Responses of the Soil Microbial Community to Weathering of Ore Minerals

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Simister, R.L., Winterburn, P.A. and Crowe, S.A. (2018): Responses of the soil microbial community to weathering of ore minerals; *in* Geoscience BC Summary of Activities 2017: Minerals and Mining, Geoscience BC, Report 2018-1, p. 57–68.

Introduction

As global population grows and modernizes, demand for mineral resources is expanding (Kesler, 2007). At the same time, existing orebodies are being exhausted, while the frequency of new discoveries of exposed or partially exposed deposits diminishes. Demand for mineral resources must therefore be met through the discovery and development of buried or concealed mineral deposits. Although mineral resource extraction supported the core of the Canadian economy for over a century—currently contributing $56 billion to Canada's GDP and providing 19% of its goods exports (The Mining Association of Canada, 2017)—its ability to do so relies on continued discovery of mineral deposits that may be concealed by overburden. Finding these mineral deposits beneath exotic overburden consisting of glacial and preglacial sediments remains a fundamental and widespread challenge to mineral exploration in Canada (Anderson et al., 2012; Ferbey et al., 2014).

New and innovative techniques that complement, enhance or even surpass traditional techniques to define the surface expression of buried ore mineralization could minimize the cost of exploration and help in targeting drilling activities (Kelley et al., 2006). Several recent studies in British Columbia (BC) have demonstrated the potential for new surface geochemical techniques to lead to the discovery of concealed orebodies. These include indicator minerals (Plouffe et al., 2013a, b), soil partial leach and selective extraction geochemistry on multiple soil horizons (Van Geffen et al., 2009; Bissig and Riquelme, 2010; Heberlein and Samson, 2010), halogen element detection (e.g., Heberlein et al., 2017), till geochemistry (Cook et al., 1995) and biogeochemistry (Dunn, 1986; Reid and Hill, 2010). Each geochemical technique and media type has both strengths and weaknesses in identifying buried mineralization:

- Indicator minerals (e.g., Plouffe et al., 2013a; Plouffe and Ferbey, 2016) and biogeochemistry (e.g., Dunn et al., 2015; Jackaman and Sacco, 2016) have demonstrated success in targeting at a regional reconnaissance scale, but additional tools are still required to define final drill targets.
- Surface geochemical techniques (e.g., soil and till) for near-source detection have not reached a level of robustness to generate high-confidence drill targets. Specifically, geochemical signatures generated from orientation surveys over known mineral deposits are noisy (i.e., poor resolution of anomalies against background; Stanley, 2003), show poor precision and have element patterns that are often difficult to reconcile with mineral-deposit chemistry and expected element mobility (Heberlein and Samson, 2010).
- Unfortunately for mineral explorers, published research has led to marketing of a range of competitive commercial analytical methodologies loosely grouped as 'selective or partial extraction techniques', many of which are proprietary to specific companies. The interpretation of the data is often ambiguous, especially if it is undertaken without consideration of the heterogeneity of the sampled mineralogy, organic-matter character, element dispersion and host (Cameron et al., 2004; Anand et al., 2016).
- Organic geochemical techniques for direct detection of deposits are dominated by the proprietary Spatiotemporal Geochemical Hydrocarbons (SGH®) method. As with the selective extractions, application of the SGH® technique is dominated by the junior exploration industry. The major exploration companies generally do not apply the technique due to concerns with robustness, repeatability of survey results and lack of a demonstrable link between the compounds analyzed and the mineralization at depth (Noble et al., 2013). There is effectively no fundamental understanding of how and where the hydrocarbon signatures are generated.

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The lack of fundamental mechanistic understanding of these techniques beyond their broadest concepts has led to inappropriate application by the mineral exploration industry with minimal return on investment. The failure of the commercial techniques to repeat the performance shown in orientation surveys over known mineralization has, in large part, resulted in their abandonment by major exploration companies. Despite these issues, there is sufficient empirical evidence to indicate causative links between mineralization beneath transported cover and the presence of geochemical gradients in the surface environment (Hamilton, 1998; Smee, 1998; Kelley et al., 2006; Nordstrom, 2011). Although much less explored, biological anomalies may be more robust indicators of buried mineralization (Kelley et al., 2006; Leslie et al. 2013), and such anomalies may be detectable through low-cost, high-throughput geobiological[1] surveys.

Microbial-Community Fingerprinting as a Mineral Exploration Tool

Micro-organisms kinetically enhance and exploit thermodynamically favourable geochemical reactions, including the dissolution and formation of diverse minerals, to support their metabolism and growth in nearly every low-temperature geological setting (Newman and Banfield, 2002; Falkowski et al., 2008). They are acutely sensitive, often rapidly responding to the dynamics of chemical and physical gradients in the environment. Subtle changes in mineral bioavailability, for example, can be reflected in dramatic shifts in composition and activity of the microbial community (Newman and Banfield, 2002; Fierer, 2017). This can be seen at the global scale as marine phytoplankton communities respond to traces of iron in seawater, a process that can be viewed as chlorophyll plumes via remote sensing (O'Reilly et al., 1998; Fuhrman et al., 2008). Application of modern sequencing technologies allows high-throughput profiling of the taxonomic diversity and metabolic potential of soil microbial communities across subtle, and often poorly resolved, geochemical gradients.

