Available online at www.sciencedirect.com
SCIENCE DIRECT
Talanta 69 (2006) 251–258
Talanta
www.elsevier.com/locate/talanta

Extraction of arsenic compounds from lichens

Tanja Mrak *, Zdenka Šlejkovec, Zvonka Jeran

Department of Environmental Sciences, Jožef Stefan Institute, Jamova 39, 1000 Ljubljana, Slovenia

Received 19 April 2005; received in revised form 2 September 2005; accepted 4 October 2005
Available online 15 November 2005

Abstract

Different extraction procedures were applied to improve the extraction efficiency of arsenic compounds from lichens. Two lichen species were chosen from an arsenic-contaminated environment: epiphytic *Hypogymnia physodes* (L.) Nyl. and terricolous *Cladonia rei* Schaer. Samples were extracted with water at temperatures of 20, 60 and 90 °C, using mixtures of methanol/water (9:1, 1:1 and 1:9), Tris buffer and acetone and the extracts speciated. Water and Tris buffer showed the best extraction efficiency of all extractants used; however, the extraction efficiency was still less than 23%. Since a major fraction of arsenic appeared to be associated with trapped soil particles, a sequential extraction procedure originally designed for soils (extraction steps: (1) 0.05 mol l^{-1} $(NH_4)_2SO_4$; (2) 0.05 mol l^{-1} $(NH)_4H_2PO_4$; (3) 0.2 mol l^{-1} NH_4-oxalate buffer, pH 3.25; (4) mixture of 0.2 mol l^{-1} NH_4-oxalate buffer and 0.1 mol l^{-1} ascorbic acid, pH 3.25; (5) 0.5 mol l^{-1} KOH) was applied and found to remove 45% of the total arsenic from *H. physodes* and 83% from *C. rei*. The lipid-soluble fraction of arsenic was estimated by k_0-INAA analysis of diethylether extracts and was found to be negligible. An HPLC-UV-HGAFS system was used to determine the arsenic compounds extracted. In both lichen species, arsenous acid, arsenic acid, monomethylarsonic acid, dimethylarsinic acid, arsenobetaine, trimethylarsine oxide and glycerol-ribose were detected. In addition, phosphate-ribose was found in *H. physodes*.

Keywords: Lichens; Arsenic compounds; Extraction efficiency; Sequential extraction; Bioindication

1. Introduction

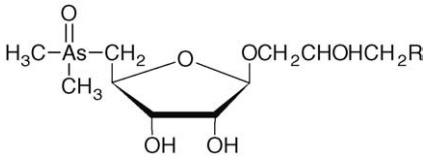
According to the International Association for Lichenology, a lichen is an association between a fungus and a photosynthetic symbiont, which results in a stable thallus of specific structure. Lichens are able to acquire substances from the environment through the whole surface of the thallus in two ways: (a) by entrapment of particles from the air or substrate and (b) by extra- and/or intracellular uptake of ions from solution [1–6]. As at least epiphytic lichens are supposed to reflect atmospheric concentrations of pollutants [6–8], they are often used as bioindicators of air pollution. Usually total concentrations of pollutants are monitored in lichens, and studies dealing with this problem are numerous (e.g. [8–14]). However, in this way no information on the chemical form of elements present in the environment is obtained. It is also not known how the compounds from the environment are reflected in lichens, if they are taken up selectively by the lichens, and if they are in any way metabolized in this association. Therefore, the study of speciation of chemical compounds is necessary, not only in lichens, but also in the bioavailable portions of the environment from where lichens are able to acquire those compounds (e.g. soil and air). To improve our knowledge of lichen behaviour in relation to their environment, efficient isolation/extraction of compounds of the elements of interest from samples of lichens is needed.

Investigations dealing with arsenic in lichens are few in number and mostly do not concentrate predominantly on lichens. Due to the chemical complexity of lichens, the extraction efficiencies of arsenic are low and therefore limiting in the study of arsenic speciation, defined as the determination of arsenic compounds (for arsenic compounds, see Table 1). In most cases, methanol/water (1:1 and 9:1) has been used as extractant with lichens [15–17]. Extraction efficiencies obtained in those cases ranged from 1.1 to 42% and were lichen species dependent. The highest extraction efficiency was achieved for an undetermined species from the genus *Cladonia* (42%) [16]. Kuehnelt et al. [17] intercompared the extraction of two lichen samples of different species (*Alectoria ochroleuca* and *Usnea articulata*) with methanol/water (9:1) and pure water at 25 °C

* Corresponding author. Tel.: +386 1 5885 354; fax: +386 1 5885 346.
E-mail address: tanja.mrak@ijs.si (T. Mrak).

0039-9140/$ – see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.talanta.2005.10.011

Table 1
Arsenic compounds investigated in this study

	Abbreviation	Formula
Anionic arsenic compounds		
Arsenous acid	As(III)	H_3AsO_3
Arsenic acid	As(V)	H_3AsO_4
Monomethylarsonic acid	MMAA	$CH_3AsO(OH)_2$
Dimethylarsinic acid	DMAA	$(CH_3)_2AsO(OH)$
Cationic arsenic compounds		
Arsenobetaine	AsB	$(CH_3)_3As^+CH_2COOH$
Arsenocholine	AsC	$(CH_3)_3As^+CH_2CH_2OH$
Tetramethylarsonium ion	TETRA	$(CH_3)_4As^+$
Trimethylarsine oxide	TMAO	$(CH_3)_3As^+OH$
Arsenosugars		
Glycerol-ribose (R = OH)		
Phosphate-ribose (R = $OPO_3HCH_2CHOHCH_2OH$)		
Sulfate-ribose (R = SO_3H)		
Sulfonate-ribose (R = OSO_3H)		

and found higher extraction yields for extraction with pure water (7 and 25%, respectively). The better extractability with water was explained by the statement that glycerol-ribose is better removed by water than by a 9:1 methanol/water mixture. Sequential extraction with Milli-Q water–$CaCl_2$–H_3PO_4 was applied by Farinha et al. [18] to transplants of *Parmelia sulcata*, where extraction efficiencies of 0.7–9.3% were achieved.

