ELUCIDATING A BIOSYNTHETIC PATHWAY FOR ARSENOBETAINE IN HIGHER TERRESTRIAL FUNGI

À LA QUÊTE D'UNE VOIE BIOSYNTHÉTIQUE DE L'ARSÉNOBÉTAÏNE DANS LES CHAMPIGNONS SUPÉRIEURS TERRESTRES

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by

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Abstract

Nearing, Michelle M. Ph.D. Royal Military College of Canada, January, 2015. Elucidating a Biosynthetic Pathway for Arsenobetaine in Higher Terrestrial Fungi. Supervised by Dr. Kenneth J. Reimer and Dr. Iris Koch.

The toxicity of arsenic is greatly dependent on its chemical form and only one arsenic compound is considered to be non-toxic, arsenobetaine (AB). The formation pathway for AB is still unknown despite being found in high concentrations and proportions in many marine organisms. AB is found in fewer organisms and at lower concentrations and proportions in the terrestrial environment with the exception of the fruiting bodies (FBs), or mushrooms, of terrestrial fungi. The present study interrogated AB formation pathways in the complete fungal organism (fungus and associated microbes) through a combination of fruiting body and substrate analysis, and culture experiments. The analytical methods used were reviewed and selected to include complementary high performance liquid chromatography – inductively coupled plasma mass spectrometry (HPLC-ICP- MS) and X-ray absorption spectroscopy (XAS), specifically x-ray absorption near edge structure (XANES) and XAS 2-dimensional imaging.

A comprehensive survey of arsenic compounds in the mushrooms of 46 different fungus species over a diverse range of phylogenetic groups were collected from Canadian grocery stores and background (sites with lower arsenic concentrations) and arsenic-contaminated areas. The major arsenic compounds in mushrooms were found to be similar among phylogenetic groups, and AB was generally absent in log-growing mushrooms, suggesting the microbial community may influence arsenic speciation in mushrooms. The high proportion of AB in mushrooms with puffball or gilled morphologies may suggest that AB acts as an osmolyte in certain mushrooms to help maintain fruiting body structure. The mycelia of three different fungi species (targeting those containing AB, including *Agaricus bisporus*) were cultured axenically and exposed to AB, arsenate (As(V)) and dimethylarsinoyl acetic acid (DMAA, a potential AB precursor) for 60 days. The mycelia of all fungi species accumulated all arsenic compounds with two species (those whose fruiting bodies contain mostly AB) accumulating significantly more AB than other compounds, but few biotransformations were observed. Thus AB biosynthesis by fungus mycelium is unlikely. To investigate the involvement of reproductive life stage of *Agaricus* species (an AB-containing fungus) in AB formation growth substrate and fungi were collected and analyzed during the commercial growth of *A. bisporus*. AB was found to be the major arsenic compound in the fungus at the earliest growth stage of fruiting (the primordium). AB was revealed by two-dimensional imaging to be present only in fungal tissue and not in embedded substrate of an *A. bisporus* primordium grown on As(V) treated substrate, and in a mature *A. campestris* fruiting body stalk from arsenic contaminated mine

tailings. These findings indicate AB formation is likely associated with the reproductive life stage of the fungus. To interrogate the contribution to AB formation of the microbial communities associated with mushrooms, 16s pyrosequencing techniques were used to reveal that phyla diversity in the growth material for the commercial cultivation of *A. bisporus* increased at the time of FB initiation, coinciding with the appearance of AB. Microbial communities from similar locations were found to have similar microbial structures, but were different between log materials and soil substrates. Ten bacteria species associated with FB initiation and AB containing FBs did not transform As(V), dimethylarsinic acid (DMA) and DMAA under axenic culture. Similarly a consortia of microbes isolated from FB-associated soil did not produce AB. Overall, AB formation appears to be associated with the reproductive life stage of the fungus and likely involves a combination of organisms, perhaps those symbiotically associated with each other.

Keywords: Arsenic**,** arsenobetaine, fungus, biotrasformation, mycelium, fruiting body, microbial community, biosynthetic pathway

Résumé

Nearing, Michelle M. Ph.D. Collège militaire royal du Canada. Janvier 2015. À la quête d'une voie biosynthétique de l'arsénobétaïne dans les champignons supérieurs terrestres. Superviseurs: M. Kenneth J. Reimer, Ph.D. et Mme Iris Koch, Ph.D.

Le niveau de toxicité de l'arsenic dépend largement de sa forme chimique, et un seul composé d'arsenic, l'arsénobétaïne (AB), est considéré non-toxique. Bien que l'AB existe en concentrations et proportions élevées dans plusieurs organismes marins, son mécanisme de synthèse demeure inconnu. En milieu terrestre, l'AB se trouve en plus faibles concentrations et proportions, à l'exception des fructifications, ou champignons, d'organismes fongiques terrestres. Le présent ouvrage a examiné les voies de synthèse de l'AB dans l'organisme fongique complet (qui inclut à la fois le champignon et les microbes qui y sont associés) par le biais d'analyses des fructifications (les fruits du champignon) et des substrats, ainsi que par des expériences de cultures champignonnières. Diverses méthodes d'analyse ont été évaluées et les méthodes complémentaires suivantes ont été retenues : chromatographie liquide à haute performance – spectrométrie de masse avec plasma à couplage inductif (CLHP-SM-PCI), et spectroscopie par absorption de rayons-X (SAX), plus particulièrement la spectroscopie de structure près du front d'absorption de rayons-X (SSPAX), et l'imagerie en deux dimensions de la SAX.

Un relevé exhaustif des composés d'arsenic se trouvant dans les champignons de 46 espèces d'organismes fongiques différents couvrant un vaste éventail des groupes phylogénétiques a été complété à partir de spécimens provenant de supermarchés canadiens, de sites de référence (où les concentrations d'arsenic sont faibles), et de régions contaminées à l'arsenic. Les principaux composés d'arsenic dans les champignons sont similaires parmi les groupes phylogénétiques, et l'AB est généralement absent chez les champignons qui poussent sur des troncs d'arbres, ce qui suggère une influence de la communauté microbienne dans la spéciation de l'arsenic par le champignon. Les grandes proportions d'AB dans les champignons de morphologies charnue ou lamellée peuvent suggérer que l'AB joue un rôle osmotique dans le maintien structurel de la fructification. Les mycéliums de trois différentes espèces d'organismes fongiques (choisies parmi celles qui contiennent de l'AB, dont l'*Agaricus bisporus*) ont été cultivés en milieu axénique et exposé à l'AB, à l'arséniate (As(V)) et à l'acide acétique de diméthylarsinoyle (AADM, un précurseur potentiel de l'AB) durant une période de 60 jours. Ces composés d'arsenic ont été accumulés dans le mycélium de chaque espèce de champignon, et deux de ces espèces (celles dont les fructifications contenaient principalement de l'AB) ont accumulé une plus grande quantité d'AB que les autres composés (de façon significative), mais comportant peu de biotransformations. Il est donc peu

probable que le mycélium fongique procède à la biosynthèse de l'AB. Pour enquêter sur la synthèse de l'AB durant le cycle de reproduction des espèces *Agaricus* (champignons contenant de l'AB), des échantillons de substrat et de champignons ont été prélevés et analysés au cours de la croissance de *A. bisporus* en production commerciale. Le composé d'arsenic principal s'est avéré être l'AB dans le champignon au tout début de son cycle de fructification (étape primordiale). Une analyse par imagerie en deux dimensions a révélé que l'AB ne se trouve que dans la matière fruitière et non dans les composantes du substrat d'un thalle primordial de *A. bisporus* dont le substrat a été imprégné d'As(V), ainsi que dans la tige d'un champignon *A. campestris* ayant complété sa croissance dans des résidus miniers contaminés à l'arsenic. Ces résultats indiquent que la formation de l'AB est probablement associée au cycle de reproduction des champignons. Des techniques de pyroséquençage 16s ont été employées fin d'élucider la contribution de l'AB à la formation des communautés microbiennes associées aux champignons. Celles-ci ont révélé que, dans le substrat de croissance utilisé en culture commerciale du *A. bisporus*, la diversité phylétique est plus importante à l'amorce de la fructification, ce qui coïncide avec l'apparition de l'AB. Les communautés microbiennes de milieux similaires se ressemblent, et sont différentes des communautés associées aux troncs d'arbres et aux substrats de terre. Des dix espèces bactériennes associées à l'initiation de la fructification et à des organismes fongiques contenant de l'AB, aucune n'a transformé l'As(V), l'acide diméthylarsinique (ADMA), ou l'AADM en culture axénique. De même manière, un consortium microbien isolé des sols associés à la fructification n'a pas produit d'AB. En fin de compte, il semble que la synthèse d'AB soit liée au cycle de reproduction champignons et implique probablement une combinaison d'organismes, dont certains fonctionnent en association symbiotique.

Mots clés : Arsenic, arsénobétaïne, champignon, organisme fongique, biotransformation, mycélium, fructification, communauté microbienne, voie biosynthétique

Co-authorship statement

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 implementation and completion of experiments, including field work, and analytical work completed both at the Advanced Photon Source (Chicago, IL) and at the Royal Military College of Canada (Kingston, ON)
- Principal author on 4 papers

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Chapter 4: Nearing, M. M.; Koch, I.; Reimer, K. J., Uptake and transformation of arsenic during the vegetative life stage of terrestrial fungi. *Environmental Pollution.* **2015,** *197*, (0), 108-115.

Chapter 5: Nearing, M. M.; Koch, I.; Reimer, K. J., Uptake and transformation of arsenic during the reproductive life stage of *Agaricus bisporus* and *A. campestris*. (Submitted 2015).

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List of Abbreviations

Abbreviation	Definition
µXANES	micro X-ray absoprtion near edge structure
AB	Arsenobetaine
AC	Arsenocholine
ADP	Adenosine di-phosphate
A_L	Human-hamster hybrid cell line
ANOVA	Analysis of variance
APS	Advanced Photon Source
As(Glu)3	Arsenic glutathione
As(III)	Arsenite
As(III)-PC2	Arsenite Phytochelatin 2
As(III)-PC3	Arsenite Phytochelatin 3
As(III)-S	Arsenite sulfur compound
As(V)	Arsenate
As-GSH	Arsenic glutathione
AsS	Arsenosugar
ATCC	American Type Culture Collection
ATP	Adenosine tri-phosphate
BC	British Columbia
bp	Base piar
CACing	Compost at casing
CE	Capillary electrophoresis
CF	Concentration factor
CID	Collision induced dissociation
CR	Column recovery
CRM	Certified reference material
CYS	Cysteine
DDW	Distilled deionized water
DM	Dry Mass
DMA	Dimethylarsinic acid
DMA(III)	Dimethylarinous acid
DMAA	Dimethylarsinoyl acetic acid
DMAE	Dimethylarsinoyl ethanol
DMAP	Dimethylarsinoyl propionate
DMTA	Dimethylthioarsinic acid
DMMTA	Dimethylmonothioarsinic acid
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DOE	Department of Energy
EE	Extraction Efficiency
ESI-MS	Electrospray ionization mass spectrometry
EXAFS	Extended X-ray absorption fine structure
FB	Fruiting Body
GC	Gas chromatography
GC-EI-qMS	Gas chromatography-electron ionization-quadrupole mass spectrometry
GLU	Glutamic acid
GLY	Glycine

Abbreviation	Definition
GSH	Glutathione
HPLC	High performance liquid chromatography
iAs	Inorganic arsenic
ICP-MS	Inductively coupled plasma mass spectrometry
IUPAC	International Union of Pure and Applied Chemistry
LC	Liquid chromatography
LD_{50}	Lethal Dose 50%
LOD	Limit of detection
LOQ	Limit of quantification
$Me_2AsDMPS$	Dimethylarsenic 2,3-dimercapto-1-propane sulfonic acid sodium salt
MeAsDMPS	Monomethylarsenic 2,3-dimercapto-1-propane sulfonic acid sodium salt
MMA	Monomethylarsonic acid
MMA(III)	Monomethylarsonous acid
MMTA	Monomethylmonothioarsonic acid
MS	Mass spectrometry
NMR	Nuclear magnetic resonance imaging
NSERC	Natural Sciences and Engineering Research Council of Canada
NT	Northwest Territories
oAs	Organic arsenic
ON	Ontario
OUT	Operational taxonomic unit
PC	Phytochelatin
PCR	Polymerase chain reaction
PNC	Pacific Northwest Consortium
QA/QC	Quality Control / Quality Assurance
QC	Quality Control
RPD	Relative percent difference
RSD	Relative standard deviation
SAM	S-adenosylmethionine
SIM	Selective ion monitoring
SRM	Selected reaction monitoring
SR-TXRF	Synchrotron-radiation-induced total reflection x-ray fluorescence
TETRA	Tetramethylarsonium ion
TMAO	Trimethylarsine oxide
TMAS	Trimethylarsine sulfide
TOF-MS	Time of flight mass spectrometry
UK	Unknown
US	United States
WM	Wet mass
χ^2	Reduce chi squared
XANES	X-ray absorption near-edge structure
XAS	X-ray absorption spectroscopy
XRF	X-ray fluorescence
XSD	X-ray Science Division
YK	Yellowknife

Chapter 1

1 Introduction

It is currently estimated that over 50 arsenic compounds exist in the environment. The chemical form, or speciation, of arsenic is of interest because its toxicity is greatly dependent on its oxidation state and chemical form [1]. Arsenobetaine (AB) is the only arsenic compound that is considered to be non-toxic [2]. This form of arsenic has been frequently found in high concentrations in marine organisms where it comprises the majority of human arsenic exposure through food. In the terrestrial environment most organisms, and food products, contain the more toxic forms of arsenic including inorganic and simple methylated arsenic compounds [1]. The exception to this trend is the fruiting bodies, or mushrooms, of terrestrial fungi.

The formation pathway for AB is unknown in both the marine and terrestrial environment. The terrestrial environment provides a simpler study system for the formation of AB because it is limited to certain fungus species and their biological environment, whereas in the marine environment AB is found in many organisms and in many trophic levels. That is, the production of AB in the fungi of interest likely occurs either in the fungus or in the surrounding microbial community, whereas even the trophic level at which AB production occurs in the marine environment is still unknown. The purpose of this thesis research is to study terrestrial fungus systems and associated microbes to elucidate a potential formation pathway for AB.

1.1 Introduction to Arsenic and Arsenic Compounds

Arsenic is a metalloid with a complex chemistry demonstrating the properties of both metals and non-metals. Arsenic commonly exists in four oxidation states: -3, 0, +3 and +5, although more can be found in minerals. The +3 and +5 states are those most commonly found in biological tissues and the existence of the -3 state in the environment has been questioned [4]. The predominant form of inorganic arsenic in aqueous aerobic, neutral environments is arsenate (usually as $AsO(OH)_2O^-$/$AsO(OH)O_2^-$) abbreviated as As(V)), while arsenous acid ($As(OH)_3$, abbreviated as As(III) and often referred to as arsenite) is the predominant form of inorganic arsenic in anoxic, neutral environments [3]. Both of these inorganic arsenicals are toxic and can interconvert with changes in redox conditions and pH [4]. Arsenic is mobilized into the aqueous and atmospheric environment naturally through the weathering of rocks and minerals, volcanic activities and biological processes [5]. Arsenic can also be introduced to the environment through anthropogenic inputs such as pesticide use, mining and wood preservation [3, 6].

Arsenic may also be found in organoarsenic compounds, defined as those containing arsenic-carbon bonds (a less precise term is organic arsenic). Methylated organoarsenic compounds are most commonly found in natural waters as breakdown/excretion products from aquatic biota, or as urinary excretions of animals [3, 4]. In addition to methylated organoarsenic compounds, arsenic can form lipid-based compounds. The affinity of arsenic for sulfur means that compounds with As-O components can also exist with As-S bonds; for example, As(III) can bond with sulfhydryl groups of proteins [1]. It is currently estimated that there are over 50 arsenic compounds found in the environment. The structures for some common arsenic compounds are shown in Chapter 2, Figure 2.1.

1.2 Arsenic Toxicity

The toxicity of arsenic compounds varies according to oxidation state and chemical form, as well as factors such as particle size, solubility, uptake and elimination rates [7]. Acute exposure to some arsenic compounds can cause death, and chronic exposure can lead to various cancers, cardiovascular and vascular disorders, neurological disorders, skin diseases and liver disorders [8]. The oxidation state and chemical form of arsenic are important factors that affect toxicity and they may be changed by biological processes. As a general rule, the trivalent analog of a compound is more toxic than the pentavalent form [4] but organisms usually possess ample reduction capacity to convert the higher oxidation state. Complex organoarsenic compounds with pentavalent arsenic generally have lower toxicities than inorganic and simple methylated compounds. Arsenosugars were thought to be of limited toxicity but one of their degradation products, dimethylarsinic acid (DMA(V)), has been found to be a cancer promoter [9]. The only arsenic compound that is non-toxic and can be consumed at high doses without harmful effects is arsenobetaine (AB). This conclusion is based on the inability to establish an LD_{50} for AB: a maximum of 10 g of AB/kg body weight was administered to mice (equivalent to 4260 mg As/kg body weight) with no adverse effects observed [2]. It is assumed that no more AB could have been administered in this study.

The mode of arsenic toxicity appears to be similar in all studied organisms and can occur through cytotoxic and genotoxic mechanisms [7, 10]. Trivalent species such as As(III), monomethylarsonous acid (MMA(III)), and dimethylarsinous acid (DMA(III)) are more broadly toxic because of their affinity for binding to sulfhydryls, which are often located at enzyme active sites resulting in inactive enzymes such as pyruvate hydrogenase preventing the generation of ATP in the citric acid cycle [11]. Arsenic can act as a genotoxin by causing gene mutations and chromosomal alterations. Specifically, As(III) has been shown to induce the deletion of millions of base pairs at the CD59 locus in human-hamster hybrid cell lines (A_L) demonstrating the ability to produce large mutations that result in nonviable cells [12, 13]. As(III) has

also been found to demonstrate clastogenicity, causing breaks in chromosomal DNA, and increase levels of free radicals in A_L cells, which can induce DNA lesions consistent with oxidative damage [14, 15]. The trivalent forms of MMA and DMA may also cause DNA damage by increasing levels of reactive oxygen species [8].

1.3 Arsenic in the Biotic Environment

Arsenic speciation in the biotic environment is typically divided into marine and terrestrial (including freshwater) ecosystems. Arsenic speciation in the marine environment has been extensively studied since 1977 with the discovery of AB in the tail muscle of a western rock lobster, *Panulirus longipes cygnus* [16]. The marine ecosystem is well suited to the study of arsenic speciation in biota since arsenic found in organisms is typically in the mg/kg range despite average arsenic concentrations of 0.5 to 0.2 µg/L in the surrounding water [17]. In contrast, organisms from terrestrial ecosystems, unless from a contaminated location, contain arsenic concentrations at the µg/kg level requiring more sensitive analytical methods.

It was once thought that simple methylated compounds marked the endpoint of arsenic metabolism in terrestrial organisms and only marine organisms produced more complex organoarsenic compounds such as AB [18]. With the use of more powerful separation systems and more sensitive analytical methods this hypothesis has since been revised.

1.3.1 Simple Methylated Compounds

Simple methylated compounds are formed as a result of biomethylation, where biomethylation refers to an enzymatic transfer of a methyl group from a donor atom to an acceptor atom within a living organism [19]. The biomethylation of arsenic compounds is best described by the pathway suggested by Challenger (1945), shown in Figure 1.1, which involves a series of alternating reduction and oxidative methylation reactions mediated by arsenic methyltransferase enzymes and S-adenosylmethionine (SAM), a near universal methyl donor in biological systems [20]. Within this model As(V) is first reduced to As(III) before being methylated and oxidized to form MMA [21]. The reduction and oxidative methylation steps are repeated producing the trivalent and pentavalent forms of MMA and DMA and finally trimethylarsine [20]. DMA marks the endpoint of arsenic metabolism in humans and rodents, with 60 – 80% of ingested arsenic detected as DMA in humans [22]. The majority of plants surveyed contain lower proportions of MMA and DMA with the majority of arsenic retained as inorganic arsenic [19, 23].

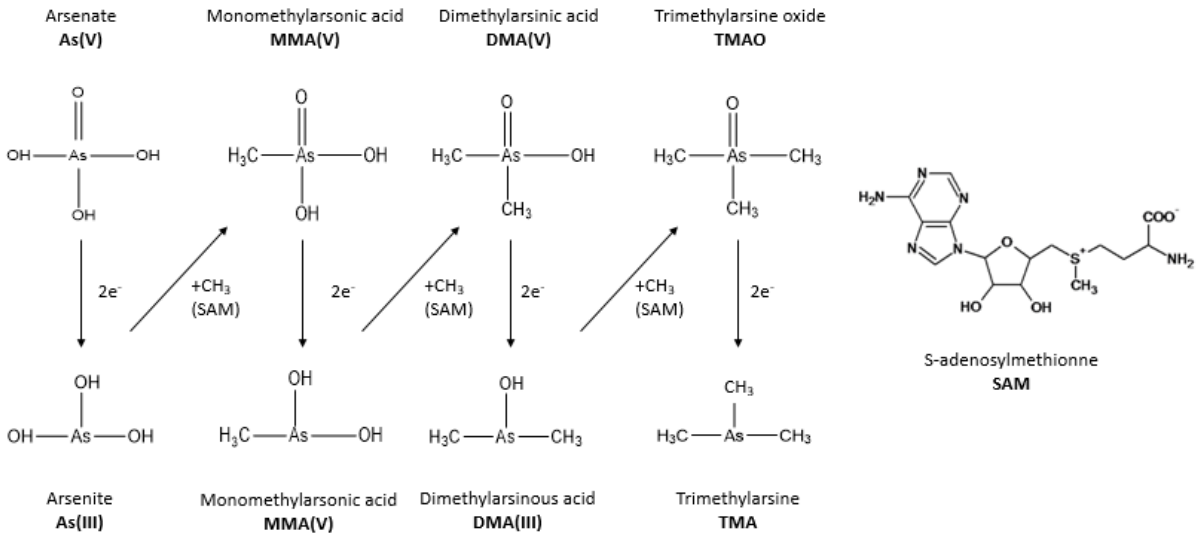

Figure 1.1: A schematic of the Challenger pathway.

Terrestrial fungi, on the other hand, display a larger array of organoarsenic compounds, especially in the fruiting bodies, but in a 2003 review [17] DMA was found to comprise the majority of arsenic compounds for 26% of the reviewed species of fruiting body-forming fungi, and as a secondary metabolite (1- 50% of extractable arsenic) in 72% of species. MMA was also found in a number of these species as a secondary metabolite, but at a much lower frequency than DMA. Other arsenic compounds were more complex organoarsenic compounds (see next sections).

1.3.2 Other Organoarsenic Compounds

Other more complex organoarsenic compounds have been widely reported in marine ecosystems; however the presence of these compounds is not limited to this environment [17]. Arsenosugars, a group of complex carbohydrates containing arsenic, can be found in freshwater phytoplankton and some freshwater fish species [24, 25]. The tetramethylarsonium ion (TETRA) has been reported in frogs, mushrooms and some plant species [1]. The formation of arsenosugars and TETRA has been proposed to partially follow the Challenger pathway. In the formation of arsenosugars, arsenate is thought to be transformed to DMA(V) via the Challenger pathway and then converted to an arsenosugar by a nucleoside or N-(4-(dimethylarsinoyl)butanol)taurine intermediate [26]. TETRA has been proposed to be formed from a final methylation of trimethylarsine in the Challenger pathway [27]; however this would

involve a volatile trimethylarsine intermediate which is unlikely to be found in an organism [1]. TETRA may also be formed as a degradation product of arsenosugars or arsenobetaine [28, 29].

1.3.3 Arsenobetaine

Following the trend of most complex organoarsenic compounds, AB is found in a greater number of marine organisms and at higher concentrations than in terrestrial organisms. The major group of organisms in which AB is found in the terrestrial environment are the fruiting bodies of terrestrial fungi. Of 49 species that had been reported in the literature by 2003, 67% contained AB as the major arsenic compound (>50% of extractable arsenic) [17]. It has been hypothesized that the more complex organoarsenic compounds, and especially AB, are produced by more highly evolved fungi [30]. Low levels of AB have been reported in birds, fox, earthworms, and some plant species [1]. The higher concentrations of AB in marine organisms has been attributed to the osmolytic properties of the molecule as it resembles the osmoregulator glycine betaine [31], and osmolytic compounds are required in saline environments. The presence of AB in terrestrial fungi may also support this theory, since the fruiting bodies rely on osmotic regulation for spore dispersal in sexual reproduction [32].

Currently, the main hypothesis for AB formation is that it is formed from the degradation products of dimethylated arsenosugars (Pathway 1 and 2, Figure 1.2). Dimethylated arsenosugars are thought to be precursors for the formation of AB because the dietary sources for marine organisms, such as phytoplankton and marine kelp, contain elevated levels of arsenosugars [33]. It has been proposed that the formation of AB may occur from the degradation of dimethylated arsenosugars to arsenocholine (AC) which is then converted to AB (Pathway 1, Figure 1.2). The conversion of AC to AB has been demonstrated with many laboratory studies with various bacteria, mice, rats and rabbits [34, 35]. However the degradation of arsenosugars to AC has not been as well demonstrated. Studies with shrimp showed that dimethylated arsenosugars remained unchanged or transformed to trace amounts of DMA(V) suggesting the formation pathway from arsenosugars to AB does not involve AC as an intermediate [35]. Another compound, dimethylarsinoyl acetic acid (DMAA), has been proposed as a potential intermediate similar to AC (Pathway 2, Figure 1.2). In contrast to AC, DMAA has been demonstrated to be a major degradation product of arsenosugars, second to DMA(V), in sheep [29]. DMAA has also been shown to be a precursor in the formation of AB in laboratory studies involving lysed bacteria extracts [26]. However when DMAA was fed to fish, no AB was formed [36]. The third proposed formation pathway for AB (Pathway 3 in Figure 1.2), offers a more direct route by simple methylated compounds involving dimethylarsinous acid (DMA(III)) and 2-oxo acids, glyoxylate and pyruvate, to form DMAA and then AB [37].

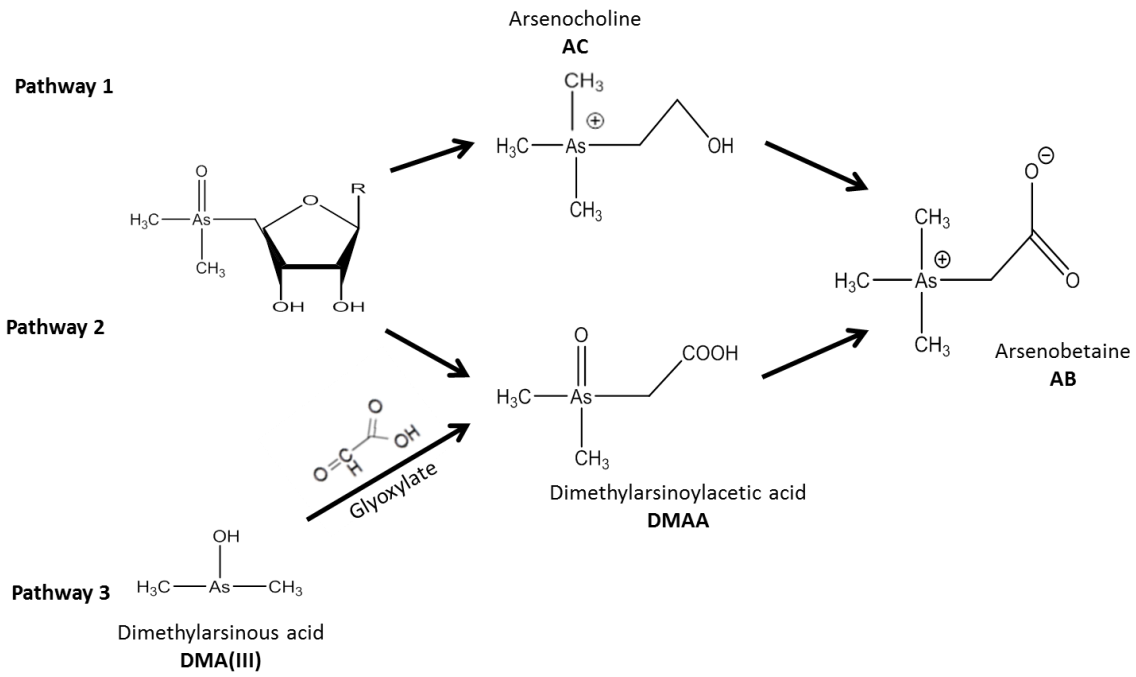

Figure 1.2: Proposed formation pathways for arsenobetaine.

1.3.4 Arsenobetaine Formation in Terrestrial Fungi

The abundance of AB in terrestrial fungi provides a simpler study system for the formation of AB than marine organisms would. The life stage at which the fungus produces AB is still unknown, as is whether the fungus, alternatively, accumulates AB from the surrounding environment. That is, the surrounding microbial community in the soil may produce AB or its precursors and these may be selectively taken up by the fungus. *Agaricus placomyces* mycelium grown on a media containing a variety of arsenic compounds, under axenic conditions, was found to selectively accumulate arsenic compounds during its vegetative life stage [38]. *Agaricus bisporus*, the commonly cultivated white button mushroom, has been shown to contain AB when grown in compost treated with arsenate solutions. The arsenate treated compost with no fungus present was found to contain TMAO as the major arsenic compound, indicating the activity of the microbial community, but no AB [39].

1.4 Higher Terrestrial Fungi and Associated Microbes

1.4.1 Basidiomycetes lifecycle

Higher fungi contain the classes Ascomycetes and Basidiomycetes that can include freshwater, marine and terrestrial species. The Basidiomycetes class contains about 30,000 described species [40]. Species in the class Basidiomycetes are defined as fungi that produce basidia, cells on which sexual spores are produced, on or in a basidiocarp. Terrestrial species in this class of fungi can grow through asexual reproduction, the vegetative life stage, and through sexual reproduction, the reproductive life stage. The reproductive life stage produces fruiting bodies, or mushrooms, for spore dispersal. The fruiting body is typically composed of a stalk that elevates the basidiocarp above the ground, a cap, and lamella on which the basidia are found. Mature basidia in most cases develop four haploid basidiospores for release. The released spores may germinate under optimal environmental conditions and form monokaryon hyphae that will develop to form mycelium through asexual reproduction. Two compatible monokaryon hyphae can fuse to form dikaryon hyphae, the form in which the fungus will spend most of its life. Environmental conditions specific to each fungi species trigger fruiting body development, which allows the fungus to reproduce sexually once again [41]. In the commercial cultivation of mushrooms, these conditions have been optimized to produce greater harvests.

1.4.2 Symbiotic Relationships: Influence of microbial activity on arsenic speciation in fungi

Microbes associated with terrestrial fungi may influence arsenic speciation in fruiting bodies through two roles. The first addresses the symbiotic role that bacteria are thought to play in the production of fruiting bodies, and the second addresses the metabolic role in which microbes are involved, with respect to the biogeochemical cycle of arsenic, affecting arsenic speciation and mobility [42]. The commonly cultivated *A. bisporus* has been used most often as a model system for examining the involvement of bacteria in fruiting body initiation, schematic of *A. bisporus* cultivation in shown in Figure 1.3. In the commercial growth of *A. bisporus* it has been reported that after spawning, bacterial populations are significantly higher in the casing (a layer of peat moss and calcium limestone material that facilitates fruiting) than in the compost material under the casing. The same study also demonstrated that after the addition of spawned compost (compost inoculated with the fungus culture) to the casing layer there was a significant increase in the bacterial populations and in the number of fruiting bodies formed compared to the unamended casing [43]. Electron scanning microscope studies of *A. bisporus* mycelium were able to show morphological differences in mycelium grown in sterile casing compared to casing containing the bacterial community from commercial casing material. Mycelium from the sterile casing was found to be thin and surrounded by calcium oxalate crystals thought to inhibit fruiting body initiation. The mycelium grown in the non-sterile casing material was not surrounded by these crystals and was found to have rod

and coccid shaped bacteria in close proximity or attached to mycelium. The mycelium in non-sterile casing also formed thicker strands that were able to form fruiting bodies. In studies when *A. bisporus* was grown on agar plates the presence of *Pseudomonas putida* and *Pseudomonas flourescens* significantly stimulated fruiting body initiation. In addition to stimulating fruiting body initiation, bacteria associated with ectomycorrhizal fungi are shown to help increase nutrient availability to the fungus by mobilizing recalcitrant forms of nutrients, specifically nitrogen [44].

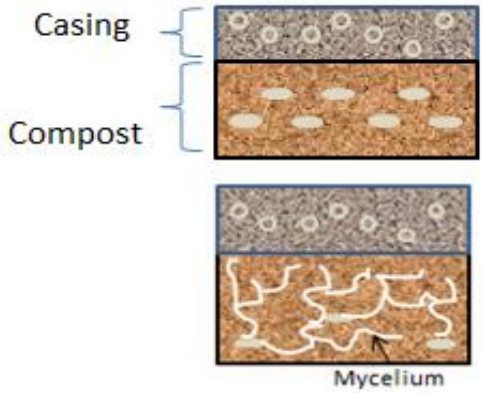

Figure 1.3: Schematic for the cultivation of A. bisporus. A) In the commercial cultivation of A. bisporus compost inoculated with mycelium is covered with a casing material after 3 days of growth. B) After 5 to 7 days the mycelium will grow upwards into the casing

Cultivation studies of associated microbes from soil can be limited because less than 1% of soil bacteria can be cultivated in this way [45]. The use of DNA extraction from soil with 16s pyrosequencing are beneficial techniques that can characterize a large proportion of associated bacteria, providing a more detailed understanding of the microbial community structure. This form of sequencing can provide information on the microbial community structure and identify differences in species distribution or abundance in soil samples from different sources. Studies that have characterized bacterial communities in compost used in the commercial cultivation of mushrooms mainly focus on the spent substrate to assess its viability for use as a fertilizer. Bacterial community characterization using amplified 16s rDNA restriction analysis and sequencing has shown that microbial diversity of the compost varies greatly with the source of the compost [46]. Soil microbial communities from soils affected by arsenic contamination have been characterized, also with a focus on their viability as agricultural soils, using 16s pyrosequencing [47, 48]. The microbial community for mushrooms growing in the wild has yet to be characterized with this method. Mainly only cultivable bacteria associated with wild fruiting bodies have been characterized and studied [49-52].

Many bacteria species have evolved mechanisms for coping with the toxicity of arsenic in the environment. Genetically, the ars operon constitutes the most ubiquitous and important factor in arsenic tolerance in bacteria. Bacteria are capable of reducing As(V) to As(III) with use of the ArsC gene and removing this As(III) with protein pumps encoded by the ArsB gene [42]. Bacteria are also thought to methylate arsenic through the Challenger pathway. The bacterial methyltransferase gene, arsM, has been found to determine all the SAM-dependent methylation steps in bacteria and has been identified in more than 200 prokaryotic species [42]. Catalysis of As(III) to methylated intermediates and the volatile end product trimethylarsine by arsM in *E. coli* has been shown to decrease arsenic concentrations in the cells and growth media. It is thus hypothesized that for some bacterial species this methylation pathway is a detoxification mechanism. However this does not mean that methylation is a detoxification mechanism for other organisms [53]

Methylation of arsenic by microbes associated with mushrooms and plants has been observed in two studies. In the first study, the microbial community in mushroom compost treated with arsenate solution transformed the majority of this inorganic arsenic to TMAO [39]. In the second study, the microbial community associated with various plant species grown in arsenate treated soils were shown to be the source of methylated arsenic compounds detected in these plants [54]. Specifically, rice, tomato and red clover plants grown in sterile cultures were found to accumulate arsenate, MMA and DMA but were unable to produce MMA and DMA from arsenate. In contrast, when rice seedlings were grown in non-sterile soils treated with arsenate, they contained these methylated compounds. The treated control soil, containing no plants, also contained these methylated compounds and showed the presence of the arsM gene [54]. These studies suggest that associated microorganisms may produce methylated arsenic compounds that can be subsequently taken up by organisms. Therefore the microbial community must be considered in the potential formation of AB.

1.5 Potential Formation Pathways for AB

Although the finding of AB in terrestrial fungi provides at least the general ecosystem and organisms responsible for AB formation, there are still a number of interacting factors in the environment to consider. These factors include the life stages of the fungus and the associated soil microbes. The purpose of this thesis is to elucidate a potential biosynthetic pathway for AB in terrestrial fungi by studying the life stages of terrestrial fungi and the surrounding microbial community in order to determine the arsenic biotransformations that occur at each of these levels. This thesis will specifically examine five potential

biosynthetic pathways for the formation of arsenobetaine in *A. bisporus*. The potential biosynthetic pathways are described below:

Biosynthetic Pathway 1 (Figure 1.4a): The vegetative life stage of the fungus, or the mycelium, may form AB *de novo* from As(V) or As(III), the most common forms in the soil environment, and the AB is then retained within the fungus until the fruiting body is developed during the reproductive life stage; the AB then moves to the fruiting body.

Biosynthetic Pathway 2 (Figure 1.4b): The vegetative life stage of the fungus, or the mycelium, may accumulate AB precursors produced from the surrounding soil microbial community. The *A. bisporus* mycelium would then, similar to biosynthetic pathway 1, biotransform the precursors to form AB which would be retained until fruiting body development and movement to the fruiting body.

Biosynthetic Pathway 3 (Figure 1.4 c): The soil microbial community associated with the fungus may accumulate inorganic arsenic or AB precursors found in the surrounding soil and biotransform these arsenic compounds to form AB. The AB formed by the microbial community would then be accumulated by the nearby mycelium for retention or immediate transport to the fruiting bodies.

Biosynthetic Pathway 4 (Figure 1.4 d): The formation of AB in *A. bisporus* could occur *de novo* during fruiting body development. Inorganic arsenic or AB precursors from the surrounding soil may be accumulated and biotransformed during the different growth stages in fruiting body development, including primordia initiation and development.

Biosynthetic Pathway 5 (Figure 1.4 d): AB may be formed *de novo* by the mature fruiting body where this stage of the fruiting body would accumulate inorganic arsenic or AB precursors from the surrounding soil and biotransform this form of arsenic to produce AB.

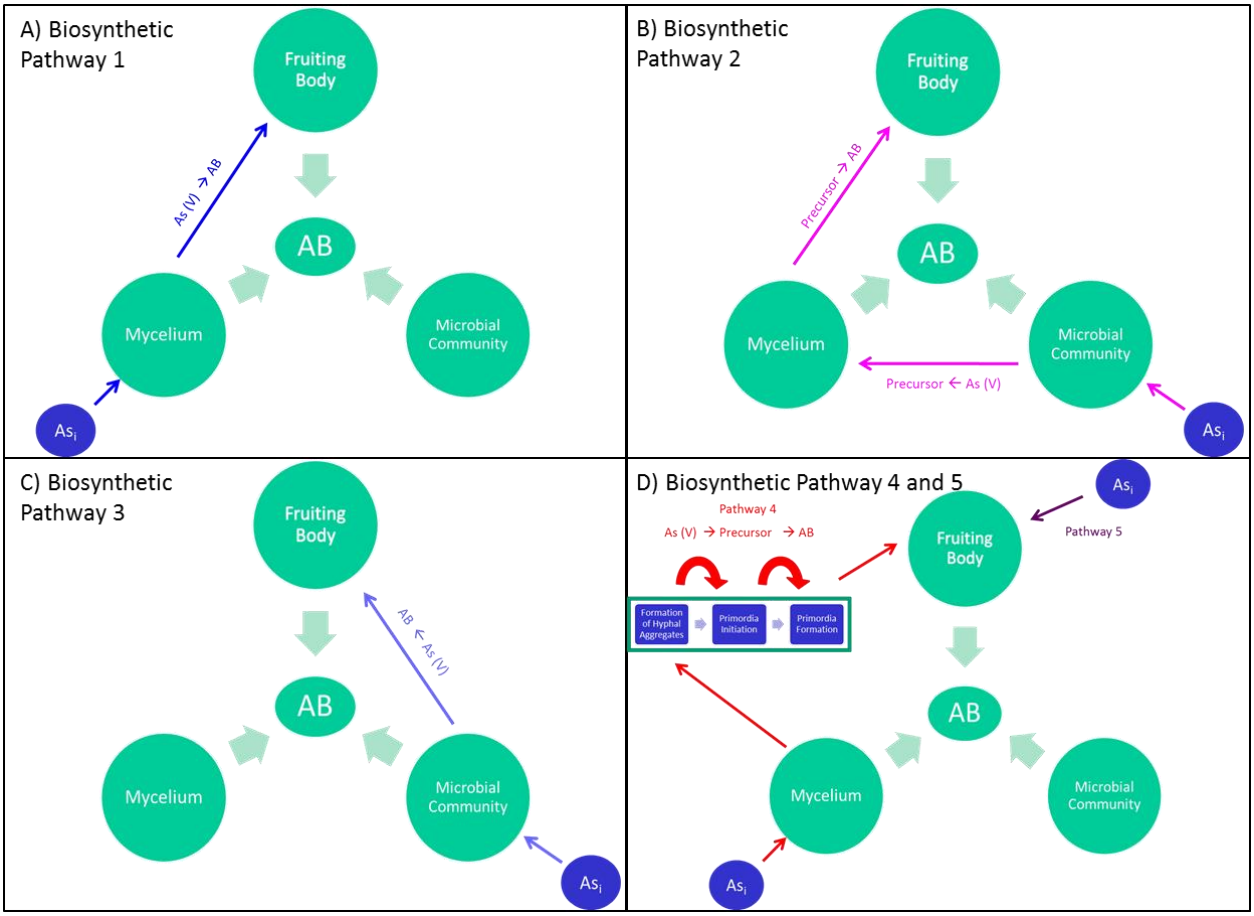

Figure 1.4: Potential biosynthetic pathways for the formation of arsenobetaine (AB) in the fungus involving the vegetative life stage (mycelium), reproductive life stage (fruiting bodies) and associated soil microbes.

1.6 Objectives and scope of work

The toxicity of arsenic is greatly dependent on its chemical form and therefore accurate determination of arsenic speciation is a crucial step in understanding its chemistry and potential risk. Currently high performance liquid chromatography with inductively coupled mass spectrometry (HPLC-ICP-MS) is the most common analysis used for arsenic speciation but it has major limitations. Chapter 2 describes the objective to identify and review complementary arsenic speciation methods that have the ability to address these disadvantages.

Although many mushrooms have been found to contain a high proportion of AB, studies have included a limited phylogenetic diversity and focused on mushrooms with higher total arsenic concentrations. Chapter 3 summarizes studies aimed to address the objective of determining the arsenic speciation in different fungus species collected from Canadian grocery stores, and background and arsenic contaminated areas, in order to provide trends in arsenic speciation and to help elucidate the role or

potential formation of AB in these mushrooms. Another aim in Chapter 3 was also to identify fungus species for further culturing experiments (Chapter 4 and 5).

The objectives described Chapters 4, 5 and 6 are to test the potential biosynthetic pathways outlined in Figure 1.4. The objective described in Chapter 4 is to test biosynthetic pathways 1 and 2 (Figure 1.4) to determine the role of the vegetative life stage of fungus in the potential formation of AB, specifically the uptake and transformation of arsenic at this stage of three fungus species exposed to various arsenic compounds. The objective described in Chapter 5 is to test biosynthetic pathways 4 and 5 (Figure 1.4) to determine the role of the reproductive life stage of the fungus, using complementary arsenic speciation methods to determine the arsenic uptake and transformations during this stage of AB containing mushrooms.

Before biosynthetic pathways 2 and 3 can be tested the microbial communities associated with various mushrooms require characterization, which is one of the objectives described in Chapter 6, utilizing 16s pyrosequencing techniques to characterize and compare microbial communities associated with different species of fungi. Another objective described in Chapter 6 iss to use the characterization of these communities to select bacteria species for cultivation and exposure to arsenic compounds in order to test biosynthetic pathways 2 and 3.

The overall aim of this thesis research is to elucidate a potential formation pathway for AB using the combined results of the comprehensive study of arsenic compounds in mushrooms (Chapter 3) and the results of the biosynthetic pathway testing (Chapters 4 to 6).

Chapter 2[1]

2 Complementary Arsenic Speciation Methods: A Review

2.1 Abstract

The toxicity of arsenic greatly depends on its chemical form and oxidation state (speciation) and therefore accurate determination of arsenic speciation is a crucial step in understanding its chemistry and potential risk. High performance liquid chromatography with inductively coupled mass spectrometry (HPLC-ICP-MS) is the most common analysis used for arsenic speciation but it has two major limitations: it relies on an extraction step (usually from a solid sample) that can be incomplete or alter the arsenic compounds; and it provides no structural information, relying on matching sample peaks to standard peaks. The use of additional analytical methods in a complementary manner introduces the ability to address these disadvantages. The use of X-ray absorption spectroscopy (XAS) with HPLC-ICP-MS can be used to identify compounds not extracted for HPLC-ICP-MS and provide minimal processing steps for solid state analysis that may help preserve labile compounds such as those containing arsenic-sulfur bonds, which can degrade under chromatographic conditions. On the other hand, HPLC-ICP-MS is essential in confirming organoarsenic compounds with similar white line energies seen by using XAS, and identifying trace arsenic compounds that are too low to be detected by XAS. The complementary use of electrospray mass spectrometry (ESI-MS) with HPLC-ICP-MS provides confirmation of arsenic compounds identified during the HPLC-ICP-MS analysis, identification of unknown compounds observed during the HPLC-ICP-MS analysis and further resolves HPLC-ICP-MS by identifying co-eluting compounds. In the complementary use of HPLC-ICP-MS and ESI-MS, HPLC-ICP-MS helps to focus the ESI-MS selection of ions. Numerous studies have shown that the information obtained from HPLC-ICP-MS analysis can be greatly enhanced by complementary approaches.

Keywords: *Arsenic speciation, complementary, HPLC-ICP-MS, XAS, ESI-MS*

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[1]: Nearing, M. M.; Koch, I.; Reimer, K. J., Complementary arsenic speciation methods: A review. *Spectrochimica Acta Part B: Atomic Spectroscopy* 2014, 99, (0), 150-162.

2.2 Introduction

Arsenic is a naturally occurring element in the earth's crust and can be mobilized into the aqueous and atmospheric environments through the weathering and erosion of rocks and minerals, volcanic activities and biological processes [5]. Arsenic can also enter the environment as a result of anthropogenic activities such as pesticide use, mining and wood preservation [3], [6]. Arsenic exists most commonly in the +3 and +5 oxidation states and these are the ones that are most commonly found in biological tissues and environmental samples [4]. The predominant form of inorganic arsenic in aqueous aerobic environments is arsenate (As(V)), while arsenite (As(III)) is the predominant form in anoxic, neutral environments [3]. Both of these aqueous compounds of inorganic arsenic are toxic and can interconvert with changes in redox condition and pH [4].

Arsenic may also be found in organometallic compounds; organoarsenic compounds are defined as containing arsenic-carbon bonds. Structures and abbreviations of some of the more common arsenicals discussed in the present review are given in Figure 2.1. Methylated organoarsenic compounds are most commonly found in natural waters (e.g., monomethylarsonic acid (MMA) and dimethylarsinic acid (DMA)) as the breakdown products from aquatic biota or as urinary excretions of animals [3, 4]. More complex organoarsenicals (e.g., arsenobetaine, AB, and arsenosugars, AsS) are often found in tissues of marine biota. Arsenic can also form lipid-based and thiolated compounds (thiolarsenicals) [1]. For example, arsenic, usually as As(III), is often found bound to glutathione (GSH) compounds which serve as intracellular metal binding thiol ligands in many organisms [55]. Phytochelatins (PCs) are groups of peptides found typically in plants that are derived from GSH, and the synthesis of these complexes usually occur under metal stress [56]. It is currently estimated that over 50 arsenic compounds exist in the environment and biological systems [4].

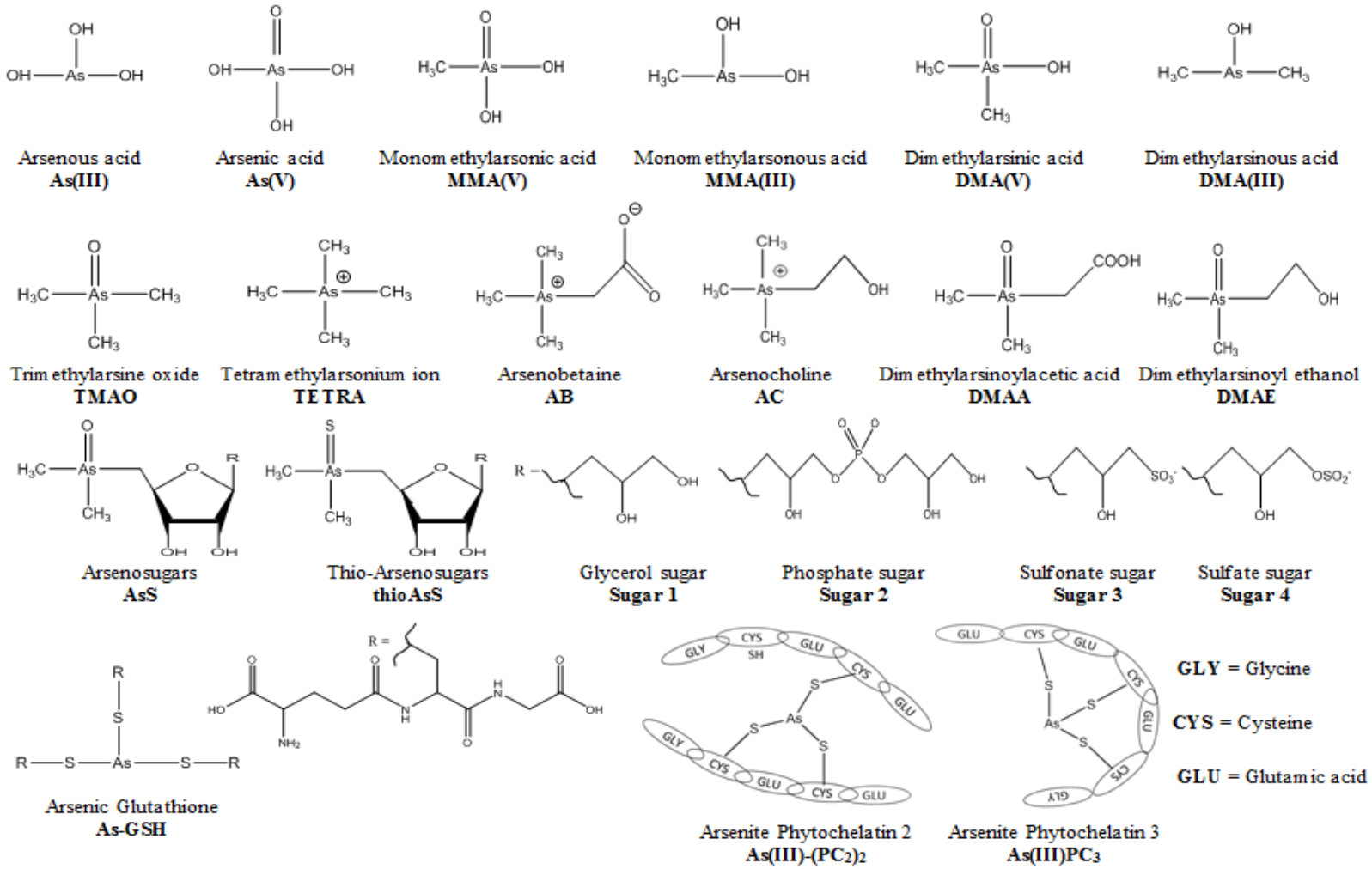

Figure 2.1: Structures and abbreviations for various arsenic compounds. Two PC complexes are shown, but many more exist. * Indicates where an oxygen (O) is replaced with a sulfur (S).

The toxicity of arsenic compounds varies according to oxidation state, chemical form and physical state and factors such as solubility, uptake and elimination rates [7]. The oxidation state and chemical form of arsenic are important factors that affect toxicity and they may be changed by biological processes. As a general rule, the trivalent analog of a compound is more toxic than the pentavalent form. Complex organoarsenic compounds generally have lower toxicities than inorganic and simple methylated compounds. Arsenosugars were thought to be of limited toxicity but one of their degradation products, DMA, has been found to be a cancer promoter [9]. The only arsenic compound that is non-toxic and can be consumed at high doses without harmful effects is AB. This conclusion is based on the fact that no LD_{50} has been established for AB: a maximum of 10 g of AB/kg body weight was administered to mice (equivalent to 4260 mg As/kg body weight) with no adverse effects observed [2]. It is assumed that no more AB could have been administered in this study.

The mode of arsenic toxicity appears to be similar in all studied organisms and can occur through cytotoxic and genotoxic mechanisms [7, 10]. A common starting point for toxicity is the affinity of trivalent species such as As(III), monomethylarsonous acid (MMA(III)), and dimethylarsinous acid (DMA(III)) for binding to sulfhydryls, or thiols, which are often located at enzyme active sites. Over 50 proteins have been identified as binding to trivalent arsenic [57], although the effects of this are still being studied.

Analytical measurements to distinguish and quantify arsenic species in a sample, that is, speciation analysis, is an important component in understanding the biogeochemistry, metabolism and toxic mechanisms of arsenic. It has long been realized that accurate identification of arsenic compounds from environmental samples is a necessary step in determining the potential environmental impacts and risk posed by the arsenic content. Recent media attention to arsenic in food or other consumer products has resulted in many regulatory bodies also stressing the importance of arsenic speciation. A summary of the proportions of sample types analyzed using HPLC-ICP-MS in journal articles published in 2012 is shown in Figure 2.2. Food comprises the largest proportion of sample types reported, followed by human blood/urine/tissues, and then followed by soils/sediments/minerals.

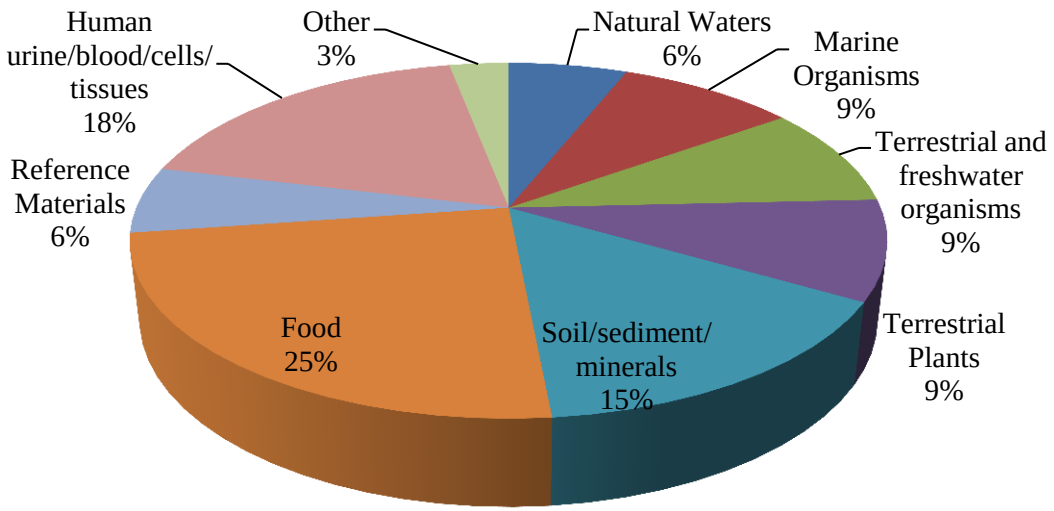

Figure 2.2: Proportion of sample types or sample matrices analyzed by HPLC-ICP-MS in 2012.

The accurate identification of arsenic compounds is dependent on the analysis method selected. In this review we adopt the convention of IUPAC, considering only methods that can directly distinguish arsenic compounds, and we do not include selective extraction techniques (also known as fractionation) [58]. Most studies of arsenic speciation utilize some form of separation system, such as liquid chromatography (LC), and pair this with an element-specific detection system like inductively coupled plasma mass spectrometry (ICP-MS). Traditionally arsenic speciation studies have only used one type of analysis method, meaning there are usually gaps in the arsenic speciation analysis for those samples that are affected by the limitations of the analytical method of choice.

The use of additional analytical methods in a complementary manner introduces the ability to address disadvantages that another method may have. Countless journal articles allude to the importance or need for complementary analysis methods for arsenic speciation but only one review of complementary methods in general metal speciation is available [59]. The purpose of the present review is to examine current complementary analysis methods used in speciation analysis of arsenic specifically, and to assess how the use of these complementary methods can provide insights on complete arsenic speciation in samples. The methods reviewed here will be limited to those that differ and complement each other in fundamentals such as detection characteristics or sample introduction.

The two most common analysis methods that have been used to date to complement arsenic speciation analysis with HPLC-ICP-MS include X-ray absorption spectroscopy (XAS) and electrospray mass spectrometry (ESI-MS) and will therefore be the focus of this review. Capillary electrophoresis (CE) has also been used in place of liquid chromatography as a separation technique for arsenic compounds with a number of different detection systems [60-62]. The main advantages of CE are that anionic and cationic compounds may be separated in one run with high resolution and the use of small sample volumes. CE has been used in complementary analysis studies with ICP-MS and ESI-MS for the identification of arsenic compounds in fish samples [63]. The use of CE has increased in recent years (although not as prevalent as HPLC); method development has addressed some of the traditional limitations associated with CE such as interfacing with detection systems [61, 64]. Electrochemical methods have been infrequently combined with other methods to obtain a comprehensive view of the dissolution of arsenic, (e.g., the electrochemical dissolution of arsenopyrite [65]) as well as the behaviour of soluble arsenic, such as the interaction of inorganic arsenic species (As(V) and/or As(III)) with carbonate [66, 67]).

2.3 Arsenic Speciation Analysis - HPLC-ICP-MS

A synopsis of methods used in arsenic speciation is provided in past reviews [68] and it is clear that the most commonly used method in arsenic speciation research today is HPLC-ICP-MS. There have been numerous studies and reviews pertaining to arsenic speciation analysis by HPLC-ICP-MS [69],[70]. The popularity of HPLC-ICP-MS for arsenic speciation analysis has resulted from its wide dynamic range (allowing for analysis of a diverse mixture of arsenic compounds at concentrations varying by orders of magnitude), high selectivity, and low detection limits (as low as 50 pg) [71]. The wide variety of HPLC separation methods available for arsenic (ion-exchange [72-74], reversed–phase ion-pairing [75-79] and micellar methods [80], etc.), which are easily linked to ICP-MS, are a key advantage. The versatility of HPLC separation methods allows “complementary” analysis where different HPLC methods are used (e.g., the use of ion exchange and ion pairing [81]); additional options for sample pre-treatment (e.g., hydride generation [82], oxidation with H_2O_2 [83]) have also been reported. This type of complementary analysis will not be included here as they are beyond the stated scope of this review (i.e., methods do not differ in fundamentals since HPLC-ICP-MS is used in these approaches).

As its name suggests, HPLC-ICP-MS requires liquid samples, which rely on extraction procedures to extract arsenic compounds, often from a solid sample, into an aqueous phase. Organoarsenic compounds are typically easily extracted from biological samples with common extraction procedures such as the use of methanol/water and water extractions. It has been commonly observed that methanol/water extractions are more efficient for marine samples, giving extraction efficiencies (EEs) of 70-100%, compared to other freshwater and terrestrial tissue types, giving EEs of 55 to 80% [1, 70]. A more aggressive sequential

extraction step is often performed following a methanol/water extraction to further extract any remaining arsenic compounds which are often found to be inorganic arsenic compounds [84]. Inorganic arsenic compounds are often found in terrestrial plants and in the residues of tissues previously extracted with methanol/water. Methanol/water extractions of plants and terrestrial invertebrates can often produce low EEs of less than 50% [85, 86]. Heated extractions using dilute nitric acid have proven useful in the extraction of inorganic species from plant samples. For rice samples this method results in EEs over 90% [87].

Such aggressive extraction schemes might change inorganic arsenic complexation in samples, such as unstable arsenic-sulfur compounds in plants (e.g., As-PCs, Figure 2.1). Studies trying to minimize the loss of these compounds during the extraction of plant samples have used 1% formic acid extractions that yield EEs from 70 to 100% [88]. The variability seen in the literature suggests that to achieve the highest possible extraction efficiencies, matrix specific extraction processing should be used. If some arsenic compounds are more easily extracted than others, and this depends on the extraction method selected and the sample matrix, the meaningfulness of the extraction technique comes into question and the results are operationally defined.

Another disadvantage of HPLC-ICP-MS is that it relies on matching chromatographic peaks to standards and provides no structural information about the separated compounds. The two factors, incomplete extraction and loss of molecular information, highlight the need to use complementary analysis methods to gain a better understanding of the arsenic chemistry occurring in the environment and biological samples. The two main methods that address the analytical limitations of HPLC-ICP-MS are XAS and ESI-MS.

2.4 X-Ray Absorption Spectroscopy and HPLC-ICP-MS

X-ray absorption spectroscopy (XAS) offers a solid state analytical technique for arsenic speciation. To obtain the necessary detection limits for analysis of arsenic in environmental samples, synchrotron-based radiation is used (giving typical detection limits of around 1-10 mg/kg, depending on experimental conditions). XAS of arsenic in environmental samples generally uses the principles of x-ray fluorescence; that is, incoming X-rays with energy at or higher than the binding energy of a core electron of the arsenic compound (E_O) are absorbed, ejecting a core electron and allowing another electron from the outer shells to fill the hole, emitting florescence [89]. X-ray absorption spectra are characterized by a sharp increase in absorption at specific X-ray photon energies giving rise to an absorption edge that is characteristic of the absorbing element [90]. The absorption edge corresponds to the energy required to eject a core electron; when a 1s electron is ejected this is called a K-edge. The white line energy is the most intense feature of

the absorption edge (energy at which the absorption is highest), Figure 2.3 [91]. For arsenic compounds the white line energy is taken to be representative of the K absorption edge.

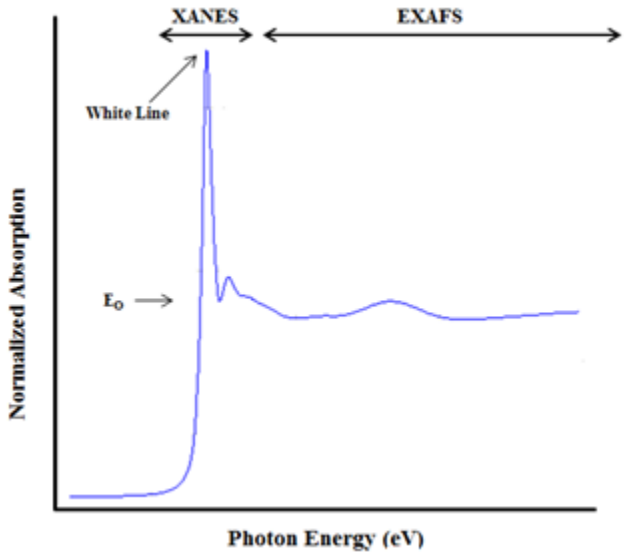

Figure 2.3: An example of an XAS spectrum.

White line energies for arsenic compounds are given in Table 2.1. It is immediately apparent that the energy is the same for arsenic compounds with the same nearest-neighbour environments, which is one of the limiting factors of XAS. For example AB, AC and TETRA have the same white line energy (11872.6) and may be misidentified without comparison with the HPLC-ICP-MS analysis. White line energies of the initial absorption edge are found in the X-ray absorption near-edge structure (XANES) region. The XANES region spans from a few eV below an element's edge to approximately 30 eV above the edge. The extended X-ray absorption fine structure (EXAFS) region is 30 eV up to 1000 eV above the K-edge and allows for the determination of direct structural information [91]. The most common use of XAS for arsenic speciation is XANES analysis, which reveals the most probable chemical environment around the arsenic, through the matching of spectral characteristics, including the white line energy, to those of standard compounds.

Table 2-1: Mean white line and E_O energies in eV for various arsenic standards. The white line or E_O energies for the aqueous and solid state are given for standards that exhibited different white line energies based on the state the standard was prepared. Extracted from [92-94].

