Quantitative Arsenic Speciation in Two Species of Earthworms from a former Mine Site

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The relationship between the total arsenic concentration and the chemical speciation of arsenic in two species of earthworm (*Lumbricus rubellus* and *Dendrodillus rubidus*) in relation to the host soil, was investigated for 13 sites of varying arsenic content, including a background level garden soil and a former mine site at the Devon Great Consols, UK. Earthworms were collected with the host soil (As soil concentration range 16 - 12,466 mg kg^{-1} dry weight) and measured for their total arsenic (concentration range 7 - 595 mg kg^{-1} dry weight) using inductively coupled plasma mass spectrometry (ICP-MS). A methanol-water mixture was used to extract arsenic species from the earthworms prior to determination of the individual arsenic species by a combination of anion and cation exchange high performance liquid chromatography coupled to inductively coupled plasma mass spectrometry (HPLC-ICP-MS). A gradient elution anion exchange method is presented whereby nine arsenic species could be measured in one sample injection. Arsenic species were identified by comparison of retention times and sample spiking with known standards and a fully characterised seaweed extract. Arsenic was generally present in the earthworm as arsenate (As^V) or arsenite (As^{III}) and arsenobetaine (AB). Methylarsonate (MA), dimethylarsinate (DMA) and three arsenosugars (glycerol, phosphate, sulphate) were present as minor constituents. These results are discussed in relation to the mechanisms for coping with exposure to soil bound arsenic.

Introduction

Sentinel organisms such as earthworms are often employed as an alternative risk assessment method for the biomonitoring of anthropogenic and geogenic toxins, particularly arsenic in the terrestrial environment[1]. Earthworms are of particular use in monitoring the potential exposure of single or multiple soil contaminants as they maintain constant contact with both the solid and liquid phase of soil. In this way, earthworms can be used in partitioning and chemical speciation models to assess bioavailability[2-5] and the basis for toxic effects[6, 7].

The study of the occurrence, accumulation and speciation of arsenic in the earthworm can help to provide more accurate assessments of the risk of exposure in both an ecological and human health context[8]. The behavioural response of earthworms to soil contaminants directly influences the degree to which exposure results in toxic effects. For example, *Lumbricus rubellus* reside in the upper soil horizon (~20 cm) and has been shown to discriminate between soils with concentration of sodium arsenate above 5000 mg kg^{-1} [9]. *Dendrodillus rubidus* is known to inhabit arsenic contaminated soils and mine waste[10-12], such as at the Devon Great Consols (DGC) in Devon, UK[9, 13]. The varied toxicities of arsenic compounds in the terrestrial environment emphasises the need to provide more information about the chemical form of arsenic in both the sentinel organism and soil in order to assess the risk of exposure to arsenic.

A major problem of identifying and quantifying arsenic species in solid samples, is an appropriate extraction method that will avoid transformations and maintain the integrity of the arsenic species. The extraction recovery may be influenced by the matrix or be species dependent. The lack of certified reference materials for arsenic species is a major hindrance for the development and validation of extraction methods. Methanol-water extractions have been widely used for the extraction of arsenic species from biota samples, including earthworms[14-17]. Arsenite and arsenate were demonstrated as the main extractable inorganic arsenicals in L. rubellus [15, 18]. In most cases arsenobetaine (AB) is present as the primary organic arsenic species with methylarsonate (MA) present at trace levels [14, 18]. However, Geiszinger *et al*. (2002)[19] found that a major arsenic containing compound in the earthworm extract (9:1 v/v methanol:water) was the phosphate arsenosugar, accounting for approximately 55 % of the total arsenic in the extract of the earthworm, with the glycerol arsenosugar, dimethylarsinate (DMA), methylarsonate (MA), arsenate (As^V) and arsenite (As^{III}) present as minor constituents. The two other arsenosugars were also detected in the cast extract, with some As^{III} and As^V.

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† Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here]. See http://dx.doi.org/10.1039/b000000x/

The measurement of arsenic species in environmental and biological samples at trace levels generally requires an on-line approach to the separation of arsenic species. HPLC is generally used for analyte separation, commonly using anion or cation exchange for organic compounds to identify arsenosugars[33]. ICP-MS is commonly coupled to HPLC [15, 20-22] for analyte detection. ICP-MS is suitable for aqueous samples and aqueous extracts of environmental and biological samples[23]. ICP-MS as the elemental detector offers the possibility of multielemental, multispecies determinations within the same sample measurement[24-27].

The aim of this study was to characterise the speciation of arsenic in earthworm populations from highly contaminated and uncontaminated soils, by determining the arsenic metabolites and potential differences between different species of earthworms. The speciation analysis of the samples was carried out using a high throughput HPLC system, incorporating anion exchange gradient elution and cation exchange isocratic elution.