Regarding speciation of arsenic in lichens, the literature was reviewed by Dembitsky and Rezanka [19] and by Dembitsky and Levitsky [20]. In lichens, both inorganic and organic arsenic compounds were recorded. Besides the dominant inorganic arsenic, Koch et al. [15] reported phosphate-ribose and trace amounts of DMAA and glycerol-ribose in lichen samples from the genera *Bryoria*, *Alectoria* and *Cladonia*. Arsenite and arsenate were the major arsenic compounds in lichens (mostly species from the genus *Cladonia*) from Yellowknife, Canada, and represented as much as 62–93% of total extracted arsenic. In this study, arsenobetaine was reported for the first time for lichens and was present in all analysed samples [16]. Besides that, glycerol-ribose was detected in two lichen samples and the presence of an unknown compound was recorded. Kuehnelt et al. [17] reported arsenobetaine as the major compound in the lichen *A. ochroleuca* and as one of the major compounds in the lichen *U. articulata*. Glycerol-ribose was the major compound in *Usnea*. Farinha et al. [18] found only anionic arsenic compounds (inorganic arsenic, DMAA and MMAA) in samples of the transplanted lichen *P. sulcata*. Variations in arsenic compounds present in lichens studied so far were explained by two possibilities: (a) differences in lichen species and therefore the metabolism of arsenic and (b) differences in their environments [16].

Our present study was aimed to improve the extraction efficiency of arsenic in lichens. Comparison was made between two species of lichens, one corticolous (*Hypogymnia physodes* (L.) Nyl.) and one terricolous (*Cladonia rei* Schaer.). A soil sample from the area where the lichens were obtained was also examined, to see if arsenic content of lichens could be directly ascribed to soil. In extracts, arsenic speciation was investigated to see the influence of extractant on speciation and to get information on the arsenic compounds present.

2. Experimental

Samples of the two lichen species *H. physodes* and *C. rei* collected in April 2003, and one representative soil sample collected in September 2004, were taken. Samples were collected in Žerjav, Slovenia, close to the former lead–zinc mine and smelter, where preliminary k_0-INAA analyses have shown an increased content of arsenic in samples from the environment.

2.1. Sample preparation

Lichen samples were moistened and substrate was removed by nylon tweezers. Subsequently, they were freeze-dried and made brittle by immersion in liquid nitrogen and then crushed and ground in a mortar. For *Cladonia*, only podetia (=vertical parts of thalli) were used to make a powder in order to avoid contamination from soil. The soil sample was freeze-dried and then sieved at 0.5 mm mesh and divided into fractions >0.5 mm (soil 1) and <0.5 mm (soil 2). Fractions were homogenized in liquid nitrogen.

2.2. Determination of total As

For determination of total As in samples, the k_0-INAA method was employed. About 100 mg of homogenized soil and about 180 mg of powdered lichen were pressed into tablets, sealed into polyethylene ampules and irradiated in the TRIGA Mark II Reactor, Ljubljana, at a neutron fluence rate of

Table 2
Extractant media and extraction conditions for the extraction of arsenic from lichens

Extractant	Extraction conditions	Sample:extractant ratio (w/v)
Water	20 °C, 16 h, shaking bath	1:50
Water	60 °C, 16 h, shaking bath	1:50
Water	90 °C, 6 h, shaking bath	1:50
Methanol/water, 9:1	20 °C, 16 h, shaking bath	1:50
Methanol/water, 1:1	20 °C, 16 h, shaking bath	1:50
Methanol/water, 1:9	20 °C, 16 h, shaking bath	1:50
Tris ($C_4H_{11}NO_3$), 10 mmol l^{-1}, pH 7.6	20 °C, 16 h, shaking bath	1:50
Acetone	20 °C, 16 h, shaking bath	1:50

1.1×10^{12} n cm^{-2} s^{-1} for 20 h. The k_0-method [21] was used to calculate the total concentrations of arsenic.

2.3. Extraction procedure

Various extractant media and various extraction conditions were applied to extract arsenic from lichens and soil (Tables 2 and 3). All chemicals used in the procedure were of analytical grade quality. For water solution preparations, Milli-Q Plus water (Millipore) was employed (18.2 MΩ cm).

When enough material was available, 0.5 g of homogenized sample was weighed into a 50 ml centrifuge tube. An appropriate volume of extractant was added to achieve the desired sample to extractant ratio (see Table 2). The tubes were immersed into liquid nitrogen and afterwards in hot water three times to break down the cells. Then, they were mounted into an end-to-end shaking water bath (Julabo SW 22) and after incubation (for times and temperatures, see Table 2) they were centrifuged at 3200 rpm for 10 min. Extracts were decanted. Aqueous extracts were freeze-dried immediately. Where methanol and acetone were used as extractants, extracts were dried in a rotary evaporator at a water bath temperature of 40 °C. Dry extracts were resuspended in a small amount of water (about 3–5 ml) and filtered through a Hydrophilic PVDF 0.45 μm membrane filter (Millipore Millex HV). The final mass of the extracts was recorded before filtering. All extractions were performed in duplicate.

In the case of the sequential extraction procedure according to Wenzel et al. [22], 1 g of dry material was used. The amounts of extractants added can be seen in Table 3. After centrifugation, extracts were decanted and filtered immediately, omitting the drying procedure. The last extraction step with KOH was applied only to lichen samples, as a high enough extraction efficiency for soil samples was achieved already with the former extraction steps.

