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Arsenic accumulation and thiol status in lichens exposed to As(V) in controlled conditions

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Abstract Thalli of epiphytic lichen *Hypogymnia physodes* (L.) Nyl. and terricolous *Cladonia furcata* (Huds.) Schrad., collected from an area with background arsenic concentrations, were exposed to 0, 0.1, 1 and 10 μg mL^{-1} arsenate (As(V)) solutions for 24 h. After exposure they were kept in the metabolically active state for 0, 24 and 48 h in a growth chamber. In the freeze dried samples glutathione (GSH), glutathione disulphide (GSSG), cysteine (Cys) and cystine were analysed and induction of phytochelatin (PC) synthesis measured by reversed-phase high-performance liquid chromatography in combination with fluorescence detection or UV spectrometry. Total arsenic content in thalli was measured by instrumental neutron activation analysis (INAA). In *H. physodes*, which contained higher amounts of arsenic compared to *C. furcata*, total glutathione content significantly decreased in samples exposed to 10 μg mL^{-1} As(V), whereas in *C. furcata* a significant increase was observed. In both species PC synthesis was induced in thalli exposed to 10 μg mL^{-1}.

Keywords Lichens · Arsenic · Glutathione · Phytochelatins · Half-cell reduction potential · *Hypogymnia physodes* · *Cladonia furcata*

Abbreviations

As(V)	Arsenate
As(III)	Arsenite
GSH	Glutathione
GSSG	Glutathione disulphide
tGSH	Total glutathione
Cys	Cysteine
tCys	Total cyst(e)ine
$PC_{(n)}$	Phytochelatin (n = number of γ-Glu-Cys units)
$E_{GSSG/2GSH}$	Half-cell reduction potential for GSSG/2GSH couple

Introduction

Lichens possess several properties that allow them to survive extreme environmental factors. As poikilohydric organisms they are adapted to rapid changes of their water content. In the desiccated state they can survive long periods of drought, extreme temperatures and deleterious radiation. With their capability to absorb and accumulate elements from the environment across the whole surface of the thallus, they can grow

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even on nutrient deficient substrates (Nash 1996; Kranner et al. 2008). In polluted environments the ability to accumulate elements may become disadvantageous (Ahmadjian 1993). However, some lichens are able to survive in environments with high concentrations of metals and metalloids (Purvis and Halls 1996). The ability to survive high concentrations of metals mainly arises from the nature of the metals—most of them occur in cationic form and therefore can be efficiently retained or detoxified in the apoplastic space of lichens (e.g. Sarret et al. 1998; McLean et al. 1998; Purvis et al. 1987; Sanità di Toppi et al. 2005), while intracellular uptake is very slow and selective (Brown and Beckett 1985). Metals and metalloids that occur in anionic form may pose a bigger problem to lichen metabolism, because they are not efficiently retained in the apoplastic space due to their negative charge. Besides, some anionic metals are chemically very similar to essential anions and are easily taken up by specific transporters of the cell membrane and they may also interfere with lichen metabolism. Arsenate (As(V)), the main form of arsenic in aerobic environments (EHC 2001), is taken up into cells by phosphate transporters (Rosen 2002). In controlled conditions it was found that part of the As(V) taken up into lichen thalli is reduced to arsenite (As(III)) and partly excreted (Mrak et al. 2008), as is known for bacteria, yeasts (Rosen 2002), the mycorrhizal fungus *Hymenoscyphus ericae* (Read) Korf&Kernan (Sharples et al. 2000) and some freshwater unicellular algae (Hasegawa et al. 2001; Levy et al. 2005), while in mammalian cells it is excreted as a complex with glutathione ($As(GS)_3$) (Rosen 2002). As(III) that is retained inside lichen cells undergoes a methylation process. The extent of methylation in lichens was found to be dependent on the concentration of arsenic taken up and the duration of metabolic activity. The efficiency of methylation in lichens was found to decrease with increasing quantity of arsenic in thalli and increase with duration of metabolic activity (Mrak et al. 2008). Methylation was reported to occur in many microorganisms, algae, plants and animals, but is not universal (Bentley and Chasteen 2002). Arsenic that is left inside cells either as As(V) or As(III) may have many toxic effects on lichen metabolism. As(V) substitutes for phosphate in ATP synthesis, while As(III) has a very high affinity for binding to thiol (SH) groups of biomolecules. Toxic reactive oxygen species (ROS) may also form during the metabolic transformations of As(V), causing lipid peroxidation and DNA damage (EHC 2001). Thiol compounds seem to have a very important role in cells coping with arsenic intoxication. The key antioxidant glutathione (GSH): (1) is involved both in reduction of As(V) to As(III) and further methylation (EHC 2001); (2) may bind As(III) to form $As(GS)_3$, which is either transported into the vacuole (yeasts) or out of the cells (mammals) (Rosen 2002) and (3) may combat the toxic effects of ROS. GSH is also a substrate for the synthesis of phytochelatins (PCs), small metal-binding peptides of the general structure $(\gamma\text{-Glu-Cys})_n\text{-Gly}$, $n = 2$–11. Synthesis of PCs is induced by a wide range of metal ions, including arsenic (As(V) and As(III)). PCs are supposed to be involved in detoxification and homeostasis of metal ions (Grill et al. 1985, 1987, 1989; Maitani et al. 1996). PCs are also capable of complexation with arsenic (Schmöger et al. 2000; Raab et al. 2004, 2005, 2007) and their role as detoxifying agents for arsenic has been thoroughly researched. In higher plants, mixed complexes of PCs and GSH were also reported (Raab et al. 2004, 2005, 2007). Low-molecular-weight antioxidants, such as GSH are supposed to be a first line of defense against toxic metals in lichen alga *Trebouxia impressa* Ahmadjian, similarly to higher plants (Sanità di Toppi et al. 2008).

