APPLIED ORGANOMETALLIC CHEMISTRY, VOL. 8, 313–324 (1994)

Bioaccumulation and Excretion of Arsenic Compounds by a Marine Unicellular Alga, *Polyphysa peniculus*

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Polyphysa peniculus **was grown in artificial seawater in the presence of arsenate, arsenite, monomethylarsonate and dimethylarsinic acid. The separation and identification of some of the arsenic species produced in the cells as well as in the growth medium were achieved by using hydride generation–gas chromatography–atomic absorption spectrometry methodology. Arsenite and dimethylarsinate were detected following incubation with arsenate. When the alga was treated with arsenite, dimethylarsinate was the major metabolite in the cells and in the growth medium; trace amounts of monomethylarsonate were also detected in the cells. With monomethylarsonate as a substrate, the metabolite is dimethylarsinate.** ***Polyphysa peniculus*** **did not metabolize dimethylarsinic acid when it was used as a substrate. Significant amounts of more complex arsenic species, such as arsenosugars, were not observed in the cells or medium on the evidence of flow injection–microwave digestion–hydride generation–atomic absorption spectrometry methodology. Transfer of the exposed cells to fresh medium caused release of most cell-associated arsenicals to the surrounding environment.**

Keywords: Arsenic species, arsenic uptake, arsenic methylation, ***Polyphysa peniculus*****, arsenic bioaccumulation, non-reducible arsenicals**

INTRODUCTION

Algae, located at the bottom of the aquatic food web, have often been the subject of arsenic metabolic studies because of their ecological and nutritional importance. Through bioaccumulation, algae exhibit concentrations of arsenic which are much higher than those of the surrounding water.[1,2] Studies of the interaction of marine algae with arsenicals are relevant because arsenic compounds produced by algae are generally believed to be the source of the arsenic compounds found in marine animals, although it is not well established how and when these transformations take place.

In seawater, arsenate [As(V)] is the predominant arsenic species and is present at approximately 1.0–2.0 ppb.[3–7] However, significant amounts of arsenite [As(III)], monomethylarsenicals [probably monomethylarsonic acid (MMAA)], and dimethylarsenicals [probably dimethylarsinic acid (DMAA)], have also been observed and are believed to be a consequence of the biological activity of marine algae.[6,7] It is believed that arsenate is readily taken up by algae from the water by using phosphate transport systems located in the algal cell membranes.[8–10] Once inside the algal cell, arsenate can remain as such or it can be reduced, or transformed to a variety of organic arsenic compounds.[8–11]

Most of the arsenic in marine macroalgae exists in complex forms and a variety of arsenosugar derivatives have been isolated and characterized.[12–15] The first successful isolation of arsenosugars from the marine alga *Ecklonia radiata* was achieved by Edmonds and Francesconi.[12,13] The arsenicals were extracted with methanol, subsequently isolated by column purification and preparative TLC, and characterized by microanalysis and spectroscopic techniques, especially ^{1}H and ^{13}C NMR spectroscopy.

Edmonds and Francesconi[16] proposed a pathway for the biotransformation of arsenate by marine algae (Scheme 1). The initial steps follow those of the mechanism outlined by Challenger (Scheme 2).[17,18] However, there is a difference in the final steps where the adenosyl group of the methylating agent *S*-adenosylmethionine (SAM) is transferred to the arsenic atom of dimethyl-

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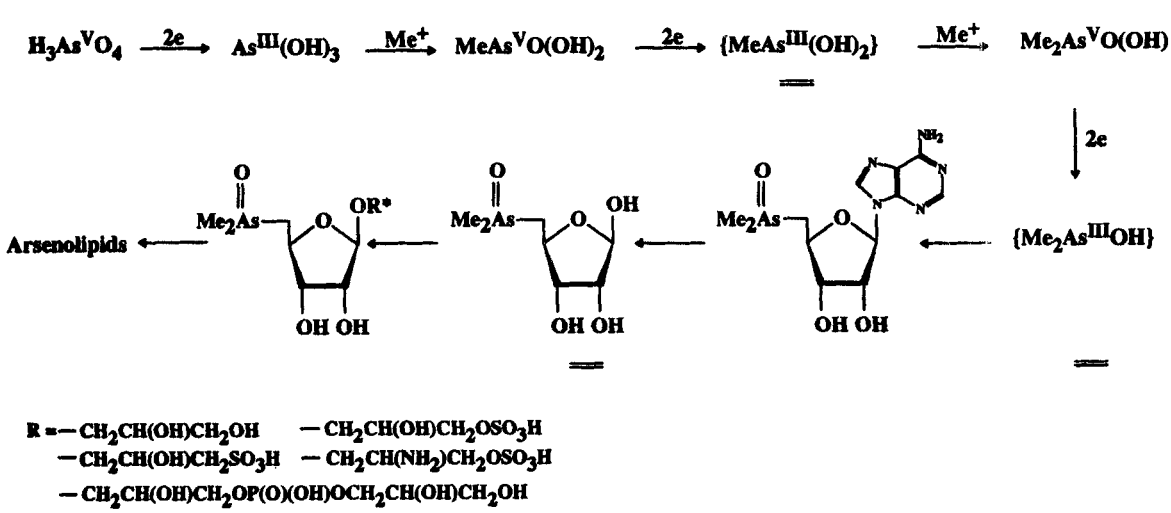
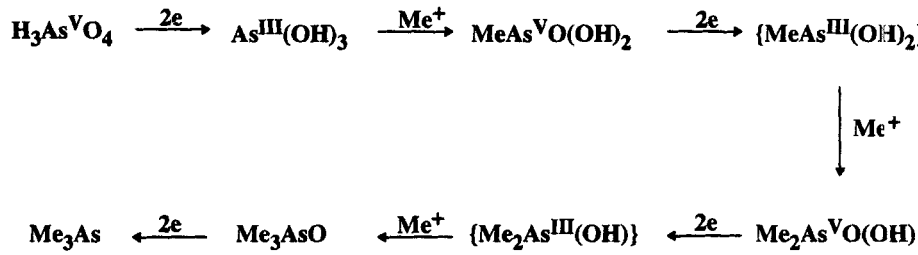
CCC 0268–2605/94/040313–12

Received 28 February 1994
Accepted 29 March 1994



Scheme 1 Proposed mechanism for transformation of arsenic compounds in marine algae.[16] Unidentified compounds are double underlined.



