GENOMIC INSIGHTS INTO THE PSYCHROTROPHIC MICROBIAL LEACHING OF LOW SULFIDE WASTE ROCK IN KINETIC TESTING SYSTEMS.

by

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ABSTRACT

The microbial activity occurring within mine wastes is a known driver of the development and perpetuation of acid generation from mining waste products, including waste rock. The acid generating capacity of waste rock can pose a great environmental liability to surrounding watersheds, which often prompts substantial management of large volumes of waste rock throughout the development, operation, and closure of a mining operation. Kinetic testing systems are a standard means of predicting the acid generating potential of waste rock, but are dependent on abiotic or artificial biological amendments to measure this key parameter. Waste rock from mine sites can be classified as potentially acid generating (PAG), which suggests uncertainty around the acid generating status of the waste rock in question. Temperature is a key control on the weathering of waste rock as the acceleration of abiotic and biotic sulfide oxidation at warmer temperatures can induce faster weathering rates. Nonetheless, the relationship between PAG rock geochemistry in colder regions and its native microbial communities have not been well characterized in the field or in kinetic testing systems.

This thesis aims to address the microbial contribution to humidity cell and field leach bin testing systems of low sulfur waste rock native to Boreal climate. A laboratory-based humidity cell experiment was conducted over an extended 28 week period where the microbial community was surveyed over time and compared to weekly leachate geochemistry. The same rock was subjected to a field bin experiment, where the microbial community and leachate geochemistry was profiled over 11 months.

Both humidity cell and field bin experiments remained at near neutral conditions over the course of the experiment and exhibited significant neutralization potential. The elemental release rates differed between the humidity cell and field bin experiments, although all experiments predicted the time to acid generation to be under 75 years. Temperature exerted a greater influence on element release in the field bin experiments. Additionally, those elements in the humidity cell experiments that exhibited significant differences in their release rates between temperature treatments were not released at the same rates as they were in the field bin experiments. Microbial communities in both experiments were analyzed through a combination of 16S rRNA gene sequencing and shotgun metagenomics. The original, untreated, waste rock from the mine site contained a microbial community dominated by sulfur oxidizers. After being applied to field and laboratory scale leaching experiments, the initial microbial community transitioned into communities unique to each experiment with clear shifts related to the duration of the experiment and temperature treatments. Both field and laboratory experiments supported microbial communities which were dominated by heterotrophs, with a less abundant community of acidophilic chemolithotrophs that persisted from the initial rock community under warm and cold temperatures. Metagenomic analysis also concluded that microogranisms living on the waste rock have the capability to function in cool temperatures. The findings of this work present a first glimpse into the microbial communities existing on low sulfide waste rock and how they influence the behavior of kinetic testing systems at relevant seasonal temperatures.

KEY WORDS:

Kinetic tests, waste rock, acid rock drainage, 16S rRNA sequencing, metagenomics, psychrotroph

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATED TERMS

AP= Acid potential

ARD= Acid rock drainage

Csp= Cold shock protein

MEND= Mining environment neutral drainage

ML= Metal leaching

MPN= Most probable number

NP=Neutralizing potential

ORP= Oxidation reduction potential

PAG= Potentially acid generating rock

PWQO= Provincial water quality objective

RISC= Reduced inorganic sulfur compound

SEM-EDS= Scanning electron microscopy- energy dispersive X-ray spectrophotometry

GENERAL INTRODUCTION

Waste rock from mining operations is an economic and environmental liability due to its ability to generate acid rock drainage (ARD) (Price, 2009). The process of acid generation from the oxidation of sulfide minerals is a complex biogeochemical process that is compounded by site specific geological, climatic, and biological features (Amos et al., 2015). Not all waste rock is identified as posing an immediate risk of acid generation, and predictions for when potentially acid generating (PAG) rock becomes acid generating, if ever, hold major implications in the selection of a site's waste rock management practices (Kevin A Morin & Hutt, 1998; Price, 2009).

Waste rock situated in arctic and subarctic climates, much of which is not uncommonly classified as PAG rock, is exposed to seasonal extremes of warm and cold temperatures (Dawson, Morin, Canada Centre for Mineral and Energy Technology, Canada. Indian Affairs and Northern Development, & MEND (Canada), 1996). Microorganisms play a large role in waste rock geochemistry (Baker & Banfield, 2003), but are particularly enigmatic in their ecology within PAG rock systems in arctic and subarctic climates. This thesis examines microbial communities originating from low sulfur waste rock of a nickel- copper- mixed element mine in northern Ontario to infer how their structure and metabolic potential supports acid generating predictions in kinetic testing systems (Fig. 1.1).

Chapter one is a literature review of the current understanding of waste rock weathering from both geochemical and microbiological perspectives. Challenges presented by low

temperature regions and their influence on waste rock weathering are also discussed. Additionally, the waste rock used throughout this study and its features are introduced.

Chapter two reviews a 28 week humidity cell experiment where waste rock is weathered at warm (20°C) and cold temperatures (5°C). Leachate geochemistry is compared between the two temperature treatments. The geochemistry of this artificial system is contrasted with the microbial community structures at the mid- and end-points of the experiment. A snapshot of the metabolic potential of the communities is also constructed at the conclusion of the experimental time period.

Chapter three follows the weathering of waste rock through the first 11 months of a field bin kinetic test. The effects of site climate are naturally exploited to observe changes in leachate geochemistry over time, as well as the succession of the microbial community present at the mid- and end-points of the experiment. Key metabolic pathways present in the microbial community in the 11^{th} month are discussed and supported by ARD prediction.

Chapter four summarizes the results of the lab and field experiments described in previous chapters to show the influence of different kinetic tests on the resulting geochemical and microbiological conclusions assisting with ARD prediction of the waste rock. The impact of each test on the changes in microbial communities from the initial unweathered substrate and their potential ability to promote waste rock geochemistry is discussed. Finally, implications of temperature on the geochemistry and microbiology of the system is highlighted with hopes to provide a better understanding of experimental and natural waste rock systems.

CHAPTER 1: MULTIDISCIPLINARY PERSPECTIVES OF WASTE ROCK WEATHERING IN TEMPERATE CLIMATES

1.1 Process and consequences of the oxidation of sulfidic waste rock.

Mineral exploitation endeavors are one of the driving forces of the World economy. Canadian mining operations exclusively have produced trillions of tonnes of waste materials which continue to be generated at a rate of 460 million tonnes per year (Winfield, Coumans, Newman Kuyek, Meloche, & Taylor, 2002). These materials exist as waste rock, involving low grade rock excavated in order to access a deposit of value, as well as tailings, which are finely ground residuals of smelting and other mineral extraction procedures (Lottermoser, 2010; Morin & Hutt, 2001). Waste materials contain reactive minerals which are the cause of extensive environmental liability in the form of metal leaching (ML) and acid rock drainage (ARD) (Lottermoser, 2010; Price, 2009). As these minerals often weather in the environment for thousands of years (Price, 2005), active mines in Canada are required by law to plan for the maintenance of their waste materials throughout the course of the mine's lifetime and must produce extensive closure plans to successfully reclaim the mine site to nearly pristine conditions (Office of the Auditor General of Ontario, 2014; Price, 2005). However, many mines that have long since ceased operation have left behind tonnes of reactive waste materials which now fall into the hands of government agencies to manage (Office of the Auditor General of Ontario 2014). The reclamation costs for mine wastes are extremely costly, and are currently predicted to be in excess of 15 billion dollars for abandoned Canadian sites alone (Mining Watch Canada, 2008).

The sulfidic nature of waste rock the root cause of ecological disturbances resulting from mine waste (Price, 2009). The majority of base metal and precious metals, uranium, diamond, and coal mines are based in sulfidic rock (Price, 2009). The most abundant sulfide mineral on Earth is pyrite, which weathers upon exposure of to water and oxygen as a series of oxidation reactions (eqn 1- 4) (D.Barrie Johnson & Hallberg, 2003; Price, 2009). Reaction (1) occurs through abiotic or by microbial oxidation as iron sulfides are exposed to oxygen and moisture, which along with Fe^{3+}, release sulfoxy anions and Fe^{2+} from the rock (Akcil & Koldas, 2006; D. Leduc, Leduc, & Ferroni, 2002). The subsequent transformation of ferric to ferrous iron tends to be microbially mediated below pH 4.5 and can be considered the rate limiting step in this equation (Singer, 2010). In the final steps, ferrous iron is oxidized into ferric iron and precipitated iron hydroxides as the reactions proceed at low pH, (Akcil & Koldas, 2006; D.Barrie Johnson & Hallberg, 2003). Additionally, iron- oxidizing microbial species or ferric iron will dissolve pyrite, generating substantial acidity and regenerating the cycle (eqn 1, 4) (Baker & Banfield, 2003). The acidic solution generated from these oxidation reactions known as acid rock drainage (ARD) or acid mine drainage (AMD), is one of the largest environmental liabilities to mining industry (Akcil & Koldas, 2006; D.B. Johnson, 1995).

1) $FeS_2 + 7/2\ O_2 \rightarrow Fe^{2+} + SO_4^{2-} + 2\ H^+$

2) $Fe^{2+} + 1/4\ O_2 + H^+ \rightarrow Fe^{3+} + 1/2\ H_2O$

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 weathering of waste rock takes place over three stages, causing a flux in the waste rock mineralogy and subsequent drainage chemistry. The first stage is characterized by the initial

dissolution of reactive primary minerals and consequent formation of secondary minerals, followed by the dissolution of secondary minerals existing as precipitates, and finally unreactive minerals (Kevin A Morin & Hutt, 1998). The extent and rate at which weathering occurs is a balance of the acid generating potential provided mainly by sulfide minerals, as well as neutralization potential from carbonates, aluminosilicates, and iron/aluminum ferric hydroxides (Lottermoser, 2010). The elements which are the most reactive and persist the longest will dictate the trajectory of the weathering process (Morin & Hutt, 2001). Acid generation from highly reactive sulfide minerals such as pyrrhotite and galena can be offset by reactive neutralizing minerals, such as carbonates, as seen in equation 5 (Lindsay et al., 2015; Nordstrom, 2011). Less reactive compounds such as aluminosilicates are also useful in attenuating acid generation, but are not readily available until the later stages of the weathering process (Jamieson, 2011). As smaller, more exposed particles will be more available to participate in weathering, the size and distribution of these acid and neutralization generating minerals within the waste rock will also influence the trajectory of waste rock weathering (Strömberg & Banwart, 1999).

5) $$CaCo_3 + H^+ \rightarrow Ca^{2+} + HCO_3^-$$

The ultimate problem of acid generation in mine wastes is its ability to often promote the solubilization of metal and metalloid species, thereby leaching potentially toxic metals into the surrounding environment (Morin & Hutt, 2001; Price, 2009). It is important to note that metal and metalloid mobility is primarily influenced by pH, redox conditions, and the mineral complex involved, among other factors including climate and temperature (Lindsay et al., 2015; Morin &

Hutt, 2001; Nordstrom, 2011). Metal and metalloid leaching therefore occurs on an element and site- specific basis, and will fluctuate based on how the rock has weathered. In effect, while numerous elements of concern including, but not limited to, Cd, Al, Pb, and Cu are released in ARD scenarios, metal and metalloid leaching of elements such as As, Zn, and Se from neutral mine drainage, can be equally problematic (Jamieson, 2011; Price, 2009).

1.2 Assessment practices for the determination of waste rock acid generating status.

The establishment of the acid generating potential of a mine's waste rock is a critical parameter in determining the scope of management practices needed throughout the existence of the waste material. The correct interpretation of results from acid generation predictive tests is reliant on a solid understanding of the current site conditions and waste rock material (McDonald et al., 2012; Morin & Hutt, 2001; Wim & Ulrich, 1988). Detailed mineralogical, physical, total element, and soluble constituent analyses are to be conducted on multiple samples from areas of the site which will be disturbed (Price, 2009). Further resampling and classification of the materials provides improved estimates of the acid generating status of the materials (Morin & Hutt, 2001). This first step allows for the general prediction of acid generating potential of the waste rock, improved interpretation of static and kinetic test results, and also provides a baseline condition for the material as it is monitored over its lifetime (Morin & Hutt, 2001; Price, 2009).

Static tests are conducted on waste materials at a single point in time to measure geochemical, physical, and mineralogical properties of a waste material which can be extrapolated to predict future characteristics of waste materials (Morin & Hutt, 2001). Many rapid and inexpensive tests are available for use in acid generation prediction, the most common

of which being the EPA 600 acid- base accounting test (ABA) (Morin & Hutt, 2001; Parbhakar-Fox & Lottermoser, 2015). ABA testing evaluates general parameters of interest for waste rock including: I) paste pH to provide the aqueous pH of the sample, II) the sulfur species present in the sample, III) the acid potential of the sample, and IV) the neutralization potential of the sample (Morin & Hutt, 2001; Price, 2009). From the results of the aforementioned tests, the neutralizing potential ratio (NPR) can be calculated to determine if the waste material has no remaining neutralization potential and is therefore acid generating (AG, NPR > 2), the waste material has no remaining acid generating sulfur species and is not potentially acid generating (non-PAG, NPR < 1), or is potentially acid generating (PAG, 1< NP:AP < 2) (Price, 2009). Careful attention to pre-existing knowledge of the mineralogy of the rock needs to be taken into account to correctly tailor ABA testing methods and calculations to material- specific attributes (Parbhakar-Fox & Lottermoser, 2015; Price, 2009). The scope of ABA testing is still limited in its scope as it does not deliver the rate of acid and neutralizing reactions, nor does it account for site specific factors such as temperature, particle size and grain distribution which can drastically affect the outcomes of rock weathering (Jambor, Dutrizac, Groat, & Raudsepp, 2002; Parbhakar-Fox & Lottermoser, 2015).

Kinetic testing systems are a more advanced form of assessing how waste materials will weather. Tests are ran for long periods of time, during which materials can be doused with water to collect leachate for analysis (Price, 2009). Although they are expensive and time intensive to complete, the benefit of kinetic testing systems is that reaction and depletion rates of materials can be calculated for specific samples which have often been selected based on their ABA results (Ferguson & Erickson, 1988; Morin & Hutt, 2001; Price, 2009). Kinetic tests can range from

more controlled laboratory tests, where reaction rates can be artificially altered, such as humidity cells and leach columns, to field leach bins and leach pads experiments, which are conducted outside on the mine site to capture more variation from environmental factors (Ferguson & Erickson, 1988; K. Lapakko, 2002). As reaction rates will vary based on which test methods are conducted, ideally a combined approach in which multiple kinetic tests, as well as other static and geological tests are conducted should provide an optimal estimation of a site's waste rock evolution (Benzaazoua, Bussière, Dagenais, & Archambault, 2004; Price, 2009).

1.3 Implications of microorganisms in waste rock weathering.

The ubiquitous nature of microorganisms on Earth predisposes microorganisms to be intimately connected to the biogeochemical cycling of elements. Microorganisms are the dominant form of life on the planet that can access energy from organic sources (heterotrophy), inorganic compounds (lithotrophy), or a combination of the two (mixotrophy). The adaptive and resilient metabolisms of microbes allows them to survive the extremes conditions present in different environments, such as those found in mine wastes (Baker & Banfield, 2003; Méndez-García et al., 2015). Waste rock piles in particular can be hostile environments to exist within, yet microbial communities composed of bacteria, archaea, and fungi still manage to establish themselves as a part of their ecosystem (Jones et al., 2011; Zhang, Niu, Liang, Liu, & Yin, 2016). Rock piles are not a homogeneous environment; surface rock is highly exposed to the exterior climate while the interior of the heaps are more stable environments but are often more prone to extreme conditions generated by the weathering of the waste rock (Amos et al., 2015; Morin & Hutt, 2001). Cells that survive in these rock piles must overcome a series of stresses including extremes in temperature, pH, metal toxicity, and extremes of moisture content (Baker

& Banfield, 2003; Zhang et al., 2016). Temperatures can range from very moderate temperatures that reflect exterior climactic temperatures to temperatures in excess of 50°C if wastes contain high concentrations of sulfides (Pham et al., 2013; Schippers et al., 2010). The acidic environment generated from highly sulfidic wastes may select for communities capable of resisting osmotic stress and metal species that are in high concentrations in the pore water (Zhang et al., 2016). Osmotic stress is also a concern with respect to moisture content, which can be saturated in the porewater, but low at the surface of exposed rocks (Mayer, Frind, & Blowes, 2002).

Waste rock is an oligotrophic environment in contrast to others such as soils, sediments, or an animal host. In aerobic regions of this environment, cells rely on metal and metalloid elements, and sulfur compounds as energy sources, which are carefully obtained by adhering directly to the surface of minerals or sequestering them by means of siderophores from pore water (Baker & Banfield, 2003; Méndez-García et al., 2015). Iron oxidizers tend to be acidophiles such as *Acidithiobacillus spp.*, *Leptospirillum spp.* and *Sulfolobus spp.* (Bellenberg et al., 2015; Remonsellez et al., 2009; Sand, Gehrke, Jozsa, & Schippers, 2001; Valdés et al., 2008). Many sulfur oxidizers can also oxidize iron, however many will only transform sulfur species, including *Thiobacillus spp*., and *Thiomonas spp*. (Dockrey et al., 2014; Schippers et al., 2010). Carbon is commonly obtained by fixation by chemolithtrophs and chemophototrophs, as access to organic carbon is generally limited to that available from the succession of the microbial community itself (Cárdenas, Valdés, Quatrini, Duarte, & Holmes, 2010; Marín et al., 2016). Organisms capable of utilizing organic carbon include *Acidiphilium spp*., members of the *Xanthomonadaceae* and the *Chloroflexi,* which simultaneously support the growth of dominant

chemolithotrophs by removing organic carbon from the ecosystem (Brierley, 1999; Liljeqvist et al., 2015; Méndez-García et al., 2015). Moreover, recent studies of circumneutral waste rock environments have suggested that microbial communities may be composed of a larger proportion of heterotrophic organisms than previously assumed (Blackmore et al., 2018; Jones et al., 2017). Nitrogenous compounds are derived from remnants of explosive materials left from the blasting of waste rock, as well as from other microorganisms and byproducts of their metabolism (Morin & Hutt, 2001). In more acidic environments *Leptospirillum* can be mainly responsible for nitrogen fixation but *Acidithiobacillus* and *Alicyclobacillus spp.* can also complete this function (Tyson et al., 2005; Valdés et al., 2008). The metabolic contributions of individual microorganisms within the waste rock environments support the survival of the community as a whole and thus provide a basis for dominant chemolithotrophic organisms to drive changes in the ecosystem's underlying geochemistry.

It is well documented that microorganisms are not only capable of metabolizing metal and sulfur species, but play major roles in the biogeochemical transformation of these elements. With regard to sulfur oxidation in mine wastes and the subsequent generation of AMD as previously discussed, microorganisms are culprits in the initiation and perpetuation of these reactions. The initial oxidation of pyrite in equation 1 occurs at circumneutral pH as ferrous iron attacks pyrite to release sulfoxy anions (Fig. 1.2) and Fe^{2+} (Morin & Hutt, 2001). Neutrophilic sulfur oxidizers such as *Thiobacillus spp.* adsorb to the mineral surface to transform the sulfoxy anions into sulfate and acid through a series of intermediate reactions involving thiosulfate, trithionate, and tetrathionate (Dockrey et al., 2014; Nordstrom & Southam, 1997). At this point, when the pH is above 4.5, Fe^{2+} can be spontaneously oxidized to Fe^{3+} and then to iron hydroxide

precipitates, which coats bacterial cells in biofilms on the pyrite (Dockrey et al., 2014). As sufficient Fe^{3+} partially re-solubilizes from the iron hydroxide, it attacks pyrite to continue regenerating the afore mentioned cycle of oxidation reactions and generates substantial quantities of acid within the biofilm's immediate environment (Southam & Beveridge, 1993). Sulfur oxidizers continue to oxidize sulfur species to clear the surface area of precipitated sulfur compounds, thus allowing further iron oxidation of the pyritic mineral by means of iron oxidizing microorganisms or abiotic reactions (Dopson & Lindström, 1999).

When acidity builds to a pH of 4.5 or lower, Fe^{2+} can no longer favourably be oxidized to Fe^{3+} by oxygen, significantly slowing the oxidation reaction (Morin & Hutt, 2001). Pyrite will then reduce Fe^{3+} faster than it can be produced by the abiotic oxidation of Fe^{2+} and the system will maintain a more neutral state (Moses & Herman, 1991). This oxidation reaction is the rate limiting step in AMD generation (Moses & Herman, 1991). However, in the presence of acidophilic iron oxidizers, who can metabolize ferrous iron as a means of generating energy such as *Acidithiobacillus spp.*, the reaction can continue at a rate sufficient to perpetuate the generation of acid (Bacelar-nicolau & Johnson, 1999). Sulfur oxidizing bacteria will continue to allow sufficient contact surfaces for access to pyrite and the continuation of AMD generation (Dopson & Lindström, 1999; Schippers et al., 2010), but will instead favour acidophilic species metabolizing elemental sulfur, pentathionate, and sulfate (Dockrey et al., 2014). If the right balance of acidophilic microbial iron and sulfur oxidizing species is present, along with sufficient sulfur minerals and oxygen, then the perpetuation of acid generation from chemical and microbial reactions can result in AMD if not suppressed by neutralizing minerals (Amos et al., 2015). Ultimately, the microbial activities within pyritic waste rock are responsible for

accelerating abiotic reactions by five to six orders of magnitude (Baker & Banfield, 2003), making them responsible for 75% of the AMD reaction process (Edwards, Bond, & Banfield, 2000).

Morin and Hutt's ARD wheel approach (Fig. 1.3) is a generally respected overview of the components needed to assess the weathering of waste rock with respect to ARD generation (Kevin A Morin & Hutt, 1998; Parbhakar-Fox & Lottermoser, 2015). Microbial communities have been long known to be fundamental in the production of ARD (Baker & Banfield, 2003; D.Barrie Johnson, 1998; Méndez-García et al., 2015), yet they are not included in the wheel approach. Morin and Hutt, amongst other texts, present biological oxidation of sulfides as a key factor in ARD production, yet little information tends to be included in the models and guidelines they provide for ARD management (Ferguson & Erickson, 1988; Price, 2009; Rimstidt & Vaughan, 2003). The reasoning in defense of refraining from further incorporating the contributions of microbes into mine waste weathering models is largely due to first, the lack of a concrete understanding of microbial communities and their functions within mine waste. Additionally, the absence of accurate, precise detection methods for microorganisms within waste materials makes it difficult to identify and monitor microbes within waste rock systems. Furthermore, it has been argued that mining materials are not maintained in sterile environments during geochemical testing and are in reality therefore already incorporated into such models (K A Morin & Hutt, 2010). This limiting attitude towards mine waste geomicrobiology is valid given that a degree of caution is necessary to avoid the consequences that can arise from incorrect modelling of ARD systems, but is short-sighted given the trajectory of the advancement of knowledge in geomicrobiology and known ecological principles.

Current models of ARD generation, both mathematical and empirical, are not exclusively based in the geological sciences but can downplay or misconstrue the realistic role of microbiology in the system (Nordstrom & Southam, 1997). Models that do not explicitly include microorganisms may still have some microorganisms present on the materials but the representation of the native microbial community of those materials and the time scales and conditions they are forced to exist within is often not realistic (Olson, Brierley, & Brierley, 2003; Parbhakar-Fox & Lottermoser, 2015; Rawlings & Johnson, 2007). Of the studies that attempt to reconcile the abiotic and biotic conditions, many experiments have utilized single organisms (ie: the model organisms for this system, *Acidithiobacillus spp.*) (Bacelar-nicolau & Johnson, 1999; Morin & Hutt, 2001) or synthetic microbial communities to test their dynamics with laboratory static and kinetic tests (Lasse Ahonen & Tuovinen, 1991; Hesketh, Broadhurst, Bryan, Van Hille, & Harrison, 2010; Sapsford, Bowell, Dey, & Williams, 2009). Most results are conclusive that sulfide reaction rates and metal leaching is increased in the presence of microbial communities, likely providing a closer description to the true model, but is still oversimplified due to the complexity of interactions within and between microbial communities and the abiotic environment that are difficult to replicate in the laboratory (Baker & Banfield, 2003; Olson et al., 2003; Schippers et al., 2010; Wakeman, Auvinen, & Johnson, 2008). The field of geomicrobiology as a whole has expanded enormously in the past 20 years with the constant improvement of molecular techniques. Improved resolution and lower costs of tools that allow researchers to examine the genetic and metabolic contents of cells and entire communities has shifted the past focus from understanding which microbes are present to how microbes are living on an intricate scale at the individual and community levels in the lab and field (Denef, Mueller,

& Banfield, 2010; Franzosa et al., 2015; Gawad, Koh, & Quake, 2016; Goodwin, McPherson, & McCombie, 2016; Wagner & Haider, 2012). While AMD research tends to be applicable to mine wastes in general, the specifics of waste rock is less studied, than that of tailings (Dawson et al., 1996). Nevertheless, leaps in our understanding of these systems have been made in the past two years as multiple publications of in- situ research on waste rock pile and AMD microbiology have been released where little was previously available (Jones et al., 2017; J. B. Langman et al., 2016; Xiao et al., 2016; Zhang et al., 2016). Such results are promising to provide grounds for industry acceptance of microbial interactions within mining environments and a push for multidisciplinary approaches to solving mine waste issues.

1.4 Waste rock weathering at cold temperatures.

Mine sites in the northern United States, Canada, and northern Europe operate under temperate climates. While the summer months can reach temperatures above 30°C, winters bring snow and temperatures below -20°C. From a geological perspective, lower temperatures, and particularly freezing temperatures bring lower reaction kinetics due to the expansion of water volume which will cause a fold decrease in O_2 diffusion lost to freezing water and change the hydrology and oxygen availability within waste rock piles (L Ahonen & Tuovinen, 1990; Dawson et al., 1996). Furthermore, below approximately -16°C, leaching of metals is no longer dependent on conductivity of the solution and interacting rock interface but will instead be mobilized more slowly by means of diffusion towards areas of lower concentration (Dawson et al., 1996). These factors likely contribute to lower sulfide oxidation and overall mineral leaching rates which is seen consistently in humidity cell experiments ran at low temperatures (Dawson et

al., 1996; J. Langman et al., 2014), as well as field waste rock test pads in permafrost areas (J. B. Langman et al., 2016, 2017).

The Mining Environment Neutral Drainage (MEND) Program for research on mine drainage in permafrost regions has suggested that although cold temperatures may commonly reduce the rate of acid generation by approximately one order of magnitude from their research in primarily Canadian sites, sulfide oxidation is not halted (Dawson et al., 1996). It has been noted that at freezing temperatures, porewater does not freeze entirely within the rock pile due to rock surface interactions, high ionic strength of the liquid from element leaching, and insulation provided within the piles (Dawson et al., 1996). Freezing water around the exterior of the heap causes flow paths to be altered, allowing pore water underneath frozen areas to become increasingly saturated with leached elements as retention time is elongated and temperature increases the solubility of oxygen (Dawson et al., 1996; J. B. Langman et al., 2017). Zonation within rock piles becomes increasingly important, as the exposed exterior of the piles are weathered more quickly and undergoes a greater flux in its environmental conditions, while the interior of the heap maintains more stable conditions (J. B. Langman et al., 2017). For example, despite winter temperatures reaching below -30°C at the Diavik mine, a diamond mine in the Northwest Territories, temperatures within the low sulfide waste rock pile did not dip below -5°C during the first two years of operation (Pham et al., 2013). On a more drastic scale, many waste rock piles with established sulfide oxidation that is highly reactive will continue to run exothermic reactions and maintain internal heap temperatures above 40°C, as demonstrated by mining operations in Québec and Finland containing large volumes of pyrrhotite (Dawson et al., 1996; Halinen, Rahunen, Kaksonen, & Puhakka, 2009). Rock within these areas that experience

freeze-thaws during the shoulder seasons also exhibit increased breakdown of the rock from the physical stress placed on the rock known as frost shattering (Dawson et al., 1996). Alongside the other factors mentioned above This exposes increased surface area of sulfides to continue to support oxidation reactions despite a higher activation energy needed to initiate reactions at cold temperatures (Dawson et al., 1996).

From a microbial perspective, life may be slowed, but is not limited by low temperatures. The majority of microorganisms are mesophilic, meaning that they are active between 15 and 40°C. Many microorganisms native to cold environments are psychrophilic, in that they are active at temperatures at or below -20°C or more commonly, psychrotrophic where they are tolerant of temperatures below 0°C but may not be actively dividing below 7°C (Liljeqvist et al., 2015). Protection from ice nucleation, and the maintenance of enzymatic function and membrane fluidity are made possible in these organisms through the use of antifreeze proteins, specialized cold shock proteins, and increased cryoprotectants such as trehalose and the secretion of extra-polysaccharides in the cell membrane, respectively (D'Amico, Collins, Marx, Feller, & Gerday, 2006). Several psychrotrophic bacteria have been characterized, notably *Acidithiobacillus ferrivorans* (Liljeqvist, Rzhepishevska, & Dopson, 2013; Liljeqvist, Valdes, Holmes, & Dopson, 2011), *Ferrovum myxofacien*s (Moya-beltrán et al., 2014), and *Acidiphilium*- like isolates (Berthelot, Leduc, & Ferroni, 1993). *A. ferrivorans* is the best studied and understood of the organisms, being closely related to *A. ferrooxidans* (Hallberg, González-Toril, & Johnson, 2009). Unlike its related mesophilic species, *A. ferrivorans* is active at 5°C but has a doubling rate less than three times that of *A. ferrooxidans* (Kupka et al., 2007; L. G. Leduc, Trevors, & Ferroni, 1993; Mykytczuk et al., 2011). Additional studies of microbial communities have documented

microbial communities being present at high latitude mine sites where AMD is being actively generated (Auld, Mykytczuk, Leduc, & Merritt, 2017; J. B. Langman et al., 2017; Liljeqvist et al., 2015). The metagenomic study of the Kristineberg AMD stream identified that cold- stress genes were present in much higher proportions in comparison to similar thermophilic or mesophilic sites, suggesting that AMD microbial communities may adapt to function in colder climates (Liljeqvist et al., 2015).

1.5 Study site characteristic and preliminary waste rock surveys.

The waste rock used in this study site originates from a pre-operational underground Ni-Cu-precious metals (Pt, Pd, Au) mine in northern Ontario. The site is situated on the boreal shield, and experiences a yearly average temperature of 3.7°C, with maximum and minimum temperatures of 38.3°C and -39.3°C respectively, and annual precipitation measuring 899 mm. The mine is slated to produce metals of value for nine years, during which time 3.6 Mt of waste rock is expected to be generated, of which 397 000 tonnes of cut rock has already been blasted (Fig. 1.4). This study focuses on the characteristics of the generated cut rock from the surface blasting and adit development (Hamilton & Pedlar-Hobbs, personal communication, 2016).

The site's ARD metal leaching program in place has identified the dominant rock lithologies to include quartzite, metasediment, metabasalt, diabase, and rhyolite. However, the cut rock includes only metasediment, metabasalt, and diabase. Of these, sulfide minerals consisting of pyrite, pyrrhotite, and chalcopyrite have been found in all three lithologies with chalcopyrite being negligible (Table 1.1). Sulfur containing minerals are of greatest concern in the metasediment (Table 1.2), with the average concentration of sulfur- sulfides being 0.22 to

0.34 wt. % and total sulfides being 0.9 wt. % (Table 1.3). The amount of neutralizing minerals is low in comparison to most mine sites, with the metabasalt (metacrystic gabbro) having the greatest neutralization potential (Table 1.2). Quartz is highly abundant (Table 1.1) but has a low reactivity and will therefore be slow to contribute to neutralizing potential within the system. The cut rock used within the experiment is classified as PAG rock, with carbonate NP to sulfide AP ratio being 1.33 (Table 1.3).

Microbiological analyses were conducted on a 1:1 waste rock mixture of < 2" crush that had weathered in the field for under a year, and the same rock crushed to < 2 mm. The initial microbial community was surveyed using 16S rRNA pyrosequencing, which provided evidence of a simple community composed of neutrophilic sulfur oxidizers and heterotrophs (Fig. 1.5.). The community is dominated by several *Thiobacillus spp.* (Fig. 1.6.) whose presence supports the likelihood that sulfide oxidation is occurring and could be assisted by means of microbial processes within the rock.

1.6 Concluding remarks

The present understanding of waste rock weathering is the result of multidisciplinary efforts aimed at predicting and managing the consequences of waste rock biogeochemical transformations over time. Knowledge gained from previous studies can be used as a base upon which to study specific challenges unique to this study site from an academic and industry perspective. The rare collaboration of geological and microbiological tools in this study will be a valuable asset when making decisions within the mine site's waste rock management program and for broadening the general understanding of similar systems present in colder climates.

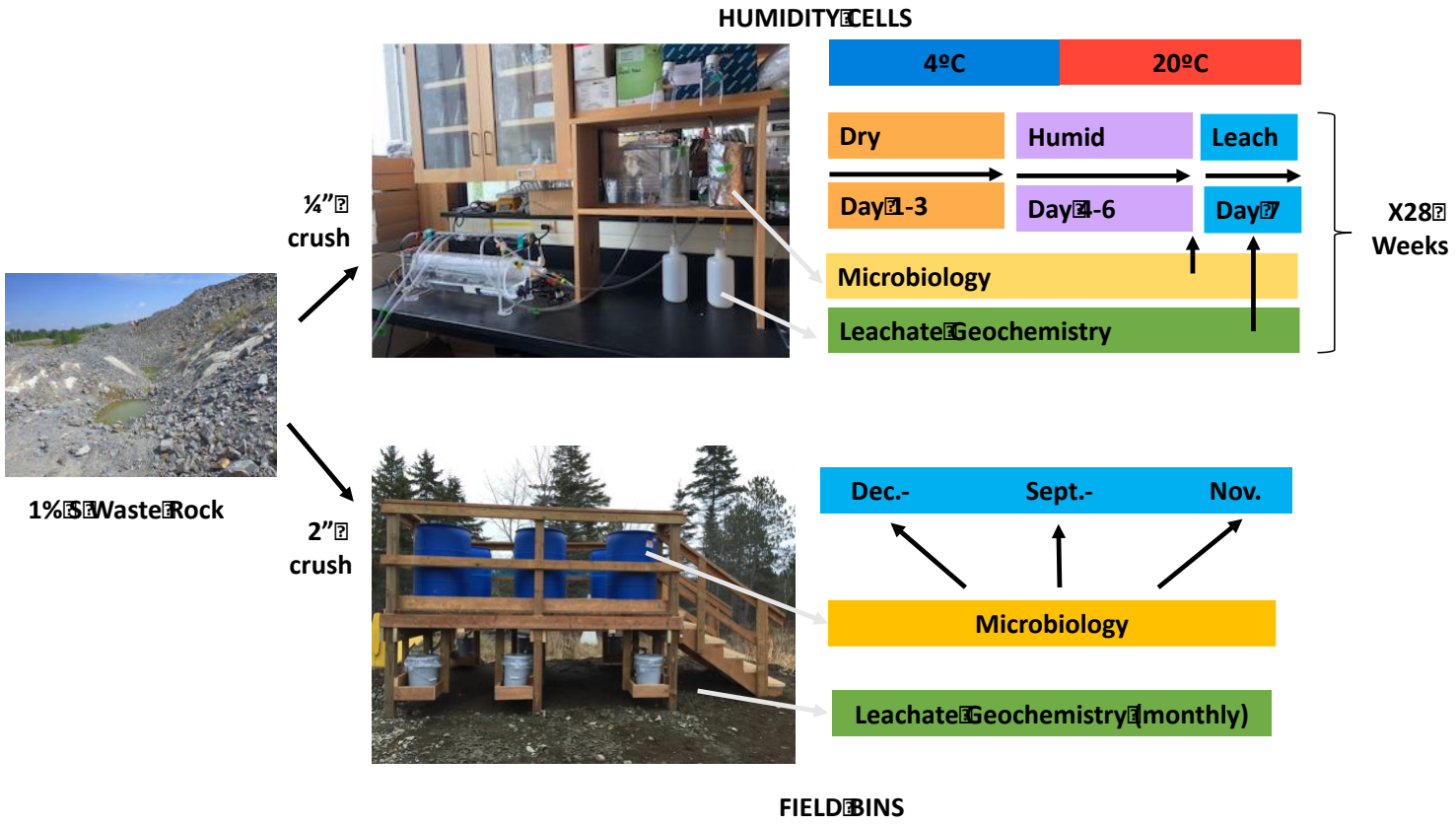


Figure 1.1.: Experimental design of humidity cell and field bin experiments analyzing geochemical and microbial leaching of low sulfur waste rock.

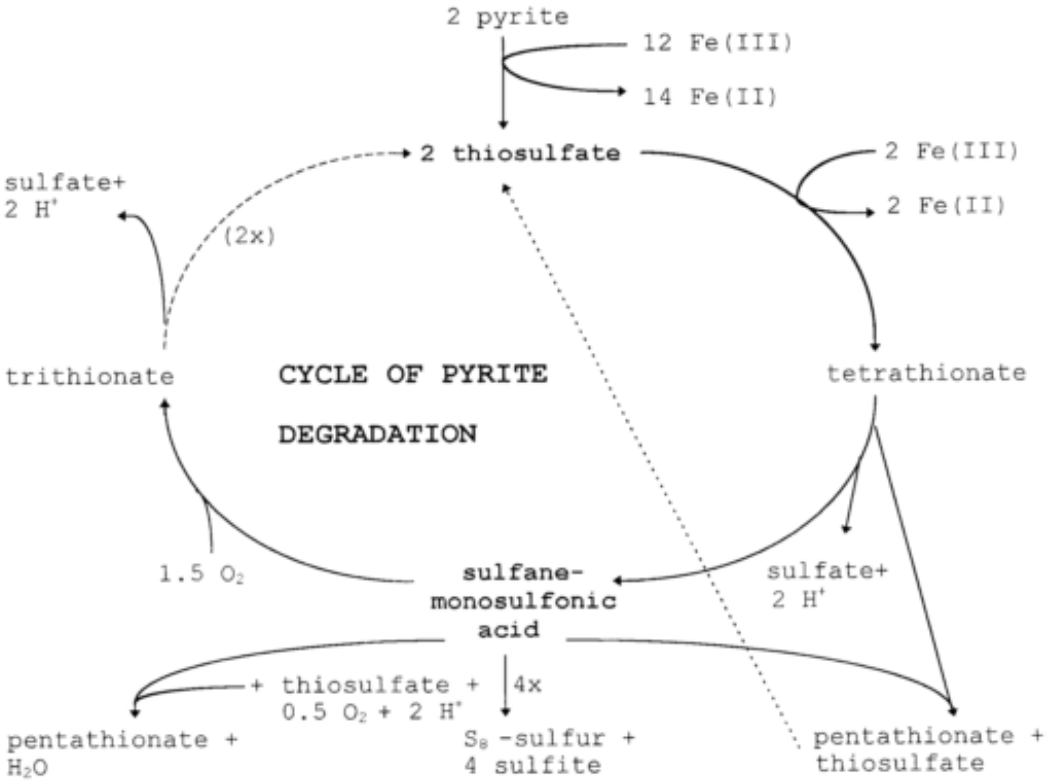


Figure 1.2.: Intermediate sulfur species metabolized from pyritic minerals by sulfur oxidizing microorganisms. From Shippers *et. Al*, 1996.

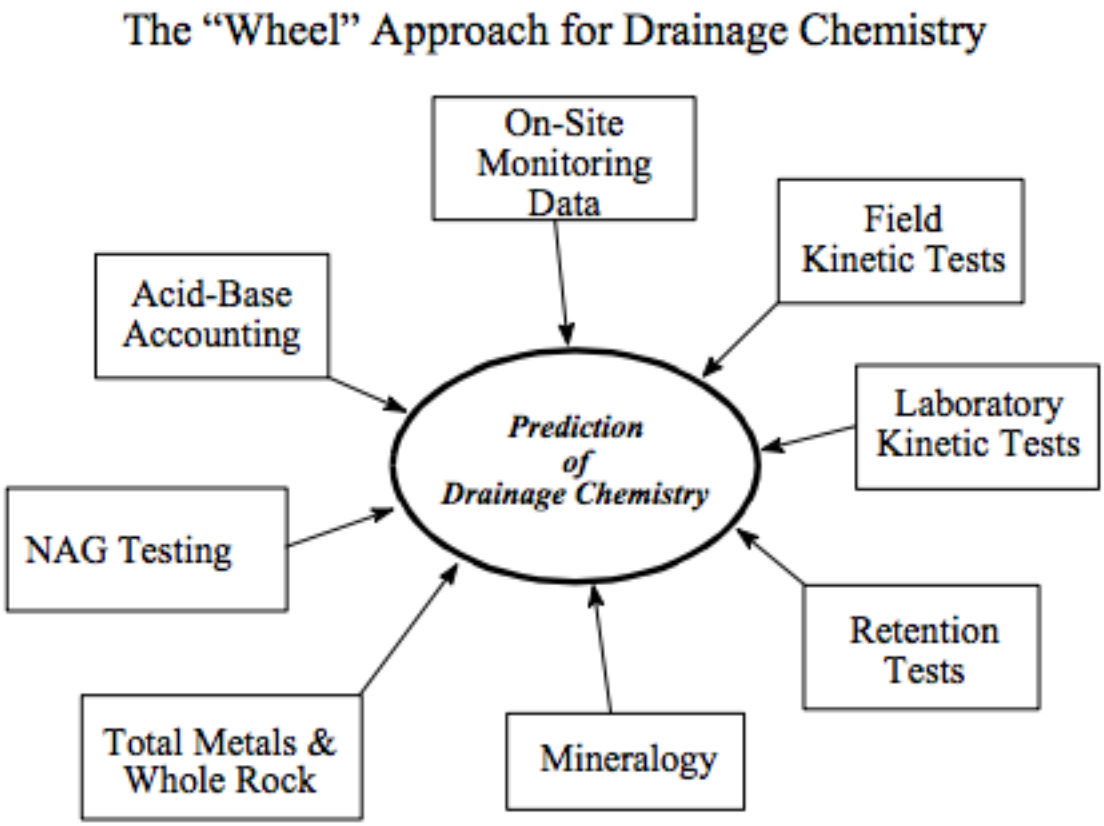


Figure 1.3.: Morin and Hutt's Wheel approach to the main inputs for the prediction of ARD generation (Kevin A Morin & Hutt, 1998).

A

B

Figure 1.4.: Site waste rock pile during early winter (A) and summer (B) months.

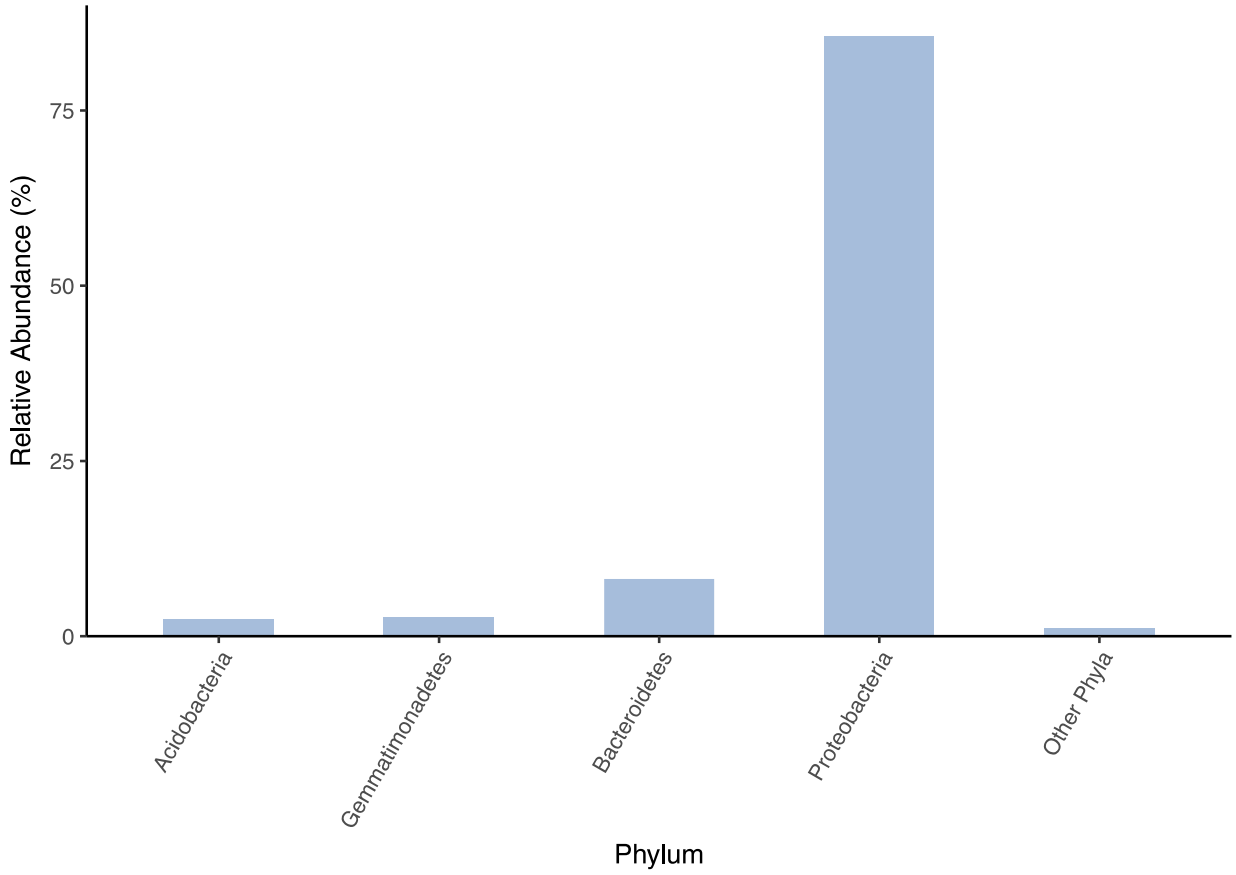


Figure 1.5.: Waste rock microbial community profile of OTUs with > 1% abundance at the phylum level as determined by 16S rRNA gene sequencing.

