



Microbial dynamics in souring oil reservoirs  
by Robert Franz Mueller

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in  
Civil Engineering  
Montana State University  
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**Abstract:**

Souring of oil formations has become a serious problem for the oil industry in recent years. Especially when seawater is injected into an oil bearing formation, a high production of hydrogen sulfide can be found in many formations after some lag time. Reservoir souring refers to the generation of hydrogen sulfide (H<sub>2</sub>S) in originally sweet oil reservoirs that have been subjected to (sea) water flooding. Industrial problems associated with hydrogen sulfide production are corrosion, plugging of the petroleum formation, and safety. Also, sulfide production increases the sulfur content of the crude oil which decreases its value and increases refining costs.

The most plausible cause of reservoir souring is the growth and activity of sulfate reducing bacteria (SRB) in the zone where seawater mixes with formation water. In this mixing zone the components supporting SRB growth -- electron donors such as organic compounds or hydrogen, electron acceptor such as sulfate, and other essential nutrients such as nitrogen, phosphate, etc.-- are present. Abiotic reactions are not considered important in the generation of hydrogen sulfide. They are important, however, in scavenging of H<sub>2</sub>S since many iron-containing minerals are capable of reacting with H<sub>2</sub>S forming iron sulfide, pyrite or pyrrhotite.

In this research the current literature on souring in oil reservoirs was reviewed. Two differently operating oil fields were sampled for microbial consortia. An innovative sampling procedure was applied. The obtained consortia were characterized for potential growth substrates for various anaerobic groups of organisms at mesophilic and thermophilic temperatures. Kinetic parameters for microbial sulfate reduction and denitrification were determined at 60°C. The obtained parameters for sulfate reducing bacteria were determined for suspended as well as for biofilm cells. Batch reactors, completely stirred tank reactors and completely mixed biofilm reactors were used to estimate the growth kinetics of thermophilic sulfate-reducing bacteria and denitrifying bacteria. The effect of nitrate addition to oilfield waters was demonstrated in laboratory experiments. Microbial sulfate reduction was inhibited when nitrate was added to fresh oil field waters without the addition of bacteria. The fundamental mechanisms for this inhibiting effect were investigated. A simulation model based on the experimentally determined growth parameters was developed and described the processes of microbial interaction between SRB and DNB with qualitative agreement to the experimental results. Based on the results of this study, a novel strategy for controlling souring in oil reservoirs undergoing secondary recovery is presented.

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This Thesis has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the College of Graduate Studies.

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## ABSTRACT

Souring of oil formations has become a serious problem for the oil industry in recent years. Especially when seawater is injected into an oil bearing formation, a high production of hydrogen sulfide can be found in many formations after some lag time. Reservoir souring refers to the generation of hydrogen sulfide ( $H_2S$ ) in originally sweet oil reservoirs that have been subjected to (sea) water flooding. Industrial problems associated with hydrogen sulfide production are corrosion, plugging of the petroleum formation, and safety. Also, sulfide production increases the sulfur content of the crude oil which decreases its value and increases refining costs.

The most plausible cause of reservoir souring is the growth and activity of sulfate reducing bacteria (SRB) in the zone where seawater mixes with formation water. In this mixing zone the components supporting SRB growth -- electron donors such as organic compounds or hydrogen, electron acceptor such as sulfate, and other essential nutrients such as nitrogen, phosphate, etc. - are present. Abiotic reactions are not considered important in the generation of hydrogen sulfide. They are important, however, in scavenging of  $H_2S$  since many iron-containing minerals are capable of reacting with  $H_2S$  forming iron sulfide, pyrite or pyrrhotite.

In this research the current literature on souring in oil reservoirs was reviewed. Two differently operating oil fields were sampled for microbial consortia. An innovative sampling procedure was applied. The obtained consortia were characterized for potential growth substrates for various anaerobic groups of organisms at mesophilic and thermophilic temperatures. Kinetic parameters for microbial sulfate reduction and denitrification were determined at 60°C. The obtained parameters for sulfate reducing bacteria were determined for suspended as well as for biofilm cells. Batch reactors, completely stirred tank reactors and completely mixed biofilm reactors were used to estimate the growth kinetics of thermophilic sulfate-reducing bacteria and denitrifying bacteria. The effect of nitrate addition to oilfield waters was demonstrated in laboratory experiments. Microbial sulfate reduction was inhibited when nitrate was added to fresh oil field waters without the addition of bacteria. The fundamental mechanisms for this inhibiting effect were investigated. A simulation model based on the experimentally determined growth parameters was developed and described the processes of microbial interaction between SRB and DNB with qualitative agreement to the experimental results. Based on the results of this study, a novel strategy for controlling souring in oil reservoirs undergoing secondary recovery is presented.

CHAPTER I  
GENERAL INTRODUCTION

Souring of oil formations has become a serious problem for the oil industry in recent years. Especially when seawater is injected into an oil bearing formation, a high production of hydrogen sulfide can be found in many formations after some lag time (Burger et al., 1992; Frazer and Bolling, 1991).

Reservoir souring refers to the generation of hydrogen sulfide ( $H_2S$ ) in originally sweet oil reservoirs that have been subjected to (sea) water flooding. Industrial problems associated with hydrogen sulfide production are corrosion, plugging of the petroleum formation, and safety. Also, sulfide production increases the sulfur content of the crude oil which decreases its value and increases refining costs (McInerney *et al.* 1991).

The total cost related to SRB mediated corrosion is estimated to be 1-2 billion U.S. dollars per year in the U.S. alone (Lee, 1990). The most plausible cause of reservoir souring is the growth and activity of sulfate reducing bacteria (SRB) in the zone where seawater mixes with formation water. In this mixing zone the components supporting SRB growth -- electron donors such as organic compounds or hydrogen, electron acceptor such as sulfate, and other essential nutrients such as nitrogen, phosphate, etc.-- are present. Abiotic reactions are not considered important in the generation of hydrogen sulfide (Curd-Ruwish *et al.* 1987). They are important, however, in scavenging of  $H_2S$  since many iron-containing minerals are capable of reacting with  $H_2S$  forming iron sulfide, pyrite or pyrrhotite (Morse *et al.* 1987, Collins, 1975b).

### SRB Associated Problems in Oilfield Water Systems

Injection of seawater into oilfield reservoirs to maintain pressure is common in Norwegian and British sectors of the North Sea as well as throughout the United States. The injection water contains large amounts of sulfate (approx. 1000 ppm  $\text{SO}_4\text{-S}$ , Herbert *et al.* 1985) as well as small amounts of other required nutrients such as nitrogen and phosphorus and trace elements such as iron or other metal elements.

In oilfield water systems SRB cause serious problems:

- Corrosion of iron in the absence of air (anaerobic corrosion) (Hamilton, 1985).
- Precipitation of amorphous ferrous sulfide and polysaccharide production by SRB or other microorganisms in the formation that causes plugging and diminishes the injectivity of water injection wells (Dewar, 1986 and Lappan and Fogler, 1990).
- Contamination of fuel gas with hydrogen sulfide (Pankhurst, 1968).
- Contamination of fuel oil with hydrogen sulfide (Burger, 1992).

Furthermore, hydrogen sulfide is extremely toxic if inhaled; it easily escapes from contaminated waters and may accumulate under poorly ventilated conditions. It is usually recognized by its distinctive smell of rotten eggs. High concentrations anesthetize the sense of smell.

### Questions Related to the Problem of Souring in Oil Reservoirs

The following questions are based on a fundamental lack of information. The answers to these questions would then enable one to understand the processes of souring in a complex system such as an oil reservoir and could build the basis for adequate reservoir simulation models and for the development of effective control strategies to prevent souring in oil formations.

- Is the souring process distributed over the whole formation or are there specific locations where almost all action occurs and other zones where nothing happens (e.g., high SRB activity at the injection well and a low activity throughout the rest of the formation versus an evenly distributed SRB activity)?

- Are the organisms distributed homogeneously or heterogeneously (e.g., planktonic organisms vs aggregated organisms) at the locations of action and does this affect the rate or stoichiometry?

- What physiologically different groups of organisms (e.g., fermentative, acetogenic, methanogenic, and sulfate reducing organisms) are present in the mixed souring consortium and can they be determined on the basis of their metabolism (e.g., methane production, sulfate reduction)?

- What substrates (e.g. sugars, hydrocarbons, long chain fatty acids, short chain fatty acids) are favored for metabolism of these groups of organisms?

- At what rates are these substrates utilized and what are the metabolic end products produced by these groups of organisms (e.g. specific substrate removal rate, rate of hydrogen sulfide production, biomass production rate)?

- How do different temperatures, pH, and pressure (as seen through the formation) influence the microbial kinetics and stoichiometry?
- What other chemical interactions between metabolic end products and reactants are likely to occur under formation temperatures?
- Can microbial interactions between SRB and other species be used for control of souring in an oil field.

#### Goals and Objective

The goal of this study was to understand and quantitatively describe the fundamental processes leading to souring of an oil reservoir and to develop strategies for its control. This goal was divided into the following objectives:

- \* Survey the current literature on microbial souring of oil formations.
- \* Sample for microbial consortia from two souring oil reservoirs and characterize the potential substrates utilized for sulfate reduction.
- \* Determine the effect of temperature on microbial sulfate reduction and substrate utilization.
- \* Determine kinetics and stoichiometry for thermophilic sulfate-reducing bacteria in suspended and biofilm culture for the most relevant substrates.

- \* Develop an efficient control strategy to prevent microbial souring. In particular investigate the effect of nitrate addition on microbial sulfate reduction in souring oil reservoirs.
  
- \* Develop a simulation model to describe the microbial interactions between sulfate-reducing bacteria and denitrifying bacteria and compare the experimental results qualitatively with the computer generated data.

### Experimental Approach

Real system behavior is studied best in real systems. However, in a complex system as a souring oil field, access to locations inside the field is impossible and alternatives had to be found. Sampling from produced water at two differently operating fields gave a good representative sample of the microorganisms present in the reservoir. Tests for potential substrates have been performed with fresh unfrozen samples. Some of these tests have been initiated at the sampling site. A wide range of temperatures are present throughout most of the oil reservoirs undergoing sea water flooding. Bacterial conversions also should be tested at a range of temperatures and environmental conditions. Suspended growth systems are compared with biofilm systems.

### Process Analysis

Process analysis was used to obtain rate parameters from experimental systems in order to describe a real system behavior quantitatively. Process analysis was applied for biofilm and suspended cultures. The rate and stoichiometry for either system can then be compared and evaluated. The fundamental processes have to be described mathematically in order to model

and simulate a system behavior in a realistic manner.

### Simulation of Microbial Interactions Between Two Species

Various models for microbial interactions between two species are available in Literature. To test these models for validity in a specific system, various simulations have to be used. A trial and error solution seems the best way to find the best fit of the simulations with a specific model for microbial interaction. In addition, the simulation model has to match the experimental data to provide a realistic picture of the actual processes in the tested system.

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## CHAPTER II

## MICROBIAL SOURING IN OIL RESERVOIRS:

## A LITERATURE REVIEW

In this chapter activity for mesophilic and thermophilic sulfate-reducing bacteria (SRB) will be reviewed. A temperature profile throughout an oil reservoir will be compared with the potential for mesophilic (m)-SRB and thermophilic (t)-SRB activity. This will provide indication where microbial souring is most likely to occur in the reservoir and where SRB growth could be controlled more efficiently at specific sites with biocides. Competition of sulfate reducing bacteria and other groups of organisms occurring in oil reservoirs such as fermenters and methanogens will be discussed.

Microbiology of SRB

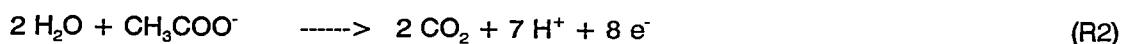
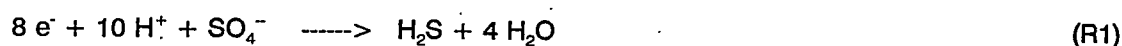
SRB were defined by Widdel (1988) as a heterogeneous assemblage of microorganisms having in common dissimilatory sulfate metabolism and obligate anaerobiosis. The production of hydrogen sulfide from sulfate in waters was recognized as a biological process in 1864 by Meyer (reviewed in Widdel 1988). But still 100 years later the understanding of that dark and mysterious group of SRB was troubling the ambitious microbiologist. "The sulphate reducing bacteria are a bizarre group of microbes of which many people, including myself know nothing. Despite their fascinating qualities they have been a somewhat neglected backwater of microbiological research: smelly, awkward to grow, intractable to isolate and count; but intriguing novelties of biochemistry and physiology to those persistent enough to stick with them." (Postgate, 1978). SRB did see much more attention in the recent years and especially Postgate and later Widdel pioneered the investigation of a variety of new species of SRB. Most of the work done at this time was at

mesophilic temperature ranges. There are still wide open areas for fundamental research with thermotolerant, thermophilic, extremely thermophilic, barophilic, and halophilic SRB species.

### SRB Metabolism

Lactate or pyruvate used to be the common and in many cases only carbon substrate for enrichment, isolation and cultivation of SRB. The capability of acetate oxidation by SRB was doubted for many years by SRB experts such as Postgate (1978). In irony Widdel and Pfennig (1981) named the sulfate reducer which almost exclusively uses acetate *Desulfobacter postgatei*. The discovery of short-chain fatty acids utilizing SRB changed the way of viewing the microbial ecology of SRB in natural systems (Sorenson, 1981).

If organic matter enters stagnant or closed water systems (such as a souring reservoir) where gas exchange with the atmosphere is limited, dissolved oxygen may be consumed completely. Biological decomposition of this organic material is carried out by fermentative bacteria. Most fermentative bacteria are facultative or obligate anaerobes. In fermentation, the overall oxidation state (e.g. energy state) of the degraded matter remains unchanged (disproportion of organic carbon). A part is converted into CO<sub>2</sub>, another part is converted to reduced products such as fatty acids, hydrogen and alcohols. In many natural anaerobic environments, the quantitatively most important fermentation products formed with CO<sub>2</sub> are H<sub>2</sub>, acetate, propionate, and butyrate (Sorensen *et al.* 1981, Lundegard and Kharaka, 1990). If sulfate is present under anaerobic conditions and the redox potential is sufficiently low ( $E_h < -100$  mV) these reduced fermentation products are used further by SRB. Sulfate is reduced to hydrogen sulfide (R1), whereas organic materials are oxidized (R2) either completely to CO<sub>2</sub> or incompletely to acetate (Widdel, 1988).

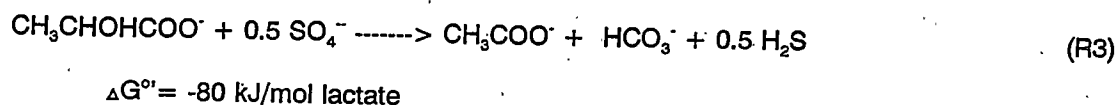


Most SRB species are much more restricted than fermentative organisms with respect to their possible electron donors, whereas some fermentative organisms can decompose complex compounds (e.g. polymers such as cellulose, proteins, or longer chain hydrocarbons). With one exception (Aeckersberg *et al.* 1991) SRB have not been shown to use polymeric substrates directly. Typical substrates for SRB are simple compounds of low molecular weight, such as fermentation products. Also hydrogen can be used by many SRB as energy source growing chemoautotrophically. Jorgensen (1989) states that hydrogen is the most important inorganic energy carrier in anaerobic pathways in nature. Therefore, SRB in natural environments depend on fermentative bacteria that cleave and ferment the more complex organics to low molecular weight compounds (Jorgensen, 1989).

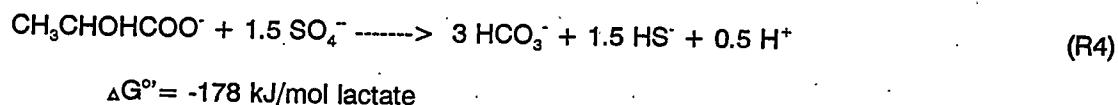
Most SRB can utilize sulfite or thiosulfate as alternative electron acceptors (Widdel, 1988; Nazina *et al.* 1988, Min and Zinder, 1990; Rosnes *et al.* 1991). Growth of SRB with elemental sulfur as e<sup>-</sup> acceptor has been observed with some isolates. *Desulfovibrio desulfuricans* and some other *Desulfovibrio* strains, *Desulfobulbus propionicus* and *Desulfobacterium catecholicum* were shown to use nitrate as electron acceptor producing ammonium (Widdel, 1988).

#### Stoichiometry of SRB metabolism

Lactate is oxidized incompletely by most SRB by producing acetate and carbonate (Table 1). R3 provides the stoichiometry for lactate oxidation by *Desulfovibrio desulfuricans*.



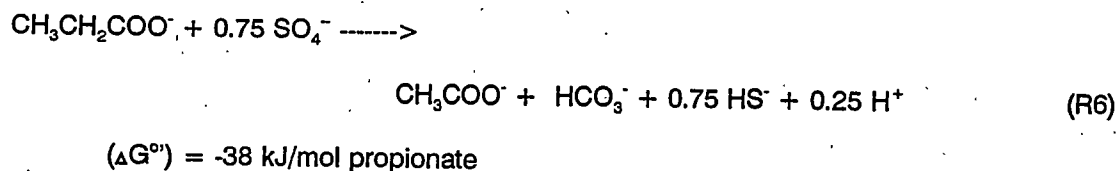
Some species of SRB such as *Desulfococcus multivorans* are capable of a complete oxidation of lactate (Table 1; R4).



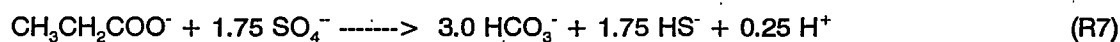
Acetate utilizing SRB exhibit lower growth rates than the lactate utilizers. Some strains use no further substrate beside acetate, other strains may use ethanol in addition (Table 2). The oxidation of acetate in *Desulfobacter postgatei* was shown to occur via the citric acid cycle (Widdel 1988) (R5).



Propionate is utilized incompletely by *Desulfobulbus* species. Growth with lactate was observed to be somewhat faster than with propionate, but only propionate allowed selective enrichment (Widdel and Pfennig, 1982).

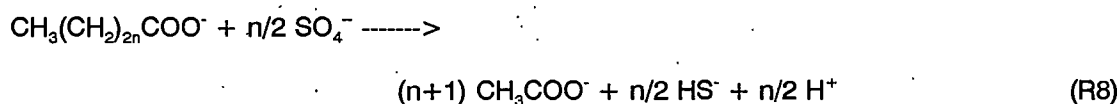


Among the complete oxidizers there are many species which oxidize propionate completely such as *Desulfococcus multivorans* (Table 1), (R7).

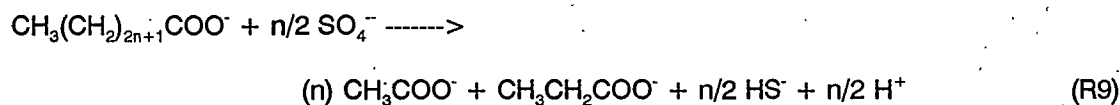


$$(\Delta G^\circ) = -86 \text{ kJ/mol propionate}$$

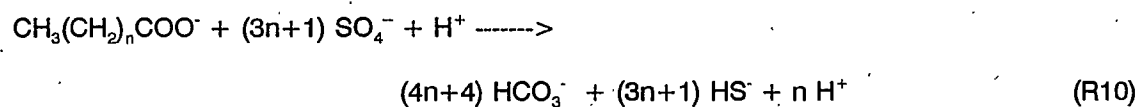
Butyrate and higher fatty acids are used by species of both the complete oxidizing and the incomplete oxidizing SRB (Table 1 and Table 2). Widdel (1988) proposes that the C-even fatty acids are degraded to acetate as the entire oxidation product (e.g. *Desulfovibrio sapovorans*) (R8). Utilization of hydrogen by these types of SRB was never observed. The degradation of these carboxylic acids follow a  $\beta$ -oxidation pathway (R8 and R9).



C-odd fatty acids are degraded to acetate and propionate (R9).



Complete oxidation of monocarboxylic acids (*Desulfosarcina variabilis*) follows R10.



Oxidation of branched chain fatty acids such as i-butyrate has only been observed with species

of completely oxidizing SRB (*Desulfonema limicola*) (R10).

Other organic compounds used as substrate for SRB are: monovalent alcohols (*Desulfovibrio* strains Braun, 1985); dicarboxylic acids (e.g. *Desulfobacterium* species, Postgate, 1984); aromatic compounds (e.g. *Desulfococcus* species, Klémps *et al.* 1985); saturated cyclic organic acids (e.g. *Desulfobacterium* strains, Widdel, 1988); and hydrocarbons (e.g. *Desulfobacterium* strain Aeckersberg *et al.* 1991).

#### Required Growth Conditions of SRB

Oxygen. Development of SRB activity can be expected wherever organic matter and sulfate containing water are combined in an oxygen limiting environment. Significant SRB activity are measured in marine sediments, because of the high sulfate concentration of seawater (2800 ppm) (Fossing and Jorgensen, 1989). The produced  $H_2S$  is a strong reducing agent that reacts with oxygen even at low temperatures. Thus, once established, the SRB can protect themselves against oxygen (Cypionka *et al.* 1985).

pH. SRB prefer neutral pH for their growth, and activity can be observed over a range of about pH5.5 to pH8.5. The metabolic products of SRB metabolism represent buffers such as  $HS^-/H_2S$  and the  $HCO_3^-/CO_2$  systems that may help to keep the pH in their surrounding environment close to neutral and protect against extreme pH changes (Crolet *et al.* 1992; Curd- Ruwisch *et al.* 1987).

Salinity. SRB species originating from fresh water may be inhibited by more than 20 to 30 g/l NaCl, in contrast, many marine SRB species are moderately halophilic (i.e. they require 10 to 30 g/l NaCl for optimum growth). The activity of most SRB declines drastically if the NaCl

concentration exceeds 50 to 100 g/l (Postgate, 1984; Widdel, 1988).

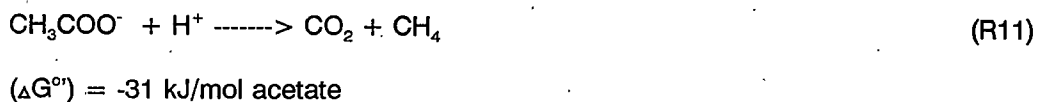
Other Nutritional Requirements. The presence and concentration of essential nutrients such as nitrogen and phosphate can affect the activity of sulfate reduction. The theoretically (stoichiometric) limiting ratio of C:N:P was found to be in the range of 1000:4:1 with a cell yield of 0.03 g cells/g lactate (Okabe *et al.* 1991, and Okabe *et al.* 1992) for *D. desulfuricans* grown on lactate as limiting nutrient. The production of extracellular polymeric substance (EPS) increased when nitrogen was limiting. Nitrogen and phosphate requirements for SRB are generally about 10 to 20 times lower than for aerobic bacteria because of the lower growth yield.

#### Biofilm formation

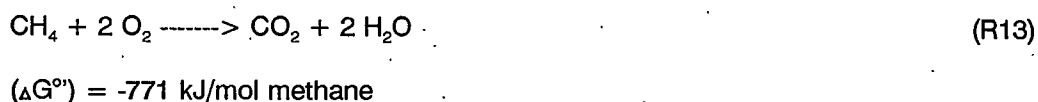
SRB biofilms have been studied by a few researchers (Nielsen, 1987; Nielsen and Hivited-Jacobsen, 1988; Yoda *et al.* 1992; Okabe *et al.* 1992). Only one study was done with a pure culture SRB biofilm using *D. desulfuricans* (Okabe *et al.* 1992). Generally the accumulation of SRB cells on substrata is slow compared to methanogens or aerobic bacteria (Yoda *et al.* 1987). The growth parameters, cell yield, and stoichiometry were not found to be significantly different in a biofilm system than in suspension, assuming the same environmental conditions for each system (Okabe *et al.* 1992). Okabe measured a volumetric sulfate reduction rate in his monopopulation biofilm 10 times higher than a mixed SRB biofilm in a sewer system (Nielsen, 1987). Neither system indicated an external mass transfer limitation and it seems safe to conclude that only a small fraction of the mixed biofilm was active in sulfate reduction, as it might be expected in a sewer system.

Thermodynamics of sulfate reduction

Thermodynamically, fermentation and sulfate reduction yield much less energy than aerobic respiration. When sulfate is used as electron acceptor (R5), the free energy change was calculated for the oxidation of 1 mol of acetate to  $\Delta G^\circ = -48$  kJ/mol acetate. For aerobic respiration of acetate the free energy change is ( $\Delta G^\circ = -837$  kJ/mol acetate) almost 20 times higher than with sulfate reduction. For methanogenesis the energy yield is even lower ( $\Delta G^\circ = -31$  kJ/mol acetate; R11):



Consequences of the relatively low free energy changes are a low growth yield of usually less than 0.1 g cells produced per g of total substrate consumed. The unoxidized products contain still the largest part of the total energy. A reoxidization of hydrogen sulfide to sulfate (e.g. *Thiobacillus*, *Beggiatoa* and *Thiothrix* species R12) or methane to carbon dioxide (e.g. *Methylomonas*, *Methylobacter*, and *Methylococcus*, R13) might occur in aerobic environments or environmental niches.



Sulfide and methane link anaerobic and aerobic processes together, analogous to hydrogen and

acetate being the major ties between fermentation and methanogenesis or sulfate reduction.

### Types of SRB

#### Types of m-SRB

M-SRB are usually found in environments with temperatures below 45°C (Herbert and Stott, 1983). Most m-SRB have a fairly broad temperature optimum between 30 and 37°C. The cell forms of m-SRB are most commonly found to be curved and oval to rod shaped with a diameter of 0.4-2 µm and 1-5 µm in length. Other forms are spheres and long multicellular filaments (Brook, 1990). Many m-SRB are flagelated and are motile. SRB can be divided into two metabolically different groups. The first group carry out an incomplete oxidation of substrate and produce acetate as end product (Table 1).

An important finding was the existence of sulfate reducers which are able to oxidize acetate (Widdel, 1988). These organisms are included in the second group which can oxidize organic substrates completely to CO<sub>2</sub> (Table 2). Most incompletely oxidizing SRB grow rather fast with  $\mu_{\max} = 0.39 \text{ h}^{-1}$  for *Desulfovibrio desulfuricans* at 30 °C (Okabe *et al.* 1991). The completely oxidizing SRB grow relatively slowly with maximum growth rates of  $\mu_{\max} = 0.02 - 0.06 \text{ h}^{-1}$  (Widdel & Pfennig, 1977; Widdel, 1986; Schauder *et al.* 1986). The completely oxidizing SRB may cooperate with incompletely oxidizers by utilizing the produced acetate.

#### Types of t-SRB

The majority of thermophilic SRB (t-SRB) have been isolated from geothermal habitats (Table 3). Their growth temperatures reflect the environment from which they are isolated (Stetter, 1990).

**Table II.1.** Characteristics of representative m-SRB for complete substrate oxydation (Widdel 1988) 1988).

Table II.1. Characteristics of representative m-SRB for complete substrate oxidation (Widdel 1988) 1988).

Species	morph.	Electron donors						growth rate and yield	
		H <sub>2</sub> /CO <sub>2</sub>	formate	lactate	acetate	other VFA	other	$\mu_{max}$ (h <sup>-1</sup> )	$Y_{XS}$ (g g <sup>-1</sup> )
<i>Desulfobacter postgatei</i>	oval	-	-	-	+	-		0.03	0.07
<i>Desulfovibrio baarsi</i>	curved	-	-	-	+	C <sub>3</sub> -C <sub>18</sub>			
<i>hydrogenophilus</i>	rod	+	-	-	+	-	ethanol	0.04	
<i>curvatus</i>	vibrio	+	-	-	+	-	ethanol	0.03	
<i>Desulfotomaculum acetoxidans</i>	rod	-	-	-	+	C <sub>4</sub> -C <sub>5</sub>	ethanol	0.03	
<i>Desulfococcus multivorans</i>	sphere	-	+	+	+	C <sub>3</sub> -C <sub>14</sub>	ethanol		
<i>niacini</i>	sphere	+	+	-	+	C <sub>3</sub> -C <sub>14</sub>	ethanol		
<i>Desulfosarcina variabilis</i>	rod	+	+	+	+	C <sub>3</sub> -C <sub>14</sub>			
<i>Desulfobacterium phenolicum</i>	oval	-	-	-	+	C <sub>4</sub>	phenol		
<i>Desulfonema limicola</i>	filamentous	+	+	+	+	C <sub>3</sub> -C <sub>12</sub>	succinate		

**Table II.2.** Characteristics of representative m-SRB for incomplete substrate oxidation (Widdel 1988).

Species	morphology	Electron donors					growth rate and yield		
		H <sub>2</sub>	formate	lactate	other VFA	other	$\mu_{\max}$ (h <sup>-1</sup> )	Y <sub>X/E</sub> (g g <sup>-1</sup> )	Y <sub>X/Lac</sub> (g g <sup>-1</sup> )
<i>Desulfovibrio</i>									
<i>desulfuricans</i>	curved	+	+	+	-	ethanol	0.40	-	
<i>vulgaris</i>	curved	+	+	+	-	ethanol	0.01	0.07	
<i>gigas</i>	curved	+	+	+	-	ethanol	0.09		0.04
<i>africanus</i>	curved	+	+	+	-	ethanol	0.06		0.02
<i>salexigens</i>	curved	+	+	+	-		0.02	0.08	
<i>sapovorans</i>	curved	-	-	+	-		0.007	0.09	
<i>Desulfotomaculum</i>									
<i>orientis</i>	rod	+	+	+	-	ethanol			
<i>ruminis</i>	rod	+	+	+	-	ethanol			
<i>Desulfobulbus</i>									
<i>propionicus</i>	oval	+	-	+	C <sub>3</sub>	ethanol			
<i>elongatus</i>	rod	+	-	+	C <sub>3</sub>	ethanol			
<i>Desulfomonas</i>									
<i>pigra</i>	rod	+	-	+	-	ethanol			

**Table II.3.** Characteristics of t-SRB

Species	morphology	Electron donors					T opt.(°C)
		H/CO <sub>2</sub>	formate	lactate	acetate	other VFA	
<i>Desulfovibrio thermophilus</i>	rod	+	+	+	-	-	65-85
<i>Desulfotomaculum nigrificans</i>	rod	+	+	+	-		ethanol 55-70
<i>kuznetsovii</i>	rod	+	+	+	+	C <sub>3</sub> -C <sub>4</sub>	ethanol 50-85
<i>thermoacetoxidans</i>	rod	+	+	+	+	C <sub>3</sub> -C <sub>5</sub>	pyruvate 45-65
<i>geothermicum</i>	rod	+	not rep	+	-	C <sub>3</sub> -C <sub>4</sub>	ethanol 54
<i>Thermodesulfobacterium commune</i>	rod	+	not rep.	+	-	-	70-85
environmental isolate strain V-16	spheres	+	+	+	-	-	pyruvate 64-92
T90A	rod	+	+	+	-	C <sub>3</sub> -C <sub>7</sub>	ethanol 43-78
T93B	rod	+	+	+	-	C <sub>3</sub> -C <sub>7</sub>	ethanol 43-78

The optimum growth temperature for thermophilic eubacterial SRB ranges from 55 to 75°C. Most of the t-SRB can even survive higher temperatures by spore formation. One of the first t-SRB isolated was *Thermodesulfobacterium commune* which grew on lactate and autotrophically on H<sub>2</sub>/CO<sub>2</sub> (Zeikus *et al.* 1983). Rosnes *et al.* (1991) isolated a spore forming t-SRB (strain T90A and strain T93B) from oil field production waters in the North Sea. The isolates grew autotrophically on H<sub>2</sub>/CO<sub>2</sub> and heterotrophically on fatty acids C<sub>1</sub>-C<sub>6</sub>, but not on acetate, pyruvate, lactate and on alcohols (C<sub>1</sub>-C<sub>3</sub>). The spores were found to be extremely heat resistant and survived up to 131°C.

The archaeobacterial t-SRB *Archaeoglobus fulgidus* (strain V-16) from thermal volcanic areas has the highest temperature optimum found yet for SRB of 83°C (Stetter *et al.* 1987). *Desulfotomaculum geothermicum* was isolated from saline geothermal groundwater (Daumas *et al.* 1988). It was able to oxidize fatty acids longer than acetate to CO<sub>2</sub> but was unable to grow on acetate. Until 1988 there were no acetate oxidizing t-SRB known. Nazina *et al.* (1988) isolated a spore forming acetate oxidizing t-SRB, strain 17, from underground thermal mineral waters. This organism has been assigned to a new species, *Desulfotomaculum kuznetsovii*, and has the ability to oxidize organic carbon completely to CO<sub>2</sub>. A medium containing propionate was most favorable for H<sub>2</sub>S formation. The cells were 1.0-1.4 µm in diameter, 3.5-5 µm long and flagellated. Two years later Min and Zehnder (1990) also reported an acetate utilizing t-SRB. *Desulfotomaculum thermoacidoxidans* is a spore forming bacterium isolated from a thermophilic (58°C) anaerobic bioreactor and capable of complete oxidation of acetate, lactate, and pyruvate.

### Inhibition of SRB

The most important inhibitors of SRB growth and activity are hydrogen sulfide and acetate or other volatile fatty acids. Both H<sub>2</sub>S and acetate are end products of SRB metabolism and can therefore reach high concentrations in the water.

#### Sulfide Inhibition

The inhibiting effect of sulfide on SRB primarily depends on the H<sub>2</sub>S concentration and on the pH in the water. Undissociated hydrogen sulfide (H<sub>2</sub>S) was found to be the most toxic sulfide species for SRB (Speece, 1983; Parkin and Speece, 1983; Reis, 1991). Okabe (1992) reported a 50 % inhibition of activity for *D. desulfuricans* grown on lactate at a concentration of total sulfide of 500 ppm. McCartney and Oleskiewicz (1991) reported a 50 % inhibition on activity of a mixed m-SRB consortium at 240 ppm total sulfide and 83 ppm undissociated hydrogen sulfide. Reis *et al.* reported a complete inhibition of SRB activity at 16.1 mM, at a pH of 6.2 to 6.7; Saleh *et al.* (1964) found total inhibition at 79 mM but did not give any pH indication; and Hauser and Holder (1986) found 100 % inhibition at a concentration of 9.4 mM. All authors reported sulfide inhibition to be reversible and SRB growth and activity can be restored as soon as the sulfide concentration is lowered. Okabe *et al.* (1992) pointed out that cell synthesis of *D. desulfuricans* was depressed at high sulfide concentrations (e.g the cell yield decreased from 0.04 at low total sulfide concentrations to 0.013 at 437 ppm-S) whereas the activity of sulfide production stayed constant up to 350 ppm H<sub>2</sub>S-S and only decreased slightly at a concentration of 437 ppm. The inhibition was described as non-competitive and the

stoichiometry for the catabolic reaction did not change in case of sulfide inhibition.

### Acetate Inhibition

Inhibition on acetate was reported to be significant for some SRB species at already low concentrations (Reis *et al.* 1991). Especially at low pH values inhibition by acetate was very effective.

### Competition Between SRB and Methanogenic Bacteria

In the absence of sulfate, methanogenesis is the dominating hydrogen oxidizing reaction. Next to acetate, which contributes about 70% of the methane formed in natural systems, hydrogen is the most important substrate for methane formation (Jorgensen, 1989).

Kinetic studies have shown that SRB have higher affinities for both hydrogen and acetate than do the methanogens (Kristjansson *et al.* 1982, Schonheit *et al.* 1982). Also, thermodynamically the SRB are at an advantage as they have a higher growth yield (Hunter 1989). However, Yoda *et al.* (1987) investigated the long term competition between methanogens and SRB in anaerobic biofilm systems using acetate as substrate. They found that SRB outcompeted the methanogens at low acetate concentrations. At high acetate concentrations, methanogens formed a biofilm faster due to a higher rate of adherence to the carrier material surfaces. And methanogens can win the competition

at high acetate concentrations (Isa *et al.* 1986). Hilton and Oleszkiewicz (1988) have shown that SRB are more sensitive to higher total sulfide concentrations when compared to methanogens, while both showed similar sensitivity to the unionized  $H_2S$  species. Therefore the authors concluded that at elevated total sulfide concentrations and high pH the methanogens should be able to outcompete the SRB for substrate. McCartney and Oleszkiewicz (1991) supported this hypothesis with the results of their experiments. They also observed an accumulation of propionate at  $H_2S$  concentrations above 3.5 mM, indicating an inhibition of propionate utilizing SRB. Reis *et al.* (1992) reported that SRB were very sensitive to low acetic acid concentrations whereas there was only an insignificant inhibition reported for low  $H_2S$  concentrations. A 50% inhibition of the SRB growth was obtained when the acetic acid concentration was 1 mM at a pH of 6.2 to 6.7. For higher pH values the inhibition was less significant. Methanogens can be adapted to very high acetate concentration (>500 mM) without showing significant inhibition effects (Temper *et al.* 1989). Therefore the environmental condition (e.g. pH; acetate and hydrogen sulfide concentration) as well as physical constraints (e.g. surface/volume ratio of the reactor system) will determine the competition outcome (Yadav and Archer, 1988).

#### Physical Factors Controlling the Rate and Activity of SRB in Souring Oil Reservoirs

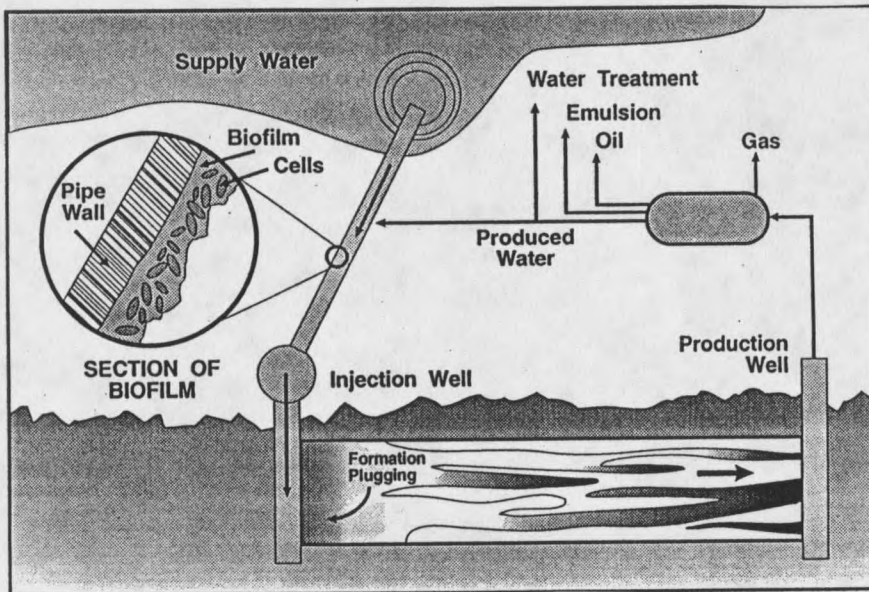
In North Sea reservoirs common pressure ranges from 200 to 500 bar and temperatures vary from 60 to 100°C (Rosnes *et al.* 1990). The initial reservoir conditions prior to well flooding at the Ninian North Sea oil field were 110-120°C and 450 bar pressure (Cochrane

*et al.* 1988). Areas close to the injection wellbores are subjected to a cooling effect and temperatures are as low as 40°C. For ARCO's Prudhoe bay oil reservoir, temperatures range from less than 20°C near seawater injection to about 100°C at the production wells. In the Kuparuk reservoir the maximum temperature is about 70°C (Burger *et al.* 1992). Hence, the environmental conditions throughout the reservoir are variable (Figure II.1 and Figure II.2).

