APPLIED ORGANOMETALLIC CHEMISTRY
Appl. Organometal. Chem. 2003; **17**: 669–674
Published online in Wiley InterScience (www.interscience.wiley.com). DOI:10.1002/aoc.498

Biotransformation of arsenate to arsenosugars by *Chlorella vulgaris*

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Received 21 February 2003; Accepted 17 April 2003

***Chlorella vulgaris* was cultivated in a growth medium containing arsenate concentration of <0.01, 10, 100 and 1000 mg l^{-1}. Illumination was carried out in 12 h cycles for 5 days. The health status of the culture was monitored by continuous pH and dissolved oxygen (DO) readings. Destructive sampling was used for the determination of biomass, chlorophyll, total arsenic and arsenic species. The chlorophyll *a* content, the DO and pH cycles were not significantly different for the different arsenate concentrations in the culture. In contrast, biomass production was significantly ($p < 0.05$) increased for the arsenic(V) treatment at 1000 mg l^{-1} compared with 100 mg l^{-1}.**

The arsenic concentration in the algae increased with the arsenate concentration in the culture. However, the bioconcentration factor decreased a hundred-fold with increase of arsenate from the background level to 1000 mg l^{-1}. The arsenic species were identified by using strong anion-exchange high-performance liquid chromatography–inductively coupled plasma mass spectrometry analysis after methanol/water (1:1) extraction. The majority (87–100%) of the extractable arsenic was still arsenate; arsenite was found to be between 1 and 6% of total extractable arsenic in the algae. In addition to dimethylarsinic acid, one unknown arsenical (almost co-eluting with methylarsonic acid) and three different arsenosugars have been identified for the first time in *C. vulgaris* growing in a culture containing a mixture of antibiotics and believed to be axenic. The transformation to arsenosugars in the algae is not dependent on the arsenate concentration in the culture and varies between 0.2 and 5% of total accumulated arsenic. Although no microbiological tests for bacterial contamination were made, this study supports the hypothesis that algae, and not associated bacteria, produce the arsenosugars. Copyright © 2003 John Wiley & Sons, Ltd.

KEYWORDS: arsenic; bioaccumulation; alga; *Chlorella vulgaris*; arsenosugars

INTRODUCTION

The current interest in arsenic and algae interactions is due to the importance of algae at the base of the aquatic food chain and their use as fertilizer[1] and also in human[2] and animal nutrition.[3] Freshwater and marine micro and macro algae have been found to take up and bioaccumulate arsenate as a phosphorus analogue during normal metabolism.[4] This has made them suitable as ecological indicators (especially for intermittent pollution), to give an indication of bioavailability and also in possible applications for the process of remediation.[5]

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Algae are thought to use the mechanism of methylation as a method of detoxifying the inorganic arsenic species. Several organo-arsenic compounds are commonly found in a wide range of marine organisms, with arsenobetaine mainly being found in animals and arsenosugars found in algae. For freshwater algae, generally methylarsonic acid (MA(V)), dimethylarsenic acid (DMA(V)) and some trimethylated species were found. Very few studies found arsenosugars. Koch *et al.*[6] detected arsenosugars in small amounts in microbial mats and green algae when investigating arsenic species in a range of freshwater biota.

It has previously been suggested[7] that the formation of arsenosugars in marine algae is a follow-on step from the methylation of inorganic arsenic, as inorganic arsenic is reduced and stepwise methylated in an oxidative methyl transfer from *S*-adenosylmethionine (SAM) to DMA(V). The

production of arsenosugars starts off with DMA(V) retained in the algal cells, which is thought to be reduced to DMA(III) and then oxidized by the addition of the adenosyl group from SAM. This nucleoside then undergoes glycosidation to produce a range of arsenosugars.