Arsenic Compounds	White Line Energies (eV)	Reference
AsS (Realgar)	11896.0	94
MeAsDMPS	11870.0	92
$As(Glu)_3$	11870.0	92
MMA (III) ***aqueous form***	11870.8	92
$Me_2AsDMPS$	11870.8	92
As (III)-O	11871.7	92
AC	11872.6	92
TETRA	11872.6	92
AB	11872.6	92
Sugar A	11873.3	92
TMAO	11873.3	92
DMA (V) ***aqueous form***	11873.3	92
MMA (V)	11874.1	92
MMA (III) ***solid form****	11874.1	92
As (V)-O	11875.3	92
	E_O Energies (eV)	
Monothioarsenate	11871.3	93
Dithioarsenate	11870.3	93
Tetrathioarsenate ***aqueous form***	11869.8	93
Tetrathioarsenate ***solid form***	11869.3	93

* MMA(III) probably oxidized to MMA(V).

In the past the majority of arsenic speciation studies by XAS have focused on arsenic interactions with major mineral phases. However, in the last decade, XAS analysis has been used for the determination of arsenic species in terrestrial and marine organisms. XAS studies of biological samples have heavily focused on plants [85, 95-97] but have also included vertebrates [98, 99], invertebrates [24, 86, 100, 101], fungi [39], human cells [102] and protozoa [103]. Many of these studies have used XAS and HPLC-ICP-MS in a complementary way, a summary is presented in Table 2.2, organized by sample matrix, and the complementary aspects highlighted in the table are further discussed in the following text.

An important characteristic of XAS analysis is the ability to analyze the original or un-extracted solid sample, as close to the in situ state as possible. This has the potential to provide insight into arsenic species that may have been transformed during the extraction and concentration step for HPLC-ICP-MS analysis and it also allows for all of the arsenic present to be speciated in the sample. Thus arsenic compounds have been identified that appear to be unstable throughout the conventional sample preparation process, such as As(III) sulfur (As(III)-S) compounds, which can include arsenic bound to GSH, PCs and other sulfhydryl groups of biomolecules like the cysteine groups of enzymes [104]. A clear trend from the reviewed studies shows that for a wide range of sample matrices, As(III)-S compounds identified through XANES analysis were not seen in the corresponding HPLC-ICP-MS analysis methods that were used. This trend has been observed in biological samples including terrestrial insects, earthworms (*Lumbricus rubellus*), blue mussels (*Mytilus edulis*), and plankton (*Daphnia pulex*) exposed to high levels of environmental arsenic [24, 86, 101, 105]. The ability to obtain HPLC-ICP-MS chromatograms for As(III)-PCs [106], which are also As(III)-S compounds, suggests that it may be possible to develop HPLC methods that can separate As(III)-S compounds, but these have not yet been developed for the afore-mentioned examples, in which primarily anion-exchange HPLC was used.

The formation of As(III)-S bonds to form compounds, such as arsenic GSH compounds, are thought to play a role in toxicity mechanisms or to provide a way of dealing with high levels of arsenic exposure. Correctly identifying their presence in biological samples is an important tool in understanding arsenic metabolism and toxicity. Using both XAS and HPLC-ICP-MS can help provide these insights. For example in studies of both terrestrial earthworms (*Lumbricus rubellus*) and marine blue mussels (*Mytilus edulis*) organisms it was observed that at lower arsenic concentrations arsenic is found to be primarily composed of organoarsenic compounds. As the arsenic concentrations increase the arsenic speciation shows a decrease in the proportion of organoarsenic compounds (determined by HPLC-ICP-MS) and an increase in the proportion of As(III)-S compounds (determined by XANES) [101, 105]. One interpretation of these findings is that organoarsenicals are accumulated in organisms to a constant concentration as needed, beyond which only inorganic arsenic is accumulated. For example a possible use

for arsenobetaine, the major organoarsenical in the *Lumbricus rubellus* and *Mytilus edulis* studied, might be as an osmoregulator [31, 107, 108]. An alternate interpretation is that the mechanisms that are in place for the accumulation of organoarsenicals are saturated by the excess arsenic in the environment and transformations of inorganic arsenic to organoarsenic compounds cannot take place.

A review of studies in the literature that use XAS and HPLC-ICP-MS to report As(III)-S compounds in plant samples shows that the stability of these compounds during HPLC-ICP-MS analysis is related to the extraction and analysis method selected. For radish plants, *Raphanus sativus*, grown in arsenic contaminated mine waste, the majority of arsenic was found to be present as As(III)-S by XANES analysis while the HPLC-ICP-MS analysis showed the majority of arsenic to be composed of As(III) and As(V) [85]. Smith et al. attributed the instability of As(III)-S compounds in plants to sample preparation methods; specifically, As(III)-S compounds decreased with additional processing steps such as grinding and drying [85]. The rationale for this is that when a sample is ground, cellular components containing As(III)-S compounds may be broken apart and mixed with other cellular components, which in turn causes the As(III)-S bonds to disassociate, and allows As(III) to be easily oxidized to form As(V); the heat and oxygen inherent in drying would assist with oxidation. The absence of As(III)-S compounds in the HPLC-ICP-MS analysis method used could be attributed to the possible disintegration of these compounds in the ammonium phosphate or formic acid buffers used in the ion-exchange chromatography methods used [88], or alternatively, retention of these compounds by some HPLC columns [109]. The absence of As(III)-S compounds in HPLC-ICP-MS analysis, but their presence in XAS analysis, has also been seen in different sections of rice plants. A 2009 study by Lombi et al. [110] was the first report that rice husks and endosperm contain a majority of As(III)-S compounds. Both HPLC-ICP-MS and XAS were performed on the dried and ground sample and XAS analysis showed that some As(III)-S compounds survived these steps; the absence of As(III)-S compounds in the HPLC-ICP-MS analysis was attributed to the As(III)-S compounds potentially being oxidized during the microwave assisted extraction with 1% nitric acid. The XAS analysis found As(III)-S compounds made up 55.6% of the arsenic in the endosperm, 52.9% in the bran and 12.9% in the husk [110].

Table 2-2: Summary of reviewed papers that use XAS to complement HPLC-ICP-MS

Sample matrix	Findings	Complementary Aspects	References
Plants	As(III)-S compounds identified by XANES not found in HPLC-ICP-MS When exposed to high levels of arsenic most plants contain majority of arsenic as As(III)-S complexes Ratio of As(III) to As(V) can change during extraction process	A. XAS identifies compounds that are not extracted for HPLC-ICP-MS B. Minimal processing steps and solid state analysis in XAS analysis may help preserve As(III)-S that may degrade with freeze drying or under chromatographic conditions C. XAS confirms As(III)-S compounds identified during HPLC-ICP-MS (when reliability of an HPLC-ICP-MS for preserving and detecting liable compounds was established) D. XAS imaging indicates where compounds are located	23, 80, 110 – 112, 117, 119
Fungi	AB is major arsenic compound in *Agaricus bisporus* exposed to As(V)	E. HPLC-ICP-MS confirms organoarsenic compounds with similar white line energies for XAS	39
Terrestrial Invertebrates	As(III)-S compounds identified by XANES not found in HPLC-ICP-MS Proportion of organoarsenic compounds decrease with increasing total arsenic concentration	A, B F. HPLC-ICP-MS identifies trace arsenic compounds that are too low to be detected by XAS	86, 101, 113

Table 2.2 Continued

Sample matrix	Findings	Complementary Aspects	References
Marine/Freshwater Invertebrates	As(III)-S compounds identified by XANES not found in HPLC-ICP-MS	A, B, D, E, F	24, 84, 100, 105, 115
	Proportion of organoarsenic compounds decrease with increasing total arsenic concentration		
	Samples with lower extractions efficiencies contain higher proportions of inorgnic arsenic		
	As(V)-O are preferentially extracted over As(III)-S compounds		
	Unextracted arsenic can contain organoarsenic compounds		
	As(III)-S compounds localized to digestive tract, while AB and sugars found in surrouninding tissues		
Vertebrates	Unextracted arsenic can contain organoarsenic compounds	A, D E, F	98, 114

XAS has been used to help determine the reliability of an HPLC-ICP-MS analysis method for plant samples by confirming the presence of As(III)-S compounds, when the HPLC-ICP-MS method is tailored to the preservation of these species and the chromatography is appropriate for them. A 2008 study by Bluemlein et al. [111] of arsenic peptides in plant roots (*Thunbergia alata*) used XANES/EXAFS analysis to show that these compounds can be extracted with formic acid and separated using a reversed phase column without significant transformations occurring during the extraction step. In contrast to the preparation of the radish plants in the previously mentioned Smith et al. [85] study, the plant roots in the Bluemlein et al. [111] study had fewer processing steps during sample preparation. The roots were extracted within two hours after harvesting as fresh samples, and analyzed within four hours of the extraction. HPLC-ICP-MS was used in parallel with electrospray ionization mass spectrometry (ESI-MS) (a technique that will be discussed in more detail in Section 2.5 of this chapter), and this method could identify the presence of As(III)-PCs, which accounted for 55 to 64% of total arsenic. XANES analysis of the fresh plant roots was used to confirm that most of the arsenic occurred as As(III)-S (53%). The remaining arsenic was As(III)-O (38%) and unchanged As(V) (9%).

In a 2007 study to optimize the extraction of arsenic from plants collected from an arsenic contaminated site, XANES analysis was used to confirm that the ratio of As(III) and As(V) was preserved throughout the sample preparation and extraction process used after plant harvest (live plants or those immediately following harvest were not analyzed). XANES results of frozen wet and dried plants were similar, indicating that the drying and grinding steps used preserved the ratio of As(V)-O/As(III)-O (As(III)-S was not a predominant compound in these plants). However, the XANES spectra of the extraction residues indicated that transformations occurred during the extraction process [23].

While XANES analysis can be used to show the reliability of a method for preserving and detecting labile compounds like As(III)-S compounds in plants, HPLC-ICP-MS analysis is typically used to confirm the presence of arsenic compounds. HPLC-ICP-MS is needed to confirm the presence of many organoarsenic compounds in biological samples that exhibit similar white line energies (e.g., AB and the tetramethylarsonium ion (TETRA)) in their XANES. In the analysis of the common white button mushroom, *Agaricus bisporus*, these complementary methods allowed the confirmation of AB in the cap, and inorganic arsenic and DMA in the stalk of the mushroom, by comparing the HPLC-ICP-MS analysis with the XANES results [39].

As stated earlier, the ability for complete speciation by XAS allows for the determination of compounds in samples with low EEs, or those that are preferentially extracted. In many cases the organisms from more highly contaminated areas have lower EEs than those from less contaminated areas, and they

conveniently also have the higher concentrations typically required for XAS analysis (generally greater than 1 mg/kg). In general, samples with lower EEs contain higher proportions of inorganic arsenic, indicating these species may be more 'difficult' to extract. In a study of rice grains using HPLC-ICP-MS and μXANES it was found that the proportion of inorganic arsenic was larger in the μXANES analysis, which was interpreted to indicate that the 14-27% of un-extracted arsenic was in this form [112]. Another example was reported for blue mussels, *Mytilus edulis*, where samples from contaminated locations had EEs of 42 ± 2% while those from uncontaminated locations had EEs of 62 ±2% and in this case XANES analysis was able to show that the major arsenic compounds present in the contaminated location samples were inorganic arsenic and As(III)-S compounds [105]. In contrast, samples with higher EEs are generally samples with lower arsenic concentrations and higher proportions of organoarsenic compounds [24, 113].

In many cases HPLC-ICP-MS analysis is needed for the detection of organoarsenic compounds because they are often found to be below the detection limits of XANES analysis. Studies of field and laboratory exposed earthworms, *Lumbricus terrestris*, show organoarsenic compounds in the HPLC-ICP-MS analysis and only inorganic arsenic in the XANES analysis [113]; similar results were seen for terrestrial invertebrates [86] and vertebrates [99], freshwater plankton [24, 100], and marine invertebrates [105].

Several studies have also recently indicated that unextracted arsenic is not solely inorganic but can include organoarsenic compounds such as MMA, DMA, arsenosugars and TMAO [84, 100, 114]. In one case the EE of the organoarsenic was half that of the inorganic arsenic [115] even though the extraction method had been optimized for the marine snail samples studied [84]. It is not understood why the EE of these organoarsenic compounds exhibit such variation.

Knowledge about unextracted arsenic in the studies summarized above relies on the comparison of the original sample arsenic speciation with that obtained from extraction and HPLC-ICP-MS. A more direct method of making this comparison is through the analysis of residues. The analysis of residues is complicated by the potential changes that can take place from the extraction and residue preparation procedure itself, which can introduce analytical artefacts, such as a glycerol form of As(V) (a pentavalent, hexa-coordinated arsenic molecule) [116]. XAS analysis of residues has been carried out only infrequently, such as when XANES was used to analyze the residues of the extraction products from both water extractions and methanol/chloroform extracts from *Daphnia pulex*. The XANES results indicated that both As(V)-O and As(III)-S compounds remained in the residues, and that As(V)-O compounds are preferentially extracted over As(III)-S compounds, which remain trapped in the final residues [24, 100]. If successful, studies of residues by XANES can prove useful in completing the arsenic speciation profile for certain samples.

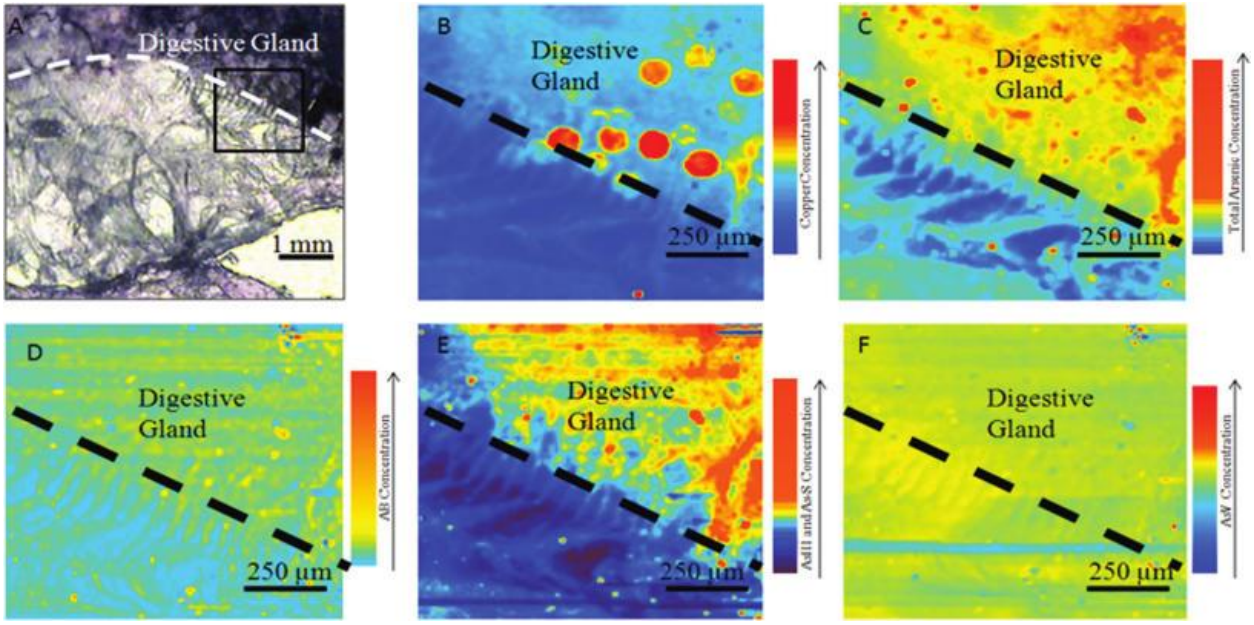

Figure 2.4: XAS map showing the distribution of different arsenic compounds in the digestive gland and surrounding tissue of a mussel (Mytilus edulis). A) Microscope photograph showing the mapped region outline in black. B) Location of digestive tubules in the digestive gland indicated by Cu (photon energy = 13696 eV). C) Total arsenic (13696 eV). D) Arsenobetaine (11872.6 eV). E) As(III) + As(III)-S (11871.7 eV). F) As(V) (11875.3 eV).Obtained from [105]with permission from the authors.

XAS can uniquely be used for imaging, which can further provide valuable complementary arsenic speciation information to HPLC-ICP-MS studies. XRF imaging techniques can be used to 'map' arsenic species in a sample, and this information can give more insight into the biological implications of arsenic in a study organism. XRF imaging studies that complement HPLC-ICP-MS analysis have examined radish plants, rice grains, blue mussels, plankton, insects and shrews [85, 86, 99, 100, 105, 112]. In these studies, XRF imaging showed the location in the sample of arsenic compounds identified in HPLC-ICP-MS and "bulk" (non-imaging) XANES. In both marine and freshwater organisms XRF imaging has shown that inorganic arsenic and As(III)-S compounds are found in the digestive tract of these organisms and organoarsenic compounds are found in other surrounding tissues [100, 105]. In an imaging study of a blue mussel (Figure 2.4), the high proportions of As(III)-S compounds concentrated within the digestive gland of the mussel (Figure 2.4e) were thought to be due to the thiol groups of proteins binding to arsenic, to sequester the metalloid and limit toxicological effects to the mussel. Arsenobetaine, identified by HPLC-ICP-MS (XAS cannot distinguish this compound from arsenocholine (AC) and TETRA) was found in small and similar concentrations in the digestive gland as well as the surrounding tissue (Figure 2.4d), suggesting AB is digested and absorbed into the surrounding tissues and distributed via the circulatory system [105]. Freshwater plankton showed a similar trend, with arsenopyrite (from diet) in the

digestive tract and organoarsenic compounds (arsenosugars and DMA, identified by HPLC-ICP-MS) in surrounding tissues [100].

The use of microbeam scanning (μXANES analysis), that is, the collection of XANES spectra at specific locations during XRF imaging, can provide information on the presence of separate compounds throughout the sample, but this does not provide accurate information on the proportion of these compounds for the whole sample [117]. Information on the overall proportions of compounds can be obtained through 'bulk' (whole sample) analysis with XANES and HPLC-ICP-MS. In a study of the distribution of arsenic and other nutrients in rice it was found using μXANES analysis that the distribution and speciation of arsenic was variable throughout the rice grain and that there were discrepancies between the μXANES and XANES/HPLC-ICP-MS analysis results. As mentioned earlier in this section, in this case the bulk XANES analysis was used to complement the bulk HPLC analysis and showed the presence of As(III)-S compounds not seen in the HPLC analysis. The proportion of DMA was consistent regardless of the method used [110].

When attempting to understand the degree to which arsenic compounds are not extracted through the examination of HPLC-ICP-MS and XAS data, a useful metric is the comparability of the two data sets. In the present context this is the degree to which such data might be expected to be the same. This is related to the uncertainty associated with each individual method. To obtain more information about this, the literature was reviewed for data pairs in which both HPLC-ICP-MS and XAS data were available, and the data were expressed as proportions of the total arsenic. In order to use the HPLC-ICP-MS data, a high extraction efficiency is needed, so that there is some confidence that the HPLC-ICP-MS data are representative of all or nearly all of the arsenic present in a sample; an extraction efficiency of >70% was selected. Data were not included in which only inorganic arsenic species had been detected by XAS, since as previously mentioned these are highly sensitive to redox and sample preparation conditions; good agreement has been seen in only a few studies [23, 111]. The data available are summarized in Table 2.3.

As can be seen in Table 2.3, few data pairs are available for this calculation and only four papers had data pairs that met the above criteria. Relative percent differences are seen to range greatly, and reflect the situations when a species was seen by one method (usually trace amounts of organoarsenicals by HPLC-ICP-MS, but also As(III)-S by XANES) but not another. For XANES analysis, most researchers consider proportions <5% to be of greater uncertainty [59]. Generally when an arsenic species (usually organoarsenic) was detected by HPLC-ICP-MS and not XANES, the HPLC-ICP-MS value was less than 10%. Therefore in the calculation of average RSDs, individual RPD values obtained from arsenic proportions less than 10% proportionally were excluded. To take into account the redox sensitivity of the

inorganic arsenic species, and the small individual proportions of the organoarsenic species, sums of inorganic arsenic and organoarsenic species were also calculated. The comparison of data resulted in average RPDs ranging from 28% (for DMA) to 56% (for arsenosugars), with the sum of inorganic arsenic (34%) and organoarsenic species (30%) around 30%. These results are entirely consistent with the uncertainty that would be inherent with extraction efficiencies as low as 70% but also with the uncertainties that might be expected in speciation analysis generally.

Therefore comparisons of XANES and HPLC-ICP-MS data should consider that a degree of uncertainty is inherent in the analysis and the comparison, which for the time being (until more data pairs are available) may be estimated to be around 30%.

XAS has proven to be useful and even crucial to understanding arsenic speciation in environmental samples, but it still suffers from several disadvantages. One limitation is that the techniques only provide semi-quantitative information about arsenic speciation; XANES spectra must be compared to known standards, and EXAFS data analysis must be accompanied by some educated guesses about the structure of the proposed compounds.

Another limitation is that the detection limits are quite high: as stated, typically in the mg/kg range. In mixtures, because of limitations in data processing software, arsenic compounds must be present in proportions greater than 5% of the total arsenic. As previously stated, proportions < 5% may be highly uncertain [59]. Longer experiment measurement times and specialized equipment can be used to improve detection limits. For example, a 2007 study by Meirer et al. [118] reports using synchrotron-radiation-induced total reflection x-ray fluorescence (SR-TXRF) analysis for XANES measurements of arsenic compounds in *Cucumis sativus* xylem sap to obtain detection limits of 30 ng/ml (µg/L) under argon atmosphere for 4.7 hrs, minimum, per xylem sap sample. Rice XANES results (<1 mg kg^{-1}) have been reported from up to 15 replicate runs [112, 119].

Longer measurement times are accompanied by another limitation that could apply to XAS analysis, which is beam damage. Beam damage can occur from the intensity of the X-ray beam and can be minimized through the use of a cryostage to allow measurements at low temperatures [120]. Additionally, moving the location for measurement of the sample for repeated runs [110, 112], while introducing sample heterogeneity as a factor into the measurement; can help to minimize beam damage.

Table 2-3: Comparison of HPLC-ICP-MS (ICP-MS) data with XANES data. Values are % of total arsenic. Data pairs were used that met the following conditions: (1) HPLC extraction efficiency >70% and (2) >10% organoarsenic by XANES. For calculation of the average RPD, only RPDs where both ICP-MS and XANES results were >10% were included. RPD = relative percent difference = 100% x │(ICP-MS – XANES)│/average of ICP-MS and XANES. Daphnia (extracted) sample XANES results were calculated by difference (unaltered sample – extraction residue). Sum iAs = sum of inorganic arsenic species = As(III)+As(V)+ As(III)-S. Sum oAs = sum of organoarsenic species. DMA+MMA+Sugars+AB+TETRA.

Sample		As(III)	As(V)	As(III)-S	DMA	MMA	Sugars	AB	TETRA	Sum iAs	Sum oAs	Reference
Daphnia extracted	ICP-MS	25	42	0	8	1	26			67	35	58
	XANES	0	31	0.4	0	0	68			31	68	58
	RPD	200	30	200	200	200	90			72	65	
Grace Lake zooplankton 180 um	ICP-MS	22	16	0	1	2	54	5		38	62	57
	XANES	0	15	18	0	0	67	0		33	67	57
	RPD	200	6	200	200	200	21	200		14	8	
Snails Site 1	ICP-MS	95		0		2	1	1	1	95	5	77
	XANES	74		4		0	11	3	0	78	14	77
	RPD	25		200		200	175	76	200	20	93	
Snails Site 3	ICP-MS	93		0		4	1	1	1	93	7	77
	XANES	48		35		0	7	9	0	83	16	77
	RPD	64		200		200	161	146	200	11	75	

Table 2-3 Continued

Sample		As(III)	As(V)	As(III)-S	DMA	MMA	Sugars	AB	TETRA	Sum iAs	Sum oAs	Reference
Snails Site 4	ICP-MS	85		0		4	2	7	2	85	15	77
	XANES	68		13		0	13	6	0	81	19	77
	RPD	22		200		200	153	16	200	5	24	
Rice R110013	ICP-MS	84	16	0						100	0	85
	XANES	0	21	78						99	0	85
	RPD	200	27	200						1		
Rice R110335	ICP-MS	16	1	0	88					17	88	85
	XANES			47	53					47	53	85
	RPD	100	100	200	50					94	50	
Rice NIES 10a	ICP-MS	53	11	0	43					64	43	85
	XANES		14	54	32					68	32	85
	RPD	100	24	200	29					6	29	
Rice R110331	ICP-MS	15	6	0	79					21	79	85
	XANES		6	35	58					41	58	85
	RPD	100	0	200	31					65	31	

Table 2-3 Continued

Sample		As(III)	As(V)	As(III)-S	DMA	MMA	Sugars	AB	TETRA	Sum iAs	Sum oAs	Reference
Rice R110015	ICP-MS	15		0	75					15	75	85
	XANES	18		9	73					27	73	85
	RPD	18		200	3					57	3	
Average RPD, only for both >10%					28		56			34	30	
N for average					4		2			10	7	

The generally high detection limits of synchrotron-based studies, combined with the lack of specificity in identifying some of the arsenic compounds, illustrates the continued need for HPLC-ICP-MS analysis for optimized identification of trace arsenic compounds present in complex environmental samples. The complementary nature of these two techniques will continue to be useful for environmental and biological studies of arsenic.

2.5 Electrospray Ionization Mass Spectrometry and HPLC-ICP-MS

Another complementary combination of arsenic speciation analysis methods is ICP-MS and ESI-MS detection following HPLC separation. As previously stated, when ICP-MS is used as a detector, molecular information is lost, since arsenic compounds are completely atomized in the plasma. ESI-MS, on the other hand, uses soft ionization to provide molecular information, usually maintaining molecular ions of macromolecules such as proteins and peptides [59].

For arsenic speciation analysis ESI-MS can be coupled to HPLC which generally increases sensitivity because the HPLC provides a sample pre-cleaning step by decreasing the amount of co-eluting matrix compounds. This can help to make detection limits more comparable to ICP-MS analysis although some species may have detection limits that are orders of magnitude higher than other species of the same element [121]. It should be noted that ESI-MS on its own does not provide a powerful speciation method for arsenic. Limitations of this analytical method for elemental speciation analysis have been previously reviewed [121]. Overall ESI-MS alone is not suited for arsenic speciation analysis because it is not element specific, some arsenic species are more difficult to ionize (e.g., DMA(III)) and ionization efficiency is suppressed by co-eluting matrices [121].

ESI-MS becomes a more powerful molecular analysis method for arsenic speciation analysis when used with a triple quadrupole mass spectrometer (tandem MS) or ion-trap systems and selected reaction monitoring (SRM) or selective ion monitoring (SIM) modes are employed. SRM can enable sensitive and selective detection and conditions can be optimized by examining measured or published collision induced dissociation (CID) breakdown curves. The CID behaviour for various organoarsenic compounds in crude extracts has been examined using HPLC-ESI with tandem MS and HPLC-ESI with ion trap MS that have given biologically relevant limits of detection (LODs) [122-127]. The use of optimized SRM or SIM conditions (generated from CID breakdown curves) for ESI with tandem MS or ion trap MS from the literature can eliminate the need for complex synthesized standards that are often not readily available when the identity of arsenic compounds are predictive. However, true unknowns cannot be identified in this way.

ESI-MS combined with HPLC and ICP-MS as a complementary method ultimately provides the best use of ESI-MS for arsenic speciation analysis. This is especially useful when the two detectors, ESI-MS and ICP-MS are used simultaneously for HPLC, by splitting the eluate from the HPLC; this instrumental arrangement is also known as "on-line" [121]. This simultaneous detection allows element-specific (i.e., As-containing compounds) and molecular information to be collected at the same time. While HPLC-ICP-MS relies on the comparison of sample extracts with arsenic standards, ESI-MS can be used to identify compounds without the need of standards. Off-line systems can also be used, where HPLC-ICP-MS is used to identify the fractions containing arsenic, which are then collected and then subjected to flow-injection ESI-MS. In this type of off-line system the fractions may be further manipulated for optimized analysis by ESI-MS. The disadvantages of this are that there is greater risk of species transformation during the process and these systems cannot be easily automated [121].

As in all arsenic speciation analysis methods the use of ESI-MS parallel to ICP-MS must be optimized. A mobile phase compatible with both HPLC-ICP-MS and ESI-MS must be selected when using these analysis methods in the on-line configuration. Finding an optimal mobile phase for both ESI-MS and ICP-MS is not straightforward. For efficient desolvation and ionization during ESI-MS analysis solvents with high volatility and a surface tension around 20 mN/m, such as acetonitrile or methanol, are often used [128]. However, these solvents can decrease plasma stability and leave deposits of reduced carbon on instrumental parts causing an increase in detection limits during the ICP-MS analysis. Chilled spray chambers (to partly condense the organic solvent vapor) in combination with addition of oxygen to the plasma (to enhance the combustion of the remaining carbon) have been used to help overcome these problems during ICP-MS analysis [129]. A 2010 study by Nygren and Bjorn [128] evaluated organic solvents for their compatibility with ESI-MS and ICP-MS analysis methods. The solvents were selected based on four criteria for optimal LC-ICP-MS performance: (1) minimal carbon deposition on ICP-MS instrumental parts, (2) minimized oxygen additions to the plasma, (3) minimal effect from increased amount of solvent (during gradient elution) on analyte signal intensity and signal-to-noise ratio; and (4) high eluent strength of the solvent so that only low solvent concentrations are needed during the separation phase [128]. It was found that n-propanol, using gradient elution and no oxygen addition to the plasma was a suitable organic solvent for the mobile phase when used for routine online use of LC-ICP-MS and ESI-MS.

However this solvent is not commonly used; the majority of online HPLC-ICP-MS and ESI-MS methods for arsenic speciation use a combination of formic acid and methanol, often with gradient elution. Diluted formic acid (1%) has been found to stabilize PCs compounds and when dilute amounts of methanol have been used the mobile phase has shown to be compatible with ICP-MS [130]. Detection of As-GSH

compounds by HPLC-ICP-MS using a mobile phase in a gradient method from 2 to 12% v/v methanol caused changes in ICP-MS sensitivity and background which resulted in differences in the LODs for the As-GSH compounds [123].

ESI-MS has been used to complement HPLC-ICP-MS arsenic speciation analysis in numerous studies, and a summary is presented in Table 2.4, organized by sample matrix. A review of these studies reveals that the methods complement in each other in three major ways: (1) ESI-MS is used to provide structural information for unknown peaks from HPLC-ICP-MS; (2) ESI-MS is used to confirm the structure of compounds identified by HPLC-ICP-MS; and (3) each system can be used to provide additional resolution power: HPLC-ICP-MS helps to focus ESI-MS selection of ions and ESI-MS is used to aid in peak resolution in mixtures with unresolved or co-eluting peaks by HPLC-ICP-MS.

One of the complementary aspects identified from studies using both HPLC-ICP-MS and ESI-MS is that ESI-MS can be used to provide the molecular information needed to identify unknown peaks found in the HPLC-ICP-MS analysis. On its own the use of HPLC-ICP-MS can be limited in identifying novel, or unknown, arsenic compounds because the compounds must be matched to a known standard, since molecular information has been lost. In the past much of the identification of unknown arsenic species was based on the use of nuclear magnetic resonance (NMR). Development of methods using ESI-MS now allow for the identification of unknown arsenic compounds in many different types of sample matrices with less sample purification [29]. The first report of DMAA as a major metabolite of arsenosugars in sheep's urine was achieved by using ESI-MS to identify a major unknown peak found during the HPLC-ICP-MS analysis of the urine samples [29]. In the same way the thiolated analogue of the DMAA compound was also first identified in sheep's urine as a minor metabolite of arsenosugars [131].

From the marine environment, trimethylarsoniopropionate was identified from an unknown peak in the HPLC-ICP-MS analysis by ESI-MS in the tissues of a sperm whale, *Physeter catodon* [27]. A number of different thiolarsenosugars were first identified in mussels, other molluscs and shellfish from the structural information provided by ESI-MS analysis for unknown peaks found during the HPLC-ICP-MS analysis [132, 133]. In a follow up to these studies two additional new thiolarsenosugars (DMThioAsSugar Sulfonate and DMAThioAsSugar-Sulfate) were identified in giant clam extracts and for the first time in algae [134]. Further study of giant clam extracts using ESI-MS to identify unknown compounds from HPLC-ICP-MS analysis also led to the first identification of three additional thiolarsenosugars [135].Three aliphatic dimethylarsinoyl compounds, thought to be potential metabolites in the biogenesis of AB, were identified as minor compounds in over 20 marine samples, and the use of ESI-MS showed for the first time the presence of DMAE and dimethylarsinoyl propionate (DMAP) in

marine samples [136]. For marine algae TETRA and arsenosugars were also identified by ESI-MS when they were not matched to a standard during the HPLC-ICP-MS analysis [137].

Unknown peaks in the analysis of freshwater snails with HPLC-ICP-MS led to the complementary use of ESI-MS which identified these unknowns as thiolarsenosugars [138].

Table 2-4: Examples of studies organized by sample matrix, in which HPLC-ICP-MS and ESI-MS were used in a complementary way.

Sample matrix	Findings	Complementary Aspects	References
Marine algae (Seaweed)	Arsenosugars, thiolarsenosugars and lipid soluble arsenic compounds First identification of 5-dimethylarsinoyl-B-ribofuranose in algae	A. ESI-MS used to confirm compounds identified by HPLC-ICP-MS. B. HPLC-ICP-MS helps to focus ESI-MS selection of ions	137, 144 - 146
Marine Organisms (Mammals Fish/Invertebrates)	Arsenosugars and thiolarsenosugars Arsine sulfide containing arsenosugars first identified in mollusc Novel thiolarsenosugars identified in the giant clam First identification of DMAA, DMAE and DMAP in marine samples (CRMs) First identification of trimethylarsoniopropionate in whale Arsenolipids identified and their fatty acid chains characterized in cod liver oil and fish meal	B C. ESI-MS used to provide structural information for unknown peaks from HPLC-ICP-MS. D. ESI-MS further resolves HPLC-ICP-MS by identifying co-eluting compounds	27, 132 – 136, 149, 154, 155

Table 2-4 Continued

Sample matrix	Findings	Complementary Aspects	References
Plants	As(III)-PC complexes	A, B, C	130, 139 - 141
	As-PCs are generally unstable during typical sample preparation steps; optimized approach provided		
	Proportion of different As-PCs differ for plants with varying tolerance levels		
	PC_2 an intermediate for the formation of PC_3		
	When PC synthesis is inhibited arsenic uptake decreases		
	Bioaccessible arsenic in rice may contain thiolarsenicals		
Freshwater Organisms	Thioarsenosugars unstable during long term storage	A, B, C	138
Biological Samples (Urine, Intestinal cultures)	Thiolarsenicals in urine samples	A, B, C	82, 29, 131, 147, 148
	Intestinal microflora transform DMTA to TMAS		
	Novel arsenosugar metabolite, DMAA, in sheep's urine		
	Ratio of protein bound arsenic to total arsenic in blood decreases as arsenic exposure increases		

In plant tissues exposed to high levels of arsenic As(III)-PCs have also often been identified using ESI-MS to identify unknown compounds from the HPLC-ICP-MS analysis. The analysis of As(III)-PC compounds using HPLC-ICP-MS and ESI-MS has already been reviewed [139, 140]. As mentioned previously, the labile nature of some As(III)-PC compounds during chromatographic analysis requires that studies of these complexed arsenic compounds in plants take precautionary steps during sample preparation and analysis to ensure the survival of these compounds. The use of offline systems with processing of the extract after HPLC-ICP-MS analysis can result in a loss of As(III)-PC compounds and on-line systems with a mobile phase with a low pH can help to stabilize these compounds [130]. Further information on how this complementary analysis has resulted in a more complete picture of arsenic compounds present, and arsenic metabolism in plants, can be found in the previously mentioned reviews [139, 140].

The use of ESI-MS to complement HPLC-ICP-MS in the enzymatic extraction of arsenic from rice (to examine the arsenic species present in the bioaccessible fraction of rice) enabled the identification of a then unknown compound observed in the HPLC-ICP-MS analysis as dimethylthioarsinic acid [141].

Unknown compounds have also been identified, when not matched to a standard, by ESI-MS/MS in a study of roxarsone in contact with soil of volcanic origin to show the major breakdown products include As(III)-containing organoarsenicals, as well as chlorinated arsenic compounds [142].

For many of the above studies, after the initial identification of the unknown compound with ESI-MS the compound was synthesized to allow additional confirmatory analysis. This typically involved matching the retention time of the synthesized standard and unknown peak by HPLC-ICP-MS analysis as well as comparing the standard and sample again with ESI-MS. For example, in the identification of DMAA in sheep's urine, DMAA was synthesized from DMAE and the identification was confirmed by matching retention times of the DMAA peak in the sample and the standard, and a sample of urine was also spiked with the DMAA standard to further confirm its presence [29]. In a similar way DMAE and DMAP were confirmed to be in marine samples [136].

In some cases an educated guess or prediction of the unknown compounds found during the HPLC-ICP-MS was made to help refine the ESI-MS analysis to target these compounds and synthesize the appropriate standards that could be used for additional confirmation. The presence of thiol analogues of some arsenic compounds can be predicted based on the behaviour of the unknown compound during changing chromatographic conditions. During the analysis of canned mussels when the extracts were left at room temperature over time, the proportion of one of the unknown compounds was found to decrease,

as the proportions of arsenosugar 1 and 2 increased; when treated with H_2O_2 the unknown disappeared indicting the possibility of thiol compound [132].

Low column recoveries of arsenic compounds can also indicate the presence of thiol compounds as they can be found to be retained on some columns [109]. Changing the pH of the mobile phase and chromatographic conditions can help to elute these compounds, another indication of the presence of thiolarsenicals [132, 133]. The thiol analogues of arsenic compounds are commonly synthesized by bubbling H_2S gas through the arsenic compound in order to be used to confirm identifications made by the initial ESI-MS analysis [131-133, 135, 138, 141]. These additional confirmation steps are often necessary for the identification of new arsenic compounds to ensure an accurate identification has been made.

The second way in which ESI-MS is used to complement HPLC-ICP-MS is to confirm the identity of compounds identified during the HPLC-ICP-MS analysis by matching retention times. For samples from the marine environment, specifically algae, ESI-MS has been used to confirm the presence of arsenosugars in the characterization of brown algae, *Fucus serratus* [143]. Studies of the chemical stability of arsenosugars, bioaccessible arsenic from seaweed, and method development for the extraction of arsenic from seaweed have all used ESI-MS as a confirmatory analysis tool for reporting the presence of arsenosugars [144-146]. For biological samples ESI-MS has been used to confirm the presence of thiolarsenosugars as a biotransformation product of arsenosugars in mouse anaerobic cecal contents, indicating this thiol analogue may be an important metabolite in the degradation pathway of arsenosugars to DMA [147].

This complementary use of HPLC methods has also aided to further elucidate arsenic methylation pathways in mammalian systems. A study of the transformations of dimethylthioarsinic acid (DMTA), a compound produced by the anaerobic microbes of mouse cecum, showed that direct methylation of this DMTA forms trimethylarsine sulfide (TMAS)[148]. The presence, for the first time, of monomethylmonothioarsonic acid (MMMTA) and dimethylmonothioarsinic acid (DMMTA) in the blood of arsenite treated rats was also confirmed by ESI-MS analysis in this way [83].

A question that arises from complementary use of ESI-MS to confirm identifications made during the HPLC-ICP-MS analysis is: when does only matching the retention of these compounds become sufficient for making identifications? The identification of arsenosugars in particular depends on well-characterized algal extracts, since authentic standards are typically not synthesized. Characterization of such algal extracts, for example, from brown algae, has included confirmation of the arsenosugars identified during the HPLC-ICP-MS analysis with ESI-MS as a form of quality control [143]. Such algal extracts are then

often used for HPLC-ICP-MS analysis of other samples, without further confirmation [100, 138], although if resources permit, peaks identified by HPLC-ICP-MS may still be confirmed with the use of ESI-MS [144, 145]. This was also the case for the identification of thiolarsenosugars [147] and trimethylarsine sulfide (TMAS) and a methylation product of dimethylthioarsinic acid in the analysis of anaerobic microbes from the cecal contents of a mouse [148].

Studies that characterize a certified reference material (CRM), usually as part of the method development, can provide speciation information for arsenic compounds found in the CRM, for which standards are not readily (commercially) available. For example, in a study examining the potential precursors for AB in marine samples, the CRM TORT-2 was found to contain DMAA, DMAE and DMAP at higher concentrations than other CRMs analyzed, and the authors suggested the possibility of using TORT-2 as a standard for these compounds in future studies, similar to the way well-characterized algae extracts are used to identify arsenosugars [136]. In a study reporting two new thiolarsenosugars (DMThioAsSugarSulfonate and DMThioAsSugar-Sulfate) in commercially available marine algae samples (Canadian kelp powder, batch no. 231-0390-15), the relatively high concentrations of these compounds led the authors to suggest its use as a standard in future studies [134]. In both these studies the arsenic compounds were determined by matching retention times with a synthetic standard and spiking the sample with the standard during the HPLC-ICP-MS analysis, and then comparing the standard and sample during the ESI-MS analysis.

The third complementary aspect of HPLC-ICP-MS and ESI-MS is the additional resolution power provided when both systems are used together. The use of two detectors for HPLC gives enhanced ability to resolve coeluting compounds; ICP-MS detection can determine which of the coleuting compounds contain arsenic. For example, ESI-MS in SRM mode was used to detect TMAO, oxo-glycerol and oxo-sulfate-arsenosugars in a certified reference material , which had co-eluted with other compounds during HPLC-ICP-MS analysis [149]. The use of ICP-MS detection also helps to focus the ESI-MS selection of ions. In marine samples ICP-MS detection was used to pin-point arsenic-containing fractions to be identified by ESI-MS, resulting in four novel thiolarsenosugars to be identified in giant clam extracts *Tridacna maxima* and *T. densa* [135]. A study of the breakdown products of roxarsone in the environment also used an online system to locate arsenic-containing fractions with ICP-MS, and to identify the major metabolites with the molecular information provided by ESI-MS [142].

The maximum information can be obtained when the three methods HPLC-ICP-MS, HPLC-ESI-MS, and XAS are combined in a complementary way. To date, only a 2008 study by Bluemlein et al.[111] has combined the three methods, showing for the first time that As-peptides can be extracted by formic acid

and chromatographically separated without significant decomposition. Only parallel use of HPLC-ICP-MS and ESI-MS could identify the presence of As(III)-PCs, which accounted for 55 to 64% of total arsenic. XANES analysis of fresh plant roots exposed to As(V) was used to confirm that most of the arsenic occurred as As(III)-S (53%), while 38% of the arsenic occurred as As(III) and only 9% remained as unchanged As(V). By combining these methods the presence of As(III)-PC complexes could confidently be reported and the important role of PCs in coping with arsenic exposure was demonstrated.

One of the major disadvantages of using ESI-MS is that it typically does not have the same low LODs offered by HPLC-ICP-MS without additional sample clean up or optimization. ESI-MS LODs are compound specific. The best LODs reported were obtained when SRM conditions were optimized and the arsenic compounds were isolated or synthesized; LODs for various arsenosugars and AB were as low as 1.6 µg/L [122]. In contrast some synthesized As-GSH compounds ESI-MS LODs are as high as 210 and 1123 µg/L [123]. However when used for the analysis of crude biological samples these LODs can be much higher. For example, in the study of arsenic compounds in seaweed and plant root extracts, there were trace unknown compounds identified by HPLC-ICP-MS that were too low to be detected or identified by complementary ESI-MS [126, 130].

Arsenolipids are a group of organoarsenicals whose original identification and present-day analysis rely heavily on a complementary approach of element-specific detection with mass spectrometric identification of molecular structure. The earliest studies depended on organic solvent extractions of marine samples, targeting lipid compounds, with hydrolysis to allow aqueous analysis of the arsenicals [150]. This resulted in a loss of information about the lipids, but identified that the arsenic was bound to the assumed lipids in dimethylated ($(CH_3)_2As(O)$-R) [151] and trimethylated ($(CH_3)_3As$-R) structures [152]. Later ICP-MS detection was modified with the introduction of oxygen to counter deposition of carbon from organic solvents, allowing the detection of arsenolipids by HPLC-ICP-MS but without identification of the compounds [153]. With ESI-MS detection, some of these compounds were identified as arsenic-containing long chain fatty acids [154] and the complementary approach has since become necessary to identify arsenolipids without standards [155]. The class of arsenolipids with long hydrocarbon chains, the arsenic-containing hydrocarbons, have been found to be suitable for GC separation and a combination of detection methods (ICP-MS, GC-EI-qMS, TOF-MS) used in a complementary fashion was emphasized in their identification [156],[157]. A comprehensive review by Sele et al. 2012 is available [158].

2.6 Conclusions

Overall, the use of two complementary analytical methods for the determination of arsenic speciation in environmental samples provides a more complete picture of arsenic chemistry in the environment, arsenic metabolism in various organisms and the potential environmental impact and risk associated with these arsenic compounds. The most commonly used method for arsenic speciation analysis, HPLC-ICP-MS, has two major disadvantages that can lead to data gaps in the accurate determination of arsenic compounds. These are that HPLC-ICP-MS relies on the extraction of arsenic compounds and provides no structural information.

Using XAS to complement HPLC-ICP-MS analysis is advantageous in that XAS can provide analysis of the solid state, or unaltered samples. This allows the identification of compounds that may be difficult to extract or labile during the chromatographic analysis, and therefore, often missed by HPLC-ICP-MS analysis. XAS imaging can also be used to show the distribution of arsenic compounds in various organisms and tissues. Complementary use of ESI-MS provides structural confirmation of compounds identified by HPLC-ICP-MS, identification of unknown arsenic compounds observed during HPLC-ICP-MS analysis and can aid in peak resolution in mixtures with unresolved or co-eluting peaks by HPLC-ICP-MS.

Complementary analysis with XAS should also be used, when total arsenic concentrations are high enough, to overcome the dependence on EEs by examining the unaltered sample. When extraction procedures have been optimized and it is predicted a sample will contain more complex arsenic compounds, specifically larger biomolecules that do not have commercially available standards, ESI-MS should be used in addition to HPLC-ICP-MS. It is important to accurately identify all arsenic compounds in a sample (not just the extractable arsenic) in order to obtain a better understanding of arsenic chemistry in the environment and accurate information when determining the potential risk associated with the arsenic. The more complete picture of arsenic speciation provided by complementary analysis has provided valuable information on the transformation and distribution of arsenic in the environment and should continue to be used, or expanded, in arsenic speciation studies.

Chapter 3[2]

3 Arsenic speciation in edible mushrooms

3.1 Abstract

The fruiting bodies, or mushrooms, of terrestrial fungi have been found to contain a high proportion of the non-toxic arsenic compound arsenobetaine (AB) but data gaps include a limited phylogenetic diversity of the fungi for which arsenic speciation is available, a focus on mushrooms with higher total arsenic concentrations, and the unknown formation and role of AB in mushrooms. To address these, the mushrooms of 46 different fungus species (73 samples) over a diverse range of phylogenetic groups were collected from Canadian grocery stores, background and arsenic-contaminated areas. Total arsenic was determined using ICP-MS, and arsenic speciation using HPLC-ICP-MS and complementary X-ray absorption spectroscopy (XAS). The major arsenic compounds in mushrooms were found to be similar among phylogenetic groups and AB was found to be the major compound in the Lycoperdaceae and Agaricaceae families but generally absent in log-growing mushrooms, suggesting the microbial community may influence arsenic speciation in mushrooms. The high proportion of AB in mushrooms with puffball or gilled morphologies may suggest that AB acts as an osmolyte in certain mushrooms to help maintain fruiting body structure. The presence of an As(III)-sulphur compound, for the first time in mushrooms, was identified in the XAS analysis. Except for *Agaricus* sp. (with predominantly AB), inorganic arsenic predominated in most of the store-bought mushrooms (albeit with low total arsenic concentrations). Should inorganic arsenic predominate in these mushrooms from contaminated areas, the risk to consumers under these circumstances should be considered.

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[2] Nearing, M. M.; Koch, I.; Reimer, K. J., Arsenic Speciation in Edible Mushrooms. *Environmental Science and Technology* 2014, 48, (24),14203-10

3.2 Introduction

A large number of different arsenic compounds have been identified in the environment and over 50 different arsenic species are thought to exist [1]. The toxicity of arsenic is largely dependent on its chemical form, with the most toxic forms generally being inorganic, including arsenite (As(III)) and arsenate (As(V)) [159-161]. Organoarsenic(V) compounds (those containing an arsenic carbon bond) are generally considered to be less toxic [9] but only arsenobetaine (AB) is considered to be non-toxic, based on the fact that an LD_{50} could not be established [2]. A large number of marine organisms contain arsenic primarily as AB [1], but this compound is less prominent in the terrestrial environment, occurring at much lower proportions in terrestrial organisms. An exception is the fruiting bodies, or mushrooms, of terrestrial fungi. Mushrooms have been found to contain a variety of organoarsenic compounds, and a higher proportion of AB [17].

In the marine environment the formation of AB is thought to be derived from the degradation of arsenosugars [1, 162, 163]. AB's structural similarity to the osmolyte betaine and the fact that its uptake and elimination in some organisms changes in response to salinity [31] has led to the hypothesis that it may be adventitiously taken up as an osmolyte in marine organisms. The role of AB in mushrooms is unknown, as is the life stage at which the fungus produces AB, or whether the fungus, alternatively, accumulates AB from the surrounding environment. The presence of arsenosugars has been reported in very few mushroom samples [164, 165], which may indicate differences in AB formation between the terrestrial and marine environments. A limited number of arsenic speciation studies for mushrooms has been reported, with the majority of studies published in the 1990s [18, 30, 38, 164-167]; more recent studies only included total arsenic concentrations with no speciation results [168-171] or incomplete speciation information (only iAs information) [170, 172]. In addition the diversity of phylogenetic groups evaluated for arsenic speciation is limited and analytical techniques of the time biased the analysis towards mushrooms containing high concentrations of arsenic [18, 30, 164, 165, 173-176].

Therefore a comprehensive study of arsenic species in a variety of fungus species from grocery stores, background areas and arsenic contaminated areas in Canada was performed to address these data gaps, and specifically to achieve two major objectives. The first objective was to analyze mushrooms over a range of arsenic concentrations that are lower than in past studies and more applicable to consumers of mushrooms as food. Consequently, mushrooms considered to be edible were largely targeted. The second objective was to identify trends associated with arsenic species occurring in a variety of mushrooms to elucidate the potential role or formation of AB in mushrooms. A total of 46 different mushroom species were collected and analyzed for total arsenic and arsenic speciation. Total arsenic was determined using

ICP-MS. Arsenic speciation was determined using HPLC-ICP-MS, and complementary X-ray absorption spectroscopy (XAS) when sufficiently high arsenic concentrations were present.

3.3 Materials and Methods

3.3.1 Chemicals and Reagents

Chemicals and reagents used for the total arsenic and arsenic speciation analysis are listed in Appendix A.1.

3.3.2 Collection and Preparation of Samples

Mushrooms were collected from local grocery stores, from background areas in Ontario (ON), British Columbia (BC) and Yellowknife (YK) in the Northwest Territories (NT) Canada. Background areas are those not known to be affected by anthropogenic activities that would introduce arsenic to the environment. Mushrooms were also collected from areas impacted by historical gold mining activities, referred to as contaminated locations. Overall there were six sampling sources for the collected mushrooms: store bought, BC (which was only background), ON background, ON contaminated, YK background and YK contaminated. Each sampling source contained multiple sampling locations that are further described in Appendix A (Table A1 and Figure A1).

Wild mushrooms picked by hand and fresh mushrooms obtained from the grocery store were stored in paper bags and kept cool until the time of processing in the laboratory (maximum 2 days), which consisted of washing thoroughly with tap water to remove soil and other particles, freezing them whole, and freeze- or oven-drying them. Dried mushrooms, including those obtained as such from the grocery store, were pulverized with a ceramic mortar and pestle and kept at room temperature until analysis.

Mushrooms were identified using the National Audubon Society Field Guide to North American Mushrooms. When a clear identification could not be made by examining physical features and collected spores the sample was sent for third party DNA sequencing for identification.

3.3.3 Arsenic Species Extraction

Duplicate extractions were carried out for each collected mushroom, following methods described by Whaley-Martin et al., 2012 [84], using 0.5 g of dried sample per replicate and 10 mL of 50% (by volume) aqueous methanol in a 50 mL Fisherbrand® disposable polypropylene centrifuge tube. The mixtures were vortexed and shaken end-over-end for 12 hours at room temperature, then placed in an ultrasonic bath for 20 min before being centrifuged at 3500 rpm for 30 min at 15 °C. The supernatant was decanted and the ultrasonication and centrifugation steps were repeated 2 more times, collecting all the supernatants together. Methanol was removed from the supernatant by evaporation at 60°C (Rapidvap, Labconco) to a

final volume of 5 mL. Extracts were syringe filtered using disposable 0.45 µm filters (Millipore® polypropylene 25 mm diameter hydrophilic PVDF durapore membrane) into 15 mL Fisherbrand® disposable polypropylene centrifuge tubes and kept frozen until analysis.

For Yellowknife samples the residues from the aqueous methanol extraction (Step 1) were sequentially extracted with 2% HNO_3 (Step 2); this additional extraction step was included since the Yellowknife samples were anticipated to contain the highest concentrations of arsenic, some of which was hypothesized to be inorganic arsenic and require a more rigorous extraction [84]. Step 2 consisted of heating the residues with 5 mL of 2 % HNO_3 for 2 hours at 70°C, placing in an ultrasonic bath for 30 min, and centrifuging at 3500 rpm for 30 min at 15 °C. The supernatant was decanted and syringe filtered and stored as before.

3.3.4 Total Arsenic Digestion

The total arsenic concentration within the mushrooms was determined by adding the total arsenic in the extracts (for Yellowknife samples, Step 1 + Step 2) and the total arsenic in the final residues from the extraction. Extraction residues were digested with 10 mL 70 % HNO_3 at 120°C until the sample was reduced to 1 -2 mL. Samples were then diluted with distilled deionised water and syringe filtered as before.

3.3.5 Instrumental Analysis

All samples (extracts and residues) were analyzed for total arsenic using ICP-MS. The ICP-MS operating conditions are described in Caumette et al., 2011 [24] (Appendix A.2). All aqueous methanol and HNO_3 extracts were analyzed for arsenic speciation using HPLC-ICP-MS (cation and anion exchange). The HPLC-ICP-MS operating conditions are described in Caumette et al., 2011 [24] (Appendix A.3).

3.3.6 Arsenic Speciation Analysis using XAS

X-ray absorption near edge structure (XANES) analysis was performed at the Advanced Photon Source (APS) Pacific Northwest Consortium/X-ray Science Division (PNC/XSD) on the bending magnet (BM) beamline, Sector 20. XANES spectra of the arsenic Kα-edge (11686 eV) were recorded in fluorescence mode using a solid state Ge detector (Canberra model GL0055PS) while monitoring incident and transmitted intensities in N_2-filled transmission ionization chambers. Dried and homogenized mushroom samples were placed in a sample holder, held between two layers of Kapton™ tape and kept at 100K using a liquid N_2 cryostat (Model 22 CTI Cryodyne Refrigerator System, Janis). A total of 5 to 7 scans were collected with a 0.5 eV step size over the edge region and averaged prior to background removal and normalization to edge jump. The Si(111) double crystal monochromator was calibrated using the first inflection point of the gold LIII absorption edge (11919.7 eV). A reference gold foil was measured

simultaneously with all samples. Additional experimental setup information can be found in Smith et al., 2005 [92]. XANES spectra of the arsenic K-edge (11868 eV) were fit within -20 to +30 eV from the arsenic E_O using Athena software. Frozen As(III), As(V) and As(V) glycerol standards [116], and liquid AB, DMA, TMAO, and arsenic glutathione ($As(Glu)_3$) [92] previously measured by our group were used for fittings.

3.3.7 Statistical Analysis

Statistical analysis was carried out using XLSTAT Version 2014.1.10 with Microsoft Excel 2010. Correlations were tested using a Pearson's R correlation coefficient test for samples from each source and for all sources combined.

3.3.8 Quality Assurance and Quality Control (QA/QC)

QA/QC results are summarized in Appendix A (Table A2) and were generally considered acceptable with some following comments. The limit of detection for total arsenic in YK samples was higher than the other samples (0.07 mg/kg) because of higher dilution factors. The average RPD was 15% for total arsenic (n=73 samples) with ten samples having an RPD greater than 30%, which may be explained by sample heterogeneity or a total arsenic concentration close to the limit of detection. The total arsenic recovery for the CRMs were also determined by adding the total arsenic in Step 1 and 2 extracts and residues and recoveries ranged from 73 – 104%, with an average recovery of 90%. Column recovery, listed in Appendix A (Table A3) (sum of species by HPLC-ICP-MS/total arsenic in extract) for the analysis of aqueous methanol extracts ranged from 63 – 131% and the average recovery was 100%. Column recovery for the analysis of HNO_3 extracts ranged from 39 - 139% and the average recovery was 89%.

3.4 Results and Discussion

3.4.1 Total Arsenic

Table 3-1: Average and range of total arsenic concentrations in collected fruiting bodies (mg/kg DM) from different sources.

Sampling Source	Number of Fungi Species Collected	n	Average	Range
Store bought	12	n = 14	0.20	0.020 - 1.3
British Columbia	4	n = 4	0.17	0.068 - 0.12
Ontario Background	13	n = 23	0.37	0.030 - 1.80
Ontario Contaminated	10	n = 11	8.18	0.39 - 59
Yellowknife Background	5	n = 5	5.25	2.3 - 14
Yellowknife Contaminated	11	n = 16	15.52	1.28 - 34

For Yellowknife samples: Total arsenic = As concentration in aqueous methanol extract + As in 2% nitric acid extract + As concentration in residue digest. For all other sources: Total arsenic = As concentration in aqueous methanol extract + As concentration in residue digest. Averages of duplicate samples and relative percent differences (RPDs) are provided in Appendix A (Table A3).

A total of 46 different fungi species across the six sampling sources were collected (73 samples in total). The average and range of total arsenic concentrations in the mushrooms from each sampling source are listed in Table 3.1 as well as the number of species and samples collected. Samples within each sampling source widely ranged in total arsenic concentrations, which is most likely a reflection of the variety of species and habitats. In spite of this variation, a general trend is that store bought and BC collected mushrooms contained lower arsenic concentrations than the other wild collected mushrooms from uncontaminated locations. Additionally, the mushrooms from arsenic-contaminated sites have higher ranges of total arsenic than those from the background locations and are comparable to mushrooms previously analyzed for total arsenic from arsenic-contaminated locations [164]. Mushrooms collected from Yellowknife background locations show a higher range of total arsenic concentrations compared with other background sites, which can be attributed to the naturally elevated concentrations of arsenic in Yellowknife soils [5]. Total arsenic accumulation was found to vary among different species collected from the same location suggesting that arsenic accumulation is dependent on the species of fungi.

Currently in Canada there are no limits for total arsenic in food, except for fish protein, bone meal and juice [177]. The only country that considers arsenic in mushrooms is China, whose Ministry of Health

currently imposes a maximum total arsenic concentration of 0.5 and 1 mg/kg for fresh and dry mushrooms, respectively [166]. Of the 12 samples (and species) of store bought mushrooms only one sample of *Cantharellus cibarius*, containing 1.3 mg/kg dry mass (DM), exceeded the Chinese guideline for arsenic in mushrooms. Another sample of the same species (from another vendor) contained 0.08 mg/kg and another related species, *Craterellus cornucopioides,* had 0.2 mg/kg, indicating that arsenic content varies among vendor sources. A large percentage (42%) of all edible mushrooms, including wild collected mushrooms, contained more than 1 mg/kg DM arsenic; most of these mushrooms originated from arsenic-contaminated locations.

3.4.2 Arsenic Speciation

The anion and cation exchange chromatographic analysis allowed for the identification of 10 different arsenic compounds in the aqueous methanol extracts of the collected fruiting bodies, anion and cation chromatographs are provided in Appendix A (Figure A2). The most prevalent arsenic compounds in the fruiting bodies were AB, inorganic arsenic (iAs = As(III) + As(V)) and DMA, and occurring less frequently were MMA, TMAO, AC and TETRA. Sugar 1, Sugar 3 and two unknown arsenic compounds visible by anion exchange (UK1 and UK2) were found in a small number of samples. The results of the HPLC-ICP-MS analysis for each sample are shown in Appendix A (Table A3), listed as the average concentration and differences between duplicates of each arsenic compound in µg/kg DM, along with the average extraction efficiency (EE) for each sample. EEs ranged from 14 to 107 % with an average EE of 70% and with EEs over 60% in the majority of samples (60%). Extraction efficiency was not correlated to total arsenic ($R^2 = 0.002$, $p > 0.05$).

Extraction efficiency was increased with a second sequential extraction step in YK mushrooms (Appendix A, Figure A3). This strategy was applied to the YK mushrooms to optimize the extraction, since previous studies have shown that the arsenic in organisms from highly contaminated locations are less efficiently extracted than those from less contaminated locations [84, 105], and YK mushrooms generally had the highest arsenic concentrations. Some studies have found that a second extraction step extracts iAs [100] but it has also been effective for organoarsenicals (oAs) like TETRA [115]. In the present study, no compound was preferentially extracted from the Yellowknife mushrooms in the first extraction step and several arsenic compounds were extracted in the second extraction step including inorganic arsenic and AB. The presence of AB in the dilute acid extract suggests that the AB in these samples is surprisingly recalcitrant to a repeated aqueous methanol extraction (Step 1).

A previous study has identified correlations of concentrations of iAs, but not oAs, with total arsenic in some marine organisms [105], and *A. bisporus* mushrooms cultivated on substrates containing high levels

of arsenic (1000 mg/kg) were found to have a higher proportion of iAs compared to those grown on the low concentration substrates (3.2 μg/g) [178]. However for the collected mushrooms in this study, neither iAs nor oAs concentrations were correlated to total arsenic ($R^2 = 0.05$ and $R^2 = 0.06$ respectively, $p > 0.05$). The concentration of AB in the mushrooms containing this compound was, however, positively correlated to total arsenic concentration ($R^2 = 0.82$, $p > 0.05$).

Edible mushrooms were targeted in the present study to ascertain trends in arsenic speciation applicable to consumers and we are the first to report results for 24 of 37 mushroom species (Figures 3.1 and 3.2). In the store bought mushrooms, iAs makes up the majority of total arsenic in many of the species (Figure 3.1); this includes the commonly purchased shiitake mushrooms, *Lentinula edodes*, which along with other shiitake products, have been previously reported to contain a majority of inorganic arsenic [166]. Only the *Agaricus* species do not contain predominantly iAs: as seen in Figure 3.1 they contain a majority of AB, corroborating results reported by others [39].

Figure 3.2 summarizes the proportion of arsenic compounds in all edible mushrooms collected for this study with total arsenic concentrations greater than 1 mg/kg DM. Half of these samples contain a majority of arsenic in the non-toxic form, AB, and they were the mushrooms from the *Agaricus* genus, regardless of location, as well as puffball mushrooms and the *Coprinus* genus. Wild edible mushrooms with pores generally contained a majority of DMA, which contrasted with the store bought pored mushrooms (*Boletus edulis*) that contained mainly iAs.

While the low levels of total arsenic in store bought mushrooms provide more relevant arsenic concentrations for typical consumers of mushrooms, the prevalence of iAs presents a potential risk for the harvesting and consumption of these mushrooms from contaminated sites, should the speciation be similar at higher concentrations.

