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Arsenic and selected elements in inter-tidal and estuarine marine algae, south-east coast, NSW, Australia

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The cycling of arsenic in marine inter-tidal and estuarine algae was examined by measuring total arsenic concentrations and arsenic species in marine inter-tidal and estuarine algae from the south-east coast, NSW, Australia. A range of elements required for metabolism in photosynthetic organisms were also measured to determine if any relationship between these elements and arsenic concentrations occurred. Total arsenic concentrations varied between classes of algae: red macro algae, 4.3–24.7 μg g^{-1}; green macro algae, 8.0–11.0 μg g^{-1}; and blue green algae, 10.4–18.4 μg g^{-1}. No significant relationships were found between arsenic concentrations and concentrations of iron, cobalt, copper, manganese, molybdenum, magnesium, phosphorus and zinc. Distinct differences between algal classes were found for the proportion of arsenic species present in the lipid and water-soluble fractions, with green algae having a higher proportion of arsenic in lipids (19–44%) than red inter-tidal (5–34%) or estuarine algae (10–24%). Acid hydrolysis of lipid extracts revealed dimethyl arsenic, glycerol arsenoribose and two unknown cation based arsenolipids. Within water-soluble extracts, red macro algae and blue green algae contained a greater proportion of arsenic as inorganic and simple methylated arsenic species compared with green macro algae, which contained predominantly glycerol arsenoribose. Arsenobetaine, arsenocholine and tetramethyl arsonium ion were also present in some water-soluble extracts, but are not normally identified with algae and are probably due to the presence of attached microscopic epiphytes. Residue extracts contained predominantly inorganic arsenic, most likely associated with insoluble constituents of the cell. Marine algae contained lipids with arsenic moieties that may be precursors for arsenobetaine. Specifically, the presence of dimethylated arsenoribose-based arsenolipids can transform to arsenobetaine via intermediates previously identified in marine organisms. Copyright © 2007 John Wiley & Sons, Ltd.

KEYWORDS: arsenic; marine algae; inter-tidal; estuarine; concentrations; species; Australia

INTRODUCTION

Arsenic concentrations and arsenic species have been reported in brown, red and green macro algae.[1–3] Previous studies have found that arsenic is present as arsenoribosides in most macro algae and the types of arsenoribosides present are dependent on the class of algal species, with more sulfonate (SO_3-ribose) and sulfate (OSO_3-ribose) arsenoribosides present in brown macro algae, with glycerol (OH-ribose) and phosphate (PO_4-ribose) arsenoribosides dominant in red and green macro algae.[2,4] The presence of as yet unidentified arsenic species in red and green macro algae may be key intermediates in arsenic metabolism and biotransformation in higher marine organisms.[1] Little is known about the arsenic species present in blue green algae (cyanobacteria) and macro algae found in inter-tidal and estuarine environments.

The estuarine environment is subjected to greater variability in salinity associated with freshwater inflows and tidal changes[5] and this is likely to affect the flux of nutrients and bioavailable elements in estuarine environments. The ratio of

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As(III) to As(V) can be greater than in the open ocean due to changing salinities and freshwater inputs.[6,7] This could influence the uptake of arsenic and formation of arsenic species in estuarine algae.

A range of elements are involved in algae metabolism, required for photosynthesis, growth and maintenance of cell structure. Phosphorus is required for oxidative phosphorylation associated with energy production within cells.[8] Elements that are associated with growth are iron, copper, manganese, molybdenum, magnesium and zinc. Magnesium is an essential component of chlorophyll[9] with elements such as iron, cobalt and molybdenum linked to chlorophyll synthesis and manganese required to maintain cell structure.[10] The influence of these elements on arsenic uptake and sequestration needs to be established to fully understand what factors may influence arsenic concentrations in marine algae.

Most studies have only examined arsenic species in water-soluble extracts, but arsenic metabolism is not restricted to water-soluble arsenic species and investigations into arsenic species present in the lipids and structural components of marine organisms also need to be undertaken. An arsenic lipid containing a dimethyl arsenoriboside moiety has been characterized in the lipids of the brown macro algae *Undaria pinnatifida*,[11] and acid-hydrolysed arsenic species [dimethylarsenic (DMA), monmethyarsenic (MA), trimethylarsenic oxide (TMAO) and PO_4-ribose] identified in the lipids of the brown macro algae *Laminaria digitata*.[12] In green and red macro algae, in particular, a large percentage of the water-soluble arsenic present remains uncharacterized while the residue-bound arsenic fraction also has not been characterized.

This study measured and compared total arsenic concentrations and arsenic species present in common marine macro algae and blue green algal species found in the inter-tidal and estuarine regions of the south-east coast, NSW, Australia. Relationships between arsenic and macro and micronutrients were examined as well as arsenic concentrations and arsenic species in the lipid-soluble, water-soluble and residue fractions.

METHODS

Study location

Inter-tidal macro algae were collected from rock pools at the northern end of the beach at North Head (South Durras), NSW, Australia. Estuarine macro and blue green algae samples were collected at four intermittently closed and open lake lagoon systems located at Joes Creek, Surf Beach, Short Beach and Saltwater Creek, NSW Australia (Fig. 1). These areas were chosen as they are known to contain algae and are uncontaminated from arsenic and trace metals. Not all algae species were present at all sites, thus a nested design to compare differences in arsenic concentrations between locations could not be undertaken.

Sample collection

Healthy algal samples with no visible signs of degradation were selected. Algae were collected by hand and placed in

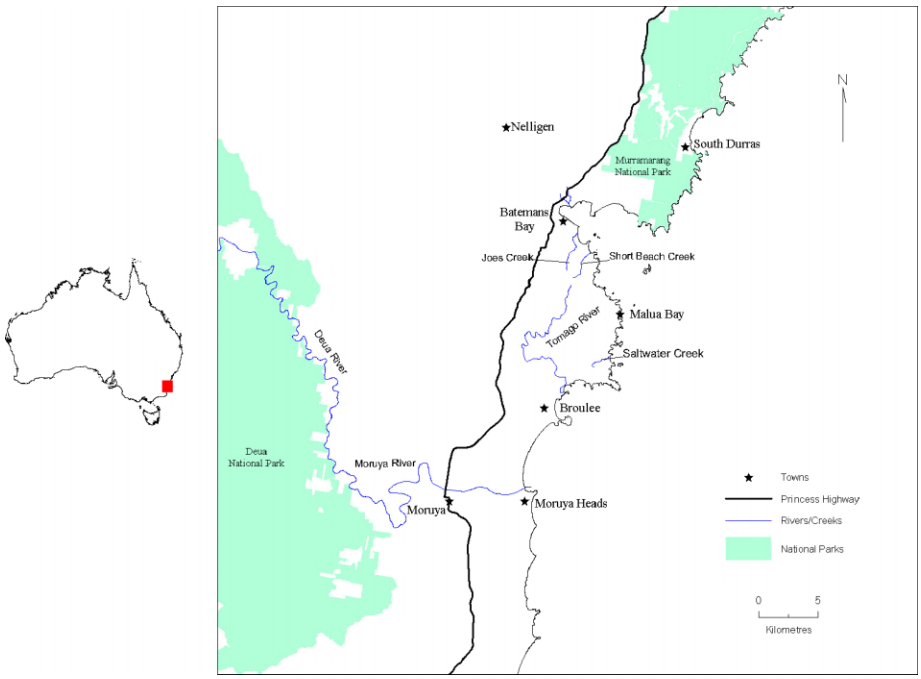

Figure 1. Sampling locations of inter-tidal and estuarine algae, south-east coast, NSW, Australia. This figure is available in colour online at www.interscience.wiley.com/AOC.

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clean zip-lock plastic bags containing water from the area. Latex gloves were worn when removing algae samples to prevent contaminating samples. Samples were pooled to obtain suitable quantities for sample analysis. All samples were transported on ice to limit decomposition and changes in arsenic species and stored in a cool room. For each species collected, a sample containing the whole plant including the thallus where possible was set aside for identification. As outlined by Womersley,[13] samples for identification were treated with 10% formalin (40% v/v formaldehyde, diluted 1 : 10 v/v with seawater) and left to soak for 1 h, placed in a plastic bag and transported as for other samples. On return to the laboratory, samples for identification were transferred to 50 ml polypropylene vials filled with 70% v/v ethanol, and stored in the dark. Algae species were identified by Dr Alan Millar (Royal Sydney Botanic Gardens).

