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Chlorella sp. and Arsenic Compounds: An Attempt to Prepare an Algal Reference Material for Arsenic Compounds

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The growth of *Chlorella Böhm*, *Chlorella kessleri* and *Chlorella* 108 in media containing sodium arsenate at 200 or 2000 mg As dm^{-3} was investigated. The cell densities in the stationary phases were 40% higher for *Chlorella Böhm* (5.6 × 10^5 cells cm^{-3}) and *Chlorella kessleri* (4.5 × 10^5 cells cm^{-3}) grown in the 2000 mg As dm^{-3}) arsenate media compared with the arsenic-free media. The growth of *Chlorella* 108 was depressed by arsenate. Dimethylarsinic acid (at concentrations never exceeding 1.2 mg As dm^{-3}) and arsenite (~0.2 mg As dm^{-3}) were detected in the growth medium. The cells harvested during the stationary phase had 2400 mg arsenic associated with 1 kg dry mass. A water/methanol (80 : 20) mixture extracted all the arsenic from the cells. Most of the arsenic (99%) was arsenate. Arsenite, methylarsonic acid and dimethylarsinic acid accounted for the remaining arsenic. Cadmium in the arsenic-containing growth media (50 μg to 100 mg dm^{-3}) reduced the yield of algal cells and induced the formation of an unidentified arsenic compound in *Chlorella Böhm*. © 1997 by John Wiley & Sons, Ltd.

Keywords: *Chlorella* sp.; biomethylation; arsenic; HPLC–ICP–MS; cadmium

INTRODUCTION

Whereas the biotransformation of arsenic in marine ecosystems including marine algae has been explored in detail,[1–5] little work has been done on terrestrial systems.[5, 6] Only a few papers have reported on the methylation of inorganic arsenic by freshwater algae.[6] Very limited information exists about the behavior of arsenic within freshwater food chains.[7] The bioaccumulation of arsenic by the arsenic-tolerant, green, freshwater alga *Chlorella vulgaris* was studied by Maeda *et al.*[8] This alga grows well in arsenate-rich media and survives even in a medium with arsenate at 10 000 mg As dm^{-3}. Arsenite is not tolerated as well as arsenate. The cells were cytolyzed at arsenite concentrations higher than 40 mg As dm^{-3}.[9] Methylated arsenic compounds were detected in algal cells cultivated in a modified Detmer medium with an arsenate concentration of 217 μg As g^{-1} of medium.[10] The ability of *Chlorella* species to reduce arsenate to arsenite[12, 13] and the competition between the uptake of phosphate and the uptake of arsenate were described.[9, 13–15]

Reference materials (RM) of various classifications (internal, IRM; certified, CRM; or standard, SRM) play a very important role in the quality assurance (QA) of chemical analyses.[16] RMs are useful for the development and optimization of analytical methods and CRMs and SRMs are indispensable for quality assessments and ascertaining accuracy. QA for the determination of arsenic compounds in marine and terrestrial organisms is hampered by lack of an appropriate RM[17] with certified concentrations of total arsenic and arsenic species.

Freshwater algae cultivated under controlled conditions in arsenic-containing media could be suitable for the preparation of reference materials certified for arsenic compounds. This paper summarizes the results of experiments leading to the establishment of conditions for the prepara-

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tion of a biological CRM for arsenic and arsenic compounds.

EXPERIMENTAL

Chemicals

Methanol, $NaAsO_2$, $Na_2HAsO_4 \cdot 7H_2O$, $Na_2HPO_4 \cdot 2H_2O$ and $NaH_2PO_4 \cdot 2H_2O$ (all p.a. quality) were purchased from Merck. Methylarsonic acid (MA, m.p. 156 °C) and dimethylarsinic acid (DMA, m.p. 190 °C) were gifts from Vineland Chemical Co. (Vineland, NJ, USA). The water used for analytical work was purified to 18.2 MΩ cm resistivity (NANOpure).

Stock solutions of the arsenic compounds containing 100 mg As dm^{-3} were prepared by dissolving 86.7 mg $NaAsO_2$, 208.2 mg $Na_2HAsO_4 \cdot 7H_2O$, 93.4 mg methylarsonic acid or 92.1 mg dimethylarsinic acid to 500 cm^3. These stock solutions were diluted with NANOpure water to the desired concentrations just before use.

The mobile phase for chromatography was prepared by dissolving 5.34 g $Na_2HPO_4 \cdot 2H_2O$ and 4.68 g $NaH_2PO_4 \cdot 2H_2O$ separately in NANOpure water to 1000 cm^3 resulting in 0.03 mol dm^{-3} solutions. To the stirred NaH_2PO_4 solution the Na_2HPO_4 solution was added, until a pH of 6 had been reached.

An indium solution containing 1000 mg In dm^{-3} was purchased from Merck [1.663 g $In(NO_3) \cdot 4.5H_2O$ per 500 mm^3 of 0.5 mol dm^{-3} nitric acid]. This solution was used as an internal standard to compensate instrumental instabilities during ICP–MS measurements of total element concentrations.

