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# A Snapshot of the Microbiome of a Portuguese Abandoned Gold Mining Area

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**Abstract:** Microbial communities are known to contribute deeply to geochemical cycles, including weathering, protection from erosion and mineral precipitation. Studies aiming to understand mining areas' microbiomes are of high relevance since they can help pinpoint the occurrence of environmental shifts, key bioremediation species, environmental metals recovery strategies, and microorganisms with relevant industrial properties. Nonetheless, so far, the study of Portuguese gold-rich areas' microbiomes has been largely neglected. The main goal of this study was to apply high-throughput sequencing methods to study the microbiome (Bacteria and Fungi) and predict their functional/metabolic profiles in an abandoned Portuguese gold mining area (considering zones without a history of mining, the tailings and the flooded mine interior). The results obtained revealed high bacterial and fungal diversities at these sites while also pinpointing the presence of relative homogenous bacterial and heterogenous fungal communities. Areas without mining history were mainly dominated by WD2101 soil groups, *Sphingomonas*, Candidatus *Solibacter*, *Helotiales*, unclassified *Fungi* and *Arxotrichum*. The tailings were mainly colonized by *Bryobacter*, WD2101 soil groups, WPS-2 genera, *Starmerella*, *Helotiales* and *Mollisia*. On the other hand, the mine area displayed a dominance of *Crossiella*, *Gemmataceae*, *Acidobacteriaceae* (Subgroup 1), *Acidiphilium*, *Mortierella*, unclassified *Fungi* and *Chaetothyriales*. Furthermore, we verified that contrary to bacteria, the fungal structural diversity is somewhat more restricted to each site. In addition, metabolic, functional and ecological profiles revealed a strong distinction for both bacterial and fungal communities while also revealing the presence of well-adapted communities to each of the particular microenvironments considered.

**Keywords:** gold mine; metabarcoding; microbiome; bacteria; fungi; tailings



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## 1. Introduction

Despite its somewhat limited mainland territorial area, Portugal holds various important inorganic hotspots, mainly congregated in three large and distinct mineralogical clusters present across the country [1,2]. When focusing on gold (Au)-rich areas/ores, multiple interesting sites have been identified, with some of them being known since the Roman occupation period [3,4]. However, for the past thirty years, solely the Panasqueira (mostly Tungsten (W)), Aljustrel (mostly Copper (Cu) and Zinc (Zn)) and Neves Corvo (mostly Cu and Zn) mines have been actively operating. Thus, Portuguese gold mines or even interest presumed prospection areas, while explored and studied in the past, have now become largely forgotten. Throughout the ages, gold has served as a critical economic

currency, shaping human civilization, society, history and development. Nonetheless, auriferous mining is known to contribute to the increase of toxic elements, heavy metals and metalloids in the environment [5–7]. Due to the high level of waste resulting from drilling and the consequent creation of dumps and tailings containing the ensuing waste stream, this activity is known for having serious detrimental environmental impacts. Arsenic (As), Zn, Cu and lead (Pb) are known to be released and accumulated during gold extraction processes, contributing to soil and water contamination and, consequently, posing several human health risks [8]. These include various skin disorders, respiratory issues and other long-term health effects (e.g., cancer) due to arsenic, mercury and cyanide food, water and land contamination, and human exposure. Moreover, these elements can also affect the microbiome of these areas and their various ecosystem functions/services.

Microbial communities are known to be key players in geochemical cycles [9–14]. Their presence, proliferation and activity contribute to various natural geomorphic roles, including weathering, protection from erosion and precipitation of particles and minerals [11–13]. Diverse microbial communities can act on fundamental geological processes, since they are able to conduct organic and inorganic transformations, element cycling, rock and mineral transformations, bioweathering, neo-mineral formation and metal/clay interactions [11,15]. In fact, a large fraction of the microorganisms at the surface and subsurface biosphere constitutes a key component of biogeochemical cycles.

The study of mining areas' soils microbiomes can contribute to (1) identifying environmental shifts; (2) recognizing key bioremediation species; (3) highlighting novel environmental metals biorecovery strategies; (4) identifying microorganisms with relevant industrial physiological properties; (5) contributing to the development of circular economies; (6) and as for the case of fungi, acting as possible geological proxies for gold prospection [16–25]. Thus, understanding the diversity and structure of microbial communities in rich metal areas, such as gold mines, is currently of high scientific, industrial and applied relevance. Nonetheless, microbial studies in such settings are still scarce, and while a considerable amount of information is available for prokaryotes, much less is known for fungal communities [6,22,26–38]. Commonly, the study of bacteria and archaea in gold mining environments focuses on their uniqueness and phylogenetic diversity in these settings [6,26,27,30,32–34,36–38]. Some physiological peculiarities have also been explored, including bacteria contribution to the sulfur cycle, the identification of mercury-resistant strains, the evaluation of heavy metal immobilization potential, and the effects of toxic metals and plant diversity on the communities' organization [6,27,30,34–36]. As for fungi, studies have mainly focused on the community structure associated with soil, boreal species colonizing mine tailings, the arsenic impact on the molecular diversity of arbuscular mycorrhizal and filamentous fungi, and some heavy metal tolerance traits (e.g., arsenic resistance) [28,29,31,34]. Nonetheless, there is still a lack of studies simultaneously focusing on prokaryotic and fungal communities in these settings. Furthermore, given that numerous mines experience flooding during and after operations, the knowledge in these cases is notably much more constrained [39]. Additionally, the settings in these areas can also lead to the formation of acid mine drainage (AMD) ecosystems. These are a result of the slow oxidation of pyrite and other sulfide minerals exposed to air and oxygenated water, being often accelerated by microorganisms with oxidation abilities [40,41]. When these minerals are exposed to oxygen and water through mining activities, they can undergo chemical reactions (oxidation and hydrolysis) that produce sulfuric acid. Similarly to general gold mine scenarios, while numerous studies on AMD bacteria and archaea are available, few have been conducted focusing on fungi, particularly with modern molecular biology techniques [40,41].

During the past years, the development, optimization and implementation of DNA-based methods to study microbial communities has revolutionized modern microbiology and microbiome analysis. The application of high-throughput sequencing (HTS) next-generation sequencing (NGS) approaches has deeply expanded the possibility to extensively characterize microbial communities [42–48] while also contributing to understanding their

metabolic functions and their active byproducts in a particular environmental context. During the last decade, Portuguese areas long known to be rich in Au, silver (Ag), Cu Zn, iron (Fe), W and lithium (Li) have been the focus of a fresh wave of interest. Nonetheless, so far, the study of the structural and functional microbiomes of Portuguese gold-rich areas has been largely neglected. Abandoned mining areas are recognized as extreme environments due to the consequences of auriferous removal, including increased concentrations of heavy metals, metalloids and other toxic elements. Flooded mines also pose additional challenges for microbial proliferation since the availability of organic matter, oxygen and light in these settings is often limited [39]. As there is still no record of the structural and functional microbiomes of Portuguese gold-rich areas, the aim of this work was to gather information regarding the bacterial and fungal communities in an abandoned gold mining area across areas without a history of mining activities, the mine tailings and the flooded mine interior. For that, we employed culture-independent methodologies (Illumina MiSeq® (Illumina, San Diego, CA, USA) 16S rRNA gene and Internal Transcribed Spacer 2 subregion targeted metabarcoding sequencing) coupled with differential bioinformatic analysis to profile these communities and their functional/ecological traits at various locations of the Escádia Grande mining complex (Góis, Portugal).

## 2. Materials and Methods

### *2.1. Sampling Site Description, Sample Collection and Processing*

Located at circa 11 km south of Góis (Coimbra, Portugal), the Escádia Grande mine is the most important mining site in the 252 $km^2$ homonymous exploration area (Au-Ag metallogenic belt). In general, the area's steep slope is covered by dense vegetation, mainly from *Eucalyptus globulus* and *Pinus pinaster*. The exploitation of mineralogical resources has been dated back to the Roman period, although recently, the mine is known to have operated from 1940 to 1952. During this period of active exploitation, it is known that prospection occurred underground on seven levels and that circa 42,878 tons of material were handled [49]. Ore deposits are composed of tabular quartz veins consisting of quartz, arsenopyrite, pyrite, rare chalcopyrite, galena, sphalerite, gold and argentite [3,50]. Au-Ag bearing quartz veins are 70 cm thick, up to 300 m long, with an average of 8 g/t of Au and 33 g/t of Ag [49,50]. Mine dumps and tailings are mainly composed of quartz and other rock pieces with various sulfides (mainly arsenopyrite and galena). Moreover, mine wastes contain up to 8090 mg/kg of As and 70.1 mg/kg of Sb with arsenic, thus being the main human health concern trace element present in the Escádia Grande mining area [51].

Three representative samples were selected for analysis: (P1) a control sample in an area located outside of the main mine zone, known to be deprived of relevant gold concentrations and without any history of past mining activities; (P2) a sample from the tailings located on the mining area hill, circa 25 m in front of the mine entrance (surrounded by dense fern vegetation); and (P3) a sample from the mine interior, collected at about 1 m next to the entrance (on one of the few lateral wall dry spots found, due to the mine being flooded) (Figure 1). Soil samples (circa 25 g) were collected to a depth of 5 to 10 cm after removal of the surface soil (±5 cm). Samples were properly conditioned to prevent contamination in individual sealed sterilized bags, cooled at 4 °C for transportation and maintained in sterile conditions (unopened and undisturbed) until further laboratory analysis. In the laboratory, samples were air-dried for 24 h and then sieved to remove the presence of plant material before environmental DNA extraction.

