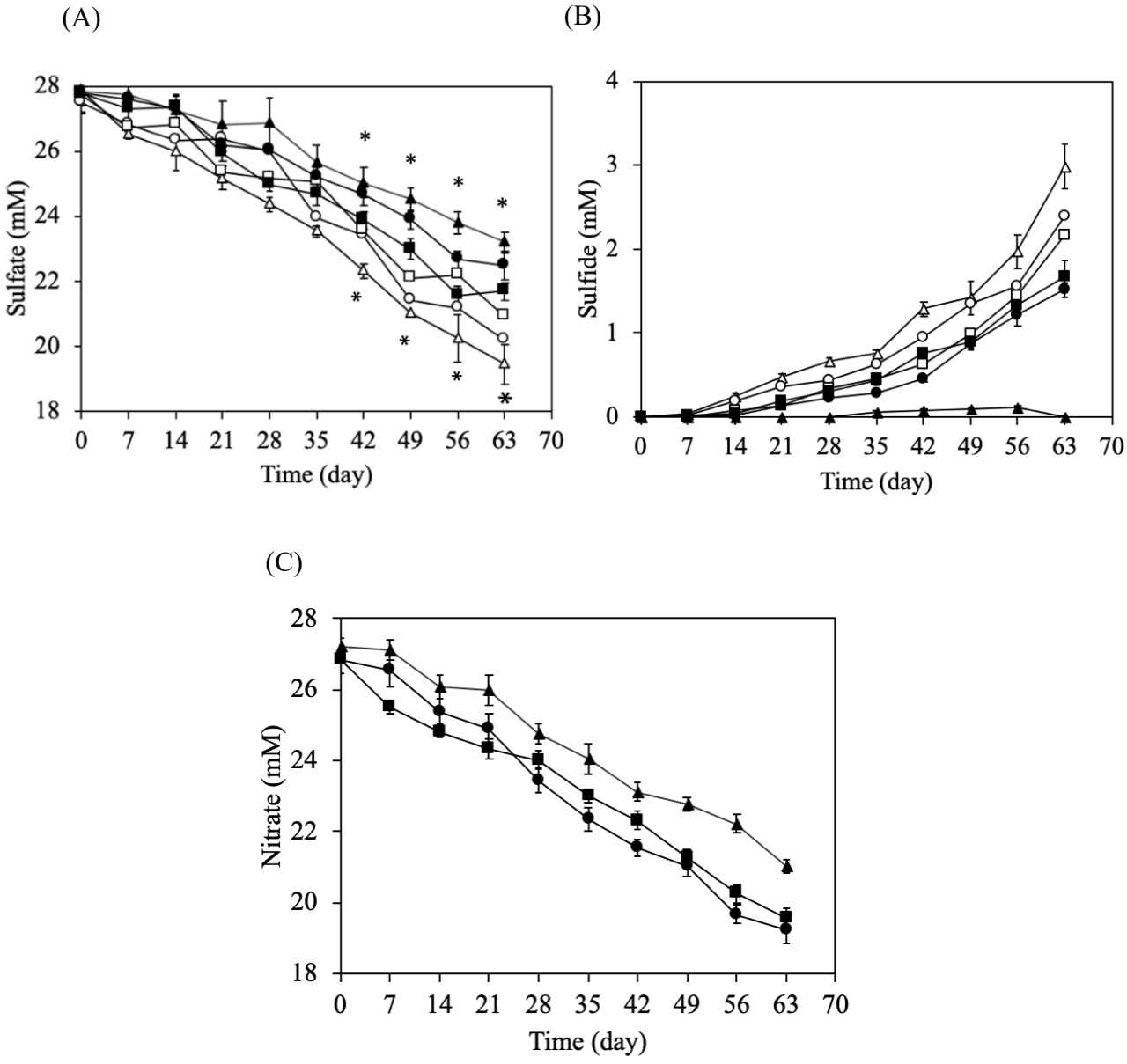

Figure 4.1 Chemical analysis of (A) sulfate reduction (B) sulfide production (C) nitrate reduction for 63-day under different conditions: YB01 (Δ), YB03 (○), YB01 + YB03 (□), YB01 + NO_3^- (▲), YB03 + NO_3^- (●), and YB01 + YB03 +NO_3^- (■).

In the YB03 with nitrate addition, 1.52 mM of sulfide was produced (Fig. 4.1B), while sulfate was reduced by 5.36 mM (Fig. 4.1A). Thus, the addition of nitrate to an YB03 culture could not control biological souring. This was confirmed by continuous sulfide production and sulfate reduction. YB03 has the gene set encoding for the assimilatory pathway of sulfate reduction (Fig. 4.2). A decreased concentration of nitrate to 7.60 mM (Fig. 4.1C) was observed. Besides, no production of nitrite could be detected. Based on the data of whole genome sequencing, this genus has the complete set of genes responsible for denitrification and dissimilatory nitrate reduction, therefore nitrite can be further reduced to ammonia and nitrogen gas. Thus, nitrite was not detected.

YB03 was identified to belong to the genus *Marinobacte*r. This bacterium has *napA*/*narG* genes encoding enzyme for nitrate reduction to nitrite. YB03 also has the gene set encoding for denitrification. So, nitrate reduction pathways of YB01 and YB03 are different (Fig. 4.3).

Addition of nitrate to co-culture of (YB01+YB03) also led to a decrease in the concentration of sulfide but at a rate lower than that observed after simultaneous addition of YB03 and nitrate. The production of sulfide was observed by 1.67 mM (Fig. 4.1B), while sulfate was reduced by 6.14 mM (Fig. 4.1A). Thus, adding only nitrate to the YB01 culture resulted in oxidation of sulfide. Oxidation of sulfide under the presence of nitrate could lead to the formation of sulfur or sulfate based on the following reaction (Tang et al. 2009; Carsoso et al. 2006):

$$1.6\ NO_3^- + S^{2-} + 1.6\ H^+ \longrightarrow SO_4^{2-} + 0.8\ N_2 + 0.8\ H_2O \qquad (1)$$

$$0.4\ NO_3^- + S^{2-} + 2.4\ H^+ \longrightarrow S^o + 0.2\ N_2 + 1.2\ H_2O \qquad (2)$$

Based on the equation (1) and (2), sulfide could be completely oxidized into sulfate or sulfur.

A decrease in nitrate concentration up to 7.25 mM (Fig. 4.1C) was observed and also no production of nitrite could be detected. The difference in nitrate reduction and nitrite production for all conditions was possibly because nitrite (an intermediate product) can be

further reduced to ammonia or nitrogen gas. This was confirmed by nitrate reduction pathway, as shown in Fig. 4.3.

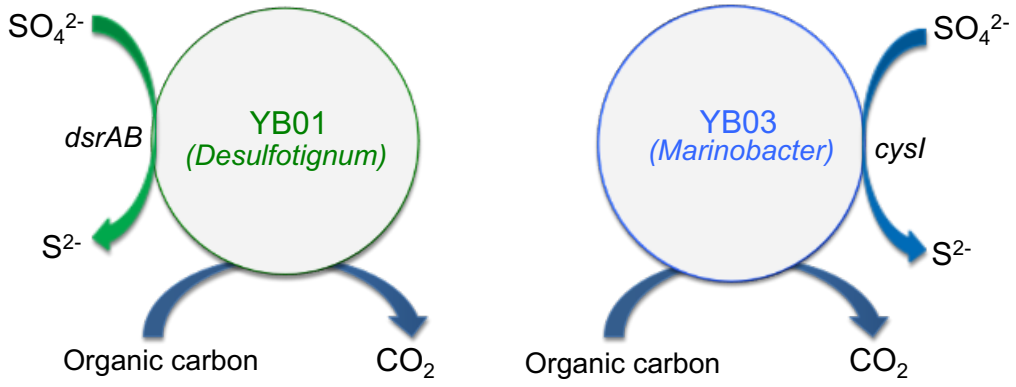

Figure 4.2 Sulfur metabolism pathway of *Desulfotignum* YB01 and *Marinobacter* YB03 without nitrate addition.

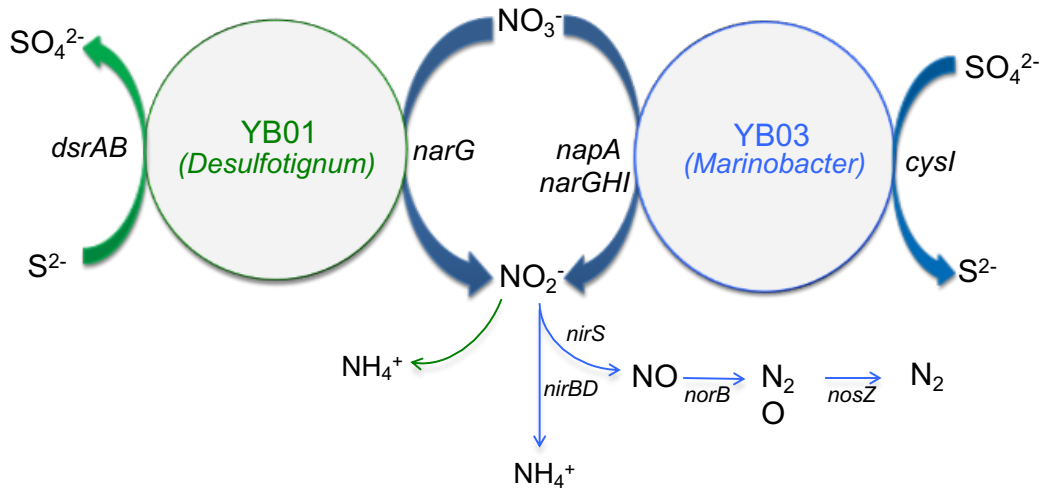

Figure 4.3 Nitrogen and sulfur metabolism pathway of *Marinobacter* YB03 and possible interaction between YB01 and YB03 with nitrate addition.

