Two Novel Thio-Arsenosugars in Scallops Identified with HPLC–ICPMS and HPLC–ESMS

Markus Kahn, Reingard Raml, Ernst Schmeisser, Birgit Vallant, Kevin A. Francesconi, and Walter Goessler

A Institute of Chemistry—Analytical Chemistry, University Graz, Universitaetsplatz 1, A-8010 Graz, Austria.
B Corresponding author. Email: walter.goessler@uni-graz.at

Environmental Context. A new group of arsenic compounds, namely thio-arsenosugars, has recently been discovered in some marine samples, including seafood products. We do not yet understand how these arsenic compounds are produced by living organisms, nor do we know their implications for human health. Their unusual chromatographic properties are the reason that they are difficult to detect by existing methods. We present a new method for finding these thio-arsenosugars, and we also report two new thio-arsenosugars.

Abstract. Two new thio-arsenosugars were identified in aqueous and methanol extracts from the gonad and the muscle of the great scallop by using high-performance liquid chromatography–inductively coupled plasma mass spectrometry (HPLC–ICPMS) and HPLC–electrospray ionization mass spectrometry (ESMS). The gonad of the scallops contained thio-arsenosugar–sulfonate [0.067 mg (As) kg\(^{-1}\); dry mass ± 3.7%] and thio-arsenosugar–sulfate [0.267 mg (As) kg\(^{-1}\); dry mass ± 12%]. The muscle also contained both thio-arsenosugar–sulfonate [0.030 mg (As) kg\(^{-1}\); dry mass ± 2.6%] and thio-arsenosugar–sulfate [0.209 mg (As) kg\(^{-1}\); dry mass ± 2.6%]. Methanol increased the extraction efficiency threefold for the two thio-arsenosugars as compared to water. Fresh and freeze-dried samples from both parts of the scallop showed no significant difference with respect to the concentration of the two thio-arsenosugars. In order to confirm the presence of the thio-arsenosugars, a new method for their determination with HPLC–ESMS was developed. Under optimized conditions [Hamilton PRP-X100; 150 by 1.0 mm; 20 mM NaHCO\(_3\) with 55% (v/v) methanol at pH 10.3] we succeeded in determining the pseudomolecular ions of thio-arsenosugar–glycerol, thio-arsenosugar–phosphate, thio-arsenosugar–sulfonate, and thio-arsenosugar–sulfate at concentrations less than 5 μg (As) dm\(^{-3}\) in standard solutions and real samples.

Keywords. arsenic — bioavailability — contaminant uptake — speciation (nonmetals)

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Introduction

Although total arsenic concentrations in seawater seldom exceed 2 μg dm\(^{-3}\), marine plants and animals may contain up to 100 mg (As) kg\(^{-1}\) (wet mass).[11] Inorganic arsenic—dominating in seawater—is almost quantitatively converted into organic compounds in marine fauna and flora.[1] Arsenosugars are major arsenic compounds in algae but are reported to be present in significant concentrations in scallops as well.[2] Altogether 15 arsenosugars have been reported, four of which (Fig. 1) are commonly found in high concentrations in the marine environment, namely, arsenosugar–glycerol, arsenosugar–phosphate, arsenosugar–sulfonate, and arsenosugar–sulfate.[3]

Recently, the thio-analogues of several known arsenic oxides were reported to be present in various samples. Thio-dimethylarsenioacetate has been found in the urine of sheep feeding on algae with high concentrations of arsenosugars,[4] while thio-dimethylarsenioacetate, thio-dimethylarsenal, and traces of thio-arsenosugar–glycerol were determined in human urine after ingestion of arsenosugar–glycerol.[5] In liver cytosol thio-arsenosugar–glycerol and thio-arsenosugar–sulfonate have been determined after incubation with arsenosugar–glycerol and arsenosugar–sulfonate.[6] Schmeisser et al. (2004) reported the presence of thio-arsenosugar–glycerol and thio-arsenosugar–phosphate in a canned mussel product,[7] and Fricke et al. (2004) detected thio-arsenosugar–phosphate in a butter clam.[8]

As part of an ongoing project investigating arsenic species in food products, several mollusc samples from the local fish market in Graz were scanned for thio-arsenosugars using HPLC–ICPMS, under conditions optimized for detection of
Total Arsenic Analysis

Arsenic concentrations in the samples were determined by ICPMS after mineralization of the samples and the reference material TORT-2 with microwave-assisted acid digestion. For the total arsenic determinations, 100 mg of the freeze-dried samples were digested with 3 cm³ HNO₃ in a microwave-heated autoclave (ultraCLAVE 2, EMSL, Leutkirch, Germany) at 250°C for 60 min. The accuracy of the measurement was tested by analysis of the reference material TORT-2 (100 mg weighed to 0.1 mg, digested in the same way) which has a certified value of 21.6 ± 1.8 mg (As) kg⁻¹ dry mass; the value we obtained was 23.0 ± 0.4 mg (As) kg⁻¹ dry mass (n = 3).

Extraction of the Arsenic Compounds from the Great Scallop

Individual great scallops (n ~ 20) were dissected to separate the gonad and the muscle. Each of the pooled gonad and muscle samples were freeze-dried (Alpha 1-4, Christ, Osterode am Harz, Germany) and ground to a homogeneous powder with an agate mortar and pestle. In order to see whether the freeze-drying process influences the specification of the sample, we extracted fresh tissue (~2 g to 5 cm³ ultra-pure water) and freeze-dried (~200 mg to 5 cm³ ultra-pure water) tissue of the gonad of the great scallop. The closed tubes were fastened to a home made cross-shaped rotator device and turned top-over-bottom for 14 h. Thereafter, the tubes were additionally sonicated for 10 min (Elma, Transsonic T700H/1, Singen, Germany) and then centrifuged for 20 min at 4500 rpm on a Jouan C2-2 centrifuge (Jouan, Saint Maurice, France). The supernatants were filtered through 0.2 µm nylon filters (LaPhyPack, Langerwehe, Germany) directly into 1 cm³ polyethylene vials. These solutions were analyzed directly with HPLC–ICPMS. In addition, the freeze-dried gonad powder was extracted with methanol using exactly the same extraction procedure as described above. The methanol extracts were used for the HPLC–ESMS measurements.

HPLC–ICPMS of Arsenicals with Emphasis on Thio-Arsenicals

An Agilent 1100 series HPLC system (Agilent, Waldbronn, Germany) with a quaternary pump, a vacuum degasser, column oven, and an autosampler with a variable 100 mm³ injection loop was used. For anion-exchange chromatography, Hamilton PRP-X100 columns (Hamilton Co., Reno, Nevada, USA) of three different dimensions but filled with the same stationary phase were used (Table 1). The outlet of the chromatographic column was directly connected to the Bainbridge nebulizer of the ICPMS with PEEK (polyetheretherketone) capillary tubing (0.125 mm i.d.) The ion intensities at m/z 75 and 77 (for possible [40Ar]³⁵Cl interferences) were monitored. The ICPMS performance was optimized with a mobile phase solution containing 10 µg (As) dm⁻³ to give maximum response on the As signal (m/z 75). The arsenic compounds were quantified with external calibration against standard solutions of arsenate. All measurements were carried out in triplicate. The results are given as mean and single standard deviation of the three individual determinations. In order to investigate whether all the arsenicals eluted from the column, flow injection experiments were performed on the extracts. For these experiments the same chromatographic system was used but the column was by-passed with a six-port injection valve. Quantification was done via standard addition with arsenate solutions. For mass balance experiments, 100 mm³ of the aqueous solution from the gonad were spiked with 10 mm³ of a 30% hydrogen peroxide solution in triplicate. For spiking experiments, authentic thio-arsenicals standards were used (2 mm³ of a 9.0 µg (As) dm⁻³ containing thio-arsenosalen–sulfonate and 2 mm³ of a 20 µg (As) dm⁻³ containing thio-arsenosalen–sulfate solution were added to 18 mm³ of the aqueous scallop extract).

HPLC–ESMS

An Agilent 1100 series HPLC system and an Agilent LCMSD single quadrupole mass spectrometer of the SL-type were used. Chromatographic conditions I and II were used for the determination of the thio-arsenicals as described in Table 1. The mass spectrometer was equipped with an atmospheric pressure ionization (API) source employing pneumatically assisted electrospray nebulization with nitrogen as the nebulizer gas.
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Table 1. Chromatographic conditions for the determination of the arsenic compounds

<table>
<thead>
<tr>
<th>Conditions</th>
<th>I</th>
<th>II</th>
<th>III</th>
</tr>
</thead>
<tbody>
<tr>
<td>Column</td>
<td>Hamilton PRP-X100</td>
<td>Hamilton PRP-X100</td>
<td>Hamilton PRP-X100</td>
</tr>
<tr>
<td>Dimensions</td>
<td>250 by 4.1 mm [i.d.]</td>
<td>100 by 4.1 mm [i.d.]</td>
<td>150 by 1.0 mm [i.d.]</td>
</tr>
<tr>
<td>Particle size</td>
<td>10 μm</td>
<td>5 μm</td>
<td>5 μm</td>
</tr>
<tr>
<td>Mobile phase</td>
<td>20 mM NH₄H₂PO₄</td>
<td>20 mM NH₄H₂CO₃ 3% (v/v) MeOH</td>
<td>20 mM NH₄H₂CO₃ 55% MeOH (v/v)</td>
</tr>
<tr>
<td>Temperature</td>
<td>40°C</td>
<td>40°C</td>
<td>40°C</td>
</tr>
<tr>
<td>pH</td>
<td>5.6</td>
<td>10.3</td>
<td>10.3</td>
</tr>
<tr>
<td>Injection volume</td>
<td>20 mm³</td>
<td>20 mm³</td>
<td>2 mm³</td>
</tr>
<tr>
<td>Flow rate</td>
<td>1.5 cm³ min⁻¹</td>
<td>1.5 cm³ min⁻¹</td>
<td>30 mm³ min⁻¹</td>
</tr>
</tbody>
</table>

Table 2. Arsenic species [μg (As) kg⁻¹ dry mass] in the aqueous extracts from the great scallop (gonad and muscle)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Total arsenic in powder mean (% RSD)</th>
<th>Arsenic in the extract (F1) mean (% RSD)</th>
<th>Extraction efficiency mean (% RSD)</th>
<th>Sum of species mean (% RSD)</th>
<th>Column recovery mean (% RSD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gonad</td>
<td>9.2 (1.8)</td>
<td>3.2 (7.6)</td>
<td>35 (3.2)</td>
<td>2.92 (3.3)</td>
<td>92 (8.7)</td>
</tr>
<tr>
<td>Muscle</td>
<td>4.8 (8.2)</td>
<td>3.2 (2.0)</td>
<td>66 (7.0)</td>
<td>2.72 (4.5)</td>
<td>87 (5.1)</td>
</tr>
</tbody>
</table>

A The extraction efficiency is based on the aqueous extracts.
B The sum of species was determined under chromatographic conditions II with an external calibration established with arsenate.

Results and Discussion

Analysis of Total Arsenic and Arsenic Species with Emphasis on Thio-Arsenosugars

The total arsenic concentration in the pooled gonad samples of the great scallop was 9.2 ± 0.2 μg (As) kg⁻¹ (Table 2). With water, 35% [3.2 ± 0.2 μg (As) kg⁻¹] of the total arsenic was extracted. The total arsenic concentration in the pooled muscle sample of the great scallop was 4.8 ± 0.4 μg (As) kg⁻¹, of which 66% [3.2 ± 0.1 μg (As) kg⁻¹] was extractable into water.

Chromatographing an aqueous extract of the gonad under conditions II (Table 1, but without methanol) produced two broad signals eluting at ~30 and ~54 min. When this extract was re-chromatographed under the same chromatographic conditions but with 3% (v/v) methanol in the mobile phase (chromatographic conditions II) the signals at ~30 and ~54 min were shifted to ~14 min (signal U1, Fig. 2a) and 30 min (signal U2, Fig. 2a). Similar signals were observed for the muscle sample. This retention behaviour (long retention times on PRP-X100 which were greatly reduced on addition of methanol to the mobile phase) was similar to the chromatographic properties of thio-arsenosugar–glycerol and thio-arsenosugar–phosphate reported by Schmieser et al.,[7] and led us to speculate that U1 and U2 represented two new thio-arsenosugars.

When the aqueous extract was treated with hydrogen peroxide the unknown compounds U1 and U2 completely disappeared within ten minutes (Fig. 2a), and the signals for arsenosugar–sulfonate and arsenosugar–sulfate increased significantly (Fig. 2b).

In order to confirm the conversion of the thio-arsenosugars to oxo-arsenosugars, we spiked the aqueous extracts with thio-arsenosugar–sulfonate and thio-arsenosugar–sulfate...
Table 3. Arsenic species [mg (As) kg⁻¹ dry mass] in the aqueous extract from the gonad of the great scallop before and after oxidation

<table>
<thead>
<tr>
<th>Sample</th>
<th>Arsenic species</th>
<th>Retention time (min)</th>
<th>Extract mean (% RSD)</th>
<th>Extract + H₂O₂ mean (% RSD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gonad</td>
<td>Arsenosugar–sulfonate</td>
<td>8.0 (I)</td>
<td>0.068 (1.7)</td>
<td>0.131 (1.5)</td>
</tr>
<tr>
<td></td>
<td>Thio-arsenosugar–sulfonate</td>
<td>14.3 (II)</td>
<td>0.067 (3.7)</td>
<td>&lt;0.02</td>
</tr>
<tr>
<td></td>
<td>Arsenosugar–sulfate</td>
<td>15.3 (I)</td>
<td>0.431 (4.2)</td>
<td>0.721 (1.7)</td>
</tr>
<tr>
<td></td>
<td>Thio-arsenosugar–sulfate</td>
<td>29.7 (II)</td>
<td>0.267 (1.2)</td>
<td>&lt;0.02</td>
</tr>
</tbody>
</table>

with synthetic standards. The spiking experiments produced suitably enhanced undistorted peaks for U1 when spiked with thio-arsenosugar–sulfonate and U2 when spiked with thio-arsenosugar–sulfate (chromatograms not shown). Mass balance calculations showed that the oxidation with hydrogen peroxide had quantitatively converted thio-arsenosugar–sulfonate and thio-arsenosugar–sulfate into their oxo-analogues (Table 3).