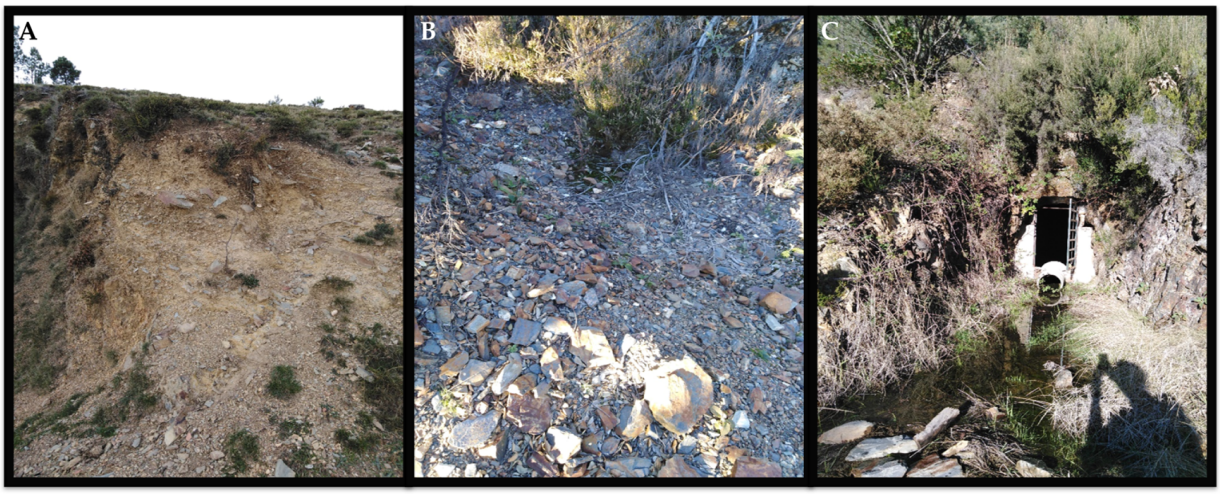


**Figure 1.** Sampling sites details of the (**A**) control, (**B**) tailings and (**C**) the mine.

### 2.2. DNA Extraction, High-Throughput Sequencing

The air-dried and sieved soil samples DNA was extracted using a combination of the Nucleospin Soil Kit (Macherey Nagel, Düren, Germany), Buffer SL1 and the Enhancer SX according to the manufacturer's instructions. Samples were then prepared for Illumina Sequencing by amplification of the 16S rRNA gene V3/V4 hypervariable regions and the Internal Transcribed Spacer 2 (ITS2) sub-regions of the microbial communities. For this purpose, the hypervariable DNA regions were initially amplified with specific primers and further reamplified with a limited-cycle PCR reaction aiming to add the sequencing adapters and dual indexes. The initial PCR reactions were individually performed using the KAPA HiFi HotStart PCR Kit (according to manufacturer suggestions), 0.3 µM of each PCR primer and 2.5 µL of template DNA in a total volume of 25 µL. The primers considered consisted of the forward primer Bakt 341F 5′–CCTACGGGNGGCWGCAG–3′ and the reverse primer Bakt 805R 5′–GACTACHVGGGTATCTAATCC–3′ for bacteria [52,53]; and a pool of forward primers ITS3NGS1 F 5′–CATCGATGAAGAACGCAG–3′, ITS3NGS2 F 5′–CAACGATGAAGAACGCAG–3′, ITS3NGS3 F 5′–CACCGATGAAGAACGCAG–3′, ITS3NGS4 F 5′–CATCGATGAAGAACGTAG–3′, ITS3NGS5 F 5′–CATCGATGAAGAACGTGG–3′ and ITS3NGS10 F 5′–CATCGATGAAGAACGCTG–3′ and the reverse primer ITS4NGS001 R 5′–TCCTSCGCTTATTGATATGC–3′ for fungi [54]. The conducted PCR reactions entailed a 3 min denaturation at 95 °C, 35 cycles of 98 °C for 20 s, 55 °C (bacterial region)/60 °C (fungal region) annealing for 30 s, extension at 72 °C for 30 s and a final extension step at 72 °C for 5 min. Indexes and sequencing adapters to both ends of the amplified target regions were added through a second PCR reaction according to the manufacturer's recommendations [55]. Negative PCR controls were considered for all amplification procedures. Amplification was evaluated by electrophoresis on a 1% (*w*/*v*) agarose gel and quantified by the Qubit dsDNA High Sensitivity Assay Kit (Invitrogen Life Technologies, Waltham, MA, USA) on the Qubit 2.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA). The obtained PCR amplicons were then one-step purified and normalized using the SequalPrep 96-well plate kit (ThermoFisher Scientific, Waltham, MA, USA) [56]. This kit allows a solid phase, high-throughput amplificon purification and normalization through DNA binding, washing (to remove contaminants) and elution at predetermined concentrations (normalization). Purified amplicons were then pooled and pair-end sequenced in the Illumina MiSeq® sequencer with the MiSeq® Reagent Kit v3, according to manufacturer's instructions (Illumina, San Diego, CA, USA) at Genoinseq (Cantanhede, Portugal).

### 2.3. Bioinformatic Data Processing

The initial sequence data was processed at Genoinseq (Cantanhede, Portugal). The obtained raw sequencing reads were extracted from the Illumina MiSeq® System in fastq

format and quality-filtered. Filtering was performed with PRINSEQ v.0.20.4 [57] and aimed to remove sequencing adapters; trimming was based on an average quality lower than 25 in a window of 5 bases and reads with less than 100 bases for the ITS2 region and 150 bases for the 16S rRNA gene were removed. Forward and reverse reads were then merged with AdapterRemoval v.2.1.5 [58] using default parameters. For bacteria, further treatment of the obtained metabarcoding data, clustering and taxonomic analyses were performed with the mothur package v.1.46.1 [59,60]. The obtained bacterial reads were subjected to quality control measures, with chimeras being detected and removed from the datasets with the VSEARCH software v.2.16.0 [61]. The remaining high-quality sequences were clustered into operational taxonomic units (OTUs) at 97% similarity with OptiClust [62] and then taxonomically annotated against the ARB-Silva taxonomic database v.138.1 [63] with the RDP classifier [64]. For fungi, the metabarcoding data treatment was performed with the SEED 2 bioinformatics package [65]. The obtained fungal reads were subjected to quality control measures, the enclosed ITSx software v.1.0.11 [66] was applied to extract the ITS2 sub-region (for all organisms) with default settings, chimeric sequences were detected and removed from the dataset, with the remaining reads being clustered into OTUs at 97% similarity applying the USEARCH/UPARSE (v.8.1.1861) approach [67]. Fungal OTUs were first BLAST-queried against the UNITE database v.8 [68]. Taxonomically unidentified OTUs were then further analyzed against the UNITE + INSD database in the PlutoF platform [69] through massBLASTer analysis.

For both taxonomic groups, the final tables were screened to remove from the datasets OTUs assigned to non-target taxonomic groups with less than three reads.

Relative abundance plots were obtained in R/R Studio v.4.1.1 with the microeco package v.0.17.0 [70] and Krona v.2.4 [71]. To evaluate individual shared OTUs between the distinct samples, Venn diagrams were constructed online with the Van de Peer Lab tool (http://bioinformatics.psb.ugent.be/webtools/Venn/, accessed on 1 January 2023). Complementarily, the diversity indexes and the coverage values were calculated with the mothur package v.1.46.1 [59,60]. Additionally, the past3 statistical package v.3.20 [72] was also employed to plot a principal component analysis (PCA) based on a correlation matrix (Biplot approach) for each taxonomic group. Both datasets were independently uploaded to MicrobiomeAnalyst v.1.0 [73,74] (https://www.microbiomeanalyst.ca/, accessed on 1 January 2023) for heat map and core microbiome analysis. Since many bacterial OTUs shared a similar "uncultured" classification at various taxonomic levels (leading to the merging of differential taxonomic groups and possible analysis incongruences), the dataset was manually adjusted by adding the last taxonomic level identified. Hierarchical clustering heat maps were conducted reflecting Pearson distance measures with Ward clustering algorithms, and the core microbiome analysis was obtained at the family level based on a 20% sample prevalence and 0.01 relative abundance. Bacterial functional profiles were obtained with the Tax4Fun tool v.1.0.0 [75] (for this case, using the original OTU table), while fungal functional traits were evaluated by screening the FungalTraits database v.1.2 [76] focusing mainly on primary lifestyle and decay substrate types. The results obtained for both the bacterial functional profiles and fungal ecological traits identified were also plotted as a heat map using ClustVis v.1.0 [77] (https://biit.cs.ut.ee/clustvis, accessed on 1 January 2023), as previously described.

## 3. Results and Discussion

### *3.1. Microbiome Diversity and Community Structural Organization*

The microbiome analysis was achieved by sequencing the V3/V4 hypervariable regions and the ITS2 subregion with the Illumina MiSeq® sequencing platform. Tables 1 and 2 highlight the metabarcoding statistical data obtained and the diversity indexes calculated for each sample considered during the course of this study.

**Table 1.** Metabarcoding statistical data and biodiversity indexes obtained for bacteria.

| Sample | nseqs | Coverage | Chao1 | Sobs | Shannon | Shannoneven | Simpson | Simpsoneven |
|---|---|---|---|---|---|---|---|---|
| **Control (P1)** | 28,226 | 0.994 | 1,785,009 | 1679 | 6282 | 0.846 | 0.006 | 0.099 |
| **Tailings (P2)** | 15,845 | 0.988 | 1,366,039 | 1256 | 5911 | 0.828 | 0.009 | 0.084 |
| **Mine (P3)** | 26,614 | 0.995 | 1,018,000 | 889 | 5244 | 0.772 | 0.015 | 0.073 |

**Table 2.** Metabarcoding statistical data and biodiversity indexes obtained for fungi.

| Sample | nseqs | Coverage | Chao1 | Sobs | Shannon | Shannoneven | Simpson | Simpsoneven |
|---|---|---|---|---|---|---|---|---|
| **Control (P1)** | 46,115 | 0.999 | 7,055,769 | 684 | 44,333 | 0.6791 | 0.034 | 0.043 |
| **Tailings (P2)** | 91,776 | 1.000 | 2,707,143 | 254 | 24,392 | 0.4405 | 0.172 | 0.023 |
| **Mine (P3)** | 47,849 | 0.999 | 383,625 | 369 | 36,553 | 0.6184 | 0.061 | 0.044 |

For bacteria, a total of 70,685 reads were obtained, with the OTU number determined for each sample fluctuating between 889 (mine) and 1679 (control). Complementarily, for fungi, 185,740 reads were obtained, with the OTU number determined for each sample fluctuating between 254 (tailings) and 684 (control). Based on the aforementioned results, it was evident that fungi constituted the most abundant group at these sites. By examining the obtained Chao1, sobs and the coverage values, it can be verified that for both taxonomic groups, their diversity was successfully recovered and analyzed. Complementarily, when considering the results obtained for the biodiversity indexes, the values demonstrated the existence of a relatively high bacterial and fungal diversity in all sites. For both cases, the uppermost diversity values were verified in the control, while the lowest were verified in the mine for bacteria and in the tailings for fungi. High evenness levels were observed for bacteria and intermediary for fungi, pointing towards the presence of a relatively homogenous bacterial taxa distribution and the presence of some dominant fungal taxa among the studied areas.

In parallel, the results also revealed the detection of 2575 OTUs for bacteria and 1039 for fungi across all samples. For bacteria, 262 OTUs were common to all sites, with the largest unique OTU number being found in the control (829), followed by the tailings (426) and the mine (333) (Figure 2). On the other hand, for fungi, 31 OTUs were found in all samples, with the largest unique OTU number being found in the control (468), followed by the mine (210) and the tailings (124) (Figure 2).

