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

Tom Sizmur[a]*, Emma L. Tilston[ab], John Charnock[c], Barbara Palumbo-Roe[d], Michael J. Watts[d] and Mark E. Hodson[a]

[a]Soil Research Centre, Department of Geography and Environmental Science, School of Human and Environmental Sciences, University of Reading, Whiteknights, Reading, RG6 6DW, U.K.

[b]Scottish Universities Environmental Research Centre, Rankine Avenue, Scottish Enterprise Technology Park, East Kilbride, Scotland, G75 0QF, U.K.

[c]School of Earth, Atmospheric and Environmental Sciences, University of Manchester, Oxford Road, Manchester, M13 9PL, U.K.

[d]British Geological Survey, Kingsley Dunham Centre, Keyworth, Nottingham, NG12 5GG, U.K.

*Corresponding author

t.p.sizmur@reading.ac.uk, Tel: +44(0) 118 378 8911, Fax: +44(0) 118 378 6666

Abstract

The introducton of earthworms into soils contaminated with metals and metalloids has been suggested to aid restoration practices. *Eisenia veneta* (epigeic), *Lumbricus terrestris* (anecic) and *Allolobophora chlorotica* (endogeic) earthworms were cultivated in columns containing 900 g 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 columns. Leachate was produced by pouring water on the soil surface to saturate the soil and generate downflow. Ryegrass was grown on the top of columns to assess metal uptake into biota. Different ecological groups affected metals in the same way by increasing concentrations and free ion activities in leachate, but anecic *L. terrestris* had the greatest effect by increasing leachate concentrations of As by 267%, Cu by 393%, Pb by 190%, and Zn by 429% compared to earthworm-free columns. 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 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. The impact of earthworms on metal mobility and availability should therefore be considered when inoculating earthworms into contaminated soils as new pathways to receptors may be created or the flow of metals and metalloids to receptors may be elevated.

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 a contaminated soil and the mechanisms responsible. 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 on day 0, see Table SI-1 for masses of earthworms. 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 0.25 mm mesh polyester netting to ensure the earthworms did not escape. A rhizon sampler (Eijkelkamp Agriresearch ,The Netherlands) was inserted 130 mm below the soil surface on day 1 and used to sample soil pore water in each column on days 12, 36, 64 and 92. On each

123 occasion the suction was applied with a syringe for 16 hours. Four columns per
124 treatment were destructively sampled on days 28, 56 and 112.
125
126 Three days before the destructive sampling of a column (days 25, 53 and 109), 296 ml
127 of ultra pure (>15 MΩ cm) water was poured onto the surface in order to saturate the
128 soil and generate downflow of soil solution through the column; leachate was
129 collected in bottles fixed to the bottom of the columns on the same day that the
130 columns were destructively sampled (days 28, 56 and 112).
131
132 Twenty eight days before a column was due to be destructively sampled (i.e. days 1,
133 28 and 84), it was seeded with 0.37 g of perennial ryegrass (*Lolium perenne* L.).
134 Twenty 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 Pore water and leachate were filtered to <0.45 μm (Whatman Cellulose nitrate
139 membrane filters) and analysed for As, Cu, Pb and Zn using an ICP-OES (Perkin
140 Elmer Optima 3000 Inductively Coupled Plasma-Optical Emission Spectrometer). As
141 and Pb were below detection limits (26 and 8 $\mu g\ L^{-1}$ respectively). Therefore, leachate
142 samples from columns destructively sampled after 112 days were analysed for As and
143 Pb with an ICP-MS (Agilent Technologies 7500 Series Inductively Coupled Plasma
144 Mass Spectrometer). Pore water and leachate samples were analysed for major anions
145 (Dionex DX-500 ion chromatograph), pH, Eh and Total Organic Carbon (TOC)
146 (Shimadzu TOC 5000).
147

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 of frozen samples from 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 in fluorescence mode following the method of Arnold *et al.* [21] with the beamline operating at 2 GeV with a current of 100-210 mA. Spectra of

sodium arsenate and sodium arsenite standards were collected at ambient temperature in transmission mode before any experimental measurements were taken. The measurements from samples were repeated between 4 and 12 times in order to obtain data to enable reliable comparisons. Background subtracted and normalised XANES spectra were modelled as linear combinations of selected standard spectra and EXAFS spectra were analysed in EXCURV98.

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% 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 ºC and raised to 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 310 ºC 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 mgAs kg^{-1} and 3.5 mgPb kg^{-1}).

