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Characterization of Thermophilic Microorganisms in Oil Reservoirs

by

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "Characterization of Thermophilic Microorganism in Oil Reservoirs" submitted by Krista Kaster in partial fulfillment of the requirements for the degree of Master of Science.

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Abstract

Thermophilic sulfate-reducing bacteria (tSRB) cause reservoir souring (sulfide production) in high temperature oil fields. In lower temperature (mesophilic) systems souring can be controlled by nitrite or nitrate. In the latter case other microbial groups e.g. nitrate-reducing bacteria (NRB) need to be present. The effects of nitrate or nitrite on different tSRB enrichments from a North Sea oil field, and from an oil storage tank were determined. Nitrate (2-15 mM) was ineffective at controlling souring in these systems. Nitrite (0.25 – 0.5 mM) proved very effective at inhibiting sulfide production in North Sea enrichments, which contained *Thermodesulforhabdus* and *Archaeoglobus* species. The oil tank consortium contained methanogenic activity. A thermophilic *Methanothrix* species forming methane from acetate was isolated. Sulfide production by the oil tank consortia was inhibited by 1 mM nitrite. The resident tSRB have not been definitively identified. Overall the results show that nitrite addition is very effective for controlling souring in high temperature systems.

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Abbreviations

BLAST	basic local alignment search tool
CSB	Colville synthetic brine adapted
CSBA	Colville synthetic brine adapted recipe
$C_3H_5O_3^-$	lactate
$C_2H_3O_2^-$	acetate
CH_4	methane
$C_{16}H_{34}$	hexadecane
CO_2	carbon dioxide
DGGE	denaturing gradient gel electophresis
DNA	deoxyribonucleic acid
Dsr	dissimilatory sulfite reductase
E_h	redox potential
FISH	fluorescent *in situ* hybridization
H_2S	hydrogen sulfide
hNRB	heterotrophic nitrate or nitrite reducing bacteria
HPLC	high-pressure liquid chromatography
mCSB	modified Coleville synthetic brine
MPN	most probable number
MS2	modified sea water #2
N_2	elemental nitrogen gas
NH_3	ammonia
NO_2^-	nitrite
NO_3^-	nitrate
NRB	nitrate or nitrite-reducing bacteria
Nrf	periplasmic nitrite reductase
NR-SOB	nitrate or nitrite-reducing, sulfide-oxidizing bacteria
PCR	polymerase chain reaction
rDNA	ribosomal deoxyribonucleic acid
rRNA	ribosomal ribonucleic acid
SDS	sodium dodecyl sulfate
SO_4^-	sulfate
sp.	species (singular)
sPGC	saline Posgate C medium
spp.	species (plural)
SRB	sulfate reducing bacteria
tSRB	thermophilic sulfate reducing bacteria
VFA	volatile fatty acids
v/v	volume per volume
w/v	weight per volume

Chapter 1: Introduction

1.1 Oil recovery

Oil is usually found in subterranean porous rock and is accessed by drilling a borehole. Initially there is a pressure gradient allowing the oil to spontaneously flow upwards (primary production). However, this pressure gradient is typically exhausted before the majority of oil is recovered; often less than 30% of reservoir oil is recovered during primary production (Davidova *et al*, 2001). The most common method for enhancing oil recovery during secondary production is water injection in order to maintain the reservoir pressure (Figure 1.1). High temperature oil reservoirs in the North Sea are usually injected with seawater, containing a high concentration of sulfate (20-30 mM; Cochrane *et al*, 1988) and other limiting nutrients that stimulate the growth of microorganisms. For example, the injected water along with the short chain organic acids already present in the oil reservoir create suitable conditions for the growth of sulfate-reducing bacteria (Beeder *et al*, 1995).

1.2. Oil reservoir microorganisms

Oil reservoirs harbour a wide variety of microorganisms. The resident temperature of an oil reservoir increases with depth by approximately 2–3 °C per 100 m below the surface (Magot *et al,* 2000). This temperature gradient creates a variety of different temperature zones for microbial growth. As a result, depending on the depth of the reservoir, the microbial communities can be mesophilic (growing at 20-50°C), thermophilic (growing at 50-80°C) or hyperthermophilic (growing at 80-100°C). Oil reservoirs contain diverse microbial communities composed of sulfate-reducing bacteria (SRB), nitrate-reducing bacteria (NRB), fermentative bacteria, methanogens and metal-

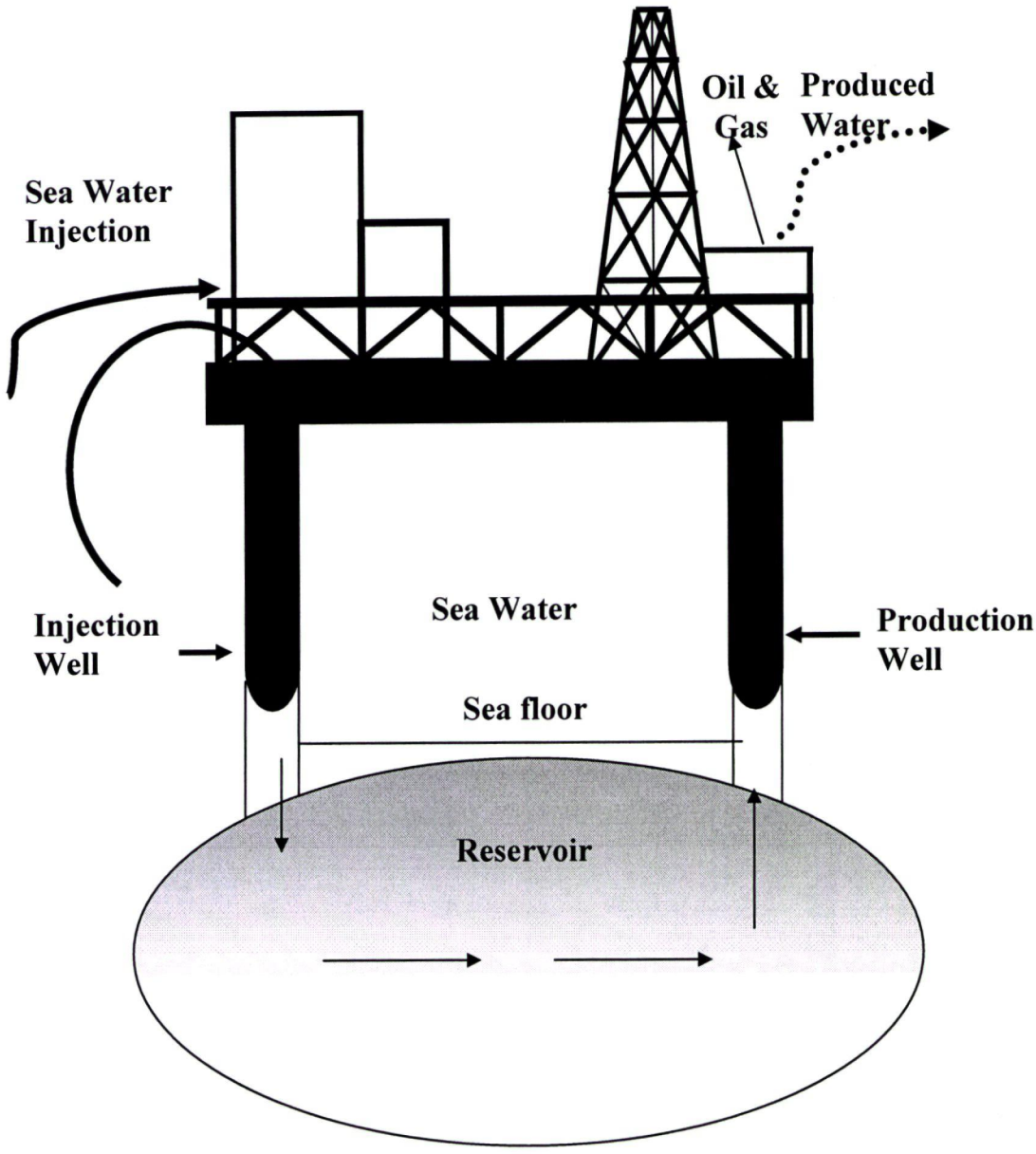

Figure 1.1 Secondary oil production. Seawater is injected into the reservoir to maintain pressure and mixes with the oil. The oil is separated from the produced water, which is cleaned and dumped back into the sea.

reducing bacteria (Watanabe *et al,* 2002; Eckford and Fedorak, 2002; Orphan *et al*, 2000; Magot *et al*, 2000; Slobodkin *et al*, 1999). Until recently, it was thought that these microorganisms were introduced at the site of drilling, however recent evidence has shown that these bacteria are not necessarily introduced but endogenous to the subsurface (L'Haridon *et al* 1995; Parkes and Cragg, 1994). Mesophilic or thermophilic SRB originating from other sources may also be introduced via the injection water (Stetter *et al*, 1993).

1.3. Reservoir souring

Water injection often leads to oil field "souring", which is the production of sulfide by SRB. Although sulfide can be produced chemically at high temperatures (above 100-150°C; Herbert, 1987), SRB activity is the main cause of sulfide production in flooded oil reservoirs.

In the Skjold oilfield in the Danish sector of the North Sea sulfide production increased from 100 kg/day in 1994 to 1000 kg/ day in 1999 due to water injection and microbial sulfate reduction (Larsen *et al,* 2000). Depending on reservoir depth, mesophilic or thermophilic SRB (tSRB) have been isolated. tSRB have been found in production facilities, injection water (Lappin-Scott *et al*, 1994), formation water and as dormant cells in seawater surrounding offshore production facilities. Both eubacterial tSRB (optimal growth temperature around 60°C) such as *Thermodesulforhabdus norvegicus* (Beeder *et al,* 1995) and hyperthermophilic archaeal sulfate-reducers (optimal growth temperature at 80°C) such as *Archaeoglobus spp.* (Stetter *et al*, 1993) have been isolated from high temperature oil reservoirs. These organisms are able to survive adverse conditions such as starvation and low temperatures for many months. Rapid

growth ensued once suitable growth conditions were provided (Lappin-Scott *et al*, 1994, Isaksen *et al*, 1994). This ability to survive in adverse conditions allows them to be introduced to oil reservoirs where conditions are suitable for their growth, via water flooding regimes (Couch, 1983). Once introduced into the reservoir, the reservoir temperature combined with the electron acceptor provided in the injection water and the electron donors in the reservoir create a suitable growth environment. SRB have been shown to use H_2 and volatile fatty acids (VFA; acetate, propionate, butyrate) as electron donors, however, some SRB have also been shown to use n-alkanes for sulfate reduction (Rueter *et al*, 1994), all of which are available in the reservoir.

1.3.1 Models for reservoir souring

Two models have been proposed for reservoir souring: the mixing model and the biofilm model. In the mixing model the SRB responsible for souring live in the mixing zone between the sulfate-rich injection water and the formation water, which contains the electron donors (Chen *et al*, 1994). The biofilm model H_2S is produced by the SRB associated with biofilms close to the injection well (Sunde *et al*, 1993). The mixing zone model proposes that sulfide production should peak with the appearance of a mixing zone in the production well and decline thereafter, whereas the biofilm model proposes that sulfide production should not occur until after several pore volumes of injection water have passed through the well. Observations from the Gullfalks field correlated more with the biofilm model than they did with the mixing zone model showing an increase in H_2S production for a longer period of time after breakthrough of water at the production well (Myhr, 2003). Souring may be the result of a combination of the two mechanisms. Also sessile SRB near the injection well have been shown to be involved in reservoir souring

(Myhr, 2003). More research is needed for a complete of understanding the mechanism of reservoir souring.

1.3.2. Problems associated with reservoir souring

For almost as long as oil has been commercially produced, companies have been battling the problems created by SRB. Sulfate reducers can use iron as the electron donor leading to the oxidation of iron (Figure 1.2). SRB are the cause of microbially induced corrosion occurring along with sulfide production leading to the corrosion of metal equipment, tanks and pipelines. Corrosion damage due to SRB creates a great deal of economic loss. SRB are estimated to be responsible for approximately 80% of all corrosion damage to oil field operating machinery (Antipov and Levashova, 2002). Sulfide in oil reservoirs leads to a variety of other problems and increases the cost of refinement. SRB can also cause oil field plugging due to the formation of metal sulfide precipitates and due to the accumulation of SRB biomass which reduces reservoir permeability (Bass *et al*, 1996). Hydrogen sulfide gas is highly toxic; 1000-2000 ppm of sulfide is lethal in a few minutes (http://www.cdc.gov/niosh/idlh/7783064.html). Sulfide on platforms can be removed via sulfide scavenging chemicals, using for example a mixture of aldehydes and amines (Myhr, 2003), however souring prevention may be the better option.

1.3.3. Prevention of reservoir souring

1.3.3.1. Sulfate removal

Sulfate can be removed from seawater using nanofiltration. Removal of sulfate by this method has proven to be effective at decreasing H_2S formation (Bakk *et al*, 1992).

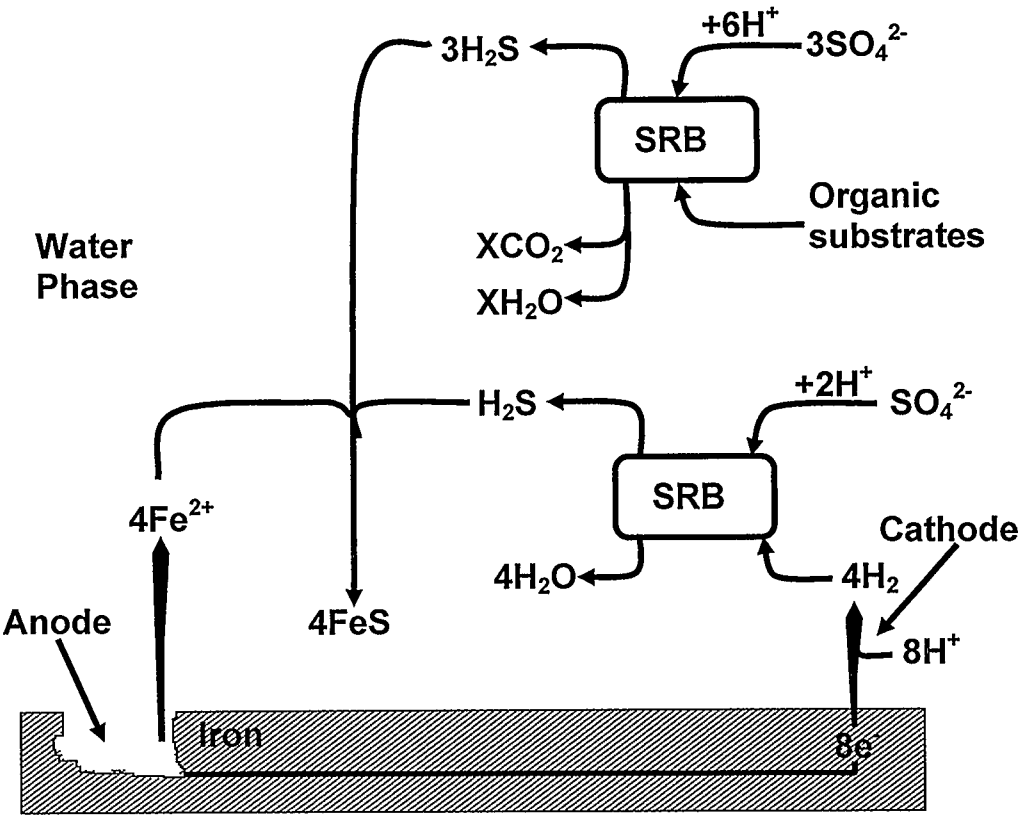

Figure1.2 The main reactions of anaerobic corrosion. SRB corrode iron via cathodic depolarization. The remaining metallic iron becomes negatively charged in water resulting from the loss of Fe^{2+} ions. The electrons in the metal then reduce the H^+ ions formed in water dissociation forming H_2 which is permanently removed from the metal via oxidation with sulfate by SRB resulting in the oxidation of iron to ferrous ions (Fe^{2+}). Ferrous ions precipitate with sulfide to form FeS.

1.3.3.2. Biocides

Common agents used in attempts to mitigate sulfide production are broad-spectrum biocides, which are added to the water prior to injection. These broad-spectrum biocides have been shown to inhibit sulfate reduction, and include glutaraldehyde and cocodiamines (Gevertz *et al.* 2000). Biocide addition to water prior to injection into reservoirs is often met with limited success. In oil reservoirs tSRB usually exist in biofilms that protect the SRB from biocides. Thus the concentration of biocide required *in situ* will likely be greater than the concentration needed to inhibit growth in laboratory batch cultures. To be successful, the concentration of biocide used must be high enough to penetrate biofilms and kill even the innermost members of these assemblages. Limited dispersal of the biocides within the reservoirs may also render them ineffective. Additionally, with continuous biocide treatment over time there is the possibility of the emergence of biocide-resistant bacteria (Telang *et al*, 1998). Furthermore, biocides are expensive and can be very toxic and harmful to oil field personnel. Some oil production occurs in environmentally sensitive areas and there is growing pressure for more environmentally friendly alternatives.

1.3.3.3. Nitrite addition

Another method that can be used to control souring is the addition of the metabolic inhibitor nitrite. Nitrite is an inhibitor of dissimilatory sulfite reductase (Dsr), the enzyme in SRB that reduces sulfite to sulfide. Dsr has a strong affinity for nitrite and can slowly reduce nitrite to ammonia (Wolfe *et al*, 1993). Thus, nitrite can function as a competitive inhibitor of Dsr. Studies by Reinsel *et al* (1996) showed that nitrite could control souring in a sand-packed column inoculated with produced water from a North

Sea oil field. Some SRB are able to overcome the inhibitory effects of nitrite through the presence of nitrite reductase (Nrf) allowing them to remove nitrite by reducing it to ammonia (Pereira *et al*, 2000).

Nitrite has been found to be an effective inhibitor of sulfate reduction. However its effectiveness is dependent on whether the SRB present in the oil reservoir have genes for Nrf and on the presence of NRB or NR-SOB in the microbial community which can also reduce nitrite, removing it from the reservoir and thus eliminating its inhibitory effect. Mid-log phase cultures of *Desulfovibrio sp.* strains Lac3 and Lac6 were inhibited by 0.5 mM nitrite whereas those of *Desulfovibrio vulgaris* strain Hildenbrough were inhibited by 10 mM nitrite (Greene *et al*, 2003). This difference between strains was found to be due to the presence or absence of Nrf. *D. vulgaris* strain Hildenbrough has a periplasmic NrfHA which confers nitrite resistance (Greene *et al*, 2003). Greene *et al* (2003) also demonstrated through Southern blotting that a Western Canadian oil field isolate, *Desulfovibrio sp.* strain Lac15, has a gene homologous to *D. vulgaris nrf* and was also able to recover from nitrite addition. Strains Lac3 and Lac6 appeared to lack Nrf.

1.3.3.4. Nitrate addition to control souring

An alternative method for controlling reservoir souring is nitrate injection. Nitrate addition has been found to be an effective method of controlling reservoir souring and has the advantage of being less toxic than biocides. Nitrate injection into an oil reservoir stimulates the activity of the endogenous NRB in the oil reservoir. Nitrate has been used since the early 1900s to control sulfide production in sewage (Carpenter, 1932; Heukelekian, 1943). Nitrate addition has since been found to be an effective method for controlling sulfide concentrations in oil reservoirs (Reinsel *et al*, 1996; Telang *et al.*

1997; Greene *et al*, 2003). Nitrate injection can control souring through a variety of mechanisms. The injection of nitrate into an oil reservoir stimulates the activity of the indigenous heterotrophic nitrate-reducing bacteria (hNRB). hNRB reduce nitrate (their electron acceptor) to nitrite, nitrogen or ammonia, while oxidizing available electron donors in the oil (e.g. acetate, propionate or butyrate) to produce energy (Figure 1.3). Thus nitrate may establish competition between hNRB and SRB for available electron donors. Nitrate reduction to nitrite (NO_2^-), ammonia (NH_3), or nitrogen (N_2), provides more Gibbs free energy than sulfate reduction (Zehnder and Strumm, 1988). Thermophilic NRB (tNRB) have been isolated from Alaskan petroleum reservoirs (Jackson and McInerney, 1996) and hyperthermophilic NRB have been isolated from hot springs (Afshar *et al*, 1998). In the presence of the injected nitrate hNRB may outcompete SRB for limited VFA electron donors. Moreover, products of nitrate reduction (e.g. nitrite) inhibit SRB. Nitrate addition can also stimulate the activity of chemolithotrophic nitrate-reducing sulfide oxidizing bacteria (NR-SOB) in oil reservoirs. NR-SOB are able to gain energy from oxidizing sulfide (Figure 1.3). Stimulation of the growth of these bacteria has the added advantage that they oxidize sulfide and thus remove it from the produced water.

Nitrate injection has proven to be a useful method for controlling souring in Western Canadian oil fields. Injecting 5 mM nitrate for 50 days into an oil field in Western Canada resulted in 50 to 100% sulfide removal (Telang *et al*, 1997; Jenneman *et al*, 1999). The sulfide removal was shown to be caused mainly by the NR-SOB *Thiomicrospira* sp. strain CVO. Nemati *et al* (2001[b]) showed that adding *Thiomicrospira*

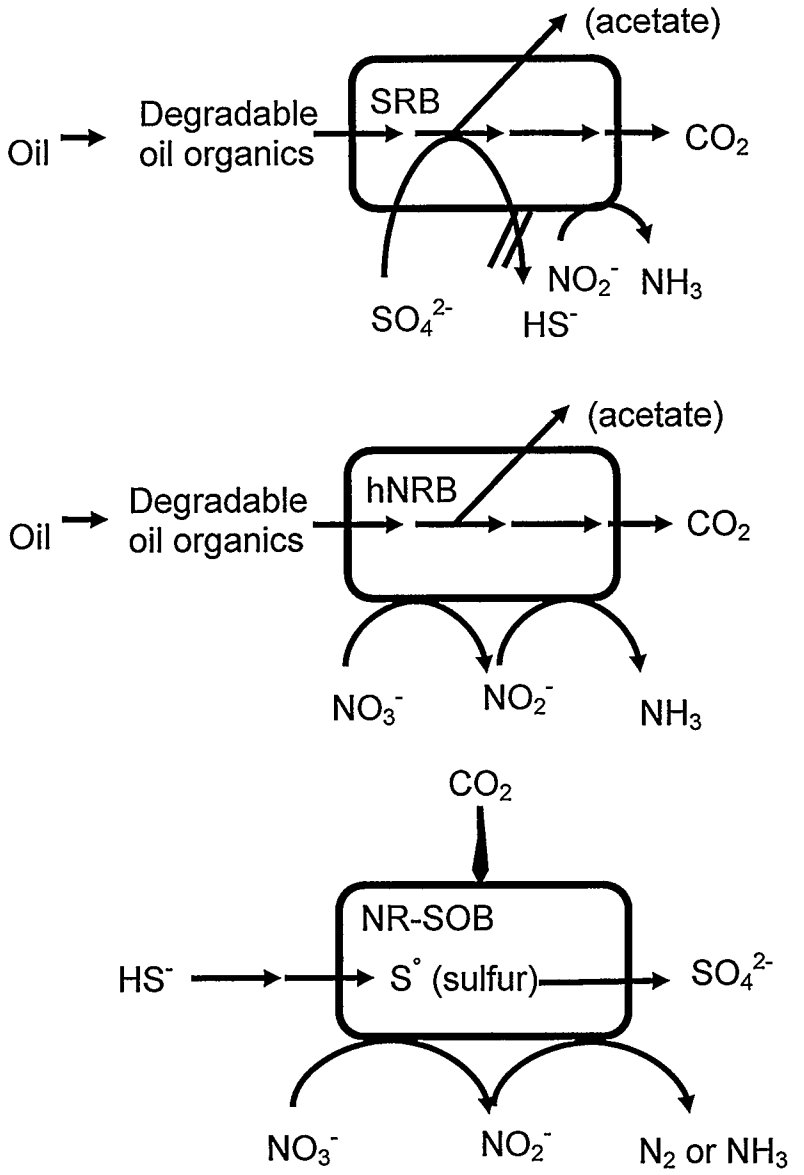

Figure 1.3 Activities of SRB, hNRB and NR-SOB. Degradable oil organics (i.e. lactate), sulfide (HS^-) and sulfur (S_0) are electron donors. Sulfate (SO_4^{2-}), nitrate (NO_3^-) and nitrite (NO_2^-) serve as electron acceptors. Sulfate reduction by SRB is inhibited by nitrite (//) produced by hNRB or NR-SOB. Some SRB possess nitrite reductase activity which allows them to overcome this inhibition by reducing nitrite to ammonia

sp. strain CVO and nitrate to a pure culture of *Desulfovibrio* sp. strain Lac6 not only inhibited this SRB directly but also removed sulfide. Like hNRB, NR-SOB use nitrate as their terminal electron acceptor and produce various end products such as nitrite, nitrous oxide and nitrogen (Gevertz *et al*, 2000). They oxidize sulfide to sulfate or elemental sulfur. In a recent study, Eckford and Fedorak (2002) enumerating NRB in Canadian oil fields found hNRB in 16 of 18 produced water samples and NR-SOB in 12 of 15 samples tested. This indicated that NRB (hNRB and NR-SOB) can be commonly found in oil fields, and that nitrate addition could stimulate the growth of NRB, potentially controlling or suppressing sulfide production.

1.4. Identification and characterization of bacteria in oil reservoirs

A great deal of recent research has attempted to characterize the microbial communities within oil reservoirs. Both culture-dependent and culture-independent methods have been used to characterize the microbial assemblages present in different environments. Culture-based methods provide information on the physiology of the organisms present in an environment, however they provide little information on composition of these communities as culturable organisms usually represent a minor component of the total microbial community (Van Hamme *et al*, 2003). Culture-independent methods provide information on the microbial groups present in the community and allow for the identification of new phylogenetic groups but reveal little about the physiology of the community members and the roles different microorganisms play.

Culture-dependent methods include the isolation and characterization of organisms, and most probable number assays (MPNs). The purpose of isolating

organisms from a community is often to identify and determine their physiological roles within a given community. With this approach organisms are cultured and isolated on liquid and/or solid media. Once isolated the organism's physiological properties in pure culture can be determined. This technique has allowed identification of many different microorganisms found in oil reservoirs and determination of their physiology (Telang *et al*, 1997; Kodama and Wantanabe, 2003; Orphan *et al*, 2000). However, even if an organism is cultured one may not be able to determine all of its roles in a community, especially how it interacts with other microbes. A further problem with this method is that since only a small portion of the total community may be cultured, the cultured organisms may not be a true representation of the entire collection of microbial activities present.

The MPN assay is culture-based and allows enumeration of bacteria in a particular environment, i.e. how many of a particular group are present and how these numbers change over time. MPN assays can be scored using a variety of criteria such as substrate utilization, growth (ie. turbity) or gas production. A limitation of this assay is that the selective media used for the MPN may not detect all of the bacteria present in the particular group that the study is trying to detect (Van Hamme *et al*, 2003). MPN assays have been used to determine the presence of methanogens and homoacetogenic bacteria in deep granitic rock aquifers (Kotelnikova and Pedersen, 1997). This assay is also employed by oil companies to monitor the number of SRB present in their reservoirs as an indicator of corrosion and souring risk using lactate sulfate medium. Recently, Eckford and Fedorak (2002) used MPN assays to enumerate SRB, hNRB and NR-SOB present in five different Western Canadian oil fields. To identify the different groups

they used three different types of media using different electron donors and acceptors; hNRB media which used nutrient broth and nitrate, NR-SOB media which used sulfide and nitrate and SRB media enumerated using lactate and sulfate. They found great variation in the numbers of the different groups between oil fields. NR-SOB were found in 7 of the 17 samples and in the positive oil fields their numbers ranged from 93 to 210 000 cells ml^{-1}, NRB were present in 16 of 18 samples and ranged from 1.5 to 23 000 cells ml^{-1} and SRB were present in 17 of 18 samples and ranged from 0.9 to 23 000 cells ml^{-1}. Such information allows one to predict how the different oil fields would respond to nitrate amendment.

Since it is thought that 90-99.9% of microorganisms remain uncultured (Edwards, 2000), culture-independent methods must also be used to obtain a more comprehensive characterization of environmental microbial communities. Culture-independent methods include fluorescence *in situ* hybridization (FISH), PCR and cloning of 16S rRNA genes, denaturing gel electrophoresis (DGGE) and reverse sample genome probing (RSGP). A large number of the culture-independent methods employ the use of PCR amplification of 16S rRNA genes. Sequencing 16S rRNA genes (about 1500 bp) is an excellent method for determining microbial diversity and phylogeny as some parts are highly conserved and others are variable (Woese, 1987). PCR primers that target the universal regions of the 16S rDNA are used to amplify this gene, which is then sequenced, allowing the determination of that organism's phylogeny. A value of 98% sequence identity or higher has been suggested to designate species similarity (Stahl, 1997).

Some caveats associated with PCR based methods include differential DNA or RNA extraction from different cells (Kent and Triplett, 2002) and also differential

amplification of the DNAs during PCR (known as PCR bias). PCR bias can be minimized by carefully selecting and designing primers which account for the more variable regions of the 16S rRNA genes and by using multiple PCR primers to analyze the microbial community. This minimizes the incidence of preferential amplification, which may cause organisms present in the community to be missed. The effects that PCR primer selection can have on the microbial diversity detected in a given microbial community was demonstrated by Watanabe *et al* (2002), who found that the PCR primers which had previously been designed to amplify an oil field community, had mismatches in the16S rDNA sequences for some bacterial groups which reduced amplification efficiency. To correct this problem they used nucleotides like inosine in the PCR primer positions they found to have the most variation in the 16S rRNA sequences. Using these redesigned primers they were able to detect phylotypes associated with *Verrucomicrobia* and candidate division OP11 in their groundwater populations which they had not previously detected using their original primers. Thus by carefully selecting primers greater microbial diversity can be uncovered.

Reverse sample genome probing (RSGP) is based upon the fact that the entire genome of a microbe can be used to probe specifically for that microorganism in the environment. Although the actual assay does not involve culturing this technique is still culture dependent such that it only describes community composition in terms of micobes that have already been cultured. Genomic DNA is extracted from different reference organisms that have been isolated from a particular environment and spotted onto a solid surface in denatured form. DNA extracted from the environment containing unknown microbial diversity is labeled and hybridized to the genomic DNAs spotted on the filter

(Voordouw *et al*, 1991). The organisms will only hybridize to genomic DNA spots of bacteria to which they are closely related. RSGP has been used to show changes in the microbial community after nitrate injection into a Western Canadian oil field and has demonstrated that after nitrate injection *Thiomicospira* sp. strain CVO (an NR-SOB) became a dominant community member (Telang *et al*, 1997).

DGGE allows one to separate identical or nearly identical length DNA fragments with different sequences of differing GC content. This method exploits the fact that the strands of DNA molecules separate, or melt at different rates when heat or a chemical denaturant is applied. The DNA fragments are separated by gel electrophoresis through a gradient of increasing chemical denaturant concentration (usually urea or formamide). This technique is commonly used to identify microbial communities in many different environments. DGGE displays community diversity by the number of different bands present and allows for the identification of these bands if they are excised, sequenced and matched against a database of known sequences. DGGE has been used recently to determine the effect of nitrate injection on the microbial community in an oil reservoir model column (Myhr, 2003)

1.5. Microbial oil biodegradation

The presence and role of microorganisms deep in the subsurface in petroleum biodegradation is of great scientific interest. Biodegraded oil is found in reservoirs up to ~4 km in depth. The level of biodegradation in reservoirs generally increases with decreasing reservoir temperatures (Head *et al*, 2003). Thus biodegraded oil has rarely been found in oil reservoirs with resident temperatures above 80 °C, i.e. below 4 km depth. This does not mean that all lower temperature oil reservoirs are highly

biodegraded; if the temperature of the reservoir was 80 – 90 °C at the time of deep burial the oil will not be biodegraded due to a proposed process called "paleopasteurization" (Wilhelms *et al*, 2000). The temperatures for oil biodegradation are much lower than the upper temperature at which life can exist, which has been found to be at 121 °C (Head *et al*, 2003). A proposed explanation for the lower temperatures of life that exists in oil reservoirs could be the extreme nutrient-limiting nature of petroleum reservoirs. All the environments that extreme hyperthermophilic organisms have been isolated from are rich in nutrients, electron acceptors and electron donors which allows these organisms to have high metabolic rates allowing them to replace heat labile cell components (Head *et al*, 2003)

Crude oil is composed largely of aromatic and saturated aliphatic hydrocarbons. The latter are dominated by *n*-alkanes. Up until the last decade research has concentrated on aerobic rather than anaerobic degradation of hydrocarbons. Recent work has revealed that crude oil hydrocarbons such as toluene, alkylbenzenes, benzene, n-alkanes and branched alkanes can be degraded anaerobically. These reactions can occur via Fe (III) reducing, sulfate-reducing, denitrifying pathways or in syntrophic consortia that contain methanogens (Van Hamme *et al*, 2003).

The nature and concentration of degradable oil organics play an important role in oil field souring. Oil biodegradation has been found to occur sequentially in reservoirs from n-alkanes, monocyclic alkananes, alkylbenzenes, isoprenoid alkanes, alkylnapthalenes, bicyclic alkanes, steranes and hopanes (Röling *et al*, 2003). The SRB and NRB may themselves degrade the oil or they may use some of the intermediates

produced during biodegradation. Some pure cultures of SRB and NRB have been found to oxidize alkanes completely to CO_2 or incompletely to acetate and CO_2.

It has been demonstrated that long chain alkanes or toluene can be degraded anaerobically to methane and CO_2 by a syntrophic consortium (Zengler *et al,* 1999). For example hexadecane would be degraded (hydrolyzed) as follows:

$4C_{16}H_{34} + 64H_2O \rightarrow 32CH_3COO^- + 32H^+ + 68H_2$ (1)

$32CH_3COO^- + 32H^+ \rightarrow 32CH_4 + 32CO_2$ (2)

$68H_2 + 17CO_2 \rightarrow 17CH_4 + 34H_2O$ (3)

Net reaction: $4C_{16}H_{34} + 30H_2O \rightarrow 49CH_4 + 15CO_2$ (4)

This net reaction was demonstrated in medium containing hexadecane or pentadecane that was inoculated with an enrichment culture obtained from anaerobic ditch sediment. The enrichment culture was found to contain a consortium composed of the following: *Syntrophus* spp., which may catalyze the conversion of alkanes to acetate (equation 1); *Methanosaeta* spp. acetotrophic methanogens, which convert acetate to methane and CO_2 (equation 2), and clone types related to the genera *Methanospirillum* spp. and *Methanoculleus* spp., which use H_2 and CO_2 to form methane (equation 3). Therefore, when water is injected into an oil reservoir, it may allow the oil organics to be degraded to methane and CO_2 through VFA intermediates. This could replenish the VFA in the oil reservoir, that can in turn be used as electron donors and as a carbon source by SRB for sulfate reduction. The problem is that the $\Delta G^{o'}$ of reaction 1 is positive under standard conditions, hence reactions 2 and 3 must be efficient to pull reaction 1 to the right. Despite all of the evidence for the biological cause of oil degradation, as of yet, few

hydrocarbon-degrading microorganisms have been isolated from oil reservoirs (Myhr, 2003).

1.6. Research objectives

More is known about mesophilic that about thermophilic bacteria associated with oil. This thesis will focus on the thermophilic microorganisms found in produced waters and oil storage tanks. Specific objectives are:

(i) To identify the presence or absence of tSRB, thNRB and tNR-SOB in samples derived from high temperature oil fields or storage sites.

(ii) To determine the nitrite reductase activity of tSRB and the effect of nitrate and nitrite addition of sulfide production in thermophilic consortia

(iii) To determine the activity of methanogens and associated oil degrading bacteria.

Chapter 2: Materials and methods

2.1 Samples

The samples, enrichments and isolates used in this study are outlined in Table 2.1. Two tSRB enrichments, NS-tSRB-1 and NS-tSRB-2, from the Ekofisk oil field in the North Sea were obtained from Dr. Gary Jenneman (ConocoPhillips, Bartlesville, OK) in July 2002 and September 2003, and maintained at 58°C. Samples ST-1, ST-2, ST-3 and ST-4 from a refined oil storage tank (50 – 60°C) were obtained from another industrial partner in February 2003. These samples were stored at room temperature in an anaerobic chamber with a gas phase of 85% (vol/vol) N_2, 10% CO_2 and 5% H_2 until use.

2.2 Enrichment and isolation of tSRB

tSRB were enriched from stored samples in liquid media containing either lactate or mixed organic acids (acetate, propionate and butyrate) as electron donors. Media used for tSRB enrichments included saline Postgate C (sPGC; Table 2.2), modified seawater 2 (MS2; Table 2.3) and modified Coleville synthetic brine (mCSB; Table 2.6). sPGC was formulated similar to Postgate (1984), with addition of 7 g/L NaCl and 1.2 g/L of $MgCl_2$ with a headspace consisting of 5% hydrogen (vol/vol), 10% CO_2 (vol/vol) and the balance made up with N_2. MS2-20 contained 20 g of NaCl/L, and M2-7 contained 7 g of NaCl/L. After sterilization by autoclaving, 1 ml EDTA-chelated trace elements solution (Table 2.4; Widdel and Bak, 1992), 1 ml selenate-tungstate solution (Table 2.5; Widdel and Bak, 1992), 30 ml 1 M $NaHCO_3$ and 1 ml vitamin B_{12} solution were added (Widdel and Bak, 1992). Either lactate (28 mM) or a mixture of organic acids (acetate, propionate and butyrate; 12, 1.2 and 0.6 mM respectively) was included in media as the electron donors.

Table 2.1 Samples, isolates and enrichments used in this study

ID[a]	Description
NS-tSRB-1	Enrichment culture from the Ekofisk oil field maintained in MS2-OA that contains acetate, propionate and butyrate
NS-tSRB-2	Enrichment culture from the Ekofisk oil field maintained in MS2-lactate
ST-1	Oil sample from the top of an oil storage tank[b]
ST-2	Oil sample from the North side of an oil storage tank[b]
ST-3	Oil/water sample from the South side of an oil storage tank[b]
ST-4	Oil/water sample taken from the West side of an oil storage tank[b]
ST-1/4	Mixture of ST-1, ST-2, ST-3 and ST-4
ST-1/4-E	Enrichment of ST-1/4 maintained in sPGC
ST-tSRB-8A	tSRB enrichment obtained from ST-1/4.
ST-tSRB-8A-2	tSRB enrichment obtained by repurifying from ST-SRB-8A on medium E then growing in sPCG
ST-FER-2	Thermophilic, non sulfate-reducing, non methanogenic presumably fermentative isolate obtained from ST-1/4-E.
ST-tMET-1	Thermophilic acetotrophic methanogenic enrichment from ST-1/4 maintained on MS2-7-acetate

[a] Identification code ST indicates oil storage tank and NS indicates a North Sea field
[b] Stored at room temperature in an anaerobic hood.

Table 2.2 Saline Postgate C (sPGC) medium

Component	(g per L)
NaCl	7.0
$MgCl_2 \cdot 6H_2O$	1.2
KH_2PO_4	0.5
NH_4Cl	1.0
Na_2SO_4	4.5
$CaCl_2 \cdot 2H_2O$	0.042
$MgSO_4 \cdot 7H_2O$	0.03
$FeSO_4 \cdot 7H_2O$	0.004
Na-citrate$\cdot 2H_2O$	0.28
Yeast extract	1.0
Na-lactate 60 % (w/w)	10.0

pH was adjusted to 7.0-7.5

Table 2.3 Modified Sea water #2 medium variations. Listing is per L of medium. pH was adjusted to approximately 7.2 using 1 M HCl

Component	MS2-20-OA	MS2-20-L	MS2-7- L	MS2-7-A	MS2-7-HD
NaCl	20.0 g	20.0 g	7.0 g	7.0 g	7.0 g
$NaSO_4$	1.5 g	1.5 g	1.5 g	-	1.5 g
$MgCl_2 \cdot 6H_2O$	3.0 g	3.0 g	3.0 g	3.0 g	3.0 g
$CaCl_2 \cdot 2H_2O$	0.15 g	0.15 g	0.15 g	0.15 g	0.15 g
KCl	0.25 g	0.25 g	0.25 g	0.25 g	0.25 g
KH_2PO_4	0.5 g	0.5 g	0.5 g	0.5 g	0.5 g
KBr	0.6 g	0.6 g	0.6 g	0.6 g	0.6 g
Trace elements*	1.0 ml	1.0 ml	1.0 ml	1.0 ml	1.0 ml
Selenite-tungstate *	1.0 ml	1.0 ml	1.0 ml	1.0 ml	1.0 ml
$NaHCO_3$ *	30 ml	30 ml	30 ml	30 ml	30 ml
Vitamin B_{12}*	1 ml	1 ml	1 ml	1 ml	1 ml
2.0 M acetate*	6 ml	-	-	10 ml	-
2.0 M propionate*	0.6 ml	-	-	-	-
1.0 M butyrate*	0.6 ml	-	-	-	-
2.8 M lactate	-	10 ml	10 ml	-	-
0.1 ml hexadecane in 1.9 ml heptamethylnonane	-	-	-	-	2 ml
1.0 M sulfide	1 ml	1 ml	1 ml	3 ml	1 ml

* Added after autoclaving

Table 2.4 Non-chelated trace element solution

Component	(per L)
HCl	12.5 ml
$FeSO_4 \cdot 7H_2O$	2.1 g
H_3BO_3	0.03 g
$MnCl_2 \cdot 4H_2O$	0.10 g
$CoCl_2 \cdot 6H_2O$	0.190 g
$NiCl_2 \cdot 6H_2O$	0.024 g
$CuCl_2 \cdot 2H_2O$	0.144g
$ZnSO_4 \cdot 6H_2O$	0.002 g
$Na_2MoO_4 \cdot 6H_2O$	0.036 g

Table 2.5 Selenite/tungstate solution

Component	(g per L)
NaOH	0.4
$Na_2SeO_3 \cdot 5H_2O$	0.006
$Na_2WO_4 \cdot 2H_2O$	0.008

Table 2.6 Modified CSB medium

Component	(per L)
NaCl	7.0 g
KH_2PO_4	0.027 g
NH_4Cl	0.02 g
$CaCl_2 \cdot 2H_2O$	0.24 g
$MgSO_4 \cdot 7H_2O$	0.68 g
$(NH_4)_2SO_4$	0.5 g
Na-acetate	0.68 g
$NaHCO_3$	1.9 g
Na-lactate 60% (w/w)	4.78 g
Micronutrient solution	50 ml
pH adjusted to 7.0-7.5	

Table 2.7 CSB micronutrient solution

Component	(per L)
nitrilotriacetic acid	2.0 g
$FeCl_3$	0.0058 g
$CaSO_4 \cdot 2H_2O$	1.2 g
$MgSO_4 \cdot 7H_2O$	2.0 g
Na_2HPO_4	1.4 g
KH_2PO_4	0.72 g
NaCl	0.16 g
$MgCl_2 \cdot 6H_2O$	0 g
Trace element mixture	10 ml

Table 2.8 CSB trace element mixture

Component	(per L)
H_2SO_4	0.5 ml
$MnSO_4 \cdot H_2O$	2.28 g
$ZnSO_4 \cdot H_2O$	0.5 g
H_3BO_3	0.5 g
$NaMoO_4 \cdot 2H_2O$	0.025 g
$CoCl_2 \cdot 6H_2O$	0.025 g
$CuSO_4 \cdot 5H_2O$	0.045 g

Following aseptic addition of 1 ml of 1 M Na_2S to reduce the media, the pH was adjusted to 7-7.5. Organic stock solutions were prepared as described by Widdel and Bak (1992). Anaerobic media were dispensed into stoppered serum bottles and flushed with anaerobic gas that was composed of 10% (vol/vol) CO_2, balance N_2. Media were dispensed into sterile serum bottles or tubes and the headspace was replaced with this anaerobic gas. The enrichment ST-tMET-1 was maintained in MS2-7-A medium (Table 2.3). All cultures were incubated at 58 – 60 °C.

ST-tSRB-8A was obtained by plating ST-1/4 on medium E agar (Table 2.9), incubated in a GasPak jar (Oxoid; from Fisher Fairlawn, NJ) and incubated at 58- 60 °C. Isolated black colonies (indicating the presence of an iron sulfide precipitate) were picked and transferred to tubes containing 1ml sPGC, then transferred to successively large volumes of sPGC until cultures in 100 ml sPGC were achieved. ST-tSRB-8A was then evaluated for growth with different by electron acceptors growing in modified CSBA (Table 2.11). Either 10 mM nitrate, sulfate or thiosulfate was added prior to inoculation with ST-tSRB-8A. Since ST-tSRB-8A was determined not to be a pure culture it was again plated on medium E agar and isolated colonies were again picked to obtain ST-tSRB-8A-2.

