APPLIED ORGANOMETALLIC CHEMISTRY, VOL. 10, 753–756 (1996)

Uptake and Reduction of Arsenate by *Dunaliella* sp.

Osamu Takimura, Hiroyuki Fuse, Katsuzi Murakami, Kazuo Kamimura and Yukiho Yamaoka
Chugoku National Industrial Research Institute, 2–2–2 Hirosuehiro, Kure, Hiroshima, 737–01, Japan

Uptake and reduction of arsenate [AS(V)] by *Dunaliella* sp. cells were determined to investigate the metabolic processes of arsenic in the alga. Cellular uptake of arsenic by *Dunaliella* sp. cells was markedly affected by the form of arsenic in the medium. The content of arsenic taken up by *Dunaliella* sp. cells increased rapidly with time on addition of As(V) to the medium. However, in the case of addition of arsenite [As(III)], the gradient of arsenic uptake by *Dunaliella* sp. cells was low, and arsenic content was small. In the water-soluble fraction of arsenic taken up by *Dunaliella* sp. cells with exposure to As(V), arsenic was in the forms of organic arsenic, As(V) and As(III). The content of As(V) in the water-soluble fraction increased with exposure time. The content of As(III) also increased with time, but remained constant after 5 h of exposure. On the other hand, organic arsenic content was small and did not increase with time. It was found that *Dunaliella* sp. takes up As(V) and readily reduces it to As(III).

Keywords: *Dunaliella* sp.; arsenate; arsenite; reduction; water-soluble fraction; gel filtration

INTRODUCTION

In general, arsenic is toxic for most organisms at high concentrations. Recently, it has been reported that microorganisms accumulate arsenic to a high extent,[1,2] and that the marine green alga *Dunaliella* sp. also accumulates arsenic[3]. The accumulation was found to depend greatly on environmental factors (e.g. pH, temperature, light intensity etc.)[4]. Furthermore, the large majority of arsenic in the cells was present in the water-soluble fraction[5]. Arsenic can occur in different forms in the marine environment.

Arsenic participates in abiological cycle by means of oxidative and reductive reactions within organisms. Reduction and methylation of inorganic arsenic by marine phytoplankton have been shown to occur[6] and arseno-compounds have been identified.[7,8] Thus, it is important to investigate the intracellular behavior of arsenic in the alga. However, there is little information on metabolic processes in the iniital phase after arsenic exposure.

In this work, we report the speciation of arsenic take-up by *Dunaliella* sp. cells and their ability to reduce arsenate in the short term after arsenic exposure.

EXPERIMENTAL

Culture of *Dunaliella* sp.

Dunaliella sp. was obtained from the Hiroshima Fisheries Experimental Station, Japan.

The medium was natural sea water, which was collected inshore and filtered (0.22 μm) to remove particulate materials. Nitrate (KNO_3; 72 mg dm^{-3}) and phosphate (KH_2PO_4; 4.5 mg dm^{-3}) were added to the medium to promote growth. Stationary-phase algae were inoculated in fresh medium to an initial concentration of 10^4 cells cm^{-3} and were grown at 23 °C under illumination with fluorescence lamps at a light intensity of 6000 lx. The growth of *Dunaliella* sp. was monitored by determining fluorescence intensity (*in vivo* chlorophyll) with a Turner fluorometer (Model 10 series). After 1 week, the cells were collected at the logarithmic phase by centrifugation at 3500 rpm for 5 min.

Uptake of arsenic by *Dunaliella* sp.

The cells harvested by centrifugation were resuspended in an artificial seawater medium

CCC 0268–2605/96/090753–04

Received 5 December 1996
Accepted 14 August 1996

(1 dm^3 distilled water; 18.0 g NaCl; 5.0 g $MgSO_4 \cdot 7H_2O$; 0.6 g KCl; 0.1 g $CaCl_2$; 1.0 g Tris. Sodium arsenate ($Na_2HAsO_4 \cdot 7H_2O$) [abbreviated as As(V)] and sodium arsenite ($NaAsO_2$) [abbreviated as As(III)] were added to the medium, respectively. The cultures were incubated under the illumination of fluorescence lamps at 5000 lx, at 23 °C and pH 8.0. Fluorescence intensity (*in vivo* chlorophyll) in *Dunaliella* sp. was measured with a Turner fluorometer as an index of growth inhibition by arsenic. After an appropriate time, the cells were collected by centrifugation at 3500 rpm for 5 min, then washed three times with an arsenic-free artificial seawater medium.