There is very limited knowledge of the responses of lichen metabolism to anionic toxicants. The aim of this study was to investigate the response of thiol compounds to arsenic exposure (arsenic as As(V)) in two morphologically and ecologically different lichen species, *Hypogymnia physo*des (L.) Nyl. and *Cladonia furcata* (Huds.) Schrad., both containing *Trebouxia* photobionts. The hypothesis was set that both species of lichens respond to low concentrations of arsenic by induction of synthesis of thiols that are known to be involved in arsenic detoxification. At the highest arsenic concentration, thiol metabolism was expected to collapse as a consequence of severe stress or even cell death.

Materials and methods

Preparation of lichen material

Thalli of the foliose lichen *H. physodes* were collected from untreated wooden fences in Gradišče v Tuhinju (590 m alt.), Slovenia, in March 2006. Thalli of fruticose *C. furcata* were collected from litter in a spruce (*Picea abies*) forest 850 m south of

Mrzli Studenec on the Pokljuka plateau (1210 m alt.), Slovenia, in October 2006. At both locations, background arsenic concentrations were expected in lichens according to Jeran et al. (2002). In the laboratory all visible extraneous material was removed from the thalli. For *C. furcata*, only vertical parts of the thallus (i.e. podetia) were used. To reestablish metabolic processes, thalli were soaked in Milli-Q water (Millipore) for 30 min, blotted dry with paper towels to remove 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 light/dark photoperiod, photon flux 250 μmol s^{-1} m^{-2} and 80% relative air humidity. Some of the material treated in this way was removed and analysed as a blank.

Arsenic exposure

Solutions of arsenic in the form of As(V) with concentrations of 0, 0.1, 1 and 10 μg mL^{-1} (0, 1.33, 13.3 and 133 μM) were prepared from a 1,000 μg mL^{-1} standard solution (Merck, Titrisol) by dilution with Milli-Q water. The pH of the solutions was adjusted to 4.6 using analytical grade NaOH and ultrapure HCl according to Richardson et al. (1984). At this pH value, the prevailing form of As(V) ($pK_a = 2.20$, 6.97, 11.53) in the solution is $H_2AsO_4^-$.

Approximately 20–25 g fresh weight of lichen thalli were put into 250 mL plastic containers and 10× their weight of As(V) solution added. For each As(V) concentration, 6 containers were filled. Containers with lichens in As(V) solution were shaken on horizontal shaker at 130 motions/min in a growth chamber. Afterwards, lichen thalli were removed from the containers, blotted dry with paper towels, washed with Milli-Q water and then blotted dry again. The volume of Milli-Q water used for washing was the same as that of the As(V) solution which was used for the exposure experiments. Blotted thalli from two containers for each concentration treatment were frozen in liquid nitrogen immediately (0 h incubation), while the rest of the thalli were spread onto moist filter paper in Petri dishes (1 container per Petri dish, $\Phi = 18$ cm), incubated for 24 (2 Petri dishes for each concentration treatment) or 48 h in the growth chamber and then frozen. All samples were freeze-dried, weighed and ground in a Retsch MM 200 ball mill. Powdered samples were put into dark plastic containers and kept at −20°C in closed plastic bags with silica gel.

Analytical procedures

Determination of total arsenic content

The total arsenic content in exposed lichens was determined by instrumental neutron activation analysis (INAA). Powdered samples were pressed into 180 mg tablets using a SPECAC press 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. An arsenic standard was irradiated together with the lichen samples. Total arsenic content in the exposure solutions was measured by flow injection on a hydride generation atomic fluorescence spectrometry system coupled to on-line UV-decomposition (UV-HGAFS). Each exposure solution was measured in three to four replicates.

Analysis of thiols and their corresponding disulphides: glutathione (GSH), glutathione disulphide (GSSG), cysteine (Cys) and cystine

Analysis of thiols and their corresponding disulphides in samples was performed at the Institut für Pflanzenwissenschaften, Karl-Franzens Universität, Graz. A slightly modified method of Kranner (1998) based on separation of monobromobimane (mBBr)-labeled thiols by reversed-phase high-performance liquid chromatography was used. Thiols were extracted from 60 mg of powdered lichen material into 3 mL of 0.1 M HCl with 60 mg of polyvinylpolypyrrolidone (PVPP) added. For determination of disulphides, free thiol groups were blocked by *N*-ethylmaleimide (NEM). The surplus of NEM was removed by washing with toluene. Subsequently, disulphides were reduced by dithiothreitol (DTT), both for determination of thiols and disulphides. Thiol groups were labeled by mBBr and the reaction was terminated by 0.25% methanesulphonic acid (MSA). 50 μL of centrifuged samples was injected onto a GROM Spherisorb ODS-2 column (5 μm, 250 × 4.6 mm) and separated by gradient elution with mobile phases H_2O-MeOH-acetic acid (1,000:50:2.5) and MeOH-H_2O (9:1), both pH 3.9, at a flow rate of 1 mL min^{-1}. Fluorescence detection of labeled thiol groups was achieved with a JASCO FP 2020 Plus detector

(excitation 380 nm, emission 480 nm). Retention times and concentrations were determined via standards for GSH (Sigma) and Cys (Biochemika). Disulphides were expressed as equivalents of their corresponding thiols. The sum of GSH and GSSG was defined as tGSH and that of Cys and cystine as tCys.

