

1 **Impacts of epigeic, anecic and endogeic earthworms on metal and metalloid**  
2 **mobility and availability**

3

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21

22

23 **Abstract**

24 The introduction of earthworms into soils contaminated with metals and metalloids has  
25 been suggested to aid restoration practices. Epigeic, anecic and endogeic earthworms  
26 were cultivated in soil with 1130, 345, 113 and 131 mg kg<sup>-1</sup> of As, Cu, Pb and Zn  
27 respectively for up to 112 days in parallel with earthworm-free controls. Different  
28 ecological groups affected metals in the same way by increasing concentrations and  
29 free ion activities in leachate, but anecic *Lumbricus terrestris* had the greatest effect  
30 by increasing concentrations of As by 267%, Cu by 393%, Pb by 190%, and Zn by  
31 429%. Ryegrass grown in earthworm-bearing soil accumulated more metal and the  
32 soil microbial community exhibited greater stress. Results are consistent with  
33 earthworm enhanced degradation of organic matter leading to release of organically  
34 bound elements. The impact of earthworms on metal mobility and availability should  
35 therefore be considered during risk assessment and when inoculating earthworms into  
36 contaminated soils.

37

38 **Keywords:** bioaccessibility, earthworms, metals, mobility, availability

39

40 **Textual abstract for the contents page**

41 Earthworms increase the mobility and availability of As, Cu, Pb and Zn in a  
42 contaminated soil.

43

## 44 **Introduction**

45 Earthworms often represent a significant proportion of the soil biomass and hence  
46 make an important contribution to the decomposition of organic matter, cycling of  
47 nutrients and pedogenesis. It has been estimated that earthworms in arable and  
48 grassland soils produce over 90 tonnes ha<sup>-1</sup> of casts annually <sup>1</sup>. Earthworms can  
49 survive and reproduce in soil anthropogenically-contaminated with metals <sup>2-4</sup>. It is  
50 their importance in soil formation, functionality and ecosystem services that has led to  
51 the introduction of earthworms to physically degraded or chemically contaminated  
52 soils during remediation activities <sup>5-7</sup>. Earthworm inoculation has the potential to  
53 become a commonly used practice during remediation and ecological restoration and  
54 is therefore being investigated as such. However, generally earthworms increase the  
55 mobility and availability of metals <sup>8</sup>. This clearly has significant implications for their  
56 use in remediation. It has been suggested that the changes in mobility and availability  
57 are a direct consequence of a reduction in soil pH or an increase in dissolved organic  
58 carbon due to earthworm activity, leading to changes in elemental speciation <sup>8</sup>.  
59 Alternatively the changes may be due to alterations to the microbial population or the  
60 sequestration of metals into earthworm tissues and their subsequent excretion <sup>8</sup>.

61

62 Earthworms can be classified into three ecological groups according to their life  
63 history strategies <sup>9</sup>. Epigeic earthworms, e.g. *Eisenia veneta* (Rosa), live in the litter  
64 layer above the mineral soil and feed on organic matter in the litter layer. Anecic  
65 earthworms, e.g. *Lumbricus terrestris* (L.), create permanent vertical burrows and  
66 feed predominantly on organic matter which they drag from the soil surface into their  
67 burrows. Endogeic species, e.g. *Allolobophora chlorotica* (Savigny), are

68 predominantly geophagous, form non-permanent horizontal burrows and feed on the  
69 organic matter in the soil and the associated microbial biomass.

70

71 The aim of this study was to determine the impact that introduced earthworms from  
72 these three different ecological groups have on metal and metalloid mobility and  
73 availability in soils and the mechanisms for this. Therefore we introduced earthworms  
74 into highly disturbed, unnatural conditions, such as they might experience if added to  
75 soil under-going remediation. Mobility and availability of metals was assessed  
76 through a combination of bioassays, pore water and leachate analysis, chemical  
77 speciation modelling and phospholipid fatty acid profiling of the soil microbial  
78 community.

79

## 80 **Experimental**

### 81 **Earthworms and Soil**

82 Earthworms were obtained from commercial sources or collected from the field.  
83 *Lumbricus terrestris* (6.0 g, SD = 0.07, n = 24) were sourced from Worms Direct,  
84 Ulting, UK., *Eisenia veneta* (1.2 g, SD = 0.03, n = 60) were sourced from Blades  
85 Biological Ltd, Edenbridge, UK and *Allolobophora chlorotica* (170 mg, SD = 4.0, n =  
86 240) were collected from the University of Reading farm at Sonning, Berkshire, UK.  
87 on the Thames floodplain. All earthworms were kept in a moist Kettering loam and  
88 Irish moss peat mixture (2:1 v/v) prior to use. They were fully clitellate (mature), and  
89 responded to physical stimulus prior to addition into test media.

90

91 Soil was collected (0-30 cm) from a grassed field (SX 423 736 GB grid) identified as  
92 a former settling pond for the separation of metal from crushed ores at Devon Great

93 Consols, an abandoned copper and arsenic mine near Gunnislake, UK <sup>10</sup>. The soil was  
94 homogenised and sieved with a 6.7 mm sieve to remove large stones and roots before  
95 addition to leaching columns.

96

97 Soil properties are shown in Table 1. Soil mineralogy was determined by X-ray  
98 Diffraction Analysis (PANalytical X'Pert series) and a Rietveld refinement <sup>11</sup> and  
99 comprised mostly quartz (38.4%) and mica (30.5%) with trace amounts of chlorite  
100 (7.0%), K-feldspar (4.4%), kaolinite (4.3%) and albite (3.0%). There was a significant  
101 quantity of amorphous material (12.4%) likely to be mostly iron oxyhydroxides and  
102 organic matter.

