ARSENIC CONTAMINATED SOILS: HUMAN EXPOSURE AND ENVIRONMENTAL TOXICOLOGY

Thesis submitted for the degree of

Doctor of Philosophy

at the University of Leicester

by

Mark Button BSc

Department of Geology

University of Leicester

April 2009

ARSENIC CONTAMINATED SOILS: HUMAN EXPOSURE AND ENVIRONMENTAL TOXICOLOGY

Mark Button

Abstract

This research details a multidisciplinary assessment of arsenic contaminated soils in terms of human exposure and environmental toxicology. Two species of earthworm (*Lumbricus rubellus* and *Dendrodrillus rubidus*) along with their host soils and excreta (casts) were collected from 24 locations at Devon Great Consols (DGC), a former arsenic mine located in the Tavistock district of Devon, UK. Total arsenic in these samples was determined via ICP-MS. The bioaccumulation of arsenic in DGC earthworms was found to be comparable to the human bioaccessible fraction of arsenic in the host soils, estimated using a physiology-based extraction test (PBET), suggesting earthworms and PBETs might be used in conjunction when assessing risk at contaminated sites. Earthworms at DGC appear to be highly resistant to arsenic toxicity. The Comet Assay revealed DNA damage levels in earthworms native to DGC were comparable to background levels in earthworms from uncontaminated sites. Non-native earthworms exposed to a contaminated DGC soil incurred high levels of DNA damage, highlighting the potential toxicity of contaminated DGC soils. Arsenic biotransformation in DGC earthworms was investigated using HPLC-ICP-MS to investigate the mechanisms by which these earthworms mitigate arsenic toxicity. Whilst toxic inorganic arsenic was transformed to less toxic organic species, the degree of transformation was limited and not related to soil total arsenic levels, suggesting this mechanism is not involved in mitigating toxicity. Human toenail samples from DGC residents were investigated as a biomarker of exposure to elevated environmental arsenic and demonstrated significantly higher levels of arsenic than a control group. These findings highlight the potential for human exposure to arsenic at contaminated sites in the southwest UK, where mining activity has led to widespread environmental arsenic contamination.

Acknowledgments

A great number of people have offered their help and support throughout the entirety of my PhD and to all of these people I am hugely grateful:

At the British Geological Survey I would like to thank my supervisor Dr Michael Watts for his unwavering supply of motivation, support and guidance. Also Dr Mark Cave and Dr Joanna Wragg for sharing their expertise in the field of medical geology and Nicholas Lloyd and Dr Simon Chenery for patiently providing answers to innumerable and completely indiscriminate questions. I would also like to thank the Analytical Geochemistry section as whole as too many of you to mention individually have helped me in completing this work.

At the University of Leicester, Department of Geology I thank my supervisor Dr Gawen Jenkin for providing insight and inspiration. The late Dr Tim Brewer for guidance and positive encouragement in the early stages of this work and the many technical and academic staff who have provided their help and support. In the department of Cancer Studies and Molecular Medicine I would like to thank Dr George, D Jones and Dr Karen Bowman for making it possible to employ the Comet Assay in this research and providing help and guidance throughout. Dr Rob Britton for helping me with a plethora of challenges and Dr Chris Harrington for his involvement during the early stages of this project.

Several other people and organisations have helped with particular aspects of this work and are specifically acknowledged where relevant at the end of chapters 4-7.

The British Geological Survey University Funding Initiative (BUFI) for funding this research and providing structured support throughout.

Thanks to my family (Mum, Dad, Phil and Jen) and friends, and finally thanks to Vicky Booth for not only putting up with me but being constantly supportive and understanding.

THANK YOU ALL!

Contents

List of Figures

List of Tables

List of abbreviations

AB	arsenobetaine
AC	arsenocholine
Ag	silver
As	arsenic
ASTDR	Agency for Toxic Substances and Disease Registry
Au	gold
As^{III}	arsenite
As^{V}	arsenate
BAF	bioaccumulation factor
CCT	collision cell technology
Cd	cadmium
CEPA	Canadian environmental protection act
CLEA	contaminated land exposure assessment model
CRM	certified reference material
Cu	copper
Defra	Department for environment food and rural affairs
DGC	Devon Great Consols
DMAA	dimethylarsinoylacetate
DMA^{III}	dimethylarsinite
DMA^{V}	dimethylarsinate
DMAE	dimethylarsinoylethanol
DNA	deoxyribonucleic acid
EA	Environment Agency
Gy	Grey unit
HBF	human bioaccessible fraction
HDPE	high density polyethylene
HPLC	high performance liquid chromatography
IARC	International Agency for Research on Cancer
ICP-AES	inductively coupled plasma-atomic emission spectrometry
ICP-MS	inductively coupled plasma-mass spectrometry
in vitro	experimentation performed outside the living organism
in vivo	experimentation using whole living organism

kg	kilogram
l	litre
LOD	limit of detection
MA^{III}	methylarsonite
MA^{V}	methylarsonate
mg	milligram
min	minute
ml	milliliter
m/z	mass to charge ratio
nd	not detected
PBET	physiology-based extraction test
PEEK	polyetheretherketone
PTFA	polytetrafluoroethylene
Re	rhenium
RF	radio frequency
ROS	reactive oxygen species
SAM	S-adenosylmethionine
Sb	antimony
Se	selenium
SGV	soil guideline value
Sn	tin
SNIFFER	Scotland and Northern Ireland forum for environmental research
Sugar 1	glycerol arsenosugar
Sugar 2	phosphate arsenosugar
Sugar 3	sulfonate arsenosugar
Sugar 4	sulfate arsenosugar
Te	tellurium
TMAO	trimethylarsine oxide
µg	microgram
v/v	volume to volume
XAS	X-ray absorption spectroscopy
XANES	X-ray absorption near-edge spectroscopy

Chapter 1 - Introduction

1.1 Background and rationale

Arsenic is highly toxic and ubiquitous in the environment. The exposure of millions of people worldwide to As, primarily in drinking water, has been described as one of the most significant human health issues of the 21st century (Kapaj *et al.* 2006). The health implications of chronic exposure to arsenic in drinking water have been studied in some detail, with the majority of exposed populations located in poorer regions such as Bangladesh, South America and South East Asia. It is now clear that chronic exposure to arsenic in drinking water can cause serious health problems including increases in various cancers, peripheral vascular disease and skin keratoses (Karagas *et al.* 2002, Yoshida *et al.* 2004). In addition to the consumption of contaminated drinking water, significant exposure can arise from the consumption of contaminated food, inhalation of dust and ingestion of contaminated soil (Oomen 2002).

In developed countries such as the UK, exposure to As contaminated drinking water is not significant due to strict regulatory guidelines (DWI 2008). However, in areas such as Devon and Cornwall where historic mining activities have led to extensive contamination of the environment with arsenic, soil ingestion is thought to represent a significant source of exposure (Oomen 2002). The potential for human exposure to arsenic in these areas has been highlighted for decades (Johnson and Farmer 1989), yet the issue has largely been ignored (Rieuwerts *et al.* 2006). There is now growing concern regarding the potential health effects in populations exposed to arsenic in these areas due to increasing evidence of exposure (Johnson and Farmer 1989, Kavanagh *et al.* 1998).

Human exposure to contaminated soils and the resulting health risks is an inherently complex and contentious issue. Quantifying the total amount of arsenic in the soil provides an understanding of the extent of the contamination problem but is not directly relevant to the health risks of exposure to the soil. The toxicity of arsenic is, to a large extent, species dependent. Whilst several inorganic and some organic species are highly toxic (Kitchin 2001), other commonly occurring organic species such as arsenobetaine and arsenocholine are reported to be of only minor toxicological significance (Gebel 2001), making consideration of speciation important to the understanding of health risks associated with exposure.

Consideration of a contaminant's bioaccessibility is also important to understanding exposure associated risk (Intawongse and Dean 2006). The bioaccessible fraction of arsenic in soil can vary widely depending on soil pH, redox potential and to which mineral phases arsenic is bound (Beak *et al.* 2006). There is now increasing interest in models that aim to simulate human bioaccessibility of soil contaminants. However, uncertainties as to whether these models provide realistic and reproducible estimations of bioaccessibility have hindered their incorporation into the contaminated land risk assessment process.

Ecosystem indicator species such as earthworms have been employed as a tool in assessing soil contamination, in particular using bioaccumulation as a guide to a contaminants bioavailability (Langdon *et al.* 2003, Marino and Morgan 1999). Whilst many studies have investigated the effects of soil contamination on earthworms (Brousseau *et al.* 1997, Qiao *et al.* 2007, Saint-Denis 1999, Spurgeon *et*

al. 2003), such ecotoxicological studies have not been adopted in better understanding the risks to human health at arsenic contaminated sites. Whilst not directly linked to potential human health effects, the use of sentinel species such as earthworms may provide a useful surrogate to human studies at contaminated sites. The use of sentinel species as a complimentary line of evidence in assessing risk at arsenic contaminated sites requires further investigation.

Biomonitoring of human populations is potentially very useful in providing quantitative estimates of exposure to arsenic at contaminated sites. Urine analysis has been employed as a biomarker of exposure in residents living close to former mine sites in Devon and Cornwall revealing higher levels of As than observed in control populations (Johnson and Farmer 1989, Kavanagh *et al.* 1998). The residence time of As in urine is only around 24 hours (Slotnick *et al.* 2007) making this biomarker not ideal for assessing exposure from soil where ingestion is likely to be sporadic. Urinary analysis is also more easily confounded by dietary exposure to organic arsenic, such as arsenobetaine in seafood, which is readily excreted in urine post consumption. Therefore alternative biomarkers that provide a longer-term measure of exposure such as hair and nails might be more appropriate for assessing exposure to arsenic contaminated soils and should be explored.

1.2 Aims

The overall aim of this thesis is to assess an integrated, multi-disciplinary approach towards investigating the human health risks of arsenic contaminated soils. This is attempted via the assessment of several available tools, specifically to:

- Investigate the interrelatedness of soil total As, the human bioaccessible As fraction in the soil and As bioaccumulation in earthworms as complementary tools in understanding risk at arsenic contaminated sites.
- Examine the toxicity of contaminated soils from a former mine site to native and non-native earthworms to assess both the relative toxicity of soils with varying levels of arsenic contamination and the possible adaptation of native earthworms to high levels of arsenic contamination.
- Determine the As species present in the contaminated soils and assess As biotransformation in earthworms with respect to potential toxicity and adaptation to toxicity.
- Quantify As exposure levels in residents living close to a highly contaminated former mine site in the southwest UK, using a suitable biomarker of exposure and investigate methods to assess a relationship between the observed levels of arsenic in the biomarker and levels of arsenic in the surrounding environment.

1.3 Outline

This thesis is a multidisciplinary study of arsenic contaminated soils in terms of human exposure and environmental toxicology.

Arsenic is a ubiquitous element with a long and fascinating history, complex chemistry and wide ranging adverse health effects. **Chapter 2** provides some background information regarding the history, chemistry, occurrence, abundance, and toxicity of arsenic. The location of the study site Devon Great Consols (DGC), where all sampling was carried out, is also detailed with a brief description of the geology and history of this former arsenic mine.

In undertaking this multidisciplinary study several analytical techniques were employed:

- ICP-MS and HPLC-ICP-MS, both well established analytical techniques for total element and speciation analysis respectively in geological and biological samples.
- Physiology-based extraction test (PBET). PBETs are increasingly employed in risk assessment to gauge the human bioaccessibility soil contaminants.
- The Comet Assay, a bio-analytical technique for assessing DNA damage in single cells from exposed organisms.

Chapter 3 provides details of these techniques in terms of practical aspects, scope and limitations.

The human health risks of arsenic contaminated soils is an inherently complex issue. The consideration of many inter-related components is required for a more holistic understanding of the problem. Quantifying the total amount of As in soil alone is the first step, but this information alone is insufficient. Estimation of the arsenic fraction bioaccessible to humans using PBETs is a more pragmatic approach to understanding risk. Soil organisms such as earthworms are important in the cycling and transformation of arsenic in the terrestrial environment. These sentinel species can be utilised to provide an estimation of As mobility. **Chapter 4** examines the interrelatedness of total As, As bioaccessibility and bioaccumulation by earthworms as multiple lines of evidence in understanding the risk to human health and the ecosystem at arsenic contaminated sites.

Earthworms are commonly employed as ecosystem indicator species in ecotoxicological studies of soil contaminants. At DGC, the native earthworm species *L. rubellus* and *D. rubidus* appear to have developed a resistance to soils highly contaminated with arsenic. **In Chapter 5** this resistance is investigated by assessing DNA damage in native earthworms collected from the site and non-native earthworms exposed to DGC soil in laboratory mesocosms.

The toxicity of arsenic is determined in part by its chemical form. By investigating the presence of different arsenic compounds in soil we can better understand the potential risks of exposure but also begin to build a picture on how the arsenic interacts with biological systems following uptake. **Chapter 6** examines the speciation of arsenic using HPLC-ICP-MS in the soils, earthworms and earthworm

excreta (casts) to build an understanding of how earthworms at DGC are able to reside in soils highly contaminated with arsenic.

Perhaps the most difficult aspect of understanding risks associated with arsenic contaminated soils is quantifying the extent to which people may or may not be exposed. Biomarkers of exposure are commonly employed to monitor arsenic in populations exposed through drinking water, but such estimation is more difficult when water is not the source of exposure. **Chapter 7** examines the use of toenails to monitor exposure to arsenic in residents at DGC. Along with the total levels of arsenic in the toenails, the amount of external contamination and As speciation was determined to help understand the extent of exposure, the levels of environmental As and how arsenic is transformed and stored following exposure.

Chapters 4 -7 are written as independent sections. **Chapter 8** amalgamates the observations and conclusions from these chapters into a discussion of the arsenic problem at Devon Great Consols as a whole. In particular, how the different aspects of this work, total elemental analysis, bioaccessibility, sentinel organisms and exposure biomarkers can be employed as multiple lines of evidence in a more holistic understanding of the risks associated with exposure to arsenic contaminated soils.

1.4 Work previously published

This thesis is submitted based on the following papers that are published or have been submitted for publication:

Chapter 4: Button, M., Watts, M.J., Cave, M., Harrington, C.F. and Jenkin, G.R.T. (2009b). Earthworms and *in vitro* physiologically-based extraction tests: complementary tools for a holistic approach towards understanding risk at arsenic-contaminated sites. *Environmental Geochemistry and Health*, 31, 273.

Chapter 5: Button, M., Jenkin, G.R.T., Bowman, k.J., Brewer, T.S., Harrington, C.F., Jones, G.D.D. and Watts, M.J. (2008). DNA damage in earthworms from highly contaminated soils: Assessing resistance to arsenic toxicity using the Comet Assay. (Under review in Mutation Research - Genetic Toxicology and Environmental Mutagenesis).

Chapter 6: Button, M., Jenkin, G.R.T. and Watts, M.J. (2008). Arsenic biotransformation in earthworms from highly contaminated soils. (Under review in the Journal of Environmental Monitoring)

Chapter 7: Button, M., Jenkin, G.R.T., Harrington, C.F. and Watts, M.J. (2009a). Human toenails as a biomarker of exposure to elevated environmental arsenic. *Journal of Environmental Monitoring*, 11, 610.

The following publications were also produced as a result of the work undertaken for this thesis and are included as appendices:

Appendix A: Button, M and Watts, M.J. (2008). Extraction and measurement of arsenic species in contaminated soils by HPLC-ICP-MS. *British Geological Survey*, IR/08/050.

Appendix B: Watts, M.J., Button, M., Brewer, T.S., Jenkin, G. R.T., and Harrington, C. F. (2008). Quantitative arsenic speciation in two species of earthworm from a former mine site. *Journal of Environmental Monitoring*, 10, 753.

Chapter 2 - Background

2.1 Arsenic

Throughout history arsenic has been characterised by its notoriety and synonymy above any other substance as a poison. The lack of colour, odour or taste in certain arsenic compounds, its ubiquitous distribution in the environment and the fact that poisoning causes few symptoms prior to death, and is therefore difficult to detect, are all factors that led to its widespread use as a poison. The first preparations of elemental arsenic are widely attributed to Albertus Magnus at around 1250 AD when he obtained pure arsenic by heating the oxide (As_2O_5) with soap (Buchanan 1962). However, the existence of arsenic containing compounds such as orpiment (As_2S_3) and realgar (As_4S_4) were known in ancient times. The name arsenic is derived from the ancient Greek word '*arsenikon*' meaning potent (Bentley and Chasteen 2002). Hippocrates recommended the use of realgar in the treatment of ulcers in the 5th century BC, a recommendation repeated later by Galen in 2nd century AD (Waxman and Anderson 2001). Since then arsenic has played an intricate role in human society with wide-ranging applications covering pigmentation of cosmetics and paints, to its use in the agricultural and electronics industry.

2.2 Chemistry of arsenic

Arsenic is a metalloid with a complex chemistry demonstrating the properties of both metals and non-metals. Arsenic is found in group 15 of the periodic table along with nitrogen, phosphorus, bismuth and the other group 15 metalloid antimony. The electronic configuration of arsenic is [Ar] $3d^{10}$ $4s^2$ $4p^3$ with 5 orbiting electrons in its outer shell. Arsenic is mono-isotopic and exists in the oxidation states –III (arsenides), V (arsenates), III (arsenites) and 0 (arsenic) although the existence of

arsenic in the environment in the –III oxidation state has been questioned (Cullen and Reimer 1989).

Arsenic exists as a solid in its elemental state but is most commonly found in compounds containing sulphur, either in isolation or alongside multiple other metals (Boyle and Jonasson 1973). Arsenic behaves in a manner chemically similar to its group 15 predecessor phosphorus, forming colourless crystalline oxides which are hygroscopic and readily soluble in water forming weak acids. Elemental arsenic demonstrates the property of allotropy whereby it is found to exist in several different forms including:

Grey arsenic - a steel grey lustrous metallic substance.

Black arsenic - formed by the slow condensation of arsenic vapour

Yellow arsenic - formed by the rapid condensation of arsenic vapour.

The densities of these allotropes vary from yellow arsenic at 1.97 g/cm^3 increasing to the more common 5.73 g/cm^3 of grey arsenic (Buchanan 1962).

The arsenic allotropes are mostly stable in dry air but undergo oxidation in the presence of moisture to form arsenious and arsenic oxides. Sublimation of arsenic occurs when heated at atmospheric pressure, an important property in the recovery of arsenic from arsenic containing ores, melting only when heated under pressure. Table 2.1 illustrates the physical and chemical properties of arsenic.

Table 2.1: The physical and chemical properties of arsenic

Arsenic	Properties
Atomic number	33
Atomic mass	74.9216 g mol^{-1}
Group	15
Period	4
Specific gravity	1.97 - 5.73 g cm^{-3}
Melting point	817°C (28 atm)
Sublimation point	613°C
Electronegativity	2.18 (Pauling) 2.2 (Allred-Rochow)
Electrons per shell	2, 8, 18, 5

2.3 Arsenic in the environment

Arsenic is a ubiquitous element that ranks 20th in abundance in the Earth's crust, 14th in the world's oceans and 12th in the human body (Bissen and Frimmel 2003, Mandal and Suzuki 2002). The human body is reported to contain an average of 10 - 20 mg of arsenic (Elsom 1992). The occurrence and distribution of arsenic in the environment is one of the most significant human health issues of the 21st century, with millions of individuals facing chronic exposure to elevated levels of arsenic via contaminated waters, soils and foodstuffs. The problem of environmental arsenic contamination ranges in scale from local to regional, affecting both developed and developing countries. The mobilisation of arsenic under natural conditions such as weathering and erosion, biological activity and volcanic emissions are the biggest cause of arsenic-related environmental problems. Anthropogenic impacts, although less widespread are also significant, particularly in areas of mining activity, fossil fuel combustion and intensive agriculture (Bissen and Frimmel 2003, Smedley and

Kinniburgh 2005). This section will look in summary at the sources behaviour and levels of arsenic that occur in the environment.

2.3.1 Minerals

Arsenic occurs naturally in over 200 different mineral forms of which 60% are arsenates, 20% sulphides and sulphosalts and the remaining 20% including arsenides, arsenites, oxides, silicates and elemental arsenic (Mandal and Suzuki 2002) consisting mainly of ore minerals or their alteration products (Smedley and Kinniburgh 2005). Arsenopyrite (FeAsS) is the most abundant As containing mineral (Mandal and Suzuki 2002) occurring most commonly in mineral veins in association with sulphide mineralisation (Smedley and Kinniburgh 2005). Other important arsenic bearing minerals include orpiment (As_2S_3), realgar (AsS), cobaltite (CoAsS) and niccolite (NiAs) (Bissen and Frimmel 2003, Smedley and Kinniburgh 2005). High arsenic concentrations are also found in many oxide minerals and hydrous metal oxides either as part of the mineral structure or as adsorbed species. Concentrations in iron oxides can also reach several percent by weight particularly where they are formed as the oxidation product of primary iron sulphides (Smedley and Kinniburgh 2005).

2.3.2 General abundances

Arsenic is less abundant in minerals than Cu and Sn but more so than Cd, Au, Ag, Sb, and Se. The total amount of arsenic in the Earth's crust is estimated to be around 4.01×10^{16} tonnes (Bissen and Frimmel 2003, Mandal and Suzuki 2002, Matschullat 2000). The average As content in the upper continental crust is estimated to be around 5 mg kg^{-1} when based on surface exposures and between around 1 and 5 mg kg^{-1} based on sedimentary data (Rudnick and Gao 2004). The total crustal

concentration of As, based on upper, middle and lower crustal compositions is estimated to be around 2.5 mg kg^{-1} (Rudnick and Gao 2004). The average concentration of As is reportedly 17 µg kg^{-1} in seawater, 13 mg kg^{-1} in shale, 20 mg kg^{-1} in pelagic clays, 140 mg kg^{-1} in manganese nodule and 15 mg kg^{-1} in marine organisms (Yuan-Hui 1991).

2.3.3 Soils

Arsenic occurs naturally in soils, particularly where pedogenesis occurs over underlying geology with arsenic containing minerals and through a variety of anthropogenic inputs. The average arsenic content of uncontaminated soils are reported in the range 1 – 40 mg kg^{-1} varying with underlying geology, climate, organic/inorganic components of the soil and redox potential (Mandal and Suzuki 2002, Smedley and Kinniburgh 2005). In the UK soil guideline values (SGVs) are prescribed by the Department for environment food and rural affairs (Defra) and the Environment Agency (EA) as indicators of potential unacceptable risk. The current SGV for As is set at 20 mg kg^{-1} (Defra 2002a). Large areas of Devon and Cornwall have background levels of arsenic in soil well above the current SGV due to both the underlying geology and contamination from historic mining activities. This makes the application of such guidelines unrealistic in certain areas.

Anthropogenic soil arsenic inputs are derived from the application of arsenic containing pesticides and herbicides, contamination by industrial and mine waste, irrigation with arsenic contaminated groundwater and atmospheric deposition from the local combustion of fossil fuel and mineral smelters (Mandal and Suzuki 2002).

Arsenate (As^{V}) and arsenite (As^{III}) are the primary arsenic species found in soils. The prevalence of either species is a function of redox potential, pH and microbial activity (Moore 1988). Under oxidising conditions arsenic is present in soils in the As^{V} oxidation state whilst in reducing conditions As^{III} predominates (Figure 2.1). The sorption of arsenic onto specific minerals in the soil is controlled by the content of amorphous iron, aluminium hydroxides, clay minerals and pH (Masscheleyn *et al.* 1991).

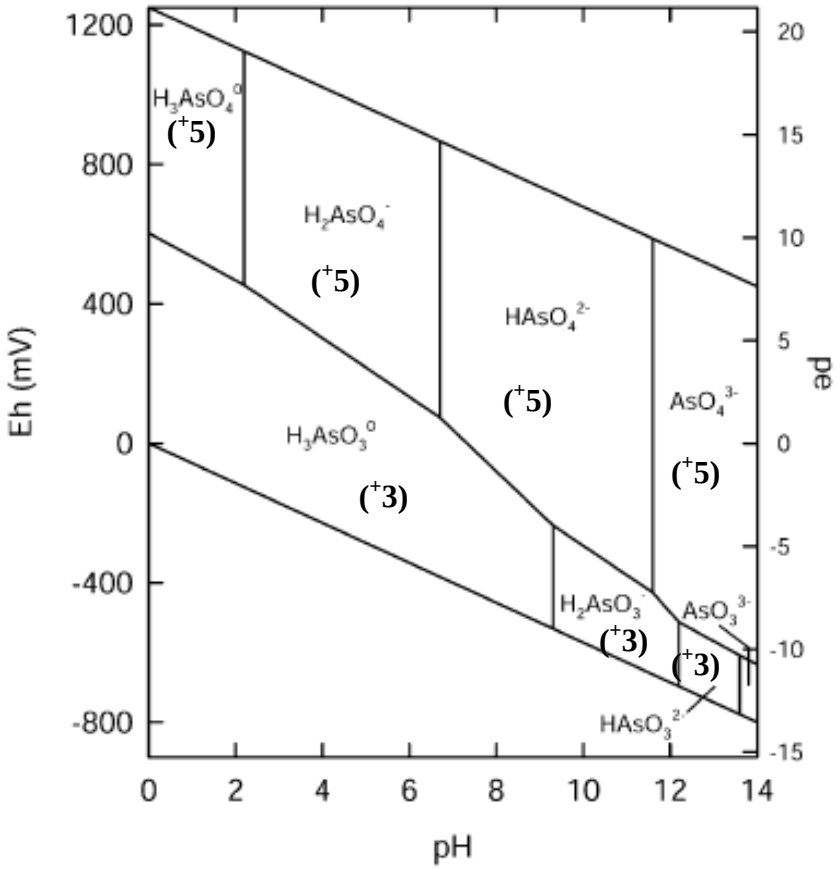


Figure 2.1: Eh-pH diagram for aqueous As species in the system As-O_2-H_2O at 25 ºC and 1 bar total pressure. Brackets denote oxidation state of As based. Adapted from Smedley and Kinniburgh (2002).

2.4 Toxicity of arsenic and its compounds

The toxicity of arsenic and arsenic containing compounds is an extensive issue complicated by the fact that arsenic can exist in different oxidation states and in a

plethora of both organic and inorganic compounds (DEFRA and EA 2002). A number of contemporary review articles cover the subject at length providing a detailed insight into the issue (ASTDR 2005a, Basu *et al.* 2001, CEPA 1993, Gebel 2001, Kapaj *et al.* 2006, Tchounwou 2004). The aim of this section is to summarise the current understanding of arsenic toxicity, modes of carcinogenicity and the role of metabolism.

Arsenic has been listed as a human carcinogen since 1980 by the International Agency for Research on Cancer (IARC) and is unique as the only known human carcinogen for which there is adequate evidence of carcinogenic risk both by inhalation and ingestion (Kapaj *et al.* 2006). Arsenic is now recognised as a carcinogen of the human skin, bladder and lung (Lantz and Hays 2006). The toxicity of arsenic containing compounds varies according to oxidation state, chemical form (organic/ inorganic), physical state and with factors such as particle size, solubility and uptake/ elimination rates (ASTDR 2000). Figure 2.2 illustrates several commonly occurring forms of arsenic in biological systems.

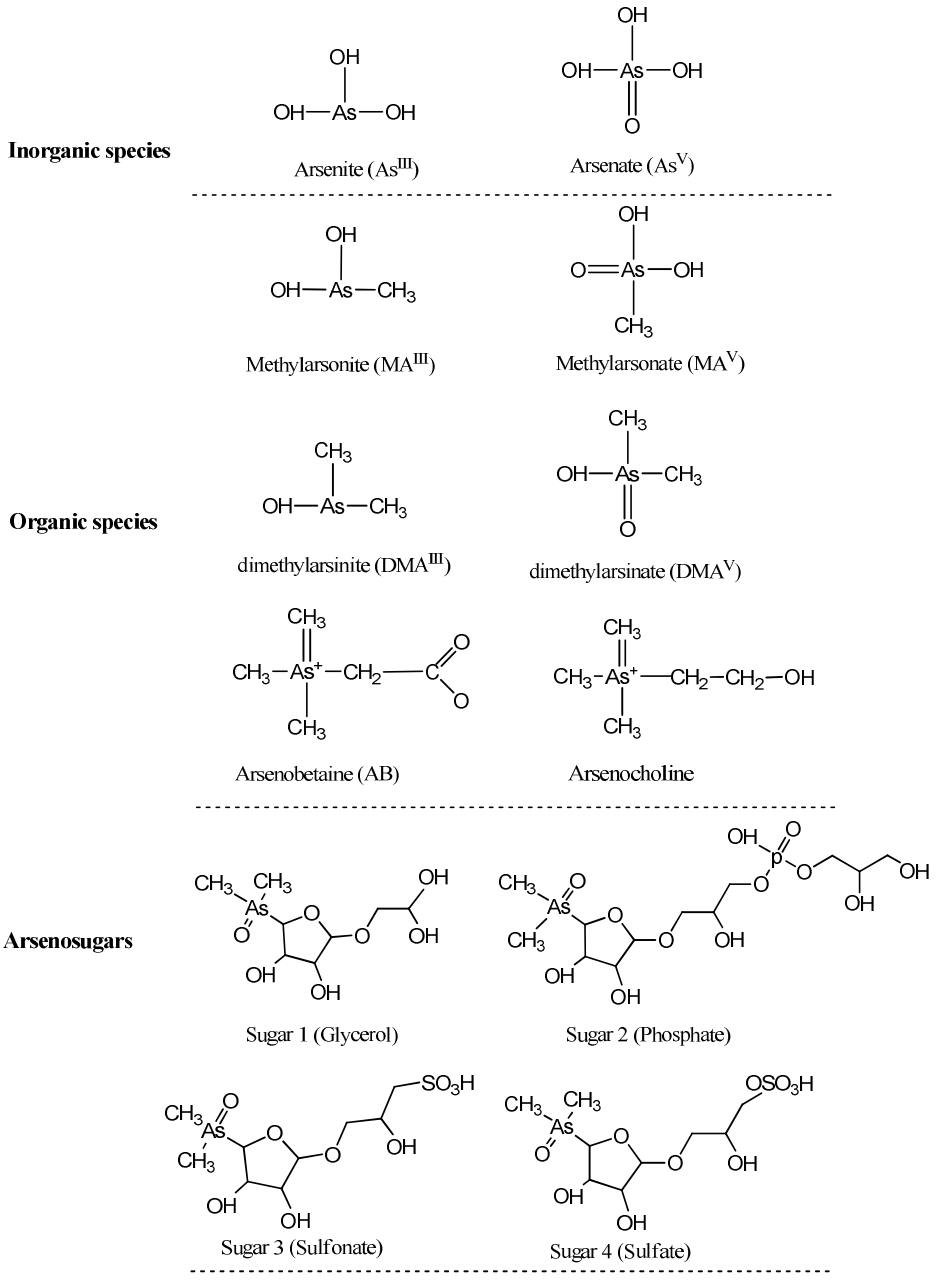


Figure 2.2: Some of the more common arsenic species found in biological systems in approximate order of decreasing toxicity. Adapted from Francesconi and Kuehnelt (2004).

2.4.1 Metabolism of arsenic

The pathway for inorganic arsenic metabolism in humans involves alternating steps of reduction of As^{V} to As^{III} followed by oxidative methylation of As^{III} to form the methylated arsenic metabolites. In brief As^{V} is first reduced to As^{III} through a process involving glutathione. As^{III} is then sequentially methylated, initially to

methylarsonate/methylarsonite ($MA^{V/III}$) then to dimethylarsinate/dimethylarsinite ($DMA^{V/III}$) via oxidative/reductive methylation steps (Adair *et al.* 2005, Gebel 2001). Until recently inorganic arsenic, particularly As^{III}, was considered the most toxic chemical form with the methylation of inorganic arsenic considered a detoxification step. This was due to an understanding based on methylated compounds being less acutely toxic, less reactive with tissue macro molecules and having increased elimination rates than inorganic compounds (Gebel 2001). However, recent research on the metabolism and biological effects of arsenic has forced our understanding on the role of metabolism in the mediation of arsenic toxicity to be reassessed (Tchounwou 2004). There is now strong evidence to suggest that the biomethylation of arsenic, in particular the production of trivalent methylated arsenic metabolites, is a process that activates arsenic as both a toxin and carcinogen (Kitchin 2001, Styblo *et al.* 2002). Methylated trivalent arsenicals (MA^{III} and DMA^{III}) are reported to be less stable and more genotoxic than their pentavalent equivalents (MA^{V} and DMA^{V}), highly reactive and at least as toxic to mammalian cells in culture as As^{III} (Aposhian 2000, Le *et al.* 2000, Mass 2001, Moore *et al.* 1997). Adair *et al.* (2005) suggest that methylated arsenicals containing As^{III} are the most reactive exceeding arsenite in both cytotoxic and genotoxic potency. In addition, two methylated arsenicals containing As^{V} (DMA^{V} and trimethylarsine oxide: TMAO) have been demonstrated as carcinogens in rats (Shen *et al.* 2003, Wei *et al.* 1999) adding further support to the idea that the methylation of inorganic As is an activation step.

2.4.2 Carcinogenicity

The precise cellular mechanism by which arsenic induces cancer is still unknown Table 2.2 outlines the possible modes of carcinogenicity proposed in the literature to

date. It seems a consensus has been reached that the mechanisms outlined in Table 2.4 are not mutually exclusive, with a strong possibility of concurrent or sequential operation (Goering *et al.* 1999). Tchounwou *et al*. (2004) suggests strongest evidence, both in experimental systems (animal and human cell) and in human tissues, for chromosomal abnormalities, oxidative stress and altered growth factors as the principal modes of carcinogenicity with less support for the remaining mechanisms, particularly from *in vivo* studies with lab animals and *in vitro* studies with cultured human cells. There is therefore a strong case for arsenic as a co-carcinogen and/or a promoter or progressor of carcinogenesis.

Table 2.2: Proposed mechanisms of arsenic carcinogenesis

Proposed mode of arsenic carcinogenesis	References
Chromosomal abnormalities: arsenicals are effective for clastogenicity (chromosome damage). Arsenic induced chromosomal aberrations reported include micronuclei and sister chromatid exchanges	(Goering *et al.* 1999, Gonsebatt *et al.* 1997, Kitchin 2001)
Oxidative stress: Reaction of minor trivalent arsenicals with molecular oxygen form a $(CH_3)_2As$ radical and superoxide anion. This radical can then add another molecule of molecular oxygen to form the $(CH_3)_2AsOO$ hydroxyl radical reactive oxygen species (ROS). Exposure of DNA to these radicals or ROS can then result in single strand breaks.	(Ahmad *et al.* 2000, Goering *et al.* 1999, Kitchin 2001)
Altered DNA repair: Trivalent arsenicals demonstrate the capability for protein binding, conformational alteration of protein structure and enzymatic inhibition causing disruption to DNA repair enzymes.	(Goering *et al.* 1999, Kitchin 2001)
Altered DNA methylation: Hyper/ hypomethylation of DNA in arsenic exposed cells could commit cells towards a carcinogenic pathway via altered gene expression	(Goering *et al.* 1999, Kitchin 2001, Mass 2001)
Altered growth factors: Arsenic exposure could increase production of ROS, activation of transcription factors and over secretion of proinflamatory and growth promoting cytokines, resulting in increased cell proliferation and finally carcinogenesis	(Kitchin 2001) (Vega *et al.* 2001)
Enhanced cell proliferation: Exposure to arsenite results in increased ornithine decarboxylase (ODC) activity, a biomarker for cell proliferation	(Goering *et al.* 1999, Kitchin 2001, Tchounwou 2004)
Enhanced cell promotion / progression: Evidence for the promotion of carcinogenesis in skin, lung bladder, kidney, liver and thyroid may be due to increased cell proliferation rates	(Tchounwou 2004) (Kitchin 2001) (Goering *et al.* 1999)
Suppression of p53: Decreased p53 protein levels and function (the "guardian of the genome") after exposure to arsenite could cause mutations to accumulate at a faster rate in exposed organisms leading to carcinogenesis.	(Kitchin 2001)
Gene amplification: Arsenic induced gene amplification has been demonstrated in mice leading to decreased cellular survival	(Kitchin 2001) (Lee *et al.* 1988)

2.5 Study site

2.5.1 Location

Devon Great Consols (DGC) is one of numerous former mining sites in Southwest England, formed in the early 19th century from the consolidation of the adjacent mines Wheal Maria, Wheal Fanny, Wheal Anna Maria, Wheal Josiah and Wheal Emma (Klinck *et al.* 2002). DGC lies on the east bank of the River Tamar in the Tavistock district of Devon (GR: 426 735), as illustrated in figure 2.3a. The mines of DGC lie east to west along the main lode of the area. The mine tailings are clearly visible in centre of figure 2.3b, surrounded by coniferous woodland and agricultural land. Although it has been many decades since mining activity ceased at DGC the high levels of arsenic in the mine wastes have prevented vegetation re-colonising the area worst affected by the contaminated mine wastes.

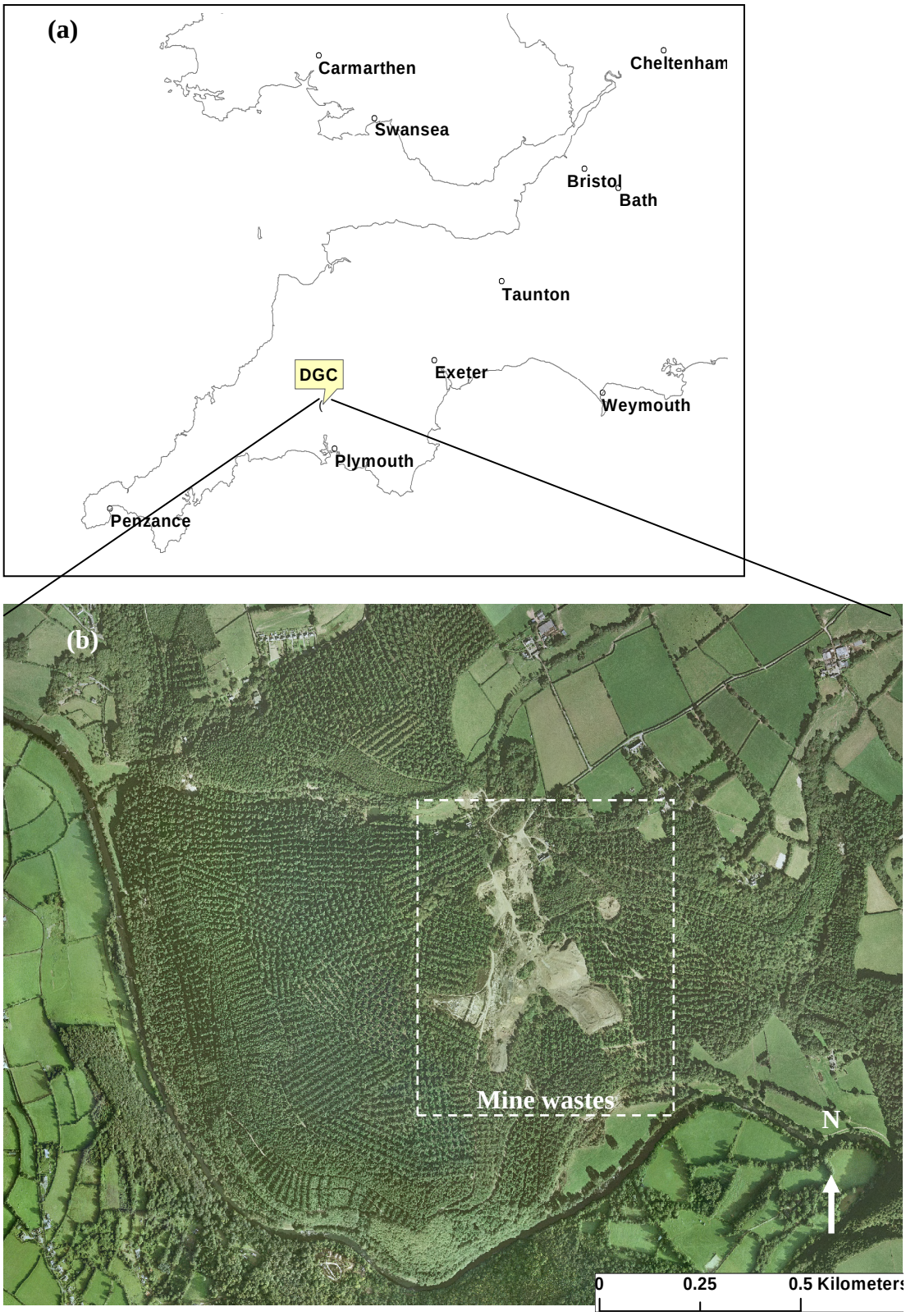


Figure 2.3a/b: Geographical location of DGC in the southwest UK and aerial view of mine wastes and surrounding woodland/ agricultural land. Image courtesy of the British Geological Survey.

2.5.2 Geology

Mineralisation at DGC is influenced by an igneous body known as the Hingston Down Granite, one of eleven satellite intrusions and six major plutons comprising the Cornubian Batholith of southwest England (Figure 2.4a). The batholith underlies the counties of Cornwall and Devon, running down the axis of the peninsula for a length in excess of 200 km (Klinck *et al.* 2002). Water, boron and other volatiles were abundant in the granites making up the batholith (Charoy 1986) enabling them to transport large quantities of metals as halogen complexes, primarily chlorine, and as complex silicic acids. Continuing crystallisation led to the concentration of incompatible elements and the subsequent formation of a series of residual volatile reservoirs in the apical sections of the various plutons (Dines 1956). Successive increases in vapour pressure led to hydraulic fracturing and the formation of high temperature greisen veins, breccia pipes and eventually, in conjunction with extensional and strike-slip faulting, the formation of the main stage lodes of the region (Le Boutillier 2001). The lodes are mainly east-west in trend, with the most productive lodes located in the metamorphic aureole rather than the granite itself (Klinck *et al.* 2002).

