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THE IMPACTS OF HISTORICAL COPPER MINING ON BACTERIAL AND FUNGAL LEAF LITTER COMMUNITIES IN STREAMS

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L'IMPACT D'ANCIENNCES MINES DE CUIVRE SUR LES COMMUNAUTÉS BACTÉRIENNES ET FONGIQUES DE DÉCOMPOSEURS DANS LES RUISSEAUX

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LIST OF ABBREVIATIONS

ASV	Amplicon sequence variant
BP	Bacterial production
Chla	Chlorophyll *a*
DGGE	Denaturing gradient gel electrophoresis
DO	Dissolved oxygen
DOC	Dissolved organic carbon
NGS	Next-generation sequencing
OTU	Operational taxonomic unit
PCoA	Principal coordinates analysis
SPC	Specific conductivity
TDS	Total dissolved solids
TN	Total nitrogen
TP	Total phosphorus

RÉSUMÉ

Les communautés microbiennes jouent un rôle essentiel dans la décomposition de la matière organique et le cycle des nutriments dans les cours d'eau. Si la décomposition dans les cours d'eau est altérée par l'activité anthropique, la capacité de ces écosystèmes à soutenir des réseaux alimentaires complexes sera réduite. Malgré la fréquence des mines abandonnées à travers les paysages, notre compréhension des impacts des anciennes mines de cuivre sur les communautés de micro-organismes décomposeurs dans les cours d'eau douce reste limitée. Notre objectif était de comprendre les impacts actuels des anciennes mines de cuivre sur les communautés de décomposeurs microbiens dans les cours d'eau douce et de mieux comprendre le rétablissement à long terme des écosystèmes lotiques après avoir été contaminés par des métaux lourds. Nous avons échantillonné les communautés microbiennes en immergeant des sacs de litière de feuilles dans trois cours d'eau touchés par l'exploitation minière et deux cours d'eau de référence au Canada. La composition et la diversité des communautés bactériennes et fongiques ont été évaluées en utilisant le séquençage de nouvelle génération des amplicons des gènes 16S rRNA et ITS. Nous avons constaté des changements dans la structure de la communauté bactérienne qui indiquent les effets négatifs potentiels des métaux lourds sur certaines classes, telles que *Deltaproteobacteria*, *Acidimicriobiia*, *Sphingobacteriia* et *Solibacteres*. Nous avons également constaté que les taxons *Chloroflexi*, *Deinococci* et *Saccharibacteria* répondaient positivement à des concentrations plus élevées de métaux lourds. À l'inverse, les communautés fongiques, ainsi que la diversité alpha bactérienne et fongique, n'ont pas été affectées par l'exploitation minière historique. Dans l'ensemble, notre étude a identifié des associations entre de nombreux taxons bactériens à différents niveaux phylogénétiques et la contamination des cours d'eau par l'exploitation minière historique. Ces groupes sont des indicateurs potentiels de l'activité minière et pourraient servir à améliorer les efforts de restauration et de surveillance des mines, mais d'autres études seront nécessaires pour mieux comprendre leurs adaptations génomiques et leur comportement écologique.

Mots-clés : Bactérie, champignon, communauté microbienne, litière de feuilles, écologie aquatique, écosystème d'eau douce, ruisseau, metabarcodage, ADN environmental, métaux lourds, ancienne mine de cuivre.

ABSTRACT

Microbial communities play an essential role in the early stages of decomposition of organic matter and nutrient cycling in streams. If decomposition in streams is impaired by anthropogenic activity, the ability of these ecosystems to support complex food webs is reduced. Despite the prevalence of abandoned mines across landscapes, our understanding of the impacts of historical contamination from copper mines on microbial decomposer communities in freshwater streams remains limited. Our goal was to understand the ongoing impacts of copper mining on microbial decomposer communities in freshwater streams and gain insight into the long-term re-establishment of stream ecosystem function following metal stress. We sampled microbial communities by submerging leaf litter bags in three mining-impacted streams and two reference streams in Canada. Bacterial and fungal community composition and diversity were assessed using next-generation sequencing of 16S rRNA and ITS gene amplicons. We found changes in bacterial community structure that indicated the potential negative effects of heavy metals on specific classes, such as *Deltaproteobacteria, Acidimicriobiia, Sphingobacteriia* and *Solibacteres.* We also found that *Chloroflexi*, *Deinococci* and *Saccharibacteria* responded positively to higher concentrations of heavy metals. Conversely, fungal communities were not impacted by historical mining, nor was bacterial and fungal alpha diversity. Overall, our study identified associations between numerous bacterial taxa at different phylogenetic levels and mining-impacted conditions in streams. These groups are potential indicators of mining activity and could be used to improve mine restoration and monitoring efforts, but further studies will be needed to better understand their genomic adaptations and ecological behaviour.

Keywords : Bacteria, fungi, microbial community, leaf litter, aquatic ecology, freshwater ecosystem, stream, metabarcoding, environmental DNA, heavy metals, historical copper mining.

INTRODUCTION

Freshwater ecosystems are exposed to multiple stressors from anthropogenic activity which threaten freshwater biodiversity and complex food chains (Tolkkinen, M. J. *et al.*, 2020). Among anthropogenic disturbances, metal mining has left a legacy of effects on aquatic landscapes (Ferreira *et al.*, 2016). Our understanding of the impact of historical metal mining on freshwater microbial community structure and diversity is still lacking (Zinger *et al.*, 2012), especially in the case of leaf litter microbial communities. Microbial communities in streams are key elements in sustaining a variety of ecosystem functions, such as nutrient cycling and decomposition of organic matter (Marks, 2019). Recent advances in metabarcoding technology have made it possible to identify changes in microbial community composition with greater taxonomic resolution (Seena *et al.*, 2010). This thesis will address the legacy effects of historical metal mining pollution on microbial diversity and composition in freshwater temperate streams. More specifically, I will focus on the diversity and composition of bacterial and fungal communities associated with the decomposition of leaf litter in streams.

0.1 The essential roles of bacteria and fungi in streams

Freshwater streams provide valuable ecosystem functions that benefit humans, such as water filtration and flood control (Yeakley *et al.*, 2016), and act as important sources of dissolved nutrients, carbon and particulate organic matter for rivers and lakes (Leff, 2019). They also contribute to regional species pools and serve as corridors that facilitate the movement of terrestrial and aquatic taxa between sites (Tolkkinen, M. J. *et al.*, 2020). Stream dynamics are influenced by many different environmental drivers and temporal changes, such as light availability, temperature and seasonal leaf input (Leff, 2019). Because of their water flow, streams are dominated by benthic organisms and cannot sustain the same planktonic food webs that are found in lakes or large rivers (Leff, 2019). Unlike lacustrine ecosystems, which can count on the autochthonous production of carbon from algae and macrophytes, the main source of energy in forested streams comes from allochthonous inputs (Wallace *et al.*, 1997). In deciduous forest streams, this input largely consists of leaf litter fallen during autumn (Abelho and Graça, 1998). This organic matter can be sequestered in stream sediments, transformed into CO_2 by microbial respiration or transferred up food webs all the way to reptiles, birds and mammals in the surrounding landscape (Marks, 2019).

Macroinvertebrates and microorganisms in streams, mainly bacteria and fungi, play a key role in the decomposition of dead organic matter for this vital energy flux to be possible. Macroinvertebrates consume larger particles of organic matter, such as leaves, and break them down into smaller particles that are consumed by microbes (Marks, 2019). Macroinvertebrates have been shown to prefer litter than has been colonized or conditioned by microbes (Suberkropp and Klug, 1980). Microbes produce extracellular enzymes that degrade plant cell walls, break down leaf litter into smaller particles, and mineralize leachate and fine particulate organic matter (FPOM) (Abelho, 2001). As microbes colonize the detritus in streams, they also become an important source of organic matter to macroinvertebrates, despite the existence of microbial taxa that produce defensive compounds to make themselves less palatable (Marks, 2019). Thus, microorganisms recycle nutrients, such as carbon and nitrogen, through the ecosystem and make them available to other levels of the food chain. Changing a link at the base of a food chain can have consequences on the energy flow of an ecosystem. If decomposition in a stream is impaired, the ecosystem's capacity to support complex food chains will be reduced (Hogsden and Harding, 2013). Microbial processes are therefore an important driver of diversity and function of higher organisms in streams (Tolkkinen, M. J. *et al.*, 2020).

0.2 Metabarcoding as a tool to identify microbial community composition and diversity

Before the emergence of molecular identification, microbial diversity was studied through microscopy and culture. However, only a narrow percentage of bacteria are adapted for growth in artificial media, leading to a major discrepancy between the number of cells counted under the microscope and those *in vitro* (Zinger *et al.*, 2012). Likewise, fungal diversity was assessed through a species' conidia development and morphology. Their identification was limited by our ability to isolate pure cultures and produce reproductive structures (Duarte *et al.*, 2013).

The development of molecular identification overcame many of the shortcomings associated with previous methods of microbial and fungal identification (Seena *et al.*, 2010). Molecular fingerprinting methods such as denaturing gradient gel electrophoresis (DGGE) are still in use today, but are increasingly being used in combination with or are being replaced by sequence-based identification techniques. Sequencing techniques have been refined over the last decade, from Sanger sequencing to high-throughput next-generation sequencing (NGS), such as

454 pyrosequencing and Illumina sequencing. Sanger sequencing produces sequence lengths between 300 and 1000 bp but is limited to only one sequence per sample. Identifying all the species in an environmental sample involves cloning, which is time-consuming and not feasible for large numbers of samples or species (Ansorge, 2009). Next-generation sequencing has shorter read lengths (35–700 bp) and higher error rates than Sanger sequencing, but it can sequence multiple samples at once in a shorter time and at lower costs (Goodwin *et al.*, 2016). These advances have led to the widespread use of NGS platforms and ensuing progress in large-scale ecological studies using environmental samples (Ansorge, 2009).

An environmental sample is a mix of organic and inorganic matter that can include microorganisms and traces of macroorganisms, such as their skin, secretions or eggs (Valentini *et al.*, 2009). Environmental DNA (eDNA) can assess the genetic diversity present in these samples (Ruppert *et al.*, 2019). It can be used to analyze diet and identify invasive species, past animal and plant communities as well as microbial communities (Valentini *et al.*, 2009). This assessment is carried out through metabarcoding, a molecular identification technique in which a specific sequence of an organism's DNA is used as a distinguishing marker or barcode. The organisms' taxonomic names are obtained by comparing the resulting sequences to a reference library, such as GenBank, UNITE and SILVA (Gardham *et al.*, 2014).

Various primers and regions can be used to identify microbiota, leading to differences in coverage, resolution and bias between taxa (Ruppert *et al.*, 2019). Although the goal of eDNA studies is often to infer relative abundances of taxa and genes from samples, these values are distorted through protocol-dependent biases (McLaren *et al.*, 2019). This implies that sequencing reads are not necessarily synonymous with biomass. The use of mock communities can help reduce biases and evaluate the sensitivity of a metabarcoding approach. Mock communities contain an isolated mixture of known species whose DNA sequences are already in a database (Duarte *et al.*, 2020). They serve as a control to help predict and correct biases in microbiome studies by calibrating their analyses. Prokaryotic microbes are typically identified using the 16S rRNA gene or the internal transcribed spacer region of rRNA genes as the barcode (Valentini *et al.*, 2009). As for fungi, the internal transcribed spacer region (ITS) of rRNA genes has been agreed on as the conventional distinguishing marker (Seifert, 2009). This region contains two highly variable spacers, ITS1 and

ITS2. Due to the short sequence length produced by NGS, it is not possible to sequence the whole ITS region, and most studies target one of the two spacers (Blaalid *et al.*, 2013).

As a result of modern sequencing and metabarcoding techniques, DNA databases encompass a growing number of sequences that cannot be associated with any functional information or morphology (Grossart *et al.*, 2019). These uncharacterized and uncultured microorganisms are known as microbial or fungal dark matter DNA (Grossart *et al.*, 2019). Because of the gap between dark matter DNA and well-annotated taxa, several studies have reported a great diversity of microorganisms while failing to connect sequences to known species, genera and orders of fungi (Duarte *et al.*, 2013). In comparison with microbial sequences, fungal representation in genomic databases is still poor (Duarte *et al.*, 2013), but the issue will likely resolve itself over time (Ruppert *et al.*, 2019). Despite these drawbacks, DNA metabarcoding remains an optimal tool to characterize communities in large-scale ecological studies (Gardham *et al.*, 2014). For example, in aquatic ecosystems, traditional techniques of biomonitoring with focus on macrobiota, are labor-intensive, may injure individuals and result in low detection during specific time periods or developmental stages (Ruppert *et al.*, 2019). DNA metabarcoding can include meio- and microbiota in biomonitoring practices and improve aquatic biodiversity surveys (Gardham *et al.*, 2014). It can also detect short DNA sequences from extinct communities that have been preserved in sediments, permafrost and ice cores (Ficetola *et al.*, 2008).

Along with improved sequencing methods, bioinformatic pipelines that transform high-throughput sequences into taxonomic units have also evolved considerably. Traditionally, sequences were clustered in operating taxonomic units (OTUs) based on a fixed sequence dissimilarity threshold. This method can be divided into two types of OTUs, *de novo* OTUs and closed-reference OTUs, each with their own limitations. *De novo* OTUs cluster sequencing reads that are similar to each other without using an external reference sequence database. Since their identification relies on their sampled relative abundance within a singular data set, *de novo* OTUs cannot be compared between different data sets (Callahan *et al.*, 2017). On the other hand, closed-reference OTUs are obtained by comparing similar reads to a reference database and can be compared between studies if they are based on same database (Callahan *et al.*, 2017). However, sequences that have not yet been identified in databases will be lost during the process. Additionally, both methods risk grouping multiple similar species into a single OTU. Amplicon sequence variants (ASVs) have

recently emerged as an alternative to OTUs. ASVs are produced using a pipeline that has improved error control and can identify unique sequencing reads without relying on their relative abundance or a reference database (Callahan *et al.*, 2017). The increased taxonomic resolution of ASVs better captures biological variation and allows for direct comparison between data sets. Although recent metabarcoding studies have used ASVs to identify taxa, some still refer to them as OTUs for convenience (Minerovic *et al.*, 2020).

0.3 Freshwater microbial metabarcoding studies

A growing number of studies have used metabarcoding to better understand freshwater ecosystems and their community dynamics (Ruppert *et al.*, 2019). Many of these studies are centred on plankton, including bacteria, archaea, algae and protozoa (Ruppert *et al.*, 2019). Microbial metabarcoding studies can also identify the communities attached to different substrates, such as sediments, biofilm and leaf litter (Ruppert *et al.*, 2019). Microbial prokaryotes have been the main focus of metabarcoding studies, while fewer studies have targeted microbial eukaryotes, such as fungi (Debroas *et al.*, 2017; Zinger *et al.*, 2012). Compared to fungi in terrestrial environments, freshwater fungal communities have received considerably less attention (Sutcliffe *et al.*, 2018). Within this smaller branch of literature, aquatic hyphomycetes have been relatively well studied (Duarte *et al.*, 2016) as they are considered to be dominant decomposer of decaying leaves (Bärlocher, 2016). In addition, freshwater eukaryotic and prokaryotic communities have been less extensively explored than their marine counterparts, and among these freshwater studies, lotic ecosystems are understudied compared to lentic waters (Debroas *et al.*, 2017; Zinger *et al.*, 2012). Freshwater bacterial communities have been recognized as taxonomically distinct from marine communities as salinity is considered to be the major environmental determinant of community composition among bacteria (Lozupone and Knight, 2007). Within lotic ecosystems, other environmental drivers of microbial community composition have been identified: metals, temperature, quantity and quality of organic matter, hydrology and nutrient concentration (Zeglin, 2015). Landscape configuration and features, such as urban, agricultural and industrial land use, have also been identified as larger-scale mechanisms that influence microbial community structure (Shu *et al.*, 2021).

