A METAGENOMIC ANALYSIS OF TAILINGS MICROBIAL COMMUNITIES FROM BOTH COLD AND HOT ENVIRONMENTS

by

Arielle Kirbie Bieniek

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Thesis Examiners/Examinateurs de thèse:

Dr. Nadia Mykytczuk
(Co-Supervisor/Co-directrice de thèse)

Dr. Thomas Merritt
(Co-Supervisor/Co-directeur de thèse)

Dr. Jeffrey Gagnon
(Committee member/Membre du comité)

Dr. Lisa Gieg
(External Examiner/Examinateur externe)

Approved for the Faculty of Graduate Studies
Approuvé pour la Faculté des études supérieures
Dr. David Lesbarrères
Monsieur David Lesbarrères
Dean, Faculty of Graduate Studies
Doyen, Faculté des études supérieures

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Abstract

Mining practices produce a substantial waste product in the form of tailings, a problematic liability particularly in materials where iron and sulfur become oxidized leading to acid mine drainage (AMD). Native microbial consortia in tailings sites accelerate this oxidation by a factor of 10^6. The oxidative capabilities of these consortia can be harnessed to bioleach low-grade, refractory metals from the waste materials while also allowing for the potential stabilization of nuisance elements. This project explores the contributions of native microbes isolated from sulfide tailings from two different climates: colder climate tailings around Sudbury, Ontario and warmer climate arsenopyrite refractory gold tailings from Ecuador (ECT). The cold community project encountered technical challenges as is summarized here as an appendix. This thesis focuses primarily on the ECT community. The ECT tailings were enriched in medium ahead of bioleaching trials and a metagenomic analysis was performed to identify key organisms responsible for driving bioleaching. The main contributors to the ECT system at the order level were *Acidithiobacillales*, *Bacillales*, *Burkholderiales*, *Clostridiales*, and *Thermoplasmatales*. The dominant organisms representing these orders were found to have complementary genetic systems that drive iron and sulfur oxidation. Understanding these key players will help optimize the conditions that the ECT culture will be applied in using stirred-tank bioreactors and will provide the baseline metagenomic information to help monitor the health of these organisms throughout bioleaching campaigns.

Keywords

Tailings, bioleaching, metagenomics, iron oxidizers, sulfur oxidizers

Co-Authorship Statement

I, Arielle Bieniek, have provided significant contribution to this project and I am the primary author of this thesis and the eventual publication of this work. Dr. Nadia Mykytczuk has provided support in project development, funding, and analytical review to this thesis. Dr. Thomas Merritt has provided contributions to study execution, funding, and analytical review to this thesis.

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List of Abbreviations

Abbreviation	Definition
°C	Degrees Celsius
µm	Micrometer
16S rRNA	Component of 30S small subunit of a prokaryotic ribosome
18S rRNA	Eukaryotic cytosolic homologue of 16S rRNA
AMD	Acid mine drainage
Aw	Region classified as tropical savanna climate
bp	Base pair
COG	Cluster of Orthologous Groups
CPM	Counts per million
Dfc	Subarctic climate
DNA	Deoxyribonucleic acid
ECT	Ecuador tailings
Fe	Iron
g	Grams
g/L	Grams per liter
g/t	Grams per ton
GC	Guanine and cytosine
gDNA	Genomic deoxyribonucleic acid or chromosomal DNA
ICP	Inductively coupled plasma
IOB	Iron oxidizing bacteria
IRB	Iron reducing bacteria
KEGG	Kyoto encyclopedia of genes and genomes
L	Liter
MG-RAST	Metagenome Rapid Annotation using Subsystem Technology

min	Minute
mL	Milliliter
mm	Millimeter
MS	Mass spectrometry
NCBI	National Center of Biotechnology Information
nm	Nanometer
ng	Nanogram
OD_{500}	Optic density at 500 nanometers
PCR	Polymerase chain reaction
QC	Quality control
RAST	Rapid Annotation using Subsystem Technology
RNA	Ribonucleic acid
rpm	Revolutions per minute
rRNA	Ribosomal ribonucleic acid
S	Sulfur
SOB	Sulfur oxidizing bacteria
SOX	Genes involved in sulfur oxidation pathway
SRB	Sulfur reducing bacteria
STR	Stirred tank reactor
TCA	Tricarboxylic acid cycle
TK medium	Tuovinen and Kelly medium
w/v	Weight per volume

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Chapter 1

1 An Overview of Mine Tailings and Solutions

1.1 Mine Waste and Acid Mine Drainage (AMD)

Conventional metal extraction of sulfide and iron ores includes numerous steps and waste products that create long-term waste management challenges for mining companies (Morin & Hutt, 2001). One of the waste products, known as tailings, are rejected at the milling stage. If the rejected minerals are rich in iron and sulfur (such as pyrite and pyrrhotite) they react with air and water, become oxidized, and can promote the release of a metal-rich seepage known as acid mine drainage (AMD) that can affect local environments and water systems (Kalin *et al.*, 2005; Zinck & MEND, 2005). AMD production is accelerated exponentially by the local microbial community, comprised of bacteria and archaea (Johnson & Hallberg, 2005) as well as fungi (Baker *et al.*, 2004), that have adapted to the unique microclimate of tailings. These microbial communities have high oxidative powers, are capable of utilizing iron and sulfur as electron donors to meet their own energy requirements, and in turn accelerate the oxidation and acid production in sulfide-rich tailings (Johnson & Hallberg, 2005; Singer & Stumm, 1970).

Acid production in sulfide tailings can be described in the following four reactions. Much of the iron and sulfur in these systems is found as pyrite (FeS_2), which upon biotic and abiotic oxidation produces ferrous iron (Fe^{2+}) and sulfate (SO_4^{2-}) (Equation 1) (Baker & Banfield, 2003). Bacterial intervention from sulfur oxidizing organisms, such as *Acidithiobacillus* and *Acidiphilium*, are contributors to the oxidization and breakdown of pyrite at this stage (Leduc *et al*., 2002). Ferrous iron can be further oxidized to ferric iron (Fe^{3+}) by iron oxidizing acidophiles, including *Acidothiobacillus* and *Leptospirillium* (Equation 2) (Baker & Banfield,

2003). As the pH lowers, the ferric iron will perform two tasks, first it will react with hydroxides and precipitate out iron hydroxides (Equation 3) (Akcil & Koldas, 2006). Second, the ferric iron acts independently as an oxidizing agent and will oxidize pyrite, generating more ferrous iron, which iron oxidizers will act upon further regenerating this cycle (Equation 4).

1. $FeS_2 + 7/2\ O_2 \rightarrow Fe^{2+} + SO_4^{2-} + 2\ H^+$
2. $Fe^{2+} + ¼\ O_2 + H^+ \rightarrow Fe^{3+} + ½\ H_2O$
3. $Fe^{3+} + 3\ H_2O \rightarrow Fe(OH)_3 + 3\ H^+$
4. $FeS_2 + 14\ Fe^{3+} + 8\ H_2O \rightarrow 15\ Fe^{2+} + 2\ SO_4^{2-} + 16\ H^+$

The solubilization of metal and metalloid species in AMD is influenced by several conditions, including pH, temperature, water and oxygen availability, and the diversity of the microbial community (Morin & Hutt, 2001; Simate & Ndlovu, 2014). The combination of these factors is site-specific, suggesting that the prediction of AMD rates, containment of environmental damage, and treatment of tailings must be addressed on an individual basis (Simate & Ndlovu, 2014).

1.2 Dealing with Mine Waste

Mining companies are required by law to maintain their mine sites post-production in an attempt to return the affected area to a socially and environmentally accepted area (Mining Watch Canada, 2000; NAOMI, 2019). However, these expectations have not grandfathered in thousands of abandoned mine sites prior to the onset of these strict regulations, consequently leaving governments with a global mine waste problem (Mining Watch Canada, 2000; NAOMI, 2019). These tailings are also repositories of low-grade ores of considerable economic value. It is estimated that in Sudbury alone, roughly 800,000 tons of nickel has been lost through traditional processing and deposited in tailings, translating to roughly $8.3 billion dollars in US currency (Peek *et al*., 2011). Although milling and smelting processes are economically viable, the

generated waste and environmental liability has encouraged the mining industry to research and implement new economical and environmentally-friendly solutions to these waste problems. Given the environmental and financial incentives of treating tailings, it is in the best interest of the mining industry to pursue low-cost, sustainable methods for treating their mine waste issues. Bioleaching can be employed as a solution to mine waste in passive applications, such as heap leaching, or in active systems, such as medium or high temperature stirred tank bioleaching using native or defined consortia of oxidizing microbes.

Colder climates, such as Northern Ontario, are classified as a subarctic climate, or Dfc, by Koppen and Geiger (Kottek *et al*., 2006). Dfc climates are characterized by short summer seasons not lasting longer than 3 months with a minimum average temperature of 10 °C, and 5-7 cold months having an average low temperature below 0 °C (Kottek *et al*., 2006). Warmer climates, such as the Camilo Ponce Enríquez region in Ecuador, are classified as a tropical savanna climate, or Aw, by Koppen and Geiger (McKnight and Hess, 2000). The mean annual temperature is 25.1 ºC with an average precipitation of 877 mm (Climate Data, 2019).

1.2.1 Passive Application: Heap Bioleaching

Heap bioleaching occurs when crushed ore is piled into heaps then doused in sulfuric acid to promote the growth of acidophilic microbes that will carry out the brunt of oxidation (Cardenas, Quatrini, & Holmes, 2016). Heap bioleaching is especially appealing thanks to its low operational costs, the relative passivity of the work being carried out, and the generation of monetary value from otherwise waste materials (Pradhan *et al.*, 2008). The microbial consortia are comprised of iron- and sulfur- oxidizing microbes, capable of oxidizing the pyrite and pyrrhotite present in many high-sulfur tailings. These microbes lower the pH of their environment (Equations 1-4) and are able to indirectly mobilize desired metals through iron and

sulfur oxidation and indirectly immobilize other metals, like arsenic, through oxidative processes.

A unique advantage of these systems is that they are not often contaminated by other microbes as the tailings environment is extreme, making these systems relatively easy to maintain in terms of contamination (Pradhan *et al.*, 2008). Alternately, some of the challenges faced by heap bioleaching are difficulties maintaining proper oxygen and carbon availability for peak microbial activity, the heterogenous nature of the piles which makes predicting reaction kinetics difficult (Baker & Banfield, 2003; Morin & Hutt, 2001), and the creation of temperature and pH gradients from the surface of the piles towards the center (Pradhan *et al.*, 2008).

Heap leaching could be of interest for re-processing materials from abandoned mine sites where either the cost of traditional processing infrastructure of treatment is too high, or where transport of the waste to secondary sites for reprocessing is not financially viable. Heap leaching has been successfully employed on low-grade ore at the Mianhuakeng uranium mine in northern Guangdong, where 88.3% uranium leaching efficiency was achieved using mixed acidophilic microbes (Wang *et al*., 2017).

In colder climates, the initiation and operation of heap leaching poses new challenges considering the seasonal temperature variations, including potentially extreme winter cold. Ideally, heap leaching would be carried out all year round, with little to no downtime during the colder months. To address this challenge, we are searching for and screening different psychrotolerant/psychrophilic acidophilic microbes that are able to carry out their oxidative capabilities at temperatures as low as 5 ºC (Liljeqvist *et al.,* 2011) and are important for initiating the oxidative process when ambient temperatures are low and for reducing edge effects in the heap during colder months. Heap leaching in colder climates has been successfully demonstrated

in Talvivaara, Finland where outside temperature fluctuations ranged between – 39 ºC and 30 ºC (Halinen *et al*., 2012). Colder temperatures and mineral oxidation progression result in decreased microbial diversity, and in this study *Acidithiobacillus ferrooxidans* dominated the microbial community (Halinen *et al*., 2012), making this a species of interest for pursuing cold heap leaching in Northern Ontario.

1.2.2 Active Application: Bioleaching with Bioreactors

Bioleaching using stirred tank reactors is a process which uses native or defined microbial consortia to extract target metals from mineral ores and their concentrates (Rawlings & Johnson, 2007). Bioreactors work by employing the biooxidative powers of iron- and sulfur-oxidizing microbes to release target metals with much faster kinetics compared to heap leaching operations (Rawlings & Johnson, 2007). These reactors operate at controlled temperatures, pH levels, oxygenation, carbon, and nitrogen sources to ensure constant and consistent results of metal release from ore or tailings. The biogeochemistry of tailings or ore materials being processed using stirred tank reactors must be evaluated to set appropriate parameters as they can change the leaching results dramatically (Cameron *et al*., 2009).

Advantages of the stirred tank reactors are that they allow for a homogenous mixture which allows for predictable kinetics, greater control over abiotic factors, and a faster model given the operational temperatures (Zhou *et al*., 2009). Cameron *et al*. (2009) performed a bioleaching campaign on low grade ultramafic nickel sulphide ore from Manitoba, containing 0.3% nickel. The different campaign parameters suggest bioleaching at a higher pH (5.0) and at lower temperature (5 ºC) increases the nickel to magnesium ratio in the leachate while reducing sulphuric acid consumption (Cameron *et al*., 2009). Contrarily, the use of moderately

thermophilic microbes from chalcopyrite mine sites in China have a copper extraction rate of 75% when using low pH (2.0) and high temperature (48 ºC) (Zhou *et al*., 2009).

A disadvantage to this method is that the process materials often contain native microbial consortia that compete with those added to the operating reactors, which can lead to some unpredictability and in some cases can significantly impact the leaching efficiency (Bryan *et al.*, 2011; Rawlings & Johnson, 2007). Stirred tank reactors generally operate at mesophilic (20-40 ˚C) or thermophilic (>45 ˚C) temperatures to improve the reaction kinetics, meaning that we must source mesophilic or thermophilic acidophiles, respectively, that are capable of carrying out their oxidative capabilities at specific temperatures (Zhou *et al*., 2009).