103

#### 104 **Experimental design**

105 Forty eight leaching columns (300 mm height, 110 mm diameter) were filled with  
106 900 g (dry wt.) of soil moistened to 80% of the water holding capacity (65% moisture  
107 content). Two *L. terrestris*, five *E. veneta* or 20 *A. chlorotica* were added to 12  
108 columns, see Table SI-1 for masses. Twelve control columns were earthworm free.  
109 Columns were maintained at constant soil moisture, arranged randomly in a constant  
110 temperature room at 18 °C in a 12 hour light-dark cycle. Earthworms were not fed  
111 during the test duration so that any effects observed were due to the activities of the  
112 earthworms and not the incorporation of organic matter. The top of the columns were  
113 covered and secured with net curtain to ensure the earthworms did not escape. A  
114 rhizon sampler was inserted 130 mm below the soil surface on day 1 and used to  
115 sample soil pore water in each column after 12, 36, 64 and 92 days. On each occasion  
116 the suction was applied for 16 hours. Four columns per treatment were destructively  
117 sampled after 28, 56 and 112 days.

118

119 Three days before the destructive sampling of a column (days 25, 53 and 109), 296 ml  
120 of ultra pure ( $>15\text{ M}\Omega$ ) water was poured onto the surface in order to saturate the soil  
121 and generate downflow of soil solution through the column; leachate was collected.  
122 Pore water and leachate were filtered to  $<45\ \mu\text{m}$  (Whatman Cellulose nitrate  
123 membrane filters) and analysed for As, Cu, Pb and Zn using an ICP-OES (Perkin  
124 Elmer Optima 3000 Inductively Coupled Plasma-Optical Emission Spectrometer). As  
125 and Pb were below detection limits ( $26$  and  $8\ \mu\text{g L}^{-1}$  respectively). Therefore, leachate  
126 samples from columns destructively sampled after 112 days were analysed for As and  
127 Pb with an ICP-MS (Agilent Technologies 7500 Series Inductively Coupled Plasma  
128 Mass Spectrometer). Pore water and leachate samples were analysed for major anions  
129 (Dionex DX-500 ion chromatograph), pH, Eh and Total Organic Carbon (TOC)  
130 (Shimadzu TOC 5000).

131

132 Twenty eight days before a column was due to be destructively sampled (i.e. day 1, 28  
133 and 84), it was seeded with 0.37 g of perennial ryegrass (*Lolium perenne* L.). Twenty  
134 one days after sowing, the grass was harvested, dried, weighed and the shoots  
135 digested in nitric acid <sup>12</sup> to determine Cu and Zn (ICP-OES) and As and Pb (ICP-MS)  
136 concentrations.

137

138 Earthworms recovered from destructively sampled columns were depurated for 48  
139 hours <sup>13</sup>. Depurate collected after 112 days exposure was frozen along with one  
140 sample of bulk soil per treatment for the determination of As speciation in the soil by  
141 X-ray Absorption Spectroscopy (XAS). Depurated earthworms were frozen before  
142 digestion in nitric acid <sup>14</sup>. Their metal and metalloid loadings were determined by

143 ICP-OES. Soil from the columns was air dried, sieved to 2 mm and pH (BS7755-3.2  
144 <sup>15</sup>) and water soluble carbon (WSC) <sup>16</sup> determined. The microbial community  
145 structure and biomass were assessed using phospholipid fatty acid (PLFA) profiles on  
146 frozen samples of the 112 day incubated soil.

147

#### 148 **Speciation modelling**

149 Speciation of Cu, Pb and Zn in porewater and leachate samples was modelled using  
150 WHAM VI <sup>17</sup>. In the absence of characterisation of the TOC fractions, we assumed  
151 that 50% of TOC was fulvic in origin and that the fulvic acid contained 50% C <sup>18</sup>. The  
152 speciation of As was modelled with PHREEQC<sub>i</sub> <sup>19</sup> using the WATEQ4F database <sup>20</sup>.

153

#### 154 **X-ray Absorption Spectroscopy (XAS) experiment**

155 Station 16.5 at SRS Daresbury Laboratory, Warrington, UK was used to obtain As  
156 K-edge spectra of earthworm depurate to compare with bulk earthworm-worked soil  
157 and earthworm-free control soil. Frozen soil was ground with a pestle and mortar and  
158 mounted in an aluminium planchette for exposure to the X-ray beam at liquid nitrogen  
159 temperatures. Spectra of the control soil sample, samples of soil worked by each of  
160 the earthworm species and the depurate of each of the earthworm species were  
161 collected and analysed following the method of Arnold *et al.* <sup>21</sup>.

162

#### 163 **Phospholipid Fatty Acid (PLFA) analysis**

164 Soils were extracted using Bligh and Dyer solvent <sup>22</sup> according to Frostegård and  
165 Bååth <sup>23</sup>. Extracted phospholipids were derivatized according to Dowling *et al.* <sup>24</sup> and  
166 analysed as fatty acid methyl esters by gas chromatography (Agilent 6890N, flame  
167 ionization detector and a 30 m x 0.25 mm capillary column with a 0.25 µm film of 5%

168 diphenyl, 95% dimethyl siloxane) according to Frostegård *et al.*<sup>25</sup> alongside a 200 µL  
169 C19:0 internal standard. The initial oven temperature was set at 60 °C and raised to  
170 145 °C at 25 °C min.<sup>-1</sup> and then to 250 °C at 2.5 °C min.<sup>-1</sup> and finally at 10 °C min.<sup>-1</sup> to  
171 310 °C where it was held for 10 minutes. Individual fatty acid methyl esters were  
172 identified and quantified according to the retention times and peak areas in qualitative  
173 (26 bacterial FAMES, C11 to C20; Supelco, Supelco UK, Poole, UK) and quantitative  
174 (37 FAMES, C4 to C24; Supelco, Supelco UK, Poole, UK) standards. Individual  
175 PLFAs were attributed to various microbial groups according to Zelles<sup>26</sup>, Frostegård  
176 and Bååth<sup>23</sup> and Kaur *et al.*<sup>27</sup>. Fatty acid nomenclature follows Frostegård *et al.*<sup>28</sup>.

