Impact of gut passage and mucus secretion by the earthworm *Lumbricus terrestris* on mobility and speciation of arsenic in contaminated soil

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16 **Abstract**

17 Earthworms inhabiting arsenic contaminated soils may accelerate the leaching of As into surface and
18 ground waters. We carried out three experiments to determine the impact of passage of As contaminated
19 soil (1150 mg As kg^{-1}) through the gut of the earthworm *Lumbricus terrestris* on the mobility and
20 speciation of As and the effects of earthworm mucus on As mobility. The concentration of water soluble
21 As in soil increased (from 1.6 to 18 mg kg^{-1}) after passage through the earthworm gut. Casts that were
22 aged for 56 days still contained more than nine times greater water soluble As than bulk earthworm
23 inhabited soil. Changes were due to increases in As(V) mobility, with no change in As(III). Dilute
24 mucus extracts reduced As mobility through the formation of As-amino acid-iron oxide ternary
25 complexes. More concentrated mucus extracts increased As mobility. These changes, together with
26 those due to the passage through the gut, were due to increases in pH, phosphate and soluble organic
27 carbon. The mobilisation of As from contaminated soils in the environment by cast production and
28 mucus secretion may allow for accelerated leaching or uptake into biota which is underestimated when
29 bulk soil samples are analysed and the influence of soil biota ignored.

30

31 **Keywords**: Cast, Risk Assessment, Ternary complexes, Water soluble organic carbon, pH

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1. Introduction

Anthropogenically induced increases in arsenic concentrations in soil above background levels due to past mining activities can lead to toxic effects on soil biota and plant life. Migration of As from such soils to surface or ground waters can result in contaminated drinking water [1]. Upon entering the pedosphere As interacts with the soil biota and may therefore undergo changes in bioavailabilty and chemical speciation which affect its environmental fate. To improve the risk assessment of As contaminated soils and better protect the environment and human health, a greater understanding on how soil biota influence the mobility and speciation of As in soil is required. Earthworm biomass in most soils exceeds that of all other soil-inhabiting invertebrates [2] and earthworms are found in soils containing elevated levels of As [3].

Lumbricus terrestris is a common anecic earthworm native to Europe but widely distributed around the world in woodland and pasture soils. Earthworms increase the mobility of metals and metalloids in soils [4]. *L. terrestris* increases the leaching of As from soil columns [5] and the mobility of As is greater in the casts of *L. terrestris* than the surrounding soil [6]. However, the longevity of such increases in the soil environment are unknown. In addition, despite the mobility and bioavailability of As in soil being greatly dependent on speciation, little is known about how this is affected by passage through the earthworm gut. The earthworm gut is an anoxic environment [7] leading to the suggestion that reduction of As(V) to As(III) may be responsible for some of the increases in mobility observed [5]. *L. terrestris* produce casts on the soil surface that are chemically, biologically and physically different to the bulk soil and they construct permanent vertical burrows leading to aestivation chambers which they line with their own faeces [8]. There is therefore potential for As to be leached out of the casts, either on the soil surface into surface waters or through earthworm burrows into ground water, at a rate greater than from bulk earthworm-free soil.

Earthworms secrete mucus from the surface of their bodies to aid locomotion through burrows in the soil and this represents a significant portion of an earthworm's carbon budget [9]. Mucus is produced in greater quantities during copulation [2] and so experiments where single earthworms are incubated in test chambers may not accurately represent the impact of earthworm mucus on As mobility. Earthworm mucus may increase the concentration of dissolved organic carbon in the soil solution resulting in greater competition between As and organic carbon for binding surfaces on positively charged soil constituents such as iron and manganese oxides [10] leading to an increase in As mobility. Alternatively, zwitterions such as amino acids in earthworm mucus may reduce the mobility of soil contaminants by complexing contaminants from the solution while simultaneously binding to soil surfaces [11].

We carried out three experiments to test the hypotheses that passage through the anoxic gut of *L. terrestris* increases the mobility of As and reduces As(V) to As(III) and that the secretion of earthworm mucus alters the mobility of As in a contaminated mine soil.

2. Experimental

2.1 Earthworms and soil

Lumbricus terrestris (L.) were sourced from Worms Direct, Ulting, UK. Devon Great Consols (DGC) (50.540851 -4.226920; WGS84) soil was collected from a grassed field adjacent to a former Cu and As mine in South-West England. Soil was collected from the top 30 cm of the soil profile and on return to the laboratory, dried (40 ºC), sieved (<2 mm), homogenised and stored until the start of the experiment. Soil pH was measured in a soil-water suspension (based on BS7755-3.2 [12]), percentage organic matter by loss on ignition (500 ºC), and soil texture by laser granulometry (Coulter LS 230 Particle Size Analyzer). Sand was classified as particles 2000-63 µm, silt as 63-2 µm and clay as < 2 µm in diameter. Pseudototal elemental composition was determined by digestion in aqua regia (based on BS7755-3.9 [13]) and cation exchange capacity was measured at pH 7 using the ammonium acetate method [14].

Soil water holding capacity was determined gravimetrically. Properties of the soil used in the experiments are given in Table 1.

