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Arsenic speciation in freshwater organisms from the river Danube in Hungary

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Abstract

Total arsenic and arsenic species were determined in a range of freshwater samples (sediment, water, algae, plants, sponge, mussels, frog and fish species), collected in June 2004 from the river Danube in Hungary. Total arsenic concentrations were measured by ICPMS and arsenic species were measured in aqueous extracts of the samples by ion-exchange HPLC-ICPMS. In order to separately determine the efficiency of the extraction method and the column recovery, total arsenic concentrations in the extracts were obtained in three ways: (i) ICPMS determination after acid digestion; (ii) flow injection analysis performed directly on the extract; (iii) the sum of arsenic species eluting from the HPLC column. Extraction efficiencies were low (range 10–64%, mean 36%), but column recovery was acceptable (generally >80%) except for the fish samples, where substantial, currently unexplained, losses were observed. The dominating arsenic species in the extracts of freshwater algae were arsenosugars, whereas arsenate [As(V)] was present only as a minor constituent. On the other hand, plant extracts contained only inorganic arsenic, except for two samples which contained trace amounts of dimethylarsinate (DMA) and the tetramethylarsonium cation (TETRA). The oxo-arsenosugar-phosphate (ca. 35% of extractable arsenic) and the oxo-arsenosugar-glycerol (ca. 20%) as well as their thio-analogues (1–10%) were found in the mussel extracts, while arsenobetaine (AB) was present as a minor species only. In general, fish extracts contained only traces of arsenobetaine, and the oxo-arsenosugar-phosphate was the major arsenic compound. In addition, samples of white bream contained thio-arsenosugar-phosphate; this is the first report of a thio-arsenical in a fish sample. The frog presented an interesting arsenic speciation pattern because in addition to the major species, arsenite [As(III)] (30%) and the tetramethylarsonium cation (35%), all three intermediate methylation products, methylarsonate (MA), dimethylarsinate and trimethylarsine oxide (TMAO), and arsenate were also present. Collectively, the data indicate that arsenobetaine, the major arsenical in marine animals, is virtually absent in the freshwater animals investigated, and this represents the major difference in arsenic speciation between the two groups of organisms.

Keywords: Arsenic speciation; Freshwater ecosystem; HPLC-ICPMS; Ion-exchange; Mass balance

1. Introduction

Investigations of the chemical constituents of aquatic organisms can provide useful information about the environment as well as toxicologically relevant data about the composition of biological species consumed by humans. It is well known that different arsenic compounds can be accumulated by aquatic biota, especially by marine algae, fish and bivalves, and at the endpoint of the food-chain contaminants can reach terrestrial animals and humans. It is also well established that the toxicity of arsenic highly depends on the chemical form in which it is present. For instance, marine fish contain mostly arsenobetaine (AB), which is non-toxic, and hence arsenic accumulated in this form represents no health hazard. On the other hand, some organisms contain considerable amounts of inorganic arsenic (known to be toxic) or arsenosugars (Fig. 1, the toxicity of which is still unknown). The use of these organisms as human food may have toxicological consequences.

During the last decades most of the arsenic speciation studies in aquatic systems have focussed on marine ecosystems, while

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reports about freshwater ecosystems are still scarce. One reason for this is that the arsenic concentrations in marine organisms are usually much higher then in freshwater organisms, which facilitates arsenic analysis in the marine ecosystem. Although "seafood" products are primarily of marine origin, there are numerous countries, like Hungary, where fish of freshwater origin form a substantial part of the local diet.

In contrast to the large amount of published data on arsenicals in marine organisms, there are relatively few data reported on arsenic speciation of freshwater organisms, and these data do not yet present a clear pattern. Shiomi et al. [1] and Slejkovec et al. [2] reported that AB was the major arsenical in freshwater fish species, whereas Zheng and Hintelmann [3] found AB only in minor concentrations, and Lawrence et al. [4] did not detect AB at all, in the freshwater fish they investigated. Consistent results, however, have been reported for freshwater mussels where arsenosugars have been reported as the major arsenicals and AB as only a minor species [5,6].

A problem with the work performed so far with freshwater organisms is that only a low percentage of the total arsenic is accounted for by HPLC-ICPMS, and hence the data provide only a part picture of arsenic speciation. This problem is exacerbated by the fact that many studies do not provide mass balances at each stage of the analysis, so it is not possible to determine where the apparent losses are occurring.

The aim of our work was to determine the arsenic speciation in several diverse species of biota collected from the same freshwater ecosystem. Additionally, mass balance calculations were carried out at each stage of the analysis to investigate possible reasons for the low overall recovery of arsenic species previously reported for freshwater organisms.

2. Materials and methods

2.1. Chemicals and reagents

All solutions were prepared with Milli-Q water (18.2 MΩ cm). Concentrated nitric acid (Merck, p.a.) was further purified in a quartz sub-boiling distillation unit. Ammonium dihydrogen phosphate (p.a.), ammonium hydrogen carbonate (p.a.), methanol (puriss p.a) and formic acid (puriss p.a.) were purchased from Fluka (Buchs, Switzerland). Aqueous ammonia solution (25%, suprapur) and pyridine (p.a.) were obtained from Merck (Darmstadt, Germany). Standard solutions (1000 μg As mL^{-1}) for the identification and quantification of arsenic species were prepared as described elsewhere [7]. Oxo-arsenosugars were isolated from natural sources and purified as described elsewhere [8]. Thio-arsenosugar-glycerol and thio-arsenosugar-phosphate were obtained by addition of a saturated aqueous solution of H_2S to a standard solution containing 100 μg As/L of the respective oxo-analogue. The stock solutions were diluted with water to the desired concentration just before use.

