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

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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 *Agaricus bisporus* and analyzed for arsenic speciation using HPLC-ICP-MS. 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 *Agaricus campestris* fruiting body collected from arsenic contaminated mine tailings, was mapped using two dimensional XAS imaging. The primordium and stalk 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* the highest proportion of AB was found in the cap, supporting the AB as an osmolyte hypothesis. The results have allowed us to pinpoint the fungus life stage at which AB formation takes place, namely reproduction, which provides a direction for further research.

Introduction

Arsenobetaine (AB) is the only arsenic compound that has no measured toxicity and is found to comprise the majority of arsenic in many marine organisms (Reimer et al., 2010). However, the 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 (Reimer et al., 2010), 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 (Nearing et al., 2014a).

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

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then converted to AB. The third currently 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 (Edmonds, 2000).

AB is structurally similar to the osmolyte glycine betaine and has been found to be adventitiously accumulated in marine organisms as an osmolyte (Clowes and Francesconi, 2004). AB has also been hypothesized to be similarly incidentally accumulated in mushrooms with other osmolytes that are used to help maintain fruiting body structure for effective spore dispersal (Nearing et al., 2014a; Smith et al., 2007). 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.

Mushrooms that have been found to contain AB as a major compound include those from the genus *Agaricus* (Nearing et al., 2014a; Šlejkovec et al., 1997). *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 (Smith et al., 2007; Soeroes et al., 2005).

In the commercial cultivation of *A. bisporus* a compost layer is inoculated with rye grain seeds overgrown with the mycelium. After the mycelium has grown into the compost layer, a casing layer (peat moss and calcium limestone) is added to the surface to facilitate fruiting body development. The growth room is aired to induce the mycelium to differentiate (*i.e.*, to form hyphal aggregations and knots), giving rise to the first growth stage of the fruiting body, the primordium. The temperature is then decreased, leading to the formation of differentiated tissues of the mature fruiting body (Eastwood et al., 2013), the cap and stipe (stalk). Gills containing the spores mature under the cap as the fruiting body enlarges (Umar and Van Griensven, 1997).

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 (Byrne et al., 1995; Koch et al., 2000; Nearing et al., 2014a; Šlejkovec et al., 1997; Slejkovec et al., 1996; Smith et al., 2007; Soeroes et al., 2005). 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 tetramethylarsonium (TETRA) and AB, but it could not synthesize AB (Slejkovec et al., 1996). *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 (Nearing et al., 2015). For the previously mentioned *A. bisporus* cultivated on arsenic treated material the growth substrate and compost material were also either collected before inoculation (Soeroes et al., 2005) or after harvesting (Smith et al., 2007). To more comprehensively investigate the formation of AB in *A. bisporus* at each part of the reproductive life cycle from the entire biosphere of the fungus, including the microbial community associated with it and the fungus itself, 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 X-ray absorption near-edge structure (micro-XANES) analysis.

1. Methods

1.1. Chemicals and reagents

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

1.2. 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 commercial growth process is further described in Appendix A. The sampling points were determined by the availability at the commercial facility and the samples collected are summarized in Table 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 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.

1.3. Cultivation of *A. bisporus*

Mushroom growing kits were purchased from White Crest Mushrooms, Putnam, ON. The kits were delivered with an

Table 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; >5 cm; mycelium
		Casing	Casing material with mycelium
		Compost	Compost material with mycelium
		Fruiting bodies[b]	Various growth stages of brown strain: <2 cm; >2 cm
		Casing	Casing material with brown strain mycelium
		Compost	Compost material with brown strain mycelium

DM: dry mass.
[a] CACing = substrate 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.

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 mg/L watering solution treatments were performed in duplicate. The kits were grown at 24°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.

1.4. 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 by Foster et al. (2007) and Nearing et al. (2014a). 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 the supplementary information.

1.5. Instrumental analysis

All samples (extracts and residues) were analyzed for total arsenic using inductively coupled mass spectrometry (ICP-MS) (DRC II, Perkin Elmer, Massachusetts, USA). The ICP-MS operating conditions are described by Caumette et al. (2011). Instrument quality control checks were found to be acceptable. All aqueous methanol extracts were analyzed for arsenic speciation using high performance liquid chromatography (HPLC) with ICP-MS (cation and anion exchange). The HPLC-ICP-MS operating conditions are described by Caumette et al. (2011) and are included in the Appendix A. Instrument quality control checks were found to be acceptable.