At DGC the main lode is heaved up to 225m in a right lateral sense by a cross course structure, as illustrated in figure 2.4b. Copper and arsenic were by far the most economically viable products of the area. The principal minerals of economic importance at DGC were arsenopyrite (FeAsS), chalcopyrite ($CuFeS_2$) and Galena (PbS). Other local ores were cassiterite (SnO_2) and stannite (Cu_2FeSnS_4) (Farago and Kavanagh 1999).

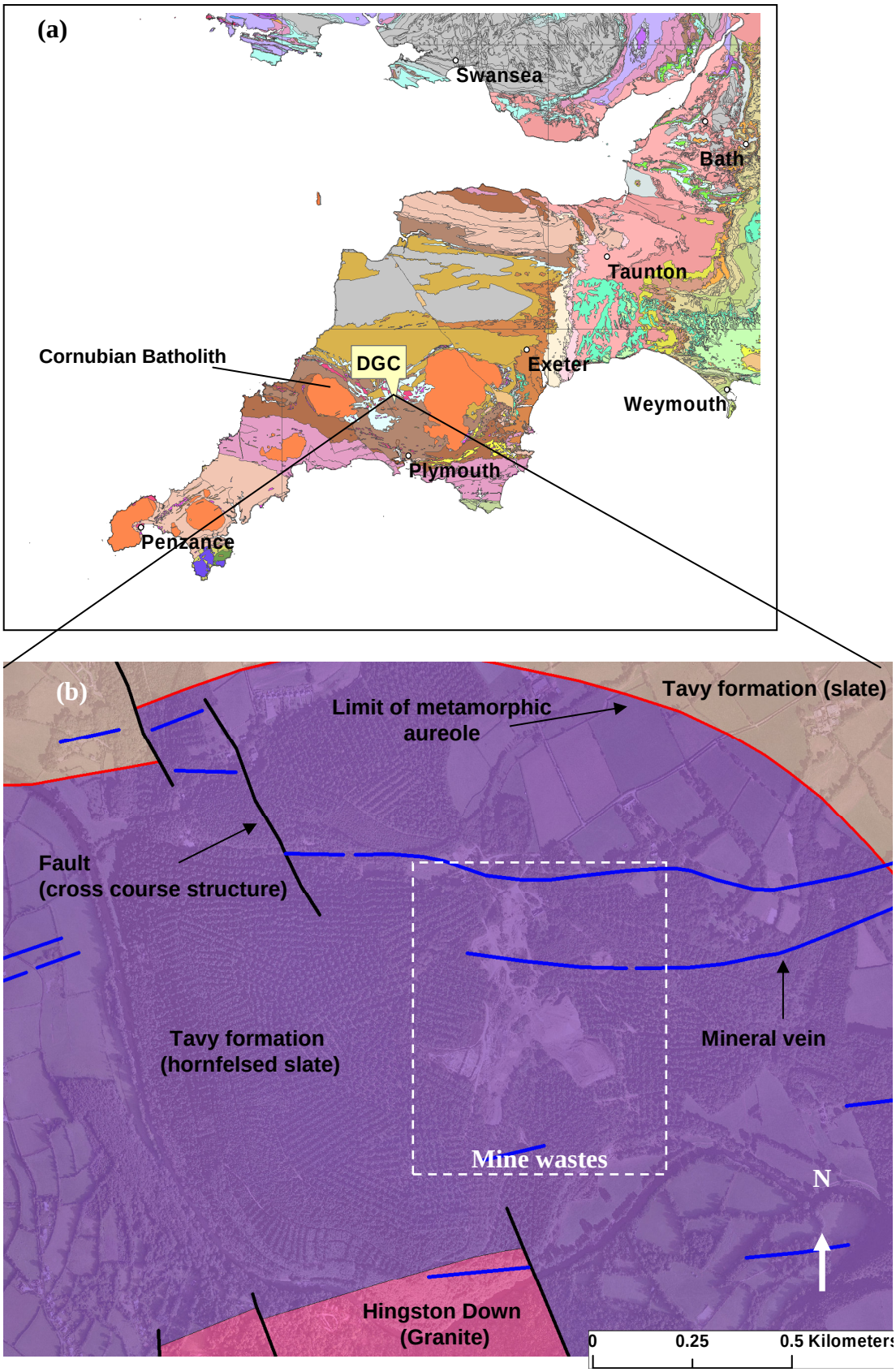


Figure 2.4: Devon/ Cornwall 1:250,000 bedrock (a) and 1:50,000 bedrock geology at DGC (b). Digital maps supplied by the British Geological Survey (DiGMapGB),© NERC.

2.5.3 History:

Throughout the 19th century DGC was the largest and richest mine in the Tamar valley. Copper production was the central activity of the mine in the early operative years until 1868 when arsenic was first commercially sold. 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 (Klinck *et al.* 2002). The total refined arsenic output from 1844 to 1902 is estimated at 72,279 tons (Barton 1964). Declining arsenic prices and loss of markets saw mining activity at DGC cease in 1930. Figure 2.5 is used to summarise the history of mining at DGC. Mining activities in the South-West, UK have caused extensive contamination of the surrounding environment with As. An area covering approximately 700 km^2 has been affected (Abrahams and Thornton 1987). Most of the contaminated area is agricultural encompassing small towns and villages (Farago and Kavanagh 1999).

Date	Activities
1844	Extensive exploration of Blanchdown wood, copper rich ore found
1845	Entire complex becomes known as DCG
1849	River Tamar used for power and transport
1856	£1.4m return on initial 12 years ore sales
1858	Railway established to Morwellham Quay with major dock excavation
1865	420,000 tons of copper ore mined and sold
1866	Construction of arsenic works near Wheal Anna Maria agreed
1868	First commercially sold arsenic
1869	160 tons of refined arsenic per month now produced (50% of worlds supply)
1871	Wheal Maria arsenic production works completed with 2,500 t/yr capacity
1888	Around 240 tons of arsenic produced per month
1891	Total arsenic produced is 5883 cwt barrels
1899	Arsenic production down to 150 tons per month
1901	Sharp decline in arsenic prices, production ceased
1903	Mine abandoned
1915	Arsenic extraction reinitiated on upper levels of Wheal Fanny
1919	Railway re-laid between Wheal Anna Maria and Bedford united mines to south
1922	Two new calciners employed as supply and quality of ore deteriorates
1925	Mining suspended as price slumps
1930	Mining activity at DGC finally ceased

Figure 2.5 Summary of mining history at DGC. Adapted from Klinck *et al*. (2002).

Chapter 3 - Analytical techniques, challenges and solutions

3.1 Inductively Coupled Plasma Mass Spectrometry (ICP-MS)

Quadrupole ICP-MS was the principal analytical technique used in this research employing the Thermoelemental PQ ExCell and Agilent 7500 models. Since the introduction of the first commercial instrument in 1983, ICP-MS has become accepted as a powerful technique for elemental analysis. ICP-MS provides rapid, multi-element and multi-isotopic analysis, high sample throughput, detection limits at single parts per trillion or below for up to 60 elements in solution (Olesik 2000) and a wide analytical range. A quadrupole ICP-MS instrument comprises (1) sample introduction system, (2) ion source (ICP), (3) interface, (4) ion focusing system, (5) mass analyser and (6) detector (Figure 3.1).

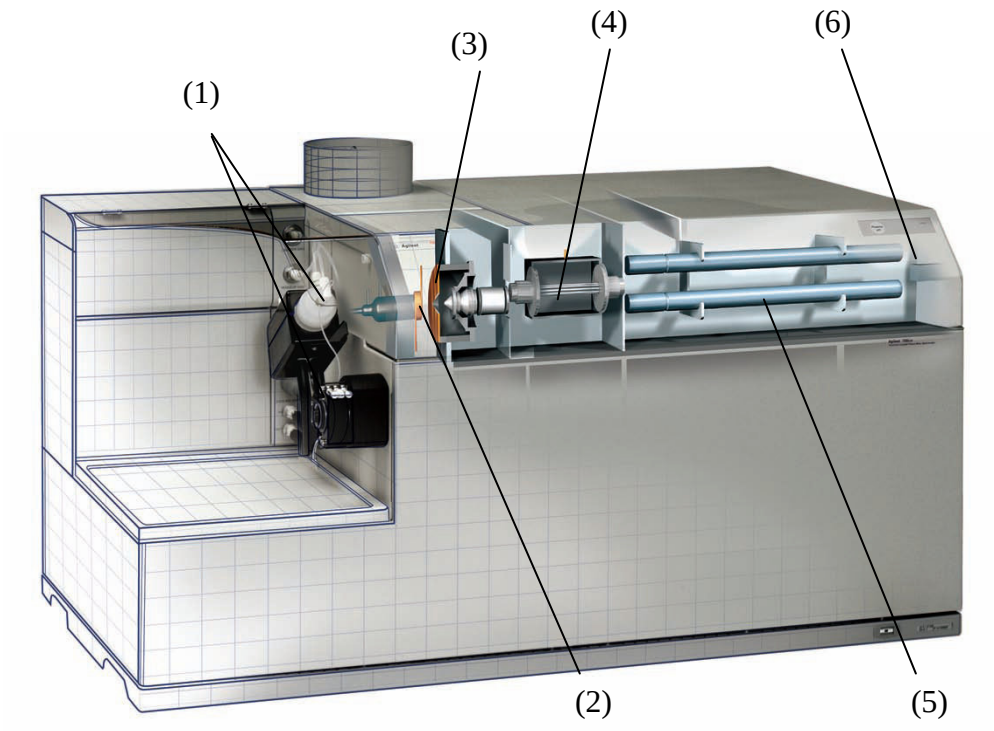


Figure 3.1: Diagram showing the components of a quadrupole ICP-MS instrument (Agilent 7500 Series). Image courtesy of Agilent, UK (www.chem.agilent.com).

3.1.1 Sample introduction

The ICP requires any sample to be introduced into the central channel gas flow as a gas, vapour or most commonly an aerosol. Most systems are fitted with an aerosol generating pneumatic nebuliser as standard. A peristaltic pump is used to deliver the sample to the nebuliser where a high velocity gas stream produces a fine droplet dispersion of the analyte solution. Larger droplets are removed by the spray chamber allowing only those below approximately 8 µm in diameter to pass on to the plasma (Jarvis *et al.* 2003). The selection of smaller droplets leads to inefficient transportation of the sample (1 to 2 % approx.) but is required firstly, because large droplets are not efficiently dissociated in the plasma and secondly, to smooth out pulses that occur during the nebulisation process caused by the peristaltic pump (Thomas 2008). The pneumatic nebuliser, of which there are many forms, remains popular due to its convenience, stability and ease of use with automated sample uptake devices.

3.1.2 Ion source (ICP)

The ICP consists of a glass torch, a radio frequency (RF) coil and an RF power supply used to generate and sustain the plasma. The ICP torch consists of a quartz tube through which an inert gas, usually argon, flows. The quartz tube is surrounded by the water-cooled RF induction coil. An intense oscillating magnetic field is developed around the coil. Ionisation of the flowing argon is initiated by a spark from a Tesla coil. Once the plasma discharge has been initiated, electrons in the plasma are accelerated by the oscillating magnetic field sustaining the plasma. Heating of the argon gas up to 8000 K is caused by collisions between electrons and argon atoms. On introduction into the plasma from the nebuliser, collisions between electrons and analyte atoms cause ionisation of the analyte (Vandecasteele and Block 1997). The

ionisation potential of an atom or molecule is described as the energy required to remove one mole of electrons from one mole of gaseous atoms or ions (McNaught and Wilkinson 1997). The majority of elements in the periodic table have first ionisation energies of < 8 eV. By comparison arsenic is reluctant to surrender an electron, with a first ionisation energy of 9.78 eV. Unstable elements like alkaline earth metals magnesium (Mg) and calcium (Ca), with ionisation energies of <6 eV are more easily ionised.

3.1.3 Interface

The role of the interface is to transport the analyte ions efficiently and consistently from the plasma to the mass analyser and to facilitate a pressure drop from atmospheric to a strong vacuum. The positively charged ions generated in the plasma at atmospheric pressure are extracted through two cones, typically made of nickel and maintained at a vacuum of 2 Torr using a rotary pump. The first cone is known as the sampler cone with an orifice diameter of 0.8 to 1.2 mm. The second cone is known as the skimmer cone with a smaller orifice of 0.4 to 0.8 mm (Thomas 2002a). To reduce the effects of the high temperature plasma on the cones the interface housing is water cooled and made from material that dissipates heats easily such as copper or aluminium (Thomas 2002a).

3.1.4 Ion optics

The function of the ion optics is to maximise transmission of positive ions from the skimmer cone into the mass analyser while minimising background noise. The ion optics consists of several electrostatically controlled lens components which steer and focus the analyte ions from the interface into the mass analyser. The ion lenses are

also used to separate ions from photons and residual neutral material. A well designed ion optic system will provide low background signal, good detection limits and a stable signal in real-world samples (Thomas 2002a).

3.1.5 Mass analyzer

The mass analyser or mass separation device is the region of the ICP-MS that separates ions according to their mass-to-charge ratio (m/z). Approximately 90 % of ICP-MS instruments use quadrupole–based systems. A quadrupole consists of four cylindrical or hyperbolic metal rods of equal length and diameter. By placing a direct current (dc) on one pair of rods and a radio frequency on the opposite pair, ions of a selected mass are allowed to pass through the rods to the detector while others are ejected from the quadrupole (Thomas 2002a). Since the plasma produces almost exclusively singly charged ions, the m/z ratio is equal to the mass of the element. By varying the current applied to the quadrupole the m/z values transmitted to the detector can be varied (Skoog and Leary 1991). The sensitivity by which an ICP-MS instrument can differentiate between m/z ratios is known as the mass resolution and can be defined as:

$$R = M / \Delta M$$

Where R is resolution, M is the mass of the isotope of interest and ΔM is the peak width of the isotope at 5% peak height. Most quadrupole mass analysers operate with an upper resolution of 400, which enables resolution of around 0.2 to 1 mass unit (O'Connor and Evans 2007), typical to both instruments used in this research.

3.1.6 Detector

Ions that pass through the quadrupole are counted by the instrument's detector. An electron multiplier type detector was fitted to both ICP-MS instruments used in this research. The detector converts the ions into electrical pulses which are then counted by its integrated measurement circuitry. The number of electrical pulses per unit time (count rate) is usually proportional to the number of analyte ions present in the ion beam. However, high ion count rates can overload the detector causing some ions to go undetected and leading to a non-linear response. This problem is overcome with the use of dual mode detectors, as used in both instruments in this research, that allow the measurement of higher count rates. Dual mode detectors use pulse counting at lower count rates (several million counts per second; CPS) then, on detection of higher count rates, switch to analogue mode measuring the current generated by the electron stream rather than the pulse derived from each individual ion impact (Agilent 2005). Quantification of trace elements in an unknown sample can then be carried out by comparing the ion signal to known calibration standards (Thomas 2002b).

3.2 Calibration

The available calibration methods suitable for elemental analyses can be divided into two categories, absolute or relative. Absolute methods produce a result that is directly traceable to SI units such as gravimetry (kg), titrimetry (moles) and coulometry (coulomb). All absolute methods are particularly sensitive to interferences making them best suited to pure samples rather than complex mixtures. Absolute methods are also generally unsuitable for the determination of trace level analytes, making them mostly unsuitable for elemental analyses. Relative methods on the other hand rely on the detection and quantification of the elemental species by comparison to a set of

calibration standards of a known concentration. Linear calibration graphs are the most practical method for use in analytical chemistry. They are obtainable by the measurement of only a few calibration standards and can be described by the following function:

$$y = mx + c$$

Where y = signal response, m = slope of the graph, x = concentration of working solution (e.g. $\mu g\ ml^{-1}$) and c = intercept on the Y-axis (Dean 1997).

The steepness of the slope represents the sensitivity of the instrument. Higher slopes give a higher signal/background ratio and therefore result in increased precision of the calibration method for a given percentage of analytical noise. Linear calibrations are not always obtained due to either non-linearity of instrument response or poor technique. The fitting of non-linear calibration curves requires the use of non-linear regression analysis. Such methods were not required in this research and are therefore beyond the scope of this text.

The repeatability of the measurement is a principal factor in the quality of the calibration, along with the trueness of standard and validity of comparison between calibrant and sample. Repeatability influences the precision of the result whilst the trueness of standard and validity of comparison will determine the accuracy. The validity of comparison between the calibrant and sample is one of the most critical factors in calibration techniques for trace level analyses with different techniques available depending on how critical the comparison is. For elemental analysis identical measuring conditions are required between the calibrant and sample.

The measurement of a range of standards of known concentration within the same analytical run is commonly used. This method can also be tailored to correct for drift in instrument response over time by measuring the calibration repeatedly throughout.

3.3 Interferences and interference correction

The measurement of an analyte by ICP-MS can be influenced by interferences leading to either enhancement or suppression of the analyte signal, in turn leading to respectively over or under-estimation of concentrations if not identified and corrected for. Interferences in ICP-MS can be classified into two major groups, spectroscopic and non-spectroscopic or matrix effects (Thomas 2008). This section will focus on those interferences relevant to arsenic determination and how they can be overcome.

Spectral overlaps provide the most serious interferences in ICP-MS. The most common are polyatomic interferences which occur when two or more atomic ions combine to give a new ion with a m/z ratio overlapping with that of the analyte (Thomas 2008). Polyatomic ions originate from a multitude of sources but are most commonly associated with the plasma and nebuliser gas, matrix components in the solvent or sample, other analyte elements or entrained oxygen or nitrogen from the surrounding air. For argon plasmas spectral overlaps caused by argon ions and combinations of argon ions with other ions are common (Olesik 2000). Arsenic, with an atomic mass of 75, is most commonly subject to interference from the polyatomic ion $^{40}Ar^{35}Cl^{+}$. For this reason matrices and solvents containing high levels of chloride are generally avoided in arsenic determination by ICP-MS. Several other potential polyatomic interferences on ^{75}As exist (Table 3.1) but in general are insignificant due to containing minor isotopes of constituent elements. For example, the polyatomic

interferences containing ^{36}Ar or ^{38}Ar are rare as these isotopes represent only 0.33 and 0.06 % of the natural abundance of Ar. Polyatomic interferences can be reduced to be negligible/ insignificant using collision/reaction cell technology (CCT). CCT uses ion-molecule collisions and reactions to cleanse the ion beam of polyatomic interferences before they enter the mass analyser (Agilent 2005). Both ICP-MS instruments used in this research (Thermo PQ Excell and Agilent 7500) are fitted with dynamic reaction cells utilising a mixture of helium and argon as the reaction gas. This type of reaction cell is highly effective at eliminating the $^{40}Ar^{35}Cl^{+}$ interference from m/z = 75 reducing its intensity to <2% of the value without the collision cell (Figure 3.2) making it possible to analyse low levels of arsenic in high chloride matrices. The gases He and H_2 are commonly employed as the reaction/ collision gases respectively either alone or in combination. Recent research indicates that He alone introduced into the collision cell at a rate of 4 l min^{-1} is most effective at removing the poly-atomic interference on ^{75}As caused by $^{40}Ar^{35}Cl$ (Dufailly *et al.* 2008). This method was employed in all analyses in this research.

Table 3.1: Potential polyatomic interferences on ^{75}As. Adapted from May and Wiedmeyer (1998).

Isotope	**Polyatomic Interference**
^{75}As	$^{59}Co^{16}O^{+}$, $^{36}Ar^{38}Ar^{1}H^{+}$, $^{38}Ar^{37}Cl^{+}$, $^{36}Ar^{39}K^{+}$, $^{43}Ca^{16}O_2{}^{+}$, $^{23}Na^{12}C^{40}Ar^{+}$, $^{12}C^{31}P^{16}O_2{}^{+}$, $^{40}Ar^{35}Cl^{+}$

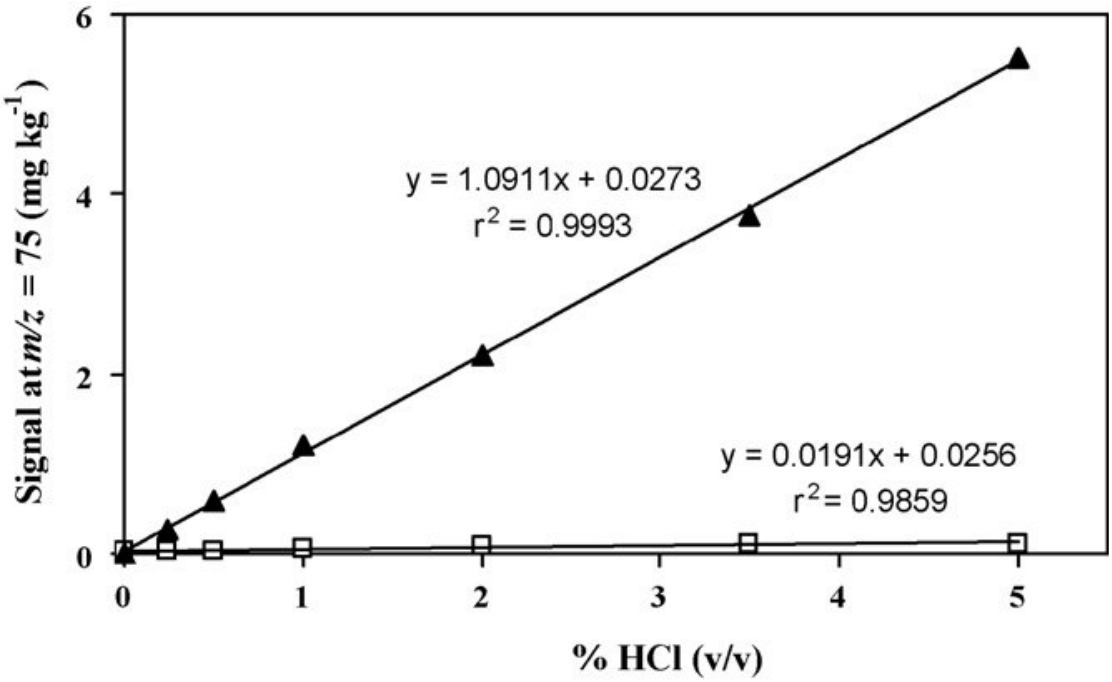


Figure 3.2: Effect on signal at m/z 75 of HCl content (% HCl (v/v)) on a blank solution in standard (▲) and collision cell made (□) modes. Adapted from Dufailly *et al.* (2008).

Matrix interferences occur for a number of reasons when the calibrant and sample matrices differ. For example; if the sample contains an organic component or high concentrations of dissolved salts, matrix-induced changes in the intensity of the ion signal might occur (Thomas 2008). Physical interferences, such as changes in the viscosity of solutions, may affect the rate of aspiration into the plasma and therefore the delivery of the analyte. Samples containing more than 10 g l^{-1} of dissolved solids are susceptible to major matrix effects. Suppression of the analyte signal is most common, although enhancement has been reported. Several instrumental factors are also important in determining the severity of possible matrix effects:

- Aperture size of sampling and skimmer cones
- Carrier gas flow rate
- RF power

- Sample uptake rate

Blockage of the sampling cone aperture by solutions containing high salt concentrations is a common matrix effect leading to a decrease in signal over time. Even without salt deposition, high salt levels in a sample can cause ionisation suppression. The introduction of an easily ionised element into the plasma, such as an alkaline earth element contributes to the electron density, shifting the ionisation equilibrium so that the analyte elements are ionised to a lesser extent (Olesik 2000).

Signal suppression is also caused by the space-charge effect. The electrostatic lens system in an ICP-MS only transmits positive ions into the detector. Without a matrix these ions are usually Ar^+ and O^+. With a matrix the number of positive ions in the extracted beam increases e.g. Na^+. These ions may repel analyte ions. The increased space charge means the beam is less easily focused. Lighter analyte ions suffer most from this effect.

Correction of matrix effects can be achieved in several ways to improve the quality of calibration in elemental analysis. The use of internal standards is perhaps the best way to compensate for matrix effects. With this method a small group of elements are mixed with the samples, standards and blanks via a T-piece attached to the sample introduction system of the ICP-MS. As the intensity of the internal standard signal changes throughout an analytical run the analyte signal can be corrected accordingly. Selection of a suitable internal standard element is important, taking into account the following: 1) they should not be present in the sample, 2) the sample matrix or analyte elements do not spectrally interfere with them and 3) they are usually grouped with analyte elements of a similar mass range and ionisation potential (Thomas

2008). Internal standards used in this research were indium (In) rhenium (Re) and tellurium (Te). Te in particular was chosen for its similar ionisation potential to As.

Maximum dilution of the sample will reduce matrix effects by increasing the purity of the sample and improving the validity of comparison between the calibrant and sample although this needs to be balanced against reduced analytical precision with decreasing concentration. Matrix matching the calibrant and standard will also improve the validity of comparison by ensuring both the calibrant and sample are subject to the same/similar matrix interferences. However the non-linearity of matrix interferences can be problematic (Dean 1997). Calibration by standard addition provides a means of overcoming problems associated with the validity of comparison between calibrant and sample by avoiding the use of both individually. In this method each sample is divided into several subsamples with an increasing, but known, concentration of the analyte spiked into the different subsamples. By comparing how the concentration of the sample has changed before and after addition of the standard the concentration in the unknown sample can be extrapolated (Figure 3.3). The calibration must therefore be linear over its entire length to avoid introducing error (Dean 1997). This type of calibration was employed for samples in this research whose matrices differed significantly from that of the calibration standards.

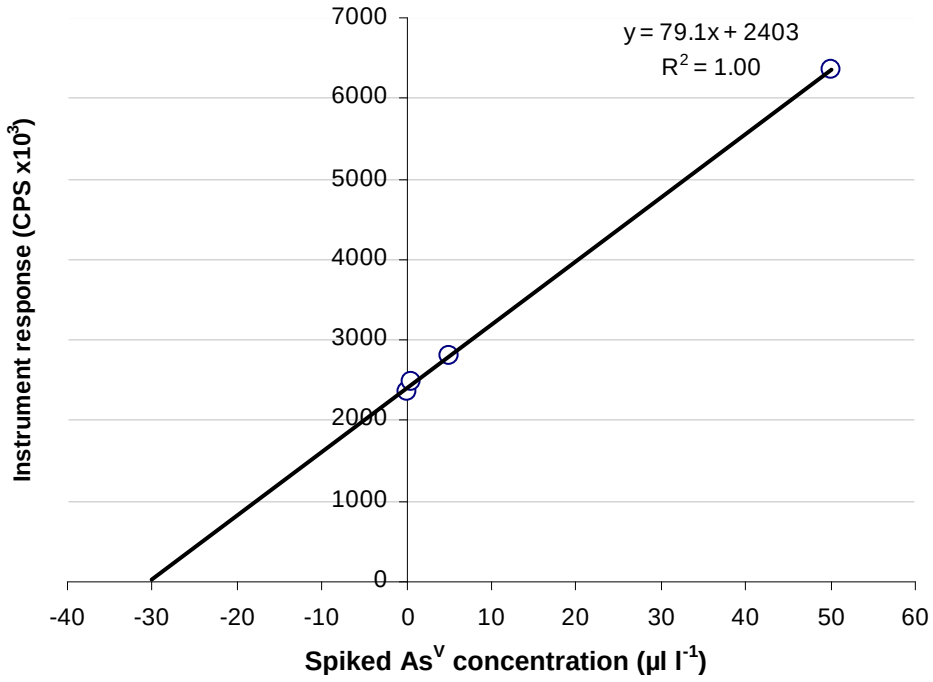


Figure 3.3: Standard addition calibration plot for a soil sample extracted using a phosphoric/ ascorbic acid matrix (Chapter 6). The point at zero spike concentration is the unknown sample alone followed by the same sample spiked with 0.5, 5 and 50 µg l^{-1} of As^V. The *x*-axis intercept is the concentration of As^V in the unknown sample, in this case 30 µg l^{-1}.

3.4 High Performance Liquid Chromatography-ICP-MS

Separation techniques are fundamental to elemental speciation analyses. ICP-MS provides highly sensitive total element detection, but it is not until a separation technique is coupled with an element specific detector that the various chemical forms (species) of an element in a sample can be determined (Ackley and Caruso 2004). High Performance Liquid Chromatographic (HPLC) separations are carried out by introducing an aqueous sample onto a chromatographic column filled with a solid stationary phase while a liquid mobile phase is continuously pumped through the column. The HPLC column is coupled directly to the nebuliser of the ICP-MS

where separated species are detected as an increase in counts (peaks in a chromatogram) for arsenic (Figure 3.4).

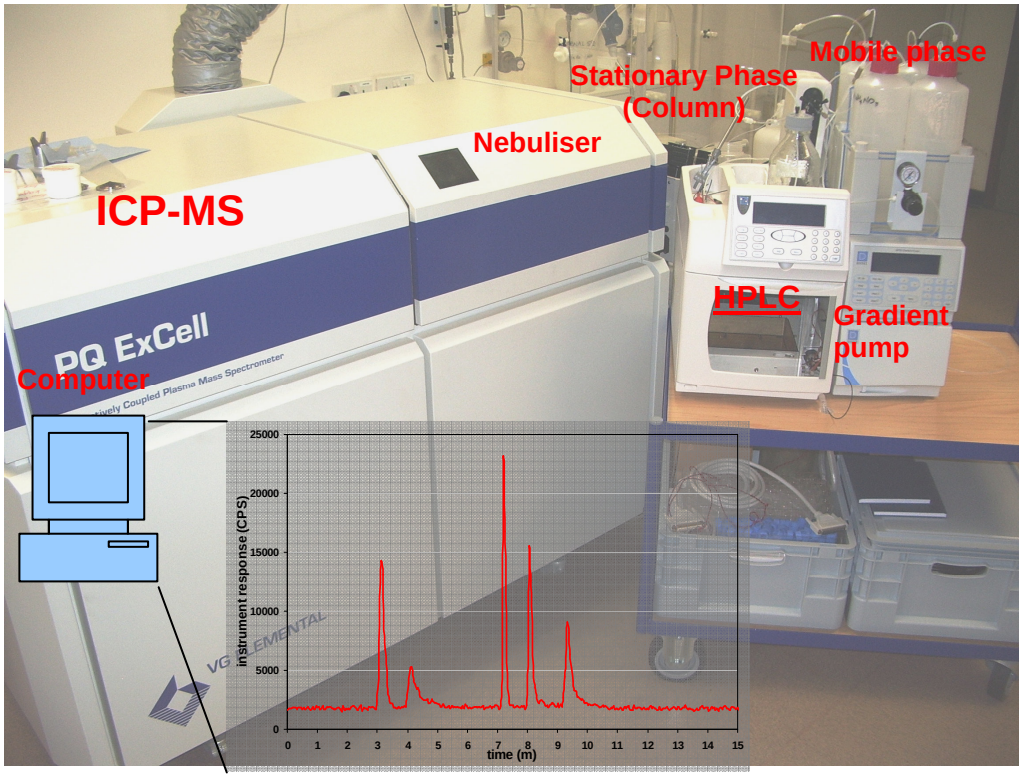


Figure 3.4: HPLC-ICP-MS instrumentation used in this research.

Interactions between an analyte and the stationary and mobile phases are based on polarity, electrical charge, pH and molecular mass (Ackley and Caruso 2004). In general, ion exchangers favour the binding of ions of higher charge and smaller radius. The stationary phase typically consists of an ionic functional group bonded to a polystyrene-based polymer or silica. The ionic sites on the stationary phase have the opposite charge to the analytes to be separated. Analytes with a charge that is opposite to that of the charge bearing functional group interact with the stationary phase electrostatically. The retention time of an analyte therefore increases with increasing electrostatic force (Ackley and Caruso 2004).

Knowledge of an analytes structure can help in predicting how it will behave during HPLC separation. The selection of a suitable stationary phase is based on the nature of the analytes to be separated. For example, a mixture of negatively charged species (anions) can be separated using a column with a positively charged anion exchange stationary phase (column). The negatively charged species are thereby retained on the positively charged sites of the anion exchange column whilst neutral and positive species are not retained. Species that are not retained elute as a mixture are referred to as the solvent front. This situation is then reversed for cation chromatography whereby a negatively charged stationary phase is used to retain positively charges cations. Other variables can be altered to achieve a suitable separation. The concentration determines the eluting power of the mobile phase by increasing or decreasing the number of counter ions available to displace retained analyte species. The pH influences the equilibrium between the mobile and stationary phase. In cation exchange an increase in pH reduces retention times whilst an increase in pH increases retention times in anion exchange (Small 1989). The pH of the mobile phase also influences speciation of the analyte in terms of overall charge and therefore the resulting retention on the column. As demonstrated in figure 2.1 in chapter 2, whilst the oxidation state of As in a particular species may be positive the overall charge of that species may be negative or neutral. The flow rate of the mobile phase can also be increased or decreased to hasten or slowdown the elution of retained analyte species respectively. During *isocratic* separations the composition of the mobile phase is maintained constant throughout the separation whereas in a *gradient* elution the composition of the mobile phase is changed during the course of the separation. Gradient elution is useful for separating mixtures of compounds that cover a wide a

wide range of chromatographic polarity. As the separation proceeds the strength of the mobile phase can be increased to speed up the elution of compounds with a stronger affinity for the column. This provides the additional benefit of combating the problem of peak broadening, associated with late eluting peaks, which can impair the resolution of individual peaks (Small 1989).

3.4.1 Species identification

Comparison of retention time allows individual peaks relating to each unknown species to be quantified by comparison to known standard solutions. The different arsenic species in aqueous sample extracts in this research were identified by comparison to five commercially available arsenic speciation standards. Inorganic As^{III} /As^{V}, and organic MA^{V} DMA^{V} and AB. In addition, four arsenosugars were isolated from a sample of marine algae (genus *Fucus*) following the method described by Madsen *et al*. (2000), as illustrated in figure 3.5.

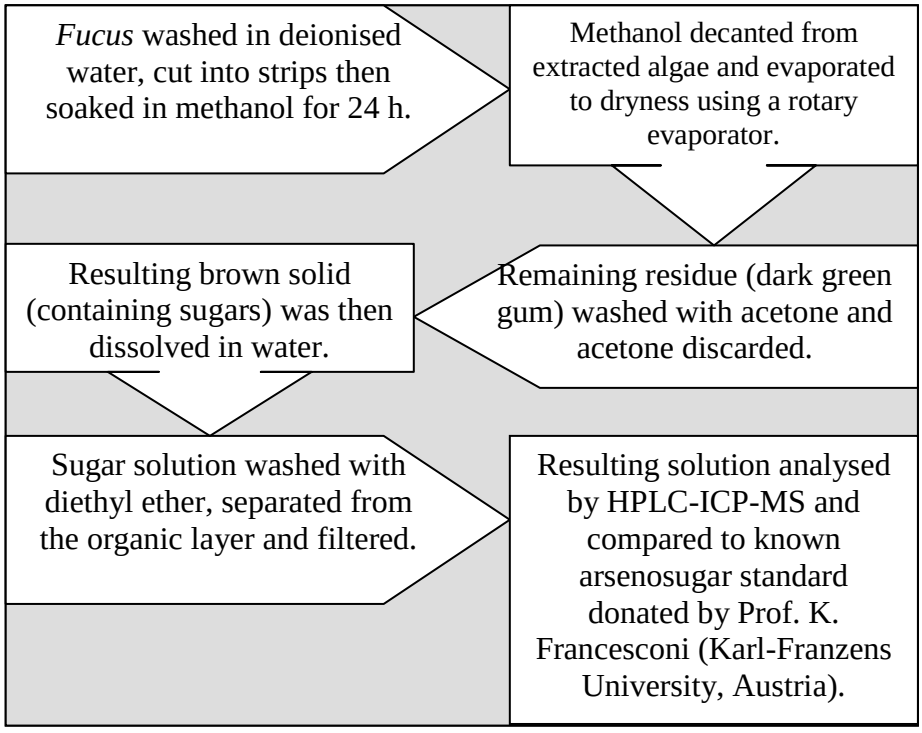


Figure 3.5: Flow chart showing steps involved in isolating the arsenosugars (1-4) from marine algae of genus *Fucus.*

Arsenic species were separated using either an anion exchange column or cation exchange column. Details of both chromatographic systems are provided in Table 3.2. The chromatographic separation of arsenic species achieved using the anion and cation systems are illustrated in figures 3.6 and 3.7 respectively. The anionic separation was based on a modified gradient elution system developed by Martinez-Bravo *et al*. (2001). This system was used to quantify As^{III} (as arsenite), DMA^{V}, MA^{V}, As^{V} (as arsenate), sugar-2 and sugar-4. The order in which the As species elute using this system is explained by both the charge of the species and pH of the solution. The PRP-X100 column provides retention of both negatively charged anions and neutral, low polarity species, due to the polymer backbone. In solution at pH 8.65, AB, sugar-1 and arsenite are all neutral causing them to elute early (figure 2.1, chapter 2). The remaining negatively charged species elute in order of increasing pH as increasing pH results in increased retention times (Francesconi 2009). The cationic separation was a modified version of the isocratic cation method developed by Geiszinger *et al.* (2002). This system was used to quantify AB (a cation, see figure 2.2, chapter 2) and sugar-1. Sugar-3 was not detected in any of the samples investigated. Sugar-1 appears later in this chromatogram (Figure 3.7) due to the neutral charge being retained by the cation exchange column, by comparison to the negative charge of sugars 2-4. This system was employed primarily to check that AB was not eluting in combination with other cations in the solvent front of the anion method. A comparison of the results obtained for AB quantification in all earthworm sample extracts is displayed in figure 3.8. The two methods gave comparable results, identical within analytical precision. Further details of the HPLC-ICP-MS methodology used in the research are included in the recent study by Watts and Button *et al*. (2008) included as **appendix B**.

Table 3.2: Details of the chromatographic systems employed in this research.

Details	Anion exchange	Cation exchange
Stationary phase (column)	PRP–X100, (250 x 4 mm, 10 µm, length, diameter, particle size)	PRP-X200, (250 x 4 mm, 10) µm
Mobile phase (eluent)	Gradient A: 4 mM NH_4NO_3 and B: 60 mM NH_4NO_3 at pH 8.65	Isocratic 10 mM pyridine at pH 2.26
Flow rate	1 ml min^{-1}	1.5 ml min^{-1}
Injection volume	100 µl	100 µl

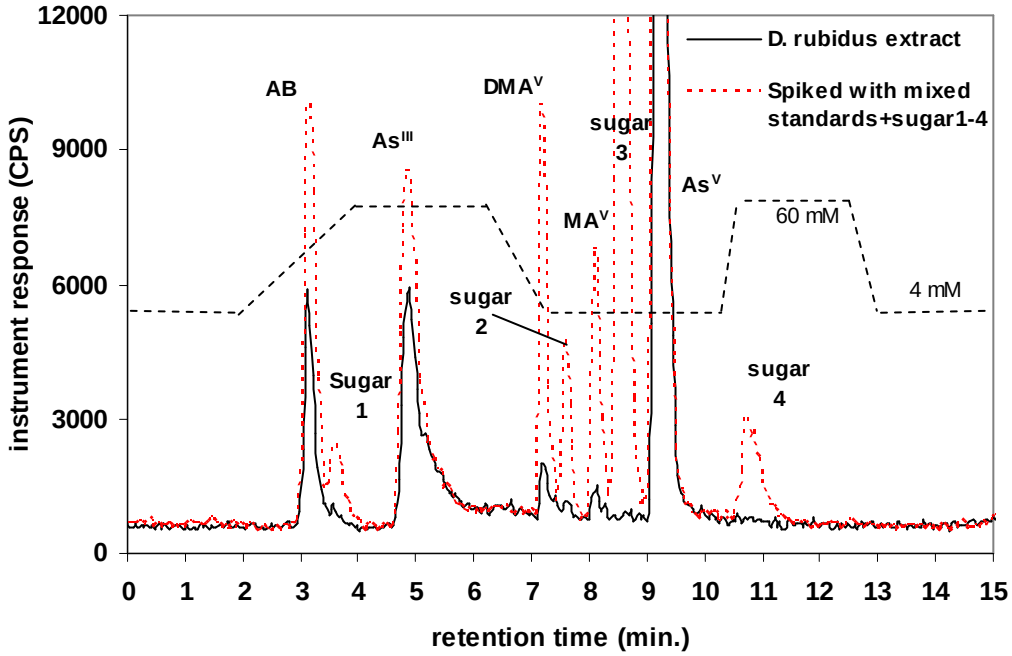


Figure 3.6: Anion gradient elution profile for a mixed standard solution (10 µg l^{-1}) and a representative earthworm extract. The dashed line indicates transitions in the mobile phase.

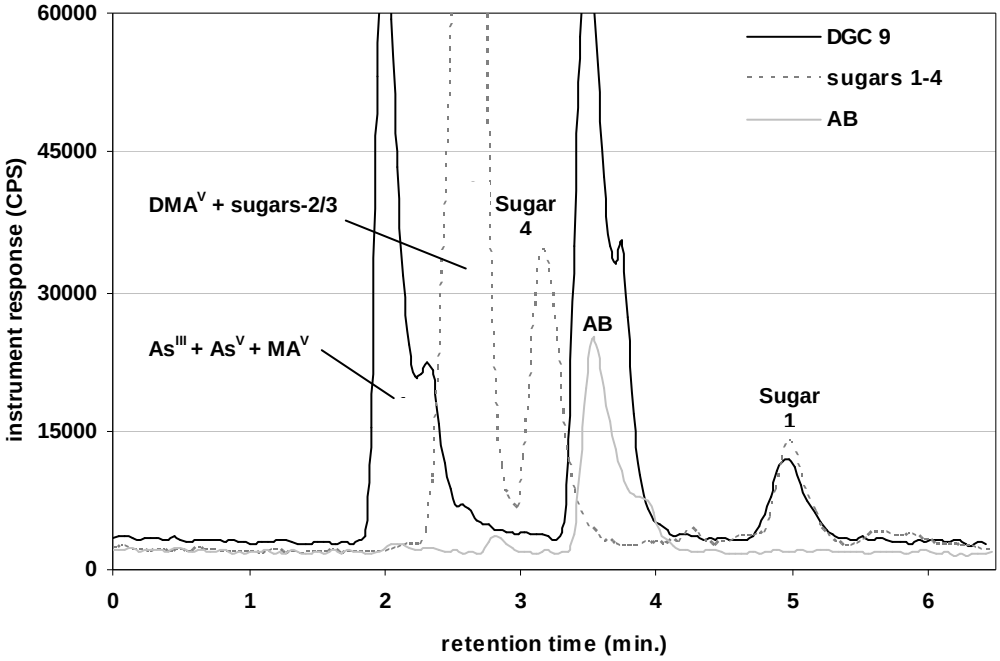


Figure 3.7: Cation isocratic elution profile for an earthworm extract (DGC 9), an algal extract containing sugars 1-4 and an AB standard at (13 µg l^{-1}).

