Seasonal Changes in Arsenic Speciation in *Fucus* Species

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Arsenic speciation in a brown alga, *Fucus gardneri*, collected in Vancouver, B.C., Canada, was carried out by using high-performance liquid chromatography–inductively coupled plasma–mass spectrometry (HPLC–ICP–MS). Hydride generation–atomic absorption spectrometry (HG–AAS) was used for total arsenic determination. The relative amounts of some arsenosugars 1 in growing tips are found to be different in comparison with the remainder of the plant. *Fucus* samples collected in summer contain 9 ppm of total arsenic. Most of the arsenic species are extractable. *Fucus* samples collected in winter contain relatively higher amounts of arsenic, 16–22 ppm, but only low amounts of this are extractable. © **1998 John Wiley & Sons, Ltd.**

Appl. Organometal. Chem. **12**, 243–251 (1998)

Keywords: arsenic; speciation; algae; *Fucus gardneri*; arsenosugar; high-performance liquid chromatography (HPLC); inductively coupled plasma–mass spectrometry (ICP–MS)

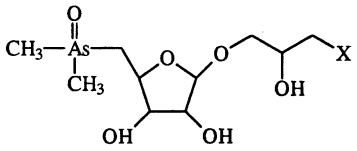
Received 12 February 1997; accepted 9 July 1997

INTRODUCTION

Arsenic is one of the most notorious elements on account of its human toxicity, and it occurs in a variety of different forms. The distribution of arsenic in natural waters can vary widely depending on the accessibility of arsenic-bearing rocks and deposits.[1,2] Generally speaking, natural waters contain arsenic concentrations ranging from 1.0 to 10 ppb with an average of 1.7 ppb: in marine environments, arsenic concentrations range from 1.5 to 5 ppb.[1–3]

The toxicity of arsenic compounds depends on their chemical form. Thus the organometallic forms of arsenic, which are widely found in marine animals and plants, are generally less toxic than the inorganic arsenicals found in the environment.[2]

Since the early 1900s, a number of different research groups have been working on the identification of the chemical forms of arsenic in the environment. But only in the past 20 years have the many complexities of arsenic speciation, in marine animals and plants, been revealed. Arsenic-containing ribofuranosides (arsenosugars) such as arsenosugars **1a, 1b** and **1c** were found in the brown alga,



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a X = OH
b X = $OPO(OH)OCH_2CH(OH)CH_2OH$
c X = SO_3H
d X = OSO_3H

Ecklonia radiata, and in a range of other marine macroalgae.[4] For example, arsenosugars **1a** and **1b** were found in the red alga *Porphyra tenera* (Nori) and the green alga *Odium fragile* (Miru). Arsenosugars **1a, 1b** and **1c** were found in brown algae *Undaria pinnatifida* (Wakame), *Eisenia bicyclis* (Arame) and *Laminaria japonica* (Makonbu).[4,5]

The present study involves an investigation of the seasonal changes in arsenic speciation in *Fucus gardneri*, particularly in the tips of the plant. *Fucus gardneri* is commonly found in British Columbia

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Contract/grant sponsor: Natural Sciences and Engineering Research Council of Canada.

 CCC 0268–2605/98/040243–09 $17.50

and the species grows on rocks in the middle and lower intertidal zones; the plant is olive–green to yellowish–green in colour. The swollen tips of the branches of *Fucus* sp., termed receptacles, are the reproductive areas. Scattered over the surface of these receptacles are minute openings, called conceptacles, in which are produced the sperm and eggs.[6,7] Receptacles are generally yellowish in colour.[8] *Fucus gardneri* is the major species growing in the sampling site, Brockton Point, Stanley Park, Vancouver. Samples were collected in July, February and May, corresponding to summer, winter and spring seasons. The same sampling site was chosen for all collections in order to minimize differences in arsenic speciation associated with geographical variations.

A combination of high-performance liquid chromatography interfaced with an inductively coupled plasma–mass spectrometer (HPLC–ICP–MS) was used to identify the arsenic species on the basis of their retention times compared with chromatographic standards.