The accuracy of the analytical data was checked with the certified reference materials BCR-62 (Olive Leaves) purchased from the EU Community Bureau of References, Brussels, Belgium, SRM 1575 (Pine Needles) purchased from NIST, Washington DC, USA, and CRM 12-02-02 (Green Algae) purchased from CHEMMEA spol. sr.o. P.O. Box 260, 81499 Bratislava, Slovakia.

Growth of ***Chlorella*** species

Nonsynchronous populations of *Chlorella vulgaris*, strain Böhm and Borns *1972* (later *Chlorella Böhm*), *Chlorella kessleri* strain *N-HM* (*Chlorella kessleri*) and *Chlorella* sp. strain 108 (*Chlorella* 108) from the algal collection of the Laboratory of Microalgal Biotechnology, Institute of Microbiology, ASCR, Trebon, Czech Republic, were grown in batches in temperature-regulated (35 °C), water-jacketed glass cylinders with a volume of 1 dm^3. The cultures were illuminated with incandescent lamps (light intensity 150 W m^{-2}) emitting photoactive radiation in the wavelength region 400–700 nm.

The concentrations of algal cells were determined by measuring the optical density at 750 nm (OD_{750}) using a Specol 11 spectrophotometer (Zeiss–Jena). The value of OD_{750} multiplied by 0.5 gives approximately the dry mass of algae present in 1 dm^3 of medium. An OD_{750} of 0.1 corresponds approximately to 1×10^7 cells cm^{-3} of algal suspension.

Air enriched to 2% CO_2 was bubbled through the growth medium, which contained (per dm^3) 2020 mg KNO_3, 340 mg K_2HPO_4, 990 mg $MgSO_4 \cdot 7H_2O$, 15.5 mg Fe–Na chelatonate, 10 mg $Ca(NO_3)_2 \cdot 4H_2O$, 3.09 mg H_3BO_3, 1.2 mg $MnSO_4 \cdot 4H_2O$, 1.4 mg $CoSO_4 \cdot 7H_2O$, 1.24 mg $CuSO_4 \cdot 5H_2O$, 1.43 mg $ZnSO_4 \cdot 7H_2O$ and 1.84 mg $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$.

For inoculation 20 cm^3 of an algal suspension with an optical density of 0.1 were used. The cells were harvested by centrifugation (6000*g* for 10 min) after having reached the stationary phase.

The algae were grown in media spiked with $Na_2HAsO_4 \cdot 7H_2O$ to 200 or 2000 mg As dm^{-3}. Aliquots (2 cm^3) of the algal suspension growing in the medium with 2000 mg As dm^{-3} were collected every 24 h. The aliquots were centrifuged and the supernatants kept in the refrigerator for the determination of arsenic compounds.

Cells of *Chlorella Böhm* grown in the medium containing 2000 mg As dm^{-3} were collected 96 h, 144 h and 168 h after inoculation by centrifugation. Cells from the inoculum were also isolated by centrifugation and then freeze-dried.

Chlorella Böhm was also grown in media spiked with arsenate at 2000 mg As dm^{-3} and cadmium. Cadmium was added as $Cd(NO_3)_2$ to achieve cadmium concentrations of 50 μg, 100 μg, 50 mg or 100 mg dm^{-3} in the growth media. The algal cells harvested after they had reached the stationary phase were freeze-dried.

Instrumentation

ICP–MS

The inductively coupled plasma mass spectrometer Plasma Quad 2 Turbo Plus (VG Elemental, Winsford, Cheshire, UK) was equipped with a Fassel-type torch, a Gilson Minipuls-3 peristaltic pump delivering solutions at 0.95 $cm^3 min^{-1}$ to a Meinhard TR-30-A3 nebulizer for the determination of total concentrations of elements, or to a Meinhard SB-30-K3 nebulizer for the determination of arsenic compounds by HPLC–ICP–MS. The aerosol was routed through a double-pass, Scott-type spray chamber cooled at 2 °C. The operating conditions are summarized in Table 1. Total arsenic was determined with an external calibration curve established with arsenite.

HPLC

The high-performance liquid chromatography system consisted of a Milton Roy CM 4000 multi-solvent delivery system and a PRP-X100 anion-exchange column (Hamilton, Reno, NV, USA; 250 mm × 4.1 mm i.d.; spherical, 10-μm particles of a styrene–divinylbenzene copolymer with trimethylammonium exchange sites; stable between pH 1 and 13; exchange capacity 0.19 meq g^{-1}). A guard cartridge (Hamilton) filled with the same stationary phase protected the analytical column. A Rheodyne (Cotati, CA, USA) six-port injection valve (type 7125) equipped with a 100-mm^3 loop was used for injection of the solutions.