Recently published studies have centred on the role of bacteria in the possible production and biodegradation of arsenosugars in the marine environment. Granchinho and coworkers[8,9] found little evidence to support the view that arsenosugars were produced by the macroalga *Fucus gardneri* and an associated fungi *Fusarium oxysporum melonis*. When this alga was grown in artificial seawater and exposed to a high level of arsenate (500 μg l^{-1}) under axenic conditions only a small increase in one arsenosugar was seen, accompanied by a decrease in the concentration of another two arsenosugars. As the total amount of arsenosugars had not increased significantly, it was concluded that it was unlikely that arsenosugars are produced by the algae. Geiszinger *et al*.,[10] however, grew *Fucus serratus* in synthetic seawater with different amounts of arsenic in the form of arsenate. They observed that the seaweed started to die at arsenate concentrations above 50 μg l^{-1}, but produced arsenosugars during the growth phase. Although the *Fucus* was cleaned from any epiphytes, the experiments have not, however, been done under axenic conditions.

In aerated seawater enriched with microbes, arsenosugars (dimethylarsinoylribofuranosides and trimethylarsinoyl ribofuranosides) were found to be unchanged over a period of 10 days (Khokiattiwong *et al*.[11]). Under anaerobic conditions microbes were able to degrade dimethylarsinoylribofuranosides to dimethylarsinoylethanol.[12]

The aim of this study was to test if *Chlorella vulgaris*, a freshwater alga, is able to accumulate arsenic and transform the inorganic arsenic species into arsenosugars at different arsenate concentrations.

EXPERIMENTAL

Chemicals and reagents

DMA(V) was obtained from Sigma chemicals and MA(V) from Chem. Service MC, West Chester UBA. Sodium arsenate (Na_2HAsO_4) and sodium arsenite ($NaAsO_2$), reagent grade, were purchased from Merck. Arsenosugar 1[13] (Fig. 1) was synthesized as reported previously. The remaining arsenosugars (2, 3 and 4, Fig. 1) were isolated from natural sources.[14] Orthophosphoric acid (85%), concentrated sulfuric acid and ammonia solution (25%) were all AnalaR® obtained from BDH Chemicals, and acetic acid (>99%) AnalaR® was from Fluka. The standard reference material IAEA 140 (common *Fucus* spp.) was used for total arsenic determination and arsenic speciation analysis. For the axenic experiments, the following mixture of antibiotics was used: Streptomycin sulfate, Nystatin, Erythromycin, Rifampicine. All these were purchased from Sigma. The Bolds Basal Medium was made up of 250 mg $NaNO_3$, 25 mg $CaCl_2{\cdot}2H_2O$, 75 mg $MgSO_4{\cdot}7H_2O$, 75 mg KH_2PO_4, 175 mg K_2HPO_4, 25 mg NaCl, 50 mg EDTA, 31 mg KOH, 5 mg $FeSO_4{\cdot}7H_2O$, 115.5 mg H_3BO_3, 9 mg $ZnSO_4{\cdot}7H_2O$, 1.6 mg $MnCl_2{\cdot}4H_2O$, 0.8 mg MoO_3, 1.6 mg $CuSO_4{\cdot}5H_2O$, 0.5 mg $Co(NO_3)_2{\cdot}6H_2O$ dissolved in 1 l of water. All chemicals were analytical grade and from Sigma.

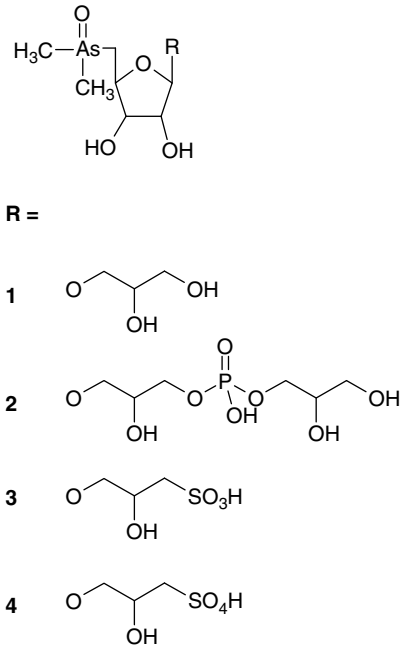

Figure 1. Structures of commonly occurring arsenosugars, termed Sugar 1, Sugar 2, Sugar 3 and Sugar 4.

