SULFATE REDUCING BACTERIA, NITRATE REDUCING BACTERIA AND THEIR INTERACTIONS IN A SOURING OFFSHORE OIL RESERVOIR SYSTEM

By

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ABSTRACT

The troublesome souring issues, especially those occurred in offshore oilfields, have plagued petroleum and environmental industries for decades. To control reservoir souring, the nitrate addition have been noticed in recognition of their safety and operational effectiveness. The interactions between sulfate reducing bacteria (SRB) and nitrate reducing bacteria (NRB) are key mechanisms in the nitrate-mediated souring control. However, much is still unknown towards the effective profiling of SRB and the detailed NRB-SRB interactions. Although NRB produced biosurfactants might be promising bio-agents affecting NRB-SRB interactions, very limited studies tackled the production of biosurfactants by natural NRB strains. Systematic investigation of their unique roles in enhancing NRB competence over SRB was not documented. This thesis targeted on filling the above stated gaps and examined SRB, NRB and their interactions in a souring offshore oil reservoir system.

A method based on phospholipid fatty acid (PLFA) profiling of microbial communities in offshore produced water was developed and optimized. The developed method was further applied to profile microorganisms and trace SRB. Biosurfactant producing NRB was isolated and the associated biosurfactant product was used for tracking NRB-SRB-biosurfactant interactions.

The outputs of this thesis include: (1) the established PLFA based protocol for profiling SRB in offshore reservoirs; (2) the successful isolation and identification of biosurfactant producing NRB coupled with subsequent biosurfactant generation and characterization; and (3) the findings to confirm, for the first time, that NRB-produced biosurfactants could significantly strengthen SRB inhibition by NRB. The thesis has resulted in promising products and scientific observations for aiding souring control in the challenging offshore reservoir environments.

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LIST OF SYMBOLS AND ABBREVIATIONS

ATR	attenuated total reflectance
BATH	bacterial adhesion to hydrocarbons
BCFA	branched saturated fatty acids
BLAST	basic local alignment search tool
CLPP	community-level physiological profiling
CMC	critical micelle concentration
CMD	critical micelle dilution
CoA	coenzyme A
CSB	Coleville synthetic brine
DCM	dichloromethane
DIC	dissolved inorganic carbon
DO	dissolved oxygen
DOC	dissolved organic carbon
DSR	dissimilatory sulfate reductase
Eh	redox potential
EI	electron ionization
EI24	emulsification index
ELDS	enhanced laser diode spectroscopy
EOR	enhanced oil recovery
FAME	fatty acid methyl ester

FID	flame ionization detector
FISH	fluorescence in situ hybridization
FT-IR	fourier transform infrared spectroscopy
GC	gas chromatography
GC-MS	gas chromatography coupled with mass spectroscopy
HE	hydrogen embrittlement
HIC	hydrogen induced cracking
hNRB	heterotrophic nitrate reducing bacteria
HRI	highly reactive intermediates
H_2S	hydrogen sulfide
LOD	limits of detection
MEOR	microbially enhanced oil recovery
MIC	microbially induced corrosion
MPN	most probable number
MUFA	monounsaturated fatty acids
NMR	nuclear magnetic resonance
NRB	nitrate reducing bacteria
NR-SOB	nitrate reducing, sulfide oxidizing bacteria
NL	Newfoundland and Labrador
NRPOP	Northern Region Persistent Pollution Control
OD	optical density
PC	phosphocholine
PC1	the first principal component

PC2	the second principal component
PCA	principal component analysis
PCR	polymerase chain reaction
PE	phosphoethanolamine
PLFA	phospholipid fatty acid
PUFA	polyunsaturated fatty acids
PWRI	produced water re-injection
r^2	coefficients of the calibration curves
RF	response factors
RNA	ribonucleic acid
RSD	relative standard deviation
SDS	sodium dodecylbenzene sulfonate
SIM	selective ion monitoring
SPE	solid phase extraction
SOB	sulfur oxidizing bacteria
SOHIC	stress orientated hydrogen induced cracking
SRB	sulfate reducing bacteria
SSCC	sulfide stress corrosion cracking
SSFAs	straight-chain saturated fatty acids
TDC	total dissolved carbon
TLC	thin layer chromatography
UV	ultra violet
VFAs	volatile fatty acids

CHAPTER 1

INTRODUCTION

1.1 Background

Oil reservoirs are of great importance to the current global economic development and thus considerable efforts have been placed to exploit and recover the petroleum resources. Generally, oil recovery activities by petroleum industry can be divided into 3 classes: primary, secondary and tertiary. These recovery methods follow a natural progression of oil production from the start to a point where it is no longer economical to produce from a hydrocarbon reservoir (Muggeridge et al. 2014). When primary oil recovery becomes no longer feasible, secondary oil recovery commences. A common method of secondary oil recovery is using water injection (Figure 1.1) allow more oil to be recovered. Water flooding techniques are frequently utilized for these operations in which seawater or other water is injected into the reservoir to maintain pressure level underneath and sweep the oil from the reservoir towards producing wells (Gieg et al. 2011). This process is where reservoir souring originates; there is sulfate in the injection water and organic electron donors in the oil phase mix in the near injection wellbore region, stimulating sulfate reducing bacteria (SRB) (Callbeck et al. 2011), which is the main cause of reservoir souring.

Reservoir souring, defined as the increase of mass of H_2S per unit mass of total produced fluids in a reservoir, is a growing concern for the petroleum production industry (Tanji et al. 2014). H_2S is a poisonous, dense gas with serious safety implications. It can lead to sudden catastrophic failure of nonresistant metallic materials from sulfide stress corrosion cracking or hydrogen-induced cracking (Usher et al. 2014). Reservoir souring can be caused by biogenic or abiotic paths occurring both in terrestrial and offshore oil production operations. As stated before, the primary cause of microbial induced offshore reservoir souring is the growth and activity of SRB near the zone of seawater injection.

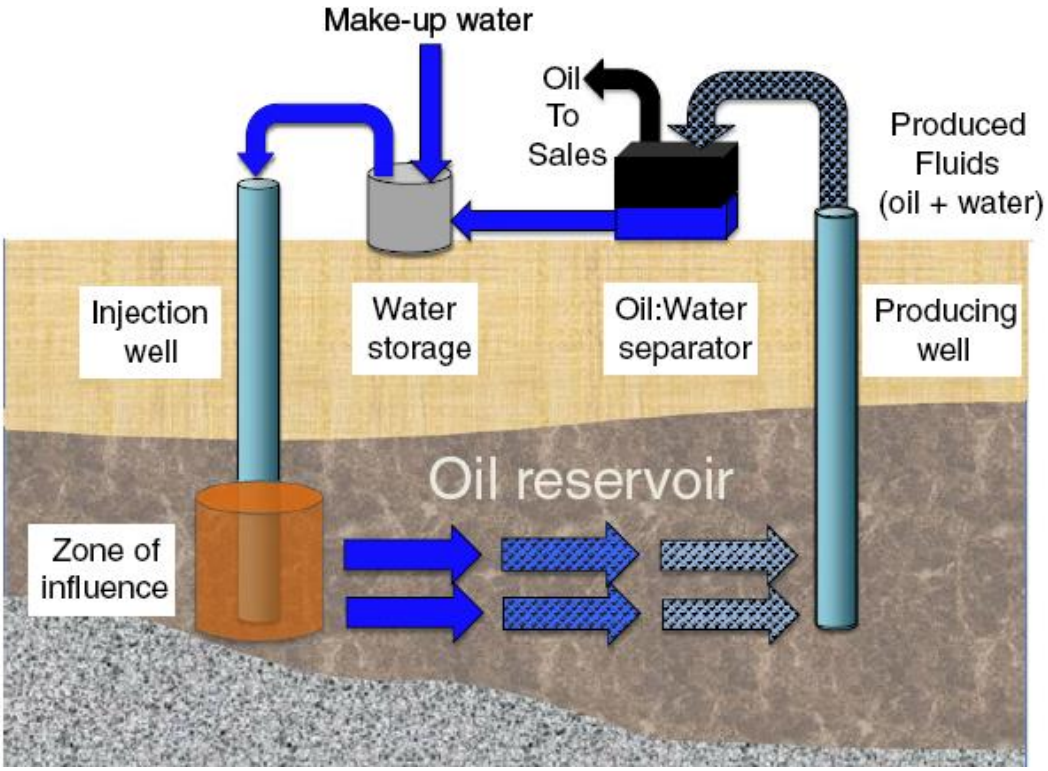


Figure 1.1 Schematics for water re-injection process

Source: (Gieg et al. 2011)

The presence of sulfate is one of the main causes as it is sufficient in offshore injected seawater. Carbon sources, as well as other nutrient sources in reservoirs, also influence the rate of sulfate reduction by SRB. Several studies show that in water flooded reservoirs, volatile fatty acids (VFAs, acetate, butyrate and propionate) and labile hydrocarbons such as alkanes and monoaromatics (e.g., toluene) are the biggest carbon sources in oilfield fluids for SRB growth (Grigoryan et al. 2009; Grigoryan et al. 2008). These readily metabolized carbon sources frequently presented in the injection water used for water flooding provide carbon sources for the SRB in the vicinity of the injection well (Cavallaro et al. 2005; Grigoryan et al. 2008). Although concentrations of nitrogen sources as nutrients are typically sufficient in oil reservoirs, phosphorous concentrations are usually low, potentially limiting *in situ* microbial metabolism (Head et al. 2003).

There are different microbial communities in each oil reservoir depending on conditions such as temperature, availability of substrates, salinity, and chemical compositions. Low-temperature reservoirs facilitate the growth of mesophilic bacteria while high-temperature reservoirs are typically dominated by thermophilic bacteria (Lin et al. 2014). If the temperature is higher than 100 ºC, the reservoir can naturally constrain the growth of any SRB (Gieg et al. 2011). Souring can still occur in hot reservoirs of >100 ºC in the vicinity of the water injection well. This is because the relatively cool water injection displaces the hot fluids from the zone of injection, resulting in more favorable conditions for SRB growth (~50-70 ºC) (Gieg et al. 2011). In addition, abiotic reactions are also of great importance in the production of H_2S due to complex reactions (Mueller and Nielsen 1996; Seto and Beliveau 2000). Specially, many iron-containing minerals are capable of reacting with H_2S forming iron sulfide, pyrite, or pyrrhotite for H_2S scavenging. The H_2S adsorption capacity of the iron-bearing rock in the offshore reservoir is closely related to the breakthrough of reservoir souring.

H_2S lowers air quality and can be lethal to humans when high concentrations are inhaled; it can easily escape the contaminated reservoir and may accumulate in a poorly vented area, such as produced water tanks, flow lines, etc. A maximum of eight hours exposure to concentrations greater than 100 ppm H_2S will cause hemorrhage and death (Ballerino-Regan and Longmire 2010). Concentrations above 600 ppm can be fatal in three to five minutes. In extreme cases of souring the well may ultimately be shut down due to dangerous levels of the gas.

Sulfide is also closely correlated with corrosion issues. SRB are commonly considered the main culprits of microbially induced corrosion (MIC), especially in anoxic, sulfate-rich environments (Enning and Garrelfs 2014). The corrosion intensity level of co-produced water from an oil reservoir can change over its lifetime. This is particularly marked in fields that are initially considered clean, but produce more H_2S in later life, in some cases at concentrations of up to many thousands of parts per million by volume in the gas phase. Whilst carbon dioxide can cause very severe corrosion (i.e., general and pitting) of steels, H_2S corrosion is more localised, and can cause sulfide stress corrosion cracking (SSCC), hydrogen embrittlement (HE), hydrogen induced cracking (HIC) or stress orientated hydrogen induced cracking (SOHIC) (Ziaei et al. 2013). Hence, increasing H_2S will not necessarily cause a pro-rata increase in general corrosion rate, but rather lay susceptible materials prone to catastrophic failure. Highly stressed, high strength steel can fail in a matter of minutes in the presence of 50 ppm H_2S. At high pressure, as little as 0.1 ppm H_2S can greatly reduce the time to failure of highly stressed, high strength steel (Amosa et al. 2013).

Besides, sulfide generation by SRB lowers oil quality and increase the cost of oil refinement. Reservoir plugging due to the precipitation of ferrous sulfide slows the flow rate of water into the reservoir reducing the efficacy of water injection as a secondary recovery method and hinders the

production speed of the well. The safety, corrosion, reduced oil quality and reservoir plugging issues will all ultimately lead to higher operational costs of offshore oil production.

Due to the negative impacts of sulfide production in the three aspects stated above, mitigation strategies are highly desired by the offshore oil and gas industry for the control of sulfide generation and its release into the environment (Tang et al. 2009). Till now, various active and passive measures have been proposed for reservoir souring mitigation, such as reverse osmosis or membrane filtration to remove sulfide and carbon nutrients in injection water, sulfide scavengers addition, the precipitation of metal sulfides and molybdate amendment (Gieg et al. 2011). The methods listed may encounter effectiveness or cost issues in offshore reservoir souring control. For this reason, biocides and nitrate/nitrite are commonly used in practical applications.

Compared with the application of nitrate/nitrite, chemical components in the reservoir may scavenge biocides through reaction and may limit the application depth of biocides (Nemati et al. 2001). In addition, biocide treatments are difficult and expensive to deliver at sufficient concentrations to the active souring zone as the enormous surface area of reservoir rock provides ample sites for biocide sorption (Ezeuko et al. 2013). In contrast, nitrate and nitrite are very mobile chemicals in subsurface environments and do not adsorb to most porous materials. Thus, nitrate/nitrite transport should not be a limiting factor. Previous studies indicate that nitrate/nitrite addition can be more effective in achieving longer periods of souring inhibition when compared to biocides (Gieg et al. 2011; Reinsel et al. 1996). Therefore, nitrate/nitrite with convenient (high solubility in water and compatibility with other chemicals), inexpensive and environmentally friendly features has attracted increased attention from researchers in offshore reservoir souring control.

The application of nitrate/nitrite could be very effective for reservoir souring control, by promoting nitrate reducing bacteria (NRB), consuming nutrients that SRB require to grow, and thus inhibiting SRB activity (Gieg et al. 2011). This treatment could be particularly effective and may extend remaining production life under conditions of relatively low VFA content in produced water and relatively short mean residence time of injection water (Stott 2012). Nitrite is more preferable than nitrate for souring prevention in some high-temperature oil fields as it reacts directly with sulfide and provides long-lasting inhibition of sulfate reduction (Kaster et al. 2007). Reinsel et al. (1996) found that glutaraldehyde (a type of biocide) did not have any long-term inhibitory effects, but instead lowered the SRB population. After glutaraldehyde removal, SRB reportedly multiplied at their original growth rate and simultaneously produced H_2S. On the other hand, H_2S concentration began to increase six days after nitrite was removed from the column. This suggests that SRB were not killed by nitrite, but rather were still inhibited after nitrite was removed; the original number of cells was still present and these were able to produce H_2S once the inhibitory effect was removed (Reinsel et al. 1996).

Effectively addressing souring-related issues requires careful consideration of the operational conditions encountered in specific offshore environments. Prior to the application of nitrate/nitrite injection treatment, a better understanding is needed of the reservoir souring process, especially the nature of NRB-SRB interactions. It's thus highly desired to track the activities of SRB and NRB, and to investigate their interactions in reservoirs, thus providing effective aid in offshore reservoir souring control.

1.2 Statement of Problems

Unlike inland conditions, offshore reservoir souring control by nitrate amendment may encounter many difficulties, which include the harsh marine environment, remote location, limited platform space and the need for remote/unmanned control systems. As a result, the approaches and technologies that are used to address souring in onshore operations may not be effective in dealing with souring in offshore operations. The major problems have been identified and stated below:

1) <u>Lack of routine determination tools for microbial analysis of offshore reservoir samples</u>

Produced water is a mixture of original water from different geological formations and the liquids injected into the hydrocarbon zone, it could be used as a mirror to reflect the undergoing chemical and biological activities beneath the seabed as a result of offshore oil and gas operations (Li et al. 2007a). SRB are responsible for the bacterial problems (Hubert and Voordouw 2007), thus the profiling of these microbial groups from produced water is highly desired for reservoir souring control. To measure microbial diversity and biomass, culture-independent methods provide obvious advantages over culture techniques. The latter ones are generally time-consuming, labor-intensive and most of the microorganisms are still recalcitrant to cultivation (Zengler 2009). As a culture-independent technique, phospholipid fatty acid (PLFA) analysis has the potential to be an inexpensive and quantitative method for microbial profiling of a large number of complex samples. PLFAs have been extensively applied as biomarkers to characterize microorganisms in a variety of solid and aqueous environmental samples (Dijkman et al. 2010; Drenovsky et al. 2010; Mills et al. 2006; Yu et al. 2009).

The performance of PLFA analysis is highly depended on the specific matrix extracted. To date, the microbial profiling of offshore produced water with a complicated matrix and high salinity using PLFA analysis is with extremely limited documentation. To examine the performance of PLFA analysis for microbial profiling of produced water, operation conditions during extraction, purification and derivatization of fatty acid methyl esters (FAMEs) in previous studies are not directly applicable and need to be further evaluated. The extraction steps, parameters of phospholipid purification as well as FAME derivatization are required to be further examined.

2) <u>Unclear SRB transformation patterns in offshore reservoir wells</u>

PLFA analysis offers a great potential of microbial community analysis in routine environmental monitoring. Boschker et al. (2001) investigated the bacterial populations and pathways involved in acetate and propionate consumption in anoxic brackish sediment. Labeled acetate and propionate (^{13}C) were incorporated into PLFAs after incubation and the results showed that they were predominantly consumed by different, specialized groups of SRB. Uranium-bearing sandstones from the Dongsheng deposit were found with the abundant presence of C15-C18 fatty acids (Jiang et al. 2012). Characteristic biomarkers of SRB *Desulfovibrio* and *Desulfobacter sp*. were found and involved in bacterial sulfate reduction to sulfide. Even though PLFA profiling has been used as SRB biomarkers in various solid and fluid samples, it is rarely applied in offshore reservoir water analysis to specifically elucidate the mechanism of reservoir souring induced by SRB. There is still a lack of basic understanding of the complex biomass and microbial community structure information from the various reservoir conditions regarding souring.

3) <u>Insufficient mechanism studies for NRB-SRB interactions</u>

NRB are well known for their denitrifying capacity in which nitrates or nitrites are converted into nitrogen-containing gases. This function enables NRB to play significant roles in the mitigation

and control of sulfide induced reservoir souring problems in offshore oil fields (Gieg et al. 2011). Although nitrate-mediated souring control has been extensively studied in the laboratory (Callbeck et al. 2011; Chen et al. 2017; Zhao et al. 2009) and in the field (Bodtker et al. 2008; Shartau et al. 2010; Voordouw et al. 2009), much is still unknown about the detailed microbial mechanisms involved in NRB-SRB interactions during nitrate/nitrite injections for reservoir souring mitigation.

Hui et al. (2012) evaluated the microbial community structure and functionally distinct groups in three kinds of produced water samples from Daqing oil reservoir. The isolates affiliated to *Pseudomonas stutzeri* PTG4-15 (DP26, BP39, and PW5) were initially identified as NRB, biosurfactant producing bacteria, and polymer-producing bacteria. Fallon et al. (2010) confirmed that biosurfactants can be naturally derived from NRB. As microorganisms capable of utilizing hydrocarbons as carbon and energy sources, NRB will produce surface-active agents as by-products to facilitate hydrophobic degradation (Ron and Rosenberg 2002). The biosurfactants can enhance the competence among species by increasing the bioavailability of entrapped organics in the porous media (Pacwa-Płociniczak et al. 2011). The specific biosurfactants might also repress the growth of certain targeted strain through their antimicrobial properties (Rodrigues et al. 2006). Besides, as possible combination agents in biofilm matrix formation (Osterreicher-Ravid et al. 2000), biosurfactants could synergistically improve the bacterial adaptation capability to harsh environments. The interactions of SRB and NRB are of great importance for reservoir souring control by nitrate amendment. Meanwhile, biosurfactants produced by natural NRB are promising bio-agents for enhancing NRB competence over SRB. However, biosurfactant producing NRB isolated from oil reservoirs have been rarely reported, and associated biosurfactant production is extremely limited in the literature (Zhao et al. 2016). Till now, there has been no published study

tackling the systematic investigation of NRB-SRB interactions with the involvement of biosurfactants produced by natural NRB.

1.3 Research Objectives

The specific objectives of this research, therefore, are to develop a monitoring technique used for routine analysis of samples with high oily and salinity properties in the marine environment, and fill knowledge and technical gaps in biosurfactant-aided nitrate injection techniques for souring remediation. The major research tasks include:

1) to develop a cost-efficient SRB quantification methodology for their profiling in offshore oil reservoirs;

2) to profile microorganisms and trace indigenous SRB populations in water samples from an offshore oil reservoir;

3) to screen NRB species from the produced water samples after the reservoir was injected with nitrate/nitrite to stimulate the growth of indigenous nitrate reducing microorganisms;

4) to isolate and evaluate the performance of biosurfactant producing NRB, and subsequently produce and characterize the biosurfactant product through the metabolism of NRB; and

5) to investigate the NRB-SRB interactions under various nitrate and biosurfactant treatments, track the associated microbial community structure changes and elucidate the mechanisms involved in the processes.

1.4 Structure of the Thesis

Chapter 2 focused on the comprehensive review of reservoir souring mechanisms, the monitoring techniques used for microbial control and detection, and the current technology advances for controlling souring in hydrocarbon reservoirs. The application of biosurfactants in reservoir system and remediation of reservoir souring was also discussed in detail.

Chapter 3 tackled the method development of PLFA analysis for profiling microbial communities in offshore produced water. The elution parameters in solid phase extraction (SPE) purification were adapted for treating the oily samples and their volumes were determined to induce a high recovery for the fraction of phospholipids. The impact of parameters including alkaline reagent, the volumes of acid used for neutralization, the time and temperature for transesterification and the analytical performance of GC-MS were studied.

Chapter 4 provided PLFA profiles of microorganisms and SRB in produced water samples from an offshore oil reservoir. The presence of SRB and SOB species and their relationship with the redox environment of the reservoir wellbores was discussed. The species distribution patterns were interpreted to elucidate the biological souring process.

Chapter 5 presented the isolation of NRB from an offshore reservoir and the associated biosurfactant production. The possible biosurfactant producers were screened and the associated biosurfactant production and characterization by the isolates were conducted.

Chapter 6 described the interactions of SRB, NRB screened from the offshore oil reservoir and NRB produced biosurfactants in microcosms under non-sour and sour conditions. Various nitrate and biosurfactant treatments were applied in the NRB-SRB interaction while using PLFA

biomarkers to trace the community responses. The potential of NRB produced biosurfactants in the nitrate-dependent suppression of SRB activities and souring control was presented.

Chapter 7 concluded this study with summarized contribution and recommendations for future research.

CHAPTER 2

LITERATURE REVIEW

2.1 Mechanisms of Offshore Reservoir Souring

2.1.1 Routes of H_2S generation in offshore reservoirs

There are two types of H_2S generation, abiotic meaning not living, and biogenic which comes from SRB. Some offshore reservoirs are sour due to non-biogenic mechanisms that include the thermophilic decomposition of sulfur containing hydrocarbons, the dissolution of pyrite or thermochemical sulfate reduction. These mechanisms are influenced by the nature of reservoir rock, oil composition and thermal maturity, high temperatures and aquathermolysis in thermal recovery operations (Frazer and Bolling 1991; Khatib and Salanitro 1997; Seto and Beliveau 2000). Most biogenic souring in offshore oil reservoirs comes from the production of H_2S through the respiration of SRB.

In iron-deficient reservoirs, particularly in carbonate reservoirs associated with evaporates, an abiotic mechanism that gives rise to H_2S presence is the thermochemical reduction of sulfate, which happens at temperatures above 100ºC. The flow of hydrogen sulfide from other geological formations to initially sweet reservoirs, either because of geomechemical processes resulting from reservoir depletion, or of failure in well cementations, are examples of abiotic H_2S appearance later in the field exploitation stage (Al-Eid et al. 2001). Reduction of the H_2S solubility in the water phase resulting from the reservoir pressure decrease can also be considered a late abiotic reservoir souring mechanism (Seto and Beliveau 2000).

Heterotrophic SRB get energy for live and growth by oxidizing carbon sources (electron donors) with the reduction of respired sulfate (electron acceptor) to sulfide. Microbial H_2S generation depends on various concurrent factors that allow the development of an active population of SRB. Adequate ranges of temperature, pressure, water salinity, pH and redox potential; availability of

sulfate and of water-soluble organic carbon; provision of other nutrients, mainly nitrogen and phosphorus, required for SRB biomass growth (Sunde and Torsvik 2005).

Thermochemical sulfate reduction

Thermochemical sulfate reduction is recognized as common and widespread geochemical mechanism for souring by many case studies and theoretical reviews (Machel 2001). Thermochemical sulfate reduction is a feasible mechanism evidenced by the Leblanc process for soda manufacture, in which a mixture of sodium sulfate and coke is heated for a considerable period at high temperature. However, the strongly endothermic process is normally operated at 1000 ºC and is not significant below 700 ºC. Sulfate is sufficiently present in injected seawater and yields the large quantities of observed H_2S in produced fluids. There are also substances present, particularly in the oil which could be reducing agents for this process and it has been shown that clay and other minerals are a rich source of catalysts for a wide variety of reactions. Perhaps under milder conditions than usually employed in the Leblanc process it is possible with this combination for such a reaction to proceed.

According to petroleum geologists, high H_2S concentration accumulated in natural environments (‘sour gas’ fields) are formed when sulfate is reduced by petroleum (mostly methane and the n-alkanes) at depths usually greater than 3000 m (Anderson and Thom 2008). Although the lower thermal limit for thermochemical sulfate reduction is somewhat controversial, many studies generally consider the minimum temperatures range from 100 to 140 ºC (Cai et al. 2003). In a temperature of below 140 ºC, thermochemical sulfate reduction is very slow or inhibited, even though the involved reactions are characterized by large negative free energy changes of reaction (Machel 2001). Under proper reservoir conditions, both elemental sulfur and polysulfides are

capable of oxidizing some organic molecules under basic conditions (Goldstein and Aizenshtat 1994). Sulfate alone will not react only if lower oxidation state sulfur is present.

Thermal decomposition

Thermal decomposition of organic sulfur compounds which are present in some crude oils is another mechanism which may lead to the production of relatively low concentration of H_2S (Worden and Smalley 1996). The disadvantage of such mechanisms is that they require high temperatures and are not associated with sulfate reduction, nor are they related to seawater injection. Generally, the H_2S production by thermal decomposition of organics appears to be proportional to the sulfur content of the oil (Ritchie et al. 1985). Organic sulfur ranging from the very stable aromatic sulfides to very unstable thiocarbamates play different roles in the kinetics of H_2S production. Under proper thermal conditions, disulfides and thiols have been recognized as the most reactive sulfur species, followed by aliphatic sulfides, thiophenic compounds (Cortese-Krott et al. 2017; Giles et al. 2003; Kelemen et al. 1991). Meawhile, benzothiophenic compounds appear to be the most stable organically bound sulfur species during thermal decomposition of organic sulfur compounds.

An example of thiocarbamate hydrolysis is:

$$RCS_2R + 3H_2O \rightarrow RCO_2H + 2H_2S + ROH \quad (2\text{-}1)$$

and thioether reduction:

$$RSR \rightarrow 2RH + H_2S \quad (2\text{-}2)$$

Dissolution of pyritic materials

Pyrite, FeS_2, is widely distributed in formation rocks and therefore has to be considered as (Gallego-Torres et al. 2015), although it is not obvious why the process should not have been proceeding during the prehistory of the field. Pyrite contained in reservoir rock can be leached out as particles of small dimension which may react with the environment according to the following reactions:

$$FeS_2 + 8H_2O \rightarrow Fe^{2+} + 2SO_4^{2-} + 16H^+ + 14e^- \quad (2\text{-}3)$$

or

$$FeS_2 + 4H^+ + 2e^- \rightarrow Fe^{2+} + 2H_2S \quad (2\text{-}4)$$

Pyrite oxidation (2-3) is known to be a slow process which requires the presence of an oxidant such as oxygen or the involvement of oxidizing bacteria (Ma and Lin 2013). The reaction leads to an acidic environment, which favors the formation of sulfide in petroleum reservoirs. Pyrite reduction (2-4) is very possible at lower pH values. The theoretical calculation of the reaction progress of (2-4) is very complex. Literature values for the pK of the solubility product for iron sulfide vary from 16.9 to 18.8, which would reflect a 100 fold variation in calculated H_2S concentration (Eden et al. 1993). The pyrite reduction under acidic conditions could be a route, and in the case of seawater injection (with the presence of sufficient sulfate) it is more likely route.

Redox reactions involving bisulfite oxygen scavengers

This mechanism has been suggested by a number of oil companies (Lasebikan et al. 2010). Oxygen scavengers used in injection waters by the oil and gas industry in water flood and production systems invariably comprise sulfite, and in many cases, ammonium bisulfite. These compounds are redox poising agents as corrosion inhibitors and are known to remove oxygen and

stimulate the growth of SRB. The rise of H_2S is not clearly associated with bisulfite injection by purely chemical reactions. However, a relationship between the content of sulfide measured and increase in ammonium bisulfite concentration in brine fluids at low pH was observed (Lasebikan et al. 2010). As only a relatively low concentration of bisulfite is injected into the reservoir, they may function as weak catalysts to give rise to high levels of H_2S. These compounds typically react completely with oxygen to yield sulfate, but the sulfate yields are negligible compared with the sulfate concentration in seawater (2650 mg/L) (Eden et al. 1993):

$$2NH_4HSO_3 + O_2 \rightarrow (NH_4)_2SO_4 + H_2SO_4 \qquad (2\text{-}5)$$

In a system where bisulfite injection are well operated, the excess ammonium bisulfite injected into reservoirs is generally less than l mg/L (Eden et al. 1993). Thus, it is not likely to be the principal sulfur source for hydrogen sulfide, although it is considerably easier to reduce to sulfide than the sulfate ion. It is more likely that the bisulfite could be involved either as a catalyst in the conversion of some other sulfur-containing substance, or that it is modifying the surface of an inert sulfur-containing solid in the reservoir so making it more reactive, thus generating H_2S (Eden et al. 1993). Since the reactivity of metal sulfide products varies significantly depending on their crystalline form and especially the nature of the surface, this possibility is promising.

2.1.2 Roles of SRB in offshore reservoir souring

Souring in offshore oilfield systems is heavily due to SRB, a diverse group of nonpathogenic, anaerobic microorganisms that respire sulfate to produce hydrogen sulfide. However in order for this to occur, there must be free electrons as an external energy present. Such biological souring is a detrimental, widespread phenomenon in the petroleum industry, occurring in offshore and onshore facilities in a wide range of growth conditions. In high temperature reservoirs most of the

souring will occur mainly in offshore facilities (topside processing of injection water) and in close proximity to the injection site where the hot reservoir fluids mix with relatively cool injection water resulting in more ideal conditions for SRB activity. Sulfate reducers exist either indigenously in deep subsurface reservoirs or can be "inoculated" into a reservoir system during oilfield development or the oilfield production phase (Gieg et al. 2011). Souring happens when microorganisms enzymatically reduce sulfate, thiosalts, or sulfur to gain energy for growth using the dissimilatory sulfate reductase (DSR) enzyme (Chang et al. 2001). Sulfide is formed directly by the activities of SRB. These consist of at least two genera (*Desulfovibrio* and *Desulfotomaculum*) of obligate anaerobes which oxidize hydrogen and organic compounds using sulfate by the DSR enzyme (Larsen et al. 2000). This DSR is apparent in mud at pond bottoms, in bogs and on the sea-bed. Sulfate concentration is high in seawater and consequently its reduction is an important factor in H_2S production. The H_2S formed in the biosphere is largely converted to sulfur: only a small part of it subsequently becomes isolated in the form of insoluble sulfides of heavy metals.

Desulfovibrio and *Desulfotomaculum* seem to be unrelated to each other and their relation to other bacterial groups is obscure. The better known genus *Desulfovibrio* is usually mesophilic and sometimes halophilic (preferring saline conditions) (Eden et al. 1993). *Desulfotomaculum* species are somewhat more difficult to isolate and purify. They are characterized by spore formation and are sometimes thermophilic.

2.2 Microbial Monitoring of Offshore Reservoir Souring

In order to effectively counter reservoir souring, its specific cause must be known. A vast majority of reservoir souring derives from microbial activity in the offshore reservoir. There are many

techniques for SRB quantification and characterizing the microbial community in general. To find the appropriate method of microbial characterization for in offshore reservoirs, details of commonly used microbiological, molecular biological and biochemical techniques must be known and compared with one another, weighing the pros and cons.

2.2.1 Microbial characterization methodologies

Microbiological methods

Metabolic assays

Community-level physiological profiling (CLPP) is a method that uses commercially available 96-well microtiter plates, usually consisting of 95 different carbon sources, nutrients, and a tetrazolium dye. The oxidation of the carbon substrate is concomitant with reduction of the dye. The carbon utilization patterns are then analyzed using mutivariant statistical techniques to evaluate the degree of similarity among environmental samples. Differences in sole carbon source utilization have been used to distinguish among different bacterial types for over 50 years (Garland 1997). The automated microbial identification system, Biolog, based mainly on aerobic metabolic activities, has contributed a great deal to our understanding of carbon source utilization (Muñiz et al. 2014). This rapid, community-level approach for assessing the utilization patterns of sole carbon sources is now being used to study microbial community dynamics. This approach has been widely used for assessing the relative similarity between aerobic heterotrophic microbial communities across spatial, temporal, and experimental gradients (Garland 1997).

Cell counting techniques

Cell counting techniques are methods of community characterization that cannot provide any information other than the number of cells in a community sample. These methods provide no information about community phylogeny, diversity or physiology when used alone. However, cell counting techniques are often used in conjunction with other methods that address these facets of community characterization to provide a more complete description of the community (Abbaci et al. 2008; Reed et al. 2002). The cell count of a community is one of the most basic characteristics of a community and is only a starting point for meaningful characterization.

Molecular biological methods

Polymerase chain reaction (PCR) -Based Gene Sequencing

PCR is a type of technique used in molecular biology to amplify segment of DNA from whole-cell extracts or from total community DNA of an environmental sample across several orders of to facilitate analysis (Spiegelman et al. 2005). DNA is exposed to a thermostable polymerase and is subject to repetitive cycles of template strand denaturation, oligonucleotide primer annealing, and polymerization of the template-primer duplex. This process results in the exponential amplification of the template DNA. The key to PCR is the use of oligonucleotide primers designed to be complementary to the desired gene or genetic region. During PCR, double-stranded DNA is separated into single strands at high temperature, a process known as denaturation. Two oligonucleotide primers then anneal to complementary regions of the denatured DNA, which flank the desired sequence. Following this, a heat-stable DNA polymerase creates a new strand of DNA by extending the primer, using the complementary strand as a template. A new cycle begins as this new double-stranded molecule is denatured again. Multiple repetitions of this cycle lead to an exponential amplification of the target gene(s) or genetic region (Kubista et al. 2006).

PCR is the simplest and currently the most widely used method to obtain 16S rRNA genes for detailed characterization of microbial communities. It is the foundation upon which most genetic polymorphism-based techniques are based. It is an effective way to inspect isolated genes of a SRB; however it must be coupled with denaturant gradient gel electrophoresis (DGGE) or other separation methods (temperature gradient gel electrophoresis, TGGE) for SRB quantification of large communities. Although the analysis of a microbial community by PCR and cloning provides a convenient and rapid alternative to some other culture-independent techniques, there are several factors that could skew diversity estimates (Farrelly et al. 1995). PCR reactions are very sensitive to reaction conditions and even duplicates might not give quantitatively identical results (Schneegurt and Kulpa, 1998). Due to the sensitivity and specificity of the PCR reactions, minor contamination can lead to false-positive signals and false-negative amplifications are often seen (Bossler and Van Deerlin 2009). Available technology does not allow for the separation of multiple bands amplified from a highly diverse bacterial community (Smith and Osborn 2009). Another concern of PCR when combined with DGGE is the assignment of particular bands to individual populations, particularly where multiple bands occur. Individual organisms could potentially contribute to multiple bands on a DGGE gel since sequences between rRNA operons of an individual organism can vary significantly (Boon et al. 2002).

Fluorescent In Situ Hybridization (FISH)

Fluorescence *in situ* hybridization (FISH) is a cytogenetic technique developed by biomedical researchers in the early 1980s (Langer-Safer et al. 1982). It enables *in situ* phylogenetic identification and enumeration of individual microbial cells by whole cell hybridization with ribosomal RNA targeted oligonucleotide probes, which are covalently mono-labeled with fluorescent dye molecules. This limits the sensitivity of the method and aggravates the use of FISH

for identification of prokaryotes with low ribosome content per cell. The intensity of fluorescent signals is correlated to cellular rRNA contents and growth rates, which provide insight into the metabolic state of the cells. FISH can be combined with flow cytometry for a high-resolution automated analysis of mixed microbial populations. The FISH method was used to follow the dynamics of bacterial populations in agricultural soils treated with s-triazine herbicides (Barra Caracciolo et al. 2010). A variety of molecular probes were used to target specific phylogenetic groups of bacteria such as α, β, γ, and δ subdivisions of *Proteobacteria* and *Planctomycetes.*

Biochemical methods

Bisbenzimidazole-CsCl-gradient fractionation

Bisbenzimidazole-CsCl-gradient fractionation is a method of DNA fractionation based on overall % G-C content, which produces a characteristic community profile of relative abundance of DNA vs. % G-C content. In this method, community DNA is exposed to bisbenzimidazole, a non-intercalating dye that preferentially binds to A + T regions of the DNA. This binding alters the buoyant density of the DNA in proportion to the amount of dye bound. This establishes a means of physically separating the DNA on the basis of G-C vs. A-T content (Holben and Harris 1995); when passed by centrifugation through a linear gradient of CsCl, the DNA–bisbenzimidazole complex separates linearly by buoyancy, i.e., by relative amount of bound bisbenzimidazole, i.e., by A-T/G-C content. Bisbenzimidazole is fluorescent under long-wave UV illumination, to a degree proportional to the amount of bisbenzimidazole present. This allows for the measurement of the relative abundance of DNA at each point in the CsCl gradient (i.e., at varying %G-C), thus establishing the parameters for the community profile.

Lipid analyses

Quinone profiling is a culture-independent lipid biomarker assay that uses the taxon-dependent specificity of microbial quinones to create a community profile with moderate taxonomic specificity. Quinones are essential lipid components of respiratory and photosynthetic electron transport systems in microorganisms. Quinone profiling is a biochemical method of fingerprinting entire microbial communities based on the distribution and relative abundance of various species of quinones in the community, a method of community analysis that predates all genetic fingerprinting techniques (Collins et al. 1979). Many molecular species of respiratory quinones can be characteristic of bacteria at the level of genera or higher-level taxa, depending on the species of molecule. Ubiquinones (a subgroup) are also used to identify genera of fungi, yeast, and yeast-like fungi (Kuraishi et al. 2000; Okada et al. 1996).

PLFAs and FAMEs are culture-independent lipid biomarker assays in which the nature and distribution of various membrane lipids are used to construct the phylogeny and metabolic activity profiles for a microbial community. Membrane lipids can potentially provide a great deal of information about the organisms from which they are derived. Microbes alter the lipid composition of their membranes in response to differing environmental conditions, for example by enhancing membrane fluidity by increasing the proportion of unsaturated fatty acids in response to cold temperatures (Okada et al. 1996; Zheng et al. 2011). As such, membrane lipids can provide information about the physiological states of a given microbe or community (Wixon and Balser 2013). Also, with the construction of reference libraries upon the variable microbial characteristics, taxonomic information can also be derived from the nature and distribution of various lipid subspecies isolated from a given microorganism.

2.2.2 PLFA with FAME quantification

2.2.2.1 Characteristics of PLFA

Phospholipids are the key component of cellular membrane in living cells. Viable microbes have an intact membrane containing fatty acids as components of its phospholipids, which are not found in storage products or in dead cells. Lipids usually make up less than 5% of the dry weight of bacteria and are both structurally and functionally diverse. The results from sediments and soils with substrate additions indicate that rapid changes in microbial community structure can be detected by changes in PLFA patterns (Frostegård et al. 2011). This suggests that PLFA analysis is suitable for detecting rapid changes in living populations. Taxonomically, fatty acids in the range C2 to C24 have provided the greatest information and are present across a diverse range of microorganisms (Banowetz et al. 2006).

Phospholipids consist of a single molecule of glycerol (3C alcohol), two OH groups of the glycerol are bound to the two fatty acid chains (hydrophobic tail) and one OH group is bonded to a phosphate group (hydrophilic head). Thus these lipids are asymmetric, having hydrophilic and hydrophobic regions and in the membrane they form a bilayer with hydrophilic ends towards the outer surface of the membrane and hydrophobic ends buried in the interior (Figure 2.1). PLFA can be classified into ester-linked phospholipid fatty acids (EL-PLFAs, 60–90% of the total) and non-ester linked phospholipid fatty acids (NEL-PLFAs, 10–40% of the total).

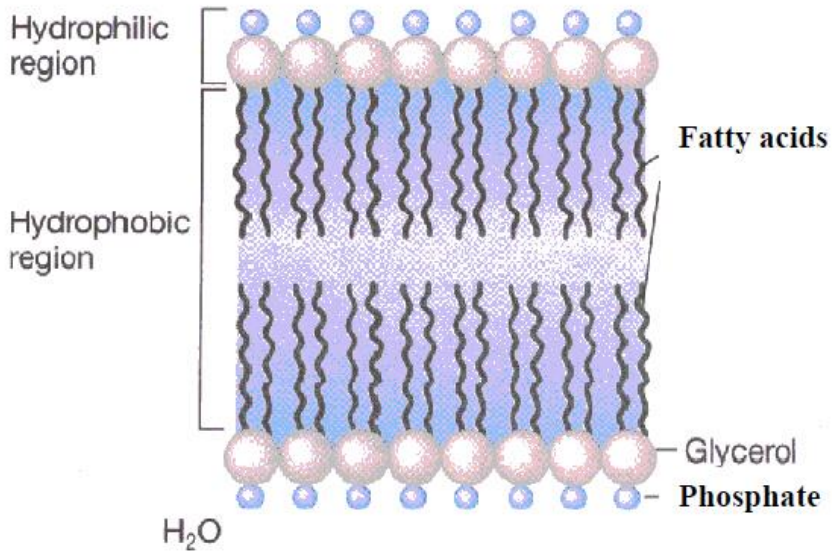


Figure 2.1 Arrangement of phospholipids in the membrane of a living cell

Source: (Kaur et al. 2005)

2.2.2.2 Evaluation of PLFA analysis

PLFA are useful biomarkers or signatures for fingerprinting the soil microbial community because of the relative abundance of certain PLFAs, which differ considerably among the specific group of microorganisms (Joergensen and Wichern 2008). PLFA with FAME quantification share the dual advantages of being rapid and inexpensive to perform. GC/MS equipment is routinely used in most analytical chemistry labs, and the cost of running individual samples is negligible. Also, PLFA profile analysis holds competitive advantage over the rest of the conventional methods (culturable technique) to study the soil microbial community structure, as it accounts for larger proportion of the soil microbial community. Digressing from the rapid and inexpensive analyses for the high number of samples needed for microbial ecology investigations, PLFA analysis has a relatively high throughput, identifies only the viable bacteria population (Øvreås 2000). The ability of PLFA and FAME to rapidly and inexpensively identify cultured isolates has been used to extensively characterize community members originally identified by less specific mechanisms of analysis (Wixon and Balser 2013).

As to the property of providing quantitative insight into the soil viable/active microbial biomass from the concentration of total PLFA, this is because the phospholipids are rapidly degraded after cell death and are not found in the storage products. A significant correlation has been observed between total phospholipid content and other methods used for measuring microbial biomass, such as acridine orange direct counts of microorganism and also with ATP content (Balkwill et al. 1988). Certain bacterial groups with specific biogeochemical activity such as SRB have already been thoroughly characterized by PLFA profiling and were found to possess several characteristic PLFA biomarkers (Córdova-Kreylos et al. 2006; Mohanty et al. 2008).

There are a few limitations associated with PLFA biomarker analysis, which may limit its use at the regional and global scale. It does not reveal any information at the species-level, archae bacteria cannot be determined using this method and databases for interpretation of biomarkers are centered on fatty acids from microorganisms from pure cultures. Since growth conditions alter the distribution of lipid species, the proper use of existing reference libraries of lipid composition requires that the samples are cultivated under the exact same conditions as the strains used to make the reference library. It is difficult to make major changes to the sample preparation method without altering the fatty acid profile of the bacteria of interest, which would then require preparation of a new reference library (Buyer 2002).

Furthermore, calibration of changes in stress biomarkers under diverse ecosystems, soil type and climate, linking of PLFA profiles with functions of ecosystems, and automation of the technique needs to be strengthened for the implementation of this bioindicator in the regional assessment of environmental impact of agriculture and its incorporation in soil quality indices. Banks et al. (2014) investigated the soil microbial community response to surfactants and herbicides in two soils and proposed the necessity of long-term microbial community studies using PLFA analysis on a wide array of soil types and management practices. The further enhancement of PLFA profiling in investigating the changes in microbial community structure of agricultural soils was also suggested (García-Orenes et al. 2013). Nevertheless, the full potential of PLFA as a bioindicator of environmental monitoring and assessment at higher scales of resolution is certainly growing as databases and novel methods focusing on functions are being developed.

2.2.2.3 PLFA with FAME for SRB quantification

Bacteria contain characteristic lipid fatty acids in the C12-C19 region which distinguish them from eukaryotic organisms and in certain cases from each other (Parkes et al. 1993). Such properties enable fatty acids to be used to study complex sedimentary communities *in situ*, thus avoiding the limitations of isolation techniques. SRB are typical bacteria in that their membranes are composed primarily of phospholipids with ester-bound fatty acids that can be analyzed as FAME. Taylor and Parkes (1983) have shown that the lipid fatty acids of the SRB *Desulfobacter, Desulfobulbus* and *Desulfovibrio desulfuricans* contain a number of characteristic acids which have the potential to act as biomarkers for these bacterial types in complex sedimentary environments. Also, the lipid composition of the Gram-negative SRB, especially polar lipid-derived fatty acids, have been studied extensively and several uncommon PLFA, e.g. cy-C17:0, 10Me C16:0, i-C17:1 (cis-10), C15:1 (cis-9) and C17:1 (cis-11), have been suggested as specific biomarkers for the different groups of SRB (Córdova-Kreylos et al. 2006; Mohanty et al. 2008). Although these PLFAs may also be found in non-SRB (e.g. 10Me C16:0 in actinomycetes), they are suitable to distinguish among the different groups of SRB since they have a high biological specificity on them and are produced only by a limited group of microorganisms. Their usage was limited under specific environments, such as sulfide-rich conditions. They were applied as biomarkers of *Desulfobacter*, *Desulfotomaculum*, and *Desulfovibrio* in uranium-contaminated subsurface sediment to evaluate the geochemical and microbial community response to ethanol amendment (Mohanty et al. 2008).

PLFA biomarkers indicative of bacterial sulfate reducers have been identified in many studies. The lipid marker Br-C17:1 (especially i-C17:1 (cis-10)) has been associated with *Desulfovibrio* (Córdova-Kreylos et al. 2006); 10Me C16:0 and C17:1 (especially C17:1 (cis-11)) were recognized as a major fatty acid component for *Desulfobacter* (Jiang et al. 2012; Kaksonen et al.

2004) and *Desulfobulbus* (Córdova-Kreylos et al. 2006), respectively. These biomarkers were determined for a small subset of isolates and may not be present in, or exclusive to, all members of the groups they are reported to represent. Caution has been urged when trying to interpret responses in the *Actionbacteria* (actino) class due to overlapping PLFAs with SRB (e.g., 10Me C16:0) (Jaatinen et al. 2006).

2.3 Offshore Reservoir Souring Control

2.3.1 Popular control techniques

2.3.1.1 Prophylaxis

Early prevention of souring (prophylaxis) can be achieved by using injection water that is naturally low in sulfate concentration, VFAs, and biomass, or by removing sulfate (as well as thiosulfate and sulfite) from injection water using technologies such as reverse osmosis or membrane filtration (Robinson et al. 2010). Although this technology is typically applied at a significant capital cost, efforts have been made in technology improvements to reduce its cost. The desulfated water is reported to be achieved through a nanofiltration membrane process and is normally used for prevention of sulfate scale in oilfields (Davis and Mcelhiney 2002). The reduction of sulfate by lowering the concentration of sulfate in reservoirs to below that of VFAs will limit production of H_2S. Aerated water in water flooding could also be considered, thus inducing more oxidizing environment to inhibit SRB. However, oxygenated seawater is corrosive to infrastructure, so this is not applicable for offshore oil wells. Prophylactic additions of nitrate/nitrite to reservoir wellbores was reported to be effective for souring control in high-temperature offshore operations (Larsen et al. 2004).

2.3.1.2 Physical and chemical treatments

Physical or chemical treatments are used for controlling microbial souring after its breakthrough. These options include using non-metallic pipes or polymer coatings inside pipelines, adding sulfide scavengers such as amines or sodium hydroxide in product streams and installing vapor recovery units on surface tank installations to remove H_2S gas. Biocides can be injected into above-ground facilities and added to the injection waters to help suppress microbial numbers and activity (discussed below). For above ground and subsea piping, biocide treatment can be coined with the use of mechanical cleaning "pigs" (Larsen et al. 2004) that are run through pipelines to disrupt and remove biologically active deposits from the pipe surface. This physical treatment helps to reduce corrosive activities by sulfidogenic biofilms and removes potential inoculum from the injection water stream. In extreme cases of souring, the best option may be to shut in the affected wells or physically isolate problematic zones of the reservoir. Jesus et al. (2015) investigated reservoir souring control using the chemical treatment of molybdate. Control of sulfate reduction by adding molybdate in short-term tests was investigated to to determine the minimum inhibitory concentration of molybdate in sulfate-rich medium. The results revealed that 0.08 mM (12.8 mg/L) molybdate, while the molar ratio molybdate/sulfate is 0.004, is sufficient to inhibit the activity of SRB for 7 days.

If these approaches are not practical or economic, chemical treatments listed below can be used to control the microbial process responsible for sulfide production. Chemicals that are widely injected into the reservoir or applied in surface facilities for microbial control can be divided into two classes: (1) non-specific biocides and (2) nitrate or nitrite, which more specifically inhibit sulfidogenesis or oxidize existing sulfide.

2.3.1.3 Biocides

Commonly used biocides include glutaraldehyde, tetrakis (hydroxymethyl) phosphonium sulfate, benzalkonium chloride, formaldehyde, sodium hypochlorite, and cocodiamines, among others (Kaur et al. 2009; Videla et al. 2005). THPS is a recently developed broad spectrum biocide with reduced environmental toxicity when compared to traditional biocides. It also has the ability to dissolve ferrous sulfide precipitates that might otherwise protect planktonic cells from biocide (Videla et al. 2005).

Biocides have the advantage of being easy to administer so they are routinely used in above-ground facilities and pipelines. However, they are difficult to push deep into the reservoir, which makes treatment of SRB communities far from the injection well challenging. Biocides have other drawbacks, they are expensive and require repeated or constant application to be effective, some are potentially hazardous to oilfield personnel and the environment, and their continued use can lead to the creation of biocide-resistant microbial populations, either through biochemical resistance or because of lack of penetration through biofilms *in situ* or in pipelines. In the latter case, cycling of biocides or combinations of two biocides is used to temporarily overcome resistance.

Recent studies have used molecular biology approaches to understand the response of SRB to biocides, particularly regarding the development of resistance. Whole genome microarrays of *Desulfovibrio vulgaris* Hildenborough (Lee et al. 2010) revealed that exposure to glutaraldehyde, a nonspecific biocide, has caused upregulation of 179 genes and downregulation of 77 genes. This diversified response was likely due to the broad range of cell targets affected by glutaraldehyde. Mutating individual genes that responded to glutaraldehyde did not rescue the mutants from

subsequent exposure to the biocide, suggesting that development of resistance to glutaraldehyde is less likely than for biocides like THPS and benzalkonium chloride. Fewer changes in gene expression were observed after exposure to these two biocides, and the genes involved differed from the glutaraldehyde response, being more specific to energy metabolism, motility, chemotaxis, solute transport, and ribosome structure (Lee et al. 2010).