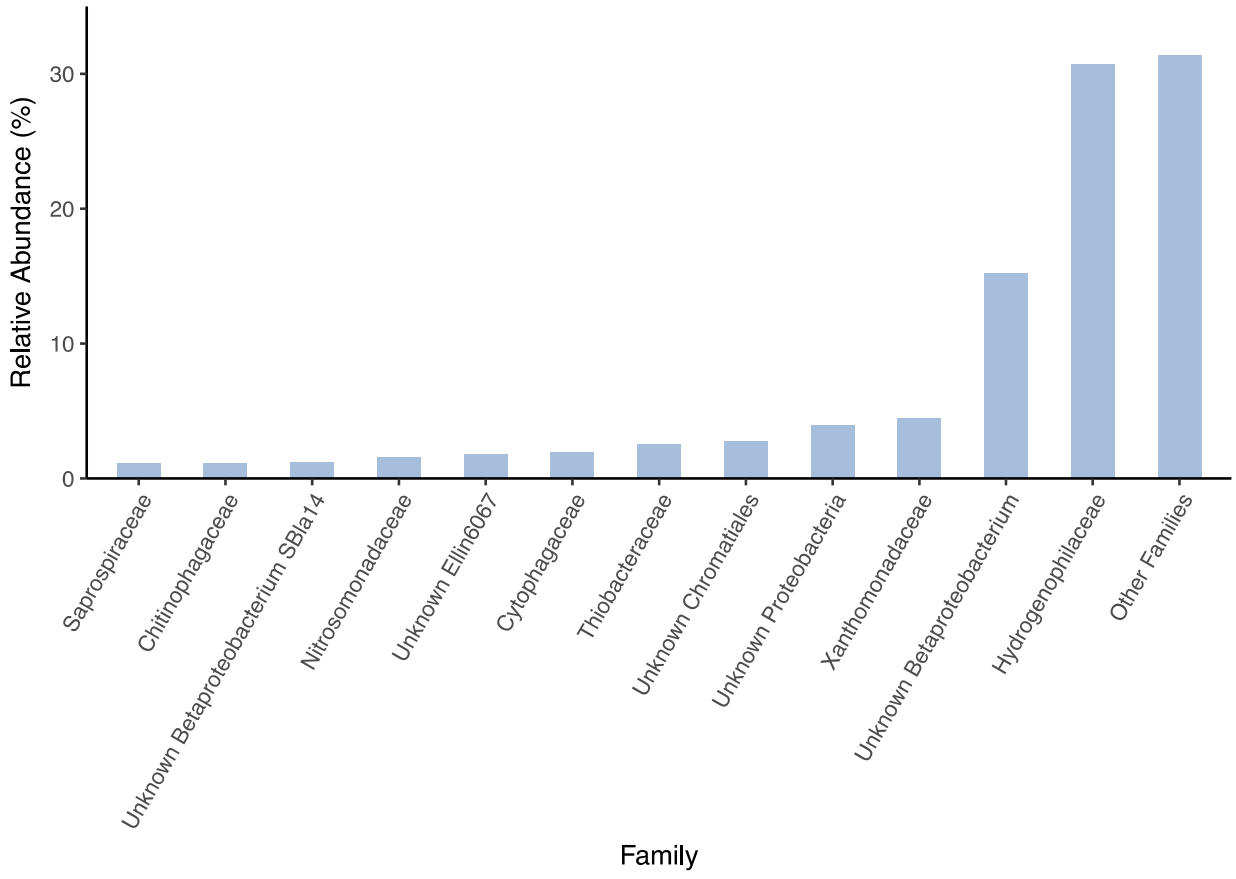


Figure 1.6.: Waste rock microbial community profile of OTUs with > 1% abundance at the family level as determined by 16S rRNA gene sequencing.

Table 1.1: Rietveld quantitative XRD analysis for field bin 1.

Mineral/ Compound	**Weight (%)**
Quartz (SiO_2)	32.9
Albite ($NaAlSi_3O_8$)	21.2
Microcline ($KAlSi_3O_8$)	1.6
Chlorite ($(Fe,(Mg,Mn)_5Al)(Si_3Al)O_{10}(OH)_8$)	12.7
Muscovite ($KAl_2(AlSi_3O_{10})(OH)_2$)	17.7
Biotite ($K(Mg,Fe)_3(AlSi_3O_{10})(OH)_2$)	2.0
Actinolite ($Ca_2(Mg,Fe)_5Si_8O_{22}(OH)_2$)	9.4
Calcite ($CaCO_3$)	1.7
Ankerite ($CaFe(CO_3)_2$	0.8

Table 1.2: Modal mineralogy of field bin 1 calculated from Rietveld XRD. The mineral mass is presented as a percent of the total minerals present by weight. The calculated estimated spherical diameter of the particles is 23.

Mineral	Mass (%)
Pyrite	0.83
Chalcopyrite	0.07
Sphalerite	0.03
Other Sulfides	0.01
Fe-Oxides	0.15
Ilmenite	0.14
Rutile	0.58
Calcite	1.04
Dolomite	0.12
Ankerite	0.04
Quartz	34.96
K-Feldspar	2.07
Plagioclase	16.97
Chlorites	13.53
Biotite	3.8
Sericite/ Muscovite	17.11
Clays	0.3
Amphibole	7.4
Titanite	0.55
Apatite	0.2
Other Silicates	0.10
Other	0.04

Table 1.3: Predicted reactivity of acid neutralizing minerals present in waste rock. Cut rock units were identified by Rietveld XRD analysis and classified by reactivity as per Morin and Hutt, 1997. Sulfide- sulfur content was determined by ABA.

Cut Rock Waste Rock Unit	Acid Neutralizing Minerals (%wt.)			Sulfide-sulfur (% wt.)
	Reactive to fast weathering	Moderately reactive	Slow weathering to inert	
Diabase	10.0	40.0	45.0	0.05
Metasediment	2.00	3.00	86.0	0.42
Metabasalt	7.00	50.0	39.0	0.05

<u>Table 1.4:</u> Neutralizing potential and acid- generating potential calculated by ABA for Field Bin 1. Net acid consuming Ca-Mg carbonates were used for the mineralogical neutralization potential (NP). Fe-sulfides were used for the mineralogical acid generation potential (AP) as they are the main sulfides to contribute to net acidity.

Mineral	**Wt. %**	**Dist'n. %**
Calcite ($CaCO_3$)	1.04	86.5
Dolomite ($CaMg(CO_3)_2$	0.12	10.1
Anketite ($Ca(Fe,Mg,Mn)(CO_3)_2$	0.04	3.42
NP from Ca-Mg Carbonates	1.20	100
Pyrite (FeS_2)	0.83	91.7
Chalcopyrite ($CuFeS_2$)	0.07	8.31
AP from Fe-sulfides	0.9	100
NP:AP ratio	1.33	1.46

CHAPTER 2: THE WEATHERING OF LOW SULFIDE WASTE ROCK IN HUMIDITY CELL EXPERIMENTS AT WARM AND COLD TEMPERATURES.

2.1 Abstract

Humidity cells are a well utilized laboratory scale kinetic weathering test in industry and academia. Triplicate humidity cell experiments were carried out over a 28 week period on low-sulfur PAG waste rock. Temperature treatments of 5°C and 20°C provided an opportunity to examine the effect of cold and warm temperatures that waste rock would be exposed to at a mine site on the evolution of waste rock mineralogy, leachate geochemistry, and microbiology. Leachate remained neutral over the course of the experiment and no elements exceeded PWQO guidelines. Alkalinity as $CaCO_3$ and Ni were preferentially leached in the cold temperature treatment, while SO_4, Al, Cu, Mo, V, and As were preferentially leached in the warm temperature treatment. These findings support the difference in the predicted time to acid generation for the two treatments, as warm humidity cells are not predicted to become acidic while the cold humidity cells will become acidic in 26 years due to higher relative release rates of carbonate to sulfide minerals. 16S rRNA gene and shotgun metagenomic analyses suggest that the humidity cells support a community of largely neutrophilic heterotrophic organisms which may not be contributing largely to sulfide oxidation. However, neutrophilic and acidophilic sulfur oxidizers are present in low abundance and have the metabolic potential to shift the community and geochemistry of the system into an ARD scenario. Humidity cell temperature treatments clustered together, with warm treatment humidity cells contained a higher microbial biomass and alpha diversity than cold humidity cells. Cold treatment humidity cells contained more hits to genes assisted with protection from cold temperatures.

2.2 Introduction

The most common kinetic test system used in a laboratory setting are humidity cells. Initially designed in the 1960s, these tests are still used as an industry standard in ARD prediction programs (ASTM, 2013; Sapsford et al., 2009). Humidity cell systems behave as microcosms in which 1 kg of waste rock is subjected to a weekly cycle of artificial weathering conditions. Humidity cells provide three benefits in comparison to other kinetic tests: I) They can predict the rate of acid generation by consolidating data from the reaction rates of sulfur and neutralizing species in primary minerals, II) The regular flushing of the cells reduces the accumulation of secondary precipitates present in the cells which can lead to incorrect constituent release rates and possibly time to AG predictions, and III) They allow a prediction of drainage chemistry from the material in question can be obtained (Morin & Hutt, 2001; Sapsford et al., 2009). It is important to note that reaction rates are reflective of the weathering of primary minerals in the material. Release rates of elements will therefore be accelerated in comparison to field values. This information can provide insight into the geochemistry of the system but is not conclusive in predicting ARD generation or elemental release rates in the field (K. A. Lapakko, 1994; Kevin A Morin & Hutt, 1998; Morin & Hutt, 2001).

The standardized protocol for humidity cells involves selecting a sample with mineralogy that has been determined to be of concern for acid generating potential and screening rock to include only material ~ < 2mm in size. 1kg of material is subjected to a weekly cycle of dry and humid air, which is completed by flooding and subsequently flushing the cell with ~ 1L of deionized water. Leachate can be collected and analyzed for dissolved constituents over time

from which release rates of the constituents and weathering of mineral compounds can be predicted. The test is run for a minimum of 20 weeks, or until rates of sulfate release have stabilized over a five week period of time (ASTM, 2013; Morin & Hutt, 2001; Price, 2009). Following the decommission of the experiment, secondary minerals and the retention of elements in the cells can be examined by Rietveld XRD analysis and sequential extractions. Humidity cells are flexible to modification, as aeration, flush volumes, microbial and solute inoculations, temperature incubations, and test duration can be altered in the experimental design depending what parameters are of interest to researchers (ASTM, 2013; Sapsford et al., 2009).

While humidity cell experiments are not standardized to assess the effect of temperature on waste rock weathering they can easily be modified to do so. By altering the temperature at which the humidity cell experiment is incubated, one can isolate the effect of temperature on waste rock weathering. Incubation at temperatures representative of those found on mine sites can be particularly useful when the mean annual site temperature is not reflected in the standard incubation temperature of 20ºC.

A notable example of utilizing temperature incubation of humidity cell experiments relevant to low sulfur waste rock in colder climates are the experiments published on the Diavik site (J. Langman et al., 2014) and the Duluth complex (Jones et al., 2017). In a series of experiments, waste rock of different acid generating classes from the Diavik diamond mine in the Northwest Territories was subjected to a five year humidity cell test at 20°C and 5°C to investigate geochemical, hydrological, thermal, biological, and temporal effects in the weathering of the site materials (Bailey, Blowes, Smith, & Sego, 2015; J. Langman et al., 2014).

Results of the geochemical investigations showed that the materials exhausted the majority of their acid generating species within the first two years of the experiment, although the material containing ~ 0.8% sulfide was capable of maintaining pH under 5 until the end of the experiment by continuing to overcome neutralization potential in the rock (J. Langman et al., 2014). The weathering of the warm cells was established to be 1.5 times that of the cold cells, as established by the ratio of specific conductivity between the two cells (J. Langman et al., 2014). Furthermore, the window in which acid was generated was smaller and lower element loads were measured in leachate of cells maintained at the colder temperature (J. Langman et al., 2014). In this study, microbiological sulfur and iron oxidizing communities were generally described by MPN methods, which found that microbial communities were responsive to pH and temperature (Bailey et al., 2015).

In a different set of studies, Lapakko *et. al* conducted a series of long term humidity cell experiments of low sulfur rock (< 1 %) with high silicate content near Duluth, MN, spanning nearly two decades at ambient temperatures (Jones et al., 2017; Lapakko & Antonson, 1994; Lapakko & Antonson, 2002). The study led by Jones *et. al* generated 16S rRNA amplicon data of DNA and RNA from two low sulfur waste rock types from different deposits, depicting active communities of mainly neutrophilic heterotrophs and sulfur oxidizers, notably, *Thiomonas spp*. and *Sulfuriferula spp*. The microbial communities involved were correlated with % S availability and leachate pH, suggesting that the microbial communities are influenced by the mineralogy and weathering of the waste rock they inhabit (Jones et al., 2014).

This thesis chapter uses humidity cell tests to examine the effects of temperature on the weathering of low sulfide waste rock as determined through leachate geochemistry and microbial community analyses. It was expected that weekly analyses of physical and elemental leachate geochemistry would be highly influenced by temperature, with cold cells exhibiting lower rates of weathering than warm cells. The microbiology of the system is also hypothesized to be reflective of the high aluminosilicate and carbonate mineralogy of the waste rock, with an expectation that neutrophilic Fe and S species would dominate the community. The microbial communities will likely also be influenced by temperature, with metabolic and taxonomic data indicating a less diverse but more established community of psychrophiles in cold cells in comparison to the warm temperature treatment. With this new information, humidity cell experiments may be better suited to include the microbiology and relevant site temperature into their design to provide improved ARD generation prediction.

2.3 Materials and methods

Experimental design

Waste rock from the site's 1.23% S rock pile that had weathered in the field for no more than a year was dried and crushed to < 0.25 inch in size at SGS (Sudbury, ON). The waste rock had previously been characterized as PAG rock by ABA accounting (Hamilton, 2015). Waste rock was not sieved to eliminate creating new mineral surfaces prior to loading the humidity cells. 1.1kg of rock was loaded into triplicate clear PVC humidity cells set on the laboratory bench at ambient temperature (20 °C ± 2°C) for the warm treatment (HW2, HW3) and in an incubator set to 5°C ± 0.5°C for the cold treatment (HC2, HC3) to contrast the effects of

temperature on weathering at warm and cool temperatures (Fig. 2.1). All cells were connected to a manifold conforming with ASTMD5744-13$^{\varepsilon 1}$ standards (ASTM, 2013). Maintenance of the cells otherwise conformed to ASTMD5744-13$^{\varepsilon 1}$ standards, with a weekly weathering regime consisting of a three day dry cycle (aeration with ten L/min/cell of room air), three day humid cycle (aeration with ten L/min/cell of humidified room air), and a final day in which cells were flooded prior to leaching (ASTM, 2013). 1L of deionized water was used to flood the cells every week and left to sit for an hour before leachate was collected from the cells and sent for geochemical analyses. Weekly flush cycles were carried out beyond the standard ASTM 20 week period to reach 28 weeks. Microbial samples were collected from the interior of the humidity cell following the humid weathering phase at mid- and end-points of the experiment to minimize edge- effects. After 28 weeks, humidity cells were leached every two weeks and geochemical data was not analyzed (Astm, 2013). Algal growth was minimized by covering the warm humidity cells in tin foil and maintaining the cold humidity cells in a dark incubator.

Leachate geochemistry

Leachate samples were collected weekly into 1L sampling bottles, stored at 4°C and processed the day following collection. Leachate mass and volume were measured from each humidity cell. Leachate collected from each of the triplicate humidity cells was mixed by shaking the vessel before pooling 250mL from each triplicate temperature treatment together and again mixing on a stir plate. 200mL and 75mL sub-samples of pooled leachate were prepared for further analyses; the 200mL subsample remained as is, and the 75mL subsample was filtered once through a 13mm 0.45µm polyethersulfane membrane filter and acidified to pH 2 with

HNO_3. These samples were sent to an external lab (SGS, Lakefield, ON) to be analyzed for total water chemistry, dissolved metals by inductively coupled plasma mass spectrometry (ICP-MS), NH_3/NH_4^+, NO_2^-, NO_3^-, and total sulfate. ORP was also measured on a pooled, unfiltered sample using a YSI Professional Plus handheld multiparameter meter in the lab (YSI Inc., Yellow Springs, OH). Analytes were compared to the Ontario Ministry of Environment and Energy Provinical Water Quality Objectives for the Protection of Aquatic Life to provide context to the concentrations of analytes released with respect to water quality standards (MOEE, 1994). Iron speciation was measured using the colorimetric assay for total and ferric iron (Karamanev, Nikolov, & Mamatarkova, 2002), however Fe concentrations appeared to be consistently under the detection limits of the assays. A pooled sample from each temperature treatment was also filtered through a 0.22µm polyethersulfone membrane and acidified to pH 2 prior to analyzing dissolved organic carbon (DOC) on a Shimadzu TOC-5000A in FPOC mode.

Mineralogy

Upon completion of the 28 week humidity cell experiment, one humidity cell from the warm and cold temperature treatments were decommissioned. After mixing the cells' contents with a spatula, a 10g sub-sample of the rock was left to dry on the bench for a month. Samples were sent for Rietveld quantitative XRD analyses completed at SGS (Lakefield, ON) to determine the resulting mineralogy of the weathered rock samples from cold and warm humidity cells, respectively. These data were used to compare differences in the mineralogy against Rietveld XRD analysis conducted on the waste rock prior to weathering in humidity cells.

Microbiology

To prevent excessive disruption to the geochemistry of the experiment, no more than a 5-10g sub-samples of waste rock were collected at a time. To collect a sample, a sterile 10mL test tube was pushed into the middle of the rock within the cell to a depth of approximately three inches to select for rock that was not in contact with the edges of the cell. A sterile scoopula was used to remove the rock from the test tubes, with the exception of the top 0.5 inch of material that was in contact with the air- rock interface of the cell. Rock samples were pooled by temperature treatment into 50mL falcon tubes and stored in RNA lifeguard (Qiagen, MD) at -20°C until they could be processed. Samples were collected from each humidity cell at the mid-point (week 19, HW2 and HC2) and end-points (week 28, HC3) on the seventh day of the cycle, prior to beginning the flush and leach of the cells. Due to low biomass in the system and difficulty extracting sufficient DNA yields for sequencing from the final humidity cell time point, additional samples were taken from the warm humidity cell treatments at 38 weeks to represent the end-point sample for the warm temperature treatment (HW3).

Samples were considered to be of low microbial biomass and limited waste rock material was available to be processed to prevent excessive disruption to the geochemistry of the experiment. Several different methods were used to maximize DNA recoveries from the low biomass samples; I) A 15g pooled waste rock sample in the RNA lifeguard solution was sonicated with a 13mm tip using a Fisher Scientific Sonic Dismembrator 500 (Fisher Scientific, CAN) for 2 min on 20 sec bursts with 2 sec breaks to dislodge biofilms. Sonicated samples were then pelleted by centrifugation at 3260 rcf for ten min. Supernatant was discarded and five g of

rock was subsampled into triplicate extraction preps of RNeasy PowerSoil DNA Elution kit (MoBio, CA), II) A 15g pooled waste rock sample was sonicated using a Fisher Scientific FS20 Ultrasonic Cleaner (Fisher Scientific, CAN) in RNA lifeguard solution for 15 min at 90 % power. Sonicated samples were then pelleted by centrifugation at 3260 rcf for 10 min. Supernatant was pipetted from the sample prior to processing and pooling the products of three preparations of a DNeasy PowerSoil kit (MoBio, CA). III) Approximately 15g of pooled waste rock sample was placed into a 50mL falcon tube and topped-up with sterile phosphate buffer (Zhou, Bruns, & Tiedje, 1996) and agitated on a vortex at top speed for 5 min. The samples were then centrifuged for 10 min at 3260 rcf, supernatant was discarded and the wash step was repeated. Supernatant was removed from the samples before continuing to extract DNA using three preparations of DNeasy PowerSoil kit. IV) 6g of waste rock samples were processed at MetagenomBio at the University of Waterloo. Two 3 g rock samples were subjected to DNA extraction utilizing membrane disruption via SDS and physical homogenization, followed by lysate clarification and purification by spin column chromatography (Jiujun Cheng, personal communication, 2017). With all of these methods, only minimal DNA (~10ng) was recovered. The end-point sample for cold humidity cells (HC3) was processed by method I, while the mid-point sample for warm humidity cells (HW2) was processed using method II, the mid-point sample for cold humidity cells (HC2) was processed using method III, and finally, the end-point sample for the warm humidity cell treatment (HW3) was processed using method IV.

DNA concentration and quality was checked by spectrophotometry using Agilent Take3 software (Agilent Technologies, CA), as well as by PCR amplification of the 16S rRNA gene using the broad eukaryotic primer set designed for the Earth Microbiome project (EMP 515F 5'-

GTGYCAGCMGCCGCGGTAA- 3', EMP 806r 5'- GGACTACNVGGGTWTCTAAT- 3') (Gilbert, Jansson, & Knight, 2014). PCR was completed using an Invitrogen Recombinant Taq DNA Polymerase and 0.6mg/mL BSA under the following conditions: initial denaturation of 4 min at 94˚C, 35 cycles at 94˚C for 45 s, 52˚C for 1 min, 72˚C for 1 min, and final elongation of 8 min at 72˚C. Library preparation for both 16S rRNA amplicons was completed by MetagenomBio at the University of Waterloo, with sequencing being completed on an Illumina Miseq using the forward primer (5'- CCTACGGGNBGCASCAG - 3') and reverse primer (5'- GACTACNVGGGTATCTAATCC - 3') (Takahashi, Tomita, Nishioka, Hisada, & Nishijima, 2014). Shotgun Metagenomic libraries were also produced for end-point samples from cold and warm treatments by MetagenomBio Inc. at the University of Waterloo.

Bioinformatic analyses

Paired end reads from 16S rRNA gene amplicon libraries were stripped of adaptors, primers and screened for PhiX contamination with BBDuk, merged with BBMerge, and bidirectionally trimmed to Q20 using BBDuk (Bushnell, 2014). Sequences were converted to fasta files using the fq2fa command in IDBA-UD (Peng, Leung, Yiu, & Chin, 2012) prior to adding QIIME labels in QIIME 1.9.1 (Caporaso et al., 2011). Dereplication, size sorting, doubleton removal, OTU clustering and a denovo chimera check was performed using the UPARSE pipeline (Edgar, 2013) in USEARCH 9.2. Taxonomy was assigned in QIIME against the Greengenes database (McDonald et al., 2012) using the RDP classifier (Cole et al., 2014) in QIIME (Caporaso et al., 2011).

Metagenomic data was processed through the MGRAST server (Meyer, Paarmann, D'Souza, & Etal., 2008) using the best hit method following minimum parameters; an e value of 5, sequence length of 15, percent identity of 60, and minimum threshold of 3 hits. Taxonomic diversity of the final time point samples was identified through the Greengenes (McDonald et al., 2012) and Refseq databases ((Pruitt, Tatusova, Brown, & Maglott, 2012). Community metabolism was also analyzed by identifying genes related to sulfur and iron cycling as well as cold stress responses using the NCBI Refseq (Pruitt et al., 2012) and KEGG databases (Kanehisa, Sato, Kawashima, Furumichi, & Tanabe, 2016) (Meyer et al., 2008).

Statistical analyses

All statistical analyses were conducted with various R packages in R 3.3.2 (R Core Team, 2016). The cumulative loadings, weekly release rates of geochemical elements over time, and depletion rates of elements were calculated in R and the former two were plotted in ggplot2 (Wickham, 2009). A maximum likelihood factor analysis was performed by factanal in the stats package (R Core Team, 2016) using three factors to determine the relationships between dominant geochemical factors. Variables that were significantly influenced by temperature were identified using a two way repeated measures analysis of variance ezANOVA script in the ez package (Lawrence, 2016) after grouping data into monthly temporal replicates and verifying that variables met the assumption of normality and equal variance. The ratio of specific conductivity between humidity cell temperature treatments was also calculated to provide an estimate of the effect of temperature of waste rock weathering (J. Langman et al., 2014). Fe: SO4 release rate ratios were also calculated to determine if leachate geochemistry was mediated

primarily by pyrite dissolution (Bowell, Sapsford, Dey, & Williams, 2006; Sapsford et al., 2009). Calculations predicting the time to acid generation were performed based on methods described by Morin and Hutt.

Manipulation of 16S rRNA gene microbial community data was facilitated by scripts included in the Phyloseq package (McMurdie & Holmes, 2013). Beta diversity of the community was interpreted by UPGMA clustering of a computed Bray- Curtis dissimilarity matrix (McMurdie & Holmes, 2013) of the raw OTU counts using the hclust and fviz_dendro scripts in the stats and Factoextra packages (McMurdie & Holmes, 2013, Kassambara & Mundt, 2017). Simpsons' and Shannon diversity were used to assess the alpha diversity of the community in the Phyloseq package (Appendix A.2) (McMurdie & Holmes, 2013). Barcharts of the relative abundance of taxa with greater than 1% or 2% abundance at the phylum and family levels, respectively were produced and visualized in ggplot2 (Wickham, 2009). As many taxa could not be identified beyond family, the top two hits of the five most abundant OTUs for each sample were identified using the NCBI BLASTn algorithm against the NCBI 16S rRNA sequences database (Altschul, Gish, Miller, Myers, & Lipman, 1990).

Absolute gene hits identified from metagenomic analysis was converted to relative abundance. Stacked bargraphs were generated of dominant taxa present at the phylum and family levels in ggplot2 (Wickham, 2009). Microorganisms identified by Refseq were grouped by their dominant form of metabolism into the following categories: sulfate and iron reducing bacteria (RB), phototrophic bacteria (Phot), neutrophilic sulfur oxidizing bacteria (nSox), neutrophilic iron oxidizing bacteria (nFeox), heterotrophic bacteria (HET), acidophilic sulfur oxidizing bacteria (aSox), and acidophilic heterotrophic bacteria (aHET). Relative abundance of COG groups, energy metabolism,

dominant organisms not involved in neutrophilic heterotrophic metabolism, and stress- related genes were also plotted as bargraphs using ggplot2 (Wickham, 2009).

2.4 Results

Geochemistry

The progression of waste rock weathering in the humidity cells followed an initial flushing of solids and all analytes through the first four weeks of the experiment. Between weeks eight and nine, a pH shift was observed from 8.16 to 7.48 in the warm treatment, and 7.90 to 7.41 in the cold treatment was observed. The pH of both temperature treatments then remained stable until ~25 weeks (Fig. 2.2A). Conductivity also decreased 40µS/cm in the warm treatment and 45µS/cm in the cold treatment at week nine (Fig. 2.2B). With this downwards shift in pH and conductivity, the rate of leaching for nearly all elements and TDS in both temperature treatments decreased(Fig 2.2, Fig 2.3, Fig 2.4). A second change in pH occurred at week 24 when pH dropped by nearly 0.5 units in both temperature treatments (Fig 2.2A). In the warm treatments, pH dropped from 7.34 to 6.88 between weeks 23 and 24, then steadily increased back to a pH of 7.38 within four weeks. In the cold treatments, pH dropped from 7.48 to 7.01 between weeks 23 and 24 but returned to pH 7.45 and 7.48 during the next 2 weeks before decreasing to pH 7.2 by the 28th week of the experiment. No large changes in mineral leaching or conductivity were noted to correlate with this inflection point in pH, with the exception of a decrease in TDS of only the warm treatment cells at weeks 23 and 24 (Fig. 2.2, Fig. 2.3, Fig. 2.4).

The analytes present in highest concentration in the leachate throughout the experiment were SO_4, alkalinity, K, Ca, Na, Si, Mg, and Al (Fig. 2.3). These analytes also contributed to the highest cumulative loads in the leachate (Fig 2.5). Factor analysis of all analytes identified SO_4, Na, K, Si, conductivity, TDS, Mg, Mo, Ca, alkalinity, and Sb significantly loading to dimension 1 (Fig. 2.7A), while those significantly loading onto dimension 2 included Al, V, As, alkalinity, and Ca (Fig. 2.7B). N species were all at or beneath the detection limit for ICP-MS; NH_3/NH_4^+ was present at <0.1mg/L, NO_2^- was present at <0.03mg/L, and NO_3^- was present at < 0.06mg/L concentrations. Dissolved organic carbon (DOC) was the only form of carbon analyzed, which was measured on the final 28th week of the experiment. DOC was measured to be 2.41mg/L in the warm humidity cell leachate and 1.104mg/L in the cold humidity cell leachate, which is high for a lithotrophic system such as the humidity cells.

A two way repeated measures analysis was used to determine which analytes were significantly associated with temperature treatments (Fig. 2.8.). SO_4, Al, Ca, Mo, V, and As were significantly associated with the warm temperature treatments while alkalinity and Ni were significantly associated with the cold temperature treatments. That the conductivity ratio of the warm: cold treatments was greater than 1.0 in all but 5 weeks of the 28 week experiment demonstrates that weathering occurred faster in the warm humidity cells, particularly after the eighth week of the experiment (Fig 2.9).

Nearly all analytes fell within acceptable PWQO weekly concentration limits (Fig. 2.11). In the cold humidity cell treatment, Cd concentrations treatment exceeded the PWQO limit of $2x10^{-4}$mg/L in only the fourth week of the experiment, by $1.4x10^{-4}$mg/L and then returned to

well below the PWQO limit for the remainder of the experiment but never exceeded the limits in the warm treatment. Aluminum exceeded the PWQO limit of $7.5x10^{-2}$ for nearly the entire experiment, especially the first 20 weeks of the experiment in both warm and cold treatments. The warm treatment humidity cells exceeded the PWQO limit for 22 weeks of the 28 week experiment by an average of 0.24mg/L while the cold treatment humidity cells exceeded the PWQO limit for 19 weeks by an average of 0.16mg/L.

Rietveld XRD analysis demonstrated that the mineralogy of the rock in both humidity cells remained similar to that of the unweathered rock, composed predominantly of silicate minerals. A small decrease in albite and chlorite and increases in muscovite, actinolite, biotite, and diopside were seen in both warm and cold humidity cells (Table 2.1). A minimal decrease in quartz was also seen only in the warm treatment. Rietveld analysis is limited in the detection of hydrated minerals, therefore undetected secondary precipitates may have formed over the 28 weeks of the experiment. Prior modal mineralogy of the waste rock identified that the rock contains < 1% S, the majority of which is contained is pyrite. As sulfide minerals were initially present in very low quantities (0.01- 0.83%) these minerals are unlikely to have been detected by the Rietveld XRD analysis. However, the Fe:SO_4 molar ratio of the humidity cells was well under 0.5 (Fig. 2.10), the stoichiometric ratio of Fe to S in pyritic minerals, in both warm and cold treatments suggesting that the presence of Fe and SO_4 in the leachate was a result of either Fe- mineral precipitation, non- ferrous sulfide dissolution, or sulfate mineral dissolution (Sapsford et al., 2009).

Over the course of the 28 week experiment, approximately 4% and 2% of the sulfur content of the waste rock was depleted in warm and cold humidity cell treatments, respectively (Table 2.2). The depletion rate of sulfur was 2mg/kg/week faster for the warm treatment, thus creating the difference in the predicted time to acid generation to be 16 years in the warm treatment vs 70 years in the cold treatment. In contrast, the time to acid generation calculated after 20 weeks of the experiment was calculated to be a similar for the warm treatment at 17 years, yet a much shorter period of 52 years for the cold treatment. As the waste rock used in this experiment is known to contain a high concentration of various neutralizing minerals, the neutralizing potential remaining was calculated in three ways to reflect the range of possible neutralization potential available within the humidity cell system (Table 2.2). Neutralizing potential was displayed in three forms, however NP was chosen to best demonstrate the neutralizing potential available within this rock as it is a function of i) the total alkalinity production, ii) neutralizing potential consumption, and iii) acid production. The calculated NP suggests that approximately 92.5% of the neutralizing potential was depleted over the 28 weeks of the experiment in both warm and cold humidity cells. The depletion rate of neutralizing potential was 2.6 mg/kg/week faster in the warm treatment, resulting in the warm treatment cells having a predicted time to neutralizing potential depletion of 28 years, while the cold cells would deplete their neutralizing potential in 30 years time. The time to depletion of neutralization potential calculated after the standard 20 weeks of the experiment was similar to that calculated after 28 weeks: 31 years for the warm treatment and 28 years for the cold treatment.

16S rRNA gene amplicon sequencing

A total of four 16S rRNA amplicon datasets were generated from both warm and cold treatments. Total sequences were provided an indirect proxy of prokaryotic biomass, indicating that in both temperature treatments the microbial biomass increased from the mid-point to end-point of the experiment, with approximately 25% increase in sequences in the warm treatment sample, and a 60% increase in sequences in the cold treatment sample. However, the number of OTUs present in > 3% abundance remained relatively constant across time points and treatments. An average of 175 OTUs were present in each sample, suggesting that the community is simple in its composition. Cluster analysis using the Bray Curtis distance metric demonstrated that the humidity cell treatment bacterial communities differed from those of the unweathered rock samples. The temperature treatments also clustered independently, regardless of their time point (Fig. 2.12).

Seven phyla were present in greater than 1% relative abundance, of which the Proteobacteria, Actinobacteria, Bacteroidetes, and Firmicutes were present in all samples. Cold temperature treatments favoured Cyanobacteria, whereas warm temperature treatments exclusively supported bacteria belonging to the Acidobacteria and Gemmatimonadetes (Fig. 2.13).

Regardless of temperature treatment, a general shift in the microbial community was seen when observing the dominant families present in the mid-point and end-point samples. The mid-point samples from both treatments had higher abundances of Oxalobacteraceae,

Micrococcaceae, Rhodobacteraceae, Hyphomicrobiaceae, Chitinophagaceae, Sphingomonadaceae, Bradyrhizobiaceae, and Comamonadaceae, while the end-point samples had higher abundances of Microbacteriaceae, Pseudomonadaceae, and Hydrogenophilaceae (Fig. 2.14).

The bacterial community present in the humidity cell system was predominantly heterotrophic, based on the taxa identified by family which were present in greater than 2% abundance (Fig. 2.14). Many heterotrophs, such as members of the Pseudomonadaceae, Caulobacteraceae, and Sphingomonadaceae, are commonly found in soil, aquatic, or rock surfaces. The non- photosynthetic unknown Cyanobacteria ML635J-21 was found in the cold humidity cell samples. Known denitrifiers among the Comamonadaceae, and nitrogen fixing bacteria within the Oxalobacteraceae, were also present. The Comamonadaceae were present in all samples, the majority of which were heterotrophic genera such as *Variovorax*, and *Hylemonella*, as well as the unique dessication tolerant *Ramlibacter* and psychrophilic *Polaromonas*. The sulfur oxidizers present in high abundance were the Hydrogenophilaceae, which were composed of *Thiobacillus spp*. *Thiobacillus spp.* were present in all samples but increased in abundance in the final time point samples. *Thiobacillus spp.* were not identified to the species level, however the closest BLAST hits suggest that the four OTUs are strains of *Thiobacillus thioparus*, *Thiobacillus starkey*, and *Thiobacillus denitrificans*. Other sulfur oxidizers which were present in ≤1% abundance in the samples included OTUs within the Comamonadaceae and Rhodobacteraceae. Extremophilic organisms capable of surviving stressors involved in cold, saline, or oligotrophic environments were also found in low abundance, including OTUs among the Haliangiaceae, and genera *Modestobacter*, and

Polaromonas. Some heterotrophic organisms present in the samples in higher abundance were human commensal organisms, such as the present as humidity cell experiments are not run as sterile microcosms, allowing the bacteria to enter the system while through human contact while handling water or equipment.

Metagenomic sequencing

Metagenome sequencing generated 823,472,738 bp (3,635,550 sequences) from the final time point of the warm humidity cell treatment (HW3) and 625,196,786 bp (4,520,594 sequences) from the final time point of the cold humidity cell treatment (HC3). Mean GC content was higher in the warm humidity cells at 64 ± 9% vs 58 ± 9% in the cold humidity cells. 79.12% of sequences from the warm humidity cells and 69.48% of sequences from the cold humidity cells contained predicted features, of which 0.31% and 0.60% were rRNA sequences in the warm and cold humidity cells, respectively (Table 2.3).

Taxonomy was assessed using 16S rRNA gene sequences identified by Greengenes and genes inferred from Refseq annotation (Fig. 2.15). Archaea represented under 0.3% of the taxa present, while Viruses were present in under 0.05% relative abundance in both warm and cold humidity cell treatments. Eukaryotes were present in 1.3% relative abundance in the warm humidity cells and 0.8 % in the cold humidity cells. In both temperature treatments, Bacteria represented 98- 99 % of taxa present. Refseq identified more taxa than Greengenes, however the following dominant phyla were constant across the two reference databases; the Actinobacteria, Bacteroidetes, Cyanobacteria, Deinococcus- Thermus, Planctomycetes, Proteobacteria, and

Spirochaetes. Taxa identified to be present in at a relative abundance of greater than 0.5 % at the family level suggests that the bacterial taxa are largely heterotrophic in nature, including dominant families such as Bradyrhizobiaceae, Microbacteriaceae, Nocardioidaceae, and Pseudomonadaceae (Fig. 2.16).

SEED subsystems (analogous to clusters of orthologous groups COGs) analyses indicated similar distribution of coding sequences in different gene categories between temperature treatments (Fig 2.17). Overall, the warm treatment had higher relative abundance of genes within protein, RNA, and DNA metabolism while the cold treatment had higher relative abundance of genes related to carbohydrate metabolism, iron acquisition, motility, membrane transport and stress response. By clustering the microbial community within general metabolic categories (sulfate reducing bacteria, phototrophic bacteria, neutrophilic sulfur oxidizing bacteria, neutrophilic iron oxidizing bacteria, heterotrophic bacteria, iron reducing bacteria, acidophilic sulfur oxidizing bacteria, and acidophilic heterotrophic bacteria) 85.29% of energy metabolism-related genes in the warm humidity cell treatment and 96.09% in the cold humidity cell treatment were found within OTUs capable of neutrophilic heterotrophic metabolism. As the metabolic pathways driving acid generation in waste rock systems are not heterotrophic in nature, we focussed on the remaining acidophilic heterotrophic, autotrophic, and chemolithotrophic forms of metabolism present within the system (Fig 2.18).

A diverse group of sulfur reducing bacteria were present in 2.6% relative abundance in the warm humidity cell treatments and 1.2% relative abundance in the cold humidity cell treatments (Fig. 2.19). Genes for dissimilatory sulfate reduction were present, however the

DsrAB gene was absent. The DsrAB subunits may be present in these organisms but likely wasn't captured in the metagenome due to the low abundance of many sulfate reducing taxa. Assimilatory sulfur reduction pathways were complete and dominated by the Hydrogenophilales and Chromatiales in the warm humidity cell treatment and by the Pseudomonadales, Sphingomonadales, and Burkholderiales in the cold humidity cell treatment. Acidophilic and neutrophilic sulfur oxidizers were present in 7.00% and 0.49% relative abundance in the warm humidity cell treatment, and 0.49% and 1.55% relative abundance in the cold humidity cell treatment, respectively. The Hydrogenophilales, composed of *Thiobacillus denitrificans,* was the dominant acidophile while members of the Chromatiales were the dominant neutrophilic organisms. The Hydrogenophilales and Chromatiales both contained genes for reduced inorganic sulfur compound (RISC) oxidation. *Thiobacillus spp.* provided the best insights into RISC oxidation, showing the ability to oxidize HS^- for entry to the cell through *Fccab,* oxidize HS to thiosulfate by the SOX genes, as well as the ability to oxidize thiosulfate to sulfate through APS reductase and ATP sulfurylase or sulfite dehydrogenase. Considerable hits to cytochrome c genes for flavocytochrome c sulfide dyhydrogenase, which is used for photochemolithotrophic sulfur oxidation were also present. Additionally, the SUOX gene for sulfite oxidation was found belonging to the Rhizobiales whom are predominantly heterotrophic organisms (Fig. 2.20).

Few iron oxidizers were present in the metagenome, representing 0.13% and 0.54% of the energy metabolism in warm and cold humidity cell treatments, respectively (Fig. 2.19). The majority of taxa were neutrophilic organisms, including *Gallionella spp*., with the exception of the Acidithiobacilli, which are acidophilic Fe-S oxidizers. Siderophores and iron transport proteins were present but rusticyanin was not identified in the metagenome. Iron reducers were

found in low abundance, such as the Deferribacterales and *Albidiferax spp*., as were genes for their metabolism within the dataset (Fig. 2.19).

All the genes needed for the complete nitrogen cycle were identified (Fig. 2.20), suggesting that N plays a large role in the microbial communities' metabolisms despite little to no N detected by ICP. Many soil and aquatic taxa that are active participants in N cycling were present. In both warm and cold treatments, the Rhizobiales and Burkholderiales were prominent contributors to nitrogen cycling, as well as the Pseudomonadales in the cold humidity cell treatment and the Hydrogenophilales in the warm humidity cell treatment (Fig 2.19).

Carbon metabolism was well represented in the humidity cell bacterial communities consistent with the predominance of heterotrophic organisms (Fig. 2.18). Autotrophic bacteria were supported by the presence of the full complement of genes to complete the reverse TCA cycle and Calvin Benson- Bassham cycle (Fig. 2.20). In the cold humidity cell treatment, dominant autotrophic taxa included the Actinomycetales, the Burkholderiales, and the Rhizobiales. In the warm humidity cell treatment, dominant autotrophs included the Hydrogenophilales, the Solibacterales, and the Burkholderiales (Fig. 2.19).

The presence of genes involved in stress responses of the microbial community reflected the challenges of their environment enriched in inorganics and varying moisture content. Genes encoding for proteins and metabolic pathways to assist with salts, metals, and acidic conditions were present, namely those categorized as under acid stress (0.02% HW3, 0.02% HC3), oxidative stress (0.46% HW3, 0.49% HC3), periplasmic stress (0.04% HW3, 0.04% HC3), and

detoxification (0.04% HW3, 0.08% HC3) (Fig. 21). Genes for oxidative stress, periplasmic stress, as well as those for osmotic stress (0.15% HW3, 0.22% HC3) and minimal genes for desiccation stress (0.00% HW3, $1.84e^{-4}$ % HC3) were also present, which may also assist organisms in surviving the fluctuating moisture content present within the humidity cells. Cold stress responses were of particular interest, as the material originated from a boreal climate and the cold temperature treatment was incubated at suboptimal temperature for most bacteria. In response, cold stress response genes were found to be in higher relative abundance in the cold treatment (0.03% HW3, 0.05% HC3) (Fig. 21). DNA/ RNA binding cold shock proteins A, B, C, D, E, F, and G were present in the metagenome, as well as pathways for trehalose uptake and utilization, a cytoplasmic solute. Genes for quorum sensing and biofilm formation were also present in warm and cold temperature treatments, with those involved in methionine biosysthesis and degradation, autoinducer-2 synthesis, and adhesion biosynthesis being the most prevalent.

2.5 Discussion

Humidity cell leaching kinetics

This study investigated the weathering of low sulfur waste rock through humidity cell experiments run under temperature conditions similar to those found in the field in a boreal climate. Leachate analyses demonstrated that the majority of analytes exhibited faster leaching rates in the first 9 weeks of the experiment, followed by a decrease in pH, ORP, and TDS. The majority of analytes were found in trace quantities in the leachate throughout the experiment with the exception of SO_4, alkalinity, Ca, K, Si, Na, and Mg, many of these can be associated

with the dissolution of fast reactive carbonate and some aluminosilicate minerals which were abundant in the waste rock. Of these elements, all fell within PWQO limits. Al concentrations were, however, above PWQO limits for 22 of the 28 weeks of the experiment and this metal has also been leached in high quantities in other leach tests of the same PAG rock (Hamilton, personal communication, 2016). Al levels may have been elevated because small aluminosilicate colloids can often pass through 0.45μm filters that are employed to prepare dissolved metal samples for ICP analysis (Kennedy & Zellweger, 1974). Interestingly, the waste rock leachate was found to be high in DOC, which is unusual for lithotrophic samples. Despite the humidity cells being a closed system with little carbon input, waste rock will be mixed with some soil as it is transported within the mine site, likely explaining the source of the vast majority of the dissolved organic carbon in the leachate.