Microbial-community profiles thus have a strong potential to resolve chemical and physical differences in sample suites that are not readily discernible through conventional geochemical and geophysical surveys. In residual terrains, for example, where chemical gradients are high, bacterial population changes have been clearly demonstrated (e.g., Southam and Saunders, 2005; Reith and Rogers, 2008). Even outdated techniques with low throughput and resolution, such as Denaturing Gradient Gel Electrophoresis (DGGE), that can produce a crude microbial-community "fingerprint" (Wakelin et al., 2012) reveal changes in bacterial communities in soils over buried volcanogenic massive-sulphide (VMS) deposits. The advent of high-throughput next-generation sequencing (NGS) platforms during the last decade has transformed our capacity to interrogate the molecular fingerprints of microbial communities (Binladen et al., 2007; Shokralla et al., 2012; Zhou et al., 2015). Application of NGS technologies thus allows profiling of the taxonomic diversity and metabolic potential of soil microbial communities across defined survey areas. Given that each soil sample comprises thousands of microbial taxa, each containing hundreds to thousands of genes (Fierer, 2017), the statistical power of this approach to identify anomalies is unprecedented. A schematic diagram of such an approach is illustrated in Figure 1.

To enhance the ability to recognize microbial fingerprints in the surface environment related to buried mineralization, a laboratory experiment has been conducted in which background soils were either amended with the copper-bearing mineral chalcopyrite or doped with copper as copper sulphate ($CuSO_4$). These soils were then incubated to test the response of the microbial community to the presence of copper amendments. Some organisms have evolved distinct extracellular acquisition and internal storage strategies to target elements that are specifically required for enzymatic or metabolic processes (Liermann et al., 2007), and the requirement for copper in some microbial species has been well documented in controlled studies (Knapp et al., 2007; Fru et al., 2011; Balasubramanian et al., 2012; Kenney and Rosenzweig, 2012). Since both chalcopyrite weathering in soils and soil microbial turnover are appreciable over timescales of several weeks, these experiments are traceable in the laboratory (Whitman et al., 1998; Kimball et al., 2010). The composition of the soil microbial community has been analyzed at initial, intermediate and end time-points, allowing identification of members of the soil microbial community that respond to the presence of ore minerals. These first bench-scale results will facilitate more detailed and controlled tests, in the future, for the presence or abundance of specific community members and their metabolic capacity in relation to buried mineral deposits.

Methodology

Soil and Ore Amendment

An archived soil sample from close to the Deerhorn porphyry, located 70 km northeast of Williams Lake in central BC, was retrieved (sample number 282140 of Rich, 2016). This sample is considered as representative of background because it has insignificant base-metal contents. The sample was collected from the upper B horizon under aseptic conditions and screened to –6 mm in the field prior to storage at ambient temperatures in double-sealed zip-lock bags. The sample was digested using a multi-acid digestion

[1] *Geobiology is the interdisciplinary science dealing with the interaction between organisms and ecosystems and their physical environment (Oxford University Press, 2017).*

and the digestate analyzed by inductively coupled plasma–mass spectrometry (ICP-MS) to determine that the soil contains 6 ppm Cu, 1 ppm As and 0.32 ppm Mo. The soil was not dried prior to the start of the experiment. Soil was dispensed aseptically into sterile containers for each treatment, with amendment concentrations chosen to represent either concentrations of copper that are routinely detected in geochemical surveys over buried mineral deposits (ambient or '(Am)') or very high levels of copper that might be expected in highly anomalous soils (high or '(Hi)'). The amendments were as follows: 1) 'Hi-ore' soil was amended with chalcopyrite ore at 600 ppm Cu; 2) 'Am-ore' soil was amended with chalcopyrite ore at 200 ppm Cu; 3) 'Hi-Cu' soil was amended with copper in the form of $CuSO_4$ (dissolved in Milli-Q®-filtered water) to 600 ppm Cu; and 4) 'Am-Cu' soil was amended with copper in the form of $CuSO_4$ to 200 ppm Cu. Soil was sampled at T = 0, T = 1 (2 weeks) and T = 2 (5 weeks).

DNA Extraction

Microbial-community DNA was extracted from samples using a MO BIO Laboratories Inc. PowerMax® Soil DNA Isolation Kit; as per manufacturer's instructions, approximately 0.25 g of soil was used. Resulting DNA was stored at –20°C. The quality and quantity of genomic DNA were measured on a ThermoFisher Scientific NanoDrop® ND-1000 spectrophotometer and by using Invitrogen™ PicoGreen™ (Quant-iT™ dsDNA Assay Kit) dye.

