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Arsenic Compounds in the Freshwater Green Microalga *Chlorella vulgaris* After Exposure to Arsenite

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The freshwater green alga, *Chlorella vulgaris*, was cultivated in a modified Detmer medium in the presence of arsenite in order to investigate tolerance, accumulation, transformation and excretion of arsenic species. When the alga was exposed to arsenite, arsenic accumulation markedly increased in the beginning of the log phase, rose to a maximum of 610 μg As g^{-1} and then decreased during the period from 40 to 120 h after inoculation. Arsenate was the major metabolite in the algal cell; trimethylarsenical species (TMA) were also found 36 h after inoculation when the alga was exposed to arsenite at levels of 70 – 100 μg As cm^{-3}. At arsenite levels of 10 – 20 μg As cm^{-3}, cell growth was higher than in an arsenic-free medium. Arsenite accumulated in *Chlorella vulgaris* was transformed to arsenate through bio-oxidation and to a small degree to methyl-, dimethyl-, and trimethyl-arsenic species through biomethylation. Furthermore, the arsenic metabolites were readily excreted under conditions undesirable for the growth of the alga. Total arsenic accumulation decreased with an increase in arsenite concentration in the medium.

Keywords: arsenic species; freshwater algae; *Chlorella vulgaris*; arsenite uptake; arsenite bioaccumulation; arsenite biotransformation

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INTRODUCTION

Studies on the biotransformation of inorganic arsenic by microalgae are important, because inorganic arsenic is taken up by algae from the aqueous phase into the aquatic food chain. Recently, many researchers have reported on the toxicity of arsenic compounds and the uptake, bioaccumulation and biotransformation of arsenic from seawater or soil. Relatively few studies, however, have been carried out on the biotransformation of arsenic in freshwater.[1,2] Bioaccumulation and excretion of several arsenic compounds by the marine unicellular alga *Polyphysa peniculus* were studied by Cullen and co-workers.[3] They reported that dimethylarsinic acid was the major metabolite and that methylarsonic acid was a minor metabolite in the cells of *Polyphysa peniculus* that were exposed to arsenite for one week. Goessler *et al.*[4] studied the transformation of arsenate by a freshwater microalga, *Chlorella sp.*, which was cultivated under controlled conditions to prepare algal reference materials for arsenic compounds.

Arsenic can exist in several oxidation states and chemical forms in aquatic environments.[5,6] These oxidation states and chemical forms of arsenic influence their biological availability, physiological and toxicological effects.[7] The toxicity decreases in the sequence $AsH_3 > H_3AsO_3 > H_3AsO_3$. Arsenous [As (III)] acid is the predominant species in anoxic river waters and groundwater[1,7] and is not easily detoxified and excreted.[8] Thermodynamically, arsenate is more stable than arsenite in aerated water.[9] Arsenite (sodium arsenite, $NaAsO_2$) is a common commercial form of trivalent arsenic compound and one of the most toxic arsenic compounds.[7,10] The freshwater green microalga *Chlorella vulgaris* tolerates arsenate much better than arsenite.[11,12] *Chlorella vulgaris* cells survived

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even in a 10 000 μg As cm^{-3} medium, but cytolyzed at levels higher than 40 μg As cm^{-3}. Fuhua *et al.*[13] also reported tolerance for another freshwater green microalga, *Scenedesmus obliguus.* The 96 h EC_{50} values of *Scenedesmus obliguus* cultivated in AGP culture medium for arsenite and arsenate are 79 μg As dm^{-3} and 160 μg As dm^{-3}, respectively.

In this paper, we focus on the biotransformation of arsenite by the freshwater green microalga *Chlorella vulgaris*, which was isolated from an arsenic-polluted environment in Japan[14] and cultured in our laboratory in freshwater under sterile conditions for more than 15 years. Further findings on arsenic speciation, transformation, tolerance, accumulation and excretion by the alga during the growth phase in an arsenite-enriched culture will be discussed.

