ARSENIC BIOTRANSFORMATION IN TERRESTRIAL ORGANISMS

A study of the transport and transformation of arsenic in plants, fungi, fur and feathers,

using conventional speciation analysis and X-ray absorption spectroscopy

by

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ABSTRACT

Arsenic taken up by plants and fungi from contaminated soils can subsequently be introduced into food chains. Given the toxic properties of some arsenic compounds, this may be a cause for concern. Much remains to be learned about how these compounds are transformed and distributed in terrestrial organisms. Radishes, white button mushrooms, fur, and feather samples were thus investigated to gain a better understanding of arsenic biotransformations in terrestrial organisms.

In this study, we utilized two analytical techniques for the detection and identification of arsenic compounds ("arsenic speciation analysis"). High performance liquid chromatography inductively coupled plasma mass spectrometry (HPLC-ICP-MS) provided a highly sensitive method for detecting low levels (ng·g^{-1}) of methanol:water extractable arsenic compounds. X-ray absorption spectroscopy (XAS) techniques provided direct arsenic speciation analysis of tissues, resulting in a more representative arsenic profile of the original organisms, without the need to extract arsenic first. Overall, the results for speciation analysis underline the complementary nature of the HPLC-ICP-MS and XAS techniques.

Mushrooms contained organic arsenic compounds which were not identified in the radish. In particular arsenobetaine (AB), which is usually found as a minor constituent of terrestrial organisms, was a predominant arsenic compound found in mushroom extracts. How AB is synthesized in the environment remains unclear; however, results presented here suggest it was a product of fungal biotransformation and we speculate it may play a role in osmoregulation. In radish, fur and feather samples, direct analysis identified arsenic(III)-sulphur compounds not observed using HPLC-ICP-MS. In plants, these

compounds are likely to be metal/metalloid binding phytochelatin proteins the formation of which has yet to be confirmed *in planta*. In radish plant vasculature, XAS imaging revealed segregation of pentavalent and trivalent arsenic compounds, suggesting differences in arsenic transport. In hair and feathers the formation of arsenic(III)-sulphur compounds may be evidence of arsenic binding to keratin proteins which has been hypothesized to occur, and may contribute to the observed reduction of exogenous arsenic contamination.

LIST OF ORIGINAL PUBLICATIONS (Co-Authorship)

The student's contributions to the thesis manuscripts are as follows:

- Participant in the initial development of research projects

- Primary researcher responsible for the successful completion of experiments, including laboratory work at the Royal Military College of Canada (Kingston, ON), and synchrotron experiments conducted at the Advanced Photon Source (Argonne, IL).

- Responsible for completing all data analysis

- Principal author on all papers

This thesis is based on the following papers:

Chapter. 3 – Smith, P. G., Koch, I., Gordon, R. A., Mandoli, D. F., Chapman, B. D. & Reimer K. J. (2005). X-ray absorption near-edge structure analysis of arsenic species for application to biological environmental samples. *Environmental Science and Technology. 39*, 248-254.

Chapter 4 – Smith, P. G., Koch I. & Reimer K. J. Uptake, transport and transformation of arsenate in radishes (*Raphinus sativus).* (submitted to *Science of the Total Environment*)

Chapter 5 – Smith, P. G., Koch I. & Reimer K. J. Arsenic speciation analysis of cultivated white button mushrooms (*Agaricus bisporus)* using high performance liquid chromatography inductively coupled plasma mass spectrometry and X-ray absorption spectroscopy. (submitted to *Environmental Science and Technology*)

Chapter 6 – Smith, P. G., Koch I. & Reimer K. J. An investigation of arsenic compounds in fur and feathers using X-ray absorption spectroscopy speciation and imaging. (submitted to *Science of the Total Environment*)

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LIST OF ABBREVIATIONS

AB	arsenobetaine
AC	arsenocholine
As	arsenic
AsGS	arsenic glutathione
AsIII	trivalent arsenic
AsV	pentavalent arsenic
APS	Advanced Photon Source
ASU	Analytical Sciences Unit (Queen's University)
C2-AB	arsenobetaine 2
C3-AB	arsenobetaine 3
Con	control
cv.	cultivar
D	Deloro (mine waste collection site)
ddH$_2$O	distilled deionized water
dil	diluted
DMA	dimethylarsinic acid
DMA(III)	dimethylarsinous acid
DMPS	2, 3-dimercapto-1-propane sulfonic acid
DM	dry mass
ESG	Environmental Sciences Group
eV	electron volt
g	gram

GSH	glutathione
h	hour
H-	hydroponic radish treatment
HPLC	high performance liquid chromatography
HG-AAS	hydride generation atomic absorption spectroscopy
ICP-MS	inductively coupled plasma mass spectrometry
ICP-OES	inductively coupled plasma optical emission spectroscopy
In	indium
in planta	occurring or made within a plant
in situ	in the original position
in vitro	made to occur in an experimental environment, outside of the organism
in vivo	occurring or made within a living organism
K-edge	energy at which unbound photoelectrons are emitted on absorption of X-rays
L	arsenic solution treatment
major species	$\geq$ 20% of the total water-soluble arsenic
MeAsDMPS	monomethylarsenic DMPS
$Me_2AsDMPS$	dimethylarsenic DMPS
MDL	method detection limit (takes into account additional steps in the analysis)
min	minute
minor species	<20% and $\geq$1% of the total water-soluble arsenic
mL	milliliter
MMA	monomethylarsonic acid

MMA(III)	monomethylarsonous acid
m/z	mass to charge ratio
MΩ	megahom
n	sample size
NAA	neutron activation analysis
ND	not detectable
n.s.	not significant
PC	phytochelatin
PNC-CAT	Pacific Northwest Consortium Collaborative Access Team
PNC-XOR	Pacific Northwest Consortium X-ray Operations and Research
QC	quality control
reporting limit	lowest concentration at which we know the arsenic and its can be detected and its concentration reported
Rh	rhodium
RMC	Royal Military College of Canada
RPD	relative percent difference
RPM	revolutions per minute
Tetra	tetramethylarsonium oxide
TKN	Total Kjehldahl Nitrogen: combination of organic bound nitrogen and ammonia
TMAO	trimethylarsine oxide
trace species	< 1% of the total water-soluble arsenic
SAM	S-adenosylmethionine
sp.	species (when referring to organisms)

Sugar A   a dimethylarsinyl riboside

μM   micromolar

U   uranium

undil   undiluted

var.   variety (e.g. Cherry Belle is a variety of radish)

v/v   volume to volume

white line   energy required for an electronic transition from an s-orbital to a
                    bound orbital with a significant amount of p-character: for arsenic
                    it is taken to be representative of the K-absorption edge position

wt   weight

XANES   X-ray absorption near edge structure

XAS   X-ray absorption spectroscopy

Yk   Yellowknife (mine waste collection site)

CHAPTER 1 - Introduction

The marine environment has always been a desirable choice for those interested in arsenic research as a result of the naturally elevated concentrations of this element, and the variety of interesting arsenic compounds discovered. However, improved analytical technologies, and public concern over soil and water contamination, have helped promote arsenic research in the terrestrial and fresh water environments.

Arsenic is of interest because arsenicals can be detrimental to human health and certain cancers have been attributed to prolonged exposure. The toxicity of arsenic is determined by its chemical form, so there is a need to be able to identify different arsenic compounds ("speciation analysis"). Some arsenic compounds have the potential for transport between ecosystem trophic levels and this is a source of concern, particularly in areas of elevated arsenic concentrations. This can occur when arsenic from the environment enters a food chain after being taken up by primary producers (e.g. plants), and primary decomposers (e.g. fungi), and is thus made available to higher trophic levels; however, limited information is available on the arsenic biotransformations involved.

Speciation analysis is often used as a starting point for understanding how arsenic is transformed by an organism. Inorganic arsenic taken up by plants usually undergoes limited biotransformation and the major compounds reported include inorganic arsenate, and arsenite. Plants are widely acknowledged to have the potential to form complexes with metal-binding proteins known as phytochelatins, and while these arsenic-sulphur compounds have been observed to form *in vitro*, direct evidence for the their *in vivo* distribution is lacking. In contrast to plants, fungi have been reported to contain a number of organic arsenic compounds. There is some speculation as to the origins of the arsenic

compounds detected; whether they are purely the result of fungal biotransformations, or if they could have been taken up from the surrounding environment. Of particular interest is arsenobetaine, a non-toxic arsenic species common in marine organisms, whose synthetic pathway is yet to be elucidated. Terrestrial animals are known to accumulate arsenic in epidermal tissues such as hair, nails and feathers, providing a record of arsenic exposure which can be used to evaluate the health of a population. However, the majority of arsenic in these tissues remains unidentified due to difficulties extracting the arsenic for conventional speciation analysis. It is theorized that arsenic can bind to sulphur-containing proteins such as keratin which is a main component of hair and feathers, though no findings of arsenic-sulphur species in these tissues has been reported. The focus of this research was to gain a better understanding of the biotransformations of arsenic in terrestrial plants, animals and fungi by investigating arsenic speciation and distribution in these organisms. To accomplish this we used both conventional and the more novel synchrotron-based analytical techniques.

In order to identify arsenic compounds using conventional methods, they must first be extracted from the solid sample. The preferred method of analyzing these extracts is high performance liquid chromatography inductively coupled plasma mass spectrometry (HPLC-ICP-MS). HPLC can separate a wide variety of arsenic compounds, and ICP-MS provides the high level of sensitivity needed to detect arsenic in terrestrial samples where concentrations of some compounds are typically low. However, extraction of arsenic (extraction efficiency) from many types of samples is not always complete, and as a result not all of the arsenic from such a sample can be identified. In addition, sample preparation involved in extraction methods may alter the native arsenic compounds.

X-ray absorption spectroscopy (XAS) offers the opportunity for direct speciation analysis of a solid sample, and thus its use in environmental and biological research is rapidly gaining popularity. Two XAS techniques were used in these studies: X-ray absorption spectroscopy near-edge structure (XANES), and XAS imaging. XANES is a speciation technique which was used to complement HPLC-ICP-MS speciation results. XAS imaging provided a means of determining the distribution of groups of arsenic compounds *in situ*. Both of these techniques are discussed in detail in section 2.0.

The overall objective of this thesis was to gain a better understanding of the fate of inorganic arsenate taken up by terrestrial organisms. Specific objectives were to:

- determine the usefulness of synchrotron techniques in identifying arsenic compounds of environmental/biological relevance (Chapter 3)

- explore how and where arsenic compounds are transformed by examining arsenic species distribution in tissues of organisms:

    - with a predominance of inorganic arsenic compounds – plant (Chapter 4)

    - with a predominance of organic arsenic compounds – fungi (Chapter 5)

- directly analyze samples of fur and feathers to distinguish externally and internally distributed arsenic compounds as an indication of the origin of the arsenic in those tissues: exogenous (from the environment), endogenous (from the body after metabolism) (Chapter 6)

CHAPTER 2 - Background

2.1 ARSENIC AT WORK

The general public indisputably perceives arsenic (As) as a poison. Historically, arsenic in the form of arsenic trioxide (As_2O_3), was used as a homicidal agent. Its popularity peaked in the fifteenth and sixteenth centuries, and this potent killer was described as the *Poison of Kings and the King of Poisons*, or *poudre de succession*, "inheritance powder" (Irgolic, 1988). Its popularity declined, however, after the development of the Marsh test (published 1836) which provided a means of detecting it in the human body (Irgolic, 1988). Already aware of the toxic properties of arsenic, humans have exploited them to produce herbicides, pesticides and exfoliants (Mutschullat, 200; Mortia and Edmonds, 1992).

Despite the common perception, arsenic has long been used for healing purposes. Early Chinese, Indian, Greek and Egyptian civilizations utilized arsenic-containing minerals in their medicines, and Hippocrates (460 – 357 B.C.) was reported to use orpiment (As_2S_3) for treating ulcers (Mutschullat, 2000; Irgolic, 1988). A number of medical formulations containing arsenic were developed in the early twentieth century, including Fowler's solution used to treat skin diseases, syphilis, digestive problems and various internal ailments. Use of many arsenical medications was phased out from 1930 to 1950 when it was realized that users had a significantly elevated incidence of some cancers. Nevertheless, in 2000 the Food and Drug Administration approved the use of arsenic trioxide for the treatment of patients with acute promyelocytic leukemia (Antaman, 2001).

Far from being limited to medicinal and homicidal uses, arsenic has found roles in a wide range of industrial and commercial enterprises. Arsenic is used to improve hardening and flight characteristics of ammunition; it is added to alloys of bronze, lead and copper to improve corrosion resistance; it was a popular green dye for wallpaper, cloth, children's toys and foodstuffs; it has been used to improve complexion; it was used in embalming and taxidermy; as a wood preservative; in the production of glass; in pyrotechnics; and in semiconductors (Moria and Edmonds, 1992; Irgolic, 1988).

2.2 A GLOBAL HEALTH PROBLEM

Arsenic in the environment is causing significant global health problems which have once again brought this element to the attention of the broad public. The United States Environmental Protection Agency (USEPA), the International Agency for Research on Cancer (IARC), and the World Health Organization (WHO) have all identified inorganic arsenic compounds as human carcinogens (Ng *et al.*, 2003; WHO, 1989). Chronic exposure to arsenic can also lead to other endpoints such as hyper/hypo-pigmentation (colour changes of the skin), keratosis (hard patches on palms and feet soles), hypertension, peripheral vascular diseases, and cardiovascular diseases. These negative health effects may arise if arsenic is present in food or drinking water. In particular, 60 – 100 million people in Bangladesh and India are exposed to inorganic-arsenic contaminated drinking water which in time may result in widespread illness in these areas. Similar situations exist in China, Taiwan, Vietnam and Nepal (Ng *et al.*, 2003; Mandal and Suzuki, 2002). It is generally accepted that arsenic concentrations in drinking water $\geq 50\ \mu g \cdot L^{-1}$ are unacceptable for daily consumption (Ng *et al.*, 2003), in Canada

and the United States the guideline is 10 $\mu g \cdot L^{-1}$. There is a pressing need for effective monitoring and measurement of arsenic, given its known toxicity, and prevalence in the environment.

2.3 ARSENIC IN THE ENVIRONMENT: SOIL AND WATER

Arsenic is a naturally occurring element that can be found in atmospheric, marine, freshwater and terrestrial environments. It is mobilized by dissolution in water and emission into the atmosphere. This is accomplished naturally through weathering of minerals and igneous rock, biological processes, and volcanic activities (including submarine volcanism). Human actions also introduce arsenic into the environment via agricultural activities, and through aerial and waste water discharge from mining and industry (Mutschullat, 2000; Cullen and Reimer, 1989; Smith *et al.*, 1998).

An important factor influencing arsenic content in soils is the parent material from which the soil is derived (Smith *et al.*, 1998; Mandal and Suzuki, 2002). Over 200 different types of mineral forms of arsenic (60% arsenates, 20% sulfides or sulfosalts) occur naturally (Mandal and Suzuki, 2002). Arsenic concentrations in soil vary considerably with geographical regions, and in the Canadian environment background values seldom exceed 15 $mg \cdot kg^{-1}$ (Smith *et al.*, 1998).

The types of arsenic compounds found in soil depend on the type of sorbing components, the pH and the redox potential of the soil (Mandal and Suzuki, 2002). Among the arsenic compounds present in soils, the inorganic species (arsenite, arsenate) dominate (Fig. 2.1). Under oxic soil conditions arsenates are the dominant species, while arsenites predominate under reducing conditions (Mandal and Suzuki, 2002; Smith *et al.*,
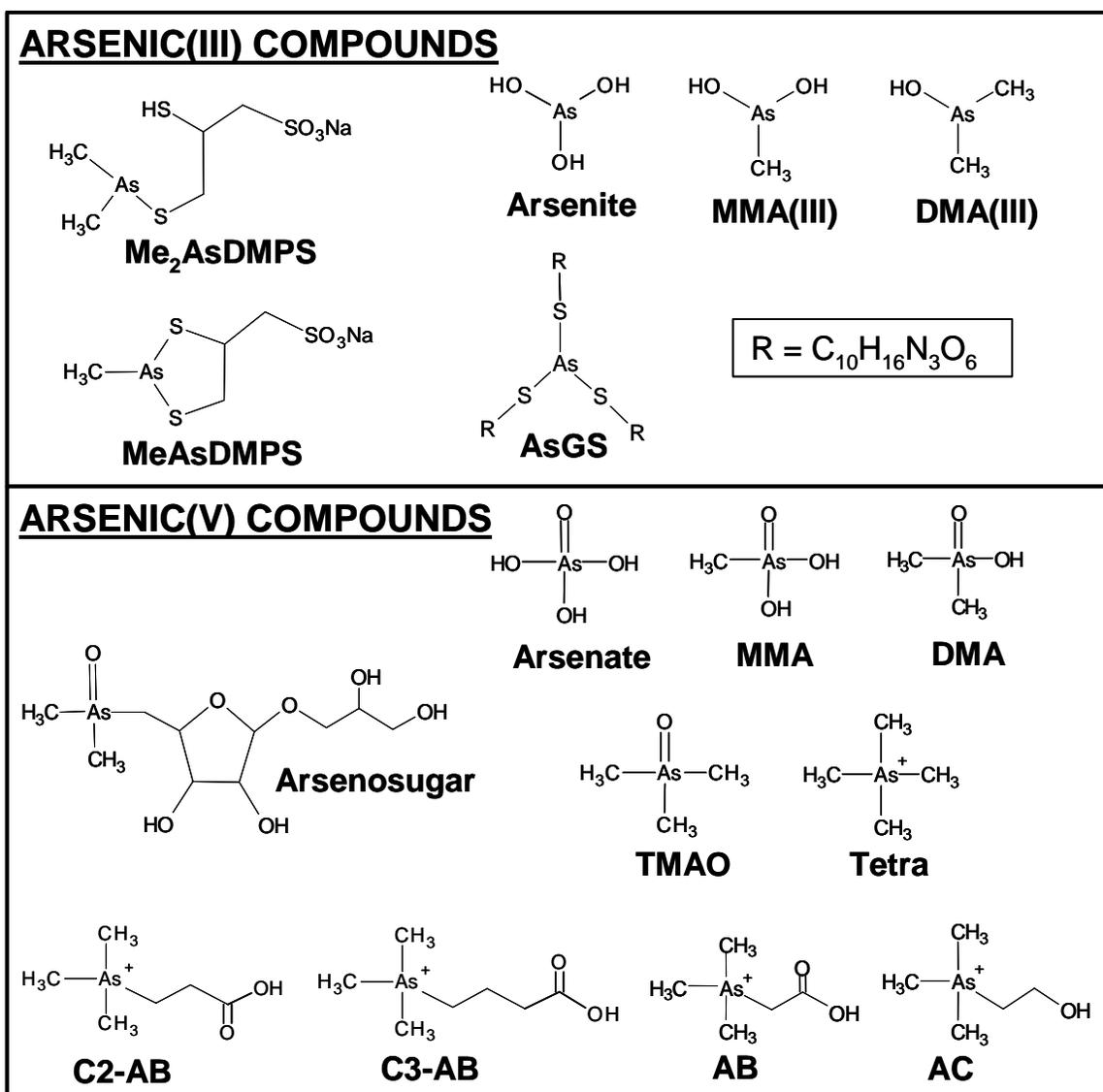
FIG. 2.1: Common inorganic and organic arsenic compounds found in biological samples. Arsenite and arsenic-glutathione (AsGS) are trivalent species. Arsenate, monomethylarsonic acid (MMA), dimethylarsinic acid (DMA) and arsenobetaine (AB) are pentavalent species.

1998). Both of these species are subject to oxidation-reduction reactions in soils, as well as microbial methylation reactions (Andrewes *et al.*, 2004; Cullen and Reimer, 1989; Smith *et al.*, 1998). In soil solution, over a normal soil pH range of 4-8, the most thermodynamically stable arsenite and arsenate species are H_3AsO_3, $H_2AsO_4^-$ and $HAsO_4^{2-}$ (Cullen and Reimer, 1989, Smith *et al.*, 1998).

The more arsenic that is present in soil solution, the more it is available to plants and microorganisms. The adsorption and desorption of arsenic to soil particles, which regulates the arsenic concentration in soil solution, is affected by soil constituents. Iron (Fe) appears to play a major role in many soil types, with iron oxyhydroxide minerals having a strong affinity for pentavalent (AsV) arsenic (Smith et al., 1998). Under reducing conditions, or when soil nitrate is depleted, the FeIII in these minerals is converted to soluble FeII, and the arsenic is released into solution (Reynolds *et al.*, 1999, Smith *et al.*, 1998). On the other hand, phosphate competes with arsenic for sorption sites in well-aerated soils (Tlustoš *et al.*, 2002; Reynolds *et al.*, 1999). Heavy additions of phosphate fertilizers can enhance arsenic mobility in soils (Smith *et al.*, 1998); this scenario can occur because many agricultural soils contain high levels of arsenic from arsenic based pesticides and herbicides, commonly used before the mid 1900s (Smith *et al.*, 1998).

Arsenic in water is also found primarily as inorganic forms of arsenate and arsenite. At neutral pHs arsenic can be stabilized as the pentavalent oxyanions $H_2AsO_4^-$, $HAsO_4^{-2}$ or neutral trivalent $As(OH)_3$ (Cullen and Reimer, 1989; Mandal and Suzuki, 2002). The total soluble arsenic in water depends on the amount of particulate matter. Turbulent waters resuspend sediments which can remove arsenic from solution by sorption.

However, these sediments can also act as a source of arsenic to the water column, and this sorption and desorption has been reported to follow a seasonal cycle (Byrd, 1988). Organic arsenic compounds, attributed to biological activities, have been reported in both fresh and marine water in trace amounts (Mandal and Suzuki, 2002).

2.4 ARSENIC IN THE ENVIRONMENT: ORGANISMS

Arsenic in soils and water is influenced by biological transformations which are considered to be the source of organic arsenic compounds in these environments. The pathway(s) for their production have two different endpoints: the first results in simple methylated compounds usually found in terrestrial organisms, and the second produces more complex organoarsenicals usually observed in marine organisms (Andreae, 1986).

2.4.1 Simple methylated compounds

Biomethylation of arsenic describes the formation of methylated arsenic compounds by the enzymatic transfer of a methyl group, to an arsenical, within a living organism (Bentely and Chasteen, 2002; Dembitsky and Rezanka, 2003). With only a few recent clarifications, the mechanism suggested by Challenger (1945) remains the best model for describing the production of arsenite, monomethylarsonic acid (MMA), monomethylarsonous acid (MMA(III)), dimethylarsinic acid (DMA), dimethylarsinous (DMA(III)) and trimethylarsine oxide (TMAO), from inorganic arsenate (Cullen and Reimer, 1989; Irgolic, 1988; Bentely and Chasteen, 2002) (Fig. 2.1). This process involves a series of alternating reduction and oxidative methylation reactions (Fig. 2.2).
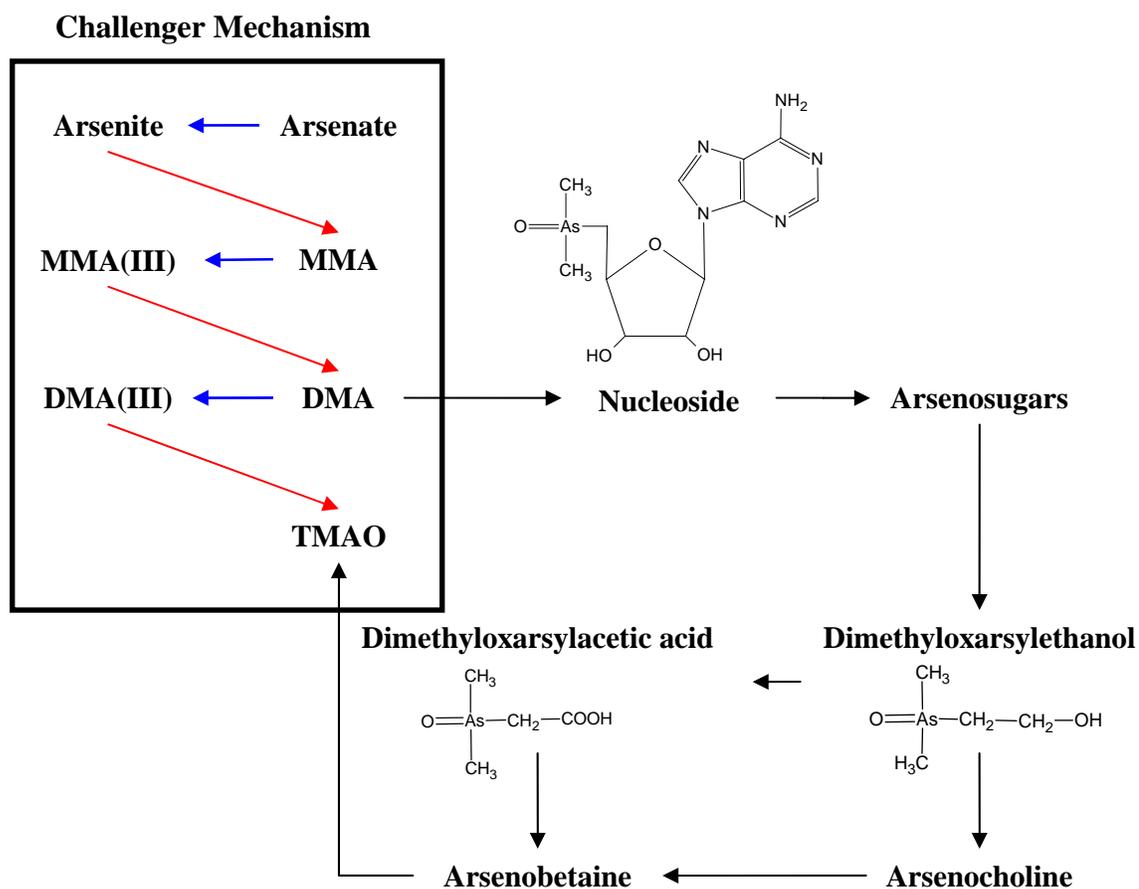
FIG. 2.2: Tentative biogeochemical pathway for the formation of arsenosugars, arsenocholine and arsenobetaine from inorganic arsenate (Adapted from Andreae, 1986; Dembitsky and Rezanka, 2003). Through a series of alternating reduction (blue arrow) and oxidative methylation (red arrow) steps (Challenger Mechanism) arsenate is converted to DMA, which in turn is converted to arsenosugars, possibly via a nucleoside intermediate. Arsenosugars are thought to be necessary for the production of arsenocholine and arsenobetaine. Structures not shown are found in Fig. 2.1.

Since arsenite is generally regarded as the inorganic compound that is methylated, arsenate must first be reduced (Irgolic, 1988). The resulting arsenite is then methylated and oxidized, a process mediated by arsenic methyltransferase enzymes, and involving the methyl donor S-adenosylmethionine (SAM) which is considered to be nearly universal in biological systems (Andreae, 1986; Mandal and Suzuki, 2002; Bentely and Chasteen, 2002). Similar reduction and oxidative methylation steps are repeated on the resulting pentavalent compounds producing trivalent and pentavalent MMA and DMA as well as TMAO (Fig. 2.2).

Simple methylated arsenic compounds have been reported in a wide variety of organisms (Andreae, 1986; Marafunte *et al.*, 1984; Mandal and Suzuki, 2002; Ng *et al.*, 2003); however, in terrestrial environments, these compounds appear to mark the endpoint of arsenic biotransformation. Humans transform a large percentage of ingested arsenic in this fashion, and under normal conditions 60-80% of ingested arsenic is routinely detected in the form of DMA, 10-20% as MMA, and only 10-30% as inorganic arsenic (Ng *et al.*, 2003; Mandal and Suzuki, 2002). Terrestrial plants are reported to contain predominantly inorganic arsenic (Dembitsky and Rezanka, 2003), although nitrogen and phosphate starved tomatoes (Nisssen and Benso, 1982), and cultures of periwinkle cells (Cullen *et al.*, 1989) have been reported to contain DMA and MMA, and in red clover arsenobetaine (AB) and arsenocholine (AC) were identified (Geiszinger *et al.*, 2002). Fungi too can contain a high percentage of organic arsenic compounds (Dembitsky and Rezanka, 2003), and it has been hypothesized that more highly evolved fungi produce more complex organoarsenicals (Šlejkovec *et al.*, 1997).

2.4.2 Other organoarsenic species

In addition to the inorganic and simple methylated arsenic compounds, marine

organisms have been found to contain additional organic arsenic species (Andreae, 1986;

Irgolic, 1988). Inorganic arsenic compounds in marine water are converted to

arsenosugars which are commonly found in phytoplankton, marine algae and kelp

(Andreae, 1986; Dembitsky and Rezanka, 2003; Hanaoka *et al.*, 1992). In brown algae, it

was concluded that arsenate was likely transformed to DMA via the Challenger method

(Fig. 2.2), and DMA was involved in the formation of an intermediate, N-[4-

(dimethylarsinoyl)butanoyl]taurine and/or nucleoside, used in the production of

arsenosugars (Geiszinger *et al.*, 2001).

Arsenosugars are thought necessary for the metabolic production of arsenobetaine

which is a dominant arsenic compound in marine invertebrates and fish (Andreae, 1986).

However, there still remains no proven pathway for the formation of arsenobetaine. Most

schemes rely on microbial breakdown products of arsenosugars to provide the precursor

compounds required for AB production. It has been theorized that because AB and

glycine betaine $[(CH_3)_3N^+CH_2COO^-]$ are structurally similar, arsenic metabolism may

parallel nitrogen metabolism, and AB may play a role in osmoregulation for some aquatic

organisms (Edmonds and Francesconi, 1988). Such a pathway may also result in the

synthesis of arsenocholine another dominant arsenic compound found in marine

invertebrates (Fig. 2.2).

2.4.3 Arsenic-sulphur compounds

It is noteworthy that both animals and plants are thought to contain a variety of

inorganic arsenic-sulphur compounds resulting from the complexation of trivalent arsenic

with proteins. Much of the evidence supporting this theory is indirect such as independent verification of arsenic and sulphur in a sample (Sorensen et al., 1982; Tu *et al.*, 2004); however, the presence of arsenic(III)-sulphur compounds in earthworms, Chinese break fern, and Indian mustard has been confirmed by direct analysis (Langdon *et al.*, 2002; Langdon *et al.*, 2006; Pickering *et al.*, 2000; Webb *et al.*, 2003).