0.4 Metabarcoding of microbial communities applied to metal contamination in freshwater

The long-term impacts of anthropogenic activity on biodiversity are increasingly the subject of research, and previous knowledge gaps regarding microbial community structure and diversity in metal-impacted freshwater ecosystems (Zinger *et al.*, 2012) are slowly being remedied thanks to various metabarcoding studies. Metal contamination can originate from urbanization, industrial and agricultural activity, as well as active or abandoned mines (Ferreira, 2016). Mining and metal-extraction processes release chemicals and heavy metals that can be dissolved in water or transported in particulate form (Hogsden and Harding, 2012). Runoff from mining sites disrupts bacterial and fungal communities by acidifying streams and increasing the concentration of metals, such as aluminum, copper, iron and zinc (Hogsden and Harding, 2012). Some species of microorganisms are more tolerant and can evolve to cope with these new conditions, while others will not survive (Pradhan et al., 2015).

0.4.1 Mesocosm-based studies

When trying to understand the impact of metal contamination on microbial communities, metabarcoding studies typically focus on naturally occurring communities in polluted waters or rely on artificial outdoor experiments by collecting microbial communities from aquatic ecosystems and exposing them to different concentrations of contaminants in mesocosms. For example, Gardham *et al.* (2014) created multiple pond mesocosms to assess the establishment of benthic eukaryote communities under different concentrations of sediment copper contamination. They found that a majority of fungi were positively affected by copper during the initial establishment of the microbial community in the mesocosms (Gardham *et al.*, 2014). In the same mesocosms, Sutcliffe *et al.* studied the response of fungal and microbial prokaryote communities from water and sediment samples as well as communities colonizing artificial leaf substitutes (Sutcliffe *et al.*, 2018; Sutcliffe *et al.*, 2019). They found that leaf litter communities differed in a concentration-dependent manner and that even at low concentrations, copper can alter overall microbial community composition. Among the leaf litter-associated phyla that differed significantly between treatments, *Acidobacteria, Deinococcus-Thermus, Ignavibacteriae* and *Verrumicrobia* showed higher relative abundance in spiked mesocosms compared to the controls, and *Planctomycetes* were in lower abundance (Sutcliffe *et al.*, 2019). In their fungal experiment, Sutcliffe *et al.* did not find any changes in diversity and evenness between treatments, despite

changes in composition. However, these changes in composition were attributed to concentrations of sulfate, sulfur, DOC and TN rather than copper concentrations. They also found differences in OTU relative abundance between treatments, but no significant differences at a phylum level (Sutcliffe *et al.*, 2018). Studies based on artificial environments such as these have helped identify the underlying mechanisms through which microorganisms are affected by heavy metals. Ultimately, some of these studies on community sensitivity aimed at improving aquatic ecosystem monitoring by highlighting the value of microbial diversity and function in quality assessments (Tlili *et al.*, 2017; Yang *et al.*, 2018). However, despite their likeness to *in situ* conditions, mesocosms still have a few drawbacks such as short exposure times, controlled conditions and the absence of naturally occurring interspecific interactions (Gardham *et al.*, 2014; Tolkkinen, M. *et al.*, 2015). In fact, several land-use stressors are difficult to replicate at realistic and temporal scales, leading to microcosm experiments that are not always considered ecologically relevant or suitable to address management issues (Tolkkinen, M. *et al.*, 2015).

0.4.2 *In situ* studies

Metabarcoding studies done in situ can focus on different types of heavy metal contamination and their real-time effects on freshwater microbiota. These studies usually target the simultaneous consequences of different types of land use and have found distinctive differences in microbial communities as a result of environmental degradation (See Table 0.1) (Emilson et al., 2016; Liao et al., 2020; Ortiz-Vera et al., 2018; Simonin et al., 2019; Wang, J. et al., 2020; Xie et al., 2016; Xie et al., 2017). When studying river sediment from agricultural and industrial regions in China, Xie et al. (2016) found certain bacterial taxa (Betaproteobacteria and Deltaproteobacteria) to be negatively correlated with cadmium and zinc, and overall bacterial community composition to be impacted by iron, nickel and zinc. At those same sites, Xie *et al.* (2017) also found eukaryotic community structure to be affected by manganese, zinc, lead and chromium. Fungi accounted for 0.5 to 26.6% of eukaryotic sequences in their samples. In another Chinese study, Wang, J. *et al.* (2020) sampled water and sediment communities from eutrophic urban rivers. They found zinc and copper to be the main factors that determine microbial community composition in eutrophic waters and noted the following classes as particularly sensitive: *Deltaproteobacteria, Acidobacteria, Gemmatimonadetes* and *Nitrospira*. In the United States, Simonin *et al.* (2019) found that

Table 0. 1. Summary of the bacterial classes impacted by metal contamination in metabarcoding studies of *in situ* freshwater environnements. Orange taxa represent positive relationships with metal-contamination and blue taxa represent negative relationships.

Substrate	Study	Metal	Impacted taxa
Leaf litter	Emilson *et al.*, 2016	Ni	*Actinobacteria* *Betaproteobacteria (Amantichitinum)* *Gammaproteobacteria (Pseudomonas)*
Water column	Wang, J. *et al.*, 2020	Cu, Zn	*Acidobacteria* *Deltaproteobacteria* *Gemmatimonadetes* *Nitrospira*
Sediment	Xie *et al.*, 2016	Cd, Zn	*Betaproteobacteria* *Deltaproteobacteria*
	Simonin *et al.*, 2019	Zn	*Acidobacteria* *Alphaproteobacteria* *Betaproteobacteria*
	Kavehei *et al.*, 2021	Cu, Pb, Zn	*Chloroflexi* *Alphaproteobacteria (Gemmobacter)* *Holophagae (Geothrix and Holophaga)*
	Yin *et al.*, 2015	As, Cd, Cr, Cu, Hg, Mn, Ni, Pb, Zn	*Actinobacteria* *Chloroflexi* *Crenarchaeota* *Firmicutes* *Proteobacteria*
	Jacquiod *et al.*, 2018	Ca, Cu, Pb, Zn	*Actinobacteria* *Alphaproteobacteria* *Betaproteobacteria* *Deltaproteobacteria* *Gammaproteobacteria* *Gemmatimonadetes* *Ignavibacteriae* *Verrucomicrobia*
	Moberly *et al.*, 2016	Fe, Mn, Pb, Zn	*Clostridia (Natronoanaerobium)* *Aquificae* *Coprothermobacteria* *Synergistia (Synergistes)*

urbanization negatively impacted sediment-dwelling families from *Acidobacteria* and *Alphaproteobacteria* in streams, while tolerant taxa were abundant within *Betaproteobacteria*. Sediment zinc concentrations were one of the five variables used to characterize urbanization in this study. Ortiz-Vera *et al.* (2018) carried out one of the rarer studies on fungal communities by sampling the water column of a Brazilian river impacted by industrial and urban activity. They found that fungal diversity was negatively impacted by high concentrations of iron and that fungal community composition changed with seasonality, physiochemical parameters and geographical distance. Among water chemistry variables, the metals that influenced fungal community composition were aluminum and iron (Ortiz-Vera *et al.*, 2018).

Additional sources of contamination in these large-scale watershed studies increase the difficulty of identifying changes in community structure caused specifically by mining activity. This challenge highlights the important contribution of studies that target the effects of heavy metals emitted from previous and current mining sites (Blanchette *et al.*, 2019; Jacquiod *et al.*, 2018; Kavehei *et al.*, 2021; Moberly *et al.*, 2016; Reis *et al.*, 2016; Yin *et al.*, 2015). For example, Blanchette *et al.* (2019) studied microbial communities sampled from water, sediment and pebble biofilms in intermittent Australian rivers previously impacted by coal mining. They found that sediment and water communities were significantly correlated with environmental variables, but biofilm assemblages did not appear impacted. In terms of metals, sediment communities were correlated with lead, chromium, aluminum, sodium, iron, magnesium and copper, and pelagic communities were correlated with chlorine, calcium, zinc, iron, lead and cobalt (Blanchette *et al.*, 2019). Another Australian study sampled sediments with elevated concentrations of copper, zinc and lead from ephemeral creeks draining two legacy mines (Kavehei *et al.*, 2021). They found that eukaryotic and prokaryotic community structure varied between sites, but only prokaryotes were good indicators of metal contamination. They also identified higher abundances of *Chloroflexi*, *Gemmobacter* (*Alphaproteobacteria*) and two genera from the class *Holophagae* in mining-impacted samples (Kavehei *et al.*, 2021). In China, Yin *et al.* (2015) sampled river sediments that had been contaminated by heavy metals (mercury, arsenic, cadmium, chromium, nickel, lead, copper, manganese and zinc) from over 30 years of mining activity (Wang, L. *et al.*, 2008). They found a change in bacterial community structure across a gradient of heavy metal contamination along with higher relative abundances of *Firmicutes, Chloroflexi* and *Crenarchaeota* as well as

lower abundances of *Proteobacteria* and *Actinobacteria* (Yin *et al.*, 2015). Jacquiod *et al.* (2018) sampled sediments in a French river contaminated with calcium, copper, lead and zinc from a century-old foundry. Contrary to expectations, they found metal contamination to have a positive effect on bacterial diversity. They also found a significant effect of the contaminated site on bacterial community dissimilarity, accompanied with drops in relative abundance of *Gammaproteobacteria, Deltaproteobacteria, Gemmatimonadetes, Ignavibacteriae and Verrucomicrobia* and an increase in *Alphaproteobacteria, Betaproteobacteria and Actinobacteria* DNA (Jacquiod *et al.*, 2018). In the United States, Moberly *et al.* (2016) sampled sediments with elevated concentrations of lead, manganese, zinc and iron in a lake impacted by decades of mining. They compared the number of families within phyla to quantify the impacts of heavy metals on bacterial communities and found higher diversity among *Natronoanaerobium* in metal-contaminated sediments and higher diversity within *Aquificae, Coprothermobacteria* and *Synergistes* in uncontaminated sites. Several candidate phyla (NC10, OP8 and LD1PA) were detected only at the contaminated site (Moberly *et al.*, 2016).

Very few metal-impacted microbial metabarcoding studies have been conducted in Canada despite our long mining history and numerous bodies of water susceptible to long-term consequences. Although a few Canadian studies have focused on the extremophilic microbes residing in acid mine drainage (e.g., Auld *et al.*, 2017; Auld *et al.*, 2013), their insight on microbial community structure is limited to particularly harsh conditions and cannot be applied to historically impacted freshwater ecosystems. To our knowledge, only one other Canadian study has used metabarcoding to assess the impact of mining on freshwater microbial communities while studying the consequences of different land-use histories (Emilson *et al.*, 2016).

0.5 Metabarcoding of leaf litter microbial communities applied to metal contamination in fresh water

Most metabarcoding studies that focused on the impacts of metal contamination on freshwater microbial communities targeted sediment communities. Out of the 18 aforementioned studies, only one was based on leaf litter communities, and two studies used artificial substrates made of cellulose paper to replicate leaf litter communities (Emilson *et al.*, 2016; Sutcliffe *et al.*, 2018; Sutcliffe *et al.*, 2019). This disparity is most likely due to the partitioning of heavy metals within freshwater sediments. Since sediments can naturally accumulate and precipitate up to 90% of

soluble metals from the water column, many studies have chosen to focus on the relationship between benthic microbial communities and their metal contamination (Jacquiod *et al.*, 2018). Although there is an overlap in community composition between sediment and leaf litter microbes, it is important to focus on decomposer communities because of their vital function within stream ecosystems. One of the rare studies of this kind was led by Emilson *et al.* (2016) on microbial communities associated with leaf litter in Canadian boreal streams exposed to different types of perturbations (wildfire, logging, industrial mining and urbanization). They found that streams impacted by industrial activity had a significantly higher concentration of nickel and lower pH, along with distinct bacterial community composition and lower relative abundance of *Amantichitinum* and *Pseudomonas* OTUs. Industrially impacted streams also showed higher relative abundances of taxa from the bacterial class *Actinobacteria* and the fungal class *Eurotiomycetes*. However, there was no difference in overall fungal composition or diversity between sites (Emilson *et al.*, 2016). This is one of few studies that used metabarcoding to assess leaf litter microbial community variation under multiple anthropogenic stressors. Other similar studies include Duarte *et al.* (2015), who studied fungal leaf litter communities in Portuguese streams impacted by urban, industrial and agricultural activity, as well as Heino *et al.* (2014) and Mykrä *et al.* (2017), who studied bacterial and fungal leaf litter communities in streams altered by intensive land use in coastal Finland.

Most studies targeting microbial decomposers in metal-impacted freshwater systems with leaf bags did so before the advent of metabarcoding. These studies found shifts in stream-dwelling leaf litter bacterial and fungal communities in response to elevated metal concentrations through various short-term microcosm experiments (Duarte *et al.*, 2007; Duarte *et al.*, 2009; Pradhan *et al.*, 2011). However, some fungal communities did not exhibit structural changes when exposed to low concentrations in the short term (Roussel *et al.*, 2008) or shortly after being released from metal stress (Duarte *et al.*, 2009). Studies such as these, which relied on fungal sporulation and denaturing gradient gel electrophoresis, do not provide a comprehensive and detailed representation of the changes in community composition due to their identification techniques, although they do offer insight into other characteristics such as Darwinian fitness. This earlier branch of research also found fungal diversity to be negatively impacted by higher concentrations of heavy metals (Duarte *et al.*, 2007; Duarte *et al.*, 2009; Medeiros *et al.*, 2010; Niyogi *et al.*, 2002; Pradhan *et al.*, 2011),

although not all studies found an obvious relationship between these two variables (Duarte *et al.*, 2004; Sridhar *et al.*, 2000). Overall, there is a notable absence of microbial metabarcoding studies that combine natural freshwater environments, metal contamination and leaf litter sampling.

0.6 A Canadian legacy of mining-impacted streams

Copper mining was an important industry in the Eastern Townships of Québec during the 19th century (Berryman *et al.*, 2003). The Capelton and Eustis mines were the largest sources of copper and sulfur among the many mines in the region. In the same area, the Weedon mine extracted copper, sulfur, iron, zinc, gold and silver (Sabina, 1992). These former mines released sulfuric acid, cadmium, copper, iron, lead and zinc into nearby streams over several decades (Painchaud, 2007). Québec's Ministère de l'Environnement mandated studies on the impact from mine tailings on these streams and commissioned various rehabilitation efforts between 1993 and 2010 (Berryman *et al.*, 2003; Painchaud, 2007; St-Onge, 1997). To evaluate the impact of these historical mines on nearby freshwater environments, these studies monitored water quality and benthic invertebrate community integrity (Berryman *et al.*, 2003; St-Onge, 1997). However, no study was done to assess the effects of the metal contamination on decomposer microorganisms in freshwater streams impacted by these mines.

0.6.1 Eustis mine

The former Eustis mining site is located in the municipality of Canton de Hatley in the Eastern Townships. The mine operated from 1865 to 1939 and was an important source of copper during the American Civil War (Berryman *et al.*, 2003). During its operation, the Eustis mine, at a depth of 2265 m, dominated Québec's production of copper and was known for many years as the deepest copper mine in Canada (Sabina, 1992). The former mining site covered a surface area of 15 ha and comprised three acid-generating tailings sites: a tailings site named Eustis 1 and two waste rock piles named Eustis 2 and Eustis 3 (MERN, 2012). Prior to its remediation, the Eustis/Capelton mining complex was considered the largest remaining source of acid mine drainage in the Eastern Townships, producing an estimated 20 000 kg of copper leachate per year (Berryman *et al.*, 2003).

In the 1990s, a section of Eustis 1 was used as an experimental remediation site. The mine tailings were covered with lime mud, pulp and paper mill deinking residue, and compost to limit infiltration, but the resulting mound was unstable and required additional work (Pelletier-Allard, 2014). In 2005, Eustis 1 underwent partial remediation; three test plots were created with deinking residue, and the riparian strip was restored (Painchaud, 2007). The main work on Eustis 1 took place between 2006 and 2008. Restoration work consisted of excavating the mine tailings, transporting them and confining them under a cover composed of deinking tailings or a geomembrane, depending on the incline. The site was then covered with soil and replanted. Wildlife habitats were also created to increase biodiversity and improve the landscape (MERN, 2012). Eustis 2 and 3 underwent mitigation work in 2003. The upstream water was redirected and the sites' surfaces were adjusted by incorporating calcite (Painchaud, 2007). The main restoration of Eustis 2 and 3 took place in 2009 and 2010. Mine tailings were placed under an impermeable cover consisting of a geomembrane, soil and plants, similar to Eustis 1 (MERN, 2012).