For speciation analysis, it is very important to determine whether all the arsenic injected onto a chromatographic system is accounted for in the effluent of the column, especially when unknown samples are analyzed. This was tested for the gonad sample: under the chromatographic conditions II, the sum of the species eluting from the column accounted for 92 ± 8.7% of the arsenic in the extract determined via FIA, and under chromatographic conditions I, the column recovery was 84 ± 2.1%. The lower recovery under chromatographic conditions I is probably a consequence of long retention times and broad signals.

Schmesser et al.[7] reported that the concentrations of thio-arsenosugar–glycerol and thio-arsenosugar–phosphate decreased upon storage of an aqueous extract at room temperature, which is in agreement with the work of Fröde et al., who reported a degradation of thio-arsenosugar–phosphate with time in a clam extract.[8] In our study, storage of aqueous extracts of the gonad sample in a freezer at −16°C did not change the concentrations of the thio-arsenosugars significantly in six months. In order to check whether the freeze-drying process influences the speciation, we compared an aqueous extract of a fresh great scallop (gonad) with an extract that had been prepared from the freeze-dried material of the same sample. The results of this comparison revealed no significant difference with respect to the concentration of the thio-arsenosugars in the gonad. The temperatures during the freeze-drying process appear to be low enough to prevent the conversion of the thio-arsenosugars into their oxo-analogues.

The muscle of the great scallop was also further examined in a manner similar to that described for the gonad sample. The chromatograms (not shown) revealed an arsenic speciation pattern similar to that for the gonad sample, albeit the two new thio-arsenosugars were present at lower concentrations. In the gonad sample, the concentration of thio-arsenosugar–sulfonate was 0.067 mg (As) kg⁻¹ (± 3.7% dry mass), whereas only 0.030 mg (As) kg⁻¹ (± 2.6% dry mass) was found in the muscle. A similar picture was obtained for 0.267 mg (As) kg⁻¹ (± 1.2% dry mass) in gonad compared to 0.200 mg (As) kg⁻¹ (± 2.6% dry mass) in the muscle.

HPLC–ESMS for the Identification of the Thio-Arsenosugars (Sulfonate and Sulfate)

Although our HPLC–ICPMS data were quite clear, we wished to demonstrate the presence of the thio-analogue arsenosugars in the great scallop extracts, by employing ESMS as a molecule-selective detector. The direct application of chromatographic conditions II for ICPMS measurements did not produce the expected signals for the [M + H]⁺ ion at m/z 409 (thio-arsenosugar–sulfonate) and m/z 425 (thio-arsenosugar–sulfate). Further, adding the authentic standards at higher concentrations to the aqueous and methanol extracts of the gonad was also not successful. Most probably the co-extracted matrix and/or the chromatographic conditions prevented the formation of [M + H]⁺. Therefore, we decided to develop chromatographic conditions suitable for the detection of these two thio-arsenosugars with ESMS. All further experiments were performed with solutions of authentic standards of all four thio-arsenosugars at a concentration of 100 μg (As) dm⁻³ each.

Method Optimization

It is well known that pseudomolecular ion formation usually improves when small amounts of sample are introduced into the ESI source.[11] For method optimization the column (PRP-X100; 100 by 4.1 mm i.d.) was changed to a microcolumn (PRP-X100; 150 by 1.0 mm i.d.). In the ESI process, formation of the pseudomolecular ions is usually enhanced when high concentrations of organic solvents are present during sample introduction. The earlier ICPMS experiments had shown that methanol does not adversely affect the separation of the thio-arsenosugars. Thus, we investigated systematically the influence of methanol on the formation of the pseudomolecular ions ([M + H]⁺ and [M − H]⁻) of the four thio-arsenosugars. The methanol concentration was varied from 40 to 70% (v/v) without significant loss of separation. The signal to noise (S/N, peak height) ratios at the different methanol concentrations are plotted in Fig. 3a and Fig. 3b. It can be clearly seen that the S/N ratios for the three acidic thio-arsenosugars (sulfate, sulfonate, and phosphate) are much higher in the negative-ion mode, whereas the S/N ratio for the thio-arsenosugar–glycerol is higher in the positive-ion mode. Use of methanol concentrations between 50 and 55% increased the S/N for thio-arsenosugar–sulfate and thio-arsenosugar–sulfonate at least five times, whereas the S/N
Two Novel Thio-Arenosugars in Scallops Identified with HPLC–ICPMS and HPLC–ESMS

![Graphs showing changes in S/N ratios with varying concentrations of MeOH](image)

**Fig. 3.** (a) Change of S/N ratios with varying concentrations of MeOH (40 to 70%) in a 20 mM NH₄HCO₃ buffer solution at pH 10.3 using a PRP-X100 (150 by 1.0 mm) anion-exchange column at a flow rate of 30 mm/min. The ion formation was monitored in the negative mode. The injection volume was 2 μL. Observed m/z: 343 (100 V); 497 (100 V); 407 (100 V); 423 (100 V). (b) Change of S/N ratios with varying concentrations of MeOH (40 to 70%) in a 20 mM NH₄HCO₃ buffer solution at pH 10.3 using a PRP-X100 (150 by 1.0 mm) anion-exchange column at a flow rate of 30 mm/min. The ion formation was monitored in the positive mode. The injection volume was 2 μL. Observed m/z: 345 (100 V); 409 (100 V); 409 (100 V); 425 (100 V).

for thio-arsenosugar–phosphate was enhanced only three times. In the negative-ion mode the S/N for thio-arsenosugar–glycerol was barely influenced by methanol. The opposite was observed in the positive-ion mode—only the S/N ratio for thio-arsenosugar–glycerol showed a significant increase when changing the methanol concentration from 70 to 40%. Although, from the trend, one could expect even better S/N ratios at lower methanol concentrations, we did not investigate methanol concentrations below 40% because of the poor S/N ratios for the other three thio-arsenosugars (phosphate, sulfonate, and sulfate). Further, the fragmenter voltages, the nebulizer pressure, and the drying gas flow were optimized for the pseudomolecular and the fragment ions. Under the optimized conditions we obtained detection limits of less than 5 μg (As) dm⁻³ (Fig. 4). Additionally, characteristic fragment ions were observed at m/z 253 (loss of aglycone) and signals for m/z 107 (As²⁻, As²⁻) and m/z 91 (AsO⁻) were observed using the positive-ion mode.

**ESMS on Real Samples**

The optimized conditions allowed direct determination of the newly discovered thio-arsenosugars to support the results obtained with HPLC–ICPMS. During this work we realised that methanol increases the extraction yields for the thio-arsenosugars threefold. To match the mobile phase composition and facilitate the detection of the pseudomolecular ions, we investigated the methanol extracts of the gonad with the optimized HPLC–ESMS method.

The chromatogram of the gonad extract showed chromatographic signals at m/z 407 and 423 at 11.5 and 13.2 min (Fig. 5a and b). The signals were slightly shifted from the retention times of the authentic standards, which we attributed to the matrix of the methanol extract. Spiking
the extracts with the thio-arsenosugar-sulfonate and the thio-arsenosugar-sulfate standard showed enhanced peaks at the retentions mentioned above (Fig. 5a and b). These experiments confirmed the correct assignment of the two thio-arsenosugars in the extracts of the great scallop.

Closing Comments

All the thio-analogues of the four major oxo-arsenosugars have now been discovered in several animal samples of terrestrial and marine origin.[12,15] The results of our experiments show that the thio-arsenosugars are present in fresh samples of the scallop, and are not just formed during storage or sample preparation. The oxygen analogue arsenosugars are dominating in marine algae, but until now thio-arsenosugars have been reported only in animal samples. The reason for this could be that until now researchers have not investigated algal samples specifically for these compounds, or that these compounds are biosynthesized only within animals. We consider it likely that these compounds are also present in algal samples, although the levels may be much lower than those found in molluscs. Whether thio-arsenosugars are present in other animal species also is currently not known, but it can be expected that researchers will have an eye on these compounds, and that publications in the future will definitely answer this question.

Acknowledgments

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References

Distribution and Speciation of Arsenic in Temperate Marine Saltmarsh Ecosystems

Simon Foster, A, B William Maher, A Anne Taylor, A Frank Krikowa, A and Kristy Telford A

A Ecochemistry Laboratory, Institute of Applied Ecology, University of Canberra, Belconnen, 2601, Australia.
B Corresponding author. Email: foster@aerg.canberra.edu.au

Environmental Context. The pathways by which arsenic is accumulated and transferred in aquatic ecosystems are relatively unknown. Examination of whole marine ecosystems rather than individual organisms provides greater insights into the biogeochemical cycling of arsenic. Saltmarshes with low ecological diversity are an important terrestrial-marine interface about which little is known regarding arsenic concentrations and species distribution. This study examines the cycling of arsenic within Australian saltmarsh ecosystems to further understand its distribution and trophic transfer.

Abstract. This paper reports the distribution of total arsenic and arsenic species in saltmarsh ecosystems located in south-east Australia. We also investigated the relationship between arsenic, iron, and phosphorus concentrations in saltmarsh halophytes and associated sediment.

Total mean arsenic concentrations in saltmarsh plants, S. quinqueflora and S. australis, for leaves ranged from 0.03 ± 0.05 to 0.67 ± 0.48 μg g⁻¹ and 0.03 ± 0.02 to 0.08 ± 0.06 μg g⁻¹, respectively, and for roots ranged from 2 ± 2.6 to 12 μg g⁻¹ and 39 ± 0.20 to 0.57 ± 1.06 μg g⁻¹ respectively. Removal of iron plaque from the roots reduced the arsenic concentration variability to 0.40–0.79 μg g⁻¹ and 0.95–1.05 μg g⁻¹ for S. quinqueflora and S. australis roots respectively. Significant differences were found between locations for total arsenic concentrations in plant tissues and these differences could be partially attributed to differences in sediment arsenic concentrations between locations. For S. quinqueflora but not S. australis there was a strong correlation between arsenic and iron concentrations in the leaf and root tissues. A significant negative relationship between arsenic and phosphorus concentrations was found for S. quinqueflora leaves but not roots.

Total mean arsenic concentrations in salt marsh animal tissues (7 ± 2–21 ± 13 μg g⁻¹) were consistent with those found for other marine animals. The concentration of total arsenic in gastropods and amphipods could be partially explained by the concentration of total arsenic in the dominant saltmarsh plant S. quinqueflora.

Of the extractable arsenic, saltmarsh plants were dominated by arsenic(III), arsenic(V) (66–99%), and glycerol arsenobis(17–35%). Arsenobetaine was the dominant extractable arsenic species in the gastropods Saltnator solda (84%) and Ophideres ornatus (89%) and the crab Neosarmatium meinerti (89%). Amphipods contained mainly arsenobetaine (44%) with some phosphate arsenobis(23%). Glycerol trimethyl arsenobis(10.7–0.8%) and the visceral mass of N. meinerti (0.1%).

These results show that arsenic uptake into plants from uncontaminated saltmarsh environments may be dependent on plant iron uptake and inhibited by high phosphorus concentrations. Arsenic in saltmarsh plants is mainly present as inorganic arsenic, but arsenic in animals that eat plant detritus is present as arsenic species, primarily arsenobetaine and arsenosugars. The presence of glycerol trimethyl arsenobis(poses the question of whether trimethylated arsenobis(are transitory intermediates in the formation of arsenobetaine.

