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## Contrasted microbial community colonization of a bauxite residue deposit marked by a complex geochemical context

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1 ***“Contrasted Microbial community colonization of a bauxite residue deposit***  
2 ***marked by a complex geochemical context.”***

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36 **Keywords:** Bauxite residue, Primary succession, Microbial communities,  
37 Physicochemical characterization, Critical metals.

38

39 **Abstract**

40 Bauxite residue is the alkaline byproduct generated during alumina extraction and is  
41 commonly landfilled in open-air deposits. The growth in global alumina production  
42 have raised environmental concerns about these deposits since no large-scale reuses  
43 exist to date. Microbial-driven techniques including bioremediation and critical metal  
44 bio-recovery are now considered sustainable and cost-effective methods to revalorize  
45 bauxite residues. However, the establishment of microbial communities and their  
46 active role in these strategies are still poorly understood. We thus determined the  
47 geochemical composition of different bauxite residues produced in southern France  
48 and explored the development of bacterial and fungal communities using Illumina  
49 high-throughput sequencing. Physicochemical parameters were influenced differently  
50 by the deposit age and the bauxite origin. Taxonomical analysis revealed an early-  
51 stage microbial community dominated by haloalkaliphilic microorganisms and strongly  
52 influenced by chemical gradients. Microbial richness, diversity and network complexity  
53 increased significantly with the deposit age, reaching an equilibrium community  
54 composition similar to typical soils after decades of natural weathering. Our results  
55 suggested that salinity, pH, and toxic metals affected the bacterial community  
56 structure, while fungal community composition showed no clear correlations with  
57 chemical variations.

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## 69 1. INTRODUCTION

70 Bauxite residue is a solid by-product generated during the production of alumina from  
71 bauxite. Alumina extraction is most often performed by the Bayer process, where  
72 bauxite is digested with large quantities of sodium hydroxide at temperatures between  
73 150 and 250 °C (Evans, 2016). Depending on the process parameters and the origin  
74 of the bauxite, 0.7 to 2 tons of bauxite residues are produced per ton of alumina  
75 (International Aluminium Institute and European Aluminium, 2015). Over the last 10  
76 years, the average annual production of alumina was  $116 \pm 15$  Mt (World Aluminium,  
77 2020), which corresponds to ~200 Mt of bauxite residue produced per year. In the  
78 absence of economically profitable large-scale applications, bauxite residues are  
79 commonly landfilled in large open-air deposit areas (BRDA), reaching a total of 4.5 Bt  
80 bauxite residue in storage at the present time (Dentoni et al., 2021).

81 Due to the physicochemical characteristics of bauxite residue i.e., high alkalinity, high  
82 salinity, high metal content and lack of nutrients, BRDAs pose a challenge to most  
83 living organisms (Di Carlo et al., 2020; Santini et al., 2015b) and represent a potential  
84 source of contamination for the surrounding terrestrial and aquatic ecosystems  
85 (Bouchoucha et al., 2019; Ren et al., 2018). Consequently, efforts have been made to  
86 remediate these deposits, usually by the addition of amendments (e.g. gypsum and  
87 organic matter) that attenuate their harsh conditions prior to the revegetation of the  
88 area (Bray et al., 2018; Khaitan et al., 2010). BRDAs have also been considered as a  
89 promising secondary source of valuable and critical metals, as these elements are  
90 concentrated by a factor of 2 in bauxite residues compared to the initial bauxite ore  
91 (Panda et al., 2021; Ujaczki et al., 2017; Vind et al., 2018). Critical metals are chemical  
92 elements characterized by their high economic relevance and supply risk (European  
93 Commission, 2020) and include metals crucial for information and energy technologies  
94 such as Co, Mg, Ba, V, Ge, Nb, Sr, Ga, platinumoids (PGMs), and rare earth elements  
95 (REEs: Lanthanides (Ln), Y, Sc).

96 Recently, microbial-driven approaches have gained attention as viable and cost-  
97 effective methods for the management and valorization of urban and industrial wastes,  
98 including bauxite residue (Lyu et al., 2021; Panda et al., 2021; Santini et al., 2019).  
99 During in-situ remediation processes, native microbial communities have been shown  
100 to decrease the pH and salinity of the bauxite residue, as well as play an essential role  
101 in soil formation and plant growth (Di Carlo et al., 2019; Tian et al., 2020; Wu et al.,

102 2019). Microbial metal recovery strategies are also promising for the dissolution and  
103 recovery of elements of interest (e.g., Fe, Al, critical metals) from waste (Baniasadi et  
104 al., 2019; Dev et al., 2020; Maes et al., 2016). Out of all these techniques, bioleaching  
105 and microbial electrochemistry are among the most-studied methods (Dominguez-  
106 Benetton et al., 2018; Srichandan et al., 2019). In bauxite residues, these techniques  
107 are still at an early experimentation stage, although some promising results for REE  
108 recovery by bioleaching have been reported (Kiskira et al., 2021; Qu et al., 2019;  
109 Zhang et al., 2020).

110 More generally, microbial-driven strategies for waste management imply the use of  
111 microbial communities native to contaminated sites, which tend to thrive better in such  
112 harsh environments due to their unique metabolisms developed through natural  
113 selection (Ghosh et al., 2018; Ma et al., 2019; Roy et al., 2018; Sajjad et al., 2020). In  
114 addition, microbial communities are known to perform complex functions and are more  
115 robust to environmental fluctuations compared to pure cultures (Perez-Garcia et al.,  
116 2016; Wang et al., 2020; Zhang et al., 2008). Metagenomics studies based on Next  
117 Generation Sequencing have significantly expanded the identification and dynamics  
118 of microbial communities involved in different bioprocesses. However, the evolution of  
119 microbial communities in metal biorecovery experiments is still limited to a few weeks  
120 (Ma et al., 2017; Sajjad et al., 2020; Wang et al., 2020). Regarding bauxite residues,  
121 the identification of pioneer microbial communities and their dynamics during primary  
122 succession have been highlighted as research needs to improve microbial-driven  
123 bioremediation (Santini et al., 2015a). Nevertheless, to date, studies on microbial  
124 diversity in bauxite residue have focused on the responses of bacterial communities  
125 to remediation strategies, overlooking their active role in the process (Banning et al.,  
126 2011; Fourier et al., 2020; Ke, 2021; Krishna et al., 2014; Schmalenberger, 2013; Wu  
127 et al., 2020).

128 This study aimed at exploring the microbial dynamics during primary succession in a  
129 BRDA from Southern France using an integrated physicochemical and biological  
130 approach. The specific objectives of this study were to (a) evaluate the effect of the  
131 deposit age and the ore origin on the geochemical characteristics of bauxite residue;  
132 (b) identify the first microbial communities colonizing BRDAs and their role in the  
133 establishment of new species; (c) determine the microbial community structure in the  
134 equilibrium stage of primary succession in BRDAs and the main geochemical factors

135 driving it; (d) explore possible implications of this study for microbial-driven  
136 bioremediation and critical metal recovery.

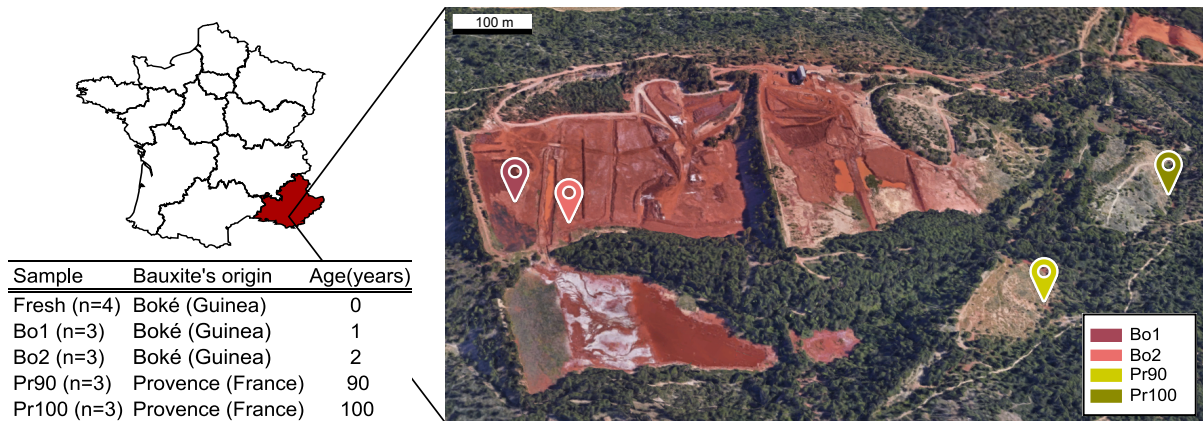
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## 138 **2. MATERIALS AND METHODS**

### 139 2.1 Site description and sampling

140 The bauxite residues come from a refinery operating the Bayer aluminum extraction  
141 process since 1894 in Gardanne, Southern France. The sampling took place at the  
142 Mange-Garri bauxite residue disposal area (BRDA) in Bouc-Bel-Air, Southern France.  
143 This region is characterized by a hot-summer Mediterranean climate, with average  
144 annual precipitation and temperature of 485 mm and 16.3°C (Copernicus Climate Data  
145 Store, 2021). We selected four distinct areas of the BRDA based on their deposit age  
146 (1, 2, 90, and 100 years) and bauxite origin (lateritic bauxite from Boké, Guinea (Bo)  
147 and karstic bauxite from Provence, France (Pr)), named Bo1, Bo2, Pr90 and Pr100  
148 (**Fig. 1**). At the Bo1 and Bo2 sites, bauxite residues were produced from Guinean  
149 bauxite and were landfilled one and two years before sampling respectively. These  
150 deposits are occasionally watered to prevent dust dispersion and were not amended  
151 at the time of sampling. The Pr90 and Pr100 sites contain bauxite residues that were  
152 produced from Provençal bauxite and were deposited around 90 and 100 years ago  
153 respectively. These sites were amended with a soil layer in the 1960s and a slight  
154 coverage with low-lying vegetation can be seen.

155 At each of the four sites, samples were collected in triplicate, gathering approximately  
156 500 g of bauxite residue at a depth of 20 to 30 cm in sterile plastic bags. At sites Pr90  
157 and Pr100, samples were collected at the edge of the deposit, where bauxite residues  
158 were distant from the added soil layer. In addition, freshly produced bauxite residue (4  
159 replicates) was also selected to analyze the initial bio-geochemical characteristics  
160 before landfill. Samples were divided into two groups based on the following analyses.  
161 Samples undergoing physicochemical analysis were oven-dried at 70°C, grounded in  
162 a mortar, passed through a 130 µm sieve, and stored in metal-free plastic tubes.  
163 Samples for microbial community analyses were stored at 4°C.



**Figure 1. Sampling site and samples description.**

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## 165 2.2 Physicochemical analyses

### 166 2.2.1 Elemental composition

167 First, pH and electrical conductivity (EC) were measured at a solid/MilliQ water ratio  
 168 of 1:5. Then, the elemental composition of the bauxite residues was determined after  
 169 alkaline fusion of the samples (Rivera et al., 2019). Briefly, 1 g of each sample was  
 170 digested with 750 mg of lithium tetraborate ( $\text{Li}_2\text{B}_4\text{O}_7$ ) at  $1000^\circ\text{C}$  for 30 min (WiseTherm  
 171 F/FH 0-1200°C) and then immediately dissolved in 40 mL of  $\text{HNO}_3$  1N. Prior to  
 172 elemental analysis, the samples were dissolved 200 times. Subsequently, inductively  
 173 coupled plasma mass spectrometry (ICP-MS, Perkin Elmer Nexlon 300X) was used  
 174 to analyze elements with concentrations up to  $1000 \mu\text{g/L}$ , while elements with  
 175 concentrations above  $1000 \mu\text{g/L}$  were analyzed by inductively coupled plasma optical  
 176 emission spectroscopy (ICP-OES, Perkin Elmer 4300 DV). All analyses were  
 177 conducted for major and trace elements commonly found in bauxite residues, namely  
 178 Fe, Ti, Al, Ca, Zr, Cr, Mn, Zn, Th, Ni, Pb, Cu, Sn, Rb, Cs as well as critical raw elements  
 179 (as defined by the European Commission (2020): P, Mg, V, Lanthanides (Ln), Sr, Y,  
 180 Nb, Ba, Sc, Ga, Hf, Ge, Co, Sb, W, Ta). Total carbon (TC) and nitrogen were  
 181 determined by dry combustion using an Elemental Analyzer (Flash EA, Thermo  
 182 Scientific). To measure the total organic carbon (TOC), samples were treated with HCl  
 183 to remove carbonates before analysis.