1.6. 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 (11,868 eV) were collected and analyzed as described in (Nearing et al., 2014a). For two-dimensional imaging whole *A. bisporus* primordia and *Agaricus 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 sec integration time for two-dimensional spectra, and the beam was focused to 5 × 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 (11,919.7 eV). A reference gold foil was measured simultaneously with all samples. Two-dimensional XAS mapping was carried out at 11,875.3 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 (11,868 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 (Koch et al., 2011), and liquid AB, DMA, trimethylarsine oxide (TMAO), and arsenic glutathione $(As(Glu)_3)$ (Smith et al., 2005) previously measured by our group were used for fittings.

1.7. 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 (Nearing et al., 2015). 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 to 6 μg/kg DM and for the mushroom growing kits samples ranged from 2 to 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 the supplementary information (Appendix A Table S1) 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.

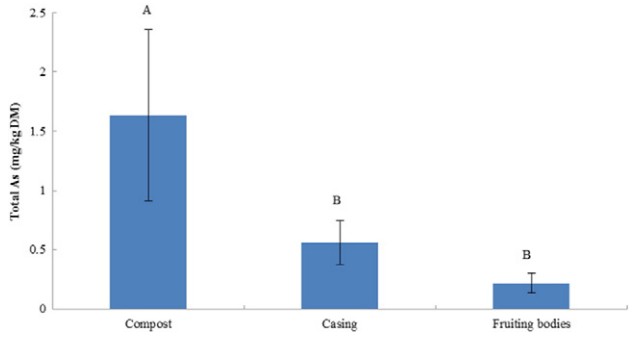

Fig. 1 – Average total arsenic concentrations in compost ($N = 18$), casing ($N = 18$) and fruiting bodies ($N = 18$) dry mass (DM) from all growth stages. Different letters indicate a significant difference between total arsenic concentrations (ANOVA, $p < 0.05$). The error bars represent one standard deviation.

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

2. Results and discussion

2.1. 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 Fig. 1. A total of 18 samples were collected for each sample type and there was significant variation in total arsenic (analysis of variance (ANOVA), F ($F(2, 51) = 52.57$, $p < 0.0001$). The compost material, which provides the nutritious substrate for fruiting body development (Straatsma et al., 2013), contained significantly higher concentrations of arsenic than the casing layer ($p < 0.0001$) and the fruiting bodies ($p < 0.0001$) indicating that arsenic is not bioconcentrated in the fruiting bodies.

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 Fig. 2 as proportions of extracted arsenic. Concentrations of the identified arsenic species, total arsenic and column recoveries for all samples are provided in Appendix A Table S2. 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 species are commonly found in other plants (Reimer et al., 2010) and were also found in the control compost before inoculation used in a previous experiment with *A. bisporus* grown on arsenic treated materials (Soeroes et al., 2005). 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 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

