A GEOMICROBIOLOGICAL STUDY OF THE RABBIT LAKE IN-PIT TAILINGS MANAGEMENT FACILITY

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Abstract

Microorganisms can strongly influence geochemical conditions and element mobility, which is an important consideration in planning for long-term stability of mine waste. In this study, I have characterized microbial communities in a uranium mine waste facility, the Rabbit Lake In-Pit Tailings Management Facility (RLITMF) in Northern Saskatchewan, Canada. The tailings have an alkaline pH (median 8.8), high Eh (median 210 mV), and low temperatures (median 2 ºC). They also contain abundant Fe(III)-oxides which control the mobility of elements such as arsenic. I am particularly interested in the potential interactions between microorganisms and arsenic and iron, which may influence arsenic mobility. Microbial diversity was investigated by analyzing high-throughput amplicon sequencing (bacterial and archaeal 16S rRNA gene, V4 region, analyzed using mothur). Geochemical data (ICP-MS analyses) and core log data were provided by the tailings facility operator, Cameco Corporation. Our results show the presence of populations related to microorganisms typically involved in iron, sulfur, and arsenic cycling. We were able to culture sulfate-reducing bacteria (SRB) from tailings samples at neutral pH; this suggests the relatives of sulfate-reducing bacteria identified in sequencing are viable. The current pH, Eh, and temperature conditions in the RMITMF are not ideal for microbial growth but if the pH and Eh decreased or the temperature increased this could promote microbial growth. Thus, this study provides evidence to support tailings management decisions that prevent microbially-driven processes from occurring in the future i.e. maintaining high pH and Eh conditions in the tailings to maintain iron oxide stability through time.

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To my parents

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List of Abbreviations

ADP	Adenosine diphosphate
AMOVA	Analysis of Molecular Variance
AMP	Adenosine monophosphate
APS	Adenosine 5'-phosphosulfate
ATP	Adenosine triphosphate
BMIT	Biomedical Imaging and Therapy
Cps	Counts per second
dsDNA	double stranded (deoxyribonucleic acid)
DNA	Deoxyribonucleic Acid
ddNTPs	Dideoxynucleotide triphosphate
dNTPs	Deoxynucleotide triphosphate
DTMF	Deilmann Tailings Management Facility
EPS	Exopolysaccharide
Eh	Reduction-oxidation potential
FAME	Fatty Acid Methyl Esters
HXMA	Hard X-Ray MicroAnalysis
Masl	Meters above sea level
NTC	No template control
PCR	Polymerase Chain Reaction
Pi	Inorganic Phosphate
qPCR	Quantitative Polymerase Chain Reaction
RLITMF	Rabbit Lake In-Pit Tailings Management Facility
rRNA	Ribosomal Ribonucleic Acid
RT-qPCR	Reverse transcription quantitative polymerase chain reaction
SRB	Sulfate-Reducing Bacteria
SRC	Saskatchewan Research Council
TMF	Tailings Management Facility
VESPERS	Very Sensitive Elemental and Structural Probe Employing Radiation from a Synchrotron

CHAPTER 1 INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

In 2012, Canada was the second largest producer of the world's mined uranium. Canadian uranium is currently mined exclusively in northern Saskatchewan from high grade ore bodies (Natural Resources Canada, 2014). Uranium extraction in northern Saskatchewan is typically carried out through sulfuric acid leaching and the addition of sodium chlorate to oxidize uranium (Donahue et al., 2003). After uranium is extracted from the ore it is neutralized through multiple lime additions phases to raise the pH (final pH varies by tailings facility) (Essilfie-Dughan et al., 2012; Moldovan et al., 2003). The waste is stored in tailings management facilities (TMFs). These wastes contain elements such as molybdenum (Mo) and arsenic (As) in high concentrations. The concentrations of these elements are variable, depending on the source ore body Arsenic mobility in the tailings is controlled by adsorption onto ferrihydrite and is influenced by pH, reduction-oxidation potential (redox, Eh), and the solid phase concentration of iron (Fe/As ratio >4) (Moldovan et al., 2003). If iron concentrations in the waste are too low iron is added to sequester arsenic.

The low temperature, alkaline pH and high redox potential typical of the uranium tailings limit the potential activity of microbial communities that are currently present in the tailings. However, the ability of microorganisms to affect the stability of constituents in the tailings through enzymatic and non-enzymatic metabolic activity is an important consideration for long-term tailings stability. For example, if the pH decreases, Eh decreases, and temperature increases, the tailings might support the growth of microbes capable of releasing mineral-bound arsenic into the porewater. At the Deilmann Tailings Management Facility (DTMF) microbiological characterization using sequencing techniques identified sulfate- and iron-reducing bacteria, and iron-reducing bacteria were also identified using culturing methods (Bondici et al., 2013). There have been no previous sequence-based analyses of microbial communities in the Rabbit Lake In-Pit Tailings Management Facility (RLITMF). The data from the DTMF site can be used in comparison with data generated from the present study on RLITMF samples to explore potential

common patterns in sequencing data from uranium mine tailings facilities in northern Saskatchewan.

1.2 Biogeochemistry of Saskatchewan Uranium Mine Wastes

1.2.1 Overview of Northern Saskatchewan Uranium Mines

Uranium was first discovered in Northern Saskatchewan in 1935 north of Lake Athabasca, where pitchblende was found at Point Nicholson (Sibbald, 1988). Uranium exploration in the Athabasca basin began in 1967 (Sibbald, 1988). In 1968, the Rabbit Lake ore deposit was the first of the major unconformity type uranium deposits in that area to be discovered (Sibbald, 1988). Exploration by airborne radiometric anomalies identified more uranium ore bodies, including the Gaertner and Deilmann ore bodies at Key Lake, discovered in 1975 and 1976, respectively. More uranium deposits were identified at Dawn Lake in 1978, McClean Lake in 1979 and Cigar Lake in 1981 (Sibbald, 1988). The uranium ore grade at these sites were found to be as high as 12% at Cigar Lake; in comparison, uranium concentrations in other uranium deposits around the world range from 0.05% to 3% depending on the deposit geology (Ceyhan, 2009; Sibbald, 1988).

A common uranium extraction method used in Saskatchewan is sulfuric acid leaching to remove uranium from the ore (Mahoney et al., 2005). The initial stages of uranium extraction includes the addition of sulfuric acid to pH 1.5 and sodium chlorate to ca Eh +450mV (Donahue et al., 2003; International Atomic Energy Agency, 1993). Uranium is soluble under these conditions and can be stripped from the solution through a solvent extraction process (International Atomic Energy Agency, 1993). The acidic liquid waste that results from the uranium extraction is called the raffinate (Essilfie-Dughan et al., 2012). Lime is added to neutralize the raffinate to between pH 7-8 for the McClean Lake Operations and pH 10 at Key Lake and Rabbit Lake. In 2000, the target pH for tailings deposited into the RLITMF was changed from pH 10 to 8.5 (Moldovan et al., 2003). The high pH is conducive to maintaining oxidized iron (III) in the tailings, and thus preventing arsenic (V) migration (Donahue et al., 2003; Essilfie-Dughan et al., 2012; Mahoney et al., 2005).

The tailings management facilities at Key Lake (DMTF) and Rabbit Lake (RLITMF) have been investigated previously to explore the potential role of microbes in the stability of constituents in these environments (Bondici et al., 2013; Wolfaardt et al., 2008).

1.2.2 Rabbit Lake In-Pit Tailings Management Facility

The RLITMF is the sampling site for this study. Previous studies have investigated the geochemistry of the site and while a study examining microbiology through culture-dependent techniques was conducted, no sequence-based characterizations were attempted in that study (Donahue et al., 2003; Donahue et al., 2000; Moldovan et al., 2003; Wolfaardt et al., 2008).

1.2.2.1 *History*

Tailings deposition at the RLITMF began in 1985 with material processed from the Rabbit Lake ore body (Donahue et al., 2000). The RLITMF is in a mined-out pit, with a pervious surround, composed of sand and crushed rock, that covers the bottom and sides of the pit (Landine, 2004). This allows for control of the hydraulic gradient (Donahue et al., 2000). Pumps allow excess water to drain and be collected; collected water can be reused in the milling or treated and released (Cameco Corporation, 2011). At the end of 2010, the RLITMF contained 7.4 million tonnes of tailings (dry weight; Government of Canada, 2011). The sources of the ore bodies include the Rabbit Lake ore body, the Collins Bay fault ore bodies composed of the B-zone (beginning in 1985), D-zone (1996), and A-zone (1997), and the Eagle Point ore body (Donahue et al., 2003). The latest processed ore layer is from the Eagle Point fault deposit, mined between 1993 and 2000 (Donahue et al., 2000; Pichler et al., 2001). The tailings stratigraphy is marked by frozen layers, at multiple depths in the boreholes, caused by tailings deposition and/or freezing during winter seasons (Donahue et al., 2000). Decreasing the permeability of tailings through consolidation limits the movement of soluble elements through the tailings; however, frozen layers within the tailings can interrupt consolidation by preventing drainage of excess water (Robertson et al., 1987).

1.2.2.2 *Geochemistry*

Arsenic geochemistry in the RLITMF has been characterized in multiple previous studies (Donahue et al., 2003; Donahue et al., 2000; Moldovan et al., 2003; Pichler et al., 2001).

Donahue *et al.* (2003) reported averages of tailings pH, Eh, and temperature in five monitoring wells. The pH averages of porewater samples from monitoring wells ranged from 9.3 to 10.3, with Eh values between +53 mV and +219 mV, and temperature ranged from -0.32 to 0.27°C based on pore water analyses (Donahue et al., 2003). Sulfate concentrations ranged between 1400 and 2000 mg/L, comparable to DTMF concentrations (Donahue et al., 2003). Arsenic concentrations in the tailings porewater were high, ranging from 11 to 67 mg/L (Donahue et al., 2003).

Donahue et al (2000) identified the minerals and constituents associated with arsenic using scanning electron microscopy (SEM) and analysis of images by electron backscatter imaging, energy dispersive spectrometry, wavelength dispersive spectrometry, and inductively coupled plasma atomic emission spectroscopy (ICP-AES) (Donahue et al., 2000). The results showed there was an association between arsenic and calcium, iron, nickel, and sulfur (Donahue et al., 2000). Iron-bearing minerals control arsenic mobility in the tailings, and this was also reported in the DTMF (Donahue et al., 2000; Essilfie-Dughan et al., 2012). The direct association between calcium and arsenic found in RLITMF tailings was not observed in DTMF tailings; instead, iron-arsenic coatings were found on gypsum in DTMF samples (Donahue et al., 2000; Essilfie-Dughan et al., 2012). The association of arsenic with calcium and iron in the RLITMF tailings appeared to be dependent on depth in the borehole; arsenic was primarily associated with iron in upper sections and calcium in the deeper sections of the borehole (Donahue et al., 2003). Ca-arsenates were both co-precipitated with gypsum and coated gypsum in the tailings (Donahue et al., 2000). This depth-dependent association was attributed to the different origins of the tailings (Donahue et al., 2003).

1.2.2.3 ***Microbiological Analyses of Site***

Microbiological analyses of the RLITMF were performed by Wolfaardt et al. (2008). Samples collected from one borehole from the RLITMF were examined for carbon metabolic profiles, lipid profiles, and quantification of culturable bacteria using Biolog GN plates (Biolog Inc., Hayward, California), Fatty Acid Methyl Ester (FAME) profiling, and culturing. They were unable to obtain FAME results (attributed to a lack of sufficient biomass). Semi-solid Postgate B medium culturing for SRB yielded too few colonies to determine colony forming units (CFUs)

for SRB in the tailings. Tryptic soy agar was used for heterotrophic CFU/g or CFU/mL calculations and they found more heterotrophic culturable bacteria near the water-tailings interface and at 0.2m deep ($1x10^5$ CFU/g) than in the water cover ($1x10^4$ CFU/g) associated with the tailings. This was similar to that of a pond upstream of the RLITMF where there was an order of magnitude higher CFU/g in the subsurface sediments than in the waters above. The CFU counts were higher near and at the water-tailings interface in comparison with the rest of the 66 m of tailings examined. No cells could be cultivated from samples at 21 and 25 meters deep. The remaining samples between 3 and 50 meters deep showed little variation in CFU counts. There was a difference in total carbon metabolic capabilities with depth based on the Biolog analyses. The authors could not identify specific individual physical or chemical factors that controlled CFU counts and metabolic differences. They theorized any differences were due to a combination of physical and chemical factors (Wolfaardt et al., 2008). Their study determined that metabolic diversity varied in the tailings while the quantity of culturable heterotrophic bacteria (generally speaking) did not vary. They were unable to quantify SRB populations or identify the members of the SRB present in the tailings (Wolfaardt et al., 2008). The authors recommended future studies use culture-independent techniques for identifying and quantifying SRB populations (Wolfaardt et al., 2008).

1.2.3 Key Lake Uranium Mine Tailings

The Deilmann Tailings Management Facility (DTMF) is another uranium tailings facility located in Northern Saskatchewan. The DTMF has been the subject of a high-throughput sequence-based microbial community study (Bondici et al., 2013). The results of the DTMF study are useful for comparing with the sequencing data from the present RLITMF study to determine if there are similarities between these two tailings environments.

1.2.3.1 *History*

The Deilmann Tailings Management Facility (DTMF) is located in the mined-out Deilmann mine pit at Key Lake in Northern Saskatchewan. Deposition of tailings into the DMTF began in 1996 (Essilfie-Dughan et al., 2012; Shaw et al., 2011). The DTMF has a water cover that is roughly 50 meters deep (Shaw et al., 2011). The acid leaching of uranium ore at the Key Lake mill generates waste containing molybdenum, iron, arsenic, and nickel. After milling the ore,

iron is added, and the waste is neutralized through a series of processes including multiple additions of lime to bring the final tailings pH to ca pH 10.5 (Essilfie-Dughan, Pickering, et al., 2011). The source of tailings changed from the Deilmann ore body to the McArthur River ore body in 1999 (Essilfie-Dughan, Pickering, et al., 2011). There is a chemical difference in the tailings dependent on the ore source. Tailings above 410 masl composed of McArthur River ore body waste had lower concentrations of arsenic, molybdenum, and selenium and was a higher grade uranium ore (Essilfie-Dughan et al., 2012; Essilfie-Dughan, Pickering, et al., 2011; Shaw et al., 2011). Deilmann ore body derived tailings (below 410 masl) had higher concentrations of arsenic, selenium, and molybdenum (Essilfie-Dughan et al., 2012; Shaw et al., 2011).

1.2.3.2 *Geochemistry*

Arsenic, molybdenum, and nickel are present in the DMTF tailings and pose a potential risk to the surrounding environment if released, so understanding their geochemistry is important. Ferrihydrite in the tailings is the primary control on the mobility of arsenic and nickel (Essilfie-Dughan, Hendry, et al., 2011; Essilfie-Dughan et al., 2012). Gypsum is also present in the waste and has been shown to sequester arsenic (Zhang et al., 2015). Iron, arsenic, and nickel are found in higher abundance in the DMTF tailings in their oxidized states Fe(III), As(V), and Ni(II) (Essilfie-Dughan et al., 2012). Micro-XRF analyses revealed that gypsum from the DMTF tailings has outer rings composed of iron associated with arsenic and/or nickel (Essilfie-Dughan et al., 2012). Arsenate (As(V)) and ferrihydrite (Fe(III)) were associated with one another in analyses of these outer rings using μ-XANES at the As and Fe K-edges (Essilfie-Dughan et al., 2012). The rings also contained As(III), but in lower quantities than As(V) (Essilfie-Dughan et al., 2012; Essilfie-Dughan, Pickering, et al., 2011; Shaw et al., 2011).

1.2.3.3 *Microbiological Analysis of Site*

Microbiological analyses of the water cover and tailings in the DTMF included culturing and molecular characterization (Bondici et al., 2014; Bondici et al., 2013). *Proteobacteria*, *Bacteroidetes*, *Actinobacteria*, and *Firmicutes* dominate the phyla composition of culture-based analyses and high-throughput amplicon sequencing of the overlying water cover (Bondici et al., 2014). Culture-dependent approaches to cell quantities showed little variation with depth and fewer culturable cells in the overlying waters than in the tailings (Bondici et al., 2014). High-

throughput amplicon sequencing of the 16S rRNA gene V5 region and clone libraries of *cpn60* identified the same four major phyla as the phyla in the water cover communities: *Proteobacteria*, *Bacteroidetes*, *Actinobacteria*, and *Firmicutes* (Bondici et al., 2013). There were lesser proportions of sequences that classified in phylum *Tenericutes*. Bacterial isolates in the same study corroborated some of these sequencing results. A large percent (48%) of isolates had an NaCl tolerance up to 7% and a tolerance for metal/metalloid salts (As(V), As(III), Ni(II), and Se(IV)). Some isolates were able to anaerobically reduce iron but were unable to reduce sulfate or arsenate, and they also could not oxidize arsenite. Some isolates were able to reduce molybdate and oxidize arsenite under aerobic conditions (Bondici et al., 2013). Analyses of the difference in community composition in the tailings between depths using Unifrac analyses found that the samples in the 0 to 20 m depths and 20 to 40 m depths were significantly different from each other. The 0 to 40 m samples were significantly different from the 40 to 60 m samples. Microbial diversity from high-throughput amplicon sequencing and *cpn60* analysis found the highest quantities of sulfate and iron-reducing bacteria sequences were in samples taken from below 40 m (Bondici et al., 2013).

1.3 Microbiology

1.3.1 General Environmental Microbiology

Microbial effects on environmental geochemistry are important in considering toxic element mobility in controlled waste management facilities. Microbes can affect the environment through pH changes, transforming elements through oxidation and reduction reactions, and concentrating and precipitating metals (Haferburg et al., 2007). The changes in the composition of the geochemical and physical environment can have long-lasting effects on the stability of the environment (Dong, 2010). Conversely, the environment can control the presence and activity of microorganisms. Microbes have specific growth requirements for pH, Eh, temperature, and other parameters (Dong, 2010). The RLITMF tailings are alkaline; thus, it is expected that pH will be a major limiting factor on microbial growth in the tailings (Donahue et al., 2003). The low temperatures of the tailings, near 0 ˚C, will also limit microbial growth (Donahue et al., 2003). Low temperature and high pH may limit activity or force microbes into dormancy (Sachidanandham et al., 2008).

Bacteria may also dissolve metals such as iron from minerals by excreting exopolysaccharides, amino acids, proteins, and organic acids that increase the solubility of minerals (Jain et al., 2004). Organic acids are important because of the diverse effects they may have in environments, including decreasing pH, complexing with metals, serving as electron donors for other microbes, and enhancing dissolution of some minerals (Jain et al., 2004).

The focus of this study is microbial influences over mobility and solubility of arsenic, minerals associated with arsenic, and the major factors influencing microbial communities in the RLITMF. The following sections discuss relevant bacterial groups and the potential implications for the RLITMF.

1.3.2 Alkaliphiles

Previous studies characterizing the RLITMF tailings reported averages of monitoring wells' pH values ranging from 9.3 to 10.3. This high pH can limit microbial activity in the tailings. There are microbes that can grow at high pH, known as alkaliphilic bacteria. Alkaliphilic bacteria grow optimally, or only, in environments with a pH greater than 9 (Preiss et al., 2015). Obligate alkaliphiles require a high pH environment to grow while facultative alkaliphiles can also grow at a lower pH (Preiss et al., 2015). Although the proteins in alkaliphiles are more adapted to function under alkaline conditions the bacteria must still maintain specific cytoplasmic pH that is lower than the surrounding environment (Preiss et al., 2015). The cytoplasmic pH can vary with species but studies of alkaliphilic *Bacillus* species demonstrated a pH 2 points lower in the cytoplasm (ca pH 8.3) than the surrounding environment (pH 10.8) (Preiss et al., 2015). The microbes accomplish this using cation pumps that transport ions such as Ca^{2+}, Na^{+}, or K^{+} out of the cell, and H^{+} ions into the cell (Preiss et al., 2015). In general, bacteria make use of proton gradients to generate energy in the form of ATP (adenosine triphosphate), this requires a higher proton concentration outside the cell i.e. lower pH (Maloney et al., 1974). ATP synthases transport protons into the cytoplasm and convert ADP (adenosine diphosphate) and P_i (inorganic phosphate) to ATP (Maloney et al., 1974). A higher pH outside of the cell poses a risk to the energy production of the cell (typically generated in bacteria via proton-coupled ATP synthases) (Preiss et al., 2015). In alkaliphiles, this is overcome through modified ATP synthases that use

sodium ions (Preiss et al., 2015). Proton-coupled ATP synthases are still found in some alkaliphilic bacteria (Preiss et al., 2015).

Alkaliphilic bacteria encompass a wide range of taxa, can use a number of different metabolic pathways to gain energy, and survive in a wide range of environmental conditions (Horikoshi, 1999).

1.3.3 Psychrophiles

Low temperatures (<15ºC) are a strong limitation on bacterial growth because they inhibit protein activity, alter cell membranes, and cause ice formation in the bacterial cell (D'Amico et al., 2006). The low temperatures of the RLITMF could then be an additional limiting factor of bacterial growth. Psychrophiles have adapted to survive under the stress of low temperature environments. Psychrophilic bacteria have been described as bacteria that can survive at temperatures lower than 15ºC (Morita, 1975). Psychrophiles can adapt to low temperature environments by producing anti-freeze proteins, cold-adapted proteins, and EPS (exopolysaccharide)-based cryoprotectants (D'Amico et al., 2006).

Psychrophilic microbes are broadly distributed across all taxa and are represented in many metabolic guilds (D'Amico et al., 2006).

1.3.4 Iron-Reducing Bacteria

The Fe(III)-bearing mineral 2-line ferrihydrite is the primary control of arsenic mobility in the RLITMF (Donahue et al., 2003; Donahue et al., 2000). Iron reduction from Fe(III) to Fe(II) could potentially release arsenic from ferrihydrite (Bissen et al., 2003; Donahue et al., 2000; Essilfie-Dughan et al., 2012). Dissimilatory iron-reducing bacteria (DIRB) use oxidized iron as a source of energy by coupling the Fe(III) reduction to the oxidation of H_2 and organic substrates (Fredrickson et al., 1996; Lonergan et al., 1996). Bacteria can reduce both crystalline and poorly crystalline iron-minerals (Glasauer et al., 2003). Iron-reducing activity occurs at or below Eh values of +100 mV (Falkowski et al., 2008). Bacteria capable of iron reduction are found in a range of environments (some can survive pH up to 8.5) (Garrity et al., 2005).