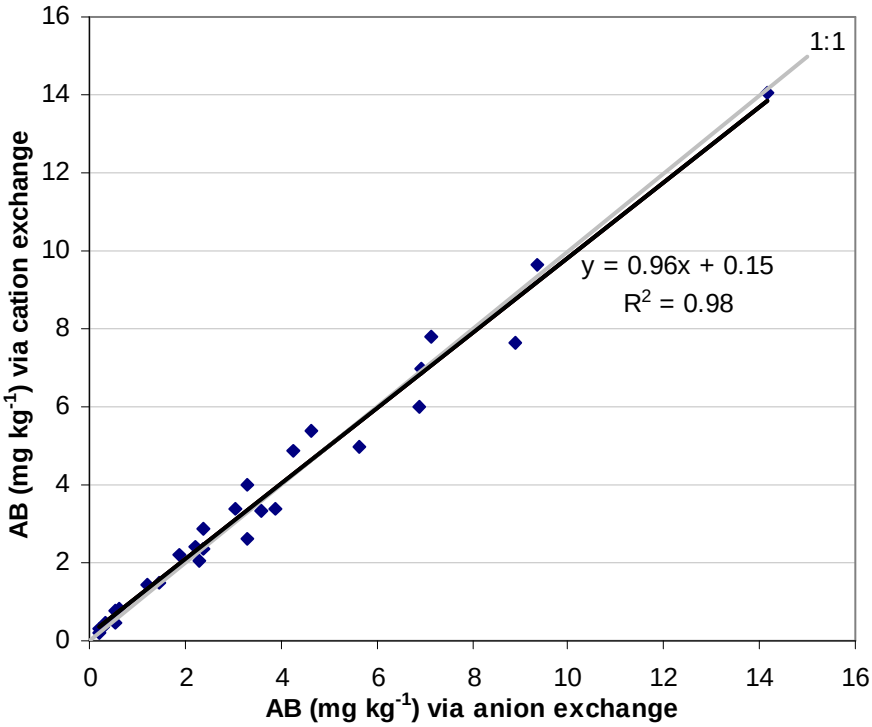


Figure 3.8: Comparison of the results obtained for AB quantification in earthworm extracts for the two chromatographic systems employed.

3.4.2 Sample extraction

The main difficulty encountered in the determination of arsenic speciation is the development of a method that provides both quantitative and reproducible recoveries whilst maintaining the species integrity of a sample. Extraction recoveries are dependent on the sample matrix, species present, extraction solvent and aggressiveness of the extraction procedure (Pizarro *et al.* 2003).

The majority of naturally occurring arsenic species identified so far are polar and highly soluble in water, suggesting water alone is the best solvent for extracting arsenic species in biological samples (Francesconi and Kuehnelt 2004). However the most common approach is to use a methanol/water mixture to improve penetration of solvent into the sample matrix. Methanol also extracts fewer non-arsenical compounds and is easily removed by evaporation where pre-concentration of a sample is required. Shakers and rotators are still the most commonly used devices to enhance the extraction of arsenic from biological samples (Van Elteren *et al.* 2007). However, for geological samples such as soils a more aggressive extraction is required as methanol/water based approaches typically extract < 5 % of the total arsenic (Francesconi and Kuehnelt 2004). Sonication, accelerated solvent extraction and microwave-assisted extraction techniques provide improved recoveries from soils by weakening the bond between arsenic species and the sample matrix. The extraction and measurement of arsenic species in soils is discussed in more detail in a report from this research (Button and Watts 2008) included as **appendix A**.

3. 5 Physiologically-based extraction tests

Physiologically-based extractions tests (PBETs) simulate the leaching of a solid matrix in the human gastrointestinal tract to determine the bioaccessibility of a particular element i.e. the total fraction that is available for adsorption during transit through the small intestine (Ruby *et al.* 1993). The PBET was designed around the paediatric gastrointestinal tract for a child of 2-3 years as this age group is believed to be at greatest risk from accidental soil ingestion (Ruby *et al.* 1993). The test is essentially a two stage sequential extraction using various acids and enzymes to simulate both the stomach and small intestine compartments. The extraction is carried out in rotating extraction vessels containing the sample and extraction solution in a water bath at 37 ºC to simulate the temperature of the human body (Figure 3.9).

Figure 3.9: Apparatus used for PBET extractions. Image courtesy of the British Geological Survey.

Numerous *in vitro* PBETs have been developed as simple, inexpensive tools to investigate the bioaccessibility of soil contaminants (Oomen 2002). Uncertainties as to whether these models produce similar estimations of bioaccessibility has hindered their incorporation into the contaminated land risk assessment process. The Bioaccessibility Research Group of Europe (BARGE 2008) undertook an

international collaborative initiative to establish a unified PBET method (the Unified BARGE Method UBM) for estimating human bioaccessibility capable of providing reproducible, robust and defensible bioaccessibility data (Cave *et al.* 2006). The UBM was used in this research to estimate the *in vitro* bioaccessibility of arsenic in soil samples. Figure 3.10 details the individual steps involved in the extraction procedure. A more detailed description of the method is provided in chapter 4.

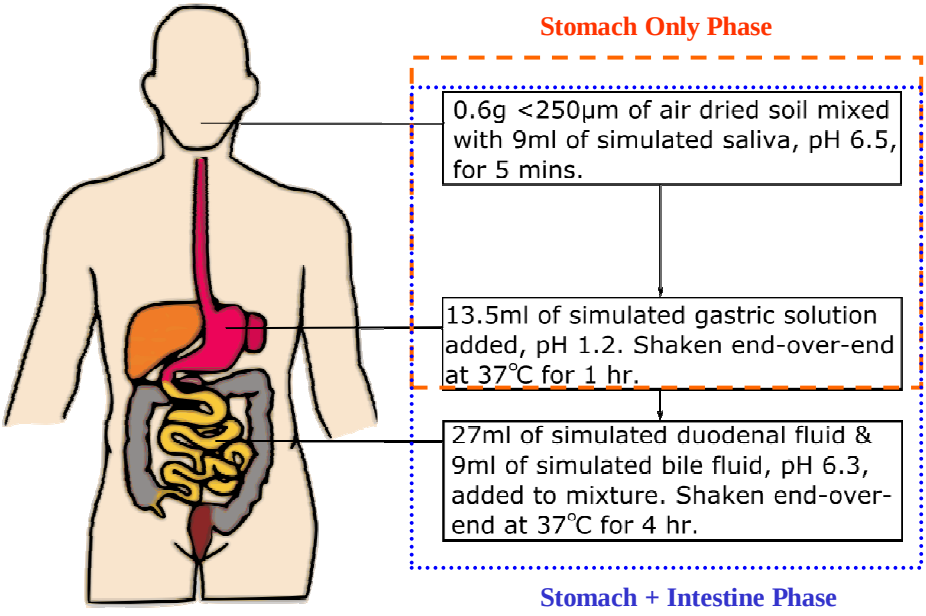


Figure 3.10: The unified BARGE bioaccessibility method (UBM) consists of a stomach only and stomach and intestine phases. Image courtesy of Dr A. Broadway.

3.6 The Comet Assay

The Comet, or SCGE (single-cell gel electrophoresis), Assay is a simple method for measuring deoxyribonucleic acid (DNA) strand breaks in eukaryotic cells. Over the past decade the Comet Assay has become one of the standard methods for assessing DNA damage in the fields of genotoxicity testing and human biomonitoring (Collins 2004). The Comet Assay begins with the immobilisation of isolated cells by

embedding them in agarose gel on a glass slide before lysis with detergent solution with a high salt content (Figure 3.11). The lysis treatment removes membranes and solubilises histones leaving behind the nucleoid body containing ribonucleic acid (RNA), proteins and supercoiled DNA (Collins 2004). The DNA is then denatured (deconstructed) by immersion in an alkaline electrophoresis buffer. Strand breaks in the denatured DNA result in supercoil relaxation (unwinding). The greater the number of strand breaks the greater the degree of unwinding. Given a sufficient degree of unwinding, the application of an electric field across the slides (electrophoresis) creates a motive force by which the charged DNA may migrate away from the immobilised nucleoid body (Lee and Steinert 2003). The resulting structure resembles a 'comet' with a bright head and fragmenting tail (Figure 3.12) Following electrophoresis the slides are rinsed in a neutral buffer and stained using a DNA binding dye (propidium iodide).

Adoption of and rigorous adherence to a standardised protocol is essential to overcome such limiting factors as discrepancies between buffers, gel thickness, temperature during preparation and exposure of cells to UV radiation. Assessment of methodological precision can be achieved to a certain extent by including standard cells in the analysis where available. For example, lymphocytes prepared from several individuals could be pooled and frozen as aliquots for future use (Collins 2004). The cells should provide a consistent measure of damage with each experiment thereby highlighting changes in the Assay when damage levels in the controls deviate widely from the normal level. Calibration of the method can be performed by irradiating samples of cells with gamma or X-rays to induce a known

number of strand breaks; comets are reported to show a linear increase in damage (% tail DNA) over a range of 0 to 8 Gy * (Collins 2004).

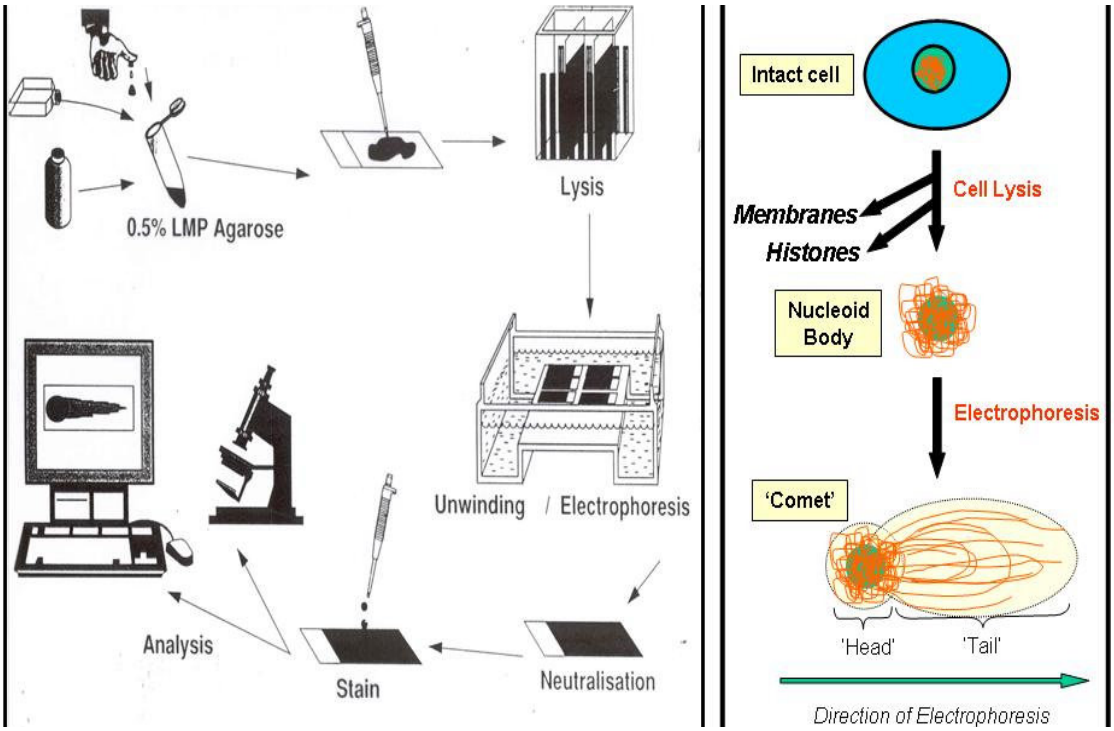


Figure 3.11: Schematic showing the various stages involved in performing the Comet Assay and a depiction of the formation of a comet following electrophoresis (Image courtesy of the Department of Cancer Studies & Molecular Medicine, University of Leicester).

* Gy = Gray unit. 1 gray is the absorption of one joule of energy, in the form of ionising radiation, by one kilogram of matter.

3.6.1 Selection and visualisation of comets

Comets must be selected for scoring without bias and must represent the whole slide. Repeated analysis of the same comets must also be avoided; therefore each slide is systematically scanned until the required numbers of comets have been recorded. The edges and areas around any air bubbles should be avoided as they often display comets with erroneously high levels of damage (Collins 2004). The number of cells in a single gel is important as cells packed too close together cannot be reliably scored.

Visualisation (scoring) is achieved using a fluorescence microscope coupled to a computer loaded with a software package that allows rapid measurement of fluorescence parameters for comets selected by the operator. Figure 3.12 displays a typical comet as imaged using Komet 5.0 software. Several parameters can be used to quantify damage levels including tail length, Tail DNA (%) and the Olive Tail Moment (OTM). Tail length (Figure 3.12) is the distance of DNA migration away from the Comet head (nucleoid body). Tail DNA (%) refers to the fraction of total DNA in the comet tail and the OTM is the product of the tail length and the fraction of the total DNA in the tail. Tail length is the most commonly employed parameter although this measure is subject to the limitation of only increasing while the tail is first becoming established at relatively low damage levels. Subsequently the tail increases in intensity but not length as damage increases (Collins 2004). Tail length is also subject to background thresholds of the imaging software as the end of the tail is defined by a certain excess of fluorescence above background (Collins 2004). Tail DNA (%) was used in this research as it bears a linear relationship to break frequency, is relatively unaffected by threshold settings and facilitates discrimination

of damage over the widest possible range. Specific details on the scoring and statistical analysis methods used in this research are provided in **chapter 5**.

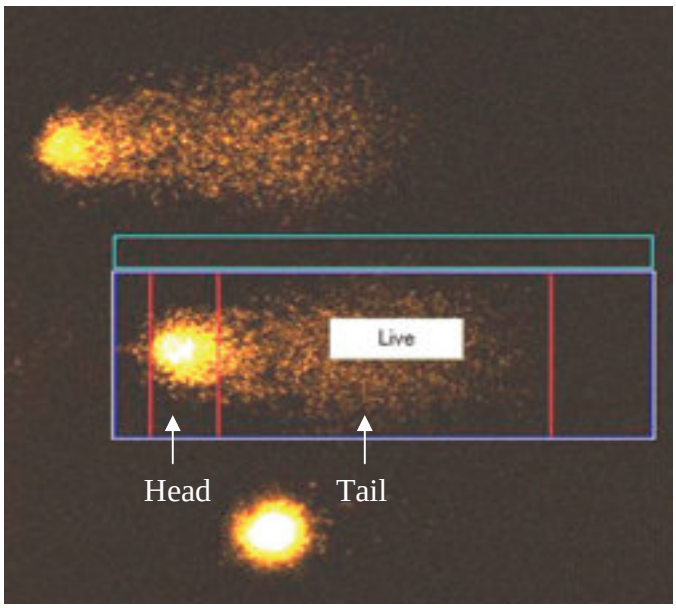


Figure 3.12: Digital fluorescence microscope image of comets with varying levels of damage. Box surrounding comet is part of Komet 5.0 auto-integration software.

Chapter 4

Earthworms and *in vitro* physiologically-based extraction tests: complementary tools for a holistic approach towards understanding risk at arsenic contaminated sites

4.1 Abstract

The relationship of the total arsenic content of a soil and its bioaccumulation by earthworms (*Lumbricus rubellus* and *Dendrodrilus rubidus*) to the arsenic fraction bioaccessible to humans, measured using an *in vitro* physiologically-based extraction test (PBET), was investigated. Soil and earthworm samples were collected at 24 sites at the former arsenic mine at the Devon Great Consols (DGC) in Southwest England (UK), along with an uncontaminated site in Nottingham, UK, for comparison. Analysis of soil and earthworm total arsenic via inductively coupled plasma-mass spectrometry (ICP-MS) was performed following a mixed acid digestion. Arsenic concentrations in the soil were elevated (204 – 9,025 mg kg^{-1}) at DGC. The arsenic Bioaccumulation Factor (BAF) for both earthworm species was found to correlate positively with the Human Bioaccessible Fraction (HBF), although the correlation was only significant ($P = \leq 0.05$) for *L. rubellus*. The potential use of both *in vitro* PBETs and earthworms as complementary tools is explored as a holistic and multidisciplinary approach towards understanding risk at contaminated sites. Arsenic resistant earthworm species such as *L. rubellus* populations at DGC are presented as a valuable tool for understanding risk at highly contaminated sites.

4.2 Introduction

Arsenic contaminated land is demanding increasing attention from environmental scientists due to its potential toxicity to humans, flora and fauna (Camm *et al.* 2004). A widely employed method for the assessment of risk to human health from contaminated land in the UK, (the Contaminated Land Exposure Assessment (CLEA) model) (Defra 2002b), is arguably preoccupied with the derivation of a single universal Soil Guideline Value (SGV) , presumably to facilitate practicality and ease of application (Hamilton 2000). The current SGV in the UK (implemented in 2002) for residential gardens and allotments is specified at 20 mg kg^{-1} dry weight (Defra 2002). In parts of the UK such as the Southwest, where arsenic contamination is widespread due to historic mining and calcination of polymetalic ores (Camm *et al.* 2004, Hutton *et al.* 2005, Van Elteren *et al.* 2006), the current SGV is unrealistic. One major criticism of the CLEA model is that contaminants are assumed to be completely available to a receptor following exposure (Hutton *et al.* 2005) leading to a potential overestimation of exposure. The primary pathways of human exposure to arsenic in soil that result in significant health effects are inhalation and oral ingestion leading to both carcinogenic and non-carcinogenic responses whilst dermal adsorption is not thought to be significant (Schultz and Biksey 2003). Consideration of a contaminant's oral bioaccessibility is important in understanding exposure associated risk (Intawongse and Dean 2006). Numerous *in vitro* physiologically-based extraction tests (PBETs) have been developed as simple, inexpensive tools to investigate the bioaccessibility of soil contaminants (Oomen 2002). Uncertainties as to whether these models produce similar estimations of bioaccessibility has hindered their incorporation into the contaminated land risk assessment process. The Bioaccessibility Research Group of Europe (BARGE 2008) undertook an international collaborative initiative to

establish a unified PBET method (the Unified Barge Method UBM) for estimating human bioaccessibility capable of providing reproducible, robust and defensible bioaccessibility data (Cave *et al.* 2006). Such efforts are likely to hasten the adoption of bioaccessibility testing in risk assessment, reinforced by the fact that the Scottish and Northern Ireland Forum For Environmental Research (SNIFFER) already propose a method for deriving site-specific human health assessment criteria for contaminants in soil that incorporates bioaccessibility testing (Fergusen *et al.* 2003).

Ecosystem indicator species such as earthworms have proven a useful tool in assessing soil contamination, particularly the accumulation of a contaminant by earthworm populations, as a guide to bioavailability (Langdon *et al.* 2001, Langdon *et al.* 2003, Mariño and Morgan 1998, Morgan and Morgan 1999). The earthworm species *Lumbricus rubellus* and *Dendrodrilus rubidus* are known to inhabit soils and mine wastes in southwest England highly contaminated with arsenic (Morgan 1994). They are thought to have developed a resistance to arsenic toxicity (Langdon *et al.* 1999), although not through avoidance of the contamination, since arsenic body burdens have been demonstrated up to 566 mg kg^{-1} (Langdon *et al.* 2002). This ability to accumulate high levels of arsenic makes these two earthworm species particularly useful tools in assessing arsenic bioavailability to indicator species in highly contaminated soils. Both earthworm species are epigeic (surface living) and therefore ideal in assessing the soil surface, the soil fraction of most concern in assessing human exposure. Whilst many studies have investigated the impact of soil contamination on soil biota, in particular earthworms (Cotter-Howells *et al.* 2005, Piearce *et al.* 2002, Van Vliet *et al.* 2006), ecological input into contaminated land risk assessment is poor. A holistic approach, whereby the geochemical, human and ecological aspects of contaminated land are employed as multiple lines of evidence in understanding risk, requires investigation.

The aim of this work is to examine the interrelatedness of available tools in understanding the risk to human health and the ecosystem at arsenic contaminated sites. Comparison of the soil total arsenic, human bioaccessible fraction and bioaccumulation by earthworms will provide insight into whether or not these complementary tools can be used in parallel for a more holistic approach towards understanding risk at contaminated sites.

4.3 Materials and methods

4.3.1 Study site

The Devon Great Consols (DGC) is situated by the River Tamar in the Tavistock district of Devon (grid reference: SX 426 735) and is one of many former mining sites in southwest England (Fig. 4.1). Soil arsenic concentrations 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} (Klinck *et al.* 2002, Langdon *et al.* 2001). Klinck *et al*. (2002) demonstrated the high potential for the release of arsenic from sulphide ore and other wastes by carrying out leaching experiments. Arsenic bioaccessibility in soils in the mine area and mine tailings have previously been shown to be well above the 20 mg kg^{-1} SGV (Cave *et al.* 2002) for gardens and allotments. Notably, concentrations up to 624 mg kg^{-1} of bioaccessible arsenic in residential areas around the mine site were reported giving cause for concern in terms of potential human exposure.

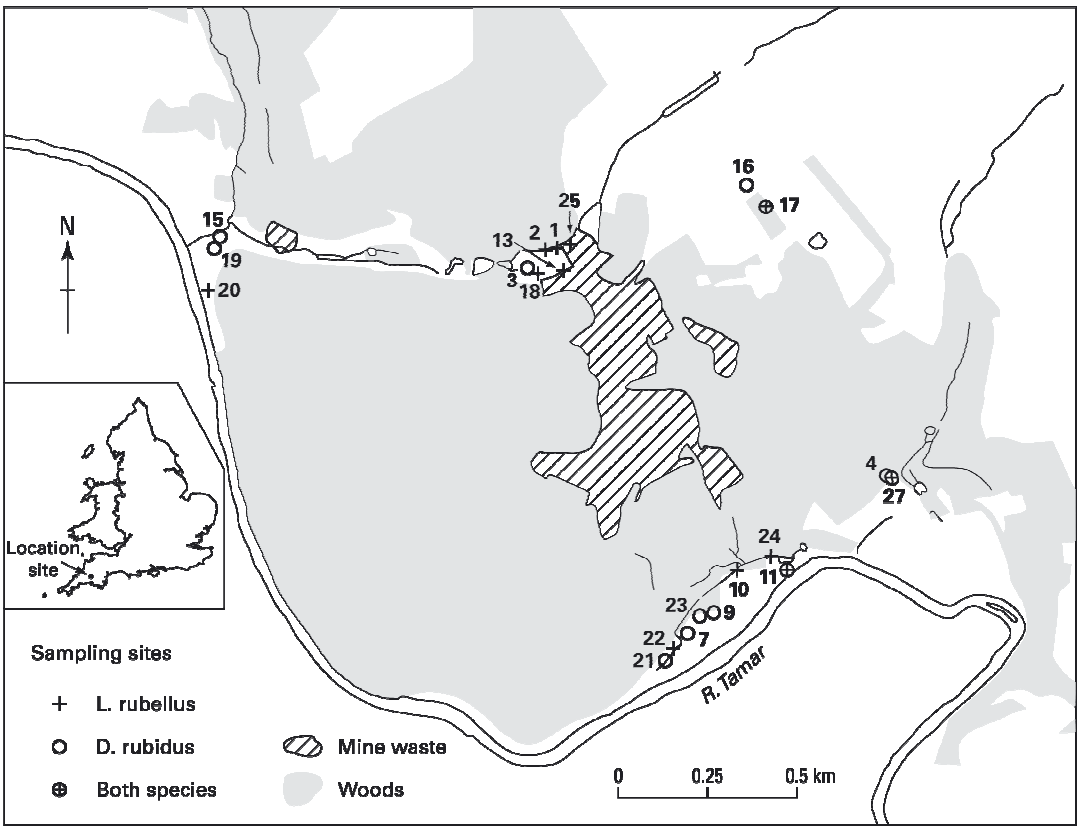


Figure 4.1: Geographical location of study area and positioning and identification number of sampling sites.

4.3.2 Sample collection and preparation

The following sample collection and preparation methods were employed at the contaminated study site (DGC) and an uncontaminated Nottingham garden. The soil surface (0 – 20 cm) with an area of ~1 m^2 was overturned with a spade allowing individual earthworms to be handpicked. Specimens were promptly separated according to species using a dichotomous earthworm key (WWC 2008), 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. 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* (Langdon, 2003). Approximately 10–25 mature earthworms (clitellum

present) were collected at each sampling point. Depurated earthworms were thoroughly rinsed with deionised water and dried in a low temperature oven (50 ˚C) before homogenisation in a ceramic pestle and mortar. A composite soil sample from the overturned surface (~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.

4.3.3 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). Concentrated HNO_3, HF, 30 % v/v H_2O_2 and $HClO_4$ (Aristar; BDH, UK) were used for the dissolution of earthworms and soil samples. $CaCl_2$ (Fisher Scientific, UK) was used for the measurement of soil pH. NaCl, KSCN, anhydrous Na_2SO_4, KCl, $CaCl_2.2H_2O$, NH_4Cl, $NaHCO_3$, KH_2PO_4, $MgCl_26H_2O$, NaOH, HCl, urea, anhydrous D+ glucose, D-glucosaminehydrochloride, pepsin (pig), Bovine Serum Albumin (BSA), pancreatin (pig), 69 % HNO_3 (Merck, UK), α-amylase (bacillus species), lipase (pig), bile salts (bovine) (Sigma, UK), NaH_2PO_4 (Baker, UK), mucin (pig) (Carl Roth, Germany), D–glucuronic acid (Fluka, Germany) and uric acid (Merck-Prolabo, UK) were used in the *in vitro* UBM PBET for estimating human bioaccessibility (Cave *et al*. 2006).

4.3.4 Total digestion of earthworm

Microwave assisted (CEM MARS5; CEM Corporation, UK) dissolution of the earthworms using a closed vessel system was performed on 0.1 g of earthworm homogenate (dry weight). Concentrated nitric acid (10 ml) and hydrofluoric acid (100

μl) was added, allowed to stand for 30 minutes and then microwaved. 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 30 % H_2O_2 was added. The vessels were sealed and the microwave cycle repeated. After cooling, the sample solutions were transferred to PTFE Savillex containers and evaporated to dryness on a hotplate (100 °C) to reduce the presence of organic compounds that could form possible polyatomic interferences on analysis by ICP-MS. Samples were reconstituted by the addition of 2 ml of 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 to that required for ICP-MS measurement (<2.5 % v/v). The procedure was monitored using a certified reference material, CRM 627 tuna fish (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 % difference (± 1 SD), between duplicate earthworm samples was 1.7 ± 0.9 % (n= 4 duplicates).

4.3.5 Soil chemistry

Soil pH was determined by adding 0.01 M aqueous $CaCl_2$ (6.25 ml) to 0.25 g of homogenised soil. Each soil slurry was 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 an in-house QC standard (pH 7.3). Loss on ignition (LOI) was also determined for each soil sample to provide an estimation of the organic matter content. One gram (dry weight) of each soil was weighed into a glass crucible before heating to 450 ºC for 4 hours.

The percentage weight reduction after heating was recorded as the estimated organic matter content.

4.3.6 Soil dissolution

Homogenised soils (0.25 g) were prepared for total elemental measurements by ICP-MS based on a mixed acid digestion approach (HF / HNO_3 / $HClO_4$) (Green *et al.* 2006). Samples were weighed directly into PTFE 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. $HClO_4$ 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 50% v/v nitric acid, heated at 50 ºC for 30 minutes and then treated with 30 % H_2O_2 (v/v) 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 2.5 % HNO_3 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. Recoveries of 98 ± 4 % (n = 6) and 91 ± 3 % (n = 3), 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 (RSC 2002). 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 (more details provided in **appendix C**).

4.3.7 Physiologically-based extraction test (PBET)

The UBM PBET (Cave *et al.* 2006) was employed in this study with the permission and assistance of BARGE members. 0.6 g of < 250 µm, dried and homogenised soil was mixed with 9 ml of simulated saliva at pH 6.5 for 5 minutes. 13.5 ml of simulated gastric solution was then added at pH 0.9 - 1.0 to give a final pH of 1.2 and shaken end over end at 37 ºC for 1 hour. This first stage constituted the stomach only phase of the extraction technique. In order to simulate the stomach and intestinal phase together, a duplicate stomach phase solution was produced and to this 27 ml of simulated duodenal fluid and 9 ml of simulated bile fluid at pH 6.3 was added to the mixture and shaken end over end at 37 ºC for 4 hours. Extraction vessels were then centrifuged at 3000 g for 5 min, 1 ml of the supernatant was then transferred to a test tube and preserved by addition of 9 ml of 1% HNO_3 (conc). Samples were analysed at a minimum 100x dilution to avoid matrix interferences. The phase giving the highest value was taken as the estimation of arsenic bioaccessibility. Certified Reference material NIST 2710 (Montana soil I) was included in each batch of samples (n = 5) along with duplicates and reagent blanks. CRM 2710 was also employed by BARGE in an inter-laboratory study (Cave *et al.* 2006) facilitating comparison of the results obtained for NIST 2710 in this study with those of BARGE. The results for arsenic (in mg kg^{-1}, errors expressed as ± 1 SD) were highly comparable. The BARGE inter-laboratory study obtained 323 ± 45 mg kg^{-1} (n = 4) for the stomach only phase and 264 ± 18 mg kg^{-1} (n = 3) for the stomach and intestine phase. In the present study 310 ± 8 mg kg^{-1} (n = 5) was obtained for the stomach only phase and 249 ± 2 mg kg^{-1} (n = 5)

for the stomach and intestine. These recoveries were well within error on the BARGE inter-laboratory values suggesting good reproducibility and accuracy of results using the UBM PBET method. The method precision expressed as the mean percentage difference (± 1 SD) between duplicate samples was 3.8 ± 3.5 % (n = 12 duplicates).

4.3.8 Instrumentation

Earthworm and soil digests were 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} dilution of 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. UBM PBET solutions were analysed using a Fisons ARL ICP-AES, with a low flow torch, Babington nebuliser and impact bead spray chamber. Simultaneous detection of analytes was employed with radial viewing of plasma at 650 W forward power. All samples were analysed at maximum dilution to minimise the occurrence of matrix effects.

4.4 Results

4.4.1 Total arsenic concentrations

Arsenic concentrations in soils were highly variable depending on their proximity to the mine tailings. Sampling sites 1 - 3, 13, 18 and 25 to the north of the study area (Fig. 4.1) and closest to the mine tailings demonstrated the highest soil arsenic

concentrations in the range 1005 – 9,025 mg kg^{-1}. Sampling sites 4, 7, 9 - 11, 21 - 24 and 27 to the south of the of the study area, closest to the River Tamar and further from mine tailings demonstrated lower soil arsenic concentrations in the range 204 – 1306 mg kg^{-1}. Sites 15, 19 - 20 and 16 - 17 to the northwest and northeast, respectively of the study area displayed soil arsenic concentrations in the range 622 – 6,308 mg kg^{-1}. The soil arsenic concentration at the uncontaminated Nottingham comparison site was 16 mg kg^{-1}, below the current SGV of 20 mg kg^{-1} (Defra 2002).

L. rubellus were found inhabiting soils covering a wide arsenic concentration range from 204 – 9,025 mg kg^{-1}, with a mean of 2,301 mg kg^{-1} (n = 12) (Table 4.1). *D. rubidus* were only found in soils up to an arsenic concentration of 3,995 mg kg^{-1} with a mean of 837 mg kg^{-1} (n = 12). Both earthworm species were found cohabiting at three sites (11, 17 and 27) where arsenic concentrations were comparatively low 289 - 622 mg kg^{-1}. The high mean soil arsenic concentrations at *L. rubellus* sites were reflected by high mean arsenic body burdens for this species (mean 287 mg kg^{-1}, n = 12). The mean arsenic body burden for *D. rubidus* was 134 mg kg^{-1} (n = 12). The arsenic body burden ranges for both earthworm species were similar (Table 4.1) and the difference between the mean values was not significant (Table 4.2). At the uncontaminated comparison site where both species of earthworm were also found residing together, the arsenic body burdens were similar (Table 4.1). A positive linear correlation was observed between the arsenic concentration in the soil and arsenic body burdens for both earthworm species (Fig. 4.2) with R^2 values of 0.73 and 0.93 for *L. rubellus* and *D. rubidus* respectively.

4.4.2 Bioaccumulation

Earthworm Bioaccumulation Factors (BAFs) were calculated as the earthworm total arsenic (mg kg^{-1}) divided by soil total arsenic (mg kg^{-1}). BAFs of <1.00 were observed at all sites indicating no enrichment above soil concentration was occurring. The mean BAF for *L. rubellus* of 0.15 (n = 12) was slightly higher than for *D. rubidus* at 0.12 (n = 12), although the BAF range for both earthworm species were similar at around 0.04 – 0.30 (Table 4.1) and the difference between the mean values was not statistically significant (Table 4.2).

Table 4.1: Mean As data presented with the range encountered across *L. rubellus* and *D. rubidus* sampling sites.

	Mean rubellus (n=12)	**min**	**max**	**Control rubellus**	**Mean rubidus (n=12)**	**min**	**max**	**Control rubidus**
Soil Total*	2301	204	9025	16	837	255	3995	16
Worm Total*	287	11	877	6.5	134	15	737	7.1
Bioaccessible*	413	36	1312	6.7	177	36	837	6.7
HBF	0.19	0.10	0.34	0.42	0.21	0.13	0.33	0.42
BAF	0.15	0.04	0.28	0.41	0.12	0.04	0.32	0.44
Soil pH	4.6	3.5	6.1	6.7	4.7	4.0	6.8	6.7
Soil LOI (%)	5.7	1.9	12	3.8	4.6	1.9	12	3.8

*(mg kg^{-1}). *HBF* Human Bioaccessible Fraction, *BAF* earthworm Bioaccumulation Factor, *LOI* loss on ignition

Table 4.2: Results of the Wilcoxon signed-rank test for significance of difference between paired groups (*L. rubellus* and *D. rubidus* sites).

Group Variable	P value
Soil total As (mg kg^{-1})	0.13
Worm total As (mg kg^{-1})	0.13
Bioaccessible As (mg kg^{-1})	0.53
Soil pH	0.82
Soil LOI (%)	0.29
HBF	0.56
BAF	0.40

Difference between group variables significant at $P < 0.05$.

4.4.3 Bioaccessibility

The estimated Human Bioaccessible Fraction (HBF) of arsenic, calculated as the bioaccessible arsenic (mg kg^{-1}) divided by total arsenic in the soil (mg kg^{-1}), varied substantially across sites from 0.10 to 0.34. The HBF at the uncontaminated comparison site was higher at 0.42. A positive linear correlation was observed between the bioaccessible arsenic and total arsenic in the soil ($R^2 = 0.93$) (Fig. 4.2) when all sites were combined. This trend did not differ when the sites were split into groups for *L. rubellus* and *D. rubidus* (Fig. 4.3). The trend was similar to that of total arsenic in both earthworm species suggesting colinearity between earthworm BAFs and the HBF at the investigated sites. Table 4.1 shows the mean arsenic bioaccessibility was higher for *L. rubellus* sites (413 mg kg^{-1}) than for *D. rubidus* (177 mg kg^{-1}), although the ranges were similar (Table 4.1) and differences between the two earthworm species were not significant (Table 4.2).

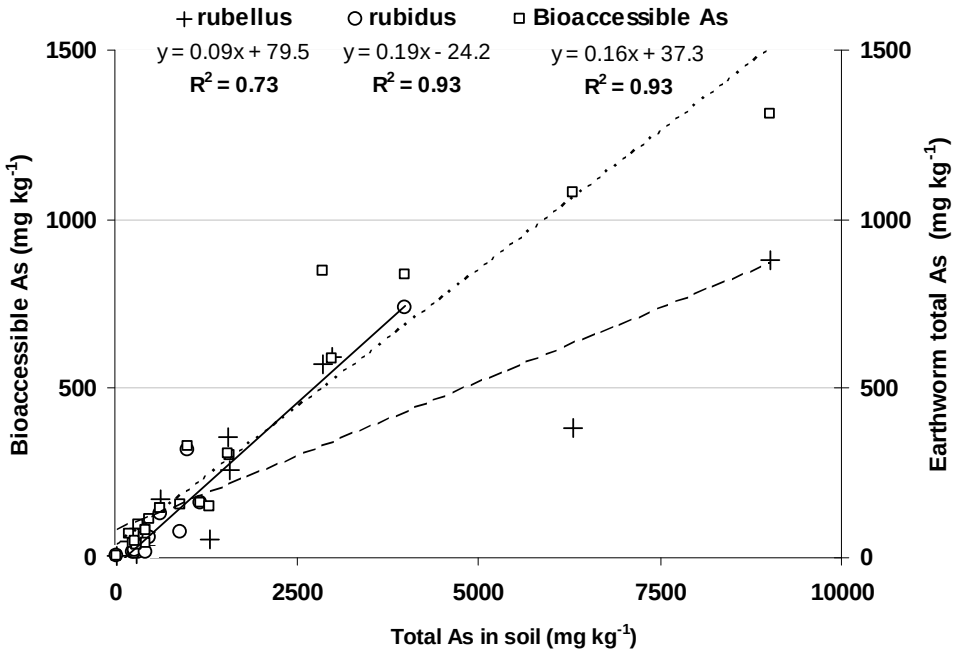


Figure 4.2: Correlations between bioaccessible As and earthworm total As to soil total arsenic for *L. rubellus* and *D. rubidus* (includes DGC and control site).

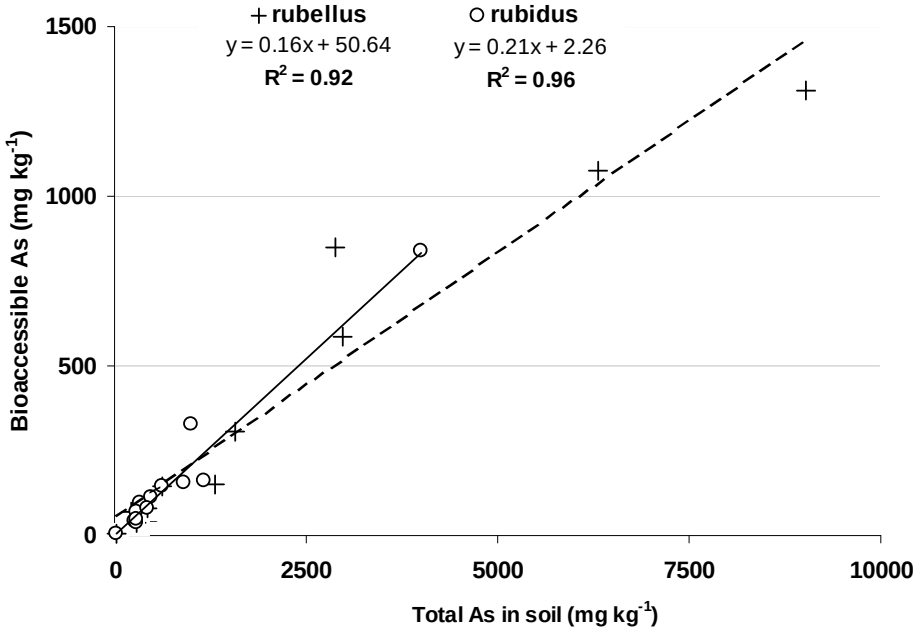


Figure 4.3: Correlations between bioaccessible As and soil total arsenic at *L. rubellus* and *D. rubidus* sites.

4.4.4 Comparability of estimated HBF and earthworm BAF

Figure 4.4 displays the HBF plotted against the BAFs of both earthworm species. The bioaccumulation of arsenic by *L. rubellus* correlates positively with the HBF at each site (R^2 = 0.75). This is reflected in similar mean values for *L. rubellus* sites of 0.19 (n = 12) for the HBF and 0.15 (n = 12) for the mean BAF (Table 4.1). The BAFs for *D. rubidus* also showed a positive correlation with the HBF at each site (R^2 = 0.52), although the correlation was not significant, as reflected by the greater difference between the means of each measure for this species (0.21 and 0.12, n = 12) for HBF and BAF, respectively.

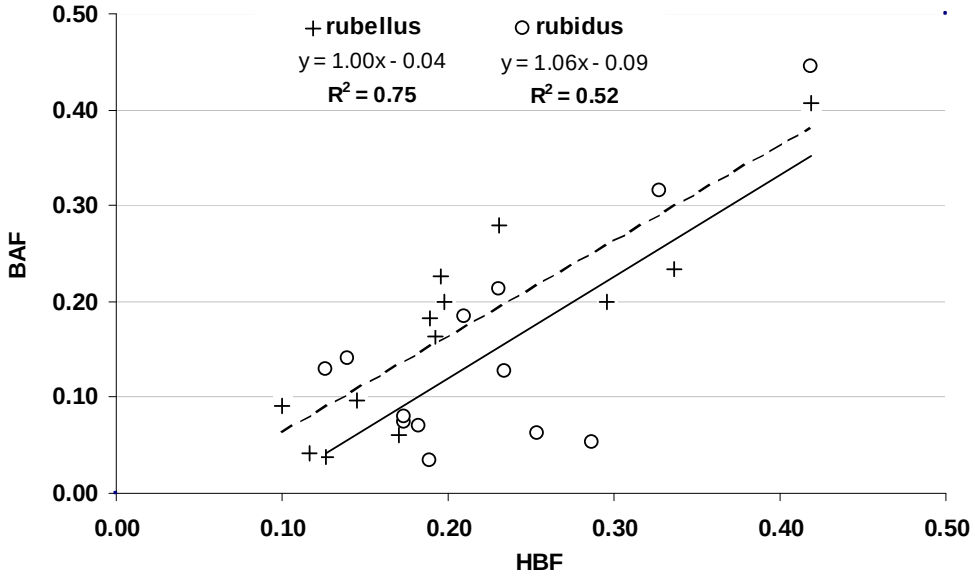


Figure 4.4: Correlations between the Human Bioaccessible Fraction (*HBF*) and earthworm Bioaccumulation Factors (*BAF*) at *L. rubellus* and *D. rubidus* sites.