Lichen samples were additionally extracted in 25 ml of diethylether for 8 h. From time to time they were shaken manually. After extraction they were centrifuged at 3200 rpm for 10 min and following decantation, the extracts were left open overnight under a fume hood to evaporate the diethylether. The dry extract was resuspended in a small amount of diethylether and transferred to a 4 ml polyethylene ampule and left to dry again. They were sealed 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. The k_0-method [21] was used to calculate total concentrations of arsenic.

2.4. Arsenic analyses in extracts

Total arsenic was determined by ICP-MS after microwave digestion of filtered extracts. 0.5–1 ml of water extract was weighed into a PTFE digestion vessel and then 6 ml 65% HNO_3 and 2 ml 30% H_2O_2 were added. The vessel was capped and placed in the turntable of a microwave system (Milestone MLS-1200 MEGA Microwave Digestion System, Shelton, CT, USA). The digestion procedure consisted of the following steps: 1 min at 250 W, 2 min at 0 W, 5 min at 250 W, 2 min at 400 W and 10 min at 600 W. After the digestion was completed, the vessel was cooled to room temperature and the digest diluted to 50 ml with water.

ICP-MS analysis for total arsenic in the digests was carried out using a Hewlett-Packard 4500 PLUS ICP-MS spectrometer equipped with a Cetac ASX-500 autosampler, Babington nebulizer and Scott spray chamber. ^{75}As masses were measured with an integration time of 0.3 s per channel (three channels per mass) in five replicates. Arsenic calibration solutions (0, 0.5, 1, 5, 10,

Table 3
Sequential extraction scheme for arsenic in soil according to Wenzel et al. [22]

Extractant	Extraction conditions	Sample:extractant ratio (w/v)
$(NH_4)_2SO_4$, 0.05 mol l^{-1}	20 °C, 4 h, shaking bath	1:25
$(NH)_4H_2PO_4$, 0.05 mol l^{-1}	20 °C, 16 h, shaking bath	1:25
NH_4-oxalate buffer, 0.2 mol l^{-1}; pH 3.25	20 °C, 4 h, shaking bath, in the dark	1:25
Wash step NH_4-oxalate, 0.2 mol l^{-1}; pH 3.25	10 min, shaking bath, in the dark	1:12.5
NH_4-oxalate buffer, 0.2 mol l^{-1} + ascorbic acid, 0.1 mol l^{-1}; pH 3.25	96 °C, 30 min, water basin	1:25
Wash step NH_4-oxalate, 0.2 mol l^{-1}; pH 3.25	10 min, shaking bath, in the dark	1:12.5
KOH, 0.5 mol l^{-1}	40 °C, 5 min, shaking bath	1:50

20, 50, 100 and 500 ng ml^{-1}) were run before and after each sample series; in-between calibration checks were performed after each four samples. Arsenic concentrations in unknown solutions were calculated based on an external calibration curve and corrected for spectroscopic interferences from ArCl. Relative standard deviations for ICP-MS measurements were below 20%.

For determination of arsenic compounds in extracts a previously developed HPLC-UV-HGAFS system was used [23]. The arsenic compounds listed in Table 1 were used as standards. Concentrations of 10–50 ng g^{-1} for anionic and cationic compounds and of 10–150 ng g^{-1} for arsenosugars were prepared fresh daily from stock solutions, as given in reference [23]. Anion (Hamilton PRP X-100, 250 mm × 4.1 mm with 15 mmol l^{-1} KH_2PO_4, pH 6.1) and cation exchange columns (Alltech Adsorbosphere SCX 5U, 250 mm × 4.6 mm with 2.5 mmol l^{-1} pyridine, pH 2.65) were used to separate anionic and cationic arsenic compounds, respectively. For the separation of arsenosugars, an anion exchange column (Hamilton PRP-X100, 250 mm × 4.1 mm with 20 mmol l^{-1} KH_2PO_4, pH 5.8) was used. Triplicate injections of each extract on each column were performed. On-line UV decomposition (3.1 m long, 0.5 mm i.d. FEP Teflon tubing coiled around an 8 W Camag UV lamp, 254 nm, with a flow of 2% $K_2S_2O_8$ in 2% NaOH) was used for the determination of arsenosugars and cationic arsenic compounds in order to decompose the non-hydride-forming compounds and compounds which form hydrides with low efficiency, prior to the hydride generation step (4.4 mol l^{-1} HCl, 3.0 ml min^{-1} and 1.5% $NaBH_4$ in 0.1% NaOH, 3 ml min^{-1}). An Excalibur (PS Analytical, Kent, UK) atomic fluorescence spectrometer was used for the detection of volatile arsenic hydrides. For safety reasons, the AFS spectrometer was placed under a fume hood to prevent toxic arsines entering the laboratory air. The presence of thio arsenosugars in samples was tested as suggested by Schmeisser et al. [24]. The analytical peaks obtained were evaluated in terms of peak area by external standard calibration. Detection limits ranged from 1 to 3 ng g^{-1} for As(III), As(V) and MMAA and were about 6 ng g^{-1} for DMAA, AsB, TMAO, glycerol-ribose and phosphate-ribose. Calibration curves were linear up to approximately 300 ng g^{-1}. Relative standard deviations for main constituents were up to 10% for *H. physodes*, up to 15% for *C. rei* and up to 5% for soil samples. Relative standard deviations for minor constituents were up to 25%. Sample chromatograms for lichens are given in Fig. 1.

2.5. Quality assurance

Since no suitable reference material is available for arsenic speciation in lichens and problems of arsenic extraction are specific to this type of sample, a lichen sample from an non-contaminated environment (*H. physodes*, total As 0.18 μg g^{-1}) was prepared in the same way as the two lichens from the arsenic-contaminated environment according to the procedure described above and incubated at 25 °C in a shaking bath for 16 h. Using HPLC-UV-HGAFS, the background lichen sample was analysed to confirm the absence of any arsenic compound, spiked with arsenic compounds of interest and analysed again.

