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## Temporal evolution of surface and sub-surface geochemistry and microbial communities of Pb-rich mine tailings during phytostabilization: A one-year pilot-scale study

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5

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9

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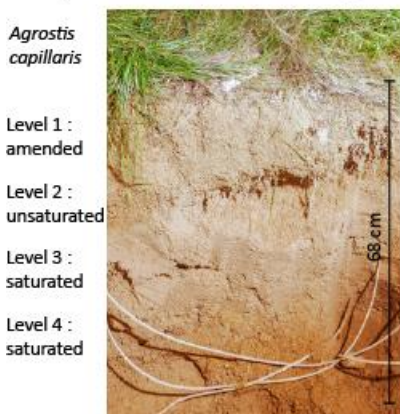
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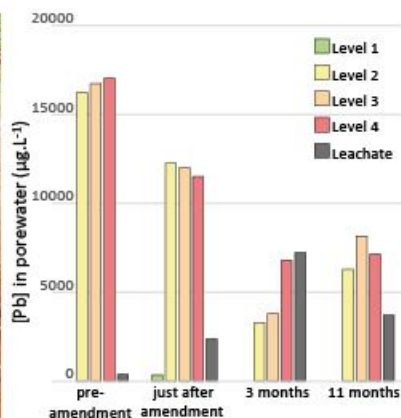
13

14 **Graphical abstract**

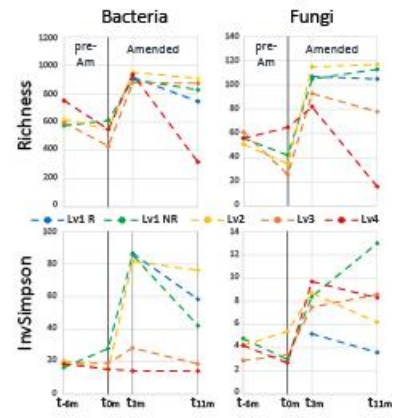
Phytostabilization of Pb tailing



Pb immobilization



Microbial diversity



15

16

17        **Abstract**

18    Old mine waste repositories can present health and/or environmental issues linked to their  
19    erosion, inducing dissemination of metals and metalloids in air and water that can be attenuated  
20    through phytostabilization. Here, the effect of this widespread phytomanagement option on the  
21    biogeochemistry of a Pb-rich mine waste was evaluated with a laboratory pilot-scale experiment  
22    giving access to the non-saturated and saturated zones below the rhizosphere compartment.  
23    Amendment of the tailings surface with biochar, manure and iron-oxide-rich ochre promoted  
24    growth of the seeded *Agrostis capillaris* plants. These events were accompanied by an increase  
25    of pH and a decrease of Pb concentration in pore water of the surface layer, and by a transient  
26    increase of Pb, Zn, and Ba concentrations in the deeper saturated levels. Macroscopic and  
27    microscopic observations (SEM) suggest that Pb was immobilized in *A. capillaris* rhizosphere  
28    through mechanical entrapment of tailing particles. Microbial taxonomic and metabolic  
29    diversities increased in the amended phytostabilized surface levels, with an increase of the  
30    proportion of heterotrophic micro-organisms. Below the surface, a transient modification of  
31    microbial communities was observed in the non-saturated and saturated levels, however 11  
32    months after seeding, the prokaryotic community of the deepest saturated zone was close to that  
33    of the initial tailings. pH and water saturation seemed to be the main parameters driving  
34    prokaryotic communities' structures. Results obtained at pilot-scale will help to precisely  
35    evaluate the impacts of phytostabilization on the temporal evolution of reactions driving the  
36    fate of pollutants inside the tailings dumps.

37

38    **Key words:** mine tailings, metals, assisted phytostabilization, laboratory pilot, microbial  
39    diversity.

## 40 **1 Introduction**

41 Unrestored mine tailing sites are a worldwide problem since they generally remain bare for  
42 decades or even centuries. For abandoned mine tailing heaps, the establishment of a  
43 spontaneous vegetation cover is usually considered beneficial as it represents a sort of  
44 phytostabilization (Houben et al., 2013). Indeed, revegetation of these degraded sites appears  
45 as a suitable long-term solution: the plant cover limits eolian dispersion, while the roots prevent  
46 water erosion and leaching, and provide a rhizosphere wherein metals can precipitate and  
47 stabilize (Mendez and Maier, 2008). However, for lead (Pb)/zinc (Zn) mine sites, metal toxicity,  
48 especially available Pb, nutrient deficiency, and poor physical structure of the substrate are  
49 major constraints for plant establishment and colonization (Ye et al., 2002). Therefore, a  
50 suitable strategy consists of aided phytostabilization by using pioneer plants (Lago-Vila et al.,  
51 2019) coupled with efficient organic and inorganic amendments (Sikdar et al., 2020).

52 In this context, numerous laboratory studies with pot experiments have recently been devoted  
53 to test the efficiency of plants and several amendments, alone or in combination, on Pb and Zn  
54 retention and plant growth in mine tailings (Ciarkowska et al., 2017; Al-Lami et al., 2019;  
55 Norini et al., 2019; Gao et al., 2020). Although different combinations may rapidly be tested,  
56 these pot experiments only focused on plant biomass and metal availability in the rhizosphere  
57 area, and are far from the field scale.

58 Other small-scale experiments include laboratory percolation tests enabling to measure/assess  
59 the transfer of contaminants through mine residues and to evaluate the effect of amendments  
60 (Rodriguez et al., 2018; Thouin et al., 2019), whereas influence of water saturation can be  
61 studied in columns (Ouanguwa et al., 2010). These systems are well constrained and they give  
62 insight on small-scale processes but it is necessary to increase the scale in order to understand  
63 interactions between different compartments and reflect field conditions.

64 In this regard, field experiments have been conducted to optimize the combination of plants and  
65 amendments in phytostabilization of metal(loid)s pollutants in mine sites (Yang et al., 2016;  
66 Acosta et al., 2018; Li et al., 2019). However, some key parameters could not be precisely  
67 controlled and accounted for, such as soil water saturation and incoming water flow. Moreover,  
68 some types of samples, such as pore water at different depths are difficult to collect during on-  
69 site studies and spatial heterogeneities of mine tailings often represent an obstacle for the  
70 interpretation of results obtained on-site (Constantinescu et al., 2019).

71 To overcome these limitations, lysimeters directly installed in the field give access to real  
72 conditions of watering, temperature and light, and allow the possibility to sample percolated  
73 water (Nicoara et al., 2014; Bilibio et al., 2017; Constantinescu et al., 2019). In such systems, ,  
74 some environmental factors cannot be controlled. Mesocosm scale laboratory pilot  
75 experiments, as an intermediary step between pot and field experiments, combine the advantage  
76 of controlled conditions of temperature, watering and water saturation with the possibility of  
77 simultaneously observing the amended and planted surface levels (Valentin-Vargas et al., 2014)  
78 and the underlying non saturated and saturated zones (Thouin et al., 2017; 2018).

79 Among the multiple parameters evaluated during these multi-scale experiments, microbial  
80 communities can give indications on the biogeochemical processes. Phytostabilization always  
81 induces modifications of the microbial diversity and metabolic profiles in the mining wastes  
82 colonized by plants, amended or not (Asemaninejad et al., 2020). The ability of diverse bacterial  
83 and fungal species to thrive in mining residues improves soil functions and encourages  
84 sustainable plant growth (Harris et al., 2009). Common effects of phytostabilization on  
85 microbial diversity were observed in diverse conditions of amendments and plants species, both  
86 in greenhouse experiments (Mendez et al., 2007, You et al., 2018) or field conditions (Li et al.,  
87 2015; Asemaninejad et al., 2020). They include increases in microbial diversity, biomass and  
88 relative increase of heterotrophic/organotrophic genera versus litho-autotrophic organisms.

89 These last organisms, mainly sulfur and iron oxidizers, are common hosts of mining wastes and  
90 contribute to environmental issues linked to production of sulfuric acid and mobilization of  
91 metals, thus a decrease of their abundance and activity in mine waste dumps, during  
92 phytostabilization, should be beneficial (Valentín-Vargas et al., 2018).

93 However, most of the previous studies provided data on the microbial communities of the  
94 surface environment, in the rhizosphere, and in the close non saturated zones. Thus, the  
95 influence of re-vegetation on the underlying microbial communities and on the main  
96 biogeochemical cycles driving the fate of pollutants in the depth of the heaps is not well  
97 documented.

98 A methodology to promote stabilization of metals and plant growth with amendments, in the  
99 surface of aged tailings from a former lead (Pb)-silver (Ag) mine tailing, was previously  
100 developed through microcosm scale experiments (Thouin et al., 2019). The objectives of the  
101 present laboratory pilot experiment were to evaluate in environmental compartments rarely  
102 studied, from the surface to the water saturated level, the influence of amendments and plants  
103 growth on temporal evolution of (1) pore water physico-chemistry, and (2) microbial diversity  
104 along the vertical profile of this tailing.

105

## 106 **2 Material and methods**

### 107 **2.1 Characteristics of the mine tailings, the amendments and the plants**

108 Tailings from the site of the former Ag and Pb mine at Pontgibaud (Puy de Dôme, France) were  
109 used for the pilot-scale experiment. Mine tailings were sampled to a depth of 0–60 cm from a  
110 mine waste heap, using a power shovel (SM1a). Residues were taken in 7 discrete zones within  
111 a 25 m<sup>2</sup> area. The material was homogenized and sieved at 2 cm; it presented the following  
112 particle size distribution: 7% (by weight) < 60 μm, 15% in the 60–315 μm range, and 78% >315

113  $\mu\text{m}$ . The detailed characteristics of this moderately acidic sandy material are given in Thouin  
 114 et al. (2019) and Table 1. The pH of the tailings – consisting mainly of quartz, orthoclase and  
 115 phyllosilicate – was 4.9 (measured in water according to ISO 10390). Tailings contain about  
 116 2.6% Pb and Pb bearing phases were detected by X-ray diffraction (XRD): anglesite ( $\text{PbSO}_4$ )  
 117 and beudantite ( $(\text{PbFe}_3(\text{AsO}_4)(\text{SO}_4)(\text{OH})_6$ ).

