Arsenic biotransformation by a cyanobacterium *Nostoc* sp. PCC 7120^{*}

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ABSTRACT

Nostoc sp. PCC 7120 (*Nostoc*), a typical filamentous cyanobacterium ubiquitous in aquatic system, is recognized as a model organism to study prokaryotic cell differentiation and nitrogen fixation. In this study, *Nostoc* cells incubated with arsenite (As(III)) for two weeks were extracted with dichloromethane/methanol (DCM/MeOH) and the extract was partitioned between water and DCM. Arsenic species in aqueous and DCM layers were determined using high performance liquid chromatography - inductively coupled plasma mass spectrometer/electrospray tandem mass spectrometry (HPLC-ICPMS/ESI/MS/MS). In addition to inorganic arsenic (iAs), the aqueous layer also contained monomethylarsonate (MAs(V)), dimethylarsinate (DMAs(V)), and the two arsenosugars, namely a glycerol arsenosugar (Oxo-Gly) and a phosphate arsenosugar (Oxo-PO₄). Two major arsenosugar phospholipids (AsSugPL982 and AsSugPL984) were detected in DCM fraction. Arsenic in the growth medium was also investigated by HPLC/ICPMS and shown to be present mainly as the inorganic forms As(III) and As(V) accounting for 29%–38% and 29%–57% of the total arsenic respectively. The total arsenic of methylated arsenic, arsenosugars, and arsenosugar phospholipids in *Nostoc* cells with increasing As(III) exposure were not markedly different, indicating that the transformation to organoarsenic in *Nostoc* was not dependent on As(III) concentration in the medium. Our results provide new insights into the role of cyanobacteria in the biogeochemical cycling of arsenic.

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1. Introduction

Arsenic is a ubiquitous and carcinogenic toxic element, and has both acute and chronic toxicity effects on humans. The bioavailability of arsenic and its resultant toxicity are influenced to a great extent by its species (Smedley and Kinniburgh, 2002; Sharma and Sohn, 2009). Inorganic arsenic, the major form of arsenic in water and soils, is transformed into organic arsenic species or in reverse in natural biological processes, and microorganisms play a critical role in arsenic biogeochemical cycle (Zhu et al., 2014; Zhang et al., 2017).

To survive in arsenic-enriched environments, organisms have evolved various mechanisms to utilize or detoxify arsenic (Stolz et al., 2006). Following the absorption of As(V) into cells via

phosphate transport systems, a two-step pathway is invoked by organisms to decrease arsenic levels in cells. As(V) is first reduced to As(III), and then As(III) is either pumped out via membrane proteins or stored in vacuoles (Tamaki and Frankenberger, 1992). Methylated arsenic species are also widely found in organisms, especially in many photosynthetic organisms (Ye et al., 2012). Although trivalent methylated arsenic, methylarsonous acid (MAs(III)) and dimethylarsinous acid (DMAs(III)), are much more toxic than As(III), As(III) methylation is considered as a process of detoxification eventually producing MAs(V), DMAs(V), and trimethylarsine oxide (TMAO) (Qin et al., 2006, 2009; Yin et al., 2011). Man-made organoarsenic (e.g. herbicide, roxarsone, nitarosone, and *p*-arsanilic acid) are widely used in the agriculture, and could be biodegraded to inorganic arsenic by microbes, worsening food and water pollution (Yoshinaga and Rosen, 2014; Yoshinaga and Cai, 2011; Chen and Rosen, 2016).

Other, more complex, organoarsenic compounds such as arsenobetaine, arsenosugars and arsenolipids are also found in

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marine (Francesconi, 2005), freshwater (Miyashita et al., 2009, 2012), and terrestrial organisms (Geislinger et al., 1998). So far, more than 20 arsenosugars have been characterized (Nischwitz and Pergantis, 2007), and arsenolipids are also now often reported in marine samples (Glabonjat et al., 2014; Rumpler et al., 2008; Taleshi et al., 2010). However, the biosynthetic processes leading to the production of arsenosugars or arsenolipids are still not well understood.