Table 1. Operating conditions for the ICP–MS

Incident power	1400 W
Reflected power	<5 W
Gas flow rates	
Plasma/coolant	13.5 $dm^3 min^{-1}$
Auxiliary	1.10 $dm^3 min^{-1}$
Nebulizer	0.74 $dm^3 min^{-1}$
Ion sampling	
Nickel sampling cone orifice	1.0 mm
Nickel skimmer cone orifice	0.7 mm
Vacuum	
Expansion stage	1.7 mbar
Intermediate stage	1.0×10^{-4} mbar
Analyser stage	1.9×10^{-6} mbar
Acquisition parameters for the determination of total element concentrations	
Dwell time	320 μs
Time/slice	0.5 s
Uptake time	60 s
Acquisition time	3 × 60 s
Wash time	60 s
Acquisition parameters for HPLC–ICP–MS investigations	
Dwell time	640 ms
Time/slice	0.54 s
Total analysis time	≈310 s

HPLC–ICP–MS

The outlet of the analytical column was connected via a 700-mm steel capillary to the Meinhard SB-30-K3 nebulizer. A 0.03 mol dm^{-3} phosphate buffer of pH 6 served as mobile phase at a flow rate of 1.5 $cm^3 min^{-1}$. The back pressure was about 150 bar. The data were acquired with the time-resolved software *TR Vision 1.a* provided by VG Elemental. The chromatograms were exported and integration was performed with software[18] written in-house.

Microwave digestion system

Digestion of samples to determine total elemental concentrations was performed with a MLS-1200 Mega (MLS GmbH, Leutkirch, Germany) microwave digestion system. The power of the microwave generating magnetron is 1200 W but only 1000 W are delivered inside the working chamber. The magnetron operates at a frequency of 2450 MHz, equivalent to a wavelength of 0.01225 m. A microprocessor controls the power from 10 W to 1000 W, in 10 W increments. Microwave emission is unpulsed in the 250 W and 1000 W mode. Other power levels are achieved with 1000 W delivered in pulses. An HPR 1000/6 digestion rotor made of polypropylene makes it possible to digest six samples simultaneously. Teflon digestion vessels (90 cm^3) were used for the mineralization of the algal samples.

Determination of total arsenic and of arsenic compounds

Total arsenic in algae

Approximately 250 mg of the freeze-dried algal cells were weighed to 0.1 mg into the Teflon digestion vessels. After addition of 3 cm^3 of conc. HNO_3 (Merck p.a., subboiled) and 0.25 cm^3 of 30% H_2O_2 (Merck p.a.), the vessels were closed and secured in the rotor. The loaded rotor was placed in the microwave oven. The digestion program was taken from the manual and is summarized in Table 2. After cooling, the solutions were quantitatively transferred into 100-cm^3 volumetric flasks. Aliquots (500 mm^3) of the 10 mg dm^{-3} indium solution were added as internal standard. The flasks were filled to the mark with NANOpure water. Total arsenic was determined in these solutions by ICP–MS.

Total arsenic in extracts from algal cells

Eight extraction agents were tested for the removal of arsenic compounds from algal bio-

Table 2. Microwave program used for the mineralization of freeze-dried algae

Step	Power (W)	Period (s)
1	250	120
2	0	30
3	250	600
4	0	30
5	450	300
6	0	30
7	600	180
8	Ventilation	120
9	Cooling (water-cooled)	1800

mass: NANOpure water; 30 mmol dm^{-3} aqueous phosphate buffer, pH = 6; mixtures of methanol/NANOpure water (20 : 80, 40 : 60, 50 : 50, 60 : 40, 80 : 20); and methanol.

An aliquot (~100 mg) of the freeze-dried algal cells was weighed to 0.1 mg into a polyethylene tube (50 cm^3). The extracting agent (20 cm^3) was added and the closed tube was shaken for 14 h. The mixture was then centrifuged for 10 minutes at 2500 rpm. An aliquot (100 mm^3) of the supernatant was pipetted into a 10-cm^3 volumetric flask to which were added 50 mm^3 of the 10 mg dm^{-3} indium solution and 100 mm^3 of conc. HNO_3. The flask was filled to the mark with NANOpure water. The concentration of total arsenic was determined in these solutions by ICP–MS.

Arsenic compounds in the growth medium

Each supernatant obtained via centrifugation of the growth medium containing algal cells was filtered through a cellulose nitrate filter (pore size 0.2 μm). Aliquots (100 mm^3) of the filtrates were chromatographed (HPLC).

Arsenic compounds in extracts from algal cells

Aliquots of the dry algal cells (200 mg) were weighed to 0.1 mg into a polyethylene tube. Methanol/water (80 : 20, cm^3) was added and the closed tube was shaken for 14 h. The mixture was centrifuged for 10 min at 2500 rpm. The supernatant was decanted into a 250-cm^3 round-bottom flask. The residue was resuspended in 10 cm^3 methanol, centrifuged for 10 min at 2500 rpm and decanted into the 250-cm^3 round-bottom flask. This solution was evaporated to dryness at room temperature in a Rotavapor under an aspirator vacuum. The residue was shaken with water (20 cm^3). The mixture was filtered through a cellulose nitrate filter (pore size 0.2 μm). Aliquots (100 mm^3) of the filtrate were chromatographed (HPLC).

