Metal-tolerant fungal communities are delineated by high zinc, lead, and copper concentrations in metalliferous Gobi Desert Soils

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Abstract

The soil fungal ecology of the southern Gobi region of Mongolia has been little studied. We utilized the ITS1 region from soil DNA to study possible influences on soil fungal community variation. In the sample network, a distinctive fungal community was closely associated with high zinc (Zn), lead (Pb) and copper (Cu) concentrations. The pattern of occurrence suggests that high metal concentrations are natural and not a product of mining activities. The metal-associated fungal community differs little from the 'normal' community in its major OTUs, and in terms of major fungal guilds and taxa, and its distinctiveness depends on a combination of many less common OTUs. The fungal community in the sites with high metal concentrations is no less diverse than in areas with normal background levels. Overall, these findings raise interesting questions of the evolutionary origin and functional characteristics of this apparently 'metal tolerant' community, and of the associated soil biota in general. It is possible that rehabilitation of metal-contaminated mined soils from spoil heaps could benefit from the incorporation of fungi derived from these areas.

Keywords: Fungi, Gobi desert, Heavy metals, Metagenetics, Soil metal, ITS1 region

1 Introduction

Heavy metals occur widely in the parent materials of soils at concentrations that are broadly dependent on lithology. During pedogenesis metals become enriched or depleted in different soil horizons, depending on the soil-forming environment and the predominant chemical reactions for each element [1]. Soil may be naturally enriched in metals due to the influence of the underlying geology and the subsequent soil forming and ecological processes, for example, through pedogenic activities in ultramafic and serpentinised geologies (e.g. [2]) or enrichment in the surface horizons following plant uptake from depth (e.g. for Cu and Pb see [3, 4]).

Soils with high concentrations of metals are associated with a distinctive microbial communities. This association is not in itself novel: there is abundant evidence of distinctive communities of plants and bacteria associated with metal-rich areas in other parts of the world. Examples include lead-, zinc- and [5] copper-rich mine spoil heaps in the UK and natural serpentine rock areas such as California and Borneo [5, 6]. Soil bacteria have been found to develop metal tolerance under laboratory conditions, by enabling energy dependent efflux of metal ions [7-9]. While metal-rich soils have occasionally been sampled by culturing individual fungal strains from them [10], there are few studies on whole metal-tolerant communities of soil fungi from anywhere in the world. Fungi are known to tolerate and detoxify metals by several mechanisms including valence transformation, extra- and intracellular precipitation and active uptake [11, 12]. Biological mechanisms implicated in fungal survival include extracellular precipitation, transformation of metals, biosorption to cell wall and pigments, decreased transport or impermeability, efflux, intracellular compartmentalization and sequestration [12-14].

The behaviour and occurrence of naturally occurring trace elements in the ecology of the soil are complex and the key metals that might influence microbial communities are poorly understood.

The present study was conducted in a naturally metal-rich area of the Gobi, a large cold desert in southern Mongolia and northern China. The desert basins of the Gobi are bounded by the Altai Mountains and the grasslands and steppes of Mongolia on the north, by the Tibetan Plateau to the southwest, and by the North China Plain to the southwest. It is the fifth largest desert in the world and the largest in Asia [15]. The climate is continental, characterized by dry and cold winters and a precipitation maximum in summer [16]. Our sampling was located in Oyu Tolgoi region, which is situated in the South Gobi desert, Mongolia. This area has only recently been discovered to be rich in metals, especially copper [17-20]. Large scale mining for copper started in 2013, with the opening of the Oyu Tolgoi mine [21]. Five major copper deposits that extend over 6 km in a north-northeast–oriented zone. These occur in a middle to late Paleozoic arc terrain and are related to Late Devonian quartz monzodiorite intrusions. The Hugo Dummett deposits are the northernmost and deepest, with up to 1,000 m of premineral sedimentary and volcanic cover rock remaining [17]. The area is characterized by sparse vegetation and large tracts of Quaternary sediments and loess. Ephemeral streams cross the area and flow for a few short periods during an average summer. Temperatures at Oyu Tolgoi range from +36°C to -25°C. Total precipitation is approximately 100 mm/year and occurs mainly in late spring and early summer [22]. Given the potential ecological sensitivity of this area, several months after the opening of Oyu Tolgoi we undertook a baseline study of the area within a 130 km radius of the mine. Given the known concentration of natural copper deposits in the southern Gobi, we specifically examined whether metal content is a key factor in the structure and composition of soil fungal communities in this area. This paper mainly aims to critically appraise apparent associations between metal-rich soils and fungal communities in SE Gobi to characterize these communities, and to consider their broader implications for microbial ecology and the study of metal enriched environments.