Keywords. arsenic — iron — phosphorus — speciation (nonmetals)

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Introduction
Saltmarshes occur from the mean high tide to the upper spring tide level.1 Saltmarshes are common features of estuaries in many parts of the world and form the interface between mangrove forests and terrestrial ecosystems. They are depositional zones that support large populations of crabs and, when in inundated, are thought to be important feeding grounds for juvenile fish.2 Saltmarshes in south-eastern Australia are dominated by only two species of halophyte plants (Sarcocornia quinqueflora and Suaeda australis), two gastropod species (Saltnator solda and Ophideres ornatus), and a crab (Neosarmatium meinerti).2, 3 Saltmarsh food webs

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are detrital based (Fig. 1) with both the gastropods and crabs being detritovores.\textsuperscript{[5,6]} Although little information is available on other invertebrates inhabiting these ecosystems it is known that supralittoral species of amphipods also occur in great numbers and are an important component of the detrital food web.\textsuperscript{[5,7]}

Marine and intertidal organisms have been shown to accumulate high concentrations of arsenic.\textsuperscript{[8]} It is well established that arsenobetaine (AB) is the dominant form of arsenic found in marine animals,\textsuperscript{[8]} while marine macroalgae\textsuperscript{[9]} and marine herbivores often contain high concentrations of dimethylarsiniclorobases (see Fig. 2 for arsenic species structures).\textsuperscript{[10-12]} To date the occurrence of AB in angiosperms from uncontaminated sites has not been conclusively demonstrated.\textsuperscript{[13-15]} The presence of AB in temperate mangrove gastropods has been reported by Kirby et al.,\textsuperscript{[12]} who found that AB made up 50–60% of the total arsenic. Similarly, Goessler et al.\textsuperscript{[16]} found AB to be the major arsenic compound in several rocky intertidal gastropods. AB has also been found in the marine amphipod \textit{Allorchestes compressa}.\textsuperscript{[17]}

The proposed pathways for the formation of arsenobetaine involve the biotransformation of arsenoriboses to AB.\textsuperscript{[8,18-20]} The main pathway for the formation of arsenobetaine is thought to be through the degradation of dimethylarsiniclorobases to dimethylarsinylethanol (DMAE), which is further converted into either dimethylarsinylacetic acid (DMAA) or arsenocholine.\textsuperscript{[8,18-20]} Further oxidation of arsenocholine at the primary alcohol group results in the formation of arsenobetaine.\textsuperscript{[21]} However, the discovery of trimethylated arsinoriboses in macroalgae\textsuperscript{[22,23]} and gastropods,\textsuperscript{[10,24]} and recently in herbivorous fish,\textsuperscript{[10]} has provided a simpler pathway for the formation of arsenobetaine. This pathway was first proposed by Francesconi and Edmonds\textsuperscript{[18]} with the view that trimethylated arsinoriboses occurred in algae, but algae have only been shown to contain trace amounts of these arseno sugars.\textsuperscript{[22,23]}

Iron and phosphorus are elements often thought to be associated with arsenic uptake in wetland plants.\textsuperscript{[25,26]} Iron oxides (plaque) are commonly formed on the roots of wetland/marsh plants.\textsuperscript{[26]} This may reduce or enhance the uptake of arsenic.\textsuperscript{[26]} Arsenic and phosphate are structurally similar species.\textsuperscript{[25,27]} They are therefore, thought to share the same uptake pathways and their concentrations are often found to be highly correlated in plant and algal tissues.\textsuperscript{[27,29]}

In this study we report the distribution of total arsenic and arsenic species in saltmarsh ecosystems located in southeast Australia. We also investigated the relationship between arsenic, iron, and phosphorus concentrations in saltmarsh halophytes and associated sediments.

**Materials and Methods**

**Sampling and Sample Preparation**

Samples were collected from three locations (Tomago River, Moruya River, and Congo Creek) on the south-east coast of New South Wales, Australia. Each of the saltmarshes was located on open estuaries and occupied areas <0.5 km$^2$. Plant samples collected were \textit{S. quinqueflora} (commonly known as Sampire) and \textit{S. australis} (commonly known as Sea Blight). Gastropod species collected were \textit{S. solida} and \textit{O. armatus}. The common saltmarsh crab \textit{N. mneisteri} was collected as well as an amphipod belonging to the family Talitridae.\textsuperscript{[30]} On a separate occasion the roots of \textit{S. quinqueflora} and \textit{S. australis} were re-sampled to determine the concentration of arsenic after the removal of iron plaque.

Plant samples were collected whole, including roots and associated sediment, and placed into acid-washed plastic zip-lock bags. Gastropods, crabs, and amphipods were collected by hand and kept alive on ice until arrival at the laboratory.

Plant samples were separated into sediment, root, and leaf tissue and rinsed in deionized water. Gastropods were cracked gently using a small bench vice and extracted whole. Gastropod tissues were rinsed clean of shell grit and placed in individual acid-washed vials. Crabs were frozen and the top shell was removed to expose the visceral mass, which was removed and placed into acid-washed vials. Muscle tissue was obtained from the claws of the crabs. Amphipods were rinsed in deionized water and 3–5 individuals placed into each acid washed vial. All samples were immediately frozen (−80°C) then freeze-dried for ~48 h (Labconco) to a constant mass. Plant tissues were homogenized using a Retsch ZM100 mill (0.2 mm stainless steel mesh, Retsch), and stored in clean polyethylene vials in a desiccator until analyzed. All other samples were homogenized using liquid nitrogen in an agate mortar and pestle due to very low sample masses (0.1–0.2 g).
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Distribution and Speciation of Arsenic in Temperate Marine Saltmarsh Ecosystems

![Chemical structures and names of arsenic species](image)

**Fig. 2.** Chemical structures, names, and abbreviations of arsenic species.

**Simple Analysis**

**Reagents and Standards**

Nitric acid (HNO₃; Arista, BDH) was used for the determination of total arsenic concentrations. Ammonium dihydrogen phosphate (Suprapur, Merck) and pyridine (Extra Pure, Merck) were used in the preparation of high-pressure liquid chromatography (HPLC) mobile phases. Formic acid (Extra Pure, Fluka) and ammonium solution (>99.9%, Aldrich) were used for the adjustment of mobile-phase pH. Methanol (HiPerSolv, BDH), phosphoric acid (AR grade, BDH), acetone (Unichrom, Ajax Laboratory Chemicals), and deionized water (18.2 MΩ cm, Millipore) were used for the extraction of arsenic species. Hydrochloric acid (HCl, Trace Pur, Merck), L-cysteine (BioChemika, Fluka), and sodium tetrahydroborate (NaBH₄) (Laboratory Chem Chemicals, APS) were used for the reduction and derivatization of arsenic species. The NaBH₄ solution was stabilized by the addition of 0.01 M sodium hydroxide (Pronalys, Selby-Biolab) in deionized water. Dithionite-citrate-bicarbonate (DCB) solution contained 0.03 M sodium citrate, 0.125 M sodium bicarbonate (AR grade, BDH) with 1.5 g of sodium dithionite (Ajax Laboratory Chemicals) added to the solution.

Stock standard solutions (1000 mg L⁻¹) of arsenous acid (As⁵⁺), arsenic acid (As⁴⁺), methylarsenic acid (MA), and dimethylarsinic acid (DMA) were prepared by dissolving sodium arsenite, sodium arsenate heptahydrate (Ajax Laboratory Chemicals), disodium methyl arsenate, and sodium dimethylarsenic (Alltech-Specialists), respectively, in 0.01 M HCl-deionized water. Synthetic arsenobetaine (BCR-626, Institute for Reference Materials and Measurements) was diluted with deionized water to desired concentration. Arsenocholine (AC), trimethylarsine oxide (TMAO), tetramethylarsonium ion (TETRA), and glycero trimethylated arsnoarobase were kindly supplied by Professor Erik...
Larsen (Danish Institute for Food and Veterinary Research, Department of Chemistry, Denmark), Professor Kevin Francesconi and Dr Walter Goessler (Institute of Food Chemistry, Karl-Franzens-University, Graz, Austria). Glycerol arsenobisulfite, sulfanate arsenobisulfite, and sulfate arsenobisulfite (OH-ribbon, SO₃-ribbon, SO₄2-ribbon respectively) were isolated in-house from the marine macroalgae certified reference material Fucus 140 (IAEA). The phosphate arsenobisulfite (PO₄-ribbon) was isolated in-house from the marine animal certified reference material Oyster 1566a (NIST). The identity of these arsenobisulfites was previously confirmed by high-performance liquid chromatography-mass spectrometry (HPLC-MS).[51]

Trimethylarsionicoproionate (TMAP) was isolated in-house from the marine animal certified reference material lobster hepatopancreas (TORT-2; NRC-CNRC).[32]

**Removal of Iron Plaque from Roots of Plant Material**

Iron plaque was removed from the roots of *S. quinqueflora* and *S. aestivallis* using dihydroxy-citrate-bicarbonate (DCB) extraction.[26] Briefly, 1-2 g of previously deionized washed root tissue was extracted with 10 mL of DCB solution for 25 min at 60°C, the supernatant was not further analyzed. After the extraction, plant root tissues were rinsed in deionized water, freeze-dried, and ground to a homogenous powder. Analysis for arsenic, iron, and phosphorus was as described below.

**Total Arsenic, Iron, and Phosphorus Analysis**

Tissues were digested using a microwave digestion procedure described previously by Baldwin et al.[33] Approximately 0.1 g of freeze-dried tissue was weighed into 7 mL Teflon polytetrafluoroethylene digestion vessels (A. I. Sciintific), and 1 mL of concentrated HNO₃ (Aristar, BDH) added. Digestion was carried out using an MDS-81D microwave oven (CEM) with a time program consisting of three steps: 2 min at 600 W, 2 min at 0 W, and 45 min at 450 W. After digestion, vessels were allowed to cool at room temperature (~25°C) for ~60 min and then diluted with deionized water to 10 mL in polyethylene vials. Total arsenic (m/z 75), iron (m/z 56/57), and phosphorus (m/z 15) concentrations were determined with a Perkin-Elmer Elman-6000 inductively coupled plasma mass spectrometer (ICP-MS). Internal standards ([54Sc, 103Rh]) were added on-line to compensate for any acid effects and instrument drift.[31] The potential interference to arsenic (m/z 75) from 40Ar35Cl⁴⁺ was determined by monitoring chloride at m/z 35, 36Cl16O⁺ at m/z 51, 37Cl17O⁺ at m/z 52 and 38Ar17O⁻ at m/z 77. Selenium was monitored at m/z 82 as a cross check on 35Ar37Cl⁴⁺. Calibration standards (0, 1, 10, 100, 1000 μg L⁻¹) for the determination of total arsenic, iron, and phosphorus were prepared daily by appropriate dilution of the multi-element calibration standard (AccuTrace, Trace Calibration Standard 2, 10 mg L⁻¹). Total arsenic in terrestrial plant tissues was determined using hydride generation-ICP-MS. Digests were diluted to 1% (v/v) HNO₃, and 1 mL of 1% (v/v) L-cysteine was added to 9 mL of digest. The carrier solution was 1% (v/v) HCl, 2% NaBH₄ stabilized with 0.01 M NaOH. Germanium(v) was used as the internal standard and monitored to determine instrument drift and hydride efficiency.[34] Calibration standards for the determination of arsenic by hydride generation were made by diluting arsenic(v) to 0, 0.01, 0.1, 1, 10 μg L⁻¹ with 1% (v/v) L-cysteine to 9 mL of standard.

Certified reference materials analyzed for arsenic were NIST SRM 1566a oyster tissue, NIST 1572 citrus leaves, NRCC DORM-2 Dogfish muscle, and NRCC PACS-2 Marine sediment. Measured arsenic concentrations (mean ± s.d.; n = 6) from the certified reference material were for oyster tissue: measured, 14.5 ± 0.3 μg (As) g⁻¹, certified 14.0 ± 0.2 μg (As) g⁻¹; citrus leaves: measured, 3.6 ± 0.1 μg (As) g⁻¹, certified 3.6 ± 0.1 μg (As) g⁻¹; DORM-2: measured, 18.0 ± 1.4 μg (As) g⁻¹, certified 18.0 ± 1.4 μg (As) g⁻¹; PACS-2: measured 27.2 ± 0.3 μg (As) g⁻¹, certified 26.2 ± 1.5 μg (As) g⁻¹.

**Arsenic Speciation**

**Acetone Extraction**

Approximately 0.1-0.2 g of homogenized freeze-dried whole-tissue was added to 50 mL propylene glycol and 10 mL of acetone added. The samples were then agitated on a mixing wheel for 1 h and the supernatant was removed after centrifuging at 3500 rpm for 15 min. The extraction procedure was repeated twice, with the supernatant removed after each centrifugation. After the final acetone extraction the residue pellet was dried to a constant mass in a fume cabinet at room temperature (~25°C).

The entire combined acetone supernatant was evaporated in a fume cabinet at room temperature (~25°C) to dryness. The residue was resuspended in 0.5 mL concentrated HNO₃, and digestion was undertaken in a hot water bath (90°C) for 1 h. Digeste acetone extracts were allowed to cool at room temperature (~25°C) and then diluted with deionized water to 5 mL in 10 mL polyethylene vials. Total arsenic concentration was determined by ICP-MS.

**Methanol/Water Extraction**

Water-soluble arsenic species were extracted from biological material by a microwave extraction procedure developed by Kirby and Maher.[32] Approximately 0.1 g of the acetone extracted pellet was weighed into 50 mL polypropylene vials and 10 mL of 50% (v/v) methanol/deionized water added. Mixtures were loaded into the carousel of an MDS-200 microwave oven (CEM) and heated to 70–75°C for 15 min. The supernatant was removed after centrifuging at 3500 rpm for 15 min. The procedure was repeated twice and the supernatants were combined.

The methanol/water supernatant (25 mL) was evaporated to dryness using an RVC 2-18 rotational vacuum concentrator (50°C; Christ, Australia) and stored in a refrigerator (~4°C) until speciation analysis. The remaining 5 mL of the methanol/water supernatant was evaporated to dryness using a RVC 2-18 rotational vacuum concentrator (50°C), and resuspended in 0.5 mL 10% HNO₃, and then further diluted to 5 mL for total arsenic analysis by ICP-MS.

**Phosphoric Acid Extraction**

Water-soluble arsenic species were extracted from sediments using phosphoric acid.[35] Sediment was extracted in 50 mL polyethylene centrifuge tubes. Approximately 0.1 g of sediment was weighed into each tube, to which 10 mL of 0.5 M phosphoric acid was added. The samples were then agitated on a mixing wheel for 1 h and the supernatant removed after centrifuging at 3500 rpm for 15 min.