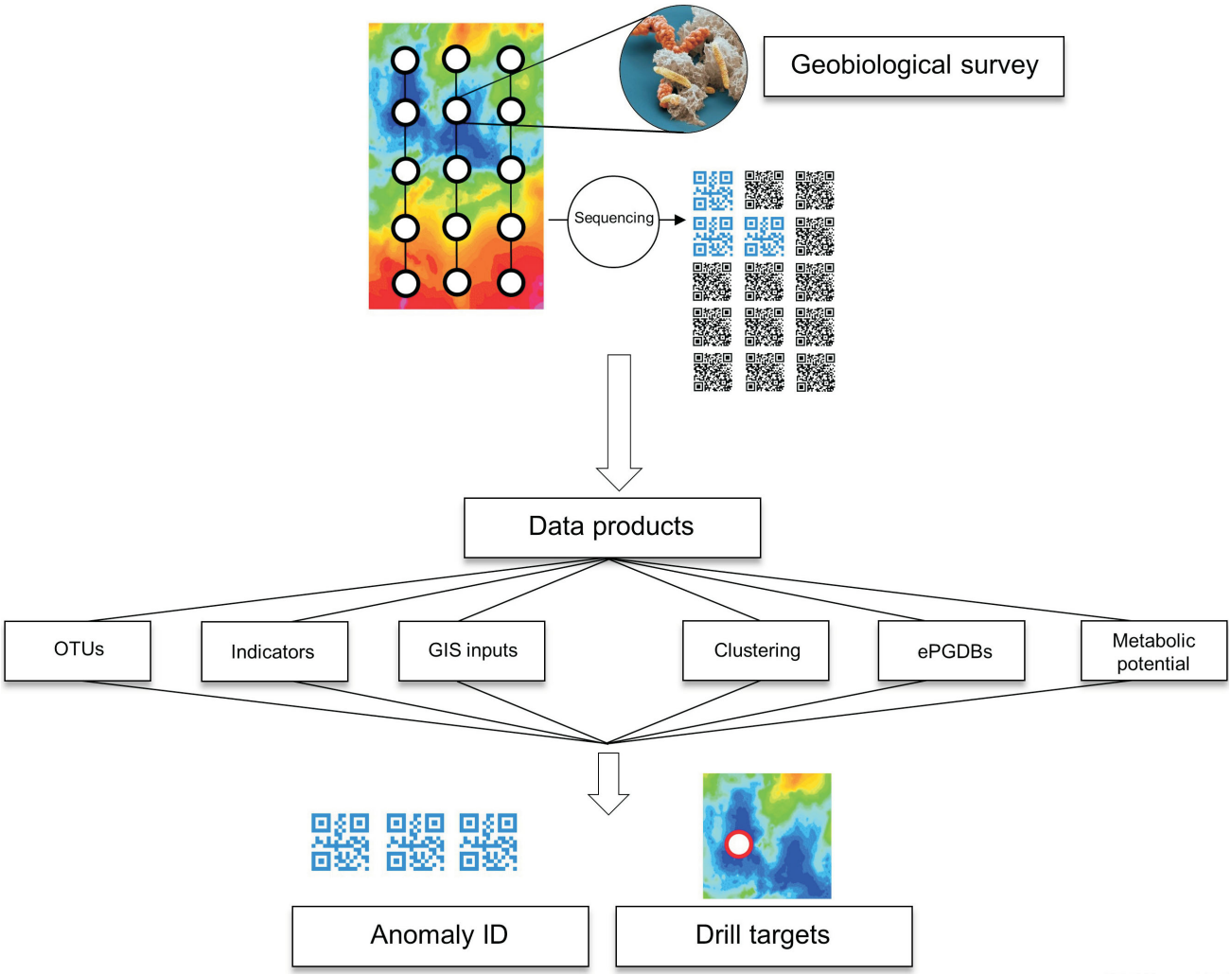

Figure 1. Schematic diagram of microbial fingerprinting applied to mineral deposit exploration. DNA is extracted and purified from soil samples taken in geobiological surveys and then sequenced to 1) generate iTag libraries of the 16S rRNA gene for community fingerprinting analysis, and 2) generate metagenomes to mechanistically link anomalous microbial communities to underlying differences in coded metabolic potential. These anomalies, reflected by hundreds to thousands of microbial species, will form unique fingerprints or barcodes that are characteristic of proximity to buried mineral resources. These barcodes will be formatted into data products such as deposit-scale exploration maps that chart microbial fingerprints (operational taxonomic units [OTUs], indicator and clustering analysis) and anomalies/ePDGBs (environmental pathway genome databases) specifically linked to mineral deposits.