Recovery was checked by adding standards for GSH, GSSG (Sigma) and Cys to selected lichen samples. Good agreement was found for determination of GSH and GSSG in both types of lichens, regardless of the arsenic concentration, while some problems occurred with Cys quantification. In *H. physodes* a very variable recovery for Cys was found, while in *C. furcata* it was low (about 50%), but constant. For this reason, only the uncorrected results for Cys in *C. furcata* are given in the results section and therefore must be treated as semi-quantitative.

Half-cell reduction potentials of GSSG/2GSH couple ($E_{GSSG/2GSH}$) were calculated using the Nernst equation as suggested by Schafer and Buettner (2001) and Kranner et al. (2006) to estimate the viability of lichens. $E_{GSSG/2GSH}$ could be used as an universal viability marker. An increase in $E_{GSSG/2GSH}$ to the zone of −180 to −160 mV correlates with viability loss and induction of programmed cell death (Schafer and Buettner 2001; Kranner et al. 2006).

Analysis of phytochelatins

Analysis of phytochelatins was performed at the Dipartimento di Biologia Evolutiva e Funzionale, Sezione di Biologia Vegetale, Università degli Studi di Parma by the method of derivatization of SH groups with Ellman's reagent after separation on a reversed-phase column. Freeze-dried samples were homogenized together with a glass filter (Whatman GF/C) in a mortar on ice to break down the cells. 5% sulphosalycylic acid (SSA) in 6.3 mM diethylentriaminopentaacetic acid (DTPA) was used as the extraction medium. Homogenized samples were transferred to Eppendorf tubes, centrifuged and filtered through 0.45 µm filter (Sartorius Minisart RC4). 200 µL samples were injected on a Merck LiChroCART 125-3 Purospher RP-18C (5 µm) column and separated by gradient elution with mobile phases of H_2O (100%) and H_2O-acetonitrile (1:1), both containing 0.05% of trifluoroacetic acid (TFA), at a flow rate of 0.7 mL min^{-1}. Thiol groups were post-column derivatized with 350 µM Ellman's reagent supplied at a flow rate of 0.35 mL min^{-1} and then detected with a Kontron Instruments HPLC Detector 430 at 412 nm. An extract of *Silene vulgaris* (Moench) Garcke, kindly donated by Prof. Meinhart Zenk, was used as an external standard to determine retention times and concentrations of phytochelatins.

Statistics

Two-factorial ANOVA was applied to statistically assess the influence of arsenic concentration and incubation time on total arsenic content and selected thiol compounds. In some cases (glutathione and cysteine components), analysis was performed despite the interdependency of parameters to facilitate the interpretation of the results. Time trends were determined by polynomic contrasts.

Results

Total arsenic content

For both species, the incubation time (i.e. duration of metabolic activity outside of the exposure solutions) within the concentration treatments did not influence the total arsenic content. However, a great difference was observed between the two species in arsenic accumulation (Table 1). In *H. physodes* the uptake of arsenic was very efficient and it increased with increasing arsenic concentration in the exposure solution. In 10 µg mL^{-1} treatments, only 21.3 ± 3.09% of arsenic was left in the exposure solutions, in 1 µg mL^{-1} 53.1 ± 3.56% and in 0.1 µg mL^{-1} treatments 63.8 ± 5.15%. In *C. furcata*, the most efficient uptake was again achieved at 10 µg mL^{-1} exposure, but 67.1 ± 0.64% of arsenic remained in the exposure solution. At 1 µg mL^{-1} 80.6 ± 0.62% and at 0.1 µg mL^{-1} 75.3 ± 1.34% of arsenic was found in the exposure solutions.

GSH and GSSG

In *H. physodes*, the tGSH and GSH contents were influenced by both arsenic concentration and time of incubation (Fig. 1; Table 2). Concentrations of tGSH and GSH significantly decreased in samples exposed

Table 1 Total arsenic content (mean ± SE) in lichen thalli exposed to As(V)

		Total As (μg g^{-1} dw)	
		H. physodes	*C. furcata*
Blank		0.28	0.13 ± 0.01
As(V) treatment (μg mL^{-1})	0	0.25 ± 0.01	0.13 ± 0.01
	0.1	0.80 ± 0.03	0.55 ± 0.01
	1	11.8 ± 0.43	3.75 ± 0.19
	10	187 ± 5.36	89.7 ± 3.47

Total arsenic content was calculated as a mean of all time treatments (0, 24 and 48 h) at one arsenic concentration since no statistically significant differences between total arsenic contents among these samples were observed

to 10 μg mL^{-1} As(V). A decrease in both parameters was also significant between 0 and 48 h of incubation, regardless of arsenic concentration. The decrease was linear with time of incubation ($P = 0.0004$ for tGSH and $P = 0.0019$ for GSH). In samples exposed to 10 μg mL^{-1}, a combination of the negative effects caused by incubation time and arsenic concentration was observed. This negative influence was especially evident in severely decreased concentrations of tGSH and GSH after 48 h of incubation (Fig. 1).

