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Uptake and biotransformation of arsenate in the lichen *Hypogymnia physodes* (L.) Nyl.

Tanja Mrak [a], Zdenka Šlejkovec [a,*], Zvonka Jeran [a], Radojko Jaćimović [a], Damijana Kastelec [b]

[a] *Department of Environmental Sciences, Jožef Stefan Institute, Jamova 39, 1000 Ljubljana, Slovenia*
[b] *Agronomy Department, Biotechnical Faculty, University of Ljubljana, Jamnikarjeva 101, 1000 Ljubljana, Slovenia*

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Lichens are able to metabolize the inorganic arsenic taken up.

Abstract

The uptake and metabolism of arsenate, As(V), as a function of time and concentration were examined in the lichen *Hypogymnia physodes* (L.) Nyl. Lichen thalli were exposed to As(V) in the form of a solution. Exponential uptake of As(V) from 4 μg mL^{-1} As(V) solution was accompanied by constant arsenite, As(III), excretion back into the solution. Arsenate taken up into the lichens from 0, 0.1, 1, 10 μg mL^{-1} As(V) solutions was partially transformed into As(III), dimethylarsinic acid (DMA) and (mono)methylarsonic acid (MA). 48 h after exposure, the main arsenic compound in the lichens was DMA in 0.1, As(III) in 1 and As(V) in 10 μg mL^{-1} treatment. The proportion of methylated arsenic compounds decreased with increasing arsenate concentration in the exposure solution. These results suggest that at least two types of As(V) detoxification exist in lichens; arsenite excretion and methylation.

Keywords: Arsenic compounds; Methylation; *Hypogymnia physodes*; Lichens

1. Introduction

Lichens are one of the most popular bioindicators and biomonitors, but usually they are employed only to follow the total concentrations of elements in the environment and not their chemical form. However, the toxicity of elements strongly depends on their chemical form. One element for which very different toxicity of its chemical forms was demonstrated, is arsenic (EHC 224, 2001).

Lichens were reported to grow in environments with elevated arsenic content, such as hot springs, mines and mining wastes (gold, lead-zinc), abandoned arsenic smelter sites and ammunition destruction sites (Koch et al., 1999,2000; Kuehnelt et al., 2000; Mrak et al., 2006; UNEP, 2003). Arsenic content in lichens can be as high as 520 and 2300 μg g^{-1} dry weight (dw), as reported by Koch et al. (2000).

In the work of Farinha et al. (2004), Mrak et al. (2006) and Machado et al. (2006) it was presumed that lichens do not act as passive biomonitors of arsenic compounds, but are able to transform them after taking them up from the environment. The biotransformation of arsenate generally occurs via reduction to arsenite and then via methylation, but disagreement exists about the metabolic pathways. The classical metabolic pathway proposes the formation of dimethylated arsenic compounds via monomethylated ones, whereas a new metabolic pathway argues for the separate formation of mono- and dimethylated arsenic compounds as end products in humans (Hayakawa et al., 2005). In some organisms, further biotransformation is possible and may result in the formation of trimethylarsine oxide (TMAO), tetramethylarsonium ion (TETRA), arsenobetaine (AB), arsenocholine, arsenosugars and arsenolipids (Meharg and Hartley-Whitaker, 2002).

* Corresponding author. Tel.: +386 1 5885 354; fax: +386 1 5885 346.
E-mail address: zdenka.slejkovec@ijs.si (Z. Šlejkovec).

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The present study was divided into two parts. In the first part, the time course of arsenate uptake from solution by the lichen *Hypogymnia physodes* (L.) Nyl. was examined. In the second part, it was hypothesized that lichens are capable of the biotransformation of arsenate taken up from the solution. Since the biotransformation may be dependent on the time available for physiological activity of the lichen and on the concentration of the arsenate taken up into the lichen, the influence of differently concentrated arsenate solutions and different incubation times after exposure to arsenate solutions on the presence of inorganic and organic arsenic compounds was tested.

2. Materials and methods

2.1. Preparation of lichen material

Thalli of the foliose lichen *H. physodes* were collected in March 2006 from untreated wooden fences in Gradišče v Tuhinju (590 m alt.), Slovenia, where background arsenic concentrations were expected (Jeran et al., 2002). In the laboratory all visible extraneous material was removed from the lichens. Experiments started 4 days after the collection of lichen material. To reestablish metabolic processes, lichen thalli were rehydrated in doubly distilled water (Milli-Q, Millipore) for 30 min, blotted with paper towels to remove remaining non-absorbed water on the outside of the thalli and incubated on moist filter paper in Petri dishes for 1 day in a growth chamber at 17 °C, a 16/8 h light/dark photoperiod, photon flux 250 μmol s^{-1} m^{-2} and 80% relative air humidity. Approximately 18 g of fresh weight (fw) of lichens treated this way was taken as the blank.

