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

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Abstract

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

Keywords: bioaccessibility, earthworms, metals, mobility, availability

Textual abstract for the contents page

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

Introduction

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

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

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

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

Experimental

Earthworms and Soil

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

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

Consols, an abandoned copper and arsenic mine near Gunnislake, UK [10]. The soil was homogenised and sieved with a 6.7 mm sieve to remove large stones and roots before addition to leaching columns.

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

Experimental design

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

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

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

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

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

Speciation modelling

Speciation of Cu, Pb and Zn in porewater and leachate samples was modelled using WHAM VI [17]. In the absence of characterisation of the TOC fractions, we assumed that 50% of TOC was fulvic in origin and that the fulvic acid contained 50% C [18]. The speciation of As was modelled with PHREEQCi [19] using the WATEQ4F database [20].

X-ray Absorption Spectroscopy (XAS) experiment

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

Phospholipid Fatty Acid (PLFA) analysis

Soils were extracted using Bligh and Dyer solvent [22] according to Frostegård and Bååth [23]. Extracted phospholipids were derivatized according to Dowling *et al*. [24] and analysed as fatty acid methyl esters by gas chromatography (Agilent 6890N, flame 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*. [25] alongside a 200 µL
169 C19:0 internal standard. The initial oven temperature was set at 60 ºC and raised to
170 145 ºC at 25 ºC min.$^{-1}$ and then to 250 ºC at 2.5 ºC min.$^{-1}$ and finally at 10 ºC min.$^{-1}$ to
171 310 ºC 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 [26], Frostegård
176 and Bååth [23] and Kaur *et al*. [27]. Fatty acid nomenclature follows Frostegård *et al*. [28].
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^{-1}). 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^{-1} and 3.5 mgPb kg^{-1}).
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

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

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

Impact of earthworms on metal and metalloid speciation

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

The vast majority (>99.99%) of the As in the leachate was modelled as As(V). The leachate from earthworm inhabited columns had a significantly ($p<0.05$) lower pH (Table 3) compared with control columns. This resulted in a modelled relative decrease in the abundance of the negatively charged $H_2AsO_4^-$ ion and an increase in the uncharged H_3AsO_4 species. We did not have the binding constants to allow us to model arsenic organic complexes in PHREEQCi. The modelled dominance of As(V) in the water soluble As is based on measured platinium electrode redox potentials. However, it may be that the AsIII/V couple is not in thermodynamic equilibrium [35]. It is possible that As(III) may form in the anoxic conditions within the earthworm gut [36] in response to thermodynamic drivers. This may be catalysed by associated or ingested dissimilatory arsenate-reducing prokaryotes [37] and be present, in a disequilibrium state, in the leachate. Reduction of As(V) to As(III) would contribute to the observed increase in As concentration in the leachate from soils containing *L. terrestris*, (Table 4), due to the higher solubility of As(III).

Impact of earthworms on metal and metalloid availability to ryegrass

Concentrations of As, Cu and Pb were significantly ($p<0.05$) greater in the shoots of ryegrass grown on columns inoculated with *L. terrestris* compared with the earthworm free control soil (Figure 1). In addition, the dry mass of the plant shoots was not significantly ($p>0.05$) different between treatments after 56 and 112 days of earthworm incubation (Table SI-3). Thus a greater mass of metals was extracted by the ryegrass from the *L. terrestris* columns. This indicates that *L. terrestris* increased the availability of these elements to ryegrass in agreement with a number of studies [30, 38, 39]. 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 [39], or the formation of organo-metal complexes
277 bringing metals into solution [40]. 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 [8]. One possibility is earthworm facilitated decomposition
288 whereby organic matter is physically and chemically conditioned for microbial and
289 enzymatic attack [41]. 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 [42]. 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 [43] 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