Scheme 2 Challenger's mechanism for the methylation of arsenic. The intermediates in {} are unknown as monomeric species. They are formulated as $(CH_3AsO)_n$ and $(CH_3As)_2O$, respectively, when prepared by conventional methods.

arsinate to form arsenosugars and arsenolipids. In order to support this proposed mechanism a number of questions need to be addressed, such as:

(1) Does this mechanism apply to all species of marine micro- and macro-algae? and

(2) Does the methylation of arsenic follow Challenger's proposed mechanism and involve the utilization of SAM as a donor of both methyl and adenosyl group?

We believe that controlled culture experiments may provide some answers.

There is only a limited number of reports that discuss the biotransformation of arsenicals by macroalgae grown in culture medium. The marine macroalgae *Fucus spiralis* (L) and *Ascophyllum nodosum* (L) assimilate arsenate to produce both water-soluble and lipid-soluble organoarsenicals,[19,20] although these compounds were not positively identified as arsenosugars. Sanders and Windom[7] used arsenate, arsenite and dimethylarsinate as substrates for cultures of a marine macroalga *Valonia macrophysa*. An increase in methylated arsenicals was detected in the cells, suggesting that more complex arsenic compounds were produced.

Studies on arsenic biotransformation in marine phytoplankton have not shown any strong evidence for the production of arsenosugars. Investigations on arsenate uptake by marine unicellular algae, mainly phytoplankton, have established that the arsenic is distributed between the MeOH–$CHCl_3$-extractable fraction and insoluble components of the cell;[7,11,21–24] however, no individual arsenic compounds have been positively identified apart from arsenite, MMAA and DMAA. Cooney and co-workers[21,25] have reported that trimethylarsoniolactate was produced by a group of unicellular algae, but this conclusion was later retracted in favor of arsenosugars.[26,27] Andreae *et al.*[11] showed that four classes of marine phytoplankton (the diatom *Skeletonema costatum*, coccolithophorid

Cricosphaera carteri, dinoflagellate *Gonyaulax polyedra*, and green alga *Platymonas cf suecica*) can transform arsenate to arsenite and subsequently to MMAA and DMAA. An increase in methylated arsenicals was observed after base digestion of the aqueous extracts, suggesting the presence of more complex arsenic compounds. The complexity of arsenic biotransformation in marine phytoplankton has been revealed in studies of arsenate uptake by the unicellular alga *Dunaliella tertiolecta*.[22,24] Wrench and Addison found that three arsenolipids, which are not related to arsenosugars, were produced when *D. tertiolecta* was treated with 0.2 MBq of [^{74}As]arsenate for 45 min.[22] They suggested that one of these is a complex between arsenite and phosphatidylinositol, the second a neutral or zwitterionic complex between arsenite and a glycolipid, and the third an unidentified phospholipid-like arsenical. However, other workers[24] have reported that about 47% of the arsenic in the same alga is present as a phospolipid (*O*-phosphatidyl trimethylarsoniumlactate, later reassigned as an arsenosugar derivative[16]) and as an unknown lipid (48% of total arsenic in cells) following exposure for 48 h to [^{74}As]arsenate. The real situation is not clear.

We have chosen to study *Polyphysa peniculus*, a unicellular marine alga which has been cultivated in our laboratories in artificial seawater under sterile conditions for more than two decades. Its cells are unusually large (4–5 cm in length, 0.4 mm in diameter), but, like many phytoplankton, it is a unicellular alga (Chlorophyta). In this paper we report on the effect of adding arsenate, arsenite, monomethylarsonate and dimethylarsinic acid to *P. peniculus* in artificial seawater. Arsenic accumulation, methylation and excretion by the alga were examined by using graphite furnace–atomic absorption spectrometry (GF AA) and hydride generation–gas chromatography–atomic absorption spectrometry (HG GC AA) methodology. The inability of *P. peniculus* to synthesize significant amounts of complex water- and lipid-soluble arsenic compounds was also established by using flow injection–microwave digestion–hydride generation–atomic absorption spectrometry methodology.

In a previous publication we reported on the effect of adding L-[$^{2}H_3$-*methyl*]methionine and arsenate to artificial seawater containing *P. peniculus*.[28] The arsenic metabolites excreted by the alga in the growth medium, principally DMAA, were identified by using hydride generation–gas chromatography–mass spectrometry (HG GC MS). This technique provided conclusive evidence of CD_3 incorporation from L-[$^{2}H_3$ – *methyl*]methionine in the dimethylarsenic species produced by *P. peniculus*, and supports the hypothesis that *S*-adenosylmethionine is the biological methyl donor.

EXPERIMENTAL

Algal cultures

Polyphysa peniculus (Dasycladales, Chlorophyta), a marine alga closely related to the better known genus *Acetabularia*, is sometimes known as *A. cliftonii* and goes by several other synonyms.[29] The *P. peniculus* culture used in this work has been maintained in sterile artificial seawater (Shephard's medium)[30] for more than two decades. The culture has not been rendered axenic, but has been maintained and handled by using sterile techniques, and treated with antibiotics if bacterial infection arose.

