ARSENIC SPECIATION IN THE EARTHWORMS LUMBRICUS RUBELLUS AND DENDRODRILUS RUBIDUS

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(Received 19 July 2002; Accepted 12 November 2002)

Abstract—Two species of earthworm, Lumbricus rubellus Hoffmeister and Dendrodrilus rubidus (Savigny) collected from an arsenic-contaminated mine spoil site and an uncontaminated site were investigated for total tissue arsenic concentrations and for arsenic compounds by liquid chromatography-mass spectrometry (LC-MS) and liquid chromatography-inductively coupled plasma-mass spectrometry (HPLC-ICP-MS). For L. rubellus, whole-body total tissue arsenic concentrations were 7.0 to 17.0 mg arsenic/kg dry weight in uncontaminated soil and 162 to 566 mg arsenic/kg dry weight in contaminated soil. For D. rubidus, whole-body tissue concentrations were 2.0 to 5.0 mg arsenic/kg dry weight and 97 to 321 mg arsenic/kg dry weight, respectively. Arsenobetaine was the only organic arsenic species detected in both species of earthworms, with the remainder of the extractable arsenic being arsenate and arsenite. There was an increase in the proportion of arsenic present as arsenobetaine in the total arsenic burden. Lumbricus rubellus and D. rubidus have similar life styles, both being surface living and litter feeding. Arsenic speciation was found to be similar in both species for both uncontaminated and contaminated sites, with dose-dependent formation of arsenobetaine. When L. rubellus and D. rubidus from contaminated sites were incubated in arsenic-free soils, the total tissue burden of arsenic diminished. Initially, L. rubellus from the tolerant populations (from the contaminated site) eliminated arsenic in the first 7 d of exposure before accumulating arsenic in tissues, whereas nontolerant populations (from the uncontaminated site) accumulated arsenic linearly. The tolerant and nontolerant L. rubellus eliminated tissue arsenic linearly over 21 d when incubated in uncontaminated soil.

Keywords—Earthworms Arsenic Arsenic resistance Speciation

INTRODUCTION

Populations of the earthworms Lumbricus rubellus Hoffmeister and Dendrodrilus rubidus (Savigny) Lumbricidae, tolerant to very high concentrations of arsenate, have colonized abandoned copper/arsenic mine spoil that contains up to 50,000 mg/kg arsenic on a soil dry weight basis [1,2]. Lumbricus rubellus and D. rubidus from uncontaminated sites suffer high mortality when exposed to arsenate [2]. Lumbricus rubellus and D. rubidus are both ubiquitous, epigeic, surface-living, litter-feeding species [3]. These species are able to survive in metal-contaminated mine spoil soils and it is probable that individuals in a population develop resistance to contaminants (either physiological acclimation or genetic adaptation) [2]. Total tissue arsenic burdens in depurated earthworms of both species, collected from the contaminated sites, were as high as 1,000 mg arsenic/kg dry weight. Further, there were clear differences in the LC50s (lethal concentration) of tolerant and nontolerant populations of L. rubellus, i.e., 1,510 and 96 mg arsenic/kg dry weight, respectively [2]. The physiological capability of these two species of earthworms to become established in arsenic-contaminated spoil must involve a mechanism for detoxifying accumulated arsenic [4].

Geiszinger et al. [5] studied arsenic speciation in lumbricid species from six sites in Styria, Austria. The authors reported that the arsenosugars found in the earthworms may have three possible sources, being biotransformation by the earthworm of inorganic arsenic, microbial alkylation of inorganic arsenic in soil ingested by the earthworm, and selective bioaccumulation from trace concentrations in the soil too low for detection. Geiszinger et al. [5] did not find organoarsenic compounds in the soil, and this supports the hypothesis that earthworms do not take up organic arsenic compounds from the soil. Langdon et al. [6] investigated arsenate metabolism in L. rubellus from Devon Great Consols and an uncontaminated site employing a range of analytical techniques. This study, in contrast with that of Geiszinger et al. [5], demonstrates the presence of substantial quantities of arsenobetaine in L. rubellus collected from uncontaminated soil and kept in contaminated soil. The arsenic was found to be predominantly bound to S in the form of arsenic (III), in a glutathione-type environment, suggesting metallothionein complexation, but was also present in smaller amounts as arsenobetaine. It is possible that, through changes in the speciation of arsenic, earthworms may be able to partially regulate concentrations of arsenic, with the elimination rate being increased at higher concentrations [5].