2.2 Experiment 1: Impact of gut passage on As mobility over time

L. terrestris were incubated at 16 ºC in 30 bags (five specimens per bag) containing 500 g of DGC soil, moist to 80% of the water holding capacity, for 7 days alongside earthworm free bags containing 50 g of soil. At the end of the incubation all of the bags were emptied and the soil in each bag homogenised. Earthworms were removed from the soil and left for 24 hours on moist filter paper to void their guts [15]. The filter papers were then sealed, moist in petri dishes, preventing evaporation, to simulate moist casts ageing in the soil environment. Bulk earthworm-inhabited soil and earthworm-free soil (circa 50g of soil) was kept in sealed plastic bags alongside petri dishes. Fresh casts (pooled from all 5 earthworms) and those aged for 1, 7, 14, 28 and 56 days, were air-dried at 30 ºC along with fresh and aged soils. One gram of air-dried soil/cast samples were extracted with 10 ml of >18.2 MΩ cm ultra pure water on a rotary shaker for 24 hours at 30 rpm at 20 °C. Soil pH was measured in the soil suspension followed by centrifuging at 3000 g for 20 min at 20 °C to produce supernatants. The supernatants were passed through 45 µm cellulose nitrate membrane filters prior to analysis. Arsenic concentration and water soluble organic carbon were determined in the supernatant by ICP-OES (Perkin Elmer Optima 7300 DV Inductively Coupled Plasma-Optical Emission Spectrometer) and a Shimadzu TOC (Total Organic Carbon) analyzer respectively.

2.3 Experiment 2: Impact of gut passage on As speciation

L. terrestris were incubated at 16 ºC in five plastic boxes (ten specimens per box) containing 1 kg of DGC soil, moist to 80% of the water holding capacity, for 7 days alongside five earthworm-free boxes of soil. At the end of the incubation the boxes were emptied and the soil in each box homogenised. Earthworms were removed from the soil and left for 48 hours on moist filter paper to void their guts

[15]. The casts were collected and air-dried at 30 ºC along with bulk earthworm-inhabited soil and earthworm-free soil. Air dried samples were transported to the Analytical Geochemistry Laboratory at the British Geological Survey, Keyworth and analysed separately to the previous experiment to ensure that freshly produced samples were analysed within 24 hours of extraction. Therefore experimental and analytical procedures differed in order to match instrument availability and adhere to local standard operating procedures. One gram of air-dried soil/cast samples were shaken at 250 rpm on an orbital shaker with 10 ml of >18.2 MΩ cm ultra pure water for 72 hours followed by centrifugation at 3000 g for 20 min at 20 °C to produce supernatants. The supernatants were passed through 45 µm nylon membrane filters prior to analysis. Arsenate (AsV), monomethylarsenic (MA), dimethylarsenic (DMA), arsenite (AsIII) and arsenobetaine (AB) species of As were then quantitatively determined in the supernatants by HPLC-ICP-MS (Dionex AS-50, GP-50 gradient pump High Performance Liquid Chromatography coupled with Agilent Technologies 7500 Series Inductively Coupled Plasma Mass Spectrometer) using the method described by Watts et al [16].

2.4 Experiment 3: Impact of mucus on As mobility

Based on the method of Zhang, et al. [17], 500 *L. terrestris* were depurated [15] for 48 hours and distributed between five 500 ml beakers to give an earthworm-free control beaker and beakers containing 50, 100, 150 and 200 earthworms. Earthworms were sprinkled evenly with 10 g quartz sand per beaker and the beakers covered with pierced parafilm. After 4 hours at 18 ºC the earthworms were removed and rinsed over the beakers with >18 MΩ cm ultra pure water. The contents of the beakers were then filtered (Whatman 540) and diluted to 250 ml. This produced five solutions, four dilute earthworm mucus solutions and a deionised water control solution. pH (Jenway 3310 pH meter), major elements (ICP-OES), major anions (Dionex DX-500 ion chromatograph) and organic carbon (Shimadzu TOC 5000) were determined in all of these solutions and are given in Table 2.

A 100 ml subsample of each of these five solutions was freeze-dried and re-dissolved in 1 ml of deuterated water. The solid, freeze-dried component of all the dilute mucus solutions did not completely dissolve in the deuterated water and therefore the subsequent analysis can only be considered qualitative. Liquid-state proton NMR (Nuclear Magnetic Resonance) spectroscopy (Bruker AVIII 700 with a TCI cryoprobe) was carried out on the five solutions and compared to amino acid standards (21 L-amino acids plus glycine; Sigma Aldrich) in order to identify amino acids present in earthworm mucus.

One gram of air-dried, DGC soil was extracted with 10 ml of each solution (replicated 5 times) by mixing on a rotary shaker for 16 hours at 30 rpm and 20 °C. pH was determined in the tubes containing the soil suspension which were then centrifuged at 3000 g for 20 min at 20 ºC. The supernatants were passed through 45 µm cellulose nitrate membrane filters and analysed for soluble organic carbon (Shimadzu TOC 5000) and soluble As (ICP-OES).

2.5 Statistical analysis

Minitab version 15 was used for all statistical analysis. Normality of data and equal variance between treatments was tested using the Kolmogorov-Smirnov test ($p>0.01$) and Bartlett's test ($p>0.01$), respectively. Where comparisons between treatments were made (e.g. between casts, bulk or control soil), one-way Analysis of Variance (ANOVA) was carried out and Fisher's Least Significant Difference test ($p<0.05$ and $p<0.01$) used to identify significant differences between individual means. When data was found to be non-parametric, the Kruskal-Wallis Test was carried out and the Mann-Whitney U test used to compare individual means.

