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Arsenate toxicity and metabolism in the halotolerant microalga *Dunaliella salina* under various phosphate regimes

Ya Wang[1], Yanheng Zheng[1], Cong Liu[1], Pingping Xu[1], Hao Li[1], Qiaoyun Lin[1], Chunhua Zhang[2] and Ying Ge[1]

[1] College of Resources and Environmental Sciences, Jiangsu Provincial Key Laboratory of Marine Biology, Nanjing Agricultural University, Nanjing 210095, China

[2] Demonstration Laboratory of Element and Life Science Research, Laboratory Centre of Life Science, College of Life Science, Nanjing Agricultural University, Nanjing, China

Corresponding author: Ying Ge

Address: College of Resources and Environmental Sciences, Jiangsu Provincial Key Laboratory of Marine Biology, Nanjing Agricultural University, Nanjing 210095, China

Telephone: 86-25-84395892

E-mail: yingge711@njau.edu.cn

Abstract

Microalgae play an important role in arsenic (As) biogeochemical cycles as they are capable to accumulate and metabolize this metalloid efficiently. This study aimed to investigate the toxicity, accumulation and transformation of arsenate (As(V)) in *Dunaliella salina*, an exceptionally halotolerant microalga, under various phosphate (PO_4^{3-}) regimes. Results of the 72-h toxicity test showed that *D. salina* was tolerant to As(V). In addition, the toxicity of As(V) was mitigated by increased PO_4^{3-} supply. *D. salina* resisted the adverse effects of As(V) through suppression of As uptake, enhancement of As reduction, methylation in the cell and excretion from the cell. Our study revealed that *D. salina* reduced As(V) toxicity using different strategies, i.e., reduction of As uptake upon acute As stress (24-h) and increase of As efflux

following chronic As exposure (9-day). Moreover, PO_4^{3-} strongly affected the adsorption, uptake and transformation of As(V) in *D. salina*. As(V) reduction, DMA production and As excretion were enhanced under P-limited conditions (0.112 mg L^{-1}) or upon higher As(V) exposure (1120 μg L^{-1}). Furthermore, PO_4^{3-} had a significant influence on the As removal ability of *D. salina*. High As removal efficiency (> 95.6%) was observed in the 5-days cultures at an initial As concentration of 11.2 μg L^{-1} and PO_4^{3-} of 0.112 and 1.12 mg L^{-1}. However, only 10.9% of total As was removed under 11.2 mg L^{-1} PO_4^{3-} after 9 days incubation. The findings of this study illustrate the pivotal roles of extracellular PO_4^{3-} in As(V) toxicity and metabolism, and the results may be relevant for future research on minimization of As contamination in algal products as well as on enhancement of As removal from the environment.

Keywords *Dunaliella salina*; Arsenate ; Phosphate; Accumulation; Transformation

Environmental impact

Certain microalga such as *Dunaliella salina* has a strong accumulation capacity of As, making the algal products prone to As contamination. On the other hand, this microalga may also be potentially used in As bioremediation. The As(V) accumulation and detoxification in this unicellular alga are affected by PO_4^{3-}, but the detailed As metabolism remain unexplored. Here, we report that PO_4^{3-} mediated As (V) toxicity, adsorption, uptake and transformation in *D. salina*. In addition, our data also indicate that the algal harvesting time and PO_4^{3-} monitoring are important in order to minimize As contamination in the algal products and to enhance As removal from environment with high alkalinity and salinity.

Introduction

Arsenic (As) is a notoriously toxic element widely distributed in all environments.[1] In natural water concentrations of arsenic have been reported in the range of <0.5 to 5000 μg L^{-1}, and may reach up to 200 mg L^{-1} in certain saline lakes.[2,3] Arsenic

contamination in groundwater occurs both under reducing and oxidizing conditions and is occurring at many sites in the world,[4-6] particularly in arid regions, where such conditions in lacustrine aquifer sediments are often concomitant with high alkalinity and/or salinity.[7,8] Elevated As concentration have been linked to increased risks of skin, lung and bladder cancer to humans who utilize groundwater for drinking and irrigation purposes.[9,10]

Dunaliella salina, a salt-tolerant unicellular alga, is occurs in low trophic levels in the ocean and saline lakes.[11] It contains large amounts of proteins, vitamins, β-carotene, and carotenoids. Therefore, this microalga is highly valuable in the production of dietary supplements and aquafeed.[11,12] However, As pollution in water may pose serious risks to the quality of these products since it has shown that *Dunaliella* could absorb As(V) and distribute it into various cellular fractions efficiently.[13-18] On the other hand, *D. salina* may be a good candidate among microalgae to be used in processes of As removal from contaminated groundwater concomitant with high alkalinity and salinity.[16,18]

Phosphate (PO_4^{3-}) is an essential nutrient for the growth of *D. salina*. The concentration of PO_4^{3-} in commercial *Dunaliella* ponds is several orders of magnitude higher (e.g., 3.1 mg L^{-1}) than that of pristine water.[19] Among the factors that influence the As accumulation in microalgae, phosphate is important since it is structurally similar to As(V) and may compete with As(V) for uptake by designated PO_4^{3-} transporters in the cell membrane.[20,21] Reductions of As uptake by *Chlorella*, *Synechocystis*, *Chlamydomonas*, etc., have been reported as the PO_4^{3-} concentration in the growth media increased.[22-24] However, the same effect of PO_4^{3-} was not observed on the As uptake by *Dunaliella*. For example, Duncan *et al.*[14] and Foster *et al.*[16] demonstrated that the accumulation of As(V) in *D. tertiolecta* was not influenced by the PO_4^{3-} level in the culture media. These findings suggest that As interaction with PO_4^{3-} may be more complicated than previously thought. In fact, As(V) may be adsorbed on the cell surface before moving across the plasma membrane of microalgae through energy-dependent active transport mechanisms.[25,26] However, this extracellular-bound As was not separated and quantified in most studies on

Dunaliella.[14-16,27] Furthermore, although As (V) adsorption on the algal cell surface may also be affected by PO_4^{3-},[28] little is known about the relationship between cell surface binding and arsenic detoxification in microalgae under different PO_4^{3-} regimes.