Shewanella spp. and *Geobacter* spp. are well characterized DIRB. *Shewanella* can use lactate as an electron donor in iron reduction resulting in the formation of acetate and Fe(II), see Equation 1 (Hansel et al., 2003; Kostka et al., 1995). Other electron donors that *Shewanella* can use for Fe(III) reduction include formate and H_2 (Kostka et al., 1999; Zegeye et al., 2010).

$$4Fe(OH)_{3(s)} + CH_3CHOHCOO^- + 7H^+ \rightarrow 4Fe^{2+} + CH_3COO^- + HCO_3^- + 10H_2O \quad \text{.....(1)}$$

Shewanella oneidensis can reduce iron by shuttling electrons from reduced compounds inside the cytoplasm to the outer membrane via an electron transport chain (Heidelberg et al., 2002). Iron reduction by *Shewanella oneidensis* is facilitated by numerous proteins, including cytochrome c enzymes (Heidelberg et al., 2002). *Geobacter sulfurreducens* can reduce iron using a similar mechanism (Methé et al., 2003).

Bacterial reduction of Fe(III) to Fe(II) in ferrihydrite may lead to abiotic formation of minerals composed of mixed Fe(III)-Fe(II) oxidation states, such as magnetite (Equation 2), or the transformation of ferrihydrite to goethite (Hansel et al., 2005; Kostka et al., 1995). Iron-reducing bacteria are able to continue to reduce the Fe(III) in minerals with mixed Fe(II) and (III) oxidation states (Kostka et al., 1995). This reduction reaction is coupled to the oxidation of carbon sources such as formate and lactate (Hansel et al., 2003).

$$2Fe(OH)_{3(s)} + Fe^{2+} \rightarrow Fe_3O_{4(s)} + 2H_2O + 2H^+ \text{.....(2)}$$

Transformation of lepidocrocite by *S. putrefaciens* to green rust and magnetite is dependent on cell aggregation density*;* more dense aggregates of bacteria leads to the formation of green rusts (Zegeye et al., 2010). Green rusts, magnetite, and other mixed Fe(II)/Fe(III) minerals are also formed via biological reduction of ferrihydrite by *S. putrefaciens* (Glasauer et al., 2003).

Many sulfate- and sulfur-reducing bacteria are can reduce iron. In the case of *Desulfuromonas* and *Desulfuromusa* species, the bacteria may couple oxidation of alcohols and organic acids to iron reduction (Vandieken et al., 2006). In culture experiments, *Desulfovibrio* was able to reduce iron independent of sulfate-reducing activities (Barton et al., 2015). This indicates that this genus has a separate mechanism for iron reduction independent of the sulfate reduction metabolic pathway or the metabolites resulting from sulfate reduction (Barton et al., 2015).

1.3.5 Sulfate- and Sulfur-Reducing Bacteria

The SRB are an important part of environmental sulfur cycling and can influence the chemistry of environments through their metabolism of sulfate and other sulfur compounds and organic acids. The SRB have diverse metabolic capabilities in addition to sulfate reduction; they can also reduce arsenate and iron (Barton et al., 2015; Braissant et al., 2007; Newman et al., 1997; Vandieken et al., 2006). The RLITMF contains high concentrations of sulfate (from 1400 to 2000 mg/L) and previous studies of arsenic in these tailings reported a co-occurrence between arsenic and gypsum (Donahue et al., 2003; Donahue et al., 2000). Sulfate reduction can occur around -200 mV and lower (Falkowski et al., 2008).

Arsenate can be incorporated into gypsum (Donahue et al., 2003; Zhang et al., 2015). Experimental work testing the incorporation and precipitation of As and gypsum at varying pH (3 to 10) found that with increasing pH more As was incorporated with gypsum (Zhang et al., 2015). Higher pH (7-10) resulted in separate calcium arsenates co-precipitating with gypsum (Zhang et al., 2015). The incorporation of As into gypsum minerals caused changes in gypsum structure and morphology that are detectable with XRD and in SEM images (Zhang et al., 2015). Reductive dissolution of gypsum by SRB may result in the release of arsenic (Ehlrich, 1996; Römer et al., 1965).

Bacteria can reduce sulfur in many forms including elemental sulfur, and thiosulfate; however, not all SRB can reduce all forms of sulfur (Barton et al., 2014; Frigaard et al., 2008; Kelly et al., 1994). The SRB have a conserved pathway for dissimilatory sulfate reduction; it is catalyzed by three enzymes, and generates H_2S (Muyzer et al., 2008). Two genes are often used to quantify SRB population size using qPCR, the genes *aps* and *dsr* (Muyzer et al., 2008). After sulfate is reduced to APS (adenosine 5'-phosphosulfate, Equation 3), the *aps* operon encodes the APS reductase which reduces APS to SO_3^{2-}, (Equation 4) (Muyzer et al., 2008). The *dsr* gene encodes the dissimilatory sulfite reductase enzyme which reduces SO_3^{2-} to H_2S, Equation 5 (Muyzer et al., 2008).

$$SO_4^{2-} + ATP \rightarrow APS + PP_i \quad (3)$$

$$APS + 2\ e^- \rightarrow SO_3^{2-} + AMP \quad (4)$$

$SO_3^{2-} + 6\ e^- \rightarrow H_2S$ ..... (5)

As mentioned in Section 1.6.2.1, SRB can transform iron and other metals; examples of SRB that can do this are *Desulfuromonas* and *Desulfosporosinus*. These include metals such as arsenic (As), chromium (Cr), iron (Fe), molybdenum (Mo), selenium (Se), and uranium (U) (Barton et al., 2015). For example, *Desulfuromonas* species have been found to respire arsenic, manganese, and iron in addition to elemental sulfur (Barton et al., 2015). The benefit of metal transformation to the microbial cells can be to detoxify, provide energy, or it may be incidental transformation caused by metabolic products. The metal transformation can occur by metal-specific enzymes or when metals are structural analogs of enzymatic targets. The production of ferrous iron or H_2S can react with the metals and catalyze reduction (Barton et al., 2015). Iron reduction by SRB can be done via a direct enzymatic mechanism that is similar to that of DIRB (Equation 1 and section 1.6.4). The SRB reduce sulfate to hydrogen sulfide, H_2S, which non-enzymatically reduces Fe(III) resulting in the dissolution of Fe(III)-bearing minerals (Equation 6, (Dos Santos Afonso et al., 1992; Lovley, 1991).

$2FeOOH(s) + H_2S + 4H^+ \rightarrow 2Fe(II) + S^0 + 4H_2O$ ..... (6)

Desulfosporosinus is a spore-forming bacterial genus that contains species capable of growing in pH 3.6 to 8.2 (Stackebrandt, 2014). This genus can grow at temperatures ranging from 4 to 40ºC. All species of *Desulfosporosinus* can reduce sulfate and some can reduce thiosulfate, sulfite, and iron (III) (Stackebrandt, 2014). Common electron donors for this genus include organic acids, sugars, and H_2 (Stackebrandt, 2014).

The *Desulfuromonas* genus membership has an optimal pH range of 6.5 to 8.5 and optimal growth temperatures between 21 and 40ºC (Garrity et al., 2005). All bacteria of *Desulfuromonas* can reduce sulfur and some can reduce iron (III) (Garrity et al., 2005). Electron donors for this genus include ethanol, organic acids, and H_2 (Garrity et al., 2005). *Desulfuromonas* benefit from growing with green sulfur bacteria, phototrophs capable of coupling H_2S oxidation to elemental sulfur reduction, which can then be used by *Desulfuromonas* as an electron acceptor (Garrity et al., 2005).

1.3.6 Arsenic-Reducing Bacteria

Aqueous arsenic is typically found in the form of the oxyanions arsenate (AsO_4^{3-}) and arsenite (AsO_3^{3-}). Bacterial reduction of As(V) generates the more mobile and toxic As(III) and may pose a risk to the surrounding environment (Langner et al., 2000). Arsenate has a greater affinity to sorb to mineral surfaces, and arsenite is more soluble (Oremland et al., 2003). Arsenite is considered more toxic because it can inhibit more enzyme activities; it can bind with a broader number of binding targets than arsenate, inhibiting the activity of the binding targets (Oremland et al., 2003).

Genes involved in arsenic reduction can be used to identify the presence and potentially quantify arsenic-reducing bacteria populations. Bacterial arsenic reduction is carried out by the proteins Arr and ArsC; these proteins govern arsenic reduction activity occurring on the exterior and interior of the cell, respectively (Gladysheva et al., 1994; Saltikov et al., 2003). The electron donors for bacterial reduction of arsenic can include organic acids, hydrocarbons, and sulfides (Gladysheva et al., 1994; Laverman et al., 1995; Song et al., 2009; Stolz & Oremland, 1999).

The *arrAB* operon encodes a transmembrane protein responsible for dissimilatory arsenate reduction that has been identified in multiple bacterial genera, including *Chrysiogenes*, *Bacillus*, *Citrobacter*, *Desulfomicrobium*, *Desulfosporosinus*, *Desulfuromonas*, *Shewanella*, *Sulfurihydrogenibium*, and *Sulfurospirillum* (Giloteaux et al., 2013; Stolz & Oremland, 1999).

Arsenate reduction in the cytoplasm occurs in a broad range of microbes via the *ars* gene. The *ars* gene has been found in bacterial chromosomes and on mobile plasmids that can move from microbe to microbe (Gladysheva et al., 1994). Interior reduction of arsenate by the *ars* operon is a mechanism to detoxify the cell of arsenate. In this mechanism, bacteria use the *ars* operon to aerobically reduce arsenate by the ArsC protein after uptake by phosphate channels. The resulting arsenite is pumped out of the cell via ArsAB proteins (Mukhopadhyay et al., 2002; Saltikov et al., 2005).

1.3.7 Heterotrophs

Heterotrophs are microbes that require organic carbon as their source of carbon for growth. Heterotrophic microbes include microbes from a wide range of phyla and include both aerobic

and anaerobic microbes. Heterotrophic microbes may also influence the environment through biosorption (Haferburg et al., 2007). While not exclusively the domain of heterotrophs, heterotroph cell surfaces can provide a location for metal accumulation and concentration. Biosorption is heavily influenced by structural features of the cell envelope (Haferburg et al., 2007).

Heterotrophic bacteria are also important to consider because of their potential hydrocarbon-degrading metabolism (Peng et al., 2008). Heterotrophic bacteria commonly use glucose to generate pyruvate for use in the tricarboxylic acid (TCA) cycle, a means of energy production in aerobic bacteria (Oren, 2010). Alternative enzymes and processes are required but hydrocarbons can be taken up by cells and eventually degraded until products can be used in the TCA cycle (Peng et al., 2008). This form of aerobic hydrocarbon degradation still requires O_2 and results in the release of CO_2 and other compounds from the TCA cycle (Peng et al., 2008).

1.4 Hypotheses and Objectives

1.4.1 Approach

The goal of this project was to identify the microbial communities of the RLITMF and prepare a conceptual biogeochemical model of the site using the microbiological data and recent geochemical data. This information will be provided to the RLITMF operators to broaden the available information on the tailings microbiology to consider in their facility management decisions. The microbial community identification was accomplished using high-throughput amplicon sequencing of genomic DNA extracted from tailings samples. Analysis of the sequence data was performed using the mothur bioinformatics software package (Schloss et al., 2009). Samples were taken at regular vertical intervals from two boreholes for comparison, and DNA was extracted and sequenced in triplicate from each sample. We identified proportions of relatives of known metal-reducing and sulfate-reducing bacteria in the sequencing data. Based on these analyses, I selectively enriched for SRB from samples with higher SRB sequence read proportions. Synchrotron tools were used for a parallel MSc project by Kendall Krepps in Dr. Derek Peak's lab (Soil Sciences, University of Saskatchewan) to characterize the geochemistry of sulfur and arsenic in the samples from the tailings facility. I compared the microbiological sequencing data and geochemical data (provided by Cameco) to look for relationships between

the geochemistry and the microbial communities in the tailings and consider the potential of microbes to influence the geochemistry in future.

1.4.2 Objectives

1. Characterize microbial communities within the tailings.
2. Enrich and isolate SRB identified in the tailings sequencing data.
3. Construct a conceptual model of the tailings environment and the potential effect of microbes on arsenic mobility, under current or changing conditions.

1.4.3 Hypotheses

I hypothesized that:

(1) There would be no significant difference in community diversity and membership between samples of genomic DNA extracted from tailings on-site and in the lab back at the University of Saskatchewan,

(2) The tailings would contain populations of microorganisms that classified as taxa known to reduce sulfate, iron, and arsenate,

(3) There would be a correlation between microbial community structure and geochemical and physical parameters in the tailings, and

(4) Sulfate-reducing bacteria in the tailings are viable.

1.5 Organization of Thesis

This thesis is organized in chapter format. Chapters 1 through 4 relate to the primary thesis project, characterizing the microbial communities of the RLITMF. Appendix C is a project conducting qPCR on samples from an oil sands centrifuged fine tailings facility; this work supplemented an MSc project by Kaitlyn Scott Heaton (Heaton, 2016).

CHAPTER 2 METHODS

2.1 Sampling

In the summer of 2014, Dr. Joyce McBeth collected samples during borehole drilling by Cameco employees at the RLITMF. Samples were taken from two boreholes, RLP-14-14 and RLP-14-17 (Figure 2-1). Samples from a range of depths in each borehole were analyzed, allowing for a comparison of data between the two locations in the tailings. Tailings were brought up in three meter sections, and the core was logged for geotechnical and physical properties. Samples were taken from the interior of the core using push cores constructed from cut-off 50 ml centrifuge tubes, and the push cores were sealed with a rubber stopper and electrical tape. Dr. Joyce McBeth extracted DNA (see procedure, section 2.5.1.1), from a number of these tailings samples in a laboratory located at the facility. These samples are referred to as "site-extracted samples" in this thesis. The push cores were sent to the University of Saskatchewan for DNA extraction and synchrotron analyses at the Canadian Light Source, Saskatoon. The synchrotron data analyses were conducted by Kendall Krepps, an MSc student in Soil Sciences under Dr. Derek Peak's supervision (methods and data from those analyses will be presented in his thesis). On-site information was collected by Cameco employees during drilling and included temperature, pH, Eh, and geotechnical characteristics of the core. Porewater and solid samples were taken by Cameco employees and sent for analyses at the Saskatchewan Research Council (SRC) using ICP-MS. Relevant ICP-MS and geotechnical and physical data was provided to us by Cameco.

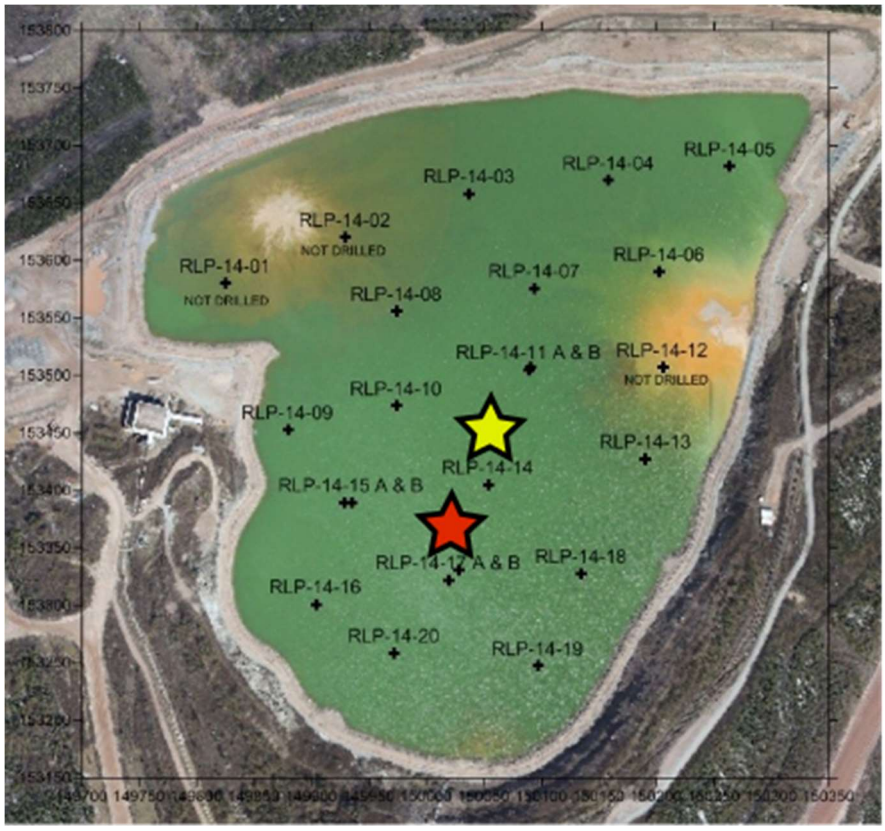

Figure 2-1. RLITMF tailings pond aerial view from 2014. The yellow and red stars highlight the boreholes that were sampled for this project, RLP-14-14 and RLP-14-17 respectively.

2.2 Radioactive sample handling

Samples from the RLITMF are radioactive and were handled and stored in accordance with approved radioactive sample handling procedures (McBeth, 2015), and all team members who handled these samples received Radiation Safety Training from the University of Saskatchewan Safety Resources Radiation Safety Officer. Samples were stored in a labelled and locked freezer/fridge in room S411, Spinks Building. Samples were identified by clear labels, in tubes closed with rubber stoppers and electrical tape, and placed in either zip-locked or sealed bags. All sample preparation was done in room 317, Geology Building (Dr. Hendry's aqueous geochemistry lab). Radon gas was vented from samples in a fume hood before any sample preparation. Work spaces were surveyed before and after sample handling using a handheld radiation monitoring device, and recorded as counts per second (cps). After sample handling, samples were closed by replacing the rubber stopper and wrapping electrical tape around the

stopper and tube. Waste from sample preparation was placed in designated radioactive waste bags and arrangements were made for disposal with USask Safety Resources.

2.3 Computing Methods

All programs used for data analyses were run using the Windows 7 (Microsoft) operating system. Microsoft Excel (2013) was used for calculations of medians, R^2, and charting data in tables. OriginPro 2017 was used for graphing and data was transferred from Excel when necessary. Graph editing and figure creation were done in Inkscape (https://inkscape.org/).

2.4 Geochemistry

Porewater and solids samples were collected by Cameco employees; porewater was pressed from tailings samples and filtered, and porewater samples for metals analyses were preserved via addition of nitric acid. Geochemical analyses of tailings pore water and solids were performed by the SRC Environmental Analytical Group (Saskatoon, SK). The SRC Environmental Analytical Group is an ISO 17025 accredited laboratory. Solid samples were digested, and both solids and porewaters were analysed to determine metal and metalloid concentrations. Other analyses included concentrations of radionuclides, major ions, nitrate, and organic and inorganic carbon. Relevant data results were provided to us by Cameco Corporation.

2.5 Microbiological Methods

2.5.1 Culture Independent Methods

2.5.1.1 *DNA Extraction and Quantification*

Genomic DNA was extracted from the samples, in triplicate, using the FastDNA® SPIN Kit for Soil (MP Biomedicals, Santa Ana, CA) with some modifications. The alterations to the standard protocol include: we used a Vortex Genie 2 for the homogenization step (5 minutes on max speed) and after adding the DNA Binding Matrix the samples were placed on the Vortex Genie 2 set to level 1 (low) for 5 minutes. These samples extracted in the lab at USask are referred to as "lab-extracted samples" in the results section.

I analyzed DNA for quantity and quality using a Qubit 2.0 Fluorometer (Invitrogen, Life Technologies, CA, USA) and an Epoch Microplate Spectrometer with Take3 plate (BioTek), respectively. Qubit quantitation uses fluorescent nucleic acid dyes that intercalate into double stranded DNA (dsDNA). I used the Qubit HS dsDNA Assay Kit (Life Technologies, CA, USA) and performed the Qubit 2.0 Fluorometer analyses according to the instructions provided by Invitrogen. The DNA samples were quantified using 5 µL of sample, but iterations of 10 and 20 µL were used if 5 µL was insufficient to detect DNA. The detection limits using Qubit and the HS reagent kit have a lower limit of 0.50 ng/mL and an upper limit of 500 ng/mL of sample in the assay tube (Life Technologies, CA, USA). The amount of DNA in the stock tube is then calculated from the volume used in the assay tube for standard analyses, and the results are used to generate a standard curve. The Epoch Spectrometer uses UV-absorbance to detect nucleic acid quality. For DNA, the ratios of 260 nm to 280 nm and 260 nm to 230 nm absorbance were used to analyze DNA quality. DNA absorbs light at 260 nm. The 260/280 ratio is used to judge the purity of the nucleic acids DNA and RNA. High purity nucleic acids will have a 260/280 ratio in the range of 1.8 to 2.0 (Thermo Scientific, 2013). Proteins and phenols absorb at 280 nm and lower the 260/280 ratio (Thermo Scientific, 2013). The 260/230 ratio can also be used to assess purity and has an expected range of 2.0-2.2 (Thermo Scientific, 2013). This ratio can be lowered by contaminants such as phenols, carbohydrates, guanidines, and glycogen (Thermo Scientific, 2013). The Epoch Microplate Spectrometer was used as instructed by the manufacturer.

2.5.1.2 *High-Throughput Amplicon DNA Sequencing*

After DNA was extracted from samples, aliquots of 60 µL were sent for high-throughput amplicon sequencing at the Research and Testing Laboratory (Lubbock, TX). Illumina MiSeq sequencing is a technique which sequences thousands of short gene fragments in the same sample in parallel (Illumina, 2010). In MiSeq sequencing, polymerase chain reaction (PCR) is used to amplify regions of genomic DNA using primers (short oligonucleotides required for DNA synthesis). PCR is also used to add adapters to the gene fragments. These adapters are composed of indices and attachment sites on either side of the regions of interest that the primers amplify. These fragments are introduced to flow cells that include adapters that are complementary to the fragment adapters, bound to the flow cell surface. After fragments bind to flow cell adapters, amplification generates DNA fragments bound to the flow cell adaptors. A

washing step removes the DNA fragments that are not anchored and leaves the DNA fragments that are anchored bound to the flow cell. Free ends of bound fragments bind to nearby complementary flow cell adapters, forming "bridge" structures. Further amplification generates many copies of the forward and reverse fragments, bound to the flow cell. The next series of steps is where the sequences reads are generated. The reverse fragments are cleaved and washed away from the flow cell. Fragments are then amplified from the end not bound to the flow cell. The four dNTPs (nucleoside triphosphate) used in this amplification step are fluorescently labelled and included during the extension phase. The four fluorescently labelled dNTPs are allowed to compete for incorporation in the growing strand. Light is used to excite the fluorescent labels after each dNTP is incorporated. The MiSeq detection system determines the dNTP that has been added based on the wavelength and intensity of fluorescence; this process is known as sequencing by synthesis. After the fragment is sequenced, the product is removed and another primer is added to sequence the first index, this is then removed. After another bridging event, the second index read is sequenced and removed. The reverse strand is then generated and the forward strand is cleaved and washed away. The sequencing of the reverse strand proceeds by the same process as for the forward strand (Illumina, 2010).

I compensated for low DNA concentration yields from extractions by sending 60 µL for each replicate (samples sent in triplicate). The universal primers 515F and 806R (Table 2-1) were used to target the V4 region of the 16S rRNA gene and sequence the archaea and bacteria in each sample community (Caporaso et al., 2011). I requested 10,000 reads per sample for the lab-extracted samples and 20,000 reads per sample for site-extracted samples to examine the consistency of sequence reads generate from the sequencing facility. Samples 1403, 1404, 1405, and 1406 were used for comparisons between samples extracted on-site and lab-extracted samples. These samples were chosen because lab-extracted DNA had quantifiable DNA in these samples, so there was a higher likelihood the samples would amplify successfully. The comparison of lab and site-extracted DNA was used to examine differences in microbial communities after transport from the site.