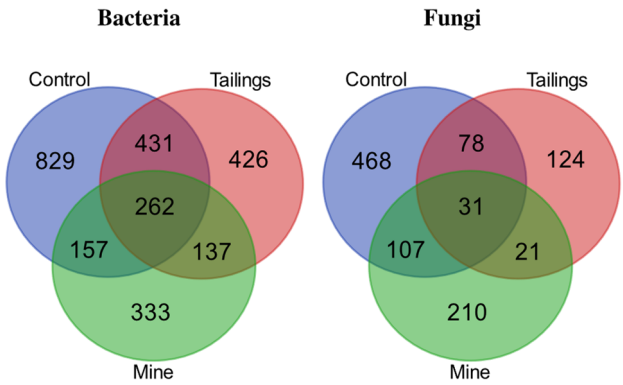


**Figure 2.** Venn diagrams displaying the number of shared bacterial and fungal OTUs across samples.

The results obtained for the PCA analysis can be verified in Supplementary Figures S1 and S2. For bacteria, the control and tailings samples clustered somewhat closer together, thus indicating a more similar composition and distribution while also implying that their structural diversity is less exclusive to either of these sites. Additionally, it could also be verified that the majority of the OTUs clustered in a somewhat dispersed pattern when taking into account the different samples. In contrast, for fungi, all the samples clustered

in a more heterogeneous manner, indicating a dissimilar composition and distribution and pointing out that their structural diversity is somewhat more restricted to each site. Furthermore, it should be noted that various distinct OTUs clustered in a more assembled pattern with each of the considered samples for this taxonomic group. Taken together, these results highlight that the overall microbiome diversity of the Escádia Grande mining area decreases inside the mine itself and at the tailings. However, bacterial and fungal diversity and distribution are affected differently, as prokaryotes displayed a relatively homogeneous taxa distribution with the lowest diversity found in the mine interior, while fungi exhibited the presence of some dominant taxa, with the lower diversity found at the tailings. Moreover, despite some commonality and the recognition that each site harbors a distinct microbial community, the data also underscores a further notorious site-specific nature of the fungal communities. In general, As, aluminum (Al), potassium (K), silicon (Si), chromium (Cr), mercury (Hg) and Pb concentrations, soil pH and total organic carbon, as well as vegetation density and the specific plant species, have been pinpointed as critical factors influencing the prokaryotic community diversity and structure in similar environments [27,30,32–36]. Moreover, it is also known that less-polluted soils exhibit increased microbial diversities and more complex community structures [30,32]. On the other hand, fungal communities' structures are known to be more similar across tailings deposits regardless of vegetation density. Moreover, fungal richness increases in a tailor-made trend with contamination level, and biodiversity is not related to the concentrations of inorganic toxicants [29]. As such, the results obtained during the course of this work are in accordance with the literature, showing that the polluted soils from the mining area shape the microbiome diversity. Additionally, our findings suggest that the presence of highly dense fern vegetation in the tailings (see Section 3.2 below for details) and the higher humidity in the mine interior may contribute to the contrasting lower fungal and bacterial diversities verified, respectively. Conversely, the lower diversities observed for a specific group of organisms at these sites might result from the documented increase in the other group, influenced by the specific settings of the location. In other words, the increase in bacterial richness in tailings was likely influenced by the presence of vegetation [35], coupled with a decrease in fungal diversity. Similarly, the observed increase in fungal diversity in the mine is likely influenced by factors such as mine settings (e.g., darkness, acidic environment) and the impact of flooding water, coupled with a decrease in bacterial abundance and richness [39].

The biodiversity results obtained from the metabarcoding analyses are presented in Figures 3 and 4 and Supplementary Files S1–S6. In general, at the Phylum level for bacteria, the control was largely dominated by *Proteobacteria* (22%), *Planctomycetota* (15%), *Acidobacteriota* (14%), *Bacteroidota* (10%) and *Actinobacteriota* (8%); the tailings were largely dominated by *Acidobacteriota* (22%), *Chloroflexi* (16%), *Proteobacteria* (15%), *Planctomycetota* (12%) and *Actinobacteriota* (7%); and lastly, the mine was largely dominated by *Acidobacteriota* (22%), *Chloroflexi* (19%), *Proteobacteria* (18%), *Actinobacteriota* (15%) and *Planctomycetota* (13%). At the family level, the great majority of reads were assigned to families: *Sphingomonadaceae* (8%), WD2101 soil group (8%), *Chitinophagaceae* (7%), *Chthoniobacteraceae* (5%) and *Gemmatimonadaceae* (3%) in the control samples; *Bryobacteraceae* (10%), *Ktedonobacteraceae* (6%), WD2101 soil group (6%), unclassified WPS-2 (Candidatus phylum *Eremiobacterota*) (6%) and an additional WPS-2 family (6%) in the tailings; and *Gemmataceae* (9%), *Pseudonocardiaceae* (9%), *Ktedonobacteraceae* (8%), *Acidobacteriaceae* (Subgroup 1) (7%) and *Acetobacteraceae* (6%) in the mine. Lastly, when considering the diversity at the genus level, a WD2101 soil group genus (8%), *Sphingomonas* (6%) and Candidatus *Solibacter* (4%) were dominant in the control sample; *Bryobacter* (10%), a WD2101 soil group genus (6%) and a WPS-2 genus (6%) were dominant in the tailings sample; and *Crossiella* (8%), uncultured *Gemmataceae* (6%), *Acidobacteriaceae* (Subgroup 1) (4%) genera and *Acidiphilium* (4%) were dominant in the mine interior.

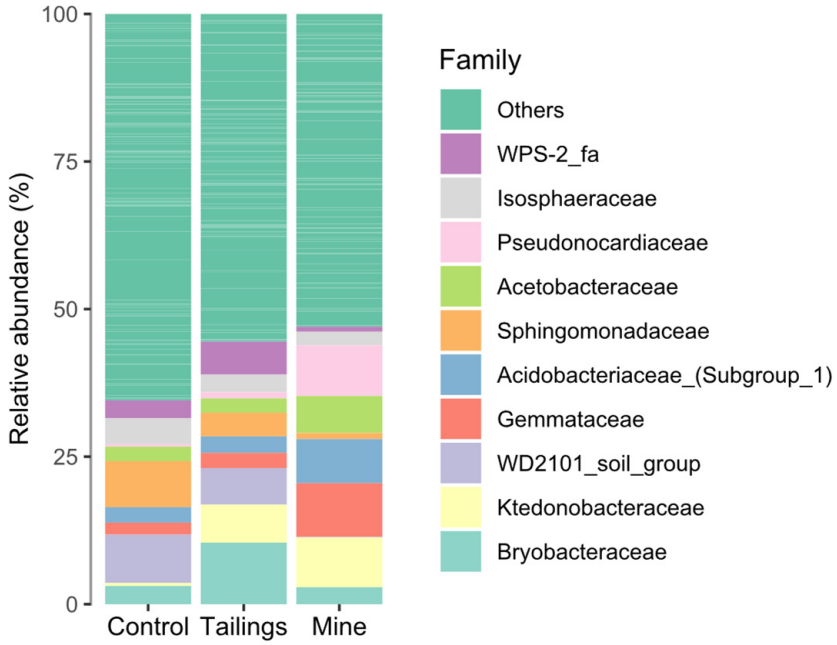


**Figure 3.** Bacteria relative abundance (Top10) at the Family level.

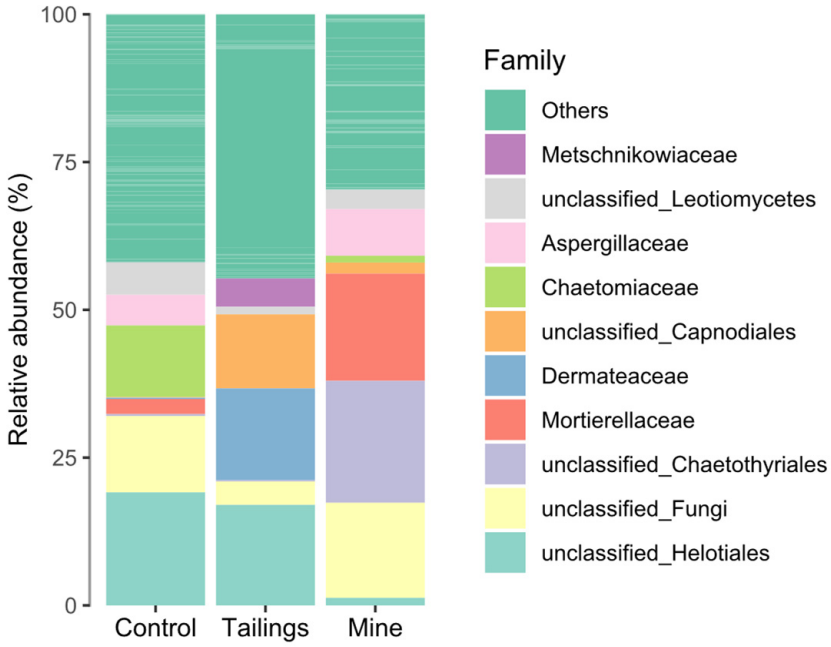


**Figure 4.** Fungi relative abundance (Top 10) at the Family level.

*Proteobacteria*, *Acidobacteria* and *Actinobacteria* are often regarded as the dominant taxa colonizing mine tailings, a consequence of low environmental pH, poor organic matter contents and high concentrations of elements such as Pb, Cd and Zn [35]. As such, our results corroborate and are in accordance with the currently available literature [35,78,79]. Moreover, it was also possible to detect a change in dominance from *Proteobacteria* to *Acidobacteriota* during the transition from the control towards the tailings and the mine areas, likely mirroring the alterations in soil pH and ion concentrations. *Acidobacteria* were also found to be dominant in soils of Linglong Gold Mine (China) [24] and have been reported as the main prokaryotes in several distinctive acidic environments [80]. As for the additional genera, the WD2101 soil group and *Gemmataceae* genus have been previously

reported in various terrestrial habitats [33,81], while *Sphingomonas*, Candidatus *Solibacter*, *Bryobacter* and *Acidiphilium* have frequently been described as present in gold mining ores and tailings [24,34,35]. Additionally, *Crossiella* has also been recently pinpointed as an *Actinomycetota* genus commonly found in the environment, including in various mineral mining soils [82].