Sample preparation

Each sample was rinsed with deionized water to remove sand, silt and salts. Filamentous algae were carefully washed in seawater to remove sediment, while stainless steel tweezers cleaned with ethanol were used to remove epiphytes such as polychaetes and amphipods. Tissue from each species was pooled, placed in clean, 2% v/v HNO_3 acid washed 50 ml polypropylene vials and immediately frozen (−80 °C). Frozen tissue samples were freeze-dried (Labconco; approx 24–48 h). Dried samples were homogenized using a Retsch ZM100 mill (0.2 mm stainless steel mesh, Retsch) and stored in clean, acid-washed polypropylene vials in a desiccator until analysed.

Total arsenic and arsenic species analysis

Reagents and standards

Nitric acid (HNO_3; Aristar, 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 ammonia solution (>99.9%, Aldrich) were used for the adjustment of mobile-phase pH. Methanol (HiPerSolv, BDH), acetone (Unichrom, Ajax Laboratory Chemicals), chloroform (Laboratory Reagent, May and Baker) and deionized water (18.2 mΩ, Millipore) were used for the extraction of arsenic species.

Stock standard solutions (1000 mg l^{-1}) of arsenous acid (As^{3+}), arsenic acid (As^{5+}), MA and DMA were prepared by dissolving sodium arsenite, sodium arsenate heptahydrate (Ajax Laboratory Chemicals), disodium methyl arsonate and sodium dimethylarsenic (Alltech-Specialists), respectively, in 0.01 M HCl–deionized water. Synthetic arsenobetaine (AB; 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 glycerol trimethylated arsonioribose were kindly supplied by Professor Kevin Francesconi and Professor Walter Goessler (Institute of Analytical Chemistry, Karl-Franz-University, Graz, Austria). Glycerol arsenoribose, sulfonate arsenoribose and sulfate arsenoribose (OH-ribose, SO_3-ribose, OSO_3-ribose respectively) were isolated in-house from the marine macro algae certified reference material *Fucus* 140 (IAEA). The phosphate arsenoribose (PO_4-ribose) was isolated in-house from the marine animal certified reference material Oyster 1566a (NIST). The identity of these arsenoriboses was previously confirmed by high-performance liquid chromatography–mass spectrometry (HPLC-MS).[14] Trimethylarsoniopropionate (TMAP) was isolated in-house from the marine animal certified reference material lobster hepatopancreas (TORT-2; NRC-CNRC).[15]

Total arsenic and element analysis

Digestion of samples for total arsenic concentrations was performed using a microwave digestion technique as outlined by Baldwin *et al.*,[16] with modifications. Approximately 0.1 g of ground sample was accurately weighed and recorded into 7 ml Teflon polytetrafluroacetate digestion vessels (A.I. Scientific) and 1 ml of concentrated nitric acid added (Aristar, BDH). Digestion vessels with sample and acid were left in the fume cupboard for approximately 1 h prior to digestion. Microwave digestion (MDS 81D, CEM, Indian Trail) program cycle was run at 2 min 600 W, 2 min 0 W, 45 min 450 W for each set of samples with certified reference materials and blanks. Samples were allowed to cool after digestion for ~60 min then diluted to 10 ml with deionized water in 10 ml polyethylene vials. Certified reference material *Ulva lactuca* (BCR 279) was treated in the same manner as samples. Total element concentrations in samples were analysed using a Perkin Elmer Elan 600 Inductive Coupled Plasma-Mass Spectrometer (ICP-MS) with an AS-90 autosampler. Internal standards were added on-line to compensate for any acid side effects and instrument drift.[14] The potential interference to arsenic (m/z 75) from $^{40}Ar^{35}Cl^+$ was determined by measuring chloride at m/z 35, $^{35}Cl^{16}O^+$ at m/z 51, $^{35}Cl^{17}O^+$ at m/z 52 and $^{40}ArCl^+$ at m/z 77. Selenium was monitored at m/z 82 as a cross check for $^{40}Ar^{37}Cl^+$. Other elements were corrected for interferences as outlined in Maher *et al.*[14]

Calibration standards using the multi element calibration standard (Accu Trace, Calibration Standards 2 and 10 mg l^{-1}) were used to produced standards (0, 1, 10, 100 and 1000 μg l^{-1}). All calibration standards were prepared daily in 1% v/v HNO_3.

Fractionation of arsenic and total arsenic analysis

Lipid extraction

Approximately 0.1 g of sample was weighed into a 50 ml polyethylene vial. The extraction process as carried out by Folch *et al.*[17] allows the separation of the lipid- and water-soluble phases. To 0.1 g sample, 5 ml of chloroform/methanol (2 : 1 v/v) was added, vortexed for 30 s to assist mixing and placed on a rotary wheel for 4 h. Samples were then centrifuged for 10 min at 5000 rpm to separate sample and supernatant. The supernatants were pipetted into 50 ml

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polypropylene vials. This procedure was repeated and the supernatant combined with the first extraction. To the supernatants, 4 ml of deionized water was added to assist in separating the lipid and water-soluble phases and left to stand overnight. The lipid- and water-soluble phases were separated and placed in 10 ml centrifuge tubes, then evaporated to dryness using a RVC 2–18 rotary vacuum concentrator (60 °C, 3000 rpm; Christ). Once dry, lipid extracts were stored in the freezer (−18 °C) until required for analysis. Prior to quantification, lipid extracts were re-suspended in 2 ml of 1% v/v HNO_3 and heated at 70 °C for 1 h.

Water extraction

The residues from the chloroform–methanol–water extractions were freeze dried (~24 h). To residues, 2 ml of hot water was added and placed in a hot water bath (100 °C) for 1 h, to remove any water-soluble arsenic species remaining after the previous extraction. The extracts were centrifuged at 5000 rpm for 10 min and the supernatants pipetted into 10 ml polyethylene tubes and combined with the water-soluble phase from the lipid extraction. The supernatants were evaporated to dryness as described for lipid extracts and stored frozen until required for analysis. Prior to analysis, the supernatants were made up to 2 ml with 1% v/v HNO_3.

Residue

The remaining residues were freeze dried (~24 h). To digest the residues, 1 ml of 2% HNO_3 was added and the solutions were heated at 70 °C for 2 h. The final extracts were made up to 1 ml with deionized water, giving a final acid concentration of 1% v/v HNO_3.

Lipid-, water-soluble and residue totals were analysed using electrothermal atomic absorption spectroscopy.[18] Arsenic was measured at a wavelength of 193.7 nm with a slit width of 0.7 nm. Ten micro-litres of sample were injected onto the surface of a pyrolytic graphite-coated tube inserted with a pyrolytic graphite L'vov platform. A palladium/magnesium matrix modifier was used for arsenic analysis.[18] Peak area was used to determine total arsenic concentrations.

Arsenic species measurement

All extracts were filtered through a 0.20 μm RC syringe filter (Millipore). Aliquots of 20 or 40 μl were injected onto a high-pressure liquid chromatography (HPLC) system consisting of a Perkin Elmer Series 200 mobile phase delivery and auto sampler system (Perkin Elmer). The eluant from HPLC columns was directed by PEEK (polyether-ether-ketone; i.d. 0.02 mm; Supelco) capillary tubing into a Ryton cross flow nebulizer of a Perkin Elmer Elan-6000 ICP-MS, which was used to monitor the signal intensity of arsenic 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 are outlined in Table 1.

Arsenic species were separated and quantified using HPLC-ICP-MS. Arsenic anions were analysed using PRP-X100 and arsenic cations were analysed using a Supelcosil LC-SCX at pH 2.6 and 3 (Table 1). External calibration curves for quantification of arsenic species were prepared by diluting As(III) for anionic species and AB for cationic species to 0, 0.5, 1, 10 and 100 μg l^{-1} daily. Peak area responses ($n = 10$) relative to AB and As(III) have been reported previously.[19] Purity of arsenic species was periodically determined by HPLC-ICP-MS.

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.

Statistical analysis

Statistical analyses were performed using SPSS (12.0). Cluster analysis and principle component analysis (PCA) by PRIMER 5; PRIMER-E[20] was used to classify groups of species with similar element concentrations and arsenic species proportions. Sigma Plot was used to report regressions (Sigma Plot, 9.0).