Separation of arsenic forms in *Dunaliella* sp. cells

The cells (about 10 mg on a dry weight basis) were resuspended in 2.0 cm^{-3} of 50 mM Tris buffer (pH 8.0), and were disrupted by a ultrasonic probe for 30 s. The sonicate was then centriguted at 18 000 rpm for 20 min at 4 °C to obtain a water-soluble fraction. Arsenic in the water-soluble fraction was fractionated by gel-filtration chromatography. The chromatography was carried out using a TOYOPEARL HW-40 column (molecular sieving range approximately 100–10 000) (Tosoh Co. Ltd, Japan), 1.5 cm i.d. × 40 cm long. The column was eluted with 100 mM NaCl and a flow rate of 0.6 cm^3 min^{-1}. The arsneic contents of the 1.5 cm^3 fractions of the eluate were determined by flame atomic absorption spectrometry.

Determination of arsenic

For the determination of arsenic in the gel-filtration chromatography fractions, aliquots were measured directly by the hydride-generation flame atomic absorption spectrometry (HG AA) method. For total arsenic in cells, the cells were digested with a mixed solution containing 3 cm^3 of concentrated nitric acid and 1 cm^3 of concentrated sulfuric acid and 1 cm^3 of 60% perchloric acid. Total arsenic in the digest was determined via the HG AA spectrometry method[9]. The basic unit of the hydride generator is composed two parts: the precision peristaltic pump, which is used to meter and mix reagents and sample solution, and the gas–liquid separator. At the gas–liquid separator a constant flow of argon strips out the hydrogen and metal hydride gases formed in the reaction and carries them to a heated quartz absorption cells, which is supported by a metal bracket mounted on top of the regular air–acetylene burner head. Wavelength and lamp current for atomic absorption spectrometry were 193.7 nm and 10 mA, respectively.

RESULTS AND DISCUSSION

Arsenic uptake and growth inhibition in *Dunaliella* sp. cells

Arsenic uptake and growth inhibition in the *Dunaliella* sp. cells were determined for As(V) and As(III). Measurement of fluorescence intensity is a reliable index of growth inhibition, quantitatively measuring only viable cells[10]. The time courses for the content of arsenic and fluorescence intensity in *Dunaliella* sp. after the addition of As(V) and As(III) are shown in Fig. 1. The cells were exposed to 10 mg dm^{-3} concentrations of arsenic in the medium in each case. There are significant differences for the two forms. The content of arsenic taken up by

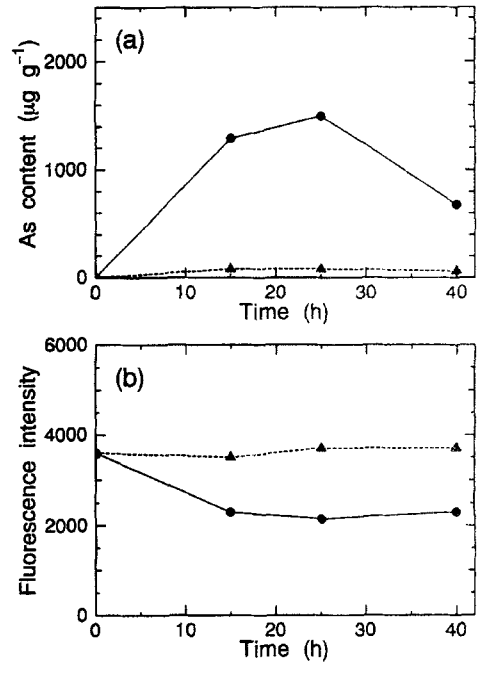

Figure 1 Time course of arsenic uptake (a) and fluorescence intensity (b) in *Dunaliella* sp. cells after exposure to arsenate (●) or arsenite (▲). The cells were exposed to 10 mg dm^{-3} in each case.

Table 1. Effect of arsenic concentration in the medium on fluorescence intensity and arsenic content in *Dunaliella* sp. cells after exposure for 15 h

	As in medium (mg dm^{-3})	Fluorescence intensity (%)[a]	As content (μg mg^{-1})
As (V)	1	78.9	0.65
	10	47.4	5.61
	100	45.6	5.68
As(III)	1	90.7	nd[b]
	10	88.9	0.50
	100	88.9	2.73

[a] Percentage of initial intensity (before exposure to arsenic).
[b] nd, Not detected.

Dunaliella sp. increased rapidly with time for As(V) and reached a maximum concentration within 25 h of the As(V) exposure. However, for As(III) exposure the content of arsenic taken up by the alga was small and did not increase with time. These results suggest that uptake of arsenic by *Dunaliella* sp. cells depends on the valence (oxidation) state of the inorganic arsenic in the medium. On the other hand, the fluoresence intensity in *Dunaliella* sp. slightly decreased with time for As(V) exposure. The fluorescence intensity at 15 h had decreased by approximately 60% compared with the initial value and, after 25 h, the intensity remained constant. However, for As(III) exposure, the fluorescence intensity was not affected. It is therefore suggested that As(V) is more sensitive than As(III) towards *Dunaliella* sp. cells. Bottino *et al.*[11] reported that arsenate has a greater toxic effect than arsenite for marine alga. On the other hand, uptake of arsenate by the marine snail *Littorina littoralis* took place twice as readily as that of the arsenite. It is concluded that the response to arsenic in *Dunaliella* sp. must be significantly different for different forms of arsenic.

The effects of arsenic concentration in the medium on the fluoresence intensity and arsenic content in *Dunaliella* sp. cells are shown in Table 1. The cells were exposed to As(V) or As(III) in the medium over a concentration range of 1–100 mg dm^{-3} for 15 h. The fluorescence intensity decreased with increase of concentration for As(V) but the intensity was not affected for As(III). On the other hand, the arsenic content in the cells after As(V) exposure was low at 1.0 mg dm^{-3} in the medium, but was much larger at 10 mg dm^{-3} and 100 mg dm^{-3} in the medium (Table 1). However, after As(III) exposure, the arsenic content in the cells was not detected at 1 mg dm^{-3} and was very low at 10 mg dm^{-3} in the medium, but the content was 2.73 mg g^{-1} in the cells at 100 mg dm^{-3} in the medium. It seems therefore that uptake of arsenic by *Dunaliella* sp. cells in high-concentration As(III) media should be further investigated.

Separation of arsenic forms in the water-soluble fraction in *Dunaliella* sp. cells

In previous work, the majority of arsenic taken up by *Dunaliella* sp. was found in the water-soluble fraction[5]. The chemical form of arsenic in the water-soluble fraction was determined by fractionation using gel filtration. The elution profile during gel filtration for the water-soluble fraction in *Dunaliella* sp. cells exposed to 10 mg dm^{-3} As(V) for 15 h is shown in Fig. 2. The elution profile was calibrated by Blue Dextran 2000 for the void volume, and by Na_2HAsO_4 [As(V)] and $NaAsO_2$ [As(III)] to fix the arsenic form of the sample. The arsenic fraction obtained by gel filtration had three peaks. The first peak (Fr. I) eluted at the void volume, as determined by Blue Dextran. In relation to the fact that the molecular sieving range of the Toyopearl HW-40 gel used in this experiment is from 100 to 10 000, the peak is confirmed as an organic arsenic compound with molecular weight over 10 000 Da. The second peak (Fr. II) corresponded to the elution peak of Na_2HAsO_4 [As(V)], and the third peak (Fr. III) was the same as the elution peak of $NaAsO_2$ [As(III)]. It is therefore deduced that the arsenic

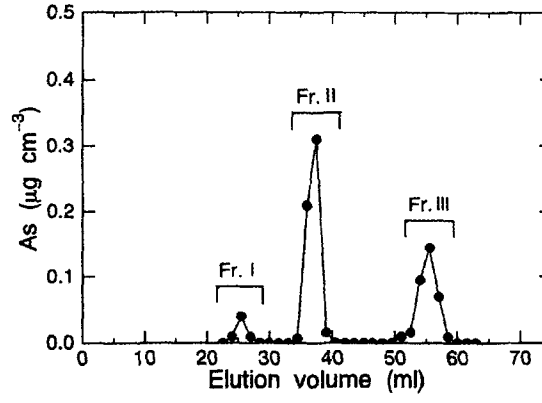

Figure 2 Gel-filtration elution profile of water-soluble fraction in *Dunaliella* sp. cells. The cells were exposed to 10 mg dm^{-3} arsenate for 15 h.

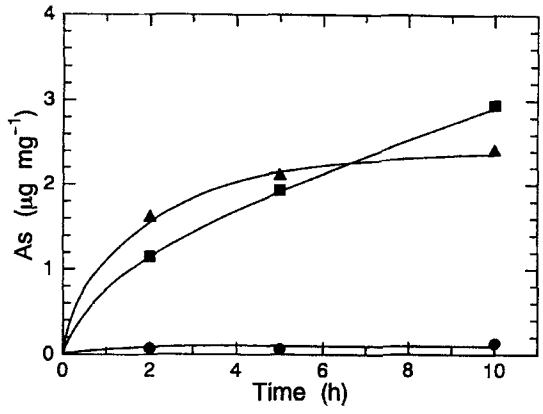

Figure 3 Time course of arsenic content in the three arsenic fractions (see Fig. 2: ●, Fr. I; ■, Fr. II; ▲, Fr. III) in *Dunaliella* sp. cells after exposure to 10 mg dm^{-3} arsenate.

in the water-soluble fraction was in the forms of organic arsenic, As(V) and As(III). As(III) is present in the cytoplasmic fraction, although As(V) is added to the medium. This result indicated that As(V) is reduced to As(III) in the cytoplasmic fraction of *Dunaliella* sp. Sanders and Windom[6] reported that in a marine phytoplankton taking up As(V), the majority of the As(V) is reduced, methylated and released to the surrounding medium. Furthermore, in the marine blue–green alga *Phomidium* sp. was readily reduced to arsenite within tissues.[1] From these results, it is probable that many organisms in the marine environment have the ability to reduce arsenate.

The time courses of the three arsenic fractions obtained by gel filtration are shown in Fig. 3. The content of Fr. I was small, did not increase with time and remained constant. The content of Fr. II increased rapidly with exposure time. The content of Fr. III also increased with time, but the content was constant after 5 h. From these results, it is deduced that reduction of arsenate to arsenite in the cytoplasmic fraction is conducted rapidly and reaches a maximum at 5 h.

The effect of arsenic concentration in the medium on the three fractions (Fr. I, Fr. II and Fr. III) in *Dunaliella* sp. cells after 15 h of exposure is shown in Table 2. In Fr. I, the arsenic content is not affected by increasing As(V) concentration in the medium. The content was small, and ranged from 0.21 to 0.33 μg mg^{-1}. It is found that the content of the arsenic-x-containing

Table 2. Effect of arsenate concentration in the medium on the three fractions (see Fig. 2) in *Dunaliella* sp. cells after exposure for 15 h

As(V) in medium (mg dm^{-3})	As content (μg mg^{-1})		
	Fr. I	Fr. II	Fr. III
1	0.21	0.12	0.32
10	0.26	4.29	1.05
100	0.33	4.36	0.99

organic compound, which is a UV-absorbing compound (absorbance data not shown), does not show a strong variation with the As(V) concentration in the medium. On the other hand, the arsenic contents of Fr. II and Fr. III both increased rapidly in the 10 mg dm^{-3} medium. However, there was no effect on the 100 mg dm^{-3} concentration compared with that with 10 mg dm^{-3} in the medium. These results suggested that As(V) reduction in *Dunaliella* sp. cells depends upon the concentration of As(V) in the medium.

REFERENCES

1. S. Mastuto, H. Kasuga, H. Okumoto and A. Takahashi, *Comp. Biochem. Physiol.* **78C**, 377 (1984).
2. S. Maeda, S. Nakashima and T. Takeshita, *Sep. Sci. Tech.* **20**, 153 (1985).
3. Y. Yamaoka and O. Takimura, *Agric. Biol. Chem.* **50**, 185 (1985).
4. O. Takimura, H. Fuse and Y. Yamaoka, *J. Chem. Sci. Jpn.* **4**, 740 (1989).
5. O. Takimura, H. Fuse and Y. Yamaoka, *Appl. Organomet. Chem.* **6**, 363 (1992).
6. J. G. Sanders and H. L. Windom, *Estuar. Coast. Mar. Sci.* **10**, 555 (1980).
7. G. Lunde, *Acta Chim. Scand.* **27**, 1586 (1973).
8. K. J. Irgolic, E. A. Woolson, R. A. Stockton, R. D. Newman, N. R. Bottino, R. A. Zingaro, P. C. Kearney, R. A. Pyles, S. Maeda, W. J. McShane and E. R. Cox, *Environ. Health Perspect.* **19**, 61 (1977).
9. M. Yamamoto, M. Yasuda and Y. Yamamoto, *Anal. Chem.* **57**, 1382 (1985).
10. D. S. Filip, T. Peters, V. D. Adams and E. J. Middlebrooks, *Water Res.* **13**, 305 (1979).
11. V. R. Bottino, R. D. Newman, E. R. Cox, R. A. Stockton, M. Hoban, R. A. Zingaro and K. J. Irgolic, *J. Exp. Mar. Biol. Ecol.* **33**, 153 (1978).
12. D. W. Klumpp, *Mar. Biol.* **58**, 265 (1980).