High-throughput sequencing results were deposited in the European Nucleotide Archive (ENA) under study accession number PRJEB20465 (sample accession numbers ERS1670082 to ERS1670129) (Appendix A, Table A-1, Table A-2).

Table 2-1. Primers for RLITMF molecular microbiology methods

Target gene	Method	Forward Primer	Reverse Primer	Reference
16S rRNA gene V4 region (for archaea and bacteria)	Illumina MiSeq	515F-5'-GTGCCAGCMGCCGCGGTAA-3'	806R-5'-GGACTACHVGGGTWTCTAAT-3'	(Caporaso et al., 2011)
16S rRNA gene (for archaea and bacteria	PCR	27F-5'-AGAGTTTGATCMTGGCTCAG-3'	1492R-5'-GGWTACCTTGTTACGACTT-3'	(Giovannoni, 1991; Lane, 1991; Suzuki et al., 2000)
16S rRNA gene fragment (for archaea and bacteria)	PCR	519F 5'-CAGCMGCCGCGGTAATWC- 3'	907R 5'-CCGTCAATTCCTTTRAGTTT-3'	(Lane, 1991)
dsrA (SRB)	PCR	DSR1F-5'-ACSCACTGGAAGCACG-3'	RH3-dsrR-5'-GGTGGAGCCGTGCATGTT-3'	(Ben-Dov et al., 2007)
arr (dissimilatory arsenate-reducing bacteria)	PCR	ArrA-D1F-5'-CCGCTACTACACCGAGGGCWWYTGGGRNTA-3'	ArrA-G2R-5'-CGTGCGGTCCTTGAGCTCNWDRTTCCACC-3'	(Kulp et al., 2006)

2.5.1.3 *Polymerase Chain Reaction (PCR)*

I used PCR to amplify DNA for Sanger sequencing, and to amplify genes of interest to determine if they are present using gel electrophoresis imaging. The PCR generates many copies of a particular DNA sequence. In PCR, a sample of DNA is added to a reaction mixture including primers for a specific gene target and a master mix (this contains dNTPs and a thermostable Taq

polymerase, the enzyme that catalyzes the DNA synthesis reaction). Reactions are performed in thermocyclers, machines that function by cycling between set temperatures. These temperatures are intended to cycle through steps that sequentially denature the dsDNA (ca 95˚C), anneal the primers to the strands of DNA (ca 55˚C), and extend from the primers along the DNA to create new dsDNA (ca 72˚C) (protocols provided in Appendix D). The PCR reactions were run using EconoTaq PLUS (Lucigen Corporation, Middleton WI) and DreamTaq PCR (Thermo Scientific, Waltham, MA, USA) master mixes on a PTC-200 Peltier Thermal Cycler (MJ Research). Primers used for PCR are listed in Table 2-1. Quality and quantity of the PCR products were assessed using the Qubit fluorometer and Take3 plate (section 2.5.1.1).

I performed gel electrophoresis using the Lonza FlashGel System (Lonza Rockland Inc, Rockland, ME) and FlashGel DNA cassettes, 1.2% agarose (Cat. No. 57023). Gel images were taken using the FlashGel camera. Gels were prepared and run according to the instructions provided by the manufacturer.

2.5.1.4 *Sanger Sequencing*

I amplified the 16S rRNA gene from DNA extractions of cultures using the 27F and 1492R primers (Table 2-1). The amplified DNA was sent to The McGill University and Genome Quebec Innovation Centre for Sanger sequencing using the sequencing primers 27F, 519F, 907R, and 1492R (Table 2-1). Sanger sequencing is a form of DNA sequencing that identifies the final base pair of DNA fragments of differing size. The fragments are generated from interruptions in the extension phase of amplification. Amplification by PCR is used to extend DNA strands from primers selected for the reaction. The extension phase is halted when fluorescently-labelled ddNTPs (dideoxynucleotide triphosphates) are randomly incorporated into the growing DNA strand. This incorporation stops the extending of the strand because dNTPs cannot be added after ddNTPs. The ddNTPs lack the 3’-hydroxyl group that is essential to the phosphate backbone of DNA strands. The random incorporation of ddNTPs in a PCR reaction produces different length fragment lengths and these fragments can then be separated by capillary electrophoresis. The capillary electrophoresis technique is sensitive to 1 bp differences in size. A laser excites the different fluorescent signals which are detected and identify each of the four NTPs as the DNA fragments pass through the capillary. A computer can then organize the fluorescent signals and

intensities into base calls by fragment length, which corresponds to position along the amplicon (Wiley, 2017).

Sequencher software (Gene Codes Corporation) and Geneious (Biomatters Limited) were used to generate contigs (sequences assembled from the reads) and to manually curate the data generated from Sanger sequencing results (e.g. checking ambiguous base calls). The final resulting sequences were classified using the NCBI-BLAST search of the Nucleotide collection database (Cole et al., 2013; Madden, 2013).

2.5.1.5 ***Bioinformatics***

The Illumina MiSeq sequencing data received from Research and Testing Laboratories (Lubbock, TX) was analyzed using the bioinformatics software package mothur (Schloss et al., 2009). I tested and optimized the pipeline using sequence data from other projects in the McBeth laboratory group (detailed pipeline provided in appendix B). Briefly, the mothur pipeline contained the following steps: contigs were formed from the paired MiSeq sequencing reads (deltaq=6, meaning the bases selected from the raw reads to form each contig are chosen by taking the base with the higher quality score, providing the difference between the quality scores is >=6); sequences were removed if they containing 8 or more homopolymers, ambiguous base pairs (bp), or sequences longer than 300bp; sequences were aligned against the V4 region of the Silva 16S rRNA gene Database and overhanging base pairs were removed; reads were pre-clustered; and chimeras were identified using UCHIME and removed by mothur (Edgar et al., 2011). I assigned taxonomy to sequences with an 80% confidence sequence classification cutoff against the Ribosome Database Project (RDP) Classifier v9 and the Silva v119 database in order to compare results from the two classifiers. Results presented in this thesis are from the Silva classifications. The Silva v119 database contains a larger number of sequences that classify unique taxa and include taxa not included in the RDP version 9 Classifier, for example the phylum *Tenericutes*.. Total sequences for each sample were subsampled to 7000 reads prior to any sequence-based beta community analyses. I used mothur to cluster sequences at 97% sequence similarity into Operational Taxonomic Units (OTUs); this similarity cutoff is ca equivalent to species-level sequence distinction. For comparison of community structures, theta Yue-Clayton calculations were used and Jaccard calculations were used for assessing community

membership (Chao et al., 2005; Yue et al., 2005). For calculations of diversity, theta Yue-Clayton values were deemed more appropriate to this study instead of Jaccard, because theta Yue-Clayton calculations of similarity incorporate the proportion of both shared and not shared species. Jaccard calculations only compare the shared species proportions and number of species between samples (Yue et al., 2005). In other words, with theta Yue-Clayton if one community has a very different quantity of unique species in comparison with another community this would be calculated as less similar. With a Jaccard calculation, these differences would not be considered. Analyses of richness and diversity were also carried out in mothur. Richness was investigated using species-observed values (number of OTUs) which provides an operationally-defined number of different species in each sample. The Inverse-Simpson index was used for diversity comparisons; here, the number of species and the number of sequences per species are combined in a calculation that allows for comparison against other samples. Good's coverage calculation was used to determine the extent of sampling coverage (Good, 1953). A high coverage index (e.g. 99%) indicates the sequencing has achieved high sampling coverage.

I conducted statistical analyses of sample diversity in mothur using NMDS, PCoA ordination plotting and Analysis of Molecular Variance (AMOVA). AMOVA provides the statistical significance of samples or sample sets' dissimilarity (Martin, 2002). Ordination plotting provides a sample community composition comparison with more similar samples being plotted closer to one another on a graph than dissimilar samples (Borg, 2005).

2.5.2 Culture-Dependent Methods

I used culturing techniques to test for viability of SRB in tailings samples. This required careful handling to prevent contaminating culture attempts. All culturing experiments were performed using standard aseptic technique. Specifically, I handled samples, bottles, syringes, and needles wearing proper personal protective equipment. I cleaned workbenches with 70% ethanol before and after working at the station. I inoculated bottles for enrichment using sterile needles and syringes and disposed of them via secured needle disposal and autoclave waste, respectively.

Samples that had sequences that classified as known sulfate reducers were selected for SRB enrichment studies. The SRB were enriched in Postgate's medium B (Postgate, 1984). Postgate's medium B is adjusted to a pH between 7 and 7.5 before degassing using N_2:CO_2 gas, aliquoting

into bottles, and autoclaved. Negative controls for the enrichment cultures were bottles of Postgate's medium B that were not inoculated, and these were stored in the same conditions as the inoculated bottles. Inoculated Postgate's medium B bottles were incubated at room temperature (about 15-20 ˚C) and in a dark place, e.g. cupboard or drawer. Inoculated bottles were considered positive for SRB growth if the media precipitate colour changed from grey to black. I attempted to isolate SRB using the dilution to extinction method (Widdel et al., 1992). Briefly, bottles deemed positive for SRB growth had a 1 mL aliquot removed to inoculate a 10 mL bottle, a further 1 mL was taken from the 10 mL inoculated bottle to inoculate another 10 mL bottle, and this was repeated a total of seven times (Figure 2-2). Subsequent dilution series consisted of 5 dilutions to save resources. Microbial cultures and isolates were observed microscopically to confirm the presence of cells. If cells were observed, I identified the cell morphology(s) and motility and took photos using a light microscope (Nikon).

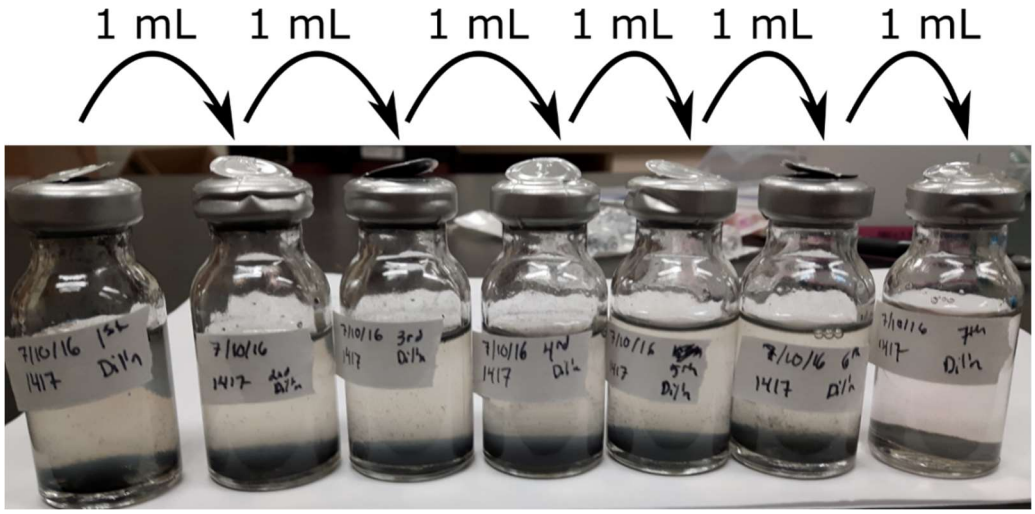

Figure 2-2 A culturing dilution to extinction series used in this study. Inoculum (1 mL) was taken from an enrichment bottle and inoculated into the 1st dilution bottle. The first dilution bottle was inverted to mix and 1 mL was taken from the 1st bottle and inoculated into the 2nd dilution bottle using a fresh needle and syringe. This process was repeated for a total of 7 dilutions.

Quantification of sulfide in enrichment bottles was used to further demonstrate the sulfate-reducing activity of the microbes cultured. The sulfide concentration in culture bottles was quantified using the methylene blue colourimetric assay (Cline, 1969) on a DR 2800 Spectrophotometer (Hach) in Dr. Matt Lindsay's lab, Spinks Building S411, on bottles deemed positive for SRB growth. Briefly, 20 mL of Postgate's B is taken and filtered from the bottle to

remove precipitates, the resulting liquid is mixed with N,N-dimethyl-p-phenylenediamine sulfate. This reagent forms methylene blue with the blue intensity corresponding to the quantity of soluble sulfide. The sample is measured at 665 nm on a spectrophotometer. This method does not include the measure of sulfide in the precipitates because the precipitates were filtered out before any reagents were added. The methylene blue method of quantification on the DR 2800 Spectrophotometer (Hach) has a detection limits of 5 (minimum) and 800 (maximum) µg/L. The Spectrophotometer was blanked using a water sample (prepared identically to the samples).

2.6 Conceptual Model

I prepared a conceptual model of the site incorporating microbes identified by my microbiological analyses, chemical and physical analyses of the tailings, and minerology of the environment based on previous literature. I used previous literature on cultured relatives of the bacteria identified by sequencing to establish the potential microbial processes of interest that may be occurring under the tailings conditions. This model will provide an organized summary of this work.

CHAPTER 3 RESULTS

3.1 Geochemical and Physical Characteristics

I constructed stratigraphic columns based on the borehole logs provided by Cameco (Appendix A, Figure A-1). The depth of the tailings varied by borehole, RL-14-14 was 98.7 m and RL-14-17 was 119 m deep. The borehole depths include the water cover of 3.8 m and 3.5 m deep, in boreholes RL-14-14 and RL-14-17, respectively. Borehole logs describe the presence of ice and frozen tailings layers throughout the depth of both boreholes. Temperature data was available only for borehole RL-14-14 and ranges from -0.1°C to +11.53°C with a median of +2.43°C (Table 3-1).

Table 3-1 Temperature, pH and reduction-oxidation Potential (Eh) by borehole and depth.

	RL-14-14				RL-14-17		
	masl*	pH	Eh (mV)	Temperature °C	masl*	pH	Eh (mV)
Region A	417.0	8.24	258.1	11.53	417.0	8.20	217.8
	414.1	8.56	254.4	5.97	414.2	8.67	196.6
	411.1	8.70	256.1	1.57	411.1	8.59	208.1
	407.9	8.93	245.1	-0.10	408.1	8.81	207.3
	404.6	8.69	242.1	1.93	405.1	8.47	216.3
	-	-	-	-	402.3	8.62	212.8
	399.1	8.37	250.6	8.47	399.3	8.60	233.4
Region B	392.9	9.12	210.3	1.43	393.2	8.29	252.2
	386.6	9.30	204.4	0.90	387.2	8.07	238.7
	381.1	8.54	223.7	1.27	381.1	10.05	174.3
	372.0	9.85	260.8	9.87	372.3	8.66	208.3
	363.1	10.06	178.3	9.93	363.2	9.86	164.7
	354.1	9.46	218.1	-0.10	354.3	9.71	170.7
	341.6	9.42	200.4	2.93	342.3	9.38	192.5
	330.1	10.80	197.9	8.53	330.3	8.96	171.7
	-	-	-	-	318.2	10.44	178.0
	-	-	-	-	306.1	9.34	197.1

*masl = meters above sea level

Table 3-1 shows the pH and Eh by depth in the tailings for boreholes RL-14-14 and RL-14-17. In 2000, the pH target for the tailings being deposited into the RLITMF changed from 10.0 to 8.5 (Moldovan et al., 2003). The change in discharge pH can be observed in the median values if samples are clustered into two zones. The borehole RL-14-14 median pH was 8.63 above 399 masl (Region A) and 9.44 below (Region B). The borehole 14-17 median pH was 8.59 above 387 masl and 9.55 below. These depths are likely the location of the discharge pH change. In this thesis, we have used these depths as major dividers in the tailings for comparing other geochemical data and the sequencing results.

The reduction-oxidation potentials (Eh) in the tailings samples were consistent for both boreholes (Table 3-1). It is important to note that as borehole samples are brought to the surface for sampling they may be exposed to air prior to or during Eh measurements and this may influence the results, particularly for samples with low Eh values. Tailings Eh was lower in samples below the position where the discharge pH changed, the lowest measured Eh value was +164.7 mV. The concentrations of arsenic, iron, molybdenum, sulfate, and sulfides are compared as median values in both boreholes in Region A and Region B (Table 3-2).

Table 3-2 A summary of various element concentrations in solids and porewater from the boreholes. Table includes the minimum, maximum, and medians (throughout the borehole, above, and below the pH change).

		RL-14-14		RL-14-17	
		Solids (µg/g)	Porewater (mg/L)	Solids (µg/g)	Porewater (mg/L)
Iron	Min	9400	0.004	8200	0.0028
	Max	30300	0.078	26900	0.11
Median	Overall	18100	0.0425	11400	0.062
	Region A	11700	0.0445	11000	0.057
	Region B	23850	0.0405	12950	0.075
		(µg/g)	(mg/L)	(µg/g)	(mg/L)
Arsenic	Min	82	0.17	43	0.28
	Max	7900	42.7	5200	54.90
Median	Overall	1700	6.03	363	2.54
	Region A	147	0.73	302	1.22
	Region B	4200	9.45	3185	10.395
		(µg/g)	(mg/L)	(µg/g)	(mg/L)
Molybdenum	Min	50	7.68	28	7
	Max	107	36.7	126	44.9
Median	Overall	68	19.05	70	16.9
	Region A	79	16.6	68	15.9
	Region B	63	26.85	79	24.15
		(%)	(mg/L)	(%)	(mg/L)
Sulfate	Min	1.7	1610	3.8	1480
	Max	9.2	3800	9.2	2640
Median	Overall	5.8	2050	4.8	2170
	Region A	3.9	2325	4.8	2230
	Region B	8.35	1830	6.5	1965
		(µg/g)		(µg/g)	
Sulfide	Min	700	n/a	400	n/a
	Max	2500	n/a	5800	n/a
Median	Overall	1700	n/a	1550	n/a
	Region A	1200	n/a	1600	n/a
	Region B	2100	n/a	800	n/a

n/a = No data available

The median iron concentrations in solids in RL-14-14 were higher in Region B (tailings below the pH change) (Table 3-2, Figure 3-1). In contrast, median porewater iron concentrations generally consistent between samples above and below the pH change (Table 3-2). Borehole RL-14-17 had little variation in median concentrations for iron in porewater and solids between the samples above and below the pH change.

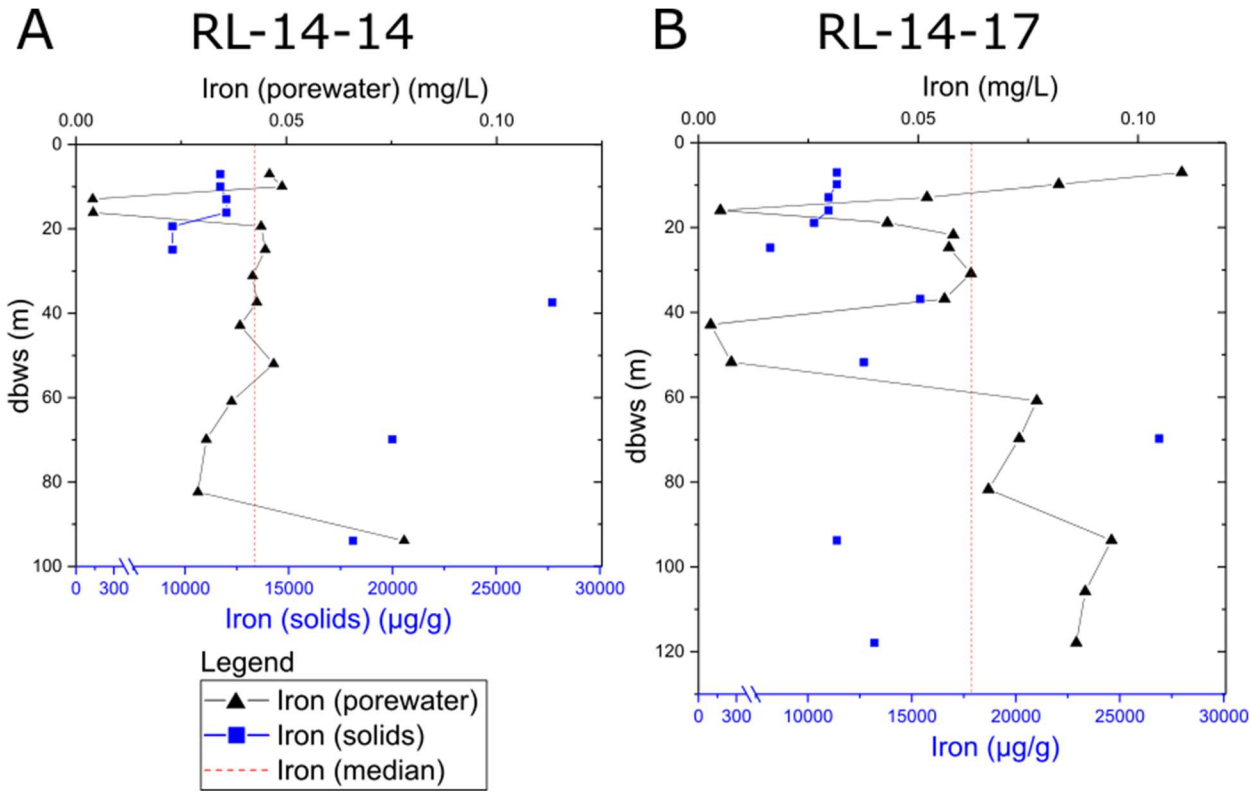

Figure 3-1 Profile of iron concentrations in the tailings based on ICP-MS analyses. Boreholes (A) RL-14-14 and (B) RL-14-17. The concentration of iron in porewater is shown with black triangles; and the concentration of iron in solids is shown with blue squares. The red dotted vertical line in both is the median concentration of iron in porewater.

The median arsenic concentrations in porewater from borehole RL-14-14 samples were much greater in Region B (ca 12x) than in Region A above the pH change (Table 3-2, Figure 3-2). The pattern was similar in the solids analyses; there was a greater median concentration in Region B samples (ca 28x). This pattern was also observed in borehole RL-14-17 data. Median porewater arsenic concentrations were greater in Region B (ca 8x) than above the pH change. The median arsenic concentrations in solids were greater in Region B (ca 10x) than above the pH change.