The Eustis stream runs near the mining site and flows into the Massawippi River, which is part of the Saint-François River watershed. For over a century, this stream collected runoff from the Eustis mine tailings, turning it a pronounced orange colour and making its junction with the Massawippi River visible for several metres (COGESAF, 2006). In 1983, the Ministère de l'Environnement sampled the Eustis stream and found the water to be highly acidic, with a pH of 2.3 (Berryman *et al.*, 2003). In 1989, copper levels in the Massawippi River's sediments were measured, and the concentrations obtained were of 630 µg/g, whereas the threshold for adverse effects is of 36 µg/g (Berryman *et al.*, 2003). The same study also found that the benthic fauna of the Massawippi River was poorer downstream of Eustis stream and that fish gave the stream's junction with the Massawippi River a wide berth and swam along the opposite bank (Berryman *et al.*, 2003). In 1997, the Ministère de l'Environnement commissioned a study on the Massawippi River following the discovery that its copper contamination was spreading to the Saint-François River (Berryman *et al.*, 2003). The study confirmed that the Eustis stream was highly acidified, with a pH of 3.3, which is well below the minimum of 6.5 required for the protection of aquatic life. There was also an exceptionally strong presence of heavy metals in the stream; concentrations of cadmium, copper, iron, lead and zinc were respectively 41, 2490, 67, 25 and 65 times higher than the criteria for the protection of aquatic life at the time. The benthic community was poor in terms of abundance,

diversity and biomass. The study also noted that benthic organisms found closer to the mine tailings sites were among the taxonomic groups most resistant to pollution (e.g., Hydropsychidae) and that taxonomic groups characteristic of less disturbed environments (e.g., Ephemeroptera) reappeared further away from the sources of contamination (Berryman *et al.*, 2003). One of the last studies conducted before the complete restoration of Eustis mine was carried out by the Comité d'hygiène et d'aménagement des rivières Magog et d'aménagement des rivières Saint-François (CHARMES) on the Massawippi River's tributaries in 2003. Its findings were consistent with prior studies: it showed that the Eustis and Capel streams still had very poor bacteriological and physicochemical water quality (COGESAF, 2006).

0.6.2 Capel Mine

The former Capelton mining site is located in the Eastern Townships, a few kilometres from the Eustis mine, and is part of the Albert, Capelton and Eustis mining complex. The Capelton copper mine began operating in 1863 and ceased operations in 1907 with the arrival of electricity, as the mine was considered too small for the installation of electricity to be a worthwhile investment (Berryman *et al.*, 2003; Lavertu, 2002). It reopened to the public as a tourist destination in 1995 (Lavertu, 2002). The Capelton mine underwent mitigation work in 2000: an alkaline product (calcite) was spread over the mine tailings slopes (Painchaud, 2007). The former Capelton mining site was characterized in 2009 and over 250 000 m^3 of metal-contaminated soil was identified (MERN, 2014). Restoration was said to have begun at the Capelton site in 2014 (MERN, 2015), but in 2021, the former mining site was still under legal validation, meaning that the provincial government was trying to verify who is responsible for its restoration, based on the history of operation and land tenure (MERN, 2021).

The Capel stream is part of the Saint-François River watershed and connects with the Massawippi River a few kilometres downstream of the Eustis stream. It runs near and through multiple mine tailings sites from the Capel mine and collected their runoff for over a century. Sampling conducted by the Ministère de l'Environnement in 1983 showed that the Capel stream had a pH of 3.6 at the time (Berryman *et al.*, 2003). Over a decade later, the ministry commissioned another study of the impact of mine tailings on the Capel stream and measured a pH of 4.2, which remained under the threshold of 6.5 for the protection of aquatic life and the threshold of 5 for acute toxicity to aquatic

life (Berryman *et al.*, 2003). They also found that the Capel stream contained concentrations of cadmium (45 µg/l), copper (5800 µg/l), iron (670 µg/l), lead (250 µg/l) and zinc (8000 µg/l) to be respectively 20, 1610, 2.25, 81 and 76 times higher than water quality guidelines for protection of aquatic life (Berryman *et al.*, 2003). Consequently, benthic macroinvertebrate abundance, diversity and biomass were measured and considered to be poor (Berryman *et al.*, 2003). In the CHARMES study of the Massawippi River's tributaries, the Capel stream was found to have very poor bacteriological and physicochemical water quality (COGESAF, 2006).

0.6.3 Weedon mine

The former Weedon mine is located in the municipality of Weedon, in the Haut-Saint-François regional municipality. The mine operated intermittently between 1910 and 1973, producing copper, sulfur, iron, zinc, gold and silver (Sabina, 1992). The mine consisted of four inclined shafts, and its mine tailings spread over more than 13 ha (Tremblay and Bedard, 1995). In 1993, the Ministère de l'Environnement excavated the mine tailings and encapsulated them on a slope, using waste rock for containment and a high-density polyethylene geomembrane cover (Tremblay and Bedard, 1995). Additional mitigation measures were undertaken in 1994–1995, 2000 and 2004 to control acidity and reduce metal leaching (Painchaud, 2007).

The Weedon stream, also known as "la rivière au Rat," is a forested stream that feeds into the Saint-François River, in the Saint-François watershed. In 1996, the Ministère de l'Environnement mandated a study to determine the impact of the abandoned Weedon mine on the quality of nearby aquatic environments following restoration work. Discharge from the mine caused the pH to reach 4.7 in the Weedon stream and concentrations of copper (180 µg/l), iron (4800 µg/l) and zinc (400 µg/l) were respectively 90, 16 and 6 times higher than the threshold for aquatic life (St-Onge, 1997). Benthic organism density, biomass and taxonomic richness were all reduced downstream of the mine tailings (St-Onge, 1997).

0.7 Thesis Objectives

The objectives of this project were to assess and compare the impacts of historical contamination from restored copper mines on bacterial and fungal leaf litter communities in streams. In doing so, my research addresses a lack of microbial metabarcoding studies that combine natural freshwater

environments, mining metal contamination and leaf litter sampling. To this end, fungal and bacterial DNA was extracted from leaf litter and characterized by next-generation sequencing at sites upstream and downstream of three streams impacted by historical copper mining and at two unimpacted reference streams in southern Québec, Canada. Impacted streams contained elevated concentrations of heavy metals, such as copper, iron and zinc, well beyond the Canadian Quality Guideline thresholds for freshwater aquatic life. My specific objectives were to 1) determine if microbial leaf litter communities were impacted by historical copper mining despite various restoration efforts and 2) compare the responses of aquatic bacterial and fungal decomposers to elevated concentrations of heavy metals caused by historical copper mining.

My first hypothesis was that bacterial and fungal diversity measures would differ significantly between mining-impacted streams and reference streams, as well as between sampling sites upstream and downstream from the mining grounds. I predicted that Shannon diversity and species richness would be lower at mining-impacted sites compared to reference sites, as well as downstream of the mining sites when compared with the upstream sites. My second hypothesis was that only bacterial community composition would differ between stream types, and to a lesser degree, between sampling site locations within mining-impacted streams. I did not predict any difference in fungal community composition. The reasoning behind these hypotheses is explained in the following chapter. The main statistical methods used to test these hypotheses were principal coordinates analyses (PCoA) and differential abundance analyses (DESeq2) for community composition comparisons, as well as linear models for comparisons of alpha diversity.

This thesis consists of an introduction, a single chapter presented as a scientific article, and a conclusion. The aim of this introduction was to provide a basic understanding of the concepts at the heart of this project and highlight important knowledge gaps that needed to be addressed. The first chapter is based on the results of sampling conducted from October to December 2014. This chapter is followed by a general conclusion that reviews the main findings of this study and suggests avenues for future research.

CHAPTER I

THE IMPACTS OF HISTORICAL COPPER MINING ON BACTERIAL AND FUNGAL LEAF LITTER COMMUNITIES IN STREAMS

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1.2 **Abstract**

Microbial communities play an essential role in the early stages of decomposition of organic matter and nutrient cycling in streams. If decomposition in streams is impaired by anthropogenic activity, the ability of these ecosystems to support complex food webs is reduced. Despite the prevalence of abandoned mines across landscapes, our understanding of the impacts of historical contamination from copper mines on microbial decomposer communities in freshwater streams remains limited. Our goal was to understand the ongoing impacts of copper mining on microbial decomposer communities in freshwater streams and gain insight into the long-term re-establishment of stream ecosystem function following metal stress. We sampled microbial communities by submerging leaf litter bags in three mining-impacted streams and two reference streams in Canada. Bacterial and fungal community composition and diversity were assessed using next-generation sequencing of 16S rRNA and ITS gene amplicons. We found changes in bacterial community structure that indicated the potential negative effects of heavy metals on specific classes, such as *Deltaproteobacteria, Acidimicriobiia, Sphingobacteriia* and *Solibacteres.* We also found that *Chloroflexi*, *Deinococci* and *Saccharibacteria* responded positively to higher concentrations of heavy metals. Conversely, fungal communities were not impacted by historical mining, nor was bacterial and fungal alpha diversity. Overall, our study identified associations between numerous bacterial taxa at different phylogenetic levels and mining-impacted conditions in streams. These groups are potential indicators of mining activity and could be used as effective bioassessment tools to improve mine monitoring efforts, but further studies will be needed to better understand their genomic adaptations and ecological behaviour.

Keywords : Bacteria, fungi, microbial community, leaf litter, aquatic ecology, freshwater ecosystem, stream, metabarcoding, environmental DNA, heavy metals, historical copper mining.

1.2 Introduction

Microbial communities play an essential role in the early stages of decomposition of organic matter and nutrient cycling in streams (Marks, 2019). Allochthonous carbon inputs, such as fallen leaves and branches, constitute the main source of energy in forested stream ecosystems, where they are transformed by bacteria and fungi into assimilable matter for higher trophic levels (Hagen, 2010). The process of organic matter decomposition in streams begins at a microscopic scale, but its outcome provides cross-ecosystem subsidies to terrestrial food webs in surrounding landscapes (Marks, 2019). If decomposition in streams is impaired by anthropogenic activity, the ability of these ecosystems to support complex food webs is reduced (Hogsden and Harding, 2013).

There is a legacy of historical and ongoing contamination of aquatic ecosystems from metal mining in many regions across the world (Schwarzenbach *et al.*, 2010). A common example of contamination in freshwater streams are tailings from copper mining. Runoff from copper mine sites, also known as *acid mine drainage*, disrupts microbial community structure and activity by acidifying neighbouring streams and increasing the concentration of heavy metals such as copper, iron, cadmium, lead and zinc (Hogsden and Harding, 2012). Despite the prevalence of abandoned mines across landscapes (Fields, 2003), our understanding of the impacts of historical contamination from copper mines on aquatic microbial decomposer communities in freshwater streams remains limited (Zinger *et al.*, 2012). By applying DNA metabarcoding to study the impact of historical copper mining on bacterial and fungal leaf litter communities, we can improve our understanding of understudied components of aquatic food webs and their response to long-term perturbations, which brings us closer to holistic restoration and monitoring efforts (Emilson *et al.*, 2016; Grossart and Rojas-Jimenez, 2016).

There is limited knowledge about how historical metal-mining pollution alters aquatic microbial decomposer communities in freshwater streams over the long term. Current research using sequencing-based approaches to quantify the effects of mining on aquatic microbial communities has focused primarily on mining-impacted water and sediment microbial communities (Blanchette *et al.*, 2019; Gardham *et al.*, 2014; Jacquiod *et al.*, 2018; Kavehei *et al.*, 2021; Reis *et al.*, 2016; Wang, J. *et al.*, 2020; Yang *et al.*, 2018; Yin *et al.*, 2015) and has largely omitted leaf litter, where microbial decomposer communities are expected to concentrate. Earlier studies that focused on

aquatic microbial decomposer communities in leaf litter relied on microscopic techniques for taxonomic identification, such as identifying released fungal spores and cultivating microbial colonies (Duarte *et al.*, 2004; Niyogi *et al.*, 2002; Roussel *et al.*, 2008; Sridhar *et al.*, 2001), or molecular fingerprinting approaches such as denaturing gradient gel electrophoresis (DGGE) to observe metal-contamination responses in microbial diversity (Duarte *et al.*, 2007; Duarte *et al.*, 2009; Fernandes *et al.*, 2009; Medeiros *et al.*, 2010; Pradhan *et al.*, 2011). These studies lacked the taxonomic resolution and overall representation of microbial diversity that can now be quantified through high-throughput sequencing of microbial barcode genes (Zinger *et al.*, 2012). The relatively few studies using sequencing-based approaches to study the response of leaf litter microbial communities to metal contamination have been conducted primarily in laboratory settings or mesocosms (copper: Sutcliffe *et al*., 2018; Sutcliffe *et al*., 2019, silver: Tlili *et al.*, 2017), limiting our understanding of the impacts of metals on leaf litter communities to the short-term impacts in controlled environments.

The objective of this study was to determine if microbial decomposer communities in streams impacted by past copper mines continue to be shaped by this historical stressor despite various restoration efforts over the last three decades (Berryman *et al.*, 2003; Painchaud, 2007; St-Onge and Richard, 1996). Our research joins the handful of studies that address the lasting impacts of anthropogenic activity on aquatic microbial decomposer community composition and, more specifically, the historical and ongoing metal-mining impacts before (Medeiros *et al.*, 2010; Niyogi *et al.*, 2002; Sridhar *et al.*, 2001; Sridhar *et al.*, 2000) and after the advent of high-throughput sequencing (Emilson *et al.*, 2016). A second objective was to compare the responses of aquatic bacterial and fungal decomposer communities to this historical and ongoing stressor. Most of the literature has focused on the responses of either bacterial or fungal leaf litter communities to elevated metal concentrations, but not both simultaneously. A few studies have used fingerprinting methods to study both bacterial and fungal community composition (Duarte *et al.*, 2007; Duarte *et al.*, 2009; Pradhan *et al.*, 2011), but this approach is limited in terms of its ability to identify fine-scale shifts in community composition, and only a handful of studies have used metabarcoding to address this question (Emilson *et al.*, 2016; Tlili *et al.*, 2017). Thus, there is still relatively little known about the fine-scale responses of these communities to mining impacts.

We used high-throughput sequencing of fungal and bacterial DNA extracted from leaf litter bags at sites upstream and downstream of three copper mining-impacted streams and at two reference streams unimpacted by metal mining in southern Québec, Canada. By comparing microbial community composition at different heights within the same streams, our study ensures that basic chemical properties remain similar while metal concentrations vary. This allows us to make more direct observations on the relationship between microbial communities and high metal concentrations, while retaining the advantages of an *in situ* study. We first predicted that bacterial and fungal diversity measures would differ significantly between mining-impacted streams and reference streams, as well as between sampling sites downstream and upstream from the mining grounds, especially following decades of exposure. This prediction was based on findings from multiple studies on leaf litter microbial communities under varying degrees of metal stress, including zinc (Niyogi *et al.*, 2002), and mixtures of zinc and copper (Duarte *et al.*, 2007; Duarte *et al.*, 2009), copper and silver (Pradhan *et al.*, 2011) and zinc, iron and manganese (Medeiros *et al.*, 2010). Secondly, we predicted that only bacterial community composition, and not fungal community composition, would differ between mining-impacted and reference stream sites. This prediction is based on findings by Emilson *et al.* (2016), in which differences in bacterial, but not fungal, community composition were detected in streams that had undergone 40 years of metal deposition from industrial mining activity. Overall, our study aims at understanding the ongoing impacts of copper mining on microbial decomposer communities in freshwater streams and gaining insight into the long-term re-establishment of stream ecosystem function following metal stress.

1.3 Materials and methods

1.3.1 Study sites

Our study focused on three metal mining-impacted streams and two unimpacted reference streams in southern Québec, Canada (Figure 1.1). The contaminated streams are located near three former copper and pyrite mines: Capelton (N 45° 19’19.6”, W 71° 54’23.1”), Eustis (N 45° 19’01.1”, W 71° 55’10.1”) and Weedon (N 45° 41’46.2”, W 71° 25’09.8”), which closed in 1907, 1939, and 1973, respectively (Berryman *et al.*, 2003; Sabina, 1992). These streams collected runoff from mine tailings for decades until the mining sites were remediated in the late 1990s and early 2000s.