4.4.5 Statistical analysis

Potential causes for the differing correlations between BAF and HBF for *L. rubellus* and *D. rubidus*, such as differing soil edaphic and geochemical factors were investigated. The non-parametric Wilcoxon signed-rank test for two related samples

was applied (SPSS 14.0) to the groups (*L. rubellus* and *D. rubidus* sites) for each of the variables listed in Table 4.2. The hypothesis that the two groups differ is significant at P values < 0.05. No significant difference was observed between the groups for any of the variables tested.

The significance of the positive correlation between BAF and HBF for both earthworm species was also investigated via a non-parametric significance test using bootstrap resampling (Efron and Tibshirani 1993) of the paired datasets. The datasets were resampled 1 x 10^4 times using a resampling statistics add-in package for Excel (Blank *et al.* 2001). For each resample the slope of the BAF to HBF least square linear fit was recalculated. The upper and lower 95% significance limits were calculated from the resampled data (97.5 and 0.025 percentiles). The 95% confidence limits for *L. rubellus* were 0.66 to 1.53, showing that the slope was significantly different from zero and therefore, a significant relationship exists. For the *D. rubidus* samples however the 95% confidence limits were -0.31 to 1.52, showing that the slope was not significantly different from zero and that there was not a significant relationship between BAF and HBF for this species of earthworm.

4.5 Discussion

Soil arsenic concentrations at the sites investigated at DGC were found to be elevated well above the current SGV (20 mg kg^{-1}). The values presented in this paper are in agreement with levels reported in previous studies (Kavanagh *et al.* 1997, Klinck *et al.* 2002, Langdon *et al.* 2002). Whilst the HBF of arsenic was never greater than 0.34 (Table 4.1) of the total arsenic in the soil, bioaccessible arsenic levels at all sites were well above the SGV. Soils at DGC are reported to show higher arsenic bioaccessibility

than other mineralised soils not affected by mining (Palumbo-Roe and Klinck 2007). Anthropogenic sources of contamination such as mine wastes are likely to give rise to higher bioaccessibility as the contaminant has relatively little time to bind to soil phases such as iron oxyhydroxides. This may also help to explain the linearity between bioaccessible and total arsenic in the soils at DGC (Fig. 4.2). The same linear trend was not observed in studies of arsenic bioaccessibility where the source of contamination was geogenic (Palumbo-Roe *et al.* 2005, Wragg *et al.* 2007). The higher bioaccessibility of arsenic at DGC is reflected in the arsenic body burdens in both *L. rubellus* and *D. rubidus* populations, which also demonstrate a degree of linearity with increasing soil concentrations (Fig. 4.2). These results differ from those in the literature where bioaccumulation of contaminants by earthworms is reported to decrease as soil concentrations increase (Neuhauser *et al.* 1995, Sample *et al.* 1999). The fact that arsenic accumulation in earthworms at DGC does not conform to models in the literature, is likely due to their reported resistance to arsenic toxicity (Langdon *et al.* 1999).

Previous studies have failed to provide firm evidence about species differentiation in terms of contaminant uptake from soils by earthworms (Marino and Morgan 1999). The correlation between BAF and HBF was statistically significant for *L. rubellus*, but not for *D. rubidus* in this study and could not be explained by any of the edaphic and geochemical soil characteristics investigated (Table 4.2). This finding agrees with the suggestion by Morgan and Morgan (1999) that no simple universal relationship exists between soil and earthworm arsenic concentrations. Further research on the ratio of earthworm body mass to the mass of ingested soil would be useful in elucidating causes for the observed difference between earthworm species. *L. rubellus* were

reported to be less sensitive to mining derived contamination than other species (Spurgeon and Hopkin 1996). The differences between the earthworm species reported here may be related, in part, to variation in sensitivity to the contamination, this may also explain differences in the distributions of earthworm species around the mine area (Fig. 4.1).

The accumulation of arsenic by both earthworm species reinforces the observed trends in bioaccessibility at DGC (Figs. 4.2 and 4.4). However, the intra-species differences in the relationship between BAF and HBF (Fig. 4.4) highlight the need for a degree of standardisation if biological receptors are to be used in conjunction with *in vitro* estimates of arsenic bioaccessibility. This need for standardisation also applies when using *in vitro* bioaccessibility models at contaminated sites if they are to be adopted in the risk assessment process. Small variations between bioaccessibility tests such as the solid-solution, pH and residence times have been shown to cause significant differences in bioaccessibilty estimates (Oomen *et al*. 2002). These differences may also impact upon the relationship of *in vitro* bioaccessibility tests to *in vivo* estimates of a contaminant's bioavailability.

For obvious reasons, earthworm species with a developed resistance to arsenic contamination are unsuitable for determining the contaminant's toxicity. However, the results presented here suggest resistant earthworm species may be more useful in the indirect assessment of bioavailability at sites with highly elevated levels of arsenic. The incorporation of earthworm BAFs alongside bioaccessibility testing at contaminated sites would provide complementary lines of evidence in support of

existing methods for assessing risk such as the CLEA (Defra 2002) and SNIFFER (Fergusen *et al.* 2003) models.

4.6 Conclusions

This study is in no way presented as an alternative to existing methods for understanding risk at contaminated sites. These results represent a focal point for discussion on more holistic, multidisciplinary approaches towards understanding risk at contaminated sites. Indirect measures of a contaminant's bioavailability, such as its accumulation by earthworms, can be used as complementary lines of evidence to reinforce site-wide trends using *in vitro* bioaccessibility, when estimating the potential for human exposure to a contaminant. Further research into the inter-relatedness of earthworm BAFs and *in vitro* PBETs at sites with differing contamination characteristics would be of benefit. This should include the study of a wider range of earthworm species to qualify the applicability of earthworms for a holistic approach towards understanding risk at contaminated sites.

Acknowledgements

The authors wish to thank the British Geological Survey University Funding Initiative (BUFI) for funding this research as part of a PhD studentship. We are also grateful to Joanna Wragg of the British Geological Survey for reviewing the manuscript and the late Tim Brewer for his guidance early on in the studentship. We would also like to thank the Tavistock estate for granting permission to access the Devon Great Consols site.

Chapter 5

DNA damage in earthworms from highly contaminated soils: Assessing resistance to arsenic toxicity using the Comet Assay

5.1 Abstract

Earthworms native to the former mine site of Devon Great Consols (DGC), UK reside in soils highly contaminated with arsenic (As). These earthworms are considered to have developed a resistance to As toxicity. The mechanisms underlying this resistance however, remain unclear. In the present study, non-resistant commercially sourced *Lumbricus terrestris* were exposed to a typical DGC soil in laboratory mesocosms. The earthworms bioaccumulated As from the soil and incurred DNA damage levels significantly above the control mesocosm (assessed using the Comet Assay). A dose response was observed between DNA damage (% tail DNA) and soil As concentration (control, 98, 183, 236, 324 and 436 mg kg^{-1}). As-resistant earthworms (*Lumbricus rubellus, Dendrodrilus rubidus and Lumbricus terrestris*) collected from contaminated soils at DGC (204 to 9025 mg kg^{-1} As) had also bioaccumulated high levels of As from their host soils yet demonstrated low levels of DNA damage compared to earthworms from uncontaminated sites. The results presented demonstrate that the As contaminated soils at DGC are genotoxic to non-native earthworms and provide further evidence of a developed resistance to As toxicity in earthworms native to DGC.

5.2 Introduction

Arsenic is well known for its toxicity, has been classified as a human carcinogen since 1980 by the International Agency for Research on Cancer (Kapaj *et al.* 2006) and is now recognised as a cause of lung, bladder and kidney cancer in humans (Lantz and Hays 2006). Arsenic occurs naturally in soils with the concentration determined mainly by the parent rock and to a lesser extent by climate, organic/ inorganic components and redox potential (Mandal and Suzuki 2002, Smedley and Kinniburgh 2005). Background concentrations of As in uncontaminated soils vary from 1 to 40 mg kg^{-1} (Mandal and Suzuki 2002, Smedley and Kinniburgh 2005). However, anthropogenic derived contamination can greatly increase the amount of As present in the soil. Historic mining activities and polymetallic ore calcination have led to widespread As contamination in the Southwest UK, (Hutton *et al.* 2005, Van Elteren *et al.* 2006) with elevated soil As concentrations reported between 204 and 34,000 mg kg^{-1} (Button *et al.* 2009, Langdon *et al.* 2001). Such highly elevated As levels give rise to concerns for human and ecosystem health.

Earthworms are important members of terrestrial ecosystems, aiding the maintenance of soil aeration, permeability, organic matter and soil structure, consuming large quantities of soil in the process (Langdon *et al.* 1999). They are also primary consumers in terrestrial food chains and are prey for many small mammals and birds (Ash and Lee 1980, Zheng *et al.* 1997). For these reasons earthworms are commonly employed as an ecosystem indicator species in ecotoxicological studies on soil contaminants (Button *et al.* 2009, Langdon *et al.* 2001, Marino and Morgan 1999, Reinecke 2004). The employment of native

earthworms to determine soil toxicity in areas contaminated with metalliferous mine waste is complicated by the fact that some populations appear to have developed a resistance to genotoxic metals in soil. The ubiquitous, epegeic 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 (Langdon *et al.* 1999, Morgan 1994). The mechanism underlying this resistance is unknown, but is thought to involve the sequestration of arsenic in the metallothionein-rich chloragogenous tissue which separates the intestine from the coelomic cavity (Morgan 1994). Whilst no physiological or behavioural side-effects have been reported in As-resistant worms at DGC, whether or not DNA damage is actually being incurred is as yet unknown.

The Comet Assay or single-cell gel electrophoresis (SCGE) has become one of the standard methods for assessing DNA damage due to its sensitivity, versatility, speed and economy (Collins 2004). The assay is essentially a method for measuring DNA strand-breaks in eukaryotic cells. One of several proposed modes of As carcinogenesis involves inhibition of the natural DNA repair process resulting in increased numbers of strand-breaks in affected cells (Aposhian 2000, Goering *et al.* 1999, Kitchin 2001, Mass 2001). The Comet Assay has been demonstrated to be effective in determining DNA damage levels in the coelomocytes of earthworms exposed to genotoxins, both *in vivo* and *in vitro*, in several studies (Di Marzio *et al.* 2005, Fourie *et al.* 2007, Qiao *et al.* 2007, Rajaguru 2003, Reinecke 2004, Salagovic *et al.* 1996). Dose-dependent DNA damage in earthworm coelomocytes has been demonstrated *in vivo* for chromium (Manerikar *et al.* 2008), cadmium (Fourie *et al.*

2007) and nickel (Reinecke 2004). As yet there are no published findings on DNA damage in the coelomocytes of earthworms exposed to As contaminated soils.

The aims of this investigation were firstly, to establish the dose-response genotoxity of a typical As contaminated DGC soil. To achieve this, non-native (As-naive) earthworms were exposed to contaminated soil in laboratory mesocosms. Resulting DNA damage was monitored using the Comet Assay. Secondly, to assess DNA damage levels in As resistant earthworms native to DGC (*L. rubellus*, *D. rubidus* and *L. terrestris*), collected from soils with varying levels of contamination and thirdly, to compare the results to background DNA damage levels observed in earthworms from uncontaminated sites.

5.3 Methods

5.3.1 Sample collection and preparation for analysis of total arsenic

All experiments were conducted in accordance with national and institutional guidelines for the protection of animal welfare. The following sample collection and preparation methods were employed for the contaminated study site (DGC) and three uncontaminated sites in the East Midlands (EM), UK. Field samples were collected in April 2007. All reagents used were analytical grade or better quality. All aqueous solutions were prepared using deionised water (18.2 MΩ Millipore, UK). Nitric acid (HNO_3. conc.), hydrogen peroxide (H_2O_2. 30%), and hydrofluoric acid (HF. conc.) (BDH Aristar, UK) were used for the digestion of samples.

5.3.2 Soil samples

The soil surface (upper 20 cm) with an area of approximately 1 m^2 was overturned with a spade. 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. Homogenised soils (0.25 g) were prepared for total elemental measurements by ICP-MS based on a mixed acid digestion approach (HF / HNO_3 / $HClO_4$) (Green *et al.* 2006). Samples were weighed directly into PFA vials, acids added (2.5 ml, 2 ml and 1 ml respectively) 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 concentrated nitric acid, heated at 50 ºC for 30 minutes and then treated with 30% H_2O_2 (v/v), before being made up to 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 digestions as a measure of quality control. These were NIST CRM 2710 Montana Soil I and NIST CRM 2711 Montana Soil II. Recoveries of 98 ± 4% (n = 6) and 91 ± 3% (n = 3), 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 (RSC 2002). The repeatability precision was found to exceed the specified fitness-for-purpose (FFP) criteria of 5% RSD on duplicate analyses (n =21).

5.3.3 Earthworms

Approximately 10 to 25 mature earthworms (clitellum present) were handpicked at each sampling point and placed in ventilated plastic boxes with approximately 2 kg of the host soil and some leaf litter. The boxes containing earthworms and soil were returned to the laboratory, stored in a cool dark room and the soils kept moist. Earthworms were separated according to species using a dichotomous earthworm key, (WWC 2008) thoroughly rinsed with deionised water and placed in ventilated 9 cm Petri dishes with moist filter paper to begin depuration of the gut contents. Filter papers were changed daily to prevent coprophagy. 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* (Langdon, 2003). Depurated earthworms were thoroughly rinsed with deionised water, humanely dispatched by rapid temperature reduction, then dried in a low temperature oven (50 ˚C) before homogenisation in a ceramic pestle and mortar. Microwave assisted (CEM MARS5, CEM Corporation, UK) digestion of the earthworms using a closed vessel system was performed on 0.1 g of earthworm homogenate (dry weight). Concentrated nitric acid (10 ml) and hydrofluoric acid (100 µl) were added to each microwave vessel, allowed to stand for 30 minutes, sealed and then heated. 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 30% H_2O_2 was added. The vessels were sealed and the microwave cycle repeated. After cooling, the sample solutions were transferred to PTFE Savillex® containers and evaporated to dryness on a hotplate (100 °C). Samples were reconstituted by the addition of 2 ml of 50% v/v nitric acid, 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). 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 earthworm samples was 1.7 ± 0.9% (n = 4 duplicates).

5.3.4 Instrumentation

Earthworm and soil digests were 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. Instrument sensitivity was optimised using a 1 µg l^{-1} dilution of 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.

5.3.5 Laboratory mesocosms

In order to assess the dose-dependent genotoxicity of a typical DGC soil to non-native earthworms, a mesocosm investigation was conducted. Adult *Lumbricus Terrestris* (Recycle Works, UK) were used as the test organism for the mesocosm investigation. A bulk soil sample (~5 kg) with a total As content of 660 ± 5 mg kg^{-1} was collected from DGC. The contaminated soil was thoroughly mixed with equal proportions of uncontaminated topsoil and John Innes No. 1® compost in increasing

amounts to give five soil mixtures (5 kg) of increasing arsenic contamination. A 1:1 mixture of uncontaminated topsoil and compost was used as the control. Clear plastic containers with drainage holes were used to house the soil mixtures and were stored in a cool dark outbuilding. Soils were kept moist with distilled water. The homogeneity of each mesocosm soil was assessed by triplicate sampling across a diagonal transect at different depths. Soil samples were prepared for analysis via ICP-MS as described above. In addition to As, mesocosm soils were analysed for a range of elements by ICP-MS to determine the presence of other potentially genotoxic elements (Al, V, Cr, Mn, Co, Ni, Cu, Zn, As, Se, Mo, Cd, Sn, Sb, Ba, Pb, Th and U) in order to assess their contribution to any DNA damage observed. Homogenous mixtures were necessary to prevent test organisms avoiding exposure to the contamination in the soils (Tomlin 1992). The pH and moisture content of each mesocosm soil was consistent across the exposure range at 5.9 ± 0.4 and 31 ± 1.3 (% moisture) respectively. Results of homogeneity testing, included as a measure of the validity of the system, are displayed in Table 5.1. Earthworms were sampled in duplicate and analysed for total As after 1, 3, 4 and 5 weeks exposure. DNA damage was assessed via the Comet assay after 5 weeks exposure.

Table 5.1: Homogeneity testing results for arsenic in mesocosm soils. Total As value is the result of triplicate samples, sampling across a diagonal transect at different depths in each mesocosm.

Mesocosm exposure	**Total** As **(mg kg^{-1})**	**% RSD (n = 3)**
control	1.0	41
1	98	8.4
2	183	17
3	236	7.4
4	324	7.5
5	436	7.7

5.3.6 Cell harvesting

All reagents used were analytical grade (Sigma-Aldrich, UK). Cell suspensions were obtained from *L. terrestris* sampled from the mesocosm soils and from earthworms from the three uncontaminated sites by dissection of the alimentary canal (Martin *et al.* 2005). Specimens were euthanised by exposure to chloroform vapour for 1 minute then mid-dorsally dissected and pinned open. The alimentary canal was opened mid-dorsally and the contents of the gut removed by rinsing with deionised water. A small piece of the intestine was then cut away below the gizzard, finely chopped using scissors and suspended in sterile 15 ml Eppendorf® tubes containing 1 ml of ice cold Phosphate Buffer Solution (PBS). Cell suspensions were filtered using 100 µm cell strainers (Falcon BD, VWR International Ltd, Lutterworth, UK) and homogeneity assessed under a microscope. Inhomogeneous samples or samples with low cell counts were rejected. For earthworms collected at DGC a more rapid method of cell collection was required due to the large numbers of specimens to be processed. For this reason a non-invasive cell extrusion method (Eyambe *et al.* 1991) was employed with the modification of excluding guaiacol glycerol ether (Di

Marzio *et al.* 2005). This method was applied to earthworms from uncontaminated sites for comparison. Individual earthworms were rinsed with cold PBS and placed on a paper towel. The lower part of the body was gently massaged with a gloved finger to expel the contents of the lower gut (Brousseau *et al.* 1997). Each worm was then placed posterior first into a 15 ml polypropylene tube containing 1 ml of cold extrusion solution (95% 0.15 M NaCl, 5% ethanol and 2.5 mg EDTA, pH adjusted to 7.5 with NaOH) and left for 3 minutes. The extrusion solution acts as an irritant causing the earthworm to secrete coelomic fluid containing coelomocytes (Reinecke 2004). The extrusion solution containing the coelomocytes was washed three times by repeated centrifugation (2000 rpm, 5 minutes at 4ºC) and re-suspension of the cell pellet in 1 ml cold PBS. Single-cell suspensions were counted using a haemocytometer and cell viability was determined by trypan blue dye exclusion; samples of too low a viability (<70%) were not studied.

5.3.7 Comet assay

All reagents used were analytical grade (Sigma-Aldrich, UK). Single clear glass slides were pre-coated with 1% agarose (normal melting point). An aliquot of counted cell suspension, sufficient to provide approximately 30,000 cells per gel, was centrifuged (2000 rpm, 5 minutes at 4ºC) and the supernatant carefully removed. The cell pellet was then suspended in 170 µl of warm (37 ºC) 0.6% agarose (low melting point) and two 80 µl aliquots placed onto a glass slide. Each aliquot was then immediately overlaid with a cover slip. Rapid solidification of the agarose was achieved by keeping the slides on a metal tray placed on ice for 5 minutes. Cover slips were then carefully removed and slides placed in lysis buffer

(100 mM EDTA, 2.5M NaCl, 10 mM Tris-HCl and 1% Triton X-100 adjusted to pH 10 with NaOH) and left overnight. Following cell lysing, slides were washed twice by submersion in ice-cold deionised water then transferred into an electrophoresis tank, containing cold electrophoresis buffer (300 mM NaOH, 1 mM EDTA, pH 13) and incubated for 20 minutes to allow unwinding of the DNA. Electrophoresis was carried out for 20 minutes at 30 V and 300 mA. Slides were removed from the tank and flooded with neutralisation buffer (0.4 M Tris-HCl, pH 7.5) then rinsed twice with deionised water.

5.3.8 Scoring and statistics

Slides were stained by flooding with approximately 1 ml of 2.5 µg ml^{-1} propidium iodide for 20 minutes then rinsed with deionised water. Comets were visualised and measured using Komet® 5.5 software under a fluorescence microscope. 50 non-overlapping comets were measured in each of the two gels on each slide, giving 100 measurements per slide (per earthworm). Only comets with similar nucleoid size and shape were scored to avoid heterogeneous response of differing cell types present in coelomic fluid (Reinecke 2004). All statistical analyses were performed using statistics package SPSS version 14 (SPSS Inc.). The paired samples T-test was used in significance testing ($P < 0.01$) for outcomes of the mesocosm experiment.

5.4 Results

5.4.1 Mesocosm investigation

A high degree of consistency was achieved for total As levels in each mesocosm soil via the mixing strategy described (Table 5.1). The high % RSD value for total As in

the control mesocosm is due to the low levels of As present in the control soil. Arsenic accumulation in *L. terrestris* increased with dose and time across the concentration range investigated (Figure 5.1). A maximum As body burden of 139 mg kg^{-1} was observed in *L. terrestris* exposed to a soil As concentration of 436 mg kg^{-1} for 5 weeks resulting in a bioaccumulation factor (BAF = total As in earthworm / total As in soil) of 0.32. Arsenic bioaccumulation was not linear across the As exposure range (Figure 5.2) with a fluctuating correlation between BAF and As concentration in the soil. However, arsenic bioaccumulation did generally increase, though not consistently, with time between 1 and 5 weeks of exposure in the contaminated mesocosms. No mortality was observed during the exposure period of 5 weeks.

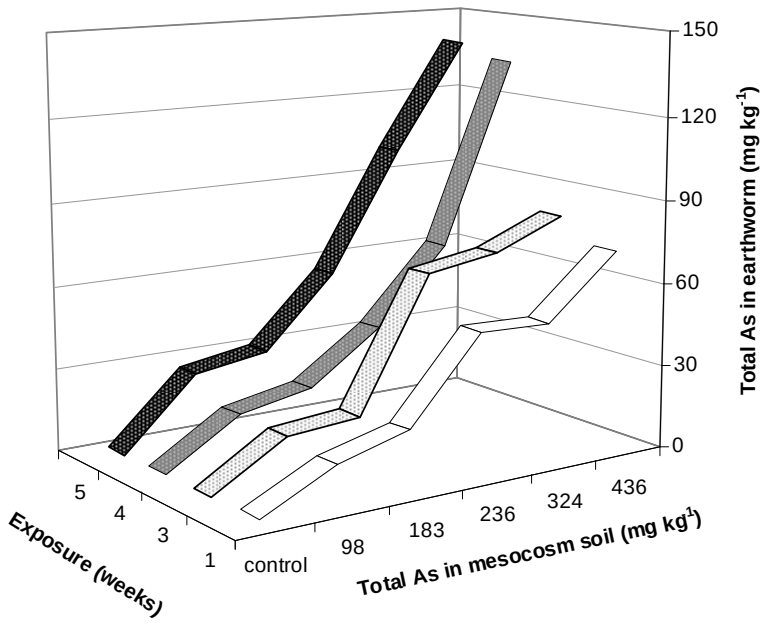


Figure 5.1: Arsenic accumulation in *L. terrestris* exposed to increasing soil As concentrations in laboratory mesocosms.

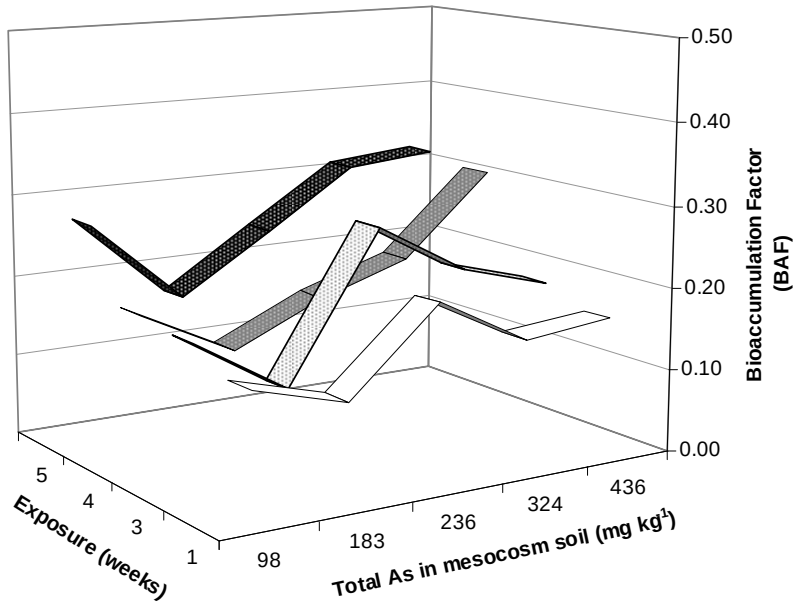


Figure 5.2: Arsenic bioaccumulation factors (BAFs) for L. *terrestris* exposed to increasing soil As concentrations in laboratory mesocosms.

The relationship of DNA damage (% tail DNA) to earthworm body burdens for multiple elements was investigated via a correlation matrix. A correlation coefficient of r^2 = >0.8 was used as the selection criteria to signify a meaningful correlation. Six elements were positively correlated to DNA damage (Table 5.2). Arsenic accumulation was by far the highest of these elements, with a maximum body burden of 139 mg kg^{-1}. A clear dose-response relationship was observed in results of the Comet after 5 weeks exposure (Figure 5.3). DNA damage levels (expressed as % tail DNA) increased with increasing concentration of arsenic in each mesocosm soil, although the trend observed was subject to slight fluctuations. DNA damage levels were significantly higher at all As concentrations investigated compared to that noted for the control mesocosm ($p < 0.01$). DNA damage levels in *L. terrestris* from the control mesocosm were comparable to the damage levels observed in the field-collected earthworms from the uncontaminated EM sites. All As exposures induced DNA damage in *L. terrestris* above the mean damage levels observed in the field collected earthworms from the uncontaminated EM sites (Figure 5.3).

Table 5.2: List of all elements analysed in earthworms exposed in mesocosm soils for 5 weeks and bioaccumulation- DNA damage correlation coefficients for potentially toxic elements with an $r^2 > 0.8$.

Elements analysed: Al, V, Cr, Mn, Co, Ni, Cu, Zn, As, Se, Mo, Cd, Sn, Sb, Ba, Pb, Th and U.		
Correlating elements	**Bioaccumulation range (mg kg^{-1})**	**Correlation with % tail DNA (r^2)**
Arsenic	0.9 - 139	0.86
Cadmium	2.5 – 5.6	0.92
Cobalt	3.8 – 9.6	0.89
Selenium	1.5 – 4.6	0.88
Tin	0.1 – 1.7	0.84
Antimony	0.1 – 0.3	0.80

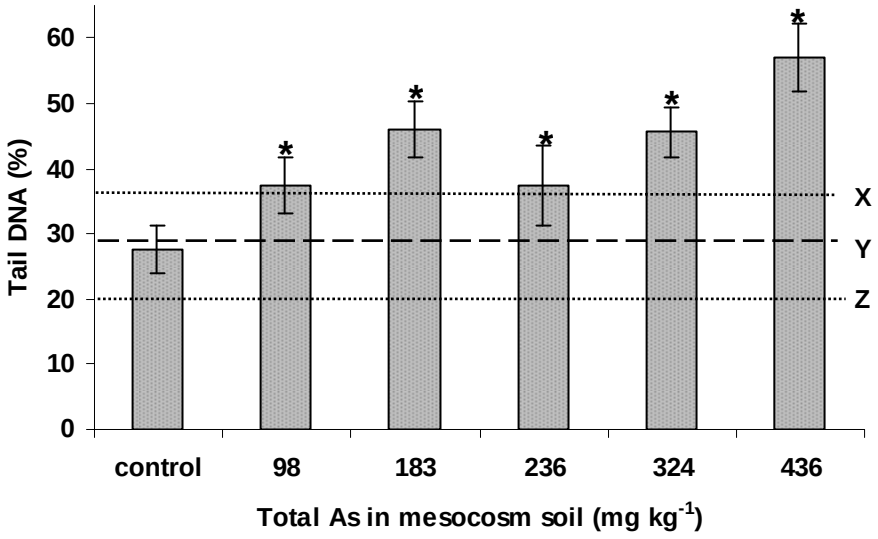


Figure 5.3: DNA damage in *L. terrestris* exposed to increasing soil As concentrations in laboratory mesocosms for 5 weeks. * = significantly different from control ($P < 0.05$). % Tail DNA based on the mean of duplicate specimens (200 comets) ± 2 SE. Lines X, Y and Z represent the mean (n = 24) 95th and 5th percentiles for DNA damage observed in earthworms collected at 3 uncontaminated sites with cell harvesting (dissection) as per the mesocosm specimens.

5.4.2 DGC earthworms

Three earthworm species were found residing at DGC (*L. terrestris*, *L. rubellus* and *D. rubidus*) inhabiting soils with total As concentrations ranging from 204 – 9025 mg kg^{-1} (Table 5.3) greatly exceeding the concentration range investigated in the mesocosm experiment. As reported in a previous study (Button *et al.* 2009), *L. rubellus* were generally found inhabiting soils with the highest As concentrations. The As body burdens in the earthworms ranged from 15 – 749 mg kg^{-1} giving rise to BAFs of 0.04 – 0.23 (Table 5.3). BAFs were generally lower in earthworms from soils with the highest As concentrations. The levels of DNA damage observed in earthworms collected at DGC were less than the lowest damage levels (5^{th} percentile) observed in the earthworms from the 3 uncontaminated EM sites (Figure 5.4) with the exception of site DGC 26 (Table 5.3), where DNA damage levels in *L. terrestris* were slightly higher than the 95^{th} percentile of damage levels observed in earthworms from the uncontaminated EM sites (Figure 5.4). No obvious correlation was observed between DNA damage (expressed as % tail DNA) and the total As concentration of the host soil (Figure 5.4). Likewise, the levels of DNA damage observed in the DGC earthworms did not correlate with the earthworm As body burdens (Table 5.3), with a correlation value of $r^2 = -0.03$.

Table 5.3: Earthworm species collected at each DGC site with the total As concentration in the earthworm, soil and resulting Bioaccumulation Factor (BAF).

Site id	Earthworm species	Earthworm total As (mg kg^{-1})	Soil total As (mg kg^{-1})	BAF
DGC22	*L. terrestris*	48	204	0.23
DGC23	*D. rubidus*	19	275	0.07
DGC27	*D. rubidus*	15	427	0.04
DGC21	*D. rubidus*	59	480	0.12
DGC17	*D. rubidus*	132	622	0.21
DGC19	*D. rubidus*	165	1173	0.14
DGC24	*L. rubellus*	50	1306	0.04
DGC18	*L. rubellus*	335	1567	0.21
DGC16	*D. rubidus*	665	3878	0.17
DGC26	*L. terrestris*	607	5760	0.11
DGC20	*L. rubellus*	374	5767	0.06
DGC20	*D. rubidus*	128	6308	0.02
DGC25	*L. rubellus*	749	9025	0.08

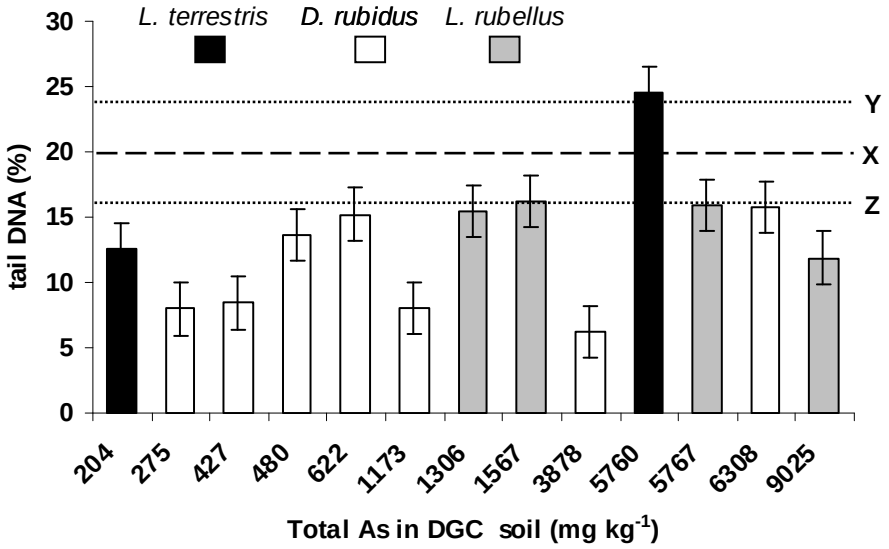


Figure 5.4: DNA damage in earthworms collected from contaminated soils at DGC. % Tail DNA represents the mean of at least 2 specimens (min. 200 comets) ± 2 SE. Lines X, Y and Z represent the mean (n = 8), 95th and 5th percentiles for DNA damage observed in earthworms collected from 3 uncontaminated EM sites with cell harvesting (extrusion) as per the DGC collected specimens.

5.5 Discussion

Homogeneity of the mesocosm soils was required to prevent *L. terrestris* specimens avoiding exposure to the As contamination. Contaminant avoidance behaviour has been reported in previous studies whereby test organisms moved away from the source of exposure (Arnold *et al.* 2003) or curled up to avoid interaction with contaminated media (Langdon *et al.* 1999). The increasing accumulation of As with increasing exposure (Figure 5.1) suggests that *L. terrestris* in the mesocosms were successfully exposed to the As contaminated soils. Arsenic BAFs did not increase linearly with dose, decreasing initially between soil As concentrations of 98 and 183 mg kg^{-1} before showing a general increase with soil As concentration (Figure 5.2). The non-linearity of earthworm bioaccumulation has been mentioned elsewhere (Sample *et al.* 1999, Watts *et al.* 2008) and may be due in part to increased excretion of a contaminant in cast material with increasing dose. Although no mortality was observed after 5 weeks exposure, it was not clear if accumulation had begun to equilibrate. Accumulation might have increased further with increasing exposure time; in a study on the time-course of metal accumulation by *L. rubellus*, an exposure period of around 9 weeks in laboratory soils was noted before metal accumulation equilibrates in relation to the substrate (Marino and Morgan 1999).

The fact that soils contaminated with metalliferous mine-waste, such as those at DGC, often contain complex mixtures of potentially toxic metals, makes it difficult to attribute any observed genotoxic effect to a particular metal/metalloid. Of the potentially toxic elements measured in the mesocosm soils only the bioaccumulation of As, Cd, Co, Se, Sn and Sb was positively correlated (r^2 >0.8) to DNA damage in

L. terrestris. The amount of As bioaccumulated from the mesocosm soils was at least 10-fold higher than the other correlating metals. The low levels at which Co, Se, Sn and Sb were present are not generally associated with adverse health effects (ASTDR 1992, 2003, 2004 and 2005b). In particular, Cd was present at levels below those that have been shown to induce DNA damage in exposed earthworms of several species (Fourie *et al.* 2007). It is therefore likely that As is the source of genotoxicity in the mesocosm soils.

A dose response relationship was observed between % tail DNA and increasing As concentration, although, the trend was subject to fluctuations. These findings are comparable with other studies in which the Comet Assay was used to investigate a dose-response in earthworms exposed to soil contaminants. Manerikar *et al.* (2008) demonstrated the genotoxicity of Cr^{VI} to *D. curgensis* showing increasing damage with dose and exposure time, also noting fluctuations in the dose-response. A non-linear dose-response between DNA damage in earthworm coelomocytes and polyaromatic hydrocarbons (PAHs) has also been demonstrated (Qiao *et al.* 2007). In the present study significant levels of DNA damage were induced in *L. terrestris* exposed to As in the mesocosm soils by comparison to the control mesocosm at all concentrations (Figure 5.3). The damage levels observed in the exposed mesocosm earthworms were generally higher than the mean (n = 24) damage levels observed in earthworms collected at three uncontaminated EM sites. Three As exposures (183, 324 and 436 mg kg^{-1} at 5 weeks) were notably higher than the 95^{th} percentile.

Published data on background or normal levels of DNA damage in earthworms is limited. The % tail DNA range reported here for earthworms from uncontaminated soils (16 – 24%) overlap with damage levels reported elsewhere (14 – 19% tail DNA) (Fourie *et al.* 2007) for control earthworms of several species where cells were harvested using a comparable cell extrusion method. Typical background % Tail DNA in human blood cells is around 9.5% ± 5.8 (Møller 2006). It is possible that earthworm cells are more susceptible to damage from the Comet Assay procedure than human cells for which the method employed was developed. Even so, any damage to cells that may have been induced due to the method employed in this study has not impeded the measurement of damage levels well above both the control mesocosm and the damage levels in earthworms from the uncontaminated sites.

Earthworms from DGC demonstrated lower levels of DNA damage than earthworms collected from the uncontaminated EM sites (Figure 5.4) despite soil As concentrations up to 9025 mg kg^{-1} and earthworm As body burdens up to 749 mg kg^{-1}. This confirms the suggestions of other studies that earthworms at DGC have developed a resistance to As toxicity (Button *et al.* 2009, Langdon *et al.* 2003b, Watts *et al.* 2008). No dose-response relationship was observed between DNA damage and As concentration in the soil. The elevated damage levels in the aneceic *L. terrestris* from site 26 with an As concentration of 5760 mg kg^{-1} may be due to a decreased As tolerance or the differing behavioural traits in this species compared to the epegeic *D. rubidus* and *L. rubellus*, whereby aneceic species may have a higher exposure risk than epegeic species (Langdon *et al.* 2003a). The bioaccumulation of

As was similar for *L. terrestris* exposed in the mesocosms and earthworms collected at DGC with little evidence that accumulation was avoided. This suggests that the mechanism for mitigating genotoxic effect in earthworms native to DGC is not behavioural, whereby interaction with contamination is avoided, as has been previously suggested (Langdon *et al.* 1999). It is probable that the tolerance to As in DGC populations is, in part, related to the sequestration of arsenic in the metallothionein-rich chloragogenous tissue (Morgan 1994). In non-resistant species toxic effects are thought to occur when the metal-binding capacity of metallothionein is exceeded (Campbell 1986). Earthworms at DGC may have an increased capacity to sequester As away from sites of adverse action, thereby mitigating the genotoxic effects observed in non resistant *L. terrestris* exposed to a typical DGC soil. It is also likely that a multi-component response mechanism is behind the resistance to arsenic toxicity observed in DGC earthworms. The low levels of DNA damage observed in DGC earthworms (Figure 5.4) could be due to an adaptive response such as the upregulation of a defence mechanism that is only present when cells are challenged with arsenic. This would also help explain why cell damage in DGC earthworms was lower than in earthworms from the uncontaminated soils. The upregulation of glutathione, a sulfhydryl tripeptide involved in the detoxification of reactive oxygen species (ROS), has been demonstrated in cultured human cells challenged with inorganic As (Schuliga *et al.* 2002). Further research is required to identify whether similar processes are involved, in part, for the resistance to arsenic toxicity in earthworms at DGC.

Collecting earthworms from the uncontaminated sites and harvesting the cells using both dissection and extrusion was intended to provide context for the damage levels observed in both the mesocosm exposed earthworms and earthworms from DGC. However, the employment of different cell harvesting methods for the mesocosm investigation (dissection) and field collected earthworms (extrusion) is a limiting factor in making direct comparison of results from the two experiments. However, in future work we propose to use just the extrusion method and this will permit direct comparability between field collected resistant species and non-resistant species exposed *in-vitro.*

5.6 Conclusions

The genotoxicity of a typical As contaminated soil from DGC has been demonstrated using the Comet Assay to detect DNA damage in coelomocytes of non-native *L, terrestris* exposed in laboratory mesocosms. A dose-response was observed between DNA damage and increasing As concentration in the soil. Multi-element correlation analysis of the dose-response between DNA damage and various metals in the DGC soil suggests As is the most likely component in the genotoxicity of the contaminated soil although genotoxic effects from Cd, Co, Se, Sn and Sb cannot be ruled out at this stage. In the earthworms *L. rubellus* and *D. rubidus* native to DGC, not only were DNA damage levels lower than levels observed in earthworms from uncontaminated sites, DNA damage showed no correlation to the As concentration in the host soil despite bioaccumulation up to 749 mg kg^{-1}. These findings provide the first DNA-based evidence of the resistance to arsenic toxicity in earthworms at the former mine site DGC.

Acknowledgements

Funding for this research was provided by the British Geological Survey University Funding Initiative (BUFI), the University of Leicester Geology department for help with storage and maintenance of mesocosms and Dr Mark Cave for his critical assessment of this manuscript.

Chapter 6

Arsenic biotransformation in earthworms from contaminated soils

6.1 Abstract

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 275 to 13080 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.

6.2 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 (Raab *et al.* 2004) 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 (Button *et al.* 2009, Langdon *et al.* 1999, Morgan 1994, Watts *et al.* 2008). 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 (Langdon *et al.* 1999), is unlikely as earthworms from DGC are known to have elevated As body burdens (Button *et al.* 2009, Langdon *et al.* 1999, Watts *et al.* 2008). The biotransformation of highly toxic inorganic As to the less toxic organic species AB has been speculated as a mode of mitigating As toxicity in DGC earthworms (Langdon *et al.* 1999, Watts *et al.* 2008). An alternative mechanism involves the sequestration of arsenic in the metallothionein-rich chloragogenous tissue which separates the intestine from the coelomic cavity (Morgan 1994). 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 (Vijver *et al.* 2004).