118 *Table 1. Main chemical characteristic of the tailings, ochre, manure and biochar. (a) pH was measured in water according to*  
 119 *ISO 10390. (b) Element concentrations in solids were analyzed by high resolution inductively coupled plasma mass*  
 120 *spectrometry and elemental flash pyrolyser. (c) Standard leaching test was performed with demineralized water according to*  
 121 *ISO/TS 21268–2. Pb, Ba and As were analyzed by oven atomic absorption spectroscopy, and Fe and Zn by flame atomic*  
 122 *absorption spectroscopy. DOC and DON were determined using a TOC elemental analyzer.*

	Tailings	Ochre	Manure	Biochar
pH <sup>a</sup>	4.9	8.1	9.9	9.5
<b>Solid chemical analyses<sup>b</sup></b>				
Pb ( $\text{mg.kg}^{-1}$ )	26432	10.1	2.82	<i>n.d.</i>
Fe (%)	1.5	26.1	<i>n.d.</i>	<i>n.d.</i>
Zn ( $\text{mg.kg}^{-1}$ )	265	14717	53.8	<i>n.d.</i>
Ba ( $\text{mg.kg}^{-1}$ )	1063	69.8	78.9	<i>n.d.</i>
As ( $\text{mg.kg}^{-1}$ )	1134	505.7	0.75	<i>n.d.</i>
C (%)	0.13	1.99	35.39	81.2
N (%)	<LQ	<LQ	2.47	0.6
S (%)	1.58	0.69	0.18	<LQ
<b>Standard leaching testing<sup>c</sup></b>				
Pb ( $\text{mg.L}^{-1}$ )	19.6	0.23	0.33	<i>n.d.</i>
Fe ( $\text{mg.L}^{-1}$ )	<LQ	0.4	<LQ	<i>n.d.</i>
Zn ( $\text{mg.L}^{-1}$ )	0.17	<LQ	0.33	<i>n.d.</i>
Ba ( $\text{mg.L}^{-1}$ )	<LQ	<LQ	0.27	<i>n.d.</i>
As ( $\mu\text{g.L}^{-1}$ )	2.3	2.5	7.0	<i>n.d.</i>
DOC ( $\text{mg.L}^{-1}$ )	63.5	44.1	1134.1	<i>n.d.</i>
DON ( $\text{mg.L}^{-1}$ )	8.1	4.4	131.1	<i>n.d.</i>

123 *DOC: dissolved organic carbon, DON: dissolved organic nitrogen, LQ: limit of quantification (SM2) and n.d.: not determined.*

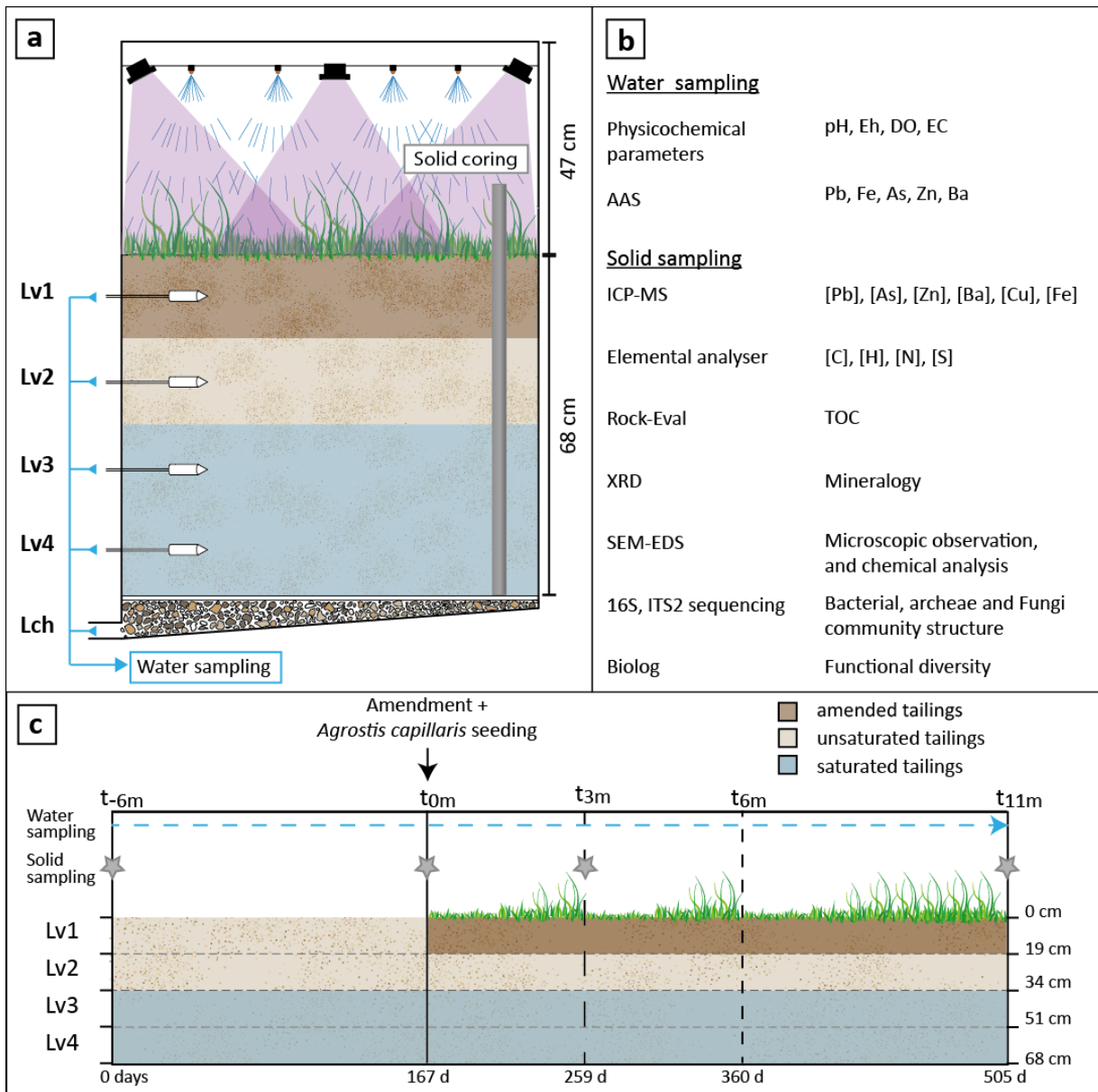
124 The first amendment used was an oxy-hydroxide sludge, called ochre, collected in a coal mine  
 125 water treatment in the south of France, mainly composed of 2-6  $\mu\text{m}$  particles. It contained 26.1  
 126 % Fe, and had a pH value in water of 8.1 (measured according to ISO 10390 ; Thouin et al.,  
 127 2019). A poorly crystalline goethite ( $\text{FeO}(\text{OH})$ ) and aragonite ( $\text{CaCO}_3$ ) were identified, and the  
 128 presence of zincite ( $\text{ZnO}$ ) was suspected (Mertz et al., 2021). The second amendment was cow  
 129 manure, collected from a farm near Orléans (France). Its pH in water was 9.9 (according to ISO  
 130 10390). The manure was dried at 30°C, ground in a mortar and sieved at 2 mm before using.  
 131 The third amendment was biochar, produced from the slow pyrolysis at 500 °C of hardwood  
 132 biomass (mixture of oak, beech, and hornbeam), followed by a sieving to obtain a particle size

133 between 0.5 and 1 mm (Norini et al., 2019). The pH in water was 9.5 measured (according to  
134 ISO 10390) and the CEC of biochar (using the 0.05 N cobaltihexamine method) was 0.65  
135 cmol.kg<sup>-1</sup>. Chemical composition of amendments are detailed in Table 1.

136 *Agrostis capillaris* naturally present on the site was selected for this study. Nandillon et al.,  
137 2019 showed that this indigenous *A. capillaris* is a metallicolous ecotype, more tolerant to the  
138 stress condition of these mine tailings than a commercial non metallicolous ecotype. The seeds  
139 of *A. capillaris* were directly sampled on the Pontgibaud mine site close to the tailings sampling  
140 site.



141 **2.2 Pilot experiment**



142

143 *Figure 1. Design of the laboratory pilot experiment. (a); Analyses performed during the experiment (b) and experimental*  
 144 *program presenting the chronology of the main events (c).*

145 The instrumented laboratory pilot device consisted of a closed stainless-steel column (100 cm  
 146 in diameter and 145 cm high), placed in a temperature-regulated room at  $23 \pm 1^\circ\text{C}$ . The pilot  
 147 design was previously described in Thouin et al. (2017). A layer of inert gravel (centimetric  
 148 quartz) and a geotextile membrane were placed at the bottom of the column to facilitate outflow  
 149 drainage without loss of tailing particles. This single metric pilot experiment (Fig. 1), represents

150 the up-scaling of the assisted phytostabilization method previously developed in multiple  
151 microcosms (Thouin et al., 2019).

152 The column was filled with 814 kg (equivalent to 670 kg of dry weight (dw.)) of the < 2 cm  
153 fraction of homogenized mine tailings (SM1b), and was equipped during the process at three  
154 levels (Lv2: 26.2 cm, Lv3: 43.7 cm, and Lv4: 61.2 cm measured from the surface and after  
155 solids settlement) with time domain reflectometry probes (TDR) (TRIME-PICO32, IMKO) to  
156 measure moisture and temperature in tailings, and with inert Polytetrafluoroethylene/Quartz  
157 porous probes (pore size 2  $\mu\text{m}$ , three probes for each depth, regularly spaced) to sample pore  
158 water. These probes were inserted into mine tailings at 30 cm from the column wall (Fig. 1a).  
159 Five horticultural light-emitting diodes (LED) at the top of the column simulated 12-h day/night  
160 cycles. Rainfall was simulated by a sprinkler system connected to a water reservoir and fed by  
161 pumps, and consisted in the watering of 5.5 L of Mont Roucouc<sup>®</sup> water each time and three  
162 times a week. This mineral water was used because of its chemical composition close to that of  
163 rainwater (pH = 6.38, electrical conductivity = 160  $\mu\text{S}\cdot\text{cm}^{-1}$ ,  $\text{Cl}^- = 56 \mu\text{M}$ ,  $\text{NO}_3^- = 24 \mu\text{M}$ ,  $\text{SO}_4^{2-}$   
164  $= 16 \mu\text{M}$ ,  $\text{Na}^+ = 109 \mu\text{M}$ ,  $\text{K}^+ = 7.7 \mu\text{M}$ ,  $\text{Ca}^{2+} = 25 \mu\text{M}$ ,  $\text{Mg}^{2+} = 21 \mu\text{M}$ ).

165 After three months of stabilization, the tailings settled by 12.5 % in height, and the thickness of  
166 the tailings in the column reached a constant value of 68 cm. The first experimental step  
167 corresponded to the control non-phytostabilized condition, which lasted 6 months ( $t_{-6\text{m}}$  to  $t_{0\text{m}}$ ).  
168 Both levels Lv4 and Lv3 were always in the saturated part of the column all along the  
169 experiment, whereas the Lv2 level was located in the unsaturated zone. The leachate was  
170 collected in a steel tank at the bottom, noted Lch, and quantified using scales. This outflow  
171 weight as well as moisture and temperature at the three levels (Lv2, Lv3 and Lv4) were  
172 continuously monitored.

173 After 6 months ( $t_{0m}$ , corresponding to 167 days after starting the monitoring of the experiment,  
174 Fig. 1c), 19 cm of tailings surface were stripped (195 kg, equivalent to 173 kg of dw.), and  
175 amended with 1 wt% ochre, 1 wt% cow manure and 1 wt% biochar (i.e. 1.73 kg of each  
176 amendment), homogenized, then replaced at the top of the column (SM1c). This tailing  
177 amended level (Lv1) was instrumented with one TDR and three porous probes at 9.5 cm from  
178 the surface, regularly spaced. The amendment was followed by the seeding of 4.1 g of *A.*  
179 *capillaris* on all the tailings surface.