Reagents

Arsenic standards were freshly prepared by serial dilutions from stock solutions (1000 ppm of elemental arsenic) of the following compounds: sodium arsenate [$Na_2HAsO_4.7H_2O$] and sodium arsenite [$NaAsO_2$] from Baker Chemical Co.; disodium methylarsonate [$CH_3AsO_3Na_2.6H_2O$] and dimethylarsinic acid [$(CH_3)_2AsO(OH)$] from Alfa Inorganics.

Solutions of 1.5% (w/v) potassium persulphate in 0.1% (w/v) NaOH, 1 M HCl, 4 M CH_3COOH, and 2% (w/v) $NaBH_4$ in 0.1% (w/v) NaOH were freshly made daily.

Instrumentation

Graphite furnace–atomic absorption spectrometry

The total amount of arsenic was measured using a Varian Techtron Model AA1275 atomic absorption spectrometer equipped with a Varian Spectra AA hollow-cathode lamp operating at 8 mA, a deuterium background corrector, and a Hewlett–Packard 82905A printer. The monochromator was set at 193.7 nm, and the slit width at 1 nm.

Table 1 Furnace operating parameters for the determination of arsenic in cell extracts

Step	Temperature (°C)	Time (s)	Gas flow ($l\ min^{-1}$)	Function
1	70	5	3.0	Dry
2	120	30	3.0	Dry
3	1200	20	3.0	Ash
4	1200	1.0	0	Ash
5	2300	1.0	0	Atomize
6	2300	1.0	0	Atomize
7	2300	2.0	3.0	Clean

For GF AA analysis, the furnace operating parameters have to be optimized for different types of samples to remove the maximum amount of matrix material and achieve the best analytical sensitivity. The GTA-95 accessory can be used to program operating parameters such as temperature, time and gas flow by using the absorbance signal during the atomization stage. The optimized program for the maximum absorbance signal of arsenic in samples applied is shown in Table 1. Argon was used as the purge gas.

The standard addition technique was used in our studies. The sample, and the arsenic standard solutions, were injected (volume 5–20 µl) into the graphite furnace by the automatic delivery system of the GTA 95 accessory. Each solution was mixed with 20 µl of palladium modifier, prepared as palladium nitrate (100 ppm) in citric acid (2% w/v), before the furnace was electrically heated.

Hydride generation–gas chromatography–atomic absorption spectrometry

A hydride generation system was used for arsine production and collection as previously described.[31] After the sample introduction was completed, the arsines trapped in liquid nitrogen were volatilized when the hydride trap was warmed in a water bath (70 °C). By using a Hewlett–Packard Model 5830A gas chromatograph with a pre-set temperature program, the arsines were then separated on a Porapak-PS column (mesh 80–100), atomized by means of a hydrogen–air flame in a quartz cuvette, and detected by using a Jarrell–Ash model 810 atomic absorption spectrometer equipped with a Varian Spectra arsenic hollow-cathode lamp set at 10 mA. The monochromator was set at 193.7 nm, and the slit width at 1 nm. Absorbances were recorded as peak areas on a Hewlett–Packard 3390A integrator.

Flow injection–microwave digestion–hydride generation–atomic absorption spectrometry

The flow injection–microwave digestion–HG AA system described by Le *et al.*[32,33] was used to determine non-hydride forming, 'hidden' arsenic compounds. The evolved arsines were carried into an open-ended T-shaped quartz tube (11.5 cm long × 0.8 cm i.d.) which was mounted in the flame of a Varian Model AA-1275 atomic absorption spectrometer equipped with a standard Varian air–acetylene flame atomizer. The signals were recorded on a Hewlett–Packard 3390A integrator.

Experimental procedure

Antibiotic-treated alga (approximately 0.6–1 g dry weight) was added to sterile Erlenmeyer flasks (2 l) each containing 1 l of sterile Shephard's medium. Sterilized arsenicals were added to the medium separately. Two concentrations, 10 ppm and 0.9 ppm, were employed in our studies. During the growth period the cultures were maintained at 20 °C. Fluorescent lamps that gave 3200 Lux intensity around the flasks were used as the light source, and the light/dark cycle was 16 h:8 h. Once each day the culture was agitated and 10 ml aliquots of the medium were removed and frozen prior to analysis. The alga was harvested on day 7 and thoroughly rinsed with sterile Shephard's medium. A half-portion of the algae was freeze-dried and stored in a freezer for future analysis. The rest of the alga was transferred to a 1 l sterile Erlenmeyer flask containing 500 ml fresh arsenic-free sterile Shephard's medium. The incubation conditions were not changed and the day of transfer is referred to as day 0 of the second cycle. The culture was handled in the same manner as in the first cycle, the medium was sampled each day, and the alga was again harvested after seven days of incubation, rinsed, freeze-dried and stored in a freezer.

The total amount of arsenic in the cells was determined following acid digestion. Freeze-dried cells (50–100 mg) were dissolved in 1 ml of concentrated nitric acid, left overnight, then boiled with 1 ml of hydrogen peroxide for 5–10 min prior to analysis. The resultant pale-yellow transparent solution was neutralized with NaOH solution, diluted to an appropriate volume, and subjected to GF AA analysis. The amount of arsenic was quantified by using a standard addition technique.

Palladium nitrate (100 ppm), prepared in citric acid (2% w/v), was used as a modifier.

For the arsenic speciation analysis, freeze-dried algal cells (0.1–0.2 g) were weighed and transferred into an Erlenmeyer flask (250 ml) containing 30 ml of mixed solvent, $CHCl_3$–MeOH–H_2O (1:1:1). The mixture was sonicated for 2 h and then stoppered with a rubber plug and left on a mechanical shaker for 24 h. It was then centrifuged and the residue was re-extracted with 10 ml of the mixed solvent for another 24 h. The extracts were combined and centrifuged to separate the aqueous fraction from the organic fraction. The colorless aqueous layer was kept at −4 °C prior to analysis. The organic extract and the residue were air-dried, digested in 4 ml of 2 M NaOH in a water bath at 95 °C for 3 h, then neutralized with 6 M hydrochloric acid prior to analysis.

