CHARACTERIZATION OF EXTREMOPHILIC SULFUR OXIDIZING MICROBIAL COMMUNITIES INHABITING THE SULFUR BLOCKS OF ALBERTA'S OIL SANDS

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ABSTRACT

This study was designed to determine if Alberta's sulfur blocks were inhabited by microorganisms which contribute to oxidation of elemental sulfur. The first objective was to elucidate a functional method of differentiating between viable and non-viable organisms in environmental samples. The second objective was to use this and other more established microbiological analyses to characterize the microbial population inhabiting the block and determine if they influence elemental sulfur oxidation.

In order to differentiate between viable and non-viable microorganisms, I relied on a DNA binding agent called ethidium monoazide bromide (EMA). Based on previous literature, I was able to test its activity in different environmental samples. Treatment with EMA inhibits the amplification of free DNA, whereas DNA protected by the membranes of viable cells is not affected. After finding that killed pure culture cells had a substantial reduction in their DNA amplification I proceeded to inoculate the same species of killed and viable cells into either soil, biofilm, or elemental sulfur samples obtained from Syncrude's Phase I sulfur block. I have found the EMA treatment to be sufficient at inhibiting amplification of DNA from non-viable cells inoculated into both the soil and sulfur samples, but not in the biofilms.

In achieving the second objective I designed experiments which tested *in vitro* and *in situ* samples of the sulfur block. Bioreactors containing microbiological inoculants from water running off the sulfur block were compared to sterile bioreactors for levels of acidity, sulfate accumulation and microbial population. Comparison between the surface block samples and the matrix samples showed a higher number of bacteria in the surface samples; however, the differences between the two bioreactor

treatments were not significant. Bioreactors which received sterile water did not increase in acidity or sulfate accumulation. The two treatments which were inoculated with 10% sulfur block run off increased by 3 and 4.3 mM sulfate, and 8.6×10^{-3} and 1.8×10^{-2} hydronium ion concentration, in the surface and matrix treatments respectively. *In situ* samples obtained by coring the sulfur blocks showed that microbial inhabitants are present throughout the block depth profile with a discontinuous pattern, which could be attributed to the fractures associated with the solidification of the block and subsequent colonization. The level of microbiological inhabitants ranged from 2.5 to 5.5 log heterotroph colony forming units g^{-1} sulfur, and 3.19×10^{1} to 1.62×10^{2} *A. thiooxidans* amplified copy numbers, and 1.23×10^{3} to 1.11×10^{4} *Eubacteria* amplified DNA copy numbers μg^{-1} of extracted DNA from EMA treated sulfur block samples. Most probable number counts for autotrophs only detected organisms along the 0-10 cm depth of the block.

The results of this study suggest that the use of ethidium monoazide bromide is a suitable method of detecting the large and varied microbial population inhabiting Alberta's sulfur blocks which can influence the level of block oxidation. The level of microorganisms present in the block is varied, which may parallel the varied pockets of air and water collected in the geomorphic fractures. Microbial communities residing in the sulfur block are partially responsible for sulfur oxidation. Methods aimed at reducing the level of sulfur oxidation must aim to reduce both the chemical and biological pathways leading to sulfur oxidation.

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LIST OF ABBREVIATIONS

SO_2	Sulfur dioxide
PCR	Polymerase chain reaction
TSB	Tryptic soy broth
EMA	Ethidium monoazide bromide
ARD	Acid rock drainage
ATP	Adenosine tri-phosphate
SAB	Special Antarctic Blend
Q-PCR	Quantitative polymerase chain reaction
DGGE	Denaturing gradient gel electrophoresis
CFU	Colony forming unit
MPN	Most probable number
ATCC	American type culture collection
SOX	Sulfur oxidizing enzyme
SOR	Sulfur oxygenase reductase
DSR	Dissimilatory sulfite reductase

1.0 INTRODUCTION

Global mining and extraction processes are responsible for mobilizing 1.5×10^{11} kg of sulfur per year (Edwards et al., 2000). Because of the current global surplus of elemental sulfur, the industry finds it more economical to store the commodity until market values improve. The expectant storage period could extend up to 100 years or more. Current methods of storing elemental sulfur, by pouring the molten form into large exposed depositories threaten to contaminate the surrounding environment with acidic drainage. Natural chemical reactions from the exposure of air and water oxidize the elemental sulfur into sulfuric acid, which risks seeping to surrounding areas of the block. Both the elemental sulfur and rain water effluent is suspected of harboring microorganisms, which may contribute to the production of sulfuric acid. Elucidating the role of the microbial inhabitants of the block will lead to the development of methods aimed at reducing sulfate accumulation and release into the environment.

One of the most significant environmental anthropogenic impacts is acid mine drainage, which is formed from the oxidation of exposed metal ores (Bridge, 2004; Leduc, 2002). Alberta's elemental sulfur blocks are enormous, man-made landmarks which are characteristic of the landscape found in the oil sands, north of Fort McMurray. Oil sweetening processes involve removing sulfur impurities from crude oil, resulting in an excess of purified elemental sulfur. When exposed to the atmosphere, elemental sulfur undergoes biological and geological oxidation producing sulfuric acid, which can leach into the soils and water surrounding the block. Stockpiles of elemental sulfur from mining and extraction processes account for approximately 50% of net river transport of sulfate to the oceans (Edwards et al., 2000). Elevated levels of sulfuric acid reduce ecosystem pH with detrimental effects on the flora and fauna of the area. Current

methods used to decrease the amount of sulfuric acid being produced involve the limitation of oxygen to the tailings areas by providing a vegetative cover (Brooks et al., 1989). However, these methods are often not rapid enough to prevent acid rock drainage and are sometimes ineffective, because the vegetative cover has very slow growing root systems which are incapable of effectively removing the oxygen containing pores (Brooks et al., 1989).

The many thin layers that form from different pouring events are exposed to the elements and windblown dust, before being sealed with the next molten sulfur coating. Solidification and settling processes cause the pure elemental sulfur blocks to fracture and shift, providing more surface area for water and gas contact. It is these surfaces which are most suspect of harboring microorganisms which can contribute to oxidation processes. Microorganisms are capable of thriving in some of the most extreme environments on the planet; therefore the possibility of certain species inhabiting the nutrient poor sulfur blocks is quite likely. Microbial communities expected within the sulfur block profile include both chemolithotrophs, capable of fixing carbon dioxide as a source of carbon, and heterotrophs, which rely on organic sources obtained from detritus and windblown fragments.

It is unknown whether microorganisms are in fact thriving in the depths of the sulfur block, and if these microorganisms are capable of a significant contribution to sulfuric acid run off. In order to develop a more successful method of limiting sulfuric acid discharge, the biological parameters involved in the process must be further investigated.

2.0 LITERATURE REVIEW

2.1 Economics of elemental sulfur

2.1.1 The global sulfur market

The environmental benefits from burning fossil fuels with decreased sulfur contents have long been realized by the petroleum industry. The burning of fuels which are high in sulfur results in elevated sulfur dioxide (SO_2) emissions, which contribute to acid rain and climate change. A high atmospheric content of sulfate particles increases the reflection of sunlight, and contributes to cooling the atmosphere (Smith et al., 2001). Since a greater awareness of global warming and concern regarding emission controls, environmental mandates have come into place forcing the hydrocarbon industry to reduce emission levels of harmful gases. Many oil companies have since invested in the development of economical methods used to detoxify combustion gases released into the environment (Bejarano et al., 2001).

Removal of impurities from fuel by the hydrocarbon industry leads to the acquisition of more sulfur than the market demands, particularly because of the increase in demand for clean fossil fuels in the last few decades. As of 2003, sulfur produced from the oil and gas industries represented two-thirds of all global sources of sulfur (Chasez, 2003). Before this massive addition to sulfur production from the hydrocarbon industry the sulfur market was relatively balanced with a controlled production of sulfur relying on mining and smelting operations (Chasez, 2003). With the vast oversupply of sulfur across the globe, environmentally and economically feasible means of indefinite storage for this commodity must be determined.

Elemental sulfur is an essential and convenient form of sulfur which can be used by a variety of different industries. Elemental sulfur can be easily converted into forms and compounds which are widely used, particularly in construction, metal refining, and agriculture (Nakamura, 2003). It is the raw material of choice for most sulfur containing products because it is easy to store and transport (Bejarano et al., 2001). The majority of the elemental sulfur trade ends in the conversion to sulfuric acid. Sulfuric acid, a mineral acid derived from elemental sulfur, is among the most economically important inorganic chemicals on the market (Bejarano et al., 2001). Sulfuric acid is commonly used in fertilizers.

The increase in demand for oil has since changed into an increase in demand for clean burning oil. The hydrocarbon industry has been steadily growing since the 1980's and with that has faced stricter environmental emission controls. Before this oil boom, the global sulfur supply retained a functional balance. Voluntary sulfur producers, which dominated the market in the past, were capable of curtailing production when market demand was low, and production costs exceeded gains (Chasez, 2003). However, the growth in the hydrocarbon industries has resulted in a growth in forced sulfur production, and an oversupply in the sulfur market. This oversupply resulted in the closure of most voluntary producers, with only a small proportion of mining operations still existing in countries such as Poland (Chasez, 2003). Canada plays a dominant role in the forced production of sulfur, achieved almost exclusively through the involuntarily production from our oil sands developments. Both Canada and the United States provide a total of 40% of global sulfur production (Chasez, 2003). Canada is the world's largest elemental

sulfur exporter, with target markets primarily in China and the United States (Chasez, 2003).

Three quarters of North American sulfur is used in the phosphate industry, and phosphate operations are projected to increase, so this may decrease the current market strain for sulfur (Chasez, 2003). Exploration of new niche markets could and should occur. However, the gross oversupply will need to be stored until such favorable changes in the market begin to take place.

The current sulfur surplus is predicted to grow as the oil and gas industry will be pressured to have even stricter regulations on sulfur content in fossil fuels and developing countries who have otherwise not controlled the sulfur content of their produced fuels will need to develop new limitations. With the growing concern in the developed world on environmental conservation, industries will find stricter regulations for production of clean burning fuels. This greater demand on refined fuels will lead to additional supplies of elemental sulfur. Future clean fuel regulations adopted by developing countries could lead to an additional global sulfur increment of 1.8 million tons per year (Nakamura, 2003).

2.1.2 Elemental sulfur storage depositories

The economic demand for sulfur has been oversupplied since the early 1990's and future predictions indicate little change in this market for the upcoming decades (McKenna, 2004). Because of this, prices have dropped significantly, forcing companies to stockpile the sulfur that they would normally sell. One of the most common methods of stockpiling sulfur is in the form of large blocks.

Canada's largest crude oil producer, Syncrude Canada Ltd., produces ~7.5 Kg of elemental sulfur for each barrel of crude oil they process (McKenna, 2004). The low market prices and high transportation costs have forced them to store the sulfur in above ground blocks. Elemental sulfur blocks can range in size between 100-400 meters long, 50-200 meters wide, and 6-15 meters high (McKenna, 2004). In 2002, Kazakhstan poured 1.4 million tonnes of sulfur, followed by Syncrude, with 0.5 million tonnes, and Iran with 0.4 million tonnes (Chasez, 2003). Syncrude's sulfur blocks are located in north eastern Alberta's Athabasca river basin, near Fort McMurray, Canada.

The sulfur is stored by the Claus process, which involves transporting and pouring molten sulfur (melted at ~150 ºC) into large moldings by free lifts, and allowing it to cool at ~115 ºC, which results in the characteristic bright yellow color (Laishley and Bryant, 1987; McKenna, 2004). The sulfur block that results from the Claus process contains 99.8% pure elemental sulfur (McKenna, 2004). An aerial photograph of some of the exposed sulfur blocks at Syncrude is shown in Figure 2.1.

The sulfur blocks are prone to cracks and fines, which are a part of the crystalline modification caused by the sulfurs monoclinic and orthorhombic forms, and occur even with proper maintenance (Clark, 2005). These cracks are of particular interest for they are likely areas of microbial growth as they are protected from harsh environmental conditions, such as direct sunlight and strong winds, and may contain an elevated level of water availability. The sulfur blocks contain black layers along the cracks and fines of the top plateau, typically one centimeter below the surface, this discoloration is believed to be attributed to bacterial activity, but has not been confirmed (McKenna, 2004).

Figure 2.1. Picture of an aerial view of exposed sulfur blocks at Syncrude taken in 1998 (Tyler Birkham).

2.2 Oxidation of elemental sulfur

2.2.1 Environmental impact of sulfuric acid accumulation

Alberta's sulfur blocks can pose a great environmental risk. The sulfate produced on and around the sulfur-blocks is transported to the surrounding environment via high intensity rainfall and leaching. Additionally, sulfur dust can be wind swept off the sulfur blocks and accumulate further down-wind, resulting in other environmental disturbances (Maynard et al., 1986; Laishley and Bryant, 1987; Crescenzi et al., 2006).

The Athabasca river basin, where Syncrude's sulfur blocks are located, experiences average temperatures ranging from -20˚C in January, to 15˚C in July, and an annual rainfall precipitation of <300 mm (Akre, 2004). The accumulation of water through the fractures of the block increases the likelihood of oxidation, and results in a highly acidic sulfate discharge from the base of the blocks. The accumulation of high levels of sulfuric acid damages the environment by increasing levels of toxic metals and decreasing essential nutrient concentrations. Toxic metals become mobilized by decreasing metal sorption to clays and as a result, metal concentrations in the environment increase, becoming toxic to the surrounding plants (Laishley and Bryant, 1987). Similarly, protons replace colloidally-bound Ca^{2+}, K^{+}, and Mg^{+} ions and these essential nutrients leach out of the system (Laishley and Bryant, 1987). Finally, plants can also become directly damaged from the acidity, and be physically incapable of taking up nutrients (Laishley and Bryant, 1987). The most direct biological impact from accumulating acidity is on the microflora, which will demonstrate decreased levels of diverse heterotrophic organisms (Laishley and Bryant, 1987).

Small particles of sulfur can be windswept off the blocks and settle kilometers away from the site. Accumulation of sulfur particles in the soil will impact microbial communities and levels of plant available sulfur. The sulfur dust off the blocks also contains sulfur oxidizing organisms which may be swept to the new areas promoting sulfur bio-oxidation by the native communities. A study performed in the Whitecourt, Alberta area showed that soils downwind from a sulfur stockpile demonstrated higher levels of sulfur oxidizing autotrophic *Acidithiobacilli* than the control sites (Laishley and Bryant, 1987); however, these results were not consistent with *in situ* experiments performed which examined the sulfur surface colonization of these species (Lawrence and Germida, 1991). Studies using Saskatchewan agricultural soils supplemented with sulfur beads have shown that the microbial population shifted toward a sulfate producing community predominantly composed of thiosulfate-oxidizing autotrophs, but sulfur oxidizing autotrophs, like *Acidithiobacilli* were not detected (Lawrence et al., 1988, Lawrence and Germida, 1991). Without the addition of sulfur, the most abundant sulfur oxidizing population of the agricultural soils is heterotrophic (Lawrence and Germida, 1991).

2.2.2 Sulfur oxidizing microorganisms

Oxidation of the elemental sulfur blocks compromises the integrity of the structures and releases environmentally harmful effluent to the surroundings. Oxidation can occur by chemical and biological means. Chemical sulfur oxidation is a spontaneous reaction requiring the presence of both water and oxygen. Biological oxidation can be much more complicated, as it varies between organisms which rely on different

enzymatic processes. The unique environment that is formed directly around and within the profile of the sulfur blocks selects for certain species of microorganisms which can benefit from the available nutrition. Sulfur oxidizing microorganisms can be divided into two main groups: autotrophs and heterotrophs. Autotrophs are capable of fixing carbon dioxide for their carbon requirements, and heterotrophs utilize carbon from organic matter. Inhabitants of the sulfur blocks are believed to predominantly include autotrophic sulfur oxidizing microorganisms, such as *Acidithiobacilli* (Table 2.1).

Microorganisms are major contributors to sulfur oxidation processes. Researchers estimate that 90% of the acid that is generated from mine tailings can be attributed to bacterial action (Brooks, 1989). These organisms have adapted unique methods for achieving acidic products, and are capable of oxidizing sulfur under aerobic or anaerobic, and light or dark conditions (Laishley and Bryant, 1987). The oxidation of inorganic sulfur to sulfate is a major component of the biological sulfur cycle, shown in Equation 2.1 (Friedrich, 2001).

$$2S + 3O_2 + 2H_2O \rightarrow 2H_2SO_4$$

Eq. 2.1

Some of the important organisms which have been found to oxidize inorganic sulfur are shown in Table 2.1. These organisms have been found in a range of environments, and they have unique mechanisms of sulfur oxidation. They are capable of sulfur oxidation when directly attached to sulfur compounds, or in certain cases, when they are free in the system (Ceškova, 2002).

Eukaryotic acidophiles have been shown to have a greater energetic cost in surviving low pHs as they require the maintenance of a very high proton gradient through their membranes (Messerli, 2005). Nonetheless, this group of organisms is found thriving in extreme acidic environments. The presence of acidic fungi may be apparent by the green slime build up along the edges of the effluent catchments around the sulfur blocks. Strings of this eukaryotic fungus are present along the run-off streams, which have been measured below a pH of zero. Though the acidophilic fungi are capable of thriving along the block and within the effluent streams, there is little evidence in the literature of their contribution to sulfur oxidation.

Sulfur oxidization is performed by Bacteria and Archaea (Johnson, 2003). Archaea are classified into either Euryarchaeota or Crenarchaeota, and these kingdoms are further divided into 8 orders, comprising of a total of 17 families (Elshahed, 2004). Archaea are generally associated with the most extremophilic environments. Archaeal sulfur oxidizers have the highest temperature tolerances and are capable of growing at the most extreme acidic pHs (Johnson, 2003). The genus *Sulfolobus* is composed of archaeal members. Archaea are distinguishable from Bacteria because they possess ether linked cell walls, lack a peptidoglycan layer and have long hydrocarbon chained cytoplasmic membranes (Laishley and Bryant, 1987). These unique physiological structures aid in withstanding high temperatures and low pHs, and render them capable of oxidizing H_2S to elemental sulfur, and subsequently sulfate, in extreme environments that other microorganisms may not tolerate (Laishley and Bryant, 1987).