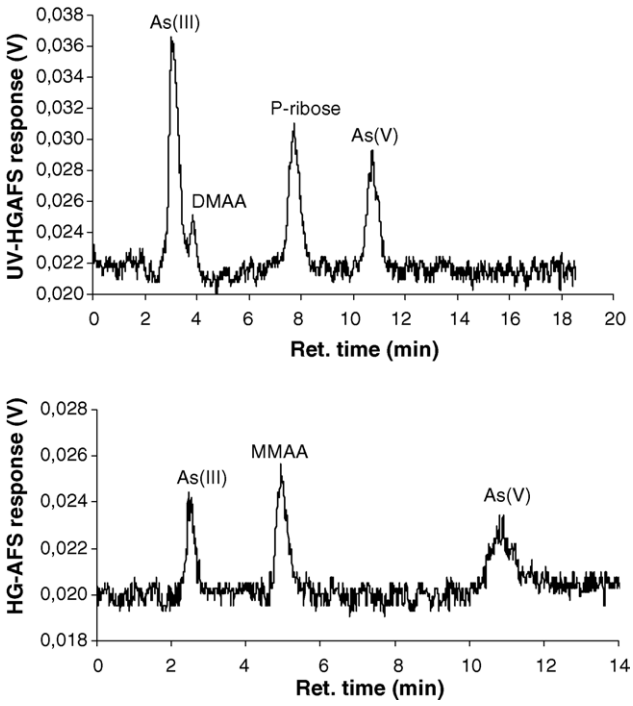


Fig. 1. Examples of anionic exchange chromatograms (HPLC-UV-HGAFS) for *H. physodes* (above; Hamilton PRP X-100, 250 mm × 4.1 mm, 20 mmol l^{-1} KH_2PO_4, pH 5.8, on-line UV-decomposition) and *C. rei* (below; Hamilton PRP X-100, 250 mm × 4.1 mm, 15 mmol l^{-1} KH_2PO_4, pH 6.1). P-ribose: phosphate-ribose.

No matrix effects were detected concerning peak areas and only minor shifts (of 10–20 s) towards shorter retention times were observed.

3. Results and discussion

3.1. Total arsenic content

The results of k_0-INAA analysis for our samples of lichens revealed concentrations of arsenic (5.04 ± 0.09 μg g^{-1} for *H. physodes* and 1.09 ± 0.08 μg g^{-1} for *C. rei*), which were above background values for arsenic for the lichen species *H. physodes* (0.51 ± 0.17 μg g^{-1}) for the year 1992 in Slovenia [12]. Further investigations of background values for Slovenia in the year 2001 showed even lower values [25]. Total concentrations of As in soil fractions were much higher than in lichens. The soil fraction >0.5 mm contained as much as 190 ± 18 μg g^{-1} of As, but concentrations in fraction <0.5 mm were even higher (322 ± 24 μg g^{-1} As). Therefore, the pool of arsenic in the investigated area of the former lead–zinc mine in Žerjav is huge, though the uptake of arsenic by a particular organism depends on the bioavailability of arsenic (which depends on its chemical form and environmental conditions), the characteristics of the organism itself and of its associations with other organisms (e.g. mycorrhizae) [26–30].

3.2. Extracted arsenic compounds

Fig. 2 summarizes the extraction of arsenic compounds from lichens and soil fractions in different extractants. The results