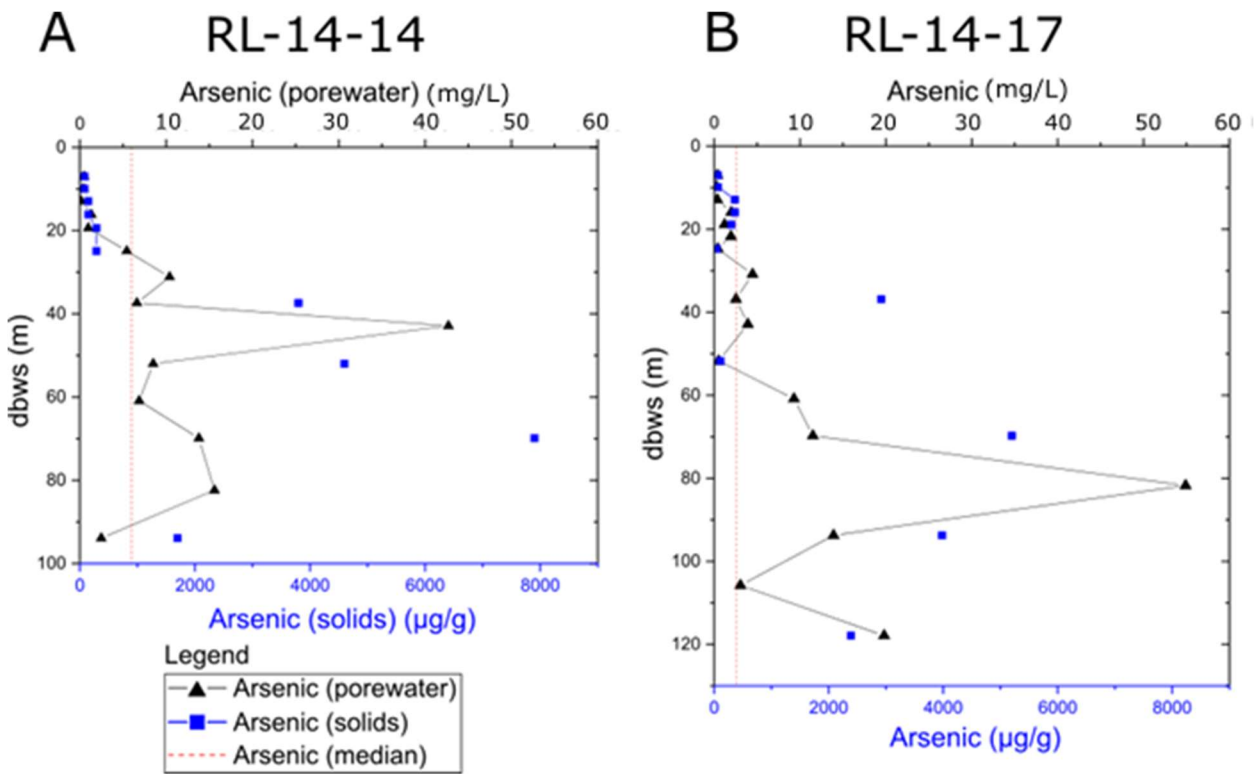

Figure 3-2 Profile of arsenic concentrations in the tailings based on ICP-MS analyses. Boreholes (A) RL-14-14 and (B) RL-14-17. The concentration of arsenic in porewater is shown with black triangles; and the concentration of arsenic in solids is shown with blue squares. The red dotted vertical line in both is the median concentration in porewater.

The concentrations of molybdenum in the tailings was generally greater in the Region B samples than in Region A above the pH change; there were only minor variations between the median concentrations of Mo in boreholes RL14-14 and RL-14-17 (Table 3-2, Figure 3-3).

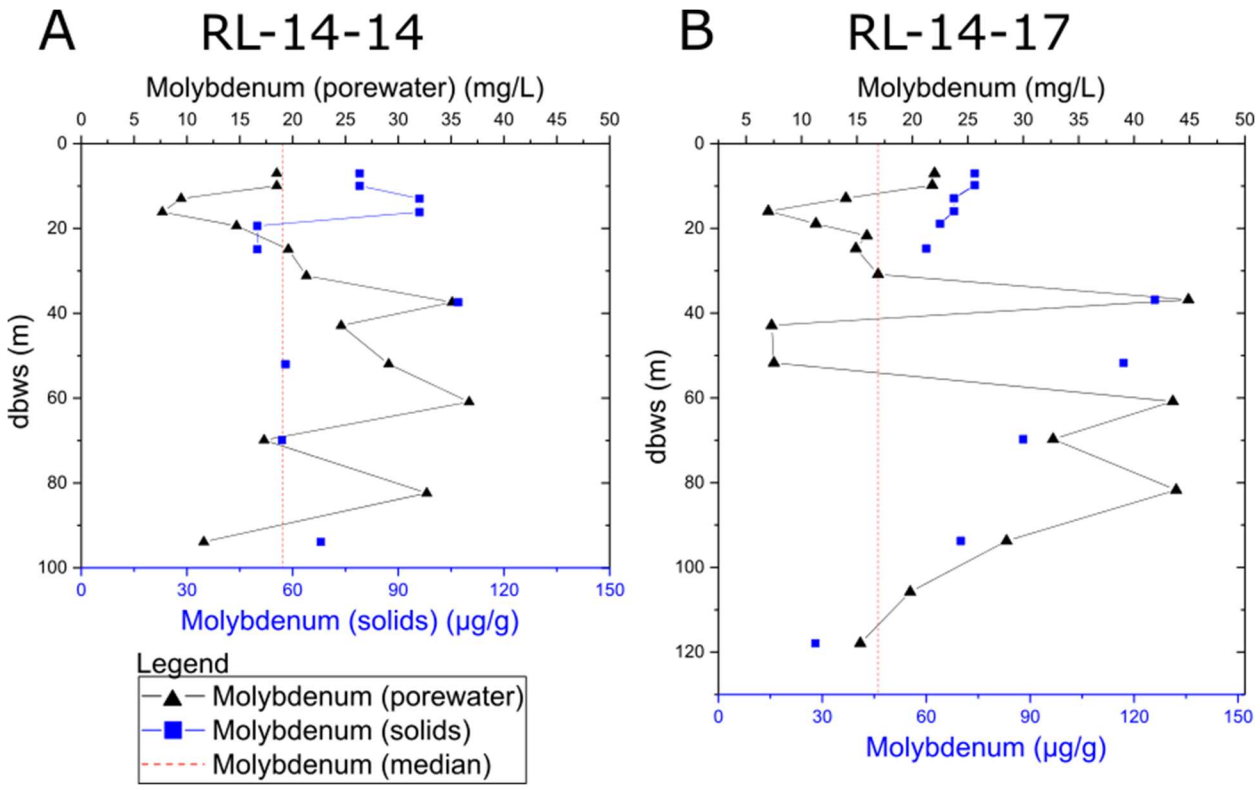

Figure 3-3 Profile of molybdenum concentrations in the tailings based on ICP-MS analyses. Boreholes (A) RL-14-14 and (B) RL-14-17. The concentration of molybdenum in porewater is shown with black triangles; and the concentration of molybdenum in solids is shown with blue squares. The red dotted vertical line in both is the median concentration in porewater.

The sulfate concentrations in the tailings were similar for boreholes RL-14-14 and RL-14-17 (Table 3-2, Figure 3-4). Both boreholes followed a similar pattern of greater sulfate concentrations in samples in Region B (below the pH change) for porewater and solids concentrations.

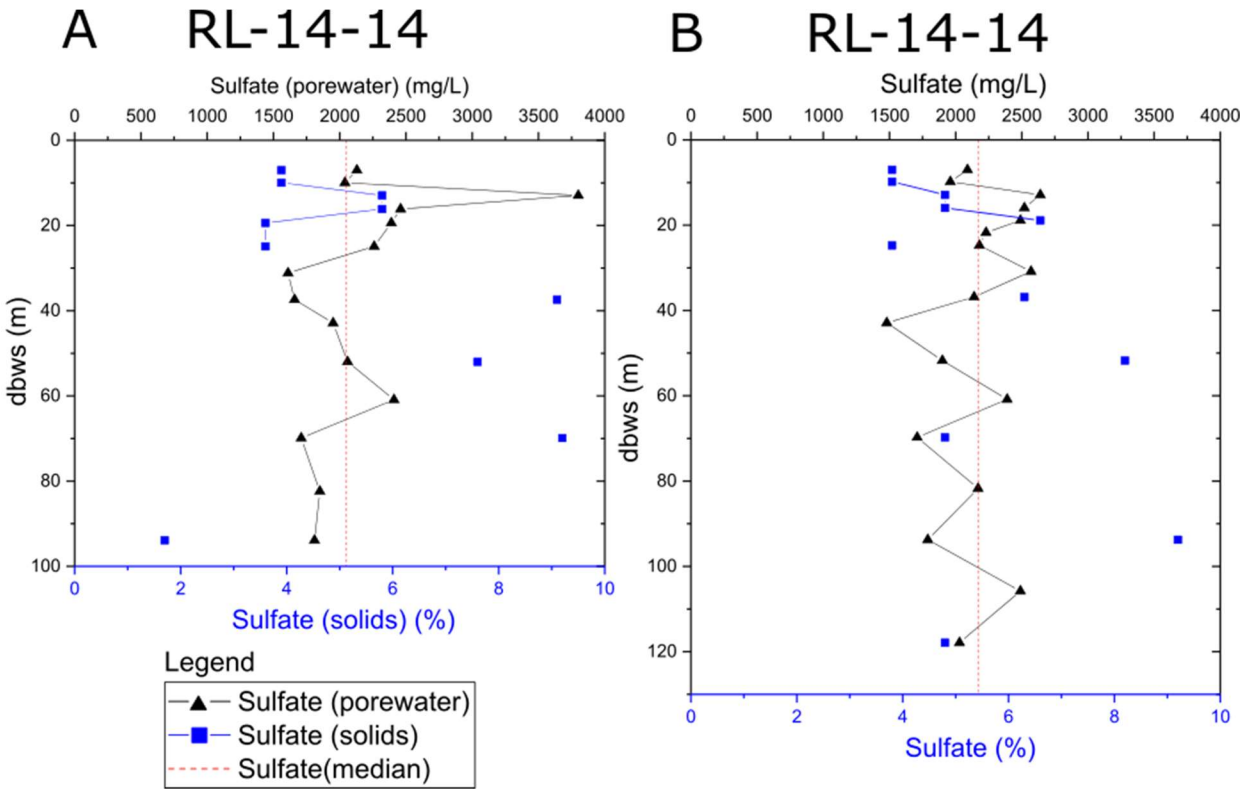

Figure 3-4 Profile of sulfate concentrations in the tailings based on ICP-MS analyses. Boreholes (A) RL-14-14 and (B) RL-14-17. The concentration of sulfate in porewater is shown with black triangles; and the concentration of sulfate in solids is shown with blue squares. The red dotted vertical line in both is the median concentration in porewater.

Borehole RL-14-14 solid phase sulfide median concentrations were greater in samples from Region B than in samples above the pH change (Table 3-2). This was different in borehole RL-14-17 where median concentrations were greater in samples from Region A than in Region B (2x).

3.2 High-Throughput Amplicon Sequencing

Sequence classification revealed that three phyla comprised 92 % of the overall bacterial and archaeal sequences: *Proteobacteria* (68 % of all sequences), *Bacteroidetes* (14 %), and *Firmicutes* (10 %). Other major phyla include *Tenericutes* (2.6 %) and *Actinobacteria* (2.4 %).

The phylum-level composition for samples 1403 to 1406 was similar for both lab and site-extracted DNA, except for sample 1404 site-extracted DNA (Figure 3-5). The site-extracted 1404 samples had a much greater proportion of *Bacteroidetes* and *Firmicutes* sequences than in the 1404 lab-extracted samples. Also, the percent of *Bacteroidetes* sequences dramatically exceeded the percent of *Proteobacteria* sequences.

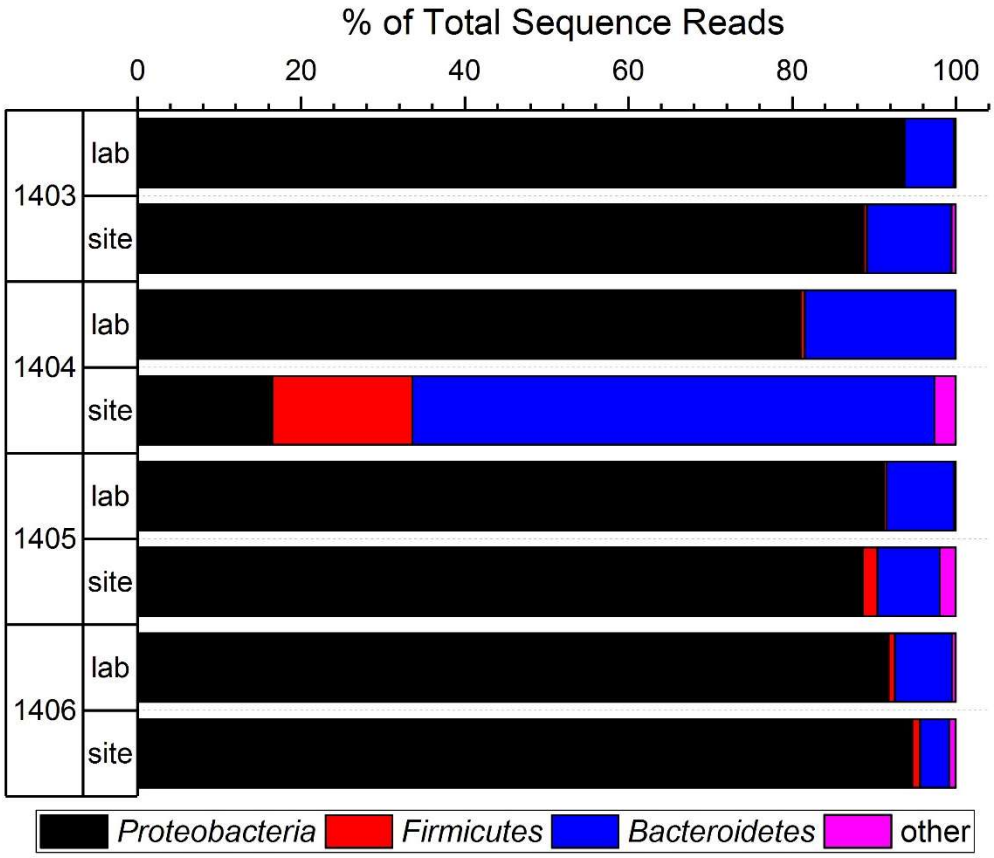

Figure 3-5 Top three phyla as a percent of total sequences for lab and site-extraction sample comparison. Samples percentages are averages of triplicates sequence reads.

This difference can be visualized with ordination plots (e.g. NMDS 3-dimentional plots), where replicates of 1404 site-extracted DNA plot separatly from the other samples (Figure 3-6). An AMOVA calculation of the difference between the 1404 site- and lab-extracted sample did not show a statistically significant difference ($p = 0.277$).

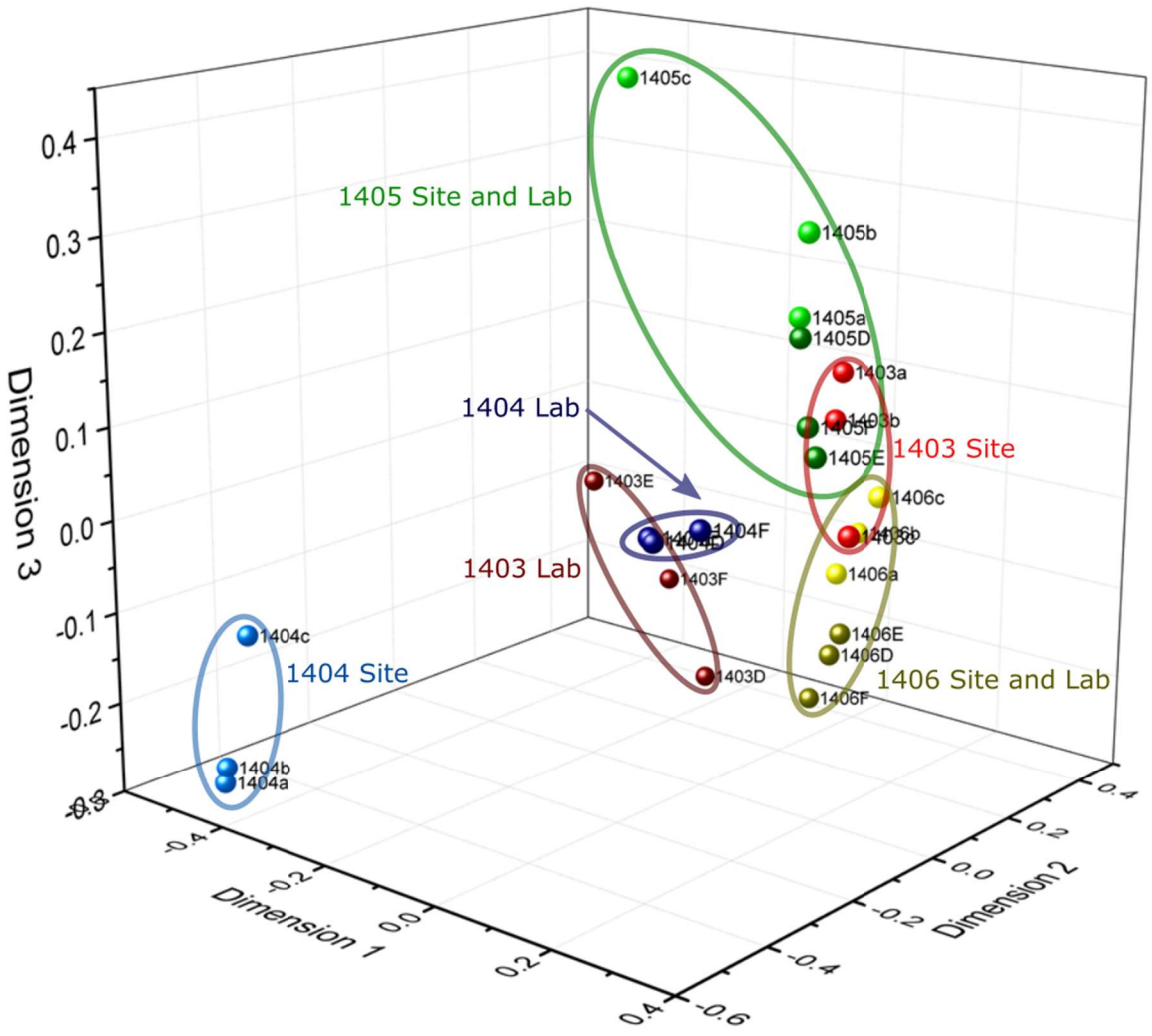

Figure 3-6 Three-dimensional NMDS plot comparing sample communities from site- and lab-extracted DNA samples. The plot had R^2 0.98 and lowest stress 0.066. Samples from the same location in the borehole are the same colour; the lighter shades represent site-extracted DNA samples and the darker shades represent lab-extracted DNA samples.

Site and lab extractions for samples 1403 to 1406 had sequences assigned to a total of 1168 OTUs (Table 3-3). Unique OTUs are OTUs that are only found in one sample. In the pair comparisons of site- and lab-extracted samples, the percent of shared OTUs varied between 19 % and 37 %, and unique OTUs make up a larger proportion of the OTUs for each sample.

Table 3-3 Unique and shared OTUs in averages of lab and site-extracted sample replicates. Data subsampled to 7000 sequence reads.

Sample ID	Unique OTUs		Shared OTUs	% Shared
	Lab	Site		
1403	49	98	47	24.2
1404	54	119	54	23.8
1405	216	128	80	18.9
1406	70	59	75	36.8

All sequence discussions that follow in this thesis are based on analyses of lab-extracted DNA. - Similar to the pH data (Table 3-1), the sequence data analyses show two zones within the tailings (Figure 3-7). These zones can be observed in the phyla level composition of samples (Figure 3-7), and PCoA ordination plotting (Figure 3-8). The presence of two distinct zones is supported by AMOVA calculations of significance ($p<0.001$). These observed differences can be seen in both boreholes. The sequencing samples we obtained for Region A consist of samples 1402, 1403, 1404, 1405, 1406, 1702, and 1704. The samples in Region A were taken from between 414 to 399 masl. From Region B, we analysed samples 1408, 1409, 1414, 1417, 1418, 1709, and 1711. The samples in Region B are from between 399 and 330 masl.

Only sample 1709 was a single data point due to the other replicates failing to sequence, all others were duplicate or triplicate (Figure 3-7). At the phylum level, the microbial communities were dominated by *Proteobacteria* and to a lesser degree *Bacteroidetes* for samples in Region A. The proportions of *Firmicutes*, *Actinobacteria*, and *Tenericutes* increased in samples in Region B. The sequence classifications of samples from borehole RL-14-17 show a similar pattern to RL-14-14. This change in community pattern occurred between samples 1704 and 1709, which fall between 408 and 387 masl.

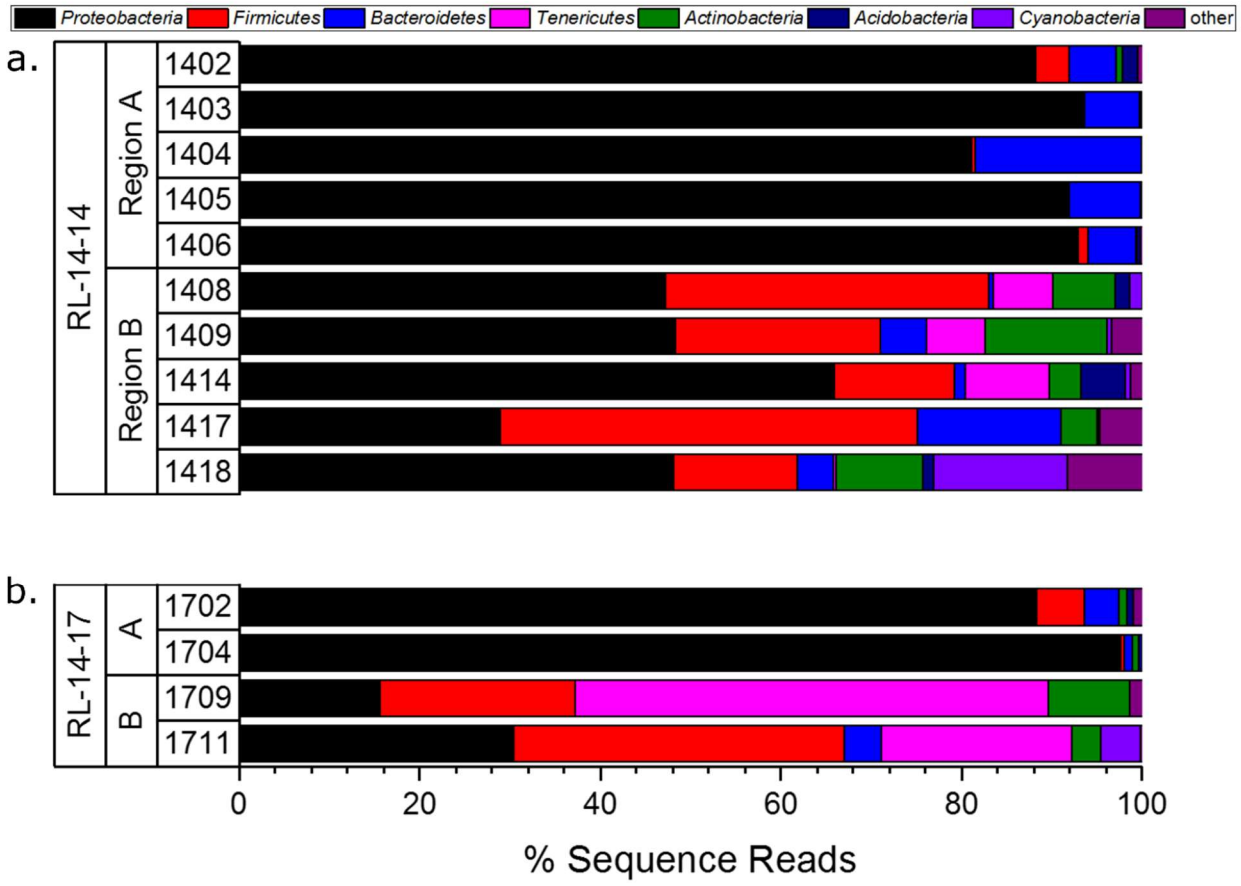

Figure 3-7 Sequences by sample from boreholes RL-14-14 and RL-14-17 as a percent of total sequences in each sample. Samples are divided into two regions, Region A and Region B (A and B in Figure 3-7 b).