180 Sampling of pore water from levels Lv1, Lv2, Lv3, Lv4 and Lch was performed (before water  
181 sprinkling) via fluorinated ethylene propylene (FEP) tubing connected to a peristaltic pump.  
182 The 3 water samples from the 3 independent regularly spaced porous probes of each level (Lv1,  
183 Lv2, Lv3 and Lv4) were pooled before determination of physicochemical and geochemical  
184 parameters. In Lv1, water sampling was limited due to the low water contents and to the  
185 competition with *A. capillaris* roots, this is why there were no water analyses after 231 days.

186 Solid sampling was performed in the column by coring at  $t_{-6m}$ ,  $t_{0m}$ ,  $t_{3m}$  and  $t_{11m}$  (at  $t_{0m}$  sampling  
187 was performed before amendment). Coring was performed with 5 cm diameter and 1m length  
188 stainless steel pipes. Only one coring was carried out at each experimental step, in order to  
189 minimize disruptive effects. After sampling, the pipes were clogged and put back in place in  
190 the tailings to fill the empty space. Each core was separated into four samples, Lv1 at a depth  
191 of 0-19 cm, Lv2 at 19-34 cm, Lv3 at 34-51 cm, and Lv4 at 51-68 cm, corresponding to the pore  
192 water sampling levels (Fig. 1c). These samples were homogenized, sub-sampled in sterile tubes  
193 stored at  $-20^{\circ}\text{C}$  for DNA extractions then stored at  $5^{\circ}\text{C}$  in tightly closed sterile jars for the  
194 determination of functional diversity of microbial communities.

195 The sampling of *A. capillaris* biomass and rhizospheric and non-rhizospheric materials were  
196 performed at  $t_{3m}$ ,  $t_{6m}$  and  $t_{11m}$ , (respectively 92, 193 and 338 days after seeding). Five plastic  
197 (polystyrene) tubes with an area of  $19.6\text{ cm}^2$  and a height of 7 cm were used to make small

198 cores of level Lv1. For each sampled *A. capillaris*, non-rhizospheric (Lv1 NR) tailings fraction  
199 was separated from rhizospheric (Lv1 R) materials by shaking, and rhizospheric-associated  
200 tailings particles were manually isolated from *A. capillaris* roots. The plants of *A. capillaris*  
201 were washed with 0.5 mM CaCl<sub>2</sub> and deionized water and plant biomass was measured after  
202 plant drying at 60°C during 48h (roots and leaves separately). The total plant cover of the  
203 tailings surface was estimated by eye in percentage and was used to extrapolate the total  
204 biomass of *A. capillaris* defined in gram of dry leaves and roots by m<sup>2</sup>. At t<sub>3m</sub> and t<sub>6m</sub>, the *A.*  
205 *capillaris* plants were cut at about 5 cm above the tailings' surface to simulate mowing and  
206 leaves were recovered.

### 207 **2.3 Water analyses**

208 Electrical conductivity (EC), redox potential (Eh), pH, dissolved oxygen (DO), and temperature  
209 of interstitial water were measured with a benchtop meter (multiparameter analyser C3040,  
210 Consort) connected to an ORP sensor (platinum electrode, with an Ag/AgCl reference  
211 electrode, 3M KCl, Eh value refers to the normal hydrogen electrode (NHE)), oxygen probes  
212 (Fisherbrand), pH electrode (Fisherbrand) and conductivity probe (Sentek) (Fig. 1b, SM2). As,  
213 Pb and Ba were analyzed by oven atomic absorption spectroscopy (AAS)  
214 (Varian AA220Z), and Zn and Fe by flame AAS (Varian AA22FS) (SM2).

### 215 **2.4 Analyses of solids**

#### 216 **2.4.1 Mineralogy and chemical composition**

217 Solid samples were dried and ground to 70 µm in an agate mortar for X-ray diffraction (XRD)  
218 and chemical analyses (SM2). XRD was performed using an INEL CPS120 diffractometer  
219 equipped with a Co anode. Measurements of total carbon (C), hydrogen (H), nitrogen (N), and  
220 sulfur (S) were obtained using an elemental flash pyrolyser analyzer (Flash 2000, Thermo  
221 Fisher Scientific) (SM2). Total organic carbon (TOC) contents were determined by Rock-Eval  
222 pyrolysis (Rock-Eval 6 Turbo, Vinci Technologies) (SM2). Elemental concentration

223 measurements of all solid samples were performed on high resolution inductively coupled  
224 plasma mass spectrometry (HR-ICP-MS ; Thermo Element-XR, details in SM2) after complete  
225 dissolution by hydrofluoric acid attack (HNO<sub>3</sub> 65%, HCl 37% and HF 40%) using a pressurized  
226 closed-vessel microwave system (MARS 6, CEM).

#### 227 **2.4.2 Optical and SEM-EDS observations**

228 Fresh tailings material sampled in Lv1 at the end of the experiment was observed with a  
229 binocular magnifier Leica 2616 APO equipped with a photography and video device and  
230 connected to a computer equipped with Leica Mycosystem software. Scanning electron  
231 microscopy coupled with energy dispersive X-ray spectroscopy (SEM-EDS) observations were  
232 performed on a TM 3000 (Hitachi) at 15 kV accelerating voltage. SEM was coupled to a  
233 SwiftED3000 X-Stream module (Hitachi). The acquisition time of EDS point analyses was 300  
234 seconds and 15 minutes for maps with a resolution of 512 x 384 pixels.

#### 235 **2.4.3 DNA extraction and next generation sequencing**

236 Total nucleic acids were extracted in duplicate from 0.5 g (wet weight) of each solid sample  
237 with a FastDNA® SPIN Kit for soil (MP Biomedicals, Illkirch, France). DNA was quantified  
238 with the Promega Quantifluor®, using the Quantus™ fluorometer (Promega, France).

239 For next generation sequencing, a fragment of the gene coding 16S rRNA (bacteria and archaea)  
240 was amplified using the barcoded, universal primer set (515WF/918WR) (Wang et al., 2009).  
241 The primers used to amplify the specific fungal barcode ITS2 were w404 and w40 (Degois et  
242 al., 2019). PCR reactions were performed using AccuStart II PCR ToughMix kit and cleaned  
243 (HighPrep PCR beads, Mokascience). Pools were submitted for sequencing on Illumina MiSeq  
244 instrument at GeT-PlaGe (Auzeville, France). The raw datasets are available on the European  
245 Nucleotide Archive system under project accession number PRJEB52405 and PRJEB52412.

246 Sequences were processed using the FROGS (v.3.0) bioinformatics pipeline (Escudié et al.,  
247 2017), implemented into the Genotoul platform of the Galaxy server of Toulouse. Fastq paired  
248 reads were merged with FLASH software, and clustering into OTU with Swarm and an  
249 aggregation distance clustering of 3. Chimera and operational taxonomic unit (OTU) with a  
250 proportion less than 0.005 % of all sequences were removed. Taxonomic affiliation was  
251 performed using BLASTn and the Silva 132 database for 16S rRNA sequences and the Unite  
252 Fungi 8.0 database for ITS2 sequences. Filtration on taxonomic affiliation was done at  
253 minimum identity of 100% and minimum coverage of 95%. The FROGS pipeline implemented  
254 Phyloseq R package was used for OTU structure visualization, rarefaction curves, and alpha  
255 diversity index calculations. Chao 1 index (non-parametric measurements) estimates the  
256 number of unobserved species from those observed 1 or 2 times while the Shannon and  
257 invSimpson diversity indexes give respectively a measure of heterogeneity of microbial  
258 community and the probability that two randomly picked individuals do not belong to the same  
259 species.

#### 260 **2.4.4 Functional diversity of heterotroph microorganisms**

261 Biolog™ Ecoplates community-level substrate utilization assays were used to study the  
262 functional diversity of microbial communities (Garland, 1999). The Biolog™ Ecoplates were  
263 incubated at 25 °C and the absorbance values (optical density (OD) at 590 nm) were monitored  
264 using an Omega SPECTROstar (BMG Labtech) microplate spectrophotometer at t=0 and 168  
265 h (7 days). The well absorbance values were adjusted by subtracting the average absorbance of  
266 the control well (water only) at t=0. After subtracting the control well, an OD<sub>590nm</sub> > 0.25 was  
267 considered as positive. Negative readings (OD < 0) were set to zero for all subsequent analyses.

#### 268 **2.5 Statistical analysis**

269 Statistical analysis were performed using R (<http://www.r-project.org/>; version 4.3.0) with the  
270 vegan package (version 2.5-7). To visualize differences in the OTUs-based community