Culturing algae

The *C. vulgaris* strain UTCC 92 was obtained from the University of Toronto culture collection and cultured under conditions of 12 h continuous illumination in sterilized glass flasks covered with cotton wool. The media used was Bolds Basal Medium[15] and all media and glassware for culturing were autoclaved at 121 °C for 30 min before use. The algae were grown for 3 days in the medium after inoculation before the start of the incubation experiment.

Incubation experiments

To the 3-day-old algae cultures, arsenate in concentrations of 0, 10, 100 or 1000 mg l^{-1} was added. The cultures were monitored from inoculation to 4 days after the start of incubation. Probes monitored the dissolved oxygen (DO) produced from photosynthesis. Oxygen concentration, temperature and pH were recorded by dataloggers. Illumination consisted of a 12 h light period (5000 lx) and a 12 h dark period. The algae cultures were cooled by pipes through which cold water was circulated and were agitated by magnetic followers. Although the algae were grown in sterilized containers, the experiments were done with and without the addition of an antiseptic and antimycotic solution containing 2 g Streptomycin sulfate,

0.5 g Nystatin, 1.5 g Erythromycin and 0.02 g Rifampicine in 4 l of medium in order to check if bacterial growth has an influence on the arsenic speciation. No microbiological plating was done in order to check for bacterial contamination, but the cultures were checked visually and neither of them had a milky appearance that indicates bacterial contamination.

The chlorophyll *a* content was determined at the start, on day 4 and at day 7 by the acetone method, after first being filtered onto a cellulose nitrate filter.[16] Briefly, 100 ml of the culture was filtered and the residue was dissolved with 7 ml of acetone. The amount of water retained by the filter after 5 min air suction was used to calculate the exact dilution of the acetone. The absorbance of the acetone was recorded at 665 nm (for the absorbance of chlorophyll *a* while turbidity was measured at 750 nm using a Hitachi U-2000 spectrophotometer.

After 7 days the algae cultures were filtered, washed with deionized water until arsenic was below the detection limit of 0.1 μg l^{-1} in the wash water, freeze dried and weighed for arsenic analysis and biomass determination. A representative algae sample for the determination of the biomass was also taken at the start of the experiment.

Extraction and digestion

The extraction method for arsenic speciation analysis in *C. vulgaris* was similar to that described previously.[3] The algae samples including the filter paper were placed in a centrifuge tube to which 10 ml of 1 : 1 (v/v) methanol/water mixture was added. The tube was first sonicated for 10 min and then centrifuged for 10 min. The extract was removed and the process repeated twice. The combined extracts were evaporated to dryness in a rotary evaporator and re-dissolved in 1.5 ml of double-distilled water (by weight).

Digestion of the residue remaining on the filter papers for total arsenic analysis was carried out in a 25 ml beaker on a hot plate. The dried filter papers and the residue were placed in beakers, and to this 3 ml of HNO_3 and 2 ml of H_2O_2 were added. The beakers were heated for approx 1–1.5 h until a clear yellowish solution was obtained. A certified reference material of freeze dried, homogenized *Fucus* spp. (common brown seaweed, IAEA 140) was also digested by this method. The extracts were made up to 25 ml and refrigerated until analysis.

Analysis of total arsenic and arsenic speciation

Total arsenic analysis was performed by inductively coupled plasma mass spectrometry (ICP-MS), using an autosampler on the mass spectrometer (Spectromass 2000). The instrument was used in standard mode with a Meinhard C-type nebulizer and a cyclonic spray chamber. Arsenic at m/z 75 was monitored and additionally at m/z 77 for $ArCl^+$ interference and indium (m/z 115), used as internal standard. The conditions can be found in Table 1.