The certified reference materials DOLT-1 (dogfish liver, National Research Council of Canada, Ottawa, Canada) and DORM-2 (dogfish muscle, National Research Council of Canada, Ottawa, Canada) were used for quality control.

2.2. Instrumentation

Samples were freeze-dried in a Christ Alpha 1-4 freeze-drying system (Christ, Osterode am Harz, Germany). The dry samples were pulverized in a coffee-mill. Samples were digested in a Milestone ultraCLAVE II microwave digestion system (EMLS, Leutkirch, Germany) for total arsenic determinations. Total arsenic determinations and arsenic speciation analyses were performed with an Agilent 7500c inductively coupled plasma mass spectrometer (ICPMS) (Agilent, Waldbronn, Germany) equipped with a PFA microconcentric nebulizer or a Babington nebulizer and a Scott double pass spray chamber.

For arsenic speciation analysis, an Agilent 1100 Series HPLC system consisting of a solvent degassing unit, a binary pump, an autosampler and a thermostatted column compartment was used as the chromatographic system. The chromatographic conditions applied are summarized in Table 1. The analytical columns were protected by guard columns filled with the same stationary phases. The outlet of the HPLC column was connected via PEEK capillary tubing (0.125 mm i.d.) to the nebulizer of the ICPMS system. The ion intensity at *m/z* 75 (^{75}As) was monitored using the 'time-resolved' analysis software. Additionally, the ion intensity at *m/z* 77 ($^{40}Ar^{37}Cl$) was monitored to detect possible argon chloride ($^{40}Ar^{35}Cl$) interference on *m/z* 75. Instrumental settings used throughout this work were described in detail elsewhere [7]. Peak areas were determined using the ICPMS chromatographic software Version C.01.00 (Agilent, Waldbronn, Germany).

An Agilent HPLC-MS system consisting of a series 1100 HPLC instrument and a G1946D series 1100 single quadrupole mass spectrometer of the SL type was used for the identification of oxo-arsenosugar-phosphate. The mass spectrometer was equipped with an electrospray ionisation source employing pneumatically assisted nebulisation with nitrogen as the nebulizer gas. Operating conditions have been previously described [9].

2.3. Sample collection and preparation

A list of the samples collected, including species names, is provided in Table 2; an overview is provided here. Water, sediment and biological samples were collected in June 2004 from the river Danube in Hungary at the City of Paks, which is one of the most important fishing-grounds along the Hungarian Danube. The water samples (five individual samples) were acidified to 1% (v/v) by addition of concentrated nitric acid and stored at 4 °C; the sediment was freeze-dried and homogenized, and stored at room temperature. The organisms collected were: one species of green alga; five species of freshwater plants, seven fish species; one species of mussels; one sponge; and one frog species. The green alga was collected in two forms: living alga collected from the surface-waters, and dead, sun-dried alga taken from the river bank. The frog was dead when collected, but no mechanical or biological changes were observed in the tissue, indicating that the frog died recently. Two samples of mussels (10 individuals in each) were collected, one from

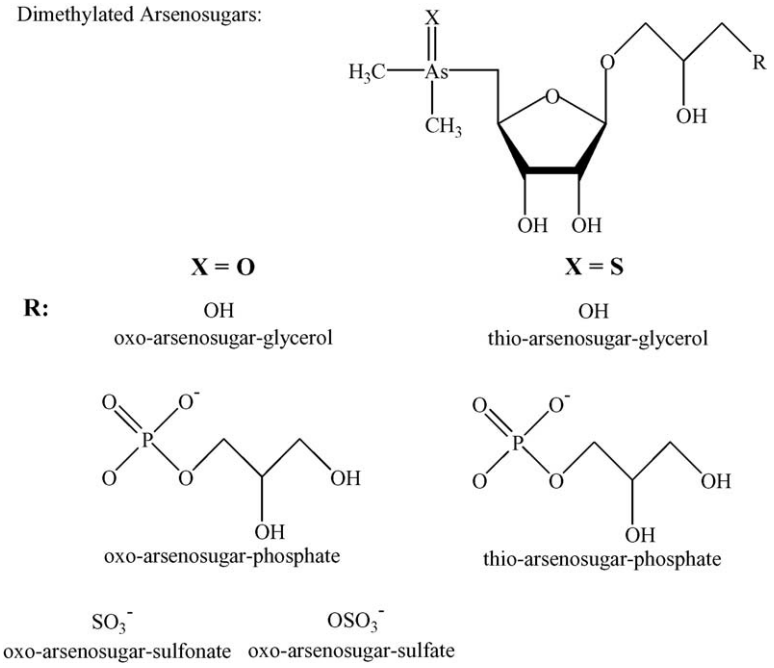

Fig. 1. Arsenosugar compounds relevant to this study (compounds are drawn in their most deprotonated form).

the river and one from a "dead-end" channel of the river; the whole wet tissues were removed and pooled for the mussels from each site. For the fish, muscle tissue was collected from all specimens; liver, eggs and scales were collected from one individual, and scales were collected from a second individual. The samples were freeze-dried, homogenized, and stored at room temperature.

