Microbial species richness under spontaneous plant colonisation in copper mine tailings

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Microbial species richness under spontaneous plant colonisation in copper mine tailings

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Abstract

We aimed to assess the species richness of the microbial communities in copper mine tailings that have been spontaneously colonised by plants. We characterised the bacterial (16S rRNA, or 16S) and fungal (ITS) sequences from samples taken from a single bench of an undisturbed mature tailings storage facility (TSF1) and samples from a reference site (Ref Site) located 1 km away. In all sampling sites, the dominant plant was a single species of grass (Saccharum spontaneum); a few other species were sometimes found with the grass in TSF1. Amplicon sequence variants (ASVs) were deduced using DADA2 from 16S rRNA gene (bacteria) and ITS2 (fungi) amplicons. Rarefaction curves suggested that the TSF1 had more ASVs on average than the Ref Site; furthermore, the TSF1 appeared to have more ASVs than were sampled. The Chao1 and Faith indices for both bacteria and fungi were higher for the TSF1 tailings. Similar bacterial orders were found in both sites, including common groups, although Rhizobiales were more common in TSF1 and Chloroflexi in the Ref Site. The most abundant bacteria found belonged to the phyla Proteobacteria, Chloroflexota, Acidobacteriota and Actinomycetota. Overall fungal diversity began to increase with plant diversity. PICRUSt2 analysis of predicted metabolic pathways showed more potential pathways at the TSF1. These results suggest a relation between microbial species richness and plant colonisation, and that succession towards greater species richness is taking place. This study may provide a baseline against which the effect of any rehabilitation intervention on the soil can be measured.

Keywords: *mine tailings, microbial communities, metabarcoding, sequences, remediation*

1 Introduction

After rock is crushed and the valuable metals extracted, the processed waste (mine tailings) has little economic value at the time of its generation. It has little organic matter, lacks key nutrients and typically has high concentrations of heavy metals (Tibbett 2024).

It may be economically viable to recover residual metal from a tailings storage facility (TSF). A facility located in Padcal, Benguet Province, Philippines, that contained tailings from the early 1980s is one example. The copper may be recovered by reprocessing the tailings. Alternatively, it might be more economical to

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inject a solvent into the tailings to extract the copper in a process called in situ leaching (ISL) (Seredkin et al. 2016). ISL using citrate as the lixiviant has been proposed for this TSF.

The Philippines Remediation of Mine Tailings (PROMT) project (2021–2025) that proposed this plan aimed to identify the effect ISL might have on ecosystem services; in particular, what the baseline microbial and plant diversity at the site looks like. Microbial species richness might reflect the plant diversity above-ground. Microbial communities drive many ecosystem functions and their richness can indicate the quality of ecosystems at the site (Trivedi et al. 2016).

To understand the potential for the tailings to develop greater species richness under increasing naturally colonised plant diversity, samples were taken from two sites that contained mine tailings deposited there in the early 1980s. The primary older site was a single flat bench at TSF1, and the secondary reference site (Ref Site) was deposited later. The sites contrasted markedly in their plant cover.

Microbial species richness was assessed using amplicon sequencing. Metabolic potential was inferred from the amplicon sequences. We aimed to see whether microbial species richness and predicted metabolic profiles indicated a potential towards increasing richness, which might be perturbed if ISL was applied to the tailings.