Moreover, the quantification of the 16S rRNA gene (Fig.4.4) under all conditions showed that the number of total bacteria dropped over time. Quantification of the 16S rRNA

gene in YB01 condition increased up to 10^8 copies/mL in the first three weeks. And then, it remained stable around 10^7 copies/mL. By contrast, the abundance of 16S rRNA gene in (YB01+NO_3^-) condition was increased up to 10^6 copies/mL in the first two weeks, and decreased by one log on the following day, indicate that the growth of YB01 was suppressed in this condition by adding nitrate. While the abundance of 16S rRNA gene in (YB03+NO_3^-) condition increased around 10^7 copies/mL, two logs higher than the initial condition (t=0). Moreover, the increased of 16S rRNA gene of (YB01+YB03+NO_3^-) condition was also observed until day 28 (10^7 copies/mL) and decreased by one log on the next day. Therefore, the addition of nitrate in YB01 condition was observed to reduce the growth of YB01. While the abundance of 16S rRNA gene of YB03 was reflecting the persistent of YB03 in the presence of nitrate concentration.

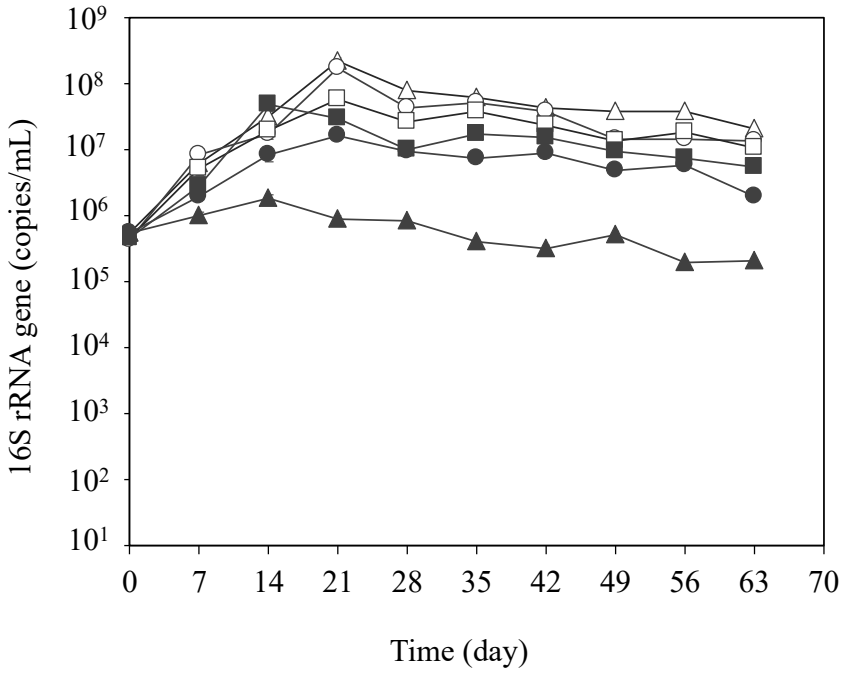

Figure 4.4 The abundance of 16S rRNA gene for 63 days under different conditions: YB01 (Δ), YB03 (○), YB01 + YB03 (□), YB01 + NO_3^- (▲), YB03 + NO_3^- (●), and YB01 + YB03 +NO_3^- (■).

Toluene was used in this study as a carbon source and electron donor. Toluene consumption by YB01 and YB03 under artificial souring experiment with and without nitrate addition was shown in the Fig.4.4. The consumption rate of toluene in all condition was different. In the condition without nitrate addition, YB01 was able to consume toluene better than YB03. By contrast, toluene consumption of YB01 in the condition with nitrate was slower than under the other conditions. In comparison with other conditions (YB01 and YB03 as independent culture), the consumption rate of toluene in the co-culture of YB01 and YB03 condition was reduced.

YB01 and YB03 reduced nitrate and sulfate, respectively, according to the following equations (Coombe et al. 2010; Sunde and Torsvik, 2005):

$$0.14\ C_7H_8 + NO_3^- + 0.03\ H^+ \longrightarrow 0.97\ HCO_3^- + 0.50\ N_2 + 0.08\ H_2O \quad (3)$$

$$0.22\ C_7H_8 + SO_4^{2-} + 0.67\ H^+ \longrightarrow 1.56\ HCO_3^- + HS^- + 2.5\ H^+ \quad (4)$$

Based on equation (3) and (4), bacteria will oxidize toluene faster when coupled with sulfate reduction compare to nitrate, which explains the phenomena in Fig.4.5.

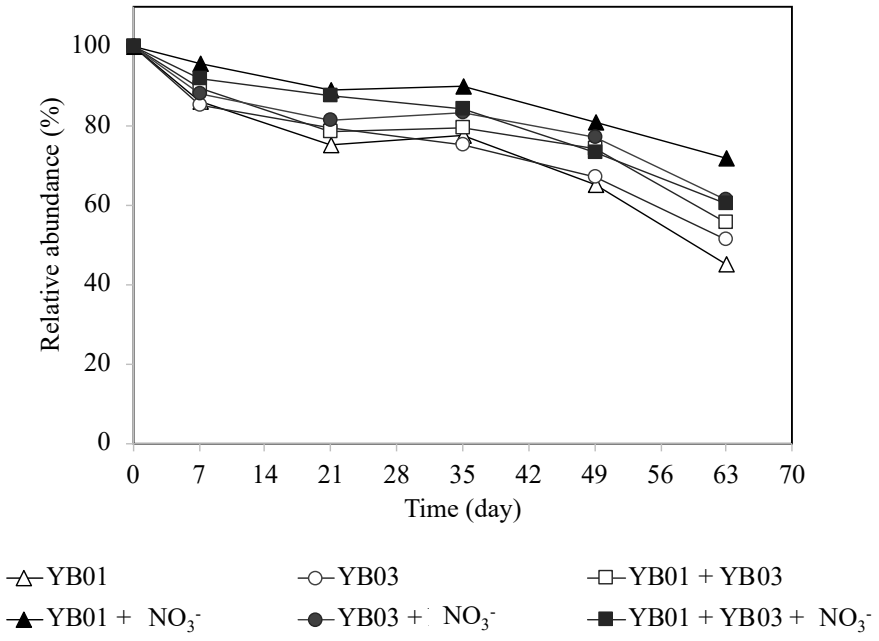

Figure 4.5 Toluene consumption for 63 days under different conditions: YB01 (Δ), YB03 (○), YB01 + YB03 (□), YB01 + NO_3^- (▲), YB03 + NO_3^- (●), and YB01 + YB03 +NO_3^- (■).

4.4 Summary

In summary, the addition of nitrate was able to repress biological souring caused by YB01. However, the production of sulfide was observed, while sulfate was reduced in the YB03 culture with nitrate addition. Thus, the addition of nitrate to YB03 culture could not control biological souring. This was confirmed by continuous sulfide production and sulfate reduction. For co-culture of (YB01+YB03) regardless of nitrate addition, sulfate reduction and sulfide production were also observed. Therefore, YB03 could not control souring caused by nitrate- and sulfate-reducing bacteria, YB01.

Chapter 5. Microbiologically influenced corrosion (MIC) of carbon steel by YB03

5.1 Introduction

Corrosion is the deterioration of materials as a result of chemical, physical and biological process. Corrosion influenced or driven by presence or activities of microorganisms is defined as biocorrosion or microbiologically influenced corrosion (MIC) (Little et al. 2000). In the oil and gas industries, water utilities and many other industries, MIC has become a serious problem. For several pipeline problems, MIC was believed to be the main factor. They include the failure and fire of the gas pipeline near Carlsbad, New Mexico on August 19, 2000 (NTSB Report, 2000). In recent years, the recognition of MIC is expanding dramatically. However, MIC mechanisms are still lacking in knowledge. The most frequently reported microbes in MIC are SRB. Biocatalytic cathodic sulfate reduction (BCSR) theory illustrates the MIC mechanism caused by SRB from a bioenergetics perspective. BCSR can be adapted to explain MIC caused by other types of microbes such as NRB in the form of biocatalytic cathodic nitrate reduction (BCNR). Nitrate injection into oil reservoir to increase NRB growth that can compete with SRB growth to suppress biogenic H_2S production which causes pipeline corrosion and reservoir souring. NRB can also cause severe corrosion on carbon steel. It was reported a nitrate reducing *B. licheniformis* biofilm was more corrosive than a sulfate reducing *D. vulgaris* biofilm on C1018 carbon steel (Xu et al. 2013). *Pseudomonas* species are facultative bacteria and can use nitrate as electron acceptor for anaerobic respiration. It is important to show that NRB biofilm will switch from organic carbon to Fe^0 as electron donor under organic carbon starvation. This will explain when and why NRB MIC happens. Yang found that the pre-grown *P. aeruginosa* (PA) biofilms became more corrosive under organic carbon starvation with weight loss data and SEM pitting images (Yang, 2016). Thus, it is necessary to investigate the NRB MIC mechanism further by studying a different NRB species.

Most people do not know that NRB can promote corrosion. If the injected nitrate in the reservoir is not entirely used, both NRB and nitrate can probably start in oil transportation pipelines. This can contribute to MIC contributed by NRB. In the field of bioenergetics, iron oxidation coupled with nitrate reduction is already recognized to provide energy for NRB respiration (Ghafari et al. 2008; Xu et al. 2013). Therefore, the objective of this chapter is to investigate the corrosion activity by YB03.

5.2 Materials and methods

5.2.1 Corrosion study in NRB culture

Bacterium, culture medium, experimental procedure

Pure culture of the YB03 was isolated from OFW in Akita (Japan). The medium used was filter-sterilized natural seawater overlaid with 1 mL of 10% diluted crude oil in HMN. The medium was prepared in a 15 mL glass tube with the addition of a carbon steel coupon, closed with a butyl cap, and autoclaved. The headspace of the glass test tube was filled with N_2 gas. 1 mL of YB03 enrichment culture was used as the bacterial inoculum. Seven conditions were set up in this study: YB03 culture, 27 mM nitrate addition, coupon, YB03 + 27 mM nitrate addition, coupon + 27 mM nitrate addition, YB03 + coupon, and YB03 + coupon + 27 mM nitrate addition. All conditions were triplicated. All tubes were incubated horizontally at 30^{o}C, in the dark, and on a shaking incubator (100 rpm). Three tubes of each condition were opened for coupon sampling every month.

