Arsenic biotransformation in earthworms from contaminated soils

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Two species of arsenic (As) resistant earthworm, *Lumbricus rubellus* and *Dendrodrillus rubidus*, their host soils and soil excretions (casts) were collected from 23 locations at a former As mine in Devon, UK. Total As concentrations, measured by ICP-MS, ranged from 255 to 13,080 mg kg^{-1} in soils, 11 to 877 mg kg^{-1} in earthworms and 284 to 4221 mg kg^{-1} in earthworm casts from a sub-sample of 10 of the 23 investigated sites. The samples were also measured for As speciation using HPLC-ICP-MS to investigate potential As biotransformation pathways. Inorganic arsenate (As^{V}) and arsenite (As^{III}) were the only species detected in the soil. As^{V} and As^{III} were also the dominant species found in the earthworms and cast material together with lower proportions of the organic species methylarsonate (MA^{V}), dimethylarsinate (DMA^{V}), arsenobetaine (AB) and three arsenosugars. Whilst the inorganic As content of the earthworms increased with increasing As body burden, the concentration of organic species remained relatively constant. These results suggest that the biotransformation of inorganic arsenic to organic species does not contribute to As resistance in the sampled earthworm populations. Quantification of As speciation in the soil, earthworms and cast material allows a more comprehensive pathway for the formation of AB in earthworms to be elucidated.

Received (in XXX, XXX) Xth XXXXXXXXX 200X, Accepted Xth XXXXXXXXX 200X
First published on the web Xth XXXXXXXXX 200X
DOI: 10.1039/b000000x

Introduction

The chemistry of As in environmental and biological systems is complex. Whilst inorganic As species are the most prevalent in abiotic environments, the uptake of inorganic As by living organisms can lead to the synthesis of organic As species[1] through biotransformation. The ubiquitous, epegeic earthworm species *L. rubellus* and *D. rubidus* are known to inhabit soils highly contaminated with As at the former mine site of Devon Great Consols (DGC), UK.[2-5] These earthworms are clearly resistant to As toxicity and several potential coping mechanisms have been proposed, yet the underlying mechanism behind this resistance is unknown. Behavioural adaptation, whereby the earthworm avoids contact with the contaminant,[4] is unlikely as earthworms from DGC are known to have elevated As body burdens.[2, 4, 5] The biotransformation of highly toxic inorganic As to the less toxic organic species arsenobetaine (AB) has been speculated as a mode of mitigating As toxicity in DGC earthworms.[2, 4] An alternative mechanism involves the sequestration of arsenic in the metallothionein-rich chloragogenous tissue which separates the intestine from the coelomic cavity.[3] With this mechanism it is proposed that inorganic As^{III} binds to the sulphur-rich metallothionein thereby sequestering ingested As in a form that is not biologically reactive.[6]

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The study of As speciation can provide important information on As biotransformation and toxicity and to date a multitude of organic As species have been identified.[7] The occurrence of organic As species and their biotransformation pathways are well documented in marine organisms such as crustaceans, molluscs, fish and algae.[8-10] However, less is known about the occurrence and behaviour of As in terrestrial organisms such as earthworms.[11] Until recently the organo-arsenic species AB was thought to be restricted to the marine environment,[12] but has now been demonstrated in terrestrial fungi[13] and earthworms.[2, 11, 14] The biotransformation pathway for the formation of AB in marine organisms is thought to involve the carbohydrate containing As compounds known as arsenosugars.[15] Langdon *et al*. (2003)[16] have proposed a biotransformation pathway for the formation of AB from ingested inorganic As in earthworms but did not include arsenosugars. It is likely that arsenosugars were not included as they were not observed in arsenic speciation studies in earthworms by the same authors.[17] In contrast, it is now clear that at least three arsenosugar species (glycerol, phosphate and sulphate) have been detected in earthworms from both uncontaminated and contaminated soils.[2, 18]

In light of the uncertainties surrounding the biotransformation pathway for AB and the role of AB and other organo-arsenicals in the mechanism underlying the resistance to As toxicity in earthworms from DGC, it seems prudent to examine the issue further. Our previous study[2] detailed total As data for soils, earthworms and casts from 12 sites at DGC plus As speciation data for the earthworms. This dataset alone was insufficient to investigate potential As biotransformation pathways. Here we present new data from an additional sampling trip, increasing the number of sampling points to 23, and providing As speciation data for

the soil, earthworm and casts components. The aims of this study are firstly, to investigate the source of AB and arsenosugars in earthworms from DGC by determining the As speciation of the host soil, earthworm and earthworm casts. Secondly, to elucidate a more comprehensive biotransformation pathway for the formation of AB in earthworms and thirdly, to examine further the role of AB and arsenosugars in the resistance to As of earthworms from DGC.