**Water-Soluble Arsenic Speciation**

Prior to chromatography, previously stored methanol/water extracted residues were resuspended in 0.5–10 mL deionized water to an arsenic concentration ~500 μg L⁻¹. All extracts were filtered through a 0.2 μm RC syringe filter (Millipore). Sediment digests were diluted 1:1 with deionized water prior to analysis. Aliquots of 100 μL were injected onto a HPLC system consisting of a Perkin-Elmer Series 200 mobile phase delivery and auto sampler system (Perkin-Elmer). The eluent from HPLC columns was directed by PEEK (polyether-ether-ketone; i.d. 0.02 mm; Supelco) capillary tubing into a Rhynon crossflow nebulizer of a Perkin-Elmer Elan-6000 ICP-MS, which was used as an arsenic-selective detector by monitoring the signal intensity at m/z 75. Potential polyatomic interferences were checked by monitoring for other ions as described for total arsenic analysis.

The column conditions used for the separation of arsenic species were:

1. A PEEK Hamilton PRP-X100 anion-exchange column (250 by 4.6 mm, 10 μm, Phenomenex) and an aqueous 20 mM NaH₂PO₄ mobile phase adjusted to pH 5.6 with aqueous ammonia (flow rate: 1.5 mL min⁻¹; temperature: 40°C) were used for the identification and quantification of arsenic(V), DMA, MA, PO₃⁴⁻, SO₄²⁻, and SO₃⁻ arsenobisulphates.

2. A Supelcosil LC-SCX cation-exchange column (250 by 4.6 mm, 5 μm, Supelco, USA) and an aqueous 20 mM pyridine mobile phase adjusted to pH 2.6 with formic acid (flow rate: 1.5 mL min⁻¹; temperature: 40°C) were used for the identification of AB, glycerol arsenobisulphate, glycerol trimethylarsenobisulphate, TETRA, AC, and TMAP.
Distribution and Speciation of Arsenic in Temperate Marine Saltmarsh Ecosystems

Arsenic was measured using identical ICP-MS conditions with the use of an in-line hydride generation system. External calibration curves for quantification of arsenic species were prepared by diluting arsenic(v) for anionic species and AB for cationic species to 0, 1, 10, 100 μg L⁻¹ daily. Purity of arsenic species was periodically determined by HPLC-ICP-MS. Typical precision for replicate analysis of arsenic species in AB (10 μg L⁻¹, coefficient of variance (CV) < 5%); TMA (0.2 μg L⁻¹, CV < 6%); AC (0.05 μg L⁻¹, CV < 8%); TETRA (0.2 μg L⁻¹, CV < 8%); arsenic(v) (0.5 μg L⁻¹, CV < 5%); DMA (0.2 μg L⁻¹, CV < 10%); MA (0.2 μg L⁻¹, CV < 5%); arsenic(v) (0.5 μg L⁻¹, CV < 6%); arsenobis(1 μg L⁻¹, CV < 15%).

The chromatography package Total Chrom (Perkin-Elmer) was used to quantify arsenic species by peak areas. Arsenic species were identified by spiking with known standards and comparisons of retention times.

The accuracy of arsenic speciation procedure was determined by the analysis of the certified reference material, DORM-2. The concentrations (mean ± s.d.) of AB (16.1 ± 0.2 μg g⁻¹) and TETRA (0.26 ± 0.04 μg g⁻¹) measured in DORM-2 tissues were similar to certified values (AB, 16.4 ± 1.1 μg g⁻¹; TETRA, 0.248 ± 0.054 μg g⁻¹).

### Data Analysis

Significant differences of total arsenic concentrations between locations were determined by single-factor ANOVA (factor = location) with a significance level of P = 0.05 applied to normalized data (SPSS 11.5). Linear regression analysis was carried out using SPSS 11.5 with a significance level of P = 0.05.

Cluster analysis and principal component analysis (PCA) were used to classify groups with similar arsenic species proportions (Primer 5; PRIMER-E, Plymouth, UK).

### Results

#### Total Arsenic Concentrations

Plants and Associated Sediments

In general, arsenic concentrations in plant tissues were highest at Tomago River, intermediate at Moruya River and lowest at Congo Creek (Table 1). Significant differences were not observed in total arsenic concentration in different plant species across the study area.

### Table 1. Total arsenic concentrations in saltmarsh sediment and plant and animal tissues

<table>
<thead>
<tr>
<th>Species</th>
<th>Tissue</th>
<th>TR¹</th>
<th>MR¹</th>
<th>CR¹</th>
<th>All locations²</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. quinqueflora</td>
<td>Leaves</td>
<td>0.67 ± 0.48</td>
<td>0.08 ± 0.05</td>
<td>0.03 ± 0.05</td>
<td>0.26 ± 0.40</td>
</tr>
<tr>
<td></td>
<td>Roots</td>
<td>6 ± 12</td>
<td>2 ± 2</td>
<td>2 ± 3</td>
<td>3 ± 7</td>
</tr>
<tr>
<td></td>
<td>Sediment</td>
<td>0.54 ± 0.16</td>
<td>1.3 ± 0.3</td>
<td>1.3 ± 0.4</td>
<td>1.0 ± 0.9</td>
</tr>
<tr>
<td>S. australis</td>
<td>Leaves</td>
<td>0.08 ± 0.06</td>
<td>0.03 ± 0.02</td>
<td>0.06 ± 0.12</td>
<td>0.06 ± 0.08</td>
</tr>
<tr>
<td></td>
<td>Roots</td>
<td>0.39 ± 0.26</td>
<td>0.45 ± 0.28</td>
<td>0.57 ± 1.06</td>
<td>0.47 ± 0.63</td>
</tr>
<tr>
<td></td>
<td>Sediment</td>
<td>1 ± 2</td>
<td>1.2 ± 0.3</td>
<td>1.3 ± 0.8</td>
<td>1.3 ± 1.1</td>
</tr>
<tr>
<td>S. solida</td>
<td>Whole</td>
<td>35 ± 15</td>
<td>15 ± 8</td>
<td>14 ± 2</td>
<td>21 ± 13</td>
</tr>
<tr>
<td>O. ornatus</td>
<td>Whole</td>
<td>31 ± 16</td>
<td>20 ± 9</td>
<td>9 ± 2</td>
<td>20 ± 14</td>
</tr>
<tr>
<td>N. meinierti</td>
<td>Muscle</td>
<td>13 ± 10</td>
<td>14 ± 5</td>
<td>9 ± 4</td>
<td>12 ± 6</td>
</tr>
<tr>
<td></td>
<td>Visceral</td>
<td>16 ± 9</td>
<td>13 ± 5</td>
<td>9 ± 1</td>
<td>13 ± 5</td>
</tr>
<tr>
<td>Taitrid amphipod</td>
<td>Whole</td>
<td>8 ± 2</td>
<td>4.9 ± 0.3</td>
<td>6.3 ± 0.2</td>
<td>7 ± 2</td>
</tr>
</tbody>
</table>

TR: Tomago River; MR: Moruya River; CR: Congo Creek.¹ n = 10; ² n = 30; ³ Turkey's post hoc analysis of significance (< 0.05) between locations. Different letters indicates significant difference (< 0.05) where a > b > c.

### Table 2. One-way analysis of variance (ANOVA) for total arsenic concentrations of saltmarsh plant and animal tissues

<table>
<thead>
<tr>
<th>Common name</th>
<th>Species</th>
<th>Tissue</th>
<th>Variable¹</th>
<th>d.f.</th>
<th>MS</th>
<th>As</th>
<th>p²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saphire</td>
<td>S. quinqueflora</td>
<td>Leaves</td>
<td>Loc</td>
<td>2</td>
<td>27.44</td>
<td>40.64</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Roots</td>
<td>Loc</td>
<td>2</td>
<td>3.36</td>
<td>2.17</td>
<td>NS³</td>
</tr>
<tr>
<td>Sea blight</td>
<td>S. australis</td>
<td>Leaves</td>
<td>Loc</td>
<td>2</td>
<td>1.99</td>
<td>4.46</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sediment</td>
<td>Loc</td>
<td>2</td>
<td>4.64</td>
<td>3.15</td>
<td>NS</td>
</tr>
<tr>
<td>Solid air-breather</td>
<td>S. solida</td>
<td>Whole</td>
<td>Loc</td>
<td>2</td>
<td>0.36</td>
<td>0.51</td>
<td>NS</td>
</tr>
<tr>
<td>Magnrove air-breather</td>
<td>O. ornatus</td>
<td>Whole</td>
<td>Loc</td>
<td>2</td>
<td>0.69</td>
<td>1.54</td>
<td>NS</td>
</tr>
<tr>
<td>Magnrove crab</td>
<td>N. meinierti</td>
<td>Visceral</td>
<td>Loc</td>
<td>2</td>
<td>2.39</td>
<td>16.32</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Land/sand hopper</td>
<td>Taitrid amphipod</td>
<td>Whole</td>
<td>Loc</td>
<td>2</td>
<td>3.03</td>
<td>3.28</td>
<td>NS</td>
</tr>
</tbody>
</table>

¹ Loc = location. ² NS = not significant. ³ Significance level was set at 0.05.
in arsenic concentrations between locations were found for *S. quinqueflora* leaves and associated sediment (Table 2).

No linear relationship was found between sediment and root ($r^2 = 0.001, P > 0.05$) or sediment and leaf arsenic concentrations ($r^2 = 0.06, P > 0.05$) of *S. quinqueflora*. The concentration of arsenic in the roots of *S. quinqueflora* was highly variable (Figs 3 and 4a); however, after the removal of iron plaque from the roots surface of these plants, variability was reduced although the overall mean arsenic concentration remained similar (Fig. 4a).

No significant difference in arsenic concentrations between locations was found for *S. australis* roots and leaves (Table 2). Only a weak linear relationship was found between sediment and root ($r^2 = 0.22, P < 0.05$) and sediment and leaf ($r^2 = 0.18, P < 0.05$) arsenic concentrations. *S. australis* roots have less iron plaque, and removal of iron plaque had a lesser effect on the variability of arsenic concentrations in the roots (Fig. 4b).

**Animals**

Arsenic concentrations in animals were in general highest at Tomago River, intermediate at Moruya River, and lowest at Congo Creek (Table 1). Significant differences in arsenic concentrations between locations were found for *S. solidus*, *O. ornatus*, and amphipods (Table 2). Arsenic concentrations increased from producers to consumers (Fig. 3). Large variability in arsenic concentrations occurred for both gastropod species (Fig. 3) with significant differences between locations.
Fig. 5. Linear relationship between total arsenic concentration in saltmarsh plant leaves and animal tissues (n = 30). Dotted lines represent 95% confidence limits. (a) S. solda; (b) O. ornatus; (c) amphipod.

Fig. 6. Linear relationship between arsenic and iron concentrations in saltmarsh plant tissues (n = 30). Dotted lines represent 95% confidence limits. (a) S. quinqueflora leaves; (b) S. quinqueflora roots; (c) S. australis leaves; (d) S. australis roots.
No significant relationships were found between the tissue arsenic concentration of *S. australis* and herbivores and detritivores. Significant linear relationships were found between the tissue arsenic concentrations of *S. quinqueflora* and *S. solida*, *O. ornatus* and the amphipods (Fig. 5a–c).

**Relationship Between Arsenic, Iron, and Phosphorus Concentrations in Plants**

Significant linear relationships between arsenic and iron concentrations were found for both *S. quinqueflora* leaves and roots (Fig. 6a,b) and *S. australis* roots (Fig. 6d), but not for *S. australis* leaves (Fig. 6c). The linear relationship between arsenic and iron concentrations in the roots of *S. quinqueflora* was still evident after the removal of iron plaque from the root surface ($r^2 = 0.86$, $P < 0.05$). The linear relationship between arsenic and iron in the roots of *S. australis* was enhanced after the removal of iron plaque from the root surface ($r^2 = 0.93$, $P < 0.01$).

A significant negative linear relationship was found between arsenic and phosphorus concentrations for *S. quinqueflora* leaves (Fig. 7a); however, no significant relationship was found for arsenic and phosphorus concentrations for *S. quinqueflora* roots (Fig. 7b). Although significant linear relationships between arsenic and phosphorus concentrations were found for the leaves and roots of *S. australis*, these relationships were weak and much of the data lie outside the 95% confidence intervals (Fig. 7c,d).

**Water-Soluble Arsenic Species**

Arsenic concentrations in acetone extracts were low ($<0.18 \pm 0.21 \mu g g^{-1}$; 0.5–3%) and not analyzed further. The water-soluble arsenic species in sediments and plant material were dominated by inorganic arsenic (Table 3). However, in leaves and roots, glycerol arsenoribose was also present at appreciable concentrations ranging from 17 to 35% of the total extractable arsenic (Table 3). Both gastropods contained mostly AB (Table 3). Glycerol trimethyl arsenioribose was present in both the gastropods and *N. meinerti* visceral mass at low concentrations (0.1–0.8%) (Table 3, Fig. 8). The presence of glycerol trimethyl arsenioribose was confirmed by spiked addition of a synthetic standard to the original sample with no distortion in peak shape found (Fig. 8). The amphipod was dominated by AB (44%), with phosphate arsenoribose making up 23% of the total extracted arsenic (Fig. 9). An arsenic peak at a retention time of 25 min was found in both *O. ornatus* (0.4%) and *N. meinerti* visceral mass (2.2%), which did not match any of our arsenic standards.