2 Methodology

2.1 Site description

The sampling area (Supplementary Fig. S1) is located in south-eastern Mongolia, close to the border with China, between the latitudes of 42°31 N to 43°36 N and longitudes of 106°34 E to 108°10 E. Samples were taken in mid-September 2013, towards the end of the vegetation growing season.

This study commissioned by the Oyu Tolgoi mine company was initially intended to establish reference data for the background state of the soils in this area for future monitoring of any effects of dust contamination, ground water alteration, or displacement of grazing in the area surrounding the mine. For this baseline study of soil chemistry and biota, we sampled a network of 34 sites (Supplementary Table S2) chosen to represent the range of natural vegetation types of the south-eastern Gobi. Thirty four separate 1 ha plot sample sites were assigned within a radius of 130 km of the Oyu Tolgoi mine, their positions chosen by selecting representative examples of different habitat types found in the Gobi, based on both local knowledge and on satellite imagery. Vegetation coverage consisted of small shrubs (mostly *Chenopodiaceae* and *Asteraceae*) and bunch grasses, with overall plant coverage on the hectare scale varying from 50% to 70% (Supplementary Table S1). Common representative plant species are *Eurotia ceratoides*, *Potaninia mongolica* and *Caragana korshinskii*. In each sampling site, we took one subsample at each corner of a hectare square and another in the center. Each subsample consisted of a core, 5 cm deep and 2 cm in diameter. The 5 subsamples were combined into one composite sample, mixed thoroughly, and brought back to the laboratory on the same day. Half of the mixed sample

was frozen at -20°C for later DNA extraction, while half was dried for soil chemical and physical analysis.

In most of the 1 ha plots, vegetation composition was recorded, with a species inventory of all vascular plants present. Due to time limitations, we were unable to make a species inventory of four of the plots.

2.2 Chemical Analysis and DNA extraction

Soil analyses were carried out in the Laboratory of Soil Science of the Institute of Geography, Mongolian Academy of Sciences, using standard protocols of the Soil Science Association of America (SSSA) [23]. Measured chemical parameters were pH, $CaCO_3$, total organic carbon (TOC), soil salinity (measured as electric conductivity, dS/m), nitrogen (N), soil texture (TX), chrome (Cr), lead (Pb), cadmium (Cd), copper (Cu) and zinc (Zn). All samples were transported to the laboratory at Oyu Tolgoi mine and frozen at -20℃ within 3 hours of sampling. Within days frozen soils were processed for DNA extraction in the Laboratory of Ecological and Evolutionary Synthesis of the National University of Mongolia. Soil was sieved with a 3 mm sieve, and 0.35 g of the sieved soil was DNA extracted using the Power Soil DNA extraction kit (MoBio Laboratories, Carlsbad, CA, USA) according to the manufacturer's protocol.

2.3 PCR and sequencing

All the extracted DNA samples were amplified for ITS1 region by using the primer pair ITSIF (5'-CTTGGTCATTTAGAGGAAGTAA-3') and ITS2 (5'-GCTGCGTTCTTCATCGATGC-3') [24, 25]. Polymerase chain reactions (PCR) were performed in 50 μl reactions using the following

temperature program: 95°C for 10 min; 30 cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 30 s; and 72°C for 7 min. The resulting amplicons were purified using the QIAquick PCR purification kit (Qiagen, CA, USA), and sequenced using Illumina Miseq platform (paired end of 2 × 300) at Macrogen Incorporation (Seoul, Korea), following standard Illumina sequencing protocols [26].