Quality assurance

Total arsenic

Certified reference material, *Ulva lactuca* (BCR 279), was used for quality assurance and was analysed in the same manner

Table 1. Column specifications for arsenic species analysis

Column	Hamilton PRP-X100 (PEEK)	Supelcosil LC-SCX	Supelcosil LC-SCX
Size	250 × 4.6 mm	250 × 4.6 mm	250 × 4.6 mm
Particle size	10 μm	10 μm	10 μm
	20 mM		
Buffer	20 mM $NH_4H_2PO_4$, 1% CH_3OH	20 mM pyridine	20 mM pyridine
pH	5.6	2.6	3
Flow rate	1.5 ml min^{-1}	1.5 ml min^{-1}	1.5 ml min^{-1}
Temperature	40 °C	40 °C	40 °C
Arsenic species	As(V), DMA, MA, PO_4-, SO_3- and OSO_3-arsenoribosides	DMAE, glycerol trimethyl arsonioribose, TETRA, AC and TMAP	AB and OH arsenoribosides

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as macro algal samples for determination of total arsenic concentrations. Measured values for arsenic (2.81 ± 0.28) were in agreement with certified values (3.09 ± 0.20 μg g^{-1}). Measured values for zinc (48 ± 4 μg g^{-1}) and copper (11.7 ± 0.5 μg g^{-1}) were similar to the certified values for zinc (51.3 ± 1.2 μg g^{-1}) and copper (13.14 ± 0.37 μg g^{-1}). *U. lactuca* contained 8865 ± 443 μg g^{-1} of magnesium but no certified value was given.

Arsenic species analysis

The accuracy of arsenic species measurement procedure was determined by the analysis of the certified reference material, DORM-2. The concentrations (mean ± SD) of AB (16.3 ± 0.5 μg g^{-1}) and TETRA (0.241 ± 0.005 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}).

RESULTS

Total arsenic and selected macro- and micronutrients

Total arsenic concentrations in algae were highly variable with no clear pattern between inter-tidal and estuarine algae (Table 2). Macro- and micronutrients were not the focus of this study and are analysed to determine relationships with arsenic concentrations in macro and blue green algae. Blue green algae had higher Fe, P and Zn concentrations compared with all other macro algae species examined (Table 2). Principle component analysis revealed four major groups (Table 3; Fig. 2). Group 1 consists of blue green algae with high zinc and magnesium concentrations. Group 2 is a mix of red and green inter-tidal and estuarine species, predominantly red algae, with high arsenic concentrations. Within this group, *Cladophoropsis hespestica* had higher copper concentrations compared to the other macro algae species examined. Group 3 contains estuarine green algae and red and green inter-tidal algal species not showing any major influence from any element in two-dimensional space. Group 4 consists of two green algae, *Caulerpa cactoides* and *Ulva rigida*, along with the red alga *Corallina officinalis* with high magnesium concentrations. Arsenic concentrations are high in red algae in Group 2, separating it from *C. herpestica* that has high copper concentrations (Table 3).

Regression analysis of arsenic concentrations vs zinc, magnesium and copper concentrations, identified by PCA as being markedly different in algal samples, was performed on all samples to determine whether element interactions are present (Fig. 3). Blue green algae were excluded from regression analysis as zinc concentrations are considerably higher than the red and green macro algae species examined and would distort results, possibly masking any relationship between other metals.

Regression analyses for concentrations of arsenic and zinc ($r^2 = 0.290$), arsenic and magnesium ($r^2 = 0.329$) and arsenic and copper [$r^2 = 0.010$; Fig. 3(a–c)] were not significant. In addition, the correlation of arsenic and phosphorus concentrations were explored as arsenic and phosphorus anions are chemically similar and arsenic is thought to be taken up via phosphates transport route; however, no significant relationship was found ($r^2 = 0.276$).

Table 2. Arsenic and selected macro and micronutrient concentrations for pooled marine inter-tidal and estuarine algal species, south-east coast, NSW, Australia

Species		As	Fe	P	Co	Cu	Mn	Zn	Mo	Mg
		Element μg g^{-1}								
Marine inter-tidal										
Rhodophyta	*Corallina officinalis*	4.3	954	450	0.51	0.75	12.7	2.6	0.67	23,365
	Martensia fragilis	11.3	1568	1159	0.75	8.4	15.8	13.7	1.6	10,626
	Laurencia obtusa	20.8	734	1585	0.97	5.0	22.9	19.3	1.2	6,653
	Laurencia sp.	24.7	338	1580	0.28	1.1	8.6	3.9	3.4	5,015
	Delisea pulchra	31.7	148	1194	0.38	1.5	6.5	17.6	1.1	4,210
Chlorophyta	*Ulva rigida*	8.7	127	1541	0.23	0.30	6.3	2.7	0.48	30,140
	Caulerpa flexilis	10.0	496	686	1.30	2.50	14.4	7.9	2.1	6,805
	Cladophoropsis herpestica	10.4	952	1002	1.54	4.27	24.4	7.6	5.4	8,068
	Caulerpa cactoides	8.9	116	964	0.28	n.d.	10.6	0.60	1.2	15,145
Estuarine										
Chlorophyta	*Rhizoclonium implexium*	11.0	1036	402	1.02	n.d.	28.7	12.7	4.4	12,501
	Cladophera subsimplex	8.0	1118	1094	1.61	0.51	316	10.3	1.9	5,573
Cyanobacterium	*Blue green alga*	10.4	2807	2805	18.2	3.60	1531	134	4.9	7,390
	Blue green alga	18.4	6424	2523	3.54	7.80	653	114	3.8	6,156

n.d., not quantifiable < 0.01 μg g^{-1}.

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Table 3. Principle component analysis of total arsenic and macro and micronutrient concentrations in marine inter-tidal and estuarine algae, south-east coast, NSW, Australia

	Eigenvalues	%Variation	Cum%Variation	Variable	Axis1 (PC1)	Axis2 (PC2)	Axis3 (PC3)
1	4.06	45.1	45.1	As	−0.055	**0.531**	**−0.564**
2	1.55	17.2	62.3	Fe	−0.163	−0.016	0.088
3	1.43	15.9	78.2	P	−0.422	−0.050	−0.235
				Co	−0.442	−0.222	0.037
				Cu	−0.068	0.401	**0.633**
				Mn	−0.466	−0.229	−0.014
				Zn	**−0.472**	−0.115	−0.080
				Mo	−0.321	0.300	0.428
				Mg	**0.220**	**−0.591**	0.164

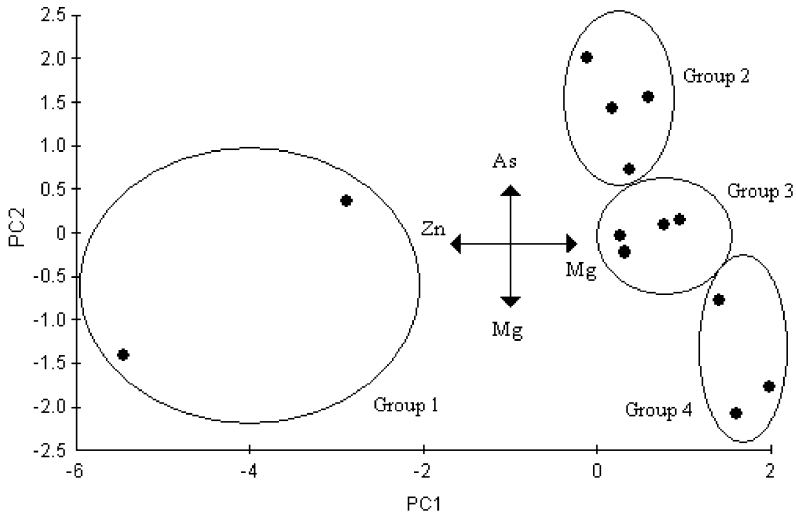

Figure 2. Principle component analysis for marine inter-tidal and estuarine algae. Group 1: Blue green algae; group 2: *Laurencia obtusa, Laurencia* sp., *Delisea pulchra* (inter-tidal red algae); *Cladophoropsis herpestica* (inter-tidal green algae); group 3: *Cladophera subsimplex, Rhizoclonium implexium* (estuarine green algae); *Caulerpa flexilis* (inter-tidal green algae); *Martensia fragilis* (inter-tidal red algae); group 4: *Caulerpa cactoides, Ulva rigida, Corallina officinalis* (inter-tidal green and red algae). Arrows indicate the factor contributing to the pattern in two-dimensional space.