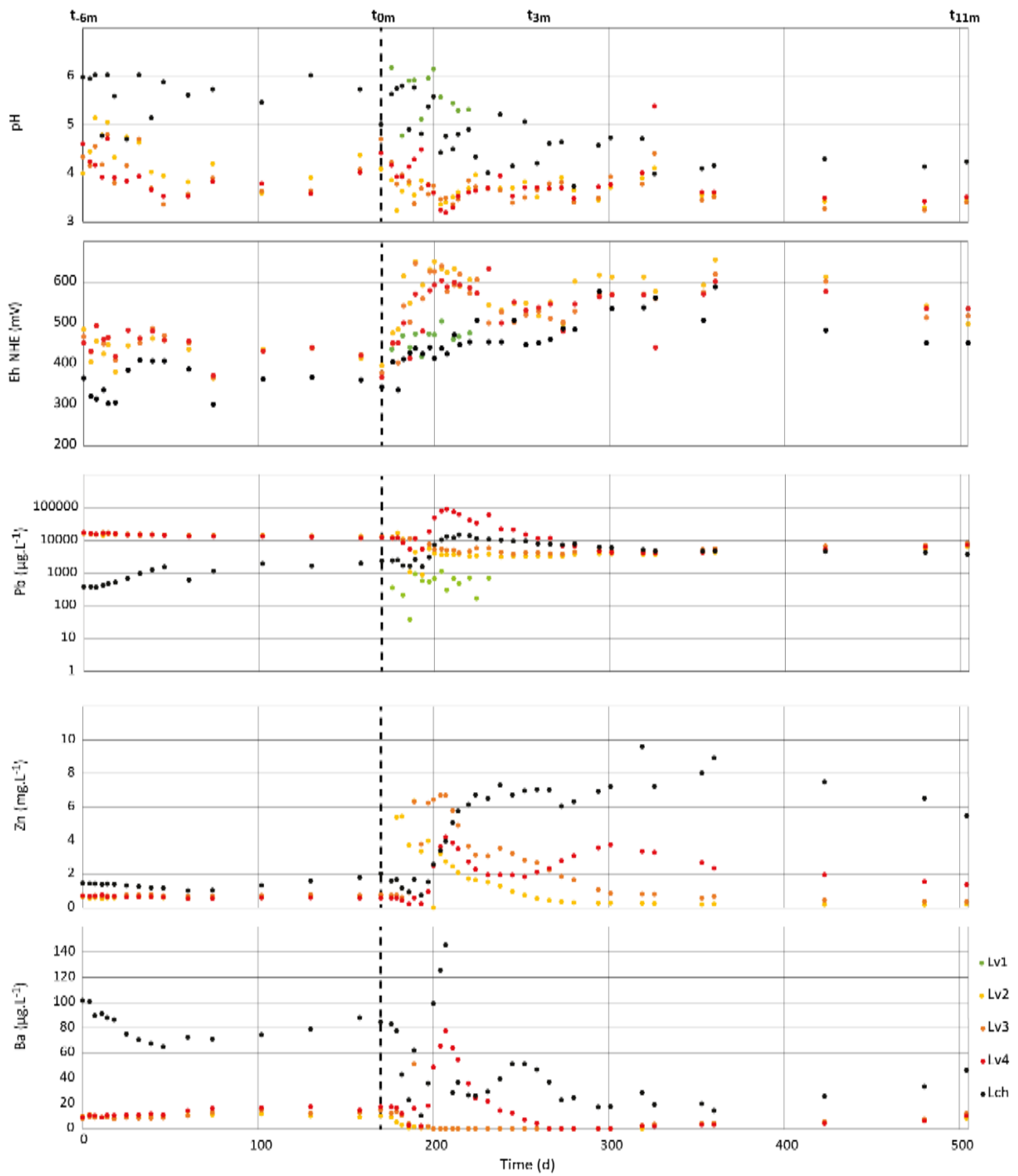
271 composition, we performed an agglomerative hierarchical clustering (AHC) on the 100 most  
272 abundant OTUs of 16S rRNA and ITS 2 sequencing data, using Bray-Curtis dissimilarity  
273 metric, and Ward's minimum variance. Redundance analysis (RDA) was performed to identify  
274 the relationships among samples, environmental variables, and relative abundance of phyla.  
275 This analysis was calculated based on Hellinger - transformed phylum abundance basis of 16S  
276 rRNA and ITS 2 data (Borcard et al., 2011). Environmental explanatory tested variables were  
277 as follows: humidity, pH, TOC, [S], [N], [Fe], [Pb], [Zn], [As], [Cu], [Ba] (solid concentrations  
278 at  $t_{0m}$ ,  $t_{3m}$ ,  $t_{11m}$ ) and were standardized. The function ordistep in vegan package was used to  
279 identify the significant explanatory forward environmental variable. The significance of the  
280 RDA model was tested by ANOVA based on Monte Carlo test with 999 permutations. This  
281 analysis helps to determine the most influential factors and the extent that various  
282 environmental parameters affected microbial composition.

## 283 **3 Results**

### 284 **3.1 Percolated water composition**

#### 285 **3.1.1 Physico-chemical parameters**

286 Pore-waters in the subsurface levels of the column (Lv2, Lv3, and Lv4) were characterized by  
287 constant pH values between 4 and 3, whether before or after the amendment (Fig. 2). In Lch,  
288 the pH was higher but decreased from 6 to 4 after amendment of the tailings. In Lv1, the pore  
289 water pH was higher than in Lv2, Lv3, and Lv4. Values of redox potential was positive ( $>300$   
290 mV NHE) before amendment, even in the saturated levels (Fig. 2). After amendment, Eh NHE  
291 increased in all sampling level and remained positive during the experiment.



292

293 *Figure 2. Evolution of water pH, Eh NHE, Pb, Zn and Ba concentrations in water all along the pilot experiment. Lv1 to Lv4*  
 294 *represent the sampling depths in the tailings. Lv1 was amended and seeded at t<sub>0m</sub>. Lch was the outlet of the pilot.*

295

296



### 297 **3.1.2 Metal(loid)s concentrations**

298 Pb was the metal with the highest concentration in tailings' water and leachate. Before  $t_{0m}$ , Pb  
299 concentrations were similar and constant in Lv2, Lv3, and Lv4 with values of  $14.4 \text{ mg.L}^{-1}$  (Fig.  
300 2). In Lch, Pb concentrations were lower and increased to reach  $2.3 \text{ mg.L}^{-1}$  just before the  
301 amendment. In the amended level Lv1, mean Pb concentration was  $532 \pm 77 \text{ } \mu\text{g.L}^{-1}$ . In Lv2 and  
302 Lv3, Pb rapidly decreased after the amendment to values close to 3 300 and 4 000  $\mu\text{g.L}^{-1}$   
303 (respectively), then between  $t_{3m}$  and  $t_{11m}$ , Pb slightly increased to reach 8 160 and 7 150  $\mu\text{g.L}^{-1}$   
304 at the end of the experiment. At the bottom of the column, in Lv4, Pb concentration rapidly  
305 increased after the amendment to reach a maximum of 88 900  $\mu\text{g.L}^{-1}$  on day (d) 207 then  
306 decreased down to 4 100  $\mu\text{g.L}^{-1}$  on d 319. As for Lv4, Pb concentration in Lch increased after  
307 the amendment to reach a maximum of 14 775  $\mu\text{g.L}^{-1}$  at d 214 and then decreased down to 3  
308 700  $\mu\text{g.L}^{-1}$  at  $t_{11m}$ .

309 During the period before the amendment, Ba concentrations were between 8.2 and 18  $\mu\text{g.L}^{-1}$  in  
310 Lv2, Lv3 and Lv4, and between 101 and 65  $\mu\text{g.L}^{-1}$  in Lch (Fig. 2). After  $t_{0m}$ , Ba concentration  
311 rapidly decreased below 2  $\mu\text{g.L}^{-1}$  in Lv2 and Lv3, while in Lv4 it increased to a maximum of  
312 77.6  $\mu\text{g.L}^{-1}$  on d 207 and decreased under 2  $\mu\text{g.L}^{-1}$  on d 300. Between d 300 and the end of the  
313 experiment, Ba concentration in Lv2, Lv3 and Lv4 slowly increased to about 10  $\mu\text{g.L}^{-1}$ . In Lch,  
314 two peaks were observed after the amendment, at 207 days (145.6  $\mu\text{g.L}^{-1}$ ) and at 252 (51.4  
315  $\mu\text{g.L}^{-1}$ ).

316 As concentrations in pore water in Lv2, Lv3 and Lv4 were less than 15  $\mu\text{g.L}^{-1}$  throughout the  
317 experiment (SM3). In Lch, As concentration decreased from 233 to 13  $\mu\text{g.L}^{-1}$  between  $t_{6m}$  and  
318  $t_{0m}$ . After the amendment As decreased below 5  $\mu\text{g.L}^{-1}$  and then increased at the end of  
319 experiment to reach 26.7  $\mu\text{g.L}^{-1}$ .

320

321 **3.2 Evolution of the solids**

322 The evolution of parameters related to organic matter and the main contaminants in solids is  
 323 presented in Table 2. Amendment slightly increased the concentration in carbon (total C, TOC)  
 324 in Lv1 and in Lv2, but not at all in the deeper levels Lv3 and Lv4. The other analysed elements  
 325 did not vary according to experimental time and depth of layer in the column. In particular, Pb,  
 326 the main contaminant, did not exhibit significant trends as a function of depth and time. One  
 327 can only note slightly lower Pb contents in the rhizospheric area compared to the non-  
 328 rhizospheric one. Zn contents slightly increased in the upper levels (Lv1 and Lv2) just after the  
 329 addition of amendments ( $t_{3m}$ ), due to the presence of Zn in ochre, then returned to values close  
 330 to that of the tailings.

331 X-ray diffractograms of the tailings sampled at different depths (Lv1, Lv2, Lv3, and Lv4) at  
 332 the end of the pilot experiment revealed no change of the mineralogy (SM4): quartz, orthoclase,  
 333 anglesite, beudantite and a phyllosilicate (presumably muscovite) were the main crystalline  
 334 phases, as previously identified in the initial tailings (Mertz et al., 2021).

335 *Table 2 Concentrations of the main interesting constituents in the solids sampled in four depths and at different periods of the*  
 336 *pilot experiment, and analyzed by <sup>a</sup> Flash pyrolyzer analyzer, <sup>b</sup> Rock-Eval 6 and <sup>c</sup> HR-ICP-MS.*

Depth	C <sup>a</sup> (%)	N <sup>a</sup> (%)	S <sup>a</sup> (%)	TOC <sup>b</sup> (%)	Fe <sup>c</sup> (mg.kg <sup>-1</sup> )	Pb <sup>c</sup> (mg.kg <sup>-1</sup> )	Zn <sup>c</sup> (mg.kg <sup>-1</sup> )	As <sup>c</sup> (mg.kg <sup>-1</sup> )	Cu <sup>c</sup> (mg.kg <sup>-1</sup> )	Ba <sup>c</sup> (mg.kg <sup>-1</sup> )
Lv1	0.10	0.06	0.68	0.09	15 085	23 817	321	1 384	124	3 631
Lv2	0.09	0.06	n.d.	0.08	19 450	24 166	327	1 398	74	3 444
Lv3	0.23	0.04	0.71	0.08	15 493	26 234	347	1 381	131	4 635
Lv4	0.04	0.02	0.72	n.d.	15 246	22 712	381	1 319	128	4 133
Lv1 R	1.00	0.05	1.12	0.96	14 166	19 722	521	1 127	119	3 452
Lv1 NR	1.04	0.04	1.05	1.02	12 793	27 594	419	969	107	7 706

	Lv2	0.99	0.06	0.65	0.97	19 000	28 479	484	1 404	69	3 974
	Lv3	0.18	0.06	n.d.	0.11	17 304	27 857	371	1 598	71	4 535
	Lv4	0.09	0.05	0.77	n.d.	14 336	20 848	345	1 235	126	4 955
<hr/>											
	Lv1 R	1.28	0.07	1.04	0.98	13 280	21 644	360	988	101	2 503
	Lv1 NR	1.00	0.07	0.68	0.85	16 912	29 835	308	1 083	52	3 340
t <sub>11m</sub>	Lv2	0.61	0.05	0.72	0.56	18 386	27 786	434	1 515	73	4 321
	Lv3	0.15	0.03	0.70	0.11	13 935	30 940	285	1 059	52	3 605
	Lv4	0.07	0.02	0.74	n.d.	12 766	27 696	368	1 054	91	3 051

337 Initial tailing composition is available in table 1. TOC: total organic carbon, Lv1 to Lv4 represent the sampling depths in the  
338 tailings. Lv1 R: solid sampled in the rhizospheric zone, Lv1 NR: solid sampled in the non rhizospheric zone, t<sub>0m</sub>: 6 months after  
339 the beginning of the monitoring without amendments and plants, t<sub>3m</sub>: 3 months after adding amendments and seeding *Agrostis*  
340 *capillaris*, t<sub>11m</sub>: end of the experiment, 11 months after adding amendments and seeding *A. capillaris*, and n.d.: not determined.

