

Cyanobacteria produce arsenosugars

Shin-ichi Miyashita,^{A,C} Shoko Fujiwara,^A Mikio Tsuzuki^{A,B}
and Toshikazu Kaise^{A,D}

^ASchool of Life Sciences, Tokyo University of Pharmacy and Life Sciences,
1432-1 Horinouchi, Hachioji, Tokyo 192-0392, Japan.

^BJapan Science and Technology Agency, CREST, 5, Sanbancho, Chiyoda-ku,
Tokyo, 102-0075, Japan.

^CCorresponding author. Present address: Environmental Standards Section, Inorganic Analytical
Chemistry Division, National Metrology Institute of Japan, 1-1-1 Umezono, Tsukuba,
Ibaraki 305-8563, Japan. Email: shinichi-miyashita@aist.go.jp

^DDeceased November 2009.

Environmental context. Although arsenic is known to accumulate in both marine and freshwater ecosystems, the pathways by which arsenic is accumulated and transferred in freshwater systems are reasonably unknown. This study revealed that freshwater cyanobacteria have the ability to produce arsenosugars from inorganic arsenic compounds. The findings suggest that not only algae, but cyanobacteria, play an important role in the arsenic cycle of aquatic ecosystems.

Abstract. Metabolic processes of incorporated arsenate in axenic cultures of the freshwater cyanobacteria *Synechocystis* sp. PCC 6803 and *Nostoc (Anabaena)* sp. PCC 7120 were examined. Analyses of arsenic compounds in cyanobacterial extracts using a high-performance liquid chromatography–inductively coupled plasma mass spectrometry system showed that both strains have an ability to biotransform arsenate into oxo-arsenosugar-glycerol within 20 min through (1) reduction of incorporated arsenate to arsenite and (2) methylation of produced arsenite to dimethylarsinic acid by methylarsonic acid as a possible intermediate product. In addition, *Synechocystis* sp. PCC 6803 cells are able to biosynthesise oxo-arsenosugar-phosphate from incorporated arsenate. These findings suggest that arsenosugar formation as well as arsenic methylation in cyanobacteria possibly play a significant role in the global arsenic cycle.

Additional keywords: arsenic biotransformation, arsenic metabolism, inductively coupled plasma mass spectrometry, *Nostoc (Anabaena)* sp. PCC 7120, photosynthetic prokaryote, *Synechocystis* sp. PCC 6803.

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Introduction

Arsenic circulates widely in the lithosphere, hydrosphere and biosphere. It is a chemically reactive element and affects the physiological and biochemical activities of other elements. Living organisms have developed specific metabolic pathways to transform As encountered in the environment.^[1,2]

Many freshwater and marine microbes can accumulate inorganic As species and reduce arsenate (As^{V} , AsO_4^{3-}) to arsenite (As^{III} , AsO_3^{3-}).^[3–5] As^{V} inhibits oxidative phosphorylation and photophosphorylation, whereas As^{III} is considered to block the SH sites of various proteins.^[6,7] Some bacteria in freshwater and marine environments have the ability to methylate inorganic As species to soluble or volatile methylated forms of As.^[8–10] Conversely, some microbes degrade organic As compounds. Trimethylarsine oxide (TMAO, $(\text{CH}_3)_3\text{AsO}$) has been reported as a product of the microbial breakdown of arsenobetaine (AB, $(\text{CH}_3)_3\text{As}^+\text{CH}_2\text{COOH}$), which is a major As species in marine fauna, under various conditions.^[11] In addition, dimethylarsinoylribosides (usually termed arsenosugars) (Fig. 1), which are the predominant As species in marine algae, can be efficiently degraded to dimethylarsinoylethanol

$((\text{CH}_3)_2\text{AsOCH}_2\text{CH}_2\text{OH})$ by microbes cultured under anaerobic conditions.^[12] Thus, microbes are involved in both the production and degradation of organic As compounds and play an important role in As circulation.

Arsenosugars are thought to play primary roles in the biotransformation and circulation of As in marine ecosystems. More than 15 types of arsenosugars have been isolated and characterised in the marine environment.^[13] The most common types, the oxo-arsenosugars (Fig. 1), contain a dimethylarsinoyl group $((\text{CH}_3)_2\text{AsO}-)$ at the C5 position of D-ribose derivatives, which is readily reduced to a tertiary alkyl arsine structure.^[14] The tertiary alkyl arsines are chemically active; for example, they could easily react with organic halogen compounds to form chemically stable quaternary alkyl arsonium compounds.^[14,15] Thus, the oxo-arsenosugars might have physiological importance. Microbes living in marine environments are likely to have an ability to produce arsenosugars,^[16,17] and several studies have centred on the role of marine microbes in the possible production and biodegradation of arsenosugars.^[18–20] Thus, identification of As metabolites in microbes grown in pure culture, such as eukaryotic microalgae and prokaryotic

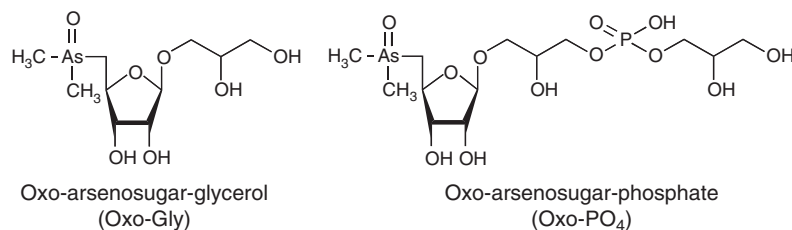


Fig. 1. Structures of the two arsenosugars found in this study.

cyanobacteria, has been performed to confirm biotransformation mechanisms of As after experimental exposure to inorganic As species. Previous reports generally indicate that microalgae (not only marine species (*Dunaliella tertiolecta* and *Phaeodactylum tricornutum*)^[17] but also freshwater ones (*Chlorella vulgaris*,^[21] *Chlorella* sp.,^[22] *Chlamydomonas reinhardtii*^[23,24] and *Monoraphidium arcuatum*^[22]) can produce arsenosugars from incorporated As^V by reduction and methylation processes, and that cyanobacteria (freshwater species, *Microcystis* sp. PCC 7806, *Nostoc* sp. PCC 7120, *Nostoc* sp. and *Synechocystis* sp. PCC 6803) can methylate incorporated As^V by a reduction process but less positively metabolise it.^[10,25,26] However, the production of arsenosugars by axenic cultures of prokaryotic microbes has not yet been proven.