2.4 Sequence processing

Miseq Illumina [24] sequence data were processed using Mothur platform [27]. In brief, the sequence data were pair assembled using make.contigs and the quality control was performed by identifying chimeric sequence via UCHIME [28] and by filtering chimera sequences. Operational Taxonomic Units (OTUs) were picked using UCLUST [29] with a threshold of 97% pairwise identity using the QIIME implementation [30]. This cut-off has often been used in fungal community analyses [31, 32]. After the process, singletons were screened in Mothur. Taxonomic classification was assigned using the UNITE database [33] using the classify command in Mothur. All of the ITS1 sequence data are available under the MG-RAST [34] project ID 17045 (http://metagenomics.anl.gov/linkin.cgi?project=17045). The FUNGuild were used to classify OTUs into trophic categories [35].

2.5 Statistical analysis

To perform the statistical analysis, all samples were standardized by random subsampling to 13,957 reads per sample. We used a *t-test* for normal data and *Wilcoxon rank-sum test* for non-normal data in R software package 2.15.2 to test whether the relative abundances of taxonomical groups were significantly different between samples in low metals sites and high metals sites.

158 OTUs richness and diversity indices were estimated using Mothur. To assess the correlation
159 between richness/diversity and environmental variables, linear regression was performed in
160 SigmaPlot v 10.0 (Systat Software, San Jose, CA). To test for spatial autocorrelation of the fungal
161 community OTU composition, we used the Mantel test (Mantel Nonparametric Test Calculator
162 2.0) [36] to compare matrices of fungal community composition in relation to geographic distance
163 between different samples.
164 An indicator species analysis [37-39] was performed using package labdsv [40] in R software to
165 identify those OTUs that are characteristics of each high and low metal-enriched sites. We used
166 the INDVAL analysis, which identifies indicator species based on OTU fidelity and relative
167 abundance [39]. Only OTUs with significant (P<0.05) and INDVAL values that were >0.5 were
168 considered as significant threshold for habitat specialization. The indicator species are defined as
169 the most characteristic species of each group, found mostly in a single group and present in the
170 majority of the samples belonging to that group. Bray-Curtis distance was calculated to analyze
171 fungal community similarity. To reduce the contribution of highly abundant OTUs in relation to
172 less abundant ones in the calculation of Bray-Curtis matrix, abundance data of OTUs were square
173 root transformed. The pairwise differences in fungal community composition were calculated by
174 analysis of similarity (ANOSIM) in relation to Bray-Curtis distance. A constrained analysis was
175 conducted using CANOCO [41] to assess the effect of environmental variables on the fungal
176 community. Forward selection was used in redundancy analysis (RDA) [42] to select significant
177 explanatory variables with 999 permutations and only significant variables ($P < 0.05$) were
178 included in the models.
179 Fungal functional guilds were assigned according to Nguyen *et al.* [35] and Tedersoo *et al.* [43]
180 using an open annotation tool (FUNGuild). Here, we only accepted the guild assignment where

confidence ranking was “probable” or “highly probable”, as recommended by Nguyen *et al.* [35]. The functional guilds of fungi detected in this study principally included three major functional groups: pathogens, saprotrophs and symbionts

3 Results

The soils in all sites were alkaline, (mean pH 8.7; range pH 8.2 – 9.3), and contained on average 56% of sand, 34% of silt and 11% of clay. Results of soil physico-chemical analysis are shown in Supplementary Table S2. In several samples, Zn metal concentrations exceeded EPA, Eco-SSL regulation (http://www.epa.gov/ecotox/ecossl/index.html) levels of 50 mg/kg, reaching 233 mg/kg in some samples. Cu and Pb contents were also above Eco-SSL, EPA level of 50 mg/kg in some samples, the highest metal concentrations measured were around 2300 mg/kg for Cu and 130 mg/kg for Pb (Fig. 1). In combination, the combined Pb, Zn and Cu concentration averaged around 689.78 mg/kg (ranging from 54.7 mg/kg to 3061 mg/kg).