Combinations of biocides and co-inhibitors such as biodegradable chelators have also been proposed. Chelators are assumed to contribute to treatment efficacy by increasing membrane permeability to the biocide. Incubating planktonic cultures of D. vulgaris ATCC 7757 and *Desulfovibrio desulfuricans* ATCC 14563 with the disodium salts of ethylenediamine disuccinate (EDDS) or N-(2-hydroxyethyl) iminodiacetic acid had no apparent effect on the cells, but cell motility was reduced when combined with either glutaraldehyde or THPS (Wen et al. 2010). A parallel test of motility inhibition exposed *D. desulfuricans subsp. aestuarii* ATCC 14563 grown on steel coupons to EDDS plus glutaraldehyde. This combination resulted in a qualitative decrease in biofilm establishment, as observed by scanning electron microscopy, and a lower concentration of glutaraldehyde required to treat established biofilms (Wen et al. 2009). Thus, the synergy between chelators and biocides might reduce the dispersal of inoculum in a pipeline or reservoir by decreasing planktonic cell motility although, notably, such a strategy does not control sulfidogenesis.

2.3.1.4 Other treatment approaches

Unconventional souring treatments recently proposed include “sterilization” of re-injection water by pulsed electric fields, and/or ultrasound (Xin et al. 2008; Xin et al. 2009). Each of these treatment methods is used for microbial inactivation and are common in the food industry for

killing bacteria and making the food safe to eat (Piyasena et al. 2003). It can be applied to souring treatment since many SRB will be rendered inactive and unable to continue conversion into sulfide thus slowing down or inhibiting reservoir souring. Inoculation of oilfield systems with a competitive microbial strain as an antagonist has also been proposed for souring control (Zuo 2007). For example, *Bacillus* sp. Strain B21 was found to outperform the biocide THPS by reducing SRB growth (Gana et al. 2011). However, the effect was apparent only during exponential growth of the antagonist, and it was tested only against planktonic SRB cultures rather than established biofilms. Whether competitive inoculation is practical and effective in real offshore reservoir environments is unknown.

In addition, Duangmanee (2009) used an innovative, low-maintenance, low-cost biological sulfide removal technology to remove sulfides simultaneously from both gas and liquid phase in a pilot-scale facility. Redox potential was used as the controlling parameter to precisely regulate air injection to the sulfide oxidizing unit attached to the digester. The micro-aeration technique provided just enough oxygen to partially oxidize sulfides to elemental sulfur without inhibiting methanogenesis. In this technology, abiotic sulfide oxidation was reported to account for 95% of overall sulfide oxidation and no inoculation of special bacteria, addition of nutrients and, or pH control chemicals are required.

2.3.2 Nitrate/Nitrite injection

Utilization of NRB for souring control was environmentally benign for oil reservoirs, because they were completely indigenous in the oil fields and highly compatible with the underground environment. Nitrate/nitrite injection has been an effective method for souring control as NRB and nitrate reducing, sulfur oxidizing bacteria (NR-SOB) outcompete and reduce SRB activity, thus

reducing H_2S production (Haghshenas et al. 2012). This technology can also be used to inhibit H_2S production within reservoirs over long terms rather instead of short terms.

2.3.2.1 Introduction

Nitrate/nitrite injection is a procedure used to inhibit the growth of SRB to help control reservoir souring. Remediation by nitrate/nitrite injection is an economically feasible, successful and relatively environmentally friendly approach to souring control (Gieg et al. 2011). NRB are stimulated by nitrate addition and yield many species during anaerobic respiration including: nitrite (NO_2^-), nitrogen gas (N_2), and ammonia (NH_4). The end products of NRB are less harmful than end products of SRB (Eq. 2-8 and 2-9) (Eckford and Fedorak 2002). Nitrate reduction also has a much more favorable Gibbs free energy than sulfate reduction (Reinsel et al. 1996). This is one of the means by which NRB inhibit SRB through the easy acquisition of energy:

$$5CH_3CO_2^- + 8NO_3^- + 3H^+ \rightarrow 10HCO_3^- + 4N_2 + 4H_2O \quad (2\text{-}8)$$

$$\Delta G = -495 \text{ KJ (mol } NO_3^-)$$

$$CH_3CO_2^- + SO_4^{2-} \rightarrow 2HCO_3^- + HS^- \quad (2\text{-}9)$$

$$\Delta G = -47 \text{ KJ (mol } SO_4^{2-})$$

NRB outcompete SRB for electron donors because of the significant thermodynamic advantage (Eckford and Fedorak 2002). Nitrate reduction provides more energy for microbial growth; therefore NRB grow faster and outcompete SRB (Haghshenas et al. 2012). Levels as low as 0.71 mM of nitrate have been shown to inhibit souring, even though high concentrations of sulfate (9.4 mM) and organic acids (2.27-4.35 mM) were present (Reinsel et al. 1996). However Kaster et al. (2007) found that nitrate alone was ineffective at inhibiting thermophilic SRB, but nitrite proved

very effective. This suggests that nitrite may be a better option for high-temperature reservoirs. Although, it is important to recognize that nitrite is a by-product of NRB respiration, and thus nitrate could be effective as long as it can be oxidized to nitrite.

Another method in which nitrate injection remediates souring is by the growth of NR-SOB. The NR-SOB gain energy by oxidizing reduced sulfur compounds to sulfate and elemental sulfur. NR-SOB are also capable of nitrate reduction with the primary end product being N_2. This removes and suppresses sulfide production (Eckford and Fedorak 2002). If NR-SOB have the same level of activity as SRB, then in theory the net rate of sulfide generation is zero (Haghshenas et al. 2012).

Nitrate/nitrite injection stimulates the growth of NRB and NR-SOB. Both oxidized forms of nitrogen aid in the mitigation of H_2S gas by biocompetitive exclusion of SRB and direct oxidation of sulfide respectively. Unlike biocides, nitrate/nitrite injection does not involve harsh chemicals and it is therefore an attractive method for H2S mitigation. It is the most viable and promising option for reservoir souring control.

2.3.2.2 Advantages/disadvantages

Nitrate/nitrite injection has many advantages and disadvantages as seen in the following table (Table 2.1). Overall, there are more advantages than disadvantages. The process is effective and cost-efficient in comparison to other options of H_2S gas inhibition.

Table 2.1 Advantages and disadvantages of nitrate/nitrite injection

Advantages	Disadvantages
Nitrate/nitrite does not adsorb to most porous materials (Reinsel et al. 1996)	Limited research for offshore oil wells using N/N injection. A new field of study.
Cost efficient: low levels of N/N needed for inhibition (Reinsel et al. 1996)	Increased concentrations of ammonia
Longer periods of inhibition than biocides (Reinsel et al. 1996)	Extra biomass may decrease permeability (Kuijvenhoven et al. 2006)
May extend production life (Reinsel et al. 1996)	Nitrate can cause pitting in steels (Kuijvenhoven et al. 2006)
End products or NRB are less harmful than end products of SRB (Eckford and Fedorak 2002)	Rapid proliferation of SRB once nitrate injection has stopped (Kuijvenhoven et al. 2006)
Reduces Corrosion (Kaster et al. 2007)	

2.3.2.3 Field applications

Kaster et al. (2007) studied the effects of nitrate/nitrite injection on sulfide production in the Ekofisk field of the Norwegian sector in the North Sea. The field is a deep reservoir where thermophilic SRB contribute to most of the sulfide production at 60°C near injection wells. It was hypothesized that nitrite may be preferable for some high-temperature oil fields as it reacts directly with sulfide. Nitrite inhibits DSR, which is an enzyme that catalyzes the reduction of sulfite to sulfide. DSR has a strong affinity for nitrite by reducing it to ammonia, thus inhibiting DSR usage by SRB (Kaster et al. 2007).

Experiments were conducted in an upflow bioreactor filled with Ekofisk Chalk inoculated with 0.1 ml/min of produced waters that had 0.1 mM of phosphate added to it. Two strains of bacteria (NS-tSRB1 and NS-tSRB2) were studied with differing additions of organic acids. Nitrate/nitrite injection did not take place until the bioreactor was stabilized with a sulfide concentration of 4-6 mM. It was observed that 2 and 10 mM of nitrate addition had little to no effect on sulfide production for both NS- tSRB1 and NS- tSRB2. It is also noted that no nitrite was detected after nitrate addition. Conversely, nitrite addition strongly inhibited sulfate reduction. 0.25 mM of nitrite inhibited sulfate reduction permanently for NS- tSRB1, and for 1100 hrs for NS- tSRB2. Results suggested that the type of organic acid used as an electron donor has an influence on the survival of SRB during nitrate/nitrite injection.

Nitrate proved effective for two high-temperature oil fields in the Norwegian and Danish sectors of the North Sea, Veslefikk and Halfdan fields, respectively (Kaster et al. 2007). Thermophilic nitrate reducing bacteria (tNRB) must have converted nitrate into nitrite, thus inhibiting tSRB of organics derived from oil in these cases. This was not observed in Ekofisk, suggesting that tNRB

are not present, or they could have been lost during enrichment with sulfate. Overall, 0.25 mM of added nitrite effectively controlled souring for the North Sea field.

Bodtker et al. (2008) studied the long-term effects of nitrate injection on SRB activity, corrosion rates, and bacterial community composition of the Veslefrikk and Gullfacks fields in the North Sea. Biocides were traditionally used to mitigate sulfide production, but nitrate injection was accepted as a more effective and environmentally safe method. Nitrate injection enriches the NRB, which outcompete SRB due to the more favorable energy potential of nitrate reduction compared to sulfate reduction. Overall, SRB are inhibited and corrosion rates decrease.

The Veslefrikk field experienced a 50–fold reduction in H_2S production (SRB activity) and a reduction in the corrosion rate immediately following continuous nitrate injection (1999-2001). NR-SOB formed major populations, and three of the four major populations that were observed before biocide treatment were no longer observed after 1 year with nitrate treatment. Sulfate reduction rate is given as the amount of H_2S produced per biofilm area per day, and remained at $\leq$0.3 μg H_2S/cm^2/day during nitrate treatment. A decrease in corrosion rate was also observed. The Gullfaks field experienced an initial decrease in number and activity of SRB, and an increase in the numbers of NRB. The SRB activity has remained low during the 8 years of nitrate injection at $\leq$0.9 μg H_2S/cm^2/day. There was also a significant reduction in corrosion rate of up to 40%. The long-term nitrate injection treatment has provided efficient inhibition of SRB activity and a decrease in corrosion rate. This has enabled a stable NRB dominated biofilm to develop. Nitrate injection has proven to be an effective and safe way to mitigate biogenic sulfide production in offshore oil wells practicing secondary oil recovery.

2.4 Biosurfactants and Reservoir Souring Control

2.4.1 Introduction of biosurfactants

2.4.1.1 Definition and classification of biosurfactants

Biosurfactants are extracellular, surface-active substances synthesized by living cells. They have the properties of reducing surface tension, stabilizing emulsions, promoting foaming and are generally non-toxic and biodegradable (Saharan et al. 2011). Biosurfactants are amphipathic molecules with hydrophilic as well as hydrophobic groups which impart their functional properties. They are high value products and efficient replacers of chemically synthesized surface-active agents due to their diversity, specific activity, ease of application, biodegradability and performance in extreme environments (Marchant and Banat 2012).

Biosurfactants are of two types, low molecular weight biosurfactants which are generally glycolipids and lipopeptides and high molecular weight biosurfactants, also called bioemulsifiers, which are generally lipopolysaccharides, lipoproteins or a combination of these (Christofi and Ivshina 2002). Generally, the former group effectively reduced the surface/interfacial tension, while the latter group tended to stabilize oil-in-water emulsions but did not reduce much surface tension (Rosenberg and Ron 1999). Most known biosurfactants are glycolipids. They are carbohydrates in combination with long-chain aliphatic acids or hydroxyaliphatic acids. Among the glycolipids, the best known are rhamnolipids, trehalolipids, and sophorolipids. Figure 2.2 shows the structure of the three classes of glycolipid biosurfactants.

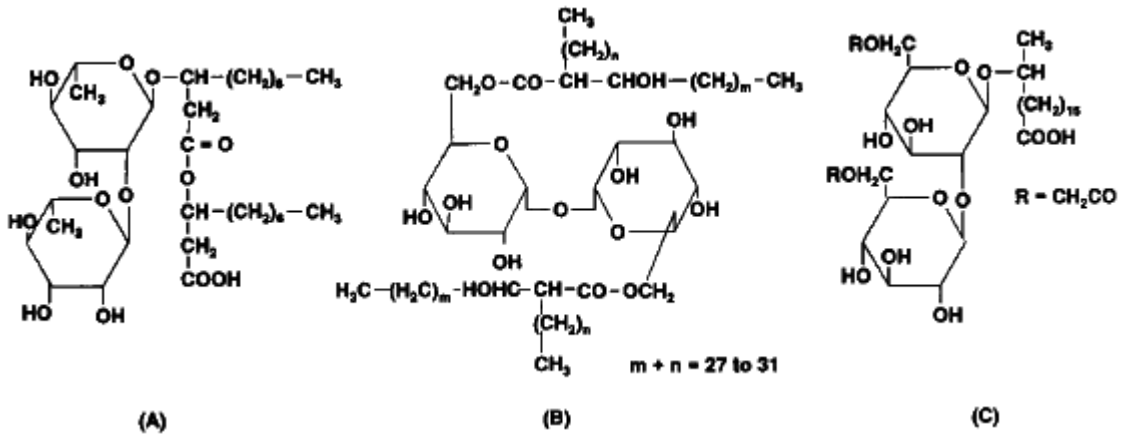


Figure 2.2 Structures of glycolipid biosurfactants (A) Rhamnolipid from *Pseudomonas aeruinosa.* (B) Trehalolipid from *Rhodococcus erythropolis.* (C) Sophorolipid from *Torulopsis bombicola.*

Source: (Desai and Banat 1997)

In addition, several bacteria and yeast produce large quantities of fatty acid and phospholipids surfactants during growth on n-alkanes (Santos et al. 2016). Emulsan, liposan, mannoprotein and polysaccharide-protein complexes are known to be the best-studied polymeric biosurfactants (Desai and Banat 1997).

2.4.1.2 Functions of biosurfactants

The basic function of a biosurfactant is to reduce the surface or interfacial tension between two mediums (e.g. water and air or oil and water). Biosurfactants are amphipathic meaning they have hydrophobic and hydrophilic parts, as explained earlier in the report. Biosurfactants can also be used to form stable emulsions, and for solubilization and desorption of hydrophobic compounds from particle surfaces (Pacwa-Płociniczak et al. 2011). These functions can shape the ecological niches for bacteria within microbial communities by altering the availability of substrates for growth and by allowing bacteria to enter into liquid phases having different levels of hydrophobicity.

The most significant role of microbial surfactants is documented for adhesion of the cells to the interfaces. Adhesion is shown to be a prerequisite for the growth of *Acinetobacter calcoaceticus* RAG-1 on liquid hydrocarbons under two conditions: low cell density and limited agitation (Rosenberg and Ron 1999). Besides, biosurfactants can enhance growth on bound substrates by desorbing them from surfaces or by increasing their apparent water solubility (Rosenberg and Ron 1999). Surfactants that lower interfacial tension are particularly effective in mobilizing bound hydrophobic molecules and making them available for biodegradation. Another important characteristic of the biosurfactants is that above the CMC, they form micelles (stable aggregates of 10 to 200 molecules), which brings about a sudden variation in the relation between the

concentration and the surface tension of the solution that can increase the solubility of hydrophobic organics (Cho et al. 2002).

According to Puchkov et al. (2002), the biosurfactants could also be an evolutionary defense strategy of microbe as evidenced by high mycocidal activity of the mycocidal complex secreted by *C. humicola*. Similar analogy can be made for the lipopeptides biosurfactant producing strains of *B. subtilis.* The lipopeptide (antibiotic) would have a strong influence on the survival of *B. subtilis* in its natural habitat, the soil and the rhizosphere (Nielsen and Sorensen 2003).

2.4.1.3 Advantages of biosurfactants

Biosurfactants have gained considerable attention in recent years due to their unique properties versus their synthetic counterparts.

Selectivity for specific interfaces

Biological molecules have been found to show more specificity as compared to the chemically synthesized materials. Microbial surfactants show a specificity due to the presence of specific functional groups, allowing specificity in the detoxification of specific pollutants, and activity under conditions of extreme temperatures, pH and salinity (De Cassia et al. 2014). For example, specificity of emulsan towards a mixture of aliphatic and aromatic hydrocarbons (Bach et al. 2003)and that of solubilizing factor of *Pseudomonas* PG1 towards pristine (Sekhon et al. 2011).

Resistance to environmental changes

Biosurfactants have chemical diversity which results in a wide variety of physico-chemical properties suited for applications (De Cassia et al. 2014). For example, biosurfactants were noted for their promising application enhanced oil recovery to remove and recover the residual oil.

Biosurfactant solutions at 0.01% and 0.05% produced by *Pseudomonas sp.* 2B removed 89% and 92% of the sand oil, respectively, while the synthetic surfactant, sodium dodecyl sulfate, removed only 63% of the contaminated oil (Aparna et al. 2012). The extracted biosurfactant product was a glycolipid type and was found to be stable over a pH range of 4 to 10, a temperature range of 4 to 121° C and salinity from 0 to 15% NaCl.

Diversity of biosurfactants

Biosurfactants have enormous diversity, and could be produced from various sources, such as industrial wastes with simple and inexpensive procedures (Muthusamy et al. 2008). A single isolate often generates chemical variations of the same surfactant, resulting in the production of a surfactant mixture with an associated characteristic surface (Bodour et al. 2003). In fact, even small variations in the structure of a surfactant can significantly affect its functions and potential industrial applications (Symmank et al. 2002).

Low toxicity and easy biodegradation

Another main advantage of biosurfactants is their lower toxicity than traditional surfactants. Sometimes the process used in the production of synthetic surfactants could release toxic byproducts, as in the case of sodium dodecylbenzene sulfonate (SDS) production, where corrosive and toxic chemicals are used and quite frequently discharged as pollutants. When focusing on environmentally sound products, potentially toxic and sparing biodegradable synthetic surfactants could be replaced by biosurfactants, which are naturally produced and nontoxic. Furthermore, microbial surfactants like all natural products are susceptible to degradations by microorganisms in water and soil (Khan and Butt 2016). Hence, concerns regarding the negative environmental risks after the application are ignorable.

2.4.1.4 Screening of biosurfactant producing microorganisms

Oil reservoirs have a great potential in producing microorganisms that may produce biosurfactants (Anandaraj and Thivakaran 2010). Currently, the understanding of biosurfactants as a class of molecules remains limited. This is partially because the present body of knowledge has been developed around a relatively small number of well-characterized biosurfactants. Contributing to this is the lack of a concerted effort to perform a comprehensive screening for biosurfactants and the microorganisms that produce them. There is a very limited amount of commercially available biosurfactants (e.g. surfactin, sophorolipids and rhamnolipids) (Santos et al. 2016; Walter et al. 2010). The development of a variety of new biosurfactants by new strains provides a solution in overcoming the economic obstacles of the production of biosurfactants. Therefore, efforts in the discovery of new biosurfactant producing microbes must be made by applying a broad range of different screening methods. The principal aim in screening for new biosurfactants is finding new structures with strong interfacial activity, low critical micelle concentration (CMC), high emulsion capacity, good solubility and activity in a broad pH range. Commercial viable biosurfactants have to be economically competitive. Additionally, another reason in screening is discovering good production strains with high yields (Walter et al. 2010).

There are eight different screening methods that have been reported as criteria to screen biosurfactant producing microbes. Namely hemolytic assay (Yoshida et al. 2015), bacterial adhesion to hydrocarbons (BATH) assay (Volchenko et al. 2007), emulsification assay (Afshar et al. 2008), drop collapse assay, oil spreading assay (Nwaguma et al. 2016), du-nouy-ring method, microplate assay (Vaux and Cottingham 2001) and CTAB agar plate. Each of these methods has their own advantages and disadvantages. Surface tension measurement is the best way to discover biosurfactant producing microorganisms in a sample (Bodour et al. 2003; Thavasi et al. 2011). The

Du-Nouy-Ring method which is a variation of surface tension measurement is the most common method used for this type of measurement. One limitation is that it needs to use a tensiometer which may not be readily available in a microbiology laboratory.

2.4.1.5 Biosurfactant production

There are numerous factors that affect biosurfactant production by microorganisms. Microorganisms depend on vital nutrients and a suitable growth environment to sustain life and thrive. Biosurfactant production is affected if the microorganisms are not getting proper nutrition or inhabit an area with poor growth conditions. The nutrients will be discussed as culture medium composition and growth conditions that will be discussed as environmental factors, both of which have significant influence on the biosurfactant production.

The use of different carbon sources changes the structure of the biosurfactants produced and, consequently, its properties. These changes may be welcomed when some properties are sought for a particular application (Amaral et al. 2010). Also, the composition and characteristics of biosurfactants are influenced by the nature of the nitrogen source as well as the presence of iron, magnesium, manganese, phosphorus and sulfur.

Main carbon sources come from 3 categories: carbohydrates, hydrocarbons and vegetable oils. Water-soluble carbon sources such as glycerol, glucose, mannitol and ethanol were all used for rhamnolipid production by *Pseudomonas* spp. However, biosurfactant production was inferior to that obtained with water-immiscible substrates such as n-alkane or olive oil (Thampayak et al. 2008). Similarly, different nitrogen compounds have been used for the production of biosurfactants, such as urea, peptone, yeast extract, ammonium sulfate, ammonium nitrate, sodium nitrate, meat extract and malt extract, etc. Different elements, such as iron and manganese, are also

reported to affect the yield of biosurfactants, for example, the addition of iron and manganese to the culture medium increased the production of biosurfactant by *Bacillus subtilis* (Gudiña et al. 2015). The ratios of different elements such as C:N, C:P, C:Fe or C:Mg mare main factors affecting biosurfactant production (Batista et al. 2010).

Environmental factors are extremely important in the yield and characteristics of the biosurfactants produced. In order to obtain large quantities of biosurfactant it is necessary to optimize the process conditions because the biosurfactant production may be induced by changes in pH, temperature, aeration or agitation speed. For instance, the effect of pH in the biosurfactant production by *Virgibacillus salarius* was investigated in the pH values varying from 5 to 12. The biosurfactant generation increased with the increase of medium pH, but maximum biosurfactant production was obtained at pH 9 for the strain *V. salarius* (KSA-T) (Elazzazy et al. 2015). The strain was found to be moderately thermophilic and the maximum biosurfactant production was observed in the temperature range of 45–50 °C. A lower culture temperature might make microoragnisms hibernate partially, and its enzyme system for biosurfactant production couldn't be activated completely. On the other hand, a higher culture temperature may have an adverse effect on the nucleic acid and the enzyme system of the strain. Aeration and agitation rates are also important factors that influence the production of biosurfactants, since they facilitate the oxygen transfer from the gas phase to the aqueous phase and it may also be linked to the physiological function of microbial emulsifiers.

2.4.1.6 Biosurfactant characterization

Physical-chemical parameters

There are several physical-chemical parameters that can be applied for the characterization of biosurfactants. Surface tension, interfacial tension and CMC are quantitatively measured in biosurfactant production. Whether these three factors are looked at individually or collectively, they can indicate if a given surfactant will be successful in its intended application.

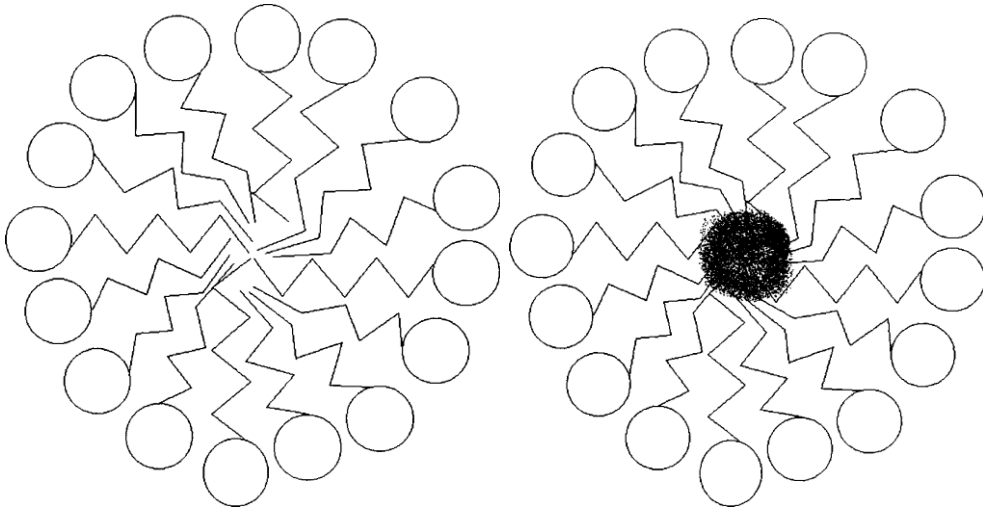
The surface tension of a liquid is the measurement of the interfacial free energy per unit area of the boundary between the liquid and the air above it (Rosen 1989). In a liquid, intermolecular forces (van der Waals) act upon each molecule in every direction by other surrounding molecules. At the surface of the liquid (in contact with air), however, there exists a difference in these forces. At the surface the molecules interact more strongly with the molecules in the interior of the liquid than they do with the widely spaced gas molecules above it (Rosen 1989). Therefore, the surface molecules are contracted or drawn into the liquid and a surface tension develops.

Surface tension is most commonly measured by a tensiometer. The surface tension is measured by the force that is required to remove the ring from the liquid or to pull the plate a given distance up while still remaining in the fluid. Surface tensions are equivalent to a force per unit length and are commonly given in units of mN/m or dyn/cm. Biosurfactants are characterized by the measure of their effectiveness and efficiency at reducing surface tensions. Effectiveness is the measure of the minimum value to which surface tension can be lowered, while efficiency is measured by the concentration of a surfactant required to produce some significant reduction in the surface tension of water (Czajka et al. 2015). The most effective biosurfactants are those that have a short, branched hydrophobic moiety. The more efficient biosurfactants have a linear, long hydrophobic moiety.

Interfacial tension is very similar to surface tension except that a liquid is in contact with another liquid phase rather than air. At the interface between the condensed phases, the dissimilar molecules in the adjacent layers facing each other across the interface also have potential energies different from those in their respective phases (Rosen 1989). Therefore, a tension develops at the boundary between the two liquids. The less miscible the two liquids are, the greater the resultant interfacial tensions will be. On the other hand, if the two liquids miscible well with each other, there will be no interfacial tension between them.

A unique phenomenon of surfactants is the self-assembly of molecules into dynamic clusters called micelles (Shchekin et al. 2016). Each singular biosurfactant molecule is termed a monomer. The monomers arrange themselves into micelles once a CMC is reached in the solution (Figure 2.3). Micelles can be spherical, cylindrical, lamellar or vesicular depending upon several factors such as temperature, hydrocarbon chain length, etc. In aqueous media, the surfactant molecules are oriented, in all of these structures, with their polar heads toward the aqueous phase and their hydrophobic tail groups away from it (Rosen 1989). In a non-polar media, the structure of the micelle is reversed with the hydrophobic tails pointing out into the media.

CMCs are different for each type of surfactant. The CMC is determined by measuring the reduction in surface tension produced by serially diluting the solution. The point at which the surface tension first begins to rise is the critical micelle dilution (CMD). The CMC is a crucial point because surface tension will decrease continually as surfactants are added until the CMC is reached. After this point, no further significant surface tension reductions will take place regardless of how much more surfactant is added. CMC is altered by metabolic acids or alcohols that are produced by organisms and subsequently react with biosurfactants and by ionic species such as salts (Palladino and Ragone 2011; Sidim and Acar 2013).



(a) (b)

Figure 2.3 Diagrams of the generalized micelle with and without oil emulsification

Source: (Rosen 1989)

One last important aspect of micelle formation is the fact that they have the ability to solubilize compounds (Figure 2.3b). Solubilization is believed to occur at a number of different sites in the micelle including: (a) on the surface of the micelle, at the micelle-solvent interface; (b) between the hydrophilic head groups; (c) in the so-called palisade layer of the micelle between the hydrophilic groups and the first few carbon atoms of the hydrophobic groups that comprise the outer-core of the micelle interior; (d) deeper inside the palisade layer; and (e) in the inner core of the micelle (Rosen 1989). The structure of the surfactant, the nature of the solubilized material, charges and temperature affect the extent of solubilization. The changes of surface tension, interfacial tension and solubilization of the surfactant over the increase of surfactant concentration are presented in Figure 2.4.

Structure characterization

For the complete structure elucidation of biosurfactants, various chromatographic and spectroscopic techniques were used. A combination of these techniques is very helpful in the characterization of the compound. Among them, thin layer chromatography (TLC) is the most important and preliminary technique for the characterization of various types of biosurfactants. The various solvent systems and developer employed in thin layer chromatography are given in Table 2.2.

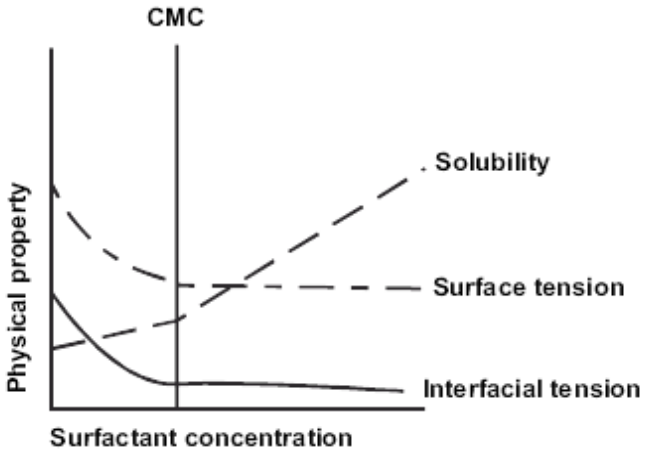


Figure 2.4 The changes of surface tension, interfacial tension and solubilization of the surfactant over the increase of surfactant concentration

Source: (Mulligan 2005)

Table 2.2 Various solvent systems and developer employed in TLC method

Biosurfactant type	*Solvent System*	*Identification of functional group*	*Developer*	*References*
Sophorolipid	Chloroform: Methanol: Water (65:15:2)	Diacylated lactone	Not mentioned	Cavalero and Cooper (2003)
Protein-lipid-polysaccharide complex	(i) Hexane: Isopropyl ether: Acetic acid (15:10:1) (ii) Chloroform: Methanol: Water (65:24:4)	Protein-40.2%, Carbohydrate-24.0% Lipid-19.5%	Ninhydrin-free amino groups Iodine vapors-Lipids α-napthol-H2SO4 - Sugar	Sarubbo et al. (2007)
Glycolipid	Chloroform: Methanol: Water (65:15:2)	Not mentioned	α-napthol	Folch et al. (1957)
Sophorolipid	Chloroform: Methanol (8:2)	Not mentioned	Anthrone/sulfuric acid	Konishi et al. (2008)
Sophorolipid	Chloroform: Methanol: Acetic acid (65:25:4)	Not mentioned	Iodine vapour and Molisch reagent	Thaniyavarn et al. (2008)

Source: Modified from Bhardwaj et al. (2013)

Additionally, GC-MS analysis for the confirmation of fatty acids, HPLC chromatogram for lipid structure elucidation, and proton nuclear magnetic resonance (^{1}H-NMR) spectrum as well as carbon nuclear magnetic resonance (^{13}C-NMR) spectrum for functional groups of biosurfactants were also used in the structure elucidation of biosurfactants (Kim et al. 1999; Konishi et al. 2008; Thaniyavarn et al. 2008). For instance, in the structure elucidation of sophorolipids produced by the *Candida bombicola*, hydroxyl-acid methyl esters were liberated by the methanolysis and were confirmed by GC-MS. The 16-hydroxydecanoic acid was confirmed by comparing the fragmentation pattern with the standard 16-hydroxyhexadecanoic acid purchased from Sigma Aldrich. Also, an isomer 15-hydroxyhexadecanoic acid was confirmed because of the availability of the same fragmentation pattern in the library (Cavalero and Cooper 2003).

2.4.2 Biosurfactant producers in reservoirs

Biosurfactant or surface-active compounds are a heterogeneous group of surface active molecules with both a lipophilic and hydrophilic moieties produced by microorganisms, which either adhere to cell surface or are excreted extracellulary in the growth medium (Santos et al. 2016). These surface active molecules reduce surface tension at air-water interfaces and interfacial tension in both aqueous solutions and hydrocarbon mixtures (Batista et al. 2006). Several types of biosurfactants have been isolated and characterized, including glycolipids, phospholipids, lipopeptides, natural lipids, fatty acids, lipopolysacharides and other fully characterized. The majority of known biosurfactants are synthesized by microorganisms grown on water immiscible hydrocarbons, but some have been produced on such water-soluble substrates as glucose, glycerol and ethanol (Tabatabaee et al. 2005). Chemically-synthesized surfactants have been used in the oil industry to aid clean up of oil spills, as well as to enhance oil recovery from oil reservoirs. These compounds are not biodegradable and can be toxic to the environment. Biosurfactants have special

advantages over their commercially manufactured counterparts because of their lower toxicity, biodegradable nature, and effectiveness at extreme temperature, pH, salinity and ease of synthesis. Due to the presence of hydrophilic and lipophilic moieties in their structure, biosurfactants are able to partition at the oil-air or the oil-water interfaces and to lower surface or interfacial tension, respectively. They are potential candidates for much commercial application in the oil recovery industries (Bordoloi and Konwar 2008; Shibulal et al. 2014).

Oil reservoirs contain diverse and metabolically active microbial communities. Knowledge of the microbial ecology of oil reservoirs can be used to stimulate the beneficial activities of microorganisms to enhance oil recovery. Microorganisms produce a variety of products that are potentially useful for enhancing oil production. Several products have become commercially viable technologies such as paraffin control. Several aerobic and anaerobic thermophiles tolerant of pressure and moderate salinity have been isolated which are able to mobilize crude oil in the laboratory. The most common biosurfactants used in microbially enhanced oil recovery (MEOR) are lipopeptides produced by *Bacillus* and some *Pseudomonas* spp., glycolipids (rhamnolipids) produced by *Pseudomonas* sp., and trehalose lipids produced by *Rhodococcus* sp. (Amani et al. 2010; Cai et al. 2014; Youssef et al. 2004). Lipopeptides and rhamnolipid biosurfactants lower interfacial tension between the hydrocarbon (crude oil or pure hydrocarbons) and aqueous phases to values of 0.1 mN/m or lower (Nguyen et al. 2008; Wang et al. 2007).

Biosurfactant producers isolated from a number of oil reservoirs are effective in mobilizing residual oil from a variety of laboratory test systems. Table 2.3 summarized the biosurfactant producers reported in the oil reservoir and related lab-scale experiments. In the oil and gas industry, NRB induced biosurfactants have been initially investigated to aid enhanced oil recovery (EOR). Fallon et al. (2010) have confirmed that biosurfactants can be naturally derived from NRB. Pure

NRB strains from ATCC (an independent, private, nonprofit biological resource center and research organization) have been purchased and their effects on EOR haven been determined. To date, there has been little relevant research related to biosurfactant-aided reservoir souring control, though its application to reservoir souring control is theoretically possible.

2.4.3 Anti-souring effects of biosurfactants

2.4.3.1 Role of biosurfactants in oil reservoir

The majority of the world's energy is still from nonrenewable fossil fuel source. The average recovery of the total oil from mature oilfields around the world is somewhere between 20% and 40% by currently used methods (Muggeridge et al. 2014). Traditional primary and secondary production methods typically recover one-third of oil in place, leaving two thirds behind. Improving oil recovery to release more trapped oil in oil fields is essential options for increased production rates. More advanced methods are desired and referred to as EOR. In Canada, for example, 70% of oil discovered was found during the earliest 20% of drilling. About 130 billion barrels have been produced to date and up to another 170 billion barrels are considered a long-term target for advanced EOR technology (Bott 1999). Given the declining reserves and the low probability of locating significant new fields, industries sought additional oil in old reservoirs, making Canada a proving ground for EOR techniques.

Table 2.3 Microorganisms and the effect of produced biosurfactants on interfacial tension and residual oil recovery in model porous systems

Microorganism	Biosurfactant	Type of experiment	Effect on IFT, wettability, and/or residual oil recovery	References
Aerobic mesophilic hydrocarbon-degrading bacteria	Unidentified	Core flood	IFT lowered; Wettability alteration	Kowalewski et al. (2006)
Isolates from Egyptian and Saudi oil fields	Unidentified	Berea sandstone core and sand-packed columns	IFT lowered; Wettability alteration Increased oil recovery	Sayyouh (2002)
Thermophilic bacterial mixtures obtained from UAE water tanks	Unidentified	Core flood under reservoir conditions	IFT of 0.07 mN/m against four crude oils; Average residual oil recovery of 15-20%	Zekri et al. (1999)
Five microorganisms from Persian reservoirs	Unidentified	Glass micromodels and carbonate rock with or without fracture	IFT reduction; Wettability alteration	Nourani et al. (2007)
Indigenous microorganisms from Persian reservoirs (45 C)	Unidentified, Lipopeptides	Core flood	Residual oil recovery of 14.3%	Abtahi et al. (2003)
Bacillus subtilis and *Pseudomonas* strain	Unidentified	Crushed limestone-packed column	IFT of 0.052 mN/m, Injection pressure decreased 5–40%, Residual oil recovery of 5–10%	Li et al. (2002)
Facultative anaerobes from Daqing oil field	Unidentified	Anaerobic core flood	IFT lowered; pH decreased; oil viscosity decreased; light alkane proportion increased; residual oil recovery of 10%	Han et al. (2001)
Anaerobic enrichments from high temperature oil reservoir	Unidentified	Sand-packed column at reservoir conditions	Residual oil recovery of 22%	Banwari et al. (2005)
Biosurfactant producing microorganisms from Indonesian oil fields	Unidentified	Native and model core floods	Residual oil recovery of 10-60%	Sugihardjo et al. (1999)
Bacillus mojavensis strain JF-2	Lipopeptide	Sand-packed columns	Residual oil recovery increased	Mcinerney et al. (1985)

Bacillus subtilis	Lipopeptide	Sand-packed columns flooded with sodium pyrophosphate	Residual oil recovery of 35%	Chang (1987)
Bacillus subtilis strain MTCC1427	Lipopeptide	Sand-packed columns with kerosene	Residual kerosene recovery of 56% with 100 ml of 1 mg/ml crude biosurfactant	Makkar and Cameotra (1998)
Bacillus subtilis strains DM03, DM04 (thermophilic)	Lipopeptide	Sand-packed column	Residual oil recovery of 56-60%	Das and Mukherjee (2007)
Bacillus subtilis 20B, *B. licheniformis* K51, B. subtilis R1, *Bacillus* strain HS3	Lipopeptide	Sand-packed columns	Residual oil recovery of 25-33%	Joshi et al. (2008)
B. subtilis	Surfactin	Adsorption to carbonates	Wettability alteration; surfactin adsorbed	Johnson et al. (2007)
Acinetobacter calcoaceticus	Unidentified	Sand-packed column at 73C	IFT lowered; residual oil recover of 36.4%	Sheehy (1992)
Enterobactercloacae and *Bacillus stearothermophilusSUCPM#14*	Unidentified	In-stu and ex-situ	IFT lowered; wettability alteration; In-situ up to 21.5% and ex-situ up to 34.1%	Sarafzadeh et al. (2014)
Engineered strains of *Pseudomonas aeruginosa* and *Escherichia coli*	Rhamnolipids	Sand-packed column	Residual oil recovery of 50% with 4 pore volumes of 250 mg/l rhamnolipid solution	Wang et al. (2007)
Pseudomonas strain	Glycolipid and phospholipids	Sand-packed column	Residual oil recovery of 52%	Okpokwasili and Ibiene (2006)
Pseudomonas strains	Glycolipid	Sand-packed column	Residual oil recovery of 64%	Das and Mukherjee (2005)
Pseudomonas aeruginosa strains	Glycolipid	Sand-packed column	Residual oil recovery of 50-60%	Bordoloi and Konwar (2008)
Rhodococcus strain	Glycolipid	Sand-packed column	Residual oil recovery of 86% with 5 pore volumes of broth	Abu-Ruwaida et al. (1991)

IFT signifies interfacial tension.

Source: Modified from Youssef et al. (2009)

Surfactant EOR represents one of the most promising advanced methods to recover a substantial proportion of the residual oil. In this technology, an aqueous surfactant formulation is injected into a mature oil reservoir. Where this solution contacts the small blobs of oil trapped in the pores of the reservoir rock, it dramatically reduces the interfacial tension and mobilizes this trapped oil. This reduces the capillary forces preventing oil from moving through rock pores (Figure 2.5). The biosurfactants act as a separating agent making the oil move more freely away from rocks and crevices so that it may travel easily out of the well. Biosurfactants can also bind tightly to the oil-water interface and form emulsion. This stabilizes the desorbed oil in water and allows removal of oil along with the injection water.

Indigenous or injected biosurfactant producing microorganisms are exploited in oil recovery in oil-producing wells. MEOR is often implemented by direct injection of nutrients with microbes that are able of producing desired products for mobilization of oil, by injection of a consortium or specific microorganisms or by injection of the purified microbial products (e.g., biosurfactants). These processes are followed by reservoir repressurization, interfacial reduction of tension/oil viscosity and selective plugging of the most permeable zones to move the additional oil to the producing wells. The main roles of biosurfactants in oil reservoirs thus lay in the enhanced oil exploitation activities.

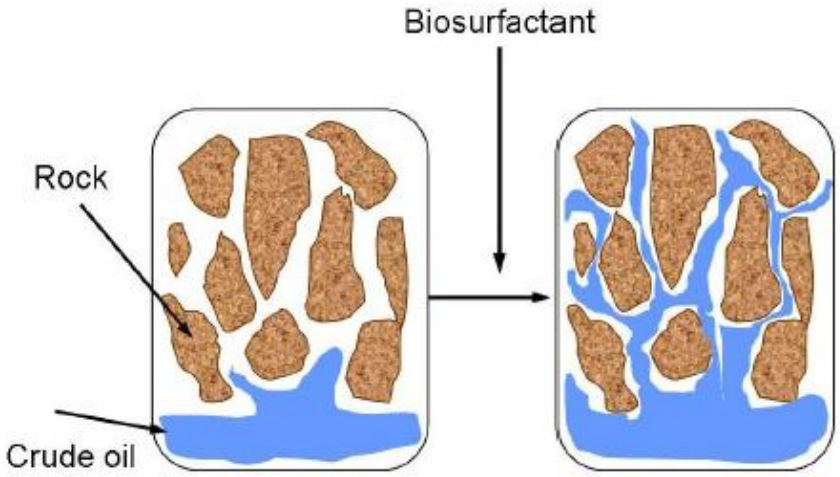


Figure 2.5 Mechanism of oil recovery enhanced by biosurfactants

Source: (Pacwa-Płociniczak et al. 2011)

2.4.3.2 Anti-souring effects of biosurfactants

Oil production by water injection often results in increased sulfide levels (souring), because SRB couple the oxidation of degradable oil organics to the reduction of sulfate to sulfide. High levels of sulfide represent increased toxicity and corrosion risk. Souring also negatively affects the quality of gas stored in subsurface reservoirs, because the H_2S must be removed prior to distribution to consumers. Souring can be prevented or reversed by the periodic, batchwise application of biocides or by the continuous, field-wide injection of nitrate. Application of nitrate to constrain souring has been especially successful for seawater injection in offshore operations.

Continuous injection of nitrate or oxygen has been reported to result in MEOR by *in situ* production of biosurfactants or of partially reduced, highly reactive intermediates, or by removal of oil-dissolved sulfur by resident bacteria. This MEOR mechanism is critical, not only for improving conventional oil production, but also for the reservoir control in a long run. Hui et al. (2012) evaluated the microbial community structure and functionally distinct groups in three kinds of produced water samples from the shallow, mesothermic and low-salinity Daqing oil reservoir using both culture-dependent and culture-independent methods. They found some isolated strains were simultaneously detected in different functional groups, and even more, from different produced water samples. The isolates affiliated to *Pseudomonas stutzeri* PTG4-15 (DP26, BP39, and PW5) were identified as NRB, biosurfactant producing bacteria, and polymer-producing bacteria. *Acinetobacter haemolyticus* BA56 represented by DT20 and BP25 was functionally related to both nitrate reducing and biosurfactant producing bacteria. In their study, the widespread occurrence of close relatives to *Pseudomonas putida* BBAL5-01 within varying functional groups (NRB, fermentative bacteria, and biosurfactants and biopolymer producing bacteria, and also, polyacrylamide degrading bacteria) was identified. It indicates that it may be a common

indigenous bacterium in petroleum reservoirs and may have a significant impact on biogeochemical cycles in oil reservoirs. Thus, anaerobic, indigenous NRB have the potential to produce specific biosurfactants in the reservoir.

NRB and SRB are both oil-degrading bacteria and they existed as competitors in the reservoir system. As oil-degrading bacteria, they can utilize only a limited group of hydrocarbons, so bacteria attached and growing on an oil droplet become nutrient-starved once this group of hydrocarbons is depleted. If the biosurfactant is cell-bound it can cause the microbial cell surface to become more hydrophobic, depending on its orientation. For example, the cell surface hydrophobicity of *Pseudomonas aeruginosa* was greatly increased by the presence of cell-bound rhamnolipid (Sotirova et al. 2009). In addition, *Acinetobacter* strains produced cell-bound emulsifier to reduce the cell-surface hydrophobicity was also reported (Patil and Chopade 2001; Vasileva-Tonkova et al. 2011). These data suggest that microorganisms can use their biosurfactants to regulate their cell-surface properties to attach or detach from surfaces of substrates according to need. As indicated by *A. calcoaceticus* RAG-1 growing on crude oil, RAG-1 utilized only relatively long-chain n-alkanes for growth after attached to the to the oil droplet (Rosenberg 1993). Afer the depletion of these compounds, RAG-1 would detach the the starved cells from the substrate although aromatics and cyclic paraffins are still enriched. In this process, the ‘emulsifier’ desorbed the cell for new fresh substrate. Meanwhile, a polymeric film on the n-alkane depleted oil droplet was formed to mark the oil droplet as “used”, as the hydrophilic outer surface is hard to be attached by the bacterium again (Garrett et al. 2008). The detachment of bacteria from the depleted oil drop enables them to move to other drops where they metabolize the specific group of utilizable hydrocarbons. This biosurfactant-induced effect will lead to the NRB more competitive to SRB while nitrate/nitrite was injected for simulating NRB.

The developed biosurfactants will exist in pore throats to stimulate the growth of targeted beneficial microorganisms (NRB) that live in all oil and gas reservoirs and improve their ability to utilize hydrocarbons (Ron and Rosenberg 2002), thus out- competing harmful SRB for basic carbon nutrients. The SRB will be inhibited from producing new hydrogen sulfide/iron sulfide, and the existing sulfides will be removed by bacterial degradation, resulting in effective control of reservoir souring.

2.5 Summary

There are copious amounts of methods for the characterization of the microbial community and only popular methodologies are discussed here. The table below (Table 2.4) summarized the characteristics of each widely applied method for microbial characterization.

Direct detection methods such as most probable number (MPN) and direct cell counting are traditional, straight-forward and culture dependent ways of estimating microbial biomass of the SRB community. But, they are labor intensive since the bacteria must be closely monitored when culturing samples and they do not provide any details on community phylogeny, diversity or physiology when used alone (Spiegelman et al. 2005). These techniques cannot be used in samples containing low SRB numbers since only 0.001-15% of the total number of visible cells can be retrieved by isolation (Xu et al. 2014) and interference of debris affects the determinations of SRB numbers in turbid samples. Direct detection need also be used together with other culture-independent methods to characterize microbial communities since they cannot provide quantitative information.

Table 2.4 Common methods of microbial characterization

Method of Characterization	Description	Community Profile	Taxonomic identification	Quantitative	Limitations	Advantages
Metabolic assay	Emerging technique to profile total metabolites produced by a community	yes	no	yes	Not yet applied to communities; no data-bases available	Great analytical potential
Cell counting techniques	Counting of stained/culturable cells	no	no	yes	Introduces viability bias in cell count	Minimal equipment required
Function PCR	Several PCR-based analyses using amplified catabolic genes; indirect functional assay	n/a	n/a	no	Limited taxonomic information; limited databases	Better-suited to specialized investigations
DGGE	Seperates amplified 16S molecules by restriction patterns	yes	possible	no	Labour-intensive; complex profiles possible	Single base-pair resolution
TGGE	Seperates amplified 16S molecules by restriction patterns	yes	possible	no	Labour-intensive; complex profiles possible	Single base-pair resolution
Probe hybridization	Identifies presence of desired sequences using labeled probes	yes	yes	possible	Probe design only as good as available sequences	Highly flexible analytical tool
Bisbenzimidazole-CsCL-gradient fractionation	DNA fractionation based on %G-C content	yes	some	yes	Taxonomic information is ambiguous	Good complementation of genetic fingerprinting profiles

PLFA + FAME	Culture-independent community profile based on the distribution of various membrane lipids	yes	some	yes	Taxonomic identification is delicate and limited	Fast and inexpensive; good for comparing communities

Modified from Spiegelman et al. (2005)

Molecular methods offer the potential of determining the whole range of prokaryotic taxa without running into the problem of selective laboratory enrichment and growth media. Also, nucleic-acid analyses are reliable, reproducible and rapid, allowing a large number of samples to be analyzed simultaneously. However, there are many drawbacks in the methodologies, including inconsistent DNA recovery, inclusion of DNA from non-viable cells, kinetic biases, high cost of sampling in complex environments and the need to develop specific primers and probes requires extensive knowledge in sequencing. In practical operations, the storage of samples prior to processing can bias results. Shifts in active functional groups of prokaryotes have been observed when samples are stored aerobically or left at room temperature (Von Wintzingerode et al. 1997). Also, there are no bias-free extraction methods currently available for DNA-based methods (Pan et al. 2010). So, this method can be difficult to apply to extremely complex communities that produce hundreds of bands on a DGGE profile, which become difficult to visualize individually.

As to the biochemical methods, from Bisbenzimidazole-CsCl-gradient fractionation, no information can be obtained below the genus level from these profiles, since related, but possibly quite different species can appear in a single % G-C peak (Holben and Harris 1995). That is, % G-C profiles give information about the base composition of DNA, but cannot, on their own, be used to determine the presence or abundance of a particular species. Quinone profiling generates fairly non-specific profiles that and cannot themselves be subject to further specific analysis (such as sequencing or probing). Thus, it was used in conjunction with other techniques as a backup or confirmatory method to reinforce the assay results.

PLFA analysis is a fast, relatively simple and inexpensive method for SRB quantification. Compared to the molecular methods used in microbial ecology, PLFAs offer a robust estimation of three important attributes of microbial communities: viable biomass, microbial community structure, and the physiological status (Piotrowska-Seget and Mrozik 2003). PLFA analysis has many advantages associated with it. Most importantly, it is cheap and has the ability to characterize large microbial communities rapidly which is optimal for our research, to develop a cost-efficient SRB quantification methodology. Chemically, phospholipids consist of a glycerol linked to one polar phosphatidyl head group and two non-polar fatty acyl side-chains. The fatty acyl side-chains vary in composition (i.e., length, alkyl branches, substituent and number of double bonds) between eukaryotes and prokaryotes, as well as among many prokaryotic groups (Joergensen and Wichern 2008). This property makes PLFAs useful as biomarkers to determine the presence and abundance of broad functional microbial groups such as fungi, Gram-positive and negative bacteria etc.

Secondary oil recovery is a process used to maintain reservoir pressure in wells all over the world. When seawater is injected into a reservoir, souring is almost inevitable. Biocides are a common treatment used to control biomass within the well, however it is slowly becoming less popular due to the high costs, high risk, and inadequate souring control. Nitrate/nitrite injection is a relatively new way in which to mitigate H_2S gas. By adding nitrate or nitrite to a reservoir, NRB and NR-SOB within the injection water and reservoir are stimulated. The growth of NRB and NR-SOB is detrimental to SRB as they are outcompeted and inhibited. NRB have a thermodynamic advantage over SRB, and NR-SOB feed directly off of the sulfide or help to increase the redox potential, which is unfavorable to SRB.