2.4. Determination of total arsenic by ICPMS

Water samples were analysed directly by ICPMS without further treatment; sediment and biological samples (freeze-dried biological samples and aqueous extracts of biological samples) were subjected to microwave-assisted acid digestion before analysis. Thus, a portion (ca. 0.2 g) of a freeze-dried sample or a certified reference material was weighed to 0.1 mg into a 12 mL quartz tube and 4.0 mL of concentrated nitric acid was added. For the aqueous extracts, a portion (1.00 mL) was transferred to the quartz tube and 2.0 mL of nitric acid was added. The tubes were then closed with Teflon caps and placed in the microwave digestion system. The system was closed, loaded with argon to 4×10^6 Pa, and the mixture was heated at 240 °C for 40 min. Thereafter, the tubes were cooled and the digests were diluted with water to 20.0 mL (freeze-dried samples) or 10.0 mL (extracts) before analysis for arsenic by ICPMS. All samples were analysed in triplicate; R.S.D.s were 5% or better in all cases except ide scale. The accuracy of the method was checked with the certified reference material DOLT-1 (certified As concentration 10.1 ± 1.4 mg kg^{-1}, measured As concentration 10.9 ± 0.5 mg kg^{-1}, $n = 3$).

Table 1
Chromatographic conditions used for arsenic speciation analyses

	Anion-exchange HPLC Hamilton PRP-X100[a]		Cation-exchange HPLC Zorbax 300-SCX[a]
	250 mm × 4.1 mm i.d. I[b]	100 mm × 4.1 mm i.d. II[b]	150 mm × 4.6 mm i.d. III[b]
Mobile phase	20 mM $NH_4H_2PO_4$	20 mM NH_4HCO_3 3% MeOH	20 mM pyridine
pH	5.6	10.3	2.6
Temperature	40 °C	40 °C	30 °C
Injection volume	20 μL	20 μL	20 μL
Flow rate	1.5 mL min^{-1}	1.5 mL min^{-1}	1.5 mL min^{-1}
Determined compounds	As(III), DMA, MA, As(V), oxo-arsenosugar-phosphate, oxo-arsenosugar-sulfonate, oxo-arsenosugar-sulfate	thio-arsenosugar-glycerol, thio-arsenosugar-phosphate	AB, oxo-arsenosugar-glycerol, TMAO, AC, TETRA

[a] Column.
[b] Condition.

Table 2
Total arsenic concentrations in the investigated samples

Sample	Total As concentration (mg kg^{-1} dry mass)[a]
Water	1.1[b] ± 0.2
Sediment	3.60
Algae	
Green algae (fresh): *Cladophora* sp.	9.33
Green algae (sun-dried): *Cladophora* sp.	5.06
Plants	
Water-milfoil: *Myriophyllum* sp.	5.42
Hornwort: *Ceratophyllum demersum*	3.38
Floating watermoss: *Salvinia natans*	4.93
Frog-bit: *Limnobium spongia*	4.40
Sedge: *Carex* sp.	1.24[c]
Animals	
Sponge: *Ephydatia fluviatilis*	8.07
Mussel (river): *Unio pictorum*	9.31
Mussel (dead channel): *Unio pictorum*	11.6
Fry (mixed fish) days old	1.37
Fry (mixed fish) months old	1.21
Silver carp: *Hypophthamichthys molitrix*	1.17
White bream A: *Blicca bjoerkna*	1.58
White bream B: *Blicca bjoerkna*	0.713
White bream C: *Blicca bjoerkna*	0.487
Roach A: *Rutilus rutilus*	0.399
Roach B: *Rutilus rutilus*	0.403
Roach C: *Rutilus rutilus*	0.372
Razorfish: *Pelecus cultratus*	0.416
Ide: *Leuciscus idus*	0.247
Pikeperch: *Stizostedion lucioperca* L.	0.256
Liver of white bream C	0.523
Pikeperch liver	1.00[c]
Ide liver	0.345
Ide egg	0.193
Pikeperch scale	0.200
Ide scale	0.11
Frog: *Rana* sp.	2.52

[a] Samples were analysed in triplicate (except for sedge and pikeperch liver) and precision (R.S.D.) was ≤5% for all samples except ide scale (R.S.D. = 10%).
[b] Result in μg L^{-1}, mean and standard deviation of five samples.
[c] Determined once only because of small quantity of material.

The total arsenic concentrations in the aqueous extracts of the biological samples were also determined without prior microwave-assisted acid digestion. These measurements were performed by flow injection analysis in the mobile phase of chromatographic condition III (Table 1) using the HPLC system for sample introduction and ICPMS for arsenic detection. The method of standard addition was applied to compensate for matrix effects whereby mixtures of the extract (27 μL) + water (3 μL) or arsenate standards (3 μL) were injected.

2.5. Determination of arsenic compounds by HPLC-ICPMS

Preliminary testing of our samples showed that meaningful arsenic speciation data were difficult to obtain when the total arsenic concentrations were below about 0.5 mg kg^{-1}. Consequently, from the total of 29 biological samples, 13 fish tissue samples (including pikeperch liver, of which sufficient material was not available) were not further examined, while the remaining 16 samples were treated as follows.

A portion (0.2 g) of the freeze-dried powder was weighed to 0.1 mg into a 15 mL polyethylene tube and 7.0 mL water was added. The mixture was sonicated for 30 s with an ultrasonic probe (Branson Sonifier 150, Branson Ultrasonics Corporation, Danbury, USA) and then diluted with water to 10.0 mL. The samples were subsequently shaken top over bottom overnight at ambient temperature. The extraction mixtures were centrifuged at 4500 rpm for 15 min and the supernatants filtered through 0.20 μm Nylon filters (LaPhaPack, Langerwehe, Germany). The extraction of the samples was performed in duplicate, and, in addition to being analysed for total arsenic after microwave-assisted digestion described in the preceding section, all extracts were analysed for arsenic species by HPLC-ICPMS performed under three different chromatographic conditions (Table 1). Flow injection analysis was performed in duplicate with one of the two extracts. The certified reference material DORM-2 was used for quality control (certified AB concentration, 16.4 ± 1.1 mg As kg^{-1}, measured AB concentration, 15.9 ± 0.3 mg As kg^{-1}, $n = 6$).