EXPERIMENTAL

High-performance liquid chromatography–inductively coupled plasma–mass spectrometry (HPLC–ICP–MS)

The HPLC system consisted of a Waters Model 510 delivery pump, a Reodyne Model 7010 injector valve with a 20 µl sample loop, and one of two reverse-phase C_{18} columns (GL Sciences Inertsil ODS, 250 mm × 4.6 mm; and Phenomenex Inertsil 5 ODS-2, 250 mm × 4.6 mm). A guard column packed with the same material (Supelco) preceded the analytical column. The HPLC system was connected to the ICP nebulizer via a PTFE tube (20 cm × 0.4 mm) and appropriate fittings.

A VG Plasma Quad 2 Turbo Plus inductively coupled plasma–mass spectrometer (VG Elemental, Fisons Instrument equipped with an SX 300 quadrupole mass analyser, a standard ICP torch, and a de Galan V-groove nebulizer, was used as the detector. The time-resolved analysis (TRA) mode was used to set the mass analyser to monitor the $m/z = 75$ signal peak corresponding to As^+. The TRA mode allowed the possibility of simultaneously monitoring more than one m/z value at a time. All signals were collected and the data (peak areas) were manipulated on a separate computer (MS Excel 5.0). A summary of the ICP–MS operating parameters is given in Table 1.

Table 1 Operating parameters for ICP–MS

Forward rf power	1350 W
Reflected power	<10 W
Outer (cooling) gas flow rate	13.8 l min^{-1}
Intermediate (auxiliary) gas flow rate	0.65 l min^{-1}
Nebulizer gas flow rate	1.002 l min^{-1}
Analysis mode	TRA, 1 s time slice
Quadrupole pressure	9×10^{-7} mbar
Expansion pressure	2.4 mbar

Hydride generation–atomic absorption spectrometry (HG–AAS)

The hydride generation system was the same as was described previously[9] except that a commercial Nafion permeation tube (Perma Pure Inc.) fitted with a nitrogen purge (90 ml min^{-1}) was used to dry the hydrides before they reached the quartz tube.

Atomic absorption measurements were made with a Jarrell Ash 810 atomic absorption spectrometer equipped with a hydrogen–air flame atomizer. A spectral band width of 1 nm and a wavelength of 193.7 nm were used. Background correction was not available. A Hewlett–Packard 3390A integrator was connected to the AA spectrometer to record signals. An open-ended T-shaped quartz absorption tube (9.5 cm × 0.8 cm i.d.) was mounted in the flame of the burner. Light from the arsenic hollow-cathode lamp (Varian) was aligned to pass through the wide end of the flame-heated quartz tube. Hydrides from the reaction of the sample with borohydride were introduced through the side arm of the quartz tube (9 cm × 0.4 cm i.d.).

Reagents and chemicals

All chemicals used were of analytical grade unless otherwise stated and included: methanol (HPLC grade, Fisher), tetraethylammonium hydroxide (TEAH, 20 wt%, Aldrich), tetrabutylammonium hydroxide (TBAH, Aldrich), malonic acid (BDH), sodium borohydride (Aldrich), hydrochloric acid (36.5%, Fisher), sulphuric acid (98%, Fisher), nitric acid (69%, sub-boiled, Seastar Chemicals) and hydrogen peroxide (30%, Fisher). Deionized water with resistivity better than 1 Ω m was used for the extractions and to make up the eluent for HPLC.

The glassware and plasticware were cleaned by soaking in 2% Extran solution overnight, rinsing

with water and deionized water, then soaking in 0.1 M HNO_3 solution overnight. They were then rinsed with deionized water and air-dried.

Samples

Sample collection

Fucus samples were collected at Brockton Point, Stanley Park, Vancouver, B.C., at mid-tide level in July 1995, February 1996 and May 1996. Oyster tissue standard reference material (SRM NIST 1566a) was purchased from the National Institute of Standards and Technology, US Department of Commerce. Kelp powder was purchased from a food store in Vancouver, B.C., Canada.

Sample treatment prior to storage

Fucus samples collected in July 1995 had obvious receptacles. The parts of the samples referred to as 'the tips' contain the receptacles with conceptacles, and the parts referred to as 'the remainder' contain the remaining parts of the plant.

