

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

Article

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1 **Metal tolerant fungal communities are delineated by high zinc, lead and copper**
2 **concentrations in metalliferous Gobi desert soils**

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28

29 **Conflict of interest:**

30 The authors declare no conflict s of interest.

31 **Abstract**

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

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

46 **1 Introduction**

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

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

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

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

92

93 2 Methodology

94 2.1 Site description

95 The sampling area (Supplementary Fig. S1) is located in south-eastern Mongolia, close to the
96 border with China, between the latitudes of 42°31 N to 43°36 N and longitudes of 106°34 E to
97 108°10 E. Samples were taken in mid-September 2013, towards the end of the vegetation growing
98 season.

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

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

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

119

120 **2.2 Chemical Analysis and DNA extraction**

121 Soil analyses were carried out in the Laboratory of Soil Science of the Institute of Geography,
122 Mongolian Academy of Sciences, using standard protocols of the Soil Science Association of
123 America (SSSA) [23]. Measured chemical parameters were pH, CaCO₃, total organic carbon
124 (TOC), soil salinity (measured as electric conductivity, dS/m), nitrogen (N), soil texture (TX),
125 chrome (Cr), lead (Pb), cadmium (Cd), copper (Cu) and zinc (Zn). All samples were transported
126 to the laboratory at Oyu Tolgoi mine and frozen at -20°C within 3 hours of sampling. Within days
127 frozen soils were processed for DNA extraction in the Laboratory of Ecological and Evolutionary
128 Synthesis of the National University of Mongolia. Soil was sieved with a 3 mm sieve, and 0.35 g
129 of the sieved soil was DNA extracted using the Power Soil DNA extraction kit (MoBio
130 Laboratories, Carlsbad, CA, USA) according to the manufacturer's protocol.

131

132 **2.3 PCR and sequencing**

133 All the extracted DNA samples were amplified for ITS1 region by using the primer pair ITSIF (5'-
134 CTTGGTCATTTAGAGGAAGTAA-3') and ITS2 (5'-GCTGCGTTCTTCATCGATGC-3') [24,
135 25]. Polymerase chain reactions (PCR) were performed in 50 µl reactions using the following

136 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;
137 and 72°C for 7 min. The resulting amplicons were purified using the QIAquick PCR purification
138 kit (Qiagen, CA, USA), and sequenced using Illumina Miseq platform (paired end of 2 × 300) at
139 Macrogen Incorporation (Seoul, Korea), following standard Illumina sequencing protocols [26].

140

141 **2.4 Sequence processing**

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

152

153 **2.5 Statistical analysis**

154 To perform the statistical analysis, all samples were standardized by random subsampling to
155 13,957 reads per sample. We used a *t-test* for normal data and *Wilcoxon rank-sum test* for non-
156 normal data in R software package 2.15.2 to test whether the relative abundances of taxonomical
157 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

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

184

185 **3 Results**

186 The soils in all sites were alkaline, (mean pH 8.7; range pH 8.2 – 9.3), and contained on average
187 56% of sand, 34% of silt and 11% of clay. Results of soil physico-chemical analysis are shown in
188 Supplementary Table S2. In several samples, Zn metal concentrations exceeded EPA, Eco-SSL
189 regulation (<http://www.epa.gov/ecotox/ecossl/index.html>) levels of 50 mg/kg, reaching 233 mg/kg
190 in some samples. Cu and Pb contents were also above Eco-SSL, EPA level of 50 mg/kg in some
191 samples, the highest metal concentrations measured were around 2300 mg/kg for Cu and 130
192 mg/kg for Pb (Fig. 1). In combination, the combined Pb, Zn and Cu concentration averaged around
193 689.78 mg/kg (ranging from 54.7 mg/kg to 3061 mg/kg).

194 From the 34 soil DNA samples, we obtained a total of 2,459,044 quality reads, which were
195 classified into 11,559 OTUs at the 97% similarity level. In presentation of results, we have
196 distinguished ‘high metal concentration’ samples as those containing >50 ppm Zn, Cu and/or Pb.