Carbon steel coupon preparation and analysis

The coupon used in this study was of JIS G3141-SPCE steel. The chemical composition (wt%) of the carbon steel coupon used in this study was, carbon (< 0.10%), manganese (< 0.45%), phosphorous (< 0.03%) and sulfur (< 0.03%). The dimension of the coupon was 1cm × 2.50 cm × 0.50 cm. The coupon was abraded sequentially with sandpaper number #320, #600,

#1200, and #2000. The coupon was then cleaned with distilled water followed by acetone and dehydrated in 90% ethanol. The coupon was dried in the oven at 80^{o}C for 1 hour. The initial dry weight (W1) was measured. Before weight loss measurement, the coupon was cleaned with an acid solution (2 M HCl + 0.7 mM hexamethylenetetramine) for removal of corrosion product for 1 hour, shaking at room temperature (Enning et al. 2012). The coupon was then washed with distilled water, followed by acetone and dehydrated in 90% ethanol. The coupon was dried in the oven at 80^{o}C for 1 hour. Finally, the final weight (W2) of the coupon was measured.

The corrosion rate was calculated using weight loss measurement and the formula for calculation was (Quan et al. 2016),

$$R = K \times (W1 - W2) / (S \times t \times D)$$

Where R is corrosion rate (μm/year), K is a constant value (8.76×10^7), W1 is the initial weight of the coupon (g), W2 is the weight of the coupon after treatment (g), S is the coupon surface area (cm^2), t is corrosion time (hour), and D is the density of carbon steel (7.86 g/cm^3).

The surface analysis was performed to analyze the detailed effect of corrosion under all conditions. The surface analysis was conducted with a laser displacement sensor KS 1100 (Keyence, Japan). The KS-measure software (Keyence, Japan) was used to analyze the scanned images.

5.2.2 Chemical analysis

The chemical analysis was done in this study includes sulfate, sulfide, nitrate, and nitrite measurements. The detailed method for chemical analysis was described previously in section 2.2.4.

5.3 Results and discussion

5.3.1 Average depth of corrosion and coupon surface analysis

Fig. 5.1 illustrates an average depth (μm) of corrosion based on weight loss measurements for 3 months. In the abiotic condition, the average depth was similarly observed in the coupon and the (coupon+NO_3^-) condition. Thus, it shows that the addition of nitrate did not accelerate chemical corrosion. Moreover, no visible pitting was found in the abiotic control coupon without inoculation, suggesting that the culture medium used was not corrosive under an anaerobic condition. The corrosion depths found under YB03 and (YB03+NO_3^-) for 3 months were 6.30 μm and 5.27 μm, respectively. The effect of the corrosion rate by weight loss was in the following order: YB03 > (YB03+NO_3^-) > NO_3^- > control.

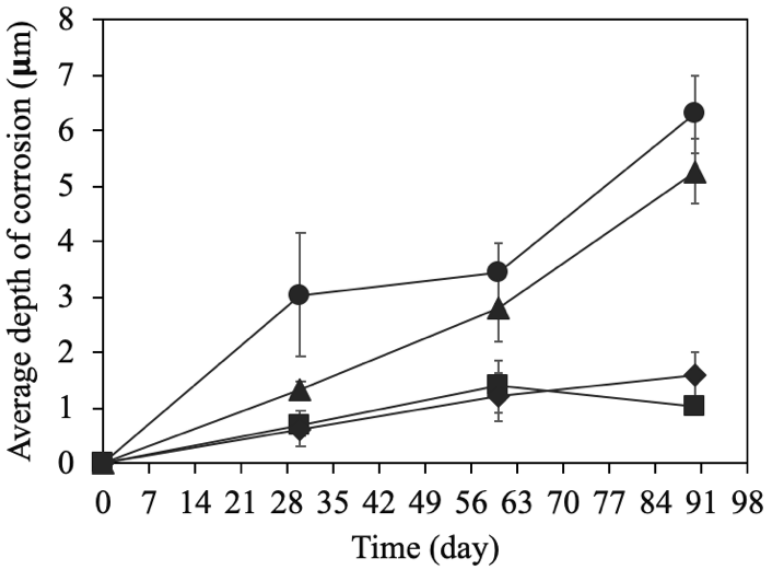

Figure 5.1 Average depth of corrosion. Coupon (■), coupon + NO_3^- (◆), YB03 + coupon (●), and YB03 + NO_3^- + coupon (▲) for 90-day.

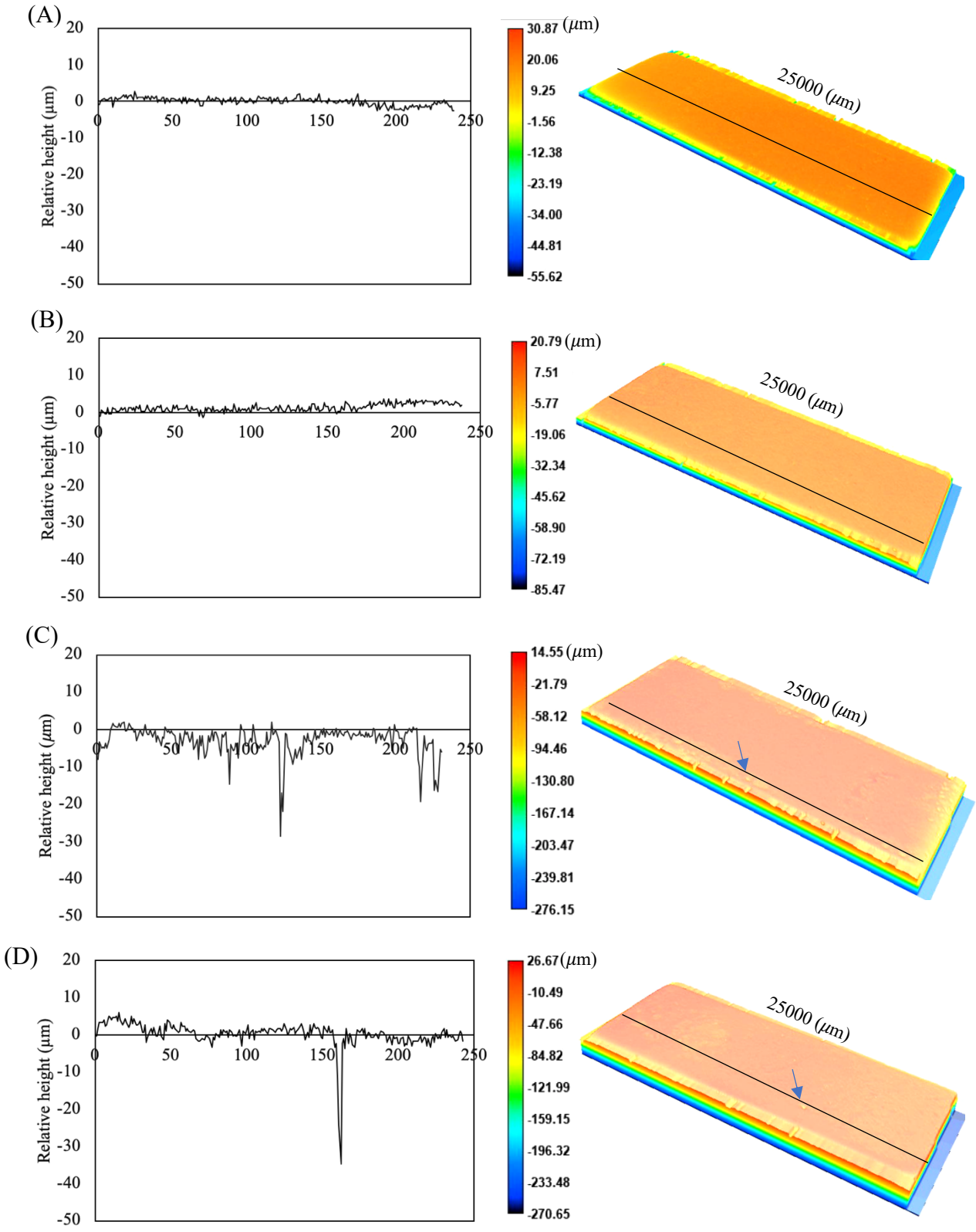

Figure 5.2 Surface analysis of carbon steel coupon under condition (A) coupon, (B) coupon+NO_3^-, (C) YB03 + coupon, and (D) YB03+NO_3^- + coupon at 90 days. X-axis: path length (×100 μm), Y-axis: pit depth (μm).

The surface analysis shown in Fig. 5.2 provides different facts. In the abiotic control, the corrosion rate was observed as 4.13 µm/year and 6.51 µm/year for the coupon and the (coupon+NO_3^-) condition, respectively. The highest corrosion rate was observed in the YB03 condition at 25.56 µm/year, followed by the (YB03+NO_3^-) condition (21.35 µm/year). The acceleration of corrosion caused by YB03 was concomitant with the production of hydrogen sulfide. Hydrogen sulfide, a highly toxic agent commonly known, and it can also be a very corrosive agent to carbon steel. It can also cause cracking corrosion of stainless steel (Bagarinao and Vetter, 1989; Kane, 1985; Little et al. 2000). Based on the surface analysis, the corrosion behavior of carbon steel caused by YB03 could be divided into two different phases: from day 30 to 60, the corrosion observed was general corrosion, and at day 90, pitting corrosion was observed. The deepest pit corrosion was observed as 28.64 µm in the YB03 condition (Fig. 5.2C). While in the (YB03+NO_3^-) condition, the deepest pit was 34.66 µm (Fig. 5.2D).