In addition, arsenic transformations outside and inside the algal cells were shown to be affected by the PO_4^{3-} concentration of the culture media.[29,30] However, dynamic variations of As accumulation and transformation in and out of *Dunaliella* cells under different phosphate regimes were not yet clarified. As such, more detailed investigations on the different pathways of As(V) in this microalga are needed. Therefore in this study, we conducted both short-term (24-h; 72-h) and long-term (9-d) experiments with the following objectives: 1) to evaluate the As resistance and As removal ability of *D. salina* at different PO_4^{3-} concentrations in the culture; 2) to assess the effect of PO_4^{3-} nutrition on the dynamic processes of As adsorption, uptake and transformation.

Materials and Methods

Cultivation and growth conditions. The marine green alga *D. salina* was bought from the Collection Centre of Marine Microalgae, Key laboratory of Marine Ecology and Environmental Science, Institute of Oceanology, Chinese Academy of Sciences, China. The microalga was transferred into Erlenmeyer flasks containing fresh sterilized f/2 medium (PO_4^{3-}: 1.12 mg L^{-1}).[15,31] The f/2 medium was prepared with deionized water and synthetic sea salt (Table S1) with salinity maintained at 35‰, filtered through 0.22 μm membrane filter to remove particulates and autoclaved at 121 °C for 30 min. Cultures were maintained in an incubator at an irradiance of 50 μmol photons m^{-2} s^{-1} on a 12 : 12 h light: dark cycle at 25 ± 2 °C. Axenic status was periodically confirmed by microscopic analysis (Optical microscopy and Scanning Electron Microscope) and streaking on LB Broth Medium.[31] The initial culture optical density (OD) was adjusted to OD_{630nm}=0.060 (approx. 5×10^5 cells mL^{-1}),[32,33] there was also 20 mmol L^{-1} 3-(N-morpholino) propanesulfonic acid (MOPS) in f/2 medium to keep its pH at 7.3 ± 0.2.

72-h As(V) toxicity experiment. Cells of *D. salina* were first acclimated in the f/2 medium (dissolved with artificial sea water) for 7 days, then collected by centrifugation (7000 × g, 2min), rinsed with fresh f/2 medium without PO_4^{3-} addition for three times and then resuspended into f/2 medium without PO_4^{3-} for 3 days to reduce the PO_4^{3-} *in vivo*.[23] Arsenate solution was prepared by dissolving $Na_3AsO_4 \cdot 12H_2O$ in deionized water. Then *D. salina* with a cell density of 5×10^5 cells mL^{-1} was exposed to the indicated concentrations of As(V) (Table S2) in 50 mL Erlenmeyer flasks containing 25 mL f/2 medium with three PO_4^{3-} treatments, namely P_1 (0.112 mg L^{-1}), P_2 (1.12 mg L^{-1}) and P_3 (11.2 mg L^{-1}). Each treatment was replicated three times. The toxicity experiment lasted 72 h. Then the algae cell numbers were counted daily in a Neubauer haemocytometer chamber after fixation with Lugol. The percentage of growth rate inhibition was calculated.[34] The concentration corresponding to 50% inhibition of the growth rate (EC50 in mg L^{-1}) in *D. salina* over the 72-h exposure was determined.[22,30] Growth rates determined by cell density *versus* the control were used as biological end points to estimate the effect of As(V) on *D. salina*. Results were fit to a sigmoidal dose-response curve equation (Eq. (1)),[22] incorporated in the software GraphPad Prism 5.

$$BR = BR_{min} + \frac{BR_{max} - BR_{min}}{1 + 10^{(\log EC50 - BR) \times Hillslope}} \quad (1)$$

where BR is the biological response; BR_{min} and BR_{max} correspond to the minimal and maximal biological response, respectively, and EC_{50} is the concentration (mg L^{-1}) corresponding to a 50% inhibition of the growth rate. Hillslope is the slope factor and describes the steepness of the curve. All fits had a Hillslope value of 1.0.

Adsorption and uptake of arsenic by *D. salina* in 24-h under different phosphate concentrations. In parallel with the 72-h toxicity experiment, the ability of *D. salina* to adsorb As on the cell surface and to take up As into the cells were investigated using the culture medium enriched with a range of As(V) concentration (1.12, 11.2, 112 and 1120 mg L^{-1}) under the above three PO_4^{3-} treatments. The exposure time was set at 24-h to reduce growth differences among different treatments. At the end of As exposure, algal cultures from each replicate were

harvested by centrifugation (7000 × g), then cleaned 3 times with 35‰ saline and further rinsed with ice-cold 0.1 mol L^{-1} phosphate buffer (pH 7.0, 35‰ salinity to maintain the osmotic balance and cell integrity) for 2 min to remove As bound on the cell surface.[22,24,30] The total and intracellular As were measured and the adsorbed As was calculated as follows: $As_{adsorbed} = As_{total} - As_{intracellular}$. The bioadsorption factor (BAF) and the intracellular accumulation factor (IAF) were calculated as the ratio of As content adsorbed on the cell surface and taken up in the dry algal biomass to the initial As concentrations in the medium .[25,35,36]

As speciation and phosphate content in medium and in cells after 9-d exposure to As(V) under different phosphate concentrations. The As adsorption, uptake and transformation by *D. salina* in the long term (9-d) were also investigated under different PO_4^{3-} treatments. *D. salina* cells were collected and processed as described above, then inoculated in 100 mL of f/2 medium and exposed to two As(V) treatments, including As_1 (11.2 μg L^{-1}) and As_2 (1120 μg L^{-1}), and three PO_4^{3-} treatments (P_1, P_2 and P_3) for 9 days. The growth of algal cells (OD_{630nm}) was measured daily using a SpectraMax M5 Microplate Reader (Molecular Devices, US). Each treatment had three replicates. Aliquots of 5 mL growth medium were taken daily by centrifugation (7000 × g, 2min), and then filtered through a 0.45 μm nylon filter and kept in the freezer at -20 °C for later determination of total As and speciation analysis in the medium. The PO_4^{3-} content in the cultures was analyzed in the same way as described above at 0, 2, 4, 8, 12 h and 1 to 9 days. After exposure to As(V) for 4h and 1, 3, 5, 7, 9 days, algal cells were harvested as described above and freeze-dried, then kept in a desiccator before As speciation analyses. After 9-d incubation, the As adsorbed and taken up by *D. salina* and intracellular phosphorus (P) were also analyzed. To investigate if extracellular secretions of *D. salina* could change As speciation under different PO_4^{3-} treatments, the algae were cultured in 0.112 and 1.12 mg L^{-1} PO_4^{3-} medium for 3 days, and then the suspension was centrifuged for 2 min at 7000 × g and at 4 °C, followed by filtration by a sterilized 0.45 μm nylon filter. The supernatant was supplemented with As(V) to a final concentration of 1120 μg L^{-1} and incubated for 9 days. Aliquots of the supernatant

samples were taken on the 1st, 3rd and 9th day of incubation and analyzed for As speciation.