Table 2.1. Important chemolithotrophic microorganisms capable of oxidizing inorganic sulfur.

Genus	Species	Description	References
Acidithiobacillus	*ferrooxidans, thiooxidans, caldus*	aerobic, γ Proteobacteria, acidophilic, optimum temperature is 28-45°C, no SOX proteins, GC content 52-63.9 mol% , not tolerant to NaCl, colorless, metal tolerant	(Ceškova et al., 2002; Friedrich et al., 2001; Friedrich et al., 2005; Harrison, 1984; Johnson and Hallberg, 2003; Kelly and Wood, 2000; Laishley and Bryant, 1987; Leduc, 2002; Suzuki et al., 1999*a*)
Thiobacillus	*denitrificans thioparus neopolitanus acidophilus*	facultative anaerobe, γ Proteobacteria, neutrophilic, transient sulfur depositor, uses thiosulfate as an electron donor	(Friedrich et al., 2001; Friedrich et al., 2005; Kelly and Wood, 2000; Leduc, 2002)
Acidianus	*ambivalens tengchongensis*	facultative anaerobe, Archaea, member of the Sulfolobales order and Crenarchaeota kingdom, acidophilic, optimum temperature is >60°C, contains SOR, GC content 31 mol%, coccoid morphology	(Friedrich et al., 2001; Friedrich et al., 2005; Fuchs et al., 1996; Johnson and Hallberg, 2003; Kletzin et al., 2004) (Fuchs et al., 1996)
Aquifex	*aeolicus pyrophilus*	aerobic, neutrophilic, optimum pH of 6.8, thermophile, incomplete SOX clusters, GC content 40 mol%, isolated from hot marine sediments	(Friedrich et al., 2001; Friedrich et al., 2005; Huber et al., 1992)
Beggiatoa		aerobic, mixotrophic, filamentous, difficult to culture, found in freshwater, rely on oxidation of H_2S and S^0 for energy, but organics for carbon	(Friedrich et al., 2001; Laishley and Bryant, 1987; Ruby et al., 1981)
Chlorobium	*tepidum acidophilium*	aerobic, phototrophic, SOX and DSR clusters, oxidizes H_2S and S^0 , maximum sulfide tolerance of 4-8 nM	(Friedrich et al., 2001; Friedrich et al., 2005; Laishley and Bryant, 1987)

	salicylatoxidans		
Rhodovulum	*sulfidophilum*	aerobic, phototrophic, SOX enzyme system	(Friedrich et al., 2001; Friedrich et al., 2005)
Rhodobacter	*capsulatus*	aerobic, phototrophic	(Friedrich et al., 2001)
Paracoccus	*denitrificans, pantotrophs*	aerobic, first characterized SOX enzyme system	(Friedrich et al., 2001; Friedrich et al., 2005)
Starkeya	*novella*	aerobic, neutrophilic, SOX enzyme system, formerly *Thiobacillus novellas*	(Friedrich et al., 2001; Friedrich et al., 2005; Ravenschlag et al., 2001)
Methylobacterium	*extorquens*	aerobic, SOX enzyme system	(Friedrich et al., 2001; Friedrich et al., 2005)
Rhodopseudomonas	*palustris*	aerobic, phototrophic, SOX enzyme system	(Friedrich et al., 2001; Friedrich et al., 2005)
Allochromatium	*vinosum*	facultative anaerobe, phototrophic, γ Proteobacteria, anoxygenic, DSR system, purple sulfur bacteria	(Friedrich et al., 2001; Friedrich et al., 2005)
Thiomicrospira	*chilensis pelophila thioparus*	originally described from estuarine environments, thiosulfate as electron donor, oxidizes H_2S and S^0, mesophilic, motile, optimum growth between 5.3 – 8.5, rod shaped	(Brinkhoff et al., 1999; Laishley and Bryant, 1987; Ruby et al., 1981)
Sulfolobus	*sulfataricus tokodaii*	aerobic, acidophilic, optimum temperature is >60°C, contains SOR, oxidizes H_2S and S^0	(Friedrich et al., 2001; Friedrich et al., 2005; Johnson and Hallberg, 2003; Kletzin et al., 2004; Laishley and Bryant, 1987)
Thermithiobacillus		aerobic	(Friedrich et al., 2001)
Thiocapsa	*roseopersicina*	aerobic, phototrophic	(Friedrich et al., 2001)
Thiomonas	*cuprina intermedia*	neutrophilic, optimum temperature is <40°C, formerly *Thiobacillus intermedia*	(Johnson and Hallberg, 2003; Leduc, 2002; Ravenschlag et al., 2001)

Hydrogenobacter	*acidophilus*	optimum temperature is 40-60°C	(Johnson and Hallberg, 2003)
Metallosphaera		optimum temperature is >60°C	(Johnson and Hallberg, 2003)
Xanthobacter		aerobic	(Friedrich et al., 2001)
Thiovirga	*sulfuroxydans*	microaerophilic, γ Proteobacteria, neutrophilic pH 7.5, mesophilic (30-34°C), 16SrRNA sequenced, GC content 47.1 mol% sensitive to NaCl, found in wastewater biofilm	(Ito, 2005)
Pseudaminobacter	*salicylatoxidans*	contains SOX enzyme system	(Friedrich et al., 2005)
Silicibacter	*pomeroyi*	contains SOX enzyme system	(Friedrich et al., 2005)
Acidiphilium	*acidiphilum*	facultative anaerobe, acidophilic, formerly known as *Thiobacillus acidophilus*	(Leduc, 2002)

2.2.2.1 Autotrophic and heterotrophic sulfur oxidizers

Autotrophic, or lithotrophic, sulfur oxidizers obtain energy from the oxidation of reduced sulfur compounds. This energy can be used to fix atmospheric CO_2, enabling these microorganisms to survive in nutrient poor, sulfur-dominated environments, such as the sulfur block. Autotrophic sulfur oxidation is performed through the transport of electrons to oxygen, generating a proton motive force which produces energy in the form of adenosine triphosphate (ATP) (Laishley and Bryant, 1987). Autotrophic microorganisms capable of oxidizing sulfur can use the electrons for respiratory chain energy transformation and for reduction of carbon dioxide (Friedrich et al., 2005). The electrons obtained from the reduced sulfur compounds flow to the electron transport chain, entering at either the flavoprotein or cytochrome c. Autotrophic fixation of carbon dioxide requires reverse electron flow through the electron transport chain which will lead to the formation of a reduced nicotinamide-adenine dinucleotide (NADH). The Calvin cycle can then use this NADH and ATP to fix the carbon dioxide.

The number of autotrophs is generally low in nutrient rich environments, such as soils, as their growth is inhibited by an abundance of organic compounds. This sensitivity has made autotrophs reliant on heterotrophs to decrease the levels of organic compounds in their surrounding environment (García et al., 1996). Consequently, environments such as soils are generally dominated by heterotrophic populations, and sulfur oxidation processes are controlled by these organisms. However, environments which had a substantial addition of reduced sulfur have shown that autotrophic microorganisms dominate oxidative activities and as such increase the rate of sulfur oxidation (Germida, 2005). Populations of autotrophic sulfur oxidizers were found to increase in agricultural soils only after the addition of elemental sulfur (Lawrence and Germida, 1991).

Acidithiobacilli, previously known as *Thiobacilli*, are key producers of sulfuric acid, as they are the most common sources of sulfate bioleaching, and also the most studied (Clark, 2005; Laishley and Bryant, 1987). There are three different sub classes of Acidithiobacilli, Type I, II and III, segregated based on their lipid compositions (Harrison, 1984). *Acidithiobacilli* have different pH optimums for biological activity, generally in the neutral or acidic pH range (Laishley and Bryant, 1987). Because of their pH preferences, these sulfur oxidizing organisms grow in species successions depending on the level of sulfate that has accumulated (Laishley and Bryant, 1987). The acid loving, *A. thiooxidans* can oxidize sulfur at a range of pH levels, but they cannot grow at a pH above 5 (Chen et al., 2004; Suzuki et al., 1999*a*). Though commonly associated with acid mine drainage environments, the presence of *A. thiooxidans* and *A. ferrooxidans* in Canadian agricultural soils is rare (Lawrence and Germida, 1991). *A. thiooxidans* and *A. ferrooxidans*, are tolerant to high levels of certain metals, however their ability to oxidize sulfur is limited by high metal concentrations (Suzuki et al., 1999*a*).

Another type of autotroph which can be capable of survival within the adverse environment of the sulfur block is the methylotrophic methanogen. Recent evidence of methane percolating through the block from the ground surface has shown concentrations approaching 1% (personal communication by Tyler Birkham). Methylotrophic methanogens are capable of surviving by obtaining energy from the minute amounts of methane that is found distributed within the block. Methylotrophic microorganisms are capable of utilizing reduced carbon substrates with no carbon-carbon bonds as their single energy source (Lidstrom, 1991). This provides evidence suggesting that the blocks contain suitable means for the growth of methylotrophs which are capable of utilizing the methane as a carbon source.

Heterotrophic organisms are dominant contributors to microbial community structure. They do not obtain any energy from sulfur oxidation (Laishley and Bryant, 1987), but are the principal sulfur oxidizers in soils low in sulfur (Lawrence and Germida, 1991). Different species of heterotrophs oxidize different forms of reduced elemental sulfur. For example, members of the following genera: *Bacillus*, *Arthrobacter*, and *Flavobacterium* can oxidize elemental sulfur or thiosulfate to make sulfate, and *Achromobacter spp.* and *Pseudomonas spp.* can oxidize thiosulfate to tetrathionate (Laishley and Bryant, 1987). Heterotrophic sulfur oxidizers, producing thiosulfate, were found to be the most abundant sulfur oxidizers in Saskatchewan agricultural soils (Lawrence and Germida, 1991).

In nutrient poor environments such as the sulfur block, heterotrophs can be nourished by the exudates of the autotrophs, which can maintain a low concentration of organic carbon in the environment (Johnson and Hallberg, 2003; Mahmoud et al., 2005). Though, they represent a lower sulfur oxidation activity in environments containing high levels of reduced sulfur, there are quite a few species of heterotrophs which are associated with sulfuric acid waste. These include the fungi *Aspergillus sp*. and *Penicillium sp*., and the bacteria *Flavobacterium acidurans*, *Bacillus sp.*, and certain heterotrophic *Acidiphilum sp*. (Leduc, 2002).

2.2.2.2 Acidophiles of the sulfur block

The extreme temperature shifts, low organic carbon, high acidity, and high purity of the elemental sulfur block make this a very unique environment. There are few similar study sites to that of the sulfur blocks, but certain qualities make it comparable to acid mine drainage sites. One of the most well studied extreme microbial communities was that of the acid mine drainage of Iron Mountain, California.

The Richmond mine at Iron Mountain has the lowest pH of acid water recorded, which approaches -4 (Bond et al., 2000; Johnson and Hallberg, 2003). The lowest pH known to support microbial growth is 0, harboring two different types of *Thermoplasmales*, which belong to the genus *Picrophilus* (Edwards et al., 2000). Organisms found around the Richmond mine area were dominated by *Eubacteria*, but low levels of rainfall during warm summer months caused archael numbers to increase by ten times their standard numbers, *Ferroplasma acidiphilum*, being the most dominant archaeon (Johnson and Hallberg, 2003). The community shift of the acid mine drainage community at Iron Mountain may be analogous to that of the sulfur block, which also experiences large temperature increases in the spring and summer months. The rain water which percolates through the cracks and crevices through the sulfur block accumulates enough sulfuric acid that the pH of the effluent at the base of the blocks has also reached levels below zero (personal communication with Tyler Birkham).

Typical acidophilic microorganisms grow at a pH optimum around 2 to 4 (Matin, 1999) but they maintain an internal pH ranging from 6 to 7 (Booth, 1985). In some instances, membrane bound enzymes of acidophilic bacteria, such as thiosulfate dehydrogenase of *A. thiooxidans,* can have optimum activity in acidic conditions (Messerli et al., 2005). Acidophiles are capable of withstanding the extreme environment because of specialized membrane structures which help them tolerate the acidity and maintain a positive internal membrane potential (Matin, 1999; Edwards et al., 2000).

An internal pH below 5 can result in the disintegration of proteins, and so maintenance of a neutral cytoplasmic pH becomes critical (Goulbourne, 1986). This can be accomplished by preventing the entrance of hydrogen ions (Goulbourne, 1986; Suzuki et al., 1999*a*; Messerli et

al., 2005) and maintained by the extrusion of protons, through regulation of cation passage and proton efflux systems (Booth, 1985; Handelsman, 2004).

Passive processes involved in membrane potential maintenance are determined by proton diffusion and the Donnan potential (Goulbourne, 1986; Matin, 1999). Factors which cause an increase in the proton diffusion rate and in turn increase the membrane potential, include a low external pH and a progressive inhibition of respiration (Booth, 1985; Matin, 1999). The Donnan potential considers the influence of positively charged macromolecules which are impermeable to the cell wall. The positive charge of these macromolecules influences the net proton influx, and in this way increases the membrane potential (Goulbourne, 1986; Suzuki et al., 1999*a*). At low pHs the positive charge of titratable molecules in the cytoplasm will increase as the carboxyl groups become protonated because of the accumulation of protons (Matin, 1999).

The environment created at the sulfur storage blocks is much more extreme than the typical acidophilic environment, suggesting that the organisms capable of surviving here must be highly specialized for the conditions. In this environment, acid tolerating physiological adaptations become critical for the organism's survival.

2.2.3 Biochemistry of sulfur oxidation

Biochemical sulfur oxidation follows the same path as chemical oxidation, however the enzymatic reactions involved add complexity to the system by changing the rate and level of chemical reactivity among the intermediates (Suzuki, 1999; Masau et al., 2001). The biochemical processes involved in sulfur oxidation differ among different sulfur oxidizing organisms, but they all demonstrate derivatives of one of two main processes either involving or not involving polythionates ($S_xO_6^{2-}$), molecules composed of a sulfur chain terminated at each end with a SO_3^- group (Kelly et al., 1997; Druschel et al., 2003a).

Sulfide (S^{2-}) oxidation is catalyzed by sulfide oxidase and yields monoclinic elemental sulfur (S^0), that spontaneously converts to orthombic elemental sulfur (S^8) (Figure 2.2) (Suzuki, 1999). Oxidation of elemental sulfur or thiosulfate ($S_2O_3^{2-}$) to sulfite (SO_3^2) is thought to occur by a sulfur oxygenase enzyme (Suzuki, 1965; Takakuwa, 1992). Some doubt exists about the importance of sulfur oxygenase because this enzyme is metabolically inefficient for autotrophs and therefore its existence is questionable in many autotrophs (Kelly, 1999). The accumulation of sulfite in the presence of sulfur results in the formation of thiosulfate, a reaction which is reversible with the help of rhodanese or thiosulfate cleaving enzyme (Laishley and Bryant, 1987; Suzuki, 1994; Suzuki, 1999).

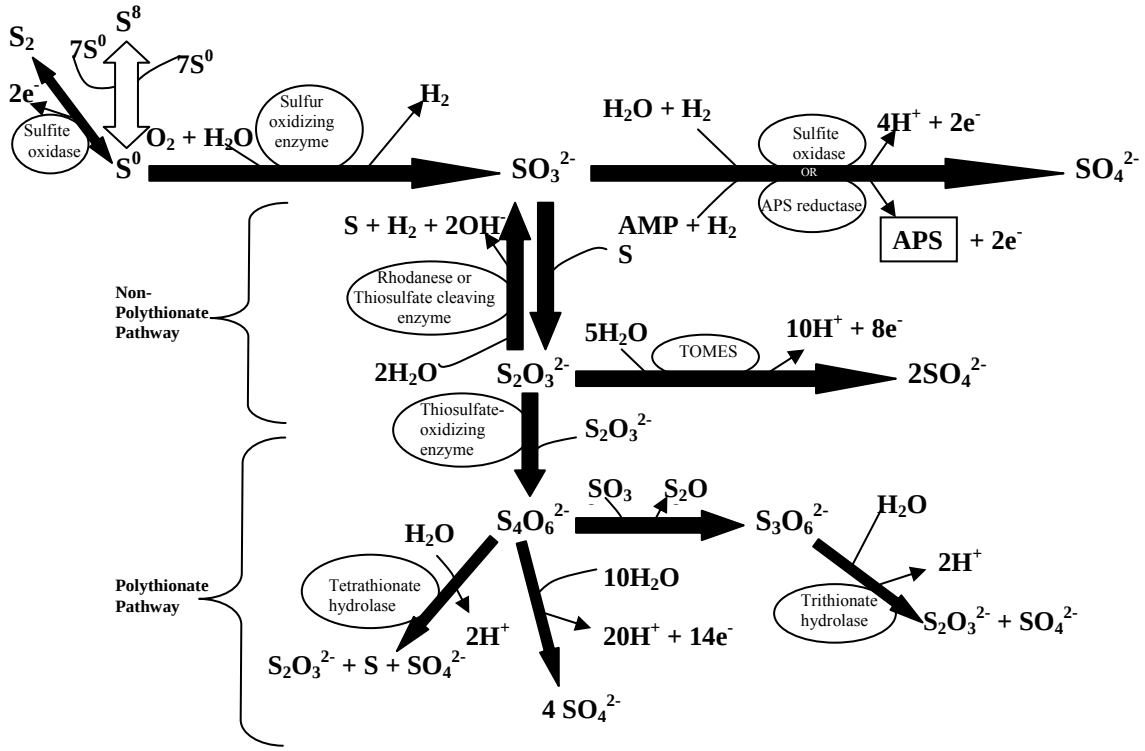

Figure 2.2.Simplified reactions involved in biological oxidation of sulfur, including the enzymatic (black arrows) and the chemical (clear arrow) reactions. The common known enzymes involved are circled and named. The route of a reaction depends on the organism and availability of substrates.