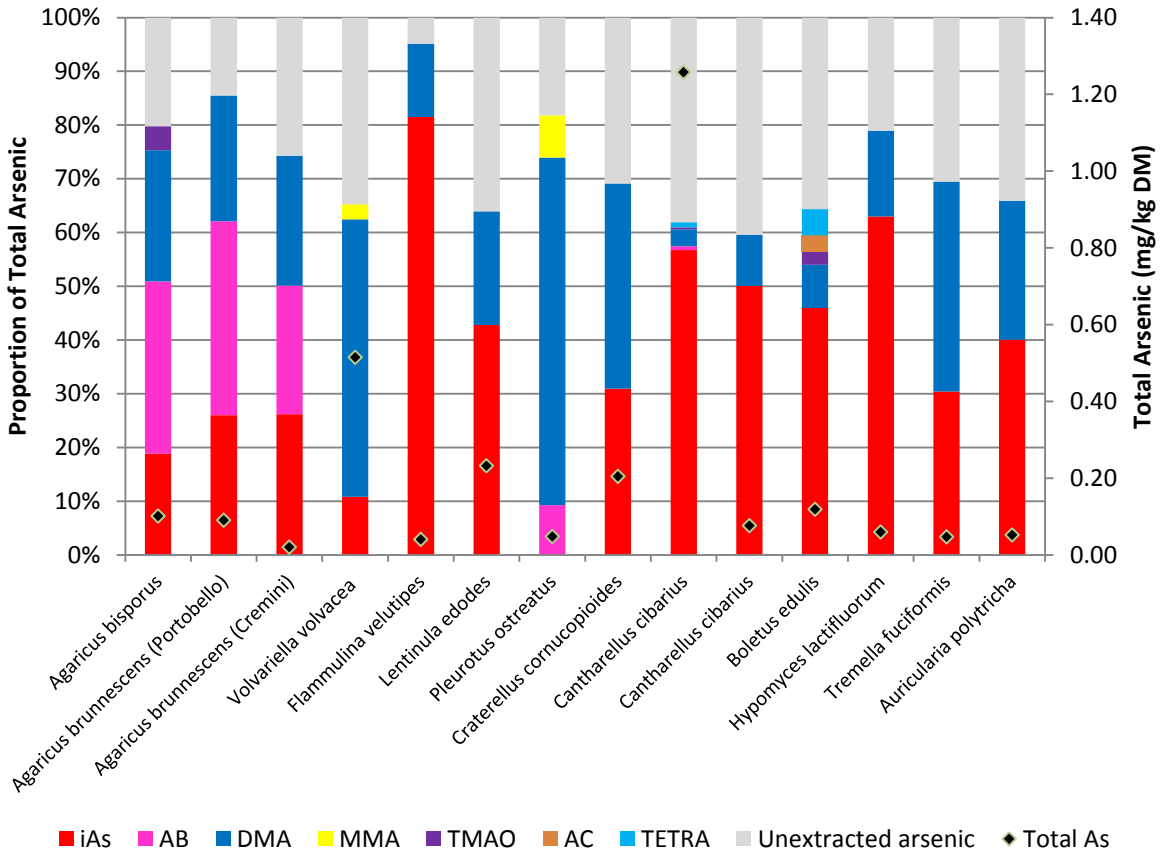


Figure 3.1: Average proportion of arsenic compounds (left axis) and total arsenic (right axis and black diamonds) in store bought mushrooms.

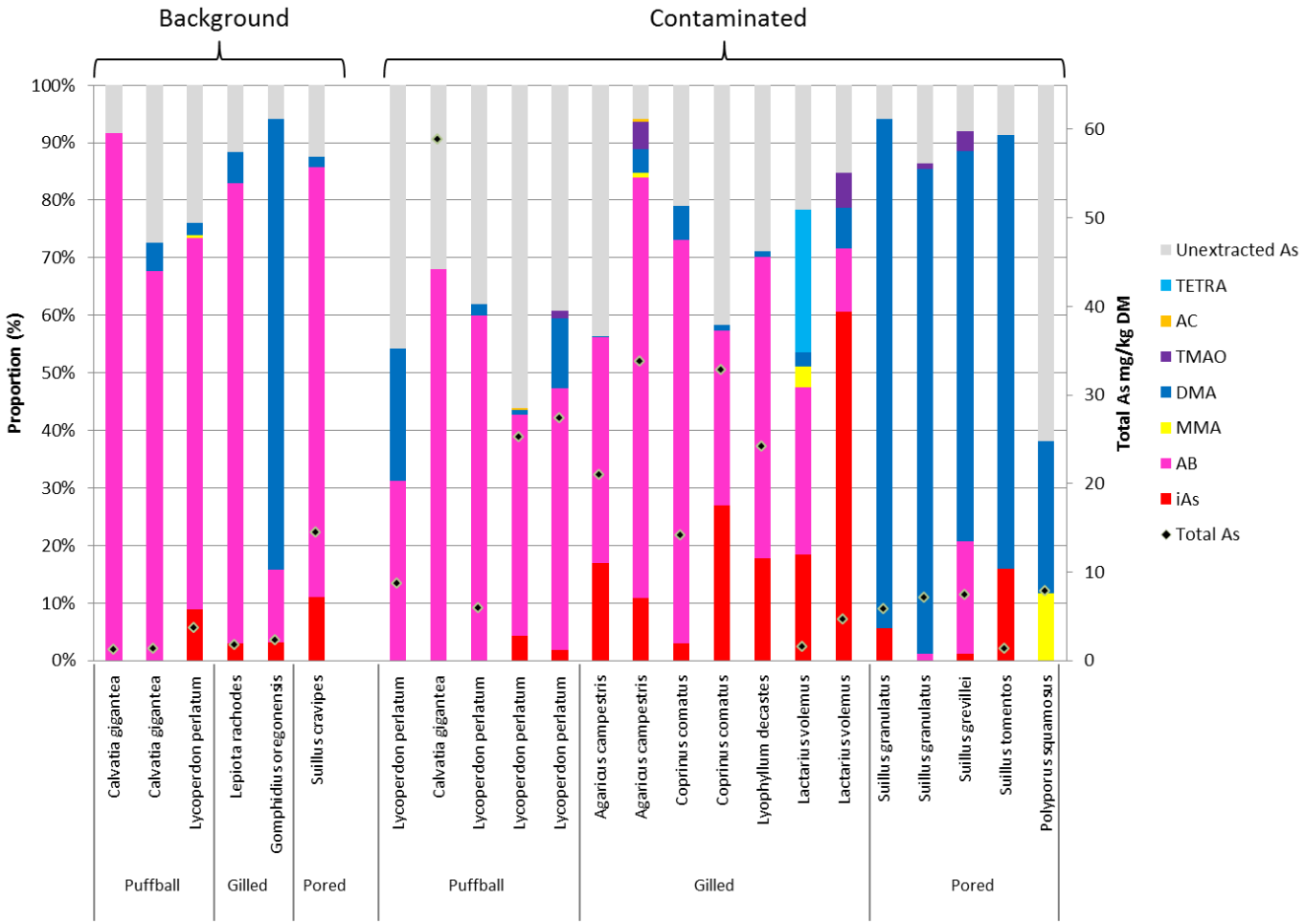


Figure 3.2: Average proportion of arsenic compounds (left axis) and total arsenic for wild edible mushrooms with a total arsenic concentration greater than 1 mg/kg DM (right axis and black diamonds). Mushroom species are grouped by fruiting body morphology (horizontal axis).

3.4.3 Phylogenetic and Morphologic Trends in Arsenic Speciation

The trends in speciation were further examined to ascertain if arsenic speciation can be predicted in certain mushroom groups and to gain insight into the potential formation and role of AB in these mushrooms.

To do this, the mushrooms were grouped according to phylogenetic placement (Appendix A, Figure A4), and include species from five different classes, nine different orders and 24 different families (compared with previous studies, which encompass only five orders [18, 30, 165, 166, 173-176]). The majority of species that were collected belong to the Agaricomycetes class, including species from the Boletales order, a large group of fungi species with pored mushrooms for which only a few species have been studied [30, 164, 167, 176]. Species from four other classes were also collected (Appendix A, Figure A4). The fruiting body morphology of each species, which generally corresponds to the order of the fungus species, is also indicated in Appendix A, Figure A4 by the red brackets.

To compare the arsenic compounds between mushroom species, the proportion of arsenic compounds were calculated by dividing the arsenic compound concentration by the sum of arsenic compounds quantified using HPLC-ICP-MS analysis. The concentrations and proportions of arsenic compounds in each fungus species are provided in Appendix A (Table A3 and A4). The average proportions for the most commonly found arsenic compounds, iAs, DMA and AB, across orders and families are summarized in Figure 3.3a and b.

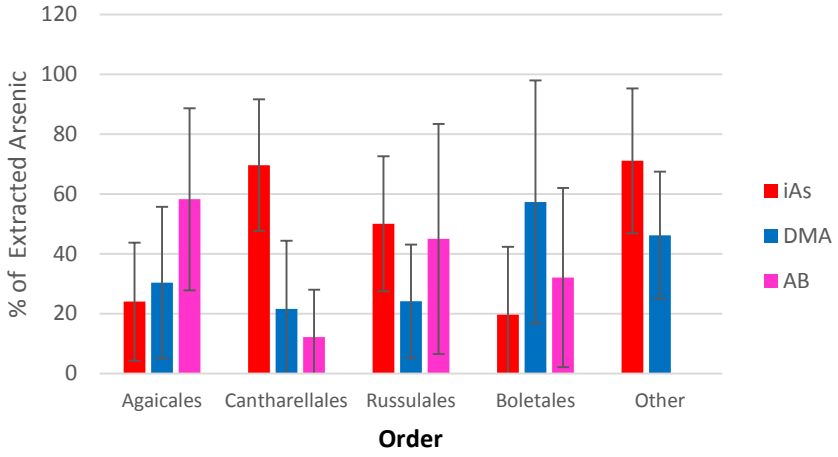
Generally species in the same order or family had the same major arsenic compound. The Agaricales order mainly contained AB as the major arsenic compound (30 of 47 mushrooms). Within the Agaricales order the Lycoperdaceae family contained a majority of AB with the exception of one *Lycoperdon pyriforme* sample, discussed in more detail later. The Agaricaceae family contained a majority of AB as the major arsenic compound (10 of 14 mushrooms); the other major compound was DMA. The Amanitaceae family was not dominated by one major arsenic compound; iAs, DMA or AB can comprise the majority of arsenic in these mushrooms across all sample sources.

The Cantharellales order mainly contained iAs as the major arsenic compound, although DMA was the major compound in one species, *Craterellus cornucopioides*. The Russulales order was found to equally contain iAs and AB as the major arsenic compound, with DMA as the major compound in one species, *Russula silvicola*. The one log growing species in the Russulales order, *Hericium coralloides*, was found to have a majority of iAs and also contained DMA.

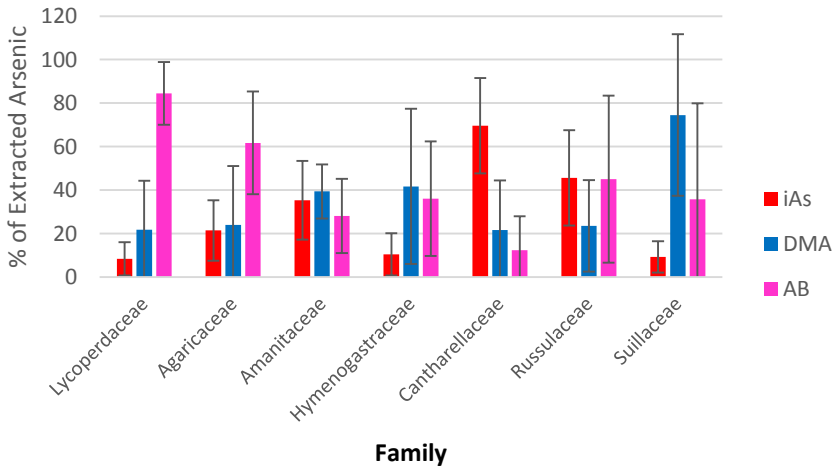
The Boletales order contained DMA as the major compound (6 of 10 mushrooms). This order is made up of mainly pored mushrooms, with the exception of *Paxillus involutus* and *Gomphidius oregonensis* which are gilled mushrooms. *Gomphidius oregonensis* contained a majority of DMA and *Paxillus involutus* contained a majority of AB. Another study of *Paxillus involutus,* also collected from Yellowknife, reported DMA as the major compound with a large proportion of the reported arsenic species occurring as a cationic unknown compound [164], which in light of the present study, may have been poorly resolved AB. Two pored mushrooms, *Suillus cavipes* and *Boletinellus meruliodes*, from YK and ON contaminated sites, respectively, contained a majority of AB, which are exceptions to the trend that pored mushrooms contain a majority of DMA.

Species from other orders, specifically Polyporales, Pezizales, Hypocreales, Tremellales, and Auriculariales, contained iAs and DMA as the major arsenic compounds. None of these species contained AB. It has been hypothesized that the phylogenetic placement of a species is responsible for the arsenic compounds it contains, and that more highly evolved species, such as puffballs, will contain more complex organoarsenic compounds [30]. However increased use of rDNA analysis in phylogenetic studies since this hypothesis was proposed [30] has resulted in changes to the phylogenetic arrangement of these species, and in some cases, some are still unresolved. For example, the Boletales order is also considered highly evolved, and while the results for 10 species from the Boletales order in this study show that some of these species contain AB, the simpler organoarsenical, DMA, is the predominant arsenic compound in this order. We propose an alternate hypothesis, that the similarity of arsenic compounds across families may be attributed to the species being physiologically similar.

A)



B)



C)

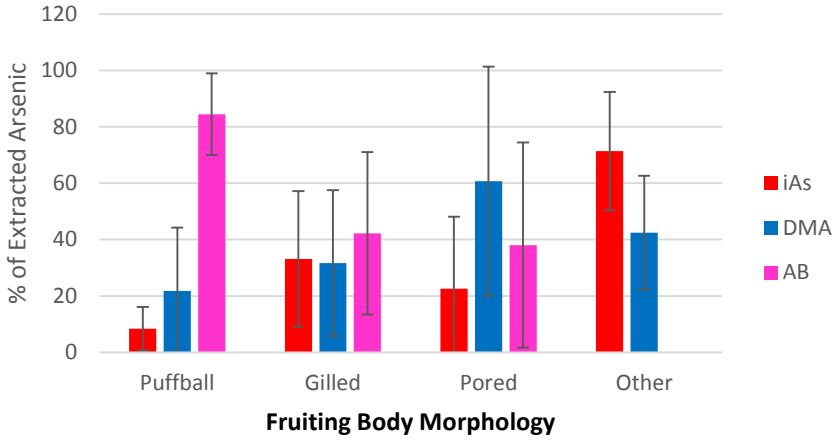


Figure 3.3: Average proportion of arsenic compounds in mushrooms across (A) orders, (B) families and (C) fruiting body morphology. The error bars represent standard deviation.

Physiological features of mushrooms may play a role in the arsenic compounds found in them, based on the role of the different compounds in the mushrooms, specifically AB. For example, AB has been hypothesized to play an osmolytic role in *Agaricus bisporus* fruiting bodies since AB localization to the cap and outer stalk edge [39] is similar to that for solutes, and this solute localization is thought to osmolytically drive water and nutrient translocation through the fruiting body [32], ultimately helping to maintain form and rigidity important for spore dispersion. Figure 3.3c summarizes the proportions of arsenic compounds across different fruiting body morphologies. The high proportions of AB in the puffball species support the hypothesis that AB may be adventitiously accumulated with other osmolytes since the structural integrity of these developing fruiting bodies, due to size and morphology, would rely on osmolytic pressure. The majority of species collected from the Agaricaceae family, gilled mushrooms, also contain AB as the major compound. Like in *Agaricus bisporus*, AB may help with osmolytic regulation in these mushrooms to ensure the cap remains turgid and elevated for effective spore dispersal. Species with a lower water content in this study included species from the Cantharelles order as well as *Hericium coralloides*, *Auricularia polytricha* and *Tremella fuciformis*; these species all contained iAs as the major arsenic compound (except for *Tremella fuciformis*, which had 44% iAs and 56 % DMA), which may also support the hypothesis of AB being accumulated as an osmolyte. It is unknown why other gilled mushrooms and pored mushrooms from the Boletales did not contain AB as the major arsenic compound more frequently; this feature, together with the variation in the proportion of arsenic compounds in gilled mushrooms could mean that only certain species accumulate AB as an osmolyte.

Thus the trends with phylogeny and morphology suggest that arsenic speciation can be predicted in mushrooms from similar groups across different locations. Puffball and gilled mushrooms from the Agaricales family contain a majority of AB as a possible osmolyte. Other gilled mushrooms in the Agaricomycetes, such as those found in the Russulales and Cantharelles order can contain a majority of iAs. Mushrooms from other classes contain a majority of iAs. Pored mushrooms can contain AB but DMA is likely the predominant compound in these mushrooms.

3.4.4 Source of AB in Mushrooms

There are a number of potential pathways for the accumulation of AB in mushrooms. The fungus may produce AB or may accumulate it from the surrounding environment. Specifically, the surrounding microbial community may produce AB or its precursors, which are then selectively taken up by the fungus. Biochemical pathways to AB have been proposed [162]: for example in the marine environment a probable pathway for AB involves the breakdown of arsenosugars. In the present study, arsenosugars were detected in only three mushrooms collected from YK contaminated sites, along with two unknown compounds (Appendix A, Table A6). Mushrooms from the same genera collected from BC and ON did

not contain arsenosugars, but overall lower arsenic content might have prevented their detection. Of the YK mushrooms containing arsenosugars, two contained AB as the major compound and one contained iAs as the major arsenic compound (13% AB). The general absence of arsenosugars in other AB-containing samples with higher arsenic concentrations (e.g., from YK and ON contaminated locations) suggests that the arsenosugar-AB formation pathway, is not likely to be the predominant AB formation pathway for mushrooms.

We consider that similar species of fungi can preferentially accumulate AB or certain arsenic compounds from the surrounding environment, since this appears to be a trend for total arsenic accumulation [30, 168], along with other elements [179]. Although the major arsenic compounds are the same across species from different locations, the proportions of all compounds present vary greatly, as seen in Figure 3.3, suggesting an external or environmental influence on arsenic speciation. We propose that the associated microbial communities in the growth substrate represent a major environmental influence on the fungus.

An example of the microbial community producing compounds that differ from those in mushrooms is when mushroom compost treated with arsenate solution transformed the majority of this inorganic arsenic to TMAO, but not AB, yet AB and only small amounts of TMAO accumulated in the fungus [39]. On the other hand for plants grown in arsenate treated soils, the associated microbial community was shown to be the source of methylated arsenic compounds detected in the plants [54]. In the present study log growing mushrooms and soil growing mushrooms provide a comparison of different associated microbial communities. The log growing mushroom species (eight species, 10 samples) in the present study contained mainly iAs and DMA and small amounts of MMA. AB is absent from all but one species of log growing mushrooms, where it was present in a trace amount (4.7 μg/kg DM). The absence of AB in the puffball species, *Lycoperdon pyriforme*, growing on a log, rather than soil like the other puffball samples, also indicates the microbial community may play a role in arsenic speciation, since the soil growing mushrooms of this species contained a majority of AB (n = 5) [17]. Further characterization of soil microbes is required to determine if the differences among microbial communities associated with soil growing mushrooms exist, and if so, their role on arsenic speciation in this environment.

From the morphological and substrate (soil vs log) observations in the present study, it is apparent that arsenic speciation is dependent on both the mushroom species and the growth environment, factors that are confounded in this environmental study; controlled laboratory study of these factors might aid in separating their effects. Nevertheless, the present findings provide insights about the arsenic species that

might be expected in mushrooms collected in the future by mushroom harvesters or researchers, as well as the potential source of AB in mushrooms.

3.4.5 Complementary XAS Analysis

Although overall EEs were reasonably high a mean EE of 70% indicates that some arsenic was still unidentified by using conventional extraction methods with HPLC-ICP-MS analysis. The solid state analysis provided by XAS can indicate which arsenic compounds were not extracted and therefore samples with sufficiently high concentrations (generally >10 ppm DM), and enough mass remaining after extraction, were also analyzed using this technique, with an example of an XAS spectra provided in Appendix A (Figure A5). The results of the XANES fitting (Table 3.2 for dried samples and Table A6 for selected fresh frozen samples) show that no arsenic compound was preferentially extracted and confirm the major compounds found in the HPLC-ICP-MS analysis. The standards selected for the fitting were selected based on the HPLC-ICP-MS analysis demonstrating the complementary nature of the two methods. The proportions for TMAO and DMA were combined because they have the same white line energy and therefore cannot be distinguished by XANES analysis. This is also true of AB and TETRA, but HPLC-ICP-MS analysis did not indicate the presence of TETRA in the samples analyzed, except in *Coprinus atramentarius* (ID 46); thus in this sample, the proportion attributed to AB may be a combination of AB and TETRA. Table 3.2 also indicates which arsenic compound was not extracted during the aqueous methanol extraction.

For the first time in mushrooms, an As(III)-S compound was identified by matching to a $As(Glu)_3$ standard (white line energy 11870.0 eV), in both *Agaricus campestris* samples and the *Coprinus atramentarius* sample collected from YK arsenic contaminated locations. The formation of As(III)-S compounds in biological organisms may initiate toxicity mechanisms or provide a way of coping with high levels of arsenic exposure [180]. The absence of As(III)-S compounds during HPLC-ICP-MS analysis have been attributed to these compounds being retained by the column [109], and/or disintegration during sample preparation or by the ammonium phosphate or formic acid buffers used in the ion-exchange chromatography methods [85, 181].

An As(V)-glycerol compound was identified in three samples. It is likely that this compound is an analytical artefact introduced during sample drying, although it has also been shown to exist authentically in few biological samples if appropriate chemical conditions exist [116].

Table 3-2: X-ray Absorption Near-Edge Structure Fitting Results for Selected Fungi Species.

#	Species	Source	EE (%)	Proportions Determined by XAS (%)						χ^2	Unextracted Compounds
				As(III)	As(V)	As(V)-Glycerol	DMA/TMAO	AB	$As(Glu)_3$		
6	Lycoperdon perlatum	ON Cont	62					100		N/A	AB
9	Lycoperdon perlatum	YK Cont	44	5	24			71		0.002	As(III)/(V)
14	Calvatia gigantea	ON Cont	68					100		N/A	AB
23	Agaricus campestris	YK Cont	94	11	2	12		63	12	0.003	As(III)/(V)
24	Agaricus campestris	YK Cont	56				1	97	2	0.003	AB
27	Coprinus comatus	YK Cont	79	3			6	88	3	0.003	
28	Coprinus comatus	YK Cont	58	16		2		82		0.003	AB
46	Coprinus atramentarius*	YK Cont	45	19			19	62		0.001	AB
47	Lyophyllum decastes	YK Cont	71	15		16		69		0.002	

Data were fit with frozen As(V) (white line energy 11875.3 eV); frozen As(III) (white line energy 11871.7 eV); As(V)-glycerol (white line energy 11876.5 eV); liquid DMA(V) (white line energy 11873.3 eV); TMAO (white line energy 11873.3 eV); frozen AB (white line energy 11872.6 eV); and $As(Glu)_3$ (white line energy 11870.0 eV). *For Coprinus atramentarius the HPLC-ICP-MS analysis indicates TETRA is present in this sample, because TETRA and AB have the same white line energy the proportion of AB in this sample is considered to be a mixture of AB and TETRA. Cont = Contaminated.

The speciation information obtained in the present study was combined with the data obtained from the literature (Figure 3.4) [18, 30, 164-166, 173-176]. As emphasized in the present study and corroborating a previous meta-analysis, it is clear that AB is prominent in mushrooms, providing an important source of this compound to the terrestrial ecosystem [17]. As Figure 3.4 demonstrates, this study contributes the majority of results for all families and orders, except for Lycoperdaceae, Agaricaceae and Tricholomataceae families, thereby providing crucial arsenic speciation data to the current literature for a large number of mushroom species over a diverse phylogenetic range.

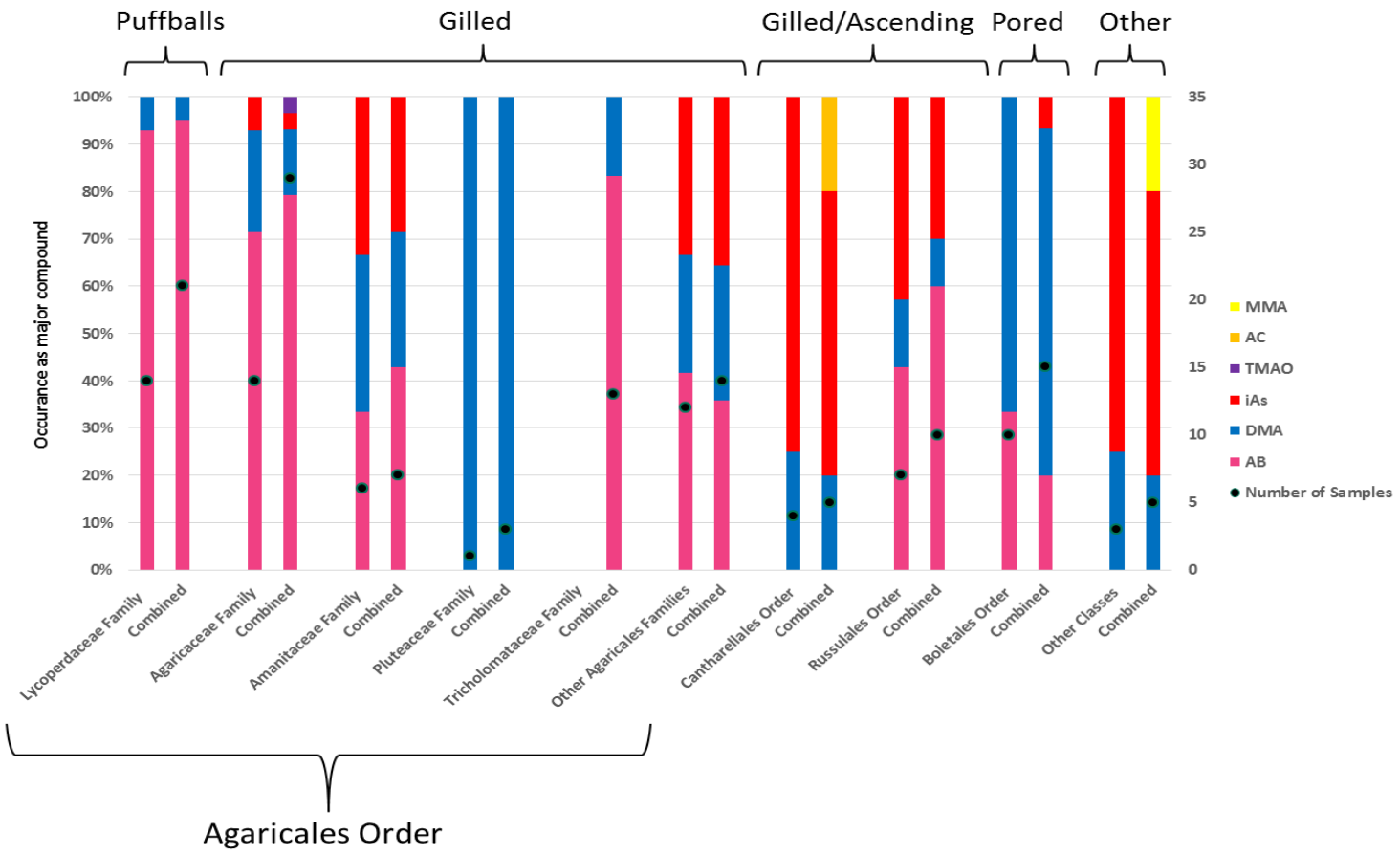


Figure 3.4: Summary of the occurrence of arsenic compounds as the major arsenic compound in species across families and orders. The results of this study are presented in the first column of each group and the results of this study combined with results from the literature are presented in the second column. The blue circle indicates the number of samples analyzed shown on the secondary axis. Note that this study presents the majority of samples for all families and order except for the Lycoperdaceae, Agaricaceae and Tricholomataceae families.

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Chapter 4[3]

4 Uptake and transformation of arsenic during the vegetative life stage of terrestrial fungi

4.1 Abstract

Many species of terrestrial fungi produce fruiting bodies that contain high proportions of arsenobetaine (AB), an arsenic compound of no known toxicity. It is unknown whether fungi produce or accumulate AB from the surrounding environment. The present study targets the vegetative life stage (mycelium) of fungi, to examine the role of this stage in arsenic transformations and potential formation of AB. The mycelia of three different fungi species were cultured axenically and exposed to AB, arsenate (As(V)) and dimethylarsinoyl acetic acid for 60 days. Agaricus bisporus was additionally exposed to hypothesized precursors for AB and the exposure time to As(V) and dimethlyarsinic acid was also extended to 120 days. The mycelia of all fungi species accumulated all arsenic compounds with two species accumulating significantly more AB than other compounds. Few biotransformations were observed in these experiments indicating that it is unlikely that the mycelium of the fungus is responsible for biosynthesizing AB.

Keywords: Arsenobetaine, arsenic, mycelium, biotransformation, concentration factor

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[3] Nearing, M. M.; Koch, I.; Reimer, K. J., Uptake and transformation of arsenic during the vegetative life stage of terrestrial fungi. *Environmental Pollution.* **2015,** *197*, (0), 108-115.

4.2 Introduction

Over 50 arsenic compounds have been identified in the environment and the toxicity of arsenic is greatly dependent on its chemical form [1], but the only arsenic compound that is considered to be non-toxic is arsenobetaine (AB) [2]. AB is predominantly found in marine organisms where it comprises a large proportion of the total arsenic. Therefore two of three current proposed pathways (Figure 4.1) for the formation of AB originate from studies of the marine environment [162].

Currently, the main hypothesis for AB formation is from the degradation products of dimethylated or trimethylated arsenosugars, although the latter are not as commonly found in the environment. Dimethylated arsenosugars are thought to be precursors because the dietary sources for marine organisms (phytoplankton and marine kelp) contain elevated levels of these compounds [33]. It has been proposed that the formation of AB may occur from the degradation of dimethylated arsenosugars to arsenocholine (AC), which is then converted to AB (Pathway 1 in Figure 4.1).The last step in Pathway 1, the conversion of AC to AB, has been demonstrated in laboratory studies within mice, rats and rabbits [34]. However shrimp and sheep did not degrade ingested dimethylated arsenosugars to AC [29, 35], and degradation in other circumstances has not yet been reported. Thus the formation pathway from arsenosugars to AB may not involve AC as an intermediate. Dimethylarsinoylacetic acid (DMAA) has been proposed as a potential intermediate similar to AC (Pathway 2 in Figure 4.1) and has been demonstrated to be a major degradation product of arsenosugars in sheep [29]. DMAA was also a precursor to AB in laboratory studies involving lysed bacteria extracts [26].Fish fed DMAA, however, did not form AB [36]. The third proposed formation pathway for AB (Pathway 3 in Figure 4.1), offers a more direct route by simple methylated compounds involving dimethylarsinous acid (DMA(III)) and 2-oxo acids, glyoxylate and pyruvate, to form DMAA and then AB [37].

In contrast to the marine environment, few terrestrial organisms contain AB and, when present, it comprises lower proportions of total arsenic. The exception to this trend is the fruiting bodies, or mushrooms, of terrestrial fungi in the Basidiomycetes class. Terrestrial species in this class of fungi can grow through asexual reproduction, producing the vegetative life stage (mycelium), and through sexual reproduction, undergoing a reproductive life stage, during which fruiting bodies, or mushrooms, are produced for spore dispersal. The fungus spends most of its life in the mycelium form until environmental conditions specific to each fungus species trigger fruiting body development [41], which is seasonal (10-14 days) in the wild [179]. Commercially cultivated species such as *Agaricus bisporus* (the button mushroom widely available in grocery stores) are grown under conditions that allow multiple fruiting body production events, or flushes [182].

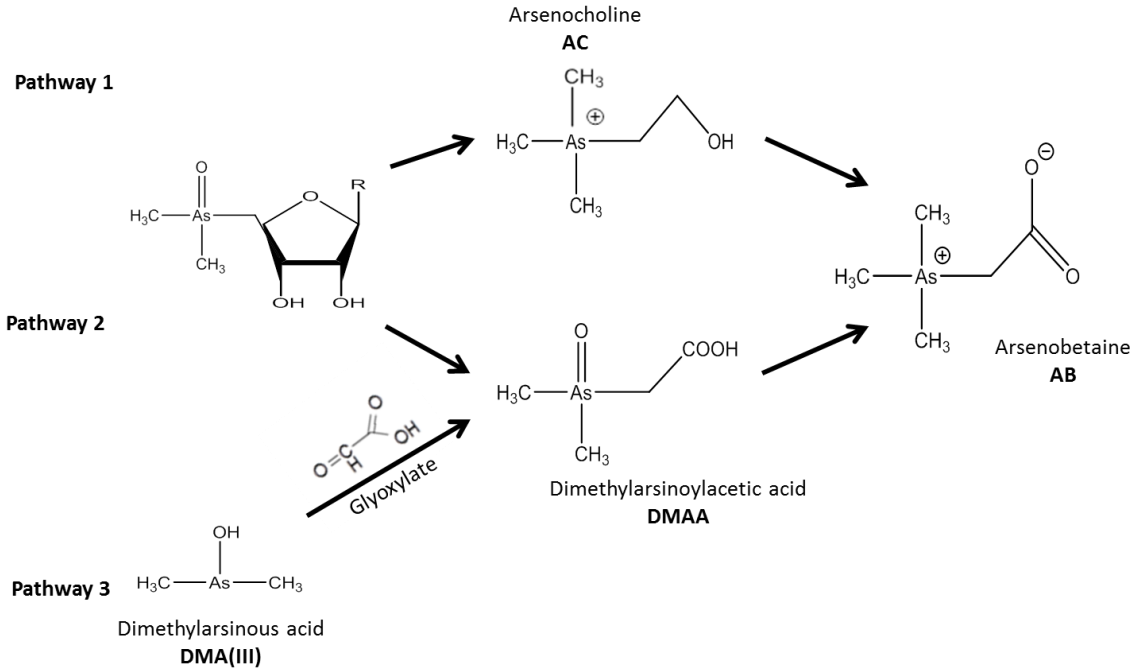


Figure 4.1: Current proposed formation pathways for arsenobetaine (AB).

Mushrooms are the main part of fungus species that have been collected and analyzed from the environment, attributable to their visibility and ease of picking and identification. They have been found to contain a variety of organoarsenic compounds, and compared with most other terrestrial organisms, a higher proportion of AB [1]. This abundance of AB in terrestrial fungi provides a simpler study system for the formation of AB than marine organisms, making them a desirable target organism for this purpose.

The role of AB in mushrooms is still unknown, but localization of AB to the cap and outer stalk has led to the hypothesis that AB may also play an osmolytic role, since the fruiting bodies rely on osmotic regulation for spore dispersal. [1]. The life stage at which the fungus produces AB is still unknown, as is whether the fungus, alternatively, accumulates AB from the surrounding environment. That is, the surrounding microbial community in the soil may produce AB or its precursors. Very few studies report the detection of arsenosugars in mushrooms [164, 165] and no arsenosugars were found in the growth material when *A. bisporus* was grown on arsenate (As(V)) treated material [39], making it unlikely that AB is produced from the degradation of arsenosugars, either through the fungus or the surrounding microbial community. In the aforementioned study [39], the fungus-free growth material contained monomethylarsonic acid (MMA), DMA and mainly trimethylarsine oxide (TMAO) likely produced by

the microbial community; we hypothesize that these compounds could then be accumulated and further transformed by the fungus.

The present study targets the vegetative, mycelium life stage of terrestrial fungi, to examine the role of this stage in arsenic transformations, especially to AB. The present study targets the mycelium life stage, grown in axenic laboratory culture, to obtain controlled conditions and to exclude the influence of the microbial community. The mycelia of three fungus species from the Basidiomycetes class were cultured with different arsenic compounds: *A. bisporus*, known to contain AB as the major arsenic compound in its fruiting bodies when cultivated; *Sparassis crispa* (common name cauliflower mushroom), known to contain AC in fruiting bodies [30]; and *Suillus luteus* (common name Slippery Jack) a species from a genus known to contain no AB but primarily DMA in fruiting bodies [17]. All mycelia were exposed to As(V), DMAA and AB . *A. bisporus* was additionally exposed to MMA, DMA and TMAO, based on previous findings on fruiting body laboratory culture of this fungus [39]. DMAA was selected to test if the fungus could transform DMAA to AB (Pathway 3 in Figure 4.1). Ideally the mycelium would also be exposed to arsenosugars to test Pathway 1 and 2 in Figure 4.1; however no authentic pure sugar standards were available to us, and algal extracts commonly used to identify arsenosugars in HPLC-ICP-MS analysis would not be suitable because they also contain As(V) and DMA [143]. AC was also not used as a treatment because it is infrequently found together with AB [30].

4.3 Methods

4.3.1 Fungus Cultures

Chemicals and reagents are listed in Appendix B. Fungus species included *A. bisporu*s ATCC# 10892, *S. crispa* ATCC# 34491 (both grown in potato dextrose broth), and *S. luteus* ATCC# MYA-4759 (yeast mold broth). Mycelia were revived and grown on suggested media until they had over grown the petri dishes. Three pieces of approximately 1 cm^2 pieces of agar with mycelium were added to the corresponding broth. Mycelia were shaken at 140 rpm at 20°C with all manipulations carried out under a biohood to prevent contamination.

Table 4-1: Summary of experimental design.

Experiment	Fungus Species	Medium	Treatments	Length of exposure (days)
A	*Agaricus bisporus*	PD	100 µg/L As(V) (n=3)	60
	Sparassis crispa	PD	100 µg/L DMAA (n=3)	
	Suillus luteus	YM	100 µg/L AB (n=3)	
B	*Agaricus bisporus*	PD	100 µg/L As(V) (n=3)	60, 90, 120
			100 µg/L DMA (n=3)	
C	*Agaricus bisporus*	PD	250 µg/L MMA (n=3)	30
			250 µg/L DMA (n=3)	
			250 µg/L TMAO (n=3)	
Controls				
A	Uninoculated negative	PD and YM	No As (n=6)	60
	Uninoculated positive	PD and YM	100 µg/L As(V) (n=3)	
			100 µg/L DMAA (n=3)	
			100 µg/L AB (n=3)	
	Agaricus bisporus negative	PD	No As (n=3)	
	Sparassis crispa negative	PD	No As (n=3)	
	Suillus luteus negative	YM	No As (n=3)	
B	Uninoculated negative	PD	No As (n=3)	60
	Uninoculated positive		100 µg/L As(V) (n=3)	60, 90, 120
			100 µg/L DMA (n=3)	
	Agaricus bisporus negative		No As (n=3)	

Uninoculated negative control = media containing no As compounds and no mycelium
Uninoculated positive control = media containing As compound and no mycelium
Inoculated negative control = media containing no As compound and mycelium
PD = potato dextrose broth; YM = yeast mold broth

Table 4-1 Continued

Experiment	Fungus Species	Medium	Treatments	Length of exposure (days)
Controls				
C	Uninoculated negative	PD	No As (n=3)	30
	Uninoculated positive		250 µg/L MMA (n=3)	
			250 µg/L DMA (n=3)	
			250 µg/L TMAO (n=3)	
	Agaricus bisporus negative		No As (n=3)	

Uninoculated negative control = media containing no As compounds and no mycelium
Uninoculated positive control = media containing As compound and no mycelium
Inoculated negative control = media containing no As compound and mycelium
PD = potato dextrose broth; YM = yeast mold broth

The experimental design is summarized in Table 4.1. All treatments were triplicated and included controls with mycelia grown in arsenic-free media (inoculated negative control); without mycelia in arsenic-containing media (uninoculated positive control); and arsenic- and mycelia-free media (uninoculated negative control). Arsenic compounds were aseptically added to the broth using a 0.22 µm Millipore ® syringe filter. The measured arsenic concentrations in the media are listed in Appendix B (Table B2). Some media concentrations were lower than expected, thought to be attributable to retention by the 0.22 µm filter of the spiking solution. *A. bisporus, S. luteus,* and *S. crispa* were grown for 60 days in media amended with As(V), DMAA and AB at a nominal concentration of 100 µg/L As (Experiment A).

A second exposure experiment was prepared in the same way, with *A. bisporus* only (Experiment B), using As(V) and DMA at 100 µg/L As amendments, carried out for a total of 120 days and sample collection at 60, 90 and 120 days . This longer time period was tested even though for most species of higher terrestrial fungi the mushroom life time is only 10 to 14 days [179] since in nature the mycelium is always present beneath the soil. As(V) was chosen to represent the inorganic arsenic available in the soil and DMA to test Pathway 3 in Figure 4.1. The media were replaced every 30 days to represent dynamic conditions in the environment and replenish nutrients.

A third exposure experiment was prepared with *A. bisporus* only, and MMA, DMA or TMAO (250 µg/L As) amendments, and a 30 day exposure (Experiment C). These compounds and length of exposure were

selected to mimic the commercial growth of this fungus using the compounds hypothesized to be precursors, based their presence in As(V) treated compost [39].

Low concentrations of arsenic (100 and 250 µg/L As) were used to promote the formation of organoarsenic compounds, since *A. bisporus* fruiting bodies produced from arsenate exposure experiments at lower arsenic concentrations (180 mg/kg As) contained predominantly AB [39], whereas AB at higher concentrations was a minor constituent [178]. The control growth substrate for *A. bisporus* fruiting bodies that produced AB in one study was found to contain approximately 113 µg/kg As wet mass (WM) total arsenic and therefore concentrations of 100 – 250 µg/L As were used to amend cultures [39].

At the end of the exposure period of each experiment 10 mL of medium from each replicate was collected and filtered (0.45 Millipore® polypropylene 25 mm diameter hydrophilic PVDF durapore membrane syringe filter) into a 15 mL Fisherbrand® disposable polypropylene centrifuge tube and kept frozen until analysis. The mycelium from each experiment was collected on a 0.22 µm Whatman filter, rinsed thoroughly with DDW, frozen and freeze dried. The dried mycelium was pulverized with a ceramic mortar and pestle and kept at room temperature until analysis.

4.3.2 Arsenic Species Extraction and Total Arsenic Digestion

For all samples 0.5 g of dried sample was extracted using a 50% aqueous methanol extraction followed by a sequential 2% HNO_3 extraction following methods described in Nearing et al., 2014 [183]. The extract residues were digested with 70% HNO_3 for total arsenic analysis. Methanol was removed from the extracts by evaporation, more details are provided in Appendix B.2 and B.3. Total arsenic was obtained as the sum of the extracted and residual arsenic.

4.3.3 Instrumental Analysis

All samples (extracts and residues) were analyzed for total arsenic using ICP-MS. The ICP-MS operating conditions are described in Caumette et al., 2011 [24]. Instrument quality control checks were found to be acceptable. All aqueous methanol extracts were analyzed for arsenic speciation using HPLC-ICP-MS (cation and anion exchange). The majority of Step 2 extracts contained total arsenic concentrations below or close to the limit of detection for the speciation analysis (3 µg/kg) and therefore were not further analyzed for arsenic species. The HPLC-ICP-MS operating conditions are described in Caumette et al., 2011 [24]. Instrument quality control checks were found to be acceptable.

4.3.4 X-ray Absorption Spectroscopy (XAS)

X-ray absorption near edge structure (XANES) analysis was performed at the Advanced Photon Source (APS) Pacific Northwest Consortium/X-ray Science Division (PNC/XSD) on the bending magnet (BM) beam line, Sector 20. XANES spectra were collected and analyzed as described in Nearing et al., 2014 [183]. Details are provided in Appendix B.4.

4.3.5 Quality Assurance and Quality Control (QA/QC)

One certified reference material, either Tuna fish (BCR- 627) (certified values: 4.8 ± 0.3 mg/kg total As, 3.9 ± 0.23 mg/kg AB and 0.15 ± 0.022 mg/kg DMA), DORM-2 (certified values: 18 ± 1.1 mg/kg total As, 16.4 ± 1.1 mg/kg AB) or DORM-3 (certified value: 6.88 ± 0.30 mg/kg total As) for extractions, and Lichen (BCR-482) (certified value: 0.85 ± 0.07 mg/kg) for total digestions, was included with every 10 samples. CRMs for extractions were those available in our laboratory at the time of analysis. DORM-3 AB and DMA recoveries were calculated with respect to reference values from a round robin experiment (Zoltan Mester, personal communication, 2012). The certified values for this were: AB value = 4.12 mg/kg, range 3.86 – 4.38 mg/kg, DMA value = 0.447 mg/kg, range 0.392 – 0.563 mg/kg (ranges calculated with 95% CIs). Method blanks were also included with every 10 samples with non-detectable arsenic. The limits of detection (LOD) for total arsenic were: Experiment A, 1 µg/L for media samples, 8.5 µg/kg wet mass (WM) for mycelium; Experiment B, 1.1 µg/L for media samples, 8.1 µg/kg WM for mycelium; and Experiment C, 1.3 µg/L for media samples and 0.38 µg/kg WM for mycelium. Higher LODs resulted from larger dilution factors. Matrix spikes (50 µg/L) were prepared immediately before analysis (10% frequency) for both arsenic total and speciation analysis. QA/QC results are summarized in the Appendix B (Table B1) and were considered acceptable (± 30 % recoveries).

4.3.6 Controls

No speciation changes occurred in any of the uninoculated positive controls indicating that the arsenic compounds remained stable in the media under the growth conditions.

The media from all experiments remained clear throughout the exposure period, suggesting that the solutions were free of bacterial contamination and axenic. For Experiment B the media were checked on nutrient agar plates with no growth observed.

All media, and mycelia for Experiments B and C, in the uninoculated negative controls contained total arsenic concentration below the detection limits. However, Experiment A mycelia for all three fungus species contained arsenic concentrations above the detection limit (8.5 µg/kg WM), which may have been accumulated from the agar on which the mycelia were grown. The WM concentrations were: *A. bisporus*, 110 ± 20 µg /kg As; *S. crispa*, 40 ± 20 µg /kg As; and *S. luteus*, 70 ± 30 µg /kg As. An analysis of

variance (ANOVA) of the concentrations indicates significant variation among the fungus species ($F(2, 6) = 6.59$, $p < 0.05$); specifically *A. bisporus* controls contained significantly higher total arsenic concentrations then the *S crispa* controls ($p < 0.05$) (even though the same type of media was used), but *S. luteus* (different medium) did not contain significantly different concentrations from *A. bisporus* ($p = 0.13$) or *S. crispa* ($p = 0.46$) (post hoc Tukey HSD test). For Experiment A, the total arsenic was averaged in each group of fungus species control mycelia and subtracted from the corresponding treatment groups to correct for the background level of arsenic in the mycelium.

No adverse effects on mycelium growth were observed in the treatment groups.

4.4 Results and Discussion

4.4.1 Total Arsenic and Concentration Factors

Total arsenic in mycelia was calculated by adding the total arsenic in the aqueous methanol extracts (aqueous methanol extracts + nitric acid extracts for Experiment A) and the residue digests. Total residue digests were not carried out for Experiment C necessitating using the extract total as the total arsenic in the sample; the total arsenic may be underestimated. Arsenic concentrations in the mycelia (WM) and concentrations in the media are listed in Appendix B (Table B2). Moisture content in the mycelia ranged from 63 – 87 % with an average of 76 % ($n = 69$). Concentration factors (CF) (total As in mycelium WM/total As in medium) for each treatment group are presented in Figure 4.2 (data in Appendix B, Table B2); all compounds were concentrated by mycelia ($CF > 1$). A two-way ANOVA of the CFs for Experiment A (Figure 4.2a, showing average values and significant differences among CFs by different letters) indicates significant variation among the fungus species and treatment groups ($F(8, 72) = 33.45$, $p < 0.0001$), with *A. bisporus* and *S. crispa* accumulating significantly more AB than DMAA and As(V) ($p < 0.0001$). In previous studies, the fruiting bodies of *A. bisporus* contained a majority of AB [39, 178] and *S. crispa* contained a majority of AC [30]. The *S. luteus* mycelium in the present study accumulated significantly more DMAA compared to the other arsenic compounds ($p < 0.0001$). DMAA, a dimethylated compound, is the most structurally similar to DMA, the major compound found most often in *Suillus* species fruiting bodies [17].

The experimental design in the present study aimed to address whether AB was made, or selectively accumulated by the fungus. The results of this study are comparable to a sole other study examining the uptake and biotransformations of arsenic compounds in the mycelium of a terrestrial fungus species, specifically, *Agaricus placomyces* mycelium grown on agar spiked plates, where tetramethylarsonium ion (TETRA) and AB were accumulated (CFs of 4-6) but all other arsenic compounds tested (As(III), As(V), MMA, DMA and TMAO) were not (CFs <1) [38]. This finding is similar to the present finding of

significantly higher bioaccumulation of AB by *A. bisporus* and *S. crispa*. The *A. placomyces* mycelium was hypothesized to preferentially accumulate AB and TETRA because of their lower toxicity compared to the other arsenic compounds used [38], but in the present study AB's lack of preferential accumulation by *S. luteus*, along with a similar accumulation of As(V) (the most toxic arsenical used in this study) ($p = 0.84$), suggests that other reasons for AB accumulation in specific fungi might exist.

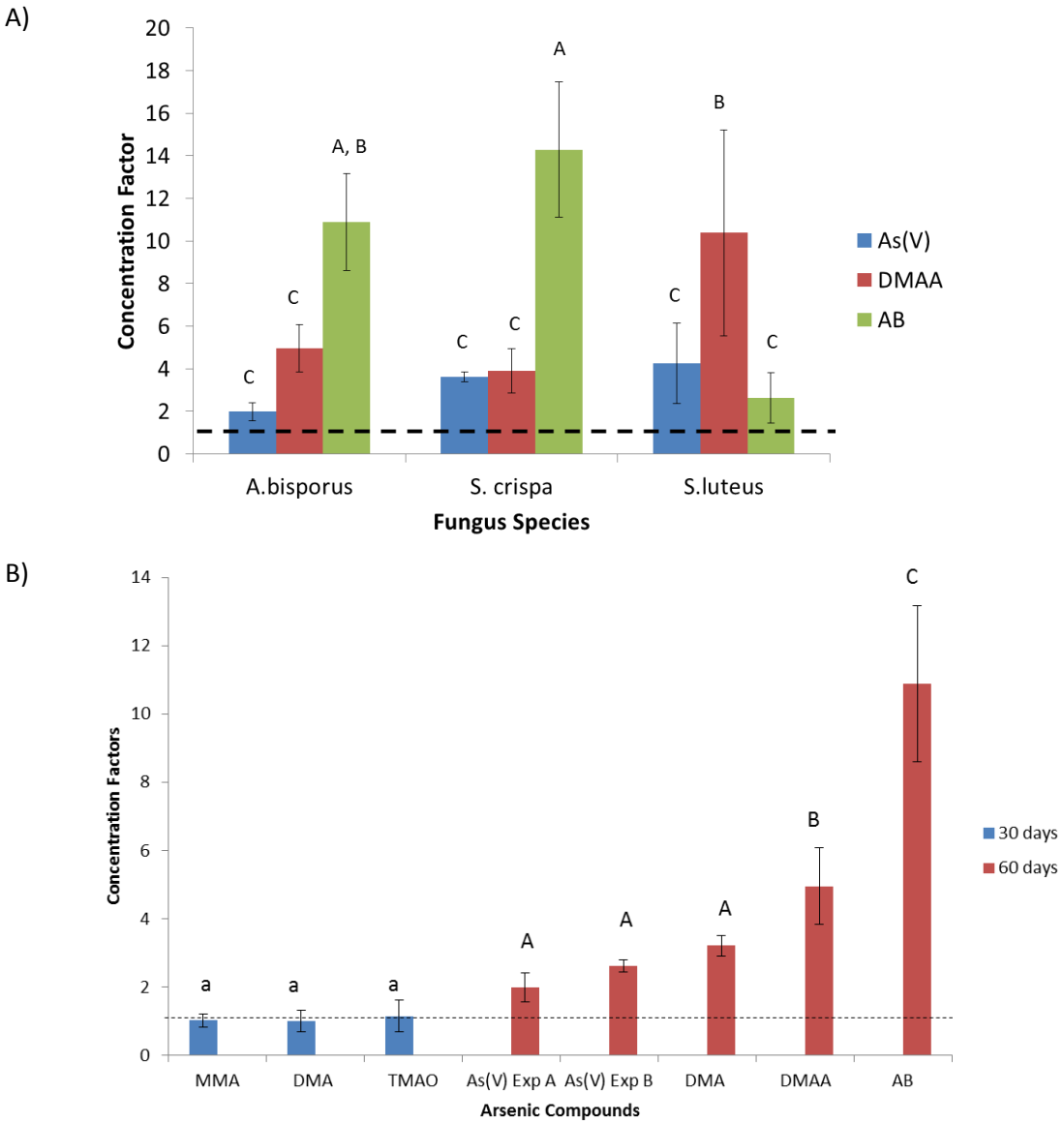


Figure 4.2: Average concentration factors (CF) for fungus species grown with arsenic compounds. Different letters above the average CF indicate a significant difference between treatments (ANOVA). The error bars represent one standard deviation of the calculated CFs. The dashed line represents a CF = 1. A) Three mushroom species treated with As(V), DMAA and AB at ~100 µg/L As for 30 days. B) A.bisporus Experiment A (As(V) Exp A, DMAA and AB at 100 µg/L As 60 days), Experiment B (As(V) Exp B and DMA at 100 µg/L As 60 days) and C (MMA, DMA and TMAO at 250 µg/L As 30 days) treatment groups. Lower case letter indicating significant differences are for Experiment C (30 days) and upper case letters are for Experiments A and B (60 days).

The mycelium's preferential accumulation of AB for a specific role in the fungus was considered by reviewing studies of arsenic speciation in fruiting bodies, which revealed that similarities in fruiting body morphologies generally corresponded with similar major arsenic compounds [17, 18, 30, 164, 165]. For example, gilled mushrooms such as *A. bisporus* contain a majority of AB. Pored mushrooms such as the *S. luteus* species mainly contain DMA and other morphologies vary. *S. crispa* is a polypore mushroom with a coral fruiting body morphology (hence its common name "cauliflower mushroom"), but in the literature the arsenic speciation information in this type of fruiting body morphology is limited. As mentioned in the introduction, AB in *A. bisporus* has been hypothesized to act, as it does in some marine organisms, as an osmolyte [31, 39], specifically in helping to maintain a rigid cap for effective spore dispersal [39]. *S. crispa* might also make use of AB for osmolytic regulation for fruiting body structure maintenance, since in nature the large fruiting bodies become very large. To date only two *S. crispa* samples have been analyzed for arsenic speciation, with AC as the major arsenical, and one sample also containing a quantifiable amount of AB [30].

A. bisporus mycelia were exposed to a more comprehensive selection of arsenic compounds than that used for other fungus species, and the comparison of CFs (ANOVA , $F(4, 40) = 86.66$, $p < 0.0001$) is shown in Figure 4.2b. The comparison included the As(V) CFs from Experiments A and B at the same conditions (60 days and 100 μg/L As), which were statistically indistinguishable ($p = 0.78$). Like in Figure 4.2a, the CF for AB is significantly higher than that for all other compounds ($p < 0.0001$) but the CF for DMAA was significantly higher than that for As(V) ($p < 0.0001$) and DMA ($p < 0.05$) (Figure 4.2b). The CFs from Experiment C for DMA, MMA and TMAO were examined separately since these results were obtained after only 30 days exposure and using a higher arsenic concentration (250 μg/L As). These factors and the possibility of underestimating total arsenic concentrations in mycelia from these experiments (since they were estimated using extracted arsenic) allow for a possible low bias in the CFs. Indeed, DMA, tested in both conditions, gave a CF after 60 days triple that after 30 days. Since no significant difference in CFs was seen for DMA and the other two compounds, MMA and TMAO, in Experiment C (ANOVA, $F(2, 6) = 0.19$, $p = 0.84$), we hypothesize that MMA and TMAO accumulation would also increase in a similar manner if the experiments had been carried out for 60 days, but would not be accumulated as much as AB, recognizing that the present study cannot discount this possibility. The 30 day time period was selected for MMA, DMA and TMAO treatments to simulate the compounds available in the growth material and length of time in the commercial growth of *A. bisporus* [39].

4.4.2 Arsenic Speciation and Transformations

The average concentrations and standard deviations of arsenic compounds found in the mycelium are listed in Table 4.2 along with column recoveries (CR) and extraction efficiencies (EE). The collected media samples were also analyzed for arsenic speciation but no changes in speciation occurred in any of the replicates across all experiments. For the majority of mycelium samples little change in arsenic speciation occurred. In *A. bisporus* mycelium treated with As(V) for 120 days, over 50% of As(V) was converted to As(III) and trace amounts of MMA and DMA appeared, indicating methylation occurs very slowly in the mycelium of this fungus species. *Agaricus placomyces* mycelium grown on agar spiked plates also reduced As(V) to As(III) but methylated MMA to DMA to a greater extent (16.8% of total arsenic) than that seen here [38].

A mixture of As(V) and As(III) was found in *S. luteus,* suggesting that reduction of As(V) to As(III) was taking place in the mycelium, but the mixture was variable; the inoculated negative control mycelium for this species also contained a mixture of As(III) and As(V). The absence of change in As(V) seen in the mycelium-free control media indicates that the mycelium is most likely responsible for reducing the As(V) to As(III).

Table 4-2: Average arsenic species concentration, column recovery (CR) and extraction efficiency (EE) of mycelium.

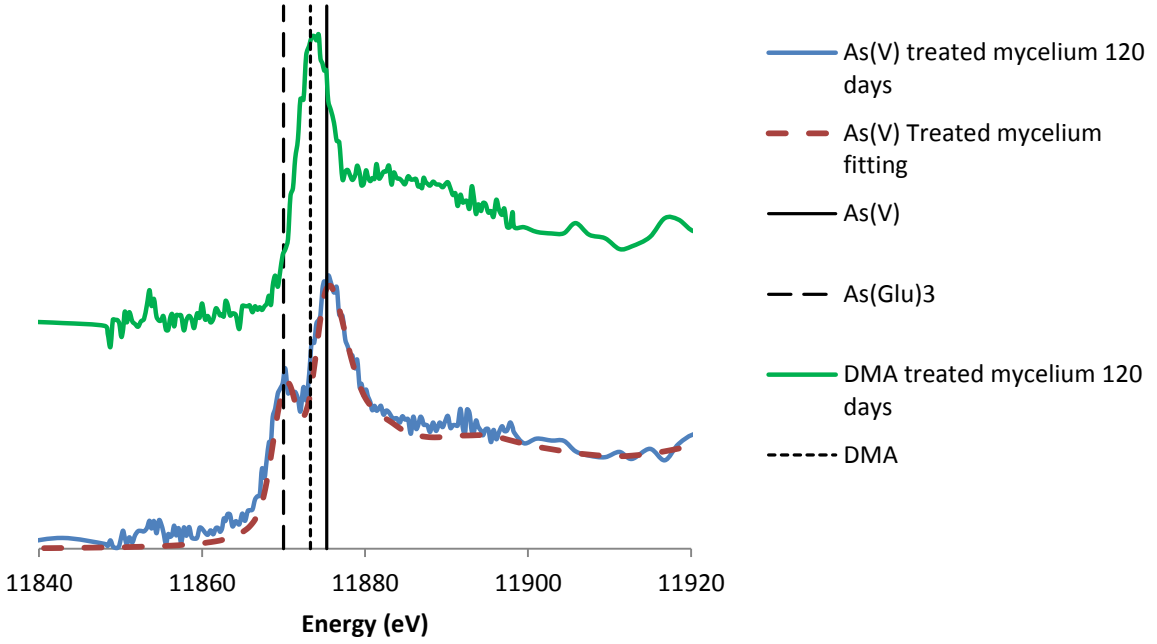
Fungus Species	Treatment	As Compounds (ug/kg wet weight)							Column Recovery (%)	Extraction Efficiency (%)
		As III	As V	MMA	DMA	TMAO	DMAA	AB		
A. bisporus	Control[A]	130 ± 9	nd	nd	nd	nd	nd	nd	140 ± 30	80 ± 20
	As(V)[A]	trace	70 ± 40	nd	nd	nd	nd	nd	120 ± 10	34 ± 10
	DMAA[A]	trace	nd	nd	nd	nd	190 ± 40	nd	110 ± 10	85 ± 8
	AB[A]	51 ± 60	nd	nd	nd	nd	nd	240 ± 40	90 ± 20	96 ± 2
	Control[B]	nd	nd	nd	nd	nd	nd	nd	NC	NC
	As(V) 60 days[B]	104.8 ± 0.8	80 ± 5	nd	nd	nd	nd	nd	110 ± 10	74 ± 20
	As(V) 90 days[B]	210 ± 20	150 ± 100	nd	nd	nd	nd	nd	80 ± 30	85 ± 11
	As(V) 120 days[B]	280 ± 100	150 ± 200	trace	trace	nd	nd	nd	90 ± 70	90 ± 3
	DMA 60 days[B]	nd	nd	nd	300 ± 9	nd	nd	nd	110 ± 4	73 ± 20
	DMA 90 days[B]	nd	nd	nd	500 ± 4	nd	nd	nd	100 ± 2	90 ± 4
	DMA 120 days[B]	nd	nd	nd	800 ± 20	nd	nd	nd	100 ± 20	68 ± 4
	Control[C]	nd	nd	nd	nd	nd	nd	nd	NC	NC
	MMA[C]	nd	nd	150 ± 10	nd	nd	nd	nd	110 ± 10	NC
	TMAO[C]	nd	nd	nd	nd	160 ± 100	nd	nd	92 ± 30	NC
	DMA 30 days[C]	nd	nd	nd	140 ± 40	nd	nd	nd	80 ± 10	NC
S. luteus	Control	trace	trace	nd	nd	nd	nd	nd	NC	82 ± 2
	As(V)	110 ± 40	trace	nd	nd	nd	nd	nd	83 ± 20	70 ± 10
	DMAA	trace	222*	nd	nd	nd	180 ± 80	nd	110 ± 20	90 ± 10
	AB	trace	nd	nd	nd	nd	nd	70 ± 30	100 ± 20	87 ± 10
S. crispa	Control	trace	60 ± 70	nd	nd	nd	nd	nd	140 ± 100	98 ± 3
	As(V)	trace	270 ± 10	nd	nd	31 ± 3	nd	nd	100 ± 7	93 ± 3
	DMAA	trace	165*	nd	nd	nd	110 ± 70	nd	110 ± 30	96 ± 1
	AB	nd	nd	nd	nd	nd	nd	580 ± 60	100 ± 7	97 ± 1

[A B C] Control mycelium (no As) corresponds to treatments with the same letter, * indicates only observed in one replicate, nd = non-detect (< 3 µg/kg), trace < 5 µg/kg, NC = not calculated

In the *S. crispa* mycelium only As(V) and TMAO (31 ± 3 µg/kg As WM) were present in the As(V) treatment, suggesting that this fungus species can produce TMAO from As(V). The absence of the possible intermediates (e.g., As(III), MMA and DMA) may be attributable to the kinetics of the various methylation steps and the time period of the exposure (30 days), reaching TMAO and possibly allowing As(III) that was produced to re-oxidize to As(V). More time and experiments would be required to determine if the fungus can further transform the arsenic to AC, the major compound in the fruiting bodies, or if the TMAO is transformed further by the surrounding microbial community. As(III) was detected in the AB treatment for *A. bisporus* mycelium but this was probably not from the degradation of AB but from background levels seen in the negative control. For one DMAA-treated *S. luteus* replicate, the majority of arsenic was in the form of As(V) indicating that the fungus may degrade the complex arsenic compound, but this transformation was not seen in the two other replicates.

