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Biomethylation and bioaccumulation of arsenic(V) by marine alga *Fucus gardneri*†

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The brown alga, *Fucus gardneri*, was collected from Vancouver Island, B.C., Canada. *Fucus* samples were acclimated in seawater and then exposed to arsenic(V) in artificial seawater, all under axenic conditions. The arsenic species in the *Fucus* samples were extracted into 1:1 methanol/H_2O and identified by using ion-pairing high-performance liquid chromatography coupled to inductively coupled plasma mass spectrometry (HPLC–ICP-MS). Anion-exchange HPLC–ICP-MS was used to identify the arsenic species in the growth medium. It was found that ~73% of the original arsenosugars in the *F. gardneri* samples were lost during the acclimation period. Arsenite [As(III)] and dimethylarsenate [DMA] concentration increased in the *Fucus* samples after exposure to arsenate [As(V)]. A small increase in the concentration of an arsenosugar was seen during this period, accompanied by a further decrease in the concentration of two other arsenosugars. The experiments provided very little evidence for the hypothesis that arsenosugars are produced by the macroalgae. Copyright © 2001 John Wiley & Sons, Ltd.

Keywords: arsenic; arsenite [As(III)]; dimethylarsenate [DMA]; accumulation; biotransformation; algae; *Fucus gardneri*; high-performance liquid chromatography (HPLC); inductively coupled plasma mass spectrometry (ICP-MS); arsenosugar

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INTRODUCTION

Arsenic is one of the better known elements because of the toxic properties of some of its compounds. Against this background it is not surprising that reports, at the beginning of the 20th century, of high levels of arsenic in marine organisms attracted attention. Since then, there has been much interest in the forms of arsenic in marine organisms, their toxicity, how they are accumulated, and what biochemical role they play.[1]

Marine algae have been the subject of many arsenic metabolic studies because of their ecological and nutritional importance. Such studies of the interaction of marine algae with arsenicals are relevant because it is possible that arsenic compounds produced by algae are the source of the arsenic compounds found in marine animals, although it is not well established how and when these transformations might take place.[1,2] Therefore, this study is concerned with elucidating this problem.

Most of the arsenic in marine macroalgae exists in complex forms, and a variety of arsenic-containing ribofuranosides derivatives (arsenosugars) have been isolated and characterized. Arsenosugars were first identified in 1981 in the brown macroalga *Ecklonia radiata* by Edmonds and Francesconi.[3,4] Further investigations of other algal species revealed the existence of 15 related

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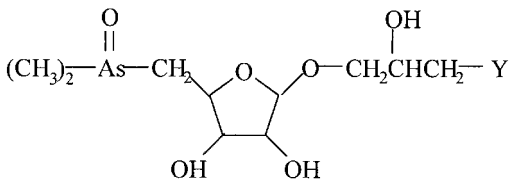

	Y
1a	-OH
b	$-OPO_3HCH_2CH(OH)CH_2OH$
c	$-SO_3H$
d	$-OSO_3H$

Figure 1 The structures of some of the arsenosugars found in the marine environment.

compounds: the most common ones are shown in Fig. 1.[4–6] Because these compounds can occur at high concentrations (several milligrams of arsenic per kilogram wet weight) in marine organisms, including those used as human food, there is considerable interest in their toxicological behaviour.[4]

The biotransformation of arsenicals by marine macroalgae in culture medium is the subject of a limited number of reports.[2,7–9] Complex arsenic compounds are produced by these algae under different conditions, but the compounds were not characterized. Therefore, this work was undertaken to determine if and how arsenosugars are produced by macroalgae, following exposure to As(V) in a controlled environment.

Fucus gardneri Silva, also known as *Fucus distichus*, was chosen for this investigation because it is abundant in the British Columbia coastal area. It grows on rocks mainly in the middle and lower inter-tidal zones. The *Fucus* is characterized by branching filamentous thallus and its colour is olive-green to yellow–green.[10–13] Mature *Fucus* possess receptacles, which are the fertile areas, at the ends of the branches. Receptacles may be enlarged and swollen when they are mature and may appear yellowish.[11]

EXPERIMENTAL

Reagents and chemicals

All chemicals used were of analytical grade or better and obtained from commercial sources, unless otherwise stated. The chemicals and reagents used were: methanol (HPLC grade, Fisher), tetraethylammonium hydroxide (20 wt%, Aldrich), malonic acid (BDH), nitric acid (68–71%, sub-boiling distilled, Seastar Chemicals), phosphoric acid (99.999%, 85 wt%, Aldrich) and ammonium hydroxide (30%, Fisher).