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 (Pergantis *et al.* 1997). The occurrence of organic As species and their

biotransformation pathways are well documented in marine organisms such as crustaceans, molluscs, fish and algae (Madsen *et al.* 2000, Nørum *et al.* 2005, Peshut *et al.* 2008). However, less is known about the occurrence and behaviour of As in terrestrial organisms such as earthworms (Geiszinger *et al.* 1998). Until recently the organo-arsenic species AB was thought to be restricted to the marine environment (Ritchie *et al.* 2004), but has now been demonstrated in terrestrial fungi (Slekovec *et al.* 1999) and earthworms (Geiszinger *et al.* 1998, Langdon *et al.* 2003b, Watts *et al.* 2008). The biotransformation pathway for the formation of AB in marine organisms is thought to involve the carbohydrate containing As compounds known as arsenosugars (Smith 2007). Langdon *et al.* (2003a) 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 (Langdon *et al.* 2002). 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 (Geiszinger *et al.* 2002, Watts *et al.* 2008).

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. The aim of this study was 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.

6.3 Methods

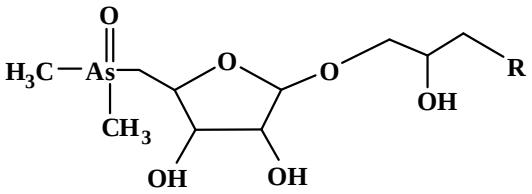
6.3.1 Study site

DGC is one of many former mining sites in southwest 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 (Klinck *et al.* 2002). 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} (Klinck *et al.* 2002, Langdon *et al.* 2001, Watts *et al.* 2008).

6.3.2 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 MA^{V} (Sigma-Aldrich, UK), 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 (Watts *et al.* 2008). These arsenosugar compounds were prepared according to methods published by Madsen *et al.* (2000) and were used for the identification of arsenosugars in earthworm extracts. Figure 6.1 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 conc.), hydrofluoric acid (HF conc.), perchloric acid ($HClO_4$ conc.) and hydrogen peroxide (H_2O_2 30 %) (BDH Aristar, UK) were used for the dissolution of earthworms, cast and soil samples.



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 6.1: Structures of the four arsenosugars

6.3.3 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 (WWC 2008), 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* (Langdon *et al.* 2003a). 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.

6.3.4 Sample digestion

6.3.4.1 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 / $HClO_4$) (Green *et al.* 2006). Samples were weighed directly into 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 50 % v/v 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 2.5 % 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.

6.3.4.2 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 the 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}.

6.3.5 Sample extraction

6.3.5.1 Soils and casts

Extraction of As from soils and earthworm casts was performed using a method developed previously (Button and Watts 2008); see **appendix A**. 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 (Pizarro *et al.* 2003), 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 (Francesconi 2003). 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 (Ruiz-Chancho *et al.* 2005).

6.3.5.2 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).

6.3.6 Instrumentation

6.3.6.1 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.

6.3.6.2 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 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 (Watts *et al.* 2008). 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).

6.4 Results

6.4.1 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 (RSC 2002). 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, see appendix B.

Total As levels in the earthworm host soils covered a wide As concentration range from 255 to 13,080 mg kg^{-1} (Table 6.1). All the soils are highly contaminated when

considered against the current UK Soil Guideline Value (SGV) for As of 20 mg kg^{-1} (Defra 2002a). 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}.

Table 6.1: 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

6.4.2 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 6.2) depending on the As concentration of the host soil. Further details of earthworm species differentiation in terms of As accumulation are presented elsewhere (Button *et al.* 2009, Watts *et al.* 2008). The methanol / water extraction procedure gave variable recoveries of As from prepared earthworm samples (Table 6.2) 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. 6.1) present at the lowest concentrations of the organic species (Table 6.2). The concentration of inorganic As showed a positive correlation with increasing As body burden in the earthworm (Fig. 6.2a). In contrast, organic As species do not demonstrate a

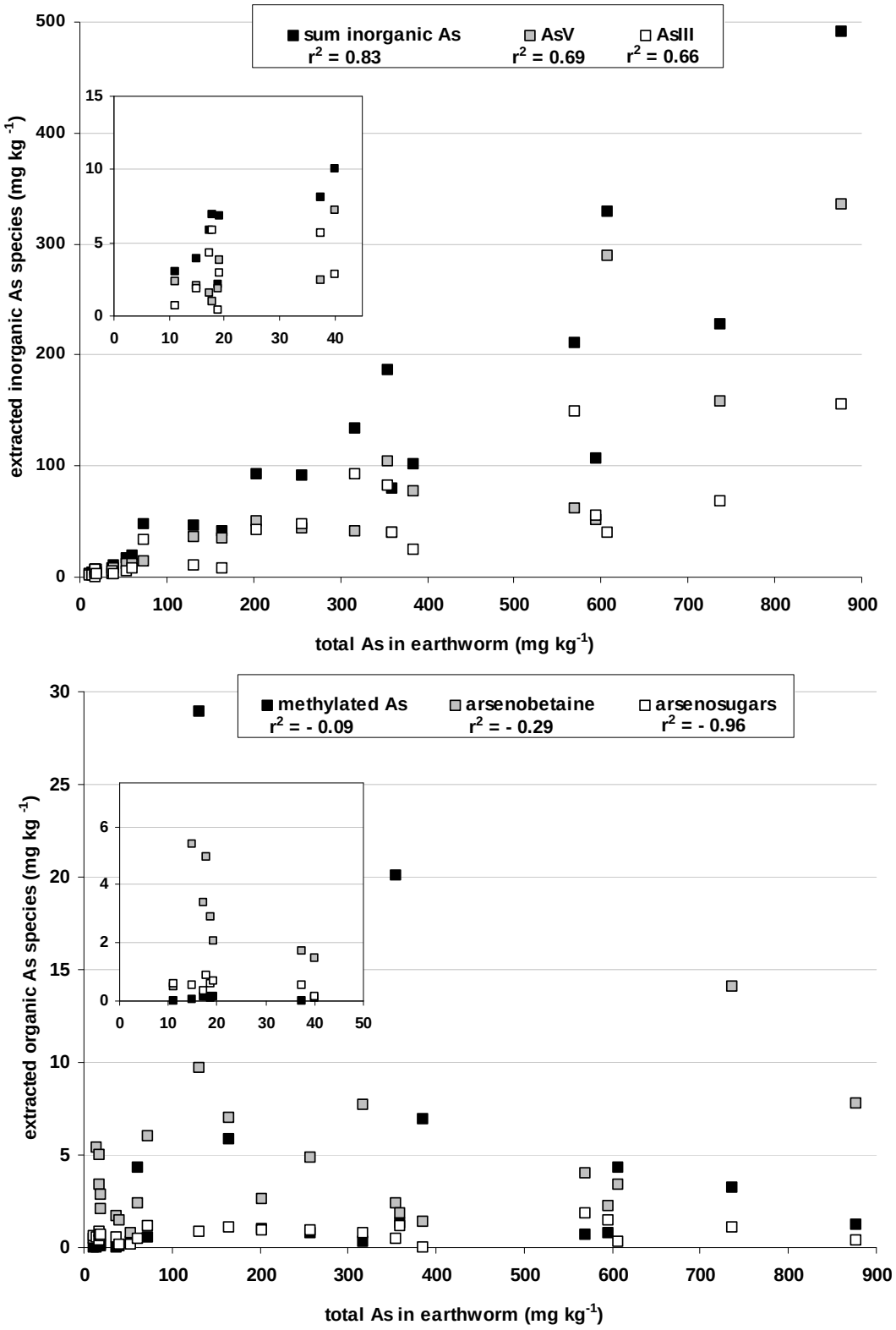
correlation with increasing As body burden (Fig. 6.2b). The organic species remain fairly constant (with some fluctuation) across the range of As body burdens observed.

6.4.3 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 6.1 and 6.3). 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.

Table 6.2: 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



Figures 6.2a/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.

Table 6.3: 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.

	Total As	Extracted As	Extraction efficiency	Speciated As (mg kg^{-1})							Column Recovery
Site id	(mg kg^{-1})	(mg kg^{-1})	(%)	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

6.5 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 (Button *et al.* 2009, Langdon *et al.* 2001, Watts *et al.* 2008). The As body burdens observed in this study are highly elevated up to a maximum of 877 mg kg^{-1} (Table 6.2) 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 (Geiszinger *et al.* 1998). Elsewhere, the biotransformation of As^{V} by soil organisms is considered the source of organic As species in the soil environment (Smith 2007). Likewise, this seems a probable source of the organic species observed in earthworms residing at DGC. The biotransformation pathway proposed by Langdon *et al*. (2003a) 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} coordinated 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 6.2) and elsewhere (Geiszinger *et al.* 2002, Geiszinger *et al.*

1998, Watts *et al.* 2008). A more comprehensive pathway for the formation of both arsenosugars and AB in earthworms (Fig. 6.3) can be hypothesised from what is known about the transformations of As by organisms in marine and freshwater environments (Murray 2003, Ritchie *et al.* 2004). It is more likely that As^{V} ingested from the soil is first converted to DMA^{V} via the Challenger pathway (Smith 2007, Thomas *et al.* 2004) (Fig. 6.3, a) which in turn is converted to arsenosugars through addition of the adenosyl group from *S*-adenosylmethionine (SAM) (Murray 2003). This nucleoside then undergoes glycosidation to produce a range of arsenosugars (Murray 2003, Smith 2007). These arsenosugars are thought to subsequently be converted to AB along a pathway involving either arsenocholine (AC) or dimethylarsinoylacetate (DMAA) (Ritchie *et al.* 2004) (Fig. 6.3, b). The tendency for AB to be more prevalent in earthworm extracts when DMA^{V} and the arsenosugars are also present (Table 6.2 and Fig. 6.2) provides some evidence for this pathway as the source of AB in DGC earthworms.

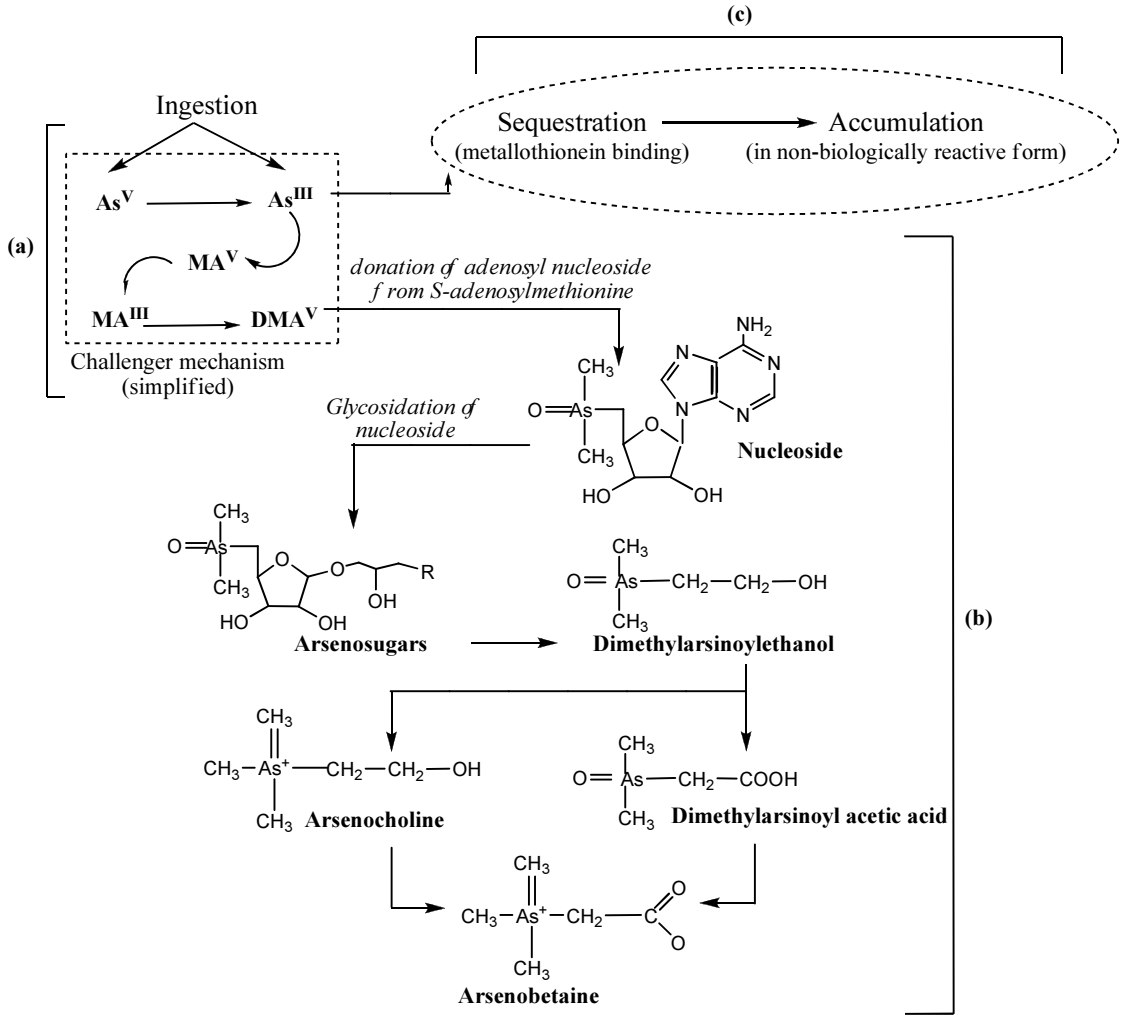


Figure 6.3: Speculative pathway for the biotransformation (a/b) and accumulation (c) of As in resistant earthworms from DGC. Adapted from (Langdon *et al.* 2003a, Murray 2003, Ritchie *et al.* 2004, Smith 2007).

The synthesis of AB in earthworms from highly contaminated soils has been speculated as a potential mechanism for mitigating As toxicity (Langdon *et al.* 2002). However, the fact that AB is only present at low concentrations between 0.5 and 14 mg kg^{-1} (Table 6.2) irrespective of the total As body burden (Fig. 6.2b) 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) 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 (Watts *et al.* 2008). 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 (Langdon *et al.* 2002, Watts *et al.* 2008). 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 (Watts *et al.* 2008), 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. 6.3, c) whereby As is thought to be sequestered via binding with sulphur-rich metallothionein within the chloragogenous tissue (Langdon *et al.* 2002, Morgan 1994, Sturzenbaum *et al.* 2004) allowing the continued accumulation of inorganic As (Fig. 6.2, a) in a form that is not biologically reactive (Vijver *et al.* 2004).

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 6.3) 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 6.3). Trace

levels of arsenosugars in earthworm casts were reported in the study by Geiszinger *et al*. (2002). 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 6.3) 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) 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 of As^{III} alone. This would provide one 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 (Amlund and Berntssen 2004). Whether this process is involved in the regulation of AB in earthworm populations at DGC requires further investigation.

6.6 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 independent processes of arsenic biotransformation occurring in these As resistant earthworms. The first process provides the mechanism for arsenic resistance and involves the sequestration of inorganic As in a form that is not biologically reactive such as binding with sulphur-rich metallothionein within the chloragogenous tissue. The second process is the biotransformation of inorganic As^V along a pathway comparable to those proposed for marine and freshwater organisms. This process is the likely source of the observed organic arsenic compounds but is not involved in the resistance to As toxicity.

Acknowledgments

Funding for this research was provided by the British Geological Survey University Funding Initiative (BUFI). We also thank the Tavistock estate for allowing us to collect samples.

Chapter 7

Human toenails as a biomarker of exposure to elevated environmental arsenic

7.1 Abstract

A pilot study was conducted to determine the applicability of toenails as a biomarker of exposure to elevated environmental arsenic (As) levels. A total of 17 individuals were recruited for the pilot study: 8 residents living near to a former As mine, Devon, UK, forming the exposed group, plus 9 residents from Nottinghamshire, UK, with no anticipated As exposure who were used for comparison as a control group. All toenail samples were thoroughly washed prior to analysis and the wash solutions retained for As determination via ICP-MS to provide an indication of the background environmental As levels for each group. Total As was determined in washed toenail samples via ICP-MS following microwave assisted acid digestion. Concentrations of total As in the toenails of the exposed group were elevated, ranging from 858 to 25,981 µg kg^{-1} (geometric mean = 5406 µg kg^{-1}), compared to the control group whose toenail As concentrations ranged from 73 to 273 µg kg^{-1} (geometric mean = 122 µg kg^{-1}). Higher levels of exogenous As contamination were present on the toenails of the exposed group (geometric mean = 506 µg kg^{-1}) compared to the control group (geometric mean = 4.0 µg kg^{-1}) providing evidence of higher environmental As levels in the exposed group. Total As concentrations in toenail samples were positively correlated to environmental As levels ($r = 0.60$, $p < 0.001$). HPLC-ICP-MS analysis of aqueous toenail extracts revealed inorganic arsenite (As^{III}) to be the dominant species extracted (~ 83 %) with lesser amounts of

inorganic arsenate (As^V) and organic dimethylarsinate (DMA^V) at ~ 13 % and ~ 8.5 % respectively. Arsenic speciation in analysed toenail extracts from the two groups was comparable. The only notable difference between groups was the presence of small amounts (<1 %) of organic methylarsonate (MA^V) in two toenail samples from the exposed group. Toenails are presented as a viable biomarker of exposure at sites with elevated environmental As, such as the former mining sites found throughout Devon and Cornwall, UK.

7.2 Introduction

Arsenic is naturally occurring and ubiquitous in the environment. Humans are exposed to As via contaminated water, food, soil and dust (Mandal and Suzuki 2002). Chronic exposure to As is associated with increases in lung, liver, bladder and kidney cancer, skin keratoses and peripheral vascular disease (Karagas *et al.* 2002, Yoshida *et al.* 2004). Biological markers (biomarkers) can be utilised to make quantitative estimates of exposure to harmful substances (Decaprio 1997) and subsequent risk of disease. There is now increasing interest in the use of human nails as a routine biomarker of As exposure. On ingestion, soluble arsenic is adsorbed from the gastro-intestinal tract and distributed to all bodily systems in the blood, accumulating in many body parts, in particular the keratin rich materials such as hair and nails (Mandal *et al.* 2003). Arsenic is thought to accumulate in hair and nails as a result of its affinity for sulphydryl groups (Wilhelm *et al.* 2005) and remains isolated from the body's metabolic processes both after nail formation and throughout nail growth (Hopps 1977). The long-term accumulation of As in human nails makes them more useful as a biomarker of chronic As exposure than blood or urine in which the residence time of As is at most a few days (Slotnick *et al.* 2007). Human toenail

samples are easy to collect, store and transport, sampling is non-invasive and they are less prone to exogenous contamination than hair samples (Slotnick *et al.* 2007), making them an attractive biomarker of exposure in arsenic contaminated areas (Wickre *et al.* 2004). The exposure–biomarker association is subject to variation due to factors such as age, gender, recreational habits and dietary intake (Slotnick and Nriagu 2006). Toenail growth rates are reportedly slower in females and the elderly (Geyer *et al.* 2004) whilst consumption of seafood or foodstuffs grown in contaminated soil can both be a significant source of exposure (Wilhelm *et al.* 2005). Investigation of these factors via questionnaire data is therefore useful when employing toenails as a biomarker of exposure. The strongest As exposure-biomarker correlations have been observed when total As levels in contaminated drinking water are employed as the measure of exposure (Slotnick and Nriagu 2006). Reported As levels in the toenails of populations considered to be exposed range from 3 µg kg^{-1}, (Slotnick *et al.* 2007) to 37,200 µg kg^{-1}, (Hinwood *et al.* 2003) (based on reported ranges). Lower levels are reported in unexposed or control populations ranging from 12 µg kg^{-1}, (Freeman *et al.* 2004) to 140 µg kg^{-1}, (Karagas *et al.* 1996), however only 3 studies report control data for toenails (Freeman *et al.* 2004, Hinwood *et al.* 2003, Karagas *et al.* 1996). There is little available data on background levels of arsenic in human toenails and levels at which adverse health effects are likely to be incurred. Arsenic levels of 1000 µg kg^{-1} in human hair have been associated with adverse health effect (Hindmarsh and McCurdy 1986). The Canadian government has employed a guideline level of 5000 µg kg^{-1} to indicate a significant increase in exposure (Pan *et al.* 1993). Some evidence has been presented of a link between an increased risk of skin cancer (squamous cell carcinoma) and high levels of toenail arsenic (350 to 810 µg kg^{-1}) (Karagas *et al.* 2001). In an

epidemiological study conducted in Iowa, US, Freeman *et al.* (2004) report toenail arsenic concentration and cutaneous melanoma to be positively correlated, the maximum reported toenail arsenic concentration was 360 µg kg^{-1} in the exposed population. However, in a similar epidemiological study no correlation was observed between toenail arsenic concentration and the risk of bladder cancer (Michaud *et al.* 2004). Correlations between the total As content of toenails and As contaminated drinking water have been demonstrated in numerous studies (Gault *et al.* 2008, Karagas *et al.* 1996, Kile *et al.* 2007). However, only a limited number of studies have investigated the speciation of As in human nails (Gault *et al.* 2008, Mandal *et al.* 2003, Sanz *et al.* 2007). The toxicity of As is known to be species dependent. A better understanding of the potential toxicity of ingested As can be gained through investigation of the resulting metabolites in human tissues. The pathway for inorganic arsenic metabolism involves alternating steps of reduction of As^{V} to As^{III} followed by oxidative methylation of As^{III} to form the methylated arsenic metabolites with S-adenosyl-methionine as the methyl donor (Gebel 2001). Until recently inorganic arsenic, particularly As^{III}, was considered the most toxic chemical form with the methylation of inorganic arsenic considered a detoxification step. There is now strong evidence to suggest that the methylation of arsenic is a process that activates arsenic as both a toxin and carcinogen (Kitchin 2001, Styblo *et al.* 2002). Therefore the identification of As speciation in toenails may be useful in understanding the potential toxicity of ingested arsenic.

The unique geology and historic mining activities of Devon and Cornwall in the UK led to widespread As contamination of the surrounding agricultural land and residential soils (Button *et al.* 2009, Camm *et al.* 2004, Van Elteren *et al.* 2006,

Watts *et al.* 2008). Several studies set out to characterise the As contamination occurring in the southwest UK (Camm *et al.* 2004), including the use of bioindicator species (earthworms) and *in vitro* bioaccessibility testing, to better understand the potential risks associated with exposure to As contaminated soils (Button *et al.* 2009, Langdon *et al.* 2003). Elevated levels of urinary As in residents of former mining sites caused by soil ingestion have been reported in Australia (Hinwood *et al.* 2004) and in two studies conducted in Devon and Cornwall in the UK (Johnson and Farmer 1989, Kavanagh *et al.* 1998). However, there are no reports to date on the sensitivity of toenails as a biomarker of exposure in residents living near to the former mining areas of Devon and Cornwall where environmental As levels are highly elevated. In particular the resulting As speciation in human toenails following exposure to As contaminated soils is yet to be investigated.

The present pilot study aims to improve understanding of the exposure-biomarker relationship between human toenails and elevated environmental As levels. Toenail samples were collected from residents living near to a former As mine in Devon, UK and a control group in Nottinghamshire, UK with no anticipated As exposure in order to: (a) assess As exposure levels in residents of a former As mine by comparison to the control group and (b) compare the resulting As speciation in the toenails of the two populations.

7.3 Methods

7.3.1 Study site

Devon Great Consols (DGC) is situated by the River Tamar in the Tavistock district of Devon (SX 426 735) and is one of many former mining sites in southwest

England. 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 (Klinck *et al.* 2002). Soil arsenic concentrations across the mine site vary significantly depending on their proximity to the main tailings, ranging from 204 - 34,000 mg kg^{-1} (Klinck *et al.* 2002, Langdon *et al.* 2001, Watts *et al.* 2008). More specifically total As in residential soils and soils adjacent to residential properties were shown in a previous study to range from 204 to 9025 mg kg^{-1} (Button *et al.* 2009). Arsenic bioaccessibility in soils in the mine area and mine tailings were previously shown to be well above the 20 mg kg^{-1} soil guideline value (SGV) (Button *et al.* 2009, Cave *et al.* 2002) for gardens and allotments. The Human Bioaccessible Fraction (HBF) estimated using an *in vitro* Physiology-Based Extraction Test (PBET) is reported to range from 10 to 30 % of the total As (Button *et al.* 2009). This equates to bioaccessible As levels between 36 and 1312 mg kg^{-1} in residential soils and soils close to residential properties at DGC (Button *et al.* 2009), giving cause for concern in terms of potential human exposure.

7.3.2 Study group

Ethical approval for this study was provided by the human ethics committee of Nottingham Trent University, UK. Residents living in properties on and around the former mining site of DGC were recruited by post. A total of 22 information packs were posted to 11 properties whose addresses were publicly available. A total of 8 residents from 6 properties volunteered to take part. Information packs contained details of the study, instructions for sample collection, a small sealable polythene sample bag and a questionnaire. Residents were asked to allow toenails to grow for at least two weeks prior to sample collection. The self administered

questionnaire was designed to provide information relevant to assessing potential exposure to As such as age, gender, time in residence at DGC, time spent outdoors around the mine site, whether or not vegetables were grown and eaten from native soil and other potential dietary sources such as seafood and alcohol consumption. A comparable number of volunteers (9) with no anticipated arsenic exposures were also recruited by word of mouth from Nottinghamshire, UK where soil As levels were below the current SGV of 20 mg kg^{-1} (Scheib and Nice 2008).

7.3.3 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). A multi-element standard and tellurium solution (SPEX CertiPrep, UK) were used as calibration standard and internal standard respectively for ICP-MS analysis. Inorganic arsenite (As^{III}, Fisher UK), arsenate (As^{V}, Fisher, UK), methylarsonate (MA^{V}, Sigma-Aldrich, UK), and dimethylarsinate (DMA^{V}, Greyhound, UK) were used for the preparation of standards for arsenic speciation analysis. Concentrated nitric acid (HNO_3) and 30 % v/v hydrogen peroxide (H_2O_2), (BDH Aristar, UK) were used for the dissolution of samples. Ammonium nitrate (> 99 %) and aqueous ammonia (Fisher, UK) were used in preparation of the mobile phase for anion exchange chromatography.

7.3.4 Preparation of toenail samples

Toenail samples were washed thoroughly following a slightly modified version of the protocol described by Slotnick *et al.* (2007), which is comparable to several published methods (Brima *et al.* 2006, Karagas *et al.* 2000, Mandal *et al.* 2003,

Wilhelm *et al.* 2005). Visible exogenous material was firstly removed using plastic forceps and a clean quartz fragment. Samples were then placed in clean glass vials and sonicated for 5 minutes using 3 ml of acetone, rinsed first with 2 ml of deionised water then 2 ml of acetone, sonicated for 10 minutes in 3 ml of deionised water then twice rinsed with 3 ml of deionised water, ensuring complete submersion of the sample during each step. The final rinse solution (3 ml) was retained for immediate analysis by ICP-MS to ensure removal of exogenous contamination was complete. The supernatants from each step of the washing procedure were combined and reduced to dryness in PFA vials (Savillex, USA) on a graphite hot block at 80ºC. The residue was then reconstituted in 3 ml of 1 % HNO_3 for analysis by ICP-MS. After washing, toenails were left to dry at room temperature in a clean laminar flow hood before freeze-drying (Christ, Germany). The freeze-dried toenails were pulverised to a fine powder using a cryogenic freezer mill in 0.5 ml stainless steel cylinders with stainless steel slugs (Spex CertiPrep, UK). Samples were stored in a silica gel dessicator prior to weighing. Certified reference materials NIES CRM 13 human hair (NIES, Onogawa Japan) with a reference As value of 100 µg kg^{-1} and NCS ZC 81002b human hair (NCS Beijing, China) with a certified As value of 198 ± 23 µg kg^{-1} were used throughout. An in-house human fingernail reference material (BGS Bulk) was also prepared using fingernail clippings collected over several months from 4 healthy volunteers not exposed to elevated environmental arsenic. The clippings were pooled and prepared into a fine powder (~ 3000 mg) using the methods outlined.

7.3.5 Quantitative arsenic determination

Toenail samples were acid digested for total As determination using a closed vessel microwave assisted digestion (MARS 5, CEM Corporation, UK). Into each vessel 4 ml of HNO_3 and 1 ml of H_2O_2 was added to accurately weighed pulverised toenail samples and left to stand for 1 hour before sealing the vessels. The average sample mass was 57 ± 29 mg and 75 ± 13 mg for the exposed and control groups respectively. Several individuals from the control group provided sufficient sample for triplicate determinations. The recommended sample mass of 200 mg was used for NCS ZC 81002b, 120 mg for CRM 13 and 100 mg for BGS Bulk. In addition, BGS Bulk was digested in triplicate using the mean sample masses of the exposed and control groups. The microwave heating programme was: 100% power (1200 W), 5 minute ramp to 100 ºC, held for 2 minutes, ramped for 5 minutes to 200 ºC then held for 30 minutes. The pressure in the system was approximately 200 psi under these conditions. This method resulted in complete sample dissolution. The solutions were transferred with MQ water to PFA vials and evaporated to dryness on a hotplate at 110 °C. Samples were reconstituted with 1 ml of 3% v/v HNO_3, heated at 50 °C for 10 minutes and then made up to 3 ml with deionised water to give a final solution of 1 % HNO_3 for direct determination via ICP-MS.

7.3.6 Extraction procedure for arsenic speciation

Aqueous extraction of As species from toenail samples was performed using a closed vessel microwave system (CEM MARS 5, CEM Corporation, UK). The extraction procedure was optimised using human hair CRM NCS ZC 81002b and the BGS Bulk fingernail sample. In addition, at each stage of the extraction optimisation, blank extraction solutions spiked with 10 µg l^{-1} of the As species As^{III}, DMA^{V}, and MA^{V}

were also heated to test species stability, specifically the formation of As^{V} under the potentially oxidising conditions of the extraction procedure. From an initial temperature of 80 ºC the extraction time was increased from 15 - 60 minutes at 15 minute intervals. From the extraction time at which As recovery peaked the temperature was ramped at 10 ºC intervals to the point at which species stability was compromised. Optimum extraction conditions were found to be a 30 minute extraction at 100 ºC. Pulverised toenail samples (30 - 80 mg) were accurately weighed into the HDPE microwave vessels and 5 ml deionised water added before shaking thoroughly by hand to ensure complete wetting of the sample. As the recommended sample mass of 200 mg was used for NCS ZC 81002b and 120 mg for CRM 13, 10 and 6 ml MQ water was added respectively to provide a comparable solid-solution ratio to that of the toenail samples. Following extraction the supernatant was recovered via syringe filtration through 0.45 µm nylon mesh filters (Pall, UK) and stored overnight in pre washed polypropylene tubes at < 4 ºC.

7.3.7 Arsenic determination by ICP-MS

Arsenic determination in toenail digests and extracts was performed by Inductively Coupled Plasma Mass Spectrometry (ICP-MS, Agilent 7500, 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 (Entwisle and Hearn 2006) by

sample spiking. The limit of detection (LOD) for the method expressed as the mean blank signal + 3SD was 0.07 µg kg^{-1}.

7.3.8 Arsenic speciation by HPLC-ICP-MS

A quaternary GP50-2 HPLC Pump and an AS-50 auto-sampler (Dionex, USA) with a 100 µl injection loop were coupled directly to the ICP-MS with PEEK tubing. The two instruments were coupled in such a way that the injection of each sample solution and its subsequent measurement was synchronised automatically using the ICP-MS ChemStation (Agilent, UK) software, enabling reproducible sample injections. Full details of the chromatographic system are published elsewhere (Watts *et al.* 2008). In brief an anion exchange column (PRP–X100, 250 x 4 mm, 10 µm) with a guard column of the same material were used to separate the arsenic species present in the toenail extracts. Ammonium nitrate was used as the mobile phase at pH 8.65 using a gradient elution between 4 and 60 mM to achieve good separation for all As species investigated (Figure 7.1).

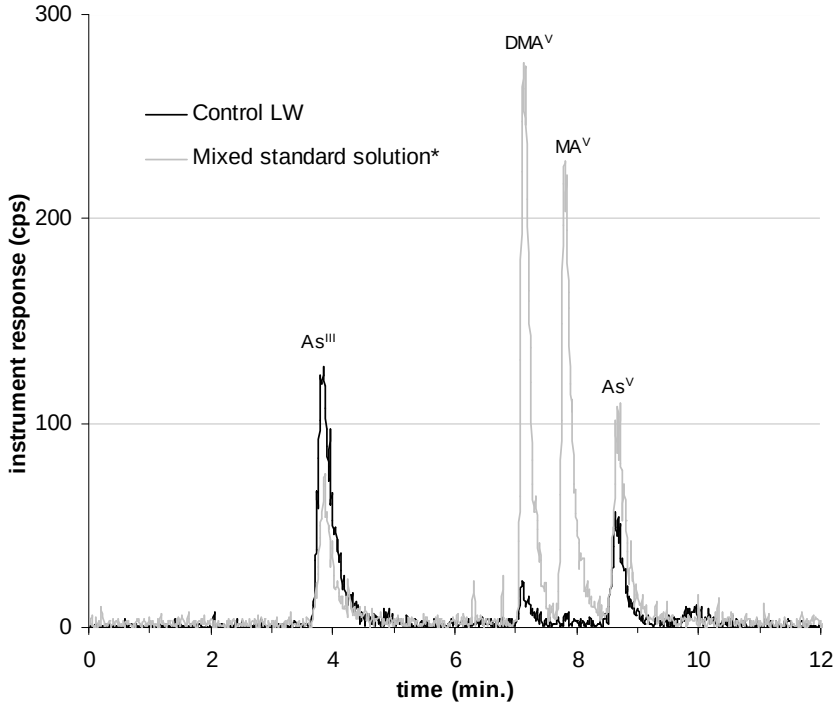


Figure 7.1: HPLC-ICP-MS chromatogram of a mixed standard solution (As^{III} and As^V at 0.2 µg l^{-1}, DMA^V and MA^V at 0.5 µg l^{-1}) and toenail extract Control LW.

7.4 Results

7.4.1 Study participants

Selected demographics for the exposed and control groups are shown in Table 7.1. All participants were white, non smokers of British origin. The mean ages of the exposed and control groups were comparable at 46 and 41 respectively. The male to female ratio for both groups was approximately 2:1. The exposed group spent more hours outdoors in the local area than the control group with mean values of 11 and 5 h/w respectively. The dietary, specifically seafood and alcohol, consumption of the two groups were comparable.

Table 7.1: Summarised demographic, toenail As concentration and exogenous toenail contamination data for the exposed and control groups. * = geometric mean.

	Exposed group			Control group		
	mean ± SD	**min**	**max**	**mean ± SD**	**min**	**max**
Age	46 ± 26	11	67	41 ± 13	25	55
Male/Female (n)	5/3			6/3		
hours outdoors (per week)	11 ± 7	3	21	5 ± 2	2	10
Toenail As ($\mu g\ kg^{-1}$)	5406 *	858	25981	122*	73	273
Exogenous toenail As ($\mu g\ kg^{-1}$)	506 *	102	3784	4.0*	2.1	13

7.4.2 Total Arsenic

All toenail samples were above the method limit of detection for As of 0.07 $\mu g\ kg^{-1}$. The accuracy of the method was assessed by determination of total As in human hair certified reference materials (Table 7.2). The As concentration determined in human hair CRM NCS ZC 81002b was 200 ± 12.8 $\mu g\ kg^{-1}$ (n = 8) well within the certified value of 198 ± 23 $\mu g\ kg^{-1}$. The As concentration determined in human hair CRM NIES 13 was 98.5 ± 6.5 $\mu g\ kg^{-1}$ (n = 6) agreeing well with the reference value of 100 $\mu g\ kg^{-1}$. The method precision was assessed by repeat analysis of the in-house BGS Bulk human fingernail sample. The overall As concentration determined in BGS Bulk was 72.8 ± 5.6 $\mu g\ kg^{-1}$ (n = 7) using a sample mass of 100 mg. Analysis of BGS Bulk using the mean sample masses of the exposed and control groups toenail samples of 57 and 75 mg gave arsenic recoveries of 79.6 ± 8.4 $\mu g\ kg^{-1}$ (n = 3) and 71.0 ± 0.2 $\mu g\ kg^{-1}$ (n = 3).

The total As concentration in toenails of the exposed group ranged from 858 to 25,981 $\mu g\ kg^{-1}$ with a geometric mean of 5406 $\mu g\ kg^{-1}$ (n = 8) (Table 7.1). Total arsenic concentrations in the control group toenails ranged from 73 to 273 $\mu g\ kg^{-1}$

with a geometric mean of 122 µg kg^{-1} (n = 9). Geometric means were used due to both datasets being positively skewed. Analysis of the wash solutions (corrected for sample mass) revealed exogenous contamination on the toenails of the exposed group ranged from 102 to 3784 µg kg^{-1} with a geometric mean of 506 µg kg^{-1}. All wash solutions for the exposed group were above the method limit of detection. Exogenous As contamination on the toenails of the control group (3 of which were below the limit of detection) ranged from 2.1 to 12.8 µg kg^{-1}, with a geometric mean of 4.0 µg kg^{-1}. Arsenic was detected in 6 out of 8 final rinse solutions for the exposed group ranging from 10 to 214 µg kg^{-1} with a geometric mean of 37 µg kg^{-1}. In the control group only 3 out of 9 final rinse solutions contained detectable levels of As ranging from 1.0 to 3.8 µg kg^{-1} with a geometric mean of 2.0 µg kg^{-1}.

Table 7.2: Data relating to the extraction and speciation of As in reference materials and toenail samples. Certified total As value for ZC 81002b and reference value for CRM 13 are shown in brackets. Extraction efficiency = extracted As / total As x 100. Column recovery = sum species / extracted As × 100. * = single determination. <LOD = below limit of detection.

Sample mean	Total As (µg kg^{-1})	Extracted As (µg kg^{-1})	Extraction efficiency (%)	As species (µg kg^{-1})				Sum species (µg kg^{-1})	Column recovery (%)
				As^{III}	DMA^{V}	MA^{V}	As^{V}		
ZC 81002b (human hair)	200 (198)	119	60	51	2.9	0.9	53	108	91
SD (n = 8)	*13*	*6.1*		*29*	*0.7*	*0.7*	*4.8*	*6.9*	
CRM 13 (human hair)	99 (100)	43	44	19	3.7	<LOD	20	43	100
SD (n = 6)	*6.5*	*2.8*		*3.1*	*1.9*		*4.13*	*4.4*	
BGS Bulk (fingernail)	74	51	70	27	6.4	<LOD	6.5	40	78
SD (n = 13)	*6.2*	*6.8*		*5.3*	*2.8*		*3.4*	*5.8*	
Control LW	85	33	39	22	2.3	<LOD	6.0	30	91
(SD (n = 3)	*37*	*7.9*		*5.2*	*0.2*		*1.5*	*4.2*	
Control JB*	99	52	52	44	3.3	<LOD	4.9	52	100
Control MW	101	59	59	51	<LOD	<LOD	2.2	53	90
SD (n = 3)	*3.1*	*9.7*		*3.2*			*2.3*	*1.7*	
Control VB	184	69	38	49	3.9	<LOD	5.8	59	86
SD (n = 3)	*28*	*19*		*17*	*1.6*		*4.8*	*14*	
Control MB	273	161	59	115	3.9	<LOD	36	155	96
SD (n = 3)	*17*	*19*		*4.3*	*0.2*		*3.2*	*10*	
Exposed 16*	2987	2031	68	1743	37	6.4	106	1892	93
Exposed 11*	25981	14176	55	11477	84	73	2899	14533	103

7.4.3 Optimisation of extraction procedure for As speciation

The impact of increased extraction time (extraction temperature = 80^{o}C) on the recovery of As species from BGS Bulk fingernail reference sample and Human hair CRM ZC81002b are displayed in Figures 7.2a and 7.2b respectively. The extraction efficiency (extracted As / total As × 100) for ZC81002b peaked at 30 minutes but continued to increase with time up to 60 minutes for BGS Bulk. The recovery of As^{V} was greatest at 30 minutes for both samples with a trend of decreasing recovery of As^{V} with time most evident in the BGS Bulk sample. Recovery of the remaining As species detected in BGS Bulk (As^{III} and DMA^{V}) showed a gradual increase with increasing extraction time. For CRM ZC 81002b extraction times above 30 minutes had little impact on the recovery of the detected As species (As^{V}, As^{III}, DMA^{V} and MA^{V}). For this reason 30 minutes was taken as the optimum extraction time for investigating the effects of increased temperature on species recovery. Figures 7.2c and 7.2d display the effects of increasing temperature on the species recovery for the same two samples. BGS Bulk fingernail sample demonstrated a gradual increase in species recovery with each temperature increment with the proportion of species remaining fairly constant. Increasing extraction temperature had a lesser impact on the recovery of species from the hair CRM ZC81002b with the exception of increased recovery of As^{III} between 80 and 100 ºC. No inter-conversion of species was observed in the blank solutions spiked with 10 µg l^{-1} of each As species and subjected to the extraction cycle (Table 7.3) with good recovery of all species under the chromatographic conditions employed. A limited supply of the BGS Bulk reference sample precluded further optimisation of the extraction procedure therefore the method applied to toenail samples, based on the present findings, was taken as a 30 minute extraction at 100 ºC.