EXPERIMENTAL

Culture of *Chlorella vulgaris*

The *Chlorella vulgaris* culture used in this experiment had been maintained under sterile modified Detmer (MD) medium [KNO_3 (1.0 g)], [$CaCl_2$ (0.1 g)], [$MgSO_4{\cdot}7H_2O$ (0.25 g)], NaCl (0.1 g), K_2HPO_4 (0.25 g), $FeSO_4{\cdot}7H_2O$ (0.02 g), H_3BO_3 (2.86 mg), $MnCl_2{\cdot}4H_2O$ (1.81 mg), $ZnSO_4{\cdot}7H_2O$ (0.22 mg), $CuSO_4{\cdot}5H_2O$ (0.08 mg), Na_2MoO_4 (0.021 mg), distilled water (1 dm^3), pH 8] for more than 15 years.

Experimental procedure

Chlorella vulgaris (6 mg dry cells) was placed in 1 dm^3 sterile MD medium. The arsenite concentration in the medium was 10, 20, 30, 50, 70 or 100 μg As cm^{-3}. During the culture period (approx. two weeks), the culture was kept at 25–30 °C under constant aeration (2 dm^3 air min^{-1}). Several fluorescent lamps provided 4000 lux around the flask for 24 h per day. At determined times, suspensions were taken for counting the living cells; the optical densities (640 nm) of the suspensions were proportional to the concentration of cells in suspension (g dry weight dm^{-3} medium). The optical density of the culture was monitored with a U-2000 spectrophotometer (Hitachi). The rest of the suspensions were separated and the cells were washed, dried and analyzed for arsenic compounds associated with *Chlorella vulgaris* after exposure to arsenite.

For the excretion experiment, the algal cells that had been cultured in MD medium containing arsenite at 50 μg As cm^{-3} were harvested, transferred to an arsenic-free MD medium or to pure water, and incubated for one or two days at cell concentrations of about 20 mg dry mass in 100 cm^3 medium. The incubation flasks were kept on a shaker (120 strokes min^{-1}) at 25–30 °C and either kept in the dark or illuminated (4000 lux, 24 h day^{-1}). After incubation the water phase was analyzed for the excretion of arsenic compounds from arsenic-containing *Chlorella vulgaris* into an arsenic-free MD medium or pure water.

For evaluating changes in extracellular arsenic species during incubation of arsenic-free *Chlorella vulgaris* exposed to arsenite, *Chlorella vulgaris* (approximately 0.1 g dry mass) was cultured in an arsenic-free MD medium, then transferred to an MD medium (300 cm^3) containing arsenite at 3.7 μg As cm^{-3}. The culture was kept on a shaker (120 strokes min^{-1}) for 15 days at 25–30 °C under conditions of shaking and continuously illumination (4000 lux, 24 h day^{-1}). The aqueous phase was sampled on days 0, 1, 2, 5, 10 and 15 after inoculation and analyzed for arsenic compounds.

Determination of arsenic species

Cells were harvested by centrifugation, washed with distilled–deionized water, and separated by centrifugation (3000 g, 10 min). The washing procedure was repeated at least twice. The washed cells were dried at 60 °C for 24 h to constant mass.

For determination of total arsenic in the cells, the dried algal cells (6–10 mg) were mineralized in the presence of 50% magnesium nitrate (2 cm^3) at 60 °C for 12 h and were ashed at 550 °C for 6 h in a furnace. The resulting ash was dissolved in 10 mol dm^{-3} hydrochloric acid (10 cm^3), 40% aqueous potassium iodide solution (1 cm^3) was added, the solution was extracted twice with chloroform (5 cm^3 each time), the chloroform phase was back-extracted with 0.02% aqueous magnesium nitrate solution (2 cm^3) and the aqueous phase was analyzed for arsenic with a Nippon Jarrell Ash graphite furnace (AA-890) equipped with an argon flameless atomizer, (FLA-1000).