197 The fungal community averaged across all the samples was dominated by Ascomycota, with 79%
198 of total reads (Fig. 2a). Basidiomycota was the next most abundant group with 7% of the total
199 reads. Relative abundance of other phyla was under 1%. There was no significant difference at the
200 phylum level when high metal samples and low metal samples were compared, with the exception
201 of certain minor phyla representing less than 1% of total reads - including Blastocladiomycota
202 ($w=77$, $P=0.02$), Chytridiomycota ($w=198$, $P=0.007$), Incertae sedis ($w=62$, $P=0.01$) and
203 Streptophycophyta ($w=172$, $P=0.01$).

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

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

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

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

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

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

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

240 An RDA (Fig. 3) showed that metal concentration (Zn, Cu, Pb individually, or all three combined)
241 was the strongest predictor of variation in fungal community composition among our samples.
242 Higher metal content samples (defined in Fig 3 as >200 mg/Kg of Zn) tended to cluster separately
243 in terms of fungal community composition (Fig 3). Together with two axes on the biplot, in an
244 accumulative variance for the interaction between communities and variables, a total of 19.3% of
245 variation was explained. Axis 1 explained 12.2% of the variation in the data, while axis 2 explained
246 15.8. Among the measured physico-chemical factors, Zn (pseudo- $F=3.7$, $P=0.001$), Pb (pseudo-
247 $=1.3$, $P=0.05$) and silt (pseudo- $F=2.0$, $P=0.001$) were significant contributors to fungal
248 community variability, and a forward test indicated that the most important factor was Zn.

249 ANOSIM performed on Bray-Curtis community matrix confirmed that samples from low metal
250 content sites and high metal content sites varied significantly from each other (Global R=0.48;
251 $P=0.001$). The Mantel test showed no effect of spatial distance on the composition of fungal
252 communities amongst the sites (Mantel statistic $r=0.091$, $P=0.15$).

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

261

262 **4 Discussion**

263 **4.1 Community characteristics and comparison with other arid environments**

264 The soil fungal community across all our sampled sites in the Gobi was dominated by Ascomycota,
265 with a much lower abundance of Basidiomycota and other phyla (Fig. 2a). This bias towards
266 Ascomycota is typical of arid environments globally – whereas Basidiomycota normally dominate
267 in forest soils [27, 45-48]. Within the phylum Ascomycota, the most abundant family across all
268 samples was *Nectriaceae* (18% of total reads), which includes a number of common pathogens,
269 but also saprobes [49]. Within this family, the genera *Gibberella* and *Fusarium* which contain both
270 plant pathogens and saprobes [50-54] were at 18% and 1% relative abundance, respectively (Fig.
271 2d). This again is typical of arid environments: for example *Fusarium* is generally one of the

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

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

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

289 In terms of trophic guilds from FUNGuild, pathotrophism was the most abundant category with
290 34% of total fungal reads, although this may reflect the difficulties of guild assignment in very
291 diverse genera such as *Giberella* and *Fusarium* known to contain saprobes [58]. Pathotroph-
292 symbiotrophism was the second abundant trophic mode with 23% of total reads. Saprotriphism
293 was the third most abundant category overall, at 19% of total reads, which agrees with the family
294 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

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

323 The 'high' metal concentrations (Zn, Pb and Cu) seen here in some of our samples are not
324 exceptionally high compared to some contaminated sites studied in Europe which have had around
325 10 times this concentration [63]. However, in their experimental studies Smolders et al. (2004)
326 observed effects of Zn on soil microbial (mostly bacterial) activity starting at concentrations
327 around 200 mg/kg of Zn, similar those we observed, which suggests that at the concentrations
328 found in the Gobi sites, microbial ecology could be significantly affected by Zn and other metals.
329 A background of previous work also suggests that soil fungi are especially susceptible to high Zn
330 concentrations, compared to bacteria. A study by Speir *et al.* [65] found that increasing Zn
331 concentrations between 0 and 400 mg/kg had a significant negative impact on enzymatic activities
332 of soil fungi (in our study the higher metal sites had around 200 mg/kg of Zn). Soil fungal
333 communities have been found to be more responsive to Zn than soil bacteria are [60, 66]. Most of
334 the samples that cluster at the higher end in terms of Zn concentrations greatly exceed
335 environmental tolerance guidelines for Zn (EPA, Eco-SSL database:
336 <http://www.epa.gov/ecotox/ecossl/index.html>, Supplementary Table S2) and the concentration
337 limits reported in Kabata-Pendias [67] who stated that the threshold upper limit value of Cu in
338 surface soils should be around 100 ppm, whereas our most Cu rich soils have concentrations of an
339 average of 1475 ppm. They also noted that upper limit for the Pb content is around 70 ppm whereas
340 our most Pb rich soils have an average concentrations of 66 ppm, and the mean Zn for worldwide