Humidity cells incubated at 4 and 20°C exhibited different leaching characteristics throughout the experiment. pH remained higher in warm humidity cells for the first eight weeks of the experiment, from which point the pH was higher in cold humidity cells. Conductivity was also similar between humidity cells until the eighth week of operation then became higher in the warm humidity cell treatments. SO_4, Al, As, Mo, V, and Cu preferentially leached from the warm humidity cells. The higher leaching rates of these elements under warm conditions can likely be explained by simple reaction kinetics generally being higher at warmer temperatures. In the cold humidity cells alkalinity was significantly higher, likely due to the higher solubility of calcium carbonate at lower temperatures. Ni also preferentially leached from cold humidity cells which may have been driven by the increase in solubility of carbonate metals with cooler temperatures (SRK Consulting, 2006).

The large neutralizing potential of the waste rock played a dominant role in the leachate geochemistry seen over the 28 week experiment. All of the minerals identified through Rietveld XRD analysis are predominantly aluminosilicates and a small number of carbonates, as the sum of sulfide minerals were present in ~1% abundance prior to rock weathering. Of those categorized as dissolving minerals (Price, 2009), calcite and ankerite were depleted by the 28th week of the experiment, as was the intermediate weathering chlorite. The pH of both temperature treatments remained relatively stable at a circumneutral pH that only exceeded pH in in the first eight weeks of the experiment. The high proportion of neutralizing minerals likely contributed to the circumneutral pH of the system (Price, 2009), yet the high sulfate loads in the leachate suggest that sulfide oxidation was occurring. Sulfide mineral dissolution likely prevented the pH from rising above circumneutral levels but may have been present primarily as hydrated secondary minerals. Rietveld XRD analysis did not identify sulfide minerals in the waste rock, although this was likely due to the fact that Rietveld XRD cannot reliably detect the presence of hydrated minerals. To gain an understanding of the weathering of the waste rock through the detection of secondary minerals, future research perform sequential leach extractions of the waste rock or employ scanning electron microscopy- energy dispersive X-ray spectrophotometry (SEM-EDS) at the end of the experiment (ASTM, 2013).

Humidity cell experiments are designed to predict the timing and extent of acid generation in mine wastes. The experiment reported here is unique in that humidity cells were extended beyond the standard 20 week experimental time period, as previous studies have suggested that 20 weeks may not provide sufficient time to see the full extent of mineral weathering. Our experiment lasted 28 weeks in duration, in which sulfate, alkalinity, and

elemental release rates did not reach steady states. The waste rock is indicated to be potentially acid generating but did not reach acidic conditions over the 28 week period, which is consistent with previous humidity cell testing results on the same waste rock over a 68 week period (Hamilton, personal communication, 2016). The conductivity ratio of the warm to cold cells indicated that the warm humidity cells weathered faster than the cold humidity cells, if not at similar rates, for the majority of the experiment. This is reflected in the difference in both S and NP depletion rates as they were 4.3X and 1.08X faster in the warm treatment than the cold treatment, respectively. In the warm cells, NP is predicted to be depleted 12 years after the depletion of S, thus acid generation is not predicted to occur. However, the lower temperatures in the cold humidity cell treatment decreased the S depletion rate more than that of NP. The reduction in S depletion rate is predicted to allows S minerals to remain for approximately 70 years, while NP may be exhausted in 30 years time. Given these results, we predict that S leaching may cause the onset of acidic conditions to begin after 26 years and continue to do so for 40 years after NP has been depleted in the cold humidity cell treatments. Humidity cell data does not accurately model some site specific factors such as precipitation, however the temperature of our cold humidity cell treatment is highly reflective of the average annual high and low site temperature of 8.8 and -1.9°C. This suggests that the weathering data from the cold humidity cells may be more relevant to the conditions occurring in the field than the industry standard experiment ran at 20°C.

Microbial community composition

Humidity cell experiments are rarely performed in conjunction with native microbial community analysis. To our knowledge, this is the second experiment in which microbial taxonomy has been collected from a humidity cell experiment (Jones et al., 2017). Despite the varied sample extraction methods, the microbial communities identified at the phylum and family levels showed good consistency for the final time point samples of 16S rRNA gene amplicon sequencing and shotgun metagenomic sequencing. Bacteria are the dominant organisms present within the humidity cell system, although all domains of life were present in the final time point samples. The majority of taxa are neutrophilic heterotrophic organisms, supported by the high DOC levels and circumneutral pH of the leachate. Similarly, Jones *et al.* (2017) and Blackmore *et al.* (2018) also found that the microbial communities found within their kinetic test experiments were also largely composed of neutrophilic heterotrophs despite having waste rock of similar but more reactive sulfur content (0.63- 1.4% S contained largely as pyrrhotite) and considerably more weathered minerals.

The microbial communities in warm and cold humidity cell treatments were found to be significantly different from each other, as well as from the unweathered waste rock. These differences are particularly evident when observing the distribution of dominant phyla, where the Cyanobacteria are present only in cold humidity cells, while the Firmicutes, Acidobacteria, and Gemmatiomonadetes are present only in warm humidity cells. Additionally, the warm humidity cells contained a larger proportion of taxa, many of which were not found in low abundance. Of interest are *Thiobacillus spp*., which were the dominant OTUs identified in the initial 16S rRNA

gene screening of unweathered rock, were more abundant in the warm humidity cells throughout the course of the experiment.

Bacterial communities differed between time points with a shift in the microbial community seen in the abundant families of microorganisms present. Both time points supported predominantly chemoorganotrophic organisms. However, bacteria found in the end-point sample were more commonly found in oligotrophic marine environments or soil while those in the mid-point sample were more commonly found primarily in soil environments.

Microbial drivers in the weathering of waste rock

Metagenomic sequencing has been extensively used in mine waste environments (Dick et al., 2009; Kantor et al., 2015; Liljeqvist et al., 2015; Zhang et al., 2016), however this is the first glimpse of the microbial communities present in humidity cell and PAG rock systems. The intent of assessing the genetic composition of the waste rock microbial community was to determine the potential metabolic contribution of microorganisms to the weathering of waste rock, as well as to determine their capacity to function within cold and potentially harsh conditions.

Given the number of neutrophilic and acidophilic heterotrophs present, the majority of energy metabolism genes were dedicated to carbon metabolism. Chemolithotrophic and phototrophic bacteria were also present in considerably lower abundance, however many of which provided genes to complete the reverse TCA cycle and Calvin Benson Bassham cycles to support carbon fixation.

Nitrogen cycling pathways for nitrogen fixation, nitrification, denitrification, ammonification, and dissimilatory nitrate fixation were present within the genome, completing the nitrogen cycle. Although nitrogen is a key energy source for many metabolic groups, we found that nitrogenous sources were present in nearly undetectable levels in the leachate using ICP. Some forms of nitrogen, such as N_2, may not be captured in the leachate, however given the extent of nitrogen cycling capacity within the bacterial community it is surprising the nitrogen concentration in the leachate was as low as it was recorded to be over the course of the experiment.

Biotic iron oxidation is an important component of sulfide mineral leaching, as iron oxidizing microorganisms can oxidize ferrous iron faster than abiotic mechanisms at low pH, leading to acid generation (Bacelar-Nicolau & Johnson, 1999). The humidity cell system has yet to become acidic due to the high neutralizing potential of the waste rock. In effect, few acidophilic iron oxidizers are present and those that are exist in low abundance. Neutrophilic iron oxidizers are also present in low abundance and while genes for iron acquisition and transport are present, those for rusticyanin are not present. At this stage in waste rock weathering, the lack of iron oxidizing bacteria suggests that abiotic oxidation is the primary source of iron oxidation occurring in the humidity cell systems.

Sulfur oxidation is the major concern of sulfidic waste rock weathering which sulfur and iron oxidizing microorganisms play a role in. Acidophilic and neutrophilic sulfur oxidizers were present, however only the Hydrogenophiliales and Chromatiales were found in no greater than

4% abundance in samples. This suggests a shift in the community to a more heterotrophic, neutrophilic community once subjected to weathering cycles, as the Hydrogenophiliales alone represented greater than 30% of the community of the unweathered rock sample. RISC oxidization pathways were fully present and best characterized in *Thiobacillus spp*., suggesting that some sulfur oxidation within the system is attributable to bacterial metabolism. The *Thiobacillus spp.* present in the metagenome was identified as *Thiobacillus denitrificans* whereas the OTUs in the 16S rRNA gene sequencing approach were also identified as *Thiobacillus thioparus*. *Thiobacillus thioparus* is strictly a sulfur oxidizer despite it carrying *nar*, *nir*, *nor* and *nos* genes for anaerobic denitrification it is not capable of doing so as an alternative form of respiration like *Thiobacillus denitrificans* (Hutt et al., 2017). Sulfur reducing bacteria were also present in low abundance along with genes for the dissimilatory sulfate reductase complex. The presence of sulfur reducers suggests that sulfate reduction was occurring in small anaerobic pockets within the waste rock profile with accessible sulfate (Muyzer, 2014).

The sum of the metabolic and taxonomic data regarding sulfur cycling is in agreement with the geochemical data, presenting a neutrophilic community of sulfur oxidizers with some sulfate reduction occurring in anaerobic pockets supported by available organic carbon. The low abundance of iron oxidizing bacteria suggests that sulfur oxidizing bacteria are the main driver of sulfidic rock weathering in the humidity cells. Should the geochemistry of the system remain at relatively stable rates, acidification is not predicted to occur. However, in the case of the cold humidity cells, neutralizing potential may decrease to a point where abiotic sulfur oxidation will increase, likely in conjunction with biological sulfur oxidation. The acid generated from the accelerated sulfide oxidation reactions should provide a more amenable environment to the

limited iron oxidizers present in the sample, such as *Acidithiobacillus* and potentially some of the unknown Betaproteobacteria (Bellenberg et al., 2015; Méndez-García et al., 2015). These iron oxidizing organisms will not only compliment the metabolism of acidophilic sulfur oxidizing bacteria, but they will also continue to maintain the rate limiting step of sulfide mineral oxidation. In effect, although there may not currently be the potential to generate substantial acid from the current microbiology and mineralogy of the system, the metabolic potential is present to potentially trigger acid generation.

Stress responses

Our humidity cell experiment presented some unique challenges to microorganisms regarding the environmental conditions they must be capable of surviving in, notably: cold and warm shock, oxidative stress, osmotic stress, and potentially acid and metal stress. Microorganisms living within mine wastes have been well documented to contain genes to guard themselves from toxic metal concentrations and acidic conditions that can cause oxidative and osmotic stress (Chen et al., 2014; Dopson, Ossandon, Lövgren, & Holmes, 2014), which were also found in our metagenomes. The dry- humid- leach cycle also likely generated drastic differences in moisture availability in the humidity cell environment, for which periplasmic stress, biofilm formation, and osmotic stress genes present within the community would provide some security against.

The ability of microorganisms to tolerate the humidity cell temperature treatments were of particular interest, as many sulfide oxidizing organisms are mesophilic or thermophilic in

nature (Ghosh & Dam, 2009). Within the metagenomics data, more cold shock responses were found in cold humidity cell treatments while a subsequently larger proportion of warm shock responses were found in warm humidity cell treatments. Within the cold shock responses, trehalose uptake and utilization, as well as cold shock proteins were highly abundant. Cold shock proteins are thought to be involved in DNA supercoiling under cold stress which are often accompanied by the intracellular solute trehalose (Horn, Hofweber, Kremer, & Kalbitzer, 2007). Different cold shock proteins can be expressed depending on the metabolic activity of the microorganism. For example, *CspA* can be expressed during cold acclimation in psychrophilic bacteria or cold shock in mesophilic bacteria (Berger et al., 1996; Inouye, 1997) while, *CspD* tends to be expressed during the stationary phase of growth (Inouye, 1997). It is difficult to confirm the specific roles the cold shock proteins are playing in different microorganisms metabolism, however their presence suggests that microorganisms within the microbial community have the ability to adapt to cold temperatures. Further metatranscriptomic or culture-based experiments could be used in the future to assess the potential psychrophilic behaviour of bacteria of interest present within the waste rock microbial community.

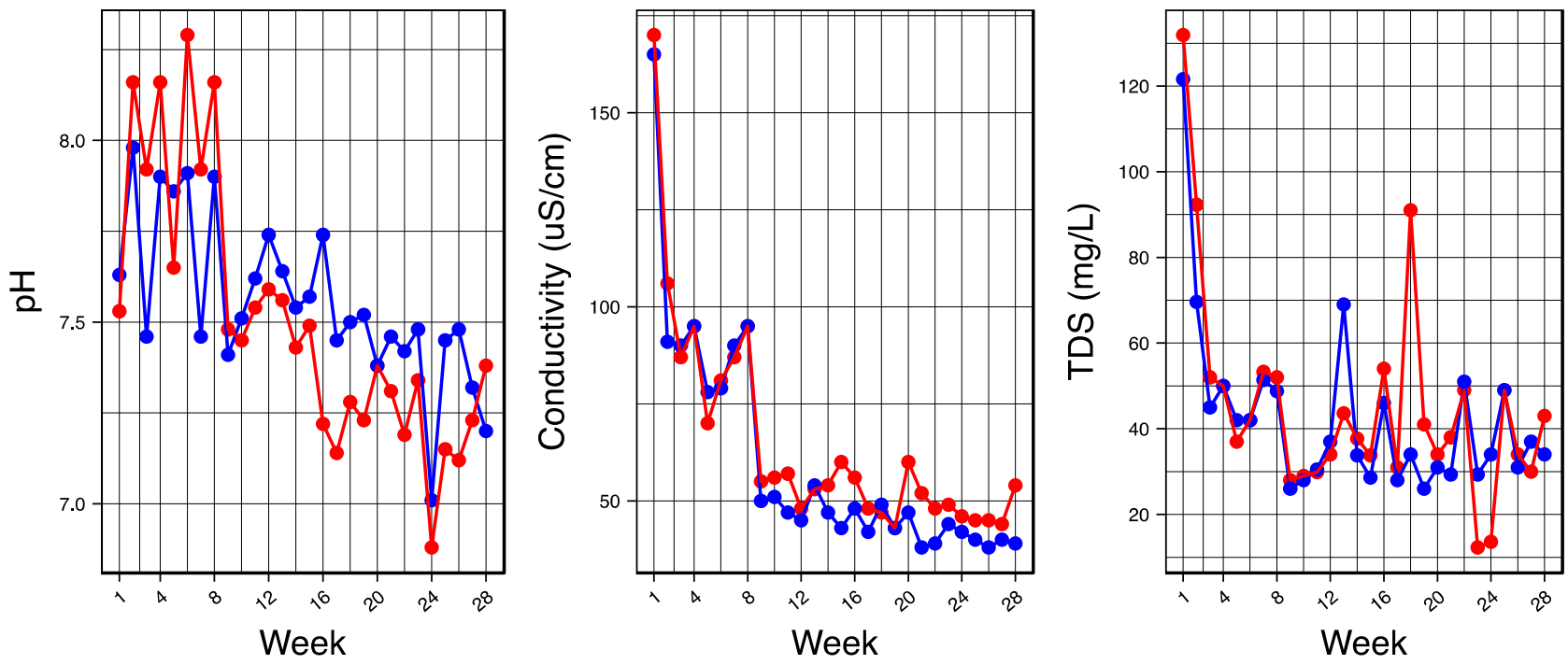
2.6. Conclusion

Humidity cell testing is designed to assess the sum of abiotic and biotic inputs on the weathering and leaching of waste rock or tailings. In this humidity cell experiment, we considered the variability in the average annual site temperature and how this might impact the weathering rate of low sulfur waste rock between 20°C and 5°C by analyzing how the geochemistry and microbiology of the humidity cell system responded. Leachate geochemistry

and the microbial community composition changed over the course of the 28 week experiment as the waste rock weathered, and also differed between warm and cold treatments. The low sulfur waste rock released circumneutral leachate with only Al being an element of concern according to PWQO guidelines. While the humidity cell system's pH remained circumneutral, the microbial community was primarily composed of heterotrophic and sulfur oxidizing bacteria. Notably, the *Thiobacillus denitrificans* that originated from the unweathered waste rock is the dominant sulfur oxidizing bacteria with some ability to resist the cold temperatures of the cold humidity cell treatment. The warm humidity cells are not predicted to become acid generating, while in the cold humidity cells neutralizing potential was expected to be exhausted 40 years prior to the depletion of sulfur due to the increase in solubility of carbonates with cooler temperatures. With the onset of abiotic acid generation, it is predicted that additional acid generated from the metabolism of sulfur oxidizing bacteria will further promote conditions to encourage the growth of acidophilic iron oxidizers found in low abundance. Therefore, although the dominant microbial community does not support immediate ARD generation, metabolic potential by a lower abundance S-cycling community could promote acid generation following neutralizing potential depletion, particularly at lower temperatures. Although humidity cells are an artificial system, they are a standard predictive tool in acid generating calculations important in the planning of mine waste management. The results of our humidity cell experiments indicate that these standard tests may need to consider relevant site temperatures (in colder climates) in addition to including microbial community analyses to provide more accurate predictions in the possible rates of ARD generation.

A **B**

<u>Figure 2.1:</u> Laboratory set up of A) warm treatment and B) cold treatment humidity cells attached to a central pump manifold during a dry cycle.



A **B** **C**

Figure 2.2: pH. conductivity, and total dissolved solids measured in leachate from warm and cold humidity cell treatments over a 28 week period.

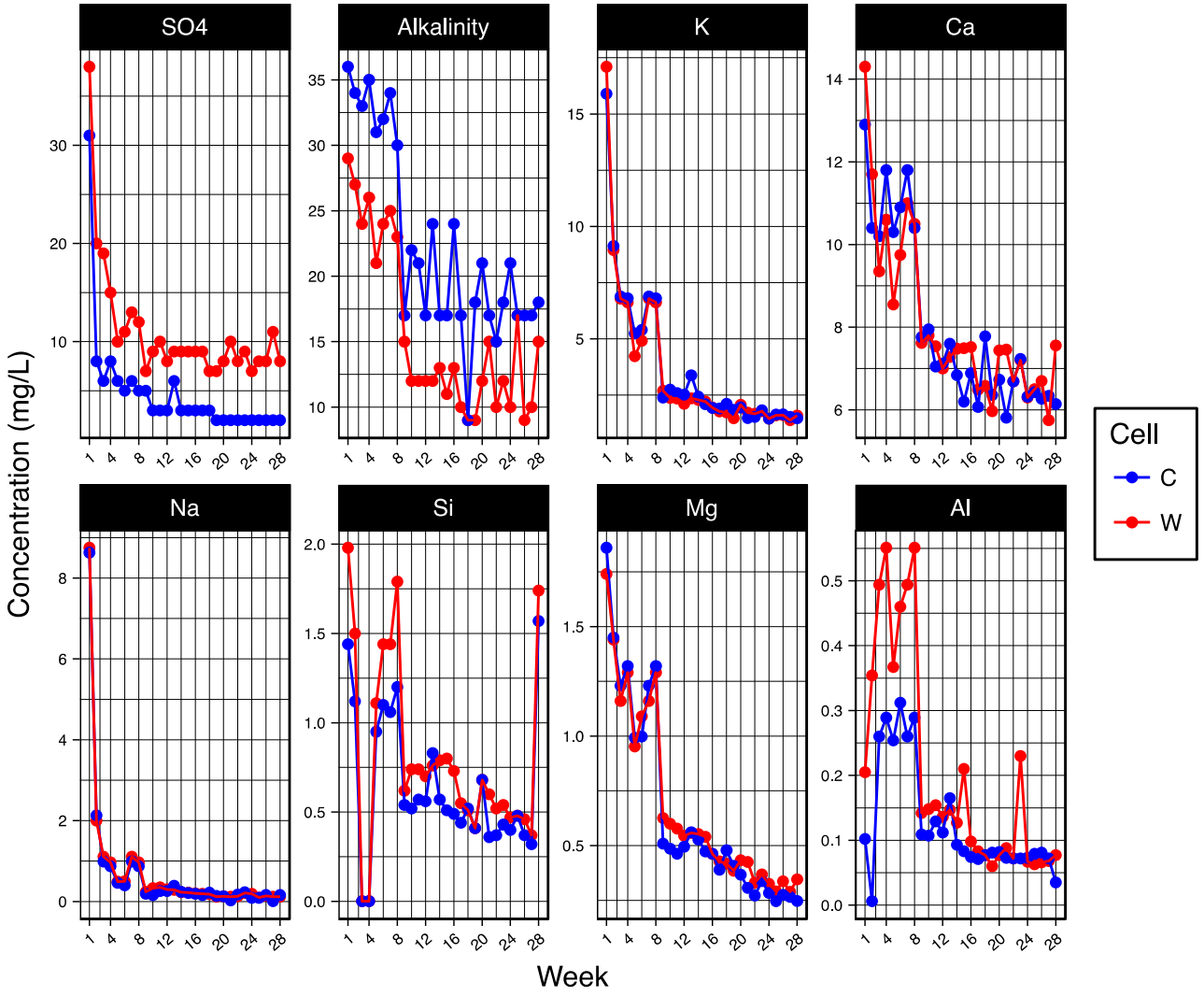


Figure 2.3: Weekly analyte concentrations of prominent analytes present in warm and cold humidity cell leachate over a 28 week period.

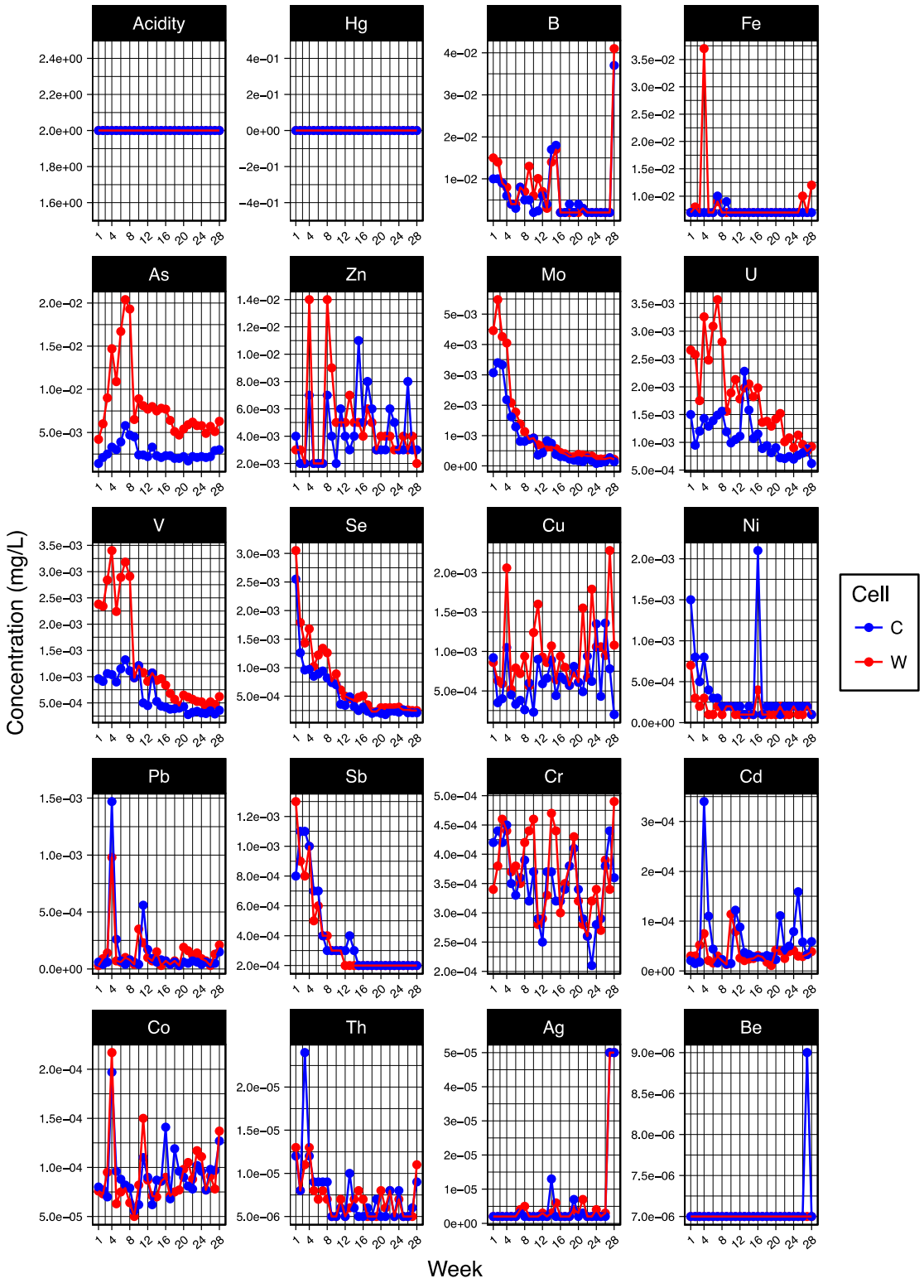
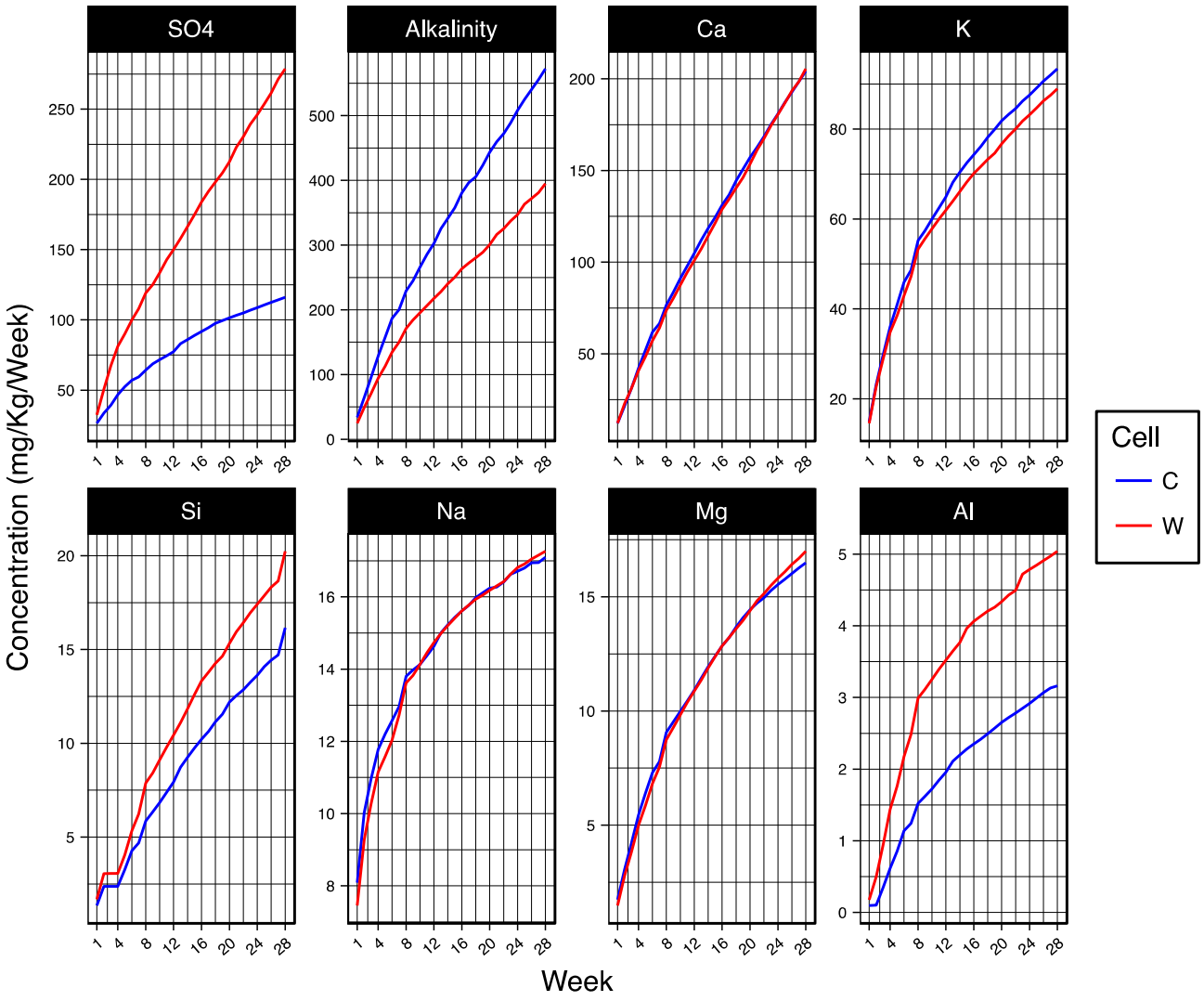


Figure 2.4: Weekly analyte concentrations of analytes present in warm and cold humidity cell leachate in low concentration over a 28 week period.



<u>Figure 2.5:</u> Cumulative analyte concentrations of prominent analytes present in warm and cold humidity cell leachate over a 28 week period.

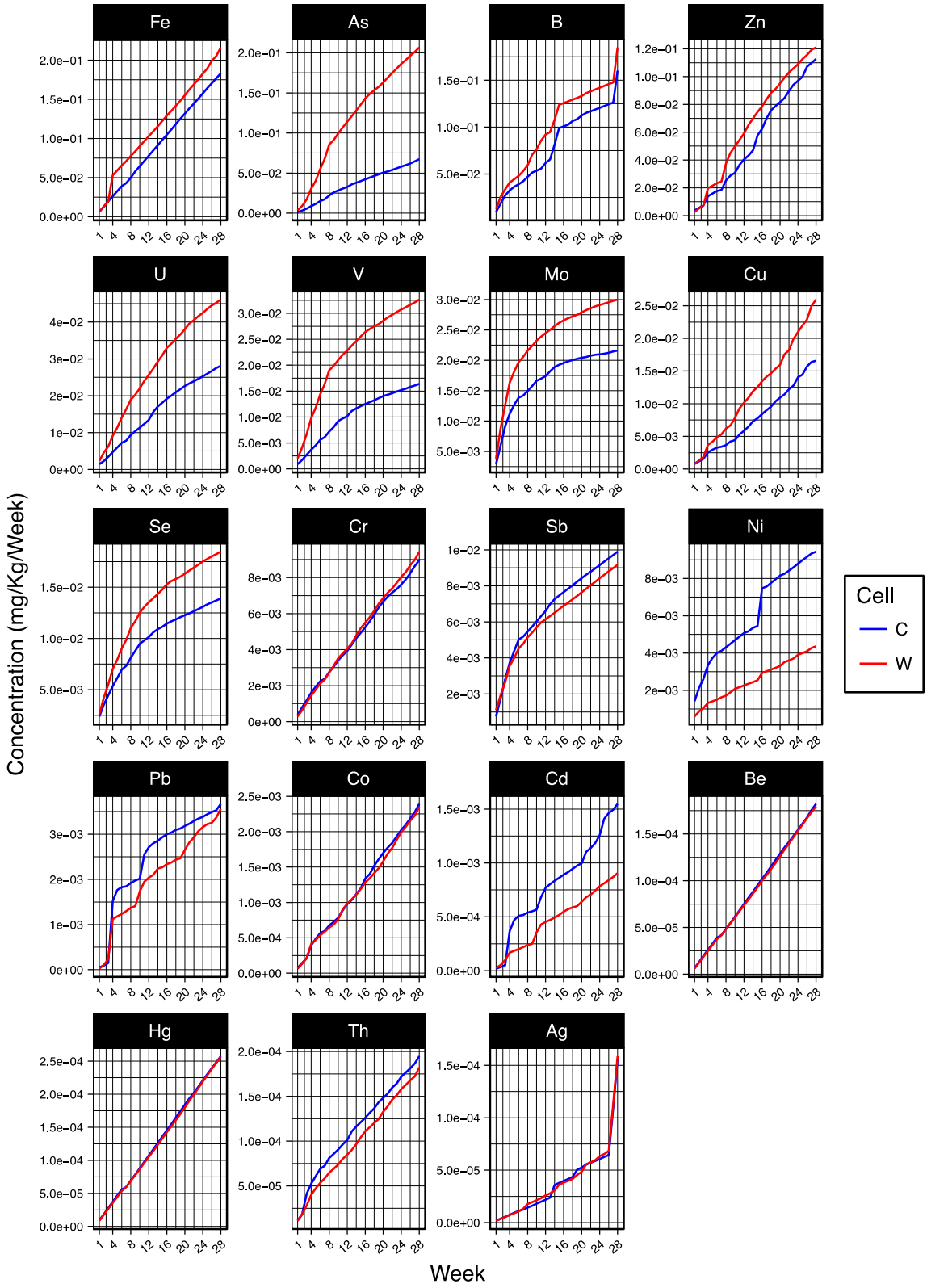
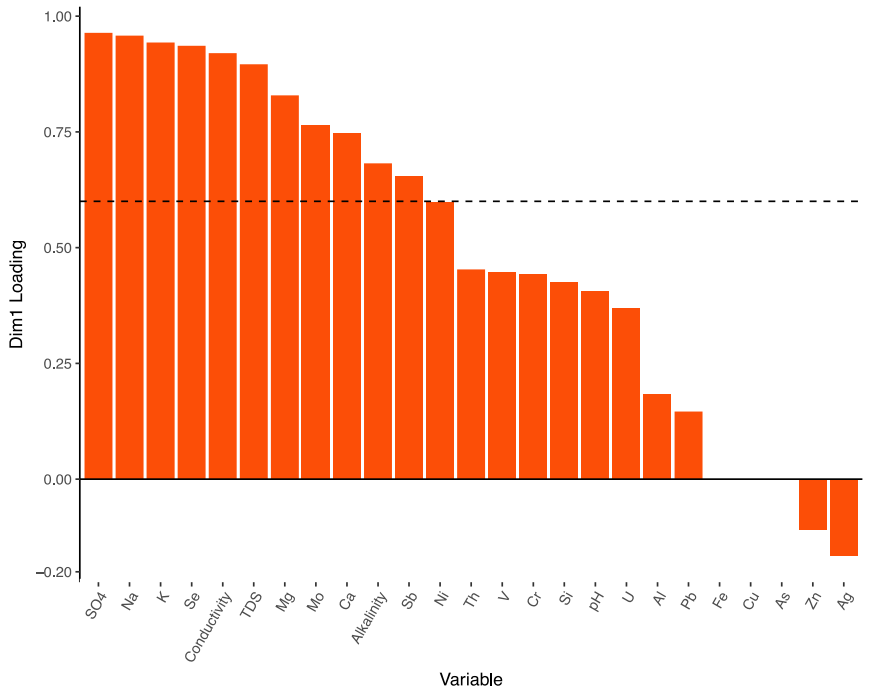
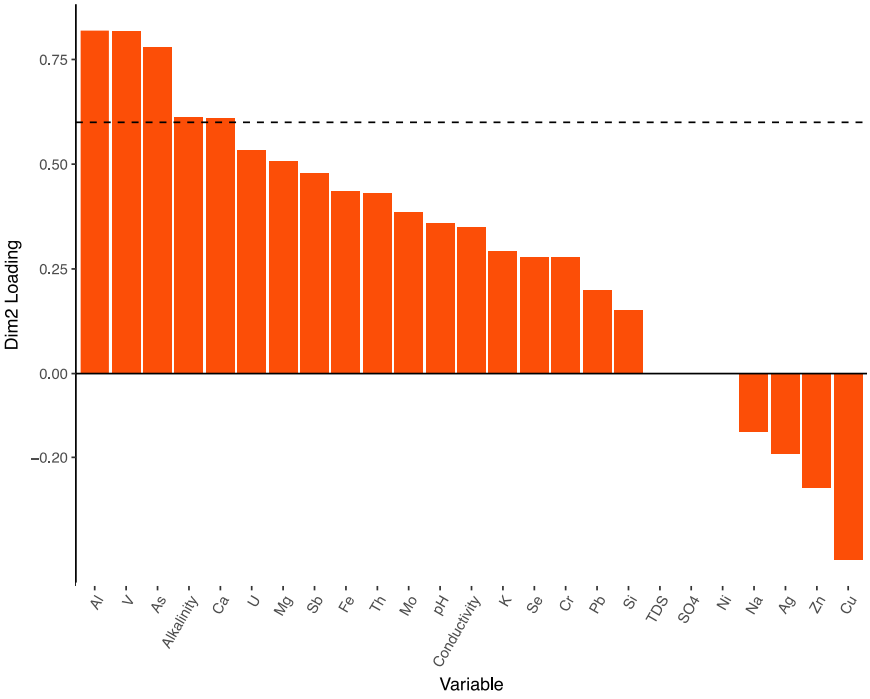


Figure 2.6: Cumulative analyte concentrations of analytes present in low concentrations in warm and cold humidity cell leachate over a 28 week period.

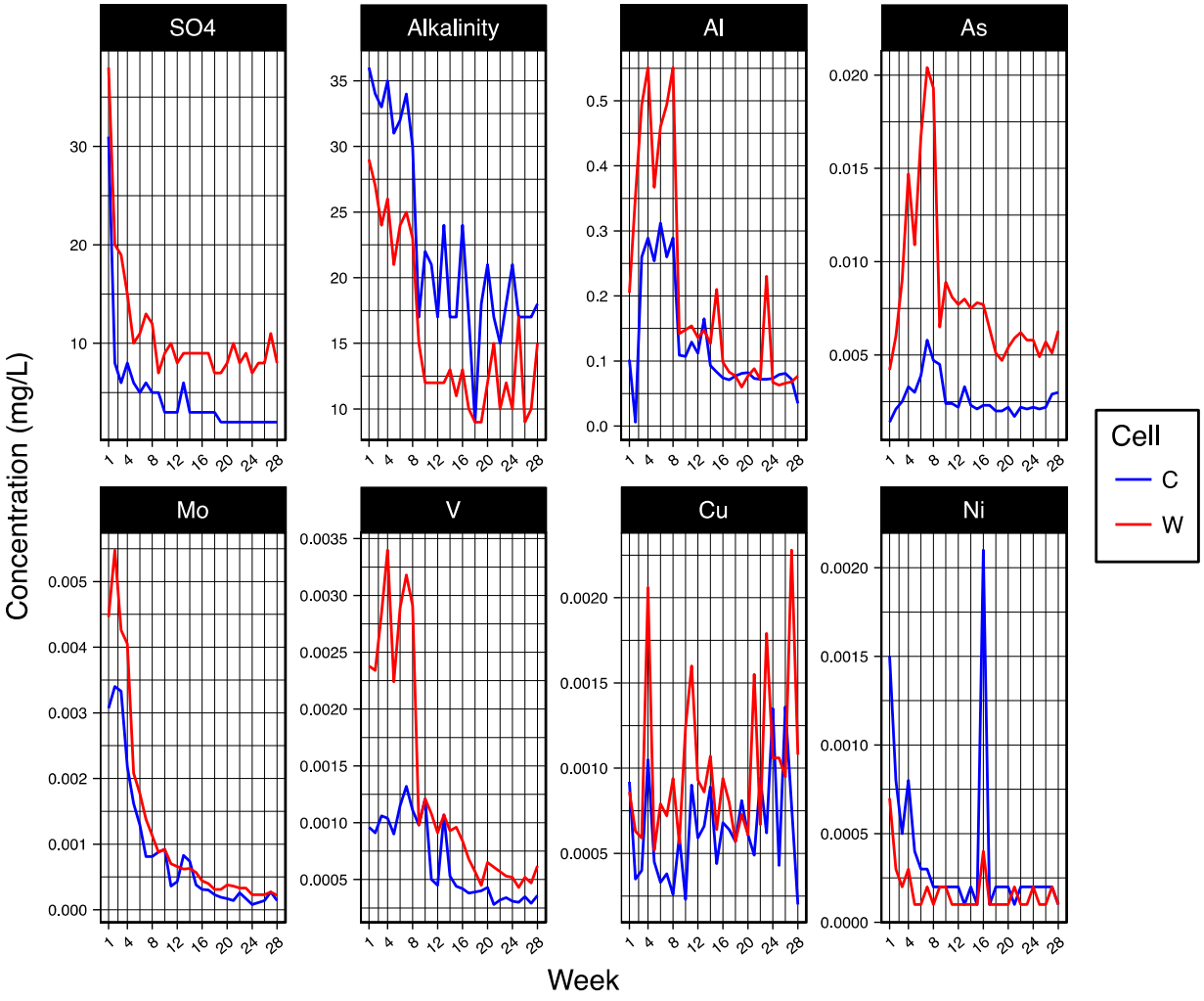


A



B

<u>Figure 2.7:</u> Maximum likelihood factor analysis of humidity cell leachate analytes using 3 factors. A) Analytes loading significantly onto Dimension 1. B) Analytes loading significantly onto Dimension 2.



<u>Figure 2.8</u>: Concentration profiles of analytes showing significantly different behaviors between temperature treatments as determined by 2 way repeated measures ANOVA<u>.</u>

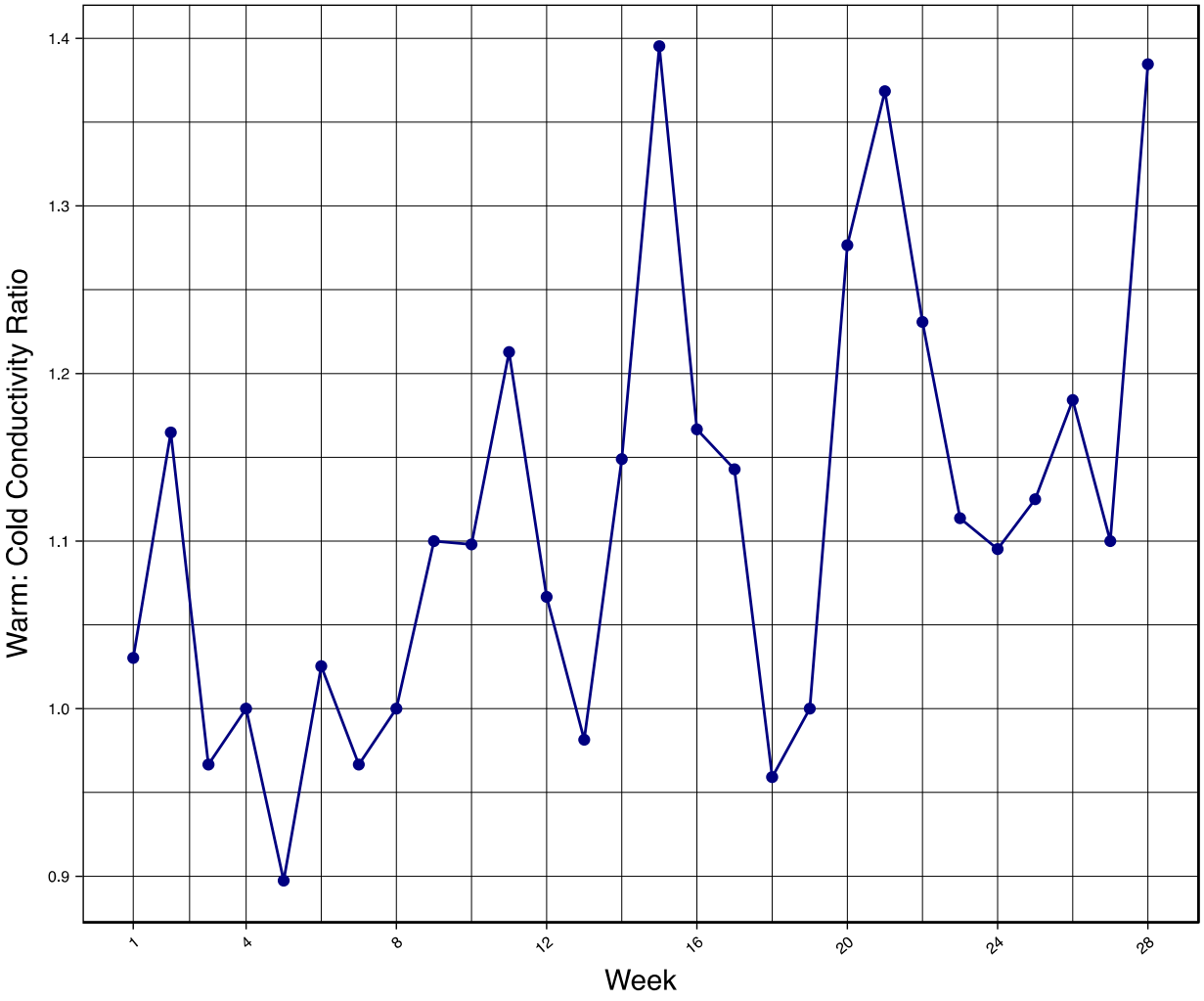


Figure 2.9: Warm: cold humidity cell treatment conductivity ratio over the 28 week experiment.