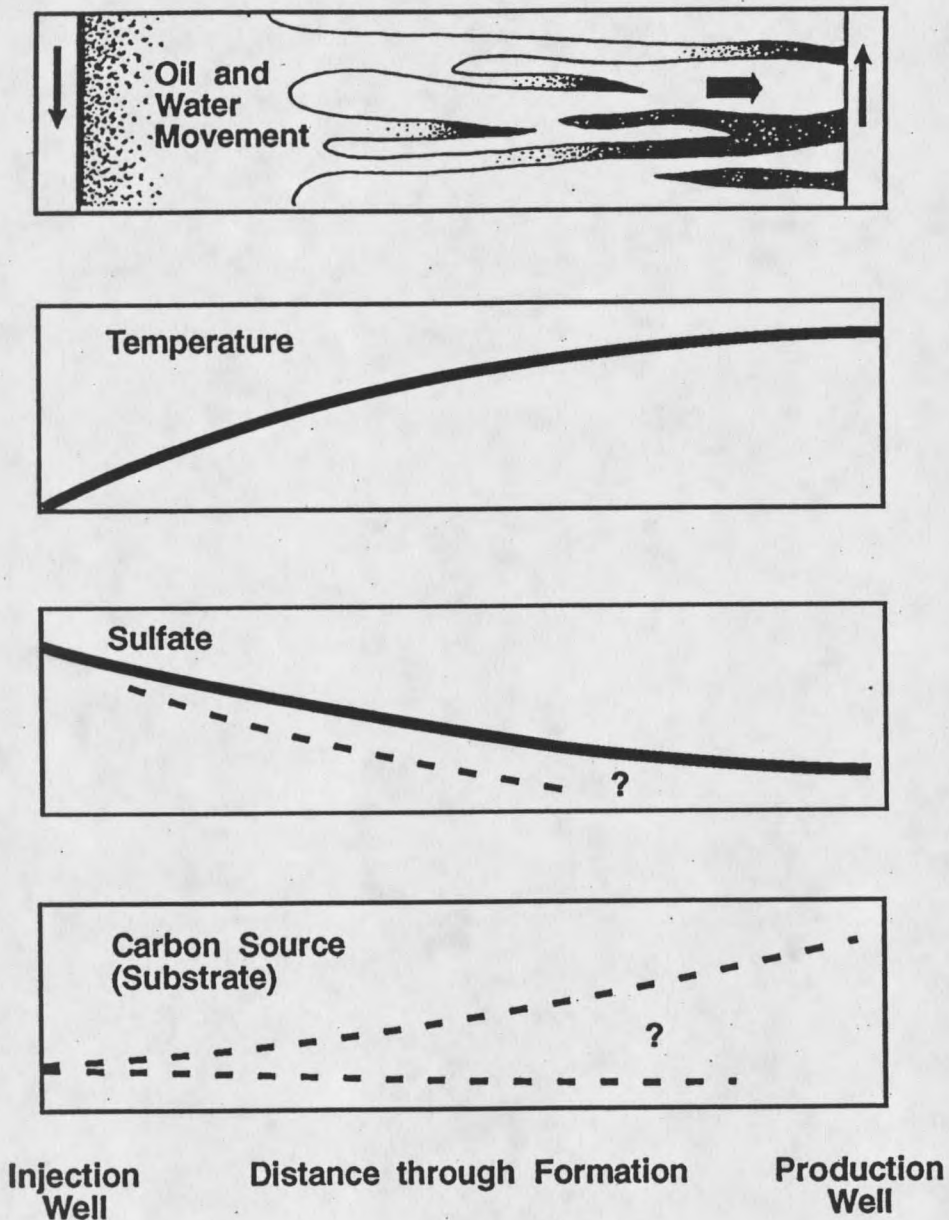
### Temperature

The temperature in the reservoirs ranges from 20°C to about 100°C, favoring m-SRB growth close to the injection well and growth of thermophilic and extremely thermophilic sulfate reducers closer to the production well. M-SRB are commonly found in seawater (Hardy and Hamilton, 1981; Burger *et al.* 1992; Herbert *et al.* 1985; Herbert and Stott, 1983). Rosnes *et al.* (1990) speculated that t-SRB may even be present in very low numbers in sea water. Injection well backflow studies have shown that SRB concentration and activity was highest around the injection wellbore and decreased with distance from the wellbore (Taylor *et al.* 1991). However, the authors found contrary data for hydrogen sulfide concentration with a seven fold higher H<sub>2</sub>S concentration further into the formation, indicating that the SRB activity occurs throughout the entire reservoir. This is supported by Herbert *et al.* (1985) who investigated the effects of mixing various ratios of formation water with injection seawater on the production of hydrogen sulfide. The SRB culture they used in their study was isolated from the North Sea. Sea water or formation water alone did not support SRB growth and activity, but when these two waters were mixed in

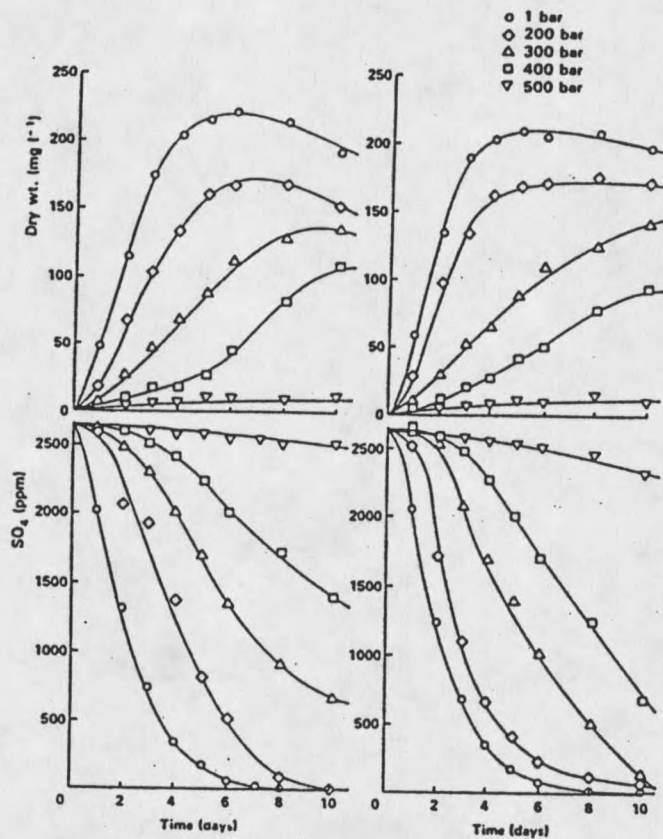
various ratios, considerable hydrogen sulfide generation was observed. The generation of  $H_2S$  was either controlled by carbon limitation (25% formation water + 75% sea water), or by sulfate limitation (75% formation water + 25% sea water). This finding supports the current understanding of souring to occur at a mixing zone, where the injection water, formation water, and the oil mix (Lighthelm *et al.* 1991, Burger *et al.* 1992, Frazer and Bolling, 1991). This mixing zone moves with the water front and therefore temperature increases as it moves toward the production well. M-SRB may be active at cooler (20 - 45°C) and t-SRB may be active at higher temperatures. Some of the SRB may be transported with the injection water, whereas others might be sessile within the formation.



**Figure II.1.** Schematic diagram of a souring oil field undergoing secondary oil recovery. Sea water is used for water injection. Produced water is separated from the oil phase and reinjected with additional sea water into the formation. The produced water is rich in organic carbon and SRB growth requirements are met. Hydrogen sulfide is produced inside the reservoir, and a considerable fraction of  $H_2S$  will be transferred to the oil phase.



**Figure II.2.** Environmental variations through an oil bearing formation under secondary recovery. Temperature, sulfate concentration and the concentration of assimilable nutrients can vary greatly between the injection well and the production well.



**Figure II.3** The effect of pressure on growth (top) and sulfate reduction (bottom) of two isolates (left and right) at 30°C using lactate as substrate.

### Pressure

There have only been a few studies on the effects of pressure in oil bearing formations (Marquis, 1976, Geverts *et al.* 1991; Herbert and Stott 1983). Marquis (1976) suggested that the basic effect of pressure is the enhancement of processes that result in volume decrease and inhibition of effects resulting in volume increase. The effect of high pressure on SRB growth and activity was investigated by Herbert and Stott (1983) Figure II.3. They used two m-SRB isolates from the Brent oil field in the N-sea. The maximum temperature

and pressure that supported growth and activity of these m-SRB were 45°C and 500 bar, respectively. The overall effect of pressure on bacteria is almost always inhibition of growth, though some individual processes may be stimulated. Many species will not grow above 200 bar and only very few species can grow above 600 bar (Herbert and Stott, 1983).

### Potential Control Mechanisms of Souring

Because of the detrimental effects of SRB as described earlier, the control of their growth and activity is important not only for the economical recovery of oil but also for many other water systems. A zero concentration of hydrogen sulfide might not even be the desirable goal in control, since H<sub>2</sub>S also acts as an excellent oxygen scavenger inside the formation.

### Chemical Control of Souring

Biocide. The most common approach is to inhibit the growth of SRB by the addition of biocides (e.g. glutaraldehyde) to the injection water (Eagar *et al.* 1988). These measures are often of limited effectiveness (Burger *et al.* 1992) since SRB might be associated in biofilms. Within a biofilm bacteria find a more protected environment for growth and activity (Van der Wende *et al.* 1989). In addition, biocides might also contain valuable nutrients (e.g. carbon, nitrogen, and phosphate) which might be utilized by SRB at lower

concentrations.

Nutrient removal is very difficult to achieve, because of the low cellular growth yield of the organism involved. Nitrogen and phosphorus concentration had to be in the ppb range to achieve the desired effect. Even if these low concentrations could be achieved by an expensive water treatment program, there might still be sources of nitrogen and phosphorus available inside the formation (Barth, 1991). The most promising direction for nutrient removal might be the removal of sulfate from the injection water. This could be achieved by chemical or biological processes. The produced hydrogen sulfide could then either be reoxidized to sulfate or to elemental sulfur (Isa *et al.* 1986; Buisman *et al.* 1990; Bos and Kuenen, 1983).

Aeration would inhibit SRB-activity, however, many SRB are capable of spore formation and can survive high oxygen levels and germinate again at more favorable environmental conditions. In addition, oxygen is a very undesirable agent in any metal bearing system due to high rates of corrosion of many metals (Tiller, 1983; Hamilton, 1986).

Product inhibition of sulfide seems to be dependent on hydrogen sulfide concentrations too high to influence the souring process effectively. The inhibition of SRB by acetate might be of interest. However, SRB are known to grow in many fields with very high acetate concentrations (up to 600 ppm as measured in the Kuparuk field).

Control of  $E_h$  or pH might be an interesting area to consider. Since pH determines the concentration of dissociated or undissociated hydrogen sulfide it will also determine its

effectiveness as inhibitor and the concentration of  $H_2S$  in the oil or gas phase. However, changing the pH in a oil formation might be a very difficult task because of strong carbonate and other buffer systems. (Barth, 1991).

Chemical scavenging of the produced  $H_2S$  might be another option to reduce the souring of oil (Littmann and McLean, 1987). Complete  $H_2S$  scavenging might be very difficult because of the poor mixing in the formation. This might also have the negative effect of actually increasing the rate of sulfide production by removing the end product which is toxic in high concentrations to the SRB.

#### Microbiological Control of Souring

Introduction of competitive bacteria was also considered (Burger *et al.* 1992). The competition of SRB and methanogens has been studied extensively for waste water treatment as well as in many natural environments (Lovley *et al.* 1982; McCartney and Oleszkiewicz, 1991; Widdel and Pfennig, 1982; Yoda *et al.* 1987, Abram and Nedwell, 1978). For specific environmental conditions, methane production can be favored over sulfate production and the methanogens could outcompete SRB for substrate. None of the competition studies however, were done at relevant environmental conditions (e.g. thermophilic temperatures and high pressure) for souring oil bearing reservoirs. Denitrifying bacteria grow more effectively and generally at a faster rate and could therefore outcompete SRB for substrate (Jenneman *et al.* 1986). A higher growth also will result in a higher cell yield which could cause extensive formation plugging. McInernay

*et al.* (1992) suggested a slightly different approach by introducing a chemoautotrophic denitrifier. *Thiobacillus denitrificans* oxidizes sulfide to sulfate using oxygen or nitrate as electron acceptor. One mutant was found to be resistant to 40 ppm glutaraldehyde and 1.5 mM total sulfide. This strain was reported to prevent the accumulation of hydrogen sulfide by *D. desulfuricans* when both strains were grown in liquid medium or in Berea sandstone cores. However, the maximum temperature this strain can grow was found to be 40°C and the maximum pressure was 120 bar. Salinity of 2% inhibited growth of the mutant strain to 80%. Even though the strain found by McInerney has many environmental restrictions which may eliminate its use in souring oil reservoirs, their approach seems promising for finding new mechanisms for the control of souring.

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CHAPTER III  
CHARACTERIZATION OF THERMOPHILIC CONSORTIA  
FROM TWO SOURING OIL RESERVOIRS

Introduction

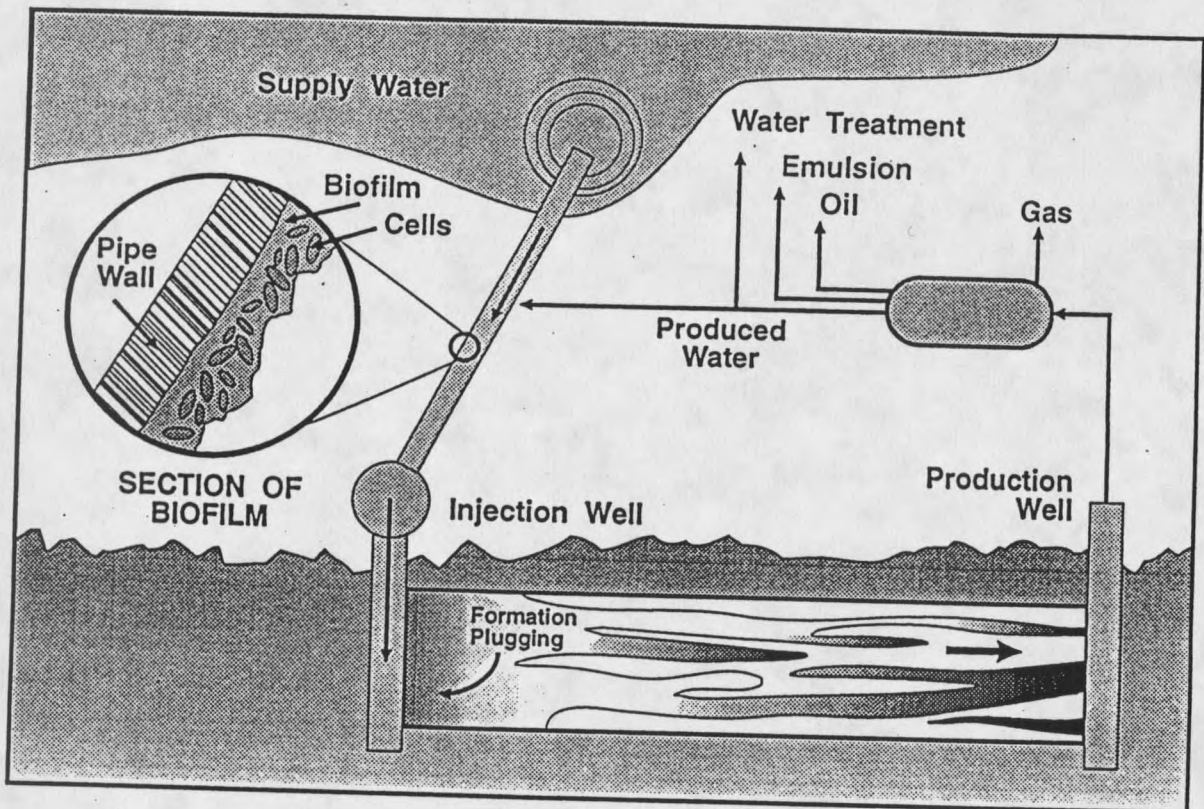
Souring of oil formations has become a serious problem for the oil industry in recent years. Injection of seawater into oilfield reservoirs for secondary oil recovery is common practice in Norwegian and British sectors of the North Sea as well as throughout the United States. The injection water contains large amounts of sulfate (approx. 1000 ppm  $\text{SO}_4\text{-S}$ , Herbert *et al.* 1985) as well as small amounts of other required nutrients such as nitrogen and phosphorus and trace elements such as iron or other metal elements. When seawater is injected into an oil bearing formation, production of hydrogen sulfide is found in many formations after some lag time. (Burger *et al.*, 1992; Frazer and Bolling, 1991).

Reservoir souring refers to the generation of hydrogen sulfide ( $\text{H}_2\text{S}$ ) in originally sweet oil reservoirs that have been subjected to (sea) water flooding (Figure III.1). Sea water is used for water injection in many fields undergoing secondary recovery, containing sulfate and other elements essential for bacterial growth. Produced water is separated from the oil phase and either reinjected with additional sea water into the formation or discharged. Especially when produced water is reinjected into the field, injection water is rich in organic carbon and SRB growth requirements are met (Tayler, 1993). Industrial problems associated with the hydrogen sulfide production are corrosion of iron in the absence of air (anaerobic corrosion) (Hamilton, 1985). The total cost related to SRB mediated corrosion is estimated to be 1-2 billion US dollars per year in the US alone (Lee, 1990). Precipitation of amorphous ferrous sulfide and polysaccharide

production by sulfate reducing bacteria or other microorganisms in the formation causes plugging and diminishes the effect of water injection wells (Dewar, 1986; Lappan and Fogler, 1990). Contamination of crude oil (Burger, 1992) and fuel gas with hydrogen sulfide (Pankhurst, 1968) decreases its value and increases refining costs (McInerney *et al.* 1991). Hydrogen sulfide is extremely toxic if inhaled; it easily escapes from contaminated waters and may accumulate under poorly ventilated conditions.

The current understanding of souring is that it occurs at a mixing zone, where the injection water, formation water, and the oil mix (Lighthelm *et al.* 1991, Burger *et al.* 1992, Frazer and Bolling, 1991). In this mixing zone the components supporting SRB growth -- electron donors such as organic compounds or hydrogen, electron acceptor such as sulfate, and other essential nutrients (such as nitrogen, phosphate, etc.) are present (Herbert *et al.* 1985). The mixing zone moves with the water front and therefore the temperature increases as it moves toward the production well. Mesophilic sulfate reducing bacteria (m-SRB) may be active at cooler temperatures (20 to 45°C) and thermophilic sulfate reducing bacteria (t-SRB) may be active at higher temperatures (45 to 80°C). Some of the bacteria may be transported with the injection water, whereas others might be sessile within the formation. Abiotic reactions are not considered important in the generation of hydrogen sulfide (Curd-Ruwish *et al.* 1987). They are important, however, in scavenging of H<sub>2</sub>S since many iron-containing minerals are capable of reacting with H<sub>2</sub>S to form iron sulfide, pyrite or pyrrhotite (Morse *et al.* 1987; Collins, 1975).

In this chapter results for substrate characterization for microbial consortia from two oilfields reservoirs at a temperature range from 35 to 75°C are presented. Competition of sulfate reducing bacteria and other groups of organisms occurring in oil reservoirs such as fermenters and methanogens will be discussed. In addition activity of thermophilic SRB is reviewed.



**Figure III.1.** Schematic diagram of a souring oilfield undergoing secondary oil recovery. Hydrogen sulfide is produced inside the reservoir, and a considerable fraction of  $H_2S$  is transferred to the oil phase. The temperature through the formation is variable, generally increasing towards the production well.

### Questions

What carbon and energy sources are present in a souring reservoir?

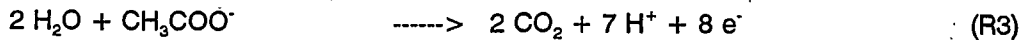
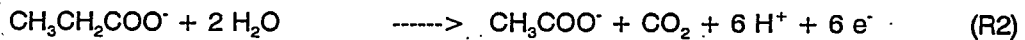
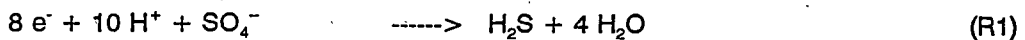
What groups of microorganisms are present in a souring oil reservoir?

What carbon and energy sources are utilized by these organisms?

How will temperature affect the rate of sulfate reduction and substrate utilization?

### SRB Metabolism

If organic matter enters stagnant or closed water systems (such as a souring reservoir) where gas exchange with the atmosphere is limited, dissolved oxygen may be consumed completely (Jorgenson, 1989). Initial biological decomposition of organic material is then carried out by fermentative bacteria. Most fermentative bacteria are facultative or obligate anaerobic. In fermentation, the overall oxidation state (e.g., energy state) of the degraded matter remains unchanged (disproportion of organic carbon). A part is converted into  $\text{CO}_2$ , and the remainder is converted to reduced products such as fatty acids, hydrogen and alcohols. In many natural anaerobic environments, the quantitatively most important fermentation products formed are  $\text{H}_2$ , formate, acetate, propionate, and butyrate (Sorensen *et al.* 1981, Lundegard and Kharaka, 1990). If sulfate is present under anaerobic conditions and the redox potential is sufficiently low ( $E_h < -100$  mV) these reduced fermentation products are used further by SRB. Sulfate is reduced to hydrogen sulfide (R1), whereas the organic materials are either oxidized incompletely to acetate and carbon dioxide (R2); or are oxidized completely to  $\text{CO}_2$  (R3; Widdel, 1988).



Most SRB can utilize sulfite or thiosulfate as alternative electron acceptors (Widdel, 1988; Nazina *et al.* 1988, Min and Zinder, 1990; Rosnes *et al.* 1991). Growth of SRB with elemental sulfur as an electron acceptor has been observed with some isolates. *Desulfovibrio desulfuricans* and some other *Desulfovibrio* strains, *Desulfobulbus propionicus* and *Desulfobacterium catecholicum* were shown to use nitrate as an electron acceptor, producing ammonium (reviewed in Widdel, 1988).

### Growth Requirements of SRB

**Oxygen:** Development of SRB can be expected wherever organic matter and sulfate in water are combined in an oxygen limiting environment. Significant SRB activity is measured in marine sediments, because of the high sulfate concentration of seawater (Fossing and Jorgensen, 1989). The produced  $H_2S$  is a strong reducing agent that reacts with oxygen at even low temperatures. Thus, once established, the SRB can protect themselves against oxygen (Cypionka *et al.* 1985).

**pH:** SRB prefer neutral pH for their growth, and activity can be observed over a range of pH5.5 to pH9. The metabolic products of SRB metabolism represent buffers such as  $HS^{-}/H_2S$  and the  $HCO_3^{-}/CO_2$  systems, which may help to keep the pH in their surrounding environment close to neutral and protect against extreme pH changes (Crolet *et al.* 1992; Curd- Ruwisch *et al.* 1987).

Salinity: SRB species originating from fresh water may be inhibited by more than 20 to 30 g l<sup>-1</sup> NaCl. In contrast, many marine SRB species are moderately halophilic (i.e. they require 10 to 30 g NaCl for optimum growth; Gevertz *et al.* 1991). The activity of most SRB declines drastically if the NaCl concentration exceeds 50 to 100 g/l (Postgate, 1984; Widdel, 1988).

### Physical Factors Controlling the

### Rate and Activity of SRB in Souring Oil Reservoirs

In North Sea reservoirs, pressure ranges from 200 to 500 bar and temperatures vary from 60 to 100°C (Rosnes *et al.* 1990). The effect of high pressure on SRB growth and metabolism was studied by Herbert and Stott, 1983. Generally, growth and activity decreased with increasing pressure. Areas close to the injection wellbores are subjected to a cooling effect and temperatures rarely exceed 40°C. For ARCO's Prudhoe bay oil reservoir, temperatures range from less than 20°C near seawater injection to about 100°C at the production wells (Burger, 1993). In the Kuparuk reservoir the maximum temperature is about 70°C (Burger *et al.* 1992). Hence, the environmental conditions throughout the reservoir are variable.

## Materials and Methods

### Sampling Procedure

The sampling was performed on site for each oil field. Approximately 200 liters of fresh, untreated produced oil/water mixture was concentrated to 2 liters of final sample volume at the Kuparuk field, and 70 liters of produced water were concentrated to 1 liter at the Ninian field. This was

achieved by using a membrane filter device (pelicon). A prefilter of 5  $\mu\text{m}$  pore size was used to eliminate oil and other particulates which would clog the filter membrane after a short time period. All sampling was performed using anaerobic techniques, including positive  $\text{N}_2$  pressure on all containers used. Approximately half of the final sample concentrate was filled into 1.6 ml vials and shock frozen in i-propanol and dry ice. All liquid transfers were carried out in an anaerobic glove box under 85/10/5%  $\text{N}_2/\text{H}_2/\text{CO}_2$  gas mixture. The frozen samples were transported in a well insulated container containing dry ice. Upon arrival in the laboratory the frozen samples were stored in a  $-70^\circ\text{C}$  freezer.

#### On-Site Test for Potential Substrates

One ml of the sample concentrate was injected directly on site into glass vials to test for utilization of various potential substrates. Residual oxygen was removed by alternation between vacuum and  $\text{N}_2$  pressurizing to remove residual oxygen from the vials. Then the vials were filled with 30 ml of 2% salinity SRB medium leaving 30 ml of headspace in the pressurized vials (2 bar). Different electron donors were added to the medium (Table III.4). Sulfate was present in the medium at an initial concentration of  $180 \text{ g m}^{-3}$  when tested for microbial sulfate reduction. Some vials did not contain sulfate to test for methanogenic activity. Four replicate vials were used for each substrate test plus a control where no organisms were added. The experiment was initiated at day 1 by incubating the on site inoculated vials at  $60^\circ\text{C}$  and terminated after day 31. Measurements for biomass (AODC), sulfate, hydrogen sulfide, formate, acetate, propionate, i-and n-butyrate, and lactate were taken on day 0, day 3, day 10, day 17, day 24 and day 31. The methane concentration in the headspace of the vials was measured on day 0, day 10, and on day 31. Redox potential and pH were measured on day 0, and on day 31. The test was called positive for microbial sulfate reduction if there was significant disappearance of sulfate, production of

hydrogen sulfide, disappearance of substrate, and production of cellular material.

#### Test for Potential Substrates using Frozen Samples

The same procedure as for on-site testing was performed when the frozen samples were tested for potential substrates. The frozen samples were defrosted in an anaerobic glove box under 85/10/5% N<sub>2</sub>/H<sub>2</sub>/CO<sub>2</sub> gas mixture. 1 ml of the defrosted sample was injected into each of the test vials.

#### Medium Preparation

The medium used was suggested by Pfennig *et al.* (1986) for marine type SRB. The medium was prepared with a variety of sterile stock solutions (see appendix). The sulfate concentration in the medium was 180 g m<sup>-1</sup> and salinity was 2% sodium chloride. A detailed medium composition is presented in the Appendix. After autoclaving, the medium was saturated with a prepurified gas mixture of 10 % CO<sub>2</sub>/ 90% N<sub>2</sub> to minimize contamination as well as to ensure an oxygen free environment. The pH was adjusted to 7.2 before use. Different electron donors were offered to the basic medium to test for substrate utilization (Table III.4). Each substrate was added at a initial concentration of 200 g m<sup>-3</sup>. Gases such as methane, hydrogen, and carbondioxide were added as a volumetric addition of 10 ml for each to the headspace of the vials.

### Microbial Analysis

Total cell counts were performed using the acridine orange direct count (AODC) method. Image analysis was used for enumeration of the cells as well as for a statistical cell size analysis (Mueller *et al.* 1992).

Most probable number (MPN) method (five tube assay) was used to differentiate between sulfate reducing bacteria (SRB), methanogenic bacteria, and general anaerobic bacteria (GAB). The media used were Postgate B with acetate, lactate, propionate, and butyrate as carbon sources, a broad methanogenic medium including hydrogen and carbon dioxide (Ward, 1977), and fluid thioglycolate (DIFCO), respectively.

Cell surface hydrophobicity. The test of bacterial adherence to hydrocarbons (BATH test), developed by Rosenberg *et al.* 1980 was adapted for use with image analysis (Mueller *et al.* 1992). Equal volumes of n-octane and hexadecane were added to the concentrated produced water sample under strict anaerobic conditions. The cell surface hydrophobicity is reported as the fraction of cells removed by hexadecane or octane, respectively, and is called the hexadecane or octane partitioning value, respectively.

### Chemical Analysis

Sulfate - 0.1 ml sample volume was added to 0.1 ml 10 % ZnAc. Distilled water was added to achieve the desired dilution. A Dionex ion chromatograph was used for analysis (Table 4). The detection limit for sulfate analysis was less than 0.1 ppm with a standard deviation smaller than 5% (Table III.1).

**Table III.1.** Ion chromatograph specification for analysis

Specification	Sulfate	vfa's
Column type	AS4A-SC	AS10
pore size	2 mm	4 mm
Detector	conduct.	conduct.
Eluent concentration	Na <sub>2</sub> CO <sub>3</sub> 5.00 mmol	K <sub>2</sub> B <sub>4</sub> O <sub>7</sub> 3.50 mmol
Regenerant concentration	none	K <sub>2</sub> B <sub>4</sub> O <sub>4</sub> 100 mmol
flow rate	0.5 ml min <sup>-1</sup>	1.0 ml min <sup>-1</sup>
suppressor liquid concentration	H <sub>2</sub> SO <sub>4</sub> 5 mmol	H <sub>2</sub> SO <sub>4</sub> 5 mmol

Hydrogen sulfide - 0.1 ml sample volume was added to 4.9 ml 1% ZnAc (1:50 dilution). 0.4 ml diamine reagent was added to the solution and after 20 minutes the absorbance was measured at 670 nm (Cline, 1969). The detection limit for H<sub>2</sub>S was smaller than 50 ppb, with a standard deviation of less than 2 % for identical samples.

Volatile fatty acids (VFA's) -The diluted and filtered sample was directly used for analysis on an Dionex ion chromatograph (Table III.1). Detection limits for acetate, propionate, i-butyrate, and n-butyrate were smaller than 2 ppm. The detection limit for formate was smaller than 4 ppm. The standard deviation among identical samples was less than 5%.

Lactate was detected using an enzymatic assay kit from Sigma (Ching-I Chen *et al.* 1993).

Methane was measured in the headspace of the test vials using a Perkin Elmer chromatograph equipped with a Flame ionization detector and a chromosorb stainless steel column. The methane concentration is reported as volume fraction of the total gas volume in the headspace.

Redox Potential was measured using a redox electrode housed in a 16 gage stainless steel needle. A double junction calomel electrode was used as a reference.

## Results

### Consortia Collection

Selection of the Sampling Sites. Arco's Kuparuk field on Alaska's North slope and Chevron's Ninian off-shore field in the North Sea were chosen for sampling of microbial consortia. Both fields are presently producing hydrogen sulfide. The rationale for choosing these sites was that previous work on characterization had been done by Arco (Burger *et al.*, 1992; Frazer and Bolling 1991) and because there were important differences in operation between the two tested sites. The Kuparuk field was H<sub>2</sub>S free from 1981 (startup) until seawater flood started in 1985. The produced water is separated from the oil phase and reinjected into the reservoir with fresh sea water. Hydrogen sulfide was first detected in 1986 in five wells with a maximum concentration of 40 g m<sup>-3</sup>. During the 1988 to 1990 period all wells with significant seawater breakthrough have soured. At the Ninian field, water flooding was used from the beginning of the operation and was accompanied with the production of hydrogen sulfide. At the Ninian field, fresh seawater is used for water flooding. The produced water is discharged after separation from the oil phase. The

initial reservoir conditions prior to well flooding at the Ninian North Sea oil field were 110-120°C and 450 bar pressure (Cochrane *et al.* 1988).

**Produced water chemistry.** The water chemistry was determined for filtered produced water from Arco's Kupa-ruk field and from Chevron's Ninian field. The water chemistry varied significantly between the two tested fields. The Kupa-ruk field produced water exhibited a ratio of acetate to propionate to butyrate of approximately 100:10:1 (Table III.2). The produced water from the Ninian field contained acetate and propionate measured at concentrations of approximately 1/10 of that from Kupa-ruk. The concentration of sulfate varied with 98 g m<sup>-3</sup> SO<sub>4</sub>-S in the produced water of the Kupa-ruk field compared to 1000 g m<sup>-3</sup> SO<sub>4</sub>-S for the Ninian field. The low sulfide concentration measured for both fields might result from precipitation in the formation and transfer of hydrogen sulfide in the water phase to the oil and gas phase. The pH was slightly higher in the Kupa-ruk field. The produced water temperature during sampling was 68°C at Kupa-ruk and 74°C at Ninian.

**Table III.2.** Chemical composition of produced water sampled from Arco's Kupa-ruk oilfield and Chevron's Ninian oilfield.

Parameter	Unit	Kupa-ruk	Ninian
Acetate	(g m <sup>-3</sup> )	600	50
Propionate	(g m <sup>-3</sup> )	74	8
Butyrate	(g m <sup>-3</sup> )	8	N.D.*
Sulfate-S	(g m <sup>-3</sup> )	98	1050
Hydrogen sulfide-S	(g m <sup>-3</sup> )	6	2
pH		7.8	7.2
Chloride	(g m <sup>-3</sup> )	13400	16000

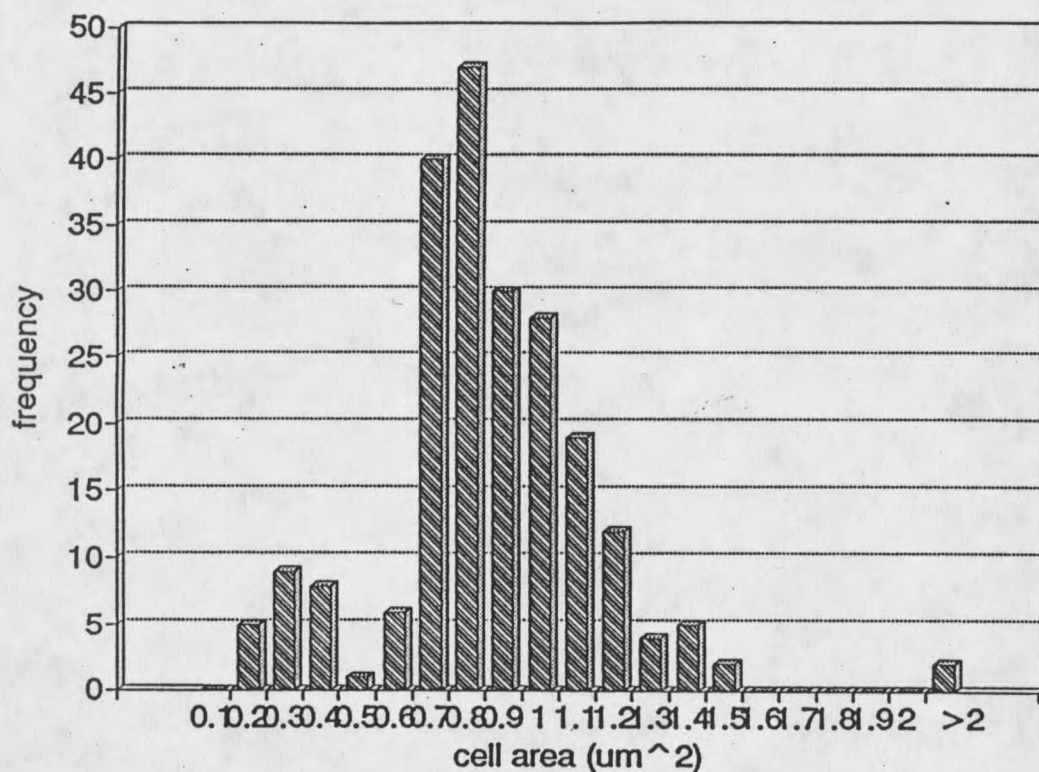
\*: Not detected with the method used for analysis

**Microbial Composition.** At both fields, the microbial consortia consist of a wide variety of anaerobic organisms, including sulfate reducing bacteria, methanogenic bacteria and general anaerobic bacteria. The microbial density (based on AODC-test) was considerably higher at Kuparuk ( $4.5 \cdot 10^4$  cells ml<sup>-1</sup>) as compared to the Ninian produced water ( $1.21 \cdot 10^3$  cells ml<sup>-1</sup>). Viable MPN-tests for general anaerobic bacteria (GAB) from Kuparuk were about a third of the total number of bacteria measured by the AODC method (Table III.3). At 35°C the numbers of methanogens were about 20 times higher than the numbers obtained for m-SRB. The concentration of thermophilic (tested at 60°C) methanogens were only slightly lower than for t-SRB. Approximately 4% of the total number of bacteria present were detected as viable t-SRB.

Statistical analysis (t-test) on cell size distribution of the Kuparuk consortium indicated a clear distinction between three morphologically different organisms (Figure III.2). The populations (pop1, pop2, pop3) are listed in order of size in Table III.3 and contributed 6.4, 92.8 and 0.8 percent, respectively, to the total cell numbers.

**Table III.3.** Microbial composition of produced water from the Kuparuk oil field.

total cell number (AODC)	$4.5 \pm 0.4 \cdot 10^4$	cells/ml
cell size distribution (pop 1)	$0.28 \pm 0.08$	$\mu\text{m}^2$
cell size distribution (pop 2)	$0.87 \pm 0.18$	$\mu\text{m}^2$
cell size distribution (pop 3)	$12.45 \pm 1.4$	$\mu\text{m}^2$
mesophilic (35°C)		
general anaerobic bacteria (MPN)	$1.4 \cdot 10^4$	cells/ml
sulfate reducing bacteria (MP)	$1.2 \cdot 10^2$	cells/ml
methanogenic bacteria (MPN)	$2.4 \cdot 10^3$	cells/ml
thermophilic (60°C)		
general anaerobic bacteria (MPN)	$1.1 \cdot 10^4$	cells/ml
sulfate reducing bacteria (MPN)	$1.6 \cdot 10^3$	cells/ml
methanogenic bacteria (MPN)	$0.9 \cdot 10^3$	cells/ml



**Figure III.2.** Histogram of the cell size distribution using acridine orange as a cell stain. The sample measured was taken from produced water at ARCO's Kuparuk river field in Alaska.

A test of bacterial adhesion to hydrocarbons (BATH test) was performed for the concentrated produced water sample from Kuparuk. Hydrocarbon partitioning was very low and statistically not significantly different from 0 for octane and hexadecane. Hence the organisms within the consortium sampled were of hydrophilic nature. Hydrophobic bacteria may have been attached to oil particles and were therefore removed by the prefilter (5  $\mu\text{m}$  pore size).

#### Characterization of Potential Substrates Supporting Microbial Activity

A variety of organic compounds as well as hydrogen/carbon dioxide were tested for their use as potential substrates for microbial sulfate reduction and methanogenesis (Table III.4). The goal was to determine which substrates favored bacterial growth and which metabolic end products (e.g.,

H<sub>2</sub>S, CH<sub>4</sub>, acetate) were formed. In addition, it was determined whether bacteria from the frozen stock cultures behaved similarly to the unfrozen cultures which were tested on-site at the Kuparuk field.

Potential substrates for microbial sulfate reduction and methanogenesis were tested for both consortia. On-site inoculum was compared to the inoculum of the same consortium as a frozen sample. Microbial growth correlated with sulfate reduction, the production of hydrogen sulfide, and the disappearance of electron donor for hydrogen/carbon dioxide, methane, formate, propionate, butyrate, and lactate. When lactate, propionate, or butyrate were utilized, acetate was produced. Acetate was not oxidized for sulfate reduction at the temperature and time period measured. Saturated fatty acids such as caprylate, stearate and palmitate were utilized for sulfate reduction, unsaturated fatty acids such as oleate or hydrocarbons such as hexadecane were not utilized for sulfate reduction or methanogenesis.

Sulfate reduction was correlated with an increase in pH and a decrease in redox potential. The pH in fresh SRB medium was adjusted at 7.2 and the redox potential was  $E_h = -80$  to  $-100$  mV. After all sulfate initially present in the medium was reduced, the pH was measured at pH 8 to 8.8 and the redox potential was between  $E_h = -340$  and  $-400$  mV.

The samples tested for methanogenic activity showed small amounts of methane produced (< 1%) in all vials inoculated directly at the Kuparuk site. When sulfate was present, methane production from hydrogen/carbon dioxide was measured at 5 to 10 % methane in the headspace (Table III.4). When no sulfate was present the methane production from H<sub>2</sub>/CO<sub>2</sub> increased up to 72% in the headspace. When butyrate or lactate were present methane production was observed to a lower degree ( 5 and 8% methane in the headspace, respectively). There was no significantly higher methane production observed between sulfate present or absent when formate or acetate

were offered as substrate.

In contrast to the Kuparuk consortium, no methanogenesis was observed in any test vials from the Ninian field.

**Table III.4.** Comparison of the on site tested (fresh) potential microbial activity with respect to various substrates.

Sample origin	Kuparuk field				Ninian field	
	SO <sub>4</sub> -reduction		CH <sub>4</sub> -production		SO <sub>4</sub> -reduction	CH <sub>4</sub> -production
culture type:	fresh	frozen	fresh	frozen	fresh	fresh
tested carbon/ energy source						
prod. water	0	n.t.	+	n.t.	0	0
formate	+	+	0	0	+	0
acetate	0	0	0	0	0	0
propionate	+	+	0	0	+	0
n-butyrate	+	+	0	0	+	0
i-butyrate	+	+	+	0	+	0
lactate	+	+	+	0	+	0
caprylate	+	n.t.	0	n.t.	n.t.	n.t.
hexadecane	0	n.t.	0	n.t.	n.t.	n.t.
oleate	0	n.t.	n.t.	n.t.	n.t.	n.t.
stearate	+	n.t.	n.t.	n.t.	n.t.	n.t.
palmitate	+	n.t.	n.t.	n.t.	n.t.	n.t.
H <sub>2</sub> /CO <sub>2</sub>	+	+	+	+	+	0
methane	+	0	n.t.	n.t.	+	n.t.

Table legend: n.t.: not tested; 0 : very little or no activity  
+ : substrate activates sulfate reduction or methanogenesis, respectively.