Methods

Study site

DGC is one of many former mining sites in south-west England situated by the River Tamar in the Tavistock district of Devon (SX 426 735). In the 1870s, DGC along with half a dozen mines from the Callington and Tavistock area were the source of an estimated 50 percent of the world's arsenic production.[19] Arsenic concentrations found in soils surrounding the mine vary significantly depending on their proximity to the main tailings, ranging from 204 - 34,000 mg kg^{-1}. [2, 19, 20]

Reagents and standards

All reagents used were analytical grade or better quality. All aqueous solutions were prepared using deionised water (18.2 MΩ Millipore, UK). Inorganic As^{III} and As^{V} (Fisher, UK), organic methylarsonate (MA^{V}, Sigma-Aldrich, UK), dimethylarsinate (DMA^{V}, Greyhound, UK) and AB (LGC, UK) were used for the preparation of standards for arsenic speciation analysis. Four arsenosugar compounds were isolated from marine algae as reported previously [2]. These arsenosugar compounds were prepared according to methods published by Madsen *et al.* (2000)[10] and were used for the identification of arsenosugars in earthworm extracts. Figure 1 illustrates the structure of the four arsenosugar compounds. Methanol (Fisher Scientific, UK), phosphoric acid and ascorbic acid (BDH Aristar, UK) were employed during sample extraction. Ammonium nitrate (Sigma-Aldrich, UK) was used as the mobile phase for gradient anion exchange separation of arsenic species. Nitric acid (HNO_3 69%), hydrofluoric acid (HF, 48%.), perchloric acid ($HClO_4$, 70%.) and hydrogen peroxide (H_2O_2 30 %) (BDH Aristar, UK) were used for the dissolution of earthworms, cast and soil samples

Sample collection and preparation

Soil and earthworm samples were collected during two sampling trips to DGC in April 2006 and April 2007. The soil surface (0 – 20 cm) with an area of approximately 1 m^2 was overturned with a spade allowing individual earthworms to be handpicked. Approximately 10 to 25 mature earthworms (clitellum present) were collected at each sampling point. *L. rubellus* and *D. rubidus* specimens were identified and separated using a dichotomous earthworm key,[21] thoroughly rinsed with deionised water and placed in ventilated plastic tubes with moist filter paper to begin depuration of the gut contents. Filter papers were changed daily to prevent coprophagy and allow collection of the cast material. Earthworms were depurated for a minimum of 48 hours, since shorter times were unlikely to remove all soil particles in larger species such as *L. rubellus*.[16] Depuration was halted when no more cast material was deposited on the filter paper. In addition, a sub-sample of depurated earthworms were dissected and the gut contents examined under a microscope to ensure depuration was complete. Depurated earthworms were dispatched humanely (rapid freezing) before being dried along with the cast material (collected during depuration) in a low temperature oven (50 ˚C) then homogenised in a ceramic pestle and mortar. A composite soil sample from the overturned surface (approximately 1 kg) was collected at each site, placed in a sealed paper bag and dried at room temperature. Soils were disaggregated in a ceramic pestle and mortar, sieved to < 250 µm, homogenised by shaking then stored in airtight containers prior to analysis.

Sample digestion

Soils and casts

Homogenised soils and earthworm casts (0.25 g) were prepared for total elemental measurements by ICP-MS based on a mixed acid digestion approach (HF / HNO_3 / $HClO_4$).[22] Samples were weighed directly into to polytetrafluoroethylene (PTFA) Savillex® 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). Once digested and evaporated the sample was taken up in 2.5 ml of HNO_3, heated at 50 ºC for 30 minutes, left to cool then treated with 1 ml H_2O_2 before being made up to volume (25 ml) with deionised water to give a final solution of 5 % HNO_3 for analysis by ICP-MS. Certified reference materials were included with each batch of soil and cast digestions as a measure of quality control. These were NIST CRM 2710 Montana Soil I and NIST CRM 2711 Montana Soil II with certified As values of 626 ± 38 mg kg^{-1} and 105 ± 8 mg kg^{-1} respectively.

Earthworms

Microwave assisted (MARS 5, CEM, UK) dissolution of the earthworms using a closed vessel system was performed on 0.1 g of earthworm homogenate (dry weight). To each vessel 10 ml HNO_3 and 100 µl HF was added before standing for 30 minutes then microwaving. Following an initial heating program (ramp to 100 ºC over 5 minutes then hold for 5 minutes, ramp to 200 ºC over 5 minutes then hold for 5 minutes) the vessels were allowed to cool (<50 ºC) and then 1 ml of H_2O_2 was added. The vessels were sealed and the microwave cycle repeated. After cooling the sample solutions were transferred to PTFA containers and evaporated to dryness on a hotplate (100 °C). Samples were reconstituted by the addition of 2 ml of 50 % v/v HNO_3, heated at 50 °C for 30 minutes and then made up to 10 ml with deionised water. The procedure was monitored using a certified reference material, CRM 627 tuna fish (BCR, Brussels) with a certified

As value of 4.8 ± 0.3 mg kg^{-1}.