### 184 2.2.2 Mineralogical composition

185 The mineralogical analyses of bauxite residue were carried out by X-ray diffraction  
 186 (XRD) using a PANalytical X'Pert Pro (Malvern Panalytical, UK) diffractometer  
 187 equipped with a cobalt tube ( $\lambda = 1.79 \text{ \AA}$ ) running at 40kV and 40mA. Samples were

188 deposited on low background silicon plates and analyzed from 8° to 80° (2θ) with a  
189 step size of 0.033° and a total counting time of 7 hours. Samples were also spun at  
190 15 rpm to improve statistics. Phase identification was performed using the X'pert  
191 Highscore plus software (PANalytical) together with the PDF-2 ICDD database  
192 (International Center for Diffraction data, Powder Diffraction Files 2). Profex software  
193 (Doebelin and Kleeberg, 2015) was used for Rietveld refinement to semi-quantify the  
194 proportions of the minerals in the bauxite residues. The following parameters were  
195 refined: zero point shift, sample displacement, cell parameters, preferred orientation  
196 and peak broadening resulting from the size of the crystallite and the micro strain.

### 197 2.3 DNA extraction, PCR amplification and sequencing

198 The total DNA was extracted from 5 g of each bauxite residue samples using the  
199 FastDNA® Spin Kit for soil (MP Biomedicals, USA) following the manufacturer's  
200 protocol. Extracted DNA was used as template in separate PCR reactions amplifying  
201 the bacterial 16S and the fungal ITS rRNA gene sequences. For bacterial diversity  
202 analysis, we used the primers 341F (5'-CCTAYGGGRBGCASCAG-3') and 806R (5'-  
203 GGACTACNNGGGTATCTAAT-3'), targeting the V3 and V4 variable regions of the  
204 bacterial 16S rRNA (Caporaso et al., 2011; Muyzer et al., 1993). For fungal diversity  
205 analysis, we used the primers fITS7 (5'-GTGARTCATCGAATCTTTG-3') and ITS4 (5'-  
206 TCCTCCGCTTATTGATATGC-3'), targeting the ITS2 region (Ihrmark et al., 2012).  
207 The amplification conditions were as follows: initial denaturation at 95 °C for 2 min;  
208 followed by 34 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s,  
209 and extension at 72 °C for 1 min; and a final extension at 72 °C for 5 min (Muller et  
210 al., 2021). PCR products were then purified using ProNex® Size-Selective Purification  
211 System (Promega, USA) and sequenced on Illumina MiSeq platform (Biofidal, Vaulx-  
212 en-Velin, France). The raw sequence reads generated from this study have been  
213 deposited in the National Center for Biotechnology Information (NCBI) Sequence  
214 Read Archive under the accession number PRJNA748554.

### 215 2.4 Sequencing data processing

216 Microbiome bioinformatics were performed by the open-source software QIIME2,  
217 version 2019.10 (<https://qiime2.org>) (Bolyen et al., 2019). Raw reads were quality-  
218 filtered, denoised and chimera-checked using DADA2 (Callahan et al., 2016). DADA2  
219 uses a parametric model to infer true biological sequences from reads. The model



220 relies on input read abundances (true reads are likely to be more abundant) and the  
221 pairwise similarity between sequences. The taxonomic annotation of the resulting  
222 sequence variants (ASVs) was assigned using the feature-classifier command with  
223 default parameters in QIIME2 and sequences were matched against the Greengenes  
224 13\_8 database (McDonald et al., 2012). Finally, scaling with ranked subsampling  
225 (SRS) curves (Beule and Karlovsky, 2020) were drawn to determine whether the  
226 sequencing depth was sufficient to represent the true diversity of the samples.

## 227 2.5 Biodiversity parameters and microbial biomarker discovery

228 Alpha diversity was explored through observed ASVs, Chao1, and Shannon. The  
229 observed ASVs and the Chao1 estimator were selected to identify community  
230 richness, and Shannon index was used to assess community diversity (Callahan et  
231 al., 2017; Hill et al., 2003). Beta diversity analysis was used to evaluate distribution  
232 patterns in samples based on bacterial and fungal ASV composition (Anderson et al.,  
233 2011; Callahan et al., 2017). For this purpose, a principal coordinate analysis (PCoA)  
234 based on weighted UniFrac distances (Lozupone et al., 2007) was conducted. All  
235 alpha and beta diversity metrics were calculated using QIIME 2 after normalization to  
236 59388 and 4778 sequences per sample for bacteria and fungi, respectively. Linear  
237 discriminant analysis effect sizes algorithm (LEfSe) (Segata et al., 2011) was  
238 performed on the Galaxy platform (<https://huttenhower.sph.harvard.edu/galaxy/>) to  
239 identify bacterial biomarkers characterizing the samples. LEfSe couples Kruskal–  
240 Wallis tests for measuring statistical significance with quantitative tests for biological  
241 consistency (Wilcoxon rank sum test).

## 242 2.6 Statistical analysis

243 All statistical analyses were performed with the open-source software R (R Core  
244 Team, 2020) using the packages “dplyr” (Wickham et al., 2021), “vegan” (Oksanen et  
245 al., 2020), “car” (Fox and Weisberg, 2019), “ggpubr” (Kassambara, 2020), and “rstatix”  
246 (Kassambara, 2021). Figures were produced with the package “ggplot2” (Wickham,  
247 2009). To study the ore-dependent differences in chemical and microbiological  
248 compositions of bauxite residue, unpaired two-sided T-tests and Wilcoxon rank sum  
249 tests were conducted respectively. Furthermore, Games-Howell post-hoc tests were  
250 used to assess the age-dependent variations in chemical and microbial compositions.  
251 Pearson correlations between each variable and the deposit age were used to further

252 explore these relationships. Also, significant differences in alpha diversity indices were  
253 tested by Wilcoxon rank sum tests.

254 Principal component analysis (PCA) was used to identify the variables that explain  
255 most of the variation in chemical composition. In addition, unsupervised hierarchical  
256 clustering was applied to the PCA to group the samples according to their chemical  
257 similarity. Both PCA and hierarchical clustering were performed using the packages  
258 “FactoMineR” (Lê et al., 2008) and “factoextra” (Kassambara and Mundt, 2020).  
259 PERMANOVA via the *adonis* function was conducted in both PCA and PCoA to test  
260 for the chemical and microbiological dissimilarities based on the deposit age and the  
261 bauxite origin. To study the factor age alone, nested PERMANOVA were calculated  
262 using the parameter *strata* to exclude the effect of bauxite origin. Furthermore, multiple  
263 co-inertia analysis (MCIA) was performed using the package “omicade4” (Meng et al.,  
264 2014) to determine the relationships between the four datasets used in this study  
265 (chemical characteristics, mineralogy, 16S rRNA sequences and ITS sequences).

## 266 2.7 Co-occurrence network construction

267 Co-occurrence analyses were implemented for a better understanding of bacterial and  
268 fungal interactions in the four bauxite residues. Co-occurrence networks were  
269 constructed based on pairwise Pearson correlations calculated between bacterial and  
270 fungal ASVs by using the base R function *cor* (Berry and Widder, 2014; Williams et  
271 al., 2014). To avoid including false positives in the network due to spurious or random  
272 interactions, the ASV table we permuted 100 times and a *p*-value for each possible  
273 pairwise interaction was calculated to test its validity. The *p*-values were then adjusted  
274 using the Benjamini-Hochberg procedure (Benjamini and Hochberg, 1995), and only  
275 edges with a *p*-value below 0.01 that corresponded to an absolute correlation higher  
276 than 0.4 were retained. To describe the topology properties of the networks, a set of  
277 network indexes including graph density, average degree of nodes, transitivity,  
278 modularity, average geodesic distance, betweenness centrality, and density were  
279 calculated with the R package “igraph” (Csardi and Nepusz, 2006). The Wilcoxon test  
280 was employed to assess significant differences in topological parameters between  
281 networks. The connectivity of the network nodes was determined by their within-  
282 module connectivity ( $Z_i$ ) and among-module connectivity ( $P_i$ ). Nodes were then  
283 classified into four categories, according to Poudel et al. (2016): peripherals ( $Z_i < 2.5$   
284 and  $P_i < 0.62$ , nodes with few links to other species), connectors ( $P_i > 0.62$ , nodes

285 that connect modules), module hubs ( $Z_i > 2.5$ , highly connected nodes within  
286 modules), and network hubs ( $Z_i > 2.5$  and  $P_i > 0.62$ , highly connected nodes among  
287 and within modules).

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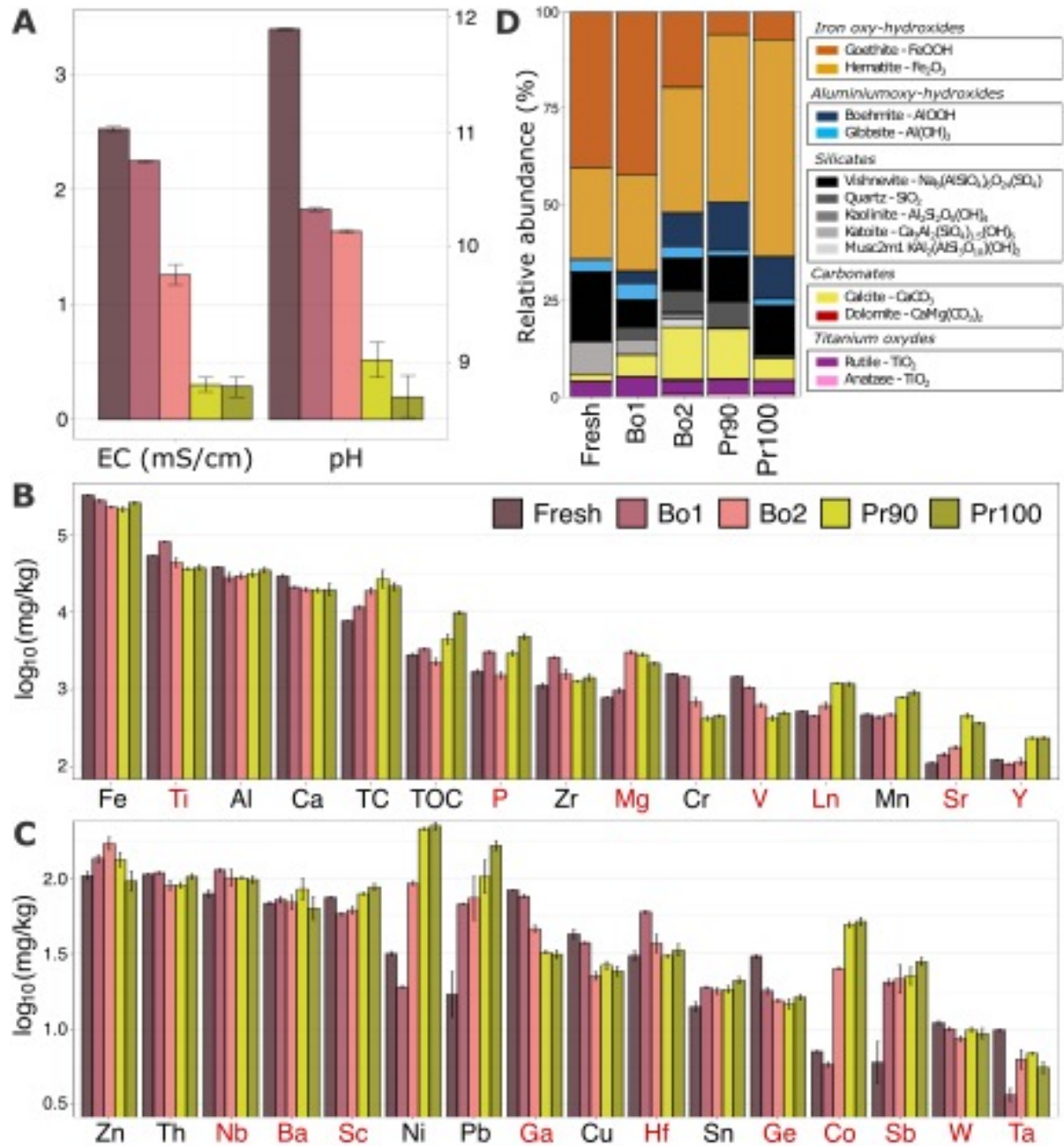
### 289 3. RESULTS

#### 290 3.1. pH, EC and geochemical composition

291 The pH of the bauxite residue ranged from  $8.70 \pm 0.34$  to  $11.90 \pm 0.01$ , with the highest  
292 value in fresh samples and the lowest in Pr100 (**Fig. 2a**, Table S1). The EC of the  
293 bauxite residue ranged from  $0.29 \pm 0.15$  mS/cm to  $2.53 \pm 0.04$  mS/cm, and again,  
294 fresh samples showed the highest value and Pr100 the lowest (**Fig. 2a**, Table S1).

