

Microbial Diversity and Potential for Biogeochemical Cycling in Heavy Metal Contaminated
Environments

by

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This thesis consists of material all of which I authored or co-authored: see Statement of Contributions included in the thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

I understand that my thesis may be made electronically available to the public.

Statement of Contributions

Xiang Chen (Molly Chen) was the primary author for the work described in this thesis. Chapters 1, 5, and 6 of this thesis were not written for publication. Chapters 2 and 3 have been published. Chapter 4 is being prepared as a single manuscript for publication. Contributions of authors other than M. Chen and L.A. Hug towards each chapter are listed below.

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Dr. Daniel Grégoire, Dr. Carolina Berdugo-Clavijo and Pascale St-Germain reviewed the writing for this chapter. Dr. Grégoire also provided additional analyses related to the thermodynamics of the study system to support predictions on metabolic activities.

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Chapter 4

This work will be described in a manuscript in preparation for submission by Chen M, Grégoire DS, Bain JG, Blowes DW, & Hug LA.

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Chapter 5

The work described in this chapter was done during a visiting student appointment in the Horsfall research group at the University of Edinburgh School of Biological Sciences. Prof. Louise Horsfall provided access to laboratory facilities, background information and data from industry collaborators. Dr. Michael Capeness provided additional background information, as well as assisted with data collection and experimental design. Prof. Horsfall and Dr. Capeness also reviewed the data analysis and writing for this chapter.

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Artificial Intelligence Tool(s): ChatGPT, May 2025; Microsoft Copilot (University of Waterloo institutional license), June 2025; *Curation*: ChatGPT and Microsoft Copilot were used to assist in writing scripts for organizing sequencing data files; *Visualization*: ChatGPT and Microsoft Copilot were used to assist in writing scripts for generating the figures shown in Chapter 4.

No generative AI was used for the conceptualization, interpretation, writing, or reviewing of any content presented in this thesis.

Abstract

Heavy metal contamination arising from resource extraction and wastes poses a threat to natural ecosystems and public health. Microorganisms influence the mobility and toxicity of metals through various resistance mechanisms and metabolic pathways. This thesis investigates microbial diversity and functional capacity across multiple metal-contaminated systems, integrating environmental sequencing data with experimental observations. The overall objective was to better understand the microbial processes involved in metal cycling, focusing on As, Fe, Cu, Ni, and Pd.

Using 16S rRNA amplicon and metagenomic sequencing, I profiled microbial communities from aquifers associated with thermally mobilized arsenic, as well as from mine tailings derived from nickel/copper ores. Sampling across a wide range of spatial and geochemical conditions revealed considerable variation in microbial taxonomic and functional diversity. Although community diversity correlated with many factors, including arsenic (in aquifers) and iron (in tailings) concentrations, metal concentrations alone were not predictive of the abundance and localization of specific populations. Metal resistance genes were widespread in all communities across sites and geochemical conditions, suggesting that metal tolerance is regulated at the gene expression level. In contrast, the distribution of taxa and genes associated with iron- and sulfur- cycling was more closely linked to environmental factors such as redox potential, pH, and/or temperature, where these variables were more selective for specific microbial guilds. Additional results from metaproteomic analyses of tailings samples complemented the metagenomics data by confirming the expression of predicted metal and sulfur-related genes.

In both environments, ‘-omics’ approaches identified many uncultivated lineages with the potential for metal and sulfur redox transformations. Sulfate-reducing bacteria were predicted to be especially relevant in heavy metal remediation, due to their ability to precipitate dissolved metals as insoluble sulfides. Building on this insight, I experimentally evaluated the ability of a model sulfate-reducing bacterium, *Oleidesulfovibrio alaskensis* G20, to selectively recover metals from simulated mining waste. *O. alaskensis* G20 was shown to form nickel sulfide and palladium nanoparticles from mixed-metal solutions, demonstrating a viable method of biologically mediated metal recovery.

Collectively, the research presented in this thesis advances our understanding of microbial diversity and biogeochemical cycling in heavy metal-contaminated environments, and highlights the potential to harness microbe-metal interactions for bioremediation and metal recovery technologies.

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I would like to thank the members of my advisory and examining committee, Dr. Josh Neufeld, Dr. David Blowes, and Dr. Jenine McCutcheon for their insights towards my research, as well as for challenging me to expand my knowledge base across a wide breadth of interdisciplinary topics. Additional thanks to Dr. Sue Baldwin for her participation as my external examiner.

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Last but not least, I would like to express my gratitude towards my family for supporting my education. To my parents, who named me after the phrase “遇到问题, 想一想” (when faced with a problem, think it through) – thank you for raising me to appreciate the value of curiosity, critical thinking, and the scientific method. And to Artem Kholodov, who provided endless encouragement, motivation, and personalized 24/7 tech support services during my studies – thank you for being the best life partner anyone could ask for.

Land Acknowledgement

I acknowledge that my research takes place at the University of Waterloo, which is located on the traditional territory of the Neutral, Anishinaabeg, and Haudenosaunee peoples. Our main campus is situated on the Haldimand Tract, the land granted to the Six Nations that includes six miles on each side of the Grand River. Furthermore, the study sites described in Chapters 2-4 are located on indigenous territories: Northeastern Alberta oil sands extraction takes place within Treaty 6 territory and the homeland of the Cree, Denesuline, and Metis peoples; Sudbury is the traditional territory of the Atikameksheng, Anishnawbek, and Wahnapiatae First Nations, within lands protected by the Robinson Huron Treaty of 1850.

As my thesis examines the environmental impacts of oil extraction and mining, I would like to specifically acknowledge the historic relationships between Canadian indigenous populations and these natural resources. In Alberta, the first documented records of oil sands use were from 1715 by a Hudson's Bay employee, who described how local First Nations communities extracted bitumen by boiling oil sands, for use in waterproofing canoes (Canada Action, 2023). Additionally, the mining and metalworking history of the Sudbury region began many thousands of years before colonial mining operations started in the late 1800's (Jewiss, 1983). Trade routes for tools, ornaments, and jewelry made of copper, silver, and other metals spanned vast distances across Northern Ontario and beyond (Jewiss, 1983). It is important to recognize and encourage awareness of this historical context when discussing issues pertaining to environmental remediation and sustainable resource use.

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List of Abbreviations

% rel. abundance	Percent Relative Abundance
°C	Degrees Celsius
ΔG	Change in Gibbs free energy
ACG	Automatic Gain Control
ACN	Acetonitrile
Alk	Alkalinity
AMD	Acid Mine Drainage
ANOSIM	Analysis Of Similarities
ANOVA	Analysis Of Variance
ASV	Amplicon Sequence Variant
ATP	Adenosine Triphosphate
BacMet	Antibacterial Biocide and Metal Resistance Genes Database
BBDuk	Software for sequence decontamination using kmers
BLAST	Basic Local Alignment Search Tool
BioCORE	Biomolecular Core Facility
C18 column	18-Carbon column
CA	Correspondence Analysis
cDNA	Complementary DNA
CheckM	Software for assessing microbial genome quality
CONCOCT	Clustering Contigs with Coverage and Composition
cryo-TEM	Cryogenic Transmission Electron Microscopy

Da	Dalton
DADA2	Divisive Amplicon Denoising Algorithm 2
DAS Tool	Dereplication, Aggregation, and Scoring Tool
DDA	Data-dependent acquisition
DiSCo	Dsr-dependent Dissimilatory Sulfur Metabolism Classification Tool
DNA	Deoxyribonucleic Acid
DOC	Dissolved Organic Carbon
DPANN	Archaeal superphylum: Diapherotrites, Parvarchaeota, Aenigmarchaeota, Nanoarchaeota, and Nanohaloarchaeota
DRAM	Distilled and Refined Annotation of Metabolism
dRep	Software for dereplicating genomes
DST	Desulfurized Tailings
DTT	Dithiothreitol
EC	Electrical Conductivity
EDS	Electron Dispersive X-ray Spectroscopy
EDTA	Ethylenediaminetetraacetic Acid
Eh	Redox Potential
FeGenie	Iron-related gene annotation tool
FDR	False Discovery Rate
FT	Fourier Transform
GTDB / GTDB-tk	Genome Taxonomy Database / GTDB-toolkit
GToTree	Tool for constructing phylogenomic trees
HDAAF-STEM	High-angle Annular Darkfield STEM
HCD	Higher-energy Collision Dissociation

HMM	Hidden Markov Model
HQ	High Quality
IAA	Iodoacetamide
IC	Ion Chromatography
ICP-OES	Inductively Coupled Plasma Optical Emission Spectroscopy
ICP-MS	Inductively Coupled Plasma Optical Mass Spectrometry
INO	Integrated Nickel Operations
iToL	Interactive Tree of Life
KEGG	Kyoto Encyclopedia of Genes and Genomes
kg / g / mg / μ g / ng	Kilograms, grams, milligrams, micrograms, nanograms
KO	KEGG Orthology
LC-MS/MS	Liquid Chromatography-Tandem Mass Spectrometry
LIB	Lithium-ion Battery
L / mL / μ L	Liter, milliliter, microliter
MAG	Metagenome-Assembled Genome
MaxBin	Metagenome binning using an expectation-maximization algorithm
MES	2-(N-morpholino)ethanesulfonic acid
MetaBAT	Metagenome Binning with Abundance and Tetra-nucleotide frequencies
ML25 / ML34	Moose Lake 25 / Moose Lake 34
MMG	Metal Metabolism Gene
M / mM / μ M	Molar / Millimolar / Micromolar
MOPS	3-(N-morpholino)propanesulfonic acid
MRG	Metal Resistance Gene

MS1 / MS2	Mass Spectrometer 1 / Mass Spectrometer 2
mV	Millivolts
NCBI	National Center for Biotechnology Information
NCE	Normalized Collision Energy
NDIS	Non-dispersive Infrared Sensor
NMDS	Non-Metric Multidimensional Scaling
NR3 / NR18	Nickel Rim 3 / Nickel Rim 18
NGS	Next Generation Sequencing
ORF	Open Reading Frame
PCA	Principal Components Analysis
PCoA	Principal Coordinates Analysis
PCR	Polymerase Chain Reaction
Pfam	Protein families database
PGMC	Postgate Media C
pH	Potential of Hydrogen (acidity/basicity)
PGM	Platinum Group Metals
ppm / ppb	Parts per million / parts per billion
QIIME2	Quantitative Insights Into Microbial Ecology 2
RefSeq	NCBI Reference Sequence Database
RISC	Reduced Inorganic Sulfur Compound
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
rRNA	Ribosomal RNA

SDS	Sodium Dodecyl Sulfate
SILVA	Taxonomic rRNA sequence database
SPAdes	St. Petersburg Assembly software
SRA	Sequence Read Archive
SRB	Sulfate-Reducing Bacteria
SSU	Small Subunit
STEM	Scanning Transmission Electron Microscopy
SWSS	Soil Water Solution Sampler
TCA	Tricarboxylic Acid
TCAG	The Center for Applied Genomics
UPLC	Ultra-Performance Liquid Chromatography
WGS	Whole Genome Shotgun
WHO	World Health Organization
wt. %	Weight Percent
x g	Relative Centrifugal Force (as a multiple of gravity)

Chapter 1

Introduction

This introductory chapter is intended to provide a broad overview of the topics and themes that unite the research described in this thesis. Separate introductions for each data chapter (Chapters 2–5) provide background information pertinent to each specific study.

1.1 Microbial interactions with heavy metals

The term ‘heavy metals’ refers to metallic elements on the periodic table that are relatively high density, and toxic to most life forms at low concentrations (Hawkes, 1997; Kiran et al., 2022). It is an informal classification that includes both metals and metalloids, and past attempts to define the category using specific criteria (*e.g.*, metals above a certain density or atomic number) have been highly debated and the subject of controversy (Appenroth, 2010; Duffus, 2002). Nonetheless, ‘heavy metals’ remains a common label for elements such as iron, copper, nickel, and arsenic in the literature when discussing their relevance to environmental contamination and ecotoxicity (Alloway, 2013; Sarkar, 2002).

Some heavy metals (*e.g.*, iron, zinc) are essential micronutrients (Rengel, 1999), whereas others (*e.g.*, lead) have no known biological function (Wani et al., 2015). In all cases, organisms have narrow tolerance ranges to intracellular metal concentrations. Free metal ions in the cytoplasm are highly cytotoxic: they can disrupt enzyme activity, interfere with DNA replication and repair, catalyze the generation of reactive oxygen species (ROS), among other effects (reviewed in Wu et al., 2016a). Metal homeostasis involves the regulation of metal bioavailability and/or toxicity, and is especially important in metal-contaminated environments. For microorganisms, genes interacting with metals may be broadly categorized into two groups: metal resistance genes (MRGs) and metal metabolism genes (MMGs) (**Figure 1.1**), along with associated regulatory genes.

MRGs encode proteins which function to protect the cell from metal toxicity, and can be subdivided based on their mechanism of action (**Figure 1.1**) (Das et al., 2016; Johnson et al., 2019; Silver, 1996). Membrane transporters (typically ATPases) export metal ions across the cytoplasm or periplasm, reducing intracellular concentrations (Argüello et al., 2007). Oxidoreductases can be used when changing the redox state of a metal reduces its toxicity (*e.g.*, from Cu^{2+} to Cu^{1+}) (Grass & Rensing, 2001), or in combination with ion-specific transporters. Metal-binding proteins or small molecules provide an alternative resistance mechanism by sequestering metal ions in the cytoplasm or

periplasm (Argüello et al., 2013; Cooksey, 1994), preventing them from interacting with and damaging cellular processes. MRG expression is regulated by accessory genes. In bacteria, these are commonly members of the two-component signal transduction family (Chang & Stewart, 1998; Nies & Brown, 1998): a sensor histidine kinase that responds to environmental stimuli (*e.g.*, metal ions) by phosphorylating a response regulator, which controls gene expression.

MMGs describe genes within energy-generating pathways involving metal ions (**Figure 1.1**). Metals are essential cofactors for many metabolic proteins, including those involved in redox catalysis and electron transport (*e.g.*, cytochromes bind Fe) during cellular respiration (Andreini et al., 2008). However, because this thesis focuses on metal biogeochemical cycling, I will be limiting the definition of ‘metal metabolism genes’ to genes from pathways that either 1) directly obtain energy through the oxidation of metal ions (*i.e.*, chemolithoautotrophy) (Hooper & DiSpirito, 2013), or 2) use metal ions as the terminal electron acceptor in an ATP-generating respiratory chain (*i.e.*, dissimilatory metal reduction) (Lovley, 1993). These pathways are unique to bacteria and archaea, with common metal substrates being iron and manganese (Osburn et al., 2014).

Other genes associated with biological metal usage (including metal metabolism) may be involved in the acquisition, transport, and/or storage of metal ions. For example, membrane transporters similar in structure to MRGs (or MRGs themselves) can be used to import metal ions when environmental concentrations are limiting (Schalk & Cunrath, 2016). Microbes may also secrete small organic ligands to assist in metal uptake; siderophores are especially important for chelating ferric (Fe^{3+}) iron, enabling its soluble transport (Kramer et al., 2020).

The biogeochemical cycling of metals is influenced by MRGs, MMGs, as well as microbial activities that influence the cycling of other elements that interact and/or form compounds with metals. Metal-bearing minerals can contain carbon (carbonates), phosphorus (phosphates), silicon (silicates), oxygen (oxides), and sulfur (sulfides and sulfates) (Krivovichev et al., 2018). Considering all of these factors is important for predicting the distribution, mobility, and toxicity of heavy metals in the environment. The biological sulfur cycle is of particular interest, because both chemolithotrophic sulfur oxidation and dissimilatory sulfate reduction pathways are widely distributed among microorganisms, inducing the dissolution or precipitation of sulfide metals (Wu et al., 2021). In this thesis, I investigate microbial communities in metal-contaminated environments and waste sources impacted by natural resource extraction, focusing on potential interactions with As, Fe, Cu, Ni, Pd, and S.

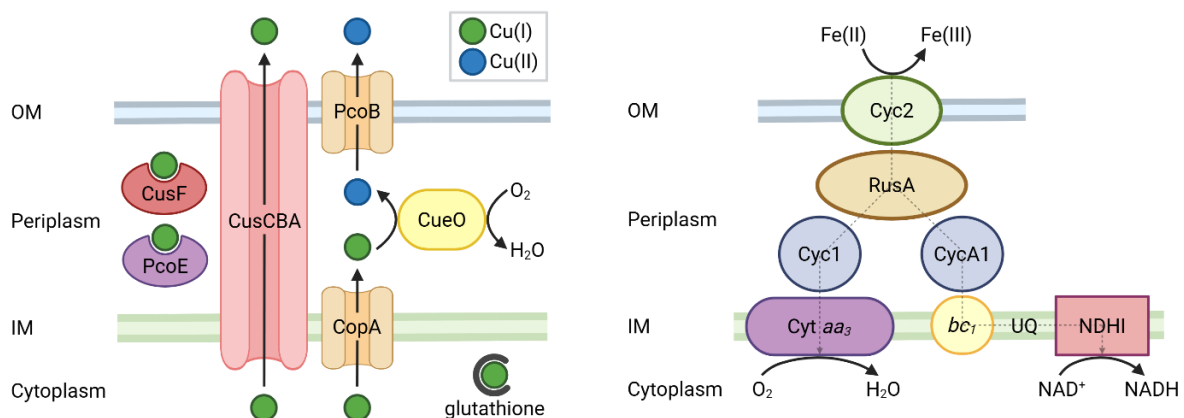


Figure 1.1: Examples of metal resistance genes (MRGs) (left) and metal metabolism genes (MMGs) (right). Left panel: MRGs encoding copper resistance pathways are selected as an example (Argüello et al., 2013). Common resistance mechanisms include efflux (*cusCBA*, *copA*, *pcoB*), oxidation/reduction (*cueO*), and sequestration in the periplasm or cytoplasm (*cusF*, *pcoE*, glutathione). Right panel: MMGs within the iron oxidation pathway in *Acidithiobacillus ferrooxidans* are shown as an example of an energy-generating pathway which uses ferrous iron as an electron source (adapted from Ilbert & Bonnefoy, 2013). Image was created with BioRender.com.

1.2 Biogeochemical cycling of arsenic in groundwater

1.2.1 Environmental arsenic distribution and toxicity

Arsenic (As) is a naturally occurring metalloid present in the atmosphere, lithosphere, hydrosphere, and biosphere. The presence of arsenic in drinking water poses a significant risk to human health (Frisbie & Mitchell, 2022), prompting the study of how this element is mobilized into terrestrial freshwater reservoirs such as lakes, rivers, and aquifers. Out of these environments, the highest concentrations of arsenic (10–5,000 µg/L) are found in aquifers, notably in localized regions of India, Bangladesh, Argentina, Chile, and the Southern United States (Frisbie & Mitchell, 2022; Smedley & Kinniburgh, 2002).

Two unique properties of arsenic influence its role as an environmental contaminant. Unlike most heavy metals, which form cations (*e.g.*, Fe^{2+} , Cu^{2+} , Ni^{2+}), inorganic arsenic primarily takes the form of an oxyanion in aqueous solutions (Smedley & Kinniburgh, 2002). Generally, the solubility of metal cations decreases with increasing pH, whereas oxyanions such as arsenate (AsO_4^{3-}) and arsenite (AsO_3^{3-}) become less strongly adsorbed by minerals with increasing pH (Dzombak & Morel, 1990) and are therefore soluble at neutral to alkaline pH ranges. Additionally, arsenic oxyanions are soluble under both oxidizing (as arsenate) and reducing (as arsenite) conditions (Smedley & Kinniburgh, 2002). The high solubility of arsenic at circumneutral pH and over a wide range of redox conditions are major factors contributing to its ability to persist in natural water sources at relatively high (in the µg/L range) concentrations (Smedley & Kinniburgh, 2002).

Although the exact mechanisms of toxicity at the cellular level are not fully understood, there are several hypotheses for how inorganic arsenic affects biological systems, based on its speciation. Pentavalent arsenic (As^{5+} , as arsenate) is structurally similar to phosphate, and may be substituted in biochemical reactions involving the latter, including those within essential metabolic pathways such as glycolysis and ATP synthesis (Hughes, 2002). The trivalent form of arsenic (As^{3+} , as arsenite) is considered more toxic than As^{5+} , likely due to higher rates of uptake and accumulation (Islam et al., 2015). As^{3+} has a high binding affinity for thiol and sulfhydryl groups on proteins, and is known to disrupt the function of at least 200 enzymes, many of which are involved in cellular respiration and DNA synthesis and repair (Ratnaike, 2003). Given arsenic's wide range of cytotoxic effects, both single and multicellular organisms have evolved a variety of MRGs for arsenic detoxification (Crognale et al., 2017; Zhang et al., 2022). Some bacteria and archaea also possess arsenic-specific

MMGs to generate energy by using inorganic arsenic as an electron donor or acceptor (Crognale et al., 2017; Shi et al., 2020; Zhu et al., 2017). A more detailed introduction to microbial arsenic MRGs and MMGs can be found in Chapter 2.

1.2.2 Natural sources of arsenic contamination

In highly reducing aquifers, often derived from alluvial sedimentary deposits, the release of As^{3+} from mineral weathering processes can lead to the accumulation of high levels of dissolved arsenite, especially if groundwater flow rates are slow (Smedley & Kinniburgh, 2002). Ferric (Fe^{3+}) iron (hydr)oxides are major sinks for environmental arsenic (Smedley & Kinniburgh, 2002). Under anoxic conditions, the reduction of Fe^{3+} to Fe^{2+} results in the solubilization of iron (hydr)oxide minerals as well as any co-precipitated or adsorbed arsenic, a process known as reductive dissolution (Sullivan & Aller, 1996). Reduction of As^{5+} to As^{3+} also increases arsenic solubility, because As^{3+} is less strongly sorbed to mineral surfaces (Masscheleyn et al., 1991). The presence of organic matter and microbial activity can accelerate reductive dissolution and desorption, as Fe^{3+} and As^{5+} can be used as alternative electron acceptors in anaerobic respiration (Smedley & Kinniburgh, 2002). Seasonal variation of arsenic concentration by 10–25 $\mu\text{g/L}$ in a New Zealand river system has been linked to increases in microbial activity during summer months (McLaren & Kim, 1995), and arsenate respiration was estimated to support the mineralization of up to 14.2% of fixed carbon in a stratified lake system (Oremland et al., 2000).

Arsenic mobilization can also occur in oxidizing environments, under which arsenate is the predominant species. These instances are common in arid climates and in neutral-to-alkaline pH waters, which promote the desorption of As^{5+} from oxide and clay minerals. Similarly, geothermal hot springs and volcanic sediments can be associated with elevated (up to 15,000 $\mu\text{g/L}$) concentrations of As^{5+} (or As^{3+} , in reducing conditions), due to the high temperatures of these environments (Thompson & DeMonge, 1996; Welch et al., 2000).

1.2.3 Anthropogenic sources of arsenic contamination

Mining-contaminated groundwaters and mine drainage may have extremely high concentrations of arsenic (up to 850,000 $\mu\text{g/L}$) (reviewed in Smedley & Kinniburgh, 2002). Arsenic is often co-localized with valuable metals such as gold, silver, or tin in ores, and can be released from oxidized waste rock and tailings via acid mine drainage (see section 1.3.1) and other weathering

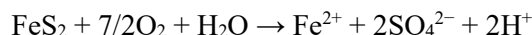
processes. The use of arsenic compounds in pesticides and fertilizers may also contribute to arsenic mobilization in agricultural runoff, although these practices have become less common in recent years (Bencko & Yan Li Foong, 2017).

Data on the distribution and biogeochemistry of arsenic associated with oil and petroleum deposits is limited, but elevated arsenic concentrations have been measured in groundwater associated with bituminous oil sands in Northeastern Alberta, Canada (Donner et al., 2017; Weinhold, 2011). *In-situ* bitumen extraction operations involving high-temperature steam injection can mobilize arsenic from sediments into surrounding aquifers, as heat conduction promotes dissolution and desorption of arsenic from minerals (see section 1.2.2) (Javed & Siddique, 2016). In Chapter 2, I describe a study on two such aquifers impacted by thermally mobilized arsenic, focusing on the microbiology of the system and potential for bioremediation.

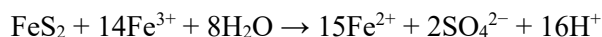
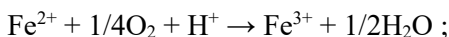
1.3 Biogeochemical cycling of metals in mining waste

1.3.1 Acid mine drainage

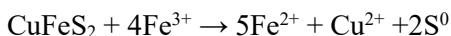
During mining operations, metal-bearing ores are excavated from an anoxic environment and exposed to air and moisture. Under these conditions, sulfide minerals (*e.g.*, pyrite, FeS_2) undergo the following reaction (Akcil & Koldas, 2006):



The generation of sulfuric acid lowers the pH of the solution, and when combined with sufficient oxidizing conditions, the ferrous (Fe^{2+}) iron ion will also oxidize to ferric (Fe^{3+}) iron. Fe^{3+} then becomes an oxidizing agent for the dissolution of additional pyrite (Akcil & Koldas, 2006):



Minerals such as pyrite are acid insoluble and undergo oxidative dissolution as shown above (Blowes et al., 2003). Ferric iron can also catalyze the oxidative dissolution of other metal-bearing minerals such as chalcopyrite (CuFeS_2) (Blowes et al., 2003):



The extent of sulfide mineral oxidation depends on its reactivity, described by its alteration index (Blowes et al., 2003). Acid soluble minerals such as chalcocite (Cu_2S) are susceptible to dissolution upon reacting with the sulfuric acid produced by sulfide oxidation (Blowes et al., 2003):



The net result of these reactions is the formation of Acid Mine Drainage (AMD): moderately ($\text{pH} < 7$) to extremely ($\text{pH} < 3$) acidic wastewater with high concentrations of heavy metals (Johnson & Hallberg, 2003). Release of AMD into soils and freshwater is highly detrimental to natural ecosystems and human health (reviewed in Simate & Ndlovu, 2014).

Microorganisms inhabiting mining wastes play various roles in the biogeochemical cycling of metals. The activity of acidophilic chemolithoautotrophs (*e.g.*, *Acidithiobacillus ferrooxidans*, *Leptospirillum ferrooxidans*, *Sulfolobus metallicus*) can significantly accelerate the rate of AMD formation by oxidizing ferrous iron (**Figure 1.1**) and reduced inorganic sulfur compounds (RISCs) (Johnson & Hallberg, 2003; Newsome & Falagán, 2021). If AMD wastewater is transported to anoxic

zones, iron-, and sulfur-reducing organisms (*e.g.*, *Acidiphilium* spp., *Desulfovibrio* spp.) may use dissolved ferric iron and sulfate as electron acceptors, resulting in reductive dissolution of secondary iron minerals and/or precipitation of sulfide minerals, depending on pH and redox conditions (Johnson & Hallberg, 2003). Heterotrophs may also be present and consume the organic compounds produced by autotrophs, which can have an indirect effect on metal cycling by detoxifying the environment, facilitating the colonization of autotrophs (Johnson & Hallberg, 2003). As a whole, mining waste microbial communities must adapt to the challenge of living in a harsh environment characterized by low pH and high concentrations of dissolved heavy metals, necessitating the use, acquisition, and/or evolutionary adaptation of MRGs.

1.3.2 Mine tailings

Mine tailings are generated when ores are crushed into smaller particles in order to extract valuable metals and minerals via flotation, density, and leaching (Blowes et al., 2003). The residual fine-grained minerals and waste rock are typically mixed with water, forming a high-volume slurry of tailings that is transported to a designated storage facility (Blowes et al., 2003). Excavated mining sites can be backfilled with tailings, and engineered structures such as dams, cover layers, and barriers are used to minimize the release of contaminants (Johnson & Hallberg, 2005).

The fate of metals in mine tailings is dependent on their exact composition and their storage conditions. Minerals in surface layers exposed to oxygen will undergo oxidative or acid dissolution, generating AMD (see section 1.3.1). Cover layers can limit oxidation and facilitate anaerobic microbial metabolisms such as sulfate reduction, promoting secondary mineral precipitation (Johnson & Hallberg, 2005). Acidic tailings can be neutralized with the addition of lime (calcium (hydr)oxides) or carbonate minerals, which also decreases the solubility of metals (Johnson & Hallberg, 2005).

Understanding the biotic and abiotic variables that control metal biogeochemical cycling in tailings is crucial to developing effective bioremediation strategies. Furthermore, there is an increasing incentive to find economically viable methods of extracting metals from wastes such as mine tailings in pursuit of a sustainable resource economy. In Chapters 3 and 4, I investigate microbial community composition, diversity, and MRG/MMG profiles along continuous vertical gradients of legacy copper/nickel mine tailings in Sudbury, Ontario. Chapter 5 explores a potential method of metal recovery from simulated waste solutions partially based on metal concentrations associated with these tailings.

1.4 Assessing microbial diversity

The development of molecular sequencing methods in the 1970s led to a revolution in our understanding of the evolution and diversity of life on Earth. Since the first phylogenetic tree of life (Pace et al., 1986), which placed 21 taxa based on SSU rRNA sequences, the amount of available genomic data has expanded to over 250,000,000 sequences, representing over 150,000 species (National Library of Medicine, 2024). Much of this growth can be attributed to environmental sequencing efforts, which enabled the discovery of many microbial lineages, including major phylum and superphylum-level radiations on the tree of life (Hug et al., 2016).

Sequencing-based approaches in environmental microbiology enable us to study species that lack cultured representatives, and to predict their roles within communities and ecosystems. Culture-independent methods can also be used to identify genes, pathways, and organisms of interest to study using culture-based methods, with various downstream applications in biotechnology and synthetic biology (reviewed in Costessi et al., 2018). A variety of methods are available, depending on the goal. 16S rRNA amplicon sequencing is a popular method for profiling bacterial and archaeal taxonomic diversity and is relatively inexpensive, because it only requires PCR amplification and sequencing of a single region of the evolutionarily conserved 16S rRNA gene (Hugerth & Andersson, 2017). Shotgun metagenomics involves untargeted sequencing of the total DNA present in a sample, followed by the optional assembly of reads into contigs, which may further be binned into metagenome-assembled genomes (MAGs) (reviewed in Quince et al., 2017). Unlike targeted marker gene sequencing, metagenomics enables the functional profiling of organisms via gene annotation, and has supported discoveries such as endosymbiotic lifestyles (Brown et al., 2015) and complete nitrification by *Nitrospira* spp. (Daims et al., 2015; van Kessel et al., 2015). Metatranscriptomics and metaproteomics can provide an even more comprehensive understanding of microbial activities in their natural environments by assessing gene expression. RNA can be converted to cDNA for sequencing, whereas protein sequencing uses liquid chromatography and tandem mass spectrometry (LC-MS/MS) of peptides to measure their mass/charge spectra; in both cases, data is typically mapped to metagenomes to identify transcripts and proteins (Bashiardes et al., 2016; Hettich et al., 2013).

16S rRNA amplicon and ‘-omics’ approaches have been crucial in advancing our understanding of microbial communities in natural environments, including those impacted by heavy

metal contamination (reviewed in Newsome & Falagán, 2021; Quince et al., 2017; Wilmes et al., 2015). Bioinformatic databases and tools for identifying MRGs (Pal et al., 2014) and MMGs (Dunivin et al., 2019; Garber et al., 2020) aid in functional profiling and prediction of microbe-metal interactions. However, environments such as arsenic-contaminated aquifers and mine tailings are highly variable in terms of the specific metals present, their concentrations, and other geochemical factors such as temperature and pH. Metal-contaminated environments are also typically low-biomass, making DNA, RNA, and protein extraction difficult, which can in turn result in downstream data being low quality and/or incomplete (Yuangen et al., 2004). The application of high-throughput sequencing and bioinformatic analyses to uncover previously uncharacterized diversity and functional potential in metal-contaminated environments is further explored in Chapters 2–4 through 16S rRNA amplicon, metagenomic, and metaproteomic sequencing.

1.5 Research objectives

The main objectives of my research were as follows:

1. Characterize microbial diversity in two heavy metal contaminated environments:
 - i. aquifer groundwater impacted by thermal arsenic mobilization (Chapter 2), and
 - ii. sulfidic copper-nickel mine tailings (Chapter 3).
2. Predict the functional capacity of microorganisms in metal biogeochemical cycling of As, Fe, Cu, and Ni in metal contaminated sites through identification of MRGs and MMGs (Chapters 2 and 4).
3. Evaluate a potential method of selective metal recovery from mining and industrial waste using the metal-cycling organism *Oleidesulfovibrio alaskensis* G20 (Chapter 5).

Chapter 2

Microbial diversity and capacity for arsenic biogeochemical cycling in aquifers associated with thermal mobilization

2.1 Introduction

Global health concerns regarding arsenic exposure are mainly tied to the contamination of groundwater used for drinking and agriculture (Mukherjee et al., 2006). Arsenic can be mobilized to groundwaters through natural sources, including hydrothermal activity and mineral weathering processes (reviewed in Masuda, 2018) and anthropogenic activities, including natural resource extraction (*e.g.*, mining) and rice agriculture. As a result, high concentrations of arsenic (above the WHO guideline of 0.01 mg/L for safe drinking water) are present in aquifers in many countries (World Health Organization, 2022). Chronic arsenic exposure is linked to cancer and skin lesions, among other adverse effects (World Health Organization, 2022). It is estimated that 94–220 million individuals are at risk of drinking arsenic-contaminated water (Podgorski & Berg, 2020).

The bioavailability and toxicity of inorganic arsenic is controlled by abiotic and biotic pathways that affect arsenic's redox state. Arsenic forms oxyanions in solution, as either the trivalent ($\text{As}^{(3+)}(\text{OH})_3$) or pentavalent ($\text{H}_2\text{As}^{(5+)}\text{O}_4^-$ or $\text{HAs}^{(5+)}\text{O}_4^{2-}$) species. As^{3+} is considered more toxic than As^{5+} (Akter et al., 2005), and is generally more mobile in natural environments due to its lower adsorption potential to minerals and sediments (Smedley & Kinniburgh, 2002). Arsenic speciation in groundwater is primarily determined by pH and redox potential: under acidic/circumneutral pH and reducing conditions, As^{3+} is the major dissolved species, while As^{5+} is predominant in high pH (>8.5) and oxidizing environments (Smedley & Kinniburgh, 2002). These variables also control whether arsenic is mobilized through reductive dissolution pathways or immobilized through precipitation and adsorption onto nearby sediments.

Microorganisms play a major role in arsenic biogeochemical cycling through direct and indirect mechanisms. Direct mechanisms involve the expression of genes that specifically interact with arsenic for detoxification or contributing to cellular metabolism (reviewed in Amend et al., 2014; Crognale et al., 2017; Zhu et al., 2017). The most common intracellular detoxification pathway involves the *ars* and/or *acr* operons, which are widely distributed in bacteria and archaea (Fu et al., 2009; Silver & Phung, 2005). The ArsB/Acr3 efflux pump expels intracellular As^{3+} and is coupled to the activity of the intracellular reductase, ArsC, which reduces As^{5+} to As^{3+} prior to efflux

(Mukhopadhyay & Rosen, 2002). Methylation of As^{3+} (by ArsM) to methylarsenite/methylarsenic acid (MAS^{3+}) is a microbial method of producing antibiotics to inhibit competitors, as MAS^{3+} is more toxic than the inorganic form (Chen & Rosen, 2020). However, As^{3+} methylation can also confer arsenic resistance if MAS^{3+} is oxidized to MAS^{5+} , which is less toxic than either inorganic form of arsenic and favoured in the presence of oxygen (Chen & Rosen, 2020).

Many bacteria and archaea are also able to obtain energy by using As^{3+} as an electron donor or As^{5+} as a terminal electron acceptor. As^{3+} oxidases include AioAB (present in various heterotrophs and chemolithoautotrophs) and ArxA (primarily in Gammaproteobacteria) (Shi et al., 2020). The dissimilatory (or respiratory) As^{5+} reductase ArrAB differs from ArsC, in that it is membrane-bound and reduces extracellular As^{5+} to As^{3+} as part of an electron transport chain (Basu et al., 2010; Malasarn et al., 2004), rather than for detoxification. Dissimilatory As^{5+} reduction is common in many microorganisms found in groundwater ecosystems (Crognale et al., 2017; Escudero et al., 2013).

Indirect mechanisms of arsenic biogeochemical cycling are connected to the iron and sulfur cycles, among others (reviewed in Zhu et al., 2017). The distribution and speciation of iron and arsenic in sediments are highly correlated, as arsenic is strongly sorbed to Fe^{3+} (oxy)hydroxides and a constituent of many Fe-bearing minerals, such as arsenopyrite (FeAsS) (Smedley & Kinniburgh, 2002). Fe^{3+} reduction and Fe^{2+} oxidation by microbes can lead to the dissolution or precipitation of these minerals, depending on pH and redox conditions, which controls the mobilization of arsenic (Zhu et al., 2017). Another important mechanism for attenuation of arsenic is dependent on sulfur cycling. Sulfate-reducing bacteria (SRB) produce biogenic sulfide, leading to the subsequent formation of arsenopyrite and other arsenic-bearing sulfide minerals (*e.g.*, realgar, As_4S_4 ; orpiment, As_2S_3) (Newman et al., 1997; Rittle et al., 1995). Although biological arsenic cycling has been well-studied in the context of natural resource extraction in mining and mine tailings (Akter et al., 2005; Pakostova et al., 2023; Zhuang et al., 2023), how microbial activities impact the fate of arsenic in petroleum extraction operations remains understudied.

We are addressing this gap by examining the biogeochemical cycling of arsenic at a model petroleum extraction site in Northern Alberta. This site uses steam to extract the bitumen from heavy oil deposits. During this process, high temperature steam ($\sim 300^\circ\text{C}$) is injected to the reservoir to liquify the bitumen and subsequently pump it to surface facilities. Neither the bitumen nor steam are

directly released into aquifers above the natural deposits during extraction. However, conductive heating of the sediments surrounding the steam injection site results in mobilization of arsenic naturally present in sediments into groundwater (Moncur, 2018). Desorption from metal oxides is the dominant mechanism of thermal arsenic mobilization (Craig et al., 2021), but reductive dissolution of arsenic-bearing minerals also accelerates under higher temperatures (Johnston et al., 2020; Weber et al., 2010). Previous work in aquifers proximal to the study sites showed that As^{3+} dominates the mineral composition in both high (>7 mg/kg) and low (<7 mg/kg) arsenic-bearing sediments (Javed et al., 2014). Broader surveys of the same region showed dominance of As^{3+} in groundwater but detected As^{5+} associated with Fe-oxyhydroxides in oxidized sediments and As^{3+} characteristic of arsenopyrite in sulfidic sediments obtained from shallower depths (i.e., <15 m) (Moncur et al., 2015). Experiments on sediment cores near *in-situ* thermal recovery operations wells showed that heating of sediments greatly increased arsenic concentrations in porewater, from a baseline of <0.025 mg/L up to a maximum of 5.20 mg/L (Javed & Siddique, 2016; Moncur et al., 2015). Dissolved arsenic was tied to the exchangeable and specifically adsorbed fraction associated with amorphous Fe-oxides, while arsenic associated with crystalline Fe-oxides, silicates, sulfides, and organic matter were not major contributors to mobilizing arsenic into the aqueous phase (Javed & Siddique, 2016). Although these studies highlight how abiotic processes associated with the steam injection process impact arsenic concentrations within the local aquifer system (Javed et al., 2014), how this mobilized arsenic affects microbial community dynamics and microbial arsenic cycling pathways remains unexplored.

In this study, we investigate microbial diversity and activities in groundwater aquifers in response to thermally mobilized arsenic from an *in-situ* bitumen extraction operation. A combined 16S rRNA amplicon and metagenomic sequencing approach was used to profile the taxonomic composition of communities and predict potential interactions between individual organisms and arsenic. We describe the contributions of microbes to direct arsenic cycling within the system (via predicted resistance mechanisms and metabolic redox transformations) as well as the biogeochemical cycling of other elements that may influence arsenic mobility (e.g., Fe and S). These findings are discussed in the context of designing effective bioremediation strategies for arsenic-contaminated aquifers.

2.2 Methods

2.2.1 Site properties and sampling

The study sites are located within Northeastern Alberta, Canada, in an area containing a series of non-saline aquifers in the Quaternary deposits. *In-situ* extraction of bitumen via high-temperature steam injection is ongoing in the bitumen reservoir, located about 350 m below the aquifers (Andriashek, 2003).

Sampling of groundwater took place in August 2021 (average monthly temperature: 17.5 °C; total monthly precipitation: 35.3 mm) (Environment and Climate Change Canada, 2024) across two transects associated with steam injection pads: Site A and Site B. The two sites are approximately 9 kilometers apart. The Site A aquifer is located approximately 80 m below ground and has a water flow rate of ~100 m/year. The aquifer has experienced historic arsenic plumes from past steam cycles (1990–2010), but these plumes have mostly dispersed. Site B's aquifer is located at a depth of ~160 m and has a much slower flow rate (<5 m/year). Site B is currently experiencing slow-moving plumes of arsenic from steaming during 2003–2013.

Water (100 L) was pumped from a series of wells along each transect (five wells along Site A, six wells along Site B), and immediately filtered through a Waterra 0.1-micron filter (CAP600X-0.1). Five wells were sampled from Site A (A-01, 02, 03, 04, 07), and six were sampled from Site B (B-02, 03, 04, 05, 06, 07); samples are numbered in order from the well closest to the site of steam injection and proceeding downstream (**Figure 2.1**). The filters were purged with air and 50 mL RNAlater (Invitrogen AM7021) was added to preserve biomass prior to filters being transported on ice to the University of Waterloo where they were stored at –80 °C.

Geochemical assessments of temperature, pH, Eh, and electrical conductivity (EC) were measured on-site during groundwater filtering. Additional groundwater was analyzed by Bureau Veritas and Brooks Applied Labs for concentrations of major cations, anions, alkalinity, and dissolved carbon (see page 4 of **Data File A1** for analysis techniques). Speciation data for dissolved iron ($\text{Fe}^{2+}/\text{Fe}^{3+}$) was measured via a colorimetric assay (SM 3500, **Data File A1**) and arsenic speciation ($\text{As}^{3+}/\text{As}^{5+}$) was measured by IC-ICP-MS (BAL–4100, **Data File A1**).

All wells were sampled again in August 2022 (average monthly temperature: 19.1 °C; total monthly precipitation: 28.7 mm) (Environment and Climate Change Canada, 2024) following the

same protocol, with the exception of the volume of water filtered (increased to a target of 150 L, with 100–160 L filtered depending on whether the filter clogged).

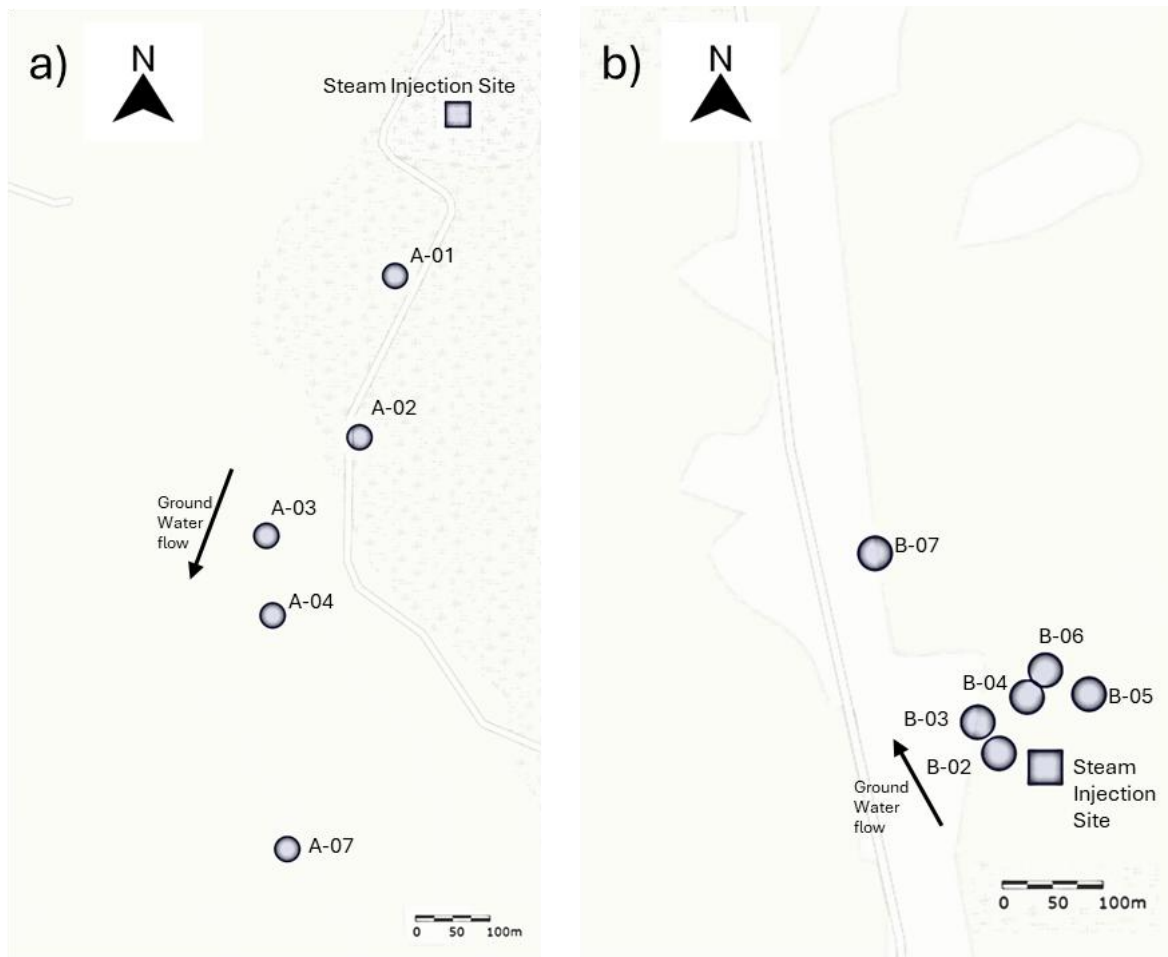


Figure 2.1: Schematic map of sampling well distribution across a) Site A and b) Site B.

Individual groundwater monitoring wells are numbered in order based on their approximate distances from the steam injection site, with respect to the water flow path (indicated with arrows). We sampled two different Northeastern Alberta aquifers, one per site. At Site A, we sampled an aquifer ~80 m below ground level, which was impacted by steaming that occurred between 1990–2010. At Site B, we sampled an aquifer ~160 m below ground level, which was impacted by steaming that occurred between 2003–2013. The two sites are separated by ~9 km distance.

2.2.2 DNA extraction

Waterra filters were thawed at room temperature for 1–2 hours. After purging the RNA later through the outlet, the filter membrane was rinsed with 50 mL of Longmire (minus SDS) buffer (0.1 M Tris-HCl, 0.1 M EDTA, 0.01 M NaCl) three times, vortexed for 30 s, and the buffer was collected out of the inlet. The combined 150 mL of buffer with resuspended biomass was filtered onto a Stericup vacuum filter (Millipore S2VPU02RE, 0.1 μ m pore size), and the filter membrane was cut into several pieces using a sterile scalpel. Approximately one-third of each filter was directly transferred to the QIAGEN DNeasy PowerSoil Pro Kit for DNA extraction; the remainder was stored in 5 mL centrifuge tubes at -80°C .

DNA was eluted in 60 μ L of 10 mM Tris and stored at -20°C . 20 μ L was used for 16S rRNA amplicon sequencing. Due to low concentrations (<3 ng/ μ L) in 6 out of 11 samples, another third of the filters were used for DNA extraction and combined with the remaining DNA from the first extraction for metagenomic sequencing. Final DNA quantities sent for metagenomic sequencing ranged from 35.2–187.2 ng.

2.2.3 16S rRNA amplicon sequencing and analysis

Samples were PCR-amplified and sequenced by MetagenomBio Life Science Inc. using the Illumina MiSeq machine (v2 reagents). Modified universal primers targeting the V4 region were used for amplification (forward primer 515FB: 5' GTGYCAGCMGCCGCGGTAA; reverse primer 806RB: 5' GGACTACNVGGGTWTCTAAT) (Walters et al., 2015).

Processing and analysis of sequencing data was performed within the QIIME2 platform (v.2022.2) (Bolyen et al., 2019). Paired-end reads were filtered, trimmed, merged, and grouped into amplicon sequence variants (ASVs) with DADA2 (Callahan et al., 2016). Taxonomy was assigned based on the SILVA 138 SSU database (Yilmaz et al., 2014). Alpha (α) and beta (β) diversity were computed using the QIIME2 core phylogenetic diversity metrics tool.

Data (including ASV abundance, taxonomy, diversity tables/matrices, and metadata) were exported for further processing in Python v3.8.8 using the pandas v1.4.4 library (McKinney, 2010). Multivariate principal components analysis (PCA) was evaluated with correspondence analysis (CA) between samples and features (ASVs). Principal coordinates (PCoA) was performed on Bray-Curtis, unweighted UniFrac, and weighted UniFrac distance matrices between samples, with respect to

features. Ordinations and statistical analyses were performed using the scikit-bio v0.5.8 package (Bradbury et al., 2022) in Python, except for environmental fitting, which was done using the vegan v2.6-4 library (Oksanen, 2010) in R v4.2 (R Core Team, 2021). Figures were generated using the seaborn (Waskom, 2021) and matplotlib v3.6.0 (Hunter, 2007) libraries.

