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

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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 dimethylarsinic 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.

Key words

Arsenobetaine, arsenic, mycelium, biotransformation, concentration factor

Capsule

Mycelia of edible mushrooms preferentially accumulate arsenobetaine but do not biosynthesize this non-toxic arsenical.

Introduction

Over 50 arsenic compounds have been identified in the environment and the toxicity of arsenic is greatly dependent on its chemical form (Reimer et al., 2010), but the only arsenic compound that is considered to be non-toxic is arsenobetaine (AB) (Kaise et al., 1985). 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 1) for the formation of AB originate from studies of the marine environment (Caumette et al., 2012).

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 (Edmonds et al., 1997). 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 1). The last step in Pathway 1, the conversion of AC to AB, has been demonstrated in laboratory studies within mice, rats and rabbits (Marafante et al., 1984). However shrimp and sheep did not degrade ingested dimethylated arsenosugars to AC (Francesconi et al., 1999; Hansen et al., 2003), 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 1) and has been

demonstrated to be a major degradation product of arsenosugars in sheep (Hansen et al., 2003). DMAA was also a precursor to AB in laboratory studies involving lysed bacteria extracts (Ritchie et al., 2004). Fish fed DMAA, however, did not form AB (Francesconi et al., 1989). The third proposed formation pathway for AB (Pathway 3 in Figure 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 (Edmonds, 2000).

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 (Stamets, 2000), which is seasonal (10-14 days) in the wild (Kalač and Svoboda, 2000). Commercially cultivated species such as *Agaricus bisporus* (the button mushroom available widely in grocery stores) are grown under conditions that allow multiple fruiting body production events, or flushes (Kües and Liu, 2000).

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 (Reimer et al., 2010). 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. (Reimer et al., 2010). 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 (Koch et al., 1999; Koch et al., 2000) and no arsenosugars were found in the growth material when *A. bisporus* was grown on arsenate (As(V)) treated material (Smith et al., 2007), 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 (Smith et al., 2007), 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 (Šlejkovec et al., 1997); and *Suillus luteus* (common name Slippery Jack) a species from a genus known to contain no AB but primarily DMA in fruiting bodies (Kuehnelt and Goessler, 2003). 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 (Smith et al., 2007). DMAA was selected to test if the fungus could transform DMAA to AB (Pathway 3 in Figure 1). Ideally the mycelium would also be exposed to arsenosugars to test Pathway 1 and 2 in Figure 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 (Madsen et al., 2000). AC was also not used as a treatment because it is infrequently found together with AB (Šlejkovec et al., 1997).

Methods

Fungus Cultures

Chemicals and reagents are listed in the supporting information. Fungus species included *A. bisporus* 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 overgrown the petri dishes. Three pieces of approximately 1 cm² 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.

The experimental design is summarized in Table 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 the supplementary information (Table S2). 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 (Kalač and Svoboda, 2000) 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 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 (Smith et al., 2007).

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 (Smith et al., 2007), whereas AB at higher concentrations was a minor constituent (Soeroes et al., 2005). 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 (Smith et al., 2007).

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.

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% HNO₃ extraction following methods described in Nearing *et al.*, in press (Nearing, 2014). The extract residues were digested with 70% HNO₃ for total arsenic analysis. Methanol was removed from the extracts by evaporation, more details are provided in the supplementary information. Total arsenic was obtained as the sum of the extracted and residual arsenic.

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 (Caumette et al., 2011). 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 (Caumette et al., 2011). Instrument quality control checks were found to be acceptable.

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.*, in press (Nearing, 2014). Details are provided in the supplementary information.

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 supplementary information (Table S1) and were considered acceptable (± 30 % recoveries).

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 than 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.

Results and Discussion

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 the supplementary information (Table S2). 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 2 (data in Table S2); all compounds were concentrated by mycelia (CF > 1).

A two-way ANOVA of the CFs for Experiment A (Figure 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 (Smith et al., 2007; Soeroes et al., 2005) and *S. crispa* contained a majority of AC (Šlejkovec et al., 1997). 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 (Kuehnelt and Goessler, 2003).

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) (Šlejkovec, 1996). 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 (Šlejkovec, 1996), 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.

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 (Byrne et al., 1995; Koch et al., 1999; Koch et al., 2000; Kuehnelt and Goessler, 2003; Šlejkovec et al., 1997). 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 (Clowes and Francesconi, 2004; Smith et al., 2007), specifically in helping to maintain a rigid cap for effective spore dispersal (Smith et al., 2007). *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 (Šlejkovec et al., 1997).