Sample extraction

Soils and casts

Extraction of As from soils and earthworm casts was performed using a method developed previously.[23] In brief, 0.2 g of each prepared sample was accurately weighed into 30 ml round-bottom Nalgene® extraction vessels, 10 ml of a 1 M phosphoric acid / 0.5 M ascorbic acid mixture was then added and the lids securely fastened. The extraction vessels were then attached to an orbital shaker and extracted for 4 hours at 200 rpm. Extractions were conducted in the dark to avoid speciation changes due to UV radiation. Following shaking the extraction vessels were placed for 5 minutes in a sonic bath [24], centrifuged for 15 minutes at 2000 rpm and the supernatant carefully removed. Only one extraction step was 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.[25] Sample extracts were stored in the dark at <4 ºC. All speciation analyses were performed within 24 hours of extraction, the maximum time period in which species are known to remain stable [26].

Earthworms

Homogenised earthworm powder (0.25 g) was weighed directly into 30 ml round bottom Nalgene® extraction vessels. 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 10 ml polypropylene bottles. The methanol was evaporated off 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^{V}, DMA^{V} and AB) was established under the extraction conditions employed 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 repeatability was monitored using the CRM 627 tuna fish tissue (BCR, Brussels).

Instrumentation

Total As analysis

All digested samples and earthworm extracts were determined for As using a Thermoelemental PQ ExCell ICP-MS (Thermo Scientific, UK). The instrument was fitted with a Meinhardt nebuliser and Scott-type spray chamber. The instrument was tuned using a 1 µg l^{-1} Claritas PPT multi-element tune solution 1 (GlenSpectra, UK). 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. Soil and cast extracts were determined for As using an Agilent 7500 ICP-MS (Agilent Technologies, UK). The instrument was fitted with a micro flow concentric nebuliser and quartz Scott-type spray chamber. The instrument response for As was optimised daily. Arsenic detection was performed in collision cell mode using He (4 l/min) to minimise potential interferences such as that of the polyatomic ion $^{40}Ar + {}^{35}Cl$. Tellurium (50 µg l^{-1}) was used as the internal standard by sample spiking.

Arsenic speciation analysis

A quaternary pump (GP50-2 HPLC Pump and an AS-50 autosampler; Dionex, USA) was directly coupled to the ICP-MS for measurement of arsenic species by connecting the analytical column to the ICP nebuliser with polyetheretherketone (PEEK) tubing. The two instruments were coupled in such a way that the injection of each sample solution via the autosampler and subsequent measurement was synchronised automatically using the ICP-MS software, enabling reproducible sample injections. Full details of the chromatographic system employed are published elsewhere.[2] In brief, anion and cation exchange columns (Hamilton PRP–X100, 250 x 4 mm, 10 µm) and (Hamilton PRP–X200, 250 x 4 mm, 10 µm) respectively, with guard columns of the same material were used to separate the arsenic species present in the sample extracts. Ammonium nitrate was used as the anion exchange mobile phase at pH 8.65 (adjusted with aqueous ammonia) using a gradient elution between 4 and 60 mM. Pyridine (10 mM isocratic) was used as the cation exchange mobile phase at pH 2.26 (adjusted using conc. formic acid). Earthworm extracts were analysed using the Thermoelemental PQ ExCell ICP-MS as the ion specific detector. Peak areas were calculated from resultant chromatograms using PeakFit V4.0 chromatography software (Seasolve Software, USA). Soil and cast extracts were analysed using the Agilent 7500 ICP-MS as the ion specific detector. Peak areas were calculated using the Agilent Chemstation LC-MS software (Agilent, UK).

Results

Soils

Recoveries of total As from CRM 2710 and CRM 2711 of 98 ± 4 % (n = 6) and 91 ± 3 % (n = 3) of the certified value, respectively, were achieved during the course of the study. The repeatability precision for the method was additionally assessed using the Thompson Howarth precision control method [27]. Thompson Howarth precision control charts are a simple graphical method for assessing and controlling repeatability precision from a moderate number of duplicated analytical results, in this case n = 21 duplicate analyses. The repeatability precision was found to exceed the specified Fitness for Purpose (FFP) criteria of 5 % RSD on the duplicate analyses.

Total As levels in the earthworm host soils covered a wide As concentration range from 255 to 13,080 mg kg^{-1} (Table 1). All the soils are highly contaminated when considered against the current UK Soil Guideline Value (SGV) for As of 20 mg kg^{-1}

[28]. The extraction procedure employed gave a mean recovery of As from the soil of 80 ± 9 %. The mean recovery of As species from the column was 97 ± 7 % of the total As in the soil extracts. Only inorganic As was present in the soil extracts with the majority being As^V with small amounts of As^{III}.

