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Effect of Glutathione on Arsenic Accumulation by *Dunaliella salina*

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The behavior of marine algae (*Dunaliella salina, Chattonella antiqua, Heteresigma akashiwo, Skeletonema costatum, chaetoceros debile* and *Thalassiosira weissflogii*) against arsenate, arsenite and DMA in a medium and the effects of glutathione that influenced the redox condition on arsenic accumulation of *D. salina* were studied. It was found that the order of growth inhibition of marine algae by arsenic species was As(III) > As(V) > DMA. The order of arsenic accumulation by *D. salina* was As(V) ≥ As(III) > DMA at a concentration of 100 mgAsdm^{-3}. A small part of the arsenic accumulated by *D. salina* was methylated *in vivo*. DMA was the major methylated arsenic compound. Methylated arsenic compounds were not present in the medium. Glutathione (GSH) treatment increased arsenic accumulation by *D. salina* at a concentration of 10–100 mg GSHdm^{-3}. Buthionine sulfoxisamine (a potent and specific inhibitor of γ-glutamylcysteine synthetase) strongly suppressed the effect of GSH on arsenic accumulation. These findings suggest that the intracellular glutathione concentration may be important for arsenic accumulation.

Keywords: arsenic; accumulation; glutathione; *Dunaliella*

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INTRODUCTION

Arsenic is a ubiquitous chemical widely distributed in food, water, air and soil. In coastal sea environments, arsenate (V), arsenite (III), monomethylarsonic acid (MMA) and dimethylarsinic acid (DMA) are usually found as the predominant forms of arsenic species in oxygenated water.[1] The abundance of one of them has been correlated with the occurrence of a particular phytoplankton group.[2] Understanding the different toxic mechanisms of As(III), As(V) and DMA is critically important in assessing the risk from arsenic exposure. The microalgae (*Chlorella*,[3] *Dunaliella*,[4] *Phoridium*,[5] *Chattonella*[6]) are capable of accumulating arsenic to concentrations several orders of magnitude higher than in the surrounding medium. Unfortunately, there is little information on the tolerance and accumulation of As(III), As(V) and DMA.[7] In a previous paper, resistance to arsenic impact and the bioaccumulation of arsenic by *D. salina* were reported.[4] *D. salina* seems to have the ability both to detoxify arsenic accumulated in the cell and to reduce it.[8] Detoxification of arsenic by the cell was probably achieved by shielding it with SH groups of algal components, e.g. proteins, or by methylating it.[7] Monomethyl- and dimethyl-arsenic compounds were found in water extracts of *Chlorella*,[7] but scarcely in *D. salina*.

Glutathione (γ)-L-glutamyl-L-cysteinylglycine) is a low-molecular-weight thiol used in a wide range of metabolic processes.[9] The functions of glutathione in higher plants include storage and transport of reduced sulfur, maintenance of protein thiol, protecting membranes against peroxidation and detoxification of xenobiotics.[10] These observations suggest that glutathione acts by mediating the response of plant cells to biological stress. The accumulated arsenic concentration in *D. salina* has been reported to be increased with an increase in arsenate(V) concentration of the aqueous phase[4] (but it is not clear how glutathione is related to arsenic accumulation).

This report describes the effects of glutathione on arsenic accumulation and growth inhibition of various marine algae *(D. salina, C. antiqua, H. akashiwo, S. costatum, C. debile* and *T. weissflogii)* with As(V), AS(III) and DMA.

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MATERIALS AND METHODS

Reagents

Trivalent sodium arsenite [$NaAsO_2$, As(III)], pentavalent sodium arsenate [$Na_2HASO_4 \cdot 7H_2O$,As(V)] and dimethylarsinic acid (DMA) were commercial products of Sigma Chemical Co. (Tokyo, Japan). Methylarsonic acid [$CH_3AsO(OH)_2$; MAA] and trimethylarsine oxide [$(CH_3)_3AsO$] was obtained from Tri Chemical Corp. (Nagano, Japan). Other reagents were purchased from Wako Pure Chemical Industries (Osaka, Japan). Analytical solutions were prepared by diluting stock solutions to the required concentration.