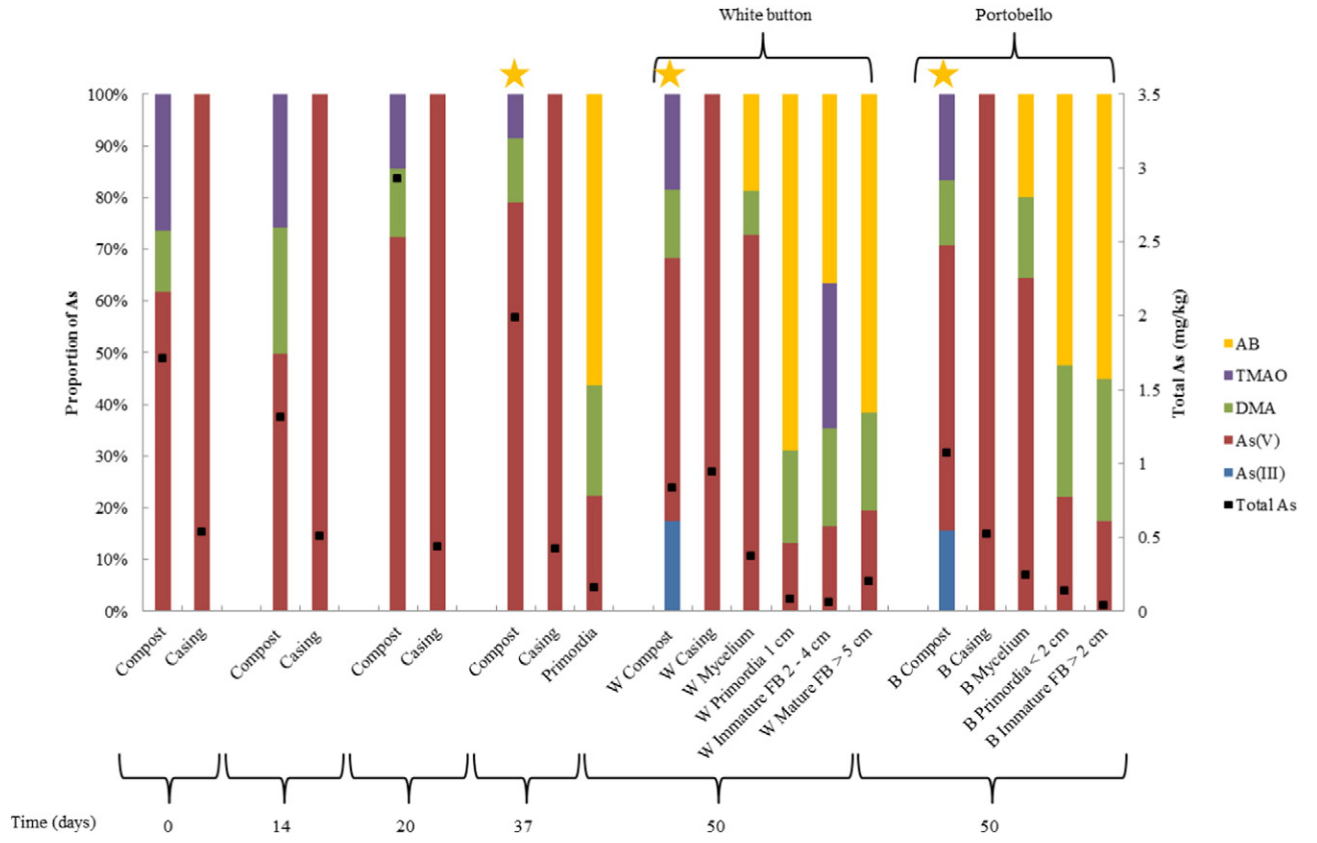

Fig. 2 – Average proportion of extracted (aqueous methanol extracts and 2% nitric acid extracts) arsenic compounds (left axis) determined by HPLC-ICP-MS and total arsenic (right axis and black squares) in compost, casing and fruiting body samples determined by ICP-MS. At day 0 there is no fungus in the 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 labeled above the graph. The yellow star indicates trace AB in sample.

fruiting bodies along with different sizes (corresponding to different growth stages of fruiting bodies) for both the white and brown strains 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, <1 cm, 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%) (Fig. 2). 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 Fig. 2) 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 (Wannet et al., 1999). Mannitol is found at much lower levels in the mycelium (1%–5% DM) compared with the fruiting bodies (Wannet et al., 1999); 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 (Nearing et al., 2015). 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. In the marine environment AB present in algae is

Table 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
Low	6 ± 2	6	4 ± 1	6	0.12 ± 0.04	3
High	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.

likely to be associated with epifauna and bacteria on the surface of the algae (Foster and Maher, 2016). 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.

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.

2.2. *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 mg/kg), *A. bisporus* fruiting bodies were grown on material treated with 10 and 100 mg/L As(V) solutions to represent a low and high treatment, respectively. *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 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 mushroom kits compared with those for the compost from the commercial operation (Appendix A Table S3). The high treatment 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.

The arsenic speciation for the collected fruiting bodies is summarized in Fig. 3, with concentrations of the identified arsenic species, total arsenic and column recoveries for all samples provided in Appendix A Table S4. 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