2.5 ARSENIC TOXICITY

The chemical form of arsenic impacts its toxicity, and we have seen that its chemical form can be changed through abiotic and biotic processes. Most of the research on arsenic toxicology deals with acute toxicity of this element to animals. Chronic toxicity is more difficult to determine but is being actively researched (B'Hymer and Caruso, 2004). Acute toxicity is dependent on the physical state of arsenic, which can affect the rate of absorption into cells and rate of elimination from the body (B'Hymer and Caruso, 2004; Mandal and Suzuki, 2002). Mouse models successfully demonstrated that inorganic arsenic compounds were carcinogens, and they are generally considered to be more acutely toxic than other arsenicals (Ng *et al.*, 2003; Ritsema *et al.*, 1998). Trivalent arsenite is considered more toxic than pentavalent arsenate (Mandal and Suzuki, 2002; Hei and Filipic, 2004), and a similar trend was observed for DMA and MMA where the trivalent forms were found to be very toxic (Styblo *et al.*, 1997; Styblo *et al.*, 2000). Despite being significantly less toxic than inorganic compounds, pentavalent MMA is a possible cancer promoter (Brown *et al.*, 1997), and pentavalent DMA was reported to be a complete carcinogen in experimental animals (Kenyon and Hughes, 2001). These reports suggest that methylation, once considered a detoxification process, may be partly

responsible for the adverse effects of arsenic. Arsenobetaine is an interesting arsenic compound, in part because it is virtually non-toxic, with a reported LD50 in mice of > 10000 mg·kg^{-1} (Andrewes *et al.*, 2004; Marafunte *et al.*, 1984). Arsenocholine, which may be a precursor of arsenobetaine, is believed to be non-toxic as well (B'Hymer and Caruso, 2004). Arsenosugars do not appear to exhibit any toxic effects, but can be broken down into some of the more toxic simple methylated compounds already discussed, and may prove detrimental to organisms chronically exposed (Andrewes *et al.*, 2004; Hansen *et al.*, 2003).

Less information is available on the toxicity of arsenic to plants. In general only arsenite, arsenate, MMA and DMA are considered in plant studies. A few reports suggest pentavalent DMA and MMA can be more phytotoxic than inorganic arsenic (Tlustoš *et al.*, 2002). This may result from more efficient uptake of these arsenicals by the species in question.

2.6 MODES OF TOXICITY

Regardless of the organism studied, the modes of arsenic toxicity are considered to be similar though the actual mechanisms remain for the most part unclear (Andrewes *et al.*, 2004). The mechanisms involved in arsenic toxicity can be both cytotoxic and genotoxic.

Cytotoxic mechanisms result in cell death and examples include the inactivation of cell enzymes, and allosteric inhibition. Cell metabolism may be inhibited if arsenate interferes with oxidative phosphorylation, the process by which adenosine tri-phosphate (ATP) is produced. ATP is a high energy molecule required for cellular function. Arsenate may form an ester with the ATP precursor adenosine di-phosphate (ADP) and

the hydrolysis of this ester (known as arsenolysis) would inhibit energy production causing cell death (Mandal and Suzuki, 2002). Trivalent arsenicals, including DMA, MMA and arsenite, inhibit key cellular enzymes by binding to sulfhydryls which are often enzyme active sites (Andrewes *et al.*, 2004; Mandal and Suzuki, 2002). For example, arsenite can inactivate pyruvate dehydrogenase preventing the generation of ATP in the citric acid cycle.

Genotoxins cause genetic mutations and chromosomal alterations. Arsenic appears to induce predominantly large mutations resulting in nonviable cells, such as those observed using a human-hamster hybrid cell (A_L) mutagenic assay, where arsenite induced the deletion of millions of base pairs at the CD59 locus (Liu *et al.*, 2001; Hei *et al.*, 1998). Arsenite is a reported clastogen, causing breaks in chromosomal DNA (Kligerman *et al.*, 2003), and also increased the levels of free radicals in A_L cells which can induce DNA lesions consistent with oxidative damage (Liu *et al.*, 2001; Kessel *et al.*, 2002). Similarly DNA damage may be caused by reactive oxygen species (ROS) produced after exposure to trivalent DMA and MMA (Nesnow *et al.*, 2002); however, the pathway for the production of these ROS is largely unknown. *In vitro* tests using synthesized arsenosugars reported that the trivalent form of the sugar studied, nicked plasmid DNA, whereas the pentavalent form did not (Andrewes *et al.*, 2004). Arsenate may inhibit DNA repair mechanisms by replacing phosphate in DNA, but there is no direct evidence to support this theory (Mandal and Suzuki, 2002).

2.7 SPECIATION ANALYSIS

It is known that to fully understand the toxic potential of arsenic it is necessary to determine not only the concentration but also to identify the individual chemical forms

(termed "speciation analysis"). This is not only necessary to understand toxicity, but also accumulation, transport, storage, and detoxification of arsenic by organisms.

2.7.1 HPLC-ICP-MS

For the past two decades high performance liquid chromatography (HPLC) paired with inductively coupled plasma mass spectrometry (ICP-MS) has been the preferred method for characterization of arsenic compounds (Fig. 2.3). HPLC-ICP-MS is a multi-element, multi-isotope technique which has the desirable attribute of a high sensitivity level provided by the MS (B'Hymer and Caruso, 2004). One of the earlier uses of this analytical method reported an absolute detection limit for arsenic of 50-300 pg (Beauchemin et al., 1989).

Arsenic must be extracted from a solid sample into solution before it can be analyzed using HPLC-ICP-MS (Fig. 2.3). A mixture of methanol:water was used in these studies to extract the water soluble compounds (Gong *et al.*, 2002; Koch *et al.*, 2001) from plants and fungi. HPLC is a favoured technique used for the separation of dissolved arsenic species (B'Hymer and Caruso, 2004). A sample is passed through a column containing stationary phase material (resin) that will interact with the arsenic and retard the passage of each compound to varying degrees. Ion-exchange chromatography is the most extensively used type of chromatography, and separates arsenic compounds based on charge. Anion exchange resins are positively charged, designed to separate negatively charged arsenic compounds, and cation resins are negatively charged, designed to separate positively charged arsenic compounds. The charge of the arsenic compound is controlled by the pH of the mobile phase passing through the column.
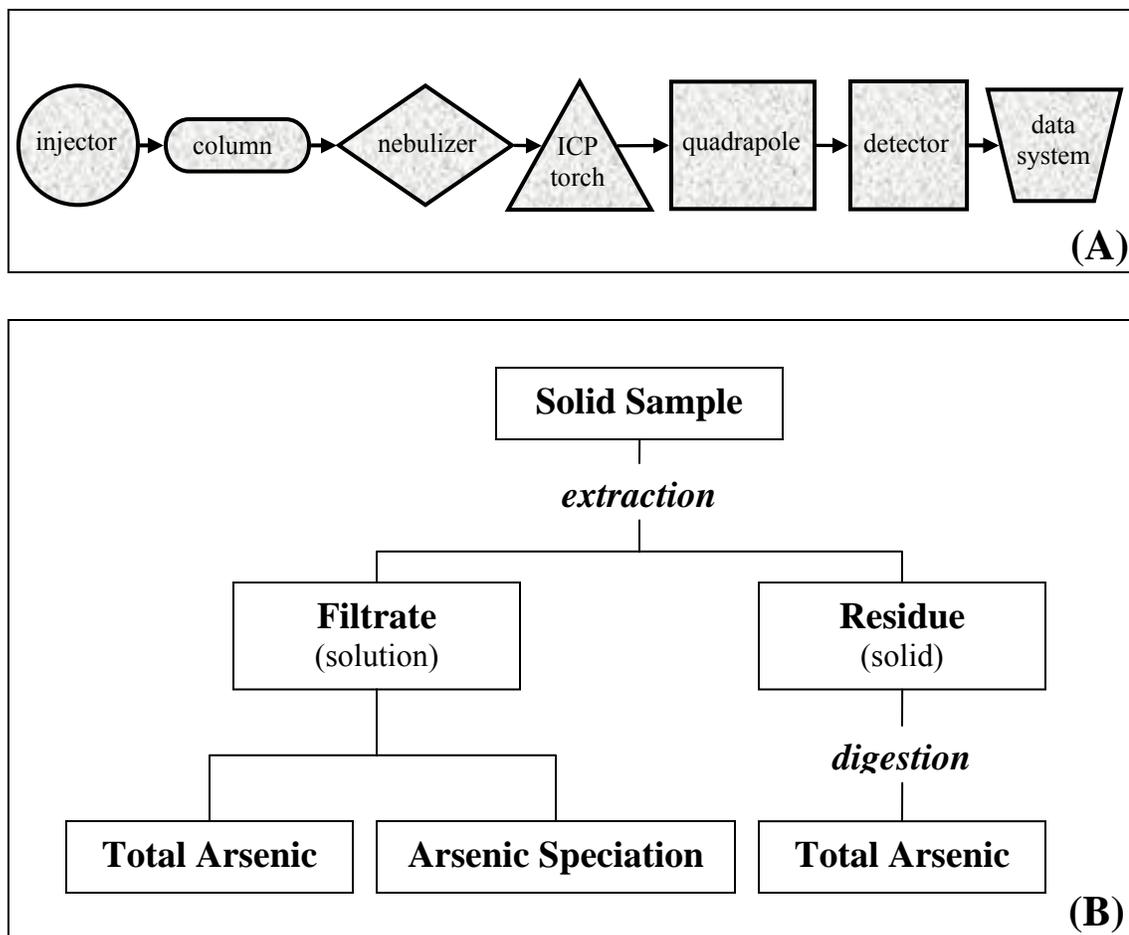
Fig. 2.3: **(A)** Schematic of a high performance liquid chromatography inductively coupled plasma mass spectrometry (HPLC-ICP-MS) set-up. Sample is introduced at the injector and separated on the column (HPLC). The nebulizer converts the liquid sample into a fine spray and introduces it into the instrument where the ICP torch converts the analyte to ions. The quadrapole sorts the ions by mass-to-charge ratio, and individual ions are "counted" by the detector. (B) Flow chart of steps involved in extraction arsenic for HPLC-ICP-MS analysis. Extraction used was equal parts methanol:water (v:v), and digestion was with nitric acid.

2.7.2 X-ray absorption spectroscopy

Spectroscopy uses light or particles which have been emitted, absorbed or scattered by matter, to investigate the properties of that matter. X-ray absorption spectroscopy (XAS), utilizes electromagnetic radiation which can range from infrared (a few eV) to hard X-rays (10^5 eV) and is called "synchrotron radiation" after the facility that produces it. A synchrotron (Fig. 2.4) is a large ring shaped facility with charged particles (e.g. electrons) continuously circulating within a magnetic field. As the path of the charged particles is bent synchrotron radiation is emitted (called beamlines). At the Advanced Photon Source (APS), where the XAS data for this thesis was collected, electrons are used as the charged particles, and X-rays are produced.

The X-rays are used to bombard a sample and eject electrons from a particular binding shell in an atom (Penner-Hahn, 1999). In XAS, inner shell electrons are excited to outer empty orbitals, leaving an inner shell "hole". When electrons from outer orbitals fill the "hole", energy is released, and emitted as radiation (fluorescence). The energy absorbed or emitted is distinctive for specific atoms, and is known as the absorption edge (Fig. 2.5). With the appropriate equipment the absorption edge can be recorded and is called the X-ray absorption near edge structure (XANES) region (Parsons *et al.*, 2002).

A XANES spectrum provides information on the identity of many arsenic compounds at one particular sample location. This information includes three-dimensional geometry, coordination environment, and oxidation state (Parsons *et al.*, 2002). By moving the sample in the path of the beam held at a constant energy representative of one particular arsenic compound, XAS can be used to "map" the location of that arsenic compound over a larger area. This "mapping" is referred to as XAS imaging and was used in this research
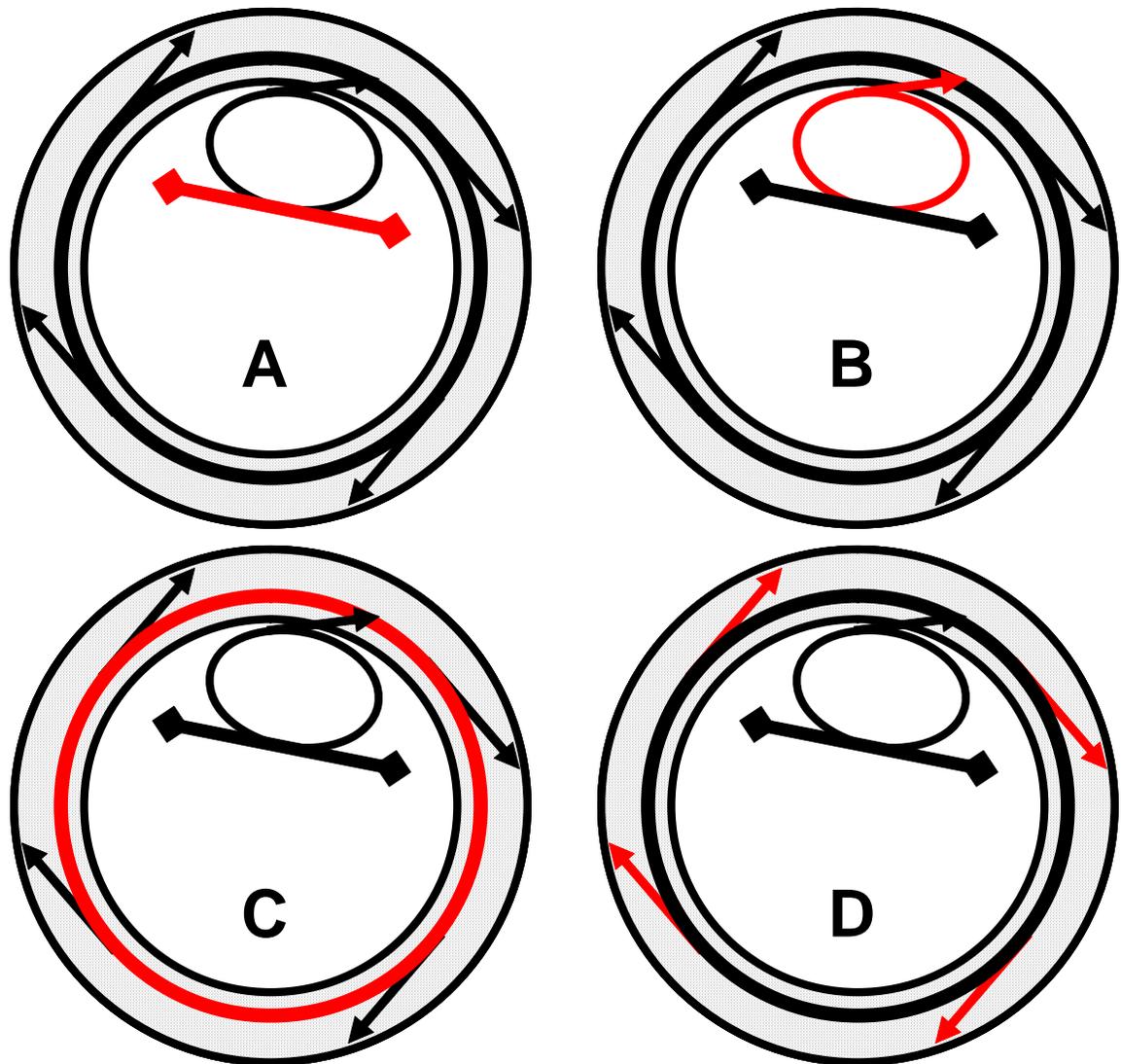
Fig. 2.4: Aerial schematic looking down onto a synchrotron facility to illustrate its operation; based on the Advanced Photon Source facility (Argonne, IL). The red lines indicate the following steps: (A) An ELECTRON GUN produces the charged particles. (B) The BOOSTER RING accelerates the electrons to approximately the speed of light. (C) Electrons from the booster ring are transferred to the STORAGE RING. (D) As the path of the electrons circling the storage ring is bent, synchrotron radiation is produced (called BEAMLINES). By varying the equipment on each beamline the radiation for experiments can be manipulated.
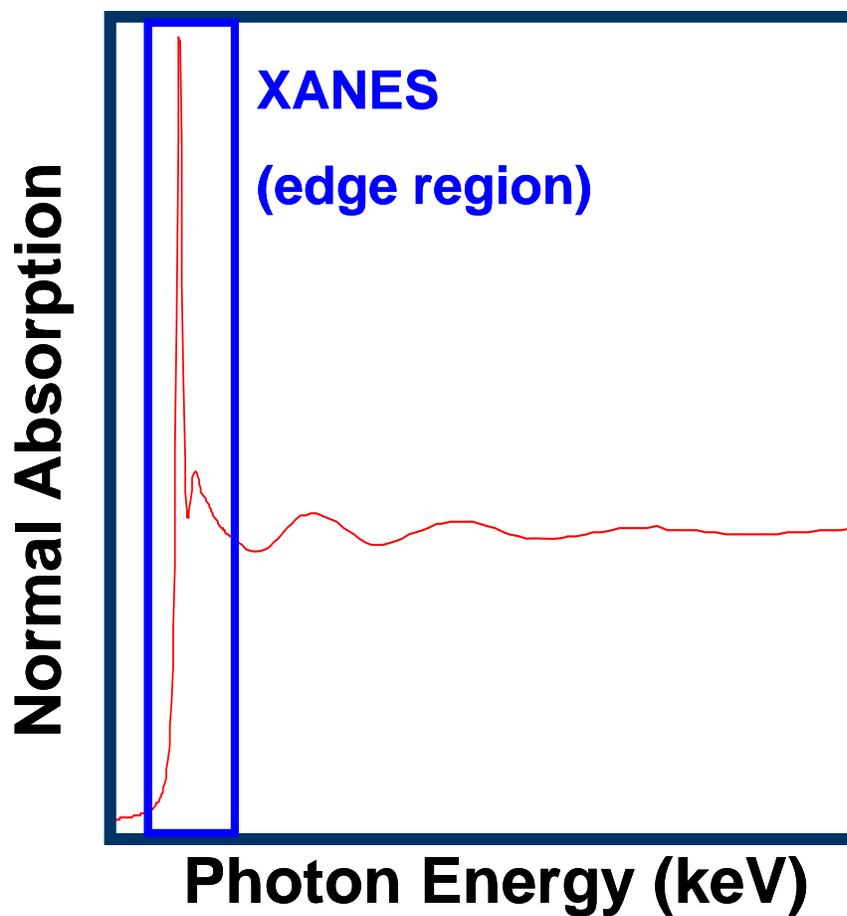
Fig. 2.5: Typical X-ray absorption near edge structure (XANES) spectra. Edge Region refers to the absorption edge. The photon energy of this feature varies with oxidation state. keV = kilo-electron volt

to localize and distinguish dominant trivalent and pentavalent arsenic compounds in a sample. XAS has become a valuable research tool with many biological and environmental applications, by making arsenic speciation analysis possible in solid phase samples, including non-crystalline matrices.

2.8 OBJECTIVES AND SCOPE OF WORK

Using both conventional and synchrotron techniques a better understanding of the interaction of arsenic compounds with biological systems was achieved. HPLC-ICP-MS is a well documented technique, providing good sensitivity for the detection of a wide variety of arsenic compounds; but complete characterization of arsenic in a sample is confounded by limitations associated with extraction of arsenic from a sample. XAS can be used for direct speciation of arsenic compounds *in situ*, but is less sensitive than HPLC-ICP-MS, and is best for the identification of groups of similar arsenic compounds. Together these techniques can identify a larger variety of arsenic compounds than individually, as well as provide distribution information for arsenic in tissues.

Ultimately, the identification of arsenic compounds using these two techniques rests on the availability of known standards be they synthetic or isolated from natural sources. To this end, arsenic compounds of biological and environmental significance were analyzed for XANES spectra, to be used for reference (Chapter 3). This work established the usefulness of XANES in identifying arsenic species, and marks the first time many of these compounds have been investigated by XAS.

These two techniques were applied to the investigation of how and where inorganic arsenic compounds are transformed and located in terrestrial plant and fungi. Among the

predominantly inorganic arsenic compounds identified in plants XANES analysis

identified an arsenic-sulphur compound previously unobserved using HPLC-ICP-MS.

The possible identity of these arsenic(III)-sulphur compounds is discussed for radish

plants, and the effects of sample preparation on arsenic speciation are considered

(Chapter 4). Unlike the radish, biotransformation of inorganic arsenic in white button

mushrooms resulted in the production of organoarsenic compounds. In particular the

possible origin and role of arsenobetaine is explored (Chapter 5). Finally, XAS imaging

was used to try to distinguish exogenous (from environment) and endogenous (from

body) sources of arsenic in fur and feathers by determining the distribution of internal

and external arsenic compounds using XAS imaging (Chapter 6).

CHAPTER 3

X-ray absorption near-edge structure analysis of arsenic species for application to biological environmental samples[1]

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3.1 ABSTRACT

Arsenic (As) is an element that is ubiquitous in the environment, and is known to form compounds with toxic, even carcinogenic properties. Arsenic toxicity is a function of its chemical form (species). Identification of arsenic species is necessary to accurately determine the transformation and fate of arsenicals as well as the actual risk posed by arsenic contamination. We report X-ray absorption near-edge structure (XANES) measurements of 16 biologically important arsenic compounds. Solid and aqueous

[1] Smith, P. G., Koch, I., Gordon, R. A., Mandoli, D. F., Chapman, B. D. & Reimer K. J. (2005). X-ray absorption near-edge structure analysis of arsenic species for application to biological environmental samples. *EnvironmentalScience and Technology. 39*, 248-254.

standards were studied for differences in XANES spectral features, white line positions, stability during exposure to the beam, and stability between two beam exposures separated by 48 hours. Samples containing AsIII (11870.0 - 11871.7 $\pm$ 0.5 eV) and AsV (11872.6 – 11875.3 $\pm$ 0.5 eV) were easily distinguished by white line energies and could be further subdivided into a total of 7 groups. Valuable examples include As(III)-sulphur compounds (11870.0 $\pm$ 0.5 eV), arsenobetaine and arsenocholine (11872.6 $\pm$ 0.5 eV), and arsenosugar A (11873.3 $\pm$ 0.5 eV). A growing number of environmental and biological studies use X-ray absorption spectroscopy (XAS) results to complement their more traditional analyses. Results provided here are intended to help make XAS more accessible to new users interested in the study of arsenic in the environment.

3.2 INTRODUCTION

Arsenic (As) is ubiquitous in the earth's crust in a variety of minerals including arsenopyrite (FeAsS), orpiment (As_2S_3) and realgar (As_4S_4). It can then be released into the environment through natural weathering. Mining, industrial activities, and to a lesser extent, agricultural practices, are common anthropogenic sources of arsenic compounds. Once introduced, the chemical form of arsenic is altered by a variety of biogeochemical processes (Cullen and Reimer, 1989; Burlo *et al.*, 1999; Francesconi *et al.*, 2002).

The two most common soluble forms of inorganic arsenic in environmental samples are arsenate (with arsenic in the +5 oxidation state, i.e. AsV) and arsenite (with arsenic in the +3 oxidation state, i.e. AsIII). In aerobic environments arsenate is thermodynamically predicted to occur as an oxyanion ($H_2AsO_4^-$ and/or $HAsO_4^{2-}$), while arsenite (as $As(OH)_3$) dominates under reducing conditions (Manning *et al.*, 2002; Cullen *et al.*, 1989; Arai *et al.*, 2001) . Organic arsenic compounds produced from the

biotransformation of these inorganic arsenicals are widely distributed in biological tissues, and to a lesser extent in soils and water. Figure 3.1 displays the chemical formula of sixteen environmentally important arsenic compounds. Each of these varies in toxicity and potential for accumulation in organisms (Cullen and Reimer, 1989). It is therefore important to identify these compounds in samples to better understand and predict the risks they may present to human, wildlife and plant populations.

While much has been learned about the distribution of inorganic and organic arsenic compounds in the environment (Appendix A), there are still a number of unanswered questions. Inorganic arsenic species are known to be converted to methylated arsenicals via biochemical pathways (Cullen *et al.*, 1989; Challenger, 1945), and methylated compounds are common in the terrestrial environment. The presence of simple methylated compounds such as MMA(III and V) (monomethylarsonous acid and monomethylarsonic acid), DMA(III and V) (dimethylarsinous and dimethylarsinic acid), TMAO (trimethylarsine oxide), or Tetra (tetramethylarsonium ion) indicates metabolism of arsenic either by microbes or more complex organisms (Cullen and Reimer, 1989; Meharg and Hartley-Whitaker, 2002). Methylated arsenic compounds formed or taken up by organisms occupying a lower trophic level (e.g. mushrooms, algae, earthworms) are thought to be the precursors for more complex organoarsenicals such as AB (arsenobetaine), or the arsenosugars that have been identified in these organisms (Cullen and Reimer, 1989; Langdon *et al.*, 2003; Lai *et al.*, 1997) but the actual pathways of, for example, arsenobetaine synthesis have yet to be elucidated. In some cases the presence of more complex organoarsenicals in organisms occupying higher trophic levels in the marine environment (e.g. fish) might indicate accumulation from an outside source, most likely through ingestion (Cullen and Reimer, 1989; Francesconi *et al.*, 1999).
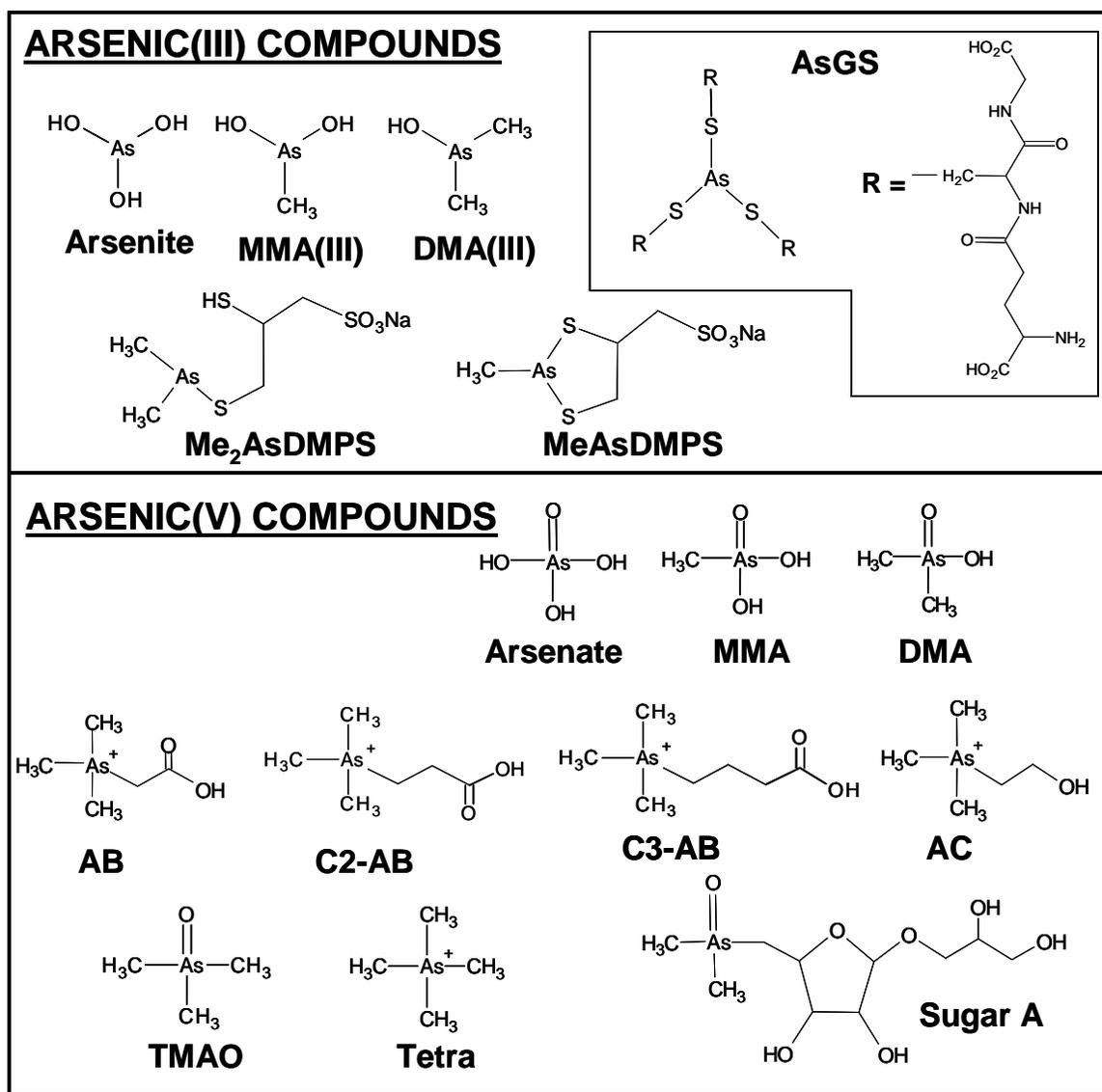
FIG. 3.1: Chemical structures of 16 arsenicals most relevant to biological systems. MMA(III) = monomethylarsonous acid; DMA(III) = dimethylarsinous acid; Me₂As (dimethylarsenic), MeAs (monomethylarsenic) DMPS = 2,3-dimercapto-1-propane sulfonic acid; AsGS = arsenic glutathione; MMA = monomethylarsonic acid; DMA = dimethylarsinic acid; TMAO = trimethylarsine oxide; Tetra = tetramethyl arsonium; AB = arsenobetaine; C2-AB = arsenobetaine 2; C3-AB = arsenobetaine 3; AC = arsenocholine; Sugar A = a dimethylarsinyl riboside.

Conversely, some compounds (e.g. TMAO) may result from catabolism of more complex arsenicals (Cullen and Reimer, 1989).

Traditionally arsenic species are identified using time consuming, indirect methods, which determine the identity of the compound through chemical manipulation. Pretreatment can involve physical homogenization and drying of samples, processes that may alter the chemical form of the arsenic. Strong acid is used to determine the total amount of arsenic in a sample, but cannot distinguish particular arsenic species. Extraction methods have therefore been designed to extract only specific arsenic species (Gong *et al.*, 2002). For example water or methanol/water is typically believed to extract the mobile component of arsenic in the sample (Gong *et al.*, 2002; Koch *et al.*, 2001), whereas hydrogen peroxide extracts arsenic bound more tightly by organic matter. Extraction methods using oxalic acid and sodium dithionite are used to release arsenic bound by metal oxy-hydrides, and diethylether removes arsenic bound by lipids. Once extracted, arsenicals are commonly separated using ion exchange, size-exclusion and reverse-phase high performance liquid chromatography (Gong *et al.*, 2002). Different arsenic compounds can be measured by element specific detection methods (e.g. atomic absorption spectrometry or inductively coupled plasma mass spectroscopy) (Gong *et al.*, 2002; Koch *et al.*, 2002). Although these techniques have the advantage of low (i.e. parts per billion) detection limits, they are often limited by incomplete extraction; this is particularly true for biological samples from the terrestrial environment (Koch *et al.*, 2002). X-ray absorption spectroscopy (XAS) is a quick means of sample analysis that minimizes sample manipulation, is indifferent to solution or solid phase samples, is element and oxidation state specific, and is sensitive to parts per million (Cutler *et al.*, 2001; Parsons *et al.*, 2002).