295 The elements with the highest concentrations in all bauxite residue samples were Fe  
296 ( $268894 \pm 45706$  mg/kg), Ti ( $51313 \pm 17317$  mg/kg), Al ( $33093 \pm 5662$  mg/kg), Ca  
297 ( $22425 \pm 4952$  mg/kg), and TC ( $17162 \pm 9439$  mg/kg) (**Fig. 2b**, Table S2). Fe  
298 represents at least  $22 \pm 3$  % of the dried mass in all samples. The main critical  
299 elements present in all samples were Ti ( $51313 \pm 17317$  mg/kg), P ( $2743 \pm 1274$   
300 mg/kg), Mg ( $1877 \pm 987$  mg/kg), V ( $849 \pm 423$  mg/kg), Ln ( $775 \pm 339$  mg/kg), Sr ( $240$   
301  $\pm 142$  mg/kg), Y ( $159 \pm 59$  mg/kg), Nb ( $99 \pm 16$  mg/kg), Sc ( $73 \pm 12$  mg/kg), Ba ( $73 \pm$   
302  $15$  mg/kg), and Ga ( $56 \pm 24$  mg/kg) (**Fig. 2c-d**, Table S2). TN concentrations were  
303 only above the machine's limit of detection (100 mg/kg) in the Provence samples, with  
304 values of  $287 \pm 183$  mg/kg for Pr90 and  $470 \pm 158$  mg/kg for Pr100 (Table S2).

305 Statistical analysis showed that Ti, Cr, V, and Ga concentrations were significantly  
306 higher in samples coming from Boké bauxite ( $p < 0.05$ , Table S2), while samples from  
307 Provence bauxite showed greater abundances of TC, TOC, P, Mg, Ln, Mn, Sr, Y, Sc,  
308 Pb, Ni, and Co ( $p < 0.05$ , Table S2). Within these two groups, some elements  
309 concentrations changed over time. In the Boké samples, a significant decrease in Fe,  
310 Ca, Cr, V, Ga, Cu, Ge, and W was observed as sample age increased ( $p < 0.05$ , Table  
311 S2) while Mg, and Sr concentrations increased with age ( $p < 0.05$ , Table S2). In the  
312 Provence samples, Mg decreased significantly with age, while P and TOC  
313 concentrations increased ( $p < 0.05$ , Table S2). These relationships between the age  
314 of the sample and their chemical properties were further confirmed by Pearson  
315 correlations ( $R \geq 0.9$ ,  $p < 0.05$ , Fig. S1).



**Figure 2. Physicochemical properties of bauxite residue samples. A)** pH and EC, **B)** Major elements concentrations, **C)** Trace elements concentrations, and **D)** Mineralogy. Critical elements are colored red.

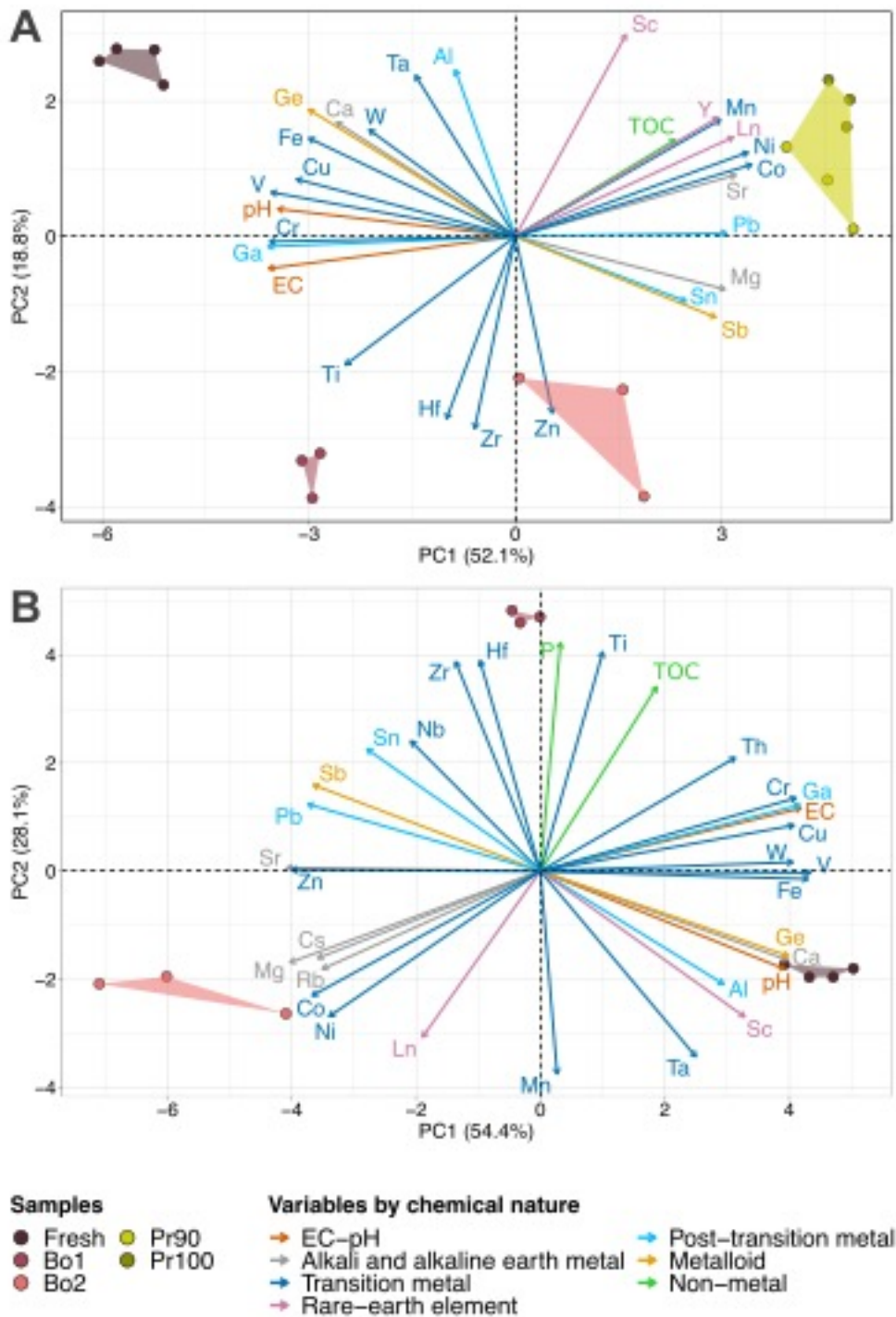
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### 317 3.1.2 Element correlation

318 PCA was used to detect patterns in the chemical dataset and describe linear relations  
 319 between the analyzed physicochemical parameters (**Fig. 3a**). The first three principal  
 320 component axis (PC1, PC2, and PC3) explained 84.3% of the data variability between  
 321 samples. PC1 explained 53.0% of the variance and included two groups: the first one

322 composed of pH, EC, Ga, Cr, V, Cu, Fe, Ge, Ca, Ti, and W whereas the second one  
323 comprised TOC, Co, Ni, Sr, Ln, Mg, Pb, Mn, Y, Sb, Cs, and Sn. PC2 explained 18.4%  
324 of the variance among samples and was mainly composed of Zr, Sc, Hf, Ta, Al, Zn,  
325 Nb, and Ti. Finally, PC3 (not shown) explained 12.9% of variance and was primarily  
326 composed of P, Th, Rb, Zn, Cs, and Sn. PERMANOVA analysis corroborated that the  
327 factors “age” ( $p < 0.01$ ) and “bauxite origin” ( $p < 0.05$ ) significantly explained the  
328 differences in chemical properties across samples. The factor “age” (tested by nested  
329 PERMANOVA to exclude the effect of the bauxite origin) explained 65.8% of the  
330 variance in chemical parameters while the factor “bauxite origin” explained 28.18%.  
331 The hierarchical cluster analysis (Fig. S2) provided statistical confirmation of the  
332 tendencies observed in the PCA and bauxite residue samples were clustered based  
333 on their chemical similarity. The first cluster contained the fresh samples, the second  
334 cluster Bo1 samples, the third cluster Bo2 samples, and the fourth cluster contained  
335 both Pr90 and Pr100 samples.

336 To further understand the effect of the factor “age” on residues generated from the  
337 same bauxite ore, a second PCA was conducted considering only the Boké samples  
338 (fresh, Bo1 and Bo2) (**Fig. 3b**). The first two dimensions explained 82.5% of the  
339 variability and the factor “age” separated the samples significantly (PERMANOVA,  $p$   
340  $< 0.01$ ). Co, Sr, Pb, Mg, and Sb were mostly associated with Bo2, while pH, EC, W,  
341 Ca, Ge, Fe, Cu, V, Cr, and Ga had their highest values in fresh samples and decreased  
342 progressively with age. In contrast, TOC, Y, Mn, Ln, Ni, Sn, Al, and Ta did not show  
343 any age-dependent trends during the first years of storage (Table S2). Globally, this  
344 statistical analysis is in line with the expected behavior of the elements, such as the  
345 colocalization of Cr and V and their correlation with Fe (Markus Gräfe et al., 2011) as  
346 well as the REEs affinity for Mn phases (Vind et al., 2018).



**Figure 3. Principal component analysis (PCA) of chemical parameters** in A) all and B) Boké bauxite residue samples. Individuals are represented by dots and colored by sample. Individuals are grouped according to hierarchical clustering results. Variables are represented by arrows and colored by chemical nature. Only variables with a  $\cos^2 > 0.5$  are shown.