Experimental

Sampling Sites

Devon Great Consols (DGC) is one of many former mining sites in the South West of England and is situated on the east bank of the River Tamar in the Tavistock district of Devon (sheet number GR: 426 735). Arsenic concentrations in soils found in and around the mine vary significantly depending on their proximity to the main tailings ranging from 249 to 34,000 mg kg^{-1} [9, 13]. Bioaccessibility of arsenic in soils at the mine is reportedly low due to arsenic binding to an iron and sulphide rich source such as arsenopyrite[13] yet still represents a significant risk to health due to the elevated arsenic levels. In addition, a Nottingham garden with soil arsnic levels below the UK Department for Food and Rural Affairs (DEFRA) Soil Guideline Value (SGV) of 20 mg kg^{-1} was also selected to provide samples of a lower concentration range.

Earthworm and Soil Sample Collection

The soil surface (0 - 20 cm) in a 1 m^2 grid was overturned with a spade and placed onto plastic sheeting allowing individual earthworms to be handpicked from their host soil. Specimens were promptly sorted according to species, thoroughly rinsed with deionised water and placed in ventilated tubes with moist filter paper to begin the depuration of gut contents. Earthworms were depurated for a minimum of 48 hours, as shorter time periods were unlikely to remove all soil particles in larger species such as L. rubellus.[10] Filter paper was changed daily and cast material collected. Depuration was halted when no more cast material was deposited on the filter paper. A small selection of earthworms was dissected and the gut contents examined under a microscope to ensure no soil particles remained. Approximately 10 to 25 earthworms were collected at each sampling. Depurated earthworms were thoroughly rinsed with deionised water, euthanased humanely and dried in a low temperature oven (50 °C) before homogenisation as a composite sample in a ceramic mortar and pestle for each collection point. All experiments were performed in compliance with the relevant laws and institutional guidelines.

The use of earthworms did not require ethical approval from any institutional committees. The host soil from the 1m^2 grid in which the earthworms were collected from was turned over using a spade to mix and , approximately 0.5 to 1 kg of a composite soil sample was placed into paper sample collection bags and dried at room temperature. Soil were sieved to < 250 μm fraction and stored in air and light sealed containers prior to analysis. Earthworm cast material was collected following depuration, dried (50 °C) and ground into a powder with a mortar and pestle.

Standards and Reagents

All reagents used were analytical grade or better quality. All aqueous solutions were prepared using deionised water (18.2 MΩ Millipore, UK). Arsenite (As(OH)$_3$), arsenate (AsO(OH)$_3$, Fisher, UK), monomethylarsonic acid (CH$_3$AsO(OH)$_2$, Sigma-Aldrich, UK), dimethylarsinic acid (CH$_3$)$_2$AsO(OH), Greyhound, UK) and arsenobetaine ((CH$_3$)$_3$As$^+$CH$_2$COO$^-$, LGC, UK) were used for the preparation of standards for arsenic speciation analysis. An algal extract containing four different arsenosugars and characterised previously by Madsen et al. (2000)[28] (kindly donated by Professor K. Francesconi, University of Graz) was used to validate the preparation of four isolated arsenosugar standards (names of arsenosugars taken from Francesconi et al., (2004)[21]: glycerol, phosphate, sulphonate, sulphate) from a seaweed extract. Figure 1 illustrates the structure of the four arsenosugar compounds. These arsenosugar standards were prepared according to methods published by Madsen et al. 2000[28]. Methanol (Fisher Scientific, UK) was employed as a solvent in the extraction procedure. Ammonium nitrate (NH$_4$NO$_3$, Sigma-Aldrich, UK) was used as the mobile phase for gradient anion exchange separation of arsenic species and pyridine (Rathburn, UK) was used for isocratic cation exchange separation. Concentrated nitric acid, hydrofluoric acid and 30 % v/v hydrogen peroxide (BDH Aristar, UK) were used for the dissolution of earthworms and soil samples. Calcium chloride (Fisher Scientific, UK) was used for the measurement of soil pH.

Total Digestion of Earthworm

Microwave assisted (CEM MARS5, CEM Corporation, UK) dissolution of the earthworms was performed on 0.1 g of earthworm homogenate (dry weight). 10 ml of concentrated nitric acid and 100 μl of hydrofluoric acid was added and allowed to stand for 30 minutes. Following an initial heating programme (ramp to 100 ºC over 5 minutes then hold for 5 minutes, ramp to 200 ºC over 5 minutes and hold for 20 minutes) the vessels were allowed to cool (<50 °C) and then 1 ml of 30 % H$_2$O$_2$ was added. The vessels were sealed and microwaved for a second cycle (same program). After cooling, the sample solutions were transferred to PTFA Savellex containers and evaporated to dryness on a hotplate (100 °C) to reduce the presence of organic compounds that could form possible polyatomic interferences by ICP-MS measurement. Samples were reconstituted by addition of 2 ml 50 % v/v nitric acid, heated at 50 °C for 30 minutes and then made up to 10 ml with deionised water. This final stage reduced the dilution of the acid content required for ICP-MS

measurement (<2.5 % v/v). The method described is a validated routine procedure for the dissolution of biological samples. The method accuracy was monitored using a certified reference material, CRM 627 tuna fish tissue (BCR, Brussels). Mean total arsenic recoveries of 96 ± 7 % (n = 6) were obtained, compared to the certified value. The method precision expressed as the mean percentage difference (± 1 SD) between duplicate samples was 1.7 ± 0.9 % (n = 4 duplicates).