The extraction efficiencies (EEs) were generally high in this study except in *A. bisporus* amended with As(V) in Experiment A (34 ±15 %), but XAS analysis, a solid state method, was used to speciate all of the arsenic present, to provide insight into arsenic species transformations during experimental manipulations required for HPLC-ICP-MS analysis [184]. The high detection limits, 1 – 5 mg/kg [184], limited the number of samples analyzed for XANES to two: the 120 day mycelia treated with As(V) and DMA (Experiment B) (Figure 4.3a). The proportions of arsenic compounds in the two measurable samples are shown in Figure 4.3b. The DMA-treated mycelium contained 100% DMA, confirming the HPLC-ICP-MS results. In the As(V)-treated mycelium the XANES analysis revealed that approximately 40% of the total arsenic was in the form of arsenic glutathione ($As(Glu)_3$, structure shown in Figure B.1). Such compounds are often not seen under HPLC-ICP-MS conditions because they can be unstable and transform to their oxygen analogues [184]. In plants, As(III) sulfur compounds are thought to be formed in plants after the uptake of inorganic arsenic from the soil, which may initiate one type of arsenic resistance mechanism [185]. The remaining 50% of the arsenic was in the form of As(V) and an As(V)-O glycerol compound (Figure B.1, a compound thought to be formed from four-coordinated As(V)-O, that is, arsenate, as a result of the sample drying process [116]), which generally agrees with the proportion of As(III) and As(V) determined by HPLC-ICP-MS analysis (Figure 4.3b).

A)



B)

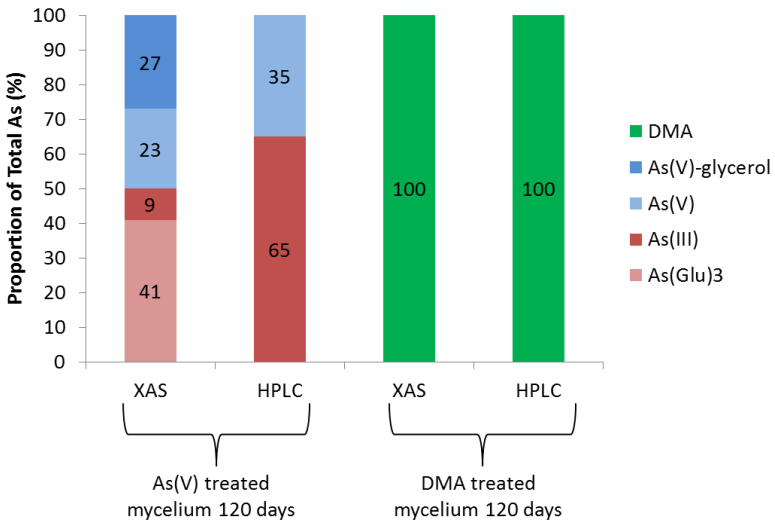


Figure 4.3: XAS spectra for As(V) and DMA treated *A. bisporus* mycelium. The red dashed line shows the fitting for the As(V) treated mycelium (reduced chi square = 0.02). The vertical lines indicate white line energies for $As(Glu)_3$ liquid (11870.0 eV), DMA(V) liquid (11873.3 eV) and As(V) solid (11875.3 eV). B) Proportion of arsenic compounds in As(V) and DMA treated *A. bisporus* mycelium determined by HPLC-ICP-MS and XAS analysis.

The lack of transformations in mycelium samples suggests that AB is not made during the vegetative life stage of the tested fungi, and specifically DMAA is not converted to AB, a transformation that was demonstrated in a previous study with lysed bacterial extracts [26].

However, for two fungi species AB is preferably accumulated at the mycelium life stage. Preferential accumulation may lead to transport of AB to the fruiting body from the mycelium. Modelling studies predict that mycelium can grow through heterogeneous environment to find necessary nutrients, for distribution to the rest of the mycelium, and axenic *A. bisporus* mycelium translocated an osmolytic precursor, trehalose, to the parts of mycelium that give rise to fruiting bodies [186, 187]. In all mushroom species it is unknown whether AB, when it is present in the fruiting body, is produced by the fungus, or if the microbial community produces AB or its precursors. The microbial community has been responsible for the presence of MMA and DMA in plant species [54], and the microbial community in compost material treated with As(V) produces methylated compounds mainly in the form TMAO [39], a potential precursor. The fact that the *S. crispa* mycelium preferentially accumulated AB, but did not transform the AB to AC suggests that when AB is available in the surrounding environment the mycelium will accumulate it. In the previously studied specimens [30], the microbial community present in the host substrate (logs) may have produced little or no AB or its precursors.

Overall, the few biotransformations, and lack of AB formed from potential precursors observed in these experiments indicate that it is unlikely that the mycelium of fungus is responsible for biosynthesizing AB. However the preferential accumulation of this compound in the species in which it is dominant in the fruiting bodies indicates that the mycelium may be selectively accumulating the compound and transporting it to the fruiting bodies. Alternatively the fruiting body itself or the microbial community (perhaps that specifically associated with the fruiting body formation) may produce AB. For most species of higher terrestrial fungi the mushroom life time is only 10 to 14 days [179], so it was presumed that the mycelium would be able to produce compounds within the 60 day experimental time period used in the present study, and if not, the transformations would have been seen in the 120 day experiment . Furthermore, in commercial growing conditions of *A. bisporus*, the fruiting bodies already contain a majority of AB after 30 days [39]. The most significant transformation occurred in the *S. crispa* mycelium treated with As(V) to produce TMAO, indicating that arsenic biotransformations vary across fungus species at the mycelium stage, in addition to the known variations in fruiting bodies [30]. In order to determine the formation pathway for AB in terrestrial fungi, the fruiting bodies and the surrounding microbial community should be individually, axenically exposed to arsenic compounds. Further exposure experiments using AC should also be performed in order to examine the possible conversion of AC to AB

as a parallel pathway to the conversion pathway of choline to betaine observed in some fungi species [188].

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Chapter 5[4]

5 Uptake and transformation of arsenic during the reproductive life stage of *Agaricus bisporus* and *A. campestris*

5.1 Abstract

Fruiting bodies from the *Agaricus* genus have been found to contain non-toxic arsenobetaine (AB) as a major compound. It is unknown whether AB is formed during the vegetative or reproductive life stages of the fungus, or by the surrounding microbial community, but AB's structural similarity to glycine betaine has led to the hypothesis that AB may be adventitiously accumulated as an osmolyte. To investigate the potential formation of AB during the reproductive life stage of *Agaricus* species growth substrate and fungi were collected during the commercial growth of *A. bisporus* and analyzed for arsenic speciation using HPLC-ICP-MS analysis. AB was found to be the major arsenic compound in the fungus at the earliest growth stage of fruiting (the primordium). The growth substrate mainly contained arsenate (As(V)). The distribution of arsenic in an *A. bisporus* primordium grown on As(V) treated substrate, and in a mature *A. campestris* fruiting body collected from arsenic contaminated mine tailings, was mapped using two dimensional XAS imaging. The primordium and stalk fungal tissue of the mature fruiting body were both found to be growing around pockets of substrate material containing higher As concentrations, and AB was found exclusively in the fungal tissues. In the mature *A. campestris* fruiting body the highest proportion of AB found in the cap, supporting the AB as osmolyte hypothesis. The presence of AB in the earliest life stage of the reproductive life cycle and preferential accumulation of AB seen in mapped fungal tissues indicates AB formation is likely associated with the reproductive life stage of the fungus.

Keywords: Arsenobetaine, arsenic, *Agaricus*, fungi, HPLC-ICP-MS, XAS imaging

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5.2 Introduction

Arsenobetaine (AB) is the only arsenic compound that is non-toxic and is found to comprise the majority of arsenic in many marine organisms [1], but its formation pathway is still unknown. Unlike in the marine environment, AB is found in only a few terrestrial organisms, and in low proportions. The exception to this trend is the fruiting bodies [1], or mushrooms, of many terrestrial fungi species from the class basidiomycetes, where AB can comprise the majority of arsenic in a wide variety of species [183].

Two of the three current hypotheses for the formation of AB are derived from studies of the marine environment and involve the degradation of arsenosugars, which are found at high levels in food sources, such as algae, for marine organisms. One proposed pathway involves the degradation of dimethylated arsenosugars to an arsenocholine (AC) intermediate, which is then converted to AB. A second proposed pathway also involves the degradation of dimethylated arsenosugars to form a dimethylarsinoylacetic acid (DMAA) intermediate, which is then converted to AB. The third current proposed pathway provides a more likely pathway for terrestrial organisms, where dimethylarsinous acid (DMA (III)) reacts with 2-oxo acids, glyoxalate and pyruvate to form a DMAA intermediate, which is then converted to AB [162].

AB is structurally similar to the osmolyte glycine betaine and has been found to be adventitiously accumulated in marine organsisms as an osmolyte [31]. AB has also been hypothesized to be similarly accumulated in mushrooms as an osmolyte to help maintain fruiting body structure for effective spore dispersal [39, 183]. It is unknown whether AB is formed by the fungus, during the vegetative or reproductive life stage, or whether it (or its precursors) is accumulated from the surrounding environment having been produced by the surrounding microbial community.

Terrestrial species in the basidiomycetes class of fungi can grow through asexual reproduction (the vegetative life stage), and through sexual reproduction (the reproductive life stage). The previously mentioned fruiting bodies, or mushrooms, are produced during the reproductive life stage and these mushrooms are used for spore dispersal. A mushroom is typically composed of a stipe (stalk) that elevates the cap above the ground. The cap contains the lamella (gills) on which the basidia (spores) are found. Spores dispersed into the environment from the gills germinate in the appropriate substrate under optimal environmental conditions and form monokaryon hyphae (containing one nucleus) that grow through asexual reproduction making up the primary mycelium, which exists in the substrate. Two compatible monokaryon hyphae can fuse to form dikaryon hyphae (containing two different nuclei) making up the secondary mycelium where the fungus will spend most of its life [41]. Environmental conditions specific to each fungi species trigger fruiting body development, which allows the fungus to reproduce sexually once again.

Mushrooms that have been found to contain AB as a major compound include those from the genus *Agaricus* [30, 183]. *Agaricus bisporus* is a commonly cultivated edible mushroom, and when cultivated on arsenic treated material, the mature mushrooms also contained a majority of AB, except when grown on material with high arsenic concentrations [39, 178].

In the commercial cultivation of *A. bisporus* the conditions for fruiting body production have been optimized. The *A. bisporus* is grown on a nutritious compost substrate usually composed of composted straw (Figure 5.1). The compost is inoculated with rye grain seeds overgrown with mycelium. The compost layer is then covered with a non-nutritious casing layer (peat moss and calcium limestone) to facilitate fruiting body development, which follows changes in environmental conditions. These changes involve airing the growth room to decrease total carbon dioxide and inhibitory gases produced by the fungus, which induces the mycelium to undergo a process known as differentiation, specifically, to form hyphal aggregations and knots that give rise to the first growth stage of the fruiting body, the primordium. A decrease in temperature then causes further differentiation of the primordium tissue to begin forming the tissues of the mature fruiting body [189]. As soon as the primordium is developed it contains two distinct tissues, the cap and stipe, with the gills containing the spores differentiating and maturing under the cap as the fruiting body enlarges [190].

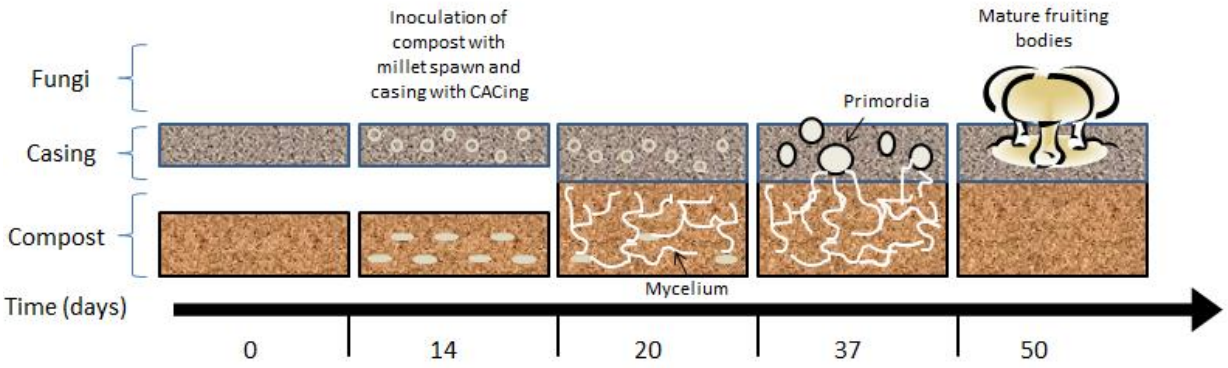

Figure 5.1: A schematic of the commercial growth process for A. bisporus. Compost at casing (CACing) is substrate fully colonized with mycelium added to the casing layer to promote uniform growth of mycelium in the casing layer.

Studies on arsenic speciation in mushroom-producing fungi have focused on the mushroom stage because the mushrooms are the part of the fungus that appear above the ground and can be identified and easily picked; this therefore limits studies to the final stage of the reproductive life cycle [18, 30, 38, 39, 164,

178, 183]. Only a few studies have been carried out at earlier stages. Two studies of *Agaricus* sp mycelium were carried out under axenic conditions to investigate the formation of AB at this stage and by the fungus alone. *Agaricus placomycetes* mycelium methylated MMA to DMA, and preferentially accumulated TETRA and AB, but it could not synthesize AB [38]. *A. bisporus* mycelium was found to produce trace amounts of MMA and DMA when exposed to As(V) and preferentially accumulate AB, but it also could not produce AB [191]. For the previously mentioned *A. bisporus* cultivated on arsenic treated material the growth substrate and compost material were also either collected before inoculation [178] or after harvesting [39]. To more comprehensively investigate the formation of AB in *A. bisporus* at each part of the reproductive life cycle, the present study aims to examine the arsenic speciation in the growth substrate and fungi at different times during the commercial growth of *A. bisporus.*

Additionally, we aimed to study the arsenic distribution in mushrooms at early life stages to interrogate AB formation further. The method used for this was solid state two dimensional X-ray absorption spectroscopy (XAS) but to use this method, higher concentrations were necessary, and these were obtained by cultivating *A. bisporus* on material from a mushroom farm that had been amended with arsenic in a laboratory setting. A mature mushroom from the *Agaricus* genus, collected from arsenic contaminated mine tailings to enable the higher required concentrations, was also mapped to examine the distribution of arsenic in the different tissues of a mature mushroom. Arsenic speciation in the different sections of the primordium and mature mushroom was also determined using micro-XANES analysis.

5.3. Methods

5.3.1 Chemicals and Reagents

Chemicals and reagents used for mushroom growing kit amendments, total arsenic and arsenic speciation analysis are listed in Appendix C.1.

5.3.2 Collection of samples from a commercial growth facility for A. bisporus

All samples were collected using a sterile scoop or tweezers. Compost, casing and fruiting bodies samples were collected from the beginning of the commercial growth process to the last harvest of the mushrooms. The sampling points were determined by the availability at the commercial facility and the samples collected are summarized in Table 5.1. Primordia and fruiting bodies were sorted by size. The white strain of *A. bisporus*, the white button mushroom, was collected from throughout the commercial growth process. The brown strain of *A. bisporus*, cremini and Portobello mushrooms, were only available for sampling at time of harvest. Fruiting bodies were washed with deionized distilled water (ddH_2O) before all samples were frozen, freeze dried, and homogenized. A stainless steel blender was used to

homogenize compost samples and a ceramic mortar and pestle was used for the casing and fruiting body samples.

Table 5-1: Summary of samples collected at a commercial growth facility for A. bisporus.

Time (days)	Process	Sample type	Description
0	Mycelium added to compost	Casing	Uninoculated casing material
		Compost	Uninoculated pasteurized compost material
14	Mycelium growth and casing material preparation	Casing	Casing material with CACing[a] added.
		Compost	Compost 2 weeks after rye grain spawn added
20	Casing added	Casing	Casing recently spread over compost layer, no mycelium in casing
		Compost	Compost material overgrown with mycelium
37	Beginning of fruiting body growth	Fruiting bodies	Primordia, < 1 cm
		Casing	Casing material with mycelium
		Compost	Compost material with mycelium
50	Mature fruiting bodies	Fruiting bodies	Various growth stages: 1 – 2 cm; 2 – 4 cm; > 5cm; mycelium
		Casing	Casing material with mycelium
		Compost	Compost material with mycelium
		Fruiting bodies [b]	Various growth stages of brown strain: < 2cm; > 2 cm
		Casing	Casing material with brown strain mycelium
		Compost	Compost material with brown strain mycelium

[a]CACing = compost at casing, which is substrate fully colonized with mycelium. The CACing is added to the casing layer to promote uniform growth of mycelium in the casing layer.
[b] Brown strain fruiting bodies(Portobello and cremini) were only available at the harvesting stage at the time of sampling at the commercial growth facility.

5.3.3 Cultivation of A. bisporus

Mushroom growing kits were purchased from White Crest Mushrooms, Putnam, ON. The kits were delivered with an inoculated compost layer covered with a top casing layer. The mycelium was growing throughout the compost layer at the time of arrival and the casing layer had already been applied. The kits were treated with arsenate by using either a 10 or 100 mg/L arsenate watering solution applied to the top of the kit and injected at multiple locations into the compost layer using a syringe. The controls (no arsenate added), 10 and 100 ppm watering solution treatments were performed in duplicate. The kits were grown at 24 ^{o}C at 90 % relative humidity. Compost and casing samples were collected after 1 week (T1) of watering before the mycelium grew into the casing. Compost, casing and mushroom samples were

collected after the first (T2, about 30 days) and second (T3, about 45 days) flushes and prepared for arsenic speciation analysis in the same way as the commercial facility samples.

5.3.4 Arsenic Species Extraction and Total Arsenic Digestion

For all samples 0.5 g of dried sample was extracted using a 50% aqueous methanol extraction followed by a sequential 2% nitric acid extraction following the methods described in Nearing et al., 2014 [183]. The samples collected from the commercial facility were extracted in triplicate. Total arsenic was obtained as the sum of extracted and residual arsenic. The complete method is provided in Appendix C.2 and C.3

5.3.5 Instrumental Analysis

All samples (extracts and residues) were analyzed for total arsenic using ICP-MS. The ICP-MS operating conditions are described in Caumette et al., 2011 [24]. Instrument quality control checks were found to be acceptable. All aqueous methanol extracts were analyzed for arsenic speciation using HPLC-ICP-MS (cation and anion exchange). The HPLC-ICP-MS operating conditions are described in Caumette et al., 2011 [24] and are included in Appendix C.4 and C.5. Instrument quality control checks were found to be acceptable.

5.3.6 X-ray Absorption Spectroscopy (XAS)

Subsamples of the dried and homogenized samples prepared for the arsenic species extractions were loaded directly into a sample holder and placed between two layers of Kapton® tape for bulk X-ray Near Edge Structure (XANES) analysis. XANES analysis was performed at the Advanced Photon Source (APS) Pacific Northwest Consortium/X-ray Science Division (PNC/XSD) on the bending magnet (BM) beam line, Sector 20. XANES spectra of the arsenic Kα-edge (11686 eV) were collected and analyzed as described in Nearing et al., 2014 [183]. For two-dimensional imaging whole *A. bisporus* primordia and *A. campestris* mushrooms were freeze-dried. Thin sections were sliced using two razors blades taped together approximately 1 mm apart. The slices were placed between two glass slides to create a sample with uniform thickness. Immediately prior to analysis the samples were removed from the glass slides, placed between two layers of Kapton® tape and mounted on the sample holder. Photographs of the mapped tissues were taken using a VistaVision stereozoom microscope. Two dimensional XAS analysis was carried out on the insertion device (ID) line, Sector 20, using fluorescence data, and samples were kept at −75 to -100 °C. Fluorescence data were collected at 40 μm steps with a 0.3 s integration time for two-dimensional spectra, and the beam was focused to 5 x 5 μm spot size. Calibration of the Si(111) double-crystal monochromators was carried out by using the first inflection point of the gold LIII absorption edge (11919.7 eV). A reference gold foil was measured simultaneously with all samples. Two-dimensional XAS mapping was carried out at 11875.3 eV. At points of interest μXANES were collected

to determine the arsenic speciation. For the µXANES collection a total of 3 to 5 scans were collected with a 0.5 eV step size over the edge region and averaged prior to background removal and normalization to edge jump. XANES spectra of the arsenic K-edge (11868 eV) were fit within -20 to +30 eV from the arsenic E_O using Athena software. Frozen As(III), As(V) and As(V) glycerol standards [116], and liquid AB, DMA, TMAO, and arsenic glutathione ($As(Glu)_3$) [92] previously measured by our group were used for fittings.

5.3.7 Quality Assurance and Quality Control (QA/QC)

One certified reference material was included with every 10 samples: for extractions, either tuna fish BCR-627 (certified values: 4.8 ± 0.3 mg/kg total As, 3.9 ± 0.23 mg/kg AB and 0.15 ± 0.022 mg/kg DMA) or dogfish muscle DORM-3 (certified value: 6.88 ± 0.30 mg/kg total As), and for total digestions, lichen BCR-482 (certified value: 0.85 ± 0.07 mg/kg). The selection of CRM for extractions was dependent on the availability in our laboratory at the time of analysis. DORM-3 AB and DMA recoveries were calculated with respect to reference values from a round robin experiment (Zoltan Mester, personal communication, 2012). The certified values for this were: AB value = 4.12 mg/kg, range 3.86 – 4.38 mg/kg, DMA value = 0.447 mg/kg, range 0.392 – 0.563 mg/kg (ranges calculated with 95% confidence intervals). Method blanks were also included for every 10 samples and returned non-detectable levels of arsenic. The limit of detection (LOD) for total arsenic in the commercial growth facility samples ranged from 4 – 6 µg/kg DM and for the mushroom growing kits samples ranged from 2 – 10 µg/kg DM. Matrix spikes, with a spiking concentration of 50 µg/L, were prepared directly prior to analysis at a frequency of 10% for both arsenic total and speciation analysis. QA/QC results are summarized in Appendix C (Table C1) and were considered acceptable (± 30 % recoveries) with the following comments. Two 2% nitric acid extract matrix spike (N =11) recoveries were below 70% with recoveries of 60 and 65%. One CRM Tuna BCR 627 extract (N = 6) recovery for AB had a recovery of 67%, and one column recovery for a CRM Tuna BCR 627 extract (N = 6) had a recovery of 140%. These values were accepted because the other QC checks in these batches were found to be in an acceptable range, ± 30 % recoveries.

No adverse effects on mushroom growth were observed in the arsenate treated mushroom growing kits.

5.4 Results and Discussion

5.4.1 Arsenic speciation during the reproductive life stages of *A. bisporus*

The average total arsenic concentrations in the growth substrate (compost and casing) and fruiting bodies from time of inoculation to time of harvesting are summarized in Figure 5.2. A total of 18 samples were

collected for each sample type and there was significant variation in total arsenic (ANOVA, F ($F(2, 51) = 52.57$, $p < 0.0001$). The compost material, which provides the nutritious substrate for fruiting body development, contained significantly higher concentrations of arsenic than the casing layer ($p < 0.0001$).

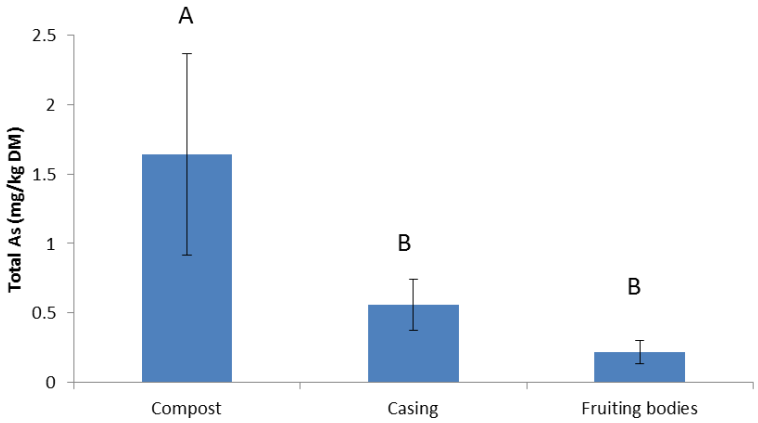

Figure 5.2: Average total arsenic concentrations in compost (N = 18), casing (N = 18) and fruiting bodies (N = 18) dry mass (DM) from all growth stages. Different letters indicate a significant difference between total arsenic concentrations (ANOVA, $p<0.05$). The error bars represent one standard deviation.

For each sampling time point the compost, casing, and fruiting bodies were analyzed for arsenic speciation, and the arsenic species for both the aqueous methanol and 2% nitric acid extracts combined are summarized in Figure 5.3 as proportions of extracted arsenic. Concentrations of the identified arsenic species, total arsenic and column recoveries for all samples are provided in Appendix C (Table C2). Day 0 results are for the pasteurized compost and freshly mixed casing, before inoculation with *A. bisporus*, although inoculation took place at day 0. The compost, a straw based material, contained a majority of As(V), TMAO and DMA at this point. These arsenic compounds are commonly found in other plant species [1] and were also found in the control compost before inoculation used in a previous experiment with *A. bisporus* grown on arsenic treated materials [178]. At day 14 and day 20 of the present study the speciation was similar, even though at both points mycelium was present in the compost (overgrown at day 20). The compost inoculated with rye grain spawn, and casing inoculated with compost at casing (CACing) material (substrate fully colonized with mycelium), had proportions of arsenic compounds (day 14 samples, Table 5.1) similar to the day 20 compost and casing samples (when compost was overgrown with mycelium and casing was added over the compost layer). When the first stage of fruiting body development, primordia, were formed at 37 days, the compost material contained proportions of As(V), DMA and TMAO similar to the earlier compost samples, but trace amounts of AB were also

detected. From day 14 and subsequently, the compost material could not be separated from the mycelium, so it is unclear whether the AB at 37 days was produced directly by the fungus or the microbial communities in the compost.

The casing material contained only As(V) throughout the entire sampling period, even though at day 14, CACing (compost-at-casing) material, or substrate fully colonized with mycelium was added. This arsenic was identified in the 2% nitric acid peaks only (HPLC peaks could not be detected in aqueous methanol extracts).

As mentioned previously, it is unknown whether AB is accumulated/produced during a specific growth stage of the fruiting body and thus the earliest possible growth stage of the fruiting bodies along with different sizes (corresponding to different growth stages of fruiting bodies) for both the white and brown strain of *A. bisporus* were examined. Only fruiting bodies of the brown strain, known commonly as cremini for smaller sizes, and Portobello for large sizes with exposed gills, were available and therefore collected. The primordia, < 1cm, were the earliest growth stage of the fruiting body that could be collected and this growth stage was found to already contain a majority of AB, with minor compounds DMA and As(V). For both the white and brown strains the proportion of AB did not increase over time, with all fruiting body growth stages containing a majority of AB and similar proportions of DMA (18 to 27 %) and As(V) (13 to 23 %) (Figure 5.3). However, the 2-4 cm fruiting bodies for the white strain also contained TMAO (28 %). The mycelium masses at the base of the fruiting bodies (labeled mycelium in Figure 5.3) were also collected, even though these samples contained a mixture of mycelium and casing material because the two could not be separated. These mycelium samples contained mainly As(V), most likely from the attached casing, but also DMA (12 %) and AB (19 %). The AB seen at trace levels in the compost (containing mycelium) and lower proportions in the mycelium samples suggest that the AB is produced within the mixture of mycelium and compost/casing. AB's hypothesized role as an osmolyte allows consideration of the findings of other mushroom osmolytes. Specifically, the osmolyte mannitol, thought to help the fungus produce primordia, is accumulated by *A. bisporus* fruiting bodies, occurring at high concentrations, up to 30% DM. Moreover, under axenic conditions the mycelium of *A. bisporus* has been found to translocate trehalose to hyphal aggregates (which eventually give rise to the fruiting bodies) where it is then used to form mannitol [187]. Mannitol is found at much lower levels in the mycelium (1 – 5 % DM) compared with the fruiting bodies [187]; this discrepancy is similar to that in the present study with higher concentrations of AB found in the fruiting bodies compared with the mycelium.

The present findings suggest that AB production is associated with the reproductive life stage, or the fruiting process, of *A. bisporus.* From a previous study, we have shown that the vegetative mycelium life

stage was not solely responsible for AB formation, since it did not transform As(V), DMA and TMAO to AB under axenic conditions [191]. However, in that study significantly higher amounts of AB were accumulated into the mycelium compared with the other compounds suggesting that any AB formed by the microbial community would be taken up by the mycelium. The microbial community in the compost should be further characterized to determine its influence on arsenic speciation and studies are underway to investigate this factor.

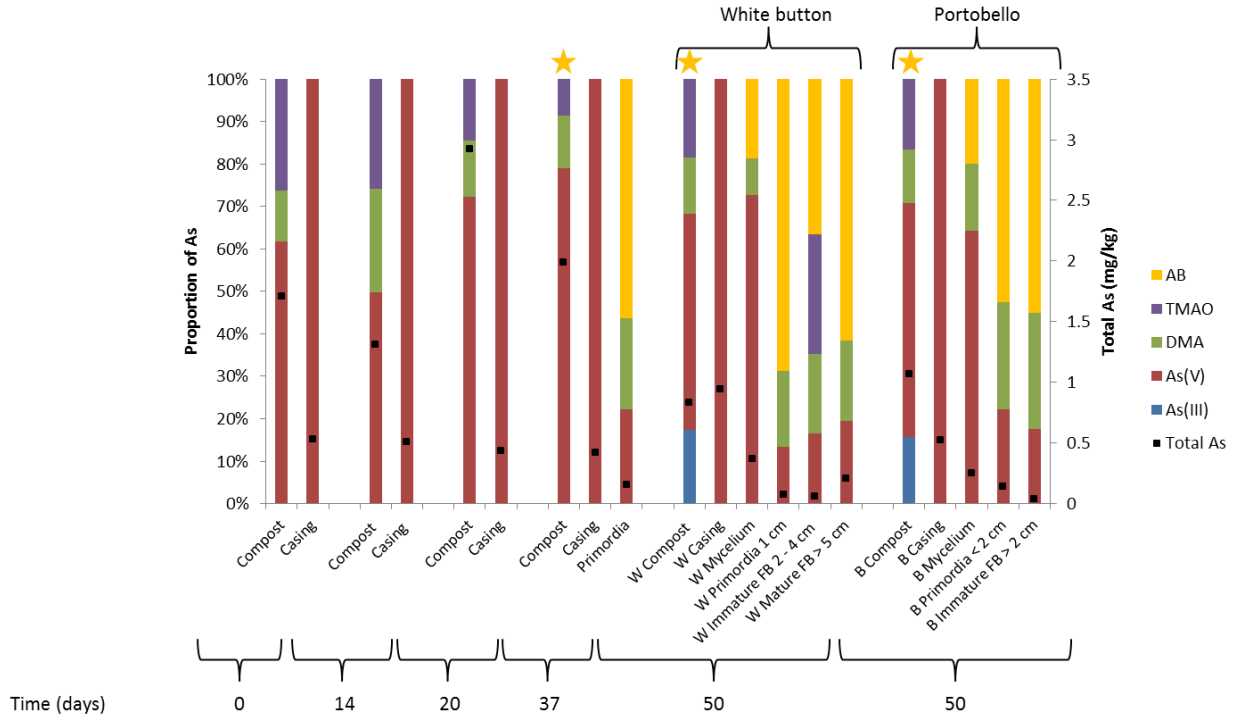

Figure 5.3: Average proportion of extracted (aqueous methanol extracts and 2% nitric acid extracts) arsenic compounds (left axis) and total arsenic (right axis and black squares) in compost, casing and fruiting body samples. At day 0 there is no fungus in the casing and compost. The time of sampling from inoculation is indicated by the numbers (in days) below the bar graph. From day 14 to 50 the fungus is present in the casing and compost. The results for the white and brown strain mushrooms are also labelled above the graph. The yellow star indicates trace AB in sample.

To determine if AB is adventitiously accumulated as an osmolyte to help elevate the cap and gills of the fruiting body, the arsenic distribution in a primordium and sections of a mature *Agaricus* sp. mushroom were mapped using two dimensional XAS imaging.

5.4.2 Arsenic uptake, transformation and distribution in *A. bisporus* and *A. campestris*

To obtain samples with sufficiently high arsenic concentrations for XAS analysis (with detection limits of approximately 1 – 5 ppm), *A. bisporus* fruiting bodies were grown on material treated with 10 and 100

mg/L As(V) solutions. *A. bisporus* fruiting bodies were grown from purchased kits that already had the mycelium growing through the compost layer, and the As(V) solutions were applied as part of the instructed watering solution. Average total arsenic concentrations for casing, compost and fruiting body samples are summarized in Table 5.2. Unlike the samples collected from the commercial production of *A. bisporus* the casing was found to contain total arsenic in concentrations that were higher than those in compost, probably because the As(V) solution added as the watering solution was mainly retained by the casing layer. The application method was also thought be the reason for the higher extraction efficiencies for the compost layer from the mushrooms kits compared with those for the compost from the commercial operation (Appendix C, Table C4). The 100 ppm treated compost material contained a total of 14 mg/kg, and the fruiting bodies collected from the duplicate treatments contained a total of 1.1 ± 0.7 mg/kg arsenic DM. The casing and compost materials were only found to contain As(V), with the exception of the control compost containing 30 – 60 % DMA and trace amounts of TMAO.

Table 5-2: Average total arsenic concentration in samples from mushroom growing kits treated with As(V) solution. The casing and compost samples were collected in duplicate at three different times (N = 6).

	Casing		Compost		Fruiting Bodies	
Treatment	Total As (mg/kg DM)	N	Total As (mg/kg DM)	N	Total As (mg/kg DM)	N
Control	2.3 ± 0.8	6	1.2 ± 0.5	6	0.103 ± 0.001	2
10 ppm	6 ± 2	6	4 ± 1	6	0.12 ± 0.04	3
100 ppm	57 ± 16	6	14 ± 4	6	1.1 ± 0.7	5

± represents standard deviation (n=6), for samples with N = 2 ± represents difference between duplicate samples

The arsenic speciation for the collected fruiting bodies is summarized in Figure 5.4, with concentrations of the identified arsenic species, total arsenic and column recoveries for all samples provided in Appendix C (Table C4). The fruiting bodies mainly contained iAs and lower proportions of DMA (3 - 30%) and AB (3 – 20 %). The control fruiting bodies contained 50 % AB and iAs. A majority of AB was not observed in the fruiting bodies from the treated kits, which may have been attributable to the higher concentrations of As(V) used in the treatment, compared with both the control and the commercial operation; higher concentrations may have inhibited some of the microbial and fungal arsenic transformations observed at the background levels for the commercial facility samples. In some marine organisms the proportion of AB has been found to decrease as total arsenic increases, and this is thought to be attributed to the biological pathways of the organism (or associated organisms) becoming saturated at the higher arsenic concentrations [105]. *A. bisporus* fruiting bodies grown on compost treated with 1000 mg/kg As(V) also contained mainly iAs compared to control group mushrooms that contained a majority of AB [178]. However mushrooms collected from arsenic-contaminated mine tailings (> 1000 mg/kg total arsenic) were found to contain a majority of AB [183] indicating that concentration alone may not have prevented

the formation of AB in the present study. The time of exposure to arsenic in the environment may also play a role in arsenic transformations; As(V) was added after inoculation of the substrate in the mushroom kits, and the already established mycelium in the kits was exposed to As(V) for a relatively short period of time, which may not have been sufficient for incorporation of As(V) into the growth substrate. Compost treated with 100 mg/L As(V) solution before inoculation with *A. bisporus*, and for a longer exposure period, produced mushrooms with AB comprising over 50% of the total arsenic in the mushroom.

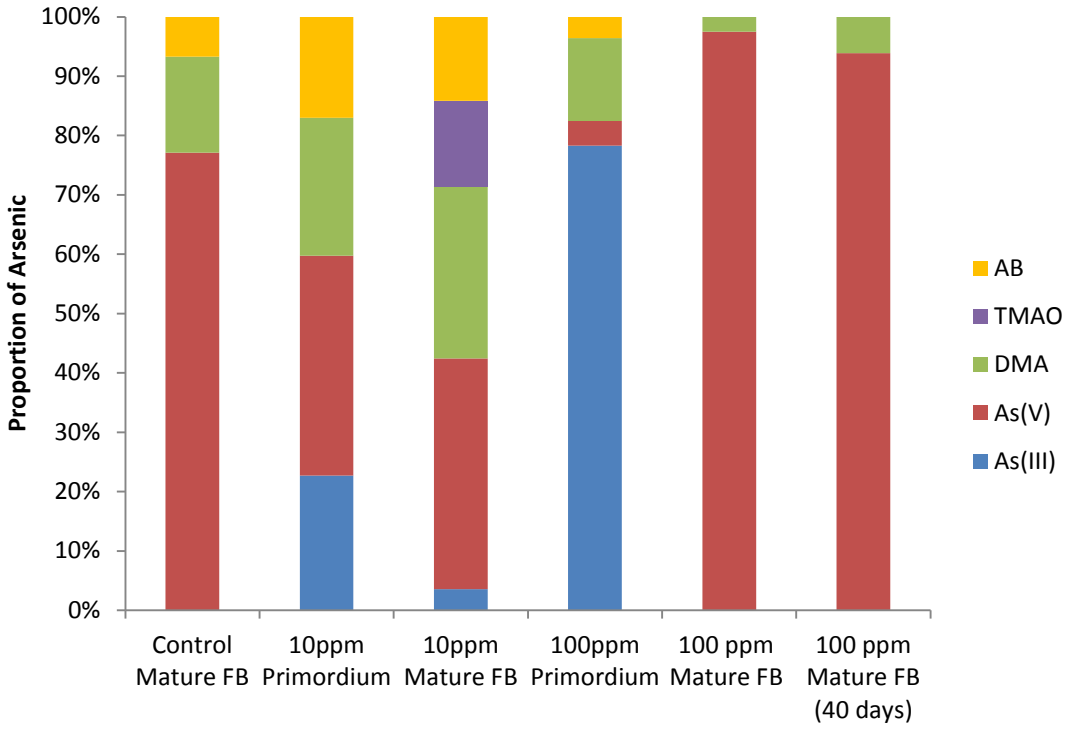

Figure 5.4: Average proportion of extracted (aqueous methanol extracts) arsenic compounds in fruiting body (FB) samples from the control and As(V) treated mushroom kits collected after 30 days.The treatment group and size of the fruiting body are indicated in the x-axis labels.

A primordium collected from the 100 ppm treated mushroom kits was selected for XAS imaging, and also analyzed in bulk by XANES. The bulk XANES analysis linear combination fitting results (Table 5.3) indicate that the majority of arsenic is comprised of iAs, specifically in the form of an As(III) sulfur compound, matched to arsenic glutathione ($As(Glu)_3$). In plants, As(III) sulfur compounds are thought to be formed after the uptake of inorganic arsenic from the soil, initiating one type of arsenic resistance mechanism [185]. This finding corresponds to the finding of predominantly As(III) by HPLC-ICPMS analysis; As(III) sulfur compounds can be oxidized to As(III) as a result of drying and grinding during

sample preparation procedures [85] and can also be degraded under chromatographic conditions during HPLC analysis [184]. The lack of DMA in the samples analyzed by XANES (but its presence seen by HPLC-ICPMS) is unclear and may be attributable to differences between the specimens analyzed.

Table 5-3: Bulk X-ray absorption near-edge structure fitting results for A. bisporus primordium and different tissue sections of A. campestris (cap, gills and stalk).

	Proportion of Arsenic (%)					
	$As(Glu)_3$	As(III)	AB	As(V)	As(V)-glycerol	Reduced chi square
Primordium	86	11	3			0.004
Cap		25.4	19.5	16	39.1	0.005
Gills	16.6	4.7		59.3	19.4	0.006
Stalk	73.8	9.5	10.1		6.6	0.004

Data were fit with frozen As(V) (white line energy 11875.3 eV); frozen As(III) (white line energy 11871.7 eV); As(V)-glycerol (white line energy 11876.5 eV); liquid DMA(V) (white line energy 11873.3 eV); TMAO (white line energy 11873.3 eV); frozen AB (white line energy 11872.6 eV); and As(Glu)3 (white line energy 11870.0 eV).

A section of the base of the primordium that was selected for mapping, and the distribution of arsenic mapped at 11875.3 eV is shown in Figure 5.5; this section was chosen because the primordium was found to grow around sections of substrate material in the microscope photograph. The section surrounding one of these pockets was mapped and μXANES were taken for the locations indicated in Figure 5.5C, with the linear combination fitting results for the collected μXANES listed in Table 5.3. Differences were expected in the mapped μXANES and bulk XANES because of the differences in locations selected for μXANES compared to the bulk (averaged or whole sample) sample. The highest arsenic concentrations were observed in the pockets of substrate material and the relative concentration decreases moving from the pockets into the surrounding tissue. μXANES spots 3, 4 and 5 (in the substrate pockets) contained mainly As(V), the main compound in the substrate material, and some As(III) and $As(Glu)_3$. Spots 2 and 6 (mostly in the primordium tissue) contained mainly As(III), some AB and DMA/TMAO (these two compounds are indistinguishable using linear combination fitting). Spots 1 and 7 (well into the primordium tissue) similarly contained mainly As(III) and a small amount of AB. At the base of the primordium it appears that the As(V) in the casing/compost material was reduced to As(III), and As(III) was found in the surrounding tissues close to the pockets of casing/compost. Moving further from the pockets AB also appears along with As(III). The higher proportion of AB observed in specific μXANES locations compared to the proportion observed in the bulk XANES indicate that AB may be produced or accumulated by this area of the primordium and further distributed to the rest of the primordium tissues where it is found at a constant low concentration. However it is clear that As(V) is reduced to As(III) by

either the fungus, or by the microbes within the substrate pocket before entering the primordium tissues. It is still unclear whether the fungus is transforming the As(III) to DMA/TMAO and AB itself or accumulating/concentrating these organoarsenic compounds.

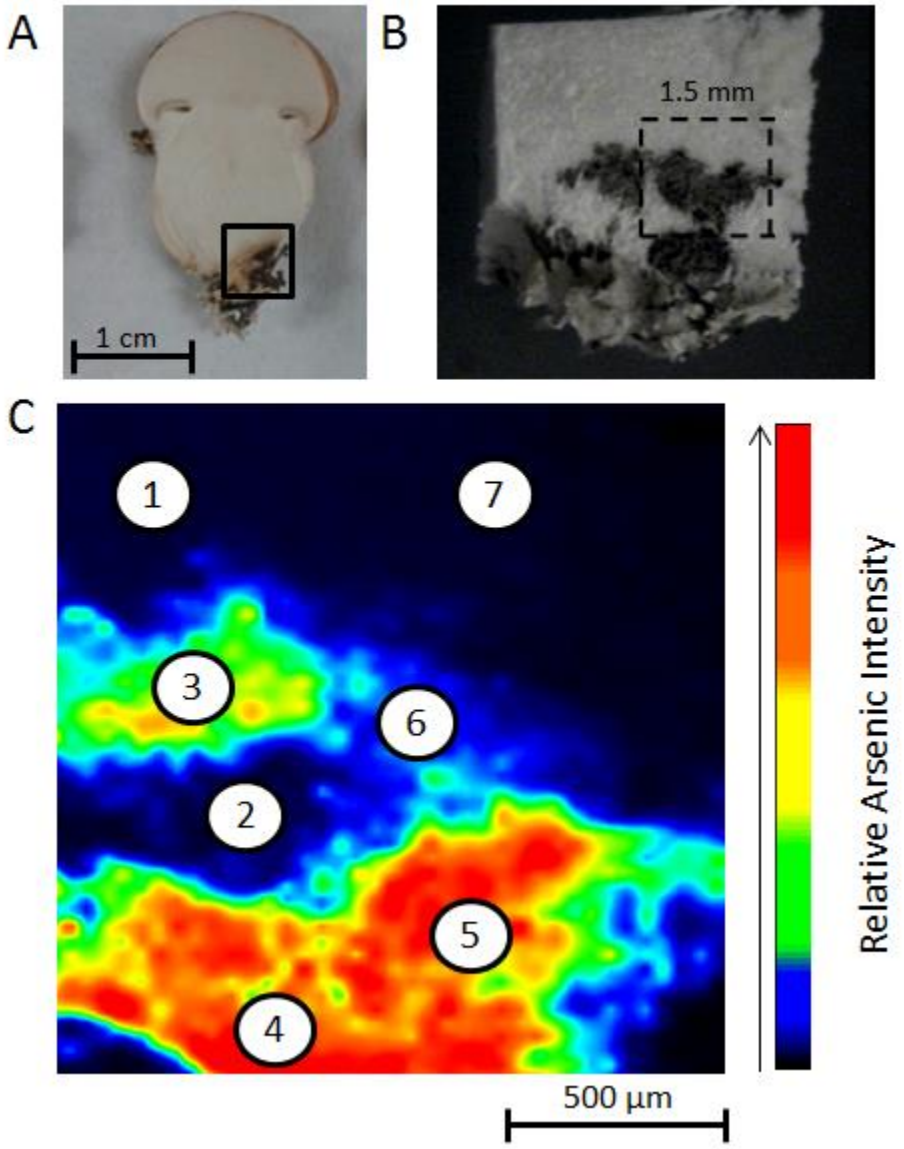

Figure 5.5: A) Cross section of A. bisporus primordium collected from 100 mg/L As(V) treated mushroom growing kit, with area sectioned for XAS imaging indicated by the black box. B) Image of *A. bisporus* primodium base, with fungus tissues growing around casing/compost material. The dashed black box indicates the area mapped. C) Map of total arsenic distribution (11875 eV) for section of *A. bisporus* primordium. The numbers indicate the locations where µXANES were collected.

Table 5-4: µXANES fitting results for locations on A. bisporus primordium total arsenic map.

Spot	Location description	Proportion of Arsenic (%)						Reduced chi square
		$As(Glu)_3$	As(III)	AB	DMA/TMAO	As(V)	As(V) glycerol	
1	Well into primordium		75.1	16.4			8.6	0.005
2	Mostly in primordium but next to substrate		45.5	12.8	29		12.6	0.0025
3	In substrate	7.1	7.5			85.4		0.008
4	In substrate	6.2	5.2			88.6		0.008
5	In substrate	1.6	15.5			82.9		0.008
6	Mostly in primordium but next to substrate		49	16.4	26		8.6	0.004
7	Well into primordium		52.1	47.9				0.02

Data were fit with frozen As(V) (white line energy 11875.3 eV); frozen As(III) (white line energy 11871.7 eV); As(V)-glycerol (white line energy 11876.5 eV); liquid DMA(V) (white line energy 11873.3 eV); TMAO (white line energy 11873.3 eV); frozen AB (white line energy 11872.6 eV); and As(Glu)3 (white line energy 11870.0 eV).

The mature fruiting body of *A. campestris* collected from arsenic contaminated mine tailings was also selected for XAS imaging and μXANES analysis. This sample was selected because it contained arsenic concentrations that were sufficiently high for XAS analysis and AB was the major arsenic compound in the bulk sample of the fruiting body [183]. The cap, gill and stalk tissues of the *A. campestris* were separated and analyzed for bulk XANES and XAS imaging, with a summary of the linear combination fittings for the bulk XANES analysis listed in Table 5.3. XANES analysis was also performed on the tailings material collected with the *A. campestris* mushroom (spectra shown in Appendix C, Figure C1), and shows the tailings are primarily comprised of As(V), indicating As(V) is the main form of arsenic to which the mycelium is exposed. Like the *A. bisporus* primordium, the stalk of the mature *A. campestris* fruiting body mainly contained $As(Glu)_3$, and lower proportions of As(III), AB, and an As(V)-O glycerol compound likely transformed from 4-coordinated As(V)-O (i.e., arsenate) during the drying process [116]. The cap contained the highest proportion of AB compared to the other tissues, supporting the hypothesis for its role as an osmolyte, but overall mainly contained inorganic arsenic compounds. The gills contained mainly As(V) compounds, which may include some tailings dust that was not washed away in the cleaning process. The proportion of AB detected in the different tissues of the mature mushroom were lower than those in whole composite samples of other *A. campestris* mushrooms collected from the same location. This could be attributable to dilution of As(V) from the gills when the whole mushroom was homogenized, since the gills make up only 15 ± 1 % of the total mass of the fruiting body. Although different fruiting bodies were analyzed they were collected within 1 m of each other and were presumably part of the same mycelium network. The lower proportion may also indicate that AB accumulation or production can vary across fruiting bodies.

The bottom of the stalk, in contact with the tailings, and the section similar to the primordium that was imaged, was selected for XAS imaging. Like the primordium, the section was mapped for total arsenic distribution at 11875.3 eV and μXANES were taken at points of interest, shown in Figure 5.6, with the results of the linear combination fitting for the collected μXANES listed in Table 5.5. Like the primordium, the stalk contained concentrated pockets of arsenic thought to be substrate material (tailings) incorporated into the fungal tissues, and the arsenic speciation in these pockets and surrounding tissues was also similar to the primordium. In the most concentrated areas, μXANES spots 3 – 5, there was a majority of As(V) and a lower proportion of As(III). Moving from the concentrated areas towards the fungal tissues, at μXANES spots 2 and 6, the proportion of As(V) decreased and As(III) increased suggesting further transformation of the As(V) to As(III) in the tissues. Further from the concentrated areas towards the cap, at μXANES spots 1 and 7, no As(V) was present and there was a mixture of

As(III) and AB. Like in the primordium, As(III) and AB were found in the less concentrated areas, and in the fungal tissues.

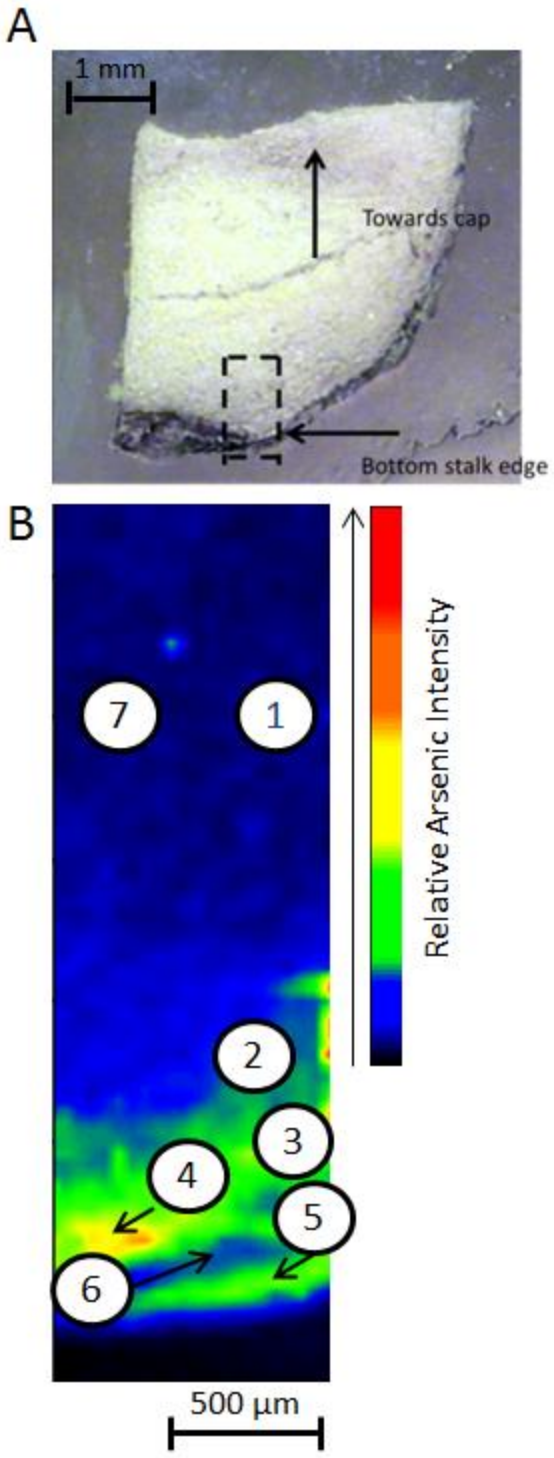

Figure 5.6: A) Cross section of A. campestris stalk collected from arsenic-contaminated mine tailings. The dashed black box indicates the area that was mapped. B) Map of total arsenic distribution (11875 eV) for section of *A. campestris* stalk. The numbers indicate the locations where µXANES were collected.

Table 5-5: µXANES fitting results for locations on A. campestris stalk total arsenic map.

	Proportion of Arsenic Compounds (%)						Reduced chi square
Spot	As(Glu)3	As(III)	AB	DMA/TMAO	As(V)	As(V) glycerol	
1		86.4	13.6				0.004
2	18.4	38.9				42.7	0.004
3	13	18			69		0.02
4		24.4			66	9.6	0.006
5		23.7			67.2	9.1	0.005
6	18.1	62.8		8.1		11	0.001
7		68.8	31.2				0.0025

Data were fit with frozen As(V) (white line energy 11875.3 eV); frozen As(III) (white line energy 11871.7 eV); As(V)-glycerol (white line energy 11876.5 eV); liquid DMA(V) (white line energy 11873.3 eV); TMAO (white line energy 11873.3 eV); frozen AB (white line energy 11872.6 eV); and As(Glu)3 (white line energy 11870.0 eV).

5.5 Conclusions

During the commercial cultivation of *A. bisporus* AB appears in the most immature form of the fruiting body. AB present in the compost and casing samples that contain the mycelium of the fungus and absence in material with no mycelium suggests that AB is also present in the mycelium when reproductive processes begin. When similar growth material was treated with high concentrations of As(V), in order to create high enough concentrations for XAS analysis, the fruiting bodies contained lower proportions of AB. This is likely due to the method of introducing As(V) and the higher concentrations used. The substrate treated with As(V) in the mushroom growing experiments did not contain AB. The map of arsenic in a primordium showed that AB is preferentially in the primordium tissue and not the substrate. However it cannot be distinguished whether the fungus has produced AB or the microbial community in the substrate has made it and it is immediately accumulated by the fungus. Mapping of an *A. campestris* stalk showed arsenic compounds and distribution similar to those in the primordium, where again the AB is in the fungus and not the substrate. AB formation is likely associated with the reproductive life stage of the fungus and occurs in the earliest stages of reproductive growth, as indicated by the primordia collected from the mushroom farm. AB's presence in the mycelium at this stage is likely to be assisted by the microbial community, as the vegetative mycelium of this species was not capable of producing AB. Further studies of the pre-fruiting stage of mycelium should be carried out to confirm that AB is associated with the reproductive life stage of the fungus. Axenic experiments are recommended, although such conditions are difficult to maintain through fruiting, and should be confirmed with plating and 16s sequencing of growth material extracts.

Acknowledgments

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Chapter 6

6 Characterization of microbial communities associated with terrestrial fungi

6.1 Abstract

Arsenobetaine (AB) is the only arsenic compound with no known toxicity and is found to comprise the majority of arsenic in the fruiting bodies (FBs) of many species of terrestrial fungi. It is unknown whether the fungus itself produces AB or if AB, or its precursors, are accumulated from the surrounding environment as a result of the associated microbial community. 16s pyrosequencing techniques were used to characterize the microbial community structure in the growth material and FBs during the commercial cultivation of *Agaricus bisporus* and identify changes that coincide with changes in arsenic speciation. This technique was also used to characterize and compare communities associated with AB containing and non-AB containing FBs from different geographical locations. The pyrosequencing revealed that the Proteabacteria phylum was the dominant phylum in all samples. Phyla diversity in the growth material for the commercial cultivation of *A. bisporus* increased at the time of FB initiation which also coincides with the appearance of AB in these samples. The phyla diversity in the *A. bisporus* FBs remained constant regardless of the time of collection. PCA analysis of all samples showed that the most clustering occurred at the family level. The field samples collected from the same location were found to cluster but these were not related to the FB species or major arsenic compound. Samples collected from logs were clustered with the arsenic containing mine tailings material samples obtained in Yellowknife, Canada. Ten bacteria species that were found to be associated with FB initiation and AB containing FBs were individually and axenically cultured and exposed to arsenate (As(V)), dimethylarsinic acid (DMA) and dimethylarsinoylacetic acid (DMAA). Few biotransformations were observed and no AB was produced. A consortia of microbes isolated from soil associated with select FBs were also cultured and exposed to As(V). All consortia were observed to reduce As(V) to arsenite (As(III)) and methylate trace amounts of monomethylarsonic acid (MMA) and DMA, however no AB was produced.

6.2 Introduction

Arsenobetaine (AB) comprises the majority of arsenic in the fruiting bodies (FBs), or mushrooms, of many terrestrial fungi species [1]. AB is the only arsenic compound with no known toxicity, but the origin of this compound – mechanism of formation, and organism responsible – is still unknown. It is possible that AB serve sas an osmolyte to help maintain the fruiting body structure for spore dispersal a function for AB, however, has been hypothesized, supported by work in this thesis [183].

A number of potential formation pathways are possible, influenced by different environmental components. The possibility of AB production by the fungi itself during the vegetative or reproductive life stage was evaluated in Nearing et al., 2014 [191], which showed with culture of the vegetative mycelium for *Agaricus bisporus*, *Suillus luteus* and *Sparasis crispa* under axenic conditions that the mycelium of these species does not produce AB. However, AB was preferentially accumulated in *A. bisporus* and *S. crispa* mycelium [191], suggesting that any AB available in the mycelium environment could be taken up by these fungus species. The formation of AB during and in association with the reproductive life stage was shown to be likely, following a study of arsenic speciation during the different stages of growth for the commercial cultivation of the button mushroom (*A. bisporus)*, where AB is found at the earliest stage of FB development but not in the growth substrate before FB initiation [192].

It remains unclear whether AB is produced by the fungus itself or if the surrounding microbial community produces AB or its precursors that are then selectively accumulated by the fungus. The bacteria communities associated with fungi have been found to increase fruiting body initiation and help fungi access more nutrients in the surrounding environment, demonstrating the important symbiotic roles and associations of bacteria communities with fungi [43, 44]. Microbial communities associated with certain plant species have been shown to be responsible for producing the methylated arsenic compounds found in those plants [54]. The microbial communities found in the growth substrate material for the commercial cultivation of *A. bisporus* are able to transform arsenate (As(V)) to trimethylarsine oxide (TMAO) when the fungus was absent but AB was only seen when the fungus was present [39]. Previous work in this thesis (Chapter 3) shows that the major arsenic compound present in the FB of a given fungus species is the same and independent of geographical location and soil arsenic concentration [183], except for log growing and soil growing mushrooms with two different microbial communities. Specifically, none of the log growing mushrooms contained AB (with the exception of one species) and a species collected from both soil and a log at the same location did not contain AB in the log growing mushroom but contained AB as the major compound in soil growing mushroom [183]. This suggests that the differences in microbial communities may influence arsenic speciation in the FB.

In the present study 16s pyrosequencing was performed to characterize the soil microbial communities associated with the wild FBs collected in Nearing et al., 2014 [183] and the growth substrate and FBs collected during the commercial cultivation of *A. bisporus* in Chapter 5. Specifically, three objectives were addressed. The first was to characterize and compare communities associated with AB producing and non-AB producing mushrooms from different geographical locations and areas of varying arsenic concentrations. The second was to characterize the microbial community structure in the growth material and FBs during the commercial cultivation of *A. bisporus* and identify changes that coincide with changes

in arsenic speciation. The third was to identify bacterial species of interest, such as those that are only associated with AB producing mushrooms, and to culture and expose these species to arsenic.

6.3 Methods

6.3.1 Sampling locations

Soils co-located with wild FBs in Nearing et al., 2014 [183] were collected. The locations are described in Appendix A (Table A1) and a map of the sampling locations is provided in Appendix A (Figure A1), with soils collected from background Ontario (ON) locations (Yarker, Y, and Odessa, O) and ON arsenic contaminated locations (Deloro, D). Soils were collected from background and arsenic contaminated areas in Yellowknife NT. Background or sites with lower arsenic concentrations in Yellowknife include Ndilo, Marina, Frame Lake and Grace Lake. Arsenic contaminated areas in Yellowknife include the Giant Mine tailings, Giant Mine water treatment area, Con Mine tailings and Con Mine residential area. Log material associated with three different fungi species was collected from Deloro and Odessa locations. Growth material and fungus samples were collected from a commercial growth facility for *A. bisporus* in Metcalfe ON (Chapter 5).

6.3.2 Collection of samples

Soils associated with the wild FBs were collected approximately 3 – 5 cm below the base of the associated FB stalk using a sterile scoop and were directly transferred to a sterile container. The time the container was open was minimized to prevent cross contamination. Soils were then frozen until the time of analysis. Log material samples were cut or chipped with a sterile stainless steel scoopula and transferred to a sterile cup or Ziploc bag and frozen until the time of analysis. A summary of collected samples associated with wild FBs is given in Table 6.1. For samples from the mushroom farm, the growth substrates (casing and compost samples) were collected following the procedure used for the soils. FBs were picked using gloves and washed with soap and water to remove substrate, rinsed with ddH_2O, placed in a sterile container and frozen until time of analysis. A summary of the samples collected from the commercial growth facility is given in Chapter 5, Table 5.1.

6.3.3 DNA Extraction

Five samples from Odessa ON and all samples from the commercial growth facility of *A. bisporus* were extracted using a salt extraction as described in [193], listed as method 1 in Table 6.1. Following the extraction, bovine serum albumin (BSA) was added to each extract (400 ng BSA / µL extract) [194]. The remaining samples from ON locations and all YK locations were extracted using a NucleoSpin Soil (Macherey-Nagel, 740780.250) following the manufacturer's instructions and using lysis buffer SL1 with 150 µL buffer SX (referred to as Method 2, Table 5.1). DNA concentrations were quantified by using a

Nanodrop UV-Vis spectrophotometer (Ultospec™ 1100 pro UV/visible Spectrophotometer; Amersham Biosciences corp., USA as well as NanoDrop-1000; Ver.3.7.1; Thermo Scientific, Wilmington, USA).

6.3.4 PCR Amplification and Pyrosequencing

Bacterial 16S rRNA genes were amplified with PCR using a Veriti®96 well Thermal Cycler (Applied Biosystems, Burlington, Canada). The PCR reaction mixture (50 µL) contained 2 µL DNA template, 0.8 µL of 10 mM dNTP mix (Vivantis technologies NP2410), 5 µL buffer solution (buffer solution = 160 mM $(NH4)_2SO_4$, 500 mM Tris-HCl at pH 9.2, 17.5 mM $MgCl_2$ and 0.1% Triton X-100), 400 ng of BSA, 2.5 µL of each primer (10 µM final concentration) and 0.25 µL of Taq polymerase 5 units/µL (Vivantis Taq DNA Polymerase (recombinant) PL1202). Amplification was performed using 519R (5'GTNTTACNGCGGCKGCTG) (Sigma) and 28F (5'GAGTTTGATCNTGGCTCAG) (Sigma) primers. PCR amplifications were run under the following cycle conditions: 5 min initial denaturing at 95°C, followed by 35 cycles of denaturing at 95°C for 1 min, annealing at 53°C for 1 min, extension at 72°C for 1 min and completed with a final extension at 72°C for 10 min. PCR products were run on a 1% agarose gel electrophoresis at 95V for 20 minutes and visualized with ethidium bromide (5 µg/mL). Positives were seen at 500 base pairs and then purified with Thermo Scientific GeneJET PCR purification kit (Thermo Fisher Scientific, FERK0702).

Sequencing of PCR extracts was performed at MR DNA using 454 pyrotag sequencing following their standard protocols (MR DNA, Shallowater, TX, USA). In summary, sequences were depleted of barcodes and primers, then sequences < 150 base pairs were removed, sequences with ambiguous base calls and with homopolymer runs exceeding 6 base pairs were also removed. Sequences were denoised, operational taxonomic units (OTUs) generated and chimeras removed. OTUs were defined by clustering at 3% divergence (97% similarity). Final OTUs were taxonomically classified using BLASTn against a curated GreenGenes database.

The Q25 sequence data derived from the sequencing process was processed using a proprietary analysis pipeline (www.mrdnalab.com , MR DNA, Shallowater, TX). Sequences are depleted of barcodes and primers, then short sequences < 200bp are removed, sequences with ambiguous base calls removed, and sequences with homopolymer runs exceeding 6bp removed. Sequences are then denoised and chimeras removed. Operational taxonomic units were defined after removal of singleton sequences, clustering at 3% divergence (97% similarity) [195-201]. OTUs were then taxonomically classified using BLASTn against a curated GreenGenes database [202] and compiled into each taxonomic level into both "counts" and "percentage" files.