Analysis of As and P in *D. salina* and quality control. The freeze-dried algal samples were digested with 2 ml of HNO_3–$HClO_4$ (4:1, v/v) at 120 °C.[37] Total As and P were determined by hydride generation atomic fluorescence spectrometry (HG–AFS, AFS-9130, Jitian, Beijing, China) and inductively coupled plasma atomic emission spectrometer (ICP-AES, PerkinElmer Optimal 2100DV, USA), respectively. The limit of detection (LOD) for As and P obtained in this study were 0.15 ± 0.07 μg kg^{-1} and 12.9 ± 1.34 mg kg^{-1}, respectively. The relative standard deviation (RSD) values were less than 3.4% for all samples.

Arsenic speciation in algal samples was analyzed. The algal samples were extracted with 10 mL 1.75% HNO_3 at 90 °C for 10 min.[38] Final algal extracts were filtered through 0.22 μm nylon syringe filters before As speciation analysis by high performance liquid chromatography coupled with hydride generation atomic fluorescence spectrometry (HPLC–HG–AFS). Arsenite (As(III)), As(V), monomethylarsonic acid (MMA(V)) and dimethylarsinic acid (DMA(V)) were separated by an anion exchange column Hamilton PRP-X100 (250 mm×4.1 mm, 10μm) with the mobile phase containing 15 mmol L^{-1} $(NH_4)_2HPO_4$ (pH 6.0). Arsenic speciation in the algae and the medium were analyzed within 24h. The above extraction method was applied and had been shown reliable as it did not change As speciation in *D. salina* upon such treatments.[38]

Standard reference material rice flour (NIST-SRM 1568b) was used to check on the accuracy of the analyses.[35]

Statistical analysis. SPSS 20.0, GraphPad Prism 5 and SigmaPlot 12.5 software were used for statistical analyses and figure preparations, respectively. One-way or two-way ANOVA and post hoc multiple comparisons (Tukey) were used to detect significance ($p < 0.05$) in the toxicity of As(V), As(V) uptake, accumulation and transformation experiments of the present study.

Results

The tolerance and accumulation of As(V) in *D. salina* was affected by the phosphate level in the culture.

To evaluate the impact of PO_4^{3-} on the acute toxicity of As(V) to *D. salina*, the growth inhibition (GI) of *D. salina* (Table S2) and values of 72-h EC50 in 0.112, 1.12 and 11.2 mg L^{-1} PO_4^{3-} levels were calculated (0.62 ± 0.06, 41.5 ± 3.8 and 258 ± 26, respectively). The As(V) toxicity on *D. salina* was clearly alleviated by the increase of P supply, since the GI was lowered significantly (Table S2) and 72-h EC50 values had an approximate 416-fold increase when *D. salina* was cultured in 0.112-11.2 mg L^{-1} PO_4^{3-}.

Although the adsorbed and absorbed As was enhanced remarkably in *D. salina* at higher concentrations of As(V) in the culture (Fig. 1), the BAF and IAF were reduced (51.2 ± 1.7 to 0.18 ± 0.01 and 30.9 ± 0.5 to 0.79 ± 0.03) irrespective of PO_4^{3-} levels (Fig. S1). In addition, the percentage of adsorbed As increased from 34.3 to 87.9% of total algal As when exposed from 1.12 to 1120 mg L^{-1} As(V) at three PO_4^{3-} levels (Fig. 1). On the other hand, at the same As(V) concentrations, the amounts of extra- and intracellular As declined significantly ($p < 0.01$) when the PO_4^{3-} levels increased (Fig. 1).

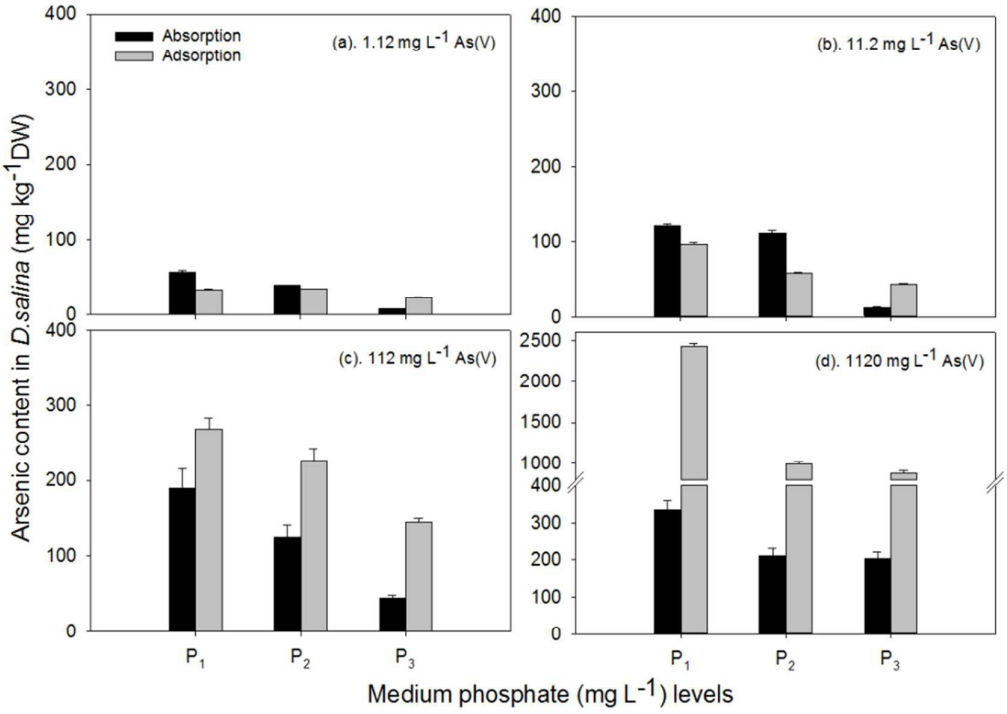

Fig. 1 Arsenic adsorption and absorption by *D. salina* after exposure to various indicated concentrations (1.12, 11.2, 112, 1120 mg L^{-1}) of As(V) in P_1, P_2 and P_3 growth medium for 24 h. P_1, P_2 and P_3 denote 0.112, 1.12 and 11.2 mg L^{-1} phosphate, respectively. Data are means ± SE (n=3).