2.6 Quality control

The aqua regia digestion of soil samples was carried out alongside an in-house reference material traceable to a certified reference material (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 of these elements were 93%, SD = 4.2, n = 2 for Pb, 90%, SD = 0.81, n = 2 for Zn and 103%, SD = 2.4, n = 2 for Cu This confirmed the efficiency of the acid digestion. Arsenic was below detection limits in the in-house reference material (<14 mg kg^{-1}) so an in-house quality control As solution was run alongside the ICP-OES analysis of As solutions. The recovery of this reference solution was 103%. The sum of As species identified by HPLC-ICP-MS was compared to total As concentrations measured in the supernatant by ICP-MS. Recoveries of the total As in the supernatant were 104% (SD = 5.2, n = 5), 101% (SD = 1.0, n = 5) and 102% (SD = 0.7, n = 5) for casts, bulk earthworm inhabited soil and control soil, respectively. The detection limits of the individual species of As were 0.10, 0.013, 0.020, 0.054 and 0.053 mg kg^{-1} for AsV, MA, DMA, AsIII and AB respectively. NMR samples were dissolved in water containing an internal reference standard (d4-trimethylsilylpropionic-acid), the presence and position of which was identified for each sample analysed.

3. Results and Discussion

3.1 Experiment 1: Impact of gut passage on As mobility over time

There was significantly ($p<0.001$) greater water soluble As, soluble organic C and soil pH in the fresh casts of *L. terrestris* and after ageing for 1, 7, 14, 28 and 56 days compared to both bulk earthworm-inhabited and earthworm-free control soil (Figure 1). There were no significant differences in water soluble As, soluble organic C or soil pH between the bulk and control soil at any of the time points. The concentration of water soluble As was significantly ($p<0.01$) greater in the fresh casts and those aged for 1 and 7 days than in the casts aged 14, 28 and 56 days. Soil pH was significantly ($p<0.01$) greater in the fresh casts and those aged 1 and 7 days than the casts aged 14 days which in turn were significantly ($p<0.01$) greater than casts aged 28 and 56 days. There were no significant differences in soluble C in casts between any of the time points.

185 The increase in water soluble As concentrations in the casts of *L. terrestris* inhabiting As
186 contaminated soil (Figure 1) agrees with previous experiments using DGC soil [6], but until now the
187 longevity of such effects has been unknown. Even after casts were aged, moist for 56 days, the
188 concentration of water soluble As was more than nine times greater than bulk earthworm inhabited soil.
189 This not only shows that passage through the earthworm gut increases the mobility of As in soil, but that
190 this effect persists in the soil environment for sufficient time for As to be leached out of the soil and
191 longer than the time after cast deposition that microbial activity is elevated [18]. As rainfall events are
192 frequent in South-West England, where DGC soil was collected, it is likely that after deposition of an
193 earthworm cast on the surface of the soil a rainfall event will take place while the mobility of As in the
194 cast is still elevated. This increases the chance that As may be leached out of the casts to water bodies.
195
196 Mineral fragments in DGC soils are coated in thin films of Fe oxyhydroxides (up to 50 μm thick),
197 which are the main carriers of As in the mine soils [19]. Increases in the pH of soils containing Fe
198 oxides and oxyhydroxides results in the soil becoming increasingly positively charged and favours the
199 desorption of oxyanions of arsenate and arsenite [20]. Increases in soluble organic carbon increases the
200 competition between dissolved organic matter and As oxy-anions for sorption sites on Fe oxides and
201 oxyhydroxides [10]. Although both increases in soil pH and increases in soluble organic carbon may be
202 responsible for the observed increases in metal mobility in this experiment, it is likely that the increase
203 in pH is responsible for the earthworm induced changes observed here because the changes in pH over
204 time more closely match the changes in water soluble As (Figure 1).
205
206 3.2 Experiment 2: Impact of gut passage on As speciation
207 The majority of the water soluble As in the bulk earthworm-inhabited and earthworm-free control
208 soils was identified as As(V) and As(III) with small quantities (<2%) of AB and DMA (Figure 2). MA
209 was not identified in any of the samples and only As(V) and As(III) were identified in the earthworm
210 casts. The total concentration of water soluble As was significantly ($p<0.001$) greater in the casts

211 compared to the bulk earthworm inhabited or control soil and there was no significant difference
212 between bulk and control soils in terms of total As, As(III) or As(V), in agreement with the observations
213 in Experiment 1 (Figure 1). There was a significantly ($p < 0.001$) greater concentration of water soluble
214 As(V) but not As(III) in the casts of *L. terrestris* compared to both the bulk earthworm-inhabited and the
215 earthworm-free control soil (Figure 2). This suggests that the increase in the mobility of As in the
216 earthworm casts observed in Experiment 1 was due to the mobilisation of As(V).
217
218 Sizmur et al. [5] suggested that the reason for increased concentrations of As in water leached through
219 columns of As-contaminated soil from DGC inhabited by *L. terrestris* may have been due to earthworm
220 facilitated decomposition whereby organic matter was physically and chemically conditioned for
221 microbial and enzymatic attack [21] leading to degradation of organically bound As and subsequent
222 release of As into the soil solution. An alternative hypothesis offered was that As(V) may be reduced to
223 As(III) in the anoxic earthworm gut [7] leading to a concurrent increase in As mobility due to the greater
224 solubility of As(III) compared to As(V). Experiments 1 and 2 from the current study support the
225 hypothesis previously suggested [5] that passage through the earthworm gut increases the pH of the soil
226 and stimulates the degradation of organic matter leading to mobilisation of organically bound As(V).
227
228 3.3 Experiment 3: Impact of mucus on As mobility
229 The concentration of As extracted with the dilute earthworm mucus solutions significantly ($p<0.001$)
230 increased with the number of earthworms used to produce the solutions (Figure 3). This was observed
231 alongside a significant ($p<0.001$) increase in the pH of the mucus-soil suspension and a significant
232 ($p<0.001$) increase in the concentration of soluble organic carbon. In addition there were greater
233 concentrations of phosphate in the mucus solutions produced using 150 or 200 earthworms (Table 2)
234 Mechanisms for the increase in extractable As could be greater desorption of As from Fe oxides and
235 oxyhydroxides as surfaces become increasingly positively charged [20] and as there is greater
236 competition between organic or inorganic (phosphate) ligands and As for sorption sites. The phosphate