Earthworms form the basis of many terrestrial food chains with predators such as birds and small mammals. Many of these animals have been shown to have elevated levels of arsenic in their tissues when their feeding ranges include arsenic-contaminated sites [7]. This has implications for food chain transfer, and it is important to understand how different species of earthworms have adapted to survival in highly contaminated sites to enable accurate assessment of possible ecological risk.
The aims of this present study were to characterize arsenic speciation in tolerant and nontolerant populations of *D. rubidus* with a view to determine how arsenic is metabolized by this species and to ascertain if this speciation is different from populations of *L. rubellus* from a similar ecological group living in highly contaminated soils. The third aim was to determine the extent of accumulation and elimination of arsenic from total earthworm tissue of contaminated and noncontaminated populations of *L. rubellus* in soils treated with the lethal concentrations to 10% of the population (LC10) of sodium arsenate (50 mg arsenic/kg dry wt of soil uncontaminated and 1,200 mg arsenic/kg dry wt of soil from Devon Great Consols and Carrock Fell, Cumbria, UK).

**METHODOLOGY**

Total earthworm tissue arsenic was measured using a graphite furnace atomic absorption spectroscopy (GF-AAS). Arsenic speciation was determined using liquid chromatography-mass spectrometry (LC-MS) and liquid chromatography-inductively coupled plasma-mass spectrometry (HPLC-ICP-MS).

**Reagents**

Sodium arsenate heptahydrate was purchased from Sigma Chemicals (St. Louis, MO, USA). Arsenobetaine was purchased from European Commission, Community Bureau of Reference, as reference material 626. Dimethylarsinic acid and reduced glutathione were obtained from Sigma Chemicals. Sodium arsenate, sodium arsenite, arsenic oxide, and arsename oxide, reagent grade, were supplied by Merck (Darmstadt, Germany). Other arsenic standards such as dimethylarsinoyl ethanol and four different dimethylated arsenoribosides were synthesized or extracted from seaweed, and some were gifts from W. Goessler (University of Graz, Graz, Austria). Tetramethylarsionium iodide, arsenocholine, and trimethylarsine oxide were graciously donated by X.C. Le (University of Alberta, Edmonton, AB, Canada).

**Experiment 1: Speciation of arsenic**

**Sample collection and preparation.** Specimens of *L. rubellus* and *D. rubidus* (*n* = 10 per species) were collected by hand sorting from arsenic-contaminated sites at Devon Great Consols, an abandoned copper and arsenic mine near Tavistock, Devon, UK. *Lumbricus rubellus* and *D. rubidus* were also collected from an uncontaminated site (*n* = 10 per species), a mixed deciduous woodland soil at Lancaster University campus (Lancaster, UK), which supported an abundant and diverse earthworm community. The collected earthworms were subjected to three soil treatments: one consisted of being placed in soil from the site of origin, another into uncontaminated soil, and a third into the uncontaminated soil treated with sodium arsenate. The uncontaminated soil was collected from the Lancaster University campus. This soil had been collected and analyzed for another study [2]. To create an arsenic-contaminated soil, a sample of campus soil was partially air dried, sieved through a 2.8-mm mesh, and rewetted to a moisture content of 53% (dry wt equivalent) with a nominal soil pH (ratio 1:2.5 aqueous H2O, ultrapure water) ± standard error (SE) was 4.71 ± 0.05. Ten specimens of each species were placed in the 20- × 25-cm polyethylene bags containing approximately 55 g moist soil for one week at 9°C for each soil treatment. Soils were partially air dried, sieved (2.8-mm mesh), and rewetted to give a moisture content of 55%.

Following the one-week incubation, in each of the soil treatments to acclimatize the earthworms and to check for any moribund specimens, earthworms were deparated overnight to avoid contamination from the gut contents by being placed individually into 9- × 10-cm polythene bags containing moist tissue paper and being kept at 9°C. Samples were homogenized by freeze fracturing through immersion in liquid nitrogen, followed by grinding with a pestle and mortar. One half of each sample was used to determine total arsenic content using GF-AAS, and arsenic speciation was investigated using the other half with HPLC-ICP-MS and HPLC-MS. The HPLC-ICP-MS analysis was only conducted on field-collected earthworms that had been incubated for one week in the soil from each population’s site of origin. Independent samples were used for HPLC-MS and HPLC-ICP-MS.