The anion concentration analysis was performed for all conditions. Under abiotic control, there were no changes of sulfate and nitrate concentration. The anion concentration analysis of all conditions is shown in Fig. 5.3. In the (YB03+coupon) condition, the production of 0.57 mM of sulfide was observed with 6.23 mM sulfate reduction, while sulfide was produced by 0.51 mM and sulfate was reduced by 5.44 mM in the (YB03+coupon+NO_3^-) condition after 90 days. Meanwhile, in the (YB03+NO_3^-) condition, the reduction of nitrate was higher than in the (YB03+coupon+NO_3^-) condition. In the (YB03+NO_3^-) condition, nitrite was produced in higher concentration than in the (YB03+coupon+NO_3^-) condition. In phase I (general corrosion), the YB03 reduced sulfate by coupled with oxidation of aromatic fraction in crude oil, which then leads to accumulation of sulfide in the bulk media (Fig.5.3B). The biogenic sulfide has been proposed as strong corrosion agent, because it can react with iron in carbon steel coupon and resulting FeS formation (Eq.1). If compared with water dissociation,

the undissociated protons in H_2S from the respiratory reduction of nitrate with organic nutrients react more rapidly than protons from or in H_2O (Eq.2) (Enning et al. 2012). Based on these equations, NRB plays an important role to accelerates corrosion by the production of sulfide. The accumulation of sulfide in the corrosion case study was less than in the souring case study, as some of the produced hydrogen sulfide reacted with the iron in the coupon resulting in FeS formation (Smith 1993).

$$H_2S + Fe \longrightarrow H_2 + FeS \qquad \Delta G^o = -72.5 \text{ kJ/mol Fe} \qquad (1)$$

$$2\ H_2O + Fe \longrightarrow Fe^{2+} + H_{2\,(g)} + 2\ OH^- \qquad \Delta G^o = -10.6 \text{ kJ/mol Fe} \qquad (2)$$

Moreover, in the presence of metal, NRB had been reported to accelerate corrosion. the production of hydrogen sulfide from the oxidation of organic carbon, as shown in Equation (3), was acting as a corrosive agent to metals.

$$0.22\ C_7H_8 + SO_4^{2-} + 0.67\ H^+ \longrightarrow 1.56\ HCO_3^- + HS^- + 2.5\ H^+ \qquad (3)$$

Therefore, the reaction between sulfide and iron on the carbon steel coupon was mainly a chemical reaction, therefore the corrosion behavior which more likely to happen is the abrasion of the metal surface after removal of corrosion products, but not pitting corrosion. The chemical reaction has been characterized as a chemical MIC (Enning and Garrelfs 2013). The formation of pitting corrosion was observed at day 90. Unlike the studies of MIC caused by SRB, there are only a few published papers related to studies of corrosion caused by NRB (Dunsmore et al. 2004; Halim et al. 2011).

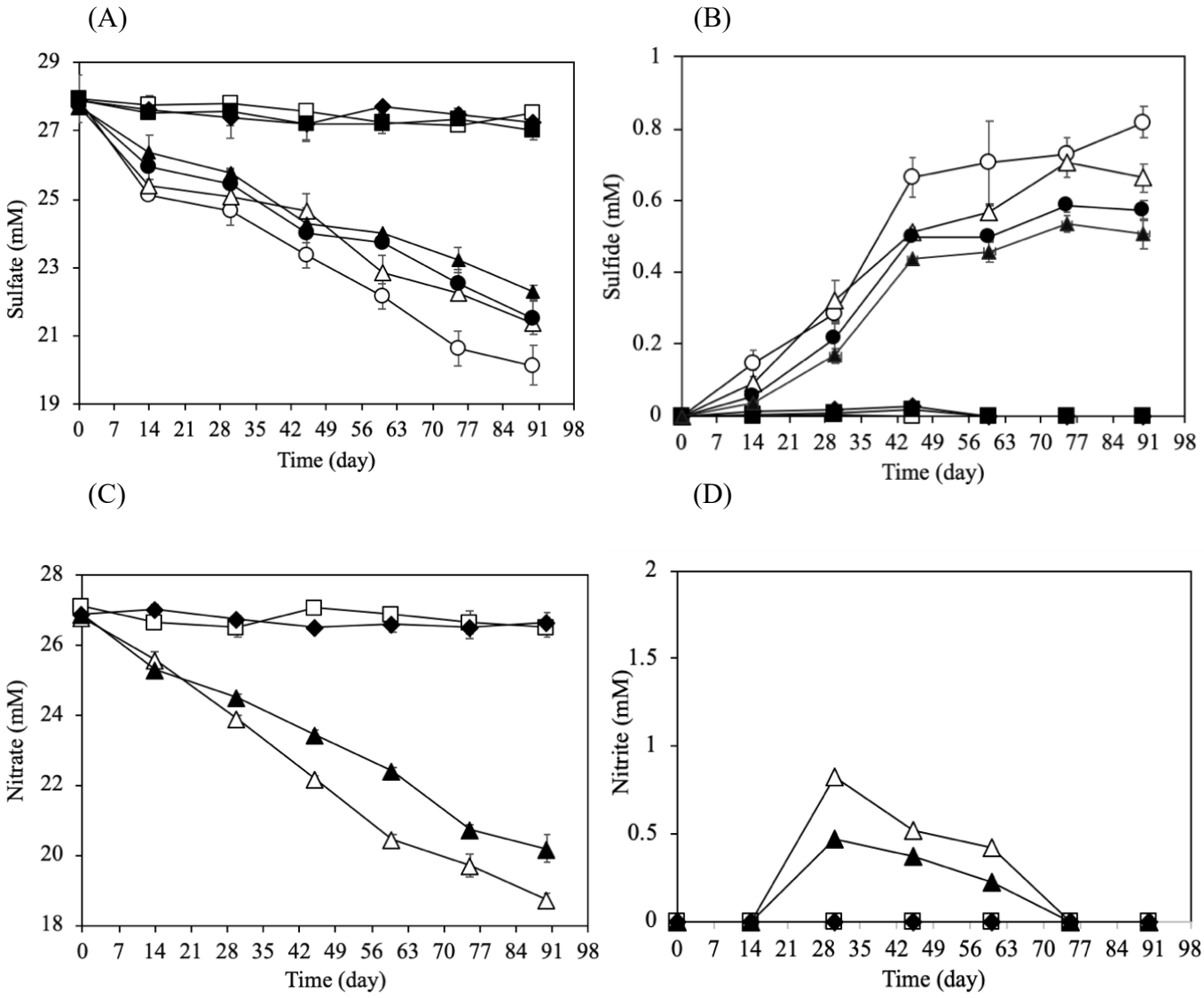

Figure 5.3 Chemical analysis of (A) sulfate (B) sulfide (C) nitrate , and (D) nitrite for 90-day under deferent conditions: YB03 (○), YB03 + NO_3^- (Δ), YB03 + coupon (●), YB03+coupon+ NO_3^- (▲), coupon (■), NO_3^- (□) and coupon + NO_3^- (◆)

Based on bioenergetics and biocatalysis, the biocatalytic cathodic sulfate reduction (BCSR) theory has been propose by Gu et al. (2009). When the nitrate is used as the terminal electron acceptor, BCSR theory can be modified to become biocatalytic cathodic nitrate reduction (BCNR) theory for MIC caused by NRB. NRB biofilm is formed on a surface of carbon steel. In the initial stage, the carbon source is available for NRB to maintain its

metabolism. In the next stage, the carbon source is limited on the steel surface because the biofilm becomes a mass transfer barrier. In the absence of organic carbon whereby the surface of a steel coupon sometimes becomes obscured by a biofilm, NRB can switch to Fe as an electron donor for energy production to sustain the deprivation of energy due to carbon starvation (Jia et al. 2017a; Jia et al. 2017b; Li et al. 2019; Xu et al. 2013; Yang, 2016). Ghafari et al. (2008) also claimed that iron oxidation coupled with nitrate reduction provides energy for NRB respiration in bioenergetics. Moreover, other mechanistic explanation of MIC under nitrate reducing conditions have also been suggested, including the formation of nitrite, consumption of cathodic hydrogen (Beeder et al. 2007; Vik et al. 2007; Dronen et al. 2014; Nemati et al. 2001; Hubert et al. 2005; Rempel et al. 2006).

Both nitrate and nitrite could enhance corrosion by oxidation of Fe to Fe^{2+} under neutral conditions from the viewpoint of thermodynamic. In addition, nitrite is known to act as both oxidizing agent and corrosion inhibitor (Hubert et a. 2004; The Institute of Petroleum, 2003). Generally, it is believed that the risk of corrosion that comes with nitrate addition may be caused by conversion of nitrate to nitrite by microbes (Fig.5.3C and 5.3D) (The Institute of Petroleum, 2003). The presence of nitrate could also control corrosion as well as souring, if the concentration of nitrite produced was sufficiently high. High nitrite concentration (460-920 mg/L) was proposed to act a corrosion inhibitor (Hubert et al. 2005). As an analog of sulfite, nitrite binds to dissimilatory sulfide reductase, preventing sulfide production. Additionally, nitrite can chemically react with sulfide forming N_2 and elemental sulfur (Reinsel et al. 1996). Under enough concentration of nitrite, mild steel can be passivated and thus corrosion could be inhibited. On the other hand, the risk of corrosion could increase due to insufficient nitrite concentration (Hubert et a. 2004; The Institute of Petroleum, 2003). This could explain why the rate of corrosion increased after longer exposure time, probably due to the consumption of nitrate and nitrite by the bacteria (Fig.5.3C and 5.3D).

Among the corrosive microbes, iron oxidizing bacteria is a special microorganism using iron as an electron donor (Byrne et al. 2015). Some studies (Bonis and Gralnick 2015, Singer et al. 2011) demonstrated that *Marinobacter subterrani* and *Marinobacter auaeolei* were iron oxidizing bacteria. Additionally, corrosion of carbon steel was promoted due to the production of Fe^{3+} from iron oxidizing bacteria, whereby they were able to rapidly oxidized Fe^{0} to Fe^{2+} (Wang et al. 2014, Liu et al. 2016, Liu et al. 2018).

5.4 Summary

In summary, in the abiotic condition, the corrosion rate was similarly observed with or without nitrate addition. Thus, it shows that the addition of nitrate did not accelerate chemical corrosion. The highest corrosion rate was observed in the YB03 condition, followed by the (YB03+NO_3^-) condition. I can summarize the effect of corrosion rate calculated by the weight loss in the following order: YB03 > (YB03+NO_3^-) > NO_3^- > control. Based on the surface analysis of coupon, the corrosion behavior of carbon steel caused by YB03 could be divided into two different phases: from day 30 to 60, the corrosion observed was general corrosion, and at day 90, pitting corrosion was observed.