3. Results and discussion

3.1. Total arsenic concentrations

The total arsenic concentrations in the samples are summarized in Table 2. Sediment collected from our sampling site at the City of Paks contained 3.6 mg As kg^{-1}. This concentration is far below the intervention limit (20 mg As kg^{-1}) posed by the Hungarian authorities for arsenic in soil [10]. Considerably higher arsenic concentrations have been recently reported in sediment from the river Szamos in Hungary [11]. Water samples contained only 1.1 ± 0.2 μg As L^{-1} ($n = 5$). This value is at the low end of the range of arsenic concentrations usually reported for river water and is well below the arsenic concentrations recently reported for surface-water in Hungary, especially those in the eastern part which typically show arsenic concentrations of 10–100 μg L^{-1} [12].

Despite the low arsenic concentrations in the water samples, many of the freshwater organisms collected from the sampling site contained appreciable concentrations of arsenic. There was, however, a considerable range of concentrations between the different types of organisms. For example, all the fish samples contained low concentrations (0.25–1.6 mg As kg^{-1}) whereas the two samples of mussels contained about 10 mg As kg^{-1}. Algae and freshwater plant samples contained intermediate concentrations, as did the single amphibian sample (frog, *Rana* sp., 2.5 mg As kg^{-1}) investigated in this study. It is of interest that the freshwater fish contain so little arsenic in comparison with other freshwater organisms—this difference does not seem to apply to marine systems because marine fish can contain high concentrations of arsenic comparable with those for organisms from other phyla [13]. We note though, that the low arsenic concentrations in fish observed in our study match the data reported so far for other freshwater fish species collected from non-polluted sites [2,3,14,15].

3.2. Mass balance and extraction efficiency of arsenic compounds

The quality and usefulness of the data from speciation analysis can often be compromised by low overall recovery of arsenic species. Arsenic speciation analysis of biological samples involves two main steps, namely extraction of species and their separation by HPLC, and it is important to know where arsenic losses are occurring. In the current study, we investigated this by determining arsenic mass balances at each stage.

Our approach was to use a simple extraction procedure with water so that we could directly inject the filtered supernatant onto the HPLC column. Our previous experience with total arsenic analyses using microwave-assisted acid digestion and ICPMS has shown that the method is accurate and has a precision of about 5%. When the method is applied to a freeze-dried sample and the aqueous extract from the sample, a reliable measure of the extraction efficiency is obtained. Because of matrix effects, however, ICPMS analysis of acid digests may not always closely match what is being measured by HPLC-ICPMS. For this reason, the arsenic concentration in the extract was also determined by flow injection analysis using a HPLC mobile phase (20 mM pyridine pH 2.6) as eluent. Thus, a measure of the arsenic content of the extract was obtained in three ways: conventional ICPMS after acid digestion, flow injection analysis performed directly on the extract and the sum of arsenic species eluting from the HPLC column (Table 3). The data had some interesting aspects, which we now discuss.

First, the extraction efficiencies for almost all samples were below 50%, and the overall mean for the 16 samples was 36%. These data contrast sharply with extraction efficiencies found for marine organisms which are usually >70% and often >95% [16,17]. Second, total arsenic data obtained from digestion/conventional ICPMS and flow injection ICPMS (without digestion) agree very well; most samples returned data within 8% for the two methods, and only the fish samples (all of which had low arsenic concentrations) showed substantial differences (e.g. silver carp 0.14 mg kg^{-1} versus 0.16 mg kg^{-1}, and fry 0.33 mg kg^{-1} versus 0.39 mg kg^{-1}). Third, for most samples the total arsenic eluting from the column (sum of quantified species) matched well the total injected onto the column. Exceptions were predominantly the fish samples which showed column recoveries of only 19–64% based on flow injection. Although this may suggest a particular problem with these fish, it may also just be a consequence of the lower arsenic concentrations in the fish samples, i.e. when a small amount of arsenic is distributed among several arsenic species, the individual species are not quantifiable and are not recorded in the sum of species. This is supported by the fact that within the fish samples, column recovery tended to be lower when arsenic concentration was low. We note that the two mussel samples also had low column recoveries (about 75%), despite their having high total arsenic concentrations. These samples, however, were unusual in other respects as well which may have compromised the quantification of the species; for example, the major arsenical in these mussel samples showed unexpected chromatographic behaviour, as discussed below.

Table 3
Mass balance

Samples	Concentration (mg As kg^{-1})[a]			Extraction yield (%)[c]	HPLC column recovery 1 (%)[d]	HPLC column recovery 2 (%)[e]
	Total As in extract (ICPMS)[b]	Flow injection (ICPMS)[b]	Sum of species (HPLC-ICPMS)			
Algae						
Green algae (fresh)	3.59	3.54	3.48	38	97	98
Green algae (sun-dried)	0.49	0.48	0.44	10	90	92
Plants						
Water-milfoil	1.42	1.39	1.18	26	83	85
Hornwort	1.03	1.10	1.01	30	98	92
Floating watermoss	1.52	1.45	1.37	31	90	94
Frog-bit	1.38	1.30	1.33	31	96	102
Sedge	0.66	0.69	0.56	53	85	81
Animals						
Sponge	2.46	2.70	2.51	30	102	93
Mussel (river)	4.76	4.84	3.47	51	73	72
Mussel (dead channel)	5.14	4.78	3.54	44	69	74
Fry (mixed fish) days old	0.35	0.38	0.23	26	66	61
Fry (mixed fish) months old	0.33	0.39	0.25	27	76	64
Silver carp	0.14	0.16	0.03	12	21	19
White bream A	0.94	0.74	0.37	59	39	50
White bream B	0.26	0.33	0.14	36	54	42
Frog	1.62	1.67	1.69	64	104	101

[a] Based on initial freeze-dried powder.
[b] Mean of two determinations of one extract; in all cases duplicates agreed within 5% of each other.
[c] Calculated from total As data obtained by the method using microwave-assisted acid digestion/ICPMS.
[d] Based on digested extract.
[e] Based on flow injection.