Principal component analysis of arsenic species proportions separated the saltmarsh organisms into three groups (Fig. 10). Group one consisted of both gastropods and *N. meinerti* muscle tissue, which were grouped together based on the similarity of AB proportion within these samples (Table 4). Although in the same group, both gastropod species are separated from *N. meinerti* muscle tissue owing to the presence of glycerol trimethyl arsenioribose (Fig. 10). Group two consisted of the amphipod and *N. meinerti* visceral
Table 3. Water-soluble arsenic species in saltmarsh sediment and plant and animal tissues from Tomago River

<table>
<thead>
<tr>
<th>Species</th>
<th>Tissue</th>
<th>Total As (µg g⁻¹)</th>
<th>Extracted As (µg g⁻¹)</th>
<th>Column recovery (%)</th>
<th>AB B</th>
<th>Tri OH-rbs</th>
<th>TMAP</th>
<th>AC</th>
<th>TETRA</th>
<th>Inorganic As</th>
<th>DMA</th>
<th>MA</th>
<th>PO₄(ribos)</th>
<th>OSO₄(ribos)</th>
<th>Uln As anion</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. quinquelora</td>
<td>Leaves</td>
<td>1.7</td>
<td>0.14 (32)</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td></td>
<td></td>
<td>0.29 (66)</td>
<td>0.01 (2.1)</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td></td>
<td>Roots</td>
<td>5.4</td>
<td>0.07 (17)</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td></td>
<td></td>
<td>0.31 (76)</td>
<td>0.02 (5.4)</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td></td>
<td>Sediment</td>
<td>7.8</td>
<td>3.9 (99)</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td></td>
<td></td>
<td>0.07 (1.1)</td>
<td>0.01 (1.5)</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>S. australis</td>
<td>Leaves</td>
<td>0.33</td>
<td>0.07 (27)</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td></td>
<td></td>
<td>0.19 (68)</td>
<td>0.01 (4.2)</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td></td>
<td>Roots</td>
<td>0.73</td>
<td>0.10 (35)</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td></td>
<td></td>
<td>0.18 (65)</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>S. solida</td>
<td>Whole</td>
<td>44</td>
<td>38 (84)</td>
<td>n.d.</td>
<td>0.36 (0.8)</td>
<td>0.44 (1.0)</td>
<td>n.d.</td>
<td>1.0 (2.3)</td>
<td>0.45 (10)</td>
<td>0.69 (1.5)</td>
<td>n.d.</td>
<td>4.0 (9.0)</td>
<td>n.d.</td>
<td>n.d.</td>
<td></td>
</tr>
<tr>
<td>O. ornatus</td>
<td>Whole</td>
<td>51</td>
<td>44 (89)</td>
<td>n.d.</td>
<td>0.36 (0.7)</td>
<td>0.22 (0.5)</td>
<td>n.d.</td>
<td>1.0 (2.1)</td>
<td>1.4 (3.0)</td>
<td>0.14 (0.3)</td>
<td>n.d.</td>
<td>2.0 (4.2)</td>
<td>n.d.</td>
<td>1.0 (0.4)</td>
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</tr>
<tr>
<td>N. meieri</td>
<td>Muscle</td>
<td>14</td>
<td>9.2 (89)</td>
<td>n.d.</td>
<td>0.22 (2.1)</td>
<td>0.01 (0.1)</td>
<td>0.05 (0.5)</td>
<td>0.58 (6.0)</td>
<td>n.d.</td>
<td>0.23 (2.3)</td>
<td>n.d.</td>
<td>n.d.</td>
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<td>n.d.</td>
<td></td>
</tr>
<tr>
<td>Visceral</td>
<td>13</td>
<td>79</td>
<td>6.0 (68)</td>
<td>n.d.</td>
<td>0.01 (0.1)</td>
<td>0.12 (1.4)</td>
<td>0.04 (0.4)</td>
<td>1.5 (16)</td>
<td>n.d.</td>
<td>0.63 (7.0)</td>
<td>0.41 (4.6)</td>
<td>0.19 (2.2)</td>
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<td>n.d.</td>
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<tr>
<td>Talitrid amphipod</td>
<td>Whole</td>
<td>6.3</td>
<td>1.2 (44)</td>
<td>n.d.</td>
<td>0.03 (1.1)</td>
<td>0.05 (0.2)</td>
<td>0.01 (0.4)</td>
<td>0.45 (16.5)</td>
<td>0.01 (0.3)</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

A Column recoveries are the total sum of species eluted from the column, n.d. = not detectable. B Arsenic quoted in µg g⁻¹ (dry mass) with the percentage of the total sum of species in brackets. C n.d. = not quantifiable <0.005 µg g⁻¹ for all species except inorganic arsenic <0.0005 µg g⁻¹.

Discussion

Phosphate accumulation is influencing the distribution of inorganic arsenic in the sediment. The lower range of the sedimentary arsenic concentrations is shown in the third dimension, while the higher range is shown in the first dimension. This is similar to the results obtained by Kirby et al. [11] in Australian mangrove sediments. The most likely explanation is that the lower range of phosphate concentrations is associated with the lower range of inorganic arsenic concentrations. The higher range of phosphate concentrations is associated with the lower range of inorganic arsenic concentrations.

Phosphate concentrations are also shown to be influencing the distribution of inorganic arsenic, which is interesting given that phosphate is known to be an important factor in the accumulation of arsenic in organisms.

The lower range of inorganic arsenic concentrations is associated with higher phosphate concentrations, while the higher range of inorganic arsenic concentrations is associated with lower phosphate concentrations. This suggests that the accumulation of inorganic arsenic is influenced by the availability of phosphate, which may be due to the presence of phosphate compounds in the sediment.

Further research is needed to determine the exact mechanism by which phosphate influences the distribution of inorganic arsenic in the sediment.

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Further research is needed to determine the exact mechanism by which phosphate influences the distribution of inorganic arsenic in the sediment.
Fig. 8. Overlay of glycerol trimethyl arsoniobise in *O. ornatus*; Supelcoasil SCX cation exchange chromatography. Solid line original 100 μL sample injection, and dotted line 100 μL injection spiked with ~20 μg L⁻¹ synthetic glycerol trimethyl arsoniobise.

Fig. 9. Phosphate arsoniobise in amphipod; Hamilton PRP-X100 anion exchange chromatography. Solid line original 20 μL sample injection, and dotted line 20 μL injection spiked with ~10 μg L⁻¹ phosphate arsoniobise.
Table 4. Principal components analysis of arsenic species proportions in saltmarsh sediment and plant and animal tissues

<table>
<thead>
<tr>
<th>Axis</th>
<th>Eigenvalues</th>
<th>%Variation</th>
<th>Cum. %Variation</th>
<th>Variable</th>
<th>Axis 1 (PC1)</th>
<th>Axis 2 (PC2)</th>
<th>Axis 3 (PC3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC1</td>
<td>5.40</td>
<td>45.0</td>
<td>45.0</td>
<td>AB</td>
<td>0.389</td>
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<td>PC2</td>
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<td>21.6</td>
<td>66.6</td>
<td>OH-ribose</td>
<td>0.288</td>
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<td>-0.538</td>
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<tr>
<td>PC3</td>
<td>1.24</td>
<td>10.3</td>
<td>77.0</td>
<td>Tri OH-ribose</td>
<td>0.233</td>
<td>0.482</td>
<td>-0.075</td>
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<td></td>
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<td>TMA</td>
<td>0.357</td>
<td>-0.071</td>
<td>0.102</td>
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<td></td>
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<td></td>
<td>AC</td>
<td>0.291</td>
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<td></td>
<td></td>
<td>TETRA</td>
<td>0.281</td>
<td>0.433</td>
<td>-0.081</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Inorganic As</td>
<td>-0.392</td>
<td>-0.133</td>
<td>0.213</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>DMA</td>
<td>-0.207</td>
<td>0.059</td>
<td>-0.433</td>
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<tr>
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<td></td>
<td></td>
<td></td>
<td>PO4-As ribose</td>
<td>0.319</td>
<td>-0.278</td>
<td>0.212</td>
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<tr>
<td></td>
<td></td>
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<td>MA</td>
<td>-0.193</td>
<td>-0.045</td>
<td>0.561</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>OSO3-ribose</td>
<td>0.235</td>
<td>-0.462</td>
<td>-0.165</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Unk As amion</td>
<td>0.182</td>
<td>-0.082</td>
<td>0.251</td>
</tr>
</tbody>
</table>

^A PC1 = x-axis; PC2 = y-axis; PC3 = z-axis. ^B Percentage of variation explained by each axis. ^C Eigenvector loadings contributing to the distribution of points on each axis.

(personal observation). For *N. meiniertii* there is no relationship between *S. quinqueflora* leaf arsenic concentrations and *N. meiniertii* total arsenic concentration. This may be due to differences in diet and/or consumption patterns. Gut contents analysis of *N. meiniertii* has shown that this species tends to consume mainly aged leaves from its environment (which it stores in its burrows); however, animal tissues, sediments, and algae have also been found in its gut.\(^{40,41}\)

**Relationship Between Arsenic, Iron, and Phosphorus Concentrations in Plants**

As plants have no active uptake system for arsenic, unlike copper and zinc,\(^{42}\) arsenic must be taken up during the transport of other essential elements such as phosphorus or another element to which it is exposed, i.e., iron from plaque coating the roots. Strong correlations were found between arsenic and iron concentrations for *S. quinqueflora* in leaf tissues, suggesting that the uptake of arsenic is clearly associated with the uptake of iron in plants leaf tissues (Fig. 6b). In some plants, iron plaque on roots has been shown to promote the uptake of iron and associated trace elements.\(^{26}\) The mechanisms of how this occurs is unclear but may be related to the inclusion of iron minerals within the root xylem. The arsenic concentrations in this plants leaf material is appreciably higher than in *S. australis*, which does not have as much iron plaque and does not demonstrate the same relationship between arsenic and iron concentrations (Fig. 6c). Iron and arsenic concentrations are clearly correlated in the root material (both with and without plaque) of both plants, reflecting the ability of iron oxides to bind and immobilize arsenic around root surfaces. Sediments in mashes and bogs outside the oxidized root zone are often water logged and organic rich, and thus, anoxic. Arsenic that is mobile under anoxic conditions can migrate to the oxidized root zone of plants where it is immobilized by iron oxides.\(^{27}\)

In *S. australis* there is little evidence that arsenic and phosphorus concentrations are correlated in either the leaf or root tissues, suggesting that if arsenic and phosphorus do share a common pathway, at these concentrations there is no effect on the uptake of one element by the other in this plant species (Fig. 7c,d). In contrast *S. quinqueflora* leaves have a significant negative correlation between leaf arsenic and phosphorus concentrations (Fig. 7b). Some workers have reported that phosphorus inhibits the uptake of arsenic\(^{43}\) and this may be occurring; however, the regression relationship ($r^2 = 0.27, P < 0.05$) explains only a small proportion of the data and does not justify a conclusion that inhibition of arsenic uptake by phosphorus is occurring.

![Fig. 10. Principal component analysis (PCA) of arsenic species proportions in saltmarsh sediment and plant and animal tissues. Arrows indicate the factor contributing to the pattern in two-dimensional space. Group 1: O. ornatus, S. solida and contained similar AB percentages; Group 2: N. meiniertii muscle and visceral mass contained sulfate arsenonibose; Group 3: S. quinqueflora and S. australis leaves, roots, and sediment were dominated by inorganic arsenic.](image-url)
Water-Soluble Arsenic Species

The PCA clearly identified three groups of organisms with markedly different arsenic species proportions (Fig. 10). The plant leaves and roots (Group 3) were dominated by inorganic arsenic and glycerol arsenobis (with traces of DMA (Table 4). This is consistent with other studies that have reported arsenic species in terrestrial plants. Studies that have examined the uptake and accumulation of arsenic in plants have reported that arsenic(v) is reduced to arsenic(III) and sequestered in vacuoles by phytochelatins (γ-glutamyl-cysteinyl-glycine). Thus, plants have a mechanism to immobilize inorganic arsenic at normal arsenic concentrations found in uncontaminated environments.

All animals (Groups 1 and 2) contained appreciable quantities of AB (Fig. 10, Table 3). This is similar to the majority of marine animals that have been analyzed and reported in the published literature. The amphipod (Group 2) contains lower concentrations of AB (44%) than normally found in marine animals and in another study that analyzed marine amphipods, Amphipods also contain high concentrations of phosphate arsenobis (23%) and sulfate arsenobis (15%). These species were not found to be present in S. guinealora or S. australis tissues, which contained glycerol arsenobis (17–35%; Table 3). Thus, it is unclear whether amphipods are either concentrating and transforming glycerol arsenobis from plant detritus or obtaining phosphate and sulfate arsenobis from other sources, such as microalgae, bacteria, or facal material. Group one (gastropods and crab muscle) were separated from Group two organisms by the presence of glycerol trimethylated arsenobis (Fig. 10). The presence of glycerol trimethylated arsenobis together with high concentrations of AB in S. solida, O. ornatus, and N. menieri compared to the amphipod suggests that at least some of the AB present may be derived from these sugars. The gastropod O. ornatus (Group 1) and N. menieri visceral tissue (Group 2) contain an unidentified anionic arsenic compound eluting in a broad peak from ~20 to 28 min. Based on the retention times for thio-arsenic sugars, this unknown arsenic compound may be a thio-arsenic compound which could be a possible intermediate in the formation of trimethyl arsenobis.

Concluding Comments

The results from this study show that arsenic uptake into saltmarsh plants from uncontaminated environments may be dependent on iron uptake and inhibited by high phosphate concentrations. The presence of glycerol trimethyl arsenobis found in gastropods of this study and in other published studies of gastropods, macroalgae-eating fish, and abalone raises the question of its importance in the formation of AB.