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

Impact of earthworms on soil microbial community composition

There were distinct differences in the PLFA profiles for the different earthworm species, as revealed by PCA. The first two components explained 58.3% and 16.5%, respectively, of the variation in the data set, with the second principal component separated the data according to the four earthworm treatments (Figure 2). The two fatty acids with greatest influence on PC2 were 18:1ω9c (negative loadings) and cy19:0 (positive loadings). The ratios of cyclopropyl fatty acids to their precursor *cis* monounsaturated fatty acids are considered to be effective indicators of stress in soil microbial communities [27, 51]. Therefore Figure 2 represents a separation of the treatments in terms of the degree to which the microbial community is stressed. Similar differences can be identified between the treatments when stress indicators (ratios of the 18:1ω9t to 18:1ω9c and cy19:0 to 18:1ω9c fatty acids) are expressed on a biomass basis (Table 6). *L. terrestris* and *E. veneta* significantly ($p<0.05$) increased

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

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

Conclusion

Our data support the hypothesis that earthworms stimulate the degradation of organic matter and release organically bound metals and metalloids into solution. The 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 ±SD.**

		Pseudototal elements[3] (mg/kg)			
pH[1] (H_2O)	LOI[2] (%)	As	Cu	Pb	Zn
4.89±0.02	15.5±0.2	1130±27	345±7	113±3	131±3

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

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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) ±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 [17].

		Cu (µg/L)	%Cu^{2+}	%Cu-FA	Zn (µg/L)	%Zn^{2+}	%Zn-FA	pH (H_2O)	DOC (mg/L)
Control	12 days	46.0±1.4	7.5	92.5	340±9.7	90.6	9.5	4.4±0.03	34.0±3.9
	36 days	94.1±5.8	45.1	54.5	639±33.8	97.5	1.9	4.5±0.04	18.2±2.3
	64 days	144±19.0	78.0	21.4	918±94.3	98.8	0.6	4.4±0.02	12.0±1.6
	92 days	201±25.0	75.0	24.5	1290±141	98.7	0.7	4.3±0.03	18.7±0.5
A. chlorotica	12 days	46.9±1.6	13.9	86.1	340±11.7	93.4	6.7	4.4±0.09	47.6±9.1
	36 days	94.6±1.3	49.8	49.8	398±42.2	97.7	1.7	4.5±0.12	19.9±2.0
	64 days	150±10.8	75.3	24.1	1170±142	98.7	0.7	4.3±0.00	15.0±4.1
	92 days	200±7.4	76.6	22.8	1460±120	98.9	0.6	4.3±0.05	24.4±6.2
L. terrestris	12 days	53.1±1.0**	20.6	79.3	330±9.3*	94.8	4.9	4.5±0.03	26.1±1.9
	36 days	143±7.6**	67.4	32.1	1000±35.9**	98.5	0.9	4.3±0.06	19.1±0.8
	64 days	211±4.6*	83.2	16.4	1530±74.6*	99.1	0.4	4.1±0.04**	13.2±0.8
	92 days	300±6.6**	83.9	15.6	2060±47.2**	99.0	0.4	4.0±0.02**	22.6±0.2
E. veneta	12 days	49.6±2.1	25.4	74.5	344±7.2	95.8	4.1	4.4±0.04	25.5±1.9
	36 days	129±14.3*	64.7	34.9	852±50.9*	98.4	1.1	4.4±0.05	17.1±0.7
	64 days	208±30.5*	84.0	15.5	1320±147*	99.1	0.4	4.2±0.02**	12.7±0.7
	92 days	279±30.9*	81.2	18.4	1810±231*	99.0	0.5	4.1±0.04**	21.9±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 ±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 [17].

		Cu (µg/L)	%Cu^{2+}	%Cu-FA	Zn (µg/L)	%Zn^{2+}	%Zn-FA	pH (H_2O)	DOC (mg/L)
Control	28 days	0.7±0.3	70.0	29.8	66.5±7.4	99.1	0.9	4.3±0.1	3.1±0.3
	54 days	1.3±0.4	81.5	18.4	137±28.7	99.5	0.4	4.1±0.03	2.4±0.4
	112 days	3.0±1.3	72.8	27.0	128±19.8	99.4	0.4	4.1±0.05	4.2±0.6
A. chlorotica	28 days	1.3±0.7	49.3	50.5	92.4±11.0	98.8	1.2	4.2±0.05	3.5±0.4
	54 days	3.0±0.7	81.8	18.0	118±14.2	99.6	0.3	4.2±0.08	2.2±0.2
	112 days	4.5±1.4	85.6	13.9	227±29.4	99.4	0.2	4.0±0.03*	3.3±0.0
L. terrestris	28 days	1.2±0.0	52.2	47.6	107±0.0	99.0	1.0	4.2±0.0	3.7±0.0
	54 days	3.1±0.9	88.9	11.0	208±54.3	99.7	0.2	3.8±0.02**	2.9±0.5
	112 days	11.8±1.0**	92.6	7.1	549±110**	99.6	0.1	3.7±0.03**	3.9±0.2
E. veneta	28 days	1.0±0.1	46.8	53.1	78.8±10.8	98.7	1.3	4.2±0.03	3.2±0.1
	54 days	2.6±0.5	84.4	15.5	158±49.0	99.7	0.3	4.1±0.06	2.2±0.1
	112 days	9.1±0.9**	85.5	14.3	257±16.0	99.7	0.2	3.9±0.04**	3.9±0.2

