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# The microbiome of a brownfield highly polluted with mercury and arsenic<sup>☆</sup>

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## ABSTRACT

Abandoned brownfields represent a challenge for their recovery. To apply sustainable remediation technologies, such as bioremediation or phytoremediation, indigenous microorganisms are essential agents since they are adapted to the ecology of the soil. Better understanding of microbial communities inhabiting those soils, identification of microorganisms that drive detoxification process and recognising their needs and interactions will significantly improve the outcome of the remediation. With this in mind we have carried out a detailed metagenomic analysis to explore the taxonomic and functional diversity of the prokaryotic and eukaryotic microbial communities in soils, several mineralogically distinct types of pyrometallurgic waste, and groundwater sediments of a former mercury mining and metallurgy site which harbour very high levels of arsenic and mercury pollution. Prokaryotic and eukaryotic communities were identified, which turned out to be more diverse in the surrounding contaminated soils compared to the pyrometallurgic waste. The highest diversity loss was observed in two environments most contaminated with mercury and arsenic (stupp, a solid mercury condenser residue and arsenic-rich soot from arsenic condensers). Interestingly, microbial communities in the stupp were dominated by an overwhelming majority of archaea of the phylum Crenarchaeota, while Ascomycota and Basidiomycota fungi comprised the fungal communities of both stupp and soot, results that show the impressive ability of these previously unreported microorganisms to colonize these extreme brownfield environments. Functional predictions for mercury and arsenic resistance/detoxification genes show their increase in environments with higher levels of pollution. Our work establishes the bases to design sustainable remediation methods and, equally important, to study in depth the genetic and functional mechanisms that enable the subsistence of microbial populations in these extremely selective environments.

## 1. Introduction

Soil pollution with metal (loid)s and mineral oil is a widespread problem in Europe (FAO and ITPS, 2015). The most common metal (loid)s in the soil are As, Hg, Cd, Cr, Cu, Ni, Pb, Zn, with mining and metallurgy being important sources of pollution. It is estimated that around 2.8 million sites across the EU are potentially contaminated, of which around 14% require urgent remediation (Payá-Pérez and Rodríguez-Eugenio, 2018). Brownfields are now abandoned, or underutilized industrial or commercial sites affected by mixtures of pollutants, which can reach high concentrations in accumulated waste and pose a long-lasting threat to human health and the surrounding environment. In this way they may require intervention before they can be returned to

beneficial use. A large-scale remediation of these lands with traditional technologies involves the use of chemical reagents and significant expenditure of energy and resources. The use of “nature-based solutions” for remediation, such as bioremediation/phytoremediation offers alternatives that, in addition to being cheaper, are also more environmentally sustainable and therefore more beneficial in the long term (Song et al., 2019).

The Asturian mining region in northern Spain was one of the largest mercury-producing regions in the world. The brownfields that remain are relevant from the point of view of their impact on the environment, as the concurrence of mining activity and metallurgical processes left a historical legacy of old and abandoned industrial facilities, where large amounts of waste accumulate on the surface with high risks of leaching

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and dispersion. One of them, known as El Terronal, in Mieres (Fig. S1), represents a paradigm of the problems associated with such sites. Therefore, it has been analysed in detail both from the geochemical and mineralogical points of view, as well as studied for the presence of organic contaminants such as polycyclic aromatic hydrocarbons (PAHs) and phenylmercury propionate (Gallego et al., 2015). The mineral of interest was cinnabar (HgS), found as inclusions in sulphide ores rich in realgar (As<sub>4</sub>S<sub>4</sub>) and orpiment (As<sub>2</sub>S<sub>3</sub>). A pyrometallurgical process was employed to oxidise the ore at high temperature, allowing to separate resulting gaseous elemental mercury from arsenic oxides (Figs. S2 and S3). It generated very large amounts of arsenic-rich soot, stupp (a solid, mercury-rich residue from mercury condensers), and highly contaminated flue dust (Loredo et al., 1999; Gallego et al., 2015). All these residues were dumped near the site together with arsenic-containing gangue from the mine, contributing to even higher environmental pollution (González-Fernández et al., 2018). After the end of mining activities in 1973, mining and metallurgy waste was left in place. While the biggest landfill containing gangue and pyrometallurgical waste was eventually covered up in 2002, highly toxic waste remaining at the site itself was left open to the elements (Fig. S4). This allowed rainwater and run-off from the nearby hills to run directly over and through waste piles before discharging into a nearby river during the wet winter season. Additionally, wind erosion affected dry waste in the summer (Gallego et al., 2015).

Mercury in the environment is found as elemental (metallic) mercury (Hg<sup>0</sup>), mercuric (Hg(II)) and mercurous (Hg(I)) compounds of chlorine, sulphur or oxygen, and organic species such as ethylmercury and methylmercury. Hg<sup>0</sup> is liquid at room temperature, and has a high vapour pressure and low solubility in water, unlike the other forms. Mercury pollution can reduce biodiversity and abundance of microorganisms and change functional community structure and taxonomic community composition in the environment (Frey and Rieder, 2013; Vishnivetskaya et al., 2018; Liu et al., 2018; Frossard et al., 2018; Zheng et al., 2022). Arsenic exists mostly as arsenites and arsenates (As(III) and As(V), respectively); the former is more mobile, more toxic, and present under reducing conditions, while the latter is normally found under oxidizing conditions. Microbial oxidation-reduction activities influence the mobility of arsenic compounds; although the effect of arsenic on microbial communities and soil functionality has been studied, it can be limited or variable (Lorenz et al., 2006; Newsome and Falagán, 2021). It has been postulated that most prokaryotes contain arsenic resistance genes and thus can participate in biogeochemical cycling of arsenic; the relative contribution of each arsenic detoxification and transformation system, however, remains poorly understood (Zhu et al., 2014).

The first stage in proposing a successful bioremediation design requires a prior study of the microorganisms present in the environment, which are assumed to have been extensively selected to survive in that environment and therefore possess adequate metabolic capacities. This will help to propose strategies best adapted to the existing species, both in biostimulation and bioaugmentation bioremediation processes (Pelaez et al., 2013; Kumar and Gopal, 2015; Das et al., 2016; Mesa et al., 2017a; Forján et al., 2020). In this sense, most studies of microbial communities in highly polluted soils deal with prokaryotes (mainly, bacteria), while studies of the diversity of eukaryotic microorganisms are scarcer, despite their important contribution to the biomass and the functioning of soil metabolic networks (Narendrula-Kotha and Nkongolo, 2017; Frossard et al., 2018; Pathak et al., 2020; Zeng et al., 2020; Vácar et al., 2021). In this work we apply Illumina sequencing of 16 S and 18 S rRNA genes to study composition and diversity of prokaryotic and eukaryotic (fungal and SAR) communities that inhabit soils, groundwater sediments, and several distinct types of the most extremely polluted pyrometallurgical waste. The abundance and characteristics of prokaryotic genes related to mercury and arsenic metabolism was also studied using functional metagenome prediction. To our knowledge, it is the first time that an exhaustive microbiological study has been carried out in an environment with metal (loid)s contamination levels as high as

those existing in the El Terronal brownfield. Additionally, the information obtained in this work will help to select and implement the most appropriate strategies for the recovery of that site.

## 2. Materials and methods

### 2.1. Study site, sampling and chemical analysis

The noteworthy geochemical characteristics and pictures of the El Terronal site and the samples analysed are described in detail in the Supplementary data. Sampling was performed at eight locations around and on the site (Fig. S5A). Soil samples were taken in the winter of 2015 from the upper 5 cm of topsoil (sample A), mixed and tilled soil from a plot attached to the industrial site facilities where a phytoremediation trial was being carried out previously (B), and soil that formed naturally over time on top of the heavily contaminated riverbank (C). Different types of pyrometallurgical waste were sampled in the abandoned industrial installations: arsenic-rich soot (samples F in the winter 2015 and FS - in the summer of 2016), stupp, the residue from the mercury condenser (D), and flue dust, the waste produced at the end of the metallurgy process (E) (Fig. S5B). Additionally, groundwater sediment samples were taken in 2016 from two monitoring wells located at the premises: one south-east of a pilot soil phytoremediation plot (Mesa et al., unpublished data), with water table at the depth of 3.5 m (sample SB), and another near the San Tirso riverbank, with water table at the depth of 2.5 m (sample SR).