2.2.4 Metagenomic sequencing, assembly, and binning

Sequencing was performed at The Center for Applied Genomics (TCAG) (Toronto, Ontario), using PCR-amplified library preparation and 2 lanes of an Illumina NovaSeq SP flowcell (PE2x150bp). Raw reads were decontaminated using BBduk (Bushnell, 2014), and sickle v1.33 (Joshi & Fass, 2011) was used to trim/filter low-quality reads.

Reads were assembled using SPAdes v3.15.5 (Bankevich et al., 2012) with the --meta flag for metagenomes. Assembled scaffolds were filtered to a minimum length of 2,500 bp using pullseq v1.0.2 (Thomas, 2015).

Read mapping was done with Bowtie2 v2.3.4.1 (Langmead & Salzberg, 2012) to generate coverage information for binning. Three binning algorithms were used: CONCOCT (Alneberg et al., 2014), MetaBAT 2 (Kang et al., 2019), and MaxBin 2.0 (Wu et al., 2016b). The dereplication, aggregation, and scoring tool (DAS Tool) (Sieber et al., 2018) was used to select the best set of high-quality non-redundant bins from the candidate bins. CheckM (Parks et al., 2015) was used to filter bins for >70% completeness and <10% contamination as the final set of high-quality bins.

2.2.5 Taxonomic classification and gene annotation

Taxonomy of MAGs was assigned using the Genome Taxonomy Database Toolkit (GTDB-Tk v2.1.0) (Chaumeil et al., 2020). A phylogenetic tree was generated using GToTree v1.6 (Lee, 2019), and visualized using the online interactive Tree of Life (iTOL) (Letunic & Bork, 2021).

Annotation of arsenic-related genes was performed with the meta-arsenic toolkit (Dunivin et al., 2019), which incorporates Hidden Markov Models (HMMs) and BLAST database information to identify the following arsenic resistance and respiratory genes: *acr3*, *aioA*, *arsB*, *arsC*, *arsD*, *arsM*, *arrA*, and *arxA*. FeGenie (Garber et al., 2020) was used to annotate iron-related genes, also using HMM databases. Distilled and Refined Annotation of Metabolism (DRAM v1.5.0) (Shaffer et al., 2020) was used to profile the overall metabolism of MAGs, with annotations labeled by Pfam (Protein families database) (El-Gebali et al., 2019) and KEGG (Kyoto Encyclopedia of Genes and

Genomes) (Kanehisa et al., 2017) identifiers. Sulfur-related genes were filtered from the resulting output for prediction of MAGs with sulfur-cycling capacities.

All data from meta-arsenic, FeGenie, and DRAM were exported to Python (pandas library). Arsenic-, iron-, and sulfur-related gene frequencies were aggregated into pathways (*e.g.*, oxidation, reduction), expressed as a binary (indicating presence/absence), and then normalized to MAG % relative abundance as calculated using average scaffold coverage data as a proxy for the prevalence of pathways in each sample. Figures were generated using matplotlib.

2.2.6 Prediction of host-symbiont interactions

To identify potential hosts for the abundant Paceibacteria MAG CLB2-064 population within well B-02, we performed a co-occurrence analysis using the R package propr v2.1.2 (Quinn et al., 2017), which calculates proportionality of compositional data (such as MAG abundances). Bowtie2 coverage information, as a proxy for abundance, for all scaffolds >10 kb from the well B-02 metagenome was compared to the average coverage of CLB2-064 scaffolds. A cutoff of $\rho=0.9$ was used to determine significant proportionality between scaffolds, which corresponded to a false discovery rate (FDR) of 0.047. An additional analysis was performed between Paceibacteria CLB2-064 abundance and the abundance of all high-quality bins within B-02. In this case, proportionality was assessed between average scaffold coverage of all bins instead of between CLB2-064 and individual scaffolds.

2.2.7 Data availability

All sequence data from this research have been submitted to the NCBI nr and WGS databases under BioProject PRJNA1111131 and BioSamples SAMN41383914-SAMN41383940. The 16S rRNA reads are available in the SRA database under accessions SRR29010669-SRR29010695. The metagenome reads are available in the SRA database under accessions SRR29054603-SRR29054613. Metagenome assembled genomes are available in the WGS database under accessions SAMN41876582-SAMN41876705 (well A-01); SAMN41876706-SAMN41876780 (well A-02); SAMN41876781- SAMN41876875 (well A-03); SAMN41876876- SAMN41876953 (well A-04); SAMN41877016- SAMN41877096 (well A-07); SAMN41877097- SAMN41877206 (well B-02); SAMN41877207- SAMN41877317 (well B-03); SAMN41877446- SAMN41877578 (well B-04); SAMN41877579- SAMN41877721 (well B-05); SAMN41877793- SAMN41877969 (well B-06); SAMN41877970- SAMN41878060 (well B-07).

2.3 Results and discussion

2.3.1 Aquifer arsenic geochemistry

Two aquifers in Northeastern Alberta were sampled along transects moving outward from steam injection sites (see **Figure 2.1** for schematic map of the transects). The Site A transect sampled an aquifer ~80 m below ground level, which was impacted by steaming occurring between 1990–2010. The Site B transect sampled an aquifer ~160 m below ground level, which was impacted by steaming occurring between 2003–2013.

All samples from Sites A and B fell within circumneutral pH (6.76–7.95). Reduction potentials (Eh) ranged from reducing (0 to +200 mV) to highly reducing (–200 to 0 mV), indicative of anoxic conditions (**Table 2.1**).

At Site A, arsenic plumes have dispersed but arsenic concentrations remain elevated above background. Site B is currently experiencing elevated arsenic concentrations at some wells. Dissolved arsenic concentrations in 2021 ranged from 0.016–0.035 mg/L in Site A and 0.016–0.25 mg/L in Site B. These values were generally stable from 2021 to 2022 (**Table 2.1**). The majority of dissolved arsenic is present as As^{3+} , which aligns with the predicted equilibrium of arsenic speciation based on pH and Eh values (Akter et al., 2005).

Table 2.1: Groundwater geochemistry measurements from sampled wells. Geochemical factors with measurable values in all wells in both 2021 and 2022, and which are predicted to influence arsenic biogeochemical cycling are included. Values are reported in mg/L unless otherwise indicated. The associated p-values from environmental fitting of factors to sample ordination scores (from PCoA; see **Figure A1**) are reported in the bottom 3 rows; significant p-values (<0.05) are indicated with an asterisk.

Sample	Year	pH	Eh (mV)	Temp. (°C)	Ammonia (as N)	Arsenic (total)	As ³⁺	As ⁵⁺	DOC	Fe ²⁺	Mn	SO ₄	Alkalinity (as CaCO ₃)
A-01	2021	7.51	-55.3	15.3	1.1	0.016	0.0145	0.00105	5.0	2.0	0.095	28	490
A-02	2021	7.48	-61.0	15.0	1.2	0.023	0.0165	0.00452	4.4	1.9	0.088	26	490
A-03	2021	7.06	-23.5	12.3	1.3	0.022	0.0195	0.00152	5.4	2.2	0.099	32	470
A-04	2021	7.32	-48.6	16.0	1.3	0.035	0.0307	0.00221	5.2	2.1	0.10	27	470
A-07	2021	6.94	35.7	11.2	1.1	0.029	0.0239	0.00380	6.5	1.5	0.12	22	470
B-02	2021	6.76	-129.9	26.3	2.9	0.17	0.155	0.00966	5.5	4.0	0.20	38	530
B-03	2021	7.23	-133.3	16.3	2.8	0.25	0.243	0.0104	5.3	2.5	0.20	48	580
B-04	2021	7.41	-110.7	32.6	2.6	0.025	0.0202	0.00170	4.8	0.28	0.035	53	550
B-05	2021	7.23	-130.3	26.0	3.1	0.08	0.0736	0.00307	5.7	1.2	0.074	52	540
B-06	2021	7.21	-47.4	22.5	2.7	0.016	0.0121	0.00293	5.7	0.12	0.084	50	570
B-07	2021	7.77	-140.1	8.6	2.2	0.036	0.0327	0.00183	4.8	1.5	0.13	59	530
A-01	2022	7.20	-99.5	14.7	1.1	0.014	0.0169	0.00105	8.1	2.1	0.090	27	510
A-02	2022	7.38	-102.8	12.7	1.3	0.018	0.0196	0.00380	7.2	1.9	0.085	28	500
A-03	2022	7.50	155.2	10.5	1.2	0.019	0.0243	0.00174	6.5	2.3	0.096	38	490
A-04	2022	7.37	160.7	14.0	1.3	0.025	0.0367	0.00283	8.2	2.2	0.10	29	480
A-07	2022	7.10	69.4	11.2	1.2	0.024	0.0289	0.00612	6.9	1.7	0.12	28	480
B-02	2022	7.16	-142.5	24.7	3.0	0.15	0.176	0.0135	8.6	4.2	0.20	44	540
B-03	2022	7.45	-150.3	15.0	3.3	0.24	0.289	0.0143	7.0	3.2	0.24	63	590
B-04	2022	7.46	-109.1	31.5	2.8	0.024	0.0266	0.00351	6.8	0.49	0.049	55	550
B-05	2022	7.70	-138.9	27.2	3.1	0.067	0.0687	0.00453	6.1	1.2	0.079	58	560
B-06	2022	7.95	44.2	23.9	2.7	0.014	0.0126	0.00329	6.0	0.10	0.077	54	570
B-07	2022	7.78	-153.0	6.9	2.4	0.029	0.0355	0.00541	5.9	1.5	0.13	67	540
p (Bray-Curtis)		0.918	0.134	0.001*	0.001*	0.06	0.085	0.125	0.975	0.736	0.822	0.011*	0.001*
p (Unweighted UniFrac)		0.797	0.029*	0.001*	0.001*	0.041*	0.051	0.059	0.847	0.882	0.561	0.004*	0.001*
p (Weighted UniFrac)		0.488	0.291	0.009*	0.035*	0.144	0.17	0.241	0.588	0.703	0.689	0.527	0.246

2.3.2 Sequencing statistics

A total of 307,306 16S rRNA amplicon reads were processed with QIIME2, resulting in 3,837 ASVs, with a total frequency of 253,383 reads, averaging 23,035 reads per sample (**Table 2.2**). Frequencies of individual ASVs across all samples ranged from 2–24,217, with a mean frequency of 66.

We identified 1,219 high-quality MAGs (ranging from 75–178 per sample, average of 107) after assembly and binning of metagenomic sequencing data (**Table 2.2**).

Table 2.2: 16S rRNA amplicon and metagenomic sequencing statistics by sample. 16S rRNA amplicon reads were processed in QIIME2 to filter, merge, and group non-chimeric sequences into amplicon sequence variants (ASVs). Assembly of metagenomic reads was performed with SPAdes3. Scaffolds were binned into metagenome-assembled genomes (MAGs) using MetaBAT2, CONCOCT, and MaxBin2, and DAS Tool was used to identify the highest quality set of non-redundant bins among the three binning algorithms. CheckM was used to filter for MAGs with >70% completeness and <10% contamination as the final set of high-quality (HQ) bins. For detailed information on each MAG see **Data File A2**.

Sample	16S rRNA amplicon			Metagenomic				
	Reads	Merged (%)	ASVs	Reads	Assembled (%)	Scaffolds	Scaffolds binned	HQ bins
A-01	32,051	82.21	26,349	98,175,715	57.26	2,254,761	23,430	124
A-02	22,597	87.53	19,779	78,424,298	59.17	1,980,164	14,751	75
A-03	21,240	82.66	17,556	80,582,181	60.94	1,747,940	16,719	95
A-04	29,182	81.22	23,702	79,079,594	37.83	2,817,451	19,902	78
A-07	33,649	78.77	25,506	76,232,243	42.54	2,404,865	12,706	81
B-02	32,971	91.20	30,068	74,120,634	75.90	1,231,811	18,924	110
B-03	32,547	82.34	26,800	80,071,928	61.69	1,838,287	22,178	111
B-04	29,191	76.45	22,316	85,422,107	58.94	2,254,958	18,571	133
B-05	16,606	83.53	13,871	75,829,097	65.89	1,678,150	25,780	143
B-06	21,952	81.73	17,941	100,182,557	56.48	2,677,247	29,888	178
B-07	35,320	80.68	28,495	94,383,591	38.46	3,412,804	17,078	91
Total	307,306	N/A	253,383	922,503,945	N/A	24,298,438	219,927	1,219

2.3.3 Microbial community overview

Community composition

Overall, the taxonomic composition of each well remained relatively stable between 2021 and 2022 samples, as determined by 16S rRNA ASV frequencies (**Figure 2.2**). The 2021 metagenomic composition data, determined by average scaffold coverage of MAGs, was also consistent with the 2021 ASV frequencies (**Figure 2.2**). Well B-07 was a slight exception, with a high abundance of Cyanobacteria ASVs (classified under f. Nostocaceae) detected in 2021 (see **Figure 2.2**). This unexpected result could be explained by contamination by plant matter, where plastid DNA was amplified during 16S rRNA amplicon library preparation.

Community composition among samples collected in Site A were generally similar at the phylum-level, which was expected based on the stability of geochemical conditions across the transect following migration of the arsenic plume downgradient (**Table 2.1**). Arsenic concentrations were relatively low in all wells but slightly above background concentrations (0.022 mg/L) in wells A-02, A-04, and A-07, as the site has not experienced recent steaming. Well A-07 was most recently impacted by the plume (2013), and had a higher proportion of Proteobacteria (now Pseudomonadota) (68% in 2022 vs. 4-38%) and lower proportion of Chloroflexota/Omnitrophota (<2% in 2022 vs. 3–36%) compared to other Site A wells (**Figure 2.2**). It is possible that the community hadn't stabilized at well A-07 at the times of sampling, or that the slightly higher redox potential (**Table 2.1**) supports different microbial guilds at this location (discussed below).

In contrast, Site B wells had high variability in both arsenic concentration and the dominant phyla present (**Figure 2.2**). B-02 and B-03 were experiencing elevated arsenic concentrations (0.15–0.25 mg/L) at the time of sampling from the most recent steam cycle, which held stable across 2021 and 2022 due to the slow flow rate (<5 m/year) of the aquifer. Well B-02 contained a high relative proportion of Patescibacteria (~80%) that was not observed in any other sample, mainly from a single population within the Class Paceibacteria. GTDB-tk classified the Paceibacteria MAG (CLB2-064) under family/order UBA6257; other members of this clade have primarily been found in groundwater environments (Zhao et al., 2022). The Patescibacteria (formerly Candidate Phyla Radiation) is a recently described phylum, whose members are characterized by small cell sizes and highly reduced genomes, with many metabolic pathways incomplete or absent (Brown et al., 2015; Fujii et al., 2022;

Tian et al., 2020). They are believed to be obligate symbionts (likely epibiotic parasites) of other bacteria and/or archaea (Kuroda et al., 2022).

Well B-03 also had a distinct community composition compared to all other samples. Well B-03 was dominated by two taxa within the phylum Nitrospirota, with MAG relative abundances of 15.43% and 24.54% respectively. Both MAGs (CLB3-019 and CLB3-097) were classified under order Thermodesulfobirionales (c. Thermodesulfobirionia), which contain obligately anaerobic, thermophilic sulfate reducers (Umezawa et al., 2021). Both MAGs were classified to unnamed families. Wells B-04, B-05, and B-06 had similar microbial compositions across both years. As with Site A, higher concentrations of arsenic previously occurred in these wells (*i.e.*, from 2005–2010), and the communities appear to have stabilized following a decrease in arsenic concentration. Notably, the community composition in Well B-07 shows similar phylum-level groups but differing abundances compared to Wells B-04, 05, and 06. Well B-07 is much farther downgradient from the steam injection site than the other wells and has not yet experienced elevated arsenic concentrations. B-07 could therefore be considered a control sample for Site B aquifer communities prior to steaming and the resultant heat and arsenic exposures. While wells B-04, B-05, and B-06 appear to be returning to this background community composition, there remains substantial disruption to communities in Site B currently experiencing elevated arsenic concentrations (B-02, B-03).

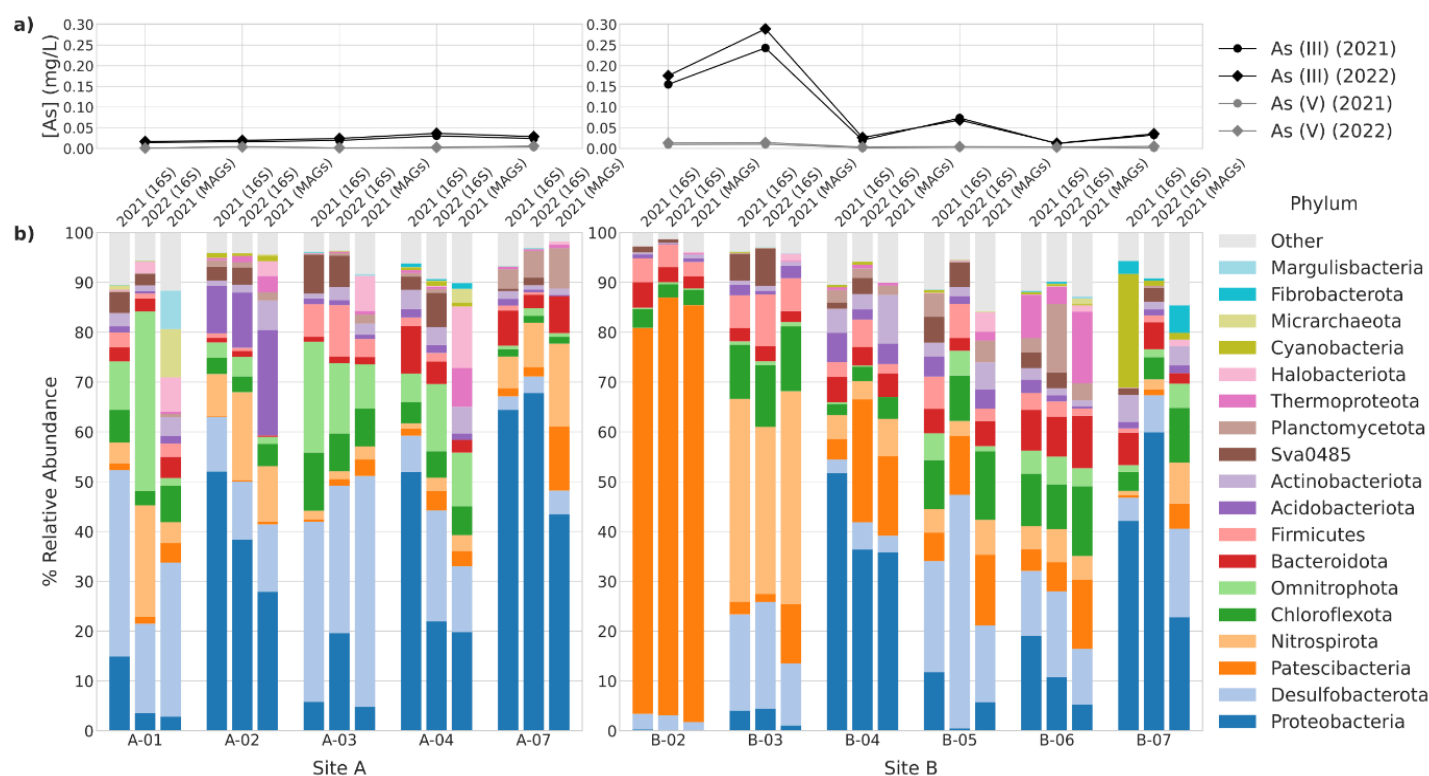


Figure 2.2: Microbial community composition and arsenic concentrations of groundwater along two aquifer transects. The concentration of dissolved arsenic species (a) for each sample is indicated in mg/L. Barplots (b) indicate phylum-level relative abundances of microbial taxa. Three bars are shown for each sample, representing the 2021 16S rRNA sequencing, 2022 16S rRNA sequencing, and 2021 metagenomic sequencing data in that order. Samples within each site are ordered left to right by the direction of water flow, starting with the well closest to the site of steam injection and proceeding downstream. Phyla that were present at a relative abundance >5% in at least one sample in any dataset are shown. Relative abundances were calculated using relative ASV frequencies for 16S rRNA amplicon data and scaffold coverage information for metagenomes. Taxonomy of ASVs were assigned using the SILVA138 database while MAGs were classified with GTDB-tk (v2.1.0). In cases where the nomenclature differed between the databases, the GTDB label is shown. Note that the phylum ‘Sva0485’ (SILVA classification) only appears in the 16S rRNA amplicon datasets. In GTDB this clade is listed under the phylum ‘SZUA-79’, which was not assigned to any of the MAGs; it is likely that the GTDB-tk classifier assigned the related MAGs’ taxonomy under Desulfobacterota, as they are closely related lineages. Note that nomenclature for the Proteobacteria (Pseudomonadota), Firmicutes (Bacillota), and Cyanobacteria (Cyanobacteriota) have been updated in more recent versions of GTDB-tk.

Diversity

All diversity metrics were calculated from 16S rRNA amplicon sequencing data, with both 2021 and 2022 samples included.

Overall, there were no significant differences in α -diversity between sites (sampled within the same year) and between samples of the same aquifer in different years (**Figure 2.3**). Well B-02 is consistently an outlier, being the lowest in α -diversity, due to the single *Patescibacteria* ASV comprising the majority of the community (**Figure 2.2**). Well B-03's α -diversity is considerably higher compared to B-02 (comparable to other samples in Site B), despite having the highest total arsenic concentration, indicating that arsenic concentration itself is not deterministic of a severe reduction in community richness/evenness.

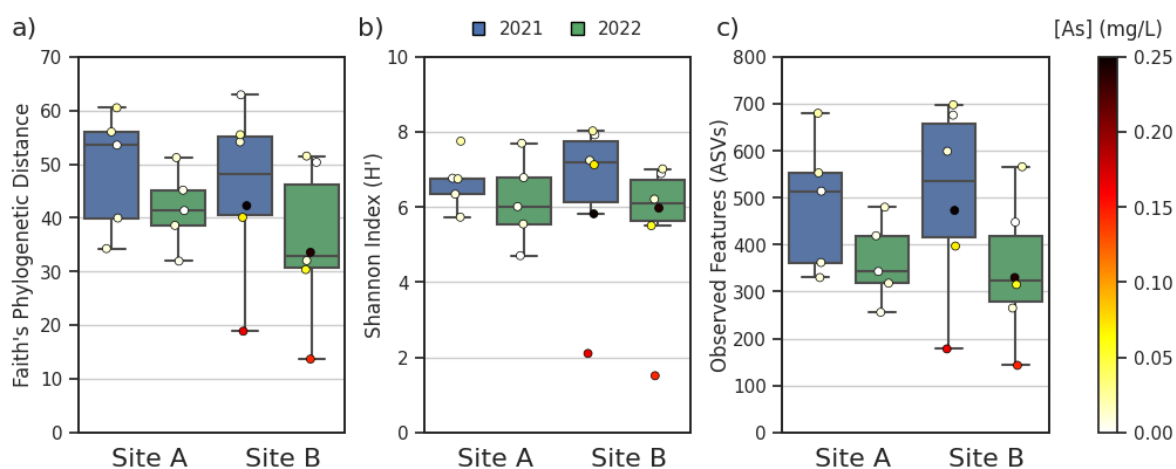


Figure 2.3: Alpha diversity of groundwater samples from two aquifer transects. Measures of Faith's phylogenetic distance (a), Shannon diversity index (b), and number of observed ASVs (c) are summarized for samples from each aquifer (Site A and Site B), taken from the same set of wells in 2021 and 2022. Data was rarefied to the minimum feature count (9,869). Pairwise comparisons of alpha diversity metrics showed no significant differences between aquifers within the same year, nor between 2021 and 2022 samples from the same aquifer (Kruskal-Wallis, $p > 0.05$). Individual samples are colored by the total arsenic concentration measured at the well.

A correspondence analysis was performed to compare the χ^2 distances between the samples and features (ASVs) (**Figure 2.4**). Correspondence analysis supports the community composition data (**Figure 2.2**) indicating that the A-07, B-02, and B-03 community compositions are highly divergent from each other, represented by the distance between the samples on the ordination axes.

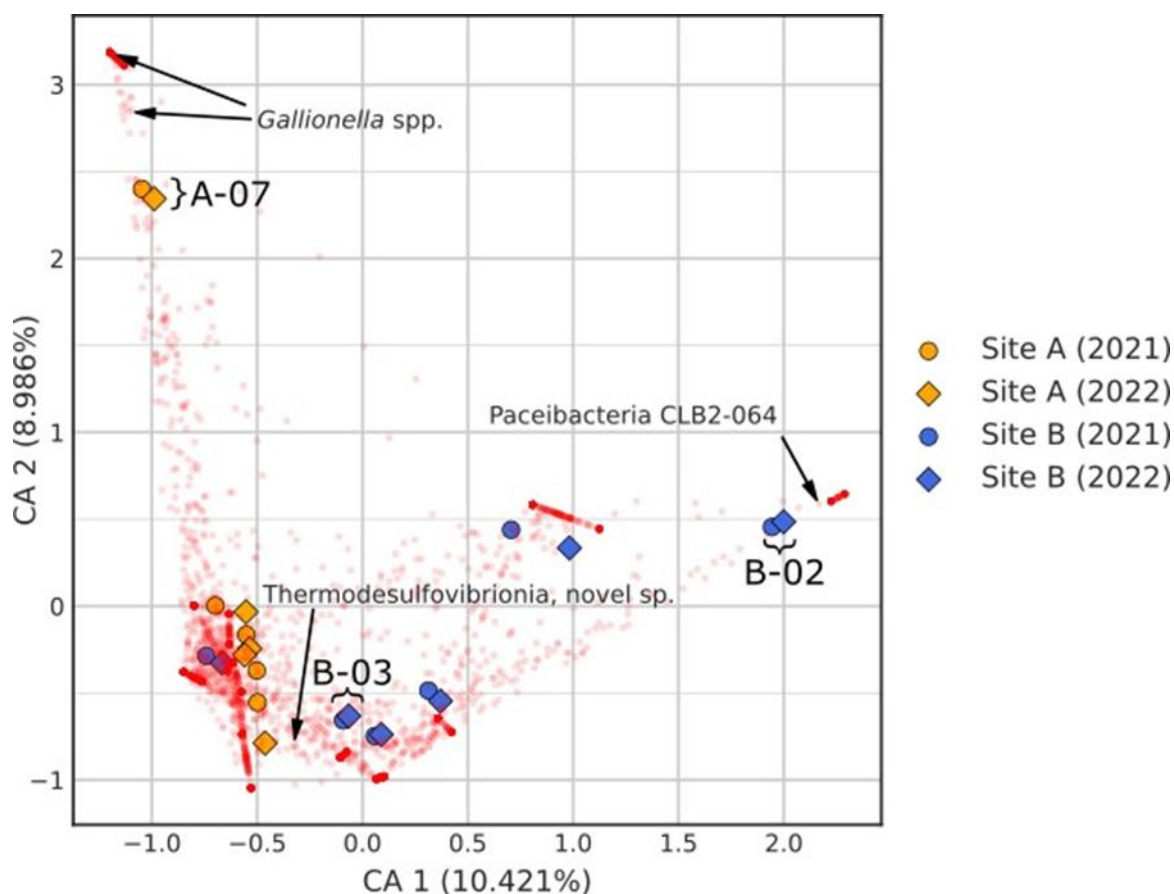


Figure 2.4: Correspondence Analysis (CA) biplot. Red points indicate the position of features (ASVs) with respect to the CA axes. Features of interest are annotated with arrows: a novel *Paceibacteria* ASV (corresponding to the metagenome-assembled genome *Paceibacteria* CLB2-064), high abundance in well B-02; a novel *Thermodesulfovibrionia* ASV, high abundance in well B-03; and various novel *Gallionella* ASVs, high abundance in well A-07. Samples of interest (A-07, B-02, B-03) associated with the indicated features are annotated with brackets.

β -Diversity was evaluated with principal coordinates analysis (PCoA) using Bray-Curtis and unweighted/weighted UniFrac distance matrices (**Figure A1**). Unlike α -diversity, all β -diversity ordinations showed a significant difference between sites (Analysis of Similarities (ANOSIM), $p < 0.05$), indicating that the specific microbial populations present at Sites A and B differ. There were no significant differences between years.

To evaluate the relationship between community composition and environmental variation across samples, we projected geochemical factors onto PCoA ordination plots as loading vectors. The unweighted UniFrac plot (**Figure 2.5**) includes a subset of geochemical variables (twelve total, listed

in **Table 2.1**), which were selected based on the following criteria: 1) variables that were measured in both 2021 and 2022, 2) values were above the detection limit in all wells, and 3) the variable was either predicted to influence arsenic biogeochemical cycling (*e.g.*, Fe, SO_4^{2-}) or major microbial metabolisms (*e.g.*, available C/N species). Significant variables were determined by environmental fitting (see section 2.2.3) of values to PCoA ordinations.

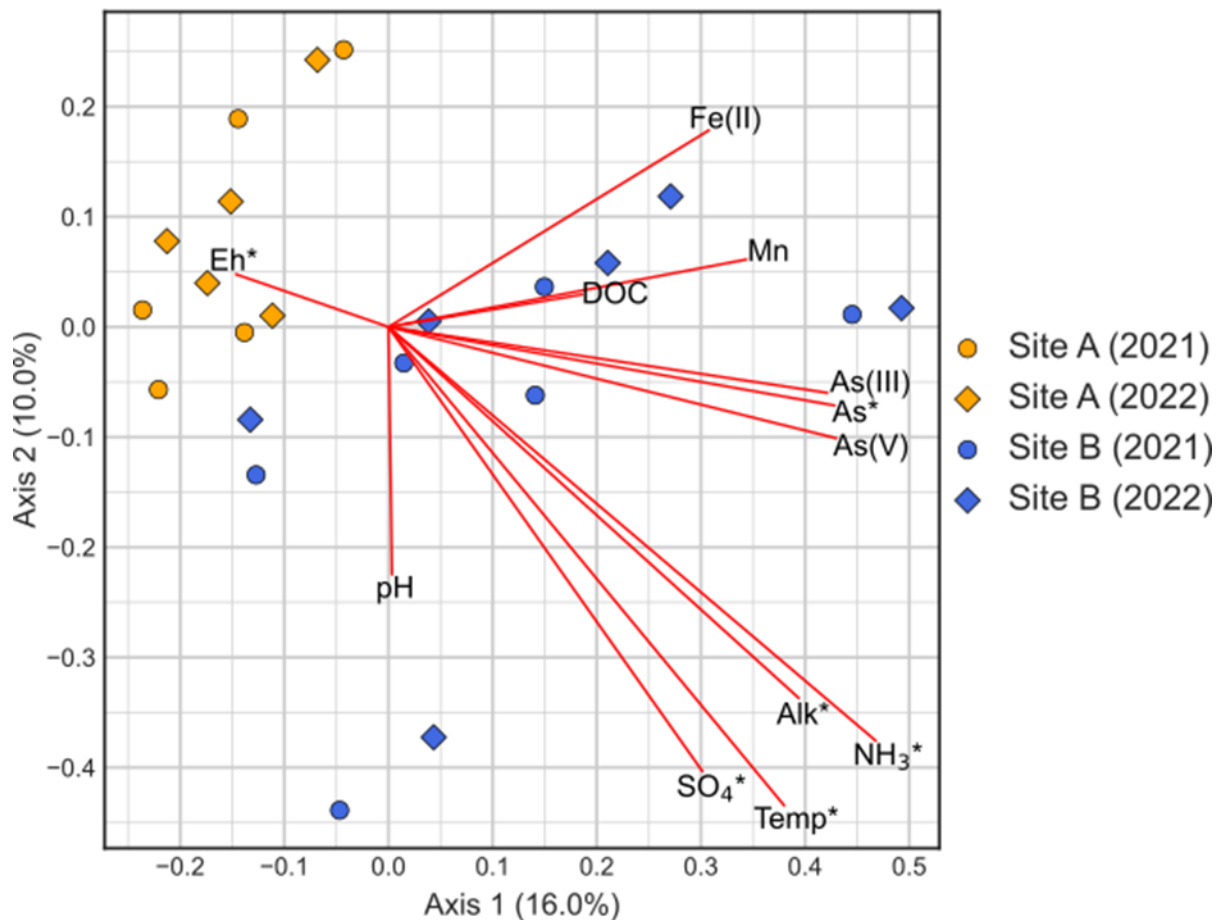


Figure 2.5: Principal Coordinates Analysis (PCoA) biplot of unweighted UniFrac dissimilarity. Samples are indicated by points, with colors representing sites and shape representing year. Axis labels indicate the proportion of variation explained. Vectors (red) indicate the projection of selected environmental factors onto the ordination, and are scaled to the maximum/minimum values of each axis. Significant variables ($p < 0.05$, see **Table 2.1**) are labelled with an asterisk. Abbreviation key: Temp=Temperature, Alk=Alkalinity (in CaCO_3), As=Total Dissolved Arsenic, DOC=Dissolved Organic Carbon. The unweighted UniFrac distance matrix was chosen over other PCoA ordinations, as the most environmental variables were found to significantly correlate with the ordination (**Table 2.1**). See **Figure A1** for ordinations that display all measured geochemical variables.

Six geochemical factors significantly correlated ($p < 0.05$) with at least one ordination: Eh, temperature, total arsenic, ammonia, alkalinity, and sulfate (**Table 2.1**). Several trace metals and other ions also showed significant correlations with β -diversity (**Data File A2**), but are not displayed in **Figure 2.5**, as they did not meet the 3rd criterion listed above. It is difficult to conclude whether these variables exert any direct influence on community diversity, as it is not clear whether their correlation with community dissimilarity is due to covariance with another environmental variable. **Figure A1** shows all ordination plots with all variables projected. Samples primarily separate based on redox potential (higher in Site A) and sulfate/ammonia/alkalinity (higher in Site B) (**Figure 2.5**). Temperature also drives separation of Sites A and B (generally higher in Site B, likely due to more recent steaming and slow water flow) (**Figure 2.5**), but also accounts for much of the variability between samples within Site B (6.9–32.6 °C in Site B vs. 10.5–15.3 °C in Site A) (**Table 2.1**). Total arsenic concentrations play a comparatively minor role in predicting community diversity between sites and samples ($0.041 \leq p \leq 0.144$, **Table 2.1**), which is consistent with the fact that arsenic resistance/metabolism genes are widely distributed among bacteria and archaea (Crognale et al., 2017; Zhu et al., 2017). Due to arsenic's high toxicity, most microbes will carry arsenic resistance genes regardless of environmental arsenic concentrations (Ben Fekih et al., 2018; Escudero et al., 2013). It appears that microbial communities undergo positive selection for arsenic resistance and thermotolerance from short-term, localized exposure to the given stressors, with temperature being a more influential factor. However, the succession of long-term, stable communities following the dispersal/migration of thermal and arsenic plumes is primarily dependent on natural aquifer redox conditions and nutrient availability.

2.3.4 Distribution and abundance of arsenic-related genes

Direct arsenic cycling genes

To predict the microbial capacity for direct arsenic cycling in each aquifer, annotation of arsenic-related genes was performed on all MAGs. Efflux pumps (*arsB/acr3*), intracellular arsenic reductase (*arsC*), and arsenic methyltransferase (*arsM*) were common in nearly all phyla (**Figure 2.6**). Exceptions to this were the Patescibacteria and members of the archaeal DPANN superphylum (Micrarchaeota and Nanoarchaeota), all lineages characterized by small, reduced genomes. Dissimilatory As^{5+} reduction, indicated by *arrA*, was uncommon and restricted to members of the Nitrospirota, Desulfobacterota, and Proteobacteria phyla (**Figure 2.6**). As^{3+} oxidation genes (*aioAB*,

arxA) were the most rare: only 7 MAGs encoded these genes, all of which were from members of the Proteobacteria (Figure 2.6).

The presence/absence of arsenic-cycling genes across MAGs (Figure 2.6) may not reflect the proportion of community members possessing these genes, due to variation in species abundance. To compare arsenic gene abundance across samples, positive hits for each gene (presence of at least one copy) were aggregated for MAGs within each sample and normalized to the relative abundance of MAGs (Figure 2.7). Across all samples, arsenic resistance and methylation genes were more abundant compared to metabolic arsenic reductase/oxidases (Figure 2.7). Efflux pumps within MAGs were mostly encoded by *acr3*, which has been previously found to be more widespread than *arsB* (Zhu et al., 2017).

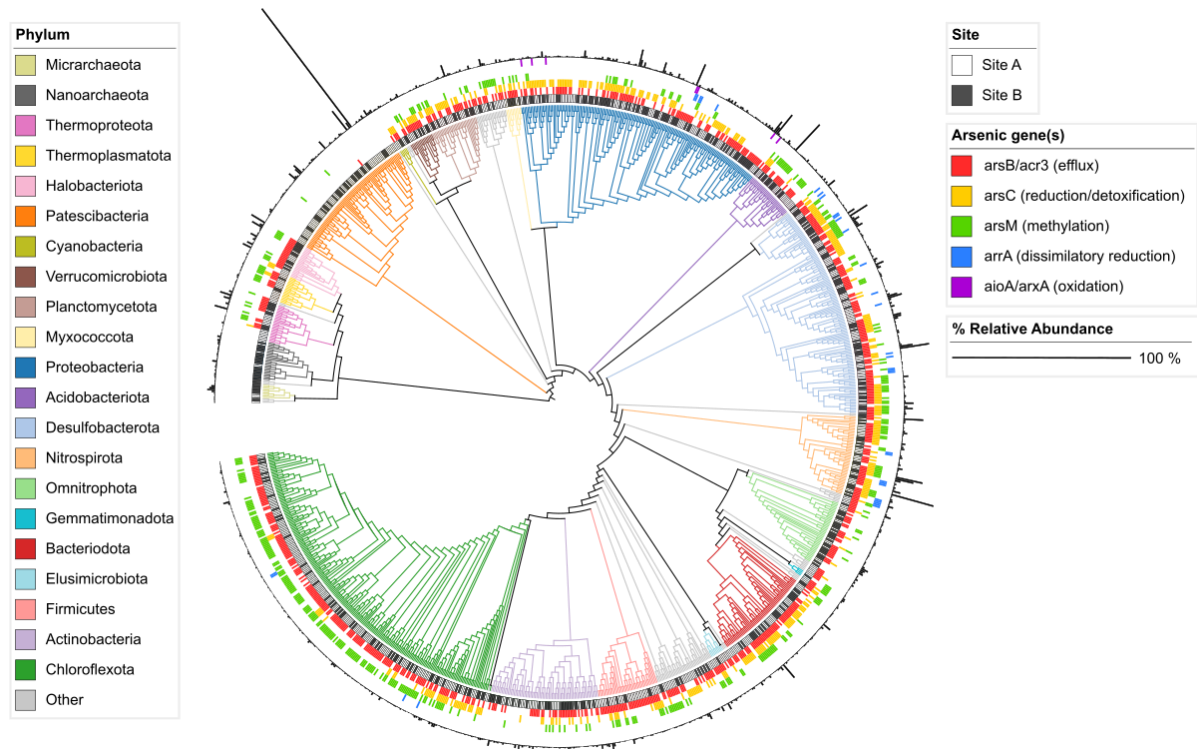


Figure 2.6: Phylogenetic distribution of arsenic-related genes across all MAGs. Tree branches are colored by phylum in clockwise order starting at the Archaea (~10 o'clock). Note that nomenclature for the Proteobacteria (Pseudomonadota), Firmicutes (Bacillota), and Cyanobacteria (Cyanobacteriota) have been updated in more recent versions (v2.1.0) of GTDB-tk. The 'Other' category represents phyla with <3 representative MAGs in the total dataset. The innermost ring designates which site the MAG originated from. Colored rings (ordered by innermost to outermost on the legend) represent presence/absence of arsenic-related genes, where at least one hit for any gene within each category was used to determine presence. The outermost ring is scaled to the % relative abundance of each MAG within its sample.

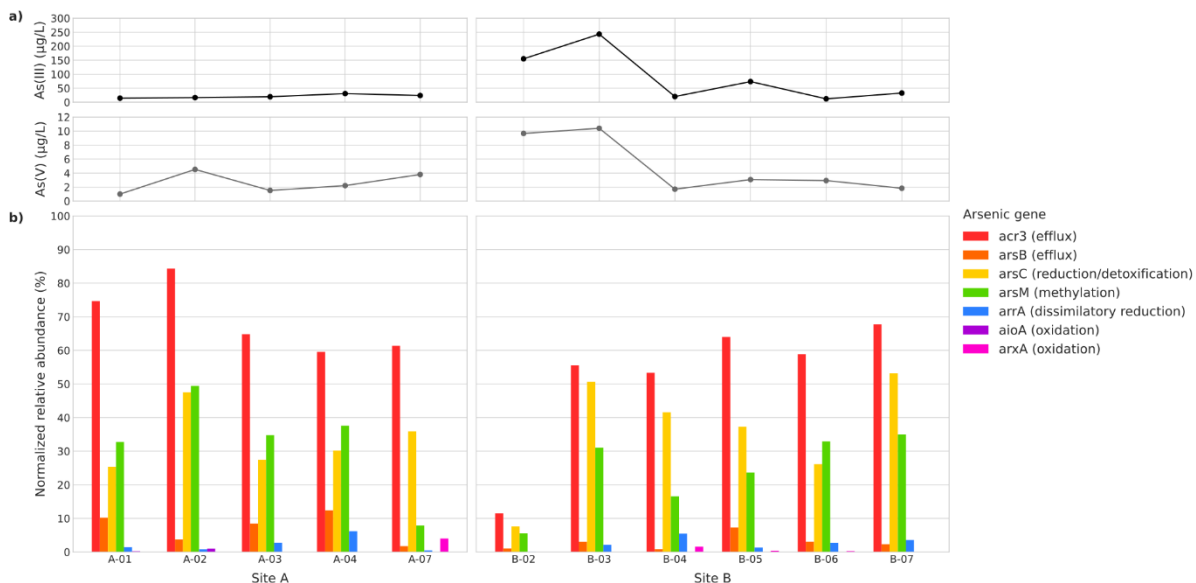


Figure 2.7: Predicted abundance of arsenic-related genes across wells. The concentrations of dissolved As^{3+} and As^{5+} are shown in (a), and abundances of arsenic-related genes in corresponding samples are shown in (b). Normalized abundances of genes were calculated by taking the sum of the relative abundances of MAGs that were positive for one or more hits of that gene. Normalized abundances therefore also represent the percentage of organisms present in each sample predicted to be capable of that function.

Arsonic concentrations within wells did not correlate with an increase in abundance of arsenic-cycling genes. Well B-02 had the second highest arsenic concentration but the lowest overall abundance of all As-cycling-associated genes (Figure 2.7). This trend is attributable to the dominant population of *Paceibacteria* (discussed further in *Potential host-symbiont interactions and arsenic resistance mechanisms of Paceibacteria CLB2-064*). B-03 shows similar gene abundance profiles compared to the rest of the aquifer despite having the highest arsenic concentration (Figure 2.7). These observations align with our whole-community analyses and suggest that arsenic concentration has a minor bearing on the distribution of arsenic-specific pathways.

In this instance, the disconnect between arsenic concentration and the distribution of arsenic cycling genes can be attributed to the fact that arsenic resistance/metabolism genes are widely distributed among bacteria and archaea (Crognale et al., 2017; Zhu et al., 2017). Additionally, the *aio*, *acr3*, and *ars* operons all have associated regulatory genes that activate or repress transcription based on $\text{As}^{3+}/\text{As}^{5+}$ concentrations (Romero et al., 2022; Shi et al., 2020; Suzuki et al., 1998). We suspect that if the microbial community within B-03 is more resistant to arsenic toxicity, this resistance may

be tied to the upregulation of *acr/ars* genes. It is also possible that As^{3+} oxidizers are expressing *aioAB/arsA* at higher rates relative to the oxidizers' abundance. Metatranscriptomic and/or metaproteomic data would be required to confirm changes in rates of arsenic cycling activity within the aquifers.

The *arsM* gene was the third most abundant arsenic-related gene detected overall (**Figure 2.7**). Biomethylation is thought to contribute to the cycling of arsenic between aquifers and surface environments, due to methylarsines being more volatile than inorganic species and more susceptible to diffusion (Maguffin et al., 2015). It is possible that microbial arsenic methylation via ArsM provides an additional mechanism of arsenic removal from the system, from volatilization of organic arsenicals (Crognale et al., 2017). However, it is unlikely to be a major factor impacting arsenic cycling at Sites A and B. If organic arsenicals were being produced in this system, we would not expect them to readily escape via volatilization given the physical barriers inherent in the aquifer (e.g., cut off from the atmosphere and slow groundwater flow). We did not detect measurable concentrations of mono- or dimethylarsenic acid in any sample (Data File A1), suggesting ArsM is not active despite its widespread distribution.

The rarity of As^{3+} oxidizers can be explained by the restricted niche space these guilds encounter in this system. While *aio/ars* genes are widely distributed across the bacterial domain, all known As^{3+} oxidizers are either aerobes (e.g., *Rhizobium*, *Thiobacillus*, *Chloroflexus*, *Alcaligenes* spp.) (Hamamura et al., 2009; Inskeep et al., 2007; Rhine et al., 2007) or anaerobes that couple As^{3+} oxidation to denitrification (e.g., *Alkalilimnicola ehrlichii* MLHE-1, *Sinorhizobium* spp.) (Oremland et al., 2002; Rhine et al., 2007; Zargar et al., 2010). Redox potential (Eh) measurements indicate that all wells are likely anoxic (**Table 2.1**). Nitrate is also below the detection limit in all wells except A-07 (0.51 mg/L), as redox potentials would favor reduced nitrogen compounds such as NH_3 (1.1–3.3 mg/L) (**Table 2.1**). Notably, A-07 showed the highest prevalence of *arsA* (encoded by a novel *Gallionella* MAG, CLA7-040, at 4.01% abundance), which supports our hypothesis that low availability of compatible electron acceptors limits the distribution of As^{3+} oxidizers.

Thermodynamic calculations for As^{3+} oxidation coupled to the reduction of other electron acceptors available (e.g., Fe^{3+} or SO_4^{2-}) indicated these metabolic processes are not thermodynamically favourable (i.e., $\Delta G > 0$) at As^{3+} concentrations and temperatures representative of this study site (i.e., 0.1–100 μM As^{3+} and 0–200 °C) (Basu et al., 2010). Although a recent study

showed that As^{3+} oxidation coupled to Fe^{3+} reduction does occur abiotically under anoxic conditions (Fang et al., 2023), there is no reported physiological evidence of microbial As^{3+} oxidation coupled to the reduction of iron-bearing minerals, suggesting it is unlikely to be occurring at this petroleum extraction site.

In summary, the overall trend of microbial activity with respect to direct arsenic cycling in both aquifers heavily favors intracellular reduction of As^{5+} and transport of As^{3+} out of the cell, which increases the relative concentration of the more toxic/mobile As^{3+} species. Although arsenic methylation pathways were widely detected, there is no geochemical evidence they are active in this system. Finally, As^{3+} oxidation pathways that could counteract the mobilization of arsenic were rare, likely due to a requirement for oxygen or nitrate in a system lacking both.

Iron- and sulfur-cycling genes

The biogeochemical cycling of arsenic is directly connected to iron and sulfur redox cycling in the environment. Due to these interactions, we considered how biological iron and sulfur redox cycling could control the fate of arsenic in a system dominated by arsenic detoxification strategies that favour the reduction of As^{5+} to As^{3+} . Annotated Fe/S redox genes were aggregated and normalized to the abundance of MAGs within each sample, reflecting the proportion of organisms within each community that encode these genes (**Figure 2.8**).

Aerobic Fe^{2+} and sulfur oxidation is unlikely to be occurring within most wells in Sites A & B, even if genes for these metabolism were detected in the metagenomes (**Figure 2.8**). Given redox potentials are below 200 mV (**Table 2.1**), it is improbable that enough oxygen is present to support the oxidation of ferrous iron and reduced inorganic sulfur compounds. Simultaneous oxidation of elemental sulfur and As^{3+} has been demonstrated in some species, but requires aerobic, oxidizing conditions such as those present in alkaline lake waters (Fisher et al., 2008), which is therefore not applicable to groundwater arsenic cycling (pH 6.76–7.95, **Table 2.1**). The maximum Fe^{3+} concentration measured was 0.41 mg/L (B-02), and the maximum H_2S concentrations measured was 0.15 mg/L (B-04), which do not correlate with abundances of iron oxidizing and sulfate reducing genes, but we acknowledge that groundwater concentrations are not indicative of $\text{Fe}^{3+}/\text{H}_2\text{S}$ production due to their poor solubility (Lux, 1963; National Center for Biotechnology Information, 2024).

The community within A-07 may be an exception, as over 50% of organisms within this sample were predicted to have iron oxidation genes (**Figure 2.8b**). Most of these genes were

represented by novel MAGs belonging to the genus *Gallionella*, which are known microaerophilic neutrophilic Fe^{2+} -oxidizing bacteria. The abundance of *Gallionella* in A-07 is the main factor driving its community dissimilarity compared to the other wells (**Figure 2.4**), as this group is largely absent from other samples. It is possible that small amounts of oxygen infiltration could support microaerophilic Fe^{2+} oxidation by *Gallionella* spp. in A-07, or that they are coupling this metabolism with NO_3^- reduction, which has been observed in members of the family Gallionellaceae (Huang et al., 2021, 2022). If this is the case, we would expect Fe^{2+} oxidizers to have low activity due to the same niche restrictions highlighted for As^{3+} oxidizers.

