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Methylation of inorganic arsenic by arsenic-tolerant freshwater algae

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Five arsenic-resistant freshwater algae which had been isolated from an arsenic-polluted environment were studied for the biotransformation of arsenic compounds accumulated by them from the aqueous phase. The algal cells bioaccumulating arsenic were digested by 2 mol dm^{-3} NaOH at 95°C, the As–C bonds except for As—CH_3 were cleaved by the treatment and the methylated arsenic compounds were reduced to the corresponding arsines by sodium borohydride (hydride generation). The arsines were chromatographically separated on the basis of their boiling-point difference and determined by atomic absorption spectrophotometry.

Methylated arsenic compounds were found in all algal cells. The predominant arsenic species in the cells, however, were non-methylated arsenic compounds which were mainly present in the residue of a chloroform–methanol extract. The non-methylated arsenic compounds were found to be not present in the free inorganic arsenic substrate and to be bound strongly with proteins or polysaccharides in the cells. Methylated arsenic compounds were found mainly in the lipid-soluble fractions and the major form was a dimethyl arsenic compound. Trimethyl- and monomethylarsenic compounds were detected but at very low level. The dimethylarsinic acid was not present in the free form in the lipid-soluble fraction and should be bound with a lipid molecule.

It was also found that the accumulation of arsenic by *Nostoc* occurred only in living cells.

Keywords: Arsenic, biomethylation, freshwater algae, bioaccumulation, metabolic inhibitor

INTRODUCTION

Marine macro-algae generally accumulate arsenic at high levels up to about 100 mg As kg^{-1} dry cell (μg g^{-1}) from seawater and biotransform it to organoarsenic compounds at high rates, ranging from a few per cent to almost 100% arsenic accumulated.[1–11] Monomethyl- and dimethylarsenic compounds were found in water extracts of macro-algae.[2,12–15] Edmonds and Francesconi isolated two arsenosugars from *Ecklonia radiata*.[15–17] Arsenobetaine is found in several higher marine organisms, but not in macro-algae. Inorganic arsenic in seawater is estimated to be first dimethylated by macro- and micro-algae and subsequently be trimethylated by the higher trophic level organisms to form arsenocholine or arsenobetaine in the sea ecosystem.

Freshwater algae also bioaccumulate and bioalkylate arsenic.[17–20] Few papers, however, have been reported on the methylation of inorganic arsenic by freshwater algae.

The authors have isolated arsenic-tolerant freshwater algae from arsenic-polluted sites.[21,22] Five pure freshwater algae were isolated, which are all tolerant to a liquid medium containing arsenic at levels of 2000 μg g^{-1} or more. In a previous paper,[21] the resistance to arsenic impact and the bioaccumulation of arsenic by *Chlorella vulgaris* Beijerinck var. *vulgaris* was reported. This paper describes the experimental results on the biotransformation and metabolism of arsenic by the five pure freshwater algae isolated by the authors. Arsenic uptake inhibition caused by metabolic inhibitor treatment to *Nostoc* sp. is also discussed.

EXPERIMENTAL

Arsenic-tolerant algae species and the pure culture

The five freshwater algae, which had been screened, isolated from micro-organisms living in arsenic-polluted sites, and identified by Isamu Umezaki (Kyoto University, Japan), were precultured pure in the following arsenic-free media: *Chlorella vulgaris* Beijerinck var. *vulgaris*—Modified Detmer;[21] *Hydrocolium* sp.—GFS;[23] *Phormidium* sp.—Modified Detmer; *Nostoc* sp.—MA;[24] *Michrochaete* sp.—Modified Detmer. The culture suspension at the stationary phase (20 cm^3) was inoculated into the respective culture medium above (5 dm^3), containing 217 $\mu g\, g^{-1}$ of elemental arsenic as Na_2HAsO_4 [abbreviated as As(V)] and cultured under standardized germ-free conditions of illumination (fluorescent light, 7000 lx, 12 h day^{-1}) at room temperature, with water-saturated air bubbling (2 $dm^3\, min^{-1}$), until the stationary phase was reached.

Chlorella, *Nostoc* and *Microchaete* species were harvested by a continuous high-speed centrifuge (CM-60RN, Tomy Seiko Co. Ltd), and *Hydrocolium* and *Phormidium* were harvested by use of glass filters. The algae were resuspended in the culture media (arsenic-free) and collected by centrifuging (Model 05P-21, Hitachi) at 4000 rpm for 20 min.