The HG GC AA system was used to analyze hydride-forming arsenicals in each of the three fractions of the cell extracts as well as in the growth medium. The flow injection–microwave digestion–HG AA technique was used to detect the existence of 'hidden' arsenic in the cells.

RESULTS

The accumulation of arsenicals in cells of *Polyphysa peniculus*

We employed two arsenic concentrations, 10 ppm and 0.9 ppm, in this study. The total amount of arsenic accumulated in *P. peniculus* was determined by using GF AA. The results are presented in Table 2. With the exception of arsenate, algae exposed to 10 ppm arsenicals accumulated more arsenic in their cells than those exposed to 0.9 ppm arsenicals. In the presence of MMAA, the arsenic accumulation in the cells was very low.

After seven days of exposure to arsenicals, the alga was transferred to an arsenic-free medium, and after another sven days only small amounts of arsenic compounds were retained in the cells (Table 2).

Table 2 Total amount of arsenic in cells of *P. peniculus* determined by using GF AA ($\mu g\,g^{-1}$, dry weight)

Arsenic exposed	Cells treated with 10 ppm arsenicals		Cells treated with 0.9 ppm arsenicals	
	A[a]	B[b]	A[a]	B[b]
Arsenate	36.0±2.5[c]	11.5±0.7	43.3±2.4	7.7±0.5
Arsenite	52.7±4.2	5.6±0.5	17.4±1.2	3.5±0.3
MMAA	18.3±1.1	4.4±0.3	3.1+0.2	Trace
DMAA	34.8±1.9	5.3±0.4	25.1±1.8	3.1±0.3

[a] Seven days after the cells were exposed to arsenicals.
[b] Seven days after the cells were transferred to fresh media.
[c] Large fraction of dead cells was present within two days of incubation

Arsenic speciation analysis in cells of *Polyphysa peniculus*

Water–methanol extracts

The hydride-forming arsenicals—arsenate, arsenite, MMAA and DMAA—in aqueous extracts were detected as AsH_3, $MeAsH_2$ and Me_2AsH, respectively, by using HG GC AA. Trimethylarsine oxide, which would have been detected as trimethylarsine, was not found in any of the samples. These arsenic speciation results are presented in Table 3. Not unexpectedly, the higher the external arsenic concentration applied, the higher the total amount of hydride-forming arsenicals was in the alga.

A large amount of inorganic arsenic, mainly arsenite (25.1 ppm), was detected in cells incubated with 10 ppm arsenate. Small amounts of DMAA (1.9 ppm) were also found in this sample. When *P. peniculus* was treated with 0.9 ppm arsenate, the amount of DMAA found in the aqueous extract of the alga is as high as 21.7 ppm and is about 45% of total hydride-forming arsenicals found in the aqueous extract. Substantial amounts of arsenite were also detected in both samples. Neither MMAA nor arsenate was found in cells incubated with 0.9 ppm arsenate. After the alga was transferred to an arsenic-free medium and incubated for seven days, only small amounts of arsenate were detected.

Four arsenicals, principally arsenite, but also arsenate, MMAA and DMAA, were found in the aqueous extracts after the alga was treated for seven days with 10 ppm and 0.9 ppm arsenite. The percentage of MMAA in both samples was about 7%. The percentage of DMAA in cells exposed to 0.9 ppm arsenite was 37% of total aqueous hydride-forming arsenicals. Little DMAA was detected when the alga was treated with 10 ppm arsenite. The arsenical content of the alga was greatly reduced after it was transferred to an arsenic-free medium.

Similar amounts of MMAA and DMAA were found in cells incubated with 10 ppm and 0.9 ppm MMAA; however, after the alga was transferred

Table 3 Arsenic distribution in aqueous extracts of the cells determined by HG GC AA ($\mu g\ g^{-1}$, dry weight)

Arsenic exposed	Arsenic species found in cells	Cells treated with 10 ppm arsenicals		Cells treated with 0.9 ppm arsenicals	
		A[a]	B[b]	A[a]	B[b]
Arsenate	Arsenate	12.8±0.8	9.7±0.7	0	5.1±0.3
	Arsenite	25.1±1.3	0	26.3±1.6	0
	MMAA	0	0	0	0
	DMAA	1.9±0.2	0	21.7±2.0	0
	Total	39.8	9.7	48.0	5.1
Arsenite	Arsenate	8.0±0.5	0	1.8±0.1	0
	Arsenite	37.7±2.5	2.3±0.2	6.5±0.4	1.2±0.1
	MMAA	3.1±0.2	0.4±0.03	1.0±0.1	0
	DMAA	0.8±0.1	0.8±0.08	5.4±0.5	0.8±0.1
	Total	49.6	3.5	14.7	2.0
MMAA	Arsenate	0	0.3±0.03	0	0
	Arsenite	0	0.9±0.07	0	0
	MMAA	5.3±0.3	0.8±0.06	1.4±0.1	0
	DMAA	6.5±0.6	2.7±0.3	1.6±0.2	0
	Total	11.8	4.7	3.0	0
DMAA	Arsenate	1.6±0.1	0	0	0
	Arsenite	0	0	0	0
	MMAA	0.6±0.04	0.1±0.01	0.7±0.04	0
	DMAA	28.1±2.5	3.6±0.3	20.4±1.8	2.5±0.3
	Total	30.3	3.7	21.1	2.5

[a] Seven days after the cells were exposed to arsenicals
[b] Seven days after the cells were transferred to fresh media

to an arsenic-free medium, small amounts of arsenicals remained in cells originally treated with 10 ppm MMAA, but none was found in the cells exposed to 0.9 ppm MMAA.