To evaluate the effect of PO_4^{3-} on the chronic toxicity of As(V), we measured the cell growth of *D. salina* under different As(V) (As_1 and As_2) and PO_4^{3-} (P_1, P_2 and P_3) treatments during a 9-d period (Fig. 2). The As_1 (11.2 μg L^{-1}) treatment did not significantly affect the algal growth at all PO_4^{3-} levels during the incubation (data not shown). However this was not the case under As_2 exposure (Fig. 2). Significant ($p < 0.01$) growth stimulation (4.2-19.1% and 13.1-24.8%, respectively) by As_2 and P_2/P_3 was observed after 3 days of culture (Fig. 2). In contrast, the algal growth was significantly ($p < 0.01$) inhibited by As_2 under P_1 treatment after 2 days incubation.

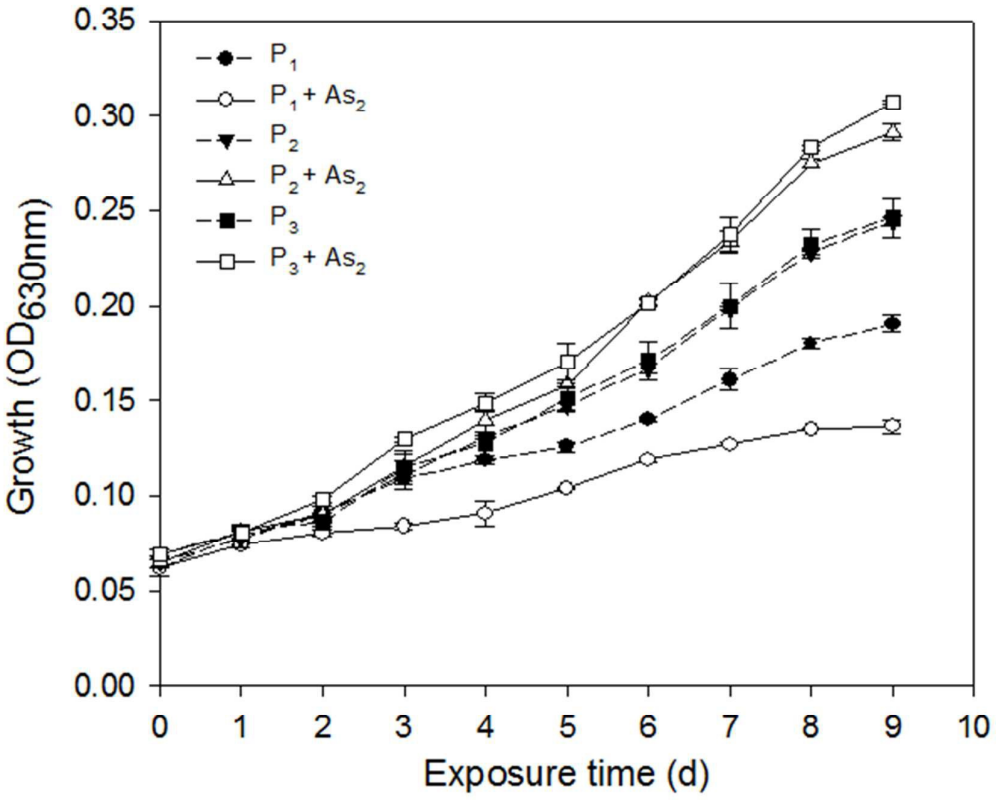

Fig. 2 Growth of *D. salina* in the f/2 medium containing the indicated concentrations of

phosphate and arsenate (0 and As_2). P_1, P_2 and P_3 denote 0.112, 1.12 and 11.2 mg L^{-1} phosphate, respectively. As_2 denote 1.12 mg L^{-1} As(V). Data are means ± SE (n=3)

As(V) reduced the uptake and accumulation of phosphate by *D. salina*.

Along with samples, standard reference material (rice flour, NIST-SRM 1568b) was detected with the same procedures. No significant loss or gain of As and P during the digestion procedures were observed. The measured values for total As (0.271 ± 0.023 mg kg^{-1}) and P (1523.5± 19.9 mg kg^{-1}), were slightly lower than the certified values (0.285 ± 0.014 mg kg^{-1} and 1530 ± 40 mg kg^{-1}, respectively; n=3).

As shown in Fig. 3, phosphate was quickly consumed by *D. salina*. At P_1 treatment, it was depleted within 2h (data not shown). The depletion of PO_4^{3-} in the medium was extended to 3 days when P_2 was applied (Fig. 3a). After 9 days of incubation under P_3 treatment, 4.8, 4.7 and 4.0 mg L^{-1} PO_4^{3-} was consumed by *D. salina* under 0, As_1 and As_2 exposure, respectively (Fig. 3b). The greatest PO_4^{3-} depletion rate in the P_2 and P_3 mediums occurred in the first 2 hours in control treatment, reaching up to 80.9 ± 2.0 and 256 ± 6.5 μg L^{-1} h^{-1}, respectively (Table S3). The depletion of PO_4^{3-} in the culture was significantly ($p < 0.01$) inhibited by As_2 under P_2 and P_3 treatments after 2 days incubation (Fig. 3). On the other hand, significant differences ($p < 0.01$) of total P in algal cells under the P_1, P_2 and P_3 treatments were observed (Table 1 and S4). Compared to the control, the intracellular P decreased by 21%, 6.6% and 11% (P_1, P_2 and P_3 treatments, respectively) when exposed to As_2.