From the 34 soil DNA samples, we obtained a total of 2,459,044 quality reads, which were classified into 11,559 OTUs at the 97% similarity level. In presentation of results, we have distinguished ‘high metal concentration’ samples as those containing >50 ppm Zn, Cu and/or Pb. The fungal community averaged across all the samples was dominated by Ascomycota, with 79% of total reads (Fig. 2a). Basidiomycota was the next most abundant group with 7% of the total reads. Relative abundance of other phyla was under 1%. There was no significant difference at the phylum level when high metal samples and low metal samples were compared, with the exception of certain minor phyla representing less than 1% of total reads - including Blastocladiomycota ($w=77$, $P=0.02$), Chytridiomycota ($w=198$, $P=0.007$), Incertae sedis ($w=62$, $P=0.01$) and Streptophycophyta ($w=172$, $P=0.01$).

Among the detected fungal classes, *Dothideomycetes* and *Sordariomycetes* (Ascomycota) were the most abundant, with a relative abundance of 48% and 25% of reads respectively amongst all samples combined. *Tremellomycetes* (Basidiomycota) represented 5% of total reads, and *Eurotiomycetes* (Ascomycota) and *Agaricomycetes* (Basidiomycota) were each at around 1% of total reads. Dominant orders were *Pleosporales* (46%), *Hypocreales* (21%), *Filobasidiales* (5%) and *Sordariales* (4%) (Fig. 2b).

As Fig. 2c shows, 9 families (These families included *Nectriaceae* (18%), *Pleosporaceae* (17%), *Pleosporales family Incertae sedis* (15%), *Sporormiaceae* (10%), *Filobasidiaceae* (5%), *Chaetomiaceae* (2%), *Leptosphaeriaceae* (2%), *Lasiosphaeriaceae* (1.5%) and *Hypocreales family Incertae sedis* (1%) made up less than 1% of the total community. Community composition at the family level did not vary in relation to metal content.

The most abundant genus across all the samples combined was *Gibberella* from the family *Nectriaceae*, representing 18% of total reads (Fig. 2d). Metal content had no statistically significant effect on genus level composition of the community (Supplementary Table S3).

Linear regression analysis showed that metal content had no significant effect on fungal diversity. Similarly, the other soil parameters (pH, soil texture, etc) did not influence the fungal diversity (Supplementary Fig. S2). The effect of metal content on fungal richness and diversity was further evaluated using multiple regression analyses. Metal content (i.e. Zn, Pb and Cu) did not show any correlation with OTU richness and with diversity indices (Supplementary Fig. S3).

Fungal diversity in the high metal samples was no lower than the normal metal samples (Supplementary Fig. S2). The heat map analysis of the 50 most abundant OTUs did not show any consistent difference between high metal and low metals sites, despite the difference of metal concentrations (Supplementary Fig. S4).

Indicator species analysis revealed fungal OTUs that sort between low and high metal sites. The OTUs classified as core community in the low metal samples were represented by 25 genera, and characterized by the genera *Phoma* (represented by the species *Phoma bulgarica*, *Phoma calidophila*, and *Phoma sp P31E4*), *Preussia*, *Giberella*, etc. There were 30 genera classified as core community in high metal samples represented most abundantly by an unclassified fungal genus previously detected in eastern US forest soils by [44] under the name species *fungal sp. 38 CC 06 28*. Also members of the genera *Cochliobolus*, *Curvularia* and *Chaetomium* are abundant examples of the core community of the metal rich sites (Table 1).

Vegetation cover (w=44.5, *P*=0.33) and plant species composition (t=-2.005, df=6.65, *P*=0.08) did not differ in relation to any measured soil characteristic among the 30 quadrat samples which had vegetation data (Supplementary Fig. S5). Multiple regression analysis showed that vegetation cover was not correlated with either Zn soil content, or Cu soil content (*P*>0.05), whereas it significantly correlated with Pb soil content (Supplementary Fig. S6).