The test for potential substrates was repeated after three months with frozen samples from the Kuparuk field. Similar results were observed for all tested substrates for sulfate reduction with one exception: The sulfate reducing methane oxidizers were damaged and we were unable to recover them from frozen cultures (Table III.4). Methanogens utilizing H<sub>2</sub> as electron donor were recovered

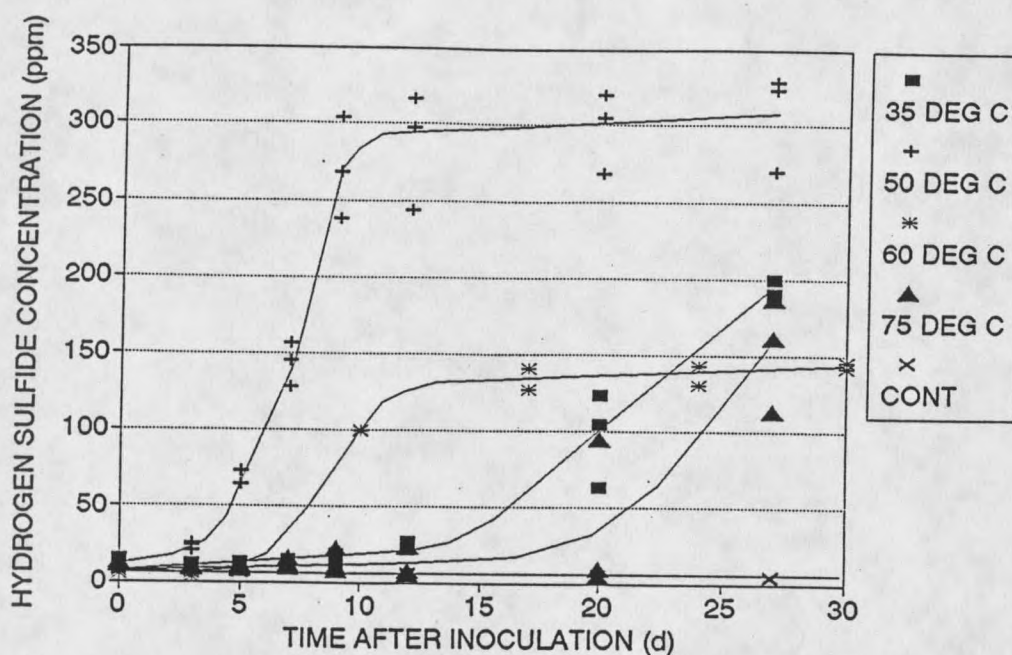
after freezing. Methane production from butyrate or lactate was not observed.

Sulfate reducers and methanogens seem to be unaffected or less damaged by the freezing process as compared to fermentative or acetogenic organisms.

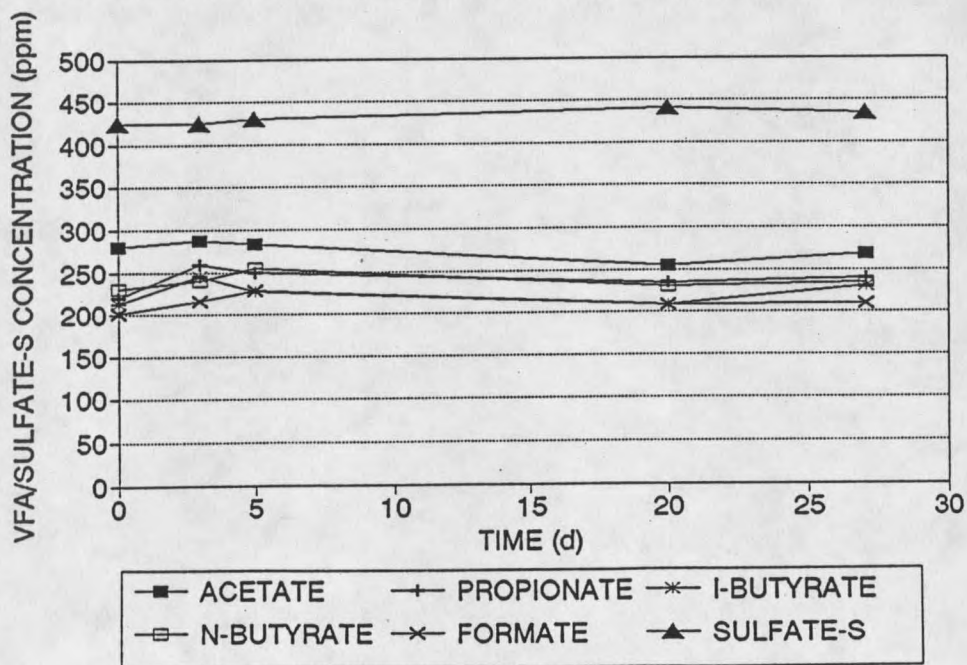
### The Effect of Temperature on Microbial

#### Sulfate Reduction and Substrate Utilization

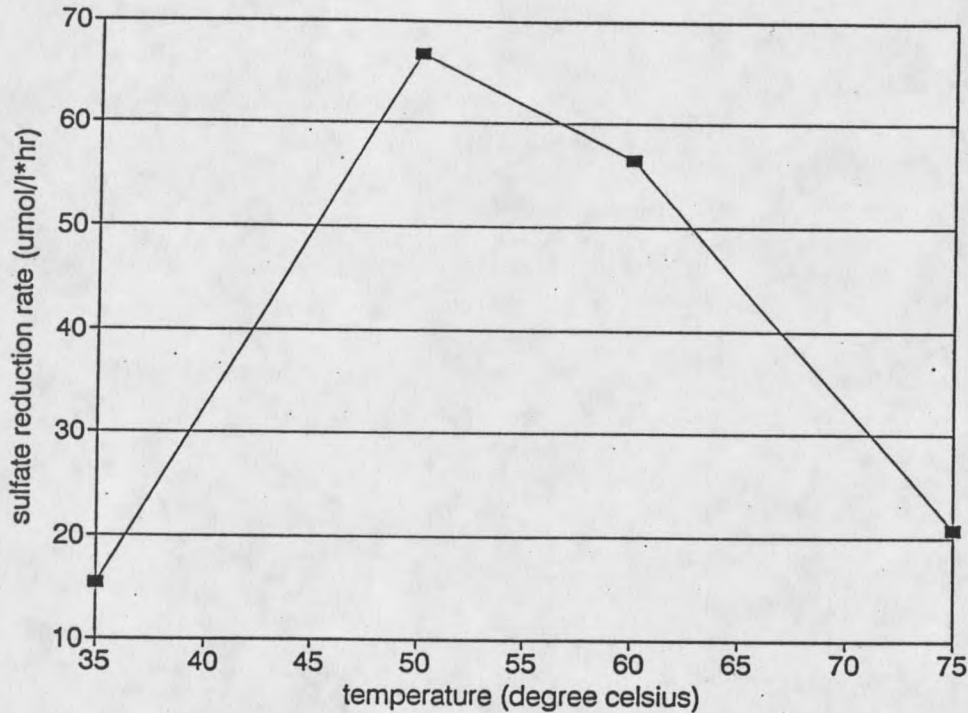
The influence of temperature on the microbial sulfate reduction was tested at 35, 50, 60, and 75 degrees Celsius. Hydrogen sulfide concentration was plotted versus time for each test vial (Figure III.3). The microbial consortium from the Kuparuk field was used for this study. Each temperature experiment was carried out in triplicate. A mixture of VFA (C1-C4) each at an initial concentration of 200 ppm were offered as substrates. One ml frozen stock culture + 1 ml from an enrichment culture growing in log phase at 60°C was injected into each vial. Abiotic controls at 75°C showed no conversions (Figure III.4). Sulfate reduction, the production of hydrogen sulfide and the uptake of vfa's was observed in all inoculated test vials and for all temperatures tested. The rate of sulfate reduction ( $H_2S$ -production rate) was influenced by the temperature (Figure III.5). The maximum rate for sulfate reduction was observed between 50 and 60°C. At lower or higher temperatures sulfate reduction occurred at a slower rate, and a longer lag phase before the maximum rate of reduction was reached. This long lag phase may indicate an adaptation period to the new temperature. The number of detected viable mesophilic SRB present in the frozen inoculum was approximately one order of magnitude lower than thermophilic SRB (Table III.3) and a longer lag phase should be expected.



**Figure III.3.** The influence of temperature on the microbial hydrogen sulfide production. A mixture of vfa's such as formate, acetate, propionate, and butyrate were offered to the mixed population from the Kuparuk produced water. The temperatures tested were 35, 50, 60, and 75°C. At 75°C a control experiment without bacteria inoculum was performed.



**Figure III.4.** No conversions were observed for sulfate or vfa's in the control experiment at 75°C.



**Figure III.5.** The rate of microbial sulfate reduction as a function of temperature.

The morphology of the microbial consortium appeared significantly different at each temperature. At 35°C rods of .3-.6 µm diameter and 1.5-3 µm length dominated, whereas at 50°C there were primarily filamentous looking organisms. At 50°C there was also more extracellular material produced. The dominant organisms at 75°C were small cocci with a diameter of 0.2 to 0.5 µm. The temperature dependent morphology may indicate a large variety of organisms present, capable of sulfate reduction at various temperatures in the consortia sampled.

Substrate Utilization at Various Temperatures. The rate of utilization of specific substrates was dependent on the temperature (Figure III.6). The rate of formate, acetate, and n-butyrate utilization varied with temperature, whereas the rate of propionate and iso-butyrate oxidation was relatively constant at all tested temperatures.

At 35°C formate was consumed at the fastest rate of  $11.3 \mu\text{mol l}^{-1} \text{h}^{-1}$ . Formate was depleted 27 days after inoculation (Figure III.7). Longer vfa were utilized at a slower rate ( $3\text{-}5 \mu\text{mol l}^{-1} \text{h}^{-1}$ ). Every vfa offered was consumed when sulfate was reduced.

At 50°C formate, n-butyrate, and sulfate were depleted 12, 20, and 27 days after bacteria inoculum, respectively (Figure III.8). The rates of formate and n-butyrate consumption were the highest measured ( $23.4$  and  $11.5 \mu\text{mol l}^{-1} \text{h}^{-1}$ , respectively). Propionate and iso-butyrate were consumed at  $4.4$  and  $1.4 \mu\text{mol l}^{-1} \text{h}^{-1}$ . Acetate was accumulating initially, but was utilized after the prime energy and carbon sources (formate and n-butyrate) were depleted. The maximum concentration of sulfide measured was 330 ppm, but even this high concentration did not indicate a slower process for sulfate reduction (Figure III.3).

At 60°C the rates of formate and n-butyrate utilization were high ( $13.6$  and  $9.3 \mu\text{mol l}^{-1} \text{h}^{-1}$ , respectively), propionate and i-butyrate were utilized at lower rates ( $4.4$  and  $1.4 \mu\text{mol l}^{-1} \text{h}^{-1}$ , respectively; Figure III.9). Acetate was produced from incomplete oxidation of butyrate and propionate at a maximum rate of  $13.4 \mu\text{mol l}^{-1} \text{h}^{-1}$ .

At 75°C all vfa's offered were utilized for sulfate reduction following a lag phase of 12 days after consortia inoculum (Figure III.10). Formate was oxidized at a maximum rate of  $20.7 \mu\text{mol l}^{-1} \text{h}^{-1}$ . Longer chain vfa's were utilized at a lower rate ( $2.0$  to  $7.4 \mu\text{mol l}^{-1} \text{h}^{-1}$ ).

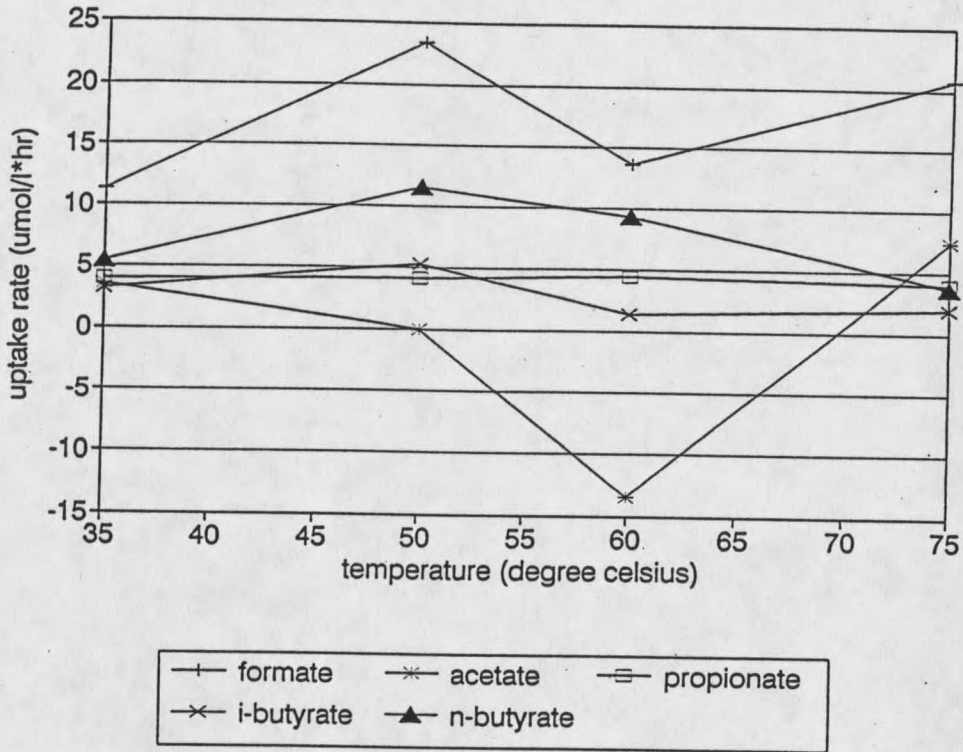


Figure III.6. The rate of substrate oxidation for formate, acetate, propionate, i-, and n-butyrate as a function of temperature.

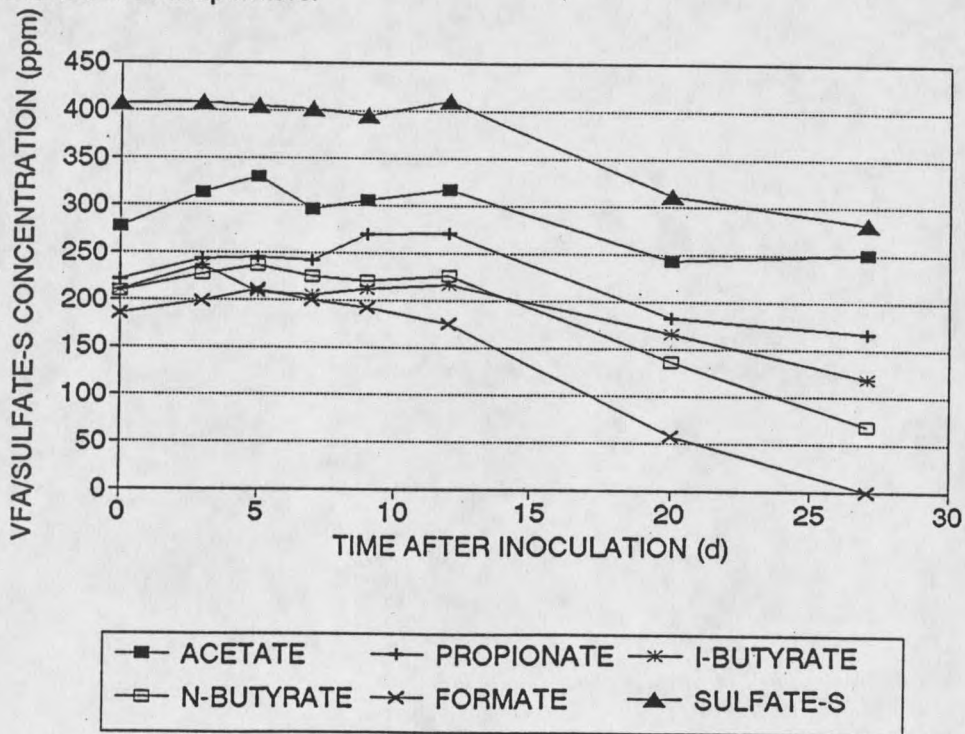
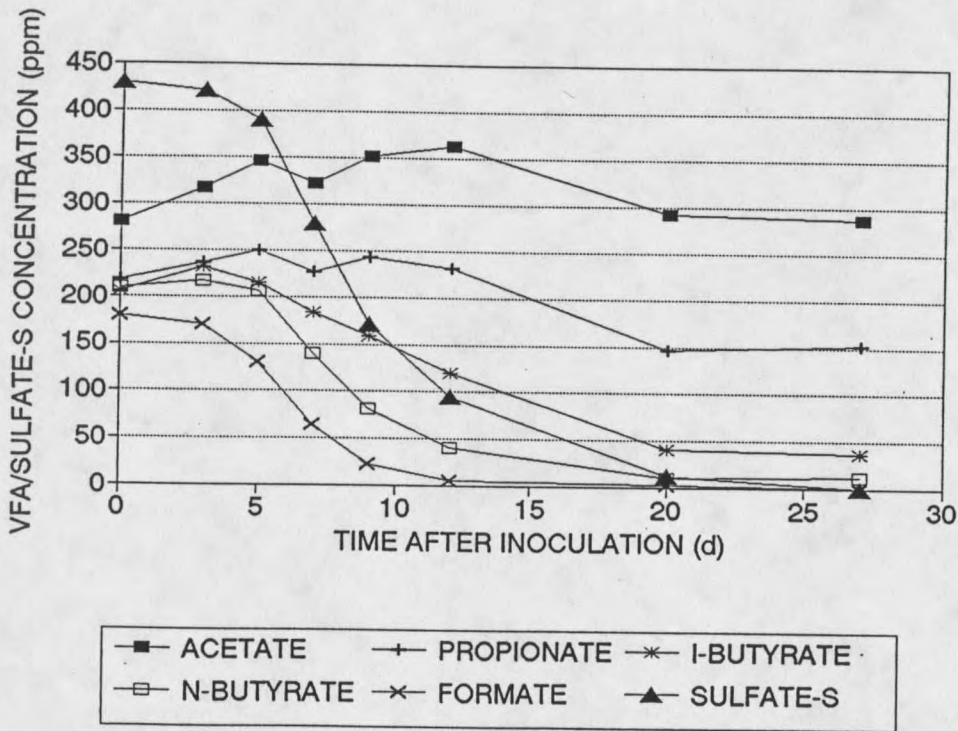
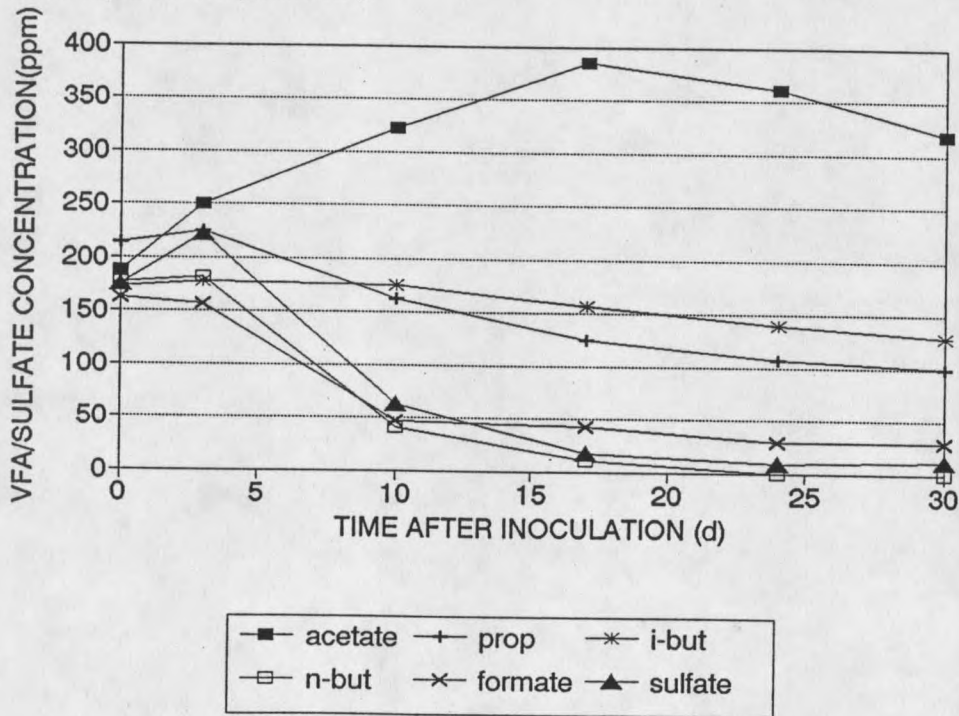


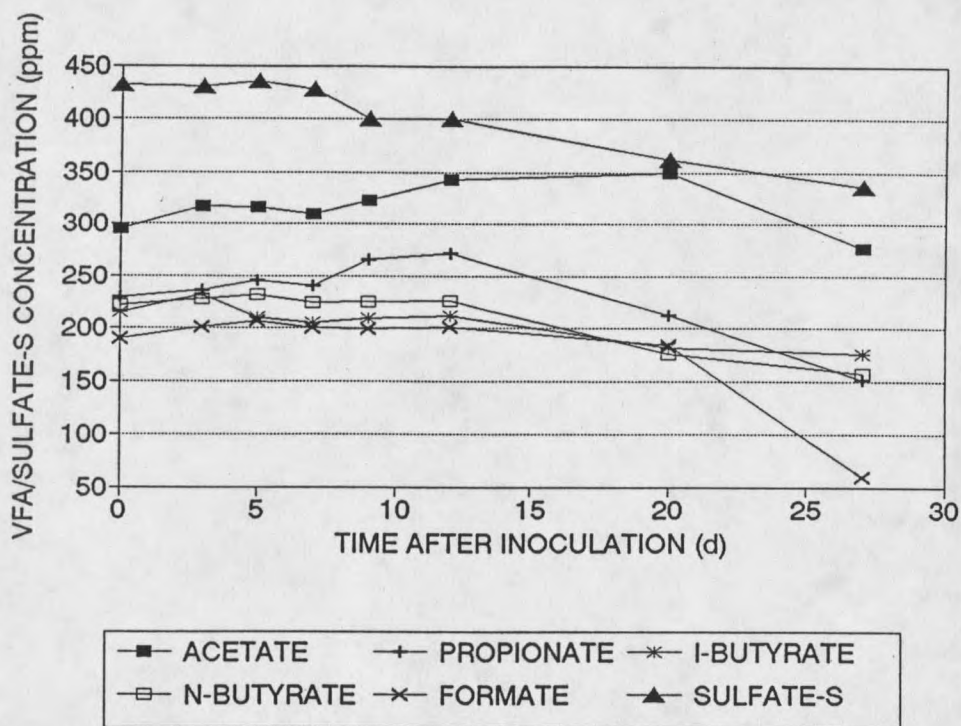
Figure III.7. Concentrations of sulfate and vfa's vs time after inoculum for 35°C (sample means from three replicative experiments).



**Figure III.8.** Concentrations of sulfate and vfa's vs time after inoculum for 50°C (sample means from three replicative experiments).



**Figure III.9.** Concentrations of sulfate and vfa's vs time after inoculum for 60°C (sample means from three replicative experiments).



**Figure III.10.** Concentrations of sulfate and vfa's vs time after inoculum for 75°C (sample means from three replicative experiments).

### Discussion

The microbial consortia sampled from both oil fields were capable of microbial sulfate reduction, producing hydrogen sulfide when the required nutrient composition was offered at relevant environmental conditions (35 and 75°C). Sulfate reduction was a proton consuming process resulting in an pH increase. Once the SRB were active they produced a reducing environment as indicated by the lowering of the redox potential. The consortium from the Kuparuk field was capable of methane production from hydrogen and carbon dioxide at mesophilic as well as thermophilic temperatures. A wide variety of substrates was used for microbial sulfate reduction at 60°C. Sulfate reduction was observed in a high salinity (2%) medium after consortia inoculation for hydrogen/carbon dioxide, lactate, formate, butyrate, propionate, and methane. Saturated

(stearic acid and palmitic acid) and unsaturated fatty acids (oleic acid) might be associated with the crude oil and dissolve in formation water due to the water/oil partitioning at the mixing zone. The utilization of these long chain fatty acids for sulfate reduction is probably not due to a direct oxidation by SRB's. Fermentative bacteria may play an important role in the utilization of these compounds. A hydrophobic substrate (hexadecane) was not consumed by any group of organisms within the microbial consortia. The reason for this may have been the specific sample procedure used in this study. Bacterial adhesion is a crucial process for degrading a hydrophobic substrate. With the sampling procedure used (prefiltration) we may have removed a large fraction of the hydrocarbon degrading bacteria.

Hydrogen, lactate, butyrate, and formate were the most favorable energy sources for sulfate reducing bacteria. When a variety of vfa's was offered to the Kuparuk consortium, butyrate was consumed prior to any other vfa. Formate was consumed prior to any other vfa by the consortium from the Ninian field.

The oxidation of butyrate and propionate was always associated with the production of acetate and we may speculate that butyrate and propionate are oxidized incompletely to acetate and carbon dioxide and donate their electrons only partly to the reduction of sulfate.

Lactate and pyruvate are commonly used as carbon substrates for enrichment, isolation and cultivation of most types of sulfate reducing bacteria. Sorenson (1981) introduced the idea of short chain fatty acids being important energy carriers for SRB in anaerobic environments. Generally, SRB are much more restricted than fermentative organisms with respect to their possible electron donors. Whereas most fermentative organisms decompose complex compounds (e.g., polymers such as cellulose, proteins, or longer chain hydrocarbons), SRB have not been shown to use polymeric substrates directly with one exception where an environmental SRB

hydrocarbons as electron source for sulfate reduction (Aeckersberg *et al.* 1991). Typical nutrients for SRB in natural systems are simple compounds of low molecular weight, such as fermentation products. Also hydrogen can be used by most SRB as energy source growing chemoautotrophically. Jorgensen (1989) states that hydrogen is the most important inorganic energy carrier in anaerobic pathways in nature. Therefore, SRB in natural environments depend on fermentative bacteria that cleave and ferment the more complex organics to low molecular weight compounds (Jorgensen, 1989).

In our experiments volatile fatty acids and hydrogen were the key substrates for microbial sulfate reduction. This was a consistent result for either field tested as well as with the results reported by others. Acetate was not consumed at the temperature and time frame tested but was produced when longer chain fatty acids were utilized. The high acetate concentration measured in the produced water from both tested oil fields might be a result from the utilization of longer chain vfa's by SRB in the reservoir. Especially at Arco's Kuparuk field, the high acetate concentration is associated with a low sulfate, low butyrate and intermediate propionate concentration. This could indicate that most of the sulfate reducing bacteria were not capable of a complete oxidation of propionate or butyrate to CO<sub>2</sub> at reservoir conditions.

Rosnes *et al.* (1991) isolated sulfate reducing bacteria from an oilfield in the North Sea. They found activity on hydrogen, lactate, ethanol, formate, and longer chain fatty acids with 3-7 carbon chains. No activity was reported on acetate. All of the reported thermophilic SRB are capable of utilizing hydrogen chemoauto-trophically (Table III.5). In addition all of the reported isolates were capable of growing on lactate and formate. Acetate utilization was only reported for two thermophilic *Desulfotomaculum* strains. *Desulfotomaculum kutznetsovii* was a spore forming isolate from underground thermal mineral waters and was the first thermophilic SRB found capable of complete organic carbon oxidation (Nazina *et al.* 1989). Two years later Min and Zehnder (1990)

also reported an acetate utilizing SRB. *Desulfotomaculum thermoacidoxidans* are spore forming bacteria isolated from a thermophilic (58°C) anaerobic bioreactor and are also capable of complete oxidation of acetate, lactate, and pyruvate. However, most thermophilic SRB seem to be capable of utilizing substrates as electron donors similar to what was found in our experiments. The most favorable electron donors for thermophilic sulfate reduction are hydrogen, lactate, formate, propionate, butyrate, and in some cases even longer chain fatty acids up to C<sub>7</sub>.

Methanogenic activity did depend on the production of hydrogen by fermenters. The produced hydrogen was used as a prime electron source for SRB and methanogens. Hence, methanogens competed with SRB's for hydrogen. The outcome of this competition could be controlled by the sulfate concentration. This competition outcome is supported by results from others (Krisjansson *et al.* 1982; Loveley *et al.* 1982; Schonheit *et al.* 1982). Conrad *et al.* 1986 determined the Gibb's free energies for microbial H<sub>2</sub> dependent exergonic reactions and found the following sequence for favorable microbial processes: sulfate reduction > methanogenesis > acetogenesis. The methanogenic activity in the absence of sulfate was high, but was suppressed with increasing sulfate concentrations. However, at a concentration of 180 g m<sup>-3</sup> SO<sub>4</sub>-S a small production of methane was still measured.

In the absence of sulfate, methanogenesis is the dominating hydrogen oxidizing reaction. Next to acetate, which contributes about 70% of the methane formed in natural systems, hydrogen is the most important substrate for methane formation (Jorgensen, 1989). Kinetic studies have shown that SRB have higher affinities for both hydrogen and acetate than do the methanogens (Krisjansson *et al.* 1982, Schonheit *et al.* 1982,). Also, thermodynamically the SRB are at an advantage as reflected in a higher growth yield. Methanogens can "win" the competition only at specifically favorable conditions. Hilton and Oleszkiewicz (1988) have shown that SRB are more sensitive to higher total sulfide concentrations when compared to methanogens, while both

**Table III.5.** Characteristics of t-SRB

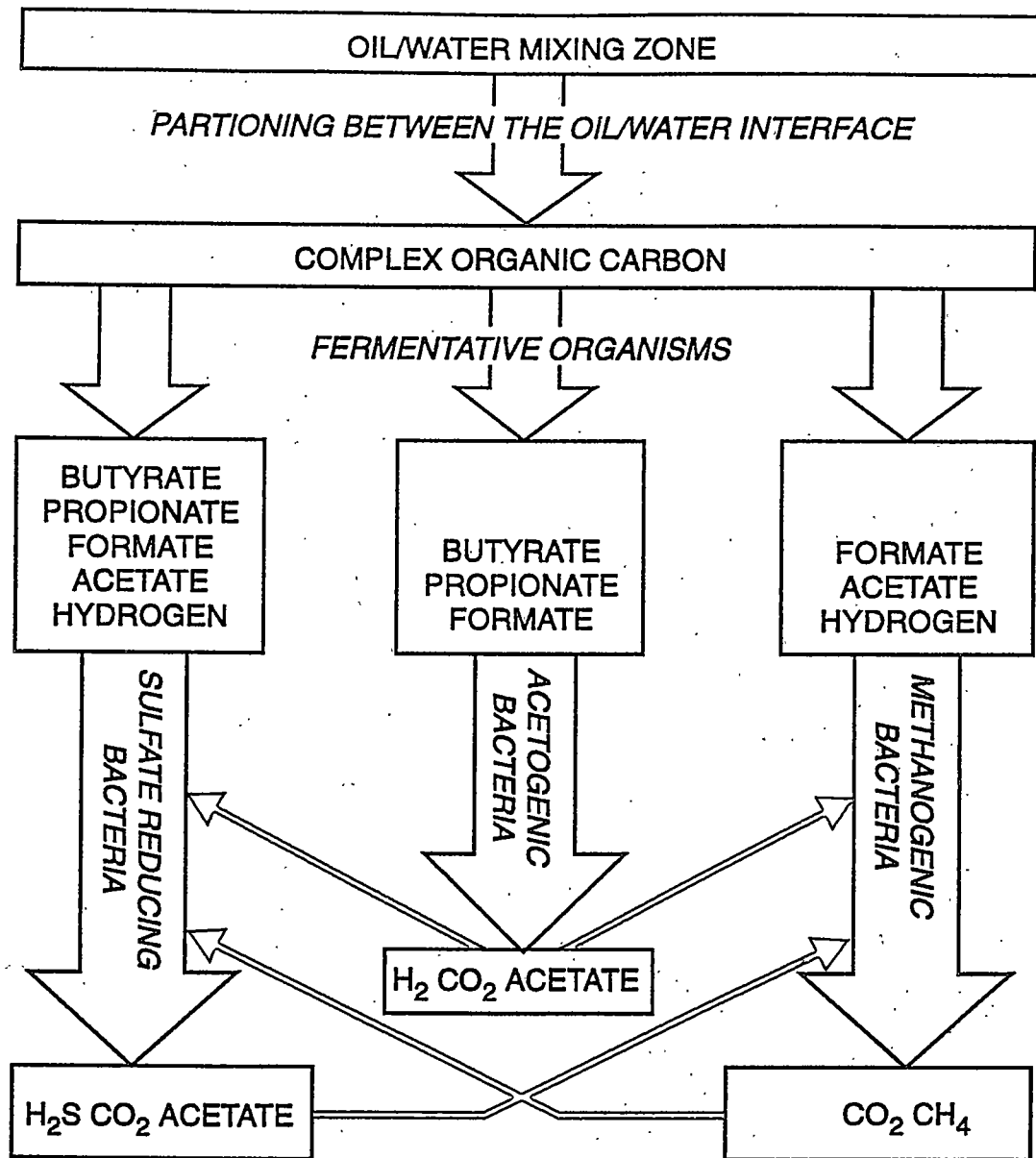
Species	morph.	Electron donors					Topt.(°C)	References
		H/CO <sub>2</sub>	formate	lactate	acetate	other VFA's		
<i>Desulfovibrio thermophilus</i>	rod	+	+	+	-	NR	65-85	Postgate, 1984
<i>Desulfotomaculum nigrificans</i>	rod	+	+	+	-	-	55-70	Postgate, 1984
<i>kuznetsovii</i>	rod	+	+	+	+	C <sub>3</sub> -C <sub>4</sub>	50-85	Nazina, <i>et al.</i> 1988
<i>thermoacetoxidans</i>	rod	+	+	+	+	C <sub>3</sub> -C <sub>5</sub>	45-65	Min and Zinder, 1990
<i>geothermicum</i>	rod	+	NR	+	-	C <sub>3</sub> -C <sub>4</sub>	54	Daumas, <i>et al.</i> 1988
<i>Thermodesulfobacterium commune</i>	rod	+	NR	+	-	-	70-85	Zeikus, <i>et al.</i> 1983
environmental isolates:								
strain V-16	sphere	+	+	+	-	-	64-92	Stetter, <i>et al.</i> 1987
T90A	rod	+	+	+	-	C <sub>3</sub> -C <sub>7</sub>	43-78	Rosnes, <i>et al.</i> 1991
T93B	rod	+	+	+	-	C <sub>3</sub> -C <sub>7</sub>	43-78	Rosnes, <i>et al.</i> 1991
Kuparuk consortium	rod	+	+	+	+/-*	C <sub>3</sub> -C <sub>4</sub>	35-75	this study
Ninian consortium	lemon shape	+	+	+	-	C <sub>3</sub> -C <sub>4</sub>	60	this study

Table legends: NR: not reported; +: utilized; -: not utilized; +/-\*: utilization was temperature dependent

showed similar sensitivity to the unionized  $H_2S$  species. Therefore the authors concluded that at elevated total sulfide concentrations and high pH the methanogens should be able to outcompete the SRB for substrate. McCartney and Oleszkiewicz (1991) supported this hypothesis with the results of their experiments. Therefore the environment specific condition (such as pH and hydrogen sulfide concentration) as well as physical constraints (e.g. surface/volume ratio of the reactor system) will determine the competition outcome (Yadav and Archer, 1988).

Figure III.11 summarizes the microbial processes in an oil reservoir environment. Due to partitioning with the oil phase, longer chain fatty acids and more complex hydrocarbons are dissolved into the water phase. Fermentative microorganisms degrade these complex organics and produce carbon dioxide, hydrogen, and shorter chain fatty acids. Butyrate, propionate, lactate, formate, acetate, and hydrogen could be utilized for sulfate reduction producing hydrogen sulfide, carbon dioxide and, in the case of incomplete oxidation, acetate. Butyrate, propionate, lactate, and formate could also be used by acetogenic bacteria producing hydrogen, carbon dioxide, and acetate. These products can then all be utilized for sulfate reduction.

Acetogenesis does not change the overall oxidative state of the electron donors available, but it does change the chemistry in the water. In the absence of sulfate, this study found that hydrogen was the only energy carrier for methanogenesis at 60°C, however at lower temperatures methanogenic bacteria are most likely to utilize formate, and acetate, also. The methane produced could then be used by sulfate reducing bacteria.



**Figure III.11.** Model of the chemical/microbial processes in a souring oil reservoir at the anaerobic water/oil mixing zone. Due to partitioning between the oil and the water phases, long chain fatty acids, hydrocarbons and more complex organic carbon compounds are dissolved into the water. These compounds could be degraded in the water phase or at the interface by fermentative bacteria. The products of this fermentation are carbon dioxide, hydrogen, and shorter chain vfa's such as butyrate, propionate, acetate and formate. These products can serve as electron donors for sulfate reduction, acetogenesis or methanogenesis. In the presence of sulfate, sulfate reduction will be the most favorable reaction occurring and sulfate reducing bacteria will become active when present. In the absence of sulfate, methanogenesis will be the next favorable process. Methane will be produced from formate, acetate and hydrogen. Butyrate and propionate will be converted by acetogenic bacteria into carbon dioxide, hydrogen, and acetate and furthermore utilized for methanogenesis.

Microbial sulfate reduction was observed over a wide range of temperature. Temperature affected the rate of microbial sulfate reduction as well as the substrate specificity and the morphology of the microorganisms present. The highest rate of specific sulfate reduction was found at 50°C. At 60°C the rate of sulfate reduction was only slightly lower. At 35 and 75° the rate of sulfate reduction was approximately one fifth of the rate for 50°C. The change in cell morphology may indicate the presence and activity of different organisms at different temperatures. The change in morphology of the microbial consortium when exposed to varying temperatures indicates adaptation due to population changes. This is supported by the long lag phases before microbial sulfate reduction became active at a new temperature. The temperature in oil reservoirs ranges from 30°C to about 100°C, favoring m-SRB growth close to the injection well and growth of thermophilic and extremely thermophilic sulfate reducers closer to the production well. M-SRB are commonly found in seawater (Hardy and Hamilton, 1981; Burger *et al.* 1992; Herbert *et al.* 1985; Herbert and Stott, 1983). Rosnes *et al.* (1990) speculated that t-SRB may even be present in very low numbers in sea water. Injection well backflow studies have shown that SRB concentration and activity was highest around the injection wellbore and decreased with distance from the wellbore (Taylor *et al.* 1991). However, they found contrary data for hydrogen sulfide concentration with a seven fold higher H<sub>2</sub>S concentration further into the formation, indicating that the SRB activity occurs throughout the entire reservoir.

### Conclusions

The sampling and storage procedures used in this study proved to be sufficient in sampling, characterizing and storing sulfate reducing and methanogenic bacteria. Fermentative organisms may have been damaged by the freezing process. The microbial consortia from produced water sampled at two differently operated oil fields were capable of sulfate reduction over a wide range

of temperature and for a variety of substrates. Temperature did influence the rate of sulfate reduction, the rate of H<sub>2</sub>S production, the rate of substrate oxidation, and the cell morphology. The highest rates of sulfate reduction and substrate oxidation were found between 50 and 60°C. Formate and n-butyrate were the most favorable carbon sources at any tested temperature. Acetate was utilized at 35°C and at 75°C but not at 50°C and was produced at 60°C.

The cell size distribution of the microbial consortium indicates a non uniform microbial composition in the original sample from the Kuparuk field. However with changing environmental conditions the microbial population changed accordingly as indicated by different morphologies and physiologies at different temperatures.

Methane producing activity was found for the Kuparuk consortium when hydrogen and carbon dioxide were present. Hydrogen and carbon dioxide were exclusive substrates for methanogenesis at the experimental conditions tested.

The microbial population as compared between on site inoculum and frozen inoculum seems to change only slightly. This comparison was based on the capability of the microbial consortium to utilize specific substrates for its carbon and energy demand.

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## CHAPTER IV

KINETICS AND STOICHIOMETRY OF MICROBIAL CONSORTIA FROM  
SOURING OIL RESERVOIRSIntroduction

The most common method used to control souring in oil fields is the use of biocide (Eagar *et al.* 1988). These measures are often of limited effectiveness since sulfate reducing bacteria (SRB) might exist in biofilms (Burger *et al.* 1992). Within a biofilm, bacteria find a more protected environment for growth and activity (Van der Wende *et al.* 1989). In addition, biocides often contain carbon, ammonia, and phosphate which might be utilized by SRB at low concentrations. The introduction of competitive organisms was considered as control mechanism by Burger *et al.* 1992 and McInernay *et al.* 1992. Different substrates and environmental conditions will result in different values for their growth parameters and the effectiveness of microbial competition will change with change as well.

Generally, a better understanding of the rate and stoichiometry at which souring occurs is needed for modelling a reservoir. This can be used to define advanced strategies for controlling souring more effectively with biocide action or the use of competitive microorganisms. In all commonly used reservoir models the kinetics of sulfate reduction are either estimated based on pure culture SRB data from literature or simply neglected (Ligthelm *et al.* 1991).

### Questions

With the optimal growth conditions, determined in chapter III of this study, what is the stoichiometry of the microbial sulfate reduction and what are the kinetic parameter values?

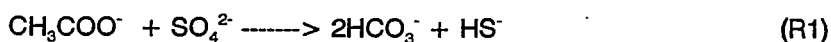
Do the SRB kinetics depend on the system configuration (e.g how do biofilm cells compare with suspended cells)?

### Rationale

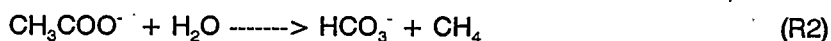
A reservoir simulation model should be able to accurately predict the individual processes of transport and microbial conversions under ground at the site specific environment. Fundamental information about the mechanisms and the rate of these microbial conversions is essential in order to simulate microbial processes in a souring reservoir accurately. Kinetic parameters will determine what type of organisms will outcompete another at various nutrient conditions relevant for competition studies.