Reductive iron and sulfur metabolisms are more favored in anoxic reducing environments, such as the deep aquifers in this study. The presence of Fe^{3+} reduction capacity (present in ~20% of organisms in some wells) presents a possible mechanism for arsenic mobilization, as reductive dissolution of Fe^{3+} -bearing minerals can lead to de-sorption of arsenic from sediments (Smedley & Kinniburgh, 2002). The production of biogenic sulfides from sulfate-reducing bacteria (SRB) can have a variety of indirect effects on the arsenic cycle. Under reducing conditions and low dissolved iron (such as this study's system), sulfide may form stable thioarsenite complexes with As^{3+} , which increases arsenic's mobility (Bostick et al., 2005; Wilkin et al., 2003). Other reactions may simultaneously occur that form arsenic sulfide minerals (AsS , As_2S_3) that limit arsenic's mobility through precipitation (Fisher et al., 2008; Newman et al., 1997; Wilkin et al., 2003). The highest arsenic well, B-03, also has the highest abundance of SRB (**Figure 2.8b**), which is attributed to the *Thermodesulfovibrionia* MAGs in that community, the most prominent populations being CLB3-097 (24.5% relative abundance) and CLB3-019 (15.4% relative abundance) (**Data File A2**). Given the comparatively low abundance of sulfur oxidizers relative to SRB (**Figure 2.8b**) and the low groundwater Eh (**Table 2.1**) in B-03, we predict that biogenic production of sulfide due to SRB may be one of the main pathways limiting arsenic's mobility in the system.

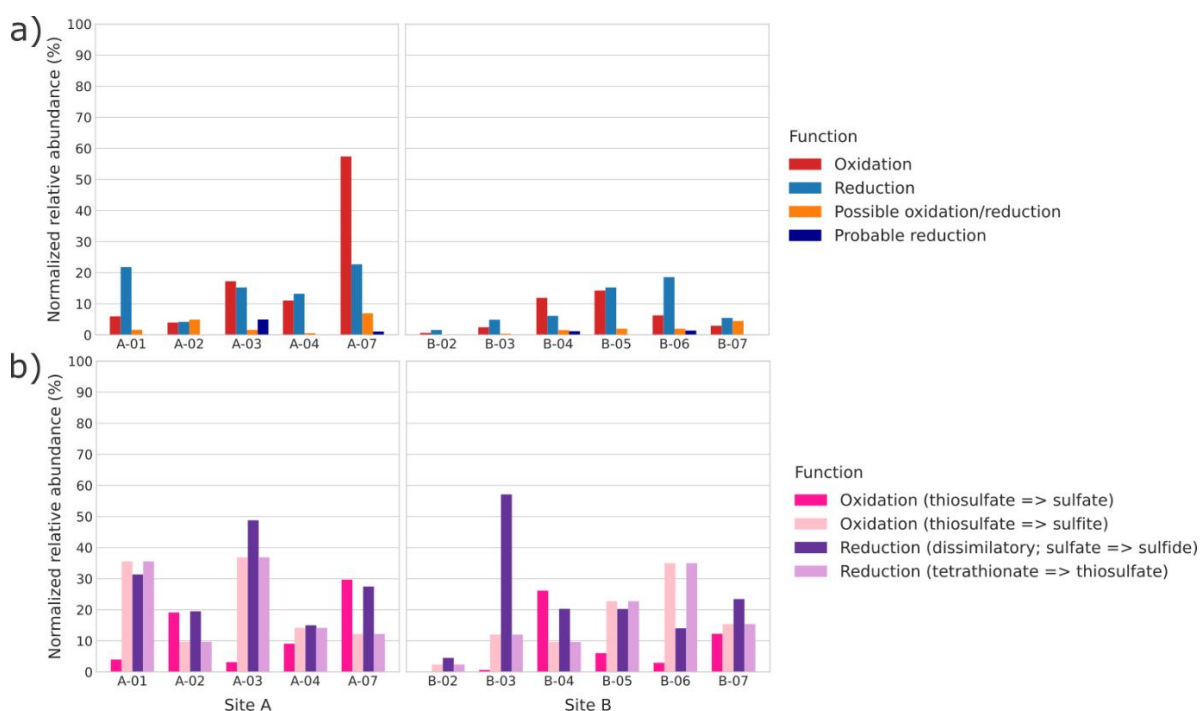


Figure 2.8: Predicted abundance of iron (a) and sulfur (b) cycling genes across wells. Genes are categorized by function (oxidation and/or reduction) for both elements. Normalized abundances for functions were calculated by taking the sum of the relative abundances of MAGs that were positive for one or more gene within the function (and therefore represent the percentage of organisms present in each sample predicted to be capable of that function). Gene families were identified and categorized using FeGenie (iron) and DRAM (sulfur). Iron oxidation genes include *cyc2*, *fox* genes, and sulfocyanin; reduction genes include *omc* and *dfe* genes. Other hypothesized (but not verified) genes for iron cycling include *mto* (possible oxidation/reduction) and *mtr* (probable reduction) genes, which are indicated separately (orange and purple, respectively). A full list of iron-related genes used in this analysis can be found in **Table A1** (Garber et al., 2020). Sulfur oxidation genes are divided into the *sox* pathway (thiosulfate to sulfate) and other pathways that produce sulfite as an intermediate. Dissimilatory reduction of sulfate is identified by *dsr* and *apr* genes, while reduction of tetrathionate to thiosulfate is identified by the *ttr* gene.

Potential host-symbiont interactions and arsenic resistance mechanisms of Paceibacteria CLB2-064

The high abundance of CLB2-064 within well B-02 was highly unexpected for two reasons. First, the genome of CLB2-064 is consistent with other members of the Paceibacteria, with pathways for glycolysis, the tricarboxylic acid (TCA) cycle, and electron transport missing or incomplete (evaluated using DRAM, **Data File A2**), suggesting an obligately symbiotic or epibiotic lifestyle. Secondly, this population was found to be dominant in a well with higher arsenic concentrations (0.17 mg/L in 2021), despite lacking any known arsenic resistance genes.

To identify potential host-symbiont interactions involving CLB2-064, proportionality (or co-occurrence) analysis was performed on CLB2-064 MAG coverage compared to the coverage of B-02 scaffolds across all samples. Scaffolds showing proportional abundance to CLB2-064 abundance could represent potential hosts for this species, with a shared abundance pattern but not shared absolute abundance levels.

All scaffolds >10 kb that correlated with CLB2-064 MAG coverage belonged to existing MAGs (**Data File A2**). A subsequent proportionality analysis was performed between the relative abundance of CLB2-064 and all other MAGs in B-02, with only one MAG identified to be proportional to CLB2-064 at the full genome rather than scaffold level (CLB2-037, **Data File A2**). Proportionally abundant scaffolds and MAGs were taxonomically diverse, and present at a maximum abundance of 1.349%. It is unlikely that any of these MAGs form a single host population for CLB2-064 given these abundances; even if multiple parasitic cells attach to a single host cell (as shown in Kuroda et al., 2022), the ratio of symbiont to host would be greater than 60:1. We initially considered the possibility that CLB2-064 dominance in 2021 samples may have been due to an event just prior to sampling that killed its host population, and the CLB2-064 population had not yet fallen in response. However, the 2022 16S rRNA amplicon data showed that the community composition remained stable over at least one year. Given these results, we hypothesize that Paceibacteria CLB2-064 can either engage in symbiosis with low host specificity and are able to parasitize multiple other species present in the aquifer, or that they parasitize a eukaryotic host that was not captured in this analysis. Patescibacteria have been identified as intracellular symbionts in ciliates, a dynamic that may be occurring in this system as well (Gong et al., 2014). Future research could use microscopy (specifically cryo-TEM as used in He et al., 2021), which is able to image Patescibacteria

mechanisms of attachment to host cells) and/or culture-based methods to elucidate the role of these understudied organisms within the aquifer community.

The ability of CLB2-064 to survive high concentrations of arsenic despite not encoding *ars* or *acr* genes may be a by-product of the characteristically reduced genomes in this clade, rather than the evolution of a completely different mechanism of arsenic resistance. The necessity for intracellular As^{5+} reductases and membrane As^{3+} efflux pumps is due to the fact that arsenic must enter the cell in order to be cytotoxic. As^{3+} has been shown to enter through aquaglyceroporins (*e.g.*, glycerol facilitator GlpF), while As^{5+} is very similar in structure to phosphate and enters through phosphate uptake systems (*e.g.*, Pit, Pst) (Crognale et al., 2017; Kruger et al., 2013). Gene annotation data for the CLB2-064 MAG showed that both glycerol and phosphate transporter genes were completely absent. We hypothesize that arsenic resistance in CLB2-064 is a byproduct of their lack of membrane transporters for As^{3+} and As^{5+} to exploit to enter their cells. Alternatively, if CLB2-064 is an intracellular parasite to a ciliate, it may rely on the host's arsenic resistance mechanisms, which also eliminates the need to encode them in their own genome.

2.3.5 Opportunities for remediation

Arsenic oxidation

As discussed in 3.4.1, As^{3+} oxidizers are rare in this study system, most likely due to the absence of electron acceptors (O_2 , NO_3^-) for As^{3+} oxidation in deep, anoxic groundwater environments (low redox potential). Leveraging As^{3+} oxidizing guilds for bioremediation would likely require supplementing the aquifers with NO_3^- and potentially introducing large exogenous populations of As^{3+} oxidizers into the aquifers. These methods are unlikely to be efficient at counteracting the mobilization of arsenic in this system because the widespread *arsC* detoxification pathways would reduce As^{5+} back to As^{3+} . Moreover, the capacity of exogenous As^{3+} oxidizers to survive the steep physicochemical gradients (*e.g.*, temperature fluxes due to in-situ bitumen recovery processes) encountered in the aquifer would need to be considered as part of whether this is feasible.

Formation of iron minerals

One Fe^{2+} -oxidizing *Gallionella* MAG (CLA7-040) was the most abundant As^{3+} oxidizer. As^{5+} and Fe^{3+} can co-precipitate as scorodite under oxidizing conditions, so stimulating both metabolisms (potentially even in the same organism) could support arsenic removal. We expect such a strategy

would find limited purchase because scorodite is relatively soluble in circumneutral pH conditions (Langmuir et al., 2006). Although scorodite formation could mitigate extremely high arsenic levels, the equilibrium of dissolved/solid phase arsenic would still be higher than regulatory standards for groundwater.

Adsorption of arsenic onto Fe^{3+} minerals (mainly iron (oxyhydr)oxides) is a more effective mechanism of controlling arsenic mobility in neutral pH groundwater. Increasing the rate of Fe^{3+} mineral formation through stimulating microbial Fe^{2+} oxidation may be possible through the addition of NO_3^- . Additional Fe^{2+} would also need to be added, as natural concentrations (0.28–2.5 mg/L) within the aquifers are low in the context of bioavailability as an electron donor (**Table 2.1**). Redox conditions within these aquifers do not currently favour biological oxidation of Fe^{2+} anywhere except A-07. For all other locations, the capacity to amend sufficient oxidized species to raise the redox potential and stimulate iron oxidation will likely be a key limitation to consider with such a strategy.

Formation of sulfide minerals

As discussed in 2.3.4, sulfide may increase arsenic mobility by forming thioarsenite complexes with As^{3+} (Bostick et al., 2005; Wilkin et al., 2003). Arsenic may also precipitate with sulfides as arsenic sulfide minerals (As_4S_4 , As_2S_3), but similar to scorodite, the equilibrium of arsenic with these minerals at pH 7 is not favored towards the solid phase (Saunders et al., 2018); the stimulation of SRB alone is therefore not reliable for large-scale remediation of thermally mobilized arsenic plumes.

If SRB activity is stimulated with the combined addition of Fe^{2+} , the formation of pyrite is more favorable – leading to subsequent sorption of As, and incorporation of As^{3+} into growing pyrite crystals (arsenopyrite) (Lee et al., 2019; Saunders et al., 2018). A field-scale remediation experiment demonstrated this principle, where researchers amended arsenic-contaminated groundwater with sulfate, ferrous iron, and organic C/N/P sources (Lee et al., 2019; Saunders et al., 2018). The treatment was effective at sequestering arsenic via the formation of biogenic pyrite, reducing dissolved concentrations from 0.5 mg/L to <0.05 mg/L. The source of arsenic contamination in the aforementioned study (agricultural runoff) was also a case of short-term, anthropogenic mobilization, so the results are likely applicable to the microbial community dynamics present in our study sites.

Sulfate concentrations within Sites A & B range from 22–59 mg/L (**Table 2.1**), making them comparable to initial concentrations in the field remediation experiment described above. To replicate their results, additional sulfate would need to be added to the aquifer (up to 300 mg/L), as well as

ferrous iron (up to 100 mg/L), and labile C/N/P sources to support the growth and metabolism of native SRB that are widely distributed in this system.

The *Thermodesulfovibrio* MAGs identified in this study (*e.g.*, *Thermodesulfovibrio* CLB3-097 and CLB3-019) may be particularly useful for remediating aquifers impacted by steam extraction of bitumen because they are able to tolerate high arsenic concentrations and heat (but appear to be abundant once temperatures have lowered back down to ~25 °C as well). Thermophilic SRB that are active at high temperatures but tolerant of lower temperatures may be better suited to counteracting arsenic plumes during active steaming and shortly after before the heat has dissipated. Mesophilic SRB that can persist through cycles of steam injection and become active once temperatures lower would be more suited for long term remediation of arsenic-impacted aquifers where steam injections have ceased but arsenic levels remain high.

In summary, the biogeochemistry of groundwater across Sites A & B favors biological sulfate reduction as a major redox reaction driving indirect arsenic cycling (**Figure 2.9**). The high arsenic well B-03 appears to support a niche containing arsenic-tolerant, thermotolerant SRB that show potential for remediation of thermally mobilized arsenic, although biostimulation via the addition of sulfate, iron, and nutrients would be required.

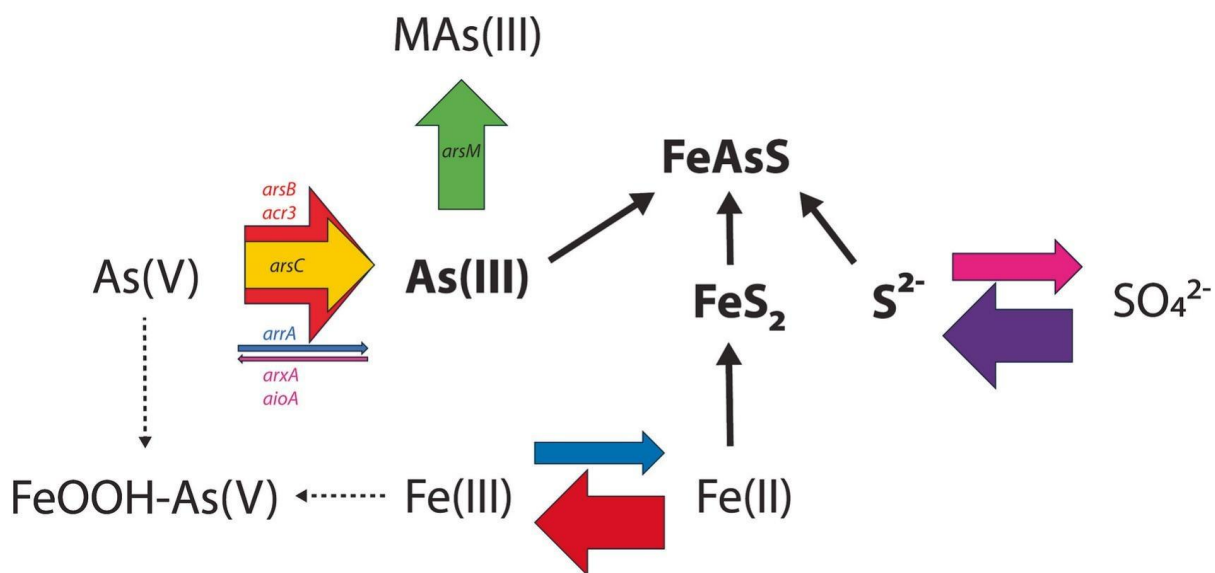


Figure 2.9: Schematic of predicted capacity of microbial arsenic cycling in groundwater impacted by thermal arsenic mobilization. Colored arrow size is scaled proportional to the maximum relative abundance of that gene (or pathway) observed across all sample sites. Black arrows represent abiotic reactions that could occur based on the substrates available from microbial activities. Bolded, solid black arrows show reactions most likely to be relevant to remediation of arsenic from this system, based on groundwater chemistry and aquifer redox conditions.

2.4 Conclusions

In this chapter, we profiled the taxonomic composition and diversity of aquifer communities experiencing recent elevated arsenic concentrations with respect to background due to *in-situ* thermal bitumen extraction. Metagenomic sequencing was used to predict the functional capacity of microbes to contribute directly to arsenic cycling and indirectly control the fate of arsenic through iron and sulfur cycling. The main goal was to identify organisms and/or consortia that could mitigate arsenic's mobility under the geochemical constraints of our study site.

Community composition was highly variable between higher-arsenic and lower-arsenic wells, although the dominant taxa within higher-arsenic wells were also different from each other within and between transects. Environmental factors such as Eh, temperature, alkalinity, $[\text{SO}_4^{2-}]$, and $[\text{NH}_3^-]$ correlated more strongly with community diversity than arsenic concentrations, indicating that groundwater communities stabilize differently after short-term arsenic exposure based on the native geochemical conditions of the aquifer.

Genes for arsenic resistance (*arsB*, *acr3*, *arsC*) and methylation (*arsM*) were widespread in all samples, regardless of current arsenic concentrations. Microbial communities predominantly contain organisms predicted to reduce intracellular As^{5+} and produce As^{3+} , which could release aqueous As^{3+} complexes back into solution. In contrast, arsenic metabolic genes (*arrA*, *aioA*, *arxA*) were rare. Arsenic oxidation is not likely supported in these environments due to limited terminal electron acceptors suitable for these metabolic reactions.

Iron and sulfur-cycling organisms were also identified that may indirectly contribute to arsenic attenuation. In more oxidizing environments (such as A-07), iron oxidizing *Gallionella* spp. could immobilize dissolved arsenic by favouring the precipitation of ferric (oxy)hydroxide minerals that strongly sorb arsenic. In reducing environments, SRB, such as the novel *Thermodesulfovibriales* MAGs within the highest arsenic well, can induce sulfide mineral formation, which could remove arsenic by co-precipitation (pyrite/arsenopyrite). Given the reducing conditions of most wells in the studied aquifer system, we predict that stimulating SRB activity with the addition of sulfate, along with ferrous iron, carbon, nitrogen, and phosphorus, is a potential strategy for bioremediation of thermally mobilized arsenic. While it is difficult to generalize these results to all sources of arsenic contamination, this study suggests that short-term mobilization of arsenic from anthropogenic

activities could be mitigated by promoting the activities of microorganisms naturally present in these environments.

An unexpected finding of a highly abundant *Paceibacteria* population in one high arsenic well could not be fully explained, as there was not a clear host population capable of supporting a symbiont present at >80% relative abundance. This population can persist at high abundances despite not encoding arsenic resistance genes. If they are endosymbionts of an unidentified eukaryote, they may benefit from arsenic resistance mechanisms of their host. Otherwise, the fact that their genome lacks genes for the membrane transporters that bring arsenic into the cell may constitute an alternative arsenic resistance strategy.

In summary, aquifer communities experiencing past and present short-term elevated arsenic consist of a wide range of organisms, due to arsenic resistance mechanisms being widespread among microbes. The high taxonomic and metabolic diversity observed across aquifer transects that differ in arsenic concentrations as well as other environmental factors present an opportunity to further explore microorganisms that directly and indirectly influence arsenic mobility in groundwater.

Chapter 3

Legacy copper/nickel mine tailings potentially harbor novel iron/sulfur cycling microorganisms within highly variable communities

3.1 Introduction

Mine tailings are the fine-grained residues left as waste as part of the extraction process of valuable metals from ores. Sulfide-rich tailings (generated from selective flotation of sulfide minerals) stored in subaerial impoundments are a major source of groundwater contamination due to release of acid mine drainage (AMD). When sulfidic minerals (*e.g.*, pyrite, FeS_2) are exposed to oxygen and water, the oxidation of ferrous iron (Fe^{2+}) and sulfur generates high amounts of Fe^{2+} , SO_4^{2-} , and H^+ . The Fe^{2+} released by sulfide mineral oxidation may be further oxidized to Fe^{3+} , which contributes to the oxidation and dissolution of additional pyrite and other metal-bearing minerals. The overall effect of these reactions includes the formation of acidic porewater with high concentrations of heavy metals. Release of AMD to natural environments and freshwater is detrimental to natural ecosystems and human health (Simate & Ndlovu, 2014).

Microbial activity can accelerate the rate of AMD formation by hundreds to millions of times compared to abiotic oxidation alone (Nordstrom et al., 2015). Acidophilic, chemolithotrophic bacteria such as *Acidithiobacillus ferrooxidans* (Falagán et al., 2017; Leduc & Ferroni, 1994) obtain energy through the oxidation of iron and sulfur compounds, releasing additional H^+ , SO_4^{2-} , and regenerating the Fe^{2+} ion. While these organisms are detrimental in the context of tailings waste management, they have also been extensively studied for industrial bioleaching applications to extract valuable metals from tailings (Falagán et al., 2017; Valdés et al., 2008).

Microbial consortia isolated from AMD environments also include acidophilic sulfate reducers that precipitate metal ions from solution as metal sulfides (Johnson et al., 2006), and species that immobilize metal ions through bioaccumulation or biosorption (Anusha et al., 2021; Bautista-Hernández et al., 2012; Mohamad et al., 2012; Tsuruta, 2011); all of these processes serve as potential remediation approaches to remove metals from tailings porewater and mitigate the negative environmental and human health impacts of mine wastes. Physical remediation strategies may also be employed to reduce acidity and metal leaching of tailings, such as by the application of cover layers (*e.g.*, fine-grained silt and clay or organic carbon) that limit oxygen and water ingress into the tailings

and facilitate the growth of vegetation (Aubertin et al., 2016; Pakostova et al., 2022; Park et al., 2019; Peppas et al., 2000). Vegetation (phytoremediation) and organic matter in organic covers also increase microbial community diversity of the tailings by promoting the establishment of organoheterotrophs and nitrogen-fixing rhizobacteria (increasing plant growth, nutrient turnover, and ecosystem productivity (Asemaninejad et al., 2020; Romero et al., 2021).

Many studies have described microbial genera common across varied AMD environments, such as the acidophilic iron and/or sulfur oxidizing *Acidithiobacillus*, *Leptospirillum*, and *Sulfobacillus* spp. (Baker & Banfield, 2003; Méndez-García et al., 2015). However, mine tailings can be diverse in composition depending on the metals targeted for extraction, resulting in substantial differences in the extent of oxidation and acidification and concentrations of contaminants. As a result, the microbial community compositions found within mine tailings are also highly heterogeneous. pH is an important driving factor in the relative abundance of several lineages, with Proteobacteria generally dominant in slightly to moderately acidic tailings (pH 4–7) and Euryarchaeota (especially *Ferroplasma* spp.) more abundant in highly acidic tailings (pH <3) (Chen et al., 2013; Pepper et al., 2012). The concentrations of metal ions (including Fe, Cu, Ni, and trace elements such as Cd, Hg, and U) select for organisms with resistance mechanisms against these contaminants (Bondici et al., 2013; Jones et al., 2017; Liu et al., 2014; Yan et al., 2020). Mine tailings also exhibit vertical stratification, with distinct zones of oxidation, neutralization, and unaltered tailings, heterogeneous physical characteristics (e.g., particle size) and temporal variability in moisture content; all of which influence the microbial guilds dominating each microenvironment (Diaby et al., 2007).

A wide variety of microbial guilds are applicable to AMD bioremediation (e.g., bioreactors with engineered sulfate-reducing consortia, *in-situ* remediation via iron-oxidizing consortia in aerobic wetlands) (Johnson & Hallberg, 2005) but making improvements in bioreactor/*in-situ* remediation efficiency and cost-effectiveness depends on understanding the appropriate isolates/consortia to leverage given the specific geochemistry of the target mine tailings. Tailings are highly selective environments that may be host to unexplored phylogenetic radiations of bacteria and archaea that have evolved unique adaptations to survive in heavy metal contaminated areas. Therefore, tailings provide an excellent starting point to identify AMD-tolerant microbes that can be used to improve the efficiency of engineered bioreactors and *in-situ* remediation strategies. Recent advances in high-

throughput sequencing methods such as 16S rRNA amplicon sequencing have enabled the high-resolution examination of the taxonomic diversity of AMD microbiomes (Lukhele et al., 2020).

In this study, we compared microbial community diversity across vertical and horizontal transects of tailings associated with two Ni/Cu mining sites in the Sudbury Basin region of Ontario, Canada. 16S rRNA amplicon sequencing was used to characterize community composition across 3–5 m depth transects at four tailings locations. A temporal comparison was made for one tailings location to assess community stability over time (2 years). Our objectives were to identify the main geochemical factors influencing microbial diversity and variability between locations, predict the contribution of microbial guilds to iron/sulfur cycling within tailings, as well as to explore microbial novelty within the context of mine tailings environments.

3.2 Methods

3.2.1 Tailings core sampling and processing

Sampling of all locations took place in November 2021. NR18 was previously sampled in November 2019, with the 2019 samples included here for an assessment of temporal variation. In November 2021, the average monthly temperature was -0.5 °C and precipitation was 0.06 mm (Environment and Climate Change Canada, 2022). Sampling in 2019 and 2021 followed the same protocol: Tailing cores of 5.0–7.6 cm (2–3 inches) in diameter were extracted to a depth of approximately 1 meter below the water table (approximately 3–5 m per location) using a similar method as described in Starr & Ingleton (1992), with a Pionjar 120 gas-powered hammer drill and aluminum pipe. Cores were capped and stored for 1–2 days on-site before being transported back to the lab, where they were stored at -20 °C prior to further processing.

Cores were sliced into subsamples at 10 cm intervals along the length of each core using a pipe cutter. The centre inch of each subsection was punched out into a sterile plastic bag using a sterile aluminum hollow rod and then stored at -80 °C prior to DNA extraction.

3.2.2 Geochemical assessments

Pore-water was collected from model 1900 soil water solution samplers (SWSS, from Soil Moisture Inc.) and piezometers installed approximately 1 week prior to sampling. Measurements of pH, Eh, alkalinity, and electrical conductivity were conducted on-site. Additional water samples were filtered (0.45 µm) and stored at 4 °C for laboratory analysis. Inductively coupled plasma-optical emission spectrometry (ICP-OES), inductively coupled plasma-mass spectrometry (ICP-MS) and ion chromatography (IC) were used to determine cation and anion concentrations respectively. Total dissolved organic carbon (DOC) was measured with a non-dispersive infrared sensor (NDIR).

Field measurements of gaseous O₂/CO₂ concentrations were taken using the QUANTEK 902P O₂/CO₂ analyzer, at approximately 10 cm depth intervals (water saturation of tailings beginning between 1–2 m in depth prevented gas quantification in deeper samples).

3.2.3 DNA extraction

Approximately 10 g of tailings were measured into a 50 mL falcon tube, washed twice with 35 mL TE buffer (10 mM Tris-HCl, 1 mM disodium EDTA, pH 8.0) to reduce inhibitor concentrations prior

to extraction, and transferred to the QIAGEN DNEasy PowerMax Soil kit. DNA was extracted following the manufacturer's instructions, with the following modifications. DNA was initially eluted with 500 μ L of elution buffer (10 mM Tris). Due to low concentrations in 23 samples (10 from NR18 and 13 from NR3), the protocol was altered in subsequent samples. DNA was eluted with 5 mL of elution buffer and subsequently concentrated to a volume of 50–100 μ L using ethanol/sodium acetate precipitation as follows: 2.5 volumes of ice-cold ethanol was added to eluted DNA, along with 1/10th volume 3 M sodium acetate. Precipitation reactions were incubated at -20 °C overnight, following which precipitated DNA was pelleted at 4,000 x g and 4 °C for 30 minutes, and washed twice with 500 μ L of 75% ethanol, spinning for 10 minutes post-wash each time at 21,000 x g held at 4 °C. The pellets were air dried and resuspended in 50–100 μ L of elution buffer. Final DNA concentrations were determined using the Thermo Fisher Qubit dsDNA High Sensitivity Assay Kit.

A total of 112 DNA samples were obtained, which were stored at -20 °C prior to sequencing.

3.2.4 16S rRNA amplicon sequencing

Samples were PCR-amplified and sequenced by MetagenomBio Life Science Inc. using the Illumina MiSeq machine (v2 reagents). Modified universal primers targeting the V4 region were used for amplification (forward primer 515FB: 5' GTGYCAGCMGCCGCGGTAA; reverse primer 806RB: 5' GGACTACNVGGGTWTCTAAT) (Walters et al., 2015).

Sequence data were processed and analyzed using the QIIME2 platform (v.2022.2) (Bolyen et al., 2019). Paired-end reads were filtered, trimmed, merged, and grouped into amplicon sequence variants (ASVs) with DADA2 (Callahan et al., 2016). Taxonomy was assigned based on the SILVA 138 SSU database (Yilmaz et al., 2014). ASV abundance and metadata were processed and visualized in Python (v3.8.8) using the pandas (v1.4.4) (McKinney, 2010) and matplotlib (v3.6.0) libraries (Hunter, 2007).

3.2.5 Diversity and statistical analyses

α -diversity was evaluated within QIIME2 (using a rarefied sampling depth of 12,000, which excluded 18 samples) using Faith's phylogenetic distance, Shannon diversity (H') index and the observed ASV count. Significant differences between locations were determined using the Kruskal-Wallis test. Plots were generated with Python using the seaborn library (Waskom, 2021).

The QIIME2 feature table and taxonomy data was exported into R (v 4.1.2) (R Core Team, 2021) using the qiime2R package (Bisanz, 2018). Bray-Curtis, weighted and unweighted UniFrac distances between samples were calculated to assess β -diversity, using the phyloseq package (McMurdie & Holmes, 2013). Ordination plots were visualized with ggplot2 (Wickham, 2016).

To map each sample to the pore-water chemistry data, the midpoint depth for each core slice was determined (after correcting for the compression factor of the core). Then, the two closest depths (above and below) this value where water chemistry was measured were taken and a weighted average of each geochemical measurement (pH, Eh, dissolved ion concentration, etc.) was calculated based on their respective distance to the midpoint of the core. For dissolved ions, only parameters with $\geq 50\%$ of measurements above the detection limit of instruments in all cores were included in statistical analyses (with the exception of dissolved organic carbon (DOC) and NO_3^- , which were included because they are key metabolic substrates required to support anaerobic respiration and heterotrophy), and values below the detection limit were set to 0.

To determine the geochemical factors affecting β -diversity, a non-metric multidimensional scaling (NMDS) analysis was performed in R using the vegan package (Oksanen, 2010). NMDS ordinations were calculated with the metaMDS function using Bray-Curtis distances. The 'envfit' function was used to fit geochemistry factors onto the ordination, using 999 permutations. Variables showing significant correlations ($p < 0.05$, Bonferroni corrected) with community composition were included in the NMDS plot.

Geochemical variables that significantly correlated with community diversity (as well as Cu, Ni, and SO_4^{2-} concentrations due to their importance as environmental contaminants) were also evaluated for their correlation to the abundance of the most dominant genera (present at a relative frequency of $\geq 15\%$ in at least one sample). Spearman's rank correlation matrices were generated using SciPy (v.1.0) (Virtanen et al., 2020) in Python and plotted using the seaborn package. As environmental factors often co-vary, a pairwise correlation matrix between geochemical variables was also calculated.

3.2.6 Data availability

All reads associated with amplicon sequencing data presented here have been submitted to the SRA under BioProject PRJNA1017420 and accessions SRR26117691-SRR26117804. All geochemical data used in this study is available in **Data File B1**.

3.3 Results and discussion

3.3.1 Sampling site and tailings core characteristics

The Sudbury Basin is a major geological structure in the Canadian Shield. Formed by a meteorite impact (French, 1967), it consists of mainly Fe-Ni-Cu sulfide deposits (*e.g.*, pyrrhotite, chalcopyrite and pentlandite), which may be enriched in precious metals (Molnár et al., 1999).

Tailings stored at two mines owned and operated by Glencore Sudbury Integrated Nickel Operations (Sudbury INO) were sampled for characterization of microbial community diversity and function: the Strathcona Waste Water Treatment System, which received tailings from 1968–2012 (Bain et al., 1998; Bain & Blowes, 2013) and Nickel Rim North tailings area, which received tailings from 1953–1958 (Johnson et al., 2000). Operations at Sudbury INO are focused primarily on extracting and processing nickel and copper, with cobalt and precious metals as by-products (Sudbury INO, 2022).

At Strathcona, two locations, Moose Lake 25 (ML25) and ML34, were chosen to assess spatial (vertical) variation. The sulfide-rich (9–18 weight percent (wt. %) S) (Blowes et al., 1999), coarse-grained tailings at ML25, which have been exposed to atmospheric weathering since deposition decades ago, are extensively oxidized. The porewater at ML25 is low pH (pH<5) and contains high concentrations of contaminants (McAlary, 2020) (**Figure 3.1b, Data File B1**). In contrast, the high sulfide tailings at ML34 are overlain by a 2 m layer of desulfurized tailings (DST) cover of <1.4 wt. % S mainly as pyrrhotite, and a 50 cm organic carbon cover composed of a mixture of a mixed municipal compost amended with biosolid fertilizer. The cover materials are not potentially acid generating, and the fine-grained DST cover maintains a high moisture content, limiting O_{2(g)} diffusion. The pH is circumneutral and concentrations of dissolved metals are lower than at ML25 (McAlary, 2020; Pakostova et al., 2022) (**Figure 3.1b, Data File B1**).

At Nickel Rim North (NR), locations NR18 and NR3 were selected for comparison. The unoxidized tailings contain on the order of 3 wt. % S mainly as pyrrhotite (Johnson, 1993). Contaminant profiles are similar at these two locations (**Figure 3.1b, Data File B1**), although NR3 has shallow water-table elevation and the shallow tailings have a high moisture content, leading to a shallower depth of oxidation and lower concentrations of dissolved metals than at NR18 (36). NR18 also has a shallow (<10 cm) organic soil, deposited in the early 1990s (Johnson, 1993).

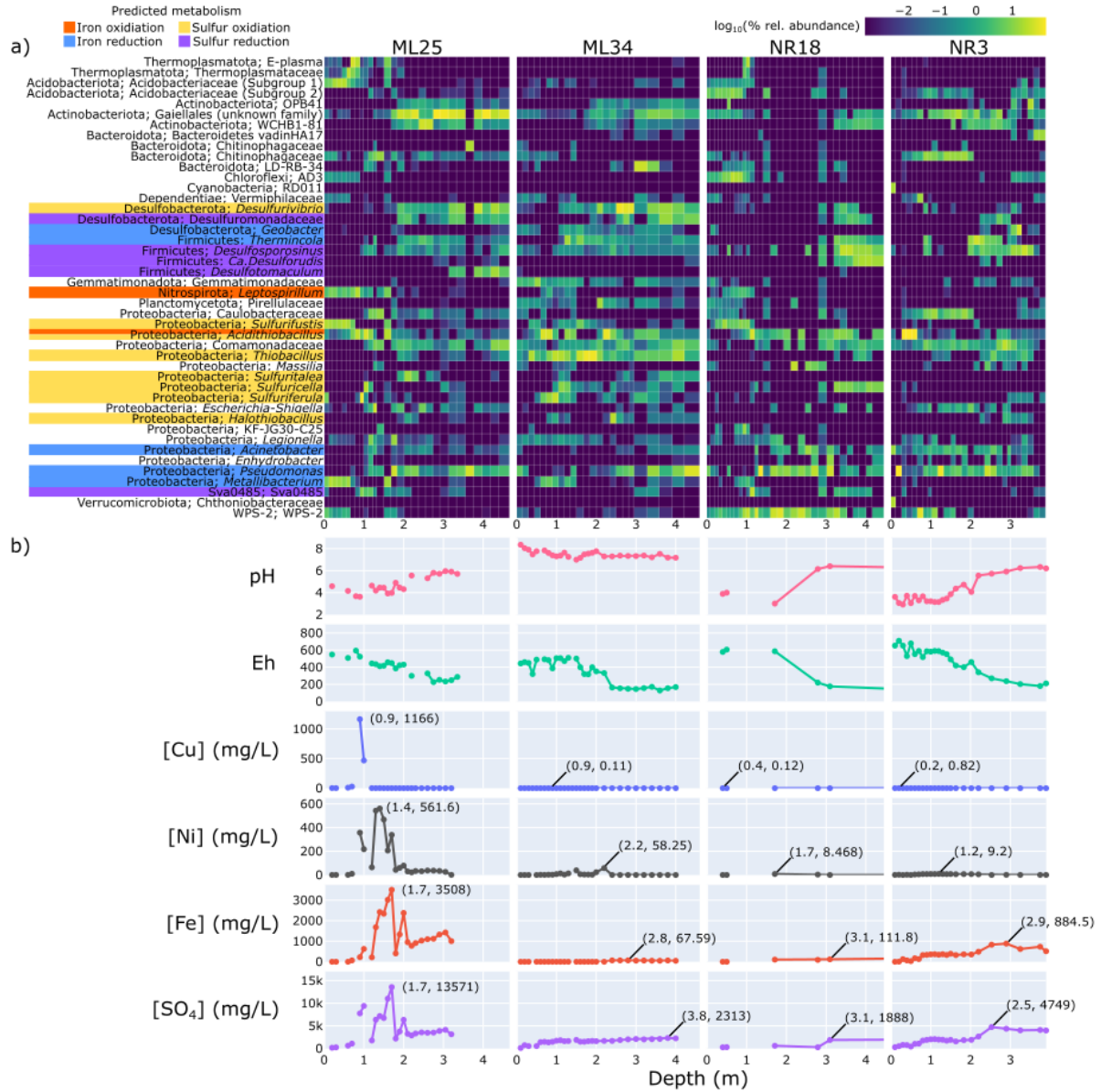


Figure 3.1: Distribution of bacterial and archaeal genera along tailing depth gradients and pore-water chemistry analyses. The heatmap (a) indicates the percent relative abundance of genera that were present at an abundance of at least 15% in at least one sample, across all depth intervals at each sampling location. The phylum and genus are labelled (if the genus was unknown, the family is shown instead). Relative abundances were log transformed, with 0% abundance values set to -2.999. Genera with predicted iron/sulfur cycling capabilities are indicated in color. The corresponding values for pH, Eh, and contaminant concentrations (Cu, Ni, Fe, SO₄) are plotted in (b). For contaminant plots, the coordinates (depth in metres, concentration in mg/L) of the peak concentration value are indicated directly on the plot.

In 2021, tailing core samples extracted from each location were collected in lengths of aluminum pipe (with inner diameters measuring 5.08 and 7.62 cm) driven into the tailings using the technique of Starr and Ingleton (1992). The core samples were subsectioned in lengths of 10 cm (except for the ends of each core, which were 10–17 cm), generating a total of 112 samples (**Table 3.1**). A core collected, frozen and processed in the same way from NR18 in 2019 was used for comparison to 2021 samples (**Table 3.1**).

Table 3.1: Length and number of subsections of tailing cores by location. The length of each core was measured by the depth below ground level that was drilled to extract the core.

Location	Max depth below ground (m)	Number of subsections
ML25	4.66	29
ML34	4.60	24
NR18 (2021)	4.45	31
NR18 (2019)	4.75	31
NR3	3.90	28

3.3.2 Sequencing statistics

Out of a total of 7,610,282 raw reads (ranging from 161 to 217,042 per sample), 6,100,701 were kept after filtering, denoising, merging, and detection of chimeric reads. From these, 11,116 amplicon sequence variants (ASVs) were identified. The median ASV frequency per sample was 50,912, though the range varied from 141–199,300, reflecting the variability in community composition within samples. The median frequency of each ASV across all samples was 29, with a maximum frequency of 546,045. ASVs with a total frequency of 100 or less were generally confined to 1 or 2 samples, while higher abundance ASVs were present across multiple cores and/or multiple depth ranges.

3.3.3 Microbial community composition

Multiple changes in the dominant phyla occur along the depth gradients for each sampled core (**Figure 3.2**). In general, upper layers are mainly comprised of Proteobacteria and Acidobacteriota, which is consistent with most studies on mine tailing communities (Chen et al., 2013; Pepper et al., 2012; Yan et al., 2020). Desulfobacterota and Firmicutes are abundant in deeper tailings; members of these phyla include anaerobic sulfate-reducing bacteria commonly found in anoxic layers of tailings

(Ayangbenro et al., 2018; Gao et al., 2022; Korehi et al., 2014; Winch et al., 2009). Actinobacteriota were highly abundant in ML25 tailings below 2 m in depth (**Figure 3.2**), but not at the other locations. While members of the Actinobacteriota phylum display a diverse range of metabolism, the genera that are commonly found in mine tailings (especially metal tolerant *Arthrobacter* spp.) (Gao et al., 2021; Lopez et al., 2017; Xie et al., 2022) are usually obligate aerobes associated with the rhizosphere of metal hyper-accumulating plants. Therefore, we would expect to see them in the surface layers of vegetated tailings (*e.g.*, ML34) as opposed to the deeper, ML25 tailings. The Actinobacteriota ASVs in ML25 appear to belong to novel clades not previously found in mine tailings; their classification is discussed in the next section.

The WPS-2 phylum is also novel in terms of its presence in mine tailings and seems to be unique to Nickel Rim samples (particularly at NR18 at depths between ~1–3 m) (**Figure 3.2**). WPS-2 (or *Ca. Eremiobacterota*) is a recently described phylum, consisting of a wide range of metabolically diverse bacteria found in many types of terrestrial environments (Ji et al., 2021). Metagenomes isolated from Antarctic desert soil suggest some WPS-2 members are acidophilic (Ji et al., 2021). Another study found that the WPS-2 were abundant (with ASV relative frequencies of up to 24%) in the unvegetated soils of extinct iron-sulfur springs in British Columbia (Sheremet et al., 2020). Given that sampling occurred during near-freezing temperature periods and the acidic, iron/sulfur contaminated nature of AMD, it is not surprising that the WPS-2 could thrive in tailing environments. However, Ji et al. (2021) and Sheremet et al. (2020) found no evidence of microaerophilic or anaerobic metabolic capacity from genomic data in their studies, meaning the WPS-2 ASVs present at Nickel Rim likely represent organisms with novel metabolic capacity within this understudied lineage.

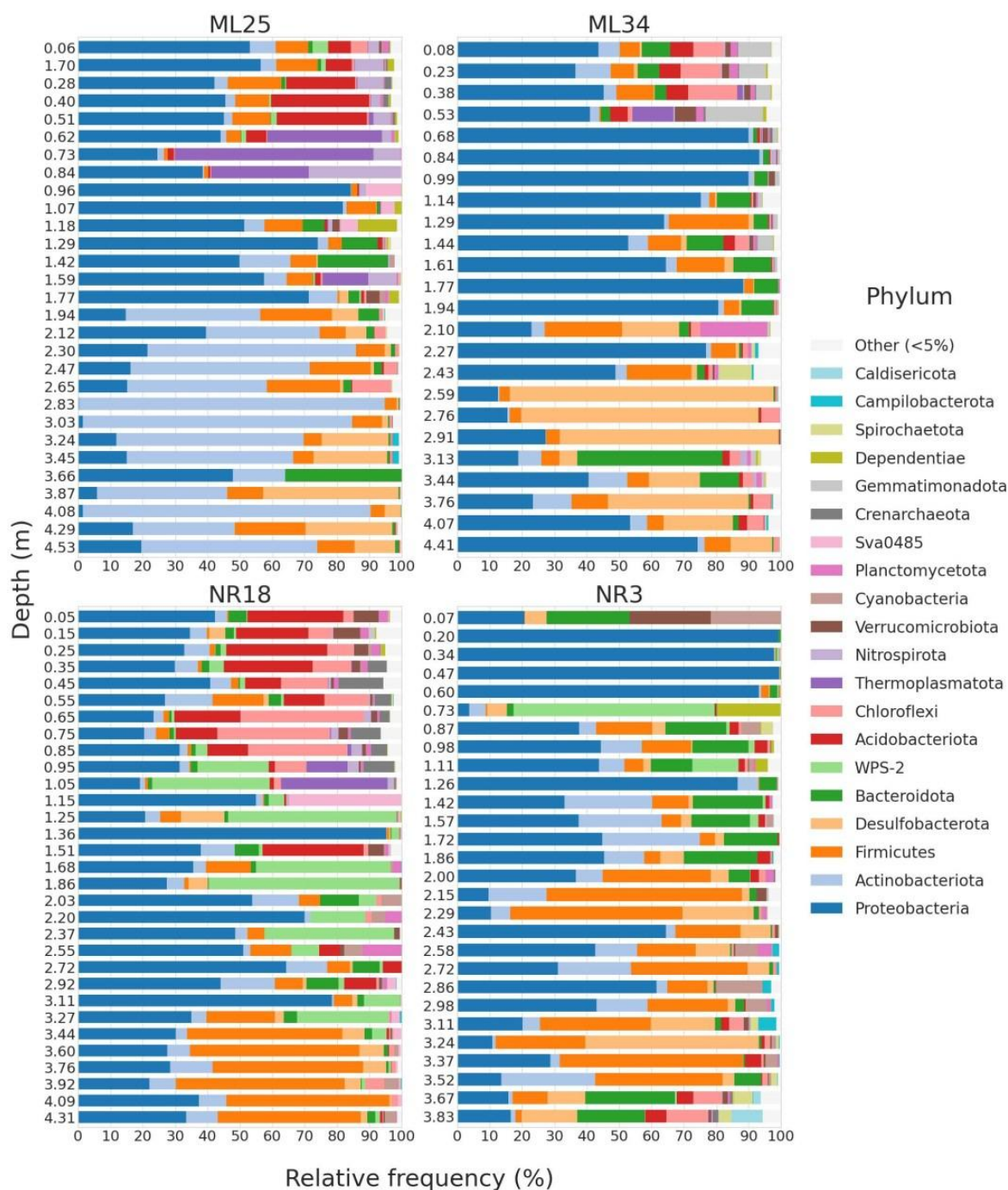


Figure 3.2: Phylum-level microbial community composition of mine tailings. Bars representing samples are grouped by location (ML25, ML34, NR18, NR3) and depth. Bars depict relative frequency of ASVs corresponding to all phyla present at a frequency of $\geq 5\%$ in at least one sample. The bar widths (y-axes) are not scaled based on the depth range covered.

3.3.4 Community change along geochemical gradients

A more detailed analysis of community composition changes (in terms of genus-level relative abundance) across tailings samples is shown in **Figure 3.1a**. The distribution of the most abundant genera ($\geq 15\%$ in at least one sample) was highly variable. Most populations did not show consistent abundance across samples and tended to be confined to and/or enriched in specific depth ranges.

To track how variation in geochemistry along cores impacted the distribution of the lineages highlighted in **Figure 3.1a**, pH/Eh and the concentration of the most environmentally relevant contaminants (Fe, Ni, Cu, SO_4) were linked to genera abundances (**Figure 3.1b**). The pH of ML25, NR18, and NR3 ranged from moderately to slightly acidic (~ 3 – 6) wherein pH increased with depth, while the pH of ML34 was slightly above 7 and more stable across all depths. The general trend of peak Fe, Ni, and Cu concentrations was similar between locations: the highest Cu concentrations were close to the surface, followed by nickel, and finally iron. This observation is consistent with previous hydrogeochemical assessments of Nickel Rim (Johnson et al., 2000). These differences are due to differences in the susceptibility of pyrrhotite ($\text{Fe}_{(1-x)}\text{S}$), pentlandite ($(\text{Fe,Ni})_9\text{S}_8$), and chalcopyrite (CuFeS_2) to oxidation, and to the formation of secondary Fe oxyhydroxide phases (*e.g.*, goethite; αFeOOH) and the secondary Cu sulfide mineral covellite (CuS) (Johnson et al., 2000). Optical examination of mineral thin sections from Nickel Rim and similar tailings impoundments have shown that pyrrhotite is the most susceptible (least resistant) to oxidation, followed by pentlandite, chalcopyrite and pyrite (Lindsay et al., 2015). Other factors influencing the localization of dissolved metals include differences in metal sorption capabilities to soil/clay particles (competitive sorption of Cu ions is greater than Ni ions, reducing transport to lower depths) (Harter, 1992; Sheikhhosseini et al., 2013) as well as pH-dependent precipitation of Fe (oxy)hydroxides (which can sorb Ni) and covellite (CuS) (Johnson, 1993). Sulfate concentrations generally followed Fe, as both are released during the dissolution of sulfide minerals. **Figure 3.3** summarizes correlations between geochemical variables.