The quantity of OTUs in the samples can be used to identify and compare richness of the samples. A total of 10993 OTUs were generated from all sample analyses. The number of unique OTUs in samples in Region A was 1946 OTUs, and there were 8162 unique OTUs in Region B samples. The number of OTUs shared between Region A and B samples was 885.

The PCoA plotting of replicates broadly separated samples into two categories (Figure 3-8). The sample communities grouped into samples in Region A and B. The AMOVA analysis between these two groups indicates a significant difference ($p < 0.001$). The AMOVA included site extractions and these clustered with their corresponding lab extracted samples in Region A.

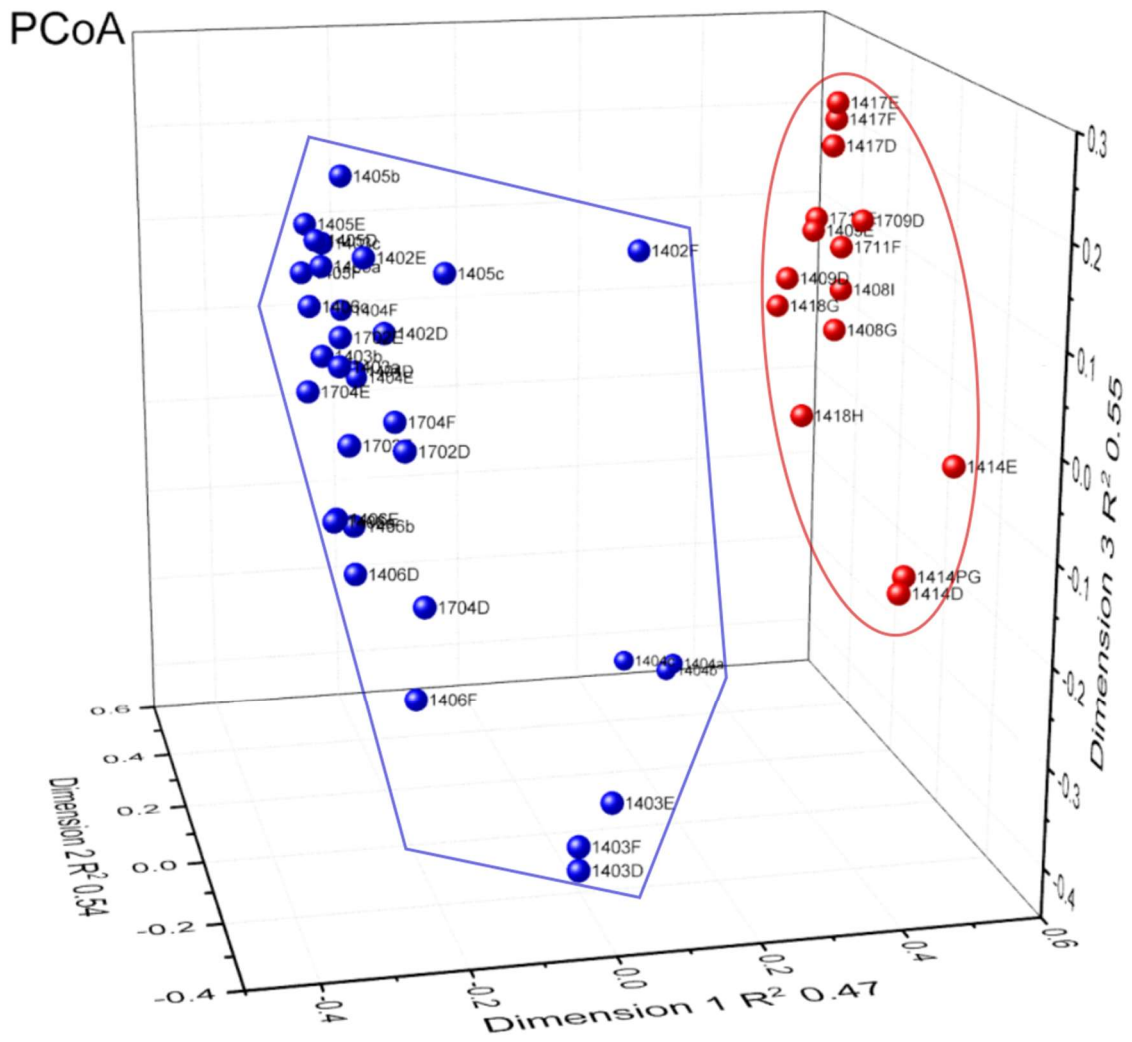

Figure 3-8 A PCoA graph for all replicates in boreholes RL-14-14 and RL-14-17. Region A replicates are blue. Region B replicates are red.

The Good's coverage calculation (subsampled to 7000 reads in each replicate) showed there was a minimum coverage of 95.7 % and a median coverage of 99.6 % (Table A-1, Table A-2). Diversity, measured with the Inverse-Simpson index, increasing with depth.

The genera *Desulfuromonas*, *Desulfosporosinus*, and *Desulfovibrio* were abundant in many samples (by sequence classifications) (Figure 3-9). These genera are known to include species with As(V)-, Fe(III)-, and sulfate/sulfur-reducing activity (Table 3-4). While we cannot be certain from 16S rRNA gene classification alone that these reads represent organisms capable of iron or sulfur species reduction, it is strong evidence that these metabolic guilds may be present in the tailings.

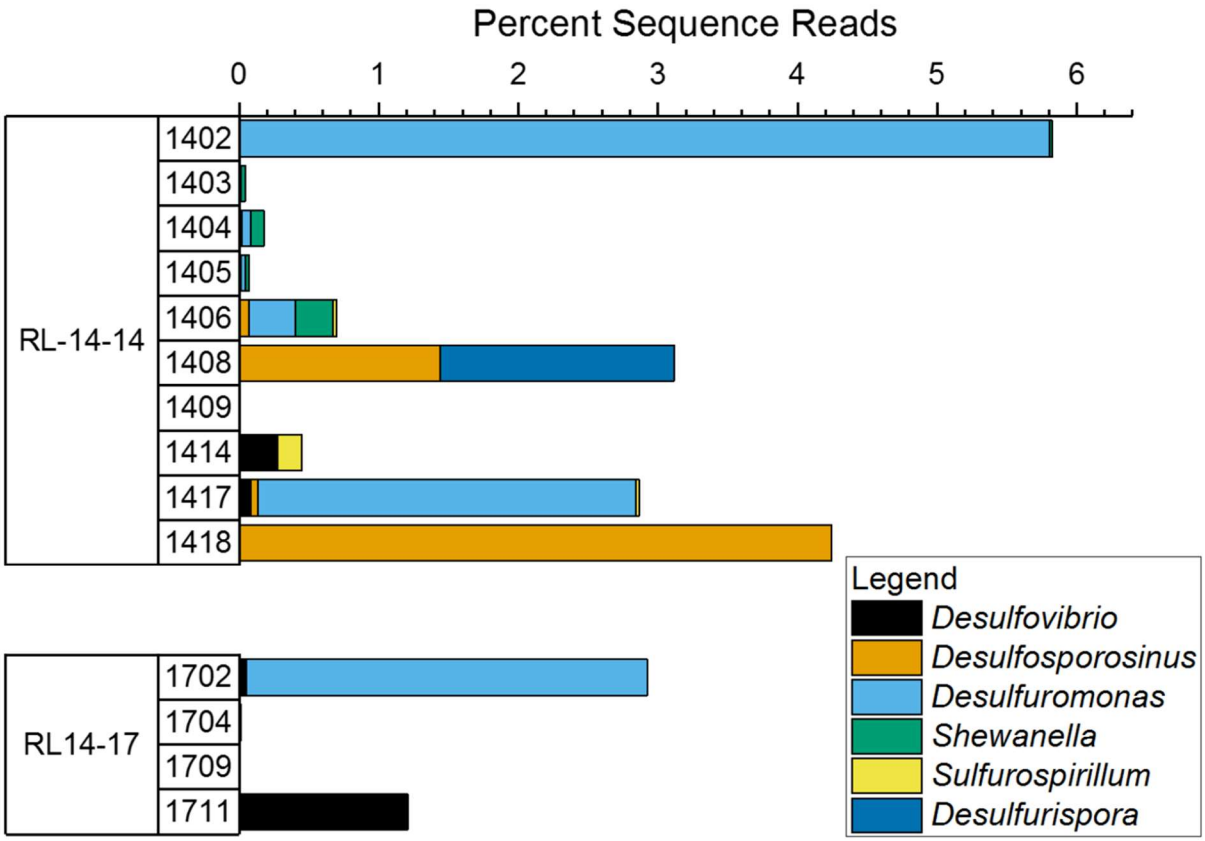

Figure 3-9 Sequences of genera of metabolic interest as a percent of total sequences in each sample, shown for each borehole. Samples are averages of replicates. The genera listed are notable because of their metabolic capabilities (e.g. sulfate- and sulfur-reduction, iron reduction, arsenic reduction). Each sample replicates was subsampled to 7000 sequences for this analysis.

Table 3-4 Genera of interest and putative metabolic reduction activities.

Genus	Putative Metabolic Reduction Activity	References
Desulfovibrio	As(V), Fe(III), Se(VI), SO_4^{2-}, Tc(VII), U(VI)	Barton, Tomei-Torres, Xu, & Zocco, 2015
Desulfosporosinus	As(V), Fe(III), SO_4^{2-}	Barton, Tomei-Torres, Xu, & Zocco, 2015
Desulfuromonas	Cr(VI), Fe(III), Mn(IV), S^0	Lojou, Bianco, & Bruschi, 1998
Shewanella	As(V), Cr(VI), Fe(III), Mn(IV), U(VI)	Nealson & Scott, 2006; Saltikov et al., 2005
Sulfurospirillum	As(V)	Stolz et al., 1999
Desulfurispora	SO_4^{2-}	Kaksonen et al., 2007

Overall, twelve of the fourteen samples I analysed contained sequences that classified as relatives of the sulfate- and sulfur-reducing bacterial genera *Desulfosporosinus*, *Desulfovibrio*, *Desulfurispora*, or *Desulfuromonas* (sequences as a percent of total sequences in a samples ranged from 1 to 6 %) (Barton et al., 2015; Kaksonen et al., 2007). The proportion of sequences classified to relatives of bacteria with sulfate-reducing activity generally increased with depth. For example, the sequences classified to *Desulfosporosinus* comprised 4.2 % of the sequence reads in sample 1418. The sequences classified to *Desulfovibrio* comprised 1.2 % of sample 1711. In sample 1408, the sequences classified to *Desulfurispora* were 1.7 % of the sequences in the sample. *Desulfuromonas* is an elemental sulfur-reducing genus and had higher percentages of sequences in both the shallow sample 1402 (5.8 %) and deeper sample 1417 (2.9 %) with smaller proportions in samples throughout borehole RL-14-14. The sequences classified to *Desulfuromonas* were also in sample 1702 with 2.9 % of the sequences in the sample. The near-surface *Desulfuromonas* may be occurring in syntrophic relationships with green sulfur bacteria as has been found in previous studies as being beneficial to *Desulfuromonas* growth (Garrity et al., 2005). We did not identify any green sulfur bacteria sequences in our data set; however, they may have been present at very low abundance.

The sequences classified to FeRB genera include *Desulfosporosinus*, *Desulfovibrio*, *Desulfuromonas*, and *Shewanella* (Barton et al., 2015; Lojou et al., 1998; Nealson et al., 2006). *Shewanella* was found only in upper samples of RL-14-14, samples 1402 to 1406, with highest abundance in sample 1406 at 0.3 %.

The sequence reads classified to known AsRB are *Desulfosporosinus*, *Shewanella*, and *Sulfurospirillum* (Barton et al., 2015; Saltikov et al., 2005; Stolz, Ellis, et al., 1999). The highest proportion of *Sulfurospirillum* was in sample 1414, at 0.2 %.

3.3 Comparison of Sequences and Select Geochemical Characteristics

I calculated correlation relationships between concentrations of elements and physical parameters (arsenic, iron, molybdenum, sulfate, and pH) and the percent of sequences in each sample for select taxa, metabolic groups, and the inverse-Simpson diversity index (Figure 3-10). The R^2 values were used as a measure of goodness of fit. Only samples with data for both geochemistry and sequencing were used for comparisons (n = 9). The concentrations are only

from porewater because of the limited number of samples available for concentrations in solids. The highest R^2 value was 0.80 between *Firmicutes* and arsenic concentrations. The SRB and pH had an R^2 of 0.67, SRB percent sequences increasing with pH. The R^2 was 0.66 between pH and inverse-Simpson index.

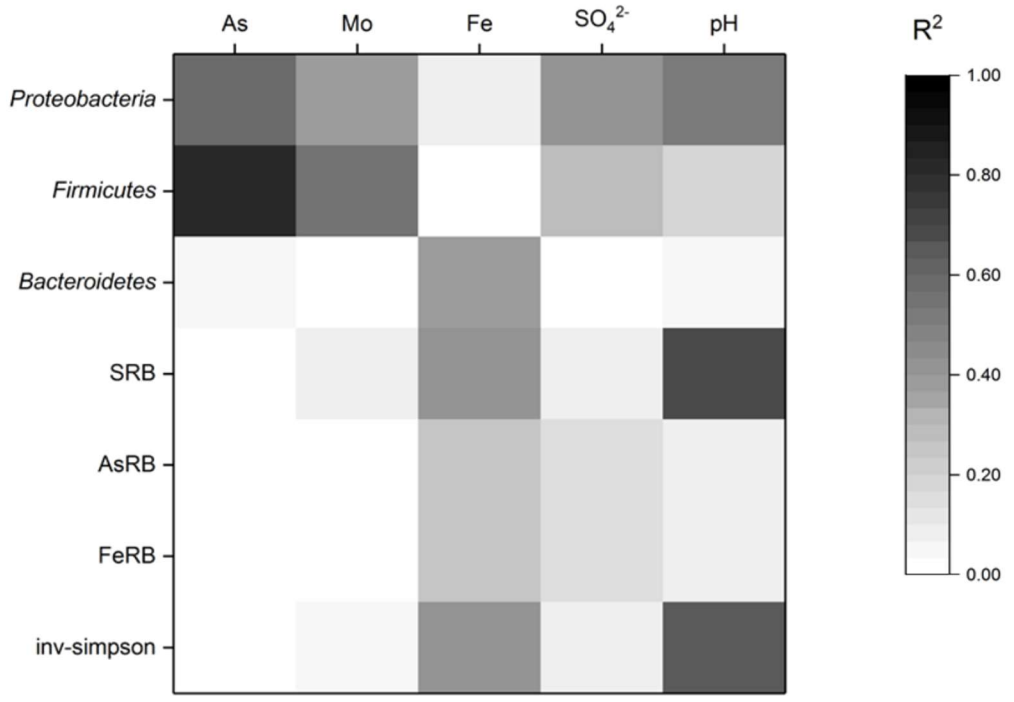

Figure 3-10 Heatmap of R^2 values for comparison of geochemical factors to inverse Simpson index and proportions of sequence reads belonging to bacterial groups.

3.4 Culturing of Sulfate-Reducing Bacteria

I selected eight samples with high SRB sequence abundance to use for culturing work, and inoculated Postgate's media B bottles with these tailings samples. A change in precipitate colour from grey to black is considered to be highly indicative of H_2S production and therefore this was used as a positive score for sulfate-reducing bacteria. The bottles with tailings from samples 1405 and 1417 resulted in potential SRB growth (Figure 3-11). The bottles with apparent growth were subsampled until calculated radioactivity was below 1 Bq per bottle. The non-radioactive bottles were moved to continue enrichment and isolation experiments in Dr. Joyce McBeth's

Kirk Hall Lab. Dilution series were prepared for samples 1405 and 1417. The bottles with sample 1405 inoculations turned black after roughly three weeks. Bottles inoculated with sample 1417 turned a dark grey after roughly two weeks. Methylene blue tests for the quantity of aqueous sulfide showed 463 µg/L in sample 1417 and 4 µg/L in 1405. The high concentrations of hydrogen sulfide in 1417 supports the interpretation that sulfate reduction is ongoing in media inoculated with tailings from sample 1417. Sulfide detection was below detection limit (5 µg/L) in media inoculated with sample 1405.

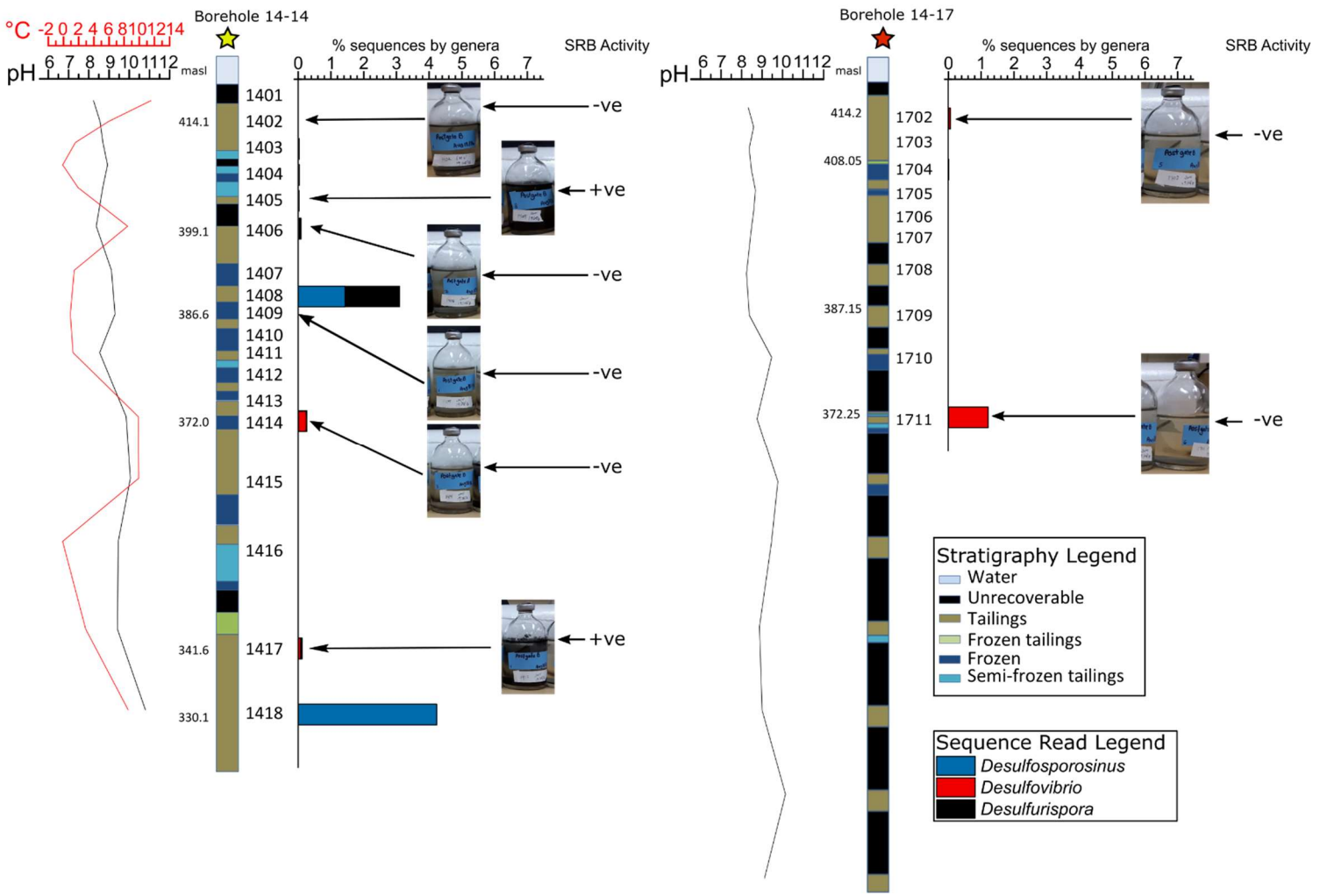

Figure 3-11 Stratigraphy, SRB sequence reads, and SRB cultures. Borehole stratigraphy is presented with the proportion of sequences of major SRB populations. Bottle images show the attempts to culture SRB using Postgate Medium B, with "+ve" indicating cultures that successfully enriched putative SRB.

I observed two morphologies of bacteria in sample 1417 during examination of samples from culture bottles under a light microscope (Figure 3-12 C and D). The bacteria I observed were motile, long rods and non-motile, short rods. The non-motile, short rods I observed were most often associated with particles. In sample 1405, I observed long rods in association with larger particles and shorter rods, sometimes found in chains of two to four cells, primarily associated with particles (Figure 3-12 A and B).