Samples were taken in duplicates and placed into sterile 50 mL plastic tubes with screw-on caps (Labbox, Spain), stored at 4 °C and processed within two days of sampling. Samples were homogenised by mixing in a sterile glass container with a sterile spatula prior to processing. Concentration of arsenic and mercury was measured by Inductively Coupled Plasma Mass Spectrometry (ICP-MS 7700, Agilent Technologies, USA) using Isotopic Dilution Analysis with a spike solution (ISC Science, Spain) from air-dried representative subsamples. High-purity standards (Charleston, USA) and Certified Reference Material (soil, ERM-CC018) were used for instrument calibration. Total carbon (TC), inorganic carbon (IC), total organic carbon (TOC) and nitrogen were measured with TOC-V CSH analyser equipped with TNM-1 Total Nitrogen Measurement unit (Shimadzu, Japan).

### 2.2. DNA extraction, 16 S and 18 S rRNA gene sequencing

DNA was extracted from 0.25 g subsamples using DNeasy® PowerSoil (Qiagen, USA) according to manufacturer's instructions. For high- and medium-biomass samples (soils, sediments, flue dust), sample replicas were retained; for low-biomass samples of arsenic-rich soot and stupp, replicas were pooled together to increase DNA yield. The V4-V5 region of prokaryotic 16 S rRNA genes was amplified using universal primers U515-532 (5'-GTGYCAGCMGCCGCGGTA-3') and U909-928 (5'-CCCCGYCAATTCMTTTRAGT-3') (Wang and Qian, 2009). Amplification of the V7-V8 region of fungal 18 S rRNA gene was performed using primers FF390 (5'-CGATAACGAACGAGACCT-3') and FR1 (5'-AICCATTCAATCGGTAIT-3') (Vainio and Hantula, 2000), that also co-amplified 18 S rRNA genes of other eukaryotic organisms such as SAR and Choanozoa (Shalchian-Tabrizi et al., 2008) that were analysed together with fungal sequences. PCR was performed on a ThermoFisher Scientific Verity thermal cycler using AmpliTaq Gold 360 polymerase (Thermo Fisher Scientific, USA). Conditions for both reactions were as follows: initial denaturation at 95 °C for 10 min, 25 cycles of 95 °C for 30 s, 52 °C for 30 s, and 72 °C for 30 s, followed by the final elongation of 7 min at 72 °C. Lack of contamination was ensured with a negative control, with deionised water as template. Illumina MiSeq sequencing (paired-end 2 × 250 bp) was performed at the GenoToul platform (Toulouse, France).

### 2.3. Sequencing data analysis

Removal of primer and adapter sequences, quality filtering, trimming, demultiplication, merging of paired-end reads, removal of chimeric sequences and generation of the Amplicon Sequence Variants (ASVs) was done using dada2 package (Callahan et al., 2016) for R (R Core Team, 2020). Primers were removed according to their length. To increase the recovery rate of ASVs with very low copy numbers, the core dada2 algorithm was run with sample pooling enabled (pool = TRUE option). Resulting ASVs and count tables were imported into QIIME2 (Bolyen et al., 2019) for further analysis.

Taxonomic affiliation of 16 S rRNA sequences was performed at 99% similarity using a Bayesian classifier trained on V4–V5 hypervariable region of 16 S rRNA genes from the SILVA 138 dataset (Yilmaz et al., 2013). For 18 S rRNA sequences, a classifier trained on the full-length 18 S rRNA genes from the SILVA 138 dataset was used; data was manually curated to conform to the classification proposed by Adl et al. (2005). Removal of plastid and mitochondrial sequences was also performed during this step. Phylogenetic trees were built by inserting ASVs into a tree build from the full-length 16 S and 18 S rRNA genes of the SILVA 138 dataset using q2-fragment-insertion plug-in (Janssen et al., 2018). Count data was normalised by scaling with ranked subsampling (Heidrich et al., 2021). To characterise alpha diversity, Hill numbers (Hill, 1973), Chao1 index (Chao, 1984) and Faith's Phylogenetic Diversity index (Faith, 1992) were calculated. Beta diversity was analysed with Principal Coordinates Analysis (PCoA) of Variance-Adjusted Weighted Normalised UniFrac (Chang et al., 2011) distance matrices. Statistical significance of the differences between samples and sample groups was determined by analysis of similarities (Clarke, 1993). ADONIS test (Anderson, 2001) was used to explore their relationship with environmental factors and variables. Functional prediction in prokaryotes was performed with PICRUSt2 (Douglas et al., 2020). Generated MetaCyc pathways and KEGG Orthologues datasets were analysed and visualised in STAMP (Parks et al., 2014), with the objective of comparing overall functional profiles of different communities and abundance of arsenic and mercury resistance genes; White's non-parametric t-test (White et al., 2009) with Šidák correction was used for statistical analysis. Predicted metabolic pathways of Archaea-dominated populations of the stupp sample (D) were not included in the analysis, due to uncertainty in the veracity of PICRUSt2 predictions for Archaea.

## 3. Results

### 3.1. Chemical parameters of the samples

The arsenic and mercury concentrations and other sample parameters analysed are summarized in Table S1, together with an explanation of the probable source of the pollutants in each sample. As expected, arsenic-rich soot (samples F and FS) contained around 550 g/kg of arsenic and almost 30 g/kg of mercury. Stupp samples (D) had lower concentration of arsenic (around 120 g/kg), but much higher concentration of mercury, almost reaching 66 g/kg. Flue dust (E) contained around 20 g/kg of arsenic and 7 g/kg of mercury. Soil samples (A, B, C) have shown high degree of arsenic and mercury pollution as well, although soil recovered at and near a phytoremediation plot (samples A and B) was contaminated to a lesser degree compared to the soil from the riverbank (C). Groundwater sediment samples (SB, SR) have shown high arsenic contamination, with sediment from the well near the river (SR) containing as much as 30 g/kg of arsenic; concentrations of mercury in the sediments were similar to the soil levels. Incidence of arsenic and mercury was correlated (Spearman correlation based on 16 samples;  $\rho = 0.79$ ,  $p = 0.003$ ), which is expected for contamination originating from the same source. Total Organic Carbon measurements indicated low organic carbon content in all waste samples except flue dust, where it was significantly higher than in soils (Table S1).

### 3.2. Diversity of microbial prokaryotic and eukaryotic communities

After merging Illumina reads 4067 unique ASVs belonging to Bacteria and Archaea and 1081 belonging to Fungi and SAR were generated. In terms of biodiversity of both Prokaryotic, Fungal and other detected Opisthokonts (SAR communities and members of the Choanozoa phylum), pronounced differences were observed between the samples (Fig. 1, Fig. 2). Topsoil (A) and soil taken from remediation plot (B) had relatively diverse microbial communities, with highest observed species richness and community evenness. All diversity metrics showed lower diversity in the rest of the samples, with pyrometallurgic waste communities (samples D, F and FS), unsurprisingly, being the least diverse.

Faith's Phylogenetic Diversity, however, indicated much lower levels of diversity in topsoil (A) and remediation plot soil (B) compared to non-phylogenetic indices, suggesting that significant contribution to community richness was done by a large number of closely-related organisms. Another noteworthy occurrence is a noticeable drop in diversity (especially in community evenness) in significantly more polluted riverbank soil (C) compared to less contaminated soils.