Fucus samples collected in February 1996 were first separated into two subsamples, young and mature. The young samples do not show observable conceptacles, whereas mature samples have observable conceptacles. The mature samples and the young samples were further separated into tips and remainder. Mature *Fucus* is usually brown in colour, and young *Fucus* is greenish–brown. Mature 'tips' were selected, as for the July collection. However, it was more difficult to decide which part of the young *Fucus* is the reproductive organ since conceptacles are not well developed. In this case, the swollen ends of the branches were picked as the tips. Occasionally, only flat non-swollen branches could be seen. For these samples, end portions of the branches, 1.5 cm in length, were regarded as the tips.

Fucus collected in May 1996 was first separated into young and mature samples, based on the presence or absence of conceptacles on the receptacles. The mature *Fucus* collected appeared to be greenish–yellow, similar to that collected in July 1995. The young *Fucus* was not subsampled because of its small size. The tips of mature *Fucus* were separated as in the July collection. Each batch of samples from many plants contained a wet weight of more than 120 g. Figure 1 shows the sampling/collecting scheme.

Sample preparation

Extraction with CH_3OH/H_2O

Freeze-dried *Fucus gardneri*, oyster tissue standard reference material, kelp powder (0.5 g dry weight) or fresh *Fucus gardneri* (5 g wet weight) was extracted by a procedure similar to that described by Shibata and Morita.[10] Each sample was weighed into a 15 ml or 50 ml centrifuge tube. To each tube was added 10 ml g^{-1} dry sample or 2 ml g^{-1} wet sample of a methanol–water mixture (1:1, v/v). The tube was sonicated for 10 min and centrifuged for 10 min. After centrifugation, the extract was

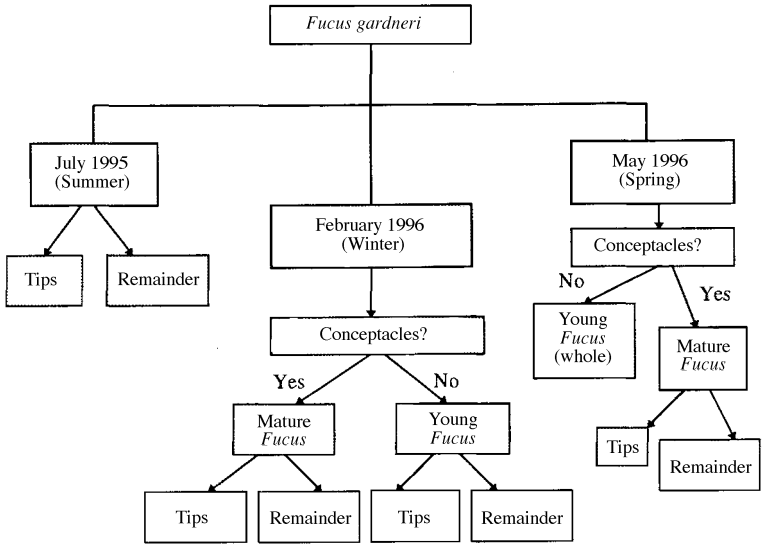

Figure 1 The sample collection scheme for *Fucus.*

Table 2 Summary of experimental conditions (HPLC)

Condition	Column	Mobile phase	Flow rate (ml min^{-1})
(1)(a)	Inertsil ODS (GL Sciences, Japan)	10 mM tetraethylammonium hydroxide (TEAH), 4.5 mM malonic acid, 0.1% CH_3OH, pH 6.8	0.8
(1)(b)	Inertsil ODS (GL Sciences, Japan)	10 mM tetrabutylammonium hydroxide (TBAH), 4.5 mM malonic acid, 0.1% CH_3OH, pH 6.8	1.0
(2)(a)	Inertsil 5 ODS-2 (Phenomenex)	10 mM tetraethylammonium hydroxide (TEAH), 4.5 mM malonic acid, 0.1% CH_3OH, pH 6.8	0.8
(2)(b)	Inertsil 5 ODS-2 (Phenomenex)	10 mM tetrabutylammonium hydroxide (TBAH), 4.5 mM malonic acid, 0.1% CH_3OH, pH 6.8	1.0

removed by means of a Pasteur pipette and placed in a round-bottomed flask. The extraction procedure was repeated an additional four times for each sample. The combined extract was evaporated to dryness and redissolved in 10 ml of deionized water before further analysis.