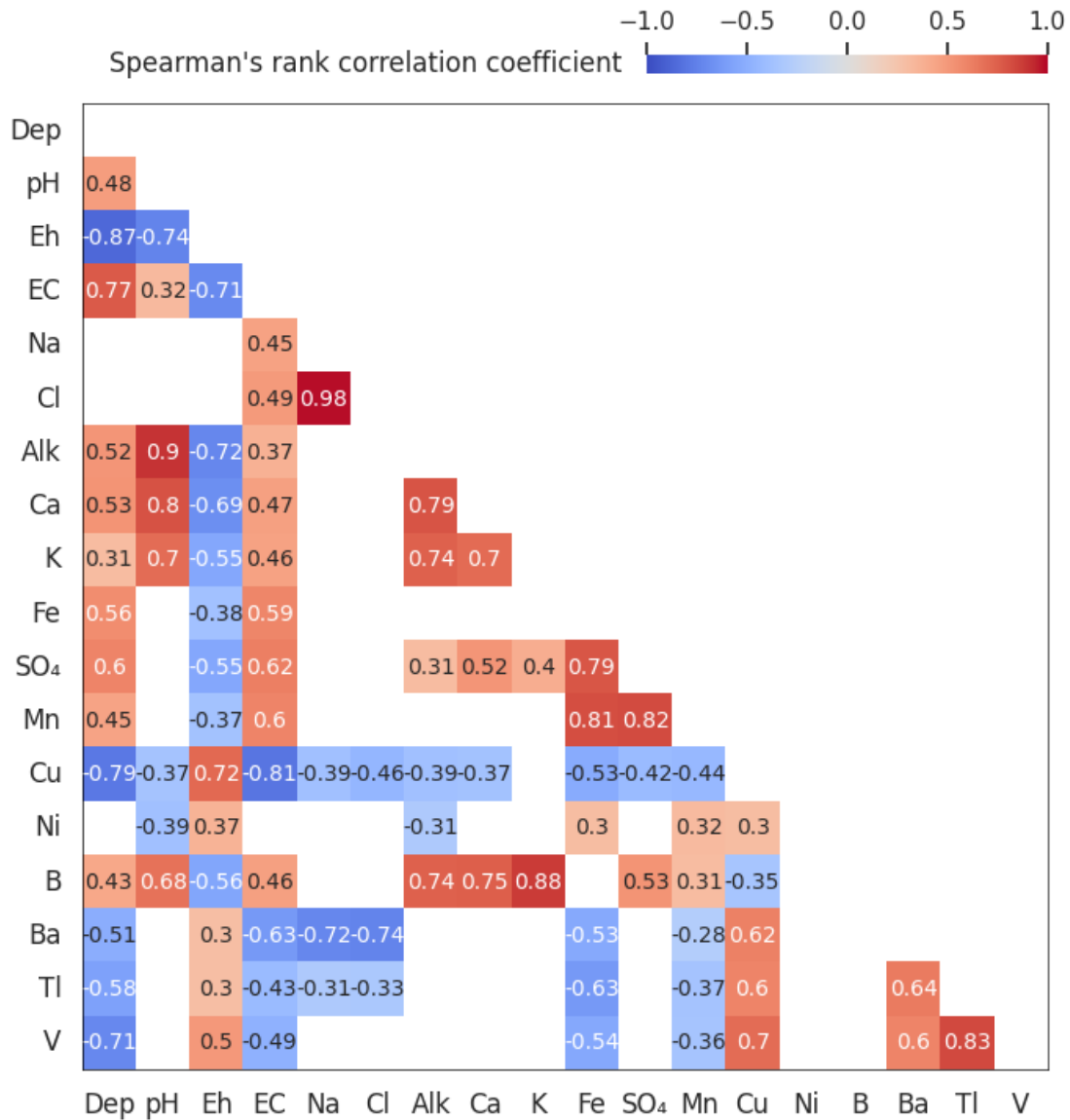


Figure 3.3: Correlation matrix of geochemical variables. Pairwise Spearman's rank correlation coefficients were calculated for each factor. Dep=depth, EC=electrical conductivity, Alk=Alkalinity. Significant correlations ($p < 0.05$, Bonferroni corrected) are displayed in the figure.

Correlations between abundance data (**Figure 3.1a**) and geochemistry identified several significant connections (**Figure 3.4**). We included Cu and Ni in our analyses despite their concentrations not having a significant impact on overall community diversity (discussed in section 3.3.5) because they are the main contaminants released from these tailings, and increased tolerance to these metals would be an asset for bioremediation-relevant organisms. Dissolved Cu concentrations were moderately correlated with the abundance of almost half of the dominant genera (21 of 44 genera, 48%). Ni showed comparatively fewer significant correlations (**Figure 3.4**). However, it was difficult to determine which specific factors were directly interacting with microorganisms, due to the interconnected nature of environmental variables (**Figure 3.3**). Correlation matrices were also individually calculated for each sampling location (**Figure B1**), which differed from the pooled dataset (most notably, ML25 had much stronger R values due to a wider range of contaminant concentrations, contributing to stronger selective forces).

Diverse consortia associated with iron and sulfur cycling were identified in each core, so their abundance and localization were specifically examined (**Figure 3.1**, **Figure 3.4**). Iron/sulfur-oxidizing genera common to AMD were typically confined to the upper 2 m of tailings. *Acidithiobacillus* ASVs (the majority of which were classified as *A. ferrooxidans*) were the most abundant predicted iron-oxidizer in the subsurface (0.5–1.5 m). The surrounding environment is likely microaerophilic at this depth, which are optimal growth conditions for *A. ferrooxidans* (Namgung & Song, 2015). Pore gas oxygen data was limited to near the tailings surface due to the low gas-filled pore space present in deeper tailings with a high water content and was shown to decrease from atmospheric levels at ground surface to <10% over 1 m in ML25, ML34, and NR18 (**Figure 3.5**). It is worth noting that *A. ferrooxidans* also exhibits considerable metabolic diversity: not only is this species able to oxidize both ferrous iron and reduced sulfur species in oxic environments, it can also use ferric iron as an electron acceptor in anoxic conditions. Iron reduction is likely the predominant metabolism supporting *A. ferrooxidans* populations under anoxic conditions below depths of 2 metres.

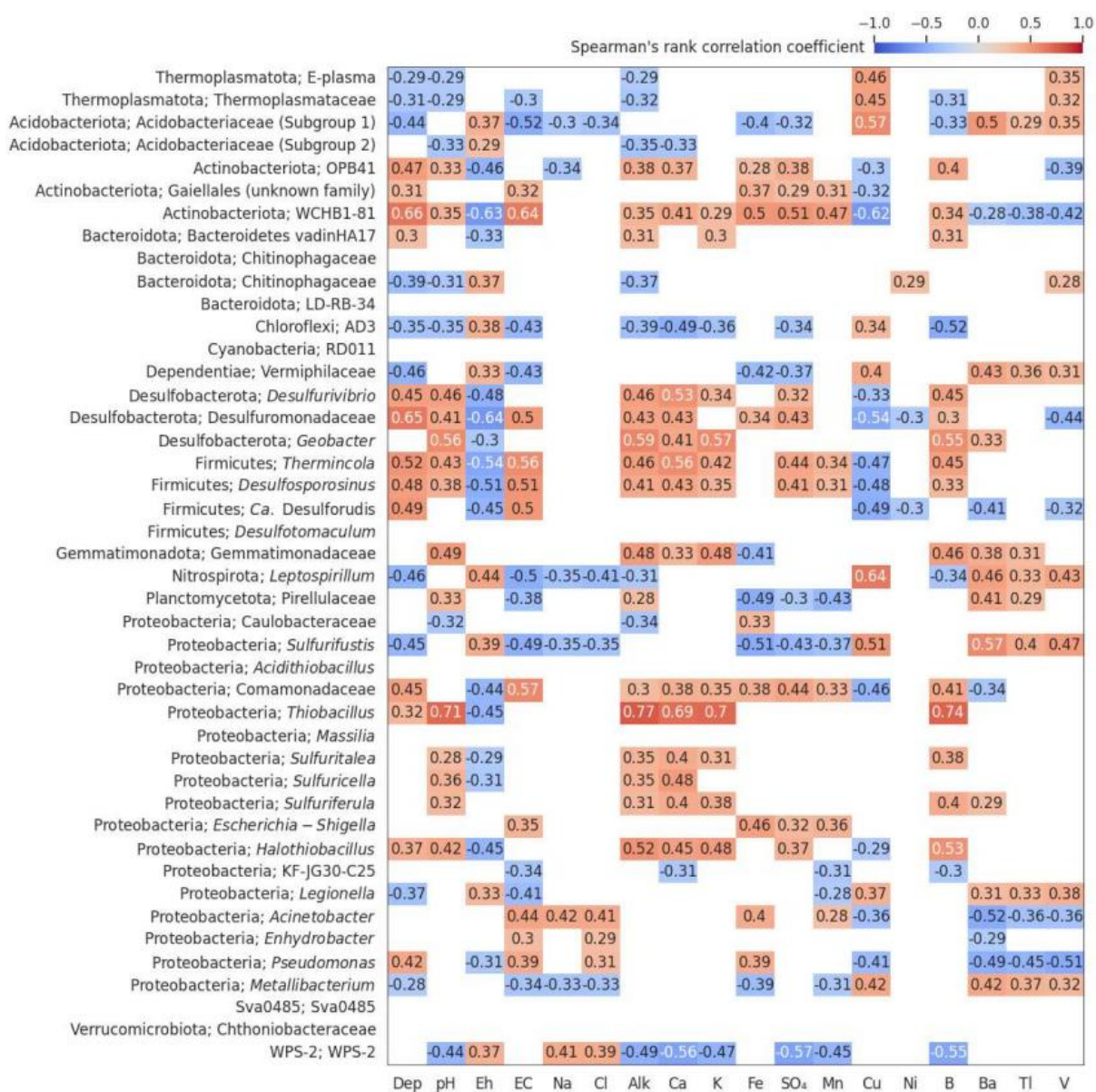


Figure 3.4: Correlation matrix of genus-level relative abundance and geochemical factors. Spearman's rank correlation coefficients were calculated between the relative abundance of the abundant genera (**Figure 3.1**) and select geochemical factors (Dep=depth, EC=electrical conductivity, Alk=Alkalinity), across all sampling locations. Significant correlations ($p < 0.05$, Bonferroni corrected) are shown. For location-specific correlations, see **Figure B1**.

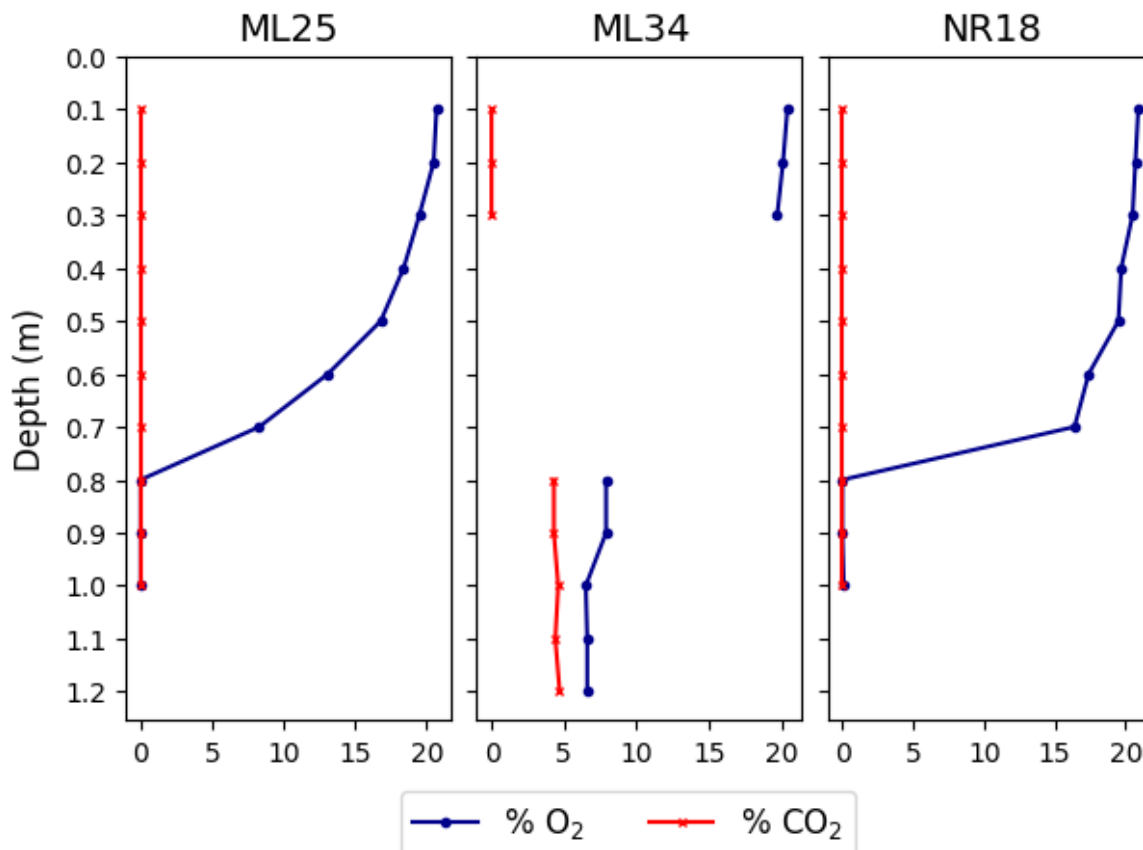


Figure 3.5: Gas measurements of oxygen and carbon dioxide in mine tailings. Oxygen decreased from atmospheric concentrations (20%) to 0.0% at ML25 and NR18 over the first 0.8 m. Depths below this point were saturated (below the water table). At ML34, depths between 0.4–0.8 m could not be measured. O₂ at ML34 dropped to 6.6% at the 1.2 depth, and further depths also could not be measured due to equipment malfunction and infiltration of atmospheric oxygen into the peristaltic pump. CO₂ was measured at 0.0% at all depth points at ML25 and NR18, while ML34 tailings showed CO₂ concentrations of 4.3–4.7% between 0.8–1.2 m, due to the dissolution of carbonate minerals present at this location.

The abundance of *Acidithiobacillus* ASVs in ML34 despite pH >7 conditions is unusual, but could be associated with the period of exposure to atmospheric oxygen and sulfide oxidation prior to the installation of the DST and organic carbon cover layers. The persistence of *Acidithiobacillus* ASVs could be leveraged for bioremediation approaches, such as in aerobic wetlands that use neutrophilic iron oxidizers to precipitate ferric iron minerals (e.g., ferrihydrite, goethite) (Blowes et al., 2003). Existing iron-oxidizers currently applied in aerobic wetlands include *Gallionella* and *Leptothrix* spp., (Johnson & Hallberg, 2005). *Acidithiobacillus* species/strains that have adapted to

survive in neutral or alkaline environments may be more efficient at immobilizing iron by oxidation while also being tolerant to higher concentrations of copper, nickel, and other toxic metals in tailings.

Leptospirillum spp. (mostly *L. ferrooxidans*), exclusive iron-oxidizers, follow a similar distribution but are much less abundant. *L. ferrooxidans* prefers lower pH ranges (0.5–0.7) than *A. ferrooxidans* (1–3) in extreme AMD environments (Schrenk et al., 1998), but was co-localized in moderately acidic environments such as ML25. *A. ferrooxidans* abundance did not significantly correlate with any factor in the combined dataset (**Figure 3.4**), but did correlate with a few factors such as pH and Eh within each sampling location (**Figure B1**). *L. ferrooxidans* abundance was moderately to strongly correlated with several environmental factors in both the combined dataset and in ML25. Based on these observations, *A. ferrooxidans* could be described as more of a ‘generalist’ species that is able to inhabit many types of mine tailings (see earlier discussion on their metabolic diversity), although they will still localize to a niche within each tailings environment based on preferences in pH, oxygen availability, etc. In contrast, *L. ferrooxidans* is a ‘specialist’ that is adapted to survive at high concentrations of metals, but is outcompeted in mine tailings with low metal contamination. *L. ferrooxidans* abundance correlates strongest with copper, $R=0.64$, although previous studies have shown that their higher affinity for ferrous iron and tolerance to higher concentrations of ferric iron is what drives niche partitioning between *A. ferrooxidans* and *L. ferrooxidans* (Blowes et al., 2003). It is also important to note that bioleaching with mixed cultures containing both *Acidithiobacillus* spp. and *Leptospirillum* spp. are more effective than pure cultures (Akcil et al., 2007; Falco et al., 2003; Fu et al., 2008; Zhang et al., 2008). Therefore, oxidation and AMD formation within ML25 tailings is expected to be higher due to the combined activity of both organisms (as evidenced by the lower pH and higher dissolved metal content) (**Figure 3.1b**), even if their total abundance is similar to single populations of *A. ferrooxidans* at other locations.

Genera predicted to exclusively be sulfur oxidizers included *Sulfurifustis*, *Thiobacillus*, *Halothiobacillus*, *Sulfuritalea*, *Sulfuriferula*, *Sulfuricella*, and *Desulfurivibrio* (**Figure 3.1a**). The *Sulfurifustis* ASV most abundant in the surface of ML25 and NR18 tailings appears to be a novel species tolerant of acidic environments, as previously isolated *Sulfurifustis* species are neutrophilic (Kojima et al., 2015). The abundance correlation for ASVs in the *Sulfurifustis* genus with geochemical factors resembles that of *Leptospirillum* rather than other sulfur oxidizers (**Figure 3.1a**), suggesting that members of the *Sulfurifustis* are also specialized for resistance to high metal concentrations (**Figure 3.4**). *Thiobacillus* and *Halothiobacillus* ASVs were also not classified to the

species level and are predicted to be novel neutrophilic sulfur oxidizers (Whaley-Martin et al., 2019) most abundant in ML34 (**Figure 3.1a**); *Thiobacillus* abundances in particular exhibit a strong positive correlation with pH ($R=0.71$) and alkalinity ($R=0.77$). The localization of *Desulfurivibrio* (and *Sulfuricella* within NR18) ASVs to the deeper levels of the tailings is in line with their expected use of sulfur oxidation pathways coupled to NO_3 reduction rather than O_2 (in the case of *Desulfurivibrio*, it is expected to use the dissimilatory sulfate reduction pathway in the reverse direction), potentially allowing these populations to co-exist with anaerobic sulfate reducers by metabolizing the sulfides generated by the sulfate reducers (Kojima & Fukui, 2010; Thorup et al., 2017).

The distribution of potential iron reducers was more varied than iron oxidizers, and included *Geobacter*, *Acinetobacter*, *Thermincola*, *Metallibacterium*, and *Pseudomonas* populations (**Figure 3.1a**) (Pan et al., 2017; Zavarzina et al., 2007; Ziegler et al., 2013). *Geobacter* and *Thermincola* generally localize to greater depths in NR18/NR3, where dissolved iron is high (**Figure 3.1a**). At pH >2.5 , ferric iron precipitates as secondary oxyhydroxide minerals (Blowes et al., 2003), and reductive dissolution is problematic in these conditions, as it re-mobilizes ferrous iron. The *Pseudomonas* and *Acinetobacter* do not display a consistent localization pattern (**Figure 3.1a**), but as these genera are highly diverse and ubiquitous in various environments, predictions about iron-reducing capabilities should only be done on the species level, which was not possible as the most abundant ASVs were only classified at the genus level. The *Metallibacterium* are a recently described genus, with one isolate (*M. scheffeleri*) from an acidic biofilm demonstrating iron reduction (Ziegler et al., 2013), although Bartsch et al. (2017) could not identify any iron-reducing genes through genomic, transcriptomic, and proteomic analyses. Unlike the other potential iron reducers, *Metallibacterium* ASVs co-localized with iron oxidizers (*L. ferrooxidans* in ML25, and *A. ferrooxidans* in ML34/NR18) (**Figure 3.1a**); as they are facultative anaerobes and only reduce iron under anoxic conditions (Ziegler et al., 2013), it is unlikely that they are actively reducing iron at these depths. However, as *Metallibacterium* have shown the potential to alkalize their surroundings through the release of ammonium (Bartsch et al., 2017), they are a promising candidate for AMD bioremediation and will be a target for investigation in future multi-omic studies.

Sulfate reducers including members of the Firmicutes (*Desulfosporosinus*, *Ca. Desulforudis*, *Desulfotomaculum*) and Desulfobacterota (unclassified Desulfuromonadaceae) were generally dominant at depths >2 m (**Figure 3.1a**), which is below the depth of oxygen ingress. As expected, the sulfate reducers were generally positively correlated with pH (most are inhibited below pH 5.5) (Hao

et al., 1996) and negatively correlated with Eh. They also show moderate negative correlations with dissolved copper concentration, as they are expected to stimulate secondary covellite (CuS) precipitation via the production of sulfide. Relative abundances were not consistent between locations, which could reflect preferences of individual taxa to specific environmental conditions, or legacy impacts from the initial subsurface community present in the ore from which the tailings were generated. The *Desulfotomaculum*, almost exclusively localized to ML25 (**Figure 3.1a**), are a genus mostly associated with deep subsurface environments (Aullo et al., 2013). *Ca. Desulforudis* (localized to NR18) (**Figure 3.1a**) is also typically found in very deep environments, the most notable being *Ca. Desulforudis audaxviator*, which was the sole organism present 2.8 km below the surface in a gold mine (Chivian et al., 2008). *Desulfosporosinus* are common in natural soil/sediments as well as mine tailings in cold climates (Abicht et al., 2011), and were more evenly distributed in Strathcona Mill tailings compared to Nickel Rim. Desulfuromonadaceae abundance followed a similar trend. Finally, the Sva0485 clade (*Ca. Acidulodesulfobacterales*) have recently been described as a dominant member of sulfate-reducing consortia in AMD and ferruginous lakes (Tan et al., 2019). While the characterized members of the Sva0485 lineage possess genes for dissimilatory sulfate reduction, they are facultative anaerobes and also encode genes for sulfur oxidation, iron cycling, and methanogenesis (Tan et al., 2019; Vuillemin et al., 2018). This varied metabolic potential could explain why their depth range was not consistent between tailings locations here, and why their abundance was not significantly correlated to geochemistry (**Figure 3.4**), although individual locations showed variable correlations between Sva0458 and salinity (**Figure B1**). Biological sulfate reduction is leveraged in a variety of AMD bioremediation strategies, including anaerobic (or compost) bioreactors, sulfidogenic bioreactors, and permeable reactive barriers (Johnson & Hallberg, 2005). All these approaches rely on biogenic sulfide production, typically in the form of H₂S, which precipitates various metal-sulfide minerals (Blowes et al., 2003; Johnson & Hallberg, 2005).

The archaeon E-plasma (and other unclassified Thermoplasmataceae ASVs) abundant at ~1 m depth at ML25 and NR18 (**Figure 3.1a**) have been identified in various AMD and non-AMD environments (Baker & Banfield, 2003; Golyshina, 2011). E-plasma were found to dominate microbial AMD communities within the Parys Mountain mine (United Kingdom), characterized by low-to-moderate temperatures and extremely low pH (Korzhnikov et al., 2019), suggesting that they are adapted to colder temperatures (unlike most members of the order Thermoplasmatales, which are mesophilic to moderately thermophilic) (Golyshina, 2011). The metabolic potential of E-plasma and

other related ‘alphabet-plasma’ lineages belonging to the Thermoplasmataceae is currently unknown, as isolation attempts have not yet been successful. However, genomic data suggests that they are heterotrophic scavengers (Korzhenkov et al., 2019).

Many of the most abundant lineages are taxonomically novel and/or previously not identified within mine tailings (**Figure 3.1a**). The Actinobacteriota (most abundant in ML25 but present at all locations) ASVs mostly belong to the order Gaiellales (common in deep sea sediments) (Chen et al., 2021), WCHB1-81 (microcystin-contaminated lakes) (Dziga et al., 2019), and OPB41 (subsurface environments) (Khomyakova et al., 2022). Other ASVs that were unclassified at the genus/species level include the Acidobacteriaceae (associated with mine tailings but highly diverse (Campbell, 2014), as shown by the fact that the two subgroups identified in this study exhibit very different localization patterns), as well as the Gemmatimonadaceae, Pirellulaceae, Comamonadaceae, and Chitinophagaceae, which have not previously been associated with mine tailings.

The high amount of unresolved microbial diversity from 16S rRNA gene sequencing data implicates that the Strathcona Mill and Nickel Rim mine tailings host currently uncharacterized microorganisms that could participate in the biogeochemical processes governing the release, mobility, and/or attenuation of contaminants associated with AMD. Nonetheless, it is important to note that most samples analyzed in this study were low in metal concentration and circumneutral pH, and therefore not representative of microbial communities that could be leveraged for the remediation of highly acidic tailings. Future multi-omic and culture-based approaches are required to elucidate the full range of microbial activities present at these sites. The ultimate goal of these combined approaches is to contribute to the development of *in-situ* bioremediation strategies for AMD, or to limit the extent of contaminant release that is accelerated by microbes (Gould et al., 1997).

3.3.5 Alpha and beta diversity

Alpha diversity

Plots comparing Faith's phylogenetic distances, Shannon indices, and Observed ASVs of samples (grouped by location) showed consistent trends across the four cores (**Figure 3.6**). Samples from ML25 consistently scored low on α -diversity metrics across all depths, although the specific taxonomic composition of samples was highly variable when comparing upper and lower depths (discussed in section 3.3.3). The α -diversity metrics of NR3 samples were overall similar to ML25, although the deeper fractions of the core tend to have higher diversity (**Figure 3.6**). It is possible that the deposition of NR3 tailings on top of native vegetation in the 1950s, the presence of nearby plants, and periodic flooding events (Johnson, 1993) account for the higher diversity at greater tailings depths, supported by the increased DOC concentrations in these samples (**Data File B1**).

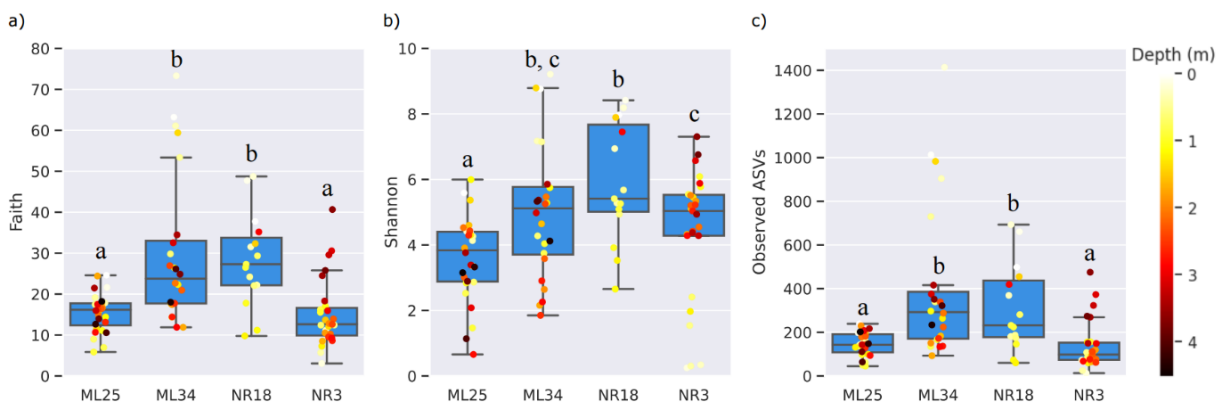


Figure 3.6: α -diversity metrics for all sampling locations and depths. Faith's phylogenetic distances (a), Shannon diversity indices (b), and observed ASVs (c) were calculated with QIIME2 using a rarefied sampling depth of 12,000 reads. Individual samples overlaid on box plots are colored according to the vertical depth below ground level. Significant differences (Kruskal-Wallis, $p < 0.05$) between locations are designated by different letters above each boxplot.

ML34 and NR18 showed significantly higher α -diversity metrics compared to ML25/NR3, which we attribute to the cover layers and vegetation present at these locations (Londry & Sherriff, 2005; Pepper et al., 2012), and is especially evident in the fact that ML34 diversity was highest in the samples closest to the surface. A similar trend was seen for NR18, but due to low read numbers in the deeper NR18 sections, most samples below 2 metres were excluded after rarefying to a minimum sampling depth of 12,000, hindering a full comparison.

Beta diversity

Samples did not appear to discretely group by location in any of the β -diversity ordinations (**Figure 3.7a, Figure B2a, Figure B3a**), indicating that the variability between samples at the same location was greater than the overall differences between locations.

The weighted UniFrac principal coordinate analysis (**Figure 3.7a**) was the most effective in mapping distances between samples, with the two principal axes explaining 91.7% of variation in the data. ML25 samples showed particularly high separation along the primary axis, which may reflect the much broader range of contaminant concentrations along the core, such as dissolved Fe, Ni, Cu, and SO₄ (**Data File B1**). Analysis of individual locations showed that weighted UniFrac PCoA was also best for mapping distances (**Figure 3.7b-e**), with the principal axes explaining 66.1–96.4% of the variation. There was some separation of samples along one or both axes based on depth, which was more apparent in the Bray-Curtis and unweighted UniFrac PCoA plots (**Figure B2b-e, Figure B3b-e**), suggesting the presence of environmental gradients along the depth of the core that influence community composition.

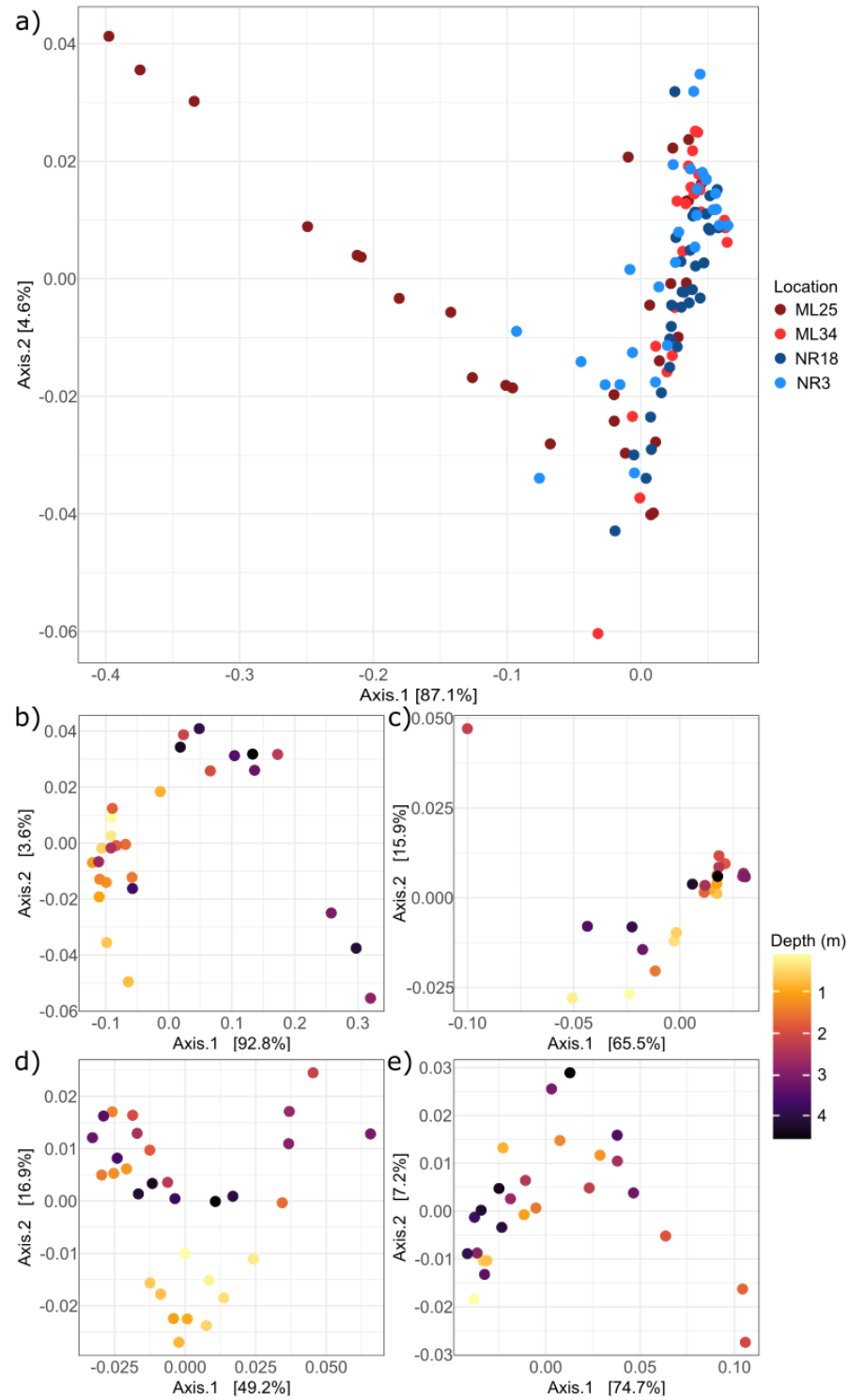


Figure 3.7: Weighted UniFrac PCoA plot for all sampling locations combined (a), ML25 (b), ML34 (c), NR18 (d), and NR3 (e). Sample points in plots b-e are colored by depth.

NMDS analysis (**Figure 3.8**) also did not show grouping of samples by location, though there was some separation between ML and NR samples along the secondary axis. Out of all geochemical variables mapped to the samples (**Data File B1**), the ones significantly correlating with community composition included pH, Eh, alkalinity, and salinity (inferred by electrical conductivity (EC) and dissolved Na, Cl, and K). It was expected that major community shifts occur across these environmental gradients, as microorganisms segregate into niches based on tolerance to selective forces (*e.g.*, stress from low pH or high salinity) (Sardinha et al., 2003; Zhahnina et al., 2015; Zhang et al., 2019) and metabolic potential (influenced by redox gradients and oxygen availability) (Chen et al., 2017; Lipson et al., 2015; Pett-Ridge & Firestone, 2005). Pore-gas oxygen measurements were taken at ML25, ML34, and NR18 tailings, but not included in the statistical analyses due to limited data points (**Figure 3.5**). However, the iron-dominated redox potential (Eh) of tailings environments can be used as a proxy to infer the metabolic capacity of samples (**Data File B1**); with aerobic respiration only supported at highly positive Eh values (above +300–500 mV) (Søndergaard, 2009), although this threshold increases with decreasing pH.

Diversity also correlated with the concentrations of Fe, Mn, B, Ba, Ca, Tl, and V. Ferrous and ferric iron availability is known to correlate with the abundance of iron oxidizing and reducing bacteria, such as members of the Actinobacteriota and Gammaproteobacteria (Liu et al., 2014). Many iron oxidizing/reducing organisms can also oxidize/reduce manganese (de Vrind-de Jong et al., 1990; Thamdrup, 2000). As for the other elements, correlation between microbial diversity and concentration is more likely due to the co-variance between ion concentration and another geochemical factor, such as pH, rather than direct interactions between the elements and microorganisms (**Figure 3.3**). For example, calcium (*i.e.*, Ca in **Figure 3.8**) concentration was strongly correlated with alkalinity (measured in mg/L CaCO₃) (R=0.79). However, it is possible that selection for resistance to Tl and V toxicity may influence community composition (She et al., 2022; Zhang et al., 2019), as these elements are highly toxic at trace concentrations. In particular, vanadium concentrations in several samples from ML25 and ML34 far exceed the recommended safe limit for drinking water (0.05 mg/L) (Environment and Climate Change Canada, 2016) so selection for vanadium resistance mechanisms such as the reduction of V⁵⁺ to V⁴⁺ would be relevant in these communities.

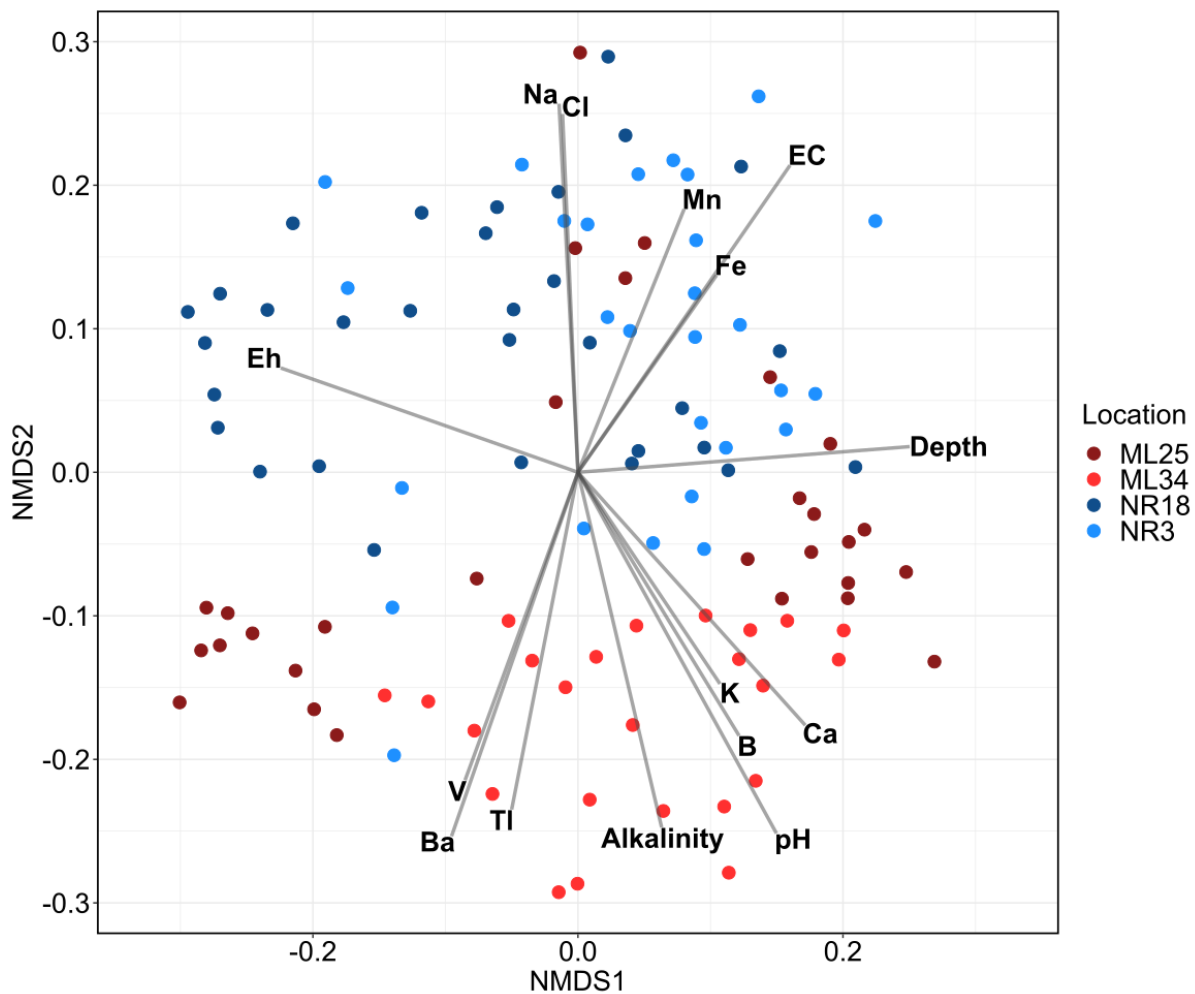


Figure 3.8: NMDS biplot of Bray-Curtis distances and environmental variables. Samples are colored by location. Environmental variables that significantly correlated with community dissimilarity ($p < 0.05$, Bonferroni corrected) were included. For a plot showing all variables, see **Figure B4**.

Interestingly, β -diversity did not significantly correlate with Ni or Cu concentrations, despite these elements being the major porewater contaminants present in these tailings besides Fe (with upper concentration limits of 1,166 mg/L for Cu and 561.6 mg/L for Ni) (**Data File B1**). A possible explanation could be that Ni/Cu tolerance is widely distributed across many microbial taxa commonly found in mine tailings, and thus the overall variance in community composition is determined by other selective forces. Ni and Cu are both micronutrients required for the function of metalloenzymes and most prokaryotes encode homeostatic mechanisms to regulate intracellular concentrations (Newsome & Falagán, 2021). Nickel efflux pumps such as RcnA have been identified in organisms

isolated from metal contaminated environments (e.g., *Cupriavidus metallidurans* strain CH34), as well as in non-Ni-resistant *E. coli* and *H. pylori*, with homologs found in other proteobacteria, cyanobacteria, and archaea (Macomber & Hausinger, 2011). Similarly, extremophiles used in industrial biomining such as *A. ferrooxidans* and *Sulfolobus metallicus* share the same ATPases and transporters for copper export (CopA and CusA) with other bacteria, archaea, and eukaryotes (Andrei et al., 2020; Antsotegi-Uskola et al., 2020; Orell et al., 2010). The presence of these pathways in Nickel Rim/Strathcona Mill tailings could be confirmed with multi-omic sequencing.

3.3.6 Temporal variation

Changes in genus-level abundance of ASVs sequenced from NR18 tailings in 2019 compared to 2021 samplings show that the abundance of most genera remained relatively stable over the two-year period (**Figure 3.9**). Both *Acidithiobacillus* and *Pseudomonas* ASVs show an overall trend of decreased abundance at various depths between 0.8–2.6 m. We note that prominent increases and decreases in abundance in adjacent core sections is more likely due to imperfect depth mapping between the two years' cores rather than large changes in community composition at NR18 as a whole. The WPS-2 relative abundance increased at multiple depth ranges between 1–2 m. Many of the NR18 vadose zone pore-water samplers were dry in 2021, and core moisture content is likely to influence WPS-2 abundance, as they are known to be common in dry, bare soil environments (Sheremet et al., 2020). Drier tailings could also explain the decrease in *A. ferrooxidans*, which are not desiccation tolerant (Díaz-Tena et al., 2017). The taxonomic representation of sulfate-reducing bacteria also changes slightly (*Ca. Desulforudis* appear to be superseded by *Desulfosporosinus* between 3–3.5 m), which may be a response to changes in an environmental factor such as pH or alkalinity (moderately positively correlated with *Desulfosporosinus* abundance but not *Ca. Desulforudis*) (**Figure 3.4**). Water samples were not collected in 2019, so we were unable to further investigate this hypothesis.

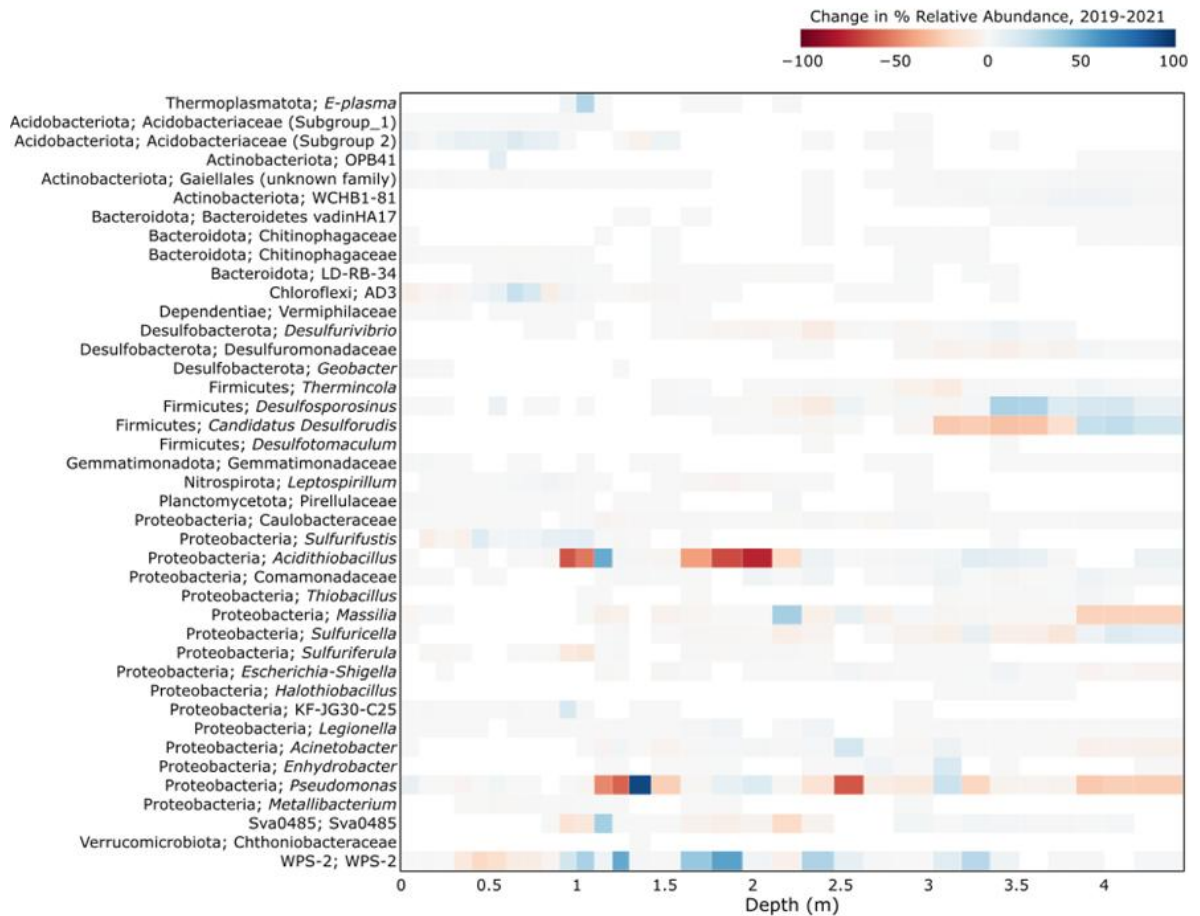


Figure 3.9: Change in relative abundance of microbial genera across varying depths at the NR18 tailings dump, 2019-2021. The genera listed in **Figure 3.1** were compared to NR18 samples taken in 2019, those present in both datasets were included in this figure. The percent relative abundance of the 2019 samples were subtracted from the NR18 2021 samples. Samples were mapped to each other based on the proximity of the midpoint depths of each core section.

3.4 Conclusions

Mine tailings present a challenge in waste management practices. AMD environments harbor taxonomically and metabolically diverse microorganisms, which have evolved a wide variety of adaptations to thrive in highly heterogeneous contaminated environments. We utilized a 16S rRNA amplicon sequencing approach to characterize microbial communities at narrow (~10 cm) depth intervals at four locations at two tailings sites, providing a high-resolution profile of community changes across tailings gradients of varying geochemical compositions.

Our research shows that microbial communities are highly diverse between and within each sampling location. Our findings suggest that future sampling efforts that capture a wide range of tailings locations and depths could contribute to the isolation of a broad range of bioremediation-relevant species. We identified that overall community composition is correlated with pH, Eh, alkalinity, salinity, and some metal ions, which surprisingly did not include the major constituents Cu and Ni. Other variables that did not correlate with overall diversity were DOC, F, SO₄, NO₃, Al, As, Co, Li, Mg, Pb, S, Si, Sr, and Zn.

The abundance of most individual lineages was also closely associated with different geochemical factors. However, due to the covarying nature of many environmental variables, it is important to frame predictions about which factors microorganisms are directly responding to, as well as the trajectory of contaminant cycling within tailings, around what is currently known about the physiology and ecology of each individual organism (or its closest known relatives).

Our results showed that amplicon sequencing data can be used to identify specific microenvironments of tailings harboring novel or currently uncultured species, although this approach is limited in its ability to predict the physiological and metabolic properties of these uncharacterized organisms. Many ASVs present in Strathcona Mill and Nickel Rim tailings could not be classified at the species level, including potential iron/sulfur oxidizers and reducers, though this result is likely influenced by the fact that most samples were circumneutral and low in metal concentration. Functional profiling of tailings known to contain these novel lineages using metagenomics and metaproteomics will be an important future direction to identify genes associated with metal (Fe, Cu, Ni) resistance and transformation. By informing on geochemical variables required to provide niche-specific conditions for growth in the lab our work also provides an insight into improved approaches for establishing enrichment cultures that target these species.

Chapter 4

‘Metal-genomes’: profiling microbial metal resistance and metabolism genes in mine tailings

4.1 Introduction

Mine tailings are fine-grained waste materials generated from the extraction of valuable metals from ores. Sulfide-rich tailings pose a major risk of environmental contamination due to the formation of acid mine drainage (AMD) from oxidation of sulfide minerals (*e.g.*, pyrite) (as previously described in sections 1.3 & 3.1). Bacteria and archaea may participate in the biogeochemical cycling of sulfur, iron, and/or other heavy metals in mining waste through their metabolic activities, with potential consequences on contaminant mobility (as previously described in sections 1.3 & 3.1).

In Chapter 3, an investigation into microbial communities inhabiting mine tailings identified high variability in community composition and diversity across four legacy Cu/Ni tailings impoundments in Sudbury, Ontario. The extent of mineral oxidation and presence of cover layer(s) varied between sampling locations, influencing the relative acidity and dissolved metal concentrations of the environments. Through 16S rRNA amplicon sequencing on 10 cm vertical sections of tailings cores, we determined a high-resolution taxonomic profile of communities across a range of geochemical gradients. Major findings of this study included correlations between the microbial community structure with pH, Eh, alkalinity, salinity, and some metal ions, which unexpectedly did not include Cu/Ni. Many lineages associated with iron/sulfur cycling in tailings were identified, as well as a number of novel taxa that have not previously been described or studied within mine tailings.

Although 16S rRNA amplicon sequencing is a valuable tool for characterizing environmental microbial diversity, it does not provide a way to directly infer the potential metabolic activities or functions of organisms (details included in section 1.4). Predicting mechanisms of heavy metal resistance and biogeochemical cycling in mine tailings is improved by the use of metagenomics to identify MRGs and MMGs, especially those that are taxonomically novel or lack isolated representatives. Additionally, the activity and subsequent ecological impact of organisms in their natural environments are not necessarily tied to their abundances. These roles can be clarified by measuring gene expression (*i.e.*, via RNA and/or proteins) (discussed in section 1.4) (Wang et al., 2016). Metagenomic, metatranscriptomic, and metaproteomic (‘-omics’) studies of AMD

environments have been crucial towards advancing our knowledge of mining waste microbiology (reviewed in Huang et al., 2016; Méndez-García et al., 2015), providing information about the importance of rare populations (Hua et al., 2015), metal resistance strategies (Ayala-Muñoz et al., 2020; Mirete et al., 2007), ecological succession (Mueller et al., 2011), and potential for biomining (Cárdenas et al., 2016; Kumar et al., 2023). Metaproteomics even enabled the discovery of a novel cytochrome essential for iron oxidation within AMD communities (Ram et al., 2005).