Arsenic adsorbed on the cell surface (except for *Nostoc* cells) was removed by repeating the procedure twice. *Nostoc* cells were subsequently resuspended in MA medium, cleaned by means of an ultrasonic wave cleaner and the extracellular peptide-glycan-free cells were collected by a centrifuge.

Determination of total and methylated arsenic

Total arsenic was determined by the dry-ashing method described in previous papers, and analysed by atomic absorption spectrophotometry.[22-24]

For the determination of the methylated arsenic compounds, the dried algal cells, *ca* 10 mg, were digested with 5 cm^3 of 2 mol dm^{-3} NaOH at 90–95°C for 3 h by use of an aluminium heating block. By this treatment, bonding of arsenic with intracellular compounds or groups except for the methyl group are cleaved and non-methyl-, monomethyl, dimethyl-, and trimethylarsenic compounds are converted to arsenate, methanearsonate, dimethylarsinate, and trimethylarsine oxide, respectively.[25] These four arsenic compounds were reduced with sodium borohydride ($NaBH_4$) to arsine, methylarsine, dimethylarsine, and trimethylarsine, respectively. The arsine gases generated were conducted with helium carrier gas into three drying tubes and then frozen out in a liquid-nitrogen-cooled U-trap which was packed with quartz wool. Their separation was accomplished by volatilization upon warming the U-trap to room temperature. The arsines successively borne out of the trap by the carrier gas were passed through a quartz tube atomizer and determined on an atomic absorption spectrometer. Figure 1 shows the spectra of arsine (A), monomethylarsine (MMA), dimethylarsine (DMA), and trimethylarsine (TMA). Disodium arsenate (Wako Pure Chemical Industries Ltd), sodium methane arsonate (Pfaltz and Bauer Inc.), dimethylarsinic acid (Wako Pure Chemical Industries) and arsenobetaine (TRI Chemicals Laboratory) were used as authentic samples for A, MMA, DMA, and TMA, respectively. Limits of the detection were approximately 1 ng and linear calibration

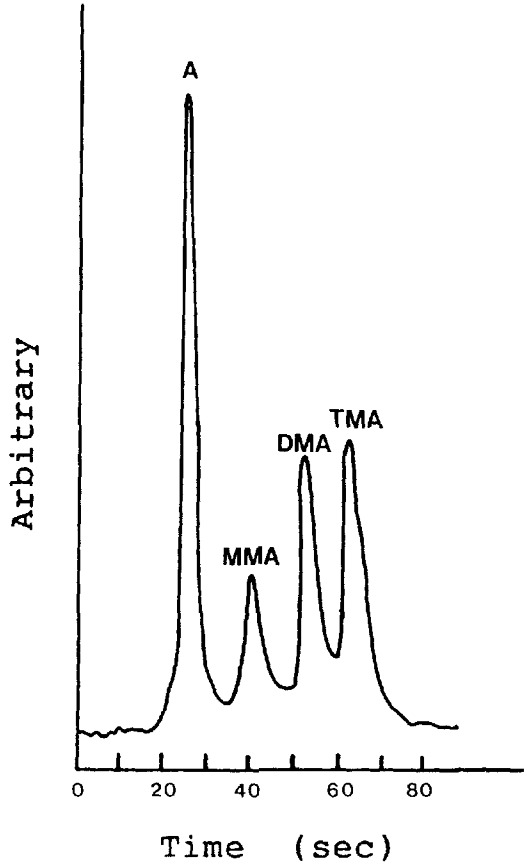


Figure 1 Atomic absorption spectra of arsine (A), monomethylarsine (MMA), dimethylarsine (DMA) and trimethylarsine (TMA) which were generated by digestion with 2N NaOH (90~95°C) and then by reduction with $NaBH_4$ of disodium arsenate, sodium methane arsonate, dimethylarsinic acid, and arsenobetaine, respectively.

curves were obtained in the range from 1 to 30 ng arsenic for each of the arsines.

Extraction of algae

Wet cells were homogenized with chloroform/methanol (2:1) using a homogenizer, the slurry was filtered under reduced pressure through a filter paper, and the residue was washed several times with the mixed solvent. The residue contained the protein, insoluble polysaccharides and nucleic acids. The filtrate was combined with the washes and shaken with one-quarter of their total volume of water, the mixture was allowed to stand at room temperature overnight, and the upper phase (water-soluble) and the lower phase (lipid-soluble) were separated and evaporated to dryness. The whole cells, the residue and the two fractions were analysed for total and methylated arsenic.