347

348

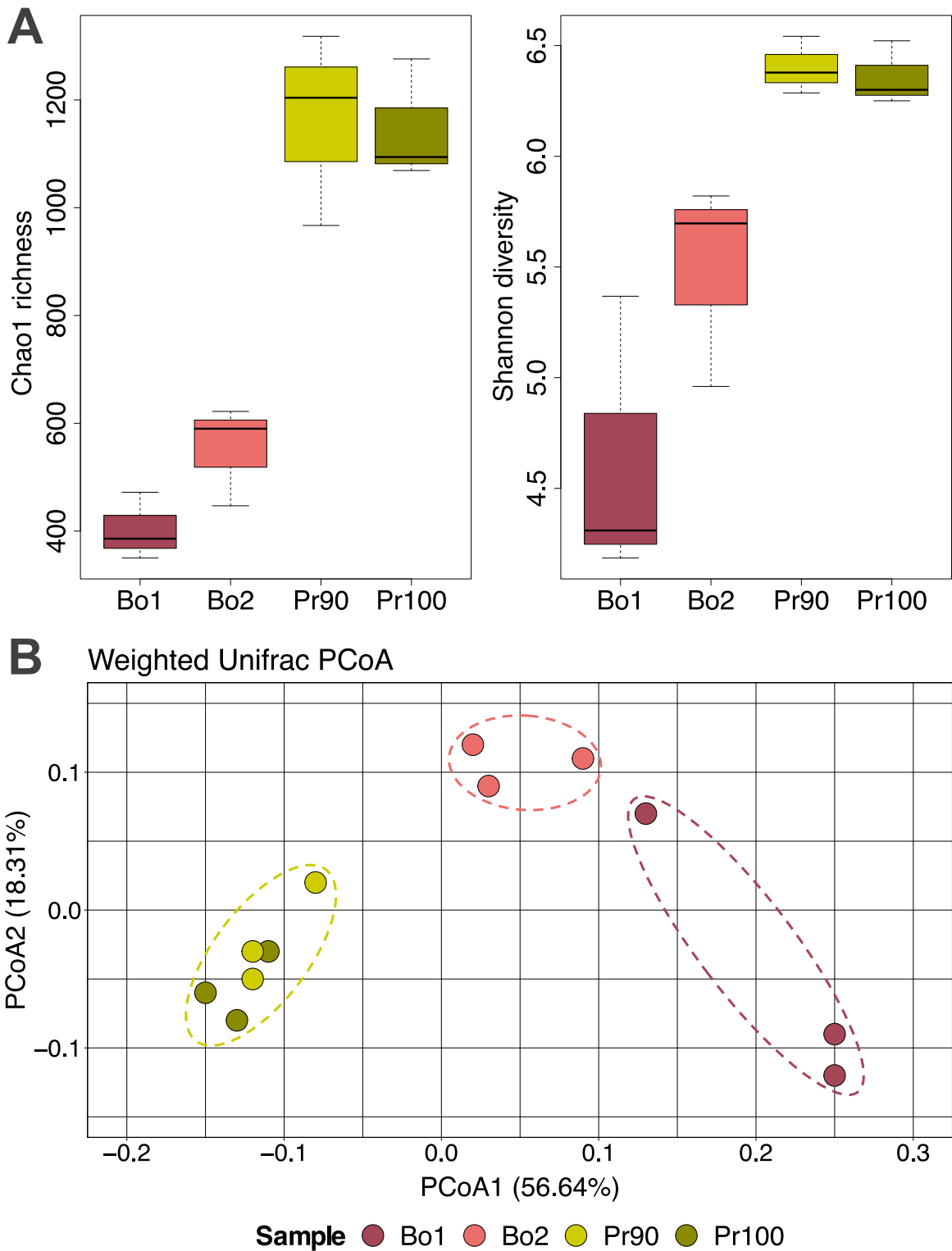
### 349 **3.1.3 Mineralogical composition**

350 XRD analysis (**Fig. 2d**, Table S3) showed that bauxite residues were principally  
351 composed of iron and aluminum oxides, hydroxides, and silicates. Goethite  
352 ( $\text{FeO}(\text{OH})$ ), hematite ( $\text{Fe}_2\text{O}_3$ ), gibbsite ( $\text{Al}(\text{OH})_3$ ), and vishnevite  
353 ( $\text{Na}_8(\text{AlSiO}_4)_6\text{O}_{24}(\text{SO}_4)$ ) are the dominant phases and accounted for at least 70 % of  
354 the crystalline fraction in all the samples. Rutile ( $\text{TiO}_2$ ) and calcite ( $\text{CaCO}_3$ ) were also  
355 detected in all samples, in the range of 3-5 % and 2-14% respectively. The hematite,  
356 anatase ( $\text{TiO}_2$ ) and boehmite ( $\text{AlO}(\text{OH})$ ) were significantly more present in Provence  
357 samples, and interestingly, neither anatase nor boehmite were found in fresh samples  
358 (Table S3). In Boké samples, a significant decrease in goethite and Katoite  
359 ( $\text{Ca}_3\text{Al}_2(\text{SiO}_4)_{1.5}(\text{OH})_6$ ) was observed as the age of the samples increased.

### 360 **3.2 Microbial community diversity, structure and dynamics**

#### 361 **3.2.1 Alpha diversity**

362 The influence of bauxite residue's age and geochemical composition on microbial  
363 communities was assessed using 16S and ITS metabarcoding. As expected, no DNA  
364 could be extracted from the fresh residue samples. For Bo1, Bo2, Pr90, and Pr100,  
365 the sequencing achieved a coverage of more than 55000 16S rRNA sequences and  
366 4700 ITS sequences per sample. The SRS curves (Fig. S3) indicated that the  
367 sequencing depth was sufficient to identify the majority of ASVs within bacterial and  
368 fungal communities in all the samples (except fresh samples). Alpha-diversity derived  
369 from the number of ASVs, Chao1 richness, and Shannon's index showed differences  
370 between samples regarding bacterial and fungal communities (Table S4). A total  
371 number of 7994 bacterial ASVs were identified, with 350 to 1318 ASVs per  
372 sample. The number of bacterial ASVs in the Provence samples (Pr90 and Pr100)  
373 was 6928, significantly greater than the 2867 ASVs found in the Boké samples (Bo1  
374 and Bo2). The Provence samples also showed the highest richness and Shannon  
375 values for bacteria, compared with Boké samples (**Fig. 4a**). For fungi, 892 ASVs  
376 ranging from 45 to 156 ASVs per sample were obtained, with again more ASVs and  
377 Chao1 richness in Provence samples. However, there were no significant changes in  
378 Shannon's index between both group of samples, indicating that the specific diversity  
379 of the fungal communities was similar in Provence and Boké samples.



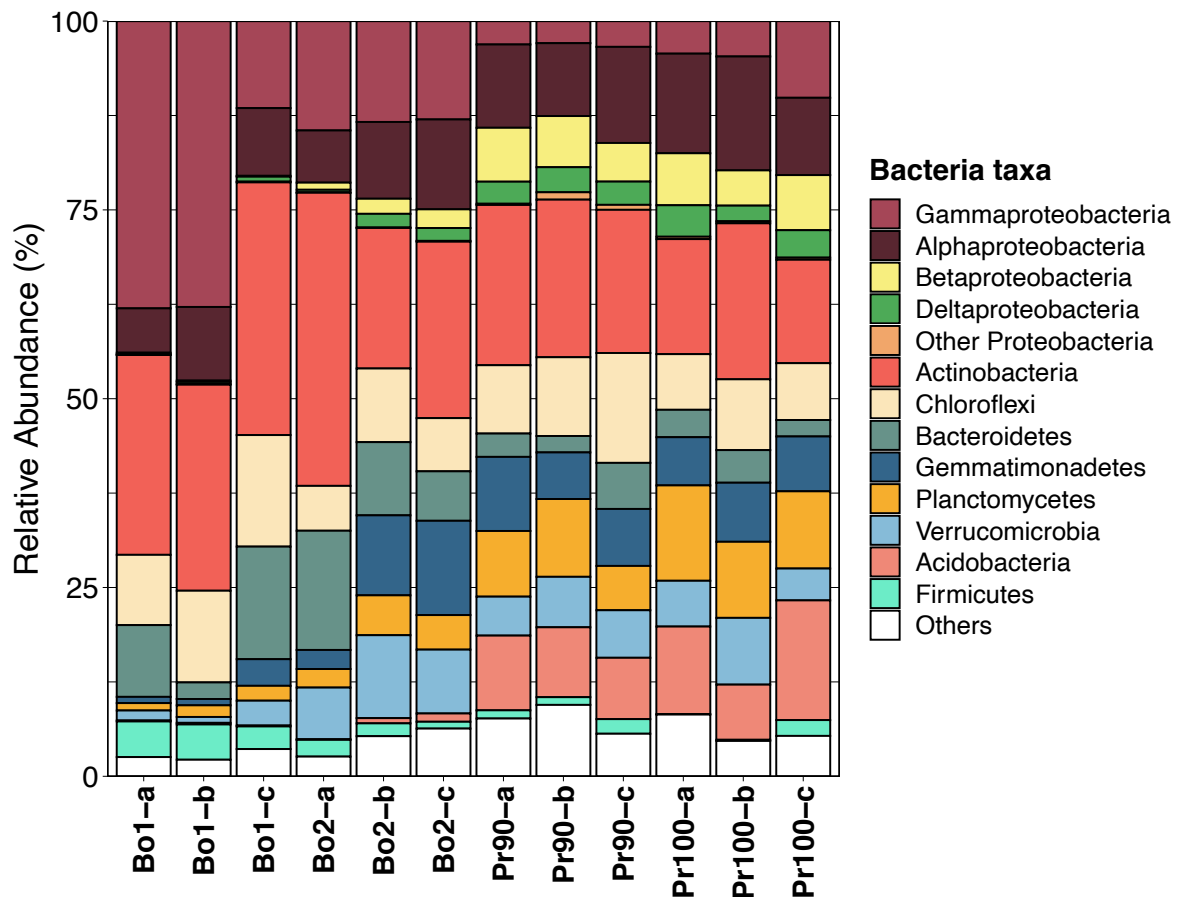
**Figure 4. Bacterial alpha and beta diversity of bauxite residues.** A) Chao1 richness and Shannon diversity index. B) Principal coordinate analysis (PCoA) based on weighted UniFrac distances between bauxite residue samples.



### 381 3.2.2 Microbial community structure

382 PCoA ordinations based on weighted UniFrac distances (**Fig. 4b**) revealed that the  
383 bacterial community structure differed between the bauxite residue samples. Samples  
384 were significantly separated based on bauxite origin (PERMANOVA,  $p < 0.05$ ), and  
385 nested PERMANOVA confirmed the difference in bacterial community structure  
386 across age ( $p < 0.001$ ). The first two axes of PCoA explained 74.95% of the community  
387 dissimilarity. Axis 1 was most correlated with EC ( $R = 0.97$ ,  $p = 1.1e-07$ ), followed by  
388 Co ( $R = 0.96$ ,  $p = 9.8e-07$ ), Ni ( $R = 0.95$ ,  $p = 1.4e-06$ ), Cr ( $R = 0.94$ ,  $p = 1.1e-07$ ), V  
389 ( $R = 0.92$ ,  $p = 2.5e-05$ ), Ti ( $R = 0.9$ ,  $p = 5.4e-05$ ), and pH ( $R = 0.9$ ,  $p = 8.1e-05$ ); while  
390 Axis 2 correlated with P ( $R = 0.75$ ,  $p = 0.005$ ). In contrast, the fungal community  
391 structure did not seem to follow a clear pattern among samples according to PCoA  
392 built on Bray-Curtis distances (Fig. S4).