Cyanobacteria are photosynthetic prokaryotes and their ecological diversity is noteworthy. They are important primary producers found in almost every conceivable habitat, from oceans to freshwater systems and from bare rock to soil. They are also found in extreme conditions of temperature, for example in thermal springs or in the desert.^[27] *Synechocystis* sp. PCC 6803 (hereafter, *Synechocystis*) is a free-living freshwater unicellular cyanobacterium and one of the most extensively studied model organisms for the analysis of photosynthetic processes. The genus *Nostoc* (*Anabaena*) consists of nitrogen-fixing filamentous cyanobacteria, and one of the freshwater strains, *Nostoc* (*Anabaena*) sp. PCC 7120 (hereafter, *Nostoc*), has become a model organism for genetic studies of photosynthesis and nitrogen fixation studies. Although commercially available *Nostoc* sp. (*Nostoc commune* var. *flagelliforme*) powder was shown to contain an arsenosugar (oxo-arsenosugar-glycerol, Oxo-Gly),^[26] this has not been demonstrated in isolated species^[25] or axenic cultures of *Nostoc*. Although Yin et al.^[10] demonstrated As methylation by an *arsM* gene product in cyanobacteria including *Synechocystis* and *Nostoc*, they have not yet investigated arsenosugar production (because their aim was to check methylated species, only samples prepared by HNO₃ and microwave treatments, in which sugars might not be preserved,^[28] were analysed). If it is substantiated that cyanobacteria have a mechanism to produce arsenosugars, their new role in the natural cycling of As in the environment as a source of arsenosugars will be elucidated. In the present study, the As toxicity, As uptake and metabolism of water-soluble As species after exposure to As^V during cyanobacterial growth were investigated for verification of the ability of cyanobacteria to produce arsenosugars.

Experimental

Materials

PlasmaCAL single-element standard solutions of 1000 µg mL⁻¹ of As and zirconium were purchased from SCP SCIENCE (Montreal, Canada). Stock solutions containing 1000 µg As mL⁻¹

of each of the following As species were prepared in water: As^{III} from sodium arsenite (NaAsO₂), As^V from disodium hydrogen arsenate (Na₂HAsO₄·7H₂O), methylarsonic acid (MA^V, (CH₃)AsO(OH)₂), dimethylarsinic acid (DMA^V, (CH₃)₂AsO(OH)), trimethylarsine oxide (TMAO, (CH₃)₃AsO), arsenocholine (AC) from AC bromide ((CH₃)₃As⁺CH₂CH₂OH) Br⁻, arsenobetaine (AB, (CH₃)₃As⁺CH₂COOH) and tetramethylarsonium (TMA) from TMA iodide ((CH₃)₄As⁺I⁻) (all the As-containing chemicals were purchased from Tri Chemical Laboratories Inc., Yamanashi, Japan). Oxo-arsenosugar-glycerol (Oxo-Gly) was synthesised following previously reported procedures.^[29] A purified extract of the brown macroalga *Fucus serratus* containing four oxo-arsenosugars, Oxo-Gly, oxo-arsenosugar-phosphate (Oxo-PO₄), oxo-arsenosugar-sulfonate (Oxo-SO₃) and oxo-arsenosugar-sulfate (Oxo-SO₄), was kindly donated by Prof. Kevin A. Francesconi (Karl-Franzens University, Graz, Austria).^[30]

Cyanobacterial culture

Axenic cultures of *Synechocystis*^[31] and *Nostoc*^[32] were obtained from E. I. du Pont de Nemours and Co., Inc. (Wilmington, DE, USA) and Kazusa DNA Research Institute (Kazusa, Chiba, Japan). *Synechocystis* cells were grown axenically in sterilised BG-11 medium (nitrogen content, 18 mM; phosphorus content, 0.2 mM)^[33] containing glucose (5 mM) with vigorous shaking at 120 rpm.^[34] *Nostoc* cells were grown axenically in sterile BG-11 medium with orbital shaking, with or without air-bubbling. The cyanobacterial cells of each strain were incubated in 300 mL of medium in 500-mL Erlenmeyer flasks at 30 and 28 °C, with continuous illumination at 40 µmol photons m⁻² s⁻¹. No other organisms were observed microscopically, and no other organisms could be cultured.

As toxicity test

An As toxicity test based on turbidity and chlorophyll (Chl) measurements was performed to determine an appropriate As^V concentration for cyanobacterial cell exposure because excessive As^V levels may disrupt the process by which As^V is metabolised to arsenosugars. A filter-sterilised 100 mM As^V solution was added to each flask to final concentrations of 0–5 mM in the 300-mL cell suspensions, except for *Nostoc* cells to be grown without air-bubbling (in this case 0–1 mM As^V was applied). At appropriate intervals, turbidity (OD₇₃₀) and Chl levels^[35] were measured.

As^V uptake

A filter-sterilised 100 mM As^V solution was added to 300 mL of a cell suspension at the log phase at the selected concentrations. Cell suspensions (15 mL) were collected from the 500-mL Erlenmeyer flask into different polypropylene centrifuge tubes at appropriate intervals, and cells were harvested by

centrifugation, and rinsed with 10 mL of As-free medium three times and once in 10 mL of water. Different subsamples were used for measurement of As^V uptake and identification of the As compounds (see below). The experiment was repeated three times. The following procedure was performed for total analysis of As in the cells. The cells were solubilised as described previously.^[24] Briefly, the cells were predigested in the presence of conc. HNO₃, and then completely solubilised in a closed-vessel microwave digestion system (speedwave MWS-3⁺; Berghof) after addition of hydrogen peroxide. Zirconium was added to the solutions as a single internal standard element, and the final HNO₃ concentrations were adjusted to ~5 % (v/v).

Total As analysis was accomplished by inductively coupled plasma-mass spectrometry (ICP-MS, ELAN DRC-e; PerkinElmer SCIEX Inc., Concord, Canada) following previously reported procedures.^[24] The following ICP-MS conditions were applied for specific determination of As (as AsO⁺ at *m/z* 91) and zirconium (as ZrO⁺ at *m/z* 106): RF power, 1600 W; nebuliser gas flow, 1.05 L min⁻¹; auxiliary gas flow, 1.4 L min⁻¹; plasma gas flow, 18 L min⁻¹; cell gas flow, 0.7 mL min⁻¹ oxygen (purity: 99.999%); rejection parameter a (RPa), 0; rejection parameter q (RPq), 0.25 (ZrO⁺) and 0.55 (AsO⁺); axial field voltage, 250 V. Dynamic reaction cell (DRC) mode was used for the speciation analysis as well as total As analysis. Both analyses were performed, after optimisation of the oxygen gas flow rate into the cell. The analytical methods were validated through analyses of two certified reference materials (CRMs) (see below). The determined As amount was normalised with culture weight and Chl amount, to determine the total amount of As accumulated into cells in the whole culture and to infer the As content per cell respectively.