**Total arsenic concentrations.** Total tissue concentrations of arsenic were measured by GF-AAS. The homogenate was weighed, dried overnight at 50°C, reweighed, and hot-acid digested in 10 ml HNO3 (Aristar grade), filtered using Whatman 540 ashless filter papers (Clifton, NJ, USA), and stored at 5°C. The samples were run on a Perkin-Elmer (Beaconsfield, Buckinghamshire, UK) flame spectrophotometer graphite furnace with a wavelength of 193.7 nm, slit width 0.7 nm, and a lamp current of 12 mA. A known set of standards was used to produce a calibration curve at the start of each analytical run. The top and bottom standard (200 and 50 ppm) were run together with a blank on each subsequent run. The methanol extracts prepared above were also analyzed using a graphite furnace, with a detection limit of 5 ng/ml.

**LC-MS analysis.** The other half of the homogenate was preweighed into 1.5-ml vials, frozen in liquid nitrogen, and thawed prior to sample preparation. To each vial, 1 ml of 50: 50 methanol:water was added and the samples were centrifuged for 5 min at 377 g. The vial contents were mixed using tweezers and centrifuged for a further 5 min at 377 g. The liquid was pipetted off into fresh vials and frozen at −73°C. Half of the methanol extract was removed for total arsenic analysis using GF-AAS (see section above). The HPLC-MS analysis of methanol extracts was performed using a Model HP1100 series HPLC with MS (Hewlett-Packard, London, UK) electrospray ionization. Three replicate samples of soils (from each population’s site of origin) in which the earthworms were kept for one week were extracted (1 g soil extracted with 2 ml of 50:50 methanol:water using the earthworm tissue extraction procedure) and the extracts were also analyzed by HPLC-MS.

Chromatographic and mass spectrometer conditions are outlined by Inoue et al. [8]. An Altech (Wolverhampton, UK) SCX 5µ, 250-mm length, 4.6-mm i.d. cation exchange column was used for separation with a mobile phase of HNO3, (8 mM)/NH4NO3. The column was maintained at a temperature of 30°C and a flow rate of 0.4 ml/min. Injection volume was 25 µl. The mass spectrometer detector was run in positive mode, with 5.0 V ion energy. A m/z range of 0 to 350 was scanned. The quadrupole temperature was 99°C and the gas temperature 300°C, with a drying gas flow rate of 10 L/min and a nebulization pressure of 45 psi. Pure monomethylarsionic acid, dimethylarsinic acid, tetramethylarsonium iodide, arsenocholine, and arsenobetaine were run as standards. The results were compared with external calibration. The deviations were 1.5%.

**HPLC-ICP-MS analysis.** Sample preparation for HPLC-
ICP-MS was identical to that for HPLC-MS. The separation and identification of the different arsenic species was achieved by using anion- and cation-exchange chromatography. A HPLC pump LKB-Bromma 2150 (Uppsala, Sweden) was used to carry out an isocratic operation and a Rheodyne® 7010 six-port injection valve (Rohnert Park, CA, USA) with a 20-μL sample loop was used for sample introduction. A Hamilton PRP X-100 (Reno, NV, USA) (250 mm × 4.1 mm, 5 μm) was used as an anion exchange column and 30 mM H₂PO₄ adjusted to pH 6.0 with NH₃ was applied with a flow rate of 1.0 ml/min. The same flow rate was used for cation exchange chromatography (Supelcosil LC-SCX Supelco, Bellefonte, PA, USA; 250 mm × 4.6 mm, 5 μm) with a mobile phase of 20 mM pyridine, adjusted to pH 2.9 with formic acid. An arsenic element-specific detector and ICP-MS (Spectromass 2000, Spectro Analytical Instruments, London, UK) was used, equipped with a water jacket spray chamber and a Meinhard nebulizer (Santa Ana, CA, USA), which could be directly coupled to the outlet of the HPLC column. The conditions are described in more detail in Feldmann et al. [9]. Pure standard solutions of the arsenic species arsenate, arsenite, arsenobetaine, arsenocholine, monomethylarsonic acid, dimethylarsinic acid, tetramethylarsonium iodide, and trimethylarsine oxide were run and the major dimethylated arsenobetaines (arsenobetaine-glycoside, -phosphate, -sulfonate, and -sulfate) were run.