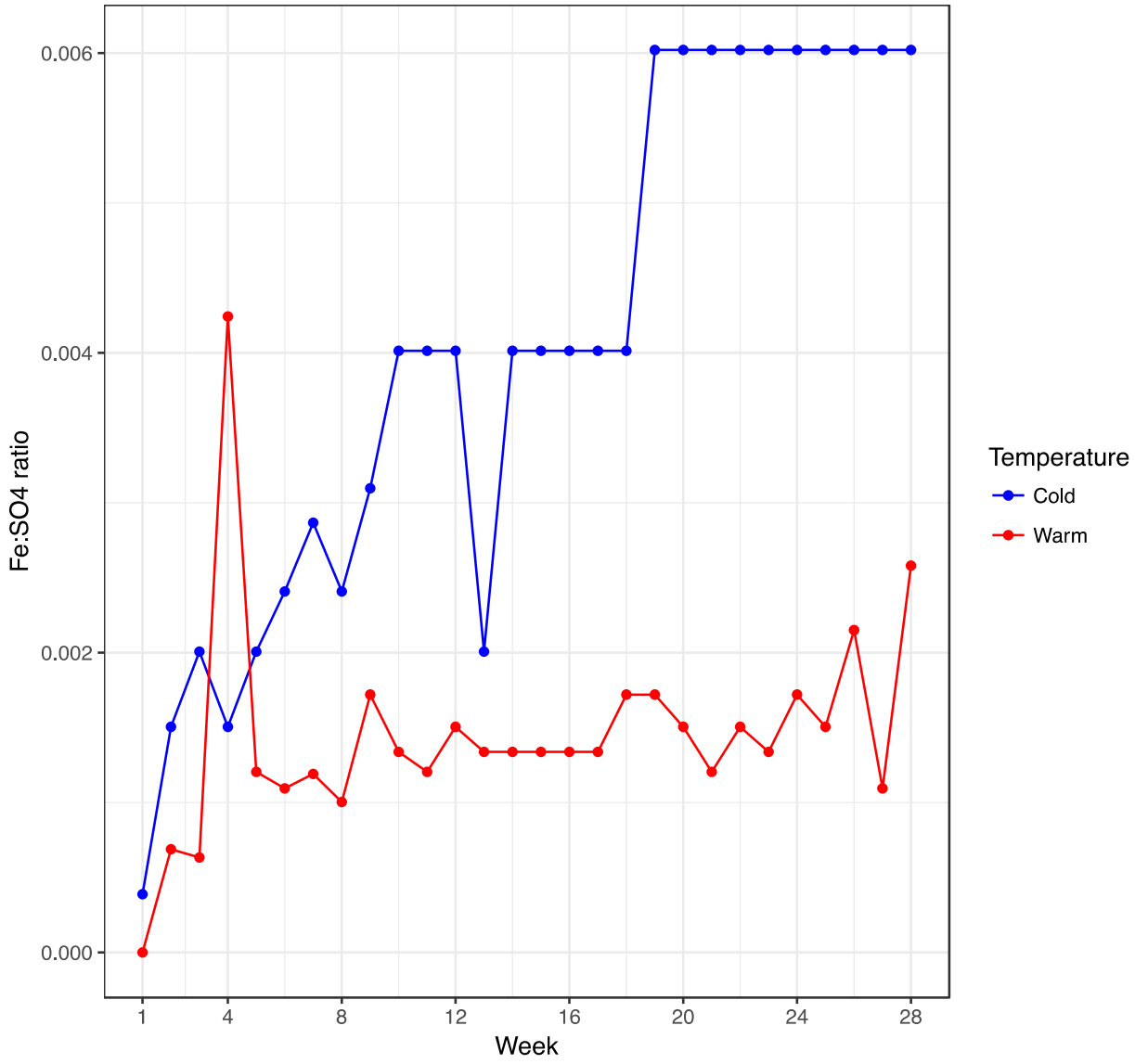
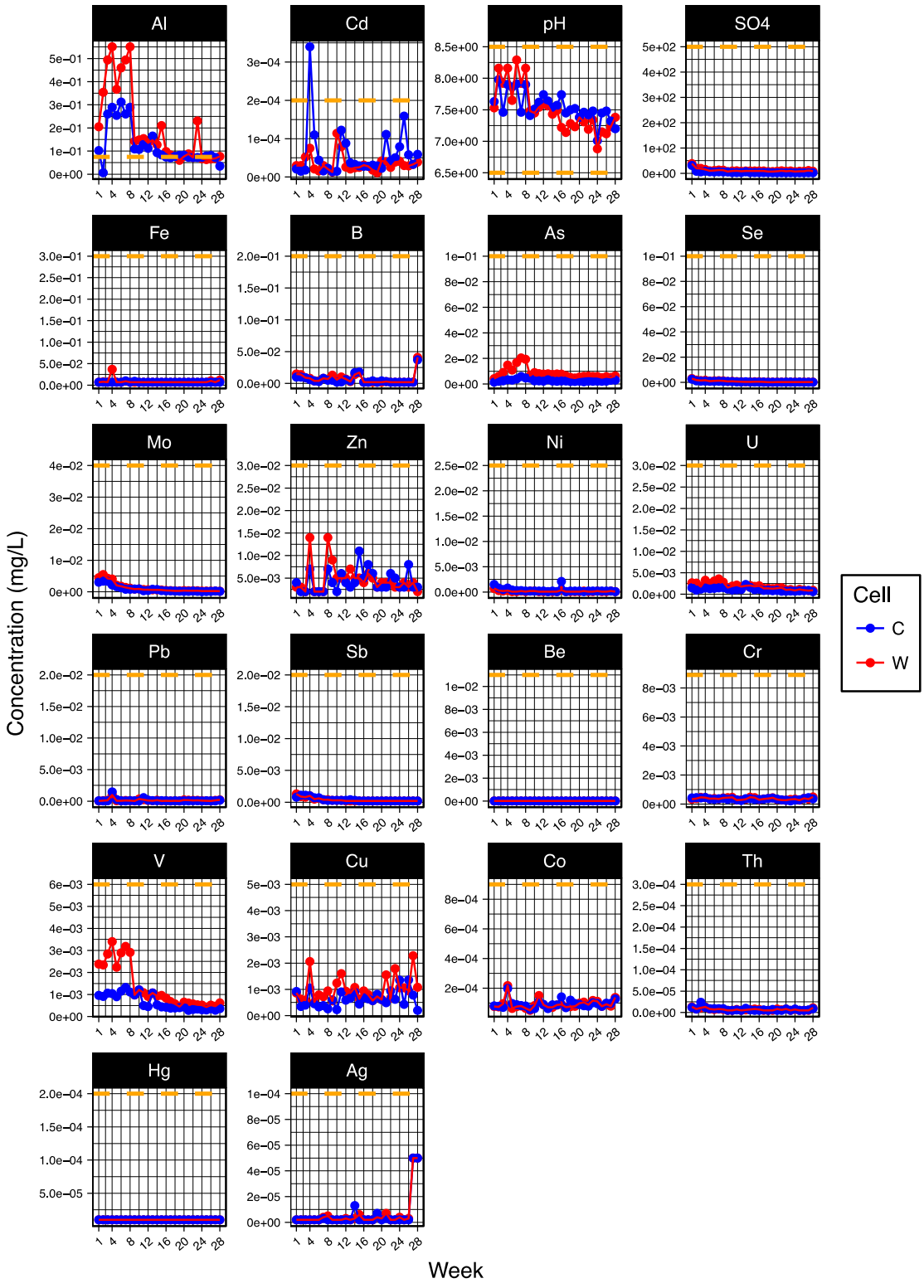


Figure 2.10: **Molar ratio of Fe:SO_4 of warm and cold treatment humidity cells over the 28 week experiment.**



<u>Figure 2.11:</u> Weekly analyte concentrations in relation to PWQO guideline limits (shown as dashed yellow line).

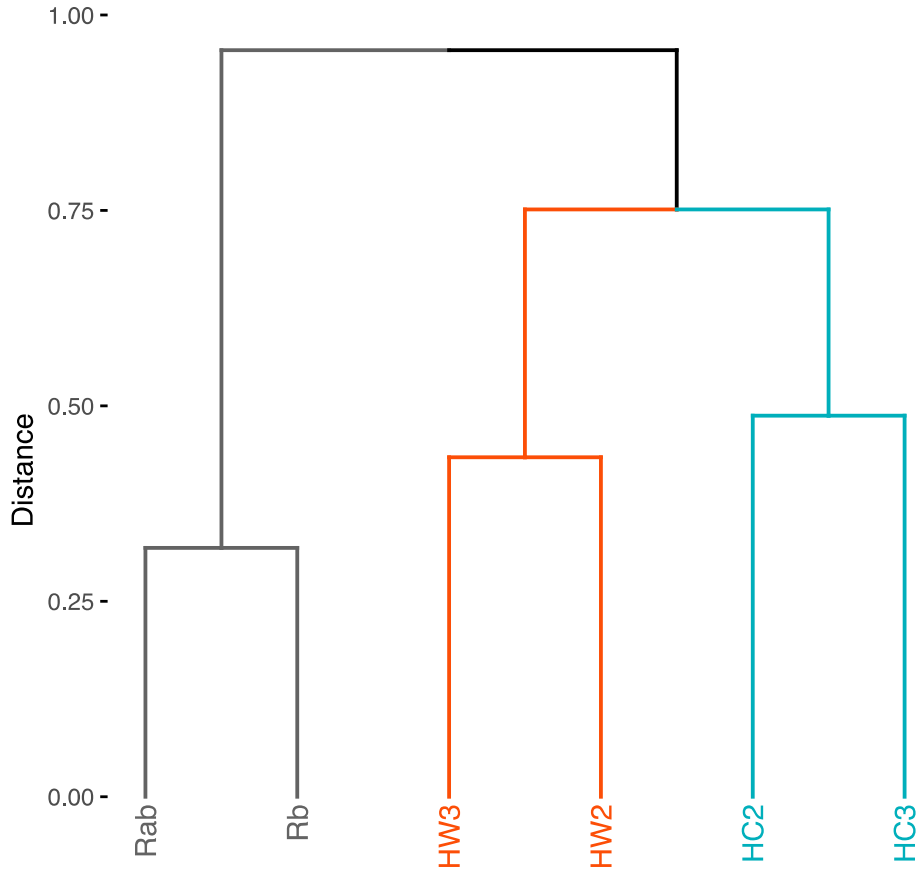
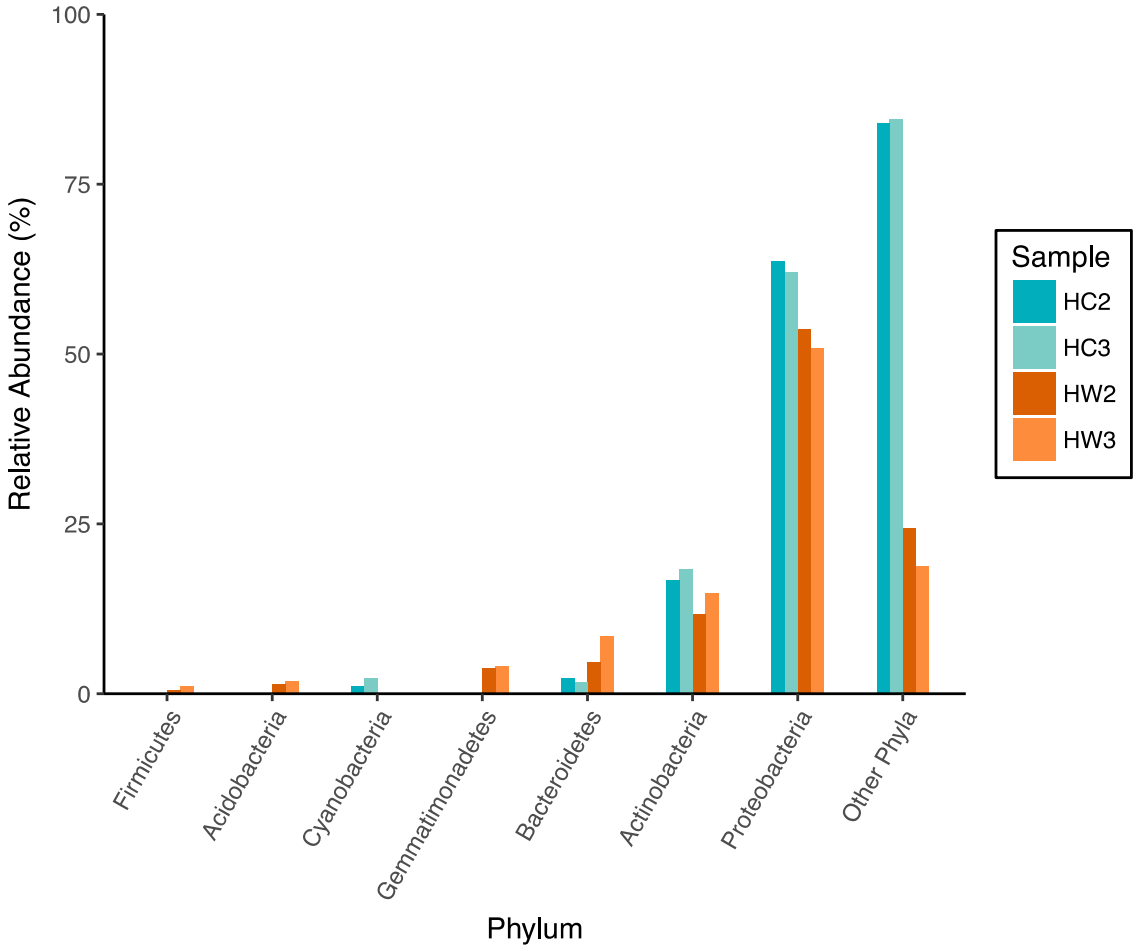
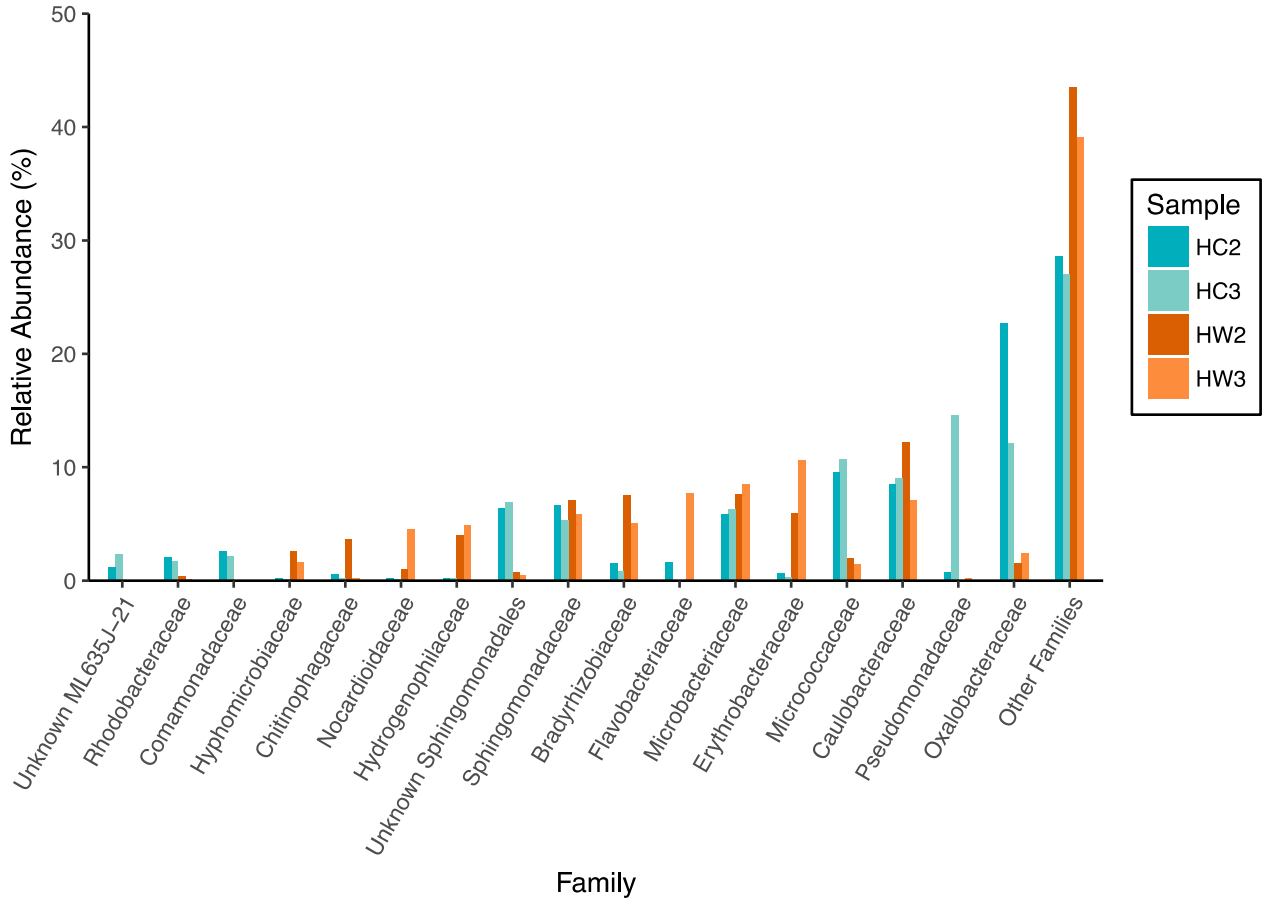


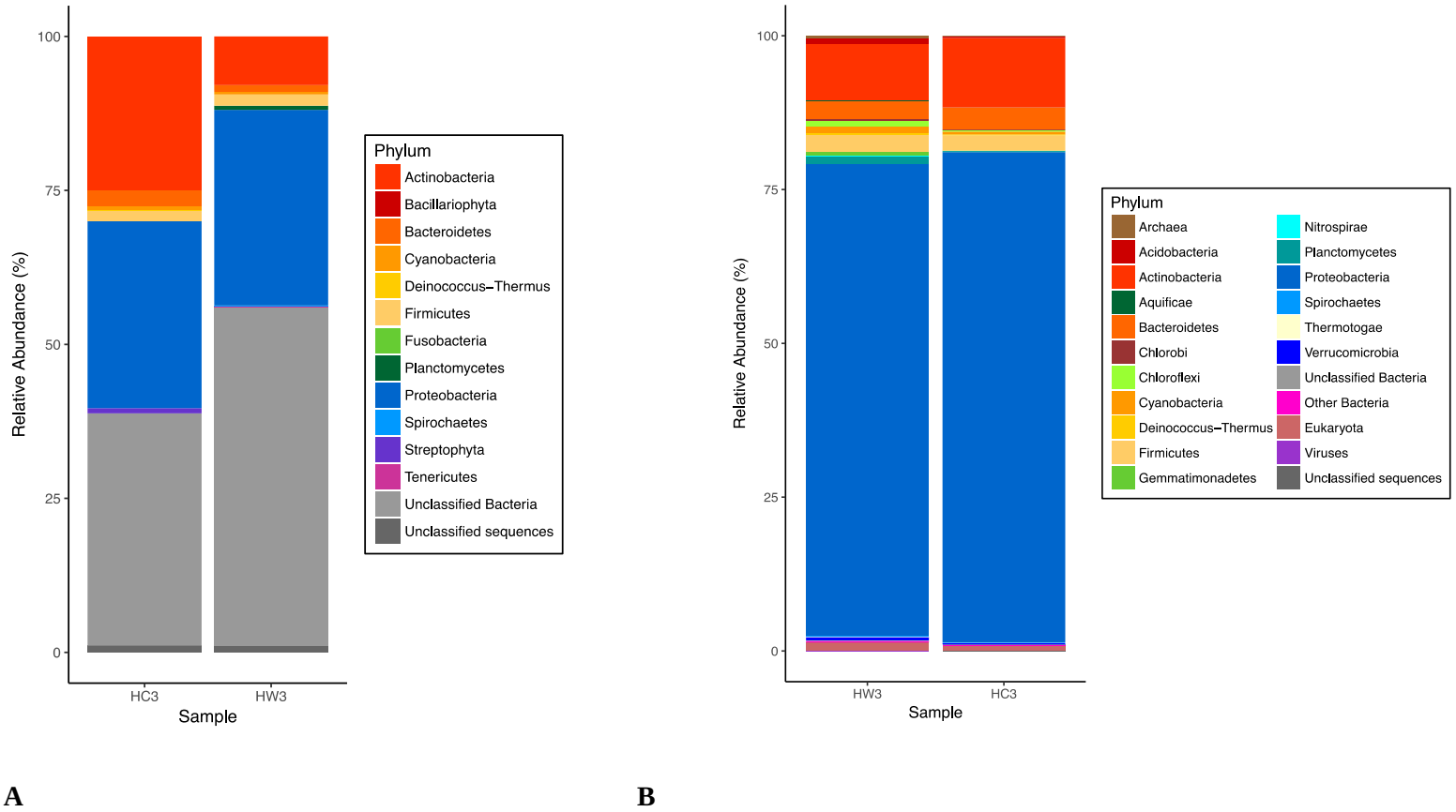
Figure 2.12: Cluster analysis by Bray Curtis distance of humidity cell time point samples by temperature treatment and initial unweathered waste rock samples.



<u>Figure 2.13:</u> Bacterial phyla present in > 1% relative abundance in humidity cell mid-point and end-point samples in warm and cold temperature treatments, as determined by 16S rRNA gene amplicon sequencing.

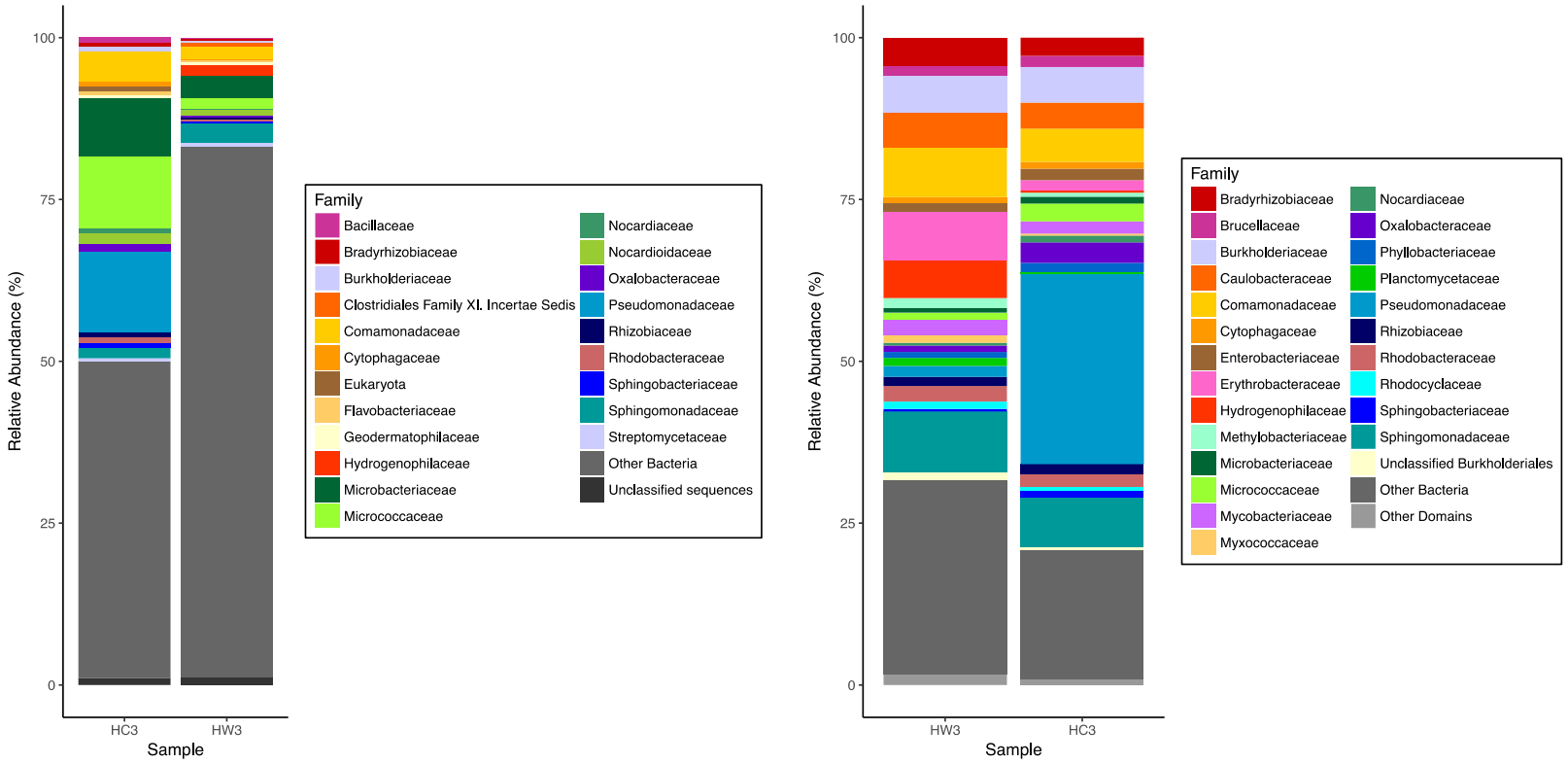


<u>Figure 2.14:</u> Bacterial families present in > 2% relative abundance in humidity cell mid-point and end-point samples in warm and cold temperature treatments, as determined by 16S rRNA gene amplicon sequencing.



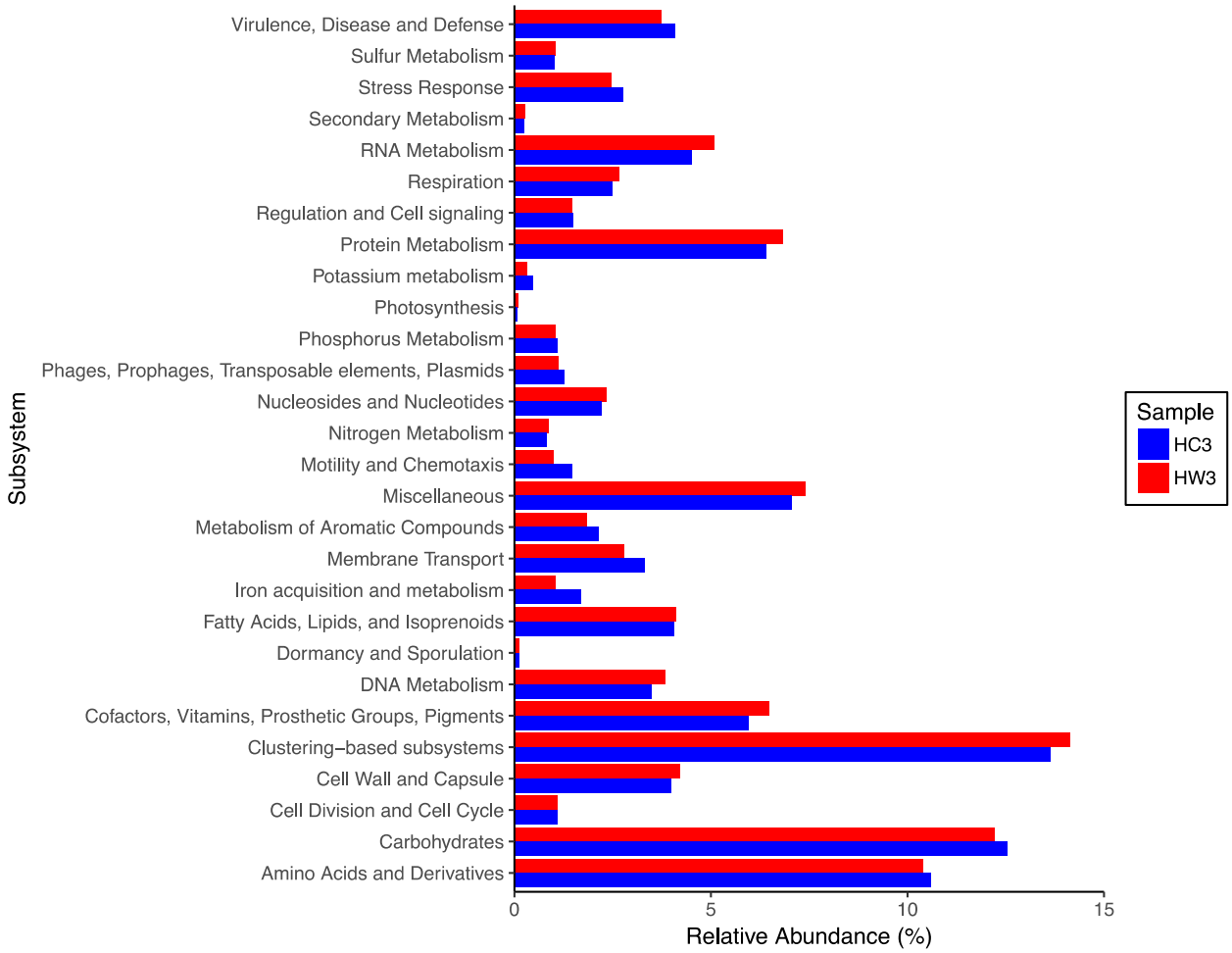
A **B**

<u>Figure 2.15:</u> Dominant microbial phyla present in humidity cell end-point metagenomes of warm and cold temperature treatments. A) Phyla present in metagenome samples identified by 16S rRNA genes annotated by Greengenes. B) Phyla present in > 0.5% relative abundance in metagenome samples identified by gene sequences annotated by the RefSeq database.

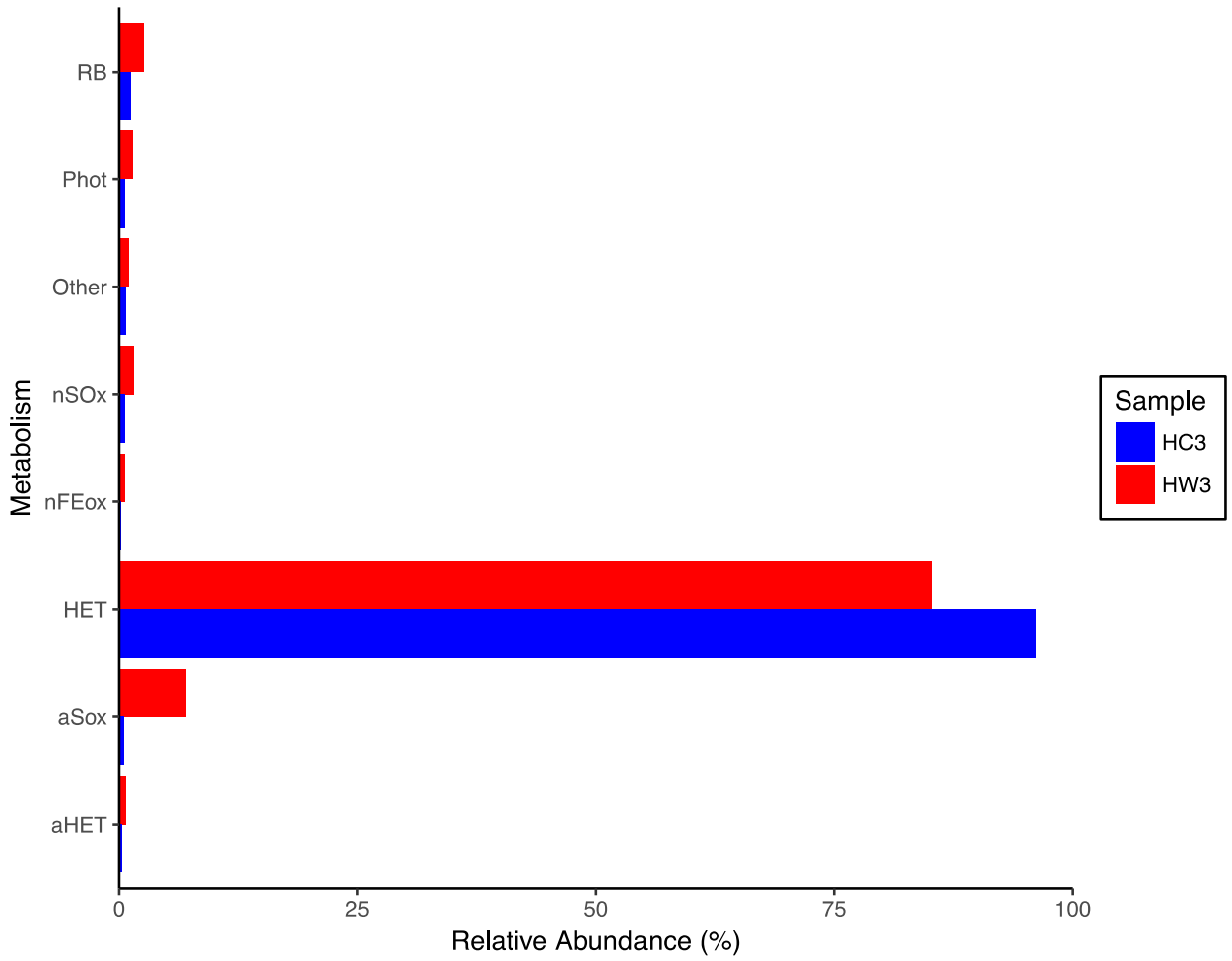


A **B**

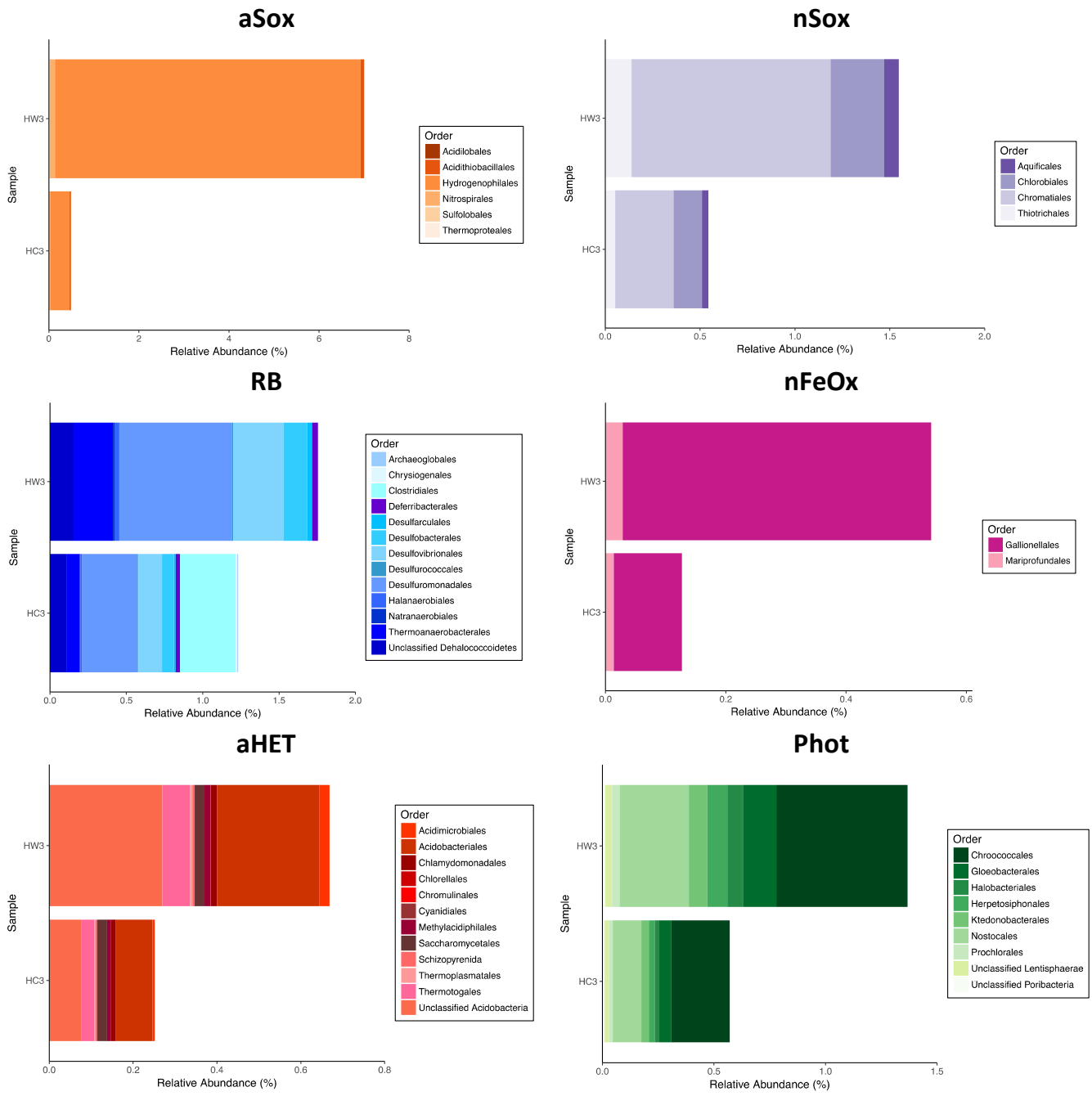
<u>Figure 2.16:</u> Dominant microbial families present in humidity cell end-point metagenomes of warm and cold temperature treatments. A) Families present in <0.5% relative abundance in metagenome samples identified by 16S rRNA genes annotated by the Greengenes database. B) Phyla present in > 1% relative abundance in metagenome samples identified by annotated gene sequences through the Refseq database.



<u>Figure 2.17:</u> Relative abundance of COGs present within warm and cold humidity cell treatments.



<u>Figure 2.18:</u> Relative abundance of microorganisms clustered by their dominant form of energy metabolism; sulfate reducing bacteria (SRB), phototrophic bacteria (Phot), neutrophilic sulfur oxidizing bacteria (nSox), neutrophilic iron oxidizing bacteria (nFeox), heterotrophic bacteria (HET), iron reducing bacteria (FeRB), acidophilic sulfur oxidizing bacteria (aSox), and acidophilic heterotrophic bacteria (aHET).



<u>Figure 2.19:</u> Relative abundance of taxa in warm and cold humidity cell end-point samples classified to order. Taxa are grouped by their dominant energetic designation: Acidophilic sulfur oxidizers (aSox), neutrophilic sulfur oxidizers (nSox), iron and sulfur reducers (RB), neutrophilic iron oxidizers (nFeOx), acidophilic heterotrophs (aHet), and phototrophs (Phot).

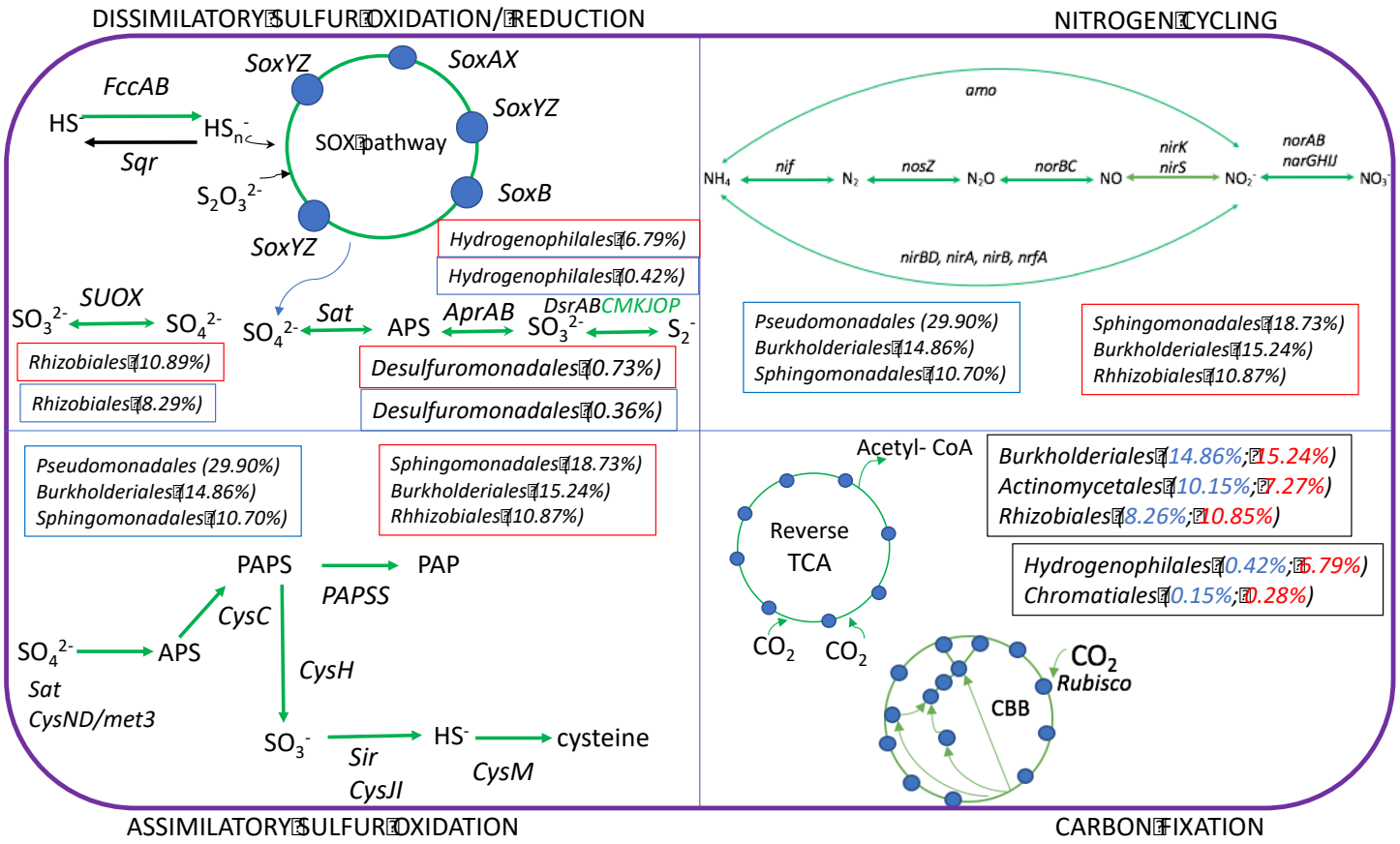
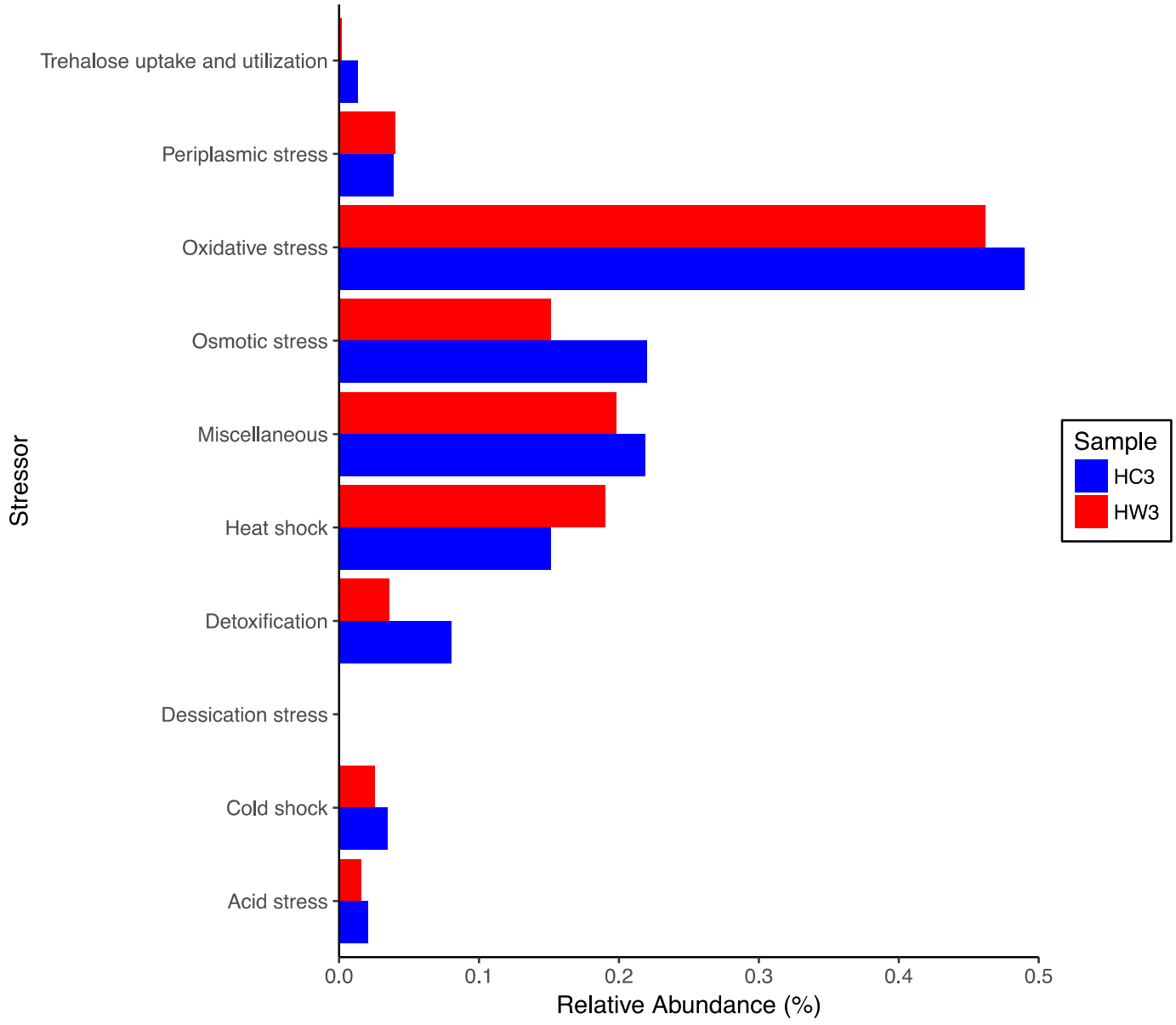


Figure 2.20: Prokaryotic sulfur oxidation and reduction, nitrogen cycling, and carbon fixation pathways in warm and cold humidity cell treatments. Red boxes indicate taxa dominant in warm humidity cell treatments, while blue boxes indicate taxa dominant in cold humidity cell treatments with relative percent abundance of genes associated with these taxa shown in brackets. Green arrows represent pathways represent in the metagenomes and black arrows represent missing pathways.