ST-FER-2 was obtained from a dilution to extinction of ST-1/4-E. First nine ten-fold dilutions were made of ST-1/4 in sPGC. As there was still growth in the most highly diluted tubes of this dilution series, 1 ml was taken from the 10^{-9} dilution and used to inoculate a series of eleven ten fold dilutions in sPGC. Growth was observed in 10 of 11 of the 10-fold dilutions in sPGC. A 5 ml sample of the last dilution with growth was used to inoculate a 100 ml bottle of sPGC. ST-FER-2 was maintained by transferring cells to fresh sPGC approximately every month. North Sea enrichments NS-tSRB-1 and

NS-tSRB-2 were maintained on MS2-20-OA and MS2-20-lactate respectively and transferred approximately once a month.

2.3 Characterization of oil storage tank samples and enumeration of tSRB

Numbers of tSRB present in samples were determined using a 3-tube most probable number (MPN) series. The MPN was conducted for each sample in 9 ml Medium B (Table 2.10), which contained lactate and sulfate (Postgate, 1984). Serial dilutions from 10^{-1} to 10^{-7} were made directly from the oil/water samples or from MS2 extracted oil samples. Oil samples were extracted in MS2-20 containing no electron donors or acceptor using 5 ml of oil sample to 5 ml MS2-20 in sterile Falcon tubes. Samples were then shaken by hand vigorously and the water phase was used to inoculate the MPN tubes. This procedure was carried out in the anaerobic chamber. Cultures were then incubated at 58 - 60°C and SRB activity was scored by the presence of a black ferrous sulfide precipitate in the medium. MPNs were scored on days 1, 3, 7 and 14, after inoculation. A set of PE tubes for all samples was also incubated at room temperature. All samples were tested for growth of NRB in CSB media (Table 2.12).

2.4 DNA extraction and purification

DNA was extracted from enrichment ST-tSRB-8A using the method described by Telang *et al* (1997). For ST-tMET-1 and ST-tSRB-8A-2 DNA was extracted by combining the methods of Ravenschlag *et al* (1999) and Telang *et al* (1997) as follows: 1000 µl of extraction buffer (1.5M NaCl, 0.1M EDTA, 0.1M Tris-HCl pH 8.0, 0.1M Na_2HPO_4) was used to resuspend pelleted cells. The resuspended cells then underwent three freeze thaw cycles (20 min at -70°C and 20 min at room temperature while rotating on a wheel). After the freeze thaw cycles 0.01 g of hexadecylmethylammonium

Table 2.9 Medium E

Component	(g per L)
KH_2PO_4	1.0
NH_4Cl	1.0
$NaSO_4$	1.0
$CaCl_2 \cdot 6H_2O$	2.0
$MgCl_2 \cdot 7H_2O$	1.0
$FeSO_4 \cdot 7H_2O$	0.5
Na-lactate 60%	4.0
yeast extract	1.0
agar	15.0

Adjust the pH to 7.0-75

Table 2.10 Medium B

Component	(per L)
KH_2PO_4	0.5 g
NH_4Cl	1.0 g
$CaSO_4$	1.0 g
$MgSO_4$	1.0 g
$FeSO_4 \cdot 7H_2O$	0.5 g
Na-lactate 60%	4.0 g
asorbic acid	0.1 g
thioglycolate	0.1 g

Adjust pH to 7.0-7.5

Table 2.11 Coleville synthetic brine adapted recipe (CSBA)

Component	(per L)
$NaSO_4$	1.7 g
NaCl	7 g
KH_2PO_4	0.2 g
$MgCl \cdot 6H_2O$	0.4 g
KCl	0.5 g
$CaCl_2 \cdot 2H_2O$	0.15 g
NH_4Cl	0.25 g
trace element solution (Table 2.4) *	1 ml
selenite–tungstate solution (Table 2.5)*	1 ml
1 M $NaHCO_3$*	30 ml
1 M NaS_2*	1 ml

* Added after autoclaving then adjust pH to 7.0-7.5

Table 2.12 Coleville synthetic brine

Component	(per L)
NaCl	7.0 g
KH_2PO_4	0.027 g
NH_4Cl	0.02 g
$CaCl_2 \cdot 2H_2O$	0.24 g
$MgSO_4 \cdot 7H_2O$	0.68 g
$(NH_4)_2SO_4$	0.13
Na-acetate	0.68 g
$NaHCO_3$	1.9 g
2 M $NaNO_3$	5 ml
Micronutrient solution (Table 2.7)	50 ml
1 M $NaS \cdot 9H_2O$	2.5 ml

bromide and 10 µl proteinase K were added and the tube was shaken at 37°C at 250 rpm for 30 min using a New Brunswick series 25 incubator shaker (Edison N.J., U.S.A.). After 100 µl of SDS were added the tubes were placed in a 65°C water bath for 2 hours; the tubes were inverted every 15 min. Following centrifugation at 6000 rpm at room temperature for 10 min, the supernatants were transferred to a fresh tube. 500 µl of extraction buffer and 100 µl of 25% SDS were added and the tubes were placed in a 65°C water bath for 10 min. The tubes were then spun at 6000 rpm for 10 min at 4°C. The supernates were then transferred to fresh tubes and 72 µl of 5 M $NaClO_4$ and 420 µl of 1:24 isoamylalcohol:CCl_4 were added and the tubes were rotated gently on a wheel for one hour. The remaining steps were carried out as described by Telang *et al* (1997). DNA was extracted from ST-FER-2 and NS-tSRB-2 using the Qiagen Dneasy^{Tm} tissue kit (Mississauga, ON).

PCR was performed on the isolated DNAs using the PCR primers shown in Table 2.13 specific for the 16S rRNA genes, as described by Watanabe *et al* (2002). The following PCR primer combinations were used: I341f with U1492r, U515f with U1492r, A25f with the reverse primers A958r, A1063r, A1392r, or U1492r, and forward primer A341f with the reverse primers A1063r or U1492r. The PCR products were purified using the QIAquick PCR purification kit (Qiagen, Germany) and automated sequencing was performed on an ABI PRISM 377 DNA Sequencer (Applied Biosystems Inc.) at University Core DNA services, at the University of Calgary using the same primers as for PCR amplification.

Table 2.13 Description of PCR primers used to amplify 16S rDNA sequences

Primer	Sequence[1]	Position (5' to 3')	Specificity
A25f	5'-CYGGTYGATYCTGCCRG-3'	9-25	Archaeal
I341f	5'-CCTACGGGIGGCIGCA-3'	341 to 356	Bacterial
A341f	5'-CCTAIGGGIGCAICAG-3'	341-357	Archaeal
U515f	5'-GTGYCAGCMGCCGCGGTAA-3'	515 to533	Universal
A958 r	5'-YCCGGCGTTGAMTCCAATT-3'	958-976	Archaeal
A1063r	5'-CGGCCATGCACCICCICTC-3'	1045-1063	Archaeal
A1392r	5'GACGGGCGGTGTGTRCA-3'	1375-1391	Archaeal
U1492r	5'-GGTTACCTTGTTACGACTT-3'	1492-1510	Universal

1) Y pyrimidine (C ,T), R purine (A,T), M amino (C, A) and I is inosine

The sequences were assembled as described by Gevertz *et al* (2000). Sequences were identified using the BLAST program (Altshul *et* al, 1990) to search the Genbank database and aligned using Clustal X version 1.8 (Thompson *et al,* 1997). The file of aligned sequences was used to generate phylogenetic trees using MEGA version 2.1 (Kumar *et al,* 2001) with 500 bootstrap replicates.

2.5. Southern blotting

Chromosomal DNA (500 ng) was digested using 2 µl *Eco*RI (5 µ/µL), 4 µl 10 X OPA buffer (Amersham, England) and 9 µl H_20 to a final volume of 15 µl and then incubating overnight at 37 °C. The DNA was then run on a 0.7% HGT gel, which was then stained by adding 5 µl ethidium bromide (5 mg/ml) in 100 ml of TAE running buffer (40 ml 50X TAE (242 g Tris-base, 57.1 ml glacial acetic acid, 16.8 g EDTA dissolved in 800 ml of H_2O the pH was then adjusted to 8.0 and the volume to 1 L) to 1960 ml H_2O). The gel was shaken for 30 min with 100 ml 0.5 M NaOH, 1.5 M NaCl (20g NaOH pellets, 87.7g NaCl dissolved in 800 ml H_2O, the volume was then made up to 1 L) and then washed 2-3 times with H_2O. After washing the gel was shaken for at least 20 min with 100 ml 1 M Tris-Cl, 1.5 M NaCl. After the solution was drained off, the gel was placed in a tray. Hybond-N which was wetted with 100 ml 10X SSC (44.1 g sodium citrate, 87.7 g sodium chloride dissolved in 800 ml H_2O, pH to 7.2 and volume to 1 L) was then placed on top of the gel. The Hybond was covered with 2 sheets of blotting paper and a stack of paper towels, weighed down and left overnight. The next day the paper towels and blotting paper were taken off. The Hybond filter was washed with with 1X SSC and air dried. Once dry the filter was UV irradiated for 3 min.

A Dsr probe was made by PCR amplifying the *dsr*A and *dsr*B genes from *Desulfovibrio vulgaris* Hildenborough using the P221r reverse primer and the P70f or P94f forward primers. The PCR mix contained per reaction 5 µl buffer, 1 µl dNTPs, 1 µl forward primer (P70f of P94f), 1 µl reverse primer (P221r), 0.2 µl Taq polymerase, 2 µl *D. vulgaris* DNA and 39.8 µl water. The following PCR cycle was then used 94 °C for 5 minutes, 94 °C for 40 seconds, 65 °C for 40 seconds and 72 °C for 2 minutes and 30 seconds. The PCR products were cleaned up using the QIAquick PCR purification kit (Qiagen, Germany).

The probe was made by adding 5 µl of the cleaned up PCR product, boiling for 3 min and then placing on ice for 3 min, then adding 6 µl of primer extension mix (PE), 2 µl Klenow and 2 µl P^{32} isotope. PE contained 44 µl 0.9 M N-[2-hydroethyl] piperazine-N'-[2-ethanesulfonic acid], 25 µl 1 M Tris-Cl (pH 7.4), 10 µl 0.1 M dithiothreitol, 4 µl of each 50 mM dGTP, dATP, dTTP and dCTP and 10 µl hexadeoxynucleotides (10 mg/ml). The probe was then kept at room temperature for 2-3 hours.

The filter was prepared for probing by incubating with 45 ml of prehybridization fluid, which contained 60 ml 20X SSC, 5 ml 10 % (w/v) SDS, 20 ml 50X Denhardt's solution (5 g Ficoll, 5 g BSA, 5 g polyvinyl pyrrolidone volume adjusted to 500 ml with H_2O), 1 ml 10 % (w/v) denatured salmon sperm DNA and 109 ml H_2O in a total volume of 200 ml, placing in a 68 °C oven and rocking for 2-3 hours. After 2-3 hours the probe prepared as described above was boiled for 3 min, placed on ice and then added to the filter and prehybridization mixture. The filter and probe were then left overnight in the 68 °C oven after which the filter was washed in 1X SSC to get rid of the excess probe. Then the filter was washed in 1X SSC and 0.2 % SDS at 68 °C, for 1 hour. Following

drying the filter was exposed to BAS III imaging plates (Fuji). Hybridization intensity was determined using a Fuji model BAS 1000 Bioimaging Analyzer.

2.6 Staining protocols

2.6.1 Gram staining

A few drops of liquid culture were placed on a microscope slide using a sterile inoculation loop. The bacteria were then allowed to dry on the slide. Cells were then heat-fixed by running the slide through a flame. The slides were first flooded with crystal violet and allowed to stain for one minute, then washed with H_2O. Slides were then flooded with Gram's iodine for one minute, rinsed with water and then decolorized, by adding 95 % alcohol, drop by drop, until the alcohol ran almost clear. After decolorizing the slide was rinsed and counterstained with safranin for 45 seconds. The safranin was rinsed off the slide which was then blotted dry (Cappuccino and Sherman, 1998).

2.6.2 Fluorescence staining

Acridine orange solution was prepared by dissolving 0.04 g acridine orange in 10 ml milli Q water which was then filtered. 200 µl of bacterial culture was added to 2 ml of milli Q and then filtered with 0.2 µm nucleopore track-etch membrane filters (Whatman) using a vacuum flask. 100 µl of the filtered acridine orange solution was placed on the filter containing the bacteria and allowed to stain for 10 minutes. After 10 min the dye was filtered off and the filter rinsed by filtering through 2 ml MQ water. Bacteria were visualized using the 60X objective lens on a Leica DSI RE2 confocal multiphoton microscope (Germany) at 450-500 nm.

2.7 Nitrate and nitrite inhibition of sulfate reduction in tSRB enrichment cultures

NS-tSRB-1 was grown in MS2-20-OA and NS- tSRB-2 was grown in MS2-20-lactate. For the oil storage tank consortium 5 ml samples were taken from ST-3 and used

to inoculate sPGC. After growing for three days the ST-3 cultures in sPGC were used to inoculate mCSB (5 ml). ST-tSRB-8A was grown in 100 ml mCSB using a 5 ml inoculum. For the inhibition experiments a 10% inoculum of the North Sea enrichments and a 5% inoculum of the oil storage tank consortia or ST-tSRB-8A were used. All cultures were then incubated at 58 °C. When the sulfide concentration reached between 4-6 mM different concentrations of nitrate or nitrite were added to the individual serum bottles.

2.8 Analytical methods

2.8.1 Chemical analysis

Sulfide, sulfate, nitrite and ammonia concentrations were determined spectrophotometrically (Cord-Ruwisch *et al*, 1985; Nemati *et al*, 2001[a]; APHA, 1992; Snell and Snell, 1949). The sulfide concentration was determined by mixing 50 µl of sample with 950 µl of 5 mM $CuSO_4 \cdot H_2O$, 50 mM HCl, measuring the optical density at 480 nm (OD_{480}) and comparing with the values obtained for a standard curve. The sulfate concentration was determined by mixing 50 µl of sample with 950 µl of conditioning agent (a 180-fold dilution in H_2O of 50 ml glycerol, 30 ml concentrated HCl, 75 g NaCl, 100 ml 95 % ethanol and 255 ml deionized water). An excess of $BaCl_2$ was added and the sample was vortexed until the $BaCl_2$ was completely dissolved. After 30 minutes the OD_{420} was measured. Nitrite concentration was determined by adding 25 µl of sample to 12 ml of deionized water, then adding 250 µl of sulfanilamide/N-(1-naphtyl)-ethylenediamine (LabChem Inc.). The samples were vortexed briefly and the absorbance at 543 nm was measured after 15 minutes. To measure the ammonia concentration, 50 µl of sample was added to 8 ml of deionized water to which 1 ml of Nessler's reagent was

added. The samples were vortexed briefly and then A_{420} was measured. Nessler's reagent consists of 50 g KI, 35.0 ml deionized water, to which a saturated aqueous solution of $HgCl_2$ is added until a slight precipitate persists, 400 ml 50% KOH solution (vol/vol) and deionized water to 1000 ml (Cappuccino and Sherman, 1998).

2.8.2 High pressure liquid chromatography and gas chromatography.

Nitrate was measured using a Waters 600E high pressure liquid chromatography unit (HPLC) equipped with a Waters 423 conductivity detector and a Waters IC-Pak A HC column and a 50 µl sample loop. Borate/gluconate eluent (Waters) was used at a flow rate of 2 ml/min as described elsewhere (Greene *et al*, 2003).

HPLC analysis was used to determine organic acid (lactate, butyrate, propionate and acetate) utilization by the tSRB enrichments. Samples were taken throughout the microbial growth curve to analyze the organic acid composition. A Waters 600E HPLC equipped with a Waters 2487 UV detector set at 220 nm was used with an Alltech Prevail Organic Acid column (250 x 4.6 mm) and an eluent of 25 mM KH_2PO_4 (pH 2.4 with a flow rate of 1 ml/min. A 20 µl sample loop was used.

The headspace of ST-tMET-1 cultures was analyzed to determine the presence of methane using a Hewlett Packard 5890 gas chromatograph(GC) equipped with a stainless steel column (0.049 cm X 5.49 m) packed with Porapak R (Supelco, Oakville, ON). The injector and the oven temperatures were both 37°C and the detector temperature was 80°C. The carrier gas was He with a flow rate of 15.6 ml/min and the reference gas was He with a flow rate of 15.8 ml/min. A Supelco (Bellefonte, PA) gas-tight syringe was used to inject 0.1 ml samples of headspace gas for analysis. A methane gas standard was used as the standard to determine the elution position of methane.

Chapter 3: Isolation and Characterization of North Sea tSRB enrichments

3.1 Introduction

Oil reservoirs contain many different microbial groups including SRB, NRB, and methanogens. Since many reservoir organisms have yet to be identified, identifying bacteria from these environments can lead to the discovery of novel genera and species. Characterizing organisms present in oil field environments will allow a better understanding of their involvement in reservoir souring and the overall functions of the microbial community. Here chromosomal DNA was extracted from two different tSRB enrichments from the North Sea and used to identify the bacteria present in these enrichments by amplifying and sequencing the 16S rRNA genes.

3.2 Methods

All methods used in this chapter were performed as described in chapter 2.

3.3. Results

3.3.1. Identification and characterization of NS-tSRB-1

NS-tSRB-1 was grown in MS-2-20-OA (Table 2.3) containing organic acid (acetate, propionate and butyrate) as electron donors. NS-tSRB-1 grew relatively slowly; using a 5% inoculum it took ca. 1 month for NS-tSRB-1 to reduce 10 mM sulfate to sulfide with a doubling time of 36 h during exponential growth (Figure 3.1). NS-tSRB-1 grew optimally with organic acid concentrations 12 mM acetate, 1.2 mM propionate and 0.6 mM butyrate (Figure 3.2: condition 4) based on these results the optimal organic acid concentrations for growth were used in the recipe for MS-20-OA. Microscopically this enrichment appeared to be composed completely of gram-negative rods. The 16S rRNA gene from

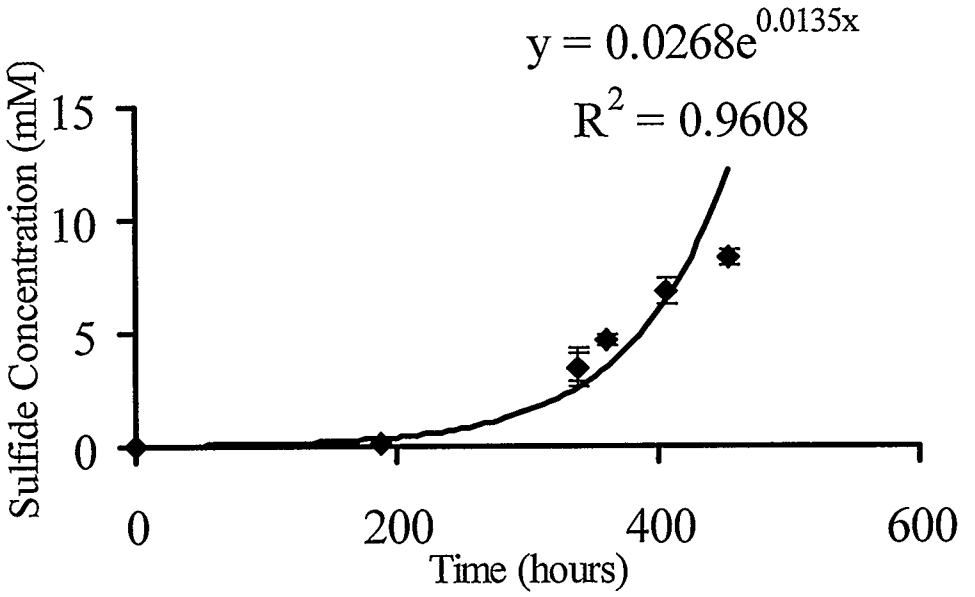

Figure 3.1 NS-tSRB-1during exponential growth phase in MS-2-20-OA when incubated at 60 °C and using a 5 % inoculum. Each point is the average of two trials with the error bars representing the standard deviation between the two points. A best fit of the data (Sulfide Concentration vs Time) is shown.

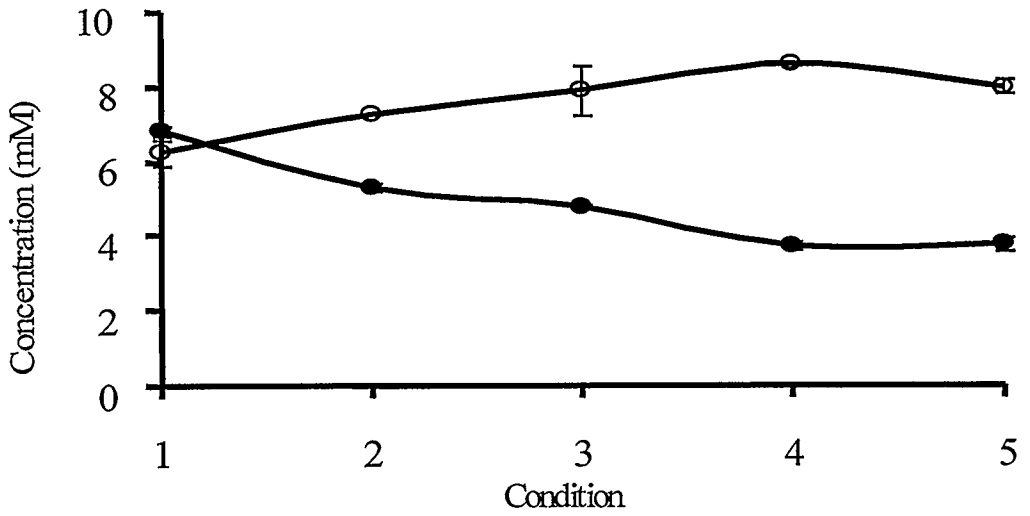

Figure 3.2 The amount of sulfate reduced to sulfide by NS-tSRB-1 in relation to the amount of carbon and energy source in the medium. At time zero the sulfate concentration in the medium was 10 mM. The concentrations of sulfate (•) and sulfide (○) were measured on day 28 after inoculation for each of the organic acid conditions 1-5 (condition 1: 3 mM acetate, 0.15 mM butyrate, 0.3 mM propionate; condition 2: 6 mM acetate, 0.3 mM butyrate; 0.6 mM propionate, condition 3: 9 mM acetate, 0.45 mM butyrate, 0.9 mM propionate; condition 4: 12 mM acetate, 0.6 mM butyrate, 1.2 mM propionate and condition 5: 15 mM acetate, 0.75 mM butyrate, 1.5 mM propionate).

Table 3.1 Results of PCR amplification of 16S rRNA genes for North Sea tSRB enrichments NS-tSRB-1 and NS-tSRB-2. All of the PCR primer sets used in this study are indicated and the size of the PCR products obtained are indicated. The primer set not used (-).

Enrichment	DNA Preparation	Primer Pair I341f & U1492r	Primer Pair U515f & U1492r	Primer Pair A341f & A1063 r
NS-tSRB-1	Telang method	1.1 kb	1 kb	-
NS-tSRB-2	Qiagen Dneasy^{Tm} kit	1.1 kb	1 kb	1.1 kb

NS-tSRB-1 was PCR amplified using the primer sets I341f U1492r and U515f U1492r (Table 3.1). Sequencing of the PCR product obtained using the primer set U515f and U1492r revealed that it was 98% identical to *Thermodesulforhabdus norvegicus,* an acetate-oxidizing, gram negative bacterium (Beeder *et al,* 1995) (Figure 3.3). *T. norvegicus* is a member of the delta proteobacteria and was originally isolated from the Norwegian sector of the North Sea. The excellent sequence template obtained from this culture, as well as the homogeneous gram stain indicates that is relatively pure.

3.3.2 Identification and characterization of NS-tSRB-2

NS-tSRB-2 was grown in MS-2 containing lactate as the electron donor. NS-tSRB- 2 grew relatively slowly also requiring one month to reduce 10 mM sulfate. DNA was isolated from this enrichment using the Qiagen Dneasy^{Tm} tissue kit (Mississauga, ON) and the 16S rRNA gene was amplified using primer sets A341f U1492r and U515f U1492r (Table 3.1). The 16S sequence obtained from this enrichment using primers U515f and U1492r was 98 % similar to that of *Archaeoglobus fulgidus,* a hyperthermophilic sulfate-reducing archaeon (Figure 3.4) growing with an optimal temperature of 80 °C although strains with lower optimal temperatures have been isolated from the North Sea (Beeder *et al,* 1994). NS-tSRB-2 had an observed doubling time of approximately 49 hours based on the doubling time of sulfide concentrations during the exponential growth phase (Figure 3.5).

3.4 Discussion

Both tSRB enrichments contained tSRB which had been previously isolated from North Sea oil fields. Based on 16S rDNA sequences NS-tSRB-1 was closely related to *Thermodesulforhabdus norvegicus* and NS-tSRB-2 was closely related

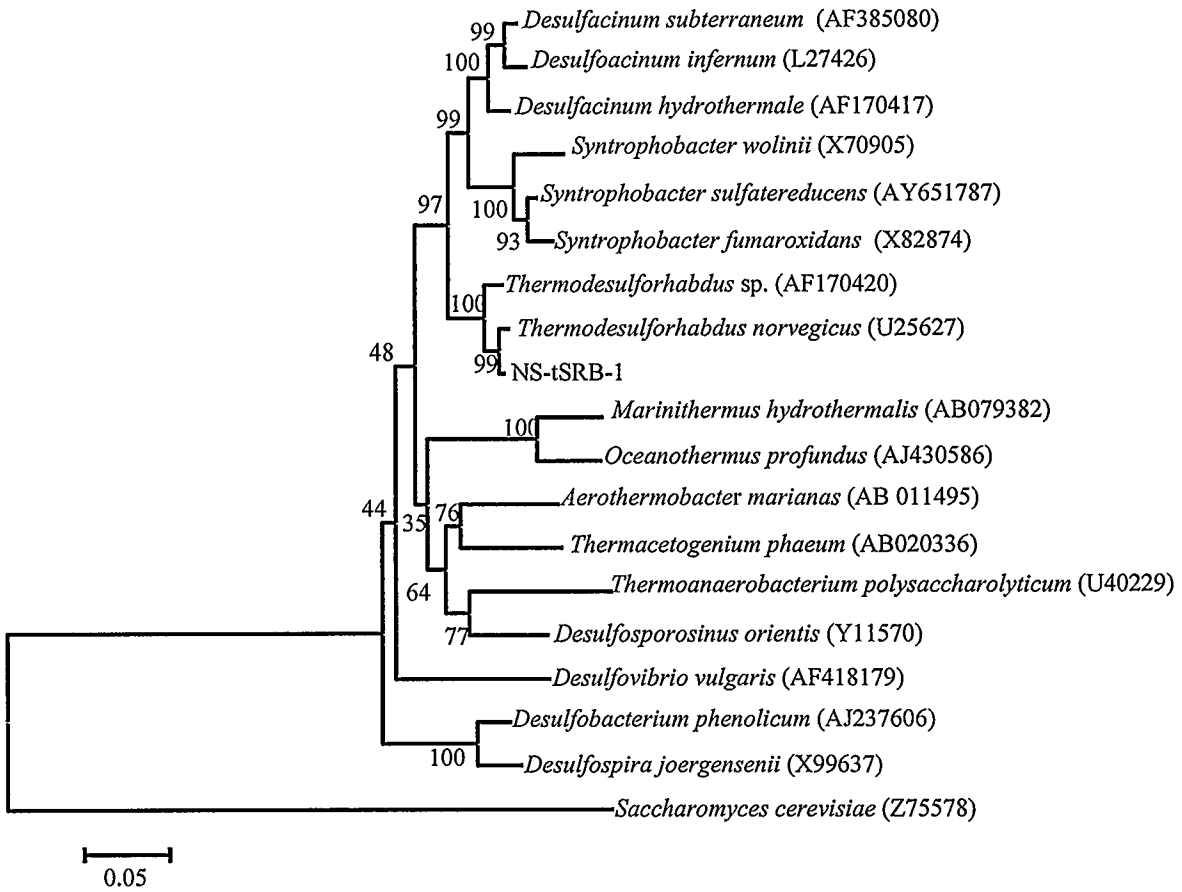

Figure 3.3 Minimum evolutionary phylogenetic tree showing the relation of 16S rDNA sequence of NS-tSRB-1 with its nearest homologs. Horizontal distances reflect pairwise sequence similarities as indicated on the scale. The numbers at the nodes represent grouping frequencies based on 500 bootstrap replicates. Nineteen sequences are included in the tree with *Saccharomyces cerevisiae* as the outgroup. Accession numbers of the sequences retrieved from the database are also indicated in parentheses

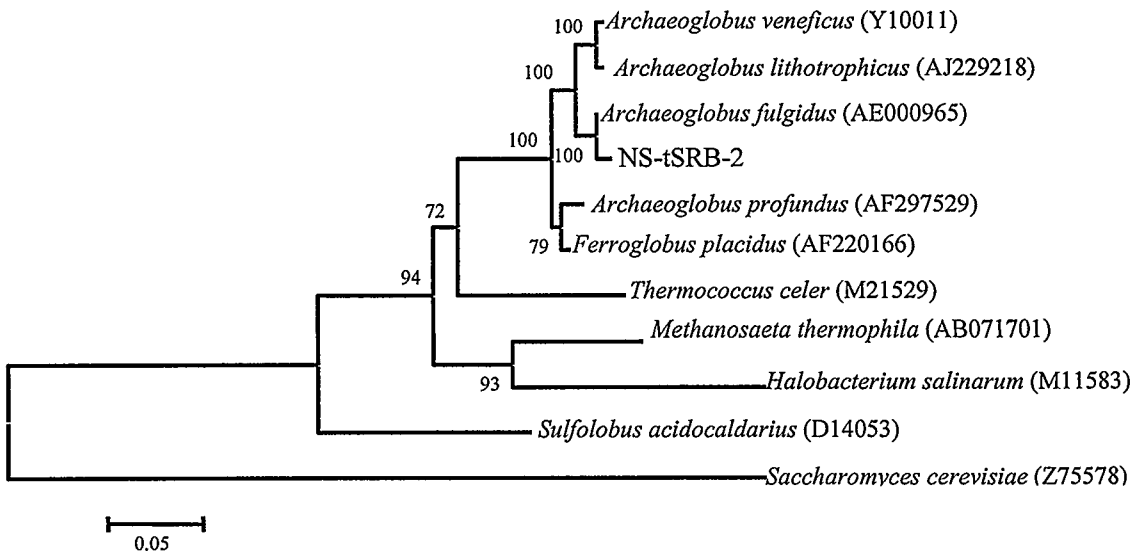

Figure 3.4 Minimum evolutionary phylogenetic tree showing the relation of the 16S rDNA sequence of NS-tSRB-2 with its nearest homologs. Horizontal distances reflect pairwise sequence similarities as indicated on the scale. The numbers at the nodes represent grouping frequencies based on 500 bootstrap replicates. Eleven sequences are included in the tree with *Saccharomyces cerevisiae* as the outgroup. Accession numbers of the sequences retrieved from the database are also indicated in parentheses

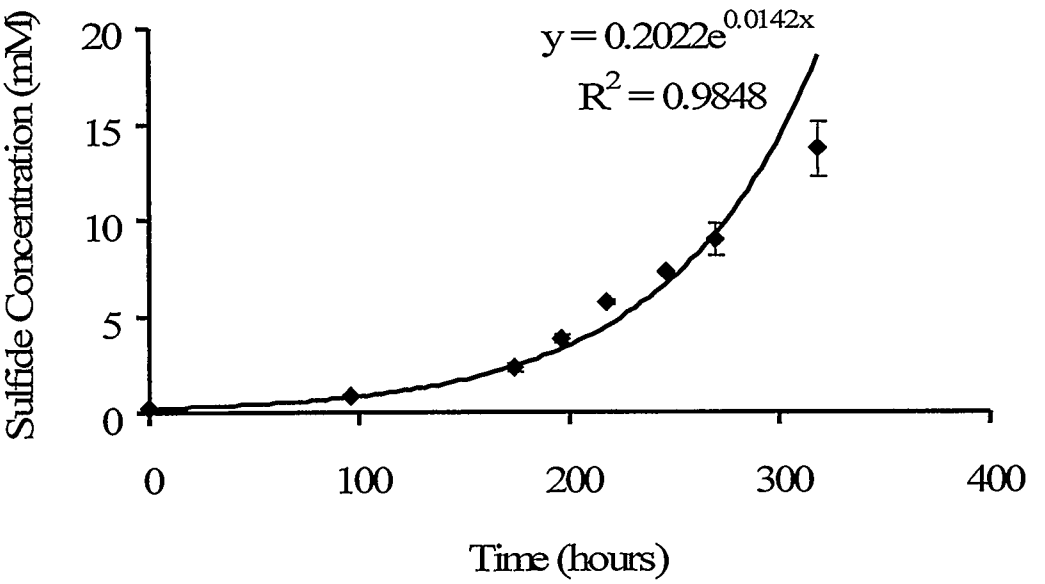

Figure 3.5 NS-tSRB-2 during exponential growth phase in MS-20-L when incubated at 60 °C and using a 5 % innoculum. Each point is the average of two trials with the error bars representing the standard deviation between the two points. A best fit of the data (Sulfide Concentration vs Time) is shown.

to *Archaeoglobus fulgidus*. These results were to be expected as both organisms have been previously isolated from the Norwegian sector of the North Sea where both of the tSRB enrichments discussed here originated as well. No NRB appear to be present in these enrichments based upon their inability to reduce nitrate. This is not surprising as both enrichments had been maintained in tSRB media prior to their being obtained for use in this study; thus an tSRB would have been preferentially selected and over time the other organisms would have been selected out.

NS-tSRB-1 was closely related to *Thermodesulforhabdus* sp., with 98 % identity with 16S rRNA identity to *Thermodesulforhabdus norvegicus,* a thermophilic sulfate-reducer (Figure 3.2). *Thermodesulforhabdus norvegicus* was first isolated from an oil reservoir in the Norwegian sector of the North Sea (Beeder *et al*, 1995). Along with *Desulfotomaculum thermoacetoxidans* (Min and Zender, 1990) and *Desulfacinum infernum* (Rees *et al*, 1995), it is one of the few completely oxidizing SRB (i.e. acetate is not a by-product and substrates are mineralized to CO_2) that have been isolated from oil reservoirs to date. NS-tSRB-1 with a doubling time of approximately 36h grew much slower than the *Thermodesulforhabus norvegicus* strains isolated by Beeder *et al* (1995) which had a doubling time of 12 hours at optimal growth at 60 °C and 16g NaCl per liter. This difference may be due to differences between strains or it may be due to differences in the media used in this study which did not contain a vitamin solution used in the media by Beeder *et al* (1995) which may affect the growth rate of NS-tSRB-1.

Archaeoglobus fulgidus was first isolated by Stetter *et al* (1988) from marine hydrothermal systems in Italy with an optimal growth temperature of 80 °C. Beeder *et al*

(1994) isolated an *Archaeoglobus fulgidus* strain from the Norwegian sector of the North Sea with an optimal growth temperature of 76 °C. Another interesting fact about the North Sea *A. fulgidus* strain was that it could not grow autotrophically whereas the Italian strain could. The North Sea strain had an optimal generation time of 20 h in lactate sulfate media. A much slower doubling time of 48 hours was observed for NS-tSRB-2 and explanation for this much slower generation time could be that the 60 °C incubation temperature is much lower then the observed optimal growth temperatures for *A. fulgidus.*

Hence the organisms present in North Sea tSRB enrichments appeared to be similar to ones previously identified from North Sea oil fields.

Chapter 4 Characterization and identification of thermophilic microorganisms in an oil storage tank

4.1 Introduction

In the previous chapter the identification and characterization of two tSRB enrichments from the North Sea were described. In this chapter a methane and sulfide-producing oil storage tank consortium, possibly capable of degrading oil, will be described. Samples containing this consortium were provided by an oil company. Gas production in the storage tank caused periodic release of odorous gas leading to complaints from residents nearby. Here a variety of techniques such as MPNs, dilution to extinction experiments and classical plate isolation, were used along with 16S rDNA sequencing to gain a better understanding of the organisms present in the oil storage tank consortium. By characterizing the organisms present in the oil storage tank consortium we will gain a better understanding of the organisms present and how they might interact to cause gas production.

4.2 Methods

The Southern blots described in this chapter were performed as described in chapter 2 using the 16S rRNA gene from ST-tSRB-8A and the *dsr* genes from *Desulfovibrio vulgaris* Hildenbrough as a probe. All other methods were as described in chapter 2.

4.3 Results

4.3.1. Enumerating SRB in the oil storage tank consortia

A three-tube MPN assay was used to determine numbers of tSRB in different oil storage tank samples. The results are summarized in Table 4.1. These results show that there

Table 4.1 Most probable numbers of lactate-utilizing SRB (boldface) in ST-1 to ST-4 samples taken from an oil storage tank, as determined by MPN assays in PB medium at 60°C. Upper and lower confidence limits are shown.

Number of SRB/ml at different incubation times					
Sample	Type	1 day	3 days	7 days	14 days
ST-1	Oil	3<**11**<36	4<**21**<47	4<**21**<47	4<**21**<47
ST-2	Oil	4<**21**<47	40<**210**<470	150<**930**<3,800	150<**930**<3,800
ST-3	oil/water	1<7<23	360<**2,400**<13,000	360<**2,400**<13,000	360<**2,400**<13,000
ST-4	oil/water	1<**9**<36	36<**240**<1,300	71<**460**<2,400	71<**460**<2,400

were significant levels of tSRB in the oil storage tank. The highest number of tSRB (2400 $cells^{-1}ml$), was detected in the ST-3 sample and the lowest level of tSRB was observed in sample ST-1 (Table 4.1), Overall, higher levels of SRB were associated with the oil/water samples than the oil samples. tNR-SOB activity as indicated by nitrite production and an increase in redox potential indicated by the resazurin turning pink in CSB (4.75 mM sulfate, 5 mM acetate, 2.5 mM sulfide) was also observed initially in ST-4 and ST-3 producing 6.39 mM and 4.15 mM nitrite respectively but these cultures did not grow when transferred to fresh CSB. No sulfide oxidation was observed in CSB using ST-1 and ST-2 as the inoculum.

A set of medium B tubes were inoculated with the oil storage tank samples and incubated at room temperature but no growth was observed, indicating all microbial activity to be obligate thermophiles.

4.3.2 Isolation and identification of ST-tSRB-8A

Serial dilutions of ST-1/4 in deionized water were plated on PE plates. Eight isolate colonies were then picked, grown in 1ml cultures of sPGC and then transferred to increasing amounts of sPGC until 100 ml cultures were achieved. DNA was then extracted from these cultures using the method described by Telang *et al* (1997). Southern blot analysis of these 8 isolates (1-7 and ST-tSRB8A) using a probe made from the PCR amplified 16SrRNA gene of enrichment ST-tSRB-8A as a gave a main hybridizing band of 2.5 kb. This revealed that all of the isolates were likely similar (Figure 4.1) to isolate ST-tSRB-8A therefore only isolate ST-tSRB-8A was studied further.

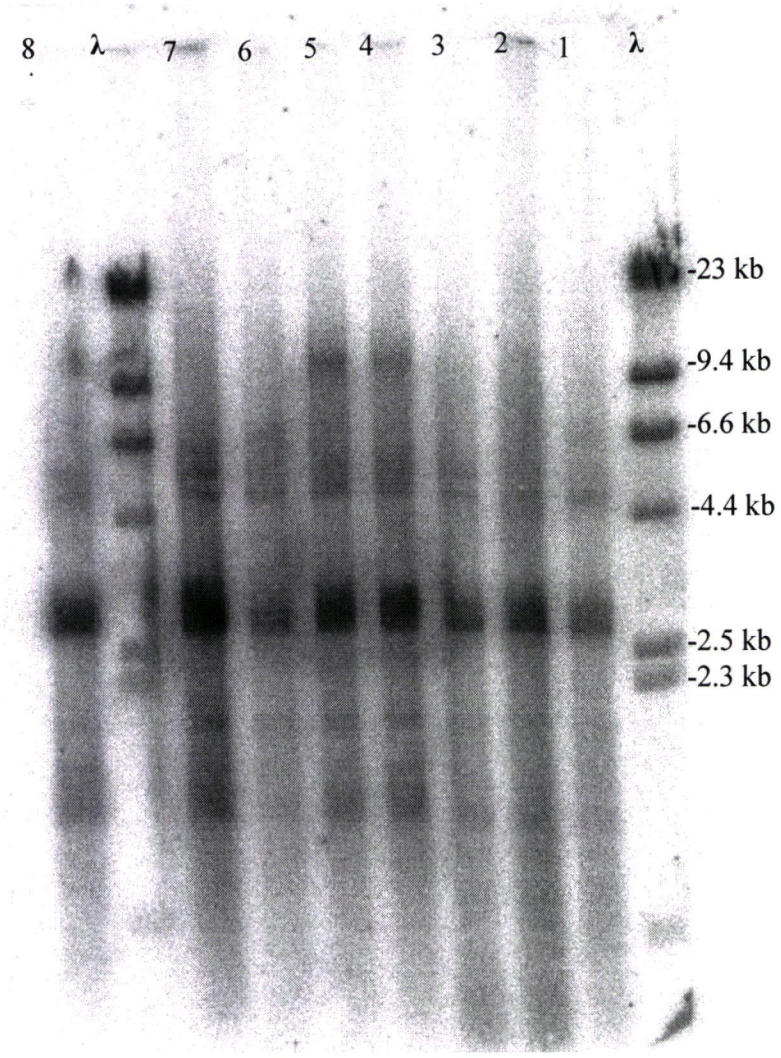

Figure 4.1 Hybridization of Southern blots of *Eco*RI-digested chromosomal DNAs from oil storage tank SRB isolates with a probe for the 16S rRNA gene amplified from ST-tSRB-8A. Lanes 1-8 are ST-tSRB isolates 1, 2, 3, 4, 5, 6, 7, and 8A respectively. Lanes λ: λ DNA digested with *Hind* III; fragment sizes from top to bottom: 23.1, 9.4, 6.6, 4.4, 2.3, 2.0, and 0.56 kilo-basepairs. λ was visualized by including λ DNA in the probe.

4.3.3 Properties of ST-tSRB-8A

Although, ST-tSRB-8A was isolated from a medium E plate containing lactate and sulfate, it also exhibited methanogenic activity indicating that it was not a pure culture (data not shown). ST-tSRB-8A was found to be able to reduce nitrate, sulfate and thiosulfate with lactate as the electron donor. ST-tSRB-8A used lactate as its electron donor with acetate as its end product, reducing sulfate to sulfide (Figure 4.2) and reduced thiosulfate to sulfide (Figure 4.3). When grown in CSBA that contained 10 mM sulfate and 38 mM lactate, 9.4 mM sulfide and 15.6 mM acetate were produced (Figure 4.2). When grown in CSBA that containing lactate and thiosulfate ST-tSRB-8A produced 3.8 mM sulfide and 7.9 mM acetate (Figure 4.3). ST-tSRB-8A was also able to reduce nitrate to ammonia. In CSBA media containing 10 mM nitrate it produced 2.8 mM ammonia after 3 days and 8.6 mM ammonia after 4 days.

Although ST-tSRB-8A is able to reduce sulfate, homology between the *dsr* genes of ST-tSRB-8A and the other oil storage tank isolates was not adequate for hybridization when probed with the *dsrA* and *dsrB* genes from *Desulfovibio vulgaris* strain Hildenbrough (Figure 4.4).