237 and arsenate oxyanion are chemically very similar and therefore compete for the same sorption sites on
238 the surfaces of soil particles which leads to increases in the desorption of As in soil solutions that
239 contain high concentrations of phosphate [22].
240
241 However, the concentrations of As extracted with the solutions made using the 50 and 100 earthworm
242 treatments were lower than the As extracted with the deionised water control solution (Figure 3), despite
243 these mucus solutions having greater pH, TOC and concentration of ions (including phosphate) in
244 solution (Table 2). This is contrary to what would be expected if the organic C in the mucus behaved
245 like the fulvic and humic acids that make up dissolved organic matter found in soils and sediments [10].
246 The formation of ternary complexes has been shown to increase the sorption of As [23], U(VI) [24] and
247 Cu and Zn [25] to iron oxides in previous studies. An explanation for the decrease in As mobility in the
248 presence of the 50 and 100 earthworm mucus solutions may be the formation of FeOH – amino acid –
249 As complexes. Amino acid zwitterions such as leucine, isoleucine, valine and lysine were identified in
250 the dilute earthworm mucus solutions used in this experiment (Figure 4), in agreement with amino acids
251 identified by Zhang et al. [26] in the mucus of the earthworm *Metaphire guillemi*. The *pKa* constants of
252 the positively charged amine groups and negatively charged carboxyl groups of these amino acids (9.6
253 and 2.4 for leucine, 9.7 and 2.4 for isoleucine, 9.6 and 2.3 for valine and, 9.0 and 2.2 for lysine
254 respectively) [27] indicate that the amino acids will act as zwitterions within the pH range of this
255 experiment (4-6). Therefore, these amino acids may act as a bridging compound between the positively
256 charged iron oxide (point of zero charge 6.5 for magnetite, 6.8 for goethite and 6.7 for hematite) [28]
257 and the negatively charged $H_2AsO_4^-$ oxyanion (*pKa* 2.20) [29]. In the case of lysine, two As oxyanions
258 may be associated with each positively charged site on the surface of iron oxide due to lysine's
259 positively charged side chain (*pKa* 10.5) [27]. In the more dilute mucus solutions produced from 50 or
260 100 earthworms this ternary complexation effect dominates over the impact of increasing pH and
261 phosphate but in the more concentrated mucus solutions produced from 150 or 200 earthworms the

effects of increasing pH and phosphate dominate, probably because the positively charged sites on the surface of the iron oxide are saturated.

3.4 Environmental Relevance

The anthropogenic input of As into the soil environment is of serious environmental concern and the migration of As from contaminated soils to receptors such as vegetation, water courses or human populations needs to be quantified and mitigated [30]. Estimates of the transfer of As from the pedosphere into the hydrosphere and biosphere [31] have not considered the effects of biological processes in the soil environment on the mobility and toxicity of As in the soil. Since the ecology of anecic earthworms results in the deposition of fresh casts on the surface of the soil, there is a risk that when rainfall events result in overland flow, As mobilised in cast material may leach out of the soil and into surface waters where toxic effects on biota and human populations can occur. In addition, the permanent vertical burrows created by anecic earthworms, provide channels of least resistance for water to percolate through the soil [32] to depths reaching the water table. During passage through the topsoil to the subsurface and eventually the groundwater, rainfall will percolate through the earthworm faeces used to line these burrows and aestivation chambers. Burrows are also lined with earthworm mucus [8] which, depending on the concentration, may either further increase or decrease the mobility of As. Owing to these environmentally relevant biogeochemical processes, the migration of As from contaminated soils to water courses may be underestimated when bulk soil or porewater samples are analysed and the impacts of soil biota are ignored.

Chapman et al. [33] discuss the use of safety factors in human and ecological risk assessment when extrapolating laboratory exposure of contaminants to field exposure, concluding that appropriate assessments of ecologically relevant endpoints be adopted in favour of safety factors. Here we provide

288 complementary evidence that chemical analyses of contaminated soils may not adequately explain the
289 bioavailability of contaminants to receptors due to the complex interactions between biota and
290 contaminants in the soil environment. We therefore recommend the assessment of appropriate,
291 ecologically relevant endpoints during the risk assessment of As in the environment, but where this data
292 is lacking, an additional safety/uncertainty factor of 10 be applied to assessments of the mobility or
293 bioavailability of As in contaminated soils where anecic earthworms are present.
294
295 **Supplementary data**
296 One figure is supplied as Supplementary data.
297
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Table 1 Mean chemical properties of soil used for earthworm experiments (n = 3, ± standard error).

	pH[1] (H_2O)	%WHC[2]	%OM (LOI)[3]	Pseudo-total elements[4] (mg kg^{-1})				CEC[5] ($cmol_c kg^{-1}$)	Texture[6]			
				As	Cu	Pb	Zn		% Sand	% Silt	% Clay	Classification[7]
DGC Soil	4.1	87.0	15.9	1150	362	109	89	21.0	41.5	54.9	3.63	Silt loam
	±0.00	±0.91	±0.03	±14	±3	±2	±1	±0.30	±1.12	±1.13	±0.12	

[1]Based on BS7755-3.2, 1995.[12] [2]Water Holding Capacity [3]Loss on ignition [4]Aqua regia extractable concentrations based on BS7755-3.9, 1995[13]. [5]Based on [14]. [6]Laser granulometry. [7]Using the United States Department of Agriculture soil texture triangle.