1.3 Microbes of Interest for Bioleaching

The microbial communities that thrive in AMD systems often change in abundance and composition based on biogeochemical compositions of tailings materials and environmental factors including temperature, pH, and water availability (Auld *et al*., 2017; Baker & Banfield, 2003). It is important to comprehensively understand the microbes being used for remediation and leaching purposes to ensure optimum results. A combination of heterotrophs, who access energy from organic sources such as carbon, lithotrophs, who access their energy from inorganic sources such as iron and sulfur, and mixotrophs, who can utilize both organic and inorganic sources for energy, are found in AMD communities. Some organisms of interest include iron- and sulfur-oxidizing bacteria (IOB, SOB) and iron- and sulfur-reducing bacteria (IRB, SRB), which contribute to these systems directly through their oxidative and reducing powers. Chemolithotrophs use inorganic compounds which act as electron donors, such as sulfur or iron, to meet their energy needs (Amils, 2014). IOB are often also acidophilic and include *Acidithiobacillus*, a dominant chemoautotrophic ferrous iron oxidizer in the pH range 1.5-2.4

(Edwards *et al*., 1999; Schrenk *et al*., 1998) and *Leptospirillum*, a dominant ferrous iron oxidizer at even lower pH than *Acidithiobacillus* (Schrenk *et al*., 1998). The rates at which different species oxidize ferrous iron differs, for example *Ferrovum* outperformed *Acidithiobacillus* in reactors by oxidizing ferrous iron twice as fast (Rowe & Johnson, 2008; Brown *et al*., 2010). This finding supports the understanding that abiotic factors play an important role in community dynamics and health; for example, pH seems to have the largest impact on the presence of specific species of acidophilic IOB (Kuang *et al*., 2012). Auld *et al*. (2017) found that the microbial community in Northern Ontario tailings changes in response to seasonal temperature shifts. Specifically, he found that the *Acidithiobacillus* genus was in greatest relative abundance throughout the winter sampling months, making this a great starting point to explore its genetic adaptations to low temperatures (Auld *et al*., 2017).

Thiobacillus (SOB) oxidizes reduced sulfur compounds but not ferrous iron and contributes to overall leaching by producing sulfuric acid (Bacelar-Nicolau & Johnson, 1999). The SOB *Sulfobacillus* oxidizes ferrous iron (Fe^{2+}) and reduced sulfur species and are able to tolerate high metal concentrations in bioreactors and heap leaching environments (Watling, Perrot, & Shiers, 2008). *Sulfobacillus* is an effective pyrite oxidizer (Baker & Banfield, 2003), is moderately sensitive to pH and temperature fluctuations, making this genus ideal to use in bioleaching efforts (Watling, Perrot, & Shiers, 2008). Acido- and themophilic *Sulfolobus* is an archaeal genus that is able to oxidize sulfur species (Brock *et al*., 1972) while *Thiomonas* is able to oxidize not only sulfur but also arsenite, making this especially interesting in removing and stabilizing pollutants in AMD (Hallberg, 2010).

Both SRB and IRB are of importance in these systems as they may play a role in other bioleaching processes that use reductive dissolution of minerals, or in the ultimate remediation of

waste sites to stabilize materials and prevent AMD. SRB and IRB can be found in the anoxic layers of tailings, and function to increase the pH in these systems (Glick, 2010). Heterotrophic *Acidiphilium* is capable of reducing ferric iron to ferrous iron (Johnson & Bridge, 2002), heterotrophic *Ferroplasma* is an archaeal genus capable of performing oxido-reduction of iron (Hallberg, 2010), while *Desulfitobacterium* have been shown to reduce both sulfite and sulfate depending on the species (Johnson *et al*., 2009; Hallberg, 2010). In heap leaching systems, detection of these microorganisms can identify undesirable processes are occurring and highlight areas that require additional aeration. It is therefore important to understand the community as a whole to create models of biological activity that can have measurable impacts on leaching rates in both heap and bioreactor systems.

1.4 Genomic Analysis

Genomic analysis tools coupled with comprehensive bioinformatic pipelines allow for high quality resolution of genomic information (Gasperskaja & Kucinskas, 2017). Genomic methodologies range from analyzing individual genes for fingerprinting identification purposes, commonly used with 16S rRNA, to more complex processes including whole metagenome high-throughput sequencing, commonly used from environmental samples (Gasperskaja & Kucinskas, 2017). It is important to understand organisms at the cellular level as well as how these organisms interact with one another as a community. Starting at the genomic level, we can gather relevant data regarding which enzymatic pathways are present or absent as well as which organisms are contributing to each of these pathways. Recognizing where processes are highly conserved and which organisms contribute to unique rate-limiting steps that drive these systems is an important part in understanding community dynamics.

Bioleaching communities are no different, these complex communities are comprised of a multitude of organisms that play important roles in the intricate oxidation and reduction processes to maintain specific and important balances. An in-depth look at individual genomes allows us to tease out who are the key players in specific AMD or bioleaching environments and predict microbial interactions (Cardenas, Quatrini, & Holmes, 2016). These data can help monitor the health of these key organisms to ensure leaching reactions are going to continue operating optimally. The use of metagenomics can be employed for species that are traditionally difficult to isolate and culture in a laboratory setting. Zhang *et al*. (2016) found that *Acidithiobacillus*, *Leptospirillum*, and *Thiobacillus* dominated in the areas of nutrient cycling and metabolism while demonstrating adaptive mechanisms to heavy metal resistance, organic solvents tolerance, and detoxification of hydroxyl radicals in a copper bioleaching heap. Hu *et al*. (2015) performed metagenomics from a heap bioleaching system and found that autotrophic *Acidithiobacillus* and heterotrophic *Acidiphilium* were both equally essential to their bioleaching process.

The connection between genomic sequences, translated proteins, enzymatic reactions, and metabolic changes all help to paint a detailed picture of what is occurring at the organism and community level in these extreme environments. The completed genomic sequence of thermoacidophilic archaeon *Metallosphaera sedula* found putative genes encoding iron and sulfur oxidation (Auernik *et al*., 2007). Combined with transcriptional data, these putative iron and sulfur oxidation genes were shown to be upregulated twofold when *M. sedula* was in the presence of ferrous sulfate, indicating this archaeal species is indeed performing oxidation in this system (Auernik *et al*., 2007). Understanding the genomic connection will help infer key

functional genes or enzymes could also be used as monitoring targets that might be easily measured by industry and help optimize the processes in either heap leaching or bioreactors.

1.5 Thesis Questions and Objectives

This thesis explores a metagenomic analysis of microbes that will be used in different leaching systems 1) a heap leaching application in a cold system located in Northern Ontario, and 2) a bioreactor application in a warm system using materials from Ecuador. The focus of this thesis is to establish a baseline metagenomic dataset to identify who the key players of these systems are and describe their functional roles. This data will be important in supporting future work where changes in community structure or function in response to temperature and other abiotic factors could be compared.

The first thesis objective was to perform a genomic analysis of cold-adapted bacterium *Acidithiobacillus ferrivorans*, an organism isolated from a nickel mine in Sudbury, ON and has been found to be abundant in cold climate mining systems. Due to time constraints of this thesis, along with insufficient biomass production, this objective was only partially carried out and is described in greater detail in Appendix A.

The second thesis objective, and the main research chapter in this thesis, was to perform a metagenomic analysis of warm-adapted bacterial community from a tailings site from a gold mine in Ecuador. Currently, the Mykytczuk Research Group is carrying out a pilot study of the native Ecuadorian microbial community in bioreactors. A genomic understanding of community dynamics is required to optimize these efforts for application in the bioleaching of arsenopyrite for optimal gold extraction and arsenic stabilization.

A summary is presented in Chapter 3 that explores future directions of these objectives when adequate time and biomass can be sustained from the Northern Ontario AMD system. The

genomic information from both cold- and warm-adapted samples will help gain better understanding of conserved processes and temperature-related differences in bioleaching communities and help advance the application of genomic data to bioleaching operations.

Chapter 2

2 A Metagenomic Overview of Ecuadorian Tailings (ECT)

2.1 Abstract

The Ecuadorian Tailings (ECT) site is comprised of an extremophilic microbial community that thrives in this sulfur-rich environment and have immense oxidation capabilities. This community is capable of breaking down the mineral surface of arsenopyrite to aid in releasing gold which can then be amenable to other forms of extraction, including cyanidation and carbon in leach. Bioleaching efforts in stirred tank reactors (STR) for ECT materials include native consortium which exponentially accelerate the oxidative powers of these microbes at high temperatures. The variation in gold recovery for ECT in the pilot STR has been attributed to our limited understanding of the native microbial consortia. A combination of classical culturing methods, metagenomic sequencing analysis, and annotation tools were used to understand the ECT community dynamics. This project explores metabolic mechanisms used by microbial consortia, including sulfur and iron oxidation, arsenic resistance, and nitrogen and carbon cycling in bioleaching processes allowing industry to optimize microbial growth for maximum gold recovery.

Keywords: *tailings, acid mine drainage (AMD), bioleaching, stirred tank reactors, metagenomics, iron oxidizers, sulfur oxidizers*

2.2 Introduction

Precious metal availability is depleting which encourages the mining industry to revisit their waste material, also known as tailings, to extract low-grade or refractory metals that were not extracted during traditional processes. The tailings also pose an environmental liability to surrounding communities (Morin & Hutt, 2001) as their content, made up often of high quantities of iron and sulfur, are oxidized over time causing acid mine drainage (AMD) (Kalin *et al.*, 2005). AMD is accelerated by the microbial consortia that thrives in these tailings systems due to their high oxidative abilities. These "bioleaching" microbes are a relatively untapped resource that can be used to solve both the loss of revenue deposited into tailings and stabilize the toxic metals that are present in these systems (Johnson & Hallberg, 2003; Johnson & Hallberg, 2005) if harnessed correctly.

Microbial diversity must be assessed on an individual basis in mine waste systems. Though there are commonalities in the consortia seen in tailings systems across the world, the unique environmental and chemical characteristics of mine waste will give rise to site-specific consortia that have adapted and thrive in that particular system (Baker & Banfield, 2003). The microbial diversity can be better understood by using modern molecular biology approaches, including genomic and metagenomic sequencing. It is therefore possible to understanding the genomic differences that drive the oxidative reactions of Fe/S oxidizing bacteria, target which species are responsible for assisting in mobilizing metals and minerals, and what makes the consortia function at optimal conditions.

The microbial consortia enriched from a tailings system in Ecuador, known as ECT, is being used in bioreactor campaigns to extract gold and stabilize arsenic from arsenopyrite tailings. Following initial leaching tests, it was evident that the amount of extracted gold and arsenic

varies substantially between different operating conditions that would be observed at full-scale operation, making for an unreliable system. To better optimize these bioleaching efforts, it is important to understand the community members of the microbial consortia. From here, differing assays can be developed to monitor the health of the consortia in the bioreactors and indicate whether or not enough nutrients are available for the entire system to consistently function at its optimal capacity.

This chapter focusses on creating a metagenomic baseline for the ECT microbial community, focusing on the overall community function and possible energy dynamics. It is important to determine which microbes are in highest abundance, which are contributing to rate-limiting steps in oxidation of iron and sulfur, which may create bi-products that inhibit the growth of important microbes, and how the system functions as a whole. Upon understanding these dynamics, a model can be drawn up to ensure the health of target microbes are being appropriately monitored to ensure high rates of success.

2.3 Methods

2.3.1 Study Site

Tailings for this project were obtained from the Ecuador Tailings (ECT) site with the help of industry partners, BacTech Environmental Inc. The tailings site is located in Camilo Ponce Enríquez, Ecuador, and is classified as a tropical savanna climate, or Aw, by Koppen and Geiger (McKnight and Hess, 2000). The mean annual temperature is 25.1 ºC with an average precipitation of 877 mm (Climate Data, 2019).

The ECT materials along with several other tailings samples were shipped to Sudbury, ON, at ambient temperature and samples were taken immediately after arrival. Sub-samples were sent total metals analyses using ICP-MS analysis (SGS Labs, Lakefield).

2.3.2 Bioreactors

The Ecuadorian tailings are being reprocessed to stabilize sulfur and arsenic while liberating the refractory gold using bioreactors (Figure 2-1). These bioreactors are able to monitor pH and temperature to keep conditions constant. The native microbial community from the tailings are present in all samples and could cause inconsistencies in gold recovery if they compete with defined consortia or could contain well-adapted Fe/S oxidizers that may be better suited for leaching than defined consortia from previous lab trials. To determine the native consortia, and possible identification of dominant bioleaching species, we targeted the initial ECT microbial community for further description.

2.3.3 Medium, Cell Culture, and Growth Rate Conditions

A 50 g sample of raw tailings material was inoculated into a 50 mL flask of Tuovinen and Kelly (TK) liquid medium ([$FeSO_4$•$7H_2O$] = 33.4 g/L; 0.3 % (w/v) each of $(NH_4)_2SO_4$; MgSO4•$7H_2O$; and K_2HPO_4, at a pH of 2.5) (Tuovinen and Kelly, 1973). Growth was monitored using ferric iron concentration as a proxy using the method described in Karamanev *et al*. (2002). Once the Fe^{3+} concentration at 500 nm reached 0.85, we inferred that the community was in late exponential phase as previously described (Mykytczuk et al., 2011; Doran *et al*., 2017). A 10 mL aliquot of ECT was inoculated into eight 1 L flasks of fresh TK medium. These eight biological replicates of ECT in TK medium were incubated on a gyratory shaker at 180 rpm and 30 ºC. Figure 2-2 is a visual representation of the work-flow of this process. Late exponential growth rate allowed for a trade-off of enough biomass to carry out downstream experimentation with the least amount of iron in solution disrupting downstream genomic analyses (Doran *et al*., 2017).

Figure 2-1: Bioreactor set-up for pilot project using Ecuadorian Tailings (ECT) starting materials.