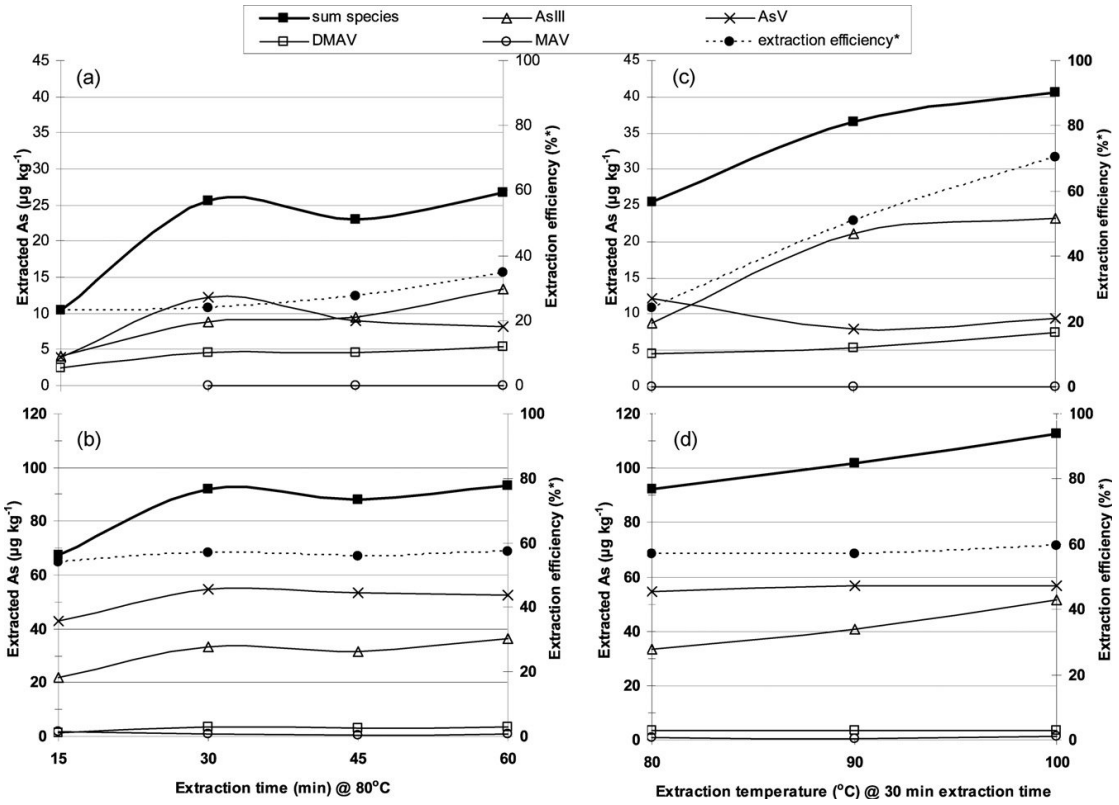


Figure 7.2: Effect of extraction time at 80 ºC on the recovery of As species from (a) BGS Bulk fingernail reference sample and (b) Human hair CRM ZC81002b. Effect of extraction temperature with a 30 minute extraction time on the recovery of As species from (c) BGS Bulk fingernail reference sample and (d) Human hair CRM ZC81002b. * = extracted As / total As x 100.

Table 7.3: Recovery of As species from blank extraction solutions spiked with 10 µg l^{-1} of each species at each step in the optimisation of extraction procedure. Errors are 1* SD (n = 3).

Extraction method	As^{III}	DMA^{V}	MA^{V}
15 min / 80°C	9.7 ± 0.4	8.9 ± 0.3	10.1 ± 0.3
30 min / 80°C	10.0 ± 0.1	9.0 ± 0.1	10.5 ± 0.3
45min / 80°C	10.1 ± 0.3	8.7 ± 0.4	10.5 ± 0.1
60min / 80°C	10.1 ± 0.1	9.0 ± 0.3	10.4 ± 0.1
30min / 90°C	9.4 ± 0.1	10.0 ± 0.2	9.9 ± 0.1
30min / 100°C	9.5 ± 0.2	10.1 ± 0.1	10.0 ± 0.1

7.4.4 As speciation in toenails

Determination of As species in toenail extracts via HPLC-ICP-MS was performed where sufficient sample material was provided by the participant. This equated to two participants in the exposed group and five participants in the control group. Table 7.2 displays data relating to the extraction and speciation of As in these toenail samples, the human hair reference materials and the BGS Bulk fingernail sample. The extraction procedure employed resulted in a mean recovery of As from toenails of 53 ± 12 % (n = 7). The highest As recovery was obtained for the BGS Bulk fingernail sample at 70 %. The two human hair reference materials ZC 81002b and CRM13 gave differing As recoveries of 60 and 44 %, respectively. The mean column recovery for toenail samples was 94 ± 7 % (n = 7). An HPLC-ICP-MS chromatogram demonstrating the peak retention and resolution for investigated As species is shown in Figure 7.1. The proportional As speciation observed in the reference materials and toenail samples under investigation are displayed in Figure 7.3. The dominant species extracted from toenail samples was inorganic As^{III} at an average proportion of 83 ± 9 % (n = 7). Inorganic As^{V} was present in all the toenail extracts but a lower mean proportion was extracted at 13 ± 8 % (n = 7). DMA^{V} was detected in 6 of the 7 toenail extracts at an average proportion of 4 ± 3 % (n = 6), with MA^{V} only detectable in the 2 samples from the exposed group at less than 1 % of the extracted As.

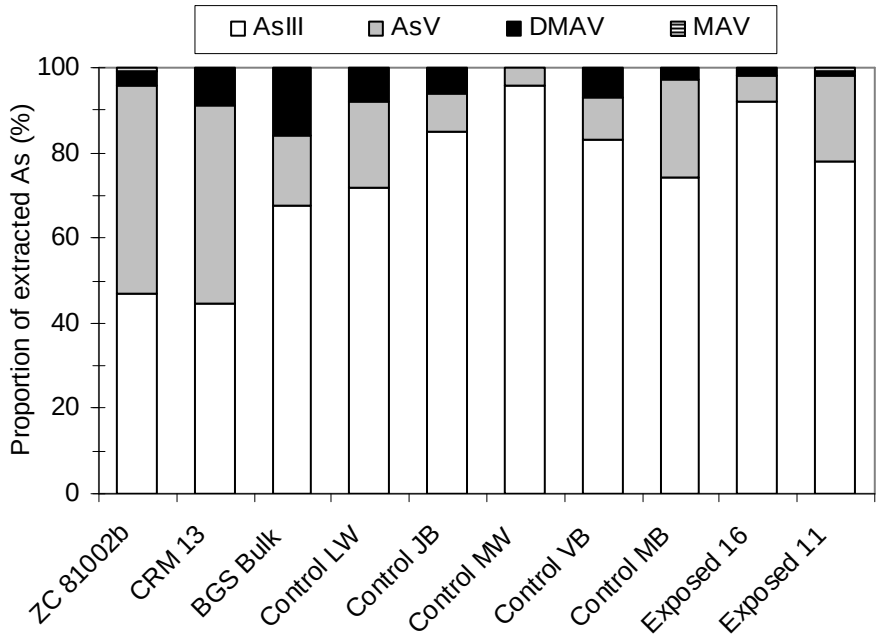


Figure 7.3: Individual As species as a percentage of the extracted As for each sample investigated. Toenail samples (Control LW onwards) are plotted in order of increasing total As.

7.5 Discussion

Total As concentrations in toenails from the exposed group are elevated compared to the control group with geometric means of 5406 and 122 µg kg^{-1} respectively. The Wilcoxon Signed Rank Test (SPSS version 14) revealed the difference between the exposed and control groups was significant ($p < 0.05$). The increased number of hours spent outdoors by the exposed population may be significant in providing increased exposure to environmental As (Table 7.1). Other potentially influential factors such as age, gender and diet were comparable between the two groups. The toenail As concentration range of the exposed group in this study (858 to 25,981 µg kg^{-1}) falls within the overall range reported in the literature (Figure 7.4) of 3 to 37,200 µg kg^{-1} for exposed populations. The geometric mean toenail As

concentration (122 µg kg^{-1}) for the control group is comparable with values reported in the literature for non-exposed individuals at 40 µg kg^{-1} (Freeman *et al.* 2004) and 140 µg kg^{-1} (Karagas *et al.* 1996). Toenail As concentrations in excess of the maximum reported in this study (25,981 µg kg^{-1}) have only been reported in the study by Hinwood *et al*. (2003), who found an upper range concentration of 37,200 µg kg^{-1} in the toenails of individuals living in areas with high environmental As concentrations. Failure to quantify the degree of exogenous contamination was cited as a limitation of the study. In the present study, quantification of exogenous As contamination revealed significantly ($p = <0.05$) higher levels of exogenous As on the toenails of the exposed group compared to the control group using the Wilcoxon Signed Rank Test. Exogenous As contamination was also positively correlated with total toenail As (Figure 7.5). Quantification of exogenous As in this way provides a useful measure of environmental As levels for the two study populations, highlighting the high levels of environmental As in the exposed population by comparison to the control group (Table 7.1). The very low levels of As in the final rinse solutions for both groups suggested that the toenail washing procedure was effective in removing exogenous contamination, however more As was present in the final rinse solutions of the exposed group. The As observed in the final rinse solutions from the toenail washing procedure may be due to very low levels being leached out during the final rinse step. Mandal *et al.* (2003) found that As was released from pre-washed fingernail samples after soaking at room temperature in deionised water although the amount of As leached was reported to be negligible at 0.4 to 1.4 % of the total As in the fingernails. In the present study the As content observed in the final rinse solutions was also negligible ranging from 0.2 to 1.6 % of the total As in the toenail samples.

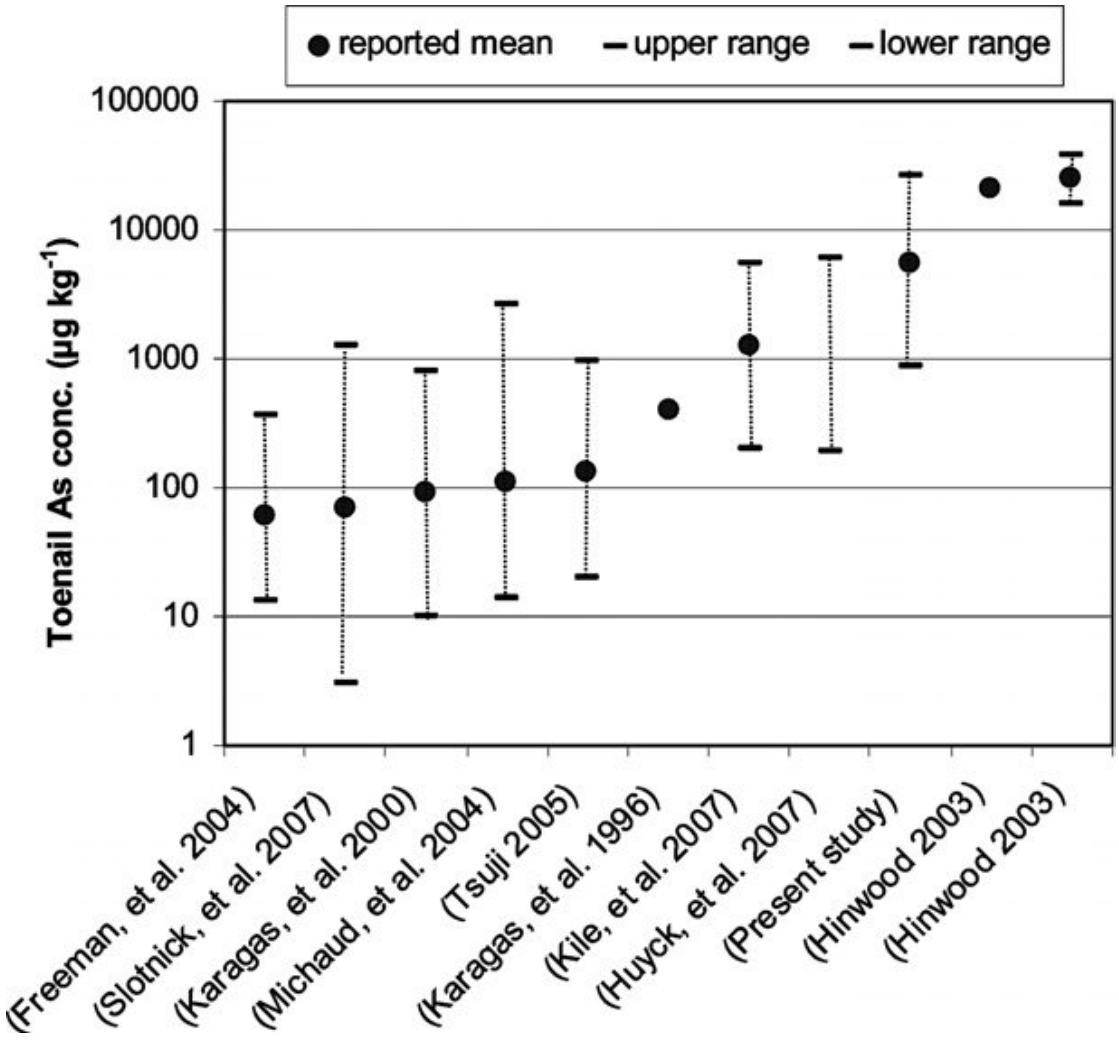


Figure 7.4: Comparison of the toenail As content of exposed populations reported in recent studies to that in the exposed group in the present study.

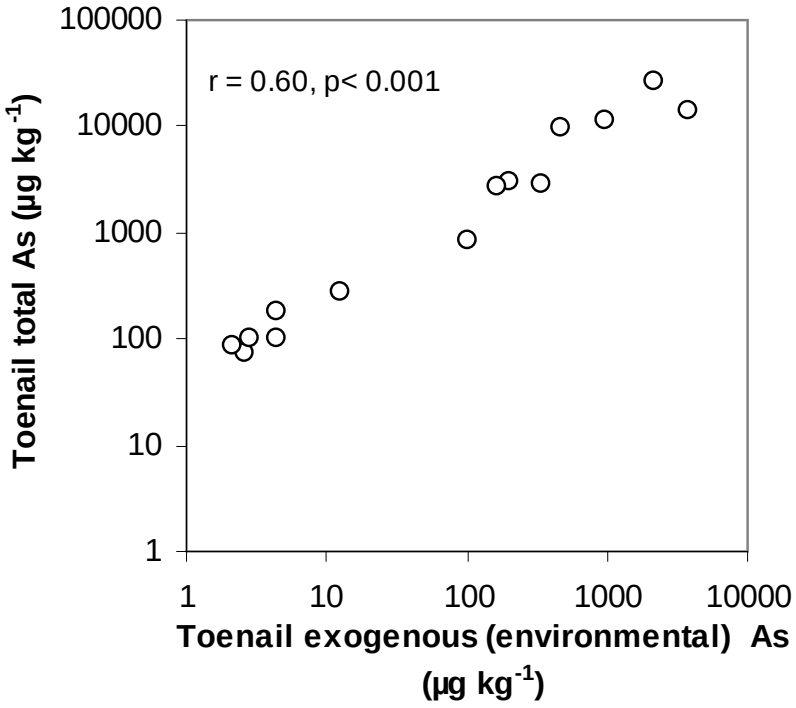


Figure 7.5: Correlation between toenail exogenous As contamination (environmental As) and toenail total As content. Based on exposed and control datasets combined.

Several previous studies have investigated As exposure in the southwest of England. In a limited study Johnson and Farmer (1989) noted slightly elevated levels of urinary As in Cornwall residents. Kavanagh *et al.* (1998) found significantly higher ($p = 0.01$) levels of total As in the urine of residents at DGC and the nearby town of Gunnislake compared to residents of an uncontaminated area. These papers conclude that the studied populations at DGC and Gunnislake were chronically exposed to As and that exposure resulted from the ingestion of As contaminated soil and dust. The use of urine as a biomarker of As exposure is more suited to assessing acute exposure since arsenic is excreted in the urine within hours of ingestion (Slotnick and Nriagu 2006), whereas toenails provide an integrated measure of exposure over a longer period, 12 - 18 months prior to sample collection

(Slotnick *et al.* 2007). For this reason toenails may represent a more suitable biomarker of exposure in populations chronically exposed to As.

During optimisation of the extraction procedure, the recovery of As^{V} from both the BGS Bulk fingernail sample and the human hair CRM ZC 81002b peaked after extracting for 30 minutes at 80 ºC (Figure 7.2a/b). It can also be observed from Figure 7.2a that after 30 minutes the recovery of As^{III} increases slightly whilst As^{V} recovery decreases. Similar trends were reported by Mandal *et al*. (2003) who found that As^{V} was reduced to As^{III} when fingernail samples were soaked at room temperature for 72 hours, suggesting the reducing capacity of fingernails in aqueous solution. Raab and Feldman (2005) suggested that boiling releases or activates strong reducing agents in human hair and reported the transformation of DMA^{V} to its sulphur analogue DMAS. The results presented here (Figure 7.2a-d) suggest that at temperatures below 100ºC, extraction time is more influential on the reduction of pentavalent arsenic species, as the recovery of As^{V} is more constant across the temperature range of 80 to 100 ºC (30 minute extraction time) than across the extraction time range of 15 to 60 minutes (80 ºC extraction temperature).

The microwave assisted extraction method developed in this study achieved a mean recovery of As from toenail samples of 53 % (Table 7.2). There are few reported studies to which this extraction efficiency can be compared. Raab and Feldman (2005) reported an extraction efficiency of 57 ± 26 % in hair samples using boiling water for 6 hours, but with species instabilities. Shraim *et al*. (2001) reported a 39 % extraction efficiency for CRM 13 when shaken for 30 minutes with water. For comparison the extraction method presented here achieved a slightly improved

recovery of As from CRM 13 of 44 % (Table 7.2). In a study on As speciation in human fingernails and hair from an As-affected area, Mandal *et al*. (2003) report a mean recovery of As in fingernails of 63 % (range 50 to 75 %) using a 30 minute water extraction at 90 ºC, slightly higher than the 53 % recovery of As from toenails reported in this study. However the extraction method developed in the present study achieved an As recovery of 70 % (Table 7.2) when applied to the BGS Bulk fingernail sample. This suggests that As is more strongly bound in toenails than fingernails.

Arsenic speciation data for human hair CRM 13 were reported in two previous studies. Mandal *et al*. (2003) reported As^{III} to be the dominant species present at 56 % with lesser amounts of As^{V} and DMA^{V} at 35 % and 9 % respectively. Shraim *et al*. (2001) reported very different proportions of each As species with As^{V} present at 66 % of extracted As, whilst the remaining As was found to be divided into similar amounts between As^{III}, DMA^{V} and MA^{V} at 16 %, 7 % and 11 % respectively. In the present study As^{III} and As^{V} were found to be present in similar proportions of 44 % and 47 % of the extracted As (Figure 7.3) with DMA^{V} present at 9 %. Species stability under the extraction procedure employed was reported in both the two previous studies and in the present study. However, only the proportion of DMA^{V} reported is comparable across the three studies. This may be due to the differing extraction procedures employed and the subsequent extraction efficiencies. Both Mandal *et al*. (2003) and Shraim *et al*. (2001) used water as the sole extractant. Mandal *et al*. (2003) heated their samples to 90 ºC for 30 minutes, achieving a 98 % As recovery. Shraim *et al*. (2001) employed mechanical shaking at ambient temperature achieving a lower extraction efficiency of 39 %. The extraction

procedure employed in the present study is more comparable to the method used by Mandal *et al*. (2003) and likewise resulted in more comparable results for As speciation in human hair CRM 13. This disparity between the reported As speciation in CRM 13 highlights the need for reproducible extraction methodologies if As speciation is to be incorporated into studies looking at biomarkers of exposure to As in human populations. In a study of As in the hair and nails of individuals exposed to As-rich groundwaters in Kandal province, Cambodia, Gault *et al*. (2008) used X-ray absorption near edge structure (XANES) spectroscopy to probe the As speciation of fingernail samples directly, precluding the necessity of an extraction step. Using XANES spectroscopy Gault *et al*. (2008) reported As^{III} to be the principal As species in fingernail samples but found considerable variability between samples and problems with the fitting of spectra were reported. In the present study inorganic As^{III} was the dominant As species in toenail samples consistently (Figure 7.3) with lesser amounts of As^{V} and DMA^{V}. Arsenic speciation was similar in the toenails of both the control and exposed samples analysed. The only notable difference was the detection of small amounts of MA^{V} in the two toenail samples from the exposed group and is likely due to the higher As concentration in the toenails of the exposed group giving rise to detectable levels of MA^{V}.

7.6 Conclusion

The As concentrations observed in the toenail samples collected from residents of the former mine site (DGC) are elevated suggesting chronic exposure to high environmental As. The quantification of exogenous As contamination on the toenails of both the exposed and control groups in this study provides a useful

measure of environmental As levels as evidence of the extent of potential As exposure. A positive correlation between exogenous toenail As (removed during the wash procedure) and total toenail As demonstrates a link between the source of exposure (environmental As) and toenail As content. Inorganic As^{III} is the dominant extractable As species in toenail samples from both the exposed and control group with lower amounts of inorganic As^{V} and DMA^{V}. The data presented highlights the sensitivity of toenails as a biomarker of exposure to high environmental As in areas such as the southwest, UK where historic mining activity has led to widespread As contamination.

Acknowledgments

Funding for this research was provided by the British Geological Survey University Funding Initiative (BUFI). We thank the participants for taking part in this research. We also thank Dr Timothy Heaton for providing assistance with sample preparation at the NERC Isotope Geosciences Laboratory (NIGL) and Dr Mark Cave for reviewing the manuscript.

Chapter 8 - General Discussion

The potential for human exposure to As contaminated soils is a significant issue in the southwest UK. An area of around 700 km^2 is reported to be contaminated due to the historic mining of As in the region (Abrahams and Thornton 1987). The average population density of the southwest is 142 people per km^2 (ONS 2002). In theory this equates to as many as 100,000 people being at risk of exposure to environmental As contamination. This figure may be exaggerative as the population density of contaminated areas is likely to be lower than average, however the likelihood is that large numbers of people are at real risk of exposure. The current SGV for As in residential soils of 20 mg kg^{-1} (Defra 2002) is unrealistic in Devon and Cornwall and requires further consideration. In order to better understand the health risks associated with As contaminated soils an improved comprehension of the actual extent of exposure is needed.

The consideration of many inter-related components is required to gain an holistic understanding of exposure and subsequent health risks. By considering only the total amount of As in the soil the current SGV for As is perhaps unnecessarily conservative. Estimating the arsenic fraction bioaccessible to humans using a PBET can provide a more realistic estimate of potential exposure, yet uncertainties surrounding the reproducibility of PBETs have hindered their incorporation into the contaminated land risk assessment process. In response to this we have investigated the use of earthworms as a complimentary line of evidence in understanding the potential for human exposure at As contaminated sites. In **chapter 4** we provided evidence of a relationship between *in vitro* human bioaccessibility and the

bioaccumulation of As by earthworms. This finding indicates the potential for the incorporation of multiple lines of evidence when assessing potential exposure at contaminated sites as a more holistic alternative to relying on a single guideline value. This research is also important in highlighting the potential for As resistant earthworm species as an indicator of As mobility at sites with highly elevated levels of arsenic. Further work is required to validate the potential of earthworms as an indicator species for this purpose. It would be revealing to calculate the accumulation of As from a known quantity of ingested soil to give a better indication of the bioavailability of As rather than the total accumulation over an indefinite period of time.

Bioaccumulated As levels of up to 877 mg kg^{-1} reported in **chapter 4** clearly show that earthworms at DGC do not avoid arsenic uptake from the soil. Despite these elevated As body burdens there have been no reports of adverse health effects in earthworms at DGC. In **chapter 5** we provide the first DNA based evidence of the resistance of DGC earthworms to arsenic toxicity via use of the Comet Assay. In this chapter it was also demonstrated that a typical DGC soil is toxic to non-native earthworms, clearly demonstrating a developed resistance to As in DGC earthworm populations. The Comet Assay may therefore be useful both in screening out resistant species to avoid their use as ecotoxicological indicators of risk at As contaminated sites and in mesocosm experiments that employ non resistant species to assess the toxicity of a contaminated soils. The use of a single harvesting method for the collection of earthworm coelomocytes throughout the Comet Assay experiments presented here would have improved comparison between mesocosm exposures and field collected earthworms. Also the incorporation of standard cell lines in each batch of experiments would have been useful as a measure of reproducibility. In future all experiments would employ the

same cell extrusion method and ideally a cultured cell line of earthworm coelomocytes for the purpose of standardisation. Further investigation of the additive or synergistic toxicity of multiple elements in the soil would also allow the relative toxicity of As alone to be better understood.

If resistant earthworm species are to be employed as a tool in understanding health risks at contaminated sites it is important to try and understand the mechanisms underlying the resistance. The results of the speciation analysis carried out in this study via HPLC-ICP-MS (**chapter 6**) show that the formation of AB and other organo-arsenic species previously undetected in DGC earthworms is not related to the level of As accumulated by the earthworm. The detected organo-arsenic species AB and arsenosugars were only present at very low levels by comparison to inorganic arsenate and arsenite. This new evidence precludes the transformation of arsenate from ingested soil to arsenobetaine in the worm as a detoxifying mechanism as has been suggested previously (Langdon *et al.* 2002). This finding is an important step towards understanding the true mechanism by which earthworms at DGC mitigate As toxicity. One limitation of this research was the inability to detect As-sulphur complexes and the more unstable trivalent methylated As species. Such information will become increasingly available with the development of techniques such as XAS and XANES (Smith 2007) that can provide in-situ speciation data.

The most important component in understanding the health risks of contaminated soils is quantifying the degree to which people are actually exposed. This can only be achieved via biomonitoring studies of exposed populations. Elevated levels of urinary As in DGC residents compared to a control group had been reported over a decade ago

(Kavanagh *et al.* 1998) yet no follow up to this study was undertaken. A single measure of urinary As is not an ideal biomarker of exposure to contaminated soils as the residence time for As in urine following ingestion is only around 24 hours (Slotnick *et al.* 2007). Any ingestion of contaminated soil is likely to be sporadic making longer-term integrated measures of exposure more suitable. Urinary As is also easily confounded by dietary sources of As, particularly organic AB from seafood. **Chapter 7** demonstrated how toenails are potentially more suitable as a biomarker of exposure to As contaminated soils by investigating a longer-term measure of exposure. Analysis by ICP-MS of toenail samples from DGC residents revealed As levels to be highly elevated compared to a control group. By analysing the external As contamination on toenail samples we were also able to provide some evidence of a link between ambient environmental As levels and toenail As content. This final chapter is significant in showing clearly that residents at DGC are exposed to elevated levels of environmental As. The study is limited by the number of participants yet the difference in toenail As content between the control and exposed group is striking. Whilst toenails can be used as biomarker of exposure to As the associated health risks remain unclear.

The emphasis of future research should be shifted from predicting potential exposure to quantifying actual exposure and subsequent health risks. Further work should therefore:

- Recruit a large number of volunteers from different areas in the southwest UK. This would help to reveal the extent of any excessive exposure, facilitate correlations with background levels of environmental As and allow the incorporation of epidemiological evidence of As related health effects such as increases in the incidence of skin cancers.
- Compare and contrast biomarkers for quantifying both long-term (toenails,

fingernails and hair) and short-term (urine, blood) exposure to As. It would also be possible to analyse a time series of As deposition in a toenail using a technique such as laser ablation-ICP-MS. In doing so we could begin to understand how toenail As levels might be influenced by factors such as growth rates and seasonality.

- Consider sources of exposure other than soil, particularly the ingestion/inhalation of windblown particulates and house dusts to gain a better understanding of the contribution of different sources of As to overall exposure. Investigating multiple elements and or isotope ratio fingerprints in the soils and dusts and comparing these to elements observed in the biomarkers could also help identify the most significant sources of exposure.
- Assess biomarkers of effect in exposed human populations. The Comet Assay could be employed to assess DNA damage in lymphocytes and correlations with exposure biomarkers.

Further research of this kind would provide much needed health risk data on the actual extent of human exposure to As in the southwest UK. Once the significance of any exposure has been established we can then begin to understand the risks associated with elevated As levels in this region of the UK and whether or not mitigation steps are required.

References

Abrahams, P.W. and Thornton, I. 1987. Distribution and extent of land contaminated by arsenic and associated metals in mining regions of southwest England. *Transactions of the Institution of Mining & Metallurgy (Section B: Applied Earth Science)*, **96**, 1-8.

Ackley, K.L. and Caruso, J.A. 2004. Separation Techniques. 147-239 In: Handbook of Elemental Speciation: Techniques and Methodology. R. Cornelis, J. A. Caruso, H. Crews and K. Heumann. Wiley.

Adair, B.M., Waters, S.B., Devesa, V., Drobna, Z., Styblo, M. and Thomas, D.J. 2005. Commonalities in metabolism of arsenicals. *Environmental Chemistry*, **2**, 161-166.

Agilent. 2005. *ICP-MS: Inductively Coupled Plasma Mass Spectrometry A Primer*. Agilent Technologies Inc, USA Publication No. 5989-3526EN.

Ahmad, S., Kitchin, K.T. and Cullen, W.R. 2000. Arsenic species that cause release of iron from ferritin and generation of activated oxygen. *Archives of Biochemistry and Biophysics*, **382**, 195-202.

Amlund, H. and Berntssen, M.H.G. 2004. Arsenobetaine in Atlantic salmon (*Salmo salar L.*): influence of seawater adaptation. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology*, **138**, 507-514.

Aposhian, H.V., Gurzau, E.S., Le, X.C., Gurzau, A., Healy, S.M., Lu, X., Ma, M., Yip, L., Zakharyan, R.A., Maiorino, R.M., Dart, R.C., Tircus, M.G., Gonzalez-Ramirez, D., Morgan, D.L., Avram, D and Aposhian, M.M. 2000. Occurrence of Monomethylarsonous Acid in Urine of Humans Exposed to Inorganic Arsenic. *Chemical Research in Toxicology*, **13**, 693-697.

Arnold, R.E., Langdon, C.J., Hodson, M.E. and Black, S. 2003. Development of a methodology to investigate the importance of chemical speciation on the bioavailability of contaminants to *Eisenia andrei*: The 7th international symposium on earthworm ecology, Cardiff, Wales, 2002. *Pedobiologia*, **47**, 633-639.

Ash, C.P.J. and Lee, D.L. 1980. Lead, cadmium, copper and iron in earthworms from roadside sites. *Environmental Pollution*, **22**, 59-67.

ASTDR. 1992. *Toxicological profile for Antimony*. Agency for Toxic Substances and Disease Registry PB/93/110641/AS.

ASTDR. 2000. *Arsenic Toxicity: Case Studies in Environmental Medicine*. Agency for Toxic Substances and Disease Registry HE-CS-2002-0003.

ASTDR. 2003. *Toxicological profile for Selenium*. Agency for Toxic Substances and Disease Registry PB2004-100005.

ASTDR. 2004. *Toxicological profile for cobalt*. Agency for Toxic Substances and Disease Registry PB 2004-10733.

ASTDR. 2005a. *Toxicological Profile for Arsenic (Draft for Public Comment)*. Agency for Toxic Substances and Disease Registry PB/93/182376/AS.

ASTDR. 2005b. *Toxicological profile for Tin*. Agency for Toxic Substances and Disease Registry PB2006-100006.

BARGE 2008. Bioaccessibility Research Group of Europe. World Wide Web Address: http://www.bgs.ac.uk/barge/home.html.

Barton, D.B. 1964. *A Historical Survey of the Mines and Mineral Railways of East Cornwall and West Devon*. Bradford Barton. Truro.

Basu, A., Mahata, J., Gupta, S. and Giri, A.K. 2001. Genetic toxicology of a paradoxical human carcinogen, arsenic: A review. *Mutation Research - Reviews in Mutation Research*, **488**, 171-194.

Beak, D.G., Basta, N.T., Scheckel, K.G. and Traina, S.J. 2006. Bioaccessibility of arsenic(V) bound to ferrihydrite using a simulated gastrointestinal system. *Environmental Science & Technology*, **40**, 1364-1370.

Bentley, R. and Chasteen, T.G. 2002. Arsenic curiosa and humanity. *Chem. Educator*, **7**, 51-60.

Bissen, M. and Frimmel, F.H. 2003. Arsenic - A Review. Part I: Occurrence, Toxicity, Speciation, Mobility. *Acta Hydrochimica et Hydrobiologica*, **31**, 9-18.

Blank, S., Seiter, C. and Bruce, P. 2001. *Resampling Stats in Excel*. Resampling Stats Inc version 2 (Arlington), VA.

Brima, E.I., Haris, P.I., Jenkins, R.O., Polya, D.A., Gault, A.G. and Harrington, C.F. 2006. Understanding arsenic metabolism through a comparative study of arsenic levels in the urine, hair and fingernails of healthy volunteers from three unexposed ethnic groups in the United Kingdom. *Toxicology and Applied Pharmacology*, **216**, 122-130.

Brousseau, P., Fugere, N., Bernier, J., Coderre, D., Nadeau, D., Poirier, G. and Fournier, M. 1997. Evaluation of earthworm exposure to contaminated soil by

cytometric assay of coelomocytes phagocytosis in Lumbricus terrestris (Oligochaeta). *Soil Biology and Biochemistry*, **29**, 681-684.

Buchanan, W.D. 1962. *Toxicity of Arsenic Compounds*. 1st Edition. Elsevier. London.

Button, M., Jenkin, G.R.T., Harrington, C.F. and Watts, M.J. 2009a. Human toenails as a biomarker of exposure to elevated environmental arsenic. *Journal of Environmental Monitoring*, **11**, 610-617.

Button, M. and Watts, M.J. 2008. Extraction and measurement of arsenic species in contaminated soils by HPLC-ICP-MS. *British Geological Survey*, IR/08/050.

Button, M., Watts, M.J., Cave, M., Harrington, C.F. and Jenkin, G.R.T. 2009b. Earthworms and in vitro physiologically-based extraction tests: complementary tools for a holistic approach towards understanding risk at arsenic-contaminated sites. *Environmental Geochemistry and Health*, **31**, 273-282.

Camm, G.S., Glass, H.J., Bryce, D.W. and Butcher, A.R. 2004. Characterisation of a mining-related arsenic-contaminated site, Cornwall, UK. *Journal of Geochemical Exploration*, **82**, 1-15.

Campbell, P.G.C., Tessier, A. 1986. Partitioning of trace metals in sediments-relaitonship with bioavailabillity. *Hydrobiologia*, **149**, 43-52.

Cave, M., Wragg, J., Klinck, B., Gron, C., Oomen, A., van de Wiele, T., Ollson, C., Koch, I., Reimer, K., Basta, N. and Tack, K. 2006. Preliminary assessment of a unified bioaccessibility method for potentially harmful elements in soils. *Epidemiology*, **17** 39-39.

Cave, M.R., Wragg, J., Palumbo, B. and Klinck, B.A. 2002. *Measurement of the Bioaccessibility of Arsenic in UK Soils*. Environment Agency P5-062/TR02.

CEPA. 1993. *Priority Substances List Assessment Report: Arsenic and its Compounds*. Canadian Environmental Protection Act Part 1 of 3.

Charoy, B. 1986. The genesis of the Cornubian Batholith (South-West England): the example of the Carnmenellis pluton. *Journal of Petrology*, **27**, 571-604.

Collins, A.R. 2004. The Comet Assay for DNA Damage and Repair: Principles, Applications and Limitations. *Molecular Biotechnology*, **26**, 249-261.

Cotter-Howells, J., Charnock, J.M., Winters, C., Kille, P., Fry, J.C. and Morgan, A.J. 2005. Metal Compartmentation and Speciation in a Soil Sentinel: The Earthworm, *Dendrodrilus rubidus*. *Environmental Science and Technology*, **39**, 7731-7740.

Cullen, W.R. and Reimer, K.J. 1989. Arsenic speciation in the environment. *Chemical Reviews*, **89**, 713-764.

Dean, J.R. 1997. *Atomic Absorption and Plasma Spectroscopy*. Second edition. John Wiley and Sons, UK. Chichester.

Decaprio, A.P. 1997. Biomarkers: Coming of age for environmental health and risk assessment. *Environmental Science and Technology*, **31**, 1837-1848.

Defra. 2002a. *Contaminants in soil: Collation of Toxicological Data and Intake Values for Humans. Arsenic*. Department for Environment Food and Rural Affairs and the Environment Agency TOX 5.

Defra. 2002b. *The contaminated land exposure assessment model (CLEA): technical basis and algorithms.* Department for Environment Food and Rural Affairs and the Environment Agency CLR 10.

Di Marzio, W.D., Saenz, M.E., Lemiere, S. and Vasseur, P. 2005. Improved single-cell gel electrophoresis assay for detecting DNA damage in *Eisenia foetida. Environmental and Molecular Mutagenesis*, **46**, 246-252.

Dines, H.G. 1956. *The metalliferous mining region of South-West England*. 1st Edition. HMSO. London.

Dufailly, V., Noël, L. and Guérin, T. 2008. Optimisation and critical evaluation of a collision cell technology ICP-MS system for the determination of arsenic in foodstuffs of animal origin. *Analytica Chimica Acta*, **611**, 134-142.

DWI. 2008. *Drinking Water 2007: Drinking Water in England and Wales*. Drinking Water Inspectorate ISBN : 978-1-905852-21-5.

Efron, B. and Tibshirani, R.J. 1993. *An introduction to the bootstrap*. Chapman & Hall. Florida.

Elsom, M.H. 1992. *Staying Healthy with Nutrition: The complete guide to diet and nutriitonal medicine*. Celestial Arts Publishing. California.

Entwisle, J. and Hearn, R. 2006. Development of an accurate procedure for the determination of arsenic in fish tissues of marine origin by inductively coupled plasma mass spectrometry. *Spectrochimica Acta - Part B Atomic Spectroscopy*, **61**, 438-443.

Eyambe, G.S., Goven, A.J., Fitzpatrick, L.C., Venables, B.J. and Cooper, E.L. 1991. A non-invasive technique for sequential collection of earthworm (Lumbricus terrestris) leukocytes during subchronic immunotoxicity studies. *Laboratory Animals*, **25**, 61-67.

Farago, M.E. and Kavanagh, P. 1999. High arsenic containing soils in SW England and human exposure. In: Geochemistry of the Earth's Surface. H. Ármannsson. Balkema. Rotterdam.

Fergusen, C., Nathanail, P., McCaffrey, C., Earl, N., Foster, N., Gillet, A. and Ogden, R. 2003. *Method for Deriving Site-Specific Human Health Assessment Criteria for Contaminats in Soil*. Scotlan and Northern Ireland Forum for Environmental Research (SNIFFER) LQ01.

Fourie, F., Reinecke, S.A. and Reinecke, A.J. 2007. The determination of earthworm species sensitivity differences to cadmium genotoxicity using the comet assay. *Ecotoxicology and Environmental Safety*, **67**, 361-368.

Francesconi, K. Personal Communication, Elution of arsenic species using PRP-X100 anion exchange column, Email. 06/03/2009.

Francesconi, K.A. 2003. Complete extraction of arsenic species: a worthwhile goal? *Applied Organometallic Chemistry*, **17**, 682-683.

Francesconi, K.A. and Kuehnelt, D. 2004. Determination of arsenic species: A critical review of methods and applications, 2000-2003. *The Analyst*, **129**, 373-395.

Freeman, L.E.B., Dennis, L.K., Lynch, C.F., Thorne, P.S. and Just, C.L. 2004. Toenail arsenic content and cutaneous melanoma in Iowa. *American Journal of Epidemiology*, **160**, 679-687.

Gault, A.G., Rowland, H.A.L., Charnock, J.M., Wogelius, R.A., Gomez-Morilla, I., Vong, S., Leng, M., Samreth, S., Sampson, M.L. and Polya, D.A. 2008. Arsenic in hair and nails of individuals exposed to arsenic-rich groundwaters in Kandal province, Cambodia. *Science of the Total Environment*, **393**, 168-176.

Gebel, T.W. 2001. Genotoxicity of arsenical compounds. *International Journal of Hygiene and Environmental Health*, **203**, 249-262.

Geiszinger, A., Goessler, W. and Kosmus, W. 2002. An arsenosugar as the major extractable arsenical in the earthworm Lumbricus terrestris. *Applied Organometallic Chemistry*, **16**, 473-476.

Geiszinger, A., Goessler, W., Kuehnelt, D., Kosmus, W. and Francesconi, K. 1998. Determination of arsenic compounds in earthworms. *Environmental Science and Technology*, **32**, 2238-2243.

Geyer, A.S., Onumah, N., Uyttendaele, H. and Scher, R.K. 2004. Modulation of linear nail growth to treat diseases of the nail. *Journal of the American Academy of Dermatology*, **50**, 229-234.

Goering, P.L., Aposhian, H.V., Mass, M.J., Cebrian, M., Beck, B.D. and Waalkes, M.P. 1999. The enigma of arsenic carcinogenesis: Role of metabolism. *Toxicological Sciences*, **49**, 5-14.

Gonsebatt, M.E., Vega, L., Salazar, A.M., Montero, R., Guzmán, P., Blas, J., Del Razo, L.M., García-Vargas, G., Albores, A., Cebrian, M.E., Kelsh, M. and Ostrosky-Wegman, P. 1997. Cytogenetic effects in human exposure to arsenic. *Mutation Research - Reviews in Mutation Research*, **386**, 219-228.

Green, K.A., Chenery, S.R., Barlow, T.S., Taylor, H. and Cook, J.M. 2006. Poster presentation. *13th Biennial National Atomic Spectroscopy Symposium*, Glasgow, UK.

Hamilton, E.I. 2000. Environmental variables in a holistic evaluation of land contaminated by historic mine wastes: A study of multi-element mine wastes in West Devon, England using arsenic as an element of potential concern to human health. *Science of the Total Environment*, **249**, 171-221.

Hindmarsh, J.T. and McCurdy, R.F. 1986. Clinical and environmental aspects of arsenic toxicity. *Critical reviews in clinical laboratory sciences*, **23**, 315-347.