Trimethyl arsenobis have been found to degrade in anaerobic environments to quantitatively produce arschnoteine, which can then be oxidized to AB. It is easier to envisage the synthesis of AB in marine animals from the trimethyl arsenobis than dimethyl arsenobis as trimethyl arsenobis only require degradation to AC and further oxidation to AB. The recent isolation of dimethyl thio-arsenobis (Fig. 2), which are less volatile than dimethyl arsenobis and probably form in anaerobic, sulfide-rich gut environments, provides a plausible transformation pathway for the formation of trimethyl arsenobis. Trimethyl arsenobis in marine organisms are possible transitory intermediates and their role in the formation of AB may be underestimated. Most studies deplete the guts of animals before analysis, often for long periods (24–48 h), and this may result in the loss of these arsenic species. As well, these arsensosugars may only be present in significant quantities while algal material is being digested. We believe gastropods (and other molluscs) contain measurable trimethyl arsenobis as they graze for long periods and have gut retention times. Perhaps other herbivores and detritivores do contain significant transient concentrations of trimethyl arsenobis, but sampling must be timed to ensure maximum likelihood of measurement of these species.

References

Distribution and Speciation of Arsenic in Temperate Marine Saltmarsh Ecosystems


these arsenicals. One of these mollusk samples, the great scallop (Pecten maximus), contained, in addition to known arsenosugars, two compounds that we have not seen in our previous studies on arsenic speciation. In the present paper we report the identification and quantification of these two unknown arsenicals using HPLC–ICPMS and HPLC–ESMS.

Experimental

Standards, Reagents, and Samples

For the quantification of arsenic compounds standard solutions, containing 1000 mg (As) dm⁻³ each, were prepared from the following compounds in Milli-Q water (18.2 MΩ cm): arsenite (As(III)) and arsenate (As(V)) prepared from NaAsO₂ and Na₂H₂AsO₄·7H₂O respectively (Merck, Darmstadt, Germany); dimethylarsinate (DMA) prepared from sodium dimethylarsinate trihydrate (Fluka, Buchs, Switzerland); methylarsonate (MA) prepared from methylarsinic acid synthesized in-house from As₂O₃ (Merck) and CH₃OH (Fluka, Buchs, Switzerland) in NaOH (Meyer reaction). In addition, four arsenosugars were used during the investigations; details of these compounds have been reported elsewhere. The thio-arsenosugars were synthesized from the common oxo-arsenosugars by adding 10 mmol of an aqueous saturated H₂S solution to 100 mm³ of a solution containing 100 μg (As) dm⁻³ of the oxo-arsenosugars (the saturated H₂S solution was prepared by bubbling gaseous H₂S through pure water). Excess H₂S was removed by purging the solution with argon.

Ammonium dihydrogen phosphate (p.a.), ammonium hydrogen carbonate (p.a.), hydrogen peroxide (33%, suprapur), and aqueous ammonia solution (25%, suprapur) were purchased from Merck. Methanol (p.a.) was purchased from Fisher Scientific (Leicester, UK) and nitric acid (concentrated, p.a.) was purchased from Roth (Karlsruhe, Germany) and further purified in a quartz sub-boiling distillation unit. The frozen scallops (Pecten maximus) were purchased at a local fish market. The certified reference material TART-2 (Lobster Hepatopancreas Reference Material for Trace Metals) was purchased from the National Research Council of Canada (NRCC, Ottawa, Canada).

Total Arsenic Analysis

Arsenic concentrations in the samples were determined by ICPMS after mineralization of the samples and the reference material TART-2 with microwave-assisted acid digestion. For the total arsenic determinations, 100 mg of the freeze-dried samples were digested with 3 cm³ HNO₃ in a microwave-heated autoclave (UltraCLAVE 2, EMLS, Leutkirch, Germany) at 250°C for 60 min. The accuracy of the measurement was tested by analysis of the reference material TART-2 (100 mg weighed to 0.1 mg, digested in the same way) which has a certified value of 21.6 ± 1.8 mg (As) kg⁻¹ dry mass; the value we obtained was 23.0 ± 0.4 mg (As) kg⁻¹ dry mass (n = 3).

Extraction of the Arsenic Compounds from the Great Scallop

Individual great scallops (n ~ 20) were dissected to separate the gonad and the muscle. Each of the pooled gonad and muscle samples were freeze-dried (Alpha 1–4, Christ, Osterode am Harz, Germany) and ground to a homogeneous powder with an agate mortar and pestle. In order to see whether the freeze-drying process influences the speciation of the sample, we extracted fresh tissue (2 to 5 cm³ ultra pure water) and freeze-dried (~200 mg to 5 cm³ ultra-pure water) tissue of the gonad of the great scallop. The closed tubes were fastened to a home made cross-shaped rotating device and turned top-over-bottom for 14 h. Thereafter, the tubes were additionally sonicated for 10 min (Elma, Transsonic T700H, Singen, Germany) and then centrifuged for 20 min at 4500 rpm on a Jouan C2-62 centrifuge (Jouan, Saint Maurice, France). The supernatants were filtered through 0.2 μm nylon filters (Laphaft, Langervelche, Germany) directly into 1 cm³ polyethylene vials. These solutions were analyzed directly with HPLC–ICPMS. In addition, the freeze-dried gonad powder was extracted with methanol using exactly the same extraction procedure as described above. The methanol extracts were used for the HPLC–ESMS measurements.

HPLC–ICPMS of Arsenicals with Emphasis on Thio-Arsenosugars

An Agilent 1100 series HPLC system (Agilent, Waldbronn, Germany) with a quaternary pump, a vacuum degasser, column oven, and a thermostated autosampler with a variable 100 mm³ injection loop was used. For anion-exchange chromatography, Hamilton PRP-X100 columns (Hamilton Co., Reno, Nevada, USA) of three different dimensions but filled with the same stationary phase were used (Table 1). The outlet of the chromatographic column was directly connected to the Nebulizer of the ICPMS with PEEK (polyetheretherketone) capillary tubing (0.125 mm i.d.). The ion intensities at m/z 74 and 77 (for possible ⁴⁰Ar³⁴Cl interferences) were monitored. The ICPMS performance was optimized with a mobile phase solution containing 10 μg (As) dm⁻³ to give maximum response on the As signal (m/z 74). The arsenic compounds were quantified with external calibration against standard solutions of arsenate. All measurements were carried out in triplicate. The results are given as mean and single standard deviation of the three individual determinations. In order to investigate whether all the arsenicals eluted from the column, flow injection experiments were performed on the extracts. For these experiments the same chromatographic system was used but the column was by-passed with a six-port injection valve. Quantification was done via standard addition with arsenate solutions. For mass balance experiments, 100 mm³ of the aqueous solution from the gonad were spiked with 10 mm³ of a 30% hydrogen sulfide solution in triplicate. For spiking experiments, authentic thio-arsenosugars standards were used (2 mm³ of a 9 μg (As) dm⁻³ containing thio-arsenosugar–sulfonate and 2 mm³ of a 20 μg (As) dm⁻³ containing thio-arsenosugar–sulfate solution were added to 18 mm³ of the aqueous scallop-extract).

HPLC–ESMS

An Agilent 1100 series HPLC system and an Agilent LC/MSD single quadrupole mass spectrometer of the SL type were used. Chromatographic conditions III were used for the separation of the thio-arsenosugars as described in Table 1. The mass spectrometer was equipped with an atmospheric pressure ionization (API) source employing pneumatically assisted electrospray nebulization with nitrogen as the
Arsenosugar Metabolism Not Unique to the Sheep of North Ronaldsay

Simon J. Martin,^A Chris Newcombe,^A Andrea Raab,^A and Jörg Feldmann^A,B

^A Department of Chemistry, College of Physical Science, University of Aberdeen, Meston Walk, Old Aberdeen, AB24 3UE, Scotland, UK.
^B Corresponding author. Email: j.feldmann@abdn.ac.uk

Environmental Context. Seaweed is enjoying a revival in farming practice, in particular by organic farmers. However, seaweed accumulates arsenic, and these arsenic compounds can enter the food chain. It is known that the arsenic is present mainly as arsenosugars, but the metabolism of these compounds by ruminants needs clarification.

Abstract. Here we describe a feeding trial with Blackface sheep conducted on an organic farm in Kintyre (Scotland), which aims to prove that the metabolism of arsenic, acquired from the consumption of seaweed, is not unique to the North Ronaldsay sheep, which are adapted to a seaweed diet. Results show that the trial sheep supplemented their diet with, on average, 20 ± 9% Laminaria digitata when given the choice. The daily arsenic intake varied greatly from sheep to sheep but on average, the sheep consumed 65 μg kg⁻¹ b.w. Total arsenic concentrations in urine, as measured by inductively coupled plasma-mass spectrometry (ICP-MS) (m/z 75) also show significant differences between the trial and control group (P < 0.0001). HPLC coupled with ICP-MS in parallel with electrospray ionization-mass spectrometry (ES-MS) for detection was used for the identification of arsenic metabolites in urine samples. Dimethylarsonic acid (DMAV) is the main metabolite in the control group as well as in the trial group. In addition, arsenic metabolites previously only found in the urine of North Ronaldsay sheep were successfully identified in the urine of the trial group of the seaweed-eating Blackface sheep: dimethylarsinioyl acetic acid (DMAA) and its thio-analogue dimethylsariniothiol acetic acid (DMAAS) as well as the monosulphide of DMAV, DMAS. However, the poor chromatographic recovery indicates that the urine contains arsenic species, which do not elute under the conditions tested.

Keywords: arsenic — contaminant metabolism — speciation (nonmetals)

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Introduction

Arsenic in drinking water is predominantly present as the two inorganic oxyanions, arsenite and arsenate, whereas arsenic in seafood comprises several organoarsenic compounds. In fish and most shellfish the predominant arsenical is arsenobetaine, the non-toxic nature of which has been clearly established. In edible seaweed (algae), the arsenic is primarily bound to carbohydrate compounds collectively termed arsenosugars. Arsenosugars can also occur at significant concentrations in marine animals feeding on algae, such as scallops.

As yet, the toxicology of arsenosugars has not been fully assessed but it is likely to be more complicated than that of arsenobetaine. While arsenobetaine is excreted rapidly and unchanged in human urine, arsenosugars appear to be metabolized to several arsenic compounds. The absorption of arsenate and arsenite through the mammalian gut is well documented, with the absorbed arsenic moving to the liver, where arsenate is reduced to arsenite. Successive oxidative methylation then converts it to methylarsonic acid (MAV) and further to dimethylarsonic acid (DMAV).

For centuries, seaweed has been used as a source of food for livestock, especially in winter when other food is in short supply. Now with increased organic farming methods, a need for natural feed supplements has once again brought seaweed back to the market place, with companies dedicated to the processing of seaweed for feed supplements as well as fertilizers. With the increased use of seaweed in this manner, there is almost certainly going to be an impact on the livestock eating these products and on the levels of arsenic entering the food chain. Hansen et al. were able to show that sheep living entirely on a diet of seaweed accumulate some arsenic and whilst most arsenic is eliminated from the blood, the arsenic concentrations in the tissue are highly elevated, although they are still far below the threshold of 1 mg kg⁻¹ fresh weight.
Arsenosugar Metabolism Not Unique to the Sheep of North Ronaldsay

The sheep of North Ronaldsay (the most northerly of the Scottish Orkney Isles) offer a unique insight into the urinary metabolites of arsenic. The sheep are a rare ancient breed of primitive sheep and their diet consists entirely of seaweed, principally Laminaria digitata, as the sheep have been forced to live on the beach for centuries and are prevented from grazing on grass further inland by a high wall that surrounds the island. Arsenic concentrations in seaweed, (bioaccumulated from seawater) is species dependent, in this case, typical concentrations in Laminaria digitata reach 70 mg kg⁻¹ (dry weight). Most seaweed-accumulated arsenic is bound as arsenosugars.

Hansen et al. carried out investigations into the effects of organoarsenical consumption by these ‘unique’ sheep. A feeding experiment with 12 adult ewes was conducted on the island. Six of the sheep were adapted to feeding on grass for 5 months before the feeding trial and the other six were kept on the usual seaweed only diet. The rationale for this trial was to assess the effects on different rumen bacterial communities on arsenic metabolism. During the trial no significant difference in seaweed/arsenic intake and arsenic excretion was found between the two groups. Analysis of the urine showed the main metabolite to be dimethylarsinic acid (DMAᵃ⁺), with smaller amounts of dimethylarsinoylethanol (DMAE), methylarsenic acid (MAᵃ⁺), tetramethylarsonium ion (TMA⁺), and arsenate (As⁵⁻) as well as seven unknown arsenic compounds. Subsequent to this work Hansen et al. have identified several of these unknown arsenic metabolites in the urine of the North Ronaldsay sheep including dimethylarsinoyl acetic acid (DMAA), dimethylarsinocetyl acetic acid (DMAAS) and dimethylarsinothioic acid (DMAS). The last two are the first thioarsenicals identified in natural environment (Fig. 1).

The work by Hansen et al., only focused on the North Ronaldsay sheep and since these sheep have shown particular metabolic anomalies towards trace elements such as copper, probably owing to their adaptation to the food, it might be possible that the identified arsenosugar metabolites are unique to this rare primitive breed of sheep. In addition, Hansen et al. stored the sheep’s urine at 4–5°C and identified that some metabolites, in particular thioarsenicals degrade after monthly storage at this temperature. Therefore, questions have been raised over whether unstable compounds could have been identified or whether the compounds identified by Hansen et al. were the result of fungal and bacterial activity in the urine samples during storage at 4–5°C. It was also found that the urine samples were not contaminated with other arsenic species, either from the soil or from other food sources.

The study found that arsenosugars are a major urinary excretion product in sheep, and that the metabolism of arsenic in these animals is unique. The findings also suggest that the arsenosugars may have a role in the detoxification of arsenic in these animals.