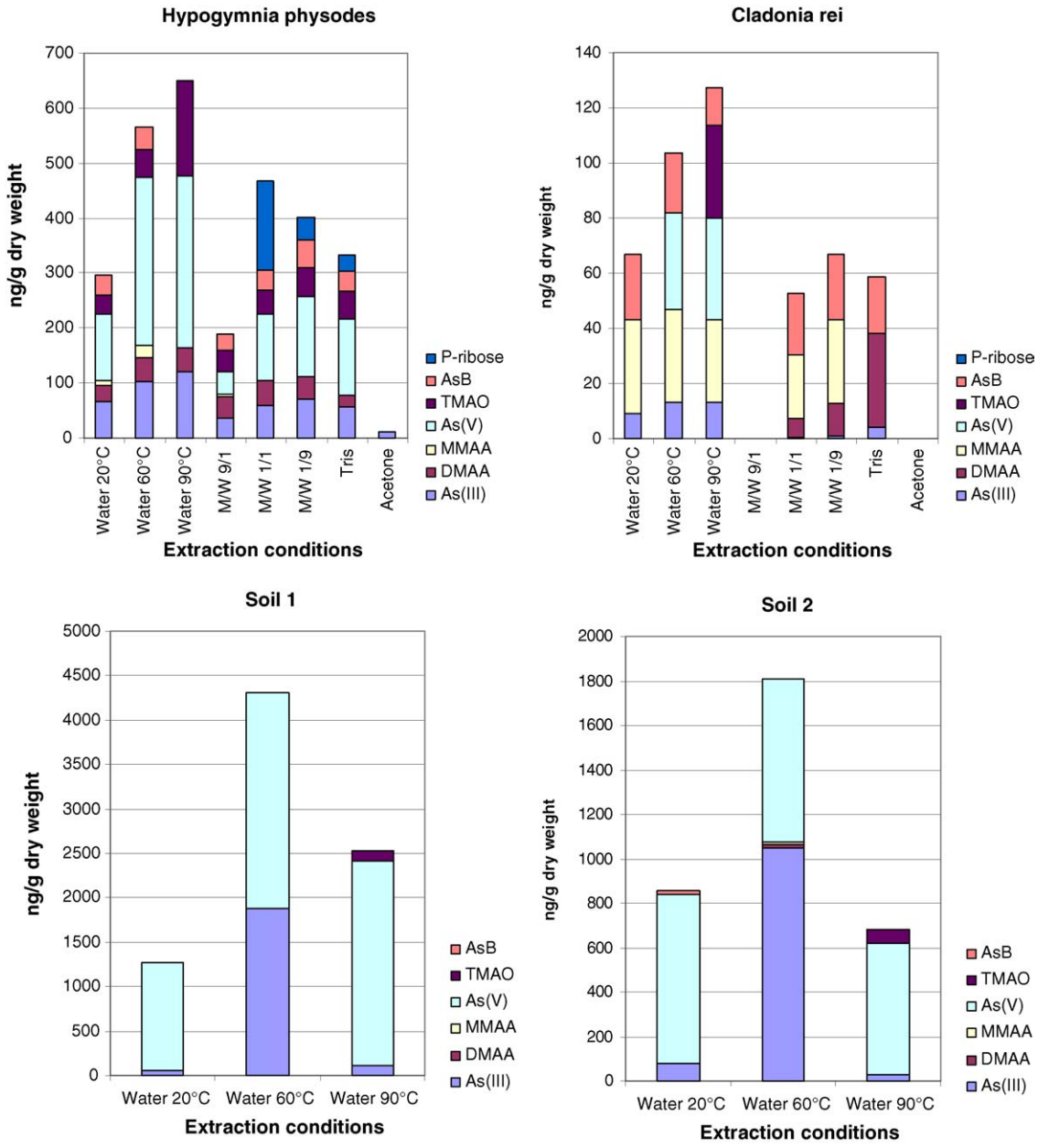


Fig. 2. Extraction of arsenic compounds from lichens and soil using different extraction conditions (note the different *y* scales). Average values of duplicate extractions and triplicate speciation analyses are shown. Glycerol-ribose is not included. P-ribose: phosphate-ribose; M/W: methanol/water.

reveal that extraction of water-soluble arsenic compounds in the two lichen species shows a different pattern, whereas soil fractions are comparable. It must be emphasized that glycerol-ribose is not presented in the figures, although it was present in both lichen species. Due to the non-optimized conditions for analysis of arsenosugars, the coelution of glycerol-ribose, As(III), TMAO and AsB occurred in the first peak of the anion exchange chromatogram. Cation exchange chromatography also suffered from a matrix effect and partial coelution of DMAA and glycerol-ribose was observed. For this reason concentrations of glycerol-ribose in extracts were only approximately estimated by subtracting As(III) (anion exchange separation without UV decomposition), TMAO and AsB (both cation exchange with UV decomposition) from the anion exchange signal (with UV decomposition). In that way, concentrations of glycerol-ribose were estimated to be about 100 ng g^{-1} dry weight for *H. physodes* and about 40–50 ng g^{-1} dry weight for *C. rei* in the case of the best extractability.

3.3. Extraction efficiency of arsenic compounds with common extractants

The extraction of As(III) and As(V) from lichens was the best applying pure water as extractant. Additions of methanol decreased the extraction efficiency of these two compounds. MMAA and DMAA were generally not influenced by the type of extractant used (except for acetone); the same is true for AsB. Only extraction at 90 °C in pure water seems to be inappropriate

Table 4
Extraction efficiency (%), calculated from total concentrations of arsenic in filtered extracts obtained by ICP-MS analysis and from total concentrations of arsenic in whole material obtained by k_0-INAA

Extractant	*H. physodes*	*C. rei*	Soil 1	Soil 2
Water 20 °C	14.2	18.0	0.9	0.3
Water 60 °C	**19.2**	17.9	**2.5**	**0.6**
Water 90 °C	nd	20.4	1.7	0.3
Methanol/water, 9:1	8.7	nd	–	–
Methanol/water, 1:1	17.0	17.4	–	–
Methanol/water, 1:9	14.3	17.3	–	–
Tris	14.7	**22.4**	–	–
Acetone	4.7	6.4	–	–

Best extractabilities are indicated in bold.

for extraction of AsB. In all samples the amounts of AsB extracted were decreased or even below the detection limit in the case when aqueous extraction was performed at 90 °C. The concentrations of TMAO increased in these conditions. Spiking experiments (an extraction mixture of background lichens was spiked with AsB) also resulted in losses of AsB (approximately 30%), but an increase of TMAO concentration was not observed and thus degradation of AsB into TMAO could not be confirmed. Phosphate-ribose, which was detected only in *H. physodes*, was extracted only in extractants with added methanol and to some extent in Tris buffer. The extraction efficiency of phosphate-ribose was the best for methanol/water (1:1) > methanol/water (1:9) > Tris buffer. However, in general, mixtures of methanol and water, which are usually applied to plant samples [31], were shown to be inferior in comparison to pure water for lichens. To check for the presence of thio arsenosugars, the extracts were oxidized with H_2O_2 as suggested by Schmeisser et al. [24]. The results remained the same as before oxidation, confirming that thio arsenosugars were absent in our samples.

Application of different extractants showed no improvement of the yield of extraction in comparison to data from the literature [15–18]; in some cases, it was even worse. Extraction efficiencies were low (Table 4). For *H. physodes* the maximum extraction yield was 19.2% using hot water as extractant and for *C. rei* 22.4% using Tris buffer as extractant. Only minor portions of total arsenic from soil fractions were extractable with pure water and thus regarded as bioavailable (2.5% or even less).

When sum of the arsenic compounds in extracts, detected by HPLC-UV-HGAFS, was compared to the total arsenic in extracts, determined by ICP-MS, it was found that in lichens some arsenic compounds must have been present that either remained bound to the chromatographic column or were invisible to the detection system used. These compounds in some cases represented as much as the half of the total arsenic in extracts.