Earthworms

Recovery of total As from CRM 627 was 96 ± 7 % (n = 6) compared to the certified value. The method precision, expressed as the mean % difference (± 1 SD), between duplicate earthworm samples was 1.7 ± 0.9 % (n= 4 duplicates). The extraction procedure employed gave a mean recovery of 77 ± 1 % (n = 3) of the total arsenic value for CRM 627 with a precision, expressed as the mean percentage difference (± 1 SD) between duplicate samples, of 2.8 ± 1.8 % (n = 4 duplicates).

Both species of earthworm had accumulated high levels of As from the host soil with As body burdens ranging from 11 to 877 mg kg^{-1} (Table 2) depending on the As concentration of the host soil. Further details of earthworm species differentiation in terms of As accumulation are presented elsewhere [2, 5]. The methanol / water extraction procedure gave variable recoveries of As from prepared earthworm samples (Table 2) with a mean extraction efficiency of 51 ± 18 %. The mean recovery of As species from the column was 90 ± 23 % of the total As in the earthworm extracts. Inorganic As^V was the dominant species extracted followed by As^{III}. In general AB was the dominant organic species extracted from the earthworms at an average concentration of 4.2 ± 3.2 mg kg^{-1}. Similar amounts of MA^V and DMA^V were present with the arsenosugars 1, 2 and 4 (Fig. 1) present at the lowest concentrations of the organic species (Table 2). The concentration of inorganic As showed a positive correlation with increasing As body burden in the earthworm (Fig. 3a). In contrast, organic As species do not demonstrate a correlation with increasing As body burden (Fig. 3b). The organic species remain fairly constant (with some fluctuation) across the range of As body burdens observed.

Casts

Total As levels in the earthworm casts ranged from 284 to 4221 mg kg^{-1} in the 10 available samples, and were similar to total As levels in the corresponding host soil (Tables 1 and 3). The extraction procedure employed gave mean recoveries for As of 84 ± 16 % of the total. The mean recovery of As species from the column was 106 ± 10 % of the total As in the cast extracts. Arsenic speciation in the earthworm casts was similar to that of the host soil with As^V present as the dominant species with lower amounts of As^{III}. In addition the organic As species AB, DMA^V, MA^V, sugar 2 and sugar 4 were present at low levels in some of the cast samples with DMA^V present in higher amounts than the other organic species. No clear relationship was observed between the organic species present in the earthworm and the subsequent organic species observed in the earthworm cast.

Discussion

The earthworm species *L. rubellus* and *D. rubidus* are able to reside in soils highly contaminated with As at the former mine site DGC, as reported here and in several other studies.[2, 5, 20] The As body burdens observed in this study are highly elevated up to a maximum of 877 mg kg^{-1} (Table 2) yet no harmful physiological side effects are evident or have been reported. These earthworms have clearly developed a biological mechanism for mitigating the toxic effects of arsenic. The only arsenic species detected in the host soils were inorganic As^{III} and As^V, of which As^V accounted for between 91 and 99% of the extracted As. This precludes selective bioaccumulation of organic species from the host soil as a potential source of the organic As species observed in the earthworms, as has been suggested previously.[11] Elsewhere, the biotransformation of As^V by soil organisms is considered the source of organic As species in the soil environment.[15] Likewise, this seems a probable source of the organic species observed in earthworms residing at DGC. The biotransformation pathway proposed by Langdon *et al.* (2003)[16] attempts to explain As accumulation via the sequestration of high levels of As through As^{III}-thiol complexing and the formation of AB via subsequent methylation of the As^{III}-thiol complexes. This pathway was proposed on the basis that AB was the only organic As species detected using HPLC-MS, whilst As^{III} co-ordinated with sulphur was detected using XAS. This pathway does not take into account the presence of both simple methylated compounds (MA^V and DMA^V) and arsenosugars in the earthworm, as reported here (Table 2) and elsewhere.[2, 11, 18] A more comprehensive pathway for the formation of both arsenosugars and AB in earthworms (Fig. 2) can be hypothesised from what is known about the transformations of As by organisms in marine and freshwater environments.[12, 29] It is more likely that As^V ingested from the soil is first converted to DMA^V via the Challenger pathway[15, 30] (Fig. 2, a) which in turn is converted to arsenosugars through addition of the adenosyl group from *S*-adenosylmethionine (SAM).[29] This nucleoside then undergoes glycosidation to produce a range of arsenosugars.[15, 29] These arsenosugars are thought to subsequently be converted to AB along a pathway involving either arsenocholine (AC) or dimethylarsinoylacetate (DMAA)[12] (Fig. 2, b). The tendency for AB to be more prevalent in earthworm extracts when DMA^V and the arsenosugars are also present (Table 2 and Fig. 2) provides some evidence for this pathway as the source of AB in DGC earthworms.

