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Article

Accepted Version

Sizmur, T. ORCID: <https://orcid.org/0000-0001-9835-7195>,
Tilston, E. L., Charnock, J., Palumbo-Roe, B., Watts, M. J. and
Hodson, M. E. (2011) Impacts of epigeic, anecic and endogeic
earthworms on metal and metalloid mobility and availability.
Journal of Environmental Monitoring, 13 (2). pp. 266-273.
ISSN 1464-0333 doi: 10.1039/C0EM00519C Available at
<https://centaur.reading.ac.uk/19279/>

It is advisable to refer to the publisher's version if you intend to cite from the work. See [Guidance on citing](#).

To link to this article DOI: <http://dx.doi.org/10.1039/C0EM00519C>

Publisher: RSC Publishing

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

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Abstract

The introduction 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.

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⁻¹ of casts annually ¹. Earthworms can
49 survive and reproduce in soil anthropogenically-contaminated with metals ²⁻⁴. 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 ⁵⁻⁷. 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 ⁸. 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 ⁸.
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 ⁸.

61

62 Earthworms can be classified into three ecological groups according to their life
63 history strategies ⁹. 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

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, UK¹⁰. 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 refinement¹¹ 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 °C 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 ensure 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 applied for 16 hours. Four columns per treatment were destructively 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 $<45\text{ }\mu\text{m}$ (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 $8\text{ }\mu\text{g L}^{-1}$ 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 ¹² 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 ¹³. 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 ¹⁴. Their metal and metalloid loadings were determined by

ICP-OES. Soil from the columns was air dried, sieved to 2 mm and pH (BS7755-3.2¹⁵) 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 VI¹⁷. 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% C¹⁸. The speciation of As was modelled with PHREEQCi¹⁹ using the WATEQ4F database²⁰.

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 solvent²² according to Frostegård and Bååth²³. 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%

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

Statistical analysis and quality control

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

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

Geochemical Exploration, Langfang, China) certified for As, Cu, Pb, and Zn. Recoveries were 94%, 106% and 89% for Cu, Pb and Zn respectively. As was below the limit of detection in the in-house reference plant material (6.3 mg kg^{-1}). The digestion of earthworm tissue in nitric acid was run alongside ERM CE278 – mussel tissue (European Commission, Institute for Reference Materials and Measurements) certified for As, Cu, Pb and Zn. Recoveries were 113% and 93% for Cu and Zn respectively. As and Pb were below the limit of detection in the mussel tissue ($9.1 \text{ mg As kg}^{-1}$ and $3.5 \text{ mg Pb kg}^{-1}$).

Results and discussion

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

Impact of earthworms on metal and metalloid mobility

Metals and metalloids in solution will be mobile in soils through diffusion and advection. In all treatments, including the earthworm-free controls, the concentration of Cu and Zn in pore water increased significantly ($p < 0.01$) with time (Table 2). However, the concentration of both Cu and Zn in pore water after 36, 64 and 92 days was significantly greater ($p < 0.05$) in the columns containing *L. terrestris* or *E. veneta* compared with the control columns. This observation indicates that the mechanism(s) 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 literature²⁹⁻³¹ 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 speciation³²⁻³⁴. 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.

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 H_2AsO_4^- ion and an increase in
 248 the uncharged H_3AsO_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 equilibrium³⁵. It
 252 is possible that As(III) may form in the anoxic conditions within the earthworm gut³⁶
 253 in response to thermodynamic drivers. This may be catalysed by associated or
 254 ingested dissimilatory arsenate-reducing prokaryotes³⁷ 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^{30,}
 267 ^{38, 39}. However, *E. veneta* and *A. chlorotica* did not significantly affect the metal or

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

Impact of earthworms on soil properties

Increases in metal mobility as a consequence of earthworm activity have been explained as being due to either reductions in pH leading to displacement of metals from binding sites on the soil surfaces³⁹, or the formation of organo-metal complexes bringing metals into solution⁴⁰. Our observation that earthworm activity decreased soil pH and water soluble carbon (Table 5) is consistent with the hypothesis that earthworm activity mobilised Cu, Pb and Zn due to a decrease in pH but not due to the formation of organo-metal complexes. The decreases in pH do not however explain the increases in As mobility because the increasing positive surface charge of the oxides with decreasing pH would facilitate the sorption of arsenate oxyanions. However, the observed increases in As mobility can be explained by reduction of As(V) to As(III) in the anoxic earthworm gut.