Hinwood, A.L., Sim, M.R., Jolley, D., de Klerk, N., Bastone, E.B., Gerostamoulos, J. and Drummer, O.H. 2003. Hair and toenail arsenic concentrations of residents living in areas with high environmental arsenic concentrations. *Environmental Health Perspectives*, **111**, 187-193.

Hinwood, A.L., Sim, M.R., Jolley, D., de Klerk, N., Bastone, E.B., Gerostamoulos, J. and Drummer, O.H. 2004. Exposure to Inorganic Arsenic in Soil Increases Urinary Inorganic Arsenic Concentrations of Residents Living in Old Mining Areas. *Environmental Geochemistry and Health*, **26**, 27-36.

Hopps, H.C. 1977. The biologic bases for using hair and nail for analyses of trace elements. *Science of the Total Environment*, **7**, 71-89.

Hutton, C., Bryce, D.W., Russeau, W., Glass, H.J., Jenkin, L.E.T., Corns, W.T. and Stockwell, P.B. 2005. Aqueous and solid-phase speciation of arsenic in Cornish soils. *Mineralogical Magazine*, **69**, 577-589.

Intawongse, M. and Dean, J.R. 2006. In-vitro testing for assessing oral bioaccessibility of trace metals in soil and food samples. *TrAC - Trends in Analytical Chemistry*, **25**, 876-886.

Jarvis, K.E., Gray, A.L. and Houk, R.S. 2003. *Handbook of Inductively Coupled Plasma Mass Spectrometry*. Viridian Publishing. Woking, U.K.

Johnson, L.R. and Farmer, J.G. 1989. Urinary arsenic concentrations and speciation in Cornwall residents. *Environmental Geochemistry and Health*, **11**, 39-44.

Kapaj, S., Peterson, H., Liber, K. and Bhattacharya, P. 2006. Human health effects from chronic arsenic poisoning - A review. *Journal of Environmental Science and Health - Part A Toxic/Hazardous Substances and Environmental Engineering*, **41**, 2399-2428.

Karagas, M.R., Morris, J.S., Weiss, J.E., Spate, V., Baskett, C. and Greenberg, E.R. 1996. Toenail samples as an indicator of drinking water arsenic exposure. *Cancer Epidemiology Biomarkers and Prevention*, **5**, 849-852.

Karagas, M.R., Stukel, T.A., Morris, J.S., Tosteson, T.D., Weiss, J.E., Spencer, S.K. and Greenberg, E.R. 2001. Skin cancer risk in relation to toenail arsenic concentrations in a US population-based case-control study. *American Journal of Epidemiology*, **153**, 559-565.

Karagas, M.R., Stukel, T.A. and Tosteson, T.D. 2002. Assessment of cancer risk and environmental levels of arsenic in New Hampshire. *International Journal of Hygiene and Environmental Health*, **205**, 85-94.

Karagas, M.R., Tosteson, T.D., Blum, J., Klaue, B., Weiss, J.E., Stannard, V., Spate, V. and Morris, J.S. 2000. Measurement of low levels of arsenic exposure: A comparison of water and toenail concentrations. *American Journal of Epidemiology*, **152**, 84-90.

Kavanagh, P., Farago, M.E., Thornton, I., Goessler, W., Kuehnelt, D., Schlagenhaufen, C. and Irgolic, K.J. 1998. Urinary arsenic species in Devon and Cornwall residents, UK. A pilot study. *Analyst*, **123**, 27-29.

Kavanagh, P.J., Farago, M.E., Thornton, I. and Braman, R.S. 1997. Bioavailability of arsenic in soil and mine wastes of the Tamar valley, SW England. *Chemical Speciation and Bioavailability*, **9**, 77-81.

Kile, M.L., Houseman, E.A., Breton, C.V., Quamruzzaman, Q., Rahman, M., Mahiuddin, G. and Christiani, D.C. 2007. Association between total ingested arsenic and toenail arsenic concentrations. *Journal of Environmental Science*

and Health - Part A Toxic/Hazardous Substances and Environmental Engineering, **42**, 1827-1834.

Kitchin, K.T. 2001. Recent advances in arsenic carcinogenesis: Modes of action, animal model systems, and methylated arsenic metabolites. *Toxicology and Applied Pharmacology*, **172**, 249-261.

Klinck, B.A., Palumbo, B., Cave, M.R. and Wragg, J. 2002. *Arsenic dispersal and bioaccessibility in mine contaminated soils: a case study from an abandoned arsenic mine in Devon, UK*. British Geological Survey RR/04/003.

Langdon, C.J., Meharg, A.A., Feldmann, J., Balgar, T., Charnock, J., Farquhar, M., Piearce, T.G., Semple, K.T. and Cotter-Howells, J. 2002. Arsenic-speciation in arsenate-resistant and non-resistant populations of the earthworm, *Lumbricus rubellus*. *Journal of Environmental Monitoring*, **4**, 603-608.

Langdon, C.J., Piearce, T.G., Black, S. and Semple, K.T. 1999. Resistance to arsenic-toxicity in a population of the earthworm *Lumbricus rubellus*. *Soil Biology & Biochemistry*, **31**, 1963-1967.

Langdon, C.J., Piearce, T.G., Meharg, A.A. and Semple, K.T. 2001. Survival and behaviour of the earthworms *Lumbricus rubellus* and *Dendrodrilus rubidus* from arsenate-contaminated and non-contaminated sites. *Soil Biology & Biochemistry*, **33**, 1239-1244.

Langdon, C.J., Piearce, T.G., Meharg, A.A. and Semple, K.T. 2003a. Interactions between earthworms and arsenic in the soil environment: a review. *Environmental Pollution*, **124**, 361-373.

Langdon, C.J., Piearce, T.G., Semple, K.T., Feldmann, J. and Meharg, A.A. 2003b. Arsenic speciation in the earthworms *Lumbricus rubellus* and *Dendrodrilus rubidus*. *Environmental Toxicology and Chemistry*, **22**, 1302-1308.

Lantz, R.C. and Hays, A.M. 2006. Role of oxidative stress in arsenic-induced toxicity. *Drug Metabolism Reviews*, **38**, 791-804.

Le Boutillier, N.G. 2001. *The tectonics of Variscan magmatism and mineralisation in southwest England*. PhD. Camborne School of Mines, University of Essex.

Le, X.C., Lu, X., Ma, M., Cullen, W.R., Aposhian, H.V. and Zheng, B. 2000. Speciation of key arsenic metabolic intermediates in human urine. *Analytical Chemistry*, **72**, 5172-5177.

Lee, R.F. and Steinert, S. 2003. Use of the single cell gel electrophoresis/comet assay for detecting DNA damage in aquatic (marine and freshwater) animals. *Mutation Research-Reviews in Mutation Research*, **544**, 43-64.

Lee, T.C., Tanaka, N., Lamb, P.W., Gilmer, T.M. and Barrett, J.C. 1988. Induction of gene amplification by arsenic. *Science*, **241**, 79-81.

Madsen, A.D., Goessler, W., Pedersen, S.N. and Francesconi, K.A. 2000. Characterization of an algal extract by HPLC-ICP-MS and LC-electrospray MS for use in arsenosugar speciation studies. *Journal of Analytical Atomic Spectrometry*, **15**, 657-662.

Mandal, B.K., Ogra, Y. and Suzuki, K.T. 2003. Speciation of arsenic in human nail and hair from arsenic-affected area by HPLC-inductively coupled argon plasma mass spectrometry. *Toxicology and Applied Pharmacology*, **189**, 73-83.

Mandal, B.K. and Suzuki, K.T. 2002. Arsenic round the world: a review. *Talanta*, **58**, 201-235.

Manerikar, R.S., Apte, A.A. and Ghole, V.S. 2008. In vitro and in vivo genotoxicity assessment of Cr(VI) using comet assay in earthworm coelomocytes. *Environmental Toxicology and Pharmacology*, **25**, 63-68.

Marino, F. and Morgan, A.J. 1999. The time-course of metal (Ca, Cd, Cu, Pb, Zn) accumulation from a contaminated soil by three populations of the earthworm, *Lumbricus rubellus*. *Applied Soil Ecology*, **12**, 169-177.

Mariño, F. and Morgan, A.J. 1998. Equilibrated body metal concentrations in laboratory exposed earthworms: can they be used to screen candidate metal-adapted populations? *Applied Soil Ecology*, **12**, 179-189.

Martin, F.L., Piearce, T.G., Hewer, A., Phillips, D.H. and Semple, K.T. 2005. A biomarker model of sublethal genotoxicity (DNA single-strand breaks and adducts) using the sentinel organism *Aporrectodea longa* in spiked soil. *Environmental Pollution*, **138**, 307-315.

Martinez-Bravo, Y., Roig-Navarro, A.F., Lopez, F.J. and Hernandez, F. 2001. Multielemental determination of arsenic, selenium and chromium(VI) species in water by high-performance liquid chromatography–inductively coupled plasma mass spectrometry. *Journal of Chromatography A*, **926**, 265-274.

Mass, M.J., Tennant, A., Roop, B.C., Cullen, W.R., Styblo, M., Thomas, D.J and Kligerman, A.D. 2001. Methylated Trivalent Arsenic Species Are Genotoxic. *Chemical Research in Toxicology*, **14**, 355-361.

Masscheleyn, P.H., Delaune, R.D. and Patrick Jr, W.H. 1991. Effect of redox potential and pH on arsenic speciation and solubility in a contaminated soil. *Environmental Science and Technology*, **25**, 1414-1419.

Matschullat, J. 2000. Arsenic in the geosphere - A review. *Science of the Total Environment*, **249**, 297-312.

May, T.W. and Wiedmeyer, R.H. 1998. A Table of Polyatomic Interferences in ICP-MS. *Atomic Spectroscopy*, **19**, 150-155.

McNaught, A.D. and Wilkinson, A. 1997. *Compendium of chemical terminology : IUPAC recommendations*. Blackwell Science. Oxford [England]; Malden, MA, USA.

Michaud, D.S., Wright, M.E., Cantor, K.P., Taylor, P.R., Virtamo, J. and Albanes, D. 2004. Arsenic concentrations in prediagnostic toenails and the risk of bladder cancer in a cohort study of male smokers. *American Journal of Epidemiology*, **160**, 853-859.

Møller, P. 2006. Assessment of reference values for DNA damage detected by the comet assay in human blood cell DNA. *Mutation Research - Reviews in Mutation Research*, **612**, 84-104.

Moore, J.N. 1988. Partitioning of arsenic and metals in reducing sulfidic sediments. *Environmental Science and Technology*, **22**, 432-437.

Moore, M.M., Harrington-Brock, K. and Doerr, C.L. 1997. Relative genotoxic potency of arsenic and its methylated metabolites. *Mutation Research - Reviews in Mutation Research*, **386**, 279-290.

Morgan, A.J., Winters, C and Yarwood, A. 1994. Speed-mapping of arsenic distribution in the tissues of earthworms inhabiting arsenious soil. *Cell Biology International*, **18**, 911-914.

Morgan, J.E. and Morgan, A.J. 1999. The accumulation of metals (Cd, Cu, Pb, Zn and Ca) by two ecologically contrasting earthworm species (*Lumbricus rubellus* and *Aporrectodea caliginosa*): Implications for ecotoxicological testing. *Applied Soil Ecology*, **13**, 9-20.

Murray, L.A., Raab, A., Marr, I.L. and Feldmann, J. 2003. Biotransformation of arsenate to arsenosugars by *Chlorella vulgaris*. *Applied Organometallic Chemistry*, **17**, 669-674.

Neuhauser, E.F., Cukic, Z.V., Malecki, M.R., Loehr, R.C. and Durkin, P.R. 1995. Bioconcentration and biokinetics of heavy metals in the earthworm. *Environmental Pollution*, **89**, 293-301.

Nørum, U., Lai, V.W.M., Pergantis, S.A. and Cullen, W.R. 2005. Arsenic compounds in the haemolymph of the Dungeness crab, *Cancer magister*, as determined by using HPLC on-line with inductively coupled plasma mass spectrometry. *Journal of Environmental Monitoring*, **7**, 122-126.

O'Connor, G. and Evans, E.H. 2007. Fundamental Aspects of Inductively Coupled Plasma-Mass Spectrometry (ICP-MS). In: Inductively Coupled Plasma Spectrometry and its Applications. S. J. Hill. Blackwell Publishing. Oxford.

Olesik, J.W. 2000. Inductively Coupled Plasma Mass Spectrometry. In: Inorganic Mass Spectrometry. C. M. Barshick, D. C. Duckworth and D. H. Smth. Marcel Decker, Inc. New York.

ONS. 2002. *Population density 2002: Regional trends 38*. Office for National Statistics D7662. Crown Copyright.

Oomen, A.G., Hack, A., Minekus, M., Zeijdner, E., Cornelis, C., Schoeters, G., Verstraete, W., Van De Wiele, T., Wragg, J., Rompelberg, C.J., Sips, A.J. and Van Wijnen, J.H. 2002. Comparison of five in vitro digestion models to study the bioaccessibility of soil contaminants. *Environmental Science and Technology*, **36**, 3326-3334.

Palumbo-Roe, B., Cave, M.R., Klinck, B.A., Wragg, J., Taylor, H., O'Donnell, K.E. and Shaw, R.A. 2005. Bioaccessibility of arsenic in soils developed over Jurassic ironstones in eastern England. *Environmental Geochemistry and Health*, **27**, 121-130.

Palumbo-Roe, B. and Klinck, B. 2007. Bioaccessibility of arsenic in mine waste-contaminated soils: A case study from an abandoned arsenic mine in SW England (UK). *Journal of Environmental Science and Health - Part A Toxic/Hazardous Substances and Environmental Engineering*, **42**, 1251-1261.

Pan, T.C., Lin, T.H., Tseng, C.L., Yang, M.H. and Huang, C.W. 1993. Trace elements in hair of blackfoot disease. *Biological Trace Element Research*, **39**, 117-128.

Pergantis, S.A., Winnik, W. and Betowski, D. 1997. Determination of ten organoarsenic compounds using microbore high-performance liquid chromatography coupled with electrospray mass spectrometry-mass spectrometry. *Journal of Analytical Atomic Spectrometry*, **12**, 531-536.

Peshut, P.J., Morrison, R.J. and Brooks, B.A. 2008. Arsenic speciation in marine fish and shellfish from American Samoa. *Chemosphere*, **71**, 484-492.

Piearce, T.G., Langdon, C.J., Meharg, A.A. and Semple, K.T. 2002. Yellow earthworms: distinctive pigmentation associated with arsenic- and copper-tolerance in *Lumbricus rubellus*. *Soil Biology & Biochemistry*, **34**, 1833-1838.

Pizarro, I., Gomez, M., Camara, C. and Palacios, M.A. 2003. Arsenic speciation in environmental and biological samples: Extraction and stability studies. *Analytica Chimica Acta*, **495**, 85-98.

Qiao, M., Chen, Y., Wang, C.X., Wang, Z. and Zhu, Y.G. 2007. DNA damage and repair process in earthworm after *in-vivo* and *in vitro* exposure to soils irrigated by wastewaters. *Environmental Pollution*, **148**, 141-147.

Raab, A. and Feldmann, J. 2005. Arsenic speciation in hair extracts. *Analytical and Bioanalytical Chemistry*, **381**, 332-338.

Raab, A., Meharg, A.A., Jaspars, M., Genney, D.R. and Feldmann, J. 2004. Arsenic-glutathione complexes - Their stability in solution and during separation by different HPLC modes. *Journal of Analytical Atomic Spectrometry*, **19**, 183-190.

Rajaguru, P., Suba, S., Palanivel, M and Kalaiselvi, k. 2003. Genotoxicity of a polluted river system measured using the alkaline comet assay on fish and earthworm tissues. *Environmental and Molecular Mutagenesis*, **41**, 85-91.

Reinecke, S.A. and Reinecke, A.J. 2004. The Comet Assay as Biomarker of Heavy Metal Genotoxicity in Earthworms. *Archives of Environmental Contamination and Toxicology*, **46**, 208-215.

Rieuwerts, J.S., Searle, P. and Buck, R. 2006. Bioaccessible arsenic in the home environment in southwest England. *Science of the Total Environment*, **371**, 89-98.

Ritchie, A.W., Edmonds, J.S., Goessler, W. and Jenkins, R.O. 2004. An origin for arsenobetaine involving bacterial formation of an arsenic-carbon bond. *FEMS Microbiology Letters*, **235**, 95-99.

RSC 2002. Royal Society of Chemistry: A simple fitness-for-purpose control chart based on duplicate results obtained from routine test materials World Wide Web Address: http://www.rsc.org/images/brief9_tcm18-25951.pdf.

Ruby, M.V., Davis, A., Link, T.E., Schoof, R., Chaney, R.L., Freeman, G.B. and Bergstrom, P. 1993. Development of an in vitro screening test to evaluate the *in vivo* bioaccessibility of ingested mine-waste lead. *Environmental Science and Technology*, **27**, 2870-2877.

Rudnick, R.L. and Gao, S. 2004. Composition of the Continental Crust. In: Treatise on Geochemistry: Volume 3 The Crust. R. L. Rudnick. Elsevier Ltd. Oxford.

Ruiz-Chancho, M.J., Sabé, R., López-Sánchez, J.F., Rubio, R. and Thomas, P. 2005. New Approaches to the Extraction of Arsenic Species from Soils. *Microchimica Acta*, **151**, 241-248.

Saint-Denis, M., Narbonne, J.F., Arnaud, C., Thybaud, E., Ribera, D. . 1999. Biochemical responses of the earthworm *Eisenia fetida andrei* exposed to contaminated artificial soil: Effects of benzo(a)pyrene. *Soil Biology and Biochemistry*, **31**, 1837-1846.

Salagovic, J., Gilles, J., Verschaeve, L. and Kalina, I. 1996. The comet assay for the detection of genotoxic damage in the earthworms : A promising tool for assessing the biological hazards of polluted sites. *Folia Biologica*, **42**, 17-21.

Sample, B.E., Suter Ii, G.W., Beauchamp, J.J. and Efroymson, R.A. 1999. Literature-derived bioaccumulation models for earthworms: Development and validation. *Environmental Toxicology and Chemistry*, **18**, 2110-2120.

Sanz, E., Munoz-Olivas, R., Camara, C., Sengupta, M.K. and Ahamed, S. 2007. Arsenic speciation in rice, straw, soil, hair and nails samples from the arsenic-affected areas of Middle and Lower Ganga plain. *Journal of Environmental Science and Health - Part A Toxic/Hazardous Substances and Environmental Engineering*, **42**, 1695-1705.

Scheib, A.J. and Nice, S.E. 2008. *Soil geochemical baseline data for the urban areas of Corby, Coventry, Derby, Leicester, Northampton, Nottingham and Peterborough in the East Midlands*. British Gelogical Survey IR/06/023.

Schuliga, M., Chouchane, S. and Snow, E.T. 2002. Upregulation of Glutathione-Related Genes and Enzyme Activities in Cultured Human Cells by Sublethal Concentrations of Inorganic Arsenic. *Toxicological Sciences*, **70**, 183-192.

Schultz, A.C. and Biksey, T.M. 2003. Arsenic Speciation and its Effect on Soil Cleanup Standards. *Environmental Claims Journal*, **15**, 107-118.

Shen, J., Wanibuchi, H., Salim, E.I., Wei, M., Konoshita, A., Yoshida, K., Endo, G. and Fukushima, S. 2003. Liver tumorigenicity of trimethylarsine oxide in male Fischer 344 rats - Association with oxidative DNA damage and enhanced cell proliferation. *Carcinogenesis*, **24**, 1827-1835.

Shraim, A., Hirano, S. and Yamauchi, H. 2001. Extraction and Speciation of Arsenic in Hair Using HPLC-ICP-MS. *Analytical Sciences*, **17**, 1729-1723.

Skoog, D.A. and Leary, J.J. 1991. *Principles of Instrumental Analysis*. 4th Edition. Saunders College Publishing.

Slekovec, M., Goessler, W. and Irgolic, K.J. 1999. Inorganic and organic arsenic compounds in Slovenian mushrooms: Comparison of arsenic-specific detectors for liquid chromatography. *Chemical Speciation and Bioavailability*, **11**, 115-123.

Slotnick, M.J., Meliker, J.R., Avruskin, G.A., Ghosh, D. and Nriagu, J.O. 2007. Toenails as a biomarker of inorganic arsenic intake from drinking water and foods. *Journal of Toxicology and Environmental Health - Part A: Current Issues*, **70**, 148-158.

Slotnick, M.J. and Nriagu, J.O. 2006. Validity of human nails as a biomarker of arsenic and selenium exposure: A review. *Environmental Research*, **102**, 125-139.

Small, H. 1989. *Ion Exchange Chromatography*. Plenum Press. New York.

Smedley, P.L. and Kinniburgh, D.G. 2002. A review of the source, behaviour and distribution of arsenic in natural waters. *Applied Geochemistry*, **17**, 517-568.

Smedley, P.L. and Kinniburgh, D.G. 2005. Arsenic in Groundwater and the Environment. 263-301 In: Essentials of Medical Geology: Impacts of the Natural Environment on Human Health. O. selinus, B. Alloway, J. A. Centino, B. R. Finkelman, R. Fuge, U. Lindh and P. L. Smedley. Elsevier Academic Press. London.

Smith, G.S. 2007. *Arsenic Biotransformation in Terrestrial Organisms*. PhD. Biology Department, Queen's University.

Spurgeon, D.J. and Hopkin, S.P. 1996. The effects of metal contamination on earthworm populations around a smelting works: Quantifying species effects. *Applied Soil Ecology*, **4**, 147-160.

Spurgeon, D.J., Weeks, J.M. and Van Gestel, C.A.M. 2003. A summary of eleven years progress in earthworm ecotoxicology. *Pedobiologia*, **47**, 588-606.

Sturzenbaum, S.R., Georgiev, O., Morgan, A.J. and Kille, P. 2004. Cadmium detoxification in earthworms: From genes to cells. *Environmental Science and Technology*, **38**, 6283-6289.

Styblo, M., Drobna, Z., Jaspers, I., Lin, S. and Thomas, D.J. 2002. The role of biomethylation in toxicity and carcinogenicity of arsenic: A research update. *Environmental Health Perspectives*, **110**, 767-771.

Tchounwou, P.B., Centeno, J.A and Patlolla, A.K. 2004. Arsenic toxicity, mutagenesis, and carcinogenesis: a health risk assessment and management approach. *Molecular and Cellular Biochemistry*, **255**, 47-55.

Thomas, D.J., Waters, S.B. and Styblo, M. 2004. Elucidating the pathway for arsenic methylation. *Toxicology and Applied Pharmacology*, **198**, 319-326.

Thomas, R. 2002a. A beginner's guide to ICP-MS. *Spectroscopy*, **17**, 36-41.

Thomas, R. 2002b. A beginner's guide to ICP-MS: Part X - Detectors. *Spectroscopy*, **17**, 34-39.

Thomas, R. 2008. *Practical Guide to ICP-MS: A Tutorial for Beginners*. Second edition. CRC Press. UK.

Tomlin, A.D. 1992. Behaviour as a source of earthworm susceptibility to ecotoxicants. In: Ecotoxicology of earthworms. P. W. Greig-Smith, H. Becker, P. J. Edwards and F. Heimbach. Intercept. Hants.

Tsuji, J.S., Van Kerkhove, M.D., Kaetzel, R.S., Scrafford, C.G., Mink, P.J., Barraj, L.M., Crecelius, E.A., and Goodman, M. 2005. Evaluation of Exposure to Arsenic in Residential Soil. *Environmental Health Perspectives*, **113**, 1735-1740.

Van Elteren, J.T., Šlejkovec, Z., Arčon, I. and Glass, H.J. 2006. An interdisciplinary physical-chemical approach for characterization of arsenic in a calciner residue dump in Cornwall (UK). *Environmental Pollution*, **139**, 477-488.

Van Elteren, J.T., Slejkovec, Z., Kahn, M. and Goessler, W. 2007. A systematic study on the extractability of arsenic species from algal certified reference material IAEA-140/TM (*Fucus sp*., Sea Plant Homogenate) using methanol/water extractant mixtures. *Analytica Chimica Acta*, **585**, 24-31.

Van Vliet, P.C.J., Didden, W.A.M., Van der Zee, S.E.A.T.M. and Peijnenburg, W.J.G.M. 2006. Accumulation of heavy metals by enchytraeids and earthworms in a floodplain. *European Journal of Soil Biology*, **42**,

Vandecasteele, C. and Block, C.B. 1997. *Modern Methods for Trace Element Determination*. Jonh Wiley and Sons. Chichester.

Vega, L., Styblo, M., Patterson, R., Cullen, W., Wang, C. and Germolec, D. 2001. Differential effects of trivalent and pentavalent arsenicals on cell proliferation and cytokine secretion in normal human epidermal keratinocytes. *Toxicology and Applied Pharmacology*, **172**, 225-232.

Vijver, M.G., Van Gestel, C.A.M., Lanno, R.P., Van Straalen, N.M. and Peijnenburg, W.J.G.M. 2004. Internal metal sequestration and its ecotoxicological relevance: A review. *Environmental Science and Technology*, **38**, 4705-4712.

Watts, M.J., Button, M., Brewer, T.S., Jenkin, G.R.T. and Harrington, C.F. 2008. Quantitative arsenic speciation in two species of earthworms from a former mine site. *Journal of Environmental Monitoring*, **10**, 753-759.

Waxman, S. and Anderson, K.C. 2001. History of the development of arsenic derivatives in cancer therapy. *Oncologist*, **6**, 3-10.

Wei, M., Wanibuchi, H., Yamamoto, S., Li, W. and Fukushima, S. 1999. Urinary bladder carcinogenicity of dimethylarsinic acid in male F344 rats. *Carcinogenesis*, **20**, 1873-1876.

Wickre, J.B., Folt, C.L., Sturup, S. and Karagas, M.R. 2004. Environmental exposure and fingernail analysis of arsenic and mercury in children and adults in a Nicaraguan gold mining community. *Archives of Environmental Health*, **59**, 400-409.

Wilhelm, M., Pesch, B., Wittsiepe, J., Jakubis, P., Miskovic, P., Keegan, T., Nieuwenhuijsen, M.J. and Ranft, U. 2005. Comparison of arsenic levels in fingernails with urinary As species as biomarkers of arsenic exposure in residents living close to a coal-burning power plant in Prievidza District, Slovakia. *Journal of Exposure Analysis and Environmental Epidemiology*, **15**, 89-98.

Wragg, J., Cave, M. and Nathanail, P. 2007. A study of the relationship between arsenic bioaccessibility and its solid-phase distribution in soils from Wellingborough, UK. *Journal of Environmental Science and Health - Part A Toxic/Hazardous Substances and Environmental Engineering*, **42**, 1303-1315.

WWC 2008. Worm Watch Canada: Key to Reproductively Mature Earthworms World Wide Web Address: http://www.naturewatch.ca.

Yoshida, T., Yamauchi, H. and Fan Sun, G. 2004. Chronic health effects in people exposed to arsenic via the drinking water: dose-response relationships in review. *Toxicology and Applied Pharmacology*, **198**, 243-252.

Yuan-Hui, L. 1991. Distribution patterns of the elements in the ocean: A synthesis. *Geochimica et Cosmochimica Acta*, **55**, 3223-3240.

Zheng, J., Goessler, W., Geiszinger, A., Kosmus, W., Chen, B., Zhuang, G., Xu, K. and Sui, G. 1997. Multi-element determination in earthworms with instrumental neutron activation analysis and inductively coupled plasma mass spectrometry: A comparison. *Journal of Radioanalytical and Nuclear Chemistry*, **223**, 149-155.

Presentations

Research contained in this thesis was presented at the following conferences and events.

Poster presentations:

Arsenic Speciation in the Earthworms *Lumbricus rubellus* and *Dendrodrilus rubidus* from a Former Arsenic Mine in Devon, UK

Mark Button, Michael J, Watts, Tim S, Brewer, Andrew D, Smith and Chris F, Harrington.

Presented at the 13th Biennial National Atomic Spectroscopy Sympossium (BNASS). Glasgow, UK. July 2006. This poster was awarded the Journal of Analytical Atomic Spectrometry (JAAS) poster prize for innovative research.

Earthworms and PBETs: Tools For Assessing Health Risks of Arsenic Contaminated Soils at a Former Arsenic Mine in Devon, UK

Mark Button, Michael J, Watts , Tim S, Brewer and Chris F, Harrington.

Presented at the Society for Environmental Geochemistry and Health (SEGH) Environment and Human Health Conference and Workshop. Liverpool, UK. June 2007.

Arsenic, Earthworms and PBETS: Understanding Risk at Contaminated Sites.

Mark Button, Michael J, Watts, Tim S, Brewer and Chris F, Harrington.

Presented at the University of Leicester Festival of Postgraduate Research. Leicester, UK. August 2007.

Oral presentations:

The Role of Arsenic Resistant Earthworms in a Multidisciplinary Approach towards Understanding Risk at a Former Mine in Devon, UK

Mark Button, Michael, J Watts, Gawen, R. T Jenkin and Chris, F. Harrington.
Presented at the 2nd International Congress, Arsenic in the Environment: Arsenic from Nature to Humans. Valencia, Spain. May 2008.

Human toenails as a biomarker of exposure to elevated environmental arsenic

Mark Button, Gawen R. T. Jenkin, Chris F. Harrington and Michael J. Watts.
Presented at Practical Applications of Medical Geology. British Geological Survey. Nottingham, UK. March 2009.

Appendix A

British Geological Survey
NATURAL ENVIRONMENT RESEARCH COUNCIL

Extraction and measurement of arsenic species in contaminated soils by HPLC-ICP-MS

Laboratory Operations

Internal Report IR/08/050

BRITISH GEOLOGICAL SURVEY

LABORATORY OPERATIONS

INTERNAL REPORT IR/08/050

Keywords

Arsenic speciation; soil

Front cover

Devon Great Consols, M J Watts (2006).

Bibliographical reference

BUTTON, M AND WATTS M J . 2008. EXTRACTION AND DETERMINATION OF ARSENIC SPECIEES IN CONTAMINATED SOILS. *British Geological Survey Internal Report*, IR/08/050. 14pp.

Extraction and measurement of arsenic species in contaminated soils by HPLC-ICP-MS

M Button and M J Watts

Contributor/editor

J M Cook

Keyworth, Nottingham British Geological Survey 2009

BRITISH GEOLOGICAL SURVEY

The full range of our publications is available from BGS shops at Nottingham, Edinburgh, London and Cardiff (Welsh publications only) see contact details below or shop online at www.geologyshop.com

The London Information Office also maintains a reference collection of BGS publications, including maps, for consultation.

We publish an annual catalogue of our maps and other publications; this catalogue is available online or from any of the BGS shops.

The British Geological Survey carries out the geological survey of Great Britain and Northern Ireland (the latter as an agency service for the government of Northern Ireland), and of the surrounding continental shelf, as well as basic research projects. It also undertakes programmes of technical aid in geology in developing countries.

The British Geological Survey is a component body of the Natural Environment Research Council.

Parent Body

Natural Environment Research Council, Polaris House,
North Star Avenue, Swindon SN2 1EU

Tel 01793 411500 Fax 01793 411501
www.nerc.ac.uk

Website www.bgs.ac.uk
Shop online at www.geologyshop.com

British Geological Survey offices

BGS Central Enquiries Desk

Tel 0115 936 3143 Fax 0115 936 3276
email enquiries@bgs.ac.uk

Kingsley Dunham Centre, Keyworth, Nottingham NG12 5GG

Tel 0115 936 3241 Fax 0115 936 3488
email sales@bgs.ac.uk

Murchison House, West Mains Road, Edinburgh EH9 3LA

Tel 0131 667 1000 Fax 0131 668 2683
email scotsales@bgs.ac.uk

London Information Office at the Natural History Museum (Earth Galleries), Exhibition Road, South Kensington, London SW7 2DE

Tel 020 7589 4090 Fax 020 7584 8270
Tel 020 7942 5344/45 email bgslondon@bgs.ac.uk

Columbus House, Greenmeadow Springs, Tongwynlais, Cardiff CF15 7NE

Tel 029 2052 1962 Fax 029 2052 1963

Forde House, Park Five Business Centre, Harrier Way, Sowton EX2 7HU

Tel 01392 445271 Fax 01392 445371

Maclean Building, Crowmarsh Gifford, Wallingford OX10 8BB

Tel 01491 838800 Fax 01491 692345

Geological Survey of Northern Ireland, Colby House, Stranmillis Court, Belfast BT9 5BF

Tel 028 9038 8462 Fax 028 9038 8461

www.bgs.ac.uk/gsni/

Acknowledgements

The authors would like to thank the BGS university Funding Initiative (BUFI) for the provision of a PhD studentship for Mark Button. The Tavistock Estate is also thanked for allowing access to the site at the Devon Great Consols.

Contents

FIGURES

TABLES

Summary

A method for the extraction and speciation of arsenic in contaminated soils was developed through modification of procedures reported in the literature. Microwave assisted extraction (MAE) and orbital shaking plus sonication were compared as extraction procedures together with different mixtures of phosphoric and ascorbic acid as the extractant. The extraction efficiency and species stability were monitored for each of the extraction procedures investigated. Quantification of arsenic species (As^{III}, As^{V}, MA^{V}, DMA^{V} and AB) was performed by HPLC (anionic column) coupled to ICP-MS using a modified ammonium nitrate gradient elution. Mean extraction efficiencies ranged from 77 to 92% of the total arsenic concentration with a MAE procedure giving the best recovery for total concentrations of arsenic. All arsenic species were stable under the extraction procedures investigated. The combined arsenic species quantified in each soil extract closely matched its total arsenic content. Arsenate (As^{V}) was the only major arsenic species detected although some As^{III}, MA and DMA were present in one soil. A novel closed vessel MAE of arsenic from soils is presented, providing both quantitative and reproducible recoveries and species stability, whilst maintaining enough simplicity for high sample throughput.

1 Introduction

The current Soil Guideline Value (SGV) in the UK for residential gardens and allotments is specified at 20 mg kg^{-1} dry weight (Defra 2002), but ignores the bioaccessibility and speciation of the contaminant. The toxicity and solubility of arsenic varies considerably depending on its chemical form (Hutton *et al.* 2005) with trivalent arsenicals thought to be the most toxic. An understanding of the bioaccessibility and speciation of arsenic in contaminated soil is therefore essential in understanding its potential risk to human health. Arsenate (As^{V}) and arsenite (As^{III}) are the primary arsenic species found in soils. The prevalence of either species is a function of redox potential, pH and microbial activity (Moore 1988). Under oxidising conditions arsenic is present in soils in the pentavalent oxidation state, whilst in reducing conditions arsenite (As^{III}) predominates. The sorption of arsenic onto specific minerals in the soil is controlled by the proportion of amorphous iron, aluminium hydroxides, clay minerals and its pH (Masscheleyn *et al.* 1991).

The main difficulty encountered in the determination of arsenic speciation is the development of a method that provides both quantitative and reproducible recoveries whilst maintaining the species integrity of a sample. Extraction recoveries are dependant on the sample matrix, species present, extraction solvent and aggressiveness of the extraction procedure (Pizarro *et al.* 2003). Table 1 outlines several approaches reported in the scientific literature. Various solvents and solvent mixtures have been employed in conjunction with agitation steps such as mechanical shaking, sonication and microwave assisted extraction (MAE) in open reflux vessels. Inorganic elemental arsenic is most susceptible to species interconversion,

particularly the oxidation of As^{III} to As^{V}. In response to this, more recent approaches have employed a reducing agent in the extraction step to prevent oxidation of As^{III} occurring. Phosphoric acid (H_3PO_4) has been used at various concentrations as the primary extractant in combination with ascorbic acid ($C_6H_6O_8$) as the reducing agent (Garcia-Manyes *et al.* 2002, Ruiz-Chancho *et al.* 2005). Table 1 summarises these methodologies in greater detail. Recoveries in the range 50 to 100% are reported using these extractants with mechanical shaking, sonication or MAE. However, species instability is still noted as a major problem (Francesconi and Kuehnelt 2004).

2. Aims of Investigation

The aim of this investigation was to develop an appropriate extraction procedure with subsequent arsenic speciation by HPLC-ICP-MS. The method developed sets out to achieve:

a) Quantitative recovery of arsenic from highly contaminated soils.

b) Species stability during the extraction procedure and analysis.

c) A suitable sample matrix for analysis via HPLC-ICP-MS, with the minimum of modification before analysis.

Table 1. Extraction methods, species stability and recoveries of arsenic from contaminated soils from recent publications.

Sample type and arsenic content	Extraction methods	As species/ recovery/ stability	Ref.
Contaminated soil (632 mg kg^{-1})	(1) 1 g of soil placed in Teflon reactor with 10 ml of extractant, (water or methanol /water mixtures) heated for 10 h @ 55ºC. Then 5 min in an ultrasonic bath. The extracts were dried down then diluted with water and filtered. (2) 0.3 g of soil with 10 ml 1 M phosphoric acid, heated to 150ºC for 3 hours. Sample evaporated to dryness and re-dissolved in 25 ml 10 mM phosphate solution pH 6.	(1) Good recoveries (total extracted) after three extractions (68-89%). Initial extraction recoveries (46-50%). (2) 99% recovery (sum species) after 3 extractions, 82% after just one extraction. Majority As^{V} with <5 % As^{III} and <10 % MA and DMA. All species reported to be stable under these conditions	(Pizarro *et al.* 2003)
Contaminated soils (15 – 780 mg kg^{-1}), CRM GBW 07405/07311 and BCR 320	0.1 g soil and 15 ml 1 M phosphoric acid and 0.1 M ascorbic acid (argon purged) placed in open reflux vessel. Maintained at 60 W for 10 min, several mls water added then filtered. Final dilution with water to 50 ml.	Recoveries of 56-101%. Majority As^{V} with <10% As^{III} and <1% MA and DMA. All species reported to be stable under these conditions. Samples deteriorated over time on storage at 4ºC.	(Garcia-Manyes *et al.* 2002)
Contaminated soils (25 – 3000 mg kg^{-1}) and CRM LGC 6138 coal.	0.2 g of soil and 10 ml of extractant (0.3 m phosphoric acid) heated in microwave for 100 min at 40% power	Spiked recoveries of 83-103% and 63-88% for soils. Majority As^{V} with <15% As^{III} , MA and DMA below detection limits. All species reported to be stable under these conditions. Interconversion occurred when heating for more than 100 min.	(Hutton *et al.* 2005)
Contaminated soils (350 – 2350 mg kg^{-1})	0.1 g of soil and 15 ml extractant (1 M phosphoric and 0.5 M ascorbic) placed in open reflux vessel. Maintained at 60 W for 10 min. several concentrations of ascorbic acid were tested. Extracts were purged with argon.	Good recoveries (~90%). Majority As^{V} with <10% As^{III} and <5% MA and DMA in some samples. Ascorbic acid said to increase extraction efficiency and stability of As^{III}. Rapid analysis after extraction recommended.	(Ruiz-Chancho *et al.* 2005)
Contaminated (spiked) soils and CRMs NIST 2711 and 2709.	0.2 g of soil with 5ml extractant. Either water, phosphoric (1 M), citrate buffer (10 mM), sodium hydroxide (0.1 M), ammonium dihydrogen phosphate (10 mM), cola and vinegar. Shaking and sonication were compared.	Sodium hydroxide and citrate gave the best recoveries of 23-98% for the individual spiked species when sonicated for 20 min. Low recoveries with phosphoric acid.	(Kahakachchi *et al.* 2004)

3 Methods

3.1 Soil Samples

Two soils collected at Devon Great Consols (DGC) in Southwest England, a low level garden soil from Tollerton, UK and contaminated soil CRM (NIST 2710) were selected for a range of arsenic levels from below the SGV (20 mg kg^{-1}) to > 5000 mg kg^{-1} (Table 2).

Table 2. Selected characteristic data for soils chosen for development work.

Sample	Total As (mg kg^{-1})	pH	Carbon (%)
Tollerton	16	6.75	6.49
2710	626	-	-
DGC 24	1306	3.94	9.99
DGC 26	5760	3.58	9.92

3. 2 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). Concentrated HNO_3, HF, 30% v/v H_2O_2 and $HClO_4$ (BDH Aristar, UK) were used for the dissolution of soil samples. H_3PO_4 (85%) and $C_6H_8O_6$ (BDH Aristar, UK) were used for the extraction of arsenic from soil samples. Inorganic arsenite (As^{III}, Fisher UK), arsenate (As^{V}, Fisher, UK), methylarsonate (MA^{V}, Sigma-Aldrich, UK), and dimethylarsinate (DMA^{V}, Greyhound, UK) were used for the preparation of standards for arsenic speciation analysis. Ammonium nitrate (> 99%) and aqueous ammonia (Fisher, UK) were used in preparation of the mobile phase for anion exchange chromatography.

3.3 Extraction of Arsenic from Soil

Based on the current literature, 1 M and 0.3 M phosphoric acid (H_3PO_4) were employed as these extractants were the ones most frequently used (Garcia-Manyes et al. 2002, Hutton et al. 2005, Kahakachchi et al. 2004, Pizarro et al. 2003, Ruiz-Chancho et al. 2005). Phosphoric acid provides a sample matrix suitable for analysis by ICP-MS, unlike commonly employed solvents such as methanol and acetone, which need to be evaporated and re-dissolved in a suitable solvent, potentially adding to the overall measurement error. Ascorbic acid ($C_6H_6O_8$) was added at concentrations of 0.5 M (Ruiz-Chancho *et al.* 2005) and 0.1 M (Garcia-Manyes *et al.* 2002) to 1 M and 0.3 M phosphoric acid respectively to act as a stabilising agent for inorganic arsenic and also, according to Ruiz-Chancho *et al*. (2005), as a way of enhancing arsenic recoveries. In summary, the two mixed extraction solutions investigated were:

1. 1 M H_3PO_4 with 0.5 M $C_6H_6O_8$

2. 0.3 M H_3PO_4 with 0.1 M $C_6H_6O_8$.