Inorganic and methylated arsenic compounds in the dry cells (6–10 mg) were determined by hydride generation – atomic absorption spectrophotometry (HG AA) after digestion with 2 mol dm^{-3} NaOH (5 cm^3) at 90–95 °C for 3 h in an aluminum heating block. The digest was treated with 5 cm^3 of 4% $NaBH_4$ in 0.1 mol dm^{-3} NaOH in pH 6.2 buffer

solution (0.125 M Tris— HCl) to reduce arsenite to arsine.[15,16] Arsenate and the methylated arsenic compounds were reduced with 5 cm^3 of 10% $NaBH_4$ in 0.1 mol dm^{-3} NaOH at pH 1. The arsines generated were cooled with liquid nitrogen and collected in a U-trap. When the U-trap was warned, the arsines volatilized in the sequence of their boiling points [AsH_3 −55 °C,CH_3AsH_2 2 °C, $(CH_3)_2AsH$ 35.6 °C (747 mmHg),$(CH_3)_3As$ 52 °C (736 mmHg)] and were passed through a quartz-tube atomizer and determined with an atomic absorption spectrometer (Nippon Jarrell Ash AA-890). Triplicate analyses were performed for each sample.

Sodium arsenite ($NaAsO_2$) and disodium arsenate (Na_2HAsO_4) were used as authentic samples for arsenite and arsenate compounds, respectively. Methylarsonic acid (MAA), dimethylarsinic acid (DMAA) and arsenobetaine (AB) were used as authentic samples for methylarsenic (MA), dimethylarsenic (DMA) and trimethylarsenic compounds (TMA), respectively. These three authentic methylated compounds (MAA, DMAA and AB) were subjected to hot-base digestion, and then hydride generation with borohydride to provider standards for methyl-, dimethyl- and trimethylarsine production. The concentrations of all arsenic compounds are expressed in μg As g^{-1} in cells (based on dry mass) and μg As cm^{-3} in the aqueous phase. The absolute detection limits for total arsenic and arsenic speciation in a single injection were 0.5 ng and 5 ng, respectively. The coefficients of variation for total arsenic and the arsenic species were below 5%.

RESULTS AND DISCUSSION

Growth curve and arsenite tolerance for *Chlorella vulgaris*

Chlorella vulgaris was cultured in an MD medium containing arsenite at concentrations ranging from 0 to 100 μg As cm^{-3} (Fig. 1). The lag phase for *Chlorella vulgaris* was approx. 24 h in the media with arsenite in the range 0–50 μg As cm^{-3}, but 50–100 hours in the media with 70–100 μg As cm^{-3}. The growth of *Chlorella vulgaris* was unaffected by arsenite up to a concentration of 50 μg As cm^{-3}. Algal growth was markedly inhibited at higher concentrations of arsenite. Surprisingly, the growth rate was 10–50% higher at 10 – 20 μg As cm^{-3} than in the arsenic-free medium. Arsenite at concentrations higher than 50 μg As cm^{-3} was toxic to *Chlorella vulgaris* and cell growth was suppressed.

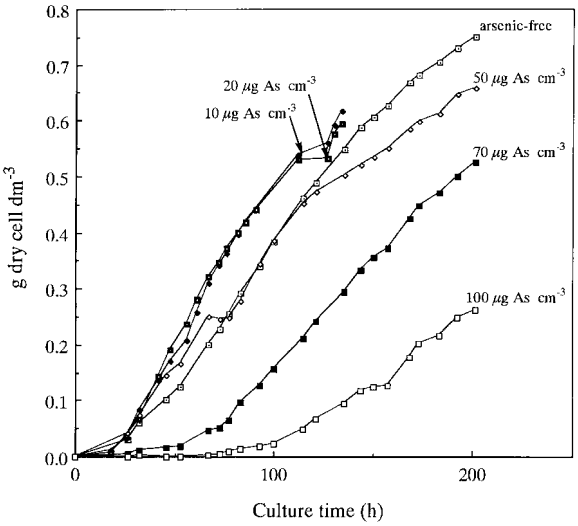

Figure 1 Growth curves for *Chlorella vulgaris* in an MD medium containing sodium arsenite at 0 – 100 μg As cm^{-3}.