341

### 342 3.3 *Agrostis capillaris* development

343 Three months after seeding (t<sub>3m</sub>), thin leaves of *Agrostis capillaris* have grown and a dense  
344 implantation with about 80% of surface cover was observed (Table 3). The biomass was about  
345 190 g.m<sup>2</sup> for leaves and stems and 57 g.m<sup>2</sup> for roots. At t<sub>3m</sub>, *A. capillaris* was cut, and 3 months  
346 later, a reduced plant cover (50%) was observed but with larger and better developed leaves.  
347 Root biomass has also increased between t<sub>3m</sub> and t<sub>6m</sub>. A second cut was made at t<sub>6m</sub>. At the end  
348 of the pilot experiment, 11 months after seeding, the biomass of aerial parts was multiplied by  
349 four (SM1d). *A. capillaris* with stolon was observed and plant cover increased between t<sub>6m</sub> and  
350 t<sub>11m</sub>. Measured root biomass seemed to drastically decrease, but only roots in the first 7  
351 centimeters were weighted whereas roots had colonized the entire thickness of the amended  
352 level and were observed up to 40 cm deep.

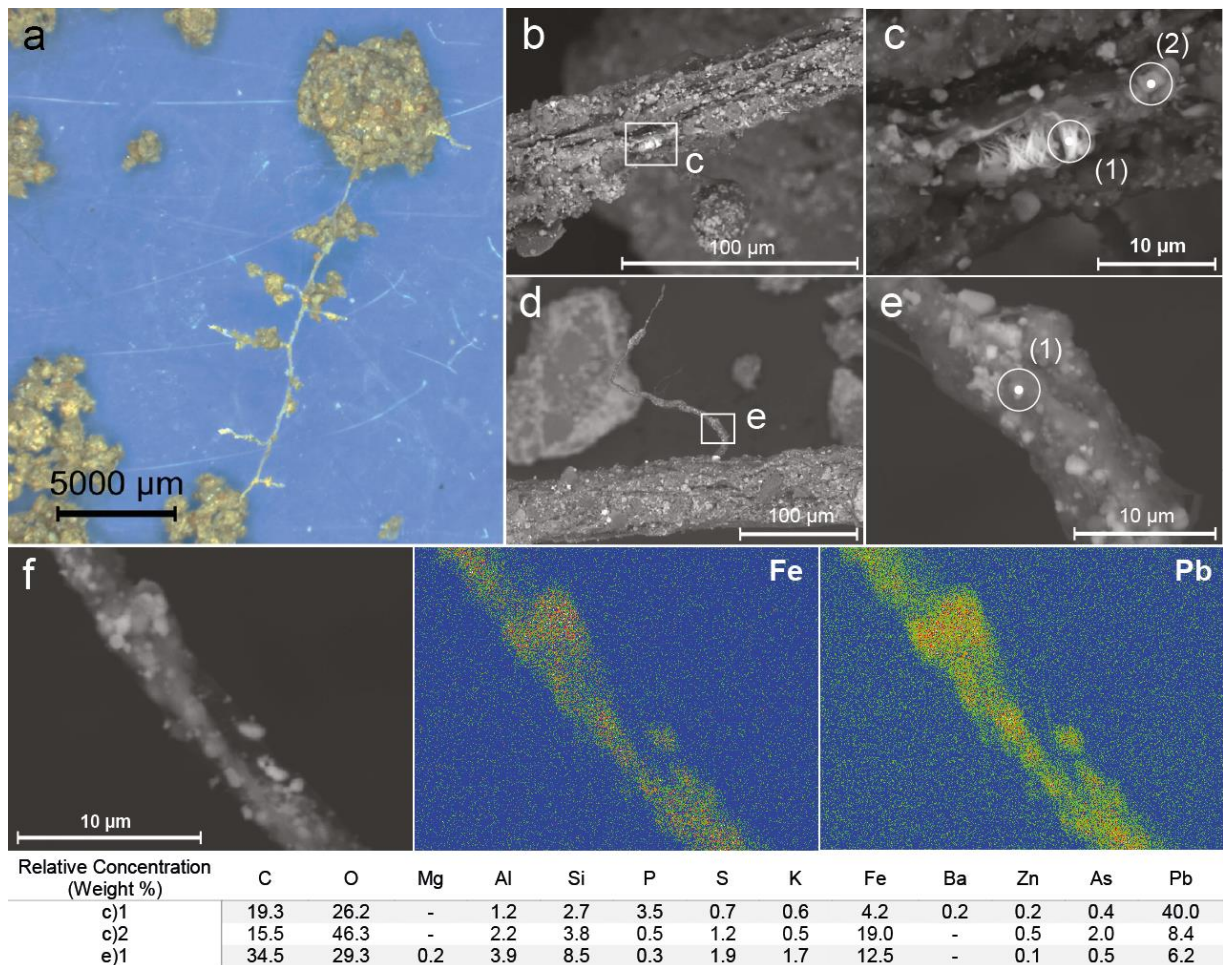
353 *Table 3 Agrostis capillaris* development. Plant cover was estimated visually and the biomass was measured for a determined  
 354 surface where the *A. capillaris* plants were well developed. The extrapolated biomass was determined with biomass and plant  
 355 cover. \* Root biomass was determined only in the first 7 cm.

	Plant cover (%)	Biomass sampling		Extrapolated biomass	
		Aerial Parts (g)	Roots* (g)	Aerial Parts (g.m <sup>2</sup> )	Roots* (g.m <sup>2</sup> )
t <sub>3m</sub>	80	2.4	0.7	191.5	57.0
t <sub>6m</sub>	50	2.4	1.2	119.7	73.3
t <sub>11m</sub>	70	9.8	0.2	700.9	11.4

356 *t<sub>3m</sub>, t<sub>6m</sub>, t<sub>11m</sub>*: respectively 3 months, 6 months and 11 months after adding amendments and seeding *A. capillaris*.

### 357 **3.4 Tailings stabilization by *Agrostis capillaris* root system**

358 The surface of the tailings at the end of the pilot experiment was visually invaded by the  
 359 *Agrostis capillaris* root system. The binocular magnifier enabled to observe solid particles  
 360 forming immobilized tailing aggregates associated with the roots (Fig. 3a). Some fragments of  
 361 roots observed with SEM-EDS presented fibrous coverage (Fig. 3b) enriched in P and Pb (Fig.  
 362 3c point 1) compared with the simple solid particles present at the root surface (Fig. 3c point  
 363 2). Fine filaments of 3-4  $\mu$ m diameter were observed in the rhizosphere (Fig. 3d, e and f). They  
 364 might be fungal hyphae. EDS mapping revealed the presence of Fe and Pb on their surface and  
 365 inside their membrane (Fig. 3e point 1, and f).



366

367 *Figure 3. Observations of Agrostis capillaris root samples at the end of the pilot experiment (t<sub>11m</sub>): (a) aggregates of tailings*  
 368 *associated with roots (binocular magnifier); (b) and (c) backscattered electron images of a root (SEM); (d) and (e)*  
 369 *backscattered electron images of a presumed fungal hyphae, and (f) backscattered electron images and elemental mapping of*  
 370 *Fe and Pb of presumed fungal hyphae; Table present the results of SEM-EDS semi-quantification of elements at the surface of*  
 371 *the (c) observed root and (e) inside hyphae membrane.*

372

### 373 3.5 Microbial diversity and structure

#### 374 3.5.1 Community species richness and diversity

375 After treatment steps, a total of 790 820 16S and 920 302 ITS 2 sequences were obtained from  
 376 the 19 samples. The rarefaction curves reach a plateau for all samples showing that the diversity  
 377 of communities was well sampled (SM5). The richness of 16S OTUs was homogeneous before  
 378 amendment at t<sub>-6m</sub> and t<sub>0m</sub>, with values varying between 431 in Lv3 t<sub>-6m</sub> and 752 in Lv4 t<sub>0m</sub>.  
 379 After amendment, an increase of richness was observed except in Lv4 t<sub>11m</sub>. Chao1 index shows

380 that the majority of OTUs was sequenced (Table 4). The richness of ITS2 sequences was lower  
 381 than 16S sequences. As with bacteria, the number of OTUs increased after amendment in all  
 382 levels except in the deepest level at the end of the experiment. The bacterial species diversity,  
 383 based on the Shannon and InvSimpson indexes (Table 4) increased after amendment in the  
 384 surface levels Lv1. In Lv2 and Lv3 an increase of diversity indexes was also observed at  $t_{3m}$   
 385 and  $t_{11m}$ . The lowest Shannon and InvSimpson indexes were observed in Lv4 at  $t_{11m}$ . For the  
 386 fungi, the evolution of Shannon and InvSimpson indexes were less obviously related to the  
 387 experimental conditions.

388 *Table 4: 16S and ITS 2 sequences diversities estimation of the solids sampled in four depths and at different periods of the pilot*  
 389 *experiment.*

16S		Richness	Chao1	Shannon	InvSimpson
$t_{-6m}$	Lv1	576	612.7	4.2	16.4
	Lv2	621	673.9	4.4	20.6
	Lv3	598	635.8	4.3	18.8
	Lv4	752	810.1	4.4	18.6
$t_{0m}$	Am	448	561.8	4.7	45.4
	Lv1	613	652.1	4.6	28.1
	Lv2	549	572.8	4.1	14.8
	Lv3	431	444.0	4.3	19.0
	Lv4	550	595.2	4.1	15.4
$t_{3m}$	Lv1 R	918	1022.2	5.4	86.6
	Lv1 NR	902	997.5	5.4	85.7
	Lv2	953	1046.9	5.4	81.8
	Lv3	880	986.0	4.7	28.5
	Lv4	938	1022.5	4.1	14.3
$t_{11m}$	Lv1 R	747	825.5	5.2	58.4
	Lv1 NR	829	927.3	4.9	42.1