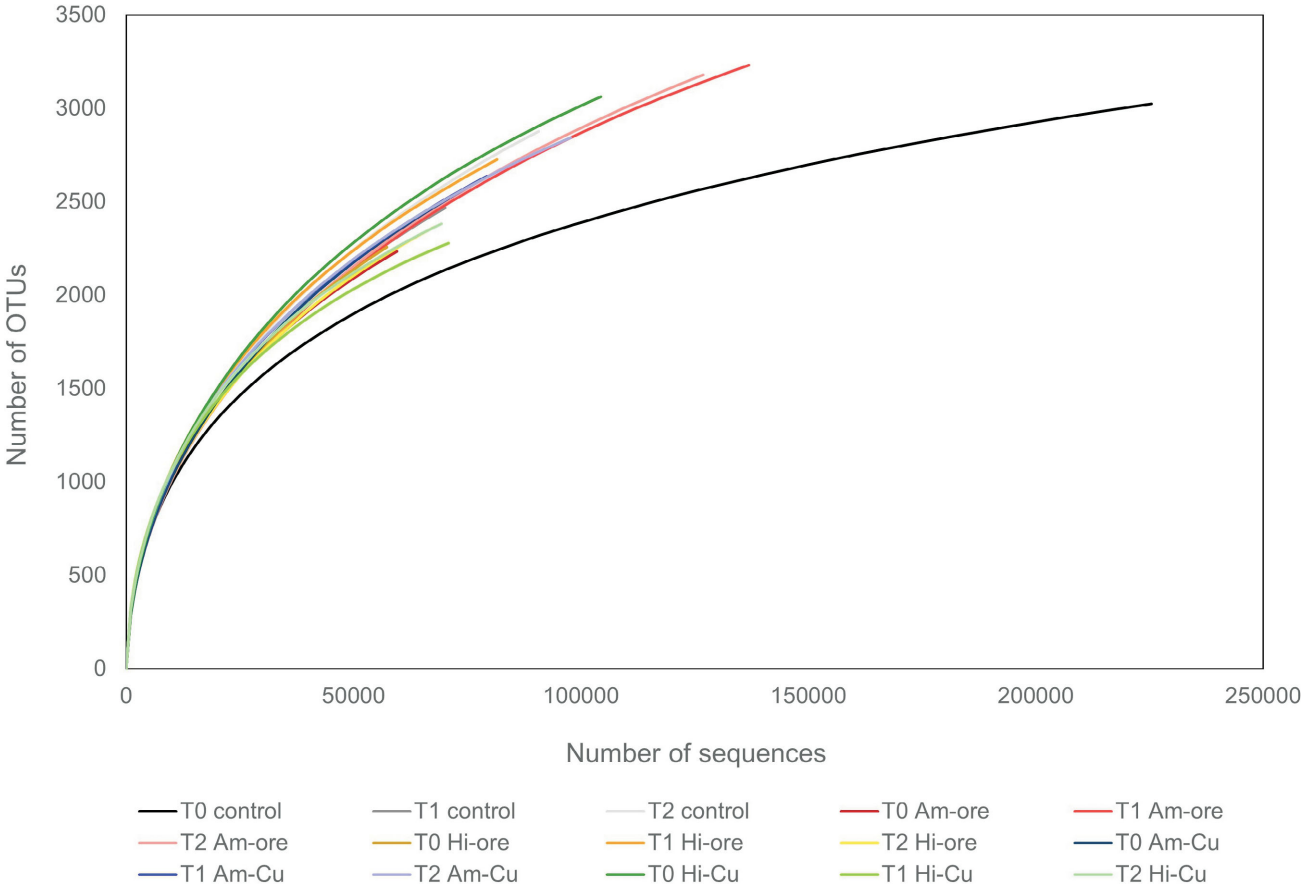

Figure 2. Bacterial diversity of samples. Rarefaction curves are based on operational taxonomic units (OTUs) at 97% sequence similarity.

Small Subunit Ribosomal RNA (SSU rRNA) Gene Amplification and iTag Sequencing

Bacterial and archaeal 16S rRNA gene fragments from the extracted genomic DNA were amplified using primers 515f and 806r (Apprill et al., 2015). Sample preparation for amplicon sequencing was performed as described by Kozich et al. (2013). In brief, the aforementioned 16S rRNA gene-targeting primers, complete with Illumina adapter, an 8-nucleotide index sequence, a 10-nucleotide pad sequence, a 2-nucleotide linker and the gene-specific primer were used in equimolar concentrations together with Deoxynucleotide triphosphate (dNTPs), Polymerase chain reaction (PCR) buffer, $MgSo_4$, 2U/µL ThermoFisher high-fidelity platinum Taq DNA polymerase and PCR-certified water to a final volume of 50 µL. PCR amplification was performed with an initial denaturing step of 95°C for 2 min, followed by 30 cycles of denaturation (95°C for 20 s), annealing (55°C for 15 s) and elongation (72°C for 5 min), with a final elongation step at 72°C for 10 min. Equimolar concentrations of amplicons were pooled into a single library. The amplicon library was analyzed on an Agilent Bioanalyzer using the High-Sensitivity DS DNA Assay to determine approximate library fragment size, and to verify library integrity. Library pools were diluted to 4 nM and denatured into single strands using fresh 0.2 N NaOH, as recommended by Illumina. The final library was loaded at a concentration of 8 pM, with an additional PhiX spike-in of 5–20%. Sequencing was conducted on the MiSeq platform at the Sequencing + Bioinformatics Consortium, The University of British Columbia, Vancouver, BC (The University of British Columbia, 2017).

Informatics

Sequences were processed using mothur (Schloss et al., 2009, Kozich et al., 2013). Briefly, sequences were removed from the analysis if they contained ambiguous characters, had homopolymers longer than 8 base pairs and did not align to a reference alignment of the correct sequencing region. Unique sequences, and their frequency in each sample, were identified and then a pre-clustering algorithm was used to further de-noise sequences within each sample (Schloss et al., 2011). Unique sequences were identified and aligned against a SILVA alignment (mothur Project, 2017a). Sequences were chimera-checked using VSEARCH (Rognes et al., 2016) and reads were then clustered into 97% operational taxonomic units (OTUs) based on uncorrected pairwise distance matrices. OTUs were classified

using the SILVA reference taxonomy database (release 128; mothur Project, 2017b).

Results and Discussion

Soil is one of the most complex and diverse microbial habitats, with merely 1 g containing up to 10^{10} cells and 10^4 bacterial species (Roesch et al., 2007; Torsvik and Øvreås, 2002). The current study's approach relies on the ability to capture this diversity through next-generation sequencing technologies. In microbiology, the assessment of diversity often involves calculation of species richness (number of species present in a sample; Magurran, 2013). The most common approach is to assign 16S rRNA sequences into operational taxonomic units (OTUs) and represent these as rarefaction curves, which plot the cumulative number of OTUs captured as a function of sampling effort, and therefore indicate the OTU richness in a given set of samples. Other common methods include nonparametric analysis, such as Chao1, which estimates the overall sample diversity (also known as alpha diversity; Hughes et al., 2001). The current study extracted microbial-community DNA from the soils amended with either chalcopyrite ore or copper, and sequenced the 16S rRNA gene. Analysis of theses sequences reveals that the number of observed OTUs (hereafter referred to as species) is 2265 ±105 (range 1993–2380), with an alpha diversity (Chao1 index) of 3438 ±327 (range 2808–3791; Table 1), indicating that the sequencing coverage was sufficient to capture 65% of the microbial-community diversity. These levels of diversity are well in line with diversity commonly observed in soils (Thompson et al., 2017). These measurements dispel dogma that extremely high diversity in soil microbial communities renders them intractable to molecular-based microbial-community analysis. Rarefaction analysis revealed that resampling of the observed OTUs approaches asymptotic values (Figure 2), confirming adequate coverage for diversity estimation. There was no pronounced difference in species richness (i.e., the number of species in a given sample) over time, due to amendment with chalcopyrite ore or copper. The study's first measurements demonstrate that soil diversity can be captured through next-generation sequencing technologies, which bodes well for the approach of imparting enormous statistical power to community profiles as anomaly indicators.