The first extraction solution was selected because it was reported to give the highest recovery and stability of species (Ruiz-Chancho *et al.* 2005). The second extraction solution was investigated on the basis of the findings of both (Hutton *et al.* 2005) who reported 0.3 M H_3PO_4 as the optimum extractant and (Garcia-Manyes *et al.* 2002) who suggested the addition of 0.1 M $C_6H_6O_8$.

3. 4 Microwave Assisted Extraction

Microwave assisted extraction (MAE) was performed using a CEM MARS 5 (CEM Corporation, UK) closed vessel microwave system. Approximately 0.2 g of dry sieved soil (<0.25 mm) was accurately weighed into the microwave vessels and 10

ml of the extraction solution was added. The vessels were then secured and heated at 50% power (300 W) to ramp the vessel temperature to 80ºC. The temperature then held at 80ºC for 15 minutes. This initial heating programme was chosen based on published methods (Garcia-Manyes *et al.* 2002, Hutton *et al.* 2005) and following advice from CEM, UK (*pers. comm.*). The vessels were then cooled to room temperature before the contents were transferred to 30 ml round bottom Nalgene extraction vessels and rinsed three times with 5 ml deionised water to ensure complete transfer. The final volume of 25 ml was then centrifuged for 15 minutes at 2000 rpm and the supernatant carefully removed. Samples were stored in the dark at <4ºC. All speciation analyses were performed within 24 hours, the maximum time period in which species are reported to remain stable (Ruiz-Chancho *et al.* 2005).

3. 5 Orbital Shaking with Sonication

A 0.2 g sub-sample of prepared soil was accurately weighed into 30 ml round bottom HDPE Nalgene vessels. 10 ml of extract solution was added and with lids securely fastened, placed on an orbital shaker for 4 hours at 200 rpm. Extractions were conducted in the dark to avoid species transformation due to UV radiation. After shaking, the extraction vessels were sonicated for 5 minutes, based on the method of Pizarro *et al*. (2003). The same centrifugation and storage procedure used in the MAE method was employed before analysis. Only one extraction step was used, since additional arsenic contained in the second and subsequent extractions has been shown to be derived from residual dissolved arsenic carried over from previous extractions (Francesconi 2003). Table 3 summarises the extraction procedures investigated.

Table 3: Summary of extraction procedures investigated.

Extraction Solution	**Extraction procedure**	
	Method 1 Closed vessel MAE	**Method 2** Orbital shaking with sonication
Solution 1	**1-1)** 1 M H_3PO_4 with 0.5 M $C_6H_6O_8$	**2-1)** 1 M H_3PO_4 with 0.5 M $C_6H_6O_8$
Solution 2	**1-2)** 0.3 M H_3PO_4 with 0.1 M $C_6H_6O_8$	**2-2)** 0.3 M H_3PO_4 with 0.1 M $C_6H_6O_8$

3. 6 Stability of Arsenic Species

Prior to undertaking extractions from soil samples, the stability of As^{III}, As^{V}, MA, DMA and AB under the proposed extraction conditions was investigated. 10 ml of each extraction solution was spiked with 100 µg l^{-1} each of four standard solutions, As^{III}, MA, DMA and AB or As^{V}, MA, DMA and AB, to give a final As concentration of 400 µg l^{-1}. All tests were carried out in duplicate. This method of spiking was employed to allow clear identification of any changes between the As^{III} and As^{V} species as only one inorganic species should be present in each solution following extraction. The extraction procedures were then carried out in full and the final solutions analysed for As species (HPLC-ICP-MS) and total arsenic (ICP-MS) following adequate dilution.

3. 7 Arsenic Speciation in Soils

A quaternary GP50-2 HPLC Pump and an AS-50 autosampler (Dionex, USA) were directly coupled to an ICP-MS (PQ ExCell, Thermoelemental, UK) for the measurement of arsenic species, by direct connection of 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 and its subsequent measurement was synchronised automatically using the ICP-MS Plasmalab software, enabling reproducible sample injections. An anion exchange column (PRP–X100, 250 x 4 mm, 10 µm) with a guard cartridge of the same material, were used to separate the extracted arsenic species present in the soils. The chromatographic separation (Figure 2) was based on a modified gradient elution system developed by (Martinez-Bravo *et al.* 2001). This method (outlined in Table 4) achieved good separation for all arsenic species investigated.

Table 4. Chromatographic parameters

Gradient	**Time**
4 mM NH_4NO_3	0 - 2 minutes
60 mM NH_4NO_3	3 - 6.5 minutes
4 mM NH_4NO_3	7.5 - 10.75 minutes
60 mM NH_4NO_3	11 - 13 minutes
4 mM NH_4NO_3	13.25 - 15 minutes
Flow rate	1 ml min^{-1}
pH	8.65

4 Results and Discussion

4. 1 Extraction of Arsenic from Soils

The results obtained for the extraction procedures investigated are shown in Figure 1. The most efficient method for the extraction of arsenic proved to be MAE with an extraction solution of 1 M H_3PO_4 / 0.5 M $C_6H_6O_8$. Under these conditions a mean

recovery of 92 ± 3% (n = 2) of the total arsenic content of the soil was achieved compared to 78 ± 7% (n = 4) when using 0.3 M H_3PO_4 / 0.1 M $C_6H_6O_8$. The same extraction solutions gave recoveries of 87 ± 3 % (n = 4) and 77 ± 2 % (n = 4) when combined with orbital shaking and sonication. The addition of 1 M H_3PO_4 gave better arsenic recoveries than 0.3 M for both MAE and shaking with sonication. However, this may be due to the higher concentration of ascorbic acid present as this reagent is reported to increase the extraction efficiency of arsenic (Ruiz-Chancho *et al.* 2005). All four methods tested gave good recoveries when compared to the recent published literature (see Table 1).

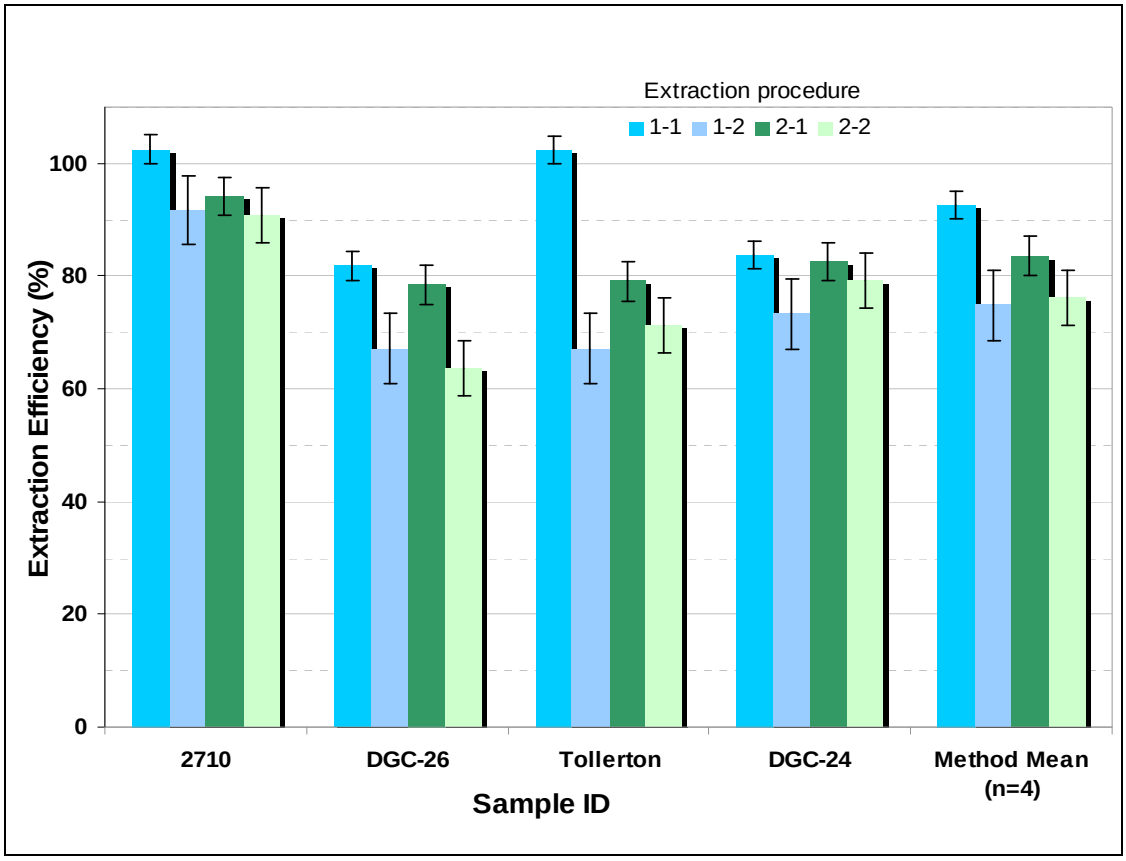


Figure 1. Recovery of arsenic (extraction efficiency) from test samples for the extraction procedures investigated. Errors are expressed as mean % RSD (n = 4).

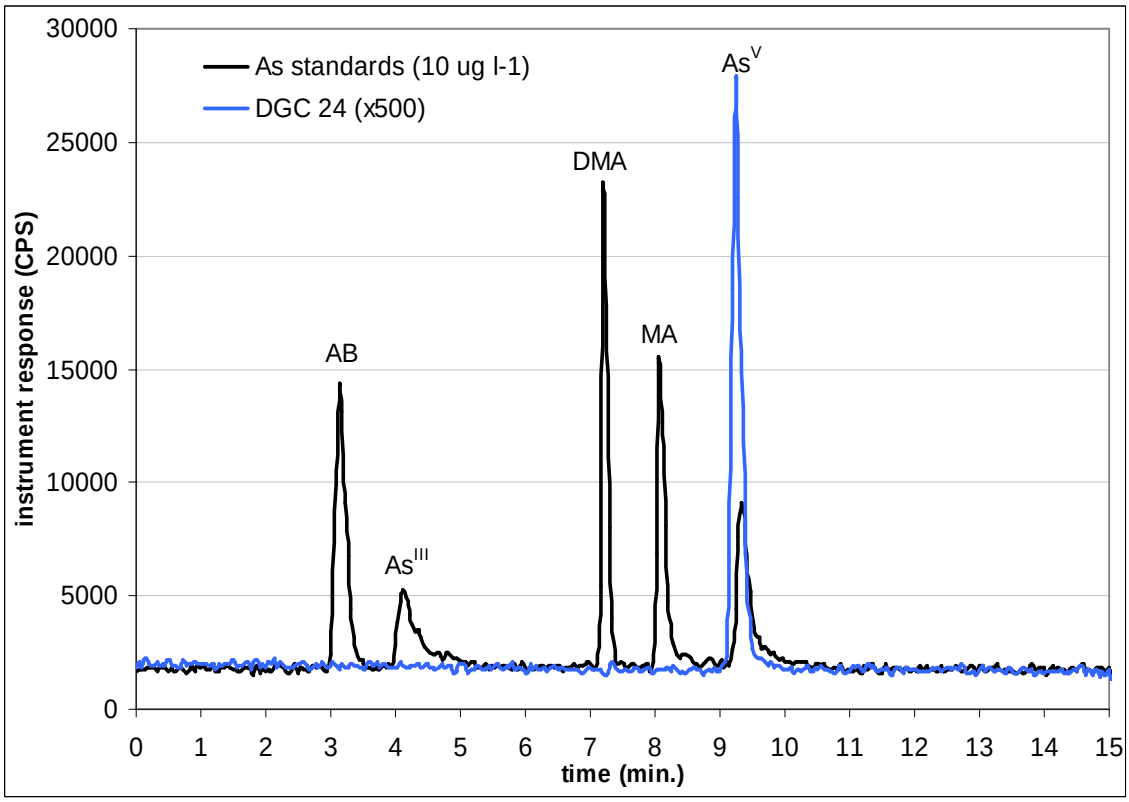


Figure 2. Anion gradient elution profile for a mixed standard solution (10 µg l^{-1}) and soil extract DGC 24 extracted using MEA with extraction solution 1.

4. 2 Stability of Arsenic Species

The recovery of arsenic species as a percentage of the total spiked arsenic (400 µg l^{-1}) and for the individual species (4 x 100 µg l^{-1}) are presented in Table 5. Good recoveries were achieved for all extraction procedures, with % RSD values on the mean value not exceeding the expected 5% RSD random error of the technique, suggesting good reproducibility. No data are available for method 2 with 1 M phosphoric acid because of problems encountered during the separation by HPLC. This was caused by the concentration of phosphoric acid in the matrix being too high, even after a four-fold dilution. Any further dilution would not have allowed the detection of the arsenic compounds present. Iserte *et al*. (2004) reported a similar problem with matrices containing greater than 0.1 M phosphoric acid. Following this discovery, all samples were run at a dilution of at least x 25. The stability of species

under MAE with 1 M phosphoric suggests the same is likely to be true for shaking and sonication, although further work would be necessary for confirmation.

Table 5. Recoveries obtained for total concentration and As species from spiked extraction solutions via HPLC-ICP-MS. Errors are expressed as % RSD.

Method-1 Closed vessel MAE	**0.3 M H_3PO_4 /** **0.1 M $C_6H_6O_8$**	**1 M H_3P0_4 /** **0.5 M $C_6H_6O_8$**
Recovery of total (%)	107 ± 8 (n = 4)	100 ± 7 (n = 4)
Recovery of individual species (%)	100 ± 13 (n = 6)	97 ± 6 n = 4
Method-2 Orbital shaking with sonication	**0.3 M H_3PO_4 /** **0.1 M $C_6H_6O_8$**	**1 M H_3PO_4 /** **0.5 M $C_6H_6O_8$**
Recovery of total (%)	105 ± 4 (n = 4)	
Recovery of individual species (%)	95 ± 13 (n = 8)	no data

4. 3 Arsenic Speciation in Test Soils

Arsenate (As^V) was the only species observed in test samples DGC 24, 26 and NIST 2710, whereas As^{III}, MA and DMA were detected in the Tollerton soil but were below the limits of quantification. In all cases the combined As species measured via HPLC-ICP-MS closely matched the total arsenic in the soil extract measured via ICP-MS (see Table 6).

Table 6. As^V as a percentage of the total As in soil extracts, calculated from the mean for all samples for each extraction procedure.

Extraction procedure	As^V (% of total As in extract)	% RSD	n
1-1	105	10	6
1-2	95	3	3
2-1	89	5	8
2-2	97	6	8

5 Conclusion

The total amount of arsenic extracted by all methods investigated was comparable to those reported in the scientific literature. In particular 1 M H_3PO_4 / 0.5 M $C_6H_6O_8$ used in conjunction with MAE or shaking and sonication gave recoveries that were adequate for quantitative determination of As species. The MAE extraction method provided the optimum conditions for arsenic extraction with good reproducibility. Chromatographic distortion caused by concentrations of phosphoric acid greater than 0.1 M was easily overcome by adequate sample dilution and would only be a problem for the analysis of uncontaminated soils. This is the first time a closed vessel microwave assisted extraction of arsenic from soils has been presented, providing both quantitative and reproducible recoveries of arsenic species, whilst ensuring species stability using a simple and rapid method. Further work will be carried out on a large batch of soils to refine the methodology as part of the PhD dissertation for Mark Button. It is anticipated that this methodology will be written up for external peer review as part of the requirements for the submission of the PhD in late 2008.

References

Defra. 2002. *The contaminated land exposure assessment model (CLEA): technical basis and algorithms.* Department for Environment Food and Rural Affairs and the Environment Agency CLR 10.

Francesconi, K.A. 2003. Complete extraction of arsenic species: a worthwhile goal? *Applied Organometallic Chemistry*, **17**, 682-683.

Francesconi, K.A. and Kuehnelt, D. 2004. Determination of arsenic species: A critical review of methods and applications, 2000-2003. *The Analyst*, **129**, 373-395.

Garcia-Manyes, S., Jimenez, G., Padro, A., Rubio, R. and Rauret, G. 2002. Arsenic speciation in contaminated soils. *Talanta*, **58**, 97–109.

Hutton, C., Bryce, D.W., Russeau, W., Glass, H.J., Jenkin, L.E.T., Corns, W.T. and Stockwell, P.B. 2005. Aqueous and solid-phase speciation of arsenic in Cornish soils. *Mineralogical Magazine*, **69**, 577-589.

Kahakachchi, C., Uden, P.C. and Tyson, J.F. 2004. Extraction of arsenic species from spiked soils and standard reference materials. *Analyst*, **129**, 714-718.

Martinez-Bravo, Y., Roig-Navarro, A.F., Lopez, F.J. and Hernandez, F. 2001. Multielemental determination of arsenic, selenium and chromium(VI) species in water by high-performance liquid chromatography–inductively coupled plasma mass spectrometry. *Journal of Chromatography A*, **926**, 265-274.

Masscheleyn, P.H., Delaune, R.D. and Patrick Jr, W.H. 1991. Effect of redox potential and pH on arsenic speciation and solubility in a contaminated soil. *Environmental Science and Technology*, **25**, 1414-1419.

Moore, J.N. 1988. Partitioning of arsenic and metals in reducing sulfidic sediments. *Environmental Science and Technology*, **22**, 432-437.

Pizarro, I., Gomez, M., Camara, C. and Palacios, M.A. 2003. Arsenic speciation in environmental and biological samples: Extraction and stability studies. *Analytica Chimica Acta*, **495**, 85-98.

Ruiz-Chancho, M.J., Sabé, R., López-Sánchez, J.F., Rubio, R. and Thomas, P. 2005. New Approaches to the Extraction of Arsenic Species from Soils. *Microchimica Acta*, **151**, 241-248.

Appendix B

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

Michael J. Watts [a], Mark Button [a,b,c], Tim S. Brewer[b], Gawen R. T. Jenkin[b] and Chris F. Harrington[c]

a British Geological Survey, Keyworth Nottingham, UK NG12 5GG. E-mail: mbutton@bgs.ac.uk or mwatts@bgs.ac.uk; Fax: +44 0115 9363200; Tel:+44 0115 9363408

b Department of Geology, University of Leicester, Leicester, UK. E-mail: grtj1@leicester.ac.uk or mb274@le.ac.uk Fax: +44 0116 2523981; Tel: +44 0116 2523934

c University of Leicester, Cancer Biomarker and Prevention Group, University Road, Leicester

Abstract

The relationship between the total arsenic concentration and the chemical speciation of arsenic in two species of earthworm (*Lumbricus rubellus* and *Dendrodrllus 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 (Spurgeon *et al.* 2003). 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 (Jager *et al.* 2003, Spurgeon *et al.* 2006, Steenbergen *et al.* 2005, Van Gestel *et al.* 1991) and the basis for toxic effects (Bundy *et al.* 2002, Langdon *et al.* 2005).

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 (Geiszinger *et al.* 1998, Langdon *et al.* 2003b, Pongratz 1998, Tukai *et al.* 2002). Arsenite and arsenate were demonstrated as the main extractable inorganic arsenicals in *L. rubellus* (Langdon *et al.* 2002, Langdon *et al.* 2003b). In most cases arsenobetaine (AB) is present as the primary organic arsenic species with methylarsonate (MA) present at trace levels (Geiszinger *et al.* 1998, Langdon *et al.* 2002). However, Geiszinger *et al*. (2002a) 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}.

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 (Francesconi and Kuehnelt 2004, Hymer and Caruso 2004, Langdon *et al.* 2003b, Pizarro *et al.* 2003) for analyte detection. ICP-MS is suitable

for aqueous samples and aqueous extracts of environmental and biological samples (Xie *et al.* 2002). ICP-MS as the elemental detector offers the possibility of multielemental, multispecies determinations within the same sample measurement (Gomez-Ariza *et al.* 1998, Iserte *et al.* 2004, Martinez-Bravo *et al.* 2001, Roig-Navarro *et al.* 2001).

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} (Klinck *et al.* 2002, Langdon *et al.* 2001). 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 (Klinck *et al.* 2002) 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

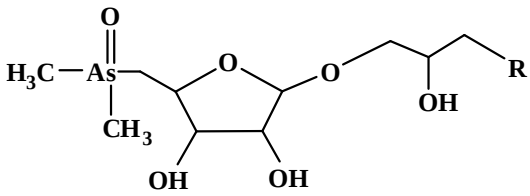
The soil surface (0 - 20 cm) in a 1 m^2 grid was overturned with a spade and placed onto plastic sheeting allowing individual earthworms to be handpicked from their host soil. Specimens were promptly sorted according to species, thoroughly rinsed with deionised water and placed in ventilated tubes with 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 (Langdon *et al.* 2003a). 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 were 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 point. Depurated earthworms were thoroughly rinsed with deionised water, euthanised 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. Soils 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 ($CH_3AsO(OH)_2$, Sigma-Aldrich, UK), dimethylarsinic acid ($(CH_3)_2AsO(OH)$, Greyhound, UK) and arsenobetaine ($((CH_3)_3As^+CH_2COO^-$, 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) (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) 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). Methanol (Fisher Scientific, UK) was employed as a solvent in the extraction procedure. Ammonium nitrate (NH_4NO_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.



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

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_2O_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 / $HClO_4$) (Green *et al.* 2006). 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 (Wei-Chun 2005), which is the usual approach for the extraction of arsenic species from marine organisms and algae (Francesconi and Kuehnelt 2004).

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.(Francesconi 2003) 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 - water 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*. (2001), 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.* (2002a) 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(Madsen *et al.* 2000). 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(Watts *et al.* 2007) 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*. (2003a) with a BAF of 0.07 in L. rubellus. At similarly high arsenic soil concentrations as that described in Langdon *et al*. (2003a) (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(Geiszinger *et al.* 1998), suggesting that elimination rates of arsenic may be increased at higher concentrations of arsenic and bioaccumulation of arsenic is non-linear (Langdon *et al.* 2002).

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**	**Total As in worm cast**	**Total As in worm**	**BAF**
L. rubellus			**(mg kg $^{-1}$)**	**(mg kg $^{-1}$)**	**(mg kg $^{-1}$)**	
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.

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-water 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*. (2002a).

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) (54 ± 20 %).

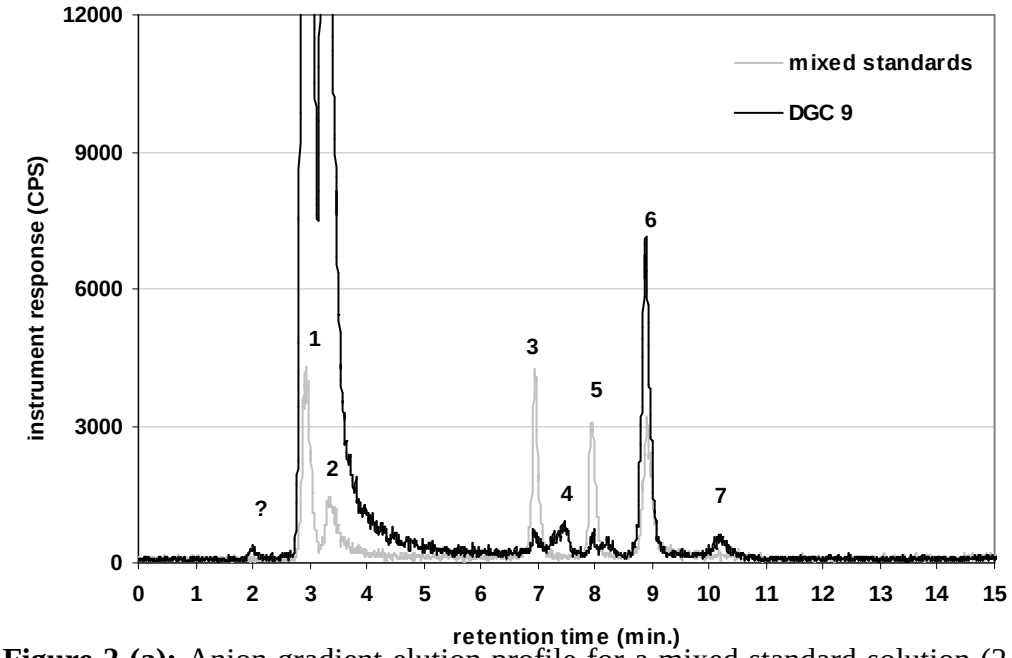


Figure 2 (a): Anion gradient elution profile for a mixed standard solution (2 µg l^{-1}) and an earthworm extract (DGC 9 *D. rubidus*). Peaks 1-7: 1 = AB, 2 = As^{III}, 3 = DMA, 4 = Sugar-2, 5 = MA, 6 = AsV, 7 = Sugar-4

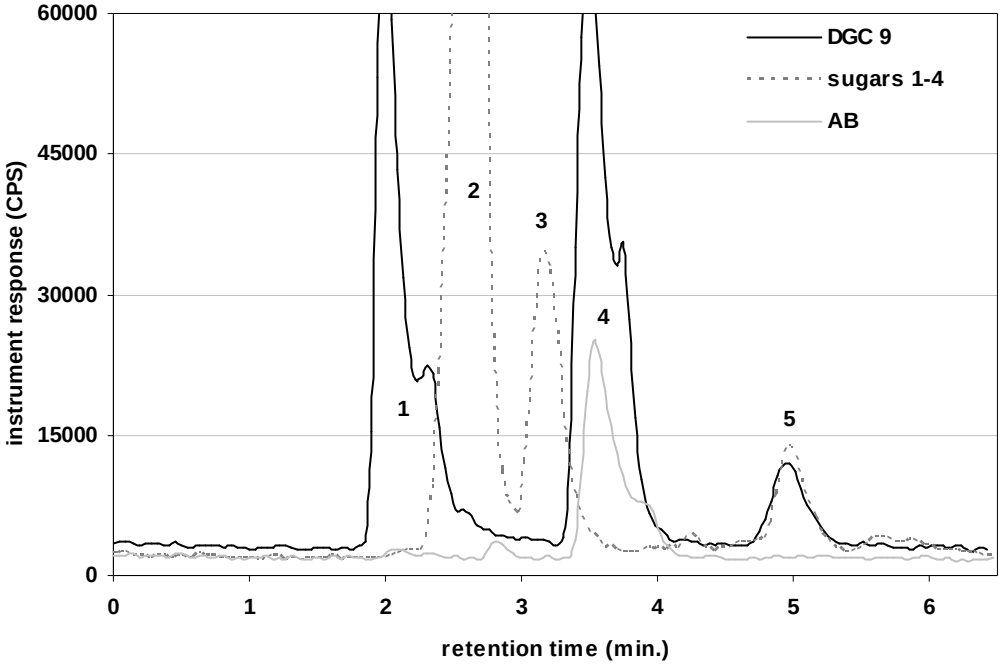


Figure 2 (b): Cation isocratic elution profile for an earthworm extract (DGC 9 *D. rubidus*), an algal extract containing sugars 1-4 and an AB standard at (13 µg l^{-1}). Peaks 1-5: 1 = solvent front containing As^{III} + As^{V} + MA, 2 = DMA + Sugars-2/3, 3 = Sugar-4, 4 = AB, 5 = Sugar 1.

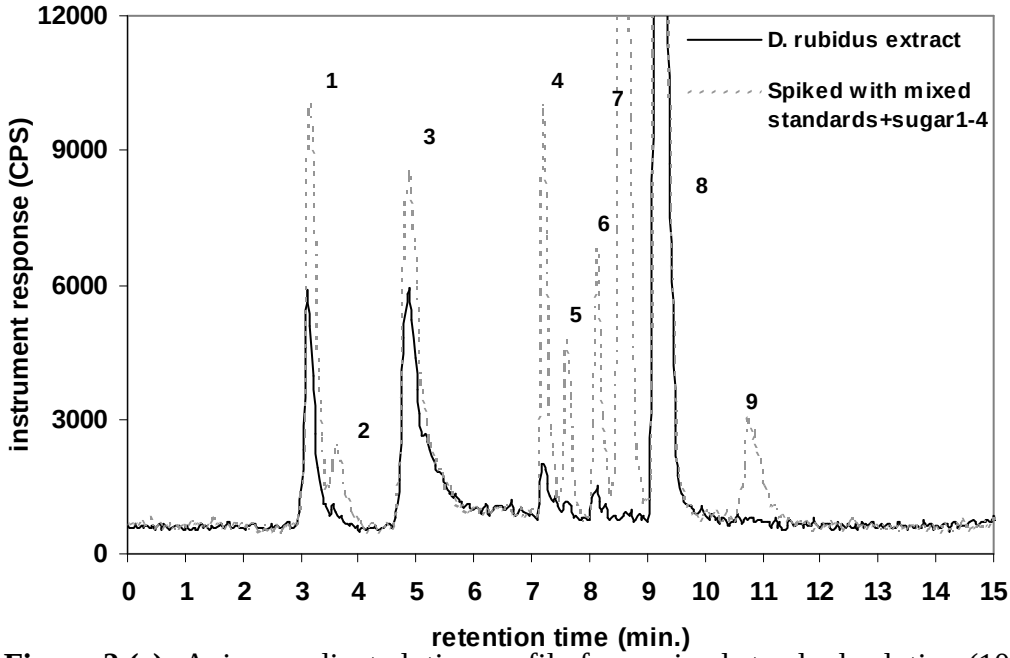


Figure 2 (c): Anion gradient elution profile for a mixed standard solution (10 µg l^{-1}) and a representative earthworm extract. Peaks 1 - 9: 1 = AB, 2 = Sugar-1, 3 = As^{III}, 4 = DMA, 5 = Sugar-2, 6 = MA, 7 = Sugar-3, 8 = As^{V}, 9 = Sugar-4.

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*. (2003b). *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*. (2002a), 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.

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	nd	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

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 (Geiszinger *et al.* 2002a, Geiszinger *et al.* 2002b, Madsen *et al.* 2000). 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 organo-arsenicals 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 organo-arsenicals 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.

Acknowledgements

The BGS-NERC university collaboration committee is thanked for the provision of a PhD studentship for Mark Button. The Tavistock Estate is also thanked for allowing access to the site at the Devon Great Consols. This paper is published with the permission of the Executive Director of the BGS. Professor Francesconi, University of Graz is thanked for provision of some of the standards used in this work.

References

Bundy, J.G., Spurgeon, D.J., Svendsen, C., Hankard, P.K., Osborn, D., Lindon, J.C. and Nicholson, J.K. 2002. Earthworm species of the genus Eisenia can be phenotypically differentiated by metabolic profiling. *FEBS Letters*, **521**, 115-120.

Francesconi, K.A. 2003. Complete extraction of arsenic species: a worthwhile goal? *Applied Organometallic Chemistry*, **17**, 682-683.

Francesconi, K.A. and Kuehnelt, D. 2004. Determination of arsenic species: A critical review of methods and applications, 2000-2003. *The Analyst*, **129**, 373-395.

Geiszinger, A., Goessler, W. and Kosmus, W. 2002a. An arsenosugar as the major extractable arsenical in the earthworm Lumbricus terrestris. *Appl. Organomet. Chem.*, **16**, 473-476.

Geiszinger, A., Goessler, W. and Kosmus, W. 2002b. Organoarsenic compounds in plants and soil on top of an ore vein. *Appl. Organomet. Chem.*, **16**, 245-249.

Geiszinger, A., Goessler, W., Kuehnelt, D., Kosmus, W. and Francesconi, K. 1998. Determination of arsenic compounds in earthworms. *Environmental Science and Technology*, **32**, 2238-2243.

Gomez-Ariza, J.L., Sanchez-Rodas, D. and Giraldez, I. 1998. Selective extraction of iron oxide associated arsenic species from sediments for speciation with

coupled HPLC-HG-AAS. *Journal of Analytical Atomic Spectrometry*, **13**, 1375-1379.

Green, K.A., Chenery, S.R., Barlow, T.S., Taylor, H. and Cook, J.M. 2006. Poster presentation. *13th Biennial National Atomic Spectroscopy Symposium*, Glasgow, UK.

Hymer, C.B. and Caruso, J.A. 2004. Arsenic and its speciation analysis using high-performance liquid chromatography and inductively coupled plasma mass spectrometry. *J. Chromatogr., A*, **1045**, 1-13.

Iserte, L.O., Roig-Navarro, A.F. and Hernandez, F. 2004. Simultaneous determination of arsenic and selenium species in phosphoric acid extracts of sediment samples by HPLC-ICP-MS. *Analytica Chimica Acta*, **527**, 97-104.

Jager, T., Fleuren, R.H.L.J., Hogendoorn, E.A. and De Korte, G. 2003. Elucidating the routes of exposure for organic chemicals in the earthworm, Eisenia andrei (oligochaeta). *Environmental Science and Technology*, **37**, 3399-3404.

Klinck, B.A., Palumbo, B., Cave, M.R. and Wragg, J. 2002. *Arsenic dispersal and bioaccessibility in mine contaminated soils: a case study from an abandoned arsenic mine in Devon, UK*. British Geological Survey RR/04/003.

Langdon, C.J., Meharg, A.A., Feldmann, J., Balgar, T., Charnock, J., Farquhar, M., Piearce, T.G., Semple, K.T. and Cotter-Howells, J. 2002. Arsenic-speciation in arsenate-resistant and non-resistant populations of the earthworm, Lumbricus rubellus. *J. Environ. Monit.*, **4**, 603-608.

Langdon, C.J., Piearce, T.G., Meharg, A.A. and Semple, K.T. 2001. Survival and behaviour of the earthworms Lumbricus rubellus and Dendrodrilus rubidus from arsenate-contaminated and non-contaminated sites. *Soil Biology & Biochemistry*, **33**, 1239-1244.

Langdon, C.J., Piearce, T.G., Meharg, A.A. and Semple, K.T. 2003a. Interactions between earthworms and arsenic in the soil environment: a review. *Environmental Pollution*, **124**, 361-373.

Langdon, C.J., Piearce, T.G., Semple, K.T., Feldmann, J. and Meharg, A.A. 2003b. Arsenic speciation in the earthworms Lumbricus rubellus and Dendrodrilus rubidus. *Environmental Toxicology and Chemistry*, **22**, 1302-1308.

Langdon, C.J., Winters, C., Sturzenbaum, S.R., Morgan, A.J., Charnock, J.M., Meharg, A.A., Piearce, T.G., Lee, P.H. and Semple, K.T. 2005. Ligand Arsenic Complexation and Immunoperoxidase Detection of Metallothionein in the Earthworm *Lumbricus rubellus* Inhabiting Arsenic-Rich Soil. *Environmental Science and Technology*, **39**, 2042-2048.

Madsen, A.D., Goessler, W., Pedersen, S.N. and Francesconi, K.A. 2000. Characterization of an algal extract by HPLC-ICP-MS and LC-electrospray MS for use in arsenosugar speciation studies. *Journal of Analytical Atomic Spectrometry*, **15**, 657-662.

Martinez-Bravo, Y., Roig-Navarro, A.F., Lopez, F.J. and Hernandez, F. 2001. Multielemental determination of arsenic, selenium and chromium(VI) species in water by high-performance liquid chromatography–inductively coupled plasma mass spectrometry. *Journal of Chromatography A*, **926**, 265-274.

Pizarro, I., Gomez, M., Camara, C. and Palacios, M.A. 2003. Arsenic speciation in environmental and biological samples: Extraction and stability studies. *Analytica Chimica Acta*, **495**, 85-98.

Pongratz, R. 1998. Arsenic speciation in environmental samples of contaminated soil. *Science of the Total Environment*, **224**, 133-141.

Roig-Navarro, A.F., Martinez-Bravo, Y., Lopez, F.J. and Hernandez, F. 2001. Simultaneous determination of arsenic species and chromium(VI) by high-performance liquid chromatography-inductively coupled plasma-mass spectrometry. *J. Chrom. A*, **912**, 319-327.

Spurgeon, D.J., Lofts, S., Hankard, P.K., Toal, M., McLellan, D., Fishwick, S. and Svendsen, C. 2006. Effect of pH on metal speciation and resulting metal uptake and toxicity for earthworms. *Environmental Toxicology and Chemistry*, **25**, 788-796.

Spurgeon, D.J., Weeks, J.M. and Van Gestel, C.A.M. 2003. A summary of eleven years progress in earthworm ecotoxicology. *Pedobiologia*, **47**, 588-606.

Steenbergen, N.T.T.M., Iaccino, F., De Winkel, M., Reijnders, L. and Peijnenburg, W.J.G.M. 2005. Development of a biotic ligand model and a regression

model predicting acute copper toxicity to the earthworm Aporrectodea caliginosa. *Environmental Science and Technology*, **39**, 5694-5702.

Tukai, R., Maher, W.A., McNaught, I.J. and Ellwood, M.J. 2002. Measurement of arsenic species in marine macroalgae by microwave-assisted extraction and high performance liquid chromatography-inductively coupled plasma mass spectrometry. *Analytica Chimica Acta*, **457**, 173-185.

Van Gestel, C.A.M., Ma, W. and Smit, C.E. 1991. Development of QSARs in terrastrial ecotoxicology: Earthworm toxicity and soil sorption of chlorophenols, chlorobenzenes and dichloroaniline. *Science of the Total Environment*, **109-110**, 589-604.

Watts, M.J., O' Reilly, J., Smiles, C.A. and Cook, J.M. 2007. *British Geological Survey Technical Report, OR/07/021.*,

Wei-Chun, L. 2005. *Arsenic Analysis and Genotoxicity in Earthworms Using Elemental and Molecular MS and the Comet Assay*. University of Leicester.

Xie, Q., Kerrich, R., Irving, E., Liber, K. and Abou-Shakra, F. 2002. Determination of five arsenic species in aqueous samples by HPLC coupled with a hexapole collision cell ICP-MS. *J. Anal. Atom. Spectrom.*, **17**, 1037-1041.

Appendix C

Thompson Howarth Precision Control Charts

Introduction

Repeatability precision can be assessed simply by conducting duplicate analyses on some or all of the test materials within an analytical run. The precision characteristics of the run are better represented by random analyses of several different, but typical, samples in duplicate then by a greater number of replicate analyses on one or two control materials (RSC 2002).

Thompson Howarth precision control charts are a simple graphical method for assessing and controlling repeatability precision from a moderate number of duplicated analytical results (RSC 2002). There are two methods for plotting the data. 1: for samples with analyte concentrations well above the detection limits (DLs) of the method the duplicate data can be plotted without taking into account DLs. 2: The second method allows the detection limits to be taken into account for samples with low analyte concentrations. In the Thompson Howarth chart the mean result is plotted against the absolute difference of the duplicate analyses then percentile lines are added. The charts outcome is dependant on the independent fitness for purpose (FFP) criterion specified by the analyst (the asymptotic RSD of the duplicate analyse) and the detection limits of the method, plotted using the following equation.

Method 1: P50* = 0.954(Bc)

Method 2: P50 = 0.954(C_L/3+Bc)

Where C_L is the repeatability detection limit, B the asymptotic RSD (FFP criteria), c the concentration of analyte on x axis and P50 the 50th percentile of the absolute difference between duplicate samples as a function of mean concentrations.

* P50 used as example. See (RSC 2002) for a detailed explanation of the method.

Duplicate samples from the Hotblock total digestion method, outlined in Chapter 4, followed by ICP-MS analysis of arsenic for DGC samples are plotted in figure 1. The FFP criterion has been set at 5% RSD for these samples. Method 1 has been employed as all samples are well above the detection limits of the method. Therefore percentile lines are plotted thus: 0.954 x (0.05 x 1000).

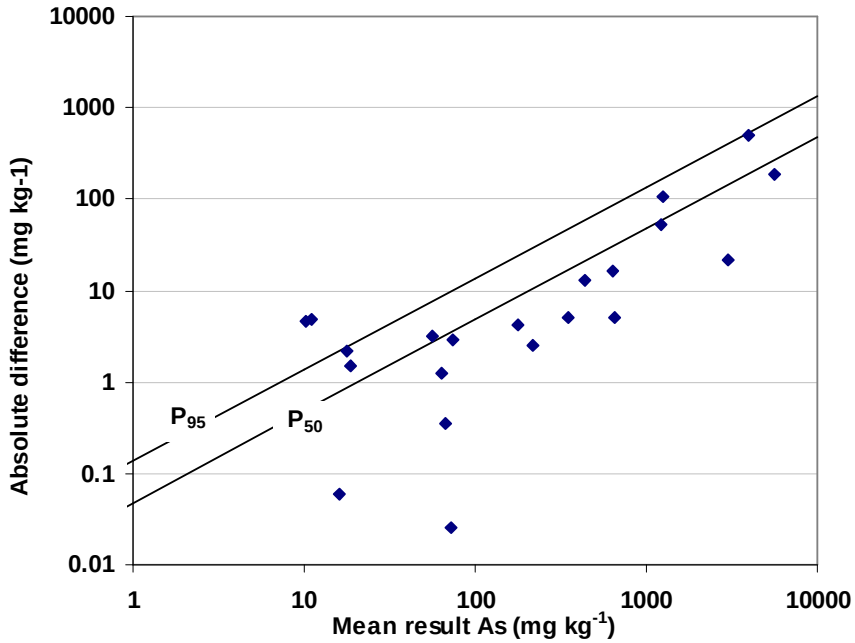


Figure 1: repeatability precision control chart (5%) for the determination of As in geological materials by hot block digestion.

In figure 1 the lines P50 and P95 are the respective 50th and 95th percentiles of the absolute difference between duplicate samples as a function of mean concentrations, assuming a normal distribution. If the duplicate data fall in line with the specified FFP criteria, in this case 5% RSD, on average 95% of the points should fall below the 95th percentile with 50% below the 50th percentile line. If the analytical precision is better a higher proportion of the points will fall below the line. In this case more than 50% of the data points (14 of 21) fall below the 50th percentile line suggesting the repeatability precision is conforming to, or is better than the specified FFP of 5% RSD on the duplicate analyses.

Reference

RSC 2002. Royal Society of Chemistry: A simple fitness-for-purpose control chart based on duplicate results obtained from routine test materials World Wide Web Address:http://www.rsc.org/images/brief9_tcm18-25951.pdf.