By convention, XAS is divided into two regions: X-ray absorption near-edge structure (XANES) and extended X-ray absorption fine structure (EXAFS). XANES analysis focuses on the absorption-edge region of the XAS spectrum from a few eV below an element's K-edge to approximately 30 eV above the edge. The K-absorption edge (K-edge), or ionization threshold, is defined as the energy required to remove an electron in a 1s orbital and eject it into the continuum. The white line energy is defined as the energy required to transfer a 1s electron to a bound orbital below the ionization threshold. Features identified in XANES analysis reveal the most probable chemical environment around the arsenic, through the identification of oxidation state, and atom type and arrangement. Higher K-edge energies generally indicate a higher oxidation state because the greater positive charge of these compounds makes it more difficult to photoionize the 1s electron. These data in turn can be used to identify and quantify phases present in a sample (Foster *et al.*, 1998). The EXAFS part of the spectrum has a typical range of 30 eV up to 1000 eV above the K-edge and provides direct structural information (e.g. near-neighbour type, distance, and coordination number).

In environmental and biological studies XAS has been used to study the interaction of metals with surfaces of major mineral phases (Arai *et al.*, 2001; Bostick *et al.*, 2003; Bostick and Fendorf, 2003; Farquhar *et al.*, 2002), the oxidation state and coordination of metals to soils and proteins, and the local structural environment of metals in living organisms (Parsons *et al.*, 2002). For the study of arsenic, XAS has been used to examine arsenic speciation in mine tailings (Foster *et al.*, 1998; Paktunc *et al.*, 2003), and in arsenic tolerant plants and earthworms (Webb *et al.*, 2003; Langdon *et al.*, 2002). XANES is used to assign chemical identity empirically (compare sample spectra to known compounds), and not quantitatively through simple mathematical formula.

Inappropriate comparisons of, for example, pure crystalline structures to molecular

structures in biological samples may make it difficult to determine the composition and

/or relative concentrations of the compounds present in the sample of interest. To

maximize the usefulness and accuracy of XAS for the identification of arsenic species

within biological samples, the scientific community needs the spectral fingerprints of a

comprehensive, expanded list of model compounds. To this end we have studied 16

individual organic and inorganic arsenic compounds (Fig. 3.1) that are frequently

detected in a variety of organisms. We have characterized their XANES for use as

standards relevant to samples taken from the environment.

3.3 MATERIALS AND METHODS

3.3.1 Standards Preparation

Standards were classified as containing arsenic either in the V or the III oxidation

states. Pentavalent starting materials included arsenate (obtained as KH_2AsO_4 - Fluka

reagent grade), monomethylarsonic acid [MMA – Pfaltz and Bauer reagent grade],

dimethylarsinic acid [DMA – Aldrich reagent grade], trimethylarsine oxide (TMAO -

synthesized), tetramethylarsonium iodide (TETRA – synthesized (Auger, 1906)),

arsenobetaine (AB – synthesized (Minhas *et al.*, 1998)), arsenobetaine 2 (C2-AB –

synthesized (Minhas *et al.*, 1998)), arsenobetaine 3 (C3-AB – synthesized (Minhas *et al.*,

1998)), arsenocholine (AC – synthesized (Minhas *et al.*, 1998)), and (R)-2,3-

dihydroxypropyl 5-deoxy-5-dimethylarsinyl-β-D-riboside (Sugar A – synthesized (Stick

et al., 2001)). Trivalent starting materials included arsenite (obtained as As_2O_3 – Fluka

reagent grade), monomethylarsenic(III) diiodide (MMA(III) – synthesized (Quick and

Adams, 1922)), dimethylarsenic(III) iodide (DMA(III) – synthesized (Roberts *et al.*,

1926)), dimethylarsenic 2,3-dimercapto-1-propane sulfonic acid sodium salt
(Me$_2$AsDMPS – synthesized (Auger, 1906)), monomethylarsenic DMPS (MeAsDMPS –
synthesized (Auger, 1906)), and arsenic glutathione (AsGS – synthesized (Serves *et al.*,
1995)). Synthesized compounds were prepared by Drs. H. Sun and W.R. Cullen using
standard methodologies. All such compounds were provided in a pure (>97%) state as
determined by nuclear magnetic resonance (NMR), elementary analysis (EA), and mass
spectroscopy (MS). Sugar A, DMA(III) and TMAO were only available as aqueous
solutions, the remaining arsenic compounds were originally obtained as solids.

Both solid samples and aqueous solutions (dissolved in distilled deionized H$_2$O) of
the arsenicals were analyzed. Solid arsenic standards were mixed with silica and sealed
into 3mm deep wells of an aluminum sample holder with Kapton™ tape on either side.
Silica was chosen as a neutral material for diluting standards (Table 3.1), however;
biological matrices are predominantly water, therefore, the XANES of the arsenic
compounds in aqueous solution are probably most relevant. Aqueous preparations were
absorbed into a piece of Kimwipe™ and then the saturated samples were mounted
between two strips of Kapton™ for XANES analysis (Table 3.1). During analysis,
samples prepared in this fashion were observed by camera and did not leak. These
samples were visually moist after analysis. Aqueous arsenic standards mounted in the
aluminum sample holder, and/or a wet cell, during an independent beamtime session
(Fig. 3.2 open circles), had similar XANES spectral shape to samples absorbed into
Kimwipe™. Undiluted standards (arsenite, arsenate, DMA, MMA) were mounted as a
thin layer of powder between two pieces of Kapton™ tape.

TABLE 3.1: List of arsenic standards for sample comparisons. Standards selected have the most relevance to biological systems. Standards were prepared as aqueous solutions (diluted with distilled deionized H_2O), and as solids (mixed with silica). pH values correspond to aqueous standards (N/A = not available). Acronyms below are defined in terms of the starting material.

Arsenic Standard	Solution Concentration ($\mu g \cdot L^{-1}$)	Solid Concentration ($\mu g \cdot L^{-1}$)	pH
Arsenite	1000	102271	9.0
MMA(III)	50	7635	0.5
DMA(III)	Immiscible	N/A	1.0
MeAsDMPS	10052	10408	4.0
Me$_2$AsDMPS	9495	10056	5.0
AsGS	10294	3766	N/A
Arsenate	10408	10936	5.0
MMA	870	10284	9.0
DMA	968	10643	6.5
TMAO	100	N/A	N/A
Tetra	10540	10000	5.0
Arsenobetaine	100	10009	2.0
Arsenobetaine 2	~1000	10000	N/A
Arsenobetaine 3	~1000	10000	N/A
Arsenocholine	12128	10817	4.0
Arsenosugar X	~100	N/A	4.5

Arsenite = As_2O_3; MMA(III) = monomethylarsenic(III) diiodide; DMA(III) = dimethylarsinic(III) idodide; Me$_2$As = dimethylarsenic, MeAs = monomethylarsenic, DMPS = 2,3-dimercapto-1-propane sulfonic acid; AsGS = arsenic glutathione; Arsenate = KH_2AsO_4; MMA = monomethylarsonic acid; DMA = dimethylarsinic acid; TMAO = trimethylarsine oxide; Tetra = tetramethylarsonium iodide; AB = arsenobetaine; C2-AB = arsenobetaine 2; C3-AB = arsenobetaine 3; AC = arsenocholine; Sugar A = a dimethylarsinyl riboside.
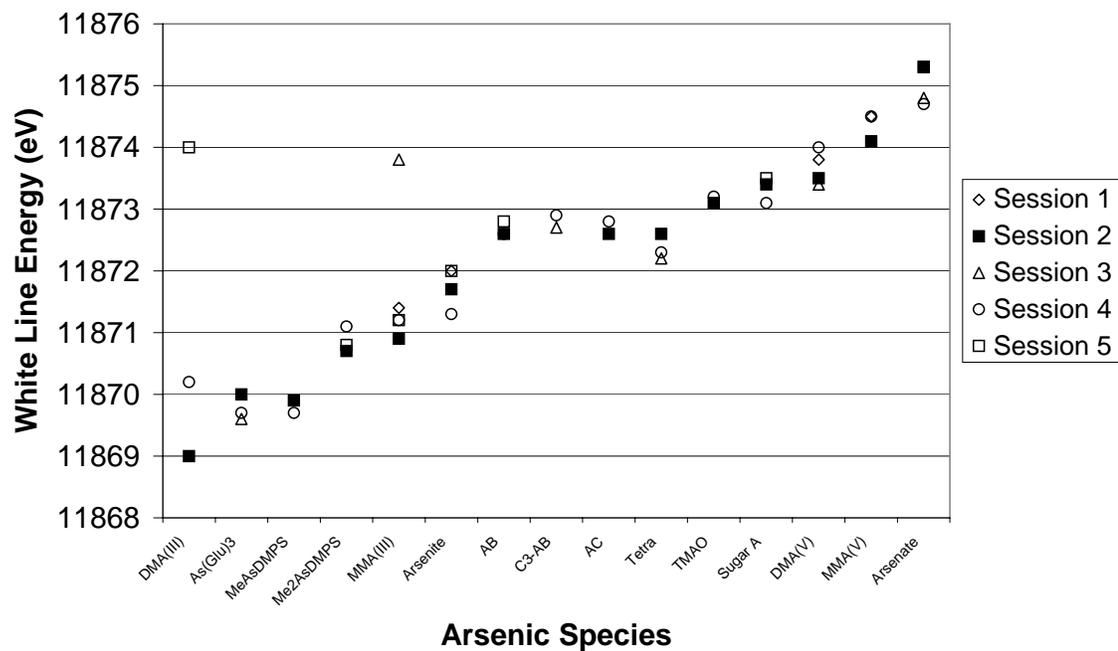
FIG. 3.2: White line (peak of the absorption edge) energies for arsenicals in aqueous arsenic solution analyzed during independent sessions at the beamline. Generally, white line energies were reproducible within 0.5 eV. DMA(III) was not reproducible. Session 5 DMA(III), and the session 3 MMA(III) may have oxidized to DMA and MMA respectively. The experiment testing both aqueous and silica standards took place during session 2.

3.3.2 Spectroscopic Techniques

XANES and EXAFS spectra were collected at the bending magnet beamline of the Pacific Northwest Consortium Collaborative Access Team (PNC-CAT), Sector 20 at the Advanced Photon Source (APS), Argonne National Laboratory (Heald *et al.*, 1999). A silicon (111) double-crystal monochromator (bandwidth ~ 5000 $\Delta E/E$ at 12 keV, detuned to 85% of maximum intensity for harmonic rejection, with 1mm slits 51 m from the source) and a rhodium-coated harmonic mirror for further planar rejection provided X-rays for measurement. The monochromator was calibrated using the first inflection point of the gold L_{III} absorption edge (11919.7 eV (Kraft *et al.*, 1996)) for measurements at the arsenic K-edge (11868 eV). A slit of 1mm vertical by 4mm horizontal was used after the mirror to define the size of the X-ray beam incident on upon the sample. Nitrogen-filled transmission ionization chambers were present before and after the samples for normalization to the incident intensity and transmission measurements, respectively. Fluorescence data for aqueous preparations were collected using a solid-state Ge(Li) detector (Canberra model GL0055PS) or an argon-filled fluorescence ionization chamber, and no self-absorption was observed for the compounds studied (Jiang and Crozier, 1998). Transmission measurements were collected for the undiluted samples, and some silica diluted samples using a nitrogen filled parallel plate transmission ion chamber. Typically, 5 scans were collected and averaged (0.5 eV step size over the edge) before background-removal and normalization-to-edge-jump. The program, WinXAS (Ressler, 1997), was used for processing the spectra.

3.4 RESULTS AND DISCUSSION

Analysis of standards was conducted to determine (a) if solid samples and aqueous solutions of the same arsenic compound had similar white line energies and XANES spectral features, (b) if standards suffered radiation-induced changes due to beam exposure, and (c) if aqueous arsenic standards were stable over a period of 48 h. XANES spectra for the arsenic compounds analyzed are given in Figure 3.3. The white line energy for arsenate was observed to be 3.6 eV greater than that of arsenite. The greater positive charge of arsenate causes the core electrons to be bound more tightly by the nucleus, therefore; more energy is required to remove one of these electrons. Generally for every electron lost there is a 2 to 3 eV increase in observed white line energy (Deschamps *et al.*, 2003). Group 1 compounds have lower white line energies than arsenite due to the difference in electronegativities of oxygen and sulphur. Core electrons of arsenic atoms in an As-S bond are more easily photoionized than the 1s electrons of arsenic in an As-O bond. For both arsenate and arsenite, peak height for the silica mixture was lower than the aqueous preparation (Fig. 3.3). This result has been reported previously for the sodium salts of arsenic versus those salts in solution (Webb *et al.*, 2003). To accurately fit and quantify the amount of arsenic compounds in a sample, appropriate measurements for white line height to post-edge height are needed, and are usually obtained in transmission mode. The XANES profile for solid arsenite, both undiluted and mixed with silica, had a unique peak feature at 11882.8 eV (Fig. 3.3). Our studies examined solid arsenite as arsenic oxide (As_2O_3), and the observed peak is attributed to the crystal structure. In comparison, solid sodium arsenite XANES spectra do not have the observed feature (Arai *et al.*, 2001).
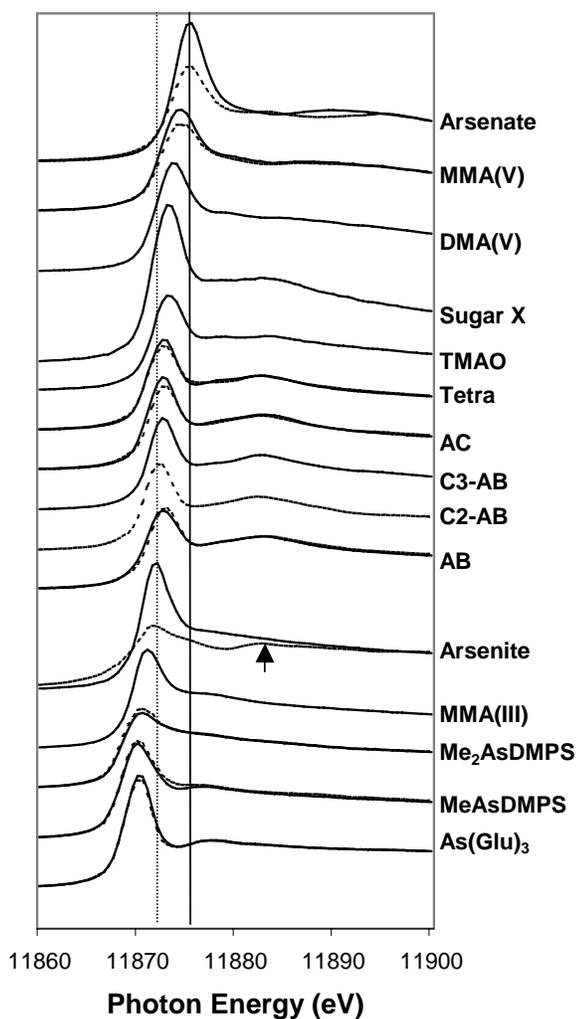
FIG 3.3: XANES spectra for 14 aqueous (solid line), and 10 solid mixed with silica (dotted line), arsenic compounds. Solid vertical line = arsenate white line position (11875.3 eV). Dotted vertical line = arsenite white line position (11871.7 eV). Arrow indicates spectral feature present in arsenite + silica sample, but not in aqueous arsenite samples.

3.4.1 White Line Comparisons

A schematic of the mean white line energy positions for the arsenic compounds is shown in Figure 3.4. White line peak positions (in eV) were obtained from the point where the first derivative of each averaged near-edge spectrum crossed zero. Arsenic species with similar white lines (<0.5 eV difference) were assigned to groups (Fig. 3.4), an example of which is group 4 (Fig. 3.5). The usefulness of using EXAFS for distinguishing arsenic species with similar XANES spectra (e.g. AB, C2-AB, C3-AB, AC,) is currently being studied. White line positions did not distinguish between sulphur-containing arsenic(III) compounds in our group 1. Data from a previous study indicated that white line energy could not be used to distinguish between FeAsS, and arsenian FeS_2, or between AsS (realgar), and As_2S_3 (orpiment) (Bostick and Fendorf, 2003). The white line energies we report for arsenite (11871.7 eV), arsenate (11875.3 eV), and group 1 compounds (11870.0 eV), fall within the values reported in the literature (Table 3.2). Spectra for a selection of aqueous standards were replicated during an independent session at APS (Fig. 3.2). White line energy values for a given arsenic compound generally varied by less than 0.5 eV between beamtime sessions. DMA(III) (session 5) and MMA(III) (session 3) appear to have oxidized to DMA and MMA respectively (Fig. 3.2).

To determine if white line energies for each arsenic compound depended on the physical state of the samples, we compared XANES spectra of solid state and aqueous standards. With the exception of MMA(III) and DMA, arsenic standards had similar white line energy positions when dissolved in water and when mixed with silica. MMA(III) and DMA had higher white line energies in the solid (i.e. mixed with silica) state than in the aqueous state (Fig. 3.4). One interpretation of this result is that the solid

Photon Energy (eV)
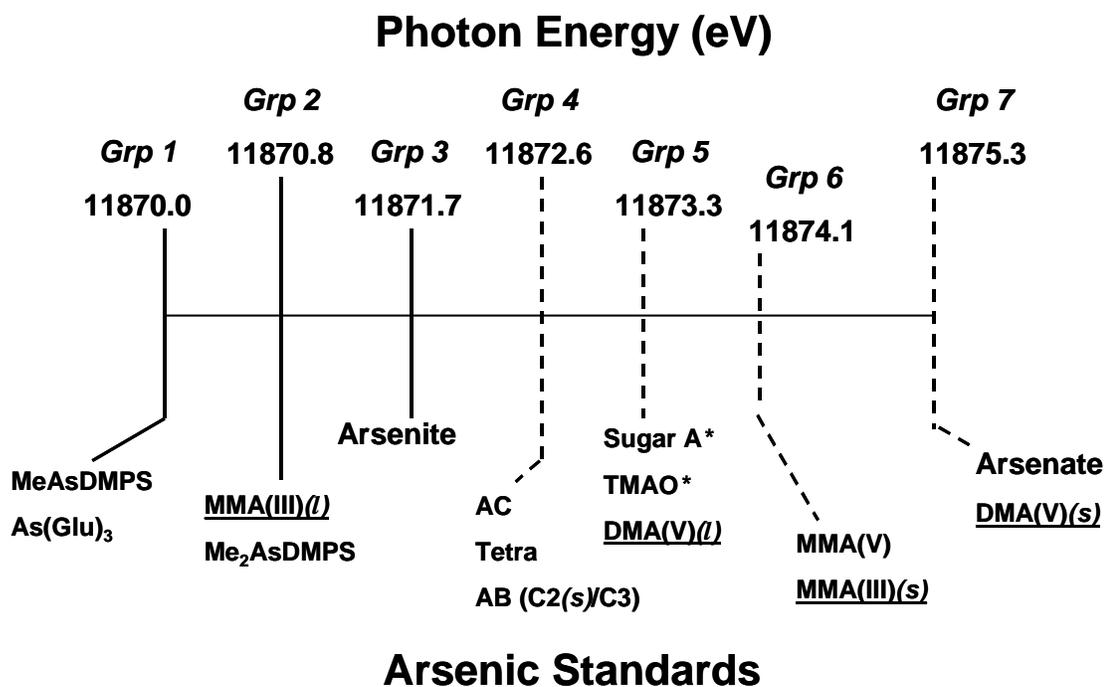
Arsenic Standards

FIG. 3.4: Mean white line (peak of the absorption edge) energies in eV for arsenical groups (Grp). Underlined standards had different white lines for aqueous preparations and solid samples mixed with silica. Asterisked (*) arsenicals were only available as aqueous solutions. For unlabelled arsenicals white line energy was independent of the phase. Dotted vertical lines represent pentavalent species (except MMA(III)(s)), solid vertical lines represent trivalent species. (s) = solid mixed with silica, (aq) = dissolved in distilled deionized H_2O. AB = arsenobetaine; C2 = AB 2; C3 = AB 3.
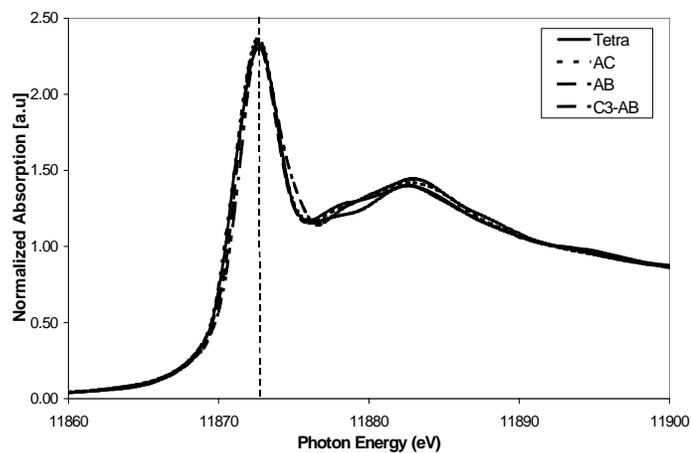
FIG. 3.5: The XANES spectra of aqueous solutions of Group 4 arsenic standards could not be distinguished by their white line energies (vertical dashed line = 11872.6 eV). AC = arsenocholine; AB = arsenobetaine, C3-AB – arsenobetaine 3; Tetra = tetramethyl arsonium.

TABLE 3.2: Summary of white line energies for arsenate, arsenite, and arsenic-sulphur compounds reported in literature.

WHITE LINE ENERGY (EV)			REFERENCES
Arsenite	**Arsenate**	**Arsenic-Sulphur**	
~11871	~11874	~11869 (AsS)	Bostick *et al.*, 2003
11871	11874	11869 (AsS)	Bostick and Fendorf, 2003
*	11875	11869 (AsGS)	Langdon *et al.*, 2002
11872	11876	*	Deschamps *et al.*, 2003
11872	11876	*	Manning *et al.*, 2003
*	~11875	*	Wang *et al.*, 2001
~11872	~11876	~11870 (AsS)	Hansel *et al.*, 2002
*	11874	*	Bull *et al.*, 2000
11871	11875	11870 (AsGS)	This paper

* no data available   ~ data estimated from graph

MMA(III) standard may have oxidized during preparation. This interpretation is consistent with the observation that the peak position of the shifted MMA(III) solid mixed with silica (11874.1 eV) corresponds to both the peak position for solid MMA mixed with silica and undiluted solid MMA. Similar reasoning does not seem plausible for DMA since the arsenic is already present in the pentavalent oxidation state. Further analysis revealed that the white line energies of undiluted solid DMA and aqueous DMA match, both occurring at a lower energy than the white line of solid DMA in silica. It is possible that this difference reflects how the hydrogen-bonded dimer form of DMA in solid phase (Paris and Brinckman, 1976) is altered by bonding to silica. Further analysis using boron nitride in place of silica may help to resolve this issue.

3.4.2 Beam Damage

Factors influencing beam damage include arsenic sample species, concentration, preparation (e.g. state), analysis conditions (e.g. temperature, pressure), and experimental parameters (e.g. beam flux level, run time). Arsenic compounds studied here were examined at room temperature and ambient pressure, as both solids and as aqueous solutions, with exposure times from 30 to 50 min. No alterations in XANES spectra, for solid or aqueous arsenic preparations, at high concentrations, were observed. Several other studies have reported no beam damage for aqueous and salt standards of both sodium-arsenate and sodium-arsenite, as well as arsenic oxide As_2O_5 (Arai *et al.*, 2001), either using similar experimental conditions, or higher photon flux beams with shorter scan time per sample, i.e. similar total number of photons per time (Strawn *et al.*, 2001).

At lower concentrations, damage attributed to total beam exposure was observed for aqueous arsenic standards. An increase in white line energy was the most commonly

observed form of beam damage. We found that for selected aqueous arsenic standards at concentrations of 10000, 1000, 100, and 10 $\mu g \cdot L^{-1}$, over ~1 h, the arsenite (100 $\mu g \cdot L^{-1}$) and MMA(III) (10 $\mu g \cdot L^{-1}$) spectra showed obvious shifts to higher photon energies, consistent with oxidation to arsenate and MMA respectively (MMA - Fig. 3.6).

Beam damage was also observed on the higher flux undulator beamline. The arsenite spectrum developed a peak feature consistent with oxidation to arsenate after only three scans. Bostick *et al.* reported oxidation from beam exposure (~ 180 minutes) for arsenite adsorbed to PbS using a high flux wiggler beamline (Parsons *et al.*, 2002). Their data indicated that the majority of oxidation occurred as a result of exposure to the X-ray source rather than atmospheric oxygen; in part because the samples were stored in an anaerobic glove box and had limited exposure to an aerobic environment before analysis (Bostick *et al.*, 2003). The literature suggests cooling samples to reduce the destructive effects of the high energy X-rays (Parsons *et al.*, 2002). In our studies, samples analyzed at -20ºC on the undulator beamline experienced decreases in signal as well as beam damage. The high energy of the undulator beam melted the sample, which was hypothesized to flow (convection) to the edge of the beam carrying the absorber with it. This loss of signal was also observed in the study of proteins using a high flux beamline (Heald, 2003).

3.4.3 Standard Stability

To determine the stability of arsenic preparations over time, under ambient conditions, the same aqueous arsenic standards were analyzed twice, with 48 h between analyses. Samples remained mounted in Kapton™ tape between analyses. With the exception of Me₂AsDMPS which appeared to oxidize over the intervening 48 hours
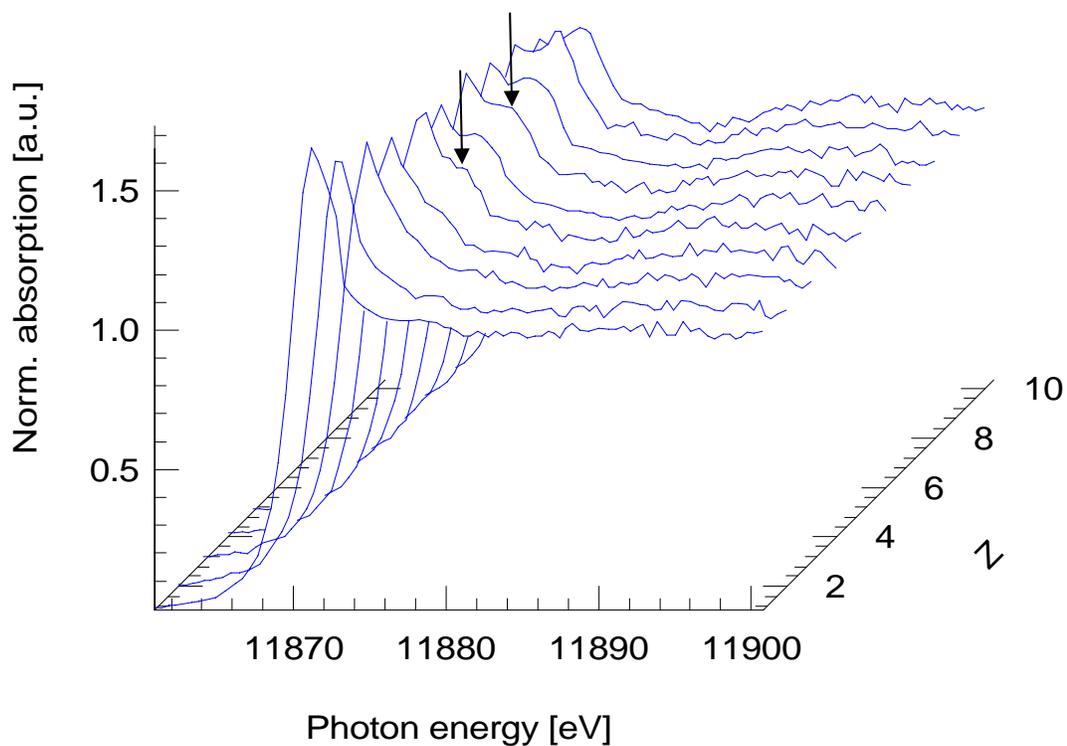
FIG. 3.6: Oxidation of aqueous 10 μg·L^{-1} MMA(III) was marked by the growth of a spectral feature (indicated by arrows) at 11874.2 eV over successive scans. This new feature has a similar white line position to that of aqueous MMA (11874.1 keV). Z = Scan Number.

(Fig. 3.7), no degradation of other aqueous preparations was observed. The behaviour of Me$_2$AsDMPS may reflect both oxidation during preparation (Strawn *et al.*, 2002), as well as lower stability under these experimental conditions.

To understand the risks posed by arsenic contaminated environments to human, wildlife, and plant populations, it is important to understand how arsenic occurs, how it is taken up, and how it is subsequently transformed and stored or accumulated by organisms (Meharg and Hartley-Whitaker, 2002). Traditional speciation analysis can result in potentially erroneous and/or incomplete identification of arsenic species. XAS identifies arsenic compounds *in situ*, which circumvents the limitations of sample preparation required with conventional methods, but it is sensitive only in the $\mu g \cdot g^{-1}$ range. Combining XAS with conventional analyses, and the use of the most appropriate standards available, will provide a more accurate and complete picture of arsenic speciation in the environment.

FIG. 3.7: Oxidation of Me₂AsDMPS was marked by the appearance of a spectral feature at 11872.9 eV (vertical dashed line) which was not present during initial XANES analysis 48 h earlier. The original Me₂AsDMPS standard remained sealed in Kapton™ at room temperature and ambient pressure for the intervening 48 h period.

CHAPTER 4

Uptake, transport and transformation of arsenate in radishes (*Raphinus sativus*)

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4.1 ABSTRACT

The localization and identification of arsenic compounds in terrestrial plants are important for the understanding of arsenic uptake, transformation and translocation within these organisms, and contributes to our understanding of arsenic cycling in the environment. High performance liquid chromatography inductively coupled plasma mass spectrometry (HPLC-ICP-MS), and X-ray absorption near edge structure (XANES) analysis identified arsenite, arsenate and arsenic(III)-sulphur compounds in leaf, stem and root sections of *Raphanus sativus* (radish) plants grown in both arsenic contaminated mine waste, and arsenic amended liquid cultures. The total arsenic distribution in leaf, stem and root sections was similar between the plants grown in mine waste and hydroponically. Arsenate was the predominant form of arsenic available in the growth medium, and after it was taken up by the roots, X-ray absorption spectroscopy (XAS) imaging indicated that some of the arsenate was transported to the shoots via the xylem. Additionally, arsenate was reduced to arsenite which further complexed to thiolate groups likely belonging to phytochelatins probably produced by the plant in response to

metalloid stress. The arsenic(III)-sulphur compound(s) accounted for the majority of arsenic in the leaf and stem of living plants. The application of synchrotron techniques permitted the identification of arsenic(III)-sulphur species which were "invisible" to conventional HPLC-ICP-MS; together these techniques led to a better understanding of the fate of inorganic arsenate taken up by radish plants.

4.2 INTRODUCTION

Arsenic can be taken up by plants and subsequently introduced into a food chain. This may be cause for concern, particularly when considering that as much as 99.7% of all biomass is found in terrestrial plants; any contamination of these organisms will have far reaching effects (Trapp and McFarlane, 1995). Plants can be exposed to elevated soil arsenic concentrations through natural geology, mining activities, contaminated irrigation water, or residues remaining from the use of arsenic based insecticides, fungicides, herbicides and desiccants. Arsenic taken up from the environment is usually converted in plants to another form and the chemical form of arsenic, also known as the "species" of arsenic, determines its toxicity. Therefore there is great interest in determining the concentration, translocation and metabolism of arsenic compounds in terrestrial plants in order to understand and better establish any risk the arsenic species identified may pose.