Microalgae

Dunaliella salina (*Chlorophyceae*) was obtained from the Culture Collection of Algae and Protozoa (University of Cambridge, UK). *Heterosigma akashiwo* (*Rhaphydophyceae*), *Skeletonema costatum* and *Chaetoceros debile* (*Bacillariophyceae*) were obtained from Global Environmental Forum (Tsukuba). *Chattonella antiqua* (*Rhaphydophyceae*) was obtained from Hiroshima Prefecture Marine Experimental Center. *Thalassiosira weissflogii* (*Bacillariophyceae*) was isolated in 1982 from a mixture of many kinds of algae collected from Harima nada. These algae were used throughout the experiments.

Cultures of algae

D. salina, *C. antiqua*, *H. akashiwo*, *S. costatum*, *Chaet. debile* and *T. weissflogii* were grown at 23 °C in an agitated, aerated, filtered and sterilized seawater medium containing Fe-EDTA 1; the various arsenic species (each at 0, 0.5, 1,5,10,50,50,100,500, 1000, 5000 and 10000 mg dm^{-3}) and (mg dm^{-3}); KNO_3 72; KH_2PO_4 4.5. The pH of each sample was adjusted by addition of dilute hydrochloric acid or sodium hydroxide to pH 8.0. The density of various algae in the medium before incubation was adjusted to 10^3 cells cm^{-3}. Cultures were grown at 23 °C with continuous light (4500 lux) and constant aeration (1dm^{-3} min^{-1}). The cultures were checked periodically for bacterial contamination by plating on nutrient agar. Each experiment was conducted twice. The cells were harvested by drying by lyophilization after cooling centrifugation (5 °C, 2500 rpm) and the content of arsenic in centrifuged cells was determined. The fluorescence intensity of the living cell suspension was found to be proportional to the cell density,[11] so measurement of the growth of the cells (g dry weight of cell per dm^3 of medium) was obtained by determination of the fluorescence intensity of the culture.

Determination of total arsenic in algae

The freeze-dried algae containing arsenic were digested with a mixed solution containing 3 cm^3 of concentrated nitric acid, 1 cm^3 of concentrated sulfuric acid and 1 cm^3 of perchloric acid (60%). The amounts of arsenic in the algae was determined by means of a hydride-generation atomic absorption spectrometer system (Jurrell Ash Co., model AA-1 MK-2).[8] Wavelength and lamp current were 193. 7 nm and 10 mA for arsenic.

Determination of MMA, DMA and TMA in algae by HPLC

Samples (1.0 g) of algae were homogenized in the presence of chloroform/methanol (2:1 v/v, 100 cm^3). The homogenate was filtered and the filtrate was then thoroughly mixed with water (50 cm^3).[12] The aqueous phase was separated and concentrated to a small volume. The concentration and evaporation were performed under reduced pressure below 40 °C throughout the experiment.

The concentrate was placed on a cation-exchange column (Dowex 50W-x8, H^+ form, 2.2 cm × 18.5 cm column) and eluted with 400 cm^3 of water, 400 cm^3 of 2.0 mol dm^{-3} pyridine and 400 cm^3 of 1.0 mol dm^{-3} HCl, successively. The concentrated fraction eluted with the pyridine solution was further applied to a Dowex 50W-x2 (200–400-mesh, pyridinium form; 1 cm × 50 cm column) equilibrated with 0.1 mol dm^{-3} pyridine–formic acid buffer (pH 3.1) and eluted with the same buffer (200 cm^3) and 0.1 mol dm^{-3} pyridine (200 cm^3).