Arsenic speciation analysis of cells exposed to DMAA shows that the accumulated arsenic exists mainly as DMAA. Trace amounts of MMAA were also detected in the aqueous extracts. Most of the accumulated arsenic was discharged from the cells after the alga was transferred to an arsenic-free medium.

In order to determine whether 'hidden' arsenic, possibly arsenosugars, existed in the aqueous extracts of the algal cells, a flow injection–microwave digestion–HG AA technique was applied. In this methodology, the 'hidden' arsenic species are decomposed and oxidized by potassium persulphate to arsenate with the aid of microwave radiation.[32,33] The product, arsenate, can be reduced easily to arsine. Thus, by comparison of the arsine absorbance before and after microwave-assisted digestion, the amount of total 'hidden' arsenic in a sample can be calculated. The results are shown in Table 4: no significant differences are apparent in the amounts of arsenic detected before and after microwave-assisted digestion. This suggests that only small amounts, if any, of 'hidden' arsenic species such as arsenosugars were produced and accumulated by the cells during growth.

Chloroform extracts

Chloroform fractions from the original $CHCl_3$–MeOH–H_2O cell extracts were air-dried,

Table 4 Arsenic distribution in aqueous extracts of the cells harvested from arsenic-enriched media before and after microwave digestion ($\mu g\ g^{-1}$, dry weight)

Arsenic exposed	Cells treated with 10 ppm arsenicals		Cells treated with 0.9 ppm arsenicals	
	Before digestion	After digestion	Before digestion	After digestion
Arsenate	37.3±2.6	38.7±2.7	45.4±3.2	47.1±3.8
Arsenite	48.7±2.9	50.6±4.1	15.9±0.8	14.7±0.9
MMAA	12.7±0.8	11.5±1.1	3.8±0.3	3.4±0.3
DMAA	32.2±2.1	33.1±2.2	20.4±1.2	20.0±1.8

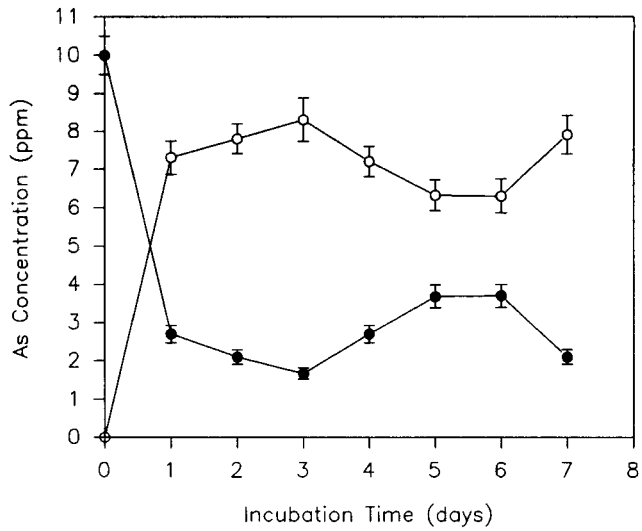


Figure 1 The charge of arsenic species in the growth medium with incubation time. The growth medium was enriched with 10 ppm arsenate before incubation. ○, Arsenite; ●, arsenate.

digested with 2 M NaOH, neutralized with concentrated HCl, and analyzed by HG GC AA. Arsenosugars, if present, would be decomposed to DMAA under these conditions and would be detected as dimethylartsine by HG GC AA. No arsenicals were detected in these $CHCl_3$ extracts of cells exposed to arsenate and arsenite. Only trace amounts of dimethylarsenic compounds were detected in cells exposed to MMAA and DMAA. Arsenic compounds were not found in the $CHCl_3$ extracts following transfer of the cells to arsenic-free media.

The flow injection–microwave digestion–HG AA technique was used to detect whether any 'hidden' arsenic compounds, which may not have been hydrolyzed by NaOH, were present in the organic extracts. There was no significant difference in the amount of hydride-forming arsenicals present before and after microwave-assisted digestion.

Insoluble residues

The residues were digested with 2 M NaOH, and were subsequently analyzed by HG GC AA. Trace amounts of monomethylarsenic and dimethylarsenic compounds were detected in cells exposed to arsenite, MMAA and DMAA. The flow injection–microwave digestion–HG AA technique did not show the presence of 'hidden' arsenic species in these digested samples.

Arsenic speciation analysis in the growth media of *Polyphysa peniculus*

Arsenic speciation analysis of the growth media was carried out by using HG GC AA. The change in the chemical form of arsenic was very dramatic in the media enriched with arsenate. When the alga was exposed to 10 ppm arsenate, more than 70% of the substrate was reduced to arsenite after one day of incubation. The concentration of arsenate and arsenite remained relatively constant until the alga was harvested (Fig. 1). Reduction of arsenate to arsenite was also observed in the growth medium of *P. peniculus* spiked with 0.9 ppm arsenate, but the reaction was slower (Fig. 2). The concentration of arsenite reached a steady state after two days of incubation. In addition to arsenate and arsenite, DMAA was detected in this culture medium on day 3. The quantity of DMAA increased slowly with time to about 0.15 ppm at the time when the experiment was terminated. No MMAA was found in either of the experiments mentioned above.

The possibility of non-metabolic reduction of arsenate by *P. peniculus* cells was studied by exposure of heat-treated or 4% formalin-treated cells to arsenate-enriched medium (4% formalin is a good reagent for killing cells but causes only minimum damage to tissue integrity[19,20]). There was no indication of arsenate reduction in these two experiments.