177

#### 178 **Statistical analysis and quality control**

179 Genstat version 9 was used for all statistical analysis. One-way analysis of variance  
180 (ANOVA) and Fisher's Least Significant Difference test were used to test significant  
181 differences between treatments. Normality was confirmed by inspecting the residual  
182 plots. Principal components analysis (PCA) was carried out on normalised PLFA data  
183 using the variance-covariance matrix.

184

185 Pseudo-total elements determined by digestion of soil in aqua regia was run alongside  
186 an in-house reference material traceable to BCR-143R - trace elements in a sewage  
187 sludge amended soil (Commission of the European Communities, Community Bureau  
188 of Reference) certified for Pb and Zn and with an indicative value for Cu. Recoveries  
189 were 90%, 99% and 91% for Cu, Pb and Zn respectively. Digestion of plant material  
190 in nitric acid was run alongside an in-house plant reference material traceable to CRM  
191 GBW 07603 - bush branches and leaves, (approved by State Bureau of Technical  
192 Supervision, The People's Republic of China, Institute of Geophysical and



193 Geochemical Exploration, Langfang, China) certified for As, Cu, Pb, and Zn.  
194 Recoveries were 94%, 106% and 89% for Cu, Pb and Zn respectively. As was below  
195 the limit of detection in the in-house reference plant material (6.3 mg kg<sup>-1</sup>). The  
196 digestion of earthworm tissue in nitric acid was run alongside ERM CE278 – mussel  
197 tissue (European Commission, Institute for Reference Materials and Measurements)  
198 certified for As, Cu, Pb and Zn. Recoveries were 113% and 93% for Cu and Zn  
199 respectively. As and Pb were below the limit of detection in the mussel tissue (9.1  
200 mgAs kg<sup>-1</sup> and 3.5 mgPb kg<sup>-1</sup>).

201

## 202 **Results and discussion**

203 Mortality data and the concentrations of As, Cu, Pb and Zn in earthworm tissue are  
204 presented in Table SI-2. *A. chlorotica* showed the greatest mortality but there was no  
205 increase in mortality over time. All the *L. terrestris* and *E. veneta* survived in the 24  
206 and 56 days treatments, but some mortality did occur in the 112 days treatment.  
207 Earthworm metal body burden increased significantly ( $p < 0.05$ ) with time for Cu, Pb  
208 and Zn (*A. chlorotica*), Pb and Zn (*L. terrestris*) and Pb (*E. veneta*).

209

### 210 **Impact of earthworms on metal and metalloid mobility**

211 Metals and metalloids in solution will be mobile in soils through diffusion and  
212 advection. In all treatments, including the earthworm-free controls, the concentration  
213 of Cu and Zn in pore water increased significantly ( $p < 0.01$ ) with time (Table 2).  
214 However, the concentration of both Cu and Zn in pore water after 36, 64 and 92 days  
215 was significantly greater ( $p < 0.05$ ) in the columns containing *L. terrestris* or *E. veneta*  
216 compared with the control columns. This observation indicates that the mechanism(s)  
217 by which the earthworms increase metal and metalloid mobility may be a process

218 already occurring in earthworm-free soils that is being accelerated by the presence of  
219 the earthworms. By day 111 the As, Cu, Pb and Zn concentrations were significantly  
220 ( $p < 0.01$ ) greater in the leachate from columns inhabited by *L. terrestris* compared  
221 with the control columns (Table 3 and 4).

222

223 These results are consistent with others in the literature<sup>29-31</sup> in which earthworm  
224 activity in soils increased the concentration of water soluble metals. Although fewer  
225 individuals of *L. terrestris* (2) were added to each column than for either *E. veneta* (5)  
226 or *A. chlorotica* (20), the ratio of earthworm biomass to soil mass was in the order *L.*  
227 *terrestris* > *E. veneta* > *A. chlorotica* (Table SI-1) and this probably accounts for *L.*  
228 *terrestris* having the greatest effect on the metal and metalloid mobility in soil.

229

### 230 **Impact of earthworms on metal and metalloid speciation**

231 The bioavailability of metals and metalloids is controlled not just by the presence of  
232 elements in solution but by their speciation<sup>32-34</sup>. Our modelling indicates that free  
233 ions and fulvic acid complexes made up over 99% of the modelled Cu, Pb and Zn  
234 species in all pore water and leachate treatments in these experiments. The decrease in  
235 pore water and leachate pH and DOC with time (Tables 2 and 3) led to a modelled  
236 increase in the abundance of Cu and Zn free ions in solution and a concurrent  
237 decrease in Cu and Zn-fulvic acid complexes (Table 2 and 3). Free ions of Cu and Zn  
238 (and Pb in leachate) were most abundant in the pore water (Table 2) and 112 day  
239 leachate (Table 3) from the *L. terrestris* and *E. veneta* inhabited columns compared  
240 with the control columns. This indicates that the *L. terrestris* and *E. veneta* were not  
241 only capable of increasing the mobility of Cu and Zn but also increasing the  
242 proportion that is in a more available form.