Chapter 6. Conclusion

Oil fields contain a variety of microbes that can be implemented for secondary oil recovery by water flooding. Injection water containing sulfate can enhance growth of sulfate-reducing bacteria (SRB) in the reservoir, resulting in the production of hydrogen sulfide (H_2S) in a process known as souring. To mitigate reservoir souring, injection of nitrate was applied to promote the growth of nitrate-reducing bacteria (NRB) to control the growth of SRB. Therefore, it is of particular interest to detect the presence of NRB and stimulate its activity for controlling souring. NRB can also cause microbiologically influenced corrosion (MIC). However, the effect of nitrate addition on reservoir souring and corrosion of carbon steel by NRB are still not well understood. In this study, the NRB strain, screened from an oil field water, was used to investigate biological souring and MIC. The results obtained are summarized as followed.

Chapter 2, NRB was isolated from OFW. According to 16S rRNA gene sequence, NRB isolate belonged to genus *Marinobacter* and was named *Marinobacter* YB03. The whole genome sequence of *Marinobacter* YB03 was determined (accession number AP019537). For nitrate metabolism of YB03, based on genome analysis, the gene sets encoding for dissimilatory nitrate reduction and denitrification were found on the draft genome of YB03. For sulfur metabolism, the YB03 has the gene sets encoding for assimilatory sulfate reduction. Based on the culture-based analysis, the carbon sources and electron donor preferences of YB03 were toluene, m-,p-xylene and VFAs. Moreover, the most favorable for VFA utilization as substrate by YB03 was acetate.

Chapter 3, in the experiment, nitrate was added at the concentration of 27mM. SRB isolated from OFW and named *Desulfotignum* YB01 was also used. With addition of nitrate, it was able to repress biological souring caused by YB01. However, the condition with and without nitrate addition, production of sulfide and reduction of sulfate in YB03 observed when

toluene was used as electron donor and carbon source. Thus, the addition of nitrate to YB03 culture could not control biological souring. For co-culture of (YB01+YB03) with nitrate and without nitrate addition, YB03 could not control souring caused by YB01as sulfate reduction and sulfide production were observed.

Chapter 4, Based on MIC assay, the corrosion behavior of YB03 against carbon steel coupon could be divided into two different phases: from day 30 to 60, the corrosion observed was general corrosion, and at day 90, pitting corrosion was observed. The highest corrosion rate was found in the YB03 condition, followed by the (YB03+NO_3^-) condition. The effect of the corrosion rate determined by the weight loss can be summarized int the following order: YB03 > (YB03+NO_3^-) > NO_3^- > control.

In this study, YB03 strain was NRB that have the ability to reduce sulfate. Therefore, this bacterium could not control souring caused by nitrate- and sulfate-reducing bacteria, *Desulfotignum* YB01. The ability of the YB03 to promote souring and corrosion was confirmed. Pitting corrosion was observed at day 90 in the conditions with and without nitrate addition.

NRB is important in controlling SRB which produces H_2S, as NRB is able to outcompete SRB. In contrast, YB03 which is an NRB was found to be able to reduce sulfate into sulfide and cause biological souring. Moreover, YB03 is able to produce nitrite which could promote corrosion, depending on nitrite's concentration and the environmental conditions. This is the first study showing that strain YB03, *Marinobacter* species which considers as NRB could cause corrosion, as observed in Chapter 5. This result also indicates the potential of YB03 in causing souring and corrosion in the oil field reservoir. Thus, it is important to monitor and keep this bacterium under constant surveillance to prevent complications caused by MIC in the future. Meanwhile, there are several problems that still has to be solved for this study. Based on the previous study (Kamarisima et al. 2018), the addition of nitrated could promote the growth of *Thalassospira*, which could suppress the

growth of SRB. *Thalassospira* became the dominant species after the addition of nitrate at 0 day. As a future plan, various growth conditions needed to be optimized for successful isolation of *Thalossospira*. Besides, the biological effect of this bacterium to metallic materials in vitro would be an interesting topic to be studied upon in the future.

References:

Aziz RK, Bartels D, Best AA, DeJongh M, Disz T, Edwards RA, Formsma K, Gerdes S, Glass EM, Kubal M, Meyer F, Olsen GJ, Olson R, Osterman AL, Overbeek RA, McNeil LK, Paarmann D, Paczian T, Parrello B, Pusch GD, Reich C, Stevens R, Vassieva O, Vonstein V, Wilke A, Zagnitko O. 2008. The RAST server: Rapid Annotations using Subsystems Technology. BMC Genomics 9:75. http://dx.doi.org/10.1186/1471-2164-9-75.

Alain, Karine, Jens Harder, Friedrich Widdel, and Karsten Zengler. 2012. "Anaerobic Utilization of Toluene by Marine Alpha- and Gammaproteobacteria Reducing Nitrate." 2946–57.

Bagarinao, T., Vetter, R.D., 1989. Sulfide tolerance and detoxification in shallow-water marine fishes. Mar. Biol. 103, 291–302.

Ballard, A.L., Ferguson, S.J., Eur. J. Biochem. 174 (1988) 207–212.

Barbaro, J.R., Barker, J.F., Lemon, L.A., Mayfield, C.I., Biotransformation of BTEX under anaerobic denitrifying conditions: field and laboratory observations, J. Contam. Hydrol. 11 (1992) 245–272.

Barton, L.L. (ed) 1995. Sulfate-Reducing Bacteria. New York: Plenum Publishing Corp.

Barton, Larry L., W. Allan Hamilton, Rudolf K. Thauer, Erko Stackebrandt, and W. Allan Hamilton. 2003. "Energy Metabolism and Phylogenetic Diversity of Sulphate-Reducing Bacteria."

Bazylinski, Dennis A. and R. P. Blakemore. 1983. "Denitrification and Assimilatory Nitrate Reduction in Aquaspirillum Magnetotacticum." 46(5):1118–24.

Beech, Iwona B. 2004. "Corrosion of Technical Materials in the Presence of Bio ÿ Lms Current Understanding and State-of-the Art Methods of Study." 53:177–83.

Beeder J, Andersen TR, Liengen T, Drønen K, Torsvik T. 2007. Corrosion as a side effect during nitrate treatment of produced water and aquifer water injection. NACE - 07512 . NACE International, Houston, TX .

Bonis, B.M., Gralnick, J.A., 2015. Marinobacter subterrani, a genetically tractable neutrophilic Fe(II)-oxidizing strain isolated from the Soudan Iron Mine.doi: 10.3389/fmicb.2015.00719

Bowman, John P., Tasmania Hobart, Thomas A. Mcmeekin, Southern Ocean Studies, and Tasmania Hobart. 2015. "Marinobacter." 1–6.

Bru, D., F. Martin-Laurent, and L. Philippot. 2008. "Quantification of the Detrimental Effect of a Single Primer-Template Mismatch by Real-Time PCR Using the 16S RRNA Gene

as an Example." *Applied and Environmental Microbiology* 74(5):1660–63.

Braker G, Ayala-del-Rio HL, Devol AH, Fesefeldt A, Tiedje JM (2001) Community structure of denitrifiers, bacteria, and archaea along redox gradients in Pacific Northwest marine sediments by terminal restriction fragment length polymorphism analysis of amplified nitrite reductase (*nirS*) and 16S rRNA genes. Appl Environ Microbiol 67:1893–1901

Byrne, J. M., Klueglein, N., Pearce, C., Rosso, K. M., Appel, E., and Kappler, A. (2015). Redox cycling of Fe (II) and Fe (III) in magnetite by Fe-metabolizing bacteria. *Science* 347, 1473–1476. doi: 10.1126/science.aaa4834

Carsoso RB, Sierra-Alvarez, Rowlette P et al. Sulfide oxidation under chemolithoautotrophic denitrifying conditions. Biotechnol Bioeng 2006; 95:1148-57.

Chakraborty, R.,Bender, K.S., Shang, C., Belchik, S.M., Coates, J.D. and Achenbach, L.A. (2005). Identification, characterization, and classification of genes encoding perchlorate reductase J Bacteriol, 187:5090-5096.

Coombe D, Jack T, Voordouw G *et al.* Simulation of bacterial souring control in an albertan heavy oil reservoir. *J Can Pet Technol* 2010;**49**:19–26.

Darling, Aaron E., Bob Mau, and Nicole T. Perna. 2010. "Progressivemauve: Multiple Genome Alignment with Gene Gain, Loss and Rearrangement." *PLoS ONE* 5(6).

Davidova, I., M. S. Hicks, P. M. Fedorak, and J. M. Suflita. 2001. "The Influence of Nitrate on Microbial Processes in Oil Industry Production Waters." *Journal of Industrial Microbiology and Biotechnology* 27(2):80–86.

Dolfing J, Zeyer J, Binder Eicher P & Schwarzenbach RP (1990) Isolation and characterization of a bacterium that mineralizes toluene in the absence of molecular oxygen. Arch. Microbiol. 154: 336–341

Dopffel, Nicole, Felix Kögler, Holger Hartmann, Paul Igor Costea, Eva Mahler, Andrea Herold, and Hakan Alkan. 2018. "Microbial Induced Mineral Precipitations Caused by Nitrate Treatment for Souring Control during Microbial Enhanced Oil Recovery (MEOR)." *International Biodeterioration and Biodegradation* 135(August):71–79.

Dou, Junfeng, Xiang Liu, Zhifeng Hu, and Dong Deng. 2008. "Anaerobic BTEX Biodegradation Linked to Nitrate and Sulfate Reduction." 151:720–29.

Drønen K, Roalkvam I, Beeder J, Torsvik T, Steen IH, Skauge A, Liengen T. 2014. Modeling of heavy nitrate corrosion in anaerobe aquifer injection water biofilm: a case study in a flow rig. Environ Sci Technol 48:8627 –8635.

Dunsmore, B., Whitfield, T., Lawson, P.A., Collins, M.D., 2004. Corrosion by Sulfate Reducing Bacteria that Utilize Nitrate, in: Corrosion/2004 Paper No. 04763. NACE

International, Houston, TX.

Eckford, R. E. and P. M. Fedorak. 2004. “Chapter 11 Using Nitrate to Control Microbially-Produced Hydrogen Sulfide in Oil Field Waters.” *Studies in Surface Science and Catalysis* 151:307–40.