The oxidation pathways which emerge from thiosulfate can be differentiated by their involvement of polythionates ($S_xO_6^{2-}$) (Kelly et al., 1997). Bio-oxidation of thiosulfate through the non-polythionate pathway results in the formation of sulfate (SO_4^{2-}) via sulfite, catalyzed by either a sulfite-oxidizing enzyme or adenosine phosphosulfate reductase (APS) (Suzuki, 1965; Suzuki et al., 1992). The formation of sulfate is the major energy generating reaction (Laishley and Bryant, 1987; Suzuki, 1999) for organisms such as *Thiobacillus denitrificans* and *T. thioparus* which are autotrophs capable of oxidizing sulfite through the use of the adenosine monophosphate (AMP) dependant APS that generates energy through substrate level phosphorylation (Takakuwa, 1992; Friedrich, 1998; Suzuki, 1999; Harahuc and Suzuki, 2001; Kletzin et al., 2004).

Alternatively, a second non-polythionate pathway exists in some facultative autotrophs, such as *Paracoccus sp*., in which thiosulfate undergoes oxidation by the periplasmic thiosulfate oxidizing multi-enzyme system (TOMES) which produces sulfate without any detectable intermediates (Kelly et al., 1997). The non-polythionate utilizing microorganisms contain a complex of proteins within TOMES which are referred to as the SOX cluster. The SOX cluster contains a repressor (SoxR), membrane proteins (SoxV), periplasmic thioredoxins (SoxS and SoxW), sulfide dehydrogenase (SoxF) and the other periplasmic proteins SoxXYZABCD which form various complexes within TOMES (Friedrich et al., 2005). One of the essential enzymes involved with TOMES is SoxB, which has been utilized as a functional marker for sulfur oxidizing bacteria (Wodara et al., 1994; Friedrich et al., 2000; Petri et al., 2001). Microorganisms which oxidize reduced sulfur compounds via TOMES include: *Paracoccus* (or

Thiobacillus) *versutus*, *P. denitrificans*, and possibly *Thiobacillus novellas* and *Xanthobacter sp*. (Kelly et al., 1997).

The polythionate pathway begins with the combination of two molecules of thiosulfate to form tetrathionate ($S_4O_6^{2-}$) (Kelly et al., 1997). Cleavage of tetrathionate by tetrathionate hydrolase produces thiosulfate, sulfur and sulfate (Meulenberg et al., 1993; DeJong et al., 1997). This reaction takes place in the periplasm under high sulfate and low pH conditions (DeJong et al., 1997; Suzuki, 1999). In the cytoplasm, tetrathionate can react with ten molecules of water and be hydrolyzed into four sulfate molecules (Kelly et al., 1997; Druschel et al., 2003b). In conditions of accumulating sulfite, tetrathionate converts to thiosulfate and trithionate ($S_3O_6^{2-}$). Trithionate can be hydrolysed by trithionate hydrolase, making thiosulfate and sulfate (Suzuki, 1999). The polythionate pathway occurs in the autotrophs: *T. acidophilus, T. aquaesulis*, *T. tepidarus*, *T. neapolitanus*, *Acidithiobacillus ferrooxidans,* and *A. thiooxidans* (Lu and Kelly, 1988; Friedrich, 1998; Suzuki, 1999). It is characteristic of obligate and facultative chemoautotrophs, but evidence of certain steps of this pathway has been found in *Klebsiella aerogenes, Bacillus globigii, B. megaterium, Pseudomonas putida, P. fluorescens, P. aeruginosa, Aeromonas* sp. and some marine and haloalkaliphilic heterotrophs (Mason and Kelly, 1988; Sorokin, 2003).

Different organisms have different variations of the reactions or components of the reactions shown in Figure 2.2 (Laishley and Bryant, 1987). For example, all thiobacilli require reduced glutathione for the activity of their sulfur-oxidizing enzymes, which are responsible for oxidizing elemental sulfur to sulfite (Suzuki, 1994). Oxygen is also a key requirement in the biochemical reaction for all thiobacilli, aside from *A. ferrooxidans* which is capable of utilizing ferrous iron in anaerobic conditions.

2.3 Microbial characterization techniques

Cultivation of acidophilic chemolithotrophs can be extremely difficult (Leduc, 2002; Gonzàlez-Toril et al., 2003). In some cases the acidophilic sulfur oxidizers, like *Acidithiobacilli* can be out-competed by other faster growing organisms like fungi, or they can be inhibited by the impurities of the medium, particularly from the agar (Harrison, 1984; Leduc, 2002). Leduc et al. (2002) used an MPN method to quantitate both neutrophilic and acidophilic sulfur oxidizing organisms, but was unsuccessful in cultivating acidophiles. Such culturing difficulties have led characterization of these fastidious microbial communities to depend on the use of molecular analysis and microscopy, in addition to, or instead of, culture based methods. Non-culturable microorganisms can represent up to 99% of microbial communities in some environments (Schloss and Handelsman, 2003). This makes molecular techniques a fundamental practice in community characterization studies, and nucleic acid probing is one of the fastest, and most precise quantitative descriptors of microbial communities (Amann and Ludwig, 2000; Mahmoud et al., 2005).

Researchers have had great success in molecular studies on extremophilic microorganisms, and there have been extensive biodiversity studies on the organisms living in acidic rock drainage environments which share similarities to the sulfur blocks. Through the use of such techniques researchers were able to determine many of the dominant communities that inhabit extremophilic environments. Past research has shown that acid mine drainage communities are dominated by only a few different species of prokaryotes (such as *Acidithiobacillus*, *Thiobacillus, Leptospirillum, Acidiphilum,* and *Thiomonas*) and Archaea (such as *Thermoplasmatales* and *Sulfolobales*) (Baker and Banfield, 2003; Gonzàlez-Toril et al., 2003; Handelsman, 2004; Tyson et al., 2004). These organisms form a pink biofilm which floats along

the surface of the mine water, generating heat and decreasing the pH of the water (Handelsman, 2004; Tyson et al., 2004). The specific conditions of each acid mine drainage system determines which organisms can survive there.

Genomic characterization of microbial communities residing in the sulfur blocks is much more feasible than it would be for more complex communities, which have complicated sequence variations from the high number of horizontal gene transfer partners. This is because of the relative physiological similarity and the low number of species diversity in extreme environments. Though the costs of such analysis can be very high, it is diminishing with further advances in sequencing technology, which also improve the rapidity and accuracy of assays (Handelsman, 2004).

Molecular methods used to quantitate and identify environmental microorganisms involve a group of methods which aim to answer different questions about the community found in a sample. The techniques used in this study include: amplifying the genetic information to workable concentrations using the polymerase chain reaction (PCR), quantifying the level of amplicons with fluorescence intensity using quantitative PCR (Q-PCR), and processing DNA fingerprints of different communities and individual species based on GC content with denaturing gradient gel electrophoresis (DGGE).

2.3.1 Polymerase Chain Reaction

Amplification of specific targets of DNA utilizes natural replication mechanisms to produce a high number of DNA copies which can be used for further analysis. The polymerase chain reaction (PCR) is one of the most widely used techniques in molecular biology, and has proven to be a highly sensitive process. There are three main steps to each PCR reaction: denaturation, annealing and extension. These are achieved by changing the temperature of the

reaction mixture. The reaction mixture must contain the template DNA, reverse primers, forward primers, DNA deoxynucleotides and a heat stable DNA polymerase. Additional reaction components may be required in order to maximize efficiency.

The primers are single strands of DNA, measuring approximately 20 nucleotides in length. The forward primer anneals to the negative DNA template strand and the reverse primer anneals to the positive strand, both primers will run towards each other, i.e. 5'to 3'. (Marcheesi, 2001). Upon denaturing the double stranded DNA, the single stranded primers can then anneal to their complementary region. The sensitivity of this process is dependent on the primer selection, reaction component concentrations, and temperature controls. The GC content is critical in the primer used. Long stretches of G or C should be avoided and overall content should not be higher than 45-65% in the primers (Marcheesi, 2001).

The DNA sequence of the 16S rRNA molecule provides species specific information. It is typically used for targeting prokaryotes, because it is structurally and genetically constant. It contains both conserved and variable regions with sufficient information for accurate statistical analysis, lacks horizontal gene transfer artifacts and has a high copy number rendering it a major component of cellular mass (Olsen et al., 1986; Moter and Göbel, 2000). Studies involving the extraction of RNA, instead of DNA from samples of interest, are useful to detect active enzymes or microorganisms. RNA molecules are relatively unstable in the environment, as they are constantly under attack by RNases. This makes RNA techniques more useful at providing information on viable microorganisms, as the molecule is protected by the cellular membranes of live cells, but it also makes RNA extractions more complex requiring the pretreatment of every object which comes in contact with the sample (Nogva et al., 2003; Rudi et al., 2005a). In order to PCR amplify the molecules using standard practices, it is necessary to first convert the RNA

into a more laboratory stable DNA form. This is achieved using the viral enzyme, reverse transcriptase. For this reason, investigation of alternative methods used to identify viable populations is of interest, and in this study the ethidium monoazide bromide (EMA) technique was optimized and used.

The chemical structure of EMA (Figure 2.3) enables it to successfully intercalate double stranded DNA by structural changes induced by high intensity light. The EMA dye can enter cells with damaged membranes and link to the DNA of those cells (Nogva et al., 2003; Rudi et al., 2005b). It is excluded from live cells by a passive process through diffusion barriers. The almost linear azide moiety is attached to the eighth position of the phenanthridinium ring, and it is positioned away from the phenyl substituent (Sternglanz et al., 1978). Experiments performed in the dark showed that both ethidium bromide and ethidium monoazide have identical activities, but upon light exposure the azide derivative showed enhanced mutation rates (Sternglanz et al., 1978). Nogva et al. (2004) have found that EMA crosslinking, using 100 μg ml^{-1} of EMA irradiated for >30 s, gave a maximum PCR signal reduction of -4.5 log units. The use of EMA with the viable and non cultivable food pathogen *Campylobacter jejuni* and *Listeria monocytogenes* has been successful with quantitative PCR methods (Rudi et al., 2005a; Rudi et al., 2005b). However, recent evidence suggests that EMA does not exclusively inhibit the amplification of DNA from dead or damaged cells of *Campylobacter jejuni* and *Listeria monocytogenes* (Flekna et al., 2007).

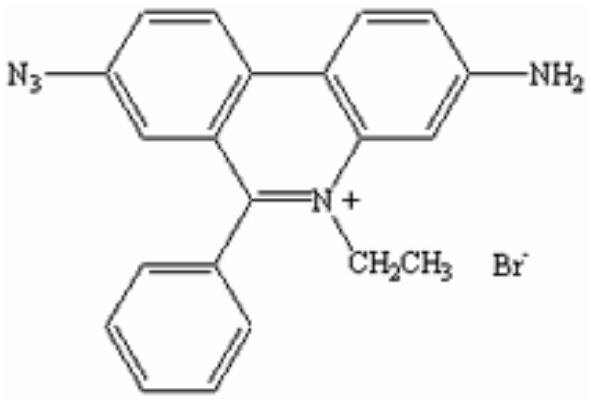

Figure 2.3. The structure of ethidium monoazide bromide.

2.3.2 Quantitative PCR

Real-time quantitative PCR (Q-PCR) is an accurate and reproducible PCR-based molecular tool, which proceeds on the assumption that each DNA molecule is duplicated once during one cycle of amplification, resulting in an exponential accumulation of product (Nogva and Rudi, 2004; Dorigo et al., 2005) . The PCR reaction is monitored by fluorescence that is measured at each stage of the reaction, and compared to that of a standard curve generated by a dilution series of known amounts of DNA targets (Rutledge and Cote, 2003; Nogva and Rudi, 2004). This method is advantageous over regular PCR because it is an automated method which detects DNA amplification during the exponential phase where there is an exact doubling of product, rather than at the end where degradation begins to take place and variability increases.

Both TaqMan and Sybr Green I Q-PCR product detectors are equally rapid and sensitive, but they differ in optimization and price (Ponchell et al., 2003). The fluorescent dye, Sybr Green I green binds to the minor groove of double stranded DNA and emits fluorescence (Pfaffl, 2001). The TaqMan dual labeled fluorogenic probe system emits fluorescence after 5' nuclease polymerase activity cleaves off the quencher allowing expression of the reporter signal. The TaqMan fluorogenic probe system is much more difficult to optimize than Sybr Green I, requiring specific buffer concentrations and reaction temperatures (Yin et al., 2001), but Sybr Green I non-specifically binds to all double stranded DNA products, including primer dimers and secondary structures. High primer concentrations can lead to increased fluorescent signals when using Sybr Green I (Ponchell et al., 2003). These potential errors in signal representation must be considered when designing an assay involving the inexpensive Sybr Green I. Three different types of products dominate in the first cycles of Q-PCR, they include the original target, undefined long products, and PCR accumulated non-specific products (Nogva and Rudi, 2004).

The standard curve is generated based on the threshold cycle (Ct) at which first detection of fluorescence occurs and the concentration of target DNA. From this curve a slope and intercept is obtained using linear regression analysis (Nogva and Rudi, 2004; Rutledge and Cote, 2003). The slope obtained from the standard curve is used to calculate the PCR reaction efficiency using the formula shown in Equation 2.2 (Klein et al., 1999; Pfaffl, 2001; Nogva et al., 2003; Fey et al., 2004; Nogva and Rudi, 2004;).

$$\text{Efficiency} = 10^{-1/\text{slope}} - 1$$

Eq. 2.2

At 100% efficiency each cycle of PCR would theoretically produce a doubling of the DNA copy number (Klein et al., 1999; Rutledge and Cote, 2003; Larionov et al., 2005). The accuracy of efficiency calculations is often disputed, as the efficiency of the PCR reaction is not uniform throughout the different stages of the reaction, being highly efficient during the exponential phase and declining in efficiency through the stationary phase (Larionov et al., 2005). The efficiencies of the Q-PCR reaction are based on various reaction parameters, including temperature and concentration of reaction components, which control the primer binding and subsequent amplification (Nogva and Rudi, 2004). Reaction efficiencies are usually below 0.9 because of factors affecting the amplification rate (Nogva and Rudi, 2004).

2.3.3 DNA Fingerprinting

DNA fingerprinting techniques used in this study include examining community and species profiles by using DGGE. The PCR products are clamped with repeated GC sequences and then run on an acrylamide gel with a DNA denaturing gradient made with urea and formamide. As the PCR products migrate toward the positive charge they encounter greater

concentrations of chemical denaturant, where weaker bonded strands begin to separate slowing the passage of the PCR products through the acrylamide matrix. The double stranded DNA products separate at different levels within the gradient based on their guanine and cytosine concentration and assortment. The GC clamps provide a molecular anchor to slow the DNA's passage through the gel. This technique is used to determine sequence variations and species diversity within a community (Muyzer et al., 1993). This is a popular method used to analyze microbial diversity and community dynamics (Dorigo et al., 2005). Lyautey et al. (2005) found that the concentration of DNA added to DGGE gels which yielded the highest amount of recoverable bands was obtained from 30-50 ng. Different banding patterns can be analyzed using the Jaccard similarity index, which considers the similarities between columns adjusted to a percentage (Fromin et al., 2002; Lyautey et al., 2005). The Jaccard similarity index equation is shown in Equation 2.3, it equals the number of bands that are present in both samples A and B (c) divided by the total number of bands in A and B (a and b respectively), minus c, the product of which is all multiplied by 100.

$$J = (c/[a+b-c])100$$

Eq. 2.3

The techniques outlined here are used to develop a means of differentiation of viable and non-viable microbial communities, and to apply those techniques to the sulfur blocks. The first objective of this study involves formulating a method, such as EMA-Q-PCR, applicable to complex environmental samples. The second objective utilizes this technique in combination with other more standard microbiological examinations to identify and quantify inhabitants of the sulfur blocks.

3.0 DIFFERENTIATION OF GENES EXTRACTED FROM NON-VIABLE VERSUS VIABLE MICROORGANISMS IN ENVIRONMENTAL SAMPLES USING ETHIDIUM MONOAZIDE BROMIDE

3.1 Introduction

Molecular analysis of bacterial communities in the environment is an important tool for the advancement of modern microbial ecology. However, it is often limited by the inability to differentiate between genes extracted from non-viable versus viable organisms. Many techniques have been developed for mono-cultural applications, such as flow cytometry, single cell sorting, and autoradiography, but are rarely successful in mixed microbial populations (O'Brien and Bolton, 1995; Rudi et al., 2005a). For functional genes such as ammonia mono-oxygenase, current methods, such as mRNA extractions, are technically challenging. Recently an intercalating ethidium bromide derivative has gained attention for use in quantitative gene assays (Lee and Levin, 2006; Nogva et al., 2003; Rudi et al., 2005a; Rudi et al., 2005b; Wang and Levin, 2006).

Ethidium monoazide bromide (EMA) (Invitrogen, Burlington, ON) is unable to enter cells with intact membranes, and so it can only attach to free DNA or DNA that is present within cells with compromised membranes. Upon exposure to high intensity visible light, EMA produces a highly reactive nitrene which covalently binds to DNA. Unbound EMA converts to a hydroxylamine derivative, rendering it incapable of further binding (Nogva et al., 2003). Thus, after intense light exposure, EMA bound to DNA becomes covalently linked and unbound EMA remaining in the solution is inactivated (Bolton and Kearns, 1978; Nogva et al., 2003). The irreversible binding of EMA to DNA prevents the DNA from being PCR amplified. The addition of EMA to

environmental samples followed by PCR analysis could allow one to differentiate between genes present in viable and non viable organisms. Such a method has already proven successful in cod fillets, drinking-water biofilms, processed chicken breasts and chicken legs spiked with common food pathogens (Lee and Levin, 2006; Nocker and Camper, 2006; Rudi et al., 2005b), but little is known about how useful the EMA protocol is in environmental samples.