RESULTS AND DISCUSSION

Concentration of arsenate and algal growth

Three algal species, *Chlorella Böhm, Chlorella kessleri* and *Chlorella* 108 were grown in media containing arsenate at a concentration of 200 or 2000 mg As dm^{-3}. The lag-phase for *Chlorella Böhm* in the media and without arsenate lasted approximately one day (Fig. 1). The stationary phase was reached within four to five days. The growth curve for algae in the arsenic-free medium is similar to the curve in the arsenate-containing medium with 200 mg As dm^{-3}. In these two experiments, approximately 4×10^8 cells were present in the stationary phase after 110 h of growth. Surprisingly, at the end of the experiment (110 h after inoculation) the cell density in the 200 mg As dm^{-3} medium was higher (4.5×10^8 cells cm^{-3}) than in the arsenic-free medium (4.1×10^8 cells cm^{-3}). In the medium with 2000 mg As dm^{-3} the logarithmic growth phase started later but lasted longer than in the 200 mg As dm^{-3} or in the arsenic-free medium. Although the growth rate in the 2000 mg As dm^{-3} (7×10^6 cells h^{-1}) was lower than in the other two media (~1.4×10^7 cells h^{-1}), the cell density in the arsenic-rich

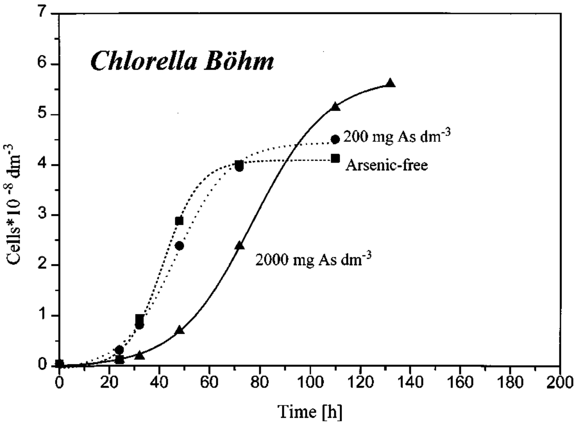

Figure 1 Growth curves for *Chlorella Böhm* in arsenate-containing growth media with 200 mg or 2000 mg As dm^{-3} and in an arsenic-free medium.

medium after four days of growth exceeded the cell density in the other two media. After 5.5 days of growth the culture in the 2000 mg As dm^{-3} medium began to enter the stationary phase with a cell density of 5.6×10^8 cells cm^{-3}. This density is 40% higher than the density in the arsenic-free medium during the stationary phase. The reproducibility of the growth experiments lies within 10%.

Qualitatively similar results although at somewhat lower cell densities were obtained with *Chlorella kessleri* (Fig. 2). The growths obtained in the arsenic-free medium and in the 200 mg As dm^{-3} medium are almost the same. The lag phase in the 2000 mg As dm^{-3} lasted almost two days, whereas a lag phase is hardly noticeable in the other two media. The growth rate during the log phase (5.9×10^6 cells h^{-1}) is higher in the arsenic-rich medium than in the control and the 200 mg As dm^{-3} media ($\sim 3.4 \times 10^6$ cells h^{-1}).

The growth curves for *Chlorella Böhm* and *Chlorella kessleri* clearly express the tolerance of these algae for arsenic. Both grow at least as well in a medium with arsenate at 200 mg As dm^{-3} as in the arsenic-free medium, judged by the rate of growth and the cell density during the entire growth period. That *Chlorella Böhm* and *Chlorella kessleri* have growth rates in media with 2000 mg As dm^{-3} similar to the rates in arsenic-free media is remarkable. Even more remarkable are the differences in cell densities in the stationary phases. Cell densities are 30–40% higher in the 2000 mg As dm^{-3} media than in arsenic-free media. Why these algae can grow so well in such arsenic-rich media is not known. Maeda *et al.*[9] obtained similar results with *Chlorella vulgaris*, a green, freshwater alga that survived even in a medium containing arsenate at 10 000 mg As dm^{-3}.

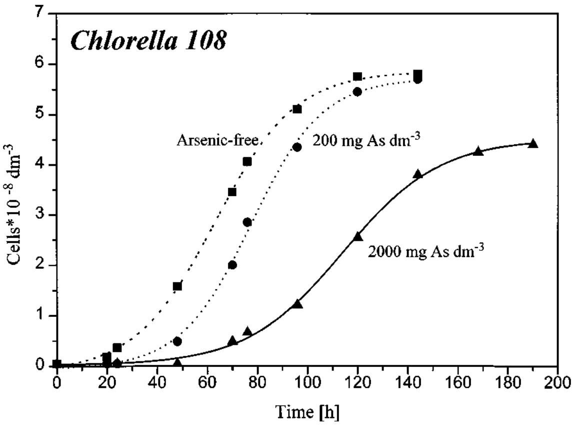

Figure 3 Growth curves for *Chlorella* 108 in arsenate-containing growth media with 200 mg or 2000 mg As dm^{-3} and in an arsenic-free medium.