The synthesis of AB in earthworms from highly contaminated soils has been speculated as a potential mechanism for mitigating As toxicity.[17] However, the fact that AB is only present at low concentrations between 0.5 and 14 mg kg^{-1} (Table 2) irrespective of the total As body burden (Fig. 3b) suggests that AB formation is not a mechanism by which earthworms at DGC mitigate As toxicity. Similar findings were interpreted differently by both Langdon *et al.* (2002)[17] who reported similar AB concentrations of 0.3 – 15 mg kg^{-1} using HPLC-ICP-MS in *L. rubellus* from contaminated soils and in a previous study.[2] When the AB

concentrations were considered as a proportion, rather than actual concentration, against the earthworm total As, a relationship dependent on the earthworm total arsenic was reported.[2, 17] Such interpretation is misleading as consistently low AB concentrations will inevitably decrease proportionally as total arsenic body burden increases. Concentrations of AB in *L. rubellus* and *D. rubidus* from uncontaminated soils of 0.3 and 0.5 mg kg^{-1} respectively have previously been reported,[2] falling within the range reported for contaminated sites. This reinforces the point that AB formation in earthworms is independent of total As burdens and not involved in mitigating As toxicity. The resistance is more likely due to a second process (Fig. 2, c) whereby As is thought to be sequestered via binding with sulphur-rich metallothionein within the chloragogenous tissue[3, 17, 31] allowing the continued accumulation of inorganic As (Fig. 3a) in a form that is not biologically reactive[6].

The egestion of As in the earthworm cast material was mainly in the form of As^V. The presence of DMA^V as the dominant organic As species in some of the cast samples (Table 3) may suggest this is the most readily excreted arsenical, although a greater sample size is required to confirm this as DMA^V was not present in all samples. Low levels of arsenosugars were detected in some of the cast samples (Table 3). Trace levels of arsenosugars in earthworm casts were reported in the study by Geiszinger *et al*. (2002).[18] However, the authors were unable to provide quantitative estimates of the concentration of arsenosugars in the earthworm casts as the methanol / water extraction was only 0.7 % efficient. The mean extraction efficiency of 84 ± 16 % presented here allows the speciation data for casts samples to be considered quantitative. Only very low levels of AB (0.1 mg kg^{-1}) were present in the cast material (Table 3) suggesting this arsenical is less readily excreted than either DMA^V (up to 7.1 mg kg^{-1}) or the arsenosugars (up to 0.7 mg kg^{-1}). This precludes egestion of AB as a possible mechanism behind the consistently low levels of this organic As species observed in earthworms with elevated As body burdens.

The use of solvent extraction separation techniques that ignore the results of in-situ studies are potentially misleading with respect to As speciation. As^{III}-sulphur complexes were detected in DGC earthworms by Langdon *et al*. (2002)[17] when the in-situ speciation technique X-ray Absorption Spectroscopy (XAS) was employed. It is possible that the solvent extraction method employed in this study may cause dissociation of As^{III}-sulphur complexes leading to the detection As^{III} alone. This would provide a possible explanation for the high levels of As^{III} determined in earthworm extracts in this study. The reason for the presence of AB at consistently low concentrations irrespective of total body burden is not clear. It would be useful to investigate AB synthesis in earthworms from soils with very low As concentrations to see if a minimum level of AB is synthesised, perhaps above soil levels, which would clarify its role in the physiological processes of earthworms. It has been suggested that AB may act as an analogue for the osmoregulant cysteine betaine in marine animals[32]. Whether this process is involved in the regulation of AB in earthworm populations at DGC requires further investigation.

Conclusions

The definitive biotransformation pathway for As in earthworms is still unclear. The data presented here suggests that the organic species are a product of As biotransformation within the worm itself as no organic species were present in the soil. The biotransformation of As^V to AB is not likely involved in the resistance to As toxicity in earthworm populations at DGC as AB concentrations are consistently low and independent of earthworm total As. It is more likely that there are two process of arsenic biotransformation occurring in these As resistant earthworms. Firstly, the sequestration of inorganic As in a form that is not biologically reactive such as binding with sulphur-rich metallothionein within the chloragogenous tissue providing the mechanism for As resistance and secondly, the biotransformation of inorganic As^V along a pathway comparable to those proposed for marine and freshwater organisms as the source of the observed organic arsenic compounds.

Acknowledgments

Funding for this research was provided by the British Geological Survey University Funding Initiative (BUFI). We also thank the University of Leicester Department of Geology for facilitating the mesocosm experiment and the Tavistock estate for allowing us to collect samples.