Total arsenic and arsenic compounds associated with *Chlorella vulgaris*

During growth in an MD medium with arsenite at 30 μg As cm^{-3}

The concentration of total arsenic and arsenic compounds associated with *Chlorella vulgaris* during growth in an MD medium with arsenite at 30 μg As cm^{-3} are summarized in Fig. 2 and Table 1. At the beginning of the log phase (36 h), the total arsenic concentration associated with the cells rose to a maximum of 610 μg As g^{-1} and then decreased during the period from approx. 40 to 120 h after inoculation.

The predominant arsenic compound associated with the cells throughout the culture period was arsenate. Arsenite had been partially biotransformed into arsenate, methylarsenic, dimethylarsenic and trimethylarsenic compounds during the first 36 h of exposure. During the period from 120 to 225 h after inoculation, the concentration of arsenite, arsenate and methylated arsenic compounds remained almost constant. Early in the log phase (36 h after inoculation), dimethylarsenic compounds had the highest concentration (61 μg As g^{-1} dry mass) among the methylated arsenic

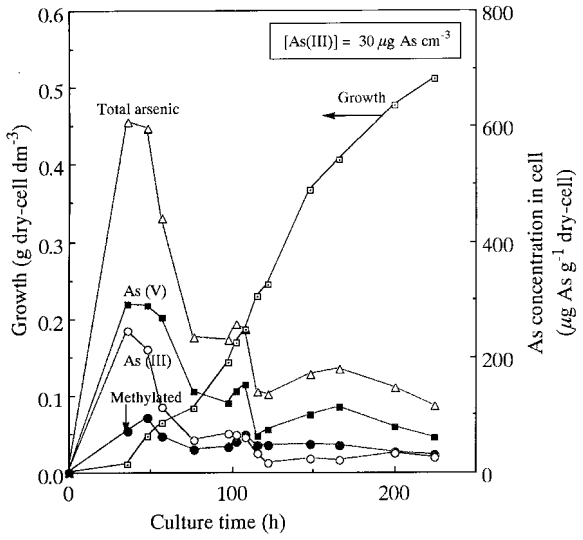

Figure 2 Growth curve for *Chlorella vulgaris* in an MD medium containing arsenite at 30 μg As cm^{-3}, and concentrations of total arsenic, arsenite, arsenate and methylated arsenic compounds in dry cells.

species. The concentration of dimethylarsenic compounds and trimethylarsenic compounds never exceeded ≈ 30 μg As g^{-1} (Table 1). Treatment with a hot solution of sodium hydroxide converts arsenobetaine to trimethylarsine oxide and arsenosugars to dimethylarsinic acid.[17–19,21] Consequently, the dimethyl- and trimethyl-arsenic compounds detected in the algal samples could also have come from these complex organic arsenic compounds. These experimental results indicate that arsenite was transformed by *Chlorella vulgaris* to arsenate through bio-oxidation, and to a small degree to methyl-, dimethyl-, and trimethyl-arsenic compounds through biomethylation.

After exposure to arsenite at 0 – 100 μg As cm^{-3}

The concentrations of arsenic compounds associated with *Chlorella vulgaris* from MD media with concentrations of arsenite in the range 0 – 100 μg As cm^{-3} are summarized in Table 2 for cells that had been exposed to arsenite for 36 h.

At 36 h, arsenate was always the major arsenic compound associated with the cells (51–83% of total arsenic associated with the cells). The arsenate concentration decreased with increasing concentration of arsenite in the medium. The highest arsenate concentration (880 μg As cm^{-3}) and the lowest arsenite concentration (90 μg As cm^{-3}) were found for *Chlorella vulgaris* grown for 36 h in a medium with 10 μg As cm^{-3}. Only mono- and di-methylarsenic species were found when *Chlorella vulgaris* was exposed to arsenite at $\leq$50 μg As cm^{-3}. Trimethylarsenic species appeared when *Chlorella vulgaris* had been cultured in media at arsenite concentration $\geq$70 μg As cm^{-3}. Total arsenic associated with the cells decreased with increasing concentration of arsenite in the medium (Table 2).