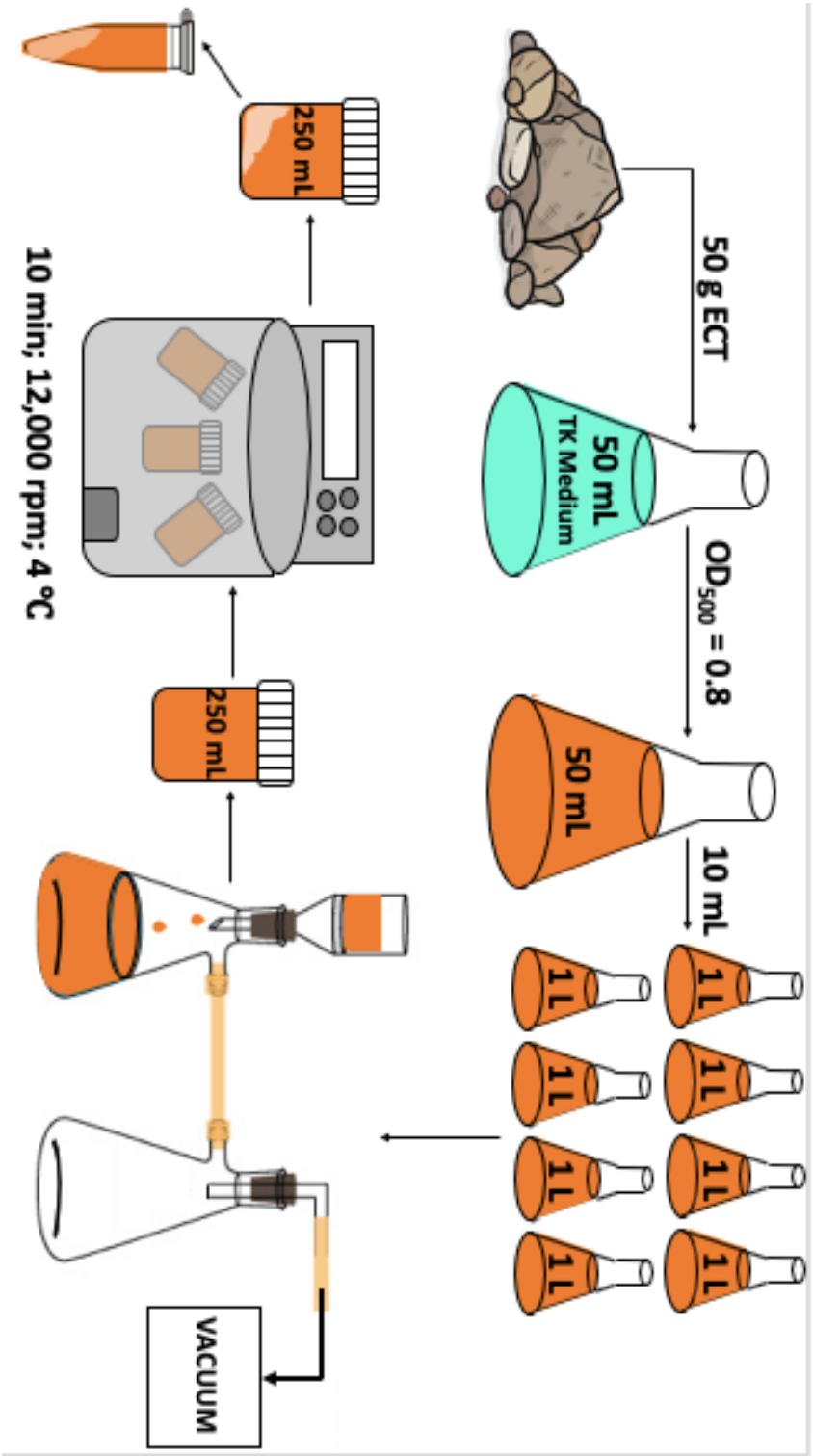

Figure 2-2: Experimental set-up and flow for Ecuadorian Tailings (ECT) project.

2.3.4 Sample Collection and DNA Extraction

Samples were vacuum filtered using Whatman No. 1 filters to remove excess ferric iron (Fe^{3+}) in solution. Filtered cultures were then centrifuged in 250 mL centrifuge tubes at 12,000 rpm for 10 minutes at 4 ºC. Cell pellets were removed from each 250 mL centrifuge tube and pooled into 1.5 mL microcentrifuge tubes then spun for an additional 10 minutes at 13,000 rpm. Additional supernatant was removed, and all cell pellets were pooled. Figure 2.2 is a visual representation of the work-flow of this process.

The pooled cell pellet was processed for DNA extraction using the DNeasy PowerSoil kit (MoBio, CA) and the Sox Soil DNA Extraction Kit (MetagenomBio, CA) following manufacturer's instructions. DNA was quantified and samples were subjected to PCR amplification to determine if positive amplification could occur without inhibition.

Iron hydroxides commonly interfere with the DNA extraction as these compounds hydrolyze DNA and further inhibit PCR amplification. This proves to be a challenge when attempting to extract sufficient, high quality DNA.

2.3.5 DNA Clean-Up, Sequencing, and Assembly

DNA samples were subjected to an ethanol precipitation purification protocol to remove possible contaminants and concentrate nucleic acids. Pure DNA was shipped to the National Research Council (Montreal, QC) on dry ice and stored at -80 °C until ready to process. Purified DNA samples were then quantified using Quant-iT PicoGreen assay (Thermo Fisher, #P7589) according to the manufacturer's procedure.

Libraries for shotgun sequencing were constructed from 2 ng of purified gDNA using Illumina Nextera XT library preparation kit (FC-131-1024), following the manufacturer's instructions. The quality of individual libraries was assessed using a TapeStation instrument and High

Sensitivity D5000 ScreenTape (Agilent, #5067-5592). Library normalization was performed by pooling equimolar amounts of libraries based on molarity values from TapeStation analysis. Prepared libraries were sent to Genome Quebec and sequenced on a HiSeq4000 system (Illumina) using the paired-end 100 bp configuration for 216 cycles.

2.3.6 Assembly and Annotation Tools

Raw sequence paired-end reads were obtained for duplicate samples of ECT DNA libraries. Sample 1 contained 5,954,660 reads, 1,190,932,000 bases, an average quality of 35, and a percent duplicate of 5.813%. Sample 2 contained 6,146,352 reads, 1,229,270,400 bases, an average quality of 35, and a percent duplicate of 5.770%. Duplicate metagenomic libraries were merged into a single sequence library using a custom Python script.

Raw sequence data was processed through a metagenomics bioinformatics pipeline (Tremblay *et al*., 2017). Sequencing adapters were removed from each read and bases at the end of reads having a quality score <30 were cut off (Trimmomatic v0.32) (Bolger, Lohse, & Usadel, 2014) and scanned for sequencing adapters contaminants reads using DUK (http://duk.sourceforge.net) to generate quality controlled (QC) reads. The QC-passed reads from each sample were co-assembled using Megahit (v1.1.2; Li *et al*., 2015) with iterative kmer sizes of 31, 41, 51, 61, 71, 81, and 91 bases. The QC-passed reads were mapped (BWA mem v0.7.15; http://bio-bwa.sourceforge.net) against contigs to assess quality of metagenome assembly and to obtain contig abundance profiles. Alignment files in bam format was sorted by read coordinates using samtools v1.2 (Li *et al*., 2009), and only properly aligned read pairs were kept for downstream steps. Each bam file (containing properly aligned paired-reads only) was analyzed for coverage of called genes and contigs using bedtools (v2.17.0; Quinlan & Hall, 2010) using a custom bed file representing gene coordinates on each contig. Only paired reads both overlapping their

contig or gene were considered for gene counts. Coverage profiles of each sample were merged to generate an abundance matrix (rows = contig, columns = samples) for which a corresponding CPM (Counts Per Million–normalized using the TMM method; edgeR v3.10.2).

Gene prediction was performed by calling genes on each assembled contig using MG-RAST (v4.0.3; Meyer *et al*., 2008) and SEED Subsystems (Kumaresan *et al*., 2017) and assignment of KEGG (Meyer *et al*., 2008) orthologs. The Greengenes database was used to identify 16S rRNA genes and assign taxonomic identification (McDonald *et al.*, 2012). The NCBI RefSeq database was used to assign taxonomic identification to assembled contigs (Pruitt *et al*., 2012). Subsystems, RefSeq, and KEGG database outputs were used and compared to assign taxonomic dentification and to identify genes related to carbon, nitrogen, sulfur, and iron metabolism. NCBI RefSeq taxanomic identification was used in conjunction with Greengenes 16S rRNA taxanomic identification. Due to the fragmentation of ECT DNA samples, the RefSeq database used the best representative hit of individual contigs to assign function, however, this likely overestimates the diversity in the metagenome. The Greengenes database uses the 16S rRNA identification gene to assign taxonomic identification, however, due to the fragmentation of ECT DNA samples, a limited subset of organisms was captured through this database. Using both RefSeq and Greengenes allows for greater confidence when assigning identification to organisms.

2.4 Results

2.4.1 ICP-MS Data

ICP-MS data is detailed in Table 2-1. The composition of ECT starting materials was determined to contain 20-25% sulfide, 4% copper, 800 g/t silver, 3 g/t gold, and ~12 % arsenic.

2.4.2 Metagenomic Sequencing Data

Metagenomic analysis generated 21,863 contigs which totaled 100,402,291 bp averaging 4,592 ± 13,300 bp in length and a mean GC percent composition of 40 ± 21% (Table 2-2).

2.4.3 Taxonomic Identification

Taxonomy was assigned to metagenomic analysis using gene sequences annotated by NCBI RefSeq database and 16S rRNA taxonomy was assigned using Greengenes database. Relative abundance at different taxonomic levels were compared between both RefSeq and Greengenes databases at the domain, phylum, order, and genus levels.

Metagenome-level classification (i.e. the taxonomic assignment of all genes in the metagenome) showed relative abundance of the following domains using the RefSeq database Bacteria (56.75%), Eukaryota (31.10%), Archaea (6.06%), Viruses (0.07%), and Other Sequences (0.01%) (Figure 2-3 A). When using only the taxonomic assignment of 16S rRNA and 18S rRNA genes as phylogenetic markers, the relative abundance changes at the domain level against the Greengenes database including Bacteria (88.00%) and Archaea (12.00%) (Figure 2-3 B). Metagenome-level classification at the phylum level identified a total of 49 distinct phyla. The phyla present in greatest relative abundance (>1%) are as follows: *Ascomycota* (36.42%), *Firmicutes* (30.09%), *Proteobacteria* (16.43%), *Euryarchaeota* (5.23%), *Actinobacteria* (3.22%), *Bacteroidetes* (2.69%), and *Chloroflexi* (1.90%) (Figure 2-4 A). These 7 phyla account for 95.98% of the diversity of ECT at the phylum level, with the remaining 42 phyla making up the final 4.02%. For phylogenetic marker genes, the relative abundance at the phylum level is made up of 5 phyla: *Firmicutes* (56.00%), *Proteobacteria* (24%), *Euryarchaeota* (12.00%), *Actinobacteria* (4.00%) and Unclassified Bacterial Phyla (4.00%) (Figure 2-4 B) with no identification of fungal marker genes.

Table 2-1: ICP-MS total metal analysis of Ecuadorian Tailings (ECT) starting materials.

Element	**Mass (g/t)**	**Element**	**Mass (g/t)**
Ag	4.0	Mn	837
Al	49,9000	Mo	<5
Au	2.52	Na	3,550
Ba	74.1	Ni	62
Be	0.12	P	246
Bi	<70	Pb	68
Ca	20,400	Sb	63
Cd	<90	Se	<30
Co	63	Sn	<20
Cr	166	Sr	26.1
Cu	1,500	Ti	4,380
Fe	139,000	Tl	<30
Hg	2.1	U	<60
K	5,030	V	253
Li	<20	Y	10.5
Mg	20,500	Zn	654

Table 2-2: MG-RAST analysis statistics of Ecuadorian Tailings (ECT) starting materials.

Uploaded sequence	Basepair Count	125,172,565 bp
	Sequences Count	21,863
	Mean Sequence Length	5,725 ± 14,340 bp
	Mean GC Percent	50 ± 8%
Artificial Duplicate Reads	Sequence Count	0
Post QC	Basepair Count	100,402,291 bp
	Sequences Count	21,863
	Mean Sequence Length	4,592 ± 13,300 bp
	Mean GC Percent	40 ± 21%
Processed	Predicted Protein Features	77,471
	Predicted rRNA Features	734
Alignment	Identified Protein Features	40,004
	Identified rRNA Features	129
Annotation	Identified Functional Categories	28 Functional Categories: As Defined by Subsystems

Metgenome-level analysis using the RefSeq database generated sequence identification for 199 distinct orders. The orders present in greatest relative abundance (>1%) are as follows: *Eurotiales* (26.13%), *Bacillales* (20.44%), *Burkholderiales* (6.19%), *Clostridiales* (6.13%), *Thermoplasmatales* (3.99%), *Actinomycetales* (2.77%), *Acidithiobacillales* (2.47%), *Pleosporales* (2.41%), *Hypocreales* (2.14%), *Onygenales* (2.08%), *Thermoanaerobacterales* (1.92%), *Sordariales* (1.26%), *Helotiales* (1.24%), *Rhizobiales* (1.23%), *Sphingobacteriales* (1.08%), and *Lactobacillales* (1.00%) (Figure 2-5 A). These 16 orders account for 82.48% of the diversity of ECT at the order level, with the remaining 183 orders making up the final 17.52%.

Metagenome-level analysis using RefSeq database was used to explore the key genera from the top three phyla, *Eurotiales*, *Bacillales*, and *Clostridiales*. *Eurotiales* was dominated by genus *Aspergillus*, accounting for 61.31% of the *Eurotiales* order and the most abundant species being *Aspergillus fumigate*, accounting for 32.80% of the *Eurotiales* order. Dominant genera from *Bacillales* included *Bacillus* and *Alicyclobacillus* accounting for 38.57% and 20.90% of the *Bacillales* order, respectively. *Burkholderiales* was dominated by genus *Ralstonia*, accounting for 58.98% of the *Burkholderiales* order and the most abundant species being *Ralstonia pickettii* accounting for 27.17% of the *Burkholderiales* order.