Collectively, these data suggest that flow injection analysis may provide a reliable measure of total arsenic injected onto HPLC columns. Data from flow injection analysis might also better account for any differences in ICPMS response for different arsenic species (although our experience suggests that such effects are minimal). Furthermore, it has the advantage of convenience and economy of time because the same solution is used for both total arsenic and arsenic speciation analysis.

It is often informative to quote the quantities of the various arsenic species detected as a percentage of total arsenic in the extract. Because our data set contains three measures of total arsenic (digestion/conventional ICPMS; flow injection ICPMS; and sum of species from HPLC-ICPMS), it is possible to express the percentages in three ways. Inspection of the data in Table 3 indicates that for most samples the three data sets are very similar. For convenience, we will use only one in the following discussion: the percentage data will be reported as the concentration of the arsenical measured by HPLC-ICPMS relative to the total arsenic concentration of the extract measured by flow injection ICPMS. We believe that on balance this approach provides the most informative picture of the arsenic speciation pattern in the extracts.

3.3. Arsenic compounds

Identification and quantification of the arsenic species in the water extracts of the samples was carried out by HPLC-ICPMS employing anion- and cation-exchange chromatography. The rationale for this approach has been previously discussed [6]. One disadvantage of the method is that arsenite cannot be definitively measured because it runs near the void volume under both cation- and anion-exchange chromatographic conditions; its concentration is determined by difference (anion-exchange front peak minus sum of cations). Thio-arsenosugars have been previously shown to be strongly retarded under chromatographic condition I (Table 1) [18]. Consequently, chromatographic condition II was employed to determine these compounds. All speciation analyses were performed in duplicate and the data agreed to within 10% in every case; the data are summarised in Table 4 and discussed below in terms of the different types of organisms examined.

3.3.1. Freshwater algae

Although arsenosugars are the major arsenic species in marine algae [19], their presence in freshwater algae is less well documented. The first report was by Lai et al. [20], who found that 93% of total arsenic in *Nostoc* sp. was oxo-arsenosugar-glycerol. Koch et al. [21] also found oxo-arsenosugar-glycerol in algae but on that occasion the dominant species was inorganic arsenate. In accordance with those previous investigations, arsenosugars were also detected in the fresh green alga *Cladophora* sp. from the Danube where oxo-arsenosugar-glycerol was the main arsenic compound in the extracts constituting 84% of the extractable arsenic. In addition, the oxo-arsenosugar-sulfonate and arsenate were also present at about 5 and 10%, respectively.

The second sample of *Cladophora* investigated in our study comprised dead specimens, washed up on the river bank, which had been naturally dried by exposure to the sun. In this case, only 10% of the total arsenic was extractable (compared with 38% extractable from fresh *Cladophora*), and arsenosugars were not detectable in these extracts. Most of the extractable arsenic was present as arsenate at a concentration comparable with that measured in the fresh green alga. It is conceivable that the arsenosugars were decomposed to non-extractable arsenic species during exposure to the natural and strong (collection was in early summer) UV radiation, or by bacterial activity on the river bank. It is also possible that the arsenosugars were trapped in impermeable cell structures after the shrinkage of the plant tissue during drying. This latter explanation implies that arsenosugars and arsenate may be present in different parts of the living plant, an unproven hypothesis that may warrant further study.

3.3.2. Freshwater plants

Arsenic speciation studies in the terrestrial environment show clearly that the dominating water soluble arsenic compounds in plant species are inorganic As(III) and As(V), but, additionally, methylated arsenic compounds such as MA and DMA are also present [21–23]. In the case of freshwater plants investigated in this work, 26–53% of the arsenic was recovered using the water extraction procedure. Arsenate was the major form in all samples constituting 49–77% of the total extractable arsenic. Additionally, a trace of DMA was found in sedge and TETRA was detected as a minor compound in frogbit and sedge.

3.3.3. River sponge

There have been only limited studies on arsenic speciation in sponges and none of them has investigated freshwater sponge. Shiomi et al. [24] found AB in marine sponge species *Halichondria okadai* and *Spirastrella insignis*, while Yamaoka et al. [25] reported AB and oxo-arsenosugar-phosphate as the major arsenic species, and oxo-arsenosugar-glycerol as a minor species, in a marine sponge. In contrast, the freshwater sponge investigated in this study contained mainly arsenate (57%) while arsenite (ca. 20%), oxo-arsenosugar-glycerol (11%) and DMA (4%) were present as minor constituents in the extract. Interestingly, AB was not detected in the sponge extract (<1%).

3.3.4. Freshwater mussels

The two samples of mussels (*Unio pictorum*) investigated in this study contained arsenosugars as the major extractable arsenic species, and arsenobetaine was present only as a minor species (1–2%) (Fig. 2). Koch et al. [5] previously reported arsenosugars as major constituents in freshwater mussel samples. The low proportion of arsenobetaine in *U. pictorum* is in contrast to the situation with marine mussels where it occurs together with arsenosugars as a significant arsenic species [26].