4.3.4 16S rRNA amplification and sequencing of ST-tSRB-8A

DNA was extracted from ST-tSRB-8A using the method described by Telang *et al* (1997). The 16S rRNA was then amplified using the PCR primer sets I341f/U1492r and U515f/U1492r (Table 4.2). This gave a sequence with 99% identity with *Garciellia nitratireducens* when using primers U515f and U1492r (Figure 4.5; Miranda-Tello *et al*,

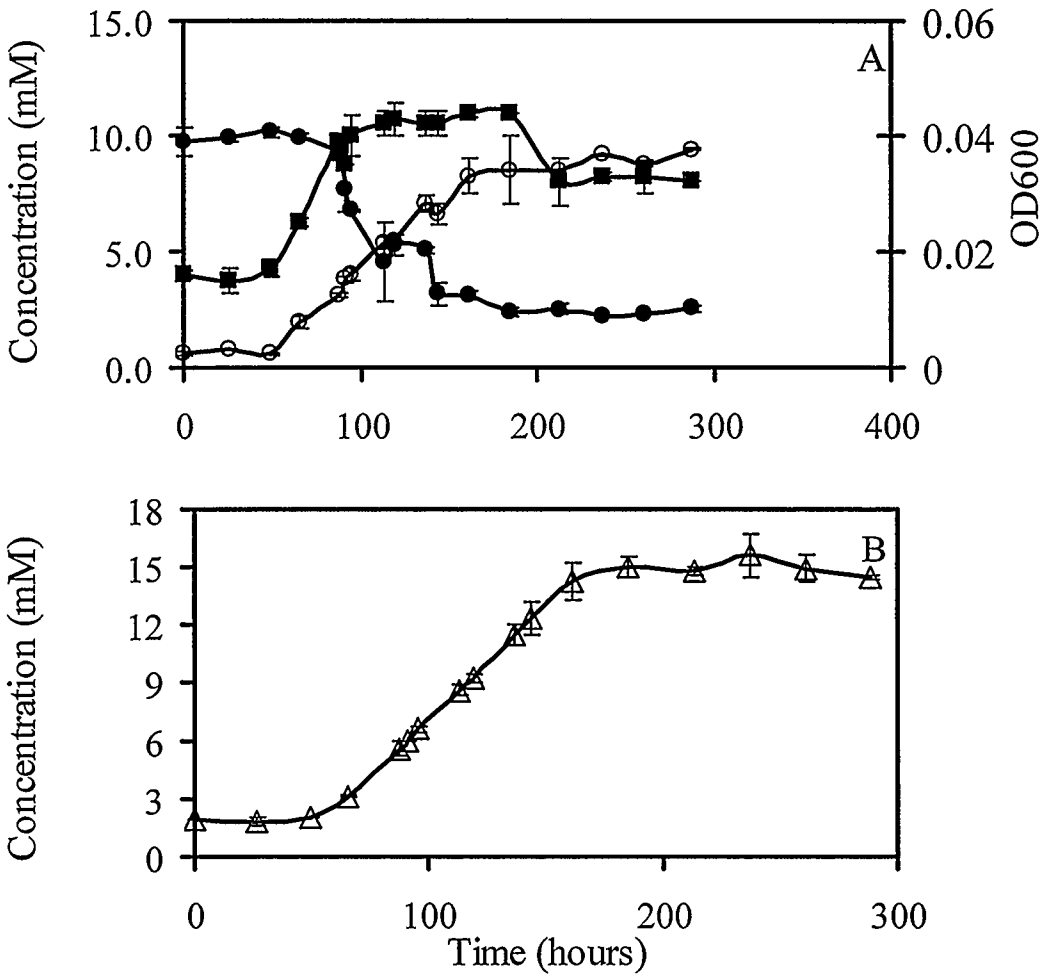

Figure 4.2 Growth of isolate ST-tSRB-8A in CSBA containing 20 mM lactate and 10 mM sulfate. (A) The concentration of sulfate (•, mM), optical density at 600 nm (■) and sulfide (○, mM) are shown as a function of time. (B) The concentration acetate (Δ, mM). Each point is the average of two trials. The error bars represent the standard deviations between two duplicate trials.

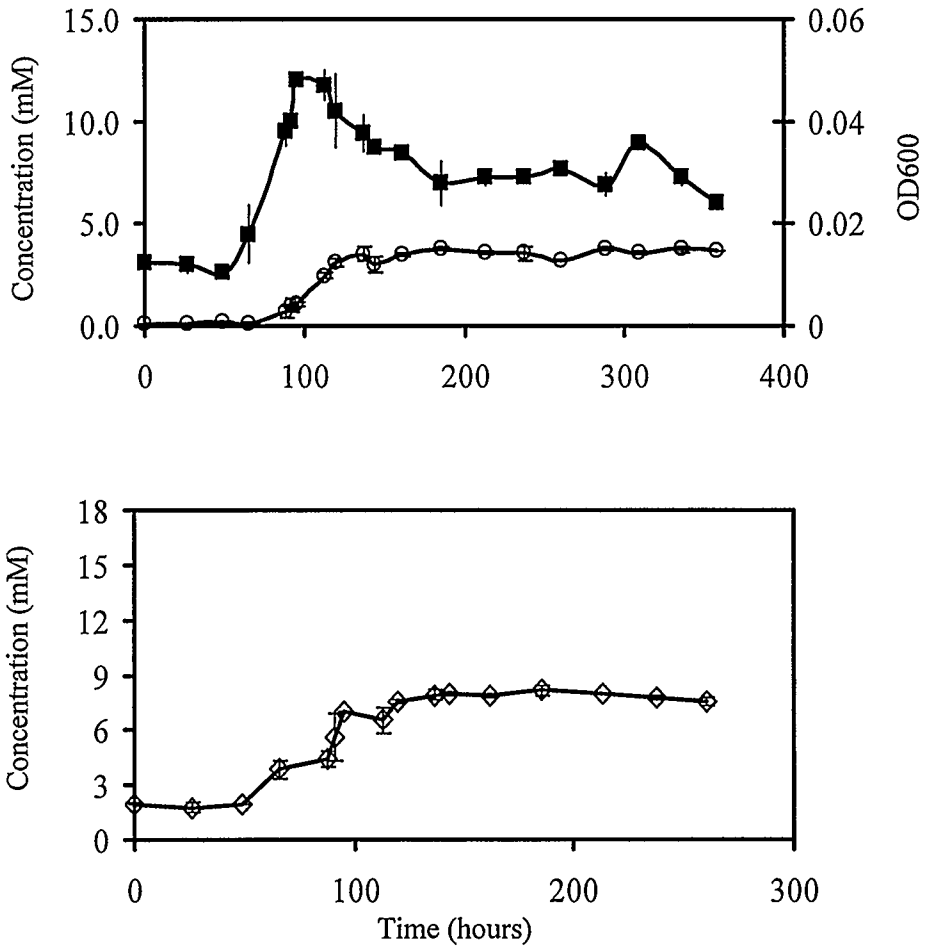

Figure 4.3 Growth of isolate ST-tSRB-8A in CSBA containing 20 mM lactate and 10 mM thiosulfate. (A) The optical density at 600 nm (■) and sulfide (○, mM) are shown as a function of time. (B) The concentration acetate (◊, mM). Each point is the average of two trials. The error bars represent the standard deviations between the two duplicate trials.

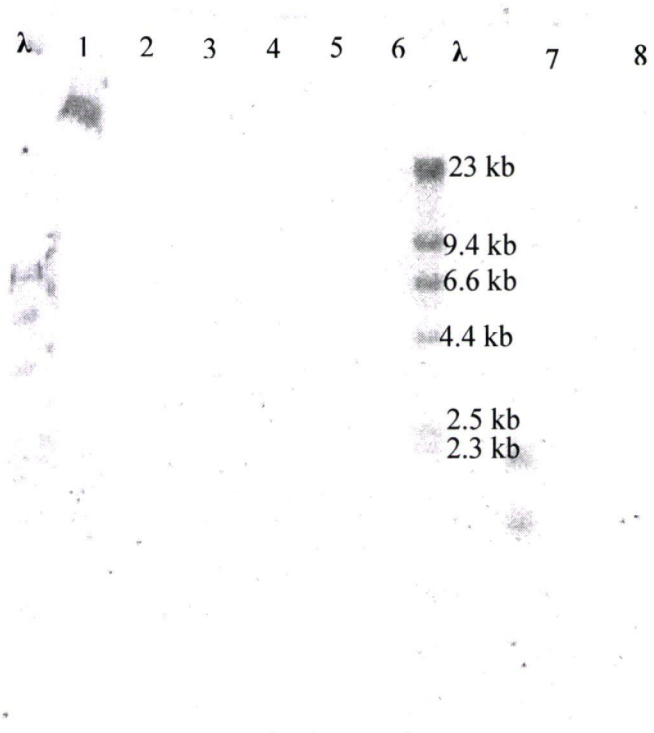

Figure 4.4 Hybridization of Southern blots of *Eco* RI digested chromosomal DNAs from oil storage tank SRB isolates with a probe for the dissimilatory sulfite reductase gene from *Desulfovibrio vulgaris* Hildenborough. Lanes 1-6 are isolates ST- tSRB-1, 2, 3, 4, 5 and 6, lane 8 is ST-tSRB-8A and lane 7 is *Desulfovibrio vulgaris* Hildenborough used as the positive control. Lanes λ: λ DNA digested with *Hind* III; fragment sizes from top to bottom: 23.1, 9.4, 6.6, 4.4, 2.3, 2.0, and 0.56 kilobase pairs.

Table 4.2 PCR of 16S rRNA gene of the oil storage tank cultures. All of the PCR pimers sets used in this study are indicated along with the method of DNA isolation and the size of the PCR products obtained. Primer sets that were not used (-) are also indicated. X indicates primer pairs where no PCR product was detected.

Enrichment	DNA preparation	Primer pair I341f & U1492	Primer pair U515f & U1492r	Primer Pair A25f & A958r	Primer Pair A25f & A1063r	Primer Pair A25f & A1392f	Primer Pair A341f & A1063r
ST-tSRB-8A	Telang method	1.1 kb	1 kb	-	-	-	-
ST-tSRB-8A-2	Ravenschlag Method	1.1 kb	1 kb	X	X	X	X
ST-FER-2	Qiagen Dneasy™ kit	1.1 kb	1 kb	-	-	-	-

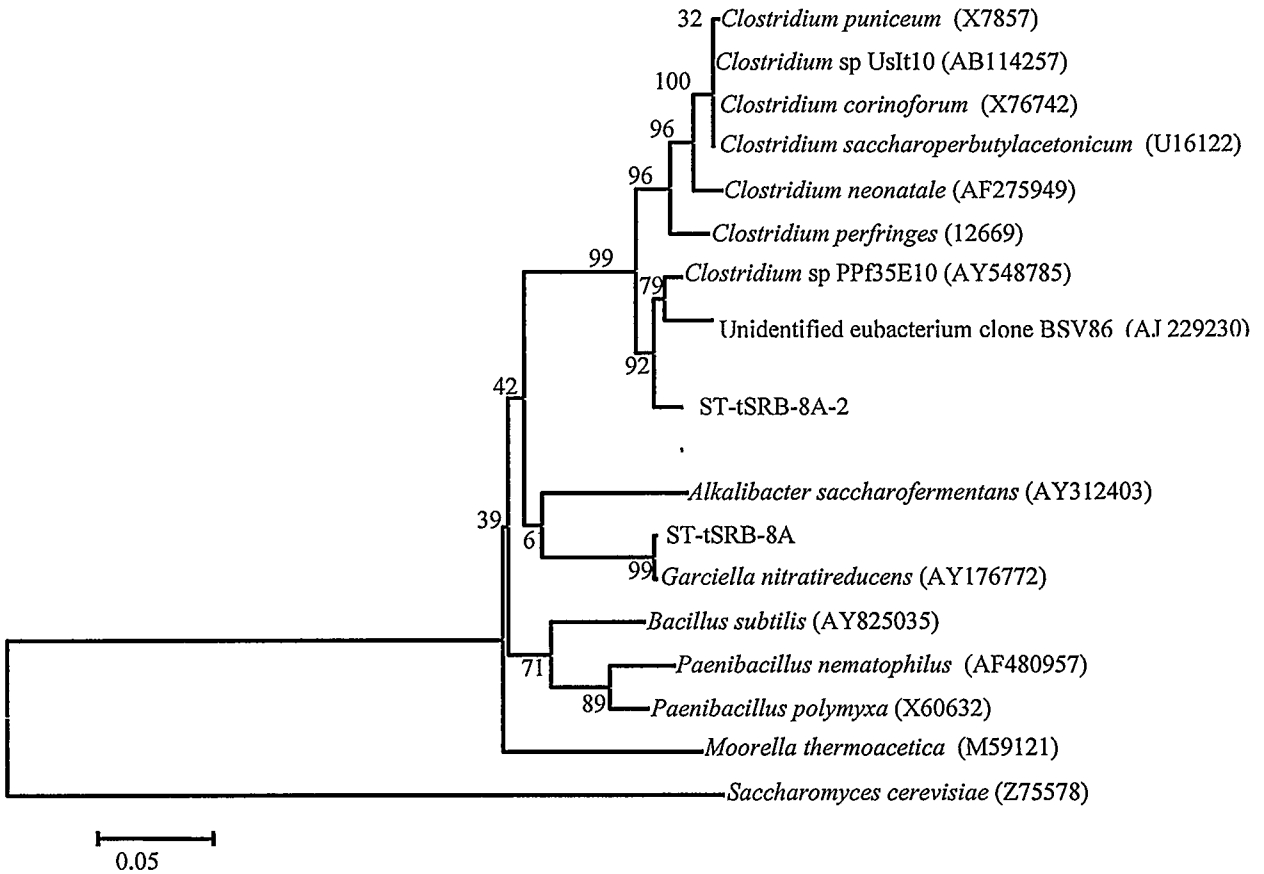

Figure 4.5 Minimum evolutionary phylogenetic tree showing 16S rDNA sequences of PCR products derived from oil storage tank enrichments ST-tSRB-8A and ST-tSRB-8A-2. Horizontal distances reflect pairwise sequence similarities as indicated on the scale. The numbers at the nodes represent grouping frequencies based on 500 bootstrap replicates. Seventeen sequences are included in the tree with *Saccharomyces cerevisiae* as the outgroup. Accession numbers of the sequences retrieved from the database are also indicated in parentheses.

(2003). *Garciellia nitratireducens* a gram positive nitrate reducer isolated from an oil field in the Gulf of Mexico (Miranda-Tello *et al*, 2003).

4.3.5 ST-tSRB-8A-2

Since ST-tSRB-8A was determined not to be a pure culture a second attempt at obtaining a pure tSRB isolate was made starting with ST-tSTB-8A cultures. Dilutions of ST-tSRB-8A were made and then spread onto medium E plates. An isolate picked and grown up in sPGC was designated ST-tSRB-8A-2. ST-tSRB-2 was able to reduce sulfate to sulfide but never produced more that 5 mM sulfide even though there was 10 mM sulfate and 30 mM lactate in the CSB medium. A variety of PCR primer sets were used in an attempt to amplify the 16S rRNA DNA sequence. However, a PCR product was only obtained with the primer sets U515f/U1492r and I341f/U1492r (Table 4.2). Based on sequences of PCR amplified 16S rRNA genes using the primers U515f and U1492r, isolate ST-tSRB-8A-2's closest matches were *Clostridium* sp. strain PPf35E10 (98% similar) and *Clostridium saccharoperbutylacetonicum* (94% similar; Figure 4.5) based on BLAST results. Even though ST-tSRB-8A was not a pure culture a very good sequence was obtained without cloning as ST-tSRB-8A-2 which was isolated from this culture was unable to be lysed using the DNA extraction method of Telang *et al.* (1997) so the method of Ravenschlag *et al* (1999).

4.3.6 Dilution to extinction experiment

Enrichment ST-1/4-E (Table 4.3) produced methane and reduced sulfate to sulfide and was maintained for one year in sPGC transferring monthly. ST-1/4-E proved to be an unstable enrichment. In February 2004 it produced a maximum sulfide concentration of between 1.2-2.6 mM whereas a year earlier these cultures produced 11.8

Table 4.3 Dilution to extinction of ST1/4-E following one year of monthly transfers in sPGC. Tubes were scored positive for growth based on visual turbity and the presence of methane. Tubes were determined to be positive for methane if the headspace contained greater than 1% methane as determined using a GC. SRB growth was determined by the production of sulfide (indicated using + or -) as indicated by a sulfide concentration greater than 1 mM (all tubes were reduced originally by adding approximately 0.7 mM sulfide).

Dilution	# of tubes positive for growth (out of 4)	Average sulfide concentration (mM)	# of tubes positive for methane (out of 4)
10^{-1}	4	2.54 (+)	4
10^{-2}	4	3.36 (+)	4
10^{-3}	4	3.25 (+)	4
10^{-4}	4	1.85 (+)	4
10^{-5}	4	1.64 (+)	4
10^{-6}	4	0.99 (+)	4
10^{-7}	4	0.78 (-)	4
10^{-8}	3	0.69 (-)	4

Table 4.4 Dilution to extinction of the 10^{-8}dilution of Table 4.2 Tubes were scored positive for growth based on visual turbity, the presence of sulfide (+ or -) and the presence of methane. Tubes were determined to be positive for methane if the headspace contained greater than 1% methane as determined using a GC.

Dilution	# of tubes positive for growth (out of 4)	Sulfide concentration of one set of tubes (mM)	# of tubes positive for methane (out of 4)
10^{-1}	4	0.79 (-)	4
10^{-2}	4	0.54 (-)	4
10^{-3}	4	0.54 (-)	4
10^{-4}	4	0.54 (-)	4
10^{-5}	4	0.79 (-)	4
10^{-6}	4	0.46 (-)	4
10^{-7}	4	0.54 (-)	3
10^{-8}	4	0.33 (-)	2
10^{-9}	3	0.31 (-)	1
10^{-10}	0	0.28 (-)	0
10^{-11}	0	0.21 (-)	0

mM sulfide. SRB activity was observed until the 10^{-6}dilution (Table 4.3). As growth was observed in the final dilution (10^{-8}) of the first series a second experiment was carried out as indicated in Table 4.4. Methanogenesis was observed until the 10^{-8}dilution in the second experiment (up to 1 % of headspace methane was detectable on the GC) growth was observed in three of the four 10^{-9} dilution tubes (Table 4.4). A 5 ml volume from one of 10^{-9}dilution tubes which was positive for growth but did not produce methane was transferred to 100 ml sPGC. Growth was observed without sulfide or methane formation and the resulting culture was designated ST- FER-2.

4.3.7 ST-FER-2

Even though ST-FER-2 was obtained from an SRB enrichment it was unable to reduce either nitrate or sulfate; therefore presumably it grows by fermenting lactate. PCR amplification of ST-FER-2 with primers I341f and U1492r and U515f and U1492r gave product. Sequencing of the PCR product from the primers U515f and U1492r for heterotrophic, fermentative strain ST-FER-2 gave a sequence which had 98% identity to *Acinetobacter radioresistens* of the gamma proteobacteria (Figure 4.6). ST-FER-2 could not be sequenced from the reverse direction as it had a long section of G and C bases which gave that region too much secondary structure for sequencing despite many trials. *Acinetobacter radioresistens* is an aerobic hydrocarbon degrader most well known for its resistance to desiccation and de-emulsification abilities (Snelling *et al,* 1998; Nadarajah *et al*, 2002).

4.4 Discussion

A variety of different microorganisms were found to be present in the oil storage tank consortium while less variety was seen in the North Sea enrichments as shown in

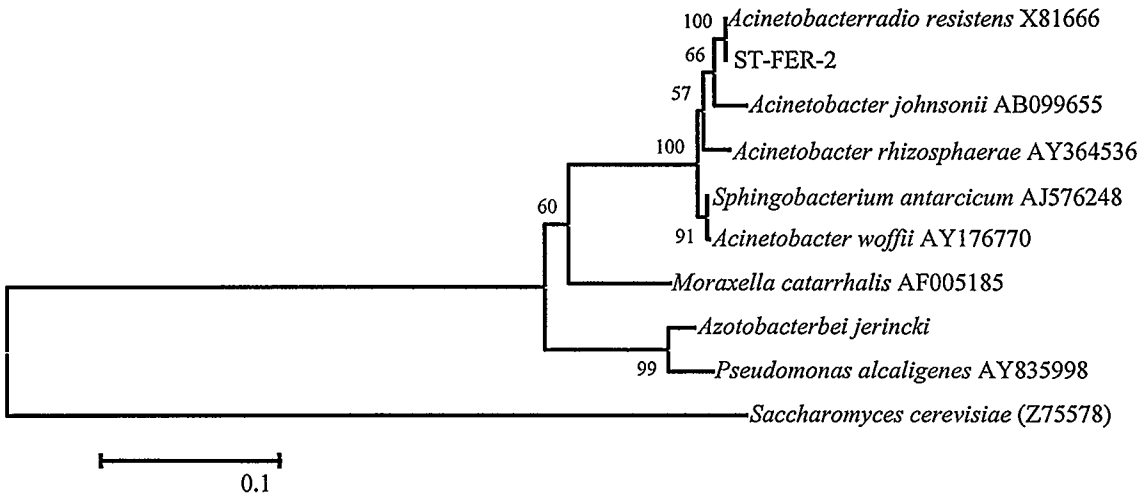

Figure 4.6 Minimum evolutionary phylogenetic tree showing 16S rDNA sequence of oil storage tank culture ST-FER-2. Horizontal distances reflect pairwise sequence similarities as indicated on the scale. The numbers at the nodes represent grouping frequencies based on 500 bootstrap replicates. Ten sequences are included in the tree with *Saccharomyces cerevisae* as the outgroup. Accession numbers of the sequences retrieved from the database are also indicated in parentheses.

Chapter 3. The types of organisms found were tSRB, tNRB, thermophilic methanogens and a fermenter as would be expected in oil field environments. It proved very difficult to obtain pure cultures from this environment and so far none have been obtained. The highest concentration of SRB in the oil storage tank were found in the water oil interface zone of ST-3 which had ten times more microbial activity as compared to ST-2 which came from an oil zone in the storage tank. Lower levels of microbial activity were found in the oil sample ST-1 and the oil/water sample ST-4 as compared to ST-2 and ST-4, still overall the oil water samples had more microbial activity. A possible explanation as to why less SRB activity would be seen is that there is less of some required nutrient in ST-1 and ST-4 compared to their counter parts ST-2 and ST-3 (Table 3.1). Higher levels of microbial activity are seen in the oil/water samples as compared to the oil samples. The actual number of lactate-utilizing tSRB present in oil samples may be higher as they had to be extracted in media before inoculation and it is highly probable that not all of the tSRB present in the oil phase were extracted into the medium. This result is what one would expect as water is required for biological activity. The higher level of microbial activity in zones of oil-water contact can also be seen in biodegraded oilfields where biodegradation gradients indicated the most biological activity to be near oil-water contacts (Larter e*t al*, 2003).

From the oil storage tank a variety of microorganisms were identified by 16S rRNA sequencing which included *Garciella nirtatireducens,* a gram positive tNRB, and a *Clostridium* sp. The *Clostridium* sp. at 92% and 94% similarity are almost sufficiently divergent at the 16S level to be considered a separate genus. Similarly, a 2% difference in sequence similarity at the 16S sequence level is grounds enough for a bacterium to be

considered a separate species (Stahl, 1997). Based on this and its ability to reduce sulfate which is not seen in other *Clostridium* spp. it is possible that the sequence obtained from ST-tSRB-8A-2 could represent a novel genus. These phylogenetic identifications were surprising as *Garciella nitratireducens* is a nitrate-reducer. While *Clostridium* spp. have been identified that can reduce nitrate, nitrite and thiosulfate (Cato *et al,* 1986) none have been isolated that are capable of reducing sulfate and nitrate. Only Leu *et al* (1998) identified a *Clostridium* sp. using cloned DNA from SRB enrichments which they hypothesized may have been capable of reducing sulfate.

Despite the fact it wasn't a pure culture a good 16S rRNA sequence was obtained from ST-tSRB-8A. An explanation for this is that the other members of the mixed culture (a methanogen and *Clostridium* sp.) could not be lysed using the Telang *et al* method (1997), which led to obtaining a pure sequence of *Garciella* DNA. *Garciella nitratireducens* is a member of the order Clostridiales (cluster XII), a thermophilic thiosulfate- and nitrate-reducing bacterium recently isolated from an oil well in the Gulf of Mexico (Miranda-Tello *et al,* 2003). ST-tSRB-8A was able to reduce sulfate and thiosulfate to sulfide and oxidize lactate to acetate. It produced 2.1 mM of acetate for every 1 mM sulfide produced from thiosulfate and produced 1.7 mM acetate for every mM of sulfide produced from sulfate, both of which are close to the theoretical values of 2 acetate being produced for every thiosulfate or sulfate reduced. It grows optimally at 55 °C (Miranda-Tello *et al,* 2003). *G. nitratireducens* is able to reduce nitrate to ammonia and thiosulfate to sulfide, and ferments glucose to butyrate, lactate and acetate.

To date only four species belonging to the *Clostridiales* have been isolated from oil fields. These include two mesophiles, *Dethiosulfovibrio peptidovorans* (Magot *et al,*

1997) and *Fusibacter paucivorans* (Ravot *et al*, 1999) and two thermophiles *Anaerobaculum thermoterrenum* (Rees *et al*, 1997) and *Garciella nitratireducens* (Miranda-Tello *et al*, 2003) all of which are phylogenetically different from ST-tSRB-8A-2. ST-tSRB-8A-2 is able to reduce sulfate to sulfide, but is inhibited by sulfide concentrations above 5 mM. This indicates that there are likely tSRB present in the consortium which are not present in ST-tSRB-8A-2 which are not inhibited by higher sulfide concentration as 10 mM sulfide gets produced in the consortium cultures. After being maintained for a year the levels of sulfate reduction in ST-1/4-E decreased. Originally these cultures were able to reduce 10 mM sulfate completely, whereas after maintenance in sPGC for one year they were only able to produce a maximum of 3 mM sulfide. A 4-tube dilution to extinction experiment after this culture had been maintained for a year indicated tSRB were were present at 10^5 cells per ml, methanogens were present at 10^8 cells per ml and presumably fermentative bacteria were 10^9cells per ml (Tables 3.2 and 3.3). Thus it can be concluded that the fermentative bacteria were present in higher numbers then the SRB in the lactate- based media but possibly not in the oil storage tank because initially there was a much higher level of sulfate reduction. When the fermentative bacterium from the dilution to extinction experiment was identified through 16S rRNA sequencing it was identified as *Acinetobacter radioresistens*, which is somewhat unexpected as *Acinetobacter* spp. are aerobes. It was also surprising to find a thermophilic fermenter in an SRB enrichment culture that displayed higher cell numbers than the SRB. A likely explanation for this is that this organism is capable of fermenting the lactate or yeast extract present in the medium. A possible explanation why a fermenter would be present in higher numbers then the SRB

in the SRB enrichment culture is that they were able to outcompete the SRB for the available lactate in the sPGC medium. *A. radioresistens* have also been shown to be affiliated with hydrocarbon-degrading consortia and with de-emulsification of oils (Nadarajah *et al*, 2002). It is also possible that *Acinetobacter* is not the dominant species in this culture but is preferentially amplified either due to biases created through lysing (these microbes were lysed using a kit) or through PCR biases. In that case ST-FER-2 would still not be a pure culture, despite having been taken through several dilution to extinction experiments.

We were able to identify a novel tSRB in the oil storage tank. We were able to show that there are organisms closely related to *Clostridium sp.* which are able to reduce sulfate as was earlier hypothesized by Leu et al (1998). Other organisms identified in this consortium have also been identified as being present in petroleum environments. Although there are many microorganisms in the storage tank consortium that have not yet been isolated or characterized the present work provides a starting point.

Chapter 5 Effects of nitrate addition on sulfate reduction

5.1 Introduction

Water flooding for enhanced oil recovery often causes reservoir souring due to the production of H_2S by SRB. "Souring" is responsible for a variety of environmental and economic problems. Broad-spectrum biocides are used to control souring but often have limited success. An alternative to biocide application is nitrate addition, which can inhibit sulfate reduction through multiple mechanisms. Nitrate addition stimulates the growth of hNRB or NR-SOB. This creates a competition between the hNRB and the SRB for available electron donors, with nitrate used preferentially as nitrate reduction is a more thermodynamically favorable reaction (Zehnder and Strumm, 1988). As well, one of the products of nitrate reduction is nitrite, an inhibitor of Dsr.

Here two tSRB enrichments and an oil storage tank consortium were used to determine the effects of nitrate addition during mid log phase on the rate of sulfate reduction.

5.2. Methods

All methods used in this chapter are as described in chapter 2.

5.3 Results

5.3.1. The effect of nitrate addition on sulfate reduction in two North Sea tSRB enrichments

10 mM nitrate added to a mid log phase culture of NS-tSRB-1 had no effect on the rate of sulfate reduction when compared to cultures where no nitrate was added (Figure 5.1). When nitrate was added to tSRB enrichment NS-tSRB-2 during mid

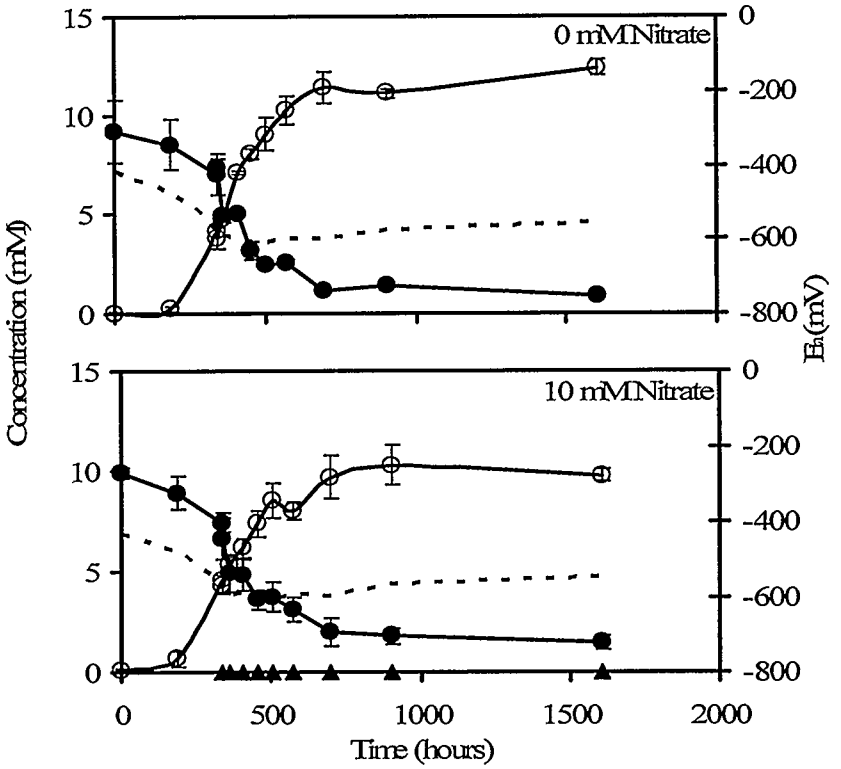

Figure 5.1 Effect of nitrate addition (↓) on sulfide production by NS- tSRB-1 in a 100 ml culture of MS2-20-OA using a 10 ml inoculum in 100 ml of medium. The concentrations of sulfate (•), sulfide (○) and nitrite (▲) were measured as a function of time. Each point represents the average of three trials with the error bars representing the standard deviation between the trials.

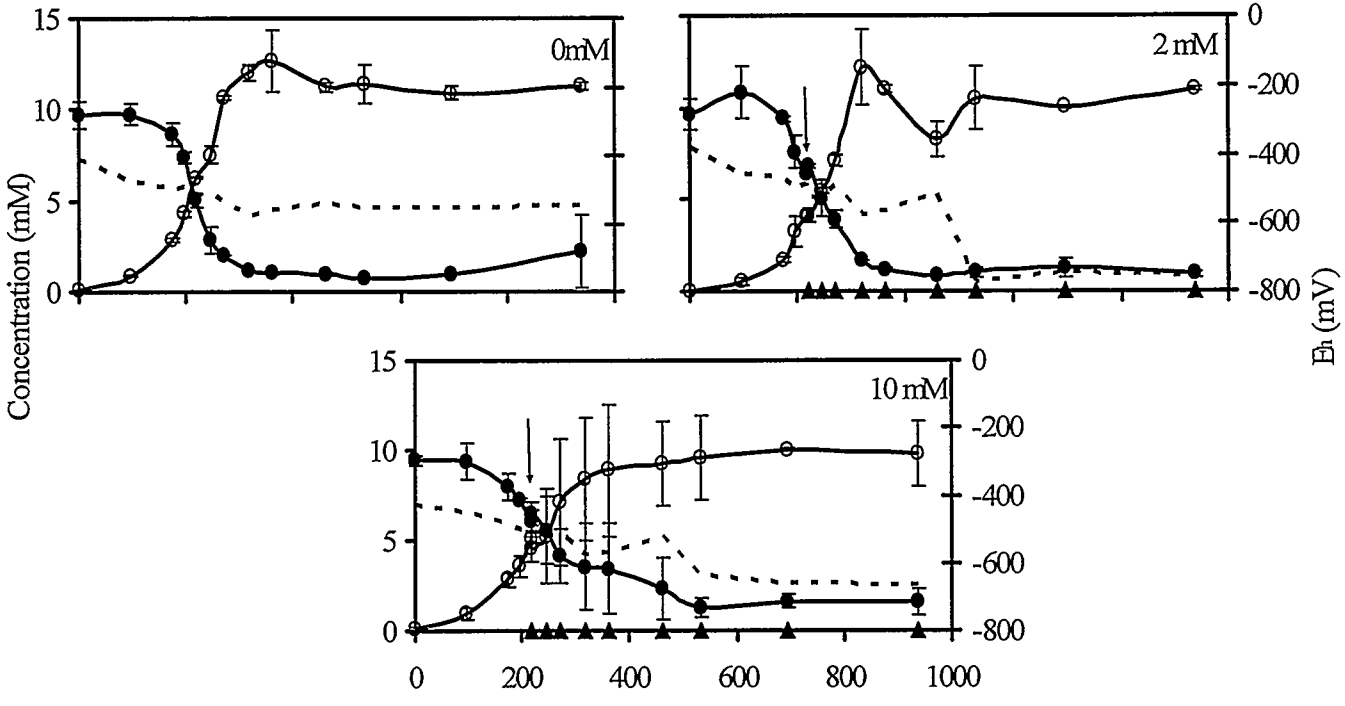

Figure 5.2 Effect of nitrate addition (↓) to a mid log phase culture on sulfide production by NS- tSRB-2. A 10 ml inoculum of NS-tSRB-2 to 100 ml of MS2-20-L was used. The concentrations of sulfate (•), sulfide (○) and nitrite (▲) were measured as a function of time. Each point is the average of three trials with the error bars representing the standard deviation between the trials.

logarithmic phase at concentrations of 2 or 10 mM, no effect was seen on the rate of sulfide production as compared to control cultures where no nitrate was added to the enrichment (Figure 5.2.). In addition no nitrite was detected in either of the NS-tSRB-1 or NS-tSRB-2 cultures after nitrate addition.

5.3.2. The effect of nitrate addition on the oil storage tank consortium

When 7.8 mM nitrate was added to mCSB at the time of inoculation, with a 5 % inoculum of ST-tSRB-8A-2, 4 mM sulfide was produced 2 days later, as was also the case in cultures that did not receive nitrate addition(data not shown). Nitrate addition (5-15 mM) to mid-log cultures of ST-3 in mCSB had no effect on the rate of sulfate reduction as compared to cultures without nitrate added (Figure 5.3). However, following addition of 10 mM and 15 mM nitrate to ST-3, the cultures turned yellow and a white precipitate was observed indicating some sulfur production. This must have been a minor fraction as there was no effect on sulfide levels.

5.4 Discussion

tSRB play an important role in the production of H_2S in high temperature oil reservoirs. Due to the problems associated with the presence of tSRB, oil companies look for strategies to mitigate the problems caused by SRB. One method used to help mitigate the problem of reservoir souring is nitrate addition. Nitrate addition has been shown to control biological sulfide production (Myhr *et* al, 2002; Telang *et al*,1997 Hitzman and Sperl, 1994; Jenneman *et al*, 1986). The addition of nitrate to oil reservoirs inhibits SRB through three main mechanisms: (i) Nitrate addition stimulates the activity of hNRB which may outcompete SRB for the available oil organics used as electron

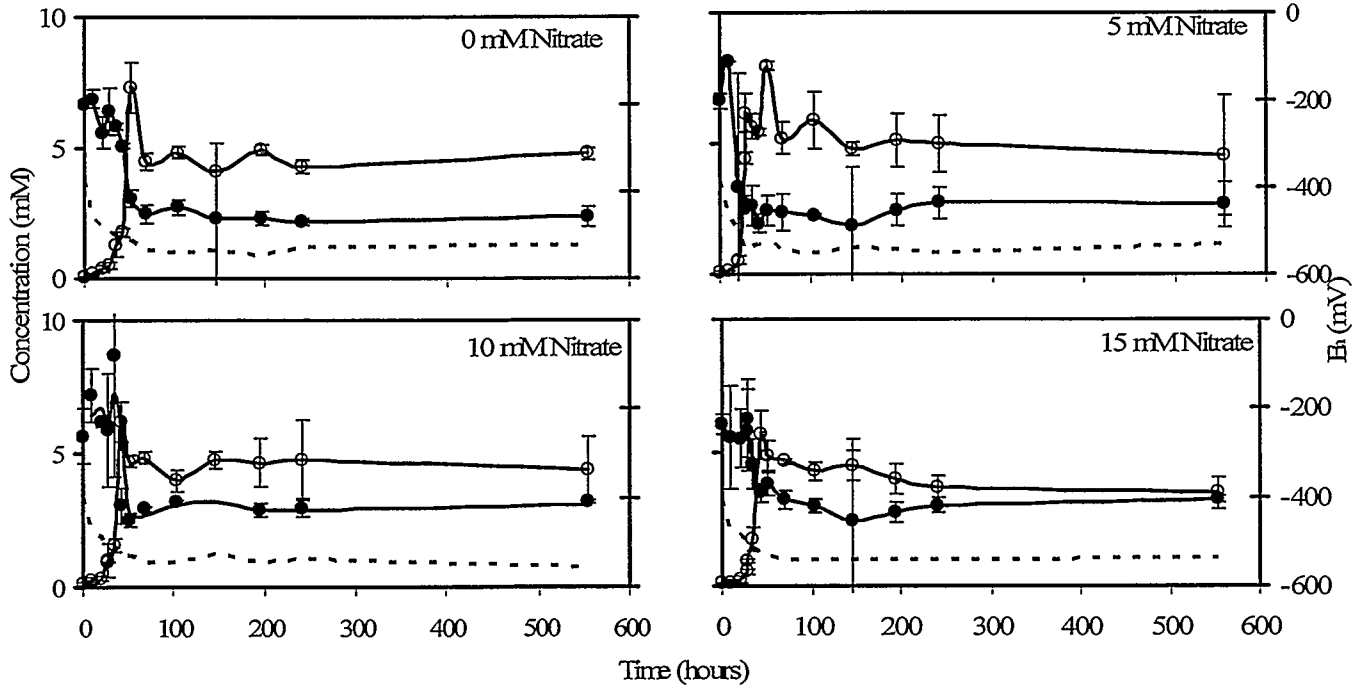

Figure 5.3 Effect of nitrate addition on sulfide production oil tank consortium ST-3 in modified Coleville synthetic brine (mCSB) with lactate as the electron donor. The concentrations of sulfate (•) and sulfide (○) were measured as a function of time. Each point is the average of three trials with the error bars representing the standard deviation between the trials.

donors by both groups as nitrate reduction is the more thermodynamically favorable reaction (Sandbeck and Hitzman,1995) (ii) Nitrate addition stimulates NR-SOB which reduce nitrate and oxidize sulfide controlling souring (Telang *et al*, 1997) (iii) Both NRB and NR-SOB have the potential of producing nitrite during nitrate reduction (Greene *et al*, 1997), which inhibits Dsr by binding to the active site of the enzyme preventing sulfite from being reduced to sulfide (Wolfe *et al*, 1993).

The main result of this study was that nitrate addition had no effect on sulfate-reduction in the cultures. While nitrate addition has proven to be effective at controlling souring in the Veslefrikk oil platform (Thorstenson *et al*, 2002) and in the Colleville oil field in Saskatchewan (Telang *et al*, 1997) nitrate addition may not always be an effective method for controlling souring. When SRB lack nitrate reduction activity the effectiveness of nitrate addition is largely dependent on whether on not NRB or NR-SOB are present in the system.

Nitrate addition was not effective at controlling souring in either of the North Sea tSRB enrichments or in the oil storage tank consortium. Even high levels of nitrate were ineffective at inhibiting sulfate reduction in these cultures. When nitrate was added to mid-log phase cultures of ST-3 or ST-tSRB-8A-2 even at concentrations of 10 mM the rate of sulfate reduction was the same as in cultures were no nitrate was added (Figure 5.2 and 5.3).

The lack of effectiveness of the nitrate addition in the North Sea tSRB enrichments is most likely due to the fact that no NRB were present in these cultures. Dr. Gary Jenneman (personal communication) was also unable to determine the presence of NRB in the Ekofisk oil field and nitrate injection was not effective in this oil field. The

lack of NRB present in the Ekofisk oil field could also explain why NS-tSRB-1 and NS-tSRB-2 have no resistance mechanism to nitrite, since these SRB would have had little previous exposure to nitrite there would be no selective advantage for nitrite resistance. With nitrate addition NRB and NR-SOB are able to inhibit sulfate reduction through a variety of mechanisms either by outcompeting the SRB for available electron donors or through the production of nitrite. In studies where nitrate addition was found to be effective nitrite was detected as being produced (Hubert *et al,* 2003; Myhr *et al*, 2002; Nemati *et al*, 2001[b]; Reinsel *et al*, 1996). After nitrate was added to the North Sea tSRB enrichments nitrite was not detected.

Although some SRB can reduce nitrate as well, this has not often been observed in oil field SRB. It has been hypothesized that if these SRB/NRB are present in oil reservoirs, upon nitrate addition they might switch from reducing sulfate to nitrate as it is a more thermodynamically favorable reaction (Sandbeck and Hitzman, 1995).

Nitrate was not effective at inhibiting sulfate reduction in the storage tank consortium. The NRB present in ST-tSRB-8A reduced nitrate to ammonia rather than nitrite (as seen in Chapter 4) which, may provide a possible explaination for the ineffectiveness of nitrate addition. Transient nitrite production has been seen in consortia where nitrate addition is effective (Hubert *et al,* 2003; Myhr *et al*, 2001; Nemati *et al*, 2001[b]; Reinsel *et al*, 1996). Hubert *et al* (2003) showed that when nitrate was added nitrite could be detected in an up-flow packed-bed bioreactor. Nitrite production was also observed during nitrate inhibition of sulfate reduction in a sandstone column containing a thermophilic sulfide producing oil consortium (Reinsel *et a*l, 1996).

Although NRB were present in the oil tank consortium, nitrate addition did not effectively inhibit sulfate reduction. Hence, the NRB present that are capable of reducing nitrate to nitrite play an important role in the inhibition of sulfate reduction. This result is not surprising since it has been shown that it is the production of nitrite a competitive inhibitor of Dsr, which is responsible for the inhibition of sulfate reduction and nitrite is a proven metabolic inhibitor of sulfate reduction.

Chapter 6 Effects of nitrite addition on sulfate reduction

6.1. Introduction

The presence of SRB in oil reservoirs and the effects of nitrite on souring was outlined in chapter 1. The nitrite concentration required for inhibition of sulfate-reduction has been found to be dependent upon whether or not nitrite-reducers are present in the community that can remove the nitrite before it inhibits the SRB or whether the SRB in the reservoir community have Nrf activity, affording them nitrite resistance. Here batch cultures containing tSRB enrichments from the North Sea and oil storage tank cultures were used to study reduction in souring in these environments through nitrite addition. Cultures were grown in MS-20 (North Sea tSRB enrichments) or mCSB (oil storage tank cultures); both contained defined sulfate and lactate or organic acid (acetate, propionate and butyrate) concentrations which allowed effective nitrite doses for inhibition of sulfate reduction to be determined.

6.2. Methods

All materials and methods used in these experiments are as described in Chapter 2.

6.3. Results

6.3.1 The effect of nitrite addition on the North Sea tSRB enrichments

Nitrite was very effective at controlling sulfate reduction in the North Sea cultures. Complete inhibition of sulfate reduction in NS-tSRB-1 cultures was achieved by 0.25 mM nitrite (Figure 6.1) Acetate utilization was also inhibited by nitrite addition (Figure 6.2) oxidation of propionate and butyrate by NS-tSRB-1 could not be determined as the concentration of propionate was too low for HPLC detection and the buyrate concentration was also below the HPLCs detectable level.