No MMAA was detected in the growth medium when 10 ppm or 0.9 ppm arsenite was used as a substrate. When the alga was treated with 10 ppm arsenite, about 90% of arsenite in the medium remained unchanged throughout the incubation period. Arsenate was detected in this medium after two days of incubation, but in a low concentration. After incubation of *P. peniculus* with 0.9 ppm arsenite for one day, small amounts of DMAA and arsenate were detected in the growth medium (Fig. 3). The concentration of DMAA increased to 0.08 ppm after five days of incubation.

Arsenic speciation analysis showed that the chemical form was not altered by the alga when MMAA and DMAA were used as substrates. Only minor changes in concentration were observed.

Arsenic efflux studies

After the cells had been exposed to arsenicals for seven days, they were washed and transferred to fresh arsenic-free medium, and incubated for a further seven days. Arsenic speciation analysis of this 'fresh' medium was carried out by using HG GC AA. Figure 4 shows that the accumulated arsenicals were rapidly excreted to the medium by the cells, usually within 1–2 days. The difference in the amounts of hydride-forming arsenicals in the cells after the first seven days and after 14 days was compared with the amounts of hydride-forming arsenicals released in the medium during the second seven-day period. The results are, in general, in agreement, indicating that 'hidden' arsenic species were not produced during this period. This conclusion is reinforced by the results obtained by using the flow injection–microwave digestion–HG AA technique.

The amount of DMAA found in the medium after 14 days from cells treated with either 10 ppm arsenate or 10 ppm arsenite was higher than that detected in the cells before the transfer. Consequently, the amount of inorganic arsenicals, mainly arsenite, was greatly decreased. In contrast, in the 0.9 ppm arsenate or 0.9 ppm arsenite experiments, a decrease in the amount of DMAA and an increase in the amount of inorganic arsenicals were observed, indicating that a demethylation process took place. After the cells which had been exposed to MMAA were transferred to an arsenic-free medium, the endocellular MMAA and DMAA were excreted to the medium. An increase in the concentration of DMAA and a decrease in the concentration of MMAA were also observed after 1–2 days.

DISCUSSION

Edmonds and Francesconi[16] have proposed the biotransformation pathway (shown in Scheme 1) to explain the production of arsenosugars found in marine macroalgae. In previous work we established that SAM is the methylating agent used for

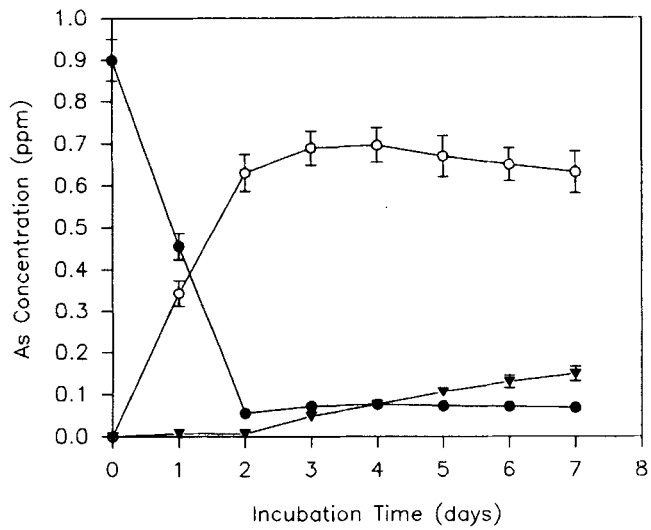


Figure 2 The change of arsenic species in the growth medium with incubation time. The growth medium was enriched with 0.9 ppm arsenate before incubation. ○, Arsenite; ●, arsenate; ▼, DMAA.

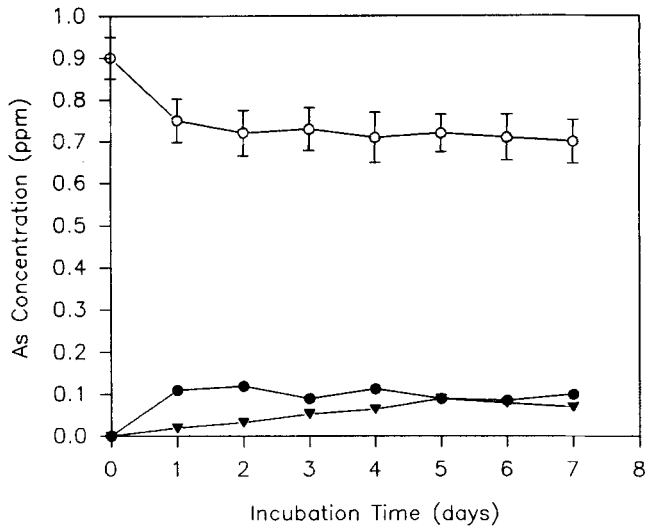


Figure 3 The change of arsenic species in the growth medium with incubation time. The growth medium was enriched with 0.9 ppm arsenite before incubation. ○, Arsenite; ●, arsenate; ▼, DMAA.

the production of the dimethylarsinate found in the growth medium following exposure of *P. peniculus* to arsenate.[28] In the present investigation, we demonstrate that arsenate, arsenite and MMAA are methylated principally to DMAA. No trimethylarsenical species are found. Exposure of *P. peniculus* to arsenate yields arsenite (in cells and in the media) and DMAA (in cells and in the medium spiked with 0.9 ppm arsenate). When the alga is treated with arsenite, MMAA and DMAA are detected in the cells; the metabolite DMAA can also be found in the growth medium spiked with 0.9 ppm arsenite. The substrate MMAA is biotransformed by *P. peniculus* to produce DMAA in the cells. When DMAA is used as a substrate, trace amounts of the demethylation product MMAA are detected in the cells. When *P. peniculus* is transferred from arsenic-enriched medium to arsenic-free medium, the accumulated arsenicals in the algal cells are excreted into the 'fresh' medium. Biotransformation of arsenic, including methylation and demethylation, also takes place in this medium.