The samples of *U. pictorum* also contained appreciable quantities of two thio-arsenosugars, namely the thio-arsenosugar-glycerol and the thio-arsenosugar-phosphate collectively accounting for about 10% of the extractable arsenic (Fig. 2). Recently, Schmeisser et al. [18] reported the thio-arsenosugar-glycerol and thio-arsenosugar-phosphate as constituents of

Table 4
Arsenic compounds in water extracts of the algae, plant and animal samples

Sample	Concentration (mg As kg^{-1})[a]												
	As(III)[b]	As(V)	MA	DMA	TMAO	AB	AC	TETRA	oxo-arsenosugar-glycerol	oxo-arsenosugar-phosphate	oxo-arsenosugar-sulfonate	thio-arsenosugar-glycerol	thio-arsenosugar-phosphate
Green algae (fresh)	n.q.	0.36	<0.03	<0.03	<0.03	<0.02	<0.02	<0.02	2.98	<0.03	0.17	<0.04	<0.04
Green algae (sun-dried)	0.13	0.29	<0.03	<0.03	<0.03	<0.02	<0.02	Trace	<0.02	<0.03	<0.08	<0.04	<0.04
Water-milfoil	0.26	0.92	<0.03	<0.03	<0.03	<0.02	<0.02	<0.02	<0.02	<0.03	<0.08	<0.04	<0.04
Hornwort	0.19	0.82	<0.03	<0.03	<0.03	<0.02	<0.02	<0.02	<0.02	<0.03	<0.08	<0.04	<0.04
Floating watermoss	0.40	1.01	<0.03	<0.03	<0.03	<0.02	<0.02	<0.02	<0.02	<0.03	<0.08	<0.04	<0.04
Frog-bit	0.25	1.00	<0.03	<0.03	<0.03	<0.02	<0.02	0.07	<0.02	<0.03	<0.08	<0.04	<0.04
Sedge	0.14[c]	0.34[c]	<0.03	Trace[c]	<0.03	<0.02	<0.02	0.05[c]	<0.02	<0.03	<0.08	<0.04	<0.04
Sponge	ca/ 0.60	1.53	<0.03	0.12	<0.03	<0.02	<0.02	<0.02	0.29	<0.03	<0.08	<0.04	<0.04
Mussel (river)	n.q.	<0.03	<0.03	<0.03	<0.03	0.11	<0.02	<0.02	0.89	1.85	<0.08	0.21	0.39
Mussel (dead channel)	n.q.	0.07	<0.03	<0.03	<0.03	0.06	<0.02	<0.02	1.07	1.69	<0.08	0.48	0.06
Fry (mixed fish) days old	<0.02	0.10	<0.03	<0.03	Trace	<0.02	<0.02	<0.02	Trace	0.12	<0.08	<0.04	<0.04
Fry (mixed fish) months old	<0.02	Trace	<0.03	<0.03	0.08	<0.02	<0.02	<0.02	<0.02	0.12	<0.08	<0.04	<0.04
Silver carp	<0.02	<0.03	<0.03	<0.03	0.03	<0.02	<0.02	<0.02	<0.02	<0.03	<0.08	<0.04	<0.04
White bream A	<0.02	<0.03	<0.03	<0.03	<0.03	Trace	<0.02	<0.02	Trace	0.24	<0.08	<0.04	0.07
White bream B	<0.02	<0.03	<0.03	<0.03	<0.03	0.03	<0.02	<0.02	<0.02	Trace	<0.08	<0.04	0.07
Frog	ca. 0.50	0.23	0.15	0.27	Trace	Trace	Trace	0.58	<0.02	<0.03	<0.08	<0.04	<0.04

n.q.: not quantified because of large contribution from co-eluting cationic arsenic compounds.

[a] Mean of two separate extractions (results agreed within 10% in all cases).

[b] As(III) values have been estimated: As in front peak (anion-exchange column)—cation compounds recorded on cation-exchange column (except green algae and mussels because of high concentration of oxo-arsenosugar-glycerol).

[c] Determined once only because of small quantity of material.