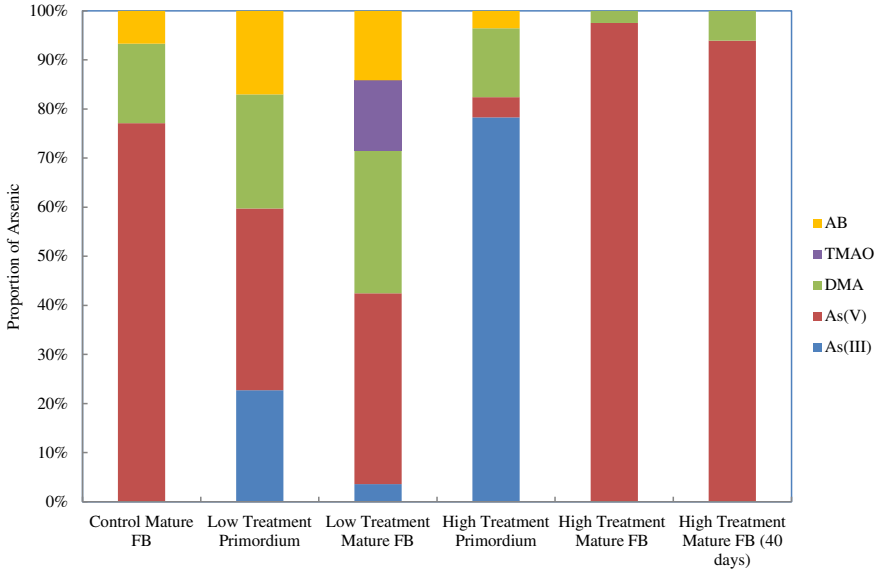

Fig. 3 – Average proportion of extracted (aqueous methanol extracts) arsenic compounds in fruiting body (FB) samples from the control and As(V) treated mushroom kits collected after 30 days determined by HPLC-ICP-MS. The treatment group and size of the fruiting body are indicated in the x-axis labels.

Table 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 (%)					Reduced chi square
	$As(Glu)_3$	As(III)	AB	As(V)	As(V)-glycerol	
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 11,875.3 eV); frozen As(III) (white line energy 11,871.7 eV); As(V)-glycerol (white line energy 11,876.5 eV); liquid DMA(V) (white line energy 11,873.3 eV); TMAO (white line energy 11,873.3 eV); frozen AB (white line energy 11,872.6 eV); and As(Glu)3 (white line energy 11,870.0 eV).

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 (Whaley-Martin et al., 2012). *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 (Soeroes et al., 2005). However mushrooms collected from arsenic-contaminated mine tailings (>1000 mg/kg total arsenic) were found to contain a majority of AB (Nearing et al., 2014a) 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.

A primordium collected from the high treatment mushroom kits was selected for XAS imaging, and also analyzed in bulk by XANES. The bulk XANES analysis linear combination fitting results (Table 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 (Zhao et al., 2009). 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 (Smith et al., 2008) and can also be degraded under chromatographic conditions during HPLC analysis (Nearing et al., 2014b). 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.

A section of the base of the primordium that was selected for mapping, and the distribution of arsenic mapped at 11,875.3 eV is shown in Fig. 4; this section was chosen because the primordium was found to grow around sections of substrate material in the microscope photograph. The section

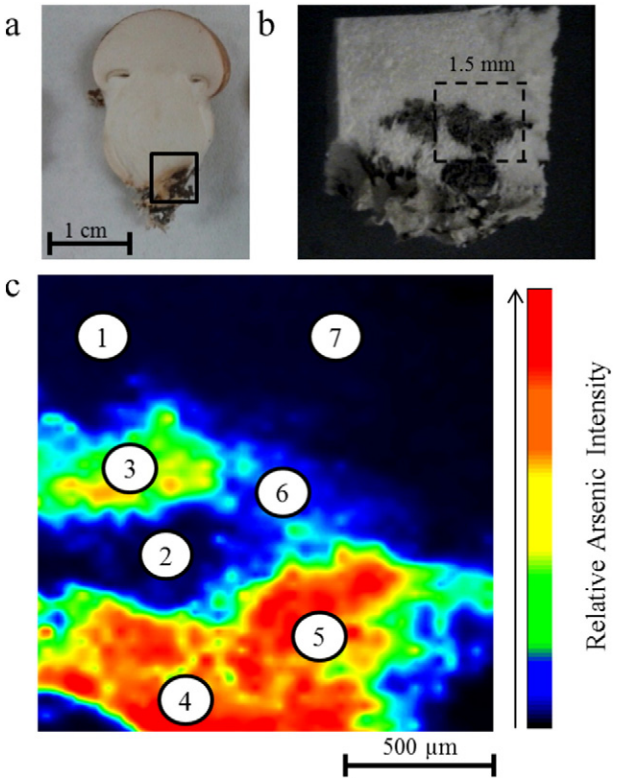

Fig. 4 – (a) Cross section of *A. bisporus* primordium collected from 100 mg/L As(V) treated mushroom growing kit, with area sectioned for XAS imaging indicated by the black box. (b) Image of *A. bisporus* primodium base, with fungus tissues growing around casing/compost material. The dashed black box indicates the area mapped. (c) Map of total arsenic distribution (11,875 eV) for section of *A. bisporus* primordium. The numbers indicate the locations where µXANES were collected.