Arsenate, arsenite, dimethylarsinic acid (DMA) and monomethylarsonic acid (MMA) are all compounds that can be taken up by a number of plant species either from solution or arsenic amended soils (Marin *et al.*, 1992; Carbonell-Barrachina *et al.*, 1998; Tu *et al.*, 2004; Tlustoš *et al.*, 2002). Generally in soils with elevated arsenic levels, it is inorganic arsenate that is the dominant phytoavailable form of arsenic, and it is well established

that plants can take up this compound via phosphate transporters (Ullrich-Eberius *et al.*, 1989; Meharg and Macnair, 1992). It is not surprising then that arsenate is often one of the main arsenic compounds reported in terrestrial plants, with arsenite, another inorganic species, frequently detected (Meharg and Hartley-Whitaker, 2002). Arsenite can be found in anaerobic soils, and it was determined that rice plants actively transport arsenite into the roots (Abedin *et al.*, 2002). In addition, arsenate can be reduced to arsenite soon after transport into the plant (Pickering *et al.*, 2000). Further evidence for the metabolism of arsenic compounds in terrestrial plants was observed in phosphate-starved tomato (*Lycopersicum esculentum*) plants and cell suspension cultures of periwinkles (*Catharanthus roseus)* which were found to methylate inorganic arsenic to some extent (Cullen *et al.*, 1989; Nissen and Benso, 1982). A pathway involving alternate reduction and oxidative methylation produces simple methylated arsenic compounds such as DMA and MMA identified in rice, grass (e.g. tufted hair), and trees (e.g. apple, cedar) (Meharg and Hartley-Whitaker, 2002). Arsenobetaine (AB) is less frequently observed in terrestrial plants, but has been reported in red clover and ribwort plantain, the latter of which was also reported to contain arsenocholine (AC) (Meharg and Hartley-Whitaker, 2002); however, the pathway for the production of these arsenic compounds is not as well understood. Many of the arsenicals noted above have been identified in both roots and shoots of plants, but the distribution of total arsenic within plants is highly variable, with the exception that fruits and seeds generally contain lower concentrations of arsenic than other parts of the plant (Marin *et al.*, 1992; Cobb *et al.*, 2000).

In addition to the metabolism of arsenic compounds by plants, there is the possibility of chelation of arsenic to biomolecules in plant cells. In particular, arsenite has a high

affinity for sulfhydryl residues found on molecules such as glutathione (GSH) and

phytochelatins (PCs). *In vitro* studies have demonstrated the complexation of arsenic to

both PCs and GSH (Schmöger *et al.*, 2000; Raab *et al.*, 2004b). Glutathione is composed

of γ-glutamate-cysteine-glycine residue (GSH = γ-Glu-Cys-Gly). In plants, the majority

of GSH is found in the chloroplasts (50-70%), where it acts as a powerful antioxidant

(Rennenberg, 1982). Phytochelatins (PCs) are synthesized from GSH and contain

additional glutamate and cysteine components ($PC_n = (γ$-Glu-$Cys)_n Gly$). PC are produced

by plants in response to metal cation (e.g. Ag^+, Cd^{2+}, Cu^{2+}, Hg^{2+}, Pb^{2+}) or metalloid (e.g.

$As^{(V)}$ oxy-anion) stress (Raab *et al.*, 2004a). A wide variety of arsenic-PC complexes

have been described, and some of the predominant complexes include: arsenite-PC_3 in *H.*

lanatus, GS-arsenite-PC_2 in *P. cretica*, PC_2-arsenite-PC_2 in *R. serpentine*, and PC_3 and

homoPC_3 in *C. arietinum* (Raab *et al.*, 2004a; Schmöger *et al.*, 2000; Gupta *et al.*, 2004).

These compounds, in combination with GSH and other acid-labile sulfhydryls found in

the cells, suggest a large number of arsenic complexes are possible *in planta*. Given the

potential role of PCs and other chelating molecules in the detoxification, transport and

compartmentalization of arsenic, in addition to possible roles in arsenic tolerance, it is

important to consider the presence of arsenic-sulphur compounds in samples.

"Elemental speciation" describes the activity of determining the actual chemical form

of arsenic in a sample, and potentially quantifying the compounds identified.

Conventionally, arsenic compounds are extracted from a sample and separated using

chromatography before detection by methods such as inductively coupled plasma mass

spectrometry (ICP-MS). These methods can detect a wide range of inorganic and organic

arsenic compounds at the parts per billion ($μg·kg^{-1}$) level. Unfortunately, extraction of

arsenic from terrestrial samples is not always complete and as much as 70% (Tlustoš *et al.*, 2002) of the arsenic compounds can remain in the solid residue which cannot be analyzed for speciation by conventional methods. Such samples can be analyzed using X-ray absorption spectroscopy (XAS), which involves direct analysis of solid material such as plant tissues or residues, and has been used to determine arsenic species at the parts per million (mg·kg^{-1}) level. This technique has been particularly useful for the detection of arsenic-sulphur compounds in terrestrial plants (Meharg and Hartley-Whitaker, 2002; Pickering *et al.*, 2000; Zhang *et al.*, 2002). XAS imaging can be used to determine the location of arsenic compounds in an unaltered plant sample, providing information on the translocation of arsenic compounds within a plant (Pickering *et al.*, 2006). Micro-X-ray absorption near edge structure (XANES) analysis can then be used to provide additional speciation data for specific points of interest identified through the imaging.

Here we present the results for a study designed to determine arsenic speciation and distribution in radish plants, grown in two sources of arsenic: mine waste and arsenic amended liquid culture. Analysis involved conventional techniques that employed a methanol:water extraction phase, as well as XANES investigation of tissues, and XAS imaging to determine arsenic distribution. The complementary nature of the analytical techniques used in this study allowed for a more comprehensive understanding of arsenic compounds in radish plants by combining results from both direct (synchrotron) and indirect (HPLC-ICP-MS) speciation analyses.

4.3 MATERIALS AND METHODS

4.3.1 Chemicals and Standard Reference Materials

Chemicals used in these studies include nitric acid (Fisher Scientific, optima), methanol (99.93% HPLC grade, Aldrich), liquid nitrogen (BOC), ortho-phosphoric acid (85% Fluka), and ammonium hydroxide 30 wt % (Sigma-Aldrich). Distilled deionized water had a resistivity better than 17.5 MΩcm (E-pure Barnstead). Arsenic compounds used as standards included: arsenate (Fluka KH_2AsO_4 solid and Aldrich ICP/DCP standard solution), arsenite (Fluka As_3O_3 solid and atomic spetrometry standard solution), monomethylarsonic acid (Pfaltz & Bauer and ChemService monosodium acid methane arsenate), dimethylarsinic acid (Aldrich cacodylic acid sodium salt hydrate), trimethylarsine oxide (synthesized), tetramethylarsonium iodide (synthesized), arsenobetaine (synthesized), arsenocholine (synthesized), arsenic(III)-glutathione (synthesized) and TuneA (Thermo Electron Corporation). 'Synthesized' compounds were prepared by Drs. H. Sun and W.R. Cullen, at the University of British Columbia, using standard methodologies which have been referenced previously (Smith *et al.*, 2005). Certified reference materials included Bush Branches and Leaves, certifying body: Institute of Geophysical and Geochemical Exploration (GWB 07603), and DORM 2 (dogfish muscle), certifying body: National Research Council of Canada.

4.3.2 Experimental Design

Liquid Cultivation of Radishes: Six treatments were used and designated H (for hydroponic) followed by a number indicating the target arsenic concentration of the growth solution. Therefore H-0 was the control treatment with a target arsenic

concentration of 0 $\mu g \cdot g^{-1}$; H-1 had a target arsenic concentration of 1 $\mu g \cdot g^{-1}$; H-2 (2 $\mu g \cdot g^{-1}$); H-5 (5 $\mu g \cdot g^{-1}$); H-15 (15 $\mu g \cdot g^{-1}$); H-30 (30 $\mu g \cdot g^{-1}$); and H-60 (60 $\mu g \cdot g^{-1}$). These designations can be found in Table 4.1 for future reference. Each treatment contained three replicates consisting of three plants each. Plants were grown in a phytotron growth chamber and experimental conditions were maintained at 12 hours dark at 12°C, 12 hours light at 18°C, and humidity 70 - 90%. Pots were watered from the bottom and plants were harvested 31 days after sowing. Radish (var. Cherry Belle) seeds were purchased from the Ontario Seed Company (Waterloo, ON) and planted in 4" pots of Turface® (Profile™ Products). Plants were supplied with a base nutrient solution (Table 4.1) without arsenic for the first 5 days, at which point they were thinned to 3 plants per pot. Arsenic treatments contained base solution prepared with KH_2AsO_4 (Fluka). Solutions were refreshed daily throughout the growth period. Plants were harvested 28 days after sowing, and sectioned into edible root, stem and leaf which were thoroughly washed and scrubbed with tap water and further rinsed with distilled water before weighing and freezing. Roots were further separated into peeled root and skin before sample preparation, to determine the possible contribution of arsenic compounds adsorbed to the outer skin.

Mine Waste Cultivation of Radishes: Mine waste was collected from the tailings area of the Miramar Con Mine in Yellowknife (NWT, Canada). Measurements were made to determine mine waste soil properties: pH, organic matter content (loss on ignition), particle size, and concentrations (measured absorbance at 780-800 nm) of total phosphorus (sample digested with 6.0 M HCl), and available phosphorous (extracted from sample with pH 8.5, 0.5 M $NaHCO_3$). Selected physical and chemical properties of the soil are presented in Table 4.1. The mine waste was sieved (Canadian metric sieve

TABLE 4.1: Selected properties of media from undiluted (Undil) and diluted (Dil) mine waste and hydroponic (H) studies. Hydroponic nutrient sources included KNO_3, KH_2PO_4, $Ca(NO_3)_2$, $MgSO_4$, Fe-chelate, $CuSO_4\text{-}5H_2O$, $MnSO_4\text{-}H_2O$, $ZnSO_4\text{-}7H_2O$, H3BO3, MoO_3 (modified from Lunney, 2007).

HYDROPONIC SOLUTION			MINE WASTE		
Treatment	Nutrient	Conc. (mg·L^{-1})	Property	Dil	Undil
Base Solution	P	15	pH	4.9	5.2
	N	150	TOC (%)	32	28
	K	91	P in soil (ug·g^{-1})	2000	3100
	Ca	180	P in solution (ug·mL^{-1})	1.1	1.6
	Mg	50	Total As (ug·g^{-1})	660	1100
	Fe	3	TKN (ug·g^{-1})	8600	6900
	Cu	0.1			
	Mn	1.0			
	Zn	0.1			
	B	1.0			
	Mo	0.4			
H-0	As	0			
H-1	As	0.8 ±0.2			
H.2	As	1.6 ±0.6			
H-5	As	4 ±1			
H-15	As	12 ±4			
H-30	As	24 ±9			
H-60	As	50 ±20			

TOC = Total organic carbon   TKN = Total Kjehldahl Nitrogen
Hydroponic arsenic concentrations = average of 5 samples over entire growth period

series, 5 mm opening) and the total arsenic concentration in the final product was ~1100 mg·kg^{-1}.

The mine waste treatments used were: undiluted and diluted. The diluted treatment was mixed with potting mix (Agro Mix Super, Fafard®) by hand until visually homogenous. A sample of soil from each treatment was taken before pots were filled for planting to determine initial soil total arsenic concentration (Table 4.1). Radish seeds (*Raphinus sativus*, var. Cherry Belle) were purchased from the Ontario Seed Company (Waterloo, ON). Plants were grown in a greenhouse during April and May, and a temperature of 24°C ±5°C was maintained. One week after planting, replicate flats were thinned to approximately 15 plants. Treatments were watered regularly with tap water. Plants were harvested ~35 days after sowing. Plants were washed thoroughly with tap water, further rinsed with distilled water, and sectioned into root, stem and leaf, before weighing. Roots were peeled before sample preparation, and the peel discarded.

4.3.3 Sample Preparation

Samples were homogenized in a mortar and pestle using liquid nitrogen, and when sufficient sample was available, a sub-sample was dried at 70°C in an Isotemp oven (Fisher Scientific) for a minimum of 12 hours, to be used for the determination of total arsenic. Between samples, homogenization equipment was washed once with soap and water, rinsed once with ~10% HNO_3, rinsed 5 times with distilled water, and rinsed 3 times with distilled deionized water before drying with Kimwipes®. Procedural blanks of distilled deionized water were subjected to the grinding procedure both before and after the samples (Table 4.2).

TABLE 4.2: Quality control (QC) results obtained through the use of blanks, standard curves, standard check solutions, duplicates and spiked samples which were run during sample analysis. SRM = Standard Reference Material: % Rec = percent recovery: RPD = relative percent difference.

		Batch Size	n	RANGE	MEDIAN
TOTALS	Blanks (ng·g⁻¹ As) — Wet Grind	5-7	26	ND	ND
	Dry Grind	5-7	24	ND	ND
	Method	10-12	16	ND	ND
	Instrument	12	8	ND	ND
	SRMs (% Rec) — DORM 2	10-12	16	84 - 123	106
	Bush Branches	10-12	8	74 - 91	84
	Spike (% Rec)	10-12	15	95-118	101
	Duplicates (RPD)	10-12	16	2.2 - 77	6.4
	QC (% Rec) — Check 1	12	9	92 -107	101
	Check 2	12	9	93 - 106	100
HPLC-ICP-MS	SRM (% Rec AB) — DORM 2	12	2	129 - 131	130
	Spike (% Rec) — Arsenate	10-12	4	74 - 101	82
	Duplicates (RPD) — Arsenite	12	2	1 – 54	27
	Arsenate	12	2	ND – 29	-
	QC (% Rec) — Arsenite	10	16	84 - 130	98
	DMA	10	16	76 - 112	92
	MMA	10	16	75 - 108	95
	Arsenate	10	16	80 - 109	98
	Spike (% Rec) — Arsenite	10-12	2	104 – 108	106
	Arsenate	10-12	2	54 - 104	79
HPLC-AAS	Duplicate (RPD) — Arsenite	10-12	1	26	-
	Arsenate	10-12	1	10	-
	QC (% Rec) — Arsenite	10-12	2	92 - 103	98
	Arsenate	10-12	2	88 - 119	103

Notes: speciation blanks contained no arsenic peaks
ND = not detectable: Batch = batch size (number of samples for which QC was run)

4.3.4 Total Arsenic Determination

Nitric Acid digestion: Two nitric acid digestion methods were used in this study in order to extract the total amount of arsenic from samples. Method *A* was used in-house, and method *B* was used by a Canadian Association for Environmental Analytical Laboratories (CAEAL) accredited commercial laboratory (Analytical Services Unit (ASU), Queen's University). Standard reference materials were used to confirm that these techniques were sufficient to extract the total arsenic from the samples (Table 4.2).

A. Sub-samples (∼500 mg) of wet or dry ground roots, stems and leaves were digested with 10 mL of concentrated HNO_3 on a hot plate at ∼120°C for a minimum of 6 hours. Samples were boiled down to ≤ 1 mL before the addition of 3 mL of 30% hydrogen peroxide. Samples were again evaporated down to < 1 mL before being made up to a final volume of 20 mL with distilled deionized water (ddH_2O). A digestion blank, standard reference materials (Bush Branches, DORM 2), and duplicates were included with each batch of ten samples.

B. Sub-samples (~500 mg) of dried, ground sample were weighed out into a Vycor® crucible and ashed in an Isotemp® Programmable muffle furnace (Fisher Scientific). Cooled samples were digested with 1 mL of concentrated nitric and 3 mL concentrated hydrochloric acid on a hotplate (Thermolyne type: 2200) for 10 minutes. Crucibles were covered with watchglasses and samples were digested for a minimum of 4 hours. Two drops of 50% hydrogen peroxide solution were then added, the hotplate setting was adjusted to 3, and the digest was evaporated to ~ 1 mL then diluted to 12.5 mL with ddH_2O. A digestion blank and calibration verification materials were included approximately every ten samples.

Analysis: Both inductively coupled plasma mass spectrometry (ICP-MS) employed in-house, and inductively coupled plasma optical emission spectrometry (ICP-OES) employed by a commercial laboratory (Analytical Services Unit (ASU), Queen's University), were used to determine total arsenic in radish samples.

ICP-MS: Approximately 500 mg of nitric acid digested sample was diluted to 10 mL with 2% nitric acid solution for analysis using a PQ-ExCell, or X7 X-series, inductively coupled plasma-mass spectrometer (Thermo Instruments). A standard ICP torch was used for atomization and ionization and the sample was introduced with a Meinhardt concentric nebulizer. The quadrapole detector monitored a mass to charge (m/z) ratio of 75 (arsenic) and an internal standard of indium (m/z 115), or scandium (m/z 45), was used to correct for plasma drift and matrix effects. The method detection limit was approximately 5 ng·g^{-1} for arsenic. Quality control is reported in Table 4.2.

ICP-OES: Analysis was conducted using a Vista AX CCD simultaneous ICP-OES. A Varian SPS 5 autosampler and a Conikal nebulizer (Glass Expansion, Australia) were used to introduce samples into the argon plasma. Results monitored at arsenic wavelengths 193.696 and 188.980 nm were averaged to determine total arsenic in samples. Scandium and indium were used as internal standards to correct for differences in viscosity and matrix effects between samples and standards. The method detection limit was established at 1 µg·g^{-1} for arsenic, and instrument QC was within 10% of accepted values.

4.3.5 Arsenic Speciation Analysis

Methanol-water Extraction: Approximately 1.0 g of wet plant material was extracted with 10 mL of methanol-water 1:1 (v/v). Samples were vortexed and placed in

a shaker (Innova 4230, New Brunswick Scientific) at 5°C and 275 RPM for ∼30 min. Samples were then placed in an ultrasonic bath (FS 140H, Fisher) for 20 min before being centrifuged (GP8R, IEC Centra) at 3500 RPM for 20 min at 15°C. Samples were filtered (Whatman P541), and a subsample of each extract was rotovapped (R-124 Buchi Rotovaper) to evaporate methanol before being made up to 5-10 mL with distilled deionized water. This extraction method resulted in 40 – 140% recovery of arsenic from leaf, stem, root, and peel of radish plants grown in both mine waste and liquid culture studies.

Individual arsenic compound analysis: Individual arsenic compounds were identified using high performance liquid chromatography hydride generation atomic absorption spectroscopy (HPLC-HG-AAS), HPLC inductively coupled plasma mass spectrometry (HPLC-ICP-MS), and X-ray absorption near edge structure (XANES) analysis.

HPLC-HG-AAS: This technique was used for samples grown in mine waste only. A Hamilton PRP-X100 anion exchange column (4.6 X 150 mm, 10 μm particle size) fitted with a matching guard column were used for HPLC separation of arsenic compounds. Arsenite, arsenate, dimethylarsinic acid (DMA) and monomethylarsonic acid (MMA) were separated based on charge. The mobile phase consisted of ortho-phosphoric acid and ammonium hydroxide, prepared to pH of 6.0. The mobile phase was pumped (Rose Scientific, HPLC pump) at 1.5 mL/min, and the pump was fitted with a Rheodyne injector and linked to a Unicam VP90 vapor generator unit. Sample effluent was combined with 1 M HCl (Varian VGA-76 peristaltic pump) and 1 % $NaBH_4$ (Unicam VP90). Nitrogen purge gas transported hydride gases separated from the liquid phase

into a quartz T-tube which was heated by the EC90 furnace of the SOLAAR 969 AAS

instrument, and positioned in the optical path of an arsenic hollow cathode lamp

(absorption wavelength of 193.7 nm). SOLAAR software was used to record data, while

calibration curves based on known arsenic standards were constructed using Peakfit™

chromatography software, and used to quantify arsenic in samples. Spikes, duplicates and

QC were included every 10 samples (Table 4.2).

HPLC-ICP-MS: The HPLC system for the speciation of the arsenic compounds

consisted of a P4000 quaternary HPLC pump, SCM100 vacuum degasser, and an AS300

autosampler (Thermo Instruments). This system was connected to the intake of the PQ

ExCell ICP-MS, or X7 X-Series ICP-MS, which monitored mass to charge (m/z) signals

of arsenic (As) 75 and rhodium (Rh) 103 for anion analysis, and arsenic, indium (In)

115, and uranium (U) 238 for cation analysis. Arsenite, arsenate, DMA and MMA were

separated using anion exchange as described above. Rh was added as an internal

standard to the 20 mM ammonium phosphate mobile phase (pH 6.0).

Trimethylarsonium oxide (TMAO), tetramethylarsonium ion (tetra), arsenocholine (AC)

and arsenobetaine (AB) were separated using a 150 x 4.5 mm Whatman SCX stainless

steel cation exchange column. Indium and uranium were added as internal standards to a

mobile phase of 20 mM pyridine formate (pH 2.7). A flow rate of 1.0 mL/min was used

for both anion and cation exchange analysis. Arsenic was quantified through external

calibration with standard solutions, and calibration curves were constructed using

Peakfit™ chromatography software. The reporting limit for arsenite, arsenate, DMA,

MMA, AB and TMAO was 5 ng·mL^{-1}. Spikes, duplicates and QC were included every

10 samples (Table 4.2).

XANES: XANES spectra of bulk samples were collected at the PNC/XOR bending magnet (BM) beamline, Sector 20 at the Advanced Photon Source (APS), Argonne National Laboratory (Heald *et al.*, 1999). A silicon (111) double-crystal monochromator (bandwidth ~ 5000 E/ΔE at 12 keV), detuned to 85% of maximum intensity to reduce harmonic content, and a rhodium-coated planar float glass mirror for further harmonic rejection provided X-rays for measurement. A slit-assembly located 51m from the source with a maximum size of 1500 μm vertical by 4000 μm horizontal was used after the mirror to define the size of the x-ray beam incident on the sample. The monochromator was calibrated using the first inflection point of the gold L_{III} absorption edge (11919.7 eV (Kraft *et al.*, 1996)) for measurements at the arsenic K-edge (11868 eV). Where experimental setup allowed, a reference gold foil was also measured in transmission during each sample scan. Nitrogen-filled transmission ionization chambers were present before and after the samples for normalization to the incident intensity and transmission measurements, respectively. Fluorescence data were collected using an argon-filled fluorescence ionization chamber (Jiang and Crozier, 1998), a single-element solid-state Ge(Li) detector (Canberra model GL0055PS), or a 13 element Ge(Li) detector (Princeton Gamma Technologies or Canberra). During data collection samples were maintained at -70ºC in a cryostat cooled with liquid nitrogen. Typically, 10 scans were collected and averaged before background-removal and normalization-to-edge-jump. Spectra were processed using WinXAS (Ressler, 1997) software. Data were compared to linear combinations of reference compounds, with the edge positions of the standards constrained to be within 0.5 eV of their initial values. The ratio of reference compounds that best resembled the data is reported and all components < 1% were excluded.

4.3.6 XAS Imaging: Arsenic Localization

X-ray fluorescence microprobe scans were performed on the sector 20 BM and ID beamlines (Heald *et al.*, 1999) at APS, Argonne National Laboratory. X-rays were focused to a of 5 x 5 square micrometer spot size using Rh-coated Kirkpatrick-Baez mirror pairs. A live radish plant were analyzed on the BM line while samples, flash frozen in liquid nitrogen, were analyzed on the ID line at approximately -20ºC using a thermoelectric cooled stage. Fluorescence data were collected with a Canberra 7-element solid state Ge-Li detector and/or a 13-element Ge(Li) detector (Princeton Gamma Technologies). For a sectioned sample on the ID-line, a motorized stage moved the sample in its cryogenic holder through the X-ray beam, and fluorescence data were collected at each 5 or 10 μm step (for coarser features or a larger image of the sample) with a 2 s integration time.

The intensities obtained were related to arsenic concentration qualitatively with higher signal corresponding to a higher arsenic concentration or thicker sample. For any one sample, microprobe images obtained at both 11.871 keV (trivalent: AsIII) and 11.875 keV (pentavalent: AsV) appeared very similar. The difference in intensities (AsV-AsIII) was used to distinguish dominant oxidation states at a particular point in a sample and produce "comparison" images.

Arsenic images were created using the SURFER8© (Golden Software) program. Micro-XANES spectra were obtained (with Au-calibration of the monochromator as above) for regions of interest identified in the microprobe images, and processed as discussed previously (section 2.5.2 XANES).

4.4 RESULTS

4.4.1 Arsenic in Growth Media

Yellowknife mine wastes contained substantially greater arsenic concentrations, 1100 mg·kg^{-1} and 660 mg·kg^{-1} for experiments using undiluted and diluted substrate respectively, than both the environmental soil quality guideline of 17 mg·kg^{-1} for agricultural soil (Canadian Council of the Ministers of the Environment, CCME), and the Yellowknife background soil arsenic levels of up to 150 mg·kg^{-1} (EHASSE, 2004). Previous field studies have shown that radish plants can tolerate elevated arsenic levels (408 mg·kg^{-1}, 748 mg·kg^{-1}) with 100% survival rate, compared to other vegetables studied (Cobb *et al.*, 2000; Warren *et al.*, 2003). X-ray absorption near-edge structure (XANES) analysis of the solid mine waste used here, indicated that the arsenic was mainly in the pentavalent oxidation state (Fig. 4.1). The peak feature position of the arsenate standard (11.875 keV) most closely reflects that of the mine waste, and is not unexpected since $HAsO_4^{-2}$ and $H_2AsO_4^-$ are the soluble forms of arsenic which predominate in most soils between pH 4 and pH 7, and are also bioavailable to plants (Mkandawire *et al.*, 2004). The liquid culture treatments (H-1, H.2, H-5, H-15, H-30, H-60) were sampled before watering and average concentrations are reported in Table 4.2. The growth solution for plants grown specifically for synchrotron analysis had an average arsenic concentration of 22.7 (±7.72) mg·L^{-1}.

4.4.2 Radish Growth

Plants grown in the diluted mine waste had approximately twice the mass of shoot material (wet mass), and three times the amount of root material (peeled, wet mass) when
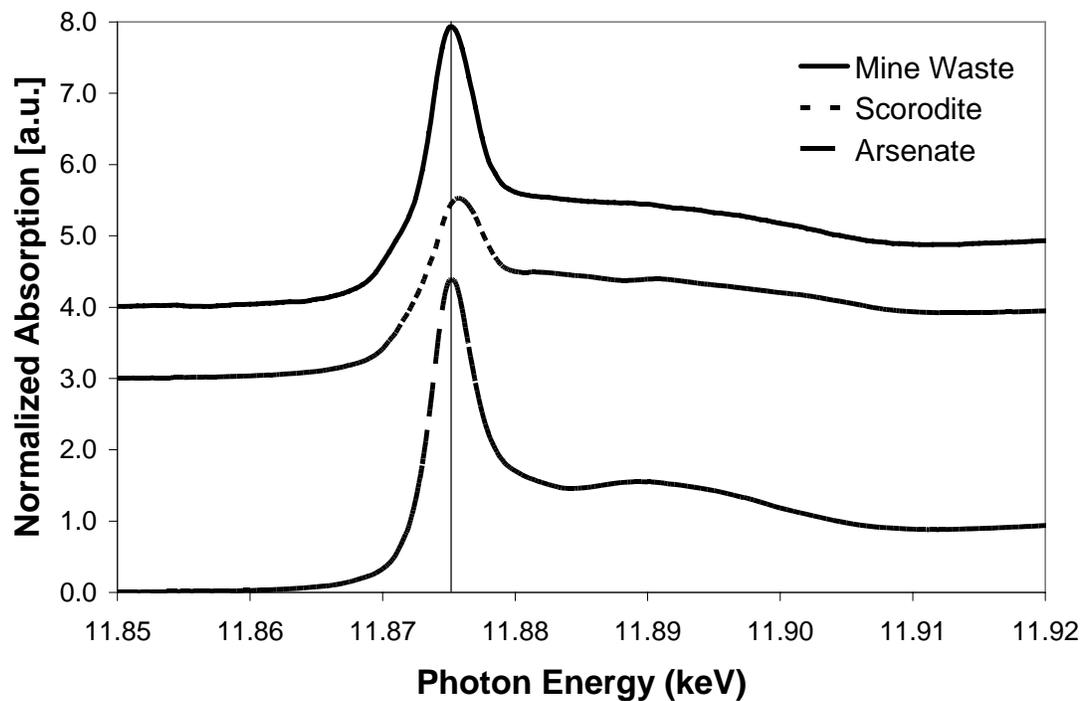
FIG. 4.1: XANES spectra for arsenate standard (solution), scorodite and mine waste collected from Miramar Con Mine Yellowknife, NWT (Canada). The majority of the arsenic was in the +5 oxidation state. While arsenate is likely the major compound present additional compounds such as scorodite may be present. The vertical line corresponds to arsenate white line energy (11.875 keV).

compared to that of plants grown in the undiluted mine waste (Table 4.3). Statistical tests were conducted using ANOVA followed by Tukey's post hoc test. Due to the small sample size variance was assumed to be equal. In general the average total wet weight mass of plants grown in both mine waste treatments was less than that of the majority (H-0, H-1, H-2, H.5, H-15) of hydroponic treatments. Growth was likely influenced by nutrient quality of the soil and the presence of contaminants other than arsenic.

It was not known at what arsenic concentration radish plants grown hydroponically would accumulate individual arsenic compounds to levels detectable by X-ray absorption spectroscopy (XAS); therefore, a range of arsenic concentrations were selected (0, 1, 2, 5, 15, 30, 60 mg·L^{-1}). Significant differences in wet weight yields were observed for shoot materials (leaf and stem), but not unpeeled root material (Table 4.3).

Plants for both types of treatments, mine waste and hydroponic, had decreased total yield (shoots + roots) with increasing arsenic concentrations (Table 4.3), though the decrease was only apparent after the H-5 treatment for hydroponic plants. Plants were grown in a 30 mg·L^{-1} treatment for analysis at APS, because the H-60-treated plants began to exhibit signs of arsenic toxicity including discolouration and necrosis of leaf tips (although all planted seeds survived). These effects, in addition to root plasmolysis and leaf wilting have been observed previously as signs of arsenic toxicity (Machlis, 1941; O'Neill, 1995).

4.4.3 Total Arsenic Concentrations

Average arsenic concentrations for leaf, stem, unpeeled root and skin samples, from mine waste and hydroponic treatments, varied from 3 µg·g^{-1} in stem and roots, to 220 µg·g^{-1} in leaves (Table 4.4). Among hydroponic treatments, a significantly greater

TABLE 4.3: Average wet weight (grams) per radish plant, for shoots (leaf, stem, cotyledon) and root (peeled) of radishes grown hydroponically (H) and in mine waste (MW). Leaf treatments marked by the same letter did not differ significantly ($\underline{F}(5,12) =$ 16.8, $p<0.05$). Stem treatments marked by the same letter did not differ significantly ($\underline{F}(5,12) = 42.3$, $p<0.05$). Root treatments did not differ significantly ($\underline{F}(5,12) = 2.59$, n.s.).