On the other hand, for fungi, at the Phylum level, the control sample was largely dominated by *Ascomycota* (69%), unclassified *Fungi* (13%) and *Basidiomycota* (9%); the tailings were largely dominated by *Ascomycota* (95%); and lastly, the mine was largely dominated by *Ascomycota* (56%), *Mortierellomycota* (18%) and unclassified *Fungi* (16%). Additionally, it could also be verified that unclassified *Helotiales* (19%), *Chaetomiaceae* (12%), unclassified *Fungi* (13%), unclassified *Leotiomycetes* (5%) and *Aspergillaceae* (5%) are the predominant family level fungal groups in the control samples; *Saccharomycetales* fam. *Incertae sedis* (34%), unclassified *Helotiales* (17%), *Dermateaceae* (16%), unclassified *Capnodiales* (13%) and *Metschnikowiaceae* (5%) are predominant in the tailings; and that unclassified *Chaetothyriales* (21%), *Mortierellaceae* (18%), unclassified *Fungi* (16%), *Aspergillaceae* (8%) and *Rhizopogonaceae* (4%) are predominant in the mine sample. Finally, when considering the diversity at the genus level, unclassified *Helotiales* (19%), unclassified *Fungi* (13%) and *Arxotrichum* (12%) were dominant in the control samples; *Starmerella* (34%), unclassified *Helotiales* (17%) and *Mollisia* (16%) were dominant in the tailings; and *Mortierella* (18%), unclassified *Fungi* (16%) and *Chaetothyriales* (21%) genera were dominant in the mine.

These results also point to a change in dominance for fungal phyla across samples, namely a strong decrease from *Basidiomycota* from the control towards the tailings and the mine. Moreover, an interesting and sharp increase of *Mortierellomycota* inside the mine was also registered. The dominance of *Mortierellomycota* (marked as *Zygomycota*) has been previously identified in highly contaminated gold mining sites, likely due to their ability to withstand significant arsenic concentrations [29]. Nonetheless, considering our differential results across different areas, they are, thus, distinct from the ones found by Crognale and colleagues [29] and Gagnon and colleagues [35], where somewhat persistent dominances of *Zygomycota* and *Basidiomycota* were verified (i.e., frequently, the fungal biodiversity is not related to the concentrations of inorganic toxicants) [29], respectively. Nonetheless, the verified *Ascomycota* dominance is in accordance (at least to some degree) with the data obtained by Gagnon and colleagues [34] while studying bulk soil, the rhizosphere and plant roots from mine tailings in Québec (Canada). As for the additional most representative genera, *Helotiales* are common in soils, fallen leaves, decaying wood and other organic matter [83], while *Arxotrichum* are common in soils [84], and their presence (in addition to dominance in the control sample) can be considered somewhat expected. Furthermore, *Mollisia* and *Mortierella* have been commonly found in gold mining ores and tailings [29,35], *Starmerella* has been highlighted to contain sophorolipids able of Cd and Pb removal [85] (that putatively can help their survival at these sites), while Chaetothyriales are saprophytes commonly found in monuments and polluted environments, being able to degrade pollutants and hydrocarbons (e.g., [86]). These are physiological characteristics that may help explain their presence and dominance at these sites. Additionally, the high verified levels of *Helotiales*, in particular *Mollisia*, in the tailings sample could also be a result of their known ability to withstand high metal concentrations and being able to proliferate in environments with auriferous mining history [35].

In an attempt to further shed light on the structural characteristics of both fungi and bacteria in these areas, a core microbiome (Figures 5A and 6A) and heatmap analyses at the family level were also constructed (Figures 5B and 6B).

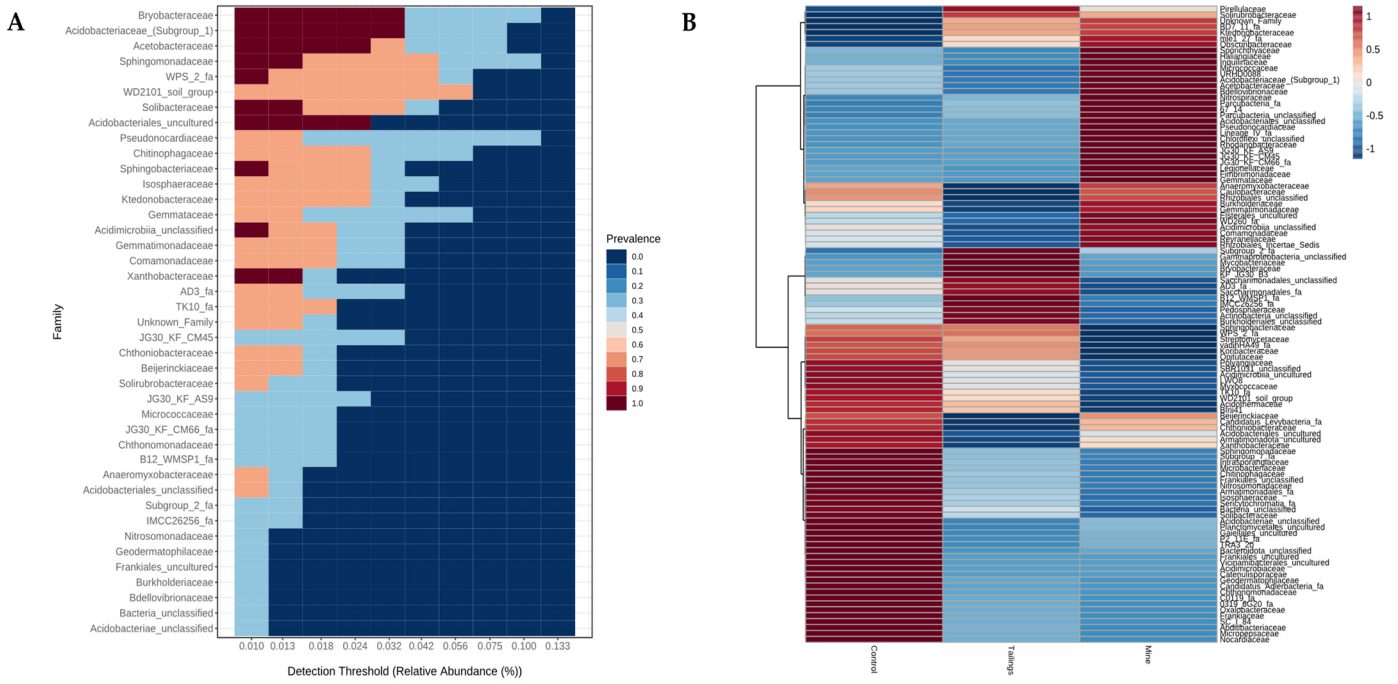


**Figure 5.** Bacteria core microbiome (**A**) and heatmap clustering (**B**) at the Family level across all samples.

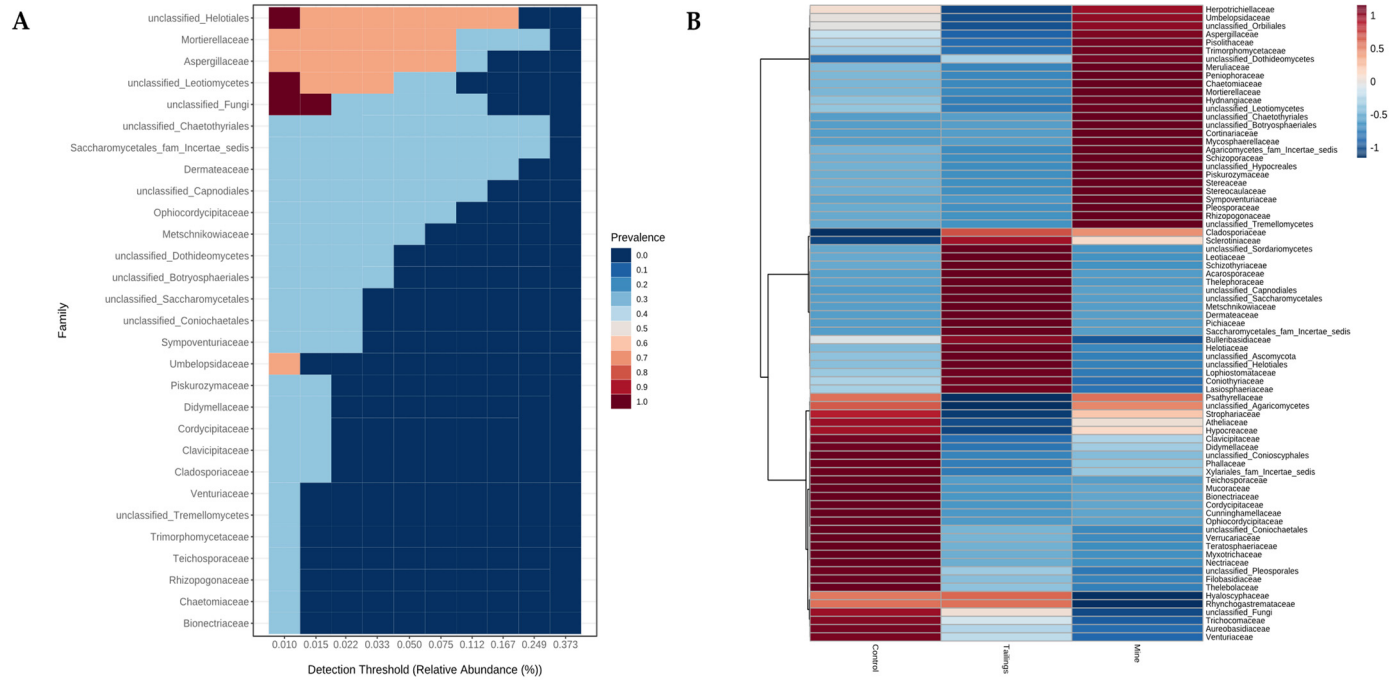


**Figure 6.** Fungi core microbiome (**A**) and heatmap clustering (**B**) at the Family level across all samples.

These results further confirm the previously verified notorious site-specific nature of the fungal communities. For bacteria, particular relevant core microbiome results are related to the families *Bryobacteraceae*, *Acidobacteriaceae* (Subgroup 1), *Acetobacteraceae*, *Sphingomonadaceae*, an unclassified family WPS-2, *Solibacteraceae*, an *Acidobacteriales* uncultured family, *Sphingobacteriaceae*, *Gemmataceae*, an unclassified *Acidimicrobiia* family and *Xanthobacteraceae*, since they denoted high prevalence and often high abundance across all samples.

On the other hand, for fungi, the most relevant core microbiome results concerned families related to unclassified *Helotiales*, unclassified *Leotiomycetes* and unclassified *Fungi*

since they were prevalent across all samples (although with somewhat lower relative abundances). Regarding the heatmaps, the results revealed that for bacteria, various families were often strongly and positively correlated with more than one location. Complementarily, for fungi, the heatmap results revealed that a more distinctive pattern was present, with multiple families being only positively and strongly correlated with one sample. Moreover, only a few families showed positive correlations in more than one sample. Overall, these results reinforce the findings previously reported and discussed for the biodiversity indexes and community structural organization while also pinpointing the presence of a more prevalent and diversified prokaryotic core microbiome rather than fungi.