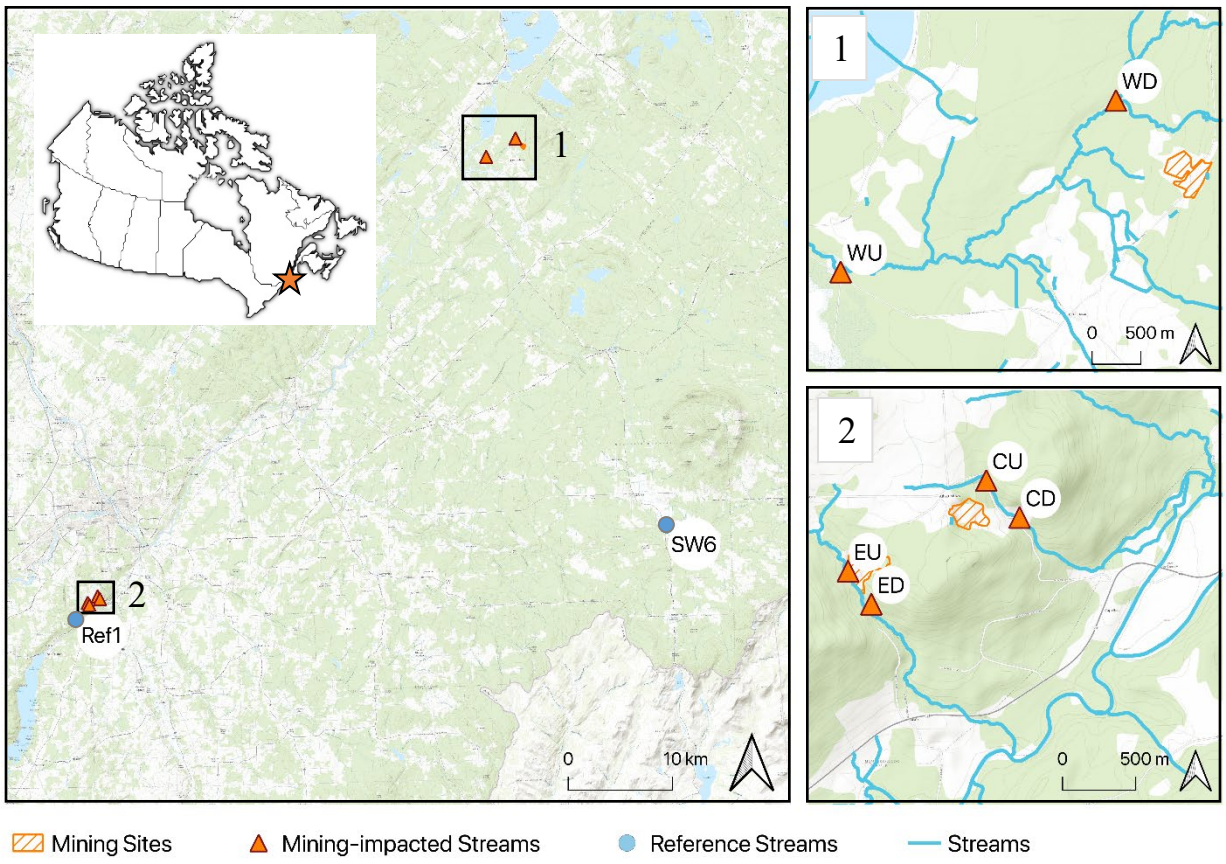

Figure 1.1. Sampling sites in southern Québec, Canada. Orange triangles represent sampling sites in mining-impacted streams and blue circles represent sampling sites in reference streams.

Site restoration consisted mainly of relocating and covering the mine tailings with various materials such as lime mud, pulp and paper mill deinking residue, compost, calcite, and geomembrane, which acts as a liner with low permeability (Painchaud, 2007; Tremblay and Bedard, 1995). Despite these measures, nearby streams remain highly contaminated by numerous heavy metals, especially downstream of the mining grounds. At each contaminated stream, leaf litter bags were incubated at two sites: one upstream and one downstream of the historical mining location (six leaf litter bags per site). The reference streams were chosen for their similar size and order, and regional proximity to the contaminated streams. Each reference stream had one sampling site (N 45° 18'23.4", W 71° 56'05.9"; N 45° 24'06.5", W 71° 14'17.4"). Leaf litter bags were incubated in the study streams in autumn 2014, from early October to early December, and microbial decomposer communities were collected from the leaf litter bags in early December 2014.

1.3.2 Stream characteristics

Study streams were described for the following physical characteristics at each of the sampling sites, at the beginning and end of the experiment: width, depth, bottom substrate, foliar coverage, water velocity and atmospheric pressure (Table 1.1). The foliage cover was estimated visually, and the dominant substrate was recorded at each site, as recommended by the Ontario Benthos Biomonitoring Network (OBBN; Ontario. Dorset Environmental Science Centre; (Jones *et al.*, 2006)). The bank width and water depth of sampling sites where leaf litter bags were placed was measured and averaged at each stream. Water velocity was calculated using a FlowTracker Handheld ADV flow meter (SonTek, San Diego, CA, USA). Water temperature was measured every hour for the duration of the seven-week experiment with two HOBO Water Temperature Pro v2 data loggers (Onset, Bourne, MA, USA) per stream, which were attached to randomly selected leaf litter bags.

Table 1.1. Average physical characteristics of mining-impacted and reference streams in southern Québec, Canada.

Site	Symbol	Latitude	Longitude	Temp (C°)	Atmospheric Pressure (mmHg)	Bank Width (m)	Water Depth (m)	Flow Velocity (m/s)	Foliar coverage (%)	Substrate composition (%)			
										Boulder	Rock	Gravel	Sand
Hatley (Reference)	Ref1	45°18’23.39 » N	71°56’05.85 » W	3.87	744.35	2.60	0.15	0.01	23	15	15	70	0
Ditton (Reference)	SW6	45°22’21.28 » N	71°12’35.91 » W	5.00	726.70	15.10	0.27	0.15	0	50	40	10	0
Weedon Upstream	WU	45°41’45.3 »N	71°25’11.2 »W	4.55	742.15	4.35	0.27	0.09	15	10	80	10	0
Weedon Downstream	WD	45°42’39.7 »N	71°22’58.8 »W	4.45	744.45	3.10	0.33	0.19	8	0	0	0	100
Capel Upstream	CU	45°19’27.9 »N	71°54’33.3 »W	6.05	741.25	1.70	0.17	0.02	30	35	45	20	0
Capel Downstream	CD	45°19’19.6 »N	71°54’23.1 »W	6.55	744.65	1.76	0.15	0.03	3	50	50	0	0
Eustis Upstream	EU	45°19’08.5 »N	71°55’17.2 »W	6.05	733.90	2.30	0.21	0.03	31	20	50	30	0
Eustis Downstream	ED	45°19’01.1 »N	71°55’10.1 »W	6.15	737.55	1.73	0.11	0.07	13	50	25	15	10

Study streams were described for water chemistry and water metal concentrations at each of the sampling sites, as well as for biological parameters of algal biomass as measured by chlorophyll *a* (chl a) and bacterial production (BP) in the water. At the beginning and end of the experiment, a YSI Professional Plus Multiparameter Water Quality Instrument (YSI Incorporated, Yellow Springs, OH, USA) was used to measure salinity, pH, conductivity, specific conductivity, total dissolved solids and dissolved oxygen. Water samples were collected mid-channel from stream sampling sites at the beginning and end of the experiment, and they were analyzed for dissolved organic carbon (DOC), total nitrogen (TN), total phosphorus (TP), chl a, and BP in the Groupe de recherche interuniversitaire en limnologie (GRIL) analytical laboratory at the Université du Québec à Montréal. DOC concentrations were measured by filtering water samples through 0.45 µm filters (surfactant-free membrane filters) after acidification (5% phosphoric acid), followed by sodium persulfate oxidation using a 1010 TOC analyzer (O.I. Analytical, College Station, TX, USA). TP was quantified by spectrophotometry using a 2 cm quartz cuvette in a BiochromUltrospec® 20100 pro spectrofluorometer and the molybdenum blue method after persulfate digestion (Griesbach and Peters, 1991). TN samples were analyzed using a continuous flow analyzer (ALPKEM Flow Solution IV©) and an alkaline persulfate digestion method with a cadmium reactor, following a standard protocol by Patton and Kryskalla (2003). Chl a was measured by filtering water through glass fibre filters (Whatman GF/F), then extracting the chl a in hot ethanol and measuring the chlorophyll spectrophotometrically on a BiochromUltrospec® 2100 pro with a 10-cm quartz (Sartory and Grobbelaar, 1984; Wintermans and De Mots, 1965). BP was estimated by measuring the rates of protein synthesis with radiolabelled leucine at the end of the experiment (Kirchman and Ducklow, 1993; Smith and Azam, 1992). These samples were incubated with 14C-leucine for one hour at ambient water temperature. Incubation was stopped by adding 100% trichloroaceticacid (TCA), then stored at 4°C. Samples were then counted in a Packard Tri Carb Liquid Scintillation Analyzer, model 2800 TR (Perkin Elmer, Waltham, MA, USA).

In early December 2014, at the end of the leaf litter stream incubation experiment, one water sample was collected from each site and analyzed for water metal concentrations: aluminum, antimony, arsenic, barium, cadmium, chromium, cobalt, copper, lead, manganese, molybdenum, nickel, selenium, silver, sodium and zinc, as well as sulfates and sulfites. All metal concentrations were

measured by inductively coupled plasma mass spectrometry (ICP/MS) at AGAT Laboratories. Sulfates were measured with ion chromatography and sulfites were measured by titration, also by AGAT Laboratories.

1.3.3 Leaf litter collection and experimental deployment

Senescent speckled alder leaves (*Alnus incana* ssp. *Rugosa*) were collected at McGill University's Gault Nature Reserve (N 45° 33'03.85", W 73° 09'18.88") in southern Québec by placing tarps under the trees and shaking them in late September 2014. Alder leaves are commonly used in decomposition experiments because of their abundance and because of their role as a high-quality natural substrate for decomposers in streams (Heeger, 2019; Pérez *et al.*, 2012). The leaves were dried at 40°C for eight hours, weighed and placed in leaf litter bags (3 g/bag). Fine mesh bags (0.5 mm mesh) were used to observe the colonization of bacteria and fungi without the influence of macroinvertebrates, and coarse mesh bags (1 cm mesh) allowed for macroinvertebrate activity. Three fine mesh bags and three coarse mesh bags were randomly distributed at each site and secured to the substrate with aluminium pegs. The mesh bags were deployed in October and retrieved in December, leaving them in the streams for an average length of 59 days. Not all leaf bags were successfully retrieved as an unprecedented ice storm extricated nearly 50% of the bags from the substrate and carried them downstream (Table A1). The leaf bags that survived the storm and remained at their experimental placements were removed from the stream, placed in individual sealed bags and then frozen at –20°C at l'Université du Québec à Montréal until they could be processed. We measured percent loss of dry matter in the leaf bags over the experimental incubation (Table A2). We also counted macroinvertebrate communities in the leaf bags using a 1Ox-dissecting microscope (SZ2-IL-ST, Olympus SZ, Japan) that was equipped with a DP21-HS Olympus camera (Olympus, Japan). Family-level identification was done using the identification key in Marshall (2006) (Table A3).

1.3.4 DNA extraction and processing

Genomic DNA was extracted using the MoBio PowerSoil DNA extraction kit (Qiagen), following the manufacturer's instructions with additional bead beating and sonication to increase DNA yields. One gram of humid leaf litter per bag was placed in thick-walled 2 ml tubes with three 2.3 mm diameter stainless steel beads (BioSpec Products, Bartlesville, OK, USA) and 250 µl of

the PowerSoil bead tube buffer. Since each leaf litter bag that was successfully retrieved represents one sample, there was an uneven number of samples among sites. Leaf litter was homogenized using a MiniBead Beadbeater-16 (BioSpec Products, Bartlesville) for 1.5 minutes. The remaining buffer from the bead beating tube was added to the resulting homogenate, sonicated at the high setting (320 W at 20 kHz) for 2 minutes and re-introduced to the bead beating tubes. Following sonication, samples were transferred back to the bead beating tubes. After sonication, the DNA extraction was performed following the manufacturer's instructions.

Following DNA extractions, all samples were cleaned using the Zymo OneStep-96 PCR inhibitor removal kit. Polymerase chain reaction (PCR) was used to amplify the targeted genes from the extracted DNA. For the bacterial samples, we used the chloroplast-excluding primers 799F and 1115R (Chelius and Triplett, 2001) to target the V5-V6 region of the 16S rRNA gene. For the fungal samples, we amplified the internal spacer region using the fungal specific primers ITS1F (Gardes and Bruns, 1993) and ITS2 (White *et al.*, 1990). Each primer also contained 1 of 20 unique barcodes and an Illumina adaptor to allow sequences to bind to the flow cell of the MiSeq sequencer. PCR was performed using 25 µl reactions prepared with 1 µl genomic DNA diluted 1:10 in molecular-grade water, 5 µl 5x HF buffer (Thermo Scientific), 0.5 µl dNTPs (10 µM each), 0.5 µl forward and reverse primer (10 µM each), 0.75 µl DMSO, 0.25 µl Phusion HotStart II polymerase (Thermo Scientific) and 16.5 µl molecular-grade water. Each reaction began with 30 seconds of denaturation at 98°C, followed by 35 cycles of the following: 15 s at 98°C, 30 s at 64°C, 30 s at 72°C and a final elongation step at 72°C for 10 minutes. Amplicons were cleaned and normalized to 0.55 ng/µl using the lnvitrogen SequelPrep normalization plate kit. After normalization, equal volumes of amplicon DNA per sample were pooled and sequenced.

1.3.5 Amplicon sequencing

16S rRNA and ITS gene amplicons were sequenced using the Illumina MiSeq platform and V3 chemistry. Illumina adapters were trimmed from the sequences using cutadapt v1.7.1 (Martin, 2011), and sequences were demultiplexed using idemp (https://github.com/yhwu/idemp). Paired-end fastq files were processed through the DADA2 pipeline v1.16.0 (Callahan *et al.*, 2016) to remove low-quality sequences, merge paired-end sequences, remove chimeras and assign taxonomy to amplicon sequence variants (ASVs). We used default parameters for all DADA2

analyses except as noted. In the bacterial dataset, forward reads were truncated at 260 nucleotides and reverse reads were truncated at 220 nucleotides. In the fungal dataset, forward reads were truncated at 240 nucleotides and reverse reads were truncated at 230 nucleotides. To remove primers, the 20 first nucleotides were trimmed from the start of the reads. Sequence variants were inferred using the pseudo-pooling method. Denoised forward and reverse reads were merged with a minimal overlap of 15. Chimeras were removed using the pooled method. Taxonomy was assigned using the SILVA version 128 sequence database for 16S gene fragments and the UNITE sequence database version 8.2 for ITS gene fragments (Glöckner *et al.*, 2017; Nilsson *et al.*, 2019; Quast *et al.*, 2013; Yilmaz *et al.*, 2014). We removed rare ASVs that appeared fewer than 10 times in all the samples and used rarefaction to normalize all samples to a common number of sequences sampled, as is necessary when calculating measures of community alpha and beta diversity (Weiss *et al.*, 2017). During rarefaction, bacterial samples that had fewer than 4900 sequences were filtered out, leading to the loss of one sample. Fungal samples with fewer than 3500 sequences were filtered out, with no sample loss. These cut-offs were chosen after visualizing rarefaction curves (Figure A1) using the function rarecurve in the vegan R package (Oksanen *et al.*, 2020).

1.3.6 Statistical analyses

Statistical analyses were performed using the software Rstudio v1.2.5042 (RStudio Team, 2020). Data was imported into the phyloseq R package for the analysis of microbiome data (McMurdie and Holmes, 2013).