For phylogenetic marker genes, the relative abundance at the order level according to Greengenes is made up of 8 orders: *Clostridiales* (50.00%), *Bacillales* (15.00%), *Thermoplasmatales* (10.00%), *Actinomycetales* (5.00%), *Hydrogenophilales* (5.00%), *Actinobacteria* (4.00%) and Unclassified Bacterial Phyla (4.00%) (Figure 2-4 B) with no identification of fungal marker genes.

Metgenome-level analysis using the RefSeq database generated sequence identification for 199 distinct orders. The orders present in greatest relative abundance (>1%) are as follows:

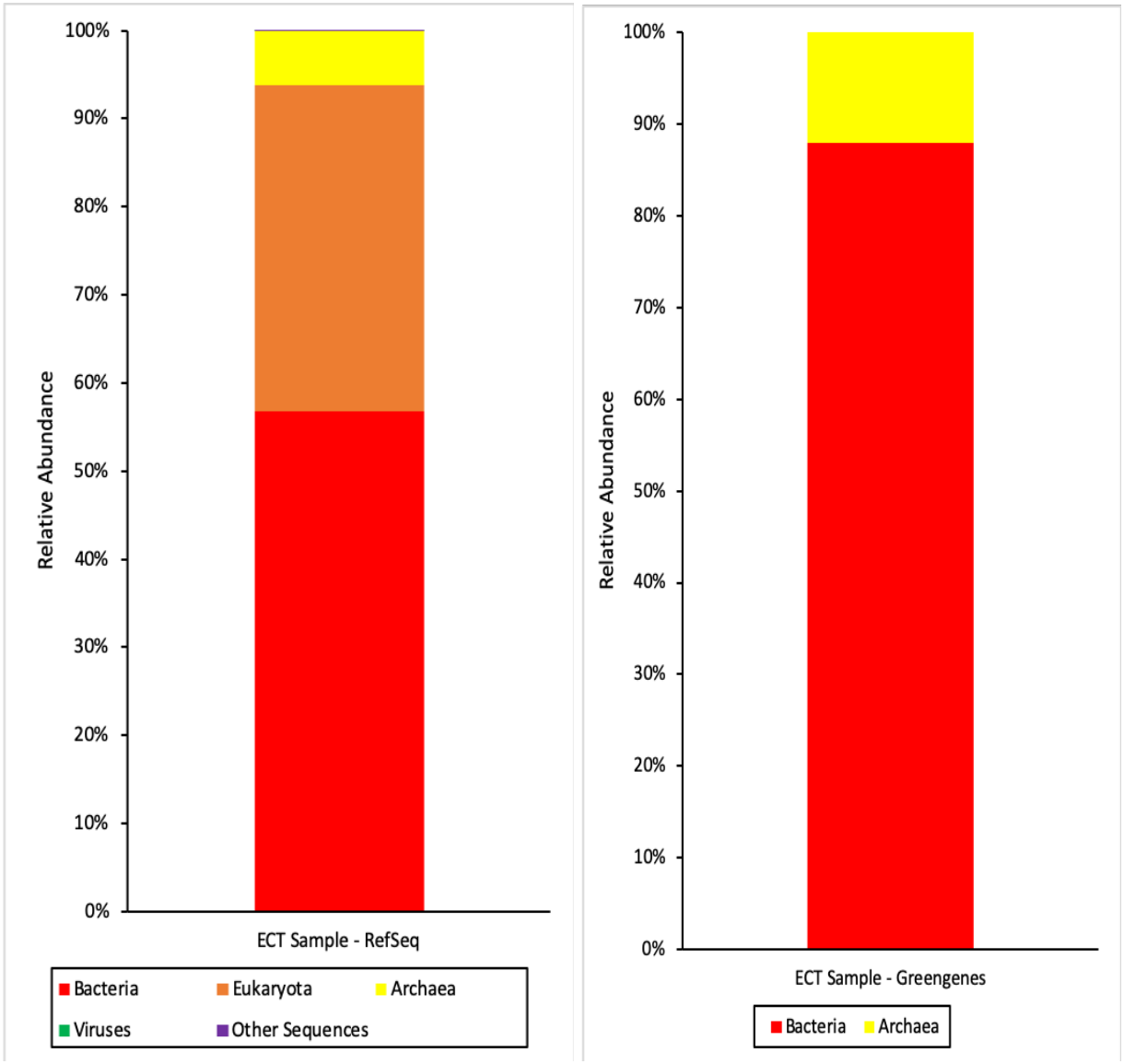

Figure 2-3: Dominant microbial domains present in metagenome of ECT. A) Domains present using metagenome level identification. B) Domains present using phylogenetic marker gene 16S rRNA.

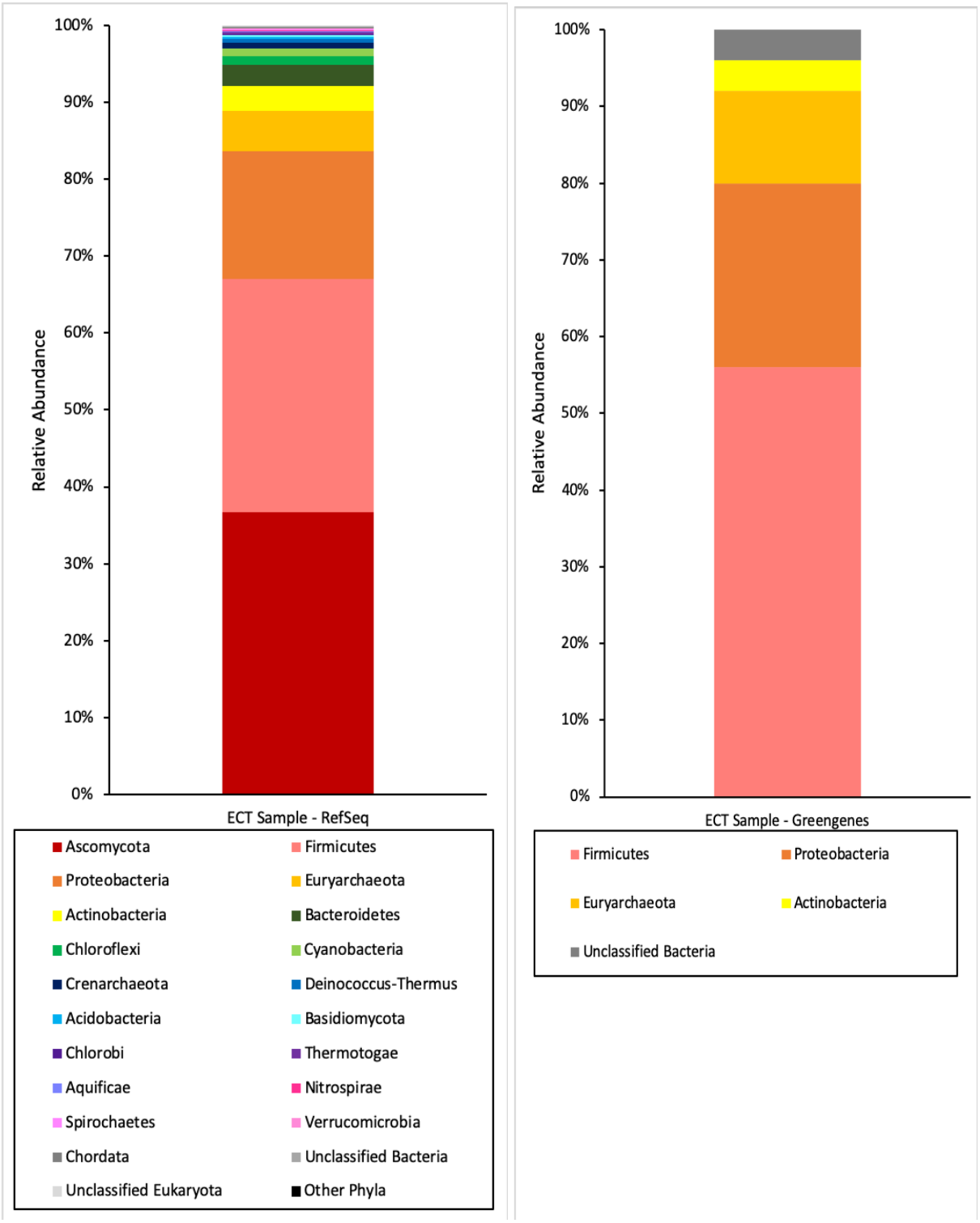

Figure 2-4: Dominant microbial phyla present in metagenome of ECT. A) Phyla present (>1% relative abundance) using metagenome level identification. B) Phyla present using phylogenetic marker gene 16S rRNA.

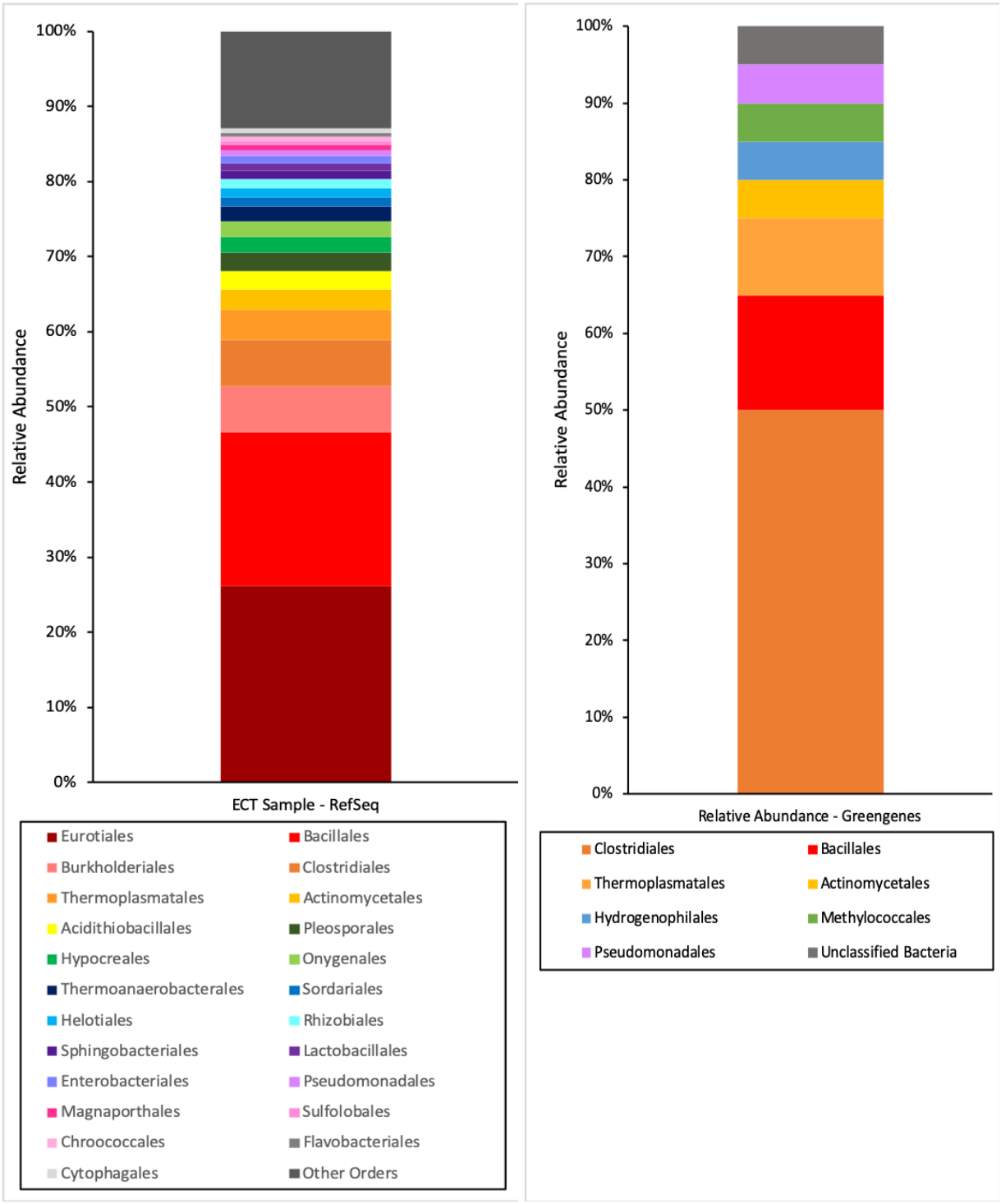

Figure 2-5: Dominant microbial orders present in metagenome of ECT. A) Orders present (>0.5% relative abundance) using metagenome level identification. B) Orders present using phylogenetic marker gene 16S rRNA.

Eurotiales (26.13%), *Bacillales* (20.44%), *Burkholderiales* (6.19%), *Clostridiales* (6.13%), *Thermoplasmatales* (3.99%), *Actinomycetales* (2.77%), *Acidithiobacillales* (2.47%), *Pleosporales* (2.41%), *Hypocreales* (2.14%), *Onygenales* (2.08%), *Thermoanaerobacterales* (1.92%), *Sordariales* (1.26%), *Helotiales* (1.24%), *Rhizobiales* (1.23%), *Sphingobacteriales* (1.08%), and *Lactobacillales* (1.00%) (Figure 2-5 A). These 16 orders account for 82.48% of the diversity of ECT at the order level, with the remaining 183 orders making up the final 17.52%. Metagenome-level analysis using RefSeq database was used to explore the key genera from the top three phyla, *Eurotiales*, *Bacillales*, and *Clostridiales*. *Eurotiales* was dominated by genus *Aspergillus*, accounting for 61.31% of the *Eurotiales* order and the most abundant species being *Aspergillus fumigate*, accounting for 32.80% of the *Eurotiales* order. Dominant genera from *Bacillales* included *Bacillus* and *Alicyclobacillus* accounting for 38.57% and 20.90% of the *Bacillales* order, respectively. *Burkholderiales* was dominated by genus *Ralstonia*, accounting for 58.98% of the *Burkholderiales* order and the most abundant species being *Ralstonia pickettii* accounting for 27.17% of the *Burkholderiales* order.