Cyanobacteria are involved in arsenic biogeochemical cycle, and have been reported to have the ability to methylate inorganic arsenic (Ye et al., 2012; Guo et al., 2016), produce arsenosugars and arsenosugar phospholipids (Xue et al., 2014a). The previous studies showed that *Nostoc* methylated As(III) to DMAs(V) and TMA(O) (Yin et al., 2011), demethylated MAs(V) and MAs(III) into As(III) (Yan et al., 2015), and produced Oxo-Gly (Miyashita et al., 2012). Compared to the extraction with water or nitric acid, the extraction with dichloromethane/methanol (DCM/MeOH) will divide arsenic in cells into water-soluble (such as inorganic arsenic, methylated arsenic, arsenosugars, etc) and lipid-soluble arsenic (such as arsenosugar phospholipids, arsenic-containing fatty acids, arsenic-containing hydrocarbons, etc) (Glabonjat et al., 2014). In this study, HPLC-ICPMS/ESIMS was used to analyze water-soluble and lipid-soluble arsenic species of *Nostoc* in order to understand arsenic biotransformation by *Nostoc* from multiple perspectives. The Oxo-PO₄ and two arsenosugar phospholipids were found in *Nostoc* treated with As(III). For the first time, arsenic demethylation and arsenolipid biosynthesis were found to co-occur in one organism. It has great implications for future studies aiming to fully understand arsenic transformation and fate in the environment.

2. Materials and methods

2.1. Cyanobacteria culture and harvesting

Axenic cultures of *Nostoc* were grown in 150 mL Erlenmeyer flasks containing 50 mL BG-11 medium without NaNO₃ (Rippka et al., 1979) at 28 °C with shaking at 120 rpm under continuous light (40 μmol photons m⁻² s⁻¹). *Nostoc* cells at stationary phase (cell number remains constant during this phase) were treated with 0.1, 1, 10, 100 μM As(III) for two weeks; each treatment was performed in triplicate. *Nostoc* was harvested by centrifuging at 4700 g for 15 min at 4 °C. The sample was separated into two parts: growth medium and cells. The growth medium was filtered using syringe filters (0.2 μm Nylon membrane) (VWR International, West Chester, PA, USA), and stored at -80 °C until arsenic species and total arsenic analysis. The cells were transferred to 15 mL polypropylene tubes with screw-caps after being washed three times with cold MES buffer (Yin et al., 2011), then freeze-dried.

2.2. Fractionation of arsenic in *Nostoc*

The fractionation of arsenic in *Nostoc* was carried out as described in Fig. 1. About 30 mg of freeze-dried cells were weighed (to a precision of 0.1 mg) directly into a centrifuge tube (15 mL, polypropylene), and 5 mL of a mixture of DCM/MeOH (2 + 1, v/v) was added. The mixture was extracted on a rotary wheel overnight, and centrifuged at 4754 g and 4 °C for 15 min.

0.5 mL of 1% aqueous NH₄HCO₃ solution was added to the supernatant (~4.5 mL), and the mixture was gently shaken. After standing for 30 min, the solution was separated into an aqueous layer (upper layer, MeOH and H₂O) and DCM-MeOH layer (lower layer). The aqueous layer was transferred to 2 mL Eppendorf tubes, and stored at -80 °C for later total arsenic analysis and water-soluble arsenic species determination.

A portion of the DCM layer (2 mL) was applied to a 150 × 5 mm

glass Pasteur pipet filled with silica gel 60 (to a height of 4 cm) that was pre-conditioned with 5 mL of 1% formic acid in acetone/MeOH (1 + 1, v/v). The column was washed with pure MeOH (5 mL) firstly, then the arsenolipids were eluted with 5 mL of pure MeOH containing 1% NH₄·H₂O. The arsenolipid fraction was evaporated to dryness, and stored at 4 °C until analysis. 300 μL of ethanol (EtOH) were added to the arsenolipid fraction, and ultrasonicated for 30 min. 200 μL of the arsenolipid fraction was used for analysis after centrifuging for 15 min and filtering with 0.2 μm Nylon filters (ProFills, Markus Bruckner Analysentechnik, Linz, Austria).

The pellet after DCM/MeOH extraction, dried using nitrogen stripping method, was added with 500 μL of H₂O, sonicated for 15 min, and the mixture subjected to repetitive freeze-thawing (6 cycles) before being centrifuged for 15 min. Arsenic species and total arsenic concentration in the supernatant were analyzed. The residue after water extraction was dried for determination of total arsenic concentration.