<u>Figure 2.21:</u> Relative abundance of stress related genes in warm and cold humidity cell treatments.

Table 2.1: Change in mineralogy of rock following a 28 week humidity cell experiment at 4 and 20°C. Green shading represents a >100% increase in the individual mineral's abundance, red shading represents a mineral which has <75% of its individual abundance remaining.

Mineral	Abundance (%)				
	Week 0	Week 28		% Remaining	
		20°C	4°C	20°C	4°C
Quartz (SiO_2)	32.9	31.8	33.3	96.7	101.2
Albite ($NaAlSi_3O_6$)	21.2	18	17.6	84.9	83.0
Microcline ($KAlSi_3O_8$)	1.6	2.3	2.0	143.8	125.0
Chlorite ($(Fe,(Mg,Mn)_5Al)(Si_3Al)O_{10}(OH)_8$)	12.7	8.0	8.5	63.0	66.9
Muscovite ($KAl_2(AlSi_3O_{10})(OH)_2$)	17.7	19.1	19.5	107.9	110.2
Biotite ($K(Mg,Fe)_3(AlSi_3O_{10})(OH)_2$)	2.0	3.3	2.8	165.0	140.0
Actinolite ($Ca_2(Mg,Fe)_5Si_8O_{22}(OH)_{22}$)	9.4	12.7	11.4	135.1	121.3
Calcite ($CaCO_3$)	1.7	1.2	1.1	70.6	64.7
Ankerite ($CaFe(CO_3)_2$)	0.8	0.0	0.0	0.0	0.0
Diopside ($CaMgSi_2O_6$)	0.0	2.7	3.0	270.0	300.0
Dolomite ($CaMg(CO_3)_2$)	0.0	0.8	0.8	80.0	80.0

Table 2.2: Predicted times to depletion of neutralizing potential of warm and cold humidity cell treatment following 28 weeks of weathering.

Treatment	20 °C			4 °C		
Parameter	**Remaining (%)**	**Depletion Rate (mg/kg/week)**	**Time for Depletion (Years)**	**Remaining (%)**	**Depletion Rate (mg/kg/week)**	**Time for Depletion (Years)**
S	95.8	2.6	16	95.8	0.6	70
NP	97.5	18.2	28	97.5	16.8	30
CaNP (SO_4)	97.4	8.2	28	98.9	1.9	119
NP (Alkalinity)	98.4	11.5	4	97.7	16.7	3

Table 2.3: Humidity cell end-point metagenome statistics.

Sample Description	HW3		HC3	
Week Sampled	Week 40		Week 28	
Sample Mass Processed (g)	3.00		15.00	
DNA Yield (ng/ uL)	30.00		10.23	
QC	**Pre**	**Post**	**Pre**	**Post**
Base Pair Count	1,446,938,825	823,472,738	77,0351,377	625,196,786
Sequences Count	4,414,826	3,635,550	5,689,285	4,520,594
Mean Sequence Length (bp)	328 ± 71	227 ± 107	135 ± 67	138 ± 60
Mean GC Percent (%)	64 ± 9	64 ± 9	56 ± 9	58 ± 9
Failed QC (Sequences, %)	-	779,276; 17.65	-	1,168,691; 20.54
Unknown Sequence (Sequences, %)	-	142,547; 3.23	-	567,787; 9.98
Predicted Feature (Sequences, %)	-	3,493,003; 79.12	-	3,952,807; 69.48
Unknown Protein (Sequences, %)	-	1,054,471; 30.19	-	1,396,701; 35.33
Annotated Protein (Sequences, %)	-	2,427,678; 69.50	-	2,532,194; 64.06
Ribosomal RNA (Sequences, %)	-	10,854; 0.31	-	23,912; 0.60

CHAPTER 3: THE WEATHERING OF LOW SULFUR WASTE ROCK IN FIELD LEACH BIN EXPERIMENTS

3.1. Abstract

Field leach bin experiments are an intermediate scale-up between laboratory scale kinetic testing systems and large-scale field leaching pads. Although less standardized than many laboratory experiments, including humidity cell testing, the utility of field leach bins is in their ability to demonstrate measurable leaching kinetics on a relatively small quantity of material as it is exposed to site climatic conditions. We monitored leachate geochemistry from 450kg bins of waste rock for an 11 month period. Additionally, we used analysis of 16S rRNA diversity to characterize the prokaryotic community on the waste rock after the first summer in the 39th and 49th week of the experiment, and shotgun metagenomic analysis of the entire microbial community in the 49th week of the experiment. The geochemistry of the system remained within a circumneutral pH range and analyte release was strongly suggestive of seasonality. Conductivity was correlated with mean site temperature, bin temperature, and bin humidity, while pH correlated strongly with site humidity and precipitation. In a multiple factor analysis, analytes also clustered together in the following groupings: Ni and alkalinity; Al, Fe, and Pb; SO_4, and salts. Most analytes respected Ontario provincial water quality objective (PWQO) guidelines, with the exception of SO_4, Ca, Al, Ni, Co, and Cd. Microbiological analyses described a heterotrophic community that showed a shift in the community composition between sampling time points. Chemolithotrophic organisms were found in low abundance, containing complete pathways for dissimilatory sulfur oxidation and carbon fixation, as well as genes for cold stress adaptation.

3.2 Introduction

Kinetic field tests are an important component of acid rock drainage prediction methods. Kinetic tests in laboratory environments are performed under controlled conditions that may not reflect the environmental conditions present on a mine site. Field testing is therefore used to confirm the results of laboratory scale experiments by exposing waste rock and tailings materials to realistic site conditions. Kinetic field tests are therefore the most accurate predictors of long-term drainage chemistry of waste rock (Parbhakar-Fox & Lottermoser, 2015). Field tests can vary in their size and design from small- scale field leach bins and field columns, to intermediate scale wall washing stations, to large-scale test pads, and finally full- scale site drainage monitoring (Morin & Hutt, 2001; Price, 2009). There is minimal standardization within each type of field test and they can differ substantially in their design, volume of materials used, rock particle size, and longevity. Operators must keep the objectives of their ARD prediction program in mind when selecting a field test to use, as different tests will provide different data, especially when observing primary and secondary mineral weathering (Morin & Hutt, 2001; Parbhakar-Fox & Lottermoser, 2015; Price, 2009).

Field leach bins and cubes have been described by Morin as an intermediate scale test which allow a relatively small volume of crushed rock to be leached by precipitation (Price, 2009). The experiment is carried out using a 220L barrel filled with waste rock of which the upper surface is left open to the atmosphere and the base is attached to a collecting tube to allow leachate that passes through the barrel to be collected for analysis. Lysimeters and thermistors may be included in the design to measure water balance and internal temperature of the bins (Parbhakar-Fox & Lottermoser, 2015). It is suggested that waste rock samples be collected

periodically from the bin for mineralogical and ABA analyses. Leachate can also be analyzed after significant precipitation events to assess elemental release, mineral weathering, and predict ARD generation over a period of years (Price, 2009). In comparison to other types of kinetic field tests, field leach bins are an intermediate scale test to undertake: they require relatively little waste rock, can be set up before the mine is fully operational, are subject to some natural water volume inputs, and are capable of providing information on primary and secondary mineral weathering (Price, 2009). As field leach bins can vary considerably to suit the needs of the operator, little published literature is available despite it being a widely used predictive test within the North American mining industry (Higgins, McGregor, & Davidson, 2018; Lorax Environmental, 2007; Stantec Consulting Ltd., 2015).

Microbial communities are recognized as an integral component of ARD generation. Waste rock and other mine wastes are known not to be sterile environments, and microorganisms have previously been identified within even the most extreme of these environments (Elberling, Schippers, & Sand, 2000; Hallberg et al., 2009; Remonsellez et al., 2009; SRK Consulting, 2006). Few studies have examined the microbiological contribution of microorganisms to the unamended weathering of waste rock, with the exception of some studies using MPN techniques or optical density measurements following inoculation in liquid media (Bailey, Blowes, Smith, & Sego, 2015; Langman et al., 2014) and more recently, a 16S rRNA DNA and RNA amplicon study of humidity cell bacterial communities (Jones et al., 2017). All of these studies were conducted on waste rock originating from polar or boreal climates. Studies from the Diavik diamond mine site in Nunavut suggested that viable neutrophilic sulfur oxidizing bacteria and some acidophilic sulfur oxidizing bacteria are present on waste rock prior to extensive

weathering, while iron oxidizing bacteria are not present when rock samples are incubated in TK media (J. Langman et al., 2014). A longer five year study of the same site concluded that neutrophilic and acidophilic sulfur oxidizing bacteria, and iron oxidizing bacteria enumerations were correlated with low pH values of the rock pile, with acidophilic sulfur oxidizing bacteria and iron oxidizing bacteria being more prevalent at pH below 3 (Bailey et al., 2015). Another study was conducted on waste rock from the pyrrhotite bearing Duluth complex rock and pyrite bearing Ely greenstone in various types of laboratory and field kinetic tests (Jones et al., 2017). The bacterial communities present were largely heterotrophic in nature, dominated by a minority of sulfur oxidizing bacteria, and largely lacking iron oxidizers. Bacterial community composition in field experiments was found to differ from those in various laboratory tests, with four abundant sulfur oxidizing taxa being present in humidity cell and field samples; *Thiobacillus*, *Thiomonas*, *Sulfuricella*, and *Sulfuriferula*. Among the field waste rock samples, the predominant sulfur oxidizer in the pH 4.7 environment of the Duluth complex rock were those of the genus *Sulfuriferula*, while *Sulfuricella* were present in the more neutral pH 7.2 environment of the Ely greenstone waste rock. The differences in microbial communities present in different field samples suggests that differences in pH and waste rock mineralogy can influence the microorganisms inhabiting waste rock (Jones et al., 2017).

This thesis chapter aimed to predict the acid generating status of low sulfur (< 1% S) waste rock when exposed to site climatic conditions in a field leach bin experiment. The geochemistry and microbial community of the waste rock were expected to evolve over the course of the 11 month experiment, with a prediction that the system would become increasingly acidic as primary mineral weathering proceeded and with the assistance of microbially mediated

sulfide oxidation. Element release was also predicted to vary with seasonal temperatures and precipitation. The high neutralizing potential of the rock predicted from the mineralogy of the waste rock was expected to prevent the system from becoming acidic within the one year time frame of the experiment, suggesting that a population of neutrophilic sulfur oxidizing microorganisms may be present on the waste rock along with a minor population of acidophilic sulfur oxidizers. Genes to support a psychrotrophic lifestyle were expected to be present within the microbial community and become more prevalent in the community during cooler seasons.

3.3 Materials and methods

Experimental design

Three 210L plastic drums were loaded with 450 kg of 2 in. minus, < 1% S PAG waste rock that had weathered in the field for under a year. The material had been previously selected at random from the rock pile and crushed to 2 in. minus for use in site construction. The waste rock had previously been characterized as PAG rock by ABA accounting (Hamilton, 2015). Drums were open to the atmosphere and set on a platform directly above a covered 20L bucket (Fig. 3.1A). The base of each drum was lined with ultra- high molecular weight polyethylene geotextile fabric with ¼" drain holes 1" centre to centre to prevent large particulate loss from the drums with leachate release. Drums were attached to 20L buckets by an outflow drain and tubing to collect bin leachate. A slotted schedule 40 PVC pipe measuring 40cm in diameter by the height of the bins was set in the middle of each bin. 2" minus waste rock of the same lithology as what was accounted for the majority of the bin volume was processed at SGS Sudbury to <1/4" crush. 10g of ¼" minus crush material was placed into nylon mesh bags for microbial sampling.

Hygrochron DS1923 data loggers (Maxim Integrated, San Jose, CA) were set to measure temperature and relative humidity of the bins every 30 min. Six nylon bags and a Hygrochron logger were attached to string and inserted into each PVC tube between packed 2" minus crush (Fig. 3.1B) so that they were accessible for periodic sampling and data logging (Smith et al., 2013).

Leachate geochemistry

A leachate sample was collected from bins monthly or after a significant precipitation event in which collecting bins were filled. Samples were not collected for the first three months of the experiment, as the temperature was too low during the winter months to allow leachate to flow within the field bins. The volume of water in the collecting bucket and volume of sediment in the bucket were manually measured and pictures of the leachate and waste rock surfaces were taken to assess changes in colour of the samples associated with mineral weathering. Leachate samples were analyzed for water temperature, pH, and conductivity in the field using a YSI Professional Plus handheld multiparameter meter (Xylem Inc., Yellow Springs, OH) in the field. From each bin, a 200mL leachate sample was collected as well as a 75mL leachate sample which was membrane filtered through a 13mm 0.45µm polyethersulfane membrane filter and acidified to pH 2 with HNO_3 in the field. These samples were sent to SGS Lakefield for total water chemistry analyses, dissolved metals, NH_3/NH_4^+, NO_2^-, NO_3^-, and sulfate by inductively coupled plasma mass spectrometry (ICP-MS). ORP was measured within an hour of sample collection upon returning to the laboratory with a YSI Professional Plus handheld multiparameter meter (Xylem Inc., Yellow Springs, OH). Analytes were compared to the Ontario Ministry of

Environment and Energy Provinical Water Quality Objectives for the Protection of Aquatic Life to provide context to the concentrations of analytes released with respect to water quality standards (MOEE, 1994). Iron speciation was attempted using the spectrophotometric colorimetric assays for total and ferric iron protocol from (Karamanev et al., 2002), however iron concentrations appeared to be largely below the detection limits of the assays. A pooled sample from each temperature treatment was also filtered through a 0.22µm polyethersulfone membrane and acidified to pH 2 prior to analyzing dissolved organic carbon (DOC) on a Shimadzu TOC-5000A in FPOC mode.

Mineralogy

Upon completion of the 11 month field bin experiment, a 10g nylon bag of ¼" minus crushed rock was taken from each field bin and pooled. After mixing the cells' contents with a spatula, a 10g sample of the rock was left to dry on the bench for a month. Rietveld quantitative XRD analysis was completed at SGS Lakefield to determine the resulting mineralogy of the weathered rock samples. We used this data to compare differences in the mineralogy against Rietveld XRD analysis conducted on the waste rock prior to weathering in humidity cells.

Microbiology

Field bins were sampled on week 39 (F2) and week 49 (F3). A 10g sample of ¼" minus crush contained in a nylon bag from each field bin was removed from the nylon bag and placed

into a 50mL falcon tube containing 30mL of RNA Lifeguard solution (Qiagen, MD). The 30g rock sample was stored at - 20ºC until further processing.

Samples were considered to be of low microbial biomass and limited waste rock material was available to be processed to prevent excessive disruption to the geochemistry of the experiment. Several different methods were used to maximize DNA recoveries from the low biomass samples; I) 60g of sample was sonicated with a Fisher Scientific FS20 Ultrasonic Cleaner (Fisher Scientific, CAN) in RNA lifeguard solution for 15 minutes at 40kHz. Sonicated samples were then pelleted by centrifugation at 3260 rcf for 10 minutes. Supernatant was pipetted from the sample prior to processing and pooling the products of four preparations of a DNeasy PowerSoil kit (MoBio, CA), II) Approximately 26g of sample was placed into a 50mL falcon tube to a third of the volume of the tube and topped with sterile phosphate buffer (Zhou et al., 1996) to fill the falcon tube to two thirds of the total tube volume. Samples were sonicated with a Fisher FS20 Ultrasonic Cleaner for 15 minutes at 40kHz. The falcon tubes were then taped to a flatbed vortex and agitated at top speed for five minutes. The samples were then centrifuged for 10 minutes at 3260 rcf and supernatant was pipetted off the samples. 20 mL of sterile phosphate buffer was again added to the falcon tubes and agitated for three min before being centrifuged as above. Supernatant was removed from the samples before continuing to extract DNA using four preparations of DNeasy PowerSoil kit (MoBio, CA). F2 samples were subjected to method I and F3 samples were subjected to method II.

DNA concentration and quality was checked by spectrophotometry using Agilent Take3 software (Agilent Technologies, CA), as well as by PCR amplification of the 16S gene using the

broad eukaryotic primer set designed for the Earth Microbiome project (EMP 515F 5'- GTGYCAGCMGCCGCGGTAA- 3', EMP 806r 5'- GGACTACNVGGGTWTCTAAT- 3') (Gilbert et al., 2014). PCR was completed using an Invitrogen Recombinant Taq DNA Polymerase and 0.6mg/mL BSA under the following conditions: initial denaturation of 4 min at 94˚C, 35 cycles at 94˚C for 45 seconds, 52˚C for 1 min, 72˚C for 1 min, and final elongation of 8 min at 72˚C. Library preparation for the F2 and F3 sample 16S rRNA amplicons was completed by MetagenomBio at the University of Waterloo, with sequencing being completed on an Illumina Miseq using the forward primer (5'- CCTACGGGNBGCASCAG - 3') and reverse primer (5'- GACTACNVGGGTATCTAATCC - 3') (Takahashi et al., 2014). Shotgun metagenomic libraries were also generated for the F3 sample by MetagenomBio at the University of Waterloo.

Bioinformatic analysis

Paired end reads from 16S rRNA amplicon libraries were stripped of adaptors, primers and screened for PhiX contamination with BBDuk, merged with BBMerge, and bidirectionally trimmed to Q20 using BBDuk (Bushnell, 2014). Sequences were converted to fasta files using the fq2fa command in IDBA-UD (Peng et al., 2012) prior to adding QIIME labels in QIIME 1.9.1 (Caporaso et al., 2011). Dereplication, size sorting, doubleton removal, OTU clustering and a denovo chimera check was performed using the UPARSE pipeline (Edgar, 2013) in USEARCH 9.2. Taxonomy was assigned in QIIME against the Greengenes database (McDonald et al., 2012) using the RDP classifier (Cole et al., 2014) in QIIME (Caporaso et al., 2011).

Metagenomic data was processed through the MGRAST server (Meyer et al., 2008) using the best hit method following minimum parameters; an e value of 5, sequence length of 15, percent identity of 60, and a minimum threshold of 3 hits. Taxonomic diversity of the final time point samples was identified through the Greengenes (McDonald et al., 2012) and Refseq databases ((Pruitt et al., 2012). Community metabolism was also analyzed by identifying genes related to sulfur and iron cycling as well as cold stress responses using the NCBI Refseq (Pruitt et al., 2012) and KEGG databases (Kanehisa et al., 2016) (Meyer et al., 2008).

Statistical analyses

All statistical analyses were conducted with various R packages in R 3.3.2 (R Core Team, 2016). The cumulative loadings, monthly release rates of geochemical analytes over time, and depletion rates of analytes were calculated in R with the former two were plotted in ggplot2 (Wickham, 2009). Site and bin temperature and humidity, as well as site precipitation were also plotted in ggplot2. Multiple factor analysis was performed by MFA in the FactoMineR package (Lê, Josse, & Husson, 2008) using three factors to determine the relationships of climate and geochemical factors. Results of the multiple factor analysis were plotted using the fviz_mfa_var and fviz_contrib scripts in the Factoextra package (Kassambara & Mundt, 2017). Linear mixed effect models were also generated for each analyte to assess their relationship with temperature and day of the experiment. Analytes were transformed as needed to meet the assumption of linearity. Linear mixed effect models were generated using the lme script in the nlme package (Pinheiro, Bates, DebRoy, Sarkar, & R Core Team, 2017). The ratio of specific conductivity between humidity cell temperature treatments was also calculated to provide an estimate of the

effect of temperature of waste rock weathering (J. Langman et al., 2014). Fe: SO4 release rate ratios were also calculated to determine if leachate geochemistry was mediated primarily by pyrite dissolution (Bowell et al., 2006; Sapsford et al., 2009). Calculations predicting the time to acid generation were performed based on methods described by Morin and Hutt.

Manipulation of 16S rRNA gene microbial community data was facilitated by scripts included in the Phyloseq package (McMurdie & Holmes, 2013). Beta diversity of the community was interpreted by UPGMA clustering of a computed Bray- Curtis dissimilarity matrix (McMurdie & Holmes, 2013) of the raw OTU counts using the hclust and fviz_dendro scripts in the stats and Factoextra packages (McMurdie & Holmes, 2013, Kassambara & Mundt, 2017). Simpsons' and Shannon diversity were used to assess the alpha diversity of the community in the Phyloseq package (Appendix A.2) (McMurdie & Holmes, 2013). Barcharts of the relative abundance of taxa with greater than 1% or 2% abundance at the phylum and family levels, respectively were produced and visualized in ggplot2 (Wickham, 2009). As many taxa could not be identified beyond family, the top two hits of the five most abundant OTUs for each sample were identified using the NCBI BLASTn algorithm against the NCBI 16S rRNA sequences database (Altschul et al., 1990).

Absolute gene hits identified from metagenomic analysis was converted to relative abundance. Stacked bargraphs were generated of dominant taxa present at the phylum and family levels in ggplot2 (Wickham, 2009). Microorganisms identified by Refseq were grouped by their dominant form of metabolism into the following categories: sulfate and iron reducing bacteria (RB), phototrophic bacteria (Phot), neutrophilic sulfur oxidizing bacteria (nSox), neutrophilic

iron oxidizing bacteria (nFeox), heterotrophic bacteria (HET), acidophilic sulfur oxidizing bacteria (aSox), and acidophilic heterotrophic bacteria (aHET). Relative abundance of COG groups, energy metabolism, dominant organisms not involved in neutrophilic heterotrophic metabolism, and stress- related genes were also plotted as bargraphs using ggplot2 (Wickham, 2009).

3.4 Results

Site conditions

The internal conditions of the field leach bins were influenced by the climatic conditions on site. The internal bin temperature was consistently ~ 5ºC cooler than the external site temperature, but followed site temperature trends (Fig. 3.2A). Bin humidity remained within a more consistent range in comparison to site humidity. Relative humidity of the bins (60-80%) was lower than site humidity in the winter and spring months after start- up. Bin humidity then rose to exceed that of the site humidity and remained consistently above 90% RH from late summer onwards (Fig. 3.2B). Precipitation was highest in the winter and early spring months, lowest in the summer, and returned to higher levels in late summer and autumn (Fig. 3.2C). Site temperature, humidity, and precipitation followed seasonal patterns which was reflected in the bin temperature, and to a lesser extent bin humidity.

Leachate geochemistry

Leachate geochemistry was dynamic over the course of the experiment and by season. pH increased minimally from 7.85 to 7.95 over the winter months and fluctuated within 0.2 units in the spring months. pH decreased over the summer months to a low of 7.3 before increasing to 7.5 by late fall (Fig. 3.3A). Trends in conductivity and TDS mirrored each other, with initial decreases over the winter to their lowest levels (288µS/cm and 210mg/L), peaking in early September (2116.67µS/cm and 2076.67mg/L) and decreasing again throughout the fall (Fig 3.3B, C). Final conductivity and TDS measurements taken in mid November were similar to those in mid- summer (375µS/cm and 434mg/L), yet still considerably higher than those present in the winter and early spring, suggesting that waste rock weathering rates generally increased over the course of the experiment.

Analytes which were consistently leached at high concentrations included: SO_4, Ca, K, Mg, Na, alkalinity, Si, Cu, Al, Ni, Co, and Cd (Fig 3.4). The majority of elements with consistently high leaching rates are also those with the highest cumulative loads in the leachate (Fig. 3.5), with the exception of Ni, Co, and Cd (Fig. 3.6). Acidity is also an analyte with a high cumulative leachate load despite showing lower weekly leaching rates (Fig. 3.5). Of the elements with high release rates, SO_4, Ca, K, Mg, Na, Si, and Cd behaved similarly; they decreased in concentration over the winter months, demonstrated an exponential increase in their release rates over the summer and then began to decrease in concentration after peaking in concentration in the fall months. Alkalinity, Ni, and Co also showed similar trends in their release rates, with the element concentrations rising over the winter months, decreasing during the spring and early

summer, stabilizing throughout the late summer, and beginning to rise again in the fall (Fig. 3.4). Fe was not present in high concentration, but was released in a higher concentration during the spring thaw (Fig. 3.7). Additionally, U was leached at steadily increasing rates over the course of the experiment but remained present in low concentration (<0.1mg/L) (Fig. 3.7). Of the nitrogen species analyzed, NO_2 and NH_3+NH_4 remained at or under the detection level for ICP at 0.03 and 0.1mg/L, respectively (Fig. 3.8). NO_3 was released in high concentration in the spring (0.47mg/L) quickly decreased in late spring (0.17mg/L) and slowly decreased to concentrations that were at or under detection levels for ICP (0.06mg/L) (Fig. 3.8). DOC was analyzed at the end of the experiment, which was measured to be high at 9.21mg/L.

Seven elements exceeded PWQO limits over the course of the experiment (Fig. 3.9). Cu and Co were elevated throughout the entire 11 month experiment. SO_4 and Cd became elevated only after the summer and late spring, respectively, and continued to be above PWQO limits for the remainder of the experiment. Al, Ni, and Zn were transiently high before returning to acceptable concentrations.

Multiple factor analysis was conducted to observe the relationships of leachate geochemistry with climate variables. MFA explained 56.1% of the total variance between leachate geochemistry and climate variables (Fig. 3.10). Conductivity, mean bin humidity, and mean site and bin temperature clustered together, as did pH, average site humidity, and precipitation. The following analytes also clustered together: I) alkalinity and Ni; II) Al, Fe, Pb; and III) SO_4, K, Na, Mg, Th, Si, Sb, Cd, Mo, Se, Cd, Ca.

As temperature and time are two factors which are substantially related to element leaching, linear mixed effect models were generated for analytes to determine their relationship with these factors (Table 3.1). Elements significantly associated with temperature at $p< 0.05$ include: SO_4, Al, As, B, Ca, Cd, Fe, K, Mo, Na, Ni, Pb, Sb, Se, Si, Th, V, alkalinity, pH, conductivity, and TDS. Of the afore mentioned analytes, Al, Co, Pb, V, Zn, alkalinity, and pH were associated negatively with temperature. Many elements were significantly associated with the date of the experiment at $p <0.05$ including: SO4, Al, Ca, Cd, Co, Fe, K, Mg, Ni, Pb, U, V, Zn, alkalinity, pH, TDS. Of the afore mentioned analytes, Al, Co, Fe, Pb, Th, V, and alkalinity were associated negatively with the date of the experiment. Cr and P were not modelled as they could not be transformed to fit the assumption of linearity.

Rietveld XRD analysis demonstrated that the mineralogy of the unweathered rock was similar to that of the waste rock that had weathered over the 11 months of the field bin experiment. The waste rock is composed predominantly of silicate minerals. A small decrease in albite, chlorite, and muscovite and increases in quartz, biotite, and diopside were seen in both warm and cold humidity cells (Table 3.2). A minimal decrease in quartz was also seen only in the warm treatment. Rietveld analysis is limited in the detection of hydrated minerals, therefore undetected secondary precipitates may have formed over the 28 weeks of the experiment. Prior modal mineralogy of the waste rock identified that the rock contains < 1% S, the majority of which is contained is pyrite. As sulfide minerals were initially present in very low quantities (0.01- 0.83%) these minerals are unlikely to have been detected by the Rietveld XRD analysis. However, the $Fe:SO_4$ molar ratio of the humidity cells was well under 0.5 (Fig. 3.11), the stoichiometric ratio of Fe to S in pyritic minerals, in both warm and cold treatments suggesting

that the presence of Fe and SO_4 in the leachate was a result of either Fe- mineral precipitation, non- ferrous sulfide dissolution, or sulfate mineral dissolution (Sapsford et al., 2009).

Field leach bin experiments are designed to be predictive tests of ARD generation while using site climatic conditions to weather waste rock. The field leach bin experiment had nearly undergone a full year of what is intended to be several years of operation. As expected, sulfate and neutralizing species did not reach steady leaching rates within the time period of the experiment. The waste rock was calculated deplete its S content after 42 years, and deplete its NP after 103 years of weathering (Table 3.3). As NP will outlive the S content of the waste rock, ARD generation is not predicted to occur.

16S rRNA gene amplicon sequencing

Bacterial communities changed substantially between the F2 and F3 time points, with 77.8% more sequences and 56.1% more OTUs present in the F3 time point. Cluster analysis using the Bray Curtis distance metric also indicated that the F2 and F3 time point bacterial communities were substantially different from the unweathered waste rock samples, and from themselves (Fig. 3.12).

Four phyla were present in > 1% relative abundance during the F2 time point which were, from highest to lowest abundance: the Bacteroidetes (51.1%), the Proteobacteria (28.3%), the Firmicutes (1.6%), and the Actinobacteria (2.3%) (Fig. 3.13). Nearly three months later at the F3 time point, the Proteobacteria became the dominant phylum present (59.2%) followed by the Actinobacteria (10.5%) and the Bacteroidetes (8.6%). The bacterial community present in >2%

abundance was similar in its composition between the F2 and F3 time points, with only the absences of the Comamonadaceae in the F2 sample and the Flavobacteria in the F3 sample. Nearly all taxa, with the exception of the Enterobacteraciae and Flavobacteriaceae, increased in abundance from the F2 to F3 time points.

The bacterial community present in the field leach bins was predominantly heterotrophic based on the taxa identified by family which were present in greater than 2% abundance (Fig. 3.14). Many heterotrophs are commonly found in soil, aquatic, or rock surfaces, such as members of the Caulobacteraceae, Chitinophagaceae, and Sphingomonadaceae. Known denitrifiers among the Comamonadaceae and Rhodobacteraceae, and nitrogen fixing bacteria within the Oxalobacteraceae and Bradyrhizobiaceae were also present. The Comamonadaceae were present only the F3 time point, the majority of which were heterotrophic genera such as *Variovorax*, and *Hylemonella*, as well as the dessication tolerant *Ramlibacter* and psychrophilic *Polaromonas*. The only sulfur oxidizer present in high abundance was the *Bosea genosp*., a genus of the Bradyrhizobiaceae. Acidophilic sulfur oxidizers were also present in low abundance, dominated by the Hydrogenophilaceae, which were composed of *Thiobacillus* spp. No Thiobacilli were identified to the species level, however the closest BLAST hits suggest that the 3 OTUs are strains of *Thiobacillus thioparus* and *Thiobacillus starkey*. Other sulfur oxidizers which were present in ≤1% abundance in the samples included OTUS within the Rhodobacteraceae. Extremophilic organisms capable of surviving stressors involved in cold, saline, or oligotrophic environments were also found in low abundance, including Paracoccus, Psychrobacter, Jeotgalicoccus, Deinococcus and the genera *Polaromonas* and *Modestobacter*. Some heterotrophic organisms present in the samples in higher abundance were animal

commensals, such as the Enterobacteraceae and Flavobacteraceae. As field leach bin experiments are not run as sterile systems, these organisms may have entered the system via birds through human contact while handling equipment.

Metagenomic sequencing

Metagenomic sequencing generated 1,431,371,709 bp (5,211,325 sequences) from the final time point of the field leach bin experiment. Mean GC content was 61 ± 10%. 79.12% of sequences from the warm humidity cells and 92.94% of sequences contained predicted features, of which 0.34% were rRNA sequences (Table 3.4).

Taxonomy was assessed using 16S rRNA gene sequences identified by Greengenes and genes inferred from Refseq annotation (Fig. 3.15). Bacteria represented 98.6% of the taxa, with the Archaea representing under 0.3% of the taxa present, Viruses representing 0.03% of the community, and Eukaryotes representing the remaining 1.07% of the microbial community. Refseq identified more taxa than Greengenes however the following dominant phyla were constant across the two reference databases; the Proteobacteria, the Bacteroidetes, the Actinobacteria, the Firmicutes, the Verrumicrobia, and the Spirochaetes. Taxa identified to be present in at a relative abundance of greater than 1% at the family level suggests that the bacterial taxa are largely heterotrophic in nature, which include dominant families such as the Sphingomonadaceae, the Comamonadaceae, the Pseudomonadaceae, the Nocardioidaceae, the Noicardioidaceae, and the Bradyrhizobiaceae (Fig. 3.16).

We analyzed metagenomic data regarding the metabolisms of all domains of life, however as the field bin microbial communities were predominantly composed of Bacteria, we focused our attention on their metabolic potential. SEED subsystems (analogous to clusters of orthologous groups COGs) analyses indicated similar distribution of coding sequences in different gene categories between temperature treatments (Fig 3.17). The most abundant COG groups identified were: clustering- based subsystems; carbohydrates; amino acids and derivatives; miscellaneous; and cofactors, vitamins, prosthetic groups, and pigments. By clustering the microbial community within general metabolic groups 95.5% of energy metabolism-related genes were dedicated to neutrophilic heterotrophic metabolism (Fig. 3.18). As the metabolic pathways driving acid generation in waste rock systems are not heterotrophic in nature, we focused on the remaining acidophilic heterotrophic, autotrophic, and chemolithotrophic forms of metabolism present within the system.

Several iron and sulfur reducing bacteria were present in 1.8% relative abundance in (Fig. 2.19). Many organisms were present in low abundance, so despite genes for dissimilatory sulfate reduction such as the DsrC and the DsrMKJOP subunit being present, the DsrAB gene was absent. Assimilatory sulfate reduction pathways were complete and dominated by the Hydrogenophilales, Chromatiales, Rhizobiales, and Burkholderiales. Acidophilic and neutrophilic sulfur oxidizers were present in 0.38% and 0.71% relative abundance, respectively. The Hydrogenophilales, composed of *Thiobacillus denitrificans,* was the dominant acidophile while members of the Chromatiales were the dominant neutrophilic organisms. The Hydrogenophilales and Chromatiales both contained genes for reduced inorganic sulfur compound (RISC) oxidation. The Thiobacilli provided the best insights into RISC oxidation,

showing the ability to oxidize HS^- through *Fccab*, oxidize HS to thiosulfate by the SOX genes, as well as the ability to oxidize thiosulfate to sulfate through APS reductase and ATP sulfurylase or sulfite dehydrogenase. Considerable hits to cytochrome c genes for flavocytochrome c sulfide dyhydrogenase, which is used for photochemolithotrophic sulfur oxidation were also present. Additionally, the SUOX gene for sulfite oxidation was found belonging to the Rhizobiales whom are predominantly heterotrophic organisms (Fig. 2.20).

Few iron oxidizers were present in the metagenome, representing 0.08% of the energy metabolism (Fig. 2.19). The majority of taxa were neutrophilic organisms, including *Gallionella spp*., with the exception of the Acidithiobacilli, which are acidophilic Fe-S oxidizers. Siderophores and iron transport proteins were present but rusticyanin was not identified in the metagenome. Iron reducers, such as the Deferribacterales and *Geobacter spp*., were also present in low abundance. (Fig. 2.19).

Many soil and aquatic taxa that are active participants in nitrogen cycling were present. Genes for nitrogen metabolism was found within the metagenomes, with nearly all genes needed for the complete nitrogen cycle identified (Fig. 2.20). However, *amo* genes for ammonia oxidation were absent in the metagenome, despite genera such as *Nitrospira* being present. Many heterotrophic organisms contributed to N cycling, such as the Rhizobiales, Actinomycetales, Burkholderiales, and Caulobacterales. Some chemolithotropic organisms found within the metagenome, such as the Hydrogenophilales, also have the potential to contribute to nitrogen cycling (Fig 2.19).

Carbon metabolism was well represented in the humidity cell bacterial communities due to the dominance of heterotrophic organisms (Fig. 2.18). Autotrophic bacteria were supported by the presence of the full complement of genes to complete the reverse TCA cycle and Calvin Benson- Bassham cycle (Fig. 2.20). Common soil taxa capable of C fixation included the Burkholderiales, Actinomycetales, and Rhizobiales. Prominent chemophototrophs and chemolithotrophs relying on carbon fixation as their carbon source included the Chromatiales, Hydrogenophilales, and Chlorobiales (Fig. 2.19).

The stress responses of the microbial community reflected the challenges of their environment enriched in inorganic compounds or metals and varying moisture content. A total of 50,976 hits to genes encoding for proteins and metabolic pathways to assist with salts, metals, and acidic conditions were present, primarily those related to acid stress, oxidative stress, periplasmic stress, and detoxification (Fig. 21). Genes for oxidative stress, periplasmic stress, and osmotic stress were identified, which may also assist organisms in surviving the fluctuating moisture conditions. Minimal genes associated with dessication stress were present. Cold stress responses were of particular interest, as the material originated from a boreal climate and the cold temperature treatment was incubated at a temperature suboptimal for most bacteria. DNA/ RNA binding cold shock proteins A, B, C, D, E, F, and G were present in the metagenome, as well as pathways for trehalose uptake and utilization, a cytoplasmic solute. Genes for quorum sensing and biofilm formation were also present, with those involved in methionine biosynthesis and degradation, and autoinducer-2 synthesis being the most prevalent.

3.5 Discussion

Field bin leaching kinetics

This study investigated the weathering of low sulfur waste rock under site climatic conditions using field leach bin experiments. As the experiment was ran for under a year, the results of this experiment provide preliminary data of the leachate geochemistry that is to develop over many years. Leachate analysis demonstrated that analytes followed seasonal patterns, with leachate conductivity being correlated with temperature and bin humidity. The majority of elements which were found in high concentration (SO_4, Ca, K, Mg, Na, Si, and Cd) as well as conductivity and TDS were present in higher concentrations in the spring and summer months and decreased during the fall and winter. Cu, Al, Ni, Co, and alkalinity were also present in high concentrations, yet Ni, Co, and alkalinity were leached in higher concentrations over the cooler fall and winter months. Linear mixed effects models identified that of the analytes accounted for in the field leach bin leachate, SO_4, As, B, Mo, Na, Ni, P, Sb, Se, Si, and Th were exclusively associated with temperature. Alternatively, Cr, U, and Zn were exclusively related to the amount of time the rock had weathered.

Additional trends in analyte release were identified by multiple factor analysis. The waste rock is composed primarily of silicate and carbonate minerals, with sulfide minerals representing in ~1% of the total waste rock composition. K, Na, Mg, Th, Sb, Cd, Mo, Se, and Cd concentrations correlated well with SO_4 and Si concentrations, suggesting that the weathering of sulfide and neutralizing minerals drives their behaviour. Ni and alkalinity were also correlated,

which may be explained by their tendancy to be released at cooler temperatures. Additionally, Al, Fe, and Pb grouped together due to their spikes in release during the spring flush and subsequent drop in concentration.

Ontario PWQO limits identified five elements of concern over the course of the experiment. Cd and SO_4 were elevated from May and June onwards, respectively, possibly due to their higher release rates with more elevated temperatures. Al has been elevated in other leaching tests on the waste rock and was present in the leachate in high concentrations over the winter months (Hamilton, personal communication, 2016). Ni concentrations were high after the spring thaw and Zn became elevated in mid November, both of which may be released at cooler temperatures due to carbonate mineral control over their solubility (SRK Consulting, 2006). Although the elevation of elements above PWQO limits indicates that these elements may be present in higher concentrations in waste rock leachate, it is not a definitive indicator of pollution. Further characterisation of the leachate based on site hydrology, background levels of elements in receiving water, and other site specific factors must be incorporated to determine the true risk of elements in discharge water from the mine site (Price, 2009).

The waste rock leachate in November was found to be high in DOC, which is unusual for lithotrophic samples. The vast majority of organic carbon likely originated from soil that became incorporated with the waste rock from surface blasting as well as cell turnover. As waste rock tends to be an oligotrophic system, soil remaining from overburden may influence the presence of heterotrophs within the system.

The large neutralizing potential of the waste rock played a dominant role in the leachate geochemistry seen over the 39 week experiment. All of the minerals identified through Rietveld XRD analysis are predominantly aluminosilicates and a small number of carbonates, as the sum of sulfide minerals represented ~1% of the waste rock prior to rock weathering. Of those categorized as dissolving minerals (Price, 2009), calcite (-35.3%) and ankerite (-100%) were depleted by the 39th week of the experiment, as was the intermediate weathering chlorite (-21%). The pH of both temperature treatments remained relatively stable at a circumneutral pH between pH 7.34 and 7.97. The high proportion of neutralizing minerals likely contributed to the circumneutral pH of the system (Price, 2009), yet sulfide oxidation was occurring given the high sulfate loads in the leachate. Sulfide mineral dissolution likely prevented the pH from rising above circumneutral levels but may have been present primarily as hydrated secondary minerals. Rietveld XRD analysis did not identify sulfide minerals in the waste rock, although this was likely due to the fact that this technique cannot reliably detect the presence of hydrated minerals. To gain an understanding of the weathering of the waste rock through the detection of secondary minerals, this can be explored in the future by performing sequential leach extractions of the waste rock in addition to SEM-EDS at the end of the experiment (ASTM, 2013).

Field leach bin programs are designed to predict the timing and extent of acid generation in mine wastes. This experiment monitored the first 11 months of a multi- year field bin experiment, during which time the PAG rock did not generate ARD. Based on our calculations after 11 months of monitoring, S should be depleted in 42 years while NP will continue to be generated for 103 years. In effect, acid generation is not expected to occur. However, field leach bins will need to be monitored for longer periods of time to determine if S or NP release rate will

remain similar to what they are now. Additionally, field leach bins cannot be directly compared to full scale waste rock piles, therefore test pads and site drainage may need to be monitored next to provide additional confidence in the ARD predictions for this waste rock.

Microbial community composition

Experiments and monitoring of mine waste rock are rarely performed in conjunction with native microbial community analysis. To our knowledge, this is the only experiment in which microbial taxonomy has been collected from a field bin experiment, and the second of which it has been collected from a field- based experiment (Jones et al., 2017). Despite the varied sample extraction methods, the microbial communities identified at the phylum and family levels showed good consistency for the final time point samples of 16S rRNA gene amplicon sequencing and shotgun metagenomic sequencing. All domains of life were present in the final time point samples, however Bacteria were the dominant organisms present within the waste rock microbial community. The majority of taxa were neutrophilic heterotrophic organisms, supported by the high DOC levels and circumneutral pH of the leachate. Similarly, *Jones et al.* (2017) also found that the microbial communities found within their field waste rock were also largely composed of neutrophilic heterotrophs despite having waste rock of similar but more reactive sulfur content (0.63- 1.4% S contained largely as pyrrhotite) and considerably more weathered minerals.

The microbial communities at the F2 and F3 time points were found to be significantly different from each other, as well as from the unweathered waste rock. This is particularly

evident when observing the increase in sequences and larger diversity of dominant OTUs in the F3 sample. A definitive turn-over in organisms from the F2 to F3 time point were seen, especially with the large decrease in Flavobacteriaceae and Enterobacteriaceae. The Flavobacteriaceae and Enterobacteriaceae can be soil or water borne organisms, however the majority of taxa present in higher abundance in the F3 sample tended to be more commonly found in soils. The least amount of precipitation was experienced in the month leading to the F3 time point, possibly explaining some of the changes in community composition to favour soil organisms over aquatic organisms.

Although most organisms existing in the field leach bins were chemoheterotrophs, chemolithotrophic organisms were of interest due to their role in ARD generation. Within this particular system, *Thiobacillus spp.* were the dominant OTUs identified in the initial 16S rRNA gene screening of unweathered rock. After weathering the waste rock in the field leach bins *Thiobacillus spp.* were present in low abundance in all samples but were more abundant in the F2 sample than the F3 sample, possibly due to resource competition from the larger number of organisms present in the F3 sample.