It can be challenging to collect sequencing data from mine tailings due to the low biomass, high volume sample matrix, and the presence of heavy metal contaminants. Currently, there are no commercial products optimized for nucleic acid or protein extraction from tailings as there are for other types of microbiomes (*e.g.*, soil, gut). Metagenomic studies on tailings communities have been conducted (Ghosh & Das, 2018; Gupta et al., 2017; Liu et al., 2021), but metaproteomic studies of mining waste have focused on biofilm samples (reviewed in Wilmes et al., 2015). There have been no proteomics analyses of biomass extracted directly from mine tailings to date, likely due to difficulties extracting from these complex matrices.

The objective of this study was to further characterize the mine tailings communities described in Chapter 3, focusing on identifying their metal resistance mechanisms and iron/sulfur cycling capabilities through the use of metagenomics and metaproteomics. In this chapter, I develop a method of protein extraction from mine tailings, and review the effectiveness of current DNA and protein extraction methods from mine tailings for ‘-omics’ level analyses. I use gene annotation tools to characterize MRG and MMGs (including sulfur-cycling genes) present in microorganisms inhabiting mine tailings. In doing so, I aim to predict organisms of interest with high metal resistance and/or metal cycling potential, and hypothesize how differences in MRG and MMGs between populations may be influenced by factors such as environmental metal concentrations.

4.2 Methods

4.2.1 Sampling, geochemical assessments, and DNA extraction

Tailings core samples and pore-water measurements were collected as described in sections 3.2.1 and 3.2.2. DNA extractions were performed as described in section 3.2.3. Separate aliquots of each DNA sample were set aside for metagenomic sequencing.

4.2.2 Metagenomic sequencing, assembly, and binning

A total of 43 samples were sent for metagenomic sequencing based on 16S rRNA sequencing results (section 3.3). Selection criteria aimed to maximize the range of geochemistry and microbial community compositions observed across samples, while minimizing redundancy. For example, if a group of adjacent samples in one core had similar concentrations of dissolved metals, pH, and relative taxon abundances, one representative sample from the group was selected for metagenomic sequencing.

Sequencing was performed at TCAG as described in section 2.2.4. Assembly and binning steps were also identical those used in section 2.2.4, with the following addition: after binning, MAGs were dereplicated across all samples with dRep (Olm et al., 2017) to generate a non-redundant set of MAGs for gene annotation.

4.2.3 Protein extraction and sequencing

The full set of samples selected for metagenomics were also subjected to metaproteomic sequencing.

The method of protein extraction was modified from a protocol described in Keiblinger et al. (2012), which compared the effectiveness of multiple protein extraction protocols on soil samples and found that an SDS-phenol extraction method (Wang et al., 2006) resulted in the highest numbers of mapped proteins from LC–MS/MS (Keiblinger et al., 2012). 10 g of tailings were measured and pre-washed as described in section 3.2.3. The following was then added to sample tubes: 5 mL each of stainless steel and garnet beads, 15 mL of 1% SDS in 50 mM Tris-HCl pH 8.0, and 15 mL of Tris-HCl equilibrated liquid phenol. Samples were vortexed for 10 min and sonicated with a probe sonicator for 30 s, with all samples stored on ice between steps. Tubes were then placed onto a horizontal shaker at 160 rpm for 1 hr at room temperature, after which the vortex and sonication steps were repeated. Samples were centrifuged for 20 min at 3500 x g at 4 °C to separate the phenol layer

(containing the solubilized proteins) from the aqueous layer. The phenol phase was transferred into a new 50 mL tube for precipitation of proteins overnight in 5 volumes of 0.1 M ammonium acetate in methanol at -20 °C.

To prepare peptides for LC-MS/MS, proteins were centrifuged for 20 minutes at 10,000 x g at 4 °C, washed with 2 mL of cold acetone, and resolubilized in 300 µL of denaturation buffer (8M urea in 50 mM Tris-HCl). Samples were then incubated with 10 mM dithiothreitol (DTT) at 60 °C for 30 mins to reduce disulfide bonds, then with 20 mM iodoacetamide (IAA) at room temperature for 30 mins to alkylate cysteines. Proteins were precipitated again in acetone, dried, and resuspended in 50 mM ammonium bicarbonate for overnight digestion with 1.5 µg trypsin at 37 °C. The digest was acidified with 1% formic acid, after which peptides were purified using Pierce C18 spin columns (Thermo Fisher #89870), dried in a vacuum centrifuge, and stored at -80 °C prior to LC-MS/MS.

LC-MS/MS was performed in the Biomolecular Core (BioCORE) Facility at Western University Schulich School of Medicine and Dentistry, London, Canada, with the following specifications: peptides were resuspended in 1% acetonitrile (ACN) and 0.1% formic acid (FA), placed in an ultrasonic bath for 20 min, and centrifuged at 21,000 x g for 7 min at room temperature. Peptide samples were injected onto a Waters Acquity Ultra-Performance Liquid Chromatography (UPLC) M-Class system (Waters, Milford, MA) coupled to a Q Exactive Plus hybrid quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific, Waltham, MA). Mobile Phase A consisted of water/0.1% FA and Mobile Phase B consisted of ACN/0.1% FA. Samples were trapped for 5 min at a flow rate of 5 µL/minute using 99% Buffer A, 1% Buffer B on a Symmetry C18 Trap Column, 5 µm, 180 µm × 20 mm (Waters). Peptides were separated using a Peptide BEH C18 Column, 130Å, 1.7 µm, 75 µm × 250 mm operating at a flow rate of 300 nL/minute at 35 °C (Waters). Samples were separated using a nonlinear gradient consisting of 1–7% B over 1 minute, 7–23% B over 135 minutes and 23–35% B over 180 minutes before increasing to 99% B, for a total time of 3 h. The Q Exactive Plus mass spectrometer was controlled by Thermo Xcalibur software (v4.0). Data were acquired in the data-dependent acquisition mode (DDA) using a Fourier Transform/Higher-Energy Collision Dissociation (FT/FT/HCD) Top 12 scheme. MS1 survey scans were performed from 375 to 1,500 m/z at a resolution of 70,000 with Automatic Gain Control (AGC) set to 3e6 and a maximum injection time of 250 ms. Multiply charged peptide ions were isolated using the quadrupole with an isolation window of 1.2 m/z and were fragmented using HCD with Normalized Collision Energy (NCE) set to 25%. MS2 scans were performed at a resolution of 17,500 with AGC set to 2e5 and a maximum

injection time of 64 ms. Dynamic exclusion was set to 40 s, and lock mass ion was enabled at 445.120025 m/z.

4.2.4 Metagenome annotation and data analysis

MAG taxonomy assignment with GTDB-tk (v2.1.0), phylogenetic tree construction with GToTree, and iron/sulfur gene annotation with FeGenie and DRAM were performed as in section 2.2.5. DiSCo (Neukirchen & Sousa, 2021) was also used for sulfur gene annotation, which can distinguish between oxidative and reductive variants of *dsr* genes.

MRG gene annotation was based on the BacMet (v2.0) antibacterial biocide- and metal-resistance genes database (Pal et al., 2014). The BacMet 2.0 database contains 753 curated, experimentally verified bacterial metal and biocide resistance genes, as well as 155,512 predicted genes based on sequence similarity to the experimentally verified database (Pal et al., 2014). Initially, MRGs were annotated with the BacMet-scan software provided with the database, which uses a Basic Local Alignment Search Tool (BLAST) algorithm to identify similar genes. Prodigal (Hyatt et al., 2010) was used to predict open reading frames (ORFs) from MAGs for BacMet-scan, and results were filtered using a $E < 0.05$ threshold.

The BacMet-scan method resulted in limited hits, so I developed an alternative method of MRG identification that would be comparable to the other gene annotation tools used in this study (FeGenie, DRAM, DiSCo) based on creating profile HMMs for MRGs of interest. Multiple sequence alignments (MSAs) were generated with MUSCLE (v3.8.31) (Edgar, 2004) for protein sequences associated with each of the 613 unique genes present in the combined BacMet experimentally verified and predicted gene databases. I verified MSAs by visual inspection in Geneious (v5.6) (Kearse et al., 2012). Profile HMMs were generated from MSAs using HMMER (v3.2.1) (Eddy, 2018), excluding genes with <5 representative sequences, resulting in 529 HMMs. The set of predicted ORFs used in the initial BacMet-scan were screened with the developed BacMet HMMs using HMMER, with $E < 0.05$ indicating a significant hit.

All data from FeGenie, DRAM, DiSCo, BacMet-scan, and BacMet HMM searches were exported to Python for analysis using the pandas (McKinney, 2010) library. Gene frequencies were aggregated into categories (*e.g.*, iron oxidation, sulfur reduction, copper resistance) and normalized to MAG relative abundance as a proxy for relative gene abundance, which was calculated from the

average scaffold coverage of each MAG divided by the total coverage of all binned scaffolds within a sample. Figures were generated using matplotlib (Hunter, 2007).

4.2.5 Metaproteome annotation and data analysis

Initial data processing was conducted by personnel at the BioCORE facility, using the PEAKS 12 software (Bioinformatics Solutions Inc., Waterloo, Canada). To map predicted proteins, a custom ORF database was generated from the metagenomic sequencing data from this study by using Prodigal to predict ORFs in assembled scaffolds above 1 kbp in length. Predicted proteins from all metagenomes were combined into a single database to search the MS data against, with the following specifications: a precursor ion mass tolerance was set to 10 ppm, and a fragment ion mass tolerance was set to 0.02 Da. Specific cleavage with trypsin was selected with a maximum of 2 missed cleavages. A fixed modification of carbamidomethylation and variable modifications of deamidation, oxidation and acetylation were added, and a maximum of two variable modifications were allowed per peptide. The peptide false discovery rate (FDR) was set to 1% for the database search. One unique peptide and an FDR of less than 1% were applied for confident protein identification.

Comparison of protein abundances across samples was not possible as all extracted peptides from each sample were injected to maximize the signal detection potential for low biomass samples, and therefore the amount of peptide injected varied between samples. Relative abundances within a sample were also not compared as the method of data collection did not account for differences in peptide detection ability by mass spectrometry. As a result, analysis of proteomic data was limited to inferring microbial activity based on the positive identification of associated proteins.

4.3 Results and discussion

4.3.1 Sequencing statistics

A total of 757 high-quality MAGs (454 non-redundant, after dereplication) were identified across 43 samples (ranging from 1–54 per sample, average of 18) after assembly and binning of metagenomic sequencing data (**Table 4.1**). The number of high-quality bins per sample was much lower than the number of ASVs identified through 16S rRNA amplicon sequencing (section 3.3.2, minimum=141). One sample (ML25_013) did not return any high-quality bins and was excluded from subsequent analyses involving MAG gene annotation. Low bin counts were often associated with a low fraction of assembled reads (**Table 4.1**), likely resulting from insufficient sequencing coverage of low biomass samples, despite PCR amplification during the metagenome library preparation step.

Positive identification of proteins from LC-MS/MS was only possible for 27 out of 43 submitted protein samples. A total of 29,069 proteins were identified (ranging from 1–8,192 per sample, average of 1,077) (**Table 4.2**), the majority of which were unique (24,033). Only 10,666 proteins (37%) mapped to binned scaffolds. A major issue impacting data collection was the presence of polyethylene glycol (PEG), a common polymer contaminant in mass spectrometry-based analyses (Keller et al., 2008). Avoiding PEG contamination is challenging, as it is ubiquitous in many commercial and laboratory products, such as cosmetics, detergents and plastics (Ahmadi & Winter, 2018). Attempts to limit contamination during protein extraction by using HPLC-grade solvents, rinsing glassware with isopropanol instead of soap, and avoiding the use of PEG-containing plastics may have led to marginal improvements in some samples, but significant PEG contamination was still detected in others. It is possible that PEG was introduced to samples from handling prior to protein extraction, such as during tailings core processing. Additional processing with detergent removal and desalting columns were performed at the BioCORE facility, but these steps did not result in an improvement on the observed relative peptide signal.

The low number of MAGs and identified proteins in this study reflect the ongoing challenges in obtaining high-quality DNA and protein samples from mine tailings. Low biomass and contaminants within the sample matrix (*e.g.*, heavy metals) limit the effectiveness of current DNA/protein extraction methods, which are optimized for soil communities. Protein samples will also be sensitive to contaminants introduced during sample processing (*e.g.*, PEG), resulting in low signal-to-noise ratios in mass spectrometry.

Table 4.1: Metagenomic sequencing statistics by sample. Scaffolds were binned into metagenome-assembled genomes (MAGs) using MetaBAT2, CONCOCT, and MaxBin2, and DAS Tool was used to identify the highest quality set of non-redundant bins among the three binning algorithms. CheckM was used to filter for bins with >70% completeness and <10% contamination as the final set of high-quality (HQ) MAGs.

Sample	Depth (m)	Reads	% Assembled	Scaffolds	Scaffolds binned	HQ bins
ML25_003	0.28	16,647,582	76.03	15,966	8,387	33
ML25_006	0.62	21,542,193	79.92	18,432	7,081	32
ML25_007	0.73	13,168,935	84.90	8,715	4,309	22
ML25_008	0.84	16,652,969	89.41	8,187	3,727	19
ML25_009	0.96	36,061,742	95.62	6,644	3,579	24
ML25_010	1.07	37,476,942	89.22	4,672	2,323	13
ML25_012	1.29	3,838,337	30.03	1,985	679	2
ML25_013	1.42	4,011,559	0.59	7	0	0
ML25_014	1.59	37,774,736	75.19	28,541	12,878	48
ML25_015	1.77	13,085,404	38.51	3,274	1,536	7
ML25_016	1.94	40,345,890	77.70	15,366	6,124	28
ML25_022	3.03	33,927,710	73.12	13,490	3,550	16
ML25_026	3.87	4,246,725	62.65	958	585	4
ML34_003	0.38	18,535,314	15.74	11,019	1,458	5
ML34_006	0.84	19,429,820	73.10	16,192	5,748	26
ML34_007	0.99	20,155,676	80.16	13,528	5,123	25
ML34_010	1.44	20,207,295	3.85	3,718	1,187	3
ML34_012	1.77	28,980,246	94.50	3,862	2,362	15
ML34_014	2.10	19,268,023	79.96	13,333	3,868	22
ML34_015	2.27	26,572,506	80.72	17,751	7,788	45
ML34_016	2.43	44,069,643	75.03	31,683	12,236	54
ML34_017	2.59	39,831,849	83.75	13,007	5,360	22
ML34_020	3.13	21,090,603	62.35	26,331	9,575	36
ML34_022	3.76	16,805,500	70.14	15,098	4,554	21
NR18_003	0.25	17,734,958	10.25	12,762	872	3
NR18_005	0.45	23,358,319	73.17	18,185	6,182	38
NR18_007	0.65	18,924,563	72.48	16,652	6,220	29
NR18_010	0.95	18,483,671	70.87	15,895	7,177	33
NR18_011	1.05	16,063,433	73.97	14,261	4,992	28
NR18_012	1.15	18,525,675	84.53	12,613	5,359	28
NR18_015	1.51	18,780,223	4.32	5,533	214	1
NR18_017	1.86	21,590,172	84.44	2,485	1,306	7
NR18_023	2.92	35,560,955	14.00	15,853	2,880	12
NR18_025	3.27	45,504,942	85.73	2,969	1,443	6
NR18_028	3.76	12,877,162	16.17	1,692	410	2
NR3_003	0.34	5,781,590	51.47	740	497	4
NR3_006	0.73	40,278,890	95.24	2,983	1,606	7
NR3_009	1.11	7,729,451	67.07	582	361	3
NR3_012	1.57	8,307,886	33.98	458	458	2
NR3_017	2.72	2,971,431	10.36	794	16	1
NR3_023	3.11	20,759,186	33.60	12,394	4,172	21
NR3_025	3.37	45,504,942	11.75	1,388	751	2
NR3_028	3.83	12,877,162	17.65	11,726	1,735	8
Total	N/A	927,847,495	N/A	441,724	160,695	757

Table 4.2: Metaproteomic sequencing statistics by sample. Peptides were identified using LC-MS/MS and mapped to a non-redundant database of predicted proteins from metagenomic sequencing data. The total number of potentially expressed proteins within a sample is reported as the Protein ID along with the total number of Peptides supporting the identified proteins (Peptide ID). A minimum of one unique peptide within a protein group (accounting for homologs and variable isoforms) and an FDR of 1% was used as the threshold for positive protein identification.

Sample	Depth (m)	Protein ID	Peptide ID	Unique Peptide ID
ML25_003	0.28	8	8	4
ML25_006	0.62	8,192	20,044	6,796
ML25_007	0.73	2,065	2,866	801
ML25_009	0.96	737	759	660
ML25_010	1.07	532	537	445
ML25_013	1.42	6	6	4
ML34_003	0.38	179	193	66
ML34_006	0.84	291	403	84
ML34_007	0.99	2,036	3,833	1,888
ML34_010	1.44	2,770	3,964	1,024
ML34_012	1.77	5,470	11,675	3,863
ML34_014	2.1	9	9	7
ML34_015	2.27	61	68	17
ML34_016	2.43	20	22	7
ML34_017	2.59	1	2	2
ML34_020	3.13	365	550	252
ML34_022	3.76	47	52	12
NR18_003	0.25	58	62	32
NR18_010	0.95	204	286	69
NR18_011	1.05	734	979	250
NR18_012	1.15	65	70	7
NR18_015	1.51	206	208	46
NR18_023	2.92	585	591	464
NR18_025	3.27	2	2	2
NR3_009	1.11	489	494	418
NR3_023	3.11	548	558	491
NR3_028	3.83	3,389	5,069	2,342
Total	N/A	29,069	53,309	20,053

4.3.2 Microbial community overview

Community profiling based on metagenomic sequencing data was low resolution due to the limited number of high-quality MAGs. In some cases, a sample appeared to be comprised of only 1–2 populations (**Figure 4.1**), but it was clear from 16S rRNA amplicon sequencing (section 3.3.2) that not all of the community diversity was captured in the assembled and binned scaffolds, especially for lower-abundance organisms. Consequently, quantitative comparisons of relative MAG abundances in this study (**Figure 4.1**) should be taken lightly, especially for samples with low bin counts (<10). However, the presence and localization of abundant taxa (>15%) generally agreed between the two datasets (**Figures 4.1, 3.1, 3.2**). The AD3, WPS-2, and Sva0485 clades under the SILVA classification system (**Figure 3.1, 3.2**) correspond to the GTDB Dormibacterota, Eremiobacteria, and SZUA-79 phyla (**Figure 4.1**) respectively. In some cases, GTDB-tk allowed for more precise taxonomic identification: SZUA-79 MAGs were classified at the genus and/or species level (*e.g.*, *Ca. Acididesulfobacter diazotrophicus*) whereas corresponding Sva0485 ASVs were not.

In terms of phylogenetic diversity, the Proteobacteria (mainly Gammaproteobacteria) and Actinobacteriota were both the most overall abundant phyla across samples (**Figure 4.1**) and had the highest number of non-redundant bins (**Figure 4.2**). Most proteobacterial MAGs belong to clades associated with iron and/or sulfur oxidation in mining waste, but there were differences in the localization of individual taxa. Within the Acidiferrobacterales, *Acidiferrobacter* spp. were unique to ML25 whereas *Sulfuricaulis* spp. were almost exclusively found in NR18. *Sulfuricella*, *Sulfuriferula*, and *Thiobacillus* spp. (o. Burkholderiales) were mainly found in ML25 and ML34, with *Thiobacillus* much more abundant in ML34 samples. *Acidithiobacillus* spp. were found in all four locations, but *A. ferrooxidans* were highly abundant in ML25, whereas *A. ferrivorans* was more common in other samples.

Unlike the Proteobacteria, Actinobacteriota MAGs were relatively novel, with most unclassified at the family or genus level, and with only one mining waste-associated Actinobacteriota (*Ferrimicrobium* spp.) present at <4% abundance. The majority of Actinobacteriota in shallow samples (<2 m) belong to the Acidimicrobiales order, and appear to be mostly organoheterotrophs. Deeper samples (>2 m) included members of the understudied anaerobic Thermoleophilia and Humimicrobiia clades, some of which may be sulfur oxidizers and/or sulfate reducers (**Figure 4.2**). Other known or potential iron/sulfur cycling organisms are discussed in 4.3.3.

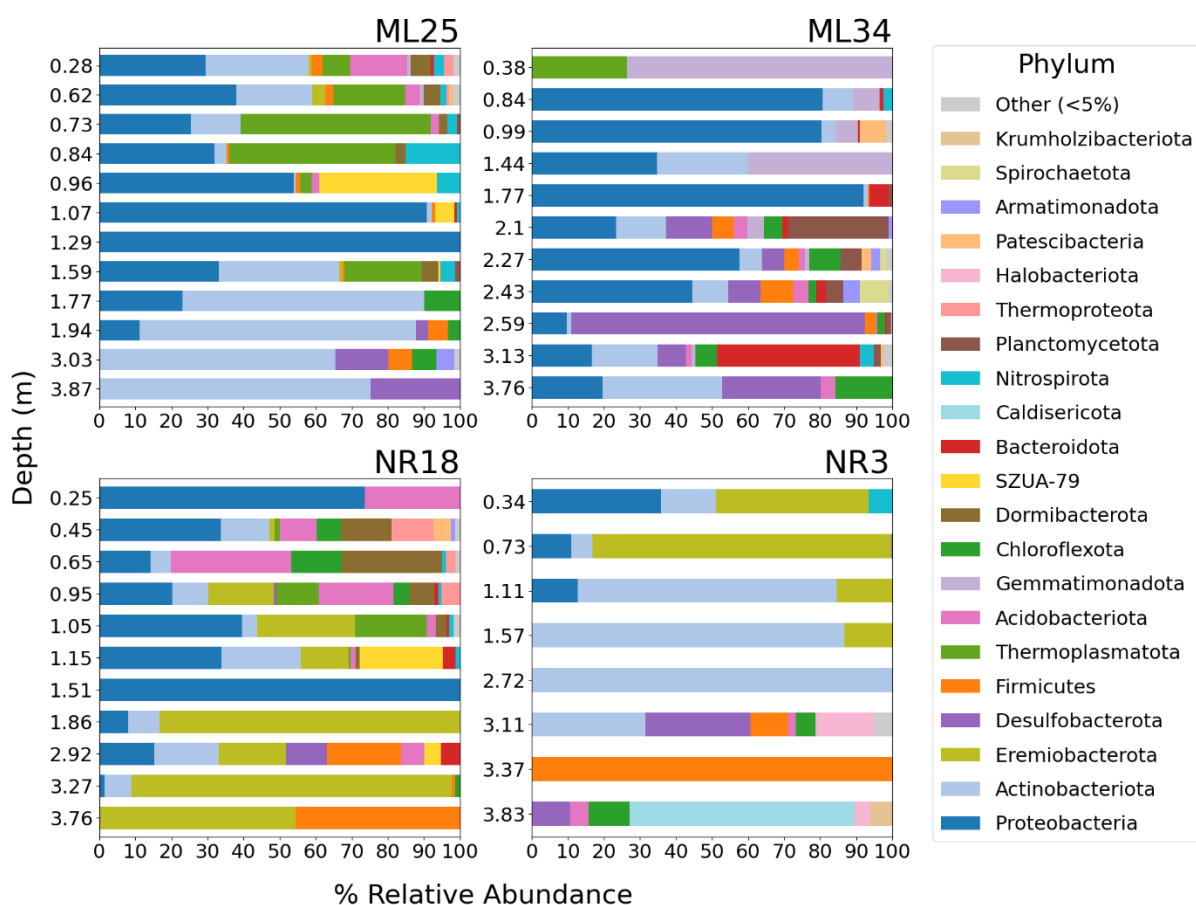


Figure 4.1: Microbial community composition across tailings cores from metagenomic sequencing data. Bars indicate phylum-level relative abundances of MAGs within each sample, based on the average depth of coverage of scaffolds within individual MAGs. Only phyla present at a relative abundance >5% in at least one sample in any dataset are shown. Phyla are ordered from most overall abundant (left) to least abundant (right). MAGs were classified with GTDB-tk (v2.1.0). Note that nomenclature for the Proteobacteria (Pseudomonadota) and Firmicutes (Bacillota) have been updated in more recent versions of GTDB-tk. The y axes indicate the sample depth below ground level and are not scaled proportionally.

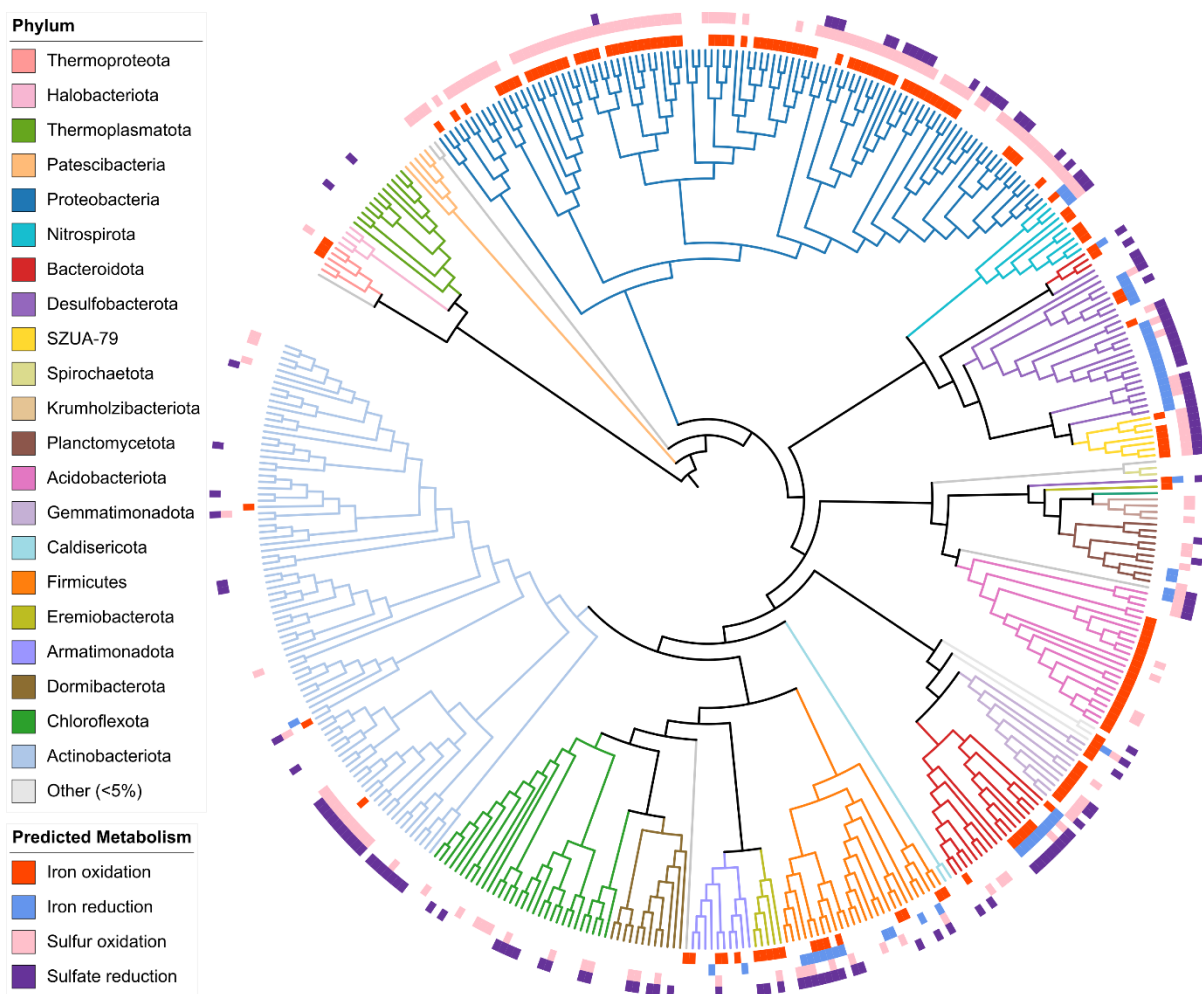


Figure 4.2: Phylogenetic distribution of sulfur and iron cycling genes across all MAGs. Tree branches are unscaled (cladogram) and colored by GTDB-tk assigned phylum in clockwise order following the legend, starting from the archaea (top left). Only phyla present at a relative abundance >5% in at least one sample in any dataset are shown. Colored rings represent presence/absence of iron and sulfur cycling genes, where at least one hit for any gene within each category was used to determine presence. Iron and sulfur metabolism genes were compiled from FeGenie, DRAM, and DiSCo annotations. Iron oxidation genes include *cyc1/cyc2*, *fox* genes, and sulfocyanin; reduction genes include *omc* and *dfe* genes. Sulfur oxidation is represented by genes in the *sox* or oxidative type *dsr* pathways; reduction genes include thiosulfate reductase, tetrathionate reductase, and reductive type *dsr* genes.

4.3.3 Distribution and expression of MRGs

BacMet-scan returned 329,520 non-redundant hits for genes within their database. Annotation using the custom BacMet HMMs returned 1,660,455 hits at the same level of statistical significance ($E < 0.05$) (**Table C1**). The BLAST-based approach used by BacMet-scan is generally considered less sensitive than Hidden Markov Models (HMMs), as it relies on pairwise sequence alignments rather than comparing queries to probabilistic models built from multiple sequence alignments (Krogh et al., 1994; Park et al., 1998). As a result, BacMet-scan may be biased toward detecting genes from closely related taxa or those associated with specific metals where sequence conservation is higher. For example, BacMet-scan identified a disproportionally higher fraction of arsenic- and mercury-associated MRGs compared to the HMM annotation method (**Table C1**). Most of these genes fall within operons (*e.g.*, *ars*, *mer*) that evolved specifically for detoxification of their respective metals (Osborn et al., 1997; Zhu et al., 2017). In contrast, HMMs can identify more evolutionarily distant homologs across gene families, enabling the detection of a broader range of genes (Park et al., 1998), including for MRGs involved in multi-metal resistance. Given these advantages, I proceeded with data analysis using results from the BacMet HMM-based gene annotations. It should be noted that this HMM set excludes genes with < 5 representative sequences in the BacMet database (84 out of 613 unique genes), leading to underrepresentation of rare genes. To partially account for this, the BacMet-scan BLAST hits for those rare genes were appended to the HMM annotations, which added 10,462 additional hits, or a 0.6% increase. Both methods also lack the ability to annotate archaeal genomes, as the database only includes bacterial sequences.

The overall abundance of MRGs was generally consistent across samples within each tailings core, with two exceptions in NR18 (1.51 m) and NR3 (3.37 m), both showing elevated proportions across MRGs (**Figure 4.3**). These samples had poor assembly and binning statistics, with only 3 total high-quality MAGs, (**Table 4.1**) and thus MRGs encoded on these MAGs are likely over-represented compared to the complete microbial communities. When comparing gene abundances by metal, zinc MRGs were the most common, followed by copper, nickel, and iron (**Figure 4.3**). Most MRGs associated with zinc exhibit broad specificity and also confer resistance to other metals (most commonly cadmium and copper) (Pal et al., 2014), which could explain the overrepresentation of zinc MRGs despite zinc not being a major contaminant in these tailings (< 9.0 mg/L) (**Data File B1**). Other multi-metal resistance genes in the BacMet database include membrane transporters for metals similar in mass and/or valence state: cadmium/zinc, tungsten/molybdenum, silver/copper, and

cobalt/nickel (Pal et al., 2014). Differences in metal toxicity may also affect the relative abundance of their associated MRGs. Resistance mechanisms for highly toxic metals such as arsenic and mercury are likely to be encoded in microbial genomes regardless of whether environmental concentrations are high, as these pathways evolved early in Earth's history and are widely distributed among bacteria and archaea (Osborn et al., 1997; Zhu et al., 2017). Likewise, copper resistance genes were more abundant than nickel and iron resistance genes (**Figure 4.3**), even though copper concentrations were lower in most samples, likely reflecting copper's greater cytotoxicity relative to iron and nickel (Fait et al., 2006; Gilani & Alibhai, 1990).

There was no significant correlation between individual concentrations of major heavy metals (Cu, Ni, Fe) and their associated MRG abundance (Pearson correlation, $p_{Cu}=0.18$, $p_{Fe}=0.79$, $p_{Ni}=0.28$). More heavily contaminated tailings (*e.g.*, ML25) had similar or lower Cu, Ni, and Fe resistance gene abundances to other locations (**Figure 4.4**, **Figure C1-C3**). There was also no correlation between metal concentrations and MRG abundances when comparing samples within a core ($0.05 \leq p \leq 0.95$). Although higher environmental metal concentrations could select for organisms with more resistance genes targeting those metals, the results from this study suggest that a broad range of MRGs are present in mine tailings communities regardless of the level of heavy metal stress they are currently experiencing.

Metal resistance in mine tailings communities could instead be regulated at the gene expression level (Chandrangsu et al., 2017; Nies & Brown, 1998), which cannot be directly inferred from metagenomic sequencing data. In samples with high protein counts, resistance genes for most metals were expressed (**Figure 4.3**), confirming that many MRGs are not only encoded in genomes, but also actively expressed by microbes inhabiting tailings environments. The presence of metal resistance proteins was detected in several samples (**Figure 4.3**), but absence in other samples, especially those with few identified proteins, cannot be confidently interpreted as a lack of MRG expression. Correlations between MRG expression levels and extracellular metal concentrations could not be determined due to the lack of quantitative mass spectrometry data. However, I hypothesize that the same factors influencing MRG abundance in metagenomes, such as the relative toxicity of individual metals and the ability of MRGs to confer multi-metal resistance, are important determinants of metal resistance protein abundance, with the added factors of metal presence and concentrations within the environment.

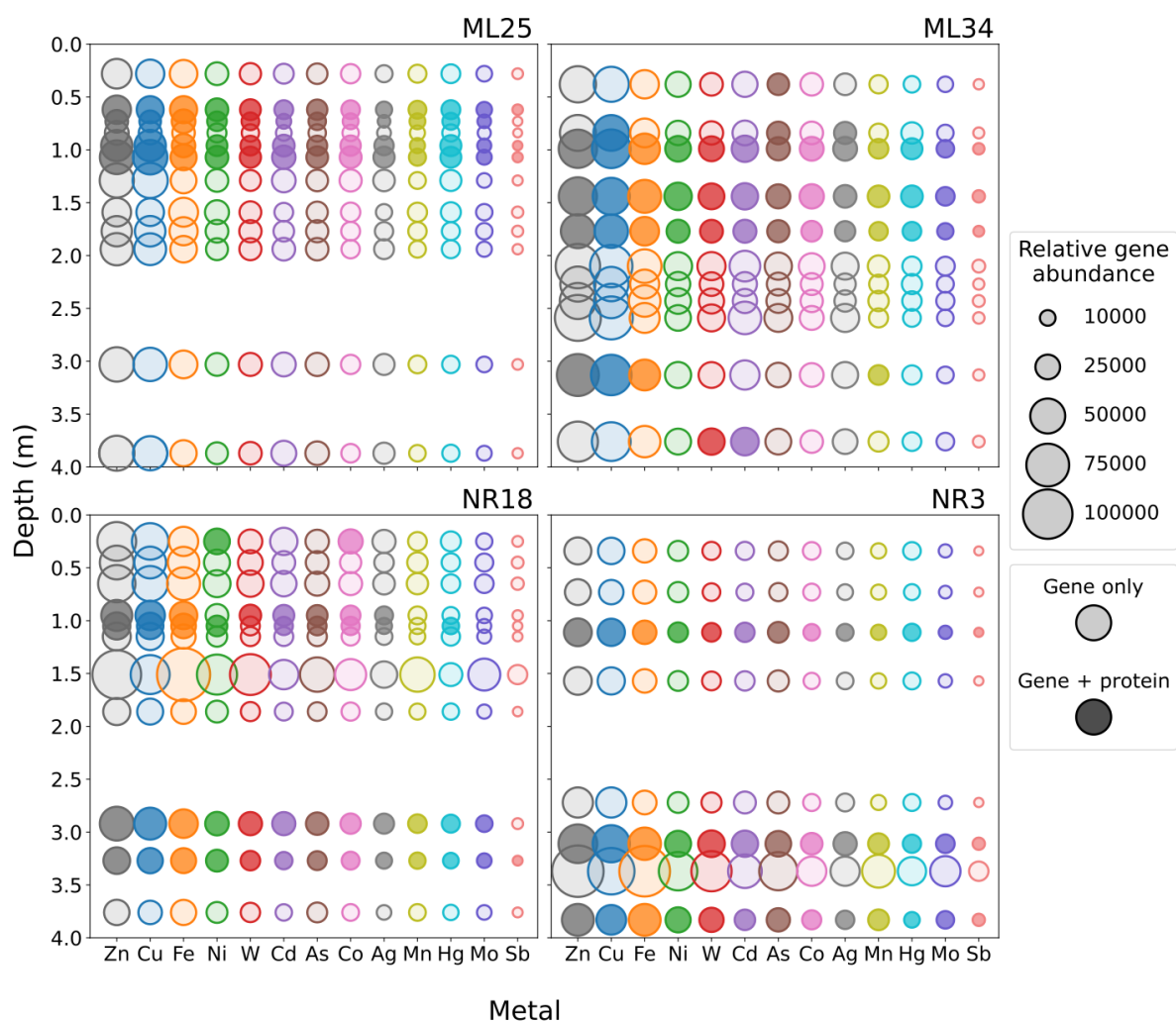


Figure 4.3: Distribution of MRGs across vertical mine tailings transects. MAGs were annotated using profile HMMs of genes in the BacMet database, and filtered for MRGs. Rare genes (<5 representatives in the BacMet database) were annotated with BacMet-scan. Circle diameters representing relative gene abundances were calculated as the number of hits multiplied by the percent relative abundance of MAGs associated with each hit. In samples where protein expression was detected, the corresponding gene(s) are indicated by circles with darker shading. MRGs are colored based on metal, ordered from highest overall abundance (Zn) on the left to lowest (Sb) on the right. Multi-metal resistance genes were counted towards multiple categories. Only categories with abundances >2% of the total are shown.

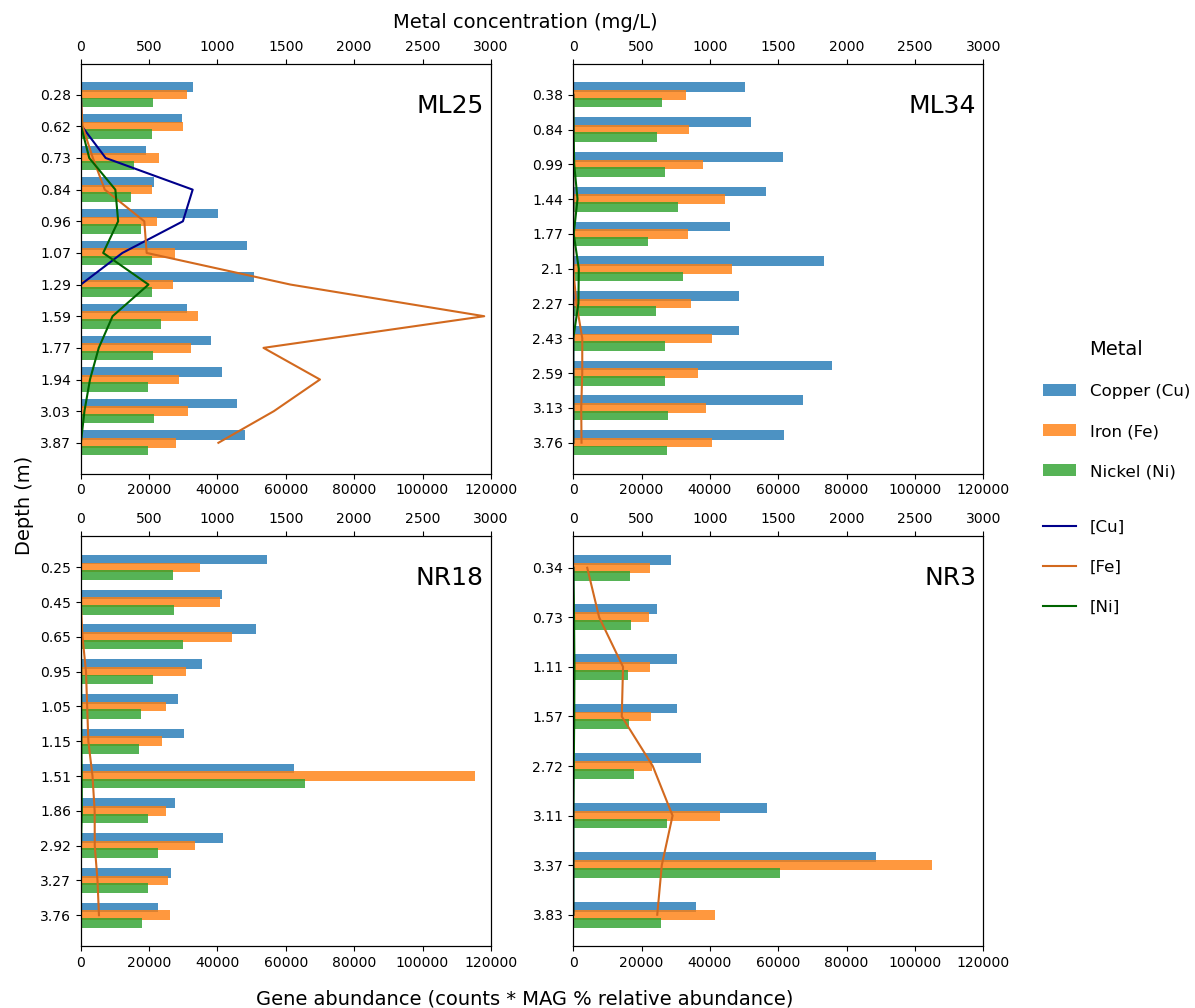


Figure 4.4: Abundance of metal resistance genes and metal concentrations along vertical mine tailings transects. Bar plots depict relative gene abundances for Cu, Fe, and Ni-associated MRGs. Line plots represent Cu, Fe, and Ni concentrations in tailings pore-water. There was no significant correlation between metal concentrations and associated MRG abundances ($p_{\text{Cu}}=0.18$, $p_{\text{Fe}}=0.79$, $p_{\text{Ni}}=0.26$). Note that the y axes are not scaled proportionally to depth.

To investigate the metal resistance mechanisms of individual bins, MAGs were ranked by the total number of predicted Cu, Ni, and Fe resistance genes they encoded (**Figure C4**), as high MRG counts may be indicative of high metal tolerance in an organism. MAGs encoding a high number of copper-resistance genes (top 200 bins) were most commonly members of the Proteobacteria (30.5%), Desulfobacterota (12.5%), Chloroflexota (10.0%), and Acidobacteria (7.5%) (**Figure C4**). The MAGs with the top 5 largest predicted copper-resistance gene complements were taxonomically novel (**Table 4.3**), unclassified at the genus level or above, and may represent interesting targets for future isolation and characterization of copper resistance mechanisms.

There was more taxonomic overlap between MAGs with high nickel- and high iron-resistance gene counts (**Figure C4**), as many genes are mapped to both functions in the BacMet database (Pal et al., 2014). Most of the top 200 highest nickel- and iron- resistance gene encoding bins were members of the Actinobacteriota (28.3%), Proteobacteria (14.75%), Chloroflexota (13.75%), and Firmicutes (9.23%) (**Figure C4**). MAGs with the top 5 highest counts of nickel and iron-associated MRGs were identical, and included *Allorhizobium* and *Bradyrhizobium* spp. (c. Alphaproteobacteria, o. Rhizobiales) (**Table 4.3**). *Bradyrhizobium* and other Rhizobiales spp. are known to be nickel tolerant and promote plant growth in Ni-rich soils, as they are nitrogen-fixing symbionts of legumes (Chaintreuil et al., 2007; Liu et al., 2022). These lineages potentially enhance tailings remediation through Ni uptake and bioaccumulation, thereby decreasing its mobility (Maier et al., 1990; Stults et al., 1987).

MAGs high in Cu, Ni, and Fe resistance genes are predicted to represent heterotrophic organisms (**Table 4.3**). However, the majority of detected metal resistance proteins mapped to *Acidithiobacillus*, *Thiobacillus*, and other autotrophic MAGs, with the exception of *Bradyrhizobium*, *Metallibacterium*, *Methylovirgula*, and *Devosia* spp. Of these bins, *Thiobacillus* spp. appeared to express the largest variety of unique protein groups related to copper, nickel, and iron resistance (**Figure 4.5**). Fewer types of metal resistance proteins were detected from taxa predominantly inhabiting highly metal-contaminated tailings such as *Acidithiobacillus*, *Metallibacterium*, and Acidiferrobacteraceae, although specific proteins/complexes such as Cme, Cus, Czn, Nik, and Nia appeared to be commonly expressed by these clades. From this data I hypothesize that microbial adaptations to heavy metal stress may involve the upregulation of a few specific pathways for metal detoxification as opposed to acquiring and utilizing many different pathways. It is also possible that the *Thiobacillus* spp. identified in ML34 could theoretically tolerate the metal concentrations in

ML25 tailings, but not other environmental stressors such as low pH. Further study is required to elucidate the differences in MRG regulation and metal tolerances between microbial populations in these environments, ideally involving comparative protein expression analyses of communities and/or isolated representatives.

Table 4.3: Metagenome-assembled genomes from mine tailings with the top five highest (99th percentile) of Cu, Ni, and Fe resistance gene counts. MAGs (Bin ID) are ranked in decreasing order of MRG counts (number of genes). The lowest assigned level of GTDB taxonomic classification is reported (c=class, o=order, f=family). MAG relative abundances within their sample (Rel. Abundance) were calculated based on average scaffold depth of coverage.

Metal	Bin ID	Taxonomic classification	# of genes	Rel. Abundance (%)
Copper (Cu)	ML34_020_30	f. Myxococcaceae	1,590	0.96
	ML34_015_36	c. Anaerolineae	1,363	0.68
	NR18_007_01	o. Aggregatilineales	1,250	1.80
	ML34_022_13	o. Deferrisomatales	1,222	1.02
	NR3_023_07	f. Pseudopelobacteraceae	1,123	7.38
Nickel (Ni)	ML34_015_29	<i>Allorhizobium</i> sp.	701	0.94
	NR18_015_01	<i>Bradyrhizobium</i> sp.	646	100
	NR3_025_02	<i>Ruminiclostridium</i> sp.	622	28.87
	NR3_023_12	<i>Mobilitalea</i> sp.	608	1.85
	NR18_005_37	o. Acidimicrobiales	594	1.96
Iron (Fe)	NR3_025_02	<i>Ruminiclostridium</i> sp.	1,197	28.88
	ML34_015_29	<i>Allorhizobium</i> sp.	1,164	0.94
	NR18_015_01	<i>Bradyrhizobium</i> sp.	1,153	100
	NR3_023_12	<i>Mobilitalea</i> sp.	1,117	1.85
	NR18_005_37	o. Acidimicrobiales	1,103	1.96

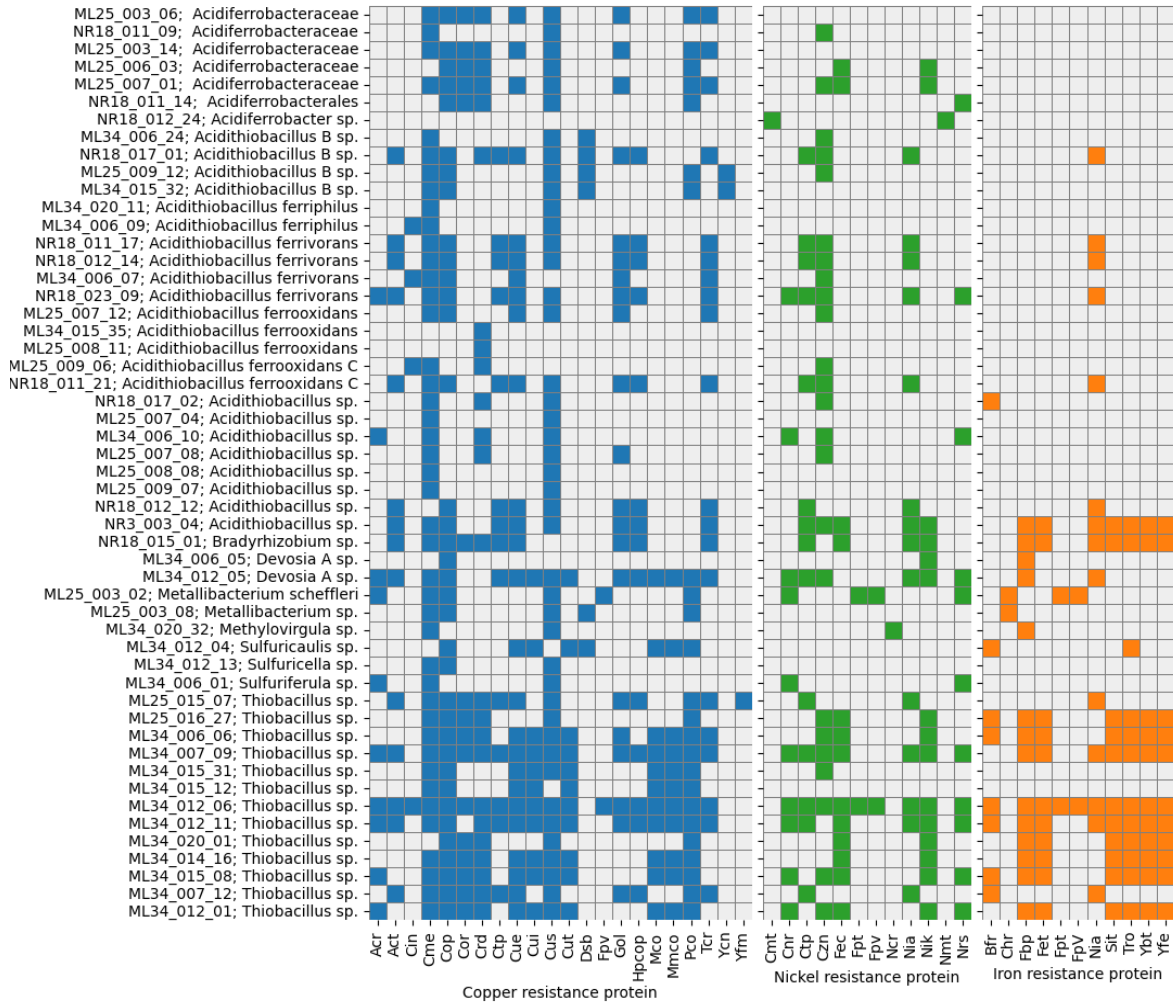


Figure 4.5: Taxonomic distribution of copper, nickel, and iron resistance proteins in tailings communities. Shaded cells represent positive detection of specific proteins associated with copper, nickel, and iron resistance. Proteins within common pathways (e.g., Cop, Cus) were aggregated. Proteins were identified by mass spectrometry and mapped to predicted proteins from metagenomic sequencing, and aggregated by the MAG from which they originated (if any). Bins are labelled by their ID, followed by their lowest level of GTDB-assigned taxonomy. Only bins with >50 unique proteins detected across all samples were included in this analysis.