### *3.2. Microbiome Functional/Ecological Traits Characteristics*

The bacterial functional predictions obtained with the Tax4fun tool can be verified in Figure 7.

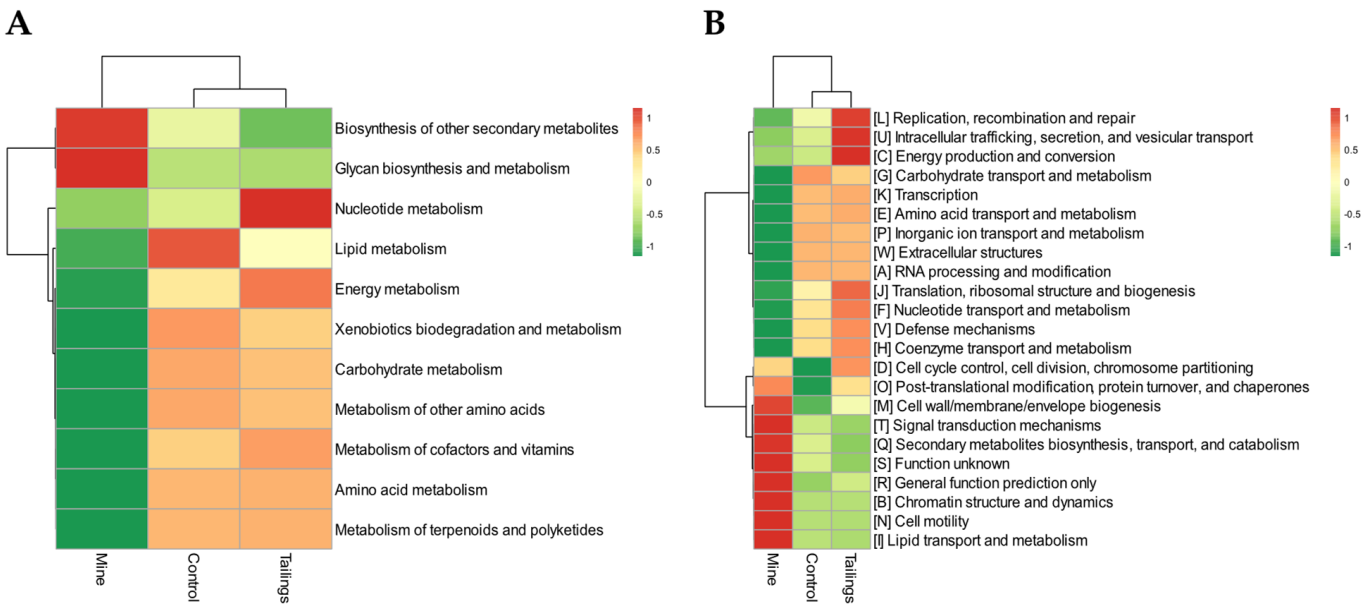


**Figure 7.** Heatmap of the bacterial KEGG metabolism classes (**A**) and COG functional categories (**B**) identified.

When considering the KEGG metabolism classes, the most relatively abundant were related to amino acid metabolism, carbohydrate metabolism, energy metabolism and the metabolism of cofactors and vitamins. Complementarily, when considering the results obtained for the COG functional categories, gene families related to metabolism were the most abundant, followed by information storage and processing and then cellular processes and signaling. Moreover, when considering the KEGG metabolism classes heatmaps obtained, the results revealed that mostly (Figure 7A) (1) lipids metabolism was strongly correlated with the control sample; (2) nucleotide and energy metabolism were sturdily correlated with the tailings sample; and that (3) the biosynthesis of other secondary metabolites and the glycan biosynthesis and metabolism were stalwartly correlated with the mine sample. For the COG functional categories heatmaps obtained, the results revealed that (Figure 7B) the [G] Carbohydrate transport and metabolism, [K] Transcription, [E] Amino acid transport and metabolism, [P] Inorganic ion transport and metabolism, [W] Extracellular structures and [A] RNA processing and modification gene families were strongly correlated with the control sample. Complementarily, gene families related to [L] Replication, recombination and repair; [U] Intracellular trafficking, secretion and vesicular transport; and [C] Energy production and conversion were strongly correlated with the tailings sample. On the other hand, the gene families connected with [M] Cell wall/membrane/envelope biogenesis, [T] Signal transduction mechanisms, [Q] Secondary metabolites biosynthesis, transport, and

catabolism, [B] Chromatin structure and dynamics, [N] Cell motility; and [I] Lipid transport and metabolism were sturdily correlated with the mine sample. Overall, from these results, it was possible to verify that the control displayed a strong association with bacteria further related to carbon metabolism (presence of carbohydrate transport and metabolism, and lignin degradation), while the tailings and the mine areas have metabolic profiles more closely associated with cell cycle maintenance and repair, energy production, xenobiotics biodegradation and various additional second-ary metabolic pathways. As such, these results point out that defense pathways and responses to environmental stress (e.g., [87–89]) are crucial for the survival of prokaryotes in metal-polluted environments [36] and thus also help explain the small variations verified for the bacterial populations.

The fungal traits results obtained can be observed in Figure 8. Overall, the results revealed that when considering primary lifestyles (1) the control was largely dominated by the categories soil saprotroph, unspecified saprotroph, litter saprotroph, animal parasite and lichenized forms; (2) the tailings sample was largely dominated by the categories nectar/tap saprotroph and plant pathogen; and (3) the mine was largely dominated by the categories, soil saprotroph, unspecified saprotroph, animal parasite, ectomycorrhizal and litter saprotroph. Moreover, when considering the primary lifestyles heatmaps obtained, the results revealed that (Figure 8A) the categories (1) lichen parasite, dung saprotroph, litter saprotroph, lichenized and sooty molds were strongly positively correlated with the control sample; (2) pollen saprotroph, nectar tap saprotroph and plant pathogen were strongly positively correlated with the tailings sample; and (3) that mainly ectomycorrhizal, mycoparasite, animal parasite and soil saprotroph were strongly positively correlated with the mine sample. On the other hand, when considering decay substrates, (1) the control was largely dominated by the categories soil, leaf/fruit/seed/soil/animal material and animal material; (2) the tailings were largely dominated by the categories sugar-rich substrates, leaf/fruit/seed and pollen; and (3) the mine was largely dominated by the category's roots/soil, soil and animal material/fungal material. Moreover, when considering the heatmaps obtained for decay, the results revealed that (Figure 8B) (1) multiple categories related to leaf/fruit/seed/animal material and soil were strongly positively correlated with the control sample; (2) various sugar-rich and pollen-related categories were strongly positively correlated with the tailings sample; and (3) that categories mainly related to dung, roots/soil, wood, animal and leaf/fruit/seed were strongly positively correlated with the mine sample.

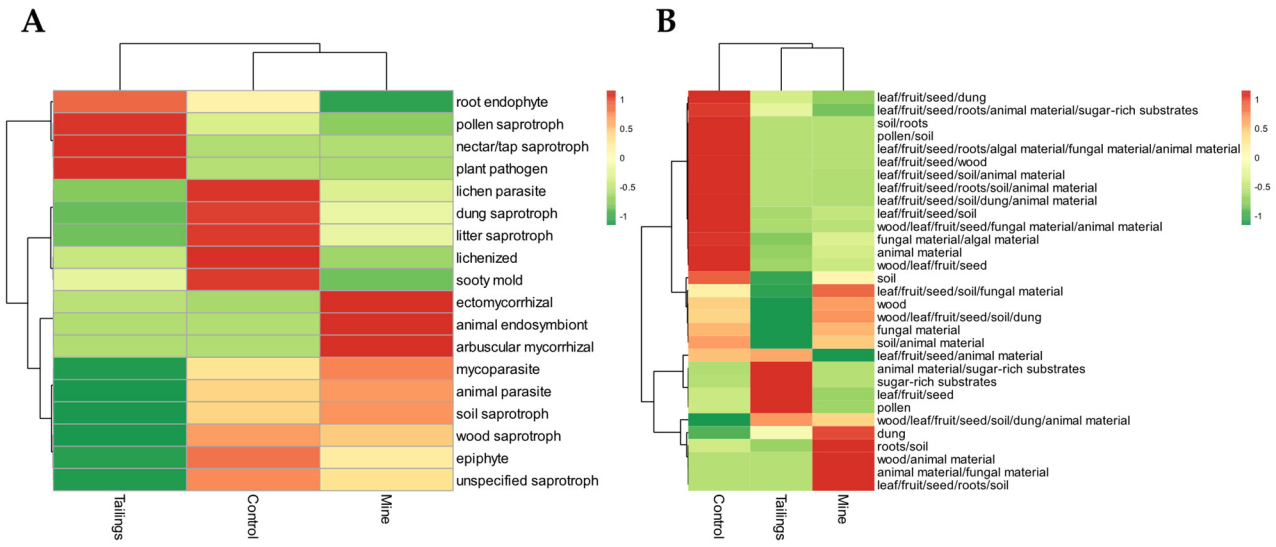


**Figure 8.** Heatmap for the fungal functional traits identified for (**A**) primary lifestyle and (**B**) decay substrates.

Often, mostly soil and wood saprotrophs, with the presence of a few rock-inhabiting/sooty molds, have been reported in mine tailings [89]. In fact, both in the control and the mine samples, strong associations with these ecological groups were detected. Nonetheless, while multiple saprotrophs were identified, some particular results were also highlighted, namely the strong associations of nectar/tap fungi with sugar-rich, leaf/fruit/seed, and pollen decay substrates preferences in the tailings. Due to the dense fern vegetation, we hypothesize that plant pollens and nectars and, consequently, the presence of a sugar-rich soil micro-environment can also impact the fungal communities in these areas (e.g., [90]). Altogether, these results also highlight the diverse ecological fungal functions at each site and allow to verify that small micro-niches present in the different sites favor further specific fungal traits based on the available substrates and distinct environmental conditions (e.g., soil pH, toxic metal concentrations, presence of plants, animals, roots and water).