Acid digestion of samples

Dry samples (0.25 g), wet samples (2.5 g), or the residue after water/methanol extraction (initially 0.5 g dry weight or 5 g wet weight) was placed in a 250 or 500 ml round-bottomed flask fitted with the special condenser, Teflon capillary, four Teflon plugs and a Teflon adaptor described by Bajo *et al.*[11] To each sample, an acid mixture of H_2SO_4:HNO_3:H_2O_2 (1:3:3, by vol.) was added[12] and the reaction mixture was refluxed for 2 h,[13] cooled and made up to 25 ml for further analysis.

Analytical procedures

HPLC–ICP–MS

The columns and eluents used are listed in Table 2. All samples were filtered (0.45 μm) before injection onto the column. In general, 20 μl samples were analysed. Arsenic compounds in the samples were identified by matching the retention times of the peaks in the chromatograms with those obtained from standards.

HG–AAS

The samples after total digestion were analysed by using HG–AAS. The samples required dilution before analysis. The blank solution was made up from the appropriate amounts of H_2SO_4, H_2O_2 and HNO_3. Standard arsenate, As(V), solutions were also made up in a similar way. After acid digestion of the samples, the only arsenic species present is the oxidized form, As(V);[12] therefore, the use of arsenate for calibration was appropriate. The optimized conditions described by Le *et al.*[14] were used for the analysis. Samples were aspirated for 30 s, corresponding to the uptake of 2 ml for each analysis. Triplicate analyses were performed for each sample.

RESULTS AND DISCUSSION

Water content

Table 3 shows the water content of the *Fucus* samples collected in summer, winter and spring between 1995 and 1996, in Brockton Point, Stanley Park. The water content of *Fucus gardneri* remains relatively constant from season to season. The tips of *Fucus*, which are sometimes swollen, contain a higher amount of water (88 to 93%) than does the remainder (80 to 82%).

Qualitative analysis by HPLC–ICP–MS

Figures 2 and 3 show chromatograms [condition(1a) Table 2] of the extracts from the tips and

Table 3 Water content of *Fucus gardneri*

Sample	Water content (%)[a]
July collection (summer)	
Tips	93
Remainder	80
February collection (winter)	
Young tips	89
Young remainder	82
Mature tips	89
Mature remainder	81
May collection (spring)	
Young whole plant	82
Mature tips	88
Mature remainder	80

[a] RSD = ± 5%.

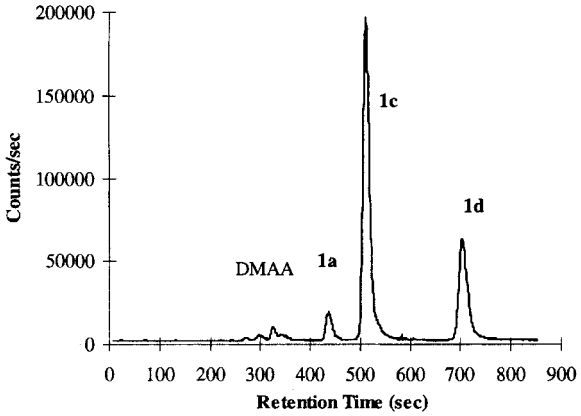

Figure 2 A chromatogram of the freeze-dried tips of *Fucus*, condition (1)(a).