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Table 1: Total and speciation data for As in soil samples. Extraction efficiency based on extracted arsenic as a percentage of total As. Column recovery refers to As species recovered from the column as a percentage of the total As in the extract. nd = no data

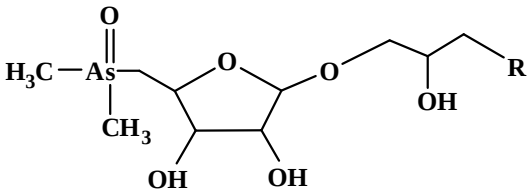
Site id	Total As (mg kg^{-1})	Extracted As (mg kg^{-1})	Extraction efficiency (%)	Speciated As (mg kg^{-1})		Column Recovery (%)
				As^{III}	As^{V}	
D1	2980	2365	79	23	2177	93
D2	1573	1113	71	5.8	973	88
D3	1005	771	77	35	734	100
D4	255	201	79	2.8	177	89
D6	13080	12434	95	76	10442	85
D7	372	400	108	36	362	100
D9	284	222	78	5.4	221	102
D10	439	326	74	4.8	302	94
D11	289	237	82	7.5	217	95
D12	5141	3713	72	89	3760	95
D13	2871	2484	87	39	2359	97
D15	913	742	81	5.6	712	97
D16	3995	3184	80	95	3044	99
D17	622	489	79	4.4	450	93
D18	1567	1188	76	19	1100	94
D19	1173	1136	97	12	905	81
D20	6308	4572	72	62	5154	109
D21	480	406	85	12	393	100
D23	275	211	77	12	192	96
D24	1306	nd	nd	nd	nd	nd
D25	9025	8097	90	111	8336	98
D26	5760	4055	70	45	4279	104
D27	427	298	70	4.9	301	97

Table 2: Total and speciation data for As in earthworm samples. Extraction efficiency based on extracted arsenic as a percentage of total As. Column recovery refers to As species recovered from the column as a percentage of the total As in the extract. < LOD = not detected or below limit of detection.

Site id	Earthworm species	Total As (mg kg^{-1})	Extracted As (mg kg^{-1})	Extraction Efficiency (%)	Speciated As (mg kg^{-1})								Column Recovery (%)
					AB	As^{III}	DMA^{V}	MA^{V}	As^{V}	sugar 1	sugar 2	Sugar 4	
D11B	*L. rubellus*	11	3.6	32	0.5	0.7	<LOD	<LOD	2.4	0.3	0.3	<LOD	115
D10	*L. rubellus*	40	11	27	1.5	2.8	<LOD	0.1	7.2	0.1	nd	<LOD	109
D24	*L. rubellus*	54	15	28	0.8	5	<LOD	0.3	11	0.1	<LOD	<LOD	114
D12	*L. rubellus*	203	163	81	2.6	42	0.1	0.8	50	0.3	nd	0.7	59
D2	*L. rubellus*	257	161	63	4.8	47	0.2	0.5	44	0.3	nd	0.6	60
D18	*L. rubellus*	355	186	52	2.4	82	10	10	104	0.3	nd	0.2	113
D6	*L. rubellus*	359	150	42	1.9	40	<LOD	1.3	40	0.2	nd	1	56
D20	*L. rubellus*	385	127	33	1.4	24	2.7	4.2	77	<LOD	nd	<LOD	86
D13	*L. rubellus*	571	366	64	4	149	0.1	0.5	61	0.8	nd	1	59
D1	*L. rubellus*	595	215	36	2.2	55	0.2	0.6	51	0.5	nd	1	52
D26	*L. rubellus*	607	345	57	3.4	39	1.5	2.8	289	0.3	<LOD	<LOD	98
D25	*L. rubellus*	877	430	49	7.8	155	<LOD	1.2	335	0.4	<LOD	<LOD	116
D27	*D. rubidus*	15	9	60	5.4	1.8	<LOD	<LOD	2.1	0.3	0.3	<LOD	110
D7	*D. rubidus*	17	9.3	54	3.4	4.3	0.1	0.1	1.6	0.3	0.1	<LOD	105
D9	*D. rubidus*	18	14	77	5	5.9	0.1	0.1	1	0.7	0.1	0	94
D4	*D. rubidus*	19	5.9	31	2.9	0.3	<LOD	0.1	1.8	0.2	0.4	<LOD	97
D23	*D. rubidus*	19	9	47	2.1	3	0.1	0.1	3.9	0.5	0.2	<LOD	108
D11A	*D. rubidus*	37	20	52	1.7	5.6	<LOD	<LOD	2.5	0.4	0.1	<LOD	53
D21	*D. rubidus*	61	27	44	2.4	7.2	3.7	0.6	11	0.3	0.2	<LOD	95
D15	*D. rubidus*	73	54	73	6	33	0.3	0.3	14	0.2	0.8	0.2	102
D17	*D. rubidus*	132	94	71	9.7	11	22	6.9	36	0.4	0.3	0.1	91
D19	*D. rubidus*	164	53	32	7	7.3	4.9	0.9	34	0.3	0.8	<LOD	104
D3	*D. rubidus*	317	260	82	7.7	93	0.1	0.1	41	0.2	0.1	0.5	55
D16	*D. rubidus*	737	237	32	14	68	1.8	1.5	159	0.8	0.3	<LOD	103

Table 3: Total and speciation data for As in cast samples. Extraction efficiency based on extracted arsenic as a percentage of total As. Column recovery refers to As species recovered from the column as a percentage of the total As in the extract. < LOD = not detected or below limit of detection.