Metal concentrations were tested for normality using Shapiro-Wilk's normality test. Since most metal concentrations were not normally distributed, we used Kruskal-Wallis tests to determine if metal concentrations differed between reference and impacted streams. We also used Kruskal-Wallis tests in a three-way comparison of metal concentrations between reference stream sites, and upstream and downstream sites in impacted streams, followed by Dunn's test using R package rstatix with the Benjamini-Hochberg method to correct for multiple comparisons (Kassambara, 2021). Metals with significant differences between stream types were illustrated using the ggplot2 package (Wickham, 2016).

To determine whether mesh size on the leaf litter sampling bags had an influence on community composition, we used a permutational multivariate analysis of variance (PERMANOVA) with sampling sites as a random factor, using 999 permutations in the vegan R package (Oksanen *et al.*, 2020). Since there was no difference in bacterial ($R^2 = 0.03$, $p = 0.35$) and fungal ($R^2 = 0.04$, $p = 0.33$) communities between fine and coarse mesh bags, both types of mesh were combined within sites for among-site comparisons.

To quantify community composition variation among streams, we analyzed rarefied community abundance data using principal coordinates analysis (PcoA). The function 28nvfit from the package vegan was used to calculate correlations between environmental variables and PcoA axes, using a randomization approach to calculate p-values for every environmental variable and fit significant ($p<0.05$) environmental variables onto the PcoA. In addition to environmental variables, we calculated total rarefied abundance of taxonomic classes for each sample, and classes with fewer than 10 sequences were discarded before using 28nvfit to calculate correlations and p-values for every class and fit significant taxa ($p<0.05$) onto the ordination.

The DESeq2 package was used to test for differential abundance of sequence data and compare taxon abundances between groups of samples (reference, mining, downstream and upstream) in order to explore which taxa were more or less abundant between groups (Love *et al.*, 2014). This method requires unrarefied community data and automatically corrects for multiple testing. Differential abundance analyses were performed on non-rarefied community data at an ASV level as well as on non-rarefied data aggregated at the class level.

Alpha diversity was assessed through Shannon's diversity index and species richness using the vegan package on rarefied community data. We used mixed models to compare diversity between upstream and downstream sites, with stream as a random effect. Linear models were used to compare diversity between mining-impacted and reference sites. Modeling was performed using the package nlme (Pinheiro *et al.*, 2020).

1.4 Results

1.4.1 Environmental conditions

Water concentrations of copper (Cu), barium (Ba), cobalt (Co) and zinc (Zn) differed between stream site types (Figure 1.2) (Cu: p=0.042, Ba: p=0.042; Co: p=0.038; Zn: p=0.044, Kruskal-Wallis tests; Figure 1.2). Metal concentrations in stream water were more elevated at sites downstream of mining grounds in comparison with reference streams (Cu: p=0.04; Ba: p=0.04; Zn: p=0.042, Dunn's test; Figure 1.2). Metal concentrations were also higher in metal-impacted streams when upstream and downstream sites were combined and compared with reference stream sites (Cu: p=0.044; Ba: p=0.044; Zn: p=0.046; Dunn's test; Figure 1.2), but there was no statistical difference in metal concentrations between upstream and downstream sites in metal-impacted streams. In this study, Ba and Zn concentrations represent a gradient in which reference streams have low concentrations, upstream sites in metal-impacted streams have intermediate concentrations, and downstream sites in metal-impacted streams have the highest concentrations (Figure 1.2). The percent loss of dry matter over the experimental incubation is presented in Table A2 and macroinvertebrate communities are presented in Table A3. Other water chemistry variables, such as nutrients, pH, dissolved organic carbon, conductivity and sulfates, as well as biotic variables, algal biomass (chl a) and bacterial production (BP), did not differ between stream site types and are presented in the supplementary material (Table B1).

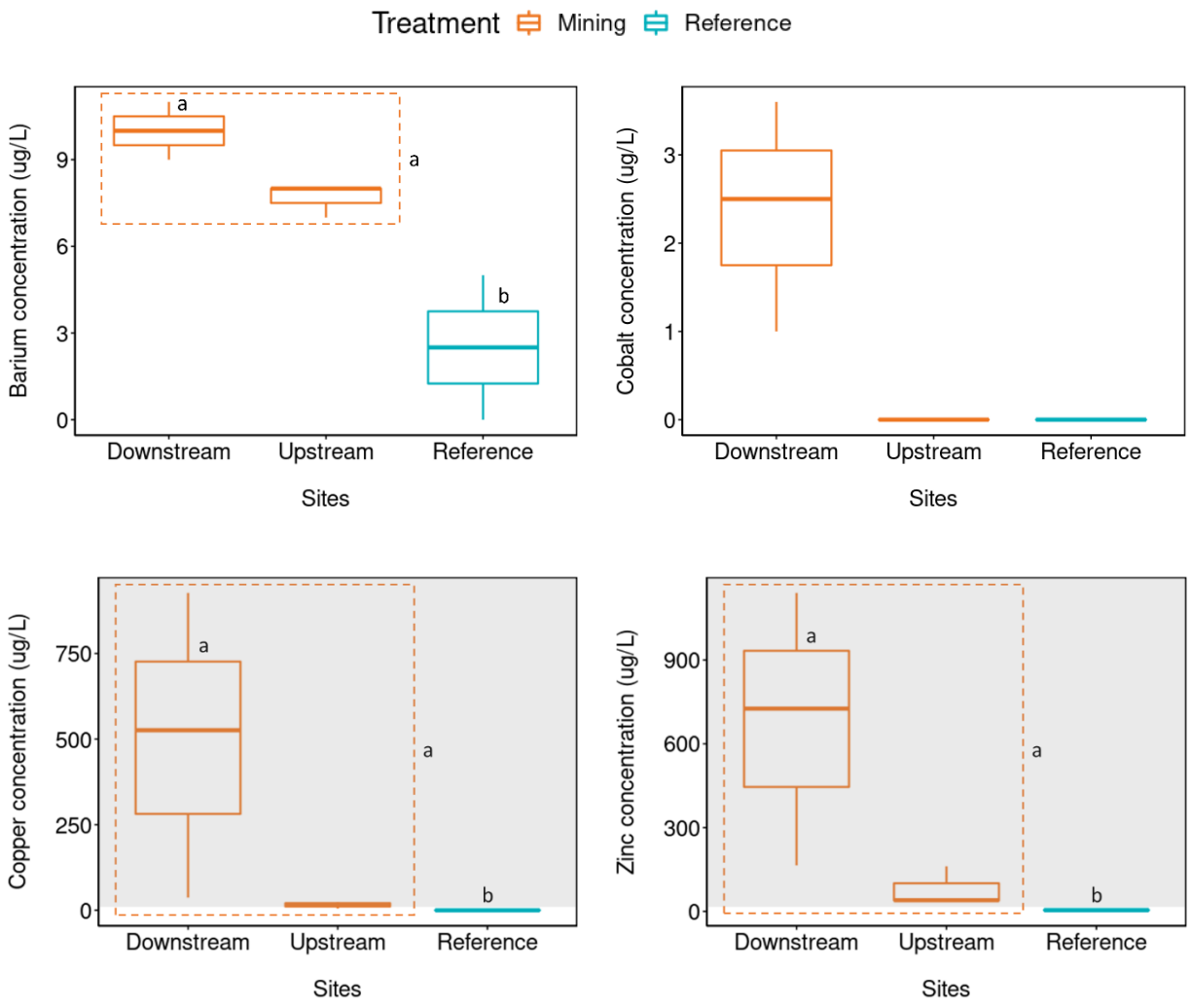

Figure 1.2. Metal concentrations that differed significantly according to Kruskal-Wallis tests between reference streams and sites upstream and downstream of mine sites. Different letters indicate a significant difference between groups according to Dunn's test and grey zones indicate values above Canadian Quality Guideline thresholds for freshwater aquatic life.

1.4.2 Bacterial and fungal communities

From our rarefied community data, 47 bacterial classes and 35 fungal classes were identified. The most abundant bacterial classes in leaf litter samples across all stream types were *Betaproteobacteria* (34% relative abundance across all samples) and *Alphaproteobacteria* (23%), followed by *Actinobacteria* (8.6%), *Sphingobacteriia* (8.5%) and *Gammaproteobacteria* (5%) in smaller proportions. The most abundant fungal classes were Agaricomycetes (30%), Leotimycetes (22%), Mortierellomycetes (17%) and Sordariomycetes (16%) (Figure 1.3). No significant differences were found in bacterial alpha diversity between upstream and downstream sites in metal-impacted streams (mixed models, Shannon: $F = 0.48$; $p = 0.50$, richness: $F = 1.05$; $p = 0.32$) or between reference and mining-impacted streams (linear models, Shannon: $F = 1.48$; $p = 0.24$, richness: $F = 0.78$; $p = 0.39$). There were also no significant differences in fungal diversity between upstream and downstream sites in metal-impacted streams (mixed models, Shannon: $F = 1.91$; $p = 0.19$, richness: $F = 1.05$; $p = 0.32$) or between reference and mining-impacted streams (linear models, Shannon: $F = 0.50$; $p = 0.49$, richness: $F = 0.07$; $p = 0.79$).

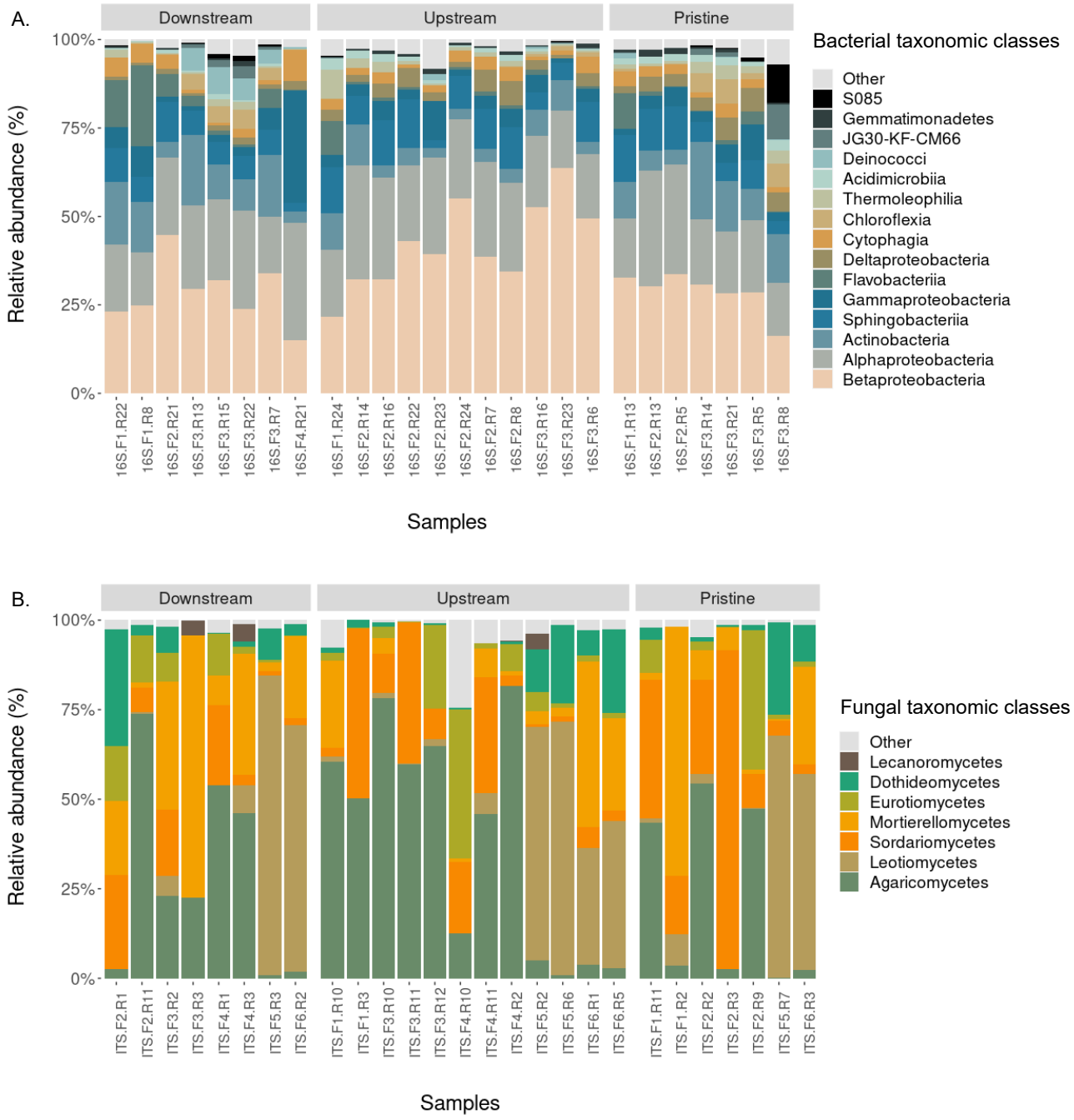

Figure 1.3. Relative abundance of bacterial (A) and fungal (B) taxonomic classes across 27 and 26 samples respectively of alder leaf litter from historically mining-impacted streams and reference streams in southern Québec, Canada. Classes containing at least 500 and 400 sequences respectively are shown, with relative abundance calculated based on rarefied community data.

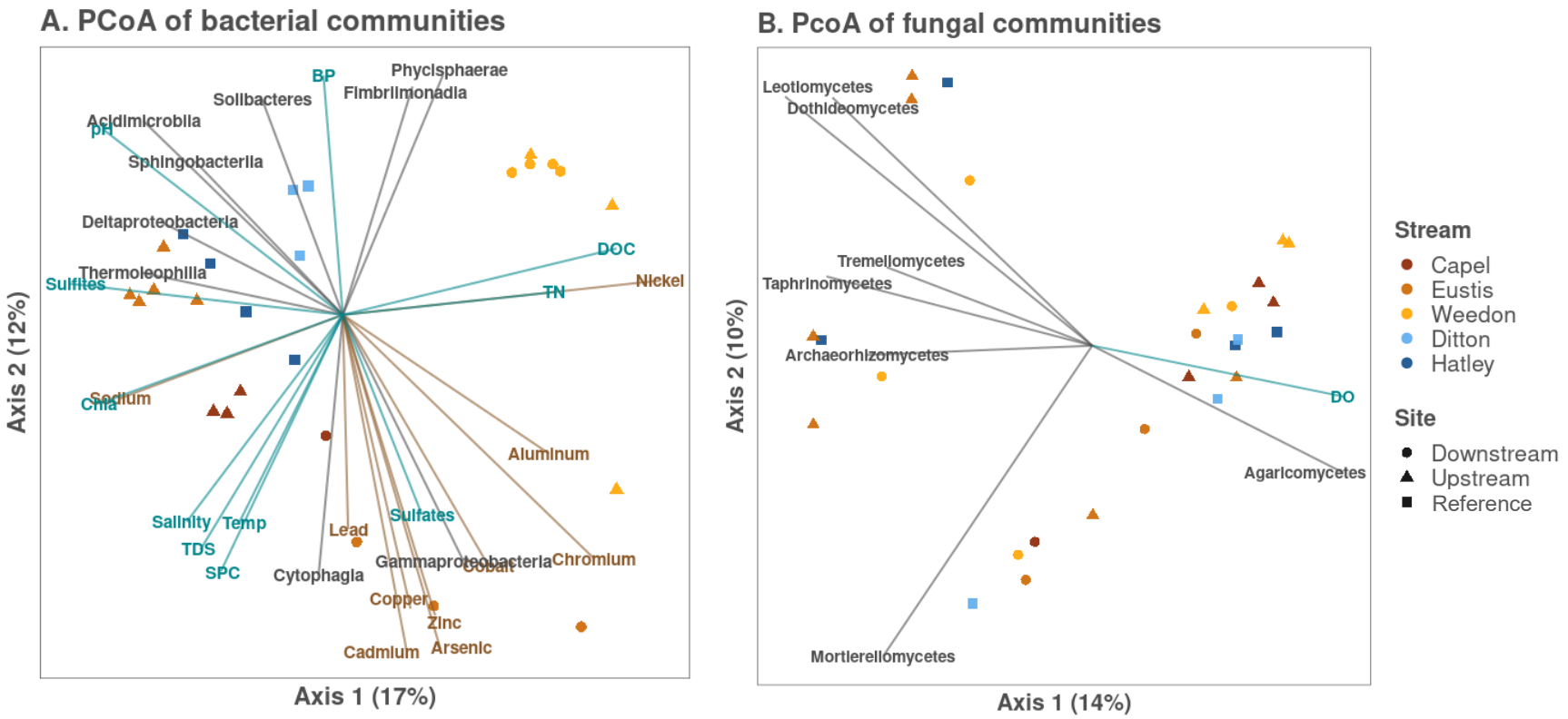

Figure 1.4. Principal coordinates analyses (PCoA) of bacterial (A) and fungal (B) communities with Bray-Curtis dissimilarity. Warm colours indicate metal-contaminated streams and cold colours indicate uncontaminated streams. Community data was rarefied prior to analysis. Rarefied community data was aggregated at the class level, and classes with fewer than 10 ASVs per sample were filtered out to plot the class arrows. Grey arrows represent the significant (adjusted p-value < 0.05) correlations between classes and the ordination axes. Blue arrows represent the significant (adjusted p-value < 0.05) correlations between water chemistry variables and the ordination axes. Orange arrows represent the significant (adjusted p-value < 0.05) correlations between metal concentrations and the ordination axes.