2 Materials and methods

2.1 Field sampling

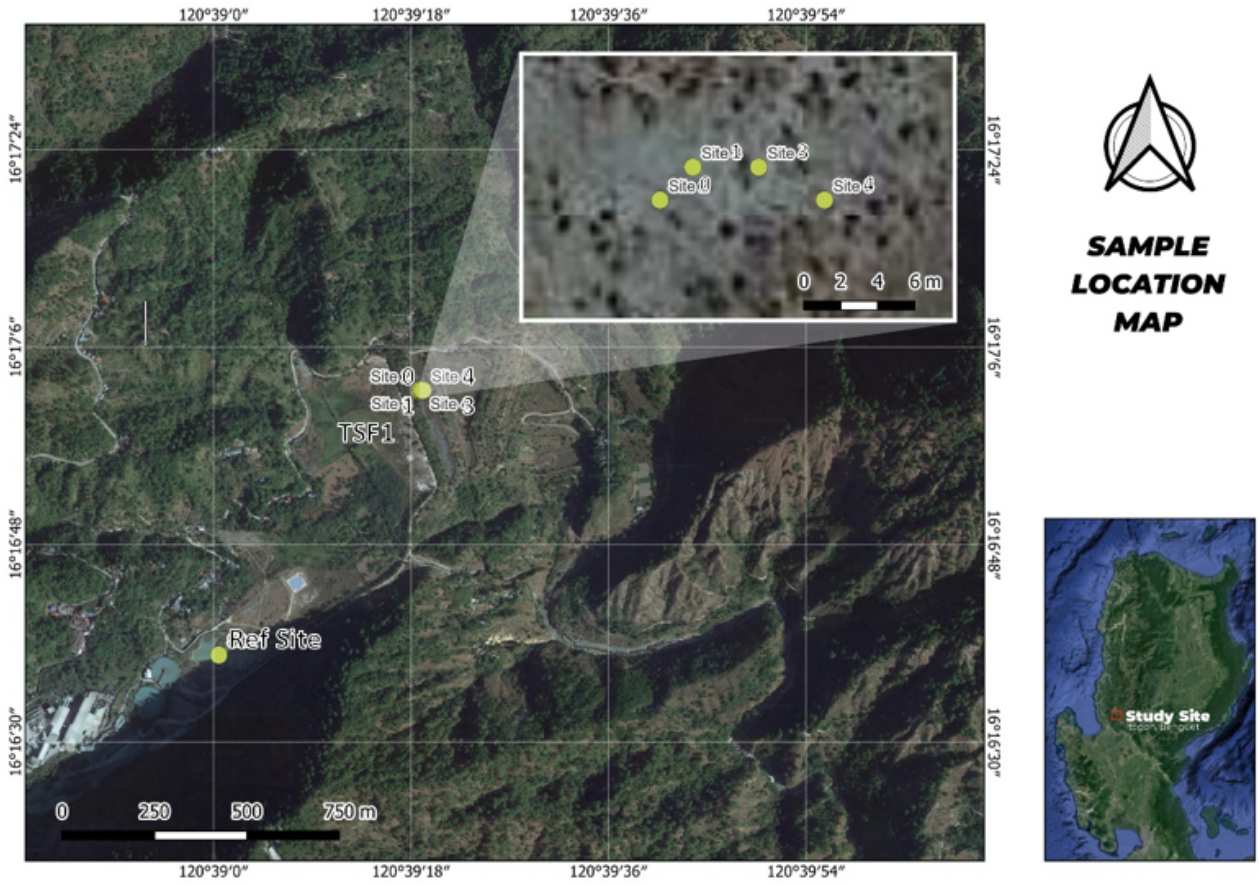

Figure 1 Sampling sites. The reference site is about 1 km from the tailings storage facility 1 (TSF1) sampling site. Within the TSF1 site are four sub-sites, 0, 1, 3 and 4, differentiated by plant cover near the point of sampling

Tailings samples were taken from two locations at the TSF containing material deposited in the early 1980s. The Ref Site was chosen due to its homogeneous distribution of grass (*Saccharum spontaneum, or S. spontaneum)* in contrast to the TSF sites which were highly heterogeneous. The sites are about 1 km apart (Figure 1). Both sites support *S. spontaneum*; the tailings at TSF1 also had a more diverse distribution of plants within patches of grass. The plants at the sites are listed in Table 1. The TSF1 samples were taken randomly from within each of four sub-sites within the TSF1 site, each sub-site differentiated from the others by the plant species diversity. These sub-sites from TSF1 were named according to the number of plant species in the patch closest to the augur drilling point, e.g. Site 0 samples were taken from within a randomly selected 1 × 1 m^2 plot within TSF1 that had no plant cover.

Samples were collected in December 2021 from a depth of 5 to 30 cm below the surface using an auger driller, placed in sterile Falcon conical tubes, transported immediately to the laboratory in an ice chest and stored at –21°C before processing.

Table 1 Tailings storage facility 1 (TSF1) sampling sites and their plant cover and composition. The sub-sites from TSF1 are named according to the number of plant species found there. Two (n = 2) samples were taken from the reference site and two samples (n = 2) from each sub-site within the TSF1 site

Site	GPS coordinates	Plant cover (% surface covered)
Reference site	N16’ 16.632”, E120’ 39.008”	*Saccharum spontaneum* (tall grass) only; covering 75% of the surface.
TSF1 sub-site 0	N16’ 17.034”, E120’ 39.023”,	No plants; 0%
TSF1 sub-site 1	N16’ 17.035”, E120’ 39.314”	*Saccharum spontaneum* (tall grass); 2%
TSF1 sub-site 3	N16’ 17.035”, E120’ 39.316”	*Saccharum spontaneum* 1 clump, *Nephrolepis* sp. 3 clumps, *Onychium siliculosum*; 31%
TSF1 sub-site 4	N16’ 17.035” E120’ 39.316”	*Psidium guajava* 1 tree, *Saccharum spontaneum* 3 clumps, *Nephrolepis* sp. 2 clumps, *Onychium siliculosum*; 44%

Metal content in the Ref Site and in TSF1 was measured by portable laser particle analyser (Malvern PanAnalytical Mastersizer 3000) following the manufacturer’s instructions. The concentrations of various metals are shown in Table 2.

Soil samples from a forested area adjacent to TSF1 which had no tailings were also taken to help assess the similarities of soil and tailings at this site. Samples were sent to the Department of Agriculture-Bureau of Soils and Water Management (DA-BSWM, Philippines) for analysis of relevant chemical properties. After air-drying of the samples, the following were determined: the content of organic carbon and organic matter (Walkley–Black titration); soil nitrogen (Kjeldahl digestion method); available sodium, potassium, calcium and magnesium (ammonium acetate pH 7.0 shaking extraction method); and available copper, iron, manganese and zinc (DTPA-TEA method) (Table 3).

2.2 DNA extraction

DNA was extracted as previously described (Verma et al. 2016) and optimised for this specific substrate. We found that commercial kits for soil did not work well with these samples.

Materials: Extraction buffer [100 mM Tris/HCl (pH 8.0)]; 100 mM EDTA (pH 8.0), 100 mM sodium phosphate buffer (pH 8.0), 1.5 M NaCl, 1% (w/v) CTAB, 100 mM CaCl2, 10 mg proteinase K/ml and 10 mg lysozyme/ml.

Method: To soil samples (15 g), 10 ml of extraction buffer was added, then incubated at 37˚C for one hour, with shaking at 200 rpm. About 2 ml of 20% SDS was added, incubated at 65˚C for two hours while invert mixing after every 10–15 minutes, then centrifuged at 7,000 g for 20 min at 4˚C. Supernatant was collected.

To the sand pellets, 6 ml of extraction buffer and 2 ml of 20% SDS were added. They were incubated at 65˚C for 15 minutes, then centrifuged. All supernatants were pooled and mixed with an equal volume of chloroform, isoamyl alcohol (24:1), then centrifuged at 14,000 g for 20 minutes at 4˚C. To the upper aqueous phase 0.1 volume of 3 M sodium acetate and 0.4 volume of 30% PEG-8000 were added, then incubated at 20˚C for two hours (or overnight). Crude DNA was pelleted by centrifugation at 14,000 g for 15 minutes at 4˚C. The pellet was washed once with 70% ethanol at room temperature. The pellet was air-dried then dissolved in 200 µL 1X TE buffer.

DNA extracts were checked for concentration using a Qubit spectrophotometer and for quality using a NanoDrop. Samples were kept to these values: Concentration >0.1 ng/µL; purity (A260/A280) >1.7; and volume >10 µL.