2.2. Arsenate treatment

Solutions of arsenic in the form of arsenate As(V) with concentrations of 0 (control), 0.1, 1, 4 and 10 μg mL^{-1} were prepared from a 1000 μg mL^{-1} standard solution (Merck, Titrisol) by dilution with Milli-Q water. The pH of the solutions was adjusted to 4.6 (Richardson et al., 1984) using analytical grade NaOH and suprapure HCl. Arsenic treatments were performed in the growth chamber.

2.2.1. Time-course of arsenate uptake and arsenic speciation

Fourteen thalli of around 0.5–1.2 g (fw) were chosen to study the time course of arsenic uptake and its variability between thalli. Thalli were placed in 50 mL plastic centrifuge tubes, 30 mL of As(V) solution with the concentration of 4 μg mL^{-1} was added, and then shaken for 24 h at 85 motions per minute (mot/min) in the growth chamber. 750 μL of exposure solution was sampled after 1, 3, 5, 7, 22 and 24 h of incubation from each centrifuge tube and transferred to a pre-weighed Eppendorf tube. To minimize the possibility of transformation of arsenic compounds, samples of exposure solutions were frozen immediately in liquid nitrogen. The frequency of sampling was chosen according to the expected highest uptake rates in the first hours of the exposure as found by Richardson et al. (1984) for the lichen *Umbilicaria muhlenbergii* (Ach.) Tuck. In samples of exposure solutions collected over 24 h at six different sampling times (14 samples of exposure solutions per each sampling time) the concentration of total arsenic was measured and arsenic compounds determined.

Subsequent to exposure, thalli were taken from the exposure solution, blotted with paper towels and quickly washed with 100 mL of Milli-Q water, blotted again, frozen immediately in liquid nitrogen, freeze-dried, weighed and milled in a Retsch MM 200 ball mill. The concentration of total arsenic in powder of selected lichen thalli ($n = 10$) was determined.

2.2.2. Concentration-dependent arsenate uptake and biotransformation of arsenate by lichens in time

Approximately 25 g fw of lichens (composite samples) were weighed into a 250 mL plastic container and 250 mL of either 0 (control), 0.1, 1 or 10 μg mL^{-1} of As(V) solution added (four concentration treatments). After 24 h-shaking at 130 mot/min in different As(V) solutions, lichens were blotted with paper towels, washed in 250 mL of Milli-Q water, blotted again and then subjected to the following three treatments according to the time of incubation:

- One third of the lichens, that is 2 composite samples of 25 g fw per each As(V) concentration treatment, were frozen immediately in liquid nitrogen (0 h incubation).
- Two thirds of the lichens, that is 4 composite samples per each As(V) concentration treatment, were transferred to moistened filter paper in Petri dishes, incubated for 24 or 48 h in the growth chamber and then frozen in liquid nitrogen (24 and 48 h incubation).

All samples were freeze dried, weighed and milled in a ball mill. The concentration of total arsenic was determined in powdered lichen samples and in their extracts. In extracts, analyses of arsenic compounds were also performed. Extraction of lichens was performed from 1 g of powdered dry material by adding 50 mL of Milli-Q water. The samples were immersed 3-times in liquid nitrogen and subsequently in hot water to break down the cells. Extraction was performed at 60 °C for 16 h in the shaking water bath (Mrak et al., 2006).

To determine the extent of non-biological transformation of inorganic arsenic due to exposure and extraction, spiking of lichen extracts, exudates and Milli-Q water with As(V) and As(III) standards was done and then both procedures imitated. Since negligible transformations were recorded for both procedures, the results are not shown here.

2.3. Analytical procedures

2.3.1. Determination of total arsenic and arsenic compounds

The concentration of total arsenic in solutions and extracts was determined by flow injection on a hydride generation atomic fluorescence spectrometry system coupled with on-line UV-decomposition, (UV)-HGAFS. Each sample was measured in three to four replicates.

Arsenic compounds were determined by high performance liquid chromatography (HPLC) coupled with (UV)-HGAFS (Šlejkovec et al., 1999). Measurements of each sample were done in two replicates. Samples were checked for the presence of anionic (As(III), DMA, MA, As(V)) and when necessary also for the presence of cationic compounds (AB, arsenocholine, TMAO, TETRA). However, the presence of cationic compounds was not detected.

During the HPLC-(UV)-HGAFS analyses of extracts the problem of unexpectedly high peaks of As(III) overlapping with much smaller peaks of DMA and MA was met and solved by precipitation of As(III) with 2.5% sodium dibenzyldithiocarbamate in methanol, adapting the method described by Van Elteren et al. (1991).