It has been well established that microorganisms can utilize hydrocarbons as a carbon and energy source. Different types of bacteria, yeasts, and fungi produce metabolic products or membrane components behaving similar to surfactants when grow on substrates insoluble in water, which are generally named biosurfactants. Biosurfactants are a diverse group of surface-active chemical compounds, which are amphiphilic molecules with both hydrophilic and hydrophobic domains. Biosurfactants have an affinity for the interface between polar and nonpolar environments where they can mediate the surface tension between two phases in a mixture such as oil and water or at the air water interface of an aqueous solution of surface-active molecules. Besides reducing the surface tension of a liquid, biosurfactants may also have emulsion-stabilizing capability. This allows the "mixing" of hydrophobic substance such as hydrocarbons in aqueous solutions. Compared with traditional chemical surfactants, biosurfactants have many advantages for further applications, such as biodegradability, surface activity and environmental friendly property.

Many factors can influence the growth of bacteria and all biosynthetic mechanisms required for biosurfactant production, e.g. carbon source, nitrogen source and amount, temperature, pH and cation availability. As a consequence, these factors affect the "growth-stage" production of biosurfactants, and may also work in concert to form a complex set of conditions favoring biosurfactant production. As the demand to identify new biosurfactant producing microbes increases, different screening methods have been developed to screen biosurfactant producing microbes. In the literature review we listed hemolytic assay, BATH assay, emulsification assay, drop collapse assay, oil spreading, du-nouy-ring method, microplate and CTAB agar plate, with advantages and disadvantages of each discussed. Physical-chemical parameters including Surface tension, interfacial tension and CMC which are quantitatively measured in biosurfactant production were discussed for biosurfactants characterization. To elucidate the complete structure

of biosurfactants, various chromatographic and spectroscopic techniques like TLC, HPLC, LC-MS and ^{13}C-NMR were listed and evaluated.

Biosurfactants can partition at the oil-water or water-air interfaces with differing polarities to reduce the interfacial and/or surface tension. Such properties make them good candidates for EOR. The appropriate biosurfactants are able to improve oil recovery processes, inhibit the production of H_2S, to be of no secondary pollution, and be operated easily and economically. In the nitrate/nitrite injection operations for reservoir souring control, biosurfactants naturally derived from NRB have potential use in characterizing NRB-SRB interaction and generating biosurfactant aided technology for reservoir souring control. Biosurfactants can be produced as intermediate products of microbial activities. The most critical issue associated with the biosurfactant development and application is to determine whether an additional biosurfactant will promote (or limit) activities of the inherent microorganisms. After playing the roles of improving media conditions and promoting microbial activities, the biosurfactant (as an organic matter) could then be used as a preferential substrate by inherent microorganisms. Ideally, a biosurfactant should be degradable by the microorganisms at a slow rate to maintain its enhancement effectiveness. Indigenous NRB would be stimulated through nitrate additions and the growth of SRB can be suppressed, thus the production of H_2S in the oil reservoirs can be well controlled. However, in this process although the anaerobic, indigenous NRB have the potential to produce specific biosurfactants in the reservoir, very little study was reported upon biosurfactants generation by NRB and their specific roles in the interaction are seldom examined. In our project, biosurfactants naturally derived from NRB will be monitored and related characterization will be conducted during the nitrate/nitrite injection batch experiments in the lab-scale reactor.

CHAPTER 3

PLFA ANALYSIS FOR PROFILING MICROBIAL COMMUNITIES IN OFFSHORE PRODUCED WATER[1]

[1] *This chapter is based on the following paper:*

F. Fan, B. Zhang, P.L. Morrill, Phospholipid fatty acid (PLFA) analysis for profiling microbial communities in offshore produced water, Marine pollution bulletin, 122 (2017) 194-206.

Role: Fuqiang Fan solely worked on this study and acted as the first author of this manuscript under the guidance of Dr. Baiyu Zhang and Dr. Penny Morrill.

3.1 Background

Offshore produced water is commonly derived from the formation water in the deep reservoir aquifer during secondary oil recovery operations. Water flooding techniques are frequently utilized for these operations in which seawater or other water is injected into the reservoir to maintain pressure level underneath and sweep the oil from the reservoir towards producing wells (Gieg et al. 2011). Since produced water is a mixture of original water from different geological formations and the liquids injected into the hydrocarbon zone, it could be used as a mirror to reflect the undergoing chemical and biological activities beneath the seabed as a result of offshore oil and gas operations. Microbial communities in offshore produced water are of great scientific significance and relevant to many industrial applications (Li et al. 2007a).

Characteristics of the deep subsurface petroleum reservoir are high temperature, high pressure, high salinity and anoxic conditions, and with multiphase fluids of oil, gas and water. Microorganisms inhabiting in such extreme harsh environments thus gained great attention in recent years (Van Hamme et al. 2003). The mesophilic and thermophilic bacteria and archaea were found in great distribution, and many of these organisms have potential to be involved in organic and inorganic compound metabolisms (Magot et al. 2000). In addition, during offshore oil productions, biological souring induced by the reduction of sulfate ions from injection water poses severe processing and environmental concerns. SRBs are responsible for the bacterial problems (Hubert and Voordouw 2007), thus the profiling of these microbial groups from produced water is highly desired for reservoir souring control. Hasegawa et al. (2014) identified crude-oil components and microorganisms in oil-field water responsible for crude-oil souring and the results indicated that the degradation of these compounds was mediated by SRB (*Desulfotignum spp.*) via

the fumarate-addition pathway. Significant activity of SRB was observed in samples from the offshore Bonga field (120 km southwest of Warri, Nigeria), although the field was amended with calcium nitrate to limit reservoir souring (Okoro et al. 2014). Tanji et al. (2014) collected a microbial consortium from an oil-water separator to investigate the mechanisms of souring, and the relative abundance of SRB was observed to increase along with souring. Thus, the microbiological analysis of offshore produced water places great importance on the investigation of biological reservoir souring control.

To measure microbial diversity and biomass, culture-independent methods provide obvious advantages over culture techniques. The latter ones are generally time-consuming, labor-intensive and most of the microorganisms are still recalcitrant to cultivation (Zengler 2009). PLFA analysis has been widely used as a culture-independent technique. Phospholipids are essential components of microbial membranes (Powl et al. 2007) and they vary between eukaryotes and prokaryotes as well as many different species among prokaryotes (Joergensen and Wichern 2008). Phospholipids decompose rapidly after cell death in the environment (Lanekoff and Karlsson 2010). PLFA analysis thus provides robust information on the microbial community structure, the abundance of viable microbial groups, and their physiological status. Nielsen and Petersen (2000) estimated that the fatty acids (FAs) yields from non-microbial sources accounted for no more than 5-10% in PLFA analysis. PLFA analysis has the potential to be an inexpensive and quantitative method for microbial profiling of a large number of complex samples. PLFAs have been extensively applied as biomarkers to characterize microorganisms in soils, drinking waters, groundwaters, sediments and biomats (Dijkman et al. 2010; Drenovsky et al. 2010; Franzmann et al. 1996; Mills et al. 2006; Yu et al. 2009). These studies showed that the performance of PLFA analysis highly depended on

the specific matrix extracted. Till now, no studies have been published for microbial profiling of offshore produced water with a complicated matrix and high salinity using PLFA analysis.

The procedures of PLFA analysis have been examined previously including extraction (Papadopoulou et al. 2011; Wu et al. 2009), purification (Mills and Goldhaber 2010) and derivatization of FAMEs for gas chromatography (GC) determination (Gómez-Brandón et al. 2008; Rosenfeld 2002) in various types of samples. In terms of the extraction, phase partition was always involved after one-phase extraction of total lipids from diverse samples (Fang et al. 2000; Fang and Findlay 1996; Sturt et al. 2004). Nevertheless, lipid extraction efficiency during the phase partition was not clearly elucidated previously and insufficient liquid-liquid extraction can lead to considerable losses of these lipids. After the extraction process, the neutral lipid, glycolipid and phospholipid fractions (F1, F2, F3) are sequentially eluted from a silica gel column with dichloromethane (DCM), acetone, and methanol, receptively. Mills and Goldhaber (2010) examined the recovery of all phospholipid classes using three commercial silica columns and concluded a methanol to silica ratio of 20:1 was sufficient to quantitatively recover the phospholipid standards. However, limited studies on phospholipid recovery were conducted on non-commercial columns and the appropriate methanol amount should be examined to adapt to the silica capacities. Furthermore, the proper volumes of DCM and acetone need to be determined for removal of non-polar oil and lipid components extracted from oily produced water.

The phospholipids in the F3 fraction need to be derivatized to FAMEs for analysis by GC. In terms of FAME preparation, the base-catalyzed method was recommended during transesterification to profile comprehensive microbial communities since fatty acids containing methyl groups could not be detected by the acid-catalyzed method (Chowdhury and Dick 2012). Zhang et al. (2007) used mild-alkali methanolysis for the generation of FAMEs and investigated the patterns of microbial

communities in paddy soil with fertilizer treatments. Different fertilizer practices were proved to have varying degrees of influence on the community structure of specific microbial groups. PLFAs were also released as FAMEs by mild alkaline transesterification and the profiles were successfully employed to illustrate the microbial community structure in drinking water biofilters fed with varied concentrations of acetate or glucose (Yu et al. 2009). Previous studies indicated that transesterification parameters such as types of methylation agent, volumes of acids for neutralization and specific reaction conditions in base-catalyzed method could significantly impact the PLFA analytical performance (Christie 1982; Ruiz-Rodriguez et al. 2010). Therefore, to examine the performance of PLFA analysis for microbial profiling of produced water, operation conditions during extraction, purification and derivatization of FAMEs in previous studies are not directly applicable and need to be further evaluated.

The objective of this study was to optimize the fatty acid extraction from offshore produced water matrix for the purpose of PLFA quantification and microbial community profiling of these fluids. The extraction steps and parameters that were studied included phase partition efficiency during extraction; volumes of DCM, acetone and methanol during purification; as well as types of methylation agent, volumes of acids and associated specific reaction conditions during FAME derivatization. This study provided a suitable and efficient GC-based analytical method for intensive detection of PLFAs in oily saline offshore produced water, which would aid the investigation of biological reservoir souring control among scientific and industrial activities.

3.2 Materials and Methods

3.2.1 Chemicals, reagents and glassware

The ultra-high purity water, silica gel (60-120 mesh), sodium hydroxide and solvents including DCM, methanol, hexane, toluene and acetone of reagent grade or higher quality were purchased from VWR® International (Mississauga, Ontario, Canada) and Fisher Scientific (Ottawa, Ontario, Canada). The commercial 3 mL SPE tubes (miniature champagne column) were bought from Supelco Inc. (Bellefonte, Pennsylvania, USA). GC supplies, including deactivated single tapered glass inlet liners and J&W Scientific DB-5MS UI fused silica capillary columns, were obtained from Agilent® Technologies Inc. (Mississauga, Ontario, Canada). Potassium phosphate monobasic, potassium phosphate dibasic and potassium hydroxide were obtained from Sigma-Aldrich (Oakville, Ontario, Canada). Analytical standards of FAMEs: Bacterial Acid Methyl Esters CP Mixture (26 methyl esters), C14:1 (cis-9) (methyl tetradecanoate, cis-9), 10Me C16:0 (methyl 10-methylhexadecanoate), C16:1 (trans-9) (methyl hexadecenoate, trans-9), C18:1 (trans-11) (methyl octadecenoate, trans-11), C19:0 (methyl nonadecanoate), and C21:0 (methyl heneicosanoate) were purchased from Matreya LLC (Pleasant Gap, Pennsylvania, USA). C18:1 (cis-11) (methyl octadecenoate, cis-11) was purchased from Sigma-Aldrich (Oakville, Ontario, Canada). Phospholipid standards C16:1 (cis-9) PC (1,2-dipalmitoleoyl-sn-glycero-3-phosphocholine), C18:1 (cis-9) PC (1,2-dioleoyl-sn-glycero-3-phosphocholine), C19:0 PC (1, 2-dinonadecanoyl-sn-glycero-3-phosphocholine) were purchased from Avanti Polar Lipids, Inc. (Alabaster, Alabama, USA). Extraction tubes and all other glassware were primarily washed with detergent and then deeply rinsed with chromic acid lotion to remove all types of oil, fingerprints, rust and other dirt.

3.2.2 Sample collection

Produced water samples were collected from a local offshore oil and gas platform in Newfoundland and Labrador, Canada after oil-water separation. Right after the collection from the offshore sampling port, 500 mL water sample was filtered through 0.2 μm nylon membrane filter (47 mm in diameter) through a vacuum pump. The filter paper was then transferred into a 10 mL amber vial with 9.5 mL extraction solvents (methanol: DCM: 125 mM phosphate buffer at pH 7.4 = 2:1:0.8) inside. Triplicated samples were collected followed by the filtration treatment and sealed with screw caps containing Teflon lined septa. All samples were stored in a freezer in the dark and then packaged with frozen ice packs while they were transported to the laboratory.

3.2.3 Extraction of lipids from water samples

3.2.3.1 Extraction solvent

PLFAs are commonly extracted from environmental samples by the modified Bligh and Dyer method following by purification with silicic acid chromatography (Axelsson and Gentili 2014). Phosphate buffer was then used to replace water in the one-phase mixture containing chloroform, methanol, and water to improve the extraction efficiency (Bossio and Scow 1998; Nielsen and Petersen 2000). Whereas further modifications involved the replacement of chloroform with less-toxic DCM to reduce the risk of carcinogenicity (threshold limit 20–25 times higher than chloroform) (Cequier-Sánchez et al. 2008). DCM has a higher density (1.3266 g/cm^3) than water and it allows the phase separation of the extraction mixture by adding more volumes of DCM and phosphate buffer. DCM was thus selected as the extraction solvent in this study. After this liquid-liquid extraction, the target lipids distribute mainly in the organic phase and this phase is collected for further treatment. Since some polar lipids extracted from microbial communities may distribute

in the water phase (methanol included) as well, insufficient liquid-liquid extraction could lead to a certain amount of loss of these lipids.

3.2.3.2 Phase separation optimization

To optimize for phase separation of lipids in the sample, total lipids were extracted using a modified Bligh and Dyer extraction method (Fang and Findlay 1996). The nylon membrane filter with suspensions from produced water samples was extracted in an amber vial with 5 mL methanol, 2.5 mL DCM and 2.0 mL phosphate buffer at pH 7.4 (2: 1: 0.8). The mixture was placed on a vortex mixer for 30 s at 3000 rpm to form fine droplets cloud and this vortex operation was further processed twice. The amber vial was left overnight in the dark at -20 ^{o}C. Then 2.5 mL DCM and 2.5 mL phosphate buffer were added for phase separation, such that the final ratio of DCM to methanol to water ratio was 1: 1: 0.9. The lower organic phase was transferred and collected in a glass tube. The membrane filter was subjected to secondary and tertiary total lipids extraction with additional 4.5 mL and 4 mL mixture of methanol, DCM and phosphate buffer (2: 1: 0.8), respectively. After the same partition procedure, all the organic phase solvents containing the extracted lipids from phase partition was collected and evaporated under a gentle stream of nitrogen. The phospholipid recovery of the phase separation was quantified by spiking the vortexed sample with phospholipid standards C16:1 (cis-9) PC, C18:1 (cis-9) PC, and C19:0 PC. All samples were extracted in triplicate.

3.2.4 SPE optimization

The total lipids were redissolved into the tube within 2 mL hexane: DCM (70:30, v/v) and then fractionated into neutral lipids, glycolipids and phospholipids with DCM, acetone and methanol respectively. SPE technology including pre-packed non-commercial columns (0.1 g silica gel) and

vacuum pump was used to assist elution. Before applying the sample, SPE tubes were initially conditioned by flushing 3 mL methanol and 3 mL DCM through them. The lipid extracts were then loaded and the column was washed sequentially with 4 mL DCM, 4 mL acetone, and 10 mL of methanol. The phospholipids were collected in the methanol fraction. The methanol fraction was then evaporated to dryness under a stream of nitrogen and stored at -20 ^{o}C before derivatization.

SPE efficiency was tested with varying volumes of DCM, acetone, methanol; as well as the solvent elution speed. The elution solvents of DCM and acetone are assumed to remove neutral lipids, glycolipids and the interference of oil components from the sample matrix. However, an excess use of these solvents may influence the final recovery of phospholipids in the specific non-commercial column. Thus three different volumes of methanol (2 mL, 4 mL and 6 mL) and two different volumes of DCM and acetone (2 mL and 4 mL) were used. The elution behavior was further studied through spiking the standards (C16:1 (cis-9) PC, C18:1 (cis-9) PC, and C19:0 PC) into 2 mL hexane: DCM (70:30, v/v). The elution from the column was separated into 8 fractions: (1) the 2 mL solution residue, (2) the 4 mL DCM, (3) the 4 mL acetone, (4) the first 4 mL methanol, (5) the second 2 mL methanol (4-6 mL), (6) the third 2 mL methanol (6-8 L), (7) the fourth 2 mL methanol (8-10 mL), and (8) the last 2 mL methanol (10-12mL). All fractions were analyzed for the spiked phospholipids standards (C16:1 (cis-9) PC, C18:1 (cis-9) PC, and C19:0 PC) after derivatization. The process control was conducted through spiking the standards (C16:1 (cis-9) PC, C18:1 (cis-9) PC, and C19:0 PC) before SPE into 0.5 mL of methanol: toluene (1:1, v/v) and further transferring them to FAMEs (the corresponding concentrations were 3.8, 3.1, and 5.1 mg/L). Four blanks were obtained through spiking C19:0 PC into 0.5 mL of methanol: toluene

(1:1, v/v) in each sample to eliminate the errors of FAMEs derived from solvents and other sources. The SPE efficiency test was conducted in triplicate to determine its reproducibility.

3.2.5 Derivatization of FAME optimization

The solvent fractions to be analyzed for phospholipids were re-dissolved in 0.5 mL of methanol: toluene (1:1, v/v) and subjected to a mild alkaline trans-methylation procedure to produce FAMEs. Then, 0.5 mL of 0.2 N KOH in methanol was added to the fraction. The solution was mixed in a vortex and heated in a water bath at 37^{o}C for 12 min. The reaction mixture was then cooled to room temperature and was neutralized with 0.5 mL of 0.2 N acetic acid. DCM and ultrapure water both at 1 mL were added for phase separation. The bottom phase containing the FAMEs was collected and the upper layer was re-extracted with 1 mL DCM. Both of the DCM phases obtained were collected and concentrated to 400 μL under nitrogen flow. To optimize the derivatization steps, the volumes of acetic acid used for neutralization (0 mL, 0.5 mL, 1.0 mL and 1.5 mL), the type of methylation agent (KOH and NaOH), the reaction time (4 min, 12min and 20 min) and temperature (22^{o}C, 37^{o}C and 52^{o}C) were investigated in the treatment. Three phospholipid standards mentioned above was spiked in the solvents before the methanolysis procedure. The derivatization of PLFA optimization test was conducted in triplicate to determine its reproducibility.

3.2.6 Gas chromatography-mass spectrometry (GC-MS) determination of FAMEs

The FAMEs were transferred into autosampler glass vials and analysed on a GC-MS system (Agilent 7890A GC system coupled with a 5975C MSD) interfaced with an Agilent 7693 auto-sampler. Data acquisition, processing and evaluation were carried out using Agilent ChemStation software Version 2.01. Twenty-nine FAMEs were separated on a 30 m × 250 μm (internal

diameter, i.d.) × 0.25μm DB-5MS UI fused silica capillary column. An electronic pressure control (7.65 psi) was utilized to maintain a constant carrier gas (Helium of ultrahigh purity) flow of 1.0 mL/min throughout the oven program. Sample injections (3μL) were conducted using a split/splitless injector (single tapered inlet liner, pulsed splitless mode) at 200 °C under a pulse pressure of 25 psi. Both full scan and selected ion monitoring (SIM) mode were used for FAME determination. The initial oven temperature was 50 ^{o}C, then it was raised to 150 ^{o}C at 20 °C/min, to 180 ^{o}C at 3°C/min, to 210 ^{o}C at 1°C/min, and finally to 280 ^{o}C at 35°C/min with a running time of 47 min. The mass spectrometer was operated in the electron impact (EI) mode at 70 eV in the scan range of 50–550 m/z.

3.2.7 Identification, quantification and validation

A mixture of 29 FAMEs was prepared and used as external standards. High resolution was achieved by the series of FAMEs that have the same primary ion and close retention times (Table 3.1). SIM mode is used to identify the methyl ester peaks and the presence of methyl esters was further confirmed by comparing their relative retention times with those of FAME standards. Fatty acids are designated by the total number of carbon atoms to the number of double bonds (e.g. a 16-carbon alkanoic acid is C16:0). The position of the double bond is indicated by a Δ number closest to the carboxyl end of the fatty acid molecule with the geometry of either cis (c) or trans (t). Prefixes i and a are given for iso- and anteiso-branched FAMEs, respectively. The suffix 10Me indicates a methyl group at the 10th C atom, while OH stands for hydroxy and the cyclopropyl group is indicated by ‘cy’.

Table 3.1 Retention time, identification and quantification ions (m/z), LODs, calibration range, linearity, recovery rates, and repeatability of the twenty-nine FAMEs included in the analysis

	FAMEs	Retention time (min)	Ions monitored for confirmation (m/z)	Ions monitored for quantization (m/z)	LODs (ng/L)	Calibration Range (mg/L)	Linearity (R2)	Recovery rates (%)	Repeatability (n=7) RSD (%)
1	C11:0	7.99	55/74/87	74	3.0	0.005-10	0.9957	81.2	4.3
2	2-OH C10:0	8.12	55/69/83	69	5.8	0.01-10	0.9974	89.2	7.2
3	C12:0	9.57	55/74/87	74	2.5	0.005-10	0.9965	82.5	3.4
4	C13:0	11.52	74/87	74	2.5	0.005-10	0.9970	83.4	3.4
5	2-OH C12:0	11.80	55/69/83/97	69	4.8	0.01-10	0.9960	80.4	7.4
6	3-OH C12:0	12.47	55/69/83/97	74	4.0	0.01-10	0.9973	78.5	7.2
7	C14:0	13.85	55/74	74	2.6	0.005-10	0.9972	79.4	3.4
8	i-C15:0	15.46	55/74/87	74	2.9	0.005-10	0.9978	95.4	4.1
9	a-C15:0	15.67	55/74/87/97	74	2.5	0.005-10	0.9980	76.7	3.6
10	C15:0	16.51	55/74/87	74	2.7	0.005-10	0.9978	84.9	4.1
11	2-OH C14:0	16.95	55/69/83/97	69	3.9	0.01-10	0.9960	99.7	2.2
12	3-OH C14:0	17.90	55/69/83/97	69	5.2	0.02-10	0.9952	75.3	2.9
13	i-C16:0	18.51	55/74/87	74	2.8	0.005-10	0.9986	85.7	3.8
14	C16:1 (cis-9)	19.02	55/69/74/83	74	3.8	0.01-10	0.9987	101.1	5.6
15	C16:1 (trans-9)	19.20	55/69/74/83	74	2.6	0.01-10	0.9982	91.1	4.1
16	C16:0	19.83	74/87/143	74	1.6	0.005-10	0.9979	100.1	2.8
17	10Me C16:0	21.48	74/87/143	74	2.2	0.01-10	0.9984	87.0	3.2
18	i-C17:0	22.33	74/87/143	74	3.7	0.01-10	0.9987	86.2	4.9
19	C17:0 Δ (all cis-9,10)	23.16	55/69/74/83	74	4.2	0.01-10	0.9988	77.6	6.2
20	C17:0	23.94	74/87/143	74	2.4	0.005-10	0.9985	89.4	5.8
21	2-OH C16:0	24.70	55/69/83/97	69	9.7	0.01-10	0.9981	100.4	5.5
22	C18:2 (all cis-9,12)	26.96	55/69/83/97	69	3.0	0.01-10	0.9980	1027	6.3
23	C18:1 (cis-9)	27.35	55/69/83/97	74	2.5	0.01-10	0.9985	102.7	4.6
24	C18:1 (cis-11)	27.72	55/69/83/97	69	0.3	0.01-10	0.9982	90.6	0.9
25	C18:1 (trans-11)	27.93	55/69/83/97	74	3.1	0.01-10	0.9980	82.5	6.5
26	C18:0	28.82	55/69/83/97	74	2.5	0.005-10	0.9985	103.1	5.6
27	C19:0 Δ (all cis-9,10)	32.89	55/69/83/97	69	3.2	0.01-10	0.9979	101.4	6.2
28	C19:0	33.75	74/87/143	74	3.4	0.005-10	0.9973	102.4	6.2
29	C20:0	37.08	74/87/143	74	1.8	0.01-10	0.9952	102.8	3.5

Quantitative analyses of individual fatty acids were performed based on the GC/MS response (peak area) relative to that of two internal standards (C14:1 (cis-9) and C21:0) with known concentrations. Method blanks were included in each set of samples and all results were blank corrected accordingly. The area responses of the characteristic m/z against concentration for each compound and internal standard were analyzed and response factors (RFs) for each compound were calculated using Equation 1 (EPA, 1999).

$$RF = \frac{(A_s)(C_{is})}{(A_{is})(C_s)} \quad (1)$$

where:

A_s = Area of the characteristic m/z for the parameter to be measured.

A_{is} = Area of the characteristic m/z for the internal standard.

C_{is}= Known concentration of the internal standard.

C_s = Known concentration of the parameter to be measured.

Then the RF value over the working range can be obtained and the concentration in the sample was calculated using the determined RF and Equation 2.

$$Concentration\ (mg/L) = \frac{(A_s)(C_{is})}{(A_{is})(RF)} \quad (2)$$

where:

A_s = Area of the characteristic m/z for the parameter or surrogate standard to be measured.

A_{is} = Area of the characteristic m/z for the internal standard.

C_{is} = Concentration of the internal standard.

The selection of internal standards was based on a pre-test of offshore water samples to confirm there were no recognizable chromatographic peaks of C14:1 (cis-9) and C21:0. Eleven calibration standards spiked with multiple concentrations were prepared by diluting the stock in DCM. The calibration range was between 0.005 and 10 mg/L. The analytical performance of the optimized methodology coupled with GC-MS was evaluated by measuring the linearity, limits of detection (LODs), recovery rates (RRs%) from the methyl esterification and relative standard deviation (RSD%). The errors in all the analyses were calculated from triplicate or higher numbers of sample analysis. Paired t tests were used for the statistical evaluation of differences in the analysis. A p value less than 0.05 was used to indicate the significant difference between the tested groups.

3.3 Results and Discussion

3.3.1 Extraction efficiency in phase partition

The one-phase mixture of methanol, DCM and phosphate buffer at pH 7.4 (2:1:0.8, v/v) was efficient in total lipids extractions, due to the presence of solvents with various polarity. Methanol was water-miscible organic solvent and was scattered in both organic and aqueous phases after the solvent partitioning when the ratio of DCM to methanol to water ratio was changed to 1: 1: 0.9. It was assumed that partitioning of solutes (the total lipids extracted) between two liquids would stay in a dynamic equilibrium (Berthod and Carda-Broch 2004) and compounds are separated based on their solubility. Phospholipids (C16:1 (cis-9) PC, C18:1 (cis-9) PC, and C19:0 PC) spiked in the vortexed samples were allowed to distribute in dynamic equilibrium in phase partition. The organic phase was collected and the aqueous phase was again processed with 1 mL DCM. The two organic phases were combined and subjected to FAME derivatization. Triplicate samples with the same amount of phospholipids were also subjected to FAME derivatization directly. Results

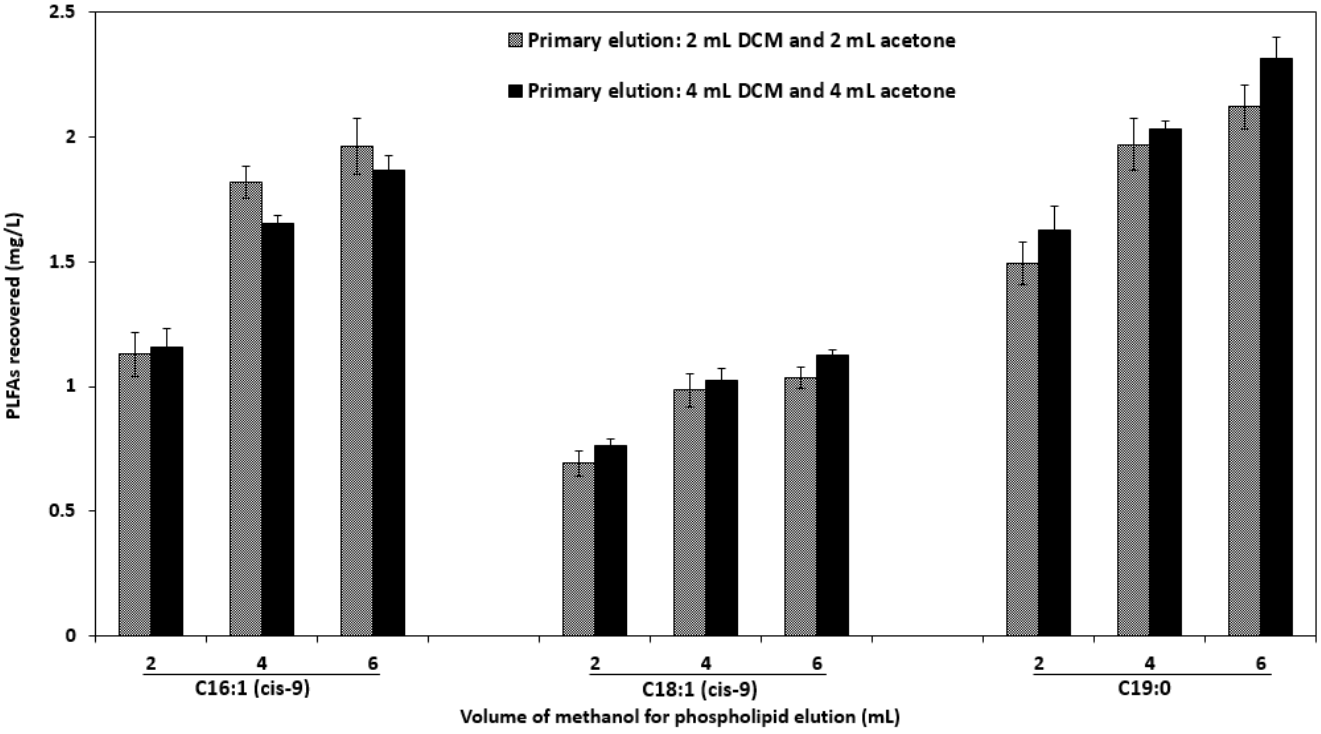
indicated that 99.7% of C16:1 (cis-9), 99.2% of C18:1 (cis-9), and 99.4% of C19:0 were recovered during the two-phase partition when the FAMEs from standards control were taken as a reference. The RSDs of the analyses were all below 7.5%. The robust recovery indicated the lipid loss in the phase separation was negligible under current experimental settings.

3.3.2 Determination of elution solvent volumes in SPE

SPE is based on the hydrophobic behavior of dissolved organic compounds on the packed column (Roubeuf et al. 2000). DCM, acetone and methanol are solvents with increasing polarity to elute increasingly polar lipids: neutral lipids, low-polarity lipids and polar lipids (Heinzelmann et al. 2014; Hutchins et al. 2008). The phospholipid isolation from the lipid extract was performed with miniature champagne tubes paced with 0.1 g silica gel. An initial column conditioning step of methanol was typically used to dehydrate the silica (Dobbs and Findlay 1993) to avoid any partial hydration, which would reduce the polarity of silicic acid and may also potentiate the chromatographic partitioning (Dickson et al. 2009). The phospholipids recoveries in methanol fractions were also observed to be reduced for the phosphoethanolamine (PE) and phosphocholine (PC) groups without methanol preconditioning (Mills and Goldhaber 2010), thus a methanol preconditioning step was necessary. Based on the polarity principle, a DCM preconditioning step was also conducted for the efficient removal of any oily organics in packed SPE tubes.

The influence of two different volumes of DCM and acetone and the elution behavior with three different volumes of methanol were shown in Figure 3.1a. While the values of parameters were selected according to previous studies (Bondia-Pons et al. 2006; Bossio and Scow 1998; Buyer and Sasser 2012; Mills and Goldhaber 2010), significant differences were detected from serial volumes of methanol for phospholipid elution. In contrast, the volumes of DCM and acetone have

little effect on the target lipid recoveries even though only 0.1 g silica gel was packed. Elution volumes of methanol seemed not to be adequate for high throughput of phospholipid fraction in the settings. Thus 4 mL, 6 mL, 8 mL, 10 mL and 12 mL volumes of methanol were used to examine elution efficiency of phospholipids. Insufficient methanol eluant may result in incomplete elution of phospholipids from silica columns and may explain the low reported recoveries (60-75%) of PC standards by Billings and Ziegler (2008). Mills and Goldhaber (2010) investigated the elution behavior of four phospholipid classes using three commercial SPE columns (packed with 0.5 g silica) and 10 mL methanol was found to be sufficient to quantitatively recover the phospholipid standards. Wu et al. (2009) also used 10 mL methanol elution on home-made SPE tubes to obtain phospholipids from two soils with different organic carbon concentrations. From the results shown in Figure 3.1b, the recoveries of phospholipids C16:1 (cis-9) PC, C18:1 (cis-9) PC, and C19:0 PC reached 92.9%, 96.3% and 92.8%, respectively, from 10 mL methanol elution and the RSDs were all below 6.5%. Thus, 10 mL methanol in phospholipid elution could provide an accurate characterization of microbial communities. Methanol elution was widely applied in quantitative phospholipid studies on SPE columns from multiple sources (Bondia-Pons et al. 2006; Bossio and Scow 1998; Chowdhury and Dick 2012; Fang and Findlay 1996), but the details of their separation recoveries were not reported. Avalli and Contarini (2005) recovered 96% of a phospholipid mixture from SPE cartridges packed with 1 g silica using 2 mL methanol followed by 2 mL methanol: chloroform: water (5:3:2 v/v/v). Polar lipids are strongly retained by hydrogen bonding and dipolar interactions within the silica column, thus more polar solvent mixture demonstrated better elution behavior. However, further phase separation was involved in this step and the complexity was increased, which prolonged the analysis procedures and may lead to potential target loss.


Primary elution: 2 mL DCM and 2 mL acetone
Primary elution: 4 mL DCM and 4 mL acetone
PLFAs recovered (mg/L)
2.5
2
1.5
1
0.5
0
2
4
6
C16:1 (cis-9)
C18:1 (cis-9)
C19:0
Volume of methanol for phospholipid elution (mL)

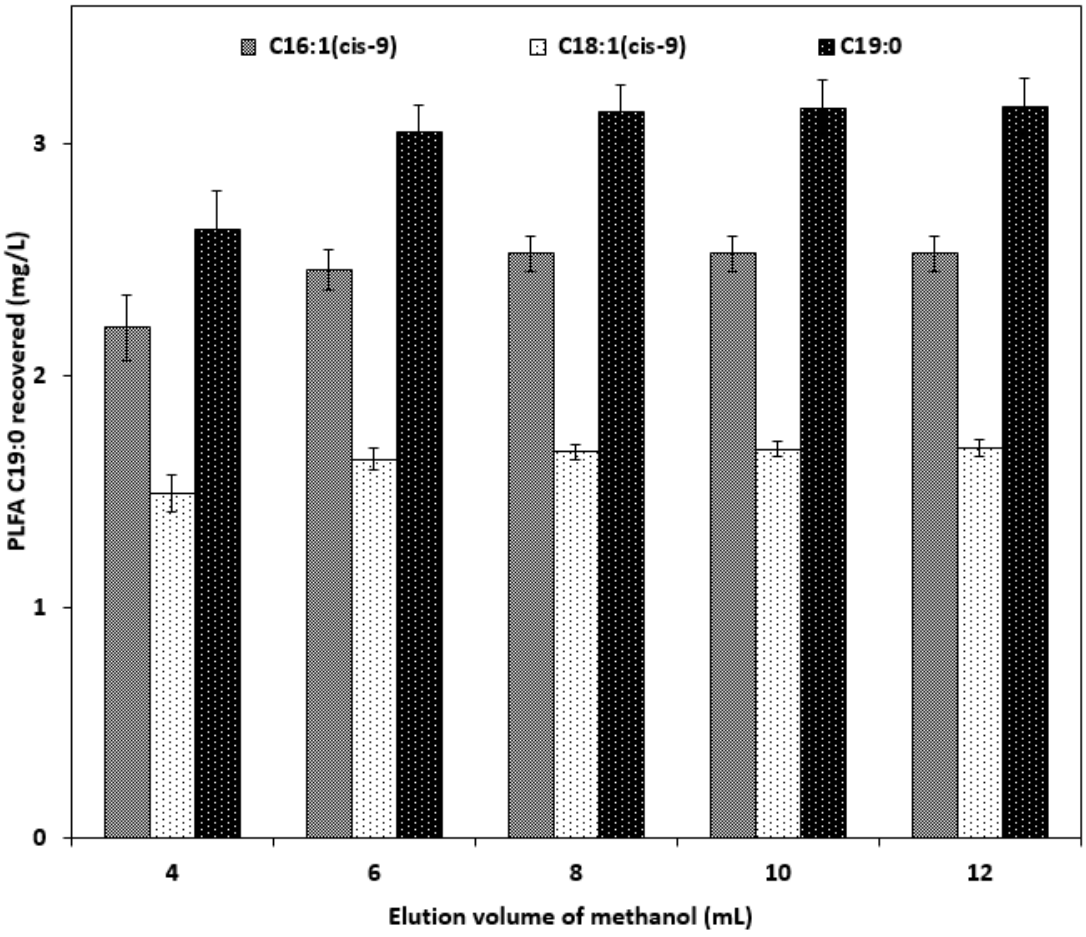


Figure 3.1 (a) Different volumes of DCM, acetone and methanol on the elution recoveries of phospholipids. (**b)** Elution recoveries of phospholipids from SPE columns using 6 mL, 10 mL and 14mL methanol.

The DCM-acetone-methanol elution protocol was further examined using two different elution rate and the results were shown in Figure 3.2. Little or no phospholipids were detected from the solution residue, DCM or acetone fraction for all the three spiked standards. It was revealed that the polar solute was transferred from the liquid phase into the silica absorbent. Elution behavior was slightly different when a higher elution rate was applied. The volume of the first fraction eluted with 4 mL methanol was reduced when elution flow rate became higher, and the volume of the subsequent phospholipid fraction recovered by the second 2 mL methanol was thus increased. This phenomenon from the first methanol fraction indicated that sufficient contact time would release more polar lipids from their interaction with the silica absorbent while applying an equivalent amount of solvent. Complete recovery of phospholipids may require more eluent if the elution speed was increased. Mills and Goldhaber (2010) used different treatments on the solvent elution rate (1 mL/min and 3 mL/min) to study the phospholipid recovery on SPE columns and found the same elution tendency in the two methanol fractions. Even though, 10 mL of methanol gave a convincing performance for the complete phospholipid recovery in this SPE procedure.

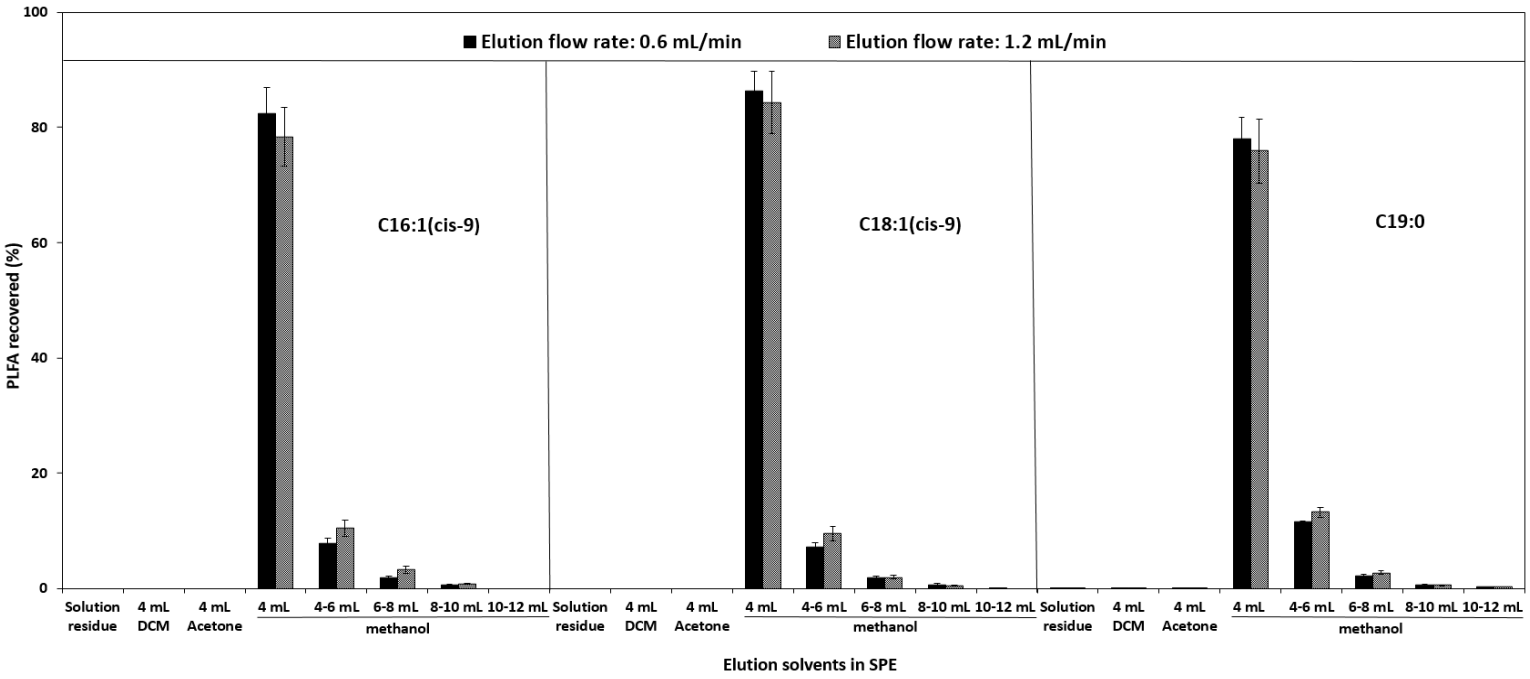


Figure 3.2 Elution behaviour of phospholipids from SPE columns under two different elution rate (0.6 mL/min and 1.2 mL/min). The phospholipids were eluted in 8 fractions continuously as stated in the methods

3.3.3 Derivatization to FAMEs

The derivatization method to FAMEs has significantly influenced the detectable concentration of fatty acids. Acid-catalyzed methylation is an effective method to esterify free fatty acids and transesterify fatty acids linked by ester bonds to glycerol or cholesterol. The acidic HCl together with methanol solvent esterify all fatty acids at approximately the same rate (Weston et al. 2008). However, its major drawback was that the PLFAs with methyl groups could not be detected, possibly due to underlying mechanisms of addition-elimination reactions that occur during esterification (Chowdhury and Dick 2012). To get a full picture of microbial profiling, a mild alkaline trans-methylation procedure was selected in this study.

Base-catalyzed methylation reaction involves the cleaving of the ester bond by an alcohol. The original ester firstly forms an anionic intermediate (b) (Figure 3.3) in the presence of a base such as an alcoholate anion (a). The intermediate (b) can dissociate back to its former state or tend to form a new ester (c) when the anion (a) was derived from a large excess of the alcohol. Due to the presence of a large number of anion (a), the equilibrium will be displaced in favor of new ester (c) production. This transesterification will virtually result in the sole product (c). Referred from the optimal molarity is 0.5 to 2 N sodium or potassium methoxide in anhydrous methanol (Demirbas 2008), 0.2 N KOH/NaOH in methanol was adopted. Since the quality of the alkaline reagent and the way it is prepared were potential factors that may affect methylation, the performance of KOH and NaOH as the catalyst was compared under stated conditions.

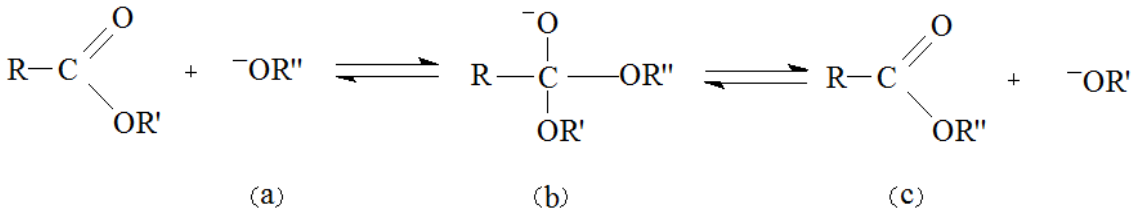
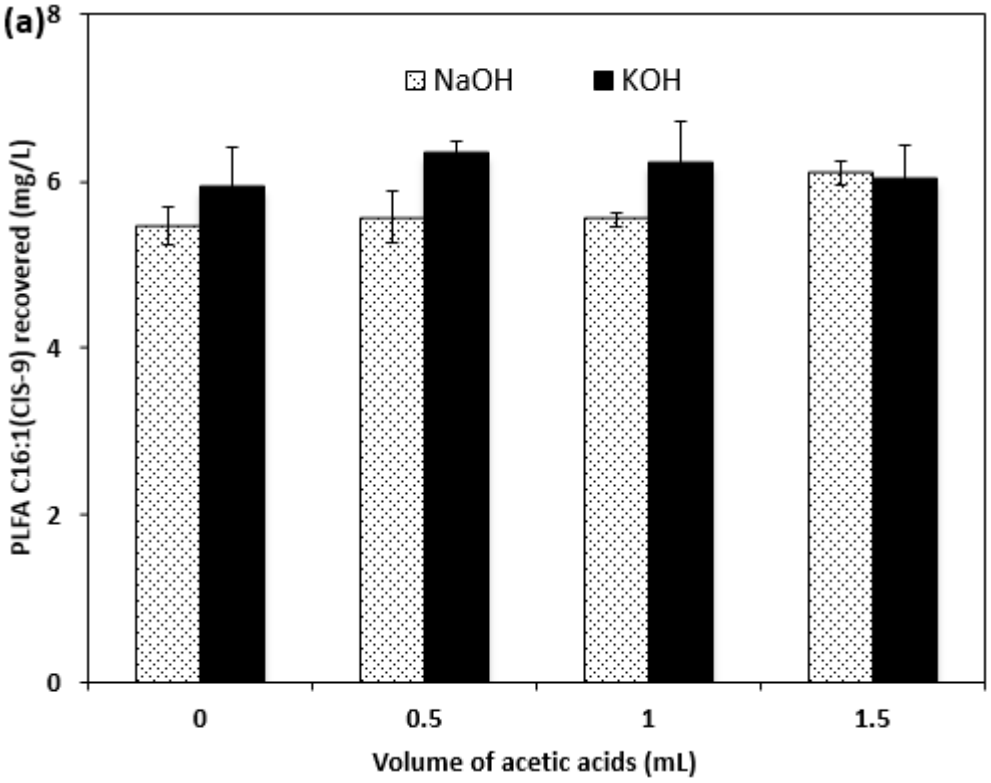
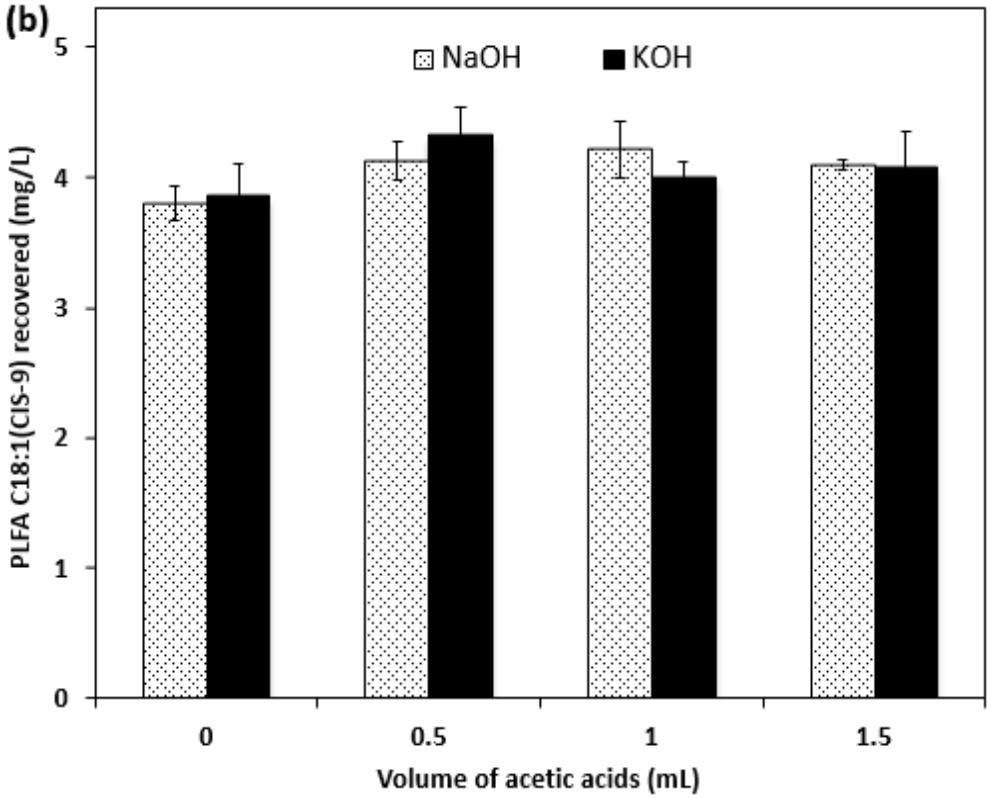


Figure 3.3 The process of base-catalyzed transesterification of lipids

After the reaction, the impact of diverse acetic acid volumes used for base neutralization on FAME yields was tested and shown in Figure 3.4. Little difference was observed by using KOH and NaOH as methylation catalyst from the results, although KOH possessed slightly better catalytic ability on C16:1 (cis-9) PC. Most of the FAME concentration changes derived from the two catalysts were less than 10% (average 4.9%). It was then believed that the two agents may have approximately the same capacity to produce alcoholate anion (a) when they have an equal molarity. The subsequent neutralization with dilute acid after the reaction was to minimize the risk of hydrolysis occurring when aqueous solutions were involved in solvent extraction (Christie 1982). Inadequate or excessive adding of the acid will lead to potential base-catalyzed hydrolysis or acid catalyzed hydrolysis of esters and in either case the output amounts of FAMEs will be reduced. The performance from serial volumes of acetic acid on FAME yields was almost the same, except that the zero use of acid produced the lowest concentrations of FAMEs. From all the KOH catalyzed outputs, 0.5 mL acetic acid produced the highest concentrations of FAMEs in all the experimental settings. This indicated that base-catalyzed hydrolysis or acid-catalyzed hydrolysis of esters was controlled within a reasonable scale. From current results, equivalent amounts of acids were recommended in lipids methylation for quantitative FAME studies to avoid or mitigate the undesirable hydrolysis effect of esters.