6.3.5 Cultivation, Exposure and Collection of Single Bacteria Species and Cultivable Bacteria Communities

Chemicals and reagents are listed in Appendix D.1. The following species were cultivated: *Methylobacterium organophilum* Patt et al. (ATCC® 27886™), *Flavobacterium sp* (ATCC® 53198), *Rhodoplanes elegans* Hiraishi and Ueda (ATCC ® 51906™), *Massilia brevitalea* (ATCC® BAA-1465™), *Hydrogenophaga taeniospiralis* (Lalucet et al.) Willems et al. (ATCC® 49743™), *Bradyrhizobium canariense* Vinuesa et al. (ATCC® BAA-1002™), *Rhizobium rhizogenes* (Riker et al) Young et al. (ATCC® 31798™), *Planctomyces brasiliensis* Schlesner (ATCC® 49425™), *Pedomicrobium manganicum* Aristovskaya (ATCC® 33121™), *Herbaspirillum aquaticum* Dobritsa et al. (ATCC® BAA-1628™). Each species was cultivated according to manufacturer instructions. Arsenic compounds were aseptically added to 50% strength nutrient broth (Difco) using a 0.22 µm Millipore ® syringe filter. Media was amended with As(V), DMA and DMAA at a nominal concentration of 100 or 200 µg/L As before aseptically transferring 1 mL of liquid bacteria culture. With the exception of *Rhodoplanes elegans* all bacteria species were cultured aerobically at their optimal growth temperature in the dark. *Rhodoplanes elegans* was cultured anaerobically in a sealed container with an anaerobic environment created with a BD GasPakan BD GasPak™. Controls included the bacteria grown in arsenic-free media (inoculated negative control); without bacteria in arsenic-containing media (uninoculated positive control); and arsenic- and mycelia-free media (uninoculated negative control).

For the cultivable soil microbial communities, soil was used to inoculate full strength nutrient broth (Difco) for one week of incubation at 25°C in the dark. Aliquots of each culture were then aseptically transferred to nutrient broth (Difco) containing 10 mg/L As(V) and incubated at 25°C in the dark for 30 days. Treatments were performed in triplicate. Controls included media containing As(V) (bacteria-free), media containing no As(V) (bacteria-free), and untreated cultures (no arsenic).

All cultures were collected using methods described in Harrington et al., 2008 [203]. Samples for speciation were diluted using ddH_2O and samples for total arsenic were acidified using 70% HNO_3.

6.3.6 Total Arsenic and Arsenic Speciation Analysis

All samples for total arsenic were analyzed using ICP-MS. The ICP-MS operating conditions are described in Caumette et al., 2011 [24] (Appendix D.2). Instrument quality control checks were found to be acceptable. The HPLC-ICP-MS operating conditions are also described in Caumette et al., 2011 [24] and Appendix D.3. Instrument quality control checks were found to be acceptable. Method blanks were also included with every 10 samples with non-detectable arsenic. The limits of detection (LOD) for total

arsenic were 0.5 µg/L and 3 µg/L for arsenic speciation. Matrix spikes (50 µg/L), were prepared immediately before analysis (10% frequency) for both total arsenic and speciation analysis. QA/QC results are summarized in Appendix D (Table D5) and were considered acceptable (± 30 % recoveries).

Table 6-1: Summary of collected soils associated with wild FBs. Samples are labelled with the major arsenic compound found in the associated fruiting body and location.

Sample ID	Associated Mushroom Species	Major As Compound in Mushroom	Location	Location Description
	Method 1			
	Calvatia gigantea	AB	Deloro, ON	ON Contaminated
	Lycoperdon perlatum	AB	Deloro, ON	ON Contaminated
	Suillus americanus	DMA	Odessa, ON	ON Background
	Calvatia gigantea	AB	Odessa, ON	ON Background
	Lycoperdon perlatum	AB	Odessa, ON	ON Background
	Method 2			
AB-Y-1	*Lepiota rachodes*	AB	Yarker, ON	ON Background
AB-O-3	*Lactarius deliciosus*	AB	Odessa, ON	ON Background
AB-O-4	*Lycoperdon perlatum*	AB	Odessa, ON	ON Background
AB-Y-2	*Lepiota rachodes*	AB	Yarker, ON	ON Background
AB-Y-3	*Calvatia gigantea*	AB	Yarker, ON	ON Background
AB-Y-4	*Lycoperdon perlatum*	AB	Yarker, ON	ON Background
DMA-O-2	*Amanita flavoconia*	DMA	Odessa, ON	ON Background
iAs-D-1	*Entoloma sinuatum*	iAs	Deloro, ON	ON Contaminated
AB-D-3	*Boletinellus meruliodes*	AB	Deloro, ON	ON Contaminated
DMA-D-1	*Polyporus squamosus*	DMA	Deloro, ON	ON Contaminated
DMA-D-2	*Leucoagaricus naucinus*	DMA	Deloro, ON	ON Contaminated
AB-D-4	*Amanita prophyria*	AB	Deloro, ON	ON Contaminated
iAs-Log-1	*Hericium coralloides*	iAs	Odessa, ON	ON Background
iAs-Log-2	*Lycoperdon pyriforme*	iAs	Odessa, ON	ON Background

Sample ID format: Major arsenic compound-Location-#
AB = arsenobetaine, DMA = dimethylarsinic acid, iAs = inorganic arsenic
O = Odessa, ON (Background), Y = Yarker, ON (Background), D = Deloro, ON (contaminated), YK = Yellowknife NT, Log = log material from log/tree growing mushroom
*Method 1 samples were not used in the combined analysis and therefore were not given sample IDs

Table 6.1 continued

Sample ID	Associated Mushroom Species	Major As Compound in Mushroom	Location	Location Description
AB-YK-1	*Coprinus comatus*	AB	YK Contaminated	NW Tailings Pond Giant Mine
AB-YK-2	*Coprinus comatus*	AB	YK Contaminated	NW Tailings Pond Giant Mine
AB-YK-3	*Coprinus comatus*	AB	YK Contaminated	Giant Mine Tailings
AB-YK-4	*Coprinus comatus*	AB	YK Contaminated	Giant Mine Tailings
AB-YK-5	*Coprinus comatus*	AB	YK Contaminated	Giant Mine Tailings
AB-YK-6	*Agaricus campestris*	AB	YK Contaminated	Con Mine Negus Tailings
AB-YK-7	*Coprinus comatus*	AB	YK Contaminated	Con Mine Negus Tailings
AB-YK-8	*Coprinus comatus mycelium*	AB	YK Contaminated	Con Mine Negus Tailings
AB-YK-9	*Agaricus campestris*	AB	YK Background	Ndilo Site 1
AB-YK-10	*Hebeloma velutipes*	AB	YK Background	Site 1 Giant Mine Marina
DMA-YK-1	*Suillus granulatus*	DMA	YK Background	Site 2 Giant Mine Marina
AB-YK-11	*Young Lycoperdon perlatum*	AB	YK Background	Con Mine Residential Site 1
AB-YK-12	*Mature Lycoperdon perlatum*	AB	YK Background	Con Mine Residential Site 1
DMA-YK-2	*Suillus tomentos*	DMA	YK Contaminated	Giant Mine Water Treatment Area
AB-YK-13	*Paxillus involutus*	AB	YK Contaminated	Giant Mine Water Treatment Area
AB-YK-14	*Agaricus campestris*	AB	YK Contaminated	Con Mine Negus Tailings (under tree)
AB-YK-15	*Lyophyluum decastes*	AB	YK Contaminated	Con Mine Negus Tailings
DMA-YK-3	*Suillus grevillei*	DMA	YK Background	Ndilo Site 2
AB-YK-16	*Suillus cavipes*	AB	YK Background	Grace Lake
DMA-YK-4	*Russula silvicola*	DMA	YK Background	Frame Lake Site 1
AB-YK-17	*Lycoperdon perlatum*	AB	YK Background	Frame Lake Site 2

Sample ID format: Major arsenic compound-Location-#
AB = arsenobetaine, DMA = dimethylarsinic acid, iAs = inorganic arsenic
O = Odessa, ON (Background), Y = Yarker, ON (Background), D = Deloro, ON (contaminated), YK = Yellowknife NT, Log = log material from log/tree growing mushroom

6.3.7 Statistical Analysis

Statistical analysis was carried out using XLSTAT Version 2014.1.10 with Microsoft Excel 2010. Principle component analysis (PCA) using a covariance matrix was carried out for relative abundance across samples at the phylum, class and order level. Both natural logarithmic (LN(x+1)) and Taylor law transformations, two transforms commonly applied in ecological data analysis [204], were used for all data sets. A description of the transformations, normality testing and PCA results for untransformed and transformed data is provided in Appendix D. The transformation applied that resulted in the highest number of normally distributed variables was selected for discussion. For common group analysis, relative abundance values < 0.5% were discarded. A phylum, class, order etc was excluded if it was not present across three or more samples. Rare groups were defined as groups with relative abundance values < 1% and were also analyzed. Table 6.2 summarizes the sample size for each location and the number of phylum, class and order variables used in the PCA.

Table 6-2: Summary of sample size for sample groups and number of phylum, class and order variable used in the PCA.

Sample Group	N	Phylum level variables	Class level variables	Order level variables
ON	14	10	21	33
YK	21	13	25	41
Commercial growth facility	18	7	11	19
ON + YK	35	13	29	49

6.4 Results and Discussion

6.4.1 Microbial Communities Associated with the Commercial Production of A. bisporus

The relative abundances of bacteria phyla were compared at different sampling times for the growth substrate (casing and compost samples) and the different growth stages of the fungus itself, as well as the fruiting bodies (FBs). At the phylum level the majority of the bacteria community, for growth substrate samples, was Proteobacteria (58% in compost and 48% for casing). In the FB samples Firmicutes was the major phylum present. The Verrucomicrobia and Planctomycetes phyla were present in both the casing and compost samples but not in any of the FB samples.

For both casing and compost material the phylum diversity (seen by the number of different phyla) increases after day 20 which is when FB growth is initiated by addition of the casing (Figure 6.1a and b); the increase in class diversity is even more pronounced at this stage This increase in phylum and class diversity also coincides with the appearance of AB in the compost and FBs [192]. Other researchers have observed an increase in bacterial population numbers after *A. bisporus* spawn was introduced [43], which

is a stage in the present study at which the diversity was not observed to change (i.e. between Day 0 and Day 14 casing). In studies when *A. bisporus* was grown on agar plates the presence of *Pseudomonas putida* and *Pseudomonas fluorescens* significantly stimulated fruiting body initiation [205], which supports the idea that the increase in diversity occurring with fruiting body initiation in the present study may help in initiating fruiting body development. In the FB samples, the phylum diversity remains fairly consistent across samples regardless of the time of collection (Figure 6.3).

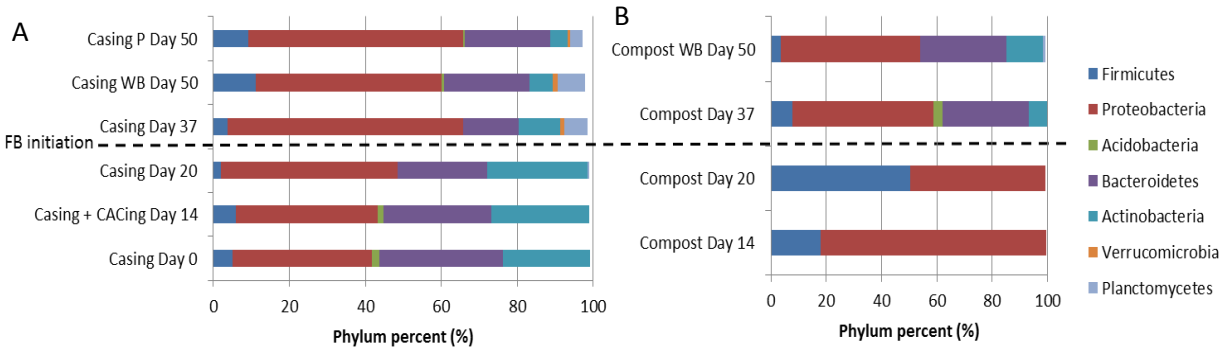

Figure 6.1: Relative abundance of A) bacteria phyla in casing material, B) bacterial phyla in compost material. Fruiting body initiation occurs after 20 days (indicated by the dashed line) and coincides with an increase in phylum diversity. P indicates that the sample was collected from an area growing the brown strain of *A. bisporus*, Portobello. WB indicates that the sample was collected from an area growing the white strain of *A. bisporus*, white button.

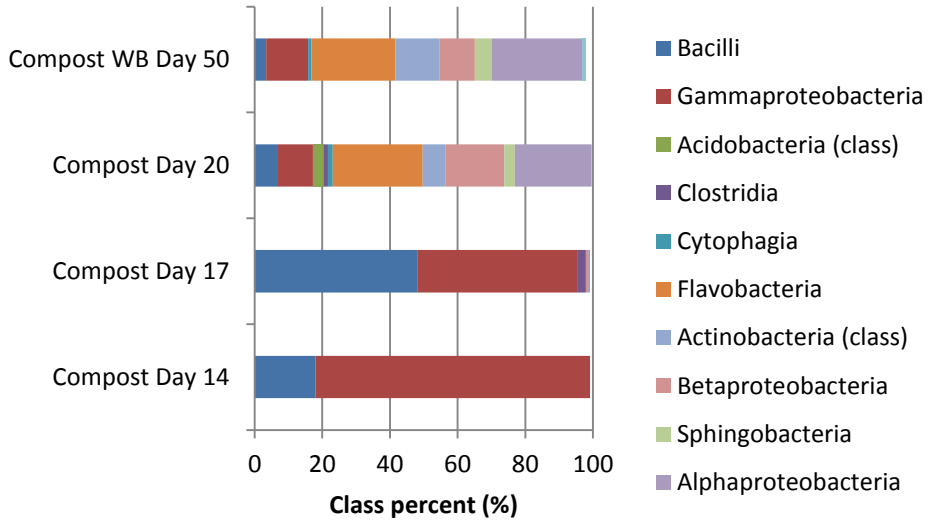

Figure 6.2: Relative abundance of bacteria class in compost samples. Fruiting body initiation occurs after 20 days and coincides with an increase in class diversity. WB indicates that the sample was collected from an area growing the white strain of *A. bisporus*, white button.

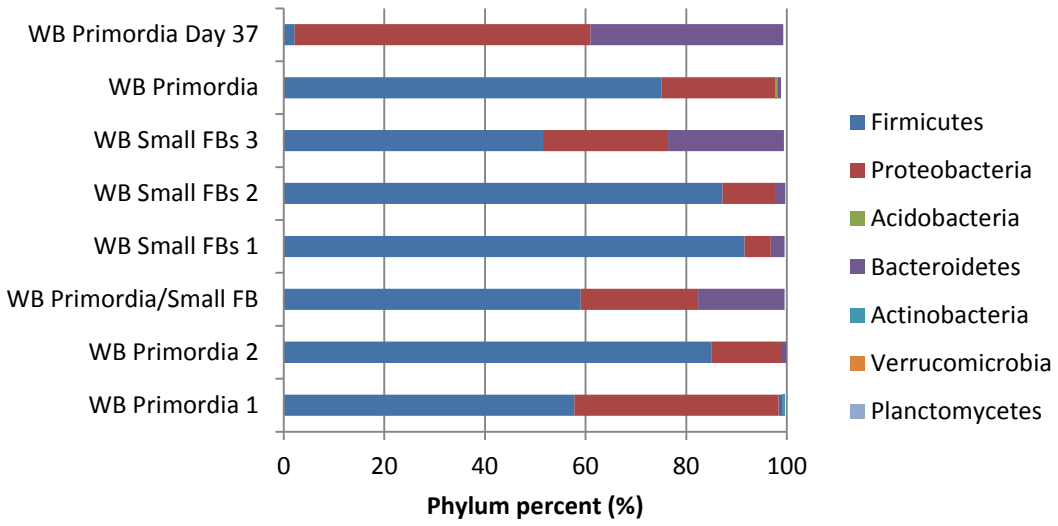

Figure 6.3: Relative abundance of bacteria phyla in the different growth stage of *A. bisporus* FBs collected. WB indicates that the sample was collected from an area growing the white strain of *A. bisporus*, white button.

A PCA of all samples collected from the commercial growth facility for the phylum level shows that phyla are similar in sample types collected at the same time (Figure 6.4). For example the FBs collected at the time of harvest are similar and are found clustered together (Figure 6.4). As seen in Figure 6.4 the bacteria communities in the casing samples with no fungus present are clustered (Casing Day 0 and Casing + CACing Day 14), as are those with fungus present (Casing Day 20 to 50). PCAs were also performed for the class and order level and showed distributions similar to that seen in the PCA for the phyla relative abundance in Figure 6.4.

One reason for the increase in bacteria diversity at the time of fruiting body initiation may be the alteration of the growth material environment to make nutrients more accessible to the fungus, similar to the edible fungus *Cantharellus cibarius'* use of the enzymatic capacity of certain bacteria species (proteases) to gain access to nutrients such as recalcitrant sources of nitrogen [44]. The bacteria may also benefit from this interaction, as some bacterial species are known to utilize carbohydrate exudates produced from the mycelium such as trehalose and mannitol [52]. The surrounding bacterial community is also thought to absorb compounds produced by mycelium that might be inhibitory to the initiation of fruiting body development [206].

To further determine whether specific species of bacteria are involved in FB initiation, we determined species that appear after the spawn addition but before fruiting body initiation, listed in Table 6.3. A total of 18 species were found to appear at the time of fruiting body initiation in the compost (but not the

casing) and 19 species were found to appear in the casing (but not the compost) layer at this time. Only 3 species were found to appear in both the casing and compost at the time of fruiting body initiation, *Shinella zoogloeoides*, *Bradyrhizobium yuanmingense* and *Sphingomonas kaistensis*.

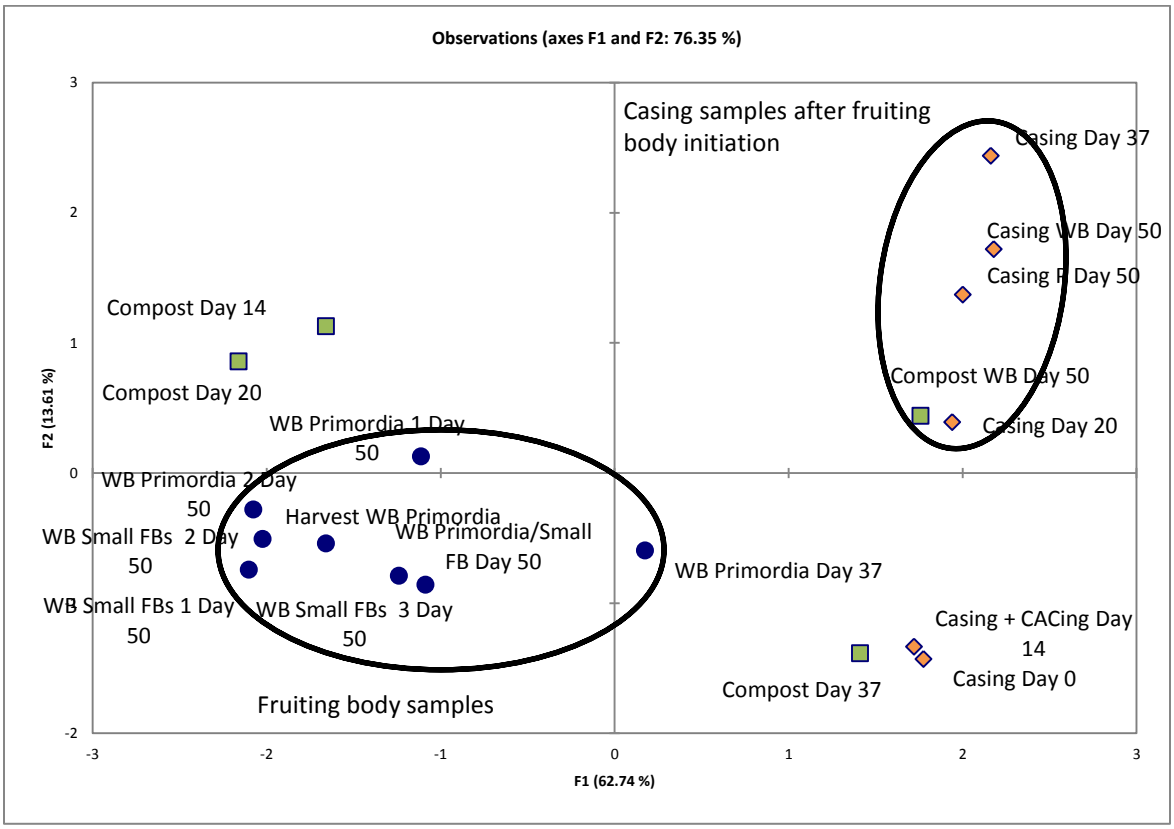

Figure 6.4: PCA ordination for Taylor transformed phylum relative abundance in all samples collected from the commercial growth facility of *A. bisporus*. The blue solid circles represent fruiting bodies (FBs), the green squares represent compost samples and the orange diamonds represent casing samples. WB indicates that the sample was collected from an area growing the white strain of *A. bisporus*, white button. Clusters of samples are indicated by the black open circles.

Table 6-3: Species present at time of fruiting body initiation in compost and casing material from the commercial growth facility for *A. bisporus.*

Species that appear in compost only at fruiting	Species that appear in casing only at fruiting	Species that appear in both compost and casing at fruiting
Asaia lannaensis	*Achromobacter sp*	*Bradyrhizobium yuanmingense*
Balneimonas sp	*Altererythrobacter sp*	*Hydrogenophaga sp.*
Bradyrhizobium liaoningense	*Asticcacaulis benevestitus*	*Methylobacterium sp.*
Bradyrhizobium yuanmingense	*Asticcacaulis excentricus*	*Planctomyces sp.*
Clostridium butyricum	*Flavobacterium aquatile*	*Rhizobium sp.*
Clostridium chromoreductans	*Flavobacterium flevense*	*Rhodoplanes sp.*
Dietzia sp	*Hahella chejuensis*	*Shinella zoogloeoides*
Enterobacter asburiae	*Mesorhizobium sp*	*Sphingomonas kaistensis*
Enterobacter sp	*Novosphingobium sp*	
Nitrosospira sp	*Oceanicola sp*	
Pantoea ananatis	*Prosthecobacter dejongeii*	
Propionibacterium sp	*Pseudoxanthomonas ginsengisoli*	
Petrimonas sp	*Prosthecobacter sp*	
Rubrimonas sp	*Roseomonas lacus*	
Shinella zoogloeoides	*Roseomonas sp*	
Sphingomonas fennica	*Sediminibacterium sp*	
Sphingomonas kaistensis	*Sphingopyxis alaskensis*	
Wolinella sp	*Sphingopyxis witflariensis*	
	Thermomonas brevis	

6.4.2 Microbial Communities Associated with ON Mushrooms

Soils associated with mushrooms collected for a study of arsenic speciation in edible mushrooms [183] were also pyrosequenced. Five of the soils were extracted using Method 1 and the remaining soils were extracted using Method 2. Figure 6.5a shows the relative abundance for bacteria phyla in ON collected mushrooms and it is apparent that the soils extracted by Method 1 (shown by the black box) have a different overall phylum distribution from the rest of the samples. All of the samples extracted using Method 1 were collected from Odessa and Deloro ON and primarily contain the Firmicutes phylum. This is in contrast to all other soils, which primarily contain the Proteobacteria phylum. A PCA of the relative abundance of bacteria phyla for all samples show that the samples extracted by Method 1 are clustered together and are driven by the Firmicutes phylum (Figure 6.5b). *Lycoperdon perlatum* collected from the same ON location was analyzed using both extraction methods and was found to have different profiles. Therefore the samples extracted using Method 1 were not included in the rest of the comparisons. Only samples extracted using the same method were compared to provide a more meaningful comparison. The majority of ON and YK soils were extracted using Method 2 and therefore combined. The commercial growth facility samples were extracted using Method 1 and will not be compared with soils collected from the field sites.

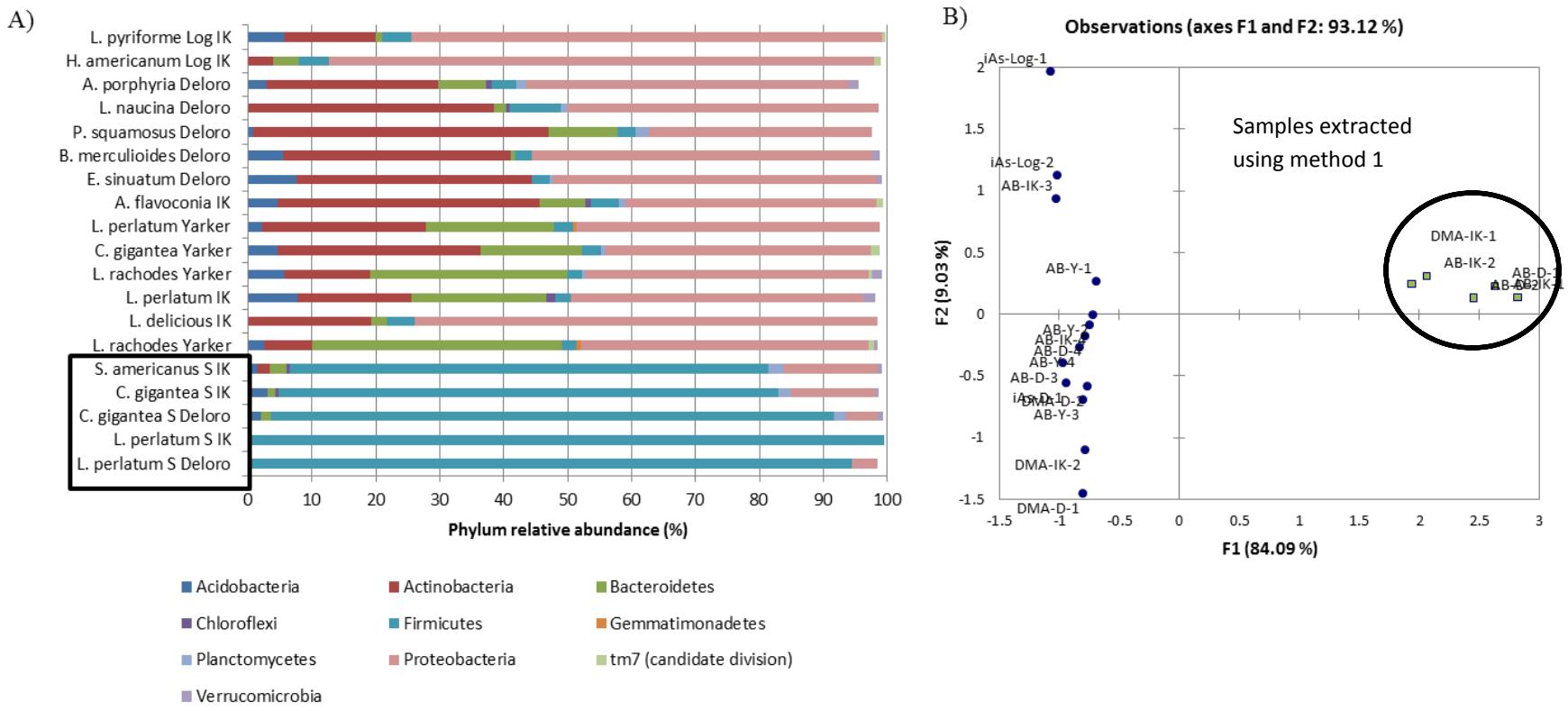

Figure 6.5: A): Relative abundance of bacteria phyla for soils associated with mushrooms collected from ON sampling sources. Samples in the black box were extracted for DNA using Method 1. B) PCA of relative abundance of bacterial phyla for soils associated with mushrooms collected from ON sampling sources. The green squares represent samples extracted using Method 1 and the blue circles represent samples extracted using Method 2. Clusters of samples are indicated by the black open circle.

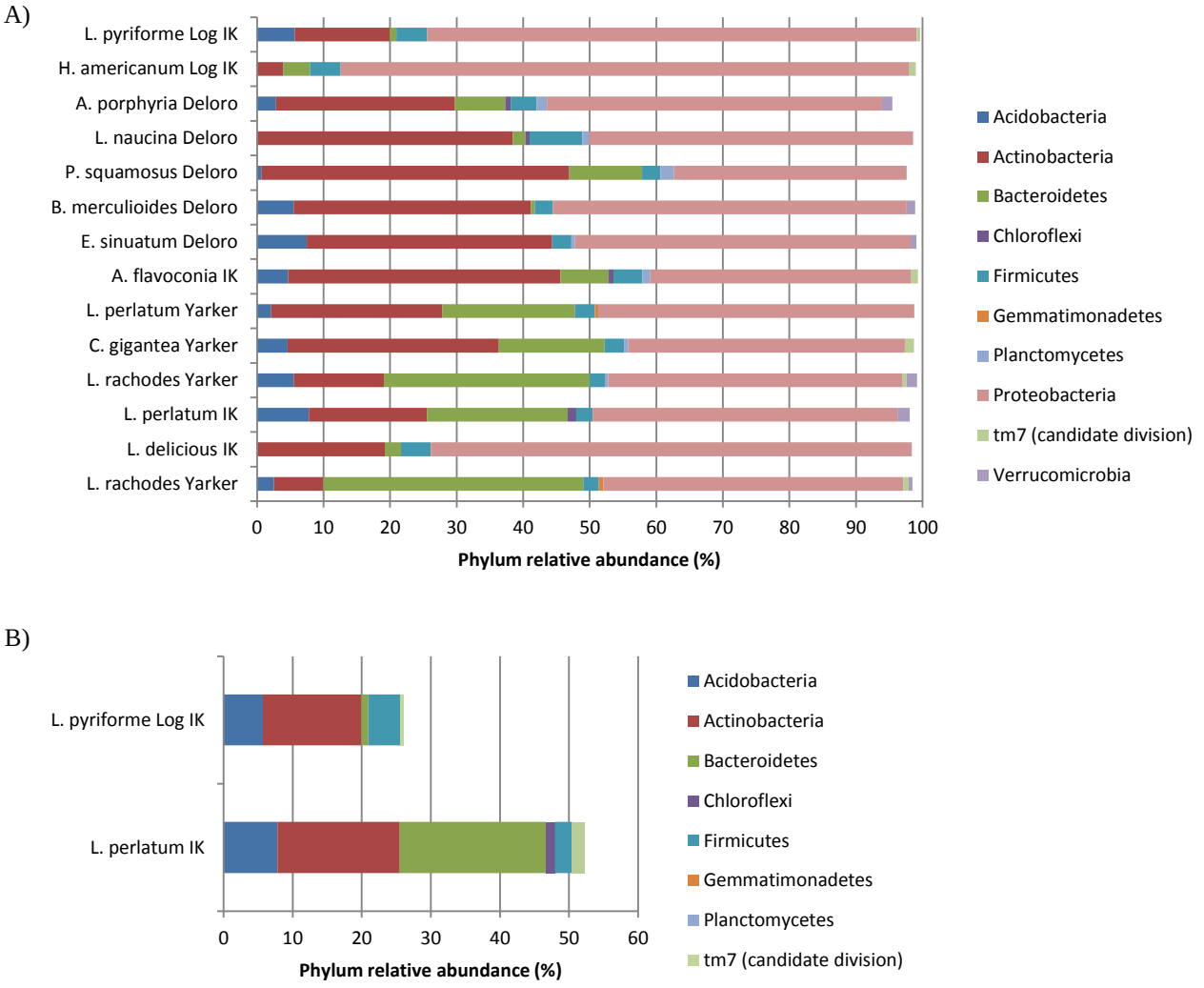

Figure 6.6: Relative abundance of bacteria phyla for soils associated with mushrooms collected from ON sampling sources. A) Samples extracted using Method 2. B) Relative abundance of bacterial phyla associated with two *Lycoperdon* species collected from the same geographical area: one from the soil and one from a log.

Figure 6.6 shows the relative abundance of bacteria phyla for soils associated with mushrooms collected from all ON sampling sources. The three most common phyla include the Proteobacteria, Bacteroidetes and Actinobacteria phyla, which are common soil bacteria phyla. Phlya with relative abundances < 0.5 % were discarded and therefore the relative abundances in Figure 6.6 appear to be less than 100%. The soils from background locations appear to be slightly more diverse than the ON contaminated soils (Deloro). However PCA analysis of phyla, classes and orders for ON mushroom samples do not show clear clustering and are generally similar across locations, as demonstrated in Figure 6.7, the PCA for LN(x+1) transformed order relative abundance. The sample labels include fungus species and the major arsenic compound found in the associated FB, and no clustering was observed for soils that were associated with mushrooms with the same major arsenic compound in the FB. Log material samples were found to be grouped together (green squares, Figure 6.7). The log material was collected for FBs found growing on decaying log material. The Verrucomicrobia phylum was absent from all log material samples (*L. pyriforme* Log O, *H americanum* Log O and *P. squamosus* D).

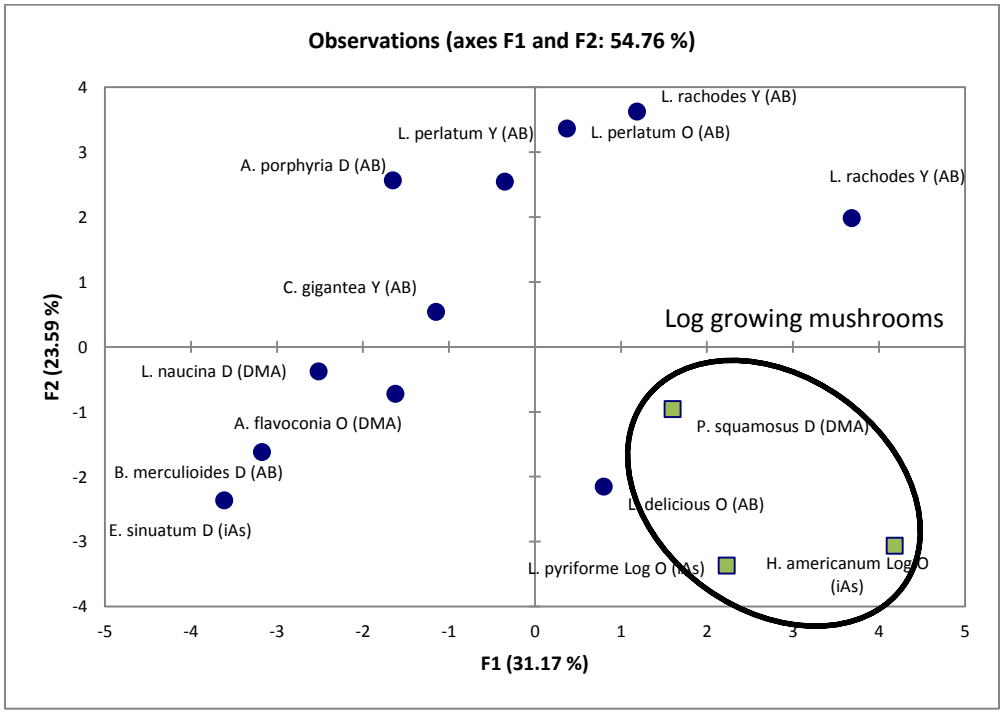

Figure 6.7: PCA of relative abundance of bacteria orders for soils associated with mushrooms collected from ON sampling sources (LN(x+1) transformed). The green squares represent log material samples associated with log growing mushrooms. The sample labels are mushroom species associated with the soil followed by the collection location (Y = Yarker, O = Odessa, D = Deloro), with the major arsenic compound found in the associated mushroom in brackets. Clusters of samples are indicated by the black open circle.

6.4.3 Microbial Communities Associated with YK Mushrooms

The most abundant phyla present in the YK soils were similar to those observed in the ON soils, with Proteobacteria also the major phylum present (Figure 6.8). Other researchers found that in a microbial community structure study of agricultural soils contaminated with different arsenic levels, the Proteobacteria phylum was more resilient and more common in soils with higher arsenic concentrations [47]. In the present study the relative abundance of the Proteobacteria phylum remains consistent in both ON and YK soils, regardless of arsenic soil concentration.

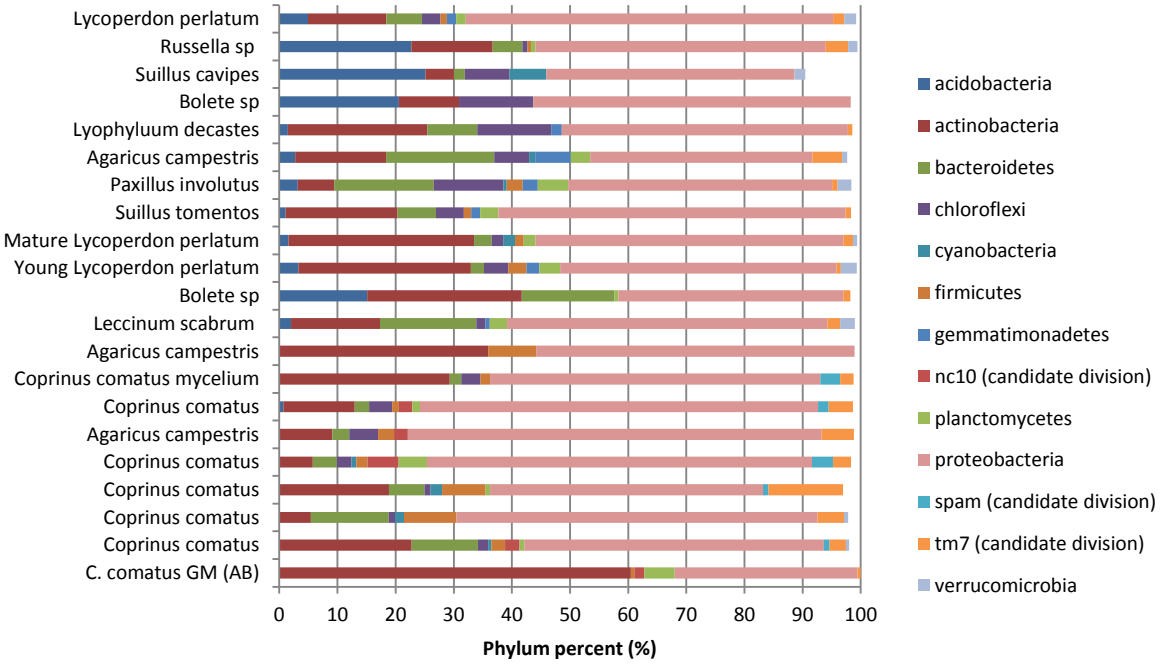

Figure 6.8: Relative abundance of bacteria phyla for soils associated with mushrooms collected from YK sampling sources.

In the PCAs, soils were labelled with the major arsenic compound found in the associated FB species (Figure 6.9). At the phylum level the microbial community structures were found to be similar in samples across YK sampling locations (Figure 6.9a). At the class level the structures were slightly more differentiated (Figure 6.9b) and the most differentiation was observed at the order level (Figure 6.9c). The species of mushroom and the major arsenic compound found in the mushroom were not observed to influence the community structure; instead the sampling location was found to influence microbial structure. For example, the soils collected from the areas with the most arsenic contamination (the tailings from both mine properties, where arsenic concentrations ranged from 1000 to 3000 mk/kg As [183]) were found to be similar. Likewise, the background sites, with lower levels of arsenic, were found to be similar to each other.

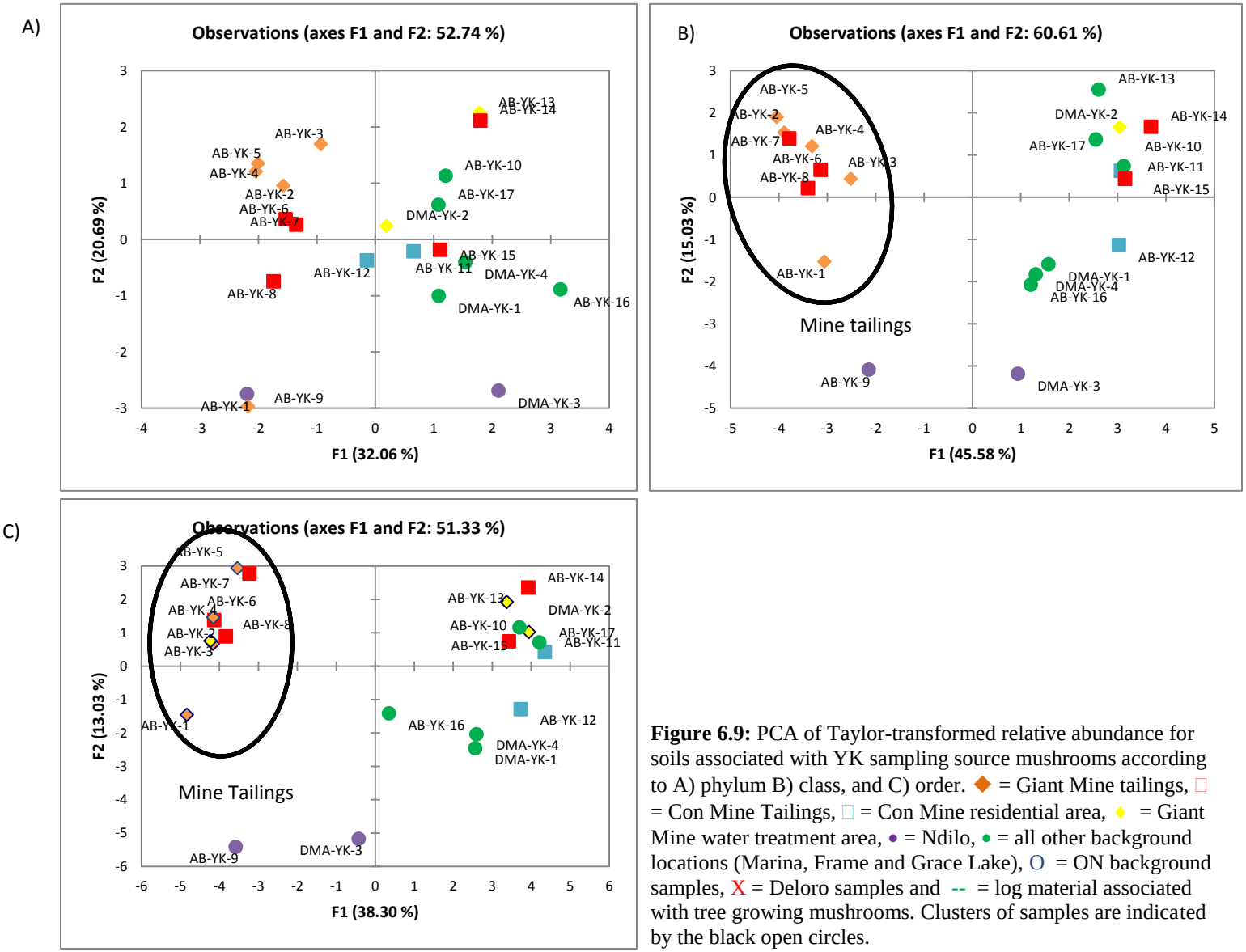

Figure 6.9: PCA of Taylor-transformed relative abundance for soils associated with YK sampling source mushrooms according to A) phylum B) class, and C) order. ◆ = Giant Mine tailings, □ = Con Mine Tailings, □ = Con Mine residential area, ♦ = Giant Mine water treatment area, ● = Ndilo, ● = all other background locations (Marina, Frame and Grace Lake), O = ON background samples, X = Deloro samples and -- = log material associated with tree growing mushrooms. Clusters of samples are indicated by the black open circles.

PCAs for YK and ON mushrooms excluding the Proteobacteria phylum, to examine the less common phyla, were also investigated but no clustering was observed (Appendix D, Figure D37 and Figure D38).

The PCAs indicate that for YK soils the location plays an important role in the microbial community. AB-YK-14 and AB-YK-15, samples located separately from other similarly located samples (Con Mine tailings), was collected from the edge of the tailings where there was vegetation, whereas the other samples were in the middle of the tailings where no plants were observed. The different levels of vegetation may explain the difference in communities. Revegetation of arsenic contaminated soils significantly increases bacteria activity and diversity of the contaminated soils compared to those that are not vegetated [48], and the bacteria present in the rhizophere of vegetated areas are beneficial for the associated vegetation as well as any ectomychorhizal fungi present [207]. The fact that the communities at the order level are so different across locations suggests that the fungus itself is producing AB, or the minor bacteria groups strictly associated with the fungus, could play an important role in arsenic speciation. The absence of AB production by the vegetative state of the fungus (Chapter 4), however, indicates that the latter scenario (bacteria groups strictly associated with the fungus), or AB produced by a combination of fungus with bacteria, is more likely.

To examine the minor or rare groups of bacteria in this study (<1% abundance), PCAs were investigated including only these bacteria; no difference in community structure was observed (Appendix D, Figure D39 and Figure D40).

6.4.4 Microbial Communities Associated with ON and YK Mushrooms Together

When the YK and ON samples were combined (all Method 2) clear clustering appeared only at the order level (Figure 6.10) with overall differentiation between the YK and ON microbial communities. The exception is the majority of the tailings samples from YK and the three log material samples from ON, which were found to be clustered together and separately from other samples at both the order and family level (Figure 6.10). These samples represent very different types of sample matrix compared with the rest of the soil samples collected. The tailings samples were sandy and contained high concentrations of arsenic with no associated vegetation and subsequent rhizosphere, and the log material samples are also not associated with a plant rhizosphere, which would be associated with the other collected soils from vegetated areas. However the log samples differed dramatically from the YK samples as they contained very low concentrations of arsenic. The microbial communities did not appear to be grouped by similar FB species or the major arsenic compound in the FBs. This is highlighted by the difference in the community structures between samples collected from tailings, compared with those collected from background locations even though arsenic speciation in FBs from the two different locations was similar.

Thus the bacteria that are predominant and driving the PCA may not be the ones associated with arsenic speciation and other organisms (minor bacteria groups) or organism combination (fungus plus bacteria) may be important for arsenic speciation.

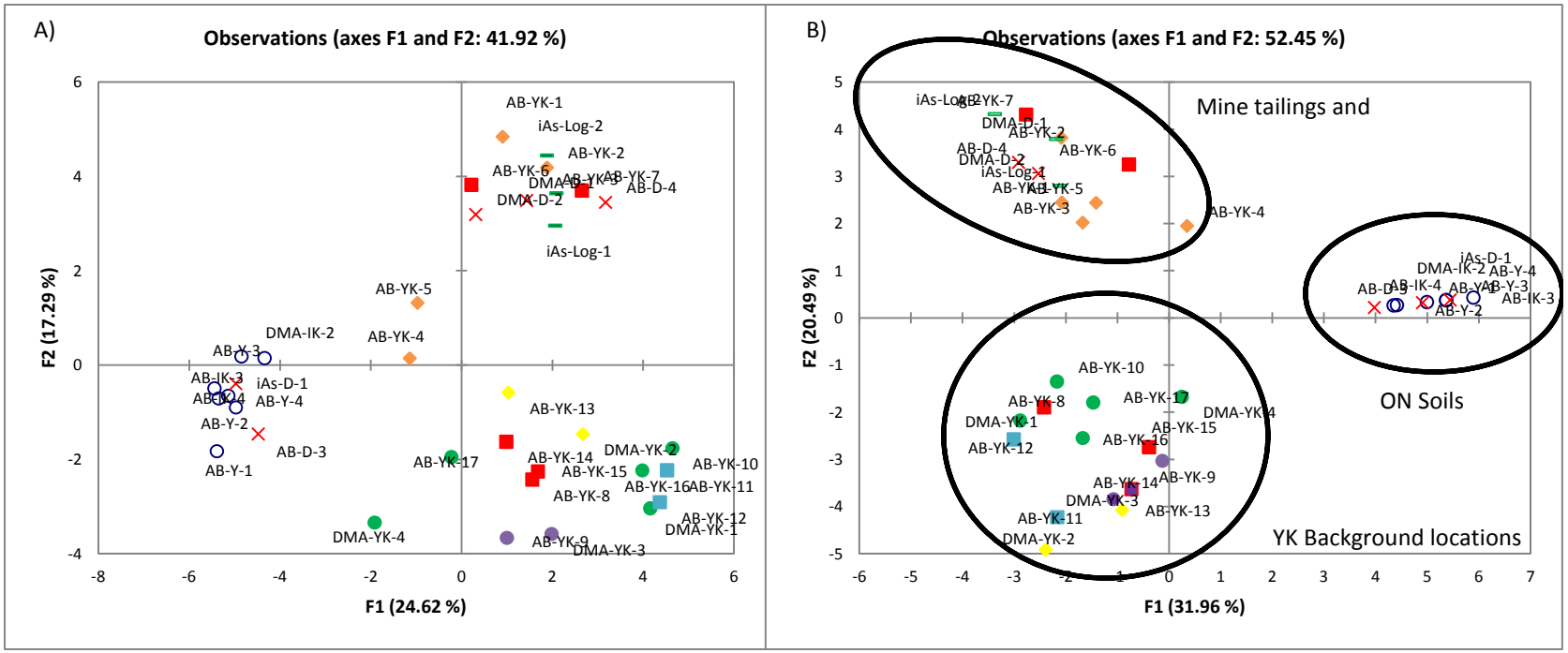

Figure 6.10: A): Order, and B) family relative abundance for Yellowknife and ON soils together (Taylor transformed). ◆ = Giant Mine tailings, □ = Con Mine Tailings, □ = Con Mine residential area, ◆ = Giant Mine water treatment area, ● = Ndilo, ● = all other background locations (Marina, Frame and Grace Lake), **O** = ON background samples, **X** = Deloro samples and -- = log material associated with tree growing mushrooms. Clusters of samples are indicated by the black open circles.

Data at the species level were filtered to search for bacteria species only associated with AB producing FBs and fruiting body initiation, regardless of the quantity of the bacteria species (i.e. presence/absence was interrogated) and are listed in Table 6.4. Of these, 10 species or genera were found in more than one group and were commercially available for purchase. These 10 species/genera were selected for cultivation experiments. The species then chosen from a selected genus were dependent on their commercial availability. These species included *Methylobacterium organophilum, Flavobacterium sp, Rhodoplanes elegans, Massilia brevitalea, Hydrogenophaga taeniospiralis, Bradyrhizobium canariense, Rhizobium rhizogenes, Planctomyces brasiliensis, Pedomicrobium manganicum,* and *Herbaspirillum aquaticum.*

Table 6-4: Bacteria species associated with FB initiation and AB containing FBs.

	Bacteria species		
Association	Casing only	Compost only	Both Casing and Compost
Associated with FB initiation	*Achromobacter sp* *Altererythrobacter sp* *Asticcacaulis benevestitus* *Asticcacaulis excentricus* *Flavobacterium aquatile** *Flavobacterium flevense** *Hahella chejuensis* *Mesorhizobium sp* *Novosphingobium sp* *Oceanicola sp* *Prosthecobacter dejongeii* *Prosthecobacter sp* *Pseudoxanthomonas ginsengisoli* *Roseomonas lacus* *Roseomonas sp* *Sediminibacterium sp* *Sediminibacterium sp* *Sphingopyxis alaskensis* *Sphingopyxis witflariensis* *Thermomonas brevis*	*Asaia lannaensis* *Balneimonas sp* *Bradyrhizobium liaoningense** *Clostridium butyricum* *Clostridium chromoreductans* *Dietzia sp* *Enterobacter asburiae* *Enterobacter sp* *Massilia sp.** *Nitrosospira sp** *Pantoea ananatis* *Petrimonas sp* *Propionibacterium sp* *Rubrimonas sp* *Shinella zoogloeoides* *Sphingomonas fennica* *Wolinella sp*	*Shinella zoogloeoides* *Bradyrhizobium yuanmingense** *Sphingomonas kaistensis* *Methylobacterium sp.** *Planctomyces sp. ** *Rhizobium sp.** *Rhodoplanes sp.** *Hydrogenophaga sp.**
	ON only	YK only	Both ON and YK
Associated with AB containing FBs	*Bradyrhizobium sp.** *Chryseobacterium indologenes* *Flavobacterium aquatile** *Halothermothrix sp.* *Hydrogenophaga sp.** *Kaistobacter sp.*	*Acidithiobacillus sp.* *Blastococcus sp.* *Brevundimonas lenta* *Hoeflea alexandrii* *Methylobacterium sp.** *Mitochondria spp.* *Nocardioides oleivorans*	*Bdellovibrio sp.* *Cytophaga sp.* *Herbaspirillum sp.** *Hoeflea sp.* *Leucothrix sp.* *Rhizobium sp.** *Pedomicrobium sp.**

* Species or genus selected for cultivation experiments. For genera tested in cultivation experiments, species were chosen based on commercial availability and are listed in the text.

Table 6.4 Continued

	Bacteria species		
	ON only	YK only	Both ON and YK
Associated with AB containing FBs	*Kaistobacter sp.* *Lysinibacillus sp* *Lysinibacillus sphaericus* *Nitrospira sp.* *Massilia sp.** *Microvirga subterranean* *Paenibacillus alginolyticus* *Pedobacter koreensis* *Pedobacter kribbensis* *Pseudonocardia halophobica* *Rhizobium etli** *Rhizobium tibeticum** *Variovorax sp.*	*Phycisphaera spp.* *Pseudoxanthomonas sacheonensis* *Quadrisphaera sp.* *Sporosarcina psychrophila*	
Associated with soil (absent in log material)	*Candidatus entotheonella sp.* *Ferruginibacter sp.* *Longispora sp.* *Rhodoplanes sp.**		

* Species or genus selected for cultivation experiments. For genera tested in cultivation experiments, species were chosen based on commercial availability and are listed in the text.

6.4.5 Microbial Cultivation and Arsenic Exposure Experiments

Pure cultures of the species in Table 6.4 that were commercially available were purchased and cultivated individually. Each species was cultivated axenically and exposed to three arsenic compounds to examine the arsenic transformations and specifically if AB could be produced by these species. An inorganic arsenic compound, arsenate (As(V)), was selected to represent the form of arsenic most likely encountered in the soil. DMA(V) was selected to test the formation pathway (Figure 4.1) where DMA(III) is transformed to (DMAA) which is then converted to AB. Finally, DMAA was selected to test if the bacteria species could convert DMAA to AB, as was observed in the lysed bacterial extract of *Pseudomonas fluorescens* [26].

Arsenic was not detected in any inoculated or uninoculated negative controls (with no arsenic compound added). All arsenic compounds were found to be stable throughout the sample preparation and incubation period in uninoculated positive controls. For the majority of bacteria species no arsenic transformations were observed (Table 6.6). *B. canariense, H. taeniospiralis, Flavobacterium sp* and *M. organophilum* were found to reduce As(V) to small amounts of As(III). *M. brevitalea* was found to transform DMA to trace amounts of As(V) and *R. rhizogenes* was found to transform DMAA to trace amounts of As(V).

Table 6-5: Single bacteria species exposure experiment controls.

Controls	Bacteria Species	Arsenic Compound	N	Arsenic Compound (µg/L)			Total Arsenic (µg/L)	Column Recovery (%)
				As(V)	DMA	DMAA		
Uninoculated positive control	None	As(V)*	3	83 ± 8	nd	nd	104 ± 20	81 ± 9
	None	DMA*	3	nd	71 ± 10	nd	93 ± 30	76 ± 30
	None	DMAA	3	nd	nd	160 ± 40	150 ± 40	106 ± 10
Uninoculated negative control	None	No arsenic	3	nd	nd	nd	nd	n/a
Inoculated negative control	*Bradyrhizobium canariense*	No arsenic	3	nd	nd	nd	nd	n/a
	Massilia brevitalea		3	nd	nd	nd	nd	n/a
	Pedomicrobium manganicum		3	nd	nd	nd	nd	n/a
	Rhodoplanes elegans		3	nd	nd	nd	nd	n/a
	Planctomyces brasiliensis		3	nd	nd	nd	nd	n/a
	Flavobacterium sp		3	nd	nd	nd	nd	n/a
	Hydrogenophaga taeniospiralis		3	nd	nd	nd	nd	n/a
	Methylobacterium organophilum		3	nd	nd	nd	nd	n/a
	Herbaspirillum aquaticum		3	nd	nd	nd	nd	n/a
	Rhizobium rhizogenes		3	nd	nd	nd	nd	n/a

* amended with 100 µg/L As

Detection limit (DL) for total As = 0.5 µg/L, DL for arsenic species = 3 µg/L, trace < 5 µg/L, nd = non-detect

n/a = not applicable

Table 6.6: Single bacteria species exposure experiment treatment groups

Bacteria Species	Treatment	N		Total As (µg/L)	Arsenic Compound (µg/L)				Column Recovery (%)	Transformation
					As(III)	As(V)	DMA	DMAA		
Bradyrhizobium canariense	As(V)	3	Average	140	trace	100	nd	nd	80	trace As(III)
			RSD	20	--	20	--	--	10	
	DMA	3	Average	150	nd	nd	130	nd	90	none
			RSD	20	--	--	20	--	2	
	DMAA	3	Average	200	nd	nd	nd	170	90	none
			RSD	30	--	--	--	10	4	
Massilia brevitalea	As(V)	3	Average	130	nd	110	nd	nd	90	none
			RSD	30	--	20	--	--	7	
	DMA	3	Average	110	nd	trace	100	nd	90	trace As(V)
			RSD	1	--	--	4	--	3	
	DMAA	3	Average	140	nd	nd	nd	130	90	none
			RSD	6	--	--	--	8	2	
Pedomicrobium manganicum	As(V)	3	Average	110	nd	90	nd	nd	80	none
			RSD	1	--	5	--	--	6	
	DMA	3	Average	120	nd	nd	110	nd	90	none
			RSD	4	--	--	9	--	5	
	DMAA	3	Average	150	nd	nd	nd	130	85	none
			RSD	20	--	--	--	20	3	
Rhodoplanes elegans	As(V)	3	Average	100	nd	80	nd	nd	80	none
			RSD	10	--	10	--	--	10	
	DMA	3	Average	110	nd	nd	105	nd	95	none
			RSD	20	--	--	20	--	8	
	DMAA	3	Average	170	nd	nd	nd	140	82	none
			RSD	8	--	--	--	3	3	

* amended with 100 µg/L As

Detection limit (DL) for total As = 0.5 µg/L, DL for arsenic species = 3 µg/L, trace < 5 µg/L, nd = non-detect, RSD = relative standard deviation

Table 6.6: Continued

Bacteria Species	Treatment	N		Total As (µg/L)	Arsenic Compound (µg/L)				Column Recovery (%)	Transformation
					As(III)	As(V)	DMA	DMAA		
Planctomyces brasiliensis	As(V)	3	Average	130	nd	100	nd	nd	76	none
			RSD	20	--	9	--	--	4	
	DMA	3	Average	210	nd	nd	190	nd	93	none
			RSD	100	--	--	60	--	20	
	DMAA	3	Average	220	nd	nd	nd	190	84	none
			RSD	20	--	--	--	10	3	
Flavobacterium sp	As(V)*	3	Average	85	70	19	nd	nd	105	reduction to As(III)
			RSD	30	30	5	--	--	10	
	DMA*	3	Average	84	nd	nd	70	nd	85	none
			RSD	3	--	--	1	--	3	
	DMAA	3	Average	150	nd	nd	nd	150	100	none
			RSD	30	--	--	--	5	20	
Hydrogenophaga taeniospiralis	As(V)*	3	Average	94	64	30	nd	nd	77	reduction to As(III)
			RSD	4	6	40	--	--	5	
	DMA*	3	Average	90	nd	nd	70	nd	78	none
			RSD	20	--	--	10	--	10	
	DMAA	3	Average	180	nd	nd	nd	180	104	none
			RSD	30	--	--	--	20	5	
Methylobacterium organophilum	As(V)*	3	Average	70	51.8	15	nd	nd	98	reduction to As(III)
			RSD	9	0.4	1	--	--	10	
	DMA*	3	Average	60	nd	nd	40	nd	76	none
			RSD	6	--	--	3	--	3	
	DMAA	3	Average	190	nd	nd	nd	200	110	none
			RSD	80	--	--	--	90	20	

* amended with 100 µg/L As

Detection limit (DL) for total As = 0.5 µg/L, DL for arsenic species = 3 µg/L, trace < 5 µg/L, nd = non-detect, RSD = relative standard deviation

Table 6.6: Continued

Bacteria Species	Treatment	N		Total As (µg/L)	Arsenic Compound (µg/L)				Column Recovery (%)	Transformation
					As(III)	As(V)	DMA	DMAA		
Herbaspirillum aquaticum	As(V)*	3	Average	120	nd	120	nd	nd	99	none
			RSD	3	--	3	--	--	1	
	DMA*	3	Average	110	nd	nd	90	nd	83	none
			RSD	8	--	--	4	--	9	
	DMAA	3	Average	190	nd	nd	nd	150	83	none
			RSD	20	--	--	--	9	6	
Rhizobium rhizogenes	As(V)*	3	Average	95	nd	70	nd	nd	78	none
			RSD	4	--	2	--	--	4	
	DMA*	3	Average	120	nd	nd	110	nd	90	none
			RSD	30	--	--	30	--	7	
	DMAA	3	Average	159.9	nd	trace	nd	150	96	trace As(V)
			RSD	0.6	--	--	--	10	8	

* amended with 100 µg/L As

Detection limit (DL) for total As = 0.5 µg/L, DL for arsenic species = 3 µg/L, trace < 5 µg/L, nd = non-detect, RSD = relative standard deviation

Culture of these individual bacteria species was the first screening for single organisms associated with FBs and their role in arsenic biotransformations. Additional species should be cultured in a similar way to continue and screen associated microbes for their potential to produce AB. The formation of AB may be reliant on a combination of bacteria species or an environment made by the microbial community and the fungus. In order to test the arsenic transformations in a consortium of bacteria the cultivable bacteria associated with FBs from ON soils were cultivated for arsenic exposure experiments. Cultivable bacteria associated with AB containing FBs *Agaricus campestris, Calvatia gigantea, Lycoperdon perlatum, Leucoargaricus naucina* and non AB containing *Suillus americanus* from Odessa ON were selected. Cultivable bacteria associated with *C. gigantea* and *L. perlatum* from Deloro ON were also selected. All community cultures were exposed to As(V) to represent the arsenic most likely encountered in the soil. The arsenic compounds found in each community after 1 month of exposure are summarized in Table 6.7.

Table 6-6: Average concentrations of arsenic compounds from media.

Soil Source of Isolated Bacteria	Arsenic Compounds ($mg \cdot L^{-1}$)				Total Arsenic ($mg \cdot L^{-1}$)	Column Recovery (%)
	As III	As V	MMA	DMA		
A. campestris	5.2 ± 1.5	2.7 ± 0.4	nd	trace	7.1 ± 3.3	110
Suillus americanus	6.6 ± 1.0	4.4 ± 0.6	nd	trace	9.9 ± 1.8	110
Calvatia gigantea	4.4 ± 1.0	3.1 ± 0.7	trace	trace	5.1 ± 1.1	150
Lycoperdon perlatum	3.7 ± 0.6	5.6 ± 0.2	nd	trace	10.2 ± 1.9	91
Leucoargaricus naucina	4.7 ± 2.1	3.3 ± 0.7	trace	trace	6.2 ± 2.4	130
Calvatia gigantea D	3.7 ± 0.8	4.6 ± 1.2	nd	trace	6.3 ± 1.4	130
Lycoperdon perlatum D	11.5 ± 0.2	0.8 ± 0.1	nd	trace	16.6 ± 0.7	75

*n = 3 refers to number of exposure experiments performed for each microbial community. nd = not detected, (± indicates standard deviation

The uninoculated media positive control was found to only contain As(V), indicating that As(V) was stable throughout the sample preparation process. Negative (blank) controls indicated no contamination occurred during the experiment. The microbial communities from the surrounding soil of all fungus species were able to reduce As(V) to As(III), which has been observed for many species of bacteria and occurs with use of the ArsC gene followed by the removal of As(III) with protein pumps encoded by the ArsB gene [42]. All communities were able to methylate As(V) to DMA in trace amounts. The community isolated from the *C. gigantea* and the *L. naucina* from the Kingston property were found to produce MMA in trace amounts as well. The microbial communities were all isolated from the surroundings of mushrooms that contained a majority of AB, except for the *S. americanus* species, which contained only DMA. However, no differences were seen in the arsenic speciation for the isolated bacteria consortia. Methylation of arsenic by soil microorganisms has been found to be the source of MMA and DMA for various plant species [54]. It remains unclear whether this symbiotic relationship

occurs in the soil microbes and fungi in the present study since only trace amounts of DMA were produced.