RESULTS AND DISCUSSION

Methylated arsenic compounds in biochemical components of algae

Yields of the extraction and concentrations of total and methylated arsenic compounds in biochemical components of the five algae are summarized in Table 1. In this Table, total arsenic is the sum of A, MMA, DMA and TMA, and was satisfactorily coincident with the total arsenic which was determined by the dry-ashing method. Data on whole cells shows that almost all of the

Table 1 Extraction yields and arsenic concentrations in biochemical components of various algae

		As concentration (mg kg^{-1} dry wt)				
Component	Yield (%)	Total As	A	MMA	DMA	TMA
Chlorella						
Whole cell	—	310	310	d	d	d
Proteins and polysaccharides	79	330	330	d	nd	nd
Lipid-soluble	17	60	10	nd	50	nd
Water-soluble	4	210	140	10	60	d
Hydrocolium						
Whole cell	—	440	—	—	—	—
Proteins and polysaccharides	98	300	300	nd	nd	nd
Lipid-soluble	1	280	140	nd	140	nd
Water-soluble	1	2520	2520	nd	nd	nd
Phormidium						
Whole cell	—	1950	1950	nd	nd	nd
Proteins and polysaccharides	94	3000	3000	nd	nd	nd
Lipid-soluble	5	110	100	nd	10	nd
Water-soluble	1	nd	nd	nd	nd	nd
Nostoc						
Whole cell	—	860	860	nd	d	nd
Proteins and polysaccharides	90	640	640	nd	nd	nd
Lipid-soluble	8	60	nd	nd	60	nd
Water-soluble	2	230	140	nd	90	nd
Microchaete						
Whole cell	—	250	250	nd	nd	nd
Proteins and polysaccharides	84	324	324	nd	nd	nd
Lipid-soluble	3	8	nd	nd	8	nd
Water-soluble	13	342	342	nd	nd	nd

Abbreviations: nd, not detected; d, detected but in a small amount; A, inorganic arsenic; MMA, DMA, TMA refer to mono-, di- and tri-methylarsenic species respectively.

arsenic was detected as arsine. The peaks of the other arsines were absorbed by the tailing of the large arsine peak.

In protein and polysaccharide fractions, the predominant form of arsenic is also non-methylated. Dimethylarsenic compounds were found in lipid-soluble and water-soluble fractions.

In the lipid-soluble fraction, 50 out of 60 (in *Chlorella*), 140 out of 280 (in *Hydrocolium*), 10 out of 110 (in *Phormidium*), 60 out of 60 (in *Nostoc*) and 8 out of 8 (in *Microchaete*) mg As kg^{-1} dry wt were found as dimethylarsenic compounds. As can be seen from Table 1, in lipid-soluble fractions of *Nostoc* and *Microchaete* all of the arsenic occurred as dimethyl compounds.

In water-soluble fractions 60 out of 210 (in *Chlorella*) and 90 out of 230 (in *Nostoc*) mg As kg^{-1} dry wt were found as dimethylarsenic compounds. In the other three algae, no DMA was detected.

TMA was detected only in *Chlorella* whole cells in trace amounts. MMA was determined only in the water-soluble fraction and was detected in the protein and polysaccharide fraction of *Chlorella*. Dimethylarsenic compounds were therefore found to be the major methylated arsenic compounds.

The above data lead to the conclusion that arsenic is biomethylated in the interior of the cell although the methylation ratio to inorganic arsenic taken up was small.

The quantity of arsenic which was detected as arsine was large, but no arsenic was thought to be present as free inorganic arsenic compounds (see below).

Effect of the extracellular peptide-glycan of *Nostoc* sp. on arsenic accumulation and methylation

Nostoc cells were cultured in MA medium containing 217 $\mu g\,g^{-1}$ of As(V) at 25–30°C for 76 days, the cells were harvested and washed with arsenic-free MA medium, and half of the cell suspension was dried in vacuum. These cells (Cells B) contain the extracellular peptide-glycan. The other half was ultrasonically cleaned and dried in vacuum. These cells (Cells A) contain no extracellular peptide-glycan. Both cells were analysed for total arsenic and methylarsenic compounds. The results are shown in Table 2.