243

244 The vast majority (>99.99%) of the As in the leachate was modelled as As(V). The  
245 leachate from earthworm inhabited columns had a significantly ( $p < 0.05$ ) lower pH  
246 (Table 3) compared with control columns. This resulted in a modelled relative  
247 decrease in the abundance of the negatively charged  $\text{H}_2\text{AsO}_4^-$  ion and an increase in  
248 the uncharged  $\text{H}_3\text{AsO}_4$  species. We did not have the binding constants to allow us to  
249 model arsenic organic complexes in PHREEQCi. The modelled dominance of As(V)  
250 in the water soluble As is based on measured platinum electrode redox potentials.  
251 However, it may be that the AsIII/V couple is not in thermodynamic equilibrium<sup>35</sup>. It  
252 is possible that As(III) may form in the anoxic conditions within the earthworm gut<sup>36</sup>  
253 in response to thermodynamic drivers. This may be catalysed by associated or  
254 ingested dissimilatory arsenate-reducing prokaryotes<sup>37</sup> and be present, in a  
255 disequilibrium state, in the leachate. Reduction of As(V) to As(III) would contribute  
256 to the observed increase in As concentration in the leachate from soils containing *L.*  
257 *terrestris*, (Table 4), due to the higher solubility of As(III).

258

### 259 **Impact of earthworms on metal and metalloid availability to ryegrass**

260 Concentrations of As, Cu and Pb were significantly ( $p < 0.05$ ) greater in the shoots of  
261 ryegrass grown on columns inoculated with *L. terrestris* compared with the  
262 earthworm free control soil (Figure 1). In addition, the dry mass of the plant shoots  
263 was not significantly ( $p > 0.05$ ) different between treatments after 56 and 112 days of  
264 earthworm incubation (Table SI-3). Thus a greater mass of metals was extracted by  
265 the ryegrass from the *L. terrestris* columns. This indicates that *L. terrestris* increased  
266 the availability of these elements to ryegrass in agreement with a number of studies<sup>30,</sup>  
267 <sup>38, 39</sup>. However, *E. veneta* and *A. chlorotica* did not significantly affect the metal or

268 metalloid concentrations of the shoots of ryegrass (Figure 1). This is probably because  
269 these species do not produce casts on the surface as anecic earthworms do. *L.*  
270 *terrestris* deposits the soil that has passed through its gut on the soil surface at the top  
271 of the column and this is what the ryegrass grew in.

272

### 273 **Impact of earthworms on soil properties**

274 Increases in metal mobility as a consequence of earthworm activity have been  
275 explained as being due to either reductions in pH leading to displacement of metals  
276 from binding sites on the soil surfaces<sup>39</sup>, or the formation of organo-metal complexes  
277 bringing metals into solution<sup>40</sup>. Our observation that earthworm activity decreased  
278 soil pH and water soluble carbon (Table 5) is consistent with the hypothesis that  
279 earthworm activity mobilised Cu, Pb and Zn due to a decrease in pH but not due to  
280 the formation of organo-metal complexes. The decreases in pH do not however  
281 explain the increases in As mobility because the increasing positive surface charge of  
282 the oxides with decreasing pH would facilitate the sorption of arsenate oxyanions.  
283 However, the observed increases in As mobility can be explained by reduction of  
284 As(V) to As(III) in the anoxic earthworm gut.

285

286 The mechanisms by which earthworm activity increases the mobility and availability  
287 of metals are unknown<sup>8</sup>. One possibility is earthworm facilitated decomposition  
288 whereby organic matter is physically and chemically conditioned for microbial and  
289 enzymatic attack<sup>41</sup>. The resultant release of organically bound metals and metalloids  
290 would account for the increases in the mobility of elements in all the treatments,  
291 including the control over time and the greater increase in the earthworm-treatments.  
292 Decreases in soil pH (Table 5) may be due to earthworm-enhanced degradation of

293 organic matter leading to the release of organic acids<sup>42</sup>. Organic matter degradation  
294 by indigenous microorganisms in the control treatments would explain the  
295 significantly ( $p < 0.01$ ) lower soil pH in the control columns after 112 days compared  
296 to 24 days (Table 5).

297

### 298 **Impact of earthworms on arsenic speciation**

299 The XANES spectra of all six earthworm-treated samples (faeces and bulk earthworm  
300 worked soil) look the same as the spectrum of the control soil sample, with an edge  
301 position characteristic of oxygen-bound As(V) (Figure SI 1). This similarity to the  
302 control sample indicates that no difference in the speciation of the arsenic in the soil  
303 between the treatments was detectable. The Fourier transform of each spectrum  
304 exhibited a large peak at ca. 1.7 Å. The EXAFS was best fitted by 4 oxygens at 1.68-  
305 1.69 Å (Table SI 4). Including As-O-O-As multiple scattering from the arsenate  
306 tetrahedron<sup>43</sup> improved the residuals and part-filled (at low  $r$ ) the second peak in the  
307 Fourier transforms at ca. 2.8 Å. Further improvements to the fits could be made by  
308 including a shell of phosphorus (or sulphur) scatterers at ca. 3.1 Å. Using heavier (e.g.  
309 Fe) or lighter (e.g. O) scatterers instead of P or S also improved the residual, but to a  
310 lesser degree. All seven EXAFS fits (one control soil, earthworm faeces for all three  
311 species and bulk earthworm-worked soil for all three species) were essentially the  
312 same (Figure SI 2) indicating that there is no evidence that the earthworms excreted  
313 As into the soil in a structure different from that present in the earthworm-free control  
314 soil.