Microbial drivers in the weathering of waste rock

Metagenomic sequencing has been extensively used in mine waste environments (Dick et al., 2009; Kantor et al., 2015; Liljeqvist et al., 2015; Zhang et al., 2016), however this is the first glimpse of the microbial communities present in field leach bins and one of few studies conducted on PAG rock systems. The intent of assessing the genetic composition of the waste

rock microbial community was to determine the metabolic contribution of microorganisms to the weathering of waste rock, as well as to determine their capacity to function within the cooler boreal climate.

Given the number of neutrophilic and acidophilic heterotrophs present, the majority of energy metabolism genes were dedicated to carbon metabolism. Chemolithotrophic and phototrophic bacteria were also present in considerably lower abundance, however many of which provided genes to complete the reverse TCA cycle and Calvin Benson Bassham cycles to support carbon fixation.

Nitrogen cycling pathways for nitrogen fixation, nitrification, denitrification, and dissimilatory nitrate fixation were present within the genome. Despite ammonia oxidizing bacteria being present in high abundance, *amo* genes were not identified in the metagenome. We found that NO_3 was present in as high as 0.46mg/L in the leachate, while NO_2 and NH_3+NH_4 were undetectable using ICP. Many bacteria present in the metagenome were capable of denitrification, thus supporting the NO_3 concentrations present in leachate. However, chemical indicators supporting the presence of considerable nitrogen fixing microorganisms may not be represented as N_2 and gaseous NH_3 also may not be captured in the leachate.

Biological iron oxidation is an important component of sulfide mineral leaching, as iron oxidizing microorganisms can oxidize ferrous iron faster than abiotic mechanisms at low pH, leading to acid generation (Bacelar-Nicolau & Johnson, 1999; Nordstrom & Southam, 1997). Neutrophilic iron oxidizers were present in low abundance and while genes for iron acquisition

and transport were present, those for rusticyanin were not present. The high pH and presence of considerable organic carbon of the field leach bins do not provide an optimal environments for acidophilic iron oxidizers, explaining their low abundance in the field leach bins. At this stage in waste rock weathering, the lack of iron oxidizing bacteria suggests that abiotic oxidation is the primary source of iron oxidation occurring in the humidity cell systems. However, as organic carbon is toxic to many chemolithotrophs (Bacelar-Nicolau & Johnson, 1999), overburden in the waste rock sampled for field leach bin construction may affected the chemolithotrophic microbial communities present as the geochemistry of the system was developing. Future microbiological studies of mining waste rock, especially those in which NP is low, should closely monitor the development of the microbial community over time to determine the dynamics of heterotrophs and Fe/S cycling microbes in waste rock weathering.

Sulfur oxidation is the major concern of sulfidic waste rock weathering which sulfur and iron oxidizing microorganisms play a role in. Acidophilic and neutrophilic sulfur oxidizers were present in very low abundance, as the most prominent sulfur oxidizer present in the 16S rRNA gene amplicon dataset, the Hydrogenophiliales were found in 0.63% F2 sample and 0.28% abundance in the F3 sample. This suggests a shift in the community to a more heterotrophic, neutrophilic community once weathering of the rock occurs, as the Hydrogenophiliales alone represented greater than 30% of the community of the unweathered rock sample. RISC oxidization pathways were fully present and best characterized in *Thiobacillus spp*., suggesting that some sulfur oxidation within the system is attributable to bacterial metabolism. The *Thiobacillus spp*. present in the metagenome was identified as *Thiobacillus denitrificans* whereas the OTUs in the 16S rRNA gene sequencing approach were also identified as *Thiobacillus*

thioparus. *Thiobacillus thioparus* is strictly a sulfur oxidizer despite it carrying *nar*, *nir*, *nor* and *nos* genes for anaerobic denitrification it is not capable of doing so as an alternative form of respiration like *Thiobacillus denitrificans* (Hutt et al., 2017). Sulfur reducing bacteria, many of which are anaerobes, were also present in low abundance. Dissimilatory sulfate reductase complex genes were also present, suggesting the opportunity to reduce sulfate in small anaerobic pockets within the waste rock profile and with accessible sulfate (Muyzer, 2014).

The sum of the metabolic and taxonomic data regarding sulfur cycling is in agreement with the geochemical data, presenting a neutrophilic community of sulfur oxidizers with some sulfate reduction occurring in anaerobic pockets supported by available organic carbon. The low abundance of iron oxidizing bacteria suggests that sulfur oxidizing bacteria are the main driver of sulfidic rock weathering in the field leach bins. If NP continues to be higher than sulfur oxidation , the waste rock is not expected to produce ARD. However, should NP release slow to a point where it is overtaken by sulfide weathering biological sulfur oxidation may increase with the increase in accessible S species and acidity. The acid generated from the accelerated abiotic and biotic sulfide oxidation reactions should provide a more amenable environment to the limited iron oxidizers present in the sample, such as *Acidithiobacillus spp.* and potentially some of the unknown Betaproteobacteria (Bellenberg et al., 2015; Méndez-García et al., 2015). These iron oxidizing organisms will not only compliment the metabolism of acidophilic sulfur oxidizing bacteria, but they will also continue to maintain the rate limiting step of sulfide mineral oxidation. In effect, although there may not currently be the potential to generate substantial acid from the current microbiology and mineralogy of the system, microbial metabolic potential is present to trigger acid generation given appropriate environmental conditions.

Stress responses

This field leach bin experiment presented some unique challenges to microorganisms regarding the environmental conditions they must be capable of surviving in, notably: cold and warm shock, oxidative stress, osmotic stress, and potentially acid and metal stress. Microorganisms living within mine wastes have been well documented to contain genes to guard themselves from toxic metal concentrations and acidic conditions that can cause oxidative, periplasmic, and osmotic stress, and biofilm formation, which were also found in our metagenomes (Chen et al., 2014; Dopson et al., 2014). These same genes could influence microbial cells' ability to obtain nutrients and maintain their cellular environment when challenged by seasonal fluxes in precipitation and temperature (Shimizu, 2013). In addition to the presence of many genes to support osmotic stress, the high internal humidity of the field bins and the lack of desiccation stress genes found in the metagenome also suggest that a considerable amount of moisture is retained in the field bins between precipitation events.

The ability of microorganisms to tolerate the cool site temperatures experienced in late fall- spring were of particular interest, as many sulfide oxidizing organisms are mesophilic or thermophilic in nature (Ghosh & Dam, 2009). Cold shock genes were found in the F3 metagenome, which was sampled in mid- November when the average monthly temperature was less than 10ºC and evening temperatures fell below freezing. Of the cold shock responses found, trehalose uptake and utilization, as well as cold shock proteins were highly abundant. Cold shock proteins are thought to be involved in DNA supercoiling under cold stress which are often accompanied by the intracellular solute trehalose (Horn, Hofweber, Kremer, & Kalbitzer, 2007).

Cold shock proteins can be expressed in situations where cold stress or cold acclimation are occurring (Berger et al., 1996; Inouye, 1997), however it is difficult to determine how microorganisms were behaving at the time of the F3 sampling without additional culture and transcriptomic data. Organisms that were present in F2 samples and initial waste rock samples, such as *Thiobacillus spp.*, suggesting that they persisted through all seasons in the field. Future studies will assess how such organisms of interest respond to seasonal temperature differences by assessing their abundance and regulation of cold shock genes with transcriptomic methods.

3.6 Conclusions

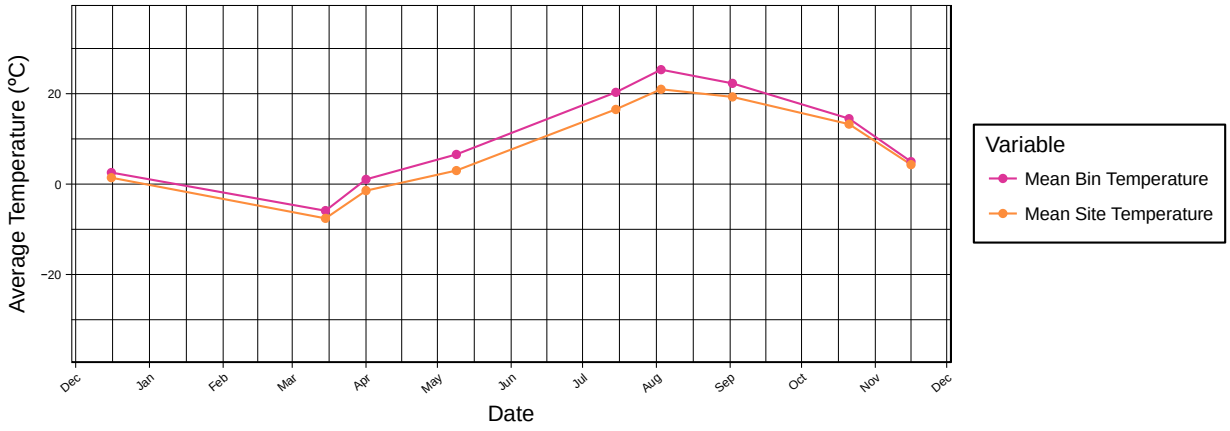
This preliminary experiment investigated the weathering of low sulfur PAG waste rock as it weathered in a boreal climate in field leach bins. Unique to this experiment was an experimental approach that used geochemistry, mineralogy, and microbial ecology to examine how the PAG rock system was functioning as a whole.

The field leach bin experiment ran for 49 weeks, spanning from December to November, during which time seasonal temperature influenced the rate at which elements were released from the waste rock. Some elements exceeded PWQO guidelines which will need to be monitored into the future including: SO_4, Al, Ni, Zn, and Cd. Weathering of the rock composed primarily of carbonate and aluminosilicates was observed, with considerable depletion of dissolving and intermediate weathering minerals. Sulfide oxidation occurred, but was largely masked by the considerable NP available in the waste rock as the pH of the system remained circumneutral throughout the 11 month period. This was supported by the dominance of neutrophilic chemoheterotrophic bacteria present in the microbial communities of the F2 and F3

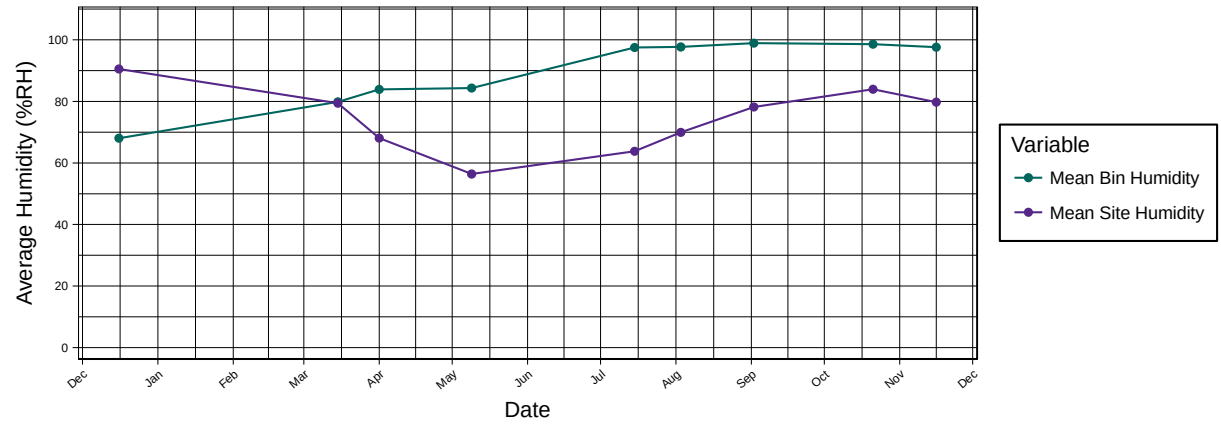
time points. Chemolithotrophic taxa and genes to support RISC oxidation, dissimilatory sulfate reduction, and neutrophilic iron oxidation were present in extremely low abundance in both time point samples, with most taxa being neutrophiles. However, the acidophilic sulfur oxidizers of the genus *Thiobacillus* continued to persist in the field bins after being the dominant taxa in the unweathered waste rock. Additionally, genes to survive stressors relevant to a cold temperature mining environment were abundant in the microbial community. As of the end of the experiment, calculations of NP and sulfur oxidation suggest that ARD will not occur. However, the microbial community contains the appropriate membership and metabolic potential to shift the system into ARD generation should abiotic sulfur oxidation increase and NP release decrease. In effect, this experiment has demonstrated the relevance of incorporating data on microbiological communities found within field leach bin experiments to provide a more complete forecast of the weathering and ARD generation of PAG waste rock.

A **B**

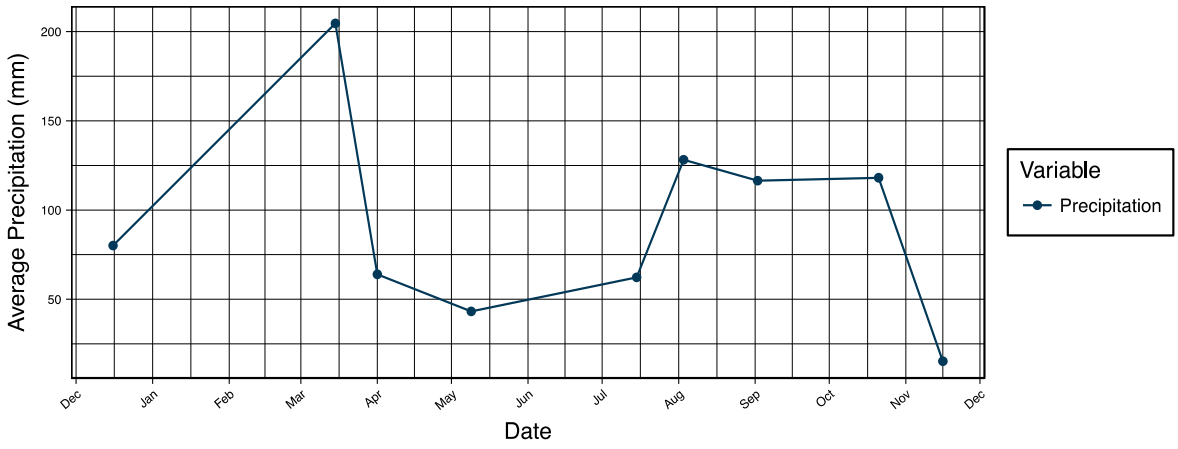
<u>Figure 3.1:</u> A) Side profile view of field leach bins; B) Top view of field leach bin with microbiology sampling bags and Hygrochron data logger removed from PVC pipe for sampling.



A

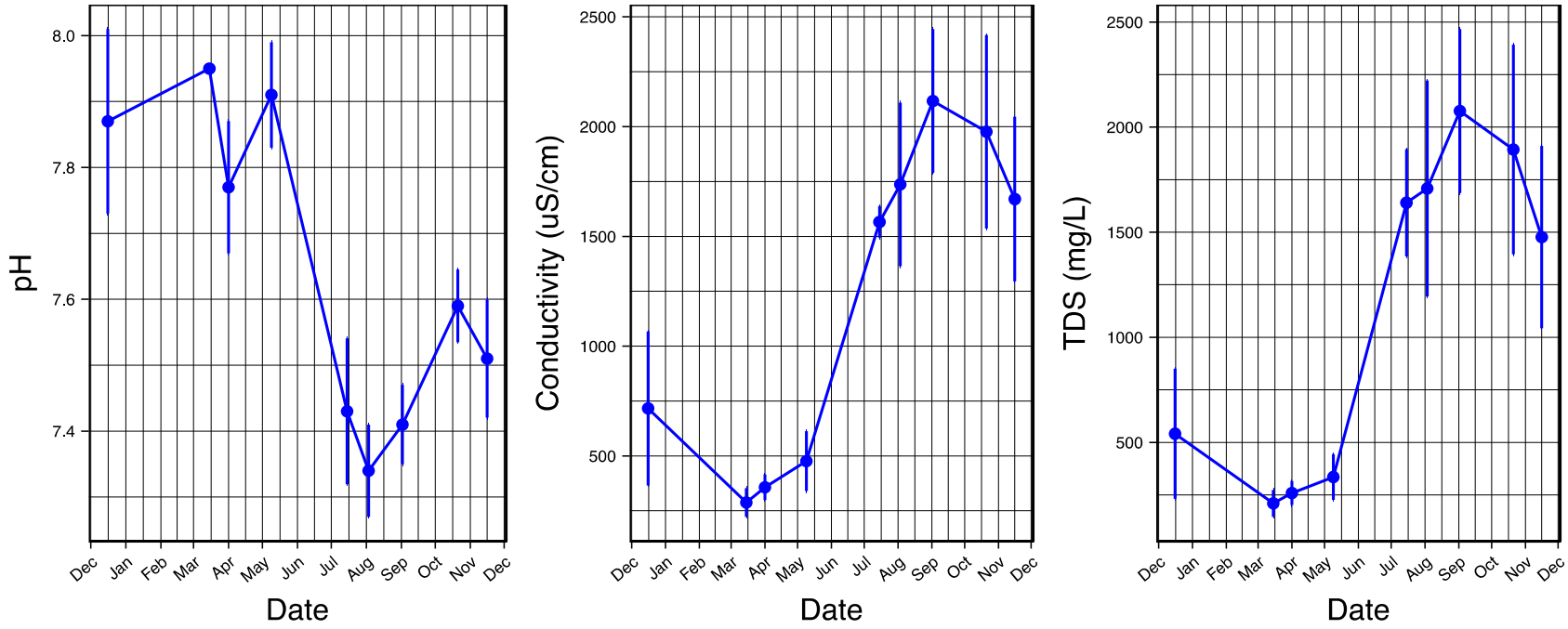


B



C

<u>Figure 3.2:</u> Mean site and field bin temperature (A) and humidity (B), and mean site precipitation (C) between sampling dates.



A **B** **C**

<u>Figure 3.3:</u> Weekly means of pH (A), Conductivity (B), and TDS (C). Error bars represent standard deviation from the mean at each sampling point.

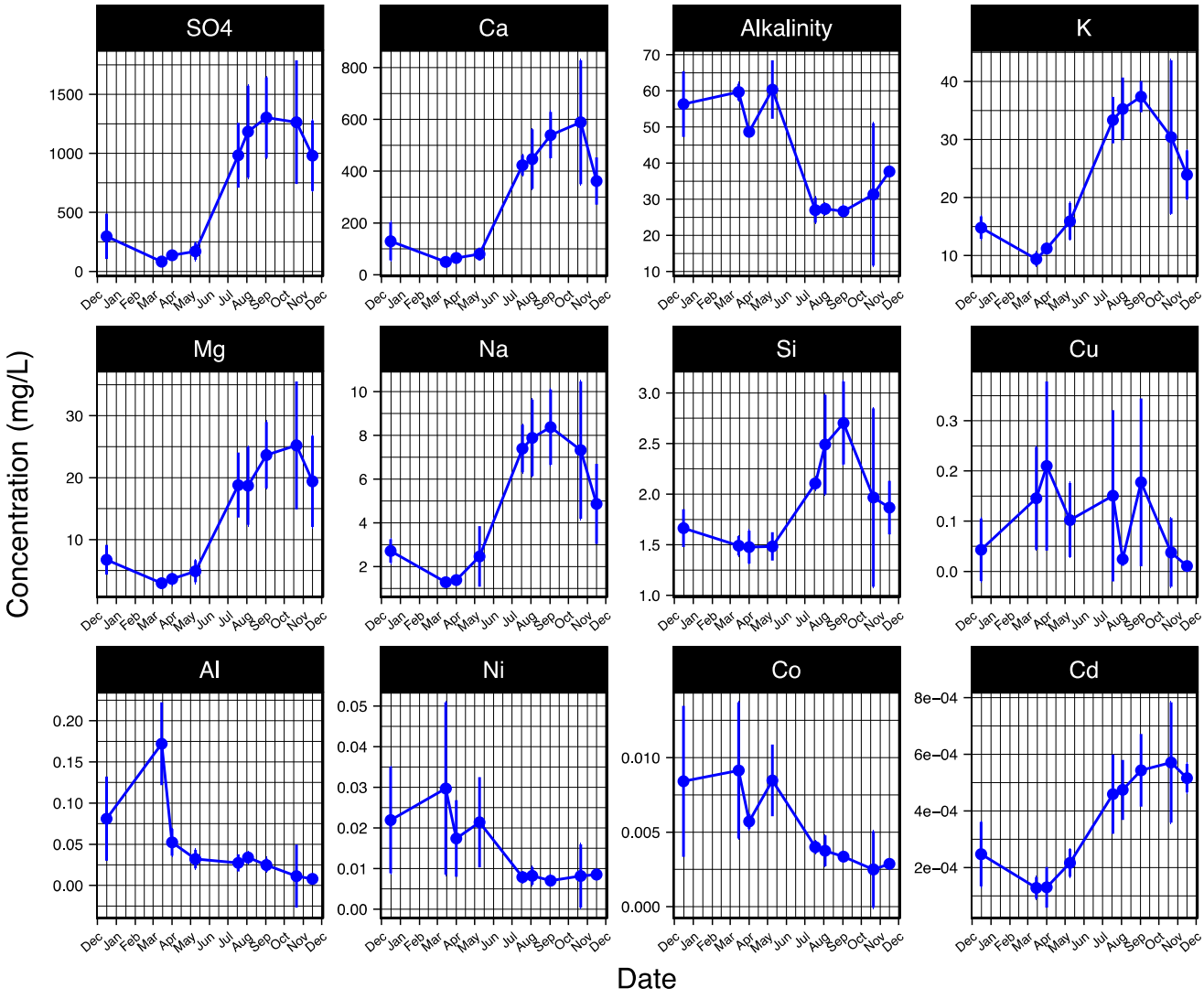
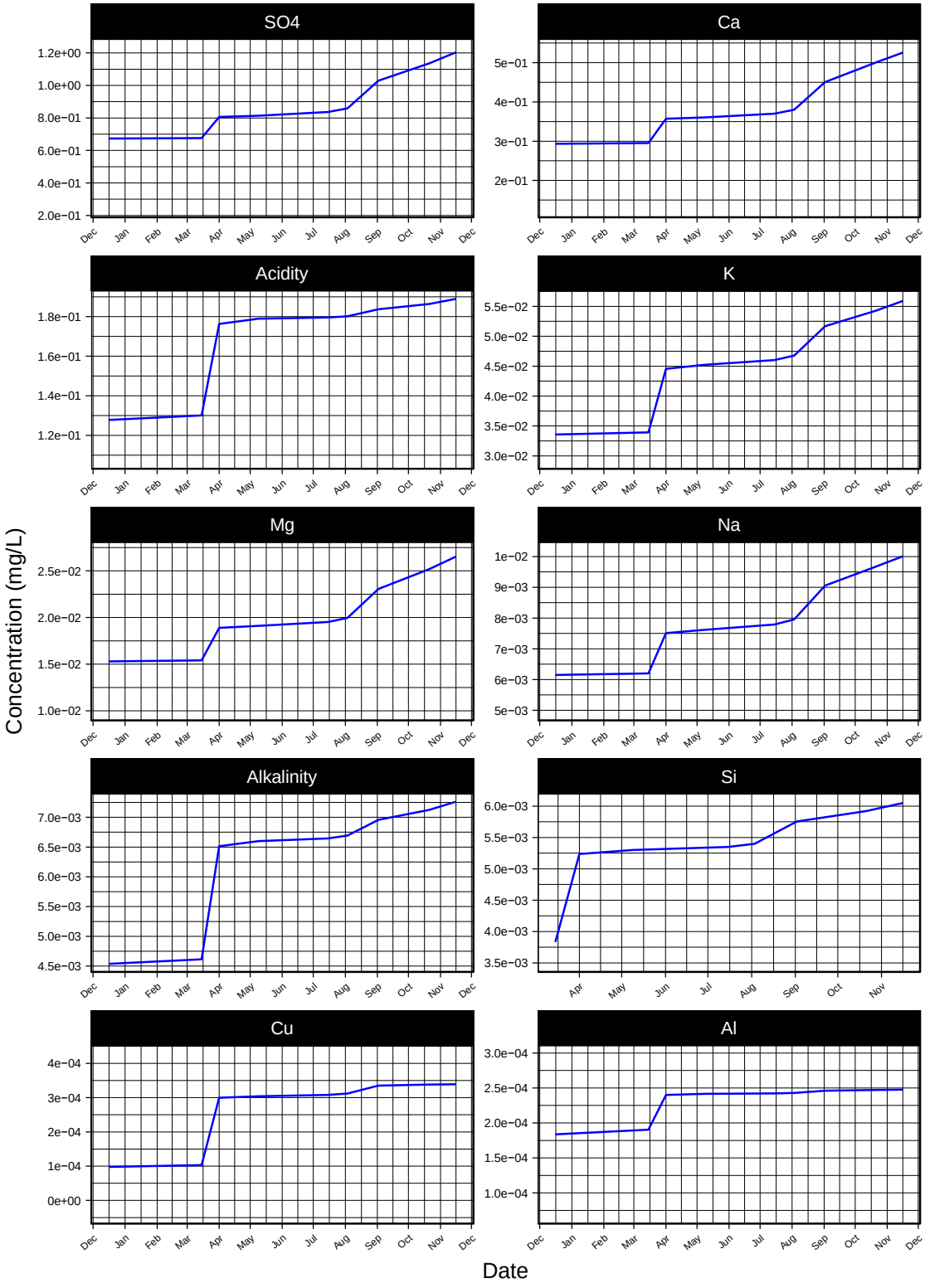
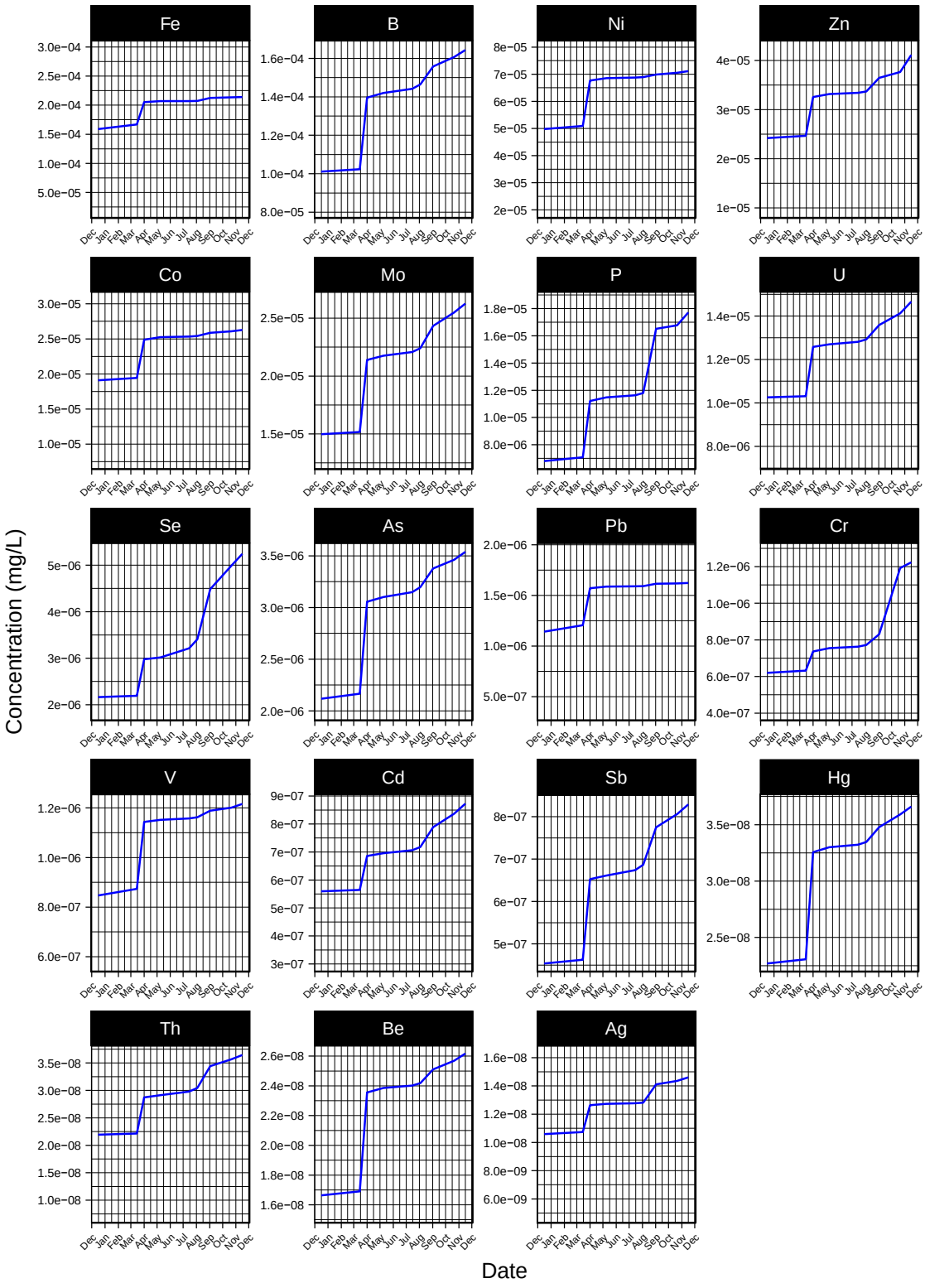


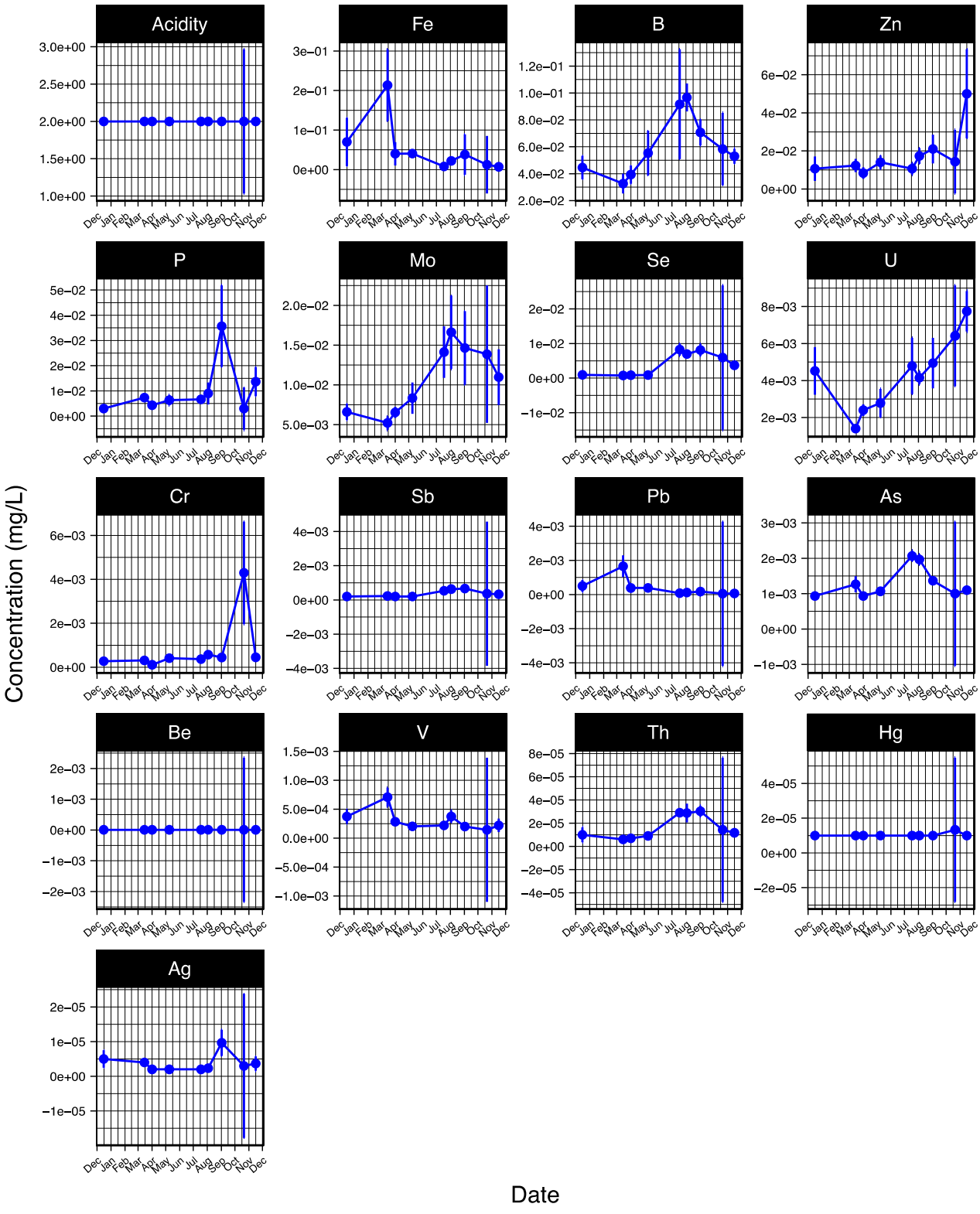
Figure 3.4: Weekly means of analyte concentrations present in high concentration. Error bars represent the standard deviation of concentration at sampling points.



<u>Figure 3.5:</u> Mean cumulative leachate concentrations of analytes found in high concentration.



<u>Figure 3.6:</u> Mean cumulative leachate concentrations of analytes found in low concentration.



<u>Figure 3.7:</u> Weekly means of analyte concentrations present in low concentration. Error bars represent the standard deviation of concentration at sampling points.

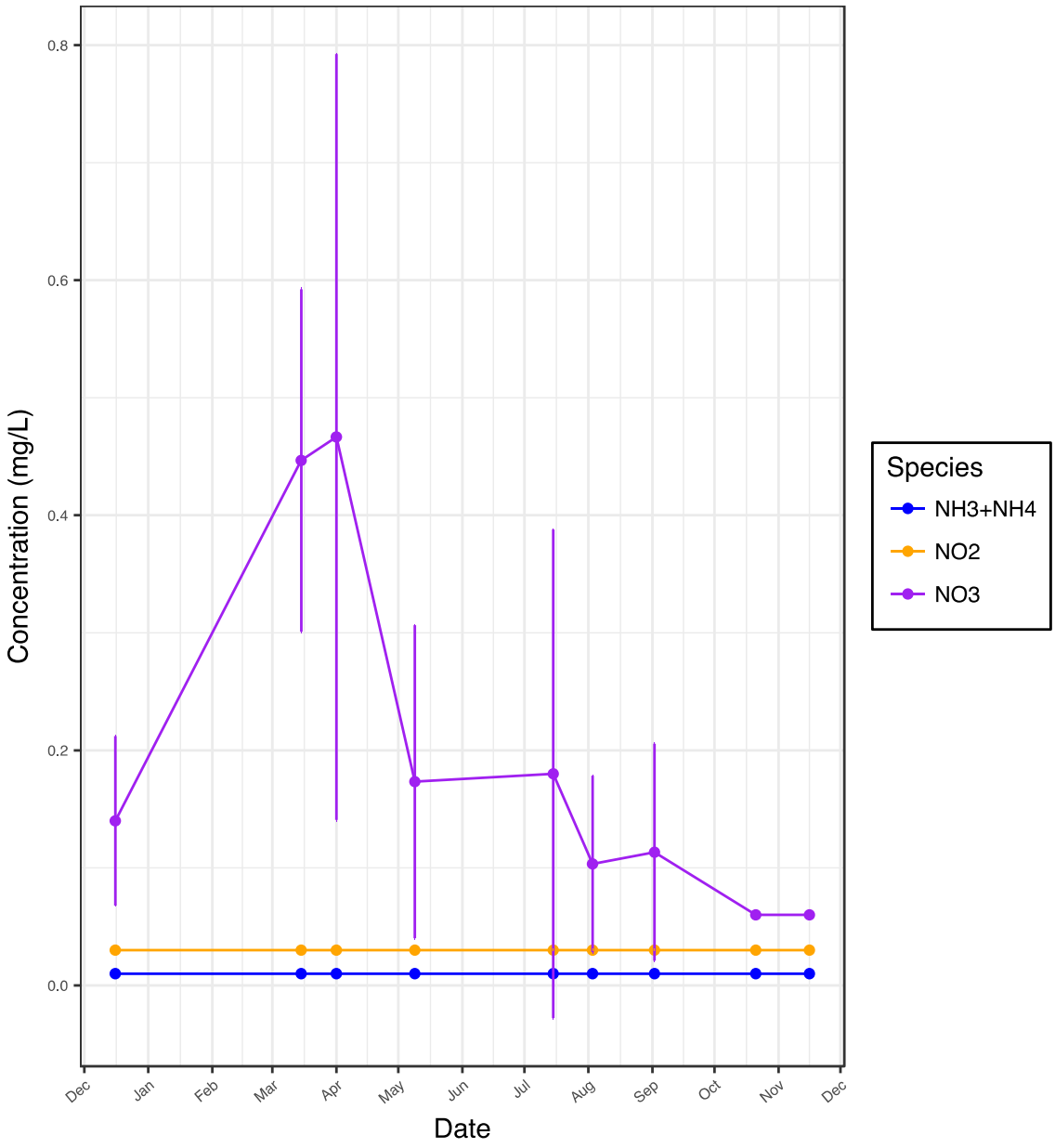


Figure 3.8: Mean concentration of N species.

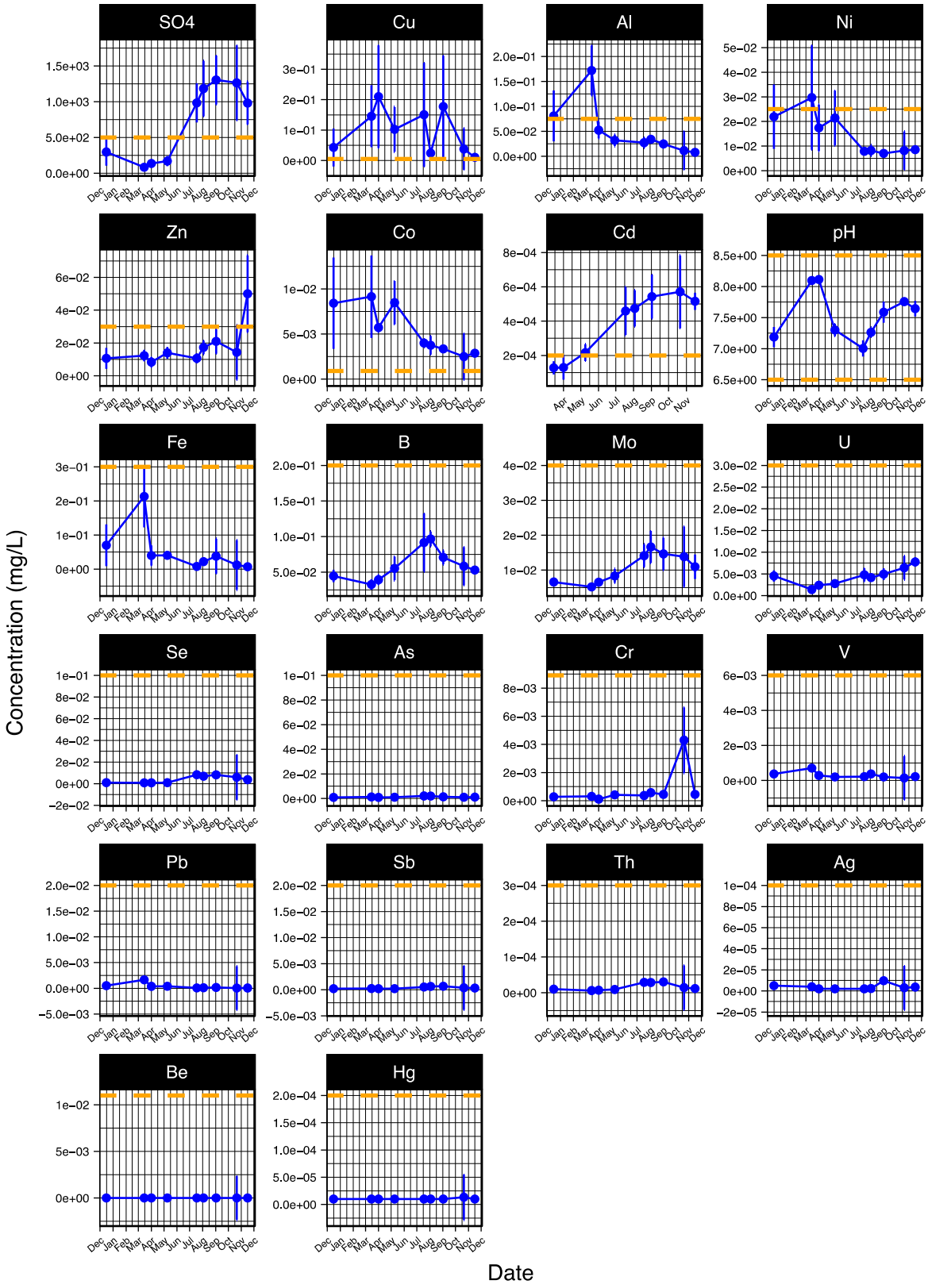
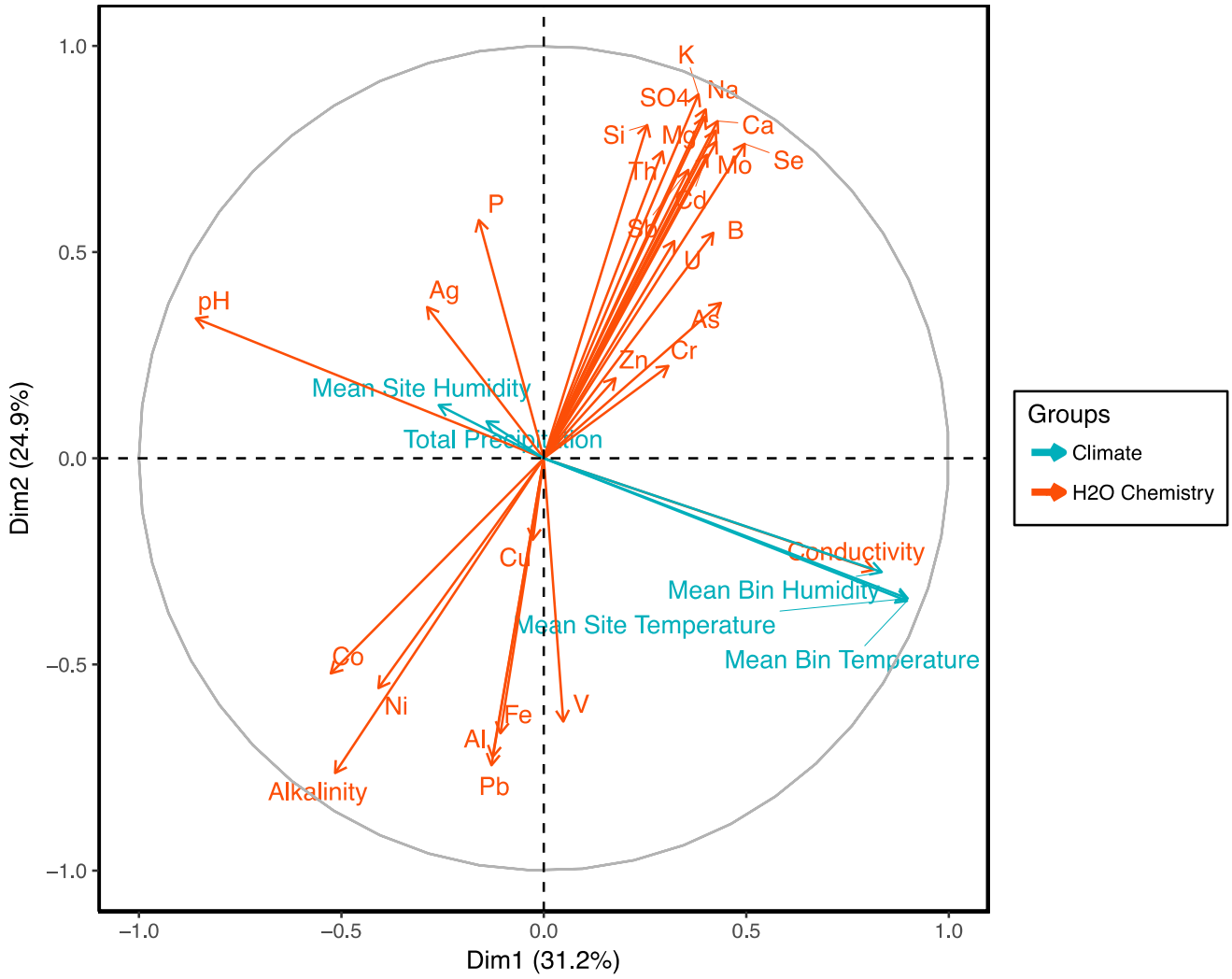


Figure 3.9: Mean weekly element concentrations with respect to PWQO limits.



<u>Figure 3.10:</u> Multiple factor analysis of leachate chemistry and climatic variables explaining 56.1% of the total variance.