After 225 h of growth, arsenate was still the

Table 1 Arsenic compounds associated with *Chlorella vulgaris* grown in MD medium containing arsenite at 30 μg As cm^{-3} during the culture period of 225 h

Culture period (h)	Dry mass of cells (g dm^{-3})	(As associated with cells μg As g^{-1} dry mass)					
		Total	Arsenite	Arsenate	MA	DMA	TMA
0	0	0	0	0	0	0	0
36	0.01	610	250	290	12	61	0
48	0.05	600	210	290	32	35	26
57	0.06	440	110	270	20	22	19
77	0.08	230	55	141	9	15	15
98	0.14	230	67	120	15	15	14
103	0.17	260	64	140	11	18	25
109	0.19	276	59	152	14	21	30
116	0.23	140	31	62	13	17	17
122	0.24	130	15	73	13	16	17
148	0.37	170	22	99	16	16	17
167	0.41	179	20	111	15	19	14
200	0.48	147	33	79	10	14	11
225	0.51	115	25	60	9	11	10

MA, methylarsenic compounds; DMA, dimethylarsenic compounds; TMA, trimethylarsenic compounds; RSD = ± 5%.

Table 2 Arsenic compounds associated with *Chlorella vulgaris* grown in MD medium containing various concentrations of arsenite after a culture period of 36 h

Arsenite concn in medium (μg As cm^{-3})	Arsenic associated with cells at 36 h (μg As g^{-1}, dry mass)					
	Total	Arsenite	Arsenate	MA	DMA	TMA
0	0	0	0	0	0	0
10	1060	90	880	44	41	0
20	910	170	640	61	38	0
30	610	250	290	11	60	0
50	610	180	340	42	48	0
70	580	180	300	52	25	27
100	650	210	350	40	21	31

MA, methylarsenic compounds; DMA, dimethylarsenic compounds; TMA, trimethylarsenic compounds; RSD = ± 5%.

Table 3 Arsenic compounds associated with *Chlorella vulgaris* grown in MD medium containing various concentrations of arsenite after a culture period of 225 h

Arsenite concn in medium (μg As cm^{-3})	Arsenic associated with cells at 36 h (μg As g^{-1}, dry mass)					
	Total	Arsenite	Arsenate	MA	DMA	TMA
0	0	0	0	0	0	0
10	120	29	78	5	11	0
20	240	55	150	11	8	15
30	115	25	60	9	11	10
50	310	41	230	16	11	14
70	630	95	490	20	13	15
100	370	25	310	15	11	11

MA, methylarsenic compounds; DMA, dimethylarsenic compounds; TMA, trimethylarsenic compounds; RSD = ± 5%.

Table 4 Excretion of arsenic into arsenic-free MD medium or pure water by *Chlorella vulgaris* exposed to arsenite

			Arsenic associated and excreted; μg As g^{-1} (%)					
			Total	Arsenite	Arsenate	MA	DMA	TMA
Arsenic associated with cells[(1)] before incubation			1100(100)	580(51)	490(44)	21(1.5)	20(1.5)	22(2.0)
Arsenic excreted after 1 days incubation	MD medium	light	560(49)	300(27)	250(23)	2.5(0.3)	2.7(0.3)	7.5(0.7)
		dark	520(47)	330(30)	170(15)	1.8(0.2)	4.1(0.4)	4(0.4)
	pure water	light	660(60)	230(21)	420(38)	1.8(0.2)	2.9(0.3)	11(1)
		dark	560(49)	220(20)	320(29)	2.8(0.3)	4(0.4)	15(1.4)
Arsenic excreted after 2 days incubation	MD medium	light	510(46)	320(29)	170(15)	3.5(0.4)	0(0)	13(1.2)
		dark	520(47)	240(22)	260(24)	0(0)	5.1(0.5)	10(0.9)
	pure water	light	450(41)	250(23)	180(16)	4.6(0.4)	3.1(0.3)	13(1.2)
		dark	710(65)	280(25)	420(38)	3.1(0.3)	2.7(0.3)	10(0.9)