3.4. Sequential extraction

Due to the lack of success in improving the extraction of As from lichens using different extractants, it was decided to apply a sequential extraction procedure used for soils [22]. Since observations under a magnifying glass revealed small soil particles on the inner and outer surfaces (hollow thalli) of both

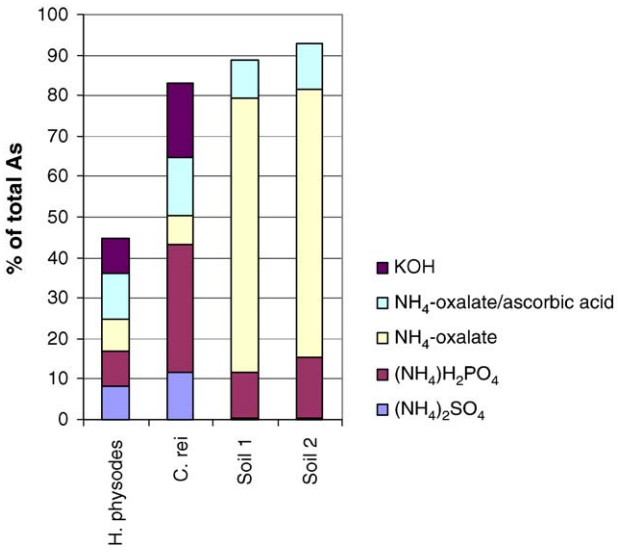


Fig. 3. Extractability of As from lichens and soil using the sequential extraction scheme according to Wenzel et al. [22]. Average values of total extracted As are shown. Standard deviations were in general less than 2%.

lichens, it could be assumed that the majority of As and other metals, which are usually analysed in bioindication studies on lichens, could be found in small soil particles trapped in thalli. It was already suggested by Koch et al. [16] that unextracted As might be in mineralized form on the outside of fungi and lichens. Applying the sequential extraction procedure according to Wenzel et al. [22], we were able to extract about 45% of the total arsenic from *H. physodes* and 83% from *C. rei* (Fig. 3). The extractability of arsenic from *C. rei* was comparable to the extractability of arsenic from soil fractions (89% for the fraction >0.5 mm and 93% for the fraction <0.5 mm). According to Wenzel et al. [22], the $(NH_4)_2SO_4$ extraction step removes from soil non-specifically sorbed As (bioavailable As), $(NH_4)H_2PO_4$ specifically sorbed As, NH_4-oxalate buffer amorphous and poorly crystalline hydrous oxides of Fe and Al, the mixture consisting of NH_4-oxalate buffer and ascorbic acid well-crystallized hydrous oxides of Fe and Al, and KOH As-sulfides. This “soil-like” arsenic in lichens could be explained by contamination of lichens with soil particles. The highest soil contamination is expected in terricolous lichens (*C. rei*), due to their place of growth (soil). Despite careful sample preparation, in which all visible contamination was removed from the lichens, it is impossible to remove soil particles, which cannot be seen with the naked eye during the cleaning procedure. Epiphytic lichens may become contaminated by wind-blown soil particles. Blow-off of soil by the wind is a common problem in degraded, metal-contaminated environments, also in the area where our samples were collected [32]. However, the pattern of soil arsenic, where the NH_4-oxalate extractable fraction (amorphous and poorly crystalline hydrous oxides of Fe and Al) prevails, differed very much from the pattern of lichen–arsenic, where NH_4-oxalate extractable fraction was the least important arsenic pool (Fig. 3). In the case of *C. rei*, the most successful removal of arsenic was the $(NH_4)H_2PO_4$ extraction step (specifically sorbed arsenic), whereas in the case of *H. physodes* the

extraction was more or less equal in all steps. Fedotov et al. [33] reported that the sequential extraction procedure underestimates the non-specifically sorbed fraction of arsenic (removable with $(NH_4)_2SO_4$) in soil and sludge samples. Differences in the extractability patterns of lichen trapped soil and soil itself may be explained by lichen transformation of the arsenic fractions in trapped soil. From Fig. 3, it is evident that the amount of easily and readily mobilizable arsenic in lichens is higher in comparison to soil. Grafe et al. [34,35] investigated the influence of dissolved organic carbohydrates (DOC) on the adsorption behaviour of arsenic on iron oxides. They found a decrease of As(V) and As(III) adsorption on goethite (well-crystallized iron oxide) in the presence of DOC (humic acid, fulvic acid and citric acid). Adsorption of As(V) onto ferrihydrite (poorly crystalline iron oxide) was decreased in the presence of citric acid, while As(III) adsorption was reduced in the presence of fulvic and citric acid. A similar influence (increased availability as a consequence of desorption activity) of water-soluble carbohydrates on arsenic adsorbed on trapped soil particles could also occur in lichens. Lichens are also known to be able to accelerate the weathering of mineral substrates (biodeterioration of the cultural heritage!), not only by physical, but also by chemical means. The mechanisms of this chemical activity are the production of respiratory CO_2, excretion of oxalic acid and lichen substances, possibly also alkaline metabolic and enzymatic reactions, e.g. [36,37]. Kasama et al. [38] have shown that lichens are able to alter mineral particles trapped inside the thallus. Sericite trapped inside the medullary part of thallus of *Trapelia involuta* contained more Fe and less K in comparison to sericite in the rock substrate. Polysaccharides were proposed to be possibly associated with the dissolution of sericite minerals.

To check whether some short-term effects of lichens on the extractability pattern of arsenic from soil could be observed, 50 mg of soil sample (soil 2) was added to an extraction mixture of water and 0.5 g of powdered *H. physodes* from the background environment (total As content 0.18 $\mu g\,g^{-1}$), pretreated at 25 °C in a shaking bath for 16 h, incubated again at 25 °C in a shaking bath for 24 h, freeze-dried to dryness and extracted according to the sequential extraction scheme [22]. As a control, a soil sample (soil 2) was extracted in the same way again. Soil incubation in the presence of lichen resulted in a changed extractability pattern of As from soil (Fig. 4). The quantity of bioavailable As greatly increased and the amount of As bound to amorphous and poorly crystalline hydrous oxides of Fe and Al decreased. Quantities of specifically sorbed As and As bound to well-crystallized hydrous oxides of Fe and Al, also decreased. These results could be explained by the influence of DOC. These are short-term effects, which can occur when an As-contaminated soil particle comes into contact with the metabolically active (wetted) lichen thallus. Long-term effects may involve further transformations (e.g. of bioavailable inorganic As into organic As compounds) and translocations of As. A study of long-term effects would require living thalli (or at least isolated bionts) in controlled conditions.