Only approximately 1% of the bacteria community from a soil sample can be cultivated [45], and thus the biotransformations observed in the cultivated consortia may only show a portion of the arsenic metabolism that might occur under natural conditions. Although such a low proportion of the bacteria community was cultured, more transformations, specifically methylation, were observed in the cultivable bacteria communities than in the single species cultured suggesting that arsenic biotransformations at this level are associated with the relationship of many bacteria species.

Additional exposure experiments should be performed using lower concentrations of arsenic and other arsenic compounds. Axenic culture of algae species exposed to As(V) produced more biotransformations at lower arsenic concentrations (2 µg/L) [208, 209] . A similar trend was also observed in the growth of *A. bisporus* FBs. FBs grown on material with lower arsenic concentrations produced more AB than those grown on material containing higher arsenic concentrations. Therefore it is predicted that more arsenic biotransformations will be observed with bacteria grown in media containing lower concentrations of arsenic.

Chapter 7

7 Conclusions and Future Direction

7.1 Summary

This thesis provides the first comprehensive investigation of the formation of the only non-toxic arsenic compound, arsenobetaine (AB).

In Chapter 2 complementary arsenic speciation methods were reviewed, specifically the use of XAS and ESI-MS to complement HPLC-ICP-MS analysis. The complementary use of ESI-MS with HPLC-ICP-MS was found to provide confirmation of arsenic compounds identified during the HPLC-ICP-MS analysis, and identification of unknown or co-eluting compounds observed during the HPLC-ICP-MS analysis. The use of XAS analysis, both XANES and XAS imaging, provide solid state analysis and is therefore independent of extraction efficiency, which yields speciation information additional to that using only HPLC-ICP-MS analysis. Thus, when possible, HPLC-ICP-MS and XAS analysis, as well as XAS imaging, were utilized in a complementary manner in this thesis (Chapter 3, 4 and 5). The findings from using these combined techniques were that no arsenic compound was preferentially extracted from mushrooms using an aqueous methanol extraction. In some mushroom samples not all AB was extracted, which is contrary the previous consensus in the literature that the inorganic forms of arsenic are more difficult to extract than organoarsenic compounds [100, 105]. XAS analysis also showed the presence of an As(III) sulfur compound for the first time in the mushrooms and the mycelium of fungus, a compound not observable by the HPLC-ICP-MS methods used in this thesis. Two dimensional XAS analysis was also able to provide information on the distribution of arsenic and arsenic compounds in the fruiting bodies.

A comprehensive survey of arsenic compounds in 46 different mushroom species was performed in Chapter 3 to determine the arsenic speciation in different fungus species collected from Canadian grocery stores, and background and arsenic contaminated areas, in order to provide trends in arsenic speciation and to help elucidate the role or potential formation of AB in these mushrooms. This survey revealed that arsenic speciation is related to both the phylogenetic placement and fruiting body morphology of the fungus. Regardless of location, species from the *Lycoperdaceae* (puffball mushrooms) and *Agariceae* (gilled mushrooms) family contained a majority of AB. Species from the *Sullicaceae* family (pored mushrooms) mainly contained DMA and other families contained inorganic arsenic compounds. A majority of AB in puffball and gilled mushrooms supports the hypothesis that AB may be adventitiously accumulated as an osmolyte to help maintain a turgid fruiting body for effective spore dispersal. Unlike

other organisms collected from arsenic contaminated locations in which the proportion of AB decreases as total arsenic increases, AB was still found to be the major arsenic compound regardless of total arsenic. The log growing mushrooms, with the exception of one species with trace amounts of AB, did not contain AB. This was demonstrated most clearly by the direct comparison that could be made with the species *Lycoperdon perlatum*, which was collected from both a log and soil in the same location: no AB was found in the log growing mushrooms while the soil growing mushrooms contained a majority of AB. The difference in speciation suggests that the microbial community may influence arsenic speciation in these mushrooms (supporting pathway 3, Chapter 1, Figure 1.4).

Another objective of Chapter 3 was to identify fungus species for further culturing experiments (Chapters 4 and 5). Fungus species from a genus identified in Chapter 3 that contained a majority of AB (*Agaricus*) and DMA (*Suillus*) were two of the fungus species selected for culture. Although Chapter 3 demonstrated that arsenic speciation differs across species with different fruiting body morphologies but it was still unclear whether the major arsenic compound, such as AB, was accumulated or produced by the fungus.

Chapter 4 targeted the vegetative, mycelium life stage of terrestrial fungi, to examine the role of this stage in arsenic transformation, especially to AB and to test pathways 1 (the mycelium *de novo* producing AB) and 2 (the mycelium producing AB from precursors provided by the microbial community). By growing the mycelia in axenic laboratory culture, controlled conditions were obtained and the influence of the microbial community was excluded. Three species of fungi were exposed to As(V) (to represent arsenic in the soil), DMAA (a potential precursor to AB), and AB. Overall few biotransformations and the lack of AB formed from the potential precursors indicate it is unlikely that the mycelium of fungus is responsible for biosynthesizing AB. However the preferential accumulation of AB in the species in which it is dominant in the fruiting bodies indicates that the mycelium may be selectively accumulating the compound and transporting it to the fruiting bodies. Alternatively the fruiting body itself or the microbial community may produce AB. Thus biosynthetic pathways 1 and 2 seem unlikely, and biosynthetic pathway 3 (where AB is taken up by the fungus) is possible.

Chapter 5 aimed to test biosynthetic pathways 4 (AB produced by the fungus during FB development) and 5 (AB produced by the mature FB) using complementary arsenic speciation methods. In order to examine the role of the reproductive life stage on arsenic transformations and potential formation of AB, the reproductive life stage of *Agaricus* species growth substrate and fungi were collected during the commercial growth of *A. bisporus* and analyzed for arsenic speciation (testing of biosynthetic pathways 3, 4 and 5). AB was found to be the major arsenic compound in the fungus at the earliest growth stage of fruiting (the primordium) with the growth substrate mainly containing arsenate (As(V)). The distribution

of arsenic in an *A. bisporus* primordium grown on As(V) treated substrate, and in a mature *A. campestris* fruiting body collected from arsenic contaminated mine tailings, was mapped using two dimensional XAS imaging. Although collected from two different locations and growth stages of fruiting body development, the primordium and stalk fungal tissue of the mature fruiting body were both found to be growing around pockets of substrate material containing higher arsenic concentrations. In both samples AB was found exclusively in the fungal tissues. The presence of AB in the earliest life stage of the reproductive life cycle and AB seen exclusively in mapped fungal tissues indicates AB formation is likely associated with the reproductive life stage of the fungus (pathways 4 and 5). It was also found that in the mature *A. campestris* fruiting body the highest proportion of AB was found in the cap, supporting the hypothesis of AB's function as an osmolyte. It is still unclear whether the microbial communities are responsible for the AB present in the fruiting bodies and therefore biosynthetic pathway 3 cannot be ruled out from these studies.

The objective of Chapter 6 was to test pathway 3 (the microbial community producing AB). The cultivable microbial communities associated with various fungus species collected from both background and contaminated ON locations were exposed to As(V). All community cultures were able to reduce As(V) to As(III) and for some communities trace amounts of DMA and MMA were found indicating that the communities associated with these fungus species can methylate inorganic arsenic. No AB was produced, making pathway 3 unlikely. However, less than 1% of soil bacteria can be cultivated in this way, and the arsenic transformations observed here represent a limited proportion of the microbial community activities. Therefore Chapter 6 also aimed to use 16s pyrosequencing methods to characterize the entire microbial communities associated with a wide variety of fungus species (from Chapter 3) from multiple locations and arsenic concentrations. Characterization of the communities associated with the growth material and the fungus itself during the commercial cultivation of *A. bisporus* was also carried out. The microbial communities from similar locations were found to be similar. For ON samples the microbial communities in log materials were found to be different in relative phyla abundance than that in soils. When the YK and ON sequencing data were combined in a PCA, mine tailing samples were found to be clustered with the log material (which itself was from both a background and contaminated location in ON). This clustering was thought to be attributable to the common freedom of these samples from having a plant rhizophere, being sourced from non-vegetated locations. Another cluster in the PCA analysis was the background YK samples with the ON samples. Statistical analysis also revealed that phylum diversity in the growth substrate of *A. bisporus* increased at the time of fruiting body initiation, coinciding with the appearance of AB.

Following the characterization of microbial communities using pyrosequencing, species of interest were identified. These included those only found associated with AB producing mushrooms and those that appear at the time of fruiting body initiation. A total of 10 bacteria species were cultivated and exposed to As(V), DMA and DMAA to further test pathway 3. Very few transformations were observed in the single species exposure experiments indicating that arsenic biotransformations in the environment may be dependent on the community or on multiple species. The study results could not definitively support or eliminate the possibility of biosynthetic pathway 3, where the microbial environment alone produces AB, which is then taken up by the fungus.

From the study of arsenic speciation in a diverse range of fruiting bodies, the vegetative and reproductive life stages, and associated microbial communities, it still remains unclear which organisms are responsible for AB formation. The lack of arsenic biotransformations and AB formation in each organism on its own suggests that the formation pathway for AB involves a combination of organisms, perhaps those symbiotically associated with each other. It can be concluded that pathways 1 and 2 are not the formation pathways for AB since AB was not formed by the mycelium from inorganic arsenic or potential AB precusors. The preferential accumulation of AB during the vegetative life stage of fungi suggests that if AB is present in the environment it would immediately be accumulated, supporting pathway 3; this pathway could not be either definitively proven or eliminated based on the microbial results. Study of arsenic transformations in the commercial production of *A. bisporus* show that AB formation is associated with the reproductive life stage of the fungus supporting pathways 4 and 5. The fact that AB is seen in the earliest growth stage of the FBs and is not restricted to the mature FBs indicates that pathway 4 is also a likely pathway for AB formation.

7.2 Future Directions

In order to continue to investigate the potential formation pathway for AB in terrestrial fungi, the reproductive life stage of a fungus species such as *A. bisporus*, that contains a majority of AB, should be cultivated under axenic conditions. Producing fruiting bodies grown under axenic conditions on arsenic treated material would determine if the fungus itself produces AB during this life stage. This study phase was included in the original study conception but attempts to produce fruiting bodies under axenic conditions have not yet been successful. The method of treatment of the growth substrate with arsenic should be considered once axenic fruiting body growth can be established. When an As(V) solution was used to amend casing and compost material that already had established mycelium, the fruiting bodies produced did not contain a majority of AB and arsenic uptake was limited. Therefore the growth substrate should be treated with the desired arsenic compounds prior to inoculation.

The cultivation of the reproductive life stage under axenic conditions should also be complemented by techniques such as plating of extracts for bacterial contamination or pyrosequencing to ensure the system is truly axenic. Few studies report methods on the growth of *A. bisporus* under axenic conditions; however with the methods used in the studies with axenic claims, it is unlikely that the system was truly axenic. The associated bacteria are thought to play a symbiotic role in fruiting body initiation by taking up inhibitory compounds secreted by the fungus. It may be possible to replace this absorptive effect with the use of activated carbon.

The importance of the bacteria community associated with the fungus for fruiting body initiation also suggests that it could play a role in AB formation. Therefore the results obtained in Chapter 6 through pyrosequencing methods should continue to be analyzed to identify more species of interest for further cultivation and exposure experiments. The combination, or a consortium, of different microbes should also be tested in this way since the formation pathway for AB may require multiple organisms. The difference in the microbial communities for log-growing mushrooms compared to soil-growing mushrooms should also be further studied. Further cultivation experiments should be performed using lower arsenic concentrations to increase the likelihood of observing arsenic transformations. Different strength media should also be investigated and its influence on arsenic uptake rates.

For specimens with sufficiently high concentrations, different sections of *A. campestris* or *A. bisporus* should be imaged by XAS for arsenic speciation. Fruiting bodies with different morphologies such as puffballs and pored mushrooms should also be imaged for arsenic distribution to further test the hypothesis that AB is adventitiously accumulated as an osmolyte hypothesis. Betaine should also be measured in a variety of mushroom species to determine if it is correlated to AB concentrations.

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APPENDICES

Appendix A

A. Additional information for Chapter 3: Arsenic speciation in edible mushrooms

A.1 Chemical and Reagents

Distilled deionized water (DDW) was prepared in-house to a minimum resistance of 18 MΩ·cm (E-pure Barnstead). For the total acid digestions trace metal grade nitric acid (~ 70%, Fisher Scientific) was used. For arsenic extractions DDW was used with HPLC grade methanol (Fisher Scientific) and 2% (v/v) Optima grade (Fisher Scientific). Total matrix spikes and calibration curves were prepared from a stock solution of 1000 µg/mL total arsenic (arsenic pentoxide, Inorganic Ventures).

For HPLC-ICP-MS analysis the anion exchange mobile phase was prepared with ammonium nitrate (99.999% purity, Aldrich) and the cation exchange mobile phase was prepared with pyridine (99% purity, Sigma).

Different sources of standards were used for the calibration curves than for quality control calibration check solutions and matrix spikes when available. For anion exchange this was possible for As(III), arsenite (90% from Fluka and 99.999% purity from Aldrich); As(V), arsenate (1000 ppm from Aldrich and 1000 ppm from Inorganic Ventures); and DMA (V), cacodylic acid (>99% from Fluka and 99% purity from City Chemical). For cation exchange this was possible for DMA (V); AB, arsenobetaine (Wako and Argus Chemicals); TMAO, trimethlyarsine oxide (Wako and Argus Chemicals). Only one source was available for AC, arsenocholine bromide (Argus Chemicals) for cation exchange and for MMA(V), monosodium acid methane arsonate sesquihydrate (99.0% purity Chemservice). TETRA, tetramethylarsonium iodide (Wako) was used for identification purposes by matching retention times, and the AC calibration curve was used for quantification of the compound when it was found in samples. Arsenosugars used as standards were extracted from brown algae (*Fucus vesiculosus)* collected in Nova Scotia, Canada. Arsenosugars were identified by matching retention times and the calibration curve of the compound closest in retention time to an identified arsenosugar was used for quantification.

For total arsenic digestions the certified reference material (CRM) BCR®-482, Lichen was obtained from European Commission Institute for Reference Materials and Measurements. For arsenic extractions the CRM BCR®- 627, Tuna Fish was obtained from the same source.

A.2 Total arsenic analysis

The instrument used was an ICP-MS DRC II from PerkinElmer (PerkinElmer, MA). The detection limit of the instrument was determined to be 0.5 µg/L and mass interferences were monitored by measuring *m/z* 77 ($ArCl^+$). Instrumental tests included instrumental blanks and calibration checks which were run every 10 samples. Instrument blanks run every 10 samples were under the instrument detection limits for total arsenic analysis. Instrument quality control checks that included a 5 µg/L and 50 µg/L solution for total arsenic were run every 10 samples and found to be within an acceptable range (80 – 120 % recovery).

A.3 Arsenic speciation analysis using HPLC-ICP-MS

Briefly, anion exchange chromatography was performed using a Hamilton PRPX100 anion exchange column with a gradient mobile phase (A: 4 mM of ammonium nitrate (NH_4NO_3) and B: 60 mM NH_4NO_3, pH = 8.65). Anion exchange was used to identify: As(III), As(V), monomethylarsonic acid (MMA), dimethylarsinic acid (DMA), glycerol arsenosugar (Sugar 1) and sulfonate arsenosugar (Sugar 3. Cation exchange chromatography was performed with a Chrompack Ionosphere C cation exchange column with a mobile phase of 20nM pyridine, pH = 2.7. Cation exchange was used to identify: DMA, AB, trimethylarsine oxide (TMAO), arsenocholine (AC) and tetramethylarsonium ion (TETRA). All chromatographic speciation data were analyzed with Peak Fit Version 4.12. HPLC blanks and calibration checks were run every 10 samples and considered acceptable (70 – 130 % recovery). All mushrooms were analyzed for arsenic speciation and all compounds that could be matched with a known standard were quantified using the calibration curve for that arsenic compound. Unknown compounds were quantified using the calibration curve for the closest eluting known compound. The limit of detection for each compound was determined to be 0.003 mg/kg, compounds 0.003 mg/kg DM to 0.005 mg/kg DM were considered as trace compounds and all compounds over 0.005 mg/kg DM were quantified.

Table A-1: Summary of mushroom sampling locations.

Source	Location	Habitat/Comments	Latitude/Longitude Coordinates	Total Arsenic in Soil
Store Bought	- Highland Mushrooms, ON Canada - Continental Mushrooms, ON Canada - Rooster Brand, China - Taiwan Roxy Brand, Taiwan - S and F Food Importers, Canada - Forest Mushrooms, Minnesota USA - Loblaws Grocery, Canada - Granville Island, BC Canada - T & T Asian Market, ON Canada	Cultivated species bought fresh: - Agaricus bisporus (White button) - Agaricus brunnescens (Portobello and cremini) Cultivated species bought dried: - Volvariella volvacea (Paddy straw) Log/tree cultivated species bought fresh: - Flammulina velutipes (Enoki) - Lentinula edodes (Shiitake) - Pleurotus ostreatus (Oyster) Log/tree cultivated species bought dried: - Tremella fuciformis (Snow fungus) - Auricularia polytricha (Black fungus) Wild species bought dried: - Hypomyces lactifluorum (Lobster mushroom) - Boletus edulis (Porcini) - Craterellus cornucopioides (Black trumpet) - Cantharellus cibarius (Chanterelle)		Unknown

Table A-1 Continued

Source	Location	Habitat/Comments	Latitude/Longitude Coordinates	Total Arsenic in Soil
ON Background	Odessa, ON Canada	Fall mushroom collection: - Grass meadow areas - Roadsides, gravelly areas - Deciduous and coniferous forest areas	44.2500° N, -76.7500° W	1.0 – 2.0 mg/kg
	Grafton, ON Canada	Fall mushroom collection: - Grass meadow areas - Roadside, gravelly areas - Under coniferous trees	43.9911° N, -78.0239° W	Unknown
	Yarker, ON Canada	Fall mushroom collection: - Grass meadow areas - Roadside, gravelly areas - Deciduous forest areas	44.3667° N, -76.7667° W	Unknown
ON Contaminated	Deloro, ON Canada	Fall mushroom collection: - Gold Mine surrounding area Deloro Village, operational late 1800s to early 1900s - Grass meadow areas - Deciduous and coniferous forest areas	44.5122° N, -77.62° W	20 – 120 mg/kg
YK Background	Frame Lake, Yellowknife NT Canada	Fall mushroom collection: - Roadside, gravelly areas - Deciduous forest areas	62.4529° N, -114.3915° W	Unknown
	Grace Lake, Yellowknife NT Canada	Fall mushroom collection: - Soil pockets on rock outcrops	62.4185°N, -114.4526°W	42 mg/kg
	Con Mine, Yellowknife NT Canada - Residential Area - Negus Tailings	Fall mushroom collection: - Grass areas - Deciduous and coniferous forest areas - Tailings	62.262°N, -114.2218°W	25 – 3195 mg/kg

Table A-1 Continued

Source	Location	Habitat/Comments	Latitude/Longitude Coordinates	Total Arsenic in Soil
YK Contaminated	Ndilo, Yellowknife NT Canada	Fall mushroom collection: - Deciduous forest area - Marsh area in forested area		17 – 1219 mg/kg
	Giant Mine, Yellowknife NT Canada - Marina area - Northwest Tailings pond - Water treatment area - Tailings area	Fall mushroom collection: - Tailings - Marsh area in forested area - Deciduous and coniferous forest area - Soil pockets on rock out crops	62.2854 to 62.3238°N, -114.1912 to -114.2234°W	50 – 1260 mg/kg
	Con Mine, Yellowknife NT Canada - Residential Area - Negus Tailings	Fall mushroom collection: - Grass areas - Deciduous and coniferous forest areas - Tailings	62.262°N, -114.2218°W	25 – 3195 mg/kg

Table A-2: Quality assurance and quality control for total arsenic and arsenic speciation analysis.

Medium	Measurement	Instrument	N	Range	Average
Method Blanks	Total Arsenic Concentration (Extracts and Residues)	ICP-MS	22	< LOD[a]	< LOD[a]
Replicates	Total Arsenic Concentration (Extracts + Residues), percent RPD[b]	ICP-MS	73 samples in duplicate	0 – 71	15
CRM BCR 482 Lichen	Percent Recovery, Total Arsenic Concentration (Residues, excluding Yellowknife)	ICP-MS	7	63 - 102	78
CRM BCR 627 Tuna Fish	Percent Recovery, Total Arsenic (Yellowknife Residues)	ICP-MS	4	81 - 92	85
Matrix Spikes	Percent Recovery, Total Arsenic Concentration	ICP-MS	21	77 - 110	93
Method Blanks	Arsenic Species Concentrations	HPLC-ICP-MS		< LOD[c]	< LOD[c]
CRM BCR 627 Tuna Fish	Percent Recovery, DMA Concentration	Anion HPLC-ICP-MS	10	64 - 110	78
CRM BCR 627 Tuna Fish	Percent Recovery, AB Concentration	Cation HPLC-ICP-MS	10	70 - 123	86
Matrix Spike	Percent Recovery, As(III) Concentration	Anion HPLC-ICP-MS	10	39 - 124	90
Matrix Spike	Percent Recovery, DMA Concentration	Anion HPLC-ICP-MS	10	37 - 116	73
Matrix Spike	Percent Recovery, MMA Concentration	Anion HPLC-ICP-MS	10	50 - 120	85
Matrix Spike	Percent Recovery, As(V) Concentration	Anion HPLC-ICP-MS	10	75 - 130	108
Matrix Spike	Percent Recovery, DMA Concentration	Cation HPLC-ICP-MS	10	82 - 127	112
Matrix Spike	Percent Recovery, AB Concentration	Cation HPLC-ICP-MS	10	81 - 124	101
Matrix Spike	Percent Recovery, TMAO Concentration	Cation HPLC-ICP-MS	10	87 - 125	105
Matrix Spike	Percent Recovery, AC Concentration	Cation HPLC-ICP-MS	10	82 – 152	110
Column Recovery	Percent Recovery ($MeOH/H_2O$ Extracts)	HPLC-ICP-MS	73	63 - 131	100
Column Recovery	Percent Recovery (HNO_3 Extracts)	HPLC-ICP-MS	18	39 - 139	89
Column Recovery	Percent Recovery, CRM BCR 627 Tuna Fish	HPLC-ICP-MS	10	70 - 125	92
Extraction Efficiency	Percent Recovery, CRM BCR 627 Tuna Fish	HPLC-ICP-MS	10	66 - 97	82

[a] ICP-MS Limit of Detection (LOD) for total arsenic is 0.01 mg/kg for all samples except YK samples, which have an LOD of 0.07mg/kg

[b] RPD = Relative percent difference (%) = 100% × (| value 1- value 2 |)/average of value 1 and value 2

[c] Arsenic species by HPLC-ICP-MS LOD = 0.003 mg/kg DM mushroom

Table A-3: Total arsenic, arsenic species concentration, extraction efficiency (EE) and column recovery (CR) of fruiting bodies collected for all locations grouped by class, order, and family. Average concentration and differences between duplicates of each sample is provided.

#	Species	Source		Total As (mg/kg)	Concentration (µg/kg DM)								
					As_i	DMA	MMA	AB	TMAO	AC	TETRA	EE(%)	CR (%)
1	*Lycoperdon pyriforme*	ON Background	Average	0.46	10	97.7	nd	220	nd	nd	nd	95.33	73
			Difference	0.02	3	0.8		9				0.06	5
2	*Lycoperdon pyriforme**	ON Background	Average	0.057	16	50	nd	nd	nd	nd	nd	100	100
			Difference	0.002	2	5						0	10
3	*Lycoperdon perlatum*	ON Background	Average	0.154	4.5	30	nd	70	nd	nd	nd	75.30	89
			Difference	0.005	0.4	4		3				0.07	3
4	*Lycoperdon perlatum*	ON Background	Average	0.37	nd	nd	nd	400	nd	nd	nd	110	100
			Difference	0.09				20				4	10
5	*Lycoperdon perlatum*	ON Contaminated	Average	9	nd	41600	nd	2000	nd	nd	nd	60	90
			Difference	4		200		200				10	60
6	*Lycoperdon perlatum*	ON Contaminated	Average	5.900	nd	130	nd	4200	nd	nd	nd	60	120
			Difference	0.005		40		400				0	1
7	*Lycoperdon perlatum*	YK Background	Average	4	560	124.0	34.0	4000	nd	nd	nd	80	100
			Difference	2	20	0.3	0.5	20				40	3
8	*Lycoperdon perlatum*	YK Contaminated	Average	27	440	3000	nd	11200	320	nd	nd	61	91
			Difference	4	70	200		2000	20			5	8
9	*Lycoperdon perlatum*	YK Contaminated	Average	25	1000	190	nd	9300	nd	93	nd	43.9	97
			Difference	4	100	5		1000		10		0.4	1
10	*Calvatia gigantea*	ON Background	Average	0.3	trace	41	nd	210	nd	nd	nd	83	110
			Difference	0.2		2		20				1	70
11	*Calvatia gigantea*	ON Background	Average	1.26	trace	trace	trace	1000	nd	nd	nd	92	87
			Difference	0.03				20				5	9
12	*Calvatia gigantea*	ON Background	Average	1.3	nd	66	nd	910	nd	nd	nd	73	100
			Difference	0.6		7		100				7	20

* indicates log growing species. iAs = As(III) + As(V). *nd = not detected, trace = < 5 µg/kg dry weight*
ON = Ontario, YK = Yellowknife

Table A-3 Continued

#	Species	Source		Total As (mg/kg)	Concentration (µg/kg DM)								
					As_i	DMA	MMA	AB	TMAO	AC	TETRA	EE(%)	CR (%)
13	*Calvatia gigantea*	ON Background	Average	0.422	8	38	nd	330	nd	nd	nd	85	100
			Difference	0.003	2	4		30				6	5
14	*Calvatia gigantea*	ON Contaminated	Average	59	nd	nd	nd	39000	nd	nd	nd	67.9	94
			Difference	0				5000				0.4	6
15	*Agaricus bisporus*	Store Bought	Average	0.102	21.8	28	nd	37	5.15	nd	nd	74.7	120
			Difference	0.007	0.5	5		2	0.03			0.5	3
16	*Agaricus brunnescens*	Store Bought	Average	0.090	28	25	nd	39	nd	nd	nd	83	71
			Difference	0.002	1	2		3				3	5
17	*Agaricus brunnescens*	Store Bought	Average	0.020	8	7	nd	7	nd	nd	nd	65	110
			Difference	0.008	3	2		1				10	30
18	*Lepiota rachodes*	ON Background	Average	0.29	26.50	35	9.6	180	nd	nd	nd	90	97
			Difference	0.05	0.09	7	0.2	30				2	10
19	*Lepiota rachodes*	ON Background	Average	1.80	51	90	nd	1400	nd	nd	nd	88	87
			Difference	0.03	3	5		100				3	8
20	*Leucoagaricus naucinus*	ON Background	Average	0.142	nd	nd	nd	25.3	nd	nd	nd	63.9	110
			Difference	0.005				0.6				0.5	9
21	*Leucoagaricus naucinus*	ON Contaminated	Average	0.55	230	210	22	180	nd	nd	48	79	100
			Difference	0.09	2	20	2	20			2	3	30
22	*Agaricus campestris*	ON Background	Average	0.2772	79	18.9	nd	200	nd	nd	nd	92	110
			Difference	0.0004	5	0.5		40				10	2
23	*Agaricus campestris*	YK Contaminated	Average	34	4100	1600	270	27700	1800	200	nd	94	89
			Difference	5	300	100	20	900	20	9		2	20
24	*Agaricus campestris*	YK Contaminated	Average	21	2400	23	nd	5600	nd	nd	nd	56	99
			Difference	10	200	50		800				10	30
25	*Coprinus comatus*	ON Background	Average	0.030	nd	16	nd	trace	nd	nd	nd	51	120
			Difference	0.004		2						3	6

* indicates log growing species. iAs = As(III) + As(V). *nd = not detected, trace = < 5 µg/kg dry weight*
ON = Ontario, YK = Yellowknife

Table A-3 Continued

#	Species	Source		Total As (mg/kg)	Concentration (µg/kg DM)								
					As_i	DMA	MMA	AB	TMAO	AC	TETRA	EE(%)	CR (%)
26	*Coprinus comatus*	ON Background	Average	0.11	22.9	29	nd	25.9	nd	nd	nd	51	100
			Difference	0.04	0.6	2		0.1				10	40
27	*Coprinus comatus*	YK Contaminated	Average	14	370	750	nd	8800	nd	nd	nd	79	89
			Difference	10	10	20		400				2	10
28	*Coprinus comatus*	YK Contaminated	Average	33	7600	280	nd	8500	nd	nd	nd	58	110
			Difference	8	300	50		3000				7	20
29	*Amanita flavoconia*	ON Background	Average	0.13	27.6	28	nd	11	nd	nd	nd	59	76
			Difference	0.02	20	8		4				9	30
30	*Amanita flavoconia*	ON Background	Average	0.092	28	19.9	5	6.9	nd	nd	nd	66.9	110
			Difference	0.006	10	0.1	4	0.5				0.8	5
31	*Amanita flavoconia*	ON Background	Average	0.25	28.1	59	6.5	60	nd	nd	10.1	59	110
			Difference	0.03	0.8	6	0.9	9			0.8	2	10
32	*Amanita flavoconia*	ON Background	Average	0.083	29	14	3.4	nd	nd	nd	nd	60	94
			Difference	0.008	4	2	0.5					10	10
33	*Amanita prophyria*	ON Contaminated	Average	4.2	180	300	24	460	nd	nd	nd	30	77
			Difference	0.4	20	30	2	40				1	2
34	*Amanita bisporigera*	ON Contaminated	Average	1.3	210	530	11.9	nd	27	59	nd	61	110
			Difference	0.2	30	60	0.6		4	1		1	3
35	*Entoloma sinuatum*	ON Background	Average	0.13	13	51	nd	24	nd	nd	nd	74	85
			Difference	0.02	1	20		9				3	30
36	*Entoloma sinuatum*	ON Contaminated	Average	0.76	170	160	nd	29	nd	nd	nd	39	120
			Difference	0.08	7	10		4				2	9
37	*Hebeloma syriense*	BC	Average	0.12	23	36	nd	56	nd	nd	nd	75	100
			Difference	0.03	2	7		2				10	2
38	*Hebeloma alpinum*	YK Contaminated	Average	9.1	420	3300	100	230	nd	nd	nd	60.5	74
			Difference	0.8	60	600	10	20				0.6	6
39	*Hebeloma velutipes*[a]	YK Contaminated	Average	18	42.8	770	nd	3400	1300	150	trace	39	88.0
			Difference	2	0.8	50		80	200	20		4	0.4
40	*Volvariella volvacea*	Store Bought	Average	0.51	32	150	9	nd	nd	nd	nd	47	81
			Difference	0.03	8	30	2					10	8

* indicates log growing species. iAs = As(III) + As(V). *nd = not detected, trace = < 5 µg/kg dry weight* [a] ***Hebeloma velutipes*** *additionally contained Sugar 3 (Average = 456.38, RPD = 12.09) and an unknown arsenic compound visible using anion exchange UK1: 2.3 mins (Average = 200, RPD = 10).*

Table A-3 Continued

#	Species	Source		Total As (mg/kg)	Concentration (µg/kg DM)								
					As_i	DMA	MMA	AB	TMAO	AC	TETRA	EE(%)	CR (%)
41	*Flammulina velutipes**	Store Bought	Average	0.040	39	6.5	trace	nd	nd	nd	nd	95	120
			Difference	0.005	3	0.6						8	10
42	*Lentinula edodes**	Store Bought	Average	0.23	130	63.4	trace	nd	nd	nd	nd	44	130
			Difference	0.02	5	0.4						3	10
43	*Pleurotus ostreatus**	Store Bought	Average	0.048	trace	32	3.9	4.7	nd	nd	nd	78	100
			Difference	0.005		1	0.7	0.5				8	9
44	*Psathyrella candolleana*	ON Contaminated	Average	0.95	210	240	nd	110	nd	21.4	nd	57	110
			Difference	0.02	10	20		8		0.2		4	4
45	*Tubaria furfuracea*	BC	Average	0.39	63	19	nd	160	nd	nd	nd	57	110
			Difference	0.04	9	2		10				7	7
46	*Coprinus atramentarius*[b]	YK Contaminated	Average	11.2	720	1900	nd	220	2100	trace	78	45.1	100
			Difference	0.2	70	50		7	10		10	0.2	3
47	*Lyophyllum decastes*	YK Contaminated	Average	24.2	3800	230	nd	11300	nd	nd	nd	71	91
			Difference	0.2	200	20		400				4	3
48	*Craterellus cornucopioides*	Store Bought	Average	0.20	51.1	63	nd	nd	nd	nd	nd	55	100
			Difference	0.04	0.9	8						2	20
49	*Cantharellus cibarius*	Store Bought	Average	1.3	490	27	trace	6.1	3.2	nd	8.0	38	110
			Difference	0.2	70	3		0.3	0.5		0.7	6	20
50	*Cantharellus cibarius*	Store Bought	Average	0.760	27	5.1	nd	nd	nd	nd	nd	32	130
			Difference	0.002	2	0.5						1	10
51	*Cantharellus xanthopus*	ON Contaminated	Average	0.39	34	5.9	4.9	14	nd	nd	nd	14	100
			Difference	0.08	2	0.9	0.3	2				2	30
52	*Russula emetica*	BC	Average	0.068	23.3	12.8	nd	nd	nd	nd	nd	57	97
			Difference	0.003	0.3	0.9						20	10
53	*Russula silvicola*	YK Background	Average	4.3	4500	5300	nd	nd	trace	nd	nd	81	120
			Difference	0.5	500	200						6	5
54	*Lactarius deliciosus*	ON Background	Average	0.253	trace	trace	trace	190	nd	nd	trace	64	120
			Difference	0.002				9				2	10

* indicates log growing species. iAs = As(III) + As(V). *nd = not detected, trace = < 5 µg/kg dry weight*

[b]***Coprinus atramentarius*** *additionally contained UK1 (Average 87.35, RPD = 5.64).*

ON = Ontario, YK = Yellowknife, BC = British Columbia

Table A-3 Continued

#	Species	Source		Total As (mg/kg)	Concentration (µg/kg DM)								
					As_i	DMA	MMA	AB	TMAO	AC	TETRA	EE(%)	CR (%)
55	*Lactarius deliciosus*	BC	Average	0.09	22	12.2	nd	28	nd	6.4	nd	58	130
			Difference	0.01	1	0.2		3		0.7		4	6
56	*Lactarius volemus* [c]	YK Contaminated	Average	1.56	210	29	41	330	nd	trace	280	78	110
			Difference	0.07	10	2	1	30			30	2	8
57	*Lactarius volemus* [d]	YK Background	Average	4.6	1800	220	trace	340	190	nd	nd	84.8	86
			Difference	0.2	200	40		8	5			0.8	4
58	*Hericium coralloides*[*]	ON Background	Average	0.21	81	31	nd	nd	nd	nd	nd	100	63
			Difference	0.02	10	20						4	4
59	*Paxillus involutus*[e]	YK Contaminated	Average	5.1	890	300	43	1300	nd	trace	800	88	110
			Difference	0.8	50	7	7	7			100	5	20
60	*Gomphidius oregonensis* [f]	YK Background	Average	2.3	51.6	1300	nd	200	nd	nd	nd	94.2	92
			Difference	0.6	0.7	90		20				0.5	20
61	*Boletus edulis*	Store Bought	Average	0.12	52	9.06	trace	nd	2.8	3.4	5.6	45	130
			Difference	0.03	2	0.05			0.4	0.5	0.7	20	20
62	*Boletinellus meruliodes*	ON Contaminated	Average	0.59	110	110	7.3	180	nd	nd	nd	58	120
			Difference	0.01	1	3	0.7	30				2	6
63	*Suillus americanus*	ON Background	Average	0.267	trace	210	nd	nd	nd	nd	nd	86	91
			Difference	0.008		10						7	10
64	*Suillus granulatus*	YK Contaminated	Average	7.2	nd	5000	nd	71.5	56	trace	trace	86	94
			Difference	0.7		600		0.7	1			7	6
65	*Suillus grevillei*[g]	YK Contaminated	Average	7.5	84	5100	nd	1500	260	nd	nd	91.9	100
			Difference	0.3	5	300		60	6			0.2	1

* indicates log growing species. iAs = As(III) + As(V). *nd = not detected, trace = < 5 µg/kg dry weight* [c] ***Lactarius volemus (56)*** *additionally contained Sugar 3 (Average = 46.72, RPD = 9.35), UK1 (Average = 234.43, RPD = 1.33), and a second unknown arsenic compound visible using anion exchange UK2: 5 mins (Average = 85.51, RPD = 5.86).* [d] ***Lactarius volemus (57)*** *additionally contained Sugar 1 (Average = 92.86, RPD = 9.56) and UK1 (Average = 32.30, RPD = 1.30).* [e] ***Paxillus involutus*** *additionally contained UK1 (Average = 1308.75, RPD = 6.24) and UK2 (Average = 133.16, RPD = 11.42).* [f] ***Gomphidius oregonensis*** *additionally contained UK1 (Average = 32.30, RPD = 1.30).* ON = Ontario, YK = Yellowknife, BC = British Columbia

Table A-3 Continued

#	Species	Source		Total As (mg/kg)	Concentration (µg/kg DM)							
					As_i	DMA	MMA	AB	TMAO	AC	TETRA	EE(%)	CR (%)
66	*Suillus tomentos*	YK Contaminated	Average	1.28	150	720	nd	nd	nd	nd	nd	91	80
			Difference	0.02	10	90						1	2
67	*Suillus granulatus*	YK Contaminated	Average	5.8	220	3600	nd	nd	nd	nd	nd	94	95
			Difference	0.5	30	200						1	9
68	*Suillus cravipes*	YK Background	Average	14	2100	330	nd	14600	nd	nd	nd	88	110
			Difference	2	90	10		1000				15	1
69	*Polyporus squamosus**	ON Contaminated	Average	8	nd	1800	790	nd	nd	nd	nd	38	87
			Difference	3		300	100					3	20
70	*Morchella esculenta*	ON Background	Average	0.08	42.5	nd	nd	nd	nd	nd	nd	69	81
			Difference	0.005	0.4							3	2
71	*Hypomyces lactifluorum*	Store Bought	Average	0.06	26	7	nd	nd	nd	nd	nd	73	75
			Difference	0.005	2	3						2	20
72	*Tremella fuciformis**	Store Bought	Average	0.05	17	22	nd	nd	nd	nd	nd	56	82
			Difference	0.002	1	7						4	6
73	*Auricularia polytricha**	Store Bought	Average	0.05	32	21	trace	nd	nd	nd	nd	48	130
			Difference	0.003	6	2						10	9

* indicates log growing species. iAs = As(III) + As(V). *nd = not detected, trace = < 5 µg/kg dry weight*
ON = Ontario

Table A-4: Average proportion of arsenic compounds in MeOH/H2O extracts.

#	Family	Species	Fruiting Body Morphology	Source	Proportion (%)							Major Compound
					iAs	DMA	MMA	AB	TMAO	AC	TETRA	
1	Lycoperdaceae	Lycoperdon pyriforme	Puffball	ON Background	4	30	nd	66	nd	nd	nd	AB
2	Lycoperdaceae	Lycoperdon pyriforme *	Puffball	ON Background	24	76	nd	nd	nd	nd	nd	DMA
3	Lycoperdaceae	Lycoperdon perlatum	Puffball	ON Background	4	31	nd	65	nd	nd	nd	AB
4	Lycoperdaceae	Lycoperdon perlatum	Puffball	ON Background	nd	nd	nd	100	nd	nd	nd	AB
5	Lycoperdaceae	Lycoperdon perlatum	Puffball	ON Contaminated	nd	43	nd	57	nd	nd	nd	AB
6	Lycoperdaceae	Lycoperdon perlatum	Puffball	ON Contaminated	nd	3	nd	97	nd	nd	nd	AB
7	Lycoperdaceae	Lycoperdon perlatum	Puffball	YK Background	12	3	1	85	nd	nd	nd	AB
8	Lycoperdaceae	Lycoperdon perlatum	Puffball	YK Contaminated	3	20	nd	75	2	nd	nd	AB
9	Lycoperdaceae	Lycoperdon perlatum	Puffball	YK Contaminated	10	2	nd	88	nd	1	nd	AB
10	Lycoperdaceae	Calvatia gigantea	Puffball	ON Background	trace	16	nd	84	nd	nd	nd	AB
11	Lycoperdaceae	Calvatia gigantea	Puffball	ON Background	trace	trace	trace	100	nd	nd	nd	AB
12	Lycoperdaceae	Calvatia gigantea	Puffball	ON Background	nd	7	nd	93	nd	nd	nd	AB
13	Lycoperdaceae	Calvatia gigantea	Puffball	ON Background	2	10	nd	88	nd	nd	nd	AB
14	Lycoperdaceae	Calvatia gigantea	Puffball	ON Contaminated	nd	nd	nd	100	nd	nd	nd	AB
15	Agaricaceae	Agaricus bisporus	Gilled	Store Bought	24	31	nd	40	6	nd	nd	AB
16	Agaricaceae	Agaricus brunnescens	Gilled	Store Bought	30	27	nd	42	nd	nd	nd	AB
17	Agaricaceae	Agaricus brunnescens	Gilled	Store Bought	35	33	nd	33	nd	nd	nd	iAs/ DMA/AB
18	Agaricaceae	Lepiota rachodes	Gilled	ON Background	11	14	4	71	nd	nd	nd	AB
19	Agaricaceae	Lepiota rachodes	Gilled	ON Background	3	6	nd	91	nd	nd	nd	AB
20	Agaricaceae	Leucoagaricus naucinus	Gilled	ON Background	nd	nd	nd	100	nd	nd	nd	AB
21	Agaricaceae	Leucoagaricus naucinus	Gilled	ON Contaminated	6	42	5	37	nd	nd	10	DMA
22	Agaricaceae	Agaricus campestris	Gilled	ON Background	27	6	nd	67	nd	nd	nd	AB
23	Agaricaceae	Agaricus campestris	Gilled	YK Contaminated	12	4	1	78	1	1	nd	AB
24	Agaricaceae	Agaricus campestris	Gilled	YK Contaminated	30	trace	nd	70	nd	nd	nd	AB
25	Agaricaceae	Coprinus comatus	Gilled	ON Background	nd	100	nd	trace	nd	nd	nd	DMA
26	Agaricaceae	Coprinus comatus	Gilled	ON Background	29	37	nd	33	nd	nd	nd	DMA
27	Agaricaceae	Coprinus comatus	Gilled	YK Contaminated	4	8	nd	89	nd	nd	nd	AB
28	Agaricaceae	Coprinus comatus	Gilled	YK Contaminated	46	2	nd	52	nd	nd	nd	AB
29	Amanitaceae	Amanita flavoconia	Gilled	ON Background	41	43	nd	16	nd	nd	nd	DMA
30	Amanitaceae	Amanita flavoconia	Gilled	ON Background	47	33	9	11	nd	nd	nd	iAs
31	Amanitaceae	Amanita flavoconia	Gilled	ON Background	17	36	4	37	nd	nd	6	AB

* Indicated a log or tree growing species. [a]Indicates species was collected at an earlier growth stage. Nd = not detected, trace = < 5 µg/kg dry weight

Table A-4 Continued

#	Family	Species	Fruiting Body Morphology	Source	Proportion (%)							Major Compound
					iAs	DMA	MMA	AB	TMAO	AC	TETRA	
32	Amanitaceae	Amanita flavoconia	Gilled	ON Background	63	30	7	nd	nd	nd	nd	iAs
33	Amanitaceae	Amanita prophyria	Gilled	ON Contaminated	18	32	2	48	nd	nd	nd	AB
34	Amanitaceae	Amanita bisporigera	Gilled	ON Contaminated	25	63	1	nd	3	7	nd	DMA
35	Entolomataceae	Entoloma sinuatum	Gilled	ON Background	15	58	nd	27	nd	nd	nd	DMA
36	Entolomataceae	Entoloma sinuatum	Gilled	ON Contaminated	47	44	nd	8	nd	nd	nd	iAs
37	Hymenogastraceae	Hebeloma syriense	Gilled	BC	20	31	nd	49	nd	nd	nd	AB
38	Hymenogastraceae	Hebeloma alpinum	Gilled	YK Contaminated	10	81	3	6	nd	nd	nd	DMA
39	Hymenogastraceae	Hebeloma velutipes	Gilled	YK Contaminated	1	12	nd	54	20	2	trace	AB
40	Pluteaceae	Volvariella volvacea	Gilled	Store Bought	17	79	4	nd	nd	nd	nd	DMA
41	Physalacriaceae	Flammulina velutipes*	Gilled	Store Bought	86	14	trace	nd	nd	nd	nd	iAs
42	Marasmiaceae	Lentinula edodes*	Gilled Ascending	Store Bought	67	33	trace	nd	nd	nd	nd	iAs
43	Pleurotaceae	Pleurotus ostreatus*	Gilled	Store Bought	trace	79	10	11	nd	nd	nd	iAs
44	Psathyrellaceae	Psathyrella candolleana	Gilled	ON Contaminated	36	41	nd	19	nd	4	nd	DMA
45	Inocybaceae	Tubaria furfuracea	Gilled	BC	26	8	nd	66	nd	nd	nd	AB
46	Psathyrellaceae	Coprinus atramentarius	Gilled	YK Contaminated	14	38	nd	4	40	trace	2	AB
47	Lyophyllaceae	Lyophyllum decastes	Gilled	YK Contaminated	25	1	nd	74	nd	nd	nd	AB
48	Cantharellaceae	Craterellus cornucopioides	Gilled Ascending	Store Bought	45	55	nd	nd	nd	nd	nd	DMA
49	Cantharellaceae	Cantharellus cibarius	Gilled Ascending	Store Bought	92	5	trace	1	1	nd	2	iAs
50	Cantharellaceae	Cantharellus cibarius	Gilled Ascending	Store Bought	84	16	nd	nd	nd	nd	nd	iAs
51	Cantharellaceae	Cantharellus xanthopus	Gilled Ascending	ON Contaminated	58	10	8	23	nd	nd	nd	iAs
52	Russulaceae	Russula emetica	Gilled	BC	65	35	nd	nd	nd	nd	nd	iAs
53	Russulaceae	Russula silvicola	Gilled	YK Background	46	54	nd	nd	trace	nd	nd	DMA
54	Russulaceae	Lactarius deliciosus	Gilled	ON Background	trace	trace	trace	100	nd	nd	trace	AB
55	Russulaceae	Lactarius deliciosus	Gilled	BC	32	18	nd	41	nd	9	nd	AB
56	Russulaceae	Lactarius volemus	Gilled	YK Contaminated	17	2	3	26	nd	trace	22	AB
57	Russulaceae	Lactarius volemus	Gillled	YK Background	69	8	trace	13	7	nd	nd	iAs
58	Hericiaceae	Hericium coralloides*	Toothed	ON Background	72	28	nd	nd	nd	nd	nd	iAs

* Indicated a log or tree growing species. [a]Indicates species was collected at an earlier growth stage. Nd = not detected, trace = < 5 µg/kg dry weight

Table A-4 Continued

#	Family	Species	Fruiting Body Morphology	Source	Proportion (%)							Major Compound
					iAs	DMA	MMA	AB	TMAO	AC	TETRA	
59	Paxillaceae	Paxillus involutus	Gilled	YK Contaminated	19	6	1	27	nd	trace	17	AB
60	Gomphidiaceae	Gomphidius oregonensis	Gilled	YK Background	3	82	nd	13	nd	nd	nd	DMA
61	Boletaceae	Boletus edulis	Pored	Store Bought	71	12	trace	nd	4	5	8	iAs
62	Boletaceae	Boletinellus meruliodes	Pored	ON Contaminated	27	26	2	45	nd	nd	nd	AB
63	Suillaceae	Suillus americanus	Pored	ON Background	trace	100	nd	nd	nd	nd	nd	DMA
64	Suillaceae	Suillus granulatus	Pored	YK Contaminated	nd	98	nd	1	1	trace	trace	DMA
65	Suillaceae	Suillus grevillei	Pored	YK Contaminated	1	71	nd	20	4	nd	nd	DMA
66	Suillaceae	Suillus tomentos	Pored	YK Contaminated	17	83	nd	nd	nd	nd	nd	DMA
67	Suillaceae	Suillus granulatus	Pored	YK Contaminated	6	94	nd	nd	nd	nd	nd	DMA
68	Suillaceae	Suillus cravipes	Pored	YK Background	13	2	nd	85	nd	nd	nd	AB
69	Polyporaceae	Polyporus squamosus*	Polypore	ON Contaminated	nd	69	31	nd	nd	nd	nd	DMA
70	Morchellaceae	Morchella esculenta	Morel	ON Background	100	nd	nd	nd	nd	nd	nd	iAs
71	Hypocreaceae	Hypomyces lactifluorum	Parasitic	Store Bought	80	20	nd	nd	nd	nd	nd	iAs
72	Tremellaceae	Tremella fuciformis*	Sponge	Store Bought	44	56	nd	nd	nd	nd	nd	DMA
73	Auriculariaceae	Auricularia polytricha*	Polypore	Store Bought	61	39	trace	nd	nd	nd	nd	iAs

* Indicated a log or tree growing species. [a]Indicates species was collected at an earlier growth stage. Nd = not detected, trace = < 5 µg/kg dry weight

Table A-5: Average proportion of arsenic compounds in MeOH/H2O extracts for the same genus or species collected from multiple sampling sources. Average concentration and standard deviation (or differences for genus or species with 2 samples) for genus and species are provided.

Genus	N	Fruiting Body Morphology	Average Proportion (%)							Major Compound
			As_i	DMA	MMA	AB	TMAO	AC	TETRA	
Lycoperdon	8	Puffball	7 ± 4	19 ± 16	0.8	79 ± 16	2	1		AB
Agaricus	6	Gilled	26 ± 8	17 ± 15	0.7	55 ± 19	5.3 ± 0.3	0.5		AB
Coprinus	5	Gilled	23 ± 19	21 ± 19		45 ± 35	41	2		AB
Amanita	6	Gilled	35 ± 18	39 ± 12	5 ± 3	28 ± 17	3	7	6	DMA
Hebeloma	3	Gilled	10 ± 10	42 ± 36	3	36 ± 26	20	2		DMA
Cantharellus	3	Ascending gilled	71 ± 18	13 ± 4	8	23				iAs
Russula	2	Gilled	46.2 ± 0.4	53.8 ± 0.3						DMA
Lactarius	4	Gilled	39 ± 27	9 ± 8	3	26 ± 14	7	9	22	iAs
Suillus	6	Pored	9 ± 7	62 ± 41		53 ± 46	4			DMA
Species										
Lycoperdon perlatum	7	Puffball	7 ± 5	16 ± 17	0.7	80 ± 15	2	0.9		AB
Calvatia gigantea	5	Puffball	2	11 ± 5		93 ± 7				AB
Leucoagaricus naucinus	2	Gilled	6	42	5	69 ± 63			10	AB
Agaricus campestris	3	Gilled	23 ± 10	4 ± 3	0.7	72 ± 5	5	0.5		AB
Coprinus comatus	4	Gilled	26 ± 21	37 ± 45		58 ± 28				AB
Lactarius deliciosus	2	Gilled	31	18		70 ± 59		9		AB
Lactarius volemus	2	Gilled	43 ± 52	5 ± 6	3	19 ± 14	7		22	iAs
Entoloma sinuatum	2	Gilled	31 ± 33	51 ± 14		18 ± 19				DMA

Table A-6: Average concentrations of additional compounds identified by anion exchange HPLC-ICP-MS.Table A6: Average concentrations of additional compounds identified by anion exchange HPLC-ICP-MS.

Sample	Source	Concentration µg/kg DM			
		Sugar 1	Sugar 3	Unknown 1 2.3 mins	Unknown 2 5.0 mins
39 *Hebeloma velutipes*	YK Contaminated		460 ± 6	210 ± 30	
46 *Coprinus atramentarius*	YK Contaminated			87 ± 5	
56 *Lactarius volemus*	YK Contaminated		47 ± 4	230 ± 3	86 ± 5
57 *Lactarius volemus*	YK Contaminated	93 ± 9		32.3 ± 0.3	
59 *Paxillus involutus*	YK Contaminated			1300 ± 80	130 ± 20
60 *Gomphidius oregonensis*	YK Contaminated			32.3 ± 0.3	
65 *Suillus grevillei*	YK Contaminated			310 ± 30	

Table A-7: X-ray absorption near-edge structure fitting results for selected fungi species

#	Species	Source	Sample Preparation	EE (%)	Proportions Determined by XAS (%)						χ^2	Unextracted Compounds
					As(III)	As(V)	As(V)-Glycerol	DMA/TM AO	AB	$As(Glu)_3$		
23	*Agaricus campestris*	YK Cont	Dried	94	11	2	12		63	12	0.003	As(III)/(V)
			Frozen		21				68	11	0.003	As(III)/(V)
28	*Coprinus comatus*	YK Cont	Dried	58	16		2		82		0.003	AB
			Frozen		10	5			85		0.002	AB
47	*Lyophyllum decastes*	YK Cont	Dried	71	15		16		69		0.002	
			Frozen		21			2	77		0.003	

Data were fit with frozen As(V) (white line energy 11875.3 eV); frozen As(III) (white line energy 11871.7 eV); As(V)-glycerol (white line energy 11876.5 eV); liquid DMA(V) (white line energy 11873.3 eV); TMAO (white line energy 11873.3 eV); frozen AB (white line energy 11872.6 eV); and $As(Glu)_3$ (white line energy 11870.0 eV). Cont = Contaminated.

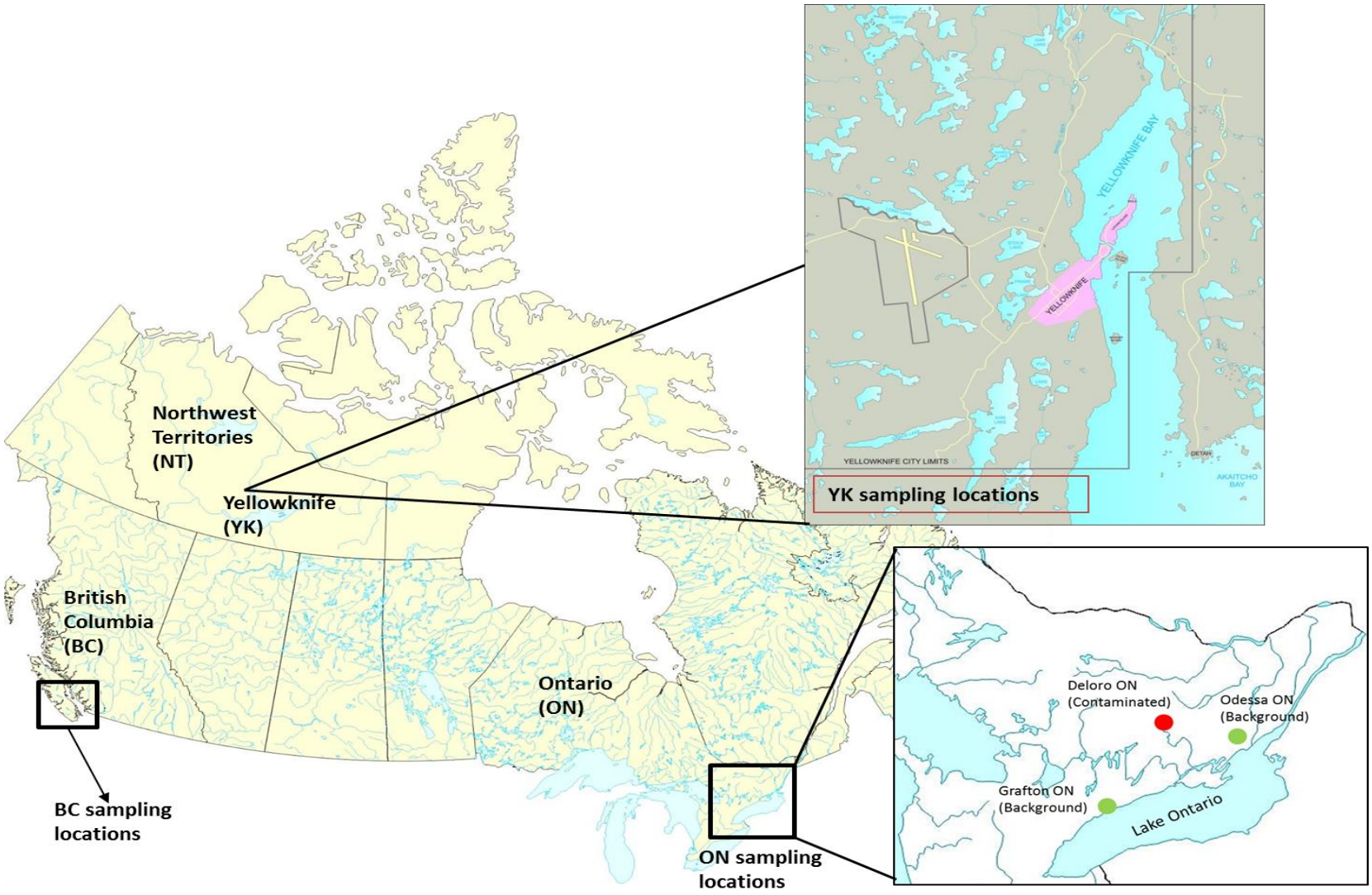
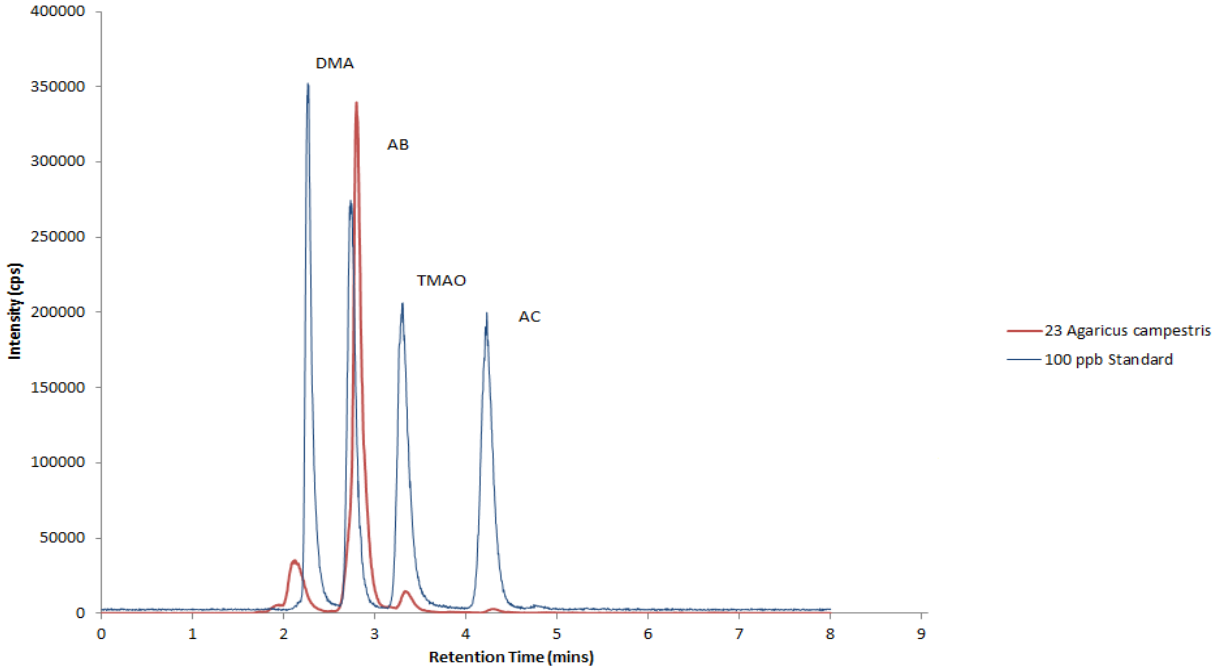


Figure A.1: A map of Canada with sampling locations indicated by the black boxes for British Columbia (BC) and Ontario ON sampling sources and red boxes for Yellowknife (YK) sampling sources.

A)



B)

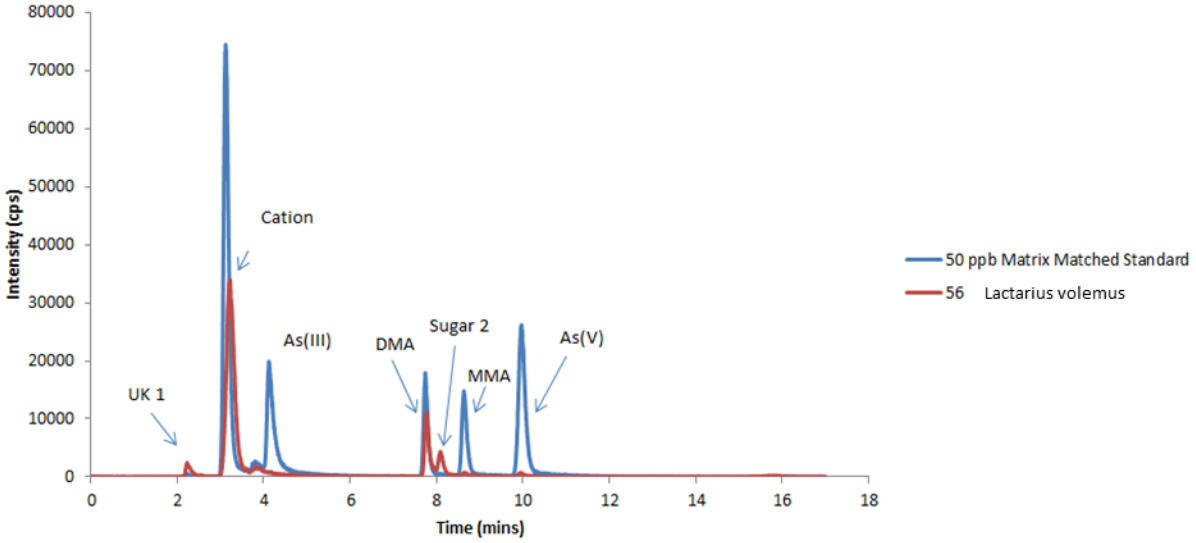


Figure A.2: Chromatographs for two mushroom MeOH/H_2O extracts from one replicate. A) Cation-exchange chromatograph for Agaricus campestris collected from a YK Contaminated location (ID 23). The blue solid line represents the 100 ppb cation standard and the red dashed line represents the extract. B) Anion-exchange chromatograph for Lactarius volemus (ID 56). The blue solid line represents the 50 ppb matrix matched anion standard and the red line represents the extract. UK 1 (2.3 mins), UK 2 (5mins) and Sugar 3 were additionally found in this sample**.**

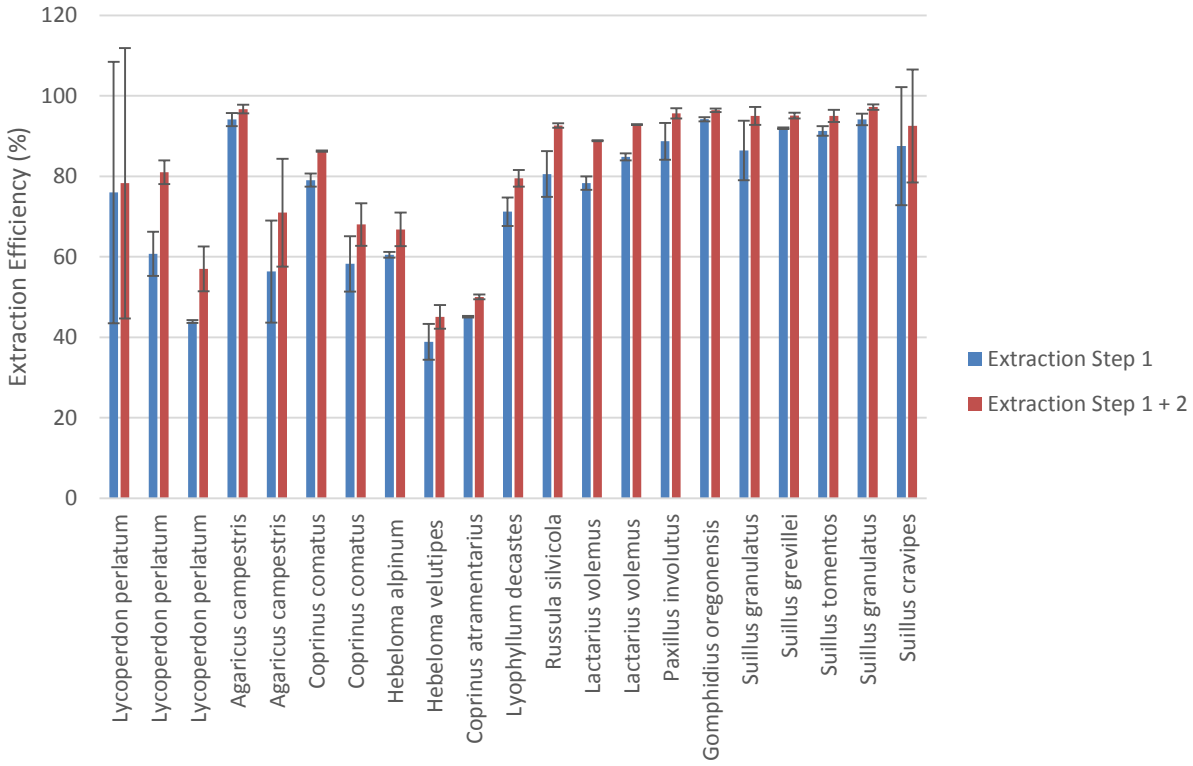


Figure A.3: Average extraction efficiencies for Extraction Step 1 and 2 for Yellowknife samples. The errors bars represent RPD.