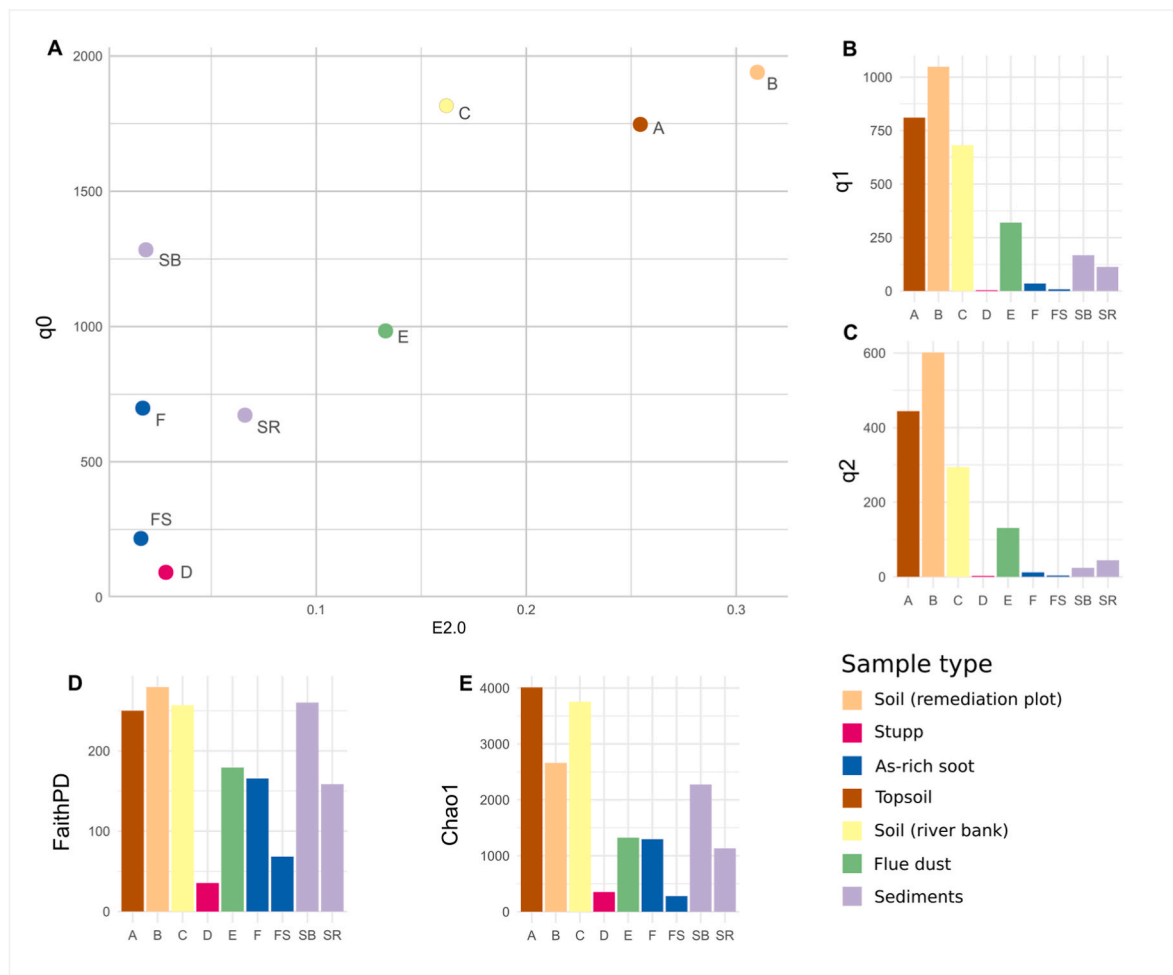
At beta diversity level, significant differences between Prokaryotic communities from different samples were observed as well (ANOSIM  $R = 0.62$ ,  $p = 0.001$ ). On a PCoA plot, the biggest observed differences were between stupp (D) and the rest of the samples (Fig. 3A). Topsoil, soil from phytoremediation plot and soil from the riverbank (sample series A, B, C) were grouped together; distances between sediment samples (SR, SB) were comparable to distances to other samples, and samples of soot taken at different times (F, FS), while not completely dissimilar, still had significant differences between each other. Flue dust samples (E), while having some similarities to soil (A, B, C), soot (FS) and sediment (SR), were distinct enough to form their own grouping.

Increase in mercury and arsenic concentrations across different samples explained about 41% of variation in UniFrac distances between samples for mercury ( $R^2 = 0.4075$ ,  $p = 0.001$ ) and 11% for arsenic ( $R^2 = 0.1134$ ,  $p = 0.01$ ) in the ADONIS test. Differences in sample type could explain 84% of variation ( $R^2 = 0.84$ ,  $p = 0.001$ ). Correlation with amounts of organic and inorganic carbon were insignificant.

Comparison of Fungi and SAR communities on a PCoA plot (Fig. 3B) has shown that soil samples (A, B, C), flue dust (E) and sediments (SR, SB) formed a pattern similar to that of Prokaryotic communities, with soils grouped together, flue dust being somewhat similar to those in soils, and sediments (SR, SB) being dissimilar to both other samples and each other. In a stark contrast to prokaryotic communities, fungal and SAR communities in samples of stupp (D) and arsenic-rich soot (F, FS) were very similar to each other, perhaps reflecting their lower sensitivity to the geochemical conditions in those samples compared to prokaryotic organisms. Differences between samples grouped into soils (A, B, C), sediments (SR, SB), flue dust (E) and highly-polluted waste (D, F, FS) were statistically significant (ANOSIM  $R = 0.81$ ,  $p = 0.006$ ). Correlation with arsenic and mercury concentrations and carbon content were not significant; differences in sample type explained 71% of inter-group UniFrac distance variation ( $R^2 = 0.7136$ ,  $p = 0.002$ ).

### 3.3. Taxonomic analysis

Annotation depth for 16 S rRNA sequences was high (up to 86.53% at the family level (Table S2). Bacterial communities in soils (A, B, C) were shown to be very similar, dominated by Gammaproteobacteria of the order Burkholderiales, Alphaproteobacteria of the order Rhizobiales, Actinobacteriota belonging mostly to orders Thermoleophilia, Actinobacteria and Acidimicrobia, and members of the phylum Acidobacteriota of the orders Vicinibacteria, Acidobacteria and Blastocatella (Fig. 4A). Large minorities of Chloroflexi, Planctomycetota, Gemmatimonadota, Methylospirae and Myxococcota were also present. Composition of bacterial communities in flue dust (E) was similar to soils, with higher amount of Gammaproteobacteria of the order Xanthomonadales, and large minorities of Verrucomicrobiota and



**Fig. 1.** Alpha diversity indices of Prokaryotic communities. Replicates of the samples were merged together. A: richness-evenness scatterplot, Hill number with diversity order ( $q$ ) of 0 used as richness metrics, and E2.0 (ratio of Hill numbers with  $q = 2$  and  $q = 0$ ) as evenness metrics. B: Hill diversity of order  $q = 1$  (equivalent to exponent of Shannon diversity). C: Hill diversity of the order  $q = 2$  (equivalent to inverse Simpson index). D: Faith's Phylogenetic Diversity. E: Chao1 diversity index.

Cyanobacteria. Groundwater sediments (SB, SR) were dominated by Proteobacteria of very similar composition (mostly orders Burkholderiales, Pseudomonadales, Xanthomonadales of Gammaproteobacteria, a variety of Alphaproteobacteria), and had large minorities of Bacteroidota, Acidobacteria, Chloroflexi, Actinobacteria and Myxococcota. Sample taken near the remediation plot (SB) also had large minorities of Nitrospirota and Planctynomycetota, while sample taken near the riverbank (SR) had sizeable minorities of Zetaproteobacteria belonging to the order Mariprofundus as well as Bdellovibrionota and Elusimicrobia.