Speciation analysis of sequential extracts (only $(NH_4)_2SO_4$, $(NH_4)H_2PO_4$ and NH_4-oxalate extractable fractions were found to be relevant for speciation analysis) of lichens and soil revealed

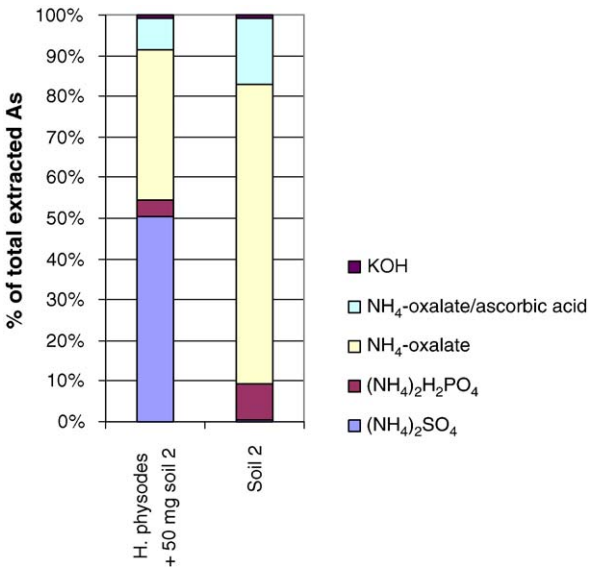


Fig. 4. Extractability of As from soil alone and soil added to an extraction mixture of powdered lichen *H. physodes* using the sequential extraction scheme according to Wenzel et al. [22]. Average values of total extracted As are shown. Standard deviations were in general less than 2%.

the presence of mainly As(V) and smaller amounts of As(III). In *C. rei*, some MMAA was detected as well in the extract of the $(NH_4)_2SO_4$ step.

3.5. Lipid-soluble arsenic

Although a part of the non-water-soluble arsenic can be ascribed to soil arsenic, we still ended up with approximately 35% of "missing" arsenic in the case of *H. physodes*. As this arsenic might be in the form of arsenolipids [15], preliminary diethylether extraction of lichen samples was performed and the arsenic content of the dried extracts investigated by INAA. The amount of lipid-soluble arsenic was found to be negligible: in the sample of *H. physodes* it was estimated to be about 55 $ng\,g^{-1}$ dry weight, whereas in *C. rei* it represented only about 3 $ng\,g^{-1}$.

4. Conclusions and outlook

Lichens are chemically complex matrices from which the isolation of arsenic compounds is certainly not an easy task. The majority of arsenic cannot be extracted using the common extractants which are usually applied to biological samples, but improvement of extraction yield was achieved employing sequential extraction as used for soils. Therefore, it can be assumed that at least part of the arsenic in lichens is in the form of small soil particles, and this "soil-like" arsenic can contribute as much as 80% of the arsenic in lichens. Regarding bioindication issues, lichens do not act simply and passively as air filters, but are able to metabolize the substances taken up, as was also confirmed by the arsenic-speciation studies of A. Marchado (personal communication) on exposed lichens (*Flavoparmelia caperata*). Consequently, lichens do not reflect the situation regarding the chemical forms present in the environment from where they were taken. It is not known how

arsenic metabolism in lichens works, what roles are played by the lichen-forming fungus and what by the algae. It might be possible that the lichen-forming fungus acts similarly to the mycorrhizal symbiont *Hymenoscyphus ericae* [27], to protect photosynthesising algae from excess of arsenic. As some of the completely non-lichen-forming ascomycetous fungi such as *Penicillium* and *Aspergillus* are supposed to derive from lichen-forming ancestors [39,40], and some of the representatives of the genera *Penicillium* and *Aspergillus* were shown to produce arsines (e.g. reviewed by Bentley and Chasteen [41]), it would be of interest if such metabolic pathways also exist in lichens.

Acknowledgements

We thank Prof. Dr. Helmut Mayrhofer (Institute for Plant Sciences, Karl-Franzens-University, Graz) for help in determination of *Cladonia rei* by providing TLC results, Dr. Johannes Teun van Elteren (National Institute of Chemistry Slovenia) for ICP-MS results and Dr. Radojko Jaćimović (Jožef Stefan Institute) for measurements of total concentrations of arsenic in samples.