Extraction and identification of the As compounds formed by the two cyanobacteria

Biotransformation of incorporated As^V in the cyanobacterial cells exposed to As^V at the concentrations selected by the As toxicity test was confirmed by analyses of water-soluble As metabolites by using a combined ICP-MS and high-performance liquid chromatography (HPLC) system (HPLC-ICP-MS) after the following extraction procedures. The cells collected as described above in the As^V uptake section were freeze-dried,^[24] because removing water completely was necessary for the following extraction carried out with a small precise amount (1 mL) of water. Each suspension in water was then homogenised on ice with a sonicator for 30 s at a power level of 50 W. This process was repeated six times with cooling intervals of 60 s between each sonication until almost all the cells were disrupted as observed by light microscopy. The homogenates were subsequently centrifuged at 1700g for 15 min at 4 °C. From these tubes, ~0.5 mL of each supernatant were filtered through a 0.45-µm membrane filter and placed in 0.6-mL polyethylene vials. The prepared samples were immediately frozen and stored in a deep freezer at -84 °C, and subjected to instrumental analysis within a few days. Extraction yields of water-soluble As metabolites were determined by comparing total As concentrations in the extracts and in the cells. The extraction yield was generally ~100 % of total As (Table S1), suggesting that extracts contained almost all the As compounds present in the cells. This high extractability might have been due to the method applied in this study, and the possibility that unstable macromolecules including As might have been broken down into soluble smaller forms by the vigorous sonication could not be ruled out.

Identification of water-soluble As compounds in the extracts was performed by HPLC-ICP-MS.^[24] A reversed-phase (RP) or strong anion-exchange (SAX) HPLC column was used to separate As compounds. For the RP-HPLC method, the following operating conditions were applied: column, Shiseido CAPCELL PAK C₁₈ MGII (4.6-mm internal diameter × 250-mm length, 5-µm particle size); guard column, Shiseido CAPCELL PAK C₁₈ MGII (4.0-mm internal diameter × 10-mm length, 5-µm particle size); column temperature, 40 °C; mobile phase, 10-mM sodium 1-butansulfonate, 4-mM tetramethylammonium hydroxide, 4-mM malonic acid and 0.05 % methanol (pH 3.0 adjusted with HNO₃); injection volume, 20 µL; flow rate, 1.0 mL min⁻¹. For the SAX-HPLC method, the following operating conditions were applied: column, Hamilton PRP-X100 (4.1-mm internal diameter × 150-mm length, 10-µm particle size); guard column, Hamilton Steel PRP-X100 (2.3-mm internal diameter × 20-mm length, 12–20-µm particle size); column temperature, 40 °C; mobile phase, 20-mM ammonium dihydrogen phosphate (pH 6.0 adjusted with aqueous ammonia); injection volume, 50 µL; flow rate, 1.5 mL min⁻¹.

For identification of As compounds in the extracts, chromatographic peaks with the retention times of eight As compounds (As^{III}, As^V, MA^V, DMA^V, TMAO, AC, AB and TMA) in standard solutions and four oxo-arsenosugars (Oxo-Gly, Oxo-PO₄, Oxo-SO₃ and Oxo-SO₄) in a purified extract of *F. serratus* (Table S2) were compared. Matrix effects seen in the retention times due to extracted cell components were evaluated by spiking standard As species into each of the samples. For quantification of As compounds in the extracts, external calibration curves of peak area versus concentration were employed in the range of 1 to 100 ng As mL⁻¹ for each standard As compound. A calibration curve for DMA^V was also used for quantification of oxo-arsenosugars.^[36,37] When arsenosugars in a *Fucus serratus* extract were quantified with the calibration curve of DMA^V, a good agreement with the reported values^[30] was obtained (reported [Oxo-Gly] = 0.10 ± 0.004 µg As kg⁻¹, obtained [Oxo-Gly] = 0.11 ± 0.008 µg As kg⁻¹ (mean ± s.d.), 8.0 % relative standard deviation (RSD), *n* = 3; reported [Oxo-PO₄] = 0.086 ± 0.002 µg As kg⁻¹, obtained [Oxo-PO₄] = 0.089 ± 0.007 µg As kg⁻¹ (mean ± s.d.), 7.6 % RSD, *n* = 3; reported [Oxo-SO₃] = 0.62 ± 0.02 µg As kg⁻¹, obtained [Oxo-SO₃] = 0.66 ± 0.06 µg As kg⁻¹ (mean ± s.d.), 9.1 % RSD, *n* = 3; reported [Oxo-SO₄] = 0.40 ± 0.01 µg As kg⁻¹, obtained [Oxo-SO₄] = 0.38 ± 0.03 µg As kg⁻¹ (mean ± s.d.), 7.7 % RSD, *n* = 3).

The RP-HPLC column was used for both identification and quantification of the various As species, whereas the SAX-HPLC column was used only for identification because faster-eluting cations (TMAO, AC and TMA) and neutral ions (As^{III}, AB and Oxo-Gly) cannot be correctly quantified by this column due to overlapping peak areas. Column recoveries of water-soluble As compounds (including unknown species, which were quantified by using standards with similar retention times) in extracts of *Synechocystis* and *Nostoc* cells exposed to As^V were reasonably low, especially in *Synechocystis* (Table S1). This might be due to decomposition or retention of some extracted compounds on the column. Because the hidden As species as a fraction is potentially interesting for this study, the time course of this non-eluting As fraction in each cyanobacterial extract is shown in Fig. S1.

The analytical methods were validated through analyses of NMIJ CRM 7402-a codfish tissue (certified [total As] = 36.7 ± 1.8 mg As kg⁻¹, found [total As] = 34.0 ± 0.34 mg As kg⁻¹