Environmentally derived samples for molecular analysis differ markedly from the more commonly studied food or body tissue samples. Many PCR inhibitors, such as humic and fulvic acids, can be present within environmental matrices and are co-extracted with the DNA. Soils and biofilms contain substantial organic matter which acts as cation exchange sites. Soils also contain clay which is another strong cation exchange site. As EMA in solution is cationic, it is possible that cation exchange sites in soil and biofilms may interfere with the concentration of EMA available to enter cells. I wished to evaluate if EMA would work in various soils, an elemental sulfur block, and three Saskatchewan River biofilms. To test the efficacy of EMA in soils, elemental sulfur and biofilms we: 1) added killed cells to all three matrices and determined the suppression of PCR amplification of a marker gene 2) correlated between a specific microbial activity, nitrification, with gene enumeration from viable populations using the EMA approach in Antarctic soil, and 3) compared soil microbial community profiles by denaturing gradient gel electrophoresis using PCR products derived from DNA, EMA treated DNA and 16S rRNA.

3.2 Materials and Methods

3.2.1 EMA Q-PCR Suppression in Pure Culture

Culture conditions. To determine if EMA is successful at suppressing PCR amplification of DNA from killed *E. coli* LKI *gfp*+ in environmental samples, it must first be shown to be effective in pure cultures. The cells contain a green fluorescent protein, which was originally isolated from the jellyfish *Aequorea victoria*, and it is used as a distinguishing marker (Chalfie et al., 1994). The *E. coli* LKI gfp+ strain was constructed by mating an isolate from a beef packing plant in Lacombe, Alberta, Canada, with a donor, *E. coli* S17-λpir, containing a mini-transposon harboring the *gfp* gene (Suarez et al., 1997; Tallon, 2006). Pure cultures of *E. coli* LKI *gfp*+ cells were grown in 20 ml of tryptic soy broth (TSB) overnight at 24°C. The final concentration of cells had an optical density of 0.828 at 650 nm. Half of the viable cultures were centrifuged and then killed by resuspending them in 70% ethanol and incubating for 20 min. This was confirmed by plate counts on tryptic soy agar to determine the colony forming units.

EMA treatment. From both the viable and killed cultures, half were subjected to ~100 μg ml^{-1} EMA treatment. This involved vortexing for 5 s, incubation in the dark for 5 min, and subsequent photoactivation by placing the sample tubes (15 ml Blue Falcon, High-Clarity Polypropylene Conical Tube, VWR Canada) 20 cm away from 500 watts of light (400-700 nm, UL Portage Worklight) for 60 s. Samples were then kept at -20 °C until time of extraction. The other half of the cultures did not receive EMA treatment.

DNA extraction. DNA was extracted from 0.5 ml of sample with three separate intervals of 30 second bead-beating in extraction buffer (5% (w/v)

hexadecyltrimethylammoniumbromide (CTAB) Sigma, 0.35 M NaCl, 120 mM K_2HPO_4 [pH 8.0]) and phenol:chloroform:isoamyl alcohol (25:24:1 v/v, Sigma, OmniPur EM Science Gibbstown, NJ) using a FastPrep^{TM} FP120 (Bio101 Savant Instruments, Holbrook, NY) homogenizer at a machine speed setting of 4.5 m s^{-1} (Griffiths et al., 2000; Kowalchuk et al., 1998). The aqueous phase was obtained after centrifuging for 3 min at 14 100 x *g* and an equal volume of chloroform:isoamyl (24:1 v/v) was used to remove the remaining phenol. Precipitation of the DNA was achieved after mixing the aqueous layer with 2 equal volumes of 30% (w/v) polyethylene glycol 6000-1.6 M NaCl and allowing it to stand at room temperature for a minimum of 2 hours. The samples were centrifuged 14100 x *g* for 10 min, and washed with ice cold 70% ethanol. The samples were then air dried, and resuspended in water. The extracted DNA was subsequently cleaned by centrifuging through a spin column filled with ~750 µl of polyvinylpyrrolidone (Aldrich, Milwaukee, WI) slurried in 20 mM K_2HPO_4 [pH 7.4] (Berthelet et al., 1996).

Q-PCR amplification. The *gfp* gene prevalence was determined using quantitative-polymerase chain reaction (Q-PCR) with Sybr Green I stain using the Quantitect SYBR Green PCR Kit (Qiagen, Mississauga, ON), the forward gfp-F (5' CCA TGG CCA ACA CTT GTC AC 3'), and the reverse gfp-R (5' CTT TCG AAA GGG CAG ATT GT 3') primers (all primers used in this study were purchased from Invitrogen). An ABI 7300 Real Time PCR system (Applied Biosystems, Foster City, CA) was used for all Q-PCR runs. The thermal cycling program consisted of an initial hotstart at 94°C for 5 min; followed by 50 cycles of 94°C for 15 s, annealing at 60°C for 40 s and elongation at 72°C for 45 s. Standard concentrations with ten fold dilutions of cleaned and spectrophotometrically (UV/Visible spectrophotometer, Ultrospec 2000,

Pharmacia Biotech, Picataway, NJ) determined GFP PCR products were used to make a standard curve with the generated fluorescence and cycle threshold (Ct). Linear regression analysis was performed on the slopes of the standards using Sequence Detection Software, 7500 System SDS Version 1.2.2 (Applied Biosystems).

3.2.2 EMA Q-PCR dose response in soil and sulfur.

Soil and sulfur samples. Subsequent to pure culture evaluation, the EMA concentration necessary to suppress PCR amplification of *gfp* in soil and elemental sulfur samples was determined. Two Saskatchewan prairie soils, a silty clay-loam with a pH of 6.6 and a loamy clay-loam texture with pH of 6.8 were used for the following experiment. Elemental sulfur was obtained from Syncrude's sulfur storage system in the northern Alberta oil sands. The sulfur was ground in a mortar and pestle and sieved through a 2 mm passing screen. Five milliliters of distilled water was used to suspend 5 independent replicates of 0.5 g of each soil type and sulfur, and this was amended with 0.1 ml of killed or viable *E. coli* LKI *gfp*+ cell suspension. Soil slurries were treated with 0, 0.5, 25, 40, 72, 90 μg ml^{-1} of EMA, and sulfur slurries were treated with 0, 0.5, 25, 40, 72, 100 μg ml^{-1} of EMA. DNA was extracted from 0.5 g of spiked soil sulfur slurry and Q-PCR amplification followed using the methods described above.

3.2.3 EMA Q-PCR suppression of *E. coli* LKI *gfp*+ in soil, sulfur and river biofilms.

Biofilm samples. After optimizing the EMA concentration required for each type of environmental sample, I assessed the effectiveness of PCR suppression by EMA treatment in environmental samples of soil, elemental sulfur, and river biofilm. Samples were amended with either live or killed *E. coli* LKI *gfp*+ cells. Sulfur and soil suspensions were prepared as described above. Scrapings of three separate river biofilms

were used for this experiment: one control river biofilm, and two river biofilms treated with 100 ppb of either Triclosan, an antimicrobial agent used in personal care products such as toothpaste, and Carbamazepine, a common pharmaceutical used for both human mental health and for convulsion prevention in epileptic patients (Lawrence et al., 2005; Morrall et al., 2004). One of the many bactericidal modes of action of Triclosan is the breakdown of membrane integrity (Russell, 2004). Biofilms were 8 week old river communities grown in rotating annular reactors fed with South Saskatchewan River water as described by Lawrence et al. (2005).

Biofilm suspensions were prepared by scraping the biofilms with sterile polypropylene silicone tubing and rinsing in distilled water making a final volume of one milliliter. They were amended with 20 µl of live or killed *E. coli* LKI *gfp*+ cells. After a brief vortexing, EMA was added to the samples. The soil samples were treated with 90 µg ml^{-1} and the sulfur and biofilm samples were treated with 100 µg ml^{-1} of EMA. Light activation and photolysis followed, and the DNA was extracted, and cleaned using polyvinylpolypyrrolidone (PVPP) spin columns (Berthelet et al., 1996; Hugenholtz and Goebel, 2001). The cleaned DNA was then used for Q-PCR specific for *gfp* quantification, as described above.

3.2.4 EMA Q-PCR suppression and amoA activity.

Nitrification activity measurements. Subsequent to determining the optimal EMA concentration for soil samples, I wished to evaluate if the EMA technique was providing information congruent with other, more established, techniques in soil microbiology. PCR detected EMA gene suppression of *amoA*, a sub-unit of ammonia mono-oxygenase, was compared to nitrification activity in soil microcosms. Nitrification

activity was measured in sub-Antarctic soil samples containing increasing levels of Special Antarctic Blend (SAB) fuel. Treatments were prepared by a 1:1 serial dilution beginning at 50 000 mg fuel kg^{-1} soil. Ten different concentrations of SAB diesel fuel spiked soil were created by mixing 220 g soil with 11 g SAB. Treated soil (110 g) was placed in a 1 L Schott bottle, covered in tinfoil, and incubated at $6 \pm 0.02^{o}C$ for 21 days before measuring activity. Activity was assessed by levels of potential nitrification, which has been shown as a highly sensitive method for detecting nitrification in soils (Gong et al., 1999). Soil samples were mixed in a 1:4 ratio with a test solution containing 4 mM $(NH_4)_2SO_4$ as growth substrate, 15 mM $NaClO_3$ for inhibition of nitrite oxidation and 1 mM KH_2PO_4 for a buffer as described by Schafer et al. (2007). Termination of ammonia oxidation was achieved by adding 2 ml of KCl. Samples were centrifuged for 3 min at 14000 rpm and filtered through 0.45 μm syringe filters before colorimetric analysis. Nitrite content was determined by linear regression of the concentration of nitrite g^{-1} soil over time as measured by absorbance at 543 nm using a Beckman DU® 650 Spectrophotometer (Mississauga, ON) (Schafer et al., 2007).

DNA extraction and Q-PCR amplification. Prior to duplicate DNA extractions, 0.5 g soil samples were suspended in 5 ml distilled water containing 72 μg EMA ml^{-1} for 10 min at room temperature shaking horizontally (100 rpm). After 10 min, EMA was inactivated by placing the sample tubes (15 ml Blue Falcon, High-Clarity Polypropylene Conical Tube, VWR Canada) 20 cm away from 500 W of light (400–700 nm, UL Portage Worklight) for 60 s. Sample tubes were then centrifuged at 800 rpm for 10 min, the supernatant was removed and DNA was extracted from the soil pellet as described above. Quantification of *amoA* was performed using amoA-1F (5' GGG GTT TCT ACT GGT GGT 3') and amoA-2R (5' CCC CTC (G/T)G(G/C) AAA

GCC TTC TTC 3') primers (Rotthauwe et al., 1997) in Q-PCR reactions using the same components as described above. The Q-PCR had the following thermal cycles repeated 35 times: 94ºC for 40 s, 60ºC for 40 s, and 72ºC for 60 s.

3.2.5 EMA treated soil community DNA fingerprinting.

If the EMA technique is successfully differentiating between viable and non-viable cells, then the viable EMA treated community should closely resemble the active population. To assess this, we compared a segment of the16S RNA gene profiles using DNA, cDNA, and DNA extracted from EMA treated samples of loamy clay-loam Saskatchewan soil. We assessed microbial community diversity using denaturing gradient gel electrophoresis. DNA from samples treated with and without 90 μg ml^{-1} of EMA was extracted in triplicate following the same procedure as described above.

RNA extraction and reverse transcription. Four separate RNA extractions were performed on the non-EMA treated samples by homogenizing 5 g of soil in 10 ml of water for 5 min, subsequent centrifugation at 800 rpm for 10 min and using the supernatant for RNA extraction using the QIAamp®Viral RNA MiniKit (Qiagen). All nucleic acids were cleaned using PVPP columns, the RNA samples were treated with Deoxyribonuclease I (Invitrogen) before a two step reverse transcription. Reverse transcription was performed using the Superscript™ II Reverse Transcriptase kit (Invitrogen) following the manufacturer's instructions.

PCR amplification. PCR amplification followed, using the *Bacteria* domain specific primers 338F (5' ACT CCT ACG GGA GGC AGC AG 3') and 518R (5' ATT ACC GCG GCT GCT GG 3') with Taq PCR Master Mix Kit (Qiagen) in a Robocycler PCR (Stratagene, La Jolla, CA) machine with 30 cycles of 94ºC for 1 min, 50ºC for 1 min, and elongation at 72ºC for 1 min. These samples, including negative controls, were

run on a 1% agarose gel for confirmation of product. The diluted ($^{1}/_{100}$) PCR products were then amplified again with the same conditions but with a GC clamped forward primer, 338F-GC.

Denaturing gradient gel electrophoresis. The products were separated on a D-Code™ Universal Mutation Detection System (16 cm, 120 V, Bio-Rad, Hercules, CA) denaturing gradient gel. The method used was a modified version of that outlined by Muyzer et al. (1993) with a 10% (w/v) polyacrylamide gel and a 40-60% denaturing gradient created by formamide and urea (Pynaert et al., 2003). The length of electrophoresis was 16.5 hours at 60ºC and 45 V in a 1x TAE (20 mM Tris, 10 mM acetate, and 0.5 mM EDTA [pH 7.4]) buffer. Then the gels were removed from the apparatus and immersed for 30 min in a 0.01% diluted solution of Sybr Green I, subsequently rinsed and photographed on a UV gel dock (GelDocMega, BioSystematica, Wales, UK) using a digital camera (Nikon CoolPix 995, Melville, NY). Statistical analysis was performed using the Jaccard band based similarity coefficient as determined by Bionumerics Applied Maths software (BioSystematica, Wales, UK).

3.3 Results and Discussion

EMA suppressed 99.99% of DNA amplification from killed pure *E. coli* LKI *gfp*+ culture with a reduction in copy number of greater than 5 logs (data not shown). The killed *E. coli* LKI *gfp*+ still produced 3700 copies of amplified DNA ng^{-1} total extracted DNA despite plate counts indicating that no culturable *E. coli* remained. This amplification of killed *E. coli* LK1 *gfp*+ may be due to cells that retained their membrane integrity after ethanol treatment. Alternatively, injured cells that were still viable may have been able to grow on solid media.

In soil and sulfur, the greatest suppression of amplified *E. coli* LKI *gfp*+ DNA was achieved with 90 μg ml^{-1} of EMA, which suppressed 100% of PCR amplification in soil, and with 100 μg ml^{-1} which suppressed 100% of PCR amplification in sulfur (Figure 3.1). The soil samples inoculated with live culture had a relatively constant average amplified DNA copy number with increasing concentrations of EMA, the numbers ranging between 6.4 and 7.8 logs. After approximately 25 μg ml^{-1} the difference between the viable and non-viable samples does not increase with increasing concentration of EMA. This indicates that if DNA amplification of all viable microorganisms within a soil sample is to be examined, a concentration of 25 μg ml^{-1} of EMA may be sufficient.

In contrast to the soil treatments, the sulfur samples inoculated with live culture decreased in amplified copy number with EMA concentrations below ~40 μg ml^{-1}, whereas the higher concentrations had amplifications closer to those without EMA treatment (Figure 3.1). The reduction of amplified copy number in the sulfur samples correlates with the findings of Rueckert et al. who found that EMA penetrated the membranes of a viable culture of *Anoxybacillus flavithermus*, crosslinking the DNA, and decreasing subsequent PCR amplification (Rueckert et al., 2005). Alternatively, the sulfur may have been toxic to the *E. coli* and thus, confounded our results. It is not clear from our results, why gene amplification of viable *E. coli* in sulfur was initially suppressed but then returned to the expected level.

Other studies have shown evidence that in certain species and exposure conditions EMA treatment can lead to a loss of extracted genomic DNA from viable cultures (Nocker and Camper, 2006) and significant staining of viable cell membranes (Nocker et al., 2006; Flekna et al., 2007). However in our hands, the amount of DNA

extracted from soil amended with killed cells treated with 90 μg ml^{-1} of EMA (146 ng DNA g^{-1} soil (standard deviation = 40)) had little difference from the non-EMA treated killed *E. coli* LKI *gfp*+ amended soil samples (92 ng DNA g^{-1} soil (standard deviation = 29)). Similarly in sulfur, 52 ng DNA g^{-1} sulfur (standard deviation = 23) was extracted from EMA treated samples compared to 76 ng DNA g^{-1} sulfur (standard deviation = 9) in non-EMA sulfur samples. Previous studies, (Nocker and Camper, 2006; Nocker et al., 2006]), did not investigate soil or elemental sulfur matrices which may be the reason why we did not observe a decrease in the amount of extracted DNA when samples were treated with EMA.