2.3. Determination of total arsenic concentration

The lyophilized *Nostoc* (about 15 mg), part of all sample extracts, the residue after water extraction, and CRM 7405-a (certified reference material) were analyzed for total arsenic concentration as follows. The samples were weighed into polypropylene digest tubes (50 mL), and 2 mL of a solution of internal standards (100 μg L⁻¹ Ge, In, Te in 1% HNO₃) and 2 mL conc. HNO₃ were added. The samples were transferred to a microwave-accelerated reaction system (Mars CEM, CEM Corporation, Matthews, NC, USA), and digested according to the following temperature program: 0–10 min, 80 °C; 10–30 min, 120 °C; 30–60 min, 160 °C. The clear digest solutions were transferred to polypropylene tubes (15 mL), and diluted with Mill-Q water to 10 mL after being cooled to room temperature.

Determination of total arsenic concentration in the digested solutions were carried out using an Agilent 7500cx ICP-MS in collision cell mode (He, 5 mL min⁻¹) to avoid interferences from ⁴⁰Ar³⁵Cl on ⁷⁵As. The digest and analytical methods were validated against the certified reference material CRM 7405-a (Hijiki), with a certified value for arsenic of 35.8 ± 0.9 mg As kg⁻¹; we obtained 36.2 ± 0.8 mg As kg⁻¹ (n = 3) in this work.

2.4. HPLC-ICPMS/ESIMS analysis of water-soluble arsenic species

Anion-exchange HPLC (Agilent 1100 HPLC system) was carried out with a PRP-X100 column (4.6 × 150 mm; 5 μm particle size;

Fig. 1. The diagram of extracting arsenic compounds from *Nostoc*.

Hamilton Company, Reno, Nevada, USA) and a Hamilton PEEK pre-column at 40 °C, and a mobile phase of (200 mM NH_4HCO_3) under gradient elution conditions: 0–6 min, 30 mM; 6–10 min, 30–200 mM; 10–15 min, 200 mM; 15.1–20 min, 30 mM to re-equilibrate the column prior to the next injection. The flow rate was 1 mL min^{-1} , and the injection volume was 20 μL . Quantification was performed using external calibration against the mixtures of As(V), MAs(V) and DMAs(V). The effluent flow from the anion-exchange column was split to the ICPMS (Agilent 7900) (10%) and to the Triple Quadrupole MS System (Agilent 6460) (90%) (Agilent Technologies, Waldbronn, Germany). Arsenic species were identified both by retention time matching experiments with standard arsenic species (As(III), As(V), MAs(V), DMAs(V)), extracts of *Fucus serratus* containing the four most common arsenosugars (Oxo-Gly, Oxo- PO_4 , sulfonate arsenosugar (Oxo- SO_3), sulfate arsenosugar (Oxo- SO_4)) by ICPMS and protonated molecular ions by ESIMSMS.

2.5. HPLC-ICPMS/ESIMSMS analysis of arsenolipids in *Nostoc* cells

Separation was performed under reversed-phase conditions using a Shodex Asahipak C8P-50 4D column (4.6 $\times$ 150 mm, 5 μm particle size), an aqueous solution containing 0.1% formic acid (A) and pure MeOH containing 0.1% formic acid (B) performed under following gradient condition: 0–20 min 50%–100% B, 20–23 min 100% B, 23.1–30 min 50% B; the flow rate was 0.5 mL min^{-1} and the injection volume was 25 μL . The column effluent was split using a passive splitter with 80% being transferred directly to the ESI-MSMS. The remaining 20% of the split flow was transferred to the ICPMS together with a support flow of water containing 1% formic acid and 20 $\mu\text{g L}^{-1}$ Ge, In, Te (0.3 mL min^{-1}) introduced through a T-piece after the splitter. Carbon compensation was performed by continuous addition of $\text{H}_2\text{O}/\text{MeOH}$ (7 + 3, v/v) delivered with an integrated sample introduction system (ISIS) pump (0.02 rpm) to ensure that a constant carbon content reached the plasma (Raber et al., 2010). Determination of arsenic in the DCM fraction was carried out by ICPMS in no gas mode, with 7% optional gas O_2 , and in platinum setup. ICPMS signal was recorded at m/z 75 (^{75}As or $^{40}\text{Ar}^{35}\text{Cl}$) at dwell times of 300 ms, at m/z 77 ($^{40}\text{Ar}^{37}\text{Cl}$, possible chloride interference), and internal standards at m/z 74 (^{74}Ge), m/z 115 (^{115}In), and m/z 125 (^{125}Te) at dwell times of 100 ms. The parameters for ESMS in the positive ion mode were as follows: gas temperature: 100 °C, gas flow: 12 L min^{-1} , nebulizer pressure: 45 psi, sheath gas temperature: 350 °C, sheath gas flow: 11 L min^{-1} , capillary voltage: 4500 V, and nozzle voltage: 500 V. Acquisition parameters: Fragmentor voltage 220 V; cell acceleration 7; MRM parameters: MRM transition m/z 985/983 to m/z 409, collision energy 30; MRM transition m/z 985/983 to m/z 237, collision energy 50; MRM transition m/z 985/983 to m/z 97, collision energy 70.