The mechanisms by which earthworm activity increases the mobility and availability of metals are unknown⁸. One possibility is earthworm facilitated decomposition whereby organic matter is physically and chemically conditioned for microbial and enzymatic attack⁴¹. The resultant release of organically bound metals and metalloids would account for the increases in the mobility of elements in all the treatments, including the control over time and the greater increase in the earthworm-treatments. Decreases in soil pH (Table 5) may be due to earthworm-enhanced degradation of

organic matter leading to the release of organic acids⁴². Organic matter degradation by indigenous microorganisms in the control treatments would explain the significantly ($p < 0.01$) lower soil pH in the control columns after 112 days compared to 24 days (Table 5).

Impact of earthworms on arsenic speciation

The XANES spectra of all six earthworm-treated samples (faeces and bulk earthworm worked soil) look the same as the spectrum of the control soil sample, with an edge position characteristic of oxygen-bound As(V) (Figure SI 1). This similarity to the control sample indicates that no difference in the speciation of the arsenic in the soil between the treatments was detectable. The Fourier transform of each spectrum exhibited a large peak at ca. 1.7 Å. The EXAFS was best fitted by 4 oxygens at 1.68-1.69 Å (Table SI 4). Including As-O-O-As multiple scattering from the arsenate tetrahedron⁴³ improved the residuals and part-filled (at low r) the second peak in the Fourier transforms at ca. 2.8 Å. Further improvements to the fits could be made by including a shell of phosphorus (or sulphur) scatterers at ca. 3.1 Å. Using heavier (e.g. Fe) or lighter (e.g. O) scatterers instead of P or S also improved the residual, but to a lesser degree. All seven EXAFS fits (one control soil, earthworm faeces for all three species and bulk earthworm-worked soil for all three species) were essentially the same (Figure SI 2) indicating that there is no evidence that the earthworms excreted As into the soil in a structure different from that present in the earthworm-free control soil.

There is evidence that earthworms sequester metals and metalloids within their 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 mechanism⁴⁸⁻⁵⁰ 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 18:1 ω 9c (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 18:1 ω 9t to 18:1 ω 9c and cy19:0 to 18:1 ω 9c 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.

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

Acknowledgements

This work was funded by a BBSRC studentship, with CASE support from BUFI-BGS. XRD analysis was carried out at BGS by Ms Doris Wagner. The XAS experiment was performed at the Daresbury synchrotron light source station 16.5, managed and assisted by Mr. Bob Bilsborrow.

Supplementary information

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

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484

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

Pseudototal elements ³ (mg/kg)					
pH ¹ (H ₂ O)	LOI ² (%)	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 ¹Based on BS7755-3.2 (1995) ¹⁵ ²Loss on ignition ³Aqua 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) $\pm$ 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 ¹⁷.**

		Cu ($\mu\text{g/L}$)	%Cu ²⁺	%Cu-FA	Zn ($\mu\text{g/L}$)	%Zn ²⁺	%Zn-FA	pH (H ₂ 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 ¹⁷.**

		Cu ($\mu\text{g/L}$)	%Cu ²⁺	%Cu-FA	Zn ($\mu\text{g/L}$)	%Zn ²⁺	%Zn-FA	pH (H ₂ 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 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 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 VI¹⁷. As speciation data is the percentage abundance of H_2AsO_4^- and H_3AsO_4 species modelled on the mean of 4 replicates using PHREEQCi¹⁹**

	Eh (mV)	As ($\mu\text{g/L}$)	% H_2AsO_4^-	% H_3AsO_4	Pb ($\mu\text{g/L}$)	% 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 ₂ 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 ω 9t / 18:1 ω 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 ω 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