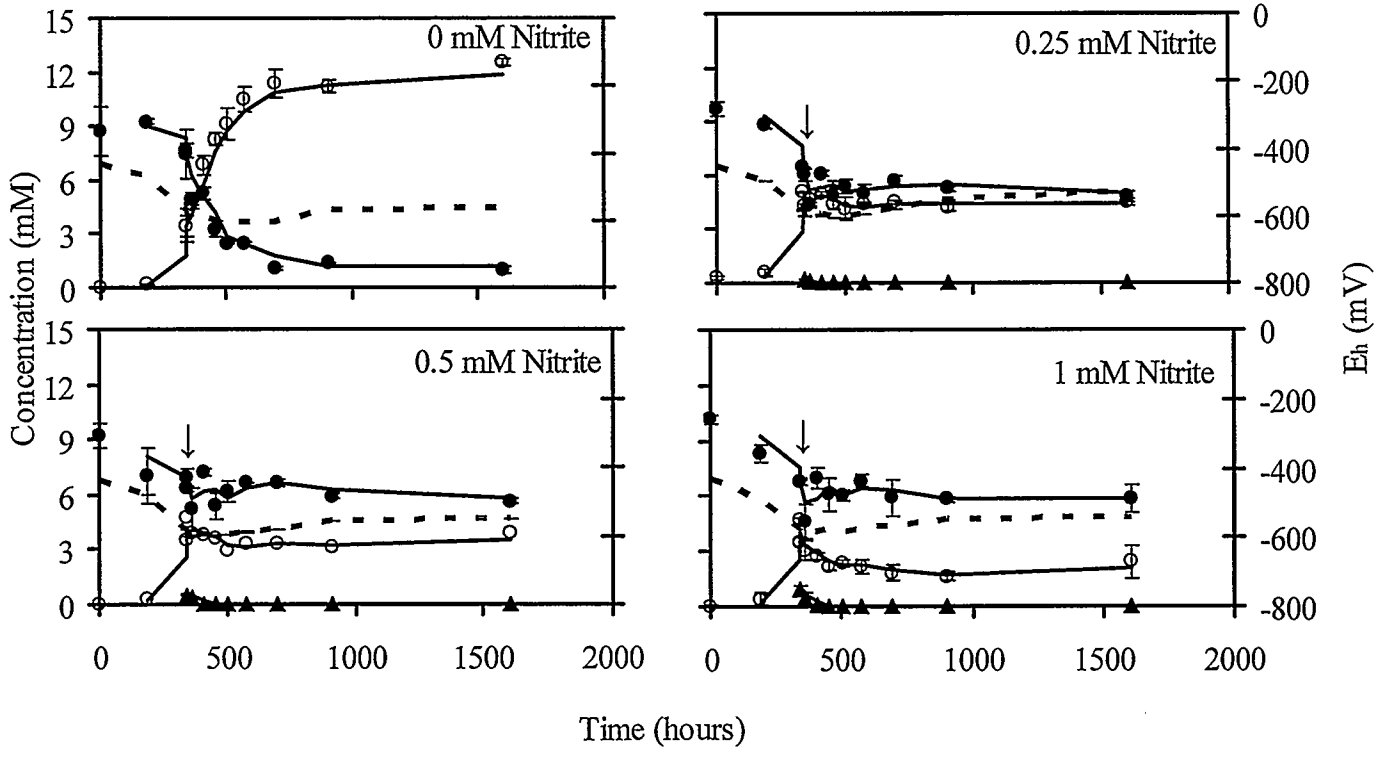

Figure 6.1 Effect of nitrite addition (↓) on sulfide production by NS-tSRB-1 in MS2-20-OA with organic acids as the electron donor and sulfate as the electron acceptor. Sulfate (•), sulfide (○), nitrite (▲) and E_h (---) were measured as a function of time. The points are the average of 3 replicates and the error bars indicate the standard deviation.

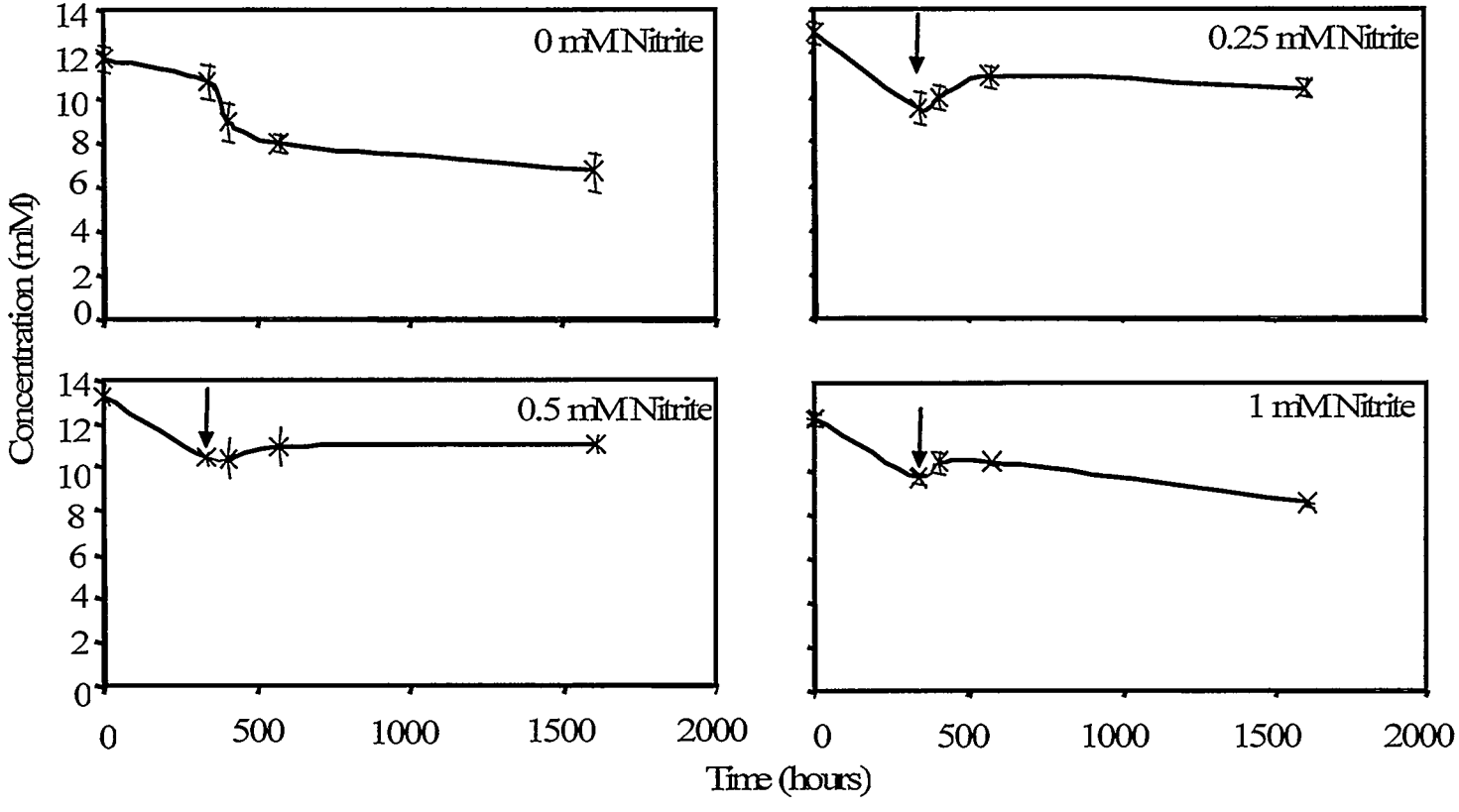

Figure 6.2 The effect of nitrite addition (↓) on acetate utilization by NS-tSRB-1 in MS2-20-OA. Acetate (×) concentration was measured as a function of time. The points are the average of three replicates and the error bars represent the standard deviation.

NS-tSRB-2 was able to completely remove up to 2 mM nitrite from the culture during mid log phase but still remained inhibited (Figure 6.3 A-E). At higher concentrations (2.5 mM or more) nitrite was not completely removed (Figure 6.3 F). NS-tSRB-2 completely oxidized lactate to CO_2 as acetate was not detected (Figure 6.4). Even though nitrite addition effectively inihibited the sulfate reduction the lactate concentration continued to decrease even after nitrite addition. Nitrite removal from these cultures coincided with sulfide oxidation. Sulfide removal corresponded to an increase in the sulfate concentration, however not all of the sulfide was oxidized to sulfate. A whitish precipitate, likely representing sulfur was seen in cultures when more then 0.25 mM nitrite was added. A nitrite concentration of 0.25 mM was effective at completely inhibiting sulfate reduction in NS-tSRB-2, which remained inhibited for 39 days post nitrite addition. In experiments where either 0.25, 0.5, 1 or 2 mM nitrite was added the sulfide concentration decreased by 1.2, 1.3, 2.5 and 4.0 mM, respectively while the sulfate concentration increased by 1.2, 1.9, 2.2 and 1.0 mM, respectively (Figure 6.3). The ratio of sulfide removed to nitrite removed varied in the cultures where the ratio found is only significant in those cultures where greater than 1 mM nitrite was added to the cultures based on the R^2 values (Figure 6.5 and Figure 6.6) for the North Sea cultures. The higher the nitrite concentration added the lower the amount of sulfide removed per nitrite. With NS-tSRB-1 with the addition of 1 mM nitrite the ratio of sulfide removed per nitrite removed was approximately 4:5 (Figure 6.5). In Figure 6.5 it appears as though the observed slope for the 0.25 mM nitrite addition condition is significant, however due to the inherent error adding this low concentration of nitrite we can not consider this data as significant (R^2=0.99). However, this slope has a different sign from that of all other

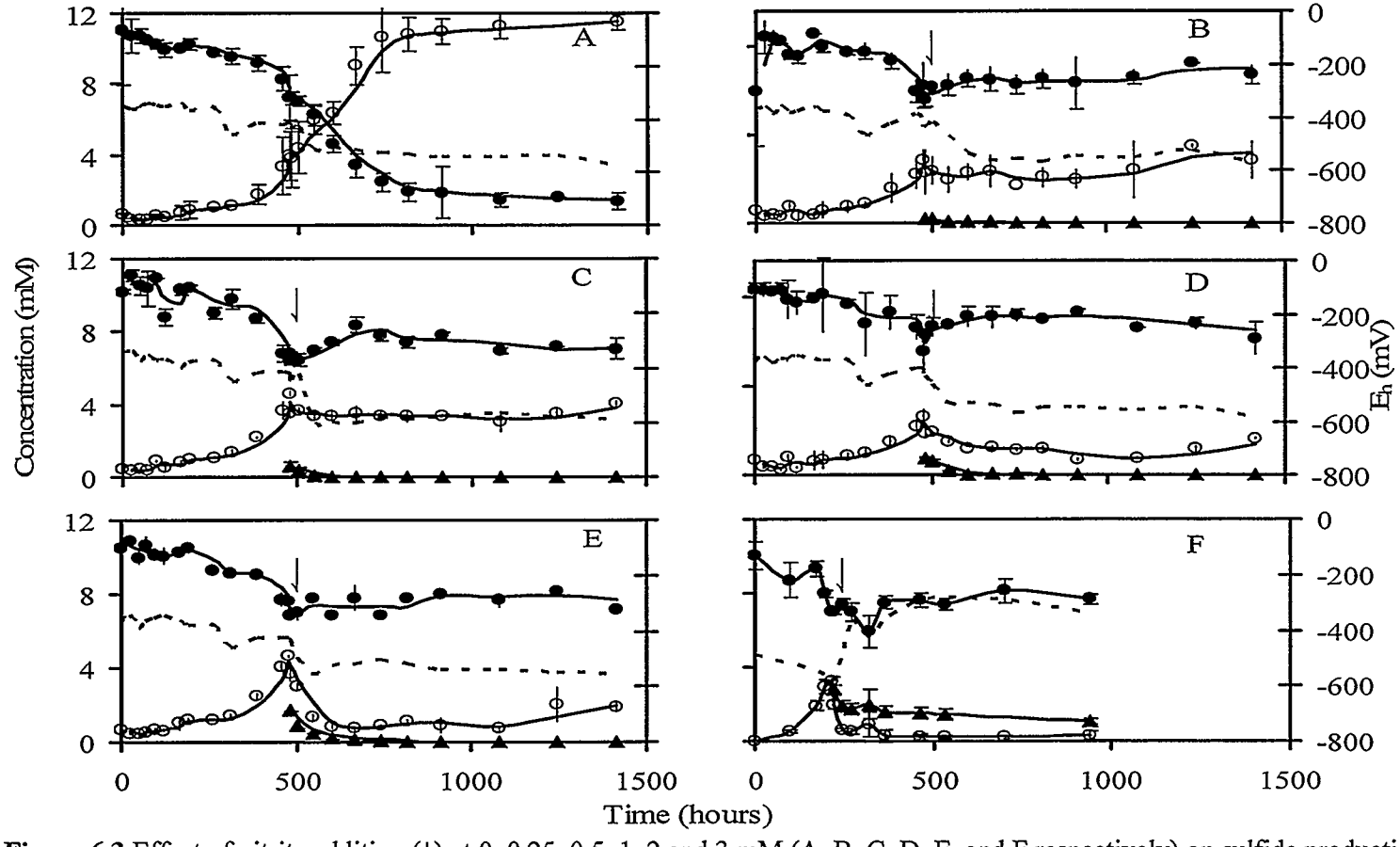

Figure 6.3 Effect of nitrite addition (↓) at 0, 0.25, 0.5, 1, 2 and 3 mM (A, B, C, D, E and F respectively) on sulfide production by NS-tSRB-2 in MS2-20-L with lactate as the electron donor and sulfate as the electron acceptor. Sulfate (•), sulfide (○), nitrite (▲) and E_h (---) were measured as a function of time. The points are the average of 3 replicates and the error bars indicate the standard deviation.

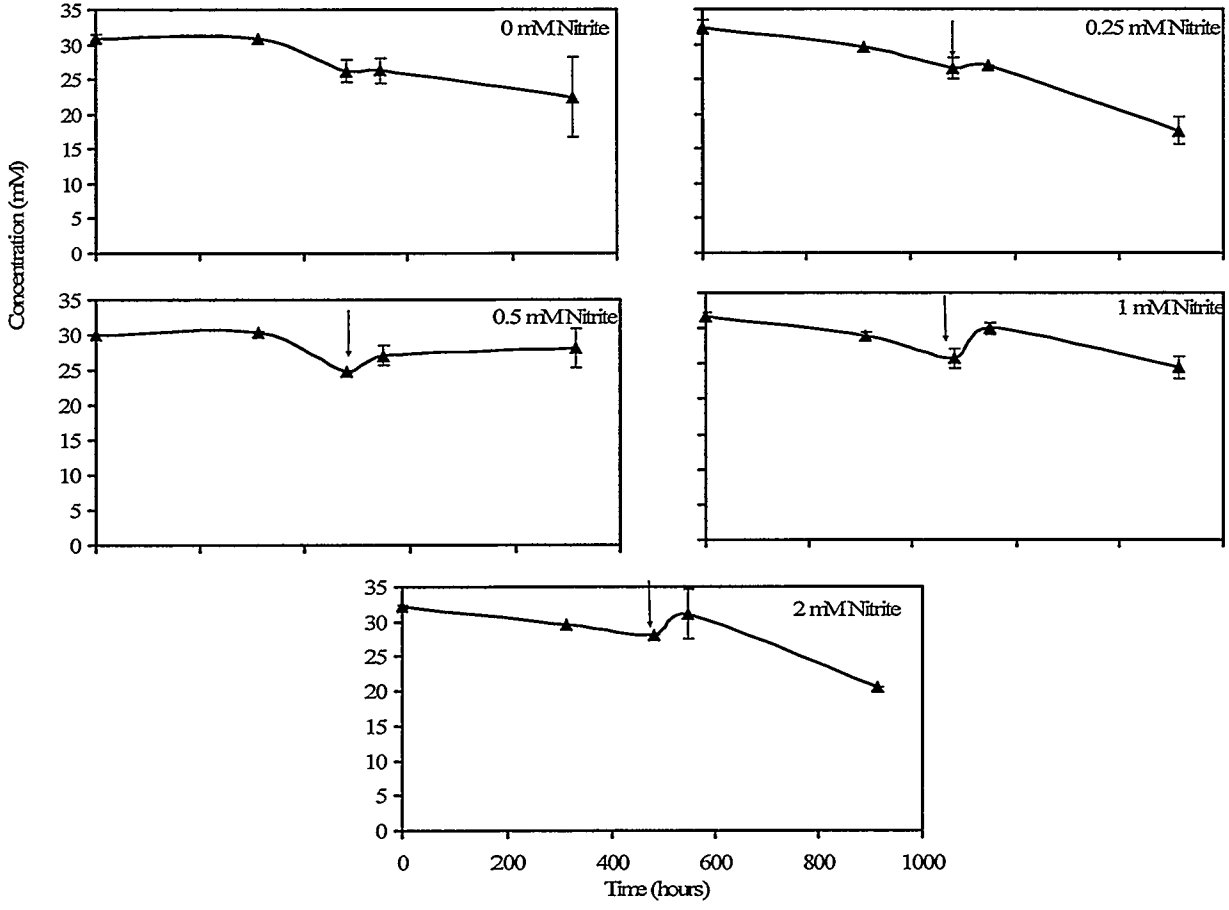

Figure 6.4 The effect of nitrite addition (↓) on lactate utilization by NS-tSRB-2 in MS2-20-OA. Lactate (▲) concentration was measured as a function of time. The points are the average of two replicates and the error bars represent the standard deviation.

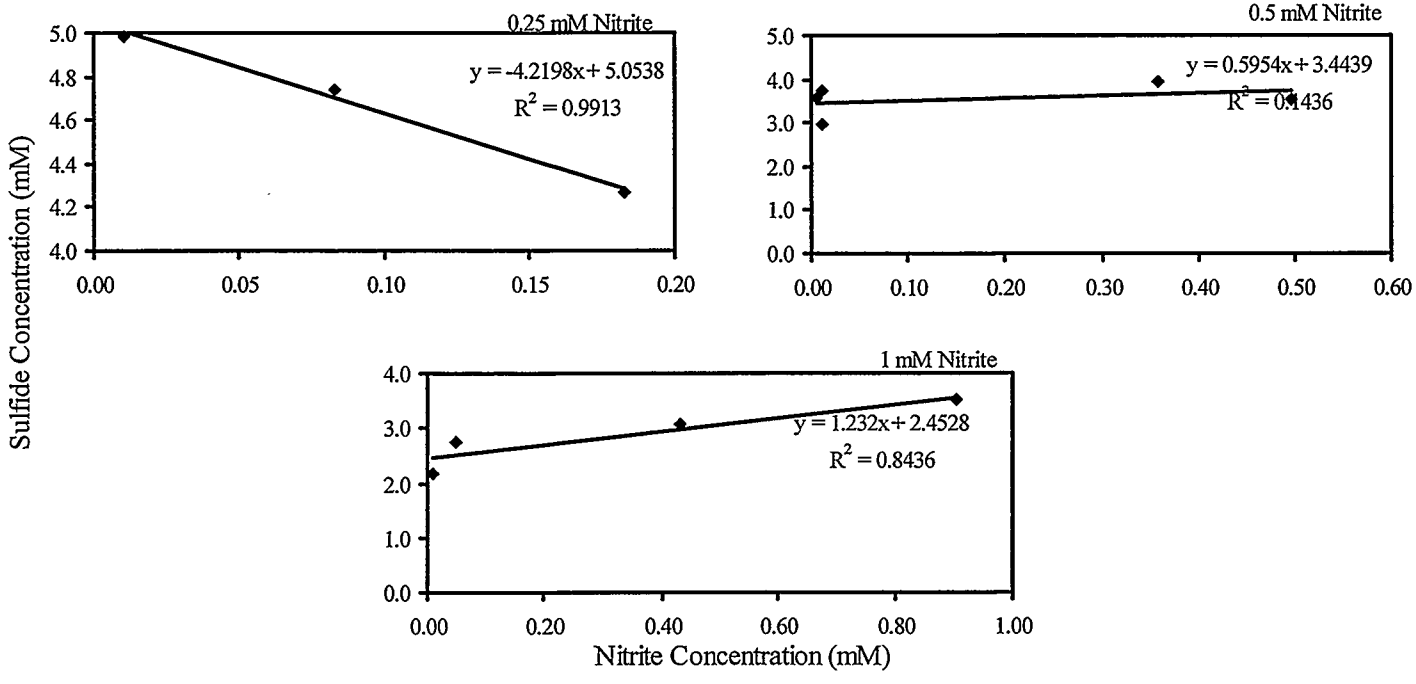

Figure 6.5 The effect of the initial concentration of nitrite on the ratio of sulfide to nitrite removed from culture NS-tSRB-1 grown in MS2-20-OA. The slope of the linear regression line showing the relationship between sulfide and nitrite removal in these cultures increases with the initial concentration of nitrite added to the culture.

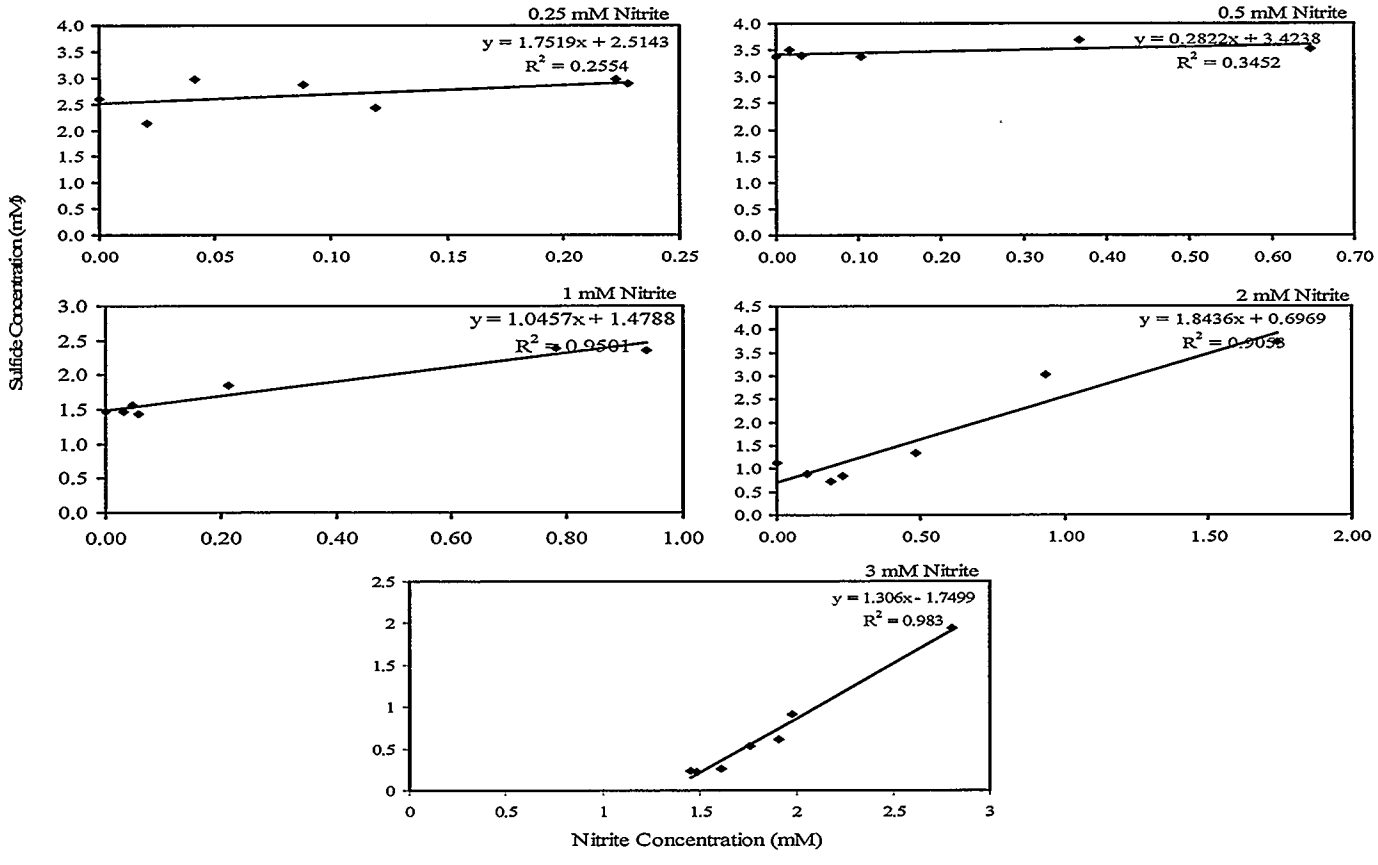

Figure 6.6 The effect of the initial concentration of nitrite added on the ratio of sulfide to nitrite removed from culture NS-tSRB-2 grown in MS2-20-OA. The slope of the linear regression line shows the relationship between sulfide and nitrite removal in these cultures.

experiments (Figures 6.5 and 6.6) Also, due to the inherent error adding this low concentration of nitrite we do not consider this data as accurate. ST-tSRB-2 has sulfide to nitrite removal ratios of approximately 1:1, 1:2, and 4:3 for the concentrations of added nitrite of 1, 2, and 3 mM (Figure 6.6) In all cases (except at 3 mM) nitrite was completely removed from the culture. Nitrite addition to mid log cultures of NS-tSRB-1 had no effect on the redox potential (E_h) of these cultures which remained -500 to -600 throughout. There was no effect on the E_h when nitrite was added to mid log phase cultures of NS-tSRB-2 up to 3 mM nitrite (Figure 6.3). However when 3 mM nitrite is added to these cultures the E_h increases (Figure 6.3) and nitrite is not completely removed. Formation of a sulfur or a polysulfide precipitate was detected in all cultures after nitrite addition as evidenced by the emergence of a yellow colour (polysulfide) or a whitish precipitate (sulfur).

6.3.2 Effect of nitrite on the oil storage tank consortium and isolate

ST-3 was inhibited by 1 mM nitrite as determined by the cessation of sulfate reduction (Figure 6.7). Interestingly, at 0 mM nitrite lactate is converted to acetate (and CO_2 which was not measured); this corresponds to the reduction of sulfate (Figure 6.6 and 6.7). Acetate is then slowly removed from the system (Figure 6.8) presumably by acetotrophic methanogens (Chapter 7). This slow conversion was not observed in the presence of 0.5 mM or 1 mM nitrite (Figure 6.8), indicating that the organisms responsible were inhibited by nitrite. Upon 0.5 mM nitrite addition inhibited acetate oxidation while 1 mM nitrite showed the inhibition of lactate utilization (Figure 6.8). Sulfate reduction was not permanently inhibited by the addition of 0.5 mM nitrite: once the nitrite was removed, ST-3 resumed sulfate reduction (Figure 6.7). When 1 mM nitrite

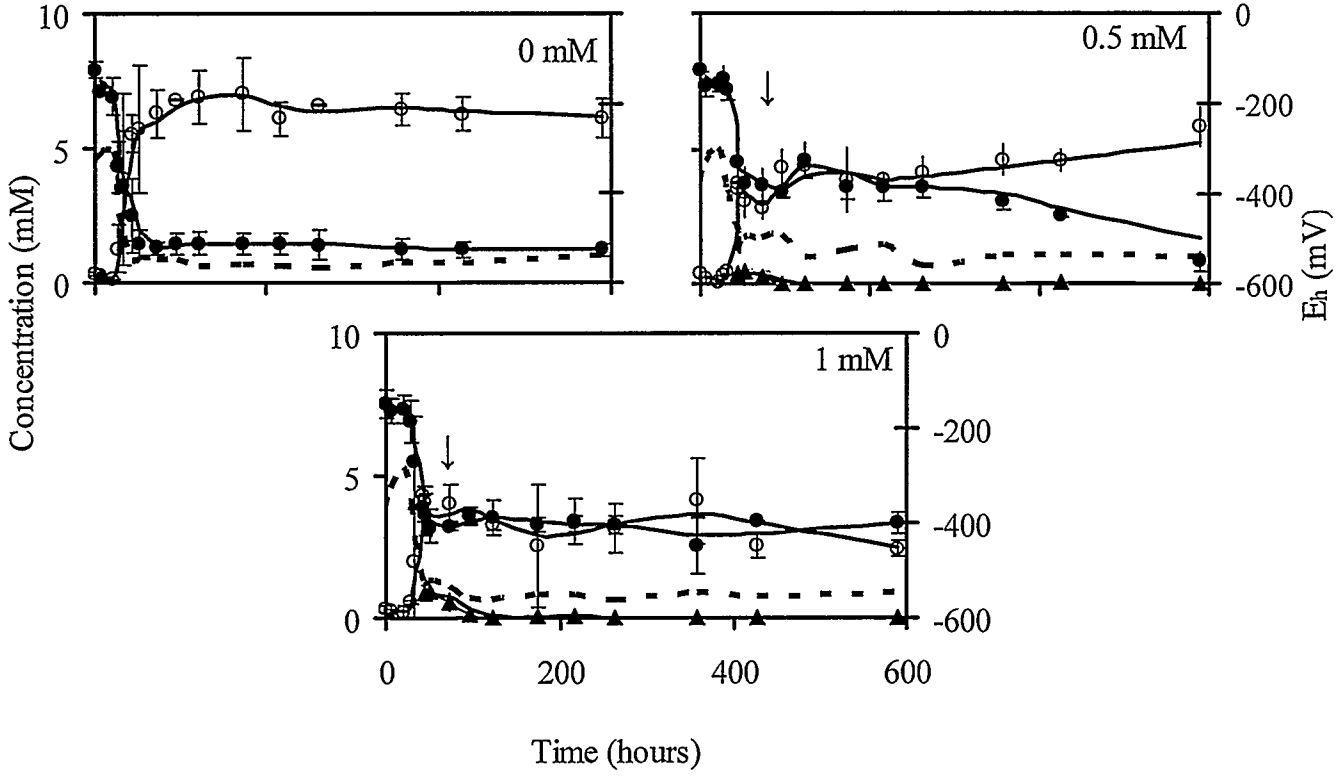

Figure 6.7 Effect of nitrite addition (↓) on sulfide production by ST-3 in mCSB with lactate as the electron donor. Sulfate (●), sulfide (○) nitrite (▲) and E_h (---) were measured as a function of time. The points are the average of 3 replicates and the error bars indicate the standard deviation.

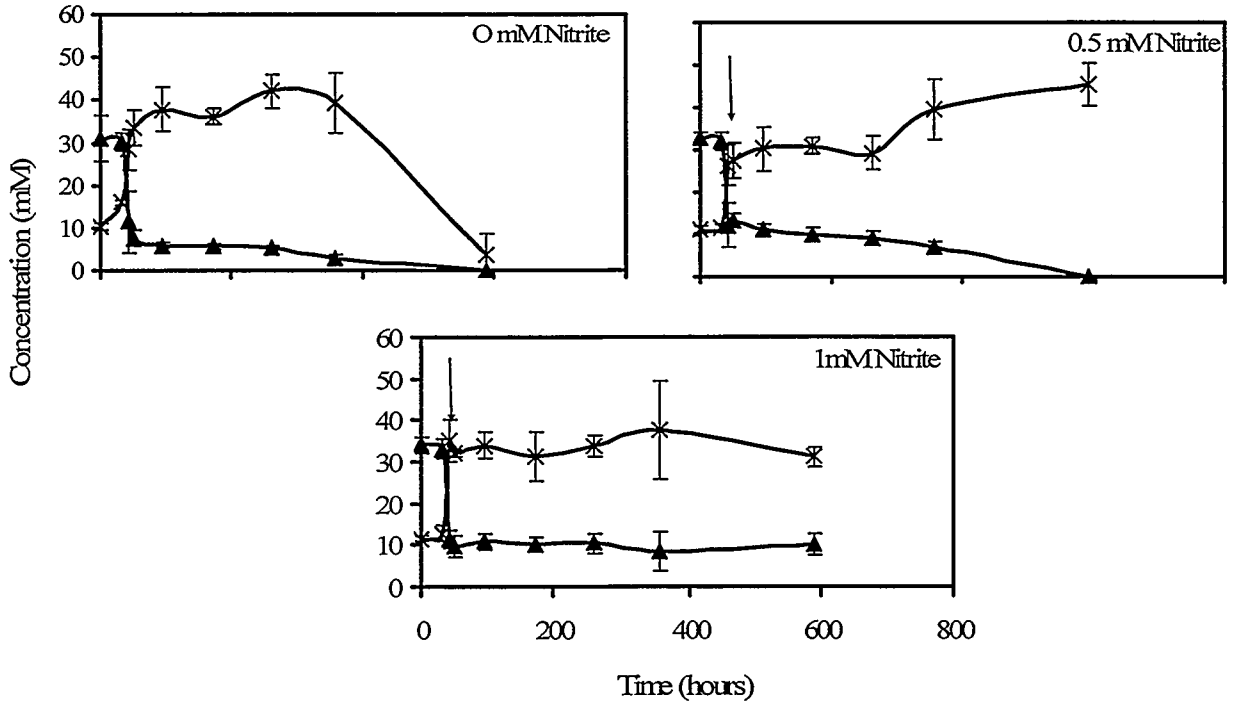

Figure 6.8 The effect of nitrite addition (↓) on lactate utilization by ST-3 in mCSB using a 5 ml inoculum in 100 ml of medium. Lactate (▲) and acetate (×) concentrations were measured as a function of time. The points are the average of three replicates and the error bars represent the standard deviation.

was added to ST-3 there was a 1.5 mM decrease in sulfide and 0.4 mM increase in the sulfate levels. This data could not be used to determine the ratio of nitrite removal to sulfide removal as the R^2 values were too low (Figure 6.9).

ST-1/4 was able to recover from the addition of 1 mM nitrite but peculiarly not from 0.5 mM nitrite (Figure 6.10). When nitrite was added to ST-1/4 there was no corresponding increase in the sulfate concentration. When 1 mM nitrite was added to ST-1/4 sulfate removal resumed and the sulfide concentration increased again after 150 hours (Figure 6.10). When 1, 2 ,4 and 5 mM nitrite was added to uninoculated bottles of mCSB that contained 5 mM sulfide the sulfide concentration decreased to 3.2, 1.6. 0.6 and 0.6 mM respectively after five days in abiotic controls (data not shown). There was no appreciable change in nitrite concentration except in the bottle where 5 mM nitrite was added; initially the nitrite concentration decreased to 4 mM (data not shown).

The effect of adding 0, 0.5, 1, 2, 4 and 5 mM nitrite to ST-tSRB-8A-2 showed that sulfate reduction was permanently inhibited by 0.5 mM nitrite (Figure 6.11). However, sulfate reduction was also inhibited in the control culture above concentrations of 5 mM sulfide. The inhibitory effect of the sulfide concentration is also seen with lactate oxidation where the lactate in no longer oxidized when the sulfide concentration is above 5 mM (Figure 6.12) Upon addition of nitrite at concentrations of 0.5, 1, 2, 4 or 5 mM the sulfate concentration increased 1.9, 1.1, 0.64, 0.74 and 0.78 mM and sulfide decreased 1.0, 3.2, 3.7, 4.0 and 3.4 mM respectively. ST-tSRB-8A-2 showed sulfide to nitrite removal ratios of approximately 1:2, 3:2, 1:1 and 1:2 for concentrations of added nitrite of 1, 2, 4, and 5 mM (Figure 6.13). When nitrite was added at concentrations of 4 and 5 mM the E_h increased as sulfide and nitrite were removed. At these high levels

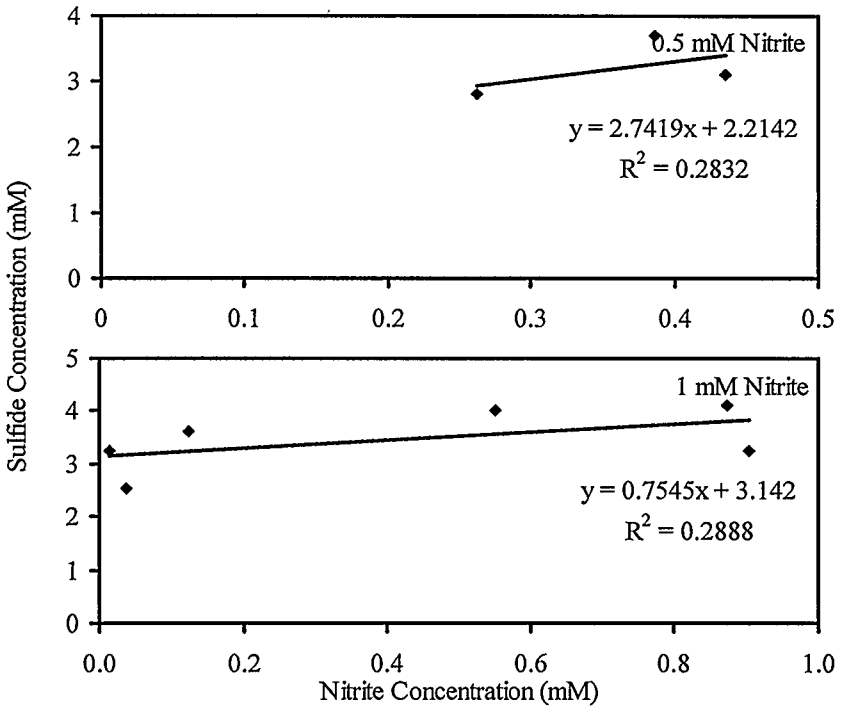

Figure 6.9 The effect of the initial concentration of nitrite addition on the ratio of sulfide to nitrite removed from culture ST-3 grown in mCSB. The slope of the linear regression line showis the relationship between sulfide and nitrite removal in these cultures.

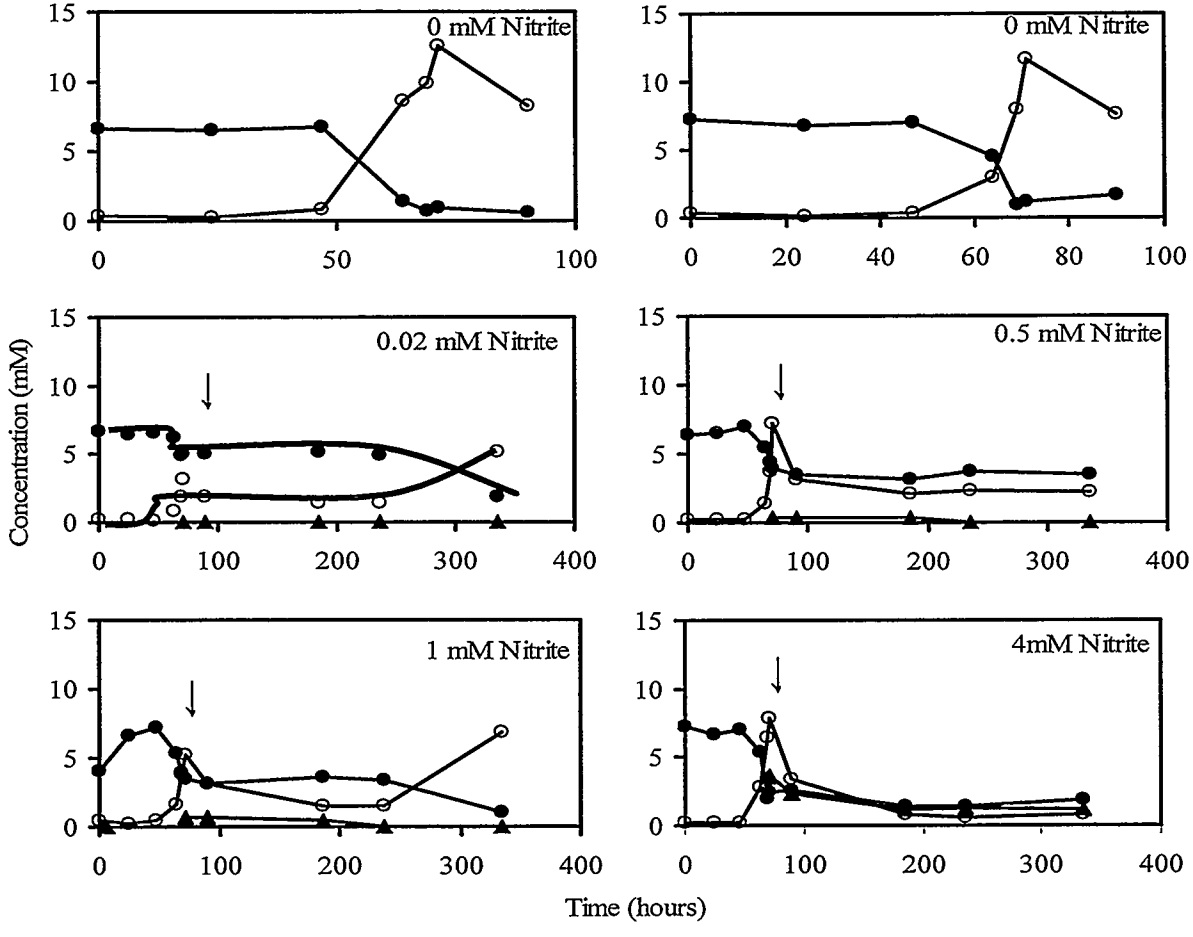

Figure 6.10 The effect of nitrite addition (↓) on ST-1/4 in mCSB with lactate as the electron acceptor. Concentrations of sulfate (•), sulfide (○) and nitrite (▲) as a function of time.

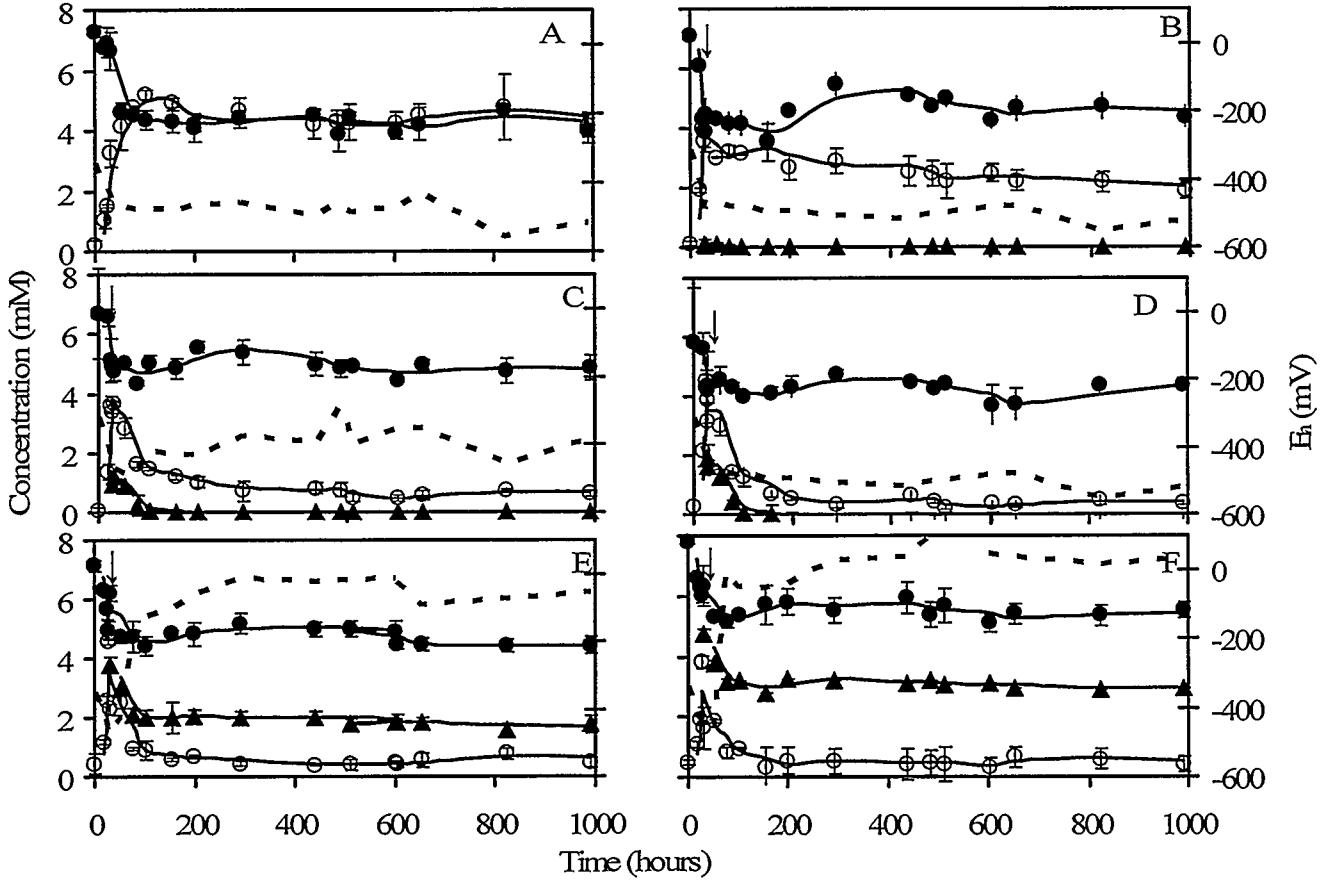

Figure 6.11 Effect of nitrite addition (↓) at concentrations of 0, 0.5, 1, 2, 4 and 5 mM (A, B, C, D, E and F respectively) on sulfide production by ST-tSRB-8A-2 in mCSB with lactate as the electron donor and sulfate as the electron acceptor. Sulfate (•), sulfide (○), nitrite (▲) and E_h (---) were measured as a function of time.The points are the average of 3 replicates and the error bars indicate the standard deviation.