Experiment 2: Accumulation and elimination of total tissue arsenic

Earthworm preparation. Specimens of mature L. rubellus were collected from an uncontaminated culture at Centre for Ecology and Hydrology (Monkswood, Huntingdon, UK) from an abandoned tungsten mine at Carrock Fell and from Devon Great Consols (see above). Soil from an uncontaminated site on the Lancaster University campus (see above) was partially air dried and sieved (2.8 mm), uniformly rewetted to a moisture content of 53% (dry wt equivalent), and placed into 60-x 40-cm polythene bags. Earthworms from each site were added to the bags (n = 10 per bag) to give a total of 50 earthworms from each site to enable elimination of any arsenic burden prior to exposure to treated soil. The bags were kept for three months in a dark room at 4°C. Frozen and thawed horse manure (20 g) (arsenic content <0.05 μg/g) was added to each bag at monthly intervals.

Sterilized Kettering loam, purchased from Barrycroft Stores (Willingham, Cambridge, UK) (arsenic content <0.05 μg/g), and Lemmington sphagnum peat (arsenic content <0.05 μg/g), purchased from Levington Horticultural (Bramford, Ipswich, Suffolk, UK), were mixed for 15 min in a mixer to give a dry ratio of 95% soil:5% peat. The mixed soil was placed into 21 individual plant pots 20 cm in diameter to a depth of 10 cm and watered to a moisture content of 45%, and 20 g of frozen and thawed horse manure were added to each pot. Seven L. rubellus were then added to each pot. A finely perforated plastic bag was secured over the top of each pot. The pots were placed in a room with a 16:8-h light:dark regime at a temperature of 15°C. They were kept for a period of nine weeks and examined at weekly intervals for drying out, deionized water being added as necessary. Earthworms were placed in the above regime to enable collection of cocoons for a culturing experiment. The L. rubellus were then transferred to three large polythene bags (one for each site) containing soil and manure as above and kept for a further three weeks at 4°C in the dark.

Soil treatments. Soil from the uncontaminated site at Lancaster University campus was partially air dried and sieved (2.8 mm). The soil was then uniformly rewetted to a moisture content of 53% (dry wt equivalent) using distilled water or a solution of sodium arsenate to give soil concentrations of the LC10 (21 d) for each site using values extracted from Langdon et al. [2]. The LC10 values (50 mg arsenic/kg uncontaminated and 1,200 mg arsenic/kg, Devon Great Consols and Carrock Fell, sodium arsenate dry wt of soil) were calculated using Probit analysis with SYSTAT® (SPSS, Ver 10, Chicago, IL, USA) statistical software from the raw data used in Langdon et al. [2]. Mean soil pH (aqueous suspension) ± SE was 5.41 ± 0.03 (n = 5). Moistened soil (69 g) was weighed into each of a series of 20 × 25-cm polythene bags.

One L. rubellus was weighed and introduced into each bag, the earthworms being assigned to treatments at random. Mean body weights ± SE were Lancaster campus L. rubellus = 0.70 ± 0.12 g, Devon Great Consols L. rubellus = 0.52 ± 0.007 g, and Carrock Fell L. rubellus = 0.38 ± 0.05 g. There were 39 earthworms per treatment. The bags were kept for 24 h at 15 ± 2°C in darkness. Three specimens, from each treatment, were removed at 0, 1, 3, 7, 14, and 21 d and analyzed for total arsenic. The remaining L. rubellus were transferred to soil prepared as above but with less than the detection limit of arsenic. The soil was then uniformly rewetted to a moisture content of 53% (dry wt equivalent) using distilled water (one L. rubellus per bag) and removed at the same time intervals as above (except day 0) and analyzed for total arsenic. Earthworms (3) were removed at each time point and analyzed for total arsenic tissue concentrations using GF-AAS.

Total tissue arsenic analysis for determination of accumulation and elimination. Three specimens of L. rubellus from each treatment at each time point were weighed and depurated for 24 h in individual polythene bags containing moist tissue, then killed by dipping in boiling water. All L. rubellus were dissected open dorsally and the gut cleaned of any soil particles using a small brush. Samples were acid digested and run in duplicate on a Perkin Elmer GF-AAS (details above).