Lv2	910	1031.9	5.4	76.2
Lv3	875	971.2	4.3	18.6
Lv4	318	366.4	3.4	14.3

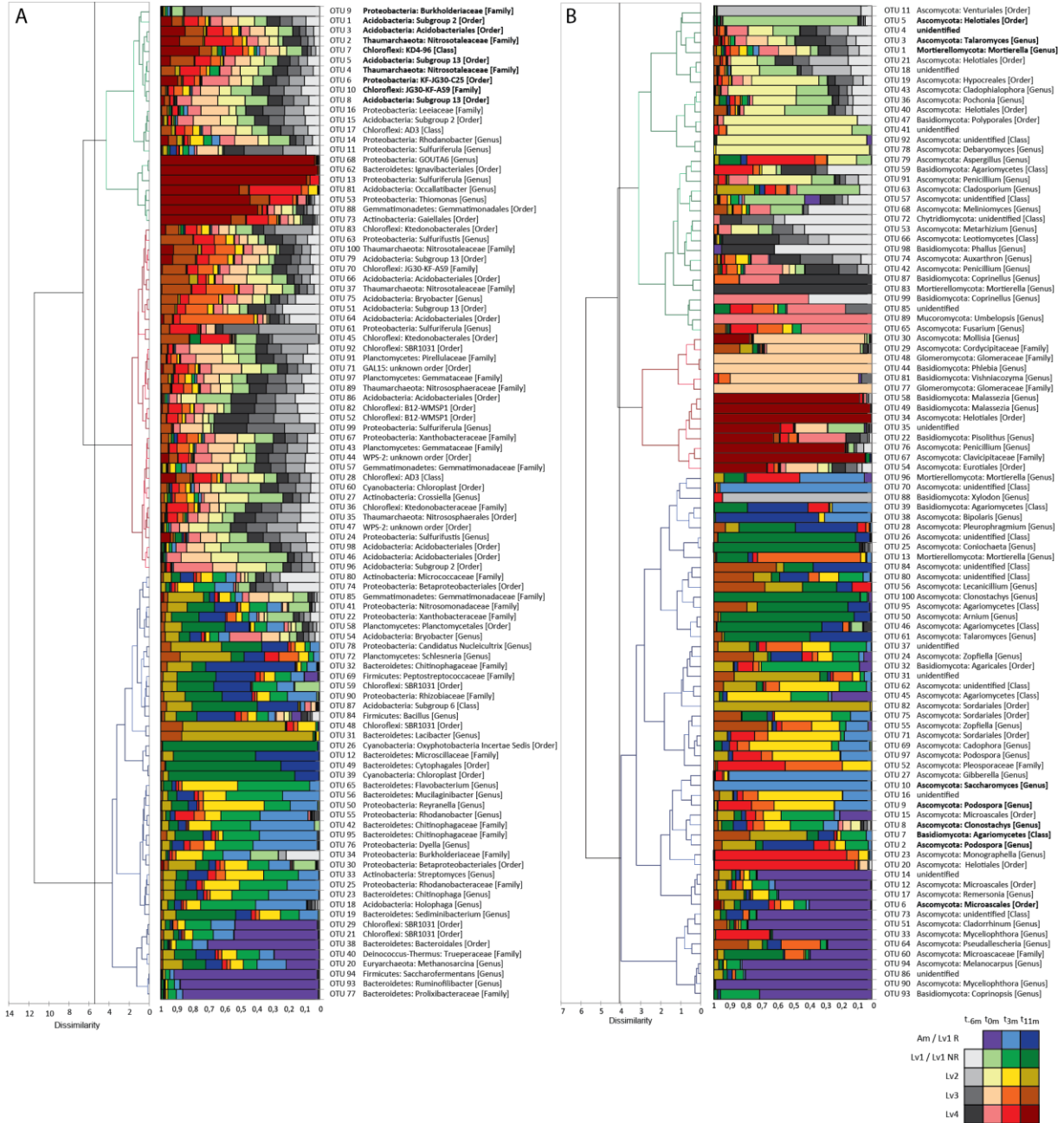
ITS 2		Richness	Chao1	Shannon	InvSimpson
t <sub>-6m</sub>	Lv1	56	61.0	2.3	4.8
	Lv2	51	58.5	1.9	4.2
	Lv3	61	61.5	1.8	2.9
	Lv4	56	63.0	2.1	4.2
t <sub>0m</sub>	Am	91	91.1	2.3	6.4
	Lv1	42	48.0	1.7	3.0
	Lv2	35	45.0	2.2	5.4
	Lv3	26	35.3	1.8	3.4
	Lv4	65	70.3	1.8	2.7
t <sub>3m</sub>	Lv1 R	107	116.2	2.2	5.2
	Lv1 NR	105	106.9	2.6	8.4
	Lv2	115	118.5	2.7	8.7
	Lv3	93	93.0	2.7	7.5
	Lv4	82	92.0	2.8	9.7
t <sub>11m</sub>	Lv1 R	105	115.5	2.1	3.6
	Lv1 NR	113	117.2	3.0	13.0
	Lv2	117	119.5	2.5	6.2
	Lv3	78	81.0	2.9	8.6
	Lv4	16	22.0	2.3	8.3

390 *Lv1 to Lv4 represent the sampling depths in the tailings. Am: tailings amended with 1 wt% ochre, 1 wt% cow manure and 1*  
391 *wt% biochar, Lv1 R: solid sampled in the rhizospheric zone, Lv1 NR: solid sampled in the non rhizospheric zone, t<sub>-6m</sub>: start of*  
392 *the experiment, t<sub>0m</sub>: 6 months after the beginning of the monitoring without amendments and plants, t<sub>3m</sub>: 3 months after adding*  
393 *amendments and seeding *Agrostis capillaris*, t<sub>11m</sub>: end of the experiment, 11 months after adding amendments and seeding *A.**  
394 *capillaris.*

### 395 3.5.2 Microbial community structure

396 Samples of solids from the pilot experiment hosted bacterial and fungal communities presenting  
397 different spatial and temporal distributions (Fig. 4). The 100 most represented phyla of  
398 procaryotes (Fig. 4A) included Proteobacteria, Acidobacteria, Chloroflexi, Bacteroidetes,  
399 Gemmatimonadetes, Actinobacteriaceae, Planctomycetes, Cyanobacteria, Firmicutes,  
400 members of the Deinococcus-Thermus group for the bacteria, then Thaumarchaeota and  
401 Euryarchaeota for the archaea. The AHC analysis, that groups the OTUs according to  
402 similarities of occurrence in samples, shows a cluster of OTUs, gathered in the upper part of  
403 the Fig. 4A, that were mainly present in the tailings before amendment ( $t_{-6m}$  and  $t_0$ ) and in the  
404 deepest levels of the column (Lv3 and Lv4) all along the experiment (OTUs n°9 to n°96). The  
405 ten most abundant procaryote OTUs (in bold) were present in this group that included  
406 Acidobacteria, Chloroflexi, Proteobacteria and Thaumarchaeota, however none of them was  
407 identified at the genus level (SM6). Procaryote OTUs more represented in surface levels from  
408  $t_{3m}$  to  $t_{11m}$ , and influenced by the amendment and plant growth, are grouped in three clusters in  
409 the bottom part of Fig. 4A. The cumulative list of the ten most represented known bacterial  
410 genera in each sample (SM7), included three sulfur-oxidizing bacteria, namely *Sulfuriferula*,  
411 *Sulfurifustis* and *Thiomonas*, and two autotrophic Fe-oxidizing bacteria, i.e. *Gallionella* (only  
412 in Lv4 at  $t_{3m}$ ) and *Sideroxydans* (only in Lv4 at  $t_{11m}$ ). All the 44 other major genera are  
413 heterotrophic organisms. A known nitrogen fixing genus, *Mesorhizobium*, was present in the  
414 list of the 10 most represented procaryote OTUs in Lv1, both in the rhizospheric and non  
415 rhizospheric samples. The contribution of the 3 sulfur-oxidizing bacteria, expressed as the sum  
416 of their relative abundance in the 10 most abundant identified genera (SM8 and SM9), shows  
417 their preponderance in the initial tailings. Their relative abundance strongly decreased in Lv1,  
418 Lv2 and Lv3 at  $t_{3m}$ . At the end of experiment their contribution rose in Lv3 and Lv4.





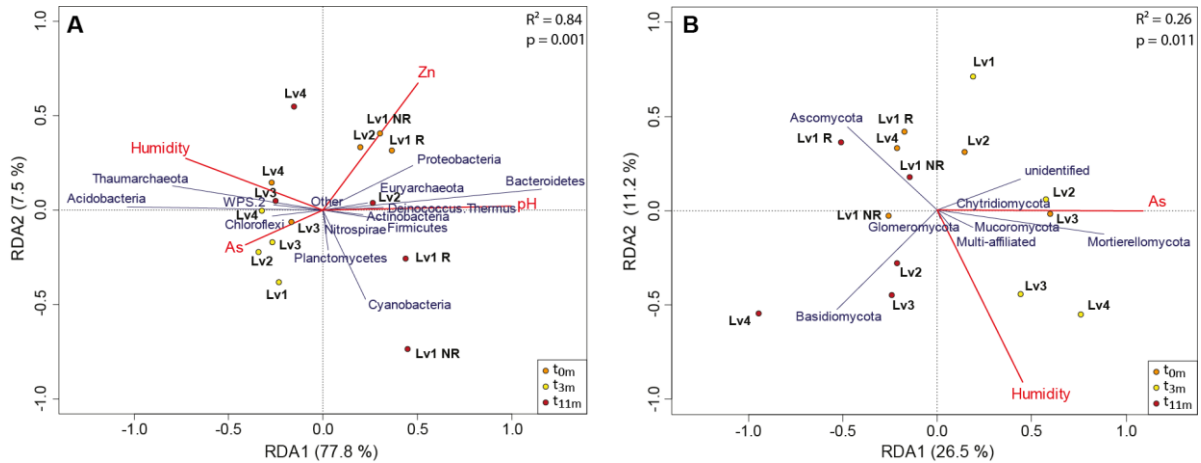
420  
 421 *Figure 4. AHC analysis of the 100 most abundant OTUs (10 most abundant in bold) in solid samples from the pilot experiment.*  
 422 *A: bacteria and archaea (based on 16S sequences), B: fungi (based on ITS sequences) (Lv1 to Lv4 represent the sampling*  
 423 *depths in the tailings, Am: tailings amended with 1 wt% ochre, 1 wt% cow manure and 1 wt% biochar, Lv1 R: solid sampled*  
 424 *in the rhizospheric zone, Lv1 NR: solid sampled in the non rhizospheric zone, t-6m: start of the experiment, t0m: 6 months after*  
 425 *the beginning of the monitoring without amendments and plants, t3m: 3 months after adding amendments and seeding *Agrostis**  
 426 *capillaris, t11m: end of the experiment, 11 months after adding amendments and seeding *A. capillaris*).*

427 The distribution of the 100 most abundant fungal OTUs (Fig. 4B) shows some tendencies  
 428 already observed with bacterial OTUs, such as a group of fungal OTUs mainly found in the  
 429 initial tailings, at t-6m in all levels, and t0m from Lv2 to Lv4 gathered at the top of Fig. 4B.

430 Another group of OTUs was mainly found in the surface levels after amendment ( bottom of  
431 Fig. 4B). The 10 most abundant fungal OTUs were distributed between these two groups (Fig.  
432 4B, SM10). A group of fungal OTUs (OTU 14 to OTU 93), probably resulting from the  
433 amendment, was found at  $t_{3m}$  and  $t_{11m}$  in deeper levels (up to Lv3 at  $t_{11m}$ ). The fungi present in  
434 the rhizospheric materials at  $t_{3m}$  and  $t_{11m}$  are different from those found in non rhizospheric Lv1  
435 samples. The classification of the ten most represented known fungal genera (SM11) shows  
436 that the genera *Mortierella* and *Talaromyces* were strongly represented in the original tailings  
437 ( $t_{-6m}$ ) and detected in all samples. The genera *Clonostachys*, *Coniochaeta* and *Pisolithus* were  
438 found among the 12 dominant fungal genera at  $t_{-6m}$ . The most represented genus in the freshly  
439 amended Am Lv1 was *Remersonia*. The genera *Zopfiella* and *Mortierella* were also found in  
440 this sample, in small proportions. At  $t_{3m}$ , the genera *Mortierella*, *Clonostachys* and *Zopfiella*  
441 were still present. The genus *Podospora* was present in all levels at this sampling time, , together  
442 with *Giberella*, emerging t in Lv1 R and Lv4. The fungal diversity of the rhizospheric Lv1 at  
443  $t_{3m}$  differed from that of the other levels, in particular because it was the only level containing  
444 *Saccharomyces* as one of the 10 dominant fungal genera. At  $t_{11m}$ , *Podospora* represented nearly  
445 50% of the fungal genera present in Lv1 R andLv4 exclusively included *Malassezia* as a  
446 dominant fungal genera.