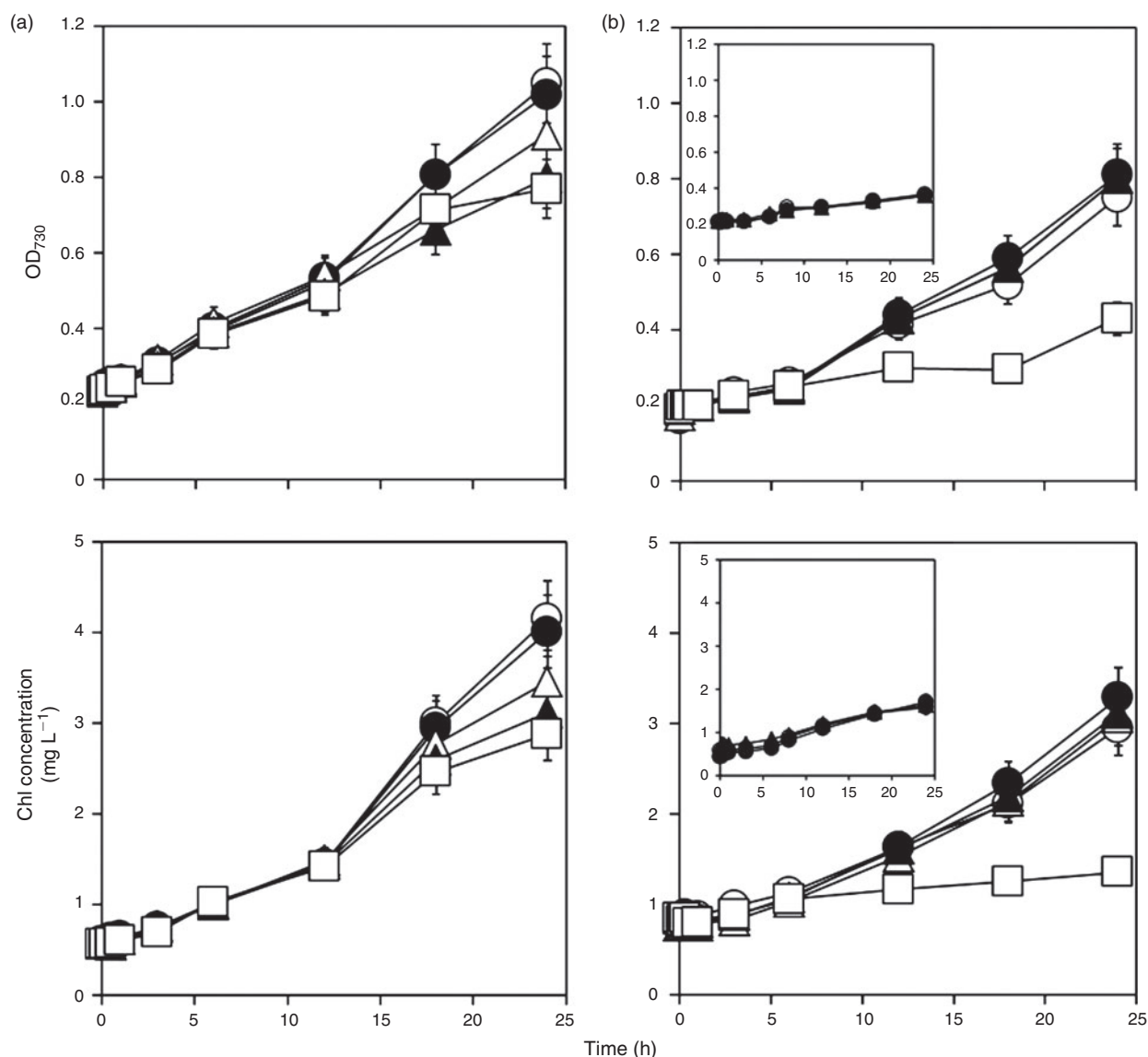


Fig. 2. Growth curves for *Synechocystis* and *Nostoc* grown in As^V-free (○, control) and As^V-containing (●, 0.1 mM; △, 0.5 mM; ▲, 1 mM; □, 5 mM) media with shaking for 24 h. Cyanobacterial growth in the liquid medium was monitored by measuring the optical density at 730 nm (OD₇₃₀) and chlorophyll-*a* (Chl) concentrations for (a) *Synechocystis* and (b) *Nostoc* (with air-bubbling). The insert shows growth curves for *Nostoc* grown without air-bubbling in As^V-free (○) and As^V-containing (●, 0.1 mM; ▲, 1 mM) media. Data are means ± s.d. for three independent experiments.

(mean ± standard deviation (s.d.)), 0.99 % RSD, $n = 3$; certified [AB] = 33.1 ± 1.5 mg As kg⁻¹, found [AB] = 30.9 ± 0.72 mg As kg⁻¹, 2.3 % RSD, $n = 3$)^[24] and NMIJ CRM 7901-a AB solution (certified [AB] = 10.27 ± 0.26 mg As kg⁻¹, found [AB] = 10.12 ± 0.07 mg As kg⁻¹, 0.7 % RSD, $n = 3$).

Results

As toxicity test

Cell growth curves of *Synechocystis* and *Nostoc* in the presence of As^V are shown in Fig. 2. In *Synechocystis* (Fig. 2a), cells exposed to 0.1 mM As^V grew at almost the same rate as the control cells cultivated in the absence of As^V ($P = 0.21$ at 24 h), whereas the cell growth was slightly depressed by 0.5–5 mM As^V after 18 h in a concentration-dependent manner ($P < 0.001$ at 18 h and < 0.002 at 24 h). The 50 % inhibitory concentration (IC₅₀) value for inhibition by As^V was > 5 mM. In *Nostoc* grown with air-bubbling (Fig. 2b), cells exposed to 0.5 and 1 mM As^V grew at almost the same rate as the control cells ($P > 0.08$ at

24 h), whereas the cell growth was slightly stimulated by 0.1 mM As^V ($P = 0.011$ at 24 h). It was strongly suppressed by 5 mM As^V ($P < 0.001$ at 24 h), but the cells survived. Without air-bubbling (Fig. 2b, inset), cells exposed to 0.1 and 1 mM As^V grew at almost the same rate as the control cells. The IC₅₀ values for inhibition by As^V were > 5 mM for air-bubbled cells and > 1 mM for non-air-bubbled cells. Accordingly, for monitoring As metabolites, As^V concentrations of ≤ 5 and ≤ 1 mM seemed to be appropriate for *Synechocystis* (without air-bubbling) and *Nostoc* (with or without air-bubbling) respectively. Thus, the cyanobacteria showed high resistance to As^V; the IC₅₀ values for *Synechocystis* and *Nostoc* were beyond the concentrations tested (> 5 or > 1 mM), whereas that of the green alga *Chlamydomonas reinhardtii* CC-125 was 0.3 mM.^[24]

As^V uptake and biotransformation of As^V

Although the incorporation of As into cells was repressed immediately after the addition of As^V in both strains (Fig. 3e, f,