Results and discussion

Mortality data and the concentrations of As, Cu, Pb and Zn in earthworm tissue are presented in Table 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

223 and 56 days treatments, but some mortality did occur in the 112 days treatment.
224 Earthworm metal body burden increased significantly ($p<0.05$) with time for Cu, Pb
225 and Zn (*A. chlorotica*), Pb and Zn (*L. terrestris*) and Pb (*E. veneta*).
226
227 **Impact of earthworms on metal and metalloid mobility**
228 Metals and metalloids in solution will be mobile in soils through diffusion and
229 advection. In all treatments, including the earthworm-free controls, the concentration
230 of Cu and Zn in pore water increased significantly ($p<0.01$) with time (Table 3).
231 However, the concentration of both Cu and Zn in pore water after 36, 64 and 92 days
232 was significantly greater ($p<0.05$) in the columns containing *L. terrestris* or *E. veneta*
233 compared with the control columns. This observation indicates that the mechanism(s)
234 by which the earthworms increase metal and metalloid mobility may be a process
235 already occurring in earthworm-free soils that is being accelerated by the presence of
236 the earthworms. By day 112 the As, Cu, Pb and Zn concentrations were significantly
237 ($p<0.01$) greater in the leachate from columns inhabited by *L. terrestris* compared
238 with the control columns (Table 4 and 5).
239
240 These results are consistent with others in the literature [29-31] in which earthworm
241 activity in soils increased the concentration of water soluble metals. Although fewer
242 individuals of *L. terrestris* (2) were added to each column than for either *E. veneta* (5)
243 or *A. chlorotica* (20), the ratio of earthworm biomass to soil mass was in the order *L.*
244 *terrestris* > *E. veneta* > *A. chlorotica* (Table SI-1) and this probably accounts for *L.*
245 *terrestris* having the greatest effect on the metal and metalloid mobility in soil.
246
247 **Impact of earthworms on metal and metalloid speciation**

248 The bioavailability of metals and metalloids is controlled not just by the presence of
249 elements in solution but by their speciation [32-34]. Our modelling indicates that free
250 ions and fulvic acid complexes made up over 99% of the modelled Cu, Pb and Zn
251 species in all pore water and leachate treatments in these experiments. The decrease in
252 pore water and leachate pH and DOC with time (Tables 3 and 4) led to a modelled
253 increase in the abundance of Cu and Zn free ions in solution and a concurrent
254 decrease in Cu and Zn-fulvic acid complexes (Table 3 and 4). Free ions of Cu and Zn
255 (and Pb in leachate) were most abundant in the pore water (Table 3) and 112 day
256 leachate (Table 4) from the *L. terrestris* and *E. veneta* inhabited columns compared
257 with the control columns. This indicates that the *L. terrestris* and *E. veneta* were not
258 only capable of increasing the mobility of Cu and Zn but also increasing the
259 proportion that is in a more available form.
260
261 The vast majority (>99.99%) of the As in the leachate was modelled as As(V). The
262 leachate from earthworm inhabited columns had a significantly ($p<0.05$) lower pH
263 (Table 4) compared with control columns. This resulted in a modelled relative
264 decrease in the abundance of the negatively charged $H_2AsO_4^-$ ion and an increase in
265 the uncharged H_3AsO_4 species. We did not have the binding constants to allow us to
266 model arsenic organic complexes in PHREEQCi. The modelled dominance of As(V)
267 in the water soluble As is primarily due to the measured platinium electrode redox
268 potentials used as model input. However, it may be that the AsIII/V couple is not in
269 thermodynamic equilibrium [35]. It is possible that As(III) may form in the anoxic
270 conditions within the earthworm gut [36] in response to thermodynamic drivers. This
271 may be catalysed by associated or ingested dissimilatory arsenate-reducing
272 prokaryotes [37] 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 5), 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 7). 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 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. *Lumbricus 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.

Mechanisms for impacts of earthworms on metal and metalloid mobility and availability

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 surfaces [39], or the formation of organo-metal complexes bringing metals into solution [40]. Our observation that earthworm activity decreased soil pH and water soluble carbon (Table 6) is consistent with the hypothesis that earthworm activity mobilised Cu, Pb and Zn due to a decrease in pH, but not due to