Anion-exchange chromatography was used for the determination of arsenic species in the algae. The high-performance liquid chromatography (HPLC) column was a Hamilton PRP-X100 directly coupled to the ICP mass spectrometer as described previously.[1] A 20 μl sample loop and a mobile phase of 30 mM H_3PO_4 adjusted to pH 6 with ammonia with a flow rate of 1 ml min^{-1} was used. Standard mixtures of arsenic(V), DMA(V), MA(V) and arsenic(III) with arsenic concentrations of 10, 20, 50 and 100 ng ml^{-1} were used for the quantification and to establish retention times. Standards were also run to determine the retention times for the arsenosugars (Sugar 1, Sugar 2, Sugar 3 and Sugar 4) (Table 2). The calibration curve used for quantification of the arsenosugars in the samples was that for the species nearest in retention time to the particular arsenosugar. For the integration of the peak area, trapezium integration was used. Standard additions were performed to corroborate retention times and to investigate any potential matrix effects.

Table 1. Operating conditions for ICP-MS speciation analysis

ICP conditions	
Generator power (W)	1350
Meinhard A-Type nebulizer (l min^{-1})	1.05
Pump speed (ml min^{-1})	1.0–1.5
Coolant gas (l min^{-1})	15
MS conditions	
Analyser stage (mbar)	1.403×10^{-6}
Operation mode (mbar)	8.55×10^{-6}
Expansion chamber (mbar)	1.423
Dwell time (ms)	500
Masses monitored	m/z 75, 77

Table 2. The retention times and percentage recovery for each spike added to the arsenic species found in the samples

Arsenic species	Retention time (s)		Spike recovery (%)
	standard	Spike	
As(III)	194	190	80
Sugar 1	209	212	56
DMA(V)	255	256	78
MA(V)	326	350[a]	80
Sugar 2	403	410	87
As(V)	587	587	97
Sugar 3	898	899	53
Sugar 4	1965	—	—

[a] Unknown peak U_{350}, elutes slightly later than MA(V).

RESULTS AND DISCUSSION

In the monitoring of the experimental tanks, DO cycling (created by the alternating 12 h illumination and darkness) continued without change after the addition of arsenate to the tanks and with this the pH continued to rise with the increase in oxygen, and fall as this was used up in respiration.

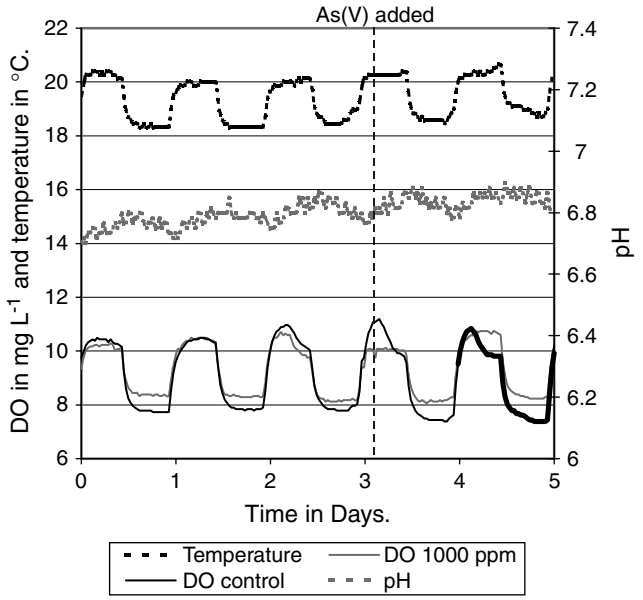

Figure 2. Data collected by dataloggers for DO for two cultures: one with no arsenate (control) and the other with 1000 mg l^{-1} arsenic(V) (1000 ppm), pH and temperature over the period of the experiment using 12 h cycles of illumination and darkness.

This indicated that, even with an arsenate addition of up to 1000 mg l^{-1}, the algae were still photosynthesizing and producing oxygen at relatively high levels (Fig. 2). No significant difference was observed for the different treatments.

The chlorophyll content on days 4 and 7 from the start for all arsenate concentrations, with no significant differences between treatments (Fig. 3). However there was some evidence of an increase in biomass production between the arsenate treatment of the 100 mg l^{-1} tanks and the tanks containing 1000 mg l^{-1} ($p < 0.05$ Fisher's pairwise comparison of least significant differences). Since the chlorophyll concentration, which did not change, is intrinsically linked to the biomass production, it is unclear why biomass is significantly higher in the cultures that contained the highest arsenate concentration. Whether this is an indication of the health of the algae or whether the arsenic has an influence on the metabolism of the algae is not clear, but chlorophyll and DO cannot be taken solely as a measure of the health of a culture.