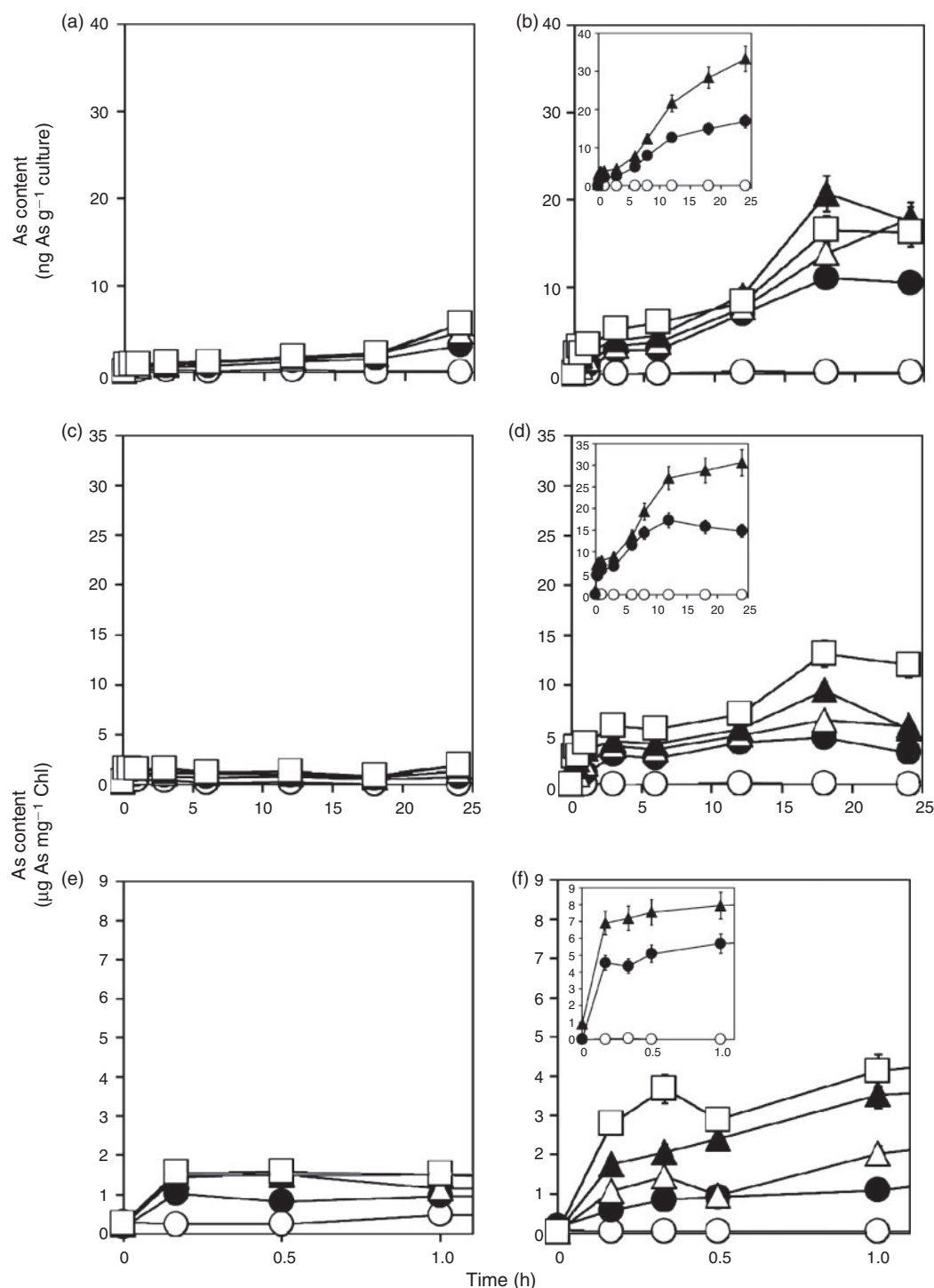


Fig. 3. Time courses of total As in (a, c) *Synechocystis* and (b, d) *Nostoc* during incubation in As^V-free (○, control) and As^V-containing (●, 0.1 mM; ▲, 0.5 mM; ▲, 1 mM; □, 5 mM) media with shaking for 24 h. The aeration conditions were the same as in Fig. 2. The insert shows time courses of total As in *Nostoc* grown without air-bubbling in As^V-free (○) and As^V-containing (●, 0.1 mM; ▲, 1 mM) media. Total As contents in the cyanobacterial cells were determined by inductively coupled plasma–mass spectrometry after microwave-assisted acid digestion. (e) and (f) are extended figures with a better time-course resolution of the early period of (c) and (d). Data are means ± s.d. for three independent experiments.

Table S1; the addition of As^V was done at time 0 in the graphs), the intracellular As content per gram of culture increased as the growth proceeded (Fig. 3a, b). Under these conditions, As molecules in the cyanobacterial extracts were analysed by HPLC-ICP-MS (Figs 4, 5, 6). The results demonstrated that the cells accumulated As^V as the most abundant As species,

followed by As^{III} (Figs 4, 5a, 6a, Table S1). In addition, arsenosugars Oxo-Gly and Oxo-PO₄ were detected along with MA^V, DMA^V and relatively low levels of unknown As compounds (U1 (eluted at 3.5 min between DMA^V and Oxo-PO₄ in RP-HPLC, and most likely, 0.84 min with As^{III} in SAX-HPLC) and U2 (eluted at 13 min after Oxo-Gly in RP-HPLC, and