298 the formation of organo-metal complexes. The decreases in pH do not, however,
299 explain the increases in As mobility, because the increasing positive surface charge of
300 the oxides with decreasing pH would facilitate the sorption of arsenate oxyanions.
301 However, the observed increases in As mobility can be explained by reduction of
302 As(V) to As(III) in the anoxic earthworm gut.
303
304 The mechanisms by which earthworm activity increases the mobility and availability
305 of metals are unknown [8]. One possibility is earthworm facilitated decomposition
306 whereby organic matter is physically and chemically conditioned for microbial and
307 enzymatic attack [41]. The resultant release of organically bound metals and metalloids
308 would account for the increases in the mobility of elements in all the treatments,
309 including the control over time and the greater increase in the earthworm-treatments.
310 Decreases in soil pH (Table 6) may be due to earthworm-enhanced degradation of
311 organic matter leading to the release of organic acids [42]. Organic matter degradation
312 by indigenous microorganisms in the control treatments would explain the
313 significantly ($p<0.01$) lower soil pH in the control columns after 112 days compared
314 to 24 days (Table 6).
315
316 **Impact of earthworms on arsenic speciation in soil**
317 The XANES spectra of all six earthworm-treated samples (faeces and bulk earthworm
318 worked soil) look the same as the spectrum of the control soil sample, with an edge
319 position characteristic of oxygen-bound As(V) (Figure SI 1). This similarity to the
320 control sample indicates that no difference in the speciation of the arsenic in the soil
321 between the treatments was detectable. All seven EXAFS fits (one control soil,
322 earthworm faeces for all three species and bulk earthworm-worked soil for all three

323 species) were essentially the same (Figure SI 2 and Table SI 2) indicating that there is
324 no evidence that the earthworms excreted As into the soil in a structure different from
325 that present in the earthworm-free control soil.
326
327 There is evidence that earthworms sequester metals and metalloids within their
328 chloragogenous tissues in two distinct structures (O-donating, phosphate-rich granules
329 and S-donating ligands) and then subsequently excrete them in a form different from
330 that ingested [8, 44-47]. It is not known whether these structures persist in the
331 environment after excretion and if they significantly impact on mobility and
332 availability. However, in the current study, there was no difference in As speciation
333 between earthworm casts, earthworm-worked soil and control soil detectable by
334 XAFS. This may be because the proportion of the As in the soil that was affected was
335 small compared with the bulk of the As and any changes in As speciation were below
336 the limits of detection using this technique. None-the-less, despite evidence that As
337 speciation is altered within earthworms as a detoxification mechanism [48-50] , we have
338 not been able to detect evidence for the persistence of these changes in the earthworm
339 worked soil.
340
341 **Impact of earthworms on soil microbial community composition**
342 There were distinct differences in the PLFA profiles for the different earthworm
343 species, as revealed by PCA. The first two components explained 58.3% and 16.5%,
344 respectively, of the variation in the data set, with the second principal component
345 separating the data according to the four earthworm treatments (Figure 2). The two
346 fatty acids with greatest influence on PC2 were 18:1ω9c (negative loadings) and
347 cy19:0 (positive loadings). The ratios of cyclopropyl fatty acids to their precursor *cis*

348 monounsaturated fatty acids are considered to be effective indicators of stress in soil
349 microbial communities [27, 51]. Therefore Figure 2 represents a separation of the
350 treatments in terms of the degree to which the microbial community is stressed.
351 Similar differences can be identified between the treatments when stress indicators
352 (ratios of the 18:1ω9t to 18:1ω9c and cy19:0 to 18:1ω9c fatty acids) are expressed on
353 a biomass basis (Table 8). *L. terrestris* and *E. veneta* significantly ($p<0.05$) increased
354 these ratios and the patterns of this stress are closely correlated to the degree to which
355 earthworms mobilise metals and metalloids.
356
357 The soils inhabited by all three species of earthworm have a lower microbial biomass
358 than the earthworm-free control soil and this is a significant difference ($p<0.05$) for
359 the soil inhabited by *A. chlorotica* (Table 8). This is evidence that different species of
360 earthworm impact the microbial community differently. Wen *et al.* [30] showed
361 increases in the microbial populations (measured by the cultivation-based dilution
362 plate method) of soils in which *Eisenia fetida* increased the mobility and
363 bioavailability of metals. However, no relationship between the size (biomass) of the
364 microbial community and the mobility or availability of metals or metalloids in the
365 soil was found in the current study. It therefore seems likely that mobilisation of
366 metals and metalloids by *L. terrestris* and *E. veneta* resulted in a toxicity-related
367 change in microbial community structure rather than the earthworms altering the
368 microbial community which in turn mobilised the elements. It can therefore be
369 concluded that increased metal availability due to earthworm activity changed the
370 microbial community to a more stressed state. It is unlikely that the presence of dead
371 earthworms in the soil had any significant affect on the PLFA profiles. Mortality
372 occurred in only half of the replicates from the *L. terrestris* and *E. Veneta* treatments

and the same effects are observed when these replicates are removed from the analysis.