Table 1. Overview of the species estimates and diversity metrics obtained per sample after quality filtering. Sample names explained in 'Soil and Ore Amendment' section. Abbreviation: OTU, operational taxonomic unit.

Sample	No. of sequences	No. of OTUs	Chao1
T0 control	57319	1993	2808
T2 control	57319	2272	3567
T3 control	57319	2380	3791
T0 Am-ore	57319	2203	3181
T2 Am-ore	57319	2277	3700
T3 Am-ore	57319	2287	3787
T0 Hi-ore	57319	2257	3364
T2 Hi-ore	57319	2369	3677
T3 Hi-ore	57319	2219	3229
T0 Am-Cu	57319	2306	3610
T2 Am-Cu	57319	2315	3606
T3 Am-Cu	57319	2317	3547
T0 Hi-Cu	57319	2418	3732
T2 Hi-Cu	57319	2129	2854
T3 Hi-Cu	57319	2229	3113

The number of reads per microbial phylum was normalized to total read number for a given sample and expressed as a percentage of the total reads from that sample (Figure 3). Most microbial-community members belong to the Proteobacteria (24–37%), Acidobacteria (13–32%) and Verrucomicrobia (11–21%) phyla (Figure 3). The relative proportions are consistent with previous studies on soil ecosystems (Choi et al., 2016; Kaiser et al., 2016). This high-level taxonomic analysis reveals strong similarities across all samples, thus giving confidence that the analyses are not overwhelmed by intersample variability arising because of the very high levels of microbial diversity and chemical and physical heterogeneity commonly found in soils. The similarity across the samples, however, suggests that discrimination between background and anomalous soils may be more sensitive with analyses at the genus or species level rather than at the phylum level. Nevertheless, when plotted relative to the unamended (control) samples, subtle changes in community composition through time can be detected even at the phylum level (Figure 4). This high-level sensitivity bodes well for application to exploration.

Differences between copper-amended and chalcopyrite ore–amended soils included a higher abundance of Chloroflexi in copper-treated soils at T_1 and T_2 (Figure 4A). The Archaeal phylum Thaumarchaeota increased in abundance relative to the control in samples amended with high levels of chalcopyrite ore (Hi-Ore) and copper (Hi-Cu; Figure 4B). The other phylum that increased over time in response to soil amendments was the Firmicutes (Figure 4C). All amendments elicited a decrease in the relative abundance of Acidobacteria, Ignavibacteria and Bacteroidetes (except for soils treated with ambient levels of chalcopyrite ore [Am-ore]) compared to control soil over time (Figure 4D–F). Relationships between treatment type (chalcopyrite ore or copper) and time point (T = 0, 1, 2) were evaluated through hierarchical-clustering analysis (Figure 5A). All control samples clustered tightly, confirming similar microbial-community compositions. Treated samples grouped apart from controls, indicating that chalcopyrite ore and copper amendments changed the composition of the microbial community and that this change was easily resolvable through standard hierarchical-clustering analysis. Hierarchal clustering separated chalcopyrite ore– and copper-treated samples, indicating that it may be possible to