447 A RDA was performed to identify the influence on microbial community structure of 12  
448 physico-chemical characteristics of solid phases (Fig. 5). For Bacteria and Archaea (Fig. 5A)  
449 the results indicated that pH, humidity, Zn, and As were explanatory variables that significantly  
450 influenced the community structure, which explained 88.6 % ( $p = 0.001$ ) of the total variance.  
451 In particular, the highest pH and lowest water content seemed to favour the presence of  
452 Bacteroidetes and Proteobacteria, whereas low pH and high water saturation seemed to induce  
453 higher proportions of Acidobacteria and Thaumarchaeota. Zn and As concentrations in the  
454 solids exerted antagonist influences. For Fungi (Fig. 5B), As and humidity were the only

455 significant explanatory variables that influenced the community structure. These parameters  
 456 significantly explain ( $p = 0.011$ ) 37.7 % of the total variance of fungal community structure.  
 457 High As concentrations seemed to favorise the development of Mortierellomycota while low  
 458 water content favoredised the presence of Ascomycota.

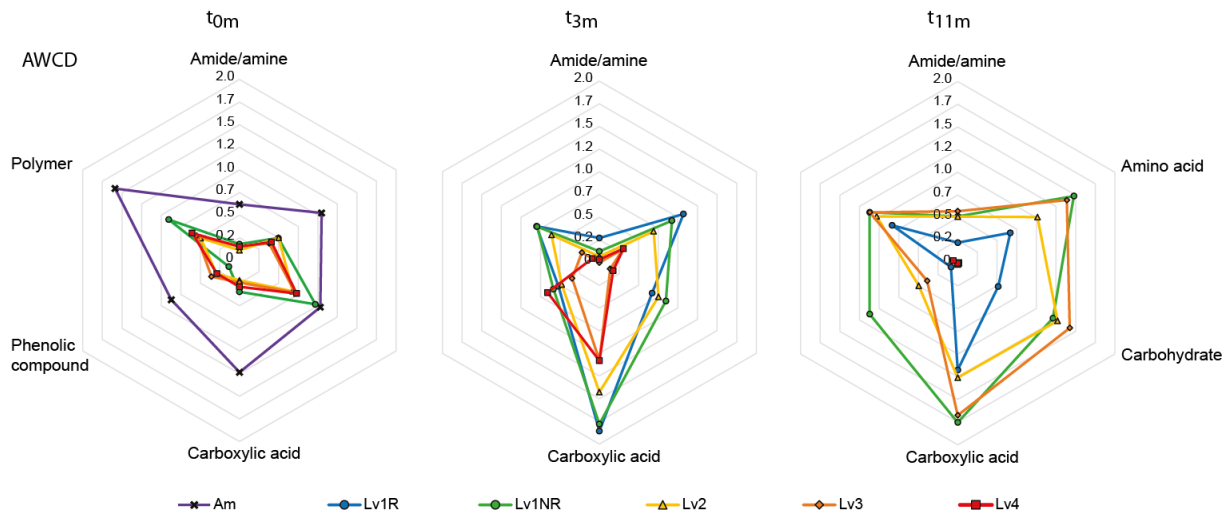


459  
 460 Figure 5. RDA of sequence abundance assigned at phylum level of microbial communities in the solids samples in relation to  
 461 environmental variables. A: Bacteria and Archaea (based on 16S sequences), B: Fungi (based on ITS sequences). Red lines  
 462 represent the significant environmental variable, blue lines represent the Phylum and circles represent the samples. (Lv1 to  
 463 Lv4 represent the sampling depths in the tailings, Lv1 R: solid sampled in the rhizospheric zone, Lv1 NR: solid sampled in the  
 464 non rhizospheric zone, t<sub>0m</sub>: 6 months after the beginning of the monitoring without amendments and plants, t<sub>3m</sub>: 3 months after  
 465 adding amendments and seeding *Agrostis capillaris*, t<sub>11m</sub>: end of the experiment, 11 months after adding amendments and  
 466 seeding *A. capillaris*).

### 467 3.5.3 Functional diversity of heterotrophic microorganisms

468 The substrate spectrum of active cultivable heterotrophs was mainly driven by polymers and  
 469 carbohydrate degradation in the initial tailings (Fig. 6). Amended surface level clearly displayed  
 470 higher heterotrophic activities and a deployment of polymer and carboxylic acids degradation  
 471 was observed at t<sub>0m</sub>. Increase of carboxylic acids biodegradation activities was shared by all  
 472 levels at t<sub>3m</sub>. A large spectrum of heterotrophic activities was detectable in non-rhizospheric  
 473 compartments Lv1, Lv2 and Lv3 at t<sub>11m</sub>, when heterotrophic activity was lower in the  
 474 rhizospheric samples, and nearly inexistent in the deepest level Lv4.

475



476

477 *Figure 6. Functional diversity of cultivable heterotroph microorganisms in solid samples from the pilot experiment (Lv1 to Lv4*  
 478 *represent the sampling depths in the tailings, Am: tailings amended with 1 wt% ochre, 1 wt% cow manure and 1 wt% biochar,*  
 479 *Lv1 R: solid sampled in the rhizospheric zone, Lv1 NR: solid sampled in the non rhizospheric zone, t<sub>0m</sub>: 6 months after the*  
 480 *beginning of the monitoring without amendments and plants, t<sub>3m</sub>: 3 months after adding amendments and seeding *Agrostis**  
 481 *capillaris, t<sub>11m</sub>: end of the experiment, 11 months after adding amendments and seeding *A. capillaris*).*

## 482 4 Discussion

### 483 4.1 Stabilization of Pb linked to the development of *Agrostis capillaris*

484 The *Agrostis capillaris* plants grown for 11 months in the column developed an abundant root  
 485 system that macroscopically retained tailing particles. *A. capillaris* was already reported as a  
 486 good candidate for phytostabilization, often present as a pioneer plant of polluted sites, resistant  
 487 to metals and metalloids including Pb and As (Bradshaw, 1952; Meharg and Macnair, 1991;  
 488 Ernst, 2006; Nandillon et al., 2019). *A. capillaris* has been found to grow spontaneously on  
 489 contaminated soils, and was recognized as an interesting candidate for the phytostabilization of  
 490 As and Pb polluted sites (Austry et al., 2013; Rodriguez-Sejjo et al., 2016). When using pioneer  
 491 plants for phytostabilization purposes, preference should be given to pseudo-metallophyte over  
 492 hyperaccumulator species to minimize the mobilization of metals (Houben et al., 2013). Lead-  
 493 rich minerals associated with roots of our *A. capillaris* plants were observed by SEM-EDS, co-  
 494 occurring with high either iron or phosphate concentrations. Pyromorphite (Pb<sub>5</sub>(PO<sub>4</sub>)<sub>3</sub>(Cl) or  
 495 (OH)<sup>-</sup> grains have been observed in the outer roots' cell-walls of *A. capillaris* grown in a mine

496 waste contaminated soil (Cotter-Howells et al., 1999). The authors suggested that the  
497 precipitation of Pb phosphate might be related to a mechanism of resistance. As a fact,  
498 Nandillon et al. (2019) found higher Pb concentrations in roots of this metallicolous ecotype of  
499 *A. capillaris* than in a non-metallicolous one, grown on Pontgibaud tailings, suggesting that Pb  
500 immobilization in roots might increase the resistance of plants to this toxic element. Apoplastic  
501 sequestration of chloropyromorphite was observed by transmission electron microscopy (TEM)  
502 in roots of Pb-resistant *Brachiaria decumbens* (Kopittke et al., 2008).

#### 503 **4.2 Impact of phytostabilization on water and solids composition**

504 Amendment of the tailings surface layer was performed to facilitate phytostabilization through  
505 the development of *A. capillaris*. This event induced a local increase of solids and pore water  
506 pH, accompanied with a decrease of Pb concentration in pore water. This phenomenon, in  
507 agreement with results of microcosm experiments (Thouin et al., 2019), was more pronounced  
508 in the planted layer, and its amplitude decreased with depth. In the water saturated deeper level  
509 Lv4, conversely, a sharp peak of Pb concentration in pore water was observed at the beginning  
510 of the phytostabilization process. This phenomenon might be linked to different processes.  
511 First, amendment brought several soluble salts releasing cations ( $\text{Ca}^{2+}$ ,  $\text{Na}^+$ ,  $\text{Mg}^{2+}$  ...) that could  
512 compete with  $\text{Pb}^{2+}$  for adsorption on mineral surfaces (Li et al., 2012). This competition might  
513 have been compensated by the pH increase in the surface layers, but not in the deeper layer  
514 whose pH was not influenced. Another phenomenon that could explain Pb release in Lv4 might  
515 be a slight pH decrease linked with the production of  $\text{CO}_2$  by the organic matter (manure)  
516 degradation. This phenomenon was observed at microcosm scale (Thouin et al., 2019) and the  
517 associated Pb mobilization was numerically simulated (Mertz et al., 2021). Again, this  
518 acidification might have been negligible in the upper levels because of the buffering effect of  
519 the amendment, that were not efficient in Lv4.

520 The redox potential in sampled water never decreased significantly, suggesting that reducing  
521 processes, such as ferric iron or sulfate bio-reduction, did not establish at a macroscopic scale,  
522 whereas they might have occurred locally in micro-environments.

523 Interestingly, Ba behaved in the same way as Pb with a peak of concentration in Lv4 observed  
524 following the amendment and seeding. Yet, both Ba and Pb form low-solubility solid phases  
525 with sulfate ( $\text{PbSO}_4$  and  $\text{BaSO}_4$ ). In a previous pot experiment (Norini et al., 2019) correlations  
526 between Ba and major ions concentrations were highlighted. This could suggest that soluble  
527 ions derived from amendment (and more precisely from biochar) induced an increase of Ba  
528 concentration in pore water. Zinc release was also observed in all levels except the surface. This  
529 might be related both with the transport of Zn-containing ochre in the underlying acidic layers,  
530 inducing release of Zn from this amendment, and/or with the release of Zn from the tailings due  
531 to competition for adsorption sites with the salts released by amendment (Voegelin et al., 2001).