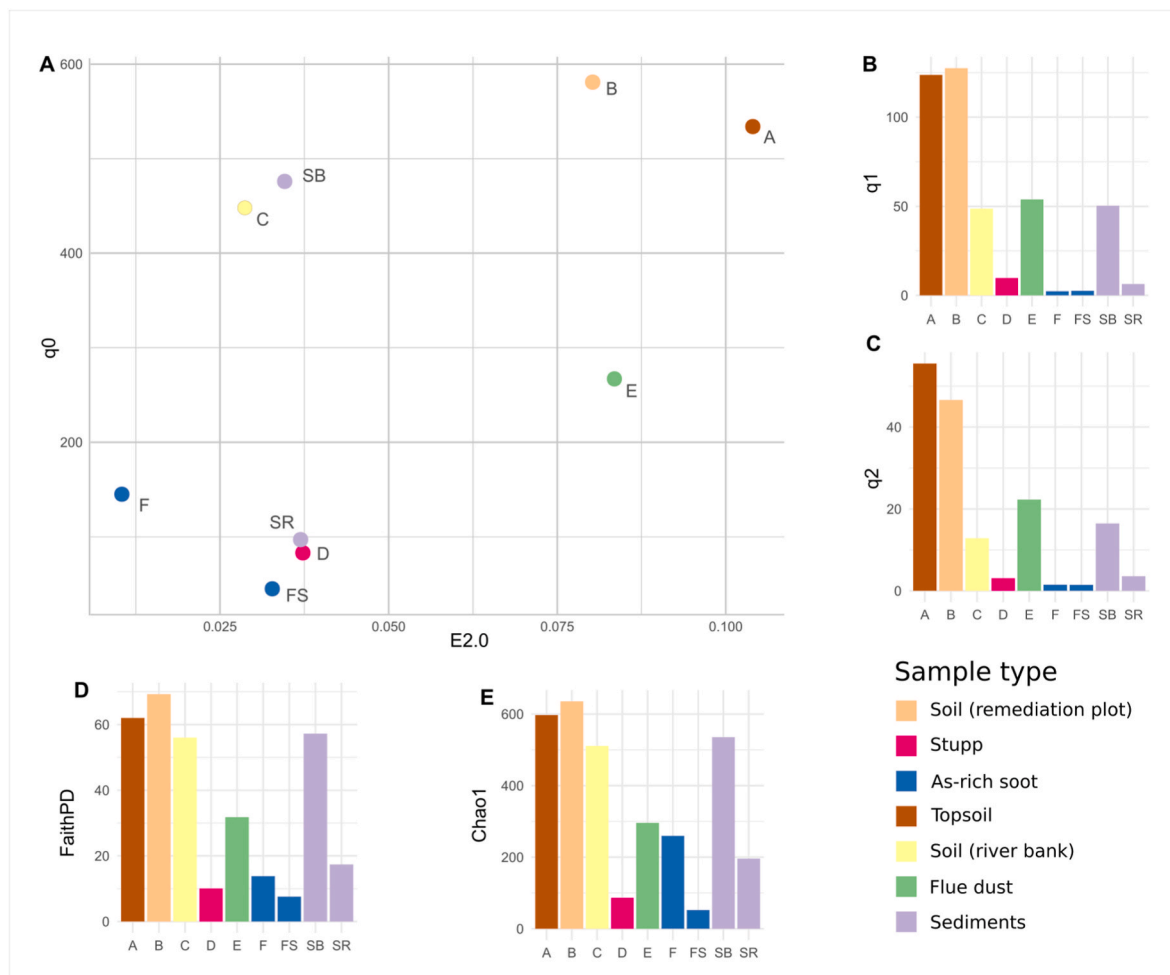
Soot sample taken in the humid winter conditions (F) was dominated by Proteobacteria, with order Burkholderiales of class Gammaproteobacteria and orders Acetobacteriales, Rhizobiales and Sphingomonadales of class Alphaproteobacteria being the most abundant; a large minority of members of phylum Firmicutes (mostly belonging to the class Bacilli) was also present. Soot sample taken in the drier summer conditions (FS) was dominated by Proteobacteria of orders Burkholderiales and Pseudomonadales (of Gammaproteobacteria) and order Acidiphilium (of Alphaproteobacteria), as well as an equally large group of Bacteroidota belonging mostly to the order Chryseobacterium. A large minority of Firmicutes was present, similarly to the sample F. In a stark contrast to the rest of the samples, a low proportion of bacteria of the class Alphaproteobacteria and phylum Firmicutes thrives in the stupp (Fig. 4A).

Various ASVs belonging to Archaea were found in all samples, but

constituted a small minority in most of them, apart from stupp (D) and groundwater sediment taken near the remediation plot (SB), where Crenarchaeota were either predominant (97%, stupp) or a large minority (20%, sediment). Archaea belonging to the phylum Thermoplasmatota in the soot sample (F) constituted a small, but noticeable minority (1.9%) (Fig. 4A).

At the ASV level, there was some degree of overlap between populations in the soot samples (F, FS), with 53 shared ASVs left after filtering out singletons, representing 63.1% and 13.68% of all ASV counts in the samples F and FS, respectively (Table S3, Fig. S6). Most abundant among them belonged to genera *Acidocella*, *Bacillus*, *Burkholderia*, *Caballeronia*, *Paraburkholderia*, and bacteria of the family of Comamonadaceae and Planococcaceae. There was, however, very little overlap between communities inhabiting soot (F, FS) and stupp (D), with only three ASVs belonging to *Bacillus*, one ASV belonging to *Micrococcus*, and one ASV identified as *Stenotrophomonas* shared between all three samples, with all ASVs encountered in very low abundance, and together representing just 0.554%, 3.343% and 1.546% of the ASV count for samples D, F and FS, respectively.

Annotation depth for eukaryotes data was lower than for bacteria and archaea, reaching 65.77% of annotated features at the phylum level, decreasing rapidly at lower taxonomic levels, reaching as low as 28.68% at the Family level (Table S2). Similarity between soil samples (A, B, C) and flue dust (E) persisted (Fig. 4B): fungal populations were represented mostly by Ascomycota, Basidiomycota and Zygomycota; Rhizaria



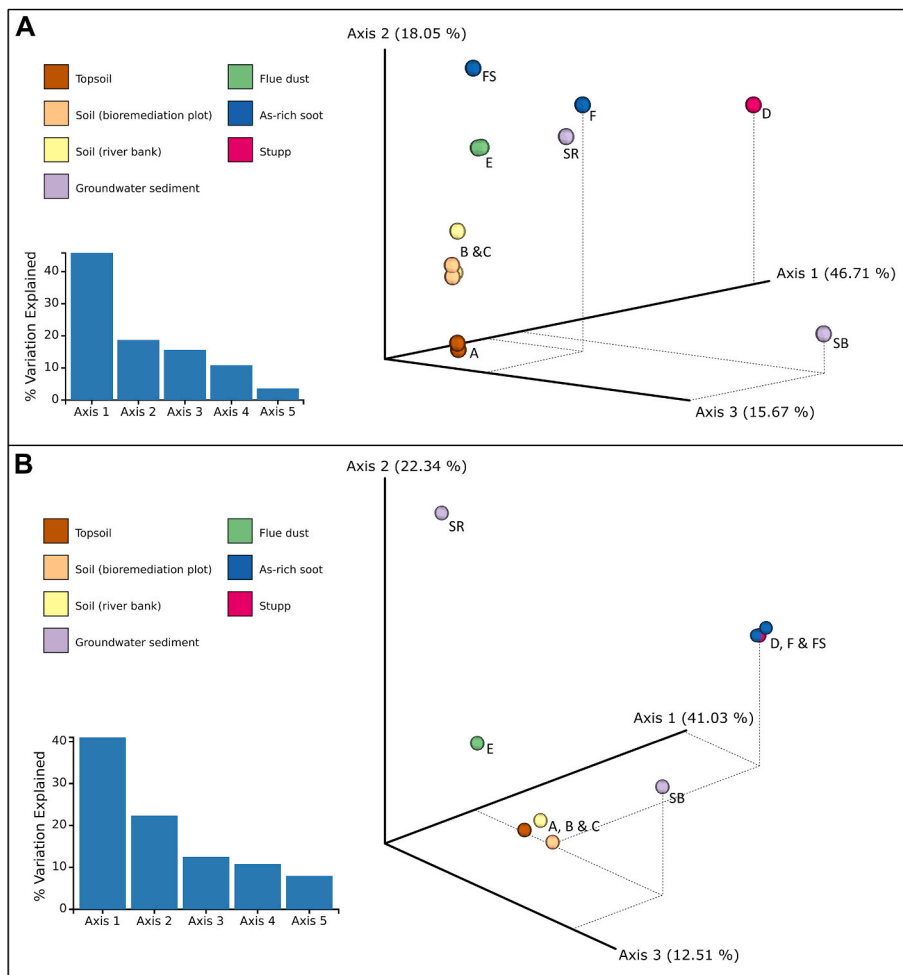
**Fig. 2.** Alpha diversity indices of fungal and SAR communities. Replicates of the samples were merged together. A: richness-evenness scatterplot, Hill number with diversity order ( $q$ ) of 0 used as richness metrics, and E2.0 (ratio of Hill numbers with  $q = 2$  and  $q = 0$ ) as evenness metrics. B: Hill diversity of order  $q = 1$  (equivalent to exponent of Shannon diversity). C: Hill diversity of the order  $q = 2$  (equivalent to inverse Simpson index). D: Faith's Phylogenetic Diversity. E: Chao1 diversity index.