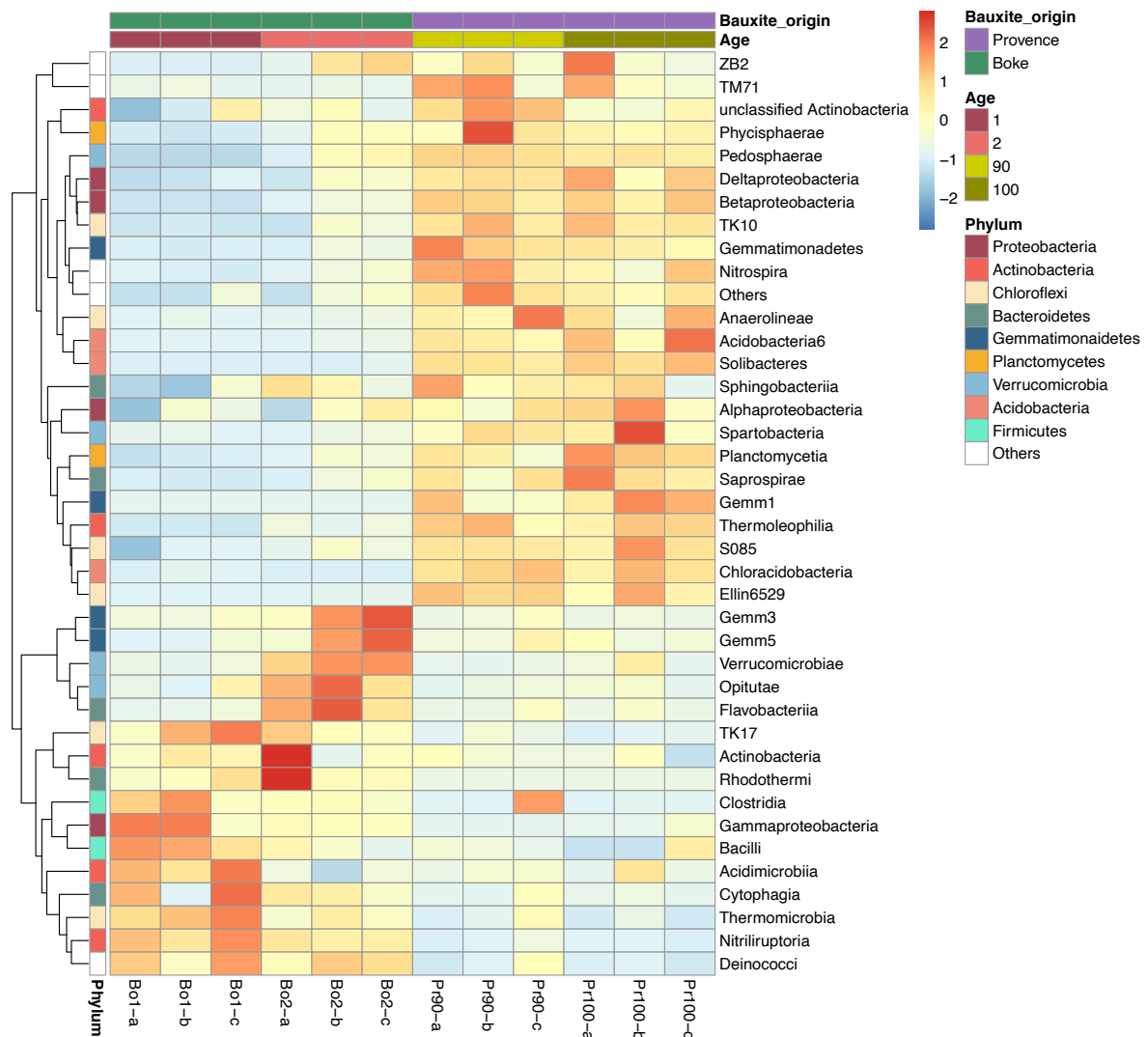
393 The bacterial phyla that dominated all bauxite residue samples were Proteobacteria,  
394 Actinobacteria, Chloroflexi, Bacteroidetes, Gemmatimonadetes, Planctomycetes,  
395 Verrucomicrobia, Acidobacteria and Firmicutes, accounting for more than 90% of the  
396 total bacterial communities (**Fig. 5**). When comparing the samples from Provence and  
397 Boké, significant taxonomic differences were observed. Actinobacteria and  
398 Gammaproteobacteria were the dominant taxa in the Boké samples with an average  
399 abundance of 28.0 % and 21.3 % respectively, followed by Chloroflexi (9.8 %) and  
400 Bacteroidetes (9.8 %). In the Provence samples, Actinobacteria (18.4 %) were still  
401 dominant while Gammaproteobacteria (4.8 %) and Bacteroidetes (3.6 %) lost  
402 prominence in favor of Alphaproteobacteria (12.0 %), Planctomycetes (9.6 %),  
403 Betaproteobacteria (6.3 %), and Deltaproteobacteria (3.2 %). Boké samples were  
404 significantly enriched in Actinobacteria, Gammaproteobacteria, Bacteroidetes and  
405 Firmicutes, whereas samples from Provence showed greater abundances of  
406 Alphaproteobacteria, Planctomycetes, Betaproteobacteria and Deltaproteobacteria ( $p$   
407  $< 0.05$ ). Acidobacteria (10.3 %), barely found in Boké samples, showed a strong  
408 increase in Provence samples ( $p < 0.05$ ). Chloroflexi, Gemmatimonadetes and  
409 Verrucomicrobia were nearly constant across samples.



**Figure 5. Relative abundance of the most abundant bacterial taxa among bauxite residue samples. Proteobacteria are divided in classes.**

410

411 At the level of bacterial classes, differences between Boké and Provence samples  
 412 were also found (**Fig. 6**). Significantly higher proportions of Gammaproteobacteria,  
 413 Nitrospirae, Thermomicrobia, Gemmatimonadetes, Bacilli, Rhodothermi, TK17, Deinococci and  
 414 Clostridia were identified in the Boké samples ( $p < 0.05$ ). Within this group, variations  
 415 between samples of different ages were also observed. Bo1 was enriched in  
 416 Thermomicrobia, Thermoleophilia, Acidimicrobia and Bacilli, whereas Ophitales,  
 417 Verrucomicrobiae and Flavobacteriia showed greater abundances in Bo2 ( $p < 0.05$ ).  
 418 In contrast, Provence samples were significantly more enriched in  
 419 Alphaproteobacteria, Planctomycetia, Acidobacteria6, Betaproteobacteria,  
 420 Thermoleophilia, Deltaproteobacteria, Gemmatimonadetes, Pedosphaerae,  
 421 Anaerolineae, Phycisphaerae, Spartobacteria, Gemm1, Chloracidobacteria,  
 422 Ellin6529, S085, Nitrospira, Saprospirae, TK10, Solibacteres and TM71 ( $p < 0.05$ ). In  
 423 the Provence samples, the only differences seen over ages were Pedosphaerae and  
 424 Actinobacteria, which were more abundant in Pr90 than in Pr100 ( $p < 0.05$ ).



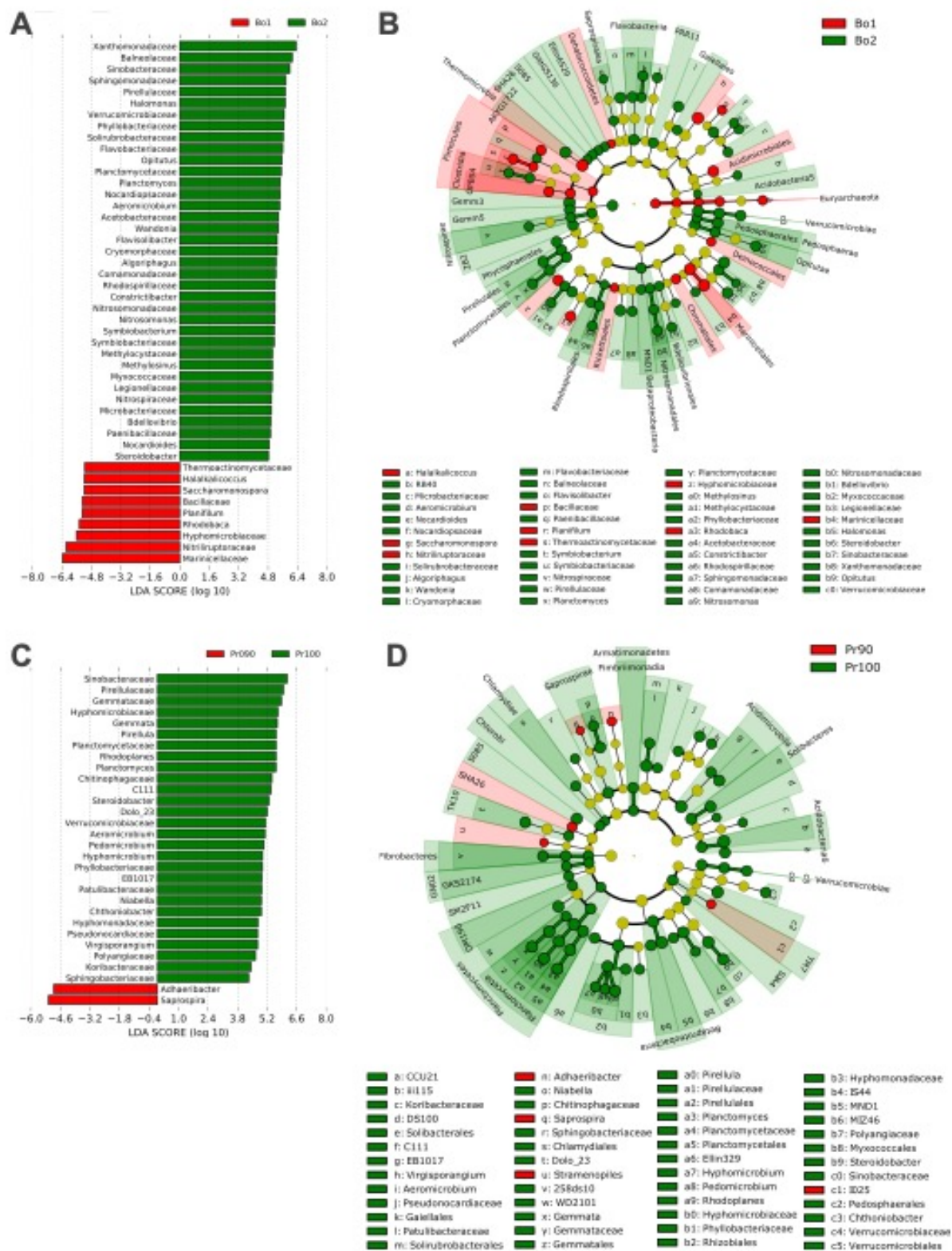
**Figure 6.** Z-score hierarchical clustering heat map representing the relative abundance of the top 40 bacterial classes in different bauxite residue samples.

425

### 426 3.2.3 Taxonomic characteristics of bacterial and fungal communities

427 To further explore these temporal scale differences, LEfSe analyses were conducted  
 428 to detect asymmetrically distributed bacterial taxa. Among the Boké samples, 9  
 429 indicator bacterial taxa were found in Bo1 against 37 in Bo2 (**Fig. 7a**). LEfSe confirmed  
 430 the highest abundance of three families of Bacilli in Bo1, as well as other taxa  
 431 belonging to the phyla Actinobacteria and Proteobacteria, and the archaeal class  
 432 Halobacteria. In line with our previous results, Opitutae, Verrucomicrobiae and  
 433 Flavobacteriia were significantly more abundant in Bo2, together with other taxa  
 434 belonging to the phyla Actinobacteria, Proteobacteria, Firmicutes, Bacteroidetes,  
 435 Planctomycetes and Nitrospirae. For the Provence samples, only two indicator

436 bacterial taxa (classified as Bacteroidetes) were identified in Pr90, against 28 in Pr100  
437 (classified as Actinobacteria, Proteobacteria, Planctomycetes, Bacteroidetes,  
438 Verrucomicrobia, and Chloroflexi) (**Fig. 7b**).



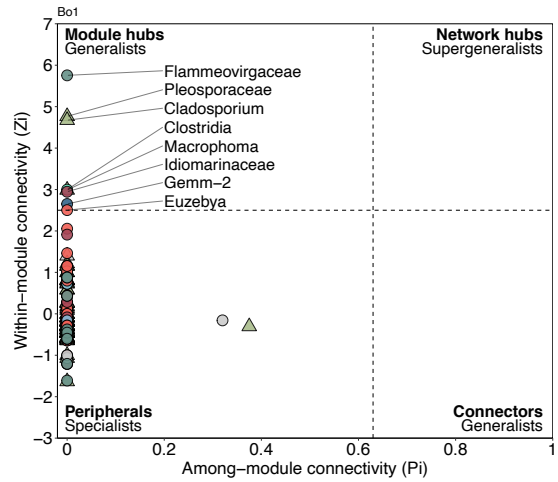
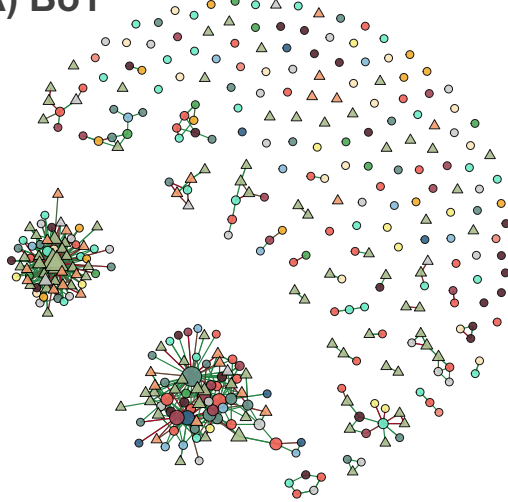
**Figure 7. LEfSe analysis of in the bauxite residue samples according to sample age.** Histograms of LDA scores of 16S gene sequences in Boké (A) and Provence (C) samples. Only taxa with a LDA score (log<sub>10</sub>) above 2.0 and a p-value lower than 0.05 for Kruskal–Wallis tests are shown. B and D Cladograms are derived from LEfSe analysis. The central point denotes the root of the tree and expanded to each ring representing the next lower taxonomic level from phylum to genus.