Fractionation of arsenic in marine inter-tidal and estuarine algae

The proportion of total arsenic in the lipid fraction across all algae species examined ranged between 5 and 44% with red inter-tidal algae containing 5–21%, green inter-tidal algae 19–44% and estuarine algae 10–24% (Table 4). The lipid-soluble arsenic content of the estuarine green algae, *Rhizoclonium implexium* and *Cladophera subsimplex* species, were within the range of lipid arsenic content measured in green algal species found in the inter-tidal region and contained a slightly higher proportion of arsenic in the lipid fraction than the estuarine blue green algae (10–18%).

A higher proportion of total arsenic was found in the water-soluble component of red inter-tidal algae (45–56%) than in green inter-tidal algae (23–38%), estuarine green algae (17–30%) and estuarine blue green algae (10–18%; Table 4).

Within the residue fraction, estuarine green and blue green algae contained a higher proportion of arsenic (46–69%) compared with red inter-tidal algae (34–39%) and green inter-tidal algae (33–45%), which had a similar proportion of arsenic (Table 4).

Arsenic species in algae

General

The cluster analysis of the proportions of arsenic species present in the algae examined shows groups that are distinct for classes of algae (Fig. 4); however, the estuarine green algae are not grouped with the other chlorophyta and *Corallina officinalis* is separated from the other red algal species due to a higher proportion of organic arsenic species in the water-soluble component (Table 7). Principle component analysis revealed five groups (Table 5, Fig. 5). Group 1 contains *C. officinalis* that has higher proportions of TETRA

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Table 4. Fractionation of arsenic in selected marine inter-tidal and estuarine algae species, south-east coast, NSW, Australia

	Location[a]	Species	Total As (μg g^{-1})	Lipid-soluble (%)	Water-soluble (%)	Residue (%)
Rhodophyta	NH	*Corallina officinalis*	4.3	21	45	34
	NH	*Laurencia obtusa*	20.8	11	55	34
	NH	*Laurencia* sp.	24.7	5	56	39
Chlorophyta	NH	*Ulva rigida*	8.7	34	30	36
	NH	*Caulerpa flexilis*	10.0	19	35	45
	NH	*Cladophoropsis herpestica*	10.4	44	23	33
	NH	*Caulerpa cactoides*	8.9	20	38	41
Estuarine	SC	*Rhizoclonium implexium*	11.0	21	17	62
	JC	*Cladophera subsimplex*	8.0	24	30	46
	SB	blue green alga	10.4	10	23	67
	SuB	blue green alga	18.4	18	13	69

[a] Location: NH, North Head; SC, Saltwater Creek; JC, Joe's Creek; SB, Short Beach; SuB, Surf Beach.

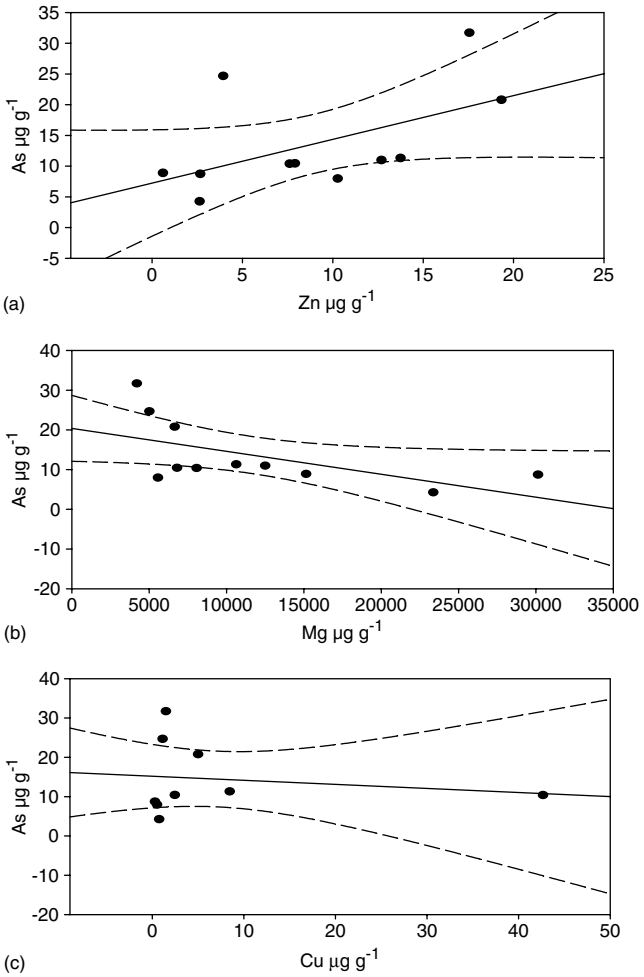

Figure 3. Regression analysis for marine inter-tidal and estuarine algae species (excluding blue green algae): (a) As and Zn; (b) As and Mg; (c) As and Cu.

and OH-ribose. Group 2 contains *Rhizoclonium implexium* that has higher proportions of TETRA and Inorganic As. Group 3 contains the red algae *Laurencia obtusa* and *Laurencia* sp. with higher proportions of inorganic As in these two species. Group 4 consists of the blue green algae and the estuarine green alga *Cladophera subsimplex* with high proportions of inorganic arsenic. The blue green algae species of group 4 can be discriminated from other algae in this group by the presence of an unknown arsenic anion. Group 5 consists of inter-tidal green algae that have higher proportions of OH-ribose.

Chromatograms of arsenic species standards are shown in Fig. 6. Chromatograms presented in Figs 7–10 are representative of the types of arsenic species present in all algae sampled in this study.

Lipid-soluble arsenic

Hydrolysed lipid extracts contained mostly two arsenic species (Table 6), OH-ribose and a large amount of an unknown compound that had the same retention characteristics of DMA on the anion column and similar retention behaviour to TMAO on the cation column under more acidic conditions (further referred to as Unk 1). This chromatographic behaviour has been shown before and is characteristic of compounds containing an dimethylarsinoyl moiety and a carboxy group.[21,22] A second unknown cation (Unk 2) at 6.5 min [Figs 7(a), 8(a), 9(b), 10(a)] was also present in some extracts. *Caulerpa flexilis* was the only algal species to contain inorganic arsenic in the hydrolysed lipid extract (Table 6).

Water-soluble arsenic

Within the water-soluble extracts the proportion of As(V) was large in the estuarine algae (23–74%) and the red inter-tidal algae *Laurencia* sp. and *L. obtusa* (43%; Table 7). Most algal species contained DMA, although it was only a minor

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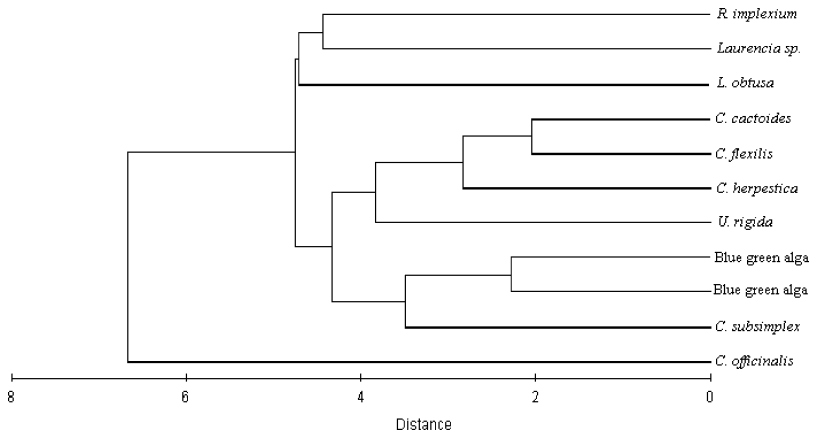

Figure 4. Cluster analysis for marine inter-tidal and estuarine algae based on proportion of arsenic species.