4.3.4 Distribution and expression of iron- and sulfur-related genes

In general, iron- and sulfur- oxidation genes were more abundant in shallow samples whereas reductive metabolisms were more abundant in deeper samples (**Figure 4.6, 4.7**), which was expected based on decreasing oxygen availability and redox potentials along vertical gradients. Most predicted iron-oxidizers were bacteria using the *cyc2* pathway (Castelle et al., 2008; McAllister et al., 2020). Iron reduction genes were more diverse, being evenly distributed among hits for *dfe_0461-0465*, *omcF/S/Z*, and other pathways (Deng et al., 2018; Santos et al., 2015). There was considerable overlap between iron oxidizing and sulfur oxidizing Gammaproteobacteria, as well as between iron reducing and sulfate reducing Desulfobacterota (**Figure 4.2**). As seen for the MRGs, corresponding proteins were only detected in a few samples (**Figure 4.6, 4.7**). In many cases some genes for a pathway were detected as proteins, but not others, and this was inconsistent between samples from similar depths in the same tailings core. Due to this low coverage, protein data should not be taken as a comprehensive profile of microbial gene expression in these tailings.

Differences in potential iron oxidation and *sox*-based sulfur oxidation were most pronounced in ML25, with gene abundances appearing to gradually increase until 1.0–1.5 m, where the microaerophilic environment favored biotic pyrite oxidation, then gradually decreasing as the environment shifted anoxic (**Figure 4.6, 4.7**). This trend was mostly attributed to *Acidithiobacillus ferrooxidans*, *Acidiferrobacter*, *Sulfuriferula*, and *Sulfuricella* spp, although many other predicted Fe and/or S oxidizers were also present, including *Leptospirillum*, *Metallibacterium*, *Thiomonas*, *Ca. Acididesulfobacter* (SZUA-79), and *Ca. Acidulodesulfobacterium* (SZUA-79) spp. The SZUA-79 (*Ca. Acidulodesulfobacteriales*) MAGs were especially abundant around 1.0 m in depth in ML25 and NR18 (up to 32.6% and 23.0%, respectively). Members of this lineage have been characterized from metagenomic sequencing data from one other AMD environment, with similar hypothesized roles in Fe/S cycling (Tan et al., 2019), but there is currently no isolated representative for this order. Cyc2 (iron oxidation) protein expression from several SZUA-79 bins was detected (**Figure 4.8**), where the SZUA-79 Cyc2 proteins were most similar to the Leptospirillaceae Cyc2 variant. Dissimilatory sulfate reduction activity was also detected from several SZUA-79 bins (**Figure 4.9**), some of which overlap with Cyc2-expressing bins (**Figure 4.8**). Iron oxidation and sulfate reduction are not compatible in the same redox environment; proteins from both pathways were detected in ML25_007 (0.73 m), but it is unlikely that sulfate reduction is supported at this depth. However, these results

may constitute evidence of *Ca. Acididesulfobacter* and *Ca. Acidulodesulfobacterium* being capable of both metabolisms depending on the availability of different electron acceptors.

Siderophore-related gene abundance followed similar depth patterns as iron oxidation gene abundance in ML25 (**Figure 4.6**), as 95% of predicted iron oxidizers co-encoded siderophore genes. Some siderophore transport proteins were detected, but not siderophore synthesis, in keeping with geochemical conditions: soluble iron is unlikely to be limiting in tailings environments.

Lower acidity in ML34 tailings supported populations of neutrophilic Fe and/or S oxidizers such as *Thiobacillus*, *Halothiobacillus*, and *Gallionella* spp.. Proteomic data indicate that *Thiobacillus* were using both Dsr and Sox pathways for sulfur oxidation (**Figure 4.9**), and also accounted for the majority of iron storage proteins (ferritin) detected (**Figure 4.8**). Given that *Thiobacillus* cannot oxidize iron for energy, they most likely use ferritin as an iron resistance mechanism (Smith, 2004) by storing excess iron released from oxidizing pyrite and other minerals containing both iron and reduced inorganic sulfur compounds (RISCs).

The expression of FecR, a protein regulating the ferric citrate uptake system, was associated with *Ca. Devosia* MAGs in ML34 (**Figure 4.8**), which also expressed a variety of Cu, Ni, and Fe-resistance proteins (**Figure 4.5**). Members of this genus are often found in soil environments contaminated with various organic toxins (Talwar et al., 2020); this study demonstrates their tolerance towards moderate levels of metal contamination as well.

The majority (68%) of predicted iron-reducing organisms (mostly from the Desulfobacterota) were identified from ML34 samples below 1.5 m in depth (**Figure 4.6**). None of the predicted iron reducers were classified below the family level. No iron reducing gene expression was detected in the proteomics, although this may be due to the fact very few proteins were identified in deeper samples, especially from the ML34 core (**Table 4.2**). An abundant (37%) Ignavibacteriaceae (Bacteroidota) MAG in the ML34_020 (3.13 m) sample encoded both iron oxidation and reduction genes, metabolisms which have not been ascribed to the only isolated representative in this genus (Iino, 2014). Cyc2 expression was detected in this sample (**Figure 4.6**), but the proteins mapped to *Gallionella*, *Ca. Acidulodesulfobacterium*, and unbinned scaffolds rather than to this Ignavibacteriaceae MAG. The geochemical conditions at 3.13 m depth are unlikely to be supporting significant rates of iron oxidation.

The *Ca. Eremiobacterota* are predicted iron oxidizers unique to NR18 and NR3 samples, and were the most abundant organisms (up to 89%) in several samples. All *Ca. Eremiobacterota* MAGs were classified under *Ca. Baltobacteraceae*, a family whose members are predicted to be metabolically versatile based on previous environmental sequencing data (Ji et al., 2021). Members of the *Baltobacteraceae* have not previously been identified in mining-associated environments, nor have they previously been predicted to capable of autotrophic iron oxidation (Ji et al., 2021; Pessi et al., 2024; Ward et al., 2019; Yabe et al., 2022).

A wide variety of taxa appear to be capable of Fe/S cycling in tailings. Vertical heterogeneity in tailings support niches for Fe/S oxidation and reduction, but the exact populations occupying these niches can be highly variable between different cores, and taxonomic composition of these guilds is likely based on selection from environmental factors such as pH and contaminant profiles (discussed in sections 3.3.3–4). Despite only assembling high-quality bins for a small fraction (4.0%) of organisms present in the 16S rRNA amplicon sequencing data, metagenomics enabled the prediction of 301 potential Fe/S cycling MAGs (**Figure 4.2**), of which 139 (46.2%) corresponded to ASVs that could not be confidently linked to these metabolisms based on taxonomic classifications (**Figure 3.1**). Although most iron- and sulfur-oxidizers identified in this study belong to well-studied genera with cultured representatives (**Figure 4.8, 4.9**), there was considerable species and strain level functional diversity within these groups that potentially influence their roles in heavy metal cycling and/or applicability towards bioleaching (Issotta et al., 2018; Kumar et al., 2019; Orell et al., 2010; Zhang et al., 2016). Furthermore, organisms encoding and/or expressing proteins for dissimilatory sulfate reduction were largely unclassified at the genus level or above (**Figure 4.9**), and would have been missed in functional predictions from 16S rRNA data. Sulfate-reducing organisms are highly relevant to bioremediation and biomining applications (discussed in Chapter 5). It is clear from this study that a considerable amount of yet uncharacterized diversity remains within Fe/S cycling consortia found in mine tailings, highlighting the benefit of deeper genomic and functional analyses in order to understand their ecological roles and evaluate their potential for heavy metal remediation.

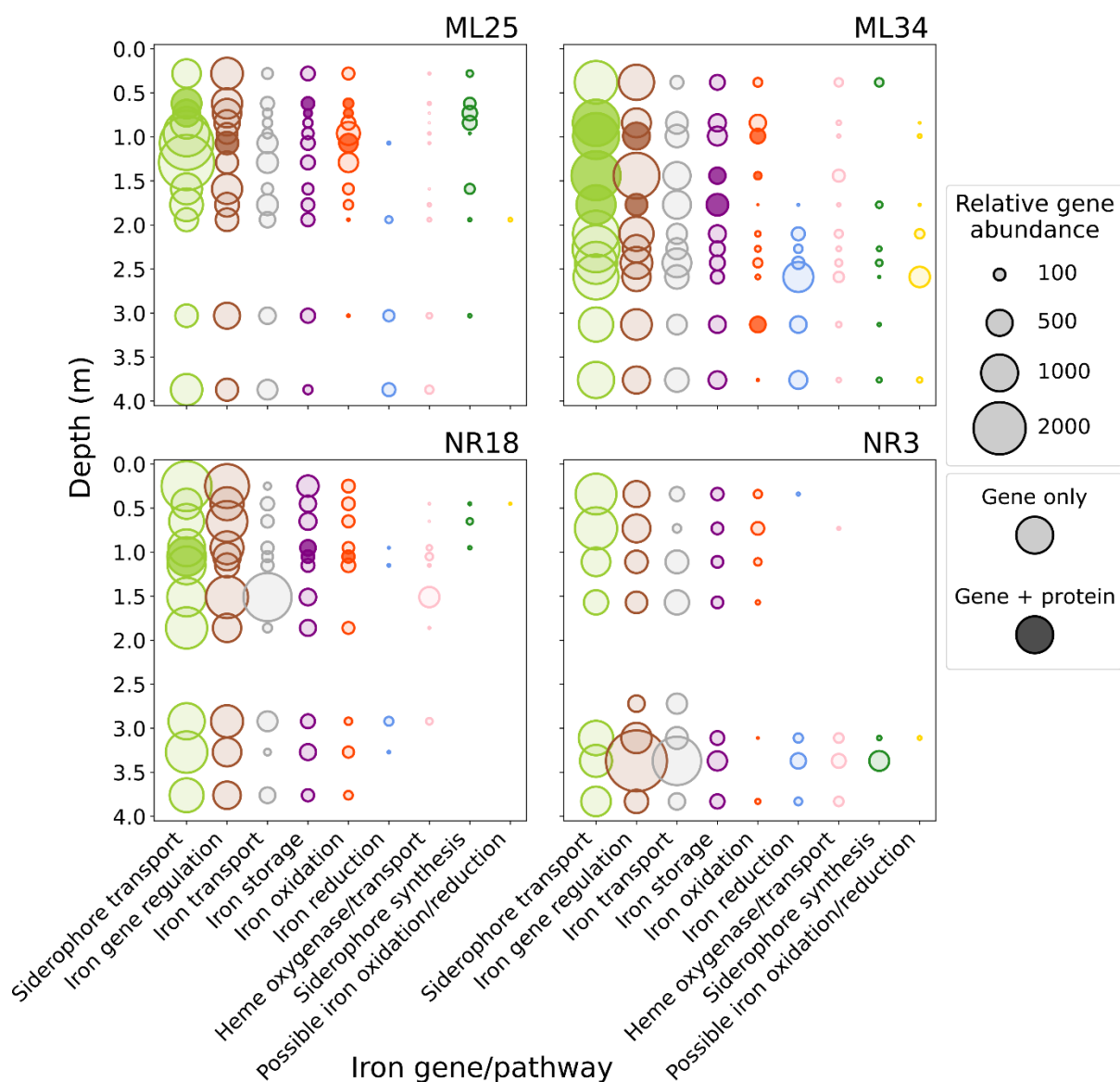


Figure 4.6: Distribution of iron-related genes across vertical tailings transects. MAGs were annotated using FeGenie. Relative gene abundances were calculated as the number of hits multiplied by the percent relative abundance of MAGs associated with each hit. In samples where protein expression was detected, the corresponding gene(s) are indicated with horizontal lines. Gene categories are colored based on function (**Table A1**) (Garber et al., 2020), ordered from highest overall abundance (Siderophore transport) on the left to lowest (Possible iron oxidation/reduction) on the right.

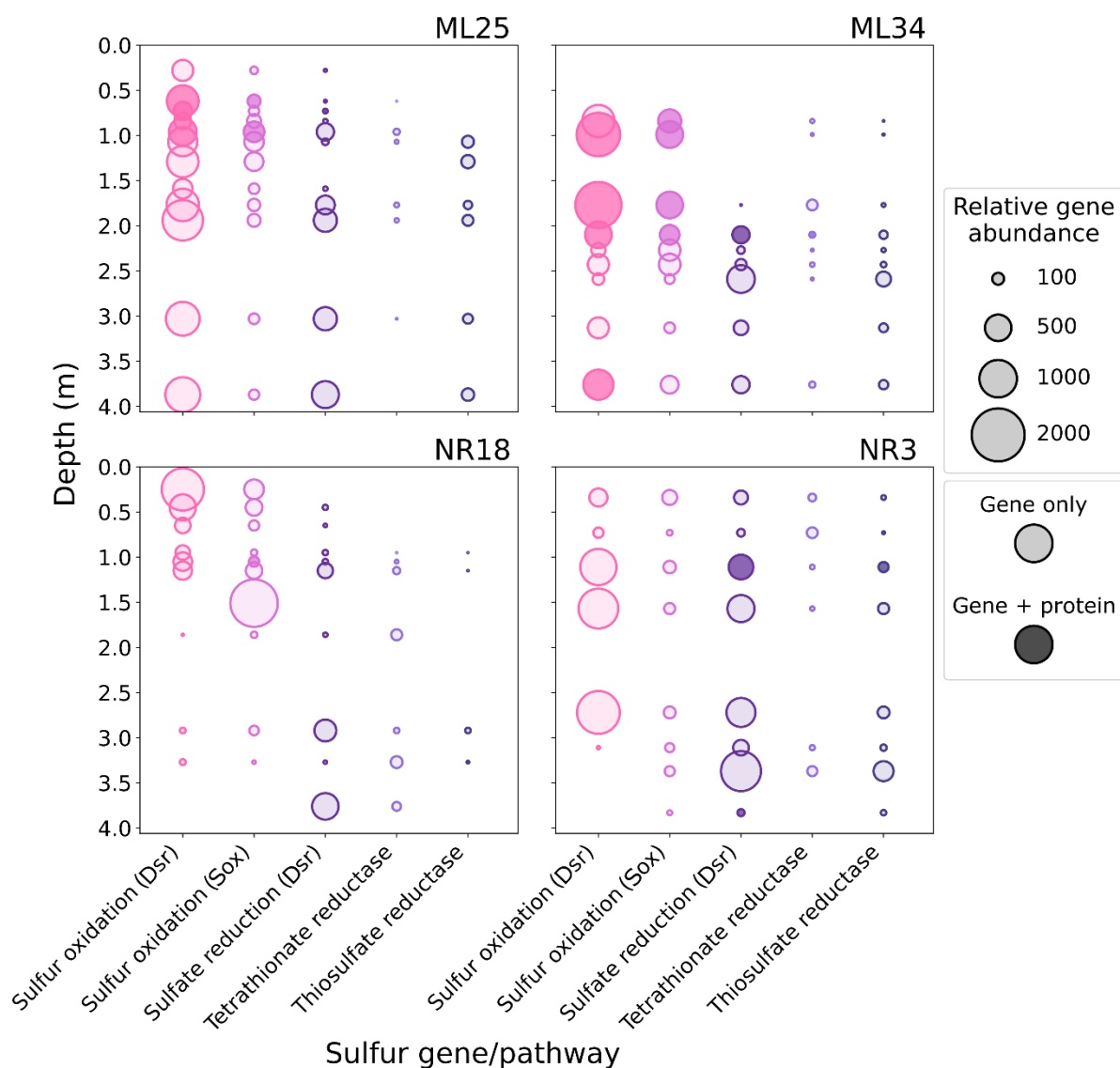


Figure 4.7: Distribution of sulfur-cycling genes across vertical tailings transects. MAGs were annotated using DRAM (*sox*, tetrathionate reductase, and thiosulfate reductase genes) and DiSCo (*dsr* genes). Relative gene abundances were calculated as the number of hits multiplied by the percent relative abundance of MAGs associated with each hit. In samples where protein expression was detected, the corresponding gene(s) are indicated with horizontal lines. Gene categories are colored based on function, ordered from highest overall abundance (Sulfur oxidation, Dsr pathway) on the left to lowest (Thiosulfate reductase) on the right.

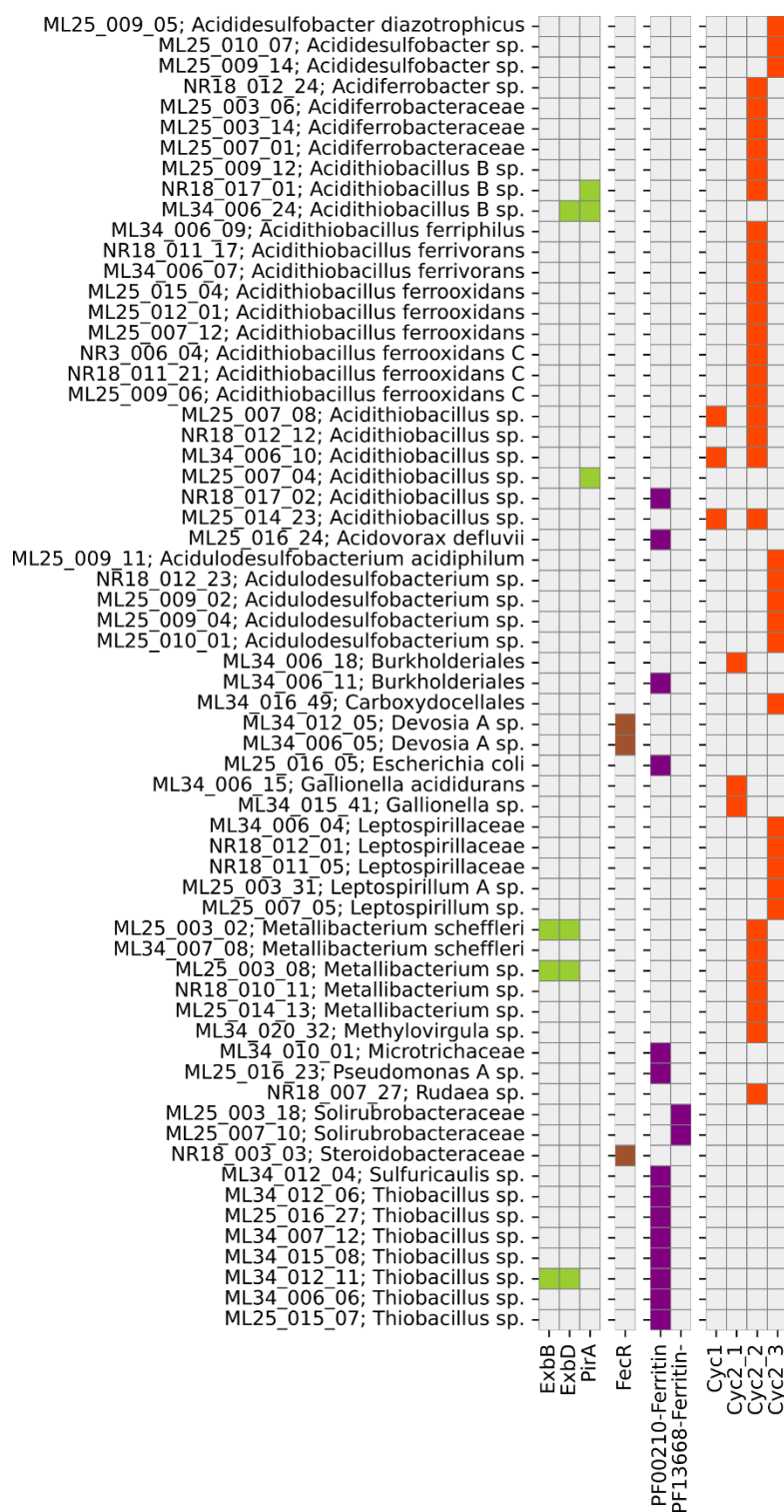


Figure 4.8: Taxonomic distribution of iron-related proteins in tailings communities. Shaded cells represent positive detection of specific proteins associated with (left to right): siderophore synthesis, iron gene regulation, iron storage, and iron oxidation. Proteins were identified by mass spectrometry and mapped to predicted proteins from metagenomic sequencing, and aggregated by the MAG from which they originated (if any). Bins are labelled by their ID, followed by their lowest classified level of GTDB-assigned taxonomy.

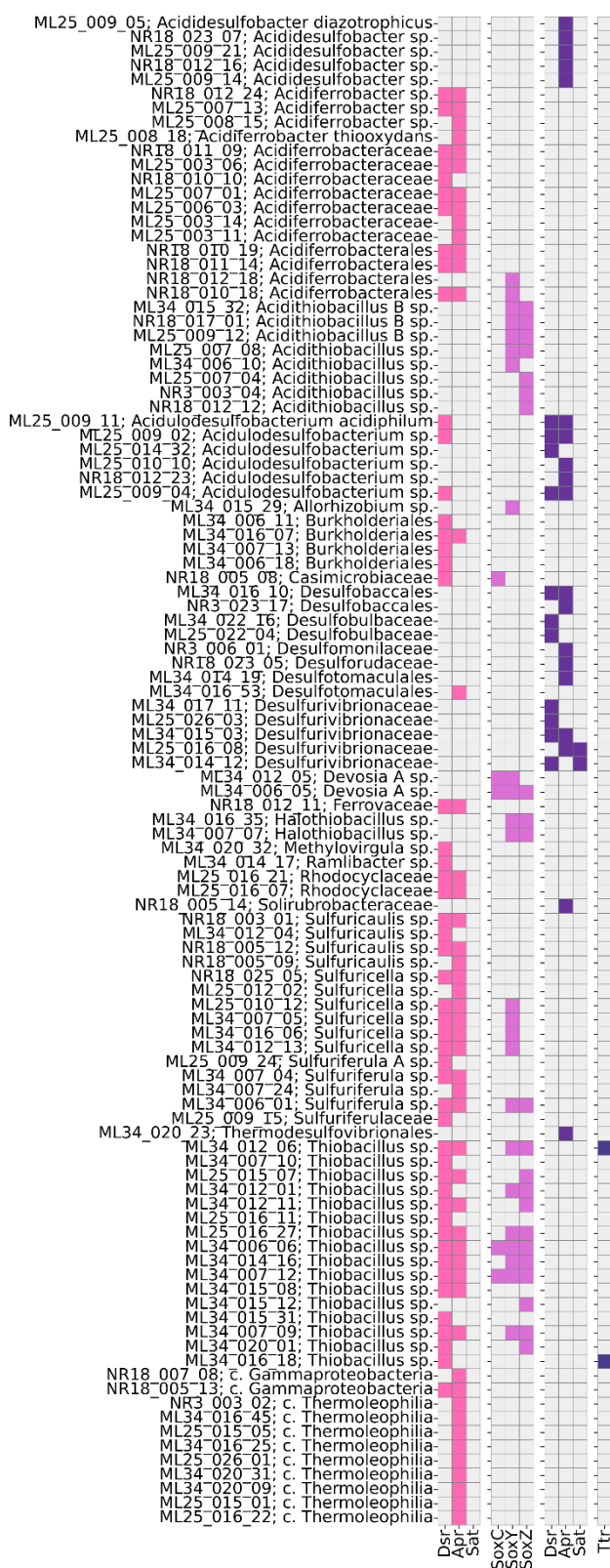


Figure 4.9: Taxonomic distribution of sulfur-related proteins in tailings communities. Shaded cells represent positive detection of specific proteins associated with (left to right): sulfur oxidation (Dsr pathway), sulfur oxidation (Sox pathway), dissimilatory sulfate reduction (Dsr pathway), and tetrathionate reduction (Ttr). Proteins were identified by mass spectrometry and mapped to predicted proteins from metagenomic sequencing, and aggregated by the MAG from which they originated (if any). Bins are labelled by their ID, followed by their lowest classified level of GTDB-assigned taxonomy.

4.4 Conclusions

To my knowledge, this is the first study to apply integrated metagenomic and metaproteomic analyses to mine tailings microbial communities. Metagenomic sequencing revealed a broad range of novel taxa, particularly within the Actinobacteriota, Desulfobacterota, and uncultured lineages such as *Ca. Eremiobacterota* and *Ca. SZUA-79*, some of which were implicated in iron/sulfur cycling processes. Functional profiling revealed distinct ecological strategies employed by differing microbial populations across geochemical gradients. In shallow, oxidized zones, iron- and sulfur-oxidizing Gammaproteobacteria were prevalent and active. Deeper, anoxic layers were dominated by potential sulfate-reducing organisms, many of which were taxonomically novel. Metal resistance mechanisms within communities as well as in individual taxa were highly diverse, reflecting potential adaptive diversity present within tailings microbiomes. The relative toxicity of metals was an important factor influencing MRG abundances in metagenomes. There were no clear correlations between MRG abundances and environmental metal concentrations, although organisms inhabiting highly contaminated tailings appeared to express similar MRGs targeting copper, nickel, and iron.

The number of high-quality MAGs was lower than anticipated, and many proteins could not be mapped back to binned genomes, indicating a lack of sequencing depth or protein modifications specific to the environment that were not captured in our mapping methods, or some combination of confounding factors. This led to an incomplete picture of the full taxonomic and functional diversity of communities. Although proteomics data was sparse and uneven, the expression of genes related to metal resistance and iron/sulfur cycling was successfully detected in some samples, confirming that the identified metal-interacting microbes in these tailings not only encode, but actively utilize, a diverse set of MRGs and MMGs. However, the low numbers of identified proteins in many samples restricted our ability to interpret the data in a systematic way, as absence of detection could not be interpreted as absence of function.

In summary, the findings presented in this chapter highlight the need to develop improved methodologies for extracting nucleic acids and proteins from challenging environmental matrices like mine tailings. However, despite substantial technical barriers towards obtaining sufficient DNA/protein for sequencing, including low biomass and interference from contaminants such as PEG, this study demonstrates the potential of multi-omics approaches to reveal new insights into microbial adaptations and activities within metal-contaminated environments.

Chapter 5

Evaluation of selective metal recovery from simulated mining and industrial waste by *Oleidesulfovibrio alaskensis* G20

5.1 Introduction

The imperative to mitigate climate change has driven a surge in technological advancements aimed at reducing carbon emissions and facilitating more sustainable resource use. With the burgeoning sustainable initiatives has come a stronger demand for a circular economy of critical metals. Critical metals are defined as metal(oids) that are vital to the function of modern technologies (especially in renewable or ‘green’ energy applications), yet are at high risk for supply chain constraints due to scarcity of natural deposits or disproportionately growing demand (reviewed in Watari et al., 2020). For example, lithium-ion batteries (LIBs) used in electric vehicles require lithium, cobalt, and nickel for their manufacture, all of which are projected to be critical raw materials for LIB production by 2050 (Sovacool et al., 2020; Weil et al., 2018). Other critical metals include platinum group metals (PGMs) such as palladium, a key component of automotive catalytic converters for reducing harmful emissions (Michalek et al., 2024). As a result, there is increasing incentive to develop efficient methods of recovering and recycling metals from mining, industrial, and consumer waste.

Existing chemical recovery methods (e.g., acid leaching, solvent extraction, electrochemical separation) of metals from waste can be costly and inefficient (Sarker et al., 2022; Whitworth et al., 2022; Wu et al., 2022). The use of microorganisms to extract metals, a process known as biomining, has emerged as a promising alternative (Sarker et al., 2022; Schippers et al., 2014; Whitworth et al., 2022). Bioleaching involves the use of acidophilic organisms (e.g., *Acidithiobacillus* spp.) to catalyze the oxidative dissolution of metal-bearing materials (Sarker et al., 2022; Whitworth et al., 2022). Subsequently, sulfidogenic organisms may be used to selectively precipitate dissolved metals (Schippers et al., 2014). Examples include several members of the genus *Desulfovibrio*: Gram negative, typically obligately anaerobic, sulfate-reducing bacteria (SRB) which have been well studied within the field of synthetic biology, where they have been engineered for bioremediation and bioenergy applications (reviewed in Keller et al., 2011).

Oleidesulfovibrio alaskensis G20 (also known as *Desulfovibrio alaskensis* G20, and formerly *Desulfovibrio desulfuricans* G20) (Hauser et al., 2011; Park et al., 2022) is a mutant *Desulfovibrio*

strain derived from the wild-type G100A strain, initially isolated from an oil well corrosion site (Wall et al., 1993; Weimer et al., 1988). G20 can transform nickel (Ni^{2+}) and cobalt (Co^{2+}) ions into nickel sulfide (NiS) (Capeness et al., 2015) and zero-valent cobalt (Echavarri-Bravo et al., 2022) nanoparticles as a method of LIB recycling. Another notable trait associated with some *Desulfovibrio* spp. and *O. alaskensis* G20 is the ability to reduce PGMs such as Pt^{4+} and Pd^{2+} in the periplasm (Capeness et al., 2015, 2019a; Mikheenko et al., 2008) and export the resulting nanoparticles out of the cell. This process is thought to be a resistance mechanism, decreasing the potentially toxic intracellular concentrations of PGM ions by making them insoluble and transporting them outside the cell, where they can act as catalysts for the further reduction of ions (Capeness et al., 2019a).

D. desulfuricans ATCC 27774 is a wild-type *Desulfovibrio* strain closely related to *O. alaskensis* G20, (Bursakov et al., 1995) and is also capable of producing PGM nanoparticles (Eggington, 2024). Although *O. alaskensis* G20 shows more potential for genetic manipulation, *D. desulfuricans* ATCC 27774 is potentially more versatile in its industrial applications, because it is more oxygen tolerant than *O. alaskensis* G20 (Lobo et al., 2007), and capable of growing on both sulfate and nitrate media (Bursakov et al., 1995). A mutant strain of *D. desulfuricans* ATCC 27774 known as *D. desulfuricans* 27774-N1 has been created through directed laboratory evolution to grow more effectively on nitrate media than the parent strain (Eggington, 2024).

Metal nanoparticles are highly valuable due to their unique physical and chemical characteristics. Their high surface area to volume ratio makes metal nanoparticles more effective as catalysts compared to bulk metal counterparts (Edmundson et al., 2014); biogenic Pd nanoparticles produced by *O. alaskensis* G20 have been shown to have especially high catalytic activity compared to chemically synthesized catalysts (Capeness et al., 2019b; Era et al., 2021, 2022). Metal nanoparticles also exhibit distinct optical, electromagnetic, and antimicrobial properties that enable their use as sensors (Era et al., 2022; Jaji et al., 2020; Kumar et al., 2021), superconductors (Jaji et al., 2020), and in biomedical technologies (Edmundson et al., 2014; Khursheed et al., 2022), among other applications.

Nickel and PGMs are often found together in mining wastes (e.g., low grade ore, tailings) and industrial waste, among other metals (e.g., iron) (Li et al., 2023; Michałek et al., 2024). However, *O. alaskensis* (and related *Desulfovibrio* spp.) have only been tested for their capacity to generate nickel and PGM nanoparticles independently; it is not known whether these organisms can effectively

generate nanoparticles when given a more complex substrate containing both metals. The presence of additional metals as well as stressors associated with exposure to metal waste (*e.g.*, low pH) may interfere with *O. alaskensis*' survival and/or nanoparticle production ability.

The main objective of the research presented in this chapter was to determine the feasibility of applying *Desulfovibrio* spp. (including *O. alaskensis* G20) towards recycling mixed-metal wastes. Specifically, I evaluated whether selective recovery of nickel and palladium as solid nanoparticles was possible with *O. alaskensis* G20 given various combinations of dissolved Ni^{2+} , Fe^{2+} , Pd^{2+} , and under different pH conditions.

5.2 Methods

5.2.1 Culture conditions

Oleidesulfovibrio and *Desulfovibrio* spp. were cultured in on Postgate Media C (PGMC) (Postgate, 1979), containing lactate as a carbon source and sulfate as an electron acceptor (Postgate, 1979). Frozen stocks of *O. alaskensis* G20 and *D. desulfuricans* 27774-N1 strains were plated onto 1% PGMC agar, and colonies were transferred to 5 mL PGMC liquid cultures after 1–2 weeks. Cultures were maintained at 30 °C in an anaerobic chamber (10% CO₂, 10% H₂, 80% N₂), and diluted (approximately 1:20) in 5–50 mL fresh PGMC media every 48–72 hours.

5.2.2 Assessment of metal removal from simulated waste solutions

Stock solutions of 100 mM NiCl₂, 1 M FeSO₄, and 100 mM Na₂PdCl₄ in diH₂O were prepared prior to experiments. NiCl₂ was stored in the anaerobic hood, whereas Na₂PdCl₄ was stored at room temperature on a lab bench, to minimize abiotic reduction. FeSO₄ solutions were prepared just prior to each experiment to minimize abiotic oxidation.

For all experiments, cells were grown in liquid culture as described in section 5.2.1 for 48 hours. Cells were left in the original growth medium, or centrifuged at 3,600 x g for 20 min, washed twice to remove H₂S, and resuspended in either 10 mM 3-(N-morpholino)propanesulfonic acid (MOPS) buffer (pH 7.5) or 10 mM acetate (NaOAc) buffer (pH 4.0). Samples were adjusted to the same optical density (OD₆₀₀) within each independent trial, ranging from 0.7–1. Combinations of metal solutions were added to samples, to the following final metal concentrations: 10 mM Ni (NiCl₂), 80 mM Fe (FeSO₄), and 1.5 mM Pd (Na₂PdCl₄). Concentrations of Ni and Fe were chosen based on the maximum values measured in Sudbury Ni/Cu mine tailings pore-water, described in Chapters 3 and 4. Ni, Fe, and Pd concentrations are also representative of industrial waste profiles (Prof. Louise Horsfall, personal communication).

To test selective Ni recovery in the presence of Fe (Experiment 1), samples were prepared as above to a final volume of 200 mL with 10 mM Ni and 80 mM Fe. After 60 minutes incubation at 30 °C, samples containing NiS and FeS precipitate were removed from the anaerobic hood and concentrated H₂SO₄ was gradually added to each while continuously stirring and measuring the pH with a pH meter. Aliquots of 1 mL were taken from the sample at 1.0 pH intervals from an initial pH of 7 to pH 2, for a total of 6 aliquots. To measure metal removal, 100 µL was sub-aliquoted from each

of the 6 aliquots, representing total metals. The remainder was centrifuged at 15,000 x g for 30 min to separate the aqueous phase, which represented the final solubilized metal concentration, from cells and precipitated metals. Both the initial sub-aliquot and 100 μ L of supernatant from the centrifugation step were digested in 1 mL 20% HNO₃ for 6 hours at 75 °C, then diluted to a final volume of 3 mL in 2% HNO₃ for ICP-OES analysis (PerkinElmer Optima 8300) with the following settings: Plasma 12, Auxillary 0.2, Nebuliser 0.6, RF Power 1500, and flow rate of 1.50 mL/min. Measurements were plotted against standard curves for each metal to determine concentration.

A separate experiment (Experiment 2) was conducted to compare metal removal in Ni-only and Fe-only solutions to the mixed Ni and Fe treatment. The experiment was performed as described in Experiment 1, with the following changes: samples were prepared to final volumes of 5 mL with either 10 mM Ni, 80 mM Fe, or both Ni and Fe, no pH adjustments were made after metal precipitation (*i.e.*, only the initial metal removal was measured), and *D. desulfuricans* 27774-N1 was used, as the G20 strain was unavailable during this time. Aliquots were prepared for ICP-OES as described above to measure Ni and Fe concentrations.

To test Pd recovery under different pH conditions (Experiment 3), metal recovery assays were prepared with *O. alaskensis* G20 and 1.5 mM Pd in either MOPS or NaOAC buffer, to a final volume of 5 mL. To test selective Pd recovery in the presence of Fe or Ni (Experiment 4), the following 3 treatments were compared using *O. alaskensis* G20 in NaOAC buffer only: 1.5 mM Pd alone, 1.5 mM Pd + 10 mM Ni, and 1.5 mM Pd + 80 mM Fe. Incubation and sample preparation for ICP-OES in Experiments 3 and 4 were identical to previous experiments.

5.2.3 Characterization of metal nanoparticles

Metal removal assays were set up as described in section 5.2.2 Experiments 2 (*O. alaskensis* G20 + Fe + Ni in growth medium) and section 5.2.3 (*O. alaskensis* G20 + Pd in MOPS or NaOAC), with 5 mL sample volumes and 60 minutes incubation at 30 °C. 5 μ L from each sample were pipetted to a 200 mesh carbon-coated copper grid, and excess liquid was removed after 5 min by blotting gently using filter paper.

Imaging of cells and nanoparticles was conducted with Scanning-Transmission Electron Microscopy (STEM) and high-angle annular darkfield STEM (HAADF-STEM) using a Zeiss Crossbeam 550, in combination with Electron Dispersive X-ray Spectroscopy (EDS) to determine elemental composition. EDS spectra were captured using a Bruker Probe.

5.2.4 *O. alaskensis* G20 spot plate assays

To assess the effect of pH on survival and growth, 5 μ L of cells from each buffer treatment (from Experiment 3) were spot plated onto PGMC agar, both upon initial resuspension and after the 60-minute incubations. MES (2-(N-morpholino)ethanesulfonic acid) buffer (pH 5.5) was tested in addition to NaOAc and MOPS. Metal-based differences in growth capacity were also tested with the NaOAc (pH 4.0) treatment. Plates were checked after one week for evidence of growth.

5.3 Results and discussion

5.3.1 Selective recovery of nickel

When *O. alaskensis* G20 cells were washed and resuspended in either MOPS or NaOAc buffer (Experiment 1), there was no evidence of nanoparticle production in any treatment containing Ni and/or Fe. However, black precipitate was immediately evident upon addition of Ni/Fe solutions to *O. alaskensis* G20 in growth media (**Figure 5.1a**), indicative of biogenic H₂S reacting with metal ions to form metal sulfides. Net metal removal was 0.54 mg/L Ni (29.00±5.82%) and 2.48 mg/L Fe (34.46±4.27%) (**Figure 5.1b**).

STEM-EDS imaging identified the precipitate as NiS/FeS nanoparticle (25–50 nm diameter) aggregates (**Figure 5.2**). Nanoparticle appearance and localization does not appear to differ based on type of metal supplied, which negates the possibility of downstream metal separation on the basis of physical characteristics such as size or density.

Metal sulfides exhibit different pH dependent solubilities (Lewis, 2010), which can be exploited to separate metals during the recovery process. Adding concentrated sulfuric acid (H₂SO₄) to samples after NiS/FeS precipitation led to the re-dissolution of metals (**Figure 5.1b**). Fe completely dissolved between pH 4–5, while approximately half of the initially removed NiS remained in the solid state (**Figure 5.1b**), which is consistent with the relative solubilities of Fe²⁺ and Ni²⁺ across this pH range (Lewis, 2010). Therefore, selective recovery of Ni²⁺ (as NiS) in the presence of Fe²⁺ is possible under pH 4.0 conditions, although the percent removal was low (13.56±2.59%) (**Figure 5.1b**). This method could be adapted to selectively recover other metals that form sulfides, such as Cu, Zn, and Mn, by adding mixed-metal solutions to *O. alaskensis* cultures and adjusting the pH during growth; however, potential metal toxicity would be a challenge with this approach (see section 5.3.3).

Although sulfide precipitation is a well-known method of selective metal recovery, it is important to note that specific reaction conditions can substantially impact the final product. The observed size, morphology and aggregation pattern of NiS/FeS nanoparticles in this study are visually different from chemically synthesized NiS nanoparticles (Molla et al., 2016; Roushdy et al., 2024). It is possible that *O. alaskensis* G20 interacts with metals in growth media during precipitation, or releases compounds other than H₂S (*e.g.*, metabolites, enzymes) that have an effect on nanoparticle physiochemical properties (*e.g.*, size, shape, electrical conductivity). Bacterial cells themselves may

also serve as nucleation sites for crystal formation, and the resulting crystalline structure of sulfide nanoparticles may be influenced by interactions between cations and negative charges on the cell surface (Hoffman et al., 2021). Nanoparticle properties are important in determining their applicability towards material technologies. Further investigation (*e.g.*, with electron diffraction) is required to fully characterize the NiS nanoparticles produced in this study to enable comparison with chemically synthesized ones, and to determine which factors (if any) associated with *O. alaskensis* G20 and/or its growth media contribute to nanoparticle characteristics.

The percent yield of Ni in Experiment 1 is much lower than previous studies, where *O. alaskensis* G20 was able to remove up to 90% of solvated Ni^{2+} (Capeness et al., 2015). The discrepancy may be due to the higher concentration of Ni^{2+} (10 mM here vs. 1 mM in Capeness et al., 2015) completely saturating the biogenic sulfides in the growth medium. When comparing *D. desulfuricans* N1 metal removal of 10 mM Ni and 80 mM Fe separately to a mixed solution of Ni/Fe (Experiment 2), the total amount of removed metal (mg/L) was higher in the Fe and mixed Ni/Fe treatment compared to the Ni treatment (**Table D1**). This suggests that there should be additional sulfide available for higher Ni precipitation yields in the *O. alaskensis* G20 Ni-only trial; however, Experiment 2 used a different *Desulfovibrionaceae* species (*D. desulfuricans* N1) and lacked replicate samples.

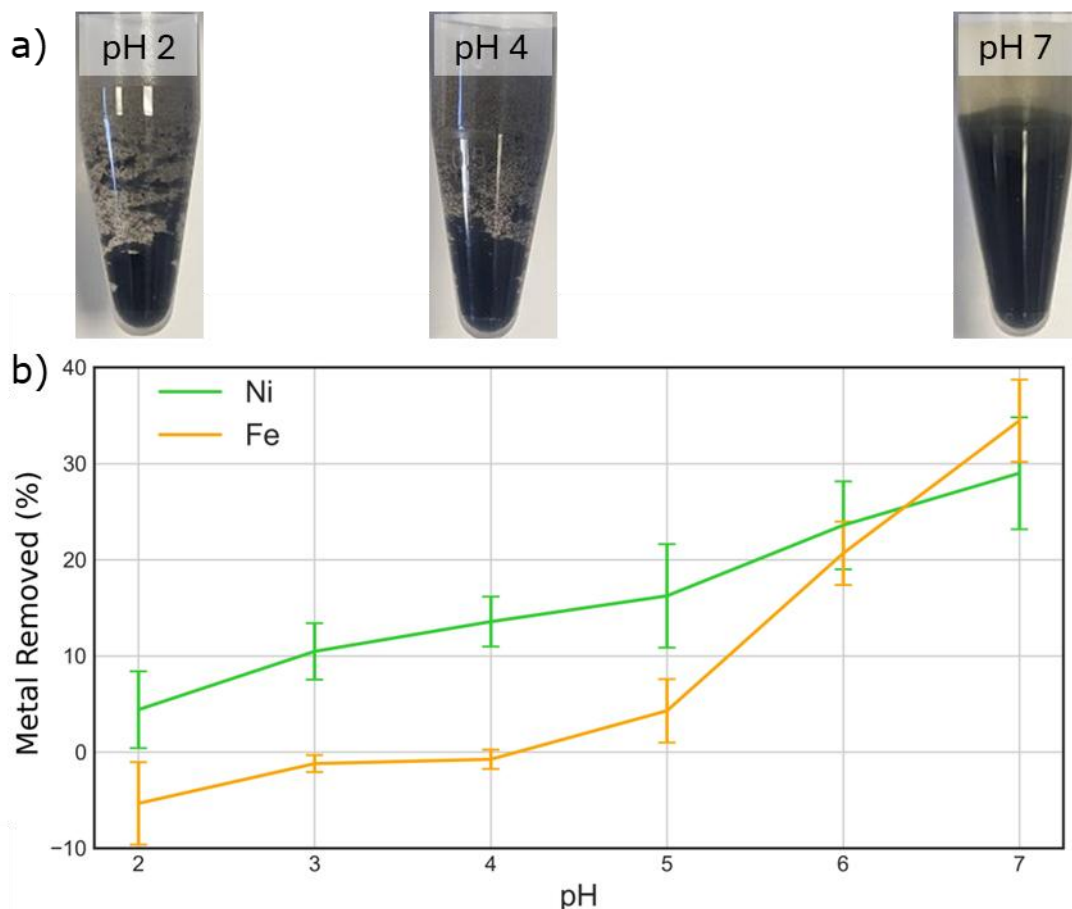


Figure 5.1: Effects of pH on relative nickel (NiS) and iron (FeS) removal. Metal precipitation (as sulfide nanoparticles) occurs when solutions containing 10 mM NiCl_2 and 80 mM FeSO_4 are mixed with *O. alaskensis* G20 growth media containing H_2S . The combined solution was titrated with H_2SO_4 from an initial starting pH of ~ 7 to pH 2. Photos in (a) show the visual appearances of samples at select pH points, and the graph in (b) indicates the percentage of metal removed, calculated by comparing the concentration of dissolved Ni or Fe in the aqueous phase post-treatment to the total amount of metal (including precipitate). Error bars represent standard deviation around the mean.

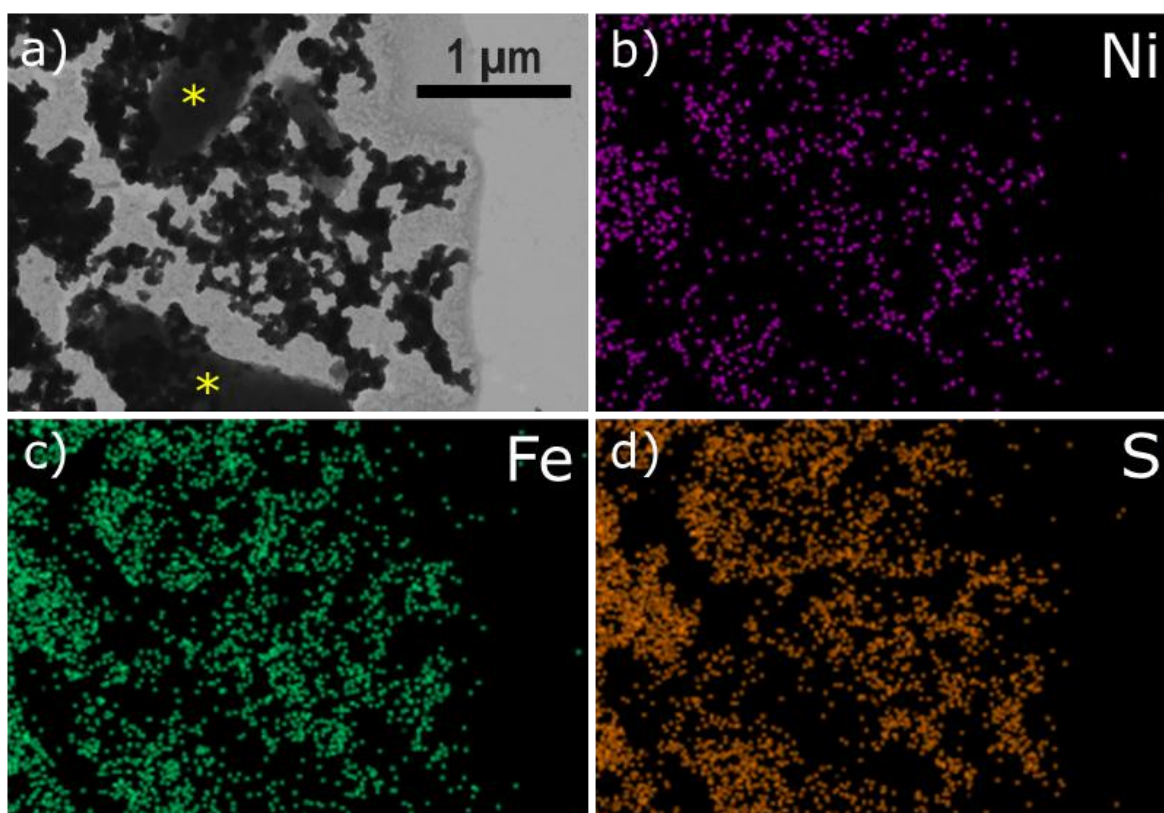


Figure 5.2: STEM micrographs of NiS/FeS nanoparticles, with EDS mapping of elemental composition. The brightfield STEM image in (a) depicts dense aggregates of metal nanoparticles <100 nm in size, among *O. alaskensis* G20 cells (marked with asterisks). EDS elemental mapping of the same region (b-d) indicate that nanoparticles are comprised of NiS and FeS; individual element maps of Ni (b) Fe (c), and S (d) are shown.