For phylogenetic marker genes, the relative abundance at the order level according to Greengenes is made up of 8 orders: *Clostridiales* (50.00%), *Bacillales* (15.00%), *Thermoplasmatales* (10.00%), *Actinomycetales* (5.00%), *Hydrogenophilales* (5.00%), *Methylococcales* (5.00%), *Pseudomonadales* (5.00%), and Unclassified Bacterial Order (5.00%) (Figure 2-5 B).

Phylogenetic marker genes using Greengenes were used to explore the key species from the top three phyla, *Clostridiales*, *Bacillales*, and *Thermoplasmatales*. *Clostridiales* was dominated by *Sulfobacillus thermosulfidooxians* accounting for 80.00% of the *Clostridiales* order and 40.00% of the total species present. *Bacillales* was dominated by *Alicyclobacillus pomorum* accounting

for 33.33% of the *Bacillales* order and 5.00% of the total species present. *Thermoplasmatales* was dominated by *Thermoplasma acidophilum* accounting for 50.00% of the *Thermoplasmatales* order and 5.00% of the total species present.

2.4.4 Subsystem Identification

Three classification algorithms for predicted gene function were used (SEED Subsystems, COG, and KEGG). Results for the SEED Subsystem (Kumaresan *et al*., 2017) identification results will be used to summarize the functional classifications in the following sections and an overall summary is shown in Figure 2-6. The most abundant groups identified (>5% relative abundance) were the following: clustering-based subsystems (11.88%), carbohydrates (11.84%), protein metabolism (9.56%), amino acids and derivatives (9.35%), cofactors, vitamins, prosthetic groups, and pigments (7.03%), miscellaneous (6.94%), and RNA metabolism (5.43%). The clustering-based subsystems groups genes believed to have similar functions while the miscellaneous subsystem group all other hypothetical genes of putative function.

Organisms of greatest functional abundance are reported on to build metabolic pathways for the following categories: carbon fixation, nitrogen cycling, sulfur metabolism, and iron metabolism. The taxa with the greatest functional abundance present were: *Bacillales* with *Bacillus* and *Alicyclobacillus* as the dominant genera, *Thermoplasmatales* with *Thermoplasma* as the dominant genus, *Clostridiales* with *Sulfobacillus* as the dominant genus, *Burkholderiales* with *Ralstonia* as the dominant genus, and *Acidithiobacillales* with *Acidithiobacillus* as the dominant genus.

The metagenome contains 11.84% of global genes that are attributed to carbon fixation in the ECT system (Figure 2-6). All genes, enzymes, and bi-products are present in both the Calvin-Benson Cycle and Reverse TCA Cycle (Figure 2-7). It is important to note that genes, enzymes,

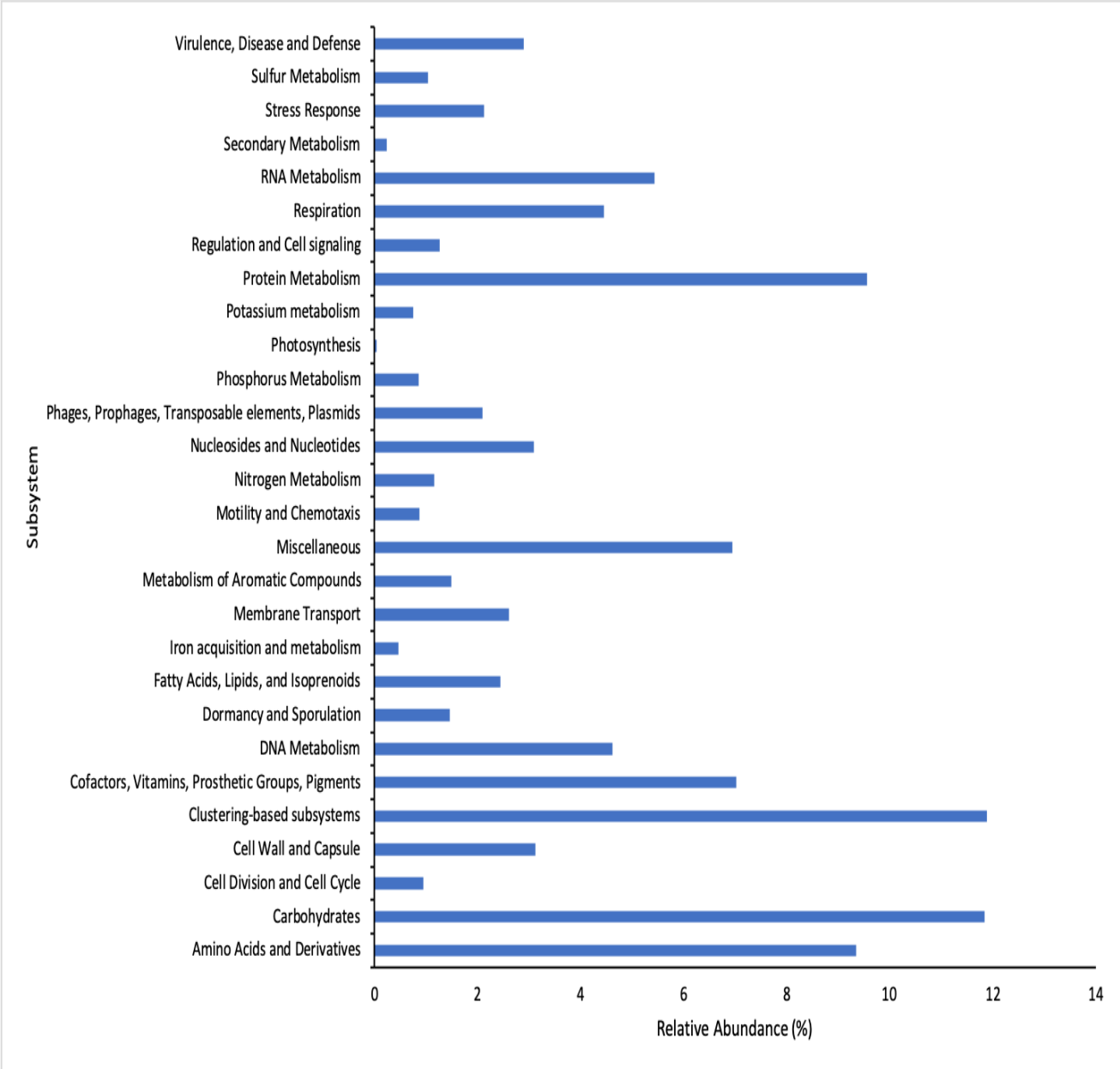

Figure 2-6: Relative abundance of COGs present in Ecuadorian Tailings (ECT) starting materials using Subsystems database.

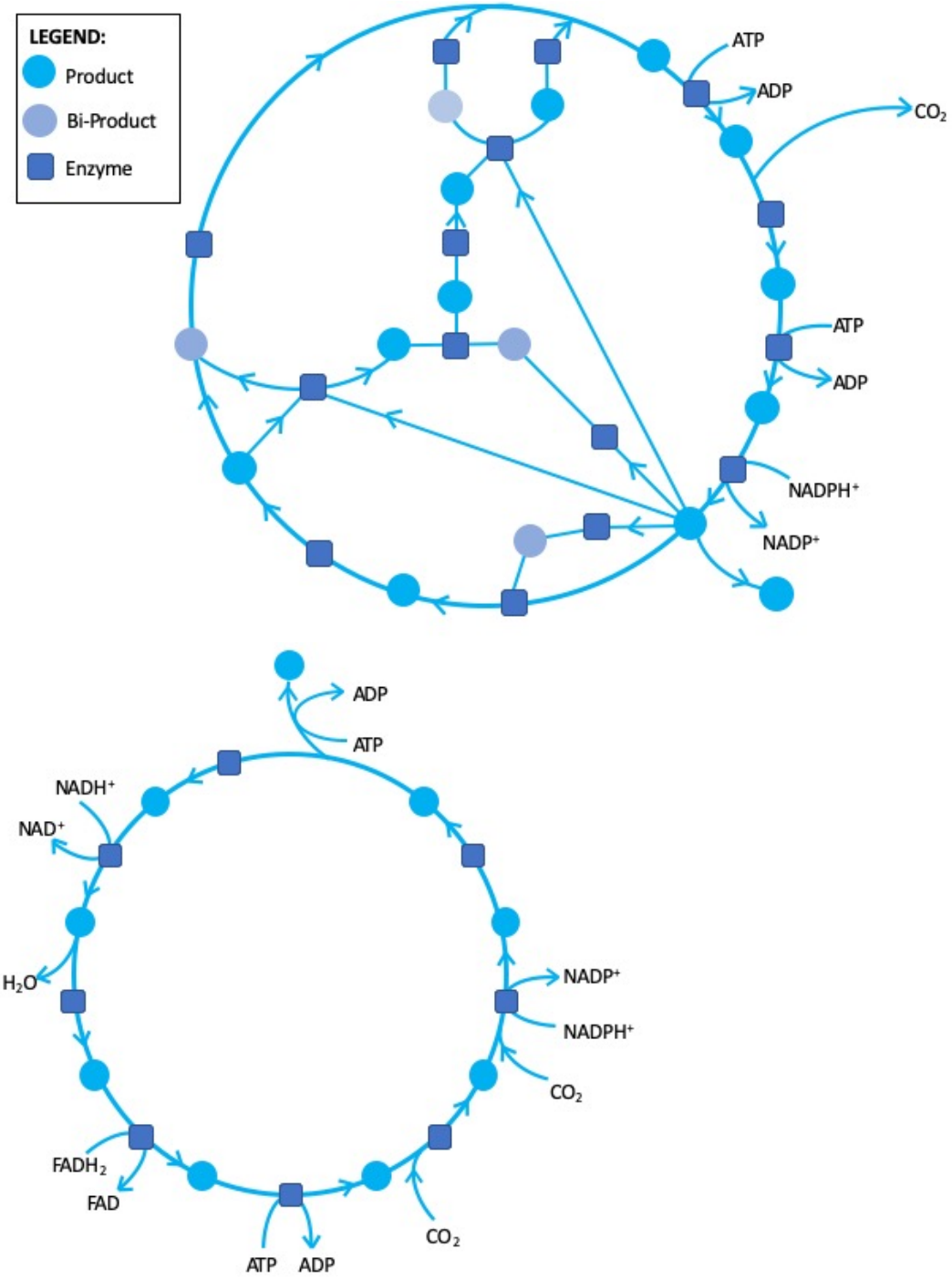

Figure 2-7: Carbon fixation diagrams for Ecuadorian Tailings (ECT) metagenome. A) Calvin-Benson Cycle contains all necessary genes and enzymes to carry out its full function. B) Reverse TCA Cycle contains all necessary genes and enzymes to carry out its full function.

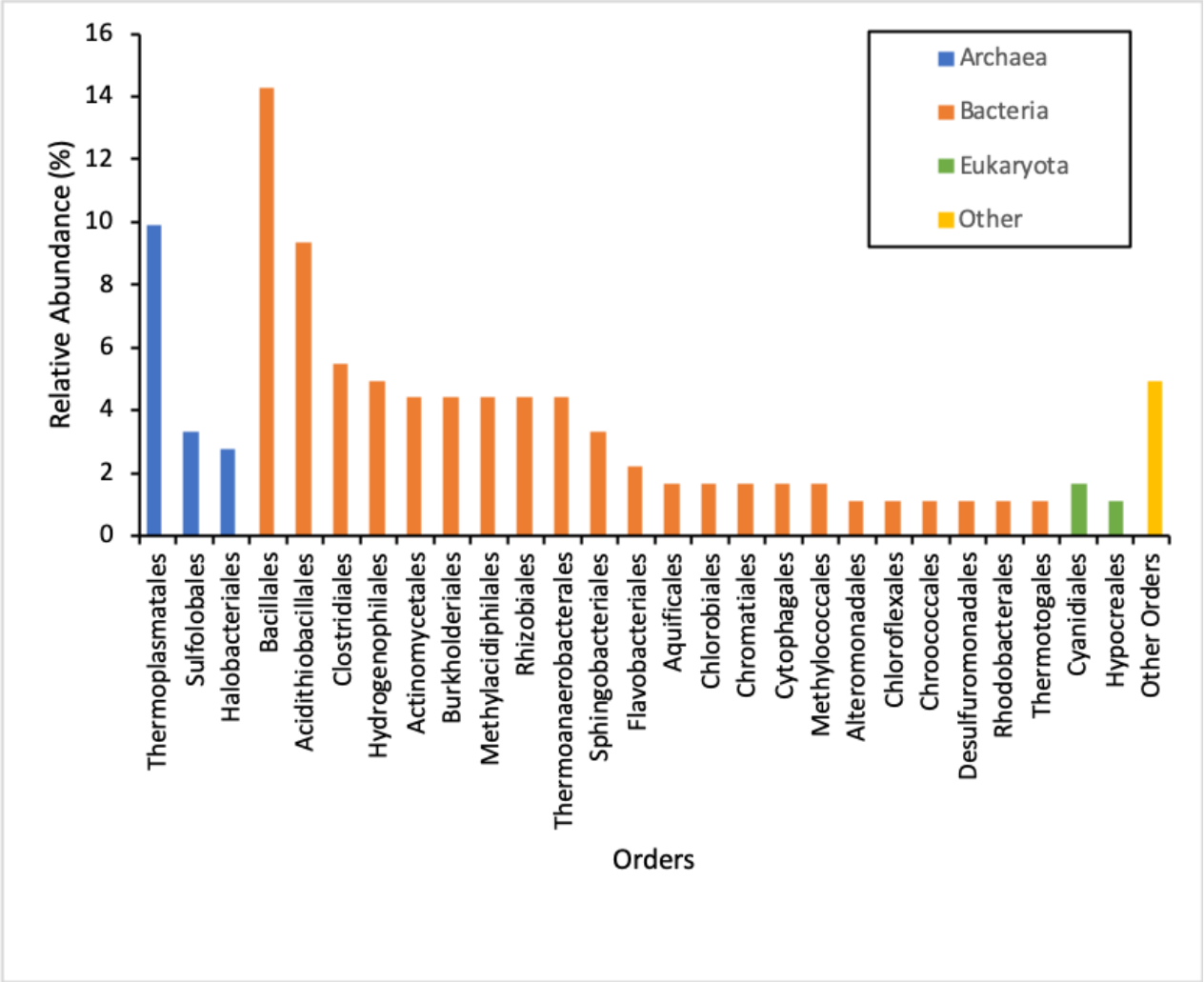

Figure 2-8: Relative abundance of Orders (>1%) involved with carbon fixation, specifically the Calvin-Benson Cycle and Reverse TCA Cycle. The dominant orders and their associated genera are: *Thermoplasmatales* (*Thermoplasma*) 9.89%, *Bacillales* (*Bacillus* and *Alicyclobacillus*) 14.29%, *Acidithiobacillales* (*Acidithiobacillus*) 9.34%, and *Clostriales* (*Sulfobacillus*) 5.50%.

and bi-products to support TCA Cycle for heterotrophs are also present, however, the focus remains on carbon metabolism for chemolithotrophs. The most abundant contributors to carbon fixation were *Bacillales (Bacillus* and *Alicyclobacillus)* (20.52%), *Thermoplasmatales (Thermoplasma)* (11.63%), *Clostridiales (Sulfobacillus)* (7.98%), and *Burkholderiales (Ralstonia)* (6.46%) (Figure 2-8).