Table 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 11,875.3 eV); frozen As(III) (white line energy 11,871.7 eV); As(V)-glycerol (white line energy 11,876.5 eV); liquid DMA(V) (white line energy 11,873.3 eV); TMAO (white line energy 11,873.3 eV); frozen AB (white line energy 11,872.6 eV); and As(Glu)3 (white line energy 11,870.0 eV).

surrounding one of these pockets was mapped and μXANES were taken for the locations indicated in Fig. 4c, with the linear combination fitting results for the collected μXANES listed in Table 4. 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.

The mature fruiting body of *A. campestris* collected from arsenic contaminated mine tailings was also selected for XRF 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 (Nearing et al., 2014a). The cap, gill and stalk tissues of the *A. campestris* were separated and analyzed for bulk XANES and XRF imaging, with a summary of the linear combination fittings for the bulk XANES analysis listed in Table 3. XANES analysis was also performed on the tailings material collected with the *A. campestris* mushroom (spectra shown in Appendix A Fig. S3), and shows the tailings are

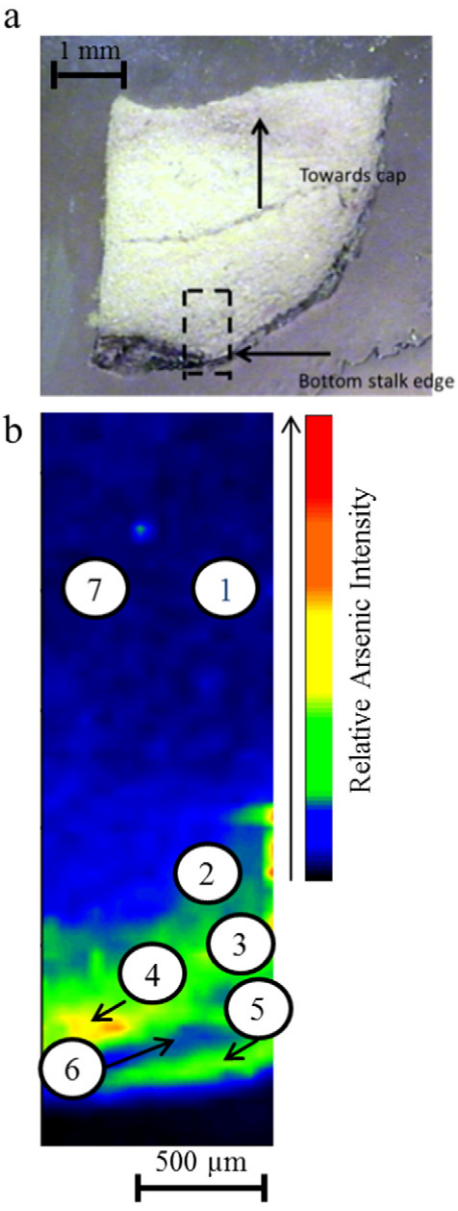

Fig. 5 – (a) Cross section of *A. campestris* stalk collected from arsenic-contaminated mine tailings. The dashed black box indicates the area that was mapped. (b) Map of total arsenic distribution (11,875 eV) for section of *A. campestris* stalk. The numbers indicate the locations where μXANES were collected.

Table 5 – μXANES fitting results for locations on *A. campestris* stalk total arsenic map.

	Proportion of arsenic (%)						
Spot	As(Glu)3	As(III)	AB	DMA/TMAO	As(V)	As(V) glycerol	Reduced chi square
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 11,875.3 eV); frozen As(III) (white line energy 11,871.7 eV); As(V)-glycerol (white line energy 11,876.5 eV); liquid DMA(V) (white line energy 11,873.3 eV); TMAO (white line energy 11,873.3 eV); frozen AB (white line energy 11,872.6 eV); and As(Glu)3 (white line energy 11,870.0 eV).

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 (Koch et al., 2011). 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 proportions 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 XRF imaging. Like the primordium, the section was mapped for total arsenic distribution at 11,875.3 eV and μXANES were taken at points of interest, shown in Fig. 5, with the results of the linear combination fitting for the collected μXANES listed in Table 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.

3. 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 (Nearing et al., 2015). 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 16 sec sequencing of growth material extracts.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jes.2016.06.021.

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