[(1)]*Chlorella vulgaris* had been cultured in MD medium containing arsenite at 50 μg As cm^{-3}; RSD = ± 5%.

major arsenic compound (52–83%) associated with the cells (Table 3). The relative concentration of arsenate increased with an increase in the arsenite concentration in the medium. All the cell samples contained arsenite, arsenate, methyl-, dimethyl- and trimethyl-arsenic compounds, except cells that had been grown in a medium containing arsenite at 10 μg As cm^{-3}. These cells contained no trimethylarsenic compounds. The concentration of total arsenic associated with the cells after 225 h of growth was much lower than after 36 h of growth.

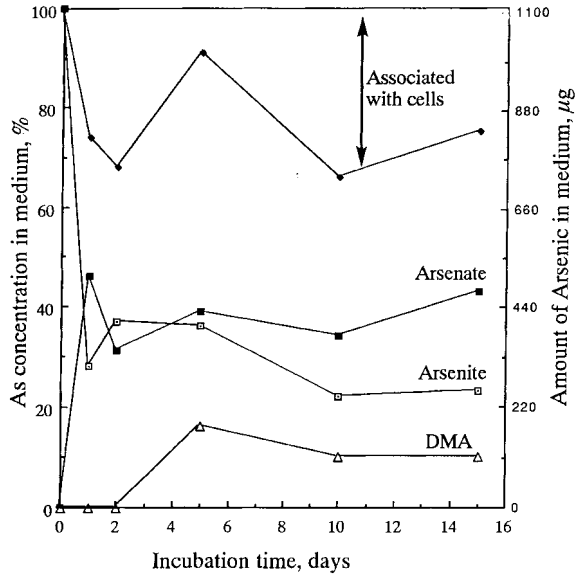

Figure 3 Arsenite, arsenate and dimethylated arsenic in 300 cm^3 MD medium containing arsenite at 3.7 μg As cm^{-3} in the presence of arsenic-free *Chlorella vulgaris.*

Excretion of arsenic compounds from arsenic-containing *Chlorella vulgaris*

The excretion of arsenic compounds from arsenic-containing *Chlorella vulgaris* into an arsenic-free MD medium or pure water are summarized in Table 4. Little growth occurred during the whole incubation period.

Arsenic associated with *Chlorella vulgaris* was excreted into pure water in preference to the MD medium (in three cases out of four) in experiments without illumination. The cells (1100 μg As g^{-1}) excreted 46–49% of the total arsenic into the MD medium irrespective of the illumination. Excretion into pure water (41–65%) under illumination was higher than in the dark after one day's incubation but was lower than in the dark after two days' incubation. These experimental results are in agreement with our previous observation that accumulated arsenic was readily excreted under conditions undesirable for growth of algae which had been exposed to arsenate.[21] Approximately 96% of the released arsenic species were arsenite and arsenate. Under conditions desirable for growth (MD medium and light), more arsenite than arsenate was present in the aqueous phase. Under undesirable conditions (pure water and dark) more arsenate than arsenite was present in the aqueous phase. Among the methylated arsenic compounds released (1.0–2.1%), trimethylarsenic was predominant (0.4–1.4%).

Change in extracellular arsenic species during incubation of arsenic-free *Chlorella vulgaris* exposed to arsenite

The algae grew hardly at all during the incubation period. During the first day the amount of arsenite in the medium decreased from 1110 μg As (100%) to approx. 310 μg As (28%) (Fig. 3). About 47% of the arsenite was oxidized to arsenate. Arsenite in the medium increased to 390 μg As (35%) on day 2 and then decreased to approx. 240 μg As (22%) on day 15. Dimethylated arsenic compounds (180 μg As, 16%) were detected on day 5 after incubation. Methylarsenic and trimethylarsenic species were not found in the medium.

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