The results for total arsenic in algal cells are given in Table 3. There was no difference between the arsenic accumulation in the cultures without and with antibiotics. The trend here is one where on increasing arsenic concentration in the culture leads to an increasing arsenic concentration in the biomass. The concentration in the biomass is always higher than the concentration in the culture, although the bioconcentration factor decreases a 100-fold from the background arsenic concentration to the highest arsenic concentration used for incubation. It should be emphasized, however, that there is a certain degree of variability within the replicates, which might result from any small imprecision in the analysis of the

Table 3. Total arsenic (per gram of dried algal cells) extracted from algal cells determined by ICP-MS

As(V) treatment (mg l^{-1})	Algal As[a] (μg g^{-1})	Bioconcentration factor
<0.1	17.2 ± 3.9 a	>172
10	100 ± 45 a	10
100	157 ± 120 a	1.5
1000	2739 ± 591 b	2.7

[a] The same letters beside the averages denote results that are not significantly different in an ANOVA and Fisher's pairwise comparison of LSDs ($p < 0.05$ significance level; $n = 2$).

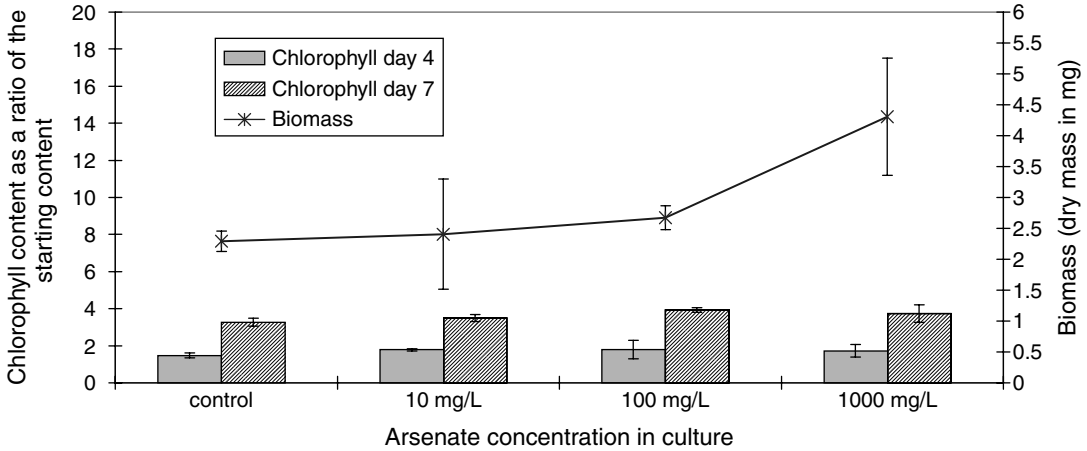

Figure 3. Chlorophyll content on days 4 and 7 and final (day 7) biomass determination of *C. vulgaris* in 4 l of culture containing different concentrations of arsenic as arsenate. The error bars indicate the standard error of three replicates.

total arsenic, since only a few milligrams of algae (dry mass) could be retrieved from the culture. This is also reflected by the determination of the standard *Fucus* certified reference material (IAEA 140), which gave a percentage recovery of 113 ± 28% and the limit of arsenic detection was calculated to be 1.03 μg l^{-1}. The bioaccumulation results confirm the results of earlier studies by Goessler *et al*.[17] and Maeda.[4]

The extraction efficiency of a 1:1 (v/v) methanol/water mixture was generally between 11 and 27% of the total arsenic, which is surprisingly low compared with extraction efficiencies for most marine algae[3] (Table 4). The extraction efficiency of the algae cultured in 1000 mg l^{-1} varies extensively, which cannot be explained. The low extraction efficiency makes it likely that the species extracted are not representative for the species present in the algal cells.