An RDA (Fig. 3) showed that metal concentration (Zn, Cu, Pb individually, or all three combined) was the strongest predictor of variation in fungal community composition among our samples. Higher metal content samples (defined in Fig 3 as >200 mg/Kg of Zn) tended to cluster separately in terms of fungal community composition (Fig 3). Together with two axes on the biplot, in an accumulative variance for the interaction between communities and variables, a total of 19.3% of variation was explained. Axis 1 explained 12.2% of the variation in the data, while axis 2 explained 15.8. Among the measured physico-chemical factors, Zn (pseudo-F=3.7, *P*=0.001), Pb (pseudo- =1.3, *P*=0.05) and silt (pseudo-F=2.0, *P*=0.001) were significant contributors to fungal community variability, and a forward test indicated that the most important factor was Zn.

ANOSIM performed on Bray-Curtis community matrix confirmed that samples from low metal content sites and high metal content sites varied significantly from each other (Global R=0.48; *P*=0.001). The Mantel test showed no effect of spatial distance on the composition of fungal communities amongst the sites (Mantel statistic *r*=0.091, *P*=0.15).

Fungal taxonomic functional analysis by FUNGuild categorized the fungal sequences into different trophic modes. 34% of all reads were identified as pathotroph, followed by pathotroph-symbiotroph (23%), saprotroph (19%), pathotroph-saprotroph (7%), and less than 1% for symbiotroph, saprotroph-symbiotroph and pathogen-symbiotroph (Fig. 4a). In this functional study, there was only significant difference in pathogen-symbiotroph (that represents less than 1% of total trophic modes) between the normal and high rich metal samples. There was no significant difference in trophic strategy composition between samples having normal metal content and samples having high metal content (Fig. 4b).

4 Discussion

4.1 *Community characteristics and comparison with other arid environments*

The soil fungal community across all our sampled sites in the Gobi was dominated by Ascomycota, with a much lower abundance of Basidiomycota and other phyla (Fig. 2a). This bias towards Ascomycota is typical of arid environments globally – whereas Basidiomycota normally dominate in forest soils [27, 45-48]. Within the phylum Ascomycota, the most abundant family across all samples was *Nectriaceae* (18% of total reads), which includes a number of common pathogens, but also saprobes [49]. Within this family, the genera *Gibberella* and *Fusarium* which contain both plant pathogens and saprobes [50-54] were at 18% and 1% relative abundance, respectively (Fig. 2d). This again is typical of arid environments: for example *Fusarium* is generally one of the

commonest fungi in desert environments globally, and members have often been found to be abundant in the presence of metals [55, 56].

The family *Pleosporaceae* (Ascomycota) was also very abundant in our samples (17% of total reads). Members of this group are typically necrotrophic pathogens and saprobes, especially associated with grasses [49]. Within the family, the genera *Mycocentrospora* (11% of total reads) and *Alternaria* (10% of total reads) were particularly abundant in these Gobi sites. *Alternaria* has also been isolated from the metal-rich soils elsewhere [56, 57]. *Mycocentrospora* forms chlamydospores which have thick walls for surviving in extreme environments, a feature that may allow it to live in the very variable water environment of the Gobi [57].

The most abundant family of Basidiomycota in our samples was *Filobasidiaceae*, belonging to the order *Filobasidiales* and the class *Tremellomycetes*. Their mean relative abundance was 4.8%, and they made up most of the Basidiomycota in these samples (the Basidiomycota averaging in total at 7% of reads). Genus *Cryptococcus* under family *Filobasidiaceae* is also known to exhibit tolerance to Cu and Zn [48]. *Filobasidiaceae* have been isolated from Antarctic ecosystems and have a very wide range of habitats. The relative abundance of Basidiomycota was much less than in typical samples in forested or damper environments, but typical of semi-arid and arid locations: generally Basidiomycota are less abundant in hot desert environments [49].