Each extract was analyzed in a high-performance liquid chromatograph (Tosoh Co. Ltd, CCP 8000 series) using a TSK Gel ODS-120T 4.5 mm × 250 mm column (Tosoh Co. Ltd with a mobile phase of 11.2 mmol dm^{-3} sodium heptanesulfonate in water–acetonitrile–acetic acid (95:5:6, by vol.; flow rate 0.8 cm^3 min^{-1}; sample size 5 mm^3).[13] Portions (10 mm^3) of each elute collected every 25 s were injected into a graphite-furnace atomic absorption spectrometer (Shimazu model AA-7600) as the arsenic-specific detector under the

following conditions: drying at 200 °C for 20 s and ashing at 500 °C for 60 s, both under an atmosphere of air, and atomization at 2400 °C for 10 s under an argon atmosphere; deuterium background correction; monochromator at 193. 7 nm.[13,14] A mixture of the authentic arsenic compounds (all with 100 mg dm^{-3} as As), including those which had been detected in the previous experiments *in vitro* on the accumulation of arsenic, was also fractionated [retention times, s: As(III) 225–300; As(V) 150–225; MMA 225–300; DMA 325–400; TMAO 725–850].

In order to determine the presence (MMA) or absence [As (V)] of a methyl group in the arsenical whose retention time was 225–300 s, atomic absorption spectrometry with hydride-generation derivatization followed by cold trapping was performed without the previous hydrolysis with NaOH.[5]

Measurement of glutathione (GSH) in algae

For GSH+GSSG measurement, algae were ground using a chilled mortar and pestle with 4 cm^3 of 20 mmol dm^{-3} EDTA and deproteinized by 1 cm^3 of 50% metaphosphoric acid (HPO_3) at 4 °C. The homogenate was centrifuged at 30 000 *g* for 15 min at 4 °C, and the supernatant was used to measure GSH+GSSG by the DTNB–GSSG reductase method.[16] The total glutathione was measured with an enzymic recycling assay based on glutathione reductase in a reaction mixture (1 cm^3) containing 5 mmol dm^{-3} potassium phosphate buffer (pH 7.4), 1 mmol dm^{-3} EDTA, 0.2 mmol dm^{-3} NADPH, 0.6 mmol dm^{-3} 5,5′-dithiobis(2-nitrobenzonic acid) (DTNB) and cell extract. After 5 min of incubation, the reaction was started by the addition of glutathione reductase (2 units, Sigma) and the reduction rate of DTNB was monitored at 412 nm for 3 min with a Shimazu double-beam spectrophotometer (Shimazu Model UV-190). GSSG was measured selectively by assaying the sample in which GSH was masked by treatment with 2-vinylpyrimidine.[17] To 500 µl of sample was added 10 µl of 1 mol dm^{-3} 2-vinylpyrimidine; this was mixed vigorously for 1 min, then left at 25 °C for 1 h. The resultant solution was assayed as described above. Under these assay conditions, the rate of increase in the absorbance at 412 nm for 3 min was linearly related to the quantity of GSSG over the range 0–0.4 nmol dm^{-3}. The difference between the total glutathione and GSSG contents was taken to be the content of GSH.

Note: All data points represent the means of three replicate experiments.

RESULTS AND DISCUSSION

Effect of arsenate, arsenite and DMA on the growth of *D. salina*

D. sanila, C. antiqua, H. akashiwo, S. costatum, Chaet. debile and *T. weissflogii* were grown in enriched seawater containing various concentrations of arsenate, arsenite and DMA for 14 days (Fig. 1). All data are expressed as percentages of the blank (weight of the algae without arsenic). On the addition of arsenate, arsenite and DMA in the growth of *D. salina* was unaffected by arsenate, arsenite and DMA in the concentration range 0–1000, 0–10 and 0–10 000 mg dm^{-3}, respectively. However, the growth of other algae was slightly affected with increasing arsenate, arsenite and DMA concentrations in the medium (at 1000, 10 and 10 000 mg dm^{-3}, respectively). It was found that the order of growth inhibition by arsenate was *C. antiqua* > *H. akashiwo* > *T. weissflogii* > *Chaet. debile* > *S. costatum* > *D. salina*, the order of growth inhibition by arsenite was *H. akashiwo* ≥ *Chaet. debile* ≥ *S. costatum* > *C. antiqua* > *T. weissflogii* > *D. salina*, and the order of growth inhibition by DMA was *C. antiqua* > *T. weissflogii* > *S. costatum* > *H. akashiwo* > *D. salina*, respectively. The results suggested that the ability to tolerate arsenic differs with different species of microalgae. Also, these results suggest that the order of growth inhibition of marine alage by arsenic was arsenite(III) > arsenate(V) > DMA.