Next-generation Illumina sequencing was done at Macrogen (South Korea). Fungal ITS2 sequences were amplified with the primer pairs ITS1/ITS4 (ITS1F: CTTGGTCATTTAGAGGAAGTAA; ITS4R: GCTGCGTTCTTCATCGATGC); and bacterial 16S rRNA gene sequences were amplified using a primer pair for 16S (V3-V4) (341F: CCTACGGGNGGCWGCAG; 805R: GACTACHVGGGTATCTAATCC) provided by Macrogen.

2.3 Sequencing and bioinformatics

The raw paired-end reads from Macrogen were run in DADA2 under QIIME2 platform version 2024.5.0 to infer amplicon sequence variants (ASVs) from ITS (ITS1-ITS4) and 16S (V3-V4) amplicon sequences (Callahan et al. 2016; Bolyen et al. 2019). One ASV roughly corresponds to one species and each ASV has an abundance value, reflected in the ASV table . Rarefaction curves were produced from the ASV table using the R package VEGAN.

The Chao1 and Faith indices were measured from the ASV table using the program PHYLOSEQ (Nagendra 2002).

ASVs were assigned to taxonomic categories (names) using the DECIPHER package. For the 16S rRNA gene sequences, SILVA_SSU_r132 (silva-138-99-nb) was used as the reference; for the ITS2 region sequences the UNITE sh_general_release_2019 (unite_ver10_dynamic) database was used. Visualisation was done using the ggplot2 package (Wickham 2016).

PICRUSt2, both as a plug-in and standalone tool, was used to infer the enrichment or depletion of metabolic pathways from the bacterial ASV table (Douglas et al. 2020).

3 Results

3.1 Tailings analysis

The percentage of selected metals are shown in Table 2. The Ref Site tailings had a higher content of Cu, Zn, and Pb than the TSF1 tailings, especially Cu. The concentrations of Fe and Mn were similar.

Table 2 Selected metals at the fresh tailings site (reference site) and sub-sites within the mature tailings site tailings storage facility 1. For each site, n = 2

Site	Cu	Mn	Fe	Zn	Pb
	% ± sd	% ± sd	% ± sd	% ± sd	% ± sd
Reference site	0.942 ± 0.008	0.084 ± 0.002	7.31 ± 0.041	0.040 ± 0.001	0.009 ± 0.001
TSF1, sub-site 0	0.116 ± 0.002	0.075 ± 0.002	6.36 ± 0.036	0.006 ± 0.001	0.002 ± 0.001
TSF1, sub-site 1	0.300 ± 0.004	0.060 ± 0.002	8.09 ± 0.044	0.001 ± 0.007	0.0002 ± 0.0008
TSF1, sub-site 3	0.270 ± 0.004	0.060 ± 0.002	6.93 ± 0.039	0.007 ± 0.001	0.0008 ± 0.0002
TSF1, sub-site 4	0.256 ± 0.004	0.058 ± 0.002	6.48 ± 0.037	0.007 ± 0.001	0.0007 ± 0.0002

Organic matter (SOM), organic carbon (SOC), total N, micronutrients (Cu, Fe and Mn) and other parameters for TSF1 sub-sites 0, 1, 3 and 4 were compared to the adjacent forest soil (high plant diversity) (Table 3). TSF1 sub-sites had comparable pH with the adjacent forest. The mine tailings samples had less than half the SOM, SOC and total N of soil from the soil in the forest.

Table 3 Soil pH, organic carbon, organic matter and total nitrogen components of the different sampling sites in tailings storage facility 1. Standard deviations, sd. For all sites, n = 3

Site	Aspect	pH	Organic C % ± sd	Organic matter % ± sd	Total N % ± sd
Adjacent forest	Forested	5.12 ± 0.60	1.85 ± 1.3	3.18 ± 2.2	0.07 ± 0.04
TSF1, sub-site 0	No plants	5.75 ± 0.08	0.22 ± 0.01	0.37 ± 0.01	0.01 ± 0.00
TSF1, sub-site 1	1 grass	5.75 ± 0.20	0.23 ± 0.04	0.39 ± 0.10	0.02 ± 0.01
TSF1, sub-site 3	1 grass, 2 ferns	5.77 ± 0.10	0.65 ± 0.40	1.11 ± 0.70	0.02 ± 0.01
TSF1, sub-site 4	Grass, 2 ferns, tree	6.61 ± 0.40	0.54 ± 0.40	0.93 ± 0.60	0.02 ± 0.00

3.2 Microbial sampling: rarefaction curves

Rarefaction curves (Figure 2) suggest that the microbial communities were well captured, although that of TSF1 may have had more ASVs than were captured. The number of bacterial ASVs was higher than the fungal ASVs.

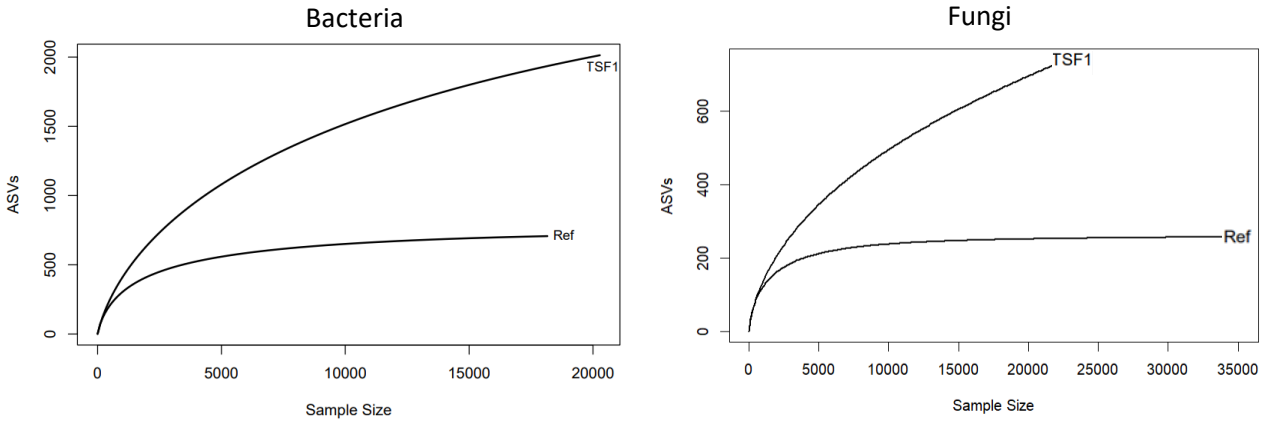

Figure 2 Rarefaction curves for bacteria 16S (a) and fungal ITS (b). The reference site had fewer amplicon sequence variants than the tailings storage facility 1 site