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

345 It is also necessary to consider the possibility that the summative effect of multiple metals in the
346 soil may be having the observed effect on fungal communities. It may also be important that the
347 metals in our sampled soils tend to co-occur at higher concentrations. In recent years, there has
348 been growing awareness of the interactive effects that multiple metals may have on soil ecology.
349 This phenomenon, known as mixture toxicity, takes place where synergies between metals arise
350 [70, 71]. Synergistic effects occur when the combined effect of two metals is greater than the sum
351 of the effect of each metal individually. For synergistic interactions to occur in the soil, interacting
352 metals have both to co-occur and to be present at concentrations high enough to induce the synergy
353 [72, 73]. It is plausible, given that Zn, Pb and Cu in our soils are strongly co-occurring, there is a
354 synergistic effect at work here in affecting the soil community.

355 An important proviso on the case that metal ions are a major direct factor in the fungal
356 ecology of this area, however, is that the soil pH in the metal-rich areas is high, and that this is
357 predicted to limit bioavailability of metal ions [74, 75]. In general – based on idealized laboratory
358 observations of the chemistry and solubility of metal salts - each unit increase in pH is forecast to
359 result in halving of available concentrations [75-78]. As the average pH of the soils which
360 contained high metals was 8.66, having a pH range between 8.43 to 8.87 (Supplementary Table
361 S2), the actual bioavailability would be predicted to be lower than in neutral or acidic soils [79,
362 80]. Exactly how much soil pH affects metal ion availability in actual soils is uncertain: Smolders
363 *et al.* [63] found that over a range of pH from 4.5 to 7.5, Zn toxicity effects on soil microbes were

364 unaffected by pH, suggesting that the theoretical limits are inaccurate. All that one can really state
365 is that while correlation suggests that Zn, Cu and Pb are affecting the fungal communities in the
366 Gobi, traditional inorganic chemistry predicts that their effects are weaker than they would be if
367 soil pH were more acidic. Protection against toxic effects of metals in soils can also occur through
368 various other mechanisms: for example organic matter content, clay content and iron oxide content
369 [78, 81-83], and it is possible that these too affect the biological availability of Zn and other metals
370 in the soil.

371

372 ***4.3 Community patterns in relation to vegetation***

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

384

385 ***4.4 High diversity of the metal-rich soil fungal communities***

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

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

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

405

406 **4.5** *Are the high metal concentrations natural or influenced by anthropogenic activity?*

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

409 mine site, and the predominant wind direction in the area is north-west to south-east [89], which
410 could imply that dust from the mine is the source. However, this seems unlikely, as the
411 concentration of Zn, Cu and Pb does not show any relationship with distance from the mine, with
412 some of the most metal rich samples being 20-80 km or more away from the mine, in areas never
413 before mined. Scattered amongst the metal rich sites were also other sites that have normal
414 background levels of metals, which would not be expected if an extensive metal-rich dust plume
415 was spreading out across the desert. Also, the mine had only recently opened, producing its first
416 ore in 2013 which was the year of sampling [21], which further implies that it is unlikely to have
417 provided such extensive contamination of Zn, Cu and Pb over such a large area, and to have
418 produced any noticeable changes in a fungal community averaged through the sampling depth of
419 0-10 cm . Additionally, a further set of very metal rich samples are found 30-40 km north-east of
420 the mine, well upwind from the mine.

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

424

425 **5 Conclusions**

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

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

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

447

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454

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681 **Figure legends**

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

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

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

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

692 **Table legend**

693 **Table 1** Results of indicator species analysis.