315

316 There is evidence that earthworms sequester metals and metalloids within their  
317 chloragogenous tissues in two distinct structures (O-donating, phosphate-rich granules

318 and S-donating ligands) and then subsequently excrete them in a form different from  
319 that ingested<sup>8, 44-47</sup>. It is not known whether these structures persist in the  
320 environment after excretion and if they significantly impact on mobility and  
321 availability. However, in the current study, there was no difference in As speciation  
322 between earthworm casts, earthworm-worked soil and control soil detectable by  
323 XAFS. This may be because the proportion of the As in the soil that was affected was  
324 small compared with the bulk of the As and any changes in As speciation were below  
325 the limits of detection using this technique. None-the-less, despite evidence that As  
326 speciation is altered within earthworms as a detoxification mechanism<sup>48-50</sup> we have  
327 not been able to detect evidence for the persistence of these changes in the earthworm  
328 worked soil.

329

### 330 **Impact of earthworms on soil microbial community composition**

331 There were distinct differences in the PLFA profiles for the different earthworm  
332 species, as revealed by PCA. The first two components explained 58.3% and 16.5%,  
333 respectively, of the variation in the data set, with the second principal component  
334 separated the data according to the four earthworm treatments (Figure 2). The two  
335 fatty acids with greatest influence on PC2 were 18:1 $\omega$ 9c (negative loadings) and  
336 cy19:0 (positive loadings). The ratios of cyclopropyl fatty acids to their precursor *cis*  
337 monounsaturated fatty acids are considered to be effective indicators of stress in soil  
338 microbial communities<sup>27, 51</sup>. Therefore Figure 2 represents a separation of the  
339 treatments in terms of the degree to which the microbial community is stressed.  
340 Similar differences can be identified between the treatments when stress indicators  
341 (ratios of the 18:1 $\omega$ 9t to 18:1 $\omega$ 9c and cy19:0 to 18:1 $\omega$ 9c fatty acids) are expressed on  
342 a biomass basis (Table 6). *L. terrestris* and *E. veneta* significantly ( $p < 0.05$ ) increased

343 these ratios and the patterns of this stress are closely correlated to the degree to which  
344 earthworms mobilise metals and metalloids.

345

346 The soils inhabited by all three species of earthworm have a lower microbial biomass  
347 than the earthworm-free control soil and this is a significant difference ( $p < 0.05$ ) for  
348 the soil inhabited by *A. chlorotica* (Table 6). This is evidence that different species of  
349 earthworm impact the microbial community differently. Wen *et al.*<sup>30</sup> showed  
350 increases in the microbial populations (measured by the cultivation-based dilution  
351 plate method) of soils in which *Eisenia fetida* increased the mobility and  
352 bioavailability of metals. However, no relationship between the size (biomass) of the  
353 microbial community and the mobility or availability of metals or metalloids in the  
354 soil was found in the current study. It therefore seems likely that mobilisation of  
355 metals and metalloids by *L. terrestris* and *E. veneta* resulted in a toxicity-related  
356 change in microbial community structure rather than the earthworms altering the  
357 microbial community which in turn mobilised the elements. It can therefore be  
358 concluded that increased metal availability due to earthworm activity changed the  
359 microbial community to a more stressed state. It is unlikely that the presence of dead  
360 earthworms in the soil had any affect on the PLFA profiles as this would have only  
361 resulted in large error bars because the *L. terrestris* and *E. Veneta* treatments involved  
362 replicate samples with both 50% and 0% mortality.

363

## 364 **Conclusion**

365 Our data support the hypothesis that earthworms stimulate the degradation of organic  
366 matter and release organically bound metals and metalloids into solution. The  
367 degradation of organic matter also releases organic acids which decrease the soil pH.

368 The earthworms do not appear to carry out a unique process, but increase the rate of a  
369 process that is already occurring. Thus, earthworms would decrease the efficiency of  
370 remediation when amendments are incorporated into soil to bind and immobilize  
371 metals and metalloids. The impact of earthworms on the mobility and availability of  
372 metals and metalloids should therefore be further quantified and considered during the  
373 risk assessment of contaminated soils or when introducing earthworms into  
374 contaminated soil as part of a land remediation scheme.

375

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381

### 382 **Supplementary information**

383 Four tables and two figures are included in the Supplementary Information.

384



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485 **Table 1 Chemical properties of the soil used in the experiments. Values are means of 12**  
 486 **replicates  $\pm$ SD.**

	Pseudototal elements <sup>3</sup> (mg/kg)					
	pH <sup>1</sup> (H <sub>2</sub> O)	LOI <sup>2</sup> (%)	As	Cu	Pb	Zn
	4.89 $\pm$ 0.02	15.5 $\pm$ 0.2	1130 $\pm$ 27	345 $\pm$ 7	113 $\pm$ 3	131 $\pm$ 3

487 <sup>1</sup>Based on BS7755-3.2 (1995) <sup>15</sup> <sup>2</sup>Loss on ignition <sup>3</sup>Aqua regia extractable concentrations based on  
 488 BS7755-3.9 (1995)<sup>52</sup>.