3.3 Diversity

Fungal and bacterial species richness varied between the Ref Site and TSF1. The higher values of the Chao1 and Faith phylogenetic diversity indices for the TSF1 site may be due to the higher numbers of plant varieties at TSF1 and to chemical and physical features that have not been examined (Figure 3).

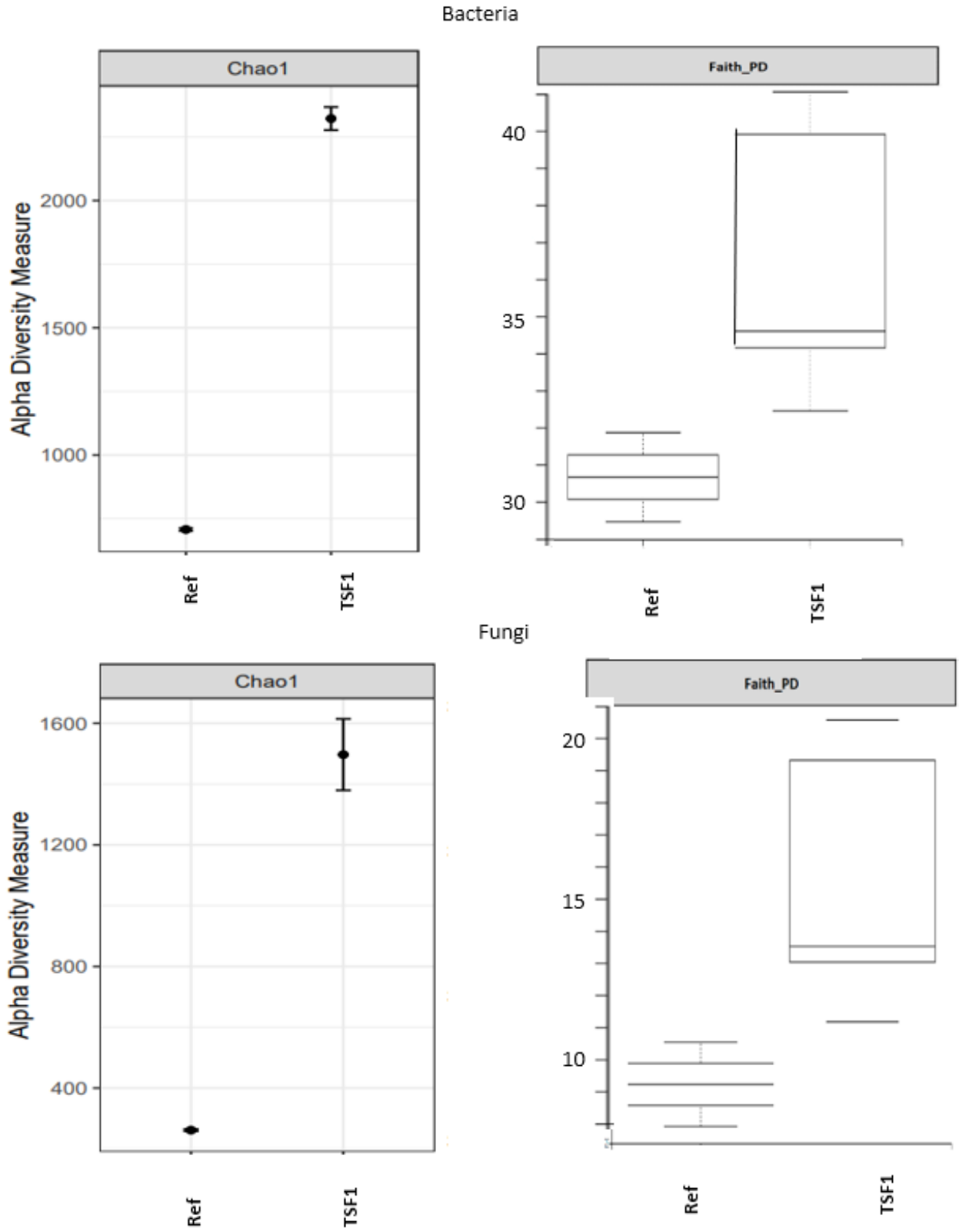

Figure 3 Alpha diversity (left) and Faith indices (right) of bacterial (top) and fungal (bottom) amplicon sequence variants in mine tailings samples. Labels on the X-axis are the reference site (n = 2) and the tailings storage facility 1 site (n = 8)

Samples were also taken from sub-sites within the TSF1 site because of the patchy distribution of plants. From a random selection of plots, four were selected that represented patches containing 1, 3 or 4 species, and one sub-site that had no plant cover.

A comparison of the microbial diversity among the sub-sites is qualitative because only two samples were taken from each sub-site. That said, there appears to be higher microbial diversity of bacteria and fungi patches of the TSF1 site that had more species of plants (Figure 4). Patches with more species, however, also covered more of the ground (Table 1).