The metagenome contains 1.16% of global genes are attributed to nitrogen cycling in the ECT system (Figure 2-6). For the nitrogen cycling pathway, all genes are present except for the following: *norB* and *norC* to convert nitrous oxide (N_2O) to nitric oxide (NO), as well as *nirK* and *nir*S to convert nitric oxide (NO) to nitrite (NO_2^-) (Figure 2-9). The most abundant contributors to nitrogen cycling were *Burkholderiales (Ralstonia)* (13.29%), *Acidithiobacillales (Acidithiobacillus)* (12.66%), *Bacillales (Bacillus* and *Alicyclobacillus)* (12.66%), and *Clostridiales (Sulfobacillus)* (8.86%) (Figure 2-10).

The metagenome contains 1.04% of global genes are attributed to sulfur metabolism in the ECT system (Figure 2.6). For general sulfur metabolism, all genes necessary to carry out sulfur metabolic functions are present (Figure 2-11). For the SOX pathway, all genes are present except for the following: *SoxA*, *SoxC*, and *SoxD* (Figure 2-11). Not noted in Figure 2-11 is the presence of both *SoxN* and *SoxL* in the metagenomic data. The most abundant contributors to sulfur metabolism were *Bacillales (Bacillus* and *Alicyclobacillus)* (19.05%), *Burkholderiales (Ralstonia)* (12.25%), *Thermoplasmatales (Thermoplasma)* (10.88%), and *Clostridiales (Sulfobacillus)* (4.08%) (Figure 2-12).

The missing genes from both the sulfur oxidation and nitrogen cycling pathways could be attributed to one of the following explanations: incomplete gene sequences due to issues with

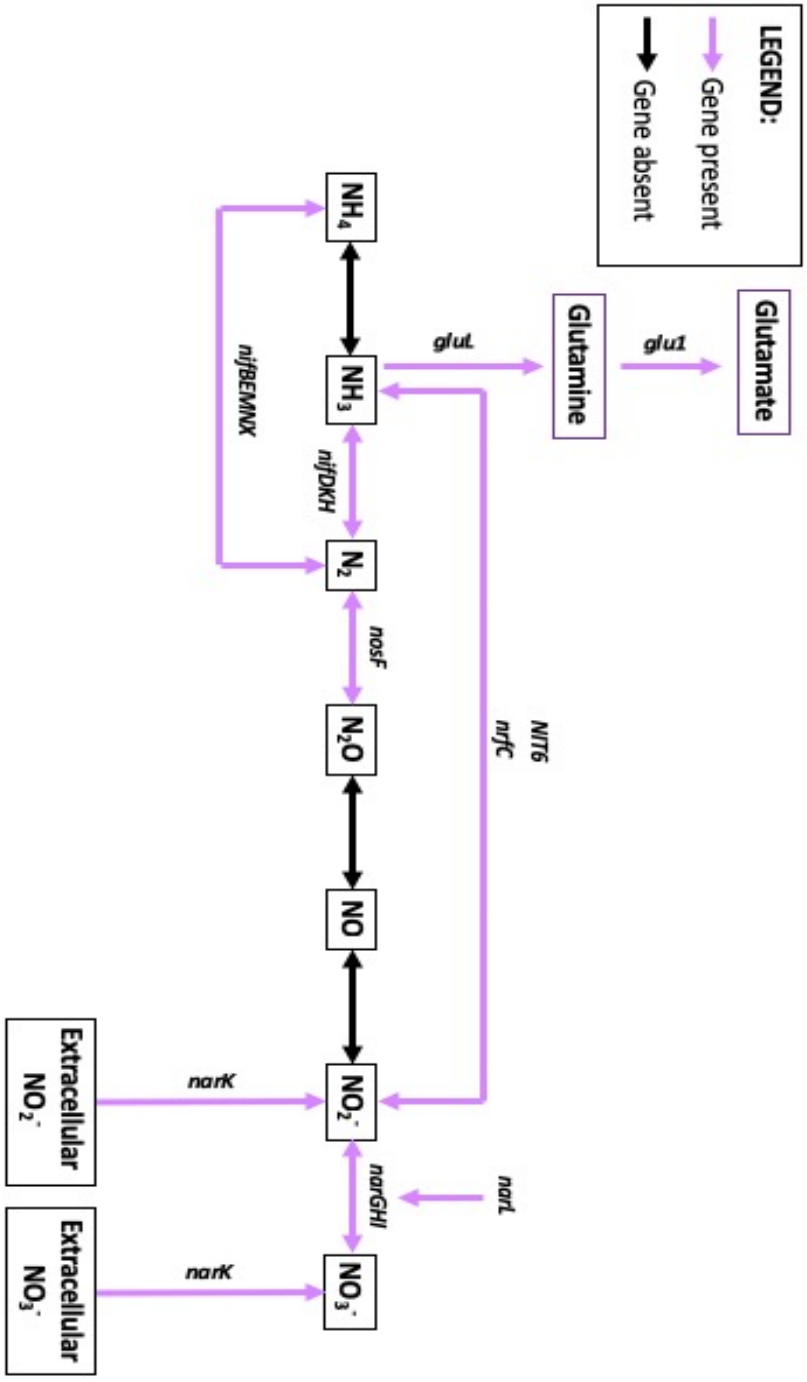

Figure 2-9: Nitrogen cycling diagram for ECT metagenome. Most genes are present to carry out nitrogen cycling including nitrogen fixation and denitrification. Arrows in black indicate missing genes, arrows in purple indicate gene is present.

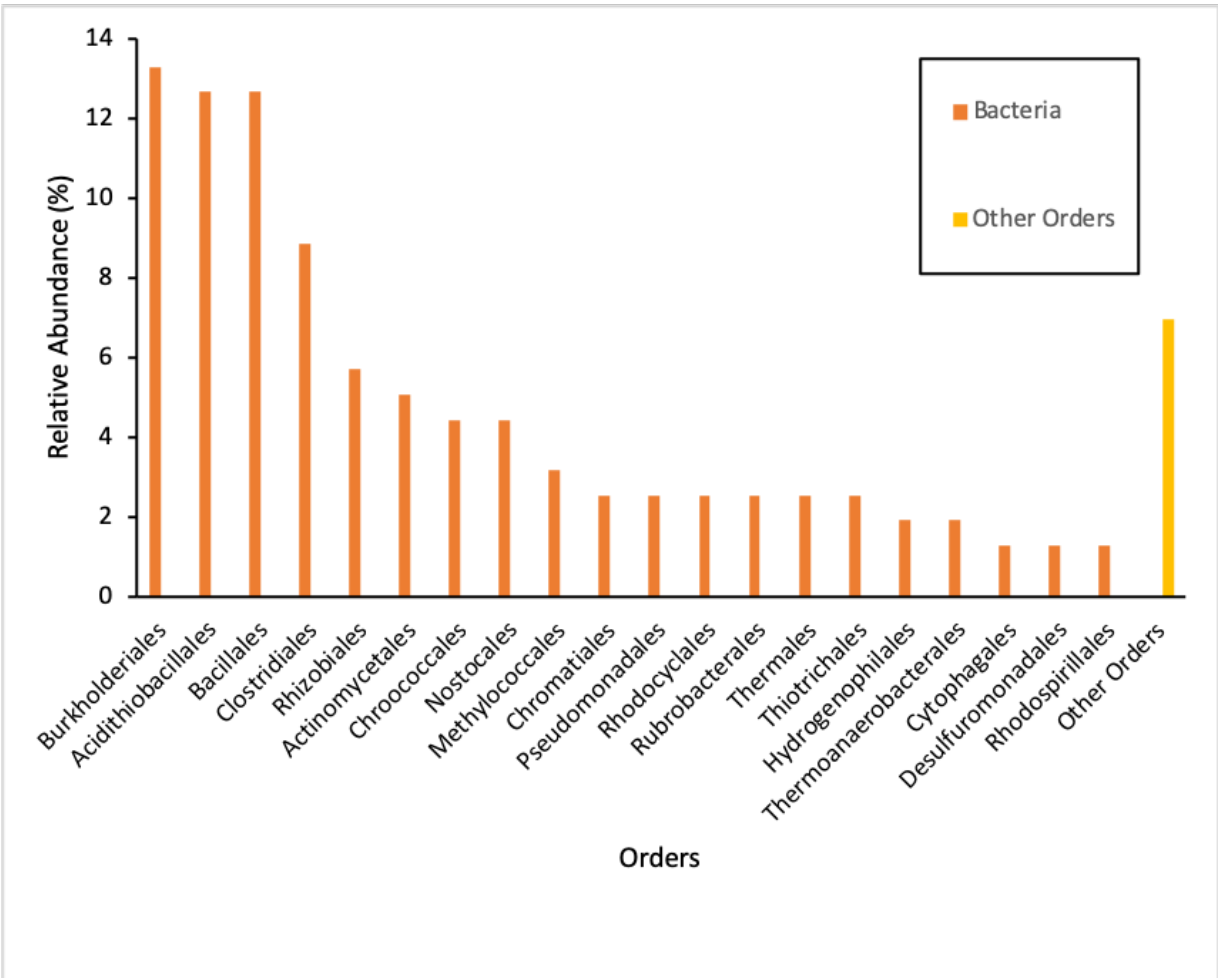

Figure 2-10: Relative abundance of Orders (>1%) involved with nitrogen cycling. The dominant orders and their associated genera are: *Burkholderiales* (*Ralstonia*) 13.29%, *Acidithiobacillales* (*Acidithiobacillus*) 12.66%, *Bacillales* (*Bacillus* and *Alicyclobacillus*) 12.66%, and *Clostridiales* (*Sulfobacillus*) 8.86%.

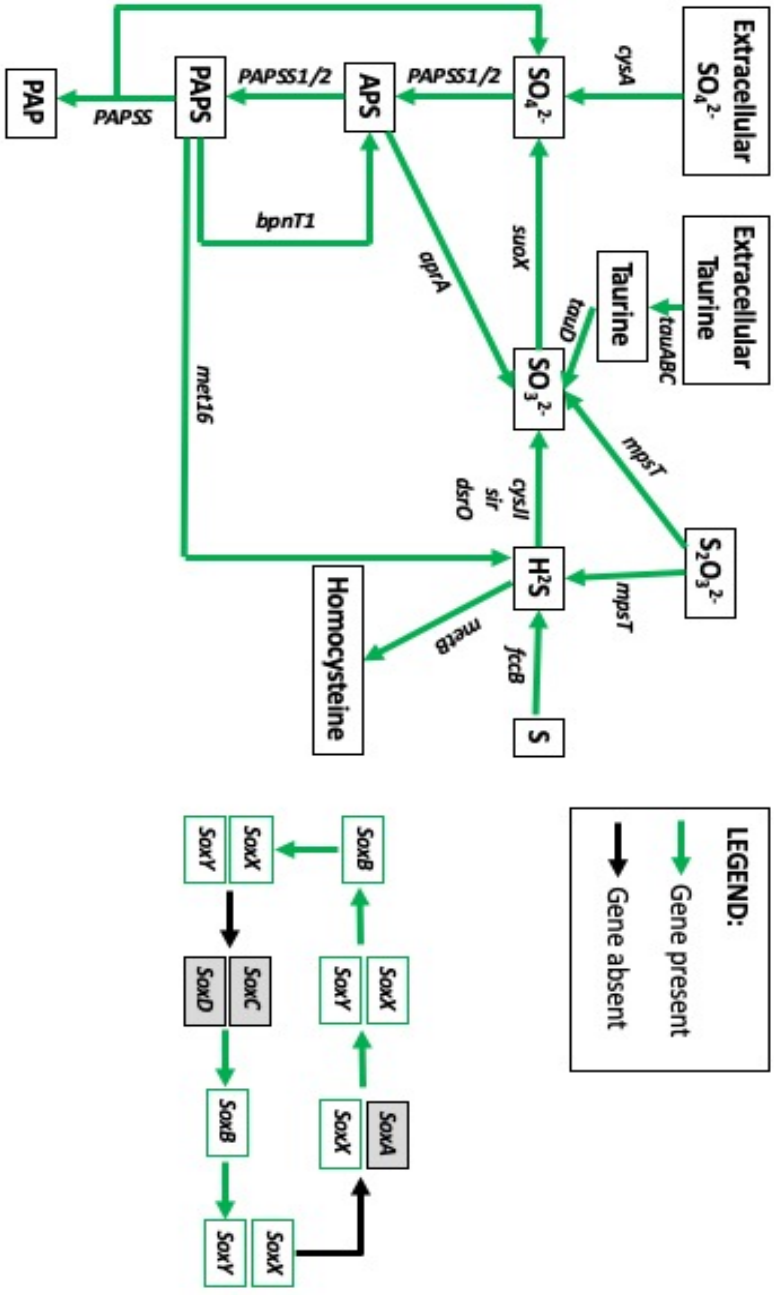

Figure 2-11: Sulfur metabolism diagram for ECT metagenome. All genes are present for sulfur metabolism except in the case of the SOX box, where *SoxA, SoxC,* and *SoxD* are not present. Arrows in black indicate missing genes, arrows in green indicate gene is present.