Deionized water, with a resistivity better than 1 MΩ cm, was used to make up all the standard solutions, the mobile phases for the HPLC system, and the sample solutions for extraction.

Because it was necessary to avoid even trace contamination, the following precautions were taken: all the glassware and plasticware were cleaned by soaking in 2% Extran solution for at least one night. They were rinsed with deionized water, and then soaked in 0.1 M HNO_3 solution for at least one night. They were then rinsed with deionized water and air-dried. All glassware used to store the standard solutions or used for the algae experiments were autoclaved.

Standards

Standard solutions of arsenite (from arsenic trioxide, As_2O_3, Fisher), arsenate (from sodium arsenate heptahydrate, $Na_2HAsO_4{\cdot}7H_2O$, Sigma), monomethylarsonic acid (Pfalz & Bauer), dimethylarsinic acid (Aldrich), and arsenobetaine (synthesized as described by Edmonds *et al.*[14]) were made up in deionized water. Standard samples of oyster tissue SRM (NIST-1566a), *Fucus* sample (IAEA-140/TM), and kelp powder (laboratory standard-Galloway's naturally kelp powder, Richmond, B.C., Canada) were also available to confirm the retention times of arsenosugars obtained from the high-performance liquid chromatography coupled to inductively coupled plasma mass spectrometry (HPLC–ICP-MS).

Medium and antibiotics

The artificial seawater that was used in the arsenic exposure experiments was prepared according to the recipe for ASP6 F2 (see Table 1), which was previously used by Fries[15] to culture members of the family Fucaceae. The pH of the artificial seawater was adjusted from 9.72 to ~7.76 (which was the pH of the seawater that was collected with the algae) by the addition of HCl. The pH reported for the ASP6 F2 artificial seawater by Fries[16] is 8.3. The seawater was prepared without the vitamins, autoclaved and stored at room temperature. The vitamin solution and the vitamin B_{12} solution were sterilized by using 0.22 μm sterile filters (Gelman)

Table 1 Contents of artificial seawater, ASP6 F2[15]

Medium ASP6 F2	
NaCl	24 g
$MgSO_4 \cdot 7H_2O$	8 g
KCl	0.7 g
$CaCl_2 \cdot 2H_2O$[a]	0.55 g
$NaNO_3$	0.1 g
Na_2-glycerophosphate	50 mg
K_2HPO_4	2 mg
KI	0.065 mg
KBr	96.9 mg
TRIS (hydroxy methyl) aminomethane	1 g
Micronutrient solution	1 ml
Vitamin solution	1 ml
B_{12}	1 μg
Distilled water	1000 ml
Micronutrient solution (per ml H_2O)	
Nitrilotriacetic acid	50 mg
Fe (as chloride)	1 mg
Zn (as chloride)	0.25 mg
Mn (as chloride)	0.5 mg
Co	5 μg
Cu	10 μg
B (as H_3BO_3)	1 mg
Mo (Na salt)	0.25 mg
Vitamin solution (per ml H_2O)	
Thiamine HCl	0.2 mg
Nicotinic acid	0.1 mg
Ca panthothenate	0.1 mg
Pyridoxin HCl	0.04 mg
p-Aminobenzoic acid	0.01 mg
Biotin	0.5 μg
Thymine	0.8 mg
Inositol	1 mg
Orotic acid	0.26 mg

[a] Original recipe has $CaCl_2 \cdot H_2O$.

prior to addition to the autoclaved seawater. The artificial seawater with the vitamins was then stored in the refrigerator at 4 °C.

The antibiotic/antimycotic solution was prepared according to the recipe for A3-antibiotics as described by Liu and Kloareg[17] (see Table 2), and was sterilized by using 0.22 μm sterile filters (Gelman) before being used. The antibiotic/antimycotic solution was added to the media to establish axenic conditions.

Samples

Sample collection

Whole young brown algae, *F. gardneri*, were collected at Bamfield marine station, Vancouver Island, B.C. All the samples were collected at low tide levels. Seawater collected at the site was later sterilized by using the autoclave and then used for washing and for the acclimation period.