The negative influence of high arsenic concentrations was also evident in the percentage of GSSG. A statistically significant increase ($P < 0.001$) in the percentage of GSSG was observed in samples exposed to 10 μg mL^{-1} (Fig. 2) where also an increase with incubation time was recorded. After 48 h the percentage of GSSG at 70.3 ± 4.1% was significantly higher than after 0 and 24 h (36.1 ± 1.8 and 41.4 ± 5.9%). The increase in percentage of GSSG resulted from a decrease in concentration of GSH and not from an increase in GSSG, since the content of GSSG even decreased after 48 h in comparison to 0 and 24 h incubation time (Fig. 1). There were no statistically significant differences in the percentages of GSSG between samples exposed to 0, 0.1 and 1 μg mL^{-1}, regardless of incubation time. Values for $E_{GSSG/2GSH}$ were ranging from −248 to −256 mV in 0, 0.1 and 1 μg mL^{-1} exposures. However, in samples exposed to 10 μg mL^{-1} and incubated for 48 h the $E_{GSSG/2GSH}$ abruptly increased to −189 mV (Fig. 1).

In *C. furcata*, the quantity of tGSH and GSH was predominantly influenced by arsenic concentration (Table 3). Concentrations of tGSH were significantly higher in lichens exposed to 10 μg mL^{-1}. The same was true for GSH (Fig. 3). The contents of tGSH and GSH in samples exposed to 10 μg mL^{-1} were significantly higher in samples incubated for 24 and 48 h compared to samples incubated for 0 h, with a quadratic trend of increase ($P = 0.0018$ for total and $P = 0.0114$ for reduced glutathione). However, the influence of arsenic concentration was far more important than the influence of time.

Compared to *H. physodes*, the percentage of GSSG was low in all treatments regardless of incubation time (less than 10%, Fig. 4), while the values for $E_{GSSG/2GSH}$ were ranging from −265 to −252 mV, Fig. 3.

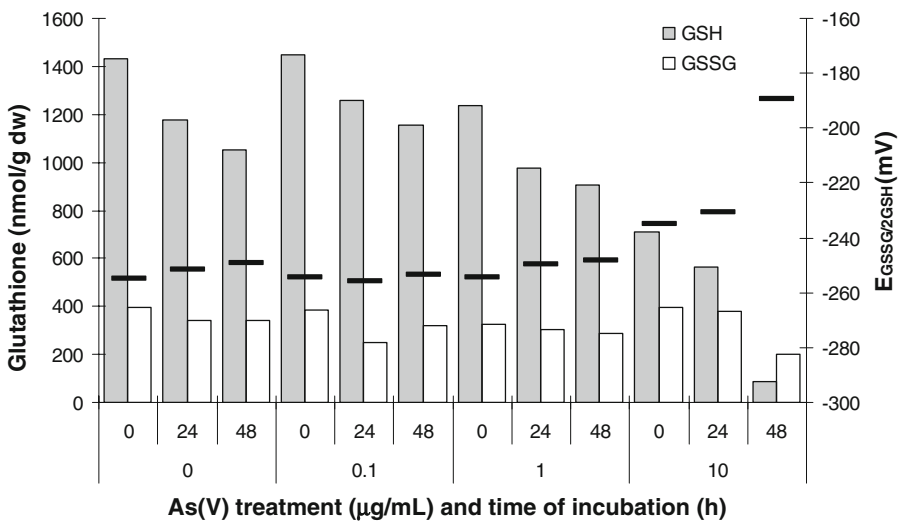

Fig. 1 Mean GSH and GSSG contents and $E_{GSSG/2GSH}$ values in *H. physodes* thalli after exposure to 0, 0.1, 1 and 10 μg mL^{-1} As(V) followed by 0, 24 and 48 h of incubation. The blank sample contained 1,340 nmol g^{-1} dw of GSH and 537 nmol g^{-1} dw of GSSG, while the $E_{GSSG/2GSH}$ was −253 mV

Table 2 Results of two-factorial ANOVA for glutathione components in arsenic-exposed *H. physodes* thalli

	As concentration ($df = 3$)		Time of incubation ($df = 2$)		As concentration × time of incubation ($df = 6$)	
	F	*P*	*F*	*P*	*F*	*P*
tGSH	20.99	0.0000*	11.80	0.0015*	1.09	0.4205
GSSG	3.00	0.0730	15.83	0.0004*	6.41	0.0032*
GSH	20.22	0.0001*	8.81	0.0067*	0.48	0.8140
% GSSG	52.00	0.0000*	11.43	0.0017*	7.01	0.0022*

df degrees of freedom

* indicates statistical significance ($P < 0.05$)

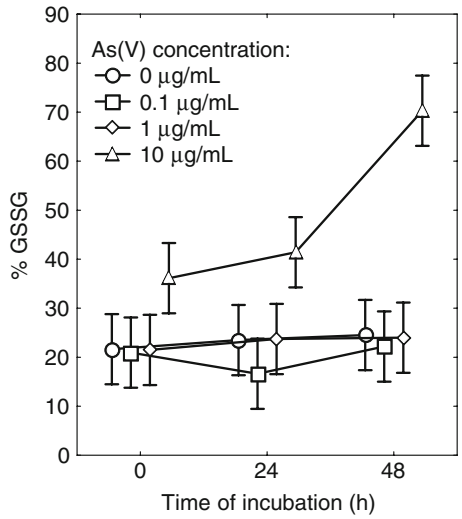

Fig. 2 Percentage of GSSG (mean ± 95% confidence interval) in *H. physodes* thalli as a function of arsenic concentration and incubation time