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**Table 2 Total Cu and Zn concentrations and the relative contributions of free ionic and fulvic acid-complexed (FA) forms, pH, and dissolved organic carbon (DOC) in pore water from control earthworm-free soil or soil inhabited by earthworms. Values are means of 12 replicates (12 and 36 days), 8 replicates (64 days) and 4 replicates (92 days)  $\pm$ SE. Asterisks indicate statistically significant differences between control and earthworm inhabited soils for the same sampling times at the 95% (\*) and 99% (\*\*) levels respectively. The percentage abundance of the free ion and fulvic acid complexes in solution was modelled on the mean of 12, 8 or 4 replicates using WHAM VI <sup>17</sup>.**

		Cu ( $\mu$ g/L)	%Cu <sup>2+</sup>	%Cu-FA	Zn ( $\mu$ g/L)	%Zn <sup>2+</sup>	%Zn-FA	pH (H <sub>2</sub> O)	DOC (mg/L)
Control	12 days	46.0 $\pm$ 1.4	7.5	92.5	340 $\pm$ 9.7	90.6	9.5	4.4 $\pm$ 0.03	34.0 $\pm$ 3.9
	36 days	94.1 $\pm$ 5.8	45.1	54.5	639 $\pm$ 33.8	97.5	1.9	4.5 $\pm$ 0.04	18.2 $\pm$ 2.3
	64 days	144 $\pm$ 19.0	78.0	21.4	918 $\pm$ 94.3	98.8	0.6	4.4 $\pm$ 0.02	12.0 $\pm$ 1.6
	92 days	201 $\pm$ 25.0	75.0	24.5	1290 $\pm$ 141	98.7	0.7	4.3 $\pm$ 0.03	18.7 $\pm$ 0.5
<i>A. chlorotica</i>	12 days	46.9 $\pm$ 1.6	13.9	86.1	340 $\pm$ 11.7	93.4	6.7	4.4 $\pm$ 0.09	47.6 $\pm$ 9.1
	36 days	94.6 $\pm$ 1.3	49.8	49.8	398 $\pm$ 42.2	97.7	1.7	4.5 $\pm$ 0.12	19.9 $\pm$ 2.0
	64 days	150 $\pm$ 10.8	75.3	24.1	1170 $\pm$ 142	98.7	0.7	4.3 $\pm$ 0.00	15.0 $\pm$ 4.1
	92 days	200 $\pm$ 7.4	76.6	22.8	1460 $\pm$ 120	98.9	0.6	4.3 $\pm$ 0.05	24.4 $\pm$ 6.2
<i>L. terrestris</i>	12 days	53.1 $\pm$ 1.0**	20.6	79.3	330 $\pm$ 9.3*	94.8	4.9	4.5 $\pm$ 0.03	26.1 $\pm$ 1.9
	36 days	143 $\pm$ 7.6**	67.4	32.1	1000 $\pm$ 35.9**	98.5	0.9	4.3 $\pm$ 0.06	19.1 $\pm$ 0.8
	64 days	211 $\pm$ 4.6*	83.2	16.4	1530 $\pm$ 74.6*	99.1	0.4	4.1 $\pm$ 0.04**	13.2 $\pm$ 0.8
	92 days	300 $\pm$ 6.6**	83.9	15.6	2060 $\pm$ 47.2**	99.0	0.4	4.0 $\pm$ 0.02**	22.6 $\pm$ 0.2
<i>E. veneta</i>	12 days	49.6 $\pm$ 2.1	25.4	74.5	344 $\pm$ 7.2	95.8	4.1	4.4 $\pm$ 0.04	25.5 $\pm$ 1.9
	36 days	129 $\pm$ 14.3*	64.7	34.9	852 $\pm$ 50.9*	98.4	1.1	4.4 $\pm$ 0.05	17.1 $\pm$ 0.7
	64 days	208 $\pm$ 30.5*	84.0	15.5	1320 $\pm$ 147*	99.1	0.4	4.2 $\pm$ 0.02**	12.7 $\pm$ 0.7
	92 days	279 $\pm$ 30.9*	81.2	18.4	1810 $\pm$ 231*	99.0	0.5	4.1 $\pm$ 0.04**	21.9 $\pm$ 2.8

**Table 3 Total Cu and Zn concentrations and the relative contributions of free ionic and fulvic acid-complexed (FA) forms, pH, and dissolved organic carbon (DOC) in leachate from control earthworm-free soil or soil inhabited by earthworms. Values are means of 4 replicates  $\pm$ SE. Asterisks indicate statistically significant differences between control and earthworm inhabited soils for the same sampling times at the 95% (\*) and 99% (\*\*) levels respectively. The percentage abundance of the free ion and fulvic acid complexes in solution was modelled on the mean of 4 replicates using WHAM VI<sup>17</sup>.**

		Cu ( $\mu\text{g/L}$ )	%Cu <sup>2+</sup>	%Cu-FA	Zn ( $\mu\text{g/L}$ )	%Zn <sup>2+</sup>	%Zn-FA	pH (H <sub>2</sub> O)	DOC (mg/L)
Control	28 days	0.7 $\pm$ 0.3	70.0	29.8	66.5 $\pm$ 7.4	99.1	0.9	4.3 $\pm$ 0.1	3.1 $\pm$ 0.3
	54 days	1.3 $\pm$ 0.4	81.5	18.4	137 $\pm$ 28.7	99.5	0.4	4.1 $\pm$ 0.03	2.4 $\pm$ 0.4
	112 days	3.0 $\pm$ 1.3	72.8	27.0	128 $\pm$ 19.8	99.4	0.4	4.1 $\pm$ 0.05	4.2 $\pm$ 0.6
<i>A. chlorotica</i>	28 days	1.3 $\pm$ 0.7	49.3	50.5	92.4 $\pm$ 11.0	98.8	1.2	4.2 $\pm$ 0.05	3.5 $\pm$ 0.4
	54 days	3.0 $\pm$ 0.7	81.8	18.0	118 $\pm$ 14.2	99.6	0.3	4.2 $\pm$ 0.08	2.2 $\pm$ 0.2
	112 days	4.5 $\pm$ 1.4	85.6	13.9	227 $\pm$ 29.4	99.4	0.2	4.0 $\pm$ 0.03*	3.3 $\pm$ 0.0
<i>L. terrestris</i>	28 days	1.2 $\pm$ 0.0	52.2	47.6	107 $\pm$ 0.0	99.0	1.0	4.2 $\pm$ 0.0	3.7 $\pm$ 0.0
	54 days	3.1 $\pm$ 0.9	88.9	11.0	208 $\pm$ 54.3	99.7	0.2	3.8 $\pm$ 0.02**	2.9 $\pm$ 0.5
	112 days	11.8 $\pm$ 1.0**	92.6	7.1	549 $\pm$ 110**	99.6	0.1	3.7 $\pm$ 0.03**	3.9 $\pm$ 0.2
<i>E. veneta</i>	28 days	1.0 $\pm$ 0.1	46.8	53.1	78.8 $\pm$ 10.8	98.7	1.3	4.2 $\pm$ 0.03	3.2 $\pm$ 0.1
	54 days	2.6 $\pm$ 0.5	84.4	15.5	158 $\pm$ 49.0	99.7	0.3	4.1 $\pm$ 0.06	2.2 $\pm$ 0.1
	112 days	9.1 $\pm$ 0.9**	85.5	14.3	257 $\pm$ 16.0	99.7	0.2	3.9 $\pm$ 0.04**	3.9 $\pm$ 0.2