Table 4 Redox potential (Eh), total As and Pb concentrations and speciations as the % abundances of $H_2AsO_4^-$ and H_3AsO_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 relicates ±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 [17]. As speciation data is the percentage abundance of $H_2AsO_4^-$ and H_3AsO_4 species modelled on the mean of 4 replicates using PHREEQCi [19]

	Eh (mV)	As (µg/L)	% $H_2AsO_4^-$	% H_3AsO_4	Pb (µg/L)	% Pb^{2+}	% Pb-FA
Control	416±3.5	0.6±0.0	98.5	1.4	1.0±0.1	95.7	4.0
A. chlorotica	417±1.4	0.8±0.1	98.1	1.8	1.0±0.1	97.0	2.0
L. terrestris	419±1.2	1.6±0.2**	96.6	3.3	1.9±0.2**	98.4	0.9
E. veneta	417±1.7	0.9±0.1	97.7	2.3	1.4±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_2O)	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
A. chlorotica	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
L. terrestris	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**
E. veneta	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 ±SE. Asterisks indicate statistically significant differences between control and earthworm inhabited soils at the 95% (*) and 99% (**) levels respectively.

	Control	*Allolobophora chlorotica*	*Lumbricus terrestris*	*Eisenia veneta*
18:1ω9t / 18:1ω9c ratio	1.3 ±0.03	1.4 ±0.02	1.5** ±0.01	1.4** ±0.01
cy19:0 / 18:1ω9c ratio	1.6 ±0.02	1.6 ±0.05	1.8** ±0.04	1.7* ±0.04
Microbial biomass (nmol/g dry soil)	46.8 ±3.4	37.6* ±2.1	39.0 ±1.3	42.0 ±2.0