## 4. Conclusions

In summary, we conducted an analysis of the microbiome (bacterial and fungal communities) diversity, structural organization and metabolic/ecological traits in an abandoned Portuguese gold mining area with metabarcoding and bioinformatic analysis. We found that the overall microbiome diversity decreases inside the mine and the tailings contrastingly for prokaryotic and fungal communities when compared with an area without previous mining activities. Prokaryotes displayed a relatively homogeneous taxa distribution, while fungi displayed the presence of some dominant taxa. Moreover, fungi exhibit a more notorious site-specific nature with a less demarked core microbiome. The predicted bacterial metabolic profiles exhibited differences when considering the control, the tailings and mine areas, establishing a shift from carbohydrate metabolism and lignin degradation towards metabolic profiles closely associated with defense pathways and responses to environmental stress (cell cycle maintenance and repair, energy production, xenobiotic biodegradation and other secondary metabolic pathways). Moreover, the fungal community's ecological characteristics were largely dominated by saprobes. Nevertheless, the tailings also displayed multiple peculiar associations with fungi able to deal with sugar-rich and pollen substrate preferences. Thus, this work provides an initial picture of the microbiomes at these sites, and further research is necessary to gain a deeper insight into their microbial ecology and the microbiome's physiological and metabolic characteristics. For instance, further studies (e.g., cultivation-dependent methodologies) on the extremophilic consortia could be focused on the identification of metal bioremediation abilities, biosynthesis of gold nanoparticles capacities, or even in gold biorecovery strategies (e.g., [23,91–98]). On the other hand, further studies employing additional -omics methodologies, such as metagenomics and metatranscriptomics, would also be advantageous since these could further allow gaining a deeper knowledge regarding these areas' microbiome composition, functional characterization, genomic description, metabolic potential, mobilome, resistome and community dynamics (e.g., [99]). Lastly, cultivation-dependent methods could also contribute to the description of novel species since extreme environments have been highlighted as requiring further studies in this regard (e.g., [100]). We hope this work can draw further attention to the study of Portuguese gold mining areas and promote additional research on microbes thriving in these extreme environments.

**Supplementary Materials:** The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/app14010226/s1, Files S1–S6: Krona charts exhibiting the overall bacterial and fungal diversity found across the distinct samples; Figure S1: Bacteria biplot PCA results; Figure S2: Fungi biplot PCA results.

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**Conflicts of Interest:** The authors declare no conflicts of interest.

## References

1. Martins, L. *Mineral Resources of Portugal*; DGEG: Lisbon, Portugal, 2012.
2. Manuel, R.; Brito, M.; Chichorro, M.; Rosa, C. Remote Sensing for Mineral Exploration in Central Portugal. *Minerals* **2017**, *7*, 184. [CrossRef]
3. Cerveira, A. Notas sobre as minas de ouro da Serra da Louzã. *Sep. Bol. Soc. Geol. Port.* **1947**, *3*, 5–15.
4. Martins, C.M.B. *A Exploração Mineira Romana e a Metalurgia do Ouro em Portugal*; Instituto de Ciências Socias da Universidade do Minho: Braga, Portugal, 2008.
5. Ferreira da Silva, E.; Zhang, C.; Serrano Pinto, L.; Patinha, C.; Reis, P. Hazard assessment on arsenic and lead in soils of Castromil gold mining area, Portugal. *Appl. Geochem.* **2004**, *19*, 887–898. [CrossRef]
6. Fashola, M.O.; Ngole-Jeme, V.M.; Babalola, O.O. Heavy Metal Immobilization Potential of Indigenous Bacteria Isolated from Gold Mine Tailings. *Int. J. Environ. Res.* **2020**, *14*, 71–86. [CrossRef]
7. Ngole-Jeme, V.M.; Fantke, P. Ecological and human health risks associated with abandoned gold mine tailings contaminated soil. *PLoS ONE* **2017**, *12*, e0172517. [CrossRef] [PubMed]
8. Donato, D.B.; Nichols, O.; Possingham, H.; Moore, M.; Ricci, P.F.; Noller, B.N. A critical review of the effects of gold cyanide-bearing tailings solutions on wildlife. *Environ. Int.* **2007**, *33*, 974–984. [CrossRef] [PubMed]
9. Ehrlich, H.L. Geomicrobiology: Relative roles of bacteria and fungi as geomicrobial agents. In *Fungi in Biogeochemical Cycles*; Gadd, G.M., Ed.; British Mycological Society Symposia, Cambridge University Press: Cambridge, UK, 2006; pp. 1–27. [CrossRef]
10. Druschel, G.K.; Kappler, A. Geomicrobiology and Microbial Geochemistry. *Elements* **2015**, *11*, 389–394. [CrossRef]
11. Gadd, G.M. Geomycology: Biogeochemical transformations of rocks, minerals, metals and radionuclides by fungi, bioweathering and bioremediation. *Mycol. Res.* **2007**, *111*, 3–49. [CrossRef]
12. Gadd, G.M. The Geomycology of Elemental Cycling and Transformations in the Environment. *Microbiol. Spectr.* **2017**, *5*, 10–1128. [CrossRef]
13. Viles, H.A. Microbial geomorphology: A neglected link between life and landscape. *Geomorphology* **2012**, *157–158*, 6–16. [CrossRef]
14. Martínez-Espinosa, R.M. Microorganisms and Their Metabolic Capabilities in the Context of the Biogeochemical Nitrogen Cycle at Extreme Environments. *Int. J. Mol. Sci.* **2020**, *21*, 4228. [CrossRef]
15. Fomina, M.; Skorochod, I. Microbial Interaction with Clay Minerals and Its Environmental and Biotechnological Implications. *Minerals* **2020**, *10*, 861. [CrossRef]
16. Bindschedler, S.; Vu Bouquet, T.Q.T.; Job, D.; Joseph, E.; Junier, P. Fungal Biorecovery of Gold From E-waste. *Adv. Appl. Microbiol.* **2017**, *99*, 53–81. [CrossRef] [PubMed]
17. Shuster, J.; Reith, F. Reflecting on Gold Geomicrobiology Research: Thoughts and Considerations for Future Endeavors. *Minerals* **2018**, *8*, 401. [CrossRef]
18. Hyde, K.D.; Xu, J.; Rapior, S.; Jeewon, R.; Lumyong, S.; Niego, A.G.T.; Abeywickrama, P.D.; Aluthmuhandiram, J.V.S.; Brahamanage, R.S.; Brooks, S.; et al. The amazing potential of fungi: 50 ways we can exploit fungi industrially. *Fungal Divers.* **2019**, *97*, 1–136. [CrossRef]
19. Dusengemungu, L.; Kasali, G.; Gwanama, C.; Mubemba, B. Overview of fungal bioleaching of metals. *Environ. Adv.* **2021**, *5*, 100083. [CrossRef]
20. Newsome, L.; Falagán, C. The Microbiology of Metal Mine Waste: Bioremediation Applications and Implications for Planetary Health. *GeoHealth* **2021**, *5*, e2020GH000380. [CrossRef]