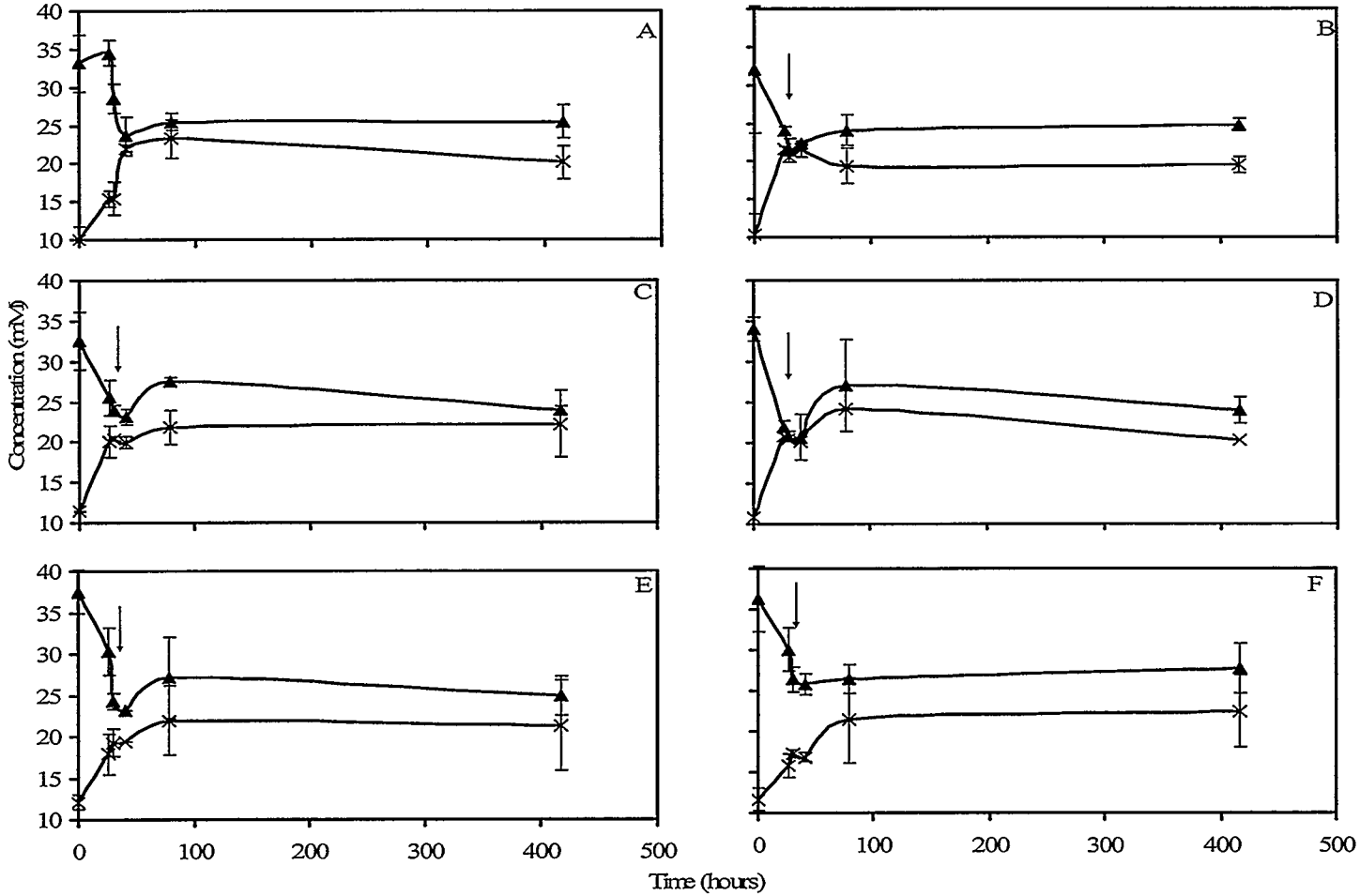

Figure 6.12 The effect of nitrite addition (↓) on lactate utilization by ST-tSRB-8-2 in mCSB using a 5 ml inoculum in a 100 ml of medium. Lactate (▲) and acetate (×) concentrations were measured as a function of time. The points are the average of three replicates and the error bars represent the standard deviation.

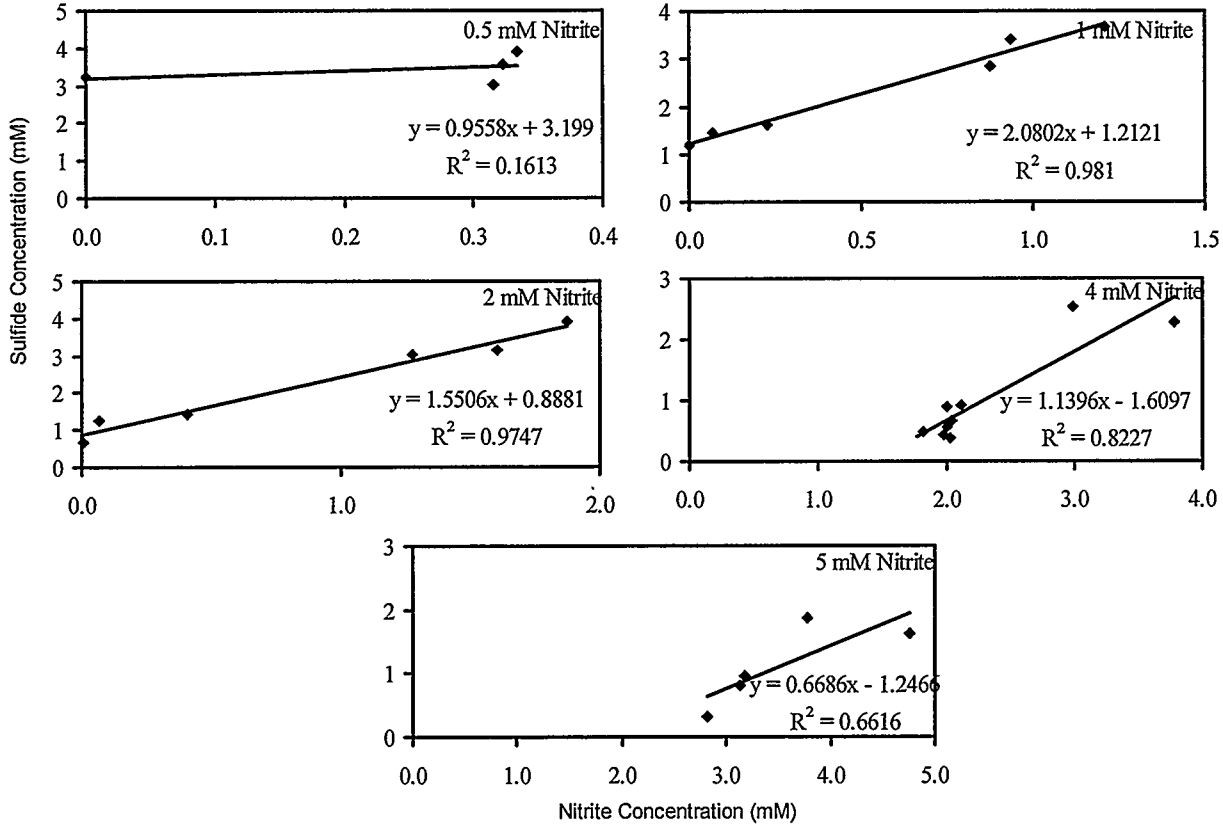

Figure 6.13 The effect of the initial concentration of added nitrite on the ratio of sulfide to nitrite removed from culture ST-tSRB-8A-2 grown in mCSB. The slope of the linear regression line shows the relationship between sulfide and nitrite removal in these cultures.

nitrite could not be completely removed from the cultures and was no longer removed once the sulfide concentrations reached zero.

6.4 Discussion

The main result of this study was that nitrite is an effective inhibitor of sulfate reduction by thermophilic SRB. Low nitrite concentrations inhibited sulfate reduction in all cultures tested. Nitrite concentrations of 0.25 mM for the North Sea tSRB enrichments, 0.5 mM for ST-tSRB-8A-2 and 1 mM for ST-3 were effective at inhibiting sulfate reduction. Following nitrite addition sulfide was removed from the cultures. It is possible that the sulfide removal was an abiotic reaction made more favorable by the high temperature at which these experiments were carried out. Lactate was present in excess in these media which allowed for the complete removal of the sulfate when sulfate reduction was not inhibited.

Nitrite addition effectively inhibited sulfate reduction in NS-tSRB-1 and NS-tSRB-2 enrichments at concentrations as low as 0.25 mM. Both NS-tSRB-1 and NS-tSRB-2 were found to be complete oxidizers since they were identified as *Thermodesulforhabdus norvegicus* and *Archaeoglobus fuldigus* respectively both of which are complete oxidizers (Beeder *et al*, 1995; Beeder *et al*, 1994). However, whereas acetate oxidation was inhibited by nitrite addition with NS-tSRB-1 (Figure 6.2), some lactate removal continued after nitrite addition in NS-tSRB-2 cultures (Figure 6.4). The nature of the process contributing to lactate removal is currently unknown. Based on these low inhibitory nitrite concentrations we can conclude that it is unlikely that the sulfate reducers present in either of these enrichments have Nrf activity. The nitrite concentrations of these enrichments are similar to the inhibitory nitrite concentration seen in *Desulfovibrio* sp. strain Lac6 which does not posses nitrite reductase (Greene *et al,*

2003). It was found that at mid log phase Lac6 was completely inhibited by 0.5 mM nitrite addition whereas *Desulfovibrio* sp. strain Hildenbrough was not. Upon further investigation using Southern blot analysis Greene *et al* (2003) found that *Desulfovibrio* sp. strain Hildenbrough has the *nrf*HA gene which allowed it to remove nitrite by converting it to ammonia whereas Lac6 did not have this gene because, like Lac6, NS-tSRB-1 and NS-tSRB-2 were inhibited by low levels of nitrite; it is likely that they also do not have the nitrite reductase gene. NS-tSRB-2 has the closest 16S rRNA gene identity with *Archaeoglobus fulgidus* DSM4304 for which we have the complete genome sequence; using a blastp search of the *Archaeoglobus fulgidus* DSM4304 genome with the *Desulfovibrio vulgaris nrf*A gene peptide sequence there are no matches, suggesting the absence of nitrite reductase.

Addition of nitrite successfully inhibited sulfate reduction in ST-3, ST-1/4 and ST-tSRB-8A, although it was less effective at inhibiting sulfate reduction in ST-1/4. Thus ST-tSRB-8A-2 likely has little or no Nrf activity while ST-1/4 may have some Nrf activity or may simply contain NRB. ST-tSRB-8A-2 shows a trend of a decreasing slope when nitrite concentration was plotted against sulfide concentration, indicating that as the nitrite concentration increases fewer sulfide are removed per nitrite (Figure 6.13). Some ways that nitrite might be removed are as follows:

	Sulfide/Nitrite	
$5H^+ + 3HS^- + 8NO_2^- \rightarrow 3SO_4^{2-} + 4N_2 + 4H_2O$	0.37	(6-1)
$4\ H_2O + 5H^+ + 4NO_2^- + 3HS^- \rightarrow 4NH_4^+ + 3SO_4^-$	0.75	(6-2)
$5H^+ + 2NO_2^- + 3HS^- \rightarrow 3S^o + N_2 + 4H_2O$	1.5	(6-3)
$5H^+ + NO_2^- + 3HS^- \rightarrow 3S^o + NH_4^+ + 2H_2O$	3.0	(6-4)

This indicates, based on the reactions above, that the bacteria would be switching from converting sulfide to sulfur which uses less nitrite per sulfide removed as compared to when the cells are converting sulfide to sulfate which requires more nitrite per sulfide removed. In an experiment by Claire Stilwell (personal communication) using an abiotic control at 30 °C it was found that the values of the slopes increased as the amount of nitrite added increased. The slope of the abiotic nitrite addtion went from 2.0 sulfide per nitrite with the addition of 5 mM sulfide to 2.7 and 2.8 sulfide per nitrite when 10 and 15 mM nitrite where added. In other abiotic nitrite reduction experiments by Stilwell (personal communication) it was found that with nitrite addition at 30 °C ammonia was transiently formed, whereas at 60 °C no significant increase in ammonia concentration was seen.

Nitrite and sulfide were removed from the cultures in this study. The sulfide removal in these experiments was most likely due to an abiotic reaction between sulfide and nitrite. When nitrite was added to uninoculated media that contained sulfide, the sulfide was after nitrite addition, although nitrite did not remain in these bottles until 5 mM nitrite was added.

It is difficult to tell from these experiments if the added nitrite or the high sulfide concentrations were responsible for inhibiting ST-tSRB-8A-2 because the lactate oxidation and the sulfate reduction data for the control culture where no nitrite was added indicate that these cultures were inhibited once the sulfide concentration reached 5 mM as both lactate oxidation and sulfate reduction ceased. An interesting result seen in this experiment is the effect that nitrite addition has on E_h in cultures of ST-tSRB-8A-2. Adding high concentrations of nitrite (5 mM) to these cultures resulted in a large E_h increase to above 0 mV (Figure 6.11). E_h increased as nitrite was being removed, and

remained high after nitrite removal was complete. The high E_h found in these cultures would prevent sulfate reduction from resuming even after the nitrite has been removed as sulfate reduction can not occur at E_h levels above –100 mV (Postgate, 1984). There was no increase in E_h when nitrite was added to either ST-3 or ST-1/4. E_h increases with nitrate or nitrite addition is a hallmark of NR-SOB activity. When nitrite was added to oil field SRB cultures containing the NR-SOB *Thiomicrospira* strain CVO a large increase in the E_h was also seen (Greene *et al*, 2003).

Both ST-3 and ST-1/4 are consortia from the oil storage tank yet they were inhibited by different nitrite concentrations, 0.5 and 1 mM nitrite respectively. There were two differences between the nitrite inhibition experiments using ST-3 and ST-1/4, which may account for the difference in nitrite inhibition thresholds. First nitrite addition to ST-1/4 was conducted one year earlier, and second, ST-1/4 was comprised of a mixture from all the oil storage tank samples while ST-3 was comprised of just one of the storage tank oil/water samples. An oil tank consortium enrichment (ST-1/4-E) was found to be unstable as demonstrated in chapter 3 by the decrease in sulfate reduction after ST-1/4 was maintained for one year. It is possible that during one year of storage in the anaerobic hood the tSRB in the consortium which possessed more nitrite reductase activity or the NRB might have died. Another possibility that could account for the differences seen in nitrite resistance in the two samples is that composition of the tSRB present in the different oil tank samples could be different, representing microbial communities with different nitrite resistance. Kolter *et al* (2004) found that microbial community composition associated with samples of fines and the community composition associated with water phase oil reservoir samples are actually quite different contrary to earlier hypotheses which stated they should be similar. It is possible that there

may be similar differences in the microbial communities in various parts of the oil storage tank.

The tSRB in both ST-3 and ST-tSRB-8A-2 appear to be incomplete oxidizers based on the fact that acetate is produced as an end product and 2 lactate are oxidized for every sulfate reduced. However in ST-3 the acetate is also subsequently used as there are a large number of acetotrophic methanogens in this storage tank consortium. As discussed in Chaper 7 the acetate is most likely being used by them. It can be seen in Figure 6.2 that acetate oxidation is inhibited by the addition of 0.5 mM nitrite whereas lactate oxidation was not inhibited in ST-3 until the addition of 1 mM nitrite. A possible explanation for this result is that the methanaogens may be more inhibited by nitrite addition than the tSRB in this consortium.

The effectiveness of nitrite for controlling reservoir souring is dependent on whether or not the SRB present have Nrf activity and whether other members of the microbial community use nitrite. A variety of different nitrite resistance levels are seen in different oil fields. In this study low levels of nitrite were found to be effective. Low nitrite doses also inhibited SRB in experiments by Myhr *et al* (2002) and Reinsel *et al* (1996). In the study by Myhr *et al* (2002) 75 μM nitrite successfully inhibited sulfate reduction in batch cultures of the dominant SRB strain obtained from a model reservoir column inoculated with produced water. Reinsel *et al* (1996) found that 0.5 mM nitrite controlled sulfate reduction in Berea sandstone columns containing a thermophilic sulfate-reducing consortium. In contrast a study by Hubert (2004) found that much higher levels of nitrite (20 mM) were required for SRB inhibition in a packed-bed bioreactor. Based on the variations seen in inhibitory nitrite concentrations, it can be concluded that

the optimal amount of nitrite required to control sulfide production in oil reservoirs depends in part on the microbial community present.

Chapter 7: Methanogenic oil degrading consortium

7.1. Introduction

Biologically produced methane has recently been hypothesized to be associated with biodegraded oil. Methanogens could be associated with oil degrading syntrophic consortia. The research presented in this chapter uses a methane producing oil-degrading consortium from an oil storage tank where the lid blew off due to methane production. The rate of methane production in this consortium using different substrates including oil.

7.2. Methods

All materials and methods used in these experiments are as described in Chapter 2.

7.3. Results

7.3.1 Acetotrophic methanogen enrichment ST-MET-2

The oil storage cultures grown in sPGC produced large amounts of an unknown gas which was identified as methane. An ST-1/4-E culture which produced methane was transferred to MS2-7-A media adapted for growth of acetotrophic methanogens. Gowth on this medium (containing 20mM acetate, and reduced with 3 mM sulfide) was able to produced 49 ml of methane in a 100 ml culture. Filamentous bacteria (approximately 1 micron in diameter) were observed microscopically (Figure 7.1). The DNA was extracted from ST-MET-2 and the 16S rRNA gene was amplified using the primer sets I341f U1492r, U515f U1492r, A25f A958r, A25f A1063r, A25f A1392r, A25f U1492r and A341f A1063r. PCR products could be obtained with primer sets A25f A1392r, U515f U1492r, and A341f A1063r; a PCR product could not be obtained with any of the other primer sets. Figure 7.2 shows a gel of PCR products amplified using A341f and A1063r. PCR products obtained with primers sets U515f U1492r, and A341f A1063r were used for sequencing.

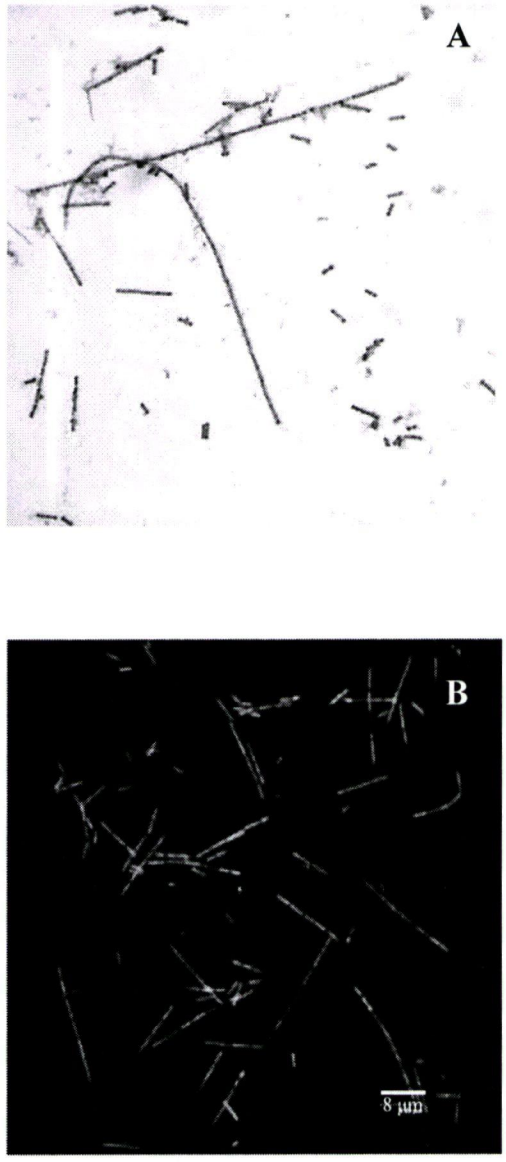

Figure 7.1 A. Gram stain of the unknown methanogen ST-MET-1 (1000 X magnification) observed using a light microscope. B. 600X magnification of the unknown methanogen ST-MET-1 stained with acridine orange and visualized using a confocal microscope.

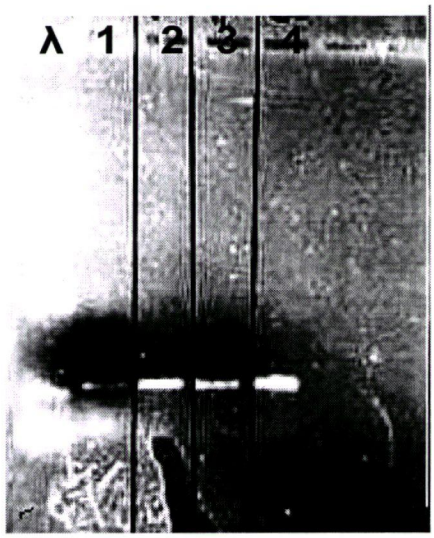

Figure 7.2 PCR amplification of the 16SrRNA genes using DNA from ST-MET-1 and PCR primers A341f and A1063r. Lanes 1-4 show the PCR products, show a band approximately 700 bp in size. Lane λ: λ DNA digested with *Hind* III; fragment sizes from top to bottom 23.1, 9.4, 6.6, 4.4, 2.3, 2.0 and 0.56 kilobase pairs.

However, a poor sequence with contained Ns throughout the sequence was obtained using primer A341f, so only the sequences obtained from the primer set U515f and U1492 and the sequence obtained using the reverse primer A1036r which gave a good sequence were used for identification A. Both U515f/U1492r (ST-MET-1) and A1063r (ST-MET-1B) had a 99 % identity with the 16S rRNA gene of *Methanothrix thermophilia* (Figure 7.3) an acetotrophic methanogen (Kamagata *et al.* 1992).

7.3.2 Methane production from oil and organic acids

The rate of methane production was determined by measuring the total gas volume produced using a syringe. The gas was determined to be methane by gas chromatography. The rate of methane production in MS2-7-OA medium containing acetate, butyrate and propionate and the rate in the MS2 medium containing oil are shown in Figure 7.1. From this figure it can be seen that the organic acid culture produced methane at a faster rate (6.1 ml/day) than did the culture using oil as a carbon and energy source (1 ml/day) (Figure 7.4). Some methane and sulfide (0.8 mM) were detected after four weeks with hexadecane as the electron donor, although production was too little to measure the gas produced using a syringe.

7.4. Discussion

The oil storage tank consortium was found to contain methanogenic activity. An acetotrophic methanogenic enrichment was obtained from the storage tank. An organism sequenced from this enrichment was 99% similar to *Methanosaeta thermophila.* The latter is known to be sheathed rods arranged in filaments. These bacteria are not susceptible to lysis by SDS or hypotonic solutions at room temperature, but can be lysed with SDS at 60^{O}C which explains the initial difficulty in lysing these cultures in order to

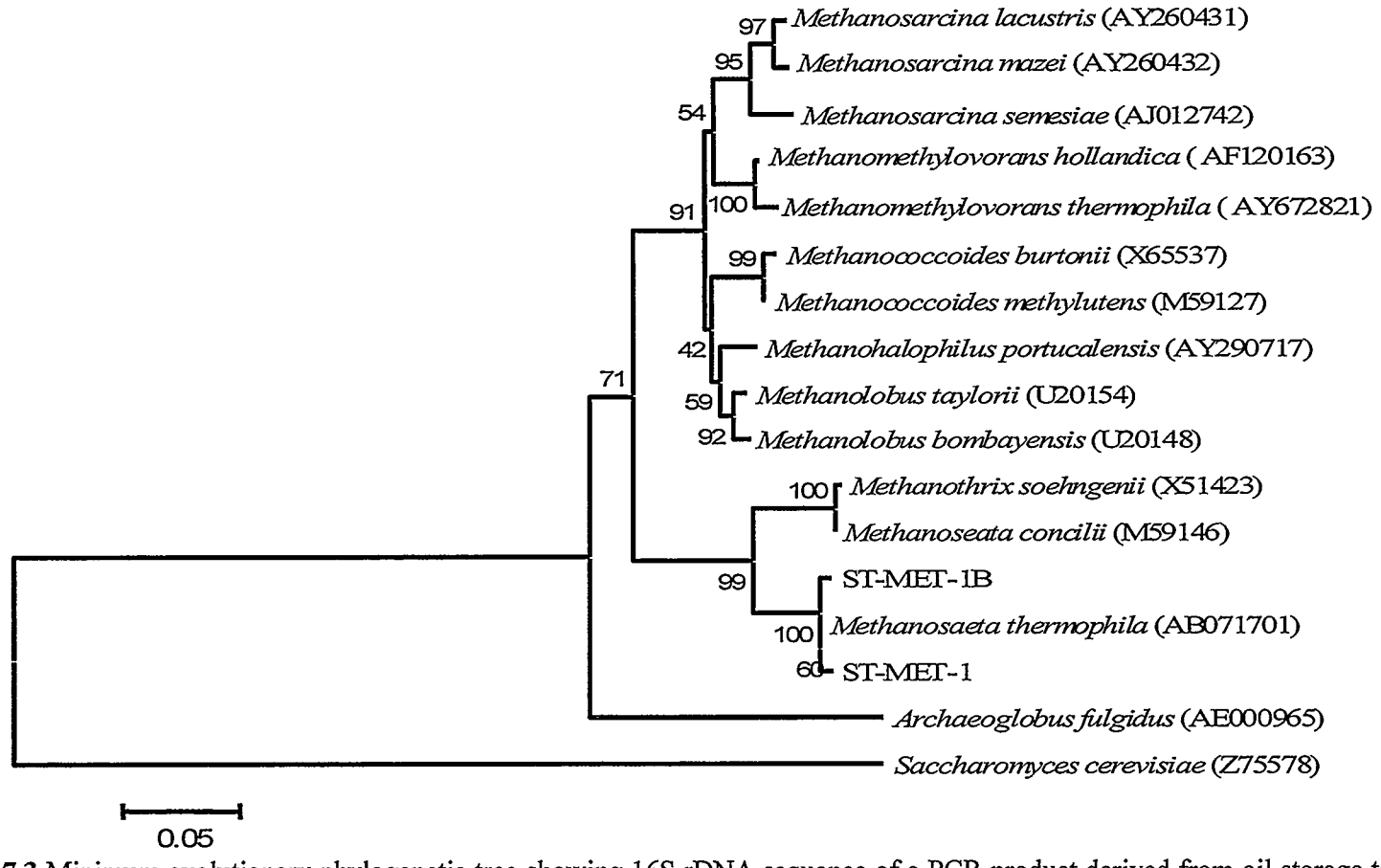

Figure 7.3 Minimum evolutionary phylogenetic tree showing 16S rDNA sequence of a PCR product derived from oil storage tank isolate ST-MET-1. Horizontal distances reflect pairwise sequence similarities as indicated on the scale. The numbers at the nodes represent grouping frequencies based on 500 bootstrap replicates. Sixteen sequences are included in the tree with *Saccharomyces cerevisae* as the outgroup. Accession numbers of the sequences retrieved from the database are also indicated in parentheses.

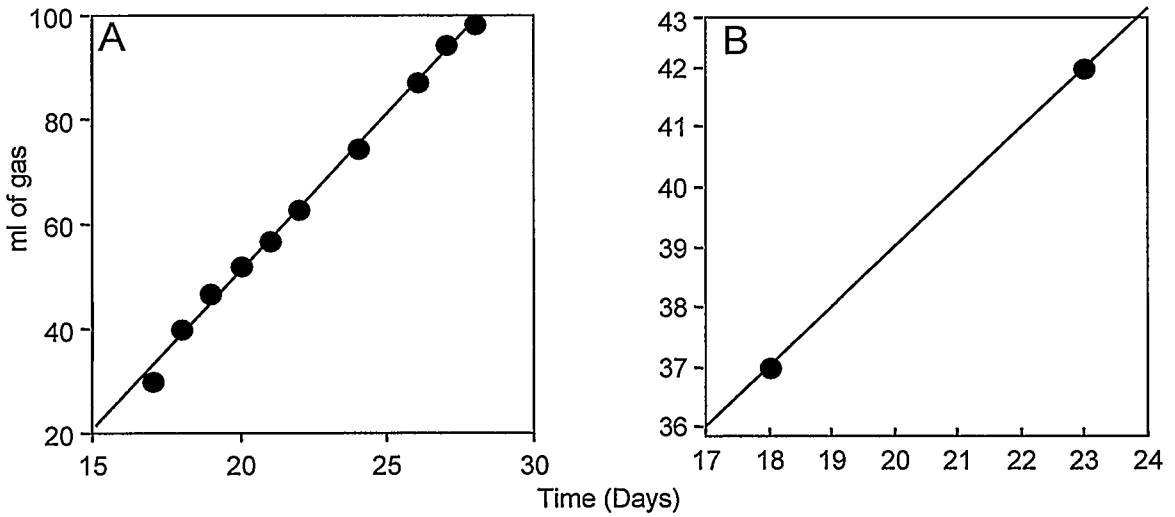

Figure 7.4 The rate of methane production by the oil storage tank consortium in MS2-7 using either (A) organic acids (acetate, propionate, butyrate) as electron donors or (B) oil.

identify them (Kamagata *et al*,1992). Acetate is the only substrate these archaea can use for growth (Kamagata *et al.* 1992). The literature states that *Methanosaeta thermophila* is inhibited by sulfide concentrations greater than 1 mM (Boone *et al*, 2001); this characteristic was not observed in ST-MET-1 which was isolated from the oil storage tank and grew well in medium containing 3 mM sulfide. Large amounts of methane production were also observed in cultures containing 10 mM sulfide.

The main result from these experiments is that the oil tank consortium was able to degrade organic acids (acetate, propionate and butyrate), oil and hexadecane to produce methane. The cultures grown using organic acids as the electron donors grew much faster than the cultures grown with oil. A possible explanation for the differences observed in Figure 7.4 is that it takes multiple microbes to produce methane from oil (Section 1.5 reactions 1, 2 and 3, with reaction 1 presumably the slowest step), whereas methane can be directly produced from acetate. This difference can be explained by the ease of degradation of each. MS-2-A contained 20 mM acetate which could be used directly by *Methanoseata thermophila* the acetotrophic methanogen that was isolated from the oil storage tank. This explains the large amount of methane produced in this culture. As well, in these cultures the tSRB would be able to utilize the butyrate and propionate to reduce sulfate to sulfide, which was detected in these cultures and likely made the environment more reduced allowing for growth of *Methanoseata thermophila.*

Because the composition of the refined oil in the storage tank was not determined it is impossible to know what compounds the bacteria were degrading, other than that it likely contained electron donors that the prokaryotes found easier to degrade than n-hexadecane. It is possible that the prokaryotes in this culture are producing CO_2, H_2 and acetate as end products which could be utilized by the methanogens for methanogensis.

By removing the acetate from the environment they would make it more suitable for the other organism like the SRB because by utilizing these end products they make the ΔG of the reaction more suitable for syntrophic growth.

Hexadecane degradation by this consortium was very slow. However, this degradation was still significant on an ecological level as oil biodegradation occurs over millions of years. The syntrophic hexadecane degrading consortium isolated by Zengler *et* al (1999) from ditch sediment produced 0.37 ml of methane per day. This was faster than the rate of hexadecane degradation determined in the oil storage tank here which produced detectable methane but never in sufficient quantities to measure the volume of gas produced using a syringe. The rate observed by by Zengler *et* al (1999) is slower than that observed for the thermophilic bacteria studied here with organic acids (6.1 ml/day) or oil (1 ml/ day).

Based on these preliminary studies it cannot be conclusively determined which mechanism(s) or organism(s) are responsible for the hydrocarbon degradation. Because MS-2 the media contained sulfate, which was reduced by the SRB in the consortium, it is possible that the hexadecane degradation was due to SRB and not a syntrophic methanogen-containing consortium. Although it is likely that SRB were not responsible for the degradation of alkanes as the previously isolated thermophilic alkane degrading SRB TD3 (Rueter *et al*, 1994) and the mesophilic strain Hxd3 (Aeckersberg *et al*, 1991) are complete oxidizers. In order to fully understand the observed oil biodegradation, more of the organisms present in this consortium will have to be identified and characterized.

Chapter 8 Conclusions

The experiments presented in this study demonstrate that nitrite can effectively control the production of sulfide from sulfate by tSRB (souring). The concentration of nitrite required for inhibition was dependent on the ability of organisms present in a given culture to remove nitrite from the system. In these same systems nitrate even when added at a much higher concentration than nitrite was found to be completely ineffective at inhibiting souring.

The effectiveness of nitrite and nitrate addition for controlling souring is dependent on the microbial community present in a given reservoir, as well as the cell biomass upon nitrite addition. In general, nitrite will be the more effective agent for souring control as it directly inhibits SRB and is not dependent on the presence of hNRB or NR-SOB. For nitrate addition to be effective not only must NRB be present in the reservoir but the NRB present must produce nitrite in significant quantities. Experiments by Nemati *et al* (2001[b]) showed that addition of nitrate with the NR-SOB *Thiomicrospira* strain CVO was very effective at inhibiting sulfate reduction by mesophilic SRB through the production of nitrite and/or increased E_h. In other studies in thermophilic systems where nitrate was added to a Berea sandstone column inoculated with produced water, nitrite production was also observed during inhibition of sulfate reduction (Reinsel *et al*, 1996). This dependence on nitrate reduction to nitrite by NRB can be seen in the oil storage tank consortium. Although NRB were detected in this culture, they most likely either reduced nitrate to ammonia or nitrogen gas because nitrite was not detected but ammonia was. Nitrite production by the storage tank organisms was never observed. In some studies that showed effective control of souring by nitrate addition, nitrite was detected after nitrate addition (Hubert *et al,* 2003; Greene *et al*, 2003; Myhr *et al,* 2002;

Reinsel *et al*, 1996). Although it has been hypothesized that NRB may outcompete SRB for available electron donors as nitrate reduction is the more theromdynamically favorable reaction, this does not appear to be the case in this study. Thus the most effective mechanism for souring control through nitrate addition is the production of nitrite.

The concentration of nitrite required for inhibition of thermophilc sulfate-reduction also depends on the community composition and physiology. Factors controlling the effectiveness of nitrite addition on the control of souring include whether or not there are NRB or NR-SOB in the community that are able to remove the nitrite, and whether the SRB have Nrf activity. The environmental conditions may also be involved in the effectiveness of nitrite as we have seen that nitrite can be removed abiotically in the presence of sulfide. Hence in environments where this reaction is more thermodynamically favorable nitrite may be less effective at inhibiting tSRB. From the results of Figure 5.4 it was seen that ST-1/4 was able to remove up to 1 mM nitrite and once the nitrite was removed sulfate reduction continued indicating some Nrf activity. ST-tSRB-8A, NS-tSRB-2 and NS-tSRB-1 were inhibited by low nitrite concentrations indicating that like *Desulfovibrio* sp. stain Lac6 they have no nitrite reductase activity, as discussed in chapter 5. ST1/4 was able to recover from 1 mM nitrite addition indicating that it had some nitrite reductase activity however less that that seen for *Desulfovibrio vulgaris* strain Hildenborough which was able to recover from concentrations of 10 mM nitrite (Greene *et al*, 2003) because ST-1/4 was not able to recover from higher nitrite concentrations.

ST-tSRB-8A-2 appears to have some NR-SOB like activity because unlike all the other cultures in this study, when higher concentrations of nitrite were added there was a

large increase in E_h however this could also be due to the large amounts of nitrite added to the cultures. Large increases in E_h are typically associated with NR-SOB and have been seen in other systems where NR-SOB are known to be present. In a study by Greene *et al* (2003) E_h increases were seen in the sulfate-reducing system known to contain NR-SOB *Thiomicrospiria* sp. strain CVO when nitrate was added.

By characterizing the thermophilic oil consortium and enrichments a better understanding of the organisms present in these systems and some of their metabolic activities can be obtained. The oil storage tank consortium was able to degrade oil organics and hexadecane. Although the hexadecane degradation was very slow this ability to degrade oil would still be significant on a geological timescale where oil biodegradation occurs over millions of years. This study did not determine whether alkane degradation occurred via tSRB or a methanogenic syntrophic consortium. From the characterization of the oil storage tank consortium we were able to determine that the isolated tSRB were incomplete oxidizers oxidizing lactate to acetate and CO_2 rather than to CO_2 only. The acetate then served as a substrate for acetotrophic methanogenesis. As well the NRB activity identified in the storage tank consortium reduced nitrate solely to ammonia or nitrogen gas indicating that this activity was not involved in the inhibition of sulfate reduction by the production of nitrite.

Overall the results of the experiments presented in this study show that nitrite is more effective at inhibiting sulfate reduction in thermophilic systems *in vitro* than nitrate.

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Appendix 1 DNA Sequences

NS-tSRB-1 Primer Set U515f and U1492r

CGTAATACGGAGGTGCGAGCGTTACTCGGAATTACTGGGCGTAAAGGGCGCGTAGGCGGCCGT
GCAAGTCTGGTGTGAAAGCCCGGGGCTTAACCCTGGAAGTGCACTGGATACTGTGCGGCTTGA
GTGCCGGAGAGGAGGGGGAATTCCCGGTGTAGCGGTGAAATGCGTAGATATCGGGAGGAATAC
CGGTGGCGAAGGCGCCCCTCTGGACGGTTACTGACGCTGAGGCGCGAAAGCGTGGGGAGCAA
ACAGGATTAGATACCCTGGTAGTCCACGCTGTAAACGATGCCCACTAGGTGTGGTGGAGGTT
ATAACTCTGCCGTGCCGTAGCTAACGCGTTAAGTGGGCCGCCTGGGGAGTACGGCCGCAAGG
TTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAT
GCAACGCGAAGAACCTTACCTGGGTTTGACATCCCCGGAACCTGGTGGAAACACTGGGGTGC
CCCTTATTGGGGAGCCGGGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATG
TTGGGTTAAGTCCCGCAACGAGCGCAACCCCTGCCTCTAGTTGCCAGCGGGTAAGGCCGGGC
ACTCTAGAGGGACTGCCGGTGATAAACCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGG
CCTTTATGCCCAGGGCTACACACGTGCTACAATGGCCGGTACAAAGGGATGCGA

NS-tSRB-2 Primer Set U515f and U1492r

TGGGCCTAAAGCGTCCGTAGCCGGGCTGGTAAGTCCTCCGGGAAATCTGGTCGCTTAACGATCAGACT
GCCGGAGGATACTGCCAGCCTAAGGACCGGGAGAGGCCGGGGGTATTCCCGGAGTAGGGGTGAAATCC
TGTAATCCCGGGAGGACCACCTGTGGCGAAGGCGCCCGGCTGGAACGGGTCCGACGGTGAGGGACGAA
GGCCAGGGGAGCGAACCGGATTAGATACCCGGGTAGTCCTGGCTGTAAACGATGCGGACTAGGTGTCA
CCGAAGCTACNAGCTTCGGTGGTGCCGGAGGGAAGCCGTTAAGTCCGCCGCCTGGGGAGTACGGCCGC
AAGGCTGAAACTTAAAGGAATTGGCGGGGGAGCACTACAACGGGTGGAGCCTGCGGTTTAATTGGATT
CAACGCCGGGAAGCTTACCGGGGGAGACAGCGGGATGAAGGTCGGGCTGAAGACCTTACCAGACTAGC
TGAGAGGTGGTGCATGGCCGCCGTCAGTTCGTACTGTGAAGCATGCTGTTAAGTCAGGCAACGAGCGA
GACCCGCGCCCCCAGTTGCCAGCGGTCCCCTTCGGGGAAGCCGGGCACACTGGGGGGACTGCCGGCGC
TAAGCCGGAGGAAGGTGCNGGCAACGGCAGGTCCGTATGCCCCGAATCCCCCGGGCTACACGCGGGCT
ACAATGGCCGGGACAATGGGTACCGACCCCGAAAGGGGTAGGTAATCCCCTAAACCCGGTCTAACCTG
GGATCGAGGGCTGCAACTCGCCCTCGTGAACCTGGAATCCGTAGTAATCGCGCCTCAAAATGGCGCGG
NGAATACGTCCCTGCTCCTTGCACACACCGCCCGTCAAGACACCCGAGTGNG

ST-tSRB-8A Primer Set U515f and U1492r

GGGGCGACGTTGTCCCTAATTACTGGGCGTAAAGGGTGCGTAGGCGGCCAAATAAGTCAGATGTGAAA
GTCCAAGGCTCAACCATGGAATAGCATTTGAAACTGTATGGCTTGAGTGCAGGAGAGGAGAGCGGAAT
TCCTAGTGTAGCGGTGAAATGCGTAGAGATTAGGAAGAACACCAGTGGCGAAGGCGGCTCTCTGGACT
GTAACTGACGCTGAGGCACGAAAGCGTGGGTAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGT
AAACGATGAGTGCTAGGTGTCGGGAGGAATCTCGGTGCCGGAGTTAACACAATAAGCACTCCGCCTGG
GGAGTACGACCGCAAGGTTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCAGCGGAGCATGTGG
TTTAATTCGAAGCAACGCGAAGAACCTTACCAGGGCTTGACATCCCTTGACGACCTAAGAGATTAGGT
GTTCTCGTTATACGGGACAAGGAGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTG
GGTTAAGTCCCGCAACGAGCGCAACCCTTATATTTAGTTGCCAGCAAGTAAAGTTGGGCACTCTAAAT
AGACTGCCGGCAAGAAGTCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGCCCTGGG
CTACACACGTGCTACAATGGTCTGTACAAAGGGAAGCGAAAGAGTGATCTGGAGCGAATCCCAGAAAG
CAGATCTCAGTTCGGATTGCAGGCTGCAACTCGCCTGCATGAAGGAGGAGTTGCTAGTAATCGCGGAT
CAGAATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCACGAGAGTTGGCAA
CACCCGAAGTCAGTGAGCCAACCTAGAAATAGGAGGCAGCTT

NS-tSRB-8A-2 U515f and U1492r

TCTTCTGGGACGATAATGACGGTACCAGAGGAGGAAGCCACGGCTAACTACGT
GCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTACTGGGC
GTAAAGGATGCGTAGGCGGATATTTAAGTCAGATGTGAAAATCCCGGGCTCAA
CTTGGGAACTGCATTTGAAACTGGGTATCTAGAGTGCAGGAGAGGAAAGCGGA
ATTCCTAGTGTAGCGGTGAAATGCGTAGAGATTAGGAAGAACACCAGTGGCGA
AGGCGGCTTTCTGGACTGTAACTGACGCTGAGGCATGAAAGCGTGGGGAGCAA
ACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAATACTAGGTGTG
GGGATTGTCATGATCTCCGTGCCGCAGTTAACACAATAAGTATTCCGCCTGGG
GAGTACGGTCGCAAGATTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGC
AGCGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCTAGACTTG
ACATCTCCTGAATTACTCGTAACGGAGGAAGCCCTTCGGGGCAGGAAGACAGG
TGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCA
ACGAGCGCAACCCTTGTCGTTAGTTGCTACCATTAAGTTGAGCACTCTAGCGA
GACTGCCGCGGTTAACGCGGAGGAAGGTGGGGTGACGTCAAATCATCATGCCC
CTTATGTCTAGGGCTACACACGTGCTACAATGGTG