**Table 4 Redox potential (Eh), total As and Pb concentrations and speciations as the % abundances of  $\text{H}_2\text{AsO}_4^-$  and  $\text{H}_3\text{AsO}_4$  and free ionic and fulvic acid-complexed forms in Day 112 leachate from control earthworm-free soil or soil inhabited by earthworms. Values are means of 4 replicates  $\pm$ SE. Asterisks indicate statistically significant differences between control and earthworm inhabited soils for the same sampling times at the 95% (\*) and 99% (\*\*) levels respectively. The percentage abundance of the free ion and fulvic acid complexes in solution was modelled on the mean of 4 replicates using WHAM VI<sup>17</sup>. As speciation data is the percentage abundance of  $\text{H}_2\text{AsO}_4^-$  and  $\text{H}_3\text{AsO}_4$  species modelled on the mean of 4 replicates using PHREEQCi<sup>19</sup>**

	Eh (mV)	As ( $\mu\text{g/L}$ )	% $\text{H}_2\text{AsO}_4^-$	% $\text{H}_3\text{AsO}_4$	Pb ( $\mu\text{g/L}$ )	% $\text{Pb}^{2+}$	% Pb-FA
Control	416 $\pm$ 3.5	0.6 $\pm$ 0.0	98.5	1.4	1.0 $\pm$ 0.1	95.7	4.0
<i>A. chlorotica</i>	417 $\pm$ 1.4	0.8 $\pm$ 0.1	98.1	1.8	1.0 $\pm$ 0.1	97.0	2.0
<i>L. terrestris</i>	419 $\pm$ 1.2	1.6 $\pm$ 0.2**	96.6	3.3	1.9 $\pm$ 0.2**	98.4	0.9
<i>E. veneta</i>	417 $\pm$ 1.7	0.9 $\pm$ 0.1	97.7	2.3	1.4 $\pm$ 0.1	97.7	2.1

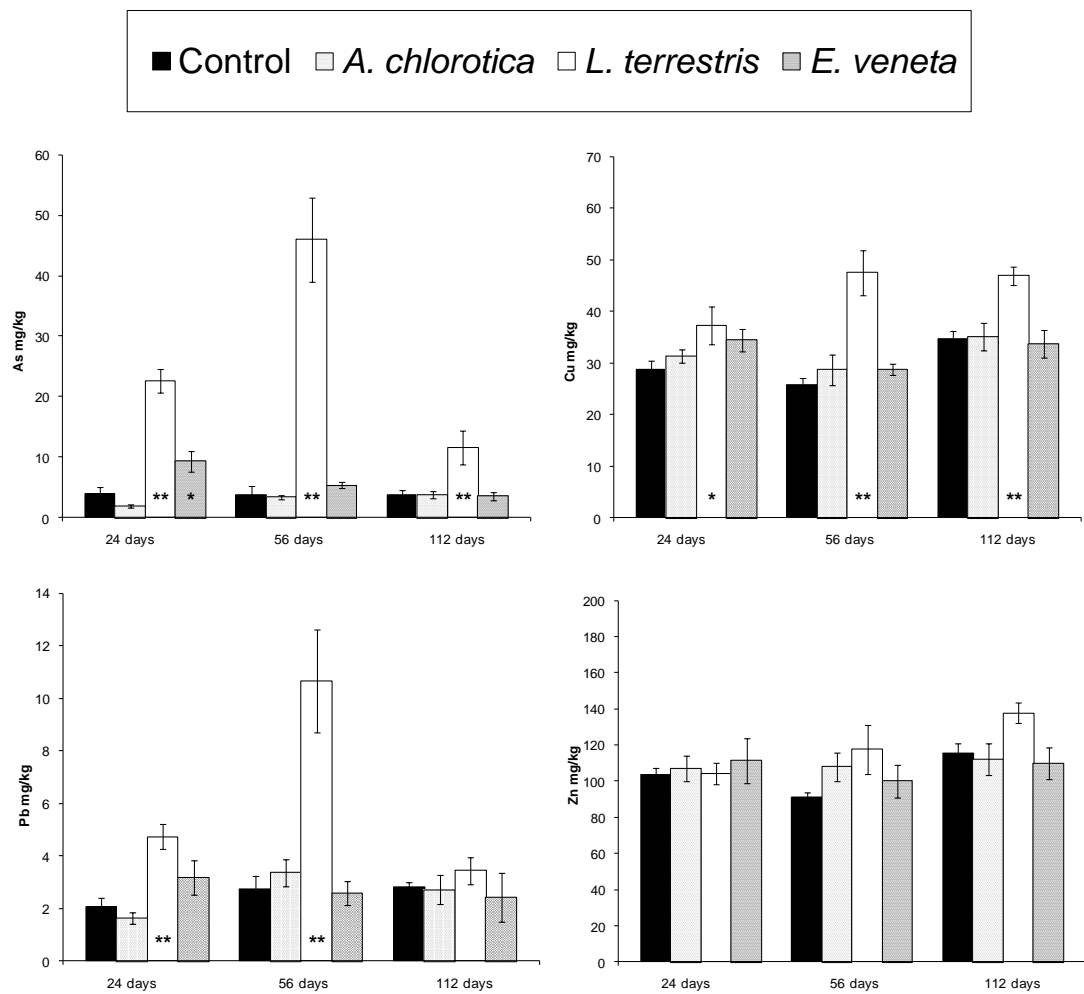
**Table 5 Soil pH and water soluble carbon (WSC) in control earthworm-free soil or soil inhabited by earthworms. Values are means of 4 replicates +SE. Asterisks indicate statistically significant differences between control and earthworm inhabited soils for the same sampling times at the 95% (\*) and 99% (\*\*) levels respectively.**