(a)
NaOH
KOH
PLFA C16:1(CIS-9) recovered (mg/L)
0
2
4
6
8
0
0.5
1
1.5
Volume of acetic acids (mL)


(b)
NaOH
KOH
PLFA C18:1(CIS-9) recovered (mg/L)
0
1
2
3
4
5
0
0.5
1
1.5
Volume of acetic acids (mL)

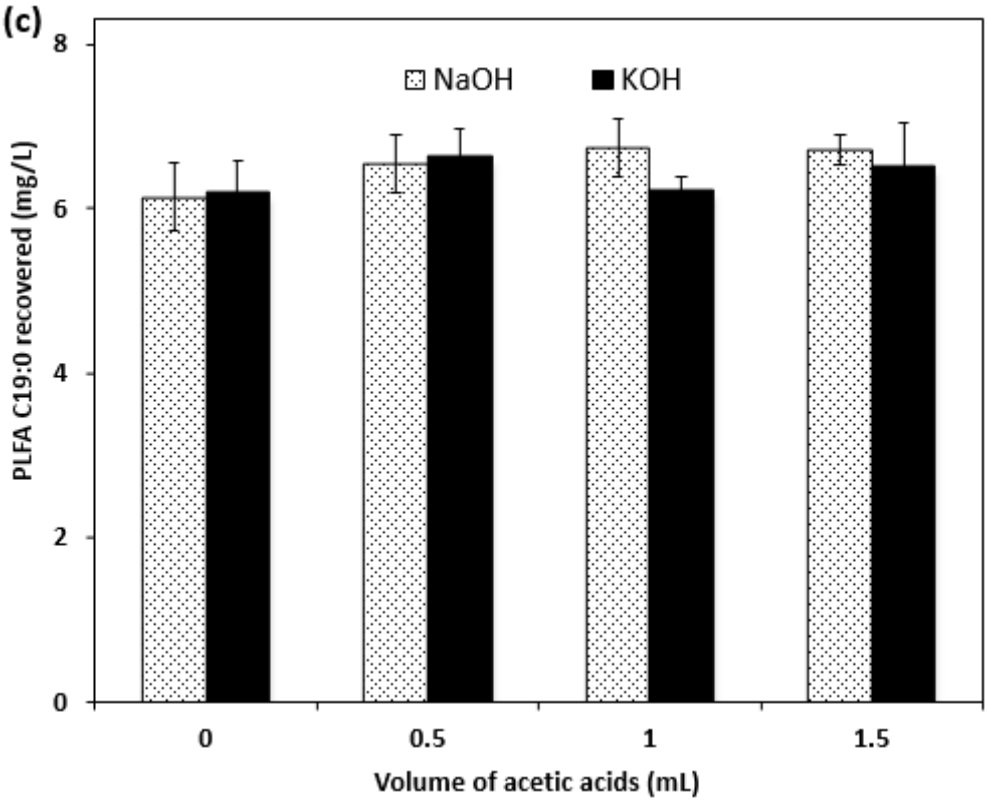
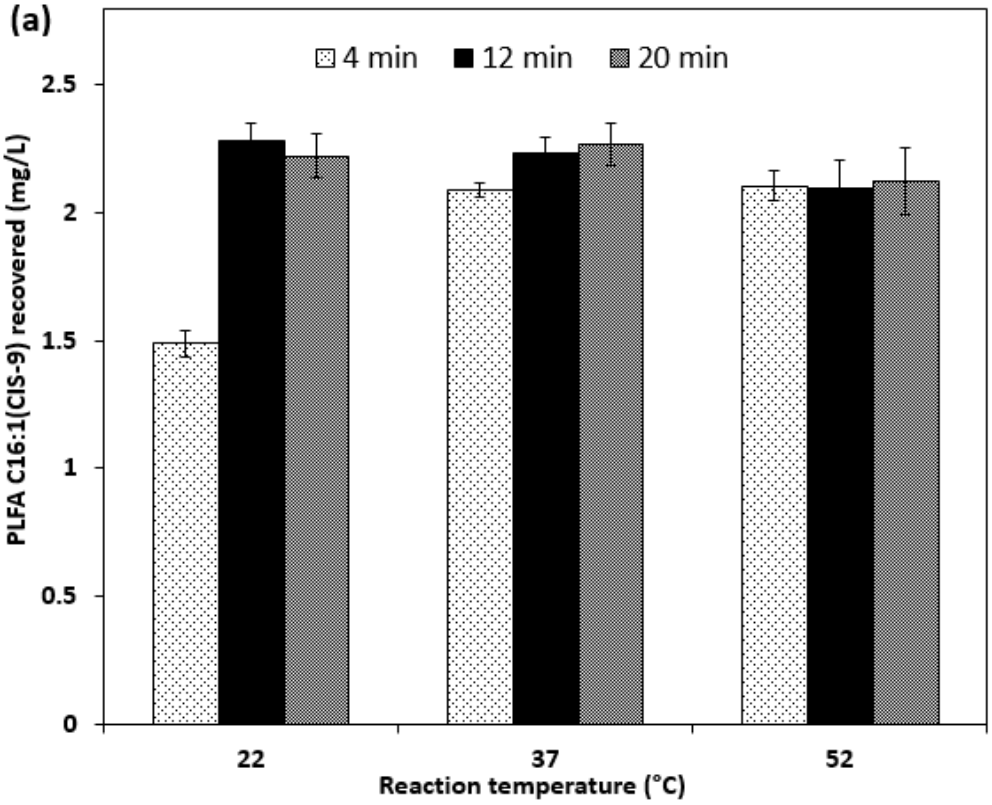
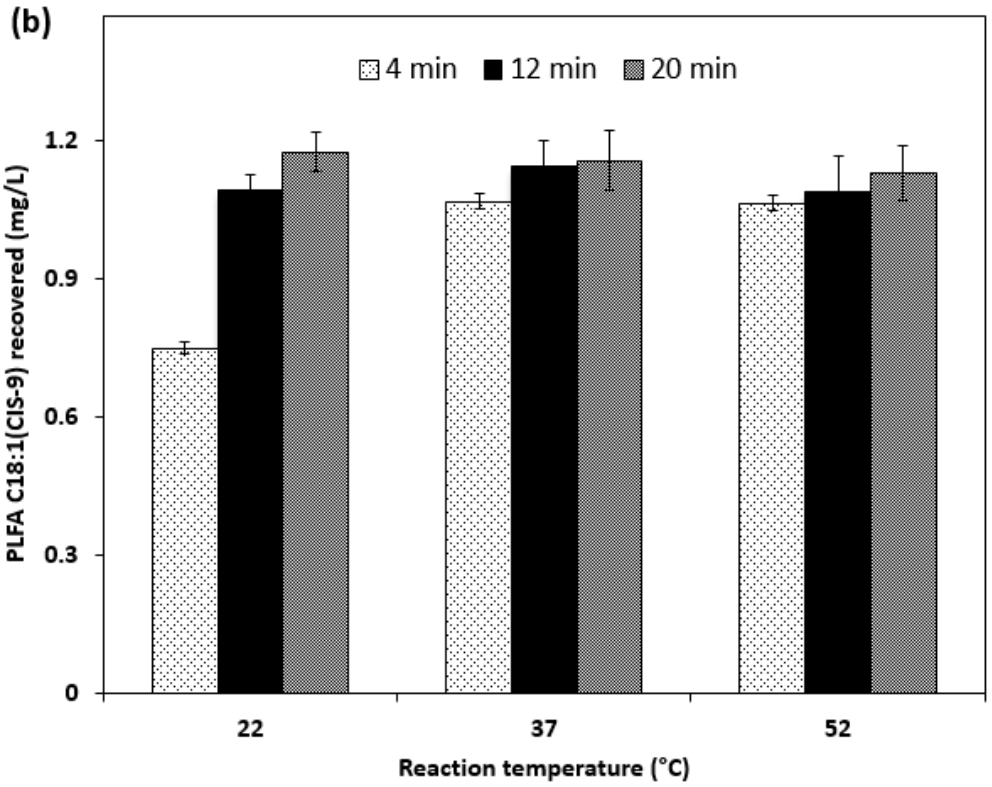


Figure 3.4 Influence of methylation agent and volumes of acetic acids used for neutralization on FAME derivatization

Methylation time and temperature are two main factors in the derivatization of FAMEs, especially the base-catalyzed transesterification is a relatively fast reaction when compared with acid catalyzed methylation. Three levels of the two parameters were investigated in this study and the results are shown in Figure 3.5. The transesterification process of lipids is very rapid. It has been reported that triglycerides can be completely transesterified in 2 to 5 minutes at room temperature while using sodium methoxide in methanol as a catalyst (Demirbas 2008). When the temperature was raised to 50 °C, triacylglycerides are completely transesterified in 10 minutes and phosphoglycerides in 5 minutes under the presence of 0.5 to 2 N sodium methoxide. Typically, the solution was maintained at 50 °C for 10 min (Christie and Han 2012). Thus, the temperature values a lot in the transesterification and yields at 37°C from our results revealed that superior performance was obtained at this point in most cases. Actually, this temperature was typically adopted in various sample treatment and method comparative studies (Chowdhury and Dick 2012; Gómez-Brandón et al. 2008;2010). FAME yields in 4 min were the lowest under the conditions of all the temperature ranges. However, this time-induced difference was greatly reduced when the temperature increased. In a contrast, the results from the other two time intervals indicated that concentrations of FAME stayed almost at the same level (Differences less than 7%). It can be estimated that transesterification of all phospholipids was completed within a certain time the amount of FAME products remain unchanged after that.


(a)
4 min
12 min
20 min
PLFA C16:1(CIS-9) recovered (mg/L)
2.5
2
1.5
1
0.5
0
22
37
52
Reaction temperature (°C)


(b)
4 min
12 min
20 min
PLFA C18:1(CIS-9) recovered (mg/L)
1.2
0.9
0.6
0.3
0
22
37
52
Reaction temperature (°C)

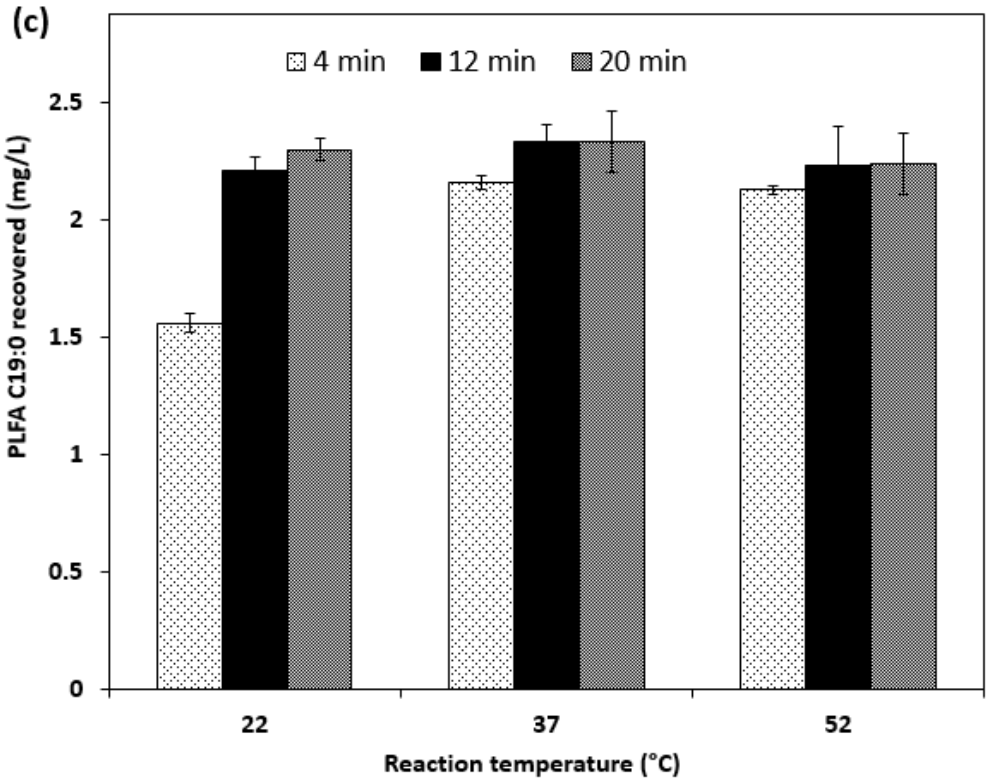


Figure 3.5 Influence of reaction temperature and reaction time on FAME derivatization

3.3.4 Analytical performance

3.3.4.1 Method linearity

The linearity of an analytical procedure is its ability (within a given range) to induce responses which are directly proportional to the concentration (amount) of analyte in the sample. The linearity of the method was verified through the measurement of serial dilutions of 29 FAME standard mixtures.

Eleven standards spiked with multiple concentrations ranging from 0.01 and 10 mg/L were used to create calibration curves and more details are shown in Table 3.1. The 11 standard concentrations were 0.005 mg/L, 0.01 mg/L, 0.02 mg/L, 0.05 mg/L, 0.1 mg/L, 0.2 mg/L, 0.5 mg/L, 1 mg/L, 2 mg/L, 5 mg/L, and 10 mg/L. Calibration curves were built based on the analytical responses of FAMEs among these concentrations. The coefficients of determination (r^2) for the obtained calibration curves were all higher than 0.995. The wide dynamic range and the high values of r-squared are robust verification of GC-MS based quantification for FAMEs derived from phospholipids. While two internal standards (C14:1 (cis-9) and C21:0) were involved in determining the values of RFs for each methyl ester, an accurate FAME determination in a wide range was obtained from RFs in the stated wide range.

3.3.4.2 Limit of detection

Detection limit is defined as the statistically calculated minimum concentration that can be measured with 99% confidence that the reported value is greater than zero (Bernal 2014). LODs were calculated by using following equation:

$$DL = s \times t_{(n-1,1-\alpha=0.99)} \quad (3)$$

Where:

s = standard deviation of replicate analyses.

$t_{(n-1,1-\alpha=0.99)}$ = the obtained t value for the 99% confidence level with n-1 degrees of freedom.

n = number of replicates.

Seven replicated samples were analyzed for LODs determination in instrumental analysis. When n = 7, $t_{(n-1,\ 1-\alpha=0.99)}$ = 3.143. The LODs of the instrument for 29 FAMEs were shown in Table 3.1 and they were as low as 0.3-9.7 ng/L.

3.3.4.3 Repeatability

Repeatability is a measurement of the precision of a method. It is often expressed as the variation of measurements achieved from independent measurement results under consistent test conditions (identical samples in the same laboratory by the same person and instrument, using the same method in a short period of time). The RSD was used to evaluate repeatability in the instrumental analysis.

A mixture of 29 FAMEs at 0.02 or 0.05 mg/L in DCM was injected 7 times and the results were used to evaluate the repeatability of GC analysis. From Table 3.1, the RSD values of 29 FAMEs were all below 7.5% (between 0.9 % and 7.4 %.) for PLFA quantification, indicating a reasonable stability in the quantitative GC-MS methodology. To investigate the whole method reproducibility, phospholipids standard C19:0 PC was spiked at the step of sample extraction (5 replicates) to allow the final yield of C19:0 methyl ester at a concentration of 1.23 mg/L, the RSD achieved was 9.0% (Figure 3.6).

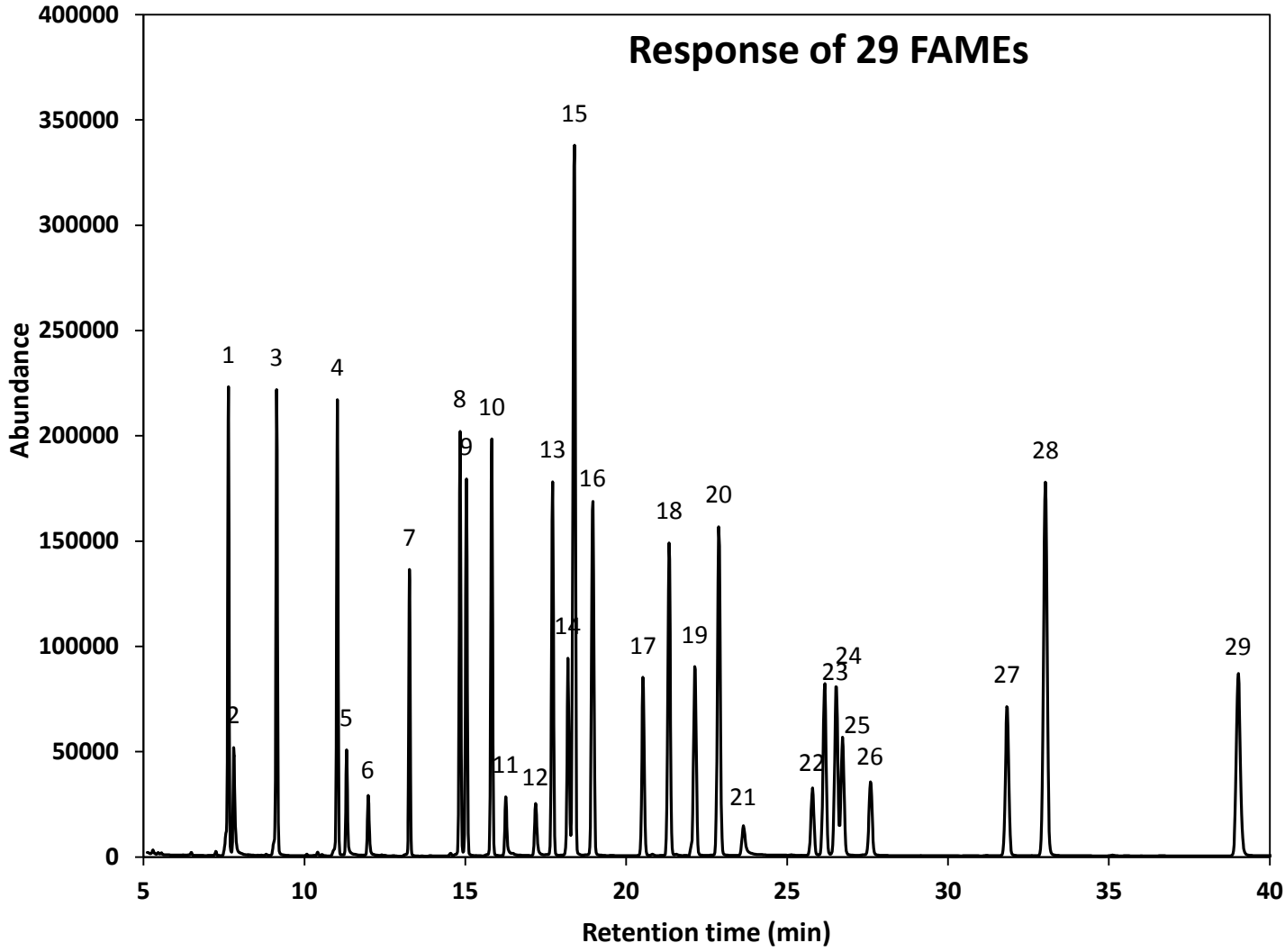


Figure 3.6 Chromatogram of standard mixture containing 29 FAMEs by GC-MS analysis on DB-5MS capillary column (number is accorded with Table 3.1)

3.3.4.4 Method recovery performance

Recovery is the detection of a known amount of an analytical parameter added to the sample matrix and included throughout the method of analysis. Since PLFA quantitative study involved the derivatization of PLFAs to FAMEs for GC analysis, the method recovery was investigated in two portions. Firstly, triplicate samples were spiked with phospholipid C19:0 PC during the treatment of sample extraction and FAME derivatization, respectively, and the concentrations of fatty acid C19:0 obtained (A and B) were used to investigate the method recovery performance. Results showed that 90.6% (A/B) of C19:0 PC was recovered before the treatment of FAME derivatization in the methodology. Solvents (toluene: methanol-1:1) were spiked with 29 FAMEs in the treatment of transesterification to test the recovery during the phase separation and concentration steps. Results indicated that all 29 FAMEs exhibited recoveries greater than 75.3%, while 24 of them showed recoveries ranging from 80.4% to 103.1% (all RSDs less than 8.8%).

3.3.5 Analysis of offshore produced water samples

Offshore produced water is characterized by its complex composition (e.g., treating chemicals, formation solids, heavy metals, salts, dissolved and dispersed oils) with high salinity and petroleum hydrocarbon content (Sirivedhin et al. 2004), and the general oil/water volume ratio is 1:3 (Fakhru'l-Razi et al. 2009). Even though, over 90% recovery of phospholipid was achieved in this study from triplicate produced water samples when C19:0 PC was spiked into the extraction solvent at the beginning of sample processing. Before spiking C19:0 PC into the samples, preliminary analysis of the reservoir samples indicated that no identifiable peaks of C19:0 were observed. After the water sampling and analysis, 14 types of PLFAs were detected from waters in a producing well of an offshore oil and gas platform and the results were illustrated in Figure 3.7.

The four most abundant fatty acids from the complex matrix were C14:0, C16:1(cis-9), C16:0 and C18:0. Other typical fatty acids C12:0, C15:0 and C18:1(cis-9) were also found in considerable amounts. The even-numbered fatty acids dominated in the PLFA profiles of offshore produced water (88.4% of total amount) and this reflected the natural pathway for their biosynthesis from the two-carbon building-block acetyl CoA (Estelmann et al. 2011). The only hydroxy fatty acid (HFA) detected was 2-OH C12:0 and the polyunsaturated fatty acid (PUFA) was solely represented by C18:2 (all cis-9, 12), which could be documented in marine hydrocarbon degrading bacteria (Zhang et al. 2012). Fatty acids i-C15:0, a-C15:0 and a-C16:0 were main branched saturated fatty acids (BCFA), but they are minor components as reflected from the whole PLFA distributions. Straight-chain saturated fatty acids (SSFAs) were major compounds in PLFA profile and were reported to be associated with swarming motility in *Proteus mirabilis* and *Serratia marcescens* (Lai et al. 2005; Liaw et al. 2004). This interesting perspective was very likely reasonable in explaining the resulting PLFA distributions in our samples, since the mobility of bacterial cells was of crucial importance for their survival in commonly porous reservoir environments.

From this point, monounsaturated fatty acids (MUFAs) and BCFAs were also associated with their indicative uses. According to previous studies, they are signature biomarkers of different microbial species to varying extents. For instance, the branched-chain fatty acids (iso, anteiso in C14-C18) are widely accepted as general indicators for gram-positive bacteria (Ruess and Chamberlain 2010). However, significant amounts of i-C15:0 were previously found in *Desulfovibrio desulfurricans* with various growth substrates, and the percentage fatty acid composition of i-C15:0 in this strain increased from 9.9% to 12.6% when substrate changed from CO_2 to lactate (Taylor and Parkes 1983). The presence of C18:1(cis-9) is common to many psychrophilic

microorganisms such as saprophytic fungi (De Deyn et al. 2011), *Pseudomonas sp.* and some *Colwellia/Vibrio sp.,* but the occurrence of C18:1(cis-9) as a major fatty acid in sulfur oxidizing bacteria (SOB) has never been reported (Guezennec et al. 1998). Specifically, a higher percentage of C18:1(cis-9) was found in SRB species *Desulfobacter curvatus* and *Desulfobacter latus* in marine mud (Kohring et al. 1994). The fatty acid C18:1(cis-9), potential marker to trace SRB activities under certain circumstances, was also chosen to represent *Desulfotomaculum* in SRB-related biogeochemical processes under the environments of subsurface sediment (Mohanty et al. 2008). The coexistence of C16:1 (cis-9) and C18:1 (cis-11) could indicate the presence of SOB. The dominance of C16:1 (cis-9) (16.7 to 37.4%) and C18:1 (cis-11) (11.8 to 16.8%) has also been observed in thiotrophic bacterial mats in the Barbados Trench (Guezennec and Fialamedioni 1996). Zhang et al. (2005) studied the metabolic functions of microbial mat in sulfide-rich marine sediments associated with gas hydrates in the Gulf of Mexico, and concluded that C16:1 (cis-9) and C18:1 (cis-11) can be used as signature biomarkers for SOB in H_2S-rich marine sediments. Our results of PLFA species in reservoir samples indicated relatively higher level of sulfate reducing activities but lower sulfur oxidizing rates. Therefore, PLFA profiles, as a significant indicator of microbial activities, could provide very reasonable interpretation and guidance towards biological reservoir souring and associated mitigation activities under continuous monitoring conditions.

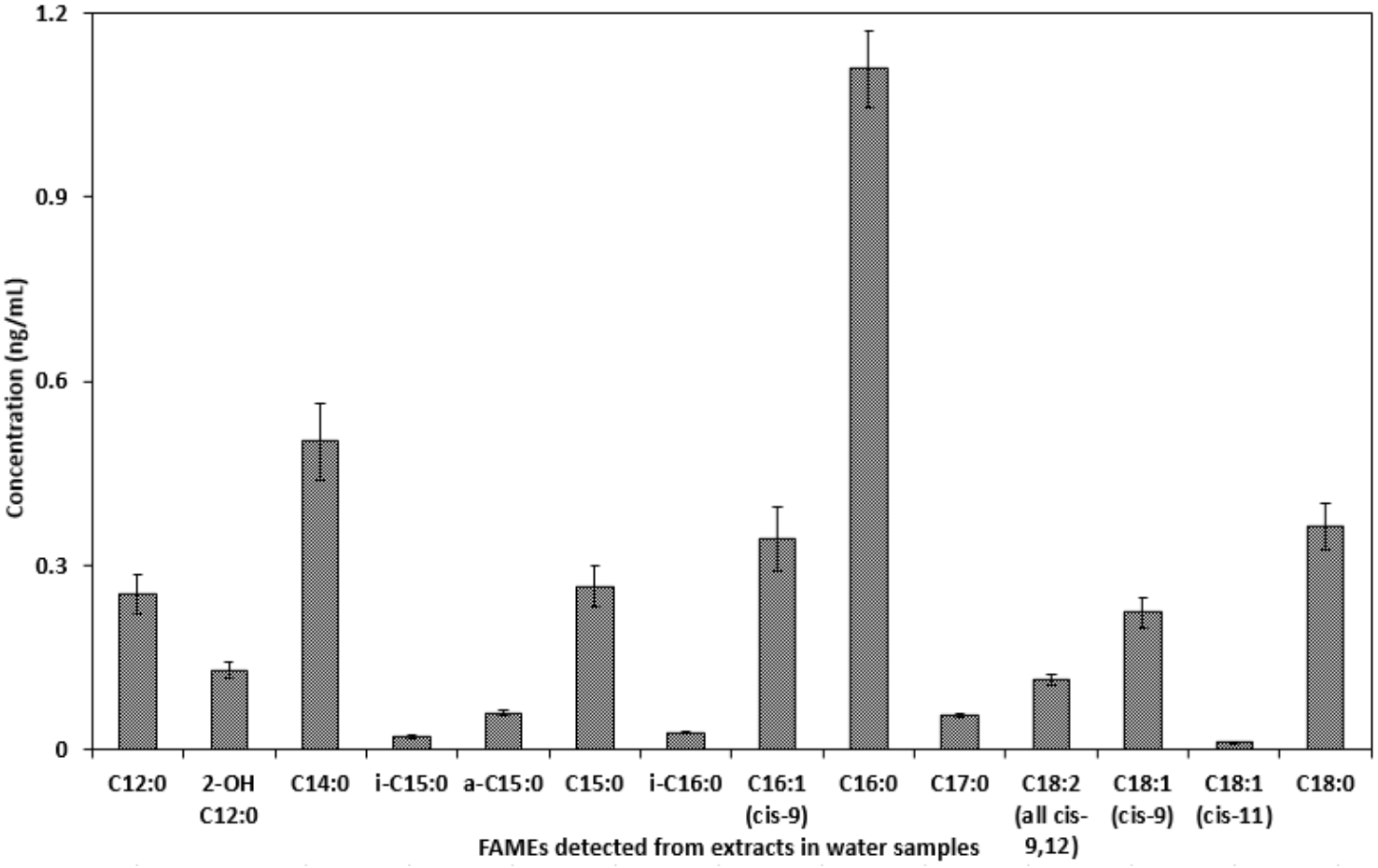
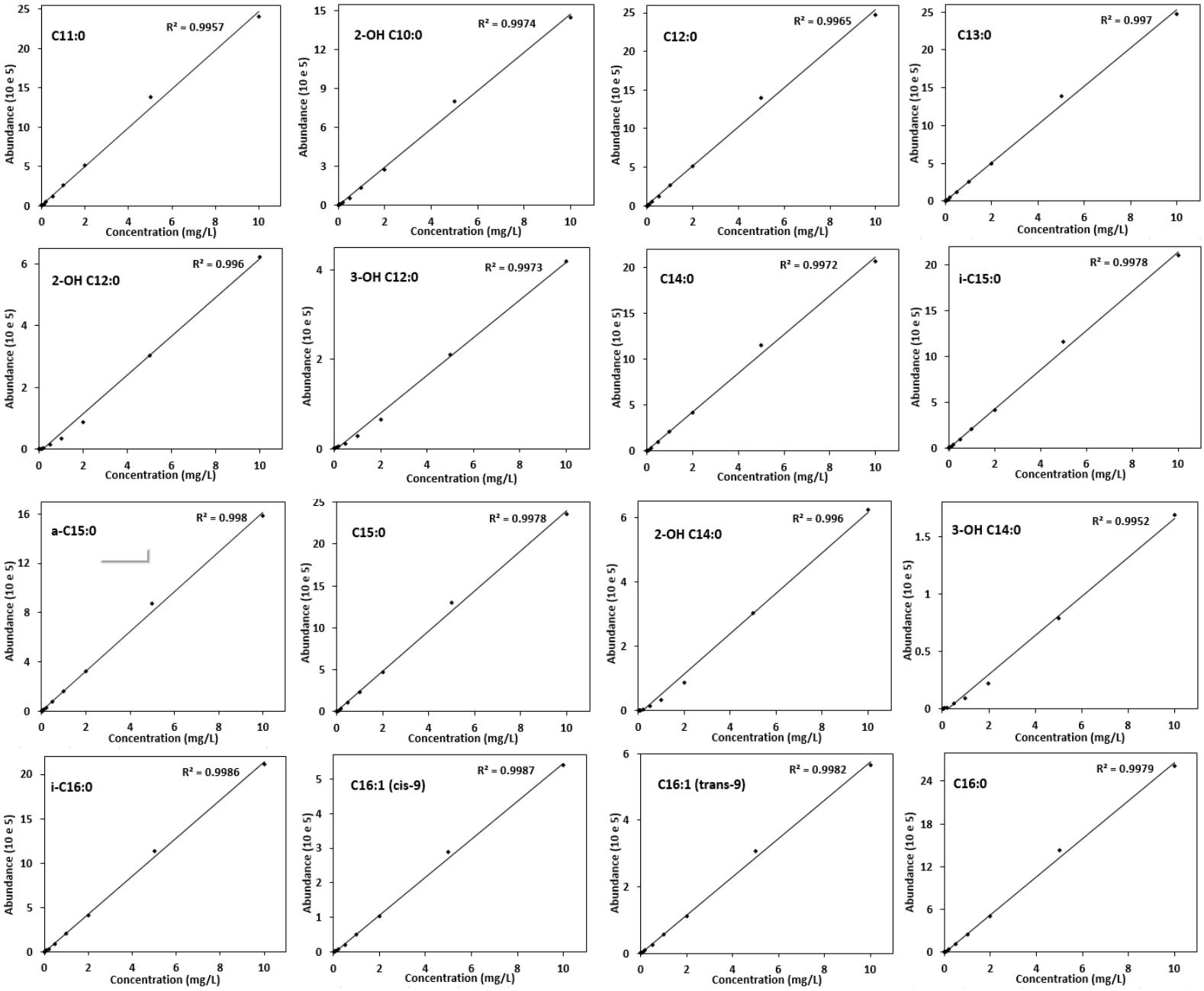


Figure 3.7 FAMEs detected from phospholipids in offshore produced water based on the optimal PLFA method

3.4 Summary

A method for the determination of PLFAs in profiling microbial communities in offshore produced water has been developed. A three-stage extraction process was confirmed and the extraction efficiency in phase partition was evaluated. The elution parameters in SPE purification were adapted for treating the oily samples and their volumes were determined to induce a high recovery for the fraction of phospholipids. The impact of parameters including alkaline reagent, the volumes of acid used for neutralization, the time and temperature for transesterification were studied. The GC-MS quantitative analysis was validated by examining the method linearity, LODs and repeatability. Method recovery performance from phospholipids preparation and FAME derivatization were studied. Results indicated that the developed method exhibited high recoveries and repeatability, remarkable selectivity and linearity, and acceptable quantification limits for PLFA analysis. With reliable accuracy and precision, the method was applied to profile microbes in offshore produced water samples. The developed method would be applicable for routine analysis of samples with high oily and salinity properties in the marine environment.

3.5 Appendix


C11:0
R² = 0.9957
2-OH C10:0
R² = 0.9974
C12:0
R² = 0.9965
C13:0
R² = 0.997
2-OH C12:0
R² = 0.996
3-OH C12:0
R² = 0.9973
C14:0
R² = 0.9972
i-C15:0
R² = 0.9978
a-C15:0
R² = 0.998
C15:0
R² = 0.9978
2-OH C14:0
R² = 0.996
3-OH C14:0
R² = 0.9952
i-C16:0
R² = 0.9986
C16:1 (cis-9)
R² = 0.9987
C16:1 (trans-9)
R² = 0.9982
C16:0
R² = 0.9979
Abundance (10 e 5)
Concentration (mg/L)

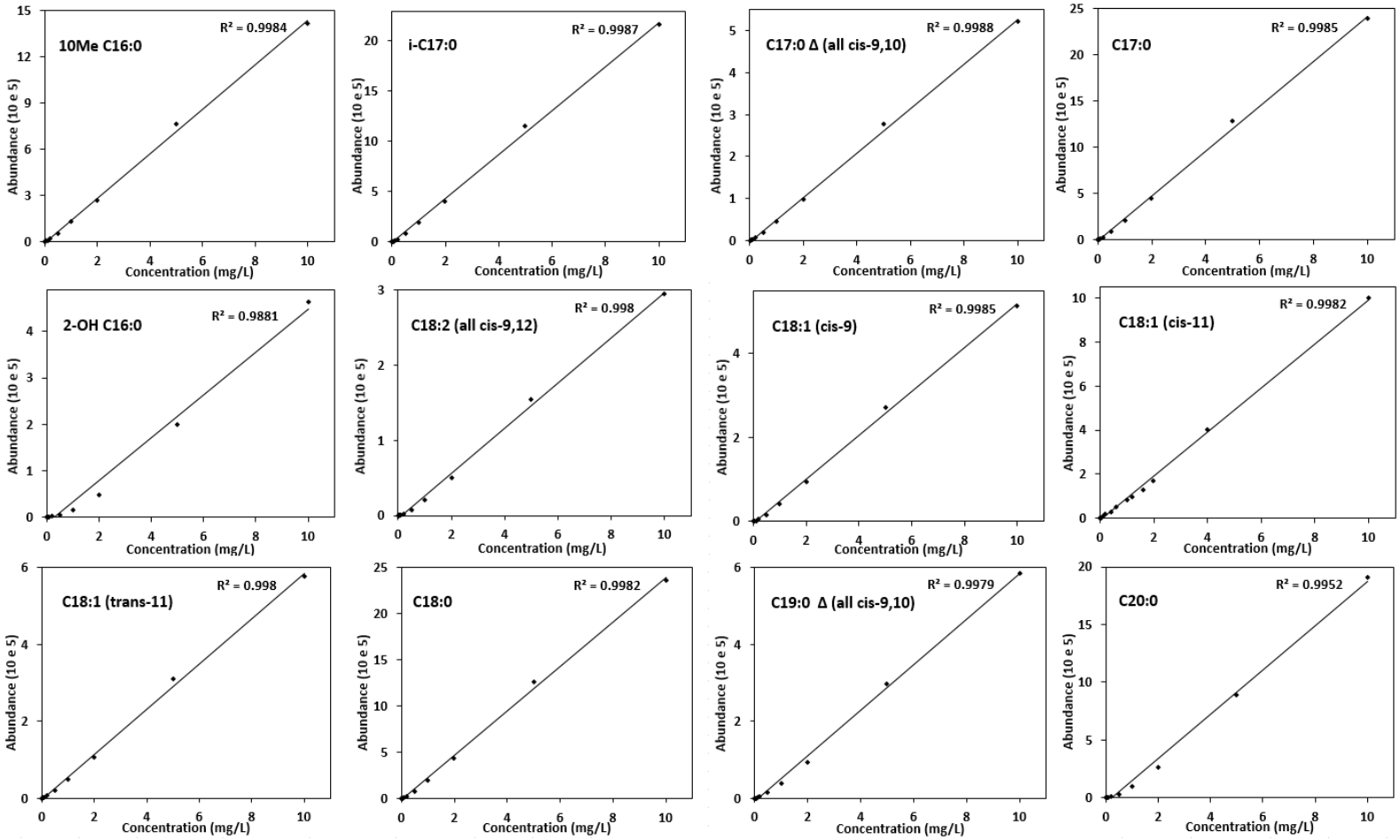


Figure 3.8 Calibration curves of 29 FAMEs from 12 levels of sample concentration ranging from 0.01 and 20 mg/L

CHAPTER 4

PROFILING OF SRB IN AN OFFSHORE OIL RESERVOIR USING PLFA BIOMARKERS[2]

[2] *This chapter is based on the following paper:*

F. Fan, B. Zhang, P.L. Morrill, T. Husain, Profiling of Sulfate-Reducing Bacteria in an Offshore Oil Reservoir Using Phospholipid Fatty Acid (PLFA) Biomarkers, Water, Air, & Soil Pollution, 228 (2017) 410

Role: Fuqiang Fan solely worked on this study and acted as the first author of this manuscript. Dr. Baiyu Zhang is my supervisor. Dr. Penny Morrill and Dr. Tahir Husain helped guide the research and polish the manuscript.

4.1 Background

The Oil and Gas industry has been well aware of the harmful activities of SRB with regards to reservoir souring and MIC for many years (Enning and Garrelfs 2014). As anaerobic microorganisms, SRB are a major concern in the petroleum industry primarily because of their ability to generate substantial amounts of hydrogen sulfide and insoluble ferrous sulfide in the presence of iron.

SRB produce hydrogen sulfide through respiration and they gain energy for growth, in which the sulfate functions as an electron acceptor and organic matter or molecular hydrogen are employed as electron donors. In secondary oil recovery activities, seawater is usually injected into reservoirs to maintain pressure level underneath and recover the remaining oil from the reservoir (Gieg et al. 2011). SRB are prevalent in many natural as well as engineered aquatic environments, and they flourish in the presence of sulfate from seawater and oil components pre-existed in the reservoir (Muyzer and Stams 2008). The subsequent undesirable production of hydrogen sulfide is closely associated with negative effects in the form of reduced quality of hydrocarbon products, increased iron sulfide scale precipitation and plugging, reduced injection efficiency in reservoir wells, and increased pipeline corrosion (Hubert and Voordouw 2007). This production of hydrogen sulfide also induces safety risks for operators and environmental concerns.

To effectively mitigate this situation, methods must be initially developed to characterize the microbial community composition during the oil production activities, thus enabling the planning for souring control solutions. Copious amounts of culture or molecular methods have been developed for the detection and enumeration of viable SRB for routine monitoring and anomaly investigation (Head et al. 1998; Kaster et al. 2009; Lenchi et al. 2013; Sun et al. 2014; Tanner

1989). Culture-dependent methods are typically laborious, time-consuming and cannot provide unequivocal results. Moreover, more than 99% of naturally occurring microbes are considered 'unculturable' using conventional cultivation techniques (Zhang et al. 2011a).

The two main culture-independent methods that are widely used for microbial profiling of environmental samples include nucleic acid and PLFA analyses. Nucleic acid analysis methods usually employ polymerase chain reaction (PCR) techniques and produce complex banding patterns of DNA fragments on a gel to fingerprint microbial species. PCR-based methods have been recognized as a highly sensitive and specific technique for species identification. However, there are many drawbacks in the methodologies, including inconsistent DNA recovery, inherent kinetic biases, the relatively high cost and the need to develop specific primers and probes (Lueders and Friedrich 2003). PCR-based methods are also not suitable for characterizing samples with complex communities (Pan et al. 2010). In contrast, PLFA analysis offers a great potential of microbial community analysis in routine environmental monitoring.

Phospholipids are essential membrane constituents of bacterial cells and their types and amounts vary from one to another (Guezennec and Fialamedioni 1996; Kaur et al. 2005). Since the content of phospholipids stays relatively constant, microorganisms with the same taxonomic composition can be identified by analyzing the diversity and specificity of their PLFAs (Kaur et al. 2005). Phospholipids are rapidly decomposed after cell death in the environment, thus PLFA analysis is widely accepted to indicate viable microbial biomass and provide a microbial community 'fingerprint' (Zelles 1999). Due to their cellular abundance and chemically diverse nature, PLFAs are commonly used as biomarkers to measure microbial biomass, nutritional/physiological status and microbial community structure in soils, surface waters, groundwaters, sediments and biomats

(Acosta-Martínez et al. 2008; Dijkman et al. 2010; Drenovsky et al. 2010; Franzmann et al. 1996; Moore-Kucera and Dick 2008; White and Ringelberg 1997; Yu et al. 2009).

Boschker et al. (2001) investigated the bacterial populations and pathways involved in acetate and propionate consumption in anoxic brackish sediment based on PLFA analysis. Labeled acetate and propionate (^{13}C) were incorporated into PLFAs after incubation and the results showed that they were predominantly consumed by different, specialized groups of SRB. Guezennec and Fialamedioni (1996) analyzed sediments from various locations in Barbados trench through PLFA analysis and concluded anaerobic bacteria and presumably SRB were also present in these sediments. Uranium-bearing sandstones from the Dongsheng deposit were also found with the abundant presence of C15-C18 fatty acids (Jiang et al. 2012). Characteristic biomarkers of SRB *Desulfovibrio* and *Desulfobacter sp*. were found and involved in bacterial sulfate reduction to sulfide.

Even though PLFA profiling has been used as SRB biomarkers in various solid and fluid samples, it is rarely applied in offshore reservoir water analysis to specifically elucidate the mechanism of reservoir souring induced by SRB. The SRB transformation patterns between the injection wells and producing wells under different redox conditions remained unclear. Furthermore, there is still a lack of basic understanding of the complex biomass and microbial community structure information from the various reservoir conditions regarding souring. In this study, we analyzed the injection water and four produced water samples with different chemical and physical properties using PLFA analysis in order to study the microbial community composition in various reducing conditions and identify possible SRB species responsible for hydrogen sulfide production.

4.2 Materials and Methods

4.2.1 Standards, reagents, apparatus and sample collection

Analytical standards of FAMEs: Bacterial Acid Methyl Esters CP Mixture (26 methyl esters), 10Me C16:0 (Methyl 10-methylhexadecanoate), Trans-9 C16:1 (Methyl hexadecenoate, trans-9), and C19:0 (Methyl nonadecanoate) were purchased from Matreya LLC (Pleasant Gap, Pennsylvania, USA). Trans-11 C18:1 (trans-11-Octadecenoic methyl ester) was purchased from Sigma-Aldrich (Oakville, Ontario, Canada). Phospholipid standards C16:1 (cis-9) PC (1,2-dipalmitoleoyl-sn-glycero-3-phosphocholine), C18:1 (cis-9) PC (1,2-dioleoyl-sn-glycero-3-phosphocholine), and C19:0 PC (1, 2-dinonadecanoyl-sn-glycero-3-phosphocholine) were purchased from Avanti Polar Lipids, Inc. (Alabaster, Alabama, USA). The ultra-high purity water, silica gel (60-120 mesh), solvents including chloroform, DCM, methanol, hexane, toluene and acetone of reagent grade or higher quality were purchased from VWR® International (Mississauga, Ontario, Canada) and Fisher Scientific (Ottawa, Ontario, Canada). The commercial 3 mL SPE tubes (miniature champagne column) were bought from Supelco Inc. (Bellefonte, Pennsylvania, USA). GC supplies, including deactivated single tapered glass inlet liners and J&W Scientific DB-5MS UI fused silica capillary columns, were obtained from Agilent® Technologies Inc. (Mississauga, Ontario, Canada). Extraction tubes and all other glassware were primarily washed with detergent and then deeply rinsed with chromic acid cleaning solution to remove all types of oil, fingerprints, rust and other dirt.

Triplicated offshore produced water and injection seawater samples were collected from four producing wells and the injection well in an offshore oil and gas platform. Gaseous H_2S concentrations were detected through enhanced laser diode spectroscopy (ELDS) technology

(Moser et al. 2017). Before sampling in each location, in-situ measurements (pH, redox potential, temperature, and dissolved oxygen) were conducted using field probes (pH, redox potential and dissolved oxygen probes respectively) and the results were recorded. After the measurements of the listed parameters, 500 mL water sample was filtered through 0.2 μm nylon membrane filter (47 mm in diameter) by a vacuum pump. The filter paper was then transferred into a 10 mL amber vial with 9.5 mL extraction solvents (methanol: DCM: 125 mM phosphate buffer at pH 7.4 = 2:1:0.8) inside. Total lipids were extracted from microbial biomass collected on the filters and the vials were sealed with screw caps containing Teflon lined septa. All samples were stored in a freezer (-20°C in the dark) before shipping and then transported to the laboratory in a package with frozen ice packs for determination.

4.2.2 Extraction of lipids and preparation of FAMEs

Total lipids were extracted using a modified Bligh and Dyer extraction method (Fang and Findlay 1996). Approximately 0.1–0.7 g of freeze-dried sediment/mat sample was subjected to vortex-assisted extraction in an amber vial with 9.5 mL extraction solvents (methanol: DCM: 125 mM phosphate buffer at pH 7.4 = 2:1:0.8). The extraction mixture was allowed to stand overnight in the dark at -20 ^{o}C. Additional 4.5 mL and 4 mL mixture of methanol, DCM and phosphate buffer (2: 1: 0.8) were used for secondary and tertiary total lipids extraction from the membrane filter. The lipids were then partitioned by adding DCM and water, such that the final ratio of DCM to methanol to water was 1: 1: 0.9. The lower organic phase was transferred and collected altogether from the extracts. Phospholipid standard C19:0 PC (1,2-dinonadecanoyl-sn-glycero-3-phosphocholine) was spiked to examine the % recovery and four blanks spiked with C19:0 PC were performed for process control.

The total lipid extract was dried under a gentle stream of nitrogen and was redissolved in hexane: DCM (70:30, v/v). Neutral lipids, glycolipids and phospholipids were eluted with 4 mL of chloroform, 4 mL of acetone and 10 mL of methanol on a home-made SPE tube (miniature champagne column) packed with 0.1 g silica gel, respectively. The fraction of phospholipids was evaporated to dryness, redissolved in 1 mL of methanol: toluene (1:1, v/v) and subjected to a mild alkaline trans-methylation procedure to produce FAMEs prior to further quantitative analysis. Potassium hydroxide with a concentration of 0.2 mol/L was used as methylation agent and the FAMEs were finally concentrated to 400 μL in chloroform.

4.2.3 GC-MS analyses

FAMEs were analyzed by GC-MS system (Agilent 7890A GC system coupled with a 5975C MSD) with an Agilent 7693 auto-sampler. Chromatographic separation was achieved by using a 30 m × 250 μm (internal diameter, i.d.) × 0.25 μm DB-5MS UI fused silica capillary column. Sample injections (3μL) were conducted using a split/splitless injector (single tapered inlet liner) in pulsed splitless mode at 200 °C under a pulse pressure of 25 psi. The oven temperature program started at 70 ^{o}C, then it was raised to 150 ^{o}C at 20 °C/min, to 180 ^{o}C at 3°C/min, to 210 ^{o}C at 1°C/min, and finally to 280 ^{o}C at 35°C/min with a running time of 47 min. Helium of ultrahigh purity was used as the carrier gas at a flow rate of 1.0 mL/min with an electronic pressure control (7.65 psi). The ion source was operated in the electron ionization (EI) mode at 70 eV and mass spectral data were acquired through the scan range of 50–550 m/z. Full scanning and the SIM mode were used simultaneously for FAME determination.

Individual compounds were identified from their mass spectra and were further confirmed by comparing their relative retention times with those of well-known FAME standards.

Quantifications of FAMEs were based on response factors (RF) derived from a mixture of 29 external standards (FAMEs from C_{10} to C_{20}) with C19:0 (methyl nonadecanoate) as an internal standard. The area responses of the characteristic m/z against concentrations (twelve different levels) for each compound were analyzed to derive RF for each compound. Replicate analyses (7×) of samples were performed to ensure reproducibility (variation of ≤6.0%) in GC-MS quantification. Fatty acids are designated by the total number of carbon atoms to the number of double bonds (e.g. a 16-carbon alkanoic acid is C16:0). The position of the double bond is indicated by a Δ number closest to the carboxyl end of the fatty acid molecule with the suffixes of either cis (c) or trans (t) as the geometric isomers. Prefixes i and a are given for iso- and anteiso-branched FAMEs, respectively. The suffix 10 ME indicates a methyl group at the 10th C atom, while OH stands for hydroxy and the cyclopropyl ring is indicated as 'cy'.

4.2.4 Statistical analysis

Triplicate samples were prepared and analyzed to ensure the reproducibility of results, and the error bars in the plotted data were derived from the standard deviations of the mean values of triplicate samples. To assess the patterns of intercorrelations among PLFA species measured, principal component analysis (PCA) was conducted using the software SPSS 18.0. The data were orthogonally transformed into a new coordinate system and orthogonal variables called principal components with the greatest variances were used as coordinates. The first principal component (PC1, 59.1%) accounts for most of the original variability and the second principal component (PC2, 27.5%) contains the second largest variance.

4.3 Results and Discussion

4.3.1 In-situ measurements

Cool seawater from the ocean was injected into the reservoir in order to maintain well pressure and sweep the oil towards production wells. Since the reservoir was characterized by high temperature and high pressure, seawater temperature was elevated when it was mixed with the formation fluids. The hot mixed fluids cooled down during the pipeline of producing wells and above-ground facilities. The temperature of produced water observed from the oil-water separator was approximately 63°C initially, however, the temperature decreased with time. In general, the intermediate zone where cool injection water was warmed to the reservoir temperature would provide favorable thermal conditions for SRB activity (Gieg et al. 2011). As seen from Table 4.1, the pH values were approximately 6.71 and showed little difference among the water samples of the injection well and producing wells. The redox potentials, however, varied from one sampling point to another were all below 100 mV and the results were accorded with low DO measurement outputs.

Table 4.1 Results for samples from the injection well and producing wells measured from the surface facilities (C2, C4, F2 and G2 were the well numbers where produced water samples were collected)

Sampling locations	Injection well	Producing wells			
		C2	C4	F2	G2
pH	6.71	6.52	6.75	6.47	6.87
DO (ppm)	<d.l.	< 1	1	< 1	< 1
Redox potential (mV)	93	-201	30	99	-145

<d.l. signifies less than the detection limit.

Water based oxygen scavengers are widely used in seawater injection systems as a tool to prevent oxygen-induced corrosion, such that DO could not be detected in injection seawater. As a result, the reducing conditions make the matrix susceptible to the growth of SRB in the reservoir (Enning and Garrelfs 2014). From redox potential results, produced water samples from wellbore C2 and G2 exhibited a more reducing environment than those from wellbore C4 and F2. The presence of small amount of oxygen (1 ppm) in water samples from wellbore C4 may illustrate the cause of its more oxidizing environment than others since DO values from other locations were all below 1 ppm. The wellbore F2, with the lowest pH value and the highest redox, has the potential to possess differential characteristic bacterial species when compared to other producing wells.

4.3.2 PLFA analytical performance

A mixture of 29 FAMEs at 0.1 or 0.01 mg/L was measured 7 times to evaluate the reproducibility of GC analysis. The results indicated that the RSD values of all the FAMEs were between 1.3 % and 5.6 % while using RF as quantification tool and C19:0 (methyl nonadecanoate) as an internal standard. Detection limit is defined as the statistically calculated minimum concentration that can be measured with 99% confidence that the reported value is greater than zero (Bernal 2014). Accordingly, the GC-MS detection limits of 29 FAMEs ranged from 0.4 to 12 ng/L. The analysis incorporated triplicate blanks by spiking phospholipid standards C16:1 (cis-9) PC and C18:1 (cis-9) PC into extraction solvents and allowed them running through the whole analytical procedure to investigate the method recovery performance. The same amounts of phospholipid standards were simultaneously spiked into FAME derivatization solvents and the results were taking as a reference. More than 90 % of the phospholipids were recovered before the treatment of FAME derivatization in the methodology and the RSDs of the blanks were all below 8.0%. The analysis incorporated triplicate controls by spiking 29 FAMEs into 1 mL of methanol: toluene (1:1, v/v)

before the treatments of transesterification and the recoveries of the methyl esters were studied as well under current experimental conditions. Results indicated that all 29 FAMEs exhibited recoveries above 72.9%, while 24 of them showed rates ranging from 80.5% to 106.8%. All of the RSDs of the tests were controlled below 8.0%. The chromatograph of the methyl esters was shown in Figure 4.1. The high sensitivity detector responded well to all the 29 compounds and their peaks were clearly separated using column chromatography with retention times from 5 min to 40 min.

4.3.3 Fatty acids profiles

Phospholipids are rapidly decomposed after cell death in the environment, thus the presence of total PLFAs are indicative of viable biomass in offshore injection seawater and produced water samples (Virtue et al. 1996; Zink et al. 2003). Fourteen kinds of PLFAs were detected in various amounts from suspended solids collected on filter membranes. The fatty acid concentrations are listed in Table 4.2. All the water samples were dominated with C12-C18 fatty acids, which include monosaturated, branched saturated, hydroxy, monounsaturated (MUFAs) and polyunsaturated (PUFAs) structures.