(of the SAR supergroup) were represented by the phylum Cercozoa; and Sarcomastigota (of Protozoa) by Choanozoa. There were some differences as well, with more contaminated riverbank soil (C) and flue dust (E) samples almost lacking populations of Glomeromycota and Chytridiomycota present in the other soil samples (A, B), and having higher relative abundance of Zygomycota fungi. Populations in stupp (D) and soot (F, FS) were comprised of Ascomycota, Basidiomycota and unidentified fungi, as well as a minority population of Choanozoa. Sediment sample from the remediation plot well (SB) had abundant fungal populations including Ascomycota, Chytridiomycota, Basidiomycota and Zygomycota, as well as members of the phylum Bigyra of the infrakingdom Halvaria (SAR) and Choanozoa protists, while populations in the sediment sample from the riverbank well (SR) consisted mainly of Cercozoa and Choanozoa (Fig. 4B).

Eukaryotes taxonomic identification and relative abundances comparisons at lower taxonomic levels were much less reliable as almost half of the ASVs below phylum level remained unassigned. Among identified fungi in soils (A, B, C) Dothideomycetes, Eurotiomycetes, Sordariomycetes, Leotiomycetes and Pezizomycetes (of the phylum Ascomycetes), Agaricomycetes (of the phylum Basidiomycota) and Mortierellales (of Zygomycota) were the most abundant. Flue dust (E) had more fungi belonging to subphylum Entomophthoromycotina (of the phylum Zygomycota) and Ustilagnomycetes (of Basidiomycota), and smaller populations of Leotiomycetes and Pezizomycetes compared to the soil samples. Predominant populations of fungi in stupp (D) and soot

(F, FS) samples consisted of Dothideomycetes of the phylum Ascomycetes, and Entomophthoromycotina (of Zygomycota) and Ustilagnomycetes (of Basidiomycota). In the soil, flue dust and sediment samples Dothideomycetes were almost equally divided into non-identified Dothideomycetes fungi, Pleosporales, Botryosphaeriales, Capnoidales and Venturiales; in contrast, in stupp (D) and soot (F, FS) samples, the non-identified Dothideomycetes ASVs, that constituted a very small minority in the rest of the samples, were much more abundant than the rest of Dothideomycetes. SAR populations in all samples were represented mostly by the members of the subphylum Monadofilosa; and while in soil samples, flue dust (E) and sediment sample SR majority of them belonged to the class Sarcomonadea (Heteromita and Cercomonas of the orders Glissomonadida and Cercomonada, respectively), in the sediment sample SB they belonged mostly to the genus Rhogostoma of the family Rhizaspidiidae, order Cryomonadida, class Thecofilosea.

At the ASV level, in contrast to the prokaryotic communities, there was a significant overlap between fungal communities of the three most-contaminated samples, with 10 shared ASVs representing 67.68% of ASV count in the stupp (D), and 16.37% and 91.12% of the ASV count in the soot samples F and FS, respectively. Those ASVs were found in very low abundance in the rest of the samples (typically at less than 1.5%, with the exception of the flue dust (sample E), where they represented ~10% of the fungal community (Table S4, Fig. S7). They belonged mostly to the Fungi of the phyla Ascomycota and Basidiomycota.



**Fig. 3.** Principal coordinates analysis (PCoA) 3D plot of variance-adjusted weighted UniFrac distance matrix generated from SRS-normalised taxon abundances of prokaryotic and fungal/SAR communities in the samples and depicting patterns of beta-diversity of Prokaryotic (A) and of Fungi and SAR communities (B). Points that are closer together are more similar; the numbers in parentheses represent percentage of variation explained by each axis. A, B, C: soil samples; E: flue dust samples; D: stupp sample; F: soot sample taken in the winter; FS: soot sample taken in the summer; SR: groundwater sediments from the well on the riverbank; SB: groundwater sediments from the well near the phytoremediation plot. Replicates of the samples were merged together. Additional barplot illustrates contribution of each principal coordinate axis to variation between samples and includes axes not shown on the PCoA plot.

### 3.4. Prokaryotic functional prediction

Principal Component Analysis of predicted abundances of MetaCyc pathways indicated clear differences in predicted metabolic profiles of prokaryotic communities inhabiting different environments at El Terrenal in a pattern similar to PCoA analysis, with the exception of sediment samples being grouped very close together, indicating a high degree of similarity at the functional level despite taxonomic differences (Fig. S8A). Predicted metabolic pathways differences between soot communities and the rest of the samples reflected taxonomic differences in community composition, with no clear pattern indicating predominant type of metabolism in the community (Fig. S8B). Proportion of predicted mercury resistance genes (KEGG orthologues) relative to the rest of the predicted metagenomes was very low (typically less than 0.01%). Since soot samples taken at different time (F, FS) showed significant inter-sample variation, comparison was made between soils (A, B, C), flue dust (E) and groundwater sediments (SR, SB) (Fig. 5). Genes encoding alkylmercury lyase *merB* (K00221) and mercuric ion transport proteins *merC*, *merE* and *merT* (K19058, K19059 and K08363, respectively) were predicted to be differentially abundant across all three groups ( $p < 0.05$ ). In all cases, predicted abundances were significantly higher in flue dust (E) compared to soils (sample series A, B, C) and groundwater sediments (SR, SB).

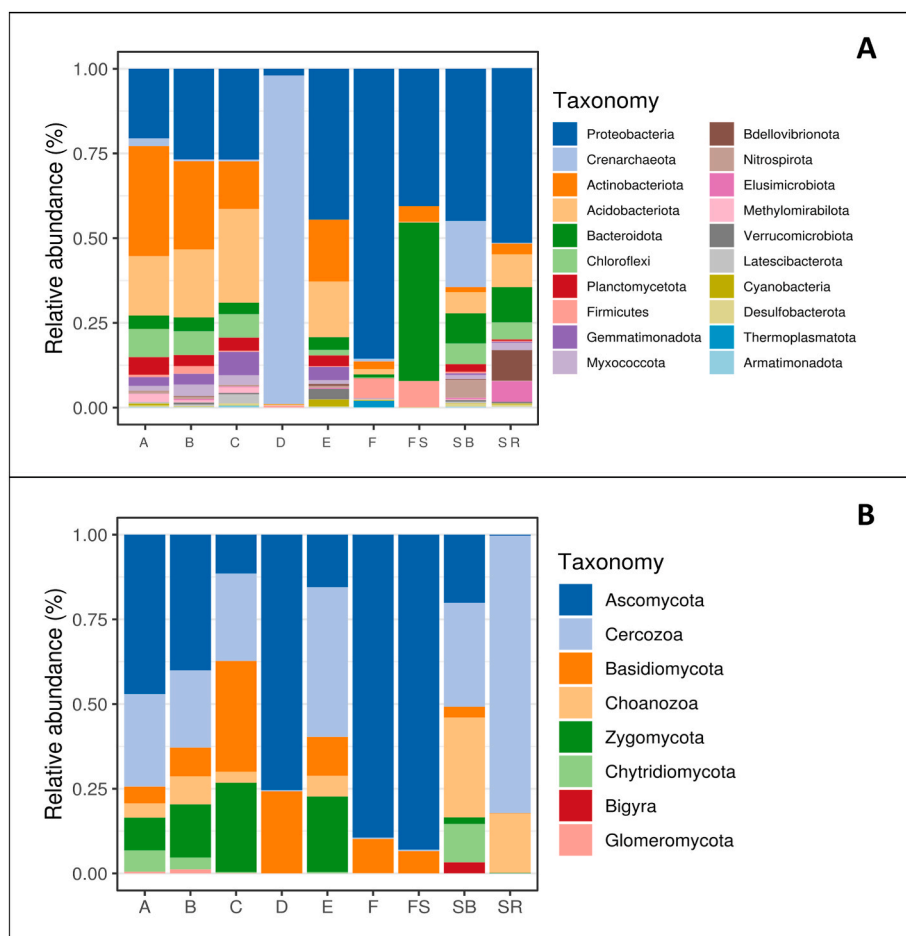
Predicted abundances of KEGG orthologues corresponding to arsenic resistance genes were compared between soils (samples A, B, C), flue dust (samples E), groundwater sediment (SR, SB) and arsenic-rich soot (F, FS) (Fig. 6). All of them represented only a small proportion of predicted genes (typically between 0.1 and 0.5%; up to 0.11% for

regulatory gene *arsR*). Four genes were differentially abundant ( $p < 0.05$ ): Arsenite pump *arsB* (K03325) was predicted to be more abundant in flue dust (E) compared to soils (A, B, C) and sediments (SR, SB), and even more abundant in soot samples F and FS. Arsenate reductase *arsC* (K00537) and organoarsenical oxidase *arsH* (K11811) were significantly more abundant in soot (F, FS) as well, with flue dust (E), sediments (SR, SB) and soils (A, B, C) showing progressively less predicted abundance. Predicted abundance of regulator/repressor genes of *arsR* type (K03892), in contrast, was significantly lower in soot (F, FS) compared to other samples.