Ehrenreich P, Behrends A, Harder J & Widdel F (2000) Anaer- obic oxidation of alkanes by newly isolated denitrifying bacteria. Arch. Microbiol. 173: 58–64

Enning, Dennis and Julia Garrelfs. 2013. “Corrosion of Iron by Sulfate-Reducing Bacteria - New Views of an Old Problem.” (December).

Enning, Dennis, Hendrik Venzlaff, Julia Garrelfs, Hang T. Dinh, Volker Meyer, Karl Mayrhofer, Achim W. Hassel, Martin Stratmann, and F. Widdel. 2012. “Marine Sulfate-Reducing Bacteria Cause Serious Corrosion of Iron under Electroconductive Biogenic Mineral Crust.” *Environmental Microbiology* 14(7):1772–87.

Eschbach, M., Schreiber, K., Trunk, K., Buer, J., Jahn, D., & Schobert, M. (2004). Long term anaerobic survival of the opportunistic pathogen Pseudomonas aeruginosa via pyruvate fermentation. Journal of Bacteriology, 186(14), 4596–4604.

Etique, M., Romaine, A., Bihannic, I., Gley, R., Carteret, C., Abdelmoula, M., Ruby, C., Jeannin, M., Sabot, R., Refait, P., Frédéric P.A. Jorand, 2018. Abiotically or microbially mediated transformations of magnetite by sulphide species: The unforeseen role of nitrate-reducing bacteria. Corrosion Science 142 (2018) 31–44.

Ezeuko, C. C., Sen, A., & Gates, I. D. (2013). Modelling biofilm-induced formation damage and biocide treatment in subsurface geosystems. *Microb Biotechnol,* 6(1), 53-66.

Fries MR, Zhou J, Chee-Sanford J. & Tiedje JM (1994) Isola- tion, characterization, and distribution of denitrifying toluene degraders from a variety of habitats. Appl. Environ. Microbiol. 60: 2802–2810

Gauthier MJ, Lafay B, Christen R, Fernandez L, Acquaviva M, Bonin P, Bertrand JC (1992) *Marinobacter hydrocarbonoclasticus* gen. nov., sp. nov., a new, extremely halotolerant, hydrocarbon-degrading marine bacterium. *Int J Syst Bacteriol* 42: 568–576.

Ghafari, S., Hasan, M., Aroua, M.K., 2008. Bio-electrochemical removal of nitrate from water and wastewater—A review. Bioresour. Technol. 99, 3965–3974.

Gieg, L. M., Jack, T. R., & Foght, J. M. (2011). Biological souring and mitigation in oil reservoirs. Applied Microbiology and Biotechnology, 92(2), 263–282.

Gittel, Antje, Ketil Bernt Sørensen, Torben Lund Skovhus, Kjeld Ingvorsen, and Andreas Schramm. 2009. “Prokaryotic Community Structure and Sulfate Reducer Activity in Water from High-Temperature Oil Reservoirs with and without Nitrate Treatment.”

75(22):7086–96.

Greene, E. A., C. Hubert, M. Nemati, G. E. Jenneman, and G. Voordouw. 2003. “Nitrite Reductase Activity of Sulphate-Reducing Bacteria Prevents Their Inhibition by Nitrate-Reducing , Sulphide- Oxidizing Bacteria.” 5:607–17.

Grein, Fabian, Ana Raquel Ramos, Sofia S. Venceslau, and Inês A. C. Pereira. 2013. “Unifying Concepts in Anaerobic Respiration: Insights from Dissimilatory Sulfur Metabolism.” *Biochimica et Biophysica Acta - Bioenergetics* 1827(2):145–60.

Grigoryan, Aleksandr A., Sabrina L. Cornish, Brenton Buziak, Shiping Lin, Adriana Cavallaro, Joseph J. Arensdorf, and Gerrit Voordouw. 2008. “Competitive Oxidation of Volatile Fatty Acids by Sulfate- and Nitrate-Reducing Bacteria from an Oil Field in Argentina.” *Applied and Environmental Microbiology* 74(14):4324–35.

Gu, T. (2014). Theoretical modeling of the possibility of acid producing bacteria causing fast pitting biocorrosion. Journal of Microbial & Biochemical Technology, 06(02), 068-074.

Gu, T., Zhao, K., & Nesic, S. (2009). A new mechanistic model for MIC based on a biocatalytic cathodic sulfate reduction theory. In Corrosion/2009 Paper No. C2009-09390. NACE International, Atlanta, Georgia.

Halim, A., Gubner, R., Watkin, E., 2011. Preliminary study on nitrate injection to control souring problem in oil reservoir:benefits and side effects on steel material, in: Corrosion/2011 Paper No. 11229. NACE International, Houston, TX.

Hamzah, E., Hussain, M. Z., Ibrahim, Z., & Abdolahi, A. (2013). Influence of Pseudomonas aeruginosa bacteria on corrosion resistance of 304 stainless steel. Corrosion Engineering, Science and Technology, 48(2), 116–120.

Hasegawa, R., Toyama, K., Miyanaga, K., Tanji, Y., 2014. Identification of crude-oil components and microorganisms that cause souring under anaerobic conditions. Appl. Microbiol. Biotechnol. 98, 1853–1861. http://dx.doi.org/10.1007/s00253- 013-5107-3.

Hauptmann, A.L., Sicheritz-Ponten, T., Cameron, K.A., Bælum, J., Plichta, D.R., Dalgaard, M., Stibal, M., 2017. Comtamination of the Arctic reflected in microbial metagenomes from the Greenland ice sheet. https://doi.org/10.1088/1748-9326/aa7445.

Hassett, D. J., Cuppoletti, J., Trapnell, B., Lymar, S. V., Rowe, J. J., Yoon, S. S., et al. (2002). Anaerobic metabolism and quorum sensing by Pseudomonas aeruginosa biofilms in chronically infected cystic fibrosis airways: rethinking antibiotic treatment strategies and drug targets. Advanced Drug Delivery Reviews, 54(11), 1425–1443.

Haner A, Ho¨hener P, Zeyer J (1995) Degradation of p-xylene by a denitrifying enrichment culture. Appl Environ Microbiol 61:3185–3188

Heider, J., Spormann, A.M., Beller, H.R., Widdel, F. Anaerobic bacterial metabolism of hyrocarbons, FEMS Microbiol. Rev. 22 (1998) 459-473.

Hendriks, J., Warne, A., Gohlke, U., Haltia, T., Ludovici, C., Lubben, M., Saraste, M., Biochemistry 37 (1998) 13102–13109.

Herbert, B.F. 1987. Reservoir Souring. In: Hill, E.C., Shennan, J.L., and Watkinson, R.J. (eds) Microbial Problems in the Off-shore Oil Industry. New York: John Wiley & Sons. pp 63-71.

Hess A, Zarda B, Hahn D, Häner A, Stax D, Höhener P & Zeyer J (1997) *In situ* analysis of denitrifying toluene- and *m*-xylene- degrading bacteria in a diesel fuel-contaminated laboratory aquifer column. Appl. Environ. Microbiol. 65: 2136–2141

Hubert, Casey, Mehdi Nemati, Gary Jenneman, and Gerrit Voordouw. 2003. "Containment of Biogenic Sulfide Production in Continuous Up-Flow Packed-Bed Bioreactors with Nitrate or Nitrite." *Biotechnology Progress* 19(2):338–45.

Hubert, Casey and Gerrit Voordouw. 2007. "Oil Field Souring Control by Nitrate-Reducing Sulfurospirillum Spp. That Outcompete Sulfate-Reducing Bacteria for Organic Electron Donors." *Applied and Environmental Microbiology* 73(8):2644–52.

Hubert C, Nemati M, Jenneman G, Voordouw G. 2005. Corrosion risk associated with microbial souring control using nitrate or nitrite. Appl Microbiol Biotechnol 68:272 – 282.

Hubert, C.,Nemati, Jenneman, M.,G., Voordouw, G. Applied Microbiology and Biotechnology 68(272)(2004)282

Hungate, R. E. 1969. "Chapter IV A Roll Tube Method for Cultivation of Strict Anaerobes." *Methods in Microbiology* 3:117–32.

Huu, N. B., Denner, E. B. M., Ha, D. T. C., Wanner, G., and Stan-Lotter, H. (1999) *Marinobacter aquaeolei* sp. nov., a halophilic bacterium isolated form a Vietnamese oil-producing well. *International Journal of Systematic and Evolutionary Microbiology, 49*(2), 367-375. doi: 10.1099/00207713-49-2-367

Jahn MK, Haderlein SB, Meckenstock RU (2005) Anaerobic degradation of benzene, toluene, ethylbenzene, and o-xylene in sediment-free iron-reducing enrichment cultures. Appl Environ Microbiol 71:3355–3358

Jia, Ru, Dongqing Yang, Dake Xu, and Tingyue Gu. 2017. "Electron Transfer Mediators Accelerated the Microbiologically Influence Corrosion against Carbon Steel by Nitrate Reducing Pseudomonas Aeruginosa Biofilm." *Bioelectrochemistry* 118:38–46.

Jia, Ru, Dongqing Yang, Jin Xu, Dake Xu, and Tingyue Gu. 2017. "Microbiologically Influenced Corrosion of C1018 Carbon Steel by Nitrate Reducing Pseudomonas

Aeruginosa Biofilm under Organic Carbon Starvation." *Corrosion Science* 127(August):1–9.

Jormakka, M., Byrne, B., Iwata, S., FEBS Lett. 545 (2003) 25–30.

Kamarisima, Kohei Hidaka, Kazuhiko Miyanaga, and Yasunori Tanji. 2018. "The Presence of Nitrate- and Sulfate-Reducing Bacteria Contributes to Ineffectiveness Souring Control by Nitrate Injection." *International Biodeterioration and Biodegradation* 129(December 2017):81–88.

Kane, R.D., 1985. Roles of H_2S in behaviour of engineering alloys. Int. Met. Rev. 30, 291–301.

Kao, C.M., Borden, R.C., Site-specific variability in BTEX biodegradation under denitrifying conditions, Ground Water 35 (1997) 305–311.