The extracts of *C. vulgaris* generally contained arsenic(V), arsenic(III), DMA(V), an unknown signal and arsenosugars (Table 5). Spiking experiments with standard compounds showed that the recovery of the species from the column was between 53 and 97% and that the retention time was not influenced by the sample matrix (Table 2, Figure 4). Spiking with MA(V) revealed that the signal eluting at 350 s is not MA(V) but an unknown arsenic species labelled as U_{350}. This unknown peak in the algal extract elutes shortly after MA(V). It shows the same retention time as dimethylarsenoyl acetate (DMAA), which was recently identified by the same chromatographic method, using ICP-MS and electrospray ionization (ESI) MS detection on the metabolites of arsenosugar ingestion found in the urine of seaweed-eating sheep.[18,19] Furthermore, DMAA was also identified as a degradation product of arsenobetaine in the marine environment.[11] However, during the time of the analysis, no DMAA standard was available to spike the extract, nor was the concentration of the peak U_{350} high enough to do ESI-MS measurements to confirm its presence in the extract.

Arsenic(V) was found to have the highest concentration in the analysis of the contents of the algal cells by HPLC–ICP-MS for all treatments. The second most abundant species produced in most of the treatments is the arsenic Sugar 2. Arsenic(III), Sugar 1 and Sugar 3 were also found to be present in this experiment. In the schema proposed by Maeda[4] for the biotransformation of arsenic in marine algae, arsenic(III) is the first stable intermediate from arsenic(V), followed by MA(V) and DMA(V). In this experiment, the intermediate species have not accumulated to as high a concentration as the sugars, and MA(V) appears to be absent. This may indicate that once the arsenic starts on this metabolic pathway the process happens quickly, and that the addition of the second methyl group takes place rapidly and/or the MA(V) is then excreted. Arsenosugars (Sugar 1 and Sugar 2) were also found previously in freshwater algae from Meager Creek hot springs in British Colombia.[6] Their concentrations in these samples were also higher than those of DMA(V) and MA(V) as reported for *C. vulgaris* here. The unknown arsenic species detected by Goessler *et al*.[17] in their

Table 4. Arsenic (per gram of dried algal cells) measured in methanol/water (1:1) extraction for speciation and nitric acid/peroxide digestion for residues of *C. vulgaris*[a]

As(V) treatment (mg l^{-1})	As concentration (μg g^{-1})			Extraction efficiency %
	$MeOH-H_2O$	$HNO_3-H_2O_2$	Total	
Series 1				
Control	3.41	9.4	12.8	26
10	8.15	60.3	68.4	11
100	14.6	57.9	72.5	20
1000	29.8	1073	1103	2.7
Series 2				
Control	1.26	9.1	10.4	12
Control	5.41	14.9	20.3	27
10	16.8	117	133	13
100	29.7	213	243	12
1000	2408	749	3157	76

[a] The experiments were run in two different series in order to cancel problems with the culture.

Table 5. Concentrations of arsenic species (per gram of freeze-dried algal cells) found in algal cells as analysed by HPLC–ICP-MS

As(V) treatment (mg l^{-1})	Arsenic species concentration[a] (μg g^{-1})						
	As(V)	As(III)	DMA(V)	U_{350}	Sugar 1	Sugar 2	Sugar 3
1000	2390	0.89	0.02	bdl	2.15	17.3	bdl
	26.0	1.95	0.13	bdl	bdl	1.44	0.30
100	25.9	0.92	0.46	0.12	bdl	2.34	bdl
	11.9	0.26	0.05	0.04	0.42	1.59	0.36
10	15.6	0.62	0.30	bdl	bdl	0.29	bdl
	3.24	0.54	0.89	0.05	0.92	2.51	bdl
<0.1	1.27	bdl	bdl	bdl	bdl	bdl	bdl
	5.10	0.28	bdl	bdl	bdl	0.03	bdl
	3.23	0.08	0.03	bdl	bdl	0.08	bdl

[a] bdl: below detection limit, 0.02 μg g^{-1}.