Arsenic concentrations in Cells B with the extracellular peptide-glycan are smaller than those in Cells A without peptide-glycan. This result means that little arsenic was adsorbed on the extracellular peptide-glycan and that arsenic was transported across the cell membrane.

Effect of extracellular arsenic concentration and arsenic oxidation state on bioaccumulation and methylation by *Nostoc* sp.

Nostoc cells were grown in MA medium containing 10 $\mu g\,g^{-1}$ of As (V) at 30°C for 14 days (Cells C) and 1 $\mu g\,g^{-1}$ of As(V) at 20°C for 10 days (Cells D). The cells were washed with arsenic-free MA but not ultrasonically cleaned and were analysed for total and methylarsenic compounds. The results are shown in Table 2. Dimethyl arsenic compounds (DMA) were detected only at

Table 2 Effects of extracellular peptide-glycan, extracellular arsenic concentration, and arsenic oxidation state on arsenic accumulation and methylation by *Nostoc* sp.

Cell sample	Peptide-glycan	Arsenic in culture medium ($\mu g\,g^{-1}$)	Arsenic concentration in whole cell (mg kg^{-1} dry wt)				
			Total As	A	MMA	DMA	TMA
A	No	As(V) 217	860	860	nd	d	nd
B	Yes	As(V) 217	470	470	nd	d	nd
C	Yes	As(V) 10	50	40	nd	10	nd
D	Yes	As(V) 1	36	34	nd	2	nd
E	Yes	As(III) 1	150	147	nd	3	nd

Abbreviations are explained under Table 1.

very small levels in Cells A, but 20 and 6% of arsenic accumulated were methylated in Cells C and Cells D, respectively. These results suggest that the quantity of methylated arsenic in the interior of the cell was little dependent on arsenic concentration in the exterior of the cell. It can be presumed that bioaccumulated inorganic arsenic was first stored by intracellular insoluble peptides or polysaccharides, and then gradually migrated into the cytoplasm, methylated and incorporated into lipids.

Trivalent arsenic [As(III)] was bioaccumulated by *Nostoc* sp. (Cells E in Table 2) at concentrations about four times larger than pentavalent arsenic [As(V), Cells D in Table 2], but the extent of dimethylation of the former was comparable with that of the latter.

Bonding mode of non-methylated arsenic in the residue of $CHCl_3$–MeOH extracts of *Chlorella* cells

It was found from Table 1 that non-methylated arsenic compounds were present without exception in large quantity in the extraction residues of the five algae tested. The non-methylated arsenic is unlikely to be present as free inorganic arsenic in the extraction residues. In order to investigate the form present, the residue of *Chlorella* cells was further fractionated in the manner shown in Fig. 2 and the fractions were analysed.

Residue A was homogenized with an aqueous solution of BAL with an As:BAL mole ratio of 1:135, and the residue obtained (Residue B) was analysed for arsenic. The results, with others, are shown in Table 3. If arsenic in Residue A was present entirely as free inorganic arsenic compound, it should be extracted effectively by the BAL aqueous solution. However, only 36% of the arsenic in Residue A was extracted.

The bonds C—O—As and C(=O)—O—As are easily hydrolysed and the mono thiol bond with arsenic, RS—As, is substitutable by BAL which has a dithiol linkage; therefore these two types of arsenic compound should be extracted by water and BAL, respectively.

Residue C in Table 3 was obtained by hot-water extraction of Residue A. The arsenic concentration decreased from 330 mg kg^{-1} in Residue A to 250 mg kg^{-1} dry wt in C.