Bacterial communities were distinguishable by their stream of origin as well as their location within-stream, i.e., upstream or downstream of the mining site (Figure 1.4). Among metal-impacted streams, Eustis showed the highest difference in community composition between upstream and downstream sampling sites, while Weedon showed a slight overlap. The Capel stream also showed distinct community composition between sampling sites, despite the uneven distribution of samples between upstream (3) and downstream (1) sites due to an unprecedented weather event. Samples associated with high concentrations of heavy metals had characteristic bacterial communities with a high abundance of *Cytophagia* and *Gammaproteobacteria*. Among these metals, copper, arsenic, cadmium, chromium, nickel and zinc showed the highest correlations with the ordination axes. Samples from mining-impacted streams were also associated with higher concentrations of sulfates, dissolved organic carbon, salinity, conductivity, total dissolved solids, total nitrogen as well as higher temperatures. Samples associated with reference stream sites and low concentrations of heavy metals contained bacterial communities composed of numerous taxa, including *Deltaproteobacteria, Acidimicriobiia, Sphingobacteriia* and *Solibacteres*. In contrast, fungal communities showed no differentiation according to sampling location, and samples did not cluster following any recognizable environmental factor. Only one environmental variable, dissolved oxygen, was correlated with the ordination axes but did not differentiate fungal communities from different streams or locations within streams.

Following a DESeq2 analysis of bacterial class abundance between upstream and downstream sites, eight bacterial classes were significantly associated with downstream sites, and one class, *Deltaproteobacteria*, was significantly associated with upstream sites (Table B2). Four of the classes associated with downstream sites (*JG30-KF-CM66, JG37-AG-4, S085* and *Chloroflexia*) belong to the phylum *Chloroflexi*. When comparing the differential abundance of bacterial classes between downstream sites of mining-impacted streams and reference streams, *JG37-AG-4* was approximately 10 times more abundant at downstream sites. (P_{adj} = 0.0001) and *Deltaproteobacteria* was 3 times more abundant at reference sites (P_{adj} = 0.0002). DESeq2 analysis of fungal class abundance between overall mining-impacted sites and reference streams revealed *Rozellomycotina cls Incertae sedis* to be almost 24 times more abundant at mining-impacted streams (log2FoldChange = –4.24; P_{adj} = 0.017). This same taxon also showed a significant association with upstream sites when comparing the differential abundance of fungal classes

between upstream and downstream sites (log2FoldChange = 5.95; P_{adj} = 6.94E-08). Exobasidiomycetes showed a similar association with upstream sites, although to a lesser degree (log2FoldChange = 2.70; P_{adj} = 0.002).

Table 1.2. DESeq2 analysis of non-rarefied community data to observe the sum of significantly differential abundant ASVs between reference and mining streams and between upstream and downstream sites (adjusted p-value < 0.05). Shading represents the number of differentially abundant ASVs, the darker the shade the higher the sum of ASVs.

Kingdom	Class	Number of ASVs associated with sites			
		Reference	Mining	Upstream	Downstream
Bacteria	Acidimicrobiia	4	0	10	1
	Acidobacteria	3	0		
	Actinobacteria	9	2	9	12
	Alphaproteobacteria	59	23	65	33
	Bacilli	1	0	1	1
	Bacteroidia	1	0	0	1
	Betaproteobacteria	27	18	39	34
	Chloroflexia	12	0	1	14
	Clostridia	1	0		
	Cytophagia	3	1	5	9
	Deinococci	2	1	0	11
	Deltaproteobacteria	17	0	20	3
	Fimbriimonadia	1	0	1	4
	Flavobacteriia	3	3	1	12
	Gammaproteobacteria	8	1	16	10
	Gemmatimonadetes	5	0	1	1
	Holophagae			0	1
	JG30-KF-CM66	4	0	0	4
	JG37-AG-4			0	1
	OPB35_soil_group	2	0	1	0
	S085			0	3
	Solibacteres	3	0		
	Sphingobacteriia	24	9	25	15
	Subgroup_2	1	0		
	Thermoleophilia	2	0	4	1
	TK10	1	0	0	1
	NA	6	2	1	10
Fungi	Agaricomycetes	3	2	5	1
	Cladochytriomycetes			1	0
	Cystobasidiomycetes			2	1
	Dothideomycetes	5	1	17	0
	Eurotiomycetes	4	4	10	4
	Lecanoromycetes			1	0
	Leotiomycetes	14	6	33	13
	Malasseziomycetes	1	0		
	Microbotryomycetes			1	0
	Mortierellomycetes			4	1
	Pezizomycotina_cls_Incertae_sedis	1	0		
	Sordariomycetes	8	0	6	5
	Tremellomycetes	1	0	4	1
	NA	6	1	16	4

Mining-impacted streams showed differences in the abundance of certain bacterial and fungal ASVs when compared to reference streams (Table 1.2, Figure 1.5). Within mining-impacted streams, the abundance of certain bacterial and fungal ASVs was also different between sampling sites upstream and downstream of the mining grounds. In total, 259 differentially abundant bacterial ASVs and 57 fungal ASVs were identified between mining-impacted and reference streams, and 382 differentially abundant bacterial ASVs and 130 fungal ASVs were identified between upstream and downstream sites. The most differentially abundant bacterial ASVs between mining-impacted and reference streams belonged to the phyla *Saccharibacteria, Proteobacteria* and *Bacteroidetes* (Figure 1.5). Among the most differentially abundant *Proteobacteria* ASVs, two were associated with mining-impacted streams (genus *Dyella*), and two were associated with reference streams (families *Sandaracinaceae* and *Rhizobiaceae*) (Table B3). Only two phyla were represented among the differentially abundant fungal ASVs between mining-impacted and reference streams: Ascomycota and Basidiomycota (Figure 1.5). The Basidiomycota ASVs with the highest differential abundance were all from the order Agaricales. Within the Ascomycota phylum, two species were highly abundant in reference streams: *Tricladium angulatum* and *Xylomyces aquaticus* (Table B3).

There was a considerable overlap between the ASVs identified when comparing mining-impacted with reference streams and the differentially abundant ASVs between upstream and downstream samplings sites within mining-impacted streams, for both bacteria and fungi. These common ASVs also showed the same trend in their associations with different sites according to heavy metal concentrations; if they were more abundant at downstream sites when compared with upstream sites, then they were also more abundant at mining-impacted streams when compared with reference streams. In fact, seven out of the ten most differentially abundant bacterial ASVs between upstream and downstream sites were also among the ASVs identified between mining-impacted and reference streams (Table B4). The most differentially abundant bacterial ASVs between upstream and downstream sites belonged to the phyla *Saccharibacteria, Proteobacteria*, *Deinococcus-Thermus* and *Actinobacteria* (Figure 1.5). The differentially abundant fungal ASVs between upstream and downstream sites belonged to four different phyla: Ascomycota, Basidiomycota, Chytridiomycota and Mortierellomycota. Fungal ASVs with the highest differential abundance between upstream and downstream sites were part of the Helotiales

order (phylum: Ascomycota, class: Leotiomycetes). *Anguillospora filiformis* was highly associated with downstream sites and *Alatospora acuminata* was highly associated with upstream sites.

A few ASVs reappeared in every comparison between sampling sites (upstream, downstream and reference) and showed the most consistent sensitivity to varying degrees of metal pollution. The two bacterial ASVs that were common to all three comparisons of differential abundance belonged to the genus *Actinoplanes* (phylum *Actinobacteria*). The three fungal ASVs shared by all three comparisons of differential abundance were part of the Asomycota phylum. One of the ASVs was identified as *Tetracladium apiense*, and the two others were identified as members of the orders Venturiales and Helotiales.

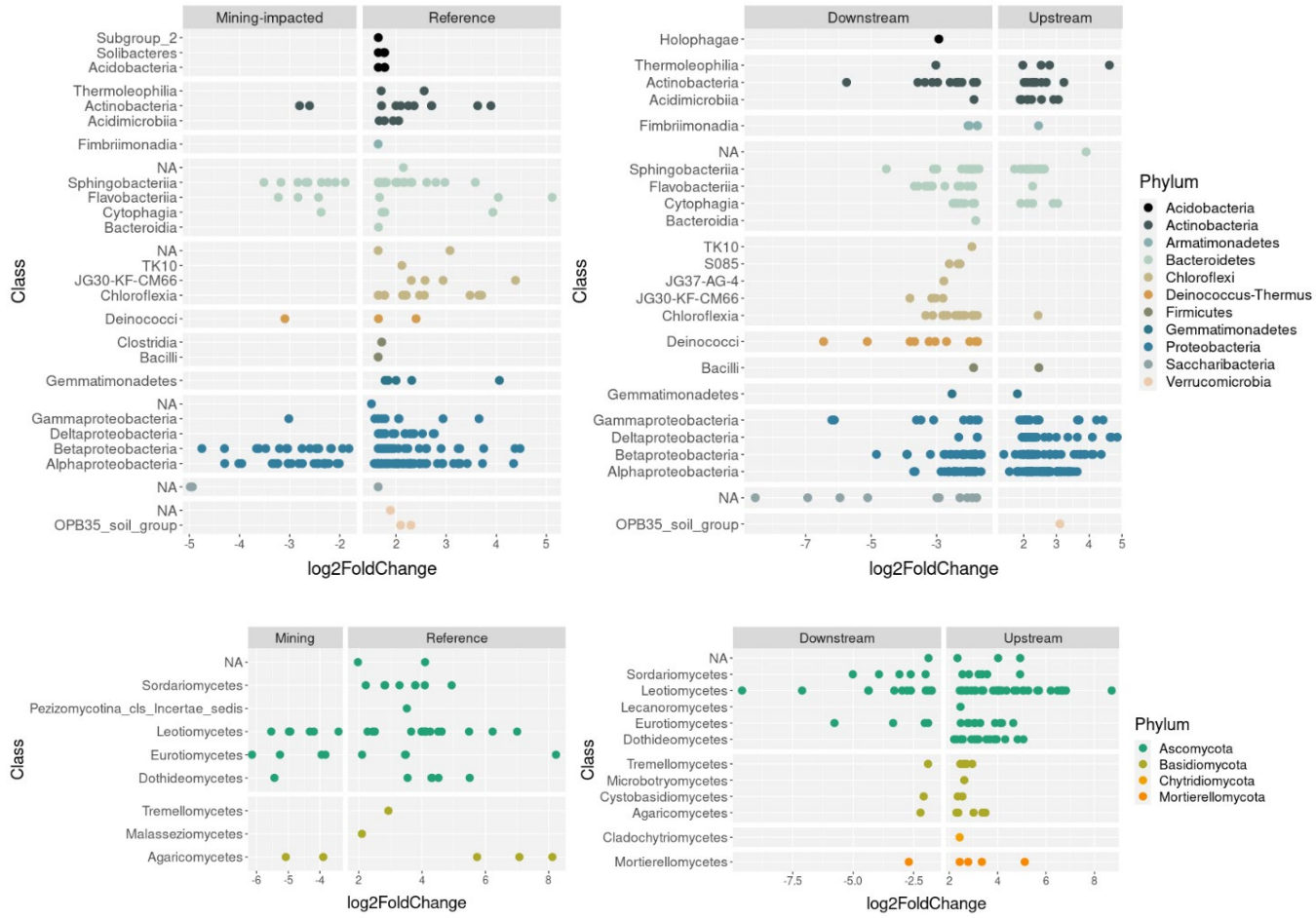

Figure 1.5. DESeq2 analysis of non-rarefied microbial (top) and fungal (bottom) community data to observe the significantly differential abundance of ASVs. Left panels are comparisons between reference and mining streams (p-value < 0.05), where positive values along the log2FoldChange axis are associated with reference streams and negatives values are associated with mining streams. Right panels are comparisons between upstream and downstream sites in mining-impacted streams (p-value < 0.05), where positive values along the log2FoldChange axis are associated with upstream sites and negatives values are associated with downstream sites. Every dot represents an ASV.

1.5 Discussion

The objectives of this study were to assess and compare the impacts of historical contamination from restored copper mines on bacterial and fungal leaf litter communities in streams. We extracted bacterial and fungal DNA from leaf litter bags submerged in five Canadian streams and used next-generation sequencing to identify changes in diversity and community composition. Our findings demonstrate the lasting impact of mining activity on aquatic leaf litter bacterial communities even decades after mine site remediation, especially downstream from mines where water concentrations of heavy metals remain elevated. Bacterial and fungal communities responded differently to higher concentrations of heavy metals in streams; bacterial communities showed marked differences in composition between reference and metal-impacted streams, while fungal communities did not respond to heavy metal concentration or differ between mining and reference streams. This difference in community response between fungi and bacteria supports our second prediction, as we did not expect fungal community composition to be strongly impacted by the presence of residual contamination from historical mining. However, the absence of significant differences in microbial diversity between contaminated and reference streams is not in line with our initial prediction. Our findings represent one of the first applications of microbial metabarcoding research on leaf litter communities under metal contamination in natural freshwater environments. By identifying compositional changes within these communities caused by historical mining and elevated heavy metal concentrations, our study contributes to an improved understanding of aquatic ecosystem responses to anthropogenic disturbances. Considering the important role of bacterial and fungal decomposers in lotic systems as well as current threats to freshwater biodiversity, a greater understanding of aquatic ecosystem responses that includes microbial processes is essential to improve restoration methods of metal-impacted environments.

1.5.1 Environmental sorting of bacterial communities

Changes in bacterial community structure covaried with several environmental variables, indicating potential negative effects of pH and elevated concentrations of heavy metals on specific classes, such as *Deltaproteobacteria, Acidimicriobiia, Sphingobacteriia* and *Solibacteres* (Figure 1.4). Our ordination also suggests that certain classes, such as *Cytophagia* and *Gammaproteobacteria,* tolerate or benefit from these conditions. Other studies have also shown shifts in stream-dwelling leaf litter bacterial communities in response to elevated metal

concentrations with short-term microcosm experiments (Duarte *et al.*, 2007; Duarte *et al.*, 2009; Pradhan *et al.*, 2011; Tlili *et al.*, 2017). However, most of these studies could not discern the taxa driving these changes in community composition due to the use of relatively low-resolution genetic fingerprinting techniques. In contrast, there is growing literature on the compositional changes in microbial sedimentary communities in aquatic environments impacted by high concentrations of heavy metals from mining or industrial activity (Blanchette *et al.*, 2019; Jacquiod *et al.*, 2018; Kavehei *et al.*, 2021; Moberly *et al.*, 2016; Reis *et al.*, 2016; Sutcliffe *et al.*, 2017; Wang, J. *et al.*, 2020; Xavier *et al.*, 2019; Xie *et al.*, 2016; Yang *et al.*, 2018; Yin *et al.*, 2015). Many of the taxa that have emerged from these sediment community studies as being significantly impacted by mining activity were also identified as such in our leaf-litter community analyses. For example, we found that *Chloroflexi*, *Deinococci* and *Saccharibacteria* responded positively to higher concentrations of heavy metals. The greater abundance of *Chloroflexi* found in our more heavily impacted sites is concordant with this phylum's frequently observed abundance in metal-contaminated river sediments (Xavier *et al.*, 2019; Xie *et al.*, 2016; Yin *et al.*, 2015). Our findings are also consistent with those of Sutcliffe *et al.* (2019), who found the phylum *Deinococcus-Thermus* to have a higher relative abundance in sediments spiked with varying copper concentrations. However, *Saccharibacteria* is a ubiquitous phylum that has only been described based on uncultivated 16S rRNA gene sequences and genome data, and very little is known about its biology (Ferrari *et al.*, 2014).