#### 440 **3.2.4 Microbial co-occurrence networks**

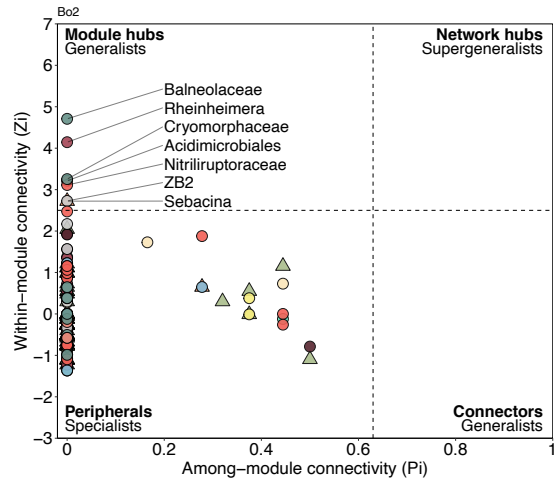
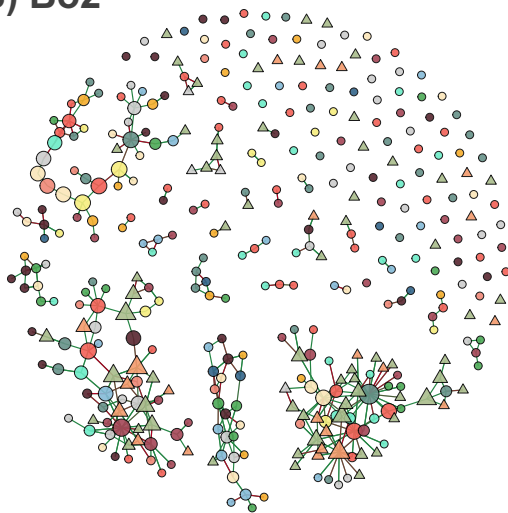
441 Co-occurrence networks were constructed using the ASV data of 16S rRNA and ITS  
442 sequences. The Boké (**Fig. 8**) and Provence (**Fig. 9**) samples did not differ significantly  
443 in network topological parameters such as average geodesic distance and modularity  
444 (Table S5). However, the network complexity (number of nodes and edges, degree of  
445 nodes, and transitivity) was different between the Boké and Provence networks (Table  
446 S5). The two Boké networks (Bo1 and Bo2) contained a similar number of nodes (366  
447 and 369), although Bo1 showed more edges (516 versus 324), higher average degree  
448 (2.46 versus 1.76), and transitivity (0.15 versus 0.11). The two Provence networks  
449 were more complex, as both Pr90 and Pr100 showed more nodes (603 and 571), more  
450 edges (1254 and 1322), higher average degree (4.16 and 4.63), and higher transitivity  
451 (0.18 and 0.12) compared to Boké.

452 Most of the nodes from the Boké and Provence networks were classified as  
453 peripherals (specialists), and few nodes fell into module hubs (generalists) (**Fig. 8-9**).  
454 Generalists are considered keystone taxa, as they are responsible for structuring the  
455 different nodes and modules into a complete community, thus determining the  
456 efficiency of energy metabolism and nutrient cycling in habitats (Wang et al., 2019). In  
457 this study, more module hubs (generalists) were identified in Pr90 and Pr100 than in  
458 Bo1 and Bo2. In Bo1 and Bo2 networks, the module hubs were mainly members of  
459 Bacteroidetes, Actinobacteria, Proteobacteria, and Firmicutes for bacteria, and  
460 Ascomycota for fungi. In contrast, the module hubs identified in Pr90 and Pr100  
461 networks belonged to Proteobacteria, Actinobacteria, Chloroflexi, Verrucomicrobia,  
462 Gemmatimonadetes, Planctomycetes and Firmicutes for bacteria, and Ascomycota  
463 and Basidiomycota for fungi.

### A) Bo1



### B) Bo2

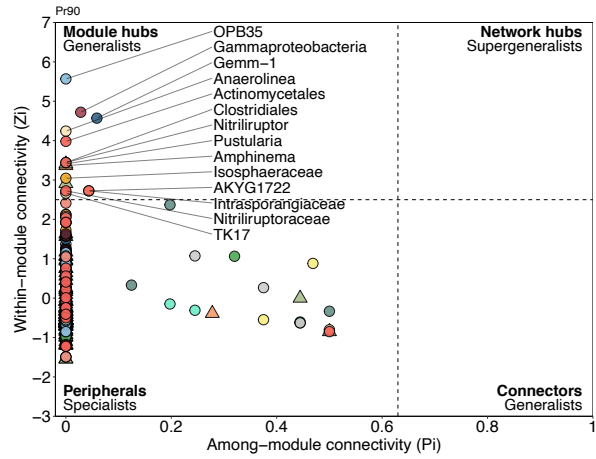
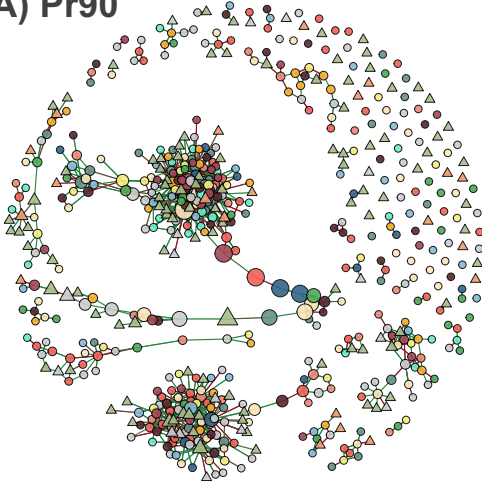


- |                        |                    |                   |                 |
|------------------------|--------------------|-------------------|-----------------|
| ● Gammaproteobacteria  | ● Actinobacteria   | ● Verrucomicrobia | ▲ Ascomycota    |
| ● Alphaproteobacteria  | ● Chloroflexi      | ● Acidobacteria   | ▲ Basidiomycota |
| ● Betaproteobacteria   | ● Bacteroidetes    | ● Firmicutes      | ▲ Other Fungi   |
| ● Deltaproteobacteria  | ● Gemmatimonadetes | ● Other Bacteria  |                 |
| ● Other Proteobacteria | ● Planctomycetes   |                   |                 |

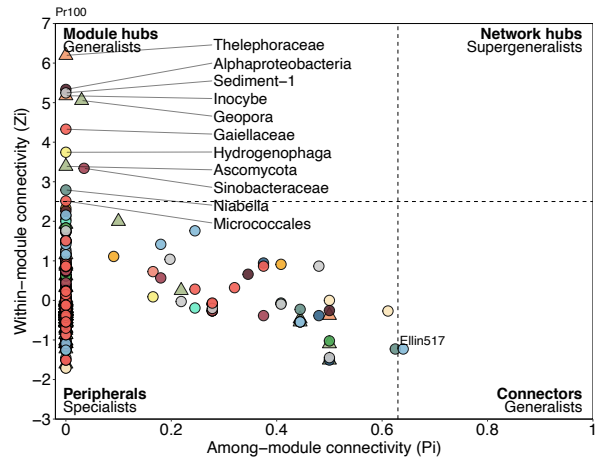
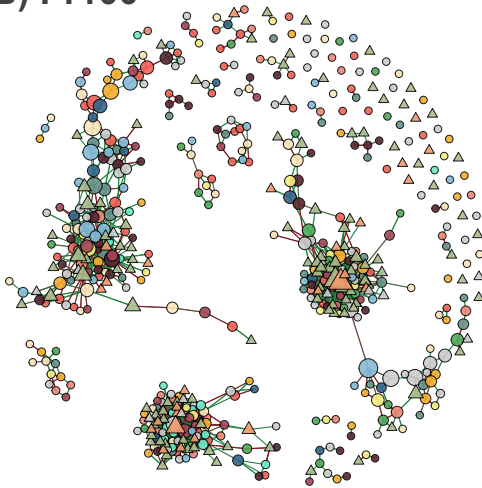
**Figure 8. Co-occurrence network and Zi-Pi plot of Bo1 (A) and Bo2 (B) bauxite residue samples.** Each node represents a bacterial class (circles) or a fungal phyla (triangles). Nodes are colored by taxonomical affiliation and their size in the network is proportional to the node betweenness. The color of each link reflects positive (red) or negative (blue) interactions. The topological role of each node is defined by within-module connectivity ( $Z_i$ ) and among-module connectivity ( $P_i$ ). According to values of  $Z_i$  (2.5) and  $P_i$  (0.62), the roles of nodes are classified into four categories: Peripherals, Module hubs, Connectors and Network hubs.



### A) Pr90



### B) Pr100



- |                        |                    |                   |                 |
|------------------------|--------------------|-------------------|-----------------|
| ● Gammaproteobacteria  | ● Actinobacteria   | ● Verrucomicrobia | ▲ Ascomycota    |
| ● Alphaproteobacteria  | ● Chloroflexi      | ● Acidobacteria   | ▲ Basidiomycota |
| ● Betaproteobacteria   | ● Bacteroidetes    | ● Firmicutes      | ▲ Other Fungi   |
| ● Deltaproteobacteria  | ● Gemmatimonadetes | ● Other Bacteria  |                 |
| ● Other Proteobacteria | ● Planctomycetes   |                   |                 |

**Figure 9. Co-occurrence network and Zi-Pi plot of Pr90 (A) an Pr100 (B) bauxite residue samples.** Each node represents a bacterial class (circles) or a fungal phyla (triangles). Nodes are colored by taxonomical affiliation and their size in the network is proportional to the node betweenness. The color of each link reflects positive (red) or negative (blue) interactions. The topological role of each node is defined by within-module connectivity (Zi) and among-module connectivity (Pi). According to values of Zi (2.5) and Pi (0.62), the roles of nodes are classified into four categories: Peripherals, Module hubs, Connectors and Network hubs.

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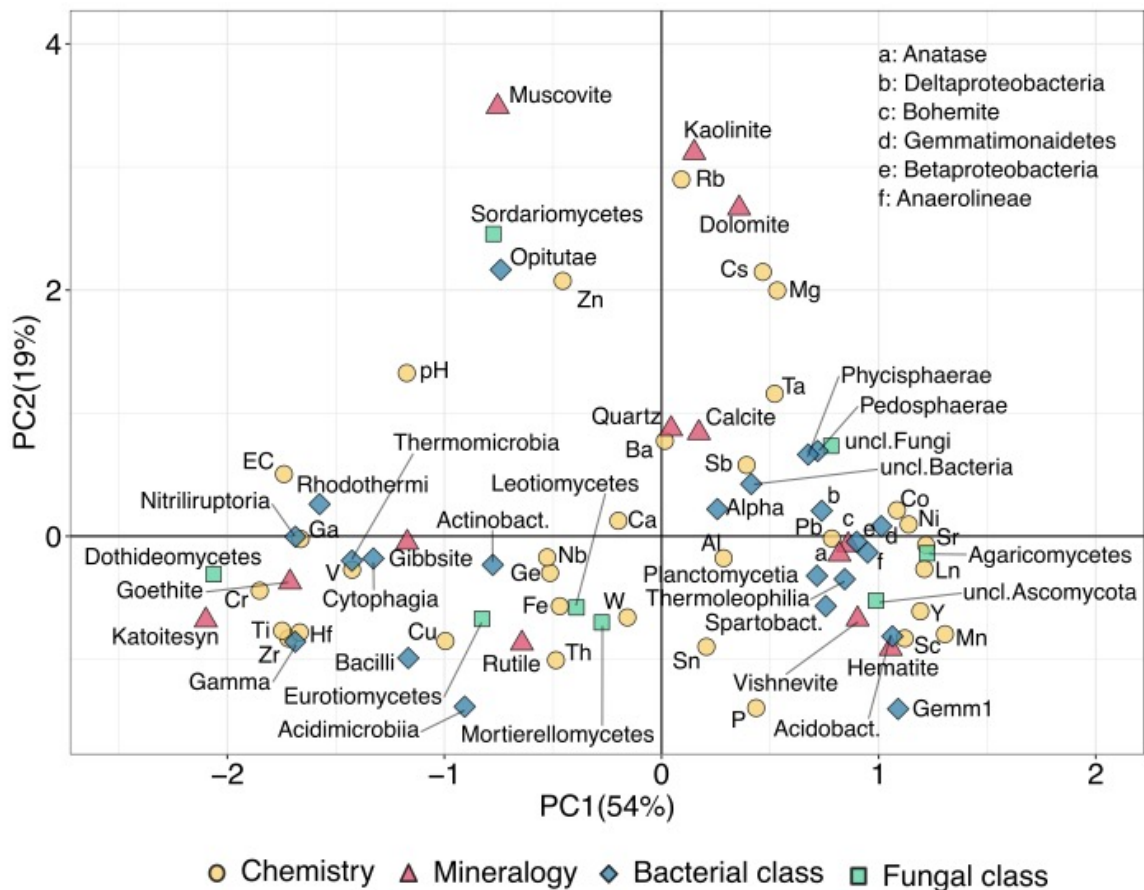
467