RESULTS

Experiment 1: Arsenic speciation

Total arsenic concentrations in the soil from Devon Great Consols were very high in comparison with levels in the uncontaminated soil (means of 8,000 mg arsenic/kg and <0.10 mg arsenic/kg, respectively). Soil percentage of organic matter was 9.93 ± 0.07 for the uncontaminated site and 10.02 ± 0.45 for Devon Great Consols, with a pH of 4.71 ± 0.05 and 5.14 ± 0.01, respectively [2]. Total arsenic concentrations determined using graphite furnace atomic absorption analysis of L. rubellus and D. rubidus from the contaminated sites ranged from 162 to 566 and 97 to 321 mg arsenic/kg dry weight, respectively, compared with 7 to 17 and 4 to 5 mg arsenic/kg dry weight, respectively, for L. rubellus and D. rubidus from the uncontaminated site (Table 1). Tissue concentrations were lower than those found in the parent soils, suggesting that there was no bioconcentration of arsenic.

Earthworm total arsenic body burdens were variable (Table 1). This variation is natural as analytical recoveries by GF-AAS using arsenobetaine certified reference material gave 101% recovery with a relative standard deviation of 3.6% (n = 3) for a single analytical run. The reason for this natural variation in body burden is not known, but it may be related...
to the degree of arsenic exposure before collection. Mine soils are heterogeneous environments, which may result in varying states of induction of arsenic metabolizing enzymes.

The only organoarsenic species found in *L. rubellus* and *D. rubidus* by LC-MS was arsenobetaine (Tables 1 and 2). The arsenobetaine was identified using its retention time and mass spectrum. The mass spectrum for arsenobetaine is distinct from other organoarsenical compounds. It has a simple spectra, a similar way at low concentrations of arsenic. The tissue arsenic dropped below 10 mg/kg, to reach a maximum of 60% in *L. rubellus*. This was also found to be the case for *D. rubidus*, although the percentages of arsenobetaine were higher, with a maximum of 90%. Earthworms of both species from uncontaminated and contaminated sites metabolized arsenic in a similar way at low concentrations of arsenic. The tissue extracts of *L. rubellus* and *D. rubidus* showed no other organic arsenic species. The inorganic species As III and As V were respectively.

*Table 1.* Concentrations of arsenobetaine (AB) in *Lumbricus rubellus* and *Dendrodrilus rubidus* tissues determined by liquid chromatography-mass spectrometry compared with total tissue arsenic concentration determined by graphite furnace atomic absorption spectroscopy in earthworms from an uncontaminated site, Lancaster (UK) (L) (less than detection limit of As dry wt of soil) and an arsenic-contaminated site, Devon Great Consols (DGC; Devon, UK) (8,000 mg As/kg dry wt soil)

<table>
<thead>
<tr>
<th>Site</th>
<th>Species</th>
<th>AB concn. (mg/kg dry wt)</th>
<th>AB (%)</th>
<th>Methanol-extracted</th>
<th>Total</th>
</tr>
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<tbody>
<tr>
<td>L</td>
<td><em>L. rubellus</em></td>
<td>0.91</td>
<td>13</td>
<td>6</td>
<td>7</td>
</tr>
<tr>
<td>L</td>
<td><em>L. rubellus</em></td>
<td>0.74</td>
<td>4</td>
<td>4</td>
<td>17</td>
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<tr>
<td>DGC</td>
<td><em>L. rubellus</em></td>
<td>20.9</td>
<td>4</td>
<td>154</td>
<td>566</td>
</tr>
<tr>
<td>DGC</td>
<td><em>L. rubellus</em></td>
<td>22.9</td>
<td>14</td>
<td>100</td>
<td>162</td>
</tr>
<tr>
<td>L</td>
<td><em>D. rubidus</em></td>
<td>0.21</td>
<td>4</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>L</td>
<td><em>D. rubidus</em></td>
<td>0.46</td>
<td>12</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>DGC</td>
<td><em>D. rubidus</em></td>
<td>4.34</td>
<td>4</td>
<td>14</td>
<td>97</td>
</tr>
<tr>
<td>DGC</td>
<td><em>D. rubidus</em></td>
<td>23.4</td>
<td>7</td>
<td>177</td>
<td>321</td>
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</tbody>
</table>

arsenobetaine was formed as a major metabolite in both of the earthworm species (Table 2). Arsenobetaine is present as a high proportion of the total arsenic burden of *L. rubellus* and *D. rubidus*. The proportion of arsenobetaine compared with total arsenic body burden was approximately 10% in *L. rubellus* and *D. rubidus* and started to increase linearly as total arsenic dropped below 10 mg/kg, to reach a maximum of 60% in *L. rubellus*. This was also found to be the case for *D. rubidus*, although the percentages of arsenobetaine were higher, with a maximum of 90%. Earthworms of both species from uncontaminated and contaminated sites metabolized arsenic in a similar way at low concentrations of arsenic. The tissue extracts of *L. rubellus* and *D. rubidus* showed no other organic arsenic species. The inorganic species As III and As V were both present in *L. rubellus* and *D. rubidus* (Table 2). There was no significant difference between *L. rubellus* and *D. rubidus* for As III or As V (*t* test, *p* = 0.358 and *p* = 0.383, respectively). *Dendrodrilus rubidus* and *L. rubellus* both showed high variation between samples in all arsenic species concentrations.