		pH (H <sub>2</sub> O)	WSC (mg/kg)
Control	28 days	4.6±0.03	320±8.3
	56 days	4.5±0.06	287±12.0
	112 days	4.1±0.03	309±18.5
<i>A. chlorotica</i>	28 days	4.4±0.01**	305±9.1
	56 days	4.3±0.04	257±17.0
	112 days	4.1±0.04	275±12.7
<i>L. terrestris</i>	28 days	4.3±0.02**	292±8.3*
	56 days	4.2±0.04**	282±24.4
	112 days	3.9±0.02**	240±12.9**
<i>E. veneta</i>	28 days	4.4±0.02**	292±9.9*
	56 days	4.3±0.04**	275±22.0
	112 days	4.0±0.06*	256±17.4*

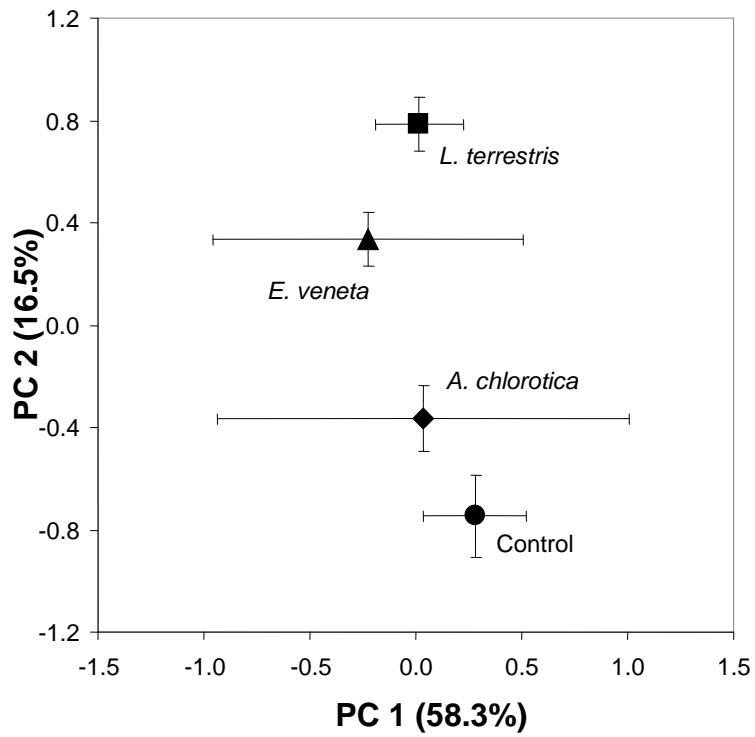
**Table 6. Phospholipid fatty acid indicators of microbial community stress and mean microbial biomass (total PLFA content) in control earthworm-free soil or soil inhabited by earthworms after 112 days. Values are means of 4 replicates  $\pm$ SE. Asterisks indicate statistically significant differences between control and earthworm inhabited soils at the 95% (\*) and 99% (\*\*) levels respectively.**

	Control	<i>Allolobophora chlorotica</i>	<i>Lumbricus terrestris</i>	<i>Eisenia veneta</i>
18:1 $\omega$ 9t / 18:1 $\omega$ 9c ratio	1.3 $\pm$ 0.03	1.4 $\pm$ 0.02	1.5** $\pm$ 0.01	1.4** $\pm$ 0.01
cy19:0 / 18:1 $\omega$ 9c ratio	1.6 $\pm$ 0.02	1.6 $\pm$ 0.05	1.8** $\pm$ 0.04	1.7* $\pm$ 0.04
Microbial biomass (nmol/g dry soil)	46.8 $\pm$ 3.4	37.6* $\pm$ 2.1	39.0 $\pm$ 1.3	42.0 $\pm$ 2.0





**Figure 1. Concentration of As, Cu, Pb and Zn in ryegrass shoots grown on columns inhabited by earthworms compared with earthworm free columns. Values are means of 4 replicates  $\pm$ SE. Asterisks indicate statistically significant differences between control and earthworm inhabited soils at the 95% (\*) and 99% (\*\*) levels respectively.**



**Figure 2.** Principal component score plot of ordination means (n = 4, error bars indicate standard errors) showing the effect of earthworm species on soil microbial community structure, as characterized by PLFA profiling of control earthworm-free soil or soil inhabited by earthworms after 112 days.