468



### 469 3.2.5 Multi co-inertia analysis

470 Multiple co-inertia analysis (MCIA) was used to determine the relationships between  
471 the chemistry, mineralogy, bacterial and fungal community composition in our bauxite  
472 residue samples. **Figure 10** displays the projection of the four datasets onto the first  
473 two principal components (PCs) of MCIA for Bo1, Bo2, Pr90 and Pr100 samples. PC1  
474 and PC2 explained 54% and 19% of the total variation respectively (Fig. S6b). The  
475 sample space (Fig. S6a) shows a good correlation between the different datasets in  
476 all samples, which could be clearly differentiated. Early-stage bauxite residues (Bo1  
477 and Bo2) were separated from equilibrium-stage samples (Pr90 and Pr100) along the  
478 PC1. Pseudo-eigenvalues (Fig. S6c) indicated that the chemistry and bacterial dataset  
479 contributed the most to the variation observed in PC1, while mineralogy was weighted  
480 in PC2. The pair-wise RV coefficient, which is multivariate generalization of the  
481 squared Pearson correlation coefficient, indicated higher global similarity between  
482 chemistry and bacterial classes (RV score = 0.86) when compared to the similarities  
483 between the chemistry and fungal classes (RV score = 0.36), and between the  
484 mineralogy and microbial classes (RV score for bacterial classes = 0.41, RV score for  
485 fungal classes = 0.22). Fungal classes contributed little to the variation of PC1 or PC2  
486 and were mostly correlated with bacterial classes (RV score = 0.38).



**Figure 10. MCA analysis of bauxite residue samples.** The figure shows the coordinates of the different data sets represented by shapes (circle: chemical data set; triangle: mineralogical data set; square: fungal classes data set; diamond: bacterial classes data set). Only bacterial and fungal classes representing at least 5% of the abundance in at least one sample are shown. Uncl. = unclassified; Alpha = Alphaproteobacteria; Gamma = Gammaproteobacteria.

487

488

#### 489 4. DISCUSSION

##### 490 4.1 BRDAs behave as primary successional environments

491 The initial geochemical conditions of the fresh bauxite residue, characterized by high  
 492 alkalinity ( $\text{pH} = 11.90 \pm 0.01$ ) and salinity ( $\text{EC} = 2.53 \pm 0.04 \text{ mS cm}^{-1}$ ) in line with  
 493 previous studies (Cusack et al., 2019; Xue et al., 2016), make BRDAs hostile  
 494 environments for most domains of life. The high pH and salinity derive from the excess  
 495 of alkaline anions (hydroxides, carbonates, aluminates, aluminum hydroxides,  
 496 silicates) and  $\text{Na}^+$  produced during the Bayer process (Cusack et al., 2019; X. Kong  
 497 et al., 2017) and thus the bauxite origin does not seem to significantly influence the  
 498 value of these parameters. Fresh and recently deposited bauxite residues (Bo1 and

499 Bo2) also contained significant amounts of toxic metals for microorganisms, namely  
500 Cr ( $1594 \pm 23$  mg/kg), V ( $1452 \pm 10$  mg/kg) and Al ( $38420 \pm 920$  mg/kg). The high  
501 alkalinity (pH > 10) of fresh, Bo1 and Bo2 bauxite residues may favor the Cr (III)  
502 oxidation to Cr(IV) as well as the predominance of V(V), both highly mobile and toxic  
503 for bacteria, fungi and plants (Chen et al., 2021; Economou-Eliopoulos et al., 2016;  
504 Liang et al., 2021; Milačić et al., 2012; Xiao et al., 2017). Regarding aluminum, only  
505 1% of the total Al content in bauxite residue has been reported to be soluble at pH  
506 values above 9, mainly as mobile  $[Al(OH)_4]^-$  (Milačić et al., 2012). Nevertheless, its  
507 impact on microbial and plant communities should not be neglected, as even small  
508 proportions of the total Al correspond to potentially toxic concentrations. Moreover,  
509 fresh and recently deposited bauxite residues were inherently poor in most  
510 macronutrients required for microbial and plant growth, such as TOC, TN, and P  
511 (Table S2). TOC accounted for less than 0.6% of the mass (0.28 % in fresh residue,  
512 0.33 % in Bo1, and 0.23 % in Bo2) which is typically attributed to desert soils (Li et al.,  
513 2013; Zech et al., 2014). TN concentrations were below the detection limit (100 mg/kg)  
514 in fresh, Bo1 and Bo2 samples in agreement with previous studies (Krishna et al.,  
515 2014; Wu et al., 2020). Finally, although the P concentrations ranged from 1500 to  
516 3000 mg/kg in fresh, Bo1, and Bo2 samples, the available phosphorus in bauxite  
517 residue is usually around 6.7 mg/kg (Santini et al., 2015a; Wu et al., 2020).

518 These extreme conditions, together with the absence of pre-existing life in the freshly  
519 produced bauxite residues, indicate that BRDAs behave as primary successional  
520 environments, similar to other well-studied natural systems (Guo et al., 2014; Schmidt  
521 et al., 2008). Primary succession is characterized by an initial short phase where the  
522 microbial community assembly is driven by stochastic dispersal processes, followed  
523 by a longer deterministic phase dominated by environmental selection and competition  
524 between species (Ortiz-Álvarez et al., 2018). In engineered environments like BRDAs,  
525 the dispersal of microorganisms is limited, and environmental selection pressure is  
526 usually stronger due to the extreme chemical characteristics of these sites. Therefore,  
527 a shorter stochastic phase and a stronger deterministic assembly is expected in such  
528 systems (Santini et al., 2015a). Given the environmental stress imposed by the  
529 geochemical properties of fresh bauxite residues, the first pioneer microorganisms to  
530 colonize BRDAs are likely to be haloalkaliphilic species able to accumulate nutrients

531 to facilitate the incorporation of new species (Santini et al., 2015a; Schmidt et al., 2014;  
532 Sun et al., 2018).

#### 533 **4.2 Geochemistry-dependent microbial community assembly in the early stages** 534 **of primary succession**

535 The bacterial community composition of the recently deposited bauxite residues (Bo1  
536 and Bo2) was similar to that observed in soda lakes, alkaline residues, and other  
537 unamended BRDAs (Bondici et al., 2013; Chakraborty et al., 2021; Kalwasińska et al.,  
538 2017; Ohlsson et al., 2019), characterized by the dominance of Actinobacteria  
539 (Actinobacteria, Nitriliruptoria, Acidimicrobiia) and Proteobacteria (especially  
540 Gammaproteobacteria) and relatively high proportions (1 to 16 %) of Bacteroidetes  
541 (Cytophagia, Rhodothermi, Flavobacteriia), Firmicutes (Bacilli, Clostridia), and other  
542 alkalophilic classes (Opitutae, Deinococci) (**Fig. 5-6**).

543 The phyla Actinobacteria, Proteobacteria and Firmicutes contain some of the  
544 prokaryotes best adapted to high alkalinity and salinity. Alkaliphilic and alkalintolerant  
545 microorganisms maintain their intracellular pH homeostasis by accumulating H<sup>+</sup> in  
546 their cytoplasm through Na<sup>+</sup>/H<sup>+</sup> antiporters (Mamo, 2020). Our results showed that  
547 Halobacteria (Archaea), Gammaproteobacteria, Nitriliruptoria and Rhodothermi were  
548 more abundant in Bo1 (**Fig. 6-7**) and correlated with EC (**Fig. 10**), corroborating the  
549 preference of these classes for high-salinity niches (Zhang et al., 2019). Extreme  
550 halophiles accumulate intracytoplasmic KCl to regulate their osmotic potential, which  
551 constrains them to be obligate halophiles and limits their dispersal (Chen et al., 2020;  
552 Shameer, 2016; Vaidya et al., 2018). Salinity (EC) decreased significantly from Bo1 to  
553 Bo2 (**Fig. 1**, Table S2), allowing the proliferation of a wider range of halophilic and  
554 halotolerant species such as Opitutae, Acidimicrobiia or the genus *Halomonas*  
555 (Gammaproteobacteria) (**Fig. 6-7**). Halotolerant bacteria can flexibly adapt to habitats  
556 with different salinities by synthesizing and accumulating compatible solutes in their  
557 cytoplasm, which in turn requires a more intensive use of energy (Gunde-Cimerman  
558 et al., 2018). Interestingly, the number of edges and network complexity was higher in  
559 Bo1 compared with Bo2 (**Fig. 8**), indicating that saline stress promoted microbial  
560 interaction as reported in other studies (Ji et al., 2019; Wang et al., 2019).

561 As pioneer colonizers, microorganisms are critical in the biogeochemical cycles and  
562 the ensuing development of soil (Schmidt et al., 2008; Zeng et al., 2016). TOC

563 concentration showed no significant trend in early-stage samples (**Fig. 2**, Table S2),  
564 suggesting that the ratio in TOC input and consumption was balanced. In primary  
565 successional environments, the main sources of TOC are airborne allochthonous  
566 organic matter and C fixed by autotrophic microbial species (Ciccazzo et al., 2016).  
567 Our results also highlighted the significant abundance of heterotrophic bacteria in the  
568 early-stage samples, especially in Bo1, enriched in Actinobacteria and Bacteroidetes  
569 (**Fig. 6-7**). MCIAs revealed that Cytophagia (Bacteroidetes) and Nitriliruptoria  
570 (Actinobacteria) correlated negatively with TOC (**Fig. 10**) and members of these  
571 classes were identified as keystone species in Bo1 (**Fig. 8**), confirming their relevance  
572 in oligotrophic habitats (Foreman et al., 2007; Gonzalez-Pimentel et al., 2018).  
573 Heterotrophic communities may represent the earliest stage of microbial assembly,  
574 degrading allochthonous organic compounds and supplying essential nutrients (TOC,  
575 N, P) for the subsequent development of autotrophic species (Hodkinson et al., 2002).  
576 Our results also indicated the presence of chemoautotrophic Proteobacteria typically  
577 involved in the primary production of alkaline environments (**Fig. 7**), including iron-  
578 oxidizing bacteria within Alphaproteobacteria (Rhodobacteraceae) and  
579 Betaproteobacteria (Comamonadaceae) (Jamieson et al., 2018; Kumaraswamy et al.,  
580 2006; Straub et al., 1996) and sulfur-oxidizing Chromatiales (Gammaproteobacteria)  
581 (Yuan et al., 2021). In tune with previous studies (Harantová et al., 2017; Schmidt et  
582 al., 2008), the abundance of non-symbiotic N-fixing bacteria, represented by members  
583 of Clostridia, Opitutae, Comamonadaceae (Betaproteobacteria), Rhodospirillales  
584 (Alphaproteobacteria) and Bacilli (Ciccazzo et al., 2016; Hingole and Pathak, 2013;  
585 Navarro-Noya et al., 2016), increased significantly after the first years of primary  
586 succession (**Fig. 5-6**). These bacteria could contribute to the slight increase in TN  
587 observed after the first years of bauxite residue weathering (Wu et al., 2020),  
588 undetectable in this study as it is expected to be less than 100 mg/kg. Nitrifying  
589 bacteria (Nitrosomonas and Nitrospiraceae) were also more prevalent in Bo2,  
590 confirming that, although the amount of ammonia derived from bacterial fixation is low  
591 at early stages of primary succession, it must be sufficient to fuel nitrification (Ollivier  
592 et al., 2011; Zeng et al., 2016). Moreover, our results suggest that the increase in N-  
593 cycle bacteria could be due to a significant decrease in EC and Cr in Bo2 (**Fig. 2**, **Fig.**  
594 **10**, Table S2), as N-cycle enzymes are particularly sensitive to high salinity (Claros et  
595 al., 2010; Herbst, 1998) and heavy metal contamination (Kim et al., 2016; Oliveira and  
596 Pampulha, 2006).