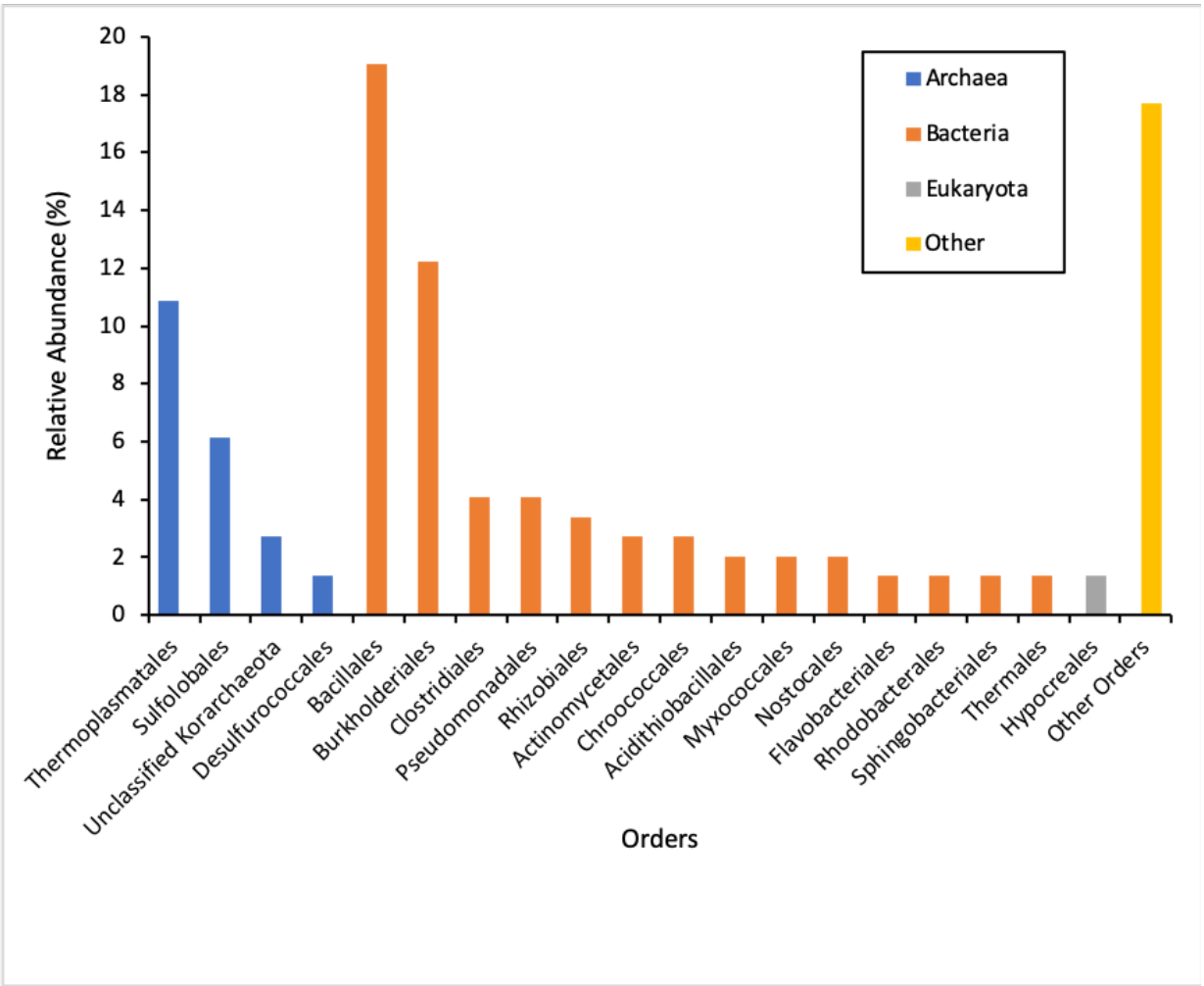

Figure 2-12: Relative abundance of Orders (>1%) involved with sulfur metabolism. The dominant orders and their associated genera are: *Thermoplasmatales* (*Thermoplasma*) 10.88%, *Bacillales* (*Bacillus* and *Alicyclobacillus*) 19.05%, and *Burkholderiales* (*Ralstonia*) 12.25%.

Table 2-3: List of iron metabolism genes found in the metagenome of Ecuadorian Tailings (ECT) starting materials. Gene function as well as number of gene hits, defined as the closest matching homologous sequence.

Gene Name	Gene Function	Number of Gene Hits
feoB	Ferrous iron transport protein B	10
tonB	Ferric siderophore transport system, periplasmic binding protein	8
corA	Magnesium and cobalt transport protein	7
etfB	Electron transfer flavoprotein beta subunit	5
bfr	Bacterioferritin	3
fecA	Ferric iron dicitrate transport protein	3
entA	2,3-dihydro-2,3-dihydroxybenzoate dehydrogenase	1
hmuU	Hemin ABC transporter permease protein	1
feoA	Ferrous iron transport protein A	1

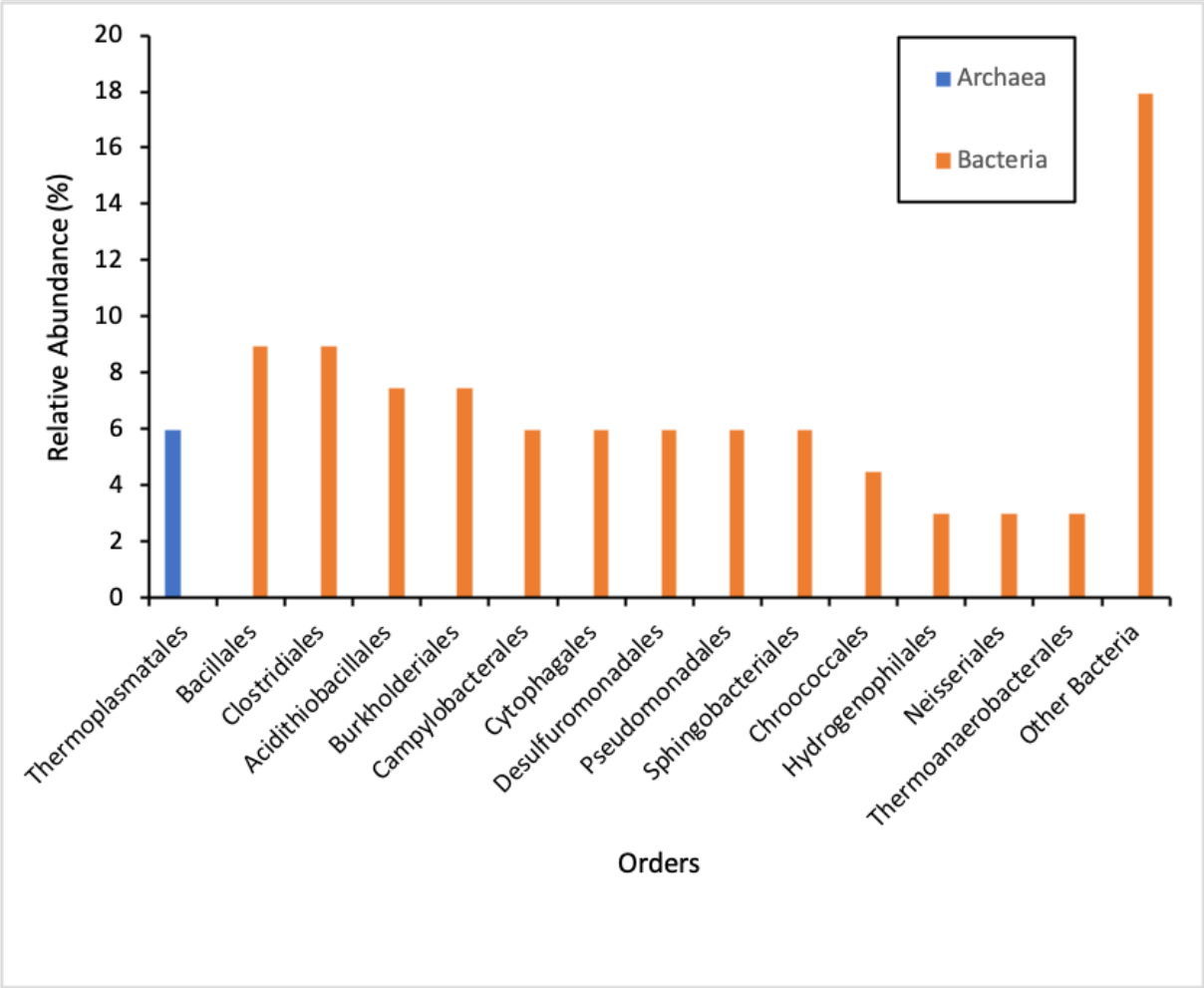

Figure 2-13: Relative abundance of Orders (>1%) involved with iron metabolism. The dominant orders and their associated genera are: *Thermoplasmatales* (*Thermoplasma*) 5.97%, *Bacillales* (*Bacillus* and *Alicyclobacillus*) 8.96%, *Clostriales* (*Sulfobacillus*) 8.96%, *Acidithiobacillales* (*Acidithiobacillus*) 7.46%, and *Burkholderiales* (*Ralstonia*) 7.46%.

DNA quality during extraction, redundancies in the pathways where another gene is performing the function of the missing gene, or the gene may not be present at all.

The metagenome contains 0.47% of global genes are attributed to iron metabolism in the ECT system (Figure 2-6). The most abundant contributors to iron metabolism were *Bacillales (Bacillus* and *Alicyclobacillus)* (8.96%), *Clostridiales (Sulfobacillus)* (8.96%), *Acidithiobacillales (Acidithiobacillus)* (7.46%), *Burkholderiales (Ralstonia)* (7.46%), and *Thermoplasmatales (Thermoplasma)* (5.97%) (Figure 2-13). Table 2-3. summarizes the names of the genes annotated for iron metabolism, their general functions, and the number of gene hits as annotated by the metagenome analysis by RefSeq. Gene hits refers to the closest matching homologous sequence.

As the bioleaching systems of interest are aiming to process arsenopyrite, we were interested in targeting arsenic resistance genes. The following genes have been found which correspond to cellular mechanisms for dealing with arsenic including: arsenate reductase (*aarA*), arsenite ATP-driven efflux pump (*arsA1*), arsenite methyltransferase (*arsM*), arsenical-resistance protein (*acr3*), arsenical resistance operon trans-acting repressor (*arsD*), and arsenical resistance operon repressor (*arsR*).

Figure 2-14 is a simplified synthesis diagram of the microbial involvement of *Acidithiobacillus*, *Alicyclobacillus*, *Aspergillus*, Bacillus, *Sulfobacillus*, *Ralstonia*, and *Thermoplasma* in the ECT system with respect to carbon, nitrogen, iron, sulfur, and arsenic metabolism.

2.5 Discussion

2.5.1 Abundant and Relevant Organisms in ECT

The most abundant organisms in the areas or carbon fixation, nitrogen cycling, sulfur metabolism, and iron metabolism were members of the *Acidithiobacillus, Alicyclobacillus, Bacillus, Ralstonia, Sulfobacillus,* and *Thermoplasma* genera.

The *Acidithiobacillus* genus aids in nitrogen fixation at low pH environments (Tyson *et al*., 2005; Valdés *et al*., 2008) and has been known to give rise to species that are predominant iron oxidizers between pH 1.5-2.4 in AMD systems (Bond *et al.*, 2000; Screnk *et al.*, 1998). *Acidithiobacillus* has been found to be dominant during cold seasons in Northern Ontario (Auld *et al*., 2017) and in Russia (Liljeqvist *et al*., 2011), making it a genus of interest in the cold project outlined in Appendix A. Although *Acidithiobacillus* was not very abundant in the starting materials (ECT) for this project, it was later added into the reactors for the pilot project due to *Acidithiobacillus*' iron oxidation powers.

Members of the *Bacillales* order have been implicated in nitrogen fixation in acidic environments (Tyson *et al*., 2005; Valdés *et al*., 2008). *Alicyclobacillus* in particular has previously been noted in AMD sites in Northern Ontario, California, and Japan (Auld *et al.*, 2013; Baker & Banfield, 2003; Joe *et al*., 2007). *Alicyclobacillus* aids in nitrogen fixation at low pH environments (Tyson *et al*., 2005; Valdés *et al*., 2008). The *Bacillus* genus has been found in abundant levels in uranium mining waste (Panak *et al*., 2009), magnetite mine tailing soils and have shown metal tolerance to nickel and zinc (Yu *et al*., 2014), both of which are present in ECT (Table 2.1).