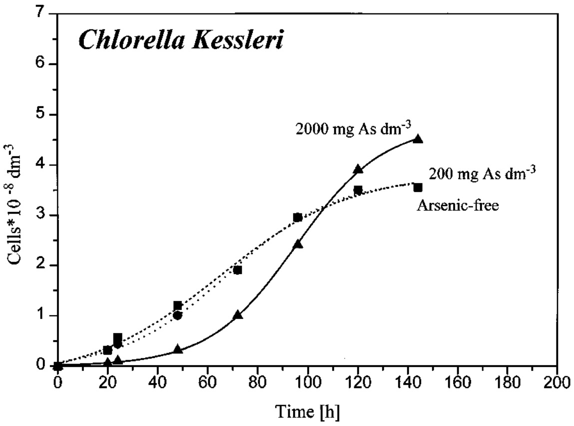

Figure 2 Growth curves for *Chlorella kessleri* in arsenate-containing growth media with 200 mg or 2000 mg As dm^{-3} and in an arsenic-free medium.

Chlorella 108 is affected by arsenate quite differently from *Chlorella Böhm* and *Chlorella kessleri*. The lag phase becomes longer with increasing arsenate concentration (Fig. 3). Whereas the growth rates ($\sim 7.2 \times 10^6$ cells h^{-1}) and the cell densities in the stationary phase ($\sim 5.8 \times 10^8$ cells cm^{-3}) are approximately the same in the arsenic-free medium and in the medium with arsenate at 200 mg As dm^{-3}, both of these values are lower in the medium with 2000 mg As dm^{-3}. The cell density in the stationary phase is about 25% lower (4.4×10^8 cells cm^{-1}) and the growth rate is about 6.2×10^6 cells h^{-1}.

Total arsenic associated with cells of *Chlorella Böhm*

Total arsenic associated with freeze-dried algal cells was determined with ICP–MS after mineralization of approximately 250 mg of the cells with a mixture of conc. HNO_3 and 30% H_2O_2 in a microwave system. After dilution of the digests to 100 cm^3, the concentrations of arsenic in the resulting solutions were at least 1000 times higher than the detection limit of the ICP–MS (0.1 ng cm^{-3}).

During the log phase the algal cells loaded themselves with arsenic. The arsenic concentration increased from 23 mg kg^{-1} (dry mass) at the beginning of the experiment to 4600 mg kg^{-1}

after 96 h (toward the end of the logarithmic growth phase). During the stationary phase (144 and 168 h after inoculation) only 2400 mg kg^{-1} were associated with the dry cells (Table 3). Even higher concentrations of arsenic (up to 50 000 mg kg^{-1}) were found by Maeda *et al.*[9] associated with *Chlorella vulgaris* grown in a Detmer medium at an arsenate concentration of 10 000 mg As dm^{-3}. Such high concentrations of arsenic are unlikely to be caused by arsenic compounds in the cytoplasma of the cells.

From 1 dm^3 of medium with an arsenic concentration of 2000 mg dm^{-3} about 1×10^9 cells of *Chlorella Böhm* were isolated, having a mass of approximately 8 g after freeze-drying and containing 20 mg (0.27 mmol) arsenic (concentration 2500 mg^{-1} kg). Each cell (diameter ~3 μm) is associated with an average of 2×10^{-11} g (2.7×10^{-13} mol) of arsenic. On the assumption that the entire volume of a cell (1.4×10^{-14} dm^3) is filled with cytoplasma, in which 2.7×10^{-13} mol of arsenic are dissolved, the concentration of arsenic within the cell would be 19 mol dm^{-3}. Even if the number of cells is two orders of magnitude larger, the concentration of arsenate within a cell would still be ~0.2 mol dm^{-3}, an arsenic concentration much too large for the survival of a cell.

Arsenic compounds extractable from freeze-dried algal cells

For the extraction of arsenic compounds from the cells of *Chlorella Böhm* grown in the 2000 mg As dm^{-3} medium water, a 30 mmol dm^{-3} phosphate buffer of pH 6, methanol and methanol/water mixtures were investigated. Total arsenic was determined in the extracts by ICP–MS after the cells had been shaken with the extraction reagents for 14 h. The recoveries ranged from 74% with NANOpure water to 100% with methanol/water mixtures with more than 40% methanol. The 30 mmol dm^{-3} phosphate buffer extracted 84% of the total arsenic (Fig. 4).

For the determination of arsenic compounds the methanol/water (80 : 20) extract was rotavaped to dryness and the residue dissolved in water. The filtered aqueous solution was chromatographed on an anion-exchange column with a 30 mmol dm^{-3} phosphate buffer of pH 6 as mobile phase. With ICP–MS as arsenic specific detector, arsenite, dimethylarsinic acid, methylarsonic acid and arsenate can be easily monitored in the column effluent, even when each compound is present in quantities equivalent to 0.1 ng arsenic (Fig. 5).