Table 2 Chemical properties of mucus solutions produced from 0, 50, 100, 150 or 200 earthworms (n = 3, ± standard error).

	0 earthworms	50 earthworms	100 earthworms	150 earthworms	200 earthworms
pH	5.49 ±0.08	7.22 ±0.02	7.15 ±0.02	7.31 ±0.01	7.28 ±0.01
Organic C (mg L^{-1})	2.25 ±0.31	6.58 ±0.12	6.45 ±0.14	12.2 ± 0.26	24.4 ±0.40
As (µg L^{-1})	< 11.6	< 11.6	< 11.6	< 11.6	< 11.6
Ca (µg L^{-1})	120 ±13	4790 ±33	4380 ±81	7740 ±140	13000 ±23
Fe (µg L^{-1})	9.14 ±3.5	27.0 ±1.8	53.8 ±1.2	125 ±5.5	378 ±11
K (µg L^{-1})	106 ±58	8250 ±27	11700 ±120	21600 ±910	29800 ±270
Na (µg L^{-1})	282 ±20	11000 ±73	21100 ±240	43800 ±750	56000 ±81
Cl^- (mg L^{-1})	0.350 ±0.050	6.80 ±0.029	17.3 ±0.19	36.2 ±0.23	42.5 ±0.060
PO_4^{3-} (mg L^{-1})	< detection	2.42 ±0.083	2.30 ±0.050	6.43 ±0.11	10.9 ±0.11
SO_4^{2-} (mg L^{-1})	< detection	9.95 ±0.029	12.63 ±0.060	22.3 ±0.017	32.8 ±0.083