Burkholderiales has been found in AMD systems found in Spain (Garcia-Mendez *et al*., 2015) and as an iron-oxidizing chemolithotroph (Fukushima *et al*., 2015). *Ralstonia* has been isolated

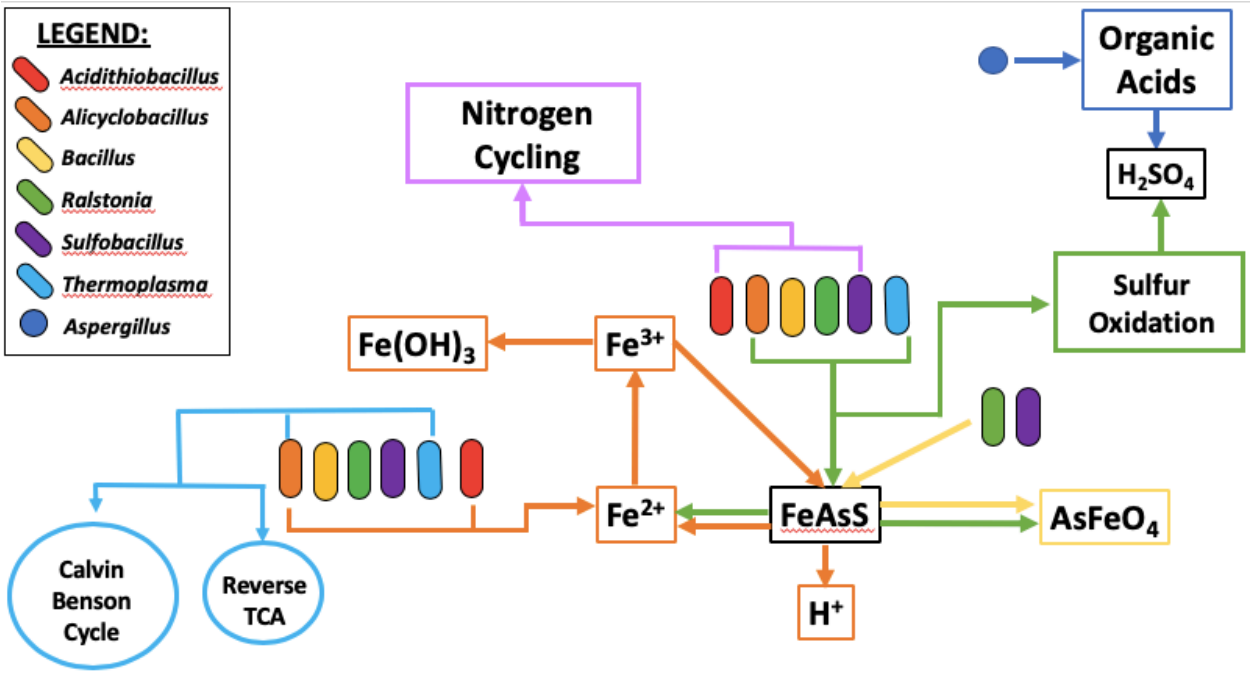

Figure 2-14: Synthesis diagram of most abundant organisms in ECT.

from copper mine tailings and has shown heavy metal tolerance to copper, lead, cadmium, and nickel (Xie *et al*., 2010). *Ralstonia* has been identified as having arsenic resistance mechanisms and has been found to oxidize arsenite (Battaglia-Brunet *et al*., 2006).

Sulfobacillus is a thermophile with an optimum growth of approximately 45 ºC and oxidizes ferrous iron and sulfur to meet its energy requirements (Mendez-Garcia *et al.*, 2015). *Sulfobacillus* can grow between pH 1.6-2.3, can oxidize iron, can enhance metal extraction from pyrite and arsenopyrite among others, and can aid in extraction of arsenic, copper, cobalt, nickel and zinc from mineral sulfides (Watling, Perrot, & Shiers, 2008). *Sulfobacillus* can attach to mineral sulfide surfaces to accelerate the sulfide oxidation and increase metal leaching (Watling, Perrot, & Shiers, 2008).

Thermoplasmatales comprises thermoacidophilic auto- or heterotrophic archaea (Huber & Stetter, 2006). *Thermoplasma* can grow between 55-60 ºC and between a pH range of 0.5-4.0 (Xie, Xiao, & Liu, 2009) and have been isolated in iron- and arsenic-rich tailings in France (Bruneel *et al*., 2008) and in pyrite-rich AMD site of California (Druschel *et al*., 2004).

Interestingly, the ECT metagenomic sample contained substantial fungal species, the *Eurotiales* order which was dominated by the *Aspergillus* genus. *Aspergillus* has been found to be effective in bioleaching hazardous heavy metals, including lead and arsenic (Seh-Bardan *et al*., 2012). *Aspergillus* has also been found to solubilize copper into solution from low-grade ores (Mulligan & Kamali, 2003).

2.5.2 Potential and Functional Relationships

Each of the dominant genera come together to play specific roles in the ECT community. *Acidithiobacillus* is able to oxidize ferrous iron, an important role in ECT and the generation of acidity in this system which liberates ferrous iron from pyrite to continue iron oxidation

(Equations 1-4). *Ralstonia* is able to oxidize arsenite while *Sulfobacillus* is able to extract arsenic from pyrite and arsenopyrite, both of which are likely to play an important role in ECT and in the liberation of gold for further downstream processing. *Sulfobacillus* also functions as a primary sulfide oxidizer in this system to make the target gold more amenable to downstream extraction. *Acidithiobacillus Ralstonia*, and *Alicyclobacillus* all contribute to nitrogen cycling in the ECT system while *Thermoplasma*, *Alicyclobacillus*, and *Bacillus* all contribute to carbon metabolism in the ECT system. Lastly, *Aspergillus* has been found to release organic acids, which when enhanced by sulfuric acid (Mulligan & Kamali, 2003) that is produced through iron oxidation (Equations 1-4), increases the leaching effect of some metals.

2.6 Conclusions

The ECT system is comprised of a diverse set of organisms including bacteria, archaea, and eukaryotes, making this a complex and interesting study system. The presence of SOB and IOB are consistent with many sulfide rich tailings and AMD systems. The main iron and sulfur oxidizers in this system were members of the *Acidithiobacillales*, *Bacillales*, *Burkholderiales*, *Clostridiales*, and *Thermoplasmatales*. These iron and sulfur oxidizers are responsible for driving the conversion of ferrous iron to ferric iron, which will attack arsenopyrite to breakdown the mineral surface of target metals, making the gold amenable to other forms of extraction, including cyanidation and carbon in leach.

Some challenges that were faced in this project include limited repetition of DNA extraction and sequence analysis due to time constraints in growing phases of the project. Our identification power using RefSeq and Greengenes was limited due to lack of genomic information. A common issue with extracting DNA from AMD systems is that the precipitated iron hydroxides hydrolyze DNA and inhibit PCR amplification making it difficult to extract high quality and

large amounts of DNA. From here, once a cell pellet is formed, it is also difficult to extract DNA due to challenges in removing as much iron as possible to halt any interaction and degradation to the DNA being sent for sequencing. Due to these iron interactions, the DNA is cut into smaller fragments, making alignment and annotation more challenging.

The goal of this project was to identify the relationships between the main contributors to the ECT system to better understand how ECT functions as a whole. Metagenomic information gained from this project created a baseline dataset which is important to understand how the ECT community will change throughout the bioleaching campaign being performed by the Mykytczuk Research Group. We can understand how the baseline community changes in relative abundance to one another as the geochemistry changes over time and develop strategies to peak health of the microbial community.

Understanding the metagenomics of tailings communities is an important first step in understanding the unique community dynamics driving oxidation and resulting in greater metal extraction. These microbial interactions are especially important to industry partners when designing bioleaching campaigns.

Chapter 3

3 Conclusions and Future Directions

3.1 Warm Project Future Directions

The ECT microbial community is being tested in a pilot benchtop bioleaching project conducted by the Mykytczuk Research Group at Cambrian College. Abiotic factors, including pH, temperature, aeration, as well as carbon and nitrogen sources and concentrations must be set to ensure the health of the microbial community is maintained. These abiotic factors may need to change in response to geochemical compositional changes throughout a leaching campaign as the availability of iron and sulfur in specific oxidative states raises and lowers. The cumulative biogeochemical parameters for a successful bioleaching campaign must be considered from a microbial standpoint. The microbial community will shift and change in response to these abiotic changes, and the health of the community, who is performing the oxidative processes to release the target gold in this project, must be monitored. Previously, we have seen a large variation in gold recovery between bioleaching campaigns, indicating that there are instances in which the microbial community, specifically the sulfur and iron oxidizers, are not performing optimally. To accurately monitor the health of the ECT community, we must first understand the dominant taxa within the ECT system and identify who is contributing to which processes. This work has been completed in conjunction with Alexandra Methé, who is conducting an *in-silico* analysis of metabolomics for the ECT system. Combining the metagenomic and metametabolomic data will provide information regarding key products that may be tested for in an industrial bioreactor setting. Rapid detection of microbial community members could be determined using a MinION

sequencer which has high taxonomic resolution (Benítez-Páez, Portune, & Sanz, 2016) or target genes or metabolites could be identified using rapid assays.

To further advance this work, lab-based transcriptomic and metabolomic analyses should be performed to detect which gene products and metabolites are present in conjunction with specific biogeochemical parameters. Using metagenomic, transcriptomic, and metabolomic data, a combination of the following can be developed to allow industry to monitor the efficacy of bioreactors: developing biosensors that indicate when specific metabolites may be below a threshold, using real-time monitoring of the system with minIONs, or having a panel of key organisms that are monitored for throughout the process.

3.2 Cold Project Future Directions

This project's challenges could not be overcome in a two-year Master's timeframe. To advance this project, the strains of *Acidithiobacillus ferrivorans* and *A. ferrooxidans* must first meet optimal growth conditions at their desired growth temperatures (20 and 30 ºC, respectively). Once these cultures are rapidly growing and producing adequate biomass, the experiment proposed in Appendix A can be carried out.

Controls growing at their normal temperature can be compared to cold-shock, in which the flasks are placed in a cold environment for an allotted amount of time, followed immediately with an RNA extraction. This can then be repeated with a longer exposure to cold temperatures to examine for long-term adaptations to the cold environment. This project will allow us to understand how these microbes are adjusting to cold temperatures on both a short-term and long-term scale. What is especially important to note is the rates at which these species are oxidizing iron and sulfur to determine if they are viable candidates to begin bioleaching at cold temperatures in Northern Ontario.

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Appendices

Appendix A: Cold Project Overview

Microbe of Interest: *Acidithiobacillus ferrivorans*

Acidithiobacillus ferrivorans is classified as a psychrotolerant acidophile which is capable of oxidizing ferrous iron and inorganic sulfur (Mykytczuk *et al*., 2010; Liljeqvist *et al*., 2011). *At. ferrivorans* is able to grow between 5 ºC – 30 ºC (Liljeqvist *et al*., 2011), a unique adaptation that is relevant for biomining and bioremediation techniques in colder climates, defined as a subarctic climate (Dfc) (Kottek *et al*., 2006), such as Northern Ontario. A unique challenge for bioremediation in Northern Ontario, and other cold climates, is to deal with legacy sites at the site of the tailings by heap-leaching. Due to the large temperature fluctuations in Northern Ontario's climate, it is important to screen for bacteria that could carry out bioremediation at colder temperatures. To better screen these bacteria and understand their limitations, we have opted to take a molecular genetics approach of sequencing target bacterium to understand their cold-adaptive mechanisms.

Goal of Cold Project

The goal of this part of the project was to compare and contrast these findings with those of a warm-climate (ECT) to determine what genomic differences were driving these adaptive capabilities. From here, we would have a strong basis to perform targeted transcriptomic analyses to understand these changes in real-time with temperature fluctuations in controlled environments. It is important to understand this, as these tailings sites often have varying microclimates within them, where temperature at the surface of these piles can be much colder than the center of the piles, meaning the microbial consortia will likely vary as we move deeper into the leach pile.

Work previously done by Mykytczuk *et al* (2010) identified strains of *Acidithiobacillus ferrivorans*, at the time the strains were still classified at *Acidithiobacillus ferrooxidans*, from several mine sites in Northern Ontario. These strains were found to oxidize iron at similar rates to their mesophilic counterparts at 10˚C and were still able to perform iron oxidation at 2-5 ˚C. These strains have been characterized for various metal tolerance and would be good candidates to initiate heap leaching at colder ambient temperatures.

Recent work by Auld *et al*. (2017) demonstrates that the *Acidithiobacillus* genus is present in greatest abundance during the winter months of sample collection at a Northern Ontario tailings site. Summer sampling indicated dominance by the *Acidophilium* genus, with *Acidithiobacillus* being a close second (Auld *et al.*, 2017).

Given these results, we decided to work with isolated *Acidithiobacillus ferrivorans* strains to better understand and test its limitations in cold-growth environments.

Methodology and Challenges

This aspect of the project was designed to run in parallel to the growing ECT strains as described in the Methodology section of this thesis. The main difference was to grow *Acidithiobacillus ferrivorans* at 5 ºC, create growth curves by testing the optic density at 500 nm, and have a standardized method of working with this strain. Once the samples had grown to $OD_{500} = 0.8$ (Doran *et al.*, 2017), the samples would have produced sufficient biomass with the least amount of precipitated iron to binds to bacterial cells.

This project was met with challenges, as the isolated strain was not growing on a timeline that was suitable for a two-year Master's time constraint. After months of inoculations, we did not produce sufficient biomass to carry out any downstream genomic analyses. The final optic density reading after 8 months of growth was $OD_{500} = 0.43$. Two of the nine samples were

centrifuged and pelleted, following the same methodology previously described. The samples were then subjected to QuBit analysis to determine whether or not there was viable DNA to send off for sequencing. QuBit analyses confirmed the sample was too low to detect any DNA present. Unfortunately, this project could not have been continued given my time constraints, however, these samples are continually growing in the lab and will be continued by a future student.

Next Steps

To improve the growth of these microbes, it would be in the best interest of future students to grow these samples in ambient temperatures (20-25 °C) to a point where sufficient iron oxidation, and therefore cellular growth, has occurred at an $0D_{500} = 0.8$. From here, microbes can be moved to a cold incubation setting (4 °C) for some period of time. Cold shock experiments can be performed to understand immediate transcriptional changes to the temperature change, while long term monitoring of transcripts can help understand the acclimation of this sample at a cold temperature.