ST-MET-1 U515f and U1492r

CCCGACTAAGTTTCTTGGGAAATCTGGCATCTCAAGTGTC
AGGCTGCCAGGGGATACTGGTCGGCTTGGGACCGGGAGAGGTGAGAGGTACCTCGGGGGT
AGGGGTGAAATCTTGTAATCCTCGAGGGACCACCAGTGGCGAAGGCGTCTCACCAGAACG
GATCCGACGGCAAGGGACGAAAGCTAGGGGCACGAACCGGATTAGATACCCGGGTAGTCC
TAGCCGTAAACGATACTCGCTAGGTGTCGGCCACGGTGCGACCGTGGTCGGTGCCGTAGG
GAAGCCGTGAAGCGAGCCACCTGGGAAGTACGGCCGCAAGGCTGAAACTTAAAGGAATTG
GCGGGGGAGCACCACAACGGGTGGAGCCTGCGGTTTAATTGGATTCAACGCCGGAAAGCT
TACCGGGGGCGACAGCAATATGAAGGTCAGGCTGAAGACCTTACCGGATTCGCTGAGAGG
TGGTGCATGGCCGTCGTCAGTTCGTACTGTGAAGCATCCTGTTAAGTCAGGCAACGAGCG
AGACCCACGCCCACAGTTGCCAGCGATCCCTCCGGGGAGGCGGGTACTCTGTGGGGACCG
CCGCTGCTAAAGCGGAGGAAGGAGTGGGCAACGGTAGGTCAGTATGCCCCGAATCCCCCG
GGCTACACGCGGGCTACAATGGTCGGTACAATGGGTATCGACCCCGAAAGGGGTAGGCAA
TCCCCTAAAACCGATCGTAGTTCGGATTGAGGGCTGAAACTCGCCCTCATGAAGCTGGAA
TCCGTAGTAATCGCGTTTCAACAGAACGCGGTGAATACGTCCCTGCTCCTTGCACACACC
GCCCGTCAAACCACCCGAGTAGGGTCCGGATGAGGGTGTATCCTCTTGATACATTCGAAT
CCGTGCTC

ST-MET-1B A341f and U1492r

CAACCGCGATAAGGGGACCTCGAGTGCTGGGTTACAAACCTGGCTGTTGGGCTGCCTAAAA
AGCAGTCTATAGCAAGGGCCGGGCAAGACCGGTGCCAGCCGCCGCGGTAACACCGGCGGCT
CGAGTGGTAACCGCTATTATTGGGTCTAAAGGGTCTGTAGCCGGCCGACTAAGTCTCTTGG
GAAATCTGGCATCTCAAGTGTCAGGCTGCCAGGGGATACTGGTCGGCTTGGGACCGGGAGA
GGTGAGAGGTACCTCGGGGGTAGGGGTGAAATCTTGTAATCCTCGAGGGACCACCAGTGGC
GAAGGCGTCTCACCAGAACGGATCCGACGGCAAGGGACGAAANCTAGGGGCACGAACCGGA
TTAGATACCCGGGTAGTCCTAGCCGTAAACGATACTCGCTAGGTGTCGGCCACGGTGCGAC
CGTGGTCGGTGCCGTAGGGAAGCCGTGAAGCGAGCCACCTGGGAAGTACGGCCGCAAGGCT
GAAACTTAAAGGAATTGGCGGGGGAGCACCACAACGGGTGGAGCCTGCGGTTTAATTGGAT
TCAACGCCGGAAAGCTTACCGGGGGCGACAGCAATATGAAGGTCAGGCTGA

ST-FER-2 U515f and U1492r

TTGTAAAGCACTTTAAGCGAGGAGGAGGCTACCTAGATTAATACTTTAGGATAGTGGACG
TTACTCGCAGAATAAGCACCGGCTAACTCTGTGCCAGCAGCCGCGGTAATACAGAGGGTG
CGAGCGTTAATCGGATTTACTGGGCGTAAAGCGTGCGTAGGCGGCCAATTAAGTCAAATG
TGAAATCCCCGAGCTTAACTTGGGAATTGCATTCGATACTGGTTGGCTAGAGTATGGGAG
AGGATGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGATG
GCGAAGGCAGCCATCTGGCCTAATACTGACGCTGAGGTACGAAAGCATGGGGAGCAAACA
GGATTAGATACCCTGGTAGTCCATGCCGTAAACGATGTCTACTAGCCGTTGGGGCCcTTG
AGGCTTTAGTGGCGCAGCTAACGCGATAAGTAGACCGCCTGGGGAGTACGGtCGCAAGAC
TAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGGAGCATGTGGTTTAATTCG
ATGCAACGCGAAGAACCTTACCTGGNCTTGACATACAGAGAACTTTCCAGAGATGGATTG
GTGNCTTCGGGAACTCTGATACAGGTGCTGCATGGCTGTCGNCAGCTCGTGNCGTGAGAT
GTTGGGTTAAGTCCCNCAACNAGCGCAACCCTTTTTCCTTATTTGCCA

Appendix 2 ST-tSRB-8A growth curve data

Trial 1 data for ST-tSRB-8A growth data grown in CSBA using a 5ml inoculum in 100 ml of media containing 10 mM sulfate, and 30 mM lactate

Time	A600	Sufate Concentration (mM)	Sulfide Concentration (mM)
0	0.015	10.57143	0.538462
26.25	0.012	10.08163	0.769231
49.25	0.015	9.857143	0.512821
65.5	0.024	10.13265	1.538462
87.75	0.04	8.857143	3.025641
91.25	0.035	6.22449	3.564103
95.25	0.045	6.836735	3.666667
113	0.045	6.989796	5.102564
119.5	0.047	5.867347	5.435897
136.5	0.045	4.857143	7.538462
143.5	0.045	3.836735	7.230769
161.5	0.045	2.918367	9.307692
185	0.044	2.183673	10.5641
213.25	0.038	2.204082	9.282051
237.25	0.034	2.244898	9.076923
260.75	0.037	2.285714	8.666667
288.25	0.032	2.387755	9.512821
309.25	0.04	2.265306	9.538462
335.75	0.03	2.469388	10.02564

Trial 2 data for ST-tSRB-8A growth data grown in CSBA using a 5ml inoculum 100 ml of media containing 10 mM sulfate, and 30 mM lactate

Time	A600	Sufate Concentration (mM)	Sulfide Concentration (mM)
0	0.016	9.734694	0.666667
26.25	0.015	9.877551	0.769231
49.25	0.017	10.17347	0.589744
65.5	0.025	9.938776	1.948718
87.75	0.039	9.22449	3.153846
91.25	0.035	7.704082	3.794872
95.25	0.04	6.765306	4.025641
113	0.042	4.55102	5.384615
119.5	0.043	5.255102	5.435897
136.5	0.042	5.071429	7.025641
143.5	0.042	3.183673	6.589744
161.5	0.044	3.142857	8.230769
185	0.044	2.438776	8.487179
213.25	0.032	2.530612	8.487179
237.25	0.033	2.234694	9.153846
260.75	0.033	2.295918	8.717949
288.25	0.032	2.561224	9.410256
309.25	0.04	2.163265	9.025641
335.75	0.03	2.744898	9.410256

Trial 3 data for ST-tSRB-8A growth data grown in CSBA using a 5ml inoculum 100 ml of media containing 10 mM sulfate, and 30 mM lactate

Time	A600	Sufate Concentration (mM)	Sulfide Concentration (mM)
0	0.023	10.87755	0.692308
26.25	0.019	10.66327	0.820513
49.25	0.013	10.73469	0.512821
65.5	0.016	11.30612	0.717949
87.75	0.029	11.20408	1.358974
91.25	0.021	10.4898	2.538462
95.25	0.027	7.897959	1.871795
113	0.039	10	2.769231
119.5	0.036	7.673469	2.846154
136.5	0.042	8.693878	3.74359
143.5	0.042	3.867347	7.051282
161.5	0.042	6.27551	5.282051
185	0.045	5.571429	6.820513
213.25	0.037	4.887755	7.461538
237.25	0.031	3.561224	7.974359
260.75	0.031	4.020408	7.769231
288.25	0.03	4.27551	8.205128
309.25	0.028	4.010204	7.717949
335.75	0.03	4.683673	8.282051

Average and Standard deviations for the growth experiment of ST-tSRB-8A in CSBA containing 10 mM Sulfate and 30mM lactate

Time	Average A600	Standard Deviation of A600	Average Sulfate Concentration	Standard Deviation of Sulfate Concentration	Average Sulfide Concentration	Standard Deviation of Sulfide Concentration
0	0.016	0.001	10.153	0.592	0.632	0.091
26.25	0.014	0.002	9.980	0.144	0.786	0.000
49.25	0.016	0.001	10.015	0.224	0.538	0.054
65.5	0.025	0.001	10.036	0.137	1.402	0.290
87.75	0.040	0.001	9.041	0.260	2.513	0.091
91.25	0.035	0.000	6.964	1.046	3.299	0.163
95.25	0.043	0.004	6.801	0.051	3.188	0.254
113	0.044	0.002	5.770	1.724	4.419	0.199
119.5	0.045	0.003	5.561	0.433	4.573	0.000
136.5	0.044	0.002	4.964	0.152	6.103	0.363
143.5	0.044	0.002	3.510	0.462	6.957	0.453
161.5	0.045	0.001	3.031	0.159	7.607	0.761
185	0.044	0.000	2.311	0.180	8.624	1.469
213.25	0.035	0.004	2.367	0.231	8.410	0.562
237.25	0.034	0.001	2.240	0.007	8.735	0.054
260.75	0.035	0.003	2.291	0.007	8.385	0.036
288.25	0.032	0.000	2.474	0.123	9.043	0.073
309.25	0.040	0.000	2.214	0.072	8.761	0.363
335.75	0.030	0.000	2.607	0.195	9.239	0.435

Acetate concentrations as determined using the HPLCof the growth of ST-tSRB-8A in CSBA containing 10mM sulfate and 30 mM lactate

Time	Acetate Concentration (mM) Trial 1	Acetate Concentration (mM) Trial 2	Average Acetate Concentration	Standard Deviation of Acetate Concentration
0		1.97	1.97	
26.25	1.94	1.58	1.82	0.254558
49.25		2.02	2.02	
65.5	3.12	3.17	3.136667	0.035355
87.75	5.76	5.22	5.58	0.381838
91.25		5.97	5.97	
95.25	6.69	6.47	6.616667	0.155563
113	8.72	8.33	8.59	0.275772
119.5	9.3	8.99	9.196667	0.219203
136.5	11.75	11	11.5	0.53033
143.5	12.72	11.53	12.32333	0.841457
161.5	14.72	13.34	14.26	0.975807
185	15.27	14.5	15.01333	0.544472
213.25	14.86	14.62	14.78	0.169706
237.25	16.15	14.57	15.62333	1.117229
260.75	15.26	14.25	14.92333	0.714178
288.25	14.53	14.38	14.48	0.106066

Trial 1 data for ST-tSRB-8A growth data grown in CSBA using a 5ml inoculum in 100 ml of media containing 10 mM thiosulfate, and 30 mM lactate

Time	A600	Sulfide Concentration (mM)
0	0.011	0.054
26.25	0.011	0.128205
49.25	0.011	0.179487
65.5	0.014	0.102564
87.75	0.036	0.461538
91.25	0.04	0.74359
95.25		1.153846
113	0.049	2.384615
119.5	0.047	3.230769
136.5	0.04	3.74359
143.5	0.036	3.282051
161.5	0.033	3.410256
185	0.025	3.717949
213.25	0.03	3.641026
237.25	0.029	3.307692
260.75	0.031	3.205128
288.25	0.029	3.820513
309.25	0.035	3.641026
335.75	0.028	3.820513

Trial 2 data for ST-tSRB-8A growth data grown in CSBA using a 5ml inoculum in 100 ml of media containing 10 mM thiosulfate, and 30 mM lactate

Time	A600	Sulfide Concentration (mM)
0	0.01	0.05
26.25	0.01	0.13
49.25	0.01	0.13
65.5	0.02	0.15
87.75	0.04	0.87
91.25	0.04	1.28
95.25	0.05	1.00
113	0.05	2.54
119.5	0.04	2.92
136.5	0.04	3.28
143.5	0.03	2.74
161.5	0.03	3.56
185	0.03	3.85
213.25	0.03	3.49
237.25	0.03	3.77
260.75	0.03	
288.25	0.03	3.69
309.25	0.04	3.49
335.75	0.03	3.64

Average and standard deviations for the growth experiment of ST-tSRB-8A in CSBA containing 10 mM Sulfate and 30mM lactate

Time	Average A600	Standard Deviation of A600	Average Sulfide Concentration	Standard Deviation of Sulfide Concentration
0	0.013	0.002	0.053	0.002
26.25	0.012	0.001	0.128	0.000
49.25	0.011	0.001	0.154	0.036
65.5	0.018	0.006	0.128	0.036
87.75	0.038	0.003	0.667	0.290
91.25	0.040	0.000	1.013	0.381
95.25	0.048		1.077	0.109
113	0.047	0.003	2.462	0.109
119.5	0.042	0.007	3.077	0.218
136.5	0.038	0.004	3.513	0.326
143.5	0.035	0.001	3.013	0.381
161.5	0.034	0.001	3.487	0.109
185	0.028	0.004	3.782	0.091
213.25	0.029	0.001	3.564	0.109
237.25	0.029	0.000	3.538	0.326
260.75	0.031	0.001	3.205	
288.25	0.028	0.002	3.756	0.091
309.25	0.036	0.001	3.564	0.109
335.75	0.029	0.001	3.731	0.127

Acetate concentrations as determined using the HPLCof the growth of ST-tSRB-8A in CSBA containing 10mM sulfate and 30 mM lactate

Time	Acetate Concentration (mM) Trial 1	Acetate Concentration (mM) Trial 2	Average Acetate Concentration	Standard Deviation of Acetate Concentration
0				
26.25	1.99	1.94	1.965	0.035355
49.25	1.55	1.99	1.77	0.311127
65.5	1.94		1.94	
87.75	3.53	4.22	3.875	0.487904
91.25	4.08	4.75	4.415	0.473762
95.25	4.71	6.48	5.595	1.251579
113	7.02		7.02	
119.5	7.03	6.07	6.55	0.678823
136.5	7.47	7.62	7.545	0.106066
143.5	7.72	8.12	7.92	0.282843
161.5	7.92	8.03	7.975	0.077782
185	7.8	7.97	7.885	0.120208
213.25	8.35	7.93	8.14	0.296985
237.25		7.94	7.94	
260.75	7.65	7.86	7.755	0.148492
288.25	7.72	7.38	7.55	0.240416

Appendix 3 Nitrate inhibition experiments

NS-tSRB-1 nitrate inhibition experiment no nitrate added bottle 7.

Time (hours)	Redox potential (E_h mV)	Sulfide concentration (mM)	Sulfate concentration (mM)
0	-456	0.03	7.40
187.5	-477	0.23	9.36
338	-584	4.13	5.93
339	-595	4.13	7.84
361	-578	4.87	4.82
407.5	-595	7.23	5.09
454	-603	7.87	3.62
504.5	-605	8.15	2.41
570.5	-610	9.74	2.46
695.5	-605	12.23	1.18
906	-568	11.00	1.35
1605	-583	12.74	0.82

NS-tSRB-1 nitrate inhibition experiment no nitrate added bottle 12.

Time (hours)	Redox potential (E_h mV)	Sulfide concentration (mM)	Sulfate concentration (mM)
0	-408	0.05	10.14
187.5	-468	0.38	7.10
338	-566	4.33	7.20
339	-588	4.05	7.06
361	-591	4.90	5.32
407.5	-596	7.13	4.96
454	-606	7.92	3.12
504.5	-605	9.51	2.36
570.5	-603	10.05	2.76
695.5	-598	11.21	1.13
906	-574	11.33	1.47
1605	-555	11.92	0.95

NS-tSRB-1 nitrate inhibition experiment no nitrate added bottle 13.

Time (hours)	Redox potential (E_h mV)	Sulfide concentration (mM)	Sulfate concentration (mM)
0	-414	0.03	10.06
187.5	-481	0.13	9.15
338	-574	3.82	8.00
339	-588	3.13	7.18
361	-590	4.56	4.68
407.5	-598	7.03	5.04
454	-604	8.38	2.76
504.5	-607	9.56	2.56
570.5	-603	11.08	2.51
695.5	-599	10.67	1.04
906	-573	11.00	1.36
1605	-557	12.56	0.96

NS-tSRB-1 nitrate inhibition experiment no nitrate average redox, sulfide and sulfate values.

Time (hours)	Average redox potential (E_h mV)	Average sulfide concentration (mM)	standard deviation of sulfide concentration	Average sulfate concentration (mM)	standard deviation of sulfate concentration
0	-415	0.03	0.014	9.20	1.56
187.5	-472	0.25	0.13	8.54	1.25
338	-567	4.09	0.26	7.04	1.04
339	-587	3.77	0.56	7.36	.042
361	-591	4.78	0.19	4.94	0.33
407.5	-595	7.13	0.10	5.03	0.07
454	-607	8.06	0.28	3.17	0.44
504.5	-609	9.08	0.80	2.44	0.11
570.5	-600	10.29	0.70	2.57	0.16
695.5	-601	11.37	0.79	1.12	0.07
906	-576	11.11	0.19	1.39	0.07
1605	-557	12.41	0.43	0.91	0.08

NS-tSRB-1 nitrate inhibition experiment 10 mM nitrate added to bottle 1. Nitrate was added at hour 338.

Time (hours)	Redox potential (E_h mV)	Sulfide concentration (mM)	Sulfate concentration (mM)
0	-471	0.23	9.94
187.5	-486	1.00	8.03
338	-567	4.44	
339	-567	4.90	5.45
361	-593	5.82	5.72
407.5	-595	6.31	3.94
454	-595	7.31	3.93
504.5	-605	8.21	4.27
570.5	-605	8.28	3.13
695.5	-601	8.49	2.27
906	-591	9.90	1.78

NS-tSRB-1 nitrate inhibition experiment 10 mM nitrate added to bottle 14. Nitrate was added at hour 338.

Time (hours)	Redox potential (E_h mV)	Sulfide concentration (mM)	Sulfate concentration (mM)
0	-422	0.03	9.65
187.5	-466	0.21	9.65
338	-561	4.41	7.79
339	-586	4.13	7.29
361	-592	5.10	3.89
407.5	-591	6.56	5.17
454	-610	8.08	3.04
504.5	-616	9.49	2.95
570.5	-594	8.28	2.44
695.5	-606	10.59	1.22
906	-580	11.41	1.41
1605	-559	9.59	1.19

NS-tSRB-1 nitrate inhibition experiment 10 mM nitrate added to bottle 15. Nitrate was added at hour 338.

Time (hours)	Redox potential (E_h mV)	Sulfide concentration (mM)	Sulfate concentration (mM)
0	-409	0.03	10.15
187.5	-488	0.77	9.07
338	-568	4.82	7.09
339	-585	4.00	7.23
361	-594	5.05	5.18
407.5	-583	5.67	5.42
454	-597	6.74	3.84
504.5	-603	7.87	4.02
570.5	-588	7.49	3.64
695.5	-601	9.95	2.51
906	-576	9.51	2.14
1605	-554	9.97	1.69

NS-tSRB-1 nitrate inhibition experiment 10 mM nitrate addition average redox, sulfide and sulfate values. Nitrate was added at hour 338.

Time (hours)	Average redox potential (E_h mV)	Average sulfide concentration (mM)	standard deviation of sulfide concentration	Average sulfate concentration (mM)	standard deviation of sulfate concentration
0	-434	0.09	0.12	9.91	0.25
187.5	-480	0.66	0.41	8.92	0.82
338	-565	4.56	0.23	7.44	0.49
339	-588	4.34	0.49	6.66	1.05
361	-594	5.32	0.43	4.93	0.94
407.5	-590	6.18	0.46	4.84	0.79
454	-604	7.38	0.67	3.60	0.49
504.5	-608	8.52	0.85	3.74	0.70
570.5	-594	8.02	0.46	3.07	0.60
695.5	-599	9.68	1.08	2.00	0.68
906	-565	10.27	1.00	1.78	0.37
1605	-546	9.78	0.27	1.44	0.35

NS-tSRB-2 nitrate inhibition experiment 0 mM nitrate added to bottle 12

Time (hours)	Redox potential (E_h mV)	Sulfide concentration (mM)	Sulfate concentration (mM)
0	-370	0.13	9.17
96	-449	0.90	10.17
174	-474	2.74	8.21
197	-492	4.18	7.57
218	-494	6.23	5.31
246	-521	7.18	2.33
270	-494	10.72	1.97
319	-581	11.77	1.15
362	-566	11.51	1.07
460	-540	11.46	0.97
532	-561	12.18	0.76
696	-556	11.15	0.96
939	-544	3.66	3.66

NS-tSRB-2 nitrate inhibition experiment 0 mM nitrate added to bottle 9

Time (hours)	Redox potential (E_h mV)	Sulfide concentration (mM)	Sulfate concentration (mM)
0	-453	0.08	10.19
96	-497	0.85	9.34
174	-513	2.95	9.10
197	-487	4.38	7.14
218	-528	6.28	4.80
246	-489	7.87	3.41
270	-578	10.59	1.99
319	-570	12.38	1.11
362	-552	13.87	0.94
460	-543	11.10	1.01
532	-548	10.69	0.79
696	-548	10.64	1.02
939		11.44	0.83

NS-tSRB-2 nitrate inhibition experiment no nitrate average redox, sulfide and sulfate values.

Time (hours)	Average redox potential (E_h mV)	Average sulfide concentration (mM)	standard deviation of sulfide concentration	Average sulfate concentration (mM)	standard deviation of sulfate concentration
0	-412	0.10	0.04	9.68	0.72
96	-473	0.87	0.04	9.76	0.59
174	-494	2.85	0.15	8.66	0.63
197	-490	4.28	0.15	7.36	0.30
218	-511	6.26	0.04	5.05	0.36
246	-505	7.53	0.49	2.87	0.76
270	-536	10.65	0.09	1.98	0.01
319	-576	12.08	0.44	1.13	0.03
362	-559	12.69	1.67	1.01	0.09
460	-542	11.28	0.25	0.99	0.03
532	-555	11.44	1.05	0.77	0.02
696	-552	10.90	0.36	0.99	0.04
939	-544	11.28	0.22	2.24	2.01

NS-tSRB-2 bottle 8 2 mM nitrate. Nitrate was added hour 218.

Time (hours)	Redox potential (E_h mV)	Sulfide concentration (mM)	Sulfate concentration (mM)
0	-395	0.05	10.20
96	-461	0.67	11.88
174	-478	1.82	9.53
197	-500	3.87	6.96
218	-478	3.87	6.29
246	-522	4.38	6.79
270	-532	5.62	4.37
319	-492	7.33	3.62
362	-573	13.67	1.65
460	-569	11.13	1.20
532	-526	8.97	0.84
696	-774	9.28	1.22
939	-714	10.03	1.60

NS-tSRB-2 bottle 4 2 mM nitrate. Nitrate was added hour 218

Time (hours)	Redox potential (E_h mV)	Sulfide concentration (mM)	Sulfate concentration (mM)
0	-366	0.05	9.07
96	-455	0.38	9.82
174	-464	1.59	9.27
197	-502	2.67	8.20
218	-494	4.31	6.45
246	-526	3.85	6.90
270	-518	5.38	5.80
319	-498	6.87	4.23
362	-581	10.77	
460	-564	10.90	1.04
532	-499	7.62	0.81
696	-768	11.72	0.82
939	-776	10.10	0.87

NS-tSRB-2 nitrate inhibition experiment 2 mM nitrate addition average redox, sulfide and sulfate values. Nitrate was added hour 218.

Time (hours)	Average redox potential (E_h mV)	Average sulfide concentration (mM)	standard deviation of sulfide concentration	Average sulfate concentration (mM)	standard deviation of sulfate concentration
0	-381	0.05	0.00	9.64	0.80
96	-458	0.53	0.20	10.85	1.46
174	-471	1.71	0.16	9.40	0.19
197	-501	3.27	0.85	7.58	0.88
218	-486	4.09	0.31	6.37	0.12
246	-524	4.12	0.38	6.84	0.08
270	-525	5.50	0.16	5.08	1.01
319	-495	7.10	0.33	3.93	0.43
362	-577	12.22	2.05	1.65	
460	-567	11.01	0.16	1.12	0.12
532	-513	8.29	0.96	0.82	0.02
696	-771	10.50	1.72	1.02	0.29
939	-745	10.06	0.05	1.23	0.52

NS-tSRB-2 bottle 10 10mM nitrate. Nitrate was added hour 218

Time (hours)	Redox potential (E_h mV)	Sulfide concentration (mM)	Sulfate concentration (mM)
0	-487	0.08	9.63
96	-439	0.72	8.68
174	-483	2.59	8.50
197	-509	4.03	7.14
218	-498	5.21	5.71
246	-521	5.21	6.06
270	-528	7.08	4.27
319	-518	9.64	3.08
362	-580	10.77	1.85
460	-573	11.46	1.66
532	-532	10.87	1.12
696	-756	11.21	0.93
939	-778	10.03	1.40

NS-tSRB-2 bottle 17 10 mM nitrate. Nitrate was added hour 218.

Time (hours)	Redox potential (E_h mV)	Sulfide concentration (mM)	Sulfate concentration (mM)
0	-369	0.21	9.21
96	-458	1.15	10.10
174		3.13	7.42
197	-492	3.18	7.27
218	-478	4.95	6.49
246	-514	4.03	6.92
270	-514	3.41	6.88
319	-502	4.62	5.21
362	-563	6.00	5.28
460	-558	6.31	5.18
532	-511	7.59	3.50
696	-517	7.92	1.61
939	-538	10.05	1.89

NS-tSRB-2 nitrate inhibition experiment 10 mM nitrate addition average redox, sulfide and sulfate values. Nitrate was added hour 218.

Time (hours)	Average redox potential (E_h mV)	Average sulfide concentration (mM)	standard deviation of sulfide concentration	Average sulfate concentration (mM)	standard deviation of sulfate concentration
0	-428	0.14	0.09	9.42	0.30
96	-449	0.94	0.31	9.39	1.00
174	-483	2.86	0.38	7.96	0.76
197	-500	3.60	0.60	7.20	0.09
218	-488	5.08	0.18	6.10	0.55
246	-517	4.62	0.83	6.49	0.61
270	-521	5.24	2.59	5.57	1.85
319	-510	7.13	3.55	4.15	1.51
362	-572	8.38	3.37	3.56	2.42
460	-566	8.88	3.64	3.42	2.49
532	-522	9.23	2.32	2.31	1.68
696	-637	9.56	2.32	1.27	0.48
939	-658	10.04	0.02	1.64	0.35

St-3 Nitrate inhibition experiment no nitrate added bottle 2

Time (hours)	Redox potential (E_h mV)	Sulfide concentration (mM)	Sulfate concentration (mM)
0	-355	0.08	6.57
9.5	-457	0.15	6.64
21	-475	0.28	6.03
28	-492	0.41	5.78
34.5	-504	0.95	5.91
42.5	-516	1.67	5.14
51	-503	7.97	2.87
69	-532	4.26	2.22
103	-540	4.67	2.52
146	-534	4.15	4.34
195	-543	4.82	2.14
240.5	-529	4.46	2.07
553.5	-515	4.95	2.09

St-3 Nitrate inhibition experiment no nitrate added bottle 4

Time (hours)	Redox potential (E_h mV)	Sulfide concentration (mM)	Sulfate concentration (mM)
0	-391	0.03	6.73
9.5	-468	0.21	7.12
21	-497	0.49	5.18
28	-494	0.62	7.02
34.5	-508	1.64	5.71
42.5	-508	1.87	5.00
51	-517	6.64	3.33
69	-538	4.69	2.71
103	-540	4.97	2.94
146	-535	4.10	
195	-547	5.08	2.51
240.5	-526	4.13	2.27
553.5	-531	4.64	2.66

ST-3 nitrate inhibition experiment no nitrate added average redox, sulfide and sulfate values.

Time (hours)	Average redox potential (E_h mV)	Average sulfide concentration (mM)	standard deviation of sulfide concentration	Average sulfate concentration (mM)	standard deviation of sulfate concentration
0	-373	0.05	0.04	6.65	0.12
9.5	-463	0.18	0.04	6.88	0.34
21	-486	0.38	0.15	5.61	0.60
28	-493	0.51	0.15	6.40	0.88
34.5	-506	1.29	0.49	5.81	0.14
42.5	-512	1.77	0.15	5.07	0.10
51	-510	7.31	0.94	3.10	0.32
69	-535	4.47	0.31	2.47	0.35
103	-540	4.82	0.22	2.73	0.30
146	-535	4.13	0.04	2.31	2.86
195	-545	4.95	0.18	2.33	0.26
240.5	-527	4.29	0.24	2.17	0.14
553.5	-523	4.79	0.22	2.38	0.40

St-3 Nitrate inhibition experiment 5 mM nitrate added bottle 1. Nitrate was added hour number 28.

Time (hours)	Redox potential (E_h mV)	Sulfide concentration (mM)	Sulfate concentration (mM)
0	-353	0	0
9.5	-455	9.5	9.5
21	-500	21	21
28	-541	28	28
28.5	-546	28.5	28.5
34.5	-533	34.5	34.5
42.5	-531	42.5	42.5
51	-498	51	51
69	-537	69	69
103	-547	103	103
146	-533	146	146
195	-551	195	195
240.5	-557	240.5	240.5
553.5	-531	553.5	553.5

St-3 Nitrate inhibition experiment 5 mM nitrate added bottle 9. St-3. Nitrate was added hour number 28.

Time (hours)	Redox potential (E_h mV)	Sulfide concentration (mM)	Sulfate concentration (mM)
0	-417	0.13	6.86
9.5	-463	0.13	8.16
21	-505	0.44	6.42
28	-542	4.28	2.48
28.5	-533	5.67	2.91
34.5	-540	5.33	3.19
42.5	-534	5.54	2.19
51	-548	8.10	2.87
69	-556	4.77	2.87
103	-551	5.13	2.30
146	-545	4.62	0.29
195	-533	4.41	2.89
240.5	-544	4.21	3.15
553.5		2.92	3.28

ST-3 nitrate inhibition experiment 5 mM nitrate added average redox, sulfide and sulfate values. Nitrate was added hour number 28.

Time (hours)	Average redox potential (E_h mV)	Average sulfide concentration (mM)	standard deviation of sulfide concentration	Average sulfate concentration (mM)	standard deviation of sulfate concentration
0	-385	0.08	0.07	6.64	0.30
9.5	-459	0.14	0.02	8.16	
21	-502	0.54	0.15	3.35	4.34
28	-542	4.44	0.22	2.51	0.04
28.5	-539	6.18	0.73	2.72	0.27
34.5	-537	5.67	0.47	2.65	0.77
42.5	-533	5.44	0.15	1.94	0.35
51	-523	7.97	0.18	2.45	0.58
69	-547	5.21	0.62	2.38	0.69
103	-549	5.90	1.09	2.28	0.03
146	-539	4.81	0.27	1.87	2.24
195	-542	5.14	1.03	2.45	0.61
240.5	-550	4.99	1.11	2.73	0.59
553.5	-531	4.55	2.30	2.66	0.87

St-3 Nitrate inhibition experiment 10 mM nitrate added bottle 8. Nitrate was added hour number 51.

Time (hours)	Redox potential (E_h mV)	Sulfide concentration (mM)	Sulfate concentration (mM)
0	-402	0.10	6.37
9.5	-467	0.15	7.88
21	-494	0.26	6.04
28	-494	0.54	6.10
34.5	-501	0.92	7.39
42.5	-511	1.74	11.87
51	-524	5.67	2.57
51.5	-527	4.87	2.31
69	-538	4.67	2.96
103	-535	4.28	3.20
146	-537	4.51	
195	-541	4.00	3.04
240.5	-537	3.72	3.17
553.5	-546	3.49	3.22

St-3 Nitrate inhibition experiment 10 mM nitrate added bottle 10. Nitrate was added hour number 51.

Time (hours)	Redox potential (E_h mV)	Sulfide concentration (mM)	Sulfate concentration (mM)
0	-397	0.18	4.93
9.5	-467	0.36	6.48
21	-489	0.38	6.29
28	-507	1.46	6.08
34.5	-498	0.92	4.40
42.5	-511	1.38	5.47
51	-522	6.74	3.50
51.5	-530	4.56	2.65
69	-542	4.97	2.86
103	-545	3.69	3.12
146	-525	4.95	
195	-545	5.28	2.69
240.5	-537	5.82	2.69
553.5	-556	5.28	3.16

ST-3 nitrate inhibition experiment 10 mM nitrate added average redox, sulfide and sulfate values. Nitrate was added hour number 51.

Time (hours)	Average redox potential (E_h mV)	Average sulfide concentration (mM)	standard deviation of sulfide concentration	Average sulfate concentration (mM)	standard deviation of sulfate concentration
0	-399	0.14	0.05	5.65	1.02
9.5	-467	0.26	0.15	7.18	0.99
21	-491	0.32	0.09	6.16	0.17
28	-500	1.00	0.65	6.09	0.01
34.5	-499	0.92	0.00	5.89	2.11
42.5	-511	1.56	0.25	8.67	4.52
51	-523	6.21	0.76	3.04	0.66
51.5	-528	4.72	0.22	2.48	0.25
69	-540	4.82	0.22	2.91	0.07
103	-540	3.99	0.42	3.16	0.06
146	-531	4.73	0.31		
195	-543	4.64	0.91	2.87	0.25
240.5	-537	4.77	1.49	2.93	0.34
553.5	-551	4.38	1.27	3.19	0.04

St-3 Nitrate inhibition experiment 15 mM nitrate added bottle 3. Nitrate was added hour number 51.

Time (hours)	Redox potential (E_h mV)	Sulfide concentration (mM)	Sulfate concentration (mM)
0	-384	0.23	6.35
9.5	-478	0.23	3.34
21	-500	0.51	6.03
28	-493	0.46	4.10
34.5	-511	0.77	6.90
42.5	-516	1.15	4.66
51	-513	5.90	3.70
51.5	-525	4.67	3.87
69	-538	4.13	3.18
103	-542	4.51	3.06
146	-531	3.72	4.62
195	-544	5.36	2.33
240.5	-542	3.18	2.90
553.5	-534	2.74	3.18

St-3 Nitrate inhibition experiment 15 mM nitrate added bottle 5. Nitrate was added hour number 51.

Time (hours)	Redox potential (E_h mV)	Sulfide concentration (mM)	Sulfate concentration (mM)
0	-403	0.18	5.64
9.5	-471	0.10	6.53
21	-502	0.54	4.27
28	-498	0.56	6.37
34.5	-508	0.69	4.60
42.5	-510	1.05	3.59
51	-520	6.15	3.66
51.5	-530	4.64	4.22
69	-545	4.26	2.93
103	-537	4.56	2.70
146	-545	3.72	
195	-543	4.21	3.01
240.5	-537	3.72	2.80
553.5	-540	3.95	3.23

St-3 Nitrate inhibition experiment 15 mM nitrate added bottle 11. Nitrate was added hour number 51.

Time (hours)	Redox potential (E_h mV)	Sulfide concentration (mM)	Sulfate concentration (mM)
0	-425	0.15	6.22
9.5	-463	0.03	6.86
21	-463	0.33	6.29
28	-495	0.69	7.03
34.5	-519	0.85	7.31
42.5	-520	1.87	5.48
51	-537	4.49	3.08
51.5	-541	5.67	3.38
69	-538	4.28	3.57
103	-543	5.15	3.18
146	-542	4.67	0.29
195	-538	4.85	2.93
240.5	-538	4.00	3.33
553.5		3.36	3.42

ST-3 nitrate inhibition experiment 15 mM nitrate added average redox, sulfide and sulfate values.

Time (hours)	Average redox potential (E_h mV)	Average sulfide concentration (mM)	standard deviation of sulfide concentration	Average sulfate concentration (mM)	standard deviation of sulfate concentration
0	-404	0.19	0.04	6.07	0.38
9.5	-470	0.12	0.10	5.57	1.95
21	-488	0.46	0.11	5.53	1.10
28	-495	0.57	0.12	5.83	1.54
34.5	-513	0.77	0.08	6.27	1.46
42.5	-515	1.36	0.45	4.58	0.95
51	-523	5.51	0.90	3.48	0.35
51.5	-532	4.99	0.58	3.82	0.43
69	-540	4.22	0.08	3.23	0.32
103	-541	4.74	0.36	2.98	0.25
146	-539	4.03	0.55	2.45	3.07
195	-542	4.80	0.58	2.76	0.37
240.5	-539	3.63	0.42	3.01	0.28
553.5	-537	3.35	0.60	3.28	0.12

Appendix 4 Nitrite Inhibition Experiments

NS-tSRB-1 nitrite inhibition experiment no nitrite added bottle 13

Time (hours)	Redox potential (E_h mV)	Sulfide concentration (mM)	Sulfate concentration (mM)
0	-414	0.03	10.06
187.5	-481	0.13	9.15
338	-574	3.82	8.00
339	-588	3.13	7.18
361	-590	4.56	4.68
407.5	-598	7.03	5.04
454	-604	8.38	2.76
504.5	-607	9.56	2.56
574.5	-603	11.08	2.51
695.5	-599	10.67	1.04
906.5	-573	11.00	1.36
1605	-557	12.56	0.96

NS-tSRB-1 nitrite inhibition experiment no nitrite added bottle 7

Time (hours)	Redox potential (E_h mV)	Sulfide concentration (mM)	Sulfate concentration (mM)
0	-456	0.03	7.40
187.5	-477	0.23	9.36
338	-584	4.13	5.93
339	-595	4.13	7.84
361	-578	4.87	4.82
407.5	-595	7.23	5.09
454	-603	7.87	3.62
504.5	-605	8.15	2.41
574.5	-610	9.74	2.46
695.5	-605	12.23	1.18
906.5	-568	11.00	1.35
1605	-583	12.74	0.82

NS-tSRB-1 nitrite inhibition experiment no nitrite added bottle 16

Time (hours)	Redox potential (E_h mV)	Sulfide concentration (mM)	Sulfate concentration (mM)
0	-432	0.05	8.73
187.5	-457	0.10	
338	-553	2.44	8.53
339	-575	3.00	7.92
361	-580	4.41	5.33
407.5	-585	6.23	5.62
454	-599	8.51	3.34
504.5	-604	9.67	2.50
574.5	-608	10.67	2.35
695.5	-615	11.18	0.99
906.5	-573	11.62	1.36
1605	-556	12.31	1.15

NS-tSRB-1 nitrite inhibition experiment no nitrite added average redox, sulfide and sulfate values

Time (hours)	Average redox potential (E_h mV)	Average sulfide concentration (mM)	standard deviation of sulfide concentration	Average sulfate concentration (mM)	standard deviation of sulfate concentration
0	-434	0.03	0.01	8.73	1.33
187.5	-472	0.15	0.07	9.26	0.14
338	-570	3.46	0.90	7.49	1.37
339	-586	3.42	0.62	7.65	0.40
361	-583	4.62	0.24	4.94	0.34
407.5	-593	6.83	0.53	5.25	0.32
454	-602	8.26	0.34	3.24	0.44
504.5	-605	9.13	0.85	2.49	0.08
574.5	-607	10.50	0.68	2.44	0.08
695.5	-606	11.36	0.80	1.07	0.10
906.5	-571	11.21	0.36	1.35	0.01
1605	-565	12.54	0.22	0.98	0.17

NS-tSRB-1 nitrite inhibition experiment 0.25 mM nitrite added bottle 2. Nitrite was added at hour number 338.

Time (hours)	Redox potential (E_h mV)	Sulfide concentration (mM)	Sulfate concentration (mM)	Nitrite concentration (mM)
0	-462	0.23	9.67	
187.5	-499	0.46	8.60	
338	-580	5.05	6.43	
339	-595	4.41	6.10	0.19
361	-575	4.56	4.51	0.10
407.5	-595		6.26	0.02
454	-600	4.15	4.01	0.00
504.5	-606	4.26	5.62	0
574.5	-595	4.33	5.52	0
695.5	-578	4.64	5.73	0
906.5	-549	4.00	5.18	0
1605	-524	4.46	4.49	0

NS-tSRB-1 nitrite inhibition experiment 0.25 mM nitrite added bottle 4. Nitrite was added at hour number 338.

Time (hours)	Redox potential (E_h mV)	Sulfide concentration (mM)	Sulfate concentration (mM)	Nitrite concentration (mM)
0	-451	0.31	9.36	
187.5	-494	0.44	8.97	
338	-583	5.03	6.49	
339	-603	4.31	5.67	0.18
361	-576	4.67	4.37	0.09
407.5	-605	4.92	5.97	0
454	-615	4.31	5.47	0
504.5	-605	3.49	5.61	0
574.5	-600	4.38	4.90	0
695.5	-580	4.56	5.44	0
906.5	-555	4.23	5.44	0
1605	-532	4.33	5.10	0

NS-tSRB-1 nitrite inhibition experiment 0.25 mM nitrite added bottle 5. Nitrite was added at hour number 338.

Time (hours)	Redox potential (E_h mV)	Sulfide concentration (mM)	Sulfate concentration (mM)	Nitrite concentration (mM)
0	-453	0.41	10.13	
187.5	-498	0.79	8.93	
338	-586	5.08	6.42	
339	-603	4.08	6.39	0.19
361	-580	5.00	4.31	0.05
407.5	-596	5.05	6.13	0
454	-605	4.82	5.22	0
504.5	-603	4.72	4.94	0
574.5	-593	4.38	4.55	0
695.5	-584	4.41	5.95	0
906.5	-554	4.49	5.21	0
1605	-532	4.74	4.82	0

NS-tSRB-1 nitrite inhibition experiment 0.25 mM nitrite added average redox, sulfide and sulfate values. Nitrite was added at hour number 338.

Time (hours)	Average redox potential (E_h mV)	Average sulfide concentration (mM)	standard deviation of sulfide concentration	Average sulfate concentration (mM)	standard deviation of sulfate concentration	Average Nitrite concentration (mM)	standard deviation of Nitrite concentration
0	-455	0.32	0.09	9.72	0.39		
187.5	-497	0.56	0.20	8.83	0.20		
338	-583	5.05	0.03	6.45	0.04		
339	-600	4.26	0.17	6.05	0.36	0.18	0.01
361	-577	4.74	0.23	4.39	0.10	0.08	0.03
407.5	-599	4.99	0.09	6.12	0.14	0	0
454	-607	4.43	0.35	4.90	0.78	0	0
504.5	-605	4.15	0.62	5.39	0.39	0	0
574.5	-596	4.37	0.03	4.99	0.49	0	0
695.5	-581	4.54	0.12	5.71	0.26	0	0
906.5	-553	4.24	0.24	5.28	0.14	0	0
1605	-529	4.51	0.21	4.80	0.31	0	0

NS-tSRB-1 nitrite inhibition experiment 0.5 mM nitrite added bottle 6. Nitrite was added at hour number 338.

Time (hours)	Redox potential (E_h mV)	Sulfide concentration (mM)	Sulfate concentration (mM)	Nitrite concentration (mM)
0	-446	0.03	9.69	
187.5	-484	0.33	5.93	
338	-588	4.69	7.27	
339	-601	3.59	6.21	0.52
361	-583	3.97	5.99	0.36
407.5	-594	3.67	7.07	0
454	-596	3.51	5.91	0
504.5	-596	3.26	5.76	0
574.5	-588	3.56	6.62	0
695.5	-583	3.23	6.78	0
906.5	-562	3.08	6.02	0
1605	-551	3.33	5.51	0

NS-tSRB-1 nitrite inhibition experiment 0.5 mM nitrite added bottle 8. Nitrite was added at hour number 338.

Time (hours)	Redox potential (E_h mV)	Sulfide concentration (mM)	Sulfate concentration (mM)	Nitrite concentration (mM)
0	-433	0.03	8.70	
187.5	-483	0.31	8.08	
338	-572	4.85	6.63	
339	-603	3.51	6.43	0.48
361	-583	3.87	4.37	0.35
407.5	-596	3.85	7.32	0
454	-597	3.62	4.89	0
504.5	-599	2.64	6.59	0
574.5	-592	3.05	6.65	0
695.5	-584	3.49	6.48	0
906.5	-552	3.23	5.84	0
1605	-556	4.36	5.70	0

NS-tSRB-1 nitrite inhibition experiment 0.5 mM nitrite added average redox, sulfide and sulfate values. Nitrite was added at hour number 338.

Time (hours)	Average redox potential (E_h mV)	Average sulfide concentration (mM)	standard deviation of sulfide concentration	Average sulfate concentration (mM)	standard deviation of sulfate concentration	Average Nitrite concentration (mM)	standard deviation of Nitrite concentration
0	-440	0.03	0.00	9.20	0.70		
187.5	-484	0.32	0.02	7.01	1.52		
338	-580	4.77	0.11	6.95	0.45		
339	-602	3.55	0.05	6.32	0.15	0.50	0.03
361	-583	3.92	0.07	5.18	1.15	0.36	0.01
407.5	-595	3.76	0.13	7.19	0.17	0	0
454	-597	3.56	0.07	5.40	0.72	0	0
504.5	-598	2.95	0.44	6.17	0.59	0	0
574.5	-590	3.31	0.36	6.64	0.02	0	0
695.5	-584	3.36	0.18	6.63	0.21	0	0
906.5	-557	3.15	0.11	5.93	0.13	0	0
1605	-554	3.85	0.73	5.61	0.14	0	0

NS-tSRB-1 nitrite inhibition experiment 1 mM nitrite added bottle 9. Nitrite was added at hour number 338.