Most of the arsenic (99%) in the extracts was arsenate. Arsenite, methylarsonic acid and dimethylarsinic acid accounted for the remaining arsenic. The concentration of arsenite reached approximately 25 mg As kg^{-1} 96 h after inoculation and decreased to approximately 7 mg As kg^{-1} in the stationary phase; the concentration of dimethylarsinic acid remained in the range 8.1–9.2 mg As kg^{-1}. The concentration of methylarsonic acid never exceeded 2.5 mg As kg^{-1} (Table 3). These results indicate that a small fraction (~0.8%) of the total arsenic associated with the cells of *Chlorella Böhm* had been transformed to arsenite through reduction, and to methylarsonic acid and to dimethylarsinic acid through methylation. These findings are in agreement with earlier observations by Maeda *et al.*[10]

The arsenic metabolites could be excreted by the cells into the growth medium. Chromatog-

Table 3. Concentration of total arsenic compounds in *Chlorella Böhm* grown in a medium containing arsenate at 2000 mg As dm^{-3}

	Concentration[a] of As (mg As kg^{-1} dry mass)			
Time elapsed after inoculation (h)	Total arsenic	Arsenite	Dimethylarsinic acid	Methylarsonic acid
0	22.7 ± 3.6	n.d.[b]	n.d.	n.d.
96	4650 ± 80	25 ± 0.7	8.1 ± 0.1	1.9 ± 0.1
144	2430 ± 70	7.3 ± 0.1	7.5 ± 1.0	2.5 ± 0.1
168	2480 ± 50	7.6 ± 0.4	9.2 ± 0.8	0.35 ± 0.06

[a] Average (n=3) and standard deviation obtained with digests from three aliquots of cells harvested during the same growing experiment.
[b] n.d. not determined.

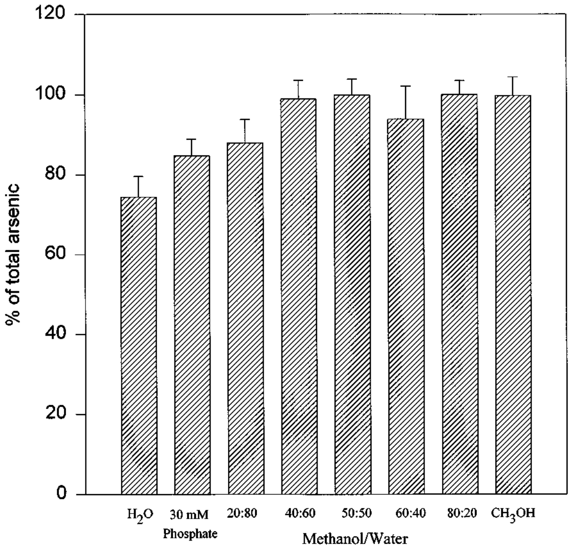

Figure 4 Average recoveries ($n=3$) of arsenic from freeze-dried cells of *Chlorella vulgaris* with 2500 mg As kg^{-1} after 14 h of shaking with eight different extraction reagents.

raphy of aliquots of the medium freed from cells by centrifugation, taken throughout the growth period, proved that arsenite and dimethylarsinic acid increased with time and reached approximately 1.2 mg As dm^{-3} for dimethylarsinic acid and approximately 0.2 mg As dm^{-3} for arsenite in the stationary phase of *Chlorella Böhm* and *Chlorella kessleri* (Fig. 6). The concentrations of the arsenic compounds in the *Chlorella* 108 medium never exceeded 0.4 mg dm^{-3}. The arsenite concentration decreased steadily during the logarithmic growth phase, whereas the concentration of dimethylarsinic acid remained constant throughout most of this period (Fig. 6).

The experiments with the three *Chlorella* species ascertained that arsenic is taken up by all three algae. The harvested, freeze-dried cells contain appreciable amounts of arsenic, most of which is present as arsenate. Arsenite, methylarsonic acid and dimethylarsinic acid are present at low concentrations. Among the three species investigated, *Chlorella Böhm* appears to be best suited for the preparation of a standard reference material, because of the excellent growth in the