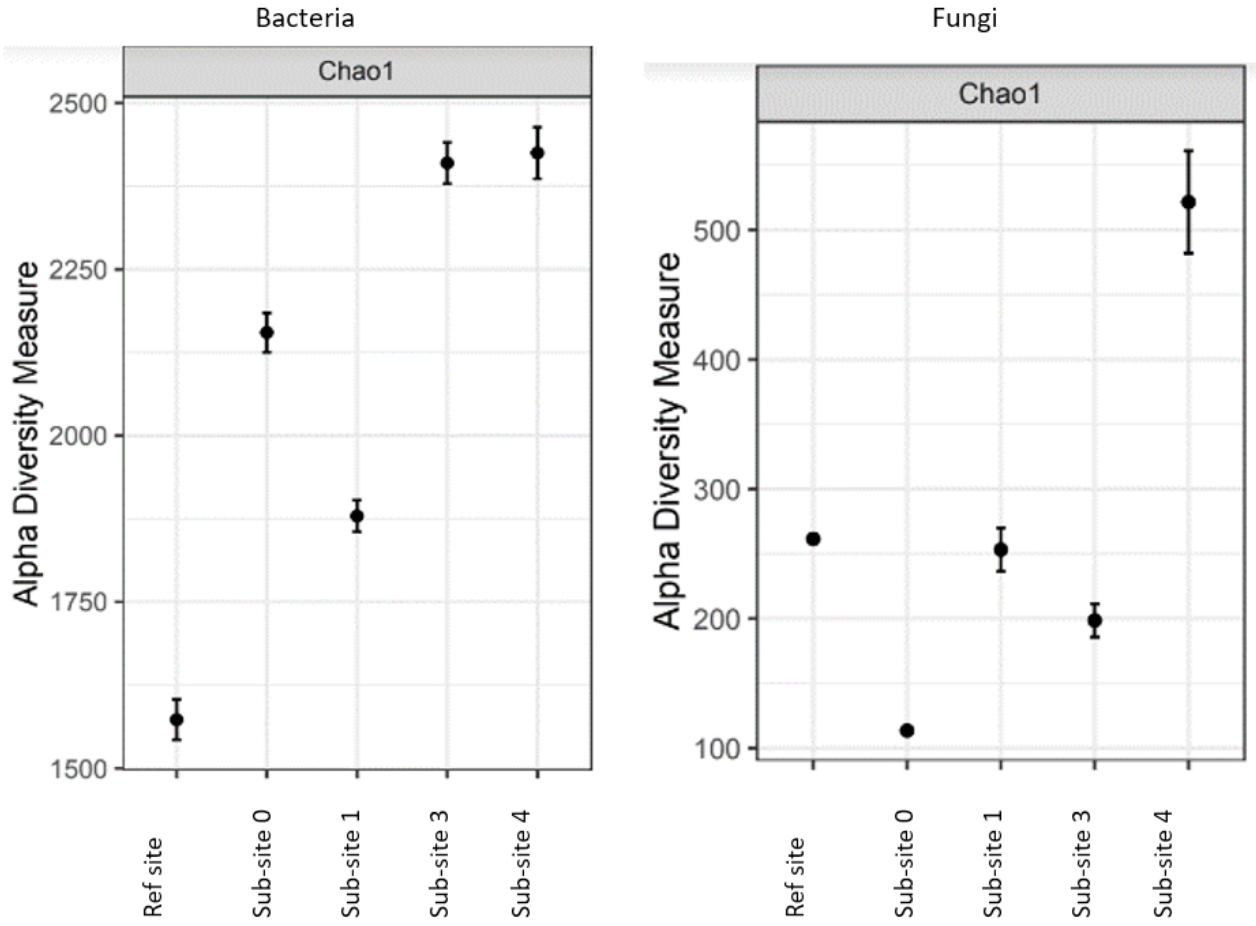

Figure 4 Alpha diversity of bacterial (left) and fungal (right) amplicon sequence variants in four tailings storage facility 1 sub-sites (for each, n = 2) and the reference site (n = 2). The naming of the sub-sites reflects the number of plant species at the point of sampling. Patches that had more plant species also covered more of the ground

3.4 Taxonomic assignation of bacteria

Of more than 3,000 bacterial ASVs most belonged to orders found in many kinds of soils (Wei et al. 2018). Figure 5 shows that the most abundant orders at TSF1 include Rhizobiales, gram-negative bacteria associated with plant roots and nitrogen-fixation (Garrido-Oter et al. 2018), acid-tolerant Acidobacteriales, and Micrococcales, also commonly found in many environments (Dastager et al. 2014). *Chloroflexi* KD4-96 were abundant in the Ref Site; Rhizobiales were abundant in the TSF1 site. Less common taxa (data not shown) included nitrite and ammonia oxidising Nitrospirota; and some uncultivable anaerobic methanotrophs known from sequence data such as Methylomirabilota (Chuvochina et al. 2023).