Soil Chemistry

Soil pH was determined by 0.01 M aqaeous $CaCl_2$ (6.25 ml) to 0.25 g soil (<250 µm particle size), mixed for 5 minutes and left to stand for 15 minutes prior to analysis using a pH meter (Orion SA720, UK). Readings were checked at the start and end of the run using a pH 7 buffer solution and in-house QC standard (pH 7.3). Total carbon in the soil was determined using a carbon / sulphur analyser (LECO CS230), 0.2 g (dry weight) of each homogenised soil sample was weighed into a ceramic crucible for each determination. All carbon analyses were performed in triplicate. Loss on ignition (LOI) was also determined for each soil sample to provide an indication of the organic matter content. 1 g (dry weight) of each soil was weighed into a glass crucible before heating to 450ºC for 4 hours. The percentage weight reduction after heating was recorded.

Soil Dissolution

Collected soils (0.25 g) and earthworm cast material (0.1 g) were prepared for total elemental measurements by ICP-MS based on a mixed acid digestion approach (HF / HNO_3 / $HClO_4$)[29]. Samples were weighed into PFA vials, acids added and heated on a temperature programmable graphite hot-block (80 ºC for 8 hrs, 100 ºC for 2 hrs, 120 ºC for 1 hr, 140 ºC for 3 hrs, 160 ºC for 4 hrs). This mixture was used, rather than the more widely used aqua regia, as the hydrofluoric acid breaks down the silicate structure, except for a few accessory minerals to give an almost total digest and hence total concentrations can be determined. Perchloric acid was used to breakdown more resistant minerals and ensure complete evaporation of the hydrofluoric acid. Once digested and evaporated, the sample was taken up in 2.5 ml of concentrated nitric acid, heated at 50 ºC for 30 minutes and then treated with 30 % (v/v) hydrogen peroxide to avoid precipitation of meta-stable hydroxyl-fluorides, before being made up to volume (25 ml) with deionised water to give a final solution of 5 % nitric acid for analysis by ICP-MS. Certified reference materials were included with each batch of soil digestions as a measure of quality control. These were NIST CRM 2710 Montana Soil I and NIST CRM 2711 Montana Soil II, and gave good recoveries of 98 ± 4 % (n = 6) and 91 ± 3 % (n = 3), respectively, during the course of the study. The method precision expressed as the mean percentage difference (± 1 SD) between duplicate samples was 3.2 ± 3.6 % (n = 4 duplicates).

Sample Extraction

The extraction of arsenic species from the earthworms was facilitated using a methanol-water mixture. The most effective ratio of methanol to water, which extracted the greatest amount of arsenic from the freeze dried earthworm powders, was determined experimentally to be a 1:1 ratio[30], which is the usual approach for the extraction of arsenic species from marine organisms and algae[31].

Homogenised earthworm powder (0.25 g) was weighed directly into 50 ml polyethylene centrifuge tubes. 10 ml of methanol:water (1:1 v/v) was then added and the tubes shaken on an orbital shaker at 175 rpm for 4 hours. The extracts were centrifuged at 3000 rpm for 10 minutes and the supernatant transferred to polypropylene bottles. A multi-step extraction was not employed as any additional arsenic contained in the second and subsequent extractions has been shown to be accountable to the residual dissolved arsenic carried over from previous extractions.[31] The sample solutions were evaporated to a syrup using a rotary evaporator before freeze drying. The freeze-dried residue was reconstituted in 10 ml of deionised water and analysed immediately. Prior to extraction of earthworm samples, the stability of arsenic species (As^{III}, As^V, MA, DMA and AB) were established under the proposed extraction conditions by separately spiking earthworm powder material with each of the arsenic species. Recoveries of spiked arsenic species were 93 ± 18 %, with no evidence of interconversion between species (particularly between As^{III} and As^V). Extraction efficiency was monitored using the CRM 627 tuna fish tissue (BCR, Brussels), A mean recovery of 77 ± 0.3 % (n = 3) of the total arsenic value was obtained under the extraction conditions described. The method precision expressed as the mean percentage difference (± 1 SD) between duplicate samples was 2.8 ± 1.8 % (n = 4 duplicates).

Instrumentation

Total Elemental Analysis

The methanol extracts reconstituted in DI water, earthworm and soil digests were all analysed for trace metal contents using a Thermoelemental PQ ExCell ICP-MS. The standard operating conditions were as follows: RF power 1350 W; gas flow rates, coolant 13 l min^{-1}, auxiliary 0.9 l min^{-1}, nebuliser 0.93 l min^{-1}; spraychamber temperature 3 ºC; Meinhardt nebuliser. The instrument was tuned using a 1 µg l^{-1} Claritas PPT multielement tune solution 1 (GlenSpectra Reference Materials, UK). Data was acquired in peak jump mode with an acquisition of 3 x 30 seconds. Indium at a concentration of 10 µg l^{-1} was used as an internal standard and was added to the sample stream via a t-piece.