Regular analysis of standard reference materials and comparison of the data for arsenic compounds with the literature values was performed as quality control of the HPLC-(UV)-HGAFS method (Šlejkovec et al., 1999).

The concentration of total arsenic in lichen powder was determined by k_0-standardized instrumental neutron activation analysis (k_0INAA) (Jaćimović et al., 2003). Powdered lichens were pressed into 180 mg tablets and irradiated in the TRIGA Mark II reactor, Ljubljana, at a neutron fluence rate of 1.1×10^{12} n cm^{-2} s^{-1} for 20 h.

2.4. Statistics

In the first experiment the dependence of arsenic concentration on time of incubation and its relationship with the mass of thalli was examined. The analysis of variance component (ANOVA) was used to estimate the portion of variability of the data expressed within different thalli and the portion of variability of the data caused by the measurement procedure. The correlation between thalli mass and arsenic concentration in the exposure solution was tested on the basis of the non-parametric Spearman correlation coefficient.

In the second experiment multivariate analysis of variance (MANOVA) was used to describe the influence of different As(V) concentrations and the influence of time of incubation on the changes in the structure of arsenic components (As(III), As(V), DMA and MA). To satisfy the assumptions of MANOVA, the data were transformed according to compositional data analysis (Aitchison, 2003). Polynomial contrasts were used to test the changes in the concentrations of arsenic components with time (linear and quadratic trend).

3. Results

3.1. Time-course of arsenate uptake and arsenic speciation

The concentration of total arsenic in exposure solutions decreased rapidly after the onset of exposure (Fig. 1, Table 1). The mean minimum concentration of arsenic was recorded already after 3 h of exposure. Data on individual thalli (not presented) have shown that exceptionally the minimum concentration was recorded after 5 or 7 h. Since no sampling was done between 7 and 22 h, it is also possible, that in this latter case minimum concentration occurred even later. Shortage of data between 7 and 22 h of exposure also prevents mathematical modeling of the uptake/release curve. After the minimum was achieved, the total concentration began to increase slowly and after 24 h 45.4% of the initial value was achieved.

Variability between arsenic concentrations in the exposure solutions could at certain times be explained by the mass of lichen thalli (Fig. 2). After 1 and 3 h, there was a negative relationship between small lichen masses (<approximately 0.17 g) and the concentration of total arsenic in the exposure solution, indicating lower arsenic uptake into lichen thalli with lower weight. After 22 h, the situation was reversed, with a positive relationship between small lichen masses and the concentration of total arsenic in the exposure solution, indicating lower arsenic release from smaller thalli.

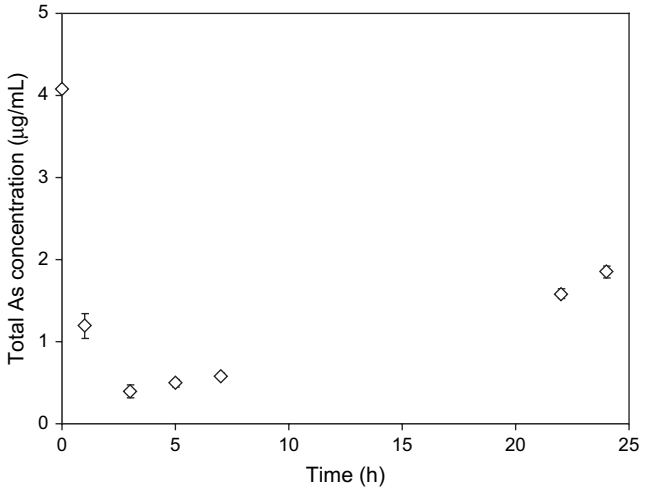

Fig. 1. Depletion diagram for the concentration of total arsenic (mean ± standard error) in exposure solutions over 24 h ($n = 14$). Total arsenic concentrations were corrected to the initial mass of the exposure solutions to allow for the removal of aliquots in the course of the experiment.

Exposure of individual thalli to arsenate resulted in the transformation of arsenate into arsenite in the exposure solution (Fig. 3). The exponential decrease of As(V) concentration was observed simultaneously with a decrease in the concentration of total arsenic, but the As(III) concentration constantly increased with time. After the minimum total arsenic was achieved, As(V) concentrations were below 5% of their initial values.

Determination of the concentration of total arsenic in selected individual thalli ($n = 10$) by k_0INAA gave a mean value of 432 ± 46 μg g^{-1} dw (min. 243, max. 696 μg g^{-1} dw). Estimations of arsenic concentration in individual thalli ($n = 10$, the same set of thalli as for k_0INAA) based on the values for arsenic concentration in exposure solutions after 24 h and the dry weight of exposed lichens gave similar results (395 ± 45 μg g^{-1} dw).