Diverging trends within certain phyla also revealed adaptations to heavy metals that appear at different phylogenetic depths. For example, *Chloroflexi* seems well-adapted to higher concentrations of heavy metals at the class level, but some individual ASVs within this class were also associated with lower metal concentrations at reference streams. Similarly, ASVs and classes from the *Proteobacteria* phylum were found in strong associations with both higher and lower concentrations of heavy metals. Since phyla like *Chloroflexi* and *Proteobacteria* are large and contain a diversity of phenotypes, their morphological and metabolic diversity (Xavier *et al.*, 2019) may explain their ubiquity at both mining-impacted and reference stream sites in our study. It is important to consider that phylum-level approaches to microbial community composition can discard valuable information when amalgamating data from different taxa, and it is difficult to generalize about the ecology of large and diverse microbial taxa using high taxonomic ranks. This

highlights one of the advantages of using high-resolution ASV-based metabarcoding approaches that identify the responses of different microbial taxonomic ranks to facilitate broader understanding of the responses of microbes to heavy metals.

Overall, our study identified associations between numerous bacterial taxa at different phylogenetic levels and mining-impacted conditions in streams. These groups are potential indicators of mining activity, and research is already underway to investigate whether these groups have genomic adaptations that can explain their tolerance to heavy metals. For example, while investigating the genetic profile of metal resistance in bacterial communities from river sediments, Xavier *et al.* (2019) found that taxa within *Proteobacteria* possess zinc and copper resistance genes. Further studies will be needed to investigate the taxa identified in our study as potential indicators of historical and on-going mining contamination to better understand their genomic adaptations and ecological behaviour.

1.5.2 Fungal community composition

In contrast with bacteria, fungal communities were not impacted by mining or elevated concentrations of heavy metals, as community dissimilarity was not linked to any of the environmental variables measured (Figure 1.4). The lack of metal-driven community dissimilarities in our fungal samples was in line with our predictions, but the absence of community patterns related to any measured variable goes against expectations. Other studies conducted at similar local scales have found several abiotic factors that shape the distribution of fungal communities in streams, including pH, temperature, conductivity and nutrients in stream water (Duarte *et al.*, 2017). Although there were no community-wide effects of heavy metals on fungi, we did identify several fungal taxa, including the classes Rozellomycotina cls Incertae sedis and Exobasidiomycetes, and several fungal ASVs that were significantly more abundant upstream of the mining sites compared to downstream sites in metal-impacted streams. Nonetheless, these differences were not associated with differences in water metal concentrations among sites.

Our findings are consistent with other research that did not find any association between fungal community composition and stream metal pollution exposure over 40 years, but we did find certain fungal taxa associated with elevated metal concentrations (Emilson *et al.*, 2016). Over the short

term (20–118 days), other studies involving microcosm experiments with heavy metal exposure have found that aquatic fungal composition varied according to metal concentration gradients and exposure time (Duarte *et al.*, 2007; Duarte *et al.*, 2009; Pradhan *et al.*, 2011; Sutcliffe *et al.*, 2019; Tlili *et al.*, 2017). However, a study that followed fungal leaf litter community composition after being released from metal stress found that 10 days later, fungal communities had recovered and there were no significant differences between treatments (Duarte *et al.*, 2009). The stochastic nature of our fungal community dissimilarities could be explained in part by the time elapsed between their initial exposure to high concentrations of mining residues and our study. This gap, which spans over a century, might have allowed the communities to readapt or shift into diverse metal-tolerant communities shaped by random processes or unaccounted factors rather than residual metal contamination. It is also possible that fungal dispersal by spores has allowed fungi to rapidly recolonize impacted streams with less metal-tolerant taxa once metal concentrations fell. This interpretation is supported by research suggesting that fungi enter stream ecosystems mainly via falling leaves, while bacteria come from sediments or the water column (Hayer *et al.*, 2021). Community origin could potentially explain differences in our bacterial and fungal responses, especially if in-water repositories of metal-tolerant bacterial taxa are responsible for the legacy effects of mining on bacterial community composition. Sampling communities from different habitats on-site, such as biofilms, sediments and deciduous leaves before they enter the streams, could help identify the source of seed taxa that colonize in-stream leaf litter in mining-impacted sites.

1.5.3 Similar diversity indices between reference and metal-impacted streams

Neither bacterial nor fungal communities showed any significant difference in diversity between stream types. Early studies on microbial decomposers that relied on fungal sporulation and DGGE found fungal diversity to be negatively impacted by higher concentrations of heavy metals (Duarte *et al.*, 2007; Duarte *et al.*, 2009; Medeiros *et al.*, 2010; Niyogi *et al.*, 2002; Pradhan *et al.*, 2011). However, not all studies found an obvious relationship between fungi and heavy metal concentrations (Duarte *et al.*, 2004; Sridhar *et al.*, 2000). Very few metabarcoding studies have measured the impact of elevated metal concentrations on bacterial decomposers, but similar studies focusing on sediment and leaf-analogue communities have found no significant difference in

microbial alpha diversity despite shifts in community composition (Sutcliffe *et al.*, 2018; Yin *et al.*, 2015).

Progress in microbial identification has allowed more accurate representations of aquatic microbial decomposer community composition and diversity. This might explain why, through high-throughput sequencing, our study identified more metal-tolerant species that could not have been detected with earlier methods. However, it is also possible that the time elapsed between the initial exposure to high concentrations of mining residues and our study (~100 years) allowed for the communities to readapt or shift into a diverse metal-tolerant community. After all, certain strains of fungi and bacteria can become resilient to copper toxicity by developing various resistance mechanisms (Cervantes and Gutierrez-Corona, 1994) that allow them to survive in contaminated aquatic environments and gain a competitive advantage (Gardham *et al*., 2014). There are also bacterial taxa whose metabolic processes, such as metal respiration, benefit from higher concentrations of copper, allowing them to thrive in these harsher conditions (Sutcliffe *et al.*, 2019).

1.5.4 Conclusion

In summary, temperate forest streams impacted by historical copper mining exhibited distinct bacterial community composition following a gradient of heavy metal contamination, while fungal community composition appeared unimpacted. Neither bacterial nor fungal alpha-diversity differed between mining-impacted and reference streams, nor did they show any variation between sites upstream and downstream of mining grounds within mining-impacted streams. Even decades after mine site restoration, we observed a lasting impact of mining contaminants on specific bacterial and fungal taxa at varying phylogenetic depths. These sensitive taxa may serve as potential indicators of degraded environments and higher concentrations of heavy metals associated with mining activity. Our study illustrates the importance of incorporating microbial decomposers in future research in order to gain a better understanding of lotic ecosystem responses to long-term perturbations and consequently improve restoration and monitoring efforts.

CONCLUSION

Abandoned metal mines are widespread across landscapes and have been contaminating aquatic ecosystems for over a century (Fields, 2003; Schwarzenbach *et al.*, 2010). Despite the prevalence of historical mines, there is limited knowledge of their long-term impacts on microbial decomposer communities in freshwater streams (Zinger *et al.*, 2012). It is essential to gain a better understanding of these ecological impacts, as freshwater streams provide valuable ecosystem services (Yeakley *et al.*, 2016), and microbial processes are essential to their function (Leff, 2019; Tolkkinen, M. J. *et al.*, 2020). Indeed, microbial communities play a crucial role in the early stages of decomposition of organic matter and nutrient cycling in streams (Marks, 2019), providing aquatic-terrestrial subsidies to surrounding ecosystems (Marks, 2019).

Previous studies on microbial decomposers in mining-impacted freshwater environments relied of microscopic and molecular identification techniques (e.g., Duarte *et al.*, 2007) and could not achieve the same in-depth analyses of community composition and diversity that are now possible through high-throughput sequencing of microbial barcode genes. However, current metabarcoding research on the effects of mining on aquatic microbial communities has focused primarily on mining-impacted water and sediment microbial communities (e.g., Blanchette *et al.*, 2019) and has largely omitted leaf litter. By applying DNA metabarcoding to study the impact of historical copper mining on bacterial and fungal leaf litter communities, we can improve our understanding of microbial decomposers in streams and their response to long-term perturbations.

The objective of this thesis was to use next-generation sequencing to determine if microbial leaf litter communities in streams contaminated by past copper mines continue to be impacted by this historical stressor despite various restoration efforts over the last three decades (Berryman *et al.*, 2003; Painchaud, 2007; St-Onge and Richard, 1996). A second objective was to compare the responses of aquatic bacterial and fungal decomposers to elevated concentrations of heavy metals caused by historical copper mining.

The main findings of this study are that bacterial community composition remain impacted by historical copper mining while fungal community composition, fungal diversity and bacterial

diversity were unimpacted after 30 years of mine reclamation. My results also singled out associations between numerous bacterial taxa at different phylogenetic levels and mining-impacted conditions in streams. Changes in bacterial community structure indicated the potential negative effects of heavy metals on specific classes, such as *Deltaproteobacteria, Acidimicriobiia, Sphingobacteriia* and *Solibacteres*. These changes also suggested that other classes, such as *Cytophagia* and *Gammaproteobacteria,* tolerate or benefit from these conditions. Comparisons of relative abundance between sites showed that *Chloroflexi*, *Deinococci* and *Saccharibacteria* responded positively to higher concentrations of heavy metals. Diverging trends within certain phyla also revealed adaptations to heavy metals that appear at different phylogenetic depths. This means that while some phyla may seem well adapted to high concentrations of heavy metals, lower taxonomic ranks and individual ASVs within a phylum can display different sensitivities to historical mining activity. Additionally, two fungal species, *Anguillospora filiformis* and *Alatospora acuminata,* showed strong associations with downstream mining sites and upstream mining sites respectively.

2.1 Limitations

No bacterial species were identified in association with mining-impacted or reference streams, but this is due to the limitations of our sequencing approach, as the primers used in 16S rRNA gene amplicon sequencing typically provide only family or genus-level taxonomy (Janda and Abbott, 2007). Another limitation of our study is the number of leaf litter samples and their uneven distribution among sites. To improve the scope of this study, a higher number of samples and sites would be necessary to even out sample distribution and improve the statistical power of certain analyses, like comparisons of heavy metals between sites. The loss of half of our community samples was caused by an unprecedented storm and therefore beyond our control, but by sampling a larger number of streams, our conclusions on bacterial and fungal diversity could be strengthened. Due to this storm, we also lost data on decomposition rates and macroinvertebrate communities, which explains why these variables were not integrated in the study. If this data had been available in full, it would have been interesting to see whether there were dissimilarities in leaf litter degradation between sites, and if they could be associated with differences found in bacterial community composition. Likewise, it would have been interesting to see whether differences in

macroinvertebrate communities had an impact on fungal community composition, since fungal communities appear unimpacted by the variables measured in this study.

2.2 Significance and future research

My results demonstrate that even a century after initial exposure to high concentrations of heavy metals, and decades after mine site restoration, aquatic leaf litter bacterial communities are still affected by residual mining contaminants in streams. Through changes in community composition, bacteria that reside on leaf litter could be good indicators of lasting mining impacts on aquatic ecosystems and their function. The bacterial taxa identified in our study in association with mining-impacted streams are potential indicators, but further studies are needed to investigate these taxa to better understand their genomic adaptations and ecological behaviour. Conversely, the lack of a consistent response to elevated metal concentrations among fungal communities suggests that this group is less useful as an aquatic biomonitoring tool. Perhaps it would be necessary to integrate other measures of fungal communities, such as their biomass, to use them efficiently in stream biomonitoring, as is exemplified in Emilson's study of historically contaminated watersheds (Emilson *et al.*, 2016).

Another interesting addition to this branch of research would be the study of community succession after mine restoration, by sampling microbial community composition and diversity before mine site restoration and at regular intervals afterwards. This could give us insight into the microbial succession and recolonization that occurs within metal-contaminated environments as concentrations of heavy metals are reduced by restoration efforts. Shifts in microbial community composition and structure were observed at different oxidation stages in acid mine drainage environments (Teng *et al.*, 2017), and it would be interesting to observe similar shifts in microbial community composition in less extreme contaminated aquatic environments as they are slowly restored. Given their essential role in energy and carbon flux within streams, it is important to integrate eDNA metabarcoding of leaf litter bacterial communities, alongside sediment and water column microbial communities, into aquatic biomonitoring and mine restoration efforts (Ruppert *et al.*, 2019).

APPENDIX A

SUPPLEMENTARY INFORMATION ON METHODS

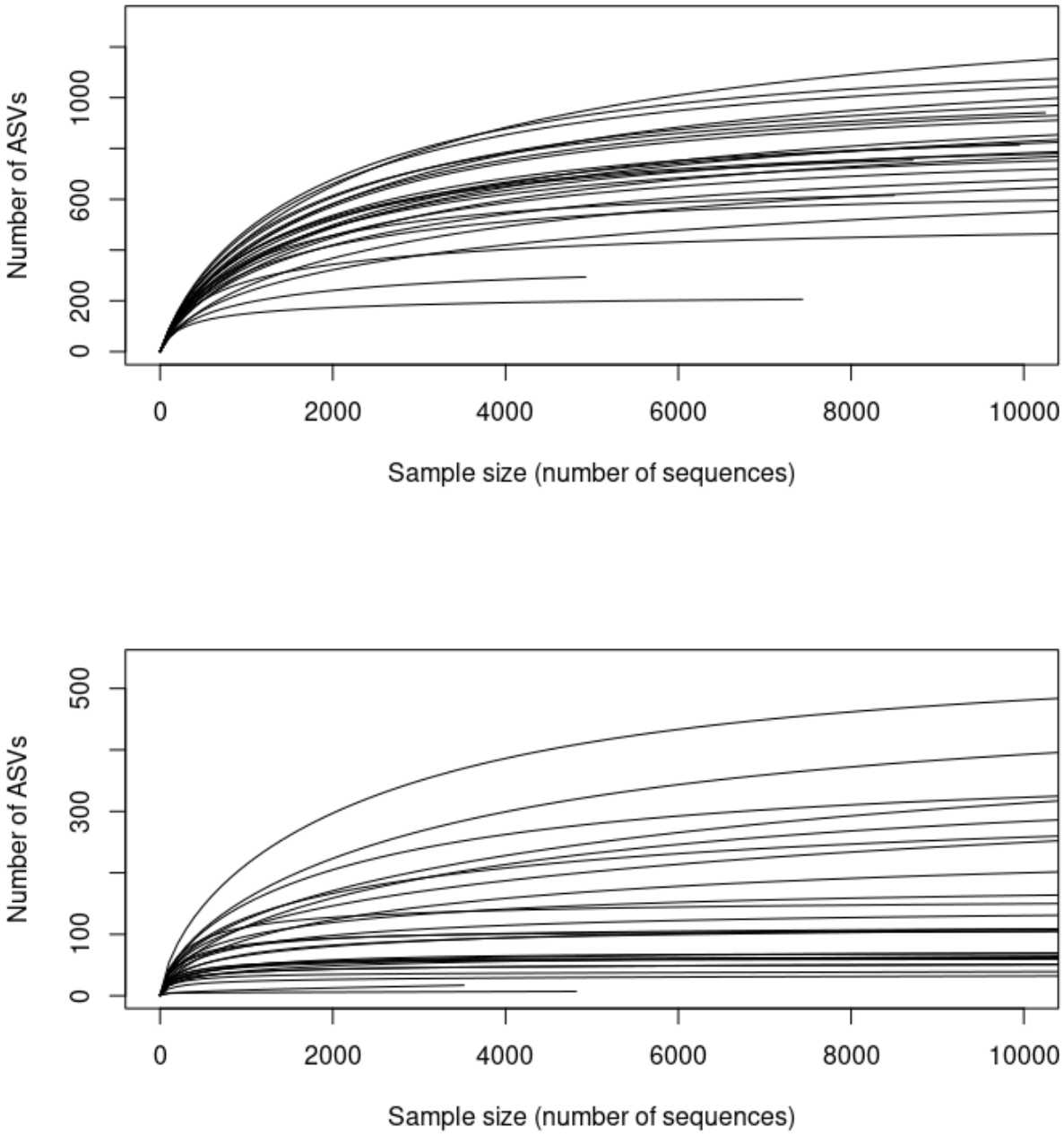

Figure A 1. Rarefaction curves of bacterial (top) and fungal (bottom) samples in mesh bags placed in mining-impacted and reference streams in southern Québec, Canada.