Arsenic Speciation

A quaternary pump (GP50-2 HPLC Pump and an AS-50 autosampler (Dionex, USA) was directly coupled to an ICP-MS (PQ ExCell, Thermoelemental, UK) for measurement of arsenic species, by connecting the analytical column to the ICP nebuliser with PEEK tubing. The two instruments were coupled in such a way that the injection of each sample solution, via the Dionex AS-50 autosampler and subsequent measurement was synchronised automatically using the ICP-

MS Plasmalab software, enabling reproducible sample injections.

Two different eluent systems were used to separate the extracted arsenic species present in the earthworms, using an anion exchange column (PRP–X100, 250 x 4 mm, 10 μm) with a guard cartridge of the same material or a cation exchange column (PRP-X200, 250 x 4 mm, 10 μm) with a guard cartridge of the same material (both Hamilton, USA). The first chromatographic separation (Figure 2a) was based on a modified gradient elution system developed by Martinez-Bravo *et al*. (2000)[24], which used a gradient between A: 4 mM NH_4NO_3 and B: 60 mM NH_4NO_3, both adjusted to pH 8.7 with ammonia. The flow rate was 1ml min^{-1}, with the gradient as follows: 100 % A 0 - 2 minutes; 100 % B 3 - 6.5 minutes; 100 % A 7.5 - 10.75 minutes; 100 % B 11 - 13 minutes; 100 % A 13.25 - 15 minutes. This method achieved good separation for As^{III}, As^V, MA, DMA, and the phosphate, sulphonate and sulphate arsenosugars. Poor separation was achieved for AB and the glycerol arsenosugar. Therefore a second chromatographic separation (Figure 2b) was employed, which was a modified version of the isocratic cation method developed by Geiszinger *et al*. (2002)[19] for the measurement of AB and the glycerol arsenosugar. The cation exchange system used 10 mM pyridine at pH 2.26, adjusted with formic acid and an isocratic flow rate of 1.5 ml min^{-1} . An injection volume of 100 μl was used for both systems. The ICP-MS when coupled to the HPLC was operated in single ion monitoring mode for m/z 75, with a dwell time of 100 ms. Peak areas were initially calculated from resultant chromatograms using PeakFit V4.0 chromatography software (Seasolve Software, USA). Quantified data was produced by inclusion of a blank (deionised water) and calibrant standards for each As species from 2 to 50 μg l^{-1} in each analytical run. Isolated arsenosugar standards were utilised for the identification of arsenosugars by retention time matching. The calibration curve of MA was used for the quantification of the phosphate, sulphonate and sulphate arsenosugars[28]. MA was used as an appropriate calibrant for these three arsenosugars, since it eluted within the same eluent concentration of 4 mM NH_3NO_3. The glycerol arsenosugar was calculated from the cation exchange method. The first method using gradient elution was validated as a routine method for measuring As^{III}, As^V, MA, DMA and AB in water samples[32] with limits of detection based on 3σ for blank values (AB: 1.3, As^{III}: 0.8, As^V: 1.5, MA: 0.7, DMA: 0.3 μg l^{-1}).

For the measurement of these arsenic species in earthworm solutions, analytical measurements were monitored using human urine CRM 18 certified reference material (NIES, Japan) giving a recovery of 97 ± 10 % and 100 ± 11 %, against certified values for AB and DMA, respectively. . The extract for CRM tuna fish tissue (BCR, Brussels) was analysed throughout each analytical run. Mean recoveries of 95 ± 7 % (n = 3) and 80 ± 0.3 % (n = 2) were obtained for AB and DMA respectively.

Results and Discussion

Total Arsenic Concentrations

Arsenic concentrations in soils found in and around the mine varied significantly depending on their proximity to the main tailings. The total arsenic concentrations in the soils from Devon Great Consols (DGC) were in the range of 255 to 12,466 mg kg^{-1}, extremely high in comparison to the control site (Notts 1) containing levels of arsenic at 16 mg kg^{-1}.

The total arsenic concentrations in the earthworms at DGC and the control site (Notts 1) are shown in Table 1. For L. rubellus the values were 11 to 595 mg kg^{-1} at the DGC site and 7 mg kg^{-1} at the control site. The values for D. rubidus at DGC were 17 to 317 mg kg^{-1} and 7 mg kg^{-1} at the control site. L. rubellus earthworms were predominantly collected from soils of higher arsenic concentration and had a higher body burden compared to D. rubidus. However, the earthworm tissue concentrations were lower than measured in the host soil providing no indication of bioconcentration of arsenic by the earthworms. L. rubellus had a median worm / soil bioaccumulation factor (BAF) of 0.09 and 0.08 for D. rubidus, marginally higher than found by Langdon *et al*. (2003)[10] with a BAF of 0.07 in L. rubellus. At similarly high arsenic soil concentrations as that described in Langdon *et al*. (2003)[10] (8,930 mg kg^{-1}, BAF 0.07) the BAF value in the current work was slightly lower (12,466 mg kg^{-1}, BAF 0.03). Earthworms from the control site with a lower soil arsenic content had relatively higher BAF compared to some of the contaminated DGC collection points (Table 1). The higher BAF values where soil arsenic is relatively low have been reported elsewhere in the literature[14], suggesting that elimination rates of arsenic may be increased at higher concentrations of arsenic and bioaccumulation of arsenic is non-linear[18].