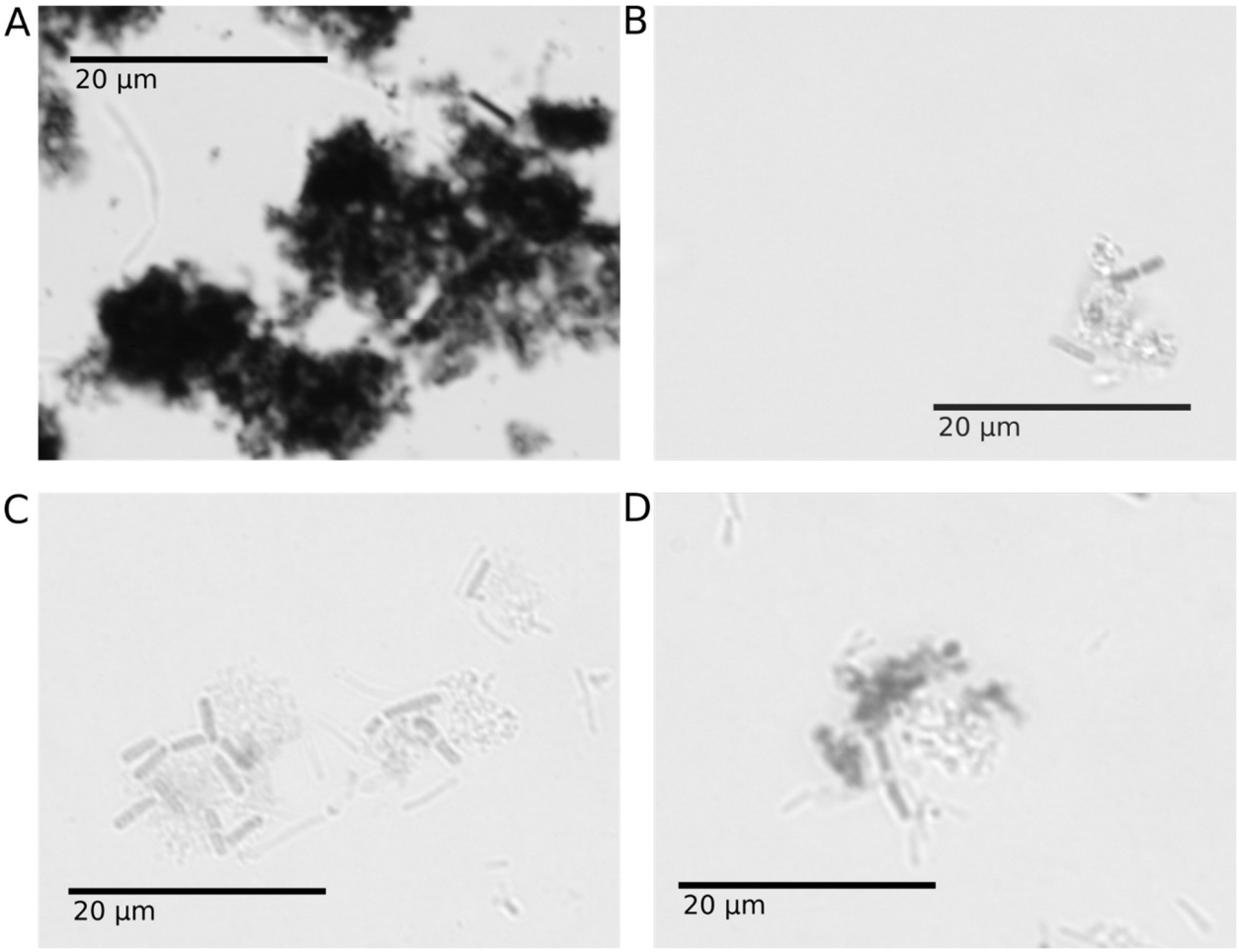

Figure 3-12 Four images obtained from light microscopy of samples 1405 and 1417 enrichment/isolation cultures. A) Sample 1405 at 14 days incubation. B) Sample 1405 at 7 days incubation. C) Sample 1417 from 100 ml bottle inoculated after third dilution series. D) Sample 1417 from the last bottle of a dilution series.

In order to identify bacteria cultured, I extracted DNA and amplified the 16S rRNA gene from the bottles deemed positive for SRB activity. Amplified 16S rRNA gene products were sent for Sanger sequencing. Sanger sequencing was successful for primers 907R and 1492R of DNA

from the putative isolate from sample 1417. The 519F, 907R, and 1492R amplifications were successful for the sample 1405 culture sample. The 27F primer amplification failed and/or sequencing was of poor quality for both samples. For future Sanger sequencing, we could try variations on the 27F or 8F primers to attempt to successfully amplify this region.

I generated a contig for sample 1417 which yielded a sequence 1381 bp long. This sequence classified according to NCBI-BLAST (Nucleotide collection) as *Pelosinus fermentans* (Table 3-5), there were no sequences classified to this genus in the high-throughput amplicon data. *Pelosinus* is not known to be a sulfate-reducing genus, however there are *Pelosinus* species known to reduce iron and it is known to outcompete sulfate-reducers in enrichment cultures (Mosher et al., 2012). I interpreted the sulfide production without sequences classifying to SRB as an indication that sample 1417 was a mixed culture because of the sulfide production and *Pelosinus* is not known to have sulfate-reducing activity. Additional DNA extractions were performed on bottles which were inoculated with media derived from the same initial culture bottle, and DNA was sent for high-throughput amplicon sequencing to attempt to identify additional community members (if present). The results of high-throughput amplicon sequencing showed 70% of sequence reads were classified as *Pelosinus*, 16% as *incertae sedis* inside the *Lachnospiraceae* family, 10% *Clostridium*, 2% *Pseudomonas*, and 2% other genera.

Table 3-5 The results of Sanger sequencing for cultures. The BLAST match is the top result as closest match, the percent and number of base pairs. BLAST match includes coverage and number of gaps required. Known reductive functions are for the species (*Pelosinus fermentans*) or are found in the genus (*Desulfosporosinus*).

Sample ID	Length	NCBI-BLAST Match	Identity Match		Sequence Coverage		Accession	Known Metabolism	References
			%	bp	%	bp (Gaps)			
JMV_1417	1381	*Pelosinus fermentans*	99	1377	100	1382 (1)	LK391730.1	Fe(III), Cr(VI), U(VI) reduction; organic acids, sugars used as electron donors	(Brown et al., 2012; Shelobolina et al., 2007)
JMV_1405	1346	Uncultured *Desulfosporosinus* sp.	98	1323	100	1346 (0)	KJ650779.1	Fe(III), SO_4^{2-}, As(V), thiosulfate, sulfite reduction; organic acids, sugars used as electron donors	(Spring et al., 2006; Stackebrandt, 2014)

The 1405 contig I generated resulted in a 1346 bp sequence. This sequence was classified as *Desulfurosporosinus* sp. – a spore-forming sulfate-reducing bacterium – by NCBI-BLAST of the Nucleotide collection database (Table 3-5).

Genomic DNA from both enrichment cultures was amplified for *dsrA* and *arrA* genes and the amplified products were run on a gel using electrophoresis. The cultures had no bands of expected size present for either gene.

CHAPTER 4 DISCUSSION AND CONCLUSIONS

4.1 Discussion

It is important to examine if the bacterial communities are the same between lab and site-extracted DNA. Due to logistical challenges, it was not possible to keep samples frozen during transport, and thus it is possible there could be some change in the communities during transport. A comparison of bacterial communities of samples that were not transported and those that were will allow identification of biases in sequencing data that arise from the transportation of samples. There was no statistical difference between the lab- and site-extracted samples when we compared them using AMOVA and observations of PCoA and phyla compositions (Figure 3-7, Figure 3-8). There was one observed difference between pairs. The phyla composition was different between sample 1404 lab and site-extractions (Figure 3-5) and this was also observed in PCoA plotting (Figure 3-8). The observed difference in phyla composition in sample 1404 (site extracted) was likely due to a difference in sampling technique; in the sampling for site extractions we avoided ice crystals but were unable to do this for samples that were transported because the ice crystals had melted. In conclusion, for the lab/site extractions comparison, there were no significant changes to the microbial communities after transporting. We used the data from the lab-extracted samples for the rest of our DNA-based analyses.

The sample 1404 site-extracted replicates grouped closer to the samples from Region B than samples in Region A in the PCoA plots (Figure 3-8). We believe the sample 1404 site-extracted replicates community difference is due to the avoidance of ice crystals during sampling. Since sample 1404 site-extracted DNA is similar to Region B this may suggest that there is a relationship between ice crystal presence and the microbial community shift. More work including additional and more detailed field sampling would be required to test this because there are frozen layers in the Region B tailings (shown in the borehole logs, Figure A-1); the frozen layers may have been missed in our Region B sampling.

The most abundant phyla identified in the tailings sequencing data (*Proteobacteria*, *Firmicutes*, *Bacteroidetes*, *Tenericutes*, and *Actinobacteria*) were identical to those identified in high-

throughput sequencing of DTMF tailings (Bondici et al., 2013), with the exception of *Tenericutes*, which had much higher proportions in RLITMF samples than in the DTMF tailings. The greater proportion of *Tenericutes* in the RLITMF data than in the DTMF data could be a result of the different databases used to classify these datasets, or the difference in primers used which amplified and sequencing different 16S rRNA gene regions. Unfortunately, it was not possible to acquire a copy of the original dataset from the DTMF work in order to do a comparison using our pipeline. *Proteobacteria* reads dropped in the deeper sections of the DTMF tailings and this is similar to our findings (Figure A-1) (Bondici et al., 2013).

The AMOVA analysis indicated that the total community difference between Region A and B is significant. The observed and calculated shift in the microbial communities occurs at a similar depth to the change observed in pH median values; both occur at ca 399 masl. A linear correlation is present between pH and diversity (as measured by inverse-Simpson index), and the $R^2 = 0.66$, which suggests a weak to moderate correlation. The number of *Firmicutes* reads and arsenic concentrations have a strong linear correlation with an R^2 value of 0.80. *Firmicutes* displayed increased representation in samples deeper in the tailings in comparison with samples closer to the surface. Tailings composition could be quite variable within the 3 m sections subsampled for each sampling point (e.g. some sections had ice lenses, some regions were sandy), and there was no identifiable pattern between tailings physical composition and the sequence analyses.

When considering sources of microbes present in the tailings, it is possible that bacteria were deposited into the tailings from the external environment (airborne dust), greywater from the camp, or from the ore. The largest proportions of SRB reads occurred in samples from the lower regions of the core, particularly samples 1408, 1418, and 1711. In the lower regions, the median pHs were 9.44 (in RL-14-14) and 9.55 (in RL-14-17). Populations of SRB in these tailings are likely dormant due to the tailings' high pH, high Eh, and low temperatures. It is possible there are lower pH micro-environments where SRB could survive and be active, however, we expect the high bulk pH would control and limit the extent of such micro-environments. Putative FeRB and AsRB were largely of the genus *Desulfuromonas* which was primarily identified in samples 1402 and 1417. Some *Desulfuromonas* species can survive up to pH 8.5 and this could explain the larger sequence reads of this genus in sample 1402. The high pH, Eh and low temperature in

the lower regions of the tailings likely also prevents growth of *Desulfuromonas*. Bacteria of the *Desulfuromonas* genus are generally strict anaerobes and their growth is likely limited by the higher Eh (ca +200 mV) reported throughout the tailings. These high Eh values inhibit strict anaerobic growth and sulfate-reducing bacterial activity, which require Eh < -100 mV (DeLaune et al., 2005; Falkowski et al., 2008). The current tailings Eh is also too high for iron-reducing activity to occur in the tailings, which generally occurs at Eh values between -50 and +100 mV (Falkowski et al., 2008). Note that we did not collect this Eh data, and the quality and accuracy cannot be confirmed or reported.

The *Desulfuromonas* and *Desulfosporosinus* genera are strict anaerobes. They are unlikely to grow and reduce arsenic, iron, or sulfate in the tailings under the reported Eh values and low temperature conditions. The pH (pH 8.56 in sample 1402) may be approaching values suitable for *Desulfuromonas* growth. The *Desulfuromonas* genus includes species of arsenic, iron, and sulfur-reducing bacteria, and was the most abundant (in terms of read numbers) of metal-reducing microbes in our samples (a maximum of 5.8 % of total reads in sample 1402). Reads of this genus were most abundant in the near surface samples 1402 and 1702 and the Region B sample 1417. Some species of *Desulfuromonas* can grow in pH as high as 8.5. Although temperature and Eh will limit *Desulfuromonas* growth, the environmental pH may be suitable for bacteria that can reduce arsenic and iron in the upper regions of the tailings. This could pose a risk to the stability of arsenic as previously described in Section 1.2.2, particularly if Eh decreases and temperature increases. *Desulfosporosinus* are spore-forming bacteria that can reduce arsenic and sulfate but are likely not active in the high pH, high Eh and low temperature conditions of the lower tailings. If the pH decreases or *Desulfosporosinus* from lower regions are mixed with other lower pH regions of the tailings, this may encourage germination, growth, and activity of this genus.

Pelosinus fermentans and *Clostridium* species were identified in the SRB enrichment cultures prepared from sample 1417. The presence of *Pelosinus*, which is not an SRB, in the enrichments may be due to incomplete isolation via dilution-to-extinction techniques, or possibly due to culture contamination. *Pelosinus* was not identified in the high-throughput sequencing data from tailings samples, so was either not present, or present at very low abundance in the tailings. *Pelosinus* species are not currently known to reduce sulfate but under certain circumstances can

outcompete SRB (Mosher et al., 2012). *Clostridium* species were found in the high-throughput amplicon sequencing of the sample 1417 culture and in the tailings samples, and could be reducing sulfate in the culture (Sallam et al., 2009). The culturing of a *Desulfosporosinus* species from sample 1405 (Region A) proves the viability of these SRB in the tailings. Though this genus was not identified in the sequencing data for sample 1405, *Desulfosporosinus* reads were identified in other samples from the tailings (samples 1404 and 1406), so it is possible they were present in sample 1405 at very low abundance. The presence of sulfide and black precipitate in the medium also provides evidence for their ability to reduce sulfate. This does not mean that this microbe is active in the tailings because the pH of the media was ca 7.5 compared to a median tailings pH of 8.63 in Region A. Further testing of this enrichment culture is required to identify the pH range where this organism can survive and grow. This expands on the information on culturability of SRB from tailings samples that was gathered by Wolfaardt *et al*. (2008), where they were able to grow a few SRB colonies on solid media but did not identify the microbes or obtain MPN data on their abundance. While I did not test for MPN, I have proven it is possible to culture and identify SRB from the tailings samples, and my sequencing analyses have improved our understanding of their spatial distribution through the tailings.

4.2 Conceptual model

I have prepared a conceptual model for geochemical processes involving metal-reducing microbes in the tailings. In the model, I consider the current environment, and potential activities in differing environmental conditions. The model includes known genera based on sequence classification placed in the context of the two regions in the tailings (Figure 4-1 A). The mineralogy is based on previous literature (Donahue et al., 2003; Donahue et al., 2000; Moldovan et al., 2003) and recent analyses (personal communication with Kendall Krepps). Under current measured pH, Eh, and temperature conditions, most bacteria of interest will not be active. I have prepared models for potential microbial growth under hypothetical conditions. If the discharge tailings pH is decreased to 7 this could encourage the growth of heterotrophic psychrotrophs that could lead to a decrease in Eh (Figure 4-1 B). If the Eh decreases to roughly +100 mV, *Desulfosporosinus* could growth and reduce iron in the upper region of the tailings. The remaining microbes are not typically psychrotrophic and would likely continue to be inactive due to the low temperatures in the tailings. The lower (Region B) tailings microbes

would likely remain inactive because of the low permeability of the tailings that would slow pH and Eh changes since the tailings are diffusion limited. If in the future the temperature of the tailings increases to above 15 º C, alkaliphiles such as heterotrophic alkaliphiles and *Desulfosporosinus* and *Desulfuromonas* could grow (Figure 4-1 C). Bacterial activity could cause the Eh to decrease and may allow for iron and later sulfate-reducing activity.

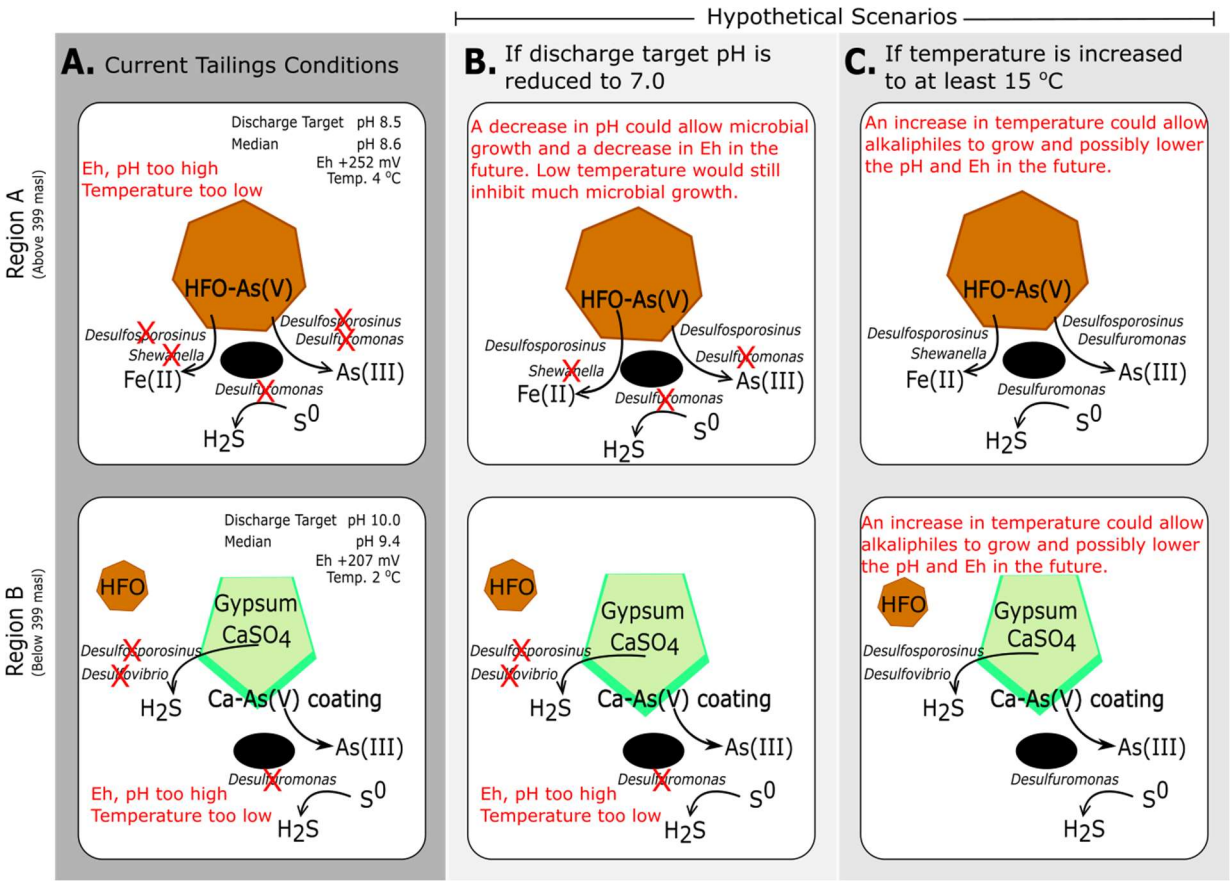

Figure 4-1 Conceptual model of hypothetical RLITMF microbial communities and processes. This model presents the possibilities of microbial interactions with arsenic-containing minerals at varying environmental conditions. (A) Shows the current conditions in the tailings. (B and C) Hypothetical scenarios and potential future conditions.

4.3 Final Conclusions

This work has shown that at various depths in the RLITMF tailings, there are microbes present that are related to SRB, FeRB, and AsRB. The source of microbes in the tailings could be from the external environment (airborne dust), greywater from the camp, or from the ore. I have successfully cultured SRB from tailings samples and maintained the culture through several

isolation-to-dilution experiments and transfers. The cultures show the viability of SRB in the tailings and their potential for growth and activity if conditions change to be more amenable to growth (i.e. circumneutral pH, lower Eh, or higher temperature). However, current conditions in the tailings are not conducive with microbial growth: the measured Eh is too high, the temperature is too low, and the pH is too high.

4.4 Future Work Recommendations

Recommendations for Sampling On-Site

Future work following from this project could include incorporating microbial community analyses to identify changes in microbial populations with time. Collecting and analysing additional geochemical and microbiological samples throughout the boreholes could provide a more precise location on where pH and microbial communities change spatially. This could provide insight into potentially subtle characteristics of the tailings that contribute to the microbial communities or other locations of community shifts. It would also be interesting to do a comparative analysis of the tailings overlying water and lakes, stream sediments, ore, camp greywater, and soils near to the tailings to provide information regarding potential sources for the bacteria present in the tailings.

A limitation of this study is that we did not assess the activity of the communities present in the tailings in situ. Active bacteria and genes can be identified through transcriptomic or proteomic analyses. This does not account for bacteria that have entered dormant states such as spores and could potentially miss important bacteria that may be active if the environment changes; therefore, ideally transcriptomic and proteomic analyses should be performed in addition to DNA-based sequencing and culture work. For transcriptomic and proteomic studies, special consideration should be taken when sampling on-site due to the susceptibility of RNA to degradation.

Recommendations for qPCR Analyses

The qPCR analyses were not performed on RLITMF samples due to technical issues (Chapter 5) and time constraints. The *dsrA* gene primers used have successfully amplified gene fragments

that have been Sanger sequenced and classified as a *dsrA* gene fragment originating from *Desulfovibrio desulfuricans* (DSMZ 642) according to NCBI BLAST. I am confident in the quality of these primers and their amplification ability. While the *dsrA* gene is not the ideal target its quantitative estimate of SRB can be compared with *aps* gene abundance to confirm the estimate's accuracy. In order to assess SRB quantity, it would be good to conduct qPCR analyses using both *dsrA* and *aps* gene primers. Other genes of interest to be targeted for qPCR analyses would include the 16S rRNA gene to get an estimate of total bacterial populations and the *arr* gene to quantify dissimilatory arsenate reductase.

The activity of bacteria could be tested by Reverse-Transcriptase (RT)-qPCR by targeting genes of interest, for example the sulfate-reducing gene *dsrA* and the arsenate reductase gene *arr*. The RT-qPCR analyses of *dsrA* and *arr* could provide useful semi-quantitative information on the activity of SRB and AsRB populations.

Recommendations for Further Culture-Dependent Work

We obtained two cultures from enrichment culturing, and it would be good to isolate the metal- or sulfate-reducers from the enrichment cultures and evaluate the physiology of these organisms. The cultures obtained from the tailings require further work to isolate. Isolation could be achieved with more serial dilutions, or using solid media kept anaerobic in an anaerobic chamber or anaerobic jars. This could be done using Postgate's media B with agar amendments that is prepared in the same manner as described in this thesis but poured into plates or tubes in an anaerobic chamber. Sample 1417 had sequence reads that classified as *Clostridium*; these are bacteria that can typically grow on any nutrient agar in anaerobic conditions. Sanger sequencing of DNA extracted from bacteria isolated from the tailings could allow for more accurate identification of viable bacteria in the tailings than high-throughput amplicon sequencing.

The cultures should be characterized for their physiology. This includes identifying optimal pH, Eh, and temperature conditions for growth, whether the cultures can form spores, which carbon sources the culture can use. The physiological characterization experiments could include examination of the bacterial growth under conditions similar to that of the tailings and identify if the culturable bacteria could be active under the tailings conditions.

The PCR targeting SRB and AsRB genes, such as *dsr* and *arr* operons, may be used to identify the potential for sulfate and arsenate-reducing abilities in isolates. The PCR products can be sent for Sanger sequencing to confirm the sequence as the gene of interest and identify the species to which it belongs. Shotgun sequencing and/or genomic sequencing could also add useful information on the isolates and their genetics. Any isolates should be submitted to a culture collection such as the DSMZ (https://www.dsmz.de/).