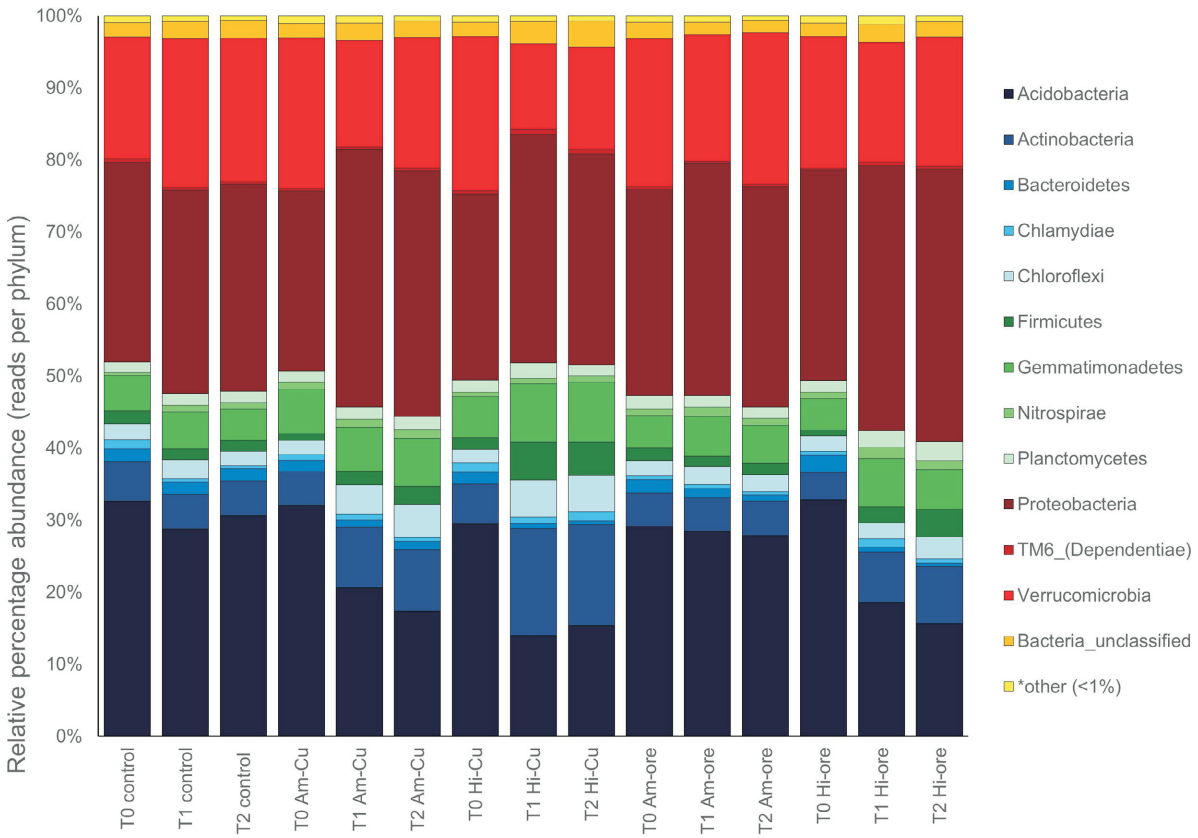

Figure 3. Distribution of 16S rRNA reads per phylum for each sample. The number of reads per phylum is calculated as a percentage of the total reads for each sample. The '*other' grouping represents summed phyla that individually contributed <0.4% of the total number of reads per sample.

determine microbial-community response to individual metals.

A number of species were appreciably enriched or depleted in response to chalcopyrite ore or copper amendment, so the relative abundance of individual species normalized to the relative abundance of the same species in the controls was plotted versus time (examples shown in Figure 5B). The species that increased in response to chalcopyrite ore and copper amendment relative to controls included *Rhodanobacteria* sp. (Koh et al., 2015), SC-I-84 sp. (Huaidong et al., 2017) and *Acidimicrobiales* sp. (Figure 5B; Hallberg et al., 2006). These species have frequently been found in relatively high abundances in materials recovered from acidic waters, sulphidic mine wastes and other mine-related environments, as well as acidic biofilms (Hallberg et al., 2006; Stackebrandt, 2014; Koh et al., 2015; Huaidong et al., 2017), anecdotally suggesting a link between the ecology of these species and the concentration of metals in their habitat. In addition to the broader community-level responses revealed through hierarchical clustering analyses, the data from this study thus imply that certain species in soil microbial communities may be useful as indicators of exposure to ore components.

Conclusions and Future Directions

This study investigated the use of soil microbial-community fingerprinting with modern DNA sequencing technologies to detect changes in soil microbial communities in response to varying levels of exposure to chalcopyrite ore and copper. It was found that soil microbial communities can be coherently sampled such that there is little variability between samples. Exposure of soil microbial communities to ore constituents elicits a response detectable on laboratory time scales of several weeks. These responses are readily resolved through standard statistical analyses, and the specific species that exhibited the strongest responses have known affinities for environments rich in heavy metals. The strong microbial responses observed are encouraging signs for the use of microbial-community fingerprinting in mineral deposit exploration. Further experiments are currently being conducted and work is ongoing to translate the approach to a real-world exploration setting. With the cooperation and permission of Consolidated Woodjam Copper Corporation, the authors have collected a suite of 150 soil samples over known copper-gold porphyry mineralization (the Deerhorn deposit) in central BC, which has