Table A 1. Distribution of fine and coarse mesh bags successfully retrieved from their experimental placements in mining-impacted and reference streams in southern Québec, Canada.

	Capel		Eustis		Weedon		Hatley (Reference)	Ditton (Reference)	Total
	Upstream	Downstream	Upstream	Downstream	Upstream	Downstream			
Fine	1	0	3	2	1	3	2	1	13
Coarse	2	1	3	1	2	1	2	2	14
Total	3	1	6	3	3	4	4	3	27

Table A 2. Percent of mass loss from leaf litter bags placed in mining-impacted and reference streams in southern Québec, Canada.

Bag ID	Site	Type of Bag	Initial Dry Weight (g)	Final Dry Weight (g)	Loss (g)	Loss (%)
25A	Ditton (Reference)	Coarse	2.97	1.95	1.02	34
27B	Ditton (Reference)	Fine	3.02	2.11	0.91	30
28A	Ditton (Reference)	Coarse	2.98	1.97	1.01	34
31A	Hatley (Reference)	Coarse	3.05	1.57	1.48	49
34A	Hatley (Reference)	Coarse	3.01	2.88	0.13	4
36A	Hatley (Reference)	Coarse	2.99	1.59	1.40	47
36B	Hatley (Reference)	Fine	3.03	2.04	0.99	33
39B	Weedon Upstream	Fine	3.04	1.86	1.18	39
40A	Weedon Upstream	Coarse	3.05	1.56	1.49	49
42A	Weedon Upstream	Coarse	3.02	0.76	2.26	75
43A	Weedon Downstream	Coarse	3.05	1.31	1.74	57
44B	Weedon Downstream	Fine	2.97	2.55	0.42	14
45B	Weedon Downstream	Fine	2.98	1.77	1.21	41
46B	Weedon Downstream	Fine	2.99	2.19	0.80	27
49A	Capel Downstream	Coarse	2.99	2.05	0.94	31
58A	Capel Upstream	Coarse	3.04	1.37	1.67	55
60A	Capel Upstream	Coarse	3.05	1.10	1.95	64
60B	Capel Upstream	Fine	3.02	1.96	1.06	35
61A	Eustis Upstream	Coarse	3.20	1.54	1.66	52
62B	Eustis Upstream	Fine	3.02	2.28	0.74	25
63B	Eustis Upstream	Fine	3.03	1.33	1.70	56
64A	Eustis Upstream	Coarse	3.00	1.43	1.57	52
66A	Eustis Upstream	Coarse	3.18	2.31	0.87	27
66B	Eustis Upstream	Fine	3.01	1.43	1.58	52
74B	Eustis Downstream	Fine	2.96	2.33	0.63	21
78A	Eustis Downstream	Coarse	3.07	2.75	0.32	10
78B	Eustis Downstream	Fine	3.00	2.18	0.82	27

Table A 3. Macroinvertebrate abundance found in coarse mesh bags placed in mining-impacted and reference streams in southern Québec, Canada.

Bag ID	Site	Coleoptera		Diptera		Ephemeroptera				Plecoptera			Trichoptera	Gastropoda	Total
		Carabidae	Elmidae	Chironimidae	Tipulidae	Isonychiidae	Baetidae	Ephemeridae	Unknown	Lectridae	Capniidae	Nemouridae	Hydropsychidae		
25A	Ditton (Reference)	0	0	0	0	0	0	0	0	23	8	0	0	0	31
27B	Ditton (Reference)	0	0	18	0	0	0	0	0	3	2	0	1	0	24
28A	Ditton (Reference)	0	0	0	0	0	0	0	0	38	25	1	0	0	64
32B	Hatley (Reference)	1	0	58	0	1	0	0	0	0	0	0	0	0	60
36B	Hatley (Reference)	0	0	51	2	0	0	0	0	0	0	0	0	0	53
39B	Weedon Upstream	0	0	6	0	0	0	0	1	1	2	0	2	0	12
40A	Weedon Upstream	0	0	0	0	0	0	0	0	7	0	0	0	0	7
44B	Weedon Downstream	0	0	6	1	0	0	0	0	1	0	0	0	0	8
45B	Weedon Downstream	0	0	1	0	0	0	0	0	0	0	0	0	0	1
48B	Weedon Downstream	0	0	2	0	0	0	0	0	0	0	0	0	0	2
49A	Capel Downstream	0	0	0	0	0	0	0	0	1	0	0	0	0	1
58A	Capel Upstream	0	0	0	0	0	0	0	0	1	0	0	0	0	1
60B	Capel Upstream	0	0	8	0	0	0	0	0	0	0	0	0	0	8
62B	Eustis Upstream	0	0	20	0	0	0	1	0	0	1	0	0	0	22
63B	Eustis Upstream	0	0	18	0	0	1	0	0	2	0	0	0	0	21
64A	Eustis Upstream	0	0	0	0	0	0	0	0	4	4	0	0	0	8
66B	Eustis Upstream	0	0	15	0	0	0	0	0	3	3	0	0	0	21
74B	Eustis Downstream	0	0	0	0	0	0	0	0	0	0	0	0	1	0
78B	Eustis Downstream	0	0	2	0	0	0	0	0	0	0	0	0	0	2

APPENDIX B

SUPPLEMENTARY RESULTS

Table B 1. Water chemistry variables measured in mining-impacted and reference streams in southern Québec, Canada. Values at beginning and end of experiment are presented chronologically. SPC, Specific conductivity; TDS, Total dissolved solids; DO, Dissolved oxygen; DOC, Dissolved organic carbon; TP, Total phosphate; TN, Total nitrogen. Sulfates and sulfites were measured only at the end of the experiment.

Site	Symbol	pH	Salinity (ppt)	SPC (uS/cm)	TDS (mg/L)	DO (mg/L)	DOC (mg/L)	TP (ug/L)	TN (ppm)	chl a (ug/L)	Bacterial Production	Sulfates (mg/L)	Sulfites (mg/L)
Reference (Hatley)	Ref1	7.7 -7.7	0.3 - 0.2	679 - 350.1	NA - 229.8	9.89 - 14.26	3.4 - 9.5	14.7 - 23.3	0.3 - 1.4	2.0 - 1.4	30358 - 13097	8.0	2.5
Reference (Ditton)	SW6	7.9 - 7.6	0.1 - 0.0	195 - 97.1	127 - 63.1	11.07 - 15.24	2.0 - 10.2	8.2 - 10.6	0.2 - 0.5	1.4 - 0.7	36946.7 - 32128.7	3.3	2.3
Weedon Upstream	WU	6.8 - 6.7	0.1 - 0.1	103.1 - 119.1	67 - 77.4	9.54 - 12.54	20.7 - 35.8	44.1 - 12.8	0.2 - 1.1	0.8 - 0.5	21920.3 - 751	14.8	0.0
Weedon Downstream	WD	7.2 - 7.6	0.1 - 0.1	278.2 - 250.4	180.7 - 162.5	8.82 - 3.12	12.8 - 21.2	25.3 - 9.3	0.2 - 1.3	0.8 - 0.5	49005.3 - 478.7	28.1	1.6
Capel Upstream	CU	8.0 - 7.5	0.3 - 0.1	711 - 280	461.4 - 291.5	9.02 - 17.24	4.5 - 5.2	21 - 10.1	0.2 - 0.3	1.2 - 0.6	17912.3 - 18609	30.0	2.5
Capel Downstream	CD	7.5 - 6.4	0.4 - 0.3	759 - 575	500.5 - 373.1	0 - 18.3	4.3 - 2.9	22.2 - 9.7	0.2 - 0.3	1.3 - 0.2	23236.7 - 598.7	82.8	0.0
Eustis Upstream	EU	7.7 - 7.5	0.2 - 0.2	473.5 - 317.4	302.9 - 206.1	9.65 - 3.8	2.5 - 19.1	70.8 - 6.7	0.2 - 0.3	0.9 - 2.1	18959.3 - 4069.3	14.8	2.5
Eustis Midstream	EM	7.9 - 7.8	0.3 - 0.2	507.7 - 331.6	330.9 - 215.2	10.93 - 13.76	2.6 - 1.7	8.5 - 11.2	0.1 - 1.3	1.2 - 0.6	15073.7 - 2975.7	20.5	1.6
Eustis Downstream	ED	6.3 - 7.1	0.3 - 0.2	613 - 654.1	399.1 - 230.8	10.13 - 13.17	0.9 - 25.5	52.4 - 10.3	0.1 - 1.3	1.8 - 0.9	5309.5 - 874.3	43.5	1.6

Table B 2. DESeq2 analysis of class-aggregated non-rarefied bacterial community data to observe their significantly differential abundance between upstream and downstream sites (adjusted p-value < 0.05). Positive values in log2FoldChange column are associated with upstream sites and negatives values are associated with downstream sites.

Class	log2FoldChange	padj
Deinococci	-3.9425	9.9E-04
JG30-KF-CM66	-2.9201	0.02
JG37-AG-4	-2.8330	9.9E-04
S085	-2.5803	0.03
Chloroflexia	-2.5086	0.03
Flavobacteriia	-2.2799	0.02
Bacteroidia	-1.9837	0.03
Holophagae	-1.8314	0.03
Deltaproteobacteria	1.7292	3.4E-03

Table B 3. DESeq2 analysis of non-rarefied community data to observe the top 10 highest differential abundance of bacterial and fungal ASVs between reference and mining-impacted streams (adjusted p-value < 0.05). Positive values along the log2FoldChange axis are associated with reference streams and negatives values are associated with mining-impacted streams.

Associated streams	ASV	log2FoldChange	padj	Kingdom	Phylum	Class	Order	Family	Genus	Species
Mining	ASV30	-8.5393	2.6E-11	Bacteria	Saccharibacteria	NA	NA	NA	NA	NA
	ASV124	-6.7110	8.0E-08	Bacteria	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	Dyella	NA
	ASV154	-6.5713	1.8E-07	Bacteria	Deinococcus-Thermus	Deinococci	Deinococcales	Deinococcaceae	NA	NA
	ASV110	-6.5087	4.8E-07	Bacteria	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	Dyella	NA
	ASV15	-6.4353	2.0E-07	Bacteria	Actinobacteria	Actinobacteria	Micrococcales	Microbacteriaceae	Lysinimonas	NA
	ASV116	-6.2863	6.1E-11	Bacteria	Saccharibacteria	NA	NA	NA	NA	NA
	ASV228	-6.0712	8.6E-07	Bacteria	Saccharibacteria	NA	NA	NA	NA	NA
Reference	ASV49	6.4834	5.0E-07	Bacteria	Actinobacteria	Actinobacteria	Micromonosporales	Micromonosporaceae	Actinoplanes	NA
	ASV132	6.1253	4.6E-06	Bacteria	Proteobacteria	Deltaproteobacteria	Myxococcales	Sandaracinaceae	NA	NA
	ASV163	6.0993	9.0E-06	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	Rhizobium	NA
Mining	ASV74	-6.1453	3.3E-04	Fungi	Ascomycota	Eurotiomycetes	Chaetothyriales	Herpotrichiellaceae	Exophiala	NA
	ASV68	-5.5391	1.2E-03	Fungi	Ascomycota	Leotiomycetes	Helotiales	Leotiaceae	Alatospora	NA
Reference	ASV152	8.2290	2.1E-12	Fungi	Ascomycota	Eurotiomycetes	Eurotiales	Trichocomaceae	Talaromyces	NA
	ASV169	8.1141	2.1E-12	Fungi	Basidiomycota	Agaricomycetes	Agaricales	NA	NA	NA
	ASV180	7.0716	1.7E-10	Fungi	Basidiomycota	Agaricomycetes	Agaricales	NA	NA	NA
	ASV163	7.0026	3.4E-09	Fungi	Ascomycota	Leotiomycetes	Helotiales	Helotiaceae	Tricladium	angulatum
	ASV208	6.8597	3.5E-10	NA	NA	NA	NA	NA	NA	NA
	ASV386	6.2268	3.4E-09	Fungi	Ascomycota	Leotiomycetes	Helotiales	Leotiaceae	Alatospora	NA
	ASV186	5.7363	6.6E-08	Fungi	Basidiomycota	Agaricomycetes	Agaricales	NA	NA	NA
	ASV292	5.5024	5.5E-06	Fungi	Ascomycota	Dothideomycetes	Jahnulales	Aliquandostipitaceae	Xylomyces	aquaticus

Table B 4. DESeq2 analysis of non-rarefied community data to observe the top 10 highest differential abundance of bacterial and fungal ASVs between upstream and downstream sites (adjusted p-value < 0.05). Positive values along the log2FoldChange axis are associated with upstream sites and negatives values are associated with downstream sites.

Associated sites	ASV	log2FoldChange	padj	Kingdom	Phylum	Class	Order	Family	Genus	Species
Downstream	ASV30	-8.5367	2.0E-16	Bacteria	Saccharibacteria	NA	NA	NA	NA	NA
	ASV116	-6.9355	9.5E-17	Bacteria	Saccharibacteria	NA	NA	NA	NA	NA
	ASV154	-6.4527	3.6E-10	Bacteria	Deinococcus-Thermus	Deinococci	Deinococcales	Deinococcaceae	NA	NA
	ASV110	-6.1989	3.8E-09	Bacteria	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	Dyella	NA
	ASV124	-6.1236	7.8E-10	Bacteria	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	Dyella	NA
	ASV228	-5.9526	3.5E-09	Bacteria	Saccharibacteria	NA	NA	NA	NA	NA
	ASV15	-5.7499	3.2E-08	Bacteria	Actinobacteria	Actinobacteria	Micrococcales	Microbacteriaceae	Lysinimonas	NA
	ASV295	-5.1174	2.2E-06	Bacteria	Deinococcus-Thermus	Deinococci	Deinococcales	Deinococcaceae	NA	NA
	ASV122	-5.1052	1.3E-06	Bacteria	Saccharibacteria	NA	NA	NA	NA	NA
Upstream	ASV219	4.8565	1.3E-04	Bacteria	Proteobacteria	Deltaproteobacteria	Myxococcales	Sandaracinaceae	NA	NA
Downstream	ASV7	-9.5928	3.7E-11	Fungi	Ascomycota	Leotiomycetes	Helotiales	Helotiaceae	Anguillospora	filiformis
	ASV82	-7.1113	1.0E-07	Fungi	Ascomycota	Leotiomycetes	Helotiales	Helotiaceae	Anguillospora	filiformis
Upstream	ASV43	8.7139	3.7E-07	Fungi	Ascomycota	Leotiomycetes	Helotiales	Leotiaceae	Alatospora	NA
	ASV141	6.8235	1.3E-04	Fungi	Ascomycota	Leotiomycetes	Helotiales	NA	NA	NA
	ASV91	6.6932	1.5E-04	Fungi	Ascomycota	Leotiomycetes	Helotiales	Leotiaceae	Alatospora	acuminata
	ASV67	6.6758	1.7E-04	Fungi	Ascomycota	Leotiomycetes	Helotiales	Leotiaceae	Alatospora	acuminata
	ASV128	6.6020	1.3E-04	Fungi	Ascomycota	Leotiomycetes	Helotiales	Leotiaceae	Alatospora	NA
	ASV13	6.4772	9.8E-04	Fungi	Ascomycota	Leotiomycetes	Helotiales	Hyaloscyphaceae	Lemonniera	NA
	ASV55	6.1958	4.7E-04	Fungi	Ascomycota	Leotiomycetes	Helotiales	Leotiaceae	Alatospora	NA
	ASV59	6.0467	2.6E-04	Fungi	NA	NA	NA	NA	NA	NA

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