Cys and cystine

Arsenic concentration strongly influenced the Cys and cystine content in *C. furcata* (Fig. 5; Table 4). The interaction of concentration and incubation time occurred due to the strong influence of concentration. When exposed to 10 µg mL^{-1}, the concentration of tCys significantly increased ($P < 0.001$) on account of both, Cys and cystine. In these samples, the percentage of cystine amounted to 60.0–70.5%. In samples exposed to lower concentrations, percentages of cystine were less reliable due to low concentrations of both Cys and cystine close to the detection limits.

PCs

In both lichen species, two forms of PCs were detected, PC_2 and PC_3 (Tables 5, 6). PC_3 was detected only in samples exposed to the highest arsenic concentration (with only one exception). On the other hand, PC_2 was also present in some of the samples exposed to 1 µg mL^{-1}. In *H. physodes* both PC_2 and PC_3 seemed to decrease with incubation time in the 10 µg mL^{-1} treatment. In *C. furcata*, incubation time influenced the content of PC_2 in the 10 µg mL^{-1} treatment significantly ($P = 0.0021$), where the content of PC_2 increased linearly with time ($P = 0.0009$). The PC_2 concentration differed significantly between lichens incubated for 0 and 48 h, and between lichens incubated for 24 and 48 h as well. The content of PC_3 was not significantly influenced by incubation time ($P = 0.2716$).

Discussion

Exposure of the two lichen species to As(V) resulted in different accumulation of total arsenic in thalli and specific effects on their thiol status, measured as glutathione, cysteine and PC components.

The difference in arsenic accumulation of the two 4 species may be a consequence of variations in morphology and anatomy, species-specificity, tolerance, phosphate nutrition, different collection seasons and different environments from which different environments of thallusprovenance. Regarding phosphate nutrition, Wang et al. (2002) reported that the uptake of As(V) in plants adequately supplied with phosphate is lower. Meharg and Macnair (1990, 1991) found that accumulation of arsenic depends on the arsenic tolerance of the plant. Due to absence of high affinity uptake for phosphate and arsenate, tolerant genotypes accumulate arsenic to a lesser extent than non-tolerant genotypes of the same species, allowing the cells to efficiently detoxify As(V).

Table 3 Results of two-factorial ANOVA for glutathione components in arsenic-exposed *C. furcata* thalli

	As concentration ($df = 3$)		Time of incubation ($df = 2$)		As concentration × time of incubation ($df = 6$)	
	F	*P*	*F*	*P*	*F*	*P*
tGSH	118.51	0.0000*	30.45	0.0000*	15.69	0.0000*
GSSG	3.36	0.0552	24.97	0.0001*	1.95	0.1531
GSH	108.78	0.0000*	21.03	0.0001*	16.00	0.0000*
% GSSG	3.71	0.0426*	11.52	0.0016*	4.28	0.0155*

df degrees of freedom

* indicates statistical significance ($P < 0.05$)

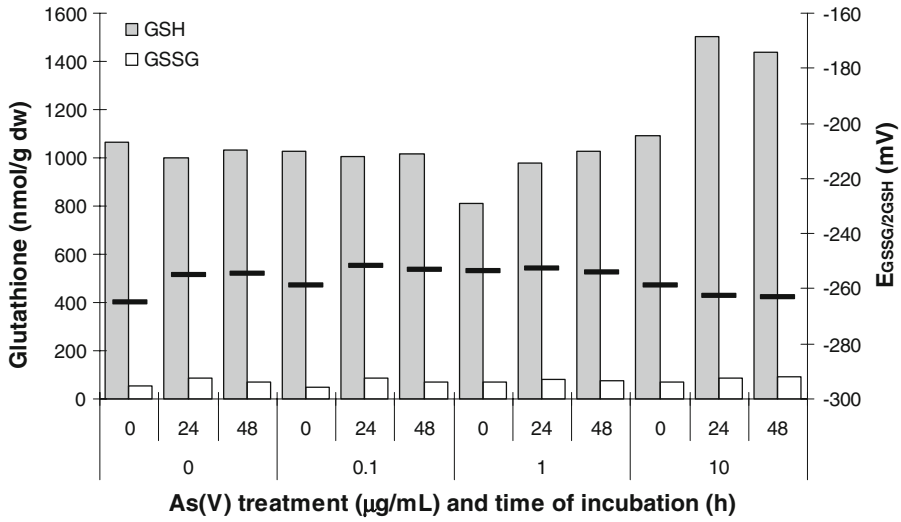

Fig. 3 Mean GSH and GSSG contents and $E_{GSSG/2GSH}$ values in *C. furcata* thalli after exposure to 0, 0.1, 1 and 10 µg mL^{-1} As(V) followed by 0, 24 and 48 h of incubation. Blank samples contained 1,160 nmol g^{-1} of GSH and 50.9 nmol g^{-1} of GSSG, while the $E_{GSSG/2GSH}$ was −275 mV