21. Das, A.P.; Ghosh, S. Role of Microorganisms in Extenuation of Mining and Industrial Wastes. *Geomicrobiol. J.* **2022**, *39*, 173–175. [CrossRef]
22. Bohu, T.; Anand, R.; Noble, R.; Lintern, M.; Kaksonen, A.H.; Mei, Y.; Cheng, K.Y.; Deng, X.; Veder, J.-P.; Bunce, M.; et al. Evidence for fungi and gold redox interaction under Earth surface conditions. *Nat. Commun.* **2019**, *10*, 2290. [CrossRef]
23. Bohu, T.; Anand, R.R.; Kaksonen, A.H.; González-Álvarez, I.; Pages, A.; Noble, R.R.P.; Lintern, M.J.; Spinks, S.C.; Zhuang, X. The role of fungi in the biogeochemical cycling of supergene gold and satellite transition metals: A potential new exploration tool. *Ore Geol. Rev.* **2022**, *140*, 104595. [CrossRef]
24. Li, M.; Tian, H.; Wang, L.; Duan, J. Bacterial Diversity in Linglong Gold Mine, China. *Geomicrobiol. J.* **2017**, *34*, 267–273. [CrossRef]
25. Li, Q.; Liu, F.; Li, M.; Chen, C.; Gadd, G.M. Nanoparticle and nanomineral production by fungi. *Fungal Biol. Rev.* **2022**, *41*, 31–44. [CrossRef]
26. Inagaki, F.; Takai, K.; Hirayama, H.; Yamato, Y.; Nealson, K.H.; Horikoshi, K. Distribution and phylogenetic diversity of the subsurface microbial community in a Japanese epithermal gold mine. *Extremophiles* **2003**, *7*, 307–317. [CrossRef] [PubMed]
27. Karnachuk, O.V.; Gerasimchuk, A.L.; Banks, D.; Frengstad, B.; Stykon, G.A.; Tikhonova, Z.L.; Kaksonen, A.; Puhakka, J.; Yanenko, A.S.; Pimenov, N.V. Bacteria of the sulfur cycle in the sediments of gold mine tailings, Kuznetsk Basin, Russia. *Microbiology* **2009**, *78*, 483–491. [CrossRef]
28. Sun, Y.; Zhang, X.; Wu, Z.; Hu, Y.; Wu, S.; Chen, B. The molecular diversity of arbuscular mycorrhizal fungi in the arsenic mining impacted sites in Hunan Province of China. *J. Environ. Sci.* **2016**, *39*, 110–118. [CrossRef] [PubMed]
29. Crognale, S.; D'Annibale, A.; Pesciaroli, L.; Stazi, S.R.; Petruccioli, M. Fungal Community Structure and As-Resistant Fungi in a Decommissioned Gold Mine Site. *Front. Microbiol.* **2017**, *8*, 2202. [CrossRef] [PubMed]
30. Ji, H.; Zhang, Y.; Bararunyeretse, P.; Li, H. Characterization of microbial communities of soils from gold mine tailings and identification of mercury-resistant strain. *Ecotoxicol. Environ. Saf.* **2018**, *165*, 182–193. [CrossRef]
31. Oladipo, O.G.; Awotoye, O.O.; Olayinka, A.; Bezuidenhout, C.C.; Maboeta, M.S. Heavy metal tolerance traits of filamentous fungi isolated from gold and gemstone mining sites. *Braz. J. Microbiol.* **2018**, *49*, 29–37. [CrossRef]
32. Sibanda, T.; Selvarajan, R.; Msagati, T.; Venkatachalam, S.; Meddows-Taylor, S. Defunct gold mine tailings are natural reservoir for unique bacterial communities revealed by high-throughput sequencing analysis. *Sci. Total Environ.* **2019**, *650*, 2199–2209. [CrossRef]
33. Fatimawali, F.; Kepel, B.J.; Gani, M.A.; Tallei, T.E. Comparison of Bacterial Community Structure and Diversity in Traditional Gold Mining Waste Disposal Site and Rice Field by Using a Metabarcoding Approach. *Int. J. Microbiol.* **2020**, *2020*, 1858732. [CrossRef]
34. Gagnon, V.; Rodrigue-Morin, M.; Tremblay, J.; Wasserscheid, J.; Champagne, J.; Bellenger, J.-P.; Greer, C.W.; Roy, S. Life in mine tailings: Microbial population structure across the bulk soil, rhizosphere, and roots of boreal species colonizing mine tailings in northwestern Québec. *Ann. Microbiol.* **2020**, *70*, 41. [CrossRef]
35. Gagnon, V.; Rodrigue-Morin, M.; Tremblay, J.; Wasserscheid, J.; Champagne, J.; Bellenger, J.-P.; Greer, C.W.; Roy, S. Vegetation drives the structure of active microbial communities on an acidogenic mine tailings deposit. *PeerJ* **2020**, *8*, e10109. [CrossRef] [PubMed]
36. Yan, C.; Wang, F.; Liu, H.; Liu, H.; Pu, S.; Lin, F.; Geng, H.; Ma, S.; Zhang, Y.; Tian, Z.; et al. Deciphering the toxic effects of metals in gold mining area: Microbial community tolerance mechanism and change of antibiotic resistance genes. *Environ. Res.* **2020**, *189*, 109869. [CrossRef] [PubMed]
37. Nosáľová, L.; Maliničová, L.; Kisková, J.; Timková, I.; Sedláková-Kaduková, J.; Pristaš, P. Cultivable Microbiota Associated with Gold Ore from the Rozália Gold Mine, Hodruša-Hámre, Slovakia. *Geomicrobiol. J.* **2021**, *38*, 415–425. [CrossRef]
38. Abrosimova, N.; Bortnikova, S.; Edelev, A.; Chernukhin, V.; Reutsky, A.; Abrosimov, N.; Gundyrev, I. Geochemical and Microbiological Composition of Soils and Tailings Surrounding the Komsomolsk Tailings, Kemerovo Region, Russia. *Bacteria* **2023**, *2*, 116–128. [CrossRef]
39. Lhoste, E.; Comte, F.; Brown, K.; Delisle, A.; Jaclin, D.; Ponsin, V.; Rosabal, M.; Lazar, C.S. Bacterial, Archaeal, and Eukaryote Diversity in Planktonic and Sessile Communities Inside an Abandoned and Flooded Iron Mine (Quebec, Canada). *Appl. Microbiol.* **2023**, *3*, 45–63. [CrossRef]
40. Mosier, A.C.; Miller, C.S.; Frischkorn, K.R.; Ohm, R.A.; Li, Z.; LaButti, K.; Lapidus, A.; Lipzen, A.; Chen, C.; Johnson, J.; et al. Fungi Contribute Critical but Spatially Varying Roles in Nitrogen and Carbon Cycling in Acid Mine Drainage. *Front. Microbiol.* **2016**, *7*, 238. [CrossRef]
41. Ou, S.; Liang, J.-L.; Jiang, X.; Liao, B.; Jia, P.; Shu, W.; Li, J. Physiological, Genomic and Transcriptomic Analyses Reveal the Adaptation Mechanisms of *Acidiella bohemica* to Extreme Acid Mine Drainage Environments. *Front. Microbiol.* **2021**, *12*, 705839. [CrossRef]
42. Quail, M.A.; Smith, M.; Coupland, P.; Otto, T.D.; Harris, S.R.; Connor, T.R.; Bertoni, A.; Swerdlow, H.P.; Gu, Y. A tale of three next generation sequencing platforms: Comparison of Ion Torrent, Pacific Biosciences and Illumina MiSeq sequencers. *BMC Genom.* **2012**, *13*, 341. [CrossRef]
43. Shokralla, S.; Spall, J.L.; Gibson, J.F.; Hajibabaei, M. Next-generation sequencing technologies for environmental DNA research. *Mol. Ecol.* **2012**, *21*, 1794–1805. [CrossRef]
44. Buermans, H.P.J.; den Dunnen, J.T. Next generation sequencing technology: Advances and applications. *Biochim. Biophys. Acta* **2014**, *1842*, 1932–1941. [CrossRef] [PubMed]

45. Goodwin, S.; McPherson, J.D.; McCombie, W.R. Coming of age: Ten years of next-generation sequencing technologies. *Nat. Rev. Genet.* **2016**, *17*, 333–351. [CrossRef] [PubMed]
46. Amarasinghe, S.L.; Su, S.; Dong, X.; Zappia, L.; Ritchie, M.E.; Gouil, Q. Opportunities and challenges in long-read sequencing data analysis. *Genome Biol.* **2020**, *21*, 30. [CrossRef] [PubMed]
47. Wang, Y.; Zhao, Y.; Bollas, A.; Wang, Y.; Au, K.F. Nanopore sequencing technology, bioinformatics and applications. *Nat. Biotechnol.* **2021**, *39*, 1348–1365. [CrossRef] [PubMed]
48. Mohammed, A.A.; Senbeta, B.; Worku, T.; Mohammed, A.A.; Senbeta, B.; Worku, T. Pacific bioscience sequence technology: Review. *Int. J. Vet. Sci. Res.* **2022**, *8*, 27–33. [CrossRef]
49. Santos Oliveira, J.M. The role of lithogeochemistry in the delineation of Au and Sn-W mineralization in schist terrains of the Gois region (Central Portugal). *Estud. Notas Trab. Direcção Geral Geol. Minas* **1991**, *33*, 9–19.
50. Pereira, V. *"Projecto Góis" Cartografia Geológica*; Divisão de Prospecção de Minérios Metálicos, Direcção Geral de Geologia e Minas, Serviços de Fomento Mineiro e Indústria Extractiva, LNEG: Lisboa, Portugal, 1984.
51. Carvalho, P.C.S.; Neiva, A.M.R.; Silva, M.M.V.G.; Santos, A.C.T. Human health risks in an old gold mining area with circum-neutral drainage, central Portugal. *Environ. Geochem. Health* **2017**, *39*, 43–62. [CrossRef]
52. Herlemann, D.P.; Labrenz, M.; Jürgens, K.; Bertilsson, S.; Waniek, J.J.; Andersson, A.F. Transitions in bacterial communities along the 2000 km salinity gradient of the Baltic Sea. *ISME J.* **2011**, *5*, 1571–1579. [CrossRef]
53. Klindworth, A.; Pruesse, E.; Schweer, T.; Peplies, J.; Quast, C.; Horn, M.; Glöckner, F.O. Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. *Nucleic Acids Res.* **2013**, *41*, e1. [CrossRef]
54. Tedersoo, L.; Bahram, M.; Põlme, S.; Kõljalg, U.; Yorou, N.S.; Wijesundera, R.; Ruiz, L.V.; Vasco-Palacios, A.M.; Thu, P.Q.; Suija, A.; et al. Global Diversity and Geography of Soil Fungi. *Science* **2014**, *346*, 1256688. [CrossRef]
55. Illumina. *16S Metagenomic Sequencing Library Preparation Preparing 16S Ribosomal RNA Gene Amplicons for the Illumina MiSeq System*; Illumina Technical Document, Part. No. 15044223 Rev. B; Illumina: San Diego, CA, USA, 2013.
56. Comeau, A.M.; Douglas, G.M.; Langille, M.G.I. Microbiome Helper: A Custom and Streamlined Workflow for Microbiome Research. *MSystems* **2017**, *2*, 10–1128. [CrossRef] [PubMed]
57. Schmieder, R.; Edwards, R. Quality control and preprocessing of metagenomic datasets. *Bioinformatics* **2011**, *27*, 863–864. [CrossRef]
58. Schubert, M.; Lindgreen, S.; Orlando, L. AdapterRemoval v2: Rapid adapter trimming, identification, and read merging Findings Background. *BMC Res. Notes* **2016**, *9*, 88. [CrossRef] [PubMed]
59. Schloss, P.D.; Westcott, S.L.; Ryabin, T.; Hall, J.R.; Hartmann, M.; Hollister, E.B.; Lesniewski, R.A.; Oakley, B.B.; Parks, D.H.; Robinson, C.J.; et al. Introducing mothur: Open-Source, Platform-Independent, Community-Supported Software for Describing and Comparing Microbial Communities. *Appl. Environ. Microbiol.* **2009**, *75*, 7537–7541. [CrossRef] [PubMed]
60. Schloss, P.D. Reintroducing mothur: 10 Years Later. *Appl. Environ. Microbiol.* **2020**, *86*, e02343-19. [CrossRef] [PubMed]
61. Rognes, T.; Flouri, T.; Nichols, B.; Quince, C.; Mahé, F. VSEARCH: A versatile opensource tool for metagenomics. *PeerJ* **2016**, *4*, e2584. [CrossRef]
62. Westcott, S.L.; Schloss, P.D. OptiClust, an Improved Method for Assigning AmpliconBased Sequence Data to Operational Taxonomic Units. *mSphere* **2017**, *2*, e00073-17. [CrossRef]
63. Quast, C.; Pruesse, E.; Yilmaz, P.; Gerken, J.; Schweer, T.; Yarza, P.; Peplies, J.; Glöckner, F.O. The SILVA ribosomal RNA gene database project: Improved data processing and web-based tools. *Nucleic Acids Res.* **2013**, *41*, D590–D596. [CrossRef]
64. Wang, Q.; Garrity, G.M.; Tiedje, J.M.; Cole, J.R. Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl. Environ. Microbiol.* **2007**, *73*, 5261–5267. [CrossRef]
65. Větrovský, T.; Baldrian, P.; Morais, D. SEED 2: A user-friendly platform for amplicon high-throughput sequencing data analyses. *Bioinformatics* **2018**, *34*, 2292–2294. [CrossRef]
66. Bengtsson-Palme, J.; Ryberg, M.; Hartmann, M.; Branco, S.; Wang, Z.; Godhe, A.; De Wit, P.; Sánchez-García, M.; Ebersberger, I.; de Sousa, F.; et al. Improved software detection and extraction of ITS1 and ITS2 from ribosomal ITS sequences of fungi and other eukaryotes for analysis of environmental sequencing data. *Methods Ecol. Evol.* **2013**, *4*, 914–919. [CrossRef]
67. Edgar, R.C. UPARSE: Highly accurate OTU sequences from microbial amplicon reads. *Nat. Methods* **2013**, *10*, 996–998. [CrossRef] [PubMed]
68. Nilsson, R.H.; Larsson, K.-H.; Taylor, A.F.S.; Bengtsson-Palme, J.; Jeppesen, T.S.; Schigel, D.; Kennedy, P.; Picard, K.; Glöckner, F.O.; Tedersoo, L.; et al. The UNITE database for molecular identification of fungi: Handling dark taxa and parallel taxonomic classifications. *Nucleic Acids Res.* **2019**, *47*, D259–D264. [CrossRef] [PubMed]
69. Abarenkov, K.; Tedersoo, L.; Nilsson, R.H.; Vellak, K.; Saar, I.; Veldre, V.; Parmasto, E.; Prous, M.; Aan, A.; Ots, M.; et al. PlutoF—A Web Based Workbench for Ecological and Taxonomic Research, with an Online Implementation for Fungal ITS Sequences. *Evol. Bioinform.* **2010**, *6*, 189–196. [CrossRef]
70. Liu, C.; Cui, Y.; Li, X.; Yao, M. microeco: An R package for data mining in microbial community ecology. *FEMS Microbiol. Ecol.* **2021**, *97*, fiaa255. [CrossRef] [PubMed]
71. Ondov, B.D.; Bergman, N.H.; Phillippy, A.M. Interactive metagenomic visualization in a Web browser. *BMC Bioinf.* **2011**, *12*, 385. [CrossRef] [PubMed]
72. Hammer, O.; Harper, D.A.T.; Ryan, P.D. PAST: Paleontological Statistics Software Package for Education and Data Analysis. *Palaeontol. Electron.* **2001**, *4*, 1.