References

[1] D.H. Brown, R.P. Beckett, Lichenologist 16 (1984) 173–188.
[2] D.H.S. Richardson, S. Kiang, V. Ahmadjian, E. Niboer, in: D.H. Brown (Ed.), Lichen Physiology and Cell Biology, Plenum Press, New York, 1985, pp. 227–246.
[3] D.H.S. Richardson, Bot. J. Linn. Soc. 96 (1988) 31–43.
[4] G. Sarret, A. Manceau, D. Cuny, C. Van Haluwyn, S. Deruelle, J.-L. Hazemann, Y. Soldo, L. Eybert-Berard, J.-J. Menthonnex, Environ. Sci. Technol. 32 (1998) 3325–3330.
[5] J. Garty, in: I. Kranner, R.P. Beckett, A.K. Varma (Eds.), Protocols in Lichenology. Culturing, Biochemistry, Ecophysiology and Use in Biomonitoring, Springer-Verlag, Berlin/Heidelberg, 2002, pp. 458–482.
[6] H.T. Wolterbeek, J. Garty, M.A. Reis, M.C. Freitas, in: B.A. Markert, A.M. Breure, H.G. Zechmeister (Eds.), Bioindicators and Biomonitors, Elsevier, Oxford, 2003, pp. 377–419.
[7] K.J. Puckett, in: D.H. Brown (Ed.), Lichen Physiology and Cell Biology, Plenum Press, New York, 1985, pp. 211–225.
[8] J.E. Sloof, Atmos. Environ. 29 (1995) 11–20.
[9] S. Loppi, S.A. Pirintsos, V. deDominicis, Environ. Monit. Assess. 58 (1999) 121–131.
[10] A. Bari, A. Rosso, M.R. Minciardi, F. Troiani, R. Piervittori, Environ. Monit. Assess. 69 (2001) 205–220.
[11] P.L. Nimis, S. Andreussi, E. Pittao, Sci. Total Environ. 275 (2001) 43–51.
[12] Z. Jeran, R. Jaćimović, F. Batič, R. Mavsar, Environ. Pollut. 120 (2002) 107–113.
[13] S. Vingiani, P. Adamo, S. Giordano, Environ. Pollut. 129 (2004) 145–158.
[14] H. Poličnik, F. Batič, C. Ribarič Lasnik, J. Atmos. Chem. 49 (2004) 223–230.
[15] I. Koch, J. Feldman, L. Wang, P. Andrewes, K.J. Reimer, W.R. Cullen, Sci. Total Environ. 236 (1999) 101–117.
[16] I. Koch, L. Wang, K.J. Reimer, W.R. Cullen, Appl. Organomet. Chem. 14 (2000) 245–252.
[17] D. Kuehnelt, J. Lintschinger, W. Goessler, Appl. Organomet. Chem. 14 (2000) 411–420.
[18] M.M. Farinha, Z. Šlejkovec, J.T. Van Elteren, H.Th. Wolterbeek, M.C. Freitas, J. Atmos. Chem. 49 (2004) 343–353.
[19] V.M. Dembitsky, T. Rezanka, Plant Sci. 165 (2003) 1177–1192.
[20] V.M. Dembitsky, D.O. Levitsky, Prog. Lipid Res. 43 (2004) 403–448.
[21] R. Jaćimović, B. Smodiš, T. Bučar, P. Stegnar, J. Radioanal. Nucl. Chem. 257 (2003) 659–663.
[22] W.W. Wenzel, N. Kirchbaumer, T. Prohaska, G. Stingeder, E. Lombi, D.C. Adriano, Anal. Chim. Acta 436 (2001) 309–323.
[23] Z. Šlejkovec, J.T. Van Elteren, U.D. Woroniecka, Anal. Chim. Acta 443 (2001) 277–282.
[24] E. Schmeisser, R. Raml, K.A. Francesconi, D. Kuehnelt, A.-L. Lindberg, C. Sörös, W. Goessler, Chem. Commun. (2004) 1824–1825.
[25] Z. Jeran, R. Jaćimović, P. Pavšič Mikuž, J. Phys. IV France 107 (2003) 675–678.
[26] W. Peijnenburg, R. Baerselman, A. de Groot, T. Jager, D. Leenders, L. Posthuma, R. Van Veen, Arch. Environ. Contam. Toxicol. 39 (2000) 420–430.
[27] J.M. Sharples, A.A. Meharg, S. Chambers, J.W.G. Cairney, Nature 404 (2000) 951–952.
[28] J.M. Sharples, A.A. Meharg, S. Chambers, J.W.G. Cairney, Plant Phys. 124 (2000) 1327–1334.
[29] A.A. Meharg, J. Hartley-Whitaker, New Phytol. 154 (2002) 29–43.
[30] A.-C. Schmidt, J. Mattusch, W. Reisser, R. Wennrich, Chemosphere 56 (2004) 305–313.
[31] Y. Bohari, G. Lobos, H. Pinochet, F. Pannier, A. Astruc, M. Potin-Gautier, J. Environ. Monit. 4 (2002) 596–602.
[32] C. Ribarič Lasnik, I. Eržen, N. Kugonič, B. Pokorny, D. Končnik, M. Svetina, B. Justin, P. Druks, M. Bole, A. Rošer Drev, M. Vetrih, J. Flis, K. Kotnik, R. Mavsar, L. Pačnik, K. Savinek, Comparative Study of Environmental Pollution in the Upper Meža Valley in the Years 1989 and 2001, Final Report, ERICo Velenje, Environmental Research & Industrial Co-operation, Velenje, Slovenia, 2002 (original in Slovene).
[33] P.S. Fedotov, W.J. Fitz, R. Wennrich, P. Morgenstern, W.W. Wenzel, Anal. Chim. Acta 538 (2005) 93–98.
[34] M. Grafe, M.J. Eick, P.R. Grossl, Soil Sci. Soc. Am. J. 65 (2001) 1680–1687.
[35] M. Grafe, M.J. Eick, P.R. Grossl, A.M. Saunders, J. Environ. Qual. 31 (2002) 1115–1123.
[36] J. Chen, H.-P. Blume, L. Beyer, Catena 39 (2000) 121–146.
[37] P. Adamo, P. Violante, Appl. Clay Sci. 16 (2000) 229–256.
[38] T. Kasama, T. Murakami, T. Ohnuki, O.W. Purvis, J. Nucl. Sci. Technol. (Tokyo, Jpn.) Suppl. 3 (2002) 943–945.
[39] A. Gargas, P.T. DePriest, M. Grube, A. Tehler, Science 268 (1995) 1492–1495.
[40] F. Lutzoni, M. Pagel, V. Reeb, Nature 411 (2001) 937–940.
[41] R. Bentley, T. Chasteen, Microbiol. Mol. Biol. Rev. 66 (2002) 250–271.