### 597 **4.3 Primary succession reaches an equilibrium in BRDA over few decades**

598 The last stage of primary succession arises after long periods of time when the habitat  
599 becomes less harsh and environmental selection loses its strength in the assembly of  
600 communities, reaching an equilibrium (Ferrenberg et al., 2013). This maturity is  
601 expected to require more time to be developed in extreme geochemical systems as  
602 BRDAs. (Santini et al., 2015a). In our study, Pr90 and Pr100 samples, which have  
603 been naturally weathered for at least 90 years, did not show significant dissimilarities  
604 in either physicochemical parameters (**Fig. 3a**, Tables S1 and S2) or bacterial and  
605 fungal community composition (**Fig. 4b**; Fig. S5), suggesting that primary succession  
606 had reached the equilibrium in these sites. In Pr90 and Pr100, pH ( $8.8 \pm 0.3$ ) and  
607 salinity ( $EC = 0.29 \pm 0.11 \text{ mS cm}^{-1}$ ) were significantly lower than in early-stage  
608 samples (**Fig. 2**, Table S1), revealing a significant dependence with the age of the  
609 samples. During bauxite residue natural weathering, salinity is generally assumed to  
610 decrease due to the dissolution of alkaline minerals by rainfall (Cusack et al., 2019;  
611 Zhu et al., 2016), while the decrease in pH is normally attributed to atmospheric and  
612 microbial carbonation (Cusack et al., 2019; Khaitan et al., 2010; Schmalenberger,  
613 2013; X. Kong et al., 2017). Moreover, Pr90 and Pr100 showed a significantly higher  
614 concentration of macronutrients such as TOC, TN and P, which are accumulated  
615 during the bauxite residue natural aging due to the action of the different  
616 microorganisms that colonize the BRDAs (Santini et al., 2015a). This negative  
617 correlation between pH-salinity and age-nutrients is characteristic of restored and  
618 amended BRDAs (Courtney et al., 2014; Wu et al., 2021). Finally, our results also  
619 indicated a significant and steady decrease in Cr and V associated with the natural  
620 aging of the samples (**Fig. 2**, Table S2). However, the concentration of these metals  
621 also seems to be determined by the bauxite type and, therefore, the effect of the ore  
622 origin should not be neglected (Gentzmann et al., 2021).

623 Both alpha and beta bacterial diversity increased significantly in the equilibrium-stage  
624 samples (**Fig. 4**), in line with the results observed in restored BRDAs (Krishna et al.,  
625 2014; Wu et al., 2021, 2020). Beta-diversity analysis confirmed that “age” was the  
626 main factor explaining the dissimilarities of microbial community between Bo1/Bo2 and  
627 Pr90/Pr100 and not the ore origin. In addition, the changes in beta-diversity could be  
628 explained by pH, EC and some metal concentration gradients, all of them strongly anti-  
629 correlated with the age of the samples (**Fig. 3**). Circumneutral pH, lower salinity and

630 metal concentration, and increase in macronutrients support the survival of a more  
631 diverse microbial community dominated by bacteria commonly found in ordinary soils  
632 and freshwater like Alphaproteobacteria, Betaproteobacteria, Planctomycetes,  
633 Gemmatimonadetes, and Acidobacteria. Acidobacteria are one of the most abundant  
634 terrestrial bacterial taxa, reaching 52% from the total bacterial community in certain  
635 soils (Kielak et al., 2016). Their abundance is positively correlated with low pH (Jones  
636 et al., 2009), and their accumulation over time in BRDAs is commonly associated with  
637 the restoration of chemical conditions in bauxite residue (Santini et al., 2015b; Wu et  
638 al., 2021). The equilibrium-stage bauxite residue also harbors a more complex  
639 microbial network, accounting for more highly connected taxa (nodes) than early-stage  
640 residues. Nutrient availability and higher microbial richness have been shown to favor  
641 microbial network complexity and the emergence of new ecological functions, making  
642 the ecosystem more stable (Qiu et al., 2021; Wagg et al., 2019).

643 In contrast, fungal biodiversity and community structure did not appear to be  
644 significantly affected by chemical gradients (Table S4, Fig. S5). The fungal community  
645 structure in early-stage bauxite residues was dominated by Ascomycota ( $80.1 \pm 9.8$   
646 % of fungal communities), and Basidiomycota ( $> 14$  %). This distribution seems to be  
647 the usual in natural and engineered haloalkaline environments, although the analysis  
648 of fungal communities in these habitats is limited to few studies (Grum-Grzhimaylo et  
649 al., 2016; Salano et al., 2017; Santini et al., 2015b). In the equilibrium-stage samples,  
650 the relative abundance of Ascomycota decreased ( $52.1 \pm 24.2$  %) while  
651 Basidiomycota (mostly Agaricomycetes) increased ( $35.0 \pm 23.3$  %). This pattern has  
652 been observed in restored BRDAs and was attributed to a decrease in total alkalinity  
653 (Santini et al., 2015b). In this study however, MCIA revealed a poor correlation  
654 between fungal communities and chemical parameters (RV score = 0.36). It is known  
655 that the fungal community assembly is more influenced by stochastic processes in  
656 primary successional environments (Schmidt et al., 2014). Unlike bacteria, climate is  
657 often considered the main environmental factor affecting the fungal community  
658 composition, rather than soil chemical properties (Egidi et al., 2019). Interestingly, all  
659 fungal keystones identified in Bo1 (**Fig. 8**) belonged to the class Dothideomycetes  
660 (Pleosporaceae, Macrophoma, Cladosporium), within the Ascomycota.  
661 Dothideomycetes constitute almost the entire fungal community in microbial mats from  
662 soda lakes and are also found in microbial mats from hypersaline and iron-rich habitats

663 (Gerea et al., 2012; Maza-Márquez et al., 2021; Salano et al., 2017). In microbial mats,  
664 fungi play a crucial role in nutrient recycling by decomposing complex carbohydrates  
665 into simpler compounds that fuel chemoheterotrophic species (Carreira et al., 2020).

#### 666 **4.4 Implications for bioremediation and metal recovery**

667 To date, only few studies have focused their efforts on an integrated chemical,  
668 physical, and biological approach for the characterization of BRDA in the optic of  
669 bioremediation or critical metal bio extraction strategies.

670 Our results highlight the key role of pH and EC for natural microbial restoration and  
671 therefore should be considered primary targets in bioremediation. If natural weathering  
672 appears to be effective in reducing both pH and EC, it is also a slow process that could  
673 be artificially accelerated. In the last few years, microbially-driven pH neutralization  
674 using organic acids and CO<sub>2</sub> produced by fermentation of added carbon sources has  
675 gained attention as a promising technique for in-situ bioremediation of BRDAs (Santini  
676 et al., 2021; Wu et al., 2019). Santini et al. (2021, 2016) proved that the efficiency of  
677 this process could be improved by decreasing the initial pH and salinity and increasing  
678 the biodiversity of the microbial inoculum. However, they also identified the shortage  
679 of nutrients (N and P) in the medium term as a limiting factor for the development of  
680 this methodology. Our results suggest that lowering EC increased biodiversity and  
681 facilitated the development of bacteria related to the N cycle. Hence, a first treatment  
682 focused on reducing salinity (and pH if possible) before bacteria-mediated pH  
683 neutralization could yield considerably better results. Few studies have investigated  
684 the effect of lowering artificially pH and EC in the optic of bioremediation of BRDAs  
685 (Courtney et al., 2014; Jones et al., 2011; Wong and Ho, 1994). As a recent example,  
686 the recent study by Fourrier et al. (2021) has reported the positive effect of gypsum  
687 addition and repeated washing on pH neutralization and EC decrease. In addition,  
688 fungal contribution to early-stage microbial assembly seems to be underrated and their  
689 role in bauxite residue remediation needs more research. Regarding microbially-  
690 driven strategies for metal bio-extraction, our study is particularly informative for  
691 selecting appropriate bauxite residue and bacteria for the selective extraction of critical  
692 metals. In agreement with literature (Rivera et al., 2019; Vind et al., 2018), our results  
693 indicated that the concentration of REEs (Ln, Sc and Y) in bauxite residue is primarily  
694 determined by the ore origin, with karst bauxite (Pr90 and Pr100) being more enriched  
695 in REEs than laterite bauxite (Fresh, Bo1 and Bo2). In addition, Y and Ln were strongly



696 correlated as expected regarding the analogy between Y and heavy REEs while Sc  
697 behaves differently to Y and Ln and is likely incorporated in iron oxyhydroxide such  
698 as goethite (Levard et al., 2018). The conventional methods to recover these metals  
699 are not environmentally acceptable due to their excessive energy consumption and  
700 the production of hazardous residues, that require further treatment and high operating  
701 costs (Baniasadi et al., 2019). Metal bio-extraction is an interesting alternative to  
702 traditional metallurgy that has still been poorly investigated for critical metals. Among  
703 the ideas that could benefit from our study, alkaline-active exoenzymes produced by  
704 bacteria found in Bo1 and Bo2 offer interesting possibilities for the valorization of  
705 bauxite residue. Despite their ability to lower their intracellular pH and salinity,  
706 haloalkaliphilic species must still be able to secrete stable and operational enzymes  
707 at elevated pH and salinity (Mamo, 2020), making BRDAs potential sources of these  
708 enzymes. Some of the haloalkaliphilic bacteria known to synthesize alkaline-active  
709 enzymes, namely Gammaproteobacteria, Actinobacteria and Firmicutes (Kalwasińska  
710 et al., 2018; Litchfield, 2011; Maharaja et al., 2018; Shameer, 2016; Shivilata and  
711 Tulasi, 2015), are also capable to modify the chemical phase and mobility of various  
712 critical metals (Chidambaram et al., 2010; Presentato et al., 2020; Wee et al., 2014),  
713 including REEs. For example, Maleke et al. (2019) reported the direct reduction of  
714  $\text{Eu}^{3+}$  to  $\text{Eu}^{2+}$  by *Clostridium* sp. (Firmicutes). In line with previous studies (Santini et  
715 al., 2015b), the majority of Gammaproteobacteria corresponded to uncharacterized  
716 lineages, highlighting the potential of this group as a phylogenetic hot spot for novel  
717 haloalkaliphilic taxa.

718

## 719 **5. CONCLUSIONS**

720 In summary, this paper presents an integrated physicochemical and biological  
721 approach to explore the composition and dynamics of bacterial and fungal  
722 communities in bauxite residues deposited at different times and produced from  
723 different bauxite ores. Our results revealed that both deposit age and ore origin affect  
724 the geochemistry of bauxite residue, although unequally. Salinity, pH, TOC, TN, and  
725 P values seem to depend predominantly on the natural aging of bauxite residue, while  
726 the content of REE is mainly influenced by the origin of the bauxite ore. Our results  
727 highlight the behavior of bauxite residue deposits as primary successional  
728 environments and bring new insights into the early stages of microbial community

729 assembly in these sites. The pioneer microbial community was dominated by  
730 haloalkaliphilic microorganisms, strongly influenced by chemical gradients.  
731 Autotrophic and heterotrophic microbial species contribute to the supply of nutrients  
732 necessary for the development of other species through C fixation and degradation of  
733 allochthonous organic compounds, respectively. After the first years of natural  
734 restoration, nitrogen-fixing bacteria increase their presence and contribute to enhance  
735 N bioavailability. Microbial richness, diversity and network complexity increased  
736 significantly with age of deposition, until primary succession equilibrium was reached  
737 decades later, characterized by a microbial community composition similar to that of  
738 typical soils and freshwater. Our results suggested that salinity, pH, nutrients, and toxic  
739 metals (mainly Cr and V) were the main factors explaining this change in microbial  
740 communities. These results confirm the key role of pH and salinity in the establishment  
741 of early microbial communities and highlight them as main targets for bioremediation.  
742 Moreover, our co-occurrence network data suggest an important role of fungal  
743 communities in structuring the early-stage microbial community during primary  
744 succession, potentially by recycling complex organic matter. We also identified  
745 bacteria with potential metal extraction abilities, such as secretion of alkaline-active  
746 enzymes that could modify the chemical phase of the metals present.

747

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768

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