Time (hours)	Redox potential (E_h mV)	Sulfide concentration (mM)	Sulfate concentration (mM)	Nitrite concentration (mM)
0	-451	0.03	10.06	
187.5	-497	0.79	8.22	
338	-576	4.79	6.94	
339	-593	3.74	6.63	0.87
361	-601	2.67	3.55	0.59
407.5	-585	2.92	7.59	0.05
454	-585	2.41	7.05	0
504.5	-584	2.54	5.68	0
574.5	-575	2.54	6.59	0
695.5	-574	1.72	6.95	0
906.5	-549	1.87	5.63	0
1605	-546	3.36	5.28	0

NS-tSRB-1 nitrite inhibition experiment 1 mM nitrite added bottle 10. Nitrite was added at hour number 338.

Time (hours)	Redox potential (E_h mV)	Sulfide concentration (mM)	Sulfate concentration (mM)	Nitrite concentration (mM)
0	-415	0.03	9.96	
187.5	-481	0.18	8.83	
338	-574	4.59	6.62	
339	-594	3.28	6.85	1.19
361	-595	2.87	5.21	0.64
407.5	-585	2.82	6.60	0.06
454	-565	1.95	6.04	0
504.5	-583	2.21	6.17	0
574.5	-576	2.15	6.65	0
695.5	-569	2.28	5.89	0
906.5	-543	1.56	5.81	0
1605	-547	2.31	5.59	0

NS-tSRB-1 nitrite inhibition experiment 1 mM nitrite added bottle 11. Nitrite was added at hour number 338.

Time (hours)	Redox potential (E_h mV)	Sulfide concentration (mM)	Sulfate concentration (mM)	Nitrite concentration (mM)
0	-435	0.05	10.45	
187.5	-488	0.26	7.92	
338	-572	4.64	6.88	
339	-593	3.54	6.79	0.65
361	-593	3.64	5.17	0.06
407.5	-583	2.54	6.78	0.03
454	-579	2.21	5.27	0.03
504.5	-579	2.26	6.15	0
574.5	-568	1.74	7.17	0
695.5	-567	1.46	4.95	0
906.5	-554	1.51	5.98	0
1605	-542	1.62	6.67	0

NS-tSRB-1 nitrite inhibition experiment 1 mM nitrite added average redox, sulfide and sulfate values. Nitrite was added at hour number 338.

Time (hours)	Average redox potential (E_h mV)	Average sulfide concentration (mM)	standard deviation of sulfide concentration	Average sulfate concentration (mM)	standard deviation of sulfate concentration	Average Nitrite concentration (mM)	standard deviation of Nitrite concentration
0	-434	0.03	0.01	10.16	0.26		
187.5	-489	0.41	0.34	8.32	0.46		
338	-574	4.68	0.11	6.81	0.17		
339	-593	3.52	0.23	6.76	0.11	0.90	0.27
361	-596	3.06	0.51	4.65	0.95	0.43	0.32
407.5	-584	2.76	0.20	6.99	0.53	0.05	0.02
454	-576	2.19	0.23	6.12	0.90	0	0
504.5	-582	2.33	0.18	6.00	0.28	0	0
574.5	-573	2.15	0.40	6.81	0.32	0	0
695.5	-570	1.82	0.42	5.93	1.00	0	0
906.5	-549	1.65	0.19	5.81	0.17	0	0
1605	-545	2.43	0.88	5.85	0.73	0	0

NS-tSRB-2 nitrite inhibition experiment no nitrite added bottle 11

Time (hours)	Redox potential (E_h mV)	Sulfide concentration (mM)	Sulfate concentration (mM)
0	-355	0.69	11.33
23.5	-361	0.36	11.00
48.5	-356	0.33	10.48
72.5	-345	0.36	10.17
96.25	-341	0.54	10.45
120.5	-351	0.41	10.17
167.5	-334	0.44	10.41
192.5	-355	0.51	10.38
262.75	-336	1.00	10.11
312	-455	1.10	9.62
384.5	-404	2.13	10.01
456	-404	2.21	8.94
479	-407	2.97	7.56
480.5	-412	2.62	7.88
502.75	-428	3.38	8.18
548	-506	5.38	6.84
601.5	-507	6.79	4.94
668.5	-513	9.79	3.08
740.5	-527	12.10	2.18
816	-540	11.46	1.39
913.75	-552	11.44	1.69
1080.5	-557	11.79	1.32
1416.75	-588	11.79	1.36

NS-tSRB-2 nitrite inhibition experiment no nitrite added bottle 11

Time (hours)	Redox potential (E_h mV)	Sulfide concentration (mM)	Sulfate concentration (mM)
0	0	0.62	10.71
23.5	23.5	0.28	10.49
48.5	48.5	0.33	10.99
72.5	72.5	0.31	10.79
96.25	96.25	0.59	9.98
120.5	120.5	0.49	9.70
167.5	167.5	0.97	9.63
192.5	192.5	1.21	10.17
262.75	262.75	1.03	9.41
312	312	1.18	9.51
384.5	384.5	1.33	8.39
456	456	4.51	7.60
479	479	4.90	6.93
480.5	480.5	5.00	6.54
502.75	502.75	5.44	5.88
548	548	6.51	5.64
601.5	601.5	5.90	4.34
668.5	668.5	8.28	3.68
740.5	740.5	9.23	2.67
816	816	10.15	2.37
913.75	913.75	10.44	2.04
1080.5	1080.5	10.79	1.51
1416.75	1416.75	11.18	1.32

NS-tSRB-2 nitrite inhibition experiment no nitrite added average redox, sulfide and sulfate values

Time (hours)	Average redox potential (E_h mV)	Average sulfide concentration (mM)	standard deviation of sulfide concentration	Average sulfate concentration (mM)	standard deviation of sulfate concentration
0	-351	0.65	0.05	11.02	0.43
23.5	-359	0.32	0.05	10.74	0.36
48.5	-356	0.33	0.00	10.73	0.36
72.5	-340	0.33	0.04	10.48	0.43
96.25	-343	0.56	0.04	10.21	0.33
120.5	-350	0.45	0.05	9.94	0.33
167.5	-341	0.71	0.38	10.02	0.55
192.5	-364	0.86	0.49	10.28	0.14
262.75	-354	1.01	0.02	9.76	0.50
312	-456	1.14	0.05	9.57	0.08
384.5	-412	1.73	0.56	9.20	1.15
456	-420	3.36	1.63	8.27	0.95
479	-411	3.94	1.36	7.24	0.45
480.5	-427	3.81	1.69	7.21	0.95
502.75	-430	4.41	1.45	7.03	1.63
548	-513	5.95	0.80	6.24	0.84
601.5	-503	6.35	0.63	4.64	0.43
668.5	-512	9.04	1.07	3.38	0.43
740.5	-525	10.67	2.03	2.43	0.35
816	-530	10.81	0.92	1.88	0.69
913.75	-542	10.94	0.71	1.87	0.25
1080.5	-539	11.29	0.71	1.41	0.14
1416.75	-536			1.57	0.09

NS-tSRB-2 nitrite inhibition experiment 0.25 mM nitrite added bottle 3. Nitrite was added at hour number 479.

Time (hours)	Redox potential (E_h mV)	Sulfide concentration (mM)	Sulfate concentration (mM)	Nitrite concentration (mM)
0	-367	0.56	5.31	
23.5	-349	0.31	11.20	
48.5	-387	0.51	10.79	
72.5	-362	0.49	10.36	
96.25	-382	0.79	9.73	
120.5	-371	0.28	9.17	
167.5	-366	0.59	10.73	
192.5	-378	0.44	10.20	
262.75	-393	0.74	9.57	
312	-461	1.08	9.44	
384.5	-409	1.44	9.52	
456	-388	2.23	7.95	
479	-437	3.23	8.67	
480.5	-443	2.03	6.70	0.22
502.75	-419	2.44	7.86	0.23
548	-458	1.92	8.23	0.02
601.5	-529	2.56	8.52	0.09
668.5	-564	2.36	8.61	0.03
740.5	-553	2.13	8.19	0.04
816	-575	2.21	8.53	0.00
913.75	-546	2.13	8.96	0.00
1080.5	-548	1.92	8.53	0.00
1245	-518	4.44	9.08	0.00
1416.75	-572	2.82	8.73	0.00

NS-tSRB-2 nitrite inhibition experiment 0.25 mM nitrite added bottle 10. Nitrite was added at hour number 479.

Time (hours)	Redox potential (E_h mV)	Sulfide concentration (mM)	Sulfate concentration (mM)	Nitrite concentration (mM)
0	-381	0.82	9.65	
23.5	-367	0.49	9.89	
48.5	-392	0.49	10.21	
72.5	-348	0.36	10.23	
96.25	-377	1.10	9.40	
120.5	-380	0.59	9.69	
167.5	-356	0.38	10.72	
192.5	-376	1.05	9.78	
262.75	-392	1.18	9.82	
312	-476	1.15	10.02	
384.5	-435	2.54	8.88	
456	-381	3.36	7.00	
479	-425	3.97	6.90	
480.5	-435	3.74	7.21	0.24
502.75	-415	3.51	7.50	0.22
548	-484	2.95	7.40	0.22
601.5	-540	3.18	7.86	0.08
668.5	-559	3.59	7.64	0.05
740.5	-563		7.49	0.00
816	-564	2.97	7.80	0.00
913.75	-545	2.85	6.86	0.00
1080.5	-553	4.15	7.95	0.00
1245	-534	4.31		0.00
1416.75	-557	4.30	8.04	0.00

NS-tSRB-2 nitrite inhibition experiment 0.25 mM nitrite added average redox, sulfide and sulfate values. Nitrite was added at hour number 479.

Time (hours)	Average redox potential (E_h mV)	Average sulfide concentration (mM)	standard deviation of sulfide concentration	Average sulfate concentration (mM)	standard deviation of sulfate concentration	Average Nitrite concentration (mM)	standard deviation of Nitrite concentration
0	-374	0.69	0.18	7.48	3.07		
23.5	-358	0.40	0.13	10.55	0.93		
48.5	-390	0.50	0.02	10.50	0.40		
72.5	-355	0.42	0.09	10.30	0.09		
96.25	-380	0.95	0.22	9.57	0.24		
120.5	-376	0.44	0.22	9.43	0.37		
167.5	-361	0.49	0.15	10.73	0.01		
192.5	-377	0.74	0.44	9.99	0.30		
262.75	-393	0.96	0.31	9.69	0.17		
312	-469	1.12	0.05	9.73	0.41		
384.5	-422	1.99	0.78	9.20	0.45		
456	-385	2.79	0.80	7.47	0.67		
479	-431	3.60	0.53	7.79	1.26		
480.5	-439	2.88	1.21	6.96	0.36	0.23	0.01
502.75	-417	2.97	0.76	7.68	0.25	0.22	0.01
548	-471	2.44	0.73	7.82	0.59	0.12	0.14
601.5	-535	2.87	0.44	8.19	0.47	0.09	0.01
668.5	-562	2.97	0.87	8.13	0.69	0.04	0.01
740.5	-558	2.13		7.84	0.50	0.02	0.03
816	-570	2.59	0.54	8.16	0.52	0.00	0.00
913.75	-546	2.49	0.51	7.91	1.49	0.00	0.00
1080.5	-551	3.04	1.58	8.24	0.41	0.00	0.00
1245	-526	4.37	0.09	9.08	0.00	0.00	0.00
1416.75	-565	3.56	1.05	8.39	0.49	0.00	0.00

NS-tSRB-2 nitrite inhibition experiment 0. 5 mM nitrite added bottle 1. Nitrite was added at hour number 479.

Time (hours)	Redox potential (E_h mV)	Sulfide concentration (mM)	Sulfate concentration (mM)	Nitrite concentration (mM)
0	-318	0.33	10.29	
23.5	-329	0.33	10.87	
48.5	-350	0.38	10.16	
72.5	-340	0.44	9.66	
96.25	-373	0.92	10.91	
120.5	-346	0.72	8.46	
167.5	-410	0.92	10.09	
192.5	-354	1.00	10.33	
262.75	-353	1.08	8.81	
312	-434	1.26	10.14	
384.5	-417	2.23	8.56	
456	-398	3.33	7.15	
479	-407	4.54	6.87	
480.5	-448	3.44	6.27	0.50
502.75	-414	3.59	6.67	0.48
548	-591	3.23	7.07	0.11
601.5	-590	3.23	7.33	0.01
668.5	-600	3.26	8.04	0.01
740.5	-578	3.23	8.04	0.00
816	-581	3.15	7.62	0.00
913.75	-565	3.44	7.63	0.00
1080.5	-553	2.67	7.06	0.00
1245	-555	3.36	7.16	0.00
1416.75	-586	3.97	7.47	0.00

NS-tSRB-2 nitrite inhibition experiment 0.5 mM nitrite added bottle 2. Nitrite was added at hour number 479.

Time (hours)	Redox potential (E_h mV)	Sulfide concentration (mM)	Sulfate concentration (mM)	Nitrite concentration (mM)
0	-361	0.51	10.10	
23.5	-344	0.41	11.33	
48.5	-390	0.46	10.87	
72.5	-367	0.38	11.05	
96.25	-373	0.85	10.92	
120.5	-383	0.41	9.08	
167.5	-381	0.77	10.48	
192.5	-397	1.03	10.49	
262.75	-389	1.13	9.21	
312	-460	1.51	9.43	
384.5	-417	2.13	8.80	
456	-430	3.97	6.47	
479	-442	4.69	6.73	
480.5	-447	3.62	6.56	0.80
502.75	-435	3.79	6.21	0.26
548	-611	3.51	6.89	0.09
601.5	-605	3.56	7.53	0.05
668.5	-599	3.77	8.66	0.02
740.5	-587	3.51	7.59	0.00
816	-594	3.51	7.17	0.00
913.75	-584	3.36	7.89	0.02
1080.5	-581	3.46	6.87	0.03
1245	-593	3.72	7.16	0.01
1416.75	-601	4.15	6.64	0.01

NS-tSRB-2 nitrite inhibition experiment 0.5 mM nitrite added average redox, sulfide and sulfate values. Nitrite was added at hour number 479.

Time (hours)	Average redox potential (E_h mV)	Average sulfide concentration (mM)	standard deviation of sulfide concentration	Average sulfate concentration (mM)	standard deviation of sulfate concentration	Average Nitrite concentration (mM)	standard deviation of Nitrite concentration
0	-340	0.42	0.13	10.19	0.13		
23.5	-337	0.37	0.05	11.10	0.32		
48.5	-370	0.42	0.05	10.52	0.50		
72.5	-354	0.41	0.04	10.36	0.98		
96.25	-373	0.88	0.05	10.91	0.01		
120.5	-365	0.56	0.22	8.77	0.44		
167.5	-396	0.85	0.11	10.29	0.27		
192.5	-376	1.01	0.02	10.41	0.12		
262.75	-371	1.10	0.04	9.01	0.29		
312	-447	1.38	0.18	9.79	0.51		
384.5	-417	2.18	0.07	8.68	0.17		
456	-414	3.65	0.45	6.81	0.48		
479	-425	4.62	0.11	6.80	0.09		
480.5	-448	3.53	0.13	6.41	0.21	0.65	0.21
502.75	-425	3.69	0.15	6.44	0.32	0.37	0.15
548	-601	3.37	0.20	6.98	0.13	0.10	0.01
601.5	-598	3.40	0.24	7.43	0.14	0.03	0.03
668.5	-600	3.51	0.36	8.35	0.44	0.02	0.01
740.5	-583	3.37	0.20	7.82	0.32	0.00	0.00
816	-588	3.33	0.25	7.40	0.32	0.00	0.00
913.75	-575	3.40	0.05	7.76	0.18	0.01	0.01
1080.5	-567	3.06	0.56	6.96	0.14	0.02	0.02
1245	-574	3.54	0.25	7.16	0.00	0.01	0.01
1416.75	-594	4.06	0.13	7.06	0.59	0.01	0.01

NS-tSRB-2 nitrite inhibition experiment 1 mM nitrite added bottle 7. Nitrite was added at hour number 479.

Time (hours)	Redox potential (E_h mV)	Sulfide concentration (mM)	Sulfate concentration (mM)	Nitrite concentration (mM)
0	-369	0.82	10.63	
23.5	-355	0.38	10.62	
48.5	-387	0.41	10.34	
72.5	-358	0.23	10.14	
96.25	-381	0.90	9.11	
120.5	-360	0.38	10.13	
167.5	-362	0.44	10.10	
192.5	-373	0.54	8.62	
262.75	-372	0.97	9.45	
312	-461	1.38	7.23	
384.5	-419	1.67	8.47	
456	-410	3.00	7.82	
479	-421	3.54	7.58	
480.5	-444	2.51	7.91	0.96
502.75	-505	2.36	7.94	0.85
548	-573	1.90	8.36	0.23
601.5	-572	1.46	8.48	0.02
668.5	-566	1.64	8.45	0.05
740.5		1.38	8.72	0.06
816	-571	1.56	8.84	0.00
913.75	-570	0.90	9.02	0.00
1080.5	-558	0.95	8.29	0.02
1245	-559	1.31	8.29	0.01
1416.75	-574	2.08	7.03	0.00

NS-tSRB-2 nitrite inhibition experiment 1 mM nitrite added bottle 8. Nitrite was added at hour number 479.

Time (hours)	Redox potential (E_h mV)	Sulfide concentration (mM)	Sulfate concentration (mM)	Nitrite concentration (mM)
0	-385	0.82	10.07	
23.5	-363	0.54	10.13	
48.5	-380	0.49	10.16	
72.5	-372	0.36	10.62	
96.25	-360	1.15	10.58	
120.5	-349	0.46	9.23	
167.5	-369	1.18	9.77	
192.5	-366	1.18	11.57	
262.75	-366	1.18	9.77	
312	-466	1.08	9.70	
384.5	-426	2.03	9.79	
456	-390	2.46	8.73	
479	-381	3.03	6.36	
480.5	-415	2.21	7.82	0.91
502.75	-419	2.41	8.66	0.71
548	-485	1.79	8.48	0.20
601.5	-488	1.46	9.29	0.04
668.5	-499	1.49	9.27	0.04
740.5	-567	1.49	9.20	0.05
816	-517	1.36	8.54	0.00
913.75	-518	0.82	9.21	0.00
1080.5	-549	0.97	8.28	0.03
1245	-535	1.59	8.73	0.01
1416.75	-595	2.03	8.29	0.00

NS-tSRB-2 nitrite inhibition experiment 1 mM nitrite added average redox, sulfide and sulfate values. Nitrite was added at hour number 479.

Time (hours)	Average redox potential (E_h mV)	Average sulfide concentration (mM)	standard deviation of sulfide concentration	Average sulfate concentration (mM)	standard deviation of sulfate concentration	Average Nitrite concentration (mM)	standard deviation of Nitrite concentration
0	-377	0.82	0.00	10.35	0.40		
23.5	-359	0.46	0.11	10.38	0.35		
48.5	-384	0.45	0.05	10.25	0.12		
72.5	-365	0.29	0.09	10.38	0.34		
96.25	-370	1.03	0.18	9.85	1.04		
120.5	-355	0.42	0.05	9.68	0.63		
167.5	-366	0.81	0.53	9.93	0.24		
192.5	-370	0.86	0.45	10.10	2.09		
262.75	-369	1.08	0.15	9.61	0.22		
312	-464	1.23	0.22	8.47	1.75		
384.5	-423	1.85	0.25	9.13	0.93		
456	-400	2.73	0.38	8.28	0.65		
479	-401	3.28	0.36	6.97	0.87		
480.5	-430	2.36	0.22	7.86	0.06	0.94	0.04
502.75	-462	2.38	0.04	8.30	0.51	0.78	0.10
548	-529	1.85	0.07	8.42	0.09	0.21	0.02
601.5	-530	1.46	0.00	8.88	0.57	0.03	0.01
668.5	-533	1.56	0.11	8.86	0.58	0.05	0.01
740.5	-567	1.44	0.07	8.96	0.34	0.06	0.01
816	-544	1.46	0.15	8.69	0.21	0.00	0.00
913.75	-544	0.86	0.05	9.12	0.14	0.00	0.00
1080.5	-554	0.96	0.02	8.28	0.01	0.03	0.01
1245	-547	1.45	0.20	8.51	0.31	0.01	0.00
1416.75	-585	2.05	0.04	7.66	0.89	0.00	0.00

NS-tSRB-2 nitrite inhibition experiment 2 mM nitrite added bottle 13. Nitrite was added at hour number 479.

Time (hours)	Redox potential (E_h mV)	Sulfide concentration (mM)	Sulfate concentration (mM)	Nitrite concentration (mM)
0	-353	0.69	9.96	
23.5	-328	0.44	10.99	
48.5	-398	0.36	9.74	
72.5	-365	0.38	10.36	
96.25	-349	0.72	9.99	
120.5	-344	0.67	9.77	
167.5	-359	1.26	10.43	
192.5	-388	1.26	10.44	
262.75	-381	1.21	9.34	
312	-460	1.38	9.05	
384.5	-424	2.41	9.16	
456	-428	3.90	7.49	
479	-429	4.64	7.28	
480.5	-439	3.44	6.77	1.74
502.75	-543	2.92	6.76	0.93
548	-553	1.49	7.68	0.49
601.5	-532	0.88	6.91	0.23
668.5	-544	0.79	7.34	0.19
740.5	-527	0.92	6.96	0.10
816	-534	1.18	7.88	0.00
913.75	-552	1.18	8.09	0.00
1080.5	-545	0.82	7.41	0.00
1245	-546	2.67	8.17	0.00
1416.75	-559	1.74	7.17	0.00

NS-tSRB-2 nitrite inhibition experiment 2 mM nitrite added bottle 13. Nitrite was added at hour number 479.

Time (hours)	Redox potential (E_h mV)	Sulfide concentration (mM)	Sulfate concentration (mM)	Nitrite concentration (mM)
0	-380	0.69	11.00	
23.5	-355	0.49	10.76	
48.5	-373	0.54	10.24	
72.5	-364	0.74	10.95	
96.25	-359	0.67	10.24	
120.5	-339	0.62	10.34	
167.5	-369	0.79	10.16	
192.5	-369	1.13	10.49	
262.75	-369	1.23	9.23	
312	-451	1.46	9.14	
384.5	-425	2.62	8.90	
456	-421	4.28	7.95	
479	-419	4.67	7.90	
480.5	-431	4.00	6.93	1.90
502.75	-454	3.13	7.21	1.14
548	-551	1.21	7.86	0.55
601.5	-514	0.79	6.80	0.32
668.5	-495	0.67	8.28	0.27
740.5	-479	0.85	6.70	0.17
816	-504	1.05	7.73	0.00
913.75	-525	0.67	7.90	0.00
1080.5	-530	0.67	7.93	0.00
1245	-548	1.41	8.15	0.00
1416.75	-552	2.05	7.17	0.00

NS-tSRB-2 nitrite inhibition experiment 2 mM nitrite added average redox, sulfide and sulfate values. Nitrite was added at hour number 479.

Time (hours)	Average redox potential (E_h mV)	Average sulfide concentration (mM)	standard deviation of sulfide concentration	Average sulfate concentration (mM)	standard deviation of sulfate concentration	Average Nitrite concentration (mM)	standard deviation of Nitrite concentration
0	-367	0.69	0.00	10.48	0.74		
23.5	-342	0.46	0.04	10.87	0.17		
48.5	-386	0.45	0.13	9.99	0.35		
72.5	-365	0.56	0.25	10.65	0.42		
96.25	-354	0.69	0.04	10.12	0.18		
120.5	-342	0.64	0.04	10.05	0.40		
167.5	-364	1.03	0.33	10.30	0.19		
192.5	-379	1.19	0.09	10.46	0.04		
262.75	-375	1.22	0.02	9.29	0.07		
312	-456	1.42	0.05	9.10	0.06		
384.5	-425	2.51	0.15	9.03	0.19		
456	-425	4.09	0.27	7.72	0.32		
479	-424	4.65	0.02	7.59	0.44		
480.5	-435	3.72	0.40	6.85	0.12	1.74	0.11
502.75	-499	3.03	0.15	6.98	0.32	0.93	0.15
548	-552	1.35	0.20	7.77	0.12	0.49	0.04
601.5	-523	0.84	0.06	6.85	0.08	0.23	0.07
668.5	-520	0.73	0.09	7.81	0.66	0.19	0.06
740.5	-503	0.88	0.05	6.83	0.18	0.10	0.04
816	-519	1.12	0.09	7.81	0.10	0.00	0.00
913.75	-539	0.92	0.36	7.99	0.14	0.00	0.00
1080.5	-538	0.74	0.11	7.67	0.37	0.00	0.00
1245	-547	2.04	0.89	8.16	0.01	0.00	0.00
1416.75	-556	1.90	0.22	7.17	0.00	0.00	0.00

NS-tSRB-2 nitrite inhibition experiment 3 mM nitrite added bottle 1. Nitrite was added at hour number 218.

Time (hours)	Redox potential (E_h mV)	Sulfide concentration (mM)	Sulfate concentration (mM)	Nitrite concentration (mM)
0	-376	9.16	0.03	
96	-440	9.50	0.46	
174	-435	8.97	2.10	
197	-480	7.77	3.36	
218	-490	7.02	3.54	
219	-508	7.18	2.13	2.66
246	-494	7.10	0.69	1.80
270	-259	6.63	0.46	1.58
319	-332	6.06	0.15	1.47
362	-332	7.60	0.05	1.40
460	-207	7.56	0.10	1.28
532	-215	4.04	0.15	1.27
696	-253	7.68	0.18	
939	-191	7.45	0.10	0.91

NS-tSRB-2 nitrite inhibition experiment 3 mM nitrite added bottle 2. Nitrite was added at hour number 218.

Time (hours)	Redox potential (E_h mV)	Sulfide concentration (mM)	Sulfate concentration (mM)	Nitrite concentration (mM)
0	-467	10.54	0.03	
96	-449	7.65	0.54	
174	-500	9.19	2.05	
197	-476	7.95	2.85	
218	-504	7.02	3.10	
219	-499	6.93	1.69	2.69
246	-233	7.70	0.49	1.72
270	-333	7.54	0.44	1.63
319	-328	5.02	1.33	1.55
362	-172	7.82	0.10	1.48
460	-185	7.32	0.18	1.33
532	-190	7.15	0.26	1.34
696	-151	8.88	0.13	
939		7.97	0.21	1.21

NS-tSRB-2 nitrite inhibition experiment 3 mM nitrite added bottle 5. Nitrite was added at hour number 218.

Time (hours)	Redox potential (E_h mV)	Sulfide concentration (mM)	Sulfate concentration (mM)	Nitrite concentration (mM)
0	-624	10.36	0.05	
96	-661	8.93	0.59	
174	-696	9.82	1.59	
197	-740	8.17	2.62	
218	-708	7.03	3.10	
219	-742	6.92	2.00	3.07
246	-701	7.45	0.64	2.19
270	-470	6.84	0.69	2.05
319	-619	6.73	1.23	2.90
362	-566	7.10	0.64	1.94
460	-474	8.02	0.38	1.83
532	-445	7.59	3.36	1.76
696	-449	7.84	0.33	
939	-487	7.60	0.56	1.03

NS-tSRB-2 nitrite inhibition experiment 3 mM nitrite added average redox, sulfide and sulfate values. Nitrite was added at hour number 218.

Time (hours)	Average redox potential (E_h mV)	Average sulfide concentration (mM)	standard deviation of sulfide concentration	Average sulfate concentration (mM)	standard deviation of sulfate concentration	Average Nitrite concentration (mM)	standard deviation of Nitrite concentration
0	-489	10.02	0.75	0.03	0.01		
96	-516	8.69	0.95	0.53	0.06		
174	-543	9.33	0.44	1.91	0.28		
197	-565	7.96	0.20	2.94	0.38		
218	-567	7.02	0.01	3.25	0.25		
219	-583	7.01	0.15	1.94	0.22	2.81	0.22
246	-476	7.42	0.30	0.61	0.11	1.90	0.25
270	-354	7.00	0.48	0.53	0.14	1.75	0.26
319	-426	5.94	0.86	0.91	0.65	1.98	0.80
362	-357	7.51	0.37	0.26	0.33	1.61	0.29
460	-289	7.63	0.36	0.22	0.15	1.48	0.31
532	-283	6.26	1.93	1.26	1.82	1.46	0.26
696	-284	8.13	0.65	0.21	0.11		
939	-339	7.67	0.27	0.29	0.24	1.05	0.15

ST-tSRB-8A-2 nitrite inhibition experiment no nitrite added bottle 16

Time (hours)	Redox potential (E_h mV)	Sulfide concentration (mM)	Sulfate concentration (mM)
0	-333	0.05	7.37
18.75	-408	1.05	6.72
26	-415	1.59	7.46
29.75	-444	3.08	6.60
52.75	-482	3.36	4.69
77.5		4.82	4.44
101.75	-465	5.36	4.00
155.75	-490	4.95	3.89
198.5	-469	4.64	3.99
290.75	-467	4.85	4.08
439	-495	4.74	4.63
486	-460	3.90	3.29
511	-484	4.92	4.36
604	-471	3.82	3.68
652.5	-418	4.87	4.01
822	-570	6.03	4.67
990	-511	4.54	3.52

ST-tSRB-8A-2 nitrite inhibition experiment no nitrite added bottle 19

Time (hours)	Redox potential (E_h mV)	Sulfide concentration (mM)	Sulfate concentration (mM)
0	-354	0.15	7.07
18.75	-398	0.79	6.83
26	-415	1.49	6.79
29.75	-453	3.74	6.06
52.75	-462	4.95	4.40
77.5	-482	4.67	4.56
101.75	-484	5.15	4.56
155.75	-478	4.74	4.49
198.5	-468	3.77	4.30
290.75	-462	4.95	4.65
439	-500	4.18	4.34
486	-453	4.44	3.96
511	-497	4.18	4.58
604	-484	4.51	4.09
652.5	-445	4.18	4.85
822	-558	4.13	4.65
990	-518	3.82	4.18

ST-tSRB-8A-2 nitrite inhibition experiment no nitrite added bottle 20

Time (hours)	Redox potential (E_h mV)	Sulfide concentration (mM)	Sulfate concentration (mM)
0	-353	0.38	7.44
18.75	-430	1.33	6.67
26	-418	1.49	6.50
29.75	-452	2.95	7.30
52.75	-456	4.15	4.83
77.5	-476	4.79	4.49
101.75	-471	5.05	4.49
155.75	-477	5.05	4.56
198.5	-468	3.87	4.39
290.75	-453	4.18	4.46
439	-494	3.74	4.55
486	-484	4.56	4.48
511	-482	3.72	4.51
604	-466	4.41	4.13
652.5	-439	4.56	3.83
822	-554	4.15	4.69
990	-530	4.08	4.30

ST-tSRb-8A-2 nitrite inhibition experiment no nitrite added average redox, sulfide and sulfate values

Time (hours)	Average redox potential (E_h mV)	Average sulfide concentration (mM)	standard deviation of sulfide concentration	Average sulfate concentration (mM)	standard deviation of sulfate concentration
0	-347	0.20	0.17	7.29	0.19
18.75	-412	1.06	0.27	6.74	0.08
26	-416	1.52	0.06	6.91	0.49
29.75	-450	3.26	0.43	6.65	0.62
52.75	-467	4.15	0.79	4.64	0.22
77.5	-479	4.76	0.08	4.50	0.06
101.75	-473	5.19	0.16	4.35	0.31
155.75	-482	4.91	0.16	4.31	0.37
198.5	-468	4.09	0.48	4.22	0.21
290.75	-461	4.66	0.42	4.40	0.29
439	-496	4.22	0.50	4.51	0.15
486	-466	4.30	0.35	3.91	0.60
511	-488	4.27	0.61	4.48	0.11
604	-474	4.25	0.37	3.97	0.25
652.5	-434	4.54	0.35	4.23	0.54
822	-561	4.77	1.09	4.67	0.02
990	-520	4.15	0.36	4.00	0.42

ST-tSRB-8A-2 nitrite inhibition experiment 0.5 mM nitrite added bottle 1. Nitrite was added at hour number 26.

Time (hours)	Redox potential (E_h mV)	Sulfide concentration (mM)	Sulfate concentration (mM)	Nitrite concentration (mM)
0	-307	0.18	7.72	
18.75	-407	1.92	6.13	
26	-448	4.10	3.96	
27	-486	3.41	3.41	0.43
29.75	-445	3.26	4.61	0.41
52.75	-429	3.08	4.17	0.41
77.5	-448	3.41	3.77	0
101.75	-467	3.15	4.24	0
155.75	-504	3.51	3.93	0
198.5	-499	2.23	4.44	0
290.75	-509	2.79	5.03	0
439	-513	2.85	5.01	0
486	-493	2.69	4.61	0
511	-490	2.64	4.69	0
604	-467	2.54		0
652.5	-475	2.54	4.50	0
822	-567	1.87	4.29	0
990	-487	1.90	3.98	0

ST-tSRB-8A-2 nitrite inhibition experiment 0.5 mM nitrite added bottle 4. Nitrite was added at hour number 26.

Time (hours)	Redox potential (E_h mV)	Sulfide concentration (mM)	Sulfate concentration (mM)	Nitrite concentration (mM)
0	-298	0.13		
18.75	-413	2.03	6.17	
26	-465	3.87	4.27	
27	-493	3.82	3.88	0.19
29.75	-492	4.62	4.92	0.28
52.75	-477	3.03	4.22	0.22
77.5	-498	3.23	4.39	0
101.75	-484	3.13	3.76	0
155.75	-504	4.15	3.95	0
198.5	-510	3.10	4.60	0
290.75	-513	3.33	5.62	0
439	-525	2.85	4.98	0
486	-502	2.72	4.74	0
511	-514	2.41	5.04	0
604	-506	2.77	4.07	0
652.5	-482	2.28	4.40	0
822	-554	2.54	5.08	0
990	-535	2.18	4.65	0

ST-tSRB-8A-2 nitrite inhibition experiment 0.5 mM nitrite added bottle 10. Nitrite was added at hour number 26.

Time (hours)	Redox potential (E_h mV)	Sulfide concentration (mM)	Sulfate concentration (mM)	Nitrite concentration (mM)
0	-333	0.03	6.53	
18.75	-397	1.82	6.08	
26	-470	4.10	4.72	
27	-473	3.51	4.42	0.35
29.75	-451	3.82	3.94	0.32
52.75	-490	2.97	4.55	0.32
77.5	-493	3.03	4.40	0
101.75	-476	3.15	4.54	0
155.75	-491	2.87	2.83	0
198.5	-471	2.72	4.64	0
290.75	-498	2.54	5.77	0
439	-517	1.95	5.31	0
486	-495	1.97	4.88	0
511	-496	1.56	5.23	0
604	-483	2.18	4.50	0
652.5	-479	1.87	5.12	0
822	-546	2.18	4.87	0
990	-541	1.64	4.54	0

ST-tSRb-8A-2 nitrite inhibition experiment 0.5 mM nitrite added average redox, sulfide and sulfate values. Nitrite was added at hour number 26.

Time (hours)	Average redox potential (E_h mV)	Average sulfide concentration (mM)	standard deviation of sulfide concentration	Average sulfate concentration (mM)	standard deviation of sulfate concentration	Average Nitrite concentration (mM)	standard deviation of Nitrite concentration
0	-313	0.11	0.08	7.13	0.84		
18.75	-406	1.92	0.10	6.13	0.05		
26	-461	4.03	0.13	4.32	0.39		
27	-484	3.58	0.21	3.90	0.51	0.32	0.12
29.75	-463	3.90	0.68	4.49	0.50	0.33	0.07
52.75	-465	3.03	0.05	4.32	0.20	0.32	0.09
77.5	-480	3.22	0.19	4.18	0.36	0	0
101.75	-476	3.15	0.01	4.18	0.40	0	0
155.75	-500	3.51	0.64	3.57	0.64	0	0
198.5	-493	2.68	0.44	4.56	0.11	0	0
290.75	-507	2.89	0.41	5.47	0.39	0	0
439	-518	2.55	0.52	5.10	0.18	0	0
486	-497	2.46	0.42	4.75	0.13	0	0
511	-500	2.21	0.57	4.99	0.27	0	0
604	-485	2.50	0.30	4.29	0.30	0	0
652.5	-479	2.23	0.34	4.67	0.39	0	0
822	-556	2.20	0.33	4.74	0.41	0	0
990	-521	1.91	0.27	4.39	0.36	0	0

ST-tSRB-8A-2 nitrite inhibition experiment 1 mM nitrite added bottle 5. Nitrite was added at hour number 26.

Time (hours)	Redox potential (E_h mV)	Sulfide concentration (mM)	Sulfate concentration (mM)	Nitrite concentration (mM)
0	-312	0.13	5.60	
18.75	-394	1.54	6.38	
26	-469	3.41	5.64	
27	-478	3.67	4.84	0.92
29.75	-470	3.72	4.53	1.35
52.75	-481	2.62	5.05	0.89
77.5	-477	1.54	4.31	0.46
101.75	-402	1.41	5.22	0.14
155.75	-413	1.13	4.58	0
198.5	-426	0.87	5.67	0
290.75	-337	0.49	5.67	0
439	-331	0.67	4.70	0
486	-251	0.59	4.63	0
511	-378	0.38	4.85	0
604	-323	0.38	4.45	0
652.5	-350	0.46	4.87	0
822	-456	0.72	4.46	0
990	-352	0.56	4.55	0

ST-tSRB-8A-2 nitrite inhibition experiment 1 mM nitrite added bottle 8. Nitrite was added at hour number 26.

Time (hours)	Redox potential (E_h mV)	Sulfide concentration (mM)	Sulfate concentration (mM)	Nitrite concentration (mM)
0	-329	0.03	7.78	
18.75	-382	1.15	6.76	
26	-453	3.82	4.60	
27	-478	3.13	4.97	0.94
29.75	-477	3.64	4.96	1.06
52.75	-489	3.08	4.99	0.86
77.5	-493	1.69	4.41	0
101.75	-436	1.49	4.83	0
155.75	-448	1.23	5.09	0
198.5	-433	1.13	5.42	0
290.75	-413	0.95	5.10	0
439	-460	0.92	5.26	0
486	-330	0.92	5.06	0
511	-423	0.59	4.95	0
604	-385	0.54	4.48	0
652.5	-355	0.67	5.08	0
822	-461	0.77	5.07	0
990	-415	0.69	5.14	0

ST-tSRb-8A-2 nitrite inhibition experiment 1 mM nitrite added average redox, sulfide and sulfate values. Nitrite was added at hour number 26.

Time (hours)	Average redox potential (E_h mV)	Average sulfide concentration (mM)	Standard deviation of sulfide concentration	Average sulfate concentration (mM)	standard deviation of sulfate concentration	Average Nitrite concentration (mM)	standard deviation of Nitrite concentration
0	-321	0.08	0.07	6.69	1.54		
18.75	-388	1.35	0.27	6.57	0.27		
26	-461	3.62	0.29	5.12	0.74		
27	-478	3.40	0.38	4.90	0.09	0.93	0.02
29.75	-474	3.68	0.05	4.74	0.30	1.21	0.21
52.75	-485	2.85	0.33	5.02	0.04	0.87	0.02
77.5	-485	1.62	0.11	4.36	0.07	0.23	0.33
101.75	-419	1.45	0.05	5.03	0.28	0.07	0.10
155.75	-431	1.18	0.07	4.84	0.36	0	0
198.5	-430	1.00	0.18	5.55	0.18	0	0
290.75	-375	0.72	0.33	5.39	0.40	0	0
439	-396	0.79	0.18	4.98	0.39	0	0
486	-291	0.76	0.24	4.85	0.30	0	0
511	-401	0.49	0.15	4.90	0.07	0	0
604	-354	0.46	0.11	4.46	0.02	0	0
652.5	-353	0.56	0.15	4.97	0.15	0	0
822	-459	0.74	0.04	4.77	0.43	0	0
990	-384	0.63	0.09	4.85	0.42	0	0

ST-tSRB-8A-2 nitrite inhibition experiment 2 mM nitrite added bottle 2. Nitrite was added at hour number 26.

Time (hours)	Redox potential (E_h mV)	Sulfide concentration (mM)	Sulfate concentration (mM)	Nitrite concentration (mM)
0	-306	0.51	7.45	
18.75	-433	1.95	6.00	
26	-453	4.23	3.84	
27	-476	3.21	4.81	1.64
29.75	-463	3.90	4.19	1.81
52.75	-494	2.67	4.11	1.22
77.5	-458	1.28	4.24	0.45
101.75	-377	1.64	3.85	0.16
155.75	-249	0.82	3.85	
198.5	-208	0.51	4.72	0
290.75	-119	0.28	4.86	0
439	-138	0.72	4.44	0
486	-123	0.36	4.17	0
511	-107	0.18	4.41	0
604	-96	0.40		0
652.5	-141	0.41	3.48	0
822	-240	0.38	4.41	0
990	-121	0.36	4.55	0

ST-tSRB-8A-2 nitrite inhibition experiment 2 mM nitrite added bottle. Nitrite was added at hour number 26.

Time (hours)	Redox potential (E_h mV)	Sulfide concentration (mM)	Sulfate concentration (mM)	Nitrite concentration (mM)
0	-281	0.18	3.80	
18.75	-418	2.69	5.86	
26	-478	4.64	4.17	
27	-498	3.08	4.18	1.53
29.75	-466	3.79	2.87	1.81
52.75	-508	3.31	4.58	1.24
77.5	-462	1.49	4.14	0.23
101.75	-408	1.13	4.00	0.05
155.75	-298	0.64	4.23	0.01
198.5	-284	0.46	4.11	0
290.75	-200	0.23	4.80	0
439	-193	0.46	4.40	0
486	-141	0.44	4.08	0
511	-164	0.18	4.37	0
604	-169	0.38	3.23	0
652.5	-153	0.33	3.37	0
822	-81	0.38	4.43	0
990	-252	0.44	4.42	0

ST-tSRB-8A-2 nitrite inhibition experiment 2 mM nitrite added bottle 7. Nitrite was added at hour number 26.

Time (hours)	Redox potential (E_h mV)	Sulfide concentration (mM)	Sulfate concentration (mM)	Nitrite concentration (mM)
0	-312	0.05	6.27	
18.75	-396	1.77	5.01	
26	-453	4.74	4.97	
27	-494	3.21	4.11	1.66
29.75	-480	4.00	5.51	2.01
52.75	-506	3.05	4.99	1.38
77.5	-447	1.51	4.50	0.54
101.75	-370	1.05	4.13	0
155.75	-348	0.59	4.20	0
198.5	-245	0.56	4.18	0
290.75	-110	0.46	4.54	0
439	-135	0.72	4.56	0
486	-130	0.51	4.47	0
511	-101	0.28	4.50	0
604	-119	0.26	4.18	0
652.5	-191	0.23	4.34	0
822	-307	0.67	4.30	0
990	-206	0.36	4.18	0

ST-tSRb-8A-2 nitrite inhibition experiment 2 mM nitrite added average redox, sulfide and sulfate values. Nitrite was added at hour number 26.

Time (hours)	Average redox potential (E_h mV)	Average sulfide concentration (mM)	standard deviation of sulfide concentration	Average sulfate concentration (mM)	standard deviation of sulfate concentration	Average Nitrite concentration (mM)	standard deviation of Nitrite concentration
0	-300	0.25	0.24	5.84	1.86		
18.75	-416	2.14	0.49	5.62	0.54		
26	-461	4.54	0.27	4.33	0.58		
27	-489	3.16	0.07	4.37	0.38	1.61	0.07
29.75	-470	3.90	0.10	4.19	1.32	1.87	0.12
52.75	-503	3.01	0.32	4.56	0.44	1.28	0.08
77.5	-456	1.43	0.13	4.30	0.18	0.41	0.16
101.75	-385	1.27	0.32	3.99	0.14	0.07	0.08
155.75	-298	0.68	0.12	4.10	0.22	0	0
198.5	-246	0.51	0.05	4.34	0.33	0	0
290.75	-143	0.32	0.12	4.73	0.17	0	0
439	-155	0.63	0.15	4.47	0.08	0	0
486	-131	0.44	0.08	4.24	0.20	0	0
511	-124	0.21	0.06	4.43	0.07	0	0
604	-128	0.35	0.08	3.71	0.67	0	0
652.5	-162	0.32	0.09	3.73	0.53	0	0
822	-209	0.48	0.16	4.38	0.07	0	0
990	-193	0.38	0.04	4.38	0.19	0	0

ST-tSRB-8A-2 nitrite inhibition experiment 4 mM nitrite added bottle 9. Nitrite was added at hour number 29.