73. Dhariwal, A.; Chong, J.; Habib, S.; King, I.L.; Agellon, L.B.; Xia, J. MicrobiomeAnalyst: A web-based tool for comprehensive statistical, visual and meta-analysis of microbiome data. *Nucleic Acids Res.* **2017**, *45*, W180–W188. [CrossRef]
74. Chong, J.; Liu, P.; Zhou, G.; Xia, J. Using MicrobiomeAnalyst for comprehensive statistical, functional, and meta-analysis of microbiome data. *Nat. Protoc.* **2020**, *15*, 799–821. [CrossRef]
75. Aßhauer, K.P.; Wemheuer, B.; Daniel, R.; Meinicke, P. Tax4Fun: Predicting functional profiles from metagenomic 16S rRNA data. *Bioinformatics* **2015**, *31*, 2882–2884. [CrossRef]
76. Põlme, S.; Abarenkov, K.; Henrik Nilsson, R.; Lindahl, B.D.; Clemmensen, K.E.; Kauserud, H.; Nguyen, N.; Kjøller, R.; Bates, S.T.; Baldrian, P.; et al. FungalTraits: A user-friendly traits database of fungi and fungus-like stramenopiles. *Fungal Divers.* **2020**, *105*, 1–16. [CrossRef]
77. Metsalu, T.; Vilo, J. ClustVis: A web tool for visualizing clustering of multivariate data using Principal Component Analysis and heatmap. *Nucleic Acids Res.* **2015**, *43*, W566–W570. [CrossRef] [PubMed]
78. Mendez, M.O.; Neilson, J.W.; Maier, R.M. Characterization of a Bacterial Community in an Abandoned Semiarid Lead-Zinc Mine Tailing Site. *Appl. Environ. Microbiol.* **2008**, *74*, 3899–3907. [CrossRef] [PubMed]
79. Hur, M.; Kim, Y.; Song, H.-R.; Kim, J.M.; Choi, Y.I.; Yi, H. Effect of Genetically Modified Poplars on Soil Microbial Communities during the Phytoremediation of Waste Mine Tailings. *Appl. Environ. Microbiol.* **2011**, *77*, 7611–7619. [CrossRef] [PubMed]
80. Chen, Y.; Li, J.; Chen, L.; Hua, Z.; Huang, L.; Liu, J.; Xu, B.; Liao, B.; Shu, W. Biogeochemical Processes Governing Natural Pyrite Oxidation and Release of Acid Metalliferous Drainage. *Environ. Sci. Technol.* **2014**, *48*, 5537–5545. [CrossRef] [PubMed]
81. Dedysh, S.N.; Beletsky, A.V.; Ivanova, A.A.; Kulichevskaya, I.S.; Suzina, N.E.; Philippov, D.A.; Rakitin, A.L.; Mardanov, A.V.; Ravin, N.V. Wide distribution of *Phycisphaera*-like planctomycetes from WD2101 soil group in peatlands and genome analysis of the first cultivated representative. *Environ. Microbiol.* **2021**, *23*, 1510–1526. [CrossRef]
82. Martin-Pozas, T.; Gonzalez-Pimentel, J.L.; Jurado, V.; Laiz, L.; Cañaveras, J.C.; Fernandez-Cortes, A.; Cuezva, S.; Sanchez-Moral, S.; Saiz-Jimenez, C. *Crossiella*, a Rare Actinomycetota Genus, Abundant in the Environment. *Appl. Biosci.* **2023**, *2*, 194–210. [CrossRef]
83. Hosoya, T. Systematics, ecology, and application of Helotiales: Recent progress and future perspectives for research with special emphasis on activities within Japan Helotiales: Recent progress and future perspectives for research with special emphasis on activities within Japan. *Mycoscience* **2021**, *62*, 1–9. [CrossRef]
84. Crous, P.W.; Wingfield, M.J.; Burgess, T.I.; Hardy, G.E.S.J.; Gené, J.; Guarro, J.; Baseia, I.G.; García, D.; Gusmão, L.F.P.; Souza-Motta, C.M.; et al. Fungal Planet description sheets: 716–784. *Persoonia Mol. Phylogeny Evol. Fungi* **2018**, *40*, 239–392. [CrossRef]
85. Qi, X.; Xu, X.; Zhong, C.; Jiang, T.; Wei, W.; Song, X. Removal of Cadmium and Lead from Contaminated Soils Using Sophorolipids from Fermentation Culture of *Starmerella bombicola* CGMCC 1576 Fermentation. *Int. J. Environ. Res. Public Health* **2018**, *15*, 2334. [CrossRef]
86. Selbmann, L.; Benkő, Z.; Coleine, C.; de Hoog, S.; Donati, C.; Druzhinina, I.; Emri, T.; Ettinger, C.L.; Gladfelter, A.S.; Gorbushina, A.A.; et al. Shed Light in the DaRk LineagES of the Fungal Tree of Life—STRES. *Life* **2020**, *10*, 362. [CrossRef] [PubMed]
87. Chen, D.; Feng, Q. Microbial Community Structure, Metabolic Function, and Phenotypic Characteristics of Sediment in Deep Coal Mine Underground Environment, China. *Water* **2023**, *15*, 2371. [CrossRef]
88. Sun, Q.; Zhang, P.; Liu, X.; Zhang, H.; Liu, S.; Sun, X.; Jiang, W. Long-term Tillage Alters Soil Properties and Rhizosphere Bacterial Community in Lime Concretion Black Soil under Winter Wheat–Summer Maize Double-Cropping System. *Agronomy* **2023**, *13*, 790. [CrossRef]
89. Nkuna, R.; Ijoma, G.N.; Matambo, T.S. Applying EDTA in Chelating Excess Metal Ions to Improve Downstream DNA Recovery from Mine Tailings for Long-Read Amplicon Sequencing of Acidophilic Fungi Communities. *J. Fungi* **2022**, *8*, 419. [CrossRef] [PubMed]
90. Mueller, T.G.; Francis, J.S.; Vannette, R.L. Nectar compounds impact bacterial and fungal growth and shift community dynamics in a nectar analog. *Environ. Microbiol. Rep.* **2023**, *15*, 170–180. [CrossRef] [PubMed]
91. Abatenh, E.; Gizaw, B.; Tsegaye, Z.; Wassie, M.; Abatenh, E.; Gizaw, B.; Tsegaye, Z.; Wassie, M. The Role of Microorganisms in Bioremediation—A Review. *Open J. Environ. Biol.* **2017**, *2*, 38–46. [CrossRef]
92. Ayilara, M.S.; Babalola, O.O. Bioremediation of Environmental Wastes: The Role of Microorganisms. *Front. Agron.* **2023**, *5*, 1183691. [CrossRef]
93. Bala, S.; Garg, D.; Thirumalesh, B.V.; Sharma, M.; Sridhar, K.; Inbaraj, B.S.; Tripathi, M. Recent Strategies for Bioremediation of Emerging Pollutants: A Review for a Green and Sustainable Environment. *Toxics* **2022**, *10*, 484. [CrossRef]
94. Cui, Z.; Zhang, X.; Yang, H.; Sun, L. Bioremediation of Heavy Metal Pollution Utilizing Composite Microbial Agent of *Mucor Circinelloides*, *Actinomucor* sp. and *Mortierella* sp. *J. Environ. Chem. Eng.* **2017**, *5*, 3616–3621. [CrossRef]
95. Natarajan, G.; Ting, Y.-P. Gold Biorecovery from E-Waste: An Improved Strategy through Spent Medium Leaching with pH Modification. *Chemosphere* **2015**, *136*, 232–238. [CrossRef]
96. Rana, S.; Mishra, P.; Wahid, Z.A.; Thakur, S.; Pant, D.; Singh, L. Microbe-Mediated Sustainable Bio-Recovery of Gold from Low-Grade Precious Solid Waste: A Microbiological Overview. *J. Environ. Sci.* **2020**, *89*, 47–64. [CrossRef] [PubMed]
97. Sheikhloo, Z.; Salouti, M.; Katiraee, F. Biological Synthesis of Gold Nanoparticles by Fungus *Epicoccum nigrum*. *J. Clust. Sci.* **2011**, *22*, 661–665. [CrossRef]
98. Tay, S.B.; Natarajan, G.; bin Abdul Rahim, M.N.; Tan, H.T.; Chung, M.C.M.; Ting, Y.P.; Yew, W.S. Enhancing Gold Recovery from Electronic Waste via Lixiviant Metabolic Engineering in *Chromobacterium violaceum*. *Sci. Rep.* **2013**, *3*, 2236. [CrossRef] [PubMed]

99. Karaolia, P.; Vasileiadis, S.; Michael, S.G.; Karpouzas, D.G.; Fatta-Kassinos, D. Shotgun Metagenomics Assessment of the Resistome, Mobilome, Pathogen Dynamics and Their Ecological Control Modes in Full-Scale Urban Wastewater Treatment Plants. *J. Hazard. Mater.* **2021**, *418*, 126387. [CrossRef]
100. Wijayawardene, N.; Phillips, A.J.L.; Tibpromma, S.; Dai, D.Q.; Selbmann, L.; Monteiro, J.S.; Aptroot, A.; Flakus, A.; Rajeshkumar, K.C.; Coleine, C.; et al. Looking for the Undiscovered Asexual Taxa: Case Studies from Lesser Studied Life Modes and Habitats. *Mycosphere* **2021**, *12*, 1290–1333. [CrossRef]