The most significant result from these studies is that no complex arsenic compounds, such as arsenosugars, are produced by *P. peniculus*. When the concentration of arsenicals varies from as high as 10 ppm to as low as 20 ppb (in the second cycle), there are no 'hidden' arsenicals in either the cells or the media as judged by the flow injection–microwave digestion–HG AA methodology which has been shown to be very effective in decomposing and converting complex arsenicals to hydride-forming species.[32,33] Results obtained by using flow injection–microwave digestion–HG AA are also in agreement with those obtained by using HG GC AA for speciation. It seems that the alga *P. peniculus* follows the microbial biomethylation pathway proposed by Challenger (Scheme 2) for the microbial process. In the case of *P. peniculus*, DMAA is the end product of this biomethylation. The reduction of arsenate to arsenite is proposed to be the first step towards methylation,[17,18] and our results are in agreement: most arsenate in the medium is reduced to arsenite by *P. peniculus* prior to the detection of DMAA in the medium. Arsenate reduction to arsenite proceeds rapidly and most of the arsenate is reduced within 1–2 days. It is possible that arsenate, being chemically similar to phosphate, is readily taken up by algae[8–10] and then reduced by thiols or dithiols as a detoxification process.[3] The reduction may be enzymic or it could occur chemically by reaction of arsenate with an enzymatically produced reducing agent.[34] Regardless of which mechanism is correct, the results show that it is necesary to have a biologically intact organism capable of generating the appropriate reducing conditions, because non-metabolizing, enzymically inactive cells do not reduce arsenate to arsenite in the growth medium.

Compared with cells exposed to 0.9 ppm arsenate, the accumulation of arsenic is much lower in cells from media containing 0.9 ppm arsenite,

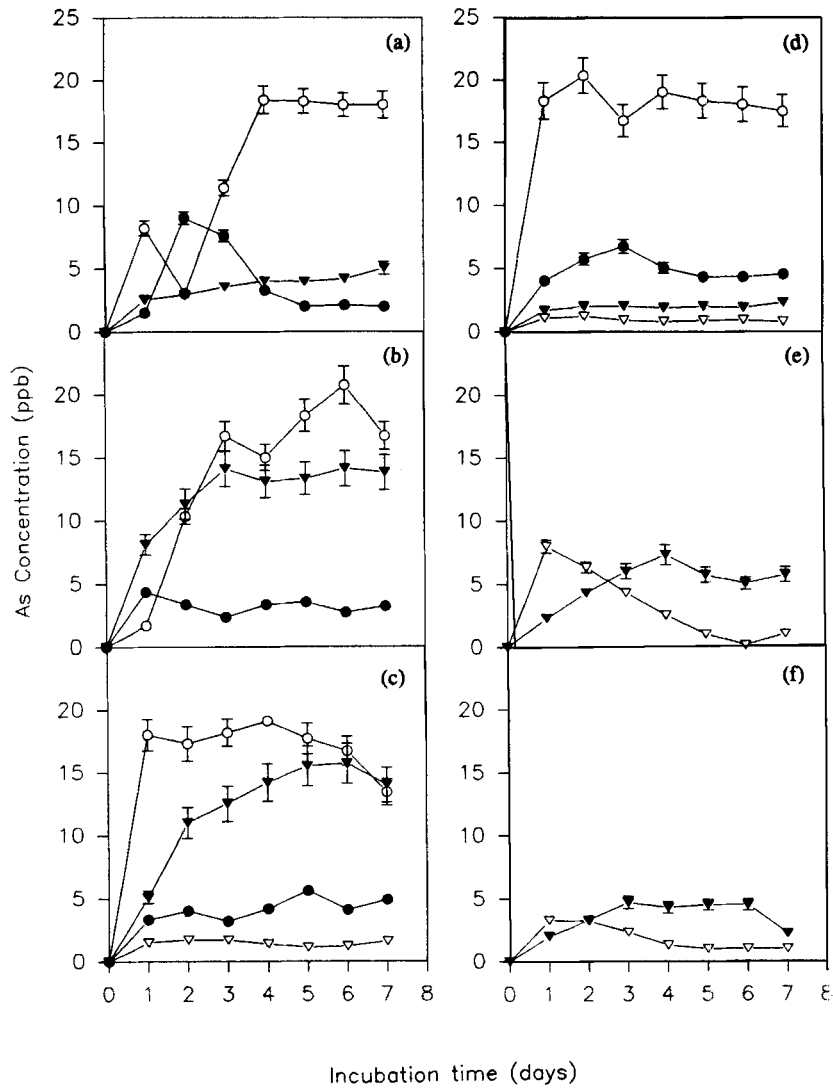
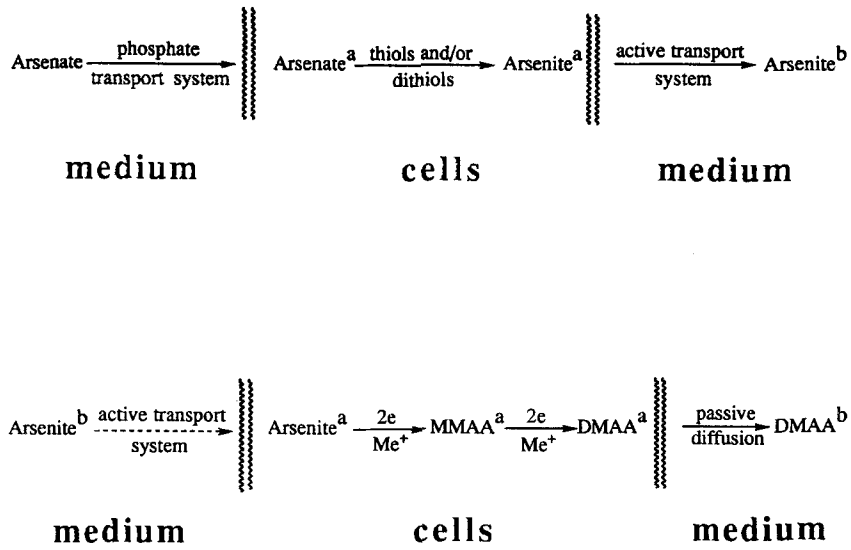