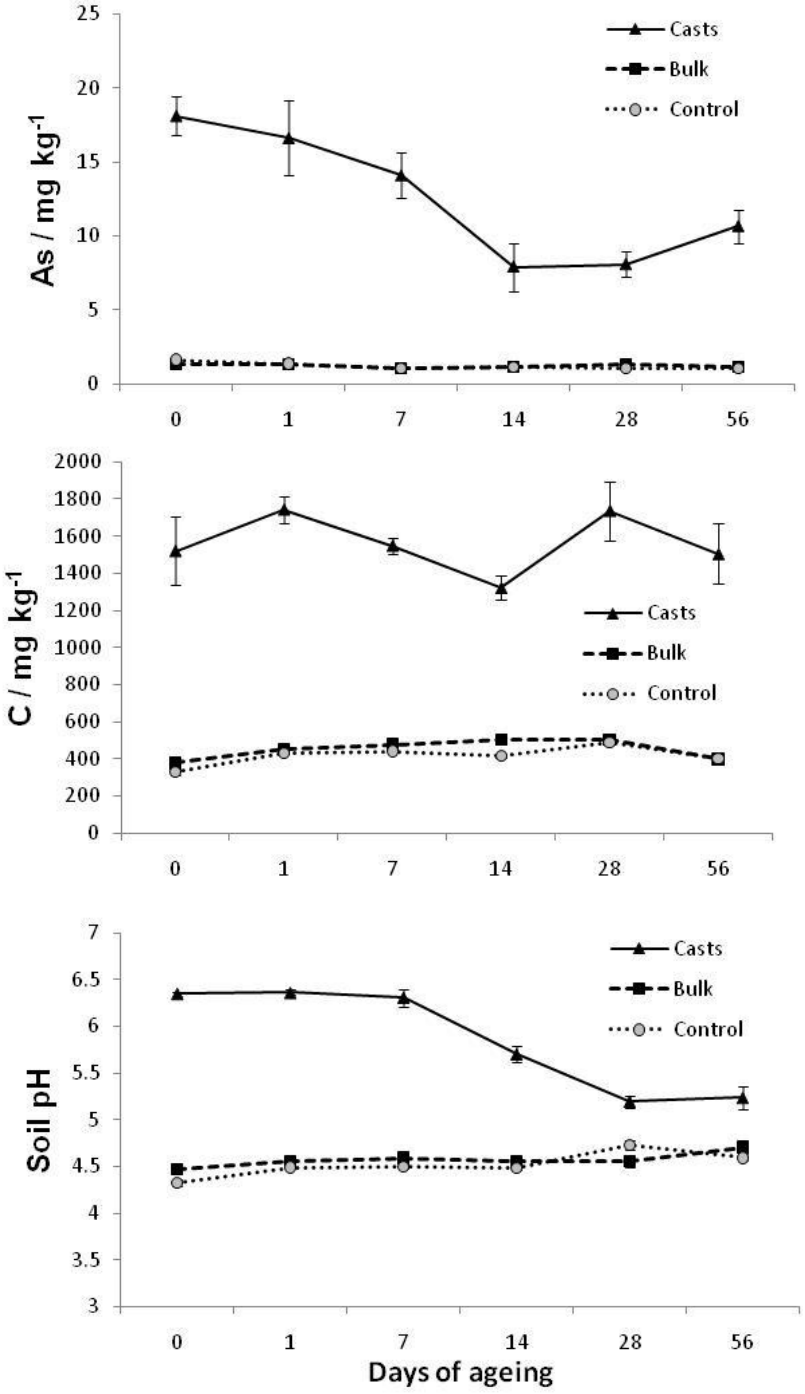

Figure 1. Water soluble arsenic, organic carbon and soil pH in *Lumbricus terrestris* casts, bulk earthworm-inhabited and earthworm-free control DGC soil after 7 days of earthworm incubation and then further ageing of 0, 1, 7, 14, 28 or 56 days. Error bars are standard errors of the mean, n = 5.

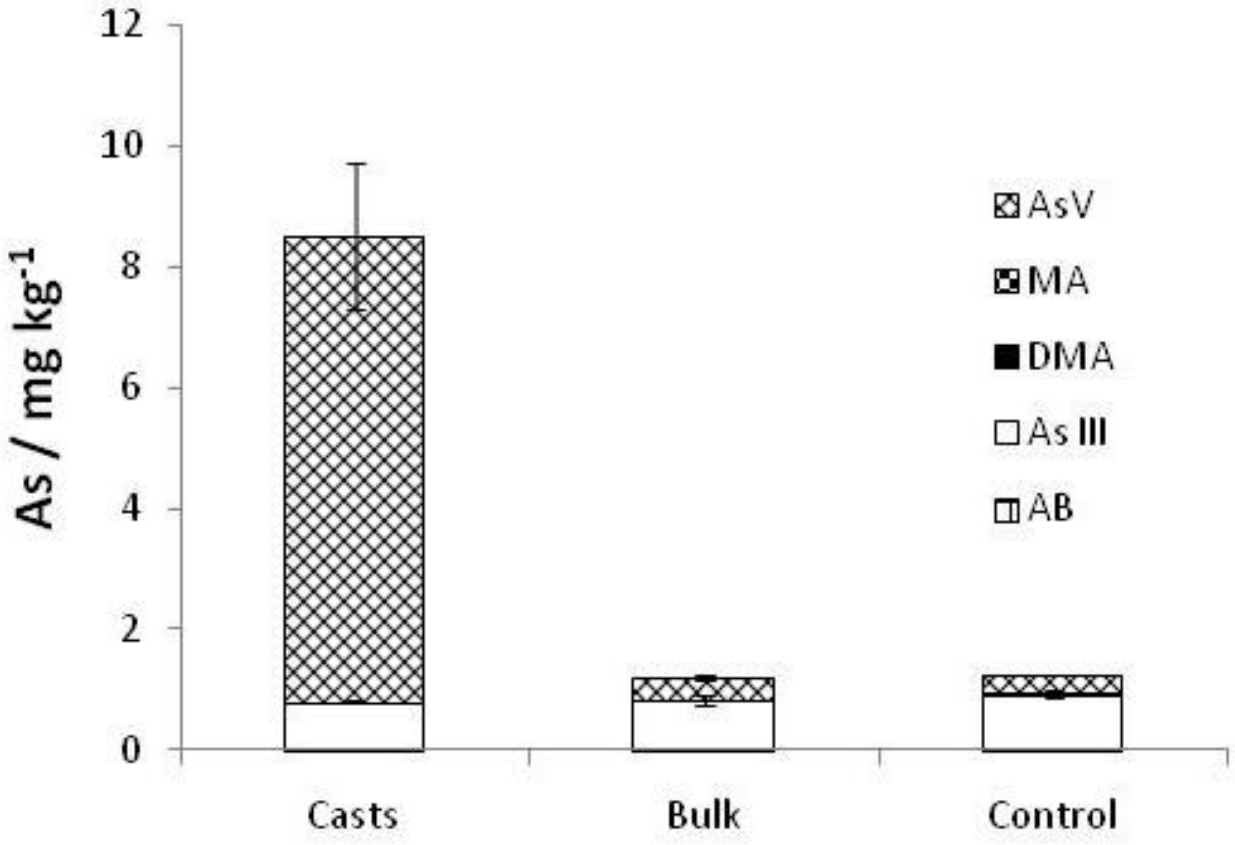

Figure 2. Concentration of water soluble arsenate (AsV), monomethylarsenic (MA), dimethylarsenic (DMA), arsenite (AsIII) and arsenobetaine (AB) in *Lumbricus terrestris* casts, bulk earthworm-inhabited and earthworm-free control DGC soil after 7 days incubation. Error bars are standard errors of the mean, n = 5.