**Experiment 2: Accumulation and elimination**

The mean initial concentrations of tissue arsenic for *L. rubellus* collected from Devon Great Consols and Carrock Fell was 614.2 mg arsenic/kg dry weight and 0.09 mg arsenic/kg dry weight for the uncontaminated population (total arsenic). During the accumulation study, *L. rubellus* from the contaminated sites showed an initial loss in tissue arsenic in response to soil treated with sodium arsenate (Fig. 1A and B). The

<table>
<thead>
<tr>
<th>Species</th>
<th>Arsenite (mg/kg dry wt)</th>
<th>Arsenate (mg/kg dry wt)</th>
<th>AB (mg/kg dry wt)</th>
<th>Total speciated As (HPLC-ICP-MS) (mg/kg dry wt)</th>
<th>As in AB/As total (%)</th>
<th>Total As in methanol/water extract (GF-AAS) (mg/kg dry wt)</th>
<th>Total As in earthworm tissue (mg/kg dry wt)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>D. rubidus</em></td>
<td>7.8</td>
<td>0.05</td>
<td>1.5</td>
<td>12.7</td>
<td>3.0</td>
<td>82.3</td>
<td>535.9</td>
</tr>
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<td><em>D. rubidus</em></td>
<td>72.4</td>
<td>10.2</td>
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<td>4.5</td>
<td>254.0</td>
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<td>0.1</td>
<td>3.1</td>
<td>15.5</td>
<td>5.8</td>
<td>83.3</td>
<td>443.9</td>
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<tr>
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<td>210.0</td>
<td>26.4</td>
<td>14.9</td>
<td>251.3</td>
<td>2.8</td>
<td>441.2</td>
<td>528.3</td>
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<tr>
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<td>61.0</td>
<td>11.5</td>
<td>10.3</td>
<td>82.9</td>
<td>4.6</td>
<td>183.3</td>
<td>225.5</td>
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<tr>
<td><em>L. rubellus</em></td>
<td>6.7</td>
<td>0.07</td>
<td>2.9</td>
<td>9.7</td>
<td>4.1</td>
<td>48.2</td>
<td>72.4</td>
</tr>
</tbody>
</table>
earthworms down-regulated arsenic in the first 7 d and then accumulating arsenic to the 21-d time point. The uncontaminated population accumulated arsenic without any initial down-regulation. *Lumbricus rubellus* from the contaminated and uncontaminated sites eliminated total tissue arsenic linearly over the 21-d period (Fig. 1A and B).

**DISCUSSION**

Oligochaete earthworms are known to accumulate toxic elements. The extent of accumulation is dependent on the type of element and the soil properties [10,11]. Meharg et al. [4] reported that arsenic was not bioconcentrated in the tissues of *L. terrestris* L. and that soil pH, organic matter, and exposure time were all found to affect arsenate accumulation and toxicity. *Lumbricus rubellus* and *D. rubidus* from Devon Great Consols showed resistance to arsenate toxicity [1,2]. This resistance may be the result of detoxification of arsenic in the earthworm body and, as both species are able to exist in the contaminated mine spoil soils, it is likely that the mechanism of detoxification is similar for both species.

**Experiment 1: Arsenic speciation**

The concentrations of arsenic in *L. rubellus* and *D. rubidus* reported here are much higher than has previously been recorded in the literature. Yeates et al. [12] reported that the earthworms *L. rubellus* and *Aporrectodea rosea* (Savigny) collected from a timber treatment site had arsenic tissue burdens of up to 40 mg/kg dry weight, with a soil arsenic of 161 mg/kg dry weight. Further, Geiszinger et al. [5] reported that, in soil arsenic concentrations ranging from 5.0 to 79.7 mg arsenic/kg dry weight, total arsenic concentration in lumbricid species ranged from 3.2 to 17.9 mg arsenic/kg dry weight.