5.3.2 Selective recovery of palladium

Effects of pH on Pd recovery

In Experiment 3, there was no significant difference in *O. alaskensis* G20 Pd removal between the pH 7.5 and pH 4.0 treatments (**Figure 5.3a**), with average yields of $54.15 \pm 10.85\%$ and $68.02 \pm 0.58\%$, respectively. Yields were also lower than were reported in past studies ($>95\%$) (Capeness et al., 2019b). However, Pd removal was much higher in the pH 4.0 treatment compared to the abiotic control, whereas abiotic precipitation appears to be favored at circumneutral (7.5) pH (**Figure 5.3a**).

The visual appearance of nanoparticles differed between treatments. At pH 4.0, many small nanoparticles (<20 nm) were seen covering the entire surface of the cells (**Figures 5.4a-c, 5.5a-b**). Larger (100–200 nm) particles with irregular surface area were also present, although these were not attached to cells (**Figure 5.4d**). Some cells appeared to have localized areas where nanoparticles formed dense ‘aggregates’ (**Figure 5.4a-b**). It is possible that the larger detached particles originate from these sites, if the biogenic nanoparticles are acting as nucleation sites for additional Pd precipitation. At pH 7.5, some (nano)particles are seen in STEM imaging (**Figures 5.4e, D1a-b**), but they are less abundant and less consistently distributed compared to the pH 4.0 treatment. Additionally, both cells and (nano)particles form aggregates in the pH 7.5 treatment.

Previous studies have found that PGM nanoparticles produced by Desulfovibrionaceae spp. exhibit autocatalytic growth, where nanoparticles attached to cell surfaces catalyze the further reduction of solvated Pt^{4+} and Pd^{2+} (Mikheenko et al., 2008; Yong et al., 2002). This process is the most likely explanation for large, detached particles seen in the pH 4.0 treatment: small biogenic Pd nanoparticles on the surface of *O. alaskensis* G20 cells undergo autocatalytic growth and detach after reaching a certain size (100–200 nm). In terms of catalysis applications, larger particles may be an undesirable product due to their lower surface area to volume ratio. It is possible that the observed irregular edges of the larger particles would mitigate this effect, which could be tested by separating the produced particles by size and evaluating catalysis rates (*e.g.*, hydrogenation, Suzuki coupling, Sonogashira reaction) (Era et al., 2022). Outside of catalysis applications, larger particles would be easier to separate and collect, if the goal is to recover pure Pd from solution.

EDS elemental mapping of (nano)particles in the pH 4.0 (**Figure 5.5b-d**) and pH 7.5 (**Figure D1b-d**) treatments indicated that the particles were comprised of elemental Pd (Pd^0) rather than PdS , confirming that they were generated through reduction of Pd^{2+} ions by *O. alaskensis* G20, although

abiotic (pH 7.5) and autocatalytic (pH 4.0) Pd²⁺ reduction mechanisms are major contributors to the net Pd removal observed under these conditions.

Effects of Fe and Ni on Pd recovery

Experiment 4, which tested Pd removal from mixed-metal solutions, was conducted using NaOAc buffer (pH 4.0), as it was previously determined that biotic Pd nanoparticle formation is favored at low pH, and abiotic precipitation of Fe and Ni sulfides is less likely to occur at low pH.

The addition of 80 mM Fe or 10 mM Ni did not significantly impact the percent removal of Pd (41–64%) (**Figure 5.3b**), nor were Fe or Ni removed from solution, demonstrating that selective recovery of Pd from mixed metal solutions is possible with *O. alaskensis* G20.

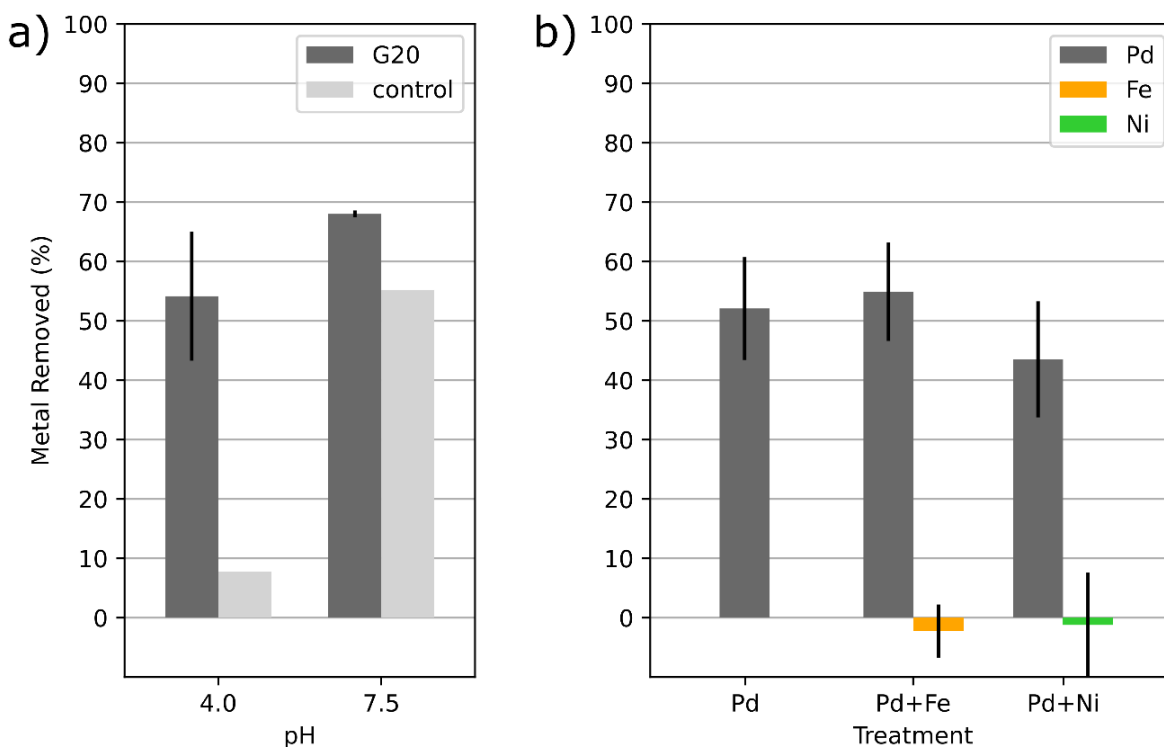


Figure 5.3: *O. alaskensis* G20 selective Pd removal. Percent metal removal (y axes) in two Pd removal experiments are shown. In (a), Pd removal was compared between different pH treatments. There was no significant difference between *O. alaskensis* G20 pH 4.0 and 7.5 groups (t-test, $p=0.21$, $n=2$), but when compared to abiotic controls ($n=1$), the proportion of biotic Pd removal appears to be higher at pH 4.0 than at pH 7.5. The effects of mixed-metal treatments with *O. alaskensis* G20, Pd, and either Fe or Ni (at pH 4.0) are shown in (b). There was no significant difference in Pd removal between treatments (one-way Analysis Of Variance (ANOVA), $p=0.33$, $n=3$).

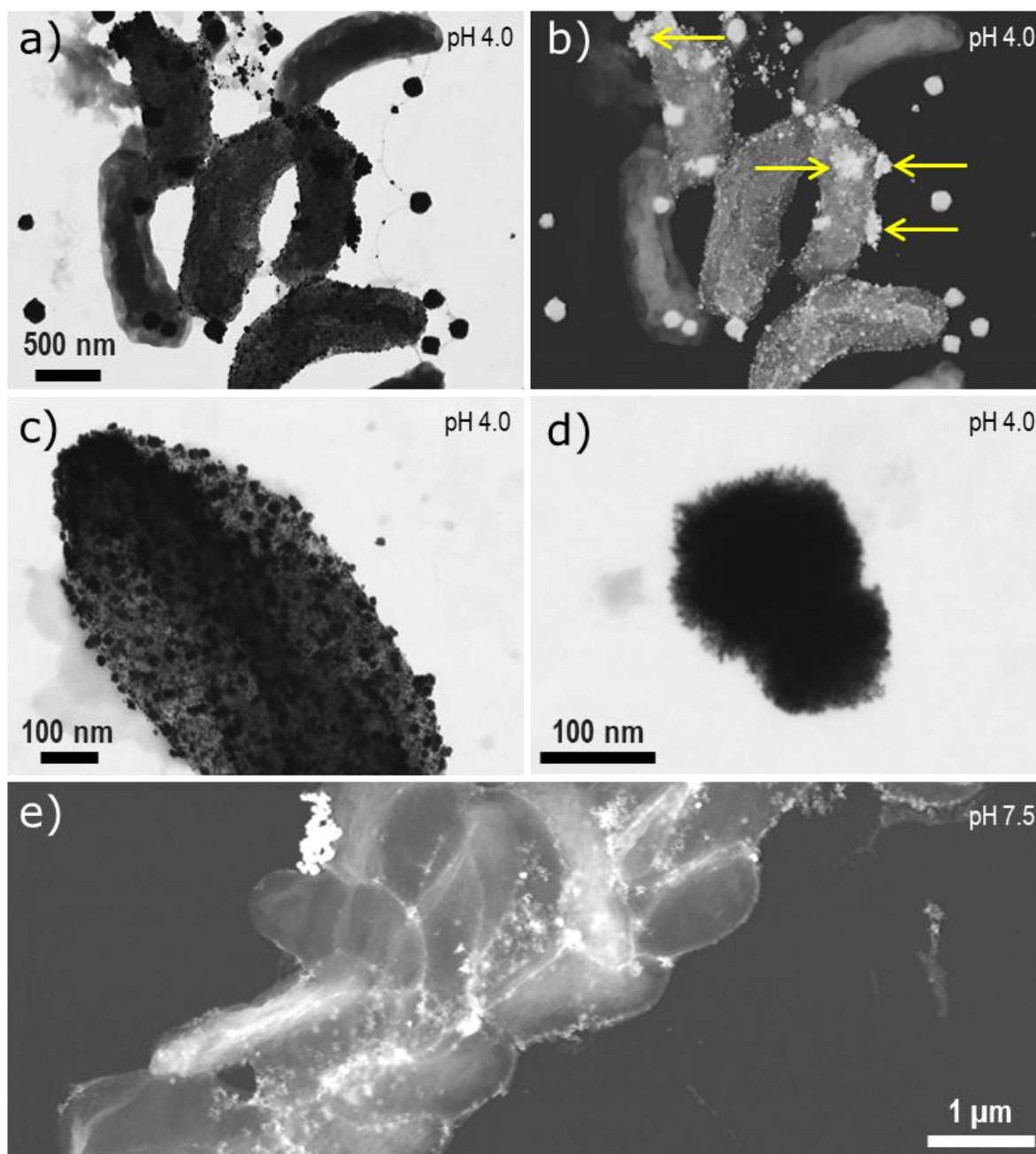


Figure 5.4: Effects of pH on Pd nanoparticle production by *O. alaskensis* G20. STEM brightfield (a) and darkfield (b) images show many small Pd nanoparticles attached to G20 cells, along with larger detached particles, in the pH 4.0 treatment. Yellow arrows in (b) indicate possible nucleation sites of particle autocatalytic growth. Higher magnification images of attached Pd nanoparticles (<100 nm) (c) and a larger detached particle (>100 nm) (d) are also shown for the pH 4.0 treatment. The darkfield STEM image of the pH 7.5 treatment (e) shows G20 cells with some (nano)particles, with both cells and nanoparticles appearing to form aggregates.

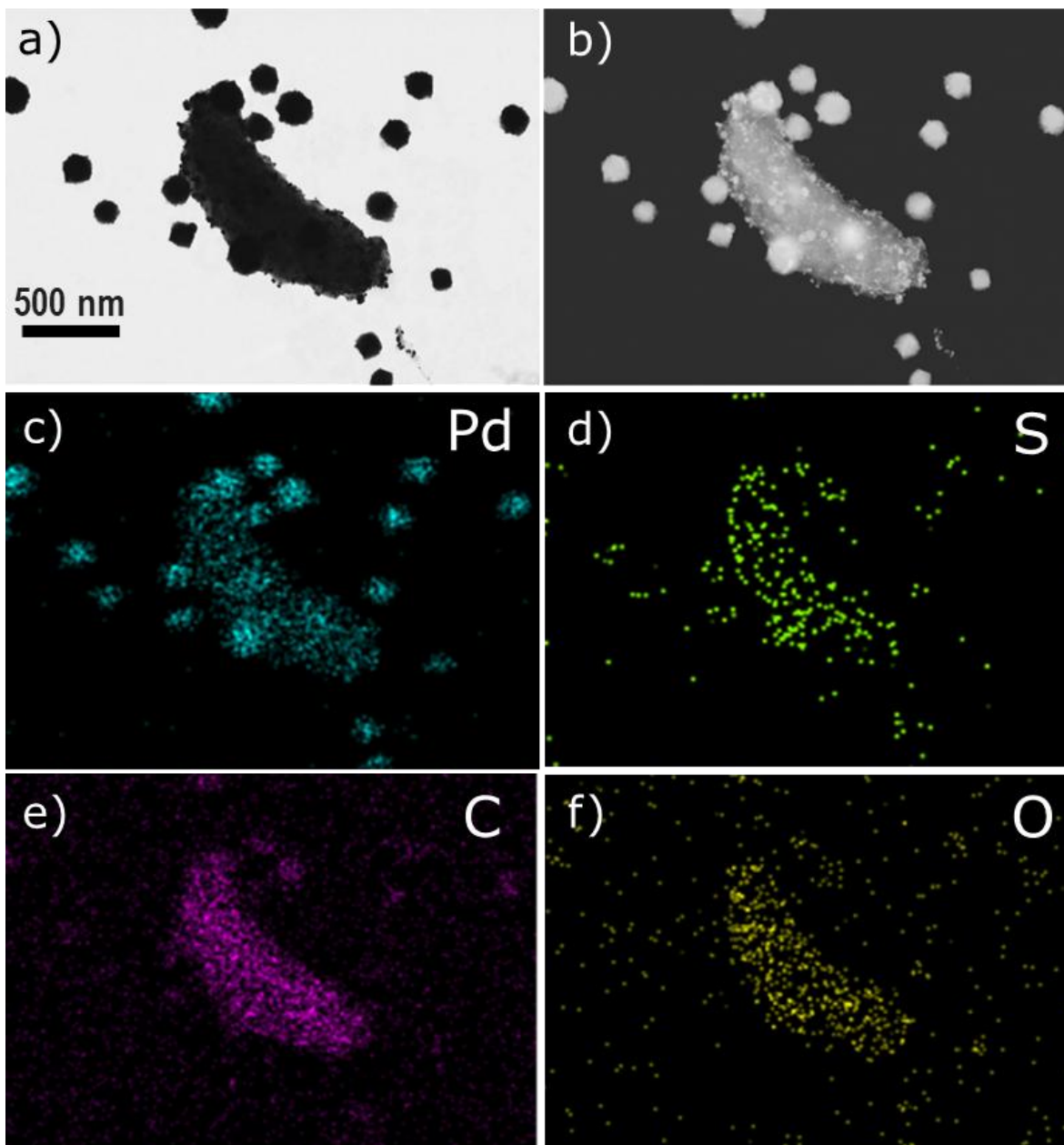


Figure 5.5: STEM - EDS micrographs of *O. alaskensis* G20 and Pd nanoparticles (pH 4.0). Images in brightfield (a) and darkfield (b) STEM show an *O. alaskensis* G20 cell with attached nanoparticles (<100 nm), as well as larger particles (>100 nm) that have detached from the cell surface. EDS elemental mapping of the same region (c-f) show that (nano)particles are enriched in Pd (c) compared to S (d). Maps of C (e) and O (f) are included for comparison.

5.3.3 Effects of pH and metals on *O. alaskensis* G20 survival and growth

Spot plates showed *O. alaskensis* G20 growth following all buffer treatments (pH 4.0–7.5) without metal (**Figure 5.6a**), but no growth following any metal treatment (**Figure 5.6b**). Possible contamination was observed in the MES and NaOAc buffers, but the colonies growing in the controls are more sparse and visually distinct compared to samples with *O. alaskensis* G20 present, so the growth observed in buffer treatments (**Figure 5.6a**) is parsimoniously attributed to *O. alaskensis* G20.

Although the results from the spot plate assays indicate that metal toxicity is severely detrimental towards *O. alaskensis* G20 survival (and/or growth), results from sections 5.3.1 and 5.3.2 suggest that long-term survival is not necessary when it comes to harnessing *O. alaskensis* G20's potential for metal recovery. Recovery of NiS and other metal sulfides does not require living cells, but rather the biogenic H₂S present in *O. alaskensis* G20 growth media. Furthermore, biotic reduction and precipitation of Pd appears to occur very quickly, and *O. alaskensis* G20 is only required for initiating the reaction at pH 4.0, following which autocatalytic growth of nanoparticles can lead to further Pd recovery without direct catalysis from *O. alaskensis* G20. It is also possible that the metal treatments are not killing cells, but inhibiting their growth on agar plates, in which case the cell fractions may be recoverable for future Pd nanoparticle formation.

Lack of growth in the 1.5 mM Pd ($\pm$ Ni or Fe) treatment is in contrast to past literature data, which found that *O. alaskensis* G20 can survive exposure of up to 5 mM Pd and up to 20 mM Ni (Capeness et al., 2015). The combination of low pH and metals may therefore be responsible for inhibiting growth, but no spot plate assays with metals at pH 7.5 were tested in these trials, prohibiting comparison. Further optimization of laboratory growth conditions for *O. alaskensis* G20 cultures would also be beneficial for ensuring consistency across experiments with respect to metal tolerance and metal removal yields.

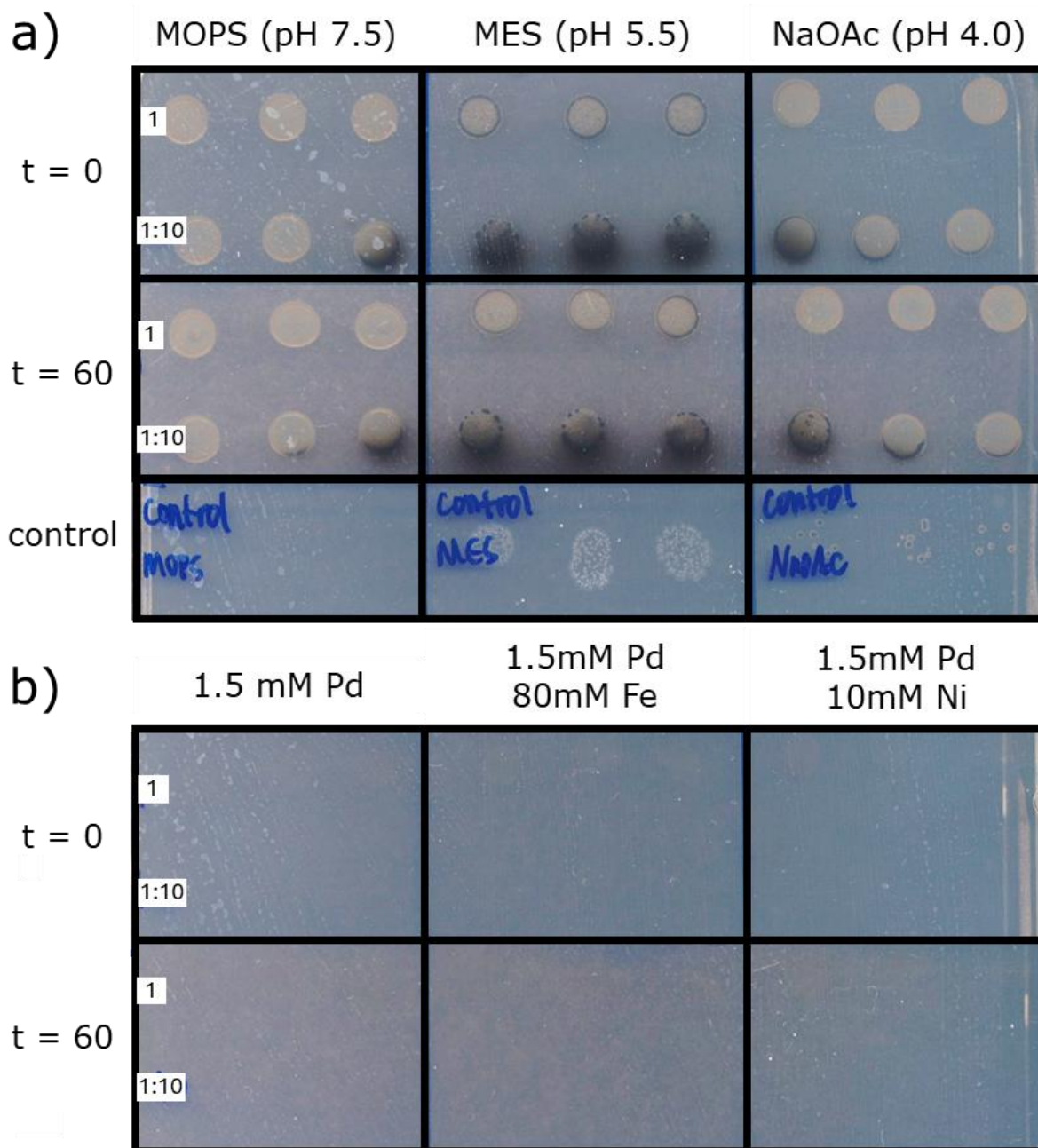


Figure 5.6: Comparison of *O. alaskensis* G20 survival and growth upon exposure to different pH conditions or different combinations of metals. Results in **(a)** show the effects of different buffers (pH 4.0–7.5) without metals. Results in **(b)** show the effects of different combinations of metals (1.5 mM Pd, 10 mM Ni, 80 mM Fe) in the same buffer (NaOAc, pH 4.0). Spot plating of 5 μ L *O. alaskensis* G20 occurred either immediately (t=0) or after 1 hour (t=60) of exposure to the given treatment. Three replicates of undiluted and a 1:10 dilution were plated for each treatment. Images represent 1 week of growth on PGMC agar.

5.3.4 Theoretical bioreactor design for *O. alaskensis* G20 selective metal recovery

Based on experimental results from sections 5.3.1–3, here I hypothesize a framework in which *O. alaskensis* G20 could be applied to selectively recover metals from mining, industrial, and other complex waste sources containing metals (**Figure 5.7**). Cells and growth media are first separated. Cells are then incubated with solvated metal waste (*e.g.*, from bioleaching reactions), allowing for separation of PGMs such as Pd from solution. The remaining waste can then be mixed with the growth media to precipitate metals such as Ni as sulfides by adjusting the pH of the solution.

Because *O. alaskensis* G20 is only needed to initiate the reactions, metal toxicity is not a major concern. Consequently, this general approach could be expanded to fit waste profiles of variable metal composition without needing to limit metal concentrations within tolerable ranges for G20. For example, *O. alaskensis* G20 could be used to recover Ni from Cu/Ni mine tailings (Chapters 3 and 4) through sulfide precipitation, despite Cu being highly toxic towards *Oleidesulfovibrio* and *Desulfovibrio* spp. (Sani et al., 2001). However, improved metal resistance may be helpful in increasing metal recovery yields and bioreactor efficiency. Engineered strains of metal-resistant *O. alaskensis* G20 could be incorporated into continuous culture systems where solvated metals are added directly to the growing culture, allowing for continuous recovery of metal without having to grow cells in a separate batch culture. To prevent sulfide precipitation of PGMs, *Desulfovibrio* spp. that are not obligate sulfate reducers such as *D. desulfuricans* 27771-N1 could be used to recover PGMs from growth media (without sulfate) in a separate reactor, before transferring the waste stream to the sulfidogenic G20 reactor for precipitation of non-PGMs. Ongoing and future work in the Horsfall research group is focused on identifying the MRG pathway(s) involved in *O. alaskensis* PGM nanoparticle production (Capenness et al., 2019a), which can be used to engineer other strains of *Desulfovibrio* that are more versatile in their metal recovery capabilities, have higher recovery yields, and/or produce higher quality nanoparticles for catalytic (or other) applications (Eggington, 2024).

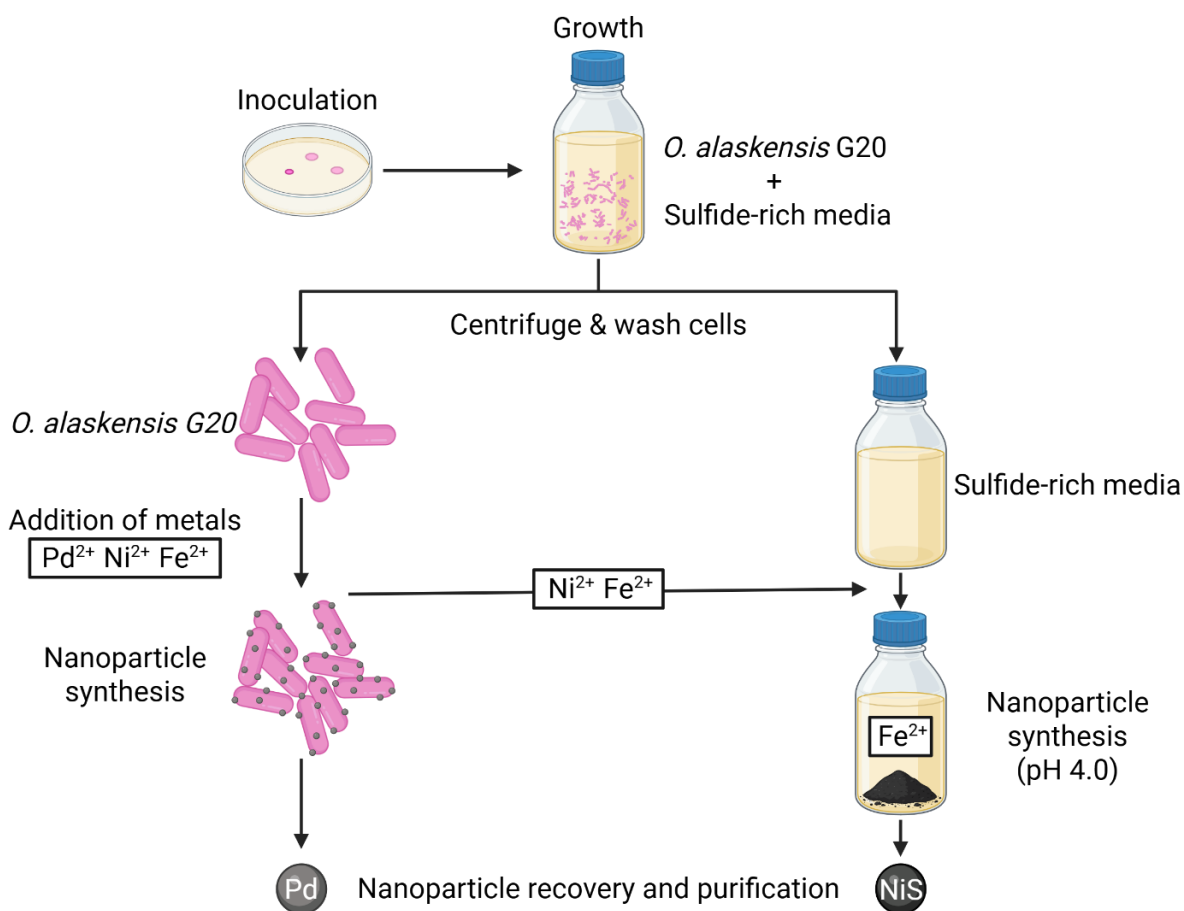


Figure 5.7: Theoretical bioreactor design for *O. alaskensis* G20 selective metal separation.

Mixed-metal waste containing PGMs (*e.g.*, Pd), Ni, and Fe can be separated in two phases, where *O. alaskensis* G20 cells are isolated and used to produce Pd nanoparticles from dissolved Pd^{2+} . Metals in the remaining aqueous waste can then be extracted using *O. alaskensis* G20 growth media, which contains sulfides from sulfate reduction. Selective separation of metals such as Ni and Fe rely on pH dependent solubility of the metal sulfides; at pH 4.0, NiS is less soluble than FeS, enabling separation. Image was created with BioRender.com.

5.4 Conclusions

In this chapter, I report the successful demonstration of *O. alaskensis* G20's ability to selectively recover nickel (as NiS) and palladium (as elemental Pd) nanoparticles from mixed-metal solutions containing Ni, Fe, and/or Pd at concentrations representative of mine tailings pore-water and industrial waste. NiS nanoparticles can be recovered through sulfide precipitation in *O. alaskensis* G20 growth media, and separated from FeS by adjusting the pH to 4.0, although the percent removal was low (<15%). Elemental Pd nanoparticles are produced by *O. alaskensis* G20 in the absence of sulfides, enabling separation from Fe and Ni. Pd removal under pH 4.0 conditions is comparable to pH 7.5, and there is less abiotic precipitation competing with biogenic nanoparticle production, ensuring higher quality nanoparticles. Evidence of autocatalytic growth of nanoparticles into larger Pd particles was observed under pH 4.0 conditions, which may have different properties than biogenic nanoparticles attached to cell surfaces. *O. alaskensis* G20 appears to be tolerant of low pH, whereas metal treatments (Pd $\pm$ Fe/Ni) inhibited all *O. alaskensis* G20 growth on spot plates. Despite this result, *O. alaskensis* G20 can still be used for selective metal recovery, as long-term survival is not required for Pd and NiS nanoparticle formation.

The work described in this chapter is intended to act as a proof-of-concept method of using a known organism to extract metals from mining and industrial waste. However, only a few combinations of metals were tested, only two metals were tested in any given trial, and the results from simulated waste solutions may not necessarily extrapolate to real waste samples. Furthermore, there were several study limitations such as the low numbers of replicate samples and lack of replicated controls that made quantitative analysis difficult, and may have contributed to the observed discrepancies in metal recovery yields when compared to past data. Additional experiments are required to optimize assay conditions and ensure consistent, reproducible results with simulated waste, before testing the feasibility of *O. alaskensis* G20 towards metal recovery from real waste samples.

Although the results of this study are preliminary, organisms such as *Oleidesulfobivrio alaskensis* G20 show excellent potential for synthetic biology approaches aimed at developing new methods of metal recycling, contributing towards the remediation of metal-bearing wastes and a more sustainable resource economy.

Chapter 6

Research summary and applications

In this thesis, I explored the diversity, function, and applied potential of microorganisms inhabiting metal-contaminated environments, spanning natural aquifers affected by anthropogenic activities, sulfidic mine tailings, and experimental systems representing aqueous metal wastes. The findings from the studies in Chapters 2–5 contributed towards a growing body of knowledge surrounding several themes: the high taxonomic and functional diversity of microbial communities, the role of microbes in biogeochemical cycling of metals, and the potential to harness microbe-metal interactions for environmental remediation and sustainable biotechnology.

6.1 Research summary

6.1.1 Microbial diversity in metal-contaminated environments

Microbial communities showed exceptional spatial heterogeneity in arsenic-impacted aquifers and legacy copper/nickel mine tailings. A total of 11,116–253,383 ASVs were identified by 16S rRNA amplicon sequencing in Chapters 2 and 3, many of which represented novel lineages. In both systems, microbial diversity correlated with the concentrations of some metals, such as arsenic (aquifers in Chapter 2) and iron (mine tailings in Chapter 3), but other geochemical gradients such as pH, redox potential, temperature, alkalinity, and sulfate concentrations were predicted to be more influential in shaping the taxonomic composition of communities and abundances of individual populations.

Through my investigations, it is clear that many highly metal-tolerant species exist within the Bacteria and Archaea, and there are likely many more which we have yet to discover. Their environmental distributions are not solely determined by metal concentrations, but by broader geochemical conditions which define ecological niches.

6.1.2 Functional profiling of microbial metal resistance and metal cycling

Metagenomic sequencing was the primary method of functional profiling of MRGs and MMGs within aquifer and tailings communities. Assembly, binning, and sequence annotation of 757–1219 high-quality MAGs anchored the analyses in Chapters 2 (aquifer samples) and 4 (mine tailings). This approach enabled more in-depth predictions of microbial metal resistance and metal cycling in these

environments than 16S rRNA amplicon sequencing alone, especially for unclassified or uncultured populations.

In Chapter 2, I focused on genes relevant to arsenic cycling within groundwater systems impacted by thermally mobilized arsenic. Despite high variability in arsenic concentrations, genes involved in direct arsenic cycling were similar in abundance across sampling locations. Arsenic resistance genes (*arsB*, *acr3*, *arsC*, *arsM*) were encoded by the majority of microorganisms, suggesting that arsenic detoxification mechanisms are widespread in aquifer communities, with a notable exception in one sample that had an unusually abundant population of *Patescibacteria*. Arsenic metabolism genes (*arxA*, *aioA*, *arrA*) were rare, commensurate with the groundwater redox conditions likely being incompatible with these functions. These findings suggest that microbes are mainly interacting with arsenic by reducing intracellular As^{5+} and exporting As^{3+} , increasing its mobility. However, iron- and sulfur-cycling genes were also identified within the community, representing potential indirect mechanisms of arsenic cycling. Novel populations of predicted sulfate reducing bacteria (o. *Thermodesulfobacterales*) in the well with the highest arsenic concentrations could be relevant to controlling arsenic mobility in this system through co-precipitation of As^{3+} minerals with biogenic sulfide (see below).

Functional profiling of copper/nickel mine tailings (Chapter 4) targeted a wider range of MRGs, which I annotated by building custom HMMs from genes in the BacMet database. The abundances of MRGs targeting key contaminants of interest (copper, nickel, iron) did not correlate with respective metal concentrations, indicating that metal resistance is likely regulated at the gene expression level. As seen in the aquifer samples from Chapter 2, arsenic resistance genes were abundant in all samples, along with MRGs targeting other highly toxic metals such as copper and mercury, implicating the ability to detoxify these metals as necessary for survival. MMG annotation revealed many potential iron- and sulfur-cycling populations, which tended to be localized to specific cores or depth ranges. Many of these populations represented taxonomically novel and/or uncultured lineages, including members of the Actinobacteria, Desulfobacterota, *Ca.* Eremiobacterota, and *Ca.* SZUA phyla. Chapter 4 also extended metagenomic insights with the use of metaproteomics. Unfortunately, poor DNA and protein yields and high contamination resulted in poor sequencing depth of coverage for both datasets, preventing a comprehensive examination of all organisms and activities present in these environments. Advancements to DNA and protein extraction methods from low-biomass, metal-rich substrates is required to be able to fully profile the taxonomic and functional

diversity of tailings microbiomes. Nevertheless, key functional proteins related to metal resistance and metal transformations were identified in multiple samples, confirming that MRGs and MMGs are both encoded by tailings communities and actively expressed *in-situ*.

Across both aquifer and tailings environments, MRGs were widespread among microbial communities. The abundance of specific MRGs at the community metagenome level are hard to predict based solely on environmental metal concentrations, and likely depend on additional factors such as the relative toxicity of individual heavy metal ions and the possibility of multiple metal interactions per protein. MMGs (including sulfur-cycling genes) are less ubiquitous, and exhibit both spatial localization (based on redox gradients) and taxonomic specificity, although the exact species predicted to be involved in iron- and sulfur-cycling can differ greatly between environments. Both chapters highlighted the contributions of microbially mediated biogeochemical processes in modulating metal mobility and fate in the environment.

6.1.3 Selective metal recovery using metal cycling microorganisms

Chapter 5 investigated microbe-metal interactions in a controlled laboratory environment using the sulfate reducing bacterium *Oleidesulfovibrio alaskensis* G20. Here, I evaluated the ability of *O. alaskensis* G20 to recover nickel and palladium nanoparticles from mixed-metal wastes. Simulated waste solutions were designed partially based on pore-water metal concentrations from mine tailings (Chapters 3 and 4). Nickel removal from mixed Ni+Fe solutions was demonstrated using *O. alaskensis* G20 growth media to induce metal sulfide precipitation, followed by separation of NiS from FeS at pH 4.0. Palladium was successfully removed from mixed Pd ± Fe/Ni solutions through *O. alaskensis* G20 reduction of Pd²⁺ ions to elemental Pd. Given the different mechanisms utilized for Ni and Pd removal, I hypothesized that Ni and Pd could be selectively recovered from the same mixed-metal waste by separation of cells from sulfide-containing growth media (**Figure 5.7**).

Due to the limited metal combinations tested, and disagreement between some results (e.g., metal removal yields) with previous literature values, more experiments are needed to verify the robustness and reproducibility of the data. Nonetheless, my work demonstrates the potential for organisms like *O. alaskensis* G20 to be used in metal recovery from wastes such as the nickel-containing mine tailings studied in Chapters 3 and 4. Given that *O. alaskensis* G20 is a sulfate-reducing bacterium, it is also possible that some of the novel SRB lineages from the Sudbury mine tailings may have similar metal recovery capabilities.

6.2 Research applications

6.2.1 Remediation of metal-contaminated environments

Studying microbial communities in arsenic-contaminated aquifers and mine tailings not only enhances our understanding of the biogeochemical processes occurring in these systems, but also helps identify candidate organisms as targets for remediation. As discussed throughout Chapters 1–4, microbial metal resistance strategies and metabolic activities can have various direct and indirect effects on metal mobility, and are thus the microbial guilds present are important considerations when designing strategies to mitigate heavy metal toxicity in anthropogenically impacted ecosystems.

Chapter 2's findings suggest that stimulating indigenous sulfate-reducing bacteria could be a viable strategy to immobilize arsenic in aquifers. Previous field studies demonstrated that amending arsenic-contaminated groundwater with ferrous iron, sulfate, and organic C/N/P enabled the co-precipitation of arsenic and ferrous iron, and biogenic sulfides as arsenopyrite (Lee et al., 2019; Saunders et al., 2018). Stimulation of naturally occurring microbial populations can be advantageous, as they are already well-adapted to the given environment, and there is less risk for unintended consequences compared to introducing non-native, cultured consortia. The research presented in this thesis has been expanded by collaborators (Grégoire lab, Carleton University) to further characterize microbial arsenic cycling in synthetic groundwater media simulating aquifer geochemical conditions. Ongoing microcosm experiments using samples directly taken from Chapter 2 study sites aim to establish a definitive link between SRB activity and arsenic cycling, and to determine the optimal amendments to increase arsenic removal rates. These findings may also be informative towards understanding and/or remediating similar systems impacted by thermally mobilized arsenic, such as geothermal waters.

The results of Chapters 3 and 4 can be used to inform decisions surrounding *in-situ* remediation strategies for mine tailings. Geochemical and microbial community variation over vertical gradients and between different tailings impoundments influences the effectiveness of a given treatment (also discussed in section 1.3.2). Depth ranges supporting acidophilic iron- and sulfur-oxidizing guilds should be controlled by preventing oxygen diffusion or encouraging the growth of competitors that do not contribute to AMD formation (*e.g.*, *Bradyrhizobium* spp. with plant symbionts) (Chaintreuil et al., 2007; Maier et al., 1990). As for the arsenic-impacted aquifer environments, biostimulation of native Desulfobacterota and other SRB populations in mine tailings

anoxic zones may offer a sustainable and low-risk method of immobilizing metals via sulfide mineral precipitation, as these lineages are already well-adapted to the local environment.

6.2.2 Development of sustainable biotechnology for metal recovery

In Chapters 2–4, SRBs consistently emerged as key contenders for application towards environmental remediation. Chapter 5 extended this perspective to include the potential for SRBs to catalyze metal recovery in *ex situ* metal waste treatments.

Mining, industrial, and consumer waste can be highly complex and variable in composition. Designing a versatile ‘microbial toolkit’ suitable for biomining requires a detailed knowledge base of the diverse range of microbial processes at our disposal. Many microorganisms currently studied for biomining applications were originally isolated from metal-contaminated environments, including bioleaching chemolithoautotrophs (*e.g.*, *Acidithiobacillus*, *Leptospirillum*, *Sulfolobus*) and metal nanoparticle-forming organisms (*e.g.*, *Shewanella*, *Morganella*, *Cupriavidus*) (Hirth et al., 2024; Punia et al., 2025; Yang et al., 2023; Yasawong et al., 2024). The discovery of novel MRG/MMG-carrying taxa in Chapters 3 and 4 opens opportunities for isolating additional metal-tolerant taxa that may carry out bioleaching or metal recovery with much greater efficiency and yields than existing species and/or strains. In an ideal scenario, bioreactors supporting different consortia could be set up in a way that mimics natural ecosystems, such that nutrients (*e.g.*, C, N, S, Fe) can be efficiently transferred through the system to support the metabolic needs of microbes at each step (see **Figure 6.1** for a conceptual schematic). Additionally, if dissolved metals still remain in the media after the recovery process, it could be recirculated within the system instead of discarded as waste, improving yields (**Figure 6.1**). I predict that future sequencing-based studies of other metal-rich environments, such as landfills, will also be valuable towards the goal of designing robust bioreactor systems supporting a circular economy of metals.

Individual MRGs/MMGs identified in ‘-omics’ studies can be the target of synthetic biology approaches to study these genes *in vitro*, providing a number of additional advantages. It is usually easier to work with existing cultured strains (*e.g.*, *E. coli*, *A. ferrooxidans*, *Desulfovibrio* spp.) than attempting to isolate new organisms from the environment, as we have a stronger understanding of their growth requirements. Cloning gene(s) of interest into plasmids also enables greater control over gene expression. Strains can be engineered for faster growth or to be able to grow on more desirable substrates (Eggington, 2024; Keller et al., 2011). Most importantly, engineered organisms could be

designed to more effectively recover metals from waste by targeting key MRG/MMGs. For example, copper resistance genes expressed by organisms living in copper-rich tailings (Chapter 4) could be cloned into *O. alaskensis* G20 (Chapter 5) to allow for selective Ni/Pd recovery from mixed-metal wastes containing otherwise toxic concentrations of copper. Other MRGs with sequence homology or predicted structural similarity to those involved in *O. alaskensis* G20 PGM transport and/or reduction (Capeness et al., 2019a) could be expressed in a model host to be evaluated for their capacity to induce other types of metal nanoparticle formation. It should be noted that the current version of the BacMet database, which was updated in 2018, does not include resistance genes for PGMs (Pal et al., 2014), and would not be a viable method of annotating these genes. Therefore, future advancements in metal recovery biotechnology will depend on continuing the development and curation of bioinformatic databases and tools, to bridge the gap between the prediction and experimental validation of metal-cycling pathways.

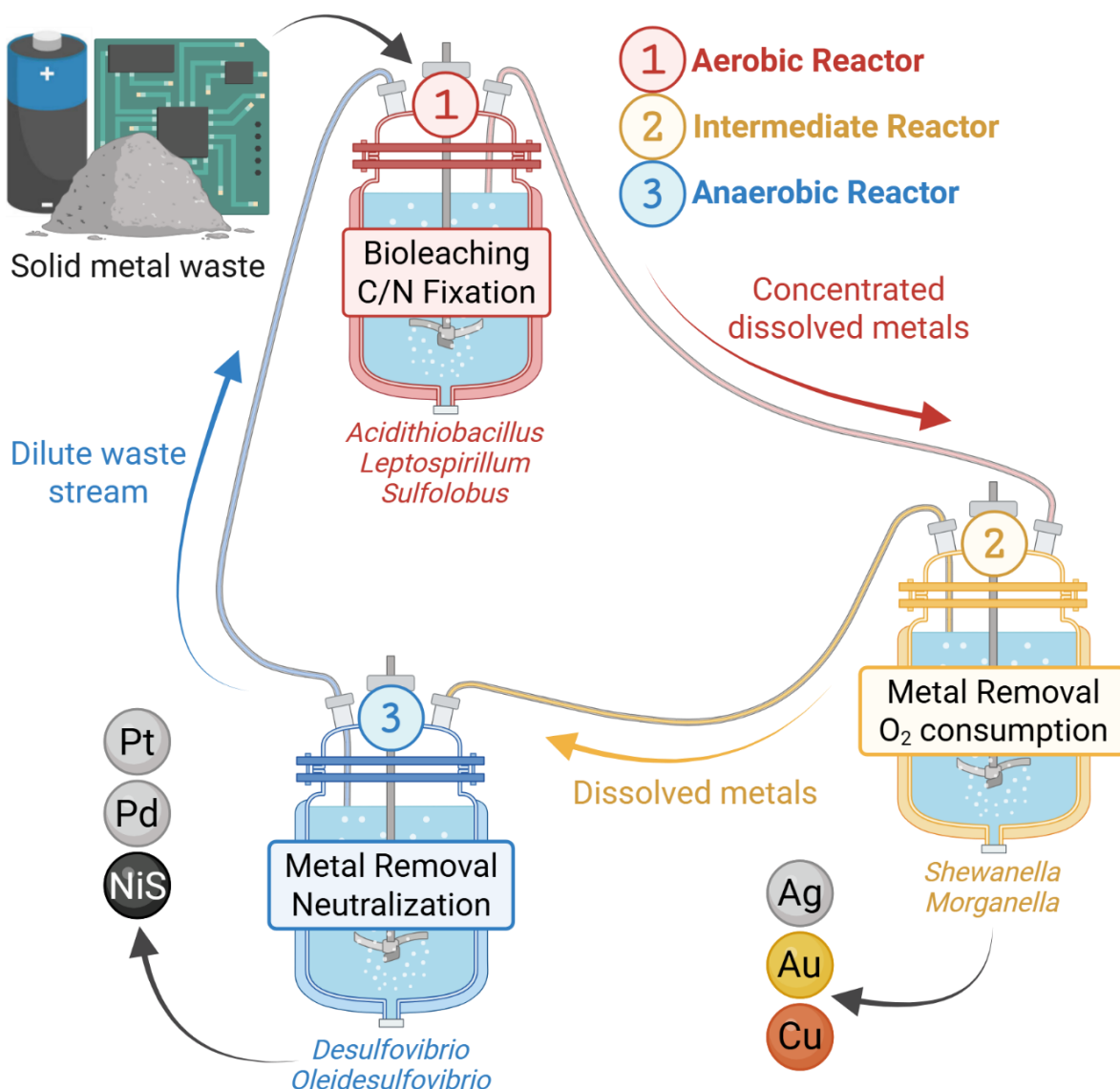


Figure 6.1: Theoretical bioreactor system for recycling of complex metal wastes. Solid wastes are introduced to the aerobic bioreactor (1), where chemolithoautotrophs solubilize metals (bioleaching). Multiple intermediate (2) and/or anaerobic (3) reactors can be incorporated depending on the specific metals targeted, each containing organisms that selectively precipitate metals. Oxygen is consumed in (2) by facultative anaerobes. Organic carbon/nitrogen are produced in (1) and consumed in (2) and (3). SRBs in (3) neutralize acids produced in (1). Under optimal conditions, it is possible to recirculate media with low concentrations of metals (dilute waste stream) from (3) back into (1), allowing for multiple rounds of metal recovery while minimizing waste. Examples of known microbial genera with bioleaching and metal recovery capabilities are listed under each type of reactor. The above schematic is intended as a conceptual illustration of how knowledge of metal cycling organisms from environmental surveys can be applied to metal recovery design, and does not represent a real example of a functional bioreactor system. Image was created with BioRender.com.

6.3 Conclusions

The necessity of implementing more sustainable practices surrounding natural resource extraction and waste management is a key and current challenge. Whether it is thermally mobilized arsenic from in-situ bitumen extraction, or acid mine drainage from oxidized tailings, heavy metal contamination resulting from human activities is a major threat to global ecosystems. Our increased reliance on electronic technologies has created an additional economic incentive to reduce the quantity of metals discarded as waste. This thesis demonstrates the value of environmental microbiological approaches for addressing these pressing challenges. By integrating 16S rRNA amplicon and ‘-omics’ sequencing data, I identified many taxonomically and functionally diverse groups of microorganisms predicted to have roles in metal biogeochemical cycling as targets for additional study. I also demonstrated an experimental approach that could be used to bridge environmental surveys with lab studies, to evaluate organisms of interest for remediating and extracting metals from waste sources.

Metal contaminated environments are reservoirs of unexplored diversity. It is without a doubt that the continued study of these habitats will yield further insights into the various ways that microorganisms have adapted to survive and thrive in these conditions. Only by understanding the complex variables controlling microbe-metal interactions can we make informed decisions regarding the responsible recovery, use, and disposal of metals and other natural resources.

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Appendix A

Chapter 2 Supplementary Materials

Data File A1 (available for download at <https://doi.org/10.1016/j.scitotenv.2025.179357>): **Groundwater analysis report.** Additional information on the laboratory methods and measured variables from groundwater samples analyzed by Brooks Applied Labs (pages 1-2) and Bureau Veritas (pages 3-4).

Data File A2 (available for download at <https://doi.org/10.1016/j.scitotenv.2025.179357>):

Tab 1: All geochemical variables. All measured values, with associated units, of groundwater samples associated with each sampling site.

Tab 2: envfit results. The output generated by environmental fitting (envfit) of geochemical variables to Bray-Curtis, unweighted UniFrac, and weighted UniFrac ordinations. Only variables that were measured in both 2021 and 2022, with values above the detection limit in all wells, were included. Significant correlations are indicated with asterisks (see Signif. codes). Note that PC1 and PC2 values do not necessarily represent the coordinates of loading vectors displayed on **Figure 5**, as vectors were scaled to the PCoA axes ranges prior to plotting.