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. Evidence suggests that in multi-element contaminated soils, such as the one used in this study, earthworms may enhance the mobility and availability of metals and As. Thus, earthworms may increase the efficiency of phytoremediation treatments when plants are employed to extract metals and metalloids from the soils and accumulate them into their tissues. Conversely, if the remedial strategy is to immobilise metals *in situ* via the addition of amendments, earthworms may decrease the efficiency of remediation. 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.

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Supplementary information

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

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500

501 **Table 1 Chemical properties of the soil used in the experiments. Values are means of 12**
502 **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

503 [1]Based on BS7755-3.2 (1995) [15] [2]Loss on ignition [3]Aqua regia extractable concentrations based on
504 BS7755-3.9 (1995)[52].
505

506

Table 2 Percentage mortality and As, Cu, Pb and Zn loadings in earthworm tissues after incubation for 24, 56 and 112 days in contaminated soil (n = 4, ± standard error).

	A. chlorotica			*L. terrestris*			*E. veneta*		
	24 days	56 days	112 days	24 days	56 days	112 days	24 days	56 days	112 days
% Mortality	30.0±6.8	31.3±4.3	25±4.1	0	0	25.0±14.4	0	0	10.0±10.0
As (mg kg^{-1})	23.0±5.9 a	23.9±4.8 a	22.3±1.5 a	117±23.5 b	152±24.9 b	108±40.5 b	24.7±8.4 c	32.3±7.1 c	33.3±4.4 c
Cu (mg kg^{-1})	20.4±1.1 a	32.6±1.5 b	44.4±2.7 c	47.4±7.6 d	72.4±9.1 d	72.3±18.5 d	36.3±4.4 e	39.2±2.6 e	41.9±3.0 e
Pb (mg kg^{-1})	7.0±0.9 a	9.8±0.6 ab	12.0±1.7 b	14.0±2.0 c	21.6±1.8 cd	26.7±5.8 d	16.6±2.9 e	22.2±2.4 e	54.8±4.7 f
Zn (mg kg^{-1})	266±8.0 a	307±7.1 b	309±4.6 b	415±42.7 c	618±48.4 d	714±40.4 d	133±4.1 e	138±4.4 e	145±8.6 e

Different letters indicate significantly different concentrations of a certain element between earthworms of the same species sampled at different exposure times at the 95% level.

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 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 4 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 5 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 6 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 7. Dry biomass of ryegrass shoots grown on the top of earthworm-free control columns or soil inhabited by earthworms for a period of 28, 56 or 12 days (n = 4, ± standard error).

		Mass (g)
Control	28 days	0.075±0.007
	56 days	0.053±0.013
	112 days	0.058±0.004
A. chlorotica	28 days	0.067±0.004
	56 days	0.050±0.011
	112 days	0.065±0.005
L. terrestris	28 days	0.057±0.005
	56 days	0.036±0.006
	112 days	0.055±0.002
E. veneta	28 days	0.072±0.004
	56 days	0.087±0.004
	112 days	0.067±0.003

* = statistically significantly different from the earthworm free control columns sampled at the same time at the 95% level.