TREATMENT	SHOOT				ROOT (including peel)	AVG TOTAL
	Leaf		Stem			
H-0	2 ±1	A	0.89 ±0.02	D	4.2 ±0.8	6.7 ±0.7
H-1	1.5 ±0.1	A	0.90 ±0.02	D	3.3 ±0.5	5.7 ±0.6
H-2	1 ±1	AB	0.7 ±0.1	E	3.7 ±0.2	5.7 ±0.2
H-5	1.3 ±0.1	AB	0.61 ±0.06	EF	4 ±1	6 ±1
H-15	1.0 ±0.1	BC	0.45 ±0.01	FG	3.2 ±0.8	4.6 ±0.9
H-30	0.6 ±1	C	0.28 ±0.03	G	2.5 ±0.6	3.3 ±0.8
H-60	0.58 ±0.05				1.2 ±0.3	1.9 ±0.3
MW Undiluted	1.3 ±0.6				0.8 ±0.5	2.1
MW Diluted	2.3 ±0.05				2.3 ±0.4	4.6

Average yield of hydroponic plants determined from three replicates (± standard deviation)
Average yield for mine waste plants determined from two replicates (± RPD)
Absence of Standard deviation indicates only one replicate.
60 µg·L^{-1} treatment not included in stats

TABLE 4.4: Average arsenic concentrations for hydroponic (H) and mine waste (undiluted (Undil), diluted (Dil)) treatments. Leaf treatments marked by the same letter did not differ significantly ($F_{(5,11)} = 25.7$, $p<0.05$). Stem treatments marked by the same letter did not differ significantly ($F_{(5,10)} = 13.1$, $p<0.05$). Root treatments marked by the same letter did not differ significantly ($F_{(5,11)} = 30.1$, $p<0.05$). Peel treatments marked by the same letter did not differ significantly ($F_{(5,11)} = 32.4$, $p<0.05$).

	Average concentration (ug·g^{-1}dry weight) of arsenic per radish section							
	LEAF		**STEM**		**ROOT**		**PEEL**	
H-0	8 ±3	A	3 ±1	C	2.9 ±0.6	F	7 ±2	H
H-1	30 ±10	A	10 ±2	CD	10 ±2	F	11 ±5	H
H-2	50 ±40	A	13 ±8	CD	10 ±3	F	9 ±1	H
H-5	60 ±20	A	60 ±30	CD	30 ±10	F	18 ±5	H
H-15	220 ±30	B	160 ±50	E	70 ±20	G	50 ±10	I
H-30	210 ±30	B	87	DE	60 ±30	G	44 ±4	I
Undil	120 ±10	-	100 ±30	-	80 ±20	-	-	-
Dil	160 ±2	-	110	-	70 ±20	-	-	-

Average arsenic concentrations for hydroponic plants determined from three replicates (± standard deviation)
Average arsenic concentration for mine waste plants determined from two replicates (± RPD)
Absence of Standard deviation indicates only one replicate.

concentration of arsenic was always found in the H-15 and H-30-treated plants, with the only exception being the stems from the H-30-treated plants (Table 4.4). Arsenic concentrations in leaf and stem sections of mine waste plants were between the concentrations obtained from the H-5 and H-15 hydroponic treatments for the same sections. On the other hand, peeled roots from the mine waste plants had the highest arsenic concentration overall.

4.4.4 Arsenic Compound Identification

In a greenhouse study by Tlustoš et al. (2002), radishes grown on soil amended with 20 mg·kg^{-1} arsenate contained only inorganic arsenite and arsenate as determined by ion-exchange HPLC-ICP-MS. The same compounds were identified in this study (Table. 4.5), using similar techniques, and arsenite was the predominant extracted arsenic compound (Table 4.5). The high (> 70%) percent recoveries calculated ((sum of species)/(nitric acid total)*100) indicated that the majority of arsenic was extracted from the samples, with the exception of leaves from both the undiluted and diluted mine waste treatments for which less than 50% of the arsenic was recovered (Table 4.5). The reason for the observed difference is not clear, but Tlustoš *et al.* (2002) also found dried and powdered samples from Duo (cv.) radishes grown in soil treated with arsenite and arsenate also had low methanol:water extraction efficiencies (4:1 v/v) for leaf material (≤ 30%). Results from XANES analysis also identified arsenic species that corresponded well to arsenite and arsenate reference solutions. In addition significant amounts of a compound resembling arsenic(III)-glutathione were found in frozen samples of radish leaf stem and root (Fig. 4.2) using XAS. Arsenic sulphur-compounds could not be detected using the HPLC-ICP-MS and HPLC-HG-AAS techniques.

TABLE 4.5: Average concentration of arsenic compounds in µg·g⁻¹ dry mass (DM), total arsenic concentration and % recovery for shoot (leaf, stem) and root (peeled root, peel) sections of radish plants grown in mine waste and liquid culture treatments. Absence of standard deviation indicates only one replicate. % recovery = (sum of species)/(nitric acid total)*100. Data for hydroponic and diluted mine waste stem samples were not analyzed in replicate.

Treatment	Plant Section	Arsenic Compounds [µg·g⁻¹] DM[a]		Total Arsenic [µg·g⁻¹] DW[b]	Average % Recovery
		Arsenite	Arsenate		
30 µg·L⁻¹ Hydroponic	Leaf	210 ±8	4 ±4	210 ±60	102
	Peeled Root	39 ±3	18 ±9	60 ±20	95
	Peel	50 ±20	5 ±8	44 ±2	120
Undiluted Mine Waste	Leaf	50 ±20	10 ±0.4	130 ±20	46
	Stem	50 ±10	20 ±8	100 ±30	70
	Peeled Root	26 ±2	30 ±20	40 ±4	140
Diluted Mine Waste	Leaf	50 ±20	10 ±0.2	160 ±4	38
	Peeled Root	50 ±70	20 ±30	70 ±20	100

[a]Speciation determined by HPLC-HG-AAS for mine waste, and HPLC-ICP-MS for liquid culture
[b]Total Arsenic determined by ICP-OES for mine waste, and ICP-MS for liquid culture
Average arsenic concentration for hydroponic plants determined from three replicates (± standard deviation)
Average arsenic concentration for mine waste plants determined from two replicates (± RPD)
Absence of Standard deviation indicates only one replicate.
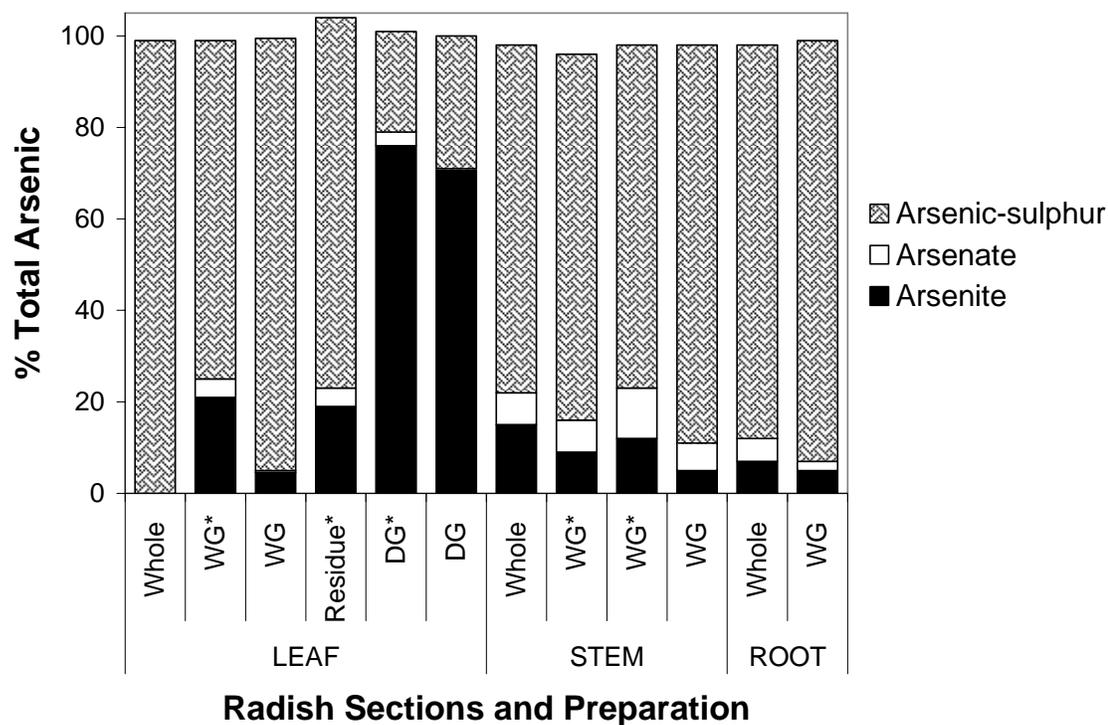
FIG. 4.2: The relative percent contribution of arsenic species for frozen leaf, stem and root samples of radish plants. Leaf samples demonstrate that sample preparation affects arsenic speciation. Samples included whole (freshly harvested before analysis), wet ground (WG, fresh material ground with liquid nitrogen), dry ground (DG, wet ground samples dried at 70°C and further ground), and residue (material remaining after methanol:water extraction). * indicates a plant grown hydroponically (30 $\mu g \cdot L^{-1}$); all other plants were grown in mine waste.

XANES analyses of leaf samples were used to determine the effects of sample preparation on arsenic speciation. The percentage of arsenic(III)-sulphur compound decreased with additional stages of sample preparation (whole leaf (~100%), wet ground leaf (~80%), leaf residue (~80%), and dry ground leaf (~30%)), while the percentage of arsenite and arsenate increased (Fig. 4.2). Arsenic speciation by XANES did not vary greatly between mine waste and liquid culture treatments (Fig. 4.2).

4.4.5 Localizing Arsenic Compounds

It is important to note that arsenic compounds which account for a small percentage (e.g. 5%) of the total arsenic in a sample may not be detectable by the XANES technique (Pickering *et al.*, 2006). However, because the beam size for XAS imaging is small (~ 5 μm), compounds which account for a small percentage of the total arsenic in a large scan area (regular XANES: 1 mm x 4 mm), may be detected during imaging. XAS images were obtained for live leaf and stem sections of a plant grown in mine waste, and a frozen root cross-section from a plant grown in approximately 30 $\mu g \cdot L^{-1}$ hydroponic solution (root rinsed not scrubbed). Only trivalent (e.g. arsenite and arsenic(III)-sulphur) and pentavalent (e.g. arsenate) arsenic species could be distinguished; therefore, additional speciation techniques (XANES or HPLC-ICP-MS) were required to further identify arsenic compounds. Extended X-ray absorption fine structure (EXAFS) analysis would also provide valuable speciation information, but arsenic concentrations were too low for data to be collected.

"Comparison" images illustrate the location of dominant AsIII and AsV compounds. In live stems this comparison revealed alternating bands of trivalent and pentavalent

arsenic compounds in a transverse section (Fig. 4.3: Comparison B). These bands appear to correspond to the location of stem vascular bundles, which contained the highest concentrations of stem arsenic, as determined by a cross-sectional scan for AsIII compounds (data not shown). This segregation was also observed in the leaf veins of a live radish plant (Fig. 4.3: Comparison C). The highest intensities for arsenic compounds in the root cross-section corresponded both to the outer edge of the root (including peel), and to AsIII compounds (Fig. 4.3: Comparison A). The highest fluorescence values for AsV species occurred in small scattered patches toward the center of the root (Fig. 4.3: Comparison A).

4.5 DISCUSSION

4.5.1 Comparison of total arsenic distribution

The total arsenic distribution in shoots and roots has been reported for a variety of radish cultivars grown hydroponically (Carbonell-Barrachina *et al.*, 1999), in greenhouse experiments using arsenic amended soil (Tlustoš *et al.*, 2002; Cobb *et al.*, 2000), and as a part of field trials (Warren *et al.*, 2003). In the greenhouse soil experiments total arsenic concentrations were highest for radish shoots in one experiment (Cobb et al., 2000), and were equal for shoots and roots in the other (Tlustoš *et al.*, 2002). In contrast, in the field trial and hydroponic experiments, the majority of arsenic was found in the radish root (including peel). In the latter experiments roots were peeled to distinguish between arsenic adsorption and absorption. High arsenic concentration for the peel suggested adsorption made a significant contribution to total root arsenic in these studies (Carbonell-Barrachina *et al.*, 1999; Warren *et al.*, 2003).

AREA IMAGED	AsIII	AsV	COMPARISON[*]
ROOT			A
STEM			B
LEAF			C
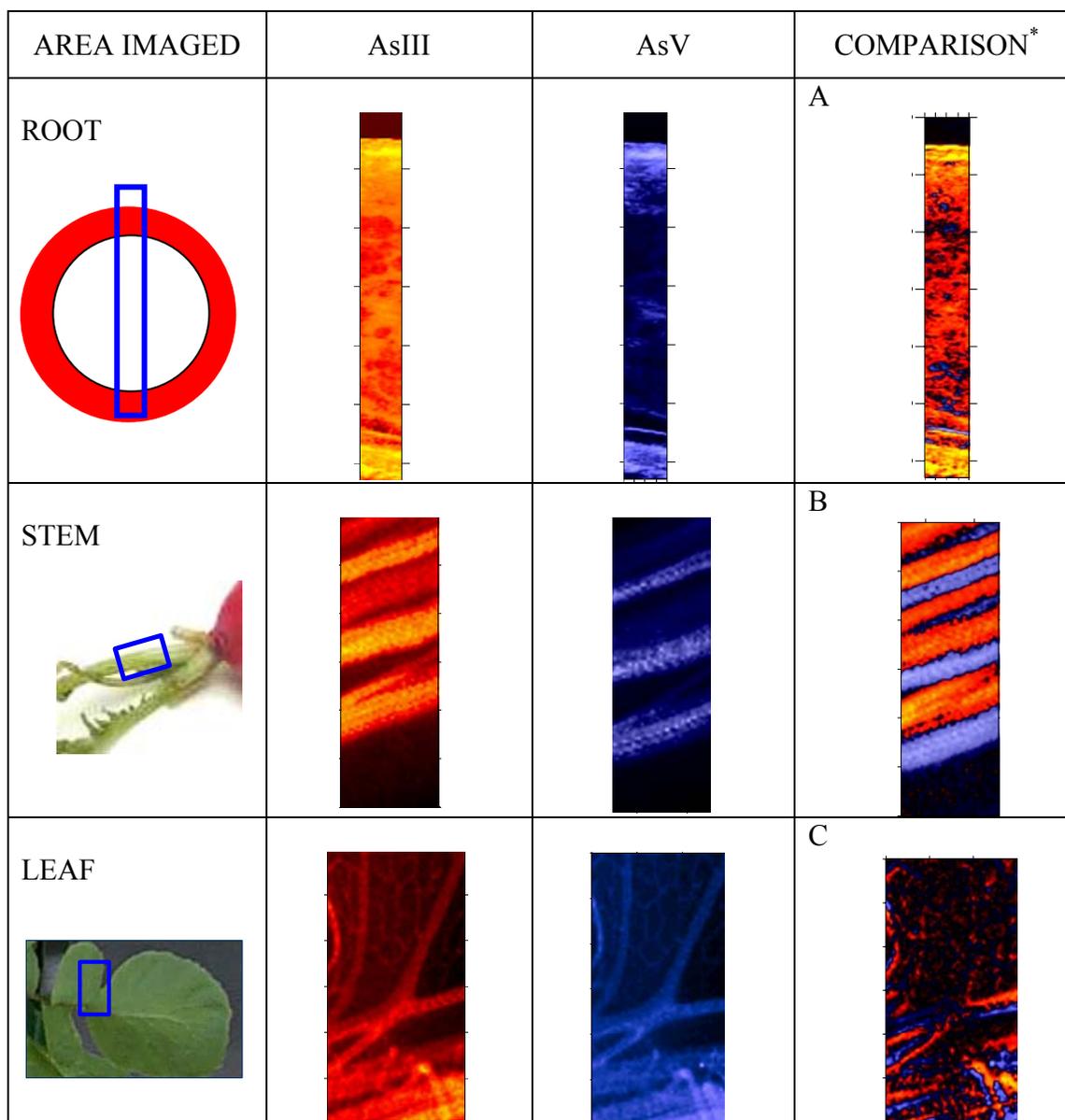
*White: AsV = AsIII
  Dark blue/Light blue (low to high concentration): AsV > AsIII
  Red/Orange/Yellow (low to high concentration): AsIII > AsV

FIG. 4.3: XAS images of radish root (frozen), stem (live) and leaf (live) samples. Individual trivalent (AsIII) and pentavalent (AsV) images were subtraction (AsV – AsIII) and revealed segregation of arsenic compounds in the vasculature of mine waste-treated leaf (COMPARISON C) and stem (COMPARISON B). The highest concentration of root arsenic (AsIII), for a 30 µg·g^{-1}-treated plant, was located around the outer edge (COMPARISON A).

Results from this experiment found the greatest total arsenic concentrations in the leaves of both hydroponic and mine waste-treated plants (Table 4.4), and these results are similar to those from the greenhouse soil experiments (Tlustoš *et al.*, 2002; Cobb *et al.*, 2000). In this study, hydroponic plants were grown in Turface® which provided plant anchorage but no nutrition; however, it also provided adsorption sites for arsenate in the nutrient solution. This is more characteristic of soil experiments than strict hydroponic work, which lacks any solid substrate, and may be an important factor influencing arsenic adsorption to the root peel. In this study the peel did not contain significantly higher levels of arsenic than the inner root (Table 4.4), potentially because some of the arsenate was adsorbed to the Turface® instead of the root peel. In addition the radish roots were scrubbed after harvest which would have removed some arsenic that was adsorbed to the peel.

4.5.2 Sample preparation affects arsenic speciation

Much of the arsenite identified in the radish extracts analyzed in this study may have originated from arsenic(III)-sulphur compounds identified *in planta* using XAS (Fig. 4.2). Physical preparation of plant material disrupts plant tissues potentially releasing compounds stored in cells. Both wet and dry ground samples were used as the starting material for the extraction experiments for these studies. The results presented as Figure 4.2 clearly show that physical grinding and drying alters the forms of arsenic, mainly through conversion of arsenic(III)-sulphur to arsenite (arsenic(III)-oxygen), which is then probably oxidized to arsenate. Indeed, the results for the whole leaf (Fig. 4.2) suggest that all of the *detectable* arsenic in the living tissue is present in an arsenic(III)-sulphur form.

The arsenic profile of the leaf extraction residues, which were prepared at the APS directly before analysis, did not vary greatly from the parent frozen wet ground leaf material, and this implies no preferential extraction or additional conversion of individual arsenite, arsenate or arsenic(III)-sulphur compounds in these samples. Yet extraction efficiencies greater than 70% (Table 4.5) show that most of the arsenic was extracted and detectable by HPLC-ICP-MS. Therefore, it is not unreasonable to hypothesize that the majority of the arsenic(III)-sulphur compound is converted during HPLC separation.

4.5.3 Biotransformation of arsenic in radishes

Arsenate was the predominant form of arsenic in both the mine waste and hydroponic growth media, and likely represents the principal form of arsenic taken up by plant roots. Other studies have found that, once inside the plant, arsenate was reduced to arsenite (Pickering *et al.*, 2000), and the presence of arsenite in radish tissues reported in this, and previous studies (Tlustoš *et al.*, 2002) support these observations. The reduction of arsenate may occur quite rapidly as evidenced by the predominance of trivalent arsenic compounds identified in the XAS images of the cross section of radish tap root (Fig. 4.3: Comparison A), which implies that arsenic does not remain in the form of arsenate long enough to be transported into the root in large quantities.

Recent papers have demonstrated the complexation of arsenite with a variety of sulphur-rich plant tissues (Schmöger *et al.*, 2000; Raab *et al.*, 2004a; Cai *et al.*, 2004). XANES analysis of Indian mustard grown hydroponically identified an arsenic(III)-sulphur compound (As[III]-*tris*-Glutathione) accounted for at least 97% of the total arsenic, in roots and leaves, of plants exposed to 250 µM arsenate for 5 days, with the remaining

arsenic in the form of arsenite (Pickering *et al.*, 2000). The presence of arsenic(III)-sulphur compounds in leaf, stem and root sections of radish plants (Fig. 4.2) indicates that such compounds play important roles in both transport and storage.

4.5.4 Transport of arsenic in radishes

Arsenic can be transported within a radish plant via the vasculature which consists of: xylem for transport of water and minerals absorbed by the roots to the photosynthetic parts of the plant, and phloem which is the principal photosynthate conducting tissue of a plant. Arsenite and arsenate were identified in the xylem sap of Indian mustard plants analyzed by XAS (Pickering *et al.*, 2000). Arsenic has also been identified in the phloem of *Pteris nervosa* using synchrotron X-ray fluorescence spectroscopy, suggesting it can be redistributed throughout the plant after transport to the leaves (Chen *et al.*, 2003).

XAS imaging described for this study not only identified arsenic compounds in radish vasculature, but indicated a segregation of AsIII and AsV compounds most likely between xylem and phloem. In leaves and stems, xylem and phloem occur together in vascular bundles (veins of leaves). It is probable that the blue colour in the leaf and stem comparison images (Fig. 4.3) depicts arsenate present in the plant xylem. The blue colour denotes an AsV compound, and speciation results identified only arsenate. Tlustoš et al. (2002) reported that arsenate appeared to be translocated to the leaves of radishes, and transport to the leaves is accomplished via the xylem. Arsenate has been identified in plant xylem of hyperaccumulating ferns both through the analysis of xylem sap (Kertulls *et al.*, 2005) and microprobe work (Pickering *et al.*, 2006). Micro-XANES analysis for both the stem and leaf strongly suggest arsenic(III)-sulphur compounds are present in the

areas dominated by trivalent arsenic. This reinforces the notion that arsenic(III)-sulphur compounds are involved in transport and storage in Cherry Belle radishes.

In the radish root, xylem and phloem are separated, with phloem located near the outer edge of the root, and xylem occurring in the centre. A few patches of pentavalent arsenic were located in the inner root area, but the low map intensity makes it difficult to draw any conclusions. Surprisingly, little AsV was observed in the root considering the possibility that arsenate is transported with water and minerals to the leaves; however, xylem sap flows relatively unimpeded through xylem cells and may have been lost when the cells were cut during sample preparation. Higher concentrations of arsenic were located at the outer edge of the root in the form of AsIII (Fig. 4.3: Comparison A). There are two possible origins this arsenic: the rapid reduction of arsenate to arsenite upon transport into the root, or the transport of AsIII compounds from other areas of the radish, to the root for storage. The second option seems plausible for the radish since the root of this plant has been modified to function in nutrient storage. Speciation data provided by micro-XANES analysis specific to the outer edge of the radish root may clarify if one or a combination of these processes is taking place.

4.6 CONCLUSION

Using HPLC-ICP-MS and complementary XAS techniques, arsenic-treated radish plants were studied to localize the arsenic compounds present, and identify possible arsenic transformations. The distribution patterns of total arsenic within the plants did not vary greatly between the mine waste and hydroponic studies, with the majority of the arsenic being located in the leaves. Arsenic was supplied predominantly as arsenate in

both studies, and was reduced to arsenite in the roots. Arsenite has a high affinity for thiolate groups, and XANES results indicate that an arsenic(III)-sulphur compound is the predominant form of arsenic in live radish leaves and shoots. It is reasonable to speculate that these sulphur-containing compounds are phytochelatins produced by the plant in response to arsenic stress. In addition XAS imaging indicated a segregation of pentavalent and trivalent arsenic compounds in plant vasculature, with arsenate chiefly in the xylem.

The results of this study provide a better understanding of the fate of inorganic arsenate taken up by plants using Cherry Belle radishes (cv.) as the model organism. XAS proved invaluable for both identifying and localizing arsenic compounds within plant sections; the presence of arsenic(III)-sulphur compounds would not have been seen using only conventional extraction based techniques. Together HPLC-ICP-MS and XAS are powerful tools which can be used to pursue the complete characterization of arsenic compounds which is necessary to understand the intake, accumulation, transport, storage, and detoxification of this element in living systems.

CHAPTER 5

Arsenic speciation analysis of cultivated white button mushrooms (*Agaricus bisporus*) using high performance liquid chromatography inductively coupled plasma mass spectrometry and X-ray absorption spectroscopy

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5.1 ABSTRACT

Agaricus bisporus mushrooms were grown in compost amended with either arsenic contaminated mine waste, or an arsenate solution, to a final concentration of approximately 200 $\mu g \cdot g^{-1}$. Fungi were cultivated at a small scale mushroom facility in Vineland (ON), where the controlled environment allowed for a large number of fruiting bodies (mushrooms) to be produced. The total arsenic concentrations as well as speciation were examined for each treatment over several harvests (breaks). Total concentrations were determined by acid digestion and inductively coupled plasma mass spectrometry (ICP-MS) detection and ranged from 2.3 $\mu g \cdot g^{-1}$ to 16 $\mu g \cdot g^{-1}$ dry mass in treatment mushrooms. Arsenic compounds were extracted from mushrooms with methanol-water (1:1 v/v), and separated by high performance liquid chromatography (HPLC, anion/cation exchange) before detection with ICP-MS. Fruiting bodies from all treatments contained arsenite, dimethylarsinic acid (DMA), and arsenobetaine (AB), and to a lesser extent arsenate and trimethylarsine oxide (TMAO). The ratio of arsenic

compounds did not vary greatly over the first three harvests. AB was absent in compost not inoculated with *A. bisporus* supporting the hypothesis that AB is a product of metabolism by the *Agaricus* fungus and not taken up by this fungus from its environment. We hypothesize that AB plays a role in nutrient translocation within the fruiting body, as well as maintaining turgor pressure to ensure the mushroom cap remains elevated for maximum spore dispersal.

5.2 INTRODUCTION

Fungi play an important role in arsenic cycling in the environment both as decomposers, and plant symbiots. Fungi of the class Hymenomycetes produce fruiting bodies (recognized as a mushroom) that have been found to contain a variety of inorganic and organic arsenic compounds. Commonly identified arsenicals include dimethylarsinic acid (DMA), arsenobetaine (AB) and minor amounts of arsenite, arsenate, monomethylarsonic acid (MMA), tetramethyl arsonium (tetra), trimethylarsine oxide (TMAO), arsenocholine (AC) (Kuehnelt *et al.*, 1997) and arsenosugar XI (Koch *et al.*, 2000), with several unidentified arsenic compounds also reported.

Despite the fact that AB has long been known as a major arsenic metabolite of marine organisms (Edmonds *et al.*, 1977), the pathway for its production has yet to be elucidated. Most hypotheses involve the biosynthesis of AB from the microbial breakdown products of arsenosugars. One suggestion is that AB is finally derived from dimethylarsinoylacetate (Ritchie *et al.*, 2004), while another considers AC to be the precursor to AB (Edmonds and Francesconi, 1988; Hanaoka *et al.*, 1992b; Hanaoka *et al.*, 1992a). The latter theory is in part based on the idea that arsenic metabolism in the

marine environment may parallel nitrogen metabolism (Edmonds and Francesconi, 1988), and arsenobetaine may play a role in osmoregulation for marine animals. Arsenobetaine is generally considered to be an end product of arsenic metabolism in the marine environment (Hanaoka *et al.*, 1992b). In contrast, terrestrial organisms appear to convert inorganic arsenic to primarily simple methylarsenic compounds (Irgolic, 1988). With the advent of better detection methods, AB has been reported at low concentrations in some plants (Meharg and Hartley-Whitaker, 2002), and terrestrial animals such as birds (Koch *et al.*, 2005). Two exceptions to this trend appear to be earthworms in which as much as 23% of the total arsenic burden was AB (Langdon *et al.*, 2002), and many terrestrial species of fungi in which AB has been reported as a major arsenic compound (Šlejkovec *et al.*, 1997). The presence of AB in both marine and terrestrial organisms may hint at a common synthetic pathway for this compound.

In spite of the identification of AB in many mushrooms, it is not entirely clear whether fungi accumulate it from the environment, or produce it through biotransformations. It is possible that terrestrial microorganisms produce AB or its precursors and these are then taken up by fungi growing in their presence. The ability of fungi to selectively take up arsenic compounds was demonstrated by *A. placomyces* which preferentially took up AB and tetramethylarsonium ion (tetra), over arsenite and MMA, from arsenic spiked agar plates (Šlejkovec *et al.*, 1996). However, in the same study, mycelium (the mass of tubular filaments forming the body of a fungus), was unable to produce AB, although this arsenical has been identified in fruiting bodies of many *Agaricus sp.* collected from arsenic contaminated sites (Šlejkovec *et al.*, 1997). Despite the results of the mycelium study, most research assumes that the

organoarsenicals that are found in mushrooms are produced by the fungus itself. This is supported by the observation that fruiting bodies from one species of mushroom often contain similar arsenicals regardless of the harvest location, and this suggests an ability to metabolize arsenic compounds (Šlejkovec *et al.*, 1997; Vetter, 1994). In addition, it has been theorized that only the more highly evolved fungi (e.g. *Geastrum* and *Agaricus*) can produce more complex arsenic compounds such as AB (Šlejkovec *et al.*, 1997).

This study examined the ability of cultivated *Agaricus bisporus* to metabolize arsenic introduced to compost as mine waste or applied as an arsenate solution. Fruiting bodies, mycelium and compost were investigated for total arsenic and speciation information, using HPLC-ICP-MS and X-ray absorption spectroscopy techniques.

5.3 MATERIALS AND METHODS

Cultivation of *Agaricus bisporus* (white button mushroom) was conducted at a unique small-scale mushroom facility belonging to the University of Guelph, Vineland (ON). Pasteurized compost was combined with 9.3 kg peat (Premier Pro Moss TBK, Professional fibrous blond sphagnum peat moss), 8.6 kg lime (Sylvite F high calcium pulverized limestone), 29 kg water (tap), and 605 g casing inoculum, and mechanically homogenized, before arsenic was added.