3.2. Concentration dependent uptake and biotransformation of arsenate by lichens

Since no statistically significant differences in concentrations of total arsenic were found between different times of incubation (not shown), data were combined together and mean values calculated (Table 2). In blank lichens, the concentration of total arsenic was similar to that of the control lichens, exposed to 0 μg mL^{-1} arsenic solution. It seems that during the exposure process very small amounts of the initial arsenic content were excreted into the exposure solutions. Lichens exposed to 1 μg mL^{-1} arsenic accumulated 21-times more arsenic than lichens exposed to 0.1 μg mL^{-1} and 16 times less than lichens exposed to 10 μg mL^{-1} (Table 2), indicating that not only passive processes are involved in the accumulation.

The extractability of the initial arsenic content from control lichens was low. If this initial (background) concentration of arsenic in lichens was neglected, then the extractability in exposed lichens amounted to 81.1–94.8% (Table 2). In extracts of control samples (0 μg mL^{-1} treatment) only trace amounts of arsenite (4.8 ± 0.5 ng g^{-1} dw) and arsenate (8.1 ± 1.1 ng g^{-1} dw) were found, whereas DMA and MA were below the detection limits. Part of the arsenate taken up into lichens from 0.1, 1 and 10 μg mL^{-1} solutions was transformed into arsenite, DMA and MA (Fig. 4). The extent of transformation varied according to the concentration of arsenate in the exposure solution and the incubation time after arsenate exposure, as shown in Tables 3a,b and Fig. 5.

In the 0.1 μg mL^{-1} treatment, the major metabolite in the first 0–24 h after arsenate exposure was arsenite. Immediately after arsenate exposure the order of arsenic compounds in decreasing concentration level was: As(III) > As(V) > DMA > MA. After 24 h of incubation the following order was recorded: As(III) > DMA > As(V) > MA. After 48 h DMA became the major metabolite together with arsenite: DMA ≥ As(III) > As(V) > MA. There were no significant changes in the ratios of As(III) to As(V) and MA to As(V) with time of incubation. In the case of the DMA to As(V) ratio, a significant increase was observed with time, being

Table 1
ANOVA results for the time course of arsenate uptake

	Time of exposure					
	1 h	3 h	5 h	7 h	22 h	24 h
Variability caused by individual thalli in the exposure solution (%)	98.3	99.5	99.1	97.0	96.9	96.0
Variability between replicates of measurement on the same sample (%)	1.7	0.5	0.9	3.0	3.1	4.0
Mean concentration (μg mL^{-1})	1.19	0.39	0.49	0.58	1.58	1.85
% of initial concentration	29.2	9.6	12.1	14.3	38.9	45.4
Coefficient of variation (%)	12.6	19.9	8.1	5.3	3.6	3.7
95% confidence limits (%)	21.8–36.6	5.8–13.5	10.1–14.1	12.7–15.8	36.1–41.7	42.0–48.8

greater in the first 24 h of the incubation, but without significant changes between 24 and 48 h of incubation.

In the 1 μg mL^{-1} treatment, the major metabolite at all sampling times was As(III), followed by As(V) > DMA > MA (Fig. 4). In the 10 μg mL^{-1} treatment, the major arsenic compound in extracts of lichens was As(V), followed by As(III) ≫ DMA > MA for the 0 h incubation, and As(III) ≫ MA > DMA after 24 and 48 h incubations. In the 1 μg mL^{-1} and 10 μg mL^{-1} treatments, the ratios of As(III) to As(V) increased linearly with time. In the case of the 1 μg mL^{-1} treatment both the MA to As(V) ratio and the DMA to As(V) ratio increased significantly with time of incubation. Whereas the increase of DMA to As(V) ratio was linear with time, the MA to As(V) ratio was already approaching its maximum with a greater difference between 0 and 24 h and a smaller, but still significant difference between 24 and 48 h. Trends in MA and DMA ratios to As(V) at 10 μg mL^{-1} were less clear and statistically insignificant. At the beginning of incubation, the quantities of MA and DMA increased, but after 48 h they were already decreased.