Site id	Total As (mg kg^{-1})	Extracted As (mg kg^{-1})	Extraction efficiency (%)	Speciated As (mg kg^{-1})							Column Recovery (%)
				AB	As^{III}	DMA^{V}	MA^{V}	As^{V}	sugar 2	sugar 4	
D1	2488	2326	94	< LOD	72	< LOD	0.3	2204	< LOD	< LOD	98
D3	994	908	91	< LOD	92	0.8	0.2	864	0.3	0.7	105
D10	284	244	86	< LOD	12	< LOD	< LOD	236	< LOD	< LOD	101
D11A	291	204	70	0.1	17	< LOD	0.1	231	< LOD	0.1	122
D12	1173	1420	121	< LOD	107	< LOD	0.7	1344	0.1	0.5	102
D16	2290	1960	86	0.1	66	5.4	0.1	1940	< LOD	0.4	103
D19	1203	1041	87	0.1	20	< LOD	0.2	1001	< LOD	< LOD	98
D20	2359	1465	62	< LOD	27	5.7	0.2	1445	< LOD	< LOD	101
D21	421	292	69	< LOD	12	7.1	< LOD	351	< LOD	< LOD	127
D26	4221	3333	79	0.1	60	< LOD	0.5	3473	< LOD	< LOD	106



Arsenosugar 1 (glycerol) R = OH

Arsenosugar 2 (phosphate) R = $OP(O)(OH)OCH_2CH(OH)CH_2OH$

Arsenosugar 3 (sulphonate) R = SO_3H

Arsenosugar 4 (sulphate) R = OSO_3H

Figure 1: Structures of the four arsenosugars.