5.3.1 Treatments

Three different arsenic treatments were applied to the compost and homogenized manually (Fig. 5.1). Treatments included mine waste material collected from Yellowknife, NT (Yk) and Deloro, ON (D), as well as an arsenate solution (L). Final

ARSENIC TREATMENTS

MICROBIAL BIOTRANSFORMATIONS	FUNGAL BIOTRANSFORMATIONS
(compost)	(mushrooms, mycelium + compost)

ConB Pot Soil   **ConC** Pot Soil   **ConD** ddH$_2$O

No Fungi Added

Fungi Added ConA

Mine Waste Yk   Mine Waste D   Arsenate Solution L

Mine Waste Y   Mine Waste D   Arsenate Solution L
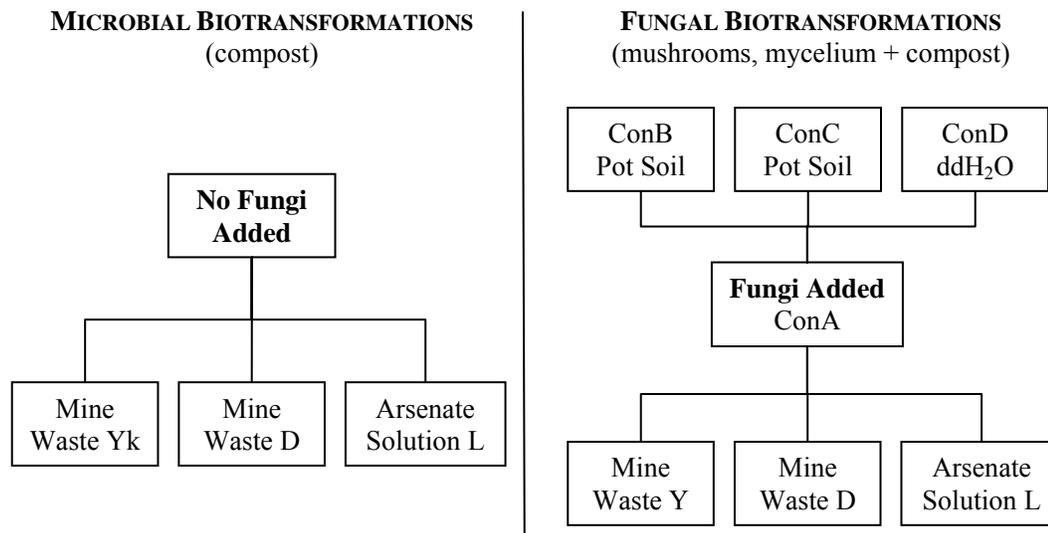
FIG. 5.1: Arsenic treatments were applied to compost without fungus (Microbial Biotransformations) and with fungus (Fungal Biotransformations). Final concentrations (dry mass) were obtained of approximately 190 µg·g^{-1}, 180 µg·g^{-1} and 360 µg·g^{-1} for mine waste Yk, arsenate solution, and mine waste D treatments respectively. Control treatments included unaltered compost (ConA), and compost amended with ddH$_2$O (ConD) or sterilized potting soil (ConB and ConC). Each treatment included 6 replicates.

arsenic concentrations were approximately 190 µg·g^{-1} and 180 µg·g^{-1} for the YK and L

treatments respectively, and 360 µg·g^{-1} for the D treatment (dry masses). Control pots

contained compost that was not amended with arsenic (ConA). To determine if the

addition of mine waste or arsenic solution influenced production of fruiting bodies three

additional control treatments were included. Adding an equivalent amount of autoclaved

potting soil to compost (ConB – 180 g, ConC – 60 g) simulated the mine waste

treatments, and distilled deionized water (ConD) simulated the addition of an arsenic

solution. All treatments were replicated 6 times. Potting soil was subjected to autoclaving

(121ºC, 20 min) twice over a 48 hour period to destroy any heat activated fungi before

addition to the compost. All liquids were applied using pump bottle sprayers.

5.3.2 Growth

Agaricus bisporus was grown in pots containing 1 kg of compost (68% moisture at

spawning). To promote fruiting, approximately 2 inches of casing consisting of 1:1 (w/w)

peat moss:calcium carbonate (lime), containing no arsenic, was added to the compost

after one week. Air temperature was maintained at 26°C, and probes were used to

monitor compost temperature which remained close to that of the air. After 4 weeks the

fungi began to fruit and mushrooms were collected over three different breaks (harvests).

The 6 replicate pots for each treatment were distributed randomly throughout the growth

chamber. Arsenic treated-compost (for each treatment) containing no fungus was used to

determine possible microbial arsenic biotransformations. These pots were maintained in a

greenhouse in conditions similar to the growth chamber. Upon harvest, samples were

cleaned of soil, rinsed with distilled water, and frozen before analysis. Samples of

mycelium with some associated compost, as well as samples of uninoculated compost were only taken from one replicate of each treatment.

5.3.3 Chemicals and Standard Reference Materials

Chemicals used included nitric acid (Fisher Scientific), methanol (99.93% HPLC grade, Aldrich), liquid nitrogen (BOC), ortho-phosphoric acid (85% Fluka), and ammonium hydroxide solution (~25% in water, Fluka). Distilled deionized water had a resistivity better than 17.5 MΩcm (E-pure Barnstead). Arsenic compounds used as standards included: arsenate (Fluka KH_2AsO_4 solid and Aldrich ICP/DCP standard solution), arsenite (Fluka As_3O_3 solid and atomic spectroscopy standard solution), monomethylarsonic acid (Pfaltz & Bauer and ChemService monosodium acid methane arsenate), dimethylarsinic acid (Aldrich cacodylic acid sodium salt hydrate), trimethylarsine oxide (synthesized), tetramethylarsonium iodide (synthesized), arsenobetaine (synthesized), arsenocholine (synthesized), arsenic(III)-glutathione (synthesized) and TuneA (Thermo Electron Corporation). 'Synthesized' compounds were prepared by Drs. H. Sun and W. R. Cullen, at the University of British Columbia, using standard methodologies which have been referenced previously (Smith *et al.*, 2005). Certified reference materials included Bush Branches and Leaves, certifying body: Institute of Geophysical and Geochemical Exploration (GWB 07603), DORM 2 (dogfish muscle), certifying body: National Research Council of Canada, Lichen, certifying body: Institute for Reference Materials and Measurements (BCR-CRM 482), and MESS 2 (marine sediment), certifying body: National Research Council of Canada.

5.3.4 Sample Preparation

Samples were kept frozen with liquid nitrogen and homogenized in a small stainless

steel blender. Sub-samples were dried at approximately 65°C in an Isotemp oven (Fisher

Scientific) for a minimum of 24 hours. Between samples, homogenization equipment was

washed once with soap and water, rinsed once with ~10% HNO_3, rinsed 5 times with

distilled water, and rinsed 3 times with distilled deionized water before drying with

Kimwipes®. Procedural blanks of distilled deionized water, included with every batch of

five samples, were subjected to the grinding procedure both before and after the samples,

and the average arsenic concentration in the undiluted blanks for each treatment did not

exceed the method detection limit for determining total arsenic concentration (5 $ng·g^{-1}$).

5.3.5 Determination of total arsenic

Aliquots of approximately 0.5 g of dried sample were digested with 10 mL of

concentrated HNO_3 on a hot plate at ~130°C for a minimum of 6 hours. Samples were

boiled down to ≤ 1 mL before the addition of 3 mL of 30% hydrogen peroxide. Samples

were again evaporated down to < 1 mL before being made up to a final volume of 10 mL

with distilled deionized water (ddH_2O). Diluted digestion blanks were below the method

detection limit. DORM 2 reference material, analyzed with every batch of 10 samples,

had 72-96% recovery; thus the nitric acid digestion method was considered sufficient for

quantifying the majority of arsenic in mushroom samples (total concentration).

Nitric acid digests were analyzed using inductively coupled plasma mass

spectrometry (ICP-MS). Approximately 2.0 mL of digested sample was diluted to 10 mL

with 2% nitric acid solution for analysis using a X7 X-series, inductively coupled plasma-

mass spectrometer (Thermo Instruments). A standard ICP torch was used for atomization

and ionization of the sample. The quadrupole detector monitored a mass to charge (m/z)

ratio of 75 (arsenic) and an internal standard of indium (m/z 115) and scandium (m/z

45), was used to correct for plasma drift and matrix effects. Instrument blanks were

below the method detection limit (5 ng·g^{-1}), and instrument blanks were below this limit.

Method duplicate relative percent differences (RPD = | difference in duplicate values | /

average of duplicate values * 100) ranged from 0.8 – 62% (median 16%). Average

percent recovery for instrument spikes (to test matrix effects) ranged from 59 – 119%

(median 92%), and for instrument QC from 77 – 100% (median 91%). These ranges

include values close to the detection limits. For each break the total arsenic determined

for the filtrate plus residue compared favourably with total arsenic determined for dried

mushroom samples: RPD values (between concentration obtained by each method) were

4 – 33% (median 8%), 18 - 86% (median 31%) and 4 – 58% (median = 22%), for first,

second and third breaks respectively.

Dried and ground samples of compost were analyzed by neutron activation analysis

(NAA). During NAA samples were irradiated at a flux of 5×10^{11} n cm^{-2} s^{-1} for 12 to 19

hours using the SLOWPOKE-2 reactor at the Royal Military College of Canada, then

cooled for 73-97 hours. A GMC HpGe detector coupled with an EG and G Ortec 919

Spectrum Mater µ-multichannel analyzer (MCA) was used to count the samples. Percent

recovery of standard reference materials for MESS-2, Lichen and Bush Branches, used as

instrument quality control, were determined to be 100%, 94% and 99% respectively.

5.3.6 Identification of arsenic compounds

HPLC-ICP-MS: Approximately 1.0 g of wet, ground, mushroom (fruiting body), or dry ground mycelium (+ compost), was extracted with 10 mL of methanol-water 1:1 (v/v). Approximately 3.0 g of wet compost was extracted with 20 mL (final volume) of distilled deionized water, since water soluble arsenic compounds were considered to be readily available to the fungus. Samples were vortexed and placed in a shaker (Innova 4230, New Brunswick Scientific) at 5°C and 275 RPM for ~30 min. Samples were then placed in an ultrasonic bath (FS 140H, Fisher) for 20 min before being centrifuged (GP8R, IEC Centra) at 3500 RPM for 20 min at 15°C. Samples were filtered (Whatman P541), and a subsample of each methanol-water extract was rotovapped (R-124 Buchi Rotovaper) to evaporate methanol before being made up to 5-10 mL with distilled deionized water. Total arsenic concentration was determined for undried residues remaining after extraction following the procedure outlined in section 5.2.5.

Extracted arsenic compounds were identified using high performance liquid chromatography inductively coupled plasma mass spectrometry (HPLC-ICP-MS). The HPLC system for the speciation of the arsenic compounds consisted of a P4000 quaternary HPLC pump, SCM100 vacuum degasser, and an AS300 autosampler (Thermo Instruments). This system was connected to the intake of the X7 X-Series ICP-MS, which monitored mass to charge (m/z) signals of arsenic (As) 75, rhodium (Rh) 103, indium (In) 115, and uranium (U) 238 as required. Rh was added as an internal standard to the anion mobile phase of 20 mM ammonium phosphate (pH 6.0), and compounds were separated with a Hamilton PRP X-100 (10μm 250 x 4.6 mm) stainless steel column. Arsenic was quantified through external calibration with standard solutions containing

arsenite, arsenate, DMA, and MMA (reporting limit 5 ng·g^{-1}). Indium and uranium were added as internal standards to the cation mobile phase of 20 mM pyridine formate (pH 2.7). Trimethylarsonium oxide (TMAO), tetramethyl-arsonium ion (tetra), arsenocholine (AC) and arsenobetaine (AB) were separated using a Chrompack 150 mm stainless steel cation exchange column. Arsenic was quantified through external calibration with standard solutions containing DMA, AB, TMAO, AC and tetra. DMA, AB and TMAO were identified in samples and had a reporting limit 5 ng·mL^{-1}. SOLAAR software was used to record data, while calibration curves based on known arsenic standards were constructed using Peakfit™ chromatography software, and used to quantify arsenic in samples. A flow rate of 1.5 mL·min^{-1} was used for both anion and cation exchange analysis. Average percent recovery of AB from DORM 2, for each treatment, ranged from 70 – 119%. Method duplicate RPD values ranged as follows: arsenite 10 - 50%, DMA 4 - 79%, AB 0.5 – 42%, and unknown cation 5 – 200 %. RPD values greater than 50% resulted from calculations using numbers within 5 times the reporting limit. Instrument spikes had average percent recoveries ranging from 70 – 130%, while instrument QC recovery ranged from 88-102% for the anion column, and 87 – 90% on the cation.

Synchrotron: X-ray absorption near edge structure (XANES) spectra were collected at the 20-ID beamline (Heald *et al.*, 1999) at APS, Argonne National Laboratory. X-rays were focused to a 5 x 5 square micrometer spot size using Rh-coated Kirkpatrick-Baez mirror pairs on both beamlines. Mushroom sections were analyzed at approximately -20ºC using a thermoelectric cooled stage. Fluorescence data were collected with a Canberra 7-element solid state Ge(Li) detector. The monochromator was calibrated using

the first inflection point of the gold L_{III} absorption edge (11919.7 eV (Kraft *et al.*, 1996))

for measurements at the arsenic K-edge (11868 eV). Where experimental setup allowed,

a reference gold foil was also measured in transmission during each sample scan.

Typically, 3 scans were collected and averaged before background-removal and

normalization-to-edge-jump. Spectra were processed using WinXAS (Ressler, 1997)

software. White line energies of arsenic standard reference materials, that best resembled

the position of peak features in XANES spectra of samples, were used to deduce possible

arsenic compounds in mushrooms.

5.4 RESULTS

5.4.1 Growth

After the compost was inoculated with spawn, mushrooms were ready to pick at 28

days for the first break (harvest), 37 days for the second break, and 47 days for the third

break. There were no visible signs that the arsenic treatments adversely effected the

growth of the mushrooms, and there was no significant difference in total yield between

treatments (Table 5.1). There was a significant difference in the total yield between

harvests (Table. 5.1). The second break produced a large number of small mushrooms;

however, it had a lower overall yield than both the first, and third. A two-factor ANOVA

and Tukey's post hoc tests were used. Due to the small sample size variance was assumed

to be equal and no significant interaction was observed between treatments and harvests

for mushroom yield.

5.4.2 Total Arsenic

Average arsenic concentrations in the control and treatment, compost and mushroom,

samples are given in Table 5.2. Our results are similar to arsenic concentrations reported

TABLE 5.1: Total mushroom yield (g, wet mass) for the mine waste (D, Yk) and arsenic solution (L) treatments. Similar capital letters across rows indicate no significant difference among harvests ($F(2,36) = 4.0$, $p<0.05$). There was no significant difference in total yield between treatments ($F(2,36) = 0.4$, n.s.).

		Harvest Yield (g wet mass)					
		First		Second		Third	
Treatment	D	150 ±70	A	80 ±20	B	150 ±60	A
	L	150 ±90	A	110 ±30	A	140 ±30	A
	YK	170 ±60	A	120 ±30	B	140 ±40	AB

TABLE 5.2: Average total arsenic dry mass concentrations ($\mu g \cdot g^{-1}$) for *Agaricus bisporus* mushroom treatments: mine waste (Yk, L), arsenic solution (L), a control without amendment (ConA), a control amended with sterilized soil (ConB, ConC), and a control amended with distilled deionized water (ConD). Similar capital letters (A, B, C) across rows indicate no significant difference among harvests for a single treatment ($\underline{F}(2,36) = 34$, $\underline{p}<0.05$). Similar capital letters down columns (D, E, F) indicate no significant difference among treatments for a single harvest ($\underline{F}(2,36) = 43$, $\underline{p}<0.05$). Average total arsenic determined from 3 or 5 treatment replicates ($\pm$ standard deviation).

	Compost[a]	Casing[a]	Total Mushroom Arsenic ($\mu g \cdot g^{-1}$, dry mass)[b]								
			1st harvest			2nd harvest			3rd harvest		
Yk	190 ±30	6 ± 1	16 ±5	A	D	5.0 ±0.2	B	D	12 ±1	C	D
L	180 ±7	8 ±2	16 ±5	A	D	5.6 ±0.6	B	D	15 ±3	A	E
D	360 ±30	3 ±1	6 ±1	A	E	2.3 ±0.1	B	E	6 ±1	A	F
ConA	0.34 ±0.05	0.72	0.12 ±0.04			0.06 ±0.02			0.14 ±0.04		
ConB	0.59 ±0.2	0.25	0.13			0.06 ±0.02			0.16 ±0.04		
ConC	0.61 ±0.5*	-	0.3 ±0.3*			0.054 ±0.003			0.14 ±0.004		
ConD	0.42 ±0.2	0.14	0.2 ±0.2			0.05 ±0.01			0.12 ±0.03		

[a]Determined by NAA
[b]Determined by ICP-MS
*value determined for total arsenic of duplicate treatments

in the literature (Byrne *et al.*, 1995; Šlejkovec *et al.*, 1997; Soeroes *et al.*, 2005) for extracts of *A. bisporus* mushrooms harvested from non-contaminated soil ($1\mu g \cdot g^{-1}$, 0.5 $\mu g \cdot g^{-1}$) and harvested from areas of elevated arsenic concentrations (22.8 $\mu g \cdot g^{-1}$, 8.7 $\mu g \cdot g^{-1}$). For arsenic contaminated samples, a two-factor ANOVA, with minimal interaction between harvests and treatments, showed a significant difference in average arsenic concentrations ($\mu g \cdot g^{-1}$ dry mass), in extracts of fruiting bodies, between harvests, $\underline{F}(2,36)$ = 43, $\underline{p} < 0.05$. This difference indicated that mushrooms collected from the first and third breaks on average contained more arsenic than second break samples (Table 5.2). A significant difference in fruiting body arsenic concentration ($\mu g \cdot g^{-1}$ dry mass) among treatments was also observed, $\underline{F}(2,36) = 34$, $\underline{p} < 0.05$. Thus the mushrooms from Yellowknife mine waste (Yk) and arsenate solution (L) treatments contained significantly more arsenic than the Deloro mine waste (D) treatment for each harvest (Table 5.2).

5.4.3 Arsenic speciation

Arsenic compounds in mushrooms are generally considered to be stable, not volatile, and not associated with proteins or other high molecular weight compounds, and therefore easily extracted with methanol:water (Byrne *et al.*, 1991; Wuilloud *et al.*, 2004). This was verified here as the 1:1 (v/v) methanol:water extraction gave average arsenic extraction efficiencies (EE) for first, second and third break mushrooms of 75 ±16% (n = 8), 129 ±38% (n = 12) and 79 ±26% (n = 15) respectively (total filtrate / nitric acid total*100); similar to that previously obtained with a 9:1 methanol:water extraction method (Šlejkovec *et al.*, 1997).

Anion and cation-exchange HPLC systems were used to effectively separate 8 known compounds. In anion-exchange HPLC, AB and TMAO signals overlapped with arsenite eluting at about 110 sec (Fig. 5.2A). DMA, MMA and arsenate eluted at approximately 150 sec, 197 sec, and 490 sec respectively. In the cation-exchange HPLC the anionic arsenic compounds eluted, first following the order arsenate, arsenite and DMA (66 sec). Though they were not always baseline separated the peaks could be used qualitatively to verify the compounds identified using the anion column (Fig. 5.2B). The cationic compounds AB, TMAO, AC and tetra eluted at approximately 150 sec, 375 sec, 575 sec and 700 sec respectively.

The quantification of DMA, MMA, arsenate, AB and TMAO posed no difficulties because the signals for these arsenic species were easily distinguished on at least one of the chromatography columns (Fig. 5.2). Quantification of arsenite however, was complicated by overlapping signals from other arsenic compounds on both columns. Therefore arsenite concentration was obtained by subtracting the AB concentration obtained using the cation-exchange, from the anion peak corresponding to the arsenite standard. This was considered acceptable since the sum of species calculated from the results for first and second break samples, was in good agreement with the total arsenic concentrations determined for the filtrate: average RPD range = 3 – 34 (median 18%). Speciation data was not available for third break mushrooms. Quantification of arsenobetaine in DORM 2 reference material indicated that average arsenic concentrations, for first and second break, determined by anion and cation columns, agreed within 30 % (RPD) of each other.
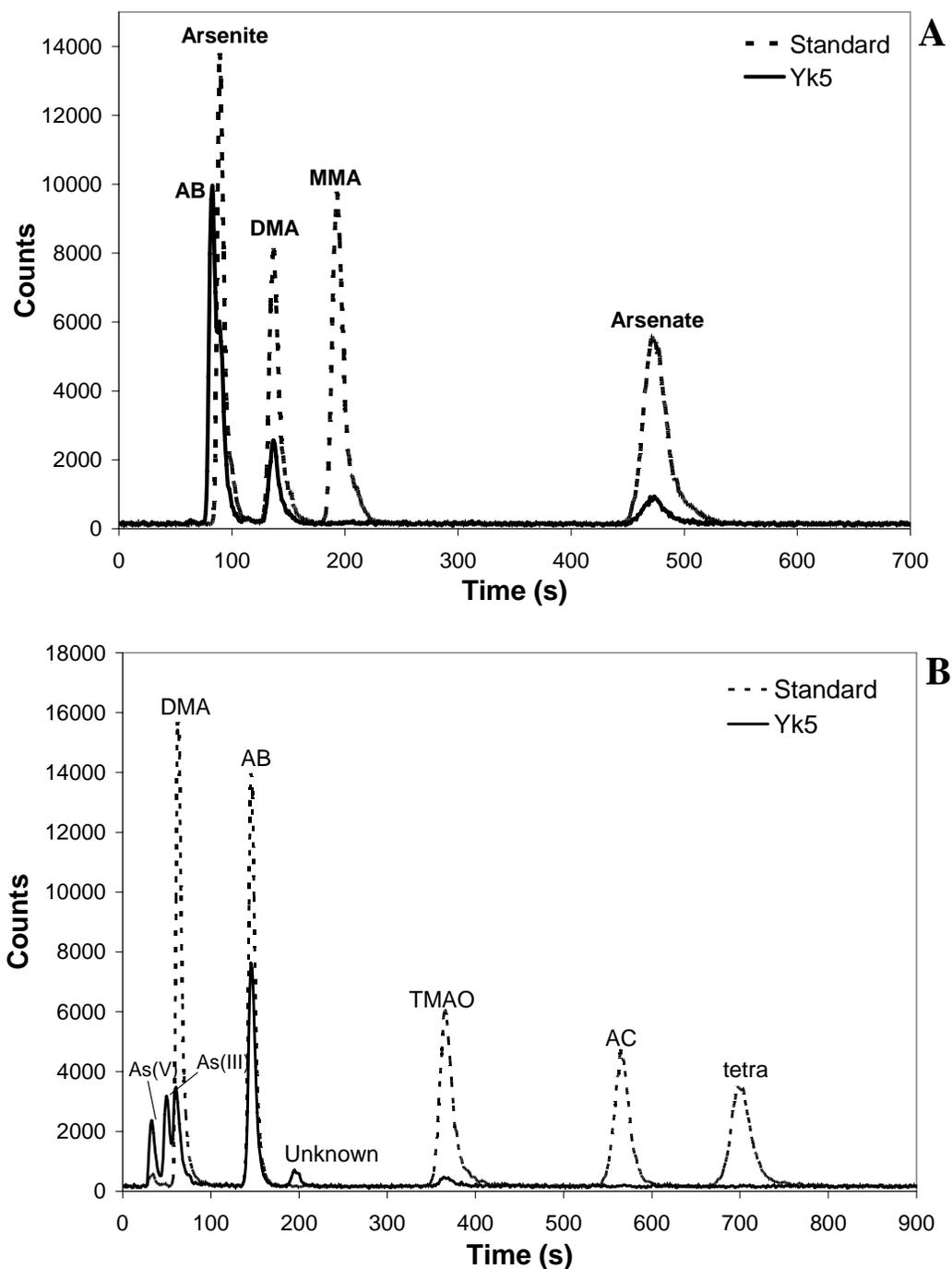
FIG. 5.2: Chromatograms for a mushroom extract from one replicate of Yellowknife treatment. A: anion-exchange showing overlap of AB and arsenite (50 ppb standard = arsenite, DMA, MMA, arsenate). B: cation-exchange showing presence of unknown arsenic compound, as well as arsenobetaine, TMAO, DMA, arsenate (As(V)) and arsenite (As(III)). Cation standard (50 ppb = DMA, AB, TMAO, AC, tetra) did not co-elute with the unknown arsenic compound.

Arsenic compounds extracted from compost without *A. bisporus* were considered to result from processes not associated with the fungus, and included TMAO, arsenite, arsenate and DMA (Fig. 5.3: Compost). In extracts of mushrooms, arsenate and TMAO account for a much smaller percentage of the total arsenic detected, and DMA, arsenite and most importantly AB were the main arsenic species present (Fig. 5.3: Mushroom). In addition, extracts from mycelium (with some adhered compost), also contained AB (data not shown). An unknown cationic arsenic compound was observed in some fruiting body extracts but was not identified in this study.

The concentrations of the individual arsenic compounds identified in mushroom extracts are presented in Table 5.3. Previous studies have identified arsenic compounds in both cultivated and collected samples of *A. bisporus*. Collected samples (Šlejkovec *et al.*, 1997) contained relative amounts of arsenic compounds (< 0.1% arsenite, 27% DMA, 55% AB, 6% MMA, 12% arsenate) similar to mushrooms in this study (Fig. 5.3), with the exception of arsenite. Control mushrooms from *A. bisporus* cultivated on heat treated substrate (Soeroes *et al.*, 2005) also contained a similar distribution of arsenic species (22% arsenite, 26% DMA, 68% AB, 2% MMA, 3% arsenate). Both the collected mushrooms (Šlejkovec *et al.*, 1997), and control mushrooms (Soeroes *et al.*, 2005), were grown in non-contaminated substrate. The arsenical distribution of cultivated mushrooms grown on arsenic treated substrate (Soeroes *et al.*, 2005) displayed a very different arsenic distribution (70 % arsenite, 1% DMA, 1% AB, <1% MMA, 28% arsenate).
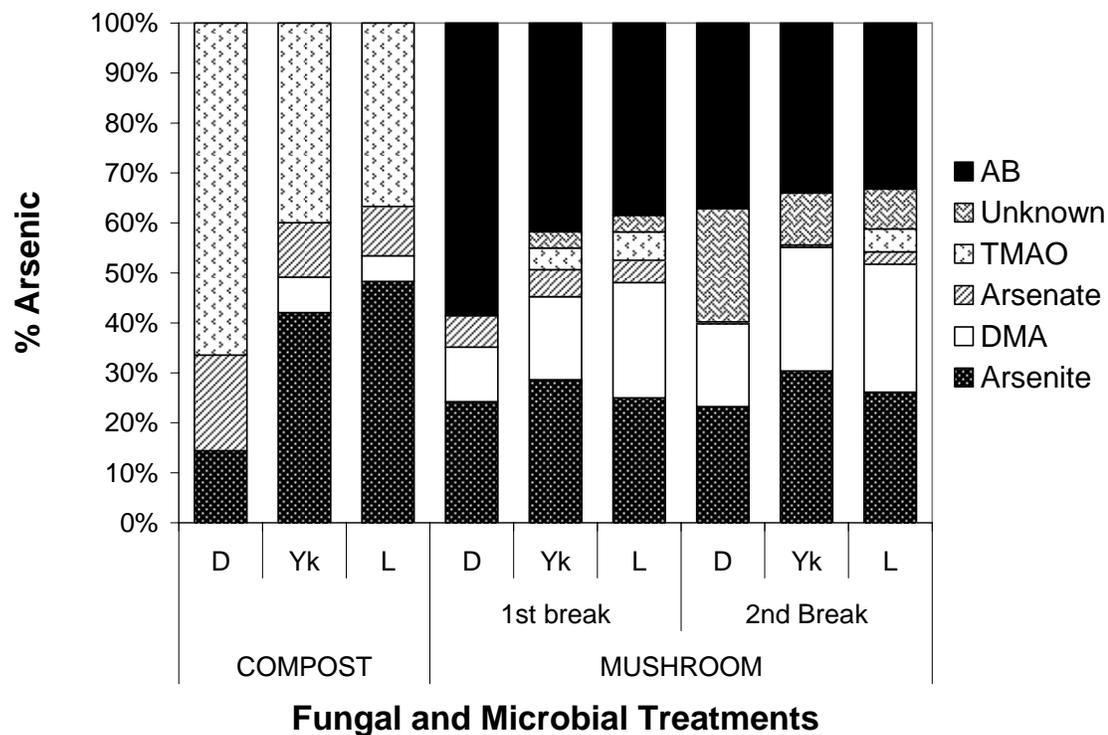
FIG. 5.3: Arsenical distribution based on arsenic concentrations ($\mu g \cdot g^{-1}$ dry mass) as determined by HPLC-ICP-MS. Arsenobetaine was not detected in compost that was lacking *Agaricus bisporus,* but the presence of TMAO and DMA suggest some microbial activity. Mine Wastes: D = Deloro, Yk = Yellowknife. L = arsenate treatment.

TABLE 5.3: Concentrations (µg·g^{-1} dry mass) of mushroom arsenic compounds, determined by HPLC-ICP-MS, for two harvests of the mine waste (Yk, D) and arsenic solution (L) treatments. Average compound concentration determined from 5 or 6 treatment replicates (± standard deviation). ND = not detectable.

Harvest		Arsenic Compounds in Mushrooms (µg·g^{-1} dry mass)					
		Arsenite[a,c]	DMA[a]	Arsenate[a]	AB[b]	Unknown[b]	TMAO[b]
1st	Yk	2 ±1	1.4 ±0.2	0.5 ±0.2	3.5 ±0.7	0.3 ±0.5	0.4 ±0.2
	L	2 ±4	2.2 ±0.3	0.4 ±0.4	4 ±2	0.3 ±0.3	0.5 ±0.4
	D	1 ±1	0.39 ±0.04	0.2 ±0.1	2.1 ±0.4	ND	ND
2nd	Yk	2.1 ±0.6	1.7 ±0.4	0.03 ±0.02	2.4 ±0.3	0.7 ±0.1	ND
	L	2.1 ±0.8	2.1 ±0.3	0.2 ±0.2	2.7 ±0.4	0.6 ±0.1	0.4
	D	0.8 ±0.4	0.6 ±0.09	0.01	1.3 ±0.2	0.8 ±0.8	ND

[a]Determined by Anion column
[b]Determined by Cation column
[c]Arsenite = Arsenite+AB from anion column – AB from cation column

5.5 DISCUSSION

5.5.1 Total arsenic comparison for treatments and breaks

Application of arsenic as either a solid (mine waste) or liquid (arsenate solution) amendment did not appear to influence arsenic uptake by the fungus. The fruiting bodies from the arsenate solution (L) and Yellowknife mine waste (Yk) treatments contained the greatest average total amounts of arsenic (Table 5.2), and did not differ significantly from each other for the first two harvests. However, mushrooms grown in Deloro mine waste (D) contained significantly less average total arsenic than mushrooms grown in Yk and L treatments (Table 5.2). The reason for this difference is not clear at present but may be related to the observed clay-like character of the D mine waste. Inorganic arsenate is strongly sorbed onto clay, reducing arsenic mobility, and consequently arsenic bioavailability (Mandal and Suzuki, 2002).

There is also evidence that larger mushrooms may accumulate higher levels of arsenic, as has been suggested for *L. laccata* and *L. amethystea* (Slekovec and Irgolic, 1996). Second break mushrooms produced smaller fruiting bodies: 7.5g lighter than the average first break mushroom and 1.2 g lighter than the average third break mushroom (wet weight). The smaller second break mushrooms contained significantly lower total arsenic concentrations ($\mu g \cdot g^{-1}$ dry mass) than both the first and third break mushrooms, supporting the observations for *L. laccata* and *L. amethystea*.