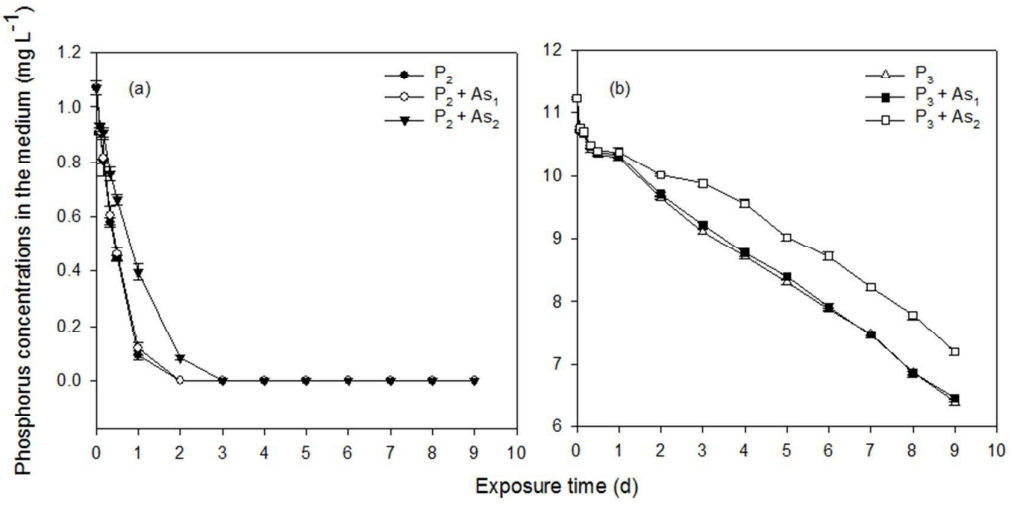

Fig. 3 Phosphate remained in the medium after exposure to 0, As_1 and As_2 in the P_2 (a) and P_3 (b) growth medium for 0, 2, 4, 8, 12h and 1 to 9 days. As_1 and As_2 denote 11.2 and 1120 μg L^{-1} As(V), respectively. P_2 and P_3 denote 1.12 and 11.2 mg L^{-1} phosphate, respectively. Data are means ± SE (n=3).

Phosphate affected the amounts of As in the culture and cells of *D. salina*.

As shown in Fig. 4, significant difference ($p < 0.01$) of As in the culture was observed from 4 h when exposed to the three PO_4^{3-} treatment. When treated with As_1 (Fig. 4a), the total As concentration in the P_3 media remained relatively constant throughout the 9-d incubation period while As(V) was observed almost depleted by *D. salina* within 3 and 5 days at the P_1 and P_2 treatments, respectively. The depletion rate of As(V) was respectively 0.13 ± 0.02 and 0.07 ± 0.03 μg L^{-1} h^{-1} in the P_1 and P_2 growth media within the 1st day (Fig. 4a and Table S5). After 1 day of incubation, most of the PO_4^{3-} in the culture was depleted (Fig. 3a), making the As uptake rate accelerated and it reached 0.20 ± 0.01 (P_1) and 0.13 ± 0.00 μg L^{-1} h^{-1} (P_2) on the 2nd day (Fig. 4a and Table S5). After 5-d incubation, 96.6% and 95.6% of the initial As(V) added to the culture was removed under the P_1 and P_2 treatments, respectively. However, the percentage of total As removed by the algal cells decreased as the concentration of As(V) in the medium increased. The highest percentage of As removed was only 7.7% when treated with the As_2 for 9 days irrespective of PO_4^{3-} levels (Fig. 4b)

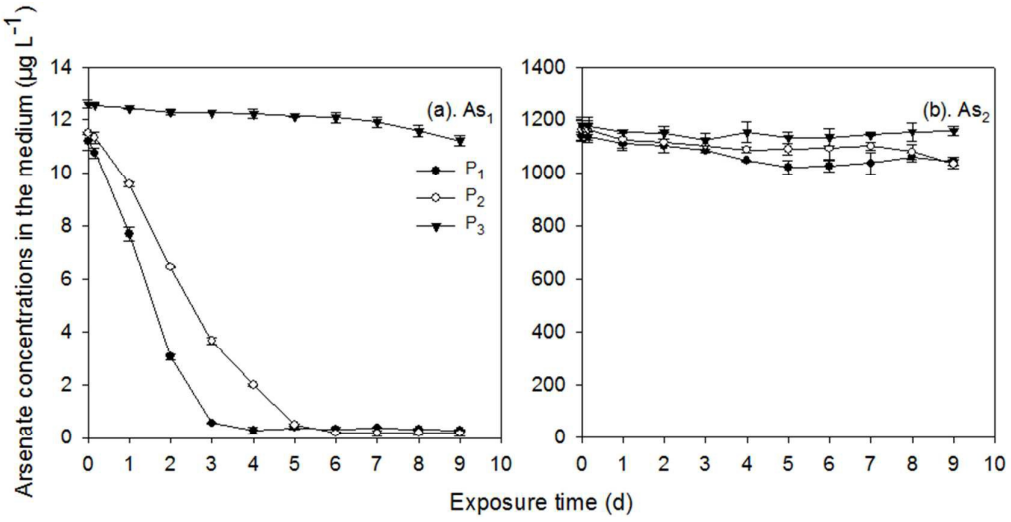

Fig. 4 Arsenic remained in the medium after exposure to As_1 (a) and As_2 (b) in P_1, P_2 and P_3 growth medium for 0, 4h and 1 to 9 days. As_1 and As_2 denote 11.2 and 1120 μg L^{-1} As(V), respectively. P_1, P_2 and P_3 denote 0.112, 1.12 and 11.2 mg L^{-1} phosphate, respectively. Data are means ± SE (n=3).

After 9-d incubation, total As in algal cells were also different under various As(V) and PO_4^{3-} treatments (Table 1 and S4). Both adsorption and uptake of As by *D. salina* increased significantly with As(V) exposure (Table 1). Besides, phosphate alone and As-P interaction had a significant effect ($p < 0.01$) on As adsorption and absorption (Table 1 and S4). When P_3 was applied, the uptake of As by *D. salina* was not detected after exposure to As_1 (Table 1 and Fig. 4a). The intracellular As increased and accounted for 8.3% of the total As in *D. salina* after exposure to As_2 (Table 1). Moreover, Arsenic uptake increased 100 times when the ratio of As/P in the culture varied from 0.1 to 10 under P_1 treatment. However, cellular As(V) levels only increased about 21 times when the As/P ratio in the medium rose from 0.01 to 1 under P_2 treatment (Table 1). Interestingly, algal As uptake in the P_2 medium was higher than that in the P_1, but the results were reversed in the As_2 treatment (Table 1 and Fig. 5 and 6).