Table 5. Principle component analysis of arsenic species proportions in marine inter-tidal and estuarine algae south-east coast, NSW, Australia

	Eigenvalues	%Variation	Cum%Variation	Variable	Axis1 (PC1)	Axis2 (PC2)	Axis3 (PC3)
1	4.02	33.5	33.5	Inorg As	0.113	**0.574**	0.063
2	2.39	19.9	53.4	DMA	0.229	−0.175	0.021
3	1.69	14.1	67.5	MA	**0.258**	0.342	−0.471
				AB	0.008	−0.400	0.174
				AC	−0.472	−0.032	−0.217
				Tri OH-ribose	−0.448	−0.082	−0.277
				TETRA	**−0.475**	−0.020	−0.213
				OH-ribose	0.212	**−0.458**	−0.033
				PO4-ribose	−0.350	0.244	0.274
				OSO_3-ribose	0.007	0.265	0.274
				Unk anion	0.227	0.034	**−0.590**
				Unk cation	0.018	0.121	**0.298**

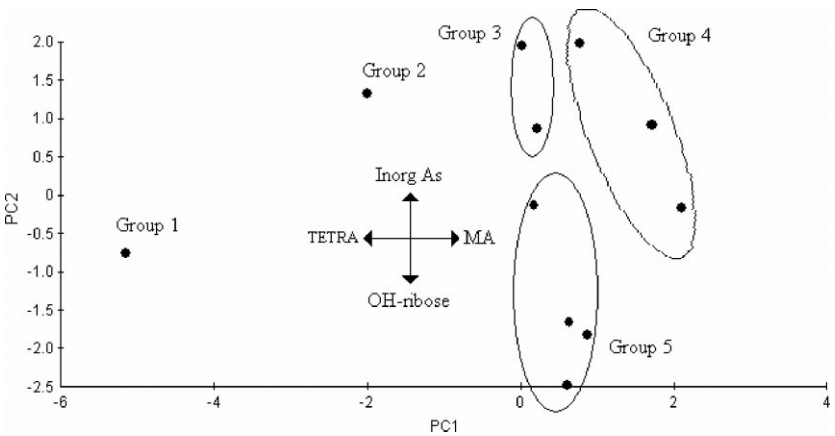

Figure 5. Principle component analysis (PCA) of arsenic species proportions in marine inter-tidal and estuarine algae. Group 1, *C. officinalis*; group 2, *R. implexium*; group 3, *L. obtusa* and *Laurencia* sp.; group 4, *C. subsimplex*, and blue green algae; group 4: *U. rigida, C. flexilis, C. cactoides, C. herpestica*. Arrows indicate the factor contributing to the pattern in two-dimensional space.

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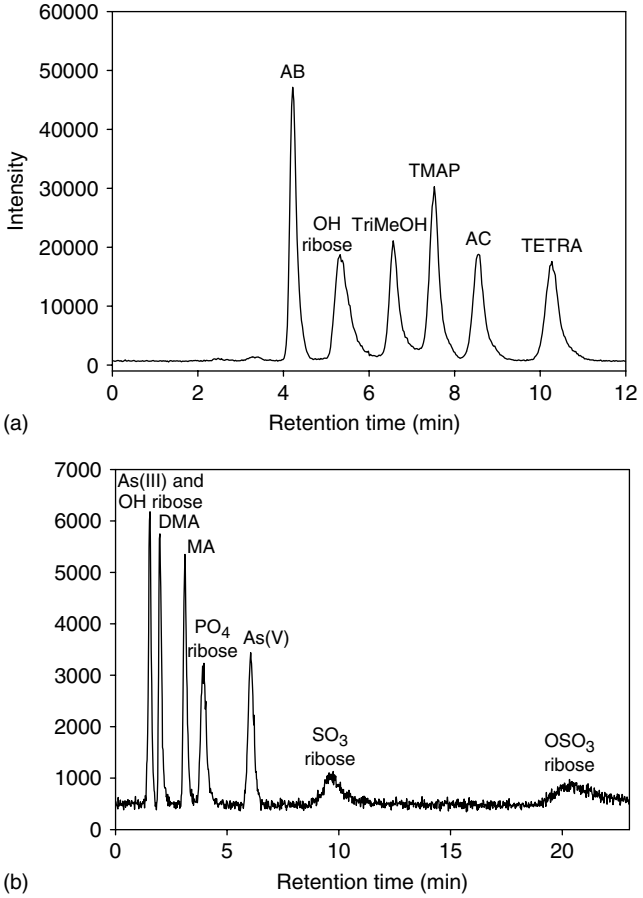

Figure 6. HPLC-ICPMS chromatograms of arsenic species standards. (a) Cationic arsenic species, determined using a Supelcosil LC-SCX cation exchange column (b) anionic arsenic species, measured using a Hamilton PRP-X100 anion exchange column.

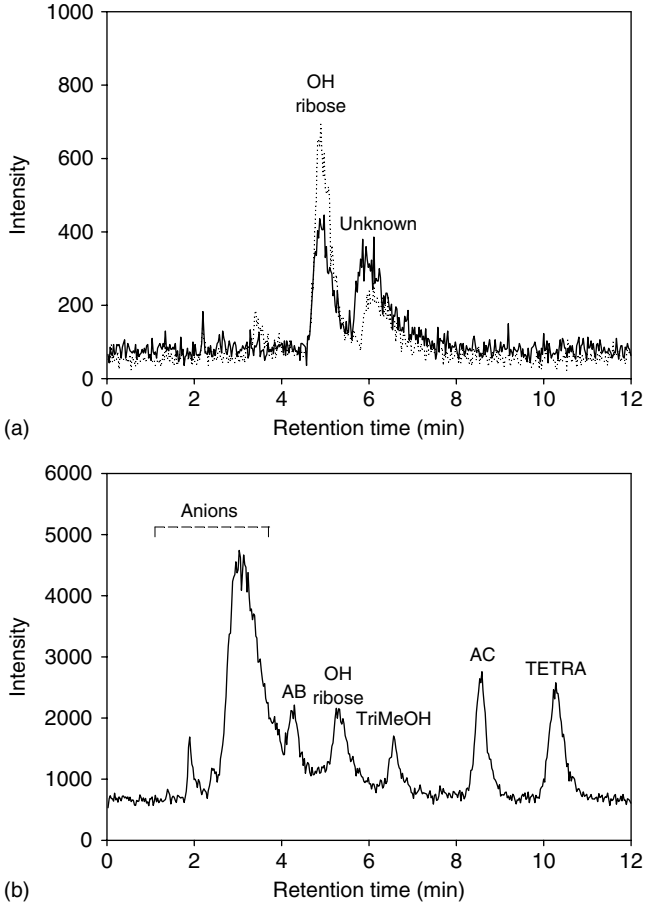

Figure 7. Arsenic species in *Corallina officinalis*: (a) lipid-soluble cations; dashed line represents spike of glycerol arsenoribose at ~5 μg l^{-1}; (b) water-soluble cations.

proportion of the total extractable arsenic (1–7%). MA was only detected in low concentrations in the *Laurencia* sp. and the estuarine algae with the exception of *R. implexium*. All algal species contained OH-ribose with the green inter-tidal algae containing a higher proportion (37–87%) compared with the red inter-tidal algae (7–15%) and estuarine green algae (11–16%). The blue green algae also contained an appreciable proportion of OH-ribose (26–50%). The PO_4-ribose was present in all algae except one species of blue green alga, with a higher proportion present in red inter-tidal algae (27–35%) compared with the other classes (2–17%). The OSO_3-ribose was present in three algal species *L. obtusa* (1%), *Laurencia* sp. (20%) and *Cladophoropsis herpestica* (1%). SO_3-ribose was not detected in any of the macro algal samples. Two green inter-tidal algae, *C. herpestica* and *Ulva rigida*, contained AB, with *U. rigida* having almost half its total water-soluble arsenic concentration as AB. *C. officinalis* was unlike other algal species examined, in that it did not contain any measurable inorganic or simple methyl arsenic species. Additionally, *C. officinalis* contained AB, AC, dimethylated and trimethylated OH-riboses and TETRA. An unknown anion was detected in blue green algae and an unknown cation was detected in *L. obtusa*.

Residue arsenic

The arsenic species present in the residues included As(III), As(V) and traces of DMA and MA (Table 8); however, most of the arsenic was inorganic.

DISCUSSION

Total arsenic and selected macro- and micronutrients

Total arsenic concentrations were consistent with those previously reported for macro algae from Australia and overseas.[1,2,23–25] Arsenic concentrations in *Laurencia* sp. were slightly higher than those reported by Tukai *et al.*[2] and Maher and Clarke,[24] but as neither sample was identified to species level, differences may be due to species or location differences.