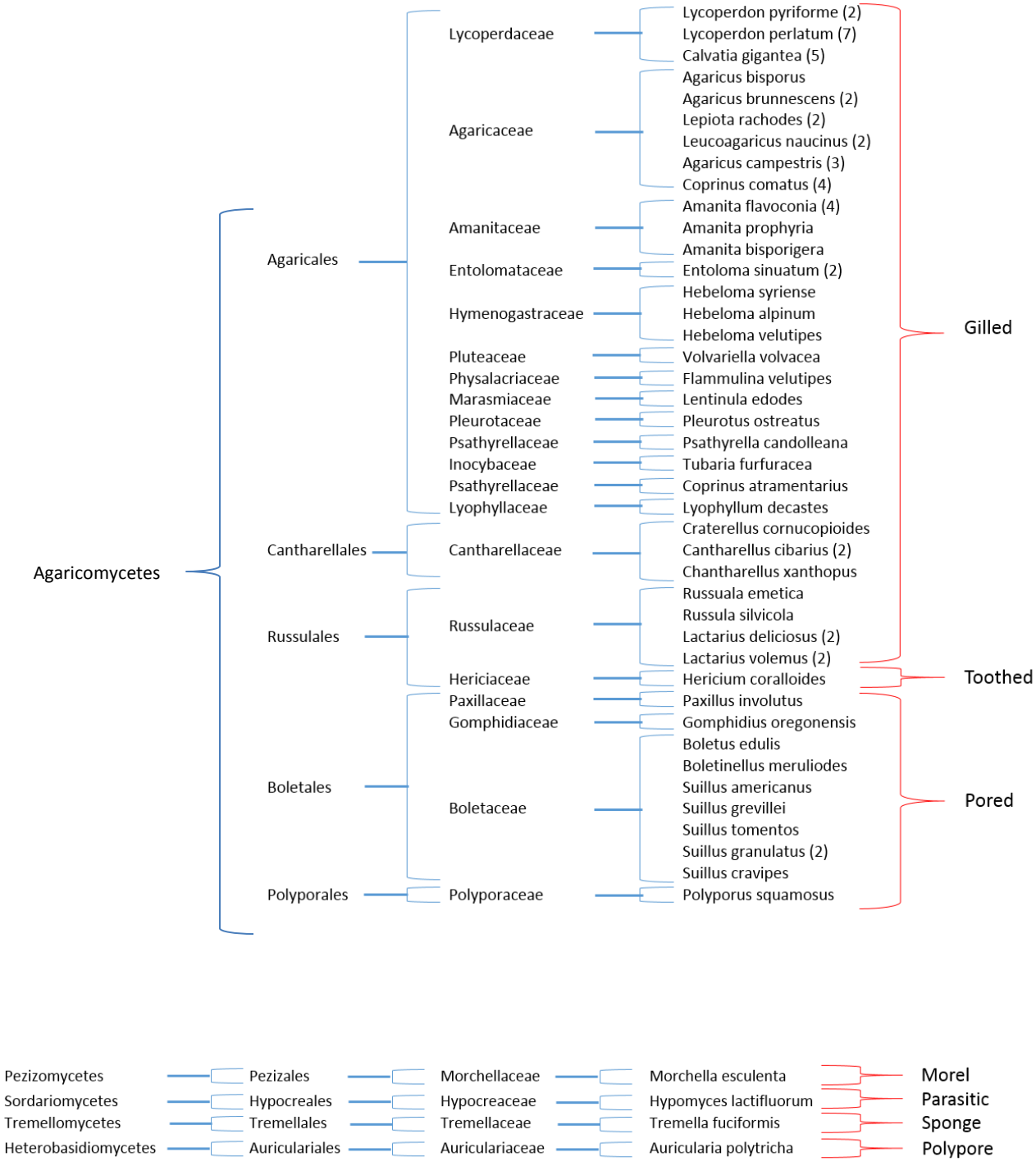


Figure A.4: Phylogenetic classifications of species collected. Class, order, family and species are shown. The fruiting body morphologies of each species are shown on the right, indicated by red brackets. For species collected from multiple sites the number of sites they were found in is indicated in brackets.

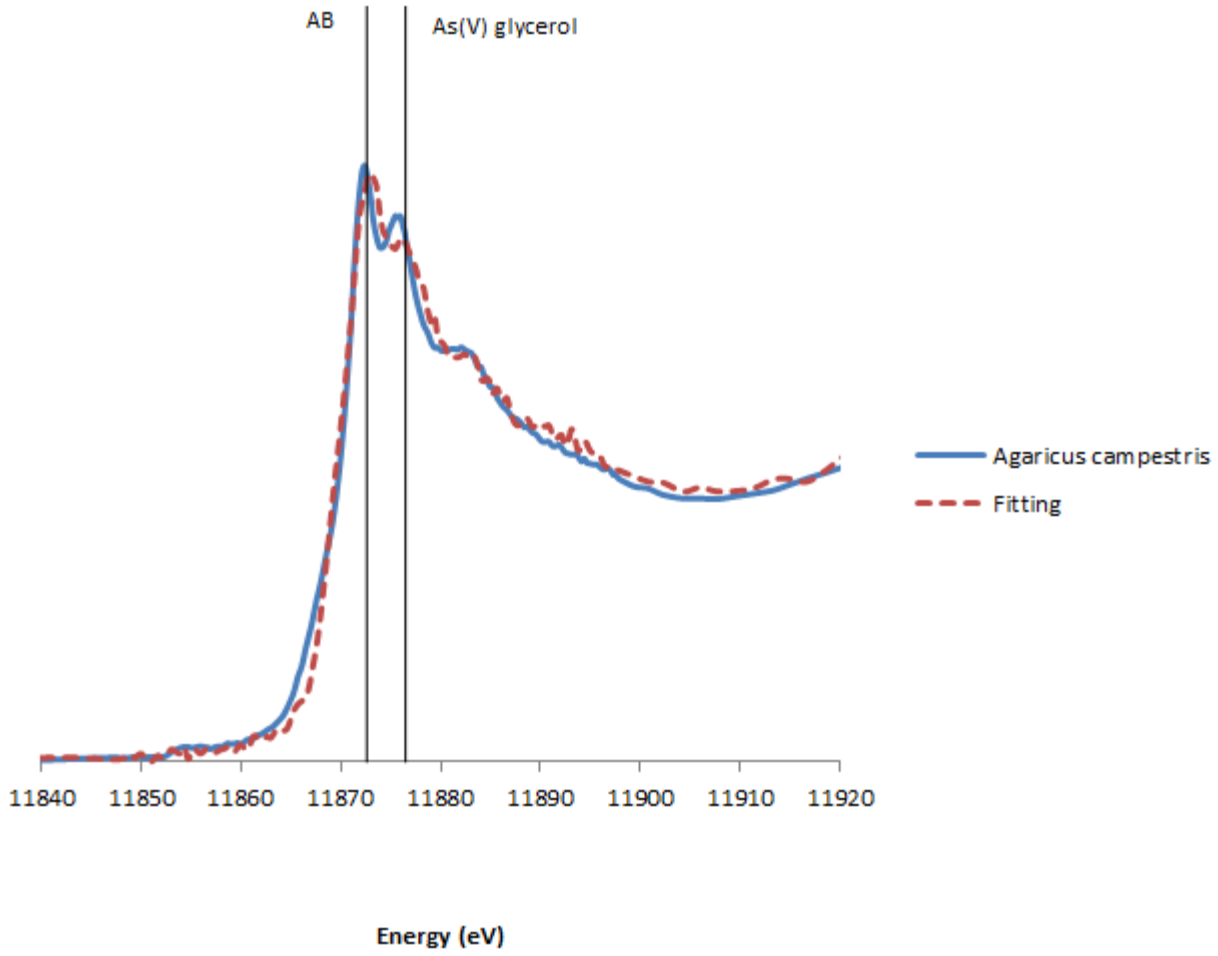


Figure A.5: XANES spectra (blue solid line) for Agaricus campestris (#23) collected from a YK contaminated location. The red dashed line represents the linear combination fit. The black vertical lines represent the white line energy for AB (11872.6 eV) and As(V)-glycerol (11876.5 eV).

Appendix B

B. Additional information for Chapter 4: Uptake and transformation of arsenic during the vegetative life stage of terrestrial fungi

B.1 Chemicals and Reagents

The solutions used to amend media were prepared from arsenic stock standards: arsenate, As(V) (1000 ppm from Inorganic Ventures); DMA(V), cacodylic acid (>99% from Fluka); MMA(V), monosodium acid methane arsonate sesquihydrate (99.0% purity Chemservice); TMAO, trimethlyarsine oxide (Wako); AB, arsenobetaine (Wako and Argus Chemicals). DMAA, dimethylarsinoylacetic acid was kindly donated by Kevin Francesconi. *A. bisporu*s ATCC# 10892 and *S. crispa* ATCC# 34491 were both grown in Potato dextrose broth (Difco, Fisher Scientific), and *S. luteus* ATCC# MYA-4759 was grown in yeast mold broth (Difco, Fisher Scientific).

For the total acid digestions trace metal grade nitric acid (~ 70%, Fisher Scientific) was used. For arsenic extractions double deionized water (DDW), prepared in-house to a minimum resistance of 18 MΩ·cm (E-pure Barnstead), was used with HPLC grade methanol (Fisher Scientific) and 2% (v/v) Optima grade nitric acid (Fisher Scientific). Total matrix spikes and calibration curves were prepared from a stock solution of 1000 µg/mL total arsenic (arsenic pentoxide, Inorganic Ventures).

For HPLC-ICP-MS analysis the anion exchange mobile phase was prepared with ammonium nitrate (99.999% purity, Aldrich) and the cation exchange mobile phase was prepared with pyridine (99% purity, Sigma). Different sources of standards were used for the calibration curves than for quality control calibration check solutions and matrix spikes, when available. For anion exchange this was possible for As(III), arsenite (90% from Fluka and 99.999% purity from Aldrich); As(V), arsenate (1000 ppm from Aldrich and 1000 ppm from Inorganic Ventures); and DMA (V), cacodylic acid (>99% from Fluka and 99% purity from City Chemical). For cation exchange this was possible for DMA (V); AB, arsenobetaine (Wako and Argus Chemicals); TMAO, trimethlyarsine oxide (Wako and Argus Chemicals). Only one source was available for AC, arsenocholine bromide (Argus Chemicals) for cation exchange and for MMA(V), monosodium acid methane arsonate sesquihydrate (99.0% purity Chemservice).

For total arsenic digestions the certified reference material (CRM) BCR®-482, Lichen was obtained from European Commission Institute for Reference Materials and Measurements. For arsenic extractions the

CRM BCR®- 627, Tuna Fish was obtained from the same source and DORM-2 and DORM-3, Dogfish Muscle was obtained from National Research Council Canada.

B.2 Arsenic Species Extraction

Extractions were carried out using methods described by Nearing et al., 2014 (submitted). The entire dried mycelium sample for each replicate (0.01 – 0.3 g) and 5 mL of 50% (by volume) aqueous methanol in a 15 mL Fisherbrand® disposable polypropylene centrifuge tube. The mixtures were vortexed and shaken end-over-end for 12 hours at room temperature, then placed in an ultrasonic bath for 20 min before being centrifuged at 3500 rpm for 30 min at 15 °C. The supernatant was decanted and the ultrasonication and centrifugation steps were repeated 2 more times, collecting all the supernatants together. Methanol was removed from the supernatant by evaporation at 60°C (Rapidvap, Labconco or Syncor®, Buchi) to a final volume of 5 mL. Extracts were syringe filtered using disposable 0.45 µm filters as above and kept frozen until analysis.

A. bisporus mycelium from Experiment B was found to have generally high extraction efficiencies with aqueous methanol water extractions. It was unknown whether the mycelium of different fungi species would have the same high extraction efficiency with only aqueous methanol water extractions, and therefore an additional extraction step was carried out for Experiment A in order to maximize the arsenic extraction efficiency. For Experiment A samples (*S. luteus, S. crispa and A. bisporus* exposed 60 days to As(V), DMAA and AB) the residues from the aqueous methanol extraction (Step 1) were sequentially extracted with 2% HNO_3 (Step 2). Step 2 consisted of heating the residues with 5 mL of 2 % HNO_3 for 2 hours at 70°C, placing in an ultrasonic bath for 30 min, and centrifuging at 3500 rpm for 30 min at 15 °C. The supernatant was decanted and syringe filtered and stored as before.

B.3 Total Arsenic Digestion

The total arsenic concentration within the mycelium was determined by summing the total arsenic in the extracts (for Experiment A samples: Step 1 + Step 2) and the total arsenic in the final residues from the extraction. Extraction residues were digested with 5 mL of 70% HNO_3 at 120°C until the sample was reduced to 1 -2 mL. Samples were then diluted to a final volume of 5 mL with distilled deionised water and syringe filtered as before.

B.4 X-ray Absorption Spectroscopy (XAS)

X-ray absorption near edge structure (XANES) analysis was performed at the Advanced Photon Source (APS) Pacific Northwest Consortium/X-ray Science Division (PNC/XSD) on the bending magnet (BM) beam line, Sector 20. XANES spectra of the arsenic Kα-edge (11686 eV) were recorded in fluorescence mode using a solid state Ge detector (Canberra model GL0055PS) while monitoring incident and

transmitted intensities in N_2-filled transmission ionization chambers. Dried and homogenized mycelium samples were placed in a sample holder, held between two layers of Kapton^{TM} tape and kept at 100 K using a liquid N_2 cryostat (Model 22 CTI Cryodyne Refrigerator System, Janis). A total of 8 scans were collected with a 0.5 eV step size over the edge region and averaged prior to background removal and normalization to edge jump. The Si(111) double crystal monochromator was calibrated using the first inflection point of the gold LIII absorption edge (11919.7 eV). A reference gold foil was measured simultaneously with all samples. Additional experimental setup information can be found in Smith et al., 2005 [92]. XANES spectra of the arsenic K-edge (11868 eV) were fit within -20 to +30 eV from the arsenic E_O using Athena software. Solid As(III), As(V) and As(V) glycerol standards [116], liquid DMA and arsenic glutathione ($As(Glu)_3$) [92] previously measured by our group were used for fittings.

Table B-1: Quality control and quality assurance control for total arsenic and arsenic speciation analysis.

Medium	Measurement	Instrument	N	Range	Average
Method Blanks	Total [As] (Extracts and Residues)	ICP-MS	7	< LOD_a	< LOD_a
CRM BCR 482	% Recovery, total [As] (Residues)	ICP-MS	7	78 - 94	87
CRM BCR 627	% Recovery, total [As] (Experiment A)	ICP-MS	3	83, 96, 99	92
Matrix Spikes	% Recovery, total [As] (Extracts, Residues, Media)	ICP-MS	18	76 - 118	93
Method Blanks	Arsenic species concentrations	HPLC-ICP-MS	7	< LOD_b	< LOD_b
CRM BCR 627	% Recovery, [DMA] (Experiment A)	Anion HPLC-ICP-MS	3	99, 119, 130	116
CRM BCR 627	% Recovery, [AB] (Experiment A)	Cation HPLC-ICP-MS	3	82,83, 85	83
DORM 2	% Recovery, [AB] (Experiment C)	Cation HPLC-ICP-MS	2	77, 78	78
DORM 3 [c]	% Recovery, [DMA] (Experiment B)	Anion HPLC-ICP-MS	2	78, 90	84
DORM 3 [c]	% Recovery, [AB] (Experiment B)	Cation HPLC-ICP-MS	2	102, 113	108
Matrix Spike	% Recovery, [As(III)]	Anion HPLC-ICP-MS	7	74 - 130	103
Matrix Spike	% Recovery, [DMA]	Anion HPLC-ICP-MS	7	84 - 99	93
Matrix Spike	% Recovery, [MMA]	Anion HPLC-ICP-MS	7	83 - 116	96
Matrix Spike	% Recovery, [As(V)]	Anion HPLC-ICP-MS	7	86 - 133	114
Matrix Spike	% Recovery, [DMA]	Cation HPLC-ICP-MS	7	79 - 130	104
Matrix Spike	% Recovery, [AB]	Cation HPLC-ICP-MS	7	78 - 96	86
Matrix Spike	% Recovery, [TMAO]	Cation HPLC-ICP-MS	7	89 - 112	94
Matrix Spike	% Recovery, [AC]	Cation HPLC-ICP-MS	7	72 - 133	100

[a] ICP-MS Limit of Detection (LOD) for total arsenic (media/mycelium): Experiment A (1.04 µg/L / 8.5 µg/kg WM); Experiment B (1.06 µg/L / 8.13 µg/kg WM); Experiment C (1.3 µg/L / 0.38 µg/kg WM)

[b] Arsenic species by HPLC-ICP-MS LOD: 3 µg/kg; Limit of quantification (LOQ): 5 µg/kg

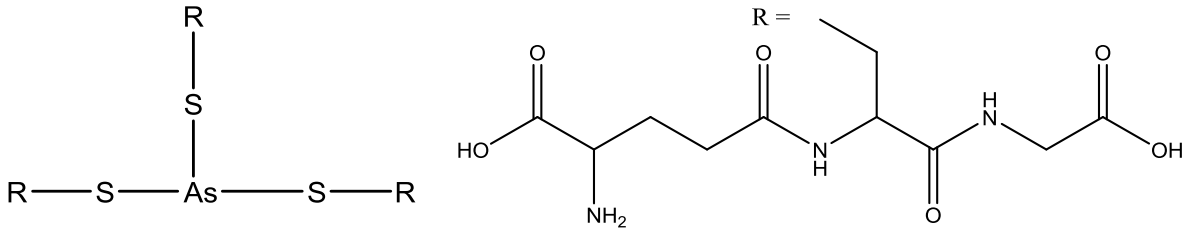
[c] Recoveries calculated with respect to reference values from a round robin experiment (Zoltan Mester, personal communication). AB value = 4.12 mg/kg, range 3.86 – 4.38 mg/kg, DMA value = 0.447 mg/kg, range 0.392 – 0.563 mg/kg (ranges calculated with 95% CIs).

Table B-2: Average total arsenic in media and mycelia, and concentration factors.

Fungus Species	Compound Added	Total As in Medium (ug/L)	Total As in Mycelium (ug/kg WM)	Concentration Factor
Agaricus bisporus	As(V)[A]	78 ± 4	45 ± 40	2.0 ± 0.4
	As(V) 60 days[B]	67 ± 3	180 ± 10	2.6 ± 0.2
	As(V) 90 days[B]	82 ± 20	450 ± 10	5 ± 1
	As(V) 120 days[B]	99 ± 10	450 ± 80	5 ± 1
	DMA 30 days[C]	140 ± 7	140.2 ± 0.3	1.0 ± 0.3
	DMA 60 days[B]	94 ± 10	300 ± 9	3.2 ± 0.3
	DMA 90 days[B]	120 ± 20	500 ± 4	4.1 ± 0.8
	DMA 120 days[B]	160 ± 30	800 ± 20	5 ± 1
	MMA[C]	150 ± 30	151.3 ± 0.2	1.0 ± 0.2
	TMAO[C]	130 ± 50	164.2 ± 0.5	1.2 ± 0.5
	DMAA[A]	42 ± 1	92 ± 20	5 ± 1
	AB[A]	42 ± 9	330 ± 20	11 ± 2
Suillus luteus	As(V)	51 ± 20	130 ± 50	4 ± 2
	DMAA	30 ± 7	200 ± 100	10 ± 5
	AB	30 ± 4	22 ± 20	3 ± 1
Sparassis crispa	As(V)	70 ± 10	240 ± 20	3.6 ± 0.2
	DMAA	42 ± 1	120 ± 30	4 ± 1
	AB	42 ± 9	540 ± 50	14 ± 3

[A B C] Indicates *A. bisporus* experiments, *S. luteus* and *S. crispa* were subjected only to Experiment A.
Concentration factor(CF) = Total As in mycelium / Total As in medium. CFs were calculated by dividing each mycelium replicate in the treatment group by each uninoculated positive control media and the average of the CFs are reported here.

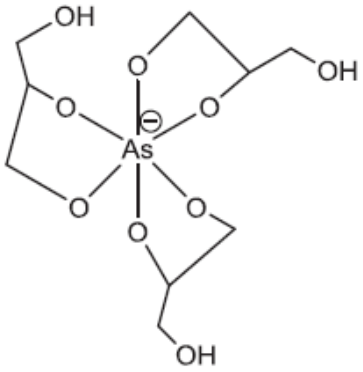
A)



Arsenic glutathione

As(Glu)$_3$

B)



As(V)-O glycerol

Figure B.1: Chemical structures for additional compounds determined with X-ray absorption spectroscopy (XAS) analysis. A) Arsenic glutathione. B) As(V)-O glycerol.

Appendix C

C. Additional information for Chapter 5: Uptake and transformation of arsenic during the reproductive life stage of *Agaricus bisporus* and *A. campestris*

C.1 Chemicals and Reagents

Distilled deionized water (DDW) was prepared in-house to a minimum resistance of 18 MΩ·cm (E-pure Barnstead). For the total acid digestions trace metal grade nitric acid (~ 70%, Fisher Scientific) was used. For arsenic extractions DDW was used with HPLC grade methanol (Fisher Scientific) and 2% (v/v) Optima grade (Fisher Scientific). Total matrix spikes and calibration curves were prepared from a stock solution of 1000 µg/mL total arsenic (arsenic pentoxide, Inorganic Ventures).

For HPLC-ICP-MS analysis the anion exchange mobile phase was prepared with ammonium nitrate (99.999% purity, Aldrich) and the cation exchange mobile phase was prepared with pyridine (99% purity, Sigma).

Different sources of standards were used for the calibration curves than for quality control calibration check solutions and matrix spikes when available. For anion exchange this was possible for As(III), arsenite (90% from Fluka and 99.999% purity from Aldrich); As(V), arsenate (1000 ppm from Aldrich and 1000 ppm from Inorganic Ventures); and DMA (V), cacodylic acid (>99% from Fluka and 99% purity from City Chemical). For cation exchange this was possible for DMA (V); AB, arsenobetaine (Wako and Argus Chemicals); TMAO, trimethlyarsine oxide (Wako and Argus Chemicals). Only one source was available for AC, arsenocholine bromide (Argus Chemicals) for cation exchange and for MMA(V), monosodium acid methane arsonate sesquihydrate (99.0% purity Chemservice). TETRA, tetramethylarsonium iodide (Wako) was used for identification purposes by matching retention times, and the AC calibration curve was used for quantification of the compound when it was found in samples.

For total arsenic digestions the certified reference material (CRM) BCR®-482, Lichen was obtained from European Commission Institute for Reference Materials and Measurements. For arsenic extractions the CRM BCR®- 627, Tuna Fish was obtained from the same source.

C.2 Arsenic Species Extraction

Extractions were carried out for each collected sample using 0.5 g of dried sample per replicate and 10 mL of 50% (by volume) aqueous methanol in a 50 mL Fisherbrand® disposable polypropylene centrifuge tube. The mixtures were vortexed and shaken end-over-end for 12 hours at room temperature, then

placed in an ultrasonic bath for 20 min before being centrifuged at 3500 rpm for 30 min at 15 °C. The supernatant was decanted and the ultrasonication and centrifugation steps were repeated 2 more times, collecting all the supernatants together. Methanol was removed from the supernatant by evaporation at 60°C (Rapidvap, Labconco) to a final volume of 5 mL. Extracts were syringe filtered using disposable 0.45 µm filters (Millipore® polypropylene 25 mm diameter hydrophilic PVDF durapore membrane) into 15 mL Fisherbrand® disposable polypropylene centrifuge tubes and kept frozen until analysis.

The residues from the aqueous methanol extraction (Step 1) were sequentially extracted with 2% HNO_3 (Step 2). Step 2 consisted of heating the residues with 5 mL of 2 % HNO_3 for 2 hours at 70°C, placing in an ultrasonic bath for 30 min, and centrifuging at 3500 rpm for 30 min at 15 °C. The supernatant was decanted and syringe filtered and stored as before.

C.3 Total Arsenic Digestion

The total arsenic concentration within the mycelium was determined by summing the total arsenic in the extracts (for Experiment A samples: Step 1 + Step 2) and the total arsenic in the final residues from the extraction. Extraction residues were digested with 5 mL of 70% HNO_3 at 120°C until the sample was reduced to 1 -2 mL. Samples were then diluted to a final volume of 5 mL with distilled deionised water and syringe filtered as before.

C.4 Total arsenic analysis

The instrument used was an ICP-MS DRC II from PerkinElmer (PerkinElmer, MA). The detection limit of the instrument was determined to be 0.5 µg/L and mass interferences were monitored by measuring *m/z* 77 ($ArCl^+$). Instrumental tests included instrumental blanks and calibration checks which were run every 10 samples. Instrument blanks run every 10 samples were under the instrument detection limits for total arsenic analysis. Instrument quality control checks that included a 5 µg/L and 50 µg/L solution for total arsenic were run every 10 samples and found to be within an acceptable range (80 – 120 % recovery).

C.5 Arsenic speciation analysis using HPLC-ICP-MS

Briefly, anion exchange chromatography was performed using a Hamilton PRPX100 anion exchange column with a gradient mobile phase (A: 4 mM of ammonium nitrate (NH_4NO_3) and B: 60 mM NH_4NO_3, pH = 8.65). Anion exchange was used to identify: As(III), As(V), monomethylarsonic acid (MMA), dimethylarsinic acid (DMA), glycerol arsenosugar (Sugar 1) and sulfonate arsenosugar (Sugar 3. Cation exchange chromatography was performed with a Chrompack Ionosphere C cation exchange column with a mobile phase of 20nM pyridine, pH = 2.7. Cation exchange was used to identify: DMA, AB, trimethylarsine oxide (TMAO), arsenocholine (AC) and tetramethylarsonium ion (TETRA). All chromatographic speciation data were analyzed with Peak Fit Version 4.12. HPLC blanks and calibration

checks were run every 10 samples and considered acceptable (70 – 130 % recovery). All mushrooms were analyzed for arsenic speciation and all compounds that could be matched with a known standard were quantified using the calibration curve for that arsenic compound. Unknown compounds were quantified using the calibration curve for the closest eluting known compound. The limit of detection for each compound was determined to be 0.003 mg/kg, compounds 0.003 mg/kg DM to 0.005 mg/kg DM were considered as trace compounds and all compounds over 0.005 mg/kg DM were quantified.

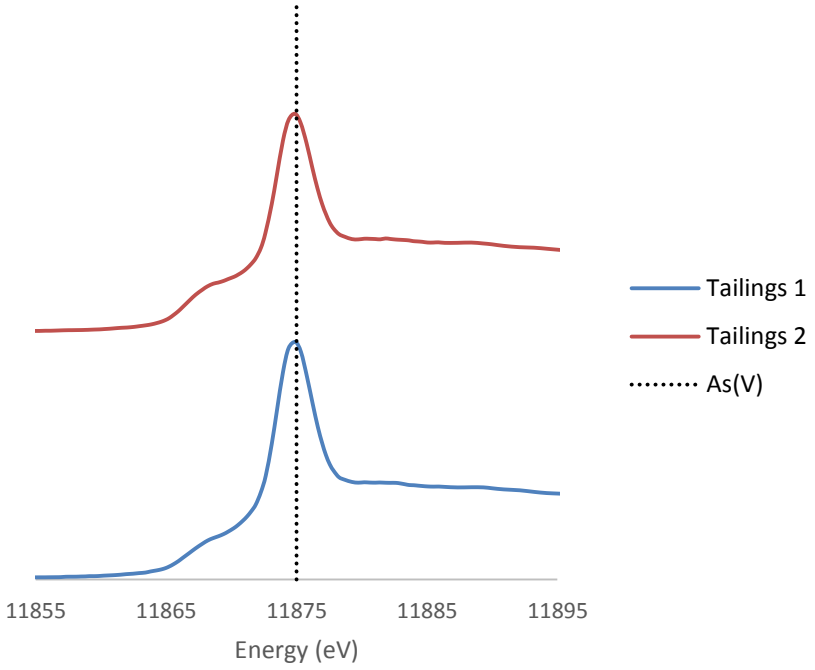


Figure C.1: XANES spectra for tailings samples collected from sampling location for *A. campestris*. The dashed vertical line indicates the white line energy for As(V)(11875.3 eV).

Table C-1: Quality control and quality assurance control for total arsenic and arsenic speciation analysis.

Medium	Measurement	Instrument	N	Range	Average
Method Blanks	Total Arsenic Concentration (Extracts and Residues)	ICP-MS	27	< LOD_a	< LOD_a
CRM BCR 482 Lichen	Percent Recovery, Total Arsenic Concentration (Residues)	ICP-MS	5	83 - 94	86
CRM DORM 3[c]	Percent Recovery, Total Arsenic Concentration (Extracts + Residues)	ICP-MS	3	70, 75, 76	74
CRM BCR 627 Tuna Fish	Percent Recovery, Total Arsenic Concentration (Extracts + Residues)	ICP-MS	5	76 - 102	84
Matrix Spikes	Percent Recovery, Total Arsenic Concentration	ICP-MS	26	60 - 122	92
Method Blanks	Arsenic Species Concentrations	HPLC-ICP-MS	8	< LOD_b	< LOD_b
CRM BCR 627 Tuna Fish	Percent Recovery, DMA Concentration	Anion HPLC-ICP-MS	5	80 - 117	99
CRM BCR 627 Tuna Fish	Percent Recovery, AB Concentration	Cation HPLC-ICP-MS	5	67 - 121	88
CRM DORM 3[c]	Percent Recovery, DMA Concentration	Anion HPLC-ICP-MS	3	73 - 103	86
CRM DORM 3[c]	Percent Recovery, AB Concentration	Cation HPLC-ICP-MS	3	76 - 105	86
Matrix Spike	Percent Recovery, As(III) Concentration	Anion HPLC-ICP-MS	12	88 - 125	110
Matrix Spike	Percent Recovery, DMA Concentration	Anion HPLC-ICP-MS	12	79 - 106	92
Matrix Spike	Percent Recovery, MMA Concentration	Anion HPLC-ICP-MS	12	81 - 115	94
Matrix Spike	Percent Recovery, As(V) Concentration	Anion HPLC-ICP-MS	12	79 - 113	94
Matrix Spike	Percent Recovery, DMA Concentration	Cation HPLC-ICP-MS	6	79 - 106	93
Matrix Spike	Percent Recovery, AB Concentration	Cation HPLC-ICP-MS	6	75 - 109	89
Matrix Spike	Percent Recovery, TMAO Concentration	Cation HPLC-ICP-MS	6	70 - 86	79
Matrix Spike	Percent Recovery, AC Concentration	Cation HPLC-ICP-MS	6	70 - 107	81
Column Recovery	Percent Recovery, CRM DORM 3	HPLC-ICP-MS	6	97 - 125	110
Column Recovery	Percent Recovery, CRM BCR 627 Tuna Fish	HPLC-ICP-MS	10	63 - 140	99

[a] ICP-MS Limit of Detection (LOD) for total arsenic: Commercial growth facility Step 1 Extracts (6 µg/L), Step 2 Extracts (4 µg/L), Step 3 Extracts (4 µg/L); Mushroom growing kit Step 1 Extracts (10 µg/L), Step 2 Extracts (2 µg/L), Step 3 Extracts (3 µg/L)

[b] Arsenic species by HPLC-ICP-MS LOD: 3 µg/kg; Limit of quantification (LOQ): 5 µg/kg

[c] Recoveries calculated with respect to reference values from a round robin experiment (Zoltan Mester, personal communication). AB value = 4.12 mg/kg, range 3.86 – 4.38 mg/kg, DMA value = 0.447 mg/kg, range 0.392 – 0.563 mg/kg (ranges calculated with 95% CIs).

Table C-2: Total arsenic, arsenic species concentration, extraction efficiency (EE) and column recovery (CR) for samples collected at a commercial growth facility for A. bisporus. Average concentration and relative standard deviation for triplicates are provided.

Sampling Time (days)	Sample Type		Total As (mg/kg DM)	Step 1 Extracts: Species Concentration (µg/kg) DM						Step 2 Extracts: Species Concentration (µg/kg) DM					Total EE (%)
				As(V)	DMA	TMAO	AB	EE (%)	CR (%)	As(III)	As (V)	DMA	EE (%)	CR (%)	
0	Compost	Average	1.71	150	150	340	nd	40	100	trace	650	trace	40	100	72.93
		RSD	0.08	7	7	50	--	4	20	--	100	--	3	20	0.04
	Casing	Average	0.53	nd	nd	nd	nd	30	--	nd	200	nd	30	120	62.8
		RSD	0.04	--	--	--	--	2	--	--	20	--	1	20	0.2
14	Compost + millet	Average	1.3	130	200	280	nd	30	140	trace	400	trace	30	100	66.4
		RSD	0.2	20	40	3	--	2	3	--	40	--	2	20	0.2
	Casing + CACing	Average	0.51	nd	nd	nd	nd	30	--	nd	150	nd	30	100	56.9
		RSD	0.04	--	--	--	--	1	--	--	10	--	5	10	0.5
37	Compost	Average	2.0	530	200	140	trace	40	100	nd	760	nd	30	110	78.7
		RSD	0.2	70	20	20	--	4	8	--	100	--	3	20	0.1
	Casing	Average	0.42	nd	nd	nd	nd	30	--	nd	110	nd	20	120	50.1
		RSD	0.03	--	--	--	--	3	--	--	10	--	3	20	0.7
	FB < 1 cm[w]	Average	0.16	40	30	nd	90	90	120	nd	trace	nd	--	--	88.7
		RSD	0.03	10	10	--	30	4	10	--	--	--	--	--	0.1
50	Compost[w]	Average	0.8	110	100	130	trace	40	100	120	260	nd	30	160	70
		RSD	0.1	30	6	2	--	3	20	10	10	--	5	50	1
	Casing[w]	Average	0.94	nd	nd	nd	nd	50	--	nd	280	trace	30	100	75.9
		RSD	0.08	--	--	--	--	3	--	--	8	--	1	10	0.2
	Mycelium/casing[w]	Average	0.4	20	10	nd	30	20	110	nd	79	nd	20	100	40
		RSD	0.2	5	3	--	2	6	10	--	4	--	6	20	3

nd = not detected < 3 µg/kg DM, trace = < 5 µg/kg DM

[w] Indicates samples collected from the white strain (button mushroom) harvest section

Total EE = Step 1 Extracts (Aqueous methanol extracts) + Step 2 Extracts (2% nitric extracts)

Table C-2 Continued

Sampling Time (days)	Sample Type		Total As (mg/kg DM)	Step 1 Extracts: Species Concentration (µg/kg) DM						Step 2 Extracts: Species Concentration (µg/kg) DM					Total EE (%)
				As(V)	DMA	TMAO	AB	EE (%)	CR (%)	As(III)	As (V)	DMA	EE (%)	CR (%)	
50	FB > 1 cm[W]	Average	0.08	46.8	60	nd	240	90	120	nd	trace	nd	6.6	--	95.33
		RSD	0.03	0.5	6	--	50	1	10	--	--	--	0.4	--	0.01
	FB 2 - 4 cm[W]	Average	0.07	50	60	90	120	90	130	nd	trace	nd	7	--	94.3
		RSD	0.07	20	10	100	30	3	20	--	--	--	1	--	0.6
	FB > 5 cm[W]	Average	0.21	40	40	nd	140	80	140	nd	trace	nd	--	--	80
		RSD	0.02	5	3	--	10	10	8	--	--	--	--	--	1
	Compost[B]	Average	1.07	150	100	130	trace	40	100	120	270	nd	30	130	65.2
		RSD	0.08	20	7	1	--	2	10	20	10	--	3	8	0.5
	Casing[B]	Average	0.52	nd	nd	nd	nd	30	--	nd	130	nd	21.2	120	51.65
		RSD	0.03	--	--	--	--	1	--	--	20	--	0.4	30	0.04
	Mycelium/casing[B]	Average	0.25	10	20	nd	20	26	80	nd	60	nd	30	90	52.5
		RSD	0.03	2	3	--	1	4	20	--	3	--	4	2	0.1
	FB < 2 cm[B]	Average	0.14	30	30	nd	60	90	100	nd	trace	nd	--	--	87.3
		RSD	0.02	2	1	--	2	5	9	--	--	--	--	--	0.3
	FB > 2 cm[B]	Average	0.04	20	40	nd	70	80	100	nd	trace	nd	6.58	--	89.69
		RSD	0.01	4	2	--	10	1	10	--	--	--	0.06	--	0.01

nd = not detected < 3 µg/kg DM, trace = < 5 µg/kg DM

[W] Indicates samples collected from the white strain (button mushroom) harvest section

[B] Indicates samples collected from the brown strain (Portobello and cremini) harvest section

Total EE = Step 1 Extracts (Aqueous methanol extracts) + Step 2 Extracts (2% nitric extracts)

Table C-3: Comparison of aqueous methanol water extraction efficiencies (EE) between the commercial growth facility material and mushroom growing kit material.

Source	Sample Type	N	EE (%)
Commercial growth facility	Compost	18	40 ± 5
	Casing	18	30 ± 7
Mushroom growing kit	Control Compost	6	70 ± 8
	Control Casing	6	50 ± 10
	10 ppm Compost	6	70 ± 10
	10 ppm Casing	6	40 ± 10
	100 ppm Compost	6	70 ± 5
	100 ppm Casing	6	30 ± 3
	Compost (control and treatments combined)	18	70 ± 8
	Casing (control and treatments combined)	18	40 ± 10

Table C-4: Total arsenic, arsenic species concentration, extraction efficiency (EE) and column recovery (CR) for samples collected from the mushroom growing kits. Average concentration and differences between replicates are provided.

Sample		Total As (mg/kg DM)	Concentration (µg/kg DM)					EE (%)	CR (%)
			As(III)	As(V)	DMA	TMAO	AB		
Control Casing T1	Average	2.0	nd	nd	nd	nd	nd	60	--
	Difference	0.2	--	--	--	--	--	2	--
Control Compost T1	Average	0.9	nd	300	160	trace	nd	80	80
	Difference	0.1	--	30	7	--	--	1	20
10 ppm Treatment Casing T1	Average	8	nd	3900	nd	nd	nd	50	100
	Difference	1	--	8	--	--	--	3	3
10 ppm Treatment Compost T1	Average	3	nd	1400	nd	nd	nd	80	70
	Difference	1	--	5	--	--	--	20	10
100 ppm Treatment Casing T1	Average	70	nd	22000	nd	nd	nd	40	90
	Difference	6	--	10	--	--	--	9	10
100 ppm Treatment Compost T1	Average	20	nd	12000	nd	nd	nd	90	100
	Difference	7	--	20	--	--	--	5	20
Control Casing T2	Average	1.5	nd	nd	nd	nd	nd	30	--
	Difference	0.1	--	--	--	--	--	20	--
Control Compost T2	Average	1.8	nd	800	340	trace	nd	80	100
	Difference	0.6	--	20	60	--	--	7	30
10 ppm Treatment Casing T2	Average	6	nd	3000	nd	nd	nd	50	100
	Difference	2	--	5	--	--	--	30	7
10 ppm Treatment Compost T2	Average	5	nd	3000	nd	nd	nd	70	90
	Difference	2	--	100	--	--	--	40	30
100 ppm Treatment Casing T2	Average	40	nd	11397.7	nd	nd	nd	40	80
	Difference	10	--	0.4	--	--	--	10	30
100 ppm Treatment Compost T2	Average	20	nd	13000	nd	nd	nd	80	120
	Difference	3	--	10	--	--	--	9	7
Control Casing T3	Average	3.2	nd	nd	nd	nd	nd	50	--
	Difference	0.3	--	--	--	--	--	10	--
Control Compost T3	Average	1.1	nd	300	370	trace	nd	70	110
	Difference	0.2	--	20	4	--	--	4	20

nd = not detected < 3 µg/kg DM, trace = < 5 µg/kg DM

Table C-4 Continued

Sample		Total As (mg/kg DM)	Concentration (µg/kg DM)					EE (%)	CR (%)
			As(III)	As(V)	DMA	TMAO	AB		
10 ppm Treatment Casing T3	Average	4.3	nd	1000	nd	nd	nd	40	90
	Difference	0.1	--	20	--	--	--	40	40
10 ppm Treatment Compost T3	Average	4	nd	2400	nd	nd	nd	80	100
	Difference	1	--	2	--	--	--	1	2
100 ppm Treatment Casing T3	Average	60	nd	1800 0	nd	nd	nd	43.7	80
	Difference	20	--	40	--	--	--	0.6	8
100 ppm Treatment Compost T3	Average	10	nd	6800	nd	nd	nd	73.4	90
	Difference	5	--	40	--	--	--	0.2	1
Control Mature FB T1	Average	0.103	nd	70	40	nd	9	90	120
	Difference	0.005	--	2	2	--	1	3	4
10 ppm Primordium T1*	Average	0.17	46	75	47	trace	34	90	120
	Difference	--	--	--	--	--	--	--	--
10 ppm Mature FB T1	Average	0.10	4	40	30	20	10	80	100
	Difference	0.01	1	5	3	1	3	4	10
100 ppm Primordium T1*	Average	0.51	520	30	93	trace	24	93	130
	Difference	--	--	--	--	--	--	--	--
100 ppm Mature FB T2	Average	0.68	nd	640	20	nd	20	90	100
	Difference	0.06	--	4	60	--	70	8	3
100 ppm Mature FB T3	Average	1.9	nd	1500	100	nd	70	100	100
	Difference	0.8	--	20	9	--	40	1	20

nd = not detected < 3 µg/kg DM, trace = < 5 µg/kg DM
FB = fruiting body
* Indicates single sample collected

Appendix D

D. Additional information for Chapter 6: Characterization of microbial communities associated with terrestrial fungi

D.1 Chemicals and Reagents

Distilled deionized water (DDW) was prepared in-house to a minimum resistance of 18 MΩ·cm (E-pure Barnstead). For the total acid digestions trace metal grade nitric acid (~ 70%, Fisher Scientific) was used. For arsenic extractions DDW was used with HPLC grade methanol (Fisher Scientific) and 2% (v/v) Optima grade (Fisher Scientific). Total matrix spikes and calibration curves were prepared from a stock solution of 1000 µg/mL total arsenic (arsenic pentoxide, Inorganic Ventures).

For HPLC-ICP-MS analysis the anion exchange mobile phase was prepared with ammonium nitrate (99.999% purity, Aldrich) and the cation exchange mobile phase was prepared with pyridine (99% purity, Sigma).

Different sources of standards were used for the calibration curves than for quality control calibration check solutions and matrix spikes when available. For anion exchange this was possible for As(III), arsenite (90% from Fluka and 99.999% purity from Aldrich); As(V), arsenate (1000 ppm from Aldrich and 1000 ppm from Inorganic Ventures); and DMA (V), cacodylic acid (>99% from Fluka and 99% purity from City Chemical). For cation exchange this was possible for DMA (V); AB, arsenobetaine (Wako and Argus Chemicals); TMAO, trimethlyarsine oxide (Wako and Argus Chemicals). Only one source was available for AC, arsenocholine bromide (Argus Chemicals) for cation exchange and for MMA(V), monosodium acid methane arsonate sesquihydrate (99.0% purity Chemservice). TETRA, tetramethylarsonium iodide (Wako) was used for identification purposes by matching retention times, and the AC calibration curve was used for quantification of the compound when it was found in samples.

D.2 Total arsenic analysis

The instrument used was an ICP-MS DRC II from PerkinElmer (PerkinElmer, MA). The detection limit of the instrument was determined to be 0.5 µg/L and mass interferences were monitored by measuring *m/z* 77 ($ArCl^+$). Instrumental tests included instrumental blanks and calibration checks which were run every 10 samples. Instrument blanks run every 10 samples were under the instrument detection limits for total arsenic analysis. Instrument quality control checks that included a 5 µg/L and 50 µg/L solution for total arsenic were run every 10 samples and found to be within an acceptable range (80 – 120 % recovery).

D.3 Arsenic speciation analysis using HPLC-ICP-MS

Briefly, anion exchange chromatography was performed using a Hamilton PRPX100 anion exchange column with a gradient mobile phase (A: 4 mM of ammonium nitrate (NH_4NO_3) and B: 60 mM NH_4NO_3, pH = 8.65). Anion exchange was used to identify: As(III), As(V), monomethylarsonic acid (MMA), dimethylarsinic acid (DMA), glycerol arsenosugar (Sugar 1) and sulfonate arsenosugar (Sugar 3. Cation exchange chromatography was performed with a Chrompack Ionosphere C cation exchange column with a mobile phase of 20nM pyridine, pH = 2.7. Cation exchange was used to identify: DMA, AB, trimethylarsine oxide (TMAO), arsenocholine (AC) and tetramethylarsonium ion (TETRA). All chromatographic speciation data were analyzed with Peak Fit Version 4.12. HPLC blanks and calibration checks were run every 10 samples and considered acceptable (70 – 130 % recovery). All mushrooms were analyzed for arsenic speciation and all compounds that could be matched with a known standard were quantified using the calibration curve for that arsenic compound. Unknown compounds were quantified using the calibration curve for the closest eluting known compound. The limit of detection for each compound was determined to be 0.003 mg/L, compounds 0.003 mg/L to 0.005 mg/L were considered as trace compounds and all compounds over 0.005 mg/L were quantified.

D.4 Statistical Analysis

The relative abundance for phylum, class and order was used in the Principle Components Analysis (PCA). PCAs were performed for untransformed, LN(x+1) and Taylor power law transformed data.

D.4.1 Normality testing

Normality of the data was evaluated following methods described in Weber et al., 2007. The kurtosis and skewness of each variable were calculated and the standard errors were calculated using Equations 1 and 2. The corresponding z values were then calculated using Equations 3 and 4. The null hypothesis, that the data follows a normal distribution, was tested using this value. A $|z|$ value > 1.96 indicates that the null hypothesis (normal distribution) can be rejected with 95% confidence. Therefore $|z|$ values < 1.96 indicate a normal distribution. Normality was evaluated by calculating the mean of the statistic across all variables as well as testing the individual variables. In this study the number of significant kurtosis and skewness values represents the number of normally distributed variables tested. The transformation that gave the highest number of normally distributed variables was used for the PCA for discussion.

$$SE_{kurtosis} = \sqrt{24/n} \quad (1)$$

n = number of observations

$$SE_{skewness} = \sqrt{6/n} \quad (2)$$

n= number of observations

The corresponding z values were then calculated as:

$$z_{kurtosis} = \frac{kurtosis}{SE_{kurtosis}} \quad (3)$$

$$z_{skewness} = \frac{skewness}{SE_{skewness}} \quad (4)$$

$|z|$ values > 1.96 indicate a non-normal distribution.

The variance ratio for each data set was also calculated as an indicator of the relative homogeneity of variance between variables (Equation 5). A lower variance ratio was considered to represent a relatively more homogenous data set

$$variance\ ratio = \frac{highest\ variance}{lowest\ variance} \quad (5)$$

D.4.2 Transformation Statistical Summary

Table D-1: Transformation statistical summary for the commercial growth facility data

n = 18	Phylum (7 variables)			Class (11 variables)			Order (19 variables)		
	Transform			Transform			Transform		
	None	LN(x+1)	Taylor	None	LN(x+1)	Taylor	None	LN(x+1)	Taylor
Mean kurtosis z-value	2.24	1.81	1.06	2.70	1.46	1.69	3.08	1.75	1.45
Number of kurtosis values within normality	4	5	6	6	10	8	11	16	16
Mean skewness z-value	1.29	1.21	0.99	1.51	0.90	0.86	1.65	0.91	0.89
Number of skewness values within normality	4	6	6	7	11	10	13	17	19
Variance ratio	5966	22.80	6.61	1527	10.51	0.007	5655	25.73	13.11

Table D-2: Transformation statistical summary for ON soils

n = 14	Phylum (10 variables)			Class (20 variables)			Order (33 variables)		
	Transform			Transform			Transform		
	None	LN(x+1)	Taylor	None	LN(x+1)	Taylor	None	LN(x+1)	Taylor
Mean kurtosis z-value	1.61	1.28	1.31	2.06	1.36	1.29	1.37	1.23	2.49
Number of kurtosis values within normality	8	9	6	14	18	17	23	30	17
Mean skewness z-value	1.70	1.47	1.38	2.03	1.25	1.33	0.96	0.71	1.06
Number of skewness values within normality	6	8	9	12	18	17	25	31	27
Variance ratio	4385	47.22	27.20	7348	41.22	12.57	3423	32.77	57.80

Table D-3: Transformation statistical summary for YK soils

n = 21	Phylum (13 variables)			Class (25 variables)			Order (41 variables)		
	Transform			Transform			Transform		
	None	LN(x+1)	Taylor	None	LN(x+1)	Taylor	None	LN(x+1)	Taylor
Mean kurtosis z-value	3.50	0.98	0.59	4.73	1.58	1.69	5.49	1.75	1.53
Number of kurtosis values within normality	7	11	13	9	19	15	8	22	27
Mean skewness z-value	3.07	1.45	1.22	2.10	1.27	1.06	2.32	1.32	1.05
Number of skewness values within normality	4	9	9	10	20	21	9	22	26
Variance ratio	177.4	32.42	9.66	3404	40.93	102.19	1152	17.80	5.98

Table D-4: Transformation statistical summary for ON and YK soils combined data set

n = 35	Phylum (13 variables)			Class (29 variables)			Order (49 variables)		
	Transform			Transform			Transform		
	None	LN(x+1)	Taylor	None	LN(x+1)	Taylor	None	LN(x+1)	Taylor
Mean kurtosis z-value	5.91	1.96	1.34	9.60	4.10	2.79	8.94	3.08	1.91371
Number of kurtosis values within normality	5	9	10	7	15	15	11	27	38
Mean skewness z-value	5.03	2.50	1.96	2.75	1.69	1.40	2.89	1.63	1.219596
Number of skewness values within normality	2	9	9	10	16	19	16	30	39
Variance ratio	226.9	22.51	6.79	5216	51.40	10.74	1574	26.58	6.07

D.4.3 Principle Components Analysis

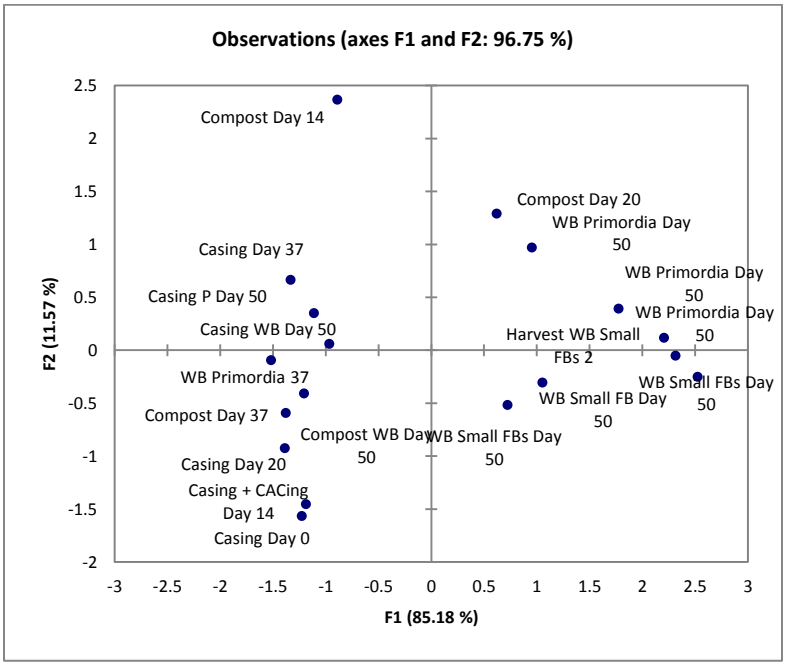


Figure D.1: PCA for untransformed phyla relative abundance in samples from the commercial growth facility for *A. bisporus.*

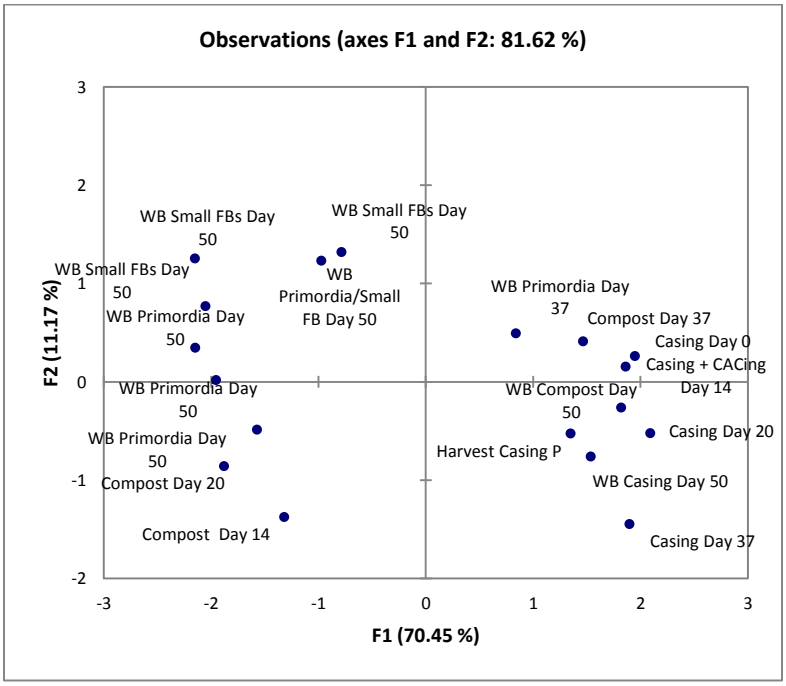


Figure D.2: PCA for LN(x+1) transformed phyla relative abundance in samples from the commercial growth facility for *A. bisporus*.

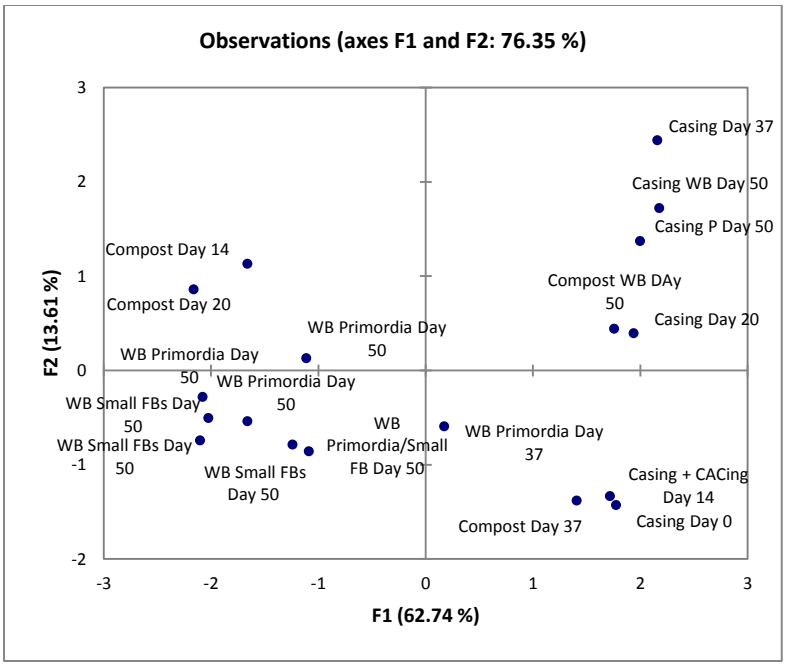


Figure D.3: PCA for Taylor transformed phyla relative abundance in samples from the commercial growth facility for *A. bisporus*.

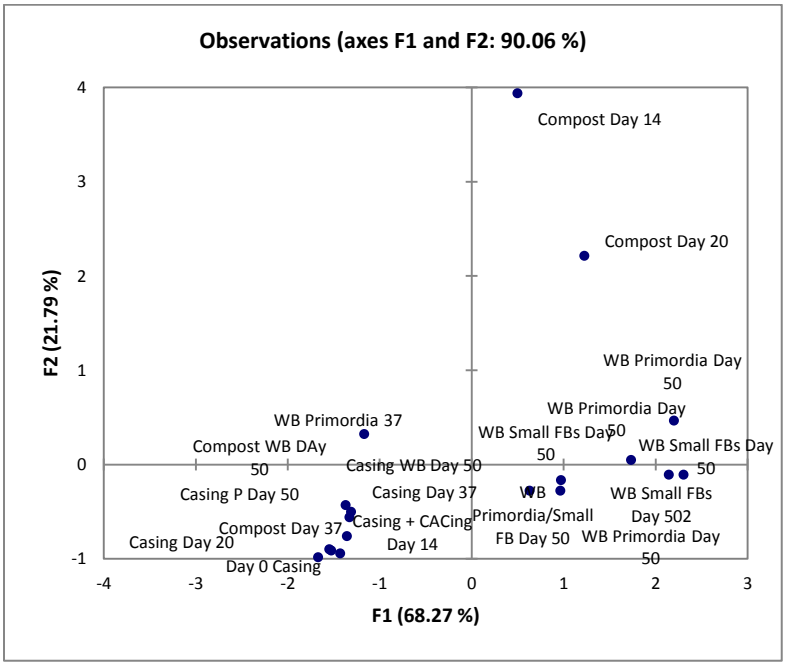


Figure D.4: PCA for untransformed class relative abundance in samples from the commercial growth facility for *A. bisporus.*

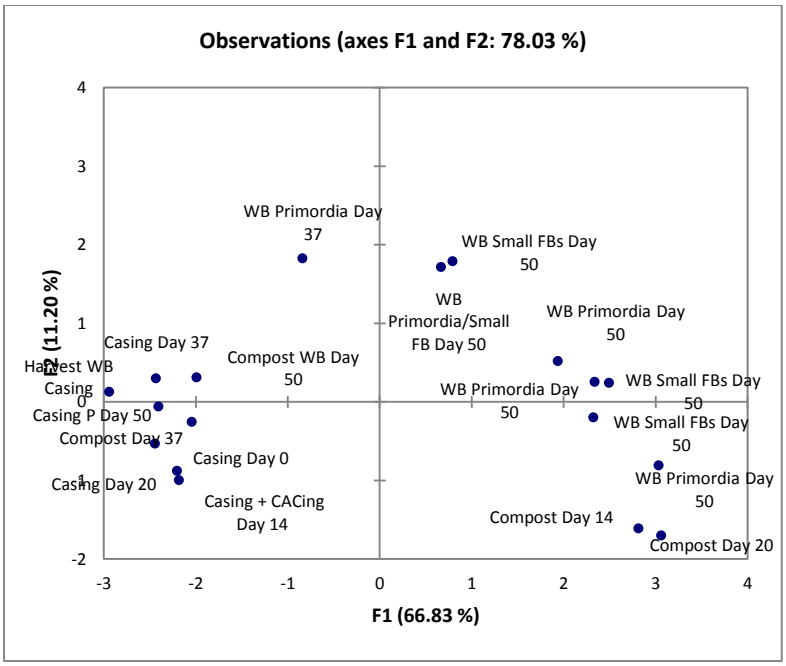


Figure D.5: PCA for LN(x+1) transformed class relative abundance in samples from the commercial growth facility for *A. bisporus*.