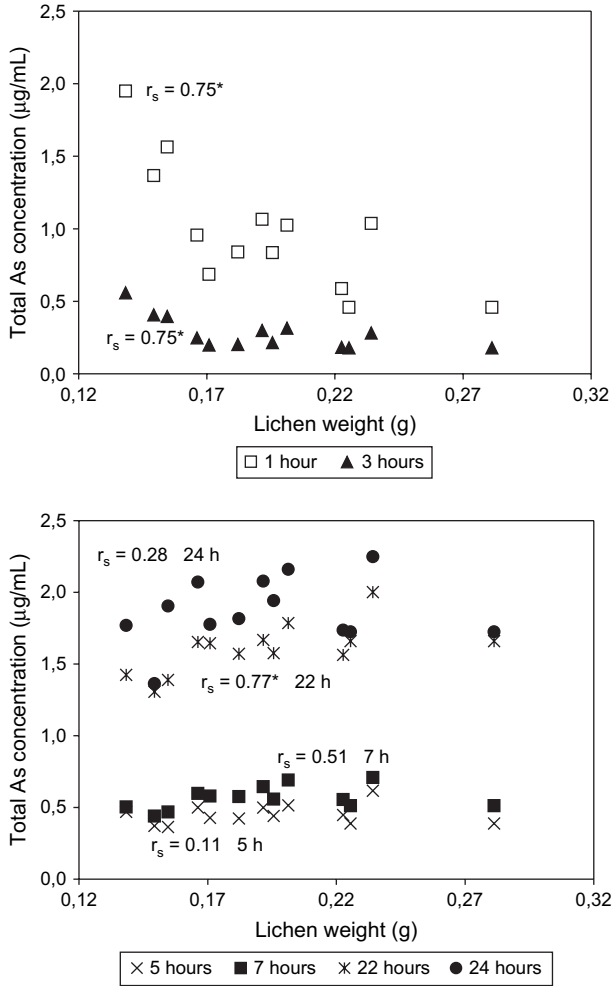

Fig. 2. Relationship between the dry weight of lichen thalli and the concentrations of total arsenic in the exposure solutions over 24 h. r_s: Spearman correlation coefficient, * denotes statistical significance at $p < 0.01$.

4. Discussion

The time-course of arsenate uptake in lichens was characterized by an unusual type of depletion/uptake curve of total arsenic, indicating an initial uptake of arsenic into the lichen thalli followed by its excretion back into the exposure medium. The few studies dealing with arsenate uptake versus time in organisms other than lichens reported an exponential type of curve (e.g. Wang et al., 2002; Cánovas et al., 2003).

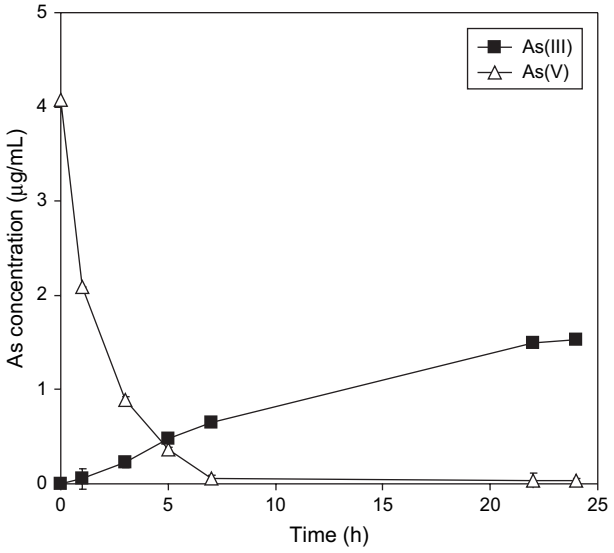

Fig. 3. Time-course of the concentrations of As(V) and As(III) in the exposure solution over 24 h, shown for one case. Concentrations were corrected to the initial mass of the exposure solution. Error bars (mean ± standard error) denote accuracy of measurements. Where not shown, they do not exceed the size of the symbols.

Table 2
Mean (±standard error) concentrations of total arsenic and of total extracted arsenic in *H. physodes* exposed to 0, 0.1, 1 and 10 μg ml^{-1} As(V) solutions ($n = 6$ composite samples)

Treatment	Total As (μg g^{-1} dw)	Total extracted As (μg g^{-1} dw)	Extractability of total As (%)	Accumulated As[a] (μg g^{-1} dw)	Extractability of accumulated As[a] (%)
Blank	0.28	0.12	42.2	–	–
0 μg mL^{-1}	0.25 ± 0.01	0.06 ± 0.01[b]	23.2[b]	0	0
0.1 μg mL^{-1}	0.80 ± 0.03	0.46 ± 0.02	57.8	0.55	84.7
1 μg mL^{-1}	11.8 ± 0.43	9.32 ± 0.19	79.3	11.5	81.1
10 μg mL^{-1}	187 ± 5.36	177 ± 2	94.6	187	94.8

[a] Values for accumulated As were calculated by subtracting values for total arsenic in control lichens (0 μg mL^{-1} treatment) from values for total arsenic in lichens exposed to 0.1, 1 and 10 μg mL^{-1}.
[b] Value for total extracted arsenic in 0 μg mL^{-1} treatment must be treated with caution due to possible overestimation of the peaks. Due to the very low concentrations of arsenic in extracts, samples were heavily concentrated. The tailing of peaks was observed in chromatograms due to matrix effects.