In terms of trophic guilds from FUNGuild, pathotrophism was the most abundant category with 34% of total fungal reads, although this may reflect the difficulties of guild assignment in very diverse genera such as *Giberella* and *Fusarium* known to contain saprobes [58]. Pathotroph-symbiotrophism was the second abundant trophic mode with 23% of total reads. Saprotrophism was the third most abundant category overall, at 19% of total reads, which agrees with the family level results discussed above. The spore-forming habit of many saprotrophs may allow their

295 survival in mostly dry soils without any physiological activity [58, 59].The least abundant trophic
296 category was saprotroph-symbiotroph, accounting for only 0.2% of reads (Fig. 4).
297
298 **4.2** ***Community patterns in relation to metal content***
299 In the RDA for the Gobi plots, the community divides very clearly into two clusters (Fig. 3). There
300 is clear tendency in the RDA for the metal rich samples (average Zn concentration in metal samples
301 of 225.44 mg/kg, 1500 mg/kg Cu and 70.39 mg/kg Pb) to cluster on one side of the ordination
302 diagram, with variation in fungal community composition mainly related to metal concentrations
303 (Fig. 3), and with Zn and Pb as the strongest predictors.
304 The RDA analysis shows a strong consistent pattern in relation to metals, especially Zn and Pb.
305 Despite the clear differentiation of the fungal community by soil metal content on the RDA
306 ordinations, at the broad taxonomic level the composition of the metal-poor and the metal-rich
307 communities at the phylum, class and family level is very similar (Fig. 2). The same dominant
308 genera are also found in all samples, both metal-rich and normal (Fig. 2d). Comparing the heat
309 maps of metal-poor and metal-rich samples, there is no clearly evident community difference in
310 terms of the 50 most abundant OTUs shown (Supplementary Fig. S4). The same major OTUs are
311 present across both sample sets. Whatever the differences that lead to the high metal samples
312 clustering separately, they presumably involve either the overall effect of many rarer OTUs, or
313 consistent but subtle differences in the abundance of both common and rare OTUs. These OTUs
314 include the ‘indicator’ taxa mentioned above.
315 It is of course necessary to ask if the close relationship we observed between metal concentrations
316 and fungal community composition is merely spurious, with these factors inter-correlating with
317 some other soil parameters that actually play the important role in determining soil fungal

community composition. Since a broad range of soil parameters was measured (Supplementary Table S2) and Zn, Pb and Cu were by far the best predictors of fungal community variation (Fig. 3), it seems unlikely that metal concentrations are merely a proxy for other soil parameters. This agrees with is a generally accepted view that high metal concentrations may lead to alteration of soil microbial community structure [61, 62].

The 'high' metal concentrations (Zn, Pb and Cu) seen here in some of our samples are not exceptionally high compared to some contaminated sites studied in Europe which have had around 10 times this concentration [63]. However, in their experimental studies Smolders et al. (2004) observed effects of Zn on soil microbial (mostly bacterial) activity starting at concentrations around 200 mg/kg of Zn, similar those we observed, which suggests that at the concentrations found in the Gobi sites, microbial ecology could be significantly affected by Zn and other metals. A background of previous work also suggests that soil fungi are especially susceptible to high Zn concentrations, compared to bacteria. A study by Speir *et al.* [65] found that increasing Zn concentrations between 0 and 400 mg/kg had a significant negative impact on enzymatic activities of soil fungi (in our study the higher metal sites had around 200 mg/kg of Zn). Soil fungal communities have been found to be more responsive to Zn than soil bacteria are [60, 66]. Most of the samples that cluster at the higher end in terms of Zn concentrations greatly exceed environmental tolerance guidelines for Zn (EPA, Eco-SSL database: http://www.epa.gov/ecotox/ecossl/index.html, Supplementary Table S2) and the concentration limits reported in Kabata-Pendias [67] who stated that the threshold upper limit value of Cu in surface soils should be around 100 ppm, whereas our most Cu rich soils have concentrations of an average of 1475 ppm . They also noted that upper limit for the Pb content is around 70 ppm whereas our most Pb rich soils have an average concentrations of 66 ppm, and the mean Zn for worldwide

soils is around 64 ppm, whereas our most of our Zn soils have concentrations of an average of 224 ppm. This is certainly suggestive that toxicity may have a role in selecting the distinctive community that is found in these places. In some previous studies, soil fungi were found to be more sensitive to Cu than Zn and tended to have tolerance of Zn [68, 69].