For the speciation analyses, HPLC-ICP-MS and LC-MS were conducted on the samples. Rarely are these two analytical methods used to cross-validate arsenic speciation in environmental samples [8]; doing so gives added confidence in the results, especially as both techniques utilized comparable cation exchange chromatography. The HPLC-ICP-MS has lower limits of detection [8] and can detect arsenate and arsenite [9]. The HPLC-ICP-MS has detection limits of about 1 ng/ml for organoarsenicals, such as the arsenobetaine, tetramethylarsionium iodide, arsenocholine, dimethylarsinioyethyl ethanol, dimethylarsinic acid, monomethylarsenic acid, and trimethylarsine oxide. None of those organoarsenicals were detected in the methanol/water extract (Table 2). The only organoarsenical species found in *L. rubellus* and *D. rubidus* by LC-MS and HPLC-ICP-MS was arsenobetaine (Tables 1 and 2). It is thought that the arsenobetaine could be biosynthesized in the earthworm by methylation of inorganic species, conversion to arsenocholine, and finally to arsenobetaine, although the evidence for this is not conclusive [13].

Chromatography (anion and cation exchange) confirmed that arsenobetaine was formed as a major metabolite in the earthworm samples. No other organoarsenicals were determined as a minor metabolite in the methanol/water extract (Table 2). Arsenobetaine is the dominant form of arsenic in marine animals [14], an analogue of the osmoregulant betaine, where arsenic substitutes for nitrogen. The arsenic zwitterion, arsenobetaine ([CH₃]₃As⁺CH₂COO⁻) is thought to be a metabolic end product in the process of detoxification of inorganic arsenic from sediments [5].

Langdon et al. [6] found that arsenobetaine can build up in *L. rubellus* tissues to high levels, with a maximum reported value of 276 mg/kg. The authors suggested that the arsenobetaine burden is regulated by total earthworm body burden. They suggested that the high concentrations and the regulation by total arsenic body burden suggest that the arsenobetaine is produced by the earthworm by metabolism of arsenate. They further indicated that, alternatively, the arsenobetaine might be produced by bacteria, either in the earthworm gut or in bulk soil. Langdon et al. [6] found trace amounts of arsenobetaine in soil determined by LC-MS, ranging between 0.01 to 0.03 mg/kg for the soils in which the earthworms had been kept for one week. No other organoarsenic compounds were detected. The levels were low and showed no relation to total soil arsenic burden. It was not known if the arsenobetaine present in the soil had been egested by the earthworm or produced by soil bacteria. It was concluded that the arsenobetaine was probably synthesized by the earthworm. Langdon et al. [6] suggested that this has yet to be shown conclusively.

The only inorganic species of arsenic found to be present in both *L. rubellus* and *D. rubidus* were As III and As V (Table 2). Geiszinger et al. [5] detected arsenosugars as minor metabolites, but a large proportion of the arsenic occurs in the extract in its inorganic forms as arsenite and arsenate. Meharg et al. [4] reported that laboratory studies showed that arsenate was bioconcentrated in the tissues of *L. terrestris* and that field
studies indicated that bioconcentration, as calculated on the basis of total arsenic residues in the soil, did not occur at the contaminated sites. *Lumbricus terrestris* is an anecic, i.e., deep-burrowing, surface-feeding species. It is possible that interspecific differences in routes of uptake (dermal or ingestion) and ecology could account for differences in speciation of arsenic. Also the existence of cocontaminants in the field containing a range of different arsenic species could affect toxicity.

Langdon et al. [6] used extended X-ray absorption fine structure and X-ray absorption near-edge structure to speciate arsenic in *L. rubellus* from the Carrock Fell site, using whole earthworms, intestine, and body wall sections. Their data suggested that arsenic was predominantly coordinated with S in the form of a -SH group, suggesting metallothionein complexation, but they also found another species present in field samples, As (V)-O (up to 30% of the body wall and 45% of the whole-earthworm arsenic). The As (V)-O phase was not found to be present in the intestine of either set of earthworms, where the majority (90–100%) of arsenic was coordinated to S. The authors suggested that the differences observed between field and laboratory samples may have been due to differences in metabolism or differences in the bioavailability of arsenic. Langdon et al. [6] observed that the dominance of As (III)-thiol complexes in the intestine agreed with Morgan et al. [15] and Yeates et al. [12], who found a close association of arsenic with S in granules within *L. rubellus*. It was suggested that these granules are mainly composed of As-S, not as a precipitate but as organic As-thiol complexes.