Other media with different metabolic enrichment targets should also be considered in future culturing attempts (e.g. using iron and arsenic-reducing media) which would allow further understanding of the potential capabilities of bacteria in the tailings. If a site visit is possible, prepared media should be brought to site to attempt to culture fresh tailings instead of inoculating the samples after transit and multiple freeze-thaw cycles. *Desulfuromonas* was the highest represented genus of metabolic interest in this study and should be considered for future culturing and isolation attempts. Elemental sulfur-based media is recommended for culturing this genus (Garrity et al., 2005).

The samples 1408 and 1418 were not sequenced until late in the project and that is the reason there was no attempt to culture SRB from these samples. The higher proportion of *Desulfosporosinus* and the proven ability to culture this genus from samples with low sequence read proportions would make both samples good candidates for further culturing attempts.

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APPENDIX A SUPPLEMENTARY FIGURES AND TABLES

Table A-1 Table of diversity and richness features of samples for borehole RL-14-14. All values were calculated based on replicates subsampled to 7000 sequences. The coverage is Good's calculated coverage (richness). Species-observed is the observed OTUs. Inverse-Simpson index is a measure of diversity. Chao1 is an estimate of richness that incorporates rarity of OTUs into calculations. The RLITMF sequence data was submitted to the European Nucleotide Archive database and the ENA accession numbers are provided for each sample.

Replicate ID	Total # Sequences	Coverage	Species-Observed	Inverse-Simpson	Chao1	ENA Accession
1402D	48483	0.993	111	6.2	217.0	ERS1670082
1402E	39668	0.991	144	4.5	247.5	ERS1670083
1402F	125945	0.987	145	5.1	446.0	ERS1670084
1403D	19837	0.997	48	4.1	86.6	ERS1670085
1403E	15398	0.997	45	6.6	81.0	ERS1670086
1403F	21980	0.997	48	5.4	72.7	ERS1670087
1403a	22783	0.997	61	4.9	88.5	ERS1670088
1403b	14861	0.996	66	4.8	96.9	ERS1670089
1403c	15218	0.996	75	2.5	104.5	ERS1670090
1404D	14566	0.997	61	3.4	87.6	ERS1670091
1404E	13722	0.996	66	3.6	104.3	ERS1670092
1404F	23466	0.997	59	2.8	88.0	ERS1670093
1404a	32224	0.996	100	2.4	131.3	ERS1670094
1404b	30603	0.995	111	2.4	152.7	ERS1670095
1404c	7403	0.999	68	5.5	71.5	ERS1670096
1405D	29214	0.990	141	4.6	270.1	ERS1670097
1405E	18065	0.991	113	3.2	236.0	ERS1670098
1405F	20176	0.990	137	4.2	263.8	ERS1670099
1405a	43870	0.995	96	4.9	145.2	ERS1670100
1405b	68444	0.996	90	4.2	124.7	ERS1670101
1405c	50904	0.997	93	9.0	115.2	ERS1670102
1406D	7857	0.998	68	3.6	77.7	ERS1670103
1406E	19227	0.998	75	3.4	86.9	ERS1670104
1406F	56589	0.996	70	3.5	110.5	ERS1670105
1406a	29654	0.996	77	3.5	102.4	ERS1670106
1406b	25824	0.997	72	4.7	97.8	ERS1670107
1406c	14083	0.999	46	3.9	51.8	ERS1670108
1408G	8715	0.997	69	6.9	116.5	ERS1670109

Replicate ID	Total # Sequences	Coverage	Species-Observed	Inverse-Simpson	Chao1	ENA Accession
1408I	11849	0.994	78	19.3	191.8	ERS1670110
1409D	47961	0.990	124	8.8	494.4	ERS1670111
1409E	101443	0.998	69	8.7	139.9	ERS1670112
1414D	43798	0.987	181	3.8	467.0	ERS1670113
1414E	25557	0.993	84	7.4	256.3	ERS1670114
1414PG	34876	0.991	130	3.4	274.6	ERS1670115
1417D	110897	0.959	421	9.0	1360.4	ERS1670116
1417E	126182	0.967	351	7.7	1076.4	ERS1670117
1417F	100833	0.957	429	9.1	1471.6	ERS1670118
1418G	28341	0.984	466	114.4	649.0	ERS1670119
1418H	17332	0.995	85	14.6	236.9	ERS1670120

Table A-2 Table of diversity and richness features of samples for borehole RL-14-17. All values were calculated based on replicates subsampled to 7000 sequences. The coverage is Good's calculated coverage (richness). Species-observed is the observed OTUs. Inverse-Simpson index is a measure of diversity. Chao1 is an estimate of richness that incorporates rarity of OTUs into calculations. The RLITMF sequence data was submitted to the European Nucleotide Archive database and the ENA accession numbers are provided for each sample.

Replicate ID	Total # Sequences	Coverage	Species-Observed	Inverse-Simpson	Chao1	ENA Accession
1702D	30921	0.993	110	6.8	251.5	ERS1670121
1702E	33880	0.992	125	5.4	223.5	ERS1670122
1702F	16140	0.995	103	5.9	186.1	ERS1670123
1704D	135847	0.996	63	1.7	105.7	ERS1670124
1704E	37527	0.995	74	2.7	143.6	ERS1670125
1704F	30169	0.994	84	4.6	159.7	ERS1670126
1709D	35408	0.997	42	3.4	98.9	ERS1670127
1711E	56451	0.997	49	2.6	131.4	ERS1670128
1711F	60882	0.998	44	14.3	98.1	ERS1670129

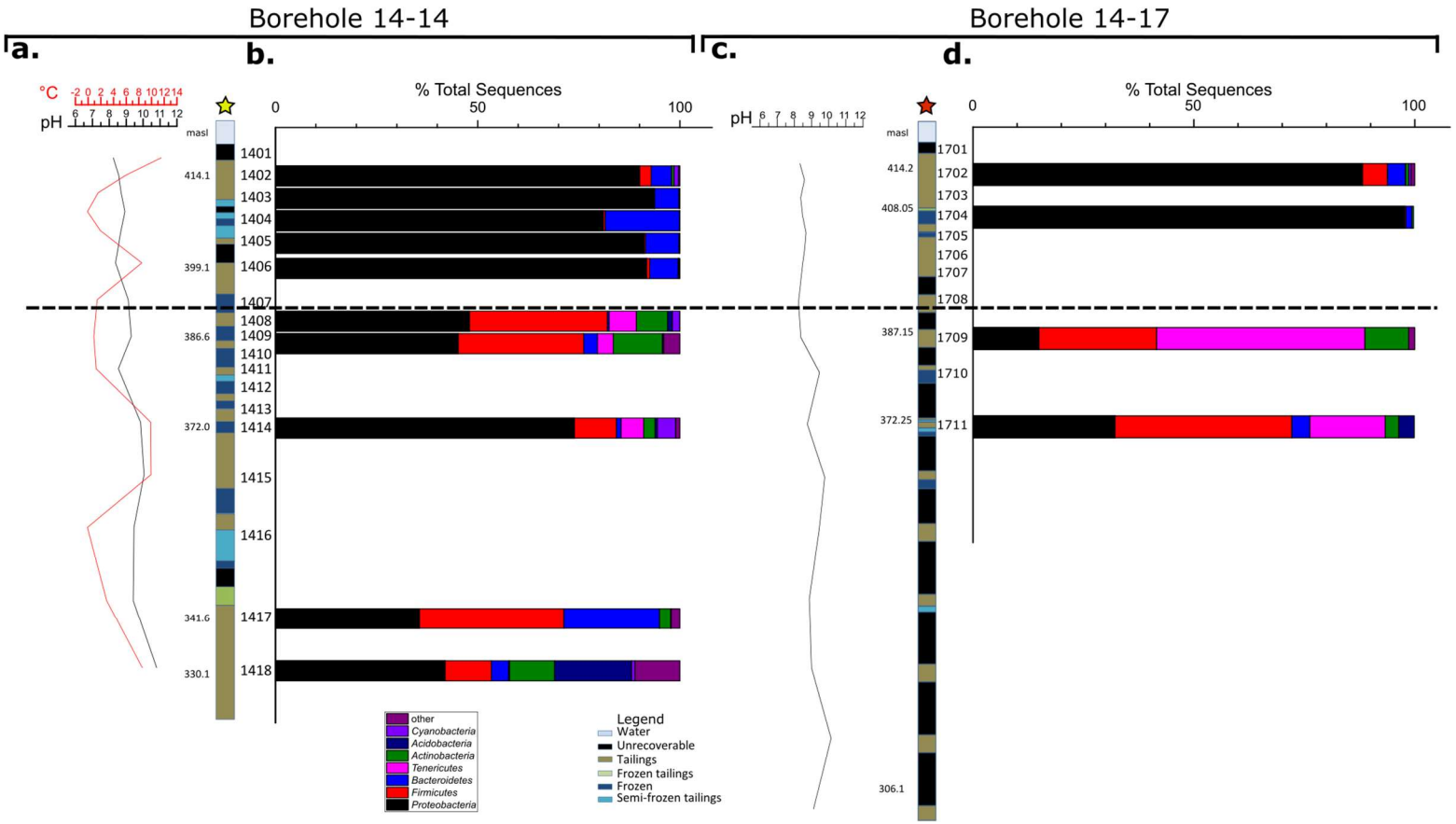

Figure A-1 Sequence data from boreholes (A) 14-14 and (B) 14-17. Phyla as a percent of total sequences in that sample averaged across triplicates when available. (C) The sequence data (b, d) in line with boreholes, stratigraphy, pH, and temperature (a, c) of 14-14

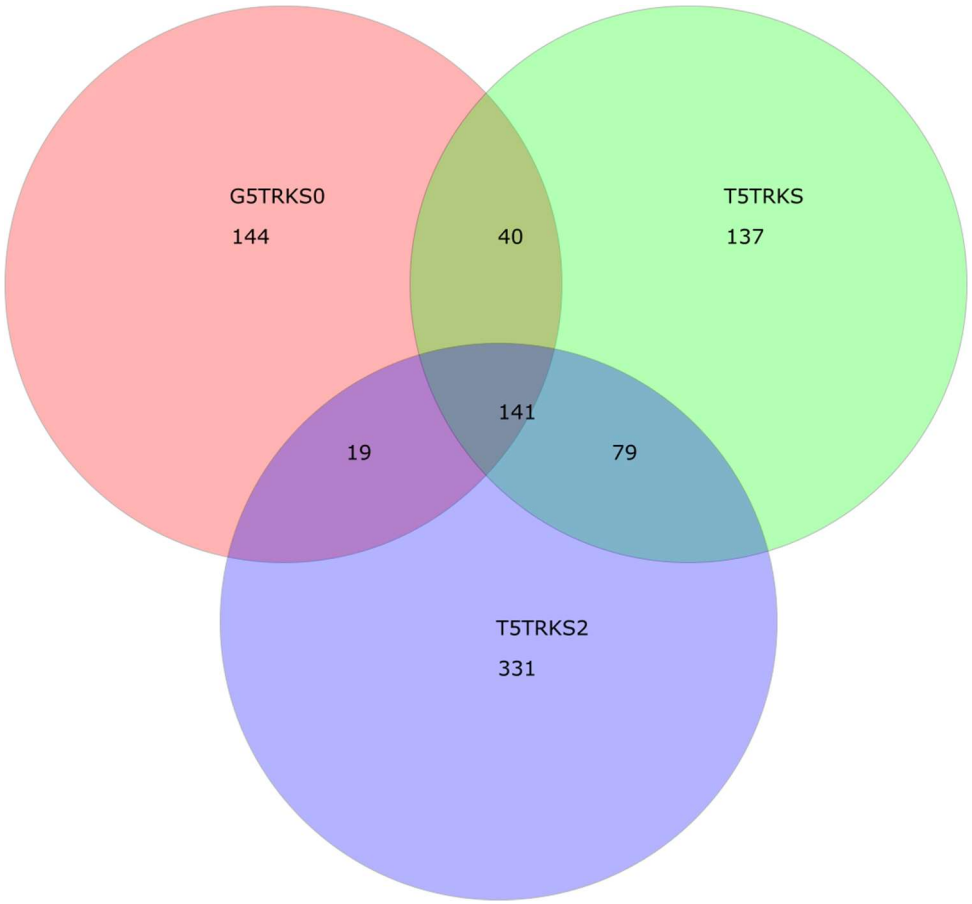

Figure A-2 Venn diagram showing the shared and unique OTUs to technical replicates sent during the three batches of Illumina MiSeq sequencing used for our high-throughput analyses. The samples were subsampled to 14250 reads. There were more OTUs shared between G5TRKS0 and T5TRKS than there were unique OTUs in either sample.

APPENDIX B DETAILED MOTHUR PIPELINE

All commands were run using mothur v 1.35.1 by Patrick D. Schloss (University of Michigan, Dept. Microbiology and Immunology). The mothur package can be downloaded from http://www.mothur.org.

Schloss, P.D., et al., Introducing mothur: Open-source, platform-independent, community-supported software for describing and comparing microbial communities. Appl Environ Microbiol, 2009. 75(23):7537-41.

Mothur is a free bioinformatics analysis software package that was used in this study to clean, classify, and statistically analyze high-throughput amplicon sequencing (Illumina MiSeq Platform) based on the mothur MiSeq SOP (version 2015). The mothur software also makes use of separate programs, such as UCHIME (Edgar et al., 2011), that can be easily incorporated into pipelines. The mothur software is flexible in what commands can be run, the variables used, and analyses performed to suit project needs.

#Initial Cleaning and Classifying of Sequences:

make.contigs(file=JV1_2_3_4.files, processors=8)

summary.seqs(fasta=JV1_2_3_4.trim.contigs.fasta)

screen.seqs(fasta=JV1_2_3_4.trim.contigs.fasta, group=JV1_2_3_4.contigs.groups, maxambig=0, maxlength=300)

get.current()

summary.seqs(fasta=current)

unique.seqs(fasta=JV1_2_3_4.trim.contigs.good.fasta)

count.seqs(name=JV1_2_3_4.trim.contigs.good.names, group=JV1_2_3_4.contigs.good.groups)

summary.seqs(count=JV1_2_3_4.trim.contigs.good.count_table)

summary.seqs(fasta=silva.nr_v119.pcr.align)

align.seqs(fasta=JV1_2_3_4.trim.contigs.good.unique.fasta, reference=silva.nr_v119.pcr.align)

summary.seqs(fasta=JV1_2_3_4.trim.contigs.good.unique.align, count=JV1_2_3_4.trim.contigs.good.count_table)

screen.seqs(fasta=JV1_2_3_4.trim.contigs.good.unique.align,
count=JV1_2_3_4.trim.contigs.good.count_table, summary=JV1_2_3_4.trim.contigs.good.unique.summary,
start=1968, end=11550, maxhomop=8, processors=4)

summary.seqs(fasta=current, count=current, processors=8)

filter.seqs(fasta=JV1_2_3_4.trim.contigs.good.unique.good.align, vertical=T, trump=., processors=8)

unique.seqs(fasta=JV1_2_3_4.trim.contigs.good.unique.good.filter.fasta,
count=JV1_2_3_4.trim.contigs.good.good.count_table)

pre.cluster(fasta=JV1_2_3_4.trim.contigs.good.unique.good.filter.unique.fasta,
count=JV1_2_3_4.trim.contigs.good.unique.good.filter.count_table, diffs=2, processors=8)

chimera.uchime(fasta=JV1_2_3_4.trim.contigs.good.unique.good.filter.unique.precluster.fasta,
count=JV1_2_3_4.trim.contigs.good.unique.good.filter.unique.precluster.count_table, dereplicate=t,
processors=6)

remove.seqs(fasta=JV1_2_3_4.trim.contigs.good.unique.good.filter.unique.precluster.fasta,
accnos=JV1_2_3_4.trim.contigs.good.unique.good.filter.unique.precluster.uchime.accnos)

summary.seqs(fasta=current, count=current)

classify.seqs(fasta=JV1_2_3_4.trim.contigs.good.unique.good.filter.unique.precluster.pick.fasta,
count=JV1_2_3_4.trim.contigs.good.unique.good.filter.unique.precluster.uchime.pick.count_table,
reference=silva.nr_v119.pcr.align, taxonomy=silva.nr_v119.tax, cutoff=80)

classify.seqs(fasta=JV1_2_3_4.trim.contigs.good.unique.good.filter.unique.precluster.pick.fasta,
count=JV1_2_3_4.trim.contigs.good.unique.good.filter.unique.precluster.uchime.pick.count_table,
reference=trainset9_032012.rdp.fasta, taxonomy=trainset9_032012.rdp.tax, cutoff=80)

remove.lineage(fasta=JV1_2_3_4.trim.contigs.good.unique.good.filter.unique.precluster.pick.fasta,
count=JV1_2_3_4.trim.contigs.good.unique.good.filter.unique.precluster.uchime.pick.count_table,
taxonomy=JV1_2_3_4.trim.contigs.good.unique.good.filter.unique.precluster.pick.nr_v119.wang.taxonomy,
taxon=Chloroplast-Mitochondria-unknown-Eukaryota)

#Analysis Commands

dist.seqs(fasta=JV1_2_3_4.trim.contigs.good.unique.good.filter.unique.precluster.pick.pick.fasta, cutoff=0.20,
processors=8)

cluster(column=JV1_2_3_4.trim.contigs.good.unique.good.filter.unique.precluster.pick.pick.dist,
count=JV1_2_3_4.trim.contigs.good.unique.good.filter.unique.precluster.uchime.pick.pick.count_table)

make.shared(list=JV1_2_3_4.trim.contigs.good.unique.good.filter.unique.precluster.pick.pick.an.unique_list.li
st, count=JV1_2_3_4.trim.contigs.good.unique.good.filter.unique.precluster.uchime.pick.pick.count_table,
label=0.03)

classify.otu(list=JV1_2_3_4.trim.contigs.good.unique.good.filter.unique.precluster.pick.pick.an.unique_list.list
, count=JV1_2_3_4.trim.contigs.good.unique.good.filter.unique.precluster.uchime.pick.pick.count_table,

taxonomy=JV1_2_3_4.trim.contigs.good.unique.good.filter.unique.precluster.pick.nr_v119.wang.taxonomy, label=0.03)

count.groups(shared=JV1_2_3_4.trim.contigs.good.unique.good.filter.unique.precluster.pick.pick.an.unique_list.shared)
s
sub.sample(shared=JV1_2_3_4.trim.contigs.good.unique.good.filter.unique.precluster.pick.pick.an.unique_list.shared, size=7000)

rarefaction.single(shared=JV1_2_3_4.trim.contigs.good.unique.good.filter.unique.precluster.pick.pick.an.unique_list.shared, calc=sobs, freq=100)

summary.single(shared=JV1_2_3_4.trim.contigs.good.unique.good.filter.unique.precluster.pick.pick.an.unique_list.shared, calc=nseqs-coverage-sobs-invsimpson, subsample=7000)

heatmap.bin(shared=JV1_2_3_4.trim.contigs.good.unique.good.filter.unique.precluster.pick.pick.an.unique_list.0.03.subsample.shared, scale=log2, numotu=50)

dist.shared(shared=JV1_2_3_4.trim.contigs.good.unique.good.filter.unique.precluster.pick.pick.an.unique_list.0.03.subsample.shared, calc=thetayc-jclass)

tree.shared(phylip=JV1_2_3_4.trim.contigs.good.unique.good.filter.unique.precluster.pick.pick.an.unique_list.0.03.subsample.thetayc.0.03.lt.dist)

pcoa(phylip=JV1_2_3_4.trim.contigs.good.unique.good.filter.unique.precluster.pick.pick.an.unique_list.0.03.subsample.thetayc.0.03.lt.dist)

nmds(phylip=JV1_2_3_4.trim.contigs.good.unique.good.filter.unique.precluster.pick.pick.an.unique_list.0.03.subsample.thetayc.0.03.lt.dist, mindim=3, maxdim=3)

amova(phylip=JV1_2_3_4.trim.contigs.good.unique.good.filter.unique.precluster.pick.pick.an.unique_list.0.03.subsample.thetayc.0.03.lt.dist, design=JV1_2_3_4.boreholes.design.group

APPENDIX C QUANTITATIVE PCR OF OIL SANDS TAILINGS

C.1. Introduction

Oil sands tailing facilities in Alberta, Canada contain high concentrations of sulfate because gypsum is added to thicken the tailings. The high concentrations of sulfate could promote SRB growth and activity since SRB use sulfate as their terminal electron acceptor. Relatives of SRB have been identified in sequencing studies of gypsum-amended tailings including from the oil sands tailings samples used in this experiment (Heaton, 2016). These SRB could produce hydrogen sulfide, a highly toxic compound, which could threaten local plant and animal life if mobilized into surrounding water systems (Lamers et al., 2013; Smith et al., 1976). Hydrogen sulfide could then slow reclamation processes by hindering plant growth in closure environments. The production of sulfides can decrease the redox potential of the environment and methanogens may then flourish because require low redox potentials (Wolfe et al., 2011). Methane is a potent greenhouse gas and an increase in methanogen populations could lead to the release of methane from the tailings (Lashof et al., 1990). An analysis of SRB populations would be useful in assessing the potential risk for SRB activity.

High-throughput amplicon sequencing can provide information regarding taxonomy, richness, and relative abundance of sequence reads classified. Semi-quantitative information from this data, however, is limited to percent abundance. In high-throughput sequencing there are amplification steps prior to and during sequencing that prevent truly quantitative analyses of the DNA. The targeting of metabolic genes of SRB (e.g. *dsrA*) in qPCR studies can provide quantitative estimates of SRB populations. Analyses using qPCR of universal bacterial target genes such as 16S rRNA gene regions can provide semi-quantitative estimates of total prokaryotes in the samples.

C.1.1 Objectives

The objectives of this study were to:

1. Quantify the gene copy numbers of total bacteria and sulfate-reducing bacteria in oil sands tailings using qPCR and calculate ratios of these gene copy numbers, and

2. Compare the ratio of *dsr*/total bacteria gene copies calculated using qPCR data to the relative abundances of SRB to total bacteria from high-throughput sequencing data results.

C.1.2 Methods

C.1.2.1 *Samples*

Samples for this project come from four CFT deposits at the Mildred Lake mine site, north of Fort McMurray, Alberta, Canada (Figure 2.1 in Heaton, 2016). The Mildred Lake mine site is operated by Syncrude Canada Ltd. Samples from the full scale deposit EV-2 are the subject of the qPCR studies presented here (Heaton, 2016).

C.1.2.2 *Quantitative Polymerase Chain Reaction (qPCR)*

Quantitative PCR provides semi-quantitative information that can support our interpretation of sequence read proportions from the Illumina MiSeq sequencing results. Select genes can be quantified using qPCR and gene-specific primers. Fluorescent dyes that bind to dsDNA can identify the number of times a gene is amplified by monitoring fluorescence during each PCR cycle to generate a fluorescence curve. The fluorescence curves can be used in conjunction with the standards' fluorescence curves for known gene copy numbers to identify the quantity of the gene in a sample (Figure C-1).