### Thermodynamics of Sulfate Reduction

Thermodynamically, anaerobic metabolism yields much less energy than aerobic respiration. When acetate is used as electron donor for sulfate reduction the free energy change is  $\Delta G^\circ = -48$  kJ/mol acetate (R1; Widdel, 1988). For aerobic respiration of acetate the free energy change ( $\Delta G^\circ = -837$  kJ/mol acetate) is almost 20 times higher than for sulfate reduction. For methanogenesis the energy yield is even lower ( $\Delta G^\circ = -31$  kJ/mol acetate; Schönheit, 1982; R2):



$$(\Delta G^\circ) = -48 \text{ kJ/mol acetate}$$



$$(\Delta G^\circ) = -31 \text{ kJ/mol acetate}$$

As a consequence of the relatively low free energy changes SRB and methanogens exhibit a low cellular growth yield of usually less than 0.1 g cells produced per g of total substrate consumed.

#### Sulfate Reduction in Biofilm Systems

Sulfate reduction in biofilm systems was of concern especially in sewer systems (Nielsen and Hvitved-Jacobsen, 1988). The biofilm system provides the sulfate reducing bacteria an anaerobic zone to grow and metabolize even when the bulk water contains oxygen in fairly high concentrations. A biofilm will establish when proper nutrients are present in the bulk water. The outer layer of this film will consist of heterotrophic bacteria depleting oxygen and developing an environment suitable for sulfate reducing bacteria to grow and metabolize if sulfate and excess organic carbon are present. Once established, the SRB will protect themselves against environmental stress by the production of  $\text{H}_2\text{S}$  which acts as an oxygen scavenger and a pH buffer in the SRB microenvironment (Crolet *et al.* 1992). Nielsen (1987) investigated these mixed population biofilms in sewer systems and found high rates of sulfate reduction in the anaerobic film layer. Okabe (1992) investigated a pure culture SRB biofilm and found even higher rates for sulfate reduction.

In a souring reservoir undergoing secondary recovery the oxygen is removed efficiently by a water treatment system prior to sea water injection. The average water flow through the formation is in the order of one meter per day and lower (Burger, 1993). Biofilms may not seem to be important at this low flow rate, since suspended bacteria are far from being washed out. In porous media experiments with thermophilic SRB from the Kuparuk consortium, Ching I Chen *et al.* (1994) reported that the attached biomass contributed over 99% to the total biomass found in the porous media reactor. Hence, biofilm processes are of crucial importance to the overall rate and stoichiometry of sulfate reduction in a oil reservoir.

### SRB Kinetics

Okabe *et al.* 1992 performed extensive studies with the sulfate-reducing bacterium *Desulfovibrio desulfuricans* in a chemostat system. In their experiments, carbon was the limiting nutrient and sulfate was in excess. If sulfate or carbon are potentially limiting the rate of SRB growth and sulfate reduction a double Monod kinetic model could be used. Half saturation coefficients for the electron donor and for the electron acceptor have to be known. The following equations express the reaction rate (corresponding material balances for a batch system) for the four constituents - SRB cell mass, substrate, sulfate, and hydrogen sulfide. Notation is defined at the end of this chapter.

$$\begin{array}{l}
 * \text{ biomass} \\
 \frac{dX}{dt} = r_a(X) = X(\mu - b) \quad (E1)
 \end{array}$$

$$\begin{array}{l}
 * \text{ substrate} \\
 \frac{dS}{dt} = r_a(S) = -X Y_{X/S}^{-1} \mu \quad (E2)
 \end{array}$$

$$\begin{array}{l} \text{* sulfate} \\ \frac{dE}{dt} \end{array} = r_a(E) = -X Y_{X/E}^{-1} \mu \quad (E3)$$

$$\begin{array}{l} \text{* hydrogen sulfide} \\ \frac{dP}{dt} \end{array} = r_a(P) = X Y_{P/E} Y_{X/E}^{-1} \mu \quad (E4)$$

$$\text{* specific growth rate } \mu = \mu_{\max} [S / (K_S + S)] [E / (K_E + E)] \quad (E5)$$

The accumulation of sulfide, and biomass can be determined for a specific system when the half saturation coefficients, the maximum growth rate and the growth yields for an organism are known. Based on the maximum growth rate and the cell yield on sulfate, the maximum sulfate reduction rate can be determined with E6:

$$V_{\max} = \mu_{\max} Y_{X/E} \quad (E6)$$

## Materials and Methods

### Kinetic Parameter Estimation

The kinetic parameters estimated were based on a double Monod kinetic model. The estimated parameters included the maximum growth rate, the half saturation constants, the cellular growth yield, and the maximum sulfate reduction rate. Two different system configurations such as batch reactors, and a completely mixed biofilm reactor (CMBR) were used to determine these parameters. Temperature was kept constant at 60°C in all experiments.

### Batch reactor

Kinetic parameters and stoichiometric ratios for SRB from both oil reservoir consortia were tested for butyrate and formate as their sole carbon and energy source. Kinetics and stoichiometry on propionate were determined for the Kuparuk consortium only. The substrates tested were the most favorable carbon and energy sources utilized by either consortium and were found to be present in the produced water (chapter III).

Experimental set up. Glass vials with a total volume of 60 ml were used to study substrate utilization. After cycling between vacuum and  $N_2$  pressure to remove all residual oxygen, the vials were filled with high salinity SRB medium (Pfennig *et al.* 1986) up to 30 ml, leaving 30 ml of headspace volume in the pressurized vials. The bacteria inoculum was added to initiate the experiment and the vials were incubated at 60°C. Substrates were offered at an initial concentration of 200 g m<sup>-3</sup>. Three replicates for each VFA were tested. To obtain an active culture for inoculum, an enrichment culture was grown on a VFA mixture. When the enrichment culture was growing in log phase, one ml from the enrichment was inoculated into each test vial. Samples were taken with a 21 gauge needle on a 1 ml syringe. The samples were analyzed for sulfate, formate, acetate, propionate, i-, and n-butyrate, and for total cell mass.

Stoichiometry. The stoichiometric ratios were obtained in batch reactors by plotting the actual VFA concentration vs acetate concentration, cell mass concentration, or sulfate concentration. Linear regression was used to determine the stoichiometric ratios.

Kinetic parameter estimation. Parameter values were determined directly from the experimental data with the aid of a simulation model. Initial substrate, sulfate, and biomass concentrations ( $S_0$ ,  $E_0$ ,  $X_0$ ) were measured at the start of each experimental run just after the consortia inoculum.

Biomass yield on substrate and sulfate ( $Y_{X/S}$ ;  $Y_{X/E}$ ) were determined from biomass production vs butyrate, propionate, and formate or sulfate consumption as described above. The maximum cellular growth rate for SRB ( $\mu_{max}$ ) was determined from the steepest slope of the cell concentration vs time (minimum doubling time  $t_d$ ). The min doubling time was converted into the maximum growth rate by  $\mu_{max} = \ln 2 t_d^{-1}$ . The half saturation constants based on substrate and sulfate concentration ( $K_s$  and  $K_e$ ) were determined by the aid of non-linear regression analysis assuming double Monod kinetics. The experimental data were compared with the computer generated simulations. The equations E1 through E4 express the material balances for the four constituents (biomass, substrate, sulfate, and hydrogen sulfide) and were the basis for the batch simulation. Finite differences for each of these constituents were computed. The values for the half saturation coefficients were determined by comparing the experimental results for biomass, substrate, and sulfate with the computer simulated data. The square of the vertical distances between the experimental data and the simulations were calculated for each experimental data point. The set of half saturation coefficients for substrate and sulfate with the minimum sum of vertical distances for the triplicate experiments and the simulated data was used for the estimated kinetic parameter values. The minimum value for the vertical distances reported was found by inspection, other minima may also be found.

#### Completely Mixed Biofilm Reactor (CMBR)

Experimental set up. A rotating annular reactor was used to perform the biofilm experiments (Table IV.1). The rationale for choosing this reactor configuration was that it has a completely mixed bulk water phase, pH, and temperature and the shear stress at the wall can be controlled independent of the flow (dilution) rate (Trulear and Characklis, 1982). Temperature and pH were controlled at 60°C and 7.6, respectively. Removable slides, located on the outer drum of the

reactor allowed biofilm sampling without interrupting the ongoing experiment. Strict anaerobic procedures were used during the course of the experiment for the reactor set up, media preparation, and acid/base addition. Purified nitrogen gas was used to purge the medium continuously and to apply a positive nitrogen gas pressure on the CMBR and all other containers connected with the experimental system. The same medium as for the sample characterization was used for this study (Pfennig *et al.* 1986). As substrates, a mixture of volatile fatty acids (such as formate, acetate, propionate, and n,- and i-butyrate) were added to the medium. The experiment was conducted for a total time of two months and data were collected for sulfate, hydrogen sulfide, volatile fatty acids (such as formate, acetate, propionate, and n-, and i-butyrate), and cell density in the water phase and in the biofilm phase. Using process analysis, cellular growth rate, the volumetric sulfate reduction rate in the biofilm, the biofilm detachment rate, the biofilm cell density, and the cellular growth yield (based on sulfate) could be determined. These coefficients determine the rate and extent of the microbial reactions within the biofilm.

**Table IV.1. CMBR Dimensions:**

		units
total liquid volume	$V = 740$	$\text{cm}^3$
total wetted surface area	$A = 1935.5$	$\text{cm}^2$
surface to volume ratio	$A/V = 2.616$	$\text{cm}^{-1}$
diameter inner drum	$d_i = 10.0$	cm
diameter outer drum	$d_o = 11.6$	cm
diameter of 4 inner tubes	$d_t = 1.0$	cm
height inner drum	$h_i = 18.5$	cm
height outer drum	$h_o = 18.5$	cm
length tubs	$h_t = 19.0$	cm
number of slides to remove	$\# = 12$	
wetted surface area per slide	$A_s = 34.2$	$\text{cm}^2$
width	$w_s = 1.8$	cm
height	$h_s = 19$	cm

The reactor start up was performed using 20 ml inoculum from enrichment cultures. The preparation of these enrichment cultures is described for the batch experimental set up. The CMBR was operated without influent for two days after inoculation of the enrichment culture followed by a sequential increase of the influent flow rate.

Kinetic Parameter Estimation. Biofilm processes can be analyzed by a set of mass conservation equations for substrate, electron acceptor, and cell mass. Notation is defined at the end of this chapter.

A) Electron acceptor (E):

$$\begin{array}{llll}
 V \frac{dE}{dt} = & F (E_i - E) & - r_a'(E) A & - r_a(E) V & (E7) \\
 \text{sulfate} & \text{rate of net} & \text{sulfate reduction} & \text{sulfate reduction} & \\
 \text{accumulation} & \text{sulfate input} & \text{in the biofilm} & \text{in the bulk water} & 
 \end{array}$$

B) Electron donor (S):

$$\begin{array}{llll}
 V \frac{dS}{dt} = & F (S_i - S) & - r_a'(S) A & - r_a(S) V & (E8) \\
 \text{butyrate} & \text{rate of net} & \text{butyrate removal} & \text{butyrate removal} & \\
 \text{accumulation} & \text{butyrate in} & \text{by biofilm cells} & \text{by suspended cells} & 
 \end{array}$$

C) Biofilm cell mass ( $\rho T$ ):

$$\begin{array}{llll}
 A \rho \frac{dL_f}{dt} = & A R_a & - A R_d & + A r_a'(E) Y_{X/E} & (E9) \\
 \text{rate of attached} & \text{rate of} & \text{rate of} & \text{rate of biofilm} & \\
 \text{cell accumulation} & \text{attachment} & \text{detachment} & \text{cell growth} & 
 \end{array}$$

D) Suspended cell mass ( $X_s$ ):

$$\begin{array}{llll}
 V \frac{dX_s}{dt} = & - F X_s & - A R_a & + A R_d & + V \mu X_s & (E10) \\
 \text{rate of suspended} & \text{rate of} & \text{rate of} & \text{rate of} & \text{rate of susp.} & \\
 \text{cell accumulation} & \text{washout} & \text{attachment} & \text{detachment} & \text{growth} & 
 \end{array}$$

E) Total cell mass (X):

$$\begin{array}{llll}
 V \frac{dX}{dt} = & A \rho \frac{dL_f}{dt} & + V \frac{dX_s}{dt} & & (E11) \\
 \text{rate of total} & \text{rate of attached} & \text{rate of suspended} & & \\
 \text{cell accumulation} & \text{cell accumulation} & \text{cell accumulation} & & 
 \end{array}$$

$$\begin{array}{llll}
 V \, dX/dt = & - F X_s & + V \mu X_s & + A \mu' \rho L_t \quad (E12) \\
 \text{rate of total} & \text{cell wash} & \text{growth of} & \text{growth of} \\
 \text{cell accumulation} & \text{out} & \text{suspended cells} & \text{attached cells}
 \end{array}$$

With some necessary assumptions, the biofilm growth yield, the specific growth rate, the detachment rate coefficient, the sulfate surface flux, and the volumetric sulfate reduction rate can be determined, based on equations E7 through E12.

The assumptions used to simplify the above rate equations were: (A1) steady state conditions (e.g., no change in substrate, sulfate, or cell mass over time); (A2) the influent cell concentration is zero (e.g., sterile medium); and (A3) that there is no cell attachment from the bulk water onto the biofilm. Since the following analysis considers suspended processes as well as biofilm processes, another simplification was made: The rate coefficient for substrate conversion and the cellular growth yield and rate are equal for biofilm and suspended bacteria (A4, A5 and A6, respectively). Mathematically these can be expressed as:

$$d/dt = 0 \quad (A1)$$

$$X_i = 0 \quad (A2)$$

$$R_a = 0 \quad (A3)$$

$$k = k' \quad (A4)$$

$$Y_{X/E} = Y_{X/E}' \quad (A5)$$

$$\mu = \mu' \quad (A6)$$

The cellular growth yield on sulfate (E) or on substrate (S) can be determined based on equation E9 with assumptions A1 and A3 (E13 and E14, respectively).

$$Y_{X/E} = R_d/r_a(E) \quad (E13)$$

$$Y_{X/S} = R_d/r_a(S) \quad (E14)$$

The detachment rate can be determined from equation E10, applying assumptions A1 and A3 to give equation E15:

$$R_d = \frac{(F/V - \mu) X_s}{A/V} \quad (E15)$$

The detachment rate coefficient can be determined with equation E16.

$$k_d = R_d / \rho L_f \quad (E16)$$

The specific growth rate of biofilm cells can be calculated from equation E12 with assumptions A1, A2, and A6 using equation E17.

$$\mu = F/V X_s / (X_s + A/V \rho L_f) \quad (E17)$$

The sulfate reduction rate (surface flux) for a fully penetrated biofilm as will be the case for thin films can be expressed as (E18; Trulear and Characklis 1982):

$$r_a'(E) = k_e L_f \quad (E18)$$

With assumption A4, the sulfate reduction rate for suspended cells can be expressed with equation E19:

$$r_a(E) = k X_s \quad (E19)$$

Applying A1 and A4 and using E7, E18, and E19 the zero order reaction rate coefficient  $k$  for sulfate reduction could be determined with E20:

$$k' = D (E_1 - E) / (e L_f (A/V) + X_s) \quad (E20)$$

The volumetric sulfate reduction rate in the biofilm is often used to express the activity of a specific biofilm system (Nielsen, 1987) and can be determined by equation E21:

$$k_{of} = k_e \quad (E21)$$

### Microbiological and Chemical Methods of Analysis

The AODC- method was used to determine the cell size and number as described in chapter III. Cell size analysis was used to convert cell numbers into cell mass, assuming a constant cell density of  $1.08 \text{ g cm}^{-3}$  (Characklis and Marshal, 1990).

Concentrations of hydrogen sulfide, sulfate, and individual VFA's were measured according to chapter III.

## Results

### Kinetics and Stoichiometry of Substrate-specific Sulfate

#### Reduction for Thermophilic SRB from Two Souring Oil Reservoirs

The goal of these experiments was to obtain stoichiometry, cell yield and kinetic parameters for sulfate-reducing bacteria from the Kuparuk and the Ninian field consortium.

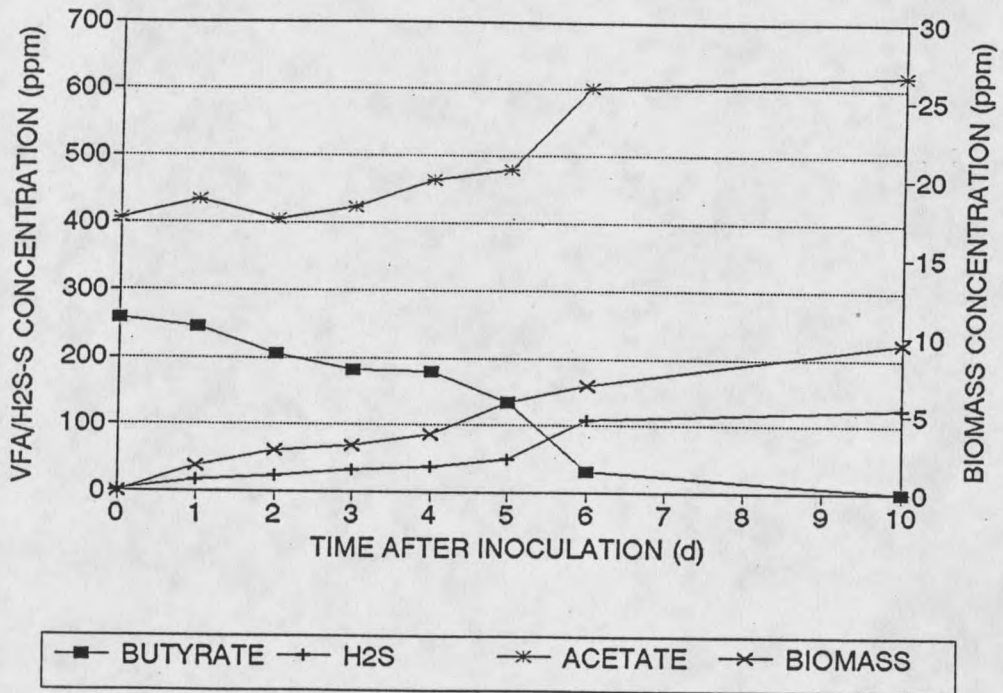
Stoichiometry for consortium from the Kuparuk field. The disappearance of butyrate correlated with the production of hydrogen sulfide and acetate (Figure IV.1). 1.4 moles of sulfate were reduced (Figure IV.2) and 1.1 moles of acetate were produced when 1 mol of butyrate was oxidized (Table IV.2).

When propionate was utilized as the main carbon source, acetate and H<sub>2</sub>S were produced (Figure IV.3). One mol of acetate was produced and 0.8 moles of sulfate were reduced per mol of propionate (Figure IV.4).

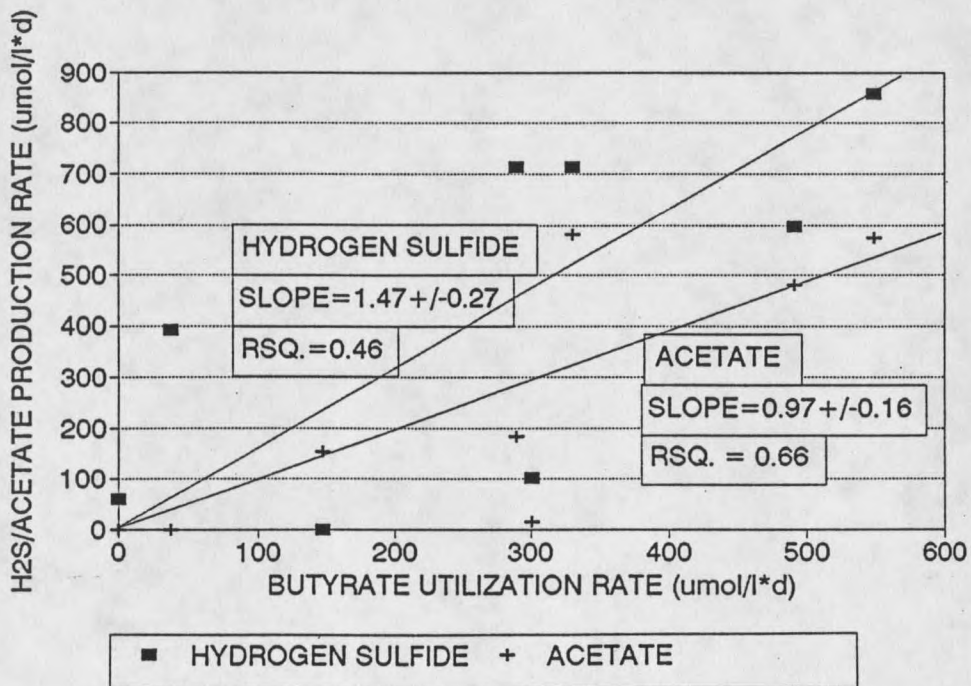
The utilization of formate was associated with production of hydrogen sulfide (Figure IV. 5). The production of hydrogen sulfide was poorly correlated to the formate utilization rate ( $r^2 = 0.34$ ; Figure IV. 6). 0.36 moles of sulfate were reduced by one mol of formate oxidation.

Stoichiometry for consortia from the Ninian field. One mole of butyrate was oxidized to one mole of acetate and two moles of carbonate. 1.2 moles of sulfate were reduced. 0.21 moles of sulfate were reduced by the oxidation of one mole of formate (Table IV.2).

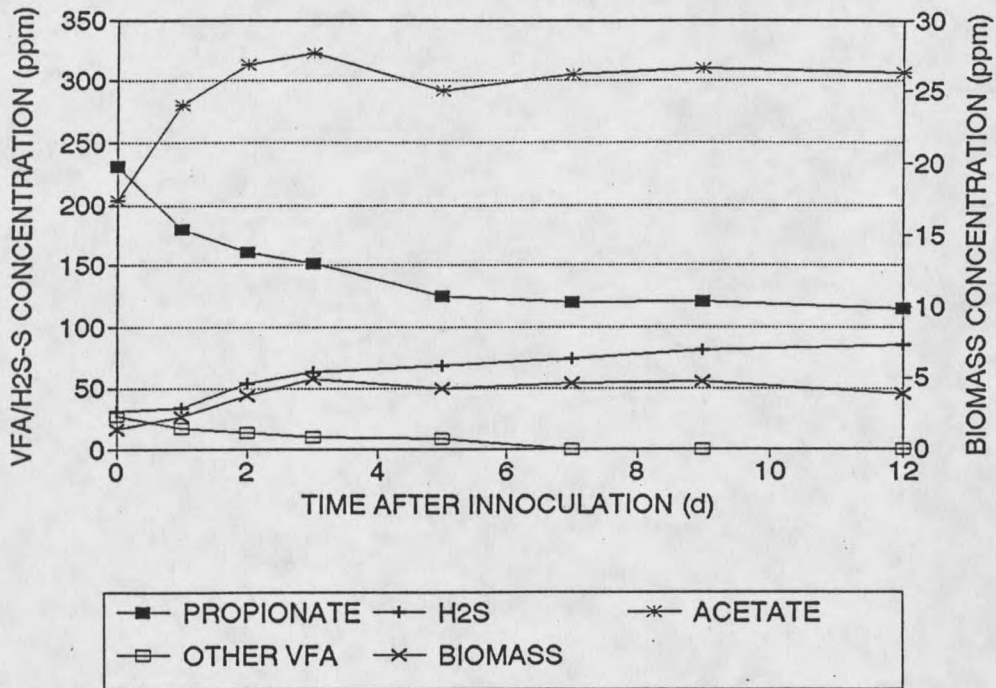
Cellular growth yield. The obtained biomass per substrate/sulfate converted was very low (Table IV.3). Less than one percent of the total amount of substrate converted for sulfate reduction was utilized for anabolic processes. This was consistent for both oil reservoir consortia tested on butyrate, propionate, and formate as carbon and energy sources (Figure IV.7, 8, and 9). The production of biomass was lower for the consortium from the Ninian field as compared to the consortium from the Kuparuk field.



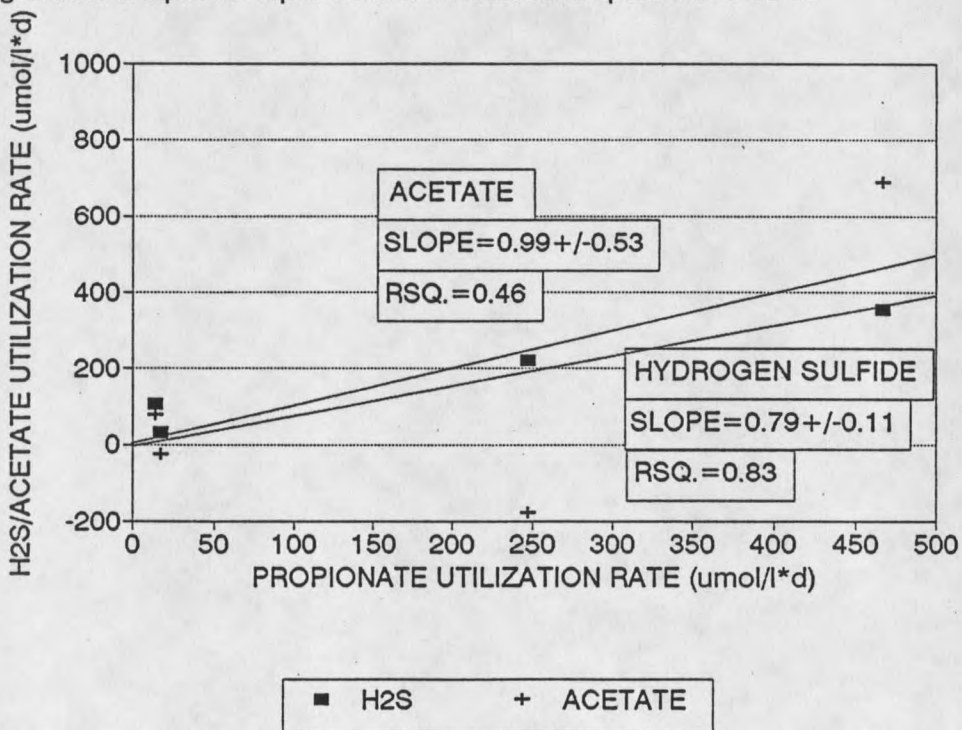
**Figure IV.1.** Butyrate utilization, H<sub>2</sub>S, acetate, and cell mass production vs time after inoculation of an actively growing mixed population from the Kuparuk oil reservoir. The data was averaged from a triplicate experiment at a constant temperature of 60°C.



**Figure IV.2.** Hydrogen sulfide and acetate production rate are plotted vs the butyrate utilization rate. The slope of the lines provide the stoichiometric ratios between acetate and butyrate and hydrogen sulfide and butyrate.



**Figure IV.3.** Propionate utilization, H<sub>2</sub>S, acetate, and cell mass production vs time after inoculation of an actively growing mixed population from the Kuparuk oil reservoir. The data was averaged from a triplicate experiment at a constant temperature of 60°C.



**Figure IV.4.** Hydrogen sulfide and acetate production rate are plotted vs the propionate utilization rate. The slope of the lines provide the stoichiometric ratios between acetate and propionate and hydrogen sulfide and propionate.

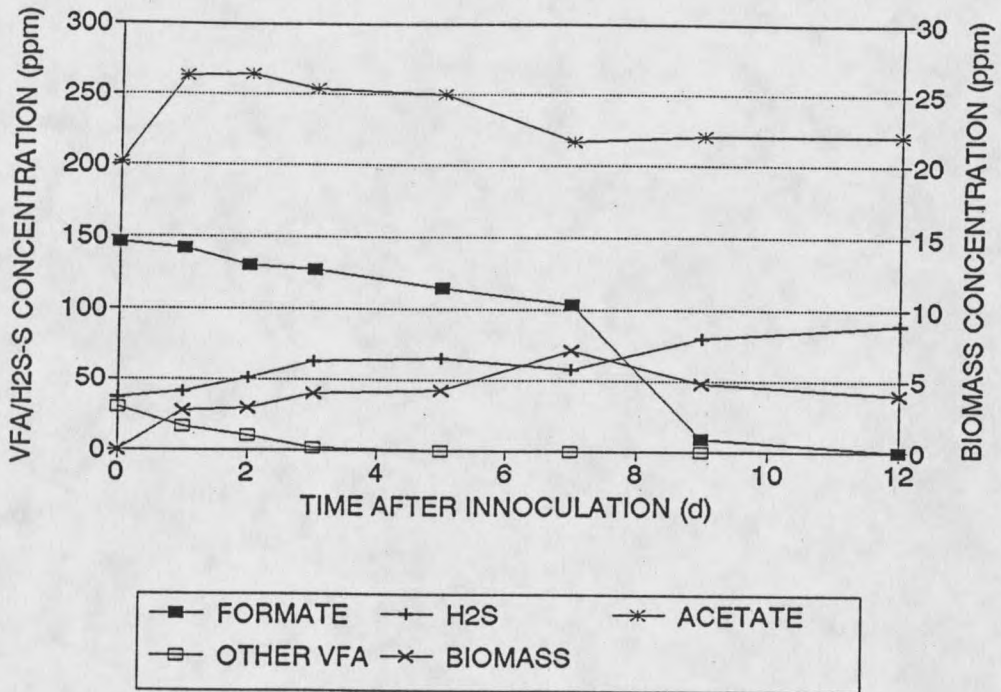


Figure IV.5. Formate utilization, H<sub>2</sub>S, acetate, and cell mass production vs time after inoculation of an actively growing mixed population from the Kuparuk oil reservoir. The data was averaged from a triplicate experiment at a constant temperature of 60°C.

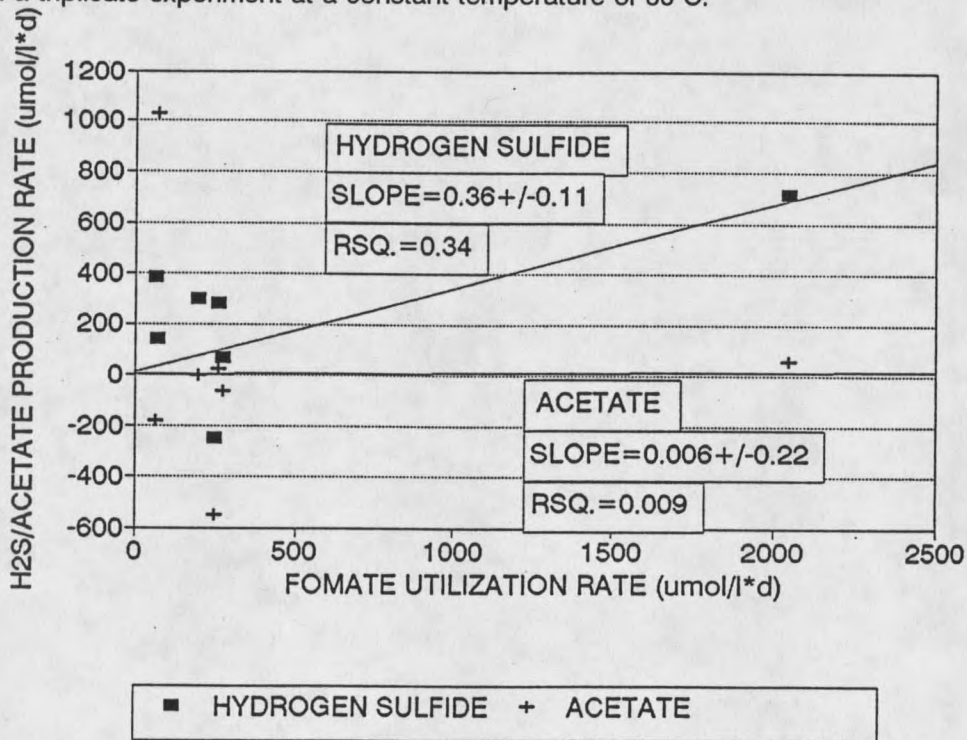
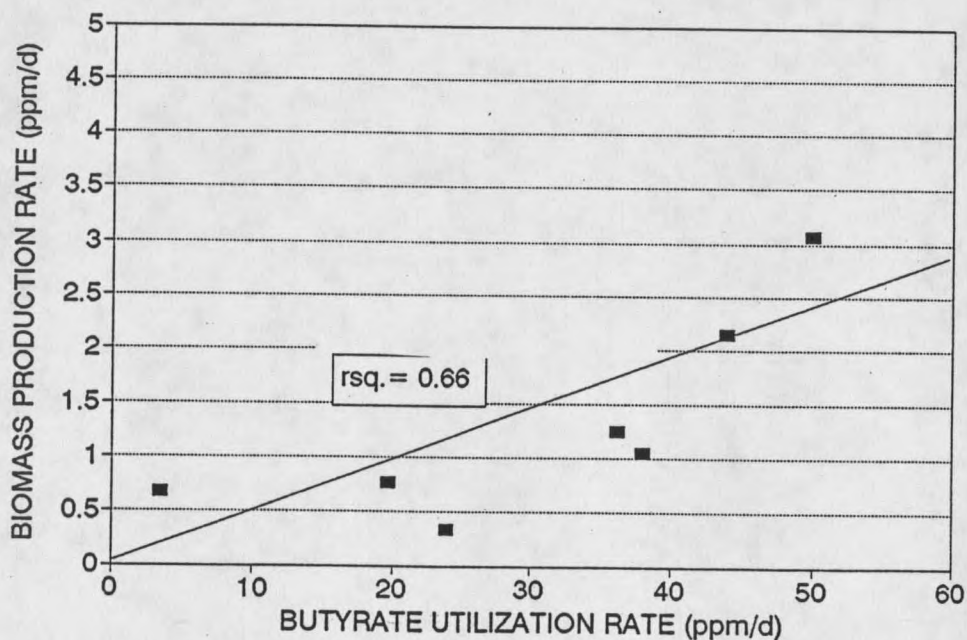
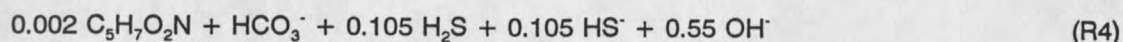
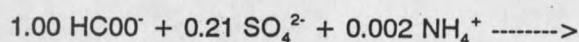
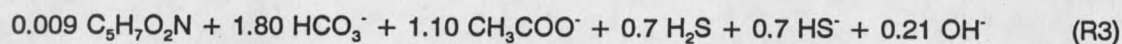
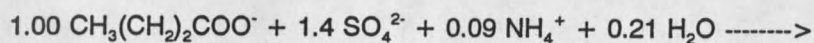


Figure IV.6. Hydrogen sulfide and acetate production rate are plotted vs the formate utilization rate. The slope of the lines provide the stoichiometric ratios between acetate and formate and hydrogen sulfide and formate.

Balanced stoichiometric equations for butyrate utilization by SRB from the Kuparuk field and formate utilization for SRB from the Ninian consortium are presented with R3 and R4, respectively. Biomass is represented as the generic formula  $C_5H_7O_2N$  (Characklis and Marshal, 1990).



**Figure IV.7.** Biomass production as a result of butyrate utilization for sulfate reduction at 60°C. Biomass (wet) production rate is plotted vs the butyrate utilization rate. The slope of the line represents the cellular growth yield of wet cell mass per butyrate consumed.

**Table IV.2.** Results of stoichiometric measurements taken at 60°C with mixed population SRB from the Kuparuk and the Ninian field. The values presented represent the mean and the standard error for three replicative experiments. Butyrate, propionate, formate were the sole carbon source and electron donor, respectively. Acetate was produced when butyrate or propionate were oxidized.

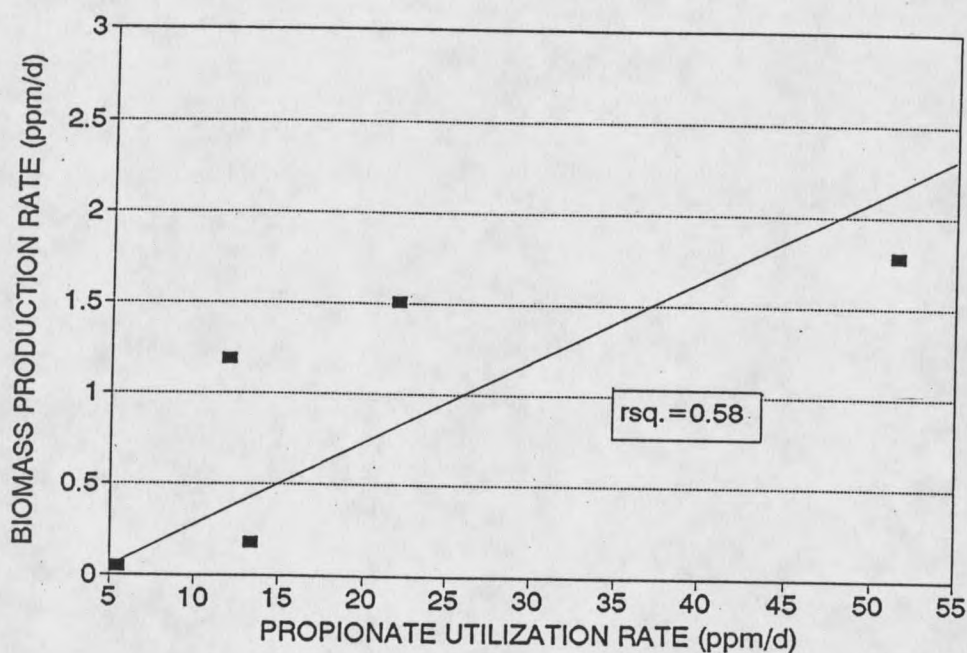
Sample origin	but. (mol m <sup>-3</sup> )	prop. (mol m <sup>-3</sup> )	for.	acetate prod. (mol m <sup>-3</sup> )	SO <sub>4</sub> -S red. (mol m <sup>-3</sup> )	dry cell mass <sup>1</sup> (mol m <sup>-3</sup> )
Kuparuk	1	-	-	1.12±0.21	1.41±0.08	0.009±0.001
Kuparuk	-	1	-	0.99±0.26	0.79±0.06	0.007±0.002
Kuparuk	-	-	1	0	0.36±0.11	0.009±0.001
Ninian	1	-	-	1.00±0.14	1.21±0.11	0.003±0.001
Ninian	-	-	1	0	0.21±0.06	0.002±0.0007

**Table IV.3.** Cellular growth yield for thermophilic SRB from two oil reservoir consortia. The total number of cells measured by AODC was converted into cell mass by using the actual cell size distribution and an average cell density of 1.08 g cm<sup>-3</sup> (Mueller *et al.* 1992).

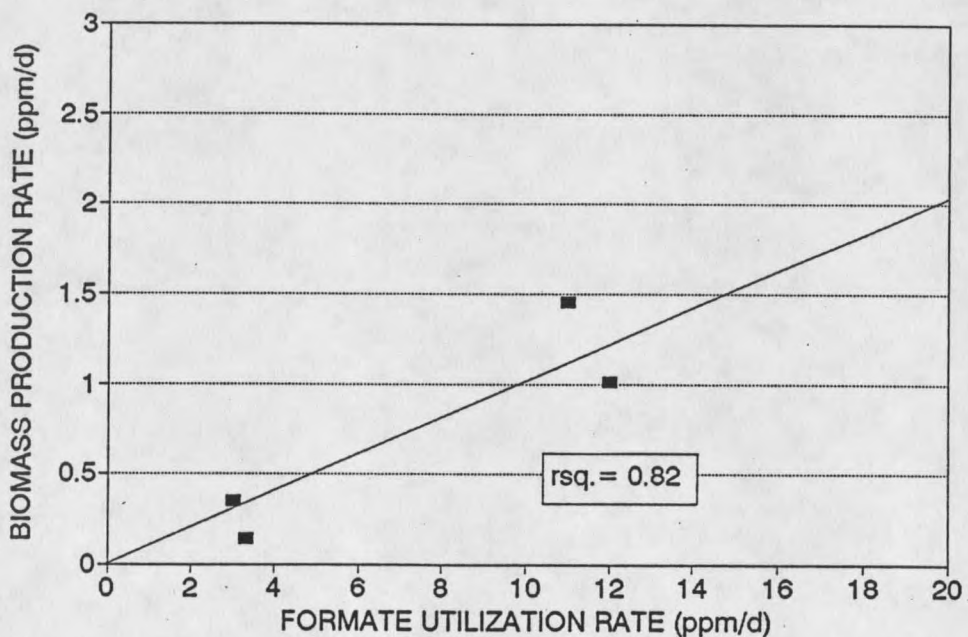
sample origin	substrate	Y (wet) (g BM g <sup>-1</sup> )	Y (dry) (g dry BM g <sup>-1</sup> )	Y (Carbon) (g-C g <sup>-1</sup> )	SD (%)
Kuparuk	butyrate	0.023	0.005	0.003	13
	sulfate <sup>2</sup>	0.038	0.008	0.004	6
Kuparuk	propionate	0.010	0.002	0.001	30
	sulfate <sup>2</sup>	0.027	0.006	0.003	27
Kuparuk	formate	0.023	0.005	0.003	8
	sulfate <sup>2</sup>	0.046	0.010	0.005	7
Ninian	butyrate	0.012	0.003	0.002	8
	sulfate <sup>2</sup>	0.027	0.006	0.003	15
Ninian	formate	0.015	0.003	0.002	11
	sulfate <sup>2</sup>	0.047	0.010	0.005	11

<sup>1</sup>cell mass was based on the generic formula C<sub>5</sub>H<sub>7</sub>O<sub>2</sub>N (Characklis and Marshal, 1990).