D. rubidus were generally collected from separate collection points to L. rubellus, with two exceptions. Earthworms of both species at the same collection point at DGC 11 with elevated arsenic levels in the soil had differing BAF values (0.04 for L. rubellus and 0.13 for D. rubidus), which might suggest differences in uptake, storage or elimination of arsenic between the two earthworm species or more likely confirm that the bioaccumulation rate is non-linear at elevated soil arsenic concentrations. This becomes apparent through the comparison of the cast material egested from the earthworm and the host soil. L. rubellus which was mainly found close to the soil surface and often within a layer of vegetation or leaf litter did not bioaccumulate arsenic to any significant degree at sites with high soil arsenic concentrations. For example, soil at DGC 6 contained arsenic at 12,466 mg kg^{-1}, whereas cast material was significantly lower at 923 mg kg^{-1}. The earthworm tissue did not contain equivalent arsenic concentrations, at 359 mg kg^{-1}. Both of these factors suggest that L. rubellus collected at DGC 6 and other sites with similar patterns (*e.g*. DGC 12 and 13) did not have a low BAF due to egestion of high arsenic concentrations in the cast material, L. rubellus was simply not exposed directly to the high soil arsenic concentrations. However, D. rubidus provided cast material with arsenic concentrations similar to the host soil at most of the sites, with cast material concentrations representing 93 ± 15 % of the host soil. This would suggest that D. rubidus was directly exposed to the host soil, particularly when compared to L. rubellus (cast material contained 55 ± 29 % of total arsenic found in host soil). These

differences between the two earthworm species were not observed at the control site (16 mg kg^{-1}) where BAFs were similar for both L. rubellus and D. rubidus at 0.41 and 0.44 respectively but significantly higher than BAFs at the contaminated sites.

Soil pH for the contaminated soils were slightly acidic and ranged from 3.9 to 5.7. Host soils from the control site had an almost neutral pH of 6.8. No significant correlation was observed between earthworm BAFs and soil pH. The organic matter content of the soil (% LOI) varied at DGC from 6 - 36% with 21% at the control site. Again, no significant correlation was observed between soil organic matter and earthworm BAFs.

Earthworm Arsenic Speciation

The methanol extraction of arsenic from earthworm tissue resulted in total recoveries of 27 to 81 % (mean 49 %) for L. rubellus and 31 to 82 % (mean 58 %) for D. rubidus. The extraction efficiency compared favourably to the 25% extraction efficiency reported by Geiszinger et al. (2002)[19].

The measurement of arsenic species was achieved using the gradient anion exchange system for DMA, MA, As^{3+}, As^{5+}, three arsenosugars (phosphate, sulphonate and sulphate) (Figure 2a) and the cation exchange system was used for AB and one arsenosugar (glycerol) (Figure 2b) due to their coelution using the gradient system. The data in Table 2 shows the sum of arsenic species measured in the earthworm methanol extracts. These values generally agree with the total arsenic measured in the extract for D. rubidus (96 ± 20 %) and L. rubellus (74 ± 24 %). The variability in the sum of speciated arsenic in the extract is significantly closer to the total arsenic content of the extract than recoveries reported by Langdon et al. (2002)[18] (54 ± 20 %).

Arsenic residing in both species of earthworm was generally in the form of As^{V} and As^{III}, with the main quantifiable organic species being AB, agreeing with the findings of Langdon et al. (2003)[15]. D. rubidus populations demonstrated a higher proportion of AB (mean 26 %) compared to L. rubellus (mean 5.5 %), although where both species of earthworm were collected from the same collection point the proportion of AB in either earthworm was within 4 % of each other. However, the occurrence of AB in the earthworms is more likely to be a factor of total arsenic concentrations in the soil and earthworm tissue rather than being dependent on earthworm species. Earthworms of both species exhibit a higher proportion of AB at the lower range of total arsenic concentrations in the earthworm tissue. This is particularly evident for D. rubidus from DGC 4, 7 and 9, where AB accounts for 50, 35 and 39 % of the arsenic speciated, respectively. The difference between earthworm species is generally due to L. rubellus residing in soils of higher arsenic concentration compared to D. rubidus and as a result exhibiting relatively less AB as a proportion of arsenic in the tissue.

MA and DMA occurred at low levels in the earthworms at all sites, generally 1 % or less of the combined sum of arsenic species. Three arsenosugars (glycerol, phosphate and sulphate) were detected as minor constituents in both L. rubellus and D. rubidus, although not necessarily all three arsenosugars for every site (Table 2). The sulphonate arsenosugar was not detected in any of the earthworm extracts.

For the control earthworm (Notts 1 site) combined organic species values accounted for 45 % of arsenic species measured in L. rubellus and 54 % in D. rubidus, and of this arsenosugars accounted for 28 and 34 %, respectively. Three individual arsenosugars were present in L. rubellus as 7 % glycerol and 21 %, phosphate and for D. rubidus, 16 % glycerol and 18 % phosphate. These findings differ from the study of Geiszinger et al. (2002)[19], who used control earthworms of similar arsenic tissue concentration (6 mg kg^{-1}), but of a different species (L. terrestris),a deep burrowing surface feeder, compared to a surface living and surface feeding species in the case of both L. rubellus and D. rubidus. Arsenic species were apportioned in L.terrestris as mainly two arsenosugars (55 % phosphate and 8 % glycerol), with 5 % DMA, 2 % MA and 18 % inorganic arsenic.