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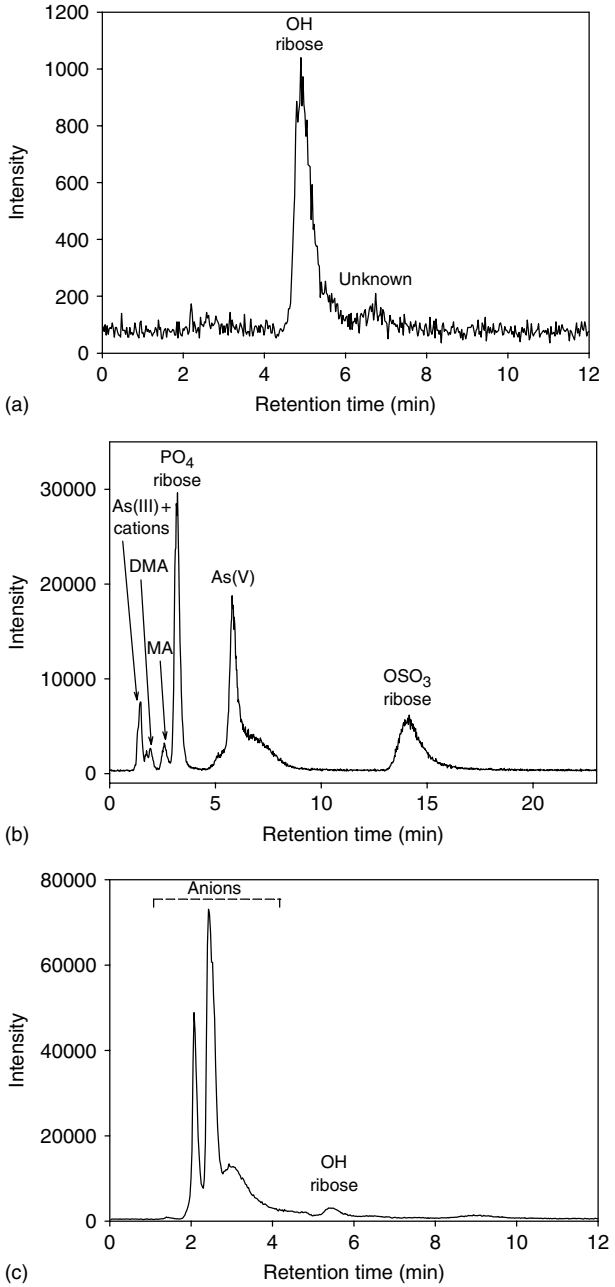

Figure 8. Arsenic species in *Laurencia* sp.: (a) lipid-soluble cations; (b) water-soluble anions; (c) water-soluble cations.

Arsenic concentrations reported for the blue green algae in this study are similar to those reported by Maher and Clarke[24] for the blue-green alga *Lyngbya* sp. that contained 6.6 μg g^{-1}.

Relationships with arsenic and macro and micronutrients were also examined (Fig. 3). No significant relationship between arsenic and phosphorus concentrations was found in this study. The chemical similarity of arsenate [$AsO(OH)_3$] and phosphate [$PO(OH)_3$] suggests that arsenic is taken up take by algae via the phosphate pathway.[25] Varying results concerning the relationship between arsenic and phosphorus concentrations have been found previously. Some studies have reported that arsenic concentrations increase with increasing phosphorus concentrations to a threshold after which arsenic uptake is inhibited by higher phosphorus concentrations.[26–28] Klumpp[26] found that phosphate at lower concentrations did not influence arsenic uptake. It was suggested that As(V) is taken up by more than one mechanism as simple competitive inhibition kinetics was not evident in the algal species studied.[28] Australian coastal waters are known to be phosphate-poor.[29] Thus, it is likely that in uncontaminated environments, such as in this study, competitive uptake between arsenic and phosphorus is not likely to occur.

Although the PCA (Table 3, Fig. 2) indicated that zinc, magnesium and copper concentrations were different in algal species, no significant correlations between arsenic and these elements were found [Fig. 3(a–c)]. It has been noted previously that a range of element concentrations are elevated in macro algae and that this may be associated with general uptake of elements rather than the uptake of specific elements to met metabolic requirements.[2]

Phosphorus, manganese, cobalt and zinc concentrations were elevated in the estuarine blue green algae with phosphorus concentrations up to six times higher and other elements one to two orders of magnitude higher than in the red and green macro algae species examined (Table 2). Arsenic concentrations in contrast were not particularly elevated relative to other algae examined (Table 2). Blue green algae are reported to resemble bacteria in that they are lacking an organized nuclei and the bluish pigment (c-phycocyanin) chemically differentiates them from most other plant species.[30] It is not known whether higher element concentrations are due to estuarine influences or a specific characteristic of blue green algae.

Fractionation of arsenic and arsenic species in inter-tidal and estuarine algae

General

Inorganic As and OH-ribose were the main arsenic species found in macro algae in this study (Table 6–8). The red inter-tidal algal species *Laurencia obtusa* and *Laurencia* sp. and the estuarine algae species contained a high proportion of inorganic arsenic and minor amounts of simple methylated compounds compared with green inter-tidal algae. In estuaries it has been shown that the ratio of As(III) to As(V) can be greater than in seawater due to changing salinity and freshwater influences,[6,7] but as algae are known to take up As(V) and convert it to As(III), it is unlikely As(III) present in estuaries would influence intracellular concentrations of As, and would only influence total arsenic concentrations by As(III) complexing with extracellular components of algae. Generally macro algae only contain minor concentrations of DMA and MA.[1,2,31] The presence of simple methyl arsenic species and arsenoribosides in all macro algae examined suggests that species are taking up As(V) from water, reducing and methylating arsenic and converting it

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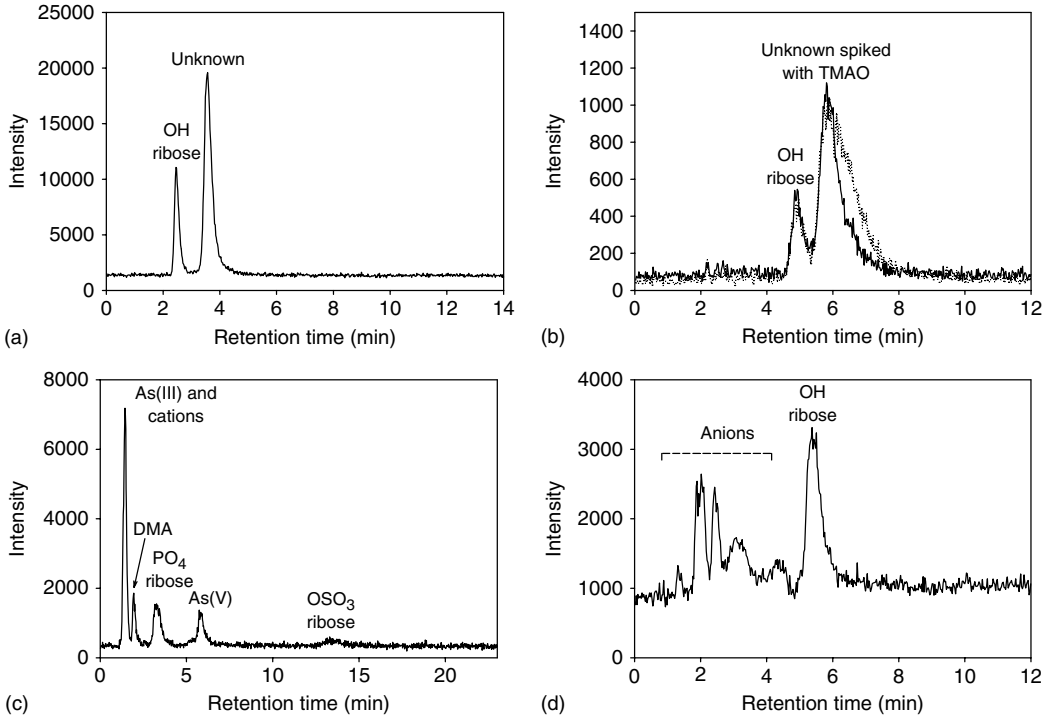

Figure 9. Arsenic species in *C. herpestica*: (a) lipid-soluble anions; (b) lipid-soluble cations; dashed line represents sample spiked with TMAO at ~5 μg l^{-1}; (c) water-soluble anions; (d) water-soluble cations.