<sup>2</sup>cellular growth yield on sulfate as g cell mass (g SO<sub>4</sub>-S)<sup>-1</sup>



**Figure IV.8.** Biomass production as a result of propionate utilization for sulfate reduction at 60°C. Biomass (wet) production rate is plotted vs the propionate utilization rate. The slope of the line represents the cellular growth yield of wet cell mass per propionate consumed.



**Figure IV.9.** Biomass production as a result of formate utilization for sulfate reduction at 60°C. Biomass (wet) production rate is plotted vs the formate utilization rate. The slope of the line represents the cellular growth yield of wet cell mass per propionate consumed.

### Kinetic Parameter Estimation in Batch Systems

Cell synthesis was a very slow process for any given condition and substrate. The highest growth rate observed was for SRB from the Kuparuk consortia growing on butyrate with minimum doubling times of 24 hours (Figure IV.10). Growth on propionate and formate was considerably slower than on butyrate (Figure IV.11 and 12, Table IV.4). Between the three tested VFA (n-butyrate, propionate, and formate) the growth rate observed for Kuparuk consortium SRB was statistically significantly different for each VFA based on a 95% level of confidence (Table IV.4).

The maximum growth rate on n-butyrate was significantly higher for the SRB from the Kuparuk consortium as compared to the Ninian consortium SRB (Figure IV.13, 14; Table IV.4) based on

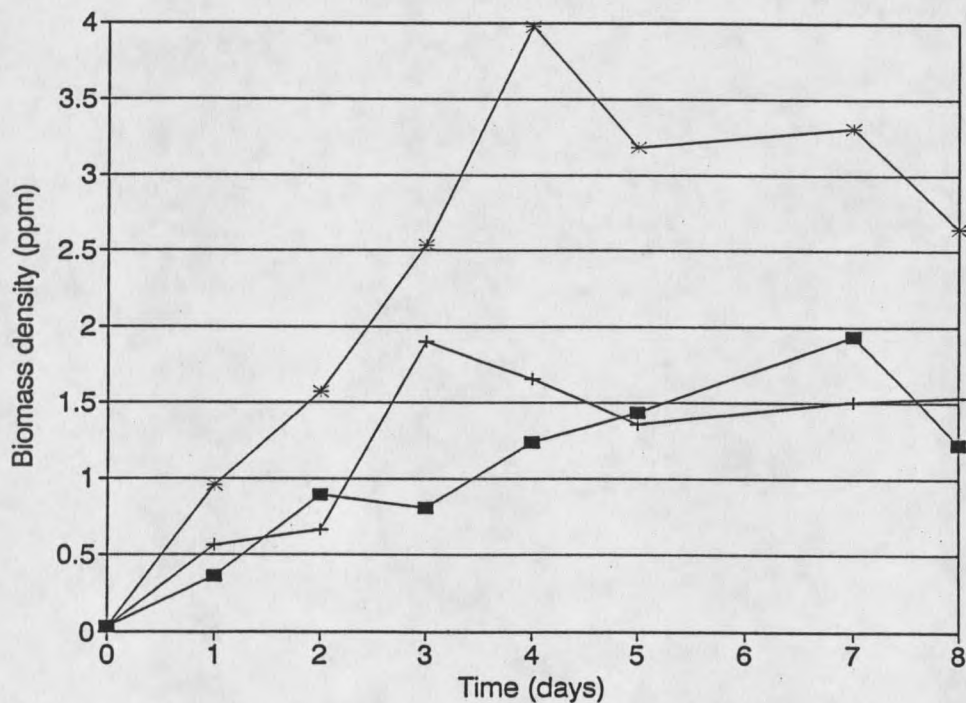
**Table IV.4.** Comparison of kinetic parameters determined for SRB from the Kuparuk and the Ninian consortium at 60°C. The growth supporting substrates were butyrate, propionate, and formate. A double Monod kinetic model was used to determine the values for the decay coefficient and the half saturation constants based on substrate and sulfate. The maximum rate of sulfate reduction was calculated based on  $\mu_{\max}$  and the yield coefficient for dry cell mass.

sample origin	carbon source	$V_{\max}$ (mmol $\text{SO}_4$ $\text{g}^{-1}$ $\text{h}^{-1}$ )	$\mu_{\max}$ ( $\text{h}^{-1}$ )	b ( $\text{h}^{-1}$ )	$K_E$ ( $\text{g m}^{-3}$ )	$K_S$ ( $\text{g m}^{-3}$ )
Kuparuk	butyrate	181±13	0.029±0.002	0.004	9.0±3.0	5.5±1.2
Kuparuk	propionate	99±16	0.019±0.003	ND <sup>3</sup>	ND <sup>3</sup>	ND <sup>3</sup>
Kuparuk	formate	31±6	0.010±0.002	ND <sup>3</sup>	ND <sup>3</sup>	ND <sup>3</sup>
Ninian	butyrate	89±16	0.017±0.003	0.004	5.0±1.3	2.0±1.8
Ninian	formate	41±6	0.013±0.002	0.004	4.0±1.4	2.0±0.7

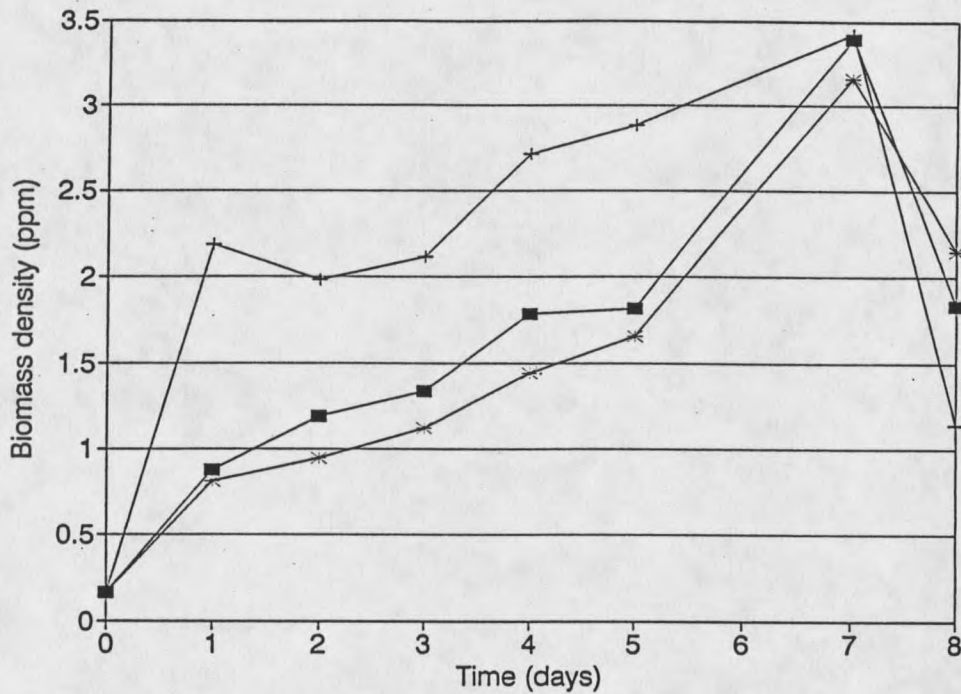
<sup>3</sup>not determined



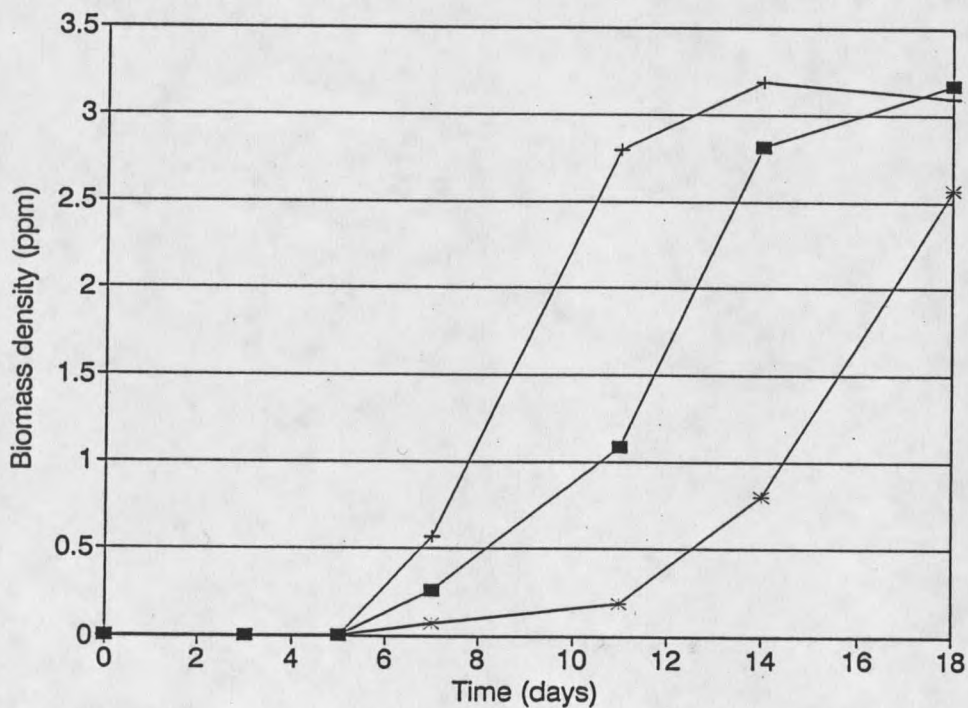
**Figure IV.10.** Growth of SRB from the Kuparuk consortium at 60°C on butyrate. At time 0 an actively growing culture was inoculated. The three different data sets represent triplicate experiments. The minimum doubling time was estimated by the maximum slope.



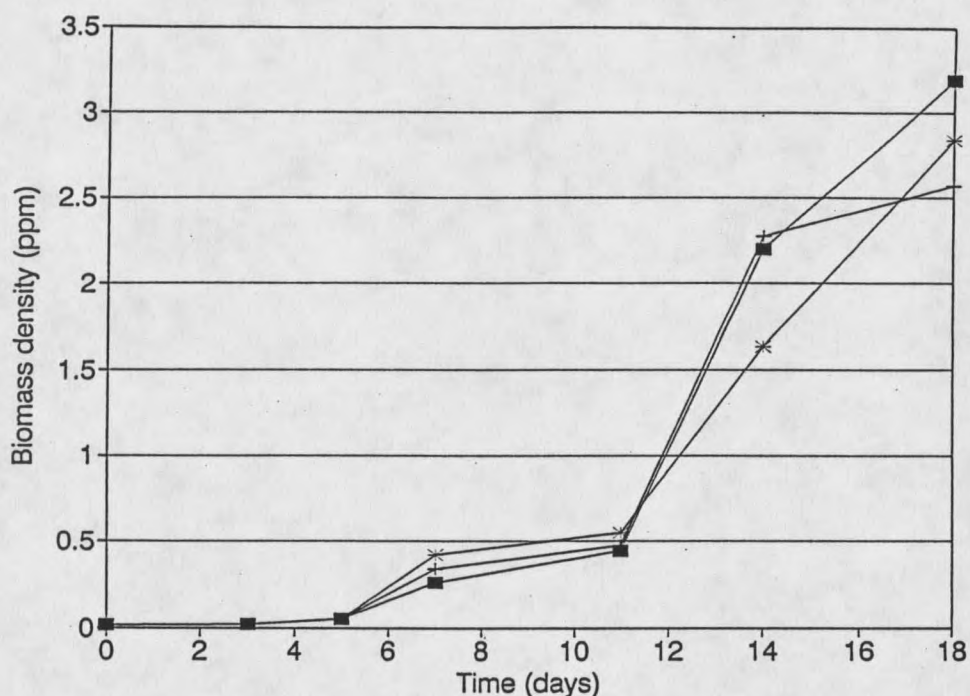
**Figure IV.11.** Growth of SRB from the Kuparuk consortium at 60°C on propionate. At time 0 an actively growing culture was inoculated. The three different data sets represent triplicate experiments. The minimum doubling time was estimated by the maximum slope.



**Figure IV.12.** Growth of SRB from the Kuparuk consortium at 60°C on formate. At time 0 an actively growing culture was inoculated. The three different data sets represent triplicate experiments. The minimum doubling time was estimated by the maximum slope.



**Figure IV.13.** Growth of SRB from the Ninian consortium at 60°C on butyrate. At time 0 an actively growing culture was inoculated. The three different data sets represent triplicate experiments. The minimum doubling time was estimated by the maximum slope.



**Figure IV.14.** Growth of SRB from the Ninian consortium at 60°C on formate. At time 0 an actively growing culture was inoculated. The three different data sets represent triplicate experiments. The minimum doubling time was estimated by the maximum slope.

Figure IV.15A presents the comparison between the simulated data and the experimental data for the Kuparuk consortium SRB growing on n-butyrate. In Figure IV.15B and C the batch simulation results are compiled for the Ninian SRB consortium growing on butyrate and formate, respectively. The half saturation constant for butyrate was significantly smaller for the Ninian field SRB (t-test,  $p=0.05$ ; Table IV.4). Since the cellular growth yields with respect to sulfate were similar for the three tested VFA's (no statistical differences on a 95 % confidence level), the sulfate reduction rates followed the same trend as the growth rates. The decay coefficient used for all the simulations was ( $b = 0.004 \text{ h}^{-1}$ ) approximately one tenth of the maximum cellular growth rate. Higher values for  $b$  resulted in a larger deviation of the simulations from the experimental results.

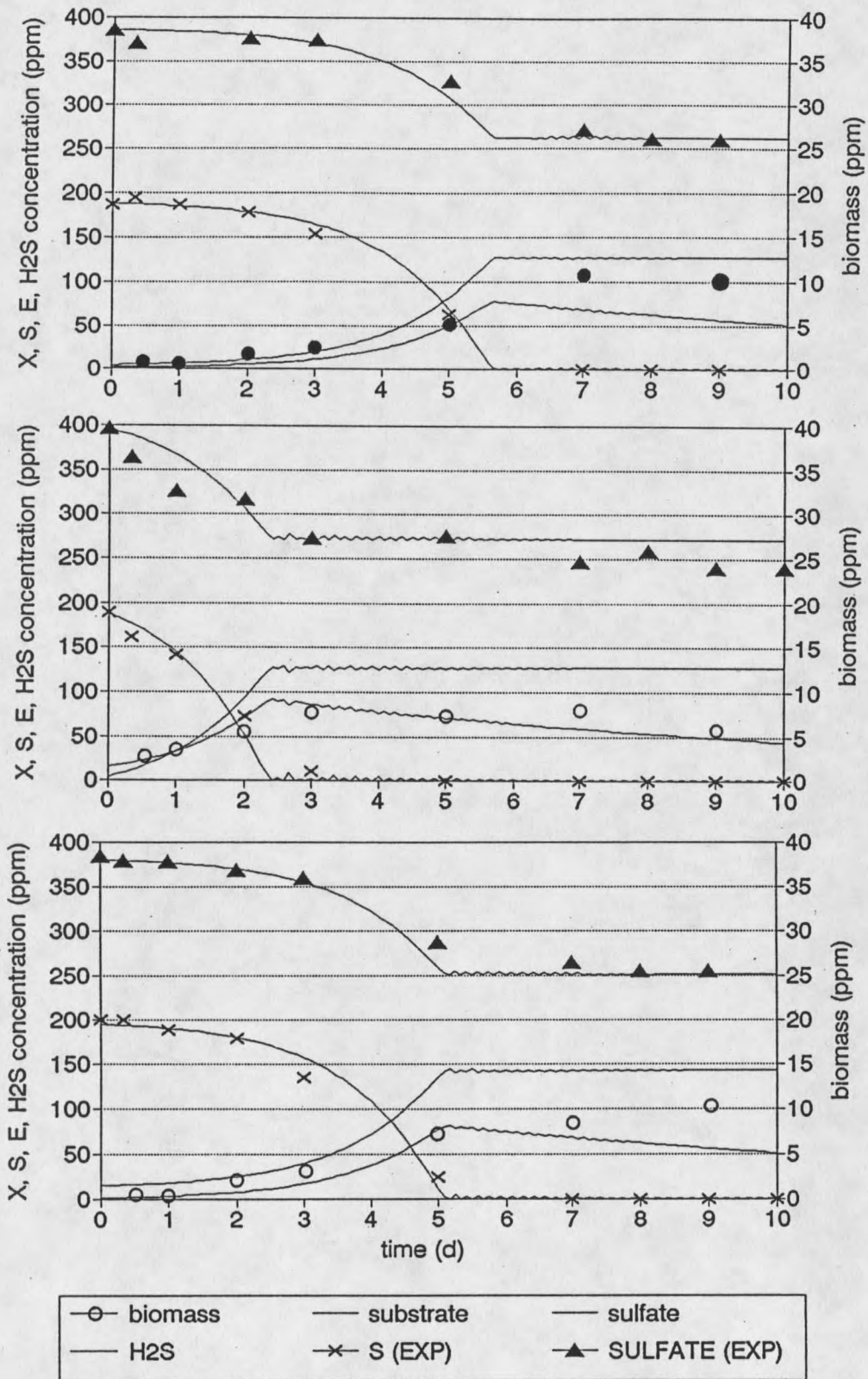


Figure IV. 15 A. Legend see Figure IV.15C.

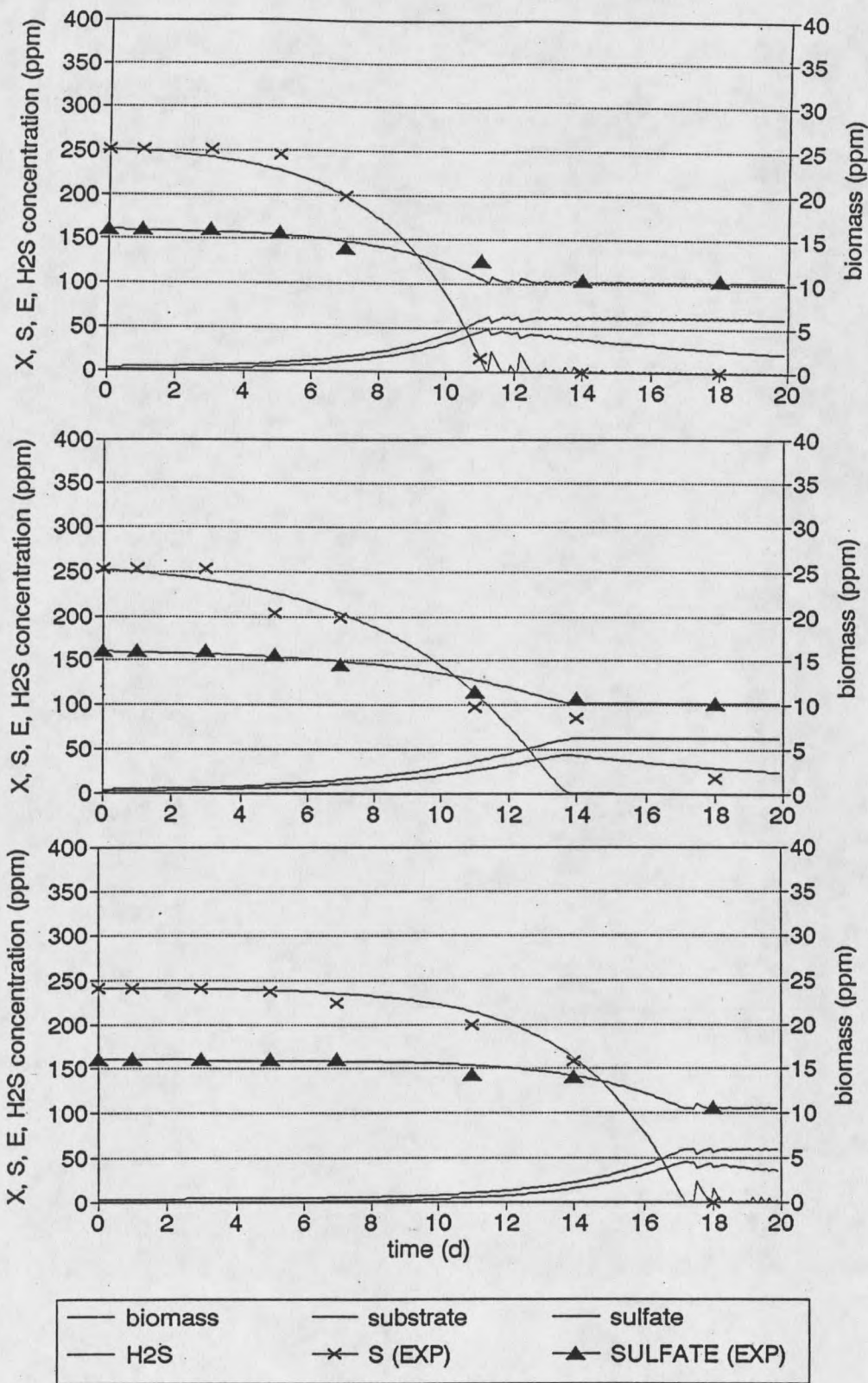
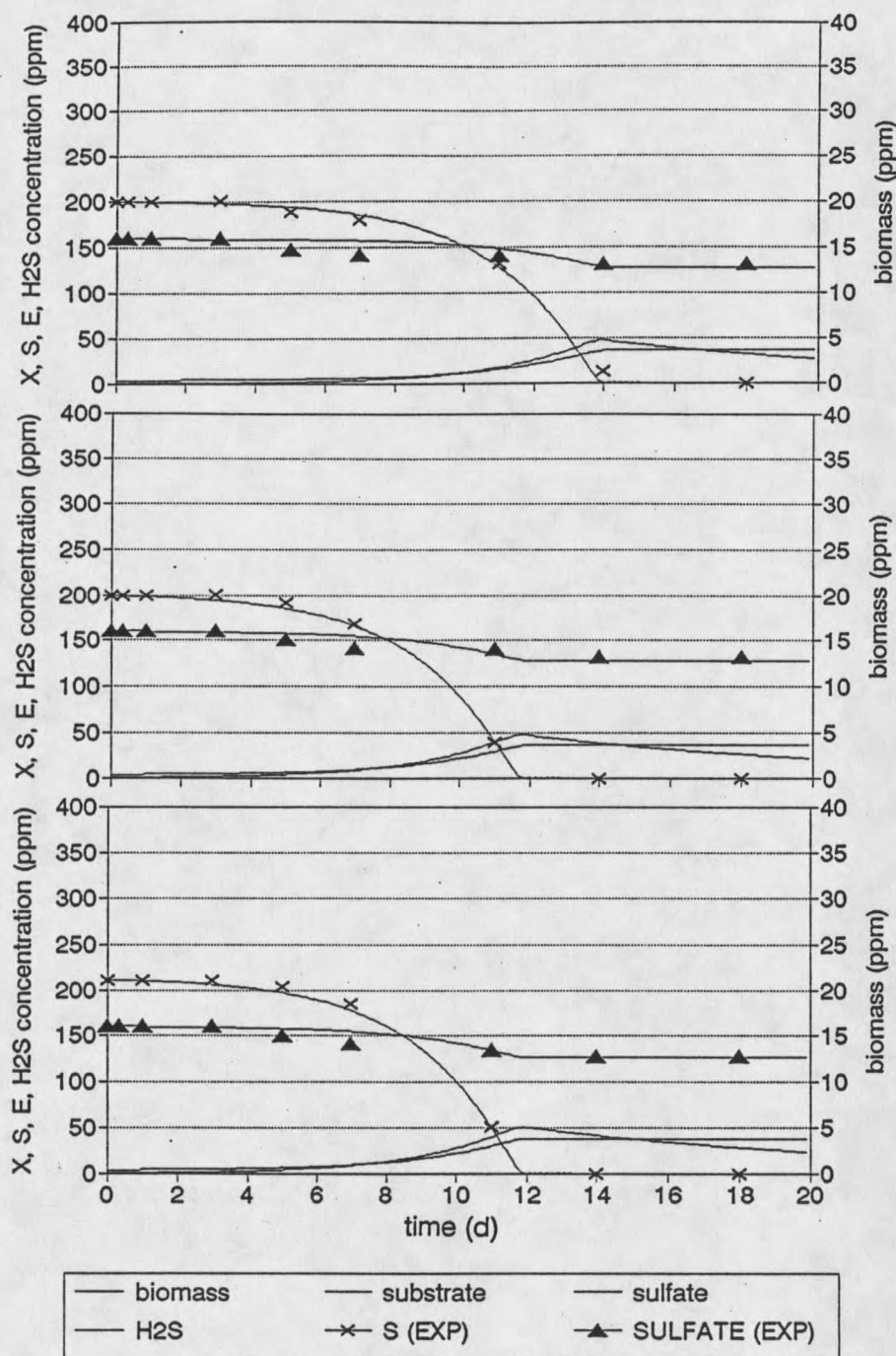


Figure IV.15B. Legend see Figure IV.15C

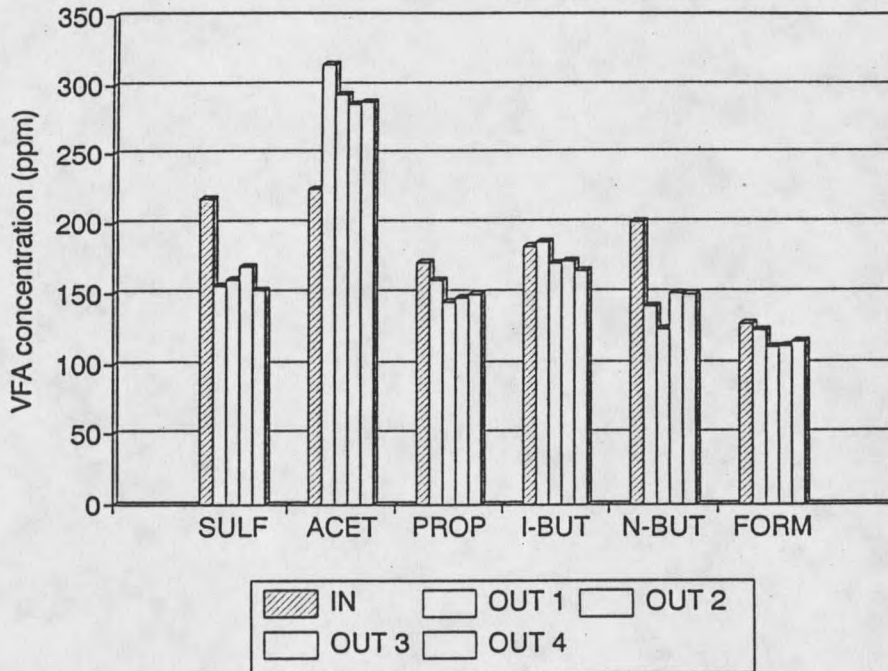


**Figure IV.15C.** The solid lines represent simulations generated by a statistical model using double Monod kinetics. The vertical distances (std. errors) were determined between each experimental data point for sulfate and butyrate and compared with the model prediction. The reported mean std. error (MSE) is the square root of the sum of the square of the std. error divided by the number of observations. The MSE in each of the three graphs was minimized to estimate the kinetic parameters  $K_S$  and  $K_E$ . The data presented in graph A is outcome of a triplicate experiment of thermophilic SRB from the Kugaruk field growing on n-butyrate. The data presented in graph B is the outcome of a triplicate experiment of thermophilic SRB from the Ninian field growing on butyrate, and graph C presents Ninian consortia SRB growing on formate.

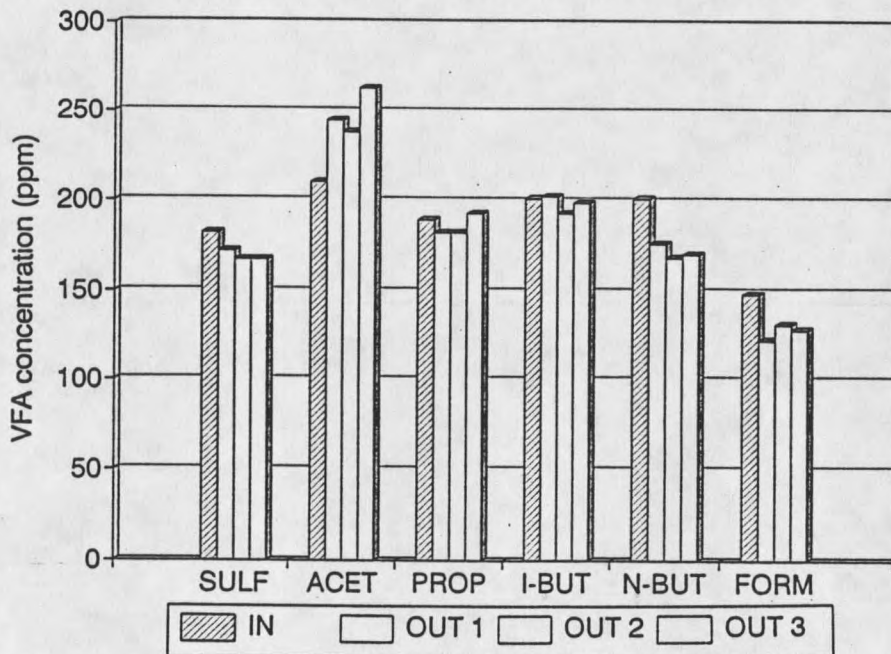
### SRB Kinetics in a Biofilm System

The goal of this study was to determine kinetics and stoichiometry in biofilms of souring oil fields. The tested organisms here were from the Kuparuk field consortium.

Biofilm formation was a very slow process and the overall biofilm thickness never exceeded much more than a monolayer. A constant sulfate and suspended cell concentration in the CMBR effluent indicated a balance between sessile and biofilm cells and steady state conditions at a dilution rate of  $0.188 \text{ h}^{-1}$  3 weeks after inoculation. One month after inoculation, the dilution rate was raised to  $1.07 \text{ h}^{-1}$ . Data was recorded for the two different dilution rates of  $0.188$  and  $1.07 \text{ h}^{-1}$ . Both dilution rates were much higher than the maximum growth rate determined from the batch studies. Suspended organisms were included in the process analysis but contributed less than 5% to the overall rate of sulfate reduction. Sulfate and VFA concentrations are compared between the influent and the reactor effluent at the two tested dilution rates of  $0.188$  and  $1.07 \text{ h}^{-1}$  (Figure IV.16 and 17, respectively). At the dilution rate of  $0.188 \text{ h}^{-1}$  the sulfate, formate, n-butyrate, and propionate effluent concentrations were significantly lower than in the influent. The concentration of acetate increased in the CMBR, and the i-butyrate concentration did not change significantly between influent and effluent, based on a 95% confidence level. At the higher dilution rate, the suspended cell concentration was lower, and differences in concentration between influent and effluent were smaller for all tested components. Propionate and i-butyrate did not change significantly in concentration between influent and effluent of the CMBR. Acetate did increase and sulfate, formate, and n-butyrate showed a significant lower concentration in the effluent as compared to the influent ( $p = 0.05$ ).



**Figure IV.16.** A comparison of influent and effluent concentrations of sulfate, acetate, propionate, i- and n-butyrate, and formate in a CMBR system at a dilution rate of  $0.188 \text{ h}^{-1}$ . The values for the influent sample were averaged for three independent measurements, the effluent samples were taken at 2 hour intervals with duplicate samples for each time.



**Figure IV.17.** A comparison of influent and effluent concentrations of sulfate, acetate, propionate, i- and n-butyrate, and formate in a CMBR system at a dilution rate of  $1.07 \text{ h}^{-1}$ . The values for the influent sample were averaged for three independent measurements, the effluent samples were taken at 2 hour intervals with duplicate samples for each time.

**Table IV.5.** Biofilm and suspended cell size analysis. The reported values are averages from  $n = 654$  (biofilm) and  $n = 233$  (suspended).

		biofilm	SD <sup>4</sup>	susp.	SD <sup>4</sup>	exp.	unit
cell volume:	$V_{\text{cell}} =$	1.25	0.42	1.03	0.27	$10^{-12}$	$\text{cm}^3$
cell length:	$L =$	3.70	0.81	3.46	0.92	$10^{-4}$	cm
cell diameter:	$d =$	0.65	0.22	0.59	0.24	$10^{-4}$	cm
wet cell mass <sup>5</sup> :	$m_w =$	1.35	0.45	1.11	0.29	$10^{-12}$	$\text{g cell}^{-1}$
dry cell mass <sup>6</sup> :	$m_d =$	3.00	1.00	2.45	0.64	$10^{-13}$	$\text{g [dry cell]}^{-1}$
carbon cell mass <sup>7</sup> :	$m_c =$	1.50	0.50	1.22	0.32	$10^{-13}$	$\text{g C cell}^{-1}$

A cell size distribution of biofilm cells at a dilution rate of  $1.07 \text{ h}^{-1}$  is compared to the suspended cells in the same system and is presented in Table IV.5. All measured cell size values show overlapping standard deviations and prove no difference on a 95% level of confidence, indicating no significant difference in cell size between biofilm cells and suspended cells within the same environment.

The measured and calculated data and coefficients with regard to each tested dilution rate are reported in Table IV.6. There was a higher overall sulfate reduction rate ( $D [E_i - E]$ ) of  $15.0 \text{ g SO}_4\text{-S m}^{-3} \text{ h}^{-1}$  observed at the higher dilution rate as compared to  $11.3 \text{ g SO}_4\text{-S m}^{-3} \text{ h}^{-1}$  at  $D = 0.188 \text{ h}^{-1}$ . This was accompanied with a higher biofilm growth rate at the

<sup>4</sup>standard deviation within the sample size

<sup>5</sup>based on a wet cell density of  $1.08 \text{ g cm}^{-3}$

<sup>6</sup>based on a dry cell density of  $0.238 \text{ g cm}^{-3}$

<sup>7</sup>based on a carbon fraction of 0.5 of the dry weight

**Table IV.6.** Thermophilic SRB biofilm kinetic parameters. Experimental results from two dilution rates are reported. The mean and standard deviation (SD) for the three runs were calculated.

Dilution rate (h <sup>-1</sup> ):	0.188	1.07	1.07	mean	SD	exp.	unit
Sulfate influent concentration (E <sub>in</sub> ):	217	180	182				g SO <sub>4</sub> -S m <sup>-3</sup>
Sulfate effluent concentration (E):	157	167	168				g SO <sub>4</sub> -S m <sup>-3</sup>
Suspended cell concentration (X <sub>s</sub> ):	6.9	4.1	3.8			10 <sup>5</sup>	cell# cm <sup>-3</sup>
Attached cell concentration (ρ L):	2.9	2.7	3.0	2.9	0.2	10 <sup>6</sup>	cell# cm <sup>-2</sup>
Biofilm thickness (L):	2.1	2.0	2.0	2.0	0.5	10 <sup>-4</sup>	cm
Biofilm cell density (ρ):	1.4	1.3	1.5	1.4	0.1	10 <sup>10</sup>	cells cm <sup>-3</sup>
Specific sulfate reduction rate (k):	4.8	6.2	6.0	5.7	0.8		g SO <sub>4</sub> -S [g dry cell h] <sup>-1</sup>
Volumetric Sulfate reduction rate (k <sub>v</sub> ):	19.6	26.0	26.9	24.2	4.0		mg SO <sub>4</sub> -S h <sup>-1</sup> cm <sup>-3</sup>
Sulfate surface flux (r <sub>a</sub> (E)):	4.1	5.2	5.4	4.9	0.7	10 <sup>-3</sup>	mg SO <sub>4</sub> -S h <sup>-1</sup> cm <sup>-2</sup> Biofilm
biofilm growth rate (μ):	0.02	0.06	0.05	0.04	0.02		h <sup>-1</sup>
Detachment rate coefficient (k <sub>d</sub> ):	0.02	0.06	0.05	0.04	0.02		h <sup>-1</sup>
cellular growth yield on sulfate (Y <sub>X/E</sub> ):	0.004	0.009	0.009	0.007	0.003		g dry cells [g SO <sub>4</sub> -S] <sup>-1</sup>
cellular growth yield on substrate (Y <sub>X/S</sub> ):	0.002	0.003	0.004	0.003	0.001		g dry cells [g VFA] <sup>-1</sup>

higher dilution rate. There was only little deviation between the attached cell concentration, the biofilm thickness and the biofilm density at both dilution rates. The biofilm thickness was consistently low for both dilution rates and did not get much thicker than a mono layer of cells ( $T = 2 \pm 1 \mu\text{m}$ ). The rate of detachment matched the rate of growth for both tested dilution rates, indicating a steady state biofilm behavior. The biofilm growth rate was low with  $0.017 \cdot \text{h}^{-1}$  at the low dilution rate and 0.04 to 0.06 at the higher dilution rate. The cellular growth yield of dry cell mass was between 0.0032 and  $0.009 \text{ g g}^{-1}$  for the low and for the high dilution rate, respectively. The cells attached to the CMBR walls were highly active for sulfate reduction as indicated by the zero order sulfate reduction rate coefficient between 5 and  $6 \text{ g SO}_4\text{-S reduced per g cell mass per hour}$ .

## Discussion

### Thermophilic Sulfate Reduction by Suspended Bacteria

The stoichiometric ratios were determined for sulfate reduction for formate, and butyrate. A comparison between the two consortia sampled did not show any significant differences for sulfate, vfa and carbonate produced. Butyrate was oxidized incompletely which was indicated by the production of one mol of acetate for each mol of butyrate consumed. Two possible pathways for the oxidation of butyrate are reported (Widdel, 1988). Butyrate could be oxidized incompletely, producing 2 moles of acetate and

donating a total of 4 electrons per mol butyrate for sulfate reduction, or butyrate could be oxidized completely, producing 4 moles of carbonate and donating a total of 20 e<sup>-</sup> per mol butyrate available for sulfate reduction.

The stoichiometry for butyrate oxidation indicates a disproportion of butyrate into 2 moles of carbonate and one mol of acetate. Per mol of butyrate utilized there is a total of 12 e<sup>-</sup> donated. The production of biomass is comparably small to the other products. 12 e<sup>-</sup> could be used to reduce 1.5 moles of sulfate. Experimentally there was a reduction of 1.4 and 1.2 moles of sulfate per mol of butyrate for SRB from the Kuparuk and the Ninian oil field consortia, respectively. Considering the production of biomass and some endogenous decay it can be shown that all butyrate was used for sulfate reduction. Whether this partial oxidation of butyrate is caused by the coexistence of a variety (2 or more) of different SRB species within the consortia or was caused by a SRB strain specific pathway for butyrate degradation cannot be concluded.

Formate is assumed to undergo a complete oxidation to carbonate, donating a total of 2 e<sup>-</sup> per mol. Therefore, 0.25 mols of sulfate could be reduced for each mol of formate oxidized. The value found experimentally for the Ninian consortium was very reasonable with a molar ratio of 0.21 (sulfate/formate). The value obtained for the Kuparuk consortia cannot be realistic with a molar ratio of 0.36 (sulfate/formate). The reason for this high value is mainly due to the presence of small amounts of longer VFA's in the formate medium which were utilized prior to formate.

Kinetics of Suspended, ThermophilicSulfate-Reducing Bacteria Found in Oil Reservoirs

The kinetic rates for the thermophilic sulfate reduction were measured at 60°C. The growth rate for any tested substrate was slow, when compared with aerobic organisms. Also the cellular yield was almost 100 times lower than what is expected for aerobic organisms. However, the values for  $\mu_{\max}$  for thermophilic SRB are similar to values reported by others for mesophilic SRB (Table IV.7 and Table IV.8). There are no kinetic parameters reported in literature for thermophilic SRB's, but the growth rates seem similar to others found for mesophilic SRB's in batch systems. The cellular yield for thermophilic SRB from both tested fields was approximately 10 times lower than most researchers reported for mesophilic pure culture SRB. The reason for this low growth yield could be an enzyme inhibition for cell synthesis due to the high temperature. An alternative reason may be a much higher decay rate (turnover rate) than assumed. Generally, the cell viability was low and cells were difficult to recover even one week after the substrates were depleted. When this concept of a high turnover rate is applied, the cellular growth rate is very difficult to estimate. For the Kuparuk consortia a drastic cell density decrease was observed after the essential nutrients were depleted with decay rates in the range of the maximum growth rates, whereas for the Ninian field consortium the cell density stayed fairly constant after nutrient depletion (Figure IV.10-14).

The maximum growth rate for the SRB from the Ninian consortium was half the rate as found for the Kuparuk SRB's. The half saturation constants for substrate and sulfate were smaller for the consortium from the Ninian field as compared to the Kuparuk field. This

may reflect the lower carbon concentration measured in the produced water of the Ninian field. At the Kuparuk field produced water is recirculated after oil water separation, resulting in a high concentration of VFA's in the produced water and in the formation water. At the Ninian field the produced water is discharged after the oil water separation and fresh sea water is used for water injection exclusively. This operating procedure results in a lower carbon (e.g., limiting nutrient content) of the formation water. The lower half saturation values for the Ninian consortium may reflect the lower nutrient conditions the organisms find in the reservoir. The values for  $K_s$  for the Kuparuk consortium as compared to the Ninian consortium were significantly different on a 95% confidence interval, whereas the values for  $K_e$  did not show a significant difference ( $p=0.05$ ) between the two tested consortia. This may indicate that in both fields sulfate is available in excess over the carbon sources.

Table IV.7. Kinetic parameters for suspended mesophilic-SRB for VFA, lactate, and hydrogen utilization in pure culture and mixed populations.