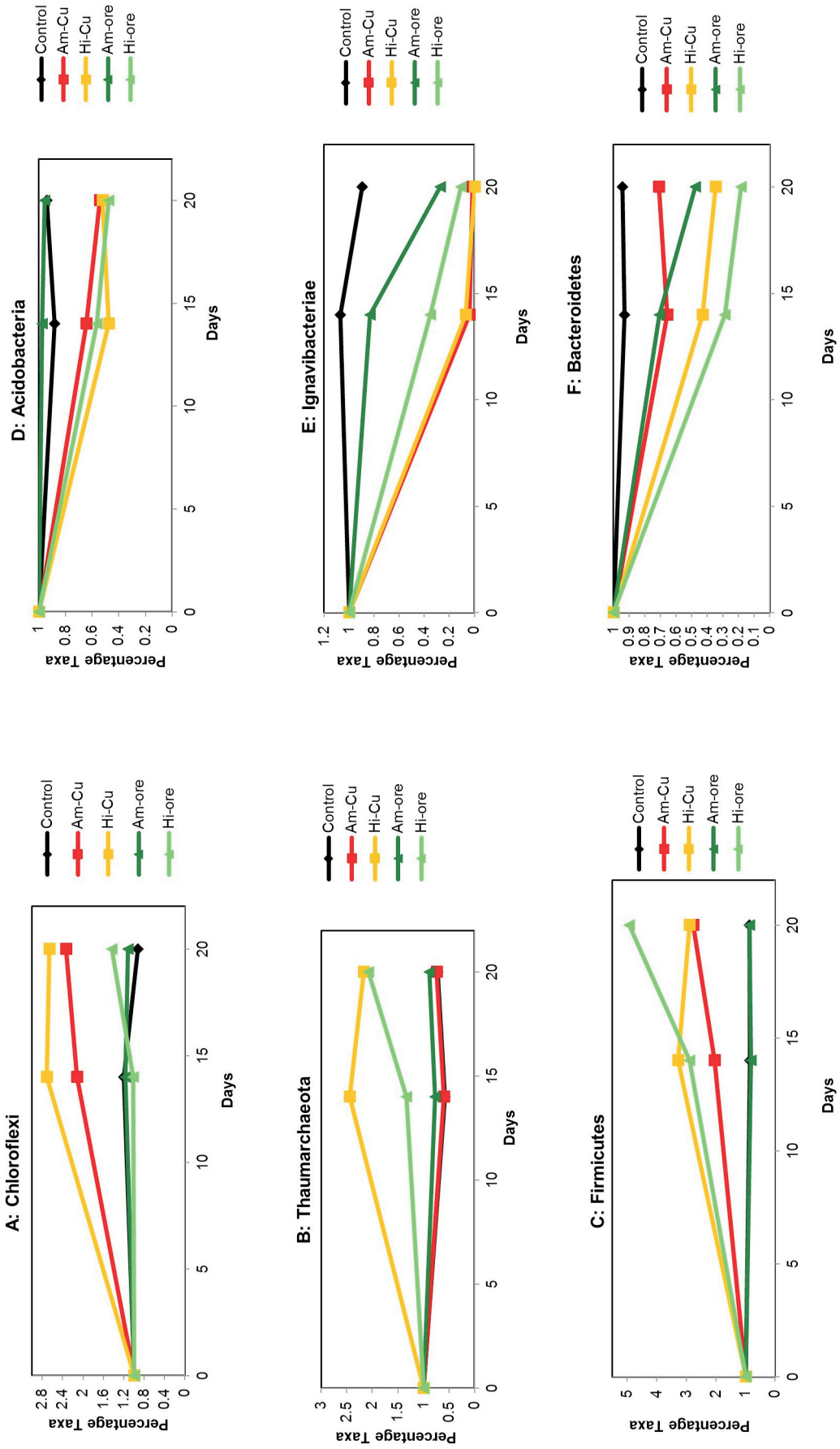

Figure 4. Examples of changes in phylum abundance over time for each sample/treatment. The number of reads per phylum is calculated as a percentage of the total reads for each sample.

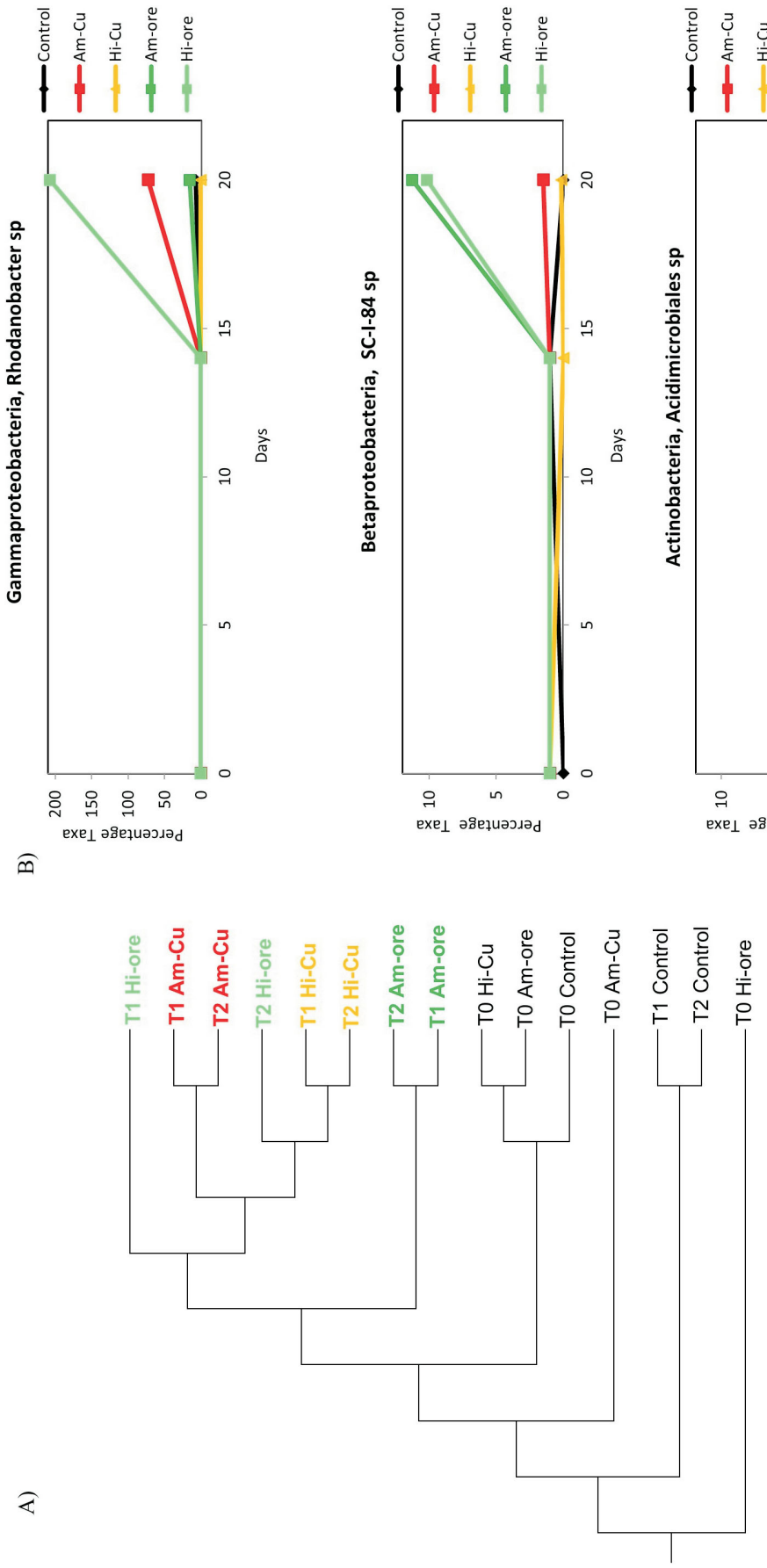

Figure 5. A) Hierarchical relationships among samples based on Euclidean distance of 16S-OTU abundances. The hierarchical relationships between samples were obtained using the unweighted pair group method with arithmetic mean (UPGMA) clustering algorithm. Node labels indicate the sample/treatment. **B)** Examples of operational taxonomic unit (OTU) 'species level' changes across treatments, over time.