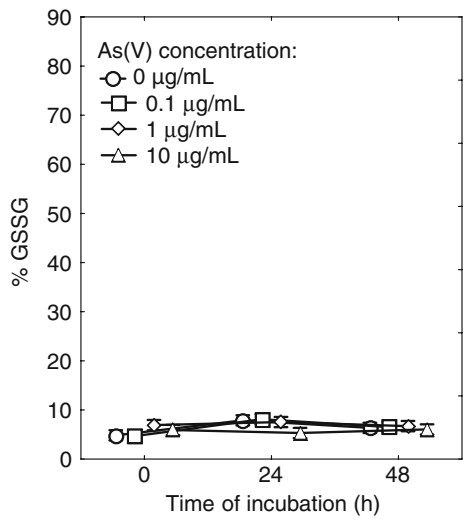

Fig. 4 Percentage of GSSG (mean ± 95% confidence interval) in *C. furcata* thalli as a function of arsenic concentration and incubation time

GSH is involved in intracellular arsenic detoxification in many organisms, while the role of PCs that are induced by arsenic is still uncertain. In lichens the role of low-molecular weight thiols in general is far from understood (Bačkor et al. 2006) and the present study is the first to investigate their response to arsenic exposure. GSH is supposed to play a key role in prevention of oxidative stress in mycobionts, which are believed to contain neither ascorbate nor its homologues or tocopherol (Kranner et al. 2005). Measurements of glutathione in lichens to a great extent reveal the response of the mycobiont (Kranner et al. 2003). Glutathione content in lichens depends on many parameters. Seasonal fluctuations with a maximal content in June and minimal in October were observed and differences related to altitude. Inside the lichen population, old thalli contain small and young thalli high amounts of glutathione (Kranner and Grill 1996). After extreme desiccation of

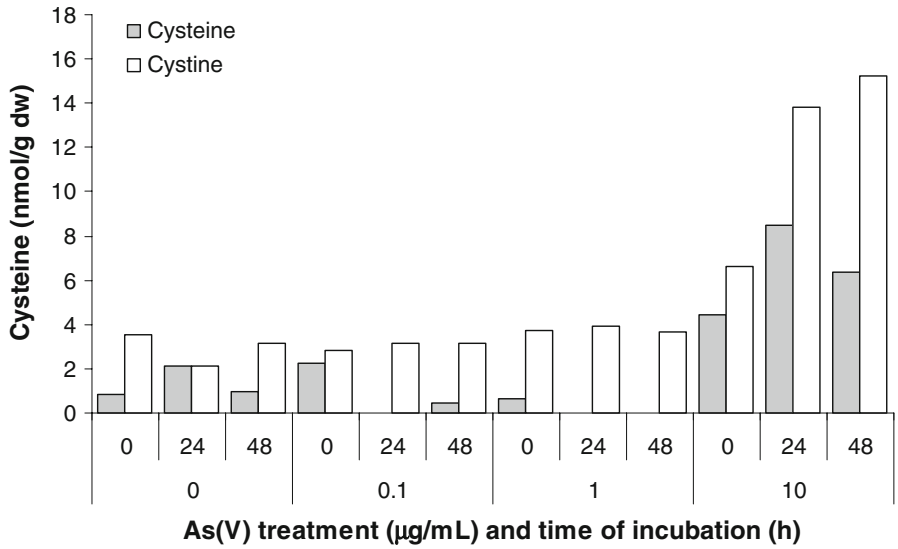

Fig. 5 Mean cyst(e)ine contents in *C. furcata* thalli after exposure to 0, 0.1, 1 in 10 µg mL^{-1} of As(V) followed by 0, 24 and 48 h of incubation. Blank samples contained 1.41 nmol g^{-1} of Cys and 2.95 nmol g^{-1} of cystine. Results are semi-quantitative

Table 4 Results of two-factorial ANOVA for cysteine components in arsenic-exposed *C. furcata* thalli

	Arsenic concentration ($df = 3$)		Time of incubation ($df = 2$)		As concentration × time of incubation ($df = 6$)	
	F	*P*	*F*	*P*	*F*	*P*
tCys	1002.73	0.0000*	37.14	0.0000*	76.34	0.0000*
Cystine	283.03	0.0000*	24.74	0.0001*	28.12	0.0000*
Cys	102.69	0.0000*	1.84	0.2017	8.19	0.0011*
% Cystine	7.34	0.0047*	2.28	0.1450	2.75	0.0644

df degrees of freedom

* indicates statistical significance ($P < 0.05$)

Table 5 Concentrations (mean ± SE) of PC_2 and PC_3 in *H. physodes* thalli, exposed to arsenic

As(V) exposure (µg mL^{-1})	Time of incubation (h)	PC_2 (nmol –SH g^{-1} dw)	PC_3 (nmol –SH g^{-1} dw)
Blank	–	–	–
0	0	–	–
	24	–	–
	48	–	–
0.1	0	–	–
	24	–	–
	48	–	–
1	0	10.4 ± 1.65	–
	24	–	–
	48	–	–
10	0	35.5 ± 0.48 a^1	10.2 ± 5.3 a
	24	17.4 b^1	11.2 a
	48	7.00 ± 1.57 c^1	1.49 ± 1.38 a