**Experimental**

A feeding trial was set up with two groups of eight Blackface male lambs (~6 months old, average weight 38 ± 3.7 kg). The trial was conducted for 7 weeks, though for the purposes of this investigation only samples taken in the first eight days are used. On the day preceding the trial the animals were separated from the main flock, weighed, numbered and penned in wooden slatted enclosures of ~2 m x 1.5 m. The control sheep whose diet comprised purely hay and water were numbered 1 to 8. The trial sheep, which were allowed a choice of hay and Laminaria digitata, the nutritional value of which has already been established were numbered 9 to 16. The day before the trial, the sheep were not fed and then were offered food and water ad libitum, with the masses carefully recorded each day. Fresh Laminaria digitata was collected every day from the local beach and cleaned of any epiphytes and epifauna before being offered to the sheep. In addition to the food and water offered to the sheep, a separate sample of hay, seaweed and water were collected and measured to correct for evaporation over the day.

**Sample Collection and Preparation**

Urine samples were collected daily from as many of the sheep as possible. In addition, a sample of hay, seaweed and water consumed during the trial was collected. All samples were frozen immediately after collection and were stored at -18°C until analysis. Urine was diluted in an approximate 1:10 ratio with 1% nitric acid with the addition of indium.
to a concentration of 20 \( \mu g \) L\(^{-1} \) as an internal standard for total arsenic determination. Analytical blanks were prepared in the same way without the addition of any sample. Samples selected for speciation were analyzed directly without dilution or acidification.

Seaweed and hay samples were ground in a mortar and pestle, while tissue samples, collected after slaughter, were homogenized with an ultra-turrax T8 homogenizer (IKA Laborteknik). A sub-sample of these homogenates, (~0.1 g) was added to a PTFE bomb with 3 mL of concentrated nitric acid and 1 mL of hydrogen peroxide. Microwave digestion (3 \( \times \) 15 min at 315 W with 15 min at 0 W between each programme) was carried out on the samples. The samples were washed from the bombs with doubly distilled water and diluted to 10.00 g. A portion of the sample was subsequently diluted to give a final acid concentration of ~1%, with the addition of indium to a final concentration of 20 \( \mu g \) L\(^{-1} \) as internal standard.

**Chemicals and Reagents**

Stock solutions (10,000 mg L\(^{-1} \)) of As\(^{III} \) and As\(^{V} \) were made up from sodium arsenite and sodium arsenate (Merck). Dimethylarsinic acid (DMA\(^{V} \)) was obtained from Sigma chemicals and methylarsionic acid (MA\(^{V} \)) from Chem. Service MC, West Chester UBA. Dimethylarsinoyl acetic acid (DMAA) was a gift from K. A. Francesconi. Dimethylarsinoyl ethanol (DMAE)\(^{[23]} \), dimethylarsinothioic acid (DMAS)\(^{[18]} \) and 2-dimethylarsiniothioic acid (DMAAS)\(^{[17]} \) were synthesized as reported previously. Normal fertilizer, SM6 containing a range of arsenosugars (As-sugar 1-4) was from stock.\(^{[23]} \) Thio-arsenosugars were produced by bubbling \( H_2S \) through a solution of arsenosugars extract (SM6) as reported previously\(^{[24]} \) and served as an in-house reference standard. All structures are shown in Fig. 1.

Other chemicals used include ammonium carbonate and methanol, Analar grade from BDH chemicals; nitric acid >69.5%, Traceselect from Fluka; hydrogen peroxide, 30% extra pure from Riedel-de Haën.

**Total Arsenic Measurement**

The urine samples were measured using an ICP-MS (7500c from Agilent Technologies, USA) equipped with a Meinhard nebulizer. The instrument was checked daily for arsenic sensitivity and optimized when necessary (Table 1). The elements monitored were arsenic (m/z 75), selenium (m/z 77 and 82), and indium (m/z 115). The calibration standards were prepared from a Merck multi element reference standard. All standards contained 20 \( \mu g \) (In) L\(^{-1} \) and ~1% nitric acid. The detection limit was determined as three times the standard deviation on the measured blanks (LOD = 0.02 \( \mu g \) L\(^{-1} \), \( n = 4 \)). As quality control, the standard reference material SRM 1640 (trace elements in natural water, National Institute of Standards and Technology, Gaithersburg, MD) was used instead of the standard urine, because the sheep urine in previous studies has shown no significant chloride interference while measuring arsenic with ICP-MS, as opposed to the

<table>
<thead>
<tr>
<th>Instrument parameters</th>
<th>Plasma power</th>
<th>Cooling gas flow (Ar)</th>
<th>Nebulizer flow (Ar)</th>
<th>Capillary voltage</th>
<th>Nebulizer pressure</th>
<th>Drying gas flow (N(_2))</th>
<th>Quadratic temperature</th>
<th>Fragmentor voltage</th>
<th>Anion exchange</th>
<th>Cation exchange</th>
</tr>
</thead>
<tbody>
<tr>
<td>ICP-MS</td>
<td>1570 W</td>
<td>15 L min(^{-1} )</td>
<td>1.27 L min(^{-1} )</td>
<td>4000 V</td>
<td>40 psi</td>
<td>12 L min(^{-1} ) (350°C)</td>
<td>100°C</td>
<td>100 V</td>
<td>20 ( \mu g ) H(_2)CO(_3)</td>
<td>5% Methanol, pH 8.0</td>
</tr>
<tr>
<td>ES-MS</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.035% v/v nitric acid</td>
</tr>
<tr>
<td>HPLC</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Hamilton PRP-X100</td>
<td>150 by 4.1 mm</td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Hamilton PRP-X200</td>
<td>150 by 4.1 mm</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

SRM 2670 which does\(^{[15]} \). The urine samples were normalized by measuring their density. Our method produced a value of 28.1 ± 1.8 \( \mu g \) L\(^{-1} \) \( n = 3 \), which was within the limits of the certified value of 26.67 ± 0.41 \( \mu g \) L\(^{-1} \) for SRM 1640. As quality control for the total digestion of seaweed, hay, and tissue samples, both IAEA-140, a dried alga (major trace elements and methyl mercury compounds in susc sample, International Atomic Energy Agency, Monaco) and TORT-2 (Lobster Hepatopancreas Reference Material for trace elements, National Research Council Canada, Ontario) were analyzed. These samples gave values of 46.0 ± 4.4 \( \mu g \) g\(^{-1} \) \( n = 3 \) and 22.3 ± 1.3 \( \mu g \) g\(^{-1} \) \( n = 3 \) arsenic respectively, both within the limits of the certified values of 44.3 ± 2.1 \( \mu g \) g\(^{-1} \) and 21.6 ± 1.8 \( \mu g \) g\(^{-1} \) respectively.

**Arsenic Speciation**

Speciation of compounds was performed using either the Hamilton PRP-X 100 (anionic) or Hamilton PRP X-200 (cationic) HPLC column. See Table 1 for HPLC system conditions. The flow from the column was split, with one part of the eluent going to the ICP-MS (7500c from Agilent) and four parts into the ES-MS (Agilent 1100 MSD, Agilent). The ES-MS was used in positive single-ion monitoring mode, with the measured m/z ratios being selected from known or expected arsenic species. These ions represent fragments known to result from certain arsenic species and so their presence in analyzed samples can help to assign peaks.

The arsenic metabolites were determined by three independent HPLC-ICP-MS methods runs, in which the parameters of successive runs were changed based on the findings from the preceding run. Samples were centrifuged at 3000 rpm for 15 min before analysis to remove any solids. Species were identified by subsequent spiking experiments using simply HPLC-ICP-MS. When ES-MS was employed parallel to ICP-MS as a detector for HPLC, the characteristic transient signals of the molecular fragments were used to confirm the species present. Quantification was performed solely by ICP-MS due to the element specific nature of the
Table 2. Average intake of hay, seaweed, and arsenic, including average urinary arsenic concentrations for individual sheep

<table>
<thead>
<tr>
<th>Sheep number</th>
<th>Average amount of hay eaten per day (g)</th>
<th>Average amount of seaweed eaten per day (g)</th>
<th>Amount of seaweed (mg/kg body weight)</th>
<th>Average daily arsenic intake (µg kg⁻¹ b.w.)</th>
<th>Average daily urinary arsenic concentration (µg L⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>583 ± 182</td>
<td>95 ± 68</td>
<td>11 ± 7</td>
<td>&lt;0.3 A</td>
<td>113 ± 34</td>
</tr>
<tr>
<td>2</td>
<td>870 ± 219</td>
<td>-</td>
<td>-</td>
<td>&lt;0.5 A</td>
<td>66 ± 29</td>
</tr>
<tr>
<td>3</td>
<td>767 ± 87</td>
<td>-</td>
<td>-</td>
<td>&lt;0.3 A</td>
<td>131 ± 77</td>
</tr>
<tr>
<td>4</td>
<td>760 ± 168</td>
<td>-</td>
<td>-</td>
<td>&lt;0.4 A</td>
<td>141 ± 69</td>
</tr>
<tr>
<td>5</td>
<td>941 ± 150</td>
<td>-</td>
<td>-</td>
<td>&lt;0.4 A</td>
<td>81 ± 32</td>
</tr>
<tr>
<td>6</td>
<td>849 ± 160</td>
<td>-</td>
<td>-</td>
<td>&lt;0.4 A</td>
<td>97 ± 41</td>
</tr>
<tr>
<td>7</td>
<td>764 ± 146</td>
<td>-</td>
<td>-</td>
<td>&lt;0.4 A</td>
<td>66 ± 19</td>
</tr>
<tr>
<td>8</td>
<td>813 ± 119</td>
<td>-</td>
<td>-</td>
<td>&lt;0.5 A</td>
<td>110 ± 35</td>
</tr>
<tr>
<td>9</td>
<td>775 ± 130</td>
<td>95 ± 68</td>
<td>11 ± 7</td>
<td>40 ± 29</td>
<td>1040 ± 995</td>
</tr>
<tr>
<td>10</td>
<td>828 ± 89</td>
<td>384 ± 232</td>
<td>24 ± 12</td>
<td>140 ± 85</td>
<td>3860 ± 2170</td>
</tr>
<tr>
<td>11</td>
<td>741 ± 77</td>
<td>94 ± 59</td>
<td>11 ± 7</td>
<td>33 ± 21</td>
<td>167 ± 57</td>
</tr>
<tr>
<td>12</td>
<td>682 ± 124</td>
<td>219 ± 159</td>
<td>20 ± 15</td>
<td>71 ± 52</td>
<td>2390 ± 1710</td>
</tr>
<tr>
<td>13</td>
<td>639 ± 239</td>
<td>158 ± 124</td>
<td>21 ± 14</td>
<td>51 ± 40</td>
<td>1210 ± 882</td>
</tr>
<tr>
<td>14</td>
<td>621 ± 147</td>
<td>280 ± 104</td>
<td>31 ± 6</td>
<td>96 ± 36</td>
<td>3760 ± 2250</td>
</tr>
<tr>
<td>15</td>
<td>699 ± 72</td>
<td>239 ± 111</td>
<td>27 ± 9</td>
<td>77 ± 36</td>
<td>1420 ± 1080</td>
</tr>
<tr>
<td>16</td>
<td>716 ± 55</td>
<td>39 ± 8</td>
<td>5 ± 1</td>
<td>14 ± 3</td>
<td>234 ± 101</td>
</tr>
</tbody>
</table>

A Values are calculated based on a detection limit of 20 µg kg⁻¹ for hay.

The variability in seaweed intake is clearly mirrored in the variability of urinary arsenic concentrations. But a significant difference in both daily arsenic intake (P < 0.01) and urinary arsenic concentrations (P < 0.0001) between control and trial sheep is clearly observed.

It is obvious that the individual variability within the group of sheep reflects their individual eating habits, since the trial sheep had a choice between hay and seaweed, in order to see how much they eat ad libitum. The large variability in the arsenic concentration in the urine from the individual sheep can be explained by the fact that arsenic is cleared very efficiently from the body. This has been reported elsewhere for humans[26] and North Ronaldsay sheep[15] and results in a large variability in the concentration of arsenic in the urine sample after a seaweed meal. Therefore not only the amount of seaweed eaten but also the sampling time after the last meal increases the variability of the arsenic concentration in the urine. Despite these factors, prediction about the sheep’s daily arsenic intake can be made when the average arsenic concentration in the urine is calculated over the course of eight days, which is shown in Fig. 2. It should, however, be pointed out that even the control sheep have significant arsenic concentrations in their urine that are only slightly lower than the two poor seaweed-eaters (sheep 11 and 16) from the trial group.

The excretion efficiency was, however, not possible to calculate from this dataset, as it was not possible to quantify total urinary output. A metabolic box set-up would be required for such calculations and unfortunately this was not available for this trial. However, in order to identify whether arsenic is accumulating during the course of the trial, the arsenic concentration in the bile as well as in the liver was measured. Figure 3 shows a box-and-whisker diagram for average arsenic concentrations in urine, bile and liver for both control and trial group sheep for the first eight days of the feeding trial.
The levels of arsenic observed in the urine of the control group seem at odds with the arsenic intake described earlier. This may well be a result of excretion of residual arsenic stored in the liver from pre-trial seaweed consumption, since sheep have been taken from a group of sheep seen at the beach eating seaweed before the trial. This postulation is supported by the levels of arsenic measured in the bile of the control group.