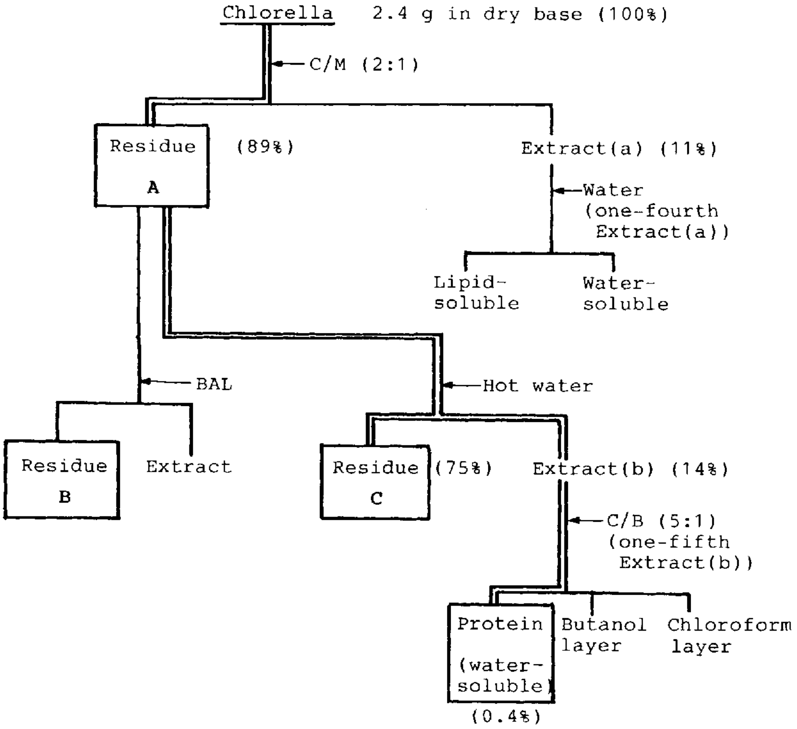


Figure 2 Fractionation of the residue of chloroform–methanol extraction of *Chlorella* cells. Solvent: C, Chloroform; M, Methanol; B, Butanol; BAL, 2,3-dimercapto-1-butanol.

Table 3 Bonding mode of non-methylated arsenic in the Residue (A) of $CHCl_3$–MeOH extracts of *Chlorella* cells

		Total As		Elemental analysis (%)		
Sample[a]	Treatment	(mg kg^{-1})	(%)[b]	H	C	N
Residue A	—	330	—	6.0	36.2	8.9
Residue B	BAL	210	−36%	—	—	—
Residue C	Water	250	−24%	5.9	36.6	9.1
Protein fraction[c]	—	1690	+512%	—	—	6.1

[a]See Fig. 2, [b]Deviation from Residue A (%), [c]Water-extractable protein.

The decrease of arsenic concentration (24%) is not so far from the decrease caused by BAL extraction (36%). The decrease in arsenic concentration by the water extraction was accompanied by a decrease in the weight of 11%.

These results mean that the arsenic decrease of 24% was not only caused by the extraction of free inorganic arsenic but also caused by the extraction of arsenic bound with water-soluble components in Residue A.

Elemental analyses (Table 3) and IR analysis of Residues A and C showed that the main components of both residues are proteins and that both have almost the same chemical components. The water-extractable proteins contain arsenic at a level of 1690 mg kg^{-1} (Table 3) which is five-fold and seven-fold larger than those of arsenic contents of Residues A and C, respectively.

The above experimental results lead to the conclusion that the arsenic in Residue A is likely to be bound (perhaps to protein) with stronger bonding forces than that of As–BAL. Suggested bonding of arsenic in Residue A is shown in Scheme 1.

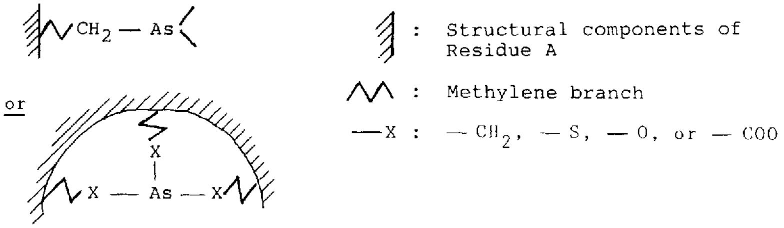
The bond CH_2—As is cleaved by wet digestion with the hot 2 mol dm^{-3} NaOH, which is verified by the digestion of arsenobetaine to produce trimethylarsenic oxide or trimethylarsenic hydroxide. The bond is quite stable to BAL, so the methylene binding arsenic is unextractable with BAL.

Analysis of lipid-soluble fraction of *Nostoc* cells

As shown in Table 1, arsenic in the lipid-soluble fraction of *Nostoc* was all in the organic (dimethyl) form. Phosphatidylarsenocholine was speculated to be in the phospholipid fraction of *Daphnia magna*.[26] An attempt was made to fractionate further the lipid-soluble fraction to simple, glyco- and phospho-lipids in order to investigate the chemical forms of the arsenic. The chloroform solution of the lipid-soluble fraction was loaded onto a silica gel ('Silicic acid', 100-mesh, Mallinckrodt Chemical Works) column and eluted successively with ten times the column volume of chloroform, acetone and methanol. The three eluted solutions were concentrated under reduced pressure to dryness and confirmed simple, glyco- and phospho-lipids respectively on the basis of IR spectroscopy. Table 4 shows the yields of the fractionation and the concentrations of methylated arsenic compounds.