Statistically significantly different ($P < 0.05$) concentrations in 10 µg mL^{-1} arsenic exposure are marked by different letters. Number [1] marks a linear trend

lichen thalli, the highest percentages of GSSG of all living cells were recorded, between 90 and 100%. During rehydration, almost all GSSG is rapidly reduced, in only 2–5 min after the uptake of water (Kranner 1998). The percentage of GSSG in hydrated lichen thalli amounts to about 10–23% (Kranner

Table 6 Concentrations (mean ± SE) of PC_2 and PC_3 in *C. furcata* thalli, exposed to arsenic

As(V) exposure (μg mL^{-1})	Time of incubation (h)	PC_2 (nmol –SH g^{-1} dw)	PC_3 (nmol –SH g^{-1} dw)
Blank	–	–	–
0	0	–	–
	24	–	–
	48	–	–
0.1	0	–	–
	24	–	–
	48	–	–
1	0	9.97 ± 8.17	0.57
	24	1.72 ± 0.00	–
	48	–	–
10	0	19.5 ± 3.0 a^1	11.2 ± 3.0 a
	24	87.5 ± 13.1 b^1	36.5 ± 30.8 a
	48	181 ± 6 c^1	65.9 ± 11.3 a

Statistically significantly different ($P < 0.05$) concentrations in 10 μg mL^{-1} arsenic exposure are marked by different letters. Number ^{1} marks a linear trend

2002), while the content of tGSH can range between <1 and 2.7 μmol g^{-1} dw, Table 7.

The initial content of tGSH in our blank samples was in the normal range compared to data from the literature (Table 7) and the initial percentages of GSSG as well. Lichens were collected in March and October, when glutathione levels do not approach their maximal values.

Exposure to arsenic followed by 0, 24 or 48 h incubation showed a more negative influence on *H. physodes* compared to *C. furcata*. In all samples of *H. physodes*, tGSH content was negatively influenced by the incubation procedure itself, while in samples exposed to 10 μg mL^{-1} As(V), a combination of negative influences arising from incubation time and high arsenic concentration was observed. A decrease in tGSH or GSH content as a result of high arsenic exposure seems to be common, although the concentration range where this happens is hard to predict. In *Aspergillus nidulans* (Eidam) G. Winter a decrease in GSH was observed when exposed to concentrations of As(V) higher than 1 mM (Cánovas et al. 2004), while the same effect on tGSH was observed in *Trifolium pratense* L. grown in soil containing 50 mg kg^{-1} As(V) (Mascher et al. 2002). A decrease in tGSH and an increase in the percentage of GSSG indicate intensive stress that leads to cell death (Tausz et al. 2004). In *H. physodes* samples exposed to 10 μg mL^{-1} As(V) the percentage of GSSG was well above the normal values for lichens (Table 7). Especially high (70.3 ± 4.1%) was the percentage of GSSG after 48 h of incubation due to the decrease in GSH. This decrease in GSH could be explained by its use in PC synthesis (Ric de Vos et al. 1992), but the

Table 7 tGSH content and percentage of GSSG reported for lichens

Lichen species	tGSH (μmol g^{-1} dw)	% GSSG	Reference
Pseudevernia furfuracea (L.) Zopf	1.7–2.7	20 ± 4	Kranner (2002)
Lobaria pulmonaria (L.) Hoffm		23 ± 7	Kranner (2002)
Peltigera polydactyla (Neck.) Hoffm		10 ± 4	Kranner (2002)
Cladonia vulcani Savicz	<1		Kranner et al. (2005)
Umbilicaria antarctica Frey & I.M. Lamb	1.9		Vráblíková et al. (2005)
Lasallia pustulata (L.) Mérat	3.3		Vráblíková et al. (2005)
Physcia adscendens (Fr.) H.Olivier	1.2		Pawlik-Skowronska et al. (2002)
Xanthoria parietina (L.) Th.Fr.	2.6		Pawlik-Skowronska et al. (2002)

content of PCs in samples exposed to 10 μg mL^{-1} As(V) decreased with incubation time as well. This might indicate toxic effects of high arsenic concentrations. Higher doses of arsenic are reported to induce oxidative stress and related damage (Mascher et al. 2002; Singh et al. 2006; Requejo and Tena 2005). Values for $E_{GSSG/2GSH}$ were approaching the zone of viability loss (−180 to −160 mV) in samples exposed to 10 μg mL^{-1} and incubated for 48 h. Kranner et al. 2006 proposed that a ROS-provoked increase in $E_{GSSG/2GSH}$ to the zone of −180 to −160 mV is part of signaling cascade that initiates programmed cell death. At 10 μg mL^{-1} exposure and 48 h incubation time also an inhibition of dimethylarsinic acid (DMA) formation was reported previously for *H. physodes*; this effect might also occur as a result of As(V) or As(III) toxicity (Mrak et al. 2008). Deleterious effects of high doses of arsenic and long incubation times are also supported by the state of photosynthetic pigments in *H. physodes,* recorded as increased phaeophytinization of chlorophylls, increased percentage of deepoxidized xanthophylls and increased chlorophyll b to a ratio (unpublished results).