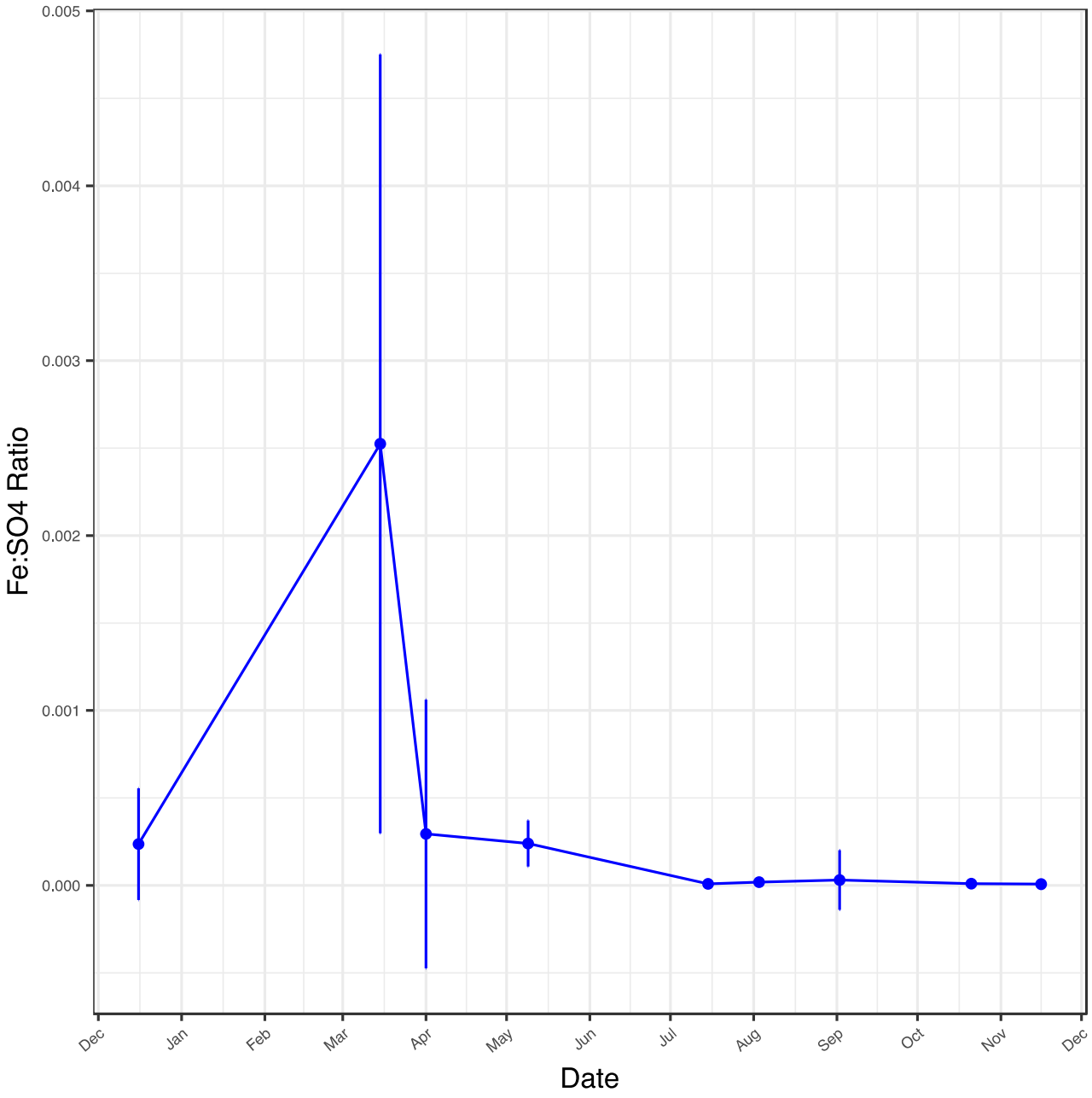
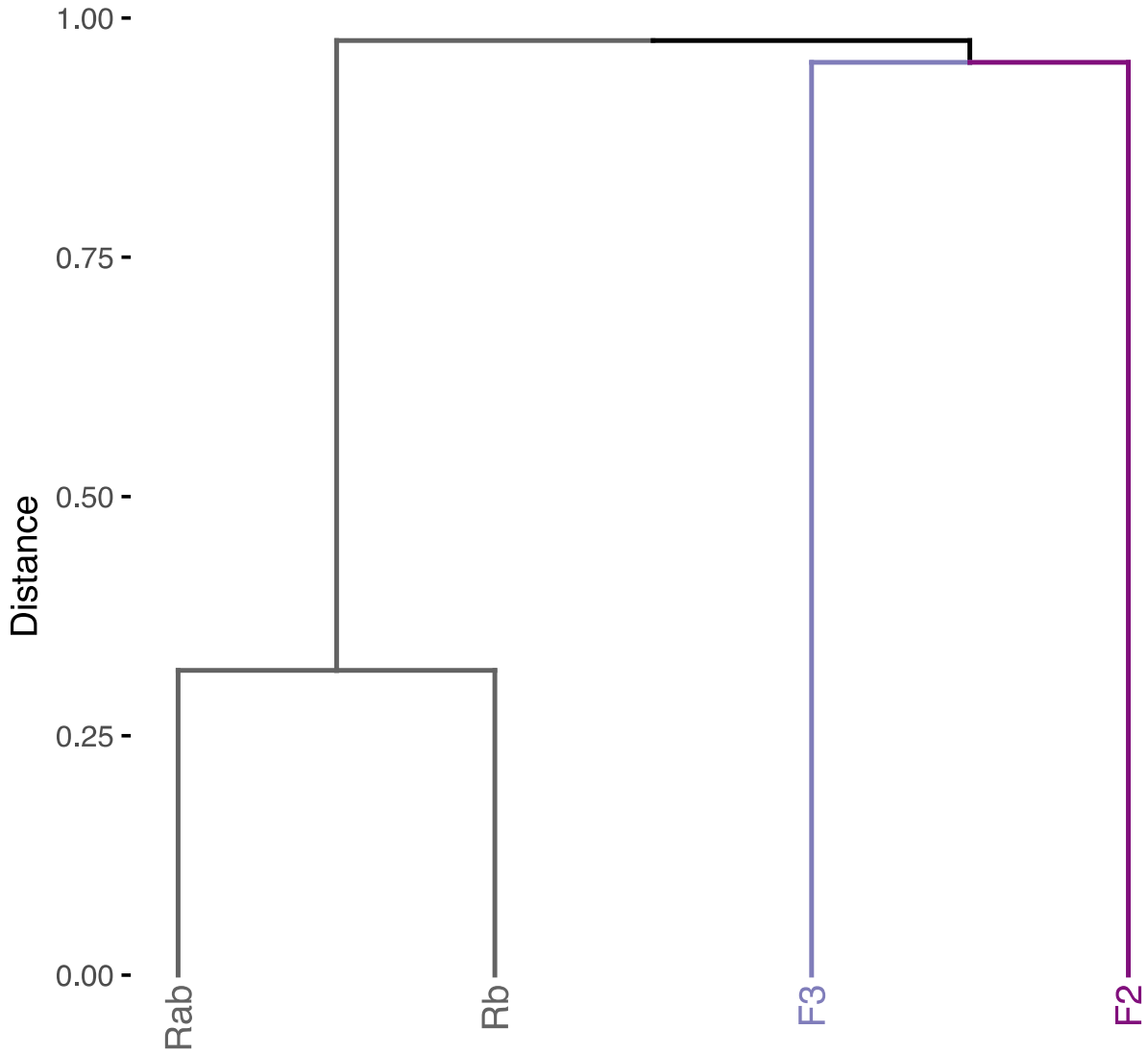


Figure 3.11: Molar ratio of Fe: SO4 over the course of the field leach bin experiment.



<u>Figure 3.12:</u> Bray Curtis cluster analysis of F2 and F3 samples from the unweathered waste rock (Ra and Rb).

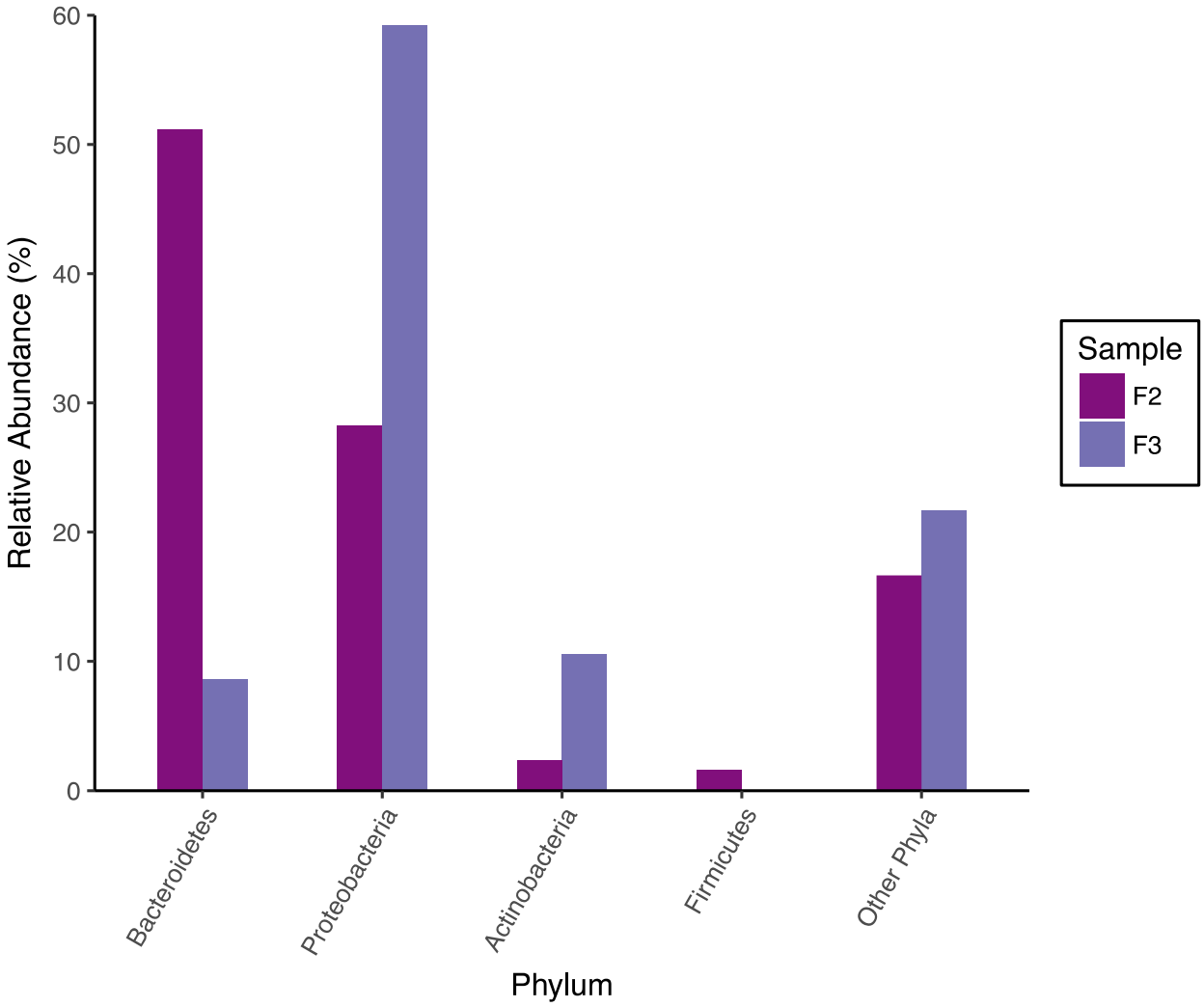
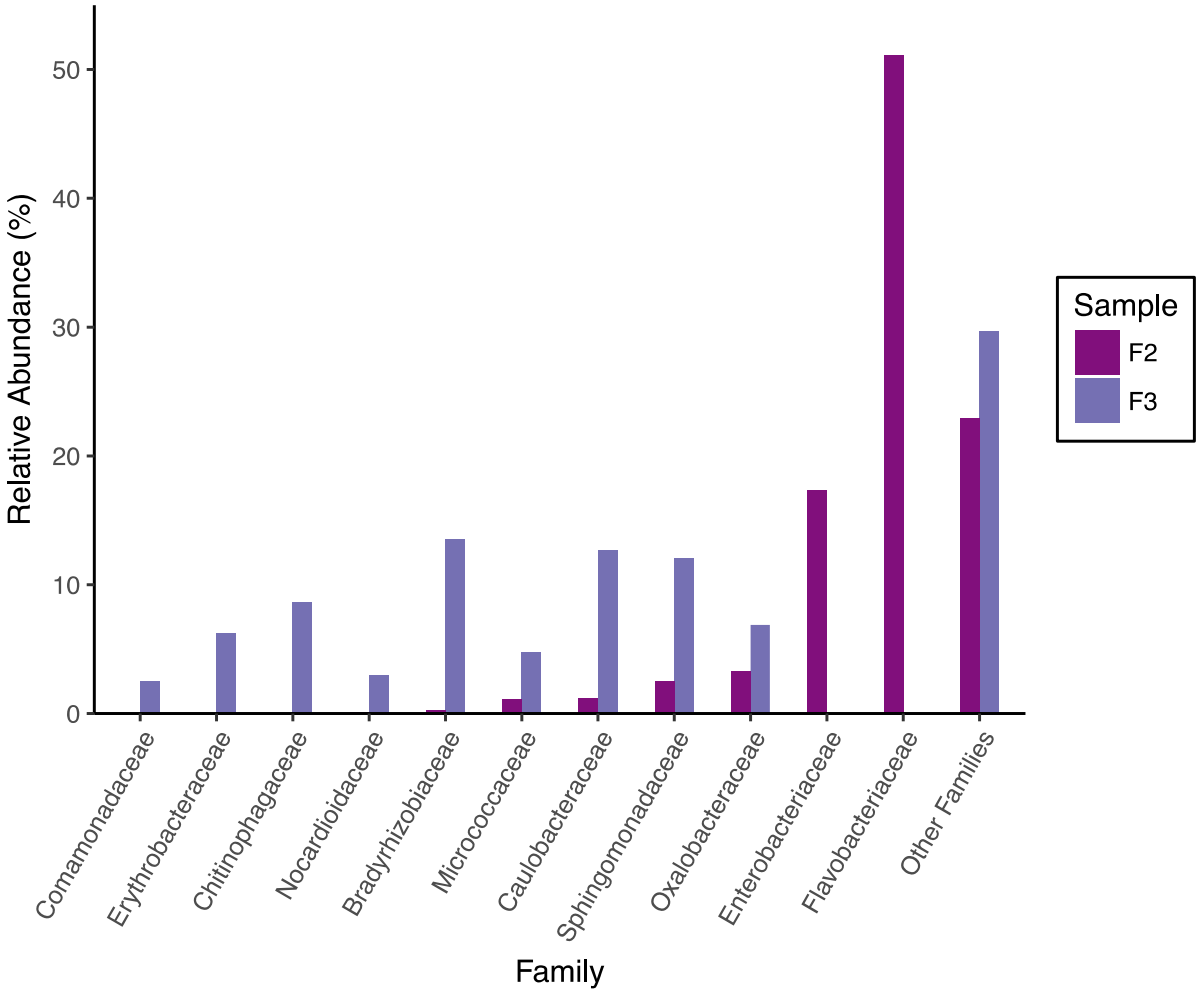
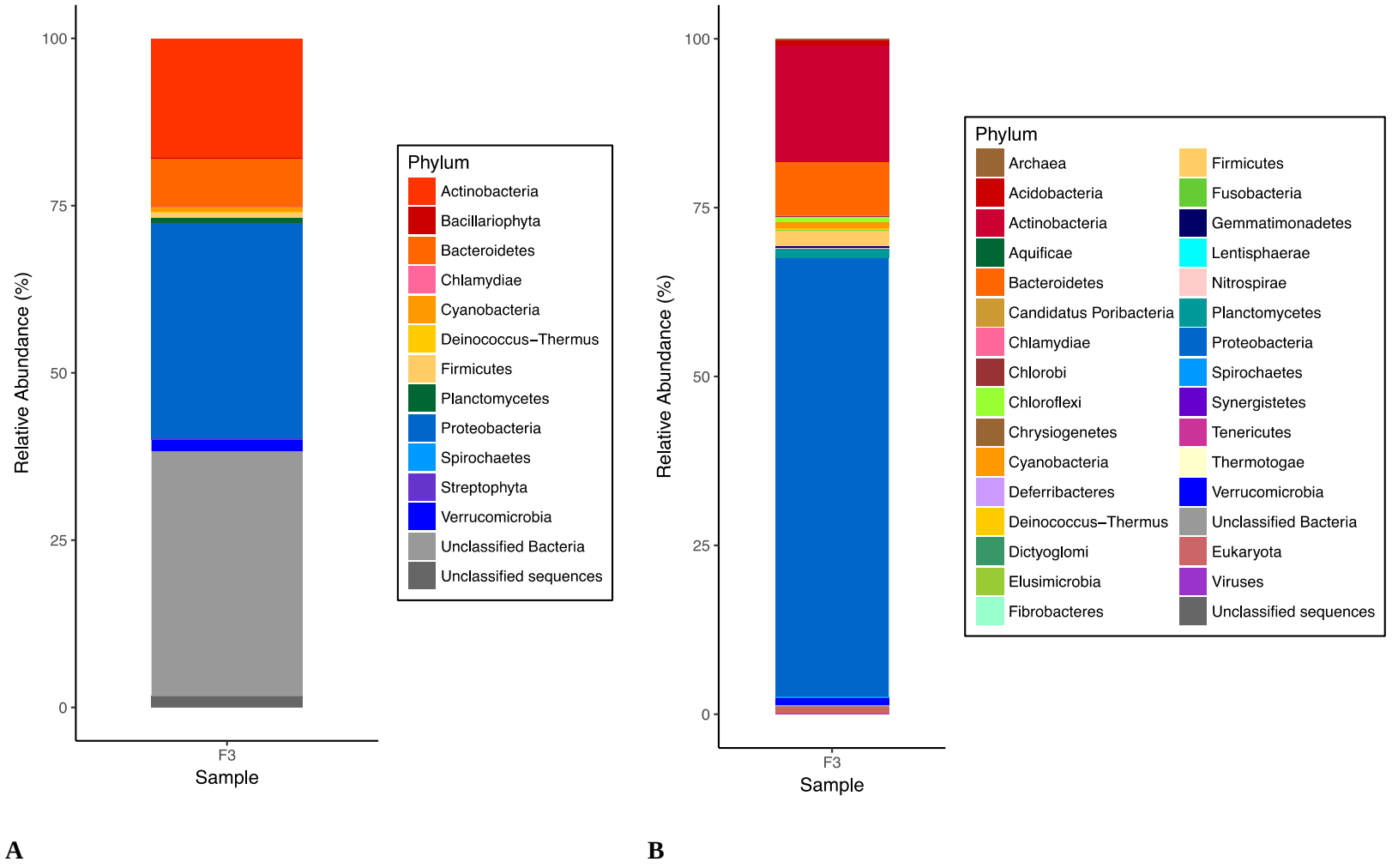


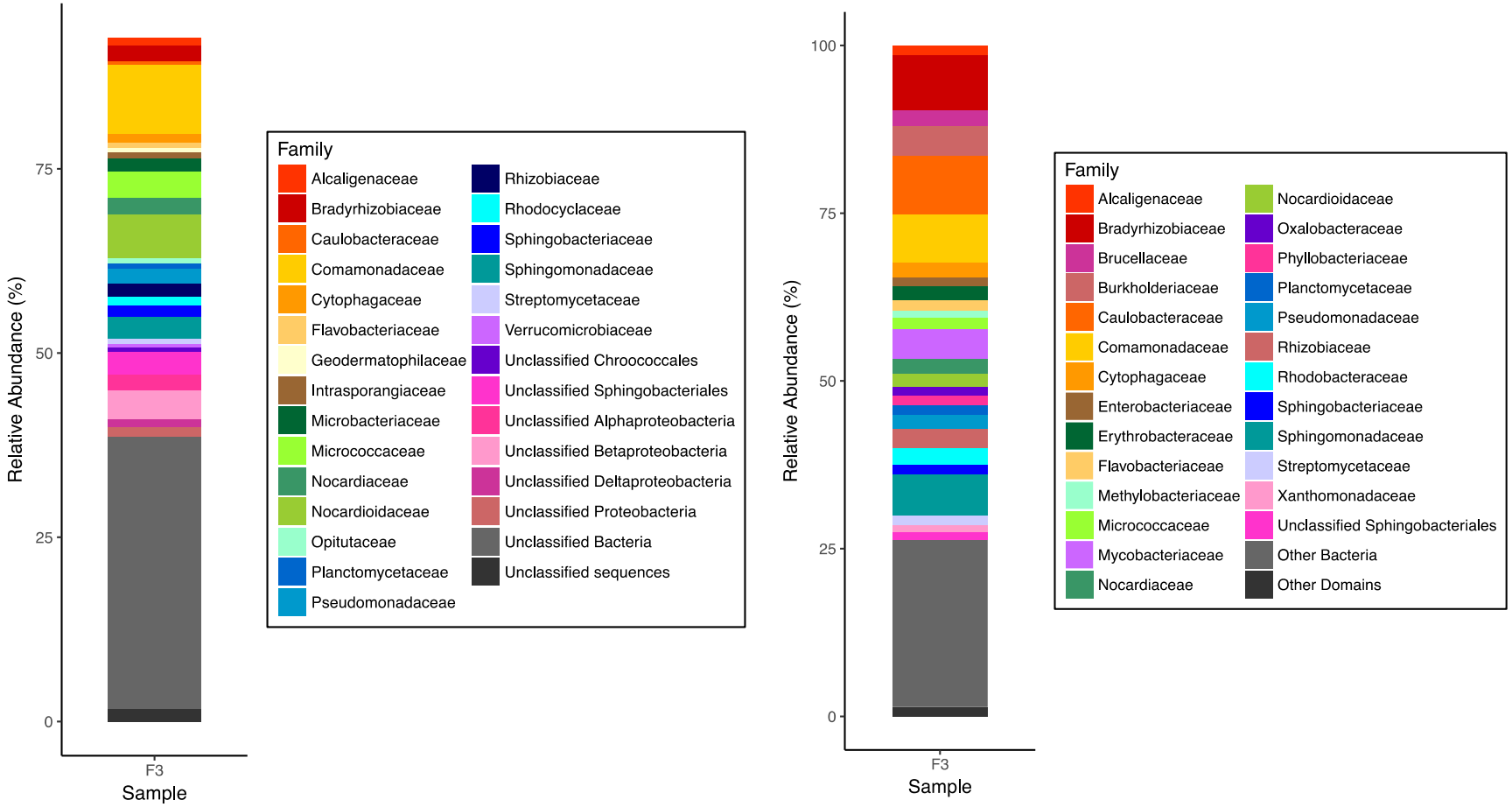
Figure 3.13: Bacterial phyla present in > 1% relative abundance in weeks 273 and 348 of the experiment determined by 16S rRNA amplicon sequencing.



<u>Figure 3.14:</u> Bacterial families present in > 2% relative abundance in weeks 273 and 348 of the experiment determined by 16S rRNA sequencing.

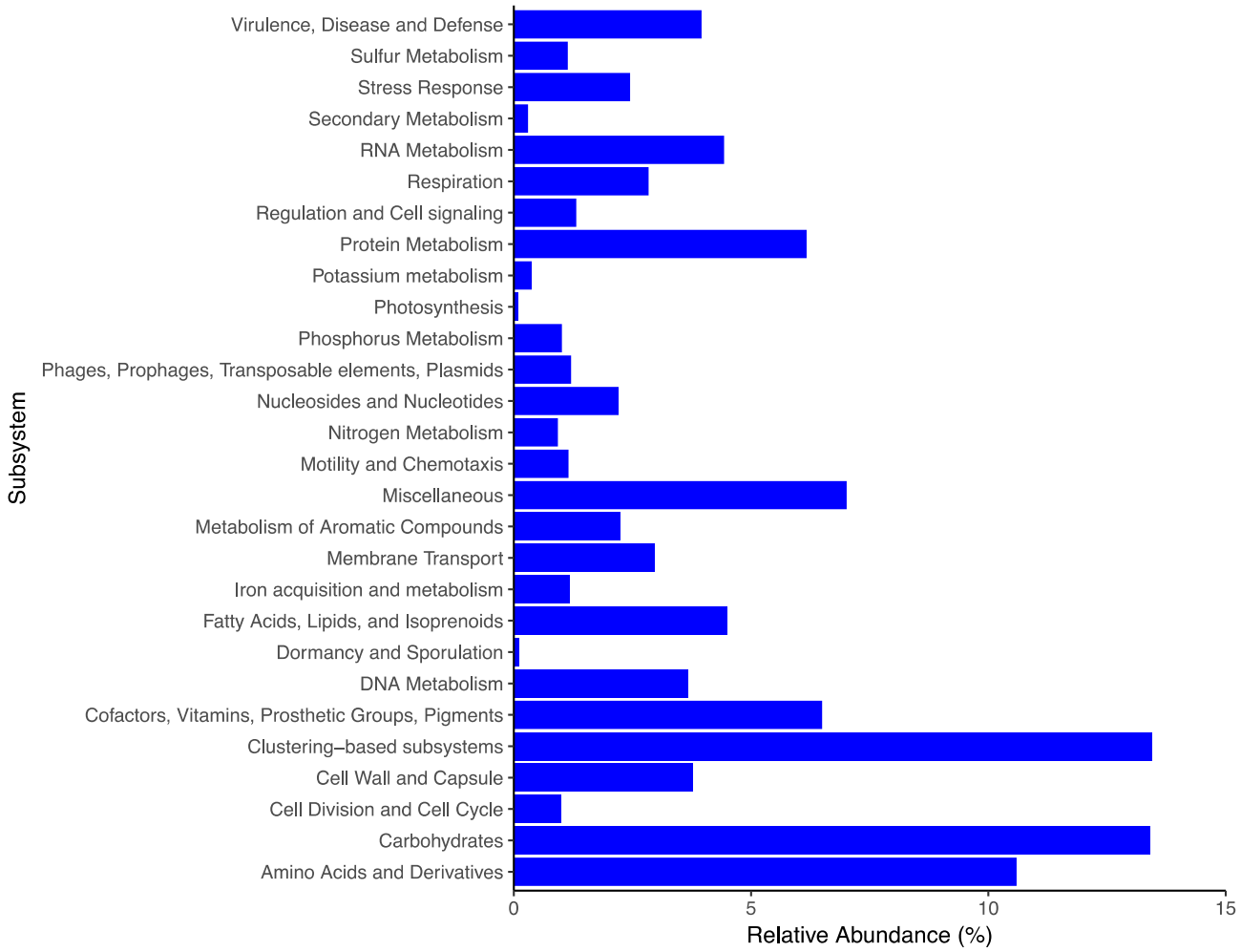


<u>Figure 3.15:</u> Microbial communities determined by metagenomic sequencing at the Phylum level by Greengenes and Refseq databases.

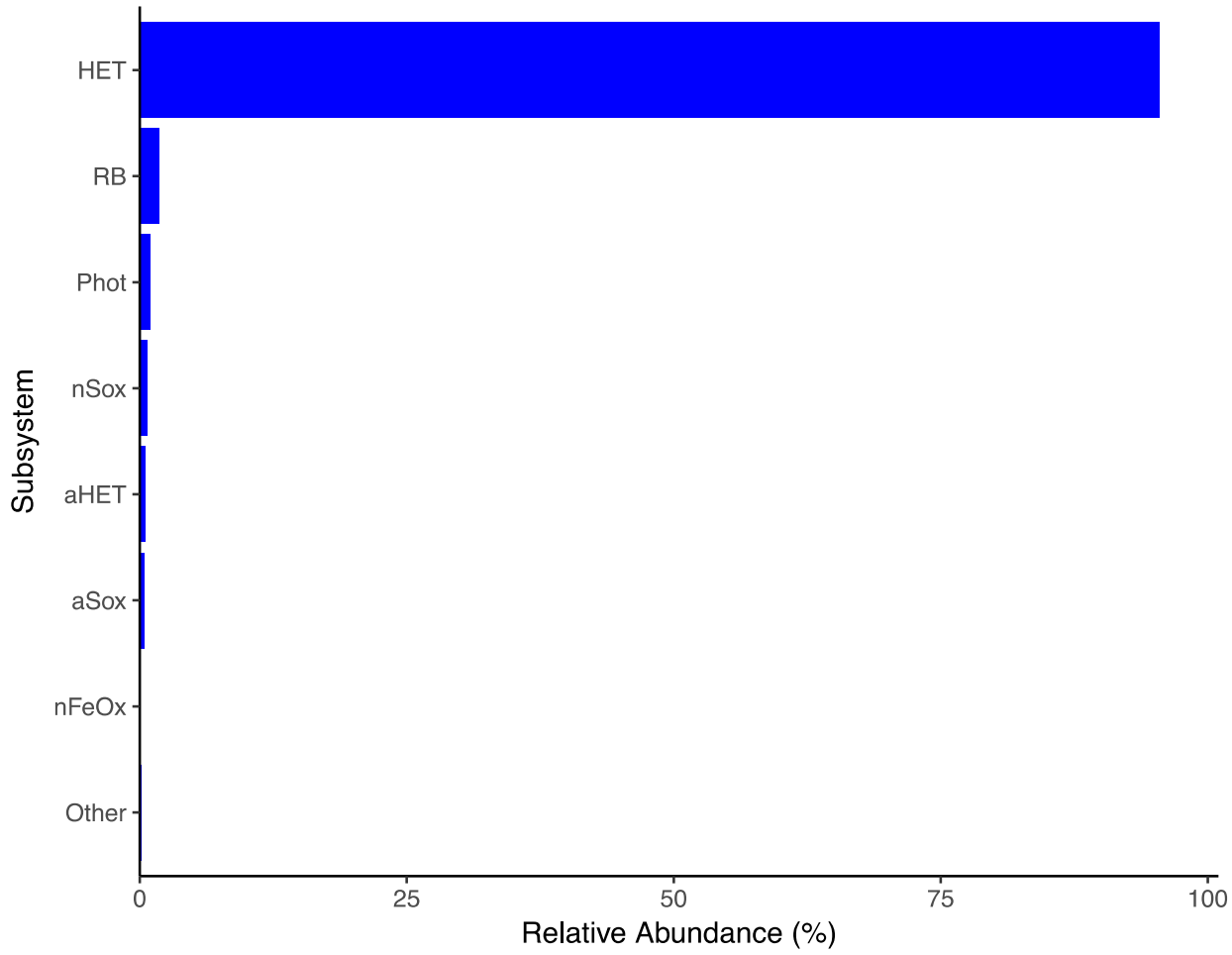


A **B**

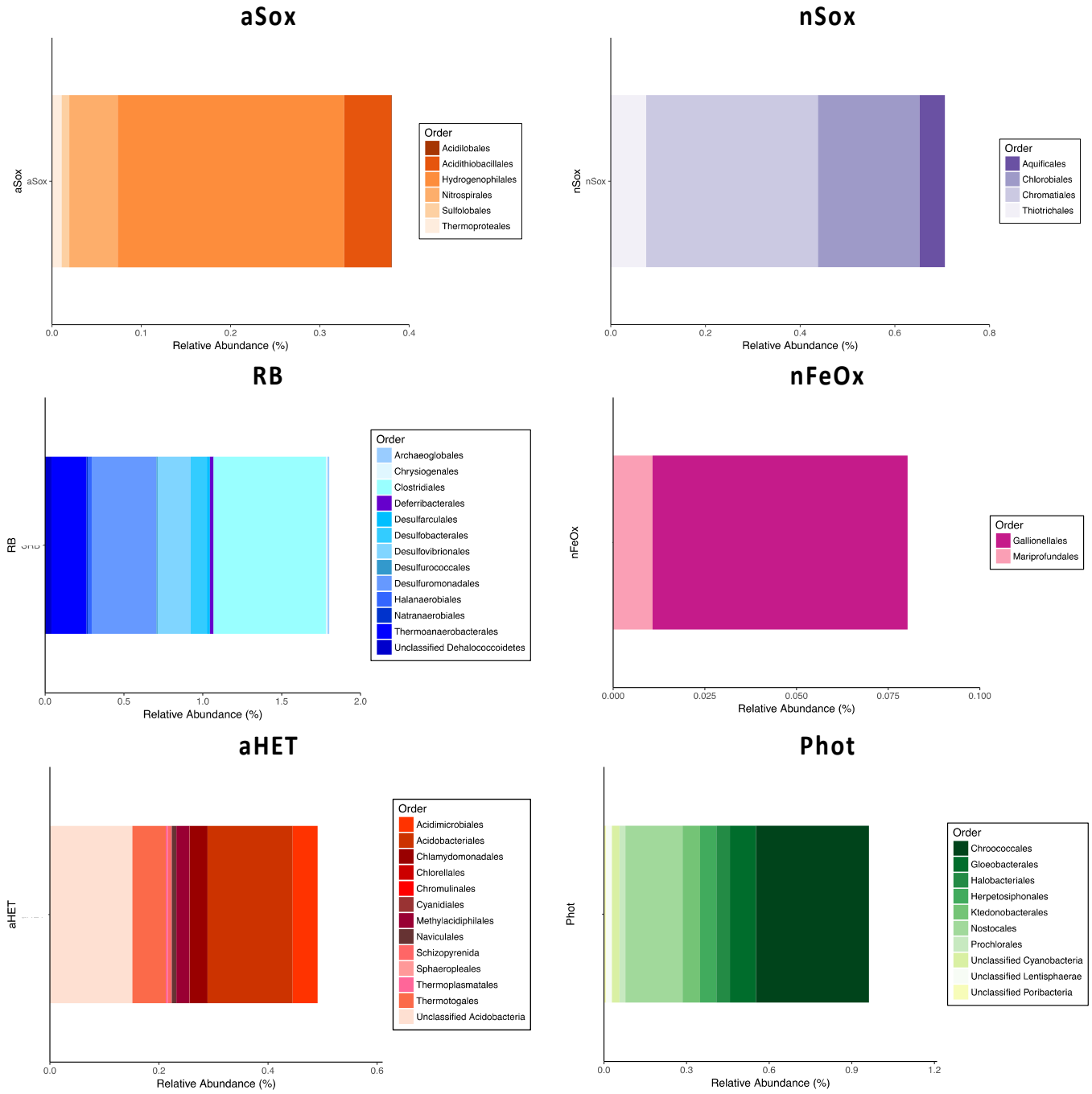
<u>Figure 3.16:</u> Microbial communities present in > 1% relative abundance determined by metagenomic sequencing at the Family level by Greengenes and Refseq databases.



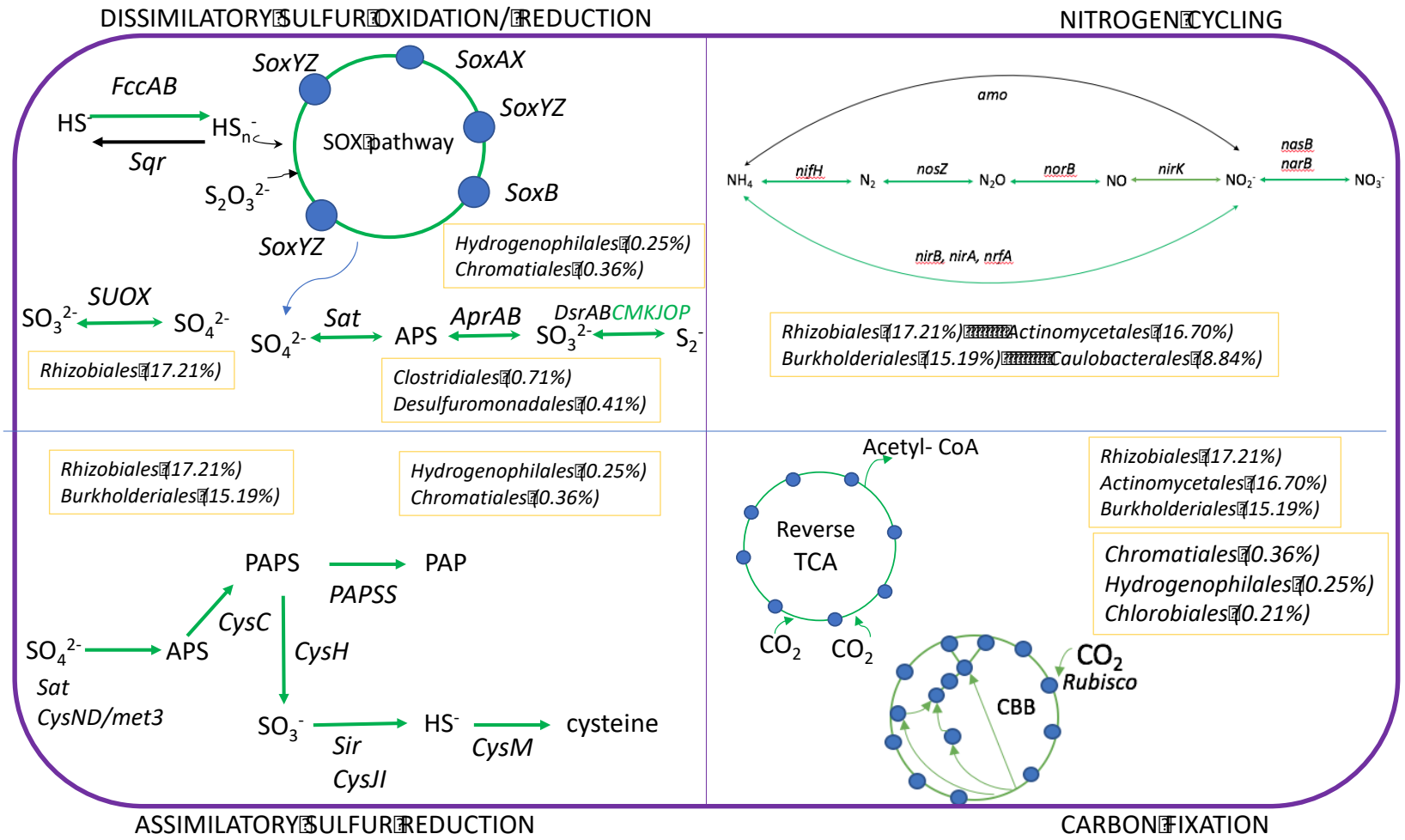
<u>Figure 3.17:</u> Relative abundance of COGs present in the F3 sample.



<u>Figure 3.18:</u> Relative abundance of microorganisms by dominant energy metabolism in the F3 sample.



<u>Figure 3.19:</u> Relative abundance of taxa in the F3 sample classified to order. Taxa are grouped by their dominant energetic designation: Acidophilic sulfur oxidizers (aSox), neutrophilic sulfur oxidizers (nSox), iron and sulfur reducers (RB), neutrophilic iron oxidizers (nFeOx), acidophilic heterotrophs (aHet), and phototrophs (Phot).



<u>Figure 3.20:</u> Prokaryotic sulfur oxidation and reduction, nitrogen cycling, and carbon fixation pathways in the F3 sample.

Green arrows represent pathways represent in the metagenomes and black arrows represent missing pathways.

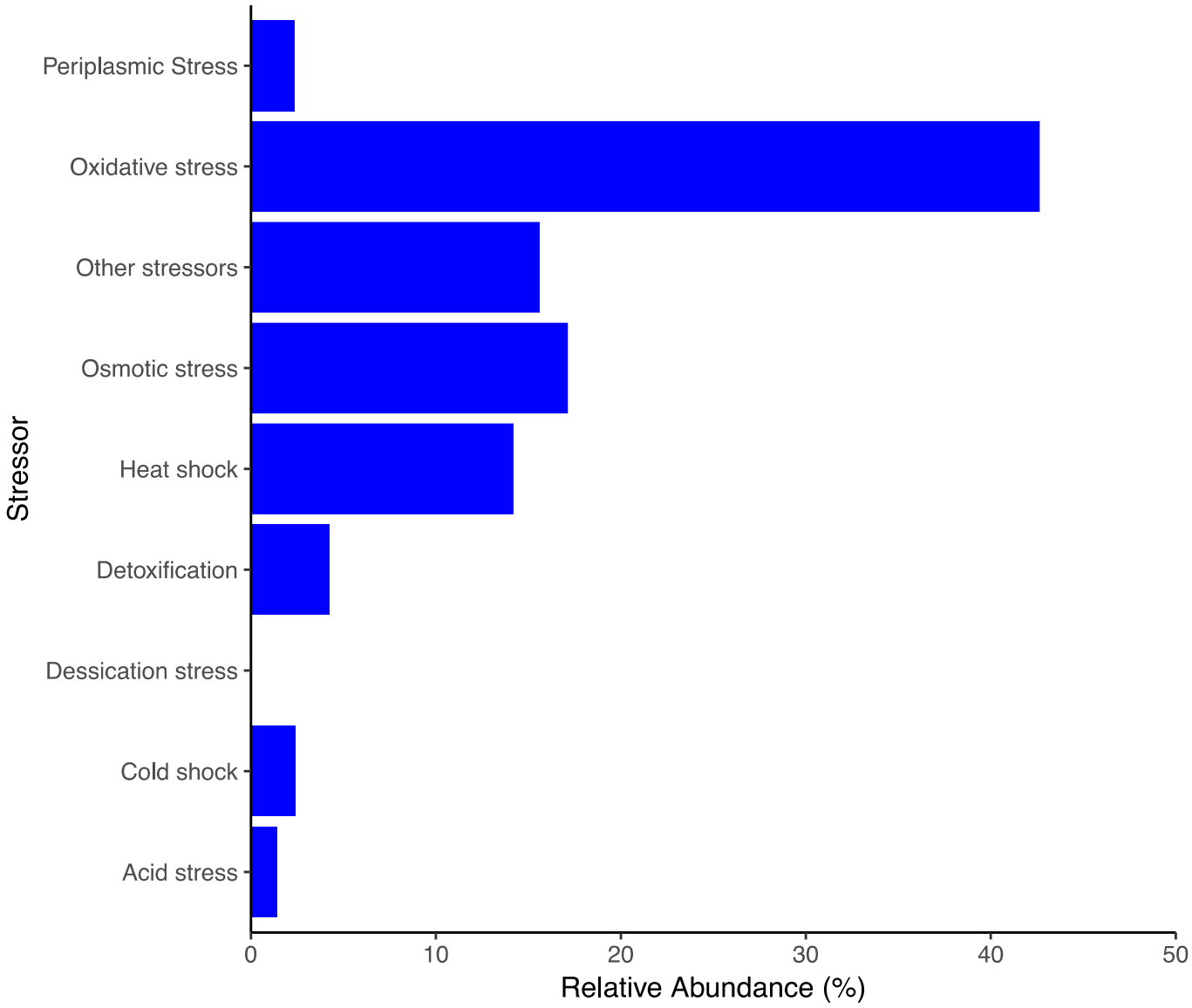


Figure 3.21: Relative abundance of stress related genes in the F3 sample.

Table 3.1: Linear mixed effects model results by analyte for the fixed effect equation (Analyte ~ Temperature + Day) and random effect equation (~1|Bin).