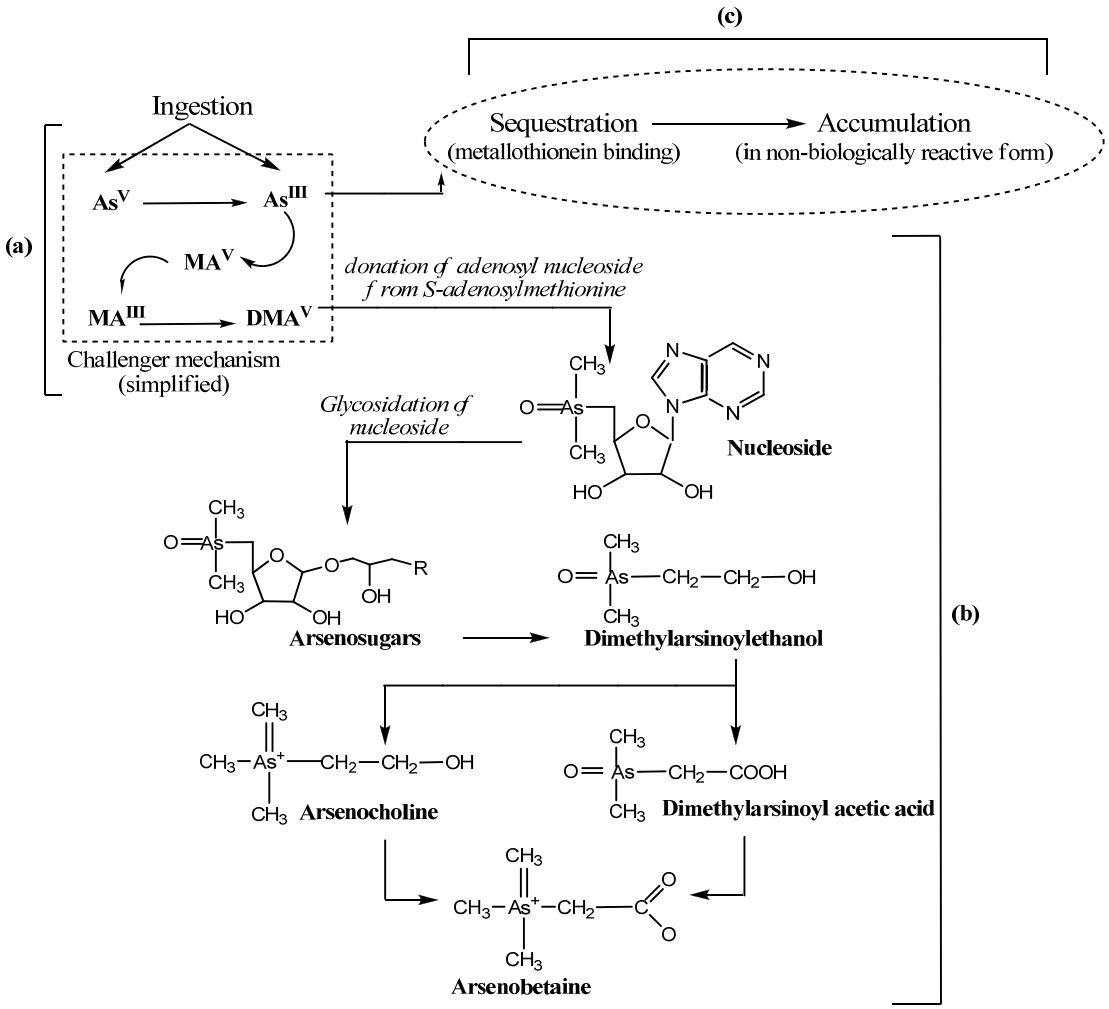
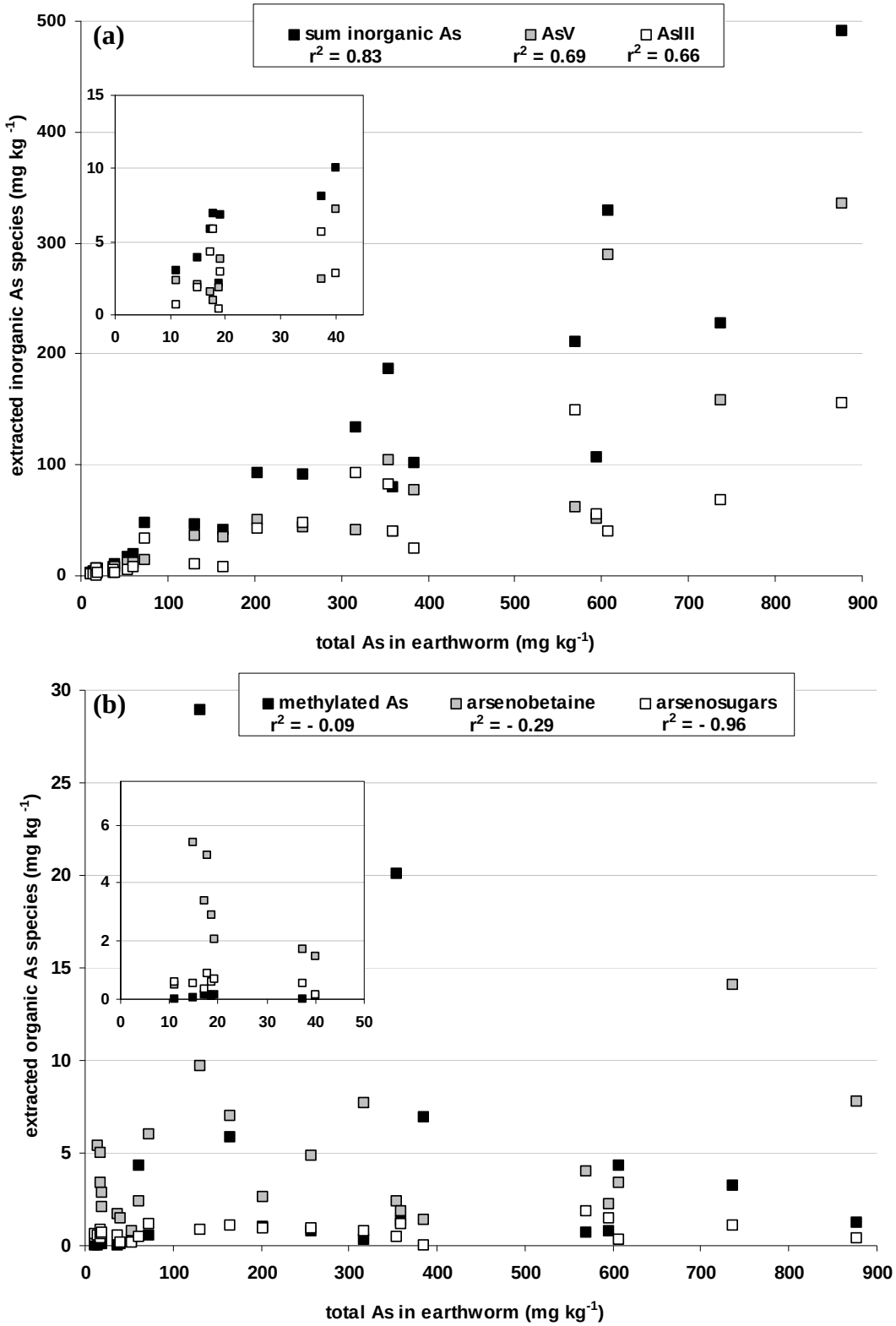


Figure 2: Speculative pathway for the biotransformation (a/b) and accumulation (c) of As in resistant earthworms from DGC. Adapted from [12, 15, 16, 29].



Figures 3a/b: Inorganic (a) and organic (b) As species extracted from earthworms plotted against increasing total As in earthworm. Methylated refers to the sum of MA^V and DMA^V. Arsenosugars refers to the sum of sugars 1, 2 and 4.