## 4. Discussion

### 4.1. Biodiversity and microbial populations

When analysing different components of diversity for both prokaryotic and fungal (and SAR) communities, both phylogenetic and non-phylogenetic richness in soil samples (A, B, C) was shown to be on the similar level regardless of the differences in arsenic and mercury concentration. This is broadly comparable to the data from arsenic-polluted soils (Gu et al., 2017; Simmler et al., 2019). Estimations of Hill diversity and evenness, however, painted a more complex picture, with most-contaminated soil (sample C) showing a significant drop in biodiversity and community evenness compared to the less contaminated soils. This negative effect may remain uncovered when using richness-dependent diversity and evenness metrics such as Shannon diversity and Pielou's J with large metagenomic datasets (McCune and Grace, 2002; Roswell et al., 2021). Alpha and beta diversity data alone



**Fig. 4.** A. Relative abundances of the twenty most abundant phyla of Bacteria and Archaea (Crenarchaeota and Thermoplasmata). Sample replicas were merged together. B. Relative abundance of the eight most abundant eukarotic phyla: Fungi (Ascomycota, Basidiomycota, Zygomycota, Chytridiomycota and Glomeromycota), Chromista SAR supergroup (Cercozoa and Bigyra) and of Protozoa (Choanozoa).

show that, while increasing arsenic and mercury contamination levels in soil did not radically alter the structure of microbial community by precluding significant number of organisms from surviving, it instead favoured some organisms over the others, most likely depending on their resistance to arsenic and mercury, decreasing overall diversity and community evenness. In contrast, heavy metals can be lethal for many microbial species when contaminant is introduced into pristine soils, and lead to lower biodiversity and significant changes in microbial community structure in contaminated soils when compared to their unaffected counterparts (Giller et al., 1998; Ji et al., 2018; Shen et al., 2019; Salam et al., 2019 and references therein). Unsurprisingly for such biomass-poor, highly polluted samples, microbial communities in groundwater sediments, flue dust, stupp and soot were significantly less diverse, and their low evenness suggested that they were heavily dominated by a very small number of organisms, where, as already mentioned, resistance to metal (loid)s likely played a part.

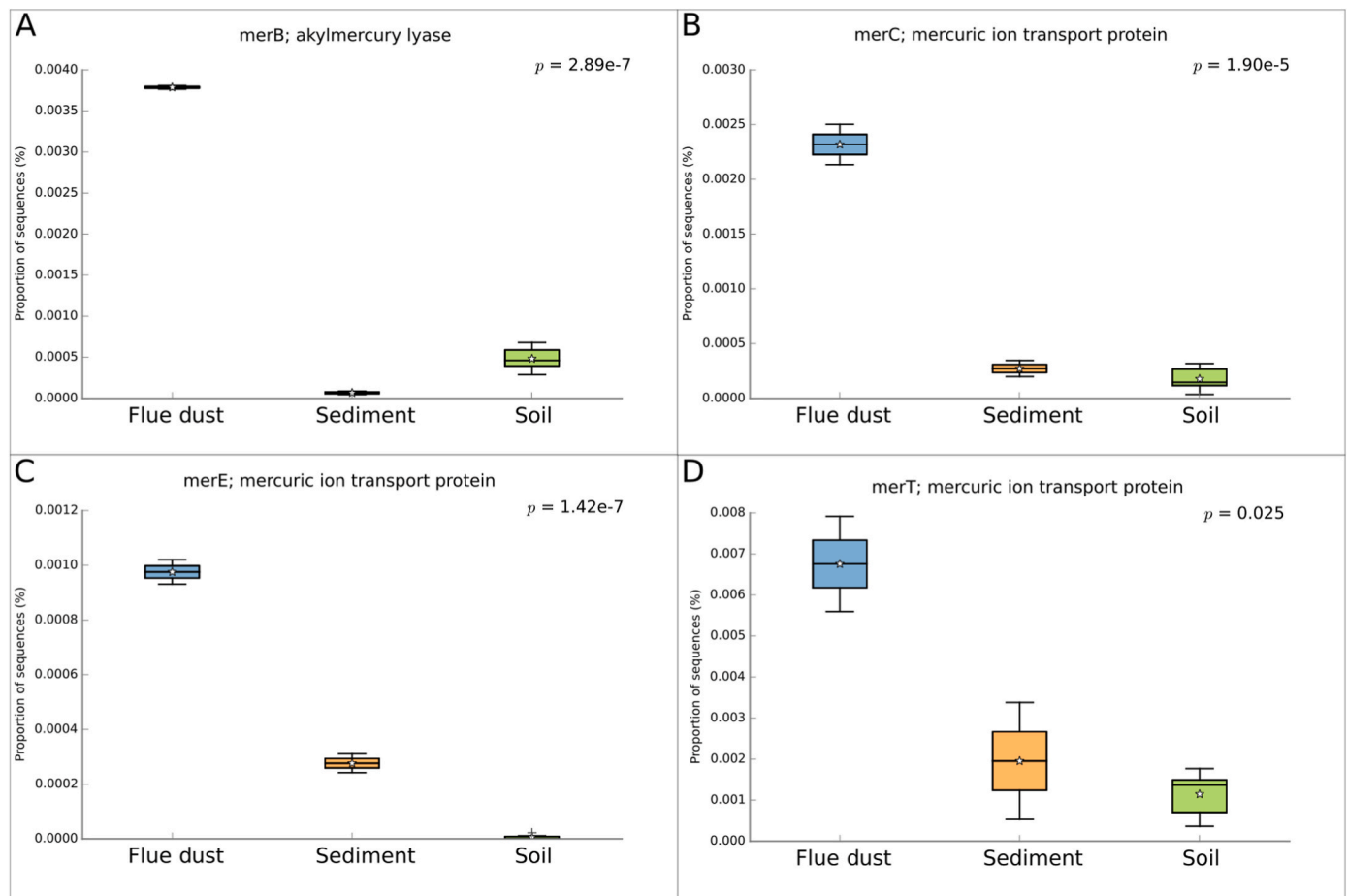
Taxonomically, prokaryotic microorganisms found in polluted soils (A, B, C) and flue dust (E) were very similar to those found by other authors in arsenic-polluted soils (Gu et al., 2017; Wu et al., 2016; Narendrula-Kotha and Nkongolo, 2017; Li et al., 2022) and mining waste undergoing natural attenuation (Bertin et al., 2011; Liu et al., 2019). Similarly to those studies, Proteobacteria, Actinobacteria and Acidobacteria were the most abundant phyla, followed by Bacteroidetes, Chloroflexi, Planctomycetes, Gemmatimonadetes and Firmicutes. In contrast, in a study of soils from the mercury mining district of Almadén, Ciudad Real (Spain), contaminated with 1710 mg/kg of total mercury, representatives of the Actinobacteria phylum were the most abundant,

followed by a much lower percentage of Alphaproteobacteria, Cyanobacteria and Acidobacteria (González et al., 2022). Soil community structure described in our study was also similar to the soil contaminated by other metals and metalloids (Liu et al., 2019; Pradhan et al., 2020). In other studies of soils contaminated with much lower mercury concentration gradients (0.25–40 mg/kg), the number of organisms from the phylum Gemmatimonadetes increased under long-term mercury exposure (Frossard et al., 2018).