Even the only existing study on arsenate uptake in lichens reported the exponential type of the curve when the lichen was exposed to 20 μg mL^{-1} (13.4 μM) arsenate solution for 21 h (Richardson et al., 1984). However, a similar phenomenon to ours was observed by Sharples et al. (2000), who reported that the initial accumulation of arsenate was followed by its decrease after 2 h of exposure in the arsenate non-resistant genotype of ericoid mycorrhizal fungus *Hymenoscyphus ericae* (Read) Korf and Kernan, exposed to 0.75 mM arsenate solution. The decrease of accumulation was explained by cell death in response to arsenate toxicity. In our experiment, the decrease of the initial concentration of arsenic to its minimum value in the exposure solution was very fast (mostly in 3 h) and was comparable to that of the arsenic hyperaccumulator fern *Pteris vittata* L., where the initial 5 μM concentration of arsenate in the exposure solution decreased to its minimal value after about 4 h (Wang et al., 2002).

The time-course curve of arsenic compounds in exposure solutions of *H. physodes* samples (Fig. 3) indicates that the reason for the unusual depletion/uptake curve is arsenite excretion. Arsenite excretion may have occurred as an active process of extrusion via membrane transporters following arsenate reduction inside the cells. For bacteria and yeasts, arsenite is excreted in its original form, but in mammals it is complexed to three molecules of gluthathione (Rosen, 2002). The excretion of arsenite was also observed in *H. ericae*, transferred into an arsenate-free medium after arsenate exposure. After 5 h in an arsenate-free medium, the arsenate-resistant genotype of *H. ericae* lost 83% of its initial arsenic concentration (71.6% in the form of arsenite), whereas for the non-resistant genotype the excretion was lower (13% of initial arsenic content after 5 h, 71.3% in the form of arsenite). Excretion of arsenite was also observed in the arsenate exposed freshwater microalgae *Closterium aciculare* T. West and *Monoraphidium arcuatum* (Korshikov) Hindák (Hasegawa et al., 2001; Levy et al., 2005). Excretion of inorganic arsenic was also reported for *Chlorella vulgaris* Beijerinck, but unfortunately no data on inorganic arsenic compounds in the excretes were given. The percentage of inorganic arsenic excretion after 3 days of incubation on arsenate-free medium was dependent on the total arsenic content in the algae and type of the incubation medium (Maeda et al., 1992). Since there were no statistically significant differences in total arsenic content in lichens incubated for 0, 24 and 48 h upon arsenate exposure, the possibility of arsenic volatilization in *H. physodes* in this timeframe was excluded. Volatilization of arsenic occurs through the reduction of various arsenic compounds to volatile arsines and was reported for several fungi (reviewed in Bentley and Chasteen, 2002), but it seems not to occur in *H. physodes*, at least not in the first 48 h after arsenic exposure.

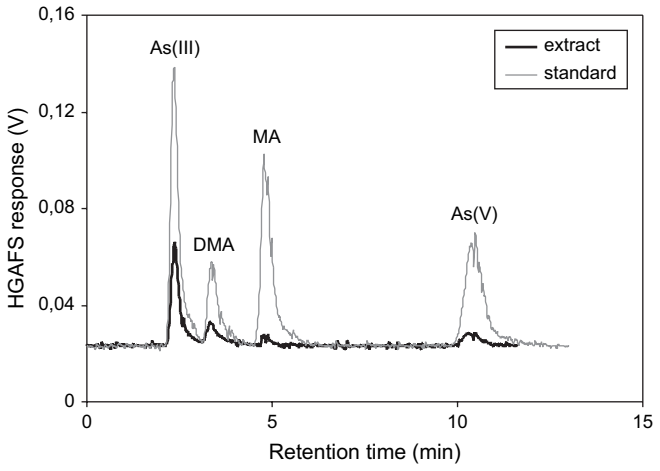

Fig. 4. HPLC-HGAFS separation (Hamilton PRP-X 100, 250 × 4.1 mm; 15 mmol L^{-1} KH_2PO_4, pH 6.1) of anionic arsenic compounds in extracts of lichens, exposed to a solution of 0.1 μg mL^{-1} As(V) for 24 h and then incubated for 48 h.