Table 2 Antibiotic solution to establish axenic cultures in marine algae[17]

Antibiotics[a]	
Streptomycin sulfate	2 g
Nystatin	0.5 g
Erythromycin	1.5 g
Rifampicine	0.02 g
Distilled water	100 ml

[a] Antibiotics were purchased at Sigma. Stock solutions were diluted with 100 volumes of medium for treating plant materials.

Sample treatment

Whole young *Fucus* samples were chosen for the exposure experiments instead of the tips because, as indicated by Fries,[15] the tips of *Fucus* grow very slowly and consequently several months would be needed to see any growth. All steps performed after the collection of the *Fucus* were done under sterile conditions in a laminar flow hood.

Samples of young *Fucus* were rinsed in a sterile beaker approximately five times with sterile seawater, followed by four washings with distilled water. The samples were then covered with a mixture of antibiotic/antimycotic solution and seawater (~2 ml per 100 ml seawater) for approximately 5 to 10 min. The mixture was removed and the *Fucus* washed with sterile seawater. The *Fucus* was separated and placed into 1-l Erlenmeyer flasks, each flask containing 400 ml seawater, 4 ml antibiotic/antimycotic solution and between 26.75 and 30.05 g of *Fucus*. The flasks were placed in an incubator (Conviron Environmental Chamber) at temperatures of 15 °C/7 °C on a 12 h day/night cycle with the light strength at 100 lux for the day cycle. The flasks were shaken once every few days to ensure adequate dissolved oxygen in the media.

After 14 days of acclimation the *Fucus* samples were clean, apart from a visible white fungal growth. Bacterial levels appeared to be minimal because the antibiotic/antimycotic-containing solution did not turn milky. The *Fucus* samples were combined and washed with sterile seawater six to eight times. The last washing also contained the antibiotic/antimycotic solution (2 ml antibiotic/antimycotic to 100 ml seawater). The *Fucus* samples were transferred to sterile flasks, each one containing 200 ml artificial seawater, 2 ml

Table 3 Medium conditions for the exposure period for *F. gardneri* collected February 1999

Flask[a]	Sample	Arsenic[b]
1	Algae from seawater	0.50 ppm As(V)
2	Algae from seawater	×
3	×[c]	0.50 ppm As(V)
4	×	×

[a] All flasks were duplicated.
[b] All flasks contained 2 ml antibiotics in 200 ml ASP6 F2 medium.
[c] Not present in the flasks.

antibiotic/antimycotic solution, 100 μg of arsenate and between 19.95 and 26.73 g of *Fucus* (Table 3). The exposure experiment was run for 28 days and 1 ml samples of the medium were taken twice a week during that period. After the exposure experiment, the *Fucus* samples were collected, frozen and freeze-dried.

Culture and medium conditions

The *Fucus* samples that were collected from the sampling sites underwent a rigorous treatment to make them as axenic as possible. Antibiotics, antimycotics and other decontamination procedures were included in an attempt to make sure no living organisms, other than the *Fucus*, were involved in the exposure experiment. However, the treatment was not completely successful, because a fungus (*Fusarium oxysporum melonis*)[18] was observed to grow with the *Fucus* during both the acclimation and exposure experiments. Nonetheless, the contamination due to bacteria was at a minimum, since no bacterial infection was in evidence. Other more rigorous techniques for cleaning the algae could have been used, but many procedures would result in destruction of the algal cells.

The *Fucus* appeared to remain intact and healthy throughout most of the experiment, although at the end of the experiment there was some wilting and the colour was darker (brownish/green) than when freshly collected. The medium (Table 3) was not changed during the acclimation period nor during the arsenic exposure period, so the algae samples were most likely experiencing starvation conditions at the end of each period. The culture medium also became increasingly coloured (ranging from light orange to dark orange) over the course of the experiment, and this was particularly noticeable for the medium containing the arsenic(V) species.

Sample preparation

Sample storage

All biomass samples collected were weighed, frozen (−20 °C) immediately, and then freeze-dried. All freeze-dried samples were kept at −20 °C until they were extracted.

All the liquid samples taken during the experiments were frozen immediately to preserve sample integrity until the time of the analysis. The liquid samples were analysed by using anion-exchange HPLC–ICP-MS.