3. Results

3.1. Arsenic accumulation in *Nostoc* cells

Increasing arsenic concentration in the medium led to an elevation of arsenic concentration in the cells. After exposure to different concentrations of As(III) for 2 weeks, the total arsenic concentrations in *Nostoc* were measured, and the results showed that *Nostoc* had a strong ability to accumulate arsenic (Table 1), total arsenic concentrations in cells ranged from 11.3 to 1523 mg kg^{-1} (dry weight) when *Nostoc* was exposed to As(III) at concentrations ranging from 0.1 μM to 100 μM . However, the concentration factors (mg As kg^{-1} dry mass of cells/exposure concentration in mg kg^{-1}) for *Nostoc* decreased with increasing arsenic exposure concentrations. For example, the concentration

factor was 555 at 0.1 μM As(III) treatment, which was markedly decreased to 75 at 100 μM As(III) treatment.

3.2. Arsenic partitioning in different fractions of *Nostoc* cells

Each sample of *Nostoc* cells was separated into four fractions: the aqueous layer, the DCM layer, the water layer, and the residue after water extraction (Fig. 1), and total arsenic in each layer was determined (Table 1). Arsenic in the aqueous layers, consisting of inorganic arsenic, methylated arsenic, and arsenosugars (Fig. 2A), accounted for 1.7%–7.1% of the total arsenic accumulated by *Nostoc* cells exposed to As(III). The total concentration of arsenosugars in *Nostoc* cells with increasing As(III) exposure were not markedly different (Fig. S1). The arsenic in DCM layer which consisted mainly of arsenosugar phospholipids (Fig. 3) accounted for 0.4%–30% of the total arsenic concentration in cells. Inorganic arsenic which was detected in the water layer after the pellet was re-extracted with water (Fig. S2A) accounted for 9.7%–29.7% of the total arsenic concentration in cells (Table 1). After being extracted with DCM/MeOH and water, more than half of the total arsenic still remained in the residues (4.0–805 mg kg^{-1}) (Table 1).

3.3. *Nostoc* produced both arsenosugars and arsenosugar phospholipids

Arsenic species in the aqueous layers were analyzed, with two arsenosugars (Oxo-Gly and Oxo- PO_4) being observed following As(III) exposure (Fig. S3). The relative proportion of arsenosugars in total aqueous arsenic species in *Nostoc* cells was 0.5%–13% for Oxo-Gly and 0.7%–8% for Oxo- PO_4 , and decreased with increasing As(III) exposure (Fig. 2A).

Arsenic-containing compounds in the DCM layers were characterized after performing a clean-up procedure with silica. HPLC/ICPMS analysis of the fraction showed the presence of arsenolipids, and analysis using ESMSMS revealed that the $[\text{M}+\text{H}]^+$ of two main arsenic-containing compounds were 983 and 985 (Fig. 3). The chromatographic behavior was the same as that reported previously for arsenosugar phospholipids ($\text{C}_{47}\text{H}_{88}\text{O}_{14}\text{AsP}$ (AsSugPL982) and $\text{C}_{47}\text{H}_{90}\text{O}_{14}\text{AsP}$ (AsSugPL984) in *Synechocystis* sp. PCC 6803 (Xue et al., 2014a) and brown macroalgae (García-Salgado et al., 2012). Fig. 4 shows the structures of arsenosugars and arsenosugar phospholipids produced by *Nostoc*.