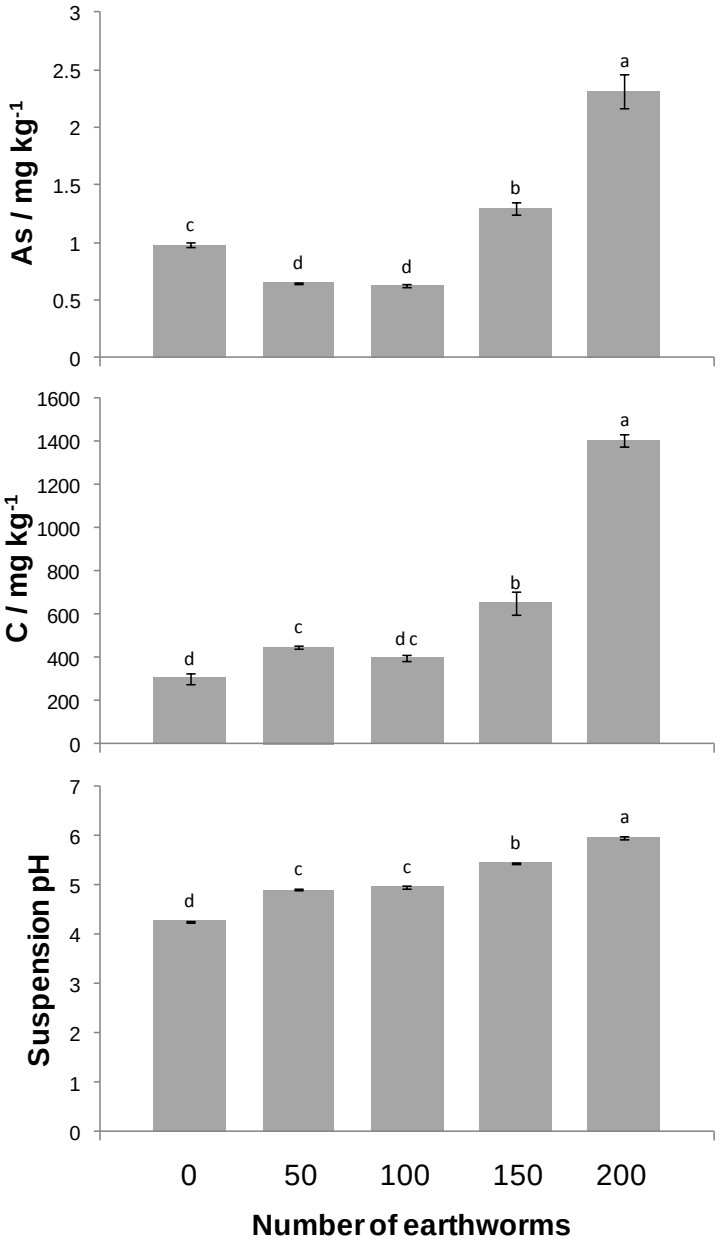

Figure 3. Soluble arsenic, organic carbon and suspension pH in DGC soil extracted with a deionised water control solution (0 earthworms) and dilute mucus solutions produced from 50, 100, 150 or 200 *Lumbricus terrestris*. Error bars are standard errors of the mean, n = 5. Bars with different letters indicate treatments that are significantly ($p<0.01$) different from one another.