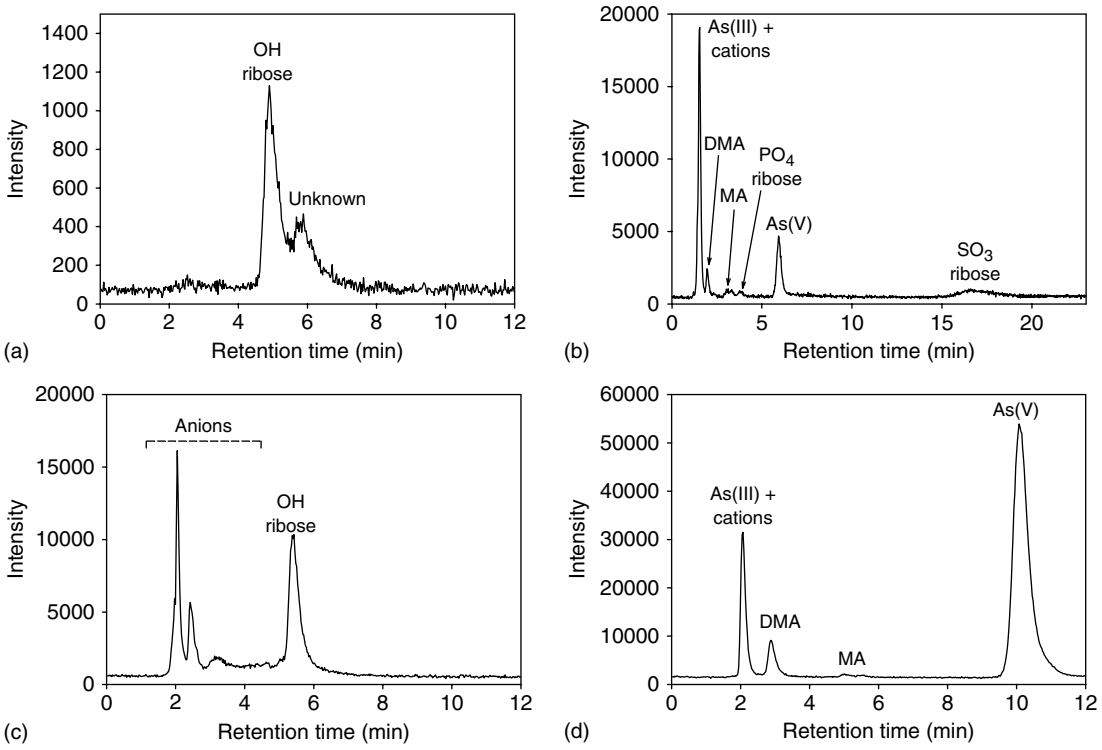

Figure 10. Arsenic species in blue green alga: (a) lipid-soluble cations; (b) water-soluble anions; (c) water-soluble cations; (d) residue anions.

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Table 6. Lipid-soluble arsenic species (μg g^{-1}) in marine inter-tidal and estuarine algae species from south-east coast, NSW, Australia (percentages in parentheses denote the percentages of the arsenic species)

Class	Species	Total As[a]	Inorg As	DMA	OH-ribose	Unk cation 1	Unk cation 2
Inter-tidal							
Rhodophyta	*Corallina officinalis*	0.9	n.q	n.q	0.12(48)	0.13(52)	n.q
	Laurencia obtusa	2.3	n.d	n.q	n.q	n.q	n.q
	Laurencia sp.	1.2	n.q	n.q	0.36(95)	n.q	0.02(5)
Chlorophyta	*Ulva rigida*	2.1	n.q	0.06(33)	0.10(56)	0.02(11)	n.q
	Caulerpa flexilis	0.9	0.02(17)	n.q	0.06(50)	n.q	0.04(33)
	Cladophoropsis herpestica	4.4	n.q	0.85(51)	0.17(10)	0.66(39)	n.q
	Caulerpa cactoides	1.6	n.q	n.q	0.15(100)	n.q	n.q
Estuarine							
Chlorophyta	*Rhizoclonium implexium*	2.4	n.q	0.78(57)	0.02(1)	0.58(42)	n.q
	Cladophera subsimplex	5.2	n.q	0.25(44)	0.09(16)	0.23(40)	n.q
Cyanobacterium	Blue green alga	1.1	n.q	0.20(33)	0.35(57)	0.06(10)	n.q
	Blue green alga	1.5	n.q	n.q	0.55(100)	n.q	n.q

n.q., not quantifiable, <0.005 μg g^{-1} for all species; n.d., no data. [a] Total As = As in pooled extracts.

to dimethylated arsenoribosides by the proposed pathway outlined by Edmonds and Francesconi.[32] Blue green algae have many arsenic species similar to the red and green algae examined in this study, yet the presence of higher inorganic arsenic in the water-soluble fraction and an as yet unidentified anion in appreciable quantities would suggest different metabolic processes compared with red and green macro algae.

Lipid arsenic species

Marine macro algae have been found to contain up to 50% dry mass arsenic in the lipid-soluble fraction.[1] In this study, 5–44% of arsenic was present in the lipid fractions of macro algae, with a higher proportion present in green macro algae (19–44%). The detection of arsenic lipids based on DMA, OH-ribose and Unk 1 in this study suggests the presence of complex As containing lipids in marine algae. DMA, MA and OH-ribose have been detected previously in the hydrolysed lipid fraction of the seaweed, *Laminaria digitata*, as major and minor constituents respectively.[12] These arsenic moieties may be precursors for AB transformation. Specifically, the presence of dimethylated arsenoribose based arsenolipids can transform to AB via intermediates previously identified in marine organisms.[3]

The presence of similar arsenic species in both the lipid and water-soluble fractions of algae (Tables 6 and 7) suggests that arsenic may be stored in the lipids in algae and released into the cytosol by degradation of lipids. The methanol–water technique generally used to extract arsenic from algae samples may draw the polar arsenic lipids into the water-soluble extract, resulting in a higher proportion of arsenic reported in the water-soluble fraction of algae samples. Practices such as acetone washing of algae prior to methanol–water extraction does not adequately remove lipids and only 'defats' samples to reduce fatty residues interfering with chromatography. Thus, the use of acetone then methanol–water extraction may be causing the over-reporting of the water-soluble component of algae and underestimation of the relative proportion of arsenic lipids. The presence of arsenic in the lipids of algae is likely to make a significant contribution to arsenic cycling in the marine environment.

Water-soluble arsenic species

The proportion of arsenic in the water-soluble fractions of algae varied with the class of algae examined (Table 4). Glycerol arsenoribose was found in all algal species and predominated in green inter-tidal algae (Table 7). Glycerol arsenoribose is the main arsenic sugar found in red and green macro algae.[1–3] However, the red inter-tidal algal species examined in this study contained more PO_4-ribose. Tukai *et al.*[2] found that *Laurencia* sp. contained 40% as As(V) in water-soluble extracts, as was found for both *Laurencia* sp. examined in this study, suggesting higher inorganic arsenic content is a genera specific response in this red algae. Low concentrations of OSO_3-ribose were found in two red algae and the green algae *Cladophoropsis herpestica* (Table 6). Higher concentrations of SO_3- and OSO_3-riboses are mostly associated with brown macro algae[1,2,33] although all three classes, red, green and brown macro algae are known to contain sulfated polysaccharides in their cell walls,[34–36] which could account for the presence of OSO_3-ribose in red and green macro algae in this study.