Extraction with MeOH/H_2O

All the freeze-dried samples collected were extracted using a procedure similar to that described by Shibata and Morita.[19] Kelp powder (a laboratory standard), oyster tissue SRM (NIST-1566a) and a *Fucus* sample (IAEA-140/TM) were similarly extracted as reference materials. The samples were weighed out on an analytical balance (∼0.5 g) into 15 ml centrifuge tubes and 5 ml methanol/water (1:1) was added to each tube. The tubes were sonicated for 10 min, centrifuged for 10 min and the liquid layer of the extracts was transferred into 250 ml round-bottomed flasks by using a Pasteur pipette. Each sample was extracted a total of five

Table 4 Summary of experimental HPLC conditions

Conditions	Column	Mobile phase	Flow rate (ml min^{-1})
Anion exchange (medium samples)	Hamilton PRP × 100	20 mM phosphoric acid, pH 6.0	1.5
Ion pairing (extract samples)	Inertsil ODS (GL Sciences, Japan)	10 mM tetraethylammonium hydroxide (TEAH), 4.5 mM malonic acid, 0.1% MeOH, pH 6.8	1.0

times. The combined extract solutions for each sample were evaporated to near dryness (~1–2 ml), and made up to 10.0 ml using a volumetric flask. The extract solutions were frozen until just before analysis. The samples were analysed by using the ion-pairing HPLC–ICP-MS method.

Instrumentation

The HPLC system consisted of a Waters Model 510 delivery pump, a Reodyne six-port injection valve with a 20 μl sample loop, an appropriate column and its corresponding guard column. The analytical column used to analyse the medium samples was an anion-exchange column (Hamilton, PRP × 100, 250 mm × 4.6 mm). The mobile phase was 20 mM phosphoric acid, adjusted to pH 6.0 by using ammonium hydroxide. The column used to analyse the extract samples was a reversed-phase C_{18} column (GL Sciences Inertsil ODS, 250 mm × 4.6 mm). The mobile phase was made up of 10 mM tetraethylammonium hydroxide, 4.5 mM malonic acid, and 0.1% MeOH, adjusted to pH 6.8 by using nitric acid. The experimental conditions, mobile phases and flow rates are summarized in Table 4. The HPLC system was connected to the ICP-MS nebulizer by using PTFE tubing (2.5 cm) with the appropriate fittings. The chromatographic columns were equilibrated with the mobile phase for about 50 min prior to analysis. All the standard samples were filtered through a 0.45 μm syringe filter (Gelman) just prior to injection onto the HPLC column, whereas all other samples were filtered through a 0.22 μm syringe filter (Gelman) before injection. Arsenic compounds in the samples were identified by matching the retention times of the peaks in the chromatograms with those of standard arsenic compounds. The mobile phases for the HPLC were filtered through a 0.45 μm filter (Millipore) after they were made up.

A VG Plasma Quad 2 Turbo Plus ICP-MS (VG Elemental, Fisons Instrument) was used as a detector for the HPLC eluent. It was also used to measure the total arsenic, via direct injection into the system. The ICP-MS was equipped with an SX 300 quadrupole mass analyser, a standard ICP torch, an argon plasma and a de Galan V-groove nebulizer. The mass analyser was operated in the time-resolved analysis (TRA) mode using TR Analysis software. The 75, 77, 82 and 103 mass to charge (*m/z*) signals were monitored. All signals were collected and the data were transferred to a computer (VG data system) for further processing with Microsoft Excel 7.0.

RESULTS

The objective of this study was to study the uptake of arsenate by axenic cultures of *Fucus* in a controlled environment and determine if and how arsenosugars are produced by the *Fucus*.

Speciation of the arsenicals found in the extracts of samples of *F. gardneri*

Acclimation period

The acclimation period, which lasted 14 days, was the period when the algae acclimated to its new environment before exposure to As(V). Chromatograms of the arsenic species extracted from the algae before and after acclimation are shown in Fig. 2. The experimental data are summarized in Table 5. The major arsenic species in fresh *F. gardneri* were arsenosugars **1a, 1c** and **1d**. After 14 days of acclimation, the total concentration of the arsenic compounds in the *Fucus* extracts decreased to ~27.5% of that of the fresh samples. Further studies are currently under way to determine how and when the arsenosugars are lost during the acclimation period.