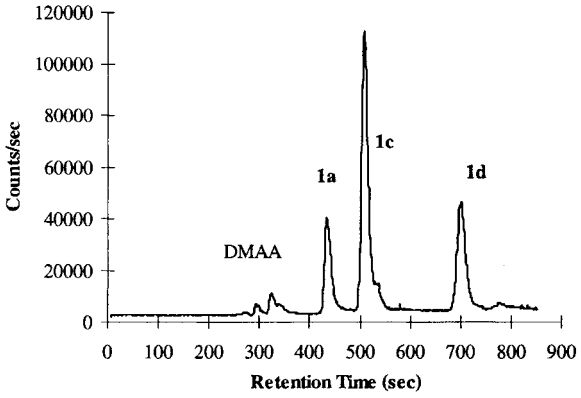

Figure 3 A chromatogram of the freeze-dried remainder of *Fucus*, condition (1)(a).

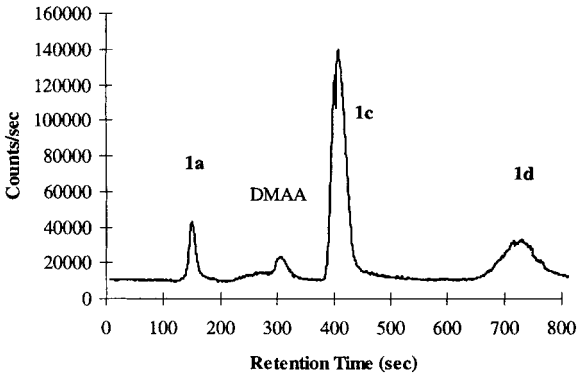

Figure 4 A chromatogram of the freeze-dried tips of *Fucus*, condition (2)(b).

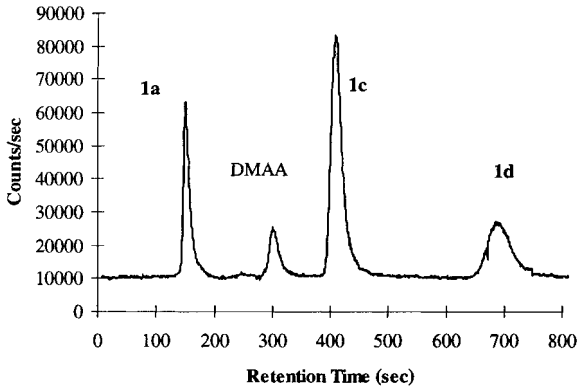

Figure 5 A chromatogram of the freeze-dried remainder of *Fucus*, condition (2)(b).

the remainder of *Fucus* from the July collection. The arsenic compounds in the extract were identified by matching the retention times of the peaks with those obtained from an oyster tissue standard reference material and a laboratory standard 'kelp powder' as described previously.[15] It can be seen that the extract from the tips (Fig. 2) contains a smaller amount of arsenosugar **1a** than the extract from the remainder (Fig. 3). The tips contain a slightly lower amount of DMAA. Figure 3 shows a shoulder at the retention time of around 520 s. The retention time of this peak is close to that of arsenosugar **1b.** However, the result is not reproducible. Other arsenic compounds, such as monomethylarsonic acid, arsenite, arsenate, arsenocholine and tetramethylarsenonium ion, if present, elute well before arsenosugar **1a** (V. W.-M. Lai, W. R. Cullen and K. J. Reimer, unpublished results).

Figures 4 and 5 show the same two samples from the July collection analysed under conditions (2b) (Table 2). With the use of TBAH as the ion-pairing reagent, arsenobetaine, if present, will co-elute with arsenosugar **1a**, and DMAA will co-elute with arsenosugar **1b.** However, no detectable amounts of arsenobetaine and arsenosugar **1b** are observed in Figs 2 and 3. The first and second major peaks in Figs 4 and 5 can be assigned to arsenosugar **1a** and DMAA respectively. Again the relative amount of arsenosugar **1a** in the *Fucus* tips (Fig. 4) is smaller than in the remainder (Fig. 5).