3.4. Arsenic species in the growth medium

Arsenic species in the growth medium with and without *Nostoc* incubation were determined after incubation with As(III) for 2 weeks (Fig. 2B and Fig. S2B). In the growth medium with *Nostoc* incubation, inorganic arsenic was the main arsenic species As(III) and As(V) accounting for 29%–38% and 29%–57% of the total arsenic respectively. The contributions of organoarsenic including DMAs and arsenosugars in the medium decreased with increasing As(III) exposure (Fig. 2B). About 16% of total arsenic in the medium containing 0.1 or 1 μM As(III) were DMAs and arsenosugars. While DMAs and arsenosugars accounted for 4% and 1.3% of total arsenic in the medium containing 10 μM As(III). Furthermore, traces of DMAs and Oxo- PO_4 were detected in the medium containing 100 μM As(III) (Fig. 2B). In the medium without *Nostoc* incubation (as the control), inorganic arsenic species alone were detected, and a certain amount of As(III) was oxidated into As(V) in the air after two weeks (Fig. S2B).

4. Discussion

Freshwater cyanobacteria can produce arsenosugar

Table 1 Total arsenic concentrations of *Nostoc* cells treated with indicated concentrations of As(III).

Treatment As(III) (μM)	Total As (mg kg^{-1})	DCM/MeOH supernatant		DCM/MeOH pellet	
		DCM layer (mg kg^{-1})	aqueous layer (mg kg^{-1})	Residue (mg kg^{-1})	Water layer (mg kg^{-1})
0.1	11.3 ± 1.8	2.9 ± 0.7	0.8 ± 0.1	4.0 ± 0.5	1.1 ± 0.3
1	101 ± 6	4.3 ± 1.3	2.8 ± 0.1	54.0 ± 3.8	17.3 ± 0.5
10	225 ± 19	4.4 ± 0.7	8.4 ± 1.4	134 ± 4.7	67.2 ± 7.2
100	1523 ± 315	5.2 ± 0.8	25.5 ± 2.9	805 ± 112	396 ± 72

The amount of arsenic is expressed as mg kg^{-1} (dry mass). The results are shown as mean $\pm$ standard deviation from triplicate culturing samples. **Fig. 2.** Arsenic species percentages in the aqueous layer (A) and the growth medium (B) after *Nostoc* was treated with 0.1, 1, 10, or 100 μM As(III) for two weeks. The error bars represent the standard error of three independent culture experiments.

phospholipids. Two species of arsenosugar phospholipids, AsSugPL982 and AsSugPL984, as previously characterized in *Synechocystis* sp. PCC 6803 (Xue et al., 2014a), were identified in *Nostoc* treated with As(III) for two weeks. In contrast to other studies (Yin et al., 2011; Miyashita et al., 2012), our study found that *Nostoc* cells exposed to As(III) produced Oxo-PO₄ and arsenosugar phospholipids. The discrepancy in the results from *Nostoc* could be related to differences in treatment time and sample extraction methods. Miyashita et al. (2012) treated *Nostoc* cells with As(V) for 24 h, and found that Oxo-Gly alone was present in *Nostoc* extracts obtained by sonicating the cells with water. Moreover, the extraction yield was generally around 100% of total arsenic suggesting that

arsenolipids were not produced by the *Nostoc* cells during the 24 h exposure to As(V). Freshwater organisms, including the green algae *Cladophora glomerata* from the Hayakawa River and *Chlamydomonas reinhardtii* incubated with As(V) for 3 days, were considered to possess water-unextractable or lipid-soluble arsenic species to some extent because the extraction yields of the water-soluble arsenic from the alga were quite low (16% for *C. glomerata*, 32% for *C. reinhardtii*) (Miyashita et al., 2009). In Yin's work (2011) with *Nostoc* cultured with As(III), arsenic-containing compounds in cells were extracted using 1% HNO₃ and microwave heating, treatment that would likely degrade arsenosugars (Nischwitz and Pergantis, 2007) and arsenosugar phospholipids.

Fig. 3. HPLC/mass chromatograms of the lipid extract (post-silica column) of *Nostoc*. ICPMS detection at m/z 75 (arsenic-selective) and represent ions selected on m/z 983 ($RT = 25.6$ min; $C_{47}H_{88}O_{14}AsP$ H^+) and 985 ($RT = 26.1$ min; $C_{47}H_{90}O_{14}AsP$ H^+), respectively. The parameters for ESMS in the positive ion mode were described in Materials and Methods.