5.5.2 Sources of methylated arsenic species

The occurrence of simple methylated arsenic compounds in compost extracts likely indicates the presence of microorganisms (Cullen and Reimer, 1989). DMA, MMA and

TMAO are considered products of biomethylation, and can be produced by many organisms, including bacteria and fungi, from inorganic arsenic by alternate reduction, and oxidative methylation steps (Irgolic, 1988, Mandal and Suzuki, 2002; Bentley and Chasteen, 2002; Šlejkovec *et al.*, 1996). The DMA and TMAO produced are available for uptake by the fungus. In the marine systems some form of microbial activity is thought necessary to covert inorganic arsenic from the environment into arsenosugars which are further converted into compounds used in AB formation by marine animals (Ritchie *et al.*, 2004; Hanaoka *et al.*, 1992a). In this study it remains unclear what role microbial activity plays in the formation of AB by terrestrial fungi. However, it is important to note the absence of AB in the compost extract, effectively eliminating microbial activity as a major source of this compound. The absence of AB in extracts of other *A. bisporus* growth substrates has also been observed (Soeroes *et al.*, 2005).

5.5.3 Arsenic compounds in fruiting bodies

Most of the arsenic compounds in the fruiting body extracts (arsenite, arsenate, DMA, AB, TMAO) found here have been noted in other studies involving *A. bisporus* (Soeroes *et al.*, 2005; Šlejkovec *et al.*, 1997). In a study by Soeroes et al. (2005), *A. bisporus* was cultivated in plastic bags, on heated treated growth medium with total arsenic concentrations (dry mass) of either 3.8 $\mu g \cdot g^{-1}$ (control) or 1000 $\mu g \cdot g^{-1}$ arsenic ('treated') . The control mushrooms contained approximately 60% AB and the distribution of arsenic compounds in these fruiting bodies reflects the results reported in this study. The AB concentration of mushrooms from 'treated' substrate however, only accounted for approximately 1% of the detected arsenic compounds with the majority of the arsenic

present as inorganic arsenite and arsenate (Soeroes *et al.*, 2005). The authors of that study

suggested a longer mycelium growth period may be required for the production of AB;

however, our study involved a similar growth period and the results clearly demonstrate

AB production. Alternatively, Soeroes *et al.* (2005) considered that the high

concentrations of arsenate in treated substrate may have negatively impacted the

production of AB. The substrate arsenic concentration in the work by Soeroes *et

al.*(2005) was five times higher than the average total arsenic concentration for all three

treatments of this study. It is possible that total arsenic concentration, as well as arsenic

species synthesis and accumulation, may be influenced by the initial level of available

contamination but further work is needed to substantiate this hypothesis.

5.5.4 Possible origins of arsenobetaine

The biosynthesis of AB was probably the result of fungal, not bacterial, processes.

The presence of AB in the mycelium and fruiting bodies, and the absence of AB in un-

inoculated compost, supports this hypothesis (Fig. 5.3). This arsenical may be produced

in the mycelium and transported to the fruiting bodies, which due to their short life span

are unlikely to be solely responsible for AB formation. *Agaricus placomyces* mycelium

grown on potato dextrose agar (PDA) media reduced arsenate to arsenite and converted

MMA to DMA (Šlejkovec *et al.*, 1996); however, the synthesis of AB was not reported

and remains to be confirmed for mycelium using sterile cultures. It is interesting to note

here that the decomposition of AB in fish (Edmonds and Francesconi, 1988) and by

microorganisms (Hanaoka *et al.*, 1992b) produces TMAO, DMA and ultimately arsenate;

similar decomposition may account for some of the methylated species identified in mushroom extracts of this study.

5.5.5 Mushroom arsenic distribution

The tertiary mycelium that makes up fruiting bodies is differentiated for specialized functions within the mushroom (e.g. spore production). It is possible that arsenic speciation may vary between the areas of specialization. In order to investigate this, X-ray absorption near edge structure (XANES) spectra were collected for L and Yk treated cap (including gills), stalk centre, and stalk edge mushroom sections (Fig. 5.4). The spectra obtained indicated that one or more of the arsenic compounds present in the stalk centre was not found in the other sections (Fig. 5.4). This pentavalent arsenic compound was indicated by the peak feature at approximately 11876 eV which is known to correspond to arsenate and possibly DMA (Smith *et al.*, 2005); both compounds were found in mushroom extracts, and the latter was found in relatively high concentrations (Fig. 5.3). All spectra contained a peak feature around 11872.5 eV corresponding to the white line energy for a group of arsenic cations all containing four As-C bonds and including AC, AB and tetra (Smith *et al.*, 2005). The compound identified by XANES is undoubtedly AB as it was the only arsenical in this group found in extracts. By a similar comparison of HPLC-ICP-MS and XANES results, the shoulder on the AB feature, which is most prominent in the cap and stalk edge (Fig. 5.4), most likely corresponds to arsenite.

The biochemical processes involved in osmoregulation, which produce compounds used to increase solute concentration in cells, provide a possible pathway for the
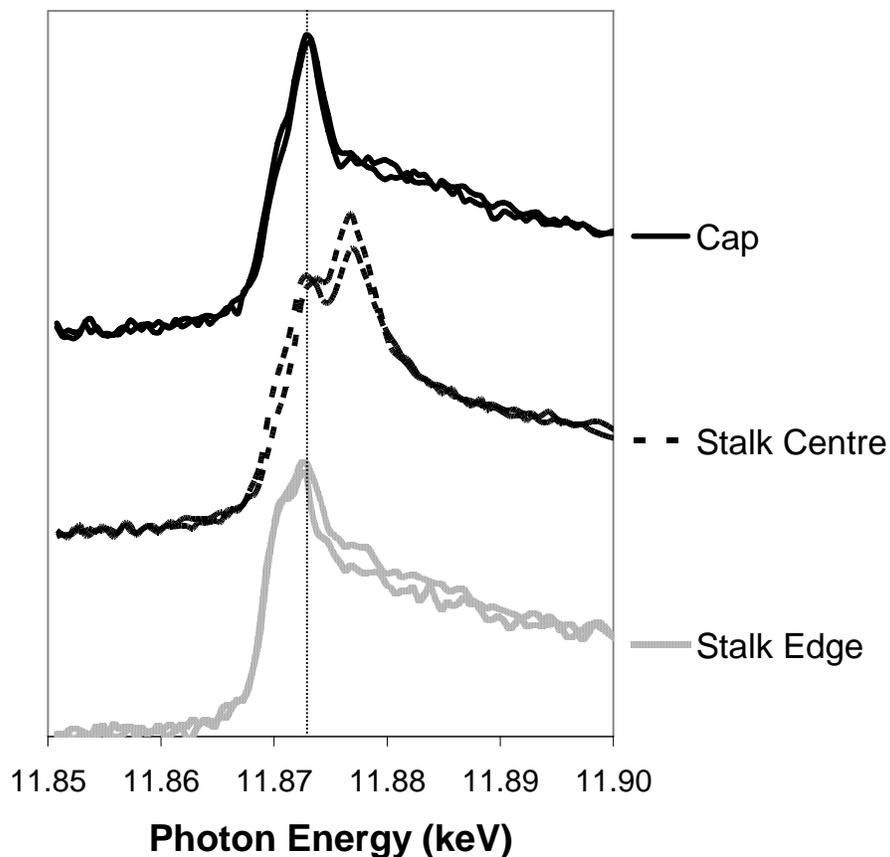
FIG. 5.4: XANES spectra for treated mushrooms indicating arsenic compounds in the cap, centre stalk and outer stalk. No distinction is made between XANES spectra collected for Yk and L-treated samples since they were similar. The AB white line energy (11872.6 eV vertical line shown) corresponds with one of the two main peak features. The shoulder on this feature is likely due to arsenite and corresponds well with its white line energy (11871.7 eV). The peak feature at approximately 11875.5 eV (stalk centre) indicates the presence of a pentavalent arsenic compound, possibly arsenate or DMA.

production of AB. In most halophytes (salt tolerant plants), as well as some algae and halophytic bacteria, a number of compounds are produced in response to osmotic stress, and these include glycine betaine and homobetaine (Bettencourt *et al.*, 1997). Water and nutrient translocation through the fruiting body are thought to be osmotically driven with greater quantities of solute accumulating in cap tissues and gills to drive the process (Beecher *et al.*, 2001). Total arsenic distribution in *Boletus sp.* was similarly dispersed in the fruiting body, with concentrations in the lamella (gills) twice that of the cap, which was in turn 2-3 times that of the stalk (Slekovec and Irgolic, 1996).

The XANES results from this study suggest that the form of arsenic that predominates in the cap and outer stalk is AB, which is hypothesized to play a role in maintaining turgor pressure. Higher turgor pressure is necessary in the cap for mechanical support of gills (location of spore formation), in the peel to reduce infection from penetration by pathogenic fungi and bacteria (Beecher *et al.*, 2001), and possibly in the outer stalk which elevates the cap for improved spore distribution.

5.6 CONCLUSION

Arsenic cycling in the marine environment has been extensively studied for many years, but comparatively little is known about the fate of arsenic in terrestrial ecosystems. An exception is found in the pathway for the production of AB by marine animals, which has yet to be elucidated, but appears dependent on the products of microbial arsenic transformations. The production of AB in the terrestrial environment remains virtually unstudied, in part because the compound is commonly found in low concentrations in

terrestrial organisms; however, this is not the case for terrestrial fungi. To gain more information on the origins of AB in terrestrial fungus, cultivated *A. bisporus* was grown.

It is expected that micro-organisms are in part responsible for simple methylated compounds identified in extracts of compost lacking fungus. However, AB was detected only in mushroom extracts suggesting the fungus itself produces this compound. There is evidence that mycelium can methylate inorganic arsenic and therefore it remains unclear whether the TMAO and DMA produced by micro-organisms were necessary precursors to fungal AB synthesis.

XANES analysis of a fruiting body indicated that AB was most prevalent in the cap and outer stalk of the mushroom. It is possible that AB is produced as one of the solutes which concentrated in the cap, and plays a role in nutrient transport (along a water gradient) and maintaining turgor pressure in the fruiting body which ensures reproductive success by maximizing spore distribution.

ACKNOWLEDGEMENTS

CHAPTER 6

An investigation of arsenic compounds in fur and feathers using X-ray absorption spectroscopy speciation and imaging

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6.1 ABSTRACT

The accumulation of arsenic in fur and feathers has been used as an indicator of environmental quality and animal health. However, there remain difficulties in distinguishing between arsenic present from external sources versus ingestion. In addition, low extraction efficiencies of conventional analytical techniques (e.g. high performance liquid chromatography inductively coupled plasma mass spectrometry, HPLC-ICP-MS) limit the complete characterization of arsenic compounds in these tissues. X-ray absorption spectroscopy (XAS) can be used to determine the speciation of arsenic compounds directly.

Inorganic arsenic is hypothesized to bind to thiol groups present in keratin-rich fur and feathers; however, arsenic-sulphur compounds have not been reported in extracts of these tissues. Here we report arsenic(III)-sulphur compounds comprising 5 – 58% of the total detected arsenic in rodent fur (vole, deer mouse, red squirrel) and bird feathers (grey jay, American tree sparrow, dark-eyed junco) as determined by X-ray absorption near

edge structure (XANES) analysis. Significant reduction of arsenic, applied to fur and feathers as arsenate solution or contaminated soil, was observed for both fur and feathers. XAS-imaging was used to localize dominant trivalent (AsIII) and pentavalent (AsV) arsenic compounds, and results were used to produce a "map" of arsenic in the sample. It is believed that the some of the reduced arsenic was internalized, while external pentavalent arsenic compounds associated with soil/dust particles were easily distinguished on goose feathers. However, distinguishing whether internal arsenic arose from exogenous (from the environment) or endogenous (from the body) sources proved difficult with this technique. XAS imaging combined with micro-XANES data highlighted the reducing potential of fur and feathers, and represent useful tools for determining the chemical speciation of arsenic in tissue samples.

6.2 INTRODUCTION

The chemical analysis of hair and feather samples from populations has been used as a means of monitoring excessive exposure of that population to a number of toxic substances (Burger and Gochfeld, 2001; Yáñez *et al.*, 2005), including arsenic. Some of the highest arsenic concentrations in an organism can be found in these tissues and the occurrence of this potentially harmful metalloid has been examined in human and bird populations (Burger and Gochfeld, 2001; Raab and Feldmann, 2005; Koch *et al.*, 2005, Kubota *et al.*, 2002).

Hair and feather tissues offer significant advantages for biomonitoring. They can be collected in relatively large quantities from a living organism, and sample collection is usually easy and atraumatic (Yáñez *et al.*, 2005; Hambidge, 1982). In addition, the ease

with which these materials can be stored and transported is attractive for survey and field studies (Yáñez *et al.*, 2005; Hambidge, 1982; Shraim *et al.*, 2001). However, there are still difficulties which confound analysis of these tissues (see review Hambidge, 1982) and these must be overcome for biomonitoring efforts to be of practical use.

One such problem is determining the origin of the arsenic: endogenous (from the body after ingestion), or exogenous (from the external environment). Common exogenous sources of arsenic include water and soil; however, arsenic from sweat, sebaceous secretions, and preengland exudates are often applied externally by animals to fur and feathers (Jasper *et al.*, 2004; Hindmarsh, 2002; Raab *et al.*, 2002) and despite their biological origins, arsenic contamination resulting from these secretions is often included with other exogenous sources. Exogenous contamination was considered an important source of arsenic for feathers from free-living, adult great tits, as indicated by significantly lower total arsenic concentrations in the least exposed tail feathers (Jasper *et al.*, 2004). However, this type of contamination is dependent on an animal's environment. For example, the primary feathers of juvenile Dunlins, formed while at a remote sub-arctic breeding ground, had only slight external contamination, implying few exogenous sources of arsenic at this location (Goede and de Bruin, 1985).

It is important to accurately distinguish exogenous and endogenous contamination in tissues, since arsenic is mainly of concern to animals when it is ingested. Animals metabolize ingested inorganic arsenic through reduction and oxidative methylation reactions (Yáñez *et al.*, 2005; Cullen and Reimer, 1989). The resulting arsenic compounds can circulate in the blood supply, and if not excreted from the body in urine or feces, they are often sequestered to sulphur-rich tissues like hair, nails and feathers

(Burger and Gochfeld, 2001; Raab and Feldmann, 2005, Jasper *et al.*, 2004). There is evidence that this deposition occurs quite rapidly in hair (Burger and Gochfeld, 2001; Curis *et al.*, 2005). The total concentration of arsenic alone does not give an accurate indication of arsenic toxicity because the toxic potential of arsenic is highly dependent on its chemical form ("species"). Arsenic species identified in extracts of hair and feather samples (Fig. 6.1) include high concentrations of inorganic arsenite and arsenate, and a small percentage of simple methylated compounds such as monomethylarsonic acid (MMA), and dimethylarsinic acid (DMA) (Yáñez *et al.*, 2005; Raab and Feldmann, 2005; Koch *et al.*, 2005). Gray jay feathers have been reported to contain the organoarsenical arsenobetaine (Koch *et al.*, 2005), which was also noted as a major arsenic compound in black-tailed gull tissues and eggs (Kubota *et al.*, 2002). It has been suggested that determining what arsenic compounds are present ("speciation analysis") in hair and feathers may provide a means of differentiating exogenous from endogenous arsenic (Yáñez *et al.*, 2005).

In the field of arsenic speciation analysis, high performance liquid chromatography inductively coupled plasma mass spectrometry (HPLC-ICP-MS) is one of the preferred analytical methods due to its high sensitivity. As with many conventional techniques, arsenic compounds must be extracted from samples prior to analysis. For hair and feathers this presents another analytical difficulty. Tissues are washed to remove external arsenic, but the technique used may not remove it all (Jasper *et al.*, 2004). Conversely, over washing may cause the leaching of some of the endogenous arsenic before the intended extraction (Jasper *et al.*, 2004; Goede and de Bruin, 1984). In addition all of the arsenic may not be removed from the tissue during extraction, leaving much of the
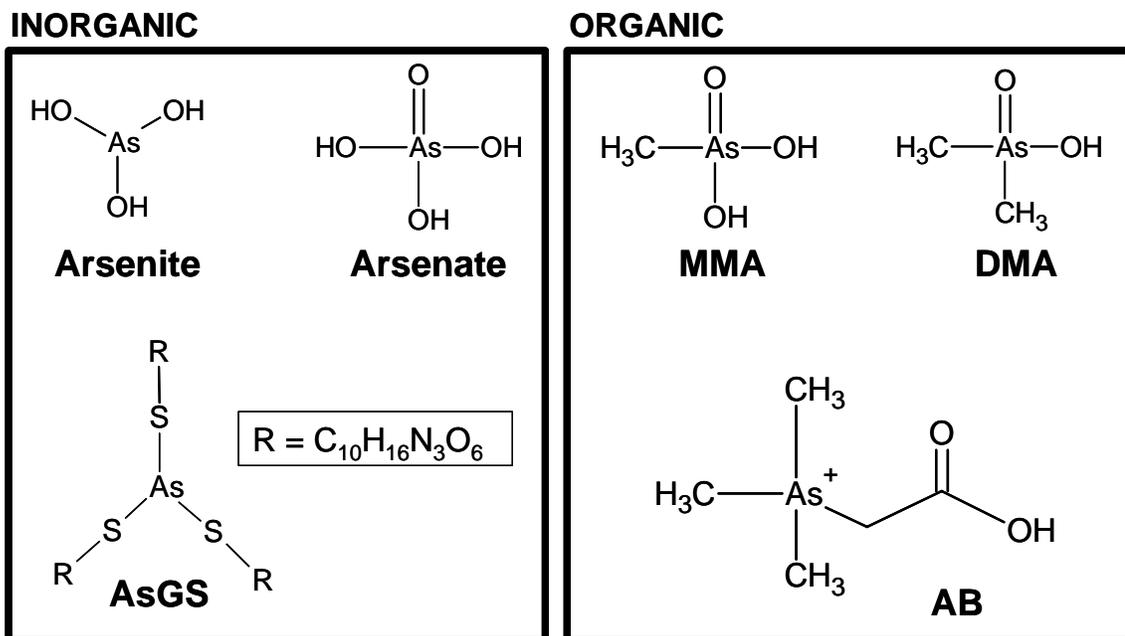
FIG. 6.1: Structure of arsenic compounds used as standards for XAS work, commonly found in extracts of hair and feather samples, with the exception of AsGS. MMA = monomethyarsonic acid: DMA = dimethylarsinic acid: AB = arsenobetaine: AsGS = arsenic(III)-glutathione.

arsenic in a solid residue and unidentified. Extraction efficiencies (EE) for hair (Yáñez *et al.*, 2005; Raab *et al.*, 2002; Mandal *et al.*, 2003) and feathers (Koch *et al.*, 2005) have been reported to be particularly low (e.g. 22% wool, ~60 % human, 8-33% feathers). X-ray absorption spectroscopy (XAS) provides a direct speciation method for studying arsenic compounds without the need for extraction. XAS imaging can be used to map the distribution of dominant pentavalent (AsV) and trivalent (AsIII) arsenic compounds within a sample. X-ray absorption near edge structure (XANES) data is used to further distinguish between arsenic compounds such as trivalent arsenite and arsenic(III)-sulphur species (Smith *et al.*, 2005). Together, HPLC-ICP-MS and XAS techniques can identify a broader range of arsenic compounds than either method individually, and provide valuable *in situ* speciation data.

A preliminary screening of samples identified a considerable amount of AsV in rodent fur and bird feathers from animals that had been living in areas with elevated soil arsenic concentrations ranging from 291 mg·kg^{-1} to 4220 mg·kg^{-1} (dry mass). This study used XAS techniques to determine the source of the AsV compounds: if they were localized externally to the samples in dust or soil particles, or arranged in a systematic fashion suggesting internalization. The presented results demonstrate the potential value of synchrotron methods in the determination of arsenic speciation in fur and feathers of animals exposed to arsenic.

6.3 MATERIALS AND METHODS

6.3.1 Chemicals and Standard Reference Materials

Distilled deionized water (ddH$_2$O) had a resistivity better than 17.5 MΩcm (E-pure Barnstead). A wide variety of standards were available for XANES analysis and these

were classified as either containing arsenic in the 5+ or 3+ oxidation states and are listed by *Smith et al.*(2005).

6.3.2 Sample Preparation

Animals were collected from Canadian sites that have naturally and anthropogenically elevated arsenic concentrations in soils. From Yellowknife, Northwest Territories, deer mice (*Peromyscus maniculatus*), red squirrel (*Tamiasciurus hudsonicus*), American tree sparrow (*Spizella arborea*), gray jay (*Perisoreus canadensis*), and dark eyed junco (*Junco hyemalis*) were obtained (Hough, 2001; Koch *et al.*, 2005). From Seal Harbour, Nova Scotia, voles (*Microtus sp.*) were collected from both background and arsenic contaminated sites (Saunders *et al.*, [in press]). Uncontaminated goose (*Anser sp.*) feathers were obtained from a farm in the Kingston area, Ontario. Animals were not sexed or aged, and it was not possible to determine the original location of the hair or feather on the body. The fur or feather was cut from the skin using sterile stainless steel scissors and washed thoroughly with ddH$_2$O. Before analysis, fur hairs and sections of feather, were sealed into Kapton™.

Vole fur and goose feathers containing arsenic concentrations below XAS detection limits were treated with dry arsenic contaminated soil (3200 mg·kg^{-1}) from Yellowknife (Yk) or a 1 µg·g^{-1} arsenate solution, as these represented forms of arsenic that birds and rodents might be in contact with in their environment. Hair and feathers were cut from the skin, and feathers were cut into approximately three additional pieces. Samples were then sealed into 50 mL centrifuge tubes with contaminated soil or arsenate solution, mixed vigorously by hand, then left at room temperature for 65 hours (fur) or 25 hours

(feather). The fur and feathers were then separated from the substrates by filtering and washed in deionized water. All fur and feathers were air dried after washing.

6.3.3 X-ray absorption spectroscopy

X-ray fluorescence microprobe scans were performed on the 20-ID beamline (Heald *et al.*, 1999) at APS, Argonne National Laboratory. X-rays were focused to 5 x 5 square micrometer spot size using Rh-coated Kirkpatrick-Baez mirror pairs. The monochromator was calibrated using the first inflection point of the gold L_{III} absorption edge (11919.7 eV (Kraft *et al.*, 1996)) for measurements at the arsenic K-edge (11868 eV). Fluorescence data were collected at ambient temperature with a Canberra 7-element solid state Ge-Li detector. A motorized stage moved the sample through the X-ray beam, and fluorescence data were collected at each 7.5 or 10 μm step (for coarser features or a larger image of the sample) with a 1 s integration time.

The difference in intensity of the pentavalent (AsV) and trivalent (AsIII) arsenic species was used to distinguish the dominant arsenic oxidation state, as a function of position. Qualitatively the higher signal corresponded to a higher arsenic concentration. Arsenic images were created using the SURFER8© (Golden Software) program. Micro-X-ray absorption near edge structure (XANES) spectra were collected for regions of interest identified in the microprobe images, as well as for bulk samples. Where experimental setup allowed, a reference gold foil was also measured in transmission during each sample scan. Typically three scans were collected and averaged before background-removal and normalization to edge jump. XANES spectra were processed using WinXAS software (Ressler, 1997) as discussed previously. Data were compared to

linear combinations of reference compounds, with the edge positions of the standards constrained to be within 0.5 eV of their initial values. The ratio of reference compounds that best resembled the data is reported as XANES results.

6.4 RESULTS AND DISCUSSION

6.4.1 Arsenic speciation in hair

Much of the speciation research in hair has been conducted on samples from humans, since there is interest in using such samples for epidemiological studies. In particular, extracts of the hair of individuals chronically exposed to inorganic arsenic, usually through drinking water, have been noted to contain predominantly inorganic arsenite, arsenate and minor amounts of DMA (Lin *et al.*, 1998; Yamato, 1988), with some reports of MMA (Yáñez *et al.*, 2005; Raab and Feldmann, 2005; Mandal *et al.*, 2003). In many cases inorganic arsenic accounts for over 90% of the extracted arsenic species, with more arsenite present than arsenate. Similar arsenic compounds were reported in the hair of victims of an acute-arsenic (poisoning) incident, but predominantly arsenate (> 70%) was identified (Shraim *et al.*, 2001). Arsenic extracted from human hair certified reference materials (CRM): CRM No. 13 (National Institute for Environmental Studies – Japan) contained mainly arsenate (66%), with the remaining arsenic present as arsenite, DMA and MMA in similar concentrations (Shraim *et al.*, 2001), and CRM GBW09101 also contained mainly arsenate (47%) with similar concentrations (~ 25%) of arsenite and DMA (Raab *et al.*, 2002). It is interesting to note that in studies using an alkaline digestion technique (Lin *et al.*, 1998; Yamato, 1988) no MMA was reported, while those using water extraction methods (Yáñez *et al.*, 2005; Raab and Feldmann, 2005; Shraim *et*

al., 2001; Mandal *et al.*, 2003) reported its presence, with the exception of CRM GBWO9101 which was extracted with water but in which no MMA was detected.

Less work has been done on arsenic speciation in animal hair. Wool collected from sheep who ingested arsenic mainly as mainly arsenosugars (from seaweed), contained chiefly DMA with minor species of arsenite, arsenate and MMA (Raab *et al.*, 2002). The simple methylated compounds (MMA, DMA) were present in both the pentavalent and trivalent forms. Hamsters given a single dose of DMA via a stomach tube, contained inorganic arsenic (arsenite and arsenate which could not be differentiated) and DMA, using an alkaline digestion technique (Yamauchi and Yamamura, 1984).

In the study reported here, hairs were obtained from field mice and voles that had been collected in areas with arsenic soil concentrations ranging from 291 to 4220 mg·kg^{-1} (Hough, 2001; Saunders *et al.*, [in press]). XANES spectra were collected for samples of fur (tufts) and the results are presented in Table 6.1. Each entry represents individual samples from different animals. The field mice were collected from four different Yellowknife study areas with a range of soil arsenic concentrations, while the voles were collected from the same Seal Harbour study area but were caught in two different traps, and the squirrels were collected from the same Yellowknife study area. Inorganic arsenic was identified in all samples, and accounted for 70% to 100% of the detectable species. MMA was also identified in samples of both mouse and vole fur. DMA was not reported in rodent hairs; however, DMA and arsenate are difficult to distinguish using XANES data alone (Smith *et al.*, 2005). With the exception of the sheep wool, DMA generally accounts for a very small percentage (< 4%) of extracted arsenic species (Yáñez *et al.*, 2005; Mandal *et al.*, 2003) and if the distribution of extracted compounds represents the

TABLE 6.1: Percent arsenic species determined by XANES (this study) and HPLC-ICP-MS (Koch *et al.*, 2005) methods of analyzing fur and feathers collected from animals living in areas with elevated arsenic concentration in the soil. HPLC-ICP-MS values are based on the concentration of each species extracted with respect to the sum of those species (not with respect to total arsenic). Each sample is taken from a different animal, with the exception of the micro-XANES which represent two different hairs (first two results are from the same hair) of the same animal. *Spizella arborea* (American Tree Sparrow); *Perisoreus Canadensis* (Gray Jay); *Junco hyemalis* (Dark Eyed Junco); Where no value is reported, levels were not within detection limits for the method used.

SPECIES	METHOD	% ARSENIC SPECIES				
		Arsenate	As(III)S	Arsenite	MMA	DMA
MICE *Peromyscus maniculatus*	XANES	46	14	41	-	-
		78	5	17	-	-
		61	20	19	-	-
		70	18	-	10	-
VOLE *Microtus sp.*	XANES	26	49	21	4	-
		38	27	28	8	-
		49	23	9	18	-
		72	-	28	-	-
SQUIRREL *Sciurus vulgaris*	XANES	44	36	20	-	-
		25	58	15	3	-
	Micro-XANES	7	75	17	-	-
		7	64	20	8	-
		7	75	18	-	-
BIRDS *Spizella arborea*	HPLC[a,b]	55	-	21	17	9
	XANES	71	18	9	-	-
Perisoreus canadensis	HPLC[c]	31	-	23	20	16
	XANES	12	52	11	24	-
Junco hyemalis	XANES	29	8	54	-	-

[a]These results were obtained from Koch *et al.* (2005)

[b]extraction efficiency = 17% (Koch *et al.*, 2005)

[c]extraction efficiency = 7.8% (Koch *et al.*, 2005), AB (1%) and TMAO (6%) detected in this sample by HPLC-ICP-MS

in situ distribution, DMA is unlikely to be detectable by XANES at such low concentrations.

Of particular interest is the presence of an arsenic(III)-sulphur compound in virtually all mouse and vole samples, identified by XANES. The arsenic(III)-sulphur finding agrees with the commonly held belief that arsenic compounds deposited in, or absorbed by the hair, can bind to the thiol groups of proteins such as keratin. To the best of our knowledge there has been no report in the reviewed literature identifying an arsenic(III)-sulphur compound in hair extracts, using alternate speciation methods.

6.4.2 Arsenic speciation in feathers

Most of the arsenic literature on birds focuses on those living in the marine environment and report total concentrations (Burger and Gochfeld, 2001; Jasper *et al.*, 2004; Goede and de Bruin, 1985), with few studies taking into account arsenic speciation (Kubota *et al.*, 2002). The work of Koch *et al.* (2005) described the arsenic speciation in birds living in a primarily terrestrial environment, with soil arsenic concentrations ranging from 291 mg·kg^{-1} to 4220 mg·kg^{-1} (dry mass). From that study (Koch *et al.*, 2005), the total arsenic concentration in tissues of grey jay, American tree sparrow and dark eyed junco birds were reported to be elevated when compared to reported values in other terrestrial birds collected from other contaminated sites. Koch *et al.* (2005) reported that arsenic speciation analysis (HPLC-ICP-MS) of feather extracts from these birds contained arsenite, arsenate, MMA, DMA and TMAO, with an unexpected finding of AB in gray jay feathers (Table 6.1). AB was one of the main arsenic compounds in gray jay breast tissue (~ 80%); previously it has only been reported in tissues of marine birds such

as the great black-backed gull and black guillemot (Mace, 2003). Only a small percentage of the arsenic could be extracted from the feathers of the Yellowknife birds (gray jay: 7%, American tree sparrow: 17%, dark eyed junco: 30%), indicating that a large portion of the arsenic remained unidentified (Koch *et al.*, 2005).

This study continued the investigation of arsenic speciation in American tree sparrow, grey jay and dark eyed junco feathers using XANES analysis to examine a bulk sample of feathers. XANES results identified arsenate, arsenite, and a hitherto unidentified arsenic(III)-sulphur compound, in feathers of these birds, while MMA was only identified in gray jay feathers (Table 6.1).