Table 1 Total As and P contents in *D. salina* under various As(V) and PO_4^{3-} concentrations in the 9-d incubation.

Treatment		As/P	Total As and P in algal cells (mg kg^{-1})		
As(V)	PO_4^{3-}		Adsorbed As	Absorbed As	P
0	P_1	0	n.d.	n.d.	1030 ± 24 ^{c}
	P_2	0	n.d.	n.d.	3261 ± 41 ^{b}
	P_3	0	n.d.	n.d.	9043 ± 119 ^{a}
As_1	P_1	0.1	2.78 ± 0.30 ^{a}	0.56 ± 0.13 ^{b}	886 ± 41 ^{c}
	P_2	0.01	1.94 ± 0.19 ^{a}	1.72 ± 0.12 ^{a}	3133 ± 32 ^{b}

	P_3	0.001	0.84 ± 0.07 ^{b}	n.d.	8268 ± 85 ^{a}
As_2	P_1	10	53.57± 1.05 ^{b}	56 ± 2.10 ^{a}	814 ± 5 ^{c}
	P_2	1	61.26 ± 0.59 ^{a}	36.44 ± 1.10 ^{b}	3046 ± 18 ^{b}
	P_3	0.1	51.30 ± 0.28 ^{b}	4.66 ± 0.35 ^{c}	8034 ± 71 ^{a}

As_1 and As_2 indicate 11.2 and 1120 μg L^{-1} As(V), respectively. P_1, P_2 and P_3 denote 0.112, 1.12 and 11.2 mg L^{-1} phosphate, respectively. All data are means ± SE (n=3). Different letters within columns indicate significant differences ($p < 0.05$) between various P levels according to Tukey's multiple range tests, n.d.: not detected, indicating As concentrations are generally at or below the LOD (0.15 μg kg^{-1}) obtained in this study.

Phosphate had differential impacts on As biotransformation in *D. salina*.

Along with algal samples, rice flour was also analyzed with the same extraction procedure. The concentrations of DMA (0.20 ± 0.03 mg kg^{-1}) and inorganic arsenic (iAs) (0.10 ± 0.04 mg kg^{-1}) were similar to the certified values (DMA 0.180 ± 0.012 mg kg^{-1}; iAs 0.092 ± 0.010 mg kg^{-1}). In the present study, the recoveries of As species in algal samples ranged between 87.4% and 104.6% by the above extraction method.

As shown in Fig. 5b, two inorganic arsenicals, As(III) and As(V), were detected in the cells when exposed to P_2 treatment. Under P_1 treatment (Fig. 5a), in addition to As(V) and As(III), DMA was detected from the 1st to 7th day with a percentage of 25.6-47.5%. Besides, during the 9-day As_1 exposure, total As taken up by the algae under the P_2 treatment were consistently higher than that under the P_1 treatment (Fig. 5 and Table 1).

Upon the As_2 treatment, arsenic concentration in the algal cells increased significantly with the culture time (Fig. 6). Arsenate was the predominant intracellular As species during the incubation, accounting for 88.3-100% of the total As. Besides, the increase of algal As(III) content was observed when the P content in the cultures decreased from 11.2 to 0.112 mg L^{-1}. Furthermore, when As exposure increased from 11.2 to 1120 μg L^{-1} under P_2 treatment (Fig. 5b and 6b), a small percentage of DMA was observed in the algal cells after 3 days incubation.

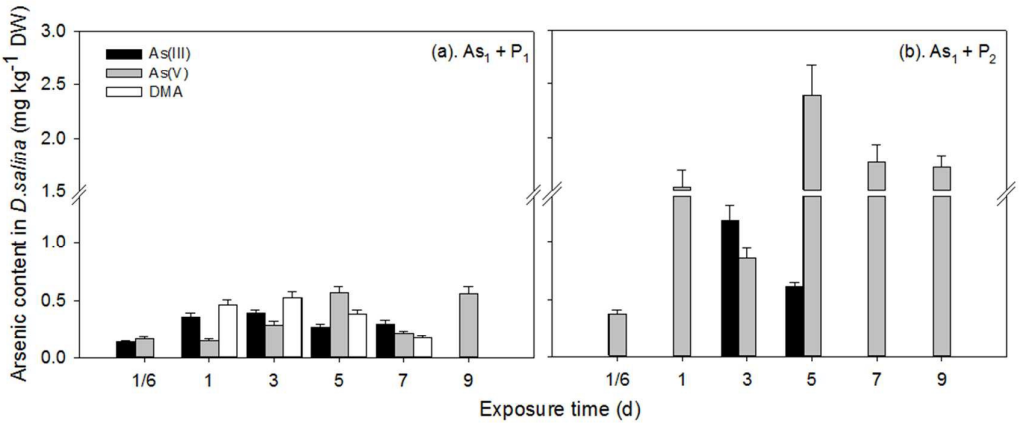

Fig. 5 Arsenic species in the algal cells after exposure to As_1 for 4h (1/6 day), 1, 3, 5, 7 and 9 days under P_1 (a) and P_2 (b) treatments. As_1 denote 11.2 μg L^{-1} As(V). P_1 and P_2 denote 0.112 and 1.12 mg L^{-1} phosphate, respectively. Data are means ± SE (n=3).