Table 4 and Figs 6–8 show summaries of the relative peak areas of arsenic compounds found in extracts from all samples collected in July, February and May. It should be noted that the response factors of all arsenosugars under present

Table 4 Relative amounts (wt% of total As) of arsenic compounds in all *Fucus* samples

Sample no.	DMAA	**1a**	**1c**	**1d**
July collection (summer)				
1. Tips	2.4	7.6	63.4	26.6
2. Remainder	4.6	16.9	52.8	25.7
February collection (winter)				
3. Mature tips	3.2	5.5	60.7	30.6
4. Mature remainder	6.4	11.4	56.4	25.8
5. Young tips	3.7	5.5	69.4	21.4
6. Young remainder	9.3	11.3	59.4	20.0
May collection (spring)				
7. Mature tips	7.8	5.2	65.6	21.4
8. Mature remainder	11.5	13.0	57.5	18.0
9. Young whole *Fucus*	8.8	8.9	66.5	15.8
Averages[a]				
All tips (1,3,5,7)	4.3	5.9	64.8	25.0
All remainders (2,4,6,8)	7.9	13.2	56.5	22.4
Mature *Fucus* from February (3,4)	4.8	8.4	58.5	28.3
Young *Fucus* from February (5,6)	6.5	8.4	64.3	20.8
Mature *Fucus* from May (7,8)	9.7	9.1	61.5	19.7
All young *Fucus* (5,6,9)	7.3	8.5	65.1	19.1
All mature *Fucus* (3,4,7,8)	7.2	8.8	60.0	24.0

[a] The numbers in parentheses are those of the samples used for calculating averages.

experimental conditions are assumed to be equal. In all three seasons, extracts of the *Fucus* tips contain relatively lower amounts of DMAA and arsenosugar **1a** and relatively higher amounts of arsenosugars **1c** and **1d** than those in the remainder of the plant (Fig. 6). The results from the February collection of *Fucus* show that there is no significant difference in the relative amounts of DMAA and arsenosugar **1a** found in young *Fucus* and mature *Fucus* (Fig. 7). When the results from young whole *Fucus* collected in May are compared with those from older plants, the relative amount of arsenosugar **1a** in this young sample is approximately the average of the relative amounts of arsenosugar **1a** in the tips and the remainder of the older specimen (Fig. 7). These results indicate that the age of *Fucus* is unlikely to be a factor affecting the relative amounts of DMAA and arsenosugar **1a** in the plant.

Figure 8 shows that the mature *Fucus* collected in February contains a slightly higher amount of arsenosugar **1d** and a lower amount of arsenosugar **1c** than are found in the young *Fucus* from the same collection. Similar results are observed from the May collection, in which mature *Fucus* contains a slightly higher amount of arsenosugar **1d** and a lower amount of arsenosugar **1c** than does the