It is also necessary to consider the possibility that the summative effect of multiple metals in the soil may be having the observed effect on fungal communities. It may also be important that the metals in our sampled soils tend to co-occur at higher concentrations. In recent years, there has been growing awareness of the interactive effects that multiple metals may have on soil ecology. This phenomenon, known as mixture toxicity, takes place where synergies between metals arise [70, 71]. Synergistic effects occur when the combined effect of two metals is greater than the sum of the effect of each metal individually. For synergistic interactions to occur in the soil, interacting metals have both to co-occur and to be present at concentrations high enough to induce the synergy [72, 73]. It is plausible, given that Zn, Pb and Cu in our soils are strongly co-occurring, there is a synergistic effect at work here in affecting the soil community.

An important proviso on the case that metal ions are a major direct factor in the fungal ecology of this area, however, is that the soil pH in the metal-rich areas is high, and that this is predicted to limit bioavailability of metal ions [74, 75]. In general – based on idealized laboratory observations of the chemistry and solubility of metal salts - each unit increase in pH is forecast to result in halving of available concentrations [75-78]. As the average pH of the soils which contained high metals was 8.66, having a pH range between 8.43 to 8.87 (Supplementary Table S2), the actual bioavailability would be predicted to be lower than in neutral or acidic soils [79, 80]. Exactly how much soil pH affects metal ion availability in actual soils is uncertain: Smolders *et al.* [63] found that over a range of pH from 4.5 to 7.5, Zn toxicity effects on soil microbes were

unaffected by pH, suggesting that the theoretical limits are inaccurate. All that one can really state is that while correlation suggests that Zn, Cu and Pb are affecting the fungal communities in the Gobi, traditional inorganic chemistry predicts that their effects are weaker than they would be if soil pH were more acidic. Protection against toxic effects of metals in soils can also occur through various other mechanisms: for example organic matter content, clay content and iron oxide content [78, 81-83], and it is possible that these too affect the biological availability of Zn and other metals in the soil.

4.3 Community patterns in relation to vegetation

Vegetation composition and percentage coverage did not correlate with fungal community composition in the sampled areas. Despite the apparent effects of these metals on the fungal community, the visual appearance of the Gobi ecosystem in the high metal areas does not suggest intense toxicity by metals. In the 30 samples which had vegetation data, the plant diversity and vegetation coverage of the most metal-rich 1 ha plots we obtained from the Gobi was no less than the 1 ha plots with normal metal concentrations, suggesting that: 1) the plants themselves have evolved local metal-tolerant ecotypes, 2) there might be evolutionary adaptation by the soil biota, perhaps in sequestering metal ions (and possibly making these metal ions unavailable to the plants in the process) or 3) due to high pH in limiting availability, the effects of high Zn or Cu or Pb concentrations are marginal in terms of plant growth and ecosystem function, despite a subtle effect that can be detected in ordination of the fungal community.

4.4 *High diversity of the metal-rich soil fungal communities*

Despite the high concentrations of metals in some of the soils, and the apparently dominant role of metal concentrations in determining variation in fungal community structure, the diversity of soil fungi in the metal-rich 1 ha plot samples was no less than in the local soils with normal background levels of metals (Supplementary Fig. S2).

Extreme environments are usually seen as being associated with lower diversity [84]. This is the case for example with soil bacterial communities of metal-polluted soils, and of extreme high and low pH conditions [85-88]. However, in a previous study of the effects of application of sewage sludge rich in metals, similarly high metal concentrations in soils still only showed minor effects on microbial diversity [60].

In the system we are studying here, there seems to be no association between diversity and 'extreme' conditions (high Zn, Cu and Pb concentrations), for soil fungi at least. It is possible that the high soil pH acts as a protectant against the worst effects of the metal ions on cell physiology preventing the diversity-suppressing effects normally associated with an extreme environment. Relatively low bioavailability could explain why fungal diversity is comparable with the other soil samples with normal background concentrations of these metals, even though the metal concentration is apparently having enough biological effect to make it a strong predictor of community variation. An alternative, or additional, explanation for why these metal-rich soils remain high in fungal diversity is that high metal concentrations in this area have existed for long geological periods, allowing a very diverse soil fungal community to build up.