Time (hours)	Redox potential (E_h mV)	Sulfide concentration (mM)	Sulfate concentration (mM)	Nitrite concentration (mM)
0	-318	0.03	6.69	
18.75	-378	1.38	6.10	
26	-452	2.85	5.42	
27	-473	4.46	5.31	
29.75	-433	2.31	6.19	4.01
52.75	-508	2.56	4.64	3.18
77.5	-271	1.08	4.20	2.10
101.75	-155	0.97	4.29	2.04
155.75	-137	0.54	4.23	2.06
198.5	-131	0.97	4.58	2.03
290.75	-22	0.28	5.27	1.97
439	-72	0.33	4.55	2.15
486	-78	0.33	4.88	-0.35
511		0.33	4.81	1.67
604	-45	0.31	4.43	1.55
652.5	-109	0.44	4.09	1.80
822	-130	0.64	4.27	1.57
990	-115	0.38	4.08	1.78

ST-tSRB-8A-2 nitrite inhibition experiment 4 mM nitrite added bottle 11. Nitrite was added at hour number 29.

Time (hours)	Redox potential (E_h mV)	Sulfide concentration (mM)	Sulfate concentration (mM)	Nitrite concentration (mM)
0	-389	1.08	7.56	
18.75	-452	1.00	6.12	
26	-456	2.64	6.20	
29	-468	4.77	4.71	
29.75	-511	2.62	5.84	3.32
52.75	-261	2.46	4.57	2.97
77.5	-115	1.03		1.97
101.75	-117	0.82	4.03	1.78
155.75	-96	0.44	4.74	1.92
198.5	24	0.51	4.70	1.86
290.75	-50	0.41	4.97	1.75
439	-8	0.44	4.92	1.96
602		0.41	4.73	1.77
511	-16	0.36	4.92	1.69
604	-32	0.64	4.38	1.95
652.5	-176	0.49	4.78	1.88
822	-25	0.67	3.77	1.69
990		0.31	4.00	1.67

ST-tSRB-8A-2 nitrite inhibition experiment 4 mM nitrite added bottle 13. Nitrite was added at hour number 29.

Time (hours)	Redox potential (E_h mV)	Sulfide concentration (mM)	Sulfate concentration (mM)	Nitrite concentration (mM)
0	-341	0.23	7.14	
18.75	-398	1.15	6.65	
26	-450	2.28	5.48	
29	-474	4.46	4.85	
29.75	-466	1.95	6.60	4.01
52.75	-497	2.56	4.92	2.81
77.5	-241	0.69	5.23	2.27
101.75	-107	0.90	4.98	2.19
155.75	-83	0.69	5.55	2.05
198.5	-78	0.54	5.16	2.24
290.75	23	0.62	5.27	2.22
439	-10	0.38	5.46	1.97
602	47	0.69	5.13	1.87
511		0.51	5.30	1.98
604	7	0.31	4.55	2.10
652.5	-1	0.74	4.48	1.90
822	-64	1.03	5.26	1.49
990	6	0.69	5.23	1.87

ST-tSRb-8A-2 nitrite inhibition experiment 4 mM nitrite added average redox, sulfide and sulfate values. Nitrite was added at hour number 26.

Time (hours)	Average redox potential (E_h mV)	Average sulfide concentration (mM)	standard deviation of sulfide concentration	Average sulfate concentration (mM)	standard deviation of sulfate concentration	Average Nitrite concentration (mM)	standard deviation of Nitrite concentration
0	-349	0.44	0.56	7.13	0.43		
18.75	-409	1.18	0.19	6.29	0.31		
26	-453	2.59	0.29	5.70	0.44		
29	-472	4.56	0.18	4.96	0.31		
29.75	-470	2.29	0.33	6.21	0.38	3.78	0.40
52.75	-422	2.53	0.06	4.71	0.18	2.99	0.19
77.5	-209	0.93	0.21	4.72	0.73	2.11	0.15
101.75	-126	0.90	0.08	4.43	0.49	2.01	0.21
155.75	-105	0.56	0.13	4.84	0.66	2.01	0.08
198.5	-62	0.68	0.26	4.82	0.31	2.04	0.19
290.75	-16	0.44	0.17	5.17	0.17	1.98	0.23
439	-25	0.38	0.05	4.98	0.46	2.02	0.11
602	-16	0.48	0.19	4.91	0.20	1.82	0.07
511		0.40	0.10	5.01	0.26	1.78	0.17
604	-23	0.42	0.19	4.45	0.09	1.86	0.28
652.5	-95	0.56	0.16	4.45	0.34	1.86	0.06
822	-73	0.78	0.22	4.43	0.76	1.58	0.10
990	-55	0.46	0.20	4.44	0.69	1.77	0.10

ST-tSRB-8A-2 nitrite inhibition experiment 5 mM nitrite added bottle 14. Nitrite was added at hour number 29.

Time (hours)	Redox potential (E_h mV)	Sulfide concentration (mM)	Sulfate concentration (mM)	Nitrite concentration (mM)
0	-335	0.49	7.65	
18.75	-390	1.10	6.64	
26	-444	2.02	6.36	
29	-436	3.74	5.68	
29.75	-467	1.72	6.56	4.52
52.75	-469	1.64	5.11	
77.5	-108	0.87	5.54	2.93
101.75	-84	1.31	5.19	3.07
155.75	-54	0.36	5.76	2.95
198.5	-54	0.56	5.62	3.06
290.75	23	0.56	5.16	2.97
439	26	0.46	5.68	2.86
486	67	0.44	5.04	3.12
511		0.31	5.53	2.85
604	35	0.41	4.99	2.92
652.5	19	0.46	5.21	2.85
822	-75	0.44	5.32	2.66
990	30	0.38	5.76	2.70

ST-tSRB-8A-2 nitrite inhibition experiment 5 mM nitrite added bottle 15. Nitrite was added at hour number 29.

Time (hours)	Redox potential (E_h mV)	Sulfide concentration (mM)	Sulfate concentration (mM)	Nitrite concentration (mM)
0	-353	0.10	8.04	
18.75	-437	1.00	6.65	
26	-439	1.92	6.65	
29	-470	3.77	6.20	
29.75	-456	1.77	6.04	4.73
52.75	-466	2.00	5.48	3.77
77.5	222	0.77	4.54	3.31
101.75	-25	0.85	5.21	2.97
155.75	-67	0.26	5.61	3.25
198.5	-38	0.49	5.50	3.22
290.75	28	0.41	5.87	3.25
439	54	0.44	6.13	3.23
486	99	0.51	5.71	3.11
511		0.33	5.54	3.06
604	49	0.18	5.06	3.03
652.5	38	0.59	5.37	2.90
822	56	0.51	5.58	2.90
990	34	0.26	5.62	2.90

ST-tSRB-8A-2 nitrite inhibition experiment 5 mM nitrite added bottle 18. Nitrite was added at hour number 29.

Time (hours)	Redox potential (E_h mV)	Sulfide concentration (mM)	Sulfate concentration (mM)	Nitrite concentration (mM)
0	-328	0.82	7.72	
18.75	-433	1.18	6.56	
26	-430	1.74	5.92	
29	-455	3.90	6.16	
29.75	-449	1.41	6.43	5.01
52.75	-452	1.97	5.32	3.81
77.5	-132	0.79	5.37	3.17
101.75	-43	0.69	5.73	3.50
155.75	-44	0.33	5.74	2.24
198.5	-33	0.54	6.26	3.53
290.75	33	0.54	5.54	3.40
439	30	0.38	5.97	3.21
486	129	0.46	5.35	3.35
511		0.67	6.02	3.17
604	55	0.36	5.23	3.35
652.5	53	0.95	5.63	3.12
822	64	0.77	5.14	3.08
990	38	0.59	5.19	3.27

ST-tSRb-8A-2 nitrite inhibition experiment 5 mM nitrite added average redox, sulfide and sulfate values

Time (hours)	Average redox potential (E_h mV)	Average sulfide concentration (mM)	standard deviation of sulfide concentration	Average sulfate concentration (mM)	standard deviation of sulfate concentration	Average Nitrite concentration (mM)	standard deviation of Nitrite concentration
0	-339	0.47	0.36	7.81	0.21		
18.75	-420	1.09	0.09	6.62	0.05		
26	-438	1.89	0.14	6.31	0.37		
29	-454	3.80	0.08	6.02	0.29		
29.75	-457	1.63	0.19	6.34	0.27	4.76	0.25
52.75	-462	1.87	0.20	5.30	0.18	3.79	0.03
77.5	-6	0.81	0.05	5.15	0.53	3.14	0.20
101.75	-51	0.95	0.32	5.38	0.31	3.18	0.28
155.75	-55	0.32	0.05	5.71	0.08	2.81	0.52
198.5	-42	0.53	0.04	5.79	0.41	3.27	0.24
290.75	28	0.50	0.08	5.52	0.35	3.21	0.22
439	37	0.43	0.04	5.93	0.23	3.10	0.21
602	98	0.47	0.04	5.37	0.34	3.19	0.13
511		0.44	0.20	5.70	0.28	3.03	0.16
604	46	0.32	0.12	5.09	0.13	3.10	0.22
652.5	37	0.67	0.25	5.40	0.21	2.96	0.14
822	15	0.57	0.17	5.35	0.22	2.88	0.21
990	34	0.41	0.17	5.52	0.30	2.96	0.29

ST-1/4 nitrite inhibition experiment no nitrite added bottle 1

Time (hours)	Sulfide concentration (mM)	Sulfate concentration (mM)
0	0.36	6.63
24	0.21	6.55
47	0.87	6.80
64	8.62	1.43
69	9.92	0.72
71	12.59	0.90
90	8.21	0.53

ST-1/4 nitrite inhibition experiment no nitrite added bottle 2

Time (hours)	Sulfide concentration (mM)	Sulfate concentration (mM)
0	0.33	7.23
24	0.15	6.76
47	0.33	7.05
64	2.97	4.48
69	8.00	0.97
71	11.67	1.19
90	7.59	1.63

ST-1/4 nitrite inhibition experiment 0.02 mM nitrite added. Nitrite was added at hour number 69.

Time (hours)	Sulfide concentration (mM)	Sulfate concentration (mM)	Nitrite concentration (mM)
0	0.26	6.65	
24	0.18	6.48	
47	0.15	6.52	
64	0.77	6.27	
69	1.82	4.93	
71	3.13	5.03	0.02
90	1.82	5.07	0.02
186	1.41	5.18	0
236	1.44	4.94	0
335	5.18	1.92	0

ST-1/4 nitrite inhibition experiment 0.5 mM nitrite added. Nitrite was added at hour number 69.

Time (hours)	Sulfide concentration (mM)	Sulfate concentration (mM)	Nitrite concentration (mM)
0	0.28	6.42	
24	0.21	6.48	
47	0.21	6.93	
64	1.38	5.52	
69	3.73	4.43	
71	7.18	3.91	0.33
90	3.13	3.49	0.36
186	2.08	3.16	0.30
236	2.28	3.73	0
335	2.15	3.44	0

ST-1/4 nitrite inhibition experiment 1 mM nitrite added. Nitrite was added at hour number 69.

Time (hours)	Sulfide concentration (mM)	Sulfate concentration (mM)	Nitrite concentration (mM)
0			
24			
47			
64			
69			
71			
90			
186			
236			
335			

ST-1/4 nitrite inhibition experiment 4 mM nitrite added. Nitrite was added at hour number 26.

Time (hours)	Sulfide concentration (mM)	Sulfate concentration (mM)	Nitrite concentration (mM)
0	0.28	7.29	
24	0.21	6.64	
47	0.21	7.06	
64	2.77	5.35	
69	6.41	2.01	3.53
71	7.87	2.48	3.59
90	3.41	2.56	2.37
186	0.77	1.43	1.15
236	0.56	1.41	1.30
335	0.77	1.92	1.21

ST-3 nitrite inhibition experiment no nitrite added bottle 6.

Time (hours)	Redox potential (E_h mV)	Sulfide concentration (mM)	Sulfate concentration (mM)
0	-357	0.28	8.13
7	-324	0.18	7.20
20	-290	0.13	7.41
27.5	-428	0.64	4.98
32	-475	1.59	5.04
43	-543	5.08	3.48
51.5	-538	4.03	1.83
72.75	-558	5.67	1.47
95.5	-542	6.77	1.71
121.5	-562	6.23	1.77
172	-553	6.05	1.76
215	-563	5.67	1.73
261.75	-561	6.56	1.81
357	-547	6.03	1.51
426.5	-559	5.82	1.44
590	-552	5.59	1.41

ST-3 nitrite inhibition experiment no nitrite added bottle 8

Time (hours)	Redox potential (E_h mV)	Sulfide concentration (mM)	Sulfate concentration (mM)
0	-343	0.36	7.83
7	-352	0.33	6.78
20	-258	0.08	7.21
27.5	-330	0.46	7.86
32	-395	0.28	7.43
43	-492	1.87	6.58
51.5	-526	4.67	2.96
72.75	-543	6.13	1.71
95.5	-543	6.03	1.83
121.5	-563	6.18	2.06
172	-547	8.23	1.97
215	-550	5.26	1.68
261.75	-562	5.92	1.92
357	-541	5.36	2.02
426.5	-549	5.97	1.77
590	-532	5.36	1.71

ST-3 nitrite inhibition experiment no nitrite added bottle 9

Time (hours)	Redox potential (E_h mV)	Sulfide concentration (mM)	Sulfate concentration (mM)
0	-309	0.38	7.70
7	-296	0.31	7.06
20	-328	0.10	6.46
27.5	-465	1.90	3.64
32	-523	6.13	2.17
43	-563	6.03	1.53
51.5	-551	7.41	1.05
72.75	-537	6.92	1.11
95.5	-553	6.82	1.14
121.5	-564	7.59	1.17
172	-568	7.97	1.19
215	-562	6.54	1.17
261.75	-572	6.64	1.01
357	-564	6.85	0.95
426.5	-557	6.72	1.00
590	-533	6.64	1.06

ST-3 nitrite inhibition experiment no nitrite added average redox, sulfide and sulfate values

Time (hours)	Average redox potential (E_h mV)	Average sulfide concentration (mM)	standard deviation of sulfide concentration	Average sulfate concentration (mM)	standard deviation of sulfate concentration
0	-333	0.33	0.07	7.92	0.30
7	-310	0.24	0.09	7.13	0.10
20	-309	0.12	0.02	6.93	0.67
27.5	-447	1.27	0.89	4.31	0.95
32	-499	3.86	3.21	3.61	2.03
43	-553	5.55	0.67	2.51	1.38
51.5	-545	5.72	2.39	1.44	0.55
72.75	-548	6.29	0.89	1.29	0.25
95.5	-548	6.79	0.04	1.43	0.40
121.5	-563	6.91	0.96	1.47	0.42
172	-561	7.01	1.36	1.47	0.40
215	-563	6.10	0.62	1.45	0.40
261.75	-567	6.60	0.05	1.41	0.56
357	-556	6.44	0.58	1.23	0.40
426.5	-558	6.27	0.63	1.22	0.31
590	-543	6.12	0.74	1.23	0.25

ST-3 nitrite inhibition experiment 0.5 mM nitrite added bottle 1. Nitrite was added at hour number 43.

Time (hours)	Redox potential (E_h mV)	Sulfide concentration (mM)	Sulfate concentration (mM)	Nitrite concentration (mM)
0	-359	0.26	7.76	
7	-313	0.15	6.62	
20	-303	0.08	7.53	
27.5	-366	0.31	6.71	
32	-378	0.64	6.60	
43	-478	4.05	4.08	
44	-528	4.15	3.68	0.41
51.5	-482	3.69	3.14	0.53
72.75	-484	3.18	3.03	0.42
95.5	-458	5.05	3.03	0
121.5	-540	4.51	3.70	0
172	-514	4.38	3.27	0
215	-492	4.10	2.91	0
261.75	-555	4.13	3.05	0
357	-535	4.72	2.93	0
426.5	-536	5.03	2.41	0
590	-543	6.49	0.87	0

ST-3 nitrite inhibition experiment 0.5 mM nitrite added bottle 2. Nitrite was added at hour number 43.

Time (hours)	Redox potential (E_h mV)	Sulfide concentration (mM)	Sulfate concentration (mM)	Nitrite concentration (mM)
0	-373	0.44	7.94	
7	-343	0.18	7.32	
20	-294	0.10	7.36	
27.5	-363	0.33	7.61	
32	-403	0.44	7.19	
43	-500	3.46	4.49	
44	-538	3.92	4.48	0.45
51.5	-508	3.08	3.74	0.37
72.75	-503	2.85	3.64	0.05
95.5	-503	4.08	3.41	0.00
121.5	-542	3.87	4.60	0.04
172	-527	2.54	3.57	0.03
215	-519	3.74	3.57	0.00
261.75	-562	3.46	3.60	0.00
357	-531	3.97	3.06	0.03
426.5	-539	4.46	2.53	0.00
590	-546	5.87	0.83	0.00

ST-3 nitrite inhibition experiment 0.5 mM nitrite added bottle 3. Nitrite was added at hour number 43.

Time (hours)	Redox potential (E_h mV)	Sulfide concentration (mM)	Sulfate concentration (mM)	Nitrite concentration (mM)
0	-380	0.54	7.80	
7	-324	0.18	7.24	
20	-300	0.05	7.03	
27.5	-357	0.38	7.20	
32	-397	0.28	7.28	
43	-508	3.10	5.42	
44	-518	3.05	5.24	0.30
51.5	-502	2.51	4.22	0.40
72.75	-504	2.41	4.18	0.31
95.5	-514	3.87	3.30	0
121.5	-545	4.74	3.33	0
172	-535	4.69	2.70	0
215	-527	3.67	2.63	0
261.75	-564	4.72	2.83	0
357	-542	5.08	2.51	0
426.5	-531	4.28	2.55	0.04
590	-529	5.05	1.50	0

ST-3 nitrite inhibition experiment 0.5 mM nitrite added average redox, sulfide and sulfate values. Nitrite was added at hour number 43.

Time (hours)	Average redox potential (E_h mV)	Average sulfide concentration (mM)	standard deviation of sulfide concentration	Average sulfate concentration (mM)	standard deviation of sulfate concentration	Average Nitrite concentration (mM)	standard deviation of Nitrite concentration
0	-371	0.41	0.14	7.83	0.10		
7	-327	0.17	0.01	7.06	0.38		
20	-299	0.08	0.03	7.31	0.25		
27.5	-362	0.34	0.04	7.18	0.45		
32	-393	0.45	0.18	7.02	0.37		
43	-495	3.54	0.48	4.66	0.69		
51.5	-528	3.71	0.58	4.47	0.78	0.39	0.08
72.75	-497	3.09	0.59	3.70	0.54	0.44	0.08
95.5	-497	2.81	0.39	3.62	0.58	0.26	0.19
121.5	-492	4.33	0.63	3.24	0.19	0.00	0.00
172	-542	4.38	0.45	3.88	0.66	0.01	0.02
215	-525	3.87	1.16	3.18	0.44	0.01	0.02
261.75	-513	3.84	0.23	3.04	0.48	0.00	0.00
357	-560	4.10	0.63	3.16	0.40	0.00	0.00
426.5	-536	4.59	0.56	2.83	0.29	0.01	0.02
590	-535	4.59	0.39	2.50	0.08	0.04	0.04

ST-3 nitrite inhibition experiment 1 mM nitrite added bottle 4. Nitrite was added at hour number 43.

Time (hours)	Redox potential (E_h mV)	Sulfide concentration (mM)	Sulfate concentration (mM)	Nitrite concentration (mM)
0	-381	0.36	7.98	
7	-349	0.21	7.56	
20	-294	0.03	6.90	
27.5	-362	0.49	6.68	
32	-460	1.18	6.24	
43	-532	4.21	3.44	
44	-532	4.49	3.77	0.81
51.5	-530	2.56	2.95	0.93
72.75	-545	3.36	3.08	0.69
95.5	-566	3.87	3.36	0.08
121.5	-559	3.31	3.55	0.00
172	-550	3.51	3.01	0.04
215	-535	4.33	3.06	0.03
261.75	-565	2.67	3.15	0.01
357	-558	3.87	2.73	0.03
426.5	-562	2.82	3.35	0.00
590	-550	2.64	3.09	0.00

ST-3 nitrite inhibition experiment 1 mM nitrite added bottle 5. Nitrite was added at hour number 43.

Time (hours)	Redox potential (E_h mV)	Sulfide concentration (mM)	Sulfate concentration (mM)	Nitrite concentration (mM)
0	-383	0.18	7.58	
7	-325	0.15	6.79	
20	-281	0.46	7.32	
27.5	-381	0.64	6.30	
32	-433	4.18	3.57	
43	-532	4.13	3.83	
44	-538	3.49	2.87	0.97
51.5	-527	3.51	2.81	0.87
72.75	-535	4.72	3.39	0.19
95.5	-564	3.33	3.48	0.00
121.5	-563	3.05	2.88	0.04
172	-554	4.05	3.43	0.03
215	-556	2.95	3.29	0.03
261.75	-561	4.13	3.04	0.00
357	-555	5.74	1.45	0.01
426.5	-551			
590	-553			

ST-3 nitrite inhibition experiment 1 mM nitrite added bottle 10. Nitrite was added at hour number 43.

Time (hours)	Redox potential (E_h mV)	Sulfide concentration (mM)	Sulfate concentration (mM)	Nitrite concentration (mM)
0	-341	0.41	7.01	
7	-300	0.33	7.48	
20	-270	0.13	7.84	
27.5	-340	0.54	7.77	
32	-405	0.49	6.58	
43	-540	4.64	4.33	
44	-535	4.31	4.32	0.84
51.5	-513	3.64	3.65	0.91
72.75	-518	3.95	3.22	0.78
95.5	-547	3.67	3.90	0.29
121.5	-562	3.38	4.09	0.00
172	-553	0.03	3.45	0.04
215	-559	2.95	3.56	0.03
261.75	-561	2.62	3.62	0.00
357	-534	2.74	3.37	0.00
426.5	-554	2.21	3.39	0.00
590	-546	2.23	3.61	0.00

ST-3 nitrite inhibition experiment 1 mM nitrite added average redox, sulfide and sulfate values. Nitrite was added at hour number 43.

Time (hours)	Average redox potential (E_h mV)	Average sulfide concentration (mM)	standard deviation of sulfide concentration	Average sulfate concentration (mM)	Standard deviation of sulfate concentration	Average Nitrite concentration (mM)	standard deviation of Nitrite concentration
0	-368	0.32	0.12	7.52	0.49		
7	-325	0.23	0.09	7.28	0.43		
20	-282	0.21	0.23	7.35	0.47		
27.5	-361	0.56	0.08	6.91	0.76		
32	-433	1.95	1.96	5.47	1.65		
43	-535	4.32	0.28	3.86	0.45		
51.5	-535	4.09	0.53	3.65	0.73	0.87	0.09
72.75	-523	3.24	0.59	3.14	0.45	0.90	0.03
95.5	-533	4.01	0.68	3.23	0.15	0.55	0.32
121.5	-559	3.62	0.27	3.58	0.28	0.12	0.15
172	-561	3.25	0.17	3.51	0.61	0.01	0.02
215	-552	2.53	2.19	3.30	0.25	0.04	0.01
261.75	-550	3.41	0.80	3.30	0.25	0.03	0.00
357	-562	3.14	0.86	3.27	0.31	0.00	0.01
426.5	-549	4.12	1.52	2.52	0.98	0.01	0.02
590	-556	2.51	0.44	3.37	0.03	0.00	0.01

Appendix 5 HPLC date for the nitrite inhibition experiments

Acetate concentration for the nitrite inhibition experiment of NS-tSRB-1 when no nitrite was added to the cultures.

Time	Acetate Concetration (mM) in Bottle 7	Acetate Concetration (mM) in Bottle 13	Acetate Concetration (mM) in Bottle 16	Average Acetate Concentration	Acetate Concentration Standard Deviation
0	12.12	12.13	11.14	11.80	0.57
339	10.18	10.46	11.57	10.74	0.74
407.5	8.13	8.93	9.82	8.96	0.84
574.5	8.17	7.52	8.17	7.95	0.38
1605	7.65	6.02	6.39	6.69	0.86

Acetate concentration for the nitrite inhibition experiment of NS-tSRB-1 with the addion of 0.25 mM nitrite to the cultures. Nitrite was added at hour number 338.

Time	Acetate Concetration (mM) in Bottle 2	Acetate Concetration (mM) in Bottle 4	Acetate Concetration (mM) in Bottle 5	Average Acetate Concentration	Acetate Concentration Standard Deviation
0	12.60	13.49	12.71	12.93	0.48
339	10.32	9.10	9.12	9.51	0.70
407.5	9.95	9.57	10.57	10.03	0.50
574.5	11.23	10.42	11.22	10.95	0.46
1605	10.34	10.09	10.82	10.42	0.37

Acetate concentration for the nitrite inhibition experiment of NS-tSRB-1 with the addion of 0.5 mM nitrite to the cultures. Nitrite was added at hour number 338.

Time	Acetate Concetration (mM) in Bottle 6	Acetate Concetration (mM) in Bottle 8	Average Acetate Concentration	Acetate Concentration Standard Deviation
0	12.42	13.26	12.84	0.59
339	10.01	10.40	10.20	0.27
407.5	9.04	10.34	9.69	0.92
574.5	12.14	10.90	11.52	0.88
1605	10.44	11.04	10.74	0.43

Acetate concentration for the nitrite inhibition experiment of NS-tSRB-1 with the addion of 1 mM nitrite to the cultures. Nitrite was added at hour number 338.

Time	Acetate Concetration (mM) in Bottle 9	Acetate Concetration (mM) in Bottle 10	Acetate Concetration (mM) in Bottle 11	Average Acetate Concentration	Acetate Concentration Standard Deviation
0	12.54	12.22	12.52	12.37	0.21
339	10.39	9.82	9.49	9.66	0.24
407.5	10.00	10.07	10.68	10.37	0.43
574.5	10.29	10.42	10.35	10.38	0.05
1605	10.62	8.40	8.56	8.48	0.11

Lactate conctration over time in the nitrite inhibition experiment of NS-tSRB-2 for the condition where no nitrite was added.

Time	Lactate Concentration (mM) Bottle 11	Lactate Concentration (mM) Bottle 12	Average Lactate Concentration (mM)	Lactate Concentration Standard Deviation
0	30.41	31.28	30.84	0.62
312	30.81	30.88	30.85	0.05
480.5	27.35	25.05	26.20	1.63
548	24.96	27.58	26.27	1.86
913.75	18.37	26.59	22.48	5.82

Lactate conctration over time in the nitrite inhibition experiment of NS-tSRB-2 for the condition where 0.25 mM nitrite was added. Nitrite was added at hour 479

Time	Lactate Concentration (mM) Bottle 3	Lactate Concentration (mM) Bottle10	Average Lactate Concentration (mM)	Lactate Concentration Standard Deviation
0	33.13	31.67	32.40	1.04
312	29.53	29.57	29.55	0.03
480.5	27.54	25.44	26.49	1.48
548	26.89	26.92	26.90	0.02
913.75	19.03	16.14	17.58	2.04

Lactate conctration over time in the nitrite inhibition experiment of NS-tSRB-2 for the condition where 0.5 mM nitrite was added. Nitrite was added at hour 479

Time	Lactate Concentration (mM) Bottle 1	Lactate Concentration (mM) Bottle2	Average Lactate Concentration (mM)	Lactate Concentration Standard Deviation
0		30.08	30.08	
312	30.32	30.57	30.44	0.17
480.5	25.01	24.71	24.86	0.21
548	26.13	28.10	27.11	1.39
913.75	26.19	30.19	28.19	2.83

Lactate conctration over time in the nitrite inhibition experiment of NS-tSRB-2 for the condition where 1mM nitrite was added. Nitrite was added at hour 479

Time	Lactate Concentration (mM) Bottle 7	Lactate Concentration (mM) Bottle8	Average Lactate Concentration (mM)	Lactate Concentration Standard Deviation
0	31.16	32.12	31.64	0.67
312	29.36	28.61	28.99	0.53
480.5	24.72	26.70	25.71	1.40
548	29.30	30.58	29.94	0.91
913.75	23.32	25.58	24.45	1.60

Lactate conctration over time in the nitrite inhibition experiment of NS-tSRB-2 for the condition where 2mM nitrite was added. Nitrite was added at hour 479

Time	Lactate Concentration (mM) Bottle 7	Lactate Concentration (mM) Bottle8	Average Lactate Concentration (mM)	Lactate Concentration Standard Deviation
0	32.31	32.0	32.13	0.25
312	29.60		29.60	
480.5	28.14		28.14	
548	33.62	28.6	31.11	3.54
913.75	20.60		20.60	

Lactate and Acetate concentrations for ST-3 nitrite inhibition experiments where no nitrite was added to the culture bottle number 6.

Time	Acetate Concentration (mM)	Lactate Concentration (mM)
0	25.14	8.17
32	30.25	13.41
43	7.89	37.72
51.5	6.21	29.68
95.5	5.21	32.46
172	5.18	32.72
261.75	5.21	42.02
357	2.77	41.91
590	0	8.08

Lactate and Acetate concentrations for ST-3 nitrite inhibition experiments where no nitrite was added to the culture bottle number 8..

Time	Acetate Concentration (mM)	Lactate Concentration (mM)
0	35.57	12.15
32	32.17	11.57
43	19.91	16.3
51.5	9.79	32.94
95.5	5.91	41.84
172	5.35	38.83
261.75	5.47	41.15
357	3.69	40.1
590	0	1.91

Lactate and acetate concentrations for ST-3 nitrite inhibition experiments where no nitrite was added to the culture bottle number 9..

Time	Acetate Concentration (mM)	Lactate Concentration (mM)
0	32.19	10.94
32	28.28	22.97
43	6.8	31.32
51.5	6.82	37.99
95.5	6.75	38.97
172	6.23	36.74
261.75	6	43.07
357	2.63	35.66
590	0	1.37

Average lactate and acetate concentrations for ST-3 nitrite inhibition experiments where no nirite was added to the cultures

Time	Average Lactate Concentration (mM)	Lactate Concentration Standard Deviation	Average Acetate Concentration	Acetate Concentration Standard Deviation
0	30.97	5.32	10.42	2.04
32	30.23	1.95	15.98	6.12
43	11.53	7.27	28.45	11.00
51.5	7.61	1.92	33.54	4.19
95.5	5.96	0.77	37.76	4.81
172	5.59	0.56	36.10	3.11
261.75	5.56	0.40	42.08	0.96
357	3.03	0.58	39.22	3.22
590	0.00	0.00	3.79	3.73

Lactate and Acetate concentrations for ST-3 nitrite inhibition experiments where 0.5 mM nitrite was added to the culture bottle number 1. Nitrite was added at hour number 43.

Time	Acetate Concentration (mM)	Lactate Concentration (mM)
0	31.68	10.57
32	29.75	11.35
43	6.64	21.41
51.5		
95.5	10.31	33.9
172	9.91	32.83
261.75	8.45	30.86
357	5.92	31.41
590	0	45.07

Lactate and Acetate concentrations for ST-3 nitrite inhibition experiments where 0.5 mM nitrite was added to the culture bottle number 2. Nitrite was added at hour number 43.

Time	Acetate Concentration (mM)	Lactate Concentration (mM)
0	36..01	12.12
32	33.91	12.59
43	12.94	30.8
51.5	11.99	30.38
95.5	11.26	24.4
172	11.71	31.01
261.75	11.3	32.2
357	8.1	43.51
590	0	50.75

Lactate and acetate concentrations for ST-3 nitrite inhibition experiments where 0.5 mM nitrite was added to the culture bottle number 3. Nitrite was added at hour number 43.

Time	Acetate Concentration (mM)	Lactate Concentration (mM)
0	33.76	11.08
32	32.3	11.33
43	17.17	27.07
51.5	14.4	24.67
95.5	12.5	32.39
172	8.52	29.16
261.75	8.06	24.85
357	7.83	44.05
590		

Average lactate and acetate concentrations for ST-3 nitrite inhibition experiments where 0.5 mM nirite was added to the cultures at hour number 43

Time	Average Lactate Concentration (mM)	Lactate Concentration Standard Deviation	Average Acetate Concentration	Acetate Concentration Standard Deviation
0	32.72	1.47	11.26	0.79
32	31.99	2.10	11.76	0.72
43	12.25	5.30	26.43	4.73
51.5	13.20	1.70	27.53	4.04
95.5	11.36	1.10	30.23	5.11
172	10.05	1.60	31.00	1.84
261.75	9.27	1.77	29.30	3.91
357	7.28	1.19	39.66	7.15
590	0.00	0.00	45.53	5.00

Lactate and Acetate concentrations for ST-3 nitrite inhibition experiments where 1 mM nitrite was added to the culture bottle number 4 at hour 43

Time	Acetate Concentration (mM)	Lactate Concentration (mM)
0	36.09	12.04
32	35.76	15.02
43	9.23	36.44
51.5	7.99	32.73
95.5	9.62	37.55
172	8.9	35.43
261.75	9.06	36.07
357	9.28	36.87
590	8.41	28.62

Lactate and Acetate concentrations for ST-3 nitrite inhibition experiments where 1 mM nitrite was added to the culture bottle number 5 at hour 43

Time	Acetate Concentration (mM)	Lactate Concentration (mM)
0	34.06	11.45
32	31.93	12.29
43	11.19	39.61
51.5	8.78	32.21
95.5	9.94	32.22
172	9.66	33.91
261.75	9.3	33.82
357	3.47	49.55
590	9.06	32.67

Lactate and acetate concentrations for ST-3 nitrite inhibition experiments where 1 nitrite was added to the culture bottle number 10 at hour 43.

Time	Acetate Concentration (mM)	Lactate Concentration (mM)
0	32.03	10.8
32	31.75	11.27
43	13.42	29.67
51.5	12.67	31.12
95.5	13.05	32.17
172	11.81	24.71
261.75	13.16	31.24
357	12.55	26.02
590	13.09	31.98

Average lactate and acetate concentrations for ST-3 nitrite inhibition experiments where 1 mM nirite was added to the cultures at hor number 43.

Time	Average Lactate Concentration (mM)	Lactate Concentration Standard Deviation	Average Acetate Concentration	Acetate Concentration Standard Deviation
0	34.06	2.03	11.43	0.62
32	33.15	2.27	12.86	1.94
43	11.28	2.10	35.24	5.08
51.5	9.81	2.51	32.02	0.82
95.5	10.87	1.89	33.98	3.09
172	10.12	1.51	31.35	5.80
261.75	10.51	2.30	33.71	2.42
357	8.43	4.60	37.48	11.78
590	10.19	2.54	31.09	2.17

Effect of nitrite addition on lacatate and acetate concentrations of ST-tSRB-8A-2 where no nitrite was added to the bottle 16 culture

Time	Acetate Concentration (mM)	Lactate Concentration (mM)
0	32.42	8.43
26	32.79	14.39
29.75	26.87	13.52
40.75	23.96	21.96
78.75	26.7	26.12
416	27	21.67

Effect of nitrite addition on lacatate and acetate concentrations of ST-tSRB-8A-2 where no nitrite was added to the bottle 19 culture

Time	Acetate Concentration (mM)	Lactate Concentration (mM)
0	37.23	11.94
26	34.68	16.4
29.75	28.09	17.76
40.75	21.06	21.67
78.75	24.47	21.49
416	24.03	18.67

Effect of nitrite addition on lacatate and acetate concentrations of ST-tSRB-8A-2 where no nitrite was added to the bottle 20 culture

Time	Acetate Concentration (mM)	Lactate Concentration (mM)
0	29.86	9.64
26	36.02	15.49
29.75	30.55	14.99
40.75	26.06	22.52
78.75	25.19	22.21
416		

Average lactate and acetate concentrations for ST-tSRB-8A-2 nitrite inhibition experiments where no nirite was added to the cultures

Time	Average Lactate Concentration (mM)	Lactate Concentration Standard Deviation	Average Acetate Concentration	Acetate Concentration Standard Deviation
0	33.17	3.74	10.00	1.78
26	34.50	1.62	15.43	1.01
29.75	28.50	1.87	15.42	2.15
40.75	23.69	2.51	22.05	0.43
78.75	25.45	1.14	23.27	2.49
416	25.52	2.10	20.17	2.12

Effect of nitrite addition on lacatate and acetate concentrations of ST-tSRB-8A-2 where 0.5 mM nitrite added to the bottle 1 culture at hour 26.

Time	Acetate Concentration (mM)	Lactate Concentration (mM)
0		
26	23.62	22
29.75	19.75	19.79
40.75	22.47	22.53
78.75	22.14	21.72
416	24.37	20.61

Effect of nitrite addition on lacatate and acetate concentrations of ST-tSRB-8A-2, 0.5 mM nitrite was added to the bottle 4 culture at hour 26.

Time	Acetate Concentration (mM)	Lactate Concentration (mM)
0	26.17	8.37
26	23.52	21.69
29.75	21.51	21.07
40.75	22.13	21.87
78.75	26.08	19.4
416	25.68	19.22

Effect of nitrite addition on lacatate and acetate concentrations of ST-tSRB-8A-2, 0.5 mM nitrite was added to the bottle 10 culture at hour 26.

Time	Acetate Concentration (mM)	Lactate Concentration (mM)
0	37.87	12.29
26	24.75	21.14
29.75	22.87	20.74
40.75	22.19	20.5
78.75	24.03	17.05
416	24.4	18.71

Average lactate and acetate concentrations for ST-tSRB-8A-2 nitrite inhibition experiments. 0.5 mM nirite was added to the cultures at hour 26.

Time	Average Lactate Concentration (mM)	Lactate Concentration Standard Deviation	Average Acetate Concentration	Acetate Concentration Standard Deviation
0	32.02	8.27	10.33	2.77
26	23.96	0.68	21.61	0.44
29.75	21.38	1.56	20.53	0.66
40.75	22.26	0.18	21.63	1.04
78.75	24.08	1.97	19.39	2.34
416	24.82	0.75	19.51	0.98

Effect of nitrite addition on lacatate and acetate concentrations of ST-tSRB-8A-2 where 1 mM nitrite added to the bottle 5 culture at hour 26.

Time	Acetate Concentration (mM)	Lactate Concentration (mM)
0	30.049	11.48
26	23.98	18.7
29.75	23.48	20.27
40.75	22.4	19.45
78.75	27.78	23.35
416	23.67	25.14

Effect of nitrite addition on lacatate and acetate concentrations of ST-tSRB-8A-2, 1 mM nitrite was added to the bottle 8 culture at hour 26.

Time	Acetate Concentration (mM)	Lactate Concentration (mM)
0	35.11	11.32
26	27.07	21.41
29.75	24.34	20.6
40.75	23.76	20.49
78.75	27.105	20.39
416	24.32	19.31

Average lactate and acetate concentrations for ST-tSRB-8A-2 nitrite inhibition experiments1 mM nirite was added to the cultures at hour 26.

Time	Average Lactate Concentration (mM)	Lactate Concentration Standard Deviation	Average Acetate Concentration	Acetate Concentration Standard Deviation
0	32.58	3.58	11.40	0.11
26	25.53	2.18	20.06	1.92
29.75	23.91	0.61	20.44	0.23
40.75	23.08	0.96	19.97	0.74
78.75	27.44	0.48	21.87	2.09
416	24.00	0.46	22.23	4.12

Effect of nitrite addition on lacatate and acetate concentrations of ST-tSRB-8A-2 where 2 mM nitrite added to the bottle 2 culture at hour 26.

Time	Acetate Concentration (mM)	Lactate Concentration (mM)
0	32.76	10.4
26		
29.75	20.44	19.85
40.75	21.14	20.12
78.75	20.91	20.08
416	22.47	20.62

Effect of nitrite addition on lacatate and acetate concentrations of ST-tSRB-8A-2, 2 mM nitrite was added to the bottle 3 culture at hour 26.

Time	Acetate Concentration (mM)	Lactate Concentration (mM)
0	33.7	10.69
26	22.53	22.5
29.75	20.56	21.42
40.75	17.65	18.16
78.75	28.23	28.85
416	23.99	21.09

Effect of nitrite addition on lacatate and acetate concentrations of ST-tSRB-8A-2, 2 mM nitrite was added to the bottle 7 culture at hour 26.

Time	Acetate Concentration (mM)	Lactate Concentration (mM)
0	35.64	11.42
26	21.45	19.08
29.75	21.54	20.22
40.75	23.36	22.25
78.75	32.12	23.59
416	25.58	19.39

Average lactate and acetate concentrations for ST-tSRB-8A-2 nitrite inhibition experiments. 2 mM nirite was added to the cultures at hour 26.

Time	Average Lactate Concentration (mM)	Lactate Concentration Standard Deviation	Average Acetate Concentration	Acetate Concentration Standard Deviation
0	34.03	1.47	10.84	0.53
26	21.99	0.76	20.79	2.42
29.75	20.85	0.60	20.50	0.82
40.75	20.72	2.88	20.18	2.05
78.75	27.09	5.69	24.17	4.41
416	24.01	1.56	20.37	0.88

Effect of nitrite addition on lacatate and acetate concentrations of ST-tSRB-8A-2 where 4 mM nitrite added to the bottle 9 culture at hour 29.

Time	Acetate Concentration (mM)	Lactate Concentration (mM)
0	40.32	13.05
26	28.08	16.8
29.75	25.13	21.11
40.75		
78.75	30.53	24.09
416	26.88	21.95

Effect of nitrite addition on lacatate and acetate concentrations of ST-tSRB-8A-2, 4 mM nitrite was added to the bottle 11 culture at hour 29

Time	Acetate Concentration (mM)	Lactate Concentration (mM)
0	36.98	11.86
26	33.54	20.73
29.75	23.28	18.9
40.75	23.21	19.4
78.75	21.44	24.75
416	22.25	26.44

Effect of nitrite addition on lacatate and acetate concentrations of ST-tSRB-8A-2, 4 mM nitrite was added to the bottle 13 culture at hour 29

Time	Acetate Concentration (mM)	Lactate Concentration (mM)
0	35.23	11.42
26	29.38	16.21
29.75	24.44	17.81
40.75		
78.75	29.45	17.09
416	25.73	15.61

Average lactate and acetate concentrations for ST-tSRB-8A-2 nitrite inhibition experiments. 5mM nirite was added to the cultures at hour 29

Time	Average Lactate Concentration (mM)	Lactate Concentration Standard Deviation	Average Acetate Concentration	Acetate Concentration Standard Deviation
0	37.51	2.59	12.11	0.84
26	30.33	2.85	17.91	2.46
29.75	24.28	0.93	19.27	1.68
40.75	23.21		19.40	
78.75	27.14	4.97	21.98	4.24
416	24.95	2.41	21.33	5.44

Effect of nitrite addition on lacatate and acetate concentrations of ST-tSRB-8A-2 where 5 mM nitrite added to the bottle 14 culture at hour 29

Time	Acetate Concentration (mM)	Lactate Concentration (mM)
0	33.21	10.5
26	29.82	15.6
29.75	24.62	16.63
40.75	25.3	17.35
78.75	27.92	15.4
416	31.26	17.5

Effect of nitrite addition on lacatate and acetate concentrations of ST-tSRB-8A-2, 5 mM nitrite was added to the bottle 15 culture at hour 29

Time	Acetate Concentration (mM)	Lactate Concentration (mM)
0	34.78	11.28
26	27.54	14.43
29.75	27.09	17.55
40.75	24.9	16.21
78.75	24.52	24.042
416	26.68	25.84

Effect of nitrite addition on lacatate and acetate concentrations of ST-tSRB-8A-2, 5 mM nitrite was added to the bottle 18 culture at hour 29

Time	Acetate Concentration (mM)	Lactate Concentration (mM)
0	40.8	13.3
26	32.8	17.35
29.75	27.44	17.56
40.75	27.34	16.98
78.75	26.9	24.85
416	25.43	24.06

Average lactate and acetate concentrations for ST-tSRB-8A-2 nitrite inhibition experiments. 5mM nirite was added to the cultures

Time	Average Lactate Concentration (mM)	Lactate Concentration Standard Deviation	Average Acetate Concentration	Acetate Concentration Standard Deviation
0	36.26	4.01	11.69	1.45
26	30.05	2.64	15.79	1.47
29.75	26.38	1.54	17.25	0.53
40.75	25.85	1.31	16.85	0.58
78.75	26.45	1.74	21.43	5.24
416	27.79	3.07	22.47	4.39