Exposure period

The *Fucus* samples were washed with fresh sterile seawater and antibiotics after 14 days of acclimation and then were incubated with 500 ppb of As(V) in ASP6 F2 medium as described in the Experimental section. The extracts of the samples obtained at the end of the arsenate exposure experiment showed an increase in the amount of arsenite, dimethylarsinate, arsenate and arsenosugar **1a** in the *Fucus* samples relative to the concentrations at the beginning of the uptake experiment, i.e. following the 14 days acclimation. A decrease in the concentration of arsenosugars **1c** and **1d** was also observed (see Fig. 3 and Table 5).

The controls for this experiment, which are the *Fucus* in media without As(V), showed a similar decrease in arsenosugars **1c** and **1d** compared with the *Fucus* samples that were exposed to arsenate (Table 5).

Speciation of the arsenicals in the medium of *F. gardneri* following exposure to arsenate

Analysis of samples of the growth medium during the exposure period was carried out by using anion-exchange HPLC–ICP-MS. The results obtained are

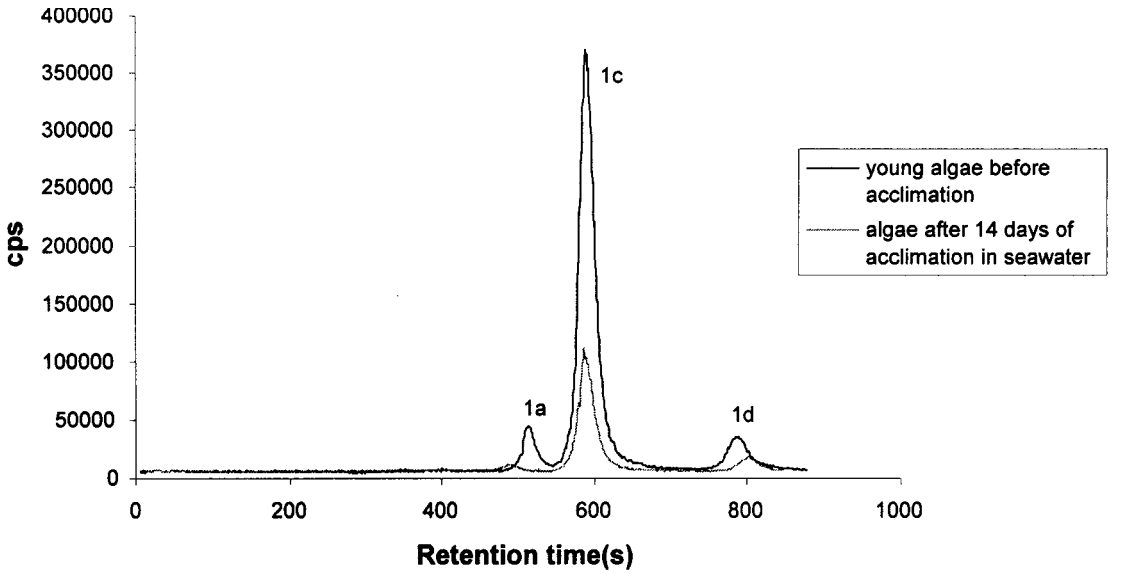

Figure 2 Extracts of *F. gardneri* before and after 14 days of acclimation in seawater.

shown in Fig. 4. The medium samples showed a rapid reduction in As(V) in the first 12 days of exposure, whereas dimethylarsenate (DMA) and As(III) increased continuously. After 12 days, the concentration of the arsenic species in the medium stayed relatively constant.