**Experiment 2: Accumulation and elimination**

*Lumbricus rubellus* from the contaminated sites retained a residual body burden of arsenic after being kept in uncontaminated soil for six months. *Lumbricus rubellus* from the contaminated sites initially lost arsenic before accumulating it into their tissues (Fig. 1A and B). The *L. rubellus* from contaminated sites kept in uncontaminated soil for six months had a residual arsenic burden in their tissues of up to 750 mg arsenic/kg. Introduction into soil treated with arsenic may have initially induced an elimination response causing initial excretion of arsenic from the tissues. After 7 d, earthworm arsenic tissue accumulation occurred in the contaminated populations, rising to a concentration comparable with the initial LC10 value of 1,200 mg arsenic/kg. Accumulation of arsenic was highest in the 14- to 21-d period for the uncontaminated population, rising to concentrations comparable with the soil LC10 value of 50 mg arsenic/kg dry weight. Fischer and Koszorus [16] found that *Eisenia fetida* (Savigny) accumulated arsenic to near lethal values of toxic arsenite (As (III)) found in tissue extracts of *L. rubellus*. The authors suggested that arsenic was predominantly coordinated with S in granules within *L. rubellus*. It was suggested that these granules are mainly composed of As-S, not as a precipitate but as organic As-thiol complexes.

Morgan et al. [17] reported that arsenic was sequestered in earthworm tissue. Evidence from other studies also suggests that, at sublethal concentrations, bioconcentration of arsenate, due to sequestration of arsenic in earthworm tissue, occurs where soil concentrations are low enough to allow survival. It is possible that the reduction and accumulation in the tolerant *L. rubellus* populations reflect the arsenic speciation found in these earthworms, arsenobetaine at low tissue concentrations and, as the arsenate is accumulated, changing to As III and As V (Table 2). Langdon et al. [6] suggested that the sulfur coordination of As (III) of this kind is not stable under nonphysiological conditions, which could explain the extremely high values of toxic arsenite (As (III)) found in tissue extracts by HPLC-ICP-MS. The authors also suggested that *L. rubellus* synthesize arsenobetaine from arsenate. This is also likely to be the case in *D. rubidus*. If the biotransformation of arsenate to arsenobetaine is considered as a detoxification mechanism at low concentrations of arsenic, the clear change in the metabolic pathway for arsenic in tolerant earthworms with high arsenic burdens (the production of As (III)-complexing S-rich proteins) must be considered as a mechanism of arsenic resistance [6].

*Lumbricus rubellus* from both the uncontaminated and contaminated sites eliminated arsenic readily from their tissues over the 21-d experimental period. This differs from the results of the study by Fischer and Koszorus [16], who found no elimination over an eight-week period in *E. fetida*. The difference between the two studies may be due to feeding behavior or availability of the arsenic in the treated soils. Fischer and Koszorus [16] suggested that the lack of elimination of arsenic may be due to restricted ability for elimination. The *L. rubellus* and *D. rubidus* from the contaminated sites are known to be tolerant to arsenic toxicity [1] and it is possible that these tolerant populations may be able to eliminate arsenic readily from their tissues when kept in uncontaminated soils. Further work on the uptake and elimination of arsenic is needed to elucidate the processes involved.

Using data obtained by standard simple chemical extraction without considering the implications in situ may be misleading with respect to arsenic speciation. Using HPLC-ICP-MS and LC-MS allows cross-validation of species of arsenic found in the earthworms. A comparison of arsenic speciation using ecologically different earthworm species (anecic, epigeic, or endogeic [3]) may highlight differences in arsenic speciation and accumulation.

This present study characterized arsenic speciation in tolerant and nontolerant populations of *D. rubidus* in an attempt to determine how arsenic is metabolized by this species. The study also examined if this speciation is different from populations of *L. rubellus* living in highly contaminated soils. The speciation of arsenic in *D. rubidus* did not differ significantly from that of *L. rubellus* characterized in a previous study [6]; however, total arsenic concentrations of *D. rubidus* were higher per gram of dry weight. Both *L. rubellus* and *D. rubidus* are epigeic species with similar ecological characteristics. These species were the only earthworms found at the contaminated sites, and it is probable that their mechanisms of tolerance to arsenic may also be similar.

**Acknowledgement**—Caroline J. Langdon was supported by a NERC CASE studentship (GT04/98/116/T3). We thank P. Clasper for her help with the accumulation and elimination experiment and the 7th Earl of Bedford for permission to sample on his Tavistock woodland estate.

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