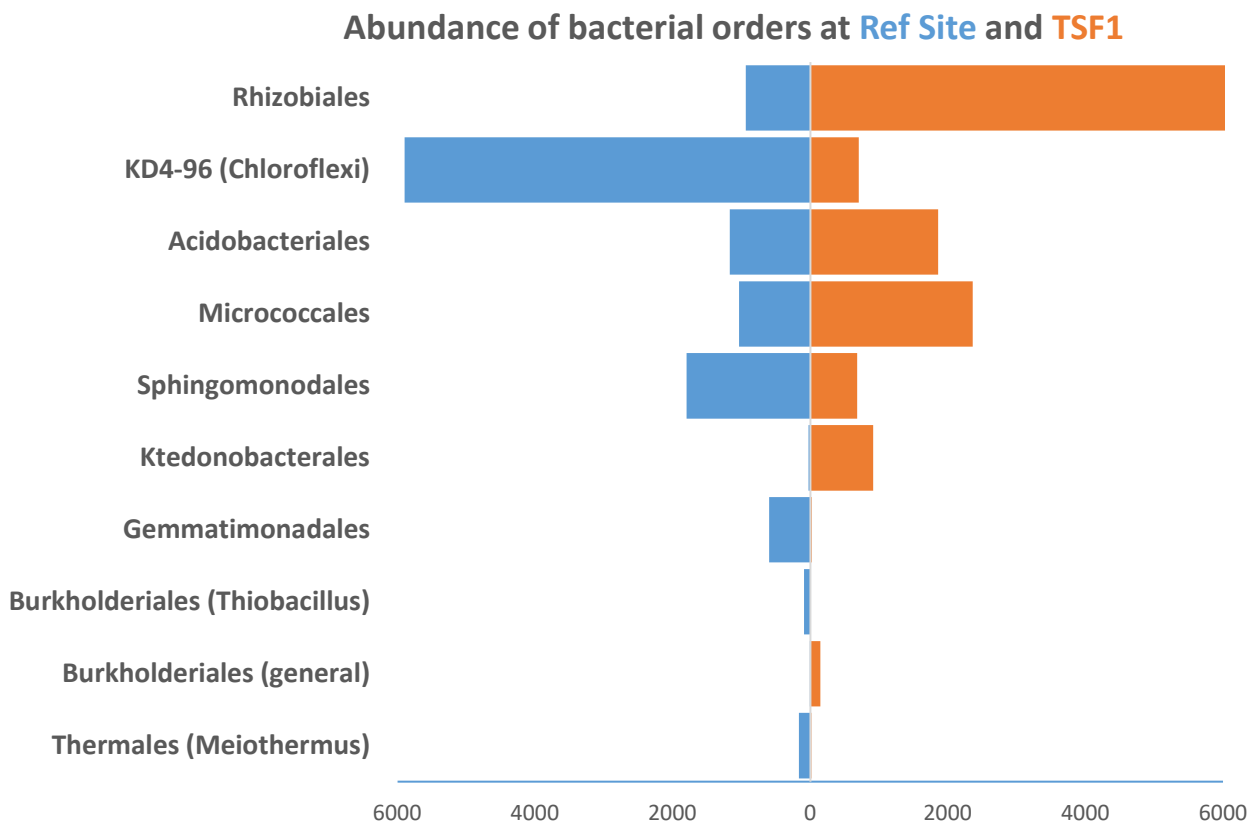

Figure 5 Top bacterial orders (right) and their relative abundances at the reference site and tailings storage facility 1. The X-axis shows the number of amplicon sequence variants averaged per sampling site. Rhizobiales are likely associated with plant roots; Sphingomonodales at the reference site may also be associated with plant roots. KD4-96 is frequently encountered in contaminated substrate (Kujala et al. 2018)

3.5 Functional diversity of bacteria

Using the ASVs as inputs, PICRUSt2 predicted 420 bacterial metabolic pathways across sites. These sites were differentiated by the metabolic pathways inferred from the ASVs (Figure 6).

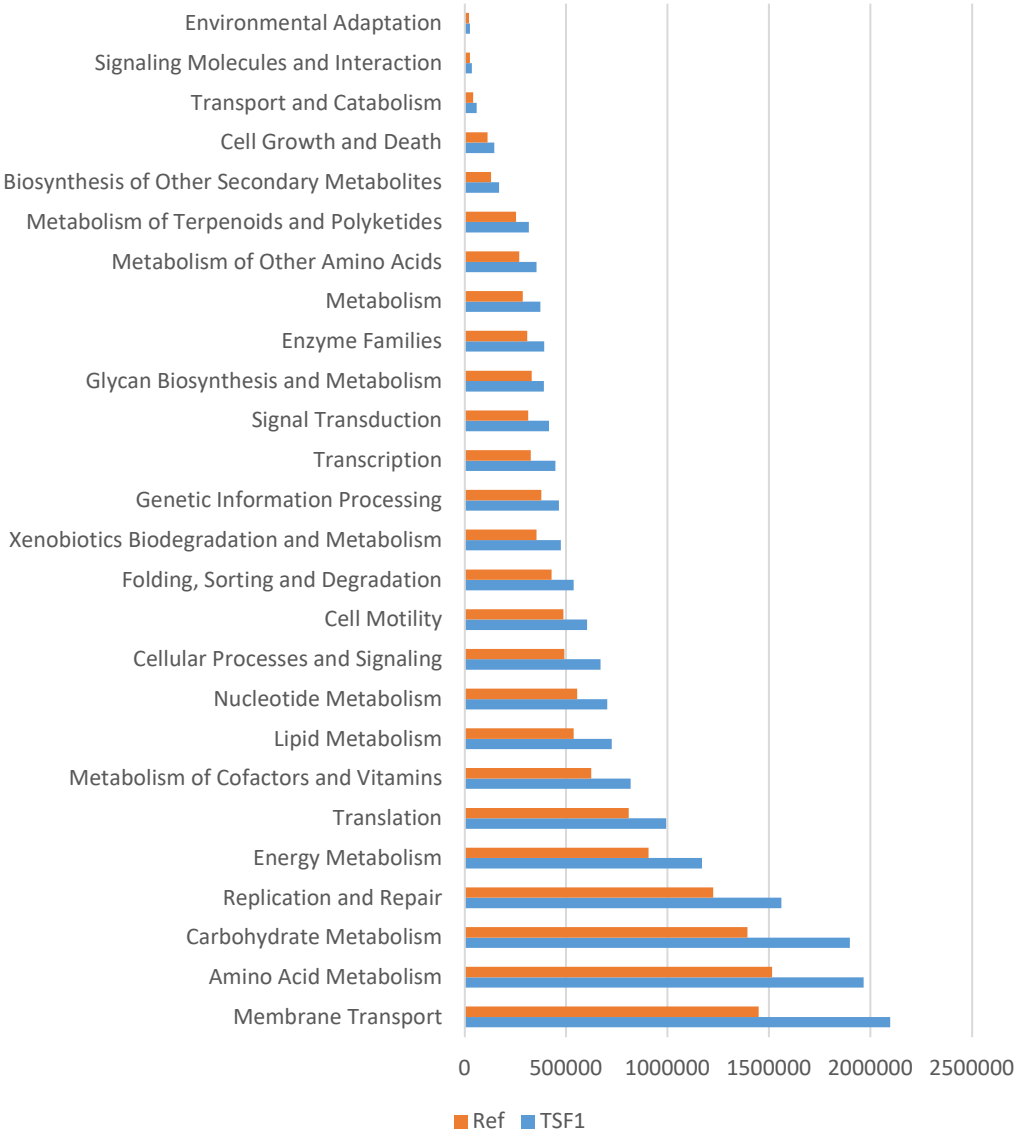

Figure 6 Categories of bacterial metabolic pathways inferred by PICRUSt2 from amplicon sequence variants (ASVs) in the reference site (red) and tailings storage facility 1 (blue). The X-axis shows the sum of the predicted functional abundance contributed by each ASV multiplied by the abundance (the number of input reads) of each ASV, then averaged per sample