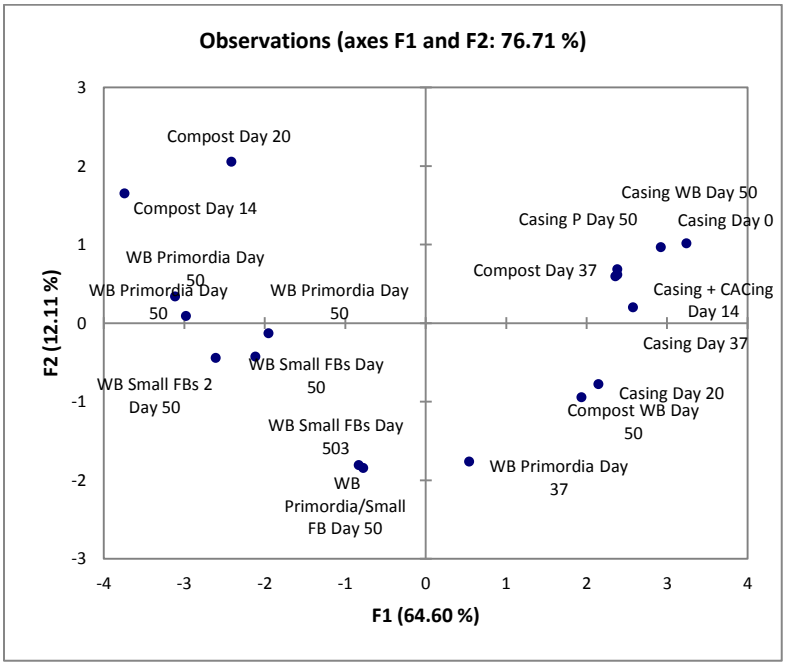


Figure D.6: PCA for Taylor transformed class relative abundance in samples from the commercial growth facility for *A. bisporus.*

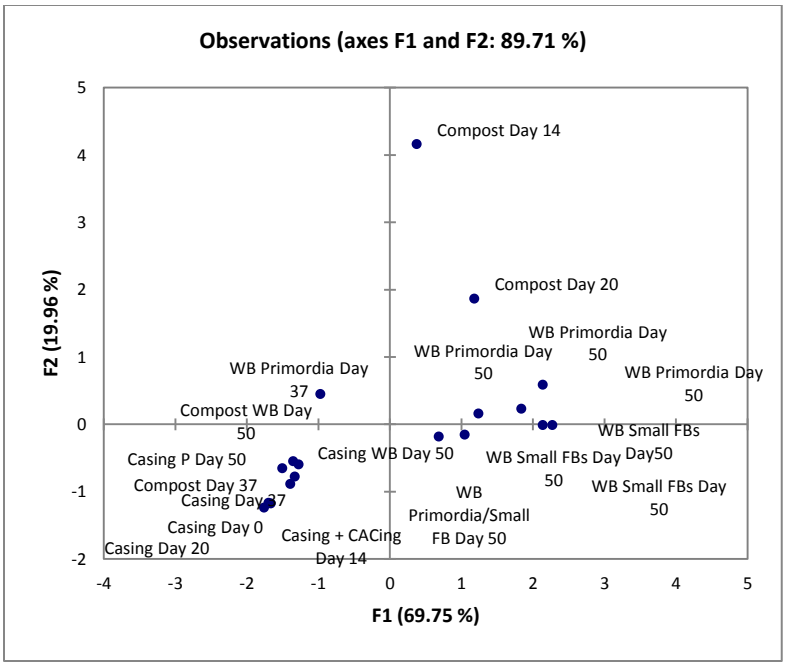


Figure D.7: PCA for untransformed order relative abundance in samples from the commercial growth facility for *A. bisporus.*

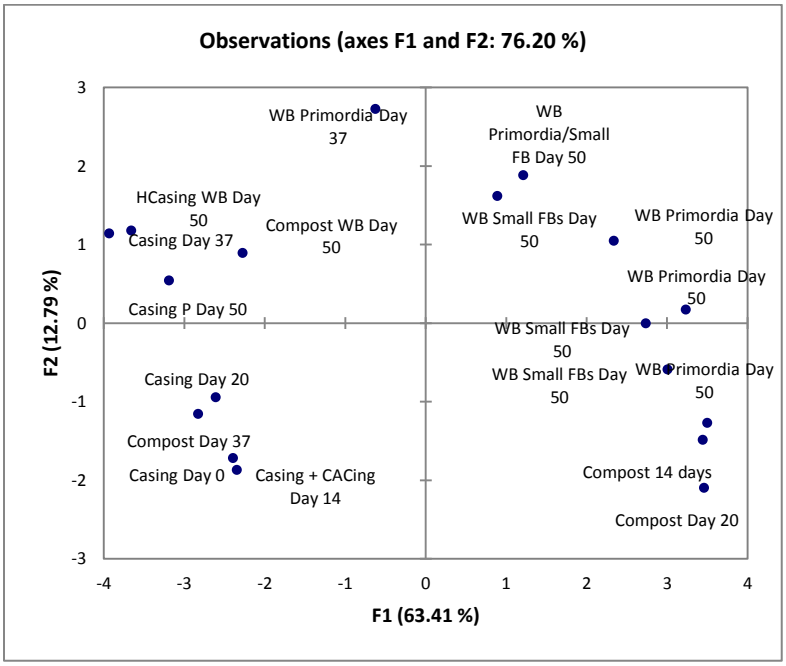


Figure D.8: PCA for LN(x+1) transformed order relative abundance in samples from the commercial growth facility for *A. bisporus*.

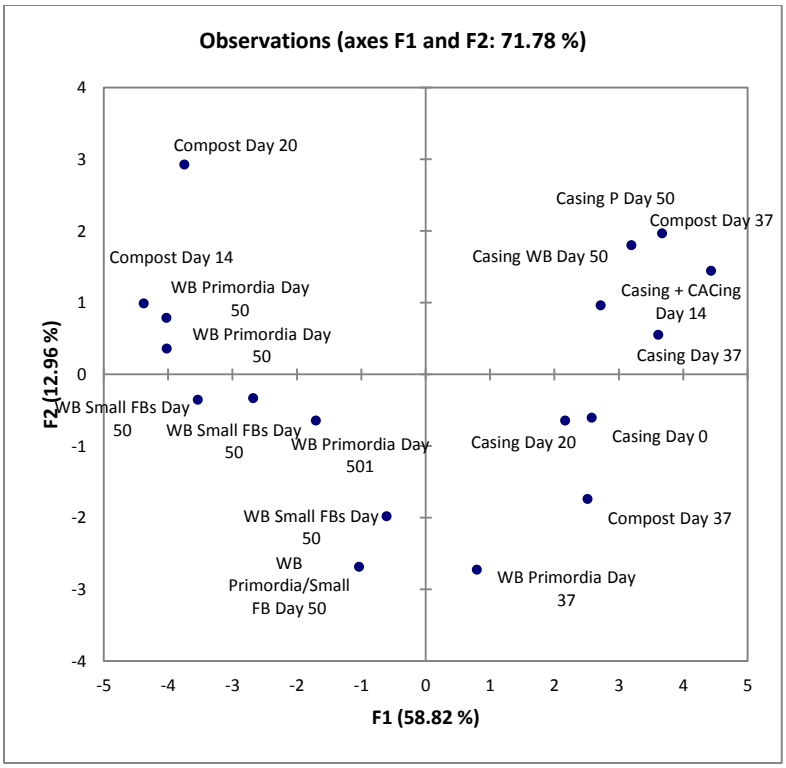


Figure D.9: PCA for Taylor transformed order relative abundance in samples from the commercial growth facility for *A. bisporus*.

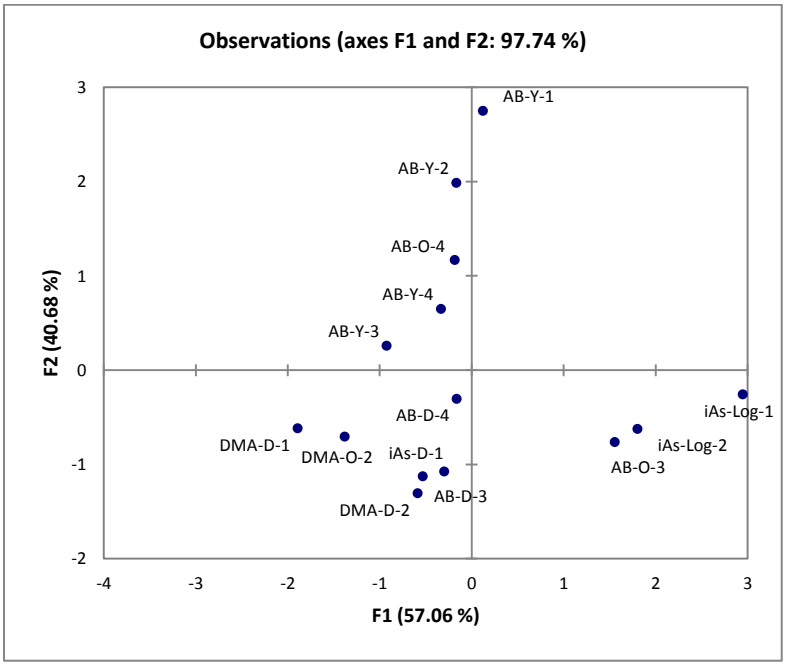


Figure D.10: PCA for untransformed phyla relative abundance in samples associated with Ontario fruiting

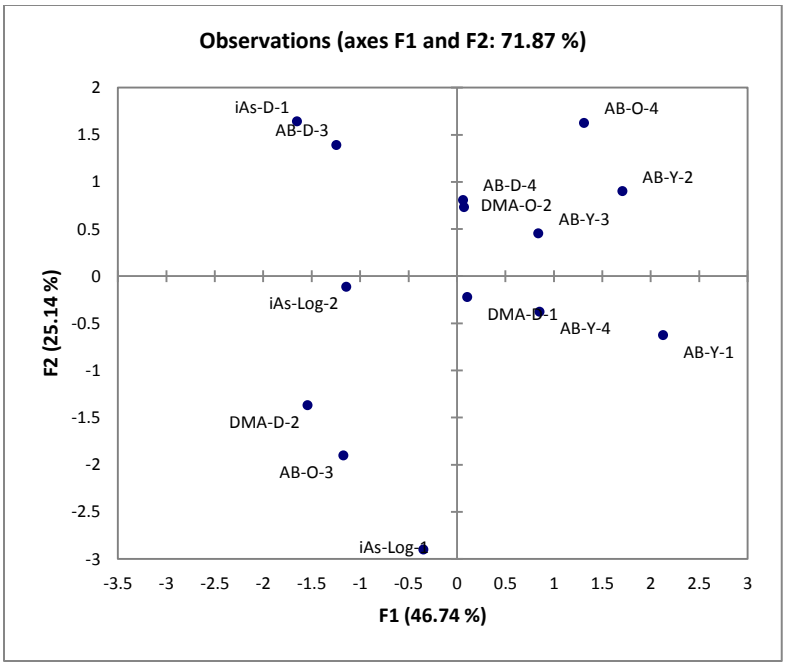


Figure D.11: PCA for LN(x+1) transformed phyla relative abundance in samples associated with Ontario fruiting

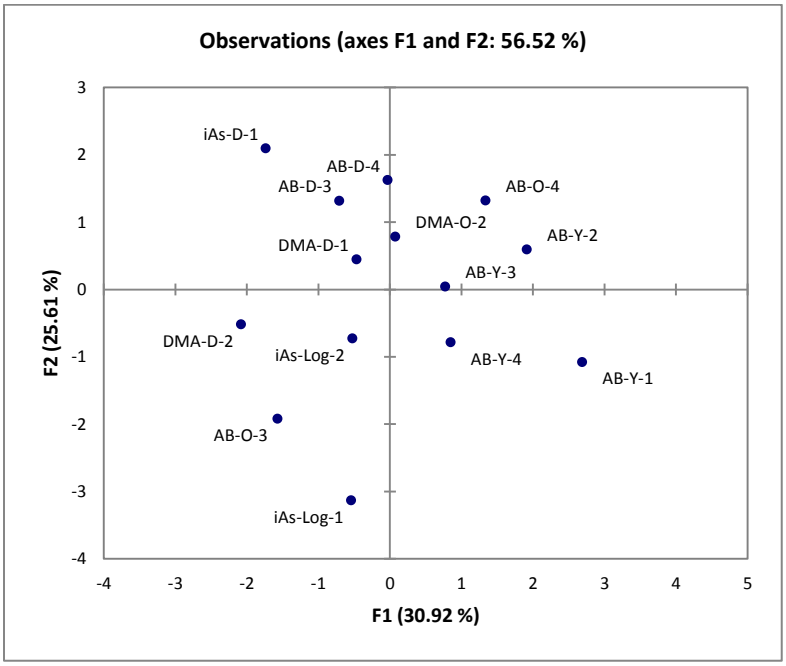


Figure D.12: PCA for Taylor transformed phyla relative abundance in samples associated with Ontario fruiting

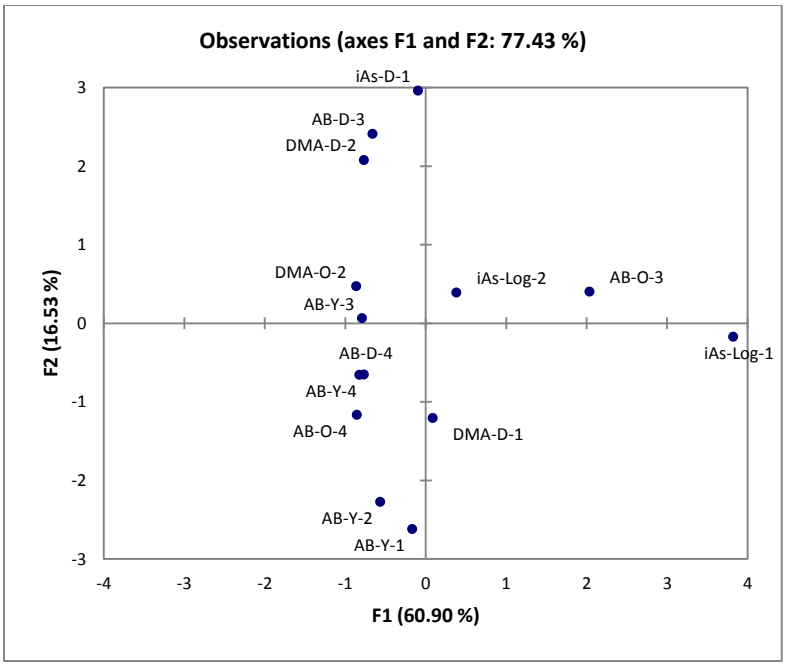


Figure D.13: PCA for untransformed class relative abundance in samples associated with Ontario fruiting

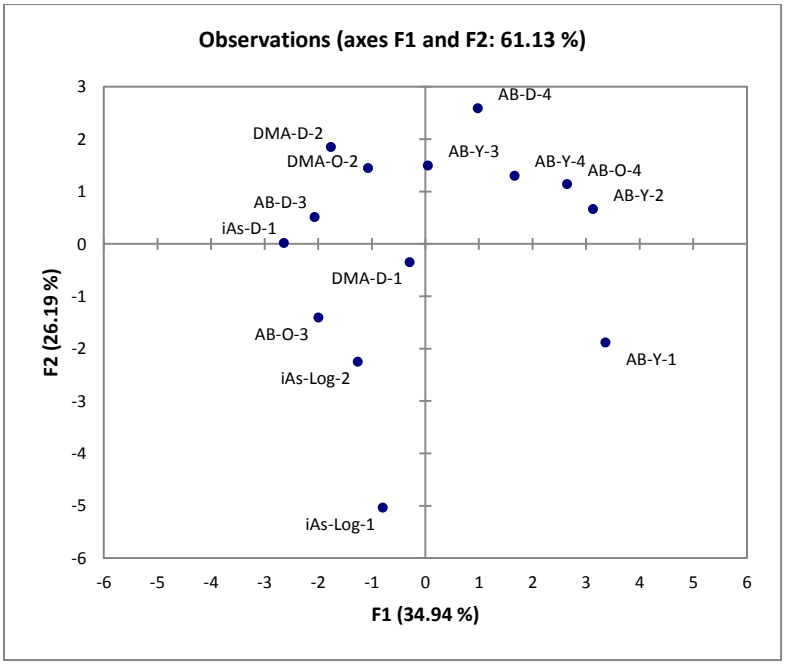


Figure D.14: PCA for LN(x+1) transformed class relative abundance in samples associated with Ontario fruiting

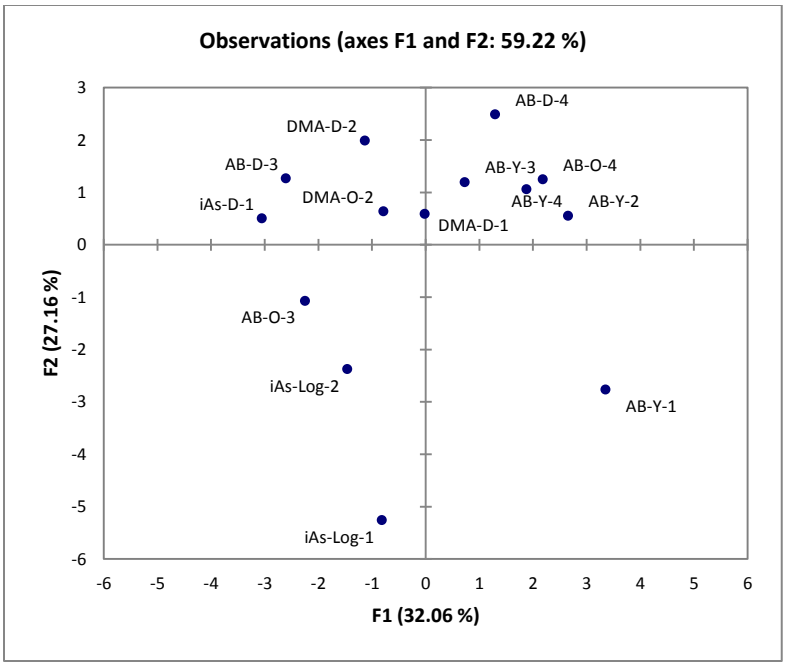


Figure D.15: PCA for Taylor transformed class relative abundance in samples associated with Ontario fruiting

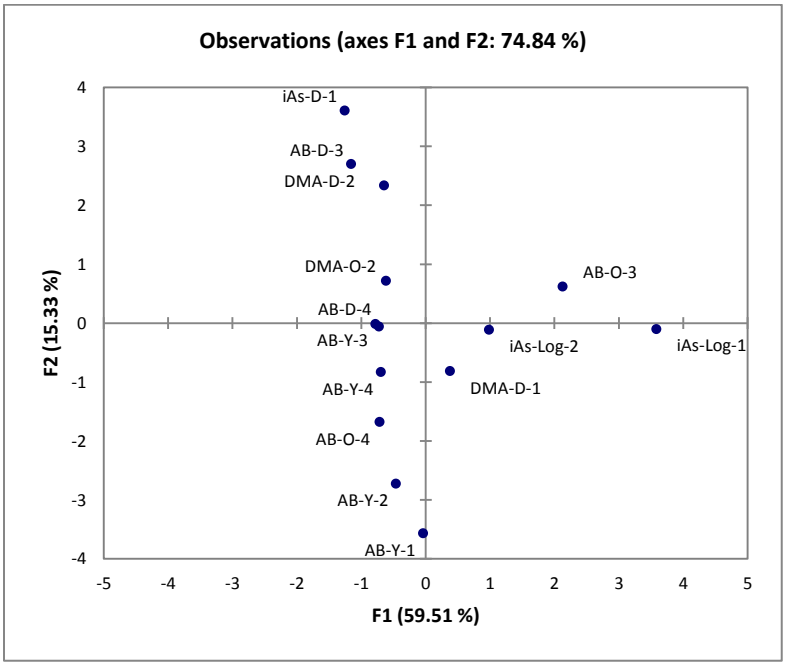


Figure D.16: PCA for untransformed order relative abundance in samples associated with Ontario fruiting

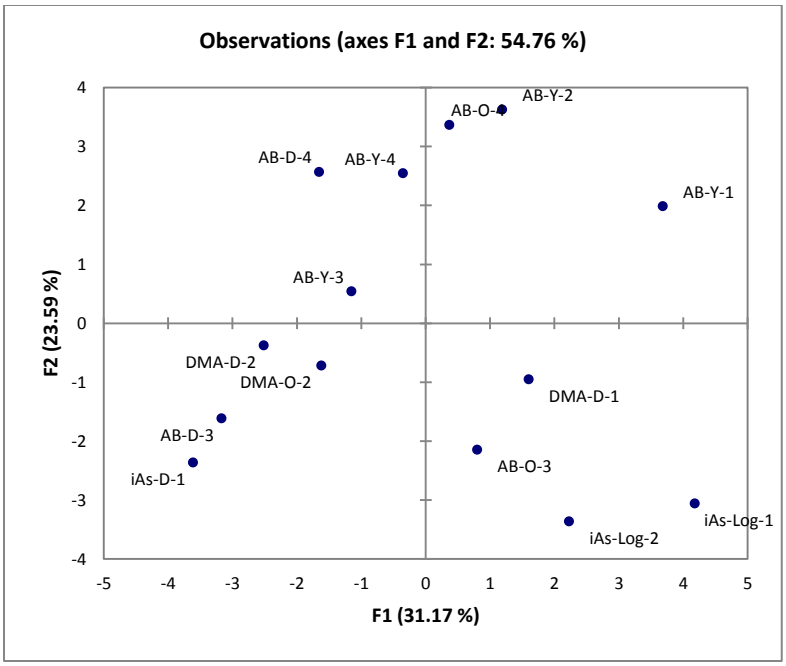


Figure D.17: PCA for LN(x+1) transformed order relative abundance in samples associated with Ontario fruiting

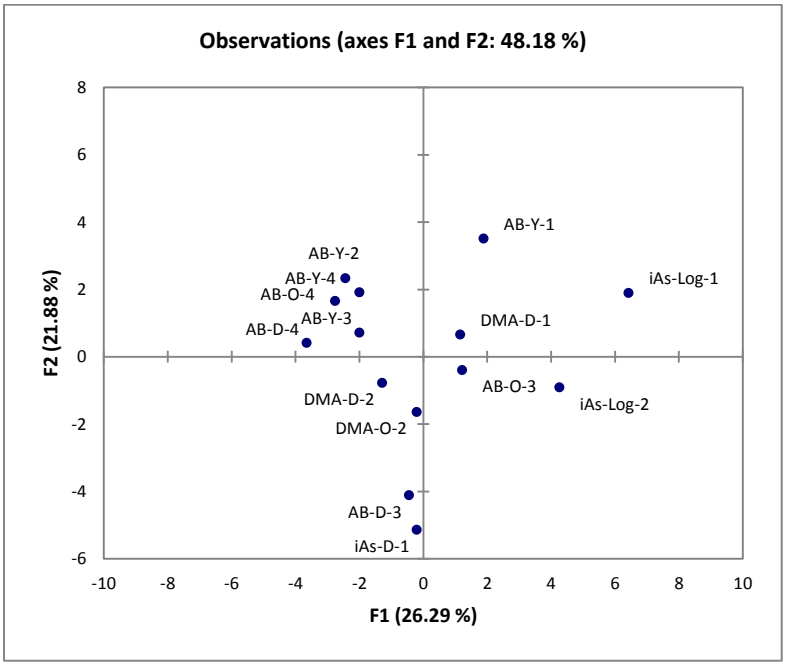


Figure D.18: PCA for Taylor transformed order relative abundance in samples associated with Ontario fruiting bodies

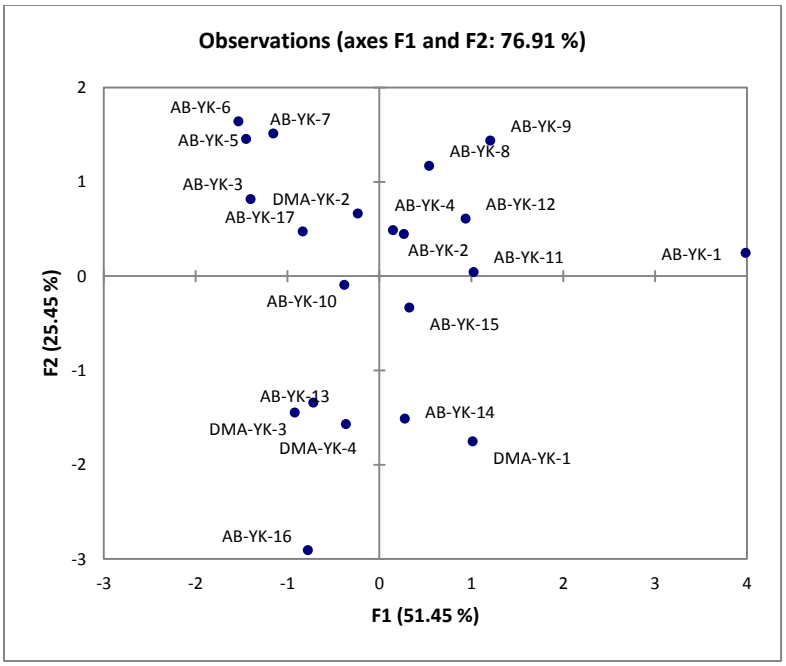


Figure D.19: PCA for untransformed phyla relative abundance in samples associated with Yellowknife fruiting bodies.

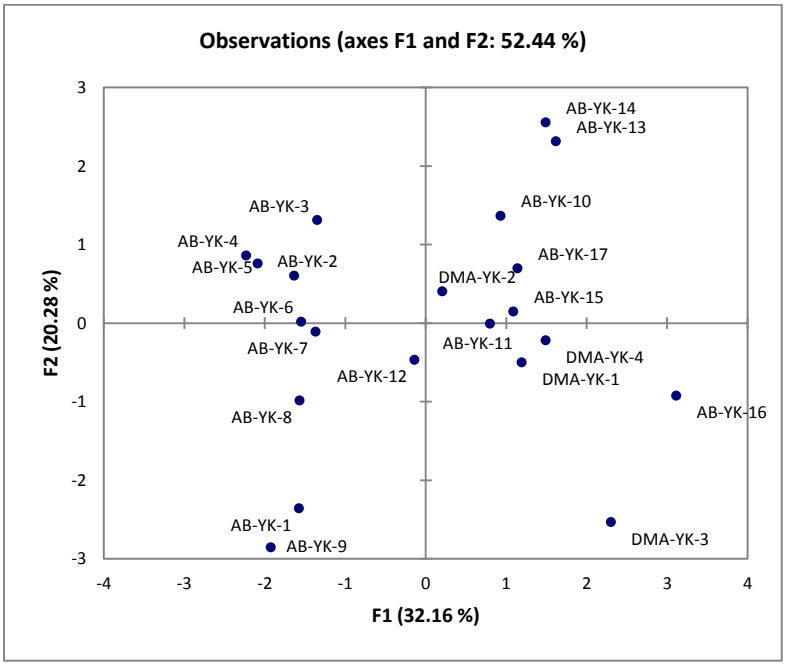


Figure D.20: PCA for LN(x+1) transformed phyla relative abundance in samples associated with Yellowknife fruiting bodies

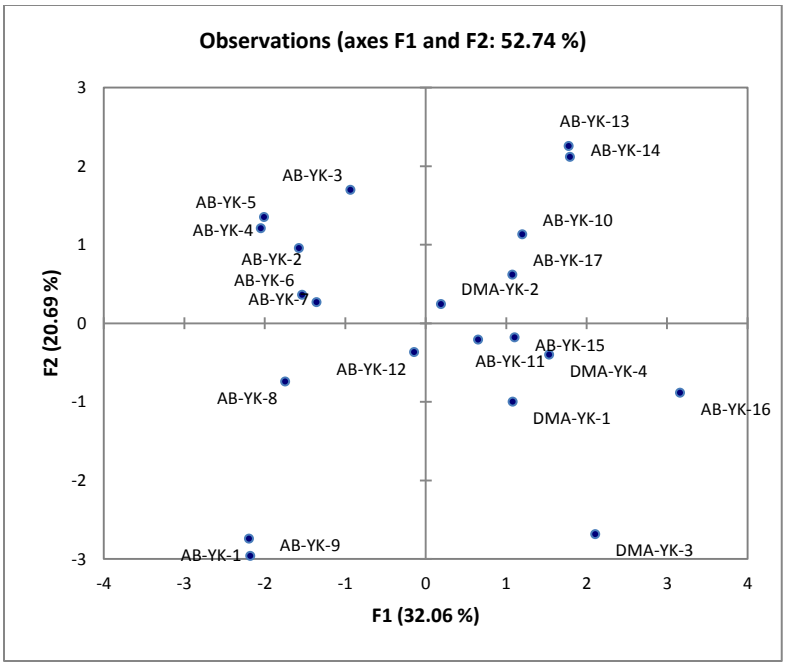


Figure D.21: PCA for Taylor transformed phyla relative abundance in samples associated with Yellowknife fruiting bodies

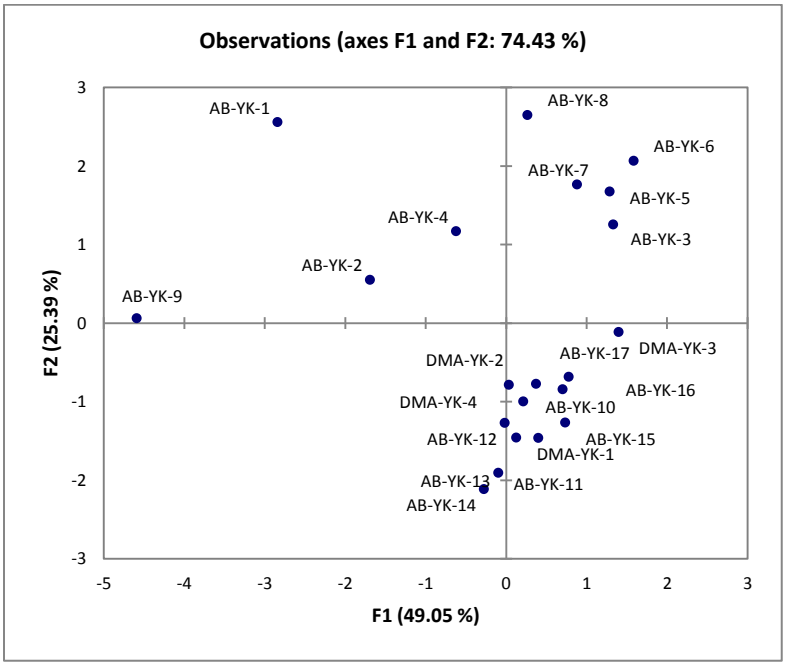


Figure D.22: PCA for untransformed class relative abundance in samples associated with Yellowknife fruiting bodies

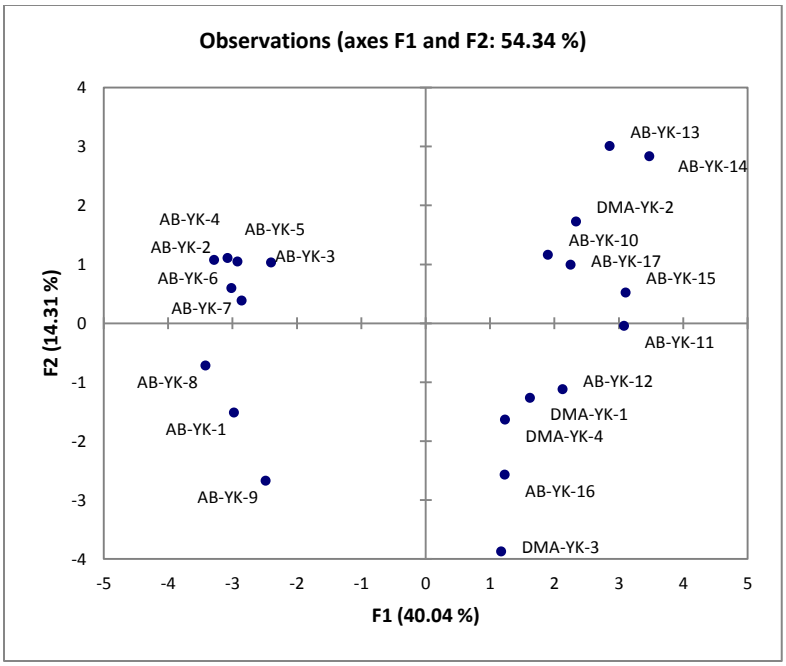


Figure D.23: PCA for LN(x+1) transformed class relative abundance in samples associated with Yellowknife fruiting bodies

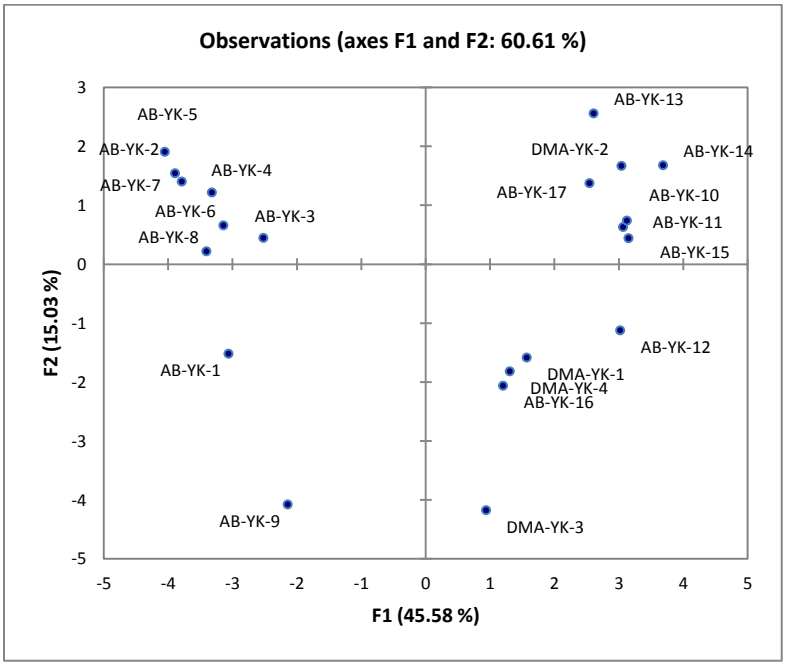


Figure D.24: PCA for Taylor transformed class relative abundance in samples associated with Yellowknife fruiting bodies

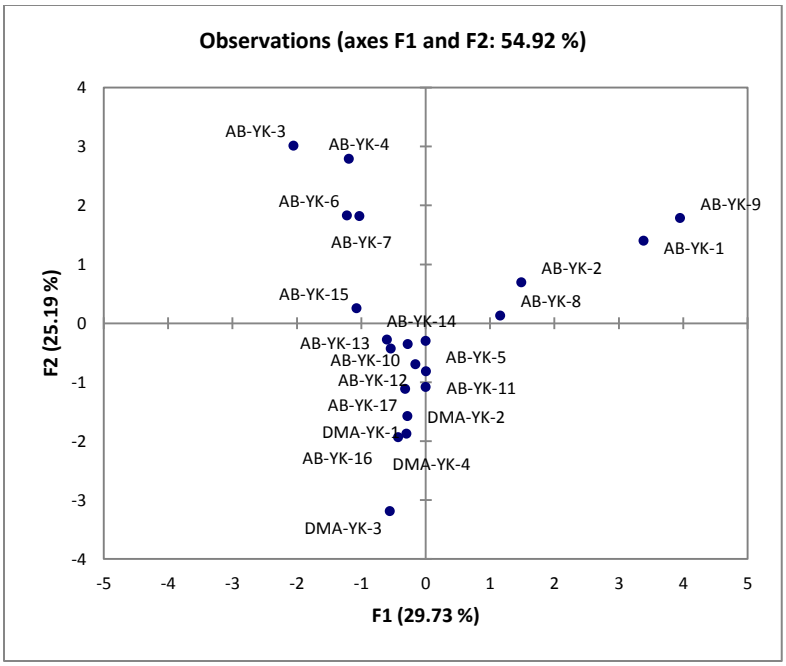


Figure D.25: PCA for untransformed order relative abundance in samples associated with Yellowknife fruiting bodies

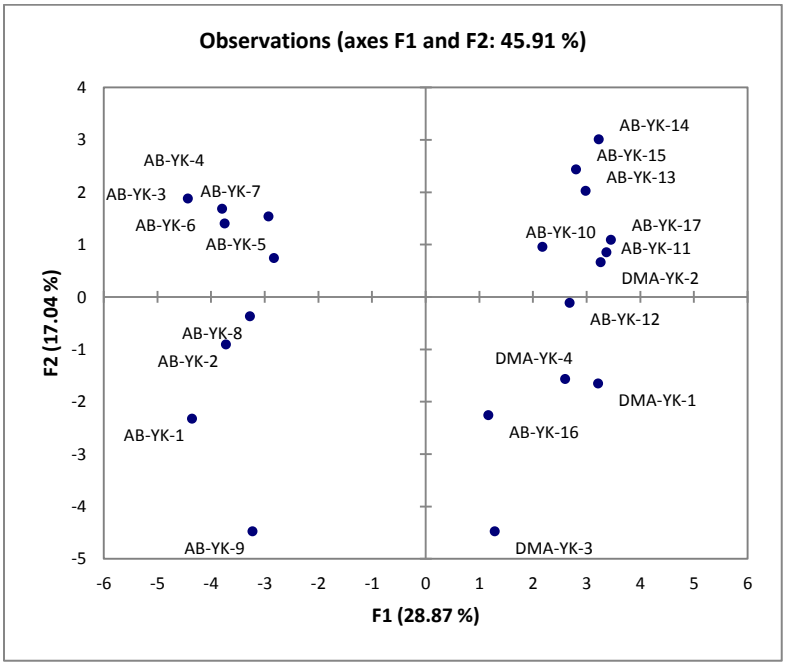


Figure D.26: PCA for LN(x+1) transformed order relative abundance in samples associated with Yellowknife fruiting bodies

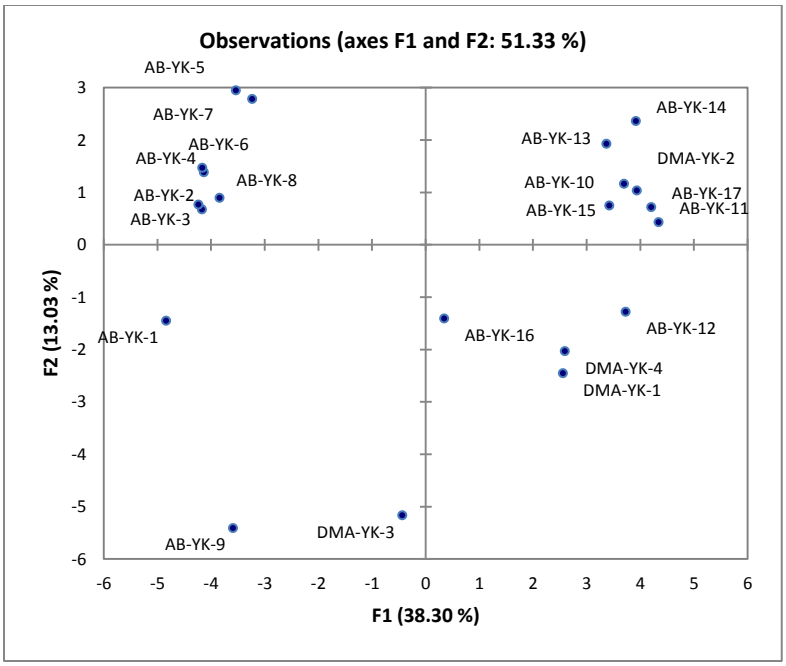


Figure D.27: PCA for Taylor transformed order relative abundance in samples associated with Yellowknife fruiting bodies

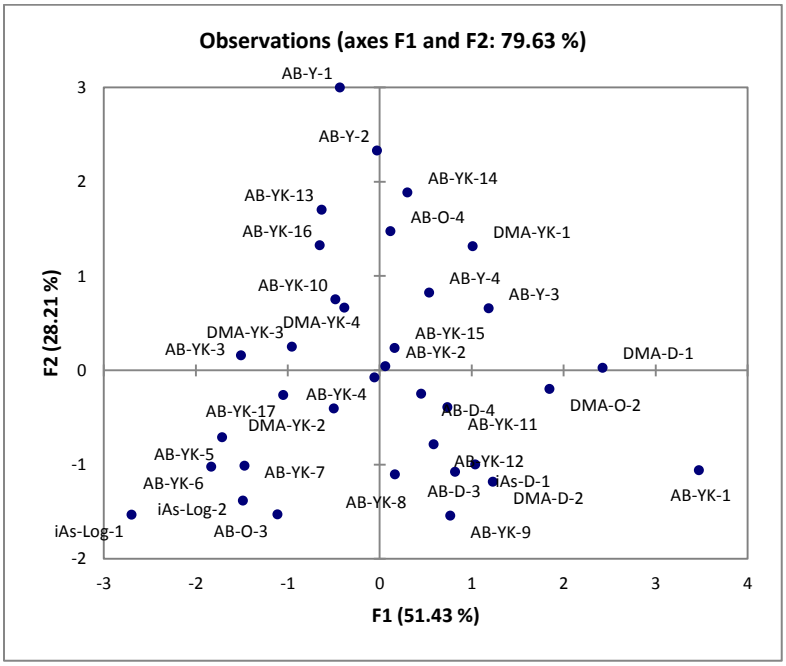


Figure D.28: PCA for untransformed phyla relative abundance in combined samples associated with Yellowknife and Ontario fruiting bodies

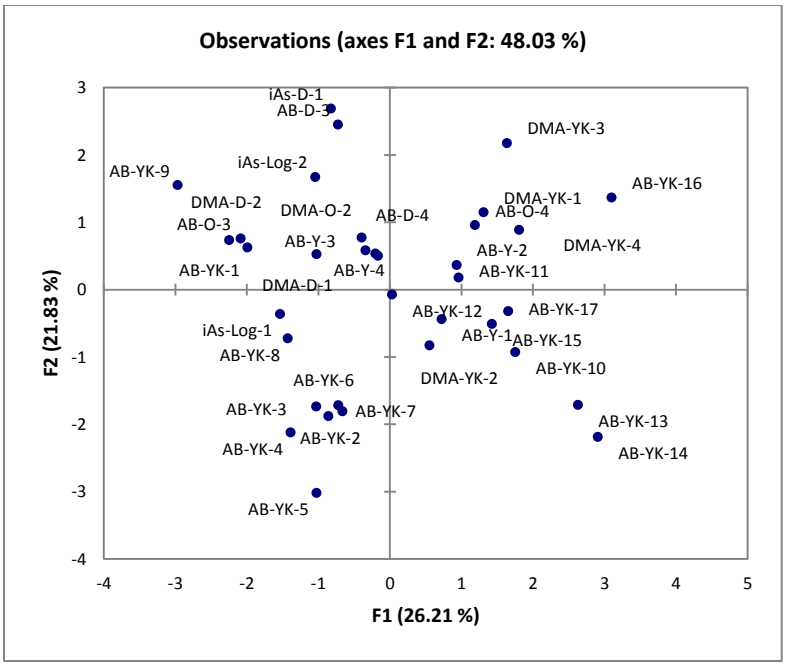


Figure D.29: PCA for LN(x+1) transformed phyla relative abundance in combined samples associated with Yellowknife and Ontario fruiting bodies

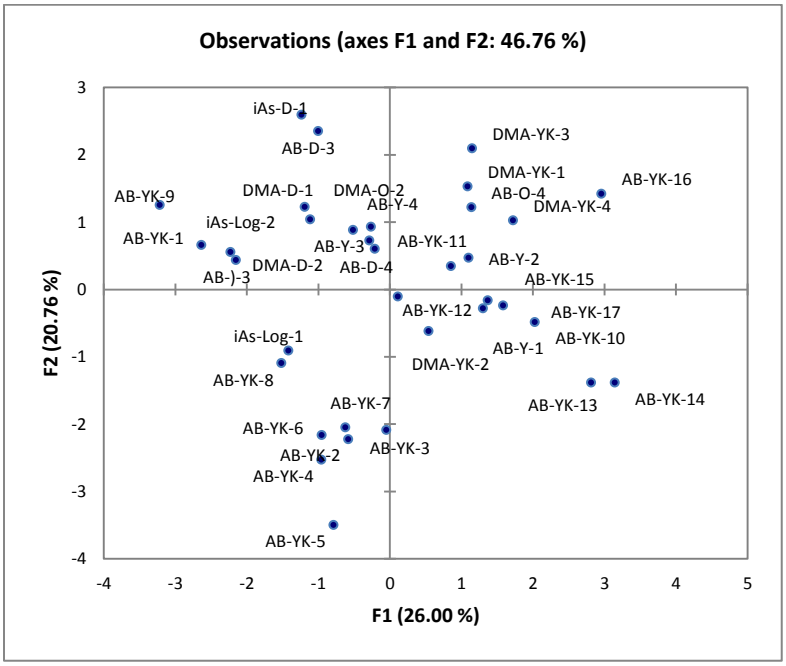


Figure D.30: PCA for Taylor transformed phyla relative abundance in combined samples associated with Yellowknife and Ontario fruiting bodies

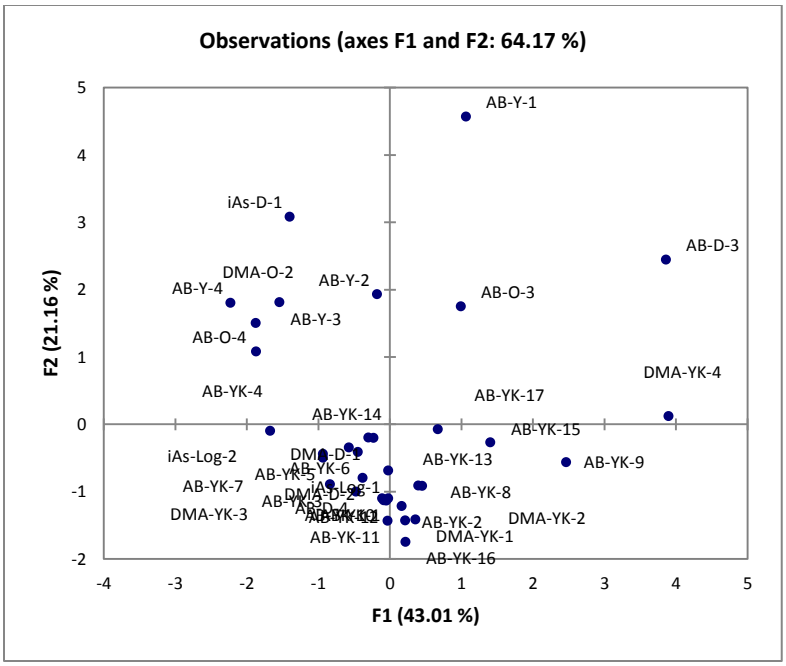


Figure D.31: PCA for untransformed class relative abundance in combined samples associated with Yellowknife and Ontario fruiting bodies

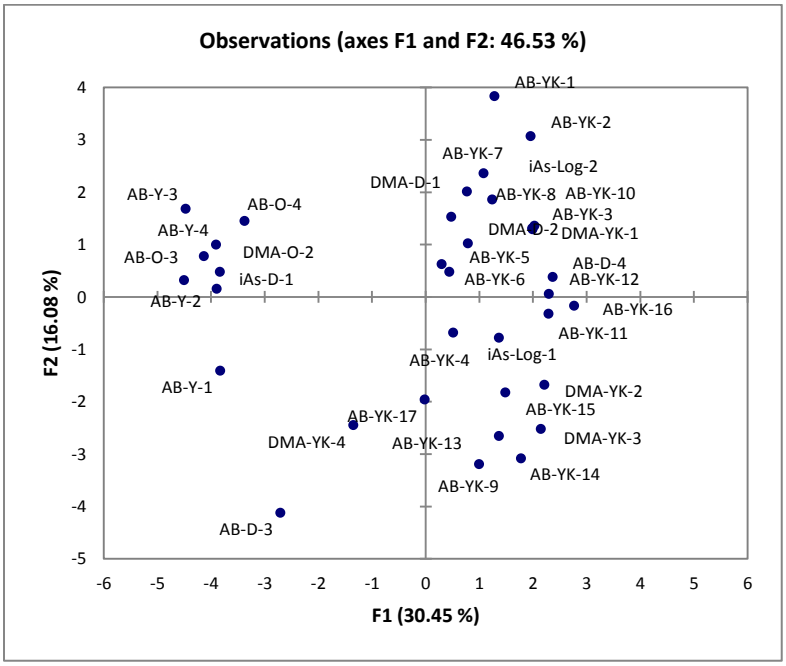


Figure D.32: PCA for LN(x+1) transformed class relative abundance in combined samples associated with Yellowknife and Ontario fruiting bodies

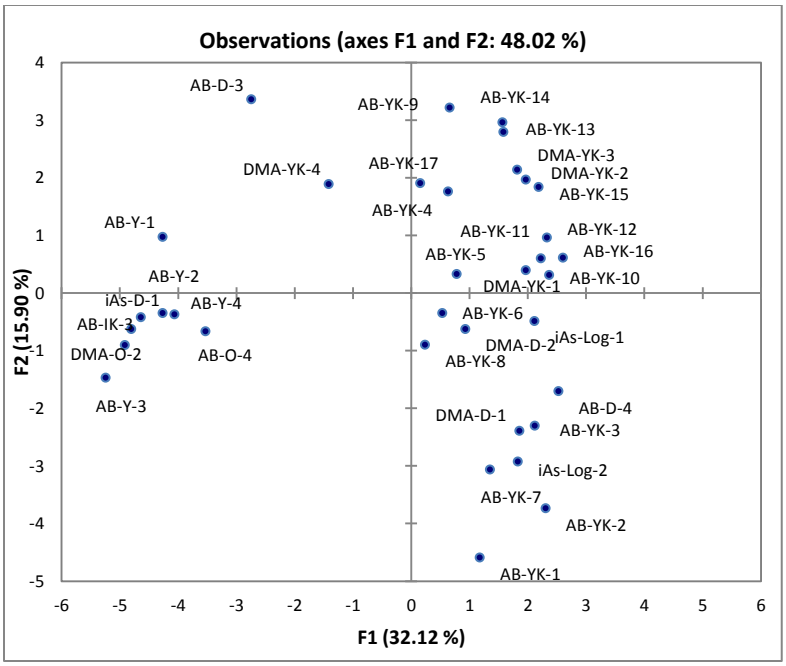


Figure D.33: PCA for Taylor transformed class relative abundance in combined samples associated with Yellowknife and Ontario fruiting bodies

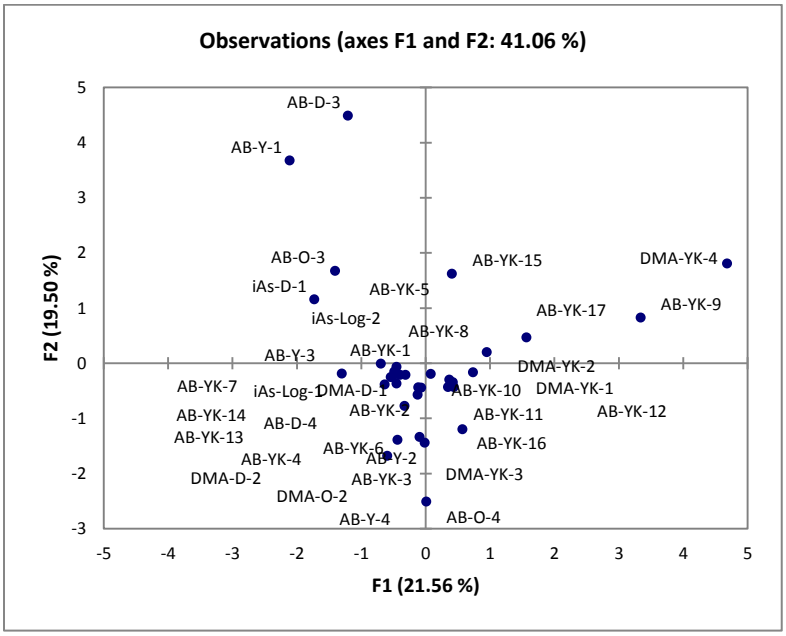


Figure D.34: PCA for untransformed order relative abundance in combined samples associated with Yellowknife and Ontario fruiting bodies

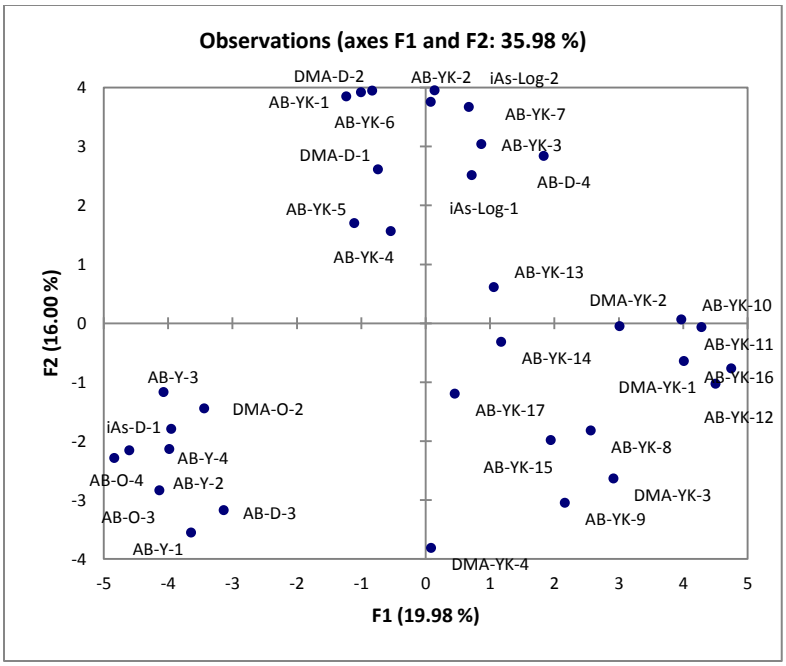


Figure D.35: PCA for LN(x+1) transformed order relative abundance in combined samples associated with Yellowknife and Ontario fruiting bodies

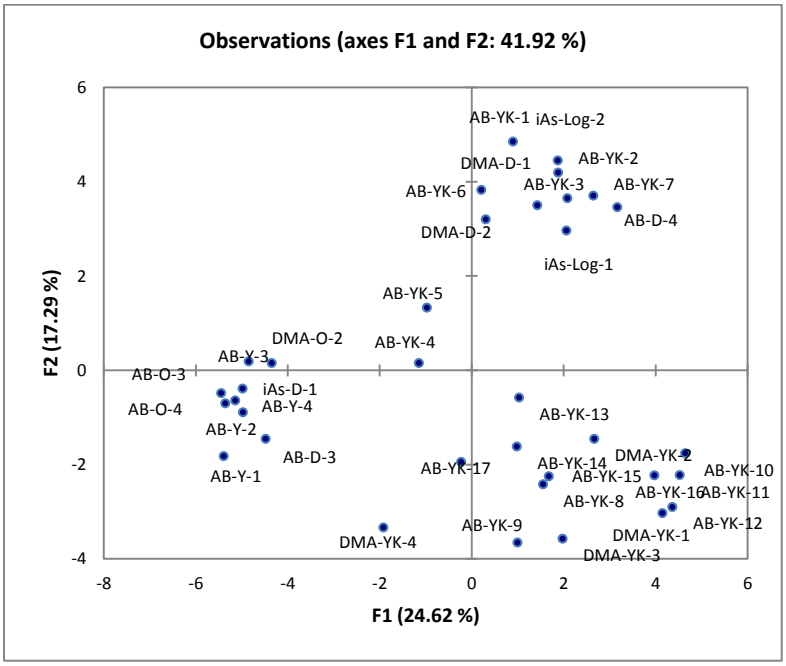


Figure D.36: PCA for Taylor transformed order relative abundance in combined samples associated with Yellowknife and Ontario fruiting bodies

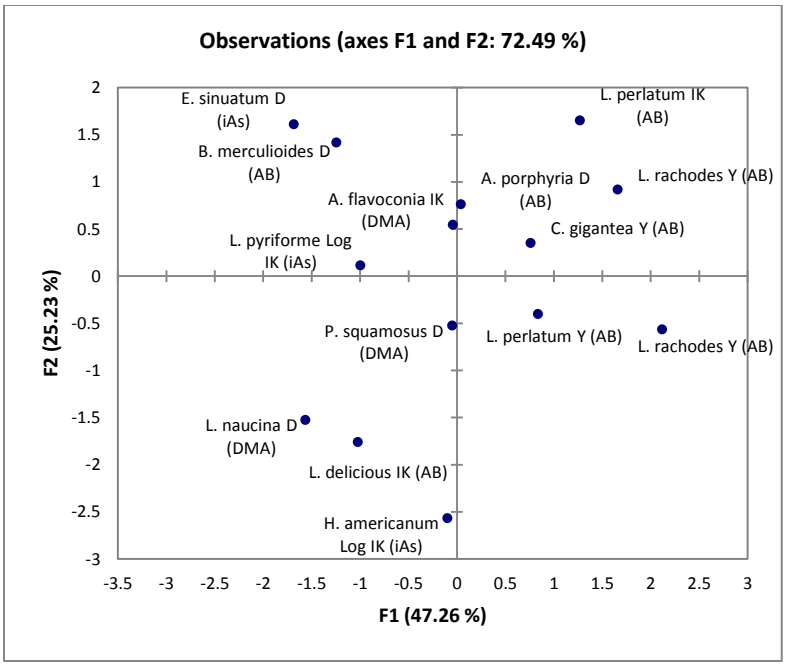


Figure D.37: PCA for LN(x+1) transformed phyla relative abundance in samples associated with Ontario fruiting bodies. Proteabacteria phylum removed prior to PCA.

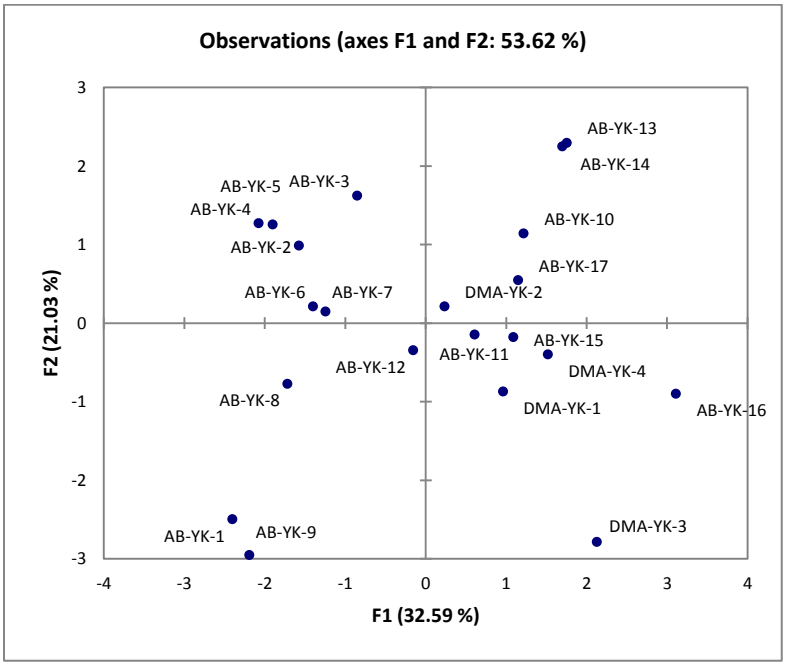


Figure D.38: PCA for Taylor transformed phyla relative abundance in samples associated with Yellowknife fruiting bodies. Proteabacteria phylum removed prior to PCA.

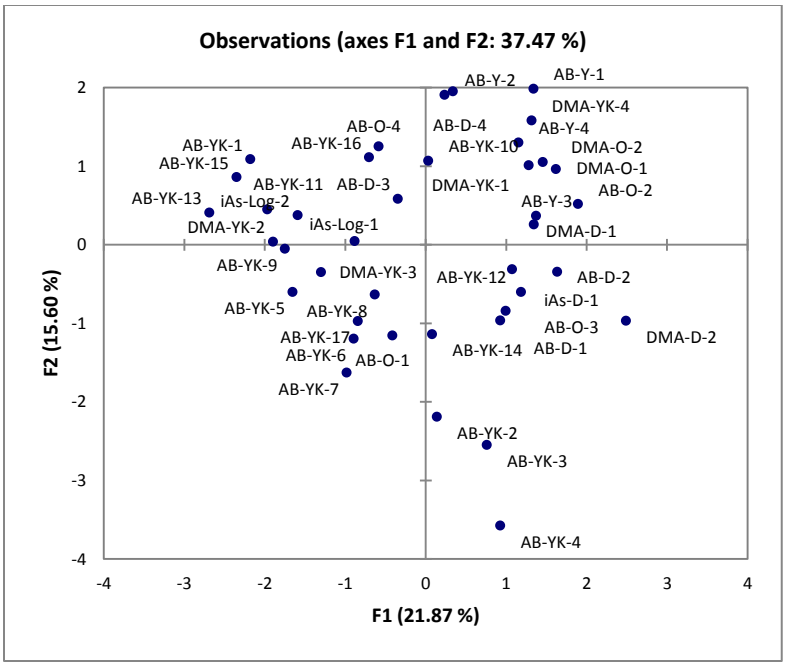


Figure D.39: PCA for Taylor transformed rare phyla relative abundance in combined samples associated with Yellowknife and Ontario fruiting bodies

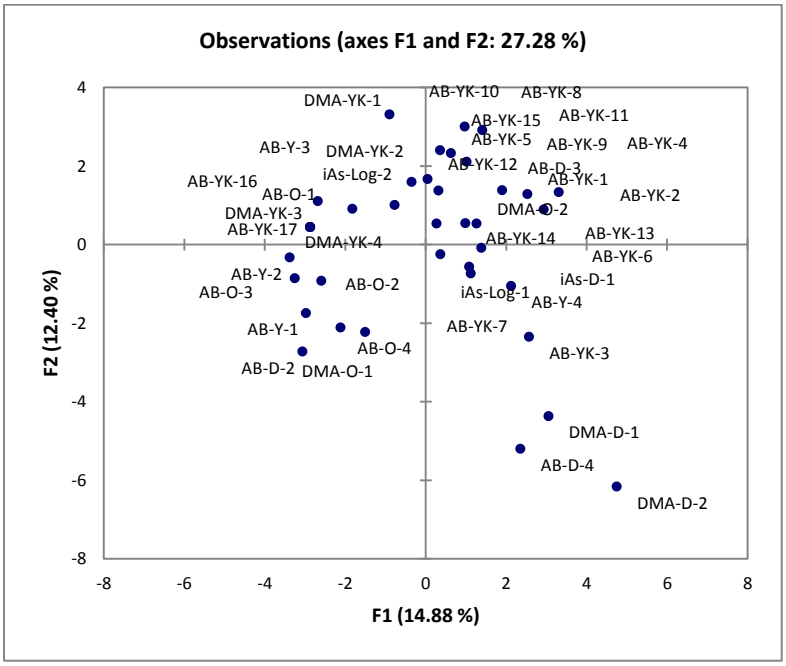


Figure D.40: PCA for Taylor transformed rare class relative abundance in combined samples associated with Yellowknife and Ontario fruiting bodies

Table D-5: Quality Control and Quality Assurance for Bacteria Exposure Experiments

Medium	Measurement	Instrument	N	Range	Average
Method Blanks	Total Arsenic Concentration	ICP-MS	20	< LOD_a	< LOD_a
Method Blanks	Arsenic Species Concentrations	HPLC-ICP-MS	20	< LOD_b	< LOD_b
Matrix Spike	Percent Recovery, As(III) Concentration	Anion HPLC-ICP-MS	10	69 - 108	93
Matrix Spike	Percent Recovery, DMA Concentration	Anion HPLC-ICP-MS	10	80 - 99	90
Matrix Spike	Percent Recovery, MMA Concentration	Anion HPLC-ICP-MS	10	68 - 104	92
Matrix Spike	Percent Recovery, As(V) Concentration	Anion HPLC-ICP-MS	10	71 - 117	100
Matrix Spike	Percent Recovery, DMA Concentration	Cation HPLC-ICP-MS	10	69 - 108	90
Matrix Spike	Percent Recovery, AB Concentration	Cation HPLC-ICP-MS	10	73 - 100	86
Matrix Spike	Percent Recovery, TMAO Concentration	Cation HPLC-ICP-MS	10	68 -99	80
Matrix Spike	Percent Recovery, AC Concentration	Cation HPLC-ICP-MS	10	71 - 113	87

[a] ICP-MS Limit of Detection (LOD) for total arsenic: 0.5
[b] Arsenic species by HPLC-ICP-MS LOD: 3 µg/L; Limit of quantification (LOQ): 5 µg/L

Curriculum Vitae

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EDUCATION

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Nearing, M. M.; Koch, I.; Reimer, K. J., Arsenic Speciation in Edible Mushrooms. *Environmental Science and Technology* 2014, 48, (24),14203-10

Nearing, M. M.; Koch, I.; Reimer, K. J., Uptake and transformation of arsenic during the vegetative life stage of terrestrial fungi. *Environmental Pollution.* **2015,** *197*, (0), 108-115.

PRESENTATIONS

Nearing, M*., Koch, I., Reimer, K.J. (2014) Uptake, transformation and fate of arsenic compounds in mushrooms. Platform Presentation. Synchrotron Environmental Science VI. Argonne National Laboratories, IL. September 12, 2014.

Nearing, M*., Koch, I., Reimer, K.J. (2014) Arsenic Speciation in Edible Mushrooms. Platform Presentation. 97th Canadian Chemistry Conference and Exhibition; Vancouver, BC. June 3, 2014.

Nearing, M*., Koch, I., Reimer, K.J. (2014) Formation of a Non-toxic Arsenic Compound in Terrestrial Fungi from Arsenic Contaminated Sites in Yellowknife, Northwest Territories. Poster. Federal Contaminated Sites Workshop; Ottawa, ON. April 14, 2014.

Nearing, M*., Koch, I., Reimer, K.J. (2011) Elucidating a Biosynthetic Pathway for the Formation of Arsenobetaine in Agaricus bisporus. Poster. The Society of Environmental Toxicology and Chemistry's 32nd Annual meeting; Boston, MA. November 17, 2011.
**** Represents presenting author***

AWARDS

Real Property Institute of Canada, Federal Contaminated Sites Student Poster Competition, 1st Place 2014

Dean's List (Queen's University) 2009 - 2010