Across all sites (contaminated sites at DGC), organic species accounted for 9 % (mean) of arsenic species for L. rubellus and 32 % (mean) for D. rubidus, with arsenosugars present as minor constituents and accounting for a mean of 3 % and 5 % of arsenic species, respectively. High levels of the phosphate arsenosugar were only found present in L. rubellus at sites DGC 11 and Notts 1 (Table 2). The sulphate arsenosugar accounted for less than 1 % of arsenic species for each earthworm species across all DGC sites.

Arsenic speciation data presented in Table 2 was compiled from the use of both anion and cation exchange chromatography, in line with common approaches reported in the scientific literature[19, 28, 33]. Co-elution of the glycerol and AB species by gradient anion exchange was confirmed by the cation exchange method. The gradient program for the anion exchange method was modified to resolve the two peaks, through adjustment of the gradient switchover times from eluent A to B. The time taken for the switchover (starting at 2 minutes) from A to B was phased over 2 minutes rather than 1 minute at the beginning of the elution program. The resulting chromatogram for a representative earthworm extract is shown in Figure 2c for the 4 arsenosugars, As^{V}, As^{III}, AB, MA and DMA measured in one solution, using one chromatographic set-up.

Conclusions

Findings suggest that earthworms accumulate arsenic, but on the whole do not bioconcentrate arsenic. Whilst the arsenic body burden mainly resides as arsenate and arsenite, the earthworms may be able to eliminate or reduce the toxicity effects when accumulating arsenic in their tissues. For example, the presence of AB and other organic arsenic species or arsenic metabolites, such as arsenosugars may point to the mechanisms for coping with exposure to soil bound arsenic. Some differences in this process and occurrence of organoarsenicals between species of earthworms is probably due to variations of exposure and uptake, such as feeding patterns and dermal uptake. The range of chromatographic measurements incorporating anion and cation exchange eluent

systems and the identification of three arsenosugars (glycerol, phosphate, sulphate) in earthworms has helped to define the metabolites, the means of arsenic elimination / sequestration / exposure patterns, and the differences between earthworm species. The majority of arsenic residing in earthworm tissue was inorganic arsenic, although for uncontaminated soil (Notts 1) up to 45 % of arsenic was present as organoarsenicals in L. rubellus and 54 % in D. rubidus. This was of particular interest, because L. rubellus generally resided in soils at the higher range of arsenic contamination compared to D. rubidus and overall bioaccumulation of arsenic was not significantly different between the two earthworm species. A chromatographic separation was demonstrated using modified anion exchange gradient elution for the separation of nine arsenic species, including four arsenosugars in one measurement / sample injection, as opposed to the usual approach of using more than one chromatographic set-up.

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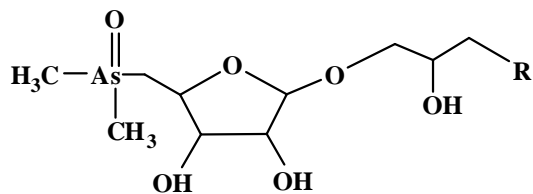
Arsenosugar 1 (glycerol)   R = OH

Arsenosugar 2 (phosphate)   R = OP(O)(OH)OCH$_2$CH(OH)CH$_2$OH

Arsenosugar 3 (sulphonate)   R = SO$_3$H

Arsenosugar 4 (sulphate)   R = OSO$_3$H

Figure 1: Structures of the four arsenosugars

Table 1: Arsenic concentrations in the host soil, earthworm cast material / tissue and bioaccumulation factor (BAF = worm total As / soil total As).

Sampling Location	Soil pH	Soil Organic Matter (%)	Total As in soil (mg kg^{-1})	Total As in worm cast (mg kg^{-1})	Total As in worm (mg kg^{-1})	BAF
L. rubellus						
Notts 1*	6.8	21	16	11	7	0.41
DGC 1	6.1	14	2980	2488	595	0.20
DGC 2	4.4	14	1573	1330	257	0.16
DGC 6	4.0	12	12466	923	359	0.03
DGC 10	4.3	13	439	284	40	0.09
DGC 11*	4.6	6	289	na	11	0.04
DGC 12	3.9	30	5141	1173	203	0.04
DGC 13	5.7	36	2871	1853	571	0.20
D. rubidus						
Notts 1*	6.8	21	16	11	7	0.44
DGC 3	4.6	15	1005	994	317	0.32
DGC 4	4.2	9	255	274	19	0.07
DGC 7	4.1	37	331	229	17	0.05
DGC 9	4.1	11	284	290	18	0.06
DGC 11*	4.6	6	289	291	38	0.13
DGC 15	5.2	10	913	720	74	0.08

* Both species of earthworm collected from the same site, na sample not available.