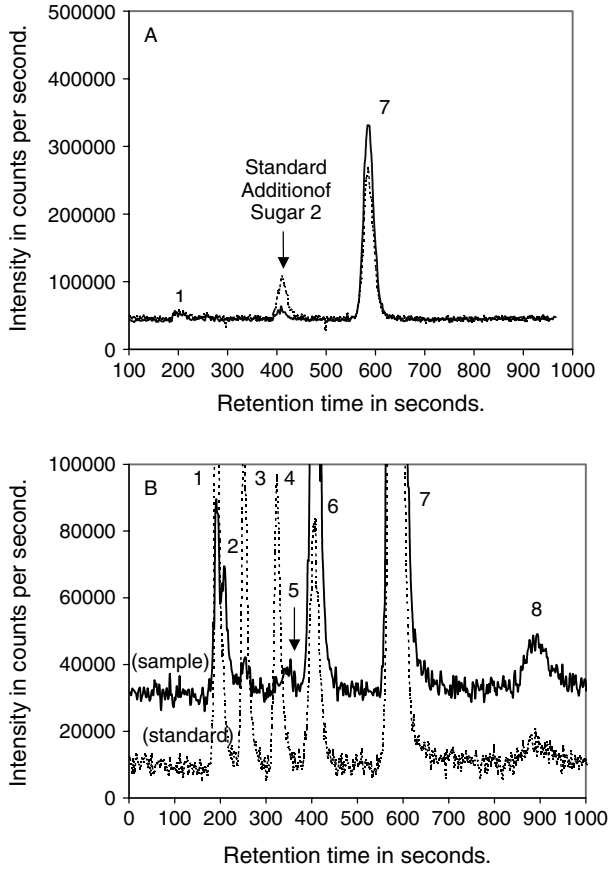

Figure 4. (A) Chromatogram of a methanol–water extract from *C. vulgaris* exposed to 1000 mg l^{-1} arsenic(V) using anion-exchange chromatography coupled to ICP-MS (*m/z* 75 trace is shown) spiked with Sugar 2; eat 7: arsenic(V). (B) Chromatogram of an extract from *C. vulgaris* exposed to 100 mg l^{-1} arsenic(V) (sample) in addition to the standard of arsenic(III), DMA(V), MA(V), arsenic(V), Sugar 2 and Sugar 3. The following arsenic species are indicated by numbers: (1) arsenic(III); (2) Sugar 1; (3) DMA(V); (4) MA(V); (5) unknown (U_{350}); (6) Sugar 2; (7) arsenic(V); (8) Sugar 3.

freshwater laboratory experiment using *Chlorella böhm* could correspond to Sugar 2, according to the reported retention time, indicating that this arsenosugar is the most common sugar found in freshwater algae. This is in common with many green and red marine algae, whereas brown marine algae frequently have significantly higher concentrations of sulfur-containing arsenosugars. Interestingly, for the first time, we also found the sulfonate arsenosugar (Sugar 3) in freshwater algae. In general, the concentration of arsenosugars in marine macroalgae are much higher than in freshwater algae, but it appears that freshwater algae can also generate arsenosugars. It has frequently been suggested that bacteria are responsible for the production of arsenosugars, but the experiments with *C. vulgaris* grown in cultures containing antiseptic and antimycotic compounds give rise to the suggestion that the arsenosugars are more likely synthesized by the algae. In contrast to marine algae, which are less tolerant to high levels of arsenate, arsenosugar production by *C. vulgaris* appeared not be hampered by a high arsenate concentration.

CONCLUSIONS

The monitoring of the oxygen production cycles, biomass and chlorophyll content all indicate that the algae *C. vulgaris* strain UTTC 92 are surviving arsenate concentrations of up to 1000 mg l^{-1}, with a possible beneficial effect on the biomass production seen in the highest concentration of arsenate treatment. The production of arsenosugars in this experiment confirms previously published studies[6] that freshwater algae metabolize arsenic in a similar manner to marine macroalgae.

Acknowledgements

The investigators thank the Department of Chemistry in the School of Engineering and Physical Sciences for the support of this study and Professor K. A. Francesconi and Dr W. Goessler for providing the arsenosugar standards.

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