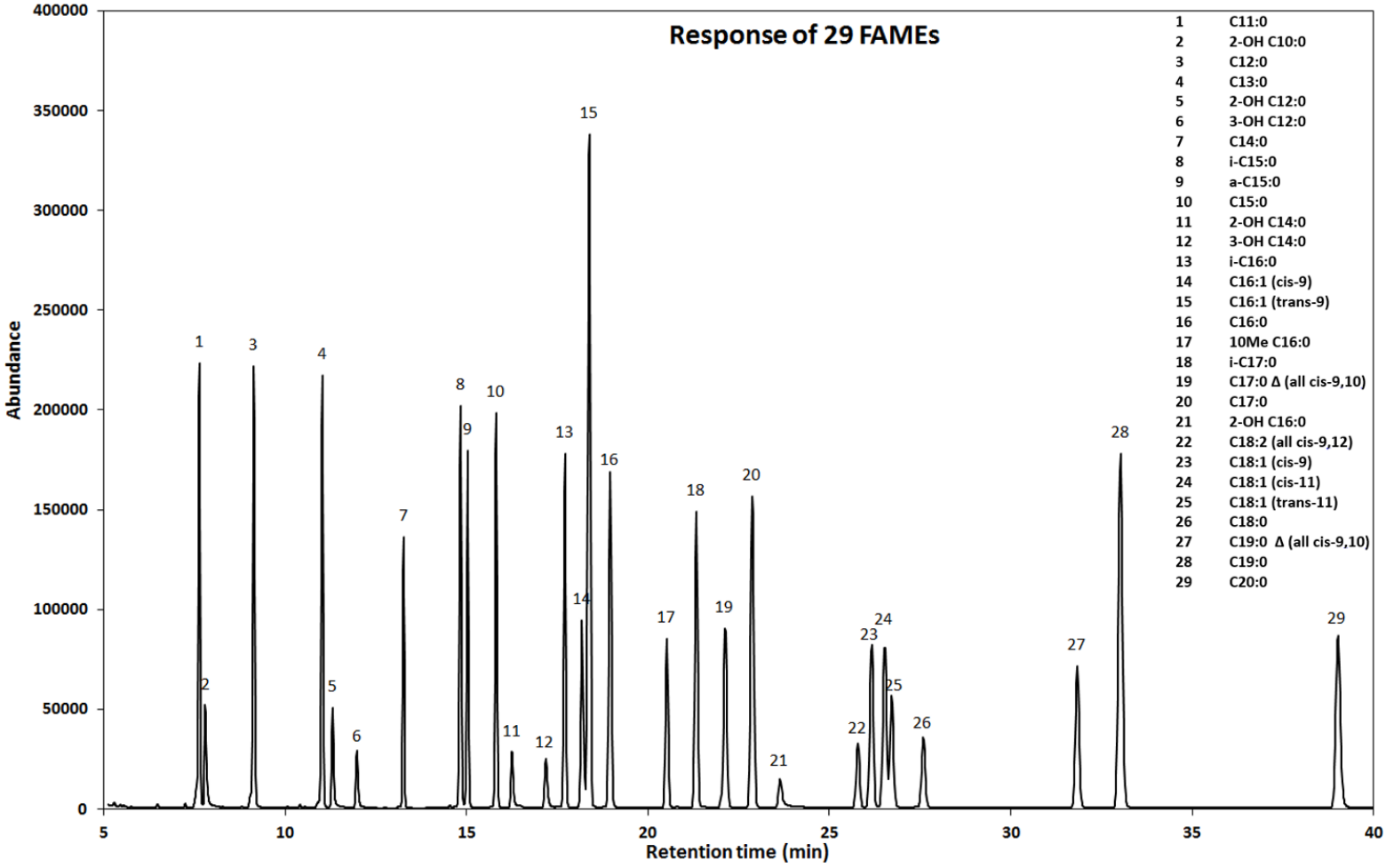


Figure 4.1 Capillary chromatograph of PLFAs (as methyl esters) from 29 external standards

Table 4.2 The absolute content and relative mole percentage of the fatty acids in water samples from the injection well and producing wells

Sampling locations	Injection well		Producing wells							
			C2		C4		F2		G2	
FAMEs	ng/L	mol%	ng/L	mol%	ng/L	mol%	ng/L	mol%	ng/L	mol%
C12:0	<d.l.		252.4	8.9	<d.l.		9.6	1.2	13.6	2.1
2-OH C12:0	<d.l.		129.5	4.3	<d.l.		177.8	21.2	136.4	19.5
C14:0	375.6	17.8	502.1	15.7	18.9	5.9	41.6	4.7	58.1	7.9
i-C15:0	62.3	2.6	20.8	0.6	<d.l.		<d.l.		12.2	1.5
a-C15:0	54.3	2.3	60.5	1.7	8.3	2.3	17.5	1.8	52.4	6.4
C15:0	25.8	1.2	266.0	7.9	10.7	3.2	24.3	2.6	21.4	2.7
i-C16:0	21.5	0.7	28.0	0.7	<d.l.		<d.l.		25.9	3.0
C16:1 (cis-9)	226.3	9.7	343.2	9.7	14.3	4.1	26.3	2.7	26.4	3.2
C16:0	528.6	22.4	1108.5	31.1	145.0	40.7	301.1	30.6	224.3	27.3
C17:0	9.6	0.4	56.8	1.5	<d.l.		8.3	0.8	11.2	1.3
C18:2 (all cis-9,12)	814.0	31.7	113.7	2.9	<d.l.		<d.l.		<d.l.	
C18:1 (cis-9)	133.0	5.1	223.7	5.7	124.3	31.9	218.0	20.2	141.0	15.6
C18:1 (cis-11)	83.0	3.0	<d.l.		<d.l.		51.4	4.8	<d.l.	
C18:0	78.7	3.0	363.7	9.2	46.9	11.9	100.7	9.3	86.2	9.5
Numbers detected	12		13		7		11		12	
Total biomass	2412.7	100	3468.9	100	368.4	100	976.7	100	809.0	100

In general, the fatty acids with carbon numbers of 15, 16 and 18 were major components in the consortium. The PLFA profiles in all the wells were characterized by even-numbered fatty acids (88%-94.9% of the total lipids) and their individual content was also higher than that of odd-numbered fatty acids. In nearly all samples, C16:0 dominated the total fatty acids, and other fatty acids with higher concentrations were C14:0, C16:1 (cis-9), C18:1 (cis-9), C18: 0, and 2-OH C12:0. Higher concentrations of C18:2 (all cis-9,12) was only observed from injection water samples. Fatty acids i-C15:0, a-C15:0 and a-C16:0 were main branched saturated fatty acids, but their concentrations were all below 62.3 ng/L. MUFAs were represented by C16:1 (cis-9) and C18:1 (cis-9), and their concentrations varied from 14.3 to 343.2 ng/L depending on the sources. The sole PUFA was C18:2 (all cis-9,12) and it was only detected in the injection well and producing wellbore C2. This PUFA was reported in great abundance in fungal fatty acids (Ruess and Chamberlain 2010), but was also found in marine hydrocarbon degrading bacteria (Zhang et al. 2012). In particular, fatty acid 2-OH C12:0, which was only detected in water samples from producing wells, was the single type of acid with hydroxy structure and interpreted as a portion of Gram-negative bacteria in the microbial community (Ruess and Chamberlain 2010; Zelles 1999). Ratios of specific PLFAs have also been used to track partly physiological change or stress responses of the microorganisms (White and Ringelberg 1997). An increase in the ratios of trans/cis PLFAs and cyclopropyl PLFAs to their monoenoic precursors (cy/pre) would typically increase with stresses caused by insufficient nutrients, low pH, and other harsh environmental conditions (Wixon and Balser 2013; Zelles 1999). The undetectable cyclopropyl fatty acids and trans MUFAs in this study revealed that the communities existed under relatively suitable conditions after long-term adaptation to the environments

The relationships between the PLFA components and the five sample units are shown in Figure 4.2. PLFA compositions in the reservoir samples of G2 and F2 exhibited similar trend while producing wells C2 and C4 were obviously different in most of the PLFA patterns. The results showed that the concentration differences in C18:2 (all cis-9,12), 2-OH C12:0, C18:1 (cis-11), and i-C15:0 were the most significant when comparing samples from the injection well with samples from the producing wells. Producing wells G2 and F2 exhibited similar PLFA compositions and their total PLFA concentration (809.0 ± 71.7 ng/L and 976.7 ± 114.3 ng/L for G2 and F2, respectively) were relatively close as well despite of their differences in redox potential. Microbial community structures changed with varying temperature, mineralization, permeability and water displacement factors in the oil reservoirs (Lin et al. 2014). The concentrations of total PLFAs observed from the wells ranged from 368.4 to 3468.9 ng/L and were believed to be greatly affected by the reservoir characteristics, e.g. geological properties and redox conditions.

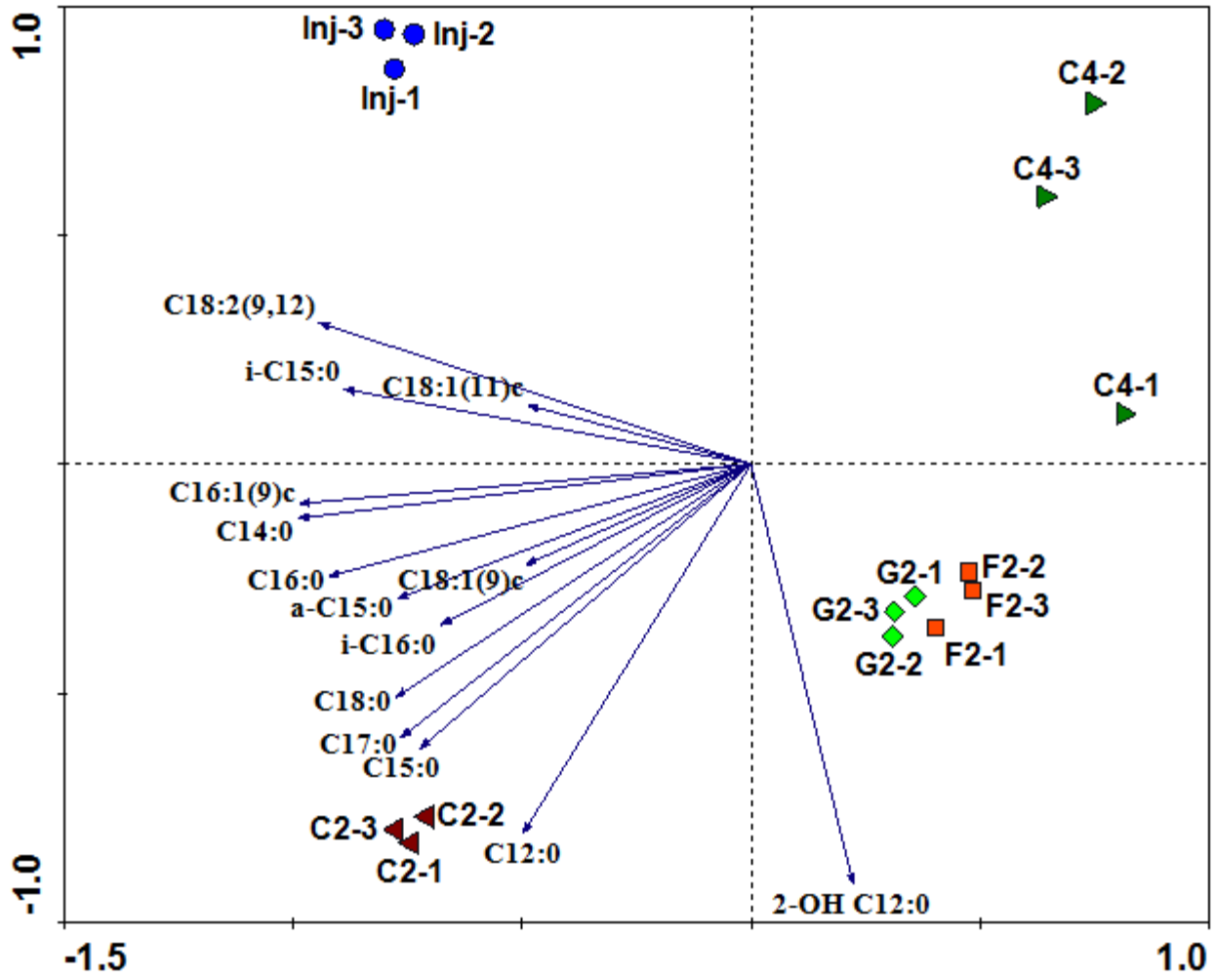


Figure 4.2 Biplot of species based on PLFA profiles and reservoir water samples of the five wells from principal component analysis

4.3.4 Identification of SRB

Based on our fatty acid results, possible SRB biomarkers and significant PLFA profiles associated with SRB were C18:1 (cis-9), C16:1 (cis-9), C18:1 (cis-11), i-C15:0, a-C15:0, C15:0, C17:0, C14:0, and C18:0. Fatty acid C18:1 (cis-9) was found in unique presence among PLFA profiles of *Desulfotomaculum acetoxidans* when Londry et al. (2004) cultivated the strain either autotrophically or heterotrophically with acetate to investigate substrate usage by SRB species. Mohanty et al. (2008) then used C18:1 (cis-9) as provisional indicative PLFA of SRB from the genera of *Desulfotomaculum* and investigated the microbial community response and associated sulfate reduction activities in the ethanol-amended slurries. Meanwhile, one species of the genus *Desulfobacter, D. latus*, also contained C18:1 (cis-9) as the dominant PLFA (31%) when cultured anaerobically (Kohring et al. 1994). This fatty acid also accounted for 15% and 14.2% in the major PLFAs of SRB species, *Desulfobacter curvatus* and *Desulfobotulus sapovorans* (Kohring et al. 1994).

SOB have been examined by several researchers with their membrane lipids and fatty acids C16:1 (cis-9) or C18:1 (cis-11) was usually predominated in these studies (Guezennec et al. 1998; Jacq et al. 1989; Katayama-Fujimura et al. 1982; Larkin 1980; Zhang et al. 2005). For instance, a chemoautotrophic SOB, *Thiomicrospira crunega*, was isolated from a deep-sea hydrothermal vent with large amounts of C16:1 (cis-9) or C18:1 (cis-11) dominant (Jannasch 1985). Two *Thioploca* species from the Peru upwelling region were also characterized using fatty acid analysis and the ratio of C16:1 (cis-9) ranged from 40.3 to 42.5% while the ratio of C18:1 (cis-11) was from 36 to 37.8% in these species (Mccaffrey et al. 1989). The dominance of C16:1 (cis-9) (16.7 to 37.4%) and C18:1 (cis-11) (11.8 to 16.8%) has also been observed in thiotrophic bacterial mats in the Barbados Trench (Guezennec and Fialamedioni 1996). It was then concluded that C16:1 (cis-9)

and C18:1 (cis-11) can be used as signature biomarkers for sulfur-oxidizing bacteria in H_2S-rich marine sediments (Zhang et al. 2005). On the other hand, Dowling et al. (1986) observed the dominance of C16:1 (cis-9) (37.3%) from one of SRB species *Desulfuromonas acetoxidans*, while the percentage of C18:1 (cis-11) was 0.8%. Interestingly, high levels of C16:1 (cis-9) (24.4%) and C18:1 (cis-11) (24.1%) were also characteristic of *Desulfotomaculum acetoxidans* (Dowling et al. 1986) and it was proposed a *Desulfotomaculum acetoxidans*-like organism or group of organisms dominated acetate-coupled sulfate reduction in estuarine and brackish sediments (Boschker et al. 1998). In our studies, the coexistence of C16:1 (cis-9) and C18:1 (cis-11) were only observed in the injection well and wellbore F2, both of which were under relative oxidizing conditions. For the other 3 production wells, only fatty acid C16:1 (cis-9) accounting for a lower ratio (3.3% -10.1%) was found and the content of C18:1 (cis-11) was beyond our detection limit. Thus, fatty acid C16:1 (cis-9) as the biomarker of SOB seemed not applicable in the reservoir wells with reducing conditions, but the coexistence of C16:1 (cis-9) and C18:1 (cis-11) could indicate the presence of SOB in the injection well and production well F2.

SRB were commonly found to contain odd-chain fatty acids, such as iso and anteiso C15:0 and C17:0 (Edlund et al. 1985; Elvert et al. 2003; Goorissen et al. 2003; Zhang et al. 2005; Zhang et al. 2002), while the relative proportions of these fatty acids might be one criterion to evaluate the possible presence of SRB. Significant amounts of C15:0 were observed when *Desulfobulbus* was cultivated with all the growth substrates (propionate, lactate and H_2/CO_2) and this fatty acid alone accounted for 23.0% of the total cellular fatty acids when on growth with propionate (Taylor and Parkes 1983). The cellular fatty acids of *Desulfovibrio desulfuricans* grown on H_2/CO_2 was dominated by branched iso fatty acids 75%, with i-C15:0 dominating (Taylor and Parkes 1983). Fatty acid i-C15:0 was also reported to account for 8.9% and 23.0% when *Desulfovibrio*

desulfuricans grown on acetate and lactate (Londry et al. 2004). Beyond *Desulfovibrio desulfuricans,* significant amounts of i-C15:0 was found in *D. vulgaris, D. baculatus, D. simplex, D. termitidis, Desulfomonas pigra* (Vainshtein et al. 1992). Moreover, a-C15:0 with various amounts (5-10%) predominanted in *Desulfovibrio* species, such as *D. alcoholovorans, D. carbinolicus, D. fructosovorans, D. giganteus, D. gigas, D. sulfodismutans* (Vainshtein et al. 1992). In addition, i-C15:0 was also reported as major compounds (48.6~68.3 in mol%) among thermophilic *Desulfotomaculum* species when the strains grew with alcohols (Goorissen et al. 2003). Specially, the branched-chain fatty acids (iso, anteiso in C14-C18) have been widely accepted as general indicators for gram-positive bacteria (Ruess and Chamberlain 2010), they (i.e. i-C15:0 and a-C15:0) thus could not specifically represent the designated genera and species of SRB. In addition, the odd chain PLFAs (e.g. C15:0 and C17:0) in bacteria are associated with the composition of hydrocarbons in the substrate but not widely used as representative biomarkers for specific microbes (Ruess and Chamberlain 2010). Therefore, the presence of these fatty acids (i.e., i-C15:0, a-C15:0, C15:0, and C17:0) could only be taken as enhanced references to describe SRB occurrence when SRB were confirmed by other biomarkers based on published PLFA profiles of SRB.

The even-number fatty acids C14:0 and C18:0 were both potential components in the PLFA profile of SRB species and could be viewed as special references in determining the biomass of microorganisms. Taylor and Parkes (1983) studied cellular fatty acid compositions of three sulfate reducing species and found the major cellular fatty acids of *Desulfobacter* sp. grown on acetate were dominated by C14:0 (23%) and C16:0 (44%). Additionally, a mole percent at 31.4% and 26.9% of fatty acid C14:0 was found in *Desulfobacter AcBa* and *Desulfoarculus baarsii,* respectively, even though its content is below 6% for most of the SRB species (Kohring et al. 1994;

Londry et al. 2004). The PLFAs were reported to be dominated by C14:0 (19.1%), C16:0 (18.1%), and C18:0 (25.9%) for *Desulfomonile tiedjei* when it was cultivated with formate/acetate and 3-chlorobenzoate (Kohring et al. 1994). Furthermore, fatty acid C18:0 was present in many SRB species with contents ranging from 0.1 to 10.6% (Kohring et al. 1994; Rütters et al. 2001; Taylor and Parkes 1983).

According to the literature and PLFA profiles from the reservoir samples, the possible presence of SRB and SOB were suggested. SRB species belonging to the genera *Desulfotomaculum* (thermophilic) and *Desulfobacter* were present in a relatively high possibility (mainly indicated by the biomarker C18:1 (cis-9)) and *Desulfovibrio* species may exist in the reservoir in a low probability. The results of reservoir samples were consistent with the simultaneous microbial community composition analysis based on DNA extraction (Okpala et al. 2017). As indicated by the coexistence of C16:1 (cis-9) and C18:1 (cis-11), SOB species distributed in the injection well and wellbore F2. Sulfidogenic communities *Desulfotomaculum and Desulfovibrio* were found in the production water samples and were involved in the souring mediated corrosion of the oil-water separation tanks in the north-eastern India oil fields (Agrawal et al. 2010). During another packed-bed bioreactor study, SRB genus *Desulfobacter* were detected from a low-temperature oilfield in Argentina through culturing and culture-independent techniques (Grigoryan et al. 2008). Difference species of *Desulfobacter, Desulfotomaculum* and *Desulfovibrio* were also 3-chlorobenzoate reported from other oilfield fluids (Grabowski et al. 2005; Lien and Beeder 1997; Miranda-Tello et al. 2003; Tardy-Jacquenod et al. 1998). The three possible SRB genera were consistent with the results of previous studies and were very likely the causes for souring in oil reservoirs.

4.3.5 Patterns of microbial community structure and SRB migration behavior in reservoir

Changes in reservoir PLFA profiles can provide valuable guidance to explore the migration behavior of target SRB communities in the course of above-ground water injection, water flooding in the reservoir and above-ground water collection (Dijkman et al. 2010; Gieg et al. 2011). SRB, notable for their utilization of sulfate as a terminal electron acceptor to produce reducing agent hydrogen sulfide (Enning and Garrelfs 2014; Hubert and Voordouw 2007), are closely associated with the redox potential of the reservoir samples. A redox potential of less than -100 mV at certain pH conditions could provide a favorable environment for the reducing sulfidogenic process (Church et al. 2007). Therefore, the redox potential of the reservoir samples can be used as a supplementary index for identifying SRB thus aiding the PLFA profile analysis.

It was noted from the results that the redox potentials of the injection well, producing wells C4 and G2 gradually decreased (Table 4.1). The varying redox potentials of the wells indicated different levels of SRB/SOB activities in the reservoir microcosms. A comparison of the associated microbial PLFA profiles thus can provide valuable information in the transformation patterns of SRB/SOB communities in these wells. From Figure 4.3, samples from the relatively oxidizing production wellbore C4 were detected with the lowest fatty acids content (368.4 ng/L) and the simplest composition. The fatty acids detected from wellbore C4, such as C14:0, C16:0, C18:1 (cis-9), C18:0, were all high-abundance components in injection seawaters. It was speculated that the main microbial components in wellbore C4 were very likely derived from microorganisms in injection seawater and the main microbial species survived in reservoir conditions due to their high biomass and adaptation to the reducing reservoir environment. As indicated by the presence of C18:1 (cis-9), a group of SRB species very likely survived.

The wellbore G2, with higher fatty acid yields (809 ng/L) than wellbore C4, revealed more obvious transformation of microbial community structure when seawater mixed with original formation water beneath the seabed. All the seven fatty acids detected from wellbore C4 (likely derived from microorganisms in seawater) were present in wellbore G2 and their content increased to various extents. The dominating fatty acids C14:0, C16:0, C18:1 (cis-9), C18:0 in wellbore C4 and injection seawater were still in great abundance in wellbore G2. However, the dominating fatty acids C16:1 (cis-9), C18:2 (all cis-9,12) and C18:1 (cis-11) in injection seawater were either not discovered or had very limited distribution in wellbore G2 and C4. Interestingly, fatty acid 2-OH C12:0, not observed in injection seawater, was found in great proportion from wellbore G2. The change in microbial community composition might be due to two mechanisms: 1) microorganisms inherited from seawater thrived in the reservoir environment, and 2) indigenous microbes previously existed in the reservoir formation water before water flooding was applied. The absence of fatty acids C18:2 (all cis-9,12) and C18:1 (cis-11) in producing wellbores G2 and C4 would be caused by the environmental change from relatively oxidizing ocean to the anaerobic reservoir. Therefore, the bacterial community structure changed to various degrees depending on the reducing conditions in the reservoir while inheriting the main microbial components from seawater.

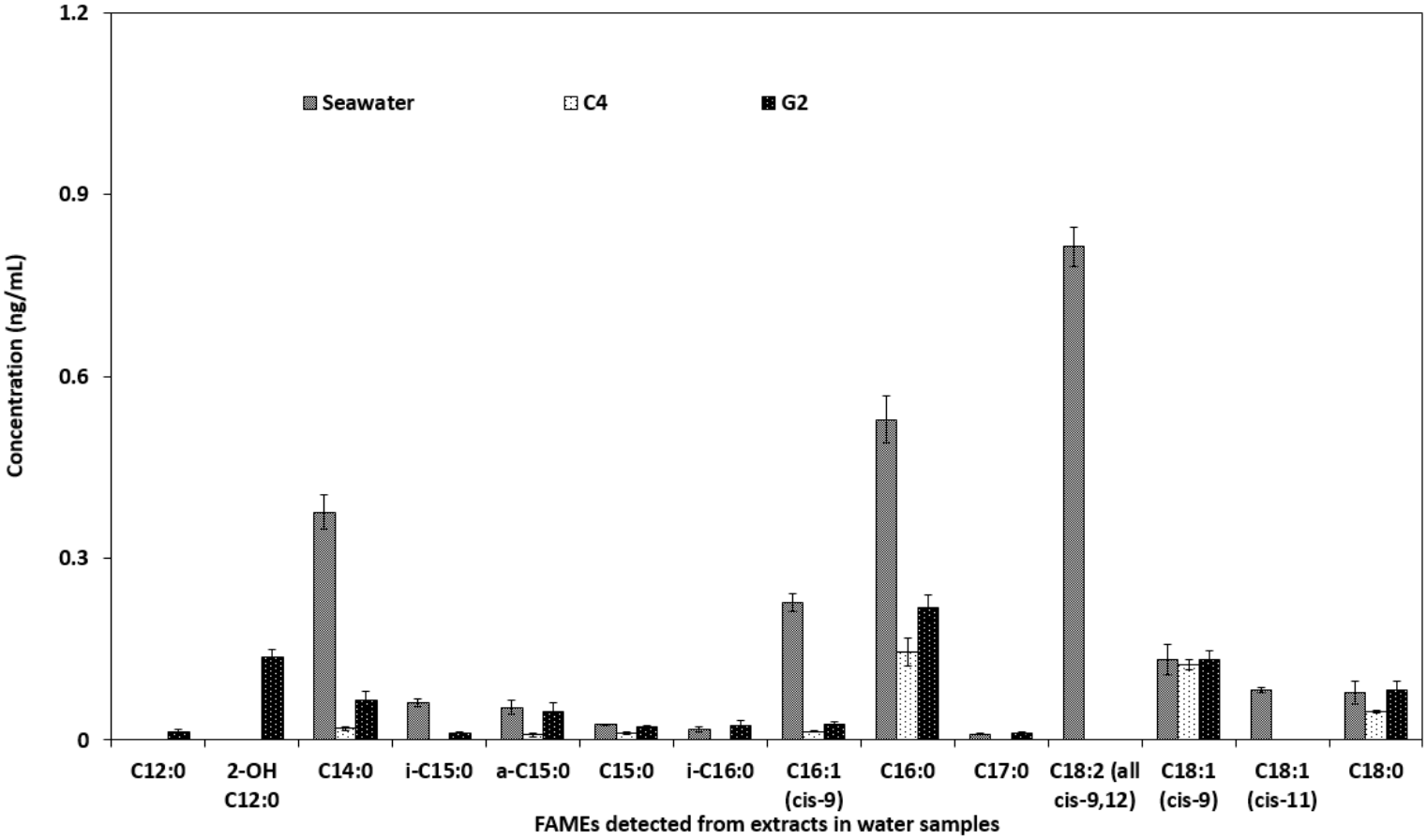


Figure 4.3 FAMEs detected from phospholipids in offshore injection water and produced water

C4 and G2

PLFA profile in the producing wellbore C2 was further compared with the microbial patterns in the original injection well, as C2 exhibited the most reducing conditions in which the greatest number of fatty acids and the highest biomass were found. Fatty acid C18:2 (all cis-9,12), not detected in other producing wells, was found in a minor presence (2.9% of the total PLFAs) in wellbore C2 even though the proportion was as high as 31.7% in seawater PLFA profiles. As shown in Figure 4.4, PLFA profiles of wellbore C2 and injection seawater also shared most of the same types of fatty acids. The geological environment near wellbore C2 was considered to be more suitable than wellbore G2 for reducing microorganisms to survive and thrive in the water flooding process. When cold seawater was mixed with hot fluids near the injection well, amenable environmental conditions with suitable temperature was created for bacterial growth and bacteria would progress into high-permeability zones and thrive there (Shibulal et al. 2014).

Two fatty acids with low-carbon numbers, C12:0 and 2-OH C12:0 were not detected from injection seawater samples, but they were detected with concentrations of 252.4 ng/L and 129.5 ng/L in wellbore C2, respectively. The amenable conditions also stimulated the activities of SRB and the H_2S would ultimately be produced by SRB through the use of sulfate in seawater and oil components existed in the reservoir (Usher et al. 2014). As revealed by the SRB biomarker C18:1 (cis-9), the most reducing condition in wellbore C2 was an indicator of the presence of H_2S (120 ppm from the gas phase at the separator), which reflected the vibrant anaerobic microbial activities (total PLFAs of 3468.9 ng/L) as well. In comparison to the pattern of PLFA concentrations in the injection seawater, fatty acids C15:0, C17:0, and C18:0 increased significantly when water was collected from wellbore C2, while compound C18:1 (cis-11) was below detection limits in this structure transformation of microbial community. This phenomenon was attributed to the disappearance of SOB species, as they are indicated by the coexistence of C16:1 (cis-9) and C18:1

(cis-11). The limited availability of oxygen and associated redox potential change are believed to be among the main factors influencing the transformation microbial community structure.

Proportions of 9 PLFA subgroups (as mol%) stated in Section 3.4 provided specific information on SRB and SOB species for the reservoir samples. As seen from Figure 4.5, while SOB species (very likely *Beggiatoa* and *Thioploca*) were indicated by the coexistence of C16:1 (cis-9) and C18:1 (cis-11) in the relatively oxidizing injection well and wellbore F2, they were below detection limits in the reducing production wells (C2, G2 and C4) according to the disappearance of fatty acid C18:1 (cis-11). The presence of SOB species could be regarded as one of the indicators reflecting the relatively oxidizing environment in the producing well F2 (99 mV).

In general, the absolute content of compound C18:1 (cis-9) from producing wells was higher than that from the injection well and this was very likely due to the increment of SRB species. Figure 4.5 indicated that the SRB biomarker C18:1 (cis-9) occupied a low proportion of total fatty acids in the injection seawater. However, its proportion raised in nearly all the produced water samples (C4, G2 and F2), a significant sign that the sulfidogenic species thrived in the warm reducing reservoir environment.

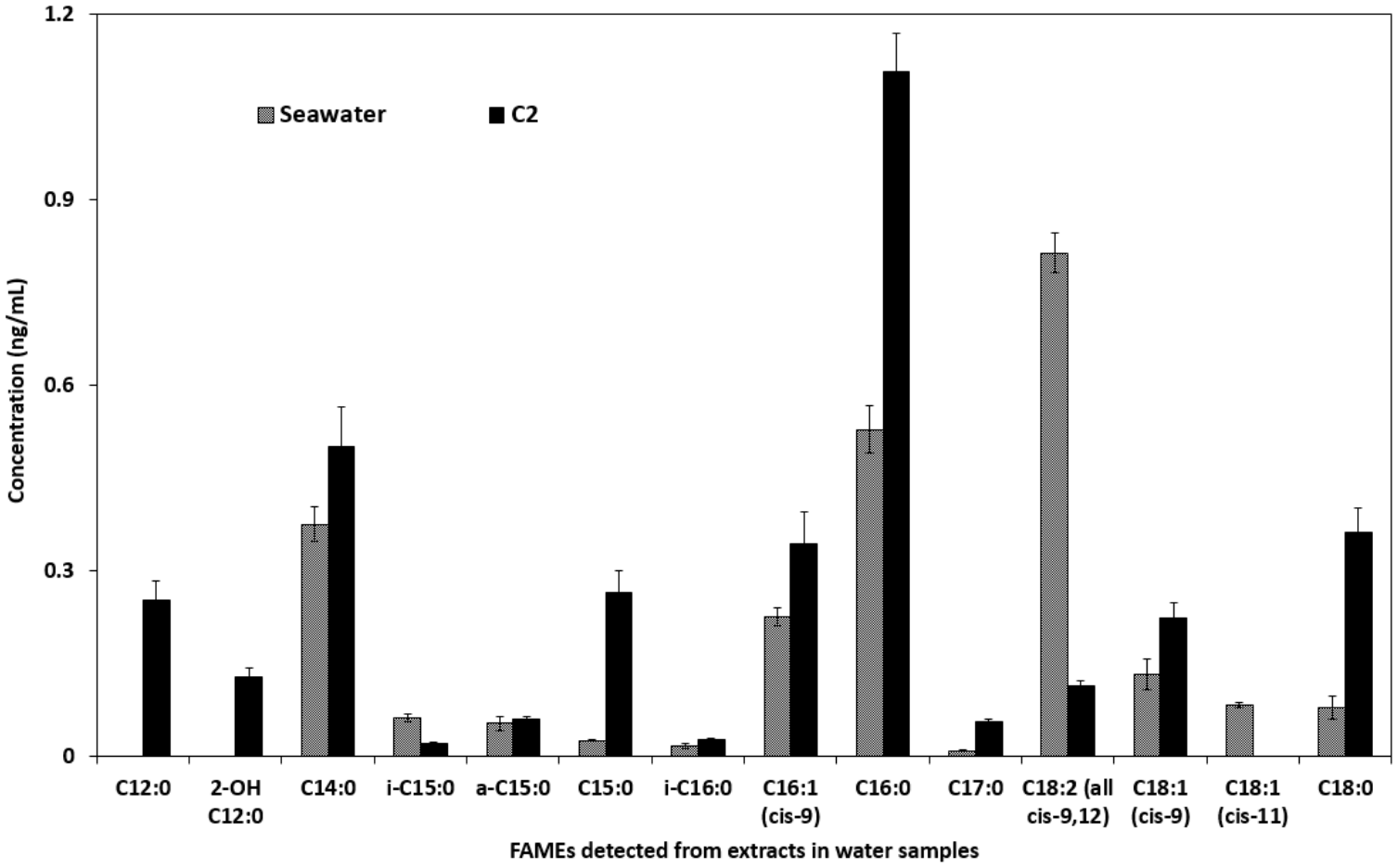


Figure 4.4 FAMEs detected from phospholipids in offshore injection water and produced water

C2

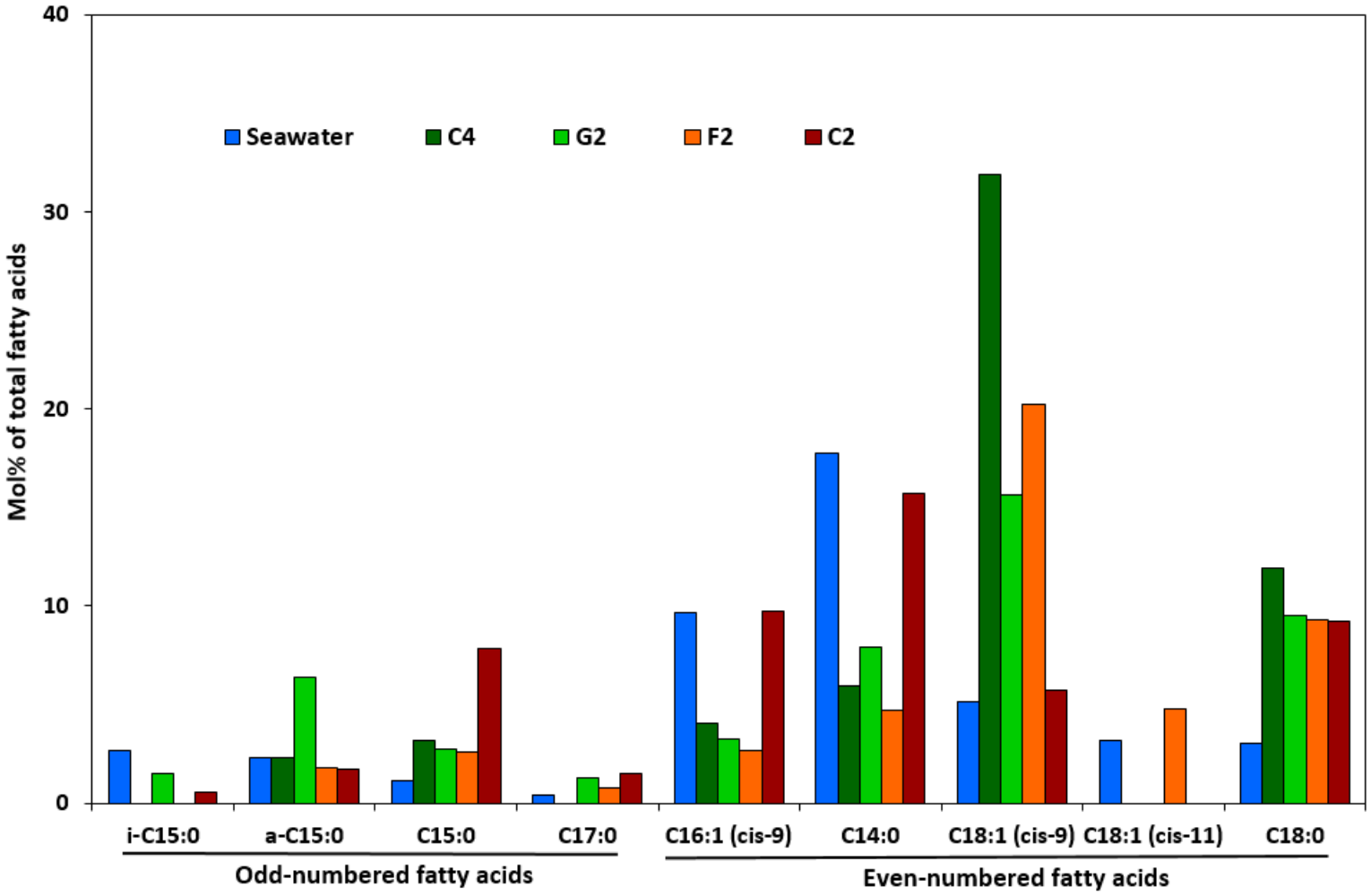


Figure 4.5 Changing patterns of possible SRB biomarkers and significant fatty acids associated with SRB in the injection water and produced water samples

The SRB flourished in the anaerobic oil reservoir due to the conducive environment provided by the removal of oxygen but the availability of sulfate from injected water, and the existence of VFAs produced by microbial degradation of crude-oil components (Hasegawa et al. 2014). The content of C18:1 (cis-9) in seawater (5.2%) and producing wellbore C2 (5.7%) were relatively low when compared to other producing wells (ranging from 15.6% to 31.9%). This phenomenon might be attributable to the relatively fierce competition for available carbon sources and other nutrients that occurs among the microorganisms, since the two samples were both detected with high biomass and the microbial consortia were diverse in the environment. Specially, C18:1 (cis-9) was possible dominating SRB biomarker in producing wellbore C4, G2 and F2, while C16:1 (cis-9) and C18:1 (cis-9) were both potential dominating SRB biomarker in producing wellbore C2. From our results, SOB species were deemed to be absent in producing wellbore C2 according to the appreciable presence of C16:1 (cis-9) but C18:1 (cis-11), and this differentiated C2 from the injection well.

In addition, all the concentrations of detectable fatty acids from the 9 compounds were summed up in Figure 4.6 to evaluate the sulfate-related microbial biomass in water samples. From the redox potential results, producing well F2 showed almost the same redox condition as that in the seawater. However, the abundance of PLFA in F2 decreased dramatically compared with the injection water likely due to the disturbance of water flooding in reservoir F2. SOB and SRB species were both detected in injection water and F2 as indicated by compounds C16:1 (cis-9), C18:1 (cis-11), and C18:1 (cis-9). The limited microbial abundance in F2 could be attributed to the restraints from harsh environments for indigenous microbes and the fact that only a small group of exogenous bacteria adapted to the reservoir environment and survived (Ren et al. 2015). Interestingly, the more reduced conditions were associated with higher bacterial abundance in

three producing wells (C4, G2, and C2) where very limited SOB biomarkers were found. Thus, the presence of sulfate reducers and SOB, coupled with their biomass were closely associated with the redox environment in the reservoir. In fact, the fatty acids analyzed in this study indicated that most of the SRB were derived from the seawater and their microbial community structure varied while the microorganisms adapted to different reservoir conditions. From Figure 4.6, a dynamic change of microbial communities was obtained based on the distribution patterns of PLFA abundance among the sample microcosms generated from the producing wells after water flooding and from the injection well before water flooding. A five-step transformation pattern indicated as (a) high SRB&SOB in seawater, (b) low SRB&SOB in F2, (c) low SRB in C4, (d) relatively high SRB in G2, and (e) high SRB in C2 was used to elucidate the souring process induced by SRB. In this regard, the PLFA profiles provided convincing reference and credible validation of SRB and SOB activities in the designated reservoir.

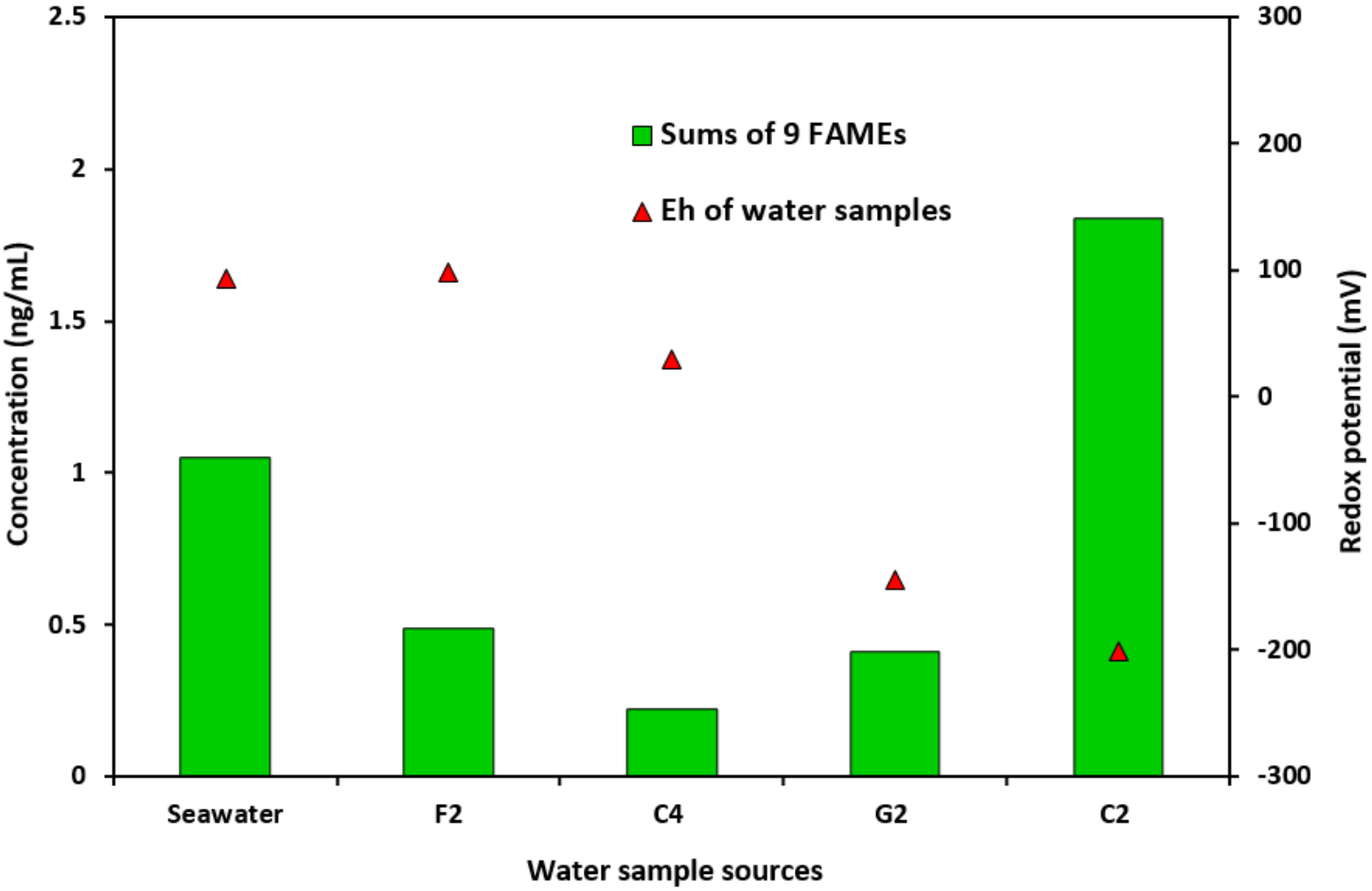


Figure 4.6 Total biomass indicated by 9 PLFAs associated with SRB and their relationship with redox potential

4.4 Summary

In this study, PLFA analysis was conducted to characterize SRB in offshore reservoir injection water and produced water samples. The in-situ measurements were performed to obtain parameters including temperature, pH, DO, and redox potential. Analytical performance in the PLFA determination was evaluated through the repeatability of GC, recoveries in the methodology and capillary chromatograph. The fatty acid profiles were elucidated between samples of the injection well and four producing wells, and the small differences with respect to the structure of the sulfate reducing community were determined. The PLFA profiles were closely related to the redox potential results and possible SRB biomarkers coupled with significant fatty acids related to SRB were selected to analyze the possible SRB species. SRB *Desulfotomaculum, Desulfobacter, Desulfovibrio* and SOB species were proposed to exist in various possibilities and their transformation patterns in the reservoir were concluded. In this case, the fatty acid analysis provided reasonable results to trace microorganisms in the offshore reservoir water samples and can be potentially used as a routine monitoring tool in the implementation of reservoir souring mitigation strategies.

CHAPTER 5

ISOLATION OF NRB FROM AN OFFSHORE RESERVOIR AND THE ASSOCIATED BIOSURFACTANT PRODUCTION[3]

[3] *The contents in the chapter will result in the potential publication:*

F. Fan, B. Zhang, P.L. Morrill, T. Husain, Isolation of nitrate-reducing bacteria from an offshore reservoir and the associated biosurfactant production, RSC Advances, 8 (2018) 26596-26609.

Role: Fuqiang Fan solely worked on this study and acted as the first author of this manuscript. Dr. Baiyu Zhang is my supervisor. Dr. Penny Morrill and Dr. Tahir Husain helped guide the research and polish the manuscript.

5.1 Background

The activity of SRB has long been a major concern in oilfield water systems and offshore petroleum reservoirs because these microorganisms are one of the main causative agents of reservoir souring as well as MIC (Xu et al. 2012). Many oilfield products (produced water, gas, etc) contain sulfides (H_2S and HS^-) as a result of the activity of SRB or other sulfidogenic bacteria. SRB reduce sulfate in the injection water to sulfide, while oxidizing degradable organic electron donors present in the offshore oil reservoir. The undesirable production of sulfides in offshore oil reservoirs results in reduced quality of produced hydrocarbons, health and safety risks for operators, and increased corrosivity of produced fluids (Hubert and Voordouw 2007; Okoro et al. 2014).

Although sulfides can be removed chemically after their production, *in situ* elimination through continuous nitrate/nitrite injection has also proven to be effective, as demonstrated both in model column (An et al. 2010; Grigoryan et al. 2008; Hubert et al. 2005), inland (Grigoryan et al. 2009; Shartau et al. 2010) and offshore field studies (Dunsmore et al. 2006; Larsen et al. 2004). Nitrate injection changes the microbial community in the subsurface from mainly SRB to one enriched in NRB, which include the nitrate reducing, sulfide oxidizing bacteria (NR-SOB) that oxidize H_2S directly and the heterotrophic NRB (hNRB) that compete with SRB for degradable organic electron donors. Additionally, both types of NRB also promote SRB inhibition via production of nitrite formed in nitrate reduction pathways (Greene et al. 2003). NRB are well known for their denitrifying capacity in which nitrates or nitrites are converted into nitrogen-containing gases. This function enables NRB to play significant roles in the global nitrogen cycle (Rothenberger et al. 2009) and mitigation and control of sulfide induced reservoir souring problems in offshore oil

fields (Gieg et al. 2011). However, understanding of the detailed microbial mechanisms involved in NRB-SRB interactions during nitrate/nitrite injections for offshore reservoirs souring mitigation is currently limited.

Anaerobic, indigenous NRB have the potential to produce specific biosurfactants in deep geological porous offshore reservoirs with diverse physiochemical *in situ* conditions. Biosurfactants are surface-active compounds with both lipophilic and hydrophilic structural moieties produced by microorganisms, which either adhere to the cell surface or are secreted extracellularly in the growth medium (Rodrigues et al. 2006). These surface active molecules reduce surface tension at air-water interfaces and interfacial tension in both aqueous solutions and hydrocarbon mixtures (Ghribi et al. 2012). Notably, biosurfactants have various advantages over their commercially manufactured counterparts because of their lower toxicity, biodegradable nature, ease of biosynthesis and the effectiveness under extreme conditions such as temperature, pH, and salinity (Mulligan 2005; Pacwa-Płociniczak et al. 2011). Fallon et al. (2010) have confirmed that biosurfactants can be naturally derived from NRB. As microorganisms capable of utilizing hydrocarbons as carbon and energy sources, NRB will produce surface-active agents as by-products to facilitate hydrophobic degradation (Ron and Rosenberg 2002). The selective surface-active agents or biosurfactants produced by NRB increase the surface area of hydrophobic water-insoluble substrates (low molecular weight biosurfactants) and increase the solubility (high molecular weight biosurfactants), thus improving the bioavailability of hydrocarbons for NRB (Pacwa-Płociniczak et al. 2011). When emulsion occurs closely to the cell surface of NRB, each cluster of cells creates its own microenvironment and stimulate the growth of NRB in oil and gas reservoirs (Ron and Rosenberg 2002). This mechanism enables NRB to out-compete harmful SRB for basic carbon nutrients. The SRB will be inhibited from producing new hydrogen sulfide/iron

sulfide, and the existing sulfides will be removed by bacterial degradation, resulting in effective control of offshore reservoir souring. Thus, at this point biosurfactants are interesting by-products involved in the SRB/NRB competition.

To investigate how biosurfactants affect NRB-SRB competition in a reservoir, successful screening of biosurfactant producing NRB and generation of associated biosurfactants are needed. Oil reservoirs could provide a unique hydrocarbon-rich environment for biosurfactant screening and the enrichment of diverse biosurfactant producers (Christofi and Ivshina 2002). The extreme conditions (e.g., high temperature, high pressure, and low oxygen concentration) in oil reservoirs would formulate a microbial community that may be distinguished from others (Grabowski et al. 2005). So far, biosurfactant producers identified from oil reservoirs are mainly from inland reservoirs and limited to the genera of *Bacillus* (Al-Bahry et al. 2013; She et al. 2011; Simpson et al. 2011; Wang et al. 2011; Youssef et al. 2007) and *Pseudomonas* (Gudiña et al. 2012; Lotfabad et al. 2009; Pruthi and Cameotra 2003). Currently, very limited marine biosurfactant producers from offshore oil and gas fields have been reported and it was unclear whether they were NRB species or not. Cai et al. (2015) isolated marine biosurfactant producers from crude oil, formation water, drilling mud, and treated produced water samples in offshore oil and gas fields. The genotype and phylogenetic relation of these isolates were investigated and biosurfactant producers were primarily found in the genera of *Rhodococcus* and *Halomonas*. However, most of these species reported are general biosurfactant producers and they were screened under aerobic conditions, which were not prevalent in the reducing reservoir environments. Hui et al. (2012) evaluated the microbial community structure and functionally distinct groups in three kinds of produced water samples from the shallow, mesothermic and low-salinity Daqing oil reservoir using both culture-dependent and culture-independent methods. The isolates affiliated to

Pseudomonas stutzeri PTG4-15 (DP26, BP39, and PW5) were initially identified as nitrate reducing bacteria, biosurfactant producing bacteria, and polymer-producing bacteria. This indicates that anaerobic indigenous NRB have the potential to produce specific biosurfactants in the reservoir.