Sediment samples taken from two different locations differed both from the soil and mining-metallurgy waste samples and between each other. Proteobacteria, Bacteroidetes, Acidobacteria and Chloroflexi were among the most abundant phyla, while minority groups differed a lot; this is similar to studies of arsenic-polluted freshwater sediments, with some of them showing significant changes in community structure of less abundant bacteria depending on location or season (Halter et al., 2011; Cavalca et al., 2019; Li et al., 2020). Unexpectedly, functional metagenomic predictions demonstrated that metabolism of microbial communities in two groundwater sediment samples from El Terronal was very similar. It should be noted, however, that comparatively high abundance of (mostly) uncultured organisms in those samples (such as Crenarchaeota and Zetaproteobacteria in the sample SB, and Elusimicrobia and Bdellovibrionota in the sample SR) could have impacted prediction accuracy due to the low number of genomes available for prediction for those species, and their poorly studied metabolism.

Samples of arsenic-rich soot and stupp were distinct from soils. Remarkably, archaea dominated in prokaryotic communities inhabiting stupp waste heap (sample D), which sets it apart from the rest of the





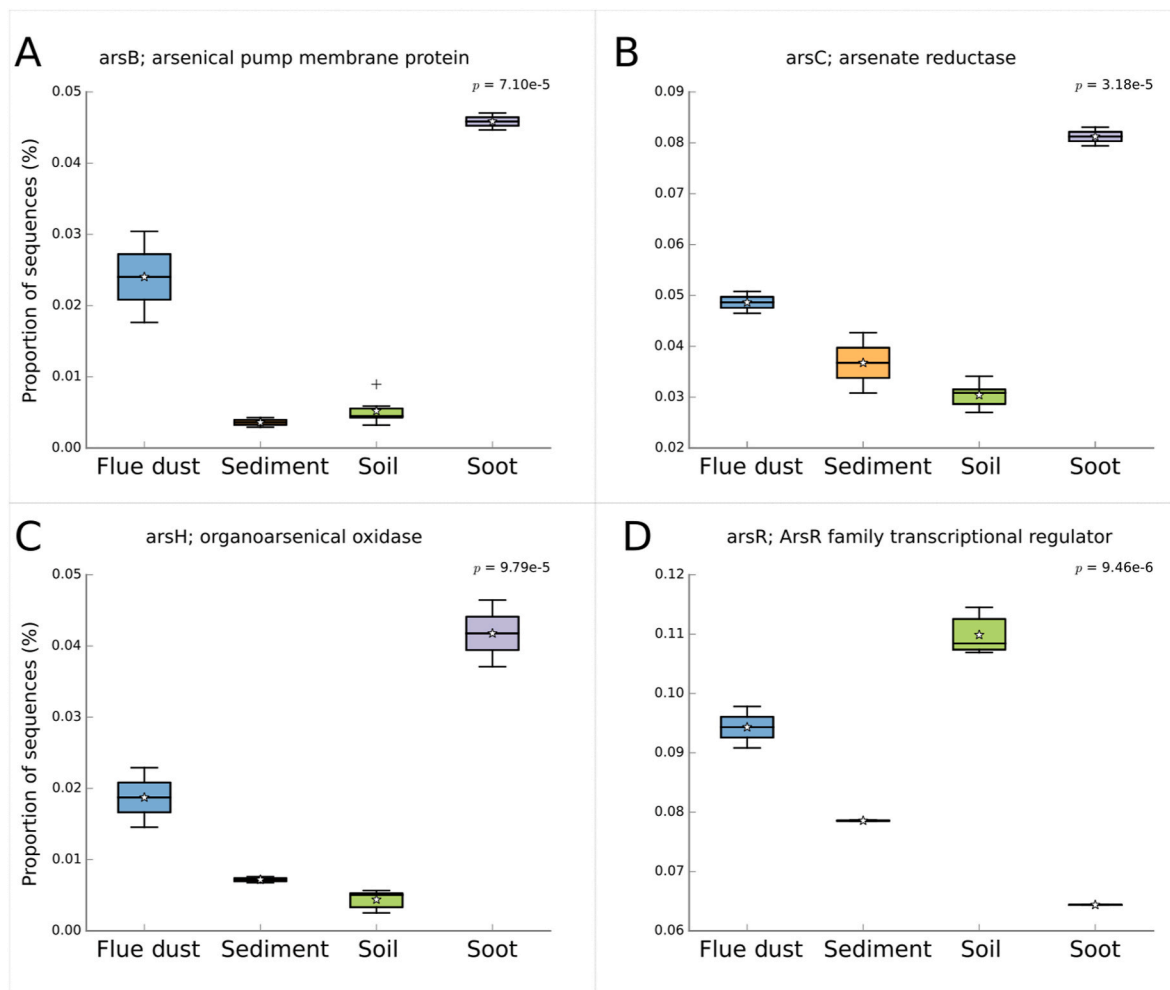
**Fig. 5.** Predicted prokaryotic mercury resistance genes differentially abundant between sample groups of soil (A, B, C), flue dust (E) and groundwater sediments (SR, SB): alkylmercury lyase merB (A), mercuric ion transport protein merC (B), mercuric ion transport protein merE (C), and mercuric ion transport protein merT (D). Significance of differences between groups were established by ANOVA.

samples, populated mostly by bacteria. It suggests that stupp is even less hospitable to life than (already extremely hostile) arsenic-rich soot (samples F, FS), with its higher mercury content and presence of PAHs and organomercurial compounds (see Supplementary material) preventing most bacteria from colonising it. Crenarchaeota are known to be present in temperate acidic forest soils, where it has been suggested to participate in the nitrogen cycle through ammonia oxidation (Kemnitz et al., 2007; Lehtovirta et al., 2009). This role was also suggested for Crenarchaeota-dominated soils contaminated by the mining of the rare earth minerals (Liu et al., 2021). Non-thermophilic Crenarchaeota were also detected in the archaean community of soils with long-term heavy metal (Cd, Cu, Ni, and Zn) contamination (Sandaa et al., 1999), and were predominant in river sediments heavily contaminated with Hg, As, Cd, Cr, Ni, Pb, Cu, Mn and Zn (Yin et al., 2015). These data and the presence of Crenarchaeota in other extreme locations, such as polluted acidic waters (Simbahan et al., 2005; Almeida et al., 2008; Gough and Stahl, 2010; Mesa et al., 2017b), support the presence of effective metal resistance determinants in those prokaryotes. Interestingly, Crenarchaeota have been suggested to contribute to the adaptation of microbial communities in soils with metal contamination by improving the environmental conditions or by cooperative interactions (Li et al., 2017; Liu et al., 2021).

Samples taken from the same arsenic-rich soot waste heap at different times (humid, winter: sample F; dry, summer: sample FS) had significant differences in composition of their prokaryotic communities, with sample F being more diverse, and dominated by Burkholderiales of Proteobacteria, while sample FS was divided very evenly between Proteobacteria and Bacteroidetes. Both samples, however, shared a

significant proportion of ASVs belonging mostly to *Burkholderia-Caballeronia-Paraburkholderia*, *Acidocella* and *Bacillus*. This hints at a presence of a stable core community surviving during seasonal changes, something that should be confirmed with more detailed studies over time. Interestingly, five ASVs were shared between all three highly contaminated waste samples (F, FS, D). Of those ASVs, one belonged to genus *Micrococcus* and three belonged to *Bacillus*, a genus that is known to have hyper-resistant species (Niane et al., 2019; Aguilar et al., 2020). The remaining ASV belonged to the genus *Stenotrophomonas*; one of the members of this genus was recently isolated from an abandoned arsenic mine and has proven to be exceptionally resistant to arsenic (Bermanec et al., 2021).