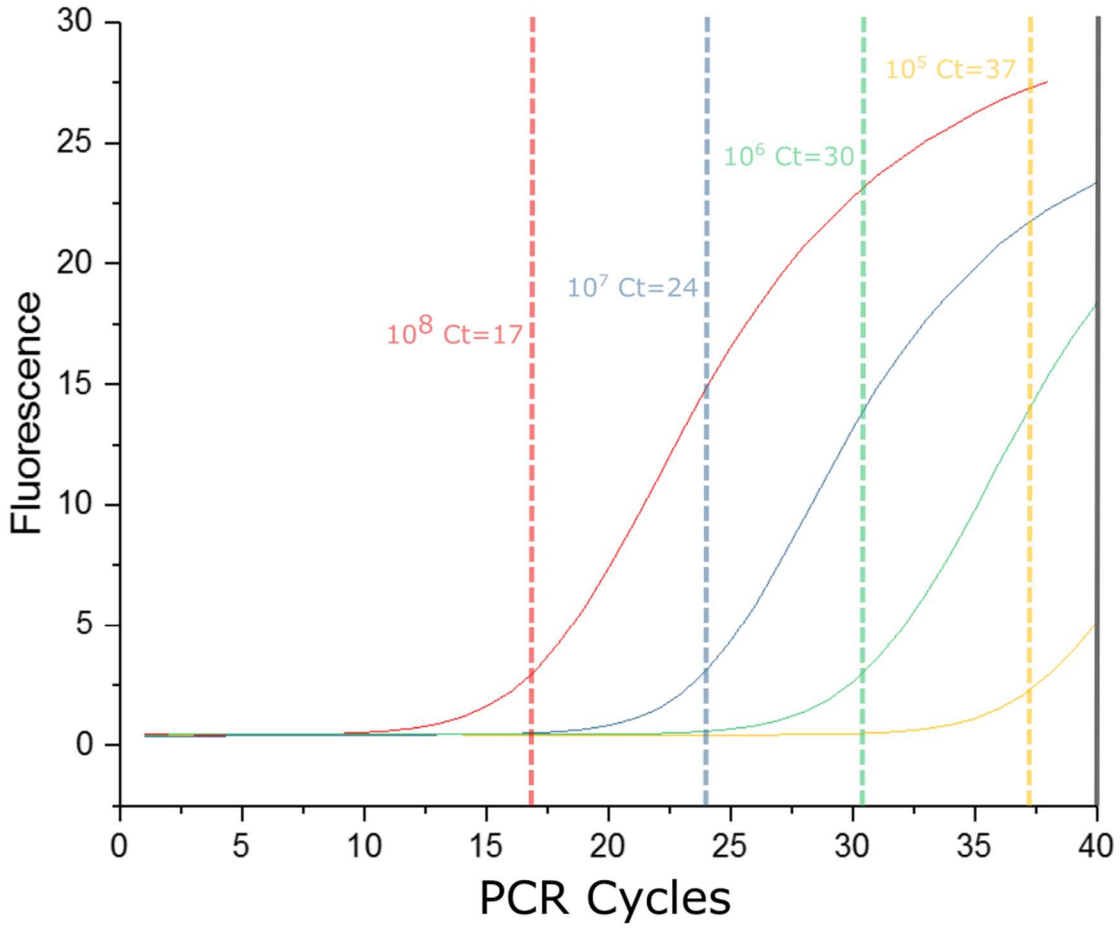

Figure C-1 An example fluorescence curve of qPCR standards. The cycle number at the beginning of the exponential phase in fluorescence signal is the cycle threshold (Ct). Each of the standards' Ct values are as the dotted coloured lines. The thick grey line at 40 PCR cycles is the maximum PCR cycles run. The standard curve is generated from the standard Ct values can be used to estimate the gene quantity in samples when Ct values are plotted against a standard curve.

I performed qPCR using a StepOne™ Real-Time PCR System (Applied Biosystems). I have used primers that target the *dsrA* gene for SRB and a 16S rRNA gene amplicon from *Desulfovibrio desulfuricans* was used as a negative control (Table C-1). The 16S rRNA gene primers were used to estimate the number of prokaryotes in each sample and a *dsr* gene amplicon from *Desulfovibrio desulfuricans* was used as a negative control. The qPCR of 16S rRNA genes can only be used as an estimate of bacteria present because many bacterial species contain multiple copies of the 16S rRNA gene. The qPCR analyses were performed in triplicate for each replicate sample, a total of nine reactions per sample point. The results from these analyses were

averaged and plotted with standard errors of the replicates. No template controls (NTCs) were reactions without any DNA added to the reaction (DNA-free PCR water was added instead). I analyzed gene quantities using a standard curve generated from dilutions of amplified 16S rRNA gene and *dsrA* genes amplified from *Desulfovibrio desulfuricans* (DSMZ 642) genomic DNA (extracted by K. Heaton; Heaton, 2016). Standards were amplified by PCR using qPCR primers (Table C-1) and run on an electrophoresis gel (1.2 %, Lonza FlashGel System) to identify band number and size to ensure amplification of the correct target amplicon (Section 2.3.1.3 method details). Standard DNA concentrations were determined using the Qubit 2.0 and dilutions were created according to the StepOne Real-Time PCR System (Applied Biosystems) recommendations for generating the standard curves. The measured concentrations and amplicon lengths were used in online calculators to determine copy numbers (http://cels.uri.edu/gsc/cndna.html, http://scienceprimer.com/copy-number-calculator-for-realtime-pcr).

Table C-1 Primers and sequences used in qPCR analyses or recommended for future work.

Target gene	Method	Forward Primer	Reverse Primer	Reference
16S rRNA gene	qPCR	uni340F 5' CCTACGGGRBGCASCAG-3'	uni806R 5' GGACTACNNGGGTATCTAAT-3'	(Takai et al., 2000)
dsrA (SRB)	qPCR	DSR1F-5'-ACSCACTGGAAGCACG-3'	RH3-dsrR-5'-GGTGGAGCCGTGCATGTT-3'	(Ben-Dov et al., 2007)
aps (SRB)	qPCR	APS7F-5'-GGGYCTKTCCGCYATCAAYAC-3'	RH2-apsR-5'-ATCATGATCTGCCAgCGgCCGGA-3'	(Ben-Dov et al., 2007)

DNA concentrations were determined as described in 2.5.1.1 by Kaitlyn Heaton; briefly, the Qubit HS dsDNA Assay Kit (Life Technologies, CA, USA) was used with the Qubit 2.0

Fluorometer and the Epoch Microplate Spectrometer with Take3 plate as instructed by the manufacturers.

C.1.3 Results and Discussion

Primers targeting the 16S rRNA gene and *dsrA* were used for qPCR analysis. The 16S rRNA gene and *dsrA* gene amplicons used as standards were successfully amplified and sequencing from amplicons to confirm gene identity, size, and organism of origin matched the source organism *Desulfovibrio desulfuricans* (DSMZ 642). Standards were made for a series of 8 dilutions, 10^1 to 10^8 gene copies in 10-fold increments, for both 16S rRNA and *dsrA* genes. After examining the standard curve for 16S rRNA gene standards, it was noted the 10^3 (10^2 for *dsrA*) to 10^1 dilutions were poorly separated by ΔCt. Thus, for the remainder of the analyses, dilutions 10^8 to 10^4 were used for 16S rRNA gene standards and 10^8 to 10^3 for the *dsrA* gene standards. 16S rRNA gene qPCR amplifications did not have amplification in NTCs or negative controls. Standards for 16S rRNA gene resulted in an R^2 value of 0.99.

The NTCs for *dsrA* gene qPCR amplification resulted in late, >30 Ct, amplifications. The NTCs are reactions without any DNA (DNA-free water is substituted for the DNA sample), and therefore there should be no amplification in the NTC samples. The amplification of NTC samples in the *dsrA* gene analyses could be due to erroneous amplification (non-specific primer binding and amplification) or contamination of the reagents. In order to further troubleshoot the issue it is important to identify the size of amplicons produced. The amplified products from qPCR amplifications were run using gel electrophoresis to examine band presence and size. NTCs run on gels resulted in bands roughly 200 bp in size which is the expected band size for positive *dsrA* gene amplification (Figure C-2). Different master mixes were used to examine whether the master mix was the source of the contamination. EconoTaq PLUS (Lucigen), used to produce the standard dilution series, and Power SYBR Green (Applied Biosystems) Master Mixes, multiple tubes of the same batch were used, (Figure C-2) were used in addition to PerfeCTa SYBR Green (Quanta Biosciences). All three were run in the PTC-200 Peltier Thermal Cycler (MJ Research) and the amplified products were run on the Lonza FastGel System. There were no bands of expected band length for the *dsr* gene in either the Power SYBR Green or the EconoTaq mixes. PerfeCTa SYBR had a roughly 200 bp band which was consistent with the

qPCR product bands when run on gels. Thus, we concluded that the PerfeCTa SYBR master mix we used was either contaminated or non-specifically amplified with the *dsrA* gene primers. We did not have the issue with 16S rRNA gene amplifications and this could mean non-specific amplification was the problem or that contamination of the master mix batch with *dsrA* gene was the problem. The qPCR results for the *dsrA* gene will not be included in the data analyses for these oil sands tailings samples because of the amplification in the NTCs.

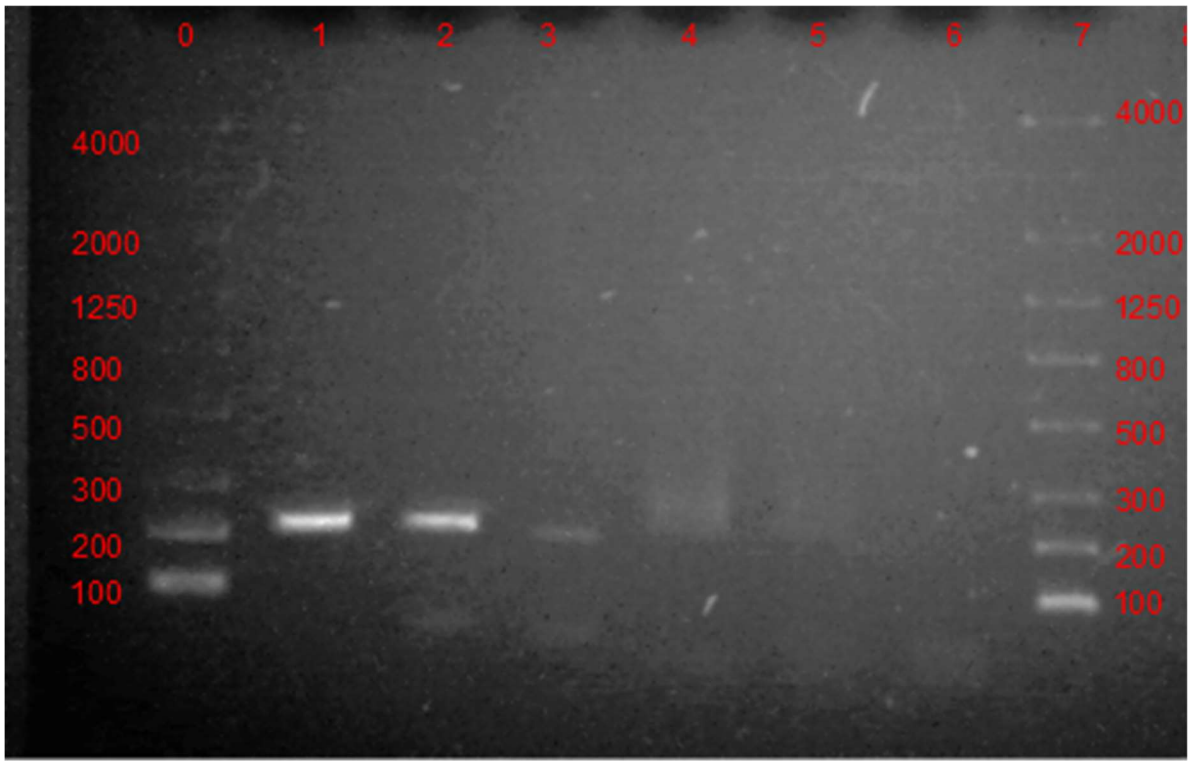

Figure C-2 Gel of *dsrA* amplifications using different master mixes. Lanes 0 and 7 are control ladders containing known lengths of DNA. Lanes 1, 2, 3 are reactions using PerfeCTa SYBR Green master mix on genomic (1), diluted standard product (2), and NTC (3). Lanes 4, 5, 6 are amplifications using Power SYBR Green master mix of genomic (4), diluted standard product (5), and NTC (6).

The resulting values are averages of nine replicates for each sample (DNA extracted in triplicate and each replicate was amplified in triplicate). The 16S rRNA gene qPCR showed the highest quantities of 16S rRNA gene copies present were in the samples at 10 cm depth from each core. Borehole EV-2-4 had the most samples for qPCR analyses. The 16S rRNA gene copy quantities in borehole EV-2-4 increases from 30 to 120 cm until a drop in copy quantity at 150 cm. The 16S rRNA gene copy quantity increases from 150 to 180 cm (Figure C-3).

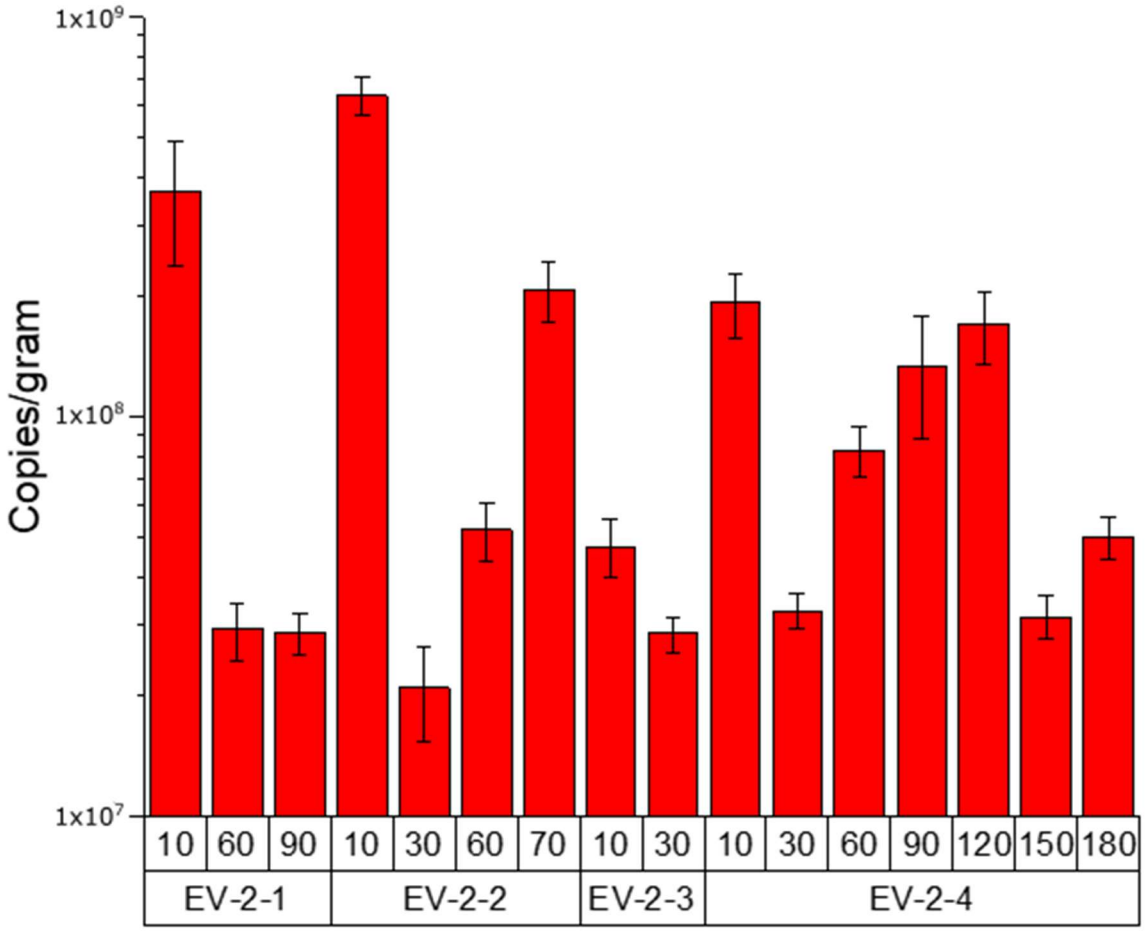

Figure C-3 Graph of 16S rRNA gene copies per wet gram tailings amplified during qPCR. Copies/gram was determined based on a target weight of sample DNA extracted of 0.5 g into 150 µL. Samples are from site EV-2 cores 1 to 4. Above sample location ID is the depth in centimeters.

A comparison of copy numbers to sample DNA concentrations (n=16) yielded an R^2 of 0.94 (Figure C-4).

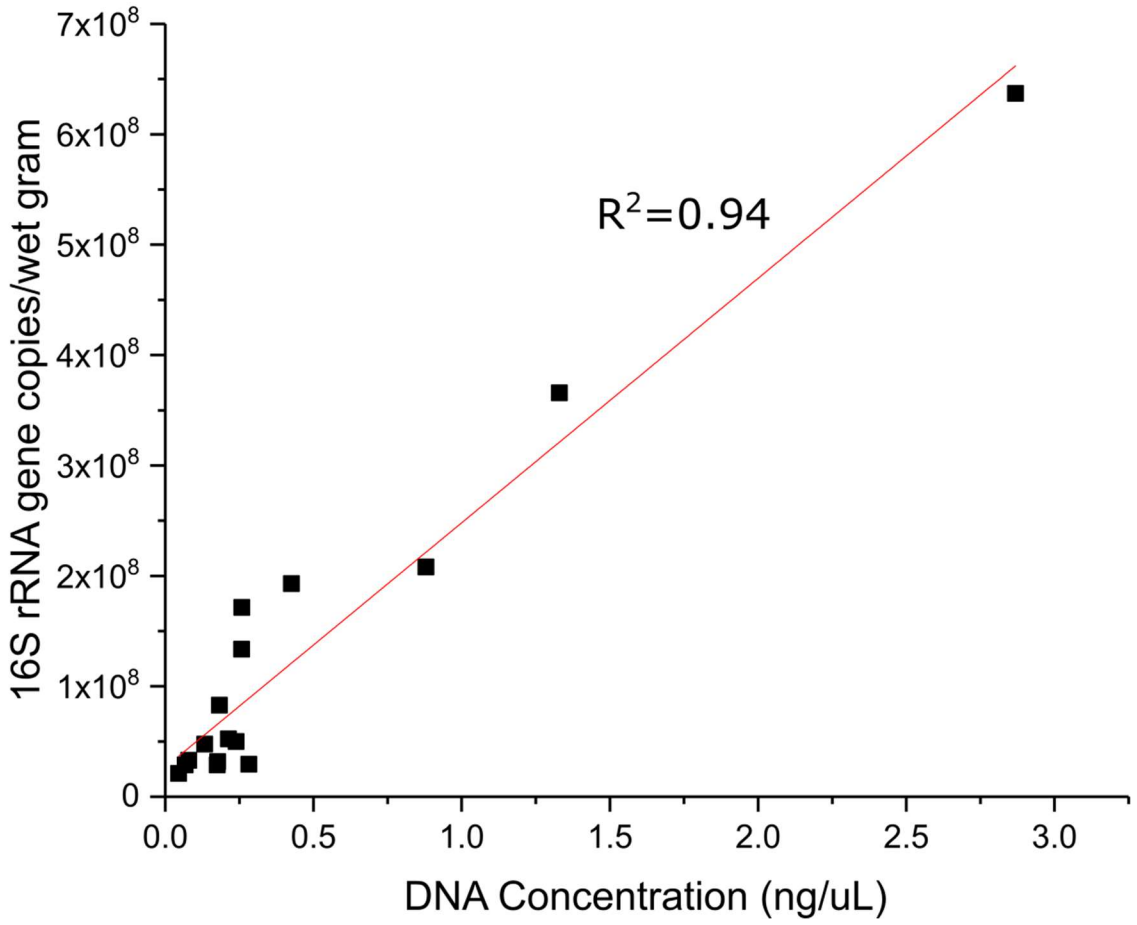

Figure C-4 Comparison of concentration and 16S rRNA gene copies per gram of wet tailings.

C.1.4 Conclusions

The amplification of product roughly 200 bp in size in NTCs using PerfeCTa SYBR Green could be due a variety of factors (Figure C-2). Amplification could be from contamination with *dsr* genes from amplified sources, potential background DNA from the master mix, or erroneous amplification of primers. Contamination with *dsr* genes from amplified sources would require systematic contamination during handling as testing multiple unopened tubes of PerfeCTA SYBR Green resulted in the same findings but the absence of 200 bp products in the other master mixes used make this explanation less likely. The random amplification of primers would likely occur in other master mixes if it was because of ambiguous primer binding. There were no issues with NTCs when amplifying 16S rRNA genes and suggests a problem related specifically to the *dsrA* gene qPCR amplification.

The successful quantification of 16S rRNA genes using qPCR and the strong correlation with DNA concentrations provides confidence in the bacterial population estimates using qPCR with these primers, despite their low concentration. If future efforts to quantify SRB are successful, then comparisons of SRB to total bacteria can be done with some confidence.

C.1.5 Future Recommendations

The future work regarding this data and samples should involve the examination of other master mixes to help identify what issues lead to the amplification in NTCs that result in product ~200 bp. This information will allow us to identify a master mix that can be used with the selected primers to conduct further, more accurate, quantification of SRB populations with master mixes that are not likely to result in NTC amplification.

Once an appropriate master mix is selected, qPCR should be continued using *dsr* gene and *aps* gene targeting primers. A comparison between the copy numbers from amplification of these genes will help verify the utility of these primers for quantification of SRB. The use of either gene as a measure of SRB and 16S rRNA gene qPCR can be used to determine proportion of SRB to total bacteria and further proportional comparisons against high-throughput amplicon sequencing can then be conducted.

APPENDIX D PCR PROTOCOLS

16S – 27F/1492R

1) 95˚C for 4 min
2) 95˚C for 30 s
3) 57˚C for 30 s
4) 72˚C for 1 min 30 s
5) Go to 2, cycle 35 times
6) 7˚2C for 10 min
7) Hold at 4˚C.

dsr

1) 95˚C for 4 min
2) 95˚C for 40 s
3) 56˚C for 40 s
4) 72˚C for 40 s
5) Go to 2, cycle 35 times
6) 72˚C for 10 min

arrA

1) 94˚C for 2 min
2) 94˚C for 30 s
3) 64˚C for 30 s
4) 72˚C for 30 s
5) Go to 2, cycle 35 times
6) 72˚C for 8 min
7) 4˚C hold

qPCR 16S uni340F/uni806R

1) 95˚C for 10 min
2) 95˚C for 15 s
3) 55˚C for 45 s
4) Go to 2, cycle 40 times

qPCR *dsrA*

1) 50˚C for 2 min
2) 95˚C for 15 min
3) 95˚C for 15 s
4) 60˚C for 1 min
5) Go to 3, cycle 40 times