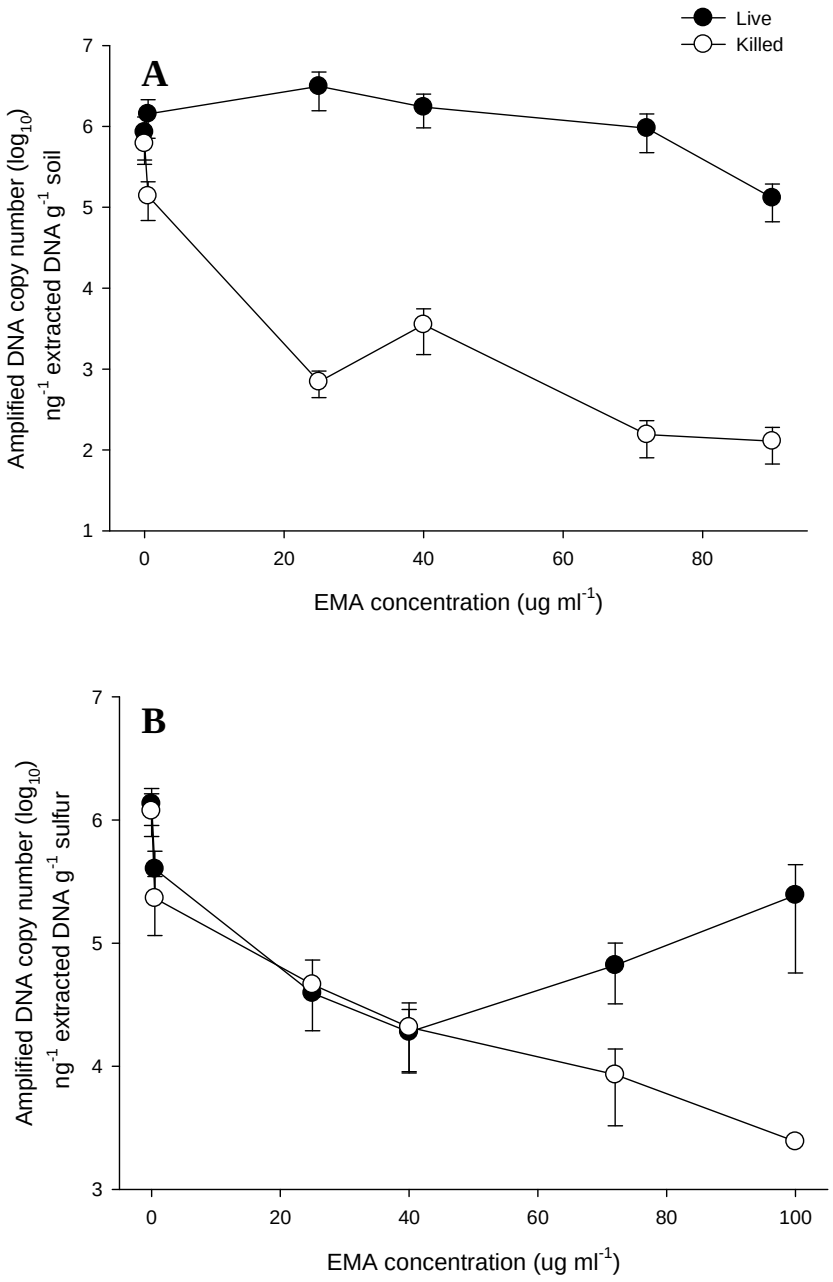

Figure 3.1. Decrease in killed *E. coli* LKI *gfp*+ DNA amplification with an increase in EMA concentration in two different Saskatchewan soils (Panel A) and elemental sulfur (Panel B). The points represent the average of amplified *gfp* copy numbers from two soils with silty clay-loam and loamy clay-loam texture. Error bars are the standard deviation of the mean.

EMA treatments decreased the amount of amplified DNA in sulfur and soil samples from killed *E. coli* LKI *gfp*+ by ~2 to 3 logs compared to live cells (Figure 3.2A). In contrast, killed and viable samples of sulfur and soil had the same amount of amplified copy number in non-EMA treated samples. EMA treated biofilm samples containing either Triclosan or Carbamazepine and spiked *E. coli* demonstrated little difference between the live and killed EMA treated samples (Figure 3.2B). The river control samples had a spike of amplification for the viable non-EMA treated samples, with a large significant error, compared to the non-viable samples. The reason for this is unclear, but it suggests that the composition of the biofilms, particularly their extracellular polymeric substances, may interfere with either DNA binding or photoactivation of EMA. This interference may be similar to the way extracellular polymers protect biofilm bound extracellular DNA from environmental nucleases (Steinberger and Holden, 2005). The presented results are in direct contrast to a recent report that 100 μg ml^{-1} EMA suppressed amplification of spiked *E. coli* in resuspended biofilm samples (Nocker and Camper, 2006). This difference may be biofilm specific and highlights the need for more detailed biofilm investigations.

Other authors have noted that EMA may reduce PCR amplification of DNA, penetrate viable organisms and thus give misleading results (Nocker and Camper, 2006; Nocker et al., 2006; Rueckert et al., 2005). Matrices studied here also demonstrated suppression of viable cultures, i.e. elemental sulfur, or resulted in no suppression of non-viable organisms DNA, i.e. biofilms supporting the observations of other investigators. However in our hands, the EMA technique appeared to be successful in soil samples. To support the Q-PCR findings, I evaluated if the EMA technique in soil was providing information congruent with other established techniques in soil microbiology.

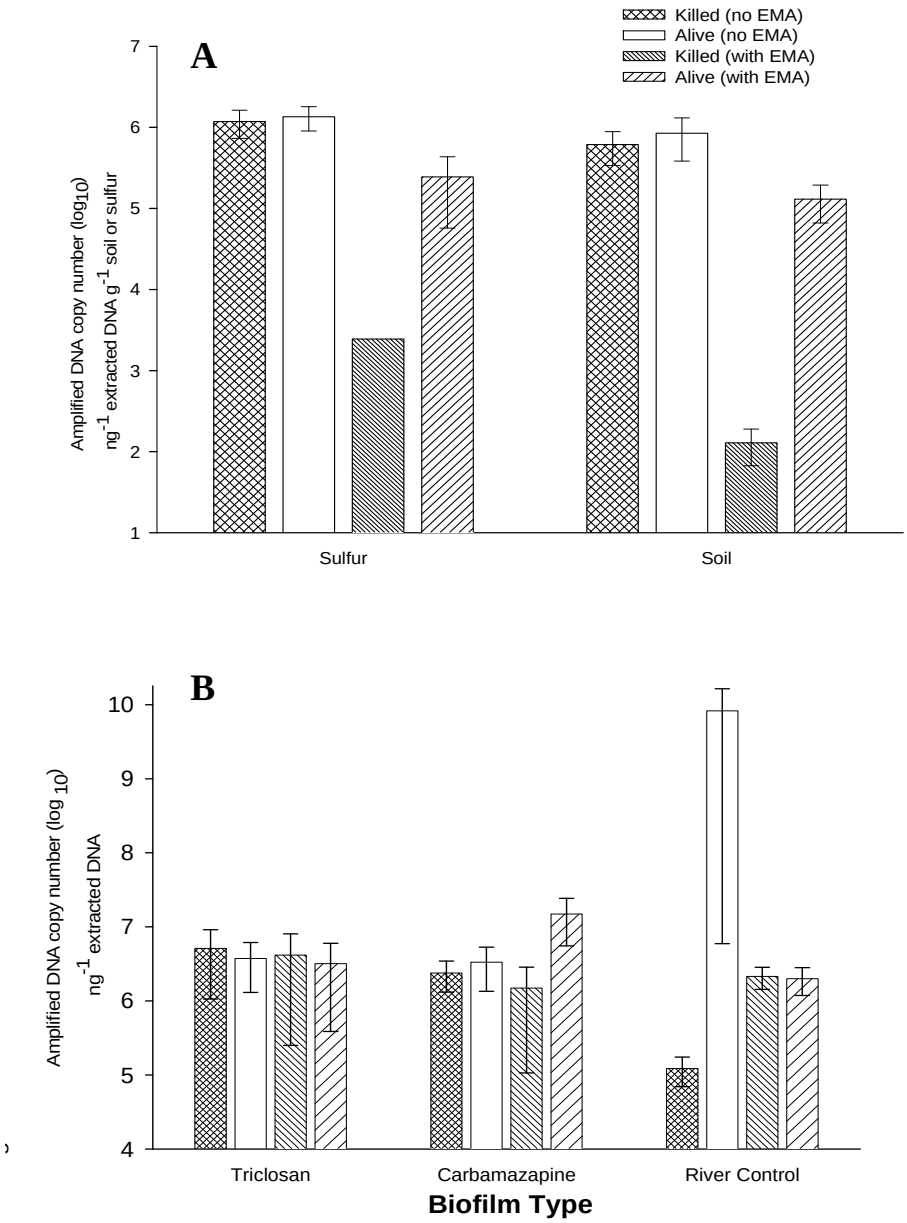

Figure 3.2. Amplification of *E. coli* LKI *gfp*+ from 99.4% pure elemental sulfur and in soil (Panel A) and three different river biofilms (Panel B). Sulfur and biofilm samples were treated with 100 µg ml^{-1} whereas soil samples were treated with 90 µg ml^{-1}. Viable or non-viable (ethanol killed) *E. coli* LKI *gfp*+ were added to each matrix type, they were EMA treated, and DNA was extracted followed by quantitative PCR. Each bar represents the average of 5 independent extractions with error bars indicating the standard error of the estimate.

Amplification of a portion of the nitrification gene, *amoA*, from the viable microbial population, as assessed by the EMA-technique, decreased in response to diesel fuel as did potential nitrification activity (Figure 3.3). In contrast, gene prevalence in total community DNA from these soils did not change with increasing fuel concentrations. Amplified prevalence of *amoA* treated with EMA was negatively correlated ($r = -0.20$) with nitrification activity. In contrast, amplified prevalence of *amoA* without EMA was not related to nitrification activity ($r = -0.001$). The decrease in nitrification activity in response to petroleum hydrocarbons has been observed previously (Deni and Penninckx, 1999) and is linked to metabolites released from the heterotrophic microorganisms which are themselves inhibited by the petroleum contamination (Deni and Penninckx, 2004). The relatively low copy number of nitrifying bacteria, as represented by the amoA gene amplification correlates with the low number of nitrifiers found in continental and maritime Antarctica soils (Wilson et al., 1997).

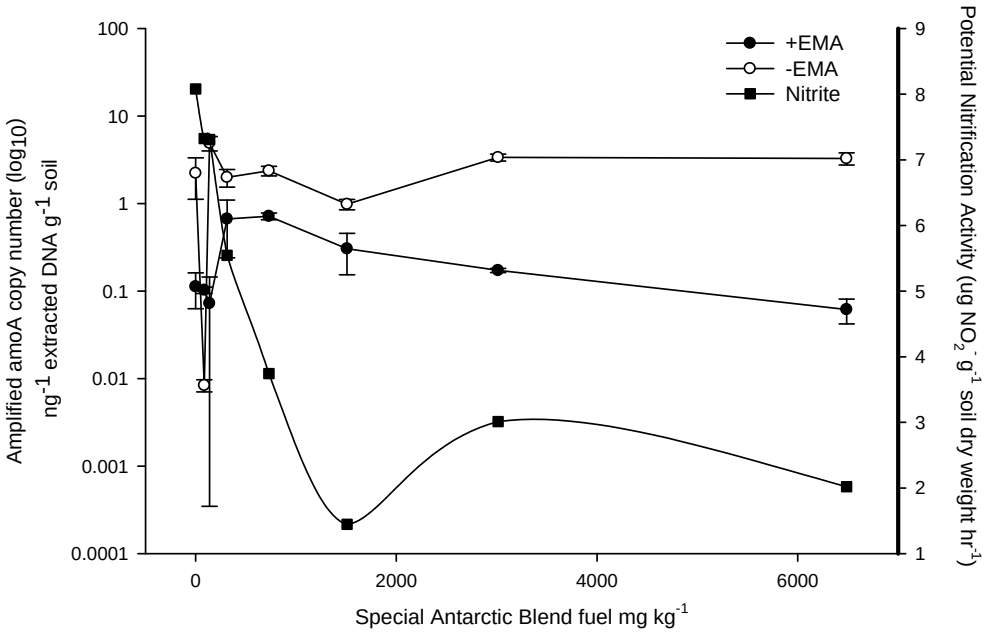

Figure 3.3. Potential nitrification activity (solid squares), EMA viable *amoA* gene prevalence (solid circles), gene prevalence of *amoA* in total community DNA (open circles), in a sub-Antarctic soil exposed to increasing concentrations of Special Antarctic Blend (SAB) diesel fuel for 21 days. Points represent the average of two determinations for gene prevalence at each SAB concentration. Error bars represent the standard error of the estimate.

Independently extracted replicates of community 16S rRNA profiles obtained with EMA treated soil samples were 47% similar to those obtained from the cDNA (Figure 3.4). In contrast, total community DNA profiles were only 27% similar to cDNA profiles. The difference between the EMA treated and cDNA fingerprints is at least partially attributable to the different extraction procedures, which may have different extraction efficiencies and biases and used different amounts of soil (0.5 g for DNA and 10 g for rRNA). However, attempts to use techniques that simultaneously extracted RNA and DNA from soils (Griffiths et al., 2000) were not successful in this soil type.

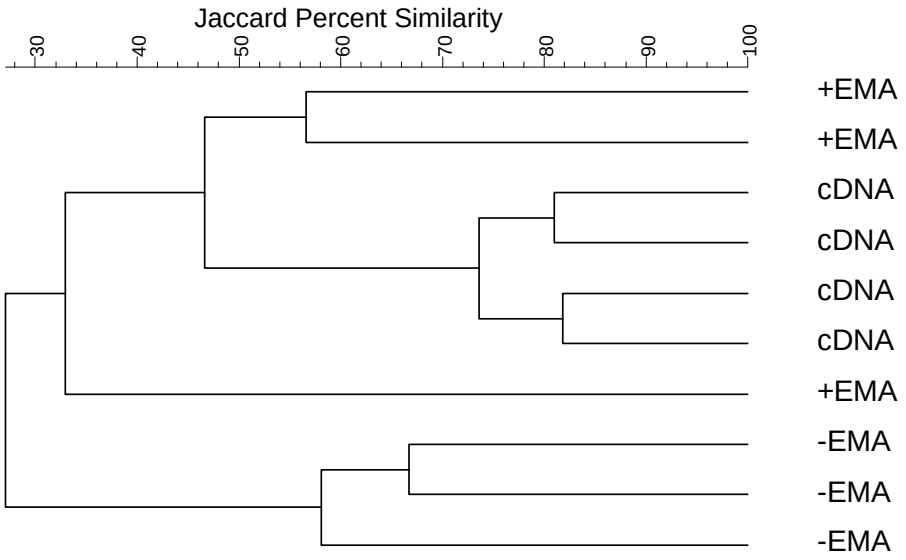

Figure 3.4. Dendrogram of Jaccard similarity analysis of 16S rRNA eubacterial community DGGE profiles obtained using primers 338f-518r. DNA was extracted from soil previously treated (+EMA) and not treated (-EMA) with 90 μg ml^{-1} of EMA. From the samples not treated with EMA, rRNA was also extracted and reverse transcribed (cDNA). Each separate branch represents one independently extracted replicate.

The use of EMA to differentiate between viable and non-viable organisms appears to be matrix dependent. Spikes of a non-viable *gfp* carrier were effectively suppressed in pure culture as well as in elemental sulfur, two different Saskatchewan soils and one sub Antarctic soil. However, the EMA based amplification suppression of spiked *E. coli* was not successful in the three different Saskatchewan River biofilms. Further, EMA appeared to partially inhibit amplification of *E. coli* LK1 *gfp*+ in elemental sulfur samples.

In two different Saskatchewan soils and one sub Antarctic soil, the EMA technique appeared to be highly successful. Using EMA to inhibit the amplification of non-viable organisms DNA, and hence non-active genes, resulted in quantitative PCR results very similar to traditional activity assessments. Further, community fingerprints obtained from DNA treated with EMA were much closer to those obtained from rRNA. It appears that the use of EMA can be an effective tool in soil to monitor viable gene prevalence in mixed communities.

4.0 DISTRIBUTION OF MICROORGANISMS WITHIN THE HIGHLY FRACTURED MATRIX OF AN ELEMENTAL SULFUR STORAGE BLOCK

4.1 Introduction

Processing of oil sands bitumen to high quality crude oil has resulted in an excessive accumulation of elemental sulfur stock, creating an increasingly large global surplus. In general, it is viewed that long-term economic storage is required until market opportunities improve. Currently, storage involves melting, concentrating, pouring and solidifying the elemental sulfur in large repositories measuring ~200 x 400 x 15 m and containing 99.8% pure elemental sulfur (McKenna, 2004). These uncovered blocks are directly exposed to precipitation and freeze-thaw cycles characteristic of the northern Canadian climate. Left exposed the blocks are at risk being degraded and oxidized by physical, chemical and biological means, which decrease the value of the blocks and threaten the surrounding environment.

Biochemical sulfur oxidation follows the same path as chemical oxidation; however, the biochemical reactions increase the rate and level of chemical reactivity among intermediates (Pepper and Miller, 1978; Suzuki, 1999; Masau et al., 2001). Non-phototrophic autotrophic sulfur oxidizing bacteria rely on sulfur oxidation as their sole source of energy for growth and proliferation, whereas heterotrophic bacteria use sulfur oxidation for supplemental energy (Mason and Kelly, 1988; Sorokin, 2003). Autotrophs and heterotrophs share many of the same enzyme pathways and there is no definitive pathway for either group of organisms. Among the different microorganisms associated with sulfur oxidation, the colorless autotrophic Gram-negative acidophile, *Acidithiobacillus thiooxidans* is a model representative of sulfur oxidation reactions (Pronk et al., 1990; Konishi et al., 1995; Kelly et al., 1997; Kelly and Wood, 2000). This

rod shaped bacterium is capable of growth in mesophilic and extremely acidic conditions, with pH levels reaching 0.5, and temperatures of up to 40ºC (Takakuwa, 1992; Konishi et al., 1995). These same conditions can be found in the rain water effluent and summer temperatures of Alberta's sulfur blocks.

Due to the extreme nature of the sulfur blocks, 99.8% pure sulfur, and the harsh northern environment in which these blocks reside, the type and importance of organisms involved in sulfur oxidation is unclear. Characterizing these organisms is an important component in the design of effective strategies to preserve sulfur block integrity and prevent acid drainage. Here, I specifically investigated the nature of the microbial community colonizing and carrying out sulfur oxidation in different regions of these large blocks of sulfur. In order to determine if native sulfur block microorganisms contribute to sulfur oxidation it is necessary to compare a system populated with microorganisms to that of a sterile system. For this purpose an *in vitro* study was designed to create sterile and non-sterile conditions, and compare the level of sulfate, acidity and microbial populations.