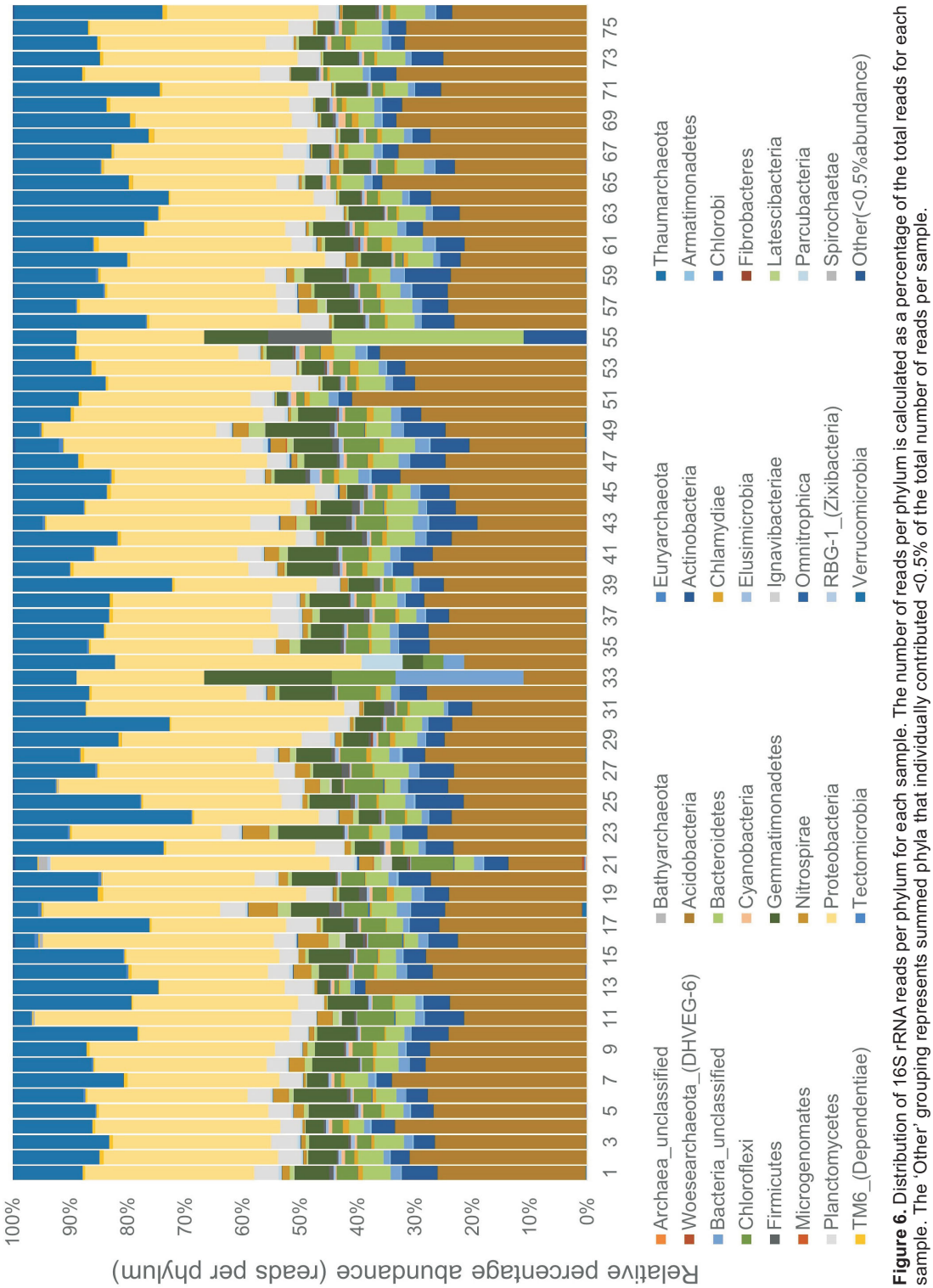

Figure 6. Distribution of 16S rRNA reads per phylum for each sample. The number of reads per phylum is calculated as a percentage of the total reads for each sample. The 'Other' grouping represents summed phyla that individually contributed <0.5% of the total number of reads per sample.

extensive geological, pedological, geochemical and geophysical metadata (Rich, 2016). Genomic DNA from nearly half of the soils collected has been extracted and tag sequencing of the 16S rRNA completed.

The first analyses of these data reveal strong similarities across the entire sample set, lending confidence to the ability to consistently sample microbial communities from the same horizon to yield a dataset from which robust comparisons can be made (Figure 6). Ongoing work includes conducting statistical analyses (hierarchical clustering and indicator-species analysis) to resolve possible patterns in the microbial-community fingerprints that could point to buried mineralization.

Acknowledgments

The authors thank S. Rich for sample collection, and P. Kenward and D. Fowle for peer review of this paper. Funding was provided by Geoscience BC.

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