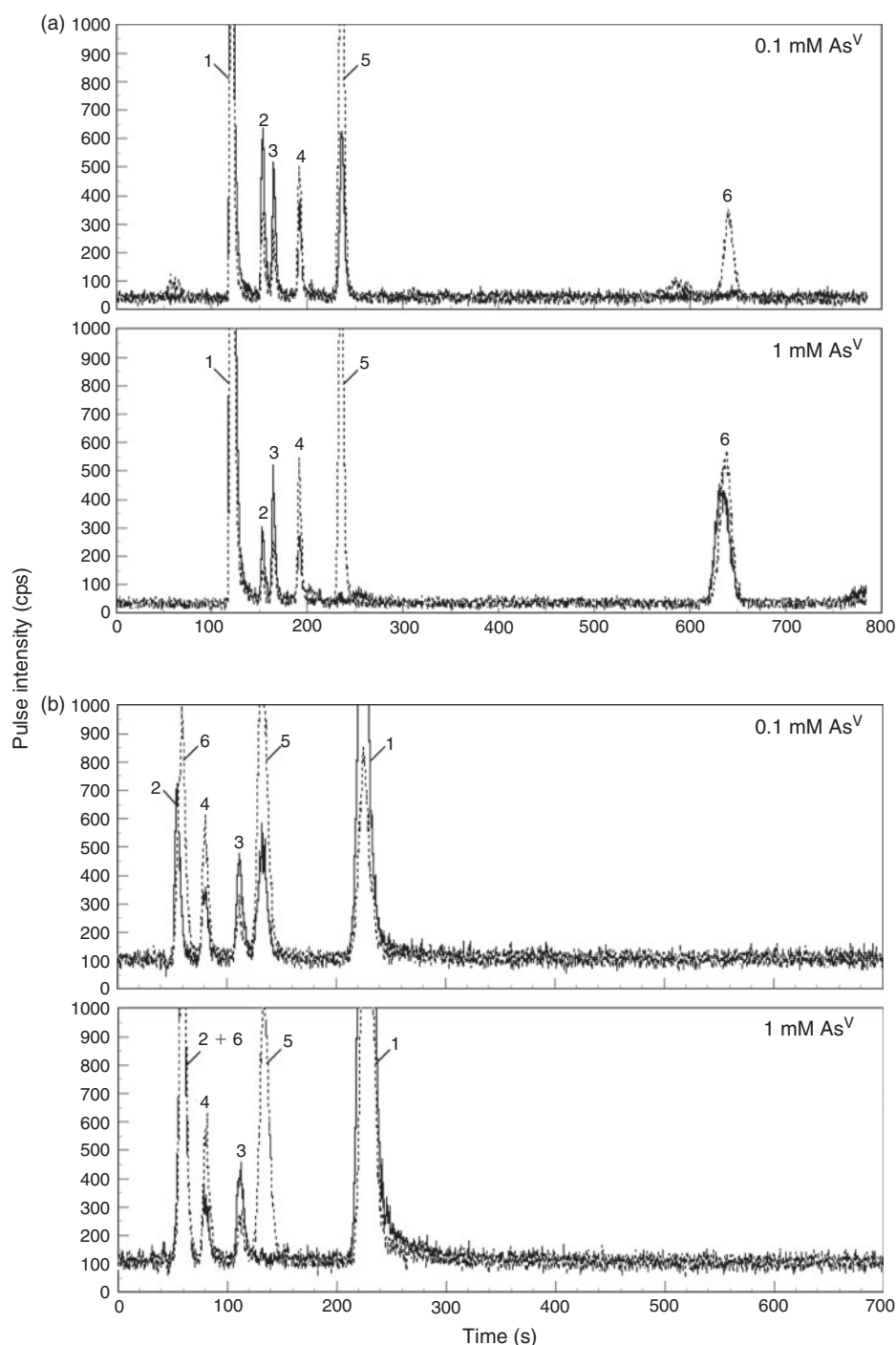


Fig. 4. Representative high-performance liquid chromatography–inductively coupled plasma–mass spectrometry (HPLC-ICP-MS) chromatograms of water extracts from *Synechocystis* after exposure to 0.1 and 1 mM As^{V} for 18 h. Chromatographic separation was carried out by (a) ion-pairing reverse-phase HPLC or (b) strong anion-exchange HPLC with ICP-MS detection (see text for details). Chromatograms of overlaid spikes are indicated by dotted lines. As species are indicated by numbers: (1) As^{V} ; (2) As^{III} ; (3) MA^{V} ; (4) DMA^{V} ; (5) Oxo- PO_4 ; (6) Oxo-Gly. The unit of intensity (cps) stands for counts per second.

presumably, 8.1 min after As^{V} in SAX-HPLC)) (Figs 4, 5, 6, Table S1). These arsenosugars as well as other As metabolites were identified by HPLC-ICP-MS not only using the RP column (Fig. 4a) but also with the SAX column (Fig. 4b).

Discussion

The results demonstrated that the freshwater cyanobacteria *Synechocystis* sp. PCC 6803 and *Nostoc* sp. PCC 7120

incorporate As into sugars as Oxo-Gly and Oxo- PO_4 . This is the first report clearly demonstrating that cyanobacteria have an ability to produce arsenosugars, which could well have important implications for freshwater lakes and hot springs occurring in the vicinity of soils rich in As (there is in fact a report of arsenosugars in hot spring mats^[38]).

The following metabolic pathway has been suggested for the formation of arsenosugars in algae.^[4,39] Initially, As^{V} is reduced

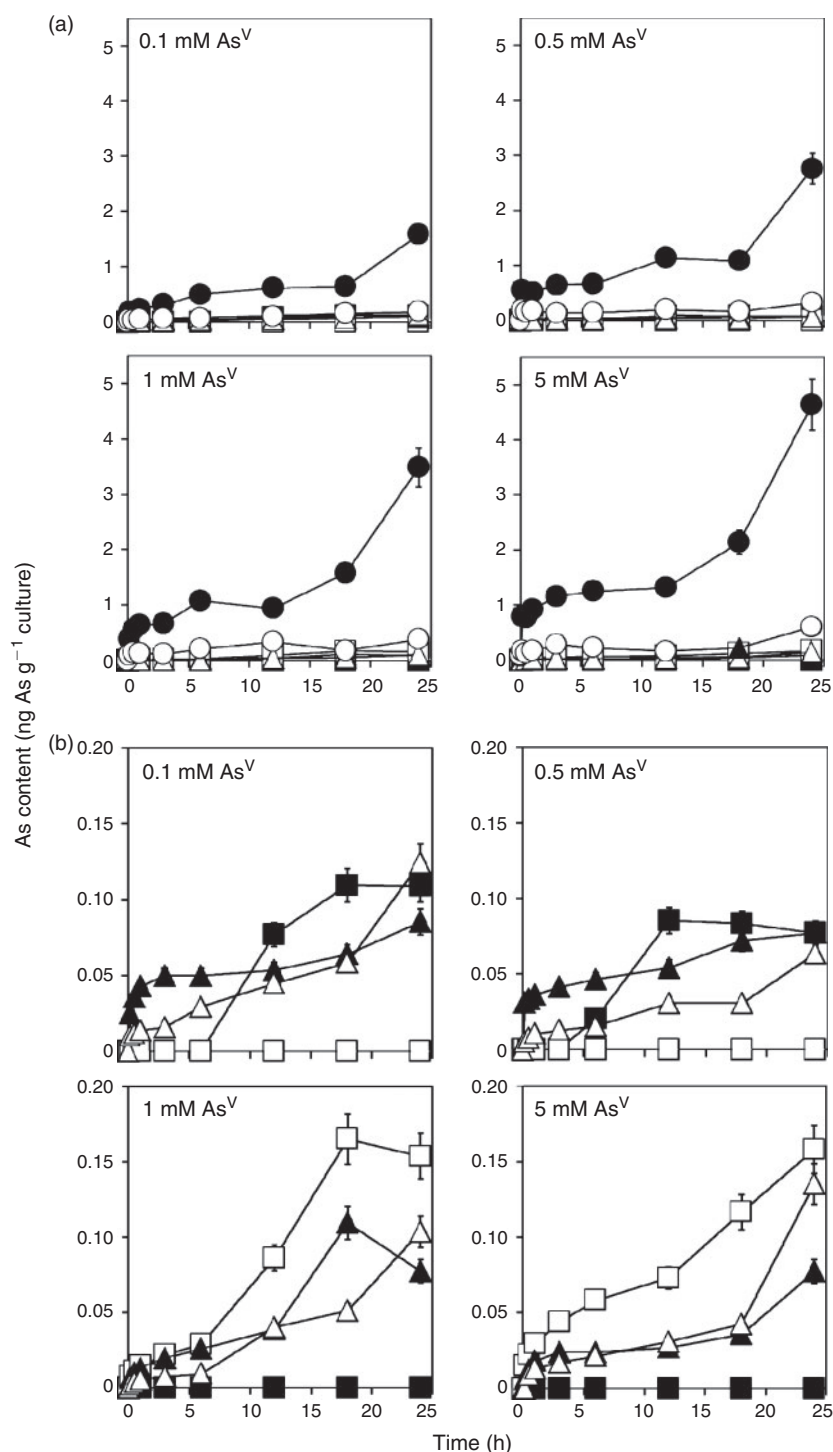


Fig. 5. Amounts per gram of culture of As species accumulated in *Synechocystis* during incubation with As^V-containing media with shaking for 24 h: (a) inorganic and organic species and (b) organic species. Chromatographic separation was carried out by ion-pairing RP-HPLC with ICP-MS detection (see text for details). Data are means $\pm$ s.d. for three independent experiments. Key: ○, As^{III}; ●, As^V; △, MA^V; ▲, DMA^V; □, Oxo-Gly; ■, Oxo-PO₄.