4.2 Materials and Methods

4.2.1 Detection of Microbial Sulfur Oxidation in Sulfur Bioreactors

Bioreactor setup. Effluent was obtained from the run off of Syncrude's Phase I sulfur block, north of Fort McMurray Alberta, and kept in clean plastic containers at 4ºC prior to use. Sulfur obtained from exposed surfaces or unexposed matrix material at the same block was granulated into a powder using a mortar and pestle, and subsequently sieved through a sieve with a 2 mm opening. Fifty grams of sulfur was submerged in 500 ml of either sterile water or 10% effluent, and incubated on a rotary shaker set at

~230 rpm and maintained between 24-27ºC. The treatments were: matrix sulfur and water, matrix sulfur and 10% effluent, sterilized matrix sulfur and water, and surface sulfur and 10% effluent. Matrix sulfur was obtained from the inner unexposed matrix of the block and surface sulfur was obtained from the exposed dusty rind of cracks and crevices of the block. The sterile sulfur was prepared by washing it with 1.8% sodium hypochlorite, followed by a sterile water rinse, and subsequent washing with 95% ethanol, and air dried under a sterile Whatman filter paper. Three replicate bioreactors of each treatment were prepared and sampled at 0, 1, 2, 4, 6, 8, 10, 16, 25, 33 and 40 weeks. Two 15 ml samples were obtained from each treatment at each time interval, and stored in blue Falcon tubes (15 ml, High-Clarity Polypropylene Conical Tube, VWR Canada) at -20ºC until chemical and microbial analyses were performed.

Sulfate concentration and pH analysis. The bioreactor samples were filtered through a 0.45 μm filter to remove any particulates. The pH was determined using an Orion glass combination electrode (Model 9102BN) which was calibrated with buffers at pH 7, 4, and 1 prior to every seventh reading depending on the level of acidity in the sample. The pH was measured in a 5 ml sample that was aseptically removed from each bioreactor. From the pH measurements hydronium ion concentration was calculated using Equation 4.1. Sulfate concentrations were determined using the standard turbidometric procedure (Clesceri et al., 1989). Calibration of the spectrophotometer was achieved with a series of known sulfate concentrations, and set at 420 nanometers (nm), with a light path at 2.5 to 10 cm.

$$[H_3O^+] = 10^{-pH} \qquad \text{Eq. 4.1}$$

Enumeration of bacteria and fungi in bioreactors. Plate counts for selected bacteria and fungi were performed at the beginning and end of the incubation period. Time 0 measurements were also taken from 10% and 100% effluent, before inoculating the bioreactors. The microbial populations in the effluent containing sulfur bioreactors were enumerated by determining colony forming unit (CFU) counts on three different media types. Three replicates of three different dilutions were spread plated on $^{1}/_{10}$ trypticase soy agar ($^{1}/_{10}$ TSA) , heterotrophic sulfur medium (HSM) (0.1% peptone, 0.05% yeast extract, 0.003% Bromophenol Blue, pH 6.8, with 0.002% 2X water rinsed and pre-autoclaved flowable sulfur (Stoller Chemicals, Burlington, ON) added after autoclaving) and acidified Czapek Dox (pH 3) agar plates to enumerate the CFUs for total heterotrophs, sulfur oxidizing heterotrophs, and total acidophilic fungi, respectively. Increased surface area of sulfur supplementation to soil has been shown to increase sulfur oxidation by up to 31% (Lawrence et al., 1988). For this reason a fine particulate flowable sulfur product was used for media preparation (Lawrence and Germida, 1991). Standard error of the mean was calculated as outlined by Koch (1994) in which the square root of the total counts was divided by the volume of solution.

Autotrophic sulfur oxidizing microorganisms were enumerated using the most probable number (MPN) technique. Five different dilutions of each bioreactor were plated on three replicate plates. The MPNs were performed on week 0 and week 40 samples. The samples from week 0 were analyzed for total sulfur oxidizing autotrophs and the samples from week 40 were analyzed for total sulfur and thiosulfate oxidizing autotrophs. The liquid mineral salts medium was modified from ATCC #125 medium designed for *Thiobacilli*, it contained the following in 1.0 L of water: 0.2 g $(NH_4)_2SO_4$, 0.5 g $MgSO_4 . 7H_2O$, 0.25 g $CaCl_2$, and 5.0 mg $FeSO_4$. After autoclaving, 5 ml of 0.22

μm filter sterilized KH_2PO_4 solution (0.3 g ml^{-1}) and 1 ml of the color indicator, a filter sterilized Bromocresol green solution (0.015 g ml^{-1}) was added. The media for thiosulfate oxidizers had 5 ml of sterilized $(NH_4)_2S_2O_3$ solution (0.5 g ml^{-1}) added after autoclaving; whereas, the media for sulfur oxidizing autotrophs had 0.002% of 2X washed sterilized flowable sulfur added.

EMA treatment and DNA extraction. After 40 weeks of incubation, 10 ml of each bioreactor was transferred to a sterile 15 ml Falcon tube which was centrifuged at 2500 x *g* for 10 min. A sterile pipette was used to remove 7.60 ml of supernatant. The remaining slurry was amended with 100 μg ml^{-1} of EMA to allow for selection for DNA from viable microbial populations (Nogva et al., 2003). EMA treatment involved vortexing for 5 sec, incubation in the dark for 5 min, and subsequent photoactivation by placing the sample tubes 20 cm away from 500 watts light (400-700 nm, UL Portage Worklight) for 60 s. Samples were then stored at -20 ºC overnight, until DNA extraction.

DNA extractions were carried out on 0.5 g of EMA treated sulfur slurry. Samples underwent three separate intervals of 30 second bead-beating in extraction buffer (5% (w/v) hexadecyltrimethylammoniumbromide (CTAB) Sigma, 0.35 M NaCl, 120 mM K_2HPO_4 [pH 8.0]) and phenol:chloroform:isoamyl alcohol (25:24:1 v/v, Sigma, OmniPur EM Science Gibbstown, NJ) using a FastPrep™ FP120 (Bio101 Savant Instruments, Holbrook, NY) homogenizer at a machine speed setting of 4.5 m s^{-1} (Griffiths et al., 2000; Kowalchuk et al., 1998). Samples were also boiled for five min between the final two homogenizations. The aqueous phase was obtained after centrifuging for 3 min at 14100 *g.* An equal volume of chloroform:isoamyl (24:1 v/v) was then added to the combined aqueous phases to remove the remaining phenol, which was achieved by inverting the tubes 3X to mix, centrifuging for 3 s, and removing the

top phase. Precipitation of the DNA was achieved after mixing the aqueous layer with 2 equal volumes of 30% (w/v) polyethylene glycol 6000-1.6M NaCl and allowing it to stand at room temperature (22 ºC) overnight. The samples were then centrifuged 14 100 x *g* for 10 min, and washed with ice cold 70% ethanol. The samples were then air dried, and resuspended in water. Triplicate extractions were pooled together. DNA extraction from the sulfur block samples provided an average yield of 150 ng DNA ml^{-1}.

Quantitative PCR analysis. Q-PCR amplification followed on uniform dilutions achieved by adding varying amounts of sterile water to 10 µl of DNA extraction to get equal final concentrations of DNA extracts among samples based on spectrophotometer readings at OD 260 (UV/Visible spectrophotometer, Ultrospec 2000, Pharmacia Biotech, Picataway, NJ). Primers used are listed in Table 4.1 and the cycle conditions listed in Table 4.2. The primers used target 16S rDNA sequences for Eubacteria, and Archaea, 18S rDNA sequences for Fungi, the conserved region of the SoxB enzyme in the multi-enzyme thiosulfate oxidizing complex and 16S rDNA of *A. thiooxidans* ATCC 19377. Each Q-PCR reaction contained 5 mM of forward and reverse primer sets, 1 x Quantitect SYBR Green PCR master mix (Qiagen, Mississauga, Ontario) and appropriately diluted DNA extract. An ABI 7300 Real Time PCR system (Applied Biosystems, Foster City, CA) was used for all Q-PCR runs. Standard concentrations with ten fold dilutions of cleaned (QIAquick PCR purification kit, Qiagen) and spectrophotometrically determined PCR products were used to make a standard curve with the generated fluorescence and cycle threshold (Ct). Positive controls for ARC344-ARC915R, 338F-518R, soxB432F-soxB1446R, EF4-EF3, Thiox1F-Thiox1R were *Halobacterium hispanicum*, *Escherichia coli* LKI gfp^{+}, *Paracoccus pantotrophus* ATCC 35512, *Penicillium bilaii*, *Acidithiobacillus thiooxidans* ATCC 19377, respectively.

Linear regression analysis was performed on the slopes of the standards using Sequence Detection Software, 7500 System SDS Version 1.2.2 (Applied Biosystems).

4.2.2 Detection of Microbial Growth in the Sulfur Blocks

Sampling conditions. Sulfur samples were obtained directly from the Phase 1 sulfur block at the Syncrude site. Three separate cores were made at locations 143-D, 144-E, and 145-D. A 2.5 inch diameter hand-held power auger was used to drill the block to a depth of up to 6 m. Samples were collected through polypropylene tubing, using a shop vacuum. Samples recovered for analysis were sub-sampled from bulk drilled depths of 0-10, 40-50, 90-100, 190-200, 290-300, 490-500, 590-600 cm depths. The auger, tubing, and collection bucket were rinsed with 95% ethanol between samples. Sub-samples were stored in sterile 50 ml Falcon tubes at -20 ºC for 24-48 hours, at which point they were transferred to a cooler with ice packs for 48 hours during transportation and subsequently placed in a -80 ºC freezer for long term storage.

CFU and MPN analysis, isolate purification, and storage. Total heterotrophic populations were enumerated using $^{1}/_{10}$ TSA, and total sulfur heterotrophs were enumerated on HSM (described above). MPN counts followed the procedure outlined in section 4.2.1. Purification of isolates of interest was achieved after 6 weeks of growth, at which time colonies were aseptically transferred to fresh media. Colonies were first inoculated onto solid media by spreading with a sterile metal loop, and those that grew were subjected to successive culturing until pure colonies were isolated, these were then transferred to 5 ml of liquid media. After one week of growth, the colonies were transferred to cryogenic storage vials with 1% glycerol, and stored at -80 ºC.

Molecular analysis. The samples from the sulfur block which had high levels of microbial counts based on the CFU plates, were selected for molecular analysis to

determine the quantity of different organisms and diversity of the resident communities. Nine samples were chosen for molecular analysis. These included: 0-10 and 590-600 cm of drill hole 143-D, 0-10, 90-100, 190-200, and 590-600 cm of drill hole 144-E, and 0-10, 40-50, and 590-600 cm of drill hole 145-D. Five grams of each sample was suspended in 5 ml sterile distilled water, and underwent EMA treatment and six separate DNA extractions (described above) which were pooled together. DNA was subjected to PCR using the primers listed in Table 4.1 and conditions outlined in Table 4.2. Successfully amplified samples were used for Q-PCR and sequence analysis.

Table 4.1. PCR primers which were used in the amplification of DNA extracted from the sulfur bioreactors and the sulfur blocks.

Primer	Target Sequence (5' to 3')	Specificity	Reference
ARC344F	ACGGGGYGCAGCAGGCGCGA	Most Archaea	(Gonzàlez-Toril et al., 2003; Koizumi et al., 2004)
ARC915R	GTGCTCCCCCGCCAATTCCT	Most Archaea	(Gonzàlez-Toril et al., 2003; Koizumi et al., 2004)
EUB338F	ACTCCTACGGGAGGCAGCAG	Most Bacteria	(Fierer et al., 2005; Gonzàlez-Toril et al., 2003)
EUB518R	ATTACCGCGGCTGCTGG	Most Bacteria	(Fierer et al., 2005)
soxB432F	GAYGGNGGNGAYACNTGG	Sulfur oxidizing enzyme	(Petri et al., 2001)
soxB1446R	CATGTCNCCNCCRTGYTG	Sulfur oxidizing enzyme	(Petri et al., 2001)
EF4F	GGAAGGGRTGTATTTATTAG	Fungi	(Anderson et al., 2003; Smit et al., 1999)
EF3R	TCCTCTAAATGACCAAGTTTG	Fungi	(Anderson et al., 2003; Smit et al., 1999)
Thiox1F	TGGGGAGCAAACAGGATTAG	*A. thiooxidans* ATCC 19377	-
Thiox1R	ACTTCCCAGGCGGAATACTT	*A. thiooxidans* ATCC 19377	-

Table 4.2**.** PCR conditions for the different primer sets. The same times and temperatures were used for those involving their corresponding GC clamps (for DGGE).

Primers	Denaturation		Annealing		Elongation		# of cycles
	Temp. (ºC)	Time (min.)	Temp. (ºC)	Time (min.)	Temp. (ºC)	Time (min.)	
ARC344F-ARC915R	94	2	56	1.5	72	2	30
EUB338F-EUB518R	94	1	50	1	72	1	30
soxB432F-soxB1446R	94	0.5	55	0.66	72	0.5	10
	94	0.5	47	0.66	72	0.5	25
EF3F-EF4R	94	1	48	1	72	3	40
Thiox1F-Thiox1R	95	1	56	1	72	1	43

DNA Cloning. PCR products which have demonstrated successful amplification with the Eubacterial primers were ligated into pCR®2.1-TOPO® vector (Invitrogen) and cloned into One Shot® Mach1™-T1^{R} competent cells using TOPO TA Cloning Kit (Invitrogen) as per manufacturer's instructions. The cloned cell suspensions were transferred to 1.5 ml Eppendorf tubes and shaken at 200 rpm for 1 hour at 37 ºC. The cultures were subsequently spread plated on pre-warmed low salt LB + kanamycin plates (1% Tryptone, 0.5% Yeast Extract, 0.5% NaCl, 1.5% agar, 0.25 μg ml^{-1} kanamycin, pH 7.5) and incubated at 37 ºC for up to 2 days until visible colonies were formed. Using sterile toothpicks the white and light blue colonies were used to inoculate 200 μl of SOC (2% Tryptone, 0.5% yeast extract, and 0.05% NaCl, 2.5 mM KCl, adjusted to pH 7.5 with NaOH). After autoclaving sterile solutions of $MgCl_2$, glucose and kanamycin were added at final concentrations of 10 mM, 20 mM and 25 μg ml^{-1} respectively. Clones were incubated at 37 ºC for two days. Confirmation of successful clones was achieved by PCR using M13-R (5' CAG GAA ACA GCT ATG AC 3') and M13-F (5' GTA AAA CGA CGG CCA G 3') primers and the following reaction conditions: initial hot-start at 97 ºC for 15 min, 35 cycles of 94 ºC for 40 s, 53 ºC for 60 s, 72 ºC for 60 s and a final 10 min elongation at 72 ºC. PCR reactions were performed as described above. The entire PCR reactions were analyzed using 1% agarose gel electrophoresis, to confirm the presence of the correct sized DNA insert. Clones which contained the DNA insert were sent for high-throughput sequencing to the Plant Biotechnology Research Institute, National Research Council, Saskatoon, SK.

DNA sequencing. DNA sequences of all successful clones were analyzed using the following web based bio-informatics software: NCBI Blast, GeneDoc, CLustalX, Bioedit, and Phylip (http://www.uk.plbio.kvl.dk/bioinfo.htm).

4.3 Results

4.3.1 Detection of Microbial Sulfur Oxidation in Sulfur Block Bioreactors

Bioreactors amended with 10% of the block effluent produced greater concentrations of sulfate (ca. 5.1 mM) than bioreactors amended with sterile water (0.007 mM) (Figure 4.1A). Surface sulfur inoculated with 10% block effluent began with an average of 1.4 mM sulfate (standard error = 0.37) and increased to 4.4 mM sulfate (standard error = 1.78). Matrix sulfur samples produced greater amounts of sulfate, beginning at 1.5 mM sulfate (standard error = 0.06) and reaching 5.8 mM (standard error = 2.75) after 40 weeks. There was no significant difference between the surface and matrix sulfur samples. The highest sulfate concentration, 11.3 mM, was found after 40 weeks of incubation in one of the replicates of the matrix sulfur, while the other two matrix sulfur reactors were 3.1 mM and 2.9 mM. The surface sulfur showed a similar pattern, in which one of the replicates attained 7.9 mM and the other two replicates were 2.0 mM and 3.3 mM.

Throughout the incubation, the concentration of hydronium ions steadily increased for the bioreactors inoculated with 10% effluent (Figure 4.1B). Non-sterile bioreactors containing the matrix sulfur began with a 6.69×10^{-3} (standard error = 7.82×10^{-4}) hydronium ion concentration, and increased by ~1.8×10^{-2}, reaching 2.47×10^{-2} (standard error = 3.08×10^{-2}). The non-sterile bioreactors which contained the surface sulfur began with 5.95×10^{-3} (standard error = 1.01×10^{-3}) but increased by only ~8.6 x

10^{-3} reaching 1.46 x 10^{-2}. These surface sulfur bioreactors reached a final hydronium ion concentration of 1.46 x 10^{-2} (standard error = 5.56 x 10^{-3}). The average hydronium ion concentration throughout the entire incubation period of the bioreactors containing sterilized matrix sulfur amended with sterile water was 1.6 x 10^{-7} (standard error = 9.2 x 10^{-8}) and for non-sterile matrix sulfur amended with sterile water was 1.8 x 10^{-5} (standard error = 8.40 x 10^{-6}). The lowest pH reached 1.22, an equivalent to 6.0 x 10^{-2} hydronium ion concentration, which was measured in one of the replicates of the matrix sulfur and 10% effluent bioreactor. Similar to the sulfate release, there was large variation between the different replicates of bioreactors.

When expressed in terms of hydronium and sulfate production per unit sulfur block, the surface sulfur samples produced ten times less, 6.17 x 10^{-7} [H_3O^+] per gram sulfur per day, than matrix sulfur samples, 1.28 x 10^{-6} [H_3O^+] g^{-1} day^{-1}. However, there was no statistically significant difference in hydronium ion concentration between the surface and matrix bioreactors at the final sampling period, where the t-test probability was 0.618 ($p<0.05$). Sulfate production was at 2.11 x 10^{-1} μM sulfate per gram sulfur produced per day by surface samples and 2.83 x 10^{-1} μM g^{-1} day^{-1} for matrix sulfur samples. Statistical analysis performed on the matrix and surface samples containing the 10% effluent at the final sampling period showed that the difference in the mean values of the two groups is not significant ($p<0.05$).