There did not seem to be a big difference in the categories of bacterial pathways inferred at the Ref Site and at TSF1; the differences in the predicted functional abundances reflect the differences in the variety of ASVs. Pathways for methanogenesis, bacterial photosynthetic pathways, degradation pathways for lignin and hemicellulose, and autotrophic CO_2 fixation were well distributed, as were primary pathways like the Krebs cycle and the variant tricarboxylic acid cycle V found in many obligate autotrophs, microaerophiles, and methanotrophic bacteria and archaea (Wood et al. 2004; Zhang & Bryant 2011).

3.6 Taxonomic assignation of fungi

More than 90% of the identified fungi in TSF1 belonged to the phylum Ascomycota, in particular the genus *Coniochaeta* (Figure 7). Ascomycota, Basidiomycota and Rozellomycota were more evenly distributed in the Ref Site.

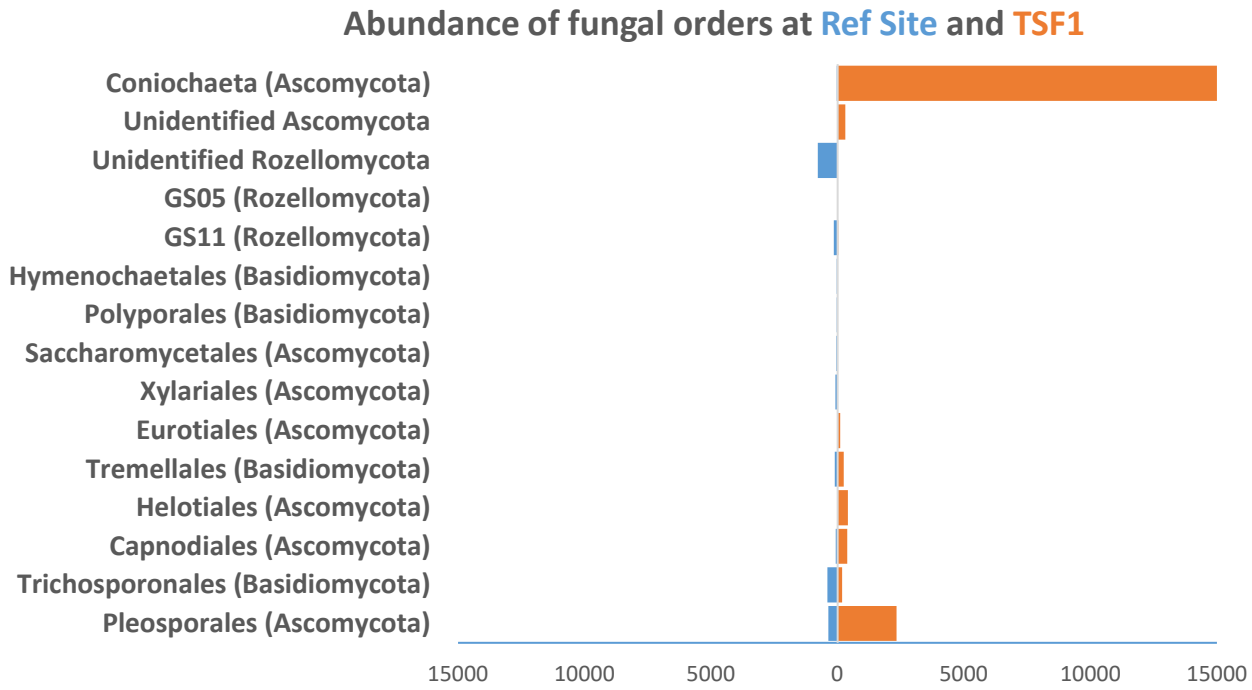

Figure 7 Most abundant fungal orders and their relative proportions in mine tailings samples. The X-axis are the number of amplicon sequence variants averaged per sampling site. Some orders are not identified or named (e.g. GS05, GS11)

4 Discussion

This study found a higher species richness and comparable metabolic functions in TSF1 samples compared to the Ref Site samples. Both sites had been spontaneously colonised by one species of tall grass, *S. spontaneum*. The Ref Site, the more recent, was overgrown by the grass. In the TSF1 site the plants had a patchier distribution but had more species associated with the grass. The tailings were acidic (ca. pH 5) except for sub-site 4, colonised by the greatest species diversity where pH was ameliorated by one unit (ca. pH 6). Organic matter in the tailings was approximately an order of magnitude less the surrounding forest soils, indicating a poor level of ecosystem and soil development (George et al. 2010). Nitrogen concentrations were found to be low and nutrient availability is known to be a key factor limiting revegetation, potentially more important than metal toxicities (Macdonald et al. 2017). Overall copper concentrations were similar to other older copper tailings known to be phytotoxic and reported in the literature (Degani et al. 2022). Tailings at the Ref Site were also found to have more than three times the copper concentration at TSF1, as well as higher concentrations of zinc and lead.

Overall microbial species diversity was loosely related to plant diversity above-ground. However, we cannot show any definitive statistical relationship due to limited sample numbers. Nonetheless we can show details of the community composition of bacterial and fungal communities in the tailings, along with their potential functional attributes.