Multiple arsenic speciation biotransformation pathways co-occur in *Nostoc*. Microbes have evolved various mechanisms to utilize or detoxify arsenic. Known mechanisms include arsenic redox changes, arsenic methylation and demethylation, As(III) efflux, and the production of complex organoarsenic. The previous proteomics analysis of *Nostoc* under As(V) stress showed that the expression of two genes, *alr1097* (encoding an As(III) efflux protein; *arsB*) and *alr1105* (encoding an As(V) reductase; *arsC*) (Pandey et al., 2013), was up-regulated (Pandey et al., 2012), illustrating that *Nostoc* was capable of performing As(V) uptake, As(V) reduction, and As(III) excretion. Yin et al. (2011) investigated the ability of

arsenic methylation by *Nostoc* and arsenic (III) S-adenosylmethionine methyltransferase (ArsM) from *Nostoc* by chemotrapping volatile TMAs(III). Moreover, our other study showed that *Nostoc* was able to demethylate MAs(III) rapidly to As(III) using ArsI that is a C-As lyase responsible for MAs(III) demethylation, and also could demethylate MAs(V) slowly to As(III) (Yan et al., 2015), suggesting that MAs(V) reduction and MAs(III) demethylation occurred in *Nostoc*. However, MAs(III) oxidation in *Nostoc* cannot be regarded to be catalyzed by an enzyme because ArsH homologous compound oxidizing MAs(III) (Chen et al., 2015a) was not found via blasting against the *Nostoc* proteome with ArsH of *Synechocystis* sp. PCC 6803 (Xue et al., 2014b), and we did not ensure that there was As(III) oxidation catalyzed by As(III) oxidase in *Nostoc* because most of As(III) in the culture medium was oxidized by oxygen after long time culture (Fig. S2B) and there was not As(III) oxidase identified in *Nostoc*. In addition, *Nostoc* was found to produce low quantities of arsenosugars and arsenolipids (Table 1).

An abbreviated biosynthesis pathway of arsenosugar phospholipids in *Nostoc* was hypothesized as described in Fig. 5. The methyl groups from S-adenosyl-L-methionine (SAM) are transferred to As(III) (which is absorbed directly by cells or arises from As(V) reduced by ArsC), by ArsM to produce trivalent methylated arsenic. Some of MAs(III) and DMAs(III) bound to ArsM are further methylated into DMAs(III) and TMAs(III). In addition, the adenosyl group from SAM is transferred to DMAs(III) falling off ArsM to generate the key intermediate of arsenosugar synthesis, namely dimethylarsinyladenosine (AsAd). AsAd undergoes glycosidation to produce Oxo-Gly which acts as a precursor to Oxo-PO₄ produced later by cyanobacteria. Finally, fatty acids were added to Oxo-PO₄ to produce arsenosugar phospholipids by unknown enzymes.

Up to now, arsenic methylation is the most thoroughly studied arsenic biotransformation pathway. Cullen et al. (1994, 1995) verified that SAM was the methyl donor in the arsenic biological methylation in a marine alga *Polyphysa peniculus* and a microorganism *Apiotrichum humicola* by adding L-methionine-methyl-d₃ in the culture medium. Marapakala et al. (2012) applied protein fluorescence of a single-tryptophan mutant to report the binding rate of trivalent arsenic species with ArsM from the thermophilic eukaryotic alga *Cyanidioschyzon* sp. 5508, and analyzed the crystal structures of co-crystallizing ArsM with the aromatic arsenicals PhAs(III) or Rox (III) successively (Marapakala et al., 2015). A model was proposed for the catalytic mechanism of ArsMs in which the products were maintained in the trivalent state by utilizing four conserved cysteine residues of ArsM in a disulfide-bond cascade, and DMAs(III) was accumulated as the principal product (Marapakala et al., 2015). The current results with other ArsMs, such as from *Methanosarcina acetivorans* C2A (Wang et al., 2014) and *Spirulina platensis* (Guo et al., 2016), can be explained by the above model. Moreover, part of DMAs(III) bound to ArsM was

Fig. 4. The structures of arsenosugars and arsenosugar phospholipids found in *Nostoc*.

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