Practically all the arsenic accumulated during the exposure phase of the experiment could be extracted from the lichens. This high extraction efficiency of accumulated arsenic is in contrast to the very low extraction efficiency from lichens exposed to sources of arsenic in-situ. Very low extractability of arsenic was thus observed for control lichens. The low extractability of arsenic from in-situ lichens was found to be related to the presence of arsenic bound to lichen-trapped soil particles (Mrak et al., 2006).

Reduction and methylation in *H. physodes* were detected very soon after arsenate exposure. They occurred already during the 24-h period of arsenate exposure, which is not consistent with the results for transplanted lichens where methylation was reported to occur with some delay, possibly as a reaction to the transplantation (Machado et al., 2006). It

Table 3a
Mean concentrations (ng g^{-1} dw) and coefficients of variation, CV, for arsenic compounds in lichens, exposed to As(V) and afterwards incubated for different times

Treatment As(V) concentration	Incubation time (h)	As(III)		DMA		MA		As(V)	
		Mean	CV (%)	Mean	CV (%)	Mean	CV (%)	Mean	CV (%)
0.1 μg mL^{-1}	0	329	2.3	33.9	30.0	28.6	8.0	69.9	20.7
	24	206	19.0	121	14.6	19.9	10.1	46.7	4.9
	48	160	15.9	171	4.8	19.3	5.1	52.3	4.1
1 μg mL^{-1}	0	5310	13.6	136	3.0	109	0.7	3790	4.6
	24	5800	8.8	294	12.3	167	2.1	2090	3.2
	48	7050	3.5	495	23.6	239	2.8	1750	1.8
10 μg mL^{-1}	0	34,900	8.5	102	9.9	191	10.6	138,000	1.8
	24	64,800	9.8	184	11.5	339	13.6	146,000	7.0
	48	68,500	8.9	120	2.2	233	20.4	108,000	10.3

is possible that late methylation in transplanted lichens reflects slow desorption of arsenic from particles trapped on the surface or on the inside of the lichen thalli, which is then followed by immediate biotransformation. In the present work it was shown that the reduction of arsenate and further methylation in lichens occurred due to biological activity. As for transplanted lichens (Farinha et al., 2004; Machado et al., 2006), also here only As(V), As(III), DMA and MA were detected. In the alga *C. vulgaris*, the production of trimethylarsenic compounds was found to follow a few days after the production of monomethyl- and dimethyl-arsenic compounds (Maeda et al., 1992); therefore it is possible that also in lichens further methylation is a delayed process. From in-situ samples of *H. physodes*, collected from an environment with elevated arsenic levels, TMAO, AB and arsenosugar phosphate-ribose were reported, in addition to As(V), As(III), DMA and MA (Mrak et al., 2006). In our experiment, the extent of transformation varied according to the concentration of arsenate in the exposure solution and the incubation time after arsenate exposure. For 0.1 μg mL^{-1} it seems that the degree of reduction already achieved its maximal value after 0 h incubation and later the concentration of As(III) decreased due to the formation of methylated arsenicals, especially DMA. After 48 h incubation, the content of DMA was already comparable to the content of As(III), reaching 38% of the sum of the determined arsenic compounds. In 1 μg mL^{-1} and 10 μg mL^{-1} treatments the degree of reduction increased linearly with time and did not achieve its maximum until the end of the experiment. The percentage of methylated arsenic compounds was very low: up to 7.7% in the 1 μg mL^{-1} and 0.25% of the sum of arsenic compounds in the 10 μg mL^{-1} treatment. A decrease in the relative content of methylated arsenic compounds with increasing concentration of arsenate in the exposure medium was also observed by Maeda et al. (1992). The influence of concentration on the extent of biotransformation was also reported for the brown marine macroalga *Fucus serratus* L. and explained by saturation of the alkylation process at higher exposures, leading to accumulation of arsenite and subsequent toxicity (Geiszinger et al., 2001). On the other hand, the low extent of methylation at higher exposures could also indicate that methylation is carried out by the photobiont of *H. physodes*, which quantitatively represents the minor part of the thallus volume. The quantity of DMA after 24 and 48 h of incubation following 10 μg mL^{-1} arsenic exposure was lower than in the 1 μg mL^{-1} treatment. Easterling et al. (2002) proposed a kinetic model where As(III) inhibits the formation of DMA from MA, but possibly the formation of DMA was depressed by toxic effects of As(V) or As(III). For both inorganic arsenic compounds, toxic effects on plant metabolism were reported (Meharg and Hartley-Whitaker, 2002). The relatively small and constant amount of MA in the case of the 0.1 μg mL^{-1} exposure is not in agreement with the new arsenic

Table 3b
The mean ratios of As(III), DMA and MA to As(V)