Table 2: Speciated As in the earthworm extracts of *L. rubellus* and *D. rubidus* from DGC and a low concentration site (Notts 1

Sampling Location	Sum of speciated As	Recovery of species from extract	Extraction efficiency	As^V	As^{III}	AB	DMA	MA	Sugar-1 (OH)	Sugar-2 (PO$_4$)	Sugar-4 (SO$_4$)
L. rubellus	(mg kg^{-1})	(%)*	(%)*	As species percentage (%) of total speciated							
Notts 1**	2.9	72	44	19	36	17	0.6	nd	6.5	21	nd
DGC 1	134	52	36	46	50	2	0.2	0.5	0.5	nd	0.9
DGC 2	132	62	63	45	49	5	0.2	0.5	0.3	nd	0.6
DGC 6	111	56	42	47	47	2.2	0	1.5	0.2	nd	1.2
DGC 10	17	109	27	62	24	13	0.2	0.7	1.1	nd	nd
DGC 11**	5.6	115	32	57	16	12	nd	nd	6.2	8.4	nd
DGC 12	127	61	81	51	44	2.7	0.2	0.9	0.3	nd	0.7
DGC 13	281	63	64	28	69	1.8	0.1	0.3	0.4	nd	0.5
Mean***		74	49	48	43	5.5	0.1	0.6	1.3	1.2	0.6
SD		24	19	11	18	4.9	0.1	0.5	2.2	3.2	0.4
D. rubidus											
Notts 1**	3.1	79	40	13	33	19	0.9	nd	16	18	nd
DGC 3	184	55	82	29	65	5.4	0.1	0.1	0.1	nd	0.4
DGC 4	7.07	101	31	32	6	50	0.6	1.1	3.7	6.5	nd
DGC 7	12.6	105	54	16	44	35	0.8	0.7	3	0.7	nd
DGC 9	16.3	105	77	7.9	46	39	0.4	0.7	5.5	0.8	0.3
DGC 11**	30	107	52	24	55	16	0.1	nd	3.8	0.9	0.3
DGC 15	82.9	102	73	26	60	11	0.5	0.5	0.5	1.4	0.3
Mean***		96	58	22	46	26	0.4	0.5	2.8	1.7	0.2
SD		20	19	8.0	20	15	0.4	0.7	1.7	2.2	0.3

*Recovery of species as a percentage of the total As in the extract, extraction efficiency based on total As in the extract as a percentage of total As in the worm **both species of earthworm collected from the same site, *** mean value does not include Notts 1 except recovery of species and extraction efficiency which do.
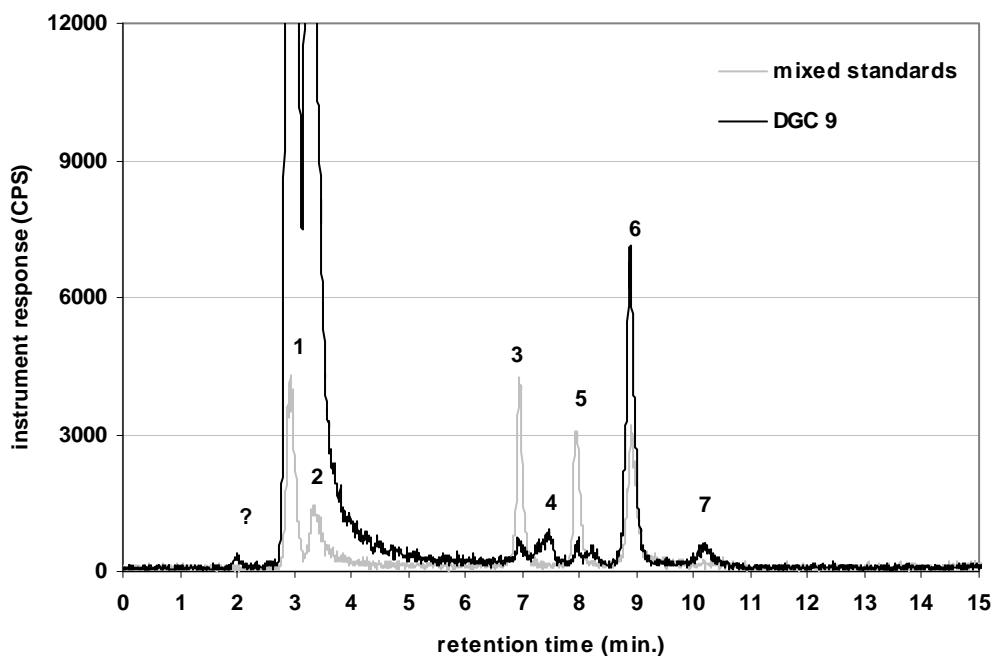nd – below limit of quantification or not detected