to As^{III} by reductase(s) and stepwise methylated in an oxidative methyl transfer from *S*-adenosyl-L-methionine (SAM) to DMA^V by methyltransferase(s).^[40] In algae, the production of arsenosugars is a subsequent step from the methylation of As^{III}.^[4,39] DMA^V is thought to be reduced to dimethylarsinous acid ((CH₃)₂As(OH)) by a reductase and then oxidised by the addition of the adenosyl group from SAM. This nucleoside,

referred to as dimethylarsinyladenosine, then undergoes glycosidation to produce a range of arsenosugars. In cyanobacteria, As^V is reduced to As^{III} and methylated to DMA^V,^[10] thereafter, DMA^V is possibly biotransformed into Oxo-Gly and Oxo-PO₄ by reduction, addition of a ribose group and glycosidation, as suggested by Lai et al.^[26] and this study. Now that the arsenosugar formation has been verified in *Synechocystis* sp. PCC

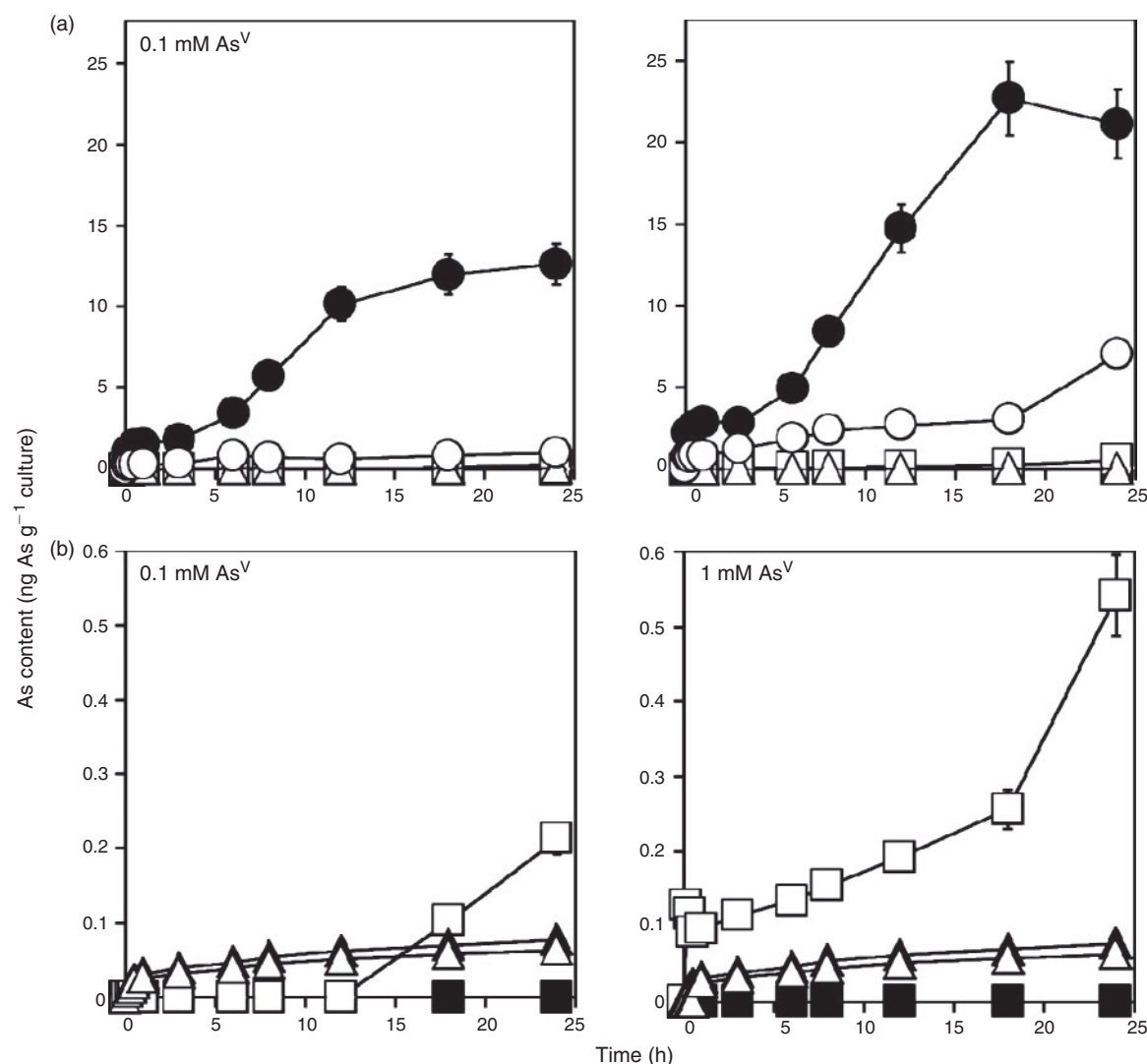


Fig. 6. Amounts per gram of culture of As species accumulated in *Nostoc* during incubation with As^{V} -containing media with shaking for 24 h: (a) inorganic and organic species and (b) organic species. Chromatographic separation was carried out by ion-pairing reverse-phase high-performance liquid chromatography with inductively coupled plasma–mass spectrometry detection (see text for details). Data are means $\pm$ s.d. for three independent experiments. Key: $\circ$, As^{III} ; $\bullet$, As^{V} ; $\triangle$, MA^{V} ; $\blacktriangle$, DMA^{V} ; $\square$, Oxo-Gly; $\blacksquare$, Oxo- PO_4 .

6803 and *Nostoc* sp. PCC 7120, it is expected that the biosynthetic mechanism will be elucidated through well developed molecular biological analytical techniques. For example, once the enzymes can be purified, the genes could be cloned reasonably easily using the genome sequences which have been completely determined, and the functions of the gene products could be analysed by both in vivo and in vitro experiments.