Table 8. 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

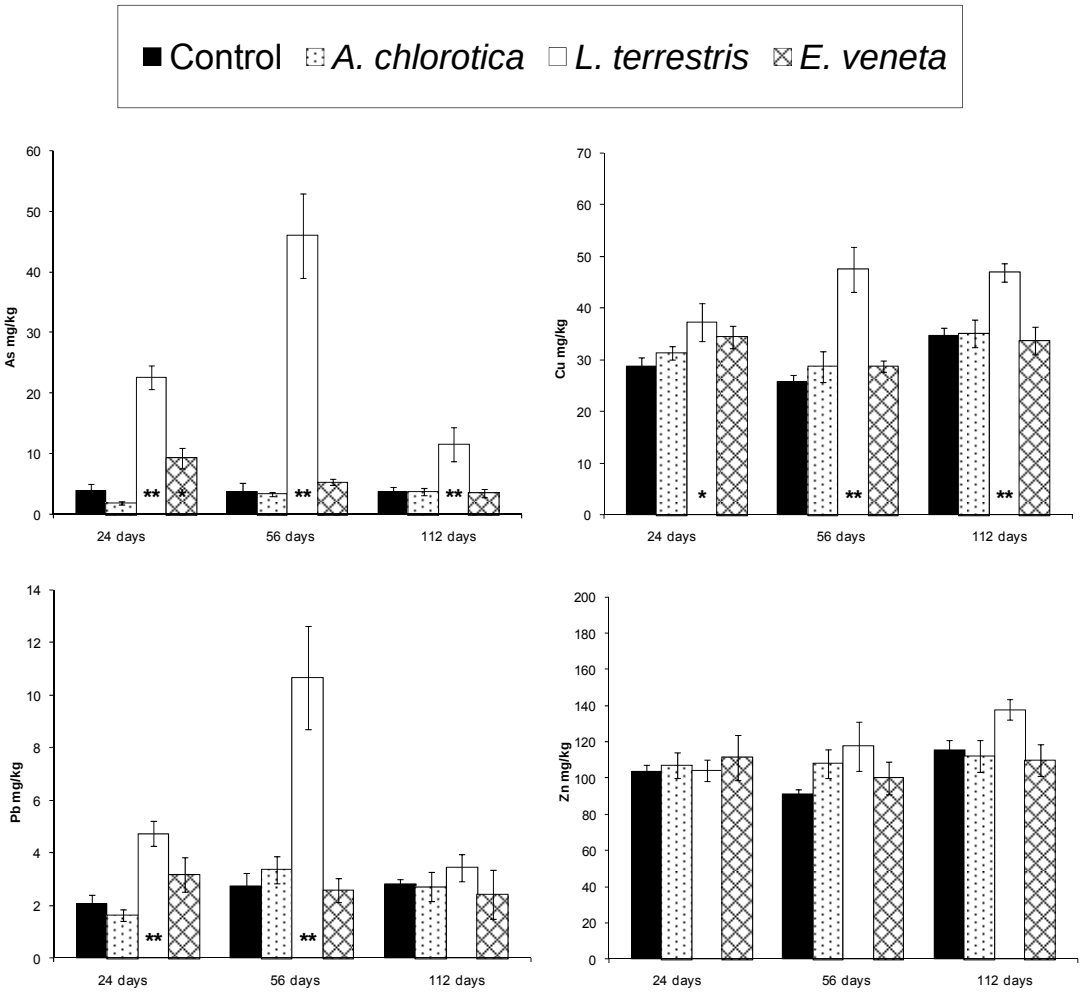

Figure 1. Concentration of As, Cu, Pb and Zn in ryegrass shoots grown on columns inhabited by earthworms compared with earthworm free columns. Values are means of 4 replicates ±SE. Asterisks indicate statistically significant differences between control and earthworm inhabited soils at the 95% (*) and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

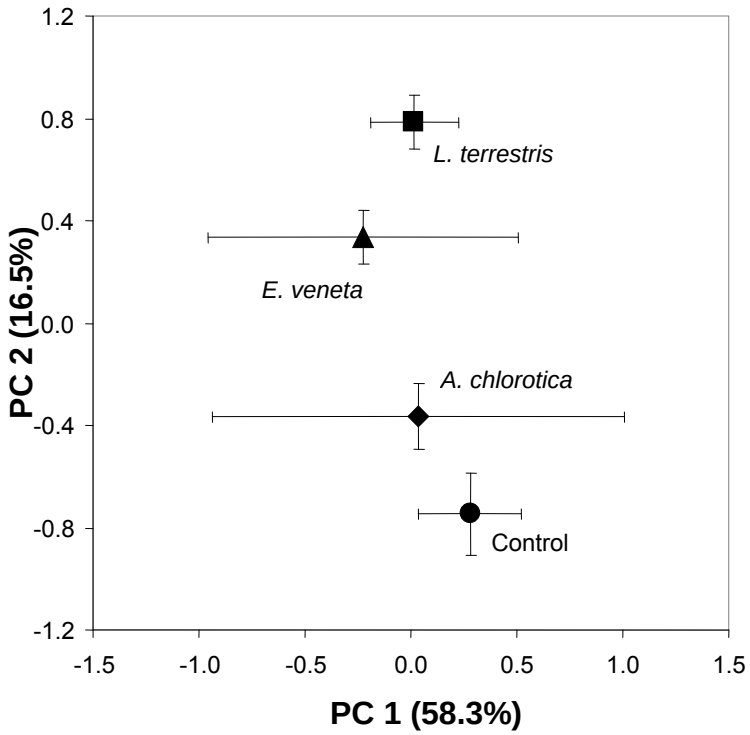

Figure 2. Principal component score plot of ordination means (n = 4, error bars indicate standard errors) showing the effect of earthworm species on soil microbial community structure, as characterized by PLFA profiling of control earthworm-free soil or soil inhabited by earthworms after 112 days.