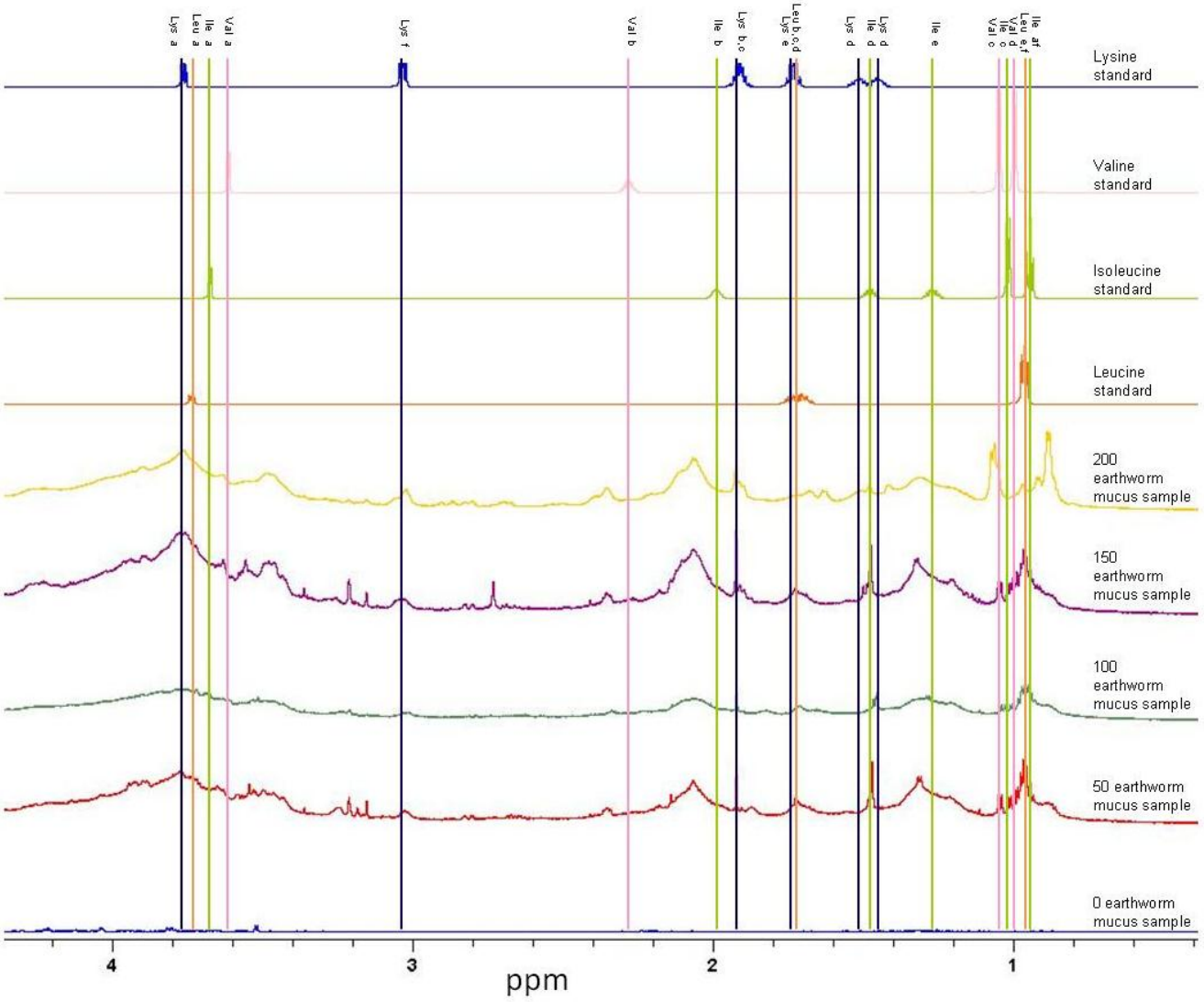

Figure 4. Proton NMR spectra in the aliphatic region (0.5 to 4.5 parts per million) of mucus samples produced using 0, 50, 100, 150 and 200 earthworms compared to spectra of selected amino acid standards. Peaks have been identified as specific functional groups (Figure S1) for Lysine, Valine, Isoleucine and Leucine. [To be presented in black and white in print but available in colour online]

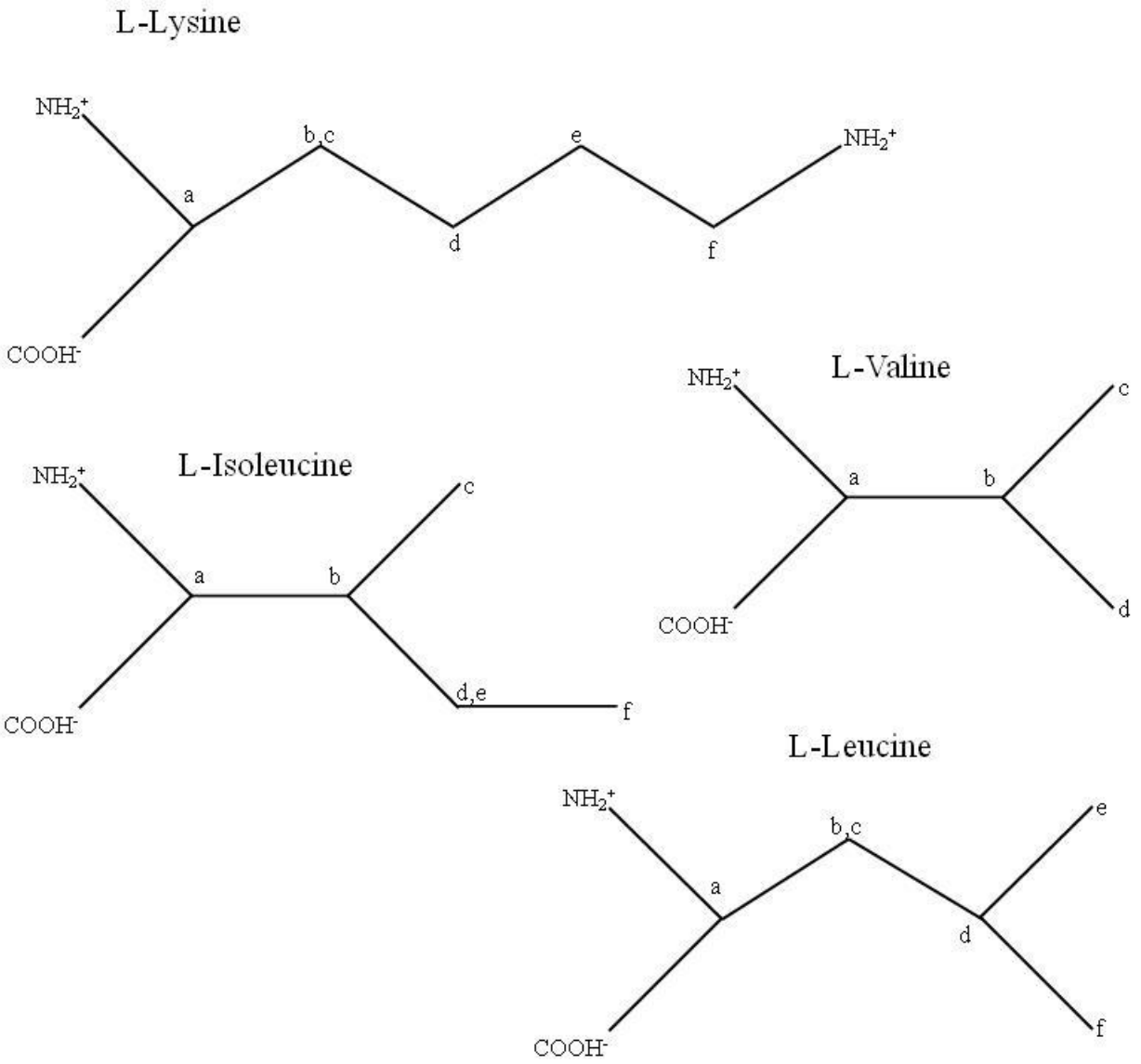

Figure S1. Identification of specific functional groups identified in NMR spectra of amino acid standards.