Interestingly, the types of oxo-arsenosugars changed, depending on the cyanobacterial species and the As^{V} concentrations (Figs 4, 5, 6, Table S1). In *Nostoc* sp. PCC 7120, Oxo-Gly was the only oxo-arsenosugar detected (Fig. 6, Table S1), as in *N. commune* var. *flagelliforme*.^[26] Thus, the genus *Nostoc* generally may not have the ability to produce Oxo- PO_4 . Because Oxo- PO_4 has been shown to be degraded to Oxo-Gly by an in vitro enzyme (glycerophosphorylcholine diesterase) reaction,^[40,41] Oxo- PO_4 might be biosynthesised from Oxo-Gly by some oppositely oriented reaction in cyanobacteria, and *Nostoc* may not have the responsible enzyme(s). Alternatively, As^{V} concentrations (≥ 0.1 mM) in the medium in this study might have been too high for *Nostoc* sp. PCC 7120 to produce

Oxo- PO_4 ; As^{V} would competitively inhibit the biosynthesis of phosphate-containing compounds such as Oxo- PO_4 . In *Synechocystis*, Oxo- PO_4 was detected at 0.1–0.5 mM, whereas Oxo-Gly was found at 1–5 mM (Figs 4, 5, Table S1). At higher concentrations of As^{V} , As^{V} possibly inhibits the biosynthesis of a phosphate-containing compound, Oxo- PO_4 , as described above. At lower concentrations of As^{V} , Oxo-Gly may not be detected because the conversion from the possible precursor Oxo-Gly into Oxo- PO_4 is not a limiting step under these conditions.

Concerning the time course of the arsenosugar production, the amount of Oxo-Gly, normalised to Chl content, continued to increase in *Nostoc*, whereas those of Oxo-Gly and Oxo- PO_4 reached a peak quickly relative to Oxo-Gly in *Nostoc* and then decreased in *Synechocystis*. This may be due to the difference in time taken for the intracellular As concentration to change; the total As amount in *Nostoc* increased gradually, whereas that in *Synechocystis* reached a plateau quickly (Fig. 3c–f). The cause of this is still unclear. However, it might be due to a difference in activities of phosphate and As^{V} transport and As efflux systems.

Table 1. Maximum As concentrations and transformation yields to organic As species in the cyanobacteria

Data are means of three independent experiments. The maximum transformation yields were calculated as ratios of all identified organic As species, methylated As species or arsenosugars to the sum of As species in the extract

Cells	As ^V concentration in the medium (mM)	Maximum As concentration in the cells ($\mu\text{g As}$ mg^{-1} Chl)	Maximum transformation yield (%)		
			All identified organic As species	Methylated As species	Arsenosugars
<i>Synechocystis</i>	0.1	1.0	36	36	10
	0.5	1.5	21	20	7.6
	1	1.8	36	23	13
	5	2.0	20	13	7.0
<i>Nostoc</i> (without air-bubbling)	0.1	17	9.8	9.8	1.5
	1	31	6.4	3.6	4.0

Or it might be simply due to the difference in cellular morphology and accessibility of As^V to each cell surface. *Nostoc* is filamentous and aggregates easily; thus, a lower exposed cell surface area may delay over-all influx of As^V into the cells, and thereby the subsequent repression of As^V uptake, which is normally seen immediately after the incorporation of As^V, may be retarded.

In *Nostoc*, the As accumulation levels relative to Chl levels in cells grown without air-bubbling were much higher than those in cells grown with air-bubbling (Fig. 3d, f). Cells grown without air-bubbling divide more slowly than air-bubbled cells (Fig. 2b). Therefore, each single cell in a non-air-bubbled culture may be exposed to more As than in the air-bubbled cultures. In addition the possibility that cells in a non-air-bubbled culture might be smaller and have more As due to a larger surface area of the cells cannot be ruled out, although cellular size did not seem to differ between the cultures microscopically.

When compared with another study^[25] using a culture of *Nostoc* sp., their results showed a similar As^V concentration-dependency in As^V uptake to the current work. In our study, the As accumulation levels relative to Chl levels in cells cultured for 24 h in 0.1-, 0.5- and 1-mM As^V-containing media were 27, 47 and 47%, shown as values relative to that in a 5-mM As^V-containing medium. In the study of Maeda et al. the As levels per dry weight in cells grown for 2 weeks in 0.14, 0.67 and 1.4 mM As^V were 10, 36 and 50%, shown as values relative to that in 6.7-mM As^V-containing medium. These findings suggest that the As accumulation is not proportionate to As^V concentration in the medium.

Biotransformation of intracellular As^V and As^{III} into simply methylated As compounds and further arsenosugars may prevent As^V from replacing the phosphate in ATP and As^{III} from reacting with the -SH group of an enzyme. In *Synechocystis* and *Nostoc*, however, the percentage of organic As compounds was reasonably low (≤ 36 and $\leq 9.8\%$ in total As in the extracts (the percentages were relatively higher at lower As concentrations in the medium); Figs 5, 6, Table 1), as in *Phormidium* sp.,^[42] suggesting that the major detoxification process in cyanobacteria is not the production of organic As compounds but reduction to As^{III} and expulsion from cells. Thus, the methylation of As and the production of arsenosugars seem to be mainly performed for a purpose(s) other than detoxification (e.g. production of lipid-soluble As compounds for membrane lipid bilayers). Many marine algae have arsenolipids including phosphatidyl dimethylarsinyl ribose (usually termed phosphatidylarsenosugar, an arsenoribosyl derivative of phosphatidyl glycerol and a derivative of Oxo-PO₄),^[43–45] and the arsenolipids

are speculated to be included in the lipid bilayer structures of the algal outer membrane.^[44] If cyanobacteria produce similar arsenolipids, they may be located at the cell membrane or thylakoid membrane, as well as phosphatidyl glycerol.

There may be a possibility that some part of the water-soluble As compounds present in extracts are not present in intact forms, due to the vigorous sonication. The stability of As species during sonication has been investigated in our previous study.^[24] The study demonstrated that our sonication procedure induced few structural changes in water-soluble As compounds, but both oxidation of As^{III} and Thio-DMA^V ((CH₃)₂AsS(OH)) and degradation of Oxo-PO₄ should be taken into account in considering the original forms of As metabolites in As^V-exposed cells. Furthermore, Oxo-Gly and Oxo-PO₄ might be partially derived from the degradation of ribose-containing, lipid-soluble As compounds, such as phosphatidylarsenosugar (its precursors possibly include Oxo-Gly or Oxo-PO₄).^[43] Freshwater organisms, including the green algae *C. reinhardtii* and *Cladophora glomerata*, possess lipid-soluble or water-unextractable As species to some extent.^[23] Therefore, analysis of such kinds of compounds should be required for understanding As behaviour in living organisms in freshwater ecosystems. In addition, the identification of other water-soluble compounds including unknown compounds U1 and U2 would be desirable. Based on their retention times in a similar chromatographic system to ours,^[18] U1 might be cationic or neutral species, such as dimethylarsinoylethanol (degradation product of arsenosugars^[40]) or trimethylated arsenosugar,^[46] and U2 might be an anionic species, such as dimethylarsinoylethylacetate and dimethylated arsenosugar.^[47]

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