Species	T (°C)	Substrate	$V_{max}$ (mmol g <sup>-1</sup> h <sup>-1</sup> )	$\mu_{max}$ (h <sup>-1</sup> )	$Y_{X/E}$ (g dry BM g-S <sup>-1</sup> )	$Y_{X/S}$ (g dry BM g <sup>-1</sup> )	$K_E$ (g m <sup>-3</sup> )	$K_S$ (g-S m <sup>-3</sup> )	Reference
<i>Desulfobacter hydrogenophilus</i>	30	acetate		0.039					Schauder et al. (1986)
<i>hydrogenophilus postgatei</i>	28	acetate		0.038					Widdel (1987)
	32	acetate	7.2 to 9.6*	0.035 to 0.046		0.07			Widdel, Pfennig (1981a)
<i>postgatei</i>	30	acetate		0.025					Schauder et al. (1986)
<i>postgatei</i>	30	acetate	5.9*	0.030	0.158		4.2		Ingovorsen et al. (1984)
<i>curvatus</i>	28	acetate		0.033					Widdel (1987)
<i>Desulfotomaculum acetoxidans</i>	36	acetate	9.5*	0.058		0.095			Widdel (1988)
<i>acetoxidans</i>	37	acetate		0.032					Schauder et al. (1986)
mixed culture	31	acetate	5.6	0.022		0.065		5.7	Middleton, Lawrence (1977)
<i>Desulfovibrio vulgaris</i>	35	hydrogen	79.0	0.23	0.091		0.32		Widdel (1988)
<i>desulfuricans</i>	37	hydrogen	5.3	0.057				3.6	Widdel (1988)
<i>Desulfobulbus propionicus</i> (1981b)	39	propionate	22.8	0.069	0.071				Widdel, Pfennig
<i>propionicus</i>	35	propionate		0.110					Nanninga et al. (1987)

Table IV.7. Continued

Species	T (°C)	Substrate	$V_{\max}$ (mmol g <sup>-1</sup> h <sup>-1</sup> )	$\mu_{\max}$ (h <sup>-1</sup> )	$Y_{X/E}$ (g dry BM g-S <sup>-1</sup> )	$Y_{X/S}$ (g dry BM g <sup>-1</sup> )	$K_E$ (g m <sup>-3</sup> )	$K_S$ (g-S m <sup>-3</sup> )	Reference
<i>Desulfotomaculum acetoxidans</i>	36	propionate		0.046					Widdel, Pfennig (1977)
mixed culture	35	propionate	74.6	0.070	0.017	0.022		90	Hunter (1989)
<i>Desulfovibrio baarsi</i>	30	butyrate		0.017					Schauder <i>et al.</i> (1986)
<i>sapovorans</i>	35	butyrate		0.066					Nanninga <i>et al.</i> (1987)
<i>Desulfovibrio desulfuricans</i>	35	lactate	69	0.37		0.030	2.9		Okabe (1992)
<i>desulfuricans</i>	30	lactate	16.4	0.36		0.343		4.4	Cappenberg (1975)
<i>vulgaris</i>	30	lactate	4.6	0.011	0.074		0.5		Ingvorson and Jorgensen (1984)
<i>salexigens</i>	30	lactate	7.9	0.021	0.083		7.4		
<i>sapovorans</i>	30	lactate	2.4	0.007	0.091		0.7		
<i>gigas</i>	30	lactate	34.2	0.092		0.042			Traore <i>et al.</i> (1982)
<i>africanus</i>	30	lactate	49.3	0.060		0.019			
<i>Desulfovibrio desulfuricans</i> biofilm study	35	lactate	37	0.1		0.015			Okabe (1992)

**Table IV.8.** Summary of the kinetic results from this study

Species	T (°C)	Substrate	$V_{max}$ (mmol g <sup>-1</sup> h <sup>-1</sup> )	$\mu_{max}$ (h <sup>-1</sup> )	$Y_{X/E}$ (g dry BM g-S <sup>-1</sup> )	$Y_{X/S}$ (g dry BM g <sup>-1</sup> )	$K_E$ (g m <sup>-3</sup> )	$K_S$ (g-S m <sup>-3</sup> )
Kuparuk consortium suspended culture	60	butyrate	181	0.029	0.005	0.008	9.0	5.5
	60	propionate	99	0.019	0.006	0.002		
	60	formate	31	0.010	0.010	0.005		
Ninian consortium suspended culture	60	butyrate	104	0.017	0.006	0.003	2.0	5.0
	60	formate	63	0.013	0.010	0.003	2.0	4.0
Kuparuk consortium biofilm culture	60	VFA mixture	177	0.041	0.007	0.003		

### Thermophilic Sulfate Reduction in a Biofilm System

At either dilution rate tested, sulfate was reduced by sessile SRB producing hydrogen sulfide. VFA's were used for donating electrons to the sulfate reduction. The results obtained for a thermophilic mixed population SRB biofilm was compared to the results with suspended cultures. The cellular growth rate in the biofilm was between 0.02 and 0.06 h<sup>-1</sup> and did not indicate a significant difference between suspended SRB from the same origin when grown at the same temperature (60°C) and on similar substrates. Butyrate and formate were the most favorable carbon sources for thermophilic biofilm SRB. Propionate was utilized to a smaller degree. The carbon sources utilized for sulfate reduction by suspended cells were identical. Acetate was produced from the incomplete oxidation of butyrate and propionate by suspended and attached SRB. At the two dilution rates of 0.188 and 1.07 h<sup>-1</sup> tested, 0.34 and 0 moles of propionate and 0.68 and 0.43 moles of n-butyrate were consumed, respectively. Applying the stoichiometry determined for suspended SRB from the Kuparuk field, 1.10±0.26 and 0.48±0.09 mol of acetate produced was calculated. The actual amount produced by biofilm SRB lay within the 95% confidence intervals of the calculated acetate production (1.20 and 0.56 moles of acetate produced, respectively). The cellular growth yield on sulfate for biofilm cells was 0.0071±0.0030 g g<sup>-1</sup> as compared to the yield for suspended SRB with values ranging from 0.010 g g<sup>-1</sup> for formate to 0.005 g g<sup>-1</sup> for butyrate. The average cellular growth yield on substrate Y<sub>XS</sub> for the biofilm SRB was 0.003 g dry cell mass per g VFA utilized, the suspended culture ranged from 0.002 to 0.005 g g<sup>-1</sup>. This indicates that there was no change in stoichiometry between suspended and biofilm cells of the tested consortium at 60°C.

The maximum sulfate reduction rate (v<sub>max</sub>) was higher for suspended SRB consortium growing on butyrate as their sole carbon and energy source than it was for SRB in a biofilm from the same consortium (Table IV.8). The biofilm culture was growing on a VFA mixture utilizing butyrate,

propionate, and formate simultaneously. The suspended culture  $v_{\max}$  for propionate and formate was considerably lower than the  $v_{\max}$  for butyrate. Thus the maximum specific sulfate reduction rate was not significantly different in a biofilm system from its value for a suspended culture when similar substrates were utilized.

In the biofilm system neither sulfate nor carbon limited cell growth, since carbon and sulfate were detected at concentrations high above their half saturation coefficient values in the effluent. The cellular growth yield for thermophilic biofilm SRB matches with the cellular growth yield of suspended cells indicating no measurable difference in cell synthesis. The biofilm thickness never exceeded a monolayer of cells. This agrees with SRB biofilms found in souring reservoirs close to the production wells (Burger 1993). This may indicate an inhibition of the microbial production of extracellular polysaccharide (EPS) at 60°C. EPS was shown to stabilize and protect cells in the biofilm microenvironment (Characklis *et al.* 1990). Okabe (1992) reported a drastic shift from cell synthesis for suspended *D. desulfuricans* to EPS synthesis when the same bacteria was present in a biofilm at 30°C. The cellular density of the thermophilic mixed population SRB biofilm had similar values to that found with a pure culture of *D. desulfuricans* biofilms. The volumetric sulfate reduction rate within the biofilm was approximately five times as high as found for pure culture biofilms of *D. desulfuricans*. Hence, thermophilic SRB biofilms seem to lack the structural integrity as found in mesophilic SRB biofilms but the activity of even very thin biofilms can be very high.

#### Thermophilic Sulfate Reduction in an Oil Reservoir Undergoing Secondary Recovery

Close to the injection well where temperatures are often 25 to 40°C (Burger *et al.* 1992) the rate of sulfate reduction may be similar to the values reported for mesophilic sulfate reducing bacteria

(Table IV.7). Further in the formation, temperature will increase up to 70 or 80°C. Thermophilic SRB will be active at these high temperature regions as long as required nutrients are available. The sulfate reduction rates at thermophilic temperatures were found to be more than 50 times higher than those reported for mesophilic SRB growing on similar substrates (Table IV.8). Hence, the rate of sulfate reduction will be many times higher at the oil water interphase than what can be expected around the injection well. Even for an oil reservoir with a high nutrient content in the injection water, as was shown for a recirculated produced water operation, the rate of sulfate reduction deep within the reservoir is of major importance. Thermophilic sulfate reduction becomes especially important since the hydrogen sulfide produced is much closer to the oil water interphase, and precipitation with iron or other inorganic components in the formation is less likely because of the close vicinity of the oil phase.

Conversely, the hydrogen sulfide produced close to the injection well must travel through most of the reservoir to meet the oil phase. It is very likely that most of the H<sub>2</sub>S produced at the injection well will precipitate as iron sulfide throughout the formation, contributing to formation plugging. The plugging effect is further enhanced by the production of EPS at the mesophilic temperature range (Dewar, 1986). In mesophilic SRB biofilms the production of EPS was found to be over 80% of all catabolic substrate conversions (Okabe, 1992). This extracellular material was also shown to protect cells from chemical stressors such as a biocide treatment at the injection well (Van der Wende, 1990). Backflow studies proved the existence of SRB close to the injection well in oil reservoirs where produced water was recirculated (Tayler, 1993). However, the concentration of hydrogen sulfide was found higher further inside the formation indicating a very active reduction of sulfate at the higher temperature zones. Butyrate and other VFA's were found throughout the formation indicating that butyrate was dissolved from the oil into the water phase.

The lack of EPS production at 60°C for thermophilic SRB enhances bacterial transport to the sites

where sulfate, VFA's, and other required nutrients are found. Many thermophilic SRB found in oil field waters are capable of spore formation and withstand temperatures of up to 130°C and become active again in a more suitable environment (Rosnes *et al.* 1991). Hence in an oil formation with recirculating produced water thermophilic SRB and their nutrient sources are constantly introduced to the reservoir and will become active wherever the environmental conditions favor their growth and metabolism.

### Conclusions

Stoichiometric ratios for SRB from both consortia are in a similar range.

Thermophilic SRB from the Kuparuk field exhibited different maximum growth rates and maximum sulfate reduction rates for different VFA's. The highest rates were found when butyrate was consumed. SRB from the Ninian field consortium did not show any significant differences in growth rate for the tested VFA's. Growing on n-butyrate at 60°C, the SRB from the Ninian oil field grew at approximately half of the maximum growth rate of the SRB from the Kuparuk field.

The lower value for  $K_s$  (electron donor) for the Ninian SRB as compared to the Kuparuk may reflect a lower nutrient availability in the formation. The half saturation constant for sulfate did not prove statistically different between the two tested consortia.

Thermophilic SRB originating from the Kuparuk oil field formed very thin biofilms (2  $\mu\text{m}$ ). The biofilm thickness was constant over time and did not vary at different dilution rates.

Thermophilic SRB from the Kuparuk field did not exhibit significant differences in cellular growth rate, growth yield, sulfate reduction rate, and stoichiometry between suspended and attached cells.

Specific microbial sulfate reduction rates are higher at thermophilic temperatures than at mesophilic temperatures. The hydrogen sulfide found in the produced oil and water phase is more likely to be produced closer to or at the oil water interface, limiting or eliminating a possible inorganic precipitation of iron sulfide.

## NOMENCLATURE

A:	wetted surface area [ $L^2$ ]
b:	decay rate [ $t^{-1}$ ]
D:	dilution-rate [ $t^{-1}$ ]
E:	sulfate concentration [ $M L^{-3}$ ]
$E_0$ :	sulfate concentration at time zero [ $M L^{-3}$ ]
$E_i$ :	sulfate inlet concentration [ $M L^{-3}$ ]
F:	liquid flow rate [ $L^3 t^{-1}$ ]
$\Delta G^0$ :	Gibbs free energy change at chemical equilibrium [ $\bar{E} \text{ mol}^{-1}$ ]
$k_i$ :	zero order sulfate reduction rate coefficient for suspended processes [ $M t^{-1} M^{-1}$ ]
$k'_i$ :	zero order sulfate reduction rate coefficient for biofilm processes [ $M t^{-1} M^{-1}$ ]
$k_{of}$ :	sulfate reduction rate per biofilm volume [ $M L^{-3} t^{-1}$ ]
$K_E$ :	half saturation constant for sulfate [ $M L^{-3}$ ]
$K_S$ :	half saturation constant for substrate [ $M L^{-3}$ ]
$\mu$ :	cellular specific growth rate of suspended cells [ $t^{-1}$ ]
$\mu'$ :	specific growth rate of biofilm cells [ $t^{-1}$ ]
$\mu_{max}$ :	cellular maximum growth rate [ $t^{-1}$ ]
P:	$H_2S$ concentration [ $M L^{-3}$ ]
$P_0$ :	$H_2S$ concentration at time zero [ $M L^{-3}$ ]
$r_a(E)$ :	rate of sulfate conversion by suspended cells per liquid volume [ $M L^{-3} t^{-1}$ ]
$r'_a(E)$ :	rate of sulfate conversion by biofilm processes per wetted surface area [ $M L^{-2} t^{-1}$ ]
$r_a(S)$ :	rate of substrate conversion by suspended cells per liquid volume [ $M L^{-3} t^{-1}$ ]
$r'_a(S)$ :	rate of substrate conversion by biofilm cells per wetted surface area [ $M L^{-2} t^{-1}$ ]
$R_a$ :	rate of cellular attachment [ $M L^{-2} t^{-1}$ ]
$R_d$ :	rate of cellular detachment [ $M L^{-2} t^{-1}$ ]
S:	substrate effluent concentration [ $M L^{-3}$ ]
$S_0$ :	substrate concentration at time zero [ $M L^{-3}$ ]
$S_i$ :	substrate inlet concentration [ $M L^{-3}$ ]
$L_f$ :	biofilm thickness [L]
t:	time [t]
$t_g$ :	cellular doubling time [t]
V:	total liquid volume [ $L^3$ ]
$V_{max}$ :	maximum sulfate reduction rate [ $M L^{-3} t^{-1}$ ]
X:	suspended cell mass concentration [ $M L^{-3}$ ]
$X_s$ :	suspended cell mass effluent concentration [ $M L^{-3}$ ]
$X_0$ :	suspended cell mass concentration at time zero [ $M L^{-3}$ ]
$X_i$ :	suspended cell mass inlet concentration [ $M L^{-3}$ ]
$Y_{XS}$ :	cellular growth yield on substrate [ $M M^{-1}$ ]
$Y_{XE}$ :	cellular growth yield on sulfate [ $M M^{-1}$ ]
$Y_{P/E}$ :	yield of hydrogen sulfide on sulfate [ $M M^{-1}$ ]

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## CHAPTER V

THE EFFECT OF NITRATE ADDITION ON MICROBIAL  
SULFATE-REDUCTION IN SOURING OIL RESERVOIRSIntroduction

The production of hydrogen sulfide in souring oil reservoirs is caused by the presence and activity of sulfate reducing bacteria in the formation. The nutrients necessary for SRB growth and metabolism are supplied with the injection water (sulfate and other growth essential trace elements) and the partitioning between the oil and the water phase (Herbert *et al* 1985). Hence, all nutritional requirements are met inside a oil producing formation under secondary recovery. High VFA concentrations can be found in produced water, especially when this produced water is recirculated and reused for injection water after oil water separation (Part 3 of this study). Biocide treatment proved ineffective to prevent souring, since the SRB around the injection well are very well protected in a biofilm (Van der Wende *et al.* 1989, Burger. *et al.* 1992). Thermophilic SRB further inside the formation are even more difficult to target with any biocide treatment because of unknown transport properties and other competitive reactants present (Burger *et al.* 1992). To date, no effective method for control of souring has been found. McInnery *et al.* 1992 suggested introducing a chemoautotrophic denitrifier into the souring reservoir. *Thiobacillus denitrificans* oxidizes sulfide back to sulfate using oxygen or nitrate as an electron acceptor. One strain was reported resistant to 50 ppm H<sub>2</sub>S and prevented the accumulation of hydrogen sulfide by *D desulfuricans* under laboratory conditions at 30°C and low salinity. Hitzman, 1994 claims that the addition of nitrate to any oil bearing formation will increase oil production due to gas and polymer production by denitrifying bacteria.

Questions

Are denitrifying bacteria present in a sea water flooded oil reservoir and what are favorable substrates for these organisms?

At what rate do DNB grow and metabolize under reservoir conditions?

How do temperature and salinity affect the rate of growth and metabolism of denitrifying bacteria present in the sampled oil reservoir consortia?

Does the addition of nitrate to a souring oil reservoir affect the growth and activity of sulfate reducing bacteria in the formation at formation temperature and salinity?

What is the mechanism for this microbial interaction (e.g., microbial competition, inhibition, change in redox, change in pH, etc.)?

What are the chemical reactions possible between reactants, intermediates or products (such as sulfate, nitrate, VFA, nitrite, hydrogen sulfide, etc.) under oil reservoir conditions?

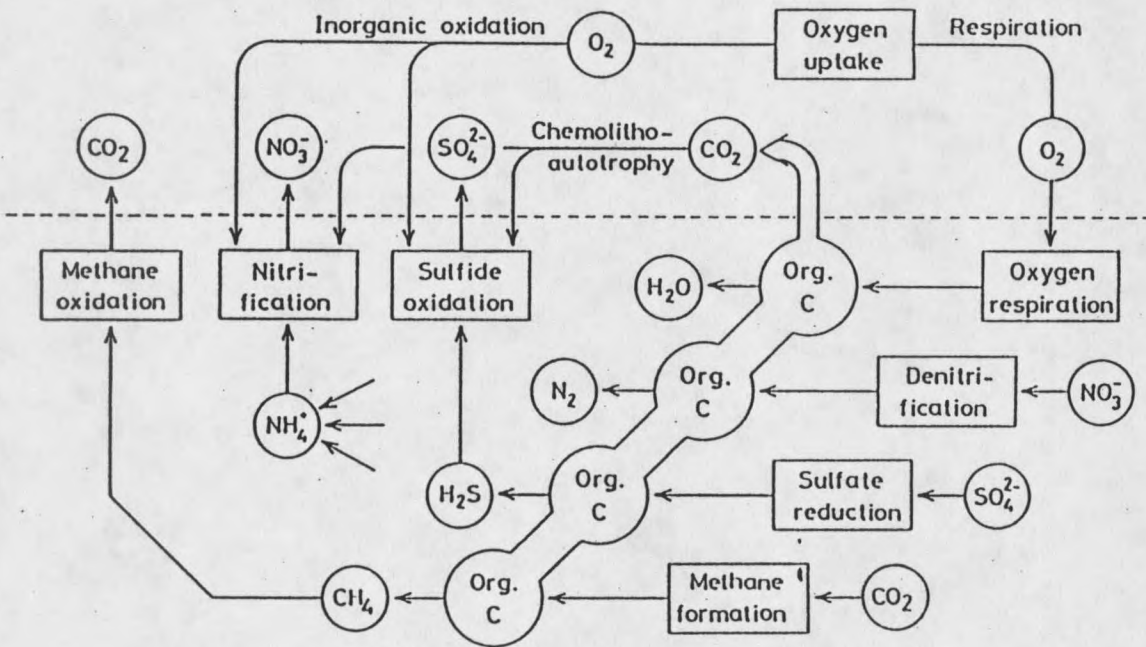
Microbial Hierarchy in Anaerobic Environments

In anaerobic environments where oxygen is absent or quickly consumed by aerobic or facultative aerobic microorganisms, several groups of anaerobes will compete for the substrate available. Depending on the availability of various electron acceptors (such as oxygen, sulfate, or nitrate) organic matter is oxidized to  $\text{CO}_2$  or other metabolic intermediates (e.g., acetate)

while  $O_2$ ,  $NO_3^-$ , or  $SO_4^{2-}$  are reduced (Figure V.1). Nitrate and sulfate are the most important electron acceptors for anaerobic respiration besides carbon dioxide. Both nitrogen as well as sulfur compounds can exist in a wide variety of oxidation states (Table V.1). Determined by the free energy change for each of the corresponding half reactions, the hierarchy of the possible processes is generally dictated by the amount of energy the organisms could gain by reducing nitrate, sulfate, or carbon dioxide (Table V.2). The reduction of nitrate will occur after all the residual oxygen is consumed in a system (Figure V.1). Nitrate will be reduced completely to nitrogen gas if no inhibitory compounds or nutrient limitations are present (Lazarova *et al.* 1994). If no nitrate is present in the system, the next energetically most favorable reaction is the reduction of sulfate and the production of hydrogen sulfide. The least energetically favorable reaction will be the production of methane which will only occur if no other electron acceptor is present or if all electron acceptors initially present are completely utilized. In the presence of oxygen all of the respiratory products such as  $H_2S$  and  $CH_4$  can be oxidized microbially to elemental sulfur or sulfate and to carbon dioxide, respectively.

**Table V.1.** Oxidation states for nitrogen and sulfur compounds (Stumm, Morgan 1981).

nitrogen compound	oxidation state	sulfur compound	oxidation state
$NH_4^+$	-III	$H_2S$	-II
$N_2$	0	$S_8$	0
$NO_2^-$	+III	$S_2O_3^{2-}$	+II
$NO_3^-$	+V	$SO_3^{2-}$	+IV
		$SO_4^{2-}$	+VI



**Figure V.1.** The hierarchy in anaerobic environments is determined by the availability of possible electron acceptors (Jorgenson, 1983). Inside a lake sediment the organic matter is oxidized to  $CO_2$  through a vertical sequence of  $O_2$ ,  $NO_3^-$ , and  $SO_4^{2-}$  respiration processes. After oxygen is depleted nitrate and then sulfate will be utilized. If these electron acceptors become depleted deeper in the sediment, methane is produced.

**Table V.2.** Free energy changes for various half reactions (Characklis, Marshal 1990).

half reaction	$\Delta G^0$ [kJ(mol e <sup>-</sup> ) <sup>-1</sup> ]
$0.2 NO_3^- + 1.2 H^+ + e^- \rightarrow 0.1 N_2 + 0.6 H_2O$	-71.66
$0.5 NO_3^- + H^+ + e^- \rightarrow 0.5 NO_2^- + 0.5 H_2O$	-39.43
$0.125 NO_3^- + 1.25 H^+ + e^- \rightarrow 0.125 NH_4^+ + 0.375 H_2O$	-39.50
$0.125 SO_4^{2-} + 1.19 H^+ + e^- \rightarrow 0.0625 H_2S + 0.0625 HS^- + 0.5 H_2O$	21.28
$0.125 CO_2 + H^+ + e^- \rightarrow 0.125 CH_4 + 0.25 H_2O$	24.11

### Control of Souring due to Microbial Interactions

In order to control souring via microbial competition, it would appear logical to investigate the competition between denitrifying bacteria and SRB, since the denitrifiers are energetically favored over SRB. Bode, 1988 found complete denitrification to  $N_2$  in a fixed film reactor treating waste water with low salinity containing more than 1000 ppm  $NO_3-N$ . The rate of denitrification was higher at  $62^\circ C$  than at mesophilic temperatures. In sea water the concentration of nitrate is relatively low, generally less than  $1 \text{ g m}^{-3}$ , whereas the concentration of sulfate is high, approximately  $1000 \text{ g m}^{-3}$ . Sulfate reducing bacteria will therefore be most active and only limited by the availability of carbon or hydrogen. In order to suppress the microbial activity of SRB's, nitrate could be added, enabling the denitrifiers to become active in metabolism and consequently growth. Depending on the level of nitrate available initially, the denitrifying bacteria should outcompete the sulfate reducers for substrate, either completely or incompletely, depending on the level of nitrate added to the system. A constant source of denitrifiers can be assumed to be present in the reservoir due to sea water injection. Denitrifiers not only tolerate oxygen, but will rapidly switch their respiratory system from using nitrate as terminal electron acceptor to oxygen if oxygen becomes available, and will return to using nitrate once oxygen is depleted (Stouthamer, 1988).

### Theory on Microbial Interaction Between Two Species

Six kinds of interactions are considered for two interacting populations: Neutralism, mutualism, commensalism, amensalism, competition, and predation (Table V.3). Neutralism means there is no change in growth rate of one species due to the presence of the other - no interaction. For mutualism each of the two species requires the other species for their growth. An example for mutualism is the growth of acetogenic bacteria and methanogens, where the acetogens

deliver acetate and hydrogen to the methanogens. Both products are essential substrates for methanogens and SRB. Since the acetogenic reactions have a positive  $\Delta G^\circ$ , a rapid product consumption by methanogens or SRB is required in order to grow and metabolize. In commensalism one species benefits from the presence and activity of another species. For example, in anaerobic environments, fermenters deliver VFA, and  $\text{CO}_2$  to acetogens, SRB and methanogens. The latter will benefit from this interaction, whereas the fermenters are unaffected. The opposite occurs in amensalism, where the growth of one species is inhibited due to the presence of another species. This could be due to the production of a toxic or inhibitory compound or due to the removal of an essential nutrient. In competition between two species, each species has a negative influence on the growth rate of the other. In predation, one species benefits at the expense of the other. Possible combinations of interactions are summarized in Table V.3.

**Table V.3.** Possible interactions of two species (Bailey and Ollis, 1986).

		Effect of species A on species B		
		-	0	+
Effect of species B on species A	-	- - competition	- 0 amensalism	- + predation
	0	0 - amensalism	0 0 neutralism	0 + commensalism
	+	+ - predation	+ 0 commensalism	+ + mutualism

### Inhibition Kinetics

In the case of amensalism, an inhibiting substance is produced by one species and the growth rate of another species present might be inhibited. Three cases of enzyme inhibition

will be considered: competitive inhibition, uncompetitive inhibition and non competitive inhibition.

Competitive inhibition. The enzyme has a single active site to which either the substrate or the inhibitor may bind. The presence of the inhibitor blocks the active site and the substrate cannot be converted. The following equation can be derived for the rate of substrate conversion (Roels, 1983):

$$v_i = \frac{v_{\max} S}{S + K_s (1 + I/K_i)} \quad (E1)$$

where:

- $v_i$ : rate of substrate conversion
- $v_{\max}$ : maximum rate of substrate conversion
- $S$ : substrate concentration
- $I$ : inhibitor concentration
- $K_s$ : half saturation constant for substrate
- $K_i$ : inhibition constant

In this type of inhibition, the inhibiting effect can always be completely overcome by increasing the concentration of substrate.

Uncompetitive inhibition. The inhibitor binds to its own effector site and not to the active site of the enzyme and only binds to the complex of enzyme and substrate (Roels, 1983). The following equation describes substrate conversion in the presence of an inhibitor:

$$v_i = \frac{v_{\max} S}{K_s + S (1 + I/K_i)} \quad (E2)$$

In this type of inhibition, a high substrate concentration cannot completely compensate for the

presence of an inhibitor. Conversely, there is relatively little effect at low substrate concentrations.

Non competitive inhibition. In non competitive inhibition the inhibitor has affinity to the inhibitor site, where the substrate also has affinity to the enzyme-inhibitor complex. The inhibition kinetics for substrate conversion can be described as follows (Aiba *et al.* 1973):

$$\mu_1 = \frac{r_{\max} S K_i}{(K_s + S) (I + K_i)} \quad (E3)$$

In non competitive inhibition, the inhibitor effects are found at all substrate concentrations.

## Materials and Methods

### Experimental Design

Two reactor systems were used to determine kinetics, yield, and stoichiometry for denitrifying bacteria. Batch reactors were used for identifying the DNB presence in the produced water from the Ninian field and the Kuparuk field using a DNB specific growth medium. In addition, a continuous stirred tank reactor (CSTR) was used to determine kinetics and stoichiometry for DNB from the Kuparuk field. The inhibiting effect of nitrite on SRB growth and activity was tested in batch reactors.

### Batch Reactor System

Glass vials with a total volume of 60 ml were used to study substrate utilization. After cycling between vacuum and  $N_2$  pressure to remove all residual oxygen, the vials were filled with the medium up to 30 ml, leaving 30 ml of headspace volume in the pressurized vials. The bacteria inoculum was added to initiate the experiment and the vials were incubated at 40 or 60°C. Samples were taken with a 21 gauge needle on a 1 ml syringe. The samples were analyzed for nitrate, nitrite, sulfate, formate, acetate, propionate, i-, and n-butyrate, and for total cell mass. When testing the inhibiting effect of nitrite on SRB, hydrogen sulfide was also assayed.

### CSTR System

The CSTR system consisted of three major components; 1) influent medium reservoir 2) the reactor vessel, and 3) effluent reservoir. Bacterial cells were grown on a VFA mixture consisting of formate, acetate, propionate and butyrate at 200 ppm of each in the influent medium. The reactor vessel was operated at a dilution rate of  $0.01 \text{ h}^{-1}$  at steady state conditions. Growth in the CSTR was nitrate limited ( $180 \text{ g NO}_3\text{-N m}^{-3}$ ) and the pH was controlled at pH 8.5. Oxygen free nitrogen gas was bubbled through the growth medium reservoir and was then used as a headspace gas for the bioreactor. The gas left the system after bubbling through the effluent reservoir. Temperature was controlled in the reactor at 60 °C. Duplicate samples were taken daily after steady state conditions were reached. The samples were analyzed for nitrate, nitrite, sulfate, formate, acetate, propionate, i-, and n-butyrate, and total cell mass.

### Sampling Procedure

Sampling was performed on site at each oil field. Approximately 200 liters of fresh, untreated produced oil/water mixture was concentrated to 2 liters of final sample volume. The particulate concentration was achieved by using a Pelicon membrane filter device. A prefilter of 5  $\mu\text{m}$  pore size was used to eliminate oil and other particulates which would clog the filter membrane after a short time period. All sampling was performed using anaerobic techniques, including positive  $\text{N}_2$  pressure on all containers used. Approximately half of the final sample concentrate was filled into 1.6 ml vials and shock-frozen in isopropanol and dry ice. The other half of the sample concentrate was injected directly on-site into prepared 30 ml vials to test for utilization by various potential substrates. All liquid transfers were carried out in an anaerobic glove box under 85/10/5%  $\text{N}_2/\text{H}_2/\text{CO}_2$  gas mixture. The bacterial consortia were characterized by measuring total count, microbial cell size distribution and MPN for various bacterial groups.

### Medium Preparation

SRB medium. The medium used was suggested for marine type SRB (Pfennig *et al.* 1986). The medium preparation was accomplished with a variety of sterile stock solutions. The complete medium composition is listed in Appendix A. After autoclaving, the medium was saturated with a prepurified gas mixture of 10%  $\text{CO}_2$  / 90%  $\text{N}_2$  to minimize contamination as well as to ensure an oxygen free environment. The pH was adjusted to 7.2 before the medium was injected into the batch vials.

DNB medium. A DNB specific medium was used to enrich for DNB from the North Sea consortia. The basic medium was described by Ieter and Ingraham (1986). A slightly modified version of this

medium was used for enrichment and growth of DNB. The composition of the basic medium used is listed in Table V.4. The medium was modified to various salinities and the nitrate concentration was lowered to  $100 \text{ g NO}_3\text{-N m}^{-3}$  for the batch experiments.

**Table V.4.** Chemical composition of the DNB specific medium

H <sub>2</sub> O	1,000	ml
EDTA	0.2	g
CaCO <sub>3</sub>	0.12	g
MgCl <sub>2</sub> ·nH <sub>2</sub> O	0.16	g
Na <sub>2</sub> HPO <sub>4</sub>	0.2	g
KH <sub>2</sub> PO <sub>4</sub>	0.2	g
KNO <sub>3</sub>	1.30	g
Yeast extract	0.1	g
NaCl	10.0	g

### Method of Analysis

Total cell counts and MPN method were performed as described in chapter III.

Sulfate, H<sub>2</sub>S, and VFA's were measured as described in chapter III.

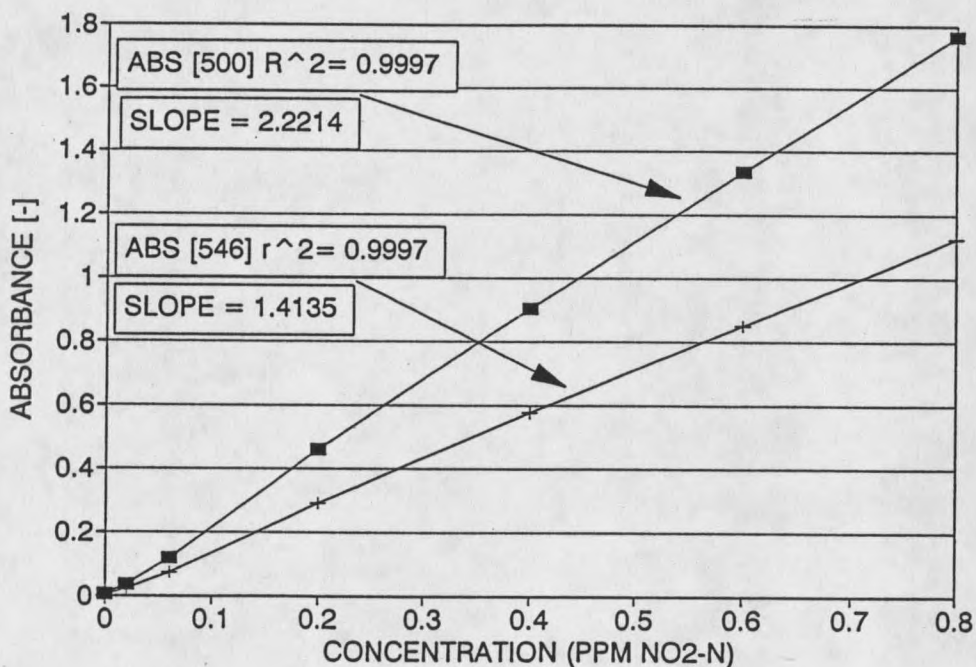
Nitrate was determined using an ion chromatograph with the identical procedure used for sulfate analysis.

Nitrite was detected with two different procedures.

A) ion chromatography: The initial problems with the use of this method were the high chloride concentration in most of the media used. Unfortunately the retention times for chloride and nitrite are only 0.25 minutes apart; hence, high concentrations of chloride covered the

nitrite peak. Low concentrations of nitrite were impossible to measure when chloride was present. To overcome this problem a silver precolumn was used for sample preparation in order to remove the chloride from the sample. This helped to increase the sensitivity for nitrite detection. For very small nitrite concentrations (<1ppm), a photometric method is used.

B) Photometric detection is described in detail Standard Methods for the Examination of Water and Waste water. After 10 minutes a red color stabilizes and a photometric measurement at 546 nm was compared to a nitrite standard curve (Figure V.2).



**Figure V.2.** Nitrite standard curve. Absorbance was measured at 500 nm and at 546 nm for 6 nitrite standard solutions containing between 0 and 0.8 ppm NO<sub>2</sub>-N.

ResultsMicrobial Composition of Oil Field Produced Waters<sup>1</sup>

At both fields, the microbial consortia consists of a wide variety of organisms, including sulfate reducing bacteria, methanogenic bacteria and general anaerobic bacteria including denitrifying bacteria. The microbial density (based on AODC-test) was considerably higher at field A ( $4.5 \cdot 10^4$  cells  $\text{ml}^{-1}$ ) as compared to field B ( $1.21 \cdot 10^3$  cells  $\text{ml}^{-1}$ ). Viable MPN-tests for general anaerobic bacteria (GAB) were approximately 55% of the total number of bacteria detected by the AODC method (Table V.5). General anaerobic bacteria included denitrifying, fermentative, sulfate-reducing, acidogenic, and methanogenic bacteria. There were similar concentrations of general anaerobic bacteria present at mesophilic and thermophilic temperatures ( $1.4$  and  $1.1 \cdot 10^4$  cells  $\text{ml}^{-1}$ , respectively). At  $35^\circ\text{C}$  the numbers of methanogens

**Table V.5.** Microbial composition of produced water from the Kuparuk oil field.

total cell number (AODC)	$4.5 \pm 0.4 \cdot 10^4$	cells $\text{ml}^{-1}$
cell size distribution (pop 1)	$0.28 \pm 0.08$	$\mu\text{m}^2$
cell size distribution (pop 2)	$0.87 \pm 0.18$	$\mu\text{m}^2$
cell size distribution (pop 3)	$12.45 \pm 1.4$	$\mu\text{m}^2$
mesophilic ( $35^\circ\text{C}$ )		
general anaerobic bacteria (MPN)	$1.4 \cdot 10^4$	cells $\text{ml}^{-1}$
sulfate reducing bacteria (MPN)	$1.2 \cdot 10^2$	cells $\text{ml}^{-1}$
methanogenic bacteria (MPN)	$2.4 \cdot 10^3$	cells $\text{ml}^{-1}$
thermophilic ( $60^\circ\text{C}$ )		
general anaerobic bacteria (MPN)	$1.1 \cdot 10^4$	cells $\text{ml}^{-1}$
sulfate reducing bacteria (MPN)	$1.6 \cdot 10^3$	cells $\text{ml}^{-1}$
methanogenic bacteria (MPN)	$0.9 \cdot 10^3$	cells $\text{ml}^{-1}$

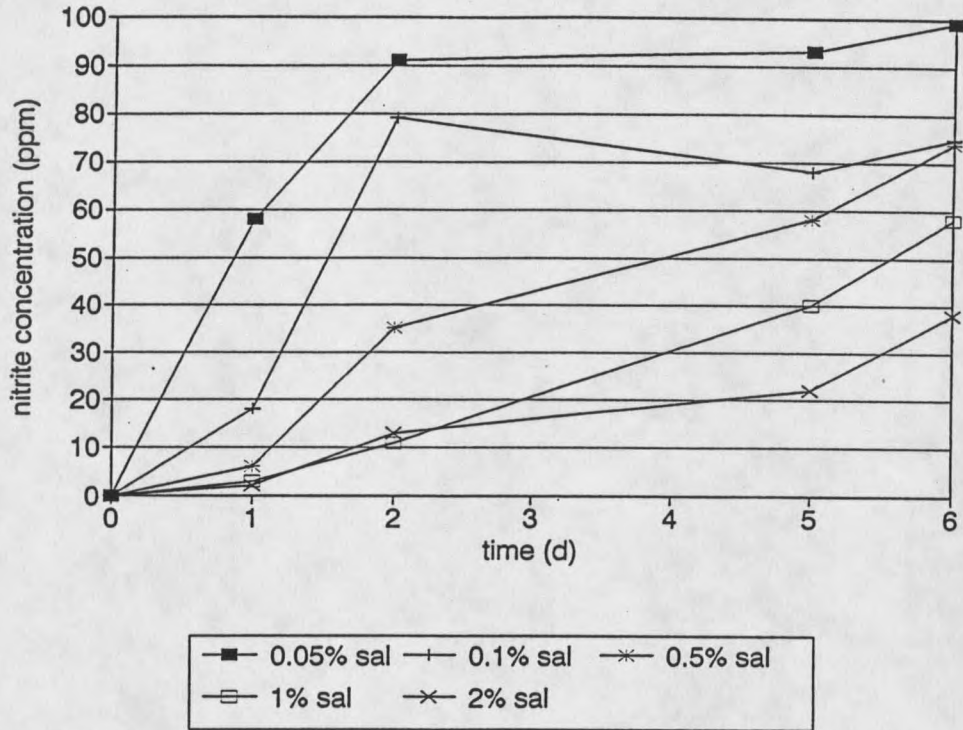
<sup>1</sup>Previously reported in chapter III and reproduced for clarity.

were one digit higher than the numbers obtained for m-SRB. The concentration of thermophilic (tested at 55°C) methanogens were only slightly lower than for t-SRB. Approximately 4% of the total number of bacteria present were detected as viable t-SRB.