6.4.3 Comparison of speciation techniques

HPLC-ICP-MS data was available only for bird feathers and thus a comparison of arsenic compounds detected by both techniques was carried out for these samples. The ratio of reference compounds that best resembled the XANES data was generally different from the HPLC-ICP-MS results obtained by Koch *et al.* (2005) (**Table 6.1**). However, conventional speciation results reported here are as the arsenic compounds extracted as a percentage of the sum of those species, and may not reflect the actual compound distribution in the original feather which was analyzed by XANES. MMA was surprisingly not found in American tree sparrow feathers by XANES, despite the fact that 17% of the extractable arsenic species detected by HPLC-ICP-MS was in this form (Table 6.1). Difficulties extracting arsenic complicates the comparison of speciation results from these different techniques. Low extraction efficiencies for arsenite in hair have been theorized to result from the presence of arsenic(III)-sulphur compounds which

were assumed to be difficult to extract (Raab and Feldmann, 2005). This may also apply to the extraction of grey jay feathers where a large quantity of arsenic(III)-sulphur (52%) was detected by XANES.

6.4.4 Rationale for presence of arsenic(III)-sulphur compounds

Arsenic species have an affinity for thiol groups, and both fur and feathers contain sulphur-rich proteins (Jones, 2001; Murphy *et al.*, 1990). Feathers contain at least a quarter of the total protein mass of the bird, and it is largely in the form of sulphur rich beta-keratin (Murphy *et al.*, 1990). Alpha-keratin is a main constituent of the hair cortex (Fig. 6.2 diagram), and contains cysteine residues, which form disulfide bridges and give the hair its abrasion resistance (toughness). While unlikely that arsenic compounds would break the existing sulphur-bridges in keratin proteins (Raab and Feldmann, 2005), they may bind any unoccupied cysteine residues. In hair, the cortex also contains an unorganized, amorphous protein matrix of high sulphur content, which could more readily bind the arsenic (Jones, 2001).

The hair cortex sometimes surrounds a central region of cells called the medulla. The medulla is considered the final destination of foreign debris and metals. In animal hair this region is well defined, continuous, and occupies greater than 1/3 of the overall diameter of the hair (Jones, 2001; Deedrick and Koch, 2004). In human hair the medulla is not always well developed, and when present, it is generally less than 1/3 of the overall hair diameter (Jones, 2001; Deedrick and Koch, 2004).

Squirrel hairs were obtained from animals collected in an area with an average soil arsenic concentration (dry mass) of 433 mg·kg^{-1} (Hough, 2001). The distribution of
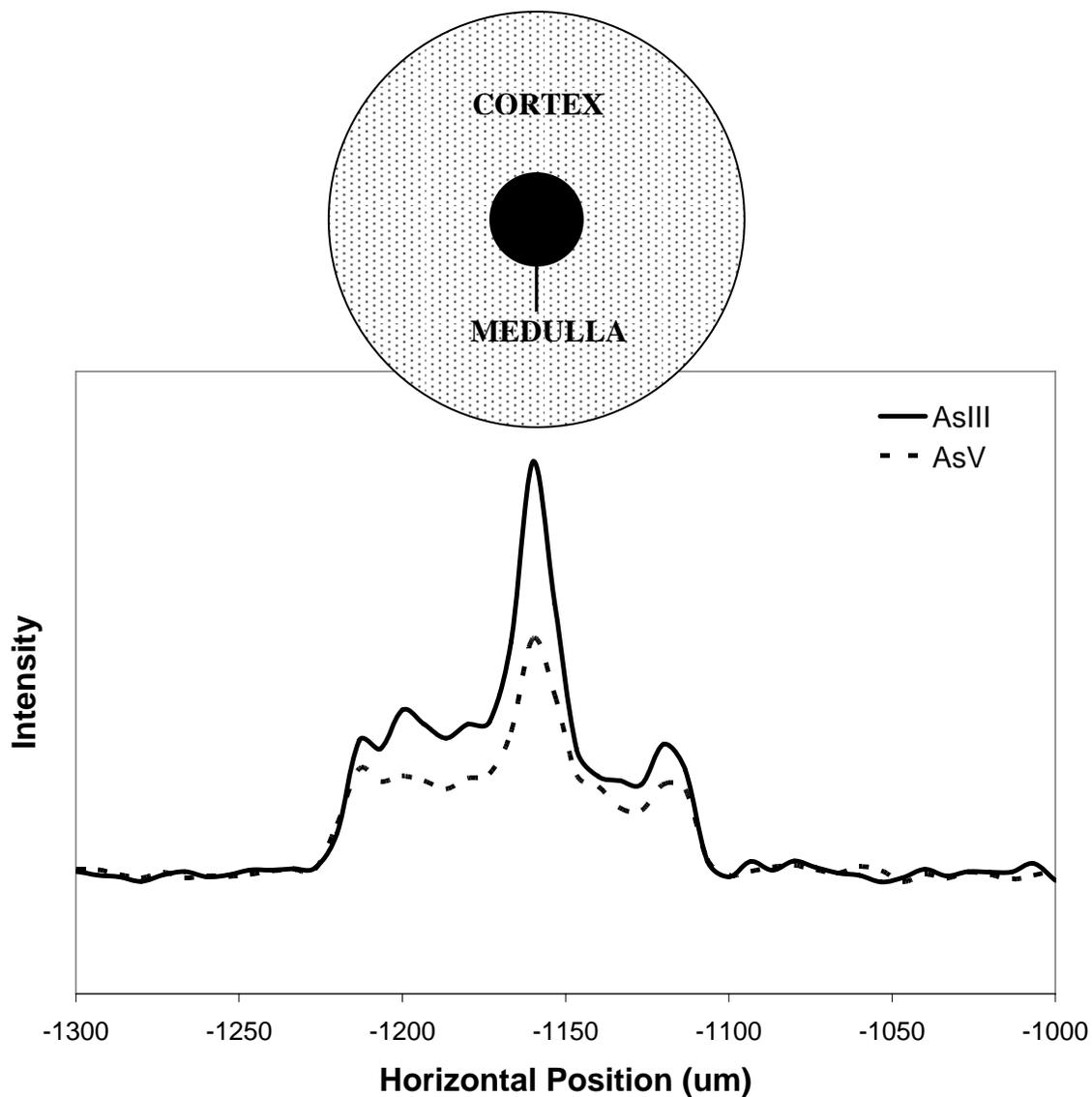
FIG. 6.2: Horizontal scans of red squirrel fur (beam perpendicular to sample), at 11.871 keV (AsIII) and 11.876 keV (AsV). Diagram of fur cross-section indicates that arsenic maximum may correspond to hair medulla.

arsenic in squirrel fur was examined using XAS imaging to scan across a hair. This technique identifies the dominant trivalent (e.g. arsenite, arsenic(III)-sulphur) and pentavalent (e.g. arsenate, DMA, MMA) compounds. Points of interest were further examined with micro-XANES analysis which can distinguish a greater number of compounds such as arsenite and arsenic(III)-sulphur species (Smith *et al.*, 2005). Arsenic appeared to be sequestered in the medulla of squirrel hair (Fig. 6.2 diagram), as indicated by a sharp peak in the horizontal scan (Fig. 6.2). Micro-XANES analyses of the centre of two squirrel hairs identified predominantly an arsenic(III)-sulphur compound with less than 30% of the arsenic present in other forms (Table 6.1). In contrast, bulk XANES scans indicated less arsenic(III)-sulphur in squirrel hairs; however, Micro-XANES are collected on a smaller sample area, whereas bulk XANES are collected on several hairs at once. Since arsenic concentrations along the hair shaft can be inconsistent (Mandal *et al.*, 2003) these differences may be sufficient to account for the variability in results from the two types of XANES scans (Hambidge, 1982).

In contrast to the squirrel results, sequestration of trivalent arsenic to the hair cortex was observed in people treated with arsenic trioxide, as determined by micro-XANES and XAS imaging (Curis *et al.*, 2005). Results from proton induced X-ray emission analysis of hair from patients taking Fowler's solution also suggest sequestration of arsenic to the cortex (Hindmarsh, 2002). Since nothing is known of the type of medulla in the human hairs of these studies (Hindmarsh, 2002; Curis *et al.*, 2005) it may be that this morphological region was absent or incomplete.

6.4.5 Distinguishing between external and internal arsenic

A first step in distinguishing between endogenous and exogenous arsenic is the determination of arsenic contamination on the exterior of the tissue. Goose feathers, naturally containing arsenic levels below ID beamline detection limits, were exposed to arsenic contaminated soil. Soil was not observed on the feathers after rinsing, but Weyers *et al.* (1988) demonstrated through electromicroscopic photographs that particles remained on the surface of blackbird feathers, even when they were washed vigorously. This was also observed in XAS images, where pentavalent arsenic present in soil/dust particles clinging to the outside of the feather was easily distinguishable (Fig. 6.3 f). The pentavalent arsenic species on the barbs did not appear to be localized to dust or soil particulates, but the pattern of distribution was difficult to ascertain due to low concentrations (Fig. 6.3 f).

6.4.6 Distinguishing between exogenous and endogenous sources

The pattern of arsenic distribution observed in the soil treated goose feather (Fig. 6.3 f) was also observed for the feather of an American tree sparrow collected from a bird that had been living in an area of elevated arsenic (Fig. 6.3 i). In the case of the American tree sparrow some of the arsenic observed in the XAS image may have originally been ingested by the bird; whereas all of the detectable arsenic in the goose feather can be attributed to exogenous sources. The distribution of AsIII and AsV in feathers does not appear to be influenced by the source of the arsenic (endogenous: American tree sparrow; exogenous: goose) for the feathers studied. However, the cut ends of the goose feather

	AsIII Scan	AsV Scan	AsV - AsIII
Goose	a	b	c
Goose	d	e	f
American Tree Sparrow	g	h	i

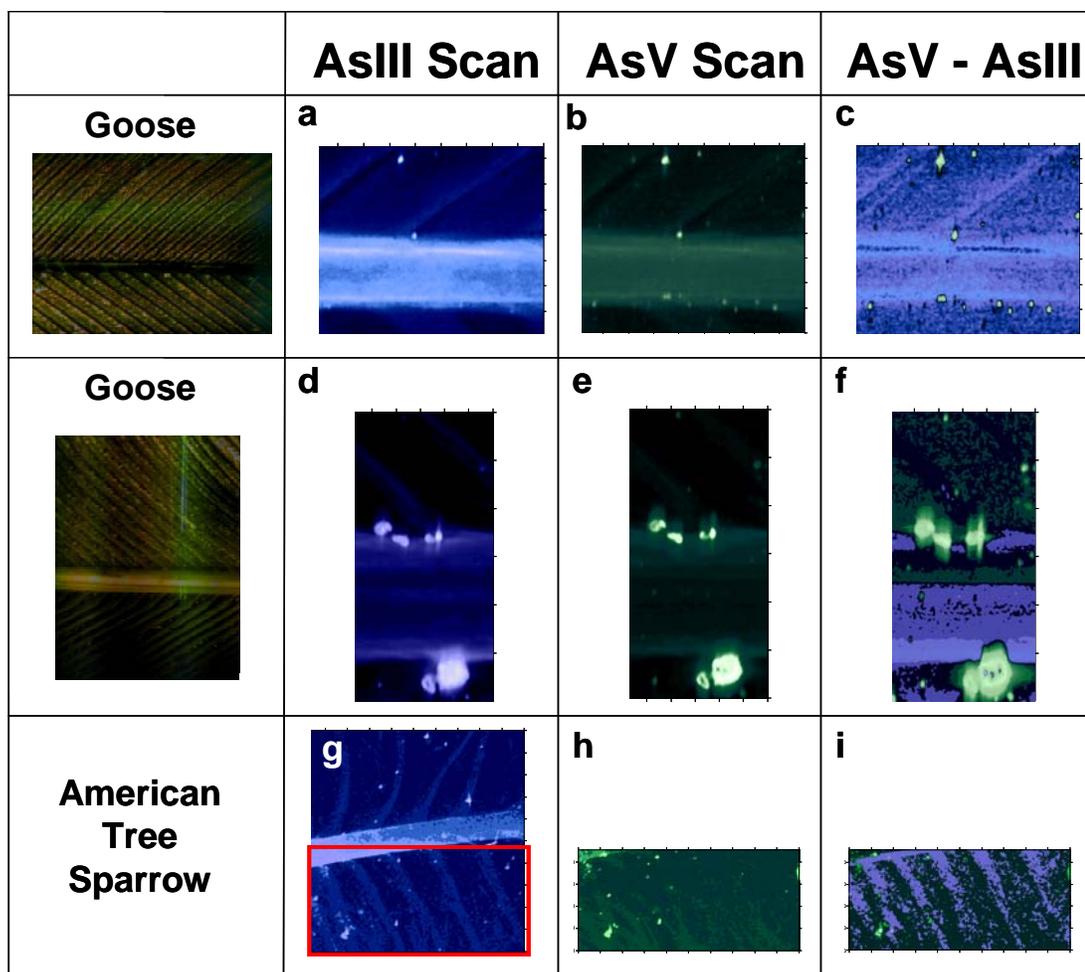**FIG. 6.3:** Goose feathers treated with an arsenate solution (c) or arsenic contaminated soil (f). American tree sparrow feather collected from a bird living in Yellowknife (i). Lighter blue indicates higher levels of AsIII (a, d, g), and lighter green indicates higher levels of AsV (b, e, h). For AsV-AsIII, black indicates areas where AsV = AsIII (c, f, i). External arsenic associated with soil particles contained AsV (f, i).

were exposed to the arsenic treatment and it is not known how this would effect the resulting arsenic distribution.

It is apparent that goose feathers treated with either arsenic solution or contaminated soil reduced the arsenate in these treatments. The XAS images clearly depict trivalent arsenic in the area of the shaft (Fig. 6.3 c,f). Furthermore, the micro-XANES spectra collected suggest the peak feature for the AsIII compound observed in the shaft (Fig. 6.4) best resembles the peak feature for an arsenic(III)-glutathione standard solution. Based on micro-XANES results there appears to be a difference in the predominant arsenic compound of the shaft (AsIII) compared to the barbs (AsV) for both treatments (Fig. 6.4). The reason for this difference is unclear, but may point to a greater reducing capability of the shaft. Alternatively if AsIII compounds are mainly located internally such compounds would dominate in a scan of the thicker shaft. However, for the second theory to be plausible the arsenic must have been internalized.

The feather shaft is hollow, and if arsenic were located inside the shaft, a scan at the centre of the shaft would pass through the least amount of material resulting in a lower signal. This was observed for the middle third of the shaft in the AsIII scans (Fig. 6.3 a,d), and most of the centre of the shaft in the AsV scans (Fig. 6.3 b,e). This implies that the exogenous arsenic was internalized, mainly in the AsIII form, and that most of the AsV may be found on or near to the exterior of the feather. However, even if this were confirmed it is unlikely that speciation analysis alone would be useful in identifying exogenous arsenic since reduction of the applied arsenical changes its chemical form.

The apparent reducing ability of the feathers is similar to that noted in arsenic absorption studies on hair (Raab and Feldmann, 2005; Raab et al., 2002; Mandal et al.,

FIG. 6.4: Micro-XANES spectra for goose feathers treated with arsenic contaminated soil (SOIL) and an arsenate solution (SOLUTION). Reduction of arsenate to a trivalent form, possibly an arsenic(III)-sulphur compound, is illustrated by the solution spectra. Approximate white line positions for various arsenic reference solutions are indicated by the vertical lines: solid = arsenic(III)-glutathione (11.8700 keV), dashed = arsenite (11.8717 keV), dashed and dotted = arsenate (11.8753 keV). All scans in a treatment are of the same feather. Soil 1, 2 = spots by shaft. Soil 3 = middle of shaft. Solution 1 = on barbs. Solution 2 = on shaft.

2003). In one study, 78% of the arsenic identified in the extract of hair exposed to an arsenate solution was in the form of arsenite (Raab and Feldmann, 2005). It is presumed that the treated tissues either released redox active compounds, or reduced arsenate to trivalent forms themselves. A similar phenomenon was observed for this study in vole hair treated with both arsenic solution and soil (Fig. 6.5). The reduction is much more pronounced for hairs exposed to arsenate solution, perhaps because the arsenic in this treatment was more accessible to the hair. The position of the AsIII peak feature is similar to that of an arsenic(III)-glutathione standard solution suggesting an arsenic-sulphur compound. The cuticle layer that surrounds a hair contains both a lipid and protein component and is rich in cystine (Jones, 2001). It is possible that cysteine residues not already part of a disulfide bond play a role in the reduction of AsV at the surface of the hair. Analysis of wool demonstrated that while speciation may not be predictable due to the observed reduction, the degree of methylation for MMA and DMA was not altered after incubation with solutions of these arsenicals (Raab *et al.*, 2002).

In addition to arsenic reducing potential, hair has been reported to absorb arsenic compounds, though the overall absorption was low (Raab *et al.*, 2002; Mandal *et al.*, 2003). This suggests that some part of exogenous arsenic sources may be absorbed by this tissue. Inorganic AsIII compounds were absorbed better than similar AsV compounds (Raab and Feldmann, 2005). The cuticle and associated intercellular material are thought to perform key roles in water and reagent penetration into the hair (Jones, 2001). Therefore, either before or after surface reduction, it is possible that arsenic compounds are transported into the interior of the hair. Once internalized, exogenous AsIII compounds may bind to the sulphur rich proteins of the cortex or potentially be
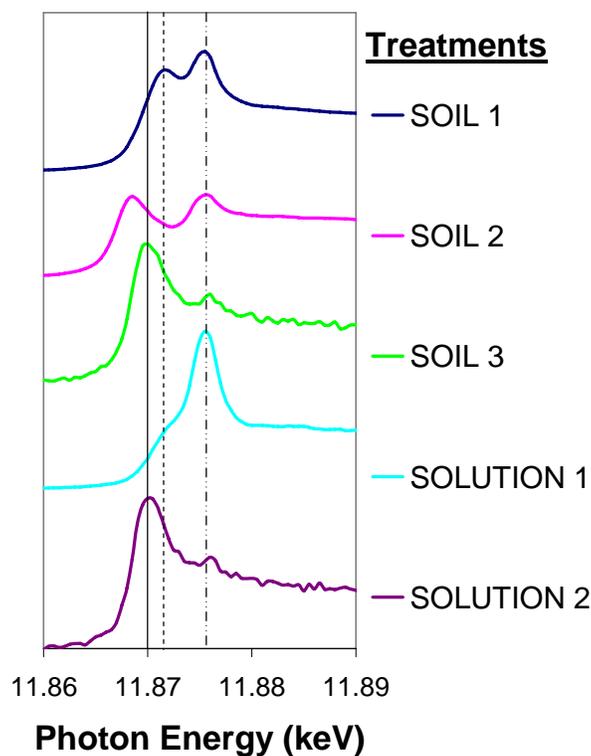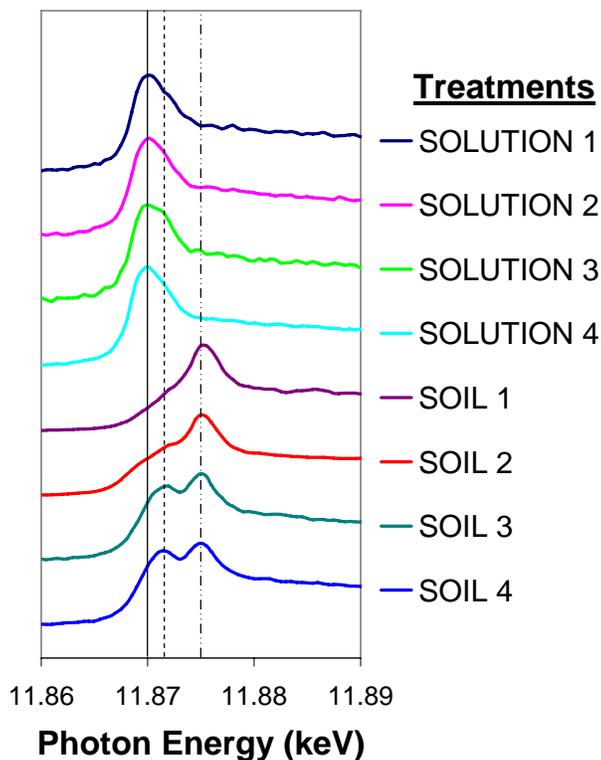
FIG. 6.5: Micro-XANES spectra for vole fur treated with arsenic contaminated soil and an arsenate solution. Reduction of arsenate to an AsIII compound is evident in the solution spectra. Approximate white line positions for various arsenic reference solutions are indicated by the vertical lines: solid = arsenic(III)-glutathione (11.8700 keV), dashed = arsenite (11.8717 keV), dashed and dotted = arsenate (11.8753 keV). All scans in a treatment are of the same hair. Numbers 1 – 4 indicate different locations where micro-XANES scans were taken.

transported to the medulla. However intercellular connections, which constitute a key means of reagent penetration, are not always observed between the cortex and the medulla (Jones, 2001). It is presumed that endogenous sources of arsenic also bind to sulphur-rich compounds in these areas. Consequently, it may be impossible to differentiate arsenic compounds from exogenous and endogenous sources, except perhaps in cases of extremely elevated arsenic levels, or where complementary tests can be performed. This was illustrated by the investigation of hair from five gold-smelter workers (Hindmarsh, 2002). Arsenic was reported inside the hair for some of these workers at levels high enough to have been lethal. Exogenous contamination of the hair by arsenic dust in the work environment was assumed to account for the majority of this arsenic, particularly since urine arsenic monitoring ensured no toxic amounts of arsenic were being ingested (Hindmarsh, 2002).

6.5 CONCLUSION

Endogenous arsenic in fur and feathers has been used in the assessment of the health of populations. There is therefore a need to distinguish between endogenous arsenic, which greatly impacts animal health, and exogenous arsenic resulting from external contamination. The use of XAS analysis allowed us to circumvent issues surrounding poor extraction efficiency of hair and feathers, and has provided evidence of arsenic(III)-sulphur compounds in these tissues. Such compounds have been anticipated in the literature due to the sulphur-rich proteins present in both hair and feathers. Using XAS imaging, external pentavalent arsenic, associated with soil/dust particles adhered to the tissues, was easily identified, and could be eliminated as endogenous in origin. The origin

of AsV observed on the barbs of goose feathers, however, was less clear. Both XAS imaging and micro-XANES results highlighted the considerable reducing potential of both fur and feathers, and there is some indication that the reduced arsenic can be internalized in feathers. For this reason it is unlikely that endogenous and exogenous arsenic can be distinguished through speciation analysis alone.

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CHAPTER 7 - General Discussion

Arsenic is present in many different compounds which can be altered as they are cycled through the environment. This is significant because different arsenic compounds exhibit different levels of toxicity, and public concern about exposure to arsenic in food and drinking water has helped heighten interest in arsenic research. Organisms themselves are an important part of arsenic cycling in the environment and biotransformations produce the main source of organic arsenic compounds; however, much is still to be learned about how arsenic is metabolized particularly in the terrestrial environment. In order to study arsenic biotransformations it is necessary to identify arsenic compounds ("speciation analysis") and their distribution in an organism. The overall objective of this thesis was to gain a better understanding of the fate of arsenic taken up by terrestrial plants, animals and fungi. Two analytical tools were available to accomplish this goal: high performance liquid chromatography inductively coupled plasma mass spectrometry (HPLC-ICP-MS) which has been established for decades as a versatile speciation tool, and X-ray absorption spectroscopy (XAS) the potential of which has only recently been recognized.

For the most part, inorganic arsenate and arsenite are reported in terrestrial plants investigated from arsenic contaminated field sites (Meharg and Hartley-Whitaker, 2002). A positive correlation between increasing arsenic concentrations and detectable thiol groups has been observed in some plant extracts (Tu *et al.*, 2004) and further characterization of the thiols determined that arsenic induced production of metal/metalloid-binding peptides known as phytochelatins (PCs) whose complexation with arsenic has been confirmed *in vitro* (Schmömger *et al.*, 2000; Raab *et al.*, 2004b).

Here we report the majority of arsenic in living radish plants was bound to sulphur (Chapter 4) and is most likely evidence of arsenic(III)-PC complex formation. Similar direct analysis has identified arsenic(III)-sulphur compounds in two other plant species: arsenic tolerant Indian mustard (Pickering *et al.*, 2000) and the hyperaccumulating Chinese break fern (Webb *et al.*, 2003). In radishes arsenic(III)-sulphur was the dominant arsenic compound in most plant tissues including the vascular phloem; however, an AsV compound thought to be arsenate was chiefly found in the xylem. This is the first *in planta* report of arsenic segregation in radish vasculature. We are aware of one other study for the Chinese break fern in which freshly harvested sections were analyzed directly and arsenate was identified in plant vasculature (Pickering *et al.*, 2006).

Unlike terrestrial plants, fungi contain a variety of organic arsenic compounds and represent some of the few terrestrial species to contain a predominance of arsenobetaine (AB). The origins of AB remain a mystery despite its prevalence in the marine environment where microbial activities are thought to play an important role in its formation (Ritchie *et al.*, 2004; Hanaoka *et al.*, 1992). The presence of AB in both terrestrial and marine organisms may suggest a common synthetic pathway for this compound. It is widely speculated that it is the fungus which produces AB, in part because it has not been found in the growth substrate of mushrooms containing it. However, AB was not reported as one of the final arsenic compounds produced by *Agaricus placomyces* mycelium grown on agar plates (Šlejkovec *et al.*, 1996).

Extracts of fruiting bodies of cultivated *Agaricus bisporus* (white button mushrooms) grown in this study contained appreciable amounts of AB, and we suggest this compound has a possible role in osmoregulation. It is hypothesized that microbes were able to

convert some of the arsenate in the compost to arsenite, dimethylarsinic acid (DMA) and trimethylarsine oxide (TMAO) which were then available for uptake by the *Agaricus* mycelium. Once inside of the fungus further biotransformations resulted in the formation of AB and these results provide some of the best evidence that the AB detected in extracts of *Agaricus bisporus* is a result of arsenic biotransformation by this organism and not accumulation from the environment. Direct arsenic speciation was carried out for different sections of the mushroom using XAS and AB was found to accumulate in the cap and outer stalk of fruiting bodies. AB along with other solutes may be used by the fungus to create an osmotic gradient which directs nutrient flow to the gills, and maintains turgor pressure required for mechanical support.

In animals, much of the arsenic ingested is excreted in urine and feces, or stored as part of the epidermal tissue (hair, nails, feathers). As a result, epidermal tissues provide a useful record of arsenic exposure and the total arsenic concentration as well as speciation data can be used in biomonitoring efforts. Mainly inorganic arsenate and arsenite with lower concentrations of DMA and MMA have been reported in these tissues; however, difficulty extracting arsenic from such samples means that the majority of the compounds remain unidentified. It has been hypothesized that arsenic is difficult to extract from hair and feathers because it can bind to keratin which is a major constituent of these tissues; however, to the best of our knowledge there have been no reports in the reviewed literature identifying the anticipated arsenic-sulphur compounds.

In this study, arsenic(III)-sulphur compounds were detected in fur and feather samples from animals that had been living in areas of elevated arsenic. This finding supports the theory that arsenic compounds deposited in, or absorbed by the hair, can

bind to the thiol groups of proteins. It is possible that sulphur-rich surface proteins may also be responsible for the reduction of AsV compounds by epidermal tissues which has been observed in this and other studies. However, this reduction phenomenon makes it is highly unlikely that endogenous (from the body) and exogenous (from the environment) arsenic can be distinguished through speciation analysis alone.

There are obvious advantages to combining results from direct arsenic speciation analysis with more conventional techniques for the investigation of arsenic biotransformations. The improved ability to detect arsenic-sulphur compounds, which appear to play important roles in various terrestrial organisms, has been highlighted by this work. The ability to perform *in situ* speciation analysis was used in these studies to localize arsenic compounds in tissues and thus provide information on where arsenic is transformed and how it is transported leading to speculations about the role of such compounds within the organism. In addition, a comparison of speciation results from *in situ* studies with those from extraction work provides an opportunity to reexamine the origins of extracted arsenic by considering the possible effects of sample preparation on arsenic speciation. These analytical methods have also been applied to the investigation of the interaction between arsenic and different surfaces and the multi-element nature of both conventional and XAS techniques may provide opportunities to gain information on interaction between arsenic and other compounds of interest.

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APPENDIX A

Data in this appendix is supporting information that was submitted with X-ray absorption near-edge structure analysis of arsenic species for application to biological environmental samples (Chapter 3) and is available online.

TABLE 1: Summary of arsenic species detected in marine organisms.

MARINE ORGANISM	DOMINANT ARSENIC SPECIES	MINOR ARSENIC SPECIES	TRACE ARSENIC SPECIES	REFERENCE
Algae	Arsenosugars	Arsenate	MMA, DMA	*1,2,3,4*
Bivalves	AB	Tetra, arsenosugars	MMA, DMA	*1,2,5*
Sponges	AB, arsenosugar	*	Arsenite, Arsenate, MMA, DMA	*6*
Sea Cucumber	AB	Tetra	*	*7*
Worms (polychaetes)	Tetra, AB	*	TMAO, AC, arsenosugars	*8*
Fish	AB	TMAO	MMA, arsenosugars	*2,9,10*
Mammals	AB	Tetra	Arsenite, Arsenate, DMA, TMAO, AC	*11,12,13,14*
Birds	AB	MMA, DMA	AC	*15*

AB = arsenobetaine; AC = arsenocholine; MMA = monomethylarsonic acid; DMA = dimethylarsinic acid; Tetra = tetramethyl arsonium; TMAO = trimethylarsine oxide
Major arsenic species ≥ 20% of the total water-soluble arsenic; Minor <20% and ≥1%; Trace < 1% (*2*)

TABLE 2: Summary of arsenic species detected in terrestrial and freshwater organisms.

TERRESTRIAL/FRESH-WATER ORGANISMS	DOMINANT ARSENIC SPECIES	MINOR ARSENIC SPECIES	TRACE ARSENIC SPECIES	REFERENCE
Plants	Arsenite, Arsenate	MMA, DMA, TMAO	Tetra, AB, AC, arsenosugars	*16,17,18,19, 20*
Fungi	AB, DMA, AC	Arsenite, Arsenate	MMA, Tetra, arsenosugars	*21,22*
Mammals	DMA	Arsenite, Arsenate, MMA	TMAO, AB	*23,24,25*
Algae	Arsenite, Arsenate	DMA, arsenosugars	TMAO	*26,27,28*
Invertebrates	TMAO, Tetra, arsenosugars, AB	Arsenite, Arsenate	MMA, DMA, AB	*17,29*
Fish	AB	Arsenite, Arsenate	MMA, DMA, TMAO, arsenosugars	*17,30,31*

AB = arsenobetaine; AC = arsenocholine; MMA = monomethylarsonic acid; DMA = dimethylarsinic acid; Tetra = tetramethyl arsonium; TMAO = trimethylarsine oxide
Major arsenic species ≥ 20% of the total water-soluble arsenic; Minor <20% and ≥1%; Trace < 1% (*2*)

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