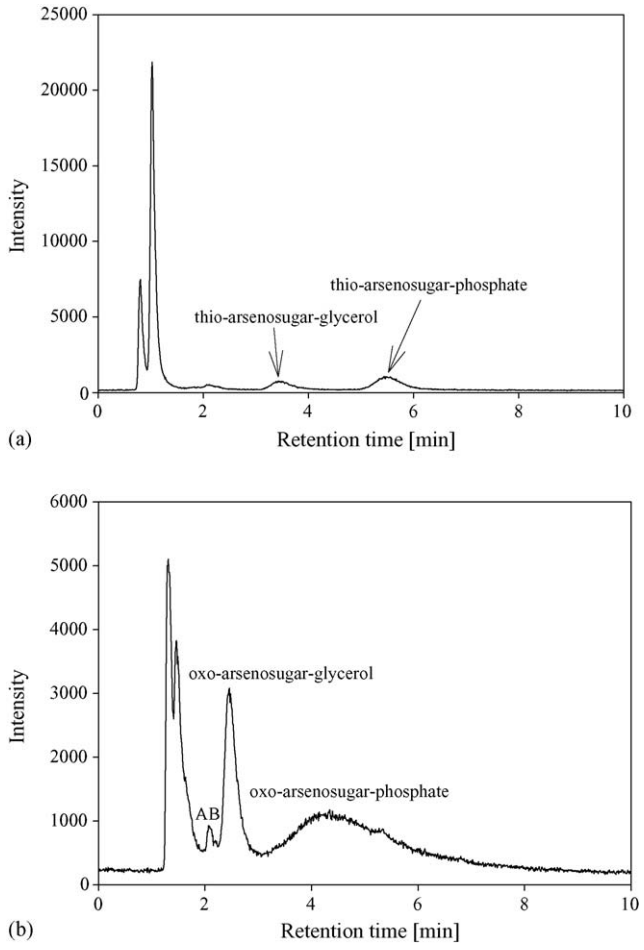

Fig. 2. HPLC-ICPMS chromatogram of an extract of freshwater mussels. Conditions were: (a) Hamilton PRP-X100 anion-exchange column at 40 °C; mobile phase of 20 mM aqueous NH_4HCO_3 at pH 10.3, 3% MeOH, 1.5 mL min^{-1} flow rate, injected volume 20 μL. (b) Zorbax 300-SCX cation-exchange column at 30 °C; mobile phase of 20 mM aqueous pyridine at pH 2.6, 1.5 mL min^{-1} flow rate, injected volume 20 μL.

marine mussels, and subsequent work [6] reported these same compounds in five species of freshwater mussel including *U. pictorum*.

The results of Soeroes et al. [6], however, were obtained from mussels, which had been stored frozen for 5 years, and the possibility remained that the thio compounds were formed from the oxo-arsenosugars during the prolonged storage. Our results were obtained on fresh mussels, which were processed within 24 h after collection, and thus indicate that thio-arsenosugars are natural constituents of the living mussels. The origin of thio-arsenosugars is not yet established. They do not appear to be significant constituents of algae despite the presence of high concentrations of oxo-arsenosugars. Possibly, they are formed primarily in vivo by the mussels from oxo-arsenosugars obtained from ingested algae.

We would like to briefly mention unexpected and previously unobserved chromatographic behaviour for the oxo-arsenosugar-phosphate present in the mussels. As shown in Fig. 2b, the mussel extract produced a broad signal between 3 and 6 min under cation-exchange conditions which we initially thought may have been a novel cationic arsenic species. We collected the peak and performed HPLC-electrospray mass spectrometry which clearly indicated that this "unknown" was actually the oxo-arsenosugar-phosphate. A subsequent check of the standard oxo-arsenosugar-phosphate on this particular cation-exchange column (Zorbax 300-SCX) showed that it also eluted as a broad peak (t_R = 3–6 min). The oxo-arsenosugar-phosphate did not show this behaviour on previous occasions (e.g. Madsen et al. [27]). It appears as though the column properties were altered during usage, possibly as a consequence of injecting solutions containing H_2S, a practice often used in our laboratory for transforming arsine oxides to their thio-analogues.

3.3.5. *Freshwater fish*

Because of the low total arsenic concentrations and small quantities of material for some fish samples, speciation analysis was performed only on the samples with more than 0.5 mg As kg^{-1} dry mass, namely two samples of fry, two samples of bream, and one sample of silver carp. Except for silver carp, the fish samples all contained oxo-arsenosugar-phosphate as the major arsenic compound while AB was present only in trace amounts. Slejkovec et al. [2] and Zheng and Hintelmann [3] did not report arsenosugars in freshwater fish, although Koch et al. [5] found traces of oxo-arsenosugar-phosphate in one (*Catostomus commersoni*) of four fish species examined. Of particular interest in our study was the presence of the thio-arsenosugar-phosphate in both samples of bream (Fig. 3). This is the first report of a thio arsenical in fish.

The single sample of silver carp was also of interest because although it contained 1.2 mg kg^{-1} total arsenic, only 12% (Table 3) was extractable into water. The recovery of arsenic after HPLC was also very low (ca. 20%), and TMAO was the only species detected. The apparent low column recovery may simply result from the arsenic being distributed among several arsenicals at concentrations below our detection limit (which ranged from 0.02 to 0.08 mg As kg^{-1} depending on the species). The

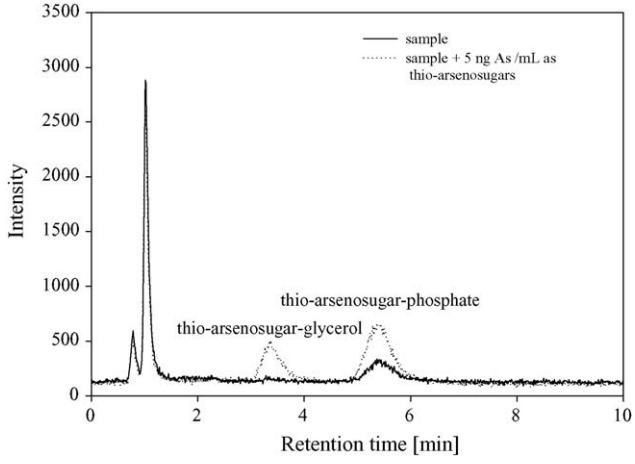

Fig. 3. HPLC-ICPMS chromatograms of an extract of white bream A (solid line) and the same extract spiked with thio-arsenosugars (dotted line). Conditions were: Hamilton PRP-X100 anion-exchange column at 40 °C; mobile phase of 20 mM aqueous NH_4HCO_3 at pH 10.3, 3% MeOH, 1.5 mL min^{-1} flow rate, injected volume 20 μL.

fish *Kyphosus sydneyanus* was also reported to contain TMAO as its major extractable arsenical [28].

TMAO and arsenate were also identified in the fry samples. From a human health view-point, it is important to note that inorganic arsenic (arsenate and arsenite) was not found in adult fish; a possible explanation for the presence of arsenate in fry fish could be that these samples were prepared whole and contained other tissues, including the gut and its contents, in addition to muscle tissue.