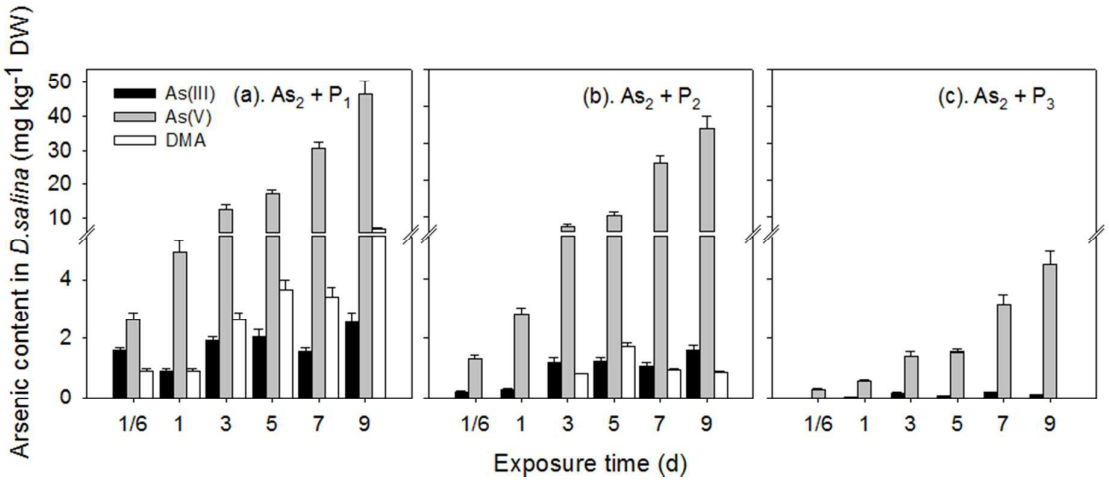

Fig. 6 Arsenic species in the algal cells after exposure to As_2 for 4h (1/6 d), 1, 3, 5, 7 and 9 days under P_1 (a), P_2 (b) and P_3 (c) phosphate treatments. As_2 denote 1.12 mg L^{-1} As(V). P_1, P_2 and P_3 denote 0.112, 1.12 and 11.2 mg L^{-1} phosphate, respectively. Data are means ± SE (n=3).

Lower phosphate in the growth media induced more As(III) and DMA excretion from *D. salina*.

Analysis of As speciation in the culture showed that As(V) remained to be the predominant (91.1-99.5% of the total As) species during the 9-d culture (Fig. 7).

Under P_1 treatment, more As(III) and DMA were found in the growth medium (Fig. 7a and b). However, when treated with P_2 and P_3, the percentage of As(III) and DMA in the medium was lower (Fig. 7b and c). Since As(V) was the only As species in the culture of the biotic control samples (supernatant after centrifugation of algal culture, representing extracellular secretion of *D. salina*, Fig. S2) and no bacteria and any other contamination were observed on the agar plates or in the liquid cultures under a Scanning Electron Microscope (Fig. S3), we could confirm that the reduction and methylation of As occurred in cells and As(III) and DMA in the culture were excreted from *D. salina*.

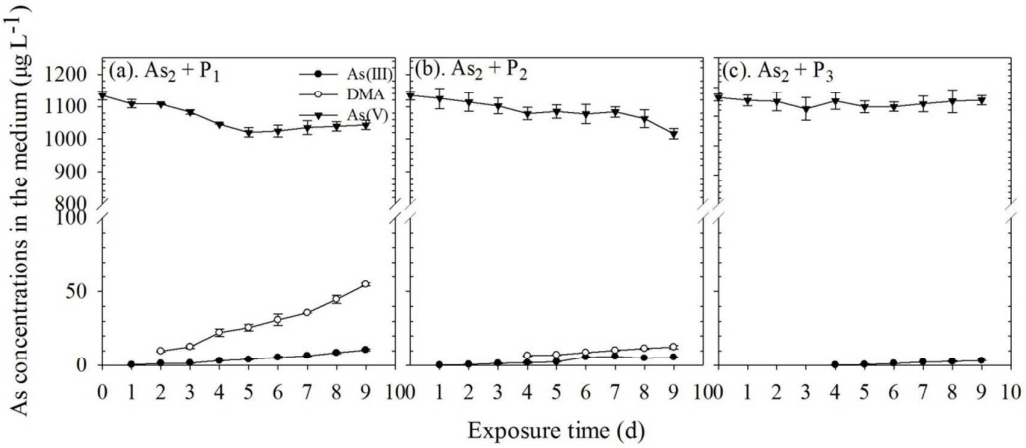

Fig. 7 Arsenic speciation in the culture after exposure to As_2 for 1 to 9 days under P_1 (a), P_2 (b) and P_3 (c) phosphate treatments. As_2 denote 1.12 mg L^{-1} As(V). P_1, P_2 and P_3 denote 0.112, 1.12 and 11.2 mg L^{-1} phosphate, respectively. Data are means ± SE (n=3).

Discussion

It has been well documented that some types of microalgae can efficiently accumulate and detoxify As.[16,18,35,39-42] As such, these microalgae may have value in use for As bioremediation from contaminated water. In the present study, when the initial PO_4^{3-} was less than or equal to 1.12 mg L^{-1}, the percentage of As removed from the 5-days cultures was more than 95.6% at an initial As concentration of 11.2 μg L^{-1} (Fig. 4a). These results indicate that *D. salina* could be a good candidate for As removal from

contaminated saline water which contained relatively low PO_4^{3-} (0.112-1.12 mg L^{-1}) and As(V) (11.2 μg L^{-1}). Additionally, algal harvesting time is important and should be taken into account in order to maximize the accumulation of As and minimize the excretion of As (Fig. 5). Since the mechanisms of As(V) resistance of *D. salina* have not been fully elucidated, we conducted batch culture studies to quantify As in various metabolic pathways (adsorption, uptake, transformation and excretion) under different P treatments.

Our results demonstrated that *D. salina* was strongly resistant to As(V). At 1.12 mg L^{-1} PO_4^{3-} level, the 72-h EC50 value of As(V) (41.5 ± 3.8 mg L^{-1}) was much higher than the values (4.53-33.5 mg L^{-1}) reported for other algal species such as *C. reinhardtii* and *Monoraphidium arcuatum* (1.5-1.6 mg L^{-1} PO_4^{3-} level).[20,30] The algal growth was even stimulated when exposed to 1120 μg L^{-1} As concentration from the 3rd to 9th day under 1.12 and 11.2 mg L^{-1} PO_4^{3-} treatments (Fig. 2). Similar growth stimulation (20-57%) by As(V) had also been reported when arsenic-resistant algae *Chlorella* sp. and *O. tauri* were exposed to 750 and 2250 μg L^{-1} As (V), respectively.[42,43] However, at 0.112 mg L^{-1} PO_4^{3-} supply, the growth of *D. salina* was depressed more severely by As, indicating that the As tolerance was partly mediated by the extracellular PO_4^{3-} level. Arsenate toxicity was aggravated (e.g., lower EC50 value (0.62 ± 0.06 mg L^{-1}), higher growth inhibition percentage (Table S2)) because there was not enough PO_4^{3-} to compete with As(V) for the phosphate transport systems.[20,30,44,45] At the same time, the algal cells synthesize more PO_4^{3-} transporters to compensate for the lack of PO_4^{3-} in the medium, leading to increased uptake of As(V) and higher As accumulation (Fig. 1 and Table 1).[20]