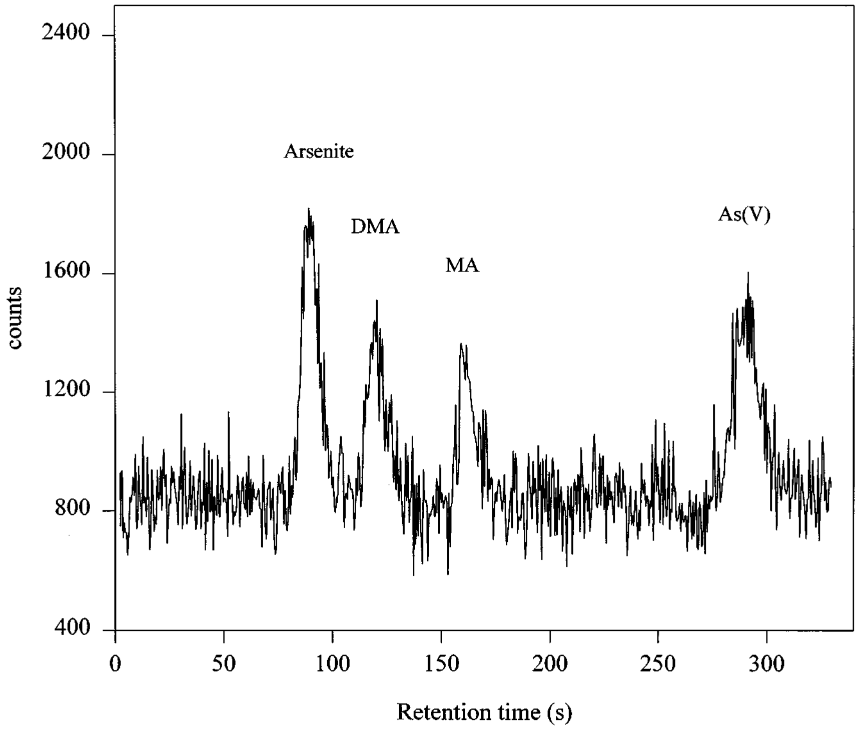

Figure 5 Typical HPLC–ICP–MS chromatogram of a mixture of arsenite, dimethylarsinic acid (DMA), methylarsonic acid (MA) and arsenate at a concentration of 1 ng As cm^{-3} of each compound (PRP-X100 anion exchange column, 0.030 mol dm^{-3} phosphate buffer of pH 6, flow rate 1.5 cm^3 min^{-1}, 100 mm^3 injected).

presence of high concentrations of arsenate (Fig. 1) and of analytically useful concentrations of arsenite (7 mg kg^{-1}), methylarsonic acid (2 mg kg^{-1}), and dimethylarsinic acid (8 mg kg^{-1}) in the freeze-dried cells. Although the high concentration of arsenate (2500 mg kg^{-1}) does not interfere with the determination of arsenite, methylarsonic acid and dimethylarsinic acid at a few milligrams per kilogram when HPLC–ICP–MS method is used, interferences will be severe when the hydride generation method without separation of the arsenic compounds by HPLC is employed. To obtain algal biomass with less divergent concentrations of the arsenic compounds, the uptake and transformation of arsenic by *Chlorella Böhm* must be studied in media with lower arsenate concentrations and in media containing arsenate and methylarsonic acid or dimethylarsinic acid.

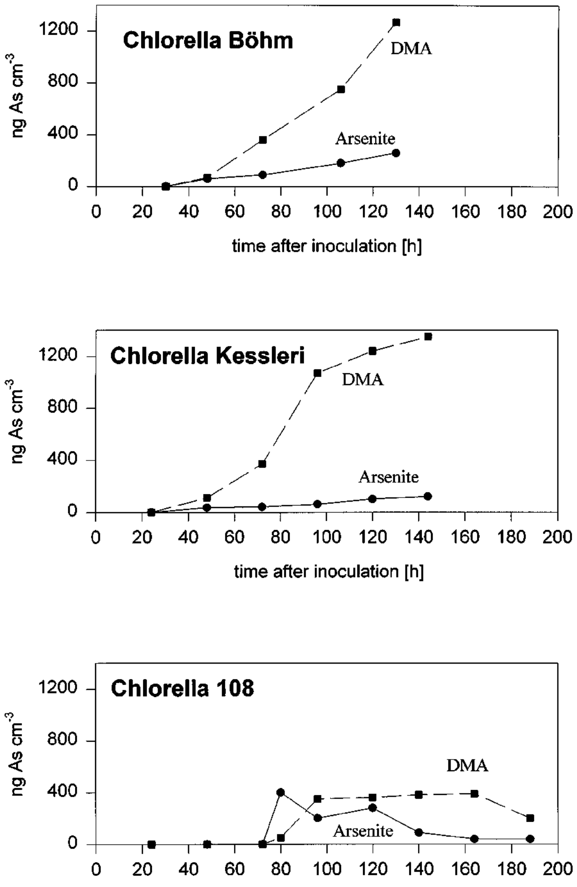

Figure 6 Arsenic compounds detected in the supernatants of media in which *Chlorella Böhm* (top), *Chlorella kessleri* (middle), and *Chlorella* 108 (bottom) had been grown (2000 mg As dm^{-3}).

The influence of cadmium on the growth of *Chlorella Böhm* and the transformation of arsenate

The usefulness of a reference material increases with the number of elements for which concentrations are certified. Therefore, the growth of *Chlorella Böhm* was investigated in media containing cadmium nitrate and sodium arsenate. Cadmium was chosen because this toxic element is frequently determined in environmental samples. The yield of algal biomass obtained in media with arsenate at 2000 mg As kg^{-1} and cadmium nitrate decreased with increasing cadmium concentration in the medium. Whereas 8 g dry biomass were obtained without cadmium in the medium, only 1 g could be harvested from the medium containing 100 mg Cd dm^{-3}. The cadmium concentrations in the dry cells increased with the concentration of cadmium in the medium (Table 4).

Aliquots of the growth media freed from algal cells by centrifugation were analyzed for arsenic compounds. Methylarsonic acid and dimethylarsinic acid were identified. Decreasing concentrations of dimethylarsinic acid were observed with increasing cadmium concentrations in the cultivation medium.