Until now, biosurfactant producing NRB isolated from oil reservoirs have been rarely reported, and associated biosurfactant production is extremely limited in the literature. No previous study tackled the isolation of biosurfactant producing NRB from offshore reservoirs and subsequent anaerobic biosurfactant production. Therefore, the aim of this study was to screen NRB strains from an offshore reservoir, and conduct biosurfactant production and characterization. The research outputs will not only help identify NRB and generate biosurfactants under anaerobic conditions, but also provide technical and methodological support for further identifying NRB-SRB interactions and generating methodologies for effective offshore reservoir souring control in the future.

5.2 Materials and Methods

5.2.1 Source and collection of inoculum

Produced water samples in an offshore water flooding reservoir were collected for screening novel biosurfactant producers. Injection wells on the platforms were injected with nitrate/nitrite to stimulate the growth of indigenous nitrate reducing microorganisms in the reservoir or exogenous NRB strains from seawater injection process. Produced water was collected in 1-liter sterile glass bottles and then sealed immediately to maintain anoxic conditions. The samples were stored in a refrigerator in darkness before shipping. Subsequently, the samples were packaged with frozen ice packs and transported to the laboratory for enrichments and bacterial isolations. The major

constituents of the produced water include (in wt/vol) chloride, 4.0%; sodium, 2.5%; sulfate, 0.13%; calcium, 0.12%; and bicarbonate, 0.076%. Water samples were stored at 4 °C and were taken for enrichment culture within one week of collection.

5.2.2 Isolation and identification of NRB

5.2.2.1 Growth media

Coleville synthetic brine (CSB) medium was selected for the NRB culturing based on the properties of produced water and NRB growth requirement. The recipe was composed of (in 1 liter) NaCl, 7.0 g; $MgSO_4 \cdot 7H_2O$, 0.68 g; $CaCl_2 \cdot 2H_2O$, 0.24 g; NH_4Cl, 0.02 g; KH_2PO_4, 0.027 g; $NaC_2H_3O_2 \cdot 3H_2O$, 0.68 g; KNO_3, 1.0 g; $NaHCO_3$, 1.9 g; resazurin, 0.0001 g; and ND trace metals (0.5 mL H_2SO_4, 2.28 g $MnSO_4 \cdot H_2O$, 0.5 g $ZnSO_4 \cdot 7H_2O$, 0.5 g H_3BO_3, 0.025 g $CuSO_4 \cdot 5H_2O$, 0.025 g $Na_2MoO_4 \cdot 2H_2O$, and 0.045 g $CoCl_2 \cdot 6H_2O$ per liter), 50 ml/liter (Gevertz et al. 2000). The medium pH was then adjusted to between 7.0 and 7.5. After autoclaving, cooling, and equilibration of the medium with chamber gas overnight, sterilized 2.5% (w/v) $Na_2S \cdot 9H_2O$ (final concentration of 0.02% (w/v)) was added to remove residual oxygen. Solid growth media were prepared by adding a certain amount of agar (2% w/v).

5.2.2.2 Enrichment and isolation

Enrichments were prepared by adding 5 ml of produced water to 125 mL medium in sterile conical flasks under anaerobic conditions. To ensure the growth of microorganisms, three enrichment recipes with various carbon sources were prepared in conical flasks, respectively. The first one was adopted from Hui et al. (2012) and contained (in 1 liter) peptone, 20 g; beef extract, 10 g, KNO_3, 1.0 g; NaCl, 0.7 g; KCl, 0.7 g; $MgCl_2 \cdot 6H_2O$, 10 g; $MgSO_4 \cdot 7H_2O$, 5.4 g; $CaCl_2 \cdot 2H_2O$, 1.0

g. The second recipe was a raw screening medium which contained (in 1 liter) peptone, 5.0 g; beef extract, 3.0 g; KNO_3, 1.0 g and trace metals (same as in CSB medium). The third recipe used citrate as the carbon source and contained L-asparagine 0.5 g; KNO_3, 1.0 g; trisodium citrate, 0.85 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; $FeCl_3 \cdot 6H_2O$, 0.05 g; $CaCl_2 \cdot 2H_2O$, 0.24 g; and KH_2PO_4, 0.05 g. Trace metals were added following the trace element solution in CSB medium. All the media pHs were buffered between 7.2 - 7.5 and further treated with sterilized $Na_2S \cdot 9H_2O$.

Enrichment was initially conducted in a chamber filled with nitrogen until observable turbidity occurred. The bacterial consortia were then inoculated into new media for further acclimatization. The average bacterial acclimatization period was 15 days. After seven periods, 5 ml of each medium broth were transferred into fresh liquid CSB medium and the citrate medium. The second round of bacterial acclimatization in liquid CSB and citrate medium were conducted through three periods. After that, the consortia were serially diluted to 10^6, 10^5, and 10^4 and then spread on solid CSB and citrate medium agar plate, respectively. The resulting plates were incubated at room temperature in a nitrogen-filled environment.

Routine growth and maintenance of broth isolates were in CSB medium. Bacterial growth status was detected by observing an increase in optical density at 600 nm (OD_{600}) or the cell dry weight filtered from medium broth.

5.2.2.3 Identification and phylogenetic characterization of isolates

The purified isolates were then subjected to 16S ribosomal RNA sequencing and amplified with universal bacterial primers 27F (5'-AGA GTT TGA TYM TGG CTC AG-3') and 16SR10 (5'-ACG GCT ACC TTG TTA CGA CT-3'). An aliquot of the each culture was used for a DNA template in a polymerase chain reaction (PCR) using the primer pair. After gel electrophoresis

confirmation of successful PCR reaction, PCR products were subjected to a clean-up process and measured by a NanoDrop spectrophotometer to determine the concentrations. Lastly, sequencing reactions with the last PCR products were conducted and measured with Applied Biosystems 3730 DNA Analyzer in Creait Network of Memorial University of Newfoundland. The obtained DNA sequences were aligned with previously published sequences from the GenBank database of National Center for Biotechnology Information (NCBI) using the Basic Local Alignment Search Tool (BLAST) program.

5.2.3 Screening of the NRB isolates for biosurfactant producers

5.2.3.1 Biosurfactant producing media

According to the morphological properties of the strains on the agar plate and associated 16S rRNA results, 5 strains were selected for subsequent biosurfactant producer screening. Two media with glycerol and glucose as carbon sources were selected for anaerobic biosurfactant production, respectively. The first one modified from Zhao et al. (2014) contained (in 1 liter) glycerol, 46.6 g; $NaNO_3$, 3.0 g; K_2HPO_4, 4.0 g; KH_2PO_4, 5.7 g; $MgSO_4 \cdot 7H_2O$, 0.4 g; $CaCl_2 \cdot 2H_2O$, 0.17 g; NaCl, 2 g; and yeast extract, 2.7 g. The second one was adjusted from a previous medium in NRB screening medium and contained (in 1 liter) glucose, 10 g; $NaNO_3$, 3.0 g; K_2HPO_4, 3.36 g; KH_2PO_4, 3.4 g; $MgSO_4 \cdot 7H_2O$, 0.68 g; $CaCl_2 \cdot 2H_2O$, 0.24 g; NaCl, 2g; yeast extract, 3.0 g; and $FeCl_3$, 0.05 g. Trace metals were also added into the two media as described previously. After autoclaving, cooling, and equilibration of the medium with chamber gas overnight, sterilized Na_2S solution was also added as mentioned above.

5.2.3.2 Biomass determination

Each isolate was incubated using the same conditions as in biosurfactant producing procedure for 228 hours. Subsequently, 10 mL culture sample was filtered through a pre-weighted 0.22 µm filter membrane and washed 2 times with 10 mL distilled water. The filter membrane was then dried in oven for 24 h and cooled down in desiccators before measuring the final weight. The biomass was determined as cell dry weight (g/L).

5.2.3.3 Parafilm test

A 25 µL aliquot of bacterial broth was added to the hydrophobic surface of a parafilm. The shape and the diameter of the droplet on the surface were inspected after 3 min. The negative control was prepared with 0.5 M phosphate buffer (Morita et al. 2007).

5.2.3.4 Drop collapsing test

Drops of the cell-free supernatant were placed on an oil-coated, solid surface. The polar water molecules are repelled from the hydrophobic surface with the absence of surfactants in the liquid and the drops remain stable whereas the droplet spread out slightly or even collapse with the presence of surfactants (Youssef et al. 2004).

5.2.3.5 Emulsification activity assay

The emulsification activity (E24, Eq.1) of the culture broth was determined by addition of 5 mL culture broth to 5 mL hexadecane or kerosene and vortexed for 2 min to create an optimum emulsion. Tests were performed in duplicate for quality assurance purpose and the results were expressed using the average of two measurements.

$$E24 = H_{EL} / H_S \times 100\% \quad \text{(Eq.1)}$$

where H_{EL} is the height of the emulsion layer and H_S is the height of the total solution.

5.2.3.6 Surface tension measurement

Culture samples were centrifuged at 10,000 rpm for 20 min to remove microbial cells and the supernatant was subject to surface activity measurements. Surface tension was determined with a surface tensiometer (DuNouy Tensiometer, Interfacial, CSC Scientific) at room temperature according to the ring method. The values reported were the mean of triplicate measurements.

5.2.4 Performance demonstration of the selected strain

According to the results, one isolate was selected to further demonstrate the performance of biosurfactant production. The two surfactant production media with glycerol and glucose as carbon sources were used to investigate the performance of biosurfactant production. The incubation was conducted at 30 °C while shaking at 200 rpm.

The isolate was firstly inoculated into the flask containing 20 mL fresh glucose medium and incubated using the above conditions for 48 h. Then a 200 µL aliquot of the culture broth was inoculated into each flask with 20 mL glycerol or glucose producing medium. The following two tests at time intervals of 0, 12, 24, 36, 48, 60, 84, 128, 156, 180, 204 and 228 h were sampled with the whole flask, respectively.

The OD_{600} of samples was employed as the index of bacterial growth (Safari et al. 2012). Absorbance was measured at λ = 600 nm using a UV−Visible spectrophotometer. All culture media were shaken for 5 s to homogenize the media before OD_{600} determination. The absorbance

of freshly autoclaved medium was adjusted to 0 as the blank control. The measurement of OD_{600} was performed in triplicate by sampling three times.

To observe the nitrate consumption by NRB, nitrate concentration in the culture was determined using a two-wavelength approach (Gopalan et al. 2005). A rapid measurement of sample absorption at 220 nm (A220) was conducted in a quartz cuvette and organic matter interference was eliminated through the second measurement at 275 nm (A275). Nitrate concentration was determined from standard curves prepared with $NaNO_3$ (0.2−8 mg/L). Additionally, surface tension changes over the period were monitored using the methodology mentioned above. CMD analysis was conducted to evaluate the concentration of biosurfactants produced during the investigation of cultivation kinetics.

5.2.5 Biosurfactant production and characterization

5.2.5.1 Extraction of the biosurfactant product

The procedures of biosurfactant extraction from the culture broth mainly followed the protocol according Silva et al. (2010) after 228 hours of incubation of CX3 in glycerol medium. The culture broth was initially centrifuged at 11000 rpm for 20 min to remove bacterial cells. The supernatant was then acidified to pH 2.0 with concentrated HCl (1 mol/L) and kept at 4°C overnight to reduce the rhamnolipid solubility. The biosurfactant product was further recovered through the addition of two volumes of chloroform: methanol (v/v, 2: 1) mixture. After shaking the mixture for 2 h, the lower organic phase was collected and evaporated to dryness by a rotary evaporator. The final precipitate was collected.

5.2.5.2 Determination of critical micellar concentrations (CMC)

CMC is defined as the surfactant concentration necessary to initiate micelle formation. The CMC of generated biosurfactant product was determined by plotting the surface tensions as a function of biosurfactant concentration and it was defined from the intercept of two straight lines extrapolated from the concentration-dependent and concentration-independent sections (De Oliveira et al. 2013).

5.2.5.3 TLC analysis

TLC analysis was conducted to preliminarily characterize the purified biosurfactant product. Ten microliter biosurfactant solution at a concentration of 200 mg/L in methanol was applied to a 20×20 silica gel TLC plate (Sigma Aldrich). The biosurfactant product was separated using $CHCl_3$:CH_3OH:H_2O (70:10:0.5, v/v/v) as developing solvent system with different color developing reagents. For detection of lipopeptide biosurfactants, ninhydrin reagent (0.5 g ninhydrin in 100 mL anhydrous acetone) was sprayed on the dry plates and red spots were visualized after keeping the plate at 105 ºC for 5 min. Anthrone reagent (1 g anthrone in 5 mL sulfuric acid mixed with 95 mL ethanol) was used to reveal the presence of glycolipid biosurfactants in yellow spots. Also, lipid content was further visualized by iodine chamber.

5.2.5.4 Lipid class determination

Lipid class composition was determined using an Iatroscan Mark VI TLC with flame ionization detector (FID), silica coated Chromarods and a three-step development method (Parrish 1999). The lipid extracts were applied to the Chromarods and focused to a narrow band using 100% acetone. The first development system was hexane:diethyl ether:formic acid (99.95:1:00.05). The rods were developed for 25 minutes, removed from the system for 5 minutes and replaced for 20

minutes. The second development was for 40 minutes in hexane:diethyl ether:formic acid (79:20:1). The final development system had two steps, the first was 100% acetone for two 15 minute time periods, followed by two 10 minute periods in chloroform:methanol:chloroform-extracted water (5:4:1). Before each solvent system the rods were dried in a constant humidity chamber. After each development system the rods were scanned in the Iatroscan and the data were collected using Peak Simple software (ver 3.67, SRI Inc). The Chromarods were calibrated using standards from Sigma Chemicals (Sigma Chemicals, St. Louis, Mo., USA).

5.2.5.5 Fatty acid analysis

For all samples, lipid extracts were transesterified using sulfuric acid and methanol for 1 hour at 100^{o}C. The FAMEs developed from the extracts were analyzed on a HP 6890 GC system with FID and a 7683 autosampler. The GC column was a ZB wax+ (Phenomenex, U.S.A.). The column length was 30m with an internal diameter of 0.32mm. The column temperature began at 65 ^{o}C and held this temperature for 0.5 minutes. The temperature ramped to 195 ^{o}C at a rate of 40 ^{o}C/min, held for 15 minutes then ramped to a final temperature of 220 ^{o}C at a rate of 2 $^{\circ}$C/min. This final temperature was held for 0.75 minutes. The carrier gas was hydrogen and flowed at a rate of 2 ml/minute. The injector temperature started at 150 ^{o}C and ramped to a final temperature of 250 ^{o}C at a rate of 120 ^{o}C/minute. The detector temperature stayed constant at 260 ^{o}C. Peaks were identified using retention times from standards purchased from Supelco, 37 component FAME mix (Product number 47885-U), Bacterial acid methyl ester mix (product number 47080-U), PUFA 1 (product number 47033) and PUFA 3 (product number 47085-U). Chromatograms were integrated using the Varian Galaxie Chromatography Data System, version 1.9.3.2. A quantitative standard purchased from Nu-Chek Prep, Inc (product number GLC490) was used to check the GC

column about every 300 samples (or once a month) to ensure that the areas returned were as expected.

5.2.5.6 Fourier transform infrared spectroscopy (FT-IR) analysis

IR spectroscopy was used for structure analysis of the extracted biosurfactant product based on the oscillation patterns of chemical bonds at characteristic frequencies. The IR absorption spectrum of the dried biosurfactant product was measured on a Bruker Tensor 27 FT-IR using 16 scans over the range of 500-4000 cm^{-1} (KBr beamsplitter). The signals were collected in transmittance mode with a Zn-Se attenuated total reflectance (ATR) spectroscopy which are commonly used for rhamnolipid analysis (Heyd et al. 2008).

5.2.5.7 Stability characterization

The effect of temperature, pH, and salinity on the surface activity of generated biosurfactants was investigated by changing surrounding conditions (Abouseoud et al. 2008b). Generally, 1 CMC of biosurfactant solution was prepared and maintained at a constant temperature of 0, 20, 40, 60, 80, 100 °C for 120 min and cooled at room temperature to determine the thermal stability of the biosurfactant. The pH influence on the biosurfactant activity was determined by adjusting the biosurfactant solutions in the range 2.0-12.0 using HCl (2 N) and NaOH (2 N) solutions, and the effect of salinity on the surface activity of the biosurfactant product was assessed by using various concentrations of sodium chloride (0.5-20% in w/v). In each case, the stability of the biosurfactant solution was evaluated by the change of surface tension values (1 CMC in all tests) and determined in triplicate.

5.3 Results and Discussion

5.3.1 Phylogenetic analysis and morphological characteristics of isolates

These isolates were incubated under anaerobic conditions with nitrate as the electron acceptor and acetate as the carbon source and electron donor. Most of the isolated NRB species were anaerobically slow-growing and mature colonies were formed around ten days on the CSB medium. As shown in Table 5.1, with sequences obtained by doing a Blast search, a similarity of 99% or 100% to *Pseudomonas stutzeri* was found by the alignment of the 16S rRNA gene sequences of all the selected five NRB strains isolated from offshore produced water.

The morphology characteristics of isolates were summarized in Table 5.1. In general, all the species could be summed into two categories: the brown one with entire margins and the light color or transparent ones with raised or curled margins. Notably, some isolates, HF5 and FX6 formed flocculants in liquid biosurfactant producing medium. The components of the flocculants were studied extensively before and were a mixture of polysaccharides, proteins, lipids, glycolipids and glycoproteins (Zheng et al. 2008). Bioflocculants could be produced by various functional microorganisms and are biodegradable, environmentally friendly and harmlessness to humans. Microorganisms with high bioflocculant-producing ability thus can be utilized to produce bioflocculants and can be used in industrial fields such as drinking and wastewater treatment, downstream processing, and fermentation processes (Salehizadeh and Shojaosadati 2001). The flocculated cells were previously reported to be immobilized inside the reactor for a continuous fermentation system without cell separation and recycling units, and the yields from the reactor were clean broths for further ethanol production (Andrietta et al. 2008). Viewed from this

perspective, the two strains have promising applications in batch, fed-batch or continuous fermentation reactors for industrial nitrate removal activities.

The strain CX3 was noticeable due to its different margin and color in the agar plate during morphology examination when compared with others (Table 5.1). During the growth on CSB medium, the isolate formed visible regular and glistening colonies with the colony diameters ranging from 1 to 3 mm. The precise taxonomic positions of the microbes were subsequently determined through the genotypic analysis on the basis of partial 16S rRNA sequencing. Unlike the other four strains, *Pseudomonas stutzeri* CX3 occupied a unique branch in the phylogenetic tree generated from the five *Pseudomonas stutzeri* strains and other *Pseudomonas* species (Figure 5.1). The selected *Pseudomonas stutzeri* strains formed a stable phyletic group within a heterogeneous cluster of *Pseudomonas xanthomarina,* and other *Pseudomonas stutzeri* strains, which distributed in ubiquitous environments and were identified among denitrifiers in natural materials (Lalucat et al. 2006). *P. stutzeri* is a highly diverse species of great physiological and ecological versatility and is of interest due to their specific activities in nitrification and denitrification processes (Sikorski et al. 2002). The strains of *Pseudomonas stutzeri* were also anaerobically isolated from various environmental samples and used in the degradation of aliphatic and aromatic hydrocarbons (Grimberg et al. 1996; Kaczorek et al. 2012).

Table 5.1 Identification of the isolated possible biosurfactant producers

Isolate ID	Query cover	Identity	Species name with the highest match	Liquid medium morphology	Agar plate morphology (shape/margin /elevation/surface texture/color)
CX1	100%	100%	*Pseudomonas stutzeri*	Turbidity	Circular/entire/convex/smooth/beige
CX3	100%	100%	*Pseudomonas stutzeri*	Turbidity	Circular/entire /convex/smooth/brown
HF5	100%	99%	*Pseudomonas stutzeri*	Flocculent	Circular/raised /convex/radiate/beige
HF6	100%	99%	*Pseudomonas stutzeri*	Turbidity	Circular/raised/convex/radiate/beige
FX8	100%	99%	*Pseudomonas stutzeri*	Flocculent	Circular/curled/convex/smooth/transparent

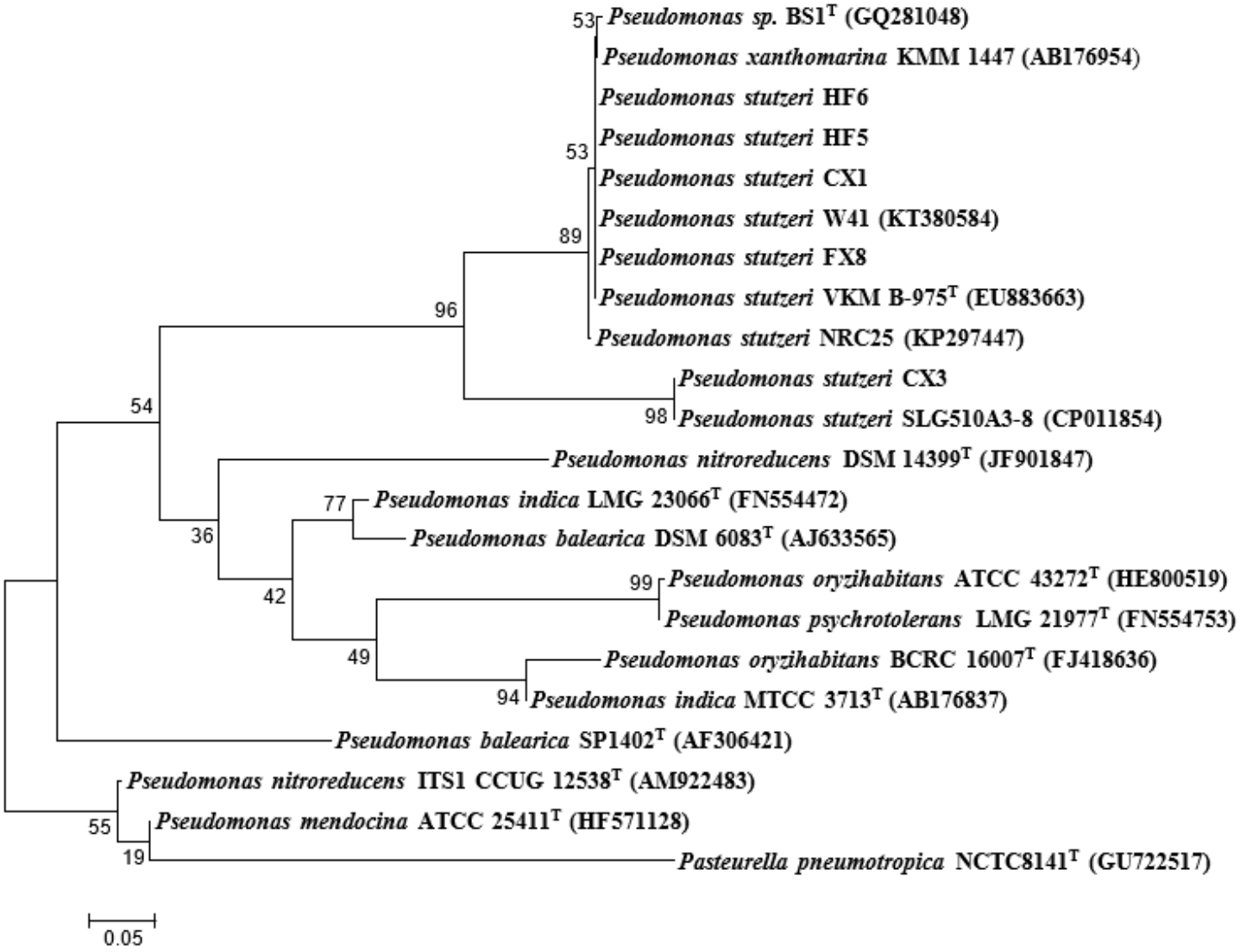


Figure 5.1 Phylogenetic tree of the isolated species from 16S rRNA gene sequences based on a neighbor-joining analysis of 1000 resampled datasets

5.3.2 Screening of biosurfactant-producing NRB

After 228 hours of incubation, cell-free broths were subjected to a parafilm test for screening of potential biosurfactant producers. The results are presented in Figure 5.2 and it was observed that the producing medium 2 yielded more flattened droplets for the *P. stutzeri* strains. The possible biosurfactant producing strains were *P. stutzeri* CX3, CX1 and FX8. As indicated in Figure 5.3a, the surface tension and E24% of the cell free culture of the 5 isolates on the two media were listed. It was observed that only the culture of *P. stutzeri* CX3 exhibited surface reducing ability on the glycerol and glucose media and lowered the surface tension to around 30 mN/m (the dotted line in Figure 5.3a indicates the surface tension of 40 mN/m). Consistent with the results from the parafilm test, all the isolates showed lower surface tension when incubated in the producing medium 2.

Although *P. stutzeri* HF5 presented the highest emulsification ability in hexadecane (13.8%) on producing medium 2, the NRB strains could not significantly emulsify n-hexadecane or kerosene. The biosurfactant producing isolate CX3 showed very limited emulsification ability on production medium 2, which suggests that the capacity of biosurfactants for surface tension reduction was not necessarily correlated with emulsification capacity for forming and stabilizing emulsions. As surface active molecules, biosurfactants can form micelles at the interface of immiscible liquids by either reduction of surface and interfacial tension, or form stable emulsions between immiscible liquids (De Sousa and Bhosle 2012). The former, which lowered surface and interfacial tensions, proved to be low-molecular-weight biosurfactants while the latter, which stabilized oil-in-water emulsions were more commonly high-molecular-weight ones and were referred as bioemulsifiers (Rosenberg and Ron 1999). The amphipathic biosurfactants produced by isolate CX3 have limited emulsification potentials and are recognized as low-molecular-weight compounds.

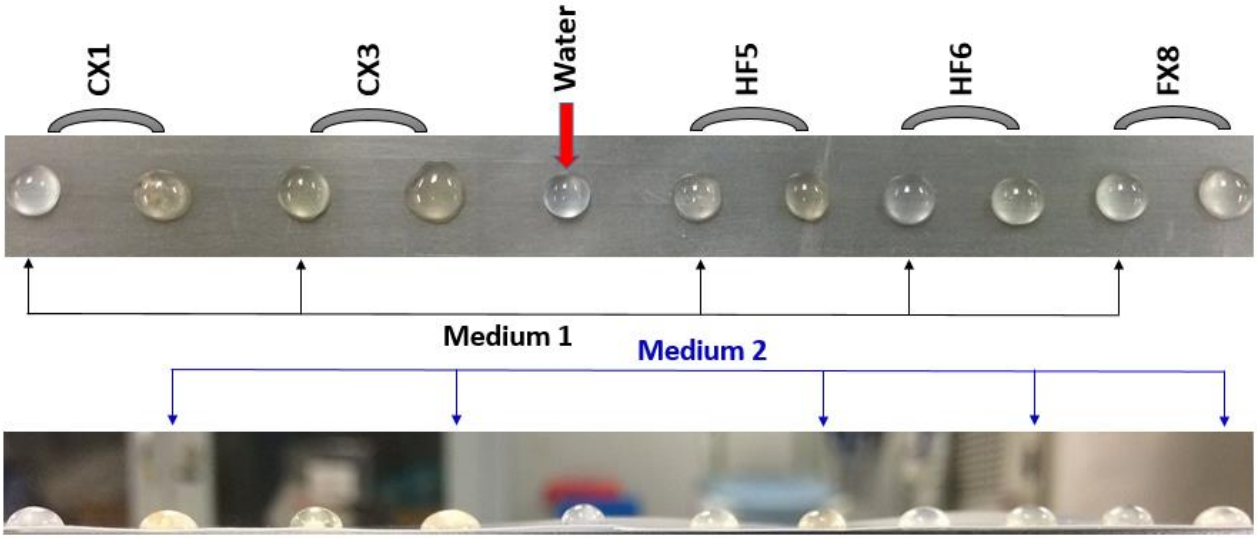
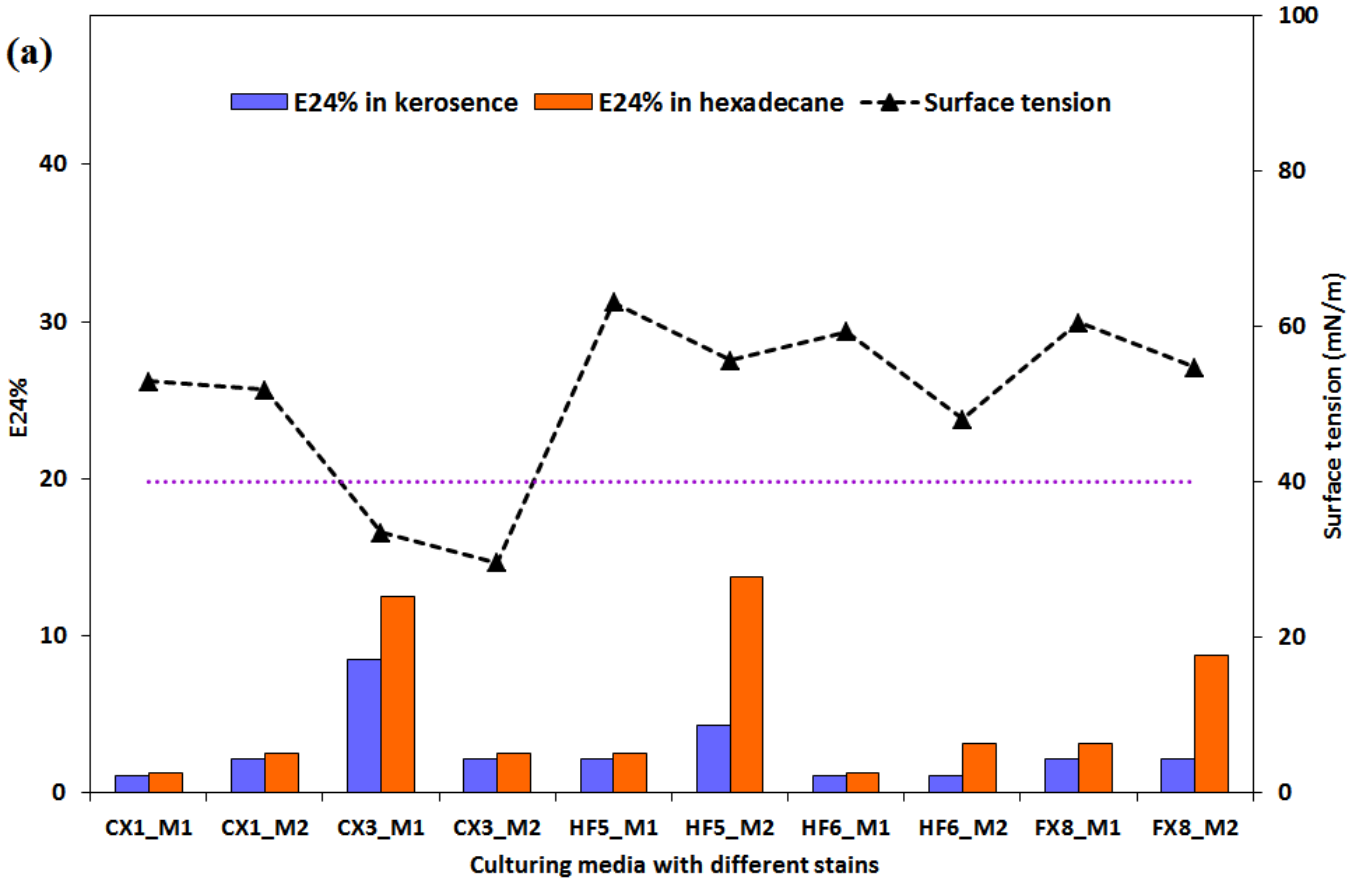


Figure 5.2 Secretion of amphipathic biosurfactants reduce the surface tension of the culture supernatant as indicated by the degree of flatness of droplet on parafilm.


(a)
E24% in kerosence
E24% in hexadecane
Surface tension
E24%
Surface tension (mN/m)
0
10
20
30
40
0
20
40
60
80
100
CX1_M1
CX1_M2
CX3_M1
CX3_M2
HF5_M1
HF5_M2
HF6_M1
HF6_M2
FX8_M1
FX8_M2
Culturing media with different stains

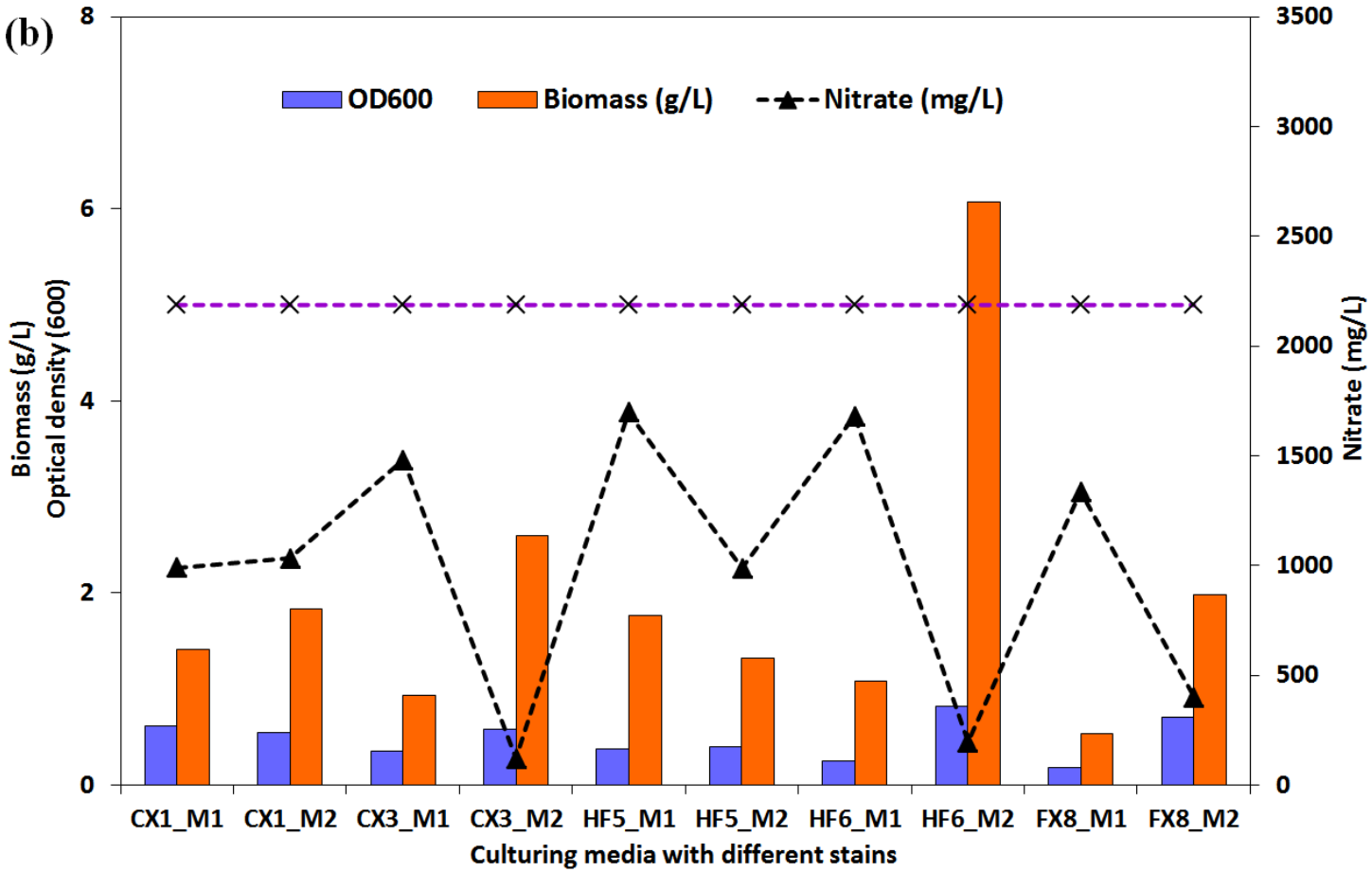


Figure 5.3 Evaluation parameters of the five *Pseudomonas stutzeri* strains in two growth media. Surface tension and E24% in kerosene and hexadecane were indicated in 2a while Biomass, OD_{600} and nitrate consumption were indicated in 2b. All the tests were conducted in duplicate and the results were expressed as the average of two measurements. The analytical errors of the methodologies on all the parameters were below 7% and the accuracy was verified through 7 consecutive measurements of one sample.

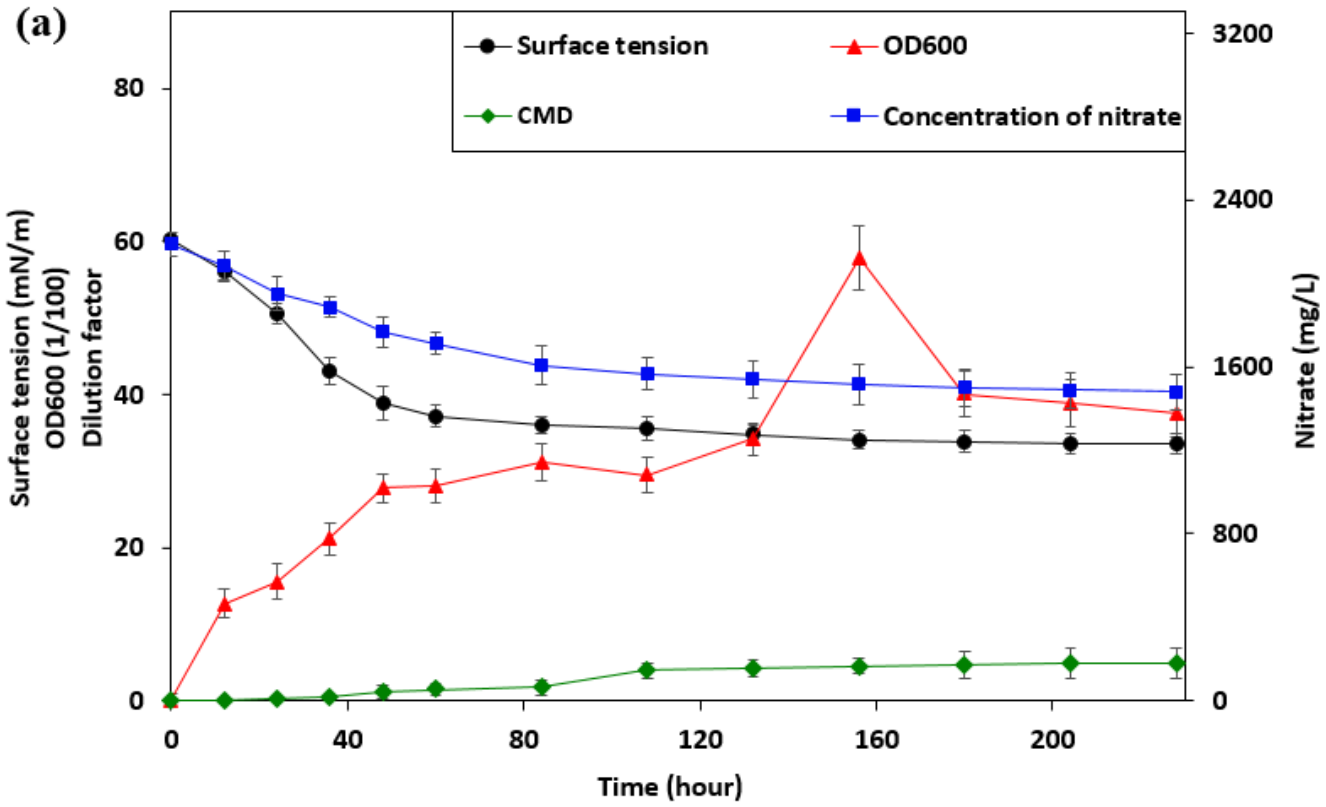
The biomass production of the selected isolates and their nitrate consumption were also summarized in Figure 5.3. The OD_{600} of broth samples was also used to evaluate the bacterial growth. As indicated by Figure 5.3b, the OD_{600} values generally accorded with the biomass results, which was the measure of dry weight of the bacterial cells (granular, flaky or flocculent) in the medium. Four of five strains consumed dramatically more nitrate when cultured on the producing medium 2, and among them CX3, HF6 and FX8 utilized 94.5%, 81.7% and 91.1% of the medium nitrate (the dotted line in Figure 5.3b indicates the initial nitrate concentration) and yielded 2.6, 2.0 and 6.1g/L biomass by dry weight, respectively. This noticeable nitrate intake by microbes was attributed to nitrate respiration as the nitrate was utilized by bacteria as a terminal electron acceptor to maintain the redox balance (nitrate dissimilation) and as a nutrient (nitrate assimilation) (Romeo et al. 2012). Compared with other isolates, the extra nitrate intake in these three strains contributed to large bacterial growth while nitrate served as a nutrient (not electron acceptor) in nitrate assimilation in all media.

5.3.3 Performance demonstration of the selected isolate

From the phylogenetic results and the morphological characters of isolates, *P. stutzeri* CX3 exhibited differential performance when compared with other strains. Only *P. stutzeri* CX3, whose 16S rRNA sequence was deposited in Genbank under the accession number KY860630, showed significant surface-tension-lowering ability on the two biosurfactant producing media. As shown in Figure 5.4a, the surface tension in culture broth gradually decreased to a plateau of around 34.0 mN/m, while the nitrate concentration gradually decreased to a plateau of around 1500 mg/L. The bacteria grew very fast during the initial 48 hours without a lag phase at a growth rate of 5.8×10^{-3} Abs/h and reached their stationary phase after around 84 hours. The lack of adaption time in the bacterial growth may be due to the fact that NRB were pre-incubated for 48 h in the glucose

medium. However, only 32.4% of the total nitrate in the medium was consumed over the study period.

CX3 grown on producing medium 2 grew more vigorously and a final nitrate consumption rate of 94.5% reached after 228 hours of incubation. From Figure 5.4b, it can be seen that the log phase of NRB growth was extended to 84 hours and the growth rate increased to 8.3×10^{-3} Abs/h compared with its growth behavior in glucose medium. During the initial 84 hours, 88.3% of nitrate was consumed at a rate of 23.0 mg/(L•h) and the biomass reached 0.702 Abs on glycerol medium. In contrast, only 26.6% of nitrate was consumed at a rate of 7.4 mg/(L•h) when CX3 grew on glucose medium. Low accumulation rates of biosurfactants (0.023 and 0.049 CMC/h on medium 1 and 2, respectively) were observed on both of the two mediums within 60 hours when the nutrients of nitrate and substrates were mainly assimilated into body cells. However, the production rate of biosurfactants increased to 0.65 CMC/h between the time range of 60 h and 156 h on medium 2 although a low rate of 0.032 CMC/h was observed on medium 1 during this period. Interestingly, the results indicated that high-speed yield of biosurfactants starts from the last stage of high rate of biomass accumulation and nitrate consumption (60 h). The surface tension of the CX3 culture broth was finally lowered to 29.6 mN/m. Zhao et al. (2014) constructed rhamnolipid-producing recombinant strain *Pseudomonas stutzeri* Rhl by cloning the rhamnosyltransferase gene rhlABRI from *Pseudomonas aeruginosa* SQ6 into a facultative anaerobic denitrifying bacterial strain *Pseudomonas stutzeri* DQ1. They utilized glycerol as carbon source for *Pseudomonas stutzeri* Rhl and similar surface tension (30.6 mN /m) was obtained through anaerobically produced rhamnolipid.


(a)
Surface tension
OD600
CMD
Concentration of nitrate
Surface tension (mN/m)
OD600 (1/100)
Dilution factor
Nitrate (mg/L)
Time (hour)
0
20
40
60
80
0
800
1600
2400
3200
0
40
80
120
160
200

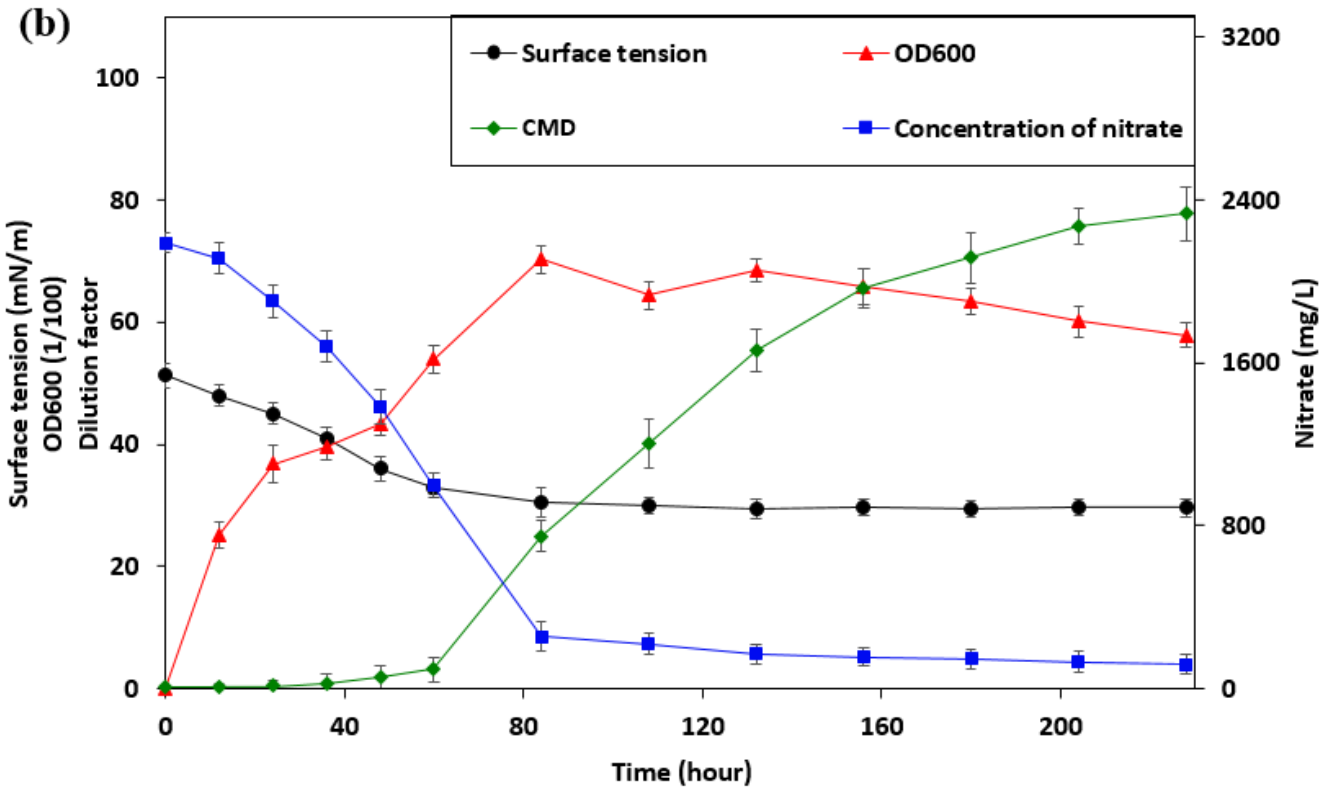


Figure 5.4 The changes of surface tension, cell growth and nitrate consumption of the *Pseudomonas stutzeri* CX3 on glucose (3a) and glycerol (3b) medium versus the incubation time

5.3.4 Characterization of NRB-generated biosurfactant product

After the extraction, the final concentrated biosurfactant product by *P. stutzeri* CX3 was with a yellow-brown colour. It could lower the surface tension of distilled water from 72.2 mN/m to 30.4 mN/m. The biosurfactant product was further characterized and the stability under various environmental conditions was also examined to facilitate its potential use in the fields.

5.3.4.1 CMC of biosurfactants

CMC is an important indicator which determines the capability of biosurfactants to mobilize crude oil from contaminated soils or the sand-oil mixture into the aqueous biosurfactant solution. Low CMC is correlated with high efficiency of the biosurfactant product (Bharali et al. 2014). The CMC value of the isolated product was determined by measuring the surface tension of different concentrated solutions of the product with a tensiometer, and a sudden change in the surface tension was observed. A lower CMC value of 35 mg/L was derived for the extracted biosurfactant product. The biosurfactant product proved to be highly efficient when compared with the typical CMC values (10-230 mg/L) previously reported for biosurfactants produced from different microbial sources (Gogoi et al. 2016; Nitschke et al. 2005).

5.3.4.2 Preliminary TLC analysis

The lipid content in the biosurfactant product was preliminarily determined through the general staining of iodine. TLC analysis also showed that light red spot on silica gel plates was generated when using ninhydrin as color developing reagent, suggesting that only a small part of lipopeptide existed in this biosurfactant. In contrast, the evident yellow spot proved the abundant presence of

glycolipid. Zhao et al. (2014) constructed an engineered strain *Pseudomonas stutzeri Rhl* and used it for heterologous production of Rhamnolipid under anaerobic conditions. Accordingly, the main components of glycolipid biosurfactants were most probable to be rhamnolipids. All the results indicated that the product was very likely to be a mixture of a small part of lipopeptides and a large part of glycolipid biosurfactants.

5.3.4.3 Lipid and fatty acid analysis

TLC-FID analysis revealed that this biosurfactant product was a mixture of seven lipid components, which was dominated by the acetone mobile polar lipids and phospholipids. As shown in Table 5.2, 46.7% of the components were acetone mobile polar lipids (mainly glycolipids), accounting for nearly half the total lipids. Phospholipids, as the most abundant lipids in cell membranes and dominating constituent in our lipids matrix (31.9 %), may be partially originated from cell debris co-precipitated with the biosurfactant product during the extraction process. The fact could also be inferred from the presence of small amounts of sterols in lipids (1%) as cholesterol is an important structural component of a phospholipid bilayer (Van Der Paal et al. 2017). Additionally, hydrocarbons, triacylglycerols and free fatty acids were also found in various amounts in the lipid mixture.

Table 5.2 The lipid components determined by TLC-FID and fatty acid profiles by GC-FID of the biosurfactant product

Components	Composition (%)		
Hydrocarbons	7.3		
Ethyl Ketones	0.7		
Triacylglycerols	4.7		
Free Fatty Acids	7.8		
Sterols	1.0		
Acetone Mobile Polar Lipids	46.7		
Phospholipids	31.9		
Fatty acids	Composition (%)	Fattay acids	Composition (%)
C14:0	2.3	C17:0	0.3
i-C15:0	2.0	C17:1	3.3
a-C15:0	13.8	C18:0	1.4
C15:0	0.2	C18:1Δ7	0.1
i-C16:0	5.5	C18:1Δ9	1.0
C16:0	15.8	C18:1Δ11	20.2
C16:1Δ5	0.3	C18:1Δ12	0.2
C16:1Δ9	11.7	C18:1Δ13	0.2
C16:1Δ11	11.2	C18:2Δ12	1.2
i-C17:0	1.7	C18:3Δ15	1.9
a-C17:0	5.3	C20:1Δ11	0.1
phytanic acid	0.2	C22:0	0.02
Saturated	20.2	PUFA	3.1
MUFA	48.5	Bacterial	32.2

Further fatty acid profiles (Table 5.2) revealed that the fatty acids were mainly monounsaturated fatty acids (MUFA) and bacterial fatty acids (48.5% and 32.2%, respectively). Polyunsaturated fatty acids (PUFA) were found in very limited amount, accounting for only 3.1% of the total. The three most abundant fatty acids from the complex matrix were C18:1w7, C16:0 and a-C15:0, whereas the long-chain acids C16 and C18 contributed up to 70.9% of the total. This phenomenon can be attributed to their metabolic pathway during which the fatty acids were biosynthesized from the stepwise addition of two-carbon units derived from the building-block acetyl-coenzyme A (CoA) to a growing chain (Estelmann et al. 2011). Similar results of fatty acid composition were obtained by Morita et al. (2007) and biosynthesis pathway is more relevant to the fatty acid compositions of products than the variations in carbon source under current situations.