3.3.6. Freshwater frog

Although amphibians form an integral part of the freshwater ecosystem, there are no data about the arsenic species distribution in these animals. In this study, we determined the arsenic species in a single specimen of the frog *Rana* sp. Aqueous extraction removed 64% of the total arsenic and recovery from the HPLC column was quantitative (101–104%). The sample showed an interesting species distribution pattern; arsenite (30% of extractable arsenic) and TETRA (35%) were major species and all three intermediate methylation products (MA, DMA, TMAO) and arsenate were also detected (Fig. 4). Thus, the frog contained all the arsenic species in the proposed arsenic biomethylation pathway which begins with arsenate and has TETRA as the ultimate end product [19]. Laboratory experiments have previously shown that polychaetes can biotransform arsenate through to TETRA, but in that study DMA was not detected in the extracts [29]. Small quantities of AB and arsenocholine (AC) were also present in the frog.

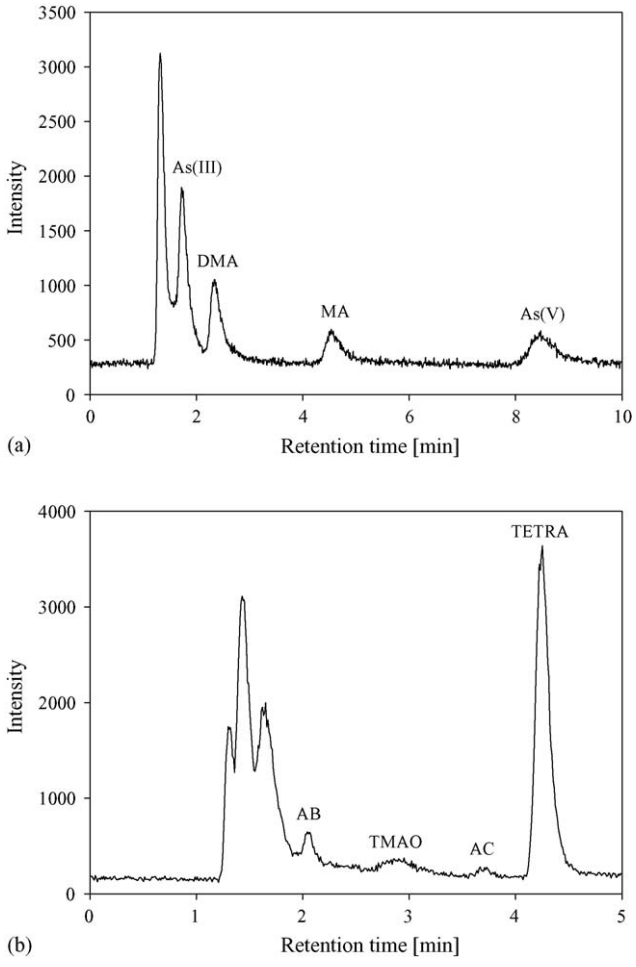

Fig. 4. HPLC-ICPMS chromatogram of an extract of frog. Conditions were: (a) Hamilton PRP-X100 anion-exchange column at 40 °C; mobile phase of 20 mM aqueous $NH_4H_2PO_4$ at pH 5.6, 1.5 mL min^{-1} flow rate, injected volume 20 μL. (b) Zorbax 300-SCX cation-exchange column at 30 °C; mobile phase of 20 mM aqueous pyridine at pH 2.6, 1.5 mL min^{-1} flow rate, injected volume 20 μL.

The pattern of arsenic compounds in the frog *Rana* is sufficiently interesting to warrant further study of other frog species. We note that the specimen in the current study was processed whole, and that additional information may come from investigating separately the major organs/tissues of frogs. Although frogs are essentially freshwater (or terrestrial) animals, some species are salt tolerant (e.g. the mangrove frog *Rana cancrivora*), and comparison of their arsenic speciation pattern with freshwater species may also be of interest.

3.4. Freshwater versus marine organisms: general comments

Although reports of arsenic speciation in freshwater organisms, the subject of our study, are still few relative to those dealing with marine organisms, some interesting similarities and differences seem to be showing through. Algae appear quite similar from both marine and freshwater systems, with arsenosugars predominating. These similarities, however, do not apply to animal samples.

It has long been considered that total arsenic concentrations in marine animals were much higher than those in freshwater animals. Inspection of the speciation data suggests that a major part of this difference lies in the relative amounts of AB. The possible role of AB as an adventitiously acquired osmolyte has been earlier discussed [19] and recent work has demonstrated that AB concentrations in mussels (*Mytilus edulis*) are proportional to the salinity of their ambient water [30]. Thus, in saline waters, osmoconforming organisms, such as molluscs, accumulate glycine betaine [$(CH_3)_3N^+CH_2COO^-$] and other structurally similar small polar molecules as protection against osmotic stress, and it is possible that AB simply joins this pool of osmolytes. High concentrations of these molecules, however, are not required by animals living in freshwater environments.

The dominance and virtual absence of AB in marine and freshwater animals, respectively, may also explain the low recoveries often reported for arsenic species in freshwater animals. AB is a polar molecule readily soluble in both methanol and water, the two most common solvents used for extraction in arsenic speciation analysis. Thus, when AB is the dominant arsenical in the sample (such as in most samples of marine animals) high extraction yields are obtained. In freshwater animals, however, AB may not be present at all, and the presence of other, non-extractable, arsenicals becomes significant. Possibly, these other arsenicals also occur in marine animals, but because they constitute just a fraction of the total arsenic, their presence is being overlooked. Future studies focusing on the currently non-extractable arsenic might be informative and perhaps reveal further similarities between arsenic species in marine and freshwater samples.

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