Analyte	Fixed Effect	LME				ANOVA	
		Value	Std. Error	t-value	P-value	F-value	P-value
SO_4	Temperature	32.03	5.80	5.52	0.000	102.35	<1.00e-4
	Day	2.18	0.53	4.13	0.0004	17.02	4.00e-4
Ag	Temperature	6.20e-08	7.16e-08	0.870	0.4	0.97	0.34
	Day	-9.18e-10	6.51e-09	-0.14	0.89	0.02	0.89
Al	Temperature	-2.37e-3	0.01	-.016	0.87	17.93	3.00e-4
	Day	-6.98e-3	1.32e-3	-5.26	0.00	27.68	<1.00e-4
As	Temperature	9.93e-3	2.68e-3	3.70	1.2e-3	16.12	6.0e-4
	Day	2.072e-4	2.44e-4	-0.85	0.40	0.72	0.40
B	Temperature	2.18e-3	3.60e-4	6.07	0.00	49.60	<1.00e-4
	Day	-1.57e-5	3.28e-5	-0.78	0.44	0.62	0.44
Ca	Temperature	0.06	9.89e-4	6.55	0.00	128.73	<1.00e-4
	Day	3.64e-3	9.00e-4	4.04	5.0e-4	16.34	5.0e-4
Cd	Temperature	8.92 e-6	2.11 e-6	4.22	3.00 e-4	80.73	<1.00e-4
	Day	9.19 e-7	1.92 e-7	4.78	1.00 e-4	22.87	1.00e-4
Co	Temperature	-0.01	8.03e-3	-1.48	0.15	26.84	<1.00 e-4
	Day	3.16 e-3	7.31e-4	-4.33	3.00 e-4	18.72	<1.0 0e-4

Analyte	Fixed Effect	LME				ANOVA	
		Value	Std. Error	t-value	P-value	F-value	P-value
Cu	**Temperature**	2.92 e-3	0.03	-0.09	0.93	0.66	0.43
	Day	2.68 e-3	2.94 e-3	-0.91	0.37	0.83	0.38
Fe	**Temperature**	-0.04	0.02	-1.57	0.13	15.28	8.00e-4
	Day	-5.16 e-3	2.04e-3	-2.52	0.02	6.36	0.02
K	**Temperature**	0.93	0.08	11.27	0.00	264.34	1.00 e-4
	Day	0.02	7.48 e-3	2.69	0.01	7.26	0.01
Mg	**Temperature**	0.05	9.45 e-3	5.41	0.00	98.20	1.00 e-3
	Day	3.47 e-3	8.60 e-4	4.03	5.00 e-4	16.31	5.00 e-4
Mo	**Temperature**	3.43 e-4	5.81 e-5	5.92	0.00	78.15	<1.00e-4
	Day	9.56 e-6	5.29 e-6	1.81	0.08	3.27	0.08
Na	**Temperature**	0.24	0.03	7.23	0.00	111.78	1.00 e-4
	Day	5.85 e-3	3.06 e-3	1.91	0.07	3.66	0.07
Ni	**Temperature**	2.122	0.65	3.29	3.4 e-3	38.72	<1.00 e-4
	Day	0.16	0.06	2.71	0.01	7.37	0.01
Pb	**Temperature**	-0.05	0.02	-2.57	0.02	32.88	1.00 e-4
	Day	-5.78 e-3	1.78 e-3	-3.25	3.70 e-3	10.57	3.7 e-3
Sb	**Temperature**	0.02	2.51 e-3	6.94	0.00	97.27	<1.00 e-4
	Day	3.35 e-4	2.29 e-4	1.47	0.16	2.15	0.16
Si	**Temperature**	0.04	6.48 e-3	6.16	0.00	62.91	<1.00 e-4
	Day	1.29 e-4	5.90 e-4	0.22	0.83	0.05	0.83

Analyte	Fixed Effect	LME				ANOVA	
		Value	Std. Error	t-value	P-value	F-value	P-value
Th	**Temperature**	1.04 e-6	1.25 e-7	8.29	0.00	90.37	1.00 e-4
	Day	-1.37	1.14	-1.20	0.24	1.45	0.24
U	**Temperature**	0.02	0.01	1.68	0.11	90.74	1.00 e-4
	Day	2.26 e-3	9.42 e-4	2.40	0.03	1.45	0.24
V	**Temperature**	-7.81 e-3	0.01	-0.72	0.48	7.43	0.01
	Day	-2.35 e-3	9.90 e-4	-2.38	0.03	5.65	0.03
Zn	**Temperature**	-0.20	0.01	-1.51	0.14	2.20	0.15
	Day	4.35 e-3	9.87 e-4	4.42	2.00 e-4	19.58	2.00 e-4
Alkalinity	**Temperature**	-0.01	1.43 e-3	-8.11	0.00	154.38	<1.00e-4
	Day	-3.76 e-4	1.30 e-4	-2.88	8.6 e-3	8.31	8.60 e-3
pH	**Temperature**	4.47 e-3	1.03 e-3	-4.36	3.00 e-4	11.89	2.30 e-3
	Day	2.48 e-4	9.33 e-5	2.66	0.01	7.09	0.01
Conductiv -ity	**Temperature**	51.89	8.18	6.34	0.00	82.14	1.00 e-4
	Day	1.04	0.74	1.40	0.17	1.97	0.17
TDS	**Temperature**	0.06	9.74 e-3	6.35	0.00	118.74	1.00 e-4
	Day	3.35 e-3	8.86 e-4	3.78	1.00 e-3	14.30	1.00 e-3

<u>Table 3.2:</u> Change in mineralogy of waste rock over 348 days. Green shading represents a >100% increase in the individual mineral's abundance, red shading represents a mineral which has <75% of its individual abundance remaining.

Mineral	Abundance (%)		
	Day 0	Day 348	% Remaining
Quartz (SiO_2)	32.9	34.3	104.3
Albite ($NaAlSi_3O_6$)	21.2	20.0	94.3
Microcline ($KAlSi_3O_8$)	1.6	2.1	131.3
Chlorite $(Fe,(Mg,Mn)_5Al)(Si_3Al)O_{10}(OH)_8$)	12.7	10.0	78.7
Muscovite ($KAl_2(AlSi_3O_{10})(OH)_2$)	17.7	16.2	91.5
Biotite $(K(Mg,Fe)_3(AlSi_3O_{10})(OH)_2)$	2.0	4.0	200.0
Actinolite $(Ca_2(Mg,Fe)_5Si_8O_{22}(OH)_{22})$	9.4	8.8	93.6
Calcite ($CaCO_3$)	1.7	1.1	64.7
Ankerite ($CaFe(CO_3)_2$)	0.8	0.0	0.0
Diopside ($CaMgSi_2O_6$)	0.0	3.1	310.0
Dolomite ($CaMg(CO_3)_2$)	0.0	0.3	30.0

Table 3.3: Time to sulfur and neutralizing potential depletion of waste rock.

Parameter	Remaining (%)	Depletion Rate (mg/kg/week)	Time for Depletion (Years)
S	99.4	1.0	42
NP	99.8	4.6	103
CaNP (SO_4)	99.7	4.6	47
NP (Alkalinity)	100.0	0.1	5,215

Table 3.4: Field bin end point metagenome statistics.

Sample Description	F3	
Day Sampled	348	
Sample Mass Processed (g)	26.00	
DNA Yield (ng/ uL)	45.10	
QC	**Pre**	**Post**
Base Pair Count	1,807,582,476	1,431,371,709
Sequences Count	5,592,162	5,211,325
Mean Sequence Length (bp)	323 ± 87	275 ± 109
Mean GC Percent (%)	61 ± 10	61 ± 10
Failed QC (Sequences, %)	-	380,837; 6.81
Unknown Sequence (Sequences, %)	-	140.7; 0.25
Predicted Feature (Sequences, %)	-	5,197,288; 92.94
Unknown Protein (Sequences, %)	-	1,704,666; 32.80
Annotated Protein (Sequences, %)	-	3,474,732; 68.86
Ribosomal RNA (Sequences, %)	-	17,890; 0.34

CHAPTER 4: GEOCHEMICAL AND MICROBIOLOGICAL FEATURES OF LOW SULFUR WASTE ROCK WEATHERING IN KINETIC TEST SYSTEMS.

4.1: Study overview

Waste rock is an inevitable feature of mine sites that can pose as an environmental and economic liability. Waste rock monitoring programs are a key component of the environmental impact assessment which are put into place as the mine is being conceptualized to mitigate the risks of neutral or acid rock drainage (Price, 2009). Sulfidic waste rock is of particular concern when assessing ARD, as even rock bearing low concentrations of sulfur can mobilize significant amounts of elements which are toxic in aquatic and downstream terrestrial environments (Gray, 1997). Kinetic testing is commonly used in the mining industry to predict the onset, duration, and extent of acid and metal leaching from waste rock (Kevin A Morin & Hutt, 1998; Parbhakar-Fox & Lottermoser, 2015). The generation of ARD is a well-studied, shared process of abiotic and microbially mediated reactions (Ferguson & Erickson, 1988; Ledin & Pedersen, 1996). Despite this well-known relationship, kinetic testing systems often exclude key factors including temperature and microbial ecology.

This study aimed to investigate the weathering of low sulfur (~1%) waste rock from a mining development in a boreal climate. Analysis of leachate geochemistry, mineralogy, 16S rRNA gene amplicons, and metagenomics were utilized to better understand how they influence the weathering of waste rock when exposed to relevant site conditions. Humidity cell experiments were conducted at standard 20ºC, as well as 5 ºC incubations to determine if sulfate

and metal leaching was slowed but not halted at cooler temperatures in the presence of psychrotrophic microorganisms. Field leach bin experiments were utilized to include site climate conditions in the analysis of waste rock leaching, which was hypothesized to drive leachate geochemistry and microbial community composition. The combination of geological and microbiological findings from this study are still relatively rare in investigations of PAG rock which enrich the current understanding of waste rock weathering and could have beneficial implications for current kinetic test designs and future biotechnological advances in mining and mine waste management.

4.2: Temperature effects on the geochemistry of kinetic test systems

Sulfide oxidation is an exothermic reaction with a lower activation energy with increasing temperatures (J. Langman et al., 2014). However, the sub-zero temperatures experienced during the winter in boreal regions drastically slows sulfide oxidation reactions while increasing the solubility of carbonates (SRK Consulting, 2006). In our humidity cell and field leach bin experiments, temperature was not only significantly associated with SO_4 and alkalinity concentrations in leachate, but it also significantly affected the leaching of many elements such as Al (increased with warmer temperatures) and Ni (increased with colder temperatures). Field bin experiments demonstrated that most elements release rates showed seasonal trends, with those more partial to warm temperatures leaching at higher rates through the late spring to early fall and those partial to cool temperatures demonstrating higher rates in the fall and early spring.

As SO_4 and alkalinity release are significantly affected by temperature, predictions of time to acid generation and the duration of acid generation were altered considerably by temperature. In the humidity cells, standard 20ºC incubations were expected to be depleted of S well before NP, therefore predicting that ARD would not be generated. In the 5ºC treatment, consistently cool temperatures increased neutralizing mineral weathering while sulfide mineral weathering was decreased to the extent where NP would be depleted earlier than S. It was effectively concluded that ARD generation could be produced after 26 years if waste rock is maintained at cooler temperatures. In the field bin experiment, which experienced seasonal temperatures from -30ºC to +30ºC, S was also expected to be depleted well before NP. Weathering likely occurred at a much slower rate in the field bin experiment, causing predictive calculations to be completed at much earlier stages in the evolution of the waste rock's weathering. Additional time to reach steady state leaching rates should be attained in future experiments to provide better predictions of the effects of temperature on ARD generation.

Although temperature appeared to be the prominent abiotic factor affecting leachate geochemistry in both humidity cell and field bin experiments, pH is a known driver of element mobility that remained relatively static in this experiment. Our statistical analyses may have overlooked the role of pH as only small fluctuations in the circumneutral pH were experienced over the courses of both experiments. Both humidity cell and field leach bin experiments were run for short periods of time yet previous studies have demonstrated that waste rock may take several years to show large pH shifts (J. Langman et al., 2014; Parbhakar-Fox & Lottermoser, 2015). In effect, in the early stages of waste rock weathering temperature may play the dominant

role in predicting the leaching of SO_4 and alkalinity while moisture availability and pH play a larger role later in the lifetime of the waste rock.

4.3: Microbial community profiles in cold temperature kinetic test systems.

This study is one of few to examine the microbial ecology of mined waste rock. Unweathered rock was dominated by *Thiobacillus spp*., as well as members of the Xanthobacteria and unknown Betaproteobacteria. All dominant OTUs in the unweathered waste rock decreased in abundance as rock was subjected to kinetic testing systems (Fig. S.1). The humidity cell experiments had higher abundances of Microbacteriaceae, Caulobaceriaceae, and Sphingomonadaceae in comparison to the unweathered waste rock. Treatment groups were significantly different from each other (Fig. S.2). Warm humidity cells had higher abundances of Erythrobacteraceae, Bradyrihizobiaceae, and Hydrogenophilaceae, while cold humidity cells had higher abundances of Micrococcaceae and Oxalobacteraceae. Alpha diversity was also highest in the humidity cell experiments, particularly in the warm humidity cells (Fig. S.3). Prokaryotic communities in mid and end point samples also differed from each other although they remained dominated by soil and aquatic taxa. Field bin experiments showed lower alpha diversity (Fig. S.3) and distinct differences between time point samples (Fig. S.2). The early fall F2 sample had Flavobacteriaceae and Enterobacteriaceae in high abundance, yet these taxa were completely absent in the late fall F3 sample and replaced with highly abundant Oxalobacteraceae, Caulobacteraceae, Sphingomonadacea, Bradyrhizobiaceae, Micrococcaceae, and Chininophagaceae. *Thiobacillus spp*. were also present in very low abundance in all field leach bin samples.

Metagenomic analysis of samples obtained from humidity cell and field bin samples at the end of their experimental periods demonstrated that the microbial communities present were dominated by heterotrophic Bacteria. Of the remaining metabolic groups, phototrophic organisms, neutrophilic sulfur oxidizing organisms, as well as S and Fe reducing organisms were present in the next highest abundance (Fig. S.4.). The afore mentioned organisms are usually present in lower abundances in inorganic environments such as sulfidic mine wastes, however the neutral pH and high DOC suggest that environmental conditions are more likely to support these types of metabolisms than acidophilic iron and sulfur oxidation. Genes for sulfur oxidation and carbon fixation pathways were complete in all samples, as demonstrated by the dominant sulfur oxidizing organism, *Thiobacillus denitrificans*. The presence of neutrophilic and acidophilic sulfur oxidizing organisms and genes for sulfur oxidation suggest that they are contributing to the weathering of sulfide minerals in the waste rock systems. However, iron oxidizing species and metabolic pathways were largely absent in samples. The absence of acidophilic iron oxidizing species, which act as a catalyst in overcoming the rate limiting step in ARD generation following substrate colonization by neutrophilic sulfur oxidizing species, supports the neutral leachate geochemistry in that sulfide oxidation is not occurring to an extent that significant acid is being generated. In effect, the microbial community has the membership and genetic potential to generate ARD should the rate of sulfide oxidation increase or NP release decrease.

The ability of the microbial community to adapt to cool temperatures was present throughout the microbial community in all samples. Genes for cold stress such as cold shock proteins and trehalose production and utilization were also in higher abundance in the field leach

bin and cold treatment humidity cells, which were exposed to temperatures near or below freezing. As the waste rock originated from a cool boreal climate it is likely that some of taxa are psychrotrophic or psychrophilic and can contribute to community metabolism even during the winter months.

4.4: Industrial implications

We demonstrated that the release of SO_4 and alkalinity, which are critical factors in the prediction of ARD generation, were affected by temperature. Our humidity cell experiment demonstrated that seasonally relevant cool temperatures can drastically change the predicted time to, and duration of acid generation which are not necessarily experienced when such experiments are conducted at standard room temperature. Additionally, we found that element release fluctuated with seasonal temperature, which can be of use when modelling leaching kinetics and predicting the release of elements that may be released in high concentration. The microbiological findings of this study supporting microbial adaption to cool temperatures also suggests that microorganisms may be capable of contributing to the weathering of the waste rock even at cold temperatures where most microorganisms would lie dormant.

The microbiological findings of this study are significant, as they are some of the first insights into the ecology of waste rock following the early stages of weathering. The microbial community is composed of a much larger proportion of heterotrophic organisms than most inorganic systems, however more organic carbon may be incorporated into these waste rock systems than previously assumed. The microbial community has the metabolic capacity to contribute to the weathering of waste rock to a limited extent while the system remains at

circumneutral pH. Microbial community contribution, largely from acidophilic and neutrophilic sulfur oxidizers, may also become more prominent should pH decrease. The presence of sulfur oxidizing species and genes to carry out this form of metabolism may need to be considered in the assessment of ARD. The results of this thesis suggests that with the contributions of future research, specific species (ie: *Thiobacillus spp.)* or threshold abundances of microorganisms possessing genes or metabolites for iron and sulfur oxidation may be used in future testing to also predict ARD generation. Furthermore, as biotechnology becomes more accepted in the mining industry biomining and bioremediation efforts, psychrotrophic organisms and genes for cold adaptation may be of use in future technological developments to be implemented in boreal and arctic mining environments (D. B. Johnson, 1999; Martinez, Vera, & Bobadilla-Fazzini, 2015; Rawlings & Johnson, 2007).

4.5: Future Directions

This study provided a basic understanding of the geochemical, mineralogical, and microbiological profile of low sulfur waste rock from a mining operation. A more complex investigation of the reaction kinetics of elements, the presence of secondary precipitates, and the availability of S, Fe, and C species should be performed to obtain a more complete picture of the weathering environment of the waste rock. Additionally, experiments should be carried out over longer periods of time and at larger scales (ie: test pads) to obtain steady state leaching rates to better predict ARD generation. The microbiology of the system should be analyzed once per season at minimum in larger replicates to better understand community dynamics over time and how they correlate with environmental variables. Transcriptomic or proteomic analyses of the microbial community or key taxa may also provide a more detailed understanding of community

metabolism at any point in time in relation to the weathering of waste rock or temperature. Furthermore, it could be of enormous value to conduct an experiment that characterizes the geochemistry and microbiology of waste rock systems utilizing waste rock with varying sulfur content and site latitude to provide definitive predictions of the interplay between microorganisms and the abiotic weathering of sulfidic waste rock.

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APPENDIX

A.1: Supplemental methods

16S rRNA analysis and processing of unweathered waste rock

16.3 g of ¼" crush waste rock sample was placed into a 50 mL falcon tube and topped-up with sterile phosphate buffer (Zhou et al., 1996). The sample was sonicated with a 13 mm tip using a Fisher Scientific Sonic Dismembrator 500 (Fisher Scientific, CAN) for 2 min on 20 second bursts with 2 s breaks to dislodge biofilms. Sonicated samples were then pelleted by centrifugation at 3260 rcf for 10 min. Supernatant was discarded and the pellet was subsampled into 4 2mL Eppendorf tubes to which 1mL of phosphate buffer was added. Samples were vortexed at max speed for 30 second and spun at 5000 rcf for 3 min. Supernatant was removed and 0.25 g of rock was subsampled into six extraction preps of DNeasy PowerSoil kit (MoBio, CA). DNA concentration and quality was checked by spectrophotometry using Agilent Take3 software (Agilent Technologies, CA), as well as by PCR amplification of the 16S rRNA gene using the broad eukaryotic primer set designed for the Earth Microbiome project (EMP 515F 5'- GTGYCAGCMGCCGCGGTAA- 3', EMP 806r 5'- GGACTACNVGGGTWTCTAAT- 3') (Gilbert et al., 2014). PCR was completed using an Invitrogen Recombinant Taq DNA Polymerase and 0.6 mg/mL BSA under the following conditions: initial denaturation of 4 min at 94 ˚C, 35 cycles at 94 ˚C for 45 s, 52 ˚C for 1 min, 72 ˚C for 1 min, and final elongation of 8 min at 72˚C. Library preparation for both 16S rRNA amplicons was completed by MetagenomBio at the University of Waterloo, with sequencing being completed on an Illumina Miseq using the

forward primer (5'- CCTACGGGNBGCASCAG - 3') and reverse primer (5'- GACTACNVGGGTATCTAATCC - 3') (Takahashi et al., 2014).

Paired end reads from 16S rRNA gene amplicon libraries were stripped of adaptors, primers and screened for PhiX contamination with BBDuk, merged with BBMerge, and bidirectionally trimmed to Q20 using BBDuk (Bushnell, 2014). Sequences were converted to fasta files using the fq2fa command in IDBA-UD (Peng et al., 2012) prior to adding QIIME labels in QIIME 1.9.1 (Caporaso et al., 2011). Dereplication, size sorting, doubleton removal, OTU clustering and a denovo chimera check was performed using the UPARSE pipeline (Edgar, 2013) in USEARCH 9.2. Taxonomy was assigned in QIIME against the Greengenes database (McDonald et al., 2012) using the RDP classifier (Cole et al., 2014) in QIIME (Caporaso et al., 2011).

Manipulation of 16S rRNA gene microbial community data was facilitated by scripts included in the Phyloseq package (McMurdie & Holmes, 2013). Barcharts of the relative abundance of taxa with greater than 1% relative abundance at the phylum and family levels were produced and visualized in ggplot2 (Wickham, 2009).

16S rRNA waste rock community comparisons

A heatmap of the changes in the relative abundances of dominant taxa in the experimental treatment in relation to that of the unweathered waste rock. The difference in the relative abundance of prokaryotic families present in >2% abundance in any sample was calculated from the relative abundance of that family in the unweathered rock sample. A

heatmap was generated using the heatmap.2 command with default parameters in the gplots package (Warnes, *et al.*, 2016).

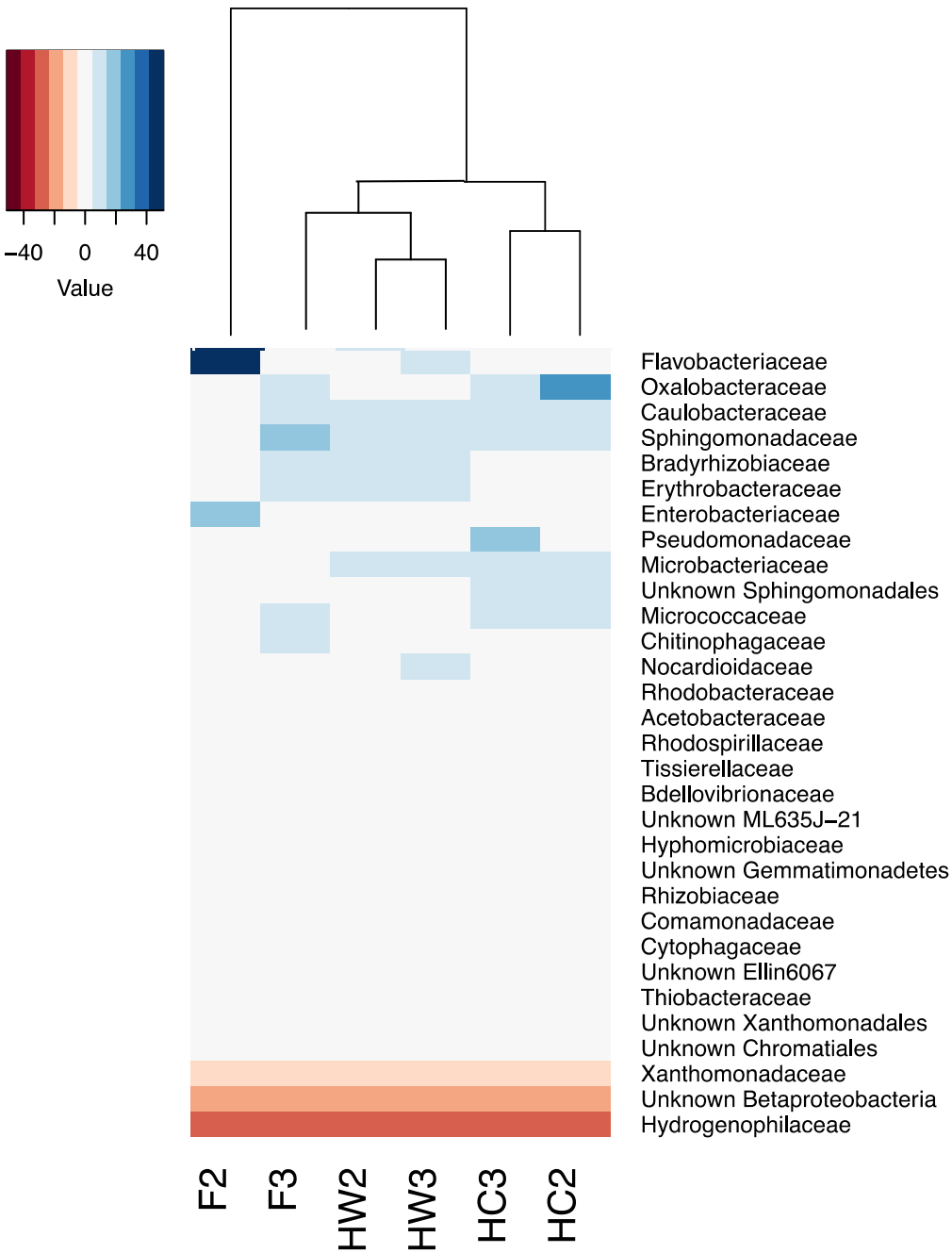
Alpha and beta diversity statistics were conducted on the unweathered rock, humidity cell, and field leach bin communities. Beta diversity was interpreted by performing NMDS on a computed Bray- Curtis dissimilarity matrix (McMurdie & Holmes, 2013) of the raw OTU counts using the metaMDS code in vegan (Oksanen *et al.*, 2013) and plotted using the fortify.metaMDS in ggvegan (Simpson, 2015) and ggplot function in ggplot2 (Wickham, 2009). Simpsons' and Shannon diversity were used to assess the alpha diversity of the community. Samples were rarefied to 3168 sequences using the rarefy_even_depth command prior to plotting and calculation of Simpson's and Shannon diversity with the plot_richness command in Phyloseq (McMurdie & Holmes, 2013)

A.2: Oxidation- neutralization curves

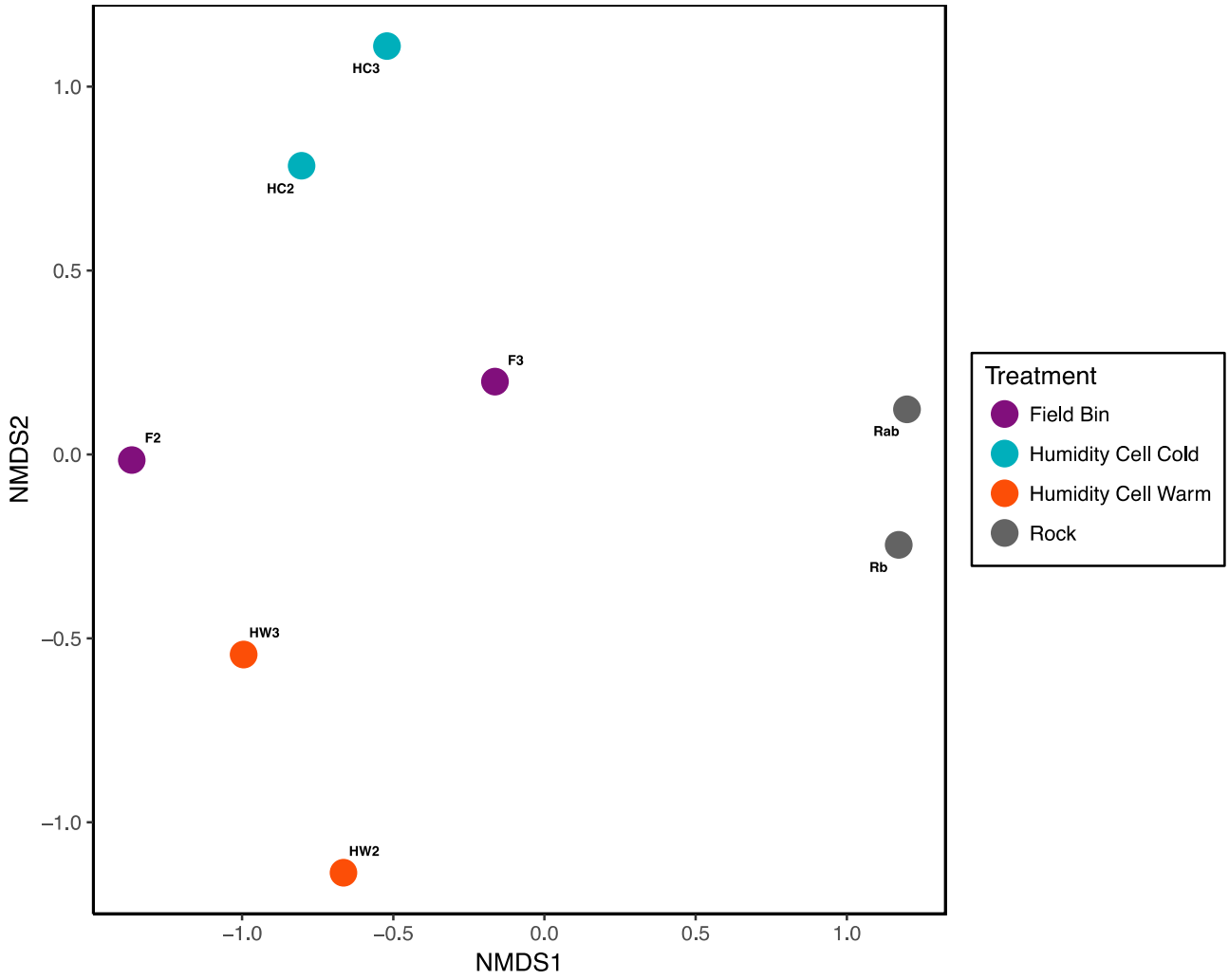
Oxidation- neutralization curves were conducted as an alternative means of predicting the acid- generation potential of samples incubated under different temperature treatments in humidity cell experiments, as well as the field bin experiment. Oxidation- neutralization curves provide an estimate of whether the material will, or will not generate acidity based on balance of cumulative element release by silicate dissolution and sulfide oxidation (Benzaazoua et al., 2004). The principal advantages of the oxidation- neutralization curve method is in its ability to predict the ARD generating status of a material from kinetic test data while avoiding the ambiguity of time frames provided by time- to acid generation predictions and visually compare the ARD predicting status of materials used in different experiments (Benzazzoua, personal

communication, 2018). Figure S.5. demonstrates that based on the assumption that weathering rates will remain constant over time, the ARD predictions are opposite to those calculated by the time to acid generation; material exposed to warm humidity cell treatments are expected to be acid generating, material exposed cold humidity cell treatments are expected to be neutral, and material exposed to the field bin experiment is expected to be acid generating.

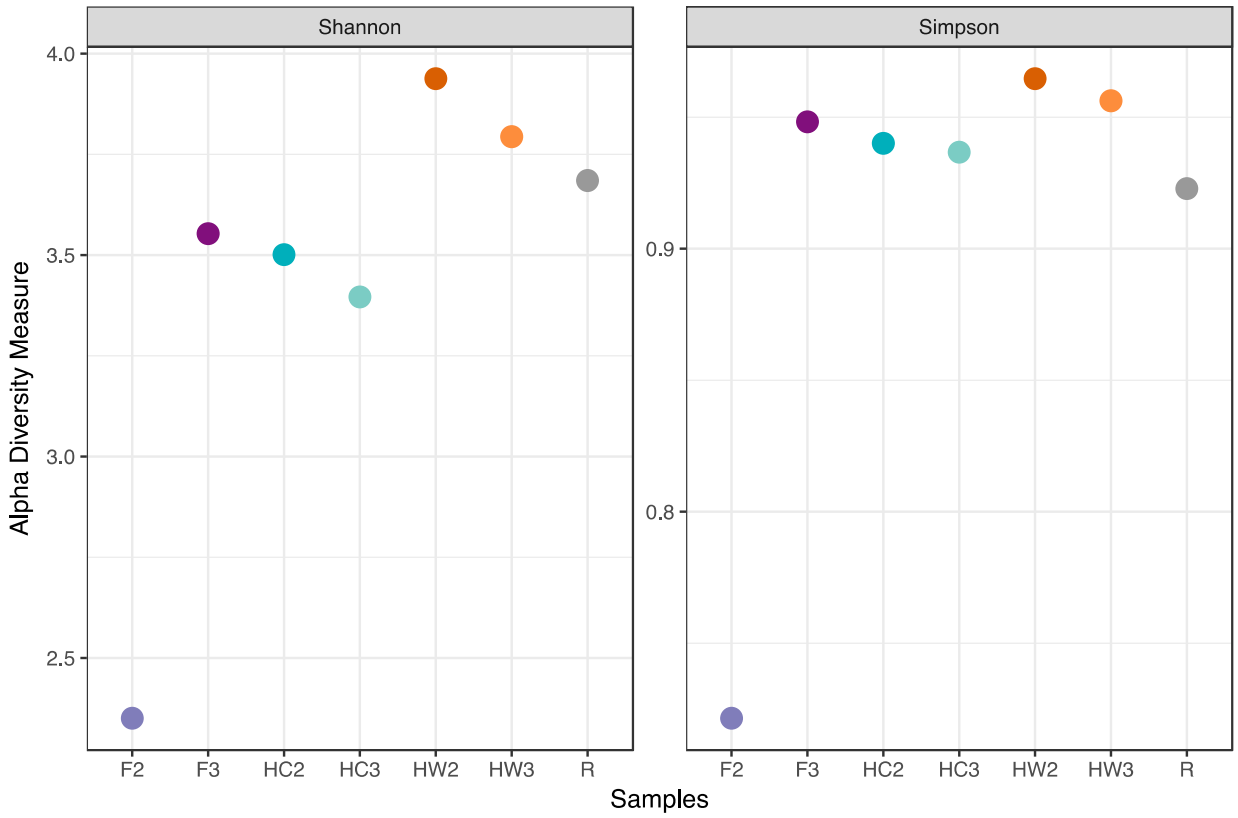
A.3: Supplemental figures and tables



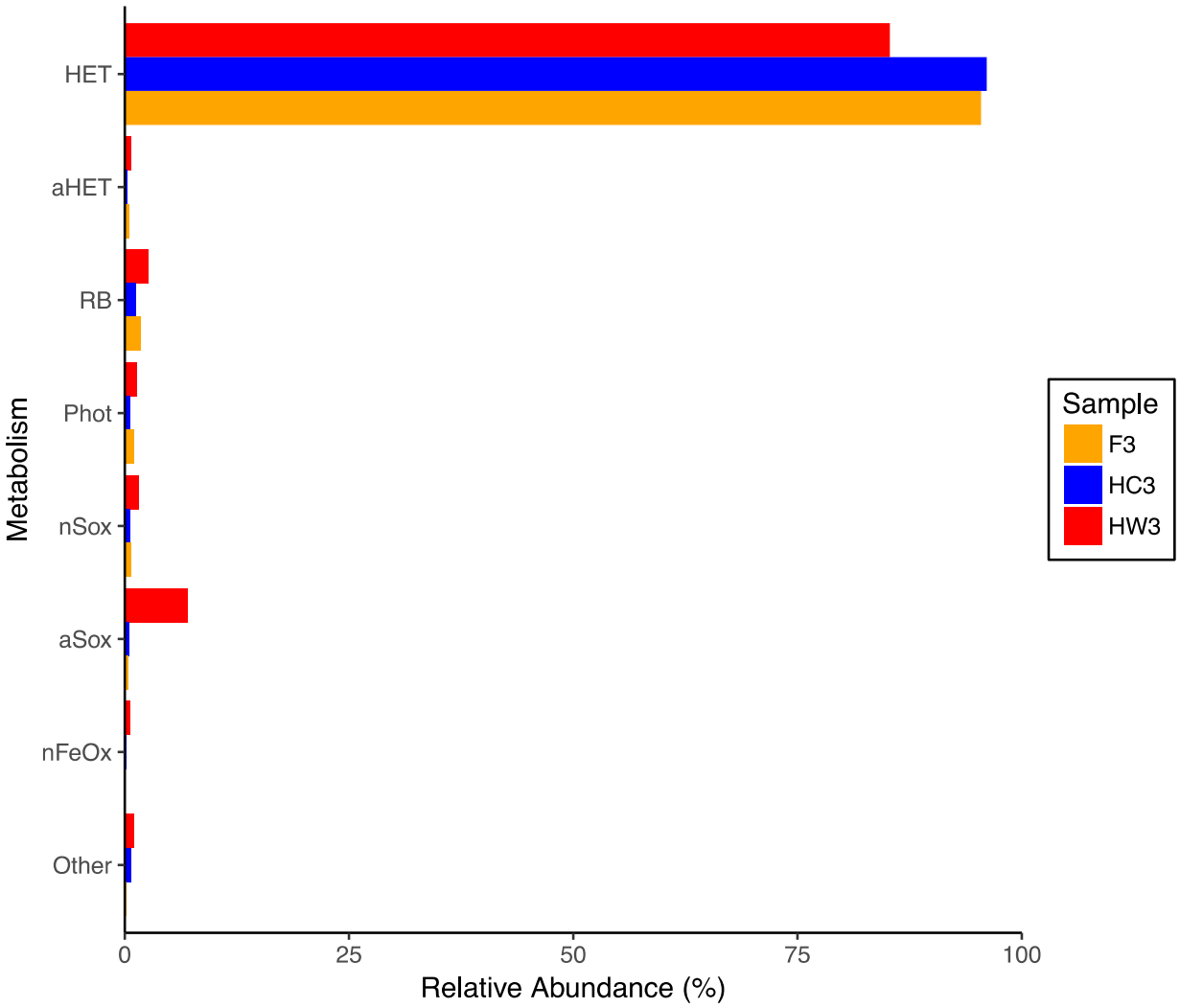
<u>Figure S.1:</u> Heatmap of the change in abundance of prokaryotic taxa present in >2% abundance at the family level relative to unweathered waste rock as determined by 16S rRNA amplicon sequencing.



<u>Figure S.2:</u> Bray- Curtis NMDS demonstrating beta diversity of unweathered waste rock, humidity cell, and field leach bin samples analyzed by 16S rRNA amplicon sequencing.



<u>Figure S.3:</u> Shannon and Simpson alpha diversity metrics of unweathered waste rock, humidity cell, and field leach bin samples analyzed by 16S rRNA amplicon sequencing.



<u>Figure S.4:</u> Relative abundance of dominant metabolic groups in humidity cell and field leach bin final time point samples as determined by metagenomic hits classified by RefSeq.

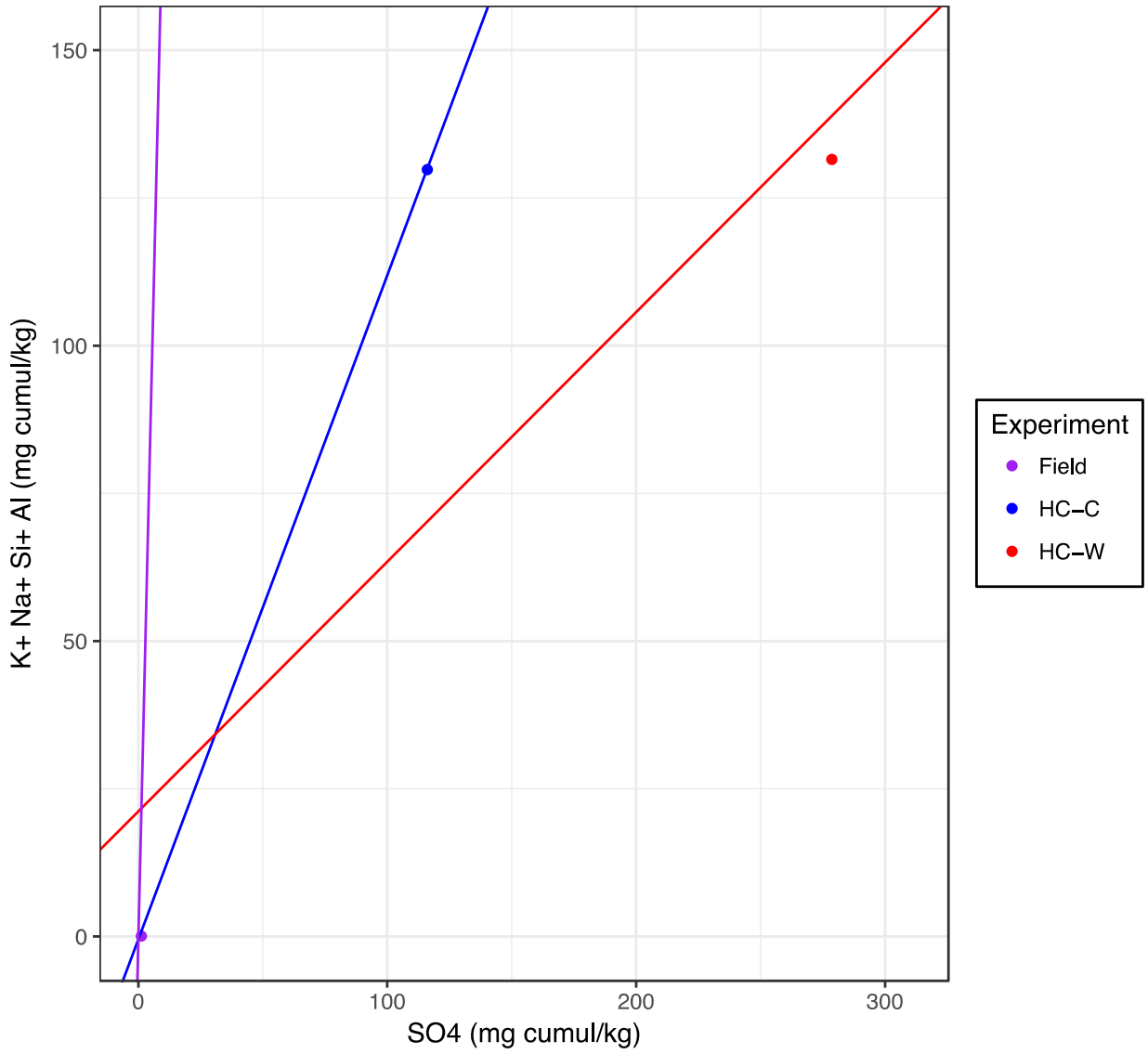


Figure S.5: Oxidization- neutralization curves for field bin (field), cold humidity cell (HC-C) and warm humidity cell (HC-W) samples. Samples with points lying above their oxidation neutralization curve are predicted to produce neutral drainage and samples with points lying below their oxidation neutralization curve are predicted to produce acidic drainage.

<u>Table S.1:</u> Top two NCBI BLAST hits to the top 5 taxa in each 16SrRNA amplicon sample from unweathered rock, humidity cell, and field bin experiments. X represents the presence of the OTU in that sample.

OTU	Sample							Lowest Greengenes Taxonomic ID	BLAST Hit #1				BLAST Hit #2			
	R	F2	F3	HW2	HW3	HC2	HC3		BLAST Hit	E Value	Coverage	%ID	Blast Hit	E Value	Coverage	%ID
2	X			X	X	X	X	Thiobacillus	NR_117864.1	0	100	97	NR_117817.1	0	100	97
3				X	X	X	X	Erythrobacteraceae	NR_108176.1	0	100	100	NR_108695.1	0	100	99
9		X			X	X		Flavobacterium succinans	NR_108520.1	0	100	99	NR_044292.1	0	100	99
10			X	X	X	X		Mycoplana	NR_133989.1	0	100	100	NR_041965.1	0	100	100
15			X	X	X	X		Bradyrhizobium	NR_145862.1	0	100	100	NR_145861.1	0	100	100
11			X	X	X	X	X	Janinthobacterium	NR_116871.1	0	100	99	NR_044274.1	0	100	99
4		X	X	X	X	X	X	Micrococcaceae	NR_117356.1	0	100	100	NR_025084.1	0	100	100
6			X			X	X	Phenylobacterium	NR_133716.1	0	99	99	NR_114055.1	0	100	98
5						X	X	Sphingomonadales	NR_117995.1	0	100	100	NR_109465.1	0	100	99
13				X	X	X	X	Microbacteriaceae	NR_115999.1	0	100	99	NR_109607.1	0	100	99
7		X					X	Pseudomonas	NR_028706.1	0	100	100	NR_117821.1	0	100	99
46		X						Enterobacteriaceae	NR_102493.1	0	100	99	NR_118556.1	0	100	99
172		X						Erwinia	NR_104943.1	0	100	99	NR_041978.1	0	100	99
32			X					Parasegitbacter luojiensis	NR_044576.1	0	100	99	NR_117796.1	2.00E-179	100	93
24			X					Sphingobium	NR_118306.1	0	100	100	NR_109535.1	0	100	99
25			X			X	X	Oxalobacteriaceae	NR_133798.1	0	100	99	NR_114175.1	0	100	99
158	X							Thiobacillus	NR_117864.1	0	100	96	NR_117817.1	0	100	96
514	X							Thiobacillus	NR_117864.1	0	100	98	NR_117817.1	0	100	98
93	X							Thiobacillus	NR_117864.1	0	100	97	NR_117817.1	0	100	97
97	X							Thiobacillus	NR_025358.1	0	100	98	NR_117864.1	0	100	97
103	X							Xanthomonadaceae	NR_109442.1	0	100	96	NR_116294.1	0	100	95

OTU	Sample							Lowest Greengenes Taxonomic ID	BLAST Hit #1				BLAST Hit #2			
	R	F2	F3	HW2	HW3	HC2	HC3		BLAST Hit	E Value	Coverage	%ID	Blast Hit	E Value	Coverage	%ID
16	X							Betaproteobacteria	NR_117864.1	0	100	95	NR_117817.1	0	100	95
82	X							Proteobacteria	NR_147747.1	3.00E-172	100	92	NR_137347.1	1.00E-162	100	90
63	X							Betaproteobacteria	NR_043249.1	2.00E-173	100	92	NR_115995.1	2.00E-172	100	92
83	X							Chromatiales	NR_112620.1	0	100	93	NR_112829.1	0	100	93
62	X							Thiobacteraceae	NR_044793.1	0	100	94	NR_117675.1	0	100	94
23			X					Geodermatophilaceae	NR_114864.1	0	100	100	NR_025925.1	0	100	100
53			X					Rhodococcus	NR_104776.1	0	100	100	NR_115708.1	0	100	100
31				X	X			Nocardioidaceae	NR_043787.1	0	100	99	NR_025777.1	0	100	98