5.3.4.4 FT-IR analysis

The molecular composition of the biosurfactant product generated by *P. stutzeri* CX3 was evaluated by FT-IR and the results were shown in Figure 5.5. The broad absorbance peak centered around 3298 cm^{-1} indicated the presence of stretching OH bonds and N-H bonds of protein. Absorption around wave numbers 2939.08 and 2885.84 cm^{-1} were assigned to the symmetric C–H stretches of $–CH_2$ and $–CH_3$ groups of aliphatic chains. The aliphatic chains were also reflected from bending vibrations at 1411.19 cm^{-1}. We also observed the protein-related bands the –C=O amide I (1632.14 cm^{-1}) and –NH/–C=O combination of the amide II bands (1536.67 cm^{-1}). However, just like the presence of phospholipids from lipid analysis results, it might be possible that the two bands at 1632.14 cm^{-1} and 1536.67 cm^{-1} were resulted from polypeptides originated from cell debris co-precipitated with the biosurfactant product during extraction process (Lotfabad et al. 2009). The absorption peaks of 1035.63 and 1107.81 cm^{-1} were expected to be stretching C–

O–C bonds, which indicated the presence of polysaccharide or polysaccharide-like substances in the biosurfactant product. The FTIR spectra provided strong evidence for the presence of glycolipids and lipopeptides in the biosurfactant product (Aparna et al. 2012; Joshi et al. 2008).

5.3.4.5 Stability studies

The applicability of biosurfactants in several fields is highly associated with their stability under various environmental conditions. Therefore, after the generation of biosurfactant product by *P. stutzeri* CX3, its stability was tested under a wide range of temperature, pH value and salinity while maintaining the biosurfactant concentrations at CMC value (35 mg/L). From the results shown in Figure 5.6, the biosurfactant activity was retained and insignificantly affected by most of the experimental settings, especially only small variations in surface tension values were determined under the wide range of temperatures between 0 and 121 °C and salinities between 0% and 20%. Biosurfactants are widely applied in petroleum, pharmaceutical, health care and food processing industries (Abouseoud et al. 2008a; Khopade et al. 2012) due to its robust stability and the findings indicated the potential use of our product in these areas. As indicated in Figure 5.6, biosurfactant solution achieved the lowest surface tension at a temperature around 40 °C, whereas heating up to 100 °C or even autoclaving (121 °C) caused no significant effect on its thermal stability. The results are accorded with the properties of rhamnolipid indicated by Abdel-Mawgoud et al. (2009), which are main components of the biosurfactant product and maintain stable structure over the temperature settings. The extreme thermal stability was reported by Seghal Kiran et al. (2010) from *Brevibacterium aureum* MSA13 and Aparna et al. (2012) from *Pseudomonas sp.* 2B, which enables the biosurfactant product to be applied in a high-temperature reservoir.

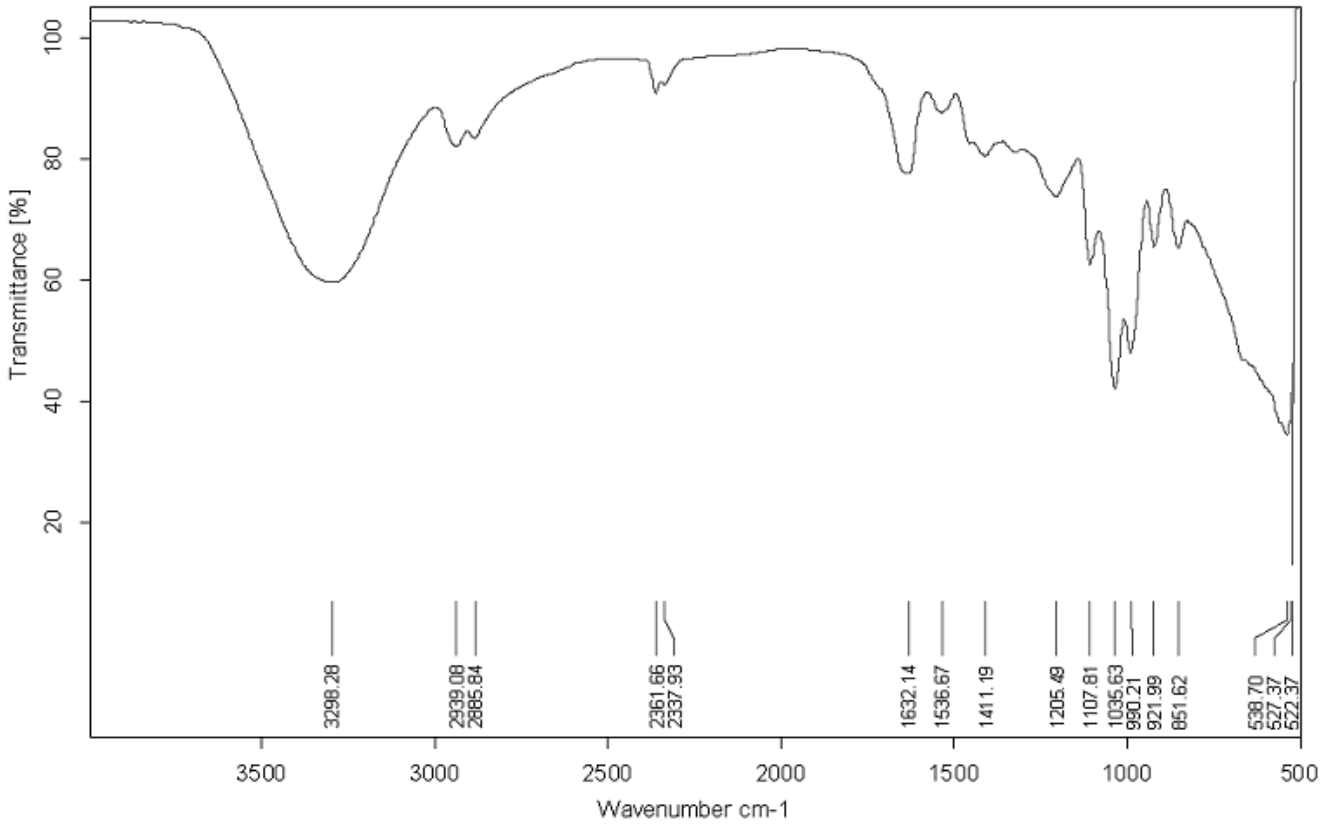
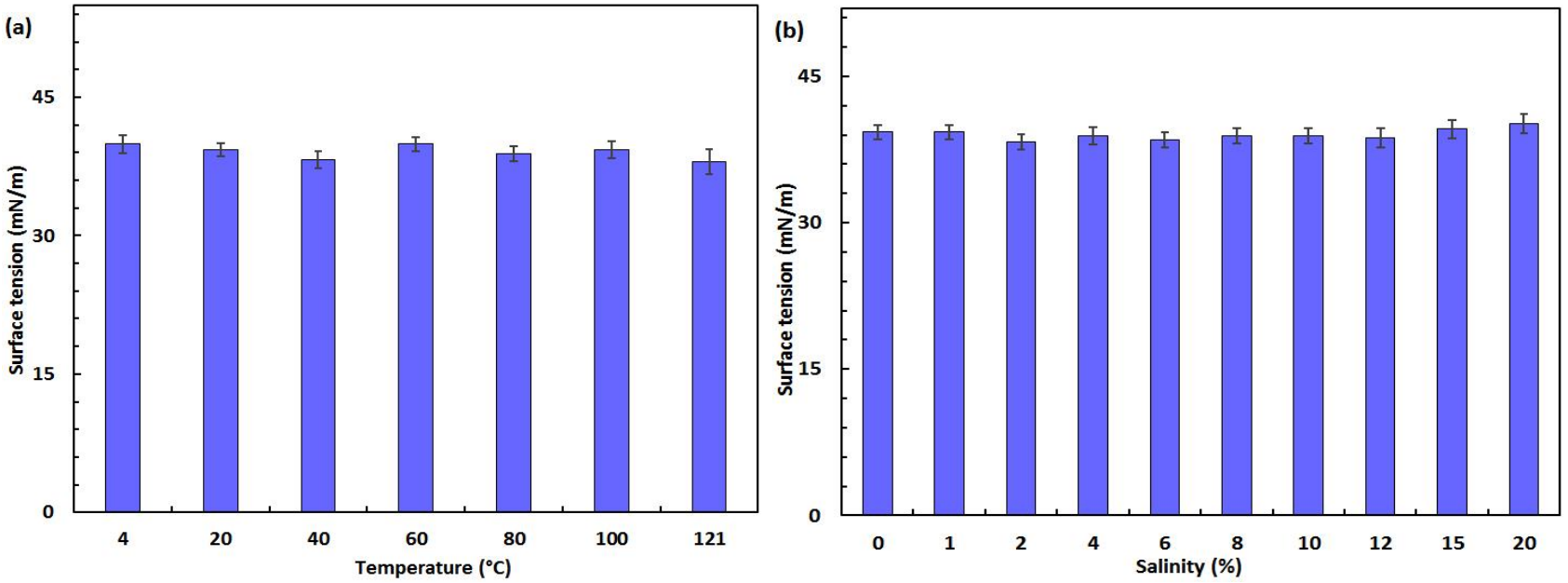


Figure 5.5 FT-IR transmittance spectrum of the extracted biosurfactant product generated by *Pseudomonas stutzeri* CX3 grown on glycerol medium


(a)
Surface tension (mN/m)
0
15
30
45
4
20
40
60
80
100
121
Temperature (°C)
(b)
Surface tension (mN/m)
0
15
30
45
0
1
2
4
6
8
10
12
15
20
Salinity (%)

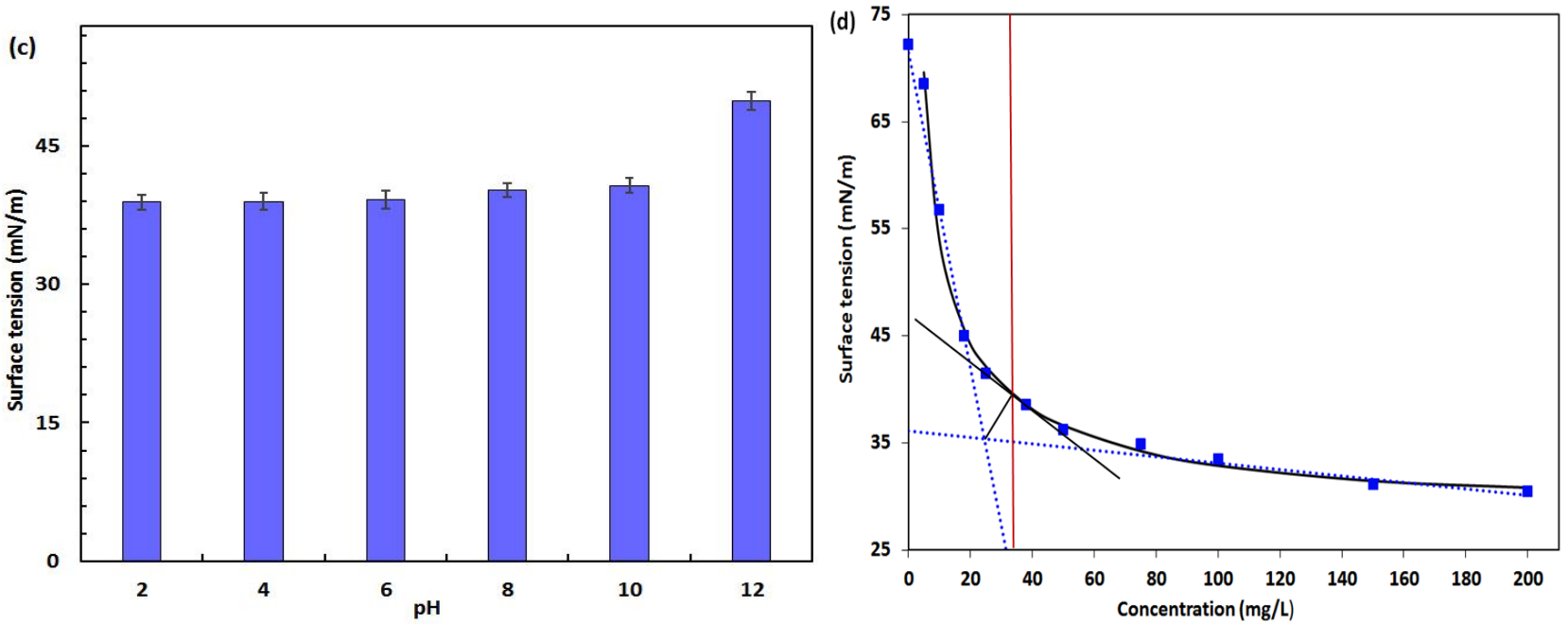


Figure 5.6 Stability of the biosurfactant product produced by *Pseudomonas stutzeri* CX3 under various environmental conditions. (a) Temperature; (b) Salinity; c) pH; (d) Critical micelle concentration (CMC) of the biosurfactant product at 35 mg/L

The effect of salinity on the biosurfactant activity was examined and negligible changes were observed in the increased concentration of NaCl up to 20% (w/v) (Figure 5.6b). Electrolytes in the bulk solutions will shield the carboxylate groups of the rhamnolipid molecules, causing them to behave more like nonionic than anionic surfactants (Helvaci et al. 2004). Although a relatively lower surface tension was obtained at 2% NaCl concentration, the differences over the wide brine concentration levels of 0 to 20% were not significant (<5%). The steady performance of surface tension in salty waters allows the product to be potentially used in reservoir MEOR or environmental bioremediations under saline environments. Singh and Tripathi (2013) isolated a strain of *Pseudomonas stutzeri* from the formation water of an Indian coalbed and the isolate produced copious amount of biosurfactant with the supplementation of coal. The biosurfactant with rhamnolipid nature showed considerable emulsifying ability and great potential for *in situ* biotransformation of coal into methane and bioremediation of PAHs from the oil-contaminated sites. Correspondingly, our product with considerable surface-tension-lowering ability will reduce the capillary force that holds oil and porous solid media together to mobilize the crude oil or oil in MEOR or environmental bioremediation activities.

The surface activity of biosurfactant solution remained relatively stable between pH 2 and 10. A negative biosurfactant performance was observed at pH 12 in which the surface tension raised to 49.9 mN/m. This detrimental effect is possibly caused by structure alteration of the biosurfactant product under extreme pH conditions. The polar head of anionic rhamnolipids was more negatively charged under more alkaline conditions (Silva et al. 2010). This is reflected by the fact of increased solubility of the product.

As amphiphilic molecules released extracellularly by microorganisms, biosurfactants are known to be beneficial for their producers in various ways. By promoting wetting, solubilization and

emulsification of various types of organics, biosurfactants could increase the surface area between the oil and water phases, thereby increasing the bioavailability of entrapped oil in the porous media (Pacwa-Płociniczak et al. 2011). Thus, biosurfactants could improve the nutrient conditions of NRB producers and enhance the competence of NRB over SRB. Additionally, several biosurfactants proved to have antibiotic effects (Rodrigues et al. 2006), which may have the potential to inhibit the growth of SRB. Moreover, biosurfactants were found to be important agents in the connection between microbial communities and biofilm formations (Osterreicher-Ravid et al. 2000), which may synergistically improve the resistance of the NRB producers to harsh environment. Therefore, the generated biosurfactant product, once injected into the soured reservoir externally, has great potential to assist NRB outcompeted SRB through these mechanisms. The effects of NRB produced biosurfactants in offshore reservoir NRB/SRB interactions, coupled with other environmental implications of this product, need to be further investigated.

5.4 Summary

In this study, the offshore petroleum-reservoir brines following nitrate/nitrite injection were used for the anaerobic screening of biosurfactant producing NRB. After periodic enrichment and sophisticated screening of the microorganisms, five typical denitrifying strains were isolated and found to be the species of *Pseudomonas stutzeri* according to their 16S rRNA sequencing results. The isolates were further screened for possible biosurfactant producers on glycerol and glucose media. The strain *P. stutzeri* CX3 was confirmed with biosurfactant production capacity through a series of biosurfactant characterization tests (e.g. drop collapsing test, parafilm test and surfaced tension determination). Better surface tension lowering ability was observed from the glycerol

medium and the consumption of nitrate by NRB was found in positive correlation with bacterial growth and surface tension reduction. CX3 was selected to further demonstrate the performance of biosurfactant production through two production media over a 228-hour monitoring. The nitrate concentrations and surface tensions on the two media were both reduced to a relatively stable level within 84 hours during which OD_{600} reached relatively high levels as well over the period. The biosurfactant product generated by *P. stutzeri* CX3 was defined with a CMC as low as 35 mg/L, and further characterized by TLC, GC-FID and FT-IR analysis. The main components of the biosurfactant were recognized as glycolipids. The biosurfactant product demonstrated stable performance during different environmental conditions with a wide range of temperature, pH values, and salinity, which indicated its potential applications in environmental bioremediation, petroleum and other various industrial fields. The successful isolation and identification of biosurfactant producing NRB from laborious screenings on offshore reservoir samples, coupled with subsequent biosurfactant characterization would provide new insight into NRB-SRB interactions for offshore reservoir souring control investigations.

CHAPTER 6

INTERACTIONS OF SRB, NRB SCREENED FROM OFFSHORE OIL RESERVOIR AND NRB PRODUCED BIOSURFACTANTS IN MICROCOSMS[4]

[4] *The contents in the chapter will result in the potential publication:*

F. Fan, B. Zhang, J. Liu, Q. Cai, W. Lin, B. Chen. Interactions of sulfate reducing bacteria (SRB), biosurfactant producing nitrate reducing bacteria (NRB) screened from an offshore reservoir and NRB produced biosurfactant in microcosms (submitted to the journal of Environmental Pollution)

Role: Fuqiang Fan solely worked on this study and acted as the first author of this manuscript. Dr. Baiyu Zhang is my supervisor. Dr. Bing Chen helped guide the research and polish the manuscript. Jibin Liu, Qinhong Cai and Weiyun Lin participated in conducting experiments.

6.1 Background

Oilfield reservoir souring caused by SRB is among the major issues faced by the petroleum industry, which occurred both in terrestrial and offshore oil production operations (Gieg et al. 2011; Voordouw et al. 2009). The injection of water in secondary oil recovery to maintain pressure and press the oil towards the production wells (Hook et al. 2014) often leads to the production of sulfide (souring) by resident SRB or other sulfidogenic bacteria. The sulfide, as a byproduct of SRB in respiration, is closely correlated with the deterioration of crude oil quality, health and safety issues, and increased corrosion risk and operating cost (Hubert and Voordouw 2007; Zhao et al. 2009). Hydrogen sulfide was also reported to inhibit the growth and metabolism of biosurfactant producing bacteria, thus exerting negative effects on MEOR (Zhao et al. 2015a).

Nitrate injection is a promising strategy for souring control due to its relatively non-toxic and cost-effective attributes and its convenience in practical distribution (Gieg et al. 2011; Xue and Voordouw 2015). The mechanism of nitrate injection includes the boost of hNRB to outcompete SRB for available nutrients (biocompetitive exclusion), the promotion of NR-SOB to directly oxidize sulfide and the SRB repression through resultant nitrite (Fida et al. 2016; Gieg et al. 2011; Hubert and Voordouw 2007). Nitrate-mediated souring control has been extensively studied in the laboratory (Callbeck et al. 2011; Chen et al. 2017; Zhao et al. 2009) and in the field (Bodtker et al. 2008; Shartau et al. 2010; Voordouw et al. 2009). The studies mainly focused on improving nitrate/nitrite inhibitory effect on SRB-induced sulfide production through varying environmental factors (An et al. 2017; Chen et al. 2017; Gadekar et al. 2006; Hubert et al. 2003; Okpala et al. 2017) or combination with other protocols (Greene et al. 2006; Nemati et al. 2001; Xue and Voordouw 2015) in the complicated matrix. Research interests of biological investigations were also mainly placed on tracing and characterizing microbial communities under complicated

biological conditions (Callbeck et al. 2011; Gieg et al. 2011; Kamarisima et al. 2018; Ontiveros-Valencia et al. 2012; Zhao et al. 2009). These studies have significantly enlightened our understanding towards effective control of reservoir souring, different NRB-SRB interaction patterns and the associated microbial community changes under various environments. However, mechanism-based studies towards specific interactions of individual NRB and SRB strains were rarely reported. Currently, much still is unknown about the detailed microbial mechanisms involved in NRB-SRB interactions during nitrate/nitrite injections for reservoir souring mitigation.

Our previous studies have proved anaerobic indigenous NRB screened from an offshore reservoir are biosurfactant producers (Fan et al. 2018). As amphiphilic molecules produced extracellularly by certain strains, biosurfactants can enhance the competence among species by increasing the bioavailability of entrapped organics in the porous media (Pacwa-Płociniczak et al. 2011). The specific biosurfactants might also repress the growth of certain targeted strain through their antimicrobial properties (Rodrigues et al. 2006). Besides, as possible combination agents in biofilm matrix formation (Osterreicher-Ravid et al. 2000), biosurfactants could synergistically improve the bacterial adaptation capability to harsh environments. Therefore, the NRB produced biosurfactants might be unique and promising agents involved in NRB-SRB interaction, which have great potential in assisting NRB to outcompete harmful SRB in reservoir environments. However, marine biosurfactant producers are relatively rarely explored (Antoniou et al. 2015; Cai et al. 2014), during which anaerobic biosurfactant producers screened from marine reservoir conditions were very limited (Domingues et al. 2017; Zhao et al. 2015b). Reports regarding biosurfactant producing NRB in marine environments are extremely limited (Zhao et al. 2016) and, to our knowledge, there has been no published study tackling the systematic investigation of NRB-SRB interactions with the involvement of biosurfactants produced by natural NRB.

In this work, NRB strain screened from a soured offshore reservoir was used to produce biosurfactants and the biosurfactants involved NRB-SRB competition mechanism was examined through multiple nitrate addition experiments in microcosm configurations. PLFA biomarkers were used to trace and signify SRB and NRB community responses.

6.2 Materials and Methods

6.2.1 Media and enrichment cultures

The NRB strain *Pseudomonas stutzeri* CX3 (Genbank accession number KY860630) (Fan et al. 2018), a natural biosurfactant producer screened from produced water of an offshore reservoir at a depth of 3200~3700 m below the sea floor (Okpala et al. 2017), was utilized for inhibition of SRB and removal of sulfide simultaneously. The SRB strain *Desulfomicrobium escambiense* ATCC 51164, which commonly exist in oil reservoirs (Gieg et al. 2011; Zapata-Peñasco et al. 2016; Zapata-Peñasco et al. 2013), was purchased from Leibniz Institute DSMZ (German Collection of Microorganisms and Cell Cultures GmbH). Routine growth and maintenance of *P. stutzeri* CX3 was conducted on a NRB medium containing NaCl, 7 g; KNO_3, 3 g; $MgSO_4 \cdot 7H_2O$, 0.68 g; $CaCl_2 \cdot 2H_2O$, 0.24 g; NH_4Cl, 0.02 g; KH_2PO_4, 0.027 g; $NaHCO_3$, 1.9 g; sodium acetate, 1.36 g; and trisodium citrate, 1.6 g. The biosurfactant (a mixture of glycolipid and lipopeptide) production of *P. stutzeri* CX3 followed a protocol described by Zhao et al. (2014). The *D. escambiense* was cultivated and maintained on a SRB growth medium containing Na_2SO_4, 4.5 g; NaCl, 5 g; KCl, 1.5 g; $MgCl \cdot 6H_2O$, 1.2 g; $MgSO_4 \cdot 7H_2O$, 0.3 g; $CaCl_2 \cdot 2H_2O$, 0.042 g; KH_2PO_4, 0.03 g; NH_4Cl, 0.6 g; sodium citrate, 3 g; sodium acetate, 2.04 g; sodium lactate 0.8 g; sodium propionate 2 g; yeast extract 1 g; and selenite-tungstate solution (Tabuchi et al. 2010), 1 mL. The pH level of all media

was between 7.2 and 7.5. The media were further treated with sterilized $Na_2S \cdot 9H_2O$. Trace metals were added to all media following the trace element receipt in CSB medium (Tang et al. 2010).

6.2.2 Experimental setup

Biosurfactant-involved NRB-SRB interaction was investigated through two rounds of experiments, which corresponded with non-sour (Round 1) and sour (Round 2) conditions.

To examine the NRB-SRB interaction patterns under various nitrate and/or biosurfactant conditions, the first round of experiments was conducted on the SRB growth medium. NRB and SRB strains were both inoculated at Time 0 in 150 mL Erlenmeyer flasks. Runs 1-4, 6 and 8 without biosurfactant addition were prepared in which KNO_3 was supplemented at concentrations of 0, 0.2, 0.5, 1, 3, and 5 g/L, respectively. In Runs 5 and 7, the KNO_3 concentrations were 1 and 3 g/L, respectively, while biosurfactants produced by NRB were added at 0.1 g/L to both flasks. The detailed setting of each run in Round 1 was listed in Table 6.1. The strains grew in sealed flasks at a shaking rate of 150 rpm. Media samples were collected on Day 5 and Day 11, respectively. Parameters including redox potential, pH, concentrations of sulfide, nitrate and microbial PLFA were determined to signify the effect of SRB growth inhibition.

To further examine the anti-souring effect of nitrate and biosurfactant treatment, the second round of experiments was carried out in 4 sealed 1000 mL Erlenmeyer flasks at 150 rpm.

Table 6.1 Experimental setting of the first round investigation of biosurfactant-involved NRB-SRB interaction

Run number	1	2	3	4	5	6	7	8
KNO_3 (mg/L)	0	0.2	0.5	1	1	3	3	5
Biosurfactants (mg/L)	0	0	0	0	0.2	0	0.2	0

SRB strain was inoculated and cultivated for 15 days beforehand to yield a mature SRB culture. At Time 0, 1 g Na_2SO_4, 1.5 g sodium citrate and KNO_3 of 1, 3, and 5 g, respectively, were added into the mature SRB culture (Runs A, B and D). Biosurfactants of 0.1 g were added in an extra run (Run C, 3g KNO_3 added) and the detailed setting of each run in Round 2 was listed in Table 6.2. The seed culture of the NRB was inoculated in all runs at Time 0. The sampling was conducted on odd days as well as on Days 6, 8 and 10. Parameters including redox potential, pH, concentrations of sulfide, sulfate, nitrate, nitrite, dissolved organic carbon (DOC), dissolved inorganic carbon (DIC) and total dissolved carbon (TDC) in NRB-SRB interactions were monitored. All the microcosm experiments were conducted under anaerobic conditions.

6.2.3 Chemical and physical analyses

Concentrations of sulfide and nitrite in all media samples were determined colorimetrically with N,Ndimethyl-p-phenylenediamine and sulfanilamide/n-(naphthyl)-ethylenediamine reagent, respectively (Bridgewater 2012). Concentration of sulfate was assayed using a Dionex ion chromatography (ICS-2100) system (Dionex Corporation, Sunnyvale, CA, USA). DOC, DIC and TDC were analyzed using a high-temperature combustion total organic carbon analyzer (Shimadzu Total Organic Carbon Analyzer (TOC-L)) (Anumol et al. 2015). The determination of nitrate followed a two-wavelength approach described by Zhang et al. (2011b) Redox potential (Eh) was measured using water proof ORPTestr 10 with a platinum band electrode. The pH measurements of the water samples were conducted using a benchtop pH meter (Mettler Toledo). All the measurements of sulfide, nitrite, nitrate, Eh and pH were carried out in triplicate and values were presented as mean ± standard deviation. The analyses of other parameters were performed in duplicate and the associated statistical analyses agreed to within 95% confidence.

Table 6.2 Experimental setting of the second round investigation of biosurfactant-involved NRB-SRB interaction

Run number	A	B	C	D
Na_2SO_4 (mg/L)	1	1	1	1
Sodium citrate (mg/L)	1.5	1.5	1.5	1.5
KNO_3 (mg/L)	1	3	3	5
Biosurfactants (mg/L)	0	0	0.2	0

6.2.4 Microbial PLFA analysis

PLFA analysis was used to profile SRB strain cultivated on SRB medium, NRB strain cultivated on NRB medium, and the microbial community structure during the first round of experiments. PLFA profiling was conducted following a modified Bligh and Dyer extraction method (Fan et al. 2017a). Phospholipid standard C19:0 PC (1,2-dinonadecanoyl-sn-glycero-3-phosphocholine; Avanti Polar Lipids) was used for determining phospholipid recovery. Internal standards C14:1 (cis-9) and C21:0 were spiked before GC-MS analysis (Ziegler et al. 2013). FAMEs were determined from five standards: Bacterial Acid Methyl Esters CP Mixture, FIM-FAME-7 Mixture, 10Me C16:0, and C16:1 (trans-9) from Matreya LLC (Pleasant Gap, Pennsylvania, USA); as well as C18:1 (trans-11) from Sigma-Aldrich (Oakville, Ontario, Canada). PCA was conducted to compare PLFA composition of different settings and illustrate their correlations. The trans/cis ratios of C16:1Δ9 and C18:1Δ11, cyclo/precursor ratios including cy-C17:0/C16:1 (cis-9) and (cy-C17:0 + cy-C19:0)/(C16:1 (cis-9) + C18:1 (cis-11)) were used as indicators of physiological or nutritional stress in bacterial communities (Moore-Kucera and Dick 2008; Wixon and Balser 2013). Triplicate measurements coupled with blank control were performed to demonstrate analytical accuracy and values were presented as mean ± standard deviation.

6.3 Results and Discussion

6.3.1 NRB-SRB interactions under various nitrate conditions

The first round of experiments were performed to determine the effects of NRB and/or biosurfactant addition on SRB growth inhibitory under various nitrate/sulfate loadings, and the associated bacterial responses. In this round, the NRB and SRB strains were inoculated in the system simultaneously, and then incubated for 11 days. Figure 6.1 presents the values of redox

potential, pH, sulfide and nitrate concentrations from samples collected on Day 5 and Day 11 in all 8 runs during the competitive NRB-SRB interactions. As shown in Figure 6.1, redox potentials in Runs 2-4 all reached below -300 mV after five days. Redox potentials in biosurfactant-added protocols (Runs 5 and 7) and Run 6 were reduced from initial -190 mV to below -400 mV. Meanwhile, the sulfide concentration was significantly increased in Runs 5-7 (26.0-113.9 mg/L). On Day 11, the redox potentials in nearly all the settings (except Run 5) rose back to above -204 mV. The lowest Eh value of -395 mV and the highest sulfide concentration (15.3 mg/L) were achieved in Run 5 on Day 11 comparing to all other runs, which indicated the environmental conditions could favorably support the growth of the SRB strain. Coincidentally, the lowest pH of 7.6 on Day 11 was also observed in Run 5, which implied the SRB growth might be inhibited by alkaline conditions in other runs. Similar to Run 5, Run 1 was also noted because of its relative lower redox potential (-204 mV) and pH value (7.8) on Day 11. Interestingly, although KNO_3 was added with various amounts (0.2- 5 g/L), the final nitrate concentrations in all Runs 2-7 reached a stable amount (~130 mg/L), resulting in diverse percentage of nitrate consumption (36.1-95.1%).

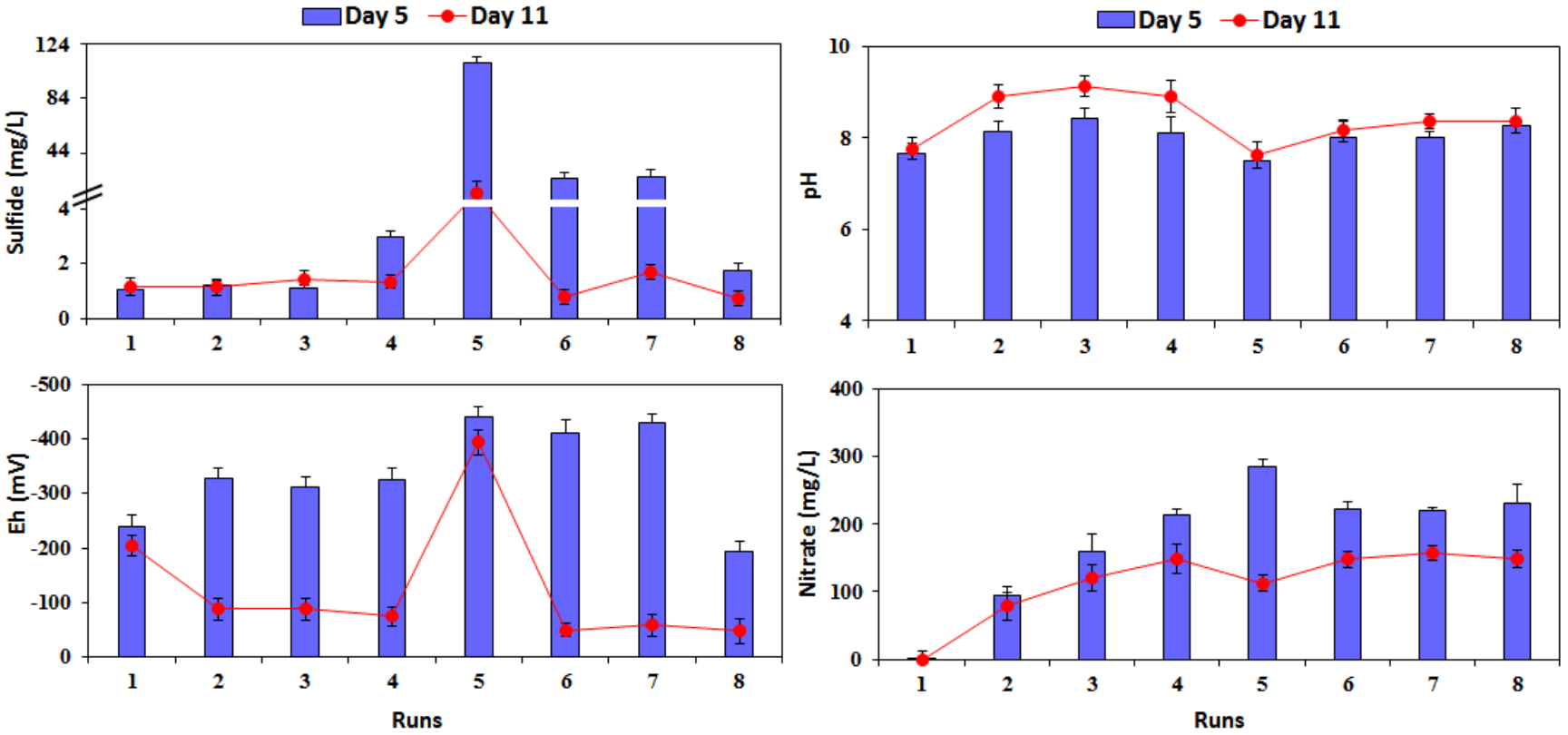


Figure 6.1 Redox potential, pH, sulfide and nitrate concentration changes in samples collected on Day 5 and Day 11 during the competitive interactions of SRB and NRB strains

The two strains (i.e., NRB *P. stutzeri* CX3 and SRB ATCC 51164) were cultivated on a sulfate-rich medium with sufficient carbon and other nutrients. The media should be more favorable to support the growth of SRB (Hubert et al. 2003; Hubert and Voordouw 2007). However, the NRB strain showed a shorter lag phase and outcompeted the SRB in nearly all runs (except Run 5), which indicated a higher adaptation ability of the NRB than the SRB strain.

The NRB in all runs except Run 5 outnumbered the SRB strain from the beginning and suppressed further massive sulfide production (< 30 mg/L). However, results from biosurfactant-involved Run 5 indicated the addition of biosurfactants promoted SRB growth under the condition of 1 mg/L KNO_3. Nitrate injection was previously reported to stimulate the proliferation of SRB under nitrogen-limiting conditions (Da Silva et al. 2014). Kamarisima et al. (2018) observed that as a stress response of nitrate addition, the SRB strain *Desulfotignum* YB01 could simultaneously reduce sulfate and nitrate, and generate biomass. In Run 5, the biosurfactant product stimulated both the SRB and NRB during the lag phase, and NRB failed to effectively inhibit the SRB growth. As the metabolic inhibitors nitrite was one of the main paths for the inhibition of SRB activities (Fida et al. 2016), insufficient nitrite produced from low nitrate concentrations was unable to inhibit SRB when the SRB population continued to reduce sulfate (Hubert and Voordouw 2007).

Moreover, although the identical amount of nitrate (1 mg/L KNO_3) was injected, a lower nitrate concentration (112.2 mg/L) was observed in Run 5 than Run 4 (148.6 mg/L) on Day 11 The biosurfactant addition increased the nitrate consumption. This implies that in a nitrogen-limiting environment, the biosurfactant product added could speed up the usage of nitrate as an alternative electron acceptor by a SRB strain (Marietou et al. 2009). Therefore, a higher nitrate concentration exceeding the stoichiometric nutrient biodegradation demands (e.g., Run 7), which will result in low

carbon/sulfate/sulfide to nitrate ratios in NRB-SRB interaction (An et al. 2010; Hubert et al. 2003), is recommended for effective souring control.

6.3.2 Analysis of microbial communities

6.3.2.1 Principal component analysis (PCA)

PLFA were used as biomarkers to identify individual microbial species, trace the community structure responses and describe changes in microbial metabolic fingerprints. The PLFA profiles of NRB grown on NRB medium (pure NRB), as well as SRB cultivated on SRB medium (pure SRB) were determined and shown in Figure 6.2. A total of 21 different PLFA biomarkers were identified from the cell membrane of the SRB strain, of which a-C15:0 (49.2%) and C16:0 (16.2%) dominated. In contrast, 16 fatty acids were observed from PLFA profiles of the NRB strain, of which C18:1 (cis-11) (50.5%) and C16:0 (19.3%) dominated. Major fatty acids identified in the NRB strain include C16:1 (cis-9) (10.4%) and cy-C19:0 (10.5%) as well. PCA of all the bacterial samples in Figure 6.3 indicated the correlation between the fatty acids and different samples. From Figure 6.3, the dominating a-C15:0 was one of characteristic PLFAs of pure SRB, and major fatty acids C16:1 (cis-9) and C18:1 (cis-11) were the representative components of pure NRB. Interestingly, fatty acids C16:1 (cis-9) and C18:1 (cis-11) are both closely correlated with the sulfide-oxidizing NRB strain, while their coexistence has been previously recognized as signature biomarkers for other sulfur-oxidizing bacteria in sulfide-rich marine sediment (Li et al. 2007b) and offshore reservoir injection water (Fan et al. 2017b). Typical SRB biomarkers 10Me C16:0, i-C17:0, C17:1 (cis-10) and i-C17:1 (cis-10) (Córdova-Kreylos et al. 2006; Mohanty et al. 2008), although detected with small amounts, all showed close relationships with pure SRB in PCA. It was suggested that the linking between a-C15:0 and these SRB biomarkers could be used as the evidence of the SRB presence in a system.

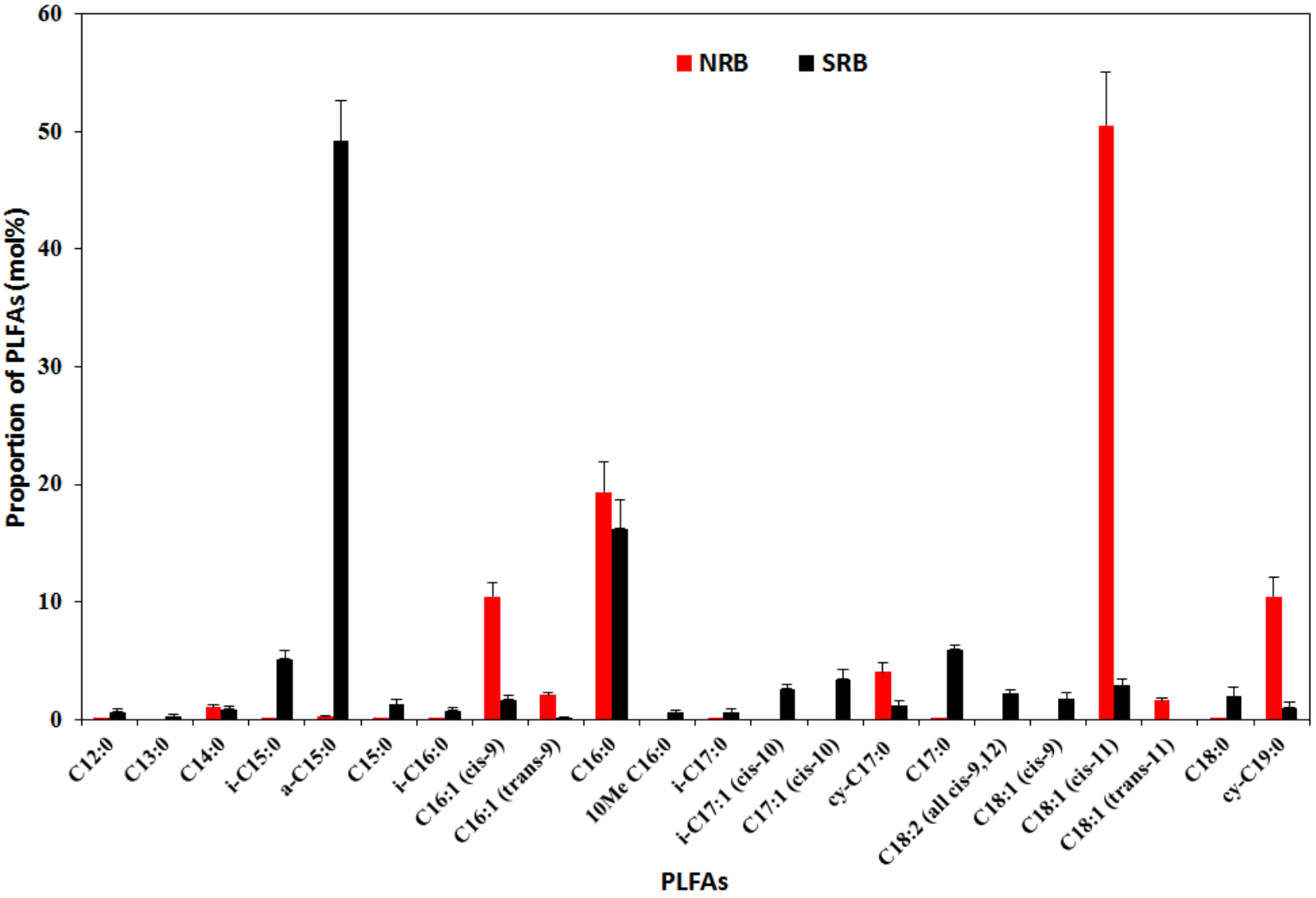


Figure 6.2 PLFA profiles of *P. stutzeri* CX3 and *D. escambiense* ATCC 51164 strains grew on NRB growth medium and SRB growth medium, respectively.

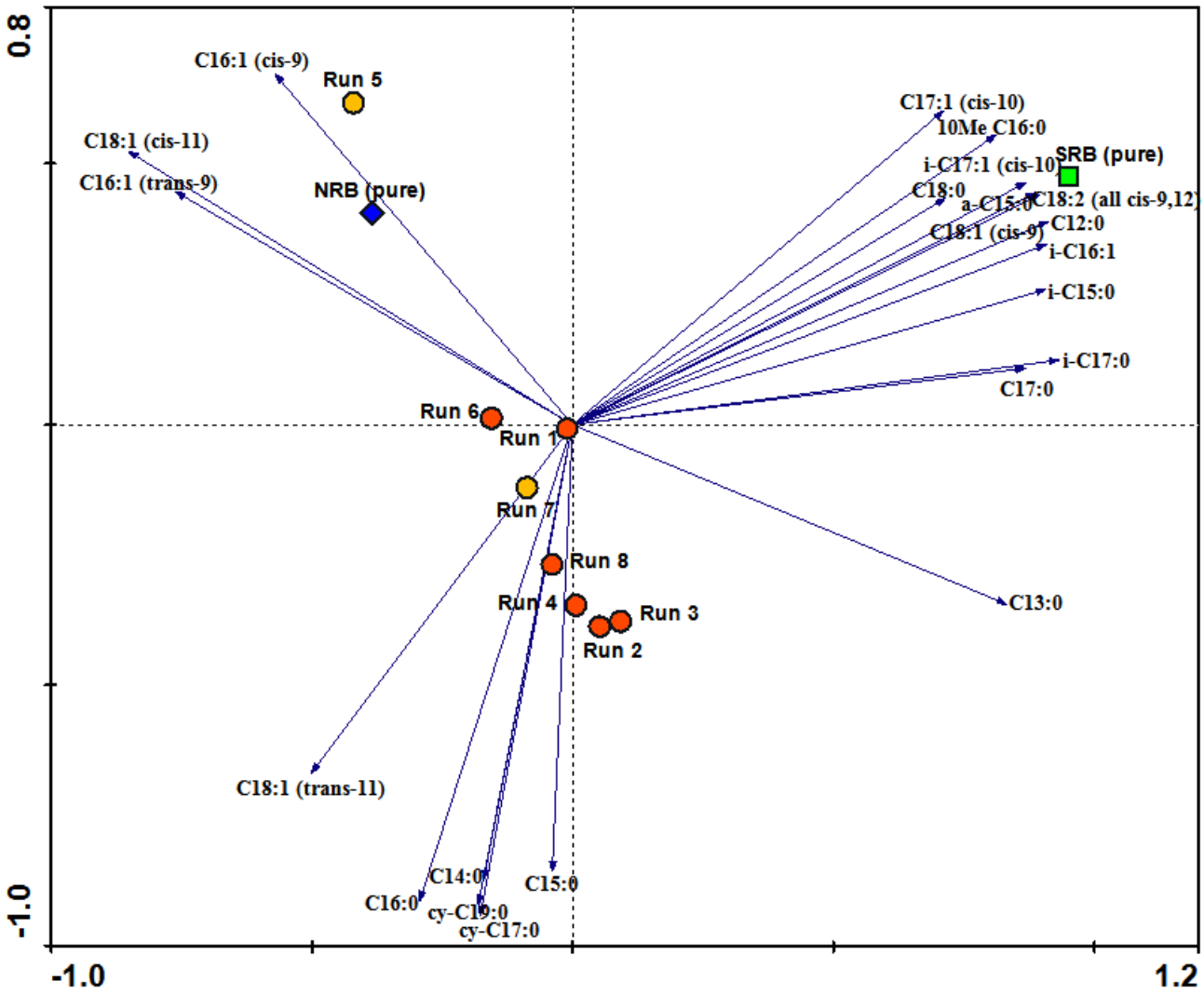
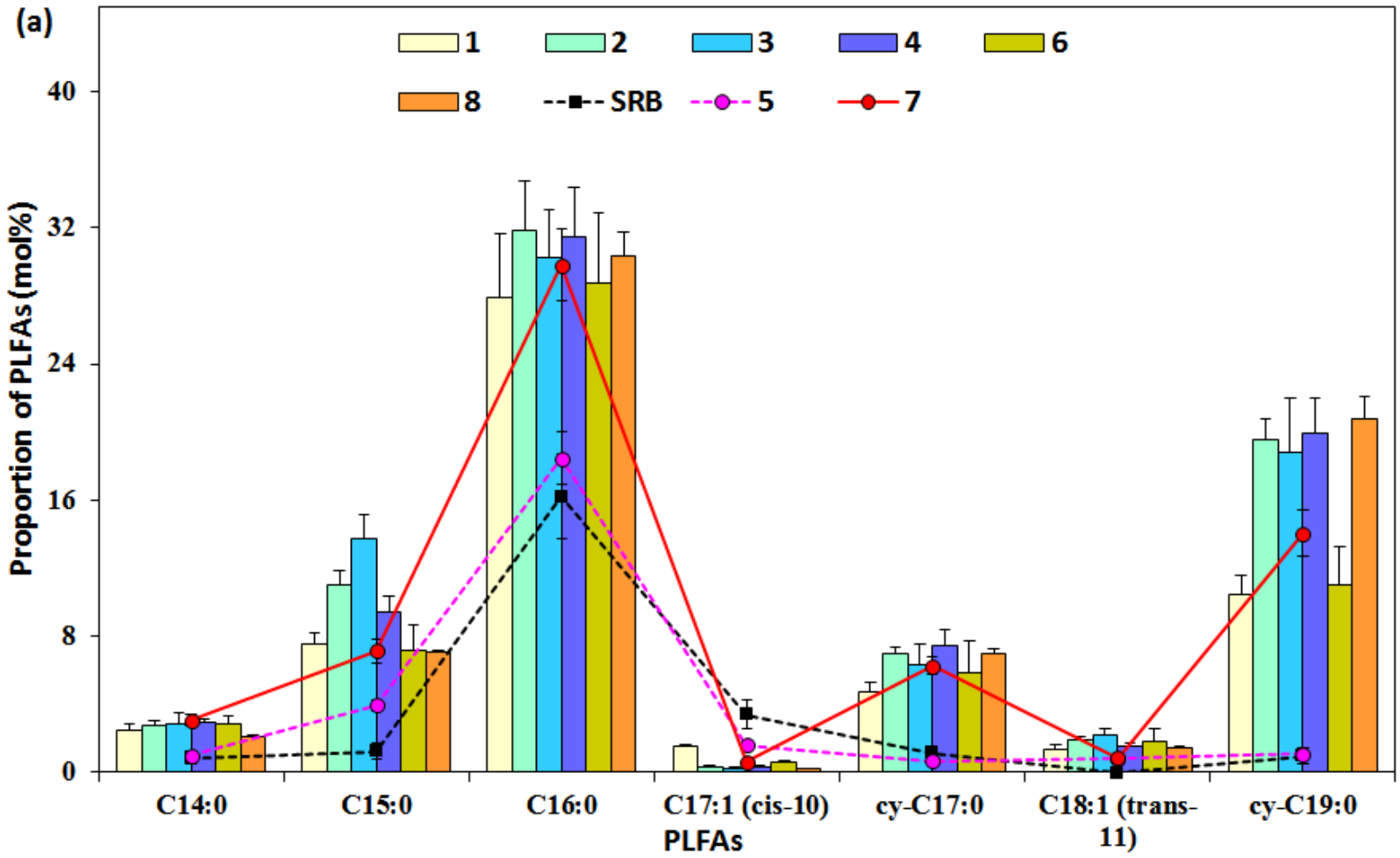


Figure 6.3 Principal component analysis (PCA) showing the effect of different nitrate and biosurfactant treatment on PLFA profiles of SRB and NRB strains on Day 11. PC1 and PC2 factors represented 61.8% and 35.2% of the total variance, respectively.

The cluster of Runs 1-4 and 6-8 in PCA suggested their similar patterns of PLFA compositions (Figure 6.3). Different from all other runs, a unique pattern was observed in Run 5, where its characteristic NRB PLFAs accumulated in PC1 and most of its representative SRB PLFAs accumulated in PC2. PCA clearly separated Run 5 with other runs dominated by the NRB strain. The PCA of PLFA profiles in Run 5 indicated the appreciable existence of the SRB in the microcosm. The results are accorded with the findings of physicochemical analysis (i.e., a much lower Eh and much higher sulfide concentration in Run 5). In addition, the predominant fatty acids C16:1 (cis-9) and C18:1 (cis-11) in Run 5 were characteristic fatty acids of the NRB strain, meanwhile the grouping of Run 5 and pure NRB were observed in PCA. The findings indicated that although the SRB community developed to a certain extent in Run 5, it was still a NRB dominated environment.

6.3.2.2 PLFA pattern changes

Ester-linked PLFA biomarkers were used as a proxy to determine the presence of individual microbial species and trace the community structure responses. As revealed by Figure 6.4, only microbial community in Run 5 showed high similarity with that of the pure SRB strain according to the fatty acid patterns of C14:0, C15:0, C16:0, C17:1 (cis-10), cy-C17:0, C18:1 (trans-11) and cy-C19:0. These PLFA biomarkers differentiated the community structure of Run 5 from that in all other runs due to well-developed SRB in the system. However, the microbial community in Run 5 had a significantly lower proportion of a-C15:0 (2.1%) than that of the pure SRB stain (49.2%), while the proportions of C16:1 (cis-9) (6.5%) and C18:1 (cis-11) (57.5%) were more likely contributed by NRB strain (Figure 6.4b). Meanwhile, the similar dominating presence of C16:0 (27.9-31.8%), C18:1 (cis-11) (15.4-32.5%), and cy-C19:0 (11.0-20.8%) in other microcosms also implied that the NRB strain dominated in all the experimental runs while the biomass of SRB strain increased to different levels after 11 days. The results quantitatively verified the PCA findings.