Cadmium in the growth medium influenced the transformation of arsenate. In the extracts from cells grown in arsenate and cadmium-containing media, arsenite, methylarsonic acid, dimethylarsinic acid, and an unknown arsenic compound were detected (Fig. 7). The concentration of arsenite was higher in cells grown with

Table 4. Concentration of total arsenic and cadmium in the green alga *Chlorella Böhm* grown in media containing arsenate at 2000 mg As dm^{-3} and cadmium nitrate at different concentrations

Concentration in growth medium		Concentration (mg kg^{-1} dry mass)	
As(mg dm^{-3})	Cd	As	Cd
0	0	11.1	0.23
2000	0	2600	0.56
2000	50 μg dm^{-3}	3100	3.89
2000	100 μg dm^{-3}	10 400	6.68
0	0	0.66	0.12
2000	0	3300	0.38
2000	50 mg dm^{-3}	4600	3330
2000	100 mg dm^{-3}	14 900	16 600

Table 5 Concentrations of arsenic compounds in algal cells

	Concentration[a] (mg As kg^{-1} dry mass)			
Concentration of Cd in medium with arsenate at 2000 mg dm^{-3}	Arsenite	Dimethylarsinic Acid	Methylarsonic Acid	Unknown
0	4.4±0.8	7.9±0.5	0.34±0.1	<DL[b]
50 μg Cd dm^{-3}	13.4±0.5	11.1±0.5	tr[c]	3.8±0.1
100 μg Cd dm^{-3}	95.0±2.8	24.7±2.8	2.9±0.3	1.1±0.01
0	11.6±0.5	9.6±0.9	0.52±0.01	<DL
50 mg Cd dm^{-3}	15.3±1.2	10.9±0.3	tr	<DL
100 mg Cd dm^{-3}	33.9±0.4	8.3±0.3	2.8±0.5	1.4±0.2

[a] Average ($n=3$) and standard deviation obtained with extracts from three aliquots of cells harvested during the same growing experiment.

[b] DL, detection limit (100 μg As kg^{-1}).

[c] tr, traces but not quantified.

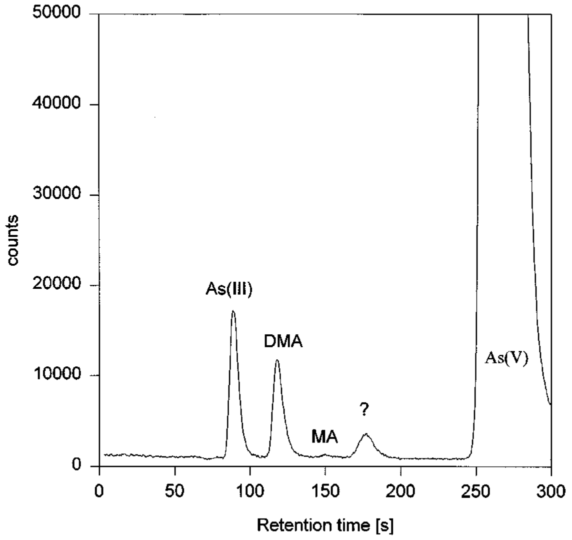

Figure 7 Typical HPLC–ICP–MS chromatogram of an extract of algal cells grown in 2000 mg As dm^{-3} and 50 μg Cd dm^{-3} medium (PRP-X100 anion exchange column, 0.030 mol dm^{-3} phosphate buffer of pH 6, flow rate 1.5 cm^3 min^{-1}, 100 mm^3 injected): As(III), arsenite; DMA, dimethylarsinic acid; MA, methylarsonic acid; ?, unknown arsenic compound; As(V), arsenate.

cadmium (95 mg As kg^{-1} at 100 μg Cd dm^{-3}, 34 mg As kg^{-1} at 100 mg Cd dm^{-3}) than in a medium without cadmium (4–11 mg As kg^{-1}) (Table 5). The concentration of dimethylarsinic acid was ~8 mg kg^{-1} in cells grown in the cadmium-free medium and ~25 mg kg^{-1} in cells from the 100 μg Cd dm^{-3} medium. At cadmium concentrations of 50 and 100 mg Cd dm^{-3} the concentration of dimethylarsinic acid was almost the same (~8 mg kg^{-1}) as in the cadmium-free medium. Methylarsonic acid and the unidentfied arsenic compound were present at low concentrations (Table 5). The unidentified arsenic compound was not detected in algal cells grown in the cadmium-free media.

Algal biomass grown in media with arsenate at 2000 mg As dm^{-3} and cadmium nitrate at 100 μg Cd dm^{-3} has concentrations of cadmium (~7 mg kg^{-1}), arsenite (~95 mg kg^{-1}), dimethylarsinic acid (~25 mg kg^{-1}), and methylarsonic acid (~3 mg kg^{-1}) that are suitable for a reference material. However, the concentration of arsenate at 10 400 mg kg^{-1} is too high. Further experiments with lower concentrations of arsenate in the presence of methylarsenic acids must be conducted in search of an algal biomass with more acceptable ratios of arsenic compounds.

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