Arsenic adsorption on the cell surface received little attention in previous studies.[20, 46] In the present study, both adsorbed and absorbed As fractions in *D. salina* were found closely related to As(V) metabolism (Table 1 and Fig. 1 and S1). Within 24 h, more As was taken up when the cells were exposed to 1.12 and 11.2 mg L^{-1} As(V), whereas more As was bound extracellularly under the higher As exposure (112 and 1120 mg L^{-1}, Fig. 1). The increase in As adsorption percentage was also evident for *M. arcuatum* when the cells were treated with higher As(V) concentrations.[30] In addition,

after 9-d of As treatments (11.2 and 1120 μg L^{-1}), arsenic absorbed in *D. salina* was generally less than that adsorbed (Table 1). These results indicate that *D. salina* may reduce As(V) toxicity using different strategies, *i.e.*, suppression of As uptake upon acute As stress (24 h, 112 and 1120 mg L^{-1}) or enhancement of As efflux following chronic As exposure (9 d, 11.2 and 1120 μg L^{-1}). Furthermore, our results revealed that As adsorption and uptake by *D. salina* were inhibited with increased P levels in all As treatments (Fig. 1). Similarly, the uptake of As(V) in many organisms (*e.g.*, algae, fungi, plants and amphibians) has been reported to be mediated by P due to their structural analogy.[20, 47-50] Our results also revealed that As(V) treatment reduced the PO_4^{3-} assimilation rate (Fig. 3 and Tables 1 and S3) from the culture, and provided further evidence for the competition between As(V) and PO_4^{3-} to be taken up by algal cells. Here it is noteworthy that PO_4^{3-} could be depleted far more quickly than As(V) by *D. salina* (Tables S3 and S5). As seen in Fig. 3b, 4.7 mg L^{-1} PO_4^{3-} was depleted by *D. salina* during 9 days of incubation when treated with 11.2 μg L^{-1} As(V). As a result, more As(V) (> 0.84 mg kg^{-1}) would be accumulated by *D. salina* when PO_4^{3-} levels become depleted (Table 1 and Fig. 6a and b). Therefore, it is necessary to continuously monitor PO_4^{3-} in commercial *Dunaliella* ponds to minimize As contamination in the algal products.

Our results demonstrated that As biotransformation and excretion was mediated by extracellular PO_4^{3-}. At conditions of 0.112 mg L^{-1} PO_4^{3-}, more As(V) was taken up into cells (Fig. 6a) through the up-regulated phosphate transport system.[23] At the same time, As(V) reduction, methylation, and As(III) and DMA excretion were accelerated and were the main pathways of As metabolism under 0.112 mg L^{-1} PO_4^{3-} treatment (Fig. 5a and 6a and 7a). These findings were supported by Hellweger *et al.* (2003) and Levy *et al.* (2005), who have shown that algae in the phosphate-limited culture were more likely to produce and efflux methylated As compounds.[29,30] However, when 1.12 and 11.2 mg L^{-1} PO_4^{3-} were supplied, As(V) dominated As speciation and was only slightly reduced to As(III) and methylated to DMA (Figs. 5b and 6b and 6c). It is possible that high concentrations of PO_4^{3-} strongly inhibited the algal uptake of As(V) and its reduction to As(III) via cytoplasmic arsenate reductase.[51] The levels of As(III)

in the culture was also negligible during the 9-d incubation (Fig. 7b and c), probably due to the more effective surface As(III) re-oxidized process with increased PO_4^{3-} levels.[23]

Our results also demonstrated that As biotransformation and efflux was mediated by intracellular P. When treated with 11.2 μg L^{-1} As, both total As and P in the 0.112 and 1.12 mg L^{-1} PO_4^{3-} treatments from the 5th to 9th day remained low (Fig. 3a and 4a), which could favor As excretion via passive diffusion.[20,29] However, As uptake in *D. salina* under 1.12 mg L^{-1} PO_4^{3-} treatment was higher than that under the 0.112 mg L^{-1} PO_4^{3-} treatment (Fig. 5 and Table 1). The difference is likely due to As efflux from *D. salina* that was enhanced at the lower intracellular P level which in turn contributed to the lower As in *D. salina* under the 0.112 mg L^{-1} PO_4^{3-} treatment (Fig. 7a and Table 1).[20]

Our results support the As biotransformation model proposed by Cullen *et al.*[52,53], in which As(V) taken up and detoxified by *D. salina* may be transformed through reduction and methylation (Fig. 5 and 6), as well as excretion of As(III) and DMA species from cells (Fig. 7). Such effects were observed in other microalgae such as *Closterium aciculare*, *Aulacoseira granulate*, *Pediastrum biwae* and *Cyanidioschyzon*.[54,55] These findings, however, were different from another *Dunaliella* species, *D. tertiolecta*, reported by Foster *et al.*[16] and Duncan *et al.*[14,15], who stated that arsenosugars to be major As components in *Dunaliella* when exposed to 2 μg L^{-1} As(V). We postulate that this discrepancy could be attributed to the difference between species and the higher As(V) exposure (> 11.2 μg L^{-1}) we used. In fact, it has been demonstrated that the formation of methylated arsenic compounds and arsenosugars by microalgae depend on arsenic concentration in the environment.[56] Inorganic As species were dominant in microalgae exposed to higher As exposures (50-200 μg L^{-1}).[14,56]

Conclusions

This study demonstrated that *D. salina* had a strong As(V) tolerance due to suppression of As uptake into the cells, detoxification of intracellular As mainly by

As(V) reduction and subsequent methylation, as well as expelling As(III) and DMA species from cells. Our results indicated that PO_4^{3-} had a significant effect on the toxicity, adsorption and uptake of As(V) by *D. salina*. Arsenate exposure also decreased PO_4^{3-} uptake in *D. salina*. Furthermore, harvesting time and PO_4^{3-} monitoring during the cultivation are important in order to minimize As contamination in the algal products and to enhance As removal from the environment using these halotolerant microalgae.

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