Fungal communities in the soil (A, B, C) and flue dust (E) were represented by Ascomycota, Basidiomycota and Zygomycota, with those belonging to the phylum Ascomycota also being overwhelmingly predominant in the most polluted stupp (D) and soot (F, FS) samples. Unlike prokaryotic communities, where populations in stupp (D) and soot (F, FS) were radically different, there was a large degree of similarity between fungal populations of those samples. It has been reported that fungal soil populations are less affected by heavy metals than bacteria (Frossard et al., 2018; Zeng et al., 2020; Njoku et al., 2020) and show higher resistance to arsenic and mercury; in fact, they are the greatest accumulators of mercury, although underlying mechanisms for that are not clear (Hiroki, 1993; Rajapaksha et al., 2004; Durand et al., 2020). The fungal species of the Ascomycota phylum are ubiquitous (Al-Sadi, 2017), and their tendency to predominate in heavy metals (Narendrula-Kotha and Nkongolo, 2017; Šimonovičová et al., 2019; Kerfahi et al., 2019; Lin et al., 2020), and specifically in mercury-contaminated



**Fig. 6.** Predicted prokaryotic arsenic resistance genes differentially abundant between sample groups of soil (samples A, B, C), fluedust (E), groundwater sediments (SR, SB) and arsenic-rich soot (F, FS): arsenite pump arsB (A), arsenate oxidase arsC (B), organoarsenical oxidase arsH (C) and arsR-type regulator/repressor gene (D). Significance of differences between groups were established by ANOVA.

soils was documented previously (Frossard et al., 2018; Pathak et al., 2020; Văcar et al., 2021). In our previous studies of acidic mine drainage water (Mesa et al., 2017b), representatives of Ascomycota phylum were also detected in abundance, which shows their extraordinary ability to adapt to diverse natural environments, including extreme ones; characteristics such as their morphology that enhances dispersal, metabolic versatility and genetic resistance to stress, including their tolerance to heavy metals, contribute to their ubiquity (Egidi et al., 2019).

An interesting result is the detection of members of the phylum Choanozoa, by co-amplifying their ASV in several of the analysed samples, especially in the flue dust (E) where it was detected in a very significant amount. Choanozoa members have been described as components of the trophic chain of degradation of vegetable carbonaceous residues (Kramer et al., 2016). The presence of these protozoa in appreciable quantity in the nutrient-poor, metal (loid)-rich flue dust (Gallego et al., 2015) raises questions about their hypothetical role in the recycling of carbon in this niche.

#### 4.2. Environmental parameters and microbial resistance to metals

As was evidenced by beta-diversity studies of both 16 S and 18 S rRNA genes and predicted KO metagenomes, differences in composition of microbial communities correlated mainly with distinct geochemical composition of each sample type (soil; three types of metallurgical waste; groundwater sediments). Individual contribution of increasing arsenic

and mercury concentrations, while not completely insignificant, could not fully explain the differences in microbial communities. This is similar to other studies employing metagenomic methods to study microbial communities in arsenic-polluted soils, such as soils contaminated by irrigation (from different geographic locations in Bangladesh and China), arable soil with high levels of geogenic arsenic from the United Kingdom (Gu et al., 2017) and floodplain soils contaminated with mining waste (Simmler et al., 2019). In fact, a joined impact of several parameters, such as oxidation-reduction potential, availability of nutrients, mineral composition, and electric conductivity and pH, in addition to the presence of heavy metals, determined the diversity and composition of bacterial and archaeal communities in soils (Lehtovirta et al., 2009; Chodak et al., 2013; Zeng et al., 2020 and references therein; Li et al., 2021) and in arsenic-contaminated groundwater treated with zero-valent iron nanoparticles (Castaño et al., 2021). In line with these hypotheses, the overwhelming predominance of archaea of the phylum Crenarchaeota in the stupp sample (D) but not in the soot samples would have to be attributed to specific environmental conditions present in the former, but not in the soot, since the levels of mercury and arsenic are quite similar in both cases.

Bacterial phyla known to carry mercury resistance determinants (Proteobacteria, Firmicutes and Actinobacteria) (Nazaret et al., 2003; Duran et al., 2008; Xu et al., 2019; Pathak et al., 2020) were abundantly represented in the El Terronal samples. When functional metagenomic predictions for mercury resistance genes as well as arsenic detoxification

and metabolism genes were carried out, data for communities found in the stupp sample (D) were excluded due to low confidence in predictions for archaea-dominated communities. Functional predictions for mercury resistance genes have shown a simple trend of higher abundance in environments with higher mercury concentration. Comparison was performed only for soils (A, B, C), sediments (SR, SB) and flue dust (E), as samples of arsenic-rich soot (F and FS) had significant inter-sample differences, most likely attributed to different community structure as a result of different sample humidity. A similar trend towards higher abundance of detoxification genes (arsenite efflux pump *arsB*, arsenate reductase *arsC*, and organoarsenical oxidase *arsH*) (Bini, 2010; Chen et al., 2015; Newsome and Falagán, 2021) in communities inhabiting environments with higher levels of arsenic concentration also emerged. The possible presence of the *arsH* gene is relevant, since it has been described in different bacterial species that it confers resistance to As(III) methylated and aromatic organic compounds by oxidizing them to As(V) (Chen et al., 2015). One possibility here is that this gene could be involved in microbial resistance to phenylmercury propionate, an extremely toxic compound detected in arsenic-rich soot (F) and stupp samples (D), and in greater quantity in flue dust (E) (Gallego et al., 2015; Supplementary material). Functional predictions for *arsR*-type regulatory/suppressor genes (Bini, 2010; Newsome and Falagán, 2021) followed an inverse trend, with predicted metagenomes of communities inhabiting arsenic-rich soot (samples F and FS) having the lowest abundance of *arsR*-type genes. This could be explained by a shift towards constitutive expression of the arsenic resistance genes as it was described for a hyper-resistant strain (Koechler et al., 2015). However, in order to determine expression of those genes in the microbial community, a further transcriptomic and/or proteomic analysis would be required.

The analysis of the El Terronal site has opened several possible lines of future research, including hypothetical novel mercury or arsenic resistance mechanisms (Boyd and Barkay, 2012; Jones et al., 2019; Christakis et al., 2021). Remediating environments heavily contaminated with mercury and arsenic such as El Terronal may require a combination of advanced physico-chemical and biological methods (Teng et al., 2020), such as nanoremediation and phytoremediation (Gil-Díaz et al., 2016; Gil-Díaz et al., 2019; discussed in more detail in the Supplementary Materials section), that will benefit from detailed knowledge of the characteristics of the site's indigenous microorganisms, and the possibility of using them in additional bio-stimulation/bioaugmentation technologies. Also, to explore the possibility of carrying out alternative bioremediation approaches, such as those related to *in situ* molecular breeding (Kumari et al., 2020) or synthetic biology (Ali et al., 2022). In the course of this work, we have been able to isolate by culture quite a number of bacteria with high levels of resistance to mercury and arsenic from all the analysed samples, except the stupp (Prosenkov et al., unpublished results); this opens up additional possibilities, such as obtaining bacteria with efficient expression systems of the *mer* operon, which would be an additional advantage for bioremediation (Priyadarshane et al., 2022). Another open line of investigation concerns the possible use of detoxification strategies mentioned previously (such as bioaccumulation and/or bio-volatilization) by the Ascomycota fungi present in this highly polluted site (Durand et al., 2020; Newsome and Falagán, 2021 and references therein).

## 5. Conclusions

The conditions at the El Terronal site range from polluted soils (with arsenic and mercury concentrations several times above permitted levels) to the extreme environments of waste heaps consisting mostly of arsenic oxides, with admixture of large amounts of mercury as well as other toxic organic compounds. For microbial life, it makes survival in these environments very challenging. Diversity and evenness of microbial communities was reduced at increasing contamination levels (e.g.,

in the most contaminated soils samples (C) and flue dust (E) compared to the rest of the soils), but the overall community structure remained very similar. Differences in microbial communities were also modulated by the local environment: we have observed a maximum selective effect for the highly contaminated samples (D and F), where diversity was extremely low. In the most polluted environment (stupp, D), the overwhelming majority of prokaryotic microorganisms were of the phylum Crenarchaeota. The almost complete prevalence of these mesophilic archaea is a very remarkable result that supports the archetypal ability of these prokaryotes to adapt and colonize extreme environments and opens new lines of research on their metabolic features and their resistance determinants.

Our study has also confirmed and extended previous evidence of the remarkable ability of fungal species of the phylum Ascomycota to colonize these environments, that are shown in this way as potentially useful biological instruments for decontamination. Using the knowledge of the genetic-molecular mechanisms of resistance to metals of the bacteria, archaea and fungi present in the studied site is a necessary step for the selection of the most effective strains/genetic determinants, their use in microbiome engineering and ultimately their application in sustainable/efficient remediation strategies.

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## Credit author statement

A.P.: Investigation, Methodology, Formal analysis, Data curation, Writing - original draft. C.C.: Supervision, Methodology, Writing - review, editing. J.L.R.G.: Resources, Funding acquisition, Supervision. A.I.P.: Conceptualization, Supervision, Validation, Writing - review, editing.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Data availability

Data will be made available on request.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.envpol.2023.121305>.

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