DISCUSSION

The results show that the marine macroalga *F. gardneri* is able to bioaccumulate and biotransform arsenic compounds that are present in the surrounding medium. The experiment was carried out under conditions that ensured a minimum of biological contamination. Even so, one fungus survived the treatment with the antibiotic/antimycotic solution and did grow on the *Fucus* during the acclimation and arsenic exposure phases of this experiment. It is conceivable that this fungus, identified as *F. oxysporum melonis* by sequencing of its 28S ribosomal RNA gene, is responsible for the biotransformation of arsenic species.[18] Independent studies showed that the isolated fungus is able to convert arsenate to arsenite and DMA;[18] however, the relative amounts of these arsenicals produced by the fungus was about 1000 times lower than that produced by the *Fucus*. Thus we conclude that, in this experiment, we are observing changes attributable mainly to the algae, even though it is difficult to predict the results of a possible joint interaction of the *Fucus* and the fungus in a symbiotic relationship.[18]

Table 5 Arsenic speciation of *F. gardneri* extracts (ppm, dry weight) following acclimation in seawater and exposure to As(V) in artificial seawater

	Arsenic species found					
Algae sample	As(III)	DMA	As(V)	**1a**	**1c**	**1d**
Before acclimation	0	0	0	0.41 ± 0.05	4.62 ± 0.6	0.51 ± 0.07
After 14 days of acclimation	0	0	0	0.06 ± 0.01	1.24 ± 0.2	0.16 ± 0.02
After exposure period						
Control— no As(V) exposure	0	0	0	0.04 ± 0.01	0.45 ± 0.06	0.13 ± 0.02
After exposure to As(V)	2.59 ± 0.3[a]	0.75 ± 0.1	2.65 ± 0.3	0.37 ± 0.05	0.50 ± 0.06	0.15 ± 0.02

[a] s_c = standard deviation from analytical results obtained with the calibration curve.

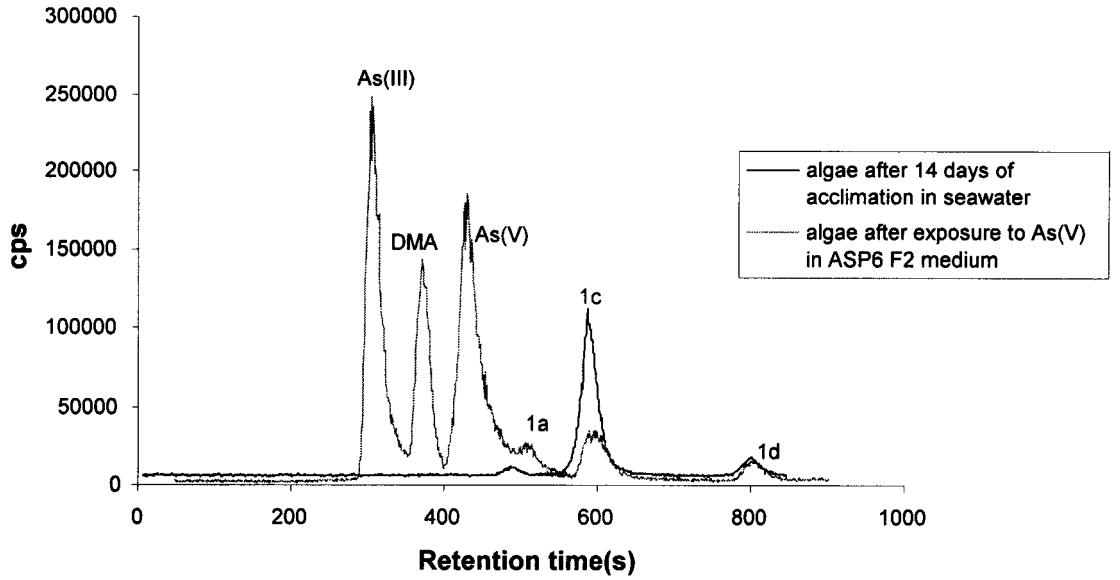

Figure 3 Extracts of *F. gardneri* after exposure to As(V) in ASP6 F2 medium.

The major species found in the growth medium and algal extracts following exposure of the acclimated *Fucus* to As(V) were As(III), DMA and As(V). Small amounts of arsenosugars were found in the extracts, but only an increase in arsenosugar **1a** was seen, with a decrease in arsenosugar **1c**. No detectable amount of MMA was found in the extract samples.