Arsenic accumulation by D. salina

As described above, it was found that survival of *D. salina* cells is unaffected by the presence of arsenic (1000 mg dm^{-3} arsenate, 10 mg dm^{-3} arsenite and 10 000 mg dm^{-3} DMA, respectively). To clarify the arsenic accumulation by *D. salina*, the content of arsenic in *D. salina* was determined using various arsenic compounds. The content of arsenic in the cells is shown in Table 1. *D. salina* did not grow at over 5 000 mg dm^{-3} arsenate, 500 mg dm^{-3} arsenite and 25 000 mg dm^{-3} DMA, respectively. The content of arsenic accumulation by *D. salina* in a medium containing arsenic compounds was greatly changed by the addition of arsenic to medium. Arsenic content in *D. salina*

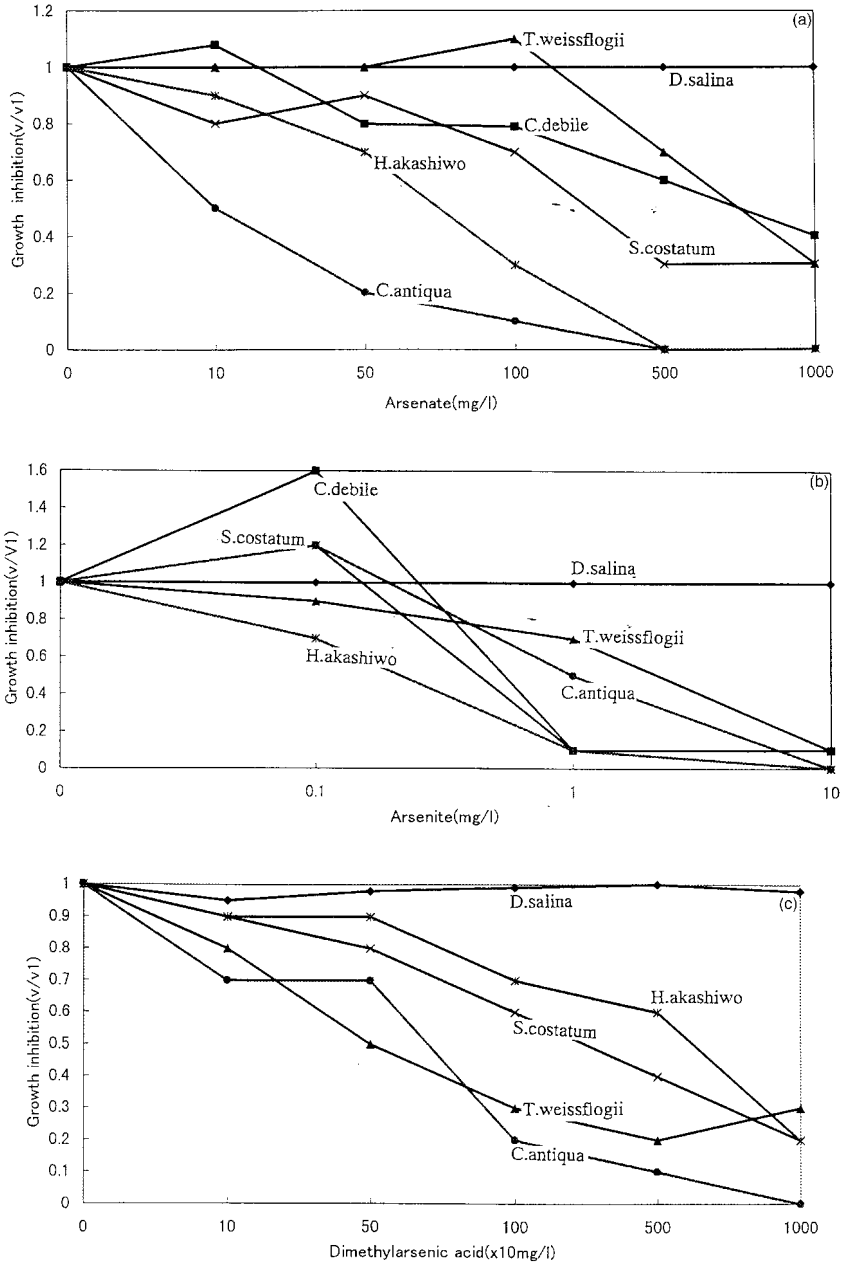

Figure 1 Impact of (a) arsenate, (b) arsenite and (c) dimethylarsenic acid on the growth of marine microalgae. Culture conditions: 23 °C; pH 8.0; KNO_3, 72 mg dm^{-3}; KH_2PO_4, 4.5 mg dm^{-3}; Fe-EDTA, 0.5 mg dm^{-3}; incubation time 14 days. Growth was monitored by measuring fluorescence *in vivo*; there was close correlation between the relative intensity of fluorescence and the biomass of the microalgae.[11] V/V_1 = sample biomass volume (mg dm^{-3})/blank biomass volume (mg dm^{-3}); ●, *C. antiqua*; ◆, *D. salina*; ▲, *T. weissflogii*; ×, *S. costatum*; ■, *Chaet. debile*; ∗, *H. akashiwo*.

Table 1 Effect of arsenic on the growth of *Dunaliella salina* and arsenic accumulation by *Dunaliella salina* after a 7-day culture[a]