4.5 *Are the high metal concentrations natural or influenced by anthropogenic activity?*

The source of the Zn and also the Cu and Pb found in many of the sites we sampled is unclear. Most of our more metal-rich sites are found across a broad radius south-east of the Oyu Tolgoi

mine site, and the predominant wind direction in the area is north-west to south-east [89], which could imply that dust from the mine is the source. However, this seems unlikely, as the concentration of Zn, Cu and Pb does not show any relationship with distance from the mine, with some of the most metal rich samples being 20-80 km or more away from the mine, in areas never before mined. Scattered amongst the metal rich sites were also other sites that have normal background levels of metals, which would not be expected if an extensive metal-rich dust plume was spreading out across the desert. Also, the mine had only recently opened, producing its first ore in 2013 which was the year of sampling [21], which further implies that it is unlikely to have provided such extensive contamination of Zn, Cu and Pb over such a large area, and to have produced any noticeable changes in a fungal community averaged through the sampling depth of 0-10 cm . Additionally, a further set of very metal rich samples are found 30-40 km north-east of the mine, well upwind from the mine.

The most plausible explanation is that the high soil metal concentrations we observe here in the south eastern Gobi are natural, a consequence of the geological enrichment of rocks in this area with metal ores [90, 91], followed by weathering to form metal-rich soils.

5 Conclusions

The area around Oyu Tolgoi appears to be an unusual system, with naturally high soil concentrations of Zn, Cu and Pb in many areas [90, 91]. The strength of correlation suggests that Zn in particular dominates the community composition of soil fungi in this area, although Cu and Pb might also play an important part since they also tend to occur at high concentrations in the same Zn-rich areas. Despite the high metal concentrations, diversity of fungi in the metal-rich

areas is as high as in areas with normal background metal concentrations, which suggests the possibility of a long history of specialized adaptation by the soil biota.

As a naturally metal-rich system, the soil ecology of Oyu Tolgoi area deserves further study. It appears to offer a natural analog to anthropogenic metal-contaminated sites associated with industrial activity around the world. Globally, there are very few known examples of naturally metalliferous soil, the few exceptions being serpentine rock outcrops (rich in Ni), and Zn-rich sites in central Europe [5, 92] – but even these are much more localized in extent than the Gobi area that we studied. To our knowledge, no naturally metalliferous soil has ever been thoroughly investigated from a microbial viewpoint. It would be interesting to know what (if any) special adaptations the fungi in these metal-enriched soils have to the presence of metal ions, and whether the distinctive community composition also extends to other soil organisms such as bacteria, soil metazoans and archaea. Further studies should also include the soil metagenome, its metatranscriptome, and soil properties such as potential soil respiration rate. Such aspects could then be compared to those of anthropogenically contaminated sites, for potential lessons in terms of the processes of community adaptation over time, and practical guidance for rehabilitation of contaminated land.

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Figure legends

Fig. 1 Metal (Zn, Cu and Pb) contents in low metal samples and high metal samples. High metal is defined by cutoff concentrations of 50 mg/kg in Zn, Pb and Cu.

Fig. 2 Relative abundance of the detected fungal taxa observed in the Gobi samples (a) at the phylum level, (b) class level, (c) Family level and (d) genus level.

Fig. 3 Redundancy Analysis (RDA) ordination plot of fungal community composition based on ITS1 gene OTUs and a vector overlay of the environmental variables. The significant environmental variables were shown in red arrows. Red dots denote samples having low heavy metal contents and blue dots denote samples having high heavy metal contents.

Fig. 4 Detected fungal tropic mode by FUNGuild. (a) Classified trophic modes and (b) Relative abundance of each trophic mode.

Table legend

Table 1 Results of indicator species analysis.