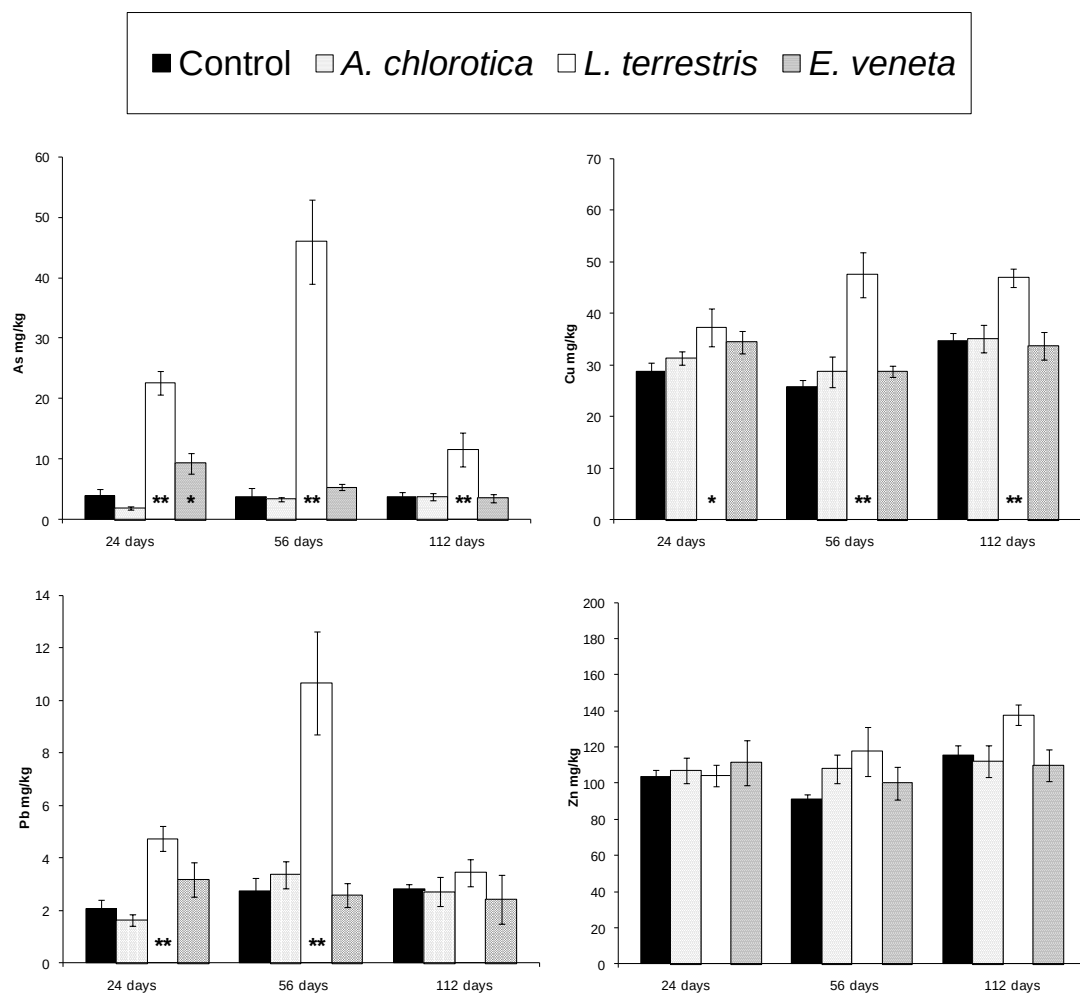


Figure 1. Concentration of As, Cu, Pb and Zn in ryegrass shoots grown on columns inhabited by earthworms compared with earthworm free columns. Values are means of 4 replicates $\pm$ SE. Asterisks indicate statistically significant differences between control and earthworm inhabited soils at the 95% (*)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

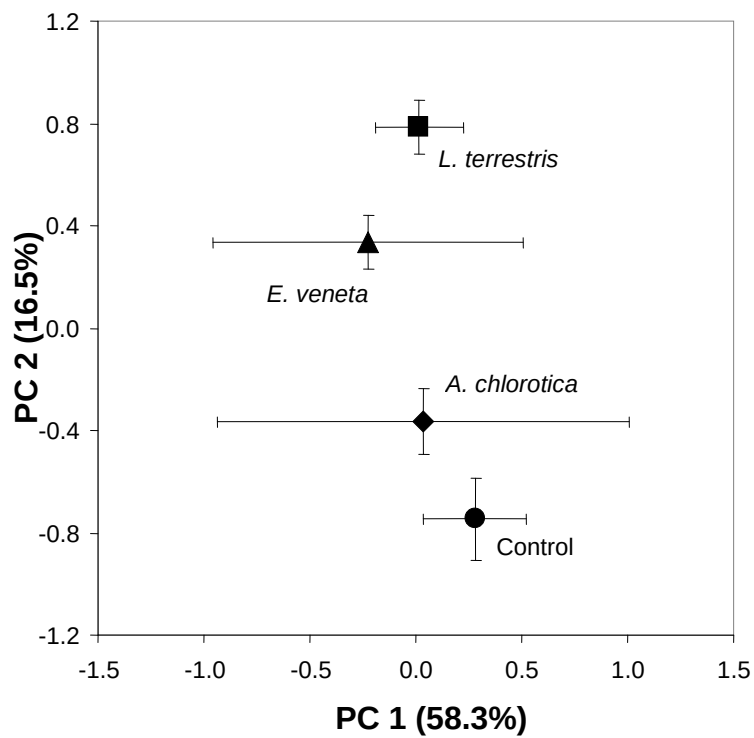


Figure 2. Principal component score plot of ordination means (n = 4, error bars indicate standard errors) showing the effect of earthworm species on soil microbial community structure, as characterized by PLFA profiling of control earthworm-free soil or soil inhabited by earthworms after 112 days.