Kjellerup, B. V., R. H. Veeh, P. Sumithraratne, T. R. Thomsen, K. Buckingham-Meyer, B. Frølund, and P. Sturman. 2005. "Monitoring of Microbial Souring in Chemically Treated, Produced-Water Biofilm Systems Using Molecular Techniques." *Journal of Industrial Microbiology and Biotechnology* 32(4):163–70.

Kodama Y, Stiknowati LI, Ueki A, Ueki K, Watanabe K. Thalassospira tepidiphila sp. nov., a polycyclic aromatic hydrocarbondegrading bacterium isolated from seawater. Int J Syst Evol Microbiol 2008;58:711–715.

Kuhn EP, Colberg PJ, Schnoor JL, Wanner O, Zehnder AJB, Schwarzenbach RP (1985) Microbial transformations of substituted benzenes during infiltration of river water to groundwater—laboratory column studies. Environ Sci Technol 19:961–968

Kumaraswamy, Raji, Sara Ebert, Murray R. Gray, Phillip M. Fedorak, and Julia M. Foght. 2011. "Molecular- and Cultivation-Based Analyses of Microbial Communities in Oil Field Water and in Microcosms Amended with Nitrate to Control H 2 S Production." 2027–38.

Lambo, Adewale, Sabrina Cornish Shartau, and Gerrit Voordouw. 2009. "Souring Remediation by Field-Wide Nitrate Injection in an Alberta Oil Field." (May).

Larsen, J. 2002. Downhole Nitrate Applications to Control Sulfate Reducing Bacteria Activity and Reservoir Souring. presented at the Corrosion 2002, Denver, Colorado, 7-11 April.

Li, Yingchao, Dake Xu, Changfeng Chen, Xiaogang Li, Ru Jia, Dawei Zhang, Wolfgang Sand, Fuhui Wang, and Tingyue Gu. 2018. "Anaerobic Microbiologically Influenced Corrosion Mechanisms Interpreted Using Bioenergetics and Bioelectrochemistry: A Review." *Journal of Materials Science and Technology* 34(10):1713–18.

Li, Zhong, Hongxia Wan, Dongdong Song, Xiaodong Liu, Ziyu Li, and Cuiwei Du. 2019.

"Corrosion Behavior of X80 Pipeline Steel in the Presence of Brevibacterium Halotolerans in Beijing Soil." *Bioelectrochemistry* 126:121–29.

Ligthelm, D.J., deBoer, R.B., Brint, J.F., and Schulte, W.M. 1991. Reservoir Souring: An Analytical Model for H2S Generation and Transportation in an Oil Reservoir Owing to Bacterial Activity. Paper SPE 23141 presented at the Offshore Europe, Aberdeen, United Kingdom, 3-6 September. DOI: 10.2118/23141-MS.

Lin, Kuen-song, Ni-bin Chang, and Tien-deng Chuang. 2008. "Nitrates in Wastewater and Groundwater Fine Structure Characterization of Zero-Valent Iron Nanoparticles for Decontamination of Nitrites and Nitrates in Wastewater and Groundwater." 6996.

Little, B.J., Ray, R.I., Pope, R.K., 2000. Relationship between corrosion and the biological sulfur cycle: a review. Corrosion 56, 433–443.

Liu, H., Gu, T., Zhang, G., Cheng, Y., Wang, H., and Liu, H. (2016). The effect of magnetic field on biomineralization and corrosion behavior of carbon steel induced by iron-oxidizing bacteria. *Corros. Sci.* 102, 93–102. doi: 10.1016/j. corsci.2015.09.023

Liu, H., Cheng, Y., Xu, D., and Liu, H. (2018). Effect of iron oxidizing bacteria biofilm on corrosion inhibition of imidazoline derivative in CO2-containing oilfield produced water with organic carbon source starvation. *J. Electrochem. Soc.* 165, C354–C361. doi: 10.1149/2.0741807jes

Lovley, D.R., Potentia lfor anaerobic bioremediation of BTEX in petroleum contaminated aquifers, J. Ind. Microbiol. Biotechnol. 18 (1997) 75–81.

Mayer, O. (1994). Functional groups of microorganisms, in: Biodiversity and Ecosystem Function: With 22 Tables. Springer-Verlag, Berlin.

McKinley, V. L., Costerton, J. W., and White, D. C. 1988. Microbial Biomass, Activity, and Community Structure of Water and Particulates Retrieved by Backflow from a Waterflood Injection Well. Applied Environmental Microbiology. 54 (6): 1383- 1393.

Meckenstock RU, Warthmann RI, Schafer W (2004) Inhibi- tion of anaerobic microbial o-xylene degradation by tol- uene in sulfidogenic sediment columns and pure cultures. FEMS Micobiol Ecol 47:381–386

Miller, Robert B., Kenton Lawson, Anwar Sadek, Chelsea N. Monty, and John M. Senko. 2018. "Uniform and Pitting Corrosion of Carbon Steel by Shewanella Oneidensis MR-1 under Nitrate-Reducing Conditions." *Applied and Environmental Microbiology* 84(12).

Morasch B, Schink B, Tebbe CC, Meckenstock RU (2004) Degradation of o-xylene and m-xylene by a novel sulfate- reducer belonging to the genus Desulfotomaculum. Arch Microbiol 181:407–417

Myhr, S., B. L. Lillebø, E. Sunde, J. Beeder, and T. Torsvik. 2002. “Inhibition of Microbial H 2 S Production in an Oil Reservoir Model Column by Nitrate Injection.” *Applied Microbiology and Biotechnology* 58(3):400–408.

Nadkarni, Mangala A., F. Elizabeth Martin, Nicholas A. Jacques, and Neil Hunter. 2002. “Determination of Bacterial Load by Real-Time PCR Using a Broad-Range (Universal) Probe and Primers Set.” *Microbiology* 148(1):257–66.

Nemati, M., Jenneman, G.E., and Voordouw, G. (2001). Mechanistic study of microbial control of hydrogen sulfide production in oil reservoirs. *Biotechnol. Bioeng*. 74:424–434.

Nemati M, Jenneman GE, Voordouw G. 2001. Impact of nitrate -mediated microbial control of souring in oil reservoirs on the extent of corrosion. Biotechnol Prog 17:852 – 859.

Nijburg, J. W., Gerards, S., & Laanbroek, H. J. (1998). Competition for nitrate and glucose between Pseudomonas fluorescens and Bacillus licheniformic under continuous or fluctuating anoxic conditions. FEMS Microbiology Ecology, 26, 345–356.

NTSB Report, 2000. Pipeline Accident Report Natural Gas Pipeline Rupture and Fire Near Carlsbad, New Mexico (No. NTSB/PAR-03/01). US National Transportation Safety Board.

Nunoura T, Nishizawa M, Kikuchi T, Tsubouchi T, Hirai M, Koide O, Miyazaki J, Hirayama H, Koba K, Takai K (2013) Molecular biological and isotopic biogeochemical prognoses of the nitrification-driven dynamic microbial nitrogen cycle in hadopelagic sediments. Environ Microbiol 15:3087–3107

Oakley BB, Francis CA, Roberts KJ, Fuchsman CA, Srinivasan S, Staley JT (2007) Analysis of nitrite reductase (*nirK* and *nirS*) genes and cultivation reveal depauperate community of denitrifying bacteria in the Black Sea suboxic zone. Environ Microbiol 9:118–130

Quan, Bian Li, Jun Qi Li, and Chao Yi Chen. 2016. “Effect of Additional Sulfide and Thiosulfate on Corrosion of Q235 Carbon Steel in Alkaline Solutions.” *International Journal of Corrosion* 2016.

Rabus, R., Wilkes, H., Schramm, A., Harms, G., Behrends, A., Amann, R. & Widdel, F. (1999). Anaerobic utilisation of alkylbenzenes and n-alkanes from crude oil in an enrichment culture of denitrifying bacteria affiliated with b-subclass of Proteobacteria. Environ Microbiol 1, 145–157.

Rabus R, Widdel F (1995) Anaerobic degradation of ethyl- benzene and other aromatic hydrocarbons by new deni- trifying bacteria. Arch Microbiol 163:96–103

Reinsel, M. A., J. T. Sears, P. S. Stewart, and M. J. Mclnerney. 1996. “Control of Microbial

Souring by Nitrate , Nitrite or Glutaraldehyde Injection in a Sandstone Column.” 128–36.

Rempel CL, Evitts RW, Nemati M. 2006. Dynamics of corrosion rates associated with nitrite or nitrate mediated control of souring under biological conditions simulating an oil reservoir. J Ind Microbiol Biotechnol 33:878 –886.

Rizk, Tony Y. 2015. “Nitrate Treatment — Effect on Corrosion and Implementation Guidelines.” *Saudi Aramco Journal of Technology*.

Rotaru AE, Probian C, Wilkes H, Harder J (2010) Highly enriched Betaproteobacteria growing anaerobically with p-xylene and nitrate. FEMS Microbiol Ecol 71:460–468

Santoro AE, Francis CA, de Sieyes NR, Boehm AB (2008) Shifts in the relative abundance of ammonia oxidizing bacteria and archaea across physicochemical gradients in a subterranean estuary. Environ Microbiol 10:1068–1079

Schwermer, Carsten U., Gaute Lavik, Raeid M. M. Abed, Braden Dunsmore, Timothy G. Ferdelman, Paul Stoodley, Armin Gieseke, and Dirk De Beer. 2008. “Impact of Nitrate on the Structure and Function of Bacterial Biofilm Communities in Pipelines Used for Injection of Seawater into Oil Fields.” *Applied and Environmental Microbiology* 74(9):2841–51.

Seitz, Hans-jiirgen and Heribert Cypionka. 1986. “Micrnbiology with Hydrogen Coupled to Ammonification of Nitrate or Nitrite.” 2:63–67.

Simon J, Klotz MG. Diversity and evolution of bioenergetic systems involved in microbial nitrogen compound transformations. *Biochim Biophys Acta - Bioenerg* 2013, DOI: 10.1016/j.bbabio.2012.07.005.

Singer, Esther, Eric A. Webb, William C. Nelson, John F. Heidelberg, Natalia Ivanova, Amrita Pati, and Katrina J. Edwards. 2011. “Genomic Potential of Marinobacter Aquaeolei, a Biogeochemical ‘Opportunitroph.’” *Applied and Environmental Microbiology* 77(8):2763–71.