4.1 Bacterial diversity

The most abundant bacteria found belonged to the phyla Proteobacteria, Chloroflexota, Acidobacteriota and Actinomycetota. These phyla are also commonly found in forest soil (Tian et al. 2017) and in soil surrounding copper mine tailings (Xia et al. 2023). This study did not find Firmicutes to be abundant, in contrast to Gupta et al. (2017) and Xia et al. (2023), who found them abundant in copper mine tailings.

The taxonomic order Rhizobiales was abundant in TSF1. Many members of this order are known to associate with plant roots. The greater variety of plants at TSF1 may have favoured the development of a complex Rhizobiales community at this site. The Ref Site was overgrown, and it also has Rhizobiales and another order known to associate with plant roots, Sphingomonodales. Both orders belong to the phylum *Proteobacteria*, a large phylum of metabolically diverse gram-negative bacteria found in many types of substrates (Felske et al. 2000; Fukuyama et al. 2020). In contrast, KD4-96, from the phylum Chloroflexota, was abundant in the Ref Site. Li et al. (2015) proposed that Chloroflexi might be a Cu bioindicator; the Ref Site had three times the copper content of the TSF1 site. KD4-96 are associated with contaminated substrates where their abundance correlates with iron concentration, though their potential function in these ecosystems is unclear (Kujala et al. 2018). Chloroflexi have been found in extreme environments as well, such as alkaline hot springs (Bennett et al. 2022). Brewer et al. *(*2019) wrote that Chloroflexi can be important in bioremediation in that they participate in organic matter degradation, nitrogen removal and biofilm aggregation.

Some orders associated with mining environments that were found in low abundance in both sites included Burkholderiales (*Thiobacillus*) and Thermales (*Meiothermus*). Gupta et al. (2020) have shown that copper mine tailings harbour characteristic species; the mix of species is different in tailings that have oxidised over time (e.g. *Sulfobacillus, Acidithiobacillus, Leptospirillum, Ferrimicrobium, Ferrithrix)* compared to disturbed tailings (*Thiobacillus, Sphingomonas, Meiothermus, Sulfurifustis,* and members of *Hydrogenophilaceae, Acidiferrobacteraceae, Gaiellales, Gemmatimonadetes, Ignavibacteriales,* KD4-96). Bacteria from Ref Site and TSF1 tailings were not enriched for highly acidophilic, iron- and sulphur-oxidising microorganisms that Gupta et al. identified in the oxidised tailings.

Taxonomic and metabolic diversity may indicate substrate health (Trivedi et al. 2016). The similarity of common phyla with constituents of forest soil suggests the tailings have a potential for developing more 'soil-like' communities and that the tailings are going through succession. However, the plant life in TSF1 is still far less rich than in the adjacent forest. How far succession will proceed in the tailings has not been assessed.

4.2 Fungal diversity

Although there appears to be more fungal species richness in TSF1, the results of fungal metabarcoding are in fact more challenging to interpret. Assigning fungal taxonomies to ITS2 region sequences was not as complete as with bacterial 16S rRNA gene sequences. Problems with using ITS sequences include small databases, varying resolution depending on species, and a lack of consensus on the type and amount of data required for species delineation (Thines et al. 2018). Their ability to disperse through spores by air or water may mean that their presence in a sample may be accidental.

Fungal diversity nonetheless appeared to increase qualitatively with plant diversity, consistent with relationships between plants and fungi which serve as extensions of root systems (Rokni et al. 2023). Fungi perform diverse ecosystem functions. Saprotrophs cycle nutrients. Symbiotrophic fungi, e.g. mycorrhizal fungi, increase nutrient capture by increasing the effective surface areas of roots. Pathotrophic fungi may cause disease but may also control other plant, insect and fungal pests. Rozellomycota, for example, are known as obligate endoparasites of other fungi (Corsaro et al. 2014). Their absence in some sites and presence in others suggests an abundance and variety of their hosts.

The dominant group, however, is *Coniochaeta* sp. — ubiquitous yeast typically associated with wood, water and soil (Weber 2002). de Andrade et al. (2022) stated that one species, *C. debeurmannianum,* produces melanin-like substances, a potential virulence factor associated with survival in extreme environments including those with metal contamination (Cordero & Casadevall 2017).

The presence of common groups Ascomycota and Basidiomycota suggests that the mine tailings are supporting succession that is leading to more soil-like properties. These fungal phyla are the most abundant in normal topsoil and the most effective in degrading plant residues (Manici et al. 2024). Basidiomycota are known for their ability to adjust to detrimental conditions and their efficiency in breaking down lignocellulosic biomass. They were found to be abundant also in copper- and cobalt-contaminated soils, with Basidiomycota being the more abundant in

the metal-contaminated soils and Ascomycota in the unimpacted reference soil (Dusengemungu et al. 2024). Manici et al. (2024) studied them as positive indicators of efficient soil carbon accrual.

Injection with a lixiviant might change the bacterial and fungal communities. We do not know whether this will perturb the succession processes and to what extent. The choice of lixiviant will matter; the altered chemistry of the tailings as well as the changes in microbial diversity will have to be examined further to assess the risk associated with ISL.

Not all clades and metabolic pathways may be required for the community to bounce back after a disruption. Sarkar et al. (2022) wrote that it is 'possible to determine a subset of microbes and microbial functions of the original population that is necessary to build a stable community'. A disrupted community will reach a steady state through a subgroup of microbes whose collective function eventually allows the community to recover.

5 Conclusion

The mine tailings at TSF1 had higher microbial species richness than samples from the Ref Site. Similar microbial taxa were found in both sites but these taxa differed in distribution. The difference in distribution is associated in part with spontaneous plant colonisation, homogenous in the Ref Site and more heterogeneous in TSF1.

The qualitative association of microbial species richness with spontaneous plant cover in TSF1 suggests 'normal' succession is taking place. Both sites appear favourable to the development of bacterial communities and increasing richness.

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