In *C. furcata*, the response to incubation and arsenic concentration was less pronounced. The percentage of GSSG was low (<10%), regardless of arsenic concentration. A weaker response can be expected because less arsenic was accumulated in *C. furcata* thalli (Table 1). Besides that, *C. furcata* as a terricolous species is better adapted to longer hydration periods in comparison to *H. physodes*, as soil retains moisture for a longer time than tree bark or bare rocks. In samples exposed to the highest arsenic concentration an increasing trend in tGSH and GSH was observed with incubation time. An increase of GSH content under stress conditions could be explained as a part of the acclimation process of the organism to stress (Tausz et al. 2004). An increased content of GSH shows adaptation of lichens to stress conditions and corresponds to the increased quantity of Cys and cystine which are needed for glutathione synthesis. Synthesis of glutathione in photobionts is supposed to occur via the classical pathway from Cys and γ-Glu-Cys, while in mycobionts glutathione is believed to be synthesized from cystine and oxidized γ-Glu-Cys. Synthesis of glutathione in aposymbiotically cultured lichen bionts is accompanied by an increase in above mentioned precursors (Kranner et al. 2005). An increase in GSH was accompanied by induction of PC_2 and PC_3 synthesis, which also increased with incubation time. Le Faucheur et al. (2006) noted that an increase in GSH content in the green alga *Scenedesmus vacuolatus* Shihira et Krauss exposed to 8 μM concentration of As(V) was accompanied by induction of PC_2 synthesis. Better viability of *C. furcata* during the exposure to high arsenic concentrations is supported by $E_{GSSG/2GSH}$ values that stayed in the range of high viability during the whole experiment and by efficient methylation of arsenic that also remained unblocked (unpublished results).

Induction of PCs by arsenic was observed for the first time in cell cultures of *Rauvolfia serpentina* (L.) Benth. ex Kurz exposed to As(V) (Grill et al. 1987). Later, the induction of PCs by As(V) and As(III) was reported for numerous species of higher plants, ferns, and lately also for some algae and fungi. As(V) is a relatively efficient inducer of PC synthesis and could be compared to Cd (Schat et al. 2002). Among algae, induction of PCs with arsenic was observed in the unicellular freshwater green alga *Stycococcus bacillaris* (Pawlik-Skowrońska et al. 2004) and *S. vacuolatus* (Le Faucheur et al. 2006) and in the unicellular marine diatom *Phaeodactylum tricornutum* (Morelli et al. 2005). Among fungi, induction of PCs by arsenic was reported for the genus *Aspergillus* (Cánovas et al. 2004). The proportion of arsenic bound to PCs is supposed to be low (Raab et al. 2004)—PCs therefore most probably do not play an important role in arsenic detoxification (Zhang et al. 2004). In plants, PCs are supposed to serve as transport agents for arsenic through the cytoplasm in relatively non-harmful form. Afterwards, arsenic is supposed to be stored in free inorganic form in the vacuole (Raab et al. 2004).

Up to date, the synthesis of PCs and des-glycyl derivatives in lichens was observed as a response to Cd, Zn and Pb exposure (18, 36 and 54 μM) in three species of lichens all containing the *Trebouxia* photobionts (Pawlik-Skowrońska et al. 2002). The presence of PCs was also reported for *Lecanora polytropa* growing in a copper mine (Pawlik-Skowrońska et al. 2006). The ability to synthesize PCs is influenced by lichen species, climatic conditions and time of sampling. Lichens from more humid areas with lower temperatures are less capable of producing PCs. In lichens, PCs are supposed to be synthesized

only by algae. For this reason, the content of PCs inside lichen thallus is dependent on the algal fraction of the lichen volume and the quantity of metal that was not bound by the mycobiont (i.e. available to induce the synthesis of PCs) (Pawlik-Skowrońska et al. 2002).

Raab et al. (2007) reported that PC synthesis is a good indicator of the negative influences of arsenic on cellular metabolism at much lower concentrations than determined by more traditional means such as growth parameters or weight. On the other hand, some authors recorded PC induction at arsenic concentrations that already impacted negatively on growth.

Our results showed that arsenic induces PC production in both lichen species when exposed to 1 and 10 μg mL^{-1} As(V), but the intensity of synthesis was dependent on the concentration of the exposure solution, the duration of metabolic activity and the lichen species. The physiological status of lichens before their collection from nature would also need consideration. In both species, arsenic predominantly induced the synthesis of PC_2, and in smaller amounts also PC_3. Induction of PC_2 and PC_3 with arsenic was also reported in the literature (Schmöger et al. 2000; Raab et al. 2004; Cánovas et al. 2004; Le Faucheur et al. 2006; Pawlik-Skowrońska et al. 2004); occasionally PC_4 and PC_5 were detected in trace amounts (Schmöger et al. 2000; Morelli et al. 2005).

In our study we have shown that the exposure of lichen thalli to low concentrations of arsenic (0.1 μg mL^{-1} As(V)) did not induce any significant changes in metabolism of selected thiol compounds. Evidently, negative effects of arsenic at low exposures were efficiently combated already by As(V) to As(III) reduction followed by methylation procedure (see Mrak et al. 2008). The role of GSH in As(V) to As(III) reduction in lichens therefore remains unknown. First changes in thiol metabolism were observed as induction of PC synthesis at intermediate exposures (1 μg mL^{-1} As(V)). Further increases in arsenic induced different responses in both species of lichens. Epiphytic *H. physodes* which was able to take up much greater quantities of arsenic in comparison to terricolous *C. furcata* has shown a progressive impairment of thiol metabolism and finally approached the state of lost cell viability, presumably due to detrimental effects of ROS. However, indices of cell death were not observed yet. *C. furcata* remained viable and has shown increased GSH and PC synthesis, presumably as a sign of acclimation to arsenic stress.

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