The increased sulfur oxidation in matrix sulfur samples seemed to parallel the distribution of culturable sulfur oxidizing heterotrophs. The matrix sulfur bioreactors contained more sulfur heterotrophs 27 CFU ml^{-1} (standard error = 3.3) and acidophilic fungi 97 CFU ml^{-1} (standard error = 6.3) compared to surface sulfur bioreactors which had only 6 CFU ml^{-1} (standard error =1.5) sulfur heterotrophs and 12 CFU ml^{-1} (standard

error = 2.2) acidophilic fungi (Figure 4.2). However, these trends were also not statistically significant. Total heterotrophs were relatively equal between the matrix and surface sulfur bioreactors containing 10% effluent.

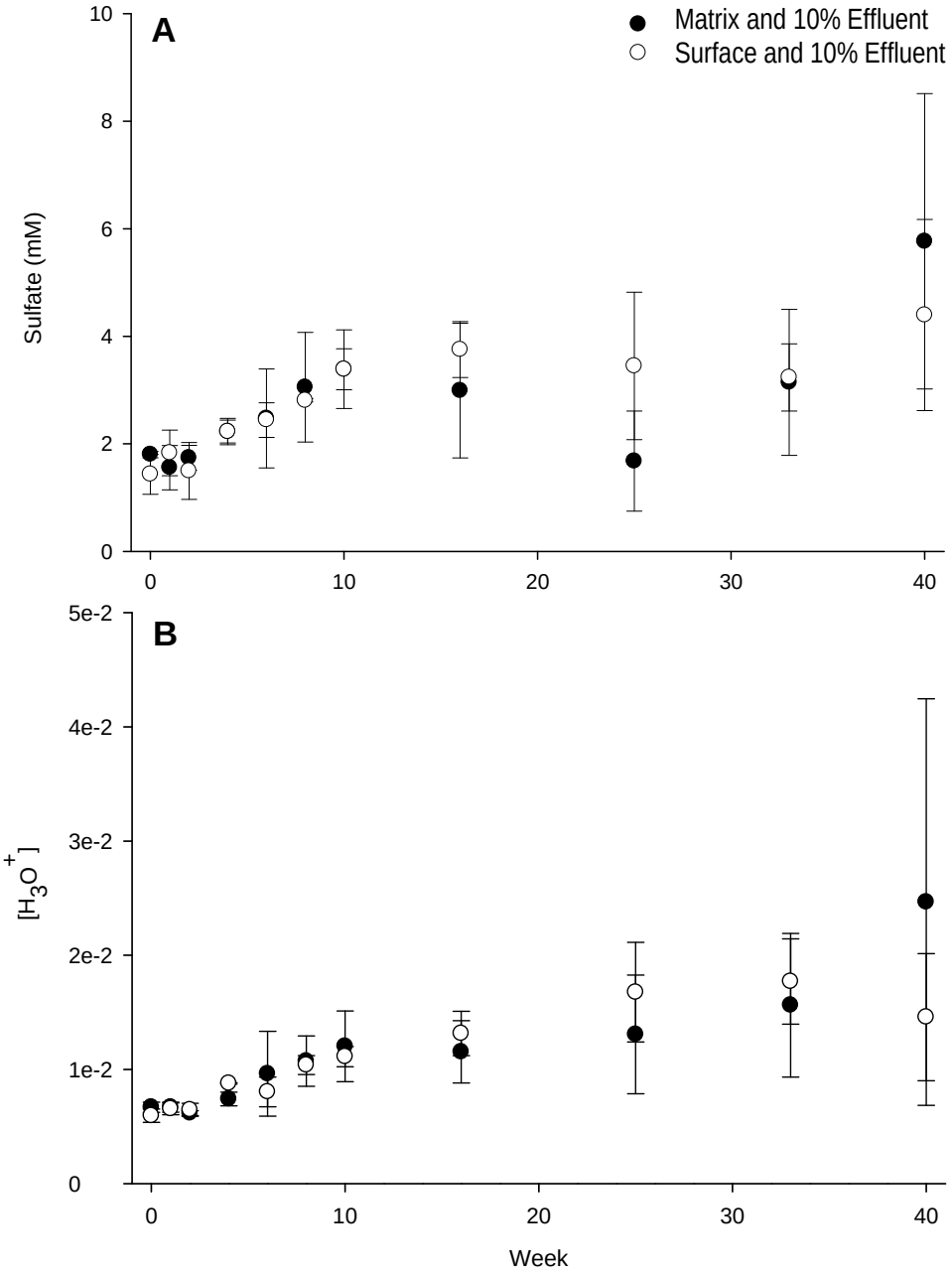

Figure 4.1. Average sulfate concentration (A) and hydronium ion concentration in the matrix (closed circles) and surface (open circles) sulfur bioreactors containing 10% effluent throughout 40 weeks of incubation. The error bars represent the standard error determined from three independent replicates.

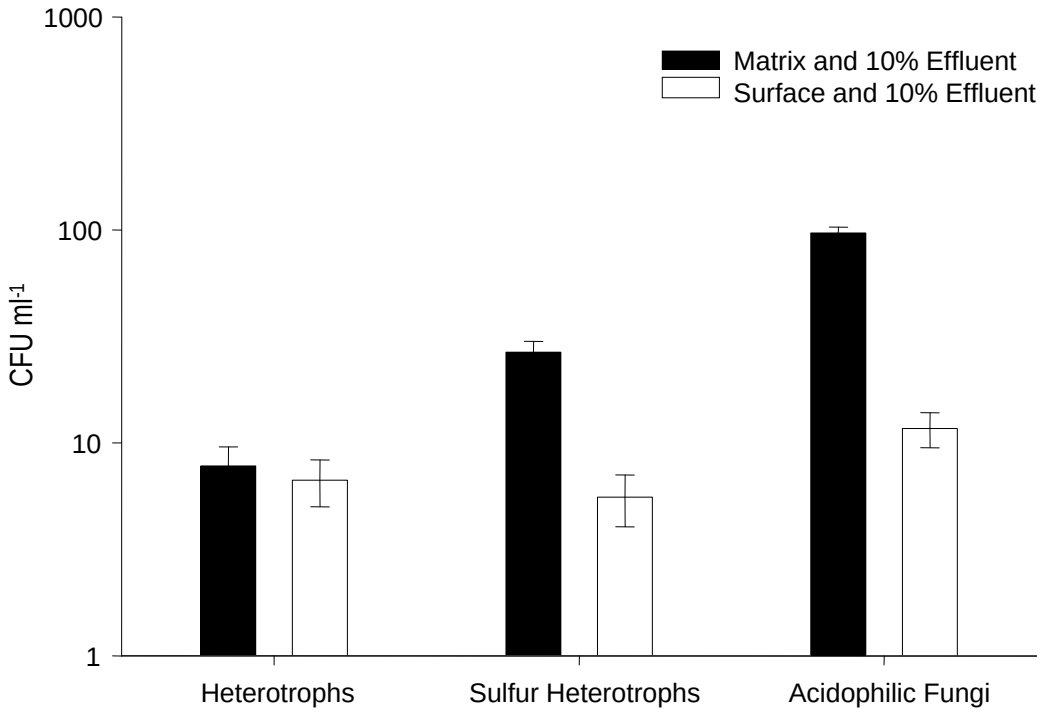

Figure 4.2. Colony forming units of different types of heterotrophs present in the matrix and surface bioreactors containing 10% effluent after 40 weeks of incubation. Error bars represent the standard error from three independent bioreactor replicates and three independent plate counts.

Autotrophic sulfur or thiosulfate oxidizing microorganisms such as *A. thiooxidans* were not detected by the MPN method. However, when the same samples were analyzed using primers designed specifically for *A. thiooxidans* ATCC 19377 there was a small proportion of these autotrophs found in all four different treatments, with the greatest DNA copy number detected in surface sulfur bioreactors (Figure 4.3). The surface sulfur bioreactors contained 17 *A. thiooxidans* DNA copy numbers g^{-1} extracted DNA ml^{-1} sulfur slurry (standard error = 2.0). A similar trend was observed with the 16S rDNA Q-PCR results for the general *Eubacterial* population, which showed that the surface sulfur bioreactors had almost double amplified *Eubacterial* DNA, with 96 DNA copy numbers g^{-1} extracted DNA ml^{-1} sulfur slurry (standard error = 1.1) compared to matrix samples which had only 55 DNA copy numbers g^{-1} extracted DNA ml^{-1} sulfur slurry (standard error = 1.5). Both the sterile water bioreactor treatments had total *Eubacterial* DNA copy number counts similar to the bioreactors containing matrix sulfur and 10% effluent suggesting that the non-sterile effluent contained within it, some organisms that initiated the sulfur oxidation. The presence of amplified DNA in the sterile water samples suggests that sulfur oxidizing bacteria may be present within the sulfur matrix, possibly associated with thin fracture surfaces.

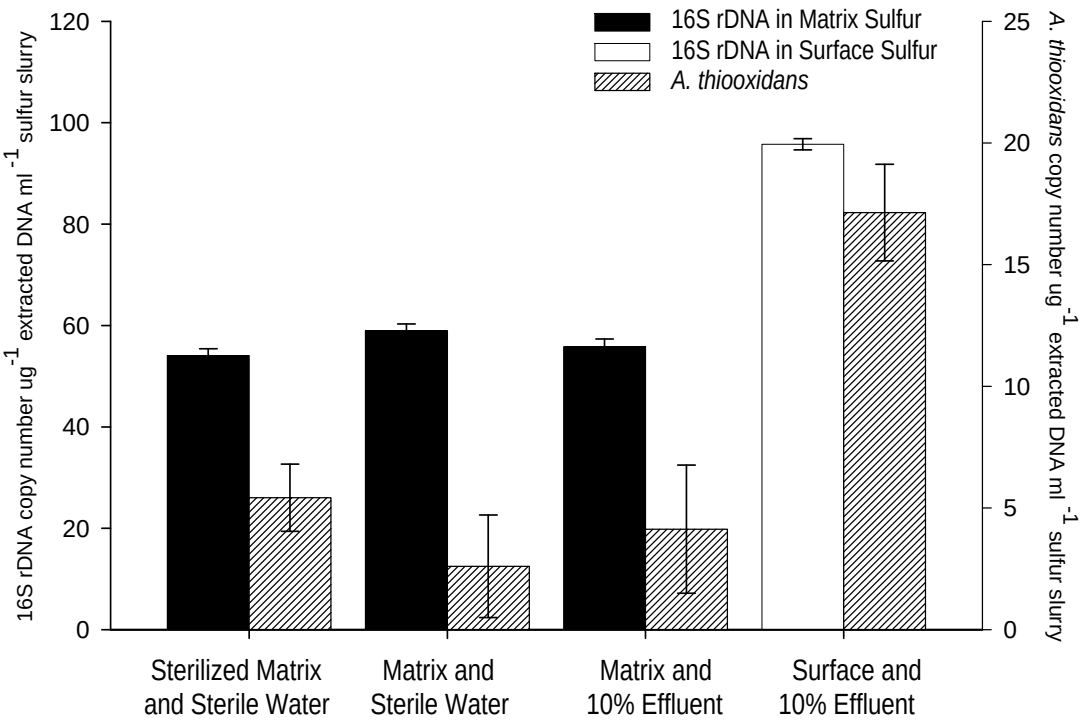

Figure 4.3. Amplified DNA copy numbers of bacterial 16S rDNA and *A. thiooxidans* 16S DNA obtained by quantitative PCR counts from the four sulfur bioreactor treatments. Error bars represent the standard error from six independent PCR reactions.

4.3.2 Detection of Microbial Growth in the Sulfur Blocks

The microbial plate counts of samples taken directly from the sulfur blocks supported the bioreactor results (Figure 4.4). Total heterotrophs were generally higher in matrix sulfur samples compared to surface samples; however, the differences between the groups were not statistically significant. The large variation between depth matrix samples could be attributed to fracture patterns within the block. As a percentage of the total heterotrophic community, sulfur heterotrophs in the bottom three samples (> 390 cm) comprised 37% of the community compared to only 3% in the top three samples (< 100 cm). The MPN technique detected 18 MPN g^{-1} sulfur (standard error = 6.4) culturable autotrophic sulfur oxidizers and 3 MPN g^{-1} sulfur (standard error = 2.0) thiosulfate oxidizers only along the 0-10 cm profile of the block after 7 weeks of incubation (Figure 4.5). There were no autotrophic organisms culturable by this method in any of the matrix sulfur samples.

Similar to the bioreactors, autotrophic *A. thiooxidans* were greatest in the surface samples, however these trends were not statistically significant due to the large variation between replicates (Figure 4.6). The highest level of *A. thiooxidans* was found in the 0-10 cm samples of the 143-D drill hole which contained 1.62×10^2 *A. thiooxidans* amplified copy numbers μg^{-1} of extracted DNA. The lowest level of *A. thiooxidans* was found in the 590-600 cm matrix samples of drill hole 145-D, which contained 3.19×10^1 *A. thiooxidans* amplified copy numbers μg^{-1} of extracted DNA. In contrast there were approximately 10 times more general *Eubacteria* present in the sulfur block than *A. thiooxidans* with values ranging from 1.23×10^3 to 1.11×10^4 amplified DNA copy numbers μg^{-1} extracted DNA.

Sequence analysis of 48 cloned Eubacterial 16S DNA amplicons did not match sequences available in GenBank that were specific for any particular organisms. Most matches were for unidentified, uncultured bacteria obtained from various environmental samples. No matches were made with known sequences of *A. thiooxidans* available with GenBank, however some of the isolates did show similarity to bacterial isolates obtained from deep sea hydrothermal vents. The relation to microorganisms that rely on oxygen and hydrogen sulfide to produce energy required to fix carbon dioxide, coincides with the metabolic properties of elemental sulfur oxidation that would be expected of the microorganisms inhabiting the block.

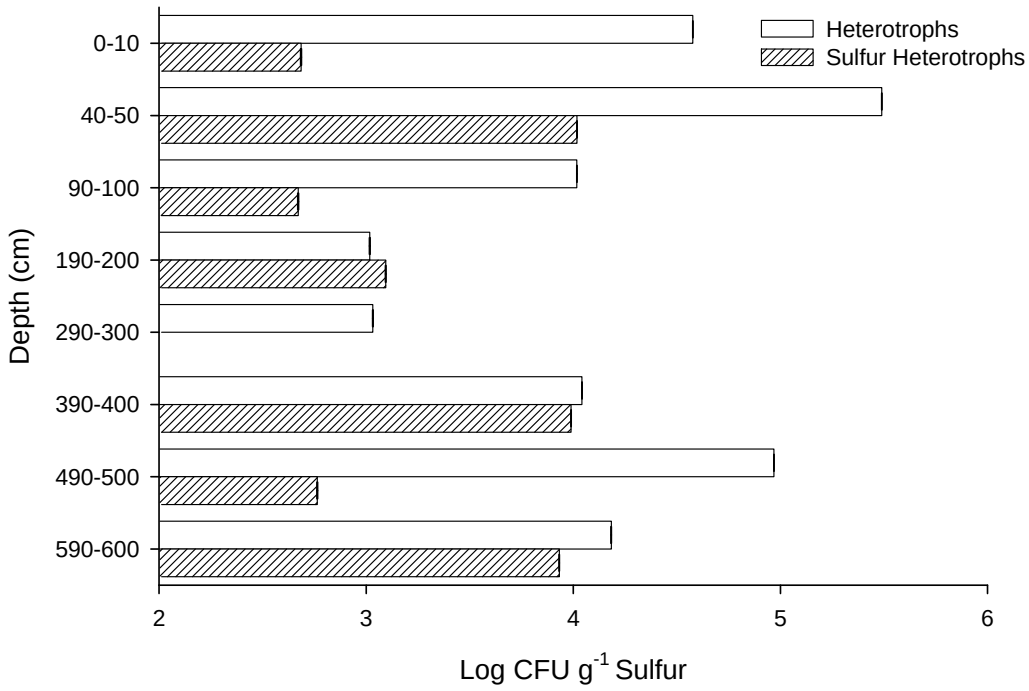

Figure 4.4. Average of heterotrophic colony forming unit counts from a matrix profile of the Phase 1 sulfur block. Standard error of the estimate is too small to be visible on the log scale.

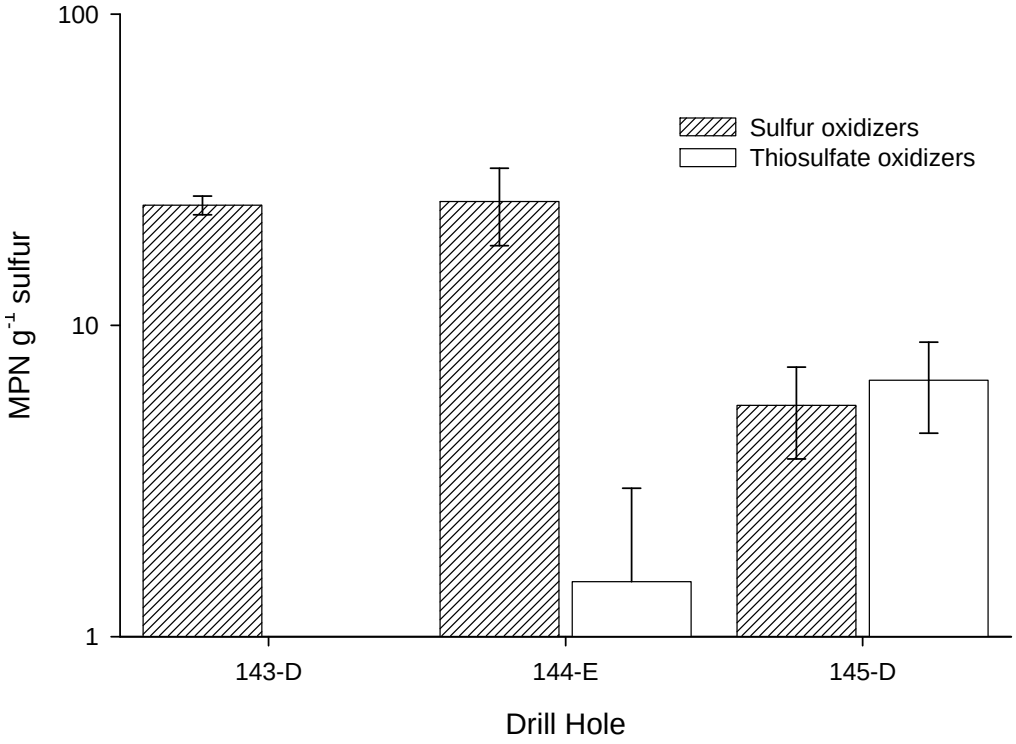

Figure 4.5. Average of most probable number of three independent replicates of sulfur and thiosulfate oxidizers found in the 0-10 cm matrix samples of the sulfur block. Error bars represent the standard error of the mean.