The elevated concentrations of arsenic in the bile and liver of the trial group confirm that the sheep do not excrete arsenic quantitatively in the urine and that the sheep accumulate arsenic in the liver. An average of 52 ± 31 ng g⁻¹ liver (fresh weight) from the Blackface sheep fed only partially on seaweed in this 7 weeks trial has only slightly lower liver arsenic concentration than the liver of the sheep which lived entirely on seaweed on North Ronaldsay all year around (94 ± 32 ng g⁻¹ fresh weight) (P = 0.039), even though they have had a 20 times higher arsenic intake than the Blackface sheep. If this effect is not due to the different genetics, sex, or eating habits of the sheep, then the concentration of

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**Fig. 2.** The average arsenic concentration in sheep's urine vs their average daily arsenic intake. The error bars represent the s.d. of the samples over the course of the 8-day feeding trial. Control sheep (1–8); trial sheep (9–16). Embedded figure shows the ranked average urine concentration for control (c) and trial (t) sheep, which emphasizes the low concentration of arsenic in the control sheep.

**Fig. 3.** Comparison of the arsenic concentration in urine, bile, and liver samples from the control group and the trial group. The data given are the mean value, and the box-and-whisker plot represents the s.d. as well as minimum and maximum values.
arsenic in the feed has a significant influence on the arsenic level in the tissue.

**Arsenic Speciation**

Standard species were measured before speciation of the urine or seaweed samples was attempted, although one has to acknowledge, that the shift in the retention time of the species in the urine samples was so severe that without spiking and/or electrospray data of the protonated molecular peak, no assignment of the peaks could have been made.

The arsenic species in the seaweed species *Laminaria digitata* are mainly comprised of As-sugar 1, 2, and 3 with considerable amounts of DMAV as shown earlier.\textsuperscript{1,3} Speciation of the urine samples from the trial group sheep realised the elucidation of several distinct species. The identifications of some of the species via spiking experiments and/or ES-MS transient signal monitoring are described here.

DMAV was identified as the main metabolite of the arsenosugars (data not shown) as is consistent with many other reports including those on human\textsuperscript{4,5} and animals.\textsuperscript{1,2,3} Identification of DMAA (Fig. 4) was achieved via the use of transient signal ions from ES-MS and confirms the previously reported identification.\textsuperscript{1,6} The As signal (m/z 75 ICP-MS) coinciding with the characteristic transient signal at m/z 181 (ES-MS) for DMAA is clearly shown and this overlaps perfectly with the corresponding transient signal for DMA (m/z 139) a fragment of the compound. The chromatographic separation of DMAA and DMAV is not satisfactory when only an ICP-MS is available. The HPLC method used here was optimized to achieve the elution of the arsenosugars, previously found in the urine of the North Ronaldsay sheep. However, by the additional use of ES-MS, the characteristic fragments (M + H\textsuperscript{+}, m/z 181) and m/z 139, can simultaneously be monitored to indicate the presence of DMAA in the shoulder of DMAV.

Figure 5 shows the results of spiking of a urine sample with DMAAS. An increase in peak intensity is observed, as highlighted, confirming the presence of DMAAS in peak 4 as one of the arsenic metabolites in the urine of the trial sheep. This metabolite is, however, not as prominent as expected from the previous study\textsuperscript{1,6} and it makes up less than 10% of the eluting arsenic (see Table 3).

The presence of the metabolite DMAAS is seen quite clearly in Fig. 6. Both transient ion signals and spiking experiments allow for clear peak assignment. The characteristic transient signal for DMAAS (m/z 197) overlaps perfectly with the ICP-MS As (m/z 75) signal. The spiking experiment produces a relative increase in intensity of the same peak, also confirming the presence of DMAAS. This arsenosugar is abundant but is generally in a lower concentration than its oxo-analogue DMAAA. However, these findings confirm again the results from our previous study\textsuperscript{3} that the arsenosugars do not arise from sample storage but are real metabolites.

Quantification of the identified species along with some unknowns in selected urine samples is summarized in Table 3, along with details of chromatographic recovery. The low chromatographic recovery indicates that half of the arsenic is not eluting from the column and is therefore not identifiable.

\[\text{Fig. 4. Identification of peak 2 as DMAA. Anion-exchange chromatogram [PRP-X100 anion-exchange column (150 by 4.6 mm), 20 mM ammonium carbonate, flow rate 1 mL min}^{-1}]\text{] of urine sample in trial sheep as detected by ICP-MS (m/z 75) and by ES-MS (m/z 139 and m/z 181) using SIM mode and a fragmentor voltage of 100 V. Embedded figure shows only the m/z 75 ICP-MS trace on a logarithmic intensity scale.}\]

\[\text{Fig. 5. Identification of peak 4 as DMAAS. Anion-exchange chromatogram [PRP-X100 anion-exchange column (150 by 4.6 mm), 20 mM ammonium carbonate, flow rate 1 mL min}^{-1}]\text{] of urine sample in trial sheep as detected by ICP-MS (m/z 75). Unknown peaks 3, 5, and 7 have not been identified.}\]

This effect of the low recovery rate confirms our observations from our study with the North Ronaldsay sheep, although subsequently we optimized the chromatographic method for recovery, which made the elution of the arsenosugars possible.\textsuperscript{3,6} Even though the arsenosugars elute here 50% of the arsenic is still missing. This points to some "hidden" arsenicals that still adsorb irreversibly onto the column. It is not clear whether this is due to the different metabolism of the breed of sheep or due to the different storage conditions of the urine.

Analysis of urine samples from the control group shows no significant signs of the arsenosugar metabolites identified within this study. Having said this, not all sheep in the trial group show the same metabolic profiles in their urine samples. This might, however, be explained by the irregular intake...
Table 3. Quantification of arsenic metabolites

Only peaks representing concentrations of 10 µg L⁻¹ or more are shown. Figures shown in parentheses express the concentrations of individual species as a percentage of the total eluted species.

<table>
<thead>
<tr>
<th>Group</th>
<th>Total As concentration (µg L⁻¹)</th>
<th>DMA (µg L⁻¹)</th>
<th>DMAA (µg L⁻¹)</th>
<th>DMAS (µg L⁻¹)</th>
<th>DMAAS (µg L⁻¹)</th>
<th>U3</th>
<th>U5</th>
<th>Chromatographic recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>58</td>
<td>15 (55)</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>46</td>
</tr>
<tr>
<td>Control</td>
<td>232</td>
<td>73 (62)</td>
<td>26 (22)</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>51</td>
</tr>
<tr>
<td>Control</td>
<td>146</td>
<td>14 (50)</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>20</td>
</tr>
<tr>
<td>Control</td>
<td>87</td>
<td>25 (86)</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>36</td>
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<tr>
<td>Control</td>
<td>161</td>
<td>38 (79)</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>30</td>
</tr>
<tr>
<td>Trial</td>
<td>4440</td>
<td>806 (74)</td>
<td>174 (16)</td>
<td>37 (3)</td>
<td>62 (6)</td>
<td>—</td>
<td>—</td>
<td>25</td>
</tr>
<tr>
<td>Trial</td>
<td>5660</td>
<td>1340 (66)</td>
<td>642 (32)</td>
<td>—</td>
<td>—</td>
<td>33 (2)</td>
<td>12 (2)</td>
<td>36</td>
</tr>
<tr>
<td>Trial</td>
<td>1400</td>
<td>305 (61)</td>
<td>175 (35)</td>
<td>12 (2)</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>36</td>
</tr>
<tr>
<td>Trial</td>
<td>2260</td>
<td>590 (71)</td>
<td>210 (25)</td>
<td>—</td>
<td>20 (2)</td>
<td>20 (2)</td>
<td>—</td>
<td>37</td>
</tr>
<tr>
<td>Trial</td>
<td>5630</td>
<td>1350 (64)</td>
<td>696 (33)</td>
<td>53 (3)</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>38</td>
</tr>
<tr>
<td>Trial</td>
<td>1460</td>
<td>397 (45)</td>
<td>88 (21)</td>
<td>84 (10)</td>
<td>150 (17)</td>
<td>10 (1)</td>
<td>—</td>
<td>60</td>
</tr>
<tr>
<td>Trial</td>
<td>1350</td>
<td>528 (61)</td>
<td>253 (29)</td>
<td>—</td>
<td>47 (5)</td>
<td>16 (2)</td>
<td>—</td>
<td>64</td>
</tr>
<tr>
<td>Trial</td>
<td>2430</td>
<td>355 (58)</td>
<td>84 (14)</td>
<td>31 (5)</td>
<td>80 (13)</td>
<td>55 (9)</td>
<td>—</td>
<td>26</td>
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<tr>
<td>Trial</td>
<td>3220</td>
<td>273 (61)</td>
<td>124 (28)</td>
<td>—</td>
<td>38 (9)</td>
<td>—</td>
<td>—</td>
<td>14</td>
</tr>
<tr>
<td>Trial</td>
<td>6020</td>
<td>1300 (70)</td>
<td>488 (26)</td>
<td>—</td>
<td>59 (3)</td>
<td>—</td>
<td>—</td>
<td>31</td>
</tr>
<tr>
<td>Trial</td>
<td>2680</td>
<td>813 (64)</td>
<td>379 (30)</td>
<td>10 (1)</td>
<td>32 (3)</td>
<td>42 (3)</td>
<td>—</td>
<td>48</td>
</tr>
<tr>
<td>Trial</td>
<td>7700</td>
<td>3100 (69)</td>
<td>600 (14)</td>
<td>27 (0.6)</td>
<td>610 (14)</td>
<td>37 (1)</td>
<td>53 (1)</td>
<td>58</td>
</tr>
<tr>
<td>Trial</td>
<td>5230</td>
<td>784 (43)</td>
<td>295 (16)</td>
<td>243 (13)</td>
<td>449 (24)</td>
<td>12 (1)</td>
<td>—</td>
<td>35</td>
</tr>
</tbody>
</table>

Fig. 6. Identification of DMAAS. Anion-exchange chromatogram [PRP-X100 anion-exchange column (150 by 4.6 mm), 20 mM ammonium carbonate, flow rate 1 mL min⁻¹] of trial sheep urine sample as detected by ICP-MS (m/z 75) and by ES-MS (m/z 197) using SIM mode and a fragmentor voltage of 100 V. Trial sheep urine and urine spiked with DMAAS were detected by ICP-MS (m/z 75).

Fig. 7. Comparison of untreated sheep urine (solid line) with the sample after treatment with H₂O₂ (dotted line). Anion-exchange chromatogram [PRP-X100 anion-exchange column (150 by 4.6 mm), 20 mM ammonium carbonate, flow rate 1 mL min⁻¹]. The embedded figure shows the same chromatogram with the emphasis on the minor compounds. 1, DMA²⁻; 2, DMAA; 3, U3; 4, DMAS; 5, U5; 6, DMAAS; 7, 7a, 8, 9 all unknown.

of seaweed and the fact that the excretion of arsenic from arsenosugar intake is between 4 and 20 h after ingestion.[135] Thus, the arsenic concentration in the urine is dependent on when the sheep last ate seaweed, and this will determine whether the excreted metabolites are present at the time of sampling.

Whether the chromatographic recovery can be increased when the urine is oxidized has been tested by addition of hydrogen peroxide. Figure 7 shows the chromatographic results of the hydrogen peroxide experiment. The overall recovery increased slightly from 58 to 64% and the speciation was influenced, as expected. It can be clearly seen that the addition of peroxide to the sample several peaks are no longer present. Most notably the peaks identified as DMAS and DMAAS along with the as yet unidentified peaks 5, 7, and 9. Interestingly, there is an observed increase in DMA²⁻, DMAA, and peak U3. This would suggest that the addition of hydrogen peroxide has converted DMAS back to DMA²⁻ and likewise with DMAAS to DMAA. These observations strongly support the identification of some of these peaks as containing sulfur.

Cation-exchange chromatography, in this case principally used for the identification of DMAE, failed to yield any definitive peak identifications.
General Discussion

The arsenosugar metabolites identified in these experiments are as follows: DMA\textsuperscript{V}, DMAA, DMAS, and DMAAS. This shows good correlation with those identified by Hansen et al.,\textsuperscript{15-17} and supports the assignments made, since, as mentioned earlier, questions were raised over whether the compounds identified by Hansen et al. were a direct result of metabolism by the sheep or if in fact they were the result of fungal and bacterial activity in the urine samples during storage at 4–5°C. Since the samples measured in this experiment were frozen immediately after collection and stored at −18°C until analysis, it is unlikely that there would be sufficient fungal or bacterial activity to produce substantial quantities of the compounds identified here. In addition, these results show that the metabolism of arsenosugars as well as being independent of rumen bacterial communities, as identified by Hansen et al., are also independent of the adaptations, sex, and genetics of the sheep. It appears that sheep are well able to cope with high levels of arsenic in their diets, metabolizing the arsenosugars via the breakdown and derivatization of the sugar rings. However, it should be pointed out that the arsenic in the liver increases significantly to 52 ng g\textsuperscript{−1} fresh tissue. The accumulation of arsenic in the liver seems higher for the Blackface sheep compared to the North Ronaldsay sheep. Although the average arsenic liver concentration of the Blackface sheep is a factor of two lower than that of the North Ronaldsay sheep, their arsenic intake is a factor of 20 lower. How the seaweed supplementation translates to levels in the food chain would require the analysis of muscle as well as kidney and heart tissues, these being the common market place products from sheep farming. In terms of worst-case scenario, taking this level of arsenic (87 ng g\textsuperscript{−1}) into account a large portion of liver (12 oz = 340 g) would account for ~30 μg As, which is still less than 20% of the WHO guidelines for maximum tolerable daily arsenic intake of 2 μg kg\textsuperscript{−1} bodyweight. Whether the arsenic present in the tissues is in a form that is of toxicological importance is a question waiting to be explored.

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References