Treatment As(V) concentration	Incubation time (h)	As(III)/As(V)		DMA/As(V)		MA/As(V)	
		Mean ratio	*p*-value	Mean ratio	*p*-value	Mean ratio	*p*-value
0.1 μg mL^{-1}	0	4.710 a	0.2598	0.486 a^{2}	0.0008	0.409 a	0.4236
	24	4.405 a		2.581 b^{2}		0.427 a	
	48	3.059 a		3.277 b^{2}		0.369 a	
1 μg mL^{-1}	0	1.404 a^{1}	0.0009	0.036 a^{1}	0.0024	0.029 a^{2}	0.0001
	24	2.779 b^{1}		0.141 b^{1}		0.080 b^{2}	
	48	4.038 c^{1}		0.284 c^{1}		0.137 c^{2}	
10 μg mL^{-1}	0	0.252 a^{1}	0.0099	0.001 a	0.0571	0.001 a	0.1448
	24	0.444 b^{1}		0.001 a		0.002 a	
	48	0.633 b^{1}		0.001 a		0.002 a	

The letters a, b and c denote statistical differences between mean ratios due to the time of incubation. There is no statistical difference between means with the same letter. [1] denotes statistically significant linear trend of ratios with time of incubation. [2] denotes statistically significant quadratic trend of ratios with time of incubation.

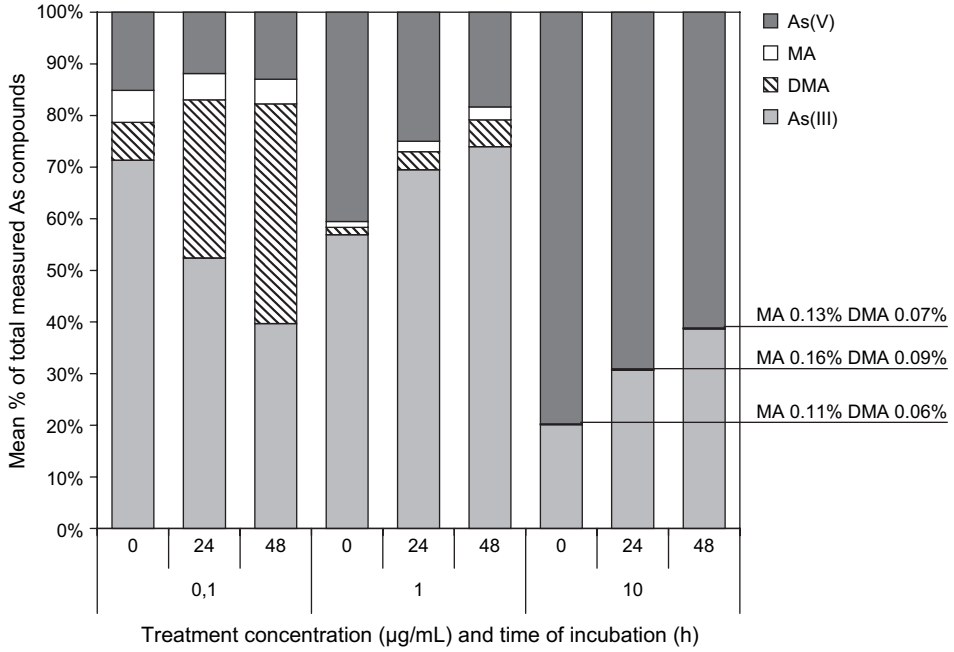

Fig. 5. Mean percentages of concentrations of arsenic compounds in lichens exposed to differently concentrated solutions of As(V) and afterwards incubated for different times.

biotransformation pathway proposed by Hayakawa et al. (2005), where both MA and DMA were the suggested end products of methylation. Our results indicate that MA occurs as an intermediate in the methylation pathway and the formation of DMA represents the limiting step that allows for the accumulation of MA. Zakharyan and Aposhian (1999) reported that rabbit liver MA-reductase was the rate-limiting enzyme in arsenic biotransformation. According to the classic biotransformation pathway, MA is transformed via MA-reductase into the intermediate MA(III), which is further methylated into DMA.

5. Conclusions

In this work the ability of lichens to actively transform arsenate was confirmed. This ability may arise from the mycobiont, the photobiont or their mutual activity, but even bacterial endophytes cannot be excluded. Apart from that, it can be concluded that at least two types of arsenic detoxification exist in lichens: (a) arsenate reduction to arsenite and then arsenite extrusion; and (b) arsenate reduction to arsenite, followed by methylation. Another possibility is the formation of arsenite-phytochelatin (PC) complexes. Possibly the role of phytochelatins is more pronounced at higher concentrations, where methylation is not efficient enough.

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