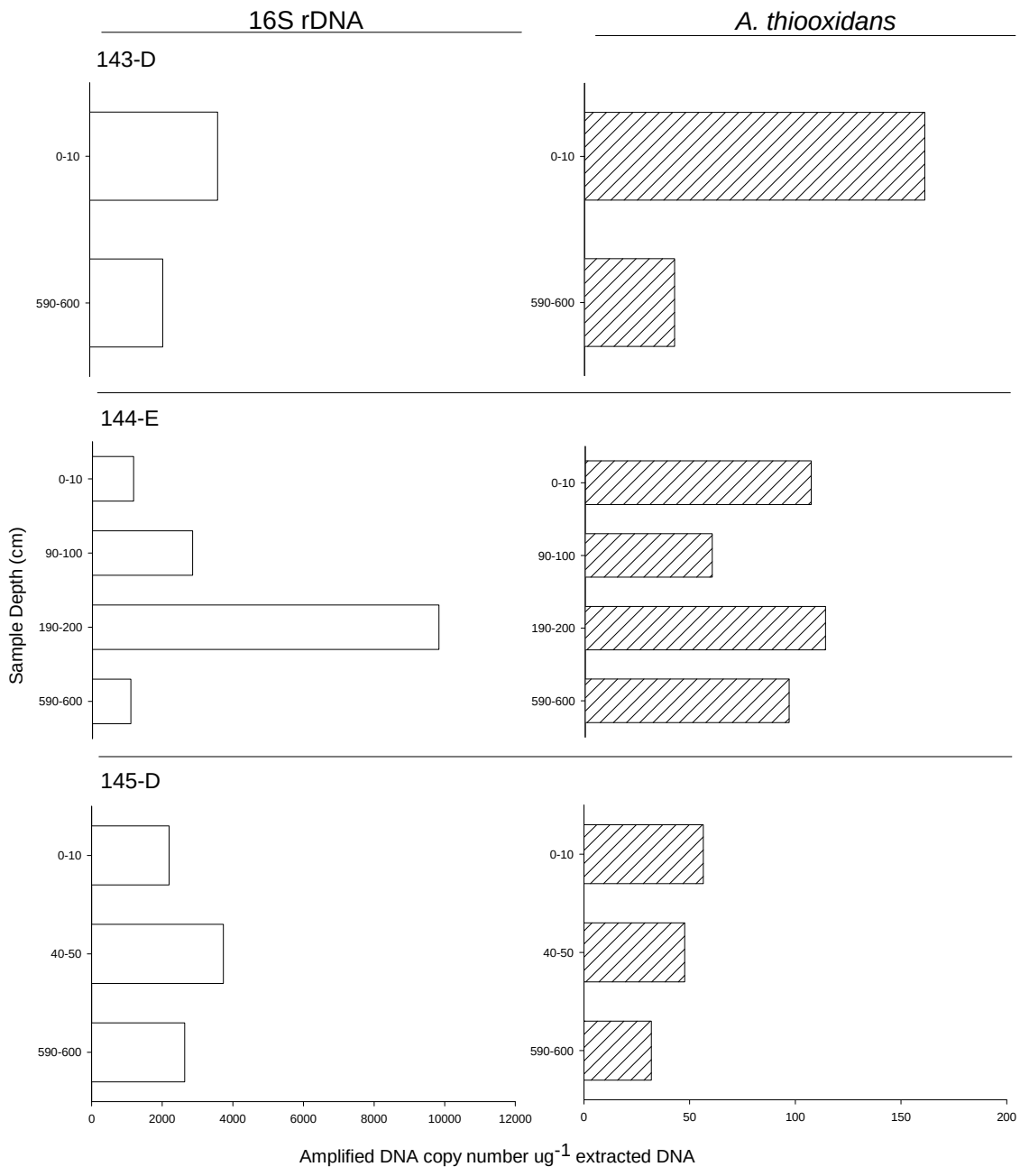

Figure 4.6. Amplified DNA copy numbers of 16S rDNA and 16S *A. thiooxidans* from different depths of the sulfur blocks.

4.4 Discussion

Here, I present for the first time, evidence that microorganisms are thriving within the depths of an elemental sulfur block and that these organisms are physiologically capable of contributing to the production of sulfuric acid. Heterotrophs dominated the oxidation of sulfur samples in laboratory bioreactors, and also numerically dominated those water and sulfur samples taken directly from the field. Autotrophic sulfur oxidizers were found in both laboratory bioreactors and sulfur block samples, however they appeared to be in greater concentration along the surface samples. Their prevalence at the surface of the block is consistent with aerobic microorganisms. Oxygen levels decrease and carbon dioxide levels increase with increasing depth through the sulfur block profile (Tyler Birkham, personal communication).

It has been well established that both heterotrophs and autotrophs contribute to the oxidation of reduced sulfur compounds in natural systems (Wainwright and Killham, 1980; Kelly, 1982; Grayston et al., 1986; Pronk et al., 1990; Jannasch et al., 1991; Hallberg et al., 1996; Friedrich et al., 2000; Friedrich et al., 2001; Sorokin, 2003; Kletzin et al., 2004). The methods of microbial sulfur oxidation remain elusive with complex pathways catalyzed by different means in different species (Kelly et al., 1997; Suzuki, 1999). It is surprising that sulfur heterotrophs were found in high numbers deep within the sulfur block, as the level of utilizable carbon substrates and available oxygen would likely be lower with increasing depth. There was only a one log difference in CFU g^{-1} sulfur between the 0-10 cm and 590-600 cm depth samples.

The sulfur blocks represent an unique environment that contain almost no carbon and extreme acidity (Crescenzi et al., 2006). Previous investigations of microbial growth in acidic environments have concentrated on acid mine drainage, which normally contains a variety of other reduced sulfur compounds, such as pyrite, not found in the sulfur block (Bond et al., 2000; Baker and Banfield, 2003; Gonzàlez-Toril et al., 2003; Johnson and Hallberg, 2003; Baker et al., 2004). Acid mine drainage often contains high concentrations of metals such as aluminum, copper, zinc and manganese, which are also not normally found in the sulfur block (Johnson and Hallberg, 2003). Microorganisms commonly associated with environments such as acid mine drainage include the autotrophs: *Leptospirillum spp*., *Ferroplasma spp.*, and *Acidithiobacillus spp*., as well as the heterotrophs: *Acidocella spp., Acidobacterium spp*., and *Acidomonas spp*. (Bond et al., 2000; Johnson and Hallberg, 2003).

The sulfur blocks are a hydrologically active environment, because of direct exposure to precipitation, and as such generate large amounts of run off or effluent. This effluent appears to be a critical component in initiating substantial sulfur oxidation. Despite also containing strains of *A. thiooxidans*, only those bioreactors which received 10% effluent had a rapid increase in acidity and sulfate production, whereas those containing sterile water did not. Typically, autotrophic microorganisms oxidize sulfur at a greater rate than heterotrophic sulfur oxidizers (Pepper and Miller, 1978). Yet in the presented experiments, surface sulfur bioreactors containing more *A. thiooxidans* than matrix sulfur bioreactors initially produced similar amounts of sulfate and hydronium ions, although after 30 weeks, matrix sulfur bioreactors began to surpass surface bioreactors in sulfur and hydronium production. Further, both surface sterilized sulfur samples and non-sterilized sulfur samples amended with sterile water produced very

little sulfate and hydronium ions. This suggests that the effluent running through the block may influence initiating and sustaining sulfur oxidation by providing the right composition of microbial population to maximize sulfur oxidation and by transporting sulfur oxidizing microorganisms to different levels of the block.

Typically, heterotrophic sulfur oxidizers play a dominant role in oxidation in habitats such as agricultural soil (Lawrence and Germida, 1988), which are considerably more complex than the relatively pure S blocks. When heterotrophs are isolated from extremely acidic environments, their survival is primarily dependent on lysis products of other organisms, such as the autotrophs which could more readily colonize the system (Johnson, 1998). One possible source of these lysis products could be fungi (Sorokin, 2003). There are large amounts of fungal hyphae in the sulfur blocks. Though little convincing evidence of fungal sulfur oxidation has been published to date, it seems possible that they can participate in oxidation reactions such as those observed in the bioreactors (Yagi et al., 1971; Wainwright and Killham, 1980; Grayston et al., 1986).

During sulfur block construction in which layers of molten sulfur are poured sequentially 2-12 cm each time, windblown debris settles upon each layer. Thus, when investigating this habitat it was essential to use a sensitive molecular technique that would only detect active organisms. I used ethidium monoazide bromide (EMA) as a dye that would only allow the amplification of the viable microbial community (Nogva et al., 2003; Rudi et al., 2005a). Ethidium monoazide bromide will also introduce some biases into the molecular results, but due to the difficulty in cultivating acidophiles, which are known to have fastidious responses to different growth media and conditions, molecular techniques can provide important supplemental information. For example, the EMA-Q-PCR method detected viable *A. thiooxidans* at low copy numbers

throughout the block, whereas the cultivation techniques could not detect any autotrophs below the surface of the sulfur block.

Through both cultivable and molecular examination of microbial communities it can be concluded that the sulfur block harbors a large number of successfully thriving and sulfur oxidizing microorganisms. The various surfaces of the block provide ideal habitats for autotrophic sulfur oxidizers. Though, autotrophs, such as *A. thiooxidans* are the major sulfur oxidizing microorganisms which could potentially grow on the block, this research has shown that it is the heterotrophs which numerically dominate the microbial population of the block. Also, the effluent from the sulfur block has been shown to have a pivotal role in contributing to sulfur oxidation and acid production. Both heterotrophic and autotrophic sulfur oxidizing organisms are associated with degradation of the sulfur block, the specific mechanisms should be further investigated to help develop technologies that minimize environmental harm and retain useful elemental sulfur for future generations.

5.0 GENERAL DISCUSSION

This study is the first in depth look at the microbiological communities inhabiting elemental sulfur repositories, providing information relevant for both oil industry operations which may be concerned with maintenance of product value and microbial ecology research concerned with sulfur oxidation. Methods developed in this study have provided valuable information for future molecular research in environmental sciences and the microbial analysis of the elemental sulfur block has explored a unique living environment where little was known before.

The use of EMA in differentiating between viable and non-viable microorganisms in environmental samples was shown to be effective at limiting the amplification of DNA from non-viable microorganisms; however the efficiency of the method appeared to be matrix dependent where it was most efficient in pure culture, soils and sulfur but not in biofilms. Nogva et al. (2003) have shown that pure DNA suspensions treated with 100 μg ml^{-1} EMA had a 4.5 log reduction in amplification compared to untreated DNA. The non-viable pure culture suspensions presented in this study agreed with these results, they showed that EMA treatment reduced the level of PCR amplification by >5 logs. Low concentrations of EMA can be used to decrease the level of amplification. Lee and Levin (2006) have shown that EMA was effective at suppressing amplification of DNA from killed cells at concentrations of 0.8 μg ml^{-1}. I have not found such dilute amounts to provide sufficient suppression in the environmental matrices tested. Inhibition of DNA amplification was not achieved until 25 μg ml^{-1} in soil slurry and 72 μg ml^{-1} in sulfur suspensions.

Unlike the published results of Nocker and Camper (2006) which showed that EMA treatment of a mixed population drinking-water biofilm containing a large proportion of non-viable cells had a community fingerprint quite different than that of the non-EMA treated portion, I was unable to adapt the method to inhibit the amplification of DNA from non-viable cells in Saskatchewan river biofilm communities. However, I did find that DGGE community fingerprints of non-EMA treated soil samples were different than those of EMA-treated soil samples and the 16S RNA profiles of the same samples.

Research which questions the efficiency of the EMA methods became available during the time of this study. Nocker et al (2006) have shown that propidium monoazide was more efficient than EMA at selectively removing DNA from non-viable cells because once intercalated the DNA becomes insoluble and is removed from suspension during DNA extraction procedures. They argued that certain species of viable cells may be ineffective at removing EMA from their cytoplasm with efflux pumps, which would lead to a misrepresentation of the true viable population. It should not be expected that activity of EMA would be the same in each type of biofilm. Biofilms are unique complex systems and the methods used in the preparation, harvest, DNA extraction and PCR amplification conditions can provide different results.

DNA amplification of killed pure cultures of *E. coli* LK1 *gfp*+ were sufficiently suppressed with EMA when extracted from soil and elemental sulfur. The different experiments performed here have confirmed the reliability of this method in these systems, and so the EMA technique could be used with assurance that PCR amplification would be limited to the DNA from a predominantly viable microbial population. The use of the EMA method is desirable for its rapidity and simplicity in comparison to other established methods aimed at examining the active microbial

populations. This method was particularly useful in elemental sulfur samples taken from the block, and so it was used in downstream research with the sulfur bioreactors and block profile samples.

The presence, distribution, and influence of microorganisms in sulfur blocks has been a serious consideration, but little work has been published which thoroughly examines this area (Clark, 2005; Crescenzi et al., 2006; Laishley and Bryant, 1987; McKenna, 2004). McKenna (2004) suspected that darkened films visible within ~2 centimeters below the surface of the block could be indicative of microbial growth, but this was not further examined. The experiments presented here did not examine the darkened films, but did show that throughout a depth profile in the matrix of the block, there was no particular area which had a significant majority of microorganisms. The relative numbers of viable microorganisms was variable and dispersed throughout the block profile up to six meters in depth.

Microorganisms were detected in bioreactors inoculated with 10% effluent, however there was little difference between the bioreactors containing sulfur from the matrix or surface of the block. Bioreactors that did not receive effluent inoculant did not change in hydronium ion and sulfate concentration within the 40 week incubation period, whereas the effluent inoculated bioreactors increased in concentration of these compounds. The only known published study which examines the microbiological properties of the runoff block effluent, simply states that the presence of microorganisms and sulfur particles has been confirmed, the extent of which was not revealed (Crescenzi et al., 2006). Researchers were also particularly interested in examining the growth of the sulfur oxidizing autotroph, *A. thiooxidans.* Crescenzi et al. (2006) have examined

the effects of acidity and ionic strength on the attachment and sulfur oxidizing ability of A. *thiooxidans* on the sulfur blocks. Such respiratory and growth inhibition was found to be strain dependent, as there have been reported *A. thiooxidans* strains which contain alophilic properties (Crescenzi et al., 2006).

The trends shown in the different bioreactors suggest that the effluent acts as a microbial inoculant for sulfur oxidation. There was extreme variation between all bioreactor samples. One of the three replicates had extreme increases in hydronium ion and sulfate concentration, whereas the other two had more subtle increases. Because of this, standard errors were high and there was no statistical difference between groups of treatments. The EMA-Q-PCR method was successful at detecting *A. thiooxidans* in all bioreactor treatments; however, there was no correlation with the number of *A. thiooxidans* and the level of acid and sulfate accumulation. This suggests that the increase in acidity and sulfate experienced in the effluent bioreactors is not achieved because of the growth of the detected strain of *A. thiooxidans*.

Microbial growth within the sulfur blocks follows a random pattern as would be expected from the natural fractures formed during elemental sulfur solidification. Growth was detected up to 6 m in depth, with sporadic pockets of uninhabited areas, believed to coincide with solid non fractured block matrix. Cracking patterns which inevitably form in the sulfur blocks during solidification processes between the two different geomorphic sulfur forms collect water through the surface fractures and hydrostatically extrude the effluent from the base (Clark, 2005). No clear definition of growth trends was observed in the block, however it is believed that it follows a distribution similar to that of oxygen and water.

The organism which was suspected of inhabiting the sulfur block and contributing to sulfur oxidation is *A. thiooxidans* (Crescenzi et al., 2006). *A. thiooxidans* was found predominantly along the surface of the block, but more sensitive detection methods have shown that there was growth of the acidophile throughout the depth profile of the sulfur block. Crescenzi et al. (2006) have found that the block can be inoculated with *A. thiooxidans,* which is capable of colonizing the surfaces. This research shows that this sulfur oxidizing acidophile is naturally present on the surfaces and throughout the depth of the block. A variety of microorganisms capable of oxidizing sulfur were found throughout the block profile.

The research presented here shows a novel molecular method which can be used in characterizing the active microbial population in Alberta's sulfur blocks. The EMA technique is suitable for studying the viable microbial populations of this extreme environment. In utilizing this and other established microbial techniques it was determined that microorganisms play an important role in biological oxidation of the block. Microbial distribution throughout the sulfur block is sporadic, likely depending on the availability of moisture and gas present throughout the fractures of the block. Also, the accumulation of sulfuric acid in the rain water effluent could be attributed to sulfur oxidizing activity of the residential microorganisms. Thus, designing methods aimed at reducing the level of sulfur block oxidation must consider the biological sulfur oxidation parameters as well as the chemical pathways.

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