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 2b. The comparison included the As(V) CFs from Experiments A and B at the same conditions (60 days and 100 $\mu\text{g/L}$ As), which were statistically indistinguishable ($p = 0.78$). Like in Figure 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 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 $\mu\text{g/L}$ 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* (Smith et al., 2007).

Arsenic Speciation and Transformations

The average concentrations and standard deviations of arsenic compounds found in the mycelium are listed in Table 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 (Šlejkovec, 1996).

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).

In the *S. crispa* mycelium only As(V) and TMAO (31 ± 3 $\mu\text{g/kg}$ 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 kinetic 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 (Nearing et al., 2014). The high detection limits, 1 – 5 mg/kg (Nearing et al., 2014), limited the number of samples analyzed for XANES to two: the 120 day mycelia treated with As(V) and DMA (Experiment B) (Figure 3a). The proportions of arsenic compounds in the two measurable samples are shown in Figure 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 S1-A). Such compounds are often not seen under HPLC-ICP-MS conditions because they can be unstable and transform to their oxygen analogues (Nearing et al., 2014). 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 (Zhao et al., 2009). The remaining 50% of the arsenic was in the form of As(V) and an As(V)-O glycerol compound (Figure S1-B, a compound thought to be formed from four-coordinated As(V)-O, that is, arsenate, as a result of the sample drying process (Koch et al., 2011)), which generally agrees with the proportion of As(III) and As(V) determined by HPLC-ICP-MS analysis (Figure 3b).

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 (Ritchie et al., 2004).

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 (Boswell et al., 2002; Wannet et al., 1999). 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 (Lomax et al., 2012), and the microbial community in compost material treated with As(V) produces methylated compounds mainly in the form TMAO (Smith et al., 2007), 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 (Šlejkovec et al., 1997), 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 (Kalač and Svoboda, 2000), 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 (Smith et al., 2007). 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 (Šlejkovec et al., 1997). 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 (Lambou et al., 2013).

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Table 1: Summary of experimental design

Experiment	Fungus species	Controls	Medium	Treatments	Length of exposure (days)
A	Uninoculated negative control (PD and YM)		PD and YM	No As (n = 6)	60
	Uninoculated positive controls (PD and YM)		PD and YM	100 µg/L As(V) (n = 3)	60
				100 µg/L DMAA (n = 3)	60
				100 µg/L AB (n = 3)	60
	<i>A. bisporus</i>	Inoculated negative control (n=3)	PD	No As (n = 3)	60
	<i>S. crispa</i>	Inoculated negative control (n=3)	PD	No As (n = 3)	60
	<i>S. luteus</i>	Inoculated negative control (n=3)	YM	No As (n = 3)	60
	Uninoculated negative control (n = 3)		PD	No As (n = 3)	60
	Uninoculated positive controls		PD	100 µg/L As(V) (n = 3)	60, 90, 120
				100 µg/L DMA (n = 3)	60, 90, 120
B	<i>A. bisporus</i>	Inoculated negative control (n=3)	PD	No As (n = 3)	60, 90, 120
	Uninoculated negative control (n = 3)		PD	No As (n = 3)	30
				250 µg/L MMA (n = 3)	
				250 µg/L DMA (n = 3)	
C	Uninoculated positive controls		PD	250 µg/L TMAO (n = 3)	30
				250 µg/L MMA (n = 3)	
	<i>A. bisporus</i>	Inoculated negative control (n=3)	PD	No As (n = 3)	30

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 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		
<i>A. bisporus</i>	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
<i>S. luteus</i>	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
	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
<i>S. crispa</i>	AB	trace	nd	nd	nd	nd	nd	70 ± 30	100 ± 20	87 ± 10
	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

Column recovery = (Sum of arsenic compounds/Total As in extract)*100, Extraction Efficiency = (Total As in extract/Total As in mycelium)*100

Figure 1: Current proposed formation pathways for arsenobetaine (AB).

A)

B)

Figure 3A: 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.

Figure 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 $\mu\text{g/L}$ As for 30 days. B) *A. bisporus* Experiment A (As(V) Exp A, DMAA and AB at 100 $\mu\text{g/L}$ As 60 days), Experiment B (As(V) Exp B and DMAA at 100 $\mu\text{g/L}$ As 60 days) and C (MMA, DMA and TMAO at 250 $\mu\text{g/L}$ 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).