Smith CJ, Nedwell DB, Dong LF, Osborn AM (2007) Diversity and abundance of nitrate reductase genes (*narG* and *napA*), nitrite reductase genes (*nirS* and *nrfA*), and their transcripts in estuarine sediments. Appl Environ Microbiol 73:3612–3622

Smith, Stephen N. 1993. “A Proposed Mechanism for Corrosion in Slightly Sour Oil and Gas Production.” *Nace* (SEPTEMBER 1993):2695–2706.

Striebich, R. C., Smart, C. E., Gunasekera, T. S., Mueller, S. S., Strobel, E. M., McNichols, B. W., & Ruiz, O. N. (2014). Characterization of the F-76 diesel and Jet-A aviation fuel hydrocarbon degradation profiles of Pseudomonas aeruginosa and Marinobacter hydrocarbonoclasticus. International Biodeterioration & Biodegradation, 93, 33–43.

Sunde, E., Torsvik, T., 2005. Microbial control of hydrogen sulfide production in oil reservoirs. In: Ollivier, B., Magot, M. (Eds.), Petroleum Microbiology. ASM, Washington DC, pp. 201–213.

Tamegai H, Aoki R, Arakawa S, Kato C (2007) Molecular analysis of the nitrogen cycle in deep-sea microorganisms from the Nankai Trough: genes for nitrification and denitrification from deep-sea environmental DNA. Extremophiles 11:269–275

Tanji, Yasunori, Kazuya Toyama, Ryo Hasegawa, and Kazuhiko Miyanaga. 2014. "Biological Souring of Crude Oil under Anaerobic Conditions." *Biochemical Engineering Journal* 90:114–20.

Tang, K., Baskaran, V., Nemati, M., 2009. Bacteria of the sulphur cycle: an overview of microbiology, biokinetics and their role in petroleum and mining industries. Biochem. Eng. J. http://dx.doi.org/10.1016/j.bej.2008.12.011.

Telang, Anita J., Sara Ebert, Julia M. Foght, D. W. S. Westlake, Gary E. Jenneman, Diane Gevertz, and Gerrit Voordouw. 1997. "Effect of Nitrate Injection on the Microbial Community in an Oil Field as Monitored by Reverse Sample Genome Probing." *Applied and Environmental Microbiology* 63(5):1785–93.

Thauer, R. K., Stackebrandt, E., & Hamilton, W. A. (2007). Energy metabolism phylogenetic diversity of sulphate-reducing bacteria (in: Barton, L.L., Hamilton, W.A., (Eds.), pp. 1–37). Cambridge, UK: Cambridge University Press.

The Institute of Petroleum, The Stimulation of Nitrate Reducing Bacteria (NRB) in Oil Field System to Control Sulphate Reducing Bacteria (SRB), Microbiologically Influenced Corrosion (MIC) and Reservoir Souring: An Introduction Review, Energy Institute, London, 200

Tsang, Monica Lik-shing. 1981. "Assimilatory Sulfate Reduction in Escherichia Coli : Identification of the Alternate Cofactor for Adenosine 3 ' - Phosphate 5 ' -Phosphosulfate Reductase as Glutaredoxin HO2i." 146(3):1059–66.

Vance, I., Thrasher, D.R., 2005. Reservoir souring: mechanism and prevention, in: Ollivier, B., Magot, M., (eds.) Petroleum Microbiology. American Society for Microbiology. ASM press: Washington, D.C., pp. 123–142.

Videla, H.A., 1996. Manual of Biocorrosion, 1st ed. CRC-Press, pp 13–45.

Vik EA, Janbu AO, Garshol FK, Henninge LB, Engebretsen S, Kuijvenhoven C, Oilphant D, Hendriks WP. 2007. Nitrate -based souring mitigation of produced water side effects and challenges from the Draugen p roduced water reinjection pilot . SPE - 106178 -MS. Society of Petroleum Engineers, Richardson, TX .

von Wolzogen Kuehr, C. A. H., & von der Vlugt, L. (1934). The graphitization, of cast iron as an electrochemical process in anaerobic soils. Water, 18, 147–165.

Wang, H., Ju, L. K., Castaneda, H., Cheng, G., and Newby, B. M. Z. (2014). Corrosion of carbon steel C1010 in the presence of iron oxidizing bacteria *Acidithiobacillus ferrooxidans*. *Corros. Sci.* 89, 250–257. doi: 10.1016/j.corsci. 2014.09.005

Windt, Wim De, Nico Boon, Steven D. Siciliano, and Willy Verstraete. 2003. "Cell Density Related H 2 Consumption in Relation to Anoxic Fe (0) Corrosion and Precipitation of Corrosion Products by Shewanella Oneidensis MR-1." 5:1192–1202.

Xu, Dake and Tingyue Gu. 2014. "Carbon Source Starvation Triggered More Aggressive Corrosion against Carbon Steel by the Desulfovibrio Vulgaris Biofilm." *International Biodeterioration and Biodegradation* 91:74–81.

Xu, Dake, Yingchao Li, Fengmei Song, and Tingyue Gu. 2013. "Laboratory Investigation of Microbiologically Influenced Corrosion of C1018 Carbon Steel by Nitrate Reducing Bacterium Bacillus Licheniformis." *Corrosion Science* 77:385–90.

Xu, D., Gu, T., 2011. Bioenergetics explains when and why more severe MIC pitting by SRB can occur, in: Corrosion/2011 Paper No 11426. NACE International, Houston, TX.

Xu, D., Li, Y.,Gu, T., Mechanistic modeling of biocorrosion caused by biofilms of sulfate reducing bacteria and acid producing bacteria, Bioelectrochemistry. 110 (2016) 52–58.

Xu, D., Li, Y., Song, F., & Gu, T. (2013). Laboratory investigation of microbiologicallyinfluenced corrosion of C1018 carbon steel by nitrate reducing bacterium Bacillus licheniformis. Corrosion Science, 77, 385–390.

Yang, D., 2016. Mechanism and mitigation of biocorrosion by nitrate reducing PA01 agiant stainless steel. https://etd.ohiolink.edu.

Yamanaka, T., and Okunuki, K., Biochim. Biophys. Acta, 67 (1963) 379.

Yoon, S. S., Hennigan, R. F., Hilliard, G. M., Ochsner, U. A., Parvatiyar, K., Kamani, M. C. et al., (2002). Pseudomonas aeruginosa anaerobic respiration in biofilms: relationships to cystic fibrosis pathogenesis. Developmental Cell, 3(4), 593–603.

Yuan, S. J., & Pehkonen, S. O. (2007). Microbiologically influenced corrosion of 304 stainless steel by aerobic Pseudomonas NCIMB 2021 bacteria: AFM and XPS study. Colloids and SurfacesB:Biointerfaces,59(1),87–99.

Yuan, S. J., Pehkonen, S. O., Ting, Y. P., Kang, E. T., & Neoh, K. G. (2008). Corrosion behavior of type 304 stainless steel in a simulated seawater-based medium in the presence and absence of aerobic Pseudomonas NCIMB 2021 bacteria. Industrial & Engineering Chemistry Research, 47(9), 3008–3020.

Yoon, M. Y., Lee, K. M., Park, Y., & Yoon, S. S. (2011). Contribution of Cell Elongation to the Biofilm Formation of Pseudomonas aeruginosa during Anaerobic Respiration. PLoS ONE, 6(1), e16105.

Zhang, Peiyu, Dake Xu, Yingchao Li, Ke Yang, and Tingyue Gu. 2015. “Bioelectrochemistry Electron Mediators Accelerate the Microbiologically in Fl Uenced Corrosion of 304 Stainless Steel by the Desulfovibrio Vulgaris Bio Fi Lm.” 101:14–21.

Zumft, W.G., Kroneck, P.M.H., Respiratory transformation of nitrous oxide (N_2O) to dinitrogen by Bacteria and Archaea, Adv. Microb. Physiol. 52 (2007) 107–227.

List of publication

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Appendix: Supplementary information

Table SI-1. Psychochemical Characteristic of Crude Oil (Akita, Japan) (Kamarisima et al. 2018)

Oil depth	350-1750 m
Sulfur contain	0.3 %
American Petroleum Institute (API) gravity	$27.0\text{-}37.2^0$

Table SI-2. Psychochemical Characteristic of Oil field water (OFW) (Akita, Japan) (Kamarisima et al. 2018)

pH	7.93
SO_4^-	20.2 mg/L
NO_3^-	15.0 mg/L
Lactate	18.3 mg/L
Formate	< 2.3 mg/L
Acetate	< 2.3 mg/L

Table SI-3. Psychochemical Characteristic of natural seawater (Ogasawara Island, Japan) (Kamarisima et al. 2018)

pH	8.2
Phosphate concentration	0.1 μM
Salt concentration	37 ‰
Specific gravity	1.027
SO_4^-	27-28 mM
Acetate	< 2.3 mg/L

Table SI-4. Trace element A

Reagent	Amount (per L)
EDTA, 2Na salt	5200 mg
H_3BO_3	10 mg
$MnCl_2 \cdot 4H_2O$	5 mg
$FeSO_4 \cdot 7H_2O$	2100 mg
$CoCl_2 \cdot 6H_2O$	190 mg
$NiCl_2 \cdot 6H_2O$	24 mg
$CuCl_2 \cdot 2H_2O$	10 mg
$ZnSO_4 \cdot 7H_2O$	144 mg
Distilled water	1000 mL

Table SI-5. Trace element B

Reagent	Amount (per L)
NaOH	400 mg
$NaSeO_3 \cdot 5H_2O$	6 mg
$NaMoO_4 \cdot 2H_2O$	36 mg
$Na_2WO_4 \cdot 2H_2O$	8 mg
Distilled water	1000 mL

Table SI-6. Vitamin mixture solution

Reagent	Amount
Sodium phosphate buffer (10mM, pH 7.1)	100 mL
4-Aminobenzoic acid	4 mg
D(+)-Biotin	1 mg
Nicotinic acid	10 mg
D(+)-Pantothenic acid, Ca-salt	5 mg
Pyridoxine dihydrochloride	15 mg

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