As species (mg dm^{-3})	Biomass (mg dry/cells dm^{-3})	Total As (mg dm^{-3} dry/cells)
Arsenate (V)		
0	53.6	1.9
10	53.6	55.0
100	58.9	388.2
1000	36.8	2740.1
2000	16.9	1226.1
5000	—	—
Arsenite (III)		
0	52.1	2.2
10	51.6	55.3
100	50.8	373.1
500	1.2	—
Dimethylarsenic acid (cacodylic acid)		
0	51.6	2.6
100	52.2	3.0
1000	49.8	6.3
5000	53.6	19.2
10000	49.7	20.1
25000	2.2	—

[a] The medium contained (mg dm^{-3}): KNO_3 72, KH_2PO_4 4.5; temperature 23 °C; illumination 4500 lux; volume of seawater 1 dm^{-3}.

increased with an increase of arsenate, arsenite and DMA concentration to 1000, 100 and 10 000 mg dm^{-3}, respectively. These results for arsenate agreed with data in previous papers.[3,4] Arsenic concentrations in algae vary significantly, ranging from 2 740 mg dm^{-3} in arsenate and 373 mg dm^{-3} in arsenite to an average of 20 mg dm^{-3} in DMA. Comparing arsenate, arsenite and DMA, the order of arsenic accumulation by *D. salina* was arsenate (V) $\geq$ arsenite(III) $>$ DMA. These results suggest that there is a significant difference in the mechanisms of accumulation of arsenical species by *D. salina*.

Methylation of inorganic arsenic by *D. salina*

D. salina (5 mg dry weight per dm^3 medium) precultured in an arsenic-free medium was inoculated in 1 dm^3 medium containing 0.100 mg As(III) and 100 mg As(V) dm^{-3} and cultured for 14 days under the general conditions (see the Experimental section).