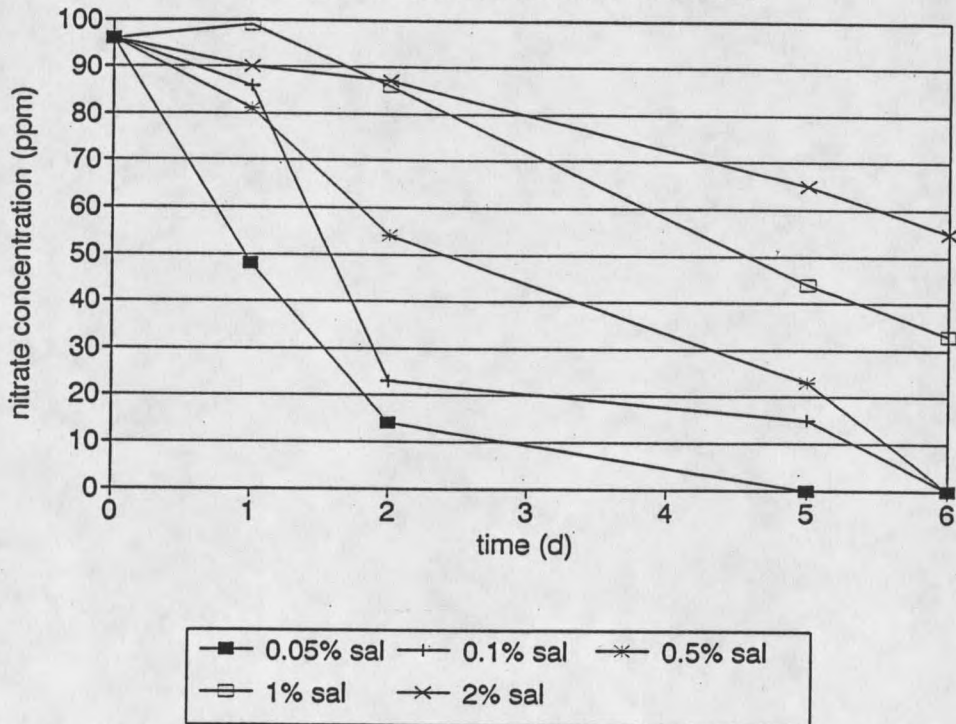
Statistical analysis (t-test) on cell size distribution of consortia from the Kuparuk field indicated three morphologically different groups of cell sizes (Table V.5). The populations (pop1, pop2, pop3) contributed 6.4, 92.8 and 0.8 percent, respectively, to the total cell numbers.

#### Growth of DNB from the North Sea Consortia in a Batch Reactor System

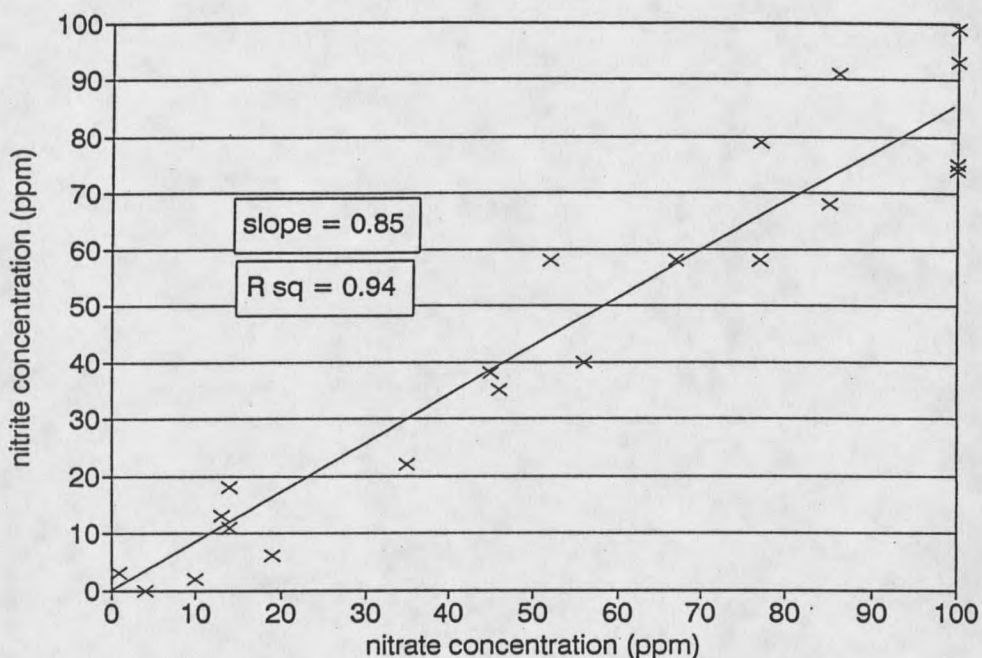
Various salinities were used to test for microbial nitrate reduction. The tested salinities were 0.05, 0.1, 0.5, 1.0, 1.0, and 2.0% NaCl in the DNB medium. At higher salinity the reduction of nitrate was slower (Figure V.3). The most vigorous growth was found at low salinity. Nitrite accumulated in all test-vials as a product from an incomplete nitrate reduction (Figure V.4). Averaged for all tested salinities,  $85 \pm 8$  % of the nitrate reduced was recovered as nitrite (Figure V.5). The most favorable substrate consumed by DNB's was acetate. Longer chain VFA's were consumed also after acetate was consumed. The averaged growth yield for DNB from the Ninian field was  $Y_{X/E} = 0.021 \pm 0.012$  g dry cell mass per g  $\text{NO}_3\text{-N}$  reduced. The large deviation for the yield coefficient was caused by a large deviation for cell size (length), upon which dry weight was based. The maximum growth rate was  $\mu_{\text{max}} = 0.06 \text{ h}^{-1}$ . At higher salinities the doubling time increased. The maximum nitrate conversion rate was found to be  $v_{\text{max}} = 204 \text{ mmol NO}_3 \text{ (g dry cell mass h)}^{-1}$ . The microbial population originating from the Ninian oil field water consortium at denitrifying conditions appeared very uniform. Filamentous organisms with various length (5-50  $\mu\text{m}$ ) and uniform thickness (0.4-0.7  $\mu\text{m}$ ) were almost exclusively present. These organisms were not apparent under sulfate reducing conditions.



**Figure V.3.** Nitrate reduction in batch reactor systems at various salinities from 0.05 to 2% NaCl. Formate and acetate were offered as carbon source. Nitrate reduction is plotted vs time after inoculation with DNB isolates from the Ninian field consortium.



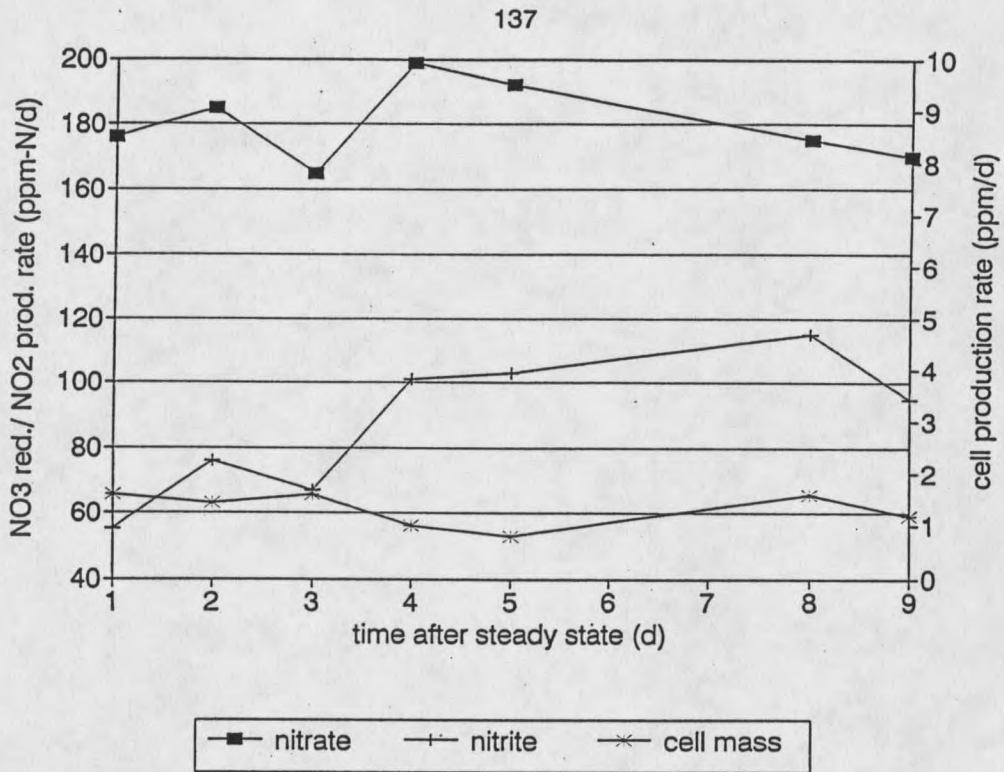
**Figure V.4.** Nitrite production at 60°C at various media salinities. Formate and acetate were the offered carbon and energy sources. The accumulation of nitrite is vs time after inoculum with DNB isolated from the Ninian consortium.



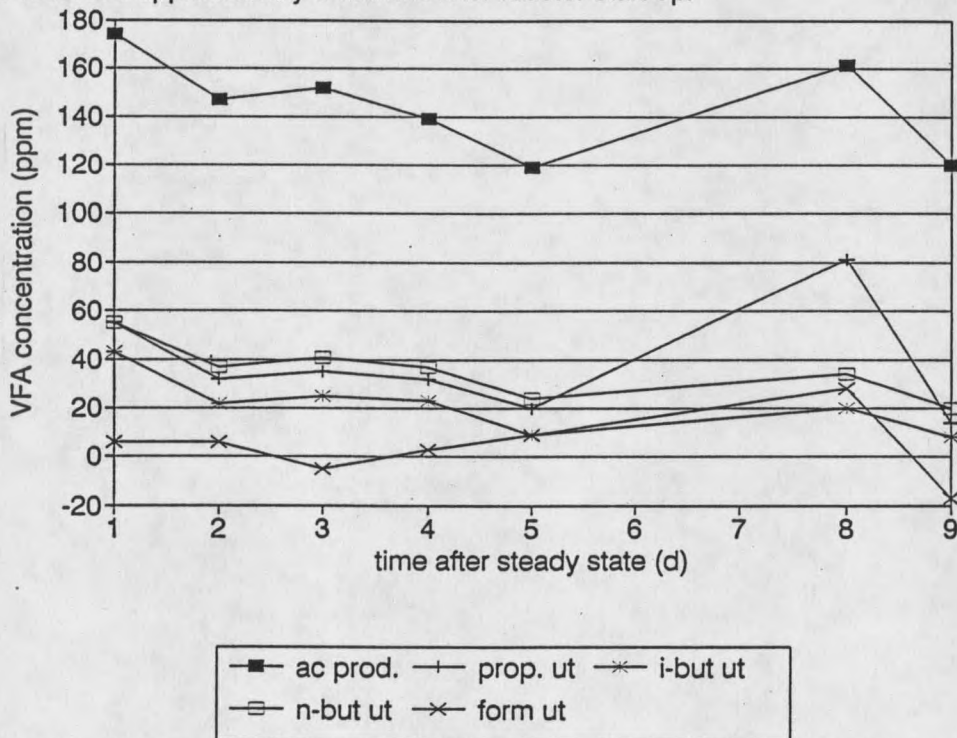
**Figure V.5.** Nitrite accumulation vs nitrate reduction (accumulated). The data for all different salinities tested was combined in this graph to determine the average stoichiometric ratio for nitrate reduction for Ninian consortia DNB.

#### Growth of DNB from Kuparuk Produced Water in a CSTR System

The reactor vessel was initially filled with fresh (not previously frozen) produced water from the Kuparuk oil field. Reduction of nitrate was observed and nitrite was produced as soon as the nitrate containing medium was added to the reactor. At steady state operation nitrate was depleted and limited cellular growth. 48% of the nitrate reduced was recovered as nitrite (Figure V.6). The cellular growth yield was low with  $Y_{X/E} = 0.0089 \pm 0.0042$  g dry cell mass per g  $\text{NO}_3\text{-N}$  reduced and  $Y_{X/S} = 0.0055$  g dry cell mass per g VFA utilized. The nitrate reduction rate was  $v = 80 \text{ mmol NO}_3 \text{ (g dry cell mass h)}^{-1}$ . A statistical analysis indicated that acetate was the most favored carbon source for Kuparuk DNB (Figure V.7). Other VFA utilized were propionate, i-, and n-butyrate. The formate concentration in the reactor effluent was not



**Figure V.6.** Steady state data on nitrate, nitrite, and cell mass production from the CSTR at denitrifying conditions. Kugaruk produced water was used to inoculate the reactor. These data were recorded approximately 2 months after reactor start up.



**Figure V.7.** Steady state data on VFA consumption from the CSTR at denitrifying conditions. Kugaruk produced water was used to inoculate the reactor. These data were recorded approximately 2 months after reactor start up.

significantly different than the influent concentration. The cells detected in the CSTR were filamentous or fragments of filaments. The filaments were often arranged in spiral or curved configurations. The filament length varied between 10 and 120  $\mu\text{m}$ . The morphology of the denitrifying bacteria from the Kuparuk field was different from SRB from the same field but similar to the DNB found within the Ninian consortia.

The Effect of Nitrite on Sulfate Reduction  
in the Ninian and the Kuparuk Consortia

Ninian consortium. The produced nitrite could exhibit inhibiting or even toxic effects on sulfate reducing bacteria. Various nitrite concentrations were added to a sulfate-reducing high salinity medium. At time zero, one ml of an actively growing SRB culture originating from the North Sea consortium was inoculated into the duplicate vials. The  $\text{NO}_2\text{-N}$  concentrations initially present in the medium were:

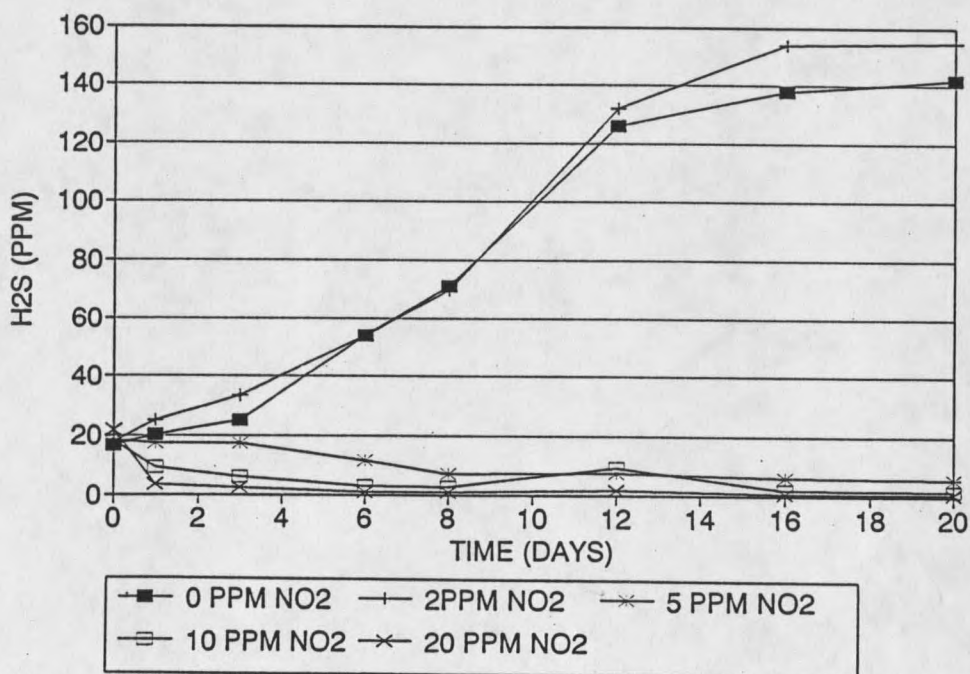
0, 2, 5, 10, and 20  $\text{g m}^{-3}$

At  $\text{NO}_2\text{-N}$  concentrations of 0 or 2  $\text{g m}^{-3}$  (e.g. 143  $\mu\text{mol}$ ), uninhibited SRB growth and metabolism were observed (Figure V.8) for the Ninian consortium DNB. At the higher nitrite concentrations no SRB growth or activity was detected. The concentration of nitrite measured in the duplicate vials where 2 ppm nitrite was added initially decreased to less than 0.2 ppm  $\text{NO}_2\text{-N}$  6 days after nitrite addition (Figure V.9). The concentration of  $\text{H}_2\text{S}$  was decreasing over time in all vials with an initial dosage of 5 ppm nitrite-N or higher. This experiment indicated that there was a simultaneous reduction occurring for nitrite and hydrogen sulfide, when both were present in the water phase.

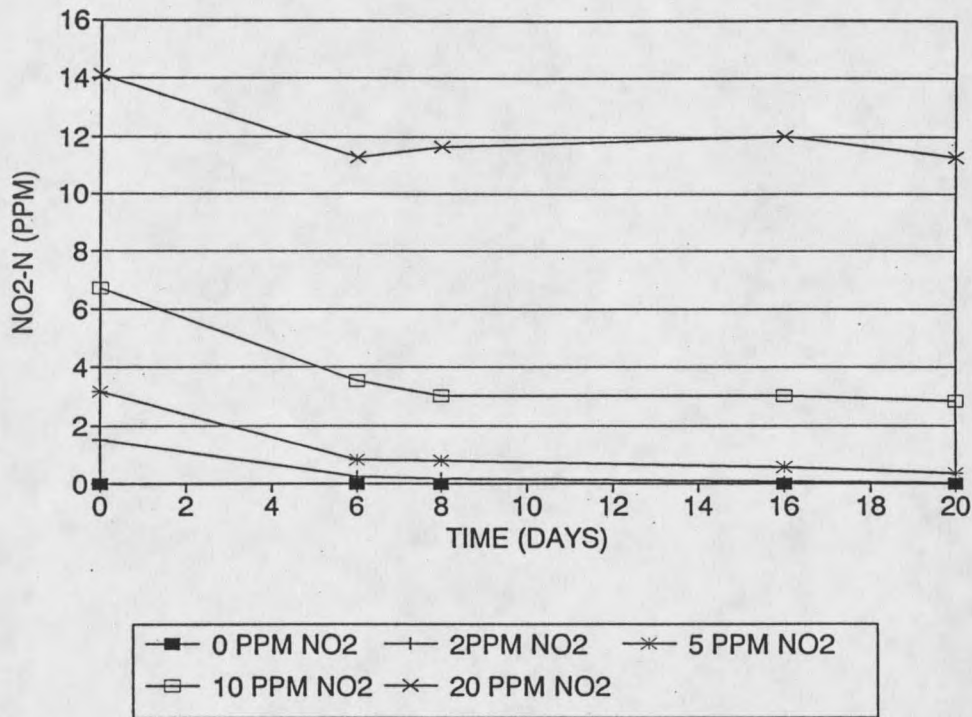
The effect of nitrite addition on redox potential. The redox potential was measured in all test vials at time 0 and at the end of the experiment. At time 0 the redox potential in the SRB medium without microbial activity was between  $E_h = -85$  to  $-99$  mV. At the end of the inhibition experiment, the redox potential was lowered in the vials where hydrogen sulfide was produced (Table V.6). In those vials where nitrite was added and no  $H_2S$  was produced the redox potential was significantly higher 20 days after inoculation than the initial value of  $E_h$ .

**Table V.6.** Change in redox potential and  $H_2S$  production after nitrite addition

initial nitrite ppm	$E_h$ (t0) mV	$E_h$ (t20) mV	$H_2S$ prod. (+/0)
0	-89	-386	+
2	-92	-370	+
5	-95	-25	0
10	-85	-10	0
20	-99	$\pm$ 0	0



**Figure V.8.** Nitrite was completely inhibiting to SRB from the Ninian field consortium at concentrations of 5 ppm  $NO_2-N$  and larger. At time 0 the inoculum was added to the prepared vials. The production of hydrogen sulfide indicates growth and activity of SRB in the control vials where no  $NO_2^-$  was added and in the vials where 2 ppm  $NO_2-N$  was added.



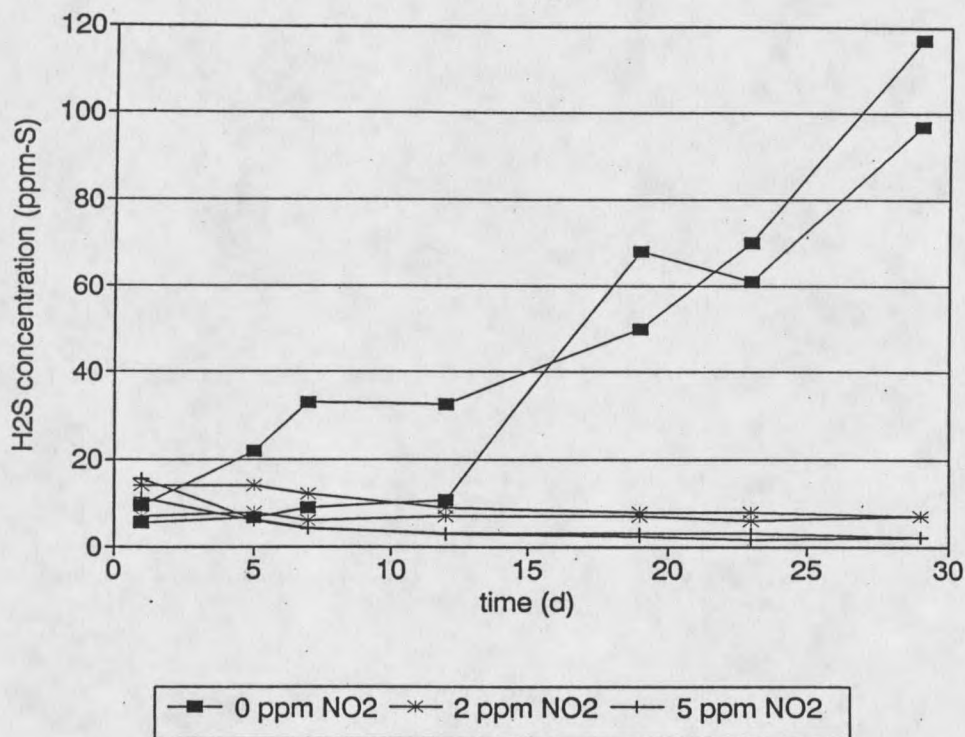
**Figure V.9.** The concentration of nitrite decreased in all the test vials where nitrite was added and hydrogen sulfide was present.

Kuparuk consortium. An identical experiment as for the Ninian field consortium was conducted with the Kuparuk consortium. No SRB growth was found at concentrations of 2 ppm  $\text{NO}_2\text{-N}$  or higher (Figure V.10).

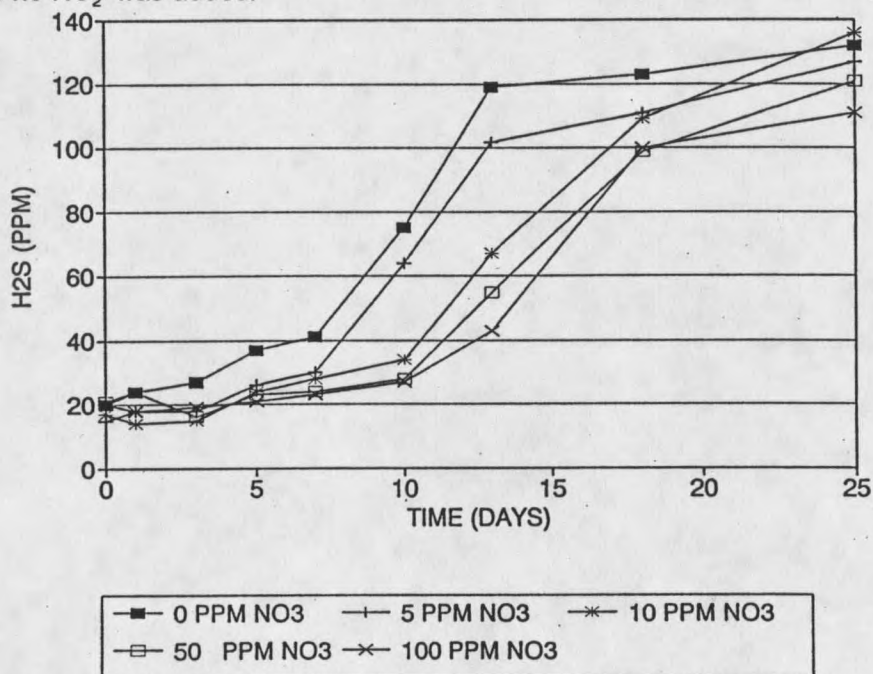
#### The Effect of Nitrate on Sulfate-Reducing

##### Bacteria from the Kuparuk Consortia

Vials were prepared with various nitrate concentrations from 0 to 100 ppm. A frozen SRB isolate from the Kuparuk field was used for inoculum. Nitrate concentrations up to 100 ppm did not affect SRB growth and activity (Figure V.11).



**Figure V.10.** Nitrite was completely inhibiting to SRB from the Kuparuk field consortium at concentrations of 2 ppm NO<sub>2</sub>-N and larger. At time 0 the inoculum was added to the prepared vials. The production of hydrogen sulfide indicates growth and activity of SRB in the control vials where no NO<sub>2</sub><sup>-</sup> was added.

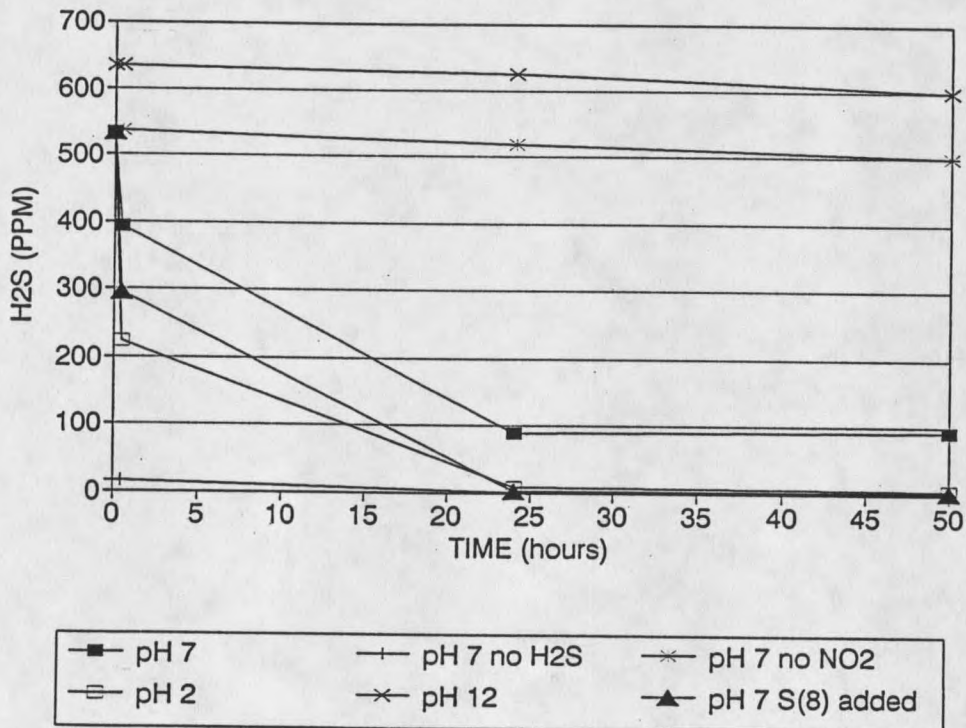


**Figure V.11.** The addition of nitrate up to 100 ppm NO<sub>3</sub>-N did not affect growth and activity of SRB isolates from the Kuparuk field consortium significantly. At time 0 the inoculum was added to the prepared vials. The production of hydrogen sulfide indicates growth and activity of SRB in all test vials where NO<sub>3</sub><sup>-</sup> was added.

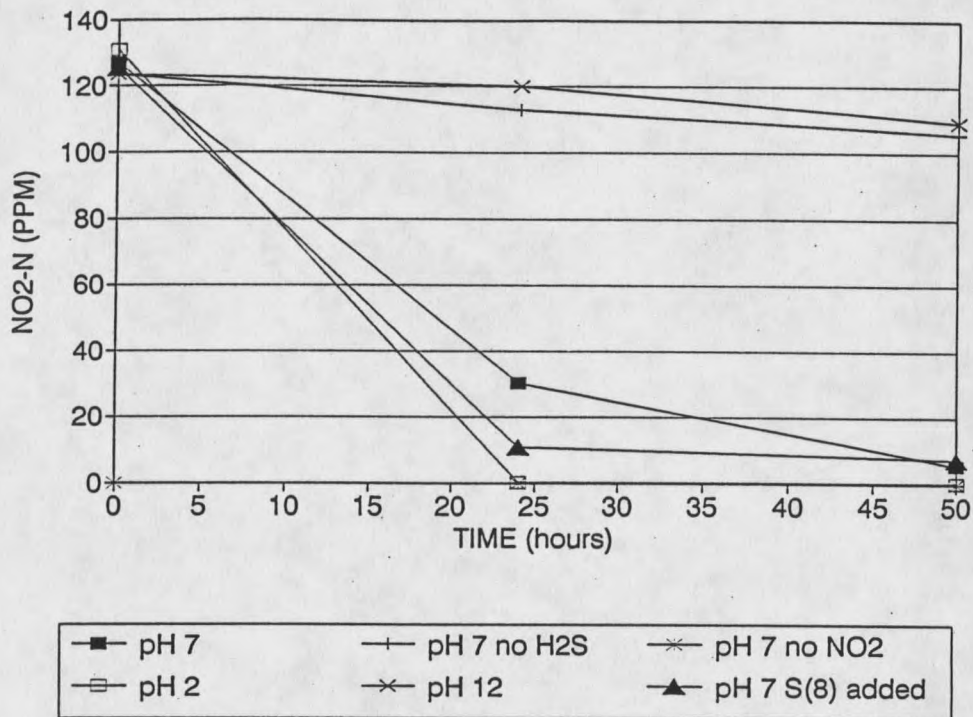
The Chemical Interaction between Hydrogen Sulfide and Nitrite

The chemical interaction between  $\text{H}_2\text{S}$  and  $\text{NO}_2^-$  was tested in a sterile environment, identical to the SRB high salinity medium used in the previous experiment. If not otherwise noted, the initial  $\text{NO}_2^-$ -N concentration in the duplicate vials was  $130 \text{ g m}^{-3}$  and the initial  $\text{H}_2\text{S}$ -S concentration was  $540 \text{ g m}^{-3}$ . A variety of conditions were tested (Figure V.10 and V.11):

- 1) pH 7;
- 2) pH 7, no  $\text{H}_2\text{S}$  added;
- 3) pH 7, no  $\text{NO}_2^-$  added;
- 4) pH 2;
- 5) pH 12;
- 6) pH 7,  $\text{S}_8^0$  added



**Figure V.12.** Abiotic interaction between hydrogen sulfide and nitrite at a variety of conditions. Different pH values were tested and elemental sulfur was added to some vials.  $\text{H}_2\text{S}$  concentration is plotted vs time after incubation at  $60^\circ\text{C}$ . All points represent the average from duplicate vials.



**Figure V.13.** Abiotic interaction between hydrogen sulfide and nitrite at a variety of conditions. Different pH values were tested and elemental sulfur was added to some vials.  $\text{NO}_2\text{-N}$  concentration is plotted vs time after incubation at  $60^\circ\text{C}$ . All points represent the average from duplicate vials.

At pH 2 the nitrite and sulfide reacted very rapidly, resulting in complete removal of the hydrogen sulfide from the water phase (Figure V.12). The concentration of nitrite was reduced correspondingly (Figure V.13). At pH 12 the rate of reaction was very slow. The presence of elemental sulfur increased the rate of nitrite reduction. Very little  $\text{H}_2\text{S}$  was lost when no nitrite was added. When no hydrogen sulfide was added all the initially available  $\text{H}_2\text{S}$  was removed and the nitrite concentration was fairly constant. The molar ratio between the hydrogen sulfide removed and the nitrite removed was calculated from #1 and #4 to:

$$\text{H}_2\text{S}/\text{NO}_2^- = 1.68 \pm 0.19 \text{ mol/mol}$$

### Microbial Interaction between

#### Sulfate-Reducing Bacteria and Denitrifying Bacteria

Microbial interaction between sulfate reducing bacteria and denitrifying bacteria was tested using fresh Kupaaruk produced water as inoculum. Three different experiments were performed. The first test was to test for microbial nitrate reduction in a DNB specific medium. For the second test nitrate and a mixture of VFA (at an initial concentration of 200 ppm each) were added to the produced water. In the third test sulfate and nitrate were added to produced water to test the outcome of the microbial interaction between denitrifying bacteria and sulfate-reducing bacteria. All of these experiments were performed at 40 and 60°C.

Microbial interaction at 40°C. When produced water was introduced into a DNB specific medium, nitrate was primarily reduced to nitrogen gas (Figure V.14). Until day 10 no nitrite was produced. After day 10 nitrite was observed in both duplicate vials. In one vial the nitrite was then depleted, while in the other vial a final nitrite concentration of 36 ppm remained.

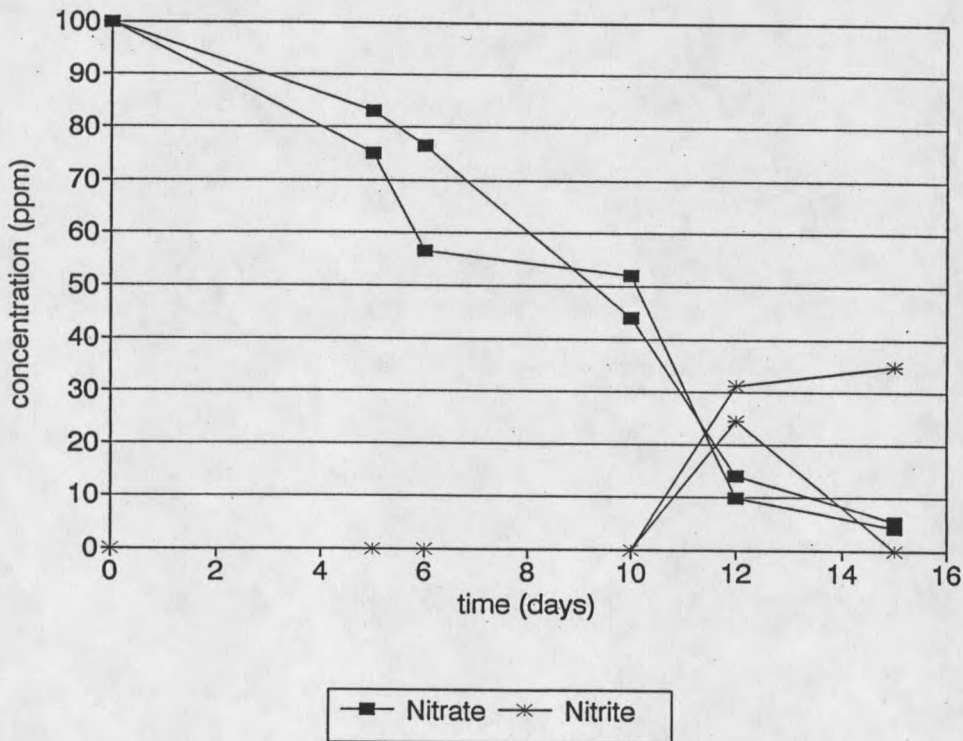
When nitrate and sulfate were added to the produced water at concentrations of 200 and 300 ppm, respectively, no sulfate was reduced and no hydrogen sulfide was produced (Figure V.15). Nitrate reduction was observed immediately and 76 to 80% of the reduced nitrate was converted into nitrite, resulting in a nitrite concentration of 75 to 80 ppm  $\text{NO}_2\text{-N}$  5 days after incubation. The concentration of nitrite decreased slowly after day 6 when nitrate reduction stopped. The remaining nitrate stayed relatively constant between 75 and 130 ppm.

Microbial interaction at 60°C. When produced water was inoculated into DNB specific medium at 60°C, nitrate was reduced to low concentrations rapidly and nitrite was produced (Figure V.16). 23 to 45% of the reduced nitrate was converted into nitrite initially. The nitrite concentration

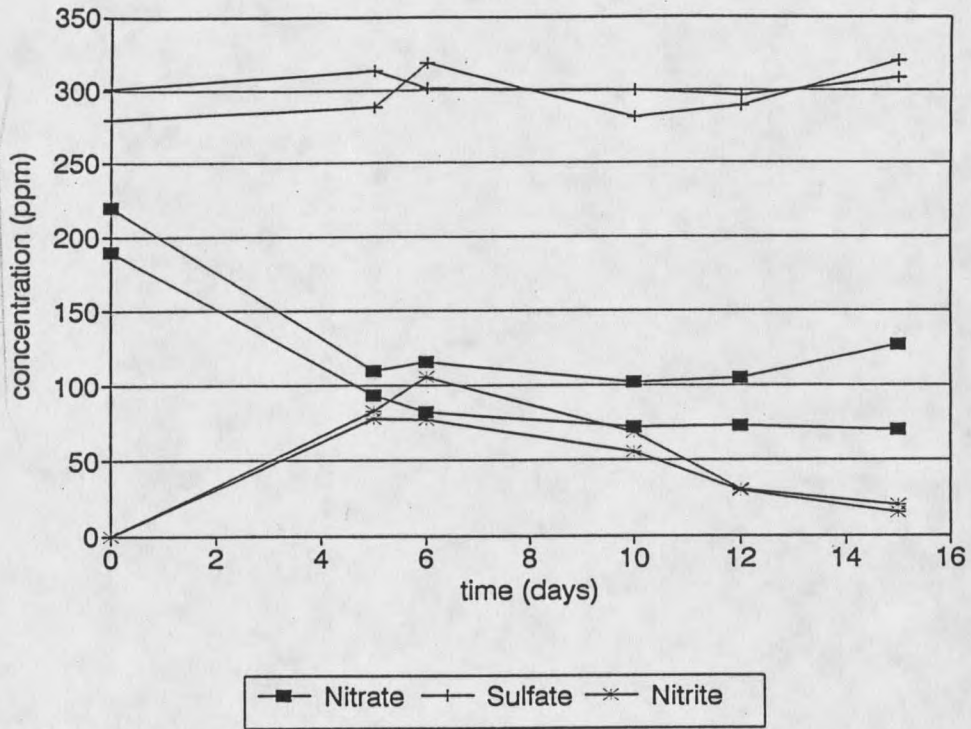
decreased to 16 to 25% of the initial nitrate present in solution. The remaining nitrate was below 10 ppm in all test vials 10 days after incubation.

A similar result was obtained when nitrate was added to Kuparuk produced water. Nitrate was reduced to nitrite at concentrations corresponding to 50 and 61% of the reduced nitrate (Figure V.17). The concentration of nitrate and nitrite remained constant 6 days after incubation, with final nitrite concentrations of 50 to 100 g NO<sub>2</sub>-N m<sup>-3</sup>.

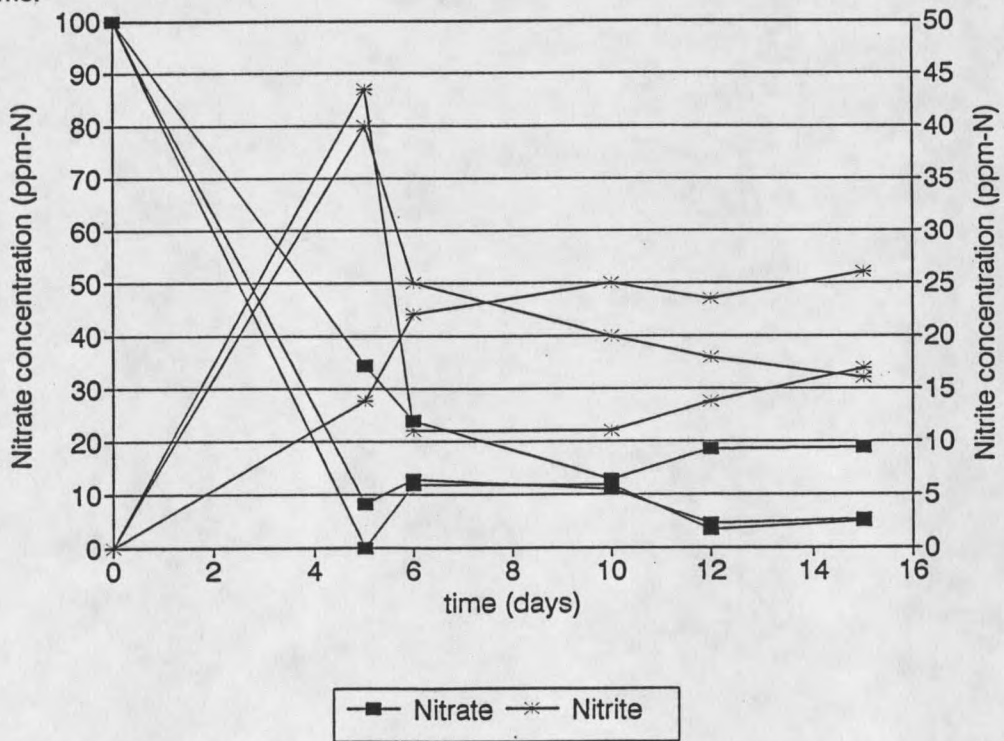
When nitrate and sulfate were present in the produced water at 60°C no sulfate was reduced and no hydrogen sulfide was produced during the entire time period tested (Figure V.18). Nitrate was reduced and simultaneously nitrite was produced. The produced nitrite was 62 to 80% of the reduced nitrate. After the nitrite concentration reached a value of over 100 ppm no nitrate or nitrite reduction occurred.



**Figure V.14.** Fresh produced water from the Kuparuk field was used for inoculation into a DNB specific medium (2% salinity). The temperature of incubation was set constant at 40°C. Nitrate and nitrite were monitored over time.



**Figure V.15.** Nitrate and sulfate were added to fresh produced water from the Kugaruk field. The temperature of incubation was set constant at 40°C. Sulfate, nitrate, and nitrite were monitored over time.



**Figure V.16.** Fresh produced water from the Kugaruk field was used for inoculation into a DNB specific medium (2% salinity). The temperature of incubation was set constant at 60°C. Nitrate and nitrite were monitored over time.