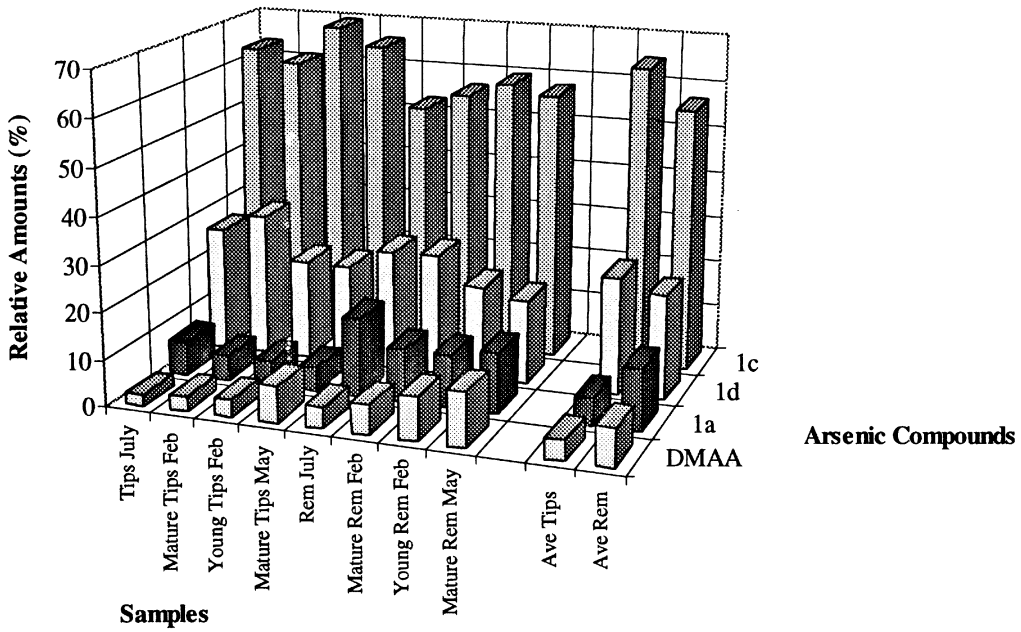

Figure 6 Relative amounts of arsenic compounds in the tips and the remainder of *Fucus.* Key: Tips, tips of *Fucus*; Rem, remainder of *Fucus*; Whole, whole plant; July, (summer) collection; Feb, February (winter) collection; May, spring collection; ave, average.

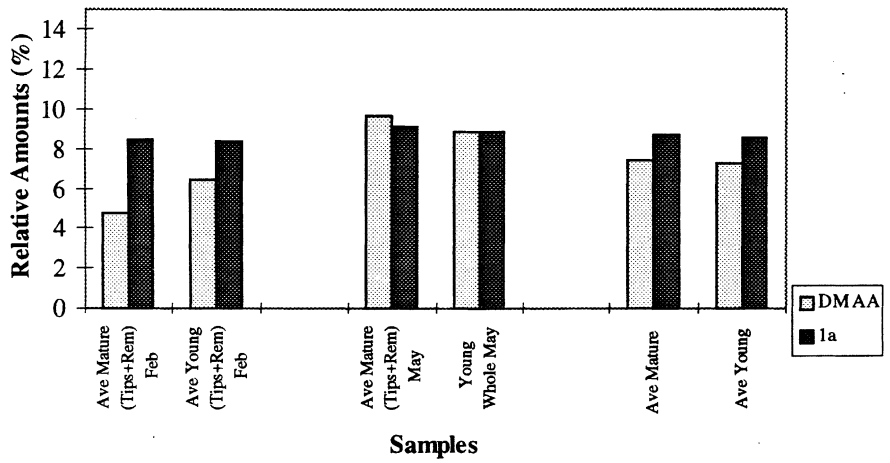

Figure 7 Relative amounts of DMAA and arsenosugar **1a** in mature and young *Fucus* samples. For key, see Fig. 6.

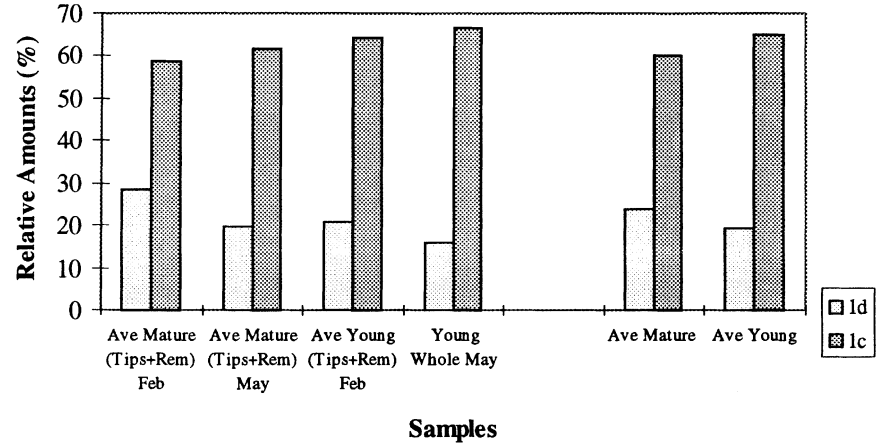

Figure 8 Relative amounts of arsenosugars **1c** and **1d** in mature and young *Fucus* samples. For key, see Fig. 6.

young whole *Fucus*. Though the differences in the relative amounts of various arsenosugars are small, they might indicate that the formation of arsenosugar **1d** is more favoured than **1c** in mature *Fucus*.

In the absence of knowledge about processes that produce the arsenosugars and the sites of this activity, it is difficult to comment on the meaning of the present results. It has been suggested that arsenosugar **1b** can be decomposed to arsenosugar **1a** and then DMAA.[5,16] On the same basis, the conversion of **1c** and **1d** to **1a** does not seem unlikely.