It is not surprising that little or no amount of MMA is detected when the algae are treated with arsenate. It has been reported that only trace amounts of MMA were produced from arsenate

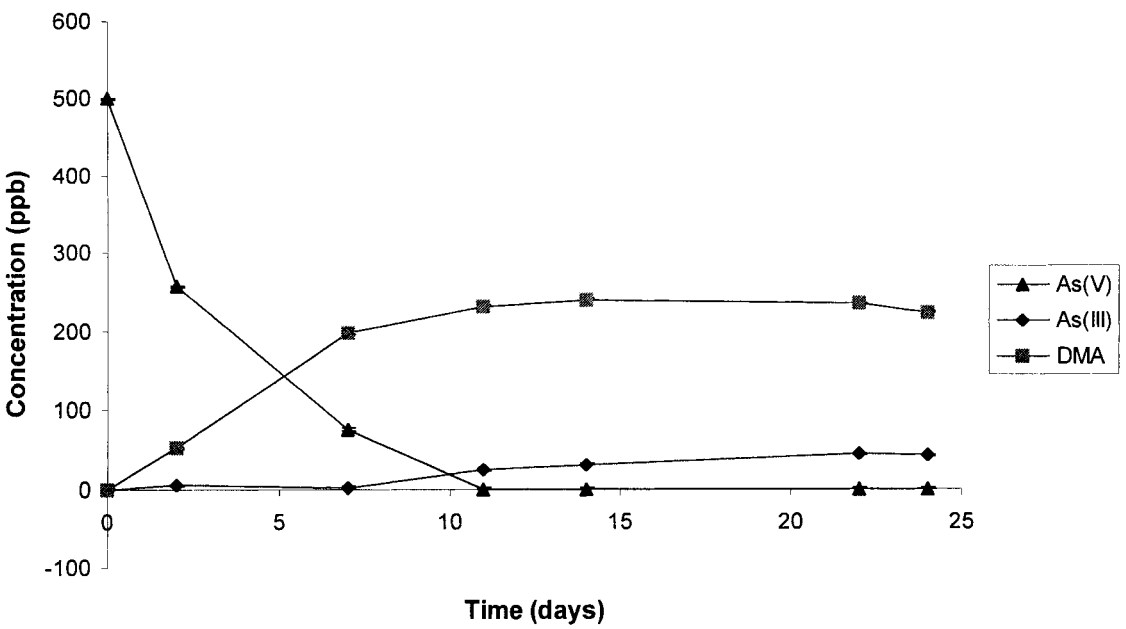

Figure 4 ASP6 F2 medium samples collected during the exposure of *F. gardneri* to As(V).

by whole-cell cultures of the fungus *Apiotrichum humicola*[9] and the marine unicellular alga *Polyphysa peniculus*,[7] and that MMA is the least transformed arsenical substrate. It was argued, on the basis of the measured diffusion coefficient, that MMA would not be lost from the cell as a free intermediate; thus the cells would of necessity metabolize the MMA to DMA for excretion into the growth medium.[7,9]

The curves in Fig. 4 are very similar to those obtained from experiments involving the marine unicellular alga *P. peniculus*.[7] The observed reduction of arsenate to arsenite is the major process in the medium. The algal cells first take up the arsenate from the medium, presumably via the phosphate transport system, reduce the arsenate to arsenite inside the cells, probably by using thiols and/or dithiols, and excrete most of the arsenite into the growth medium by means of an active transport system.[2,9,20] Some arsenite retained in the cells is methylated to MMA by using *S*-adenosylmethionine (SAM); as mentioned above, the MMA is not excreted to the growth medium, but remains in the cell where it is more likely to be reduced and further methylated to DMA. This arsenical is lost to the medium, presumably by means of passive diffusion.[21,22]

It has been suggested that any DMA retained in the algal cells can be further reduced and then oxidized by donation of the adenosyl group in SAM. The nucleoside formed undergoes glycosidation to produce the range of arsenosugars that have been identified from algal sources.[20,23,24] However, there is little evidence for this process from the present results, as the overall amount of the sugars in the algae did not change significantly during the course of the arsenic exposure experiment. Although there was some increase in the concentration of arsenosugars **1a** after exposure to As(V), this could have been associated with the loss of arsenosugar **1c** that was also seen. That is, arsenosugar **1c** could have been transformed into arsenosugar **1a** during the exposure experiment, a process that is not unproven.[23] The most significant result from this study is that it seems unlikely that complex arsenic compounds, such as arsenosugars, are produced by the macroalga *F. gardneri* when exposed to As(V). This same conclusion was reached from the study on the unicellular alga *P. peniculus*.[7] Thus, the process of how the arsenosugars accumulate in the algal cells is yet to be elucidated.

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