Table 2 shows that total arsenic, MMA, DMA and TMA were produced in the algal cell in the medium containing 100 mg dm^{-3} of arsenate and arsenite. Total arsenic level in *D. salina* was composed of 99.4% As (inorganic), 0.55% DMA, 0.01% MMA. Also, trimethylarsenic was detected in only trace amounts (below 0.1 mg dm^{-3}). The predominant accumulated arsenic species in *D. salina* was inorganic arsenic. Also, DMA were therefore the major methylated arsenic compound. However, no methylated arsenic was detected in the medium from *D. salina* exposed to either As(III) or As(V). These results show that when *D. salina*

Table 2 Arsenic compounds[a] accumulated in *Dunaliella salina cells* and excreted in the medium[b]

Arsenic in medium (mg dm^{-3})		Arsenic in *D. salina* (mg dm^{-3} dry cell wt)					Arsenic in medium (mg dm^{-3} medium)		
		Total	A	MMA	DMA	TMA	MMA	DMA	TMA
Total As	0	1.2	1.2	0	0	0	0	0	0
As (III)	100	109	108.3	0.1	0.6	tr	0	0	0
As (V)	100	62	61.8	tr	0.2	tr	0	0	0

[a] A, inorganic arsenic;MMA, DMA and TMA refer to mono-, di- and tri-metylarsenic species, respectively; tr: $>$ 0.05 mg dm^{-3}.
[b] The medium contained (mg dm^{-3}) KNO_3 72, KH_2PO_4 -4.5; temperature 23 °C; illumination 4500lux; volume of seawater 1 dm^{-3}.

Table 3 Effect of glutathione and buthionine on arsenic accumulation by *D. salina* after a 7-day culture

Medium (mg dm^{-3}) As (V)	GSH[b]	BS[b]	Biomass (mg dry cells dm^{-3})	As (mg dm^{-3} dry cells)	GSH (mg dm^{-3} dry cells)
0	0	0	44.8	3.1	1.6
100	0	0	57.2	115.2	1.9
100	10	0	57.3	125.9	3.0
100	10	10	58.1	102.2	1.3
100	100	0	45.4	921.7	5.2
100	100	10	52.6	93.5	1.1

[a] The medium contained (mg dm^{-3}): KNO_3, 72, KH_2PO_4, 4.5; temperature 23 °C; illumination 4500 lux; volume of seawater 1 dm^{-3}.
[b] GSH, glutathione; BS, buthionine sulfoxisamine.

accumulated As(V) and As(III) from the aqueous solution, a part of the arsenic was methylated in the cell, but the quantity of methylated arsenic excreted was not in proportion to that of the total arsenic accumulated. Also the above data lead to the conclusion that arsenic is biomethylated in the interior of the cell although the ratio of methylated ratio to inorganic compounds is small. *D. salina* therefore seems to have a limited methylation capacity.

Effect of glutathione on arsenic accumulation by *D. salina*

To order to demonstrate the effect of GSH on arsenic accumulation, *D. salina* was cultured in a medium containing arsenate (100 mg dm^{-3}) and GSH (0–100 mg dm^{-3} in medium) at 23 °C for seven days. The cells were washed with seawater and were analyzed for total arsenic and GSH. The results are shown in Table 3. The arsenic and GSH content in *D. salina* increased when the GSH concentration in the medium was raised from 10 to 100 mg dm^{-3}, but the biomass was decreased. On the other hand, the arsenic content in *D. salina* did not increase at GSH levels of 10 – 100 mg dm^{-3} in the medium containing 10 mg dm^{-3} of buthionine sulfoxisamine (BS). With the addition of BS, the intracellular content of GSH in *D. salina* was small, ranging from 1.1. to 1.3 mg dm^{-3}. It is known that BS is an irreversible inhibitor of γ-glutamylcysteine synthetase.[18] This result suggests that arsenic accumulation was promoted to some degree by GSH at GSH levels higher than 100 mg dm^{-3}. From the above experimental results, the medium containing 100 mg dm^{-3} GSH was recommended for the purpose of removal of arsenate by *D. salina* from the aqueous phase, as this medium accumulates about eight times more arsenic than a GSH-free medium.

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