Figure 2 (a): Anion gradient elution profile for a mixed standard solution (2 µg l^{-1}) and an earthworm extract (DGC 9 *D. rubidus*). Peaks 1-7: 1 = AB, 2 = As^{III}, 3 = DMA, 4 = Sugar-2, 5 = MA, 6 = AsV, 7 = Sugar-4
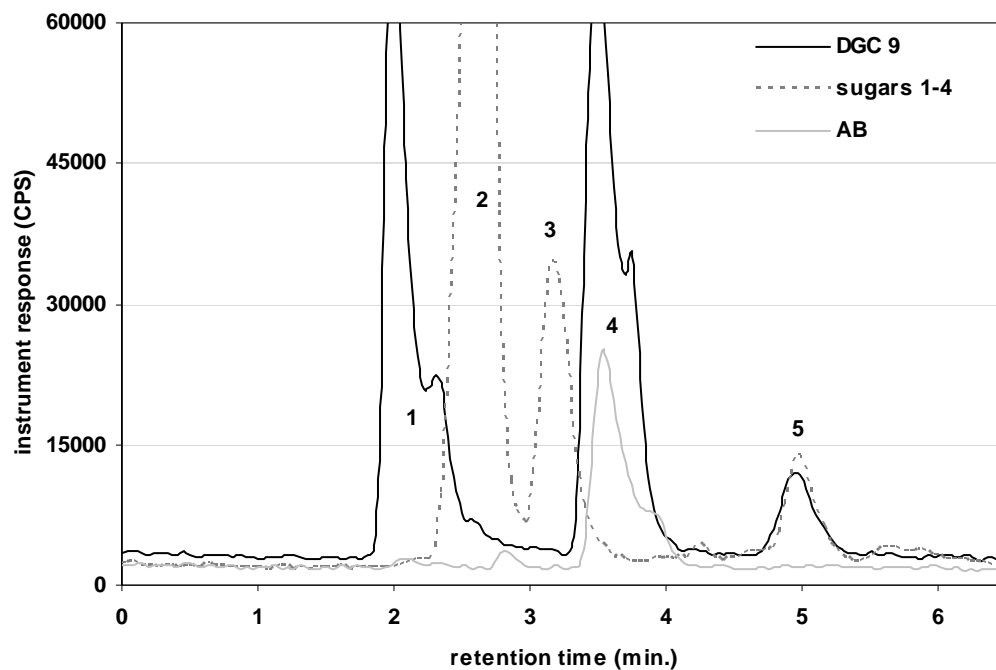
Figure 2 (b): Cation isocratic elution profile for an earthworm extract (DGC 9 *D. rubidus*), an algal extract containing sugars 1-4 and an AB standard at (13 µg l^{-1}). Peaks 1-5: 1 = solvent front containing As^{III} + As^{V} + MA, 2 = DMA + Sugars-2/3, 3 = Sugar-4, 4 = AB, 5 = Sugar 1.
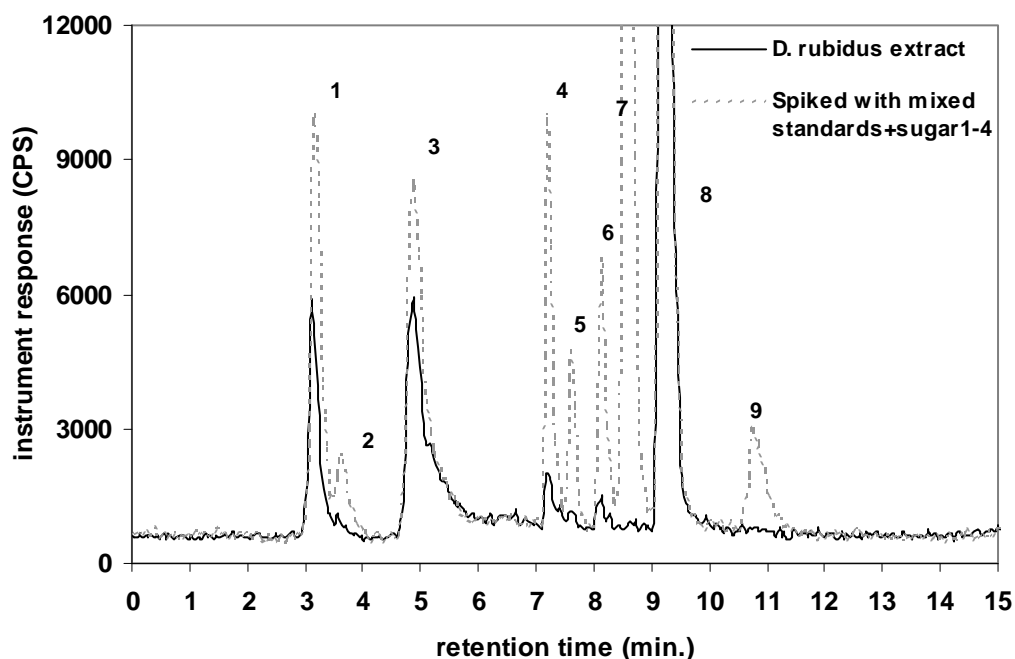

Figure 2 (c): Anion gradient elution profile for a mixed standard solution (10 µg l^{-1}) and a representative earthworm extract. Peaks 1 - 9: 1 = AB, 2 = Sugar-1, 3 = As^{III}, 4 = DMA, 5 = Sugar-2, 6 = MA, 7 = Sugar-3, 8 = As^{V}, 9 = Sugar-4.