Tab 3: Metagenome-assembled genome (MAG) details. Information on all high-quality MAGs is included. The first four characters of MAG ids (Bin) represent the site (CLA=Site A, CLB=Site B) and location/well of the sample (by number, e.g., CLB2=Site B-02). Completeness and contamination scores were calculated by CheckM. Percent relative abundance is expressed as the average scaffold coverage from Bowtie2 read mapping within a MAG, divided by the sum of all average coverage values across high-quality bins within the sample.

Tab 4: DRAM pathways. The output table generated by DRAM, where metabolic genes are aggregated into pathways for each MAG. Pathways are described with either percentage completion values (0-100%) or as true/false.

Tab 5: CLB7-064 proportionality. Summary of all MAGs with scaffolds showing proportional abundance to CLB7-064 scaffolds. Scaffold (>10 kb) proportionality was calculated using the 'rho' metric in the propr R package, with a cut-off of 0.9 indicating significant proportional abundance. No unbinned scaffolds were significantly proportional to CLB7-064. Only one MAG (indicated with an asterisk) also showed significant proportionality to CLB7-064 when comparing MAG relative abundances instead of individual scaffolds. The % of genome proportional column identifies the percent of the total genome length made up of scaffolds scoring as proportionally abundant.

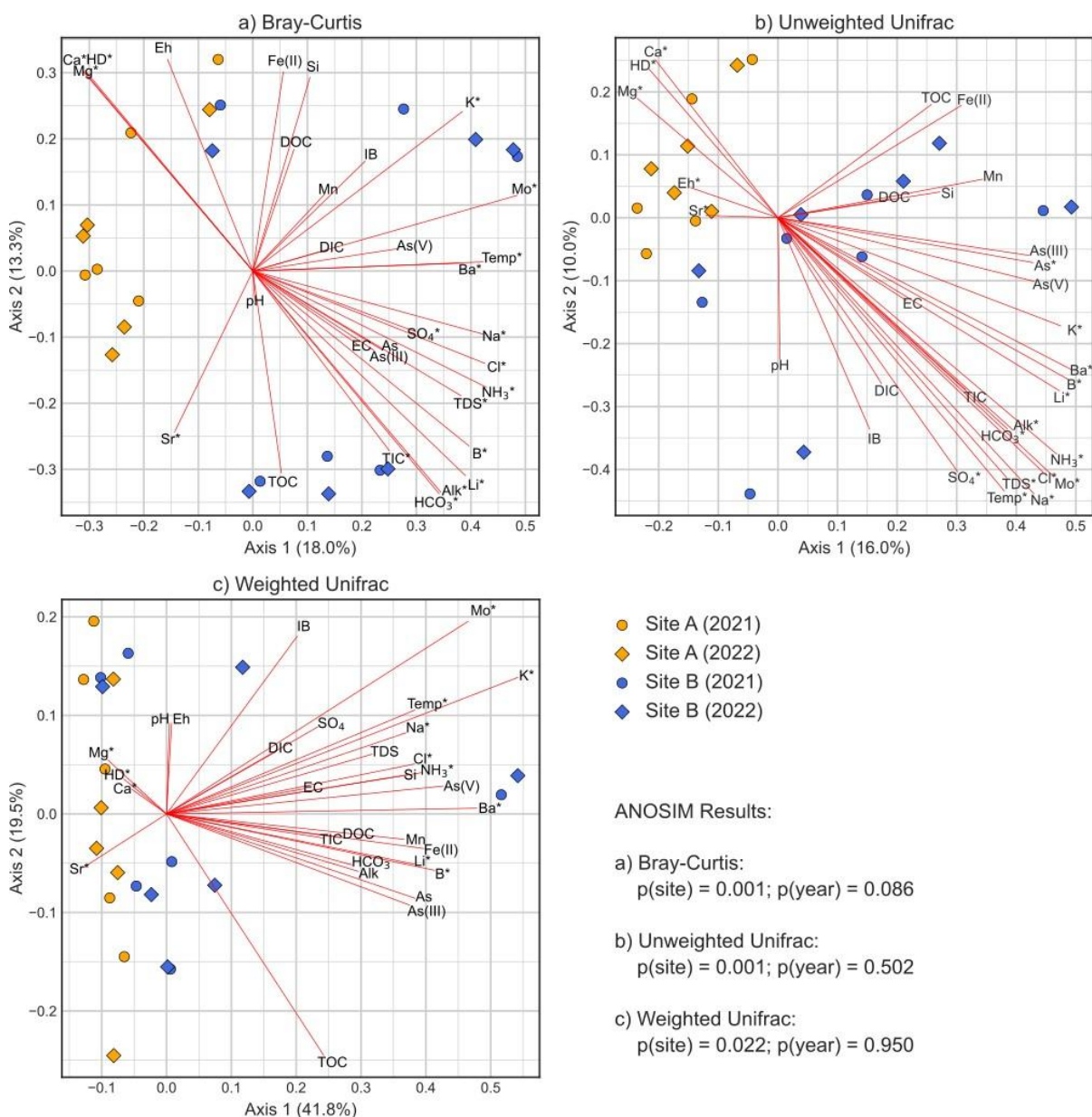


Figure A1: Principal coordinates analysis (PCoA) plots of Bray-Curtis (a), unweighted UniFrac (b), and weighted UniFrac (c) distance matrices. Points represent samples, which are colored by site (aquifer), with samples taken in different years indicated by shape. Significant differences ($p < 0.05$) were found between Sites A and B, but not between 2021 and 2022 samples (ANOSIM). Geochemical variables are projected on each ordination as loading vectors, with significant variables ($p < 0.05$, see **Table 2.1**) indicated with an asterisk. Abbreviation key: EC=Electrical Conductivity, Temp=Temperature, As=Total Arsenic, DIC=Dissolved Inorganic Carbon, DOC=Dissolved Organic Carbon, HD=Hardness, IB=Ion Balance, Alk=Alkalinity, TDS=Total Dissolved Solids, TIC=Total Inorganic Carbon, TOC=Total Organic Carbon.

Table A1: Summary of iron-related protein families that are represented as profile HMMs in FeGenie. Adapted from Table 1 in Garber et al., (2020); see the associated publication for more information, including references to publications and databases for protein families.

Function	Protein Families
Iron transport	EfeUOB, FbpABC, SfuABC, YfuABC, FeoAB(C), FutA1, FutA2, FutB, FutC, YfeABCD
Heme oxygenase	ChuS, ChuZ, HemO, PigA, HemRSTUV, HmoB, HmuO, HugZ, HupZ, Isd-LmHde, IsdG, IsdI, MhuD, PhuS (in PhuRSTUVW)
Heme transport	HasRADE(B)F, HmuRSTUV, HmuY, HmuY', HutZ, HxuCBA, IsdX1, IsdX2, PhuRSTUVW, Rv0203
Transferrin/Lactoferrin	TbpAB (LbpAB), SstABCD
Siderophore synthesis	AcsABCDEF, AmoA, AngR, AsbABCDEF, DhbACEBF, entD-fepA-fes-entF-fepECGDB-entCEBA-ybdA, IroD in IroNBCDE, IucABCD,, IutA, MbtIJABCDEFGHI, LbtA (in LbtUABC), PchABCDREFGHI, PvdQAPMNOFEDJIHLGS, PvsABCDE, VenB, Vab genes in VabR-fur-vabGA-fur-VabCEBSFH-fur-fvtA-vabD, Vib genes in VibB-vibEC-vibA-vibH-viuPDGC-vibD and viuAB-vibF, RhbABCDEF-rhrA-rhtA
Siderophore transport	BesA, CbrABCD, TonB-ExbB-ExbD, FatABCD, FecIRABCDE, FeuABC-yusV, FhuACDB, FhuF, FptABCX, FpuAB, FpuC, FpuD, FpvIR-FpvA-FpvGHJKCDEF, FvtA in VabR-fur-vabGA-fur-VabCEBSFH-fur-fvtA-vabD, HatCDB, IroNBCDE, LbtUABC, PirA, PiuA, PvuABCDE, Viu genes in VibB-vibEC-vibA-vibH-viuPDGC-vibD and viuAB-vibF, YfiZ-yfhA, YfiY, YqjH, ybdA and Fep genes in entD-fepA-fes-entF-fepECGDB-entCEBA-ybdA
Iron gene regulation	DtxR63, FecR (in FecIRABCDE), FeoC in FeoAB(C), Fur, IdeR, YqjI, RhrA in RhbABCDEF-rhrA-rhtA
Iron oxidation	Cyc1, Cyc2 (cluster 1), Cyc2 (cluster 2), FoxABC, FoxEYZ, Sulfocyanin, PioABC
Iron reduction	CymA, MtrCAB, OmcF, OmcS, OmcZ, FmnA-dmkA-fmnB-pplA-ndh2-eetAB-dmkB, DFE 0448-0451, DFE 0461-0465
Possible iron oxidation /reduction	MtoAB, Cyc2 (cluster 3)
Probable iron reduction	MtrCB, MtrAB, MtoAB-MtrC
Iron storage	Bfr, DpsA, Ftn
Magnetosome formation	MamABEKLMOPI (Note: These genes are found in all known magnetotactic microorganisms, except for <i>mamL</i> which is found in magnetite-producing magnetotactic microorganisms)

Appendix B

Chapter 3 Supplementary Materials

Data File B1 (available for download at <https://doi.org/10.1128/aem.00143-24>):

Geochemistry measurements used for NMDS environmental fitting. Data was measured from pore-water samples. Only factors which had measured values above the detection limit in >50% of samples in each location were included (with the exception of dissolved organic carbon (DOC) and NO₃, as they were expected to correlate with microbial diversity). Raw and adjusted (Bonferroni correction) p-values of environmental fitting vectors are shown in the bottom 2 rows; significant adjusted p-values (<0.05) are shaded in grey.

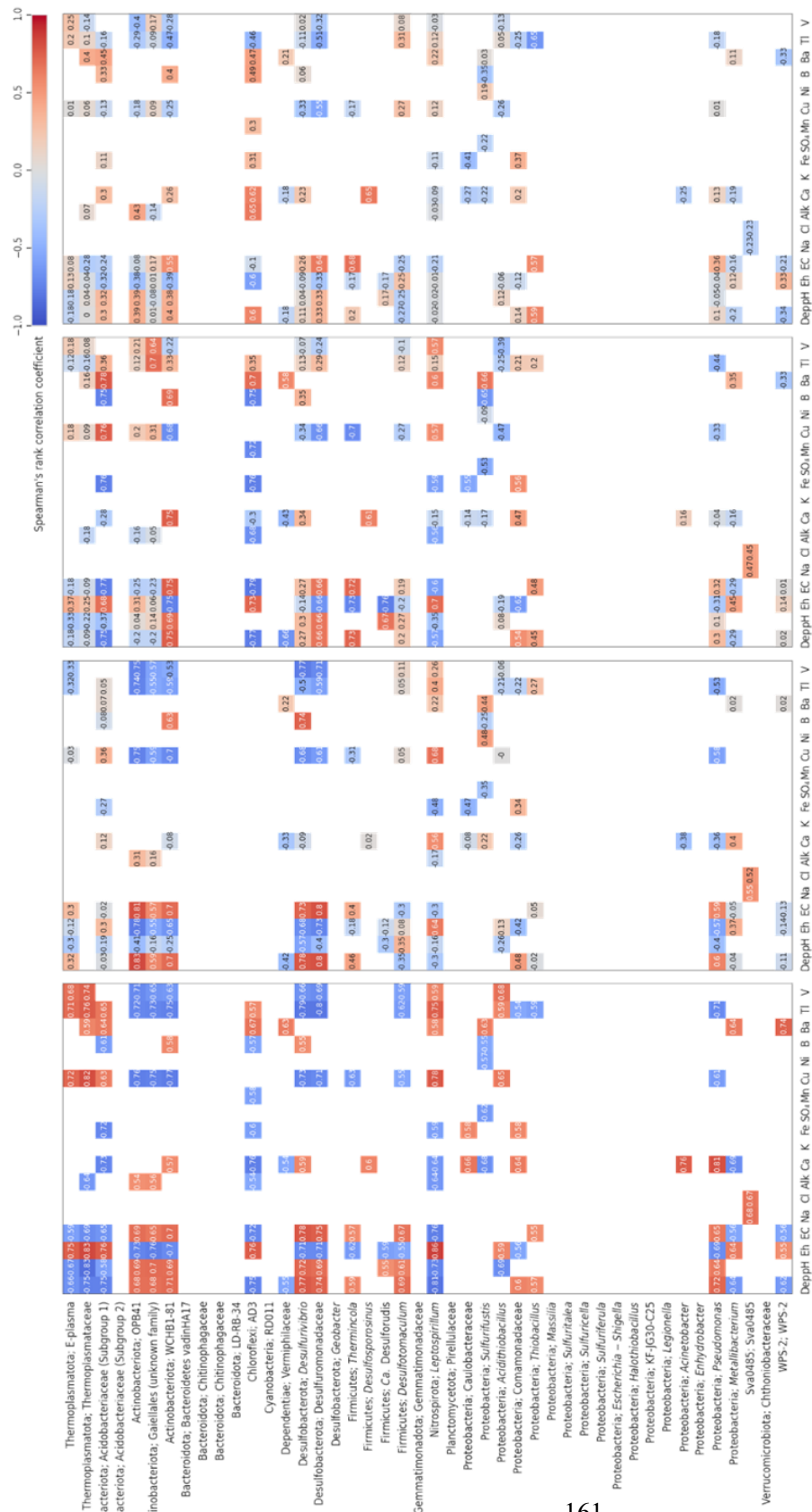


Figure B1: Correlation matrices of genus-level relative abundance and geochemical factors (Dep = depth, EC = electrical conductivity, Alk=Alkalinity), subset by location (left to right: ML25, ML34, NR18, NR3). Spearman's rank correlation coefficients were calculated between the relative abundance of the genera present at >15% relative abundance in at least one sample (profiled in Figure 3) and select geochemical factors at each sampling location. Significant correlations ($p < 0.05$, Bonferroni corrected) are shown

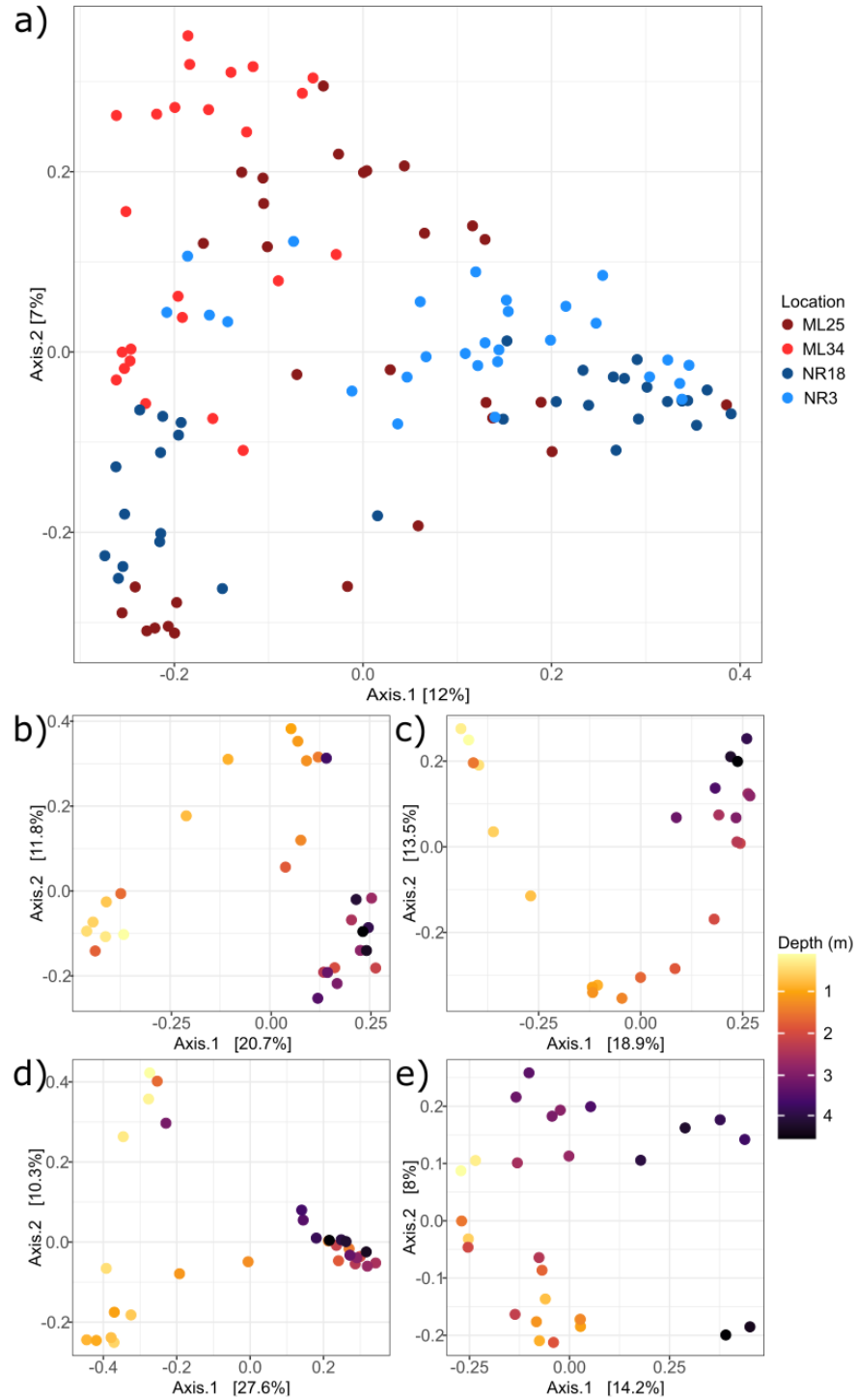


Figure B2: Unweighted UniFrac PCoA plot for all sampling locations combined (a), ML25 (b), ML34 (c), NR18 (d), and NR3 (e). Samples in plots b-e are colored by depth.

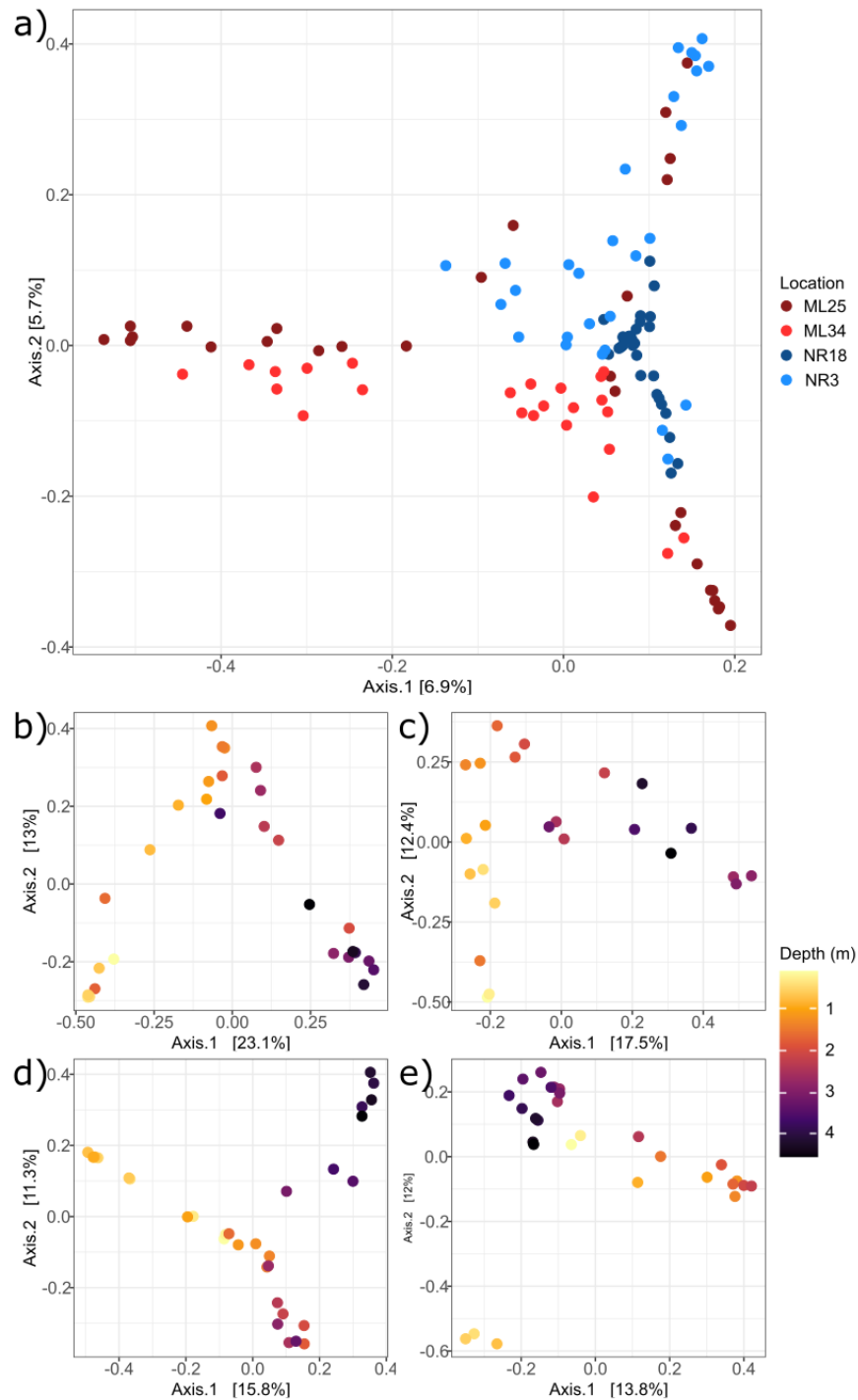


Figure B3: Bray-Curtis PCoA plot for all sampling locations combined (a), ML25 (b), ML34 (c), NR18 (d), and NR3 (e). Samples in plots b-e are colored by depth.

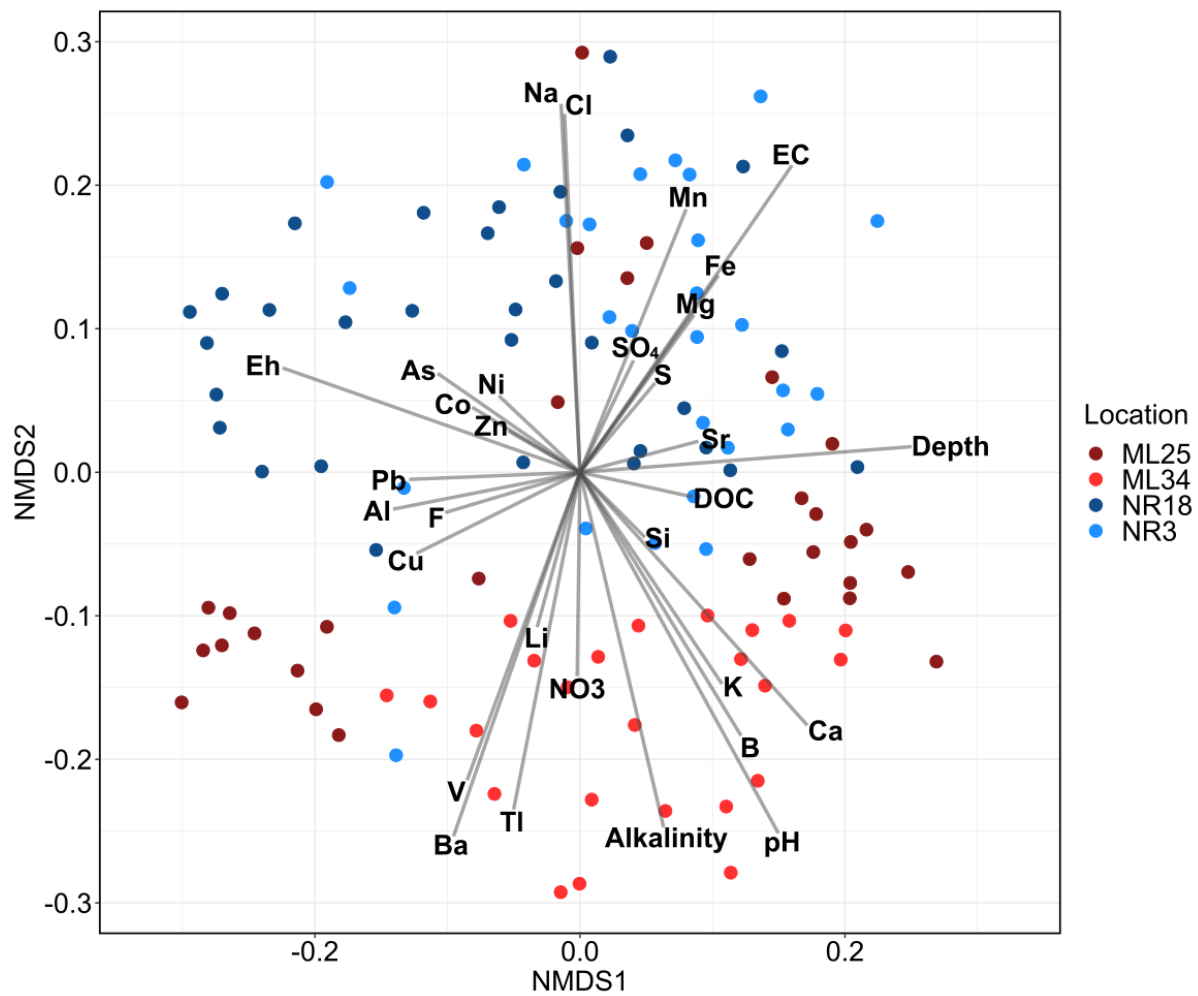


Figure B4: NMDS of Bray-Curtis distances and environmental variables. All measured variables (with detectable values in >50% of samples, except for DOC and NO₃) are included. For raw data and p-values associated with geochemical variables, see **Data File B1**.

Appendix C

Chapter 4 Supplementary Materials

Table C1: Comparison of BacMet-scan method to custom HMMs for annotation of MRGs in protein sequences from MAGs. The number and percentage of hits returned using each method are grouped based on metal. The second last row (Total) represents the sum of all MRG hits across all metals, which may include multiple hits for the same gene if it maps to multiple categories. The Total (non-redundant, including non-metals) row represents the number of unique hits returned by either method, although this may still include multiple hits for the same query sequence to more than one unique gene in the BacMet database. Note that rare genes (<5 representative sequences in the BacMet database) were not used for the HMM method; instead, the BacMet-scan results for those genes (10,462 total, included in BacMet-scan totals in this table) were added to the HMM hits for data analysis.

Metal	BacMet-scan		BacMet HMMs	
	Hits	% of Total	Hits	% of Total
Antimony (Sb)	7,687	2.18	26,031	1.69
Arsenic (As)	38,391	10.91	101,861	6.60
Bismuth (Bi)	841	0.24	2,297	0.15
Cadmium (Cd)	16,476	4.68	105,780	6.85
Chromium (Cr)	13,044	3.71	8,390	0.54
Cobalt (Co)	25,706	7.31	83,352	5.40
Copper (Cu)	45,067	12.81	209,372	13.56
Gallium (Ga)	6,856	1.95	25,150	1.63
Gold (Au)	911	0.26	10,951	0.71
Iron (Fe)	19,526	5.55	179,110	11.60
Lead (Pb)	8,084	2.30	23,364	1.51
Magnesium (Mg)	6,538	1.86	8,552	0.55
Manganese (Mn)	9,140	2.60	75,419	4.89
Mercury (Hg)	19,350	5.50	66,106	4.28
Molybdenum (Mo)	17,462	4.96	59,265	3.84
Nickel (Ni)	30,703	8.73	117,354	7.60
Selenium (Se)	6,973	1.98	3,995	0.26
Silver (Ag)	6,576	1.87	81,469	5.28
Tellurium (Te)	10,962	3.12	7,355	0.48
Tungsten (W)	27,111	7.71	114,477	7.42
Vanadium (V)	968	0.28	3,080	0.20
Zinc (Zn)	33,436	9.50	230,821	14.95
Total	351,808		1,543,551	
Total (non-redundant, including non-metals)	329,520		1,660,455	

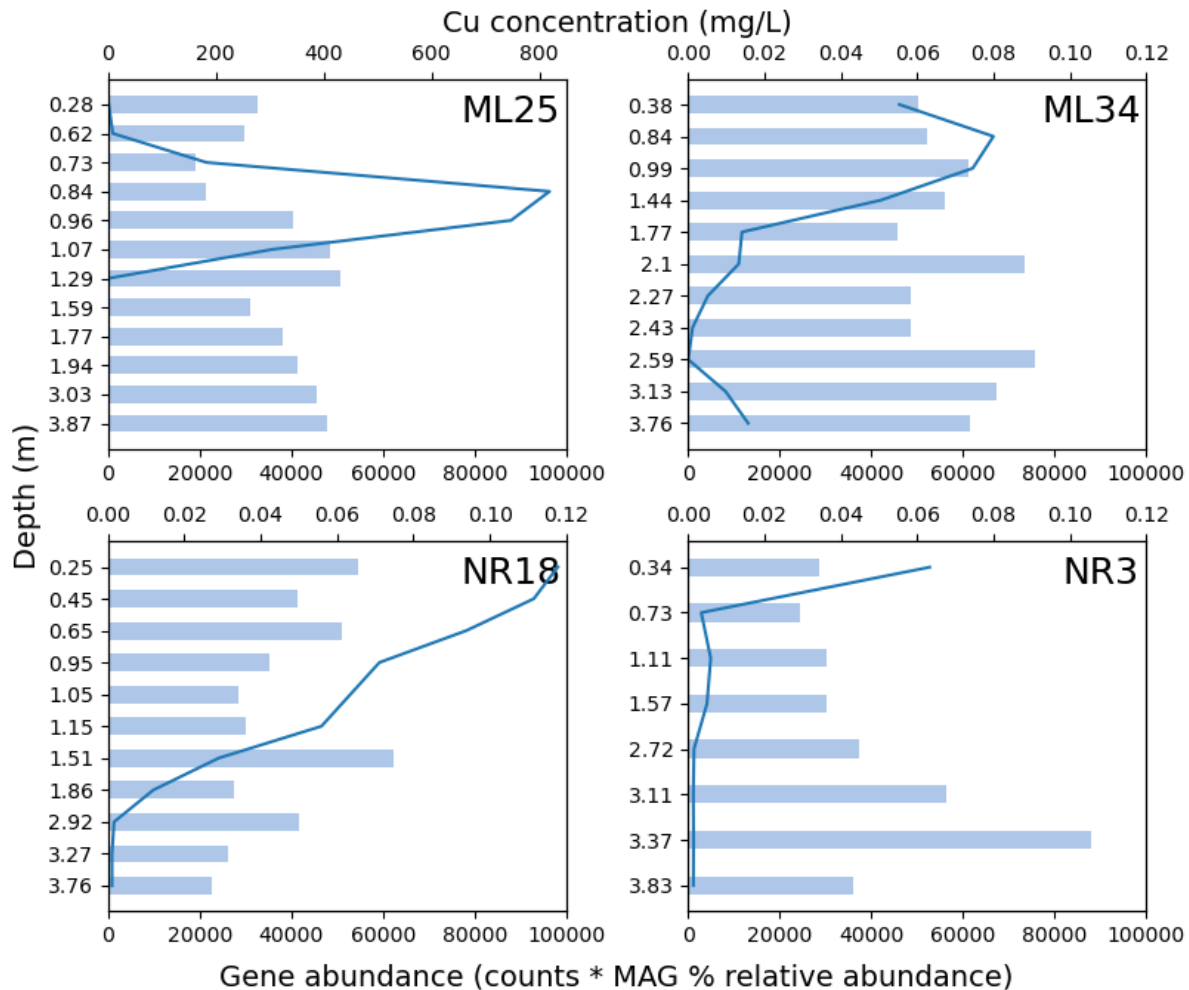


Figure C1: Abundance of copper resistance genes and copper concentrations along four vertical mine tailings cores. Bar plots (lower x-axis values) represent relative gene abundances for Cu-associated MRGs. Line plots (upper x-axis values) represent Cu concentrations in tailings pore-water. Metal concentrations in ML25 are reported on a different scale for visibility. There was no significant correlation between metal concentrations and MRG abundances across all samples ($p=0.18$) or across samples within each tailings core ($0.15 \leq p \leq 0.51$). Note that the y axes are not scaled proportionally to depth.

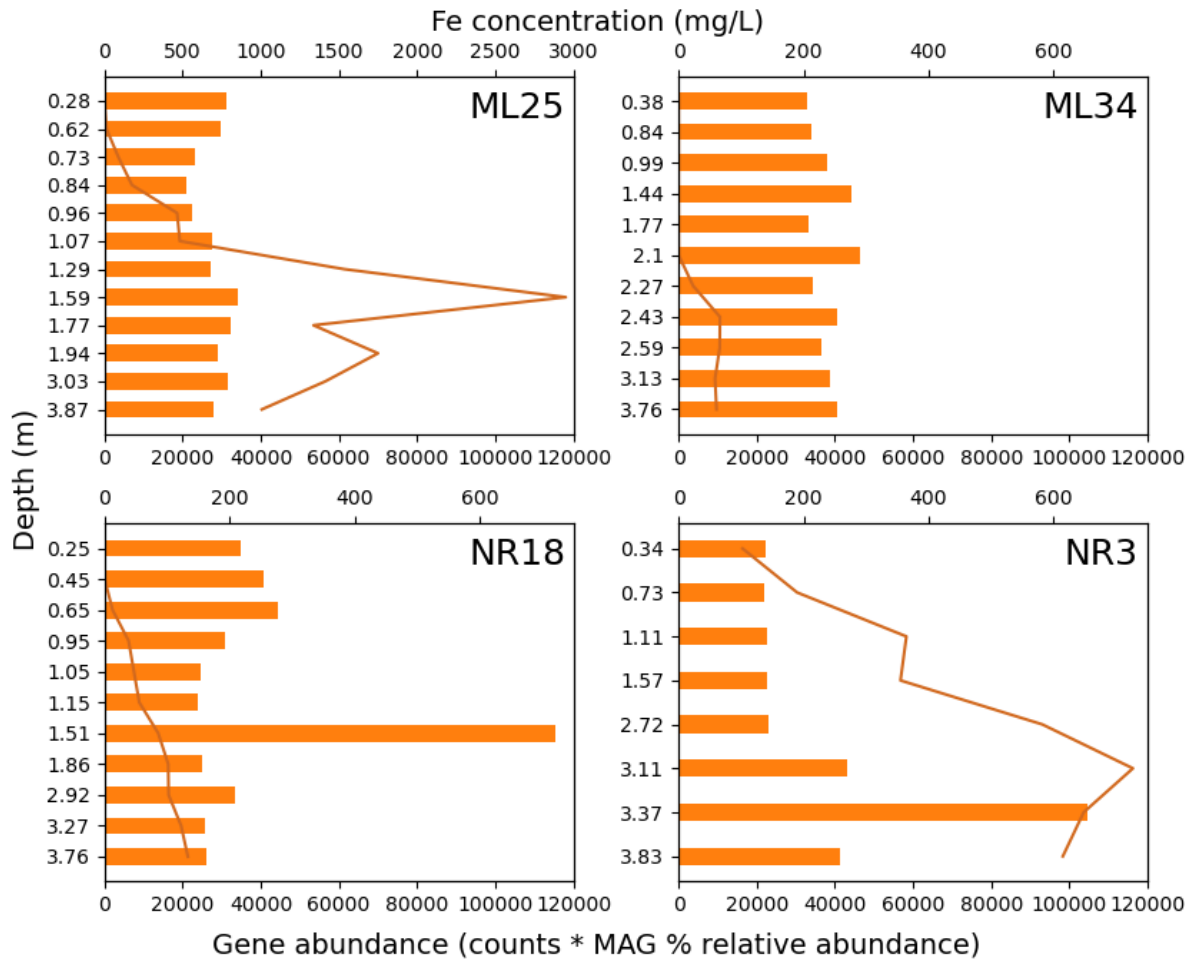


Figure C2: Abundance of iron resistance genes and iron concentrations along four vertical mine tailings cores. Bar plots (lower x-axis values) represent relative gene abundances for Fe-associated MRGs. Line plots (upper x-axis values) represent Fe concentrations in tailings pore-water. Metal concentrations in ML25 are reported on a different scale for visibility. There was no significant correlation between metal concentrations and MRG abundances across all samples ($p=0.79$) or across samples within each tailings core ($0.05 \leq p \leq 0.95$). Note that the y axes are not scaled proportionally to depth.

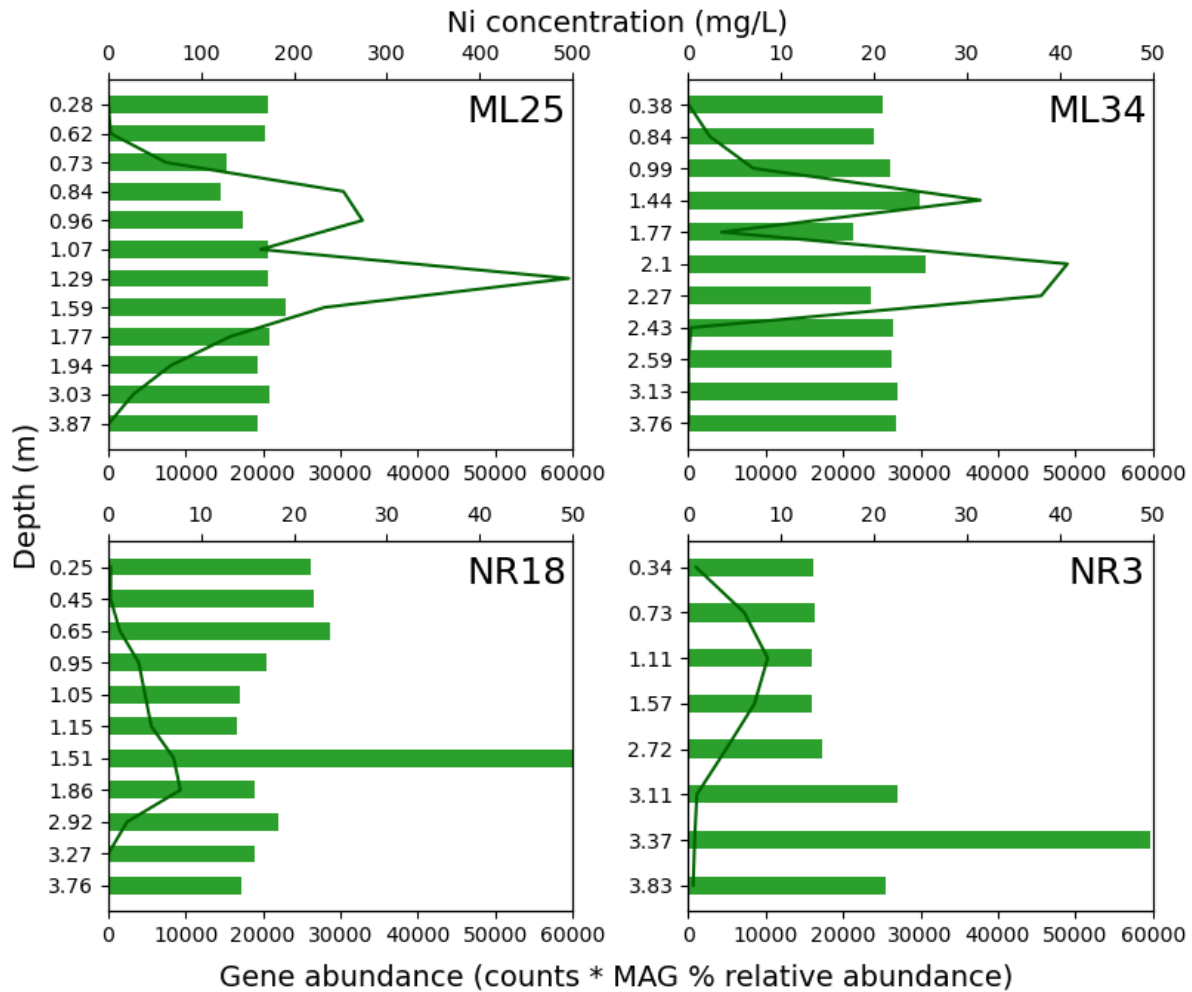


Figure C3: Abundance of nickel resistance genes and nickel concentrations along four vertical mine tailings cores. Bar plots (lower x-axis values) represent relative gene abundances for Ni-associated MRGs. Line plots (upper x-axis values) represent Ni concentrations in tailings pore-water. Metal concentrations in ML25 are reported on a different scale for visibility. There was no significant correlation between metal concentrations and MRG abundances across all samples ($p=0.26$) or across samples within each tailings core ($0.17 \leq p \leq 0.92$). Note that the y axes are not scaled proportionally to depth.

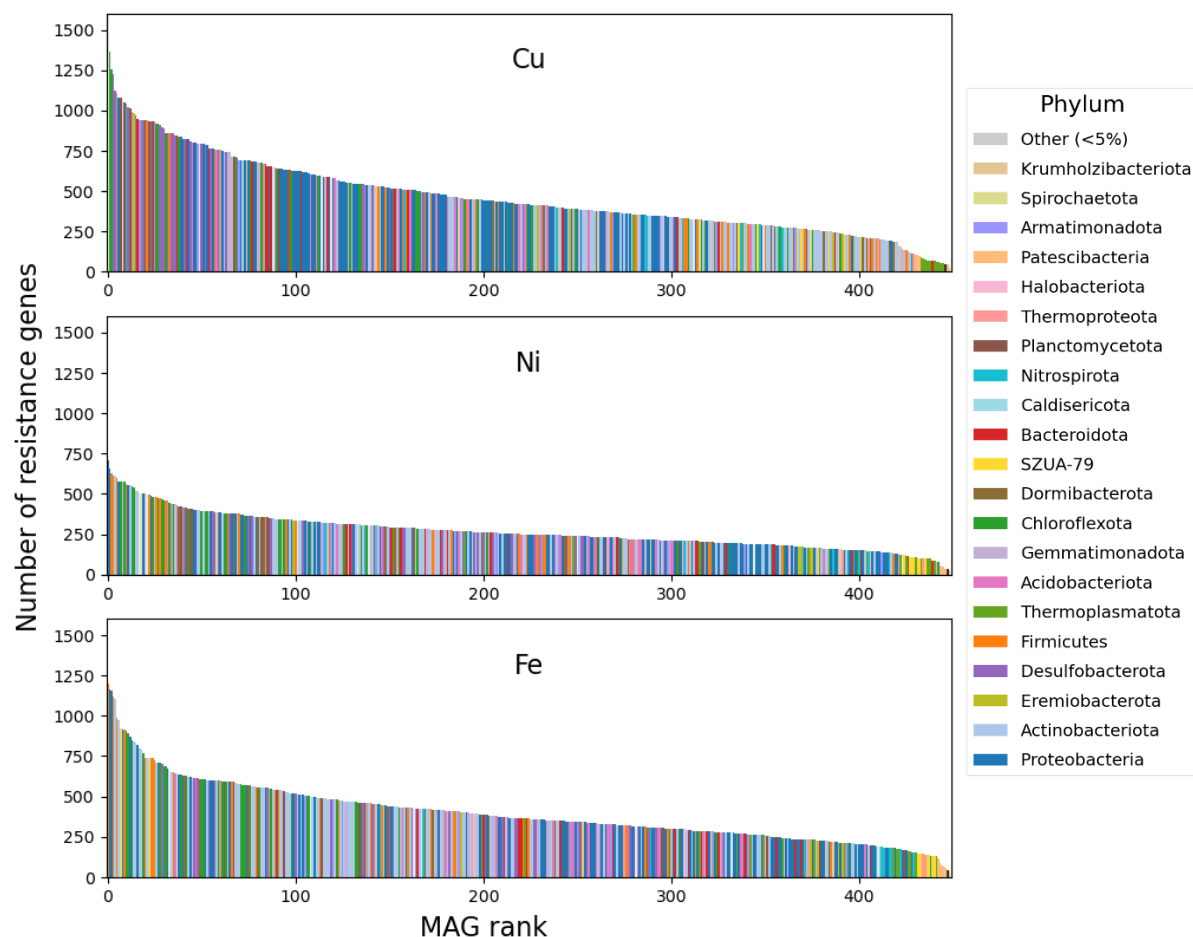


Figure C4: MAG rank abundance plot by metal resistance genes. Bars representing all non-redundant MAGs across all tailings samples are ordered from highest to lowest by counts of predicted MRGs targeting copper (top), nickel (middle), and iron (bottom). MRGs were annotated using profile HMMs based on the BacMet resistance gene database. Bars indicate the total number of MRGs encoded on the MAG, and are colored by the corresponding phylum (GTDB) of each MAG.

Appendix D

Chapter 5 Supplementary Materials

Table D1: Recovery of nickel and iron by *Desulfovibrio desulfuricans* N1. The amount of metal removed from solution, measured by ICP-OES, is reported in mg/L and as a percentage of the initial amount added to N1 growth media. Results are from a preliminary experiment with only one sample per treatment (*i.e.*, no replicates).

Treatment	Ni removed (mg/L)	Ni removed (%)	Fe removed (mg/L)	Fe removed (%)
10 mM NiCl ₂	1.18	35.13	n/a	n/a
80 mM FeSO ₄	n/a	n/a	2.7	20.87
10 mM NiCl ₂ + 80 mM FeSO ₄	0.95	25.01	2.01	17.29

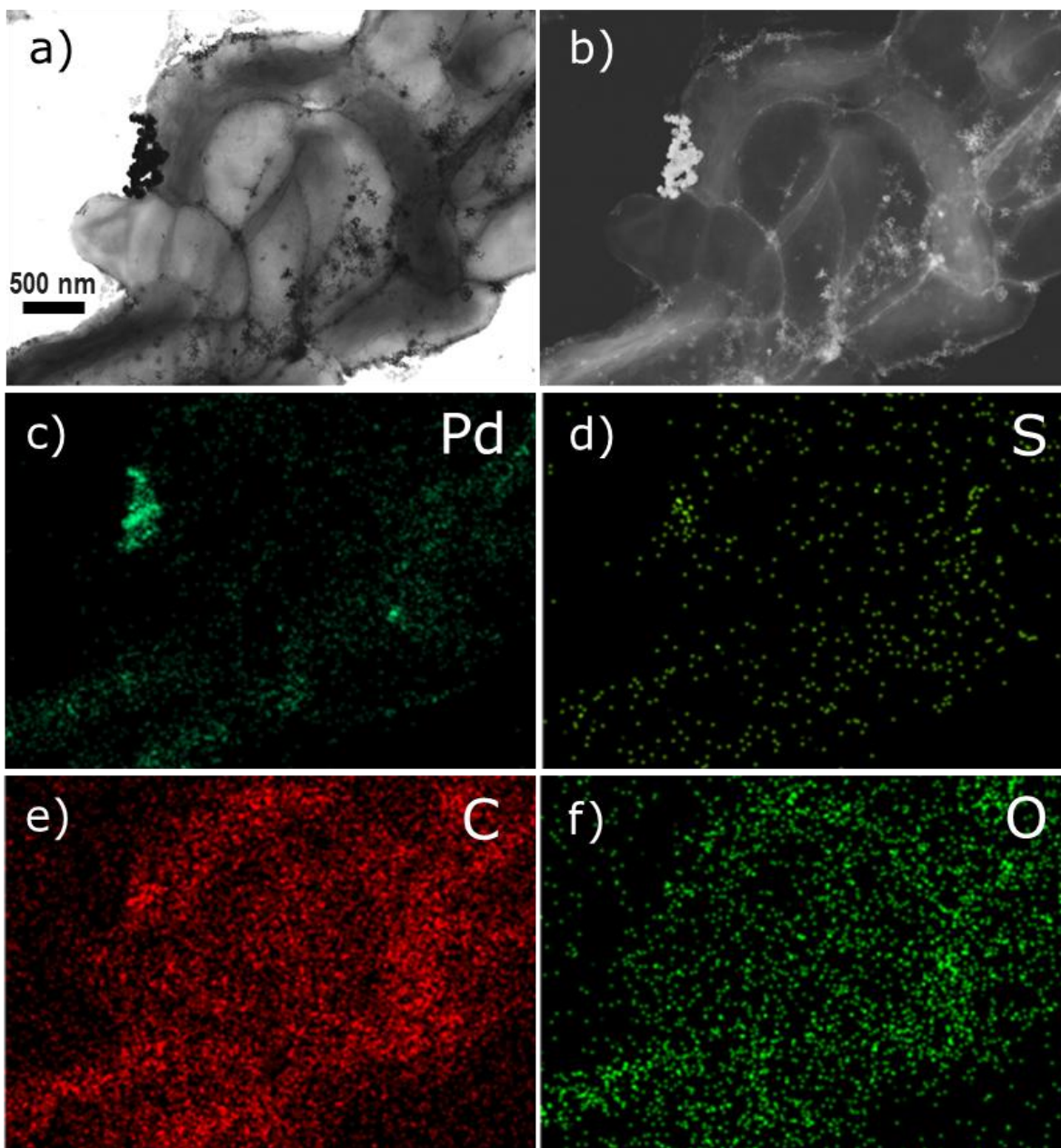


Figure D1: STEM micrographs of Pd nanoparticles (pH 7.0), with EDS mapping of elemental composition. Images in brightfield (a) and darkfield (b) STEM show a *O. alaskensis* G20 cell with attached nanoparticles (<100 nm), as well as larger particles (>100 nm) forming aggregates. EDS elemental mapping of the same region show that the particles are enriched in Pd (c) compared to S (d), suggesting that particles are comprised of elemental Pd. Maps of C (e) and O (f) are included for comparison.