Trimethyl glycerol arsenoribose was present in the red algae (*Corallina officinalis*) and a green algae (*Rhizoclonium implexium*), yet AC and TETRA were also present in these algae, suggesting a relationship with other animal organisms incorporated with the algae rather than intracellular concentrations. The presence of AB in one red and two green

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Table 7. Water-soluble arsenic species (μg g^{-1}) in marine inter-tidal and estuarine algae species from south-east coast, NSW (percentages in parentheses denote the percentages of the arsenic species)

Class	Species	Total As[a]	Inorg As	DMA	MA	AB	AC	Tri-MeOH	TETRA	Dimethyl arsenoribosides			Unk anion	Unk cation
										-OH	$-PO_4$	$-OSO_3$		
Inter-tidal														
Rhodophyta	*Corallina officinalis*	1.6	n.q.	n.q.	n.q.	0.10(6)	0.33(21)	0.13(8)	0.31(19)	0.16(10)	0.56(35)	n.q.	n.q.	n.q.
	Laurencia obtusa	6.6	2.38(43)	0.34(6)	0.08(1)	n.q.	0.10(2)	n.q.	0.07(1)	0.86(15)	1.50(27)	0.08(1)	n.q.	0.29(5)
	Laurencia sp.	7.6	2.55(43)	0.03(1)	0.14(2)	n.q.	n.q.	n.q.	n.q.	0.41(7)	1.66(28)	1.17(20)	n.q.	n.q.
Chlorophyta	*Ulva rigida*	2.6	0.03(7)	0.02(5)	n.q.	0.20(47)	n.q.	n.q.	n.q.	0.16(37)	0.02(5)	n.q.	n.q.	n.q.
	Caulerpa flexilis	1.0	0.06(10)	n.q.	n.q.	n.q.	n.q.	n.q.	n.q.	0.54(87)	0.02(3)	n.q.	n.q.	n.q.
	Cladophoropsis herpestica	2.5	0.07(8)	0.06(7)	n.q	0.06(7)	n.q.	n.q.	n.q.	0.60(66)	0.11(12)	0.01(1)	n.q.	n.q.
	Caulerpa cactoides	2.6	0.24(37)	n.q.	n.q	n.q.	n.q.	n.q.	n.q.	0.30(46)	0.11(17)	n.q.	n.q.	n.q.
Estuarine														
Chlorophyta	*Rhizoclonium implexium*	1.5	0.55(61)	0.02(2)	n.q.	n.q.	0.06(7)	0.01(1)	0.07(8)	0.14(16)	0.05(6)	n.q.	n.q.	n.q.
	Cladophera subsimplex	1.6	0.87(74)	n.q.	0.06(5)	n.q.	n.d.	n.q.	n.q.	0.13(11)	0.11(9)	n.q.	n.q.	n.q.
Cyanobacterium	Blue green alga	2.5	0.29(23)	0.06(5)	0.02(2)	n.q.	n.d.	n.q.	n.q.	0.62(50)	0.02(2)	n.q.	0.24(19)	n.q.
	Blue green alga	3.5	0.81(55)	0.07(5)	0.07(5)	n.q.	n.d.	n.q.	n.q.	0.38(26)	n.q.	n.q.	0.13(9)	n.q.

n.q., not quantifiable, <0.005 μg g^{-1} for all species except inorganic arsenic, <0.0003 μg g^{-1}. [a] Total As = As in pooled extracts.

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Table 8. Residue arsenic species (μg g^{-1}) in marine inter-tidal and estuarine algae species from south-east coast, NSW, Australia (percentages in parentheses denote the percentages of the arsenic species)

Class	Species	Total As[a]	Inorg As	DMA	MA
Inter-tidal					
Rhodophyta	*Corallina officinalis*	1.5	0.02(100)	n.q	n.q
	Laurencia obtusa	7.1	1.25(97)	0.04(3)	n.q
	Laurencia sp.	8.7	0.62(98)	0.01(2)	n.q
Chlorophyta	*Ulva rigida*	2.2	0.32(97)	0.01(3)	n.q
	Caulerpa flexilis	2.2	0.53(98)	0.01(2)	n.q
	Cladophoropsis herpestica	3.4	0.30(86)	0.05(14)	n.q
	Caulerpa cactoides	3.3	1.32(100)	n.q	n.q
Estuarine					
Chlorophyta	*Rhizoclonium implexium*	9.6	7.38(83)	1.51(17)	n.q
	Cladophera subsimplex	6.4	5.29(78)	0.07(1)	1.42(21)
Cyanobacterium	Blue green alga	7.2	2.65(95)	0.15(5)	n.q
	Blue green alga	19.9	3.76(99)	n.q	0.04(1)

n.q., not quantifiable, <0.005 μg g^{-1}.
[a] Total As = As in pooled extracts.

macro algae and AC and TETRA in two red algae and one green alga is unusual, as these arsenic species are not normally associated with marine algae. It is likely these compounds are related to microscopic epiphytes incorporated into the cellular structure of algae that are not easily removed by rinsing of samples, as reported recently by Šlejkovec *et al*.[37]

Residue bound arsenic species

Around one-third of arsenic was found in insoluble residues, with up to two-thirds of arsenic associated with the residues of estuarine algae (Table 5) as inorganic arsenic (Table 8). This indicates that a high proportion of arsenic is bound to structural cellular components such as thio-complexes, of which arsenite in particular has a high affinity.[38,39] As-polychelatin complexes have been reported previously for green micro algae, *Stichococcus bacillaris*,[40] diatoms[41] and higher plants[38,42] and may also have a role in binding inorganic arsenic in inter-tidal and estuarine algae.

Arsenic cycling in inter-tidal and estuarine algae

A general overview of the likely cycling of arsenic in marine algae is presented below.

Uptake

Algae take up arsenic from seawater as As(V), most likely via the active phosphate uptake pathway,[39,43] as no active uptake pathway has been found for arsenic. No clear relationship exists with arsenic and the uptake of other macro- and micronutrients required by algae to maintain cellular metabolism, structure and growth and it is likely that arsenic is taken up with a range of elements in response to the general metabolic requirements of algae.

Metabolism and sequestration

Algae convert As(V) to As(III), then by methylation and reduction produce simple methylated compounds such as MA and DMA. By the process of glycosidation, arsenic forms arsenoriboses, chiefly OH-ribose, although the PO_4- and OSO_3-riboses are also present in red and green algae. The majority of arsenic is present as organic arsenic species in green inter-tidal algae with a high proportion of arsenoribosides, compared with red inter-tidal algae, while estuarine green and blue green algae species contain a significant proportion of arsenic as inorganic arsenic. This is in contrast to brown macro algae, which mostly contain arsenoriboses.[1,2] Epiphytes are most likely responsible for the presence of arsenic species such as AB, AC and TETRA.

Either inorganic arsenic or the organic arsenic species formed are incorporated into lipids, most likely as complex arseno phospholipids. The majority of hydrolysed lipid arsenic species in this study were based on DMA and OH-ribose, with a minor amount of Unk 1. Inorganic arsenic binds non-specifically to cellular components or is sequestered in to the insoluble constituents of the cell, most likely into vacuoles where arsenic is probably incorporated into As(III)–polychelatins or As–SH complexes, as occurs for micro algae and higher order plants,[38,41] although this is to be confirmed for inter-tidal and estuarine algae.

Homeostasis

The similarities of AB and TMAP to nitrogen and sulfur analogues used as osmotic compounds in marine algae[44] suggest that these arsenic species may also be used in osmotic regulation of algal cells. Estuarine systems are dynamic and it is likely that osmotic regulation is critical to algae due to changes in salinity, periods of desiccation and changes in

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temperature, requiring more responsiveness of algal cells to maintain osmotic balance. These arsenic species were found in very low concentrations or not at all in the algae species examined in this study and thus it unlikely that these algal species are using arsenic compounds for osmotic regulation.

Excretion

Algae return arsenic compounds back to the environment via breakdown of plant surface, with microbial degradation converting arsenoribosides to simple methyl arsenic species, returned to water and converted to predominantly As(V). Complex lipids that are structural components of membranes could be used as a method of transporting arsenic species for excretion from cells, due to the nature of continual degradation and re-synthesis of the polar head groups of lipid compounds.[45]

In conclusion, total arsenic concentrations present in inter-tidal and estuarine algae are similar to those found in marine red and green macro algae species. Arsenic does not appear to be dependent on phosphorus or other elements required for photosynthesis.

The lipid-soluble fraction of macro algae contained appreciable quantities of arsenic compounds based on OH-ribose, DMA and an unknown arsenic compound and is likely to be a significant pathway in metabolism and sequestration of arsenic in algae. The presence of similar arsenic species in both the lipid and water-soluble fractions of algae suggests that arsenic may be stored in the lipids of algae and released into the cytosol when lipids degrade.

Glycerol arsenoribose was the main arsenoribose present in inter-tidal green and estuarine algae, while PO_4-ribose was the main arsenoribose present in the red inter-tidal algae species examined. The formation of arsenoriboses in macro algae is a general response to the uptake of arsenic. However, a high proportion of inorganic arsenic in red inter-tidal macro algae and estuarine algae suggests that metabolism of arsenic is also related to class and environmental differences. Detection of AB, AC and TETRA, which are not normally found in algae, are likely be due to the presence of epiphytic organisms such as animals and fungi, using algae as a host.

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