(a)
1
2
3
4
6
8
SRB
5
7
40
32
24
16
8
0
Proportion of PLFAs (mol%)
C14:0
C15:0
C16:0
C17:1 (cis-10)
cy-C17:0
C18:1 (trans-11)
cy-C19:0
PLFAs

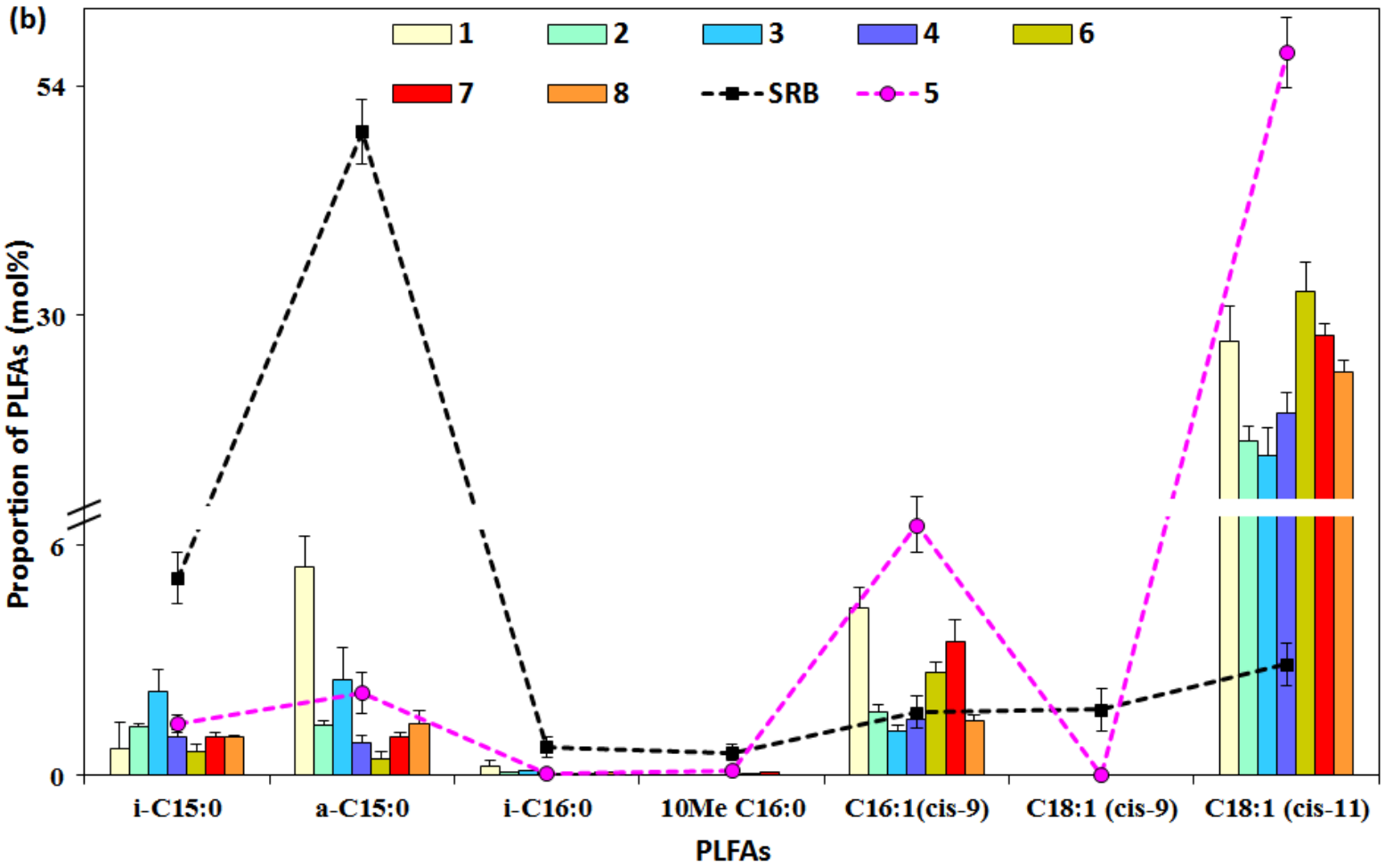


Figure 6.4 Representative PLFA patterns of microbial communities detected in microcosms with various nitrate conditions on Day 11.

Compared with other runs, microbial communities in Runs 1 and 6 have closer relationships with Run 5 in PC 2 (Figure 6.3). The PLFA profiles in Runs 1 and 6 showed relatively lower proportions of C15:0, C16:0, cy-C17:0 and cy-C19:0 (Figure 6.4a) as indicated in pure SRB strain. Notably, the highest proportion of a-C15:0 (dominating PLFA of SRB) was observed in Run 1, which indicated the presence of a small biomass of SRB. The absence of nitrate in Run 1 weakened the competence of NRB strain and its SRB inhibition effect, resulting in a slight rise of the redox potential to above -100 mV (-240 mV on Day 5 and -204 mV on Day 11), the approximate threshold Eh for sulfate reduction (Hulecki et al. 2009). In Run 6, suitable nitrate addition partially stimulated SRB growth in the culturing medium and resulted in the sulfide concentration of 26.0 mg/L on Day 5. In Runs 1, 5 and 6, lower pH values were also observed when compared with other runs. This further demonstrated that an alkaline environment might help the SRB inhibition (Guan et al. 2016).

6.3.2.3 Physiological changes

The alteration of microbial membrane fatty acid components is a natural part of microbial growth and an important survival and adaptation strategy to environmental stresses (Rowlett et al. 2017). Certain compositional PLFA patterns representing adaptive and protective responses of the microbes could help to track partly physiological change or stress responses (Wixon and Balser 2013). Physiological status was determined using the ratios of cyclopropyl PLFAs to their monoenoic precursors (cy/pre), the trans/cis ratio of C16:1Δ9, and the trans/cis ratio of C18:1Δ11 (Fig. 4). The ratio of trans/cis PLFAs indicated the environmental stress that might be caused by high pressure, low temperature or low nutrient conditions (Li et al. 2007b). In Figure 6.5, all the ratios of PLFA indicators in pure SRB or pure NRB maintained relative low values. Notably, the biosurfactant addition in Runs 5 and 7 lowered both the trans/cis ratios of C16:1Δ9 and C18:1Δ11. The biosurfactant addition was thus considered to facilitate the microbial growth in general, possibly by promoting microcolony

formation in the initial phase (Pamp and Tolker-Nielsen 2007). Very small amounts of cyclopropyl PLFAs of C17:0 and C19:0 in Run 5 was observed and the ratios of cy/pre reached nearly zero, suggesting the co-existence of the SRB and NRB in a mild environment. Overall, Runs 1-4, 6 and 8 without biosurfactant addition suffered different levels of nutritional fluctuation and environmental stress, which maybe induced by limited nitrate availability, starvation, and pH changes (Kaur et al. 2005; Willers et al. 2015). On the contrary, the indicative stress analysis revealed the biosurfactant addition significantly enhanced the action of bacterial mitigation towards the environmental stress. The beneficial effect demonstrated by biosurfactant addition acted on both of the NRB and SRB, and explained the obvious sulfide production in Run 5 but not in Run 4.

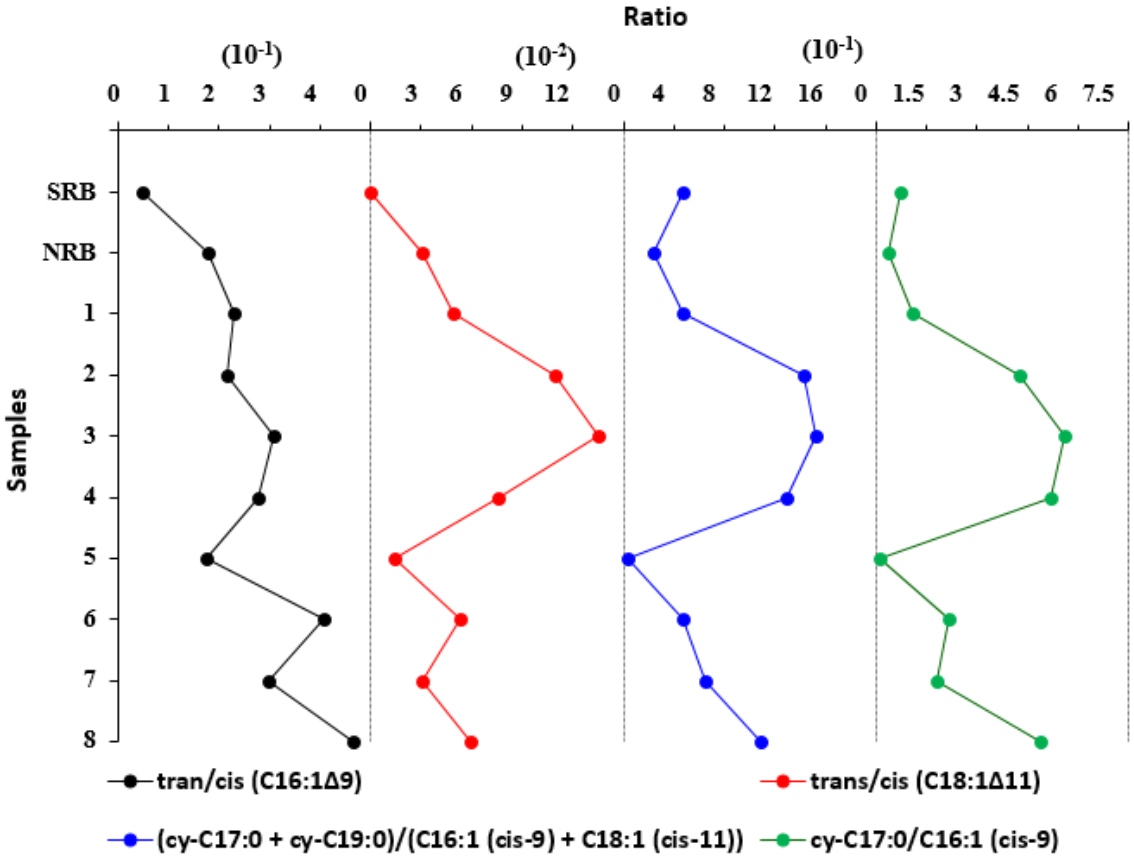
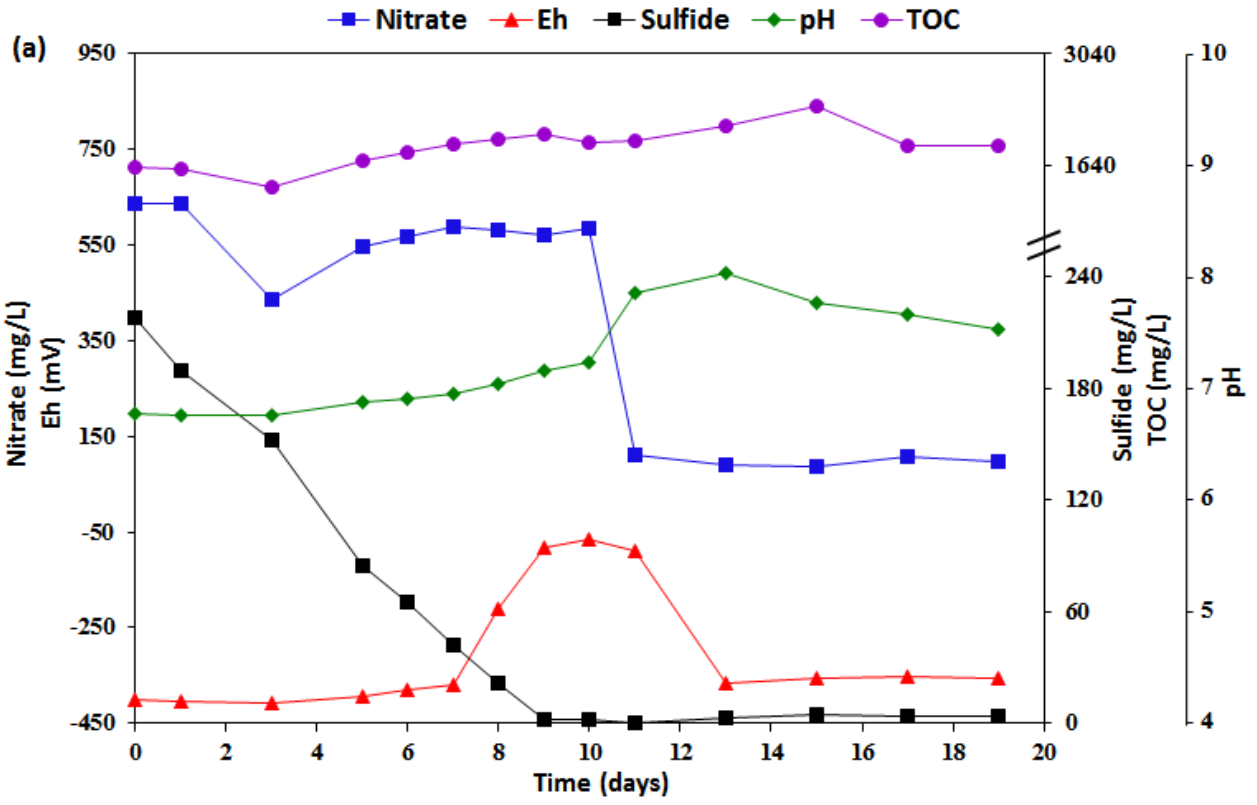


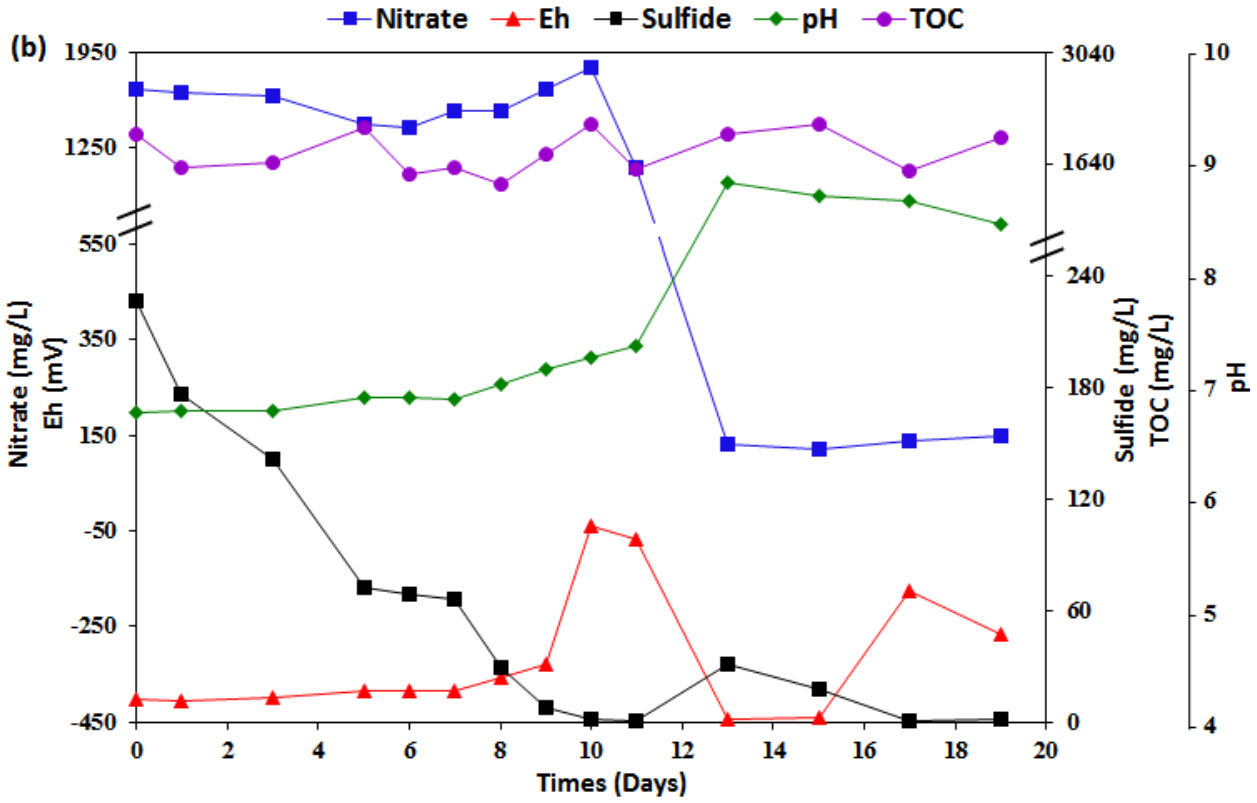
Figure 6.5 Changes in the ratios of trans/cis of C16:1Δ9 and C18:1Δ11 and the ratios of cyclopropyl PLFAs to their monoenoic precursors (cy/pre) at various growing conditions. Numbers 1-8 in samples indicate runs in the first round of experiments.

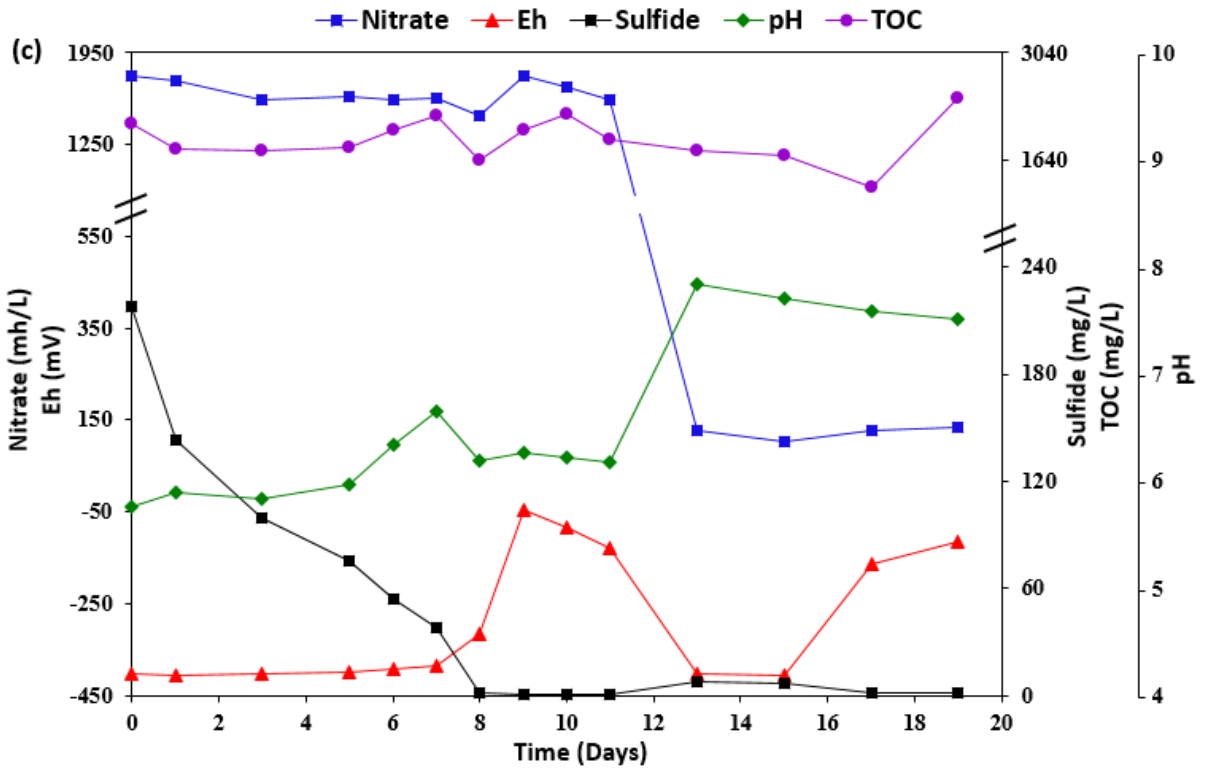
6.3.3 Souring control by nitrate and biosurfactant injection

6.3.3.1 Anti-souring effect of nitrate and biosurfactant addition

To investigate the anti-souring effect of nitrate and biosurfactant treatment, the second round of experiments was conducted. The SRB strain was inoculated in the media for 15 days in each run to create an extremely souring situation in which Eh below -400 mV was achieved and the sulfide concentration reached ~200 mg/L. In all four experimental runs (Runs A to D), the NRB strain was then introduced to the system together with nitrate (and biosurfactants in Run C) addition for souring mitigation. Figure 6.6 showed the results of changes on Eh, pH and the concentrations of sulfide, nitrate and DOC during 19 days of souring mitigation activities in all the four runs. From Figure 6.6, sulfide concentration in all runs was significantly reduced over time with values changes from ~200 mg/L to below 2 mg/L within 10 days. The pattern of sulfide concentration changes then varied in the 4 experimental runs after 10 days, whereas the final values were all below 4 mg/L. On Day 19, Run A achieved the highest sulfide concentration (3.8 mg/L) and the most reducing condition (-356 mV). The redox potentials of the Runs B to D were between -265 to -96 mV on Day 19. The pH values in Runs B to D were all elevated to above 8.5 while pH 7.6 was observed in Run A on Day 19. In spite of the varying concentrations of nitrate injection (1-5 g/L KNO_3) the final nitrate concentrations reached a relatively constant and low level (96.9-146.7 mg/L). In general, the souring condition in the microcosm was mitigated to different degrees through the various nitrate (and biosurfactant) addition in all four runs. Specially, high levels of nitrate injection (3 or 5 g/L KNO_3) resulted in effective control of souring caused by SRB activities and improved the reducing conditions in the system.


(a)
Nitrate
Eh
Sulfide
pH
TOC
Nitrate (mg/L)
Eh (mV)
Sulfide (mg/L)
TOC (mg/L)
pH
Time (days)


(b)
Nitrate
Eh
Sulfide
pH
TOC
Nitrate (mg/L)
Eh (mV)
Sulfide (mg/L)
TOC (mg/L)
pH
Times (Days)


(c)
Nitrate
Eh
Sulfide
pH
TOC
Nitrate (mh/L)
Eh (mV)
Sulfide (mg/L)
TOC (mg/L)
pH
Time (Days)

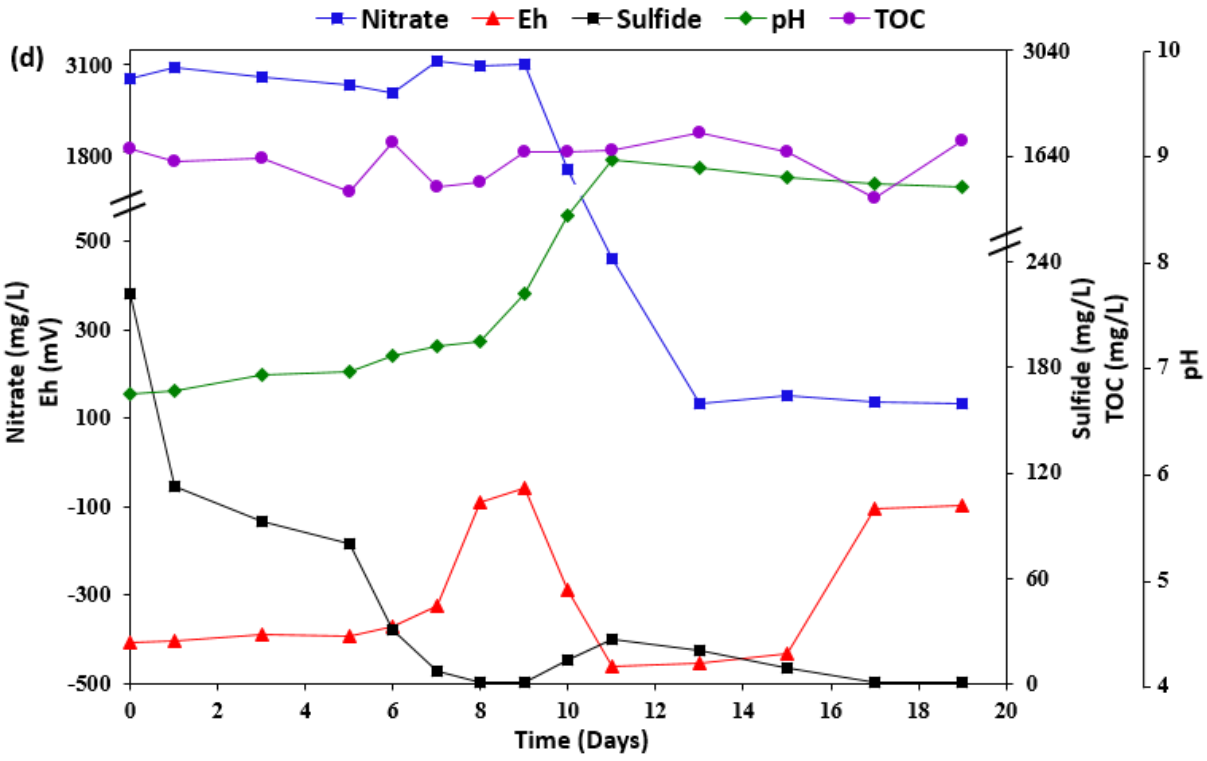

(d)
Nitrate
Eh
Sulfide
pH
TOC
Nitrate (mg/L)
Eh (mV)
Sulfide (mg/L)
TOC (mg/L)
pH
Time (Days)

Figure 6.6 Time course analyses of changes in physicochemical parameters after the inoculation of *P. stutzeri* CX3 for souring mitigation. NRB-produced biosurfactants and varying nitrate concentrations were added in the soured microcosms: (a) 1 g/L KNO_3 (b) 3 g/L KNO_3 (c) 3 g/L KNO_3 and 0.1 g/L biosurfactants (d) 5 g/L KNO_3

Fluctuating sulfide concentrations indicated the dynamic NRB-SRB interactions in the microcosms. The sulfide concentration was reduced within a shorter period of time in Runs C and D, with a removal rate of 99.4% and 99.5% on Day 8, respectively. In Runs A and B, the removal rates were 99.2% and 96.4% on Day 9, respectively. As a result, a sharp rise of redox potential was followed and the Eh value in all the microcosms reached above -70 mV accordingly. In accompany with the Eh increase, the nitrate concentration was significantly decreased and the pH level was increased obviously in all runs. On Day 19, Run A with the lowest nitrate addition resulted in the highest sulfide concentration (3.8 mg/L) and the lowest Eh value (-356 mV). Run B reached an Eh of -265 mV while the redox potentials of Runs C and D were around -100 mV on Day 19. Notably, compared with Run B, Run C with the same amount of nitrate injection (3 g KNO_3) resulted in more effective souring control. The similarity of Runs C and D in pH, redox potential and sulfide removal (99.6% for both) on Day 19 demonstrated their approximately identical capability to counter souring. Biosurfactant addition in Run C thus enhanced the NRB competence over SRB.

6.3.3.2 Mechanism

The biocompetitive exclusion process in which organotrophic NRB outcompete SRB for shared electron donors and nutrients was frequently proposed as the potential mechanism of nitrate-dependent souring control (Dolfing and Hubert 2017; Hulecki et al. 2009; Xue and Voordouw 2015). Under such a circumstance, it is expected that the SRB should experience obvious nutritional stress. However, the nutrient conditions indicated by DOC concentrations in the media (Figure 6.6) showed that no significant nutritional stress was observed (Isabelle and Natalie 2013). Hence, instead of the biocompetitive exclusion, the inhibition of SRB by nitrite seems the reasonable mechanism in our experiments (Gieg et al. 2011; Xue and Voordouw 2015). Nitrite was thus barely detected in nearly all the samples because it was consumed right after its generation in the system. The nitrite

metabolized by NRB during the initial 8-10 days was first utilized to oxidize sulfide in all runs. After sulfide was removed, redox potentials rose to values ranged from -40 to -64 mV in all the microcosm configurations. The suitable condition for stimulating nitrate reduction by NRB was thus formed and led to the subsequent sharp drop of nitrate concentration in the media. Overall, nitrate metabolism by the NRB was recognized as the major driving force for sulfide removal and nitrate concentration decrease accompanied. The subsequent elevation of redox potential and pH inhibited SRB activities in the system.

Nitrate injection is an effective tool for souring remediation; however, the effect is not permanent. Voordouw et al. (2009) observed a significant decrease of sulfide concentration in production wells in the first 5-7 weeks after nitrate injection. The sulfide levels, however, increased to original values prior to nitrate injection in many production wells afterwards. The phenomenon was caused by the penetration of SRB groups into the deep reservoir zones near the injection well where nitrate was depleted. A strategy with pulsed high-concentration injection of nitrate was then proposed to maintain the effectiveness of souring control (Callbeck et al. 2011). The strategy functioned well (Callbeck et al. 2013), but it significantly increased the quantity of injected nitrate and the associated costs. Any option for prolonging the effective duration of nitrate injected for souring control is thus promising. In this study, Run B (without biosurfactant addition) and Run C (with biosurfactant addition) were injected with the same amount of nitrate (3 g KNO_3). The effective duration of the nitrate in Run C was longer than that of Run B. Run C resulted in a higher Eh value and lower sulfide concentration after treatment, which indicated a more effective souring control than Run B. While similar anti-souring results were obtained in Runs C and D, prolonged duration time of nitrate was observed in Run C. The biosurfactant addition can thus be confirmed as an option for enhancing the performance

of nitrate injection in souring control. This is the first time that biosurfactant involved NRB-SRB interaction was investigated in a souring system.

6.3.3.3 Parameter correlation

PCA was used to visualize the relationships between the time points and the connection of the parameters monitored during the biosurfactant-involved NRB-SRB interaction in Run C (Figure 6a). The variances between nitrate and pH, while significant, were not as strong as the variances between sulfide, sulfate and Eh. This is because the major changes of nitrate and pH occurred only between Day 9 and Day 13 while dynamic changes of other 3 parameters were observed in the whole process. The inverse relationship between sulfide and sulfate was observed due to the shifts between the two chemicals (Chen et al. 2017; Voordouw et al. 2009).

A positive correlation of sulfate and DOC in PCA was also observed. This is because sulfate, as an essential nutrient for both NRB (Gadekar et al. 2006; Tang et al. 2010) and SRB, was consumed along with the metabolizing of DOC (electron donor) by the two strains. Considering the DIC (mainly CO_3^{2-}, HCO_3^-) formation increased the medium pH (Zhu and Dittrich 2016), which inhibited SRB activities and elevated the medium Eh in the microcosm, these three factors (e.g. DIC, pH and Eh) were closely related in Figure 6.7a.

The relationship of parameters including DIC, sulfide, pH and Eh on Day 19 in all runs was shown in Figure 6.7b. Sulfide concentration, as the significant factor influencing the redox potential, was negatively correlated with the redox potential. Accorded with the PCA results in Figure 6.7a, DIC and pH are both positively correlated with redox potential on Day 19 in the systems.

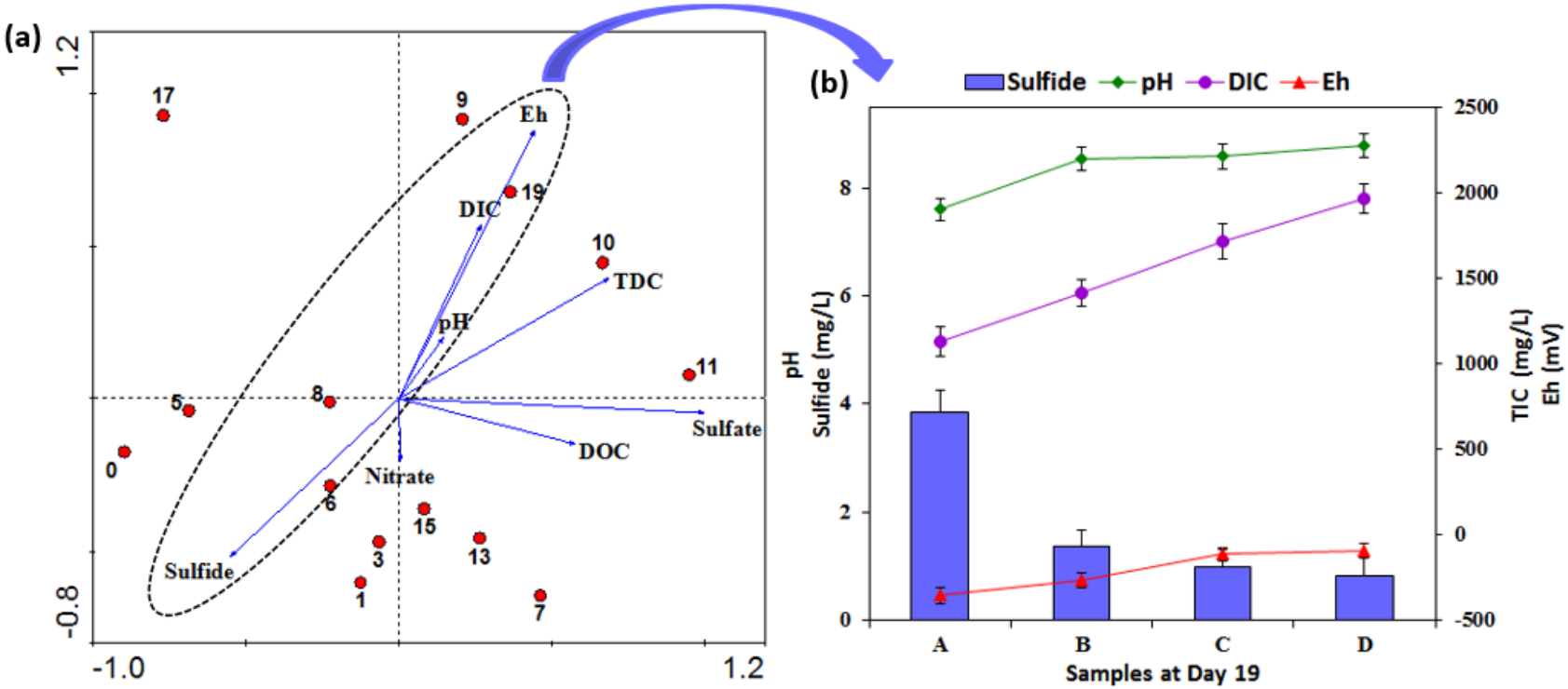


Figure 6.7 Relationships between these time points and the connection of the parameters: (a) PCA of the monitored time points and parameters during the 19 days of the biosurfactant-involved NRB-SRB interaction. PC1 and PC2 factors represented 84.8% and 7.5% of the total variance, respectively. (b) Eh, pH and the concentrations of sulfide and DIC on Day 19 in the souring mitigation activities.

6.4 Summary

SRB are the main culprits of MIC (Enning and Garrelfs 2014) and the sulfide formed will lower the value of petroleum products, increase health hazards and environmental threats (Chen et al. 2017; Hubert and Voordouw 2007). The troublesome souring issue has plagued petroleum and environmental industries for decades (Cheng et al. 2016). The individual impacts (Callbeck et al. 2013; Callbeck et al. 2011; Gieg et al. 2011; Kamarisima et al. 2018) and combining effects with other chemical agents (Greene et al. 2006; Hulecki et al. 2009; Xue and Voordouw 2015) of nitrate injection have been extensively examined owing to its convenient, inexpensive, and environmentally friendly features. However, the nitrate-mediated control of microbial sulfide production may encounter many issues, such as high nitrate demand (Da Silva et al. 2014), SRB resistance towards nitrate as a stress response (Kamarisima et al. 2018; Korte et al. 2014), the alteration of SRB zone into deeper reservoir (Callbeck et al. 2011; Voordouw et al. 2009) and incompetence of NRB due to their limited thermotolerant capabilities (Fida et al. 2016; Okpala et al. 2017).

We, for the first time, gained insight into the significant positive effect of biosurfactants produced by natural NRB *P. stutzeri* CX3 on the inhibition of SRB *D. escambiense* ATCC. Our results revealed biosurfactant addition promoted the growth of both SRB and NRB under non-sour conditions. Insufficient nitrate injection led to limited SRB inhibition due to the concurrent reduction of nitrate and sulfate by the SRB strain. Under sour conditions, the biosurfactant addition within specific nitrate levels increased the sulfide removal efficiency and significantly enhanced the nitrate inhibition of SRB by NRB. Nitrite oxidation of sulfide was the major reason for sulfide removal. Notably, The biosurfactant injection could reduce nitrate usage in souring mitigation. The prolonged effective

duration of nitrate will help nitrate/nitrite reach deeper zone in the system, resulting in long-term suppression of SRB activities and better souring control.

The natural NRB-produced biosurfactants enhanced the competence of NRB in the microcosm at the presence of soluble carbon nutrients. This enhancement may be strengthened when biosurfactants are introduced into environments where hydrophobic oil organics are main carbon sources. The amphiphilic properties of biosurfactants have the potential of helping NRB outcompete sulfidogenic microbes through the mechanism of both biocometative exclusion and nitrite suppression. This knowledge would extend the traditional perspective of biosurfactants in MEOR applications and lead to management strategies for targeted control of souring in oil fields, and thus needs to be further investigated.

CHAPTER 7

CONCLUSIONS AND RECOMMENDATIONS

7.1 Conclusions

Microbially induced reservoir souring, the undesirable production of hydrogen sulfide (H_2S) in oil reservoirs by SRB, has become a major concern during secondary oil recovery when water is injected into the reservoir to recover the remaining oil. SRB are responsible for the majority of the bacterial problems in oil production, and H_2S is produced directly by SRB as a by-product of respiration. This hazardous gas, a respiratory inhibitor, is both volatile and toxic. The unexpected production of H_2S has implications for reduced quality of produced hydrocarbons, for health and safety risks for operators, and for increased corrosivity of produced fluids. Currently, reservoir souring has occurred in approximately 70% of fields under water flooding (Elshahawi and Hashem 2005). The engineering, environmental and safety costs associated with reservoir souring have attracted great attention across the globe in recent decades. Hence, effective removal of sulfide or the inhibition of sulfide production is highly desired by the oil and gas industry, especially in offshore operations.

The injection of nitrate/nitrite could be very effective for reservoir souring control by promoting NRB to inhibit SRB activities. The possible mechanisms include the boost of hNRB to outcompete SRB for available nutrients (biocompetitive exclusion) because of its thermodynamic advantage over SRB, the promotion of nitrate reducing sulfide oxidizing bacteria (NR-SOB) to directly oxidize sulfide, and the repression of SRB through resultant nitrite. Due to its convenient, inexpensive, and environmentally friendly features, the injection of nitrate/nitrite has been widely investigated and applied under both simulated and real reservoir conditions. However, to effectively address souring-related issues, careful consideration of the operational conditions encountered in specific environments is required. The approaches and technologies that are used to address souring in onshore operations may not be effective in dealing with souring in offshore operations.

Nitrate injection has not been universally effective in reservoir souring control, the application of this technique may encounter many issues, such as high nitrate demand, SRB resistance towards nitrate as a stress response, the alteration of SRB zone into deeper reservoir and incompetence of NRB due to their limited thermotolerant capabilities. Much still is unknown about the detailed microbial mechanisms involved in NRB-SRB interactions during nitrate/nitrite injections for reservoir souring mitigation. Meanwhile, biosurfactants produced by natural NRB are promising bio-agents for enhancing NRB competence over SRB, which may assist in the enhanced suppression of SRB activities and better souring control. However, there is still a lack of routine SRB monitoring tools in offshore reservoir samples and systematic investigation of NRB-SRB-biosurfactant interactions. This thesis, therefore, has presented SRB profiling of an offshore oil reservoir, isolated NRB for possible biosurfactant producers, characterized the biosurfactant product and investigated the effect of biosurfactants in NRB-SRB interaction. A summary of the method development of biosurfactant-based nitrate injection for reservoir souring is described below:

1) A method based on PLFA analysis for profiling microbial communities in offshore produced water was optimized. A three-stage extraction process was confirmed and the extraction efficiency in phase partition was evaluated. The elution parameters in SPE purification were adapted for treating the oily samples and their volumes were determined to induce a high recovery for the fraction of phospholipids. Under the selected conditions, 92.9%, 96.3% and 92.8% of the spiked phospholipid standards C16:1 (cis-9) PC, C18:1 (cis-9) PC, and C19:0 PC were recovered, respectively, using 10 mL methanol as elution solvent on a non-commercial SPE column. Over 90% of spiked C19:0 PC was recovered before sample transesterification. Four parameters including alkaline reagent, volume of acid for neutralization, time and temperature for FAME derivatization were examined. GC-MS was used to analyze FAMEs and the method linearities, recoveries of 29 FAMEs during

transesterification, detection limits, relative standard deviations were presented. Results indicated that the developed method exhibited high recoveries and repeatability, remarkable selectivity and linearity, and acceptable quantitation limits for PLFA analysis. With reliable accuracy and precision, the method was applied to profile microbes in offshore produced water samples.

2) PLFA analysis was conducted to profile microorganisms and trace SRB in water samples from an offshore oil reservoir. From the results of spiked phospholipid standards, more than 90 % of the phospholipids were recovered before the treatment of FAME derivatization while the RSDs were below 8.0 %. The water samples from the injection well and four producing wells exhibited various reducing conditions and were further subjected to PLFA analysis. Fourteen kinds of PLFAs were detected in the five wellbores and the concentrations of total fatty acids ranged from 368.4 to 3468.9 ng/L. Possible SRB biomarkers and significant PLFAs associated with SRB including C14:0, i-C15:0, a-C15:0, C15:0, C16:1 (cis-9), C17:0, C18:1 (cis-9) and C18:1 (cis-11) were selected for determining the presence of SRB species and evaluating the sulfate-related microbial biomass. The possible existence of SRB genera *Desulfobacter*, *Desulfotomaculum*, *Desulfovibrio* and SOB in the reservoir were proposed based on PLFA profiles. The highest biomass was found in the most reducing well where very limited SOB biomarkers were found. Results indicated that the presence of SRB and SOB species was closely associated with the redox environment of the reservoir wellbores. The species distribution patterns were interpreted to elucidate the biological souring process.

3) The offshore petroleum-reservoir brines following nitrate/nitrite injection were used for the anaerobic screening of biosurfactant producing NRB. After periodic enrichment and sophisticated screening of the microorganisms, five NRB strains were screened from offshore produced water samples and all identified as *Pseudomonas stutzeri* according to their 16S rRNA sequencing results. The strain *P. stutzeri* CX3 was confirmed with biosurfactant production capacity through a series of

biosurfactant characterization tests (e.g., drop collapsing test, parafilm test and surfaced tension determination). Their biosurfactant producing abilities fed on either glucose or glycerol media were investigated. *P. stutzeri* CX3 reduced the medium surface tension to 33.5 and 29.6 mN/m, respectively, while growing on glucose or glycerol media. The CX3 strain was further inoculated to examine its growth performance, resulting in 32.4% and 94.5% of nitrate consumption over 228 hours of monitoring in two media, respectively. The nitrate concentrations and surface tensions on the two media were both reduced to a relatively stable level within 84 hours during which OD_{600} reached relatively high levels as well over the period. The composition analysis of the biosurfactant product generated by *P. stutzeri* CX3 was conducted through thin-layer chromatography, GC with FID and fourier transform infrared spectroscopy (FT-IR). The biosurfactant product was identified as a mixture of a small part of lipopeptide and a large part of glycolipid while its critical micellar concentration (CMC) was as low as 35 mg/L. The biosurfactant product demonstrated high stability over a wide range of temperature (4–121°C), pH (2-10), and salinity (0%–20% w/v) concentration.

4) The effectiveness of nitrate-mediated souring control highly depends on the interactions of SRB and NRB while biosurfactants produced by natural NRB are promising bio-agents for enhancing NRB competence over SRB. Biosurfactant-aided inhibitory control of SRB strain *Desulfomicrobium escambiense* ATCC 51164 by NRB strain *Pseudomonas stutzeri* CX3was examined in two scenarios. Under non-sour conditions, insufficient nitrate injection resulted in limited SRB inhibition due to the concurrent reduction of nitrate and sulfate by SRB. PLFA biomarkers traced the community responses and verified the existence of the two strains. Compositional PLFA patterns revealed biosurfactant addition benefitted both SRB and NRB towards stressful conditions. The NRB strain dominated in all the experimental runs while the biomass of SRB strain increased to different levels. During the investigations, an alkaline environment helped the SRB inhibition. Under sour conditions,

nitrite oxidation of sulfide, instead of the biocompetitive exclusion, proved to be the primary mechanism for sulfide removal. The subsequent elevation of redox potential and pH inhibited SRB activities. The connection of the parameters monitored during the biosurfactant-involved NRB-SRB interaction was elucidated. NRB-produced biosurfactants added at proper nitrate level significantly enhanced the nitrate inhibition of SRB activities by *P. stutzeri* CX3 and resulted in more efficient sulfide removal and effective duration of nitrate in the microcosms.

7.2 Research Contributions

According to the research findings, this study can be summarized and highlighted by the following contributions:

1) A suitable and efficient GC-based analytical method for intensive detection of PLFAs in oily saline offshore produced water was generated. Operation parameters affecting SPE purification and FAME derivatization were optimized. Characteristic biomarkers of microorganisms in offshore produced water were identified from PLFA profiles. The microbiological analysis of offshore produced water places great importance on the investigation of biological reservoir souring control among scientific and industrial activities.

2) Effective PLFA profiling was achieved in offshore reservoir water analysis to specifically elucidate the mechanism of reservoir souring induced by SRB. The complex biomass and microbial community structure information from the various reservoir conditions were revealed. The SRB and SOB species, coupled with their transformation patterns between the injection wells and producing wells under different redox conditions were proposed. The reasonable results in tracing microorganisms indicated PLFA analysis can be potentially used as routine monitoring tool in implementation of reservoir souring mitigation strategies.

3) The isolation of biosurfactant producing NRB from offshore reservoirs was achieved and subsequent anaerobic biosurfactant production was investigated for the first time. *Pseudomonas stutzeri* CX3 was identified as biosurfactant producer and the kinetic behavior of biosurfactant production was investigated. Glycolipids are major components of the biosurfactant product while high biosurfactant stability was demonstrated under various environmental conditions. The successful isolation and identification of biosurfactant producing NRB from laborious screenings on offshore reservoir samples, coupled with subsequent biosurfactant characterization provided valuable technical and methodological support for effective offshore reservoir souring control and associated EOR activities.

4) NRB-produced biosurfactants were for the first time reported to significantly strengthen SRB inhibition by NRB. Research results indicated insufficient nitrate injection resulted in ineffective SRB inhibition and biosurfactant addition benefitted both SRB and NRB towards stressful conditions. Nitrate metabolism was the major driving force for sulfide removal while the elevated redox potential and pH inhibited SRB activities. The biosurfactant injection could reduce nitrate usage in souring mitigation. The prolonged effective duration of nitrate will help nitrate/nitrite reach deeper zone in the system, resulting in long-term suppression of SRB activities and better souring control.

7.3 Selective Publications

1. **F. Fan,** B. Zhang, J. Liu, Q. Cai, et al. Interactions of sulfate reducing bacteria (SRB), biosurfactant producing nitrate reducing bacteria (NRB) screened from an offshore reservoir and NRB produced biosurfactants in microcosms, *Environmental Pollution*, 2018, under review

2. **F. Fan,** B. Zhang, P.L. Morrill, T. Husain, Isolation of nitrate-reducing bacteria from an offshore reservoir and the associated biosurfactant production, *RSC Advances*, 8 (2018) 26596-26609.

3. X. Li, **F. Fan**, K. Zhang, B. Zhang, B. Chen, PLFA based tracking of mechanisms and profiling of microbial community during enhanced soil bioremediation of hydrocarbons, *International Biodeterioration and Biodegradation*, 132 (2018) 216-225 (equally contributing 1st author).

4. X. Zhao, **F. Fan**, H. Zhou, P. Zhang, G. Zhao, Microbial diversity and activity of an aged soil contaminated by polycyclic aromatic hydrocarbons, *Bioprocess and Biosystems Engineering*, 41 (2018) 871-883.

5. **F. Fan**, X. Li, B. Zhang, Enhanced coastal soil bioremediation of petroleum hydrocarbons (PHCs) and associated microbial community transformation. Costal Zone Canada (CZC 2018), July 15-19, 2018, St. John's, Canada

6. **F. Fan**, B. Zhang, P.L. Morrill, Phospholipid fatty acid (PLFA) analysis for profiling microbial communities in offshore produced water, *Marine Pollution Bulletin*, 122 (2017) 194-206.

7. **F. Fan**, B. Zhang, P.L. Morrill, T. Husain, Profiling of Sulfate-Reducing Bacteria in an Offshore Oil Reservoir Using Phospholipid Fatty Acid (PLFA) Biomarkers, *Water, Air, & Soil Pollution*, 228 (2017) 410.

8. W. Qin, **F. Fan**, Y. Zhu, Y. Wang, X. Liu, A. Ding, J. Dou, Comparative proteomic analysis and characterization of benzo(a)pyrene removal by Microbacterium sp. strain M.CSW3 under denitrifying conditions, *Bioprocess and Biosystems Engineering*, 40 (2017) 1825-1838.

9. Y. Pi, B. Chen, M. Bao, **F. Fan**, Q. Cai, L. Ze, B. Zhang, Microbial degradation of four crude oil by biosurfactant producing strain Rhodococcus sp, *Bioresource Technology*, 232 (2017) 263-269.

10. **F. Fan**, B. Zhang, P.L. Morrill, Investigation of seasonal pattern of microbial community structure and identification of sulfate-reducing bacteria (SRB) in seawater samples. Symposium on

Persistent and Emerging Organic Pollution in cold and coastal Environments (PEOPLE 2017), October 16-17, 2017, St. John's, Canada

11. **F. Fan**, B. Zhang, P.L. Morrill, Phospholipid fatty acid analysis for identifying sulfate-reducing bacteria in seawater samples using base and acid catalyzed methylation methods. EWRI World Environmental & Water Resources Congress (EWRI 2016), May 22-26, 2016, West Palm Beach, Florida, USA

7.4 Recommendations for Future Research

The current research efforts focus on method development of PLFA profiling, the identification of SRB and associated souring mechanisms in an offshore oil reservoir. Biosurfactant producing NRB was screened and the associated biosurfactant-involved NRB-SRB interactions in microcosms were investigated. Future investigations can be carried out in the following aspects:

1) Based on the current results obtained in Chapter 3 and 4, phospholipid and FAME degradation rates during the sampling, storage and sample analysis could be further investigated. There is lack of data on other species that may produce similar PLFA profiles, thus more quantity and types of samples are recommended to be analyzed for more precise PLFA profile elucidation. The differences and similarities in geological and operation conditions among the sampling points needs to be further investigated for better elucidation of the results.

2) Carbon sources are of great importance on the production of biosurfactants and the alternation of culture conditions for the bacterial producers. The natural NRB-produced biosurfactants enhanced the competence of NRB over SRB through the mechanism of nitrite suppression at the presence of soluble carbon nutrients in the microcosm. However, as amphiphilic compounds with both hydrophilic and hydrophobic moieties, biosurfactants will increase the bioavailability of entrapped oil

in the reservoir for the bacterial producers by promoting wetting, solubilization and emulsification of various types of organics. Thus, the enhancement may be strengthened when biosurfactants are introduced into environments where hydrophobic oil organics are main carbon sources. The biosurfactants have the potential of helping NRB outcompete sulfidogenic microbes through the mechanism of both biocometative exclusion and nitrite suppression. This knowledge would extend the traditional perspective of biosurfactants in MEOR applications and lead to management strategies for targeted control of souring in oil fields, and thus needs to be further investigated.

3) NRB strain *Pseudomonas stutzeri* CX3, with the treatment of nitrate and biosurfactants, exhibited promising anti-souring potential under sour conditions. Further investigations of reservoir souring control by nitrate and biosurfactant amendment can be conducted on continuous bioreactors powered by a variable speed peristaltic pump. Under proper operation of the pumps and maintenance of the anaerobic conditions by pressurized sterilized nitrogen gas, SRB enrichment culture or field produced water can be introduced to simulate the reservoir fluids in water flooding process. Various nitrate and biosurfactant amendments can be applied with the inoculation of *Pseudomonas stutzeri* CX3. Sampling ports located near the influent and effluent regions can be used for the monitoring of parameters including nitrate, nitrite, sulfate, sulfide, carbon source and redox potential changes in the continuous reactors when nitrate/nitrite injection is conducted. The structural changes of the microbial communities under the complicated biological conditions, especially the associated SRB and NRB species, can be traced and characterized by PLFA analysis, quantitative fluorescence PCR techniques and other diagnostic tools.

4) As facultative microorganisms, NRB strains of *Pseudomonas stutzeri* were promising petroleum hydrocarbon degraders under aerobic or anaerobic conditions. The strains thus have great potential in applications of oil spill cleanup as well as hydrocarbon bioremediation & soil reclamation.

The biosurfactant producing ability of *Pseudomonas stutzeri* CX3 will help enhance the remediation efficiency by promoting the mobility and bioavailability of petroleum hydrocarbons and subsequent biodegradation. PLFA analysis can be used to evaluate the physiological status of the NRB strains and elucidate the associated biodegradation mechanisms linked to different soil treatments.

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