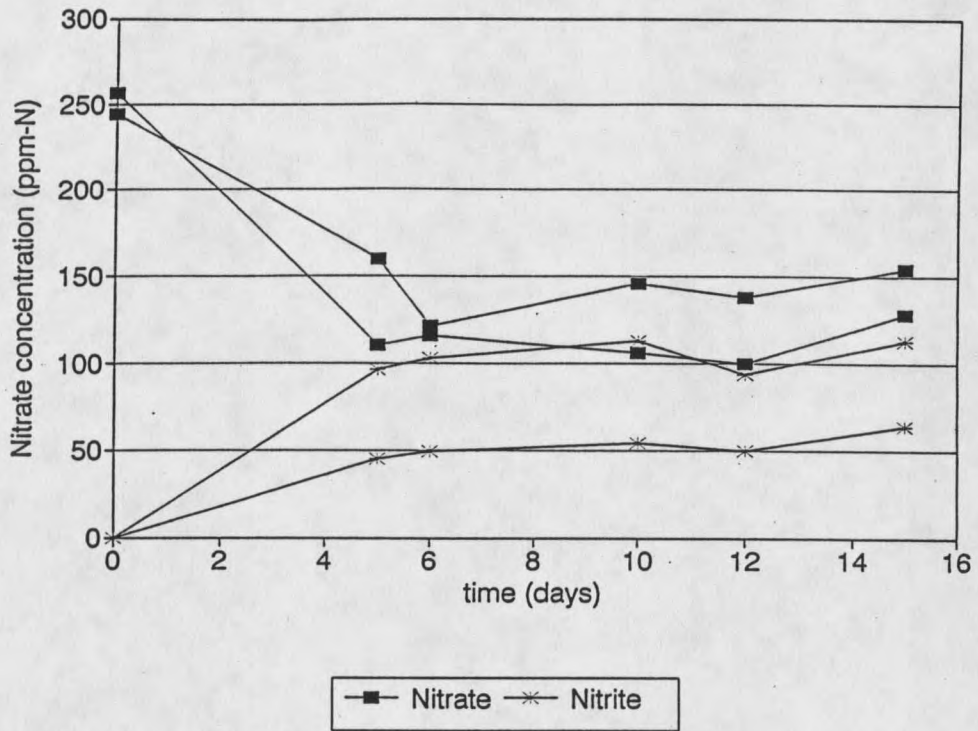


Figure V.17. Nitrate was added to fresh produced water from the Kuparuk field. The temperature of incubation was set constant at 60°C. Nitrate, and nitrite were monitored over time.

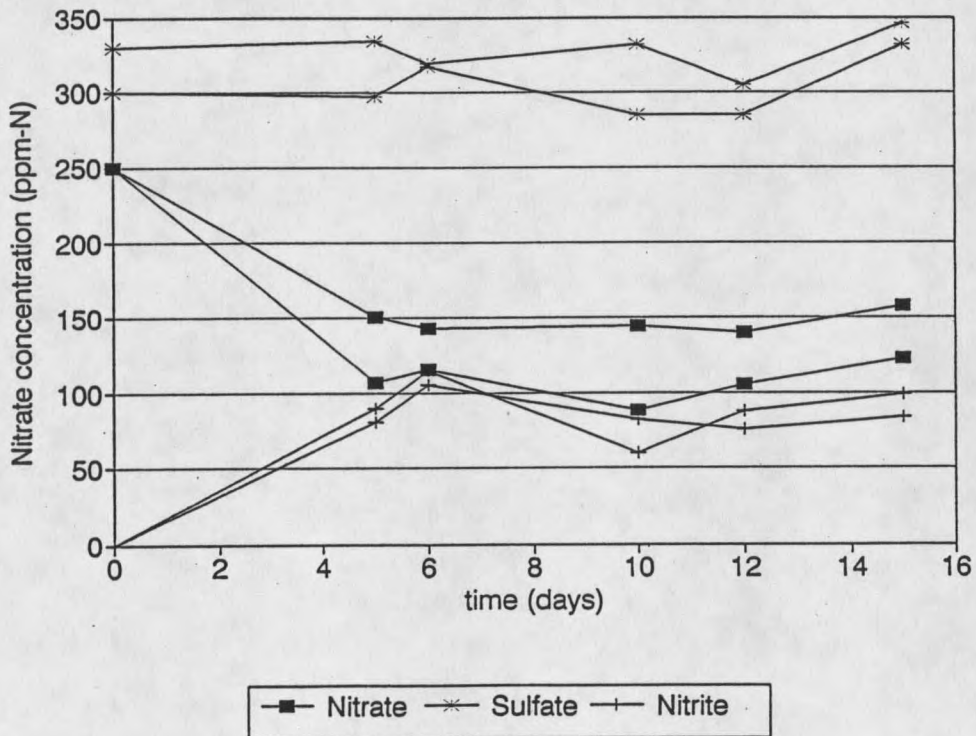


Figure V.18. Nitrate and sulfate were added to fresh produced water from the Kuparuk field. The temperature of incubation was set constant at 60°C. Sulfate, nitrate, and nitrite were monitored over time.

### Simulation of Microbial Interaction

Based on the results of this study the microbial interaction was simulated as amensalism. Species 1 (SRB) was affected negatively by the presence of species 2 (DNB). Double Monod kinetics were used to describe the cellular growth process for each species. For the sake of model simplicity, inhibition was assumed to affect only the rate of substrate conversion. Different inhibition models (competitive, uncompetitive and non competitive inhibition; equations E1, E2, and E3, respectively) were applied to simulate the growth and metabolism of the two interacting species. A qualitative comparison was used to find agreement of the simulation results with the experimental results. The kinetic parameters for species 1 (SRB) were determined in chapter IV. The kinetic parameters for DNB, such as cellular growth yield and the maximum growth rate, were determined in this chapter. The values for half saturation constants for species 2 (DNB) were extracted from literature data (Grady and Lim, 1980). The  $K_i$  values for the two species were estimated based on experimental data. Since inhibition for SRB was observed at low nitrite levels above 2 ppm for both tested consortia a value for  $K_i(1) = 2$  ppm was estimated. Nitrite inhibition of DNB was observed at much higher concentrations and a  $K_i(2) = 50$  ppm was assumed. To simulate the microbial interaction a set of mass conservation equations was used. The following chemical and microbial species were simulated as batch experiments:

Substrate (butyrate), sulfate, nitrate, nitrite, cell mass (DNB), and cell mass (SRB). The following equations were applied to simulate the microbial interactions between SRB and DNB. SRB related processes are denoted (1), DNB related processes are denoted (2).

$$* \text{ biomass (SRB)} \quad r_a(X(1)) \quad = \quad X(1) \mu(1) \quad (E4)$$

$$* \text{ biomass (DNB)} \quad r_a(X(2)) \quad = \quad X(2) \mu(2) \quad (E5)$$

$$* \text{ substrate} \quad r_a(S) \quad = \quad -X(1) Y(1)_{X/S}^{-1} \mu(1) - X(2) Y(2)_{X/S}^{-1} \mu(2) \quad (E6)$$

$$* \text{ sulfate} \quad r_a(E1) \quad = \quad -X(1) Y(1)_{X/E1}^{-1} \mu(1) \quad (E7)$$

$$* \text{ nitrate} \quad r_a(\text{E2}) \quad = -X(2) Y(2)_{X/E2}^{-1} \mu(2) \quad (\text{E8})$$

$$* \text{ nitrite} \quad r_a(\text{I}) \quad = X(2) Y(2)_{I/E2}^{-1} \mu(2) \quad (\text{E9})$$

$$* \text{ growth rate (SRB)} \quad \mu(1) \quad = \mu_{11} [E1/(K_{E1} + E1)] \quad (\text{E10})$$

$$* \text{ growth rate (DNB)} \quad \mu(2) \quad = \mu_{12} [E2/(K_{E2} + E2)] \quad (\text{E11})$$

where:

$r_a$ :	reaction rate [ $M L^{-3} t^{-1}$ ]
$\mu_{11}$ :	growth rate for SRB in the presence of inhibitor I [ $t^{-1}$ ]
$\mu_{12}$ :	growth rate for DNB in the presence of inhibitor I [ $t^{-1}$ ]
$\mu(1)$ :	specific growth rate for SRB [ $t^{-1}$ ]
$\mu(2)$ :	specific growth rate for DNB [ $t^{-1}$ ]
$Y(1)_{X/S}$ :	SRB cellular growth yield on substrate [ $M M^{-1}$ ]
$Y(2)_{X/S}$ :	DNB cellular growth yield on substrate [ $M M^{-1}$ ]
$Y(1)_{X/E1}$ :	SRB cellular growth yield on sulfate [ $M M^{-1}$ ]
$Y(2)_{X/E2}$ :	DNB cellular growth yield on nitrate [ $M M^{-1}$ ]
$Y(2)_{I/E2}$ :	yield of nitrite from nitrate reduction [-]
$K_{S1}$ :	half saturation coefficient for SRB with respect to substrate [ $M L^{-3}$ ]
$K_{S2}$ :	half saturation coefficient for DNB with respect to substrate [ $M L^{-3}$ ]
$K_{E1}$ :	half saturation coefficient for SRB with respect to sulfate [ $M L^{-3}$ ]
$K_{E2}$ :	half saturation coefficient for DNB with respect to nitrate [ $M L^{-3}$ ]
S:	substrate concentration [ $M L^{-3}$ ]
I:	inhibitor concentration [ $M L^{-3}$ ]
$K_{i1}$ :	inhibition constant for SRB with respect to nitrite [ $M L^{-3}$ ]
$K_{i2}$ :	inhibition constant for DNB with respect to nitrite [ $M L^{-3}$ ]

The equations E4 through E11, production rates for each component were set equal to accumulation rates, which were used to build finite difference equations using spreadsheet software such as Quattro Pro to calculate the changes over time in cell mass, substrate, electron acceptor, and inhibitor concentration. The model for competitive inhibition was used in the simulation presented in Figure V.19. Nitrate is depleted after 3.3 days, whereas sulfate is reduced slightly. Most of the butyrate is utilized for nitrate reduction, but butyrate was not completely removed, even though sulfate remained in the system. The rate of sulfate reduction did not increase after nitrate was depleted. Results of the simulation using uncompetitive inhibition is presented in Figure V.20 and the simulation result from the use of the non competitive inhibition model is presented in Figure V.21. The outcomes for both are identical. The reason for this is the high substrate concentration remaining in the system after nitrate depletion. At lower substrate

concentrations, the inhibiting effect would be more distinct for the non competitive inhibition model. However at the concentrations tested, the uncompetitive and the non competitive models for inhibition of substrate conversion qualitatively matched the experimental data (Figure V.18). Nitrate was reduced resulting in nitrite accumulation within 5 days after startup. The reduction of sulfate still remained insignificant 8 days after startup.

**Table V.7.** Kinetic parameters for the simulation of microbial interaction between the tested SRB and DNB from the Kuparuk oil field consortium.

X1(0) =	0.1	ppm
S(0) =	200	ppm
E1(0) =	500	ppm
E2(0) =	50	ppm
NO <sub>2</sub> /NO <sub>3</sub> =	0.8	
mumax1 =	0.04	1/h
mumax2 =	0.06	1/h
Ks1 =	5.5	ppm
Ks2 =	5	ppm
Ke1 =	9	ppm
Ke2 =	5	ppm
ki1 =	2	ppm
ki2 =	50	ppm
Yxs1 =	0.023	g cell/g substrate
Yxs2 =	0.05	g cell/g substrate
YX/E1 =	0.038	g cell/g sulfate-S
YX/E2 =	0.04	g cell/g nitrate-N

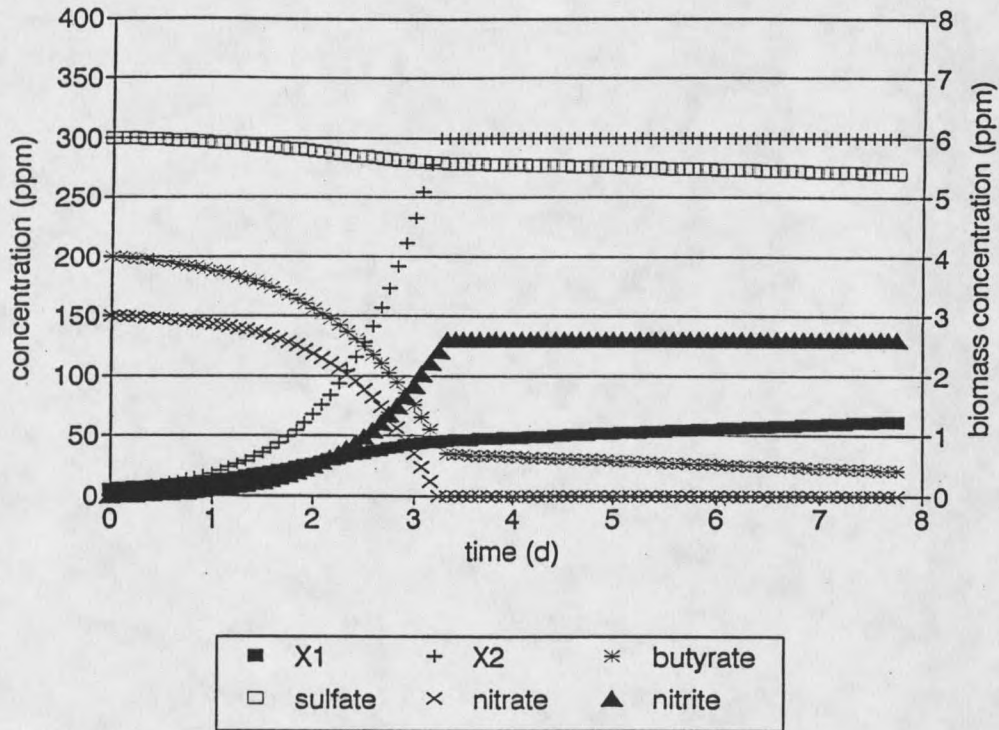


Figure V.19. Microbial interaction between SRB and DNB. Butyrate was used as a growth substrate and sulfate and nitrate were present as electron acceptors. A competitive model for inhibition of SRB and DNB was used.

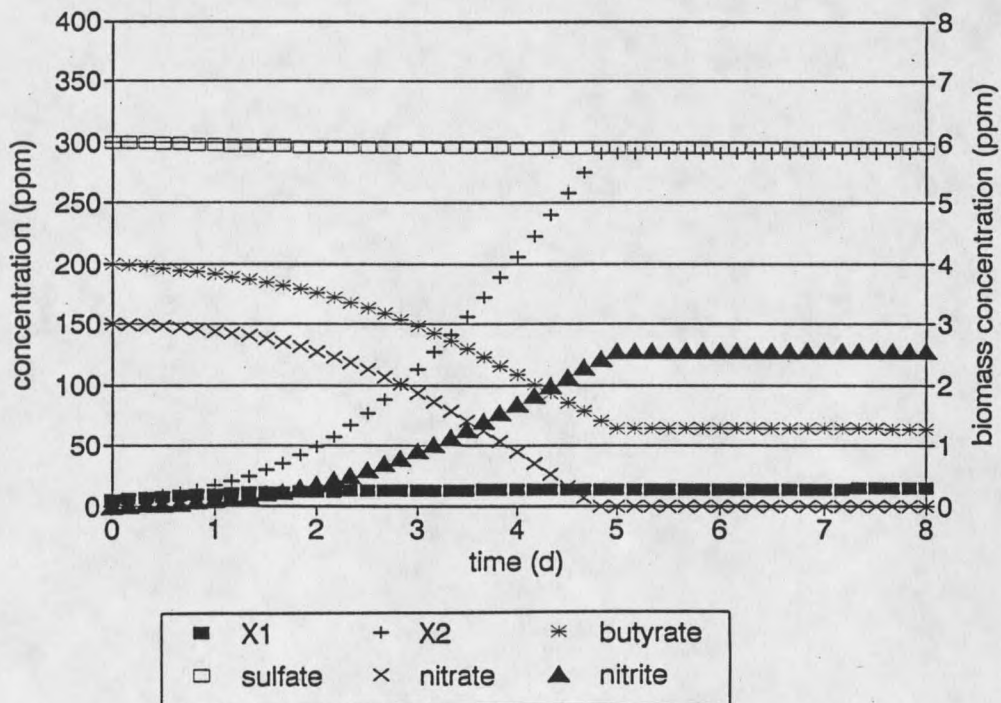
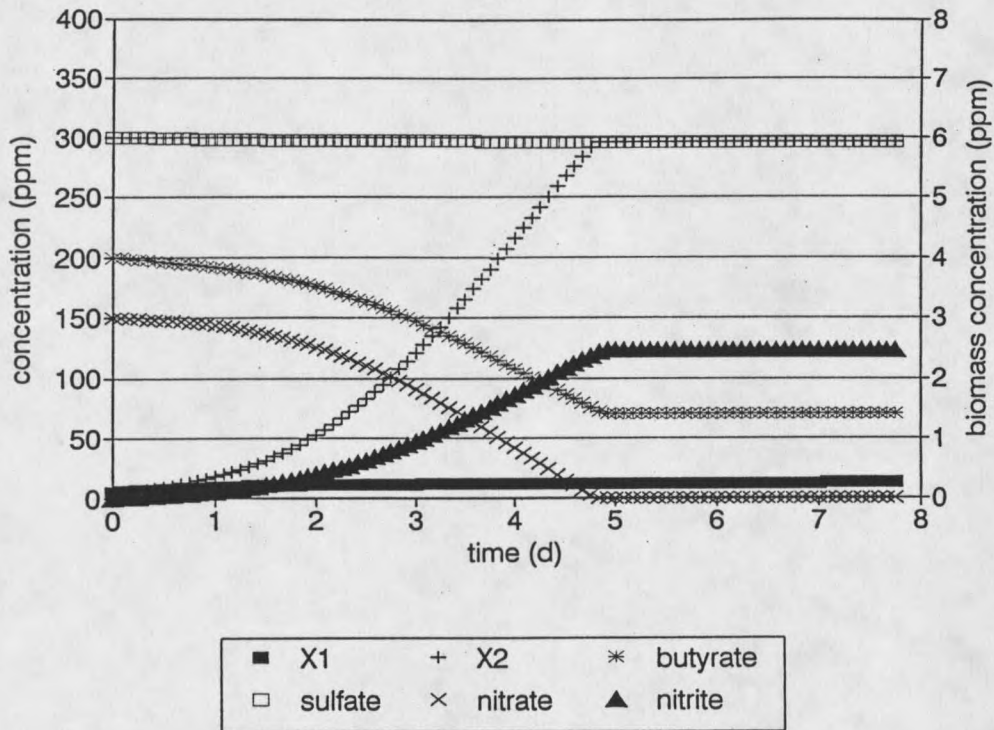


Figure V.20. Microbial interaction between SRB and DNB. Butyrate was used as a growth substrate and sulfate and nitrate were present as electron acceptors. An uncompetitive model for inhibition of SRB and DNB was used.



**Figure V.21.** Microbial interaction between SRB and DNB. Butyrate was used as a growth substrate and sulfate and nitrate were present as electron acceptors. A non competitive model for inhibition of SRB and DNB was used.

### Discussion

#### Characterization of thermophilic DNB in Oil Field Waters

Thermophilic denitrifying bacteria were present in produced water from both oil reservoirs sampled. Denitrifying bacteria became active in fresh produced water from the Kuparuk oil field as soon as nitrate was offered. Acetate was the prime carbon source utilized as electron donor for denitrification. Other VFA up to C4 were used as carbon source as well, with the exception that formate was not utilized by the Kuparuk DNB when other VFA were available. The concentration of acetate in a souring oil formation can be very high, up to 1000 ppm (chapter III). Hence, there is an excess of available organic carbon in a formation for DNB growth. Growth of DNB in a

souring oil bearing formation would therefore be limited by the amount of nitrate added to the formation. Growth of thermophilic DNB was a slow process and the cellular growth rate and yield were not significantly different from the tested SRB. The growth rate for the Ninian DNB decreased with increasing salinity. The cellular growth yields for DNB from the Kuparuk consortium were determined in a chemostat with  $Y_{X/E} = 0.0089$  g dry cell mass per g  $\text{NO}_3\text{-N}$  reduced and  $Y_{X/S} = 0.0055$  g dry cell mass per g VFA utilized. These values are uncharacteristically low when compared with data available for mesophilic denitrifying bacteria (Rusten, 1982). Generally the growth yield and growth rate values for mesophilic DNB are comparable with heterotrophic bacteria and should be expected to be orders of magnitude larger than those measured.

When all of the experimental results are compiled, in those systems where nitrate was reduced, the recovery of nitrite from the reduction of nitrate varied from 90% to zero recovery, dependent on the experimental conditions (Table V.8). Sulfate reduction was not observed in any of the experiments with nitrate reduction.

**Table V.8.** Nitrite recovery from nitrate reduction at various environmental conditions

Reactor System	sample origin	conditions	nitrite recovered (%)
Batch	Ninian	DNB medium, 60°C, salinity 0-2%	85
CSTR	Kuparuk	Steady state, 60°C, $D = 0.01 \text{ h}^{-1}$	50
Batch	Kuparuk	DNB medium, 40°C, salinity 1%	0-25
Batch	Kuparuk	DNB medium, 60°C, salinity 1%	20-30
Batch	Kuparuk	produced water + $\text{NO}_3^-$ , 40°C	20
Batch	Kuparuk	produced water + $\text{NO}_3^-$ , 60°C	75-90

This variability, even within the Kuparuk consortium, may be attributed to selection pressures induced by the varying environmental conditions for each experiment. The maximum nitrite recovery was observed when nitrate was added to oil field produced water at 60°C. Nitrite was

very effective at suppressing SRB growth and activity.

The sampled oil water consortia reflected a broad variety of microorganisms. The reduction of nitrate to nitrite might be carried out by one group, whereas another group could convert the produced nitrite further to nitrogen gas. In the case of nitrite accumulation, the rate of growth and conversion of the second group might be slower, resulting in accumulation of the intermediate. Another reason could be the absence of thermophilic nitrite reducing bacteria or an inhibition in the synthesis and/or activity of the nitrite reductase enzyme at thermophilic temperatures (Hollocher and Kristjansson, 1992).

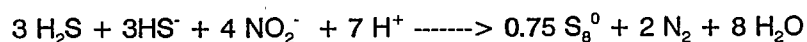
The production of nitrite was found to be a function of the concentration and quality of the available carbon sources, and the presence of other inhibitors for denitrification (Lazarova, *et al.* 1994). Acetate was shown to provide a better carbon source than sugars or longer VFA for complete denitrification. Different mechanisms are responsible for nitrite accumulation, (Wilderer *et al.* 1987). Oxygen was shown to completely inhibit the synthesis and activity of the nitrite reductase enzyme (Krul and Veeningen, 1977). Beccari *et al.* (1983) found that nitrite reduction is much more sensitive to pH changes than the reduction of nitrate. Hence, at low pH values nitrite is most likely to accumulate in the system. The pH optimum for complete nitrate reduction was found at pH 8.5 (Timmermans and Van Haute, 1983). Gas producing reactions are more likely to be inhibited at high pressure. In oil reservoirs close to the oil water interphase, pressure can be as high as 500 bar (Herbert and Stott 1983), and an accumulation of nitrite is even more likely to occur at these high pressure zones.

The Effect of Nitrite and Nitrate  
on Sulfate-Reducing Bacteria from Oil Field Waters

For both SRB consortia tested, growth and activity of thermophilic SRB were effectively zero at nitrite concentrations above 2 ppm, whereas nitrate concentrations up to 100 ppm did not affect SRB growth.

The observed nitrite inhibition was either 100% or 0. The reason for this "on/off" type behavior might be the resulting change of redox potential after nitrite addition to the SRB growth medium. The removal of reduced compounds such as H<sub>2</sub>S by nitrite from the water phase caused an increase in redox potential. The inhibiting effect on SRB's therefore cannot be concluded to be caused by nitrite directly. It is well known that sulfate-reducing bacteria are very restricted with respect to redox potential and will only grow and become active in a reduced environment (e.g. E<sub>h</sub> < -100 mV; Postgate 1984). The added or microbially produced nitrite therefore may affect SRB via two mechanisms: direct inhibition due to nitrite or indirect inhibition due to a less reduced environment (change in redox potential).

Nitrite was found to affect the redox potential of the water by reacting with reducing agents such as the produced H<sub>2</sub>S from sulfate reduction, forming elemental sulfur and nitrogen gas at low or neutral pH values. Based on the experimentally observed ratio for the redox reaction of 1.68 ± 0.19 mol NO<sub>2</sub> per mol H<sub>2</sub>S, the stoichiometry between hydrogen sulfide and nitrite can be formulated as:



The formulated stoichiometry is consistent with the observation that increasing pH slows down

or completely inhibits the reaction. Lowering the pH speeds up the reaction. Sulfur may act as an autocatalyst to the overall reaction since its presence increased the rate of reaction. The removal of hydrogen sulfide results in the production of a yellow sulfur precipitate. The precipitate is obvious to see after the reaction occurred, and its yellow color indicates that elemental sulfur was formed. One problem arising from the sulfur precipitation may be its negative effect on water injection in a reservoir. In addition, elemental sulfur is suspected to enhance the corrosion process (Nielson, 1993).

This chemical interaction between  $H_2S$  and  $NO_2^-$  indicates that higher nitrite/nitrate levels may be required to achieve the desired inhibiting effect on SRB's in an actively souring oil field. This becomes of particular importance when the pH in the formation is near pH 7 or lower. Other reducing agents present in the reservoir (e.g., dithionite) may also react with nitrite and an overall change in redox potential inside an oil bearing formation might be the final result from adding nitrite or nitrate. This increased redox potential as well as the presence of elemental sulfur may increase the corrosion potential of the injection, the formation, and finally the produced water.

The use of nitrite as a corrosion inhibitor in high concentrations (up to 0.5%) was used previously by others (Trivedi *et al.* 1992, Alonso and Andrade, 1990, Al-Borno *et al.* 1989).

#### Microbial Interaction between Sulfate-Reducing

##### Bacteria and Denitrifying Bacteria

When sulfate and nitrate were added to produced water from the Kuparuk oil field no sulfate was reduced at either 40 or 60°C. Nitrate was reduced rapidly to nitrite at both tested temperatures. At 40°C nitrite was slowly further reduced and the nitrite concentration declined over time,

whereas at 60°C nitrite accumulated to approximately 80% of the amount of nitrate reduced and remained at this level. No nitrite reduction was apparent in produced water at 60°C. As stated above, nitrite inhibits the activity of sulfate reducing bacteria at concentrations of 5 g NO<sub>3</sub>-N m<sup>-3</sup> or higher. After 5 days of incubation, the nitrite concentrations in the produced water were above 50 g NO<sub>3</sub>-N m<sup>-3</sup> in all cases tested, completely inhibiting SRB grow and activity. At nitrite concentrations between 75 and 100 g NO<sub>3</sub>-N m<sup>-3</sup> nitrate reduction stopped. Hence, at high nitrite concentrations, the reduction of nitrate might be inhibited by the intermediate product (nitrite).

The type of microbial interaction between the SRB and the DNB species could be described as amensalism. The presence and metabolism of one species (DNB) has a negative effect on the growth of the second species (SRB) by producing an inhibitory substance (nitrite). A simulation model was used to test the microbial interactions based assuming amensalism with three different inhibition kinetic models. Qualitative agreement between the computer simulated data and the experimental results were found for the uncompetitive and the non competitive inhibition models. No qualitative agreement was found when a model for competitive inhibition was used.

The addition of nitrate to prevent souring was proposed by others previously. The addition of sulfide utilizing autotrophic denitrifying bacteria (*Thiobacillus denitrificans*) to an H<sub>2</sub>S producing oil reservoir was effective in some specific cases (McJnerney *et al.* 1991; Hitzman and Sperl, 1994). The produced hydrogen sulfide could then be used as an electron donor and nitrate had to be supplied as electron acceptor. The disadvantage of adding organisms is that a very large inoculum had to be obtained to achieve any effect in a large scale system such as a souring oil reservoir. Also, the targeted strain of *Thiobacillus* is restricted to low salinities and mesophilic temperature ranges. Hence, the addition of a mesophilic denitrifier may contribute to plugging around the injection well where suitable temperatures for the growth of mesophilic denitrifiers are found, but could not prevent the H<sub>2</sub>S production at the oil water interphase at thermophilic

temperature ranges.

Adding nitrate will stimulate the denitrifying population present throughout the entire field and especially at thermophilic temperature ranges nitrite may accumulate and inhibit sulfate reduction and  $H_2S$  production. The applied concentrations for nitrate addition can be rather small, since the microbial interaction is due to amensalism and not as previously thought due to competition for substrate (Hitzman and Sperl, 1994; Jenneman *et al.* 1986).

### Conclusions

DNB were present in produced water from both tested oil fields. Acetate was the most favorable carbon source to support DNB growth at 40 and 60°C.

DNB grew at a faster rate at low salinity. High salinity inhibits the microbial conversion, but nitrate is still reduced at 2% salinity.

At reservoir conditions, most of the nitrate (>50%) is partially reduced to nitrite. The produced nitrite will chemically react with hydrogen sulfide and form nitrogen gas and elemental sulfur. This reaction is strongly pH dependent.

Complete inhibition of SRB was found for nitrite concentrations of  $5 \text{ g m}^{-3} \text{ NO}_2\text{-N}$  or higher. However, nitrite may not be the actual inhibiting agent, since it also changes the redox potential in its environment.

When nitrate and sulfate were present in oil field waters no measurable sulfate reduction occurred and approximately 80% of the nitrate initially present was converted to nitrite.

The type of microbial interaction between SRB and DNB in oil field water can be described as amensalism. The DNB produce nitrite, which was shown to be an inhibitory substance for SRB growth. A computer simulation model based on amensalism could qualitatively describe the processes of microbial interaction. The basis of this model could be uncompetitive or non competitive inhibition kinetics acting on substrate conversion.

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## CHAPTER VI

## SUMMARY

In this research the current literature on souring in oil reservoirs was reviewed. Two differently operating oil fields were sampled for microbial consortia. An innovative sampling procedure was applied. The obtained consortia were characterized for potential growth substrates for various anaerobic groups of organisms at mesophilic and thermophilic temperatures. Kinetic parameters for microbial sulfate reduction and denitrification were determined at 60°C. The obtained parameters for sulfate reducing bacteria were determined for suspended as well as for biofilm cells. Batch reactors, completely stirred tank reactors and completely mixed biofilm reactors were used to estimate the growth kinetics. The effect of nitrate addition to oilfield waters was demonstrated in laboratory experiments. Microbial sulfate reduction was inhibited when nitrate was added to fresh oil field waters without the addition of bacteria. The fundamental mechanisms for this inhibiting effect were investigated. A simulation model based on the experimentally determined growth parameters was developed and described the processes of microbial interaction between SRB and DNB with qualitative agreement to the experimental results. Based on the results of this study, a novel strategy for controlling souring in oil reservoirs undergoing secondary recovery is presented.

### General Conclusions

Based on the results of this study the following general conclusions were drawn:

The sampling and storage procedure proved to be sufficient in sampling, characterizing and storing the microbial consortia.

The cell size distribution of the microbial consortia sampled indicates a multi species microbial composition in the original sample from the Kuparuk field.

The microbial consortia sampled from two differently operated fields were capable of microbial sulfate reduction over a wide range of temperatures and for a variety of substrates. Temperature did influence the rate of microbial sulfate reduction, the rate of H<sub>2</sub>S production, the rate of substrate oxidation, and the cell morphology. The highest rate of sulfate reduction was found between 50 and 60°C.

Formate and n-butyrate were the most favorable carbon sources at any tested temperature. Acetate was utilized at 35°C and at 75°C but not at 50°C and was produced at 60°C.

Methane producing activity was found for the Kuparuk consortium when hydrogen and carbon dioxide were present. Hydrogen and carbon dioxide were exclusive substrates for methanogenesis at the conditions tested (high salinity medium at 60°C).

The microbial population as compared between on site inoculum and frozen inoculum seems to change slightly. This comparison was based on the capability of the microbial consortium to utilize specific substrates for its carbon and energy demand. Methanogens used H<sub>2</sub> and CO<sub>2</sub> exclusively as energy and carbon source. Due to the freezing process fermentative organisms present in the microbial consortium may have been damaged.

Stoichiometric ratios for sulfate, butyrate, formate and biomass for SRB from both consortia are in a similar range.

The maximum growth rates found for SRB from both oil reservoirs sampled were very slow with a  $\mu_{\max}$  of 0.02 to 0.04 h<sup>-1</sup>. At 60°C, the SRB from the Ninian oil field grew at approximately half of the maximum growth rate of the SRB from Kuparuk.

The lower values for  $K_S$  of the Ninian SRB as compared to the Kuparuk field consortium may reflect a lower nutrient availability in the formation. The  $K_E$  values were not statistically different for the consortia from the two tested oil fields.

Cellular growth yields, the growth rate and the specific sulfate reduction rate for biofilm SRB at 60 °C are statistically not different from suspended SRB from the same consortium.

The biofilms measured at 60°C were very thin and rarely exceeded a monolayer. An average thickness of  $2 \pm 1$   $\mu\text{m}$  was reported.

Denitrifying bacteria were found to be present in produced water from both tested oil fields. Acetate and longer VFA were the most favorable electron donors for nitrate reduction at 40 and at 60°C.

Denitrifying bacteria grow at a faster rate at low salinity. High salinity inhibits the microbial conversion, but nitrate is still reduced at 2% salinity.

At souring conditions (e.g., high salinity, high temperature, and hydrogen sulfide present) most of the nitrate (>50%) is partially reduced to nitrite. The produced nitrite will chemically react with hydrogen sulfide and form nitrogen gas and elemental sulfur. This reaction is strongly pH dependent.

Complete inhibition of SRB was found for nitrite concentrations of  $5 \text{ g m}^{-3} \text{ NO}_2\text{-N}$  or higher for SRB from both oil field consortia. However, nitrite may not be the actual inhibiting agent, since it also changes the redox potential in its environment.

When nitrate and sulfate are present in oil field waters from the Kuparuk field no measurable sulfate reduction occurred and approximately 80% of the nitrate present initially was converted to nitrite, inhibiting sulfate reduction.

The type of microbial interaction between SRB and DNB in oil field water can be described as amensalism. The DNB produce nitrite, which was demonstrated to be an inhibitory substance for SRB growth. A computer simulated model could describe the processes of microbial interaction. The basis of this model could be uncompetitive or non competitive inhibition kinetics acting on substrate conversion.

#### Implications and Recommendations for Future Research

Biocide treatment in the context of oil reservoir souring had very limited success. The control strategy found in this study utilizing microbial interaction between two species seems promising. However, at low temperature regions the effect of nitrate addition on SRB growth and metabolism might be of limited success, also. One would expect the added nitrate in the injection water would be readily utilized by DNB around the injection site so that little remained for transport deeper into the formation. A combination between biocide treatment and nitrate addition might be a promising strategy for the control of souring. If biocide and nitrate are applied simultaneously an inactivation of the organisms close to the injection site could be expected and the nitrate could reach locations further into the formation where thermophilic SRB are located. An addition of nitrate at

concentrations above  $100 \text{ g m}^{-3}$  may be beneficial to suppress SRB activity further in the formation. Since the conversion of nitrate to nitrite was inhibited by nitrite concentrations between 50 and  $100 \text{ g m}^{-3}$ , the unconverted nitrate would then reach further into the formation. Another option might be the addition of nitrate into the production well or to regions close to the production site. Only a very well planned field test could answer how to apply nitrate most efficiently to a complex system such as an oil reservoir. Prior to a field test demonstration different backflow strategies should be tested with a meso scale system such as a porous media column reactor. These backflow studies have to be tied together with a simulation model, which can accurately describe the behavior of the chosen meso scale system. The calibrated simulation model could then be used to develop specific conditions for the field test.

APPENDIX  
SRB MEDIUM COMPOSITION AND PREPARATION

### General Description of the Growth Medium used for SRB

This medium was suggested by Pfennig *et al.* (1986) for marine type SRB. Widdel used this medium in most of his studies and found a whole array of new mesophilic and thermophilic SRB. The medium is carbon free and therefore suited for stoichiometric tests and to test for potential substrates. The medium consists of 9 different solutions which can be made up and stored sterile for over one month.

### Medium Preparation

The basic medium (solution #1) was autoclaved and cooled under an 10% CO<sub>2</sub> / 90% N<sub>2</sub> atmosphere. Different amounts of the previously prepared stock solutions were added to the base medium as long as the medium was still warm (40°C) and purged with the CO<sub>2</sub>/N<sub>2</sub> gas mixture. After all solutions were added, the medium was transferred to the anaerobic and sterile glass vials via an 21 gage needle. The filled vials without any carbon addition were stored for many weeks at 4°C. If storage of the vials was not desired, various carbon sources were added. The best results for SRB growth was obtained with a mixture of VFA such as formate, acetate, propionate, i-, and n-butyrate at 200 ppm each. The following procedure was used for all SRB medium preparations.

- 1) Prepare 1000 ml of the base medium (solution #1).

Autoclave for 30 minutes together with a Teflon-coated magnetic stir bar and cool under an atmosphere of 90% N<sub>2</sub> and 10% CO<sub>2</sub>. Stir the medium continuously while purging.

- 2) Make 1 L of final volume of the medium by adding via syringe and 21 gage needle to Solution

#1 when solution #1 is still stirring and purging.

- \* 1 ml of Solution #2 (trace elements stock solution)
- \* 1 ml of Solution #3 ( $\text{Na}_2\text{SeO}_4$  stock solution)
- \* 30 ml of Solution #4 (bicarbonate stock solution)
- \* 3 ml of Solution #5 (sulfide stock solution)

3) Add 1 ml of each vitamin solution (7-A,B,C,D) via sterile 0.2  $\mu\text{m}$  filter, and add 1 ml of Solution #9 (dithionite stock solution).

The exact composition of each of the nine stock solutions is listed below.

SOLUTION #1

distilled $\text{H}_2\text{O}$	970 ml
$\text{Na}_2\text{SO}_4$	0.8 g (180 ppm S)
NaCl	20.0 g
KCl	0.3 g
$\text{NH}_4\text{Cl}$	0.3 g
$\text{MgCl}_2 \times 6 \text{H}_2\text{O}$	3.0 g
$\text{KH}_2\text{PO}_4$	0.2 g
$\text{CaCl}_2 \cdot 2 \text{H}_2\text{O}$	0.15 g
$\text{NaNO}_3$	0.30 g (50 ppm N)

SOLUTION #2

distilled H <sub>2</sub> O	998 ml
conc. HCl	1.6 ml
FeCl <sub>2</sub> · 4 H <sub>2</sub> O	1.5 g
H <sub>3</sub> BO <sub>3</sub>	0.060 g
MnCl <sub>2</sub> · 4 H <sub>2</sub> O	0.100 g
CoCl <sub>2</sub> · 6 H <sub>2</sub> O	0.120 g
ZnCl <sub>2</sub>	0.070 g
NiCl <sub>2</sub> · 6 H <sub>2</sub> O	0.025 g
CuCl <sub>2</sub> · 2 H <sub>2</sub> O	0.015 g
Na <sub>2</sub> MoO <sub>4</sub> · 2 H <sub>2</sub> O	0.025 g

Autoclave for 30 min and store in an airtight container.

SOLUTION #3

distilled H <sub>2</sub> O	1000 ml
NaOH	0.5 g
Na <sub>2</sub> SeO <sub>3</sub>	.003 g

Autoclave for 30 minutes and store in an airtight container.

SOLUTION #4

1) Autoclave two 120-ml vials.

2) Make solution #4 and pour into one of the vials.

(100 ml H<sub>2</sub>O + 8.5 g NaHCO<sub>3</sub>)

3) Evacuate both vials and flush with N<sub>2</sub> (4 times), then evacuate as last step.

4) Saturate both vials with CO<sub>2</sub> from syringe.

5) Transfer solution via 18 gage needle and sterilized filter from first vial to second vial.

6) Adjust CO<sub>2</sub> pressure in two vials so solution can be withdrawn from the first vial and injected into the second.

7) Store second vial as Solution #4.

#### SOLUTION #5

distilled H<sub>2</sub>O (deaerated)                      100 ml

Na<sub>2</sub>S · 9 H<sub>2</sub>O    12 g

Must wash and dry crystals of sodium sulfide before weighing and adding.

Pour solution into a 120-ml vial, evacuate and purge with N<sub>2</sub>, and autoclave for 20 minutes.

#### SOLUTION #7

a) 100 ml H<sub>2</sub>O (filter-sterilized) + .001 g biotin

b) 100 ml H<sub>2</sub>O (filter-sterilized) + .005 g p-aminobenzoic acid

c) 100 ml H<sub>2</sub>O (filter-sterilized) + .005 g vitamin B<sub>12</sub>

d) 100 ml H<sub>2</sub>O (filter-sterilized) + .010 g thiamine

Store in 120-ml vials and filter-sterilize when adding each solution.

SOLUTION #9

- 1) Filter-sterilize 100 ml of distilled H<sub>2</sub>O into autoclaved vial (or autoclave 100 ml H<sub>2</sub>O).
- 2) Evacuate and purge with N<sub>2</sub> (4 times).
- 3) Weigh 3.0 g Na<sub>2</sub>S<sub>2</sub>O<sub>4</sub> and quickly pour into purged vial.
- 4) Repeat step #2 and store as Solution #9.

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