### 532 **4.3 Impact of phytostabilization on microbial communities**

533 The development of a plant cover during phytostabilization of mine tailings is generally  
534 associated with a diversification of microbial communities in the colonized surface material.  
535 This shift can be associated with the amendments provided to help plant growth (Valentin-  
536 Vargas et al., 2014), however survey of long-term phytostabilized mine tailings shows that  
537 plants alone can induce a complexification of the microbial communities' structure  
538 (Asemaninejad et al., 2020). The observed evolutions linked with phytostabilization of mine  
539 tailings include the increase of the proportion of heterotrophic bacteria linked to a decrease of  
540 the abundance of chemo-autotrophic or mixotrophic bacteria involved in iron and sulfur cycles  
541 (Mendez et al., 2007; Valentin-Vargas et al., 2014; Gil-Loaiza et al., 2016; Li et al., 2015), and  
542 the development of nitrogen-fixing and plant growth promoting bacteria (Honeker et al., 2019;  
543 Asemaninejad et al., 2020). Our moderately acid tailings hosted three main sulfur oxidizers, i.e.  
544 *Sulfuriferula*, *Sulfurifustis* and *Thiomonas*, and no FeII-oxidizing acidophiles, whereas many

545 previous studies reported the occurrence of the genera *Leptospirillum*, *Acidithiobacillus*,  
546 *Alicyclobacillus*, *Sulfobacillus*, or *Ferrithrix* (Li et al., 2016; Li et al., 2017; Honeker et al.,  
547 2019; Asemaninejad et al., 2020). *Sulfuriferula* genus includes the species *Sulfuriferula*  
548 *plumbophilus*, formerly *Thiobacillus plumbophilus*, a chemolithotrophic bacterium growing  
549 between pH 4.0 and pH 6.5 by oxidation of galena, producing anglesite (Drobner et al., 1992).  
550 *Sulfurifustis* genus (Kojima et al., 2015) gathers autotrophic species oxidizing reduced sulfur  
551 compounds (thiosulfate, tetrathionate, elemental sulfur) at near neutral pH conditions.  
552 *Thiomonas* species are acid-tolerant mixotrophic organisms presenting optimal growth in  
553 presence of both organic substrates and sulfur compounds or arsenite (Battaglia-Brunet et al.,  
554 2011). The main Pb minerals identified by XRD in Pontgibaud tailings were the oxidized forms  
555 anglesite and beudantite. However, a minor fraction of Pb probably remained in the form of the  
556 sulfide galena. As a fact, a darker zone that was sampled from the tailing was enriched in Pb,  
557 suggesting the presence of less oxidized materials. The absence of acidophilic iron-oxidizing  
558 bacteria, that distinguishes our system from many other described autotrophic microbial  
559 communities of tailings, may be explained by the low proportion of Fe-bearing sulfides (pyrite  
560 and arsenopyrite) compared with galena. Amendment with material providing organic  
561 substrates and neutralizing acidity had an immediate effect on microbial communities'  
562 composition in the surface level, as previously observed (Valentin-Vargas et al., 2014), with an  
563 increase of the proportion of heterotrophic phyla and an activation of the metabolism of organic  
564 substrates, in terms of activities and diversity. Moreover, our pilot experiment highlighted the  
565 effect of amendments in the underlying zones, with clear changes of global community  
566 composition in the non-saturated zone below roots. The two deeper saturated levels were less  
567 influenced in terms of global bacterial diversity, however the proportion of sulfur-oxidizers in  
568 the 10 most abundant OTUs declined both in Lv2 (non-saturated) and Lv3 (saturated just below)  
569 3 months after the amendment and seeding event. Only the deepest level Lv4 always kept a

570 significant proportion of sulfur-oxidizers. However, at  $t_{11m}$ , the proportion of sulfur-oxidizers  
571 was restored in Lv3, suggesting an attenuation of the organic substrates supply from the surface.  
572 Honeker et al. (2019) already observed re-acidification of phytostabilized mine tailings during  
573 phytostabilization, in the initially amended surface levels. Here, we showed a transient  
574 influence of surface amendments and plant growth on the underlying levels, however limited  
575 spatially as the deeper saturated zone retained the characteristics of initial tailings. From the  
576 perspective of Fe cycling, the typical acidophile FeII-oxidizers *Leptospirillum* were not  
577 represented in the 100 most abundant OTUs, neither in the 10 most abundant identified genera,  
578 however they were detected by the sequencing as minor OTUs typically present in the tailings  
579 signature ( $t_{6m}$  and Lv3-Lv4). Two other less acidophile chemolithotrophic FeII-oxidizers,  
580 *Gallionella* and *Sideroxydans*, emerged (list of 10 most abundant identified genera) in the  
581 deepest level Lv4 at  $t_{3m}$  and  $t_{11m}$ . In parallel, the presence of an acidophilic FeIII-reducer,  
582 *Acidibacter*, was noticed (list of 10 most abundant identified genera) in Lv3 at  $t_{3m}$  and Lv1R at  
583  $t_{11m}$ . *Acidibacter* was previously mentioned as part of the acid-generating group in  
584 phytostabilized pyritic mine tailings (Honeker et al., 2019). Its presence in our column suggests  
585 that amendments and plant growth could induce activation of Fe cycling, including FeIII-  
586 reduction in the surface levels fueled by new organic electron donors, providing energy (FeII)  
587 for moderately acidophilic FeII-oxidizers in the deepest zones.

588 In the surface level colonized by plants, plant growth promoted bacteria of the genus  
589 *Mesorhizobium* which emerged in the 10 most abundant OTUs at  $t_{11m}$ , suggesting that  
590 conditions favorable to long-term plant maintenance were established, and that N<sub>2</sub>-fixing  
591 bacteria could take over from the manure as a source of nitrogen necessary for plant  
592 development. Asemaninejad et al. (2020) already observed groups of N<sub>2</sub>-fixing bacteria  
593 including *Mesorhizobium* in long-term vegetated tailings. Bacterial diversity of rhizospheric  
594 and non rhizospheric materials did not differ significantly, and evolved similarly from  $t_{3m}$  to



595  $t_{11m}$ , both in terms of global diversity and representation of the major known bacteria. According  
596 to Honeker et al. (2019), the microbial diversity in a phytostabilized soil can be similar in the  
597 rhizospheric and bulk soils when pH is near neutral; differences appear when the pH is acidic  
598 (<4). In our case, amendment enabled to maintain pH >4 in Lv1 along the experiment. However,  
599 differences in the metabolism of organic compounds were observed at  $t_{11m}$ , the metabolic  
600 activity being higher in the bulk than in the rhizospheric material. Whereas root exudates should  
601 feed the heterotrophic microbes (Navarro-Noya et al., 2010), here, not the organic substrate  
602 availability but other type of limitation of microbial activity might be linked to rhizospheric  
603 processes. In particular, phosphate might be depleted in the root vicinity, because of uptake by  
604 plants. In a context of low phosphate bioavailability linked to low solubility of Pb phosphate,  
605 competition between plants and microbes for this essential nutrient might occur.

606 Concerning fungi, about 90 species of *Mortierella* are known mainly from soil, the rhizosphere,  
607 and plant or animal remains in contact with soil (Domsch et al., 1980). Members of *Mortierella*  
608 (phylum Zygomycota) are rapidly growing organisms which have been suggested to be  
609 saprobes in forest ecosystems (Heitefuss, 2011) and that are important decomposers in soil. The  
610 genus *Mortierella* also comprises highly chitinolytic fungi. Due to their potentially endophytic  
611 trophic lifestyle, *Mortierella* may be tracking changes in nutrients driven by vegetation turnover  
612 (Veach et al., 2017). It is therefore not surprising that this fungal genus was found in the initial  
613 tailings that were surrounded by a forest. The adaptive capacities and oligotrophic lifestyle  
614 allowed these fungi to be present throughout the experiment. *Talaromyces* are saprotrophic  
615 Ascomyceta which contribute to the spoilage of dead or decaying organic material. They are  
616 notoriously tolerant to extreme conditions. *Talaromyces* species are commonly distributed in a  
617 wide range of substrates, mostly in soil. In an As-contaminated mine soil, Nam et al. (2019)  
618 showed that a fungal strain hyper-tolerant to arsenic of *Talaromyces* sp. efficiently removed As  
619 from aqueous medium (>70%) with significant improvement by the surface modification of the

620 fungi with Fe(III) (hydr)oxides. Thus, the hypothesis of a contribution of fungal biomass to the  
621 removal of As and/or metals from the pore water of our system cannot be excluded, as our  
622 SEM-EDS observations suggested the presence of Fe and Pb at the surface of fungal hyphae.  
623 *Pisolithus* is a genus of fungi within the family *Sclerodermataceae*. It is an ectomycorrhizal  
624 (ECM) *Gasteromycetes* able to realize symbiosis with various trees (Cairney et al., 2013). We  
625 found this genus in Lv1  $t_{0m}$ , in the amended level, but also in Lv4 at  $t_{0m}$ , thus it was not  
626 exclusively present in the amendments. This genus was detected in different levels and at  
627 different times thereafter. The spores of this ectomycorrhizal fungus were probably initially  
628 present in the tailings and were maintained throughout the experiment thanks to their great  
629 resistance.

## 630 **5 Conclusion**

631 The first objective of phytostabilization is the decrease of pollutants spreading through surface  
632 water runoff and wind erosion. However, the modification of the transfer of metals and  
633 metalloids linked with infiltration processes may have important implications in terms of impact  
634 on downstream groundwater quality or mine drainage. Here, the phytostabilization technology  
635 was examined not only in the surface zones but also from the perspective of the evolution of  
636 underlying compartments. The metallicolous *A. capillaris* grew and efficiently trapped tailing  
637 particles in its root system. The most mobile pollutant, i.e. lead, was actually stabilized in the  
638 surface tailings layer. Beyond these phenomena previously reported for planted tailing systems,  
639 our pilot experiment revealed some phenomena occurring in the underlying zones, following  
640 the amendment/seeding event, such as a transient increase of the pore water concentrations in  
641 Pb, Zn, and Ba. Geochemical data acquired at this laboratory pilot scale will find application in  
642 the development of numerical model, helpful to foresee the future evolution of pollutant fluxes  
643 in the waste heap. Meanwhile, the phytomanagement process strongly influenced the microbial  
644 diversity and metabolic activity in the surface layer but less intensely in the deeper ones. The

645 main parameters influencing bacterial communities' structure, namely pH and water saturation  
646 could be easily related to amendment in the surface compartment, and to the experimental  
647 simulation of a groundwater zone. The evolution of fungal community and their potential  
648 influence on metalloids and metals dynamics would deserve deeper investigation. Thus, the  
649 overall influence of a phytomanagement strategy should be considered and evaluated not only  
650 in the surface compartments, but also considering the potential diffusion of phytostabilization-  
651 related substances that could influence both abiotic phenomena and microbial processes, toward  
652 underlying non-saturated and saturated zones of the mining site.

### 653 **CRedit author statement**

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