Quantitative analysis by HG–AAS

Fucus samples collected in July and February and the residue after CH_3OH/H_2O extraction of the samples were digested to determine the total arsenic content, as described in the Experimental section. A summary of the total arsenic content as well as the extraction efficiency of the samples is given in Table 5. The extraction efficiency is defined as $[(As_1 - As_2)/As_1] \times 100\%$ where As_1 and As_2 are the arsenic contents (μg As/g sample) of the sample before and after extraction respectively.

Table 5 Arsenic content in *Fucus gardneri*

Sample	Arsenic content ± SD (μg g^{-1})[a]		Extraction efficiency[b]
	As_1	As_2	
July Collection			
Tips (dry)	8.9 ± 0.5	1.8 ± 0.1	79.2%
Remainder (dry)	9.0 ± 0.5	0.45 ± 0.08	95.0%
Tips (wet)	1.60 ± 0.04	0.100 ± 0.005	93.8%
Remainder (wet)	3.7 ± 0.3	0.06 ± 0.01	98.3%
February Collection			
Young Tips (dry)	17.1 ± 0.8	16.1 ± 0.2	5.8%
Young Remainder (dry)	16.2 ± 0.9	11.5 ± 0.9	29.3%
Mature Tips (dry)	22.2 ± 0.8	11 ± 1	48.7%
Mature Remainder (dry)	17.0 ± 0.9	8.9 ± 0.2	47.6%

[a] As_1 = content before extraction; As_2 = content after extraction; ±SD of triplicate analyses of duplicate sample.
[b] The extraction efficiency is defined as $(As_1 - As_2)/As_1 \times 100\%)$.

The total arsenic content in different *Fucus* samples collected in the same season is relatively constant. However, there is a wide variation in the total arsenic content of *Fucus* samples collected in different seasons (8.9–9.0 ppm in July and 16.2–22.2 ppm in February on a dry-weight basis).

In an earlier arsenic-uptake study involving *Fucus spiralis* growing in aquaria, Klumpp[17] found that the intensity of illumination and the temperature affect the short-term uptake and efflux of arsenic by *Fucus* sp. He also found that only living *Fucus* cells are able to accumulate arsenic and that the uptake of arsenic is reduced in the presence of a respiratory inhibitor. The results suggest that the accumulation of arsenic by *Fucus* is an active process dependent on respiratory energy[3] and is not associated with phosphorus uptake.[17]

In open marine environments, other factors might affect the total arsenic content and the speciation of arsenic in *Fucus.* For example, the arsenic concentration in the seawater, the reproduction and the growth rate of *Fucus* need to be considered. The arsenic concentration in seawater might change as a result of point input but generally the concentration is expected to be constant. The reproduction of the same (or similar) *Fucus* sp. in False Creek, Vancouver, peaks in autumn to winter[18] but the growth rate is lowest in winter,[19] which is when we observe higher levels of arsenic in *Fucus* (February collections); however, no biochemical role has been proposed for arsenic in these plants,[4] and until this is established the relationship between the total arsenic content in *Fucus*, the season and the growth of *Fucus* must remain unknown.

The extraction efficiency found for the *Fucus* samples collected in July (79.2–98.3%) is higher than for those collected in February (5.8–48.7%). Most of the arsenic compounds exist as water-soluble forms in July; however, the February results suggest that either the distribution of the arsenic species is different at this time or the structure of the plant changes in such a way as to hinder the extraction of the water-soluble species. These differences may also be related to plant age, because in February the mature *Fucus* samples contain higher amounts of water-soluble compounds than do the young samples. Whatever the cause of this phenomenon, the results suggest that care is needed when discussing speciation in algae. Season and age, at least, need to be considered.

Arsenic uptake experiments performed on *Fucus* growing in culture are planned in order to provide further information about the arsenic speciation and possible biological function in *Fucus.*

Acknowledgments The authors are grateful to the Natural Sciences and Engineering Research Council of Canada for financial assistance, and Mr Bert Mueller for help with ICP–MS analysis.

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