Figure 4 The change of arsenic species in the medium with incubation time after the transfer of cells that had been previously grown in (a) 10 ppm arsenate; (b) 0.9 ppm arsenate; (c) 10 ppm arsenite; (d) 0.9 ppm arsenite; (e) 10 ppm MMAA; (f) 0.9 ppm MMAA. ○, Arsenite; ●, arsenate; ▽, MMAA; ▼, DMAA.

MMAA, or DMAA; entry of MMAA and DMAA into the cell probably occurs by means of passive diffusion.[3] In particular, the uptake of MMAA seems to be inefficient. This result agrees with that reported by Cullen and Nelson[35] that MMAA has a much lower diffusion coefficient than DMAA. This study employed liposomes as model membranes and showed that the permeability coefficient of MMAA is 10 times lower than that of DMAA.

Arsenate at high concentration (10 ppm) is more toxic to *P. peniculus*, as indicated by the large percentage of dead cells found within two days of initiating the incubation. It is possible that high concentrations of arsenate inactivate the phosphate transport system and interfere with oxidative phosphorylation.[36–38] Certainly, high concentrations of arsenate affect the arsenic-accumulating ability of the alga, as shown in Table 2.

A high concentration (10 ppm) of arsenate or arsenite also seems to inhibit the biomethylating ability of *P. peniculus*. As shown in Table 3, only a small amount of methylated arsenicals is found in the cells after seven days of growth. In a lower arsenate or arsenite concentration (0.9 ppm), *P.*



Scheme 3 Proposed model for biomethylation of arsenate in marine alga *P. peniculus*. [a] Endocellular arsenicals; [b] extracellular arsenicals.

peniculus can efficiently methylate inorganic arsenic to DMAA which can either be excreted into the medium or kept in the cells. When the alga is transferred from a hostile environment, such as medium containing 10 ppm arsenate or arsenite, to a fresh arsenic-free environment, the biomethylating ability of *P. peniculus* seems to be restored. This was indicated by an increase in the amount of DMA in the new medium compared with the amount of DMAA in the cells harvested at the end of the first cycle.

It is not surprising that little or no MMAA was detected when the alga was treated with arsenate and arsenite. Cullen *et al.*[39] reported that only traces of MMAA were produced from arsenate by broken-cell homogenates of *Apiotrichum humicola* (previously known as *Candida humicola*) and that MMAA is the least transformed arsenical substrate. They tentatively concluded that MMAA is not a free intermediate in Challenger's arsenate-to-trimethylarsine pathway. Our recent study of whole-cultures of *A. humicola* also showed that only limited amounts of MMAA are found as an extracellular arsenic metabolite in the growth medium.[31] As mentioned previously, MMAA has a very low diffusion coefficient, and the cells may prefer to metabolize the endocellular MMAA to DMAA rather than excreting MMAA into the growth medium. The DMAA thus produced has a higher permeability coefficient, and can be excreted by the cells to the growth medium. This surmise seems to be consistent with what we have observed. Work on arsenic speciation in seawater showed that arsenite and DMAA are the main arsenical products of natural phytoplankton blooms; MMAA has not been detected in high concentrations.[40-42]

A variety of marine phytoplankton take up arsenate from seawater and produce arsenite, MMAA and DMAA,[7,11,24,43] and release them into the surrounding media.[7,11,43] The efflux studies of *P. peniculus* demonstrate that the excretion of arsenicals is a rapid process. No 'hidden' arsenic species were detected in the cells or in the medium indicating that *P. peniculus* does not produce arsenosugars when exposed to low concentrations of arsenic species. We suggest that the fast excretion of the reduced and/or methylated arsenic compounds to the media reduces the requirement for further detoxification processes. It seems that the cells interact not only with endocellular arsenicals but also with the excreted arsenicals, as indicated by the differences in the amount of individual arsenical species before and after the transfer. The biotransformation, the excretion, and the re-uptake of the excreted arsenicals may take place simultaneously. These interactions seem to reach steady states after 2–3 days in the 'fresh' media.

In order to put these results together, we now propose a model for the methylation of arsenate by cells of *P. peniculus* (Scheme 3). First, algal cells take up arsenate from the medium via the phosphate transport system, reduce the arsenate

to arsenite inside the cells by using thiols and/or dithiols, and excrete most of the arsenite into the growth medium by means of an active transport system. Second, endocellular arsenite is methylated to MMAA by using SAM; however, because of its low passive diffusion coefficient, the endocellular MMAA is not excreted to the growth medium. Consequently, the MMAA remains in the cells where it is more likely to be reduced and further methylated to DMAA. This arsenical, which has a greater diffusion coefficient, is then passively diffused into the growth medium. This model explains many of the results obtained in the present investigations. However, in reality, the biotransformation process is probably much more complex. For example, the uptake/excretion equilibrium between endocellular and extracellular arsenicals, and the cleavage of As–C bonds, may also be involved in the metabolic process.

Acknowledgements We thank X. C. Le for his help in operating the flow injection–microwave digestion–HG AA system, Dr B. R. Green for the original supply of the algal culture, Mr G. Donaldson for culture maintenance, and the Natural Sciences and Engineering Research Council of Canada for financial support.

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