Table 4 shows that non-methylated, monomethyl and trimethyl arsenic compounds were not present in any lipids and that dimethylarsenic alone was present predominantly in simple and glyco-lipids.

The three lipid fractions were analysed by TLC using 'Silica Gel 60 Plate F_{254} (Merck)' as a plate, chloroform–methanol–water (60:30:5) as eluent and a phosphomolybdic acid (10%)



Scheme 1

Table 4 Fractionation of lipid-soluble fractions of *Nostoc* cells and the concentration of methylated arsenic compounds

Lipid fractionated	Yield (%)	Concentration of methylated arsenic ($mg\,kg^{-1}$ dry wt)				
		Total	A	MA	DMA	TMA
Simple lipid	33	54	nd	nd	54	nd
Glycolipid	15	6	nd	nd	6	nd
Phospholipid	52	33	nd	nd	33	nd

Table 5 TLC analysis of simple, glyco-, and phospho-lipids of *Nostoc* cells

Sample	R_f value								
Simple lipid					0.63	0.73	0.79		
Glycolipid				0.51	0.63	0.72		0.82	0.85
Phospholipid			0.41		0.62	0.73	0.78	0.82	0.85
Dimethylarsinic acid	0.30								
Phosphatidylcholine		0.38							

Eluent: Chloroform–methanol–water (60:30:5), Chromogenic agent: Phosphomolybdic acid, 10% ethanol solution.

Table 6 Effects of metabolic inhibitors on arsenic accumulation by *Nostoc* cells

Metabolic inhibitor			Arsenic accumulated in cell during 15-day incubation ($mg\,kg^{-1}$ dry cell)
Compound	Concentration ($mmol\,dm^{-3}$)	Attack	
Control	—	—	94
DNP	0.1	Oxidative phosphorylation	74
NaN_3	1	Hill reaction	36
BAL	10	Succinate oxidase	0.0
EDTA	1	Metallic enzyme	63
SPP		Mg^{2+}, Mn^{2+} and Zn^{2+}-enzyme	9.1
OTC	0.1	Protein biosynthesis	3.8
GLAL	2.5(%)	Fatal	0.0
Ethanol	70(%)	Fatal	0.0

Incubation conditions: shake culture, in dark, 20–23°C, 15 days. Medium: MA medium (pH 7) containing 2.0 $\mu g\,g^{-1}$ of As(V). Inhibitors: DNP, dinitrophenol; NaN_3 sodium azide; BAL, 2,3-dimercapto-1-propanol; EDTA, ethylenediaminetetra-acetic acid; SPP, sodium pyrophosphate; OTC, oxytetracycline hydrochloride; GLAL, glutaraldehyde.

ethanol solution as a chromogenic agent, and the results were compared with those of two authentic samples of dimethylarsinic acid and phosphatidylcholine ('Phospho Lipid—PCE', Nippon Seika Ltd). The experimental results are summarized in Table 5.

The results in Tables 5 and 4 show that the three lipids contain dimethylarsenic compounds, but that no free dimethylarsinic acid was present in the lipids. The spot of R_f 0.41 in phospholipid is close to that of phosphatidylcholine, but they are assigned to different compounds because the phospholipid does not contain trimethylarsenic compounds as shown in Table 4.

Effect of metabolic inhibitors on arsenic accumulation by *Nostoc* cells

Nostoc cells which had been precultured in arsenic-free MA medium were treated with solutions containing the metabolic inhibitors listed in Table 6 at room temperature for 24 h, separated by a centrifuge and washed with pure water. The treated cells were inoculated in MA medium containing $2\,\mu g\,g^{-1}$ of As(V), incubated at room temperature for 15 days, and then the separated cells were analysed for total accumulated arsenic. The experimental results are summarized in Table 6.

As shown in Table 6, no arsenic was accumulated by cells killed with glutaraldehyde or ethanol. It was also found that even if the metabolic systems of the cells were only partially inhibited, arsenic accumulation was largely lowered. These results mean that arsenic accumulation by *Nostoc*, similarly to that by *Chlorella*,[22] occurs only under living conditions, as distinct from the uptake of heavy metals such as uranium, cadmium and copper by *Chlorella* cells.[27]

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