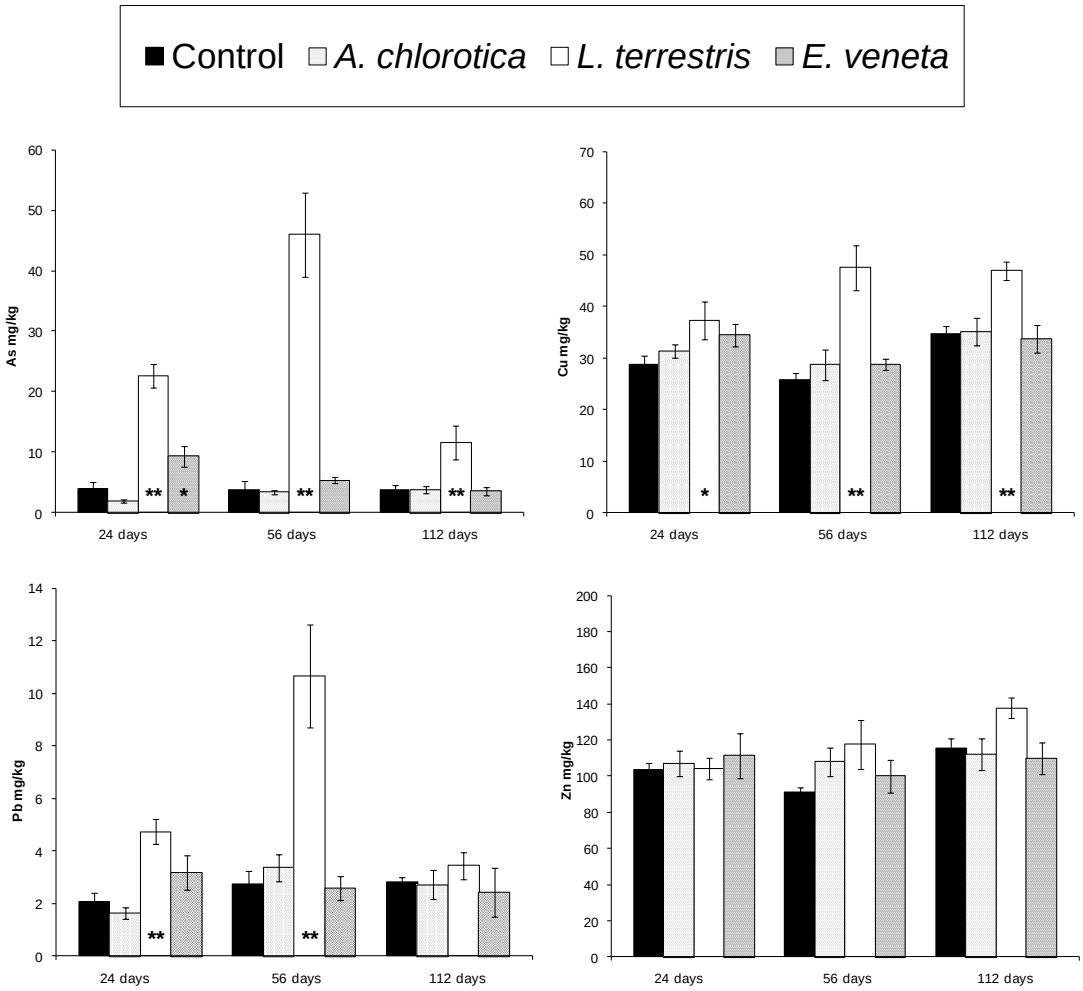

Figure 1. Concentration of As, Cu, Pb and Zn in ryegrass shoots grown on columns inhabited by earthworms compared with earthworm free columns. Values are means of 4 replicates ±SE. Asterisks indicate statistically significant differences between control and earthworm inhabited soils at the 95% (*) and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

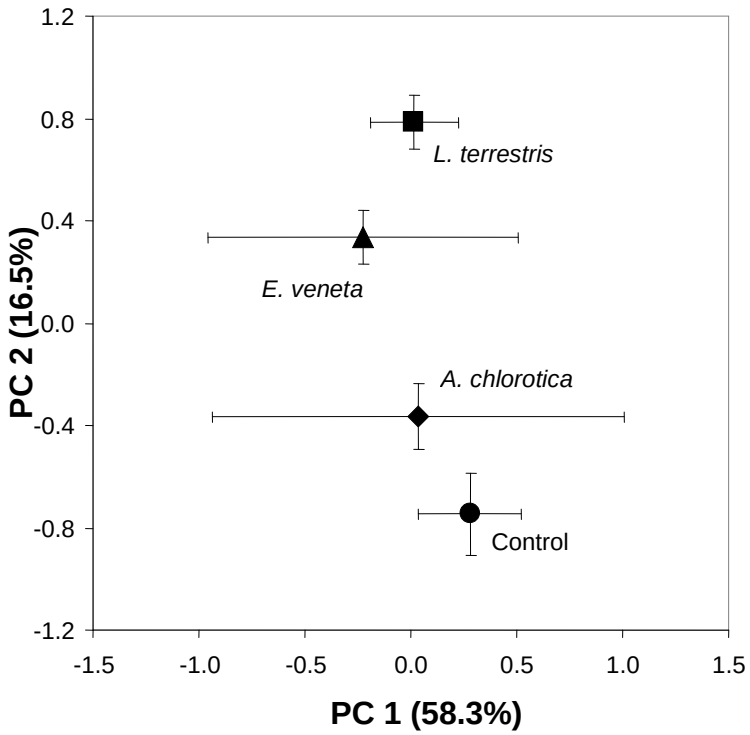

Figure 2. Principal component score plot of ordination means (n = 4, error bars indicate standard errors) showing the effect of earthworm species on soil microbial community structure, as characterized by PLFA profiling of control earthworm-free soil or soil inhabited by earthworms after 112 days.