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Effects of arsenic on the organic component of the alga *Dunaliella salina*

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The unicellular marine alga, *Dunaliella salina* 19/30 was grown in seawater containing an inorganic arsenic concentration (Na_2HAsO_4) up to 2000 mg dm^{-3}. The cells survived even at 5000 mg dm^{-3}. The arsenic concentration of the cells increased with an increase of the surrounding arsenic concentration. Arsenic in *D. salina* was also greatly affected by addition of phosphorus. The arsenic-tolerance behavior of *D. salina* seemed to suggest that the algae have a function to prevent accumulation of inorganic arsenic by increasing the β-carotene, fatty-acid ($C_{18:1}$, $C_{18:3}$) and water-extractable carbohydrate content in the cells. Arsenic accumulation also rose steadily with an increase in the nitrogen concentration in the medium.

Keywords: Arsenic, β-carotene, carbohydrate, *Dunaliella salina*, microalgae

INTRODUCTION

Arsenic is an element which is widely distributed in the biosphere. Maeda[1] investigated the bioaccumulation of arsenic by freshwater algae, which when tested were resistant to 100 mg dm^{-3} of inorganic arsenic and had a great ability to accumulate arsenic. *Dunaliella sp.* (*D.sp.*)[2] was found to tolerate exposure to 2000 mg dm^{-3} inorganic arsenic well when grown in an arsenic-enriched medium. Based on the fact that microalgae internally accumulate a large amount of arsenic, a proposal to collect arsenic in the water by this function of microalgae might seem feasible. If *D. salina* was grown in a specific environment, (i.e. high salinity, high light intensity, low nitrogen, etc.), it accumulated a higher content of β-carotene in the cells.[3] There are few reports on the characteristics of *D. salina* including arsenic accumulation, arsenic tolerance and the effects of arsenic on organic materials in cells. This report describes the effects of inorganic arsenic (Na_2HAsO_4) levels on (1) the organic material in the alga (viz. pigment, fatty acid, carbohydrate), (2) phosphate levels (3) nitrogen levels on the growth and arsenic bioaccumulation of *D. salina*.

MATERIALS AND METHODS

Microalgae

D. salina 19/30 (Chlorophyceae) obtained from the Culture Collection of Algae and Protozoa (University of Cambridge, UK) was used throughout the experiments.

Cultures of algae

D. salina was grown in 5 dm^{-3} volumes of sterile media containing appropriate nutrient concentrations at 23 °C with constant bubbling of air.

Light was supplied by a Toshiba 40 W power cool white lamp at an intensity of 5000 or 15 000 lux (at the surface of the liquid medium). The medium consisted of variants of KNO_3, 72 mg; KH_2PO_4, 4.5 mg; iron-chelated EDTA, 0.5 mg; and aged seawater, 1000 cm^3.

D. salina cells taking up arsenic were collected at the stationary growth phase by continuous centrifugation at 3000 rpm and the arsenic, carbohydrate, fatty-acid and β-carotene content in the centrifuged cells was determined. The growth of *D. salina* was monitored by a Hitachi Model 100-20 Spectrophotometer by measuring absorbance at 663 nm.[4]

Determination of β-carotene in algae

Pigments extracted with 80% acetone–water (v/v) solution (2 cm^3) were transferred to diethyl ether. This procedure was repeated three times and the total amount of extracted solution was adjusted to 10 cm^3 with acetone. Before high-performance liquid chromatography (HPLC), the extracted solution was filtered with a Millipore

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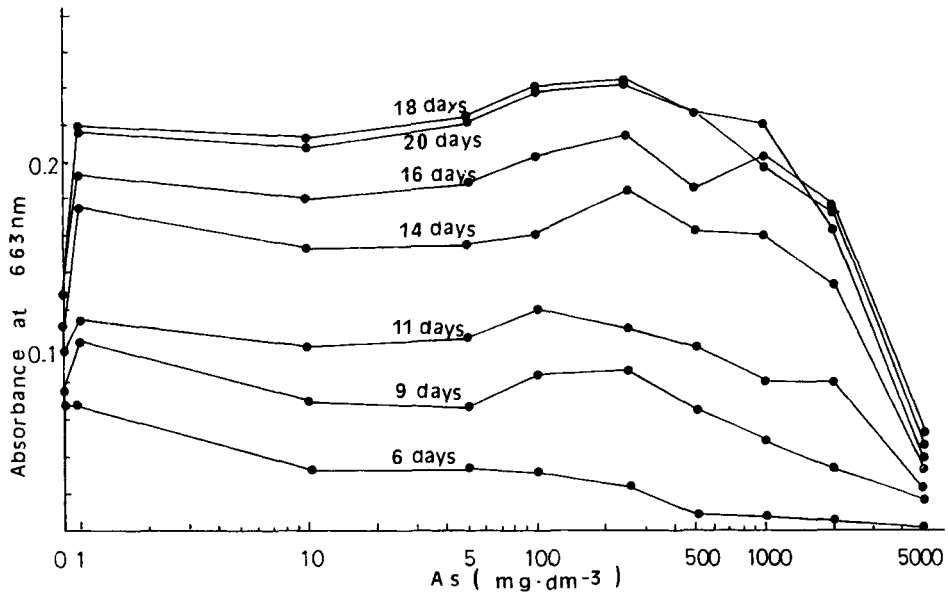

Figure 1 Effect of arsenic concentration in the medium on the growth of *Dunaliella salina*, measured in terms of absorbance at 663 nm. Conditions: KNO_3, 72 mg dm^{-3}; KH_2PO_4, 4.5 mg dm^{-3}; in aged sea water; 23 °C, 10 000 lux, 20 days

filter (diameter 0.45 μm). β-Carotene in the algae was measured by HPLC (Nippon Bunnkou Co. Ltd, Trirotar SR-2 Model) on a column of Wakosil 5C8 (i.d. 4.6 × 150 mm) with a linear gradient of methanol–acetonitrile (25:75, v/v)–water as described previously.[4] Major eluted pigments were identified by their spectral characteristics.

Determination of fatty acid in algae

Approx. 20 mg of freeze-dried cells were first homogenized with 5 cm^3 of water, then extracted twice with 25 cm^3 of chloroform–methanol (2:1, v/v) and filtered.

The concentrated material was saponified with 0.5 mol dm^{-3} potassium hydroxide–methanol solution (10 cm^3) and methylated by adding 14% boron trifluoride–methanol solution (5 cm^3).[5] The fatty-acid composition of lipids of the *D. salina* was determined by gas–liquid chromatography (GLC) using an instrument with a flame ionization detector (FID) (Shimazu model GC-14A). The column was packed with fused silica WCOT, CP-Sil 88 CB (0.2 μm, i.d. 0.22 mm × 50 m). Fatty acids in the chromatogram were identified by comparison of their retention times with known standards. Fatty-acid contents were determined by comparing their peak areas with a C_{17}:0 fatty acid (heptadecanoic acid) internal standard.

Determination of carbohydrates in algae

Freeze-dried cells (10 mg) were twice treated with distilled water (250 cm^3) at 100 °C for 1 h, and then filtered through an ultra-filter. The resulting filtrate was used for water-extractable carbohydrate determination. The residual carbohydrate was determined by the phenol–sulfuric acid method, after hydrolysis with sulfuric acid.[6]

Determination of arsenic in algae

The freeze-dried algae containing arsenic were digested with a mixed solution containing of 3 cm^3 of concentrated nitric acid, 1 cm^3 of concentrated sulfuric acid and 1 cm^3 of perchloric acid (60 %). The total arsenic content in algae was determined by a hydride-generation atomic absorption spectrometry method.[7]

RESULTS AND DISCUSSION

Relation between arsenic resistance and β-carotene content

Figure 1 shows the effect of arsenic concentration (0–500 mg) on the growth of *D. salina*. Each volume of biomass was compared between an arsenic-containing medium and an arsenic-free medium. The growth of *D. salina* was better in

Table 1 Effect of arsenate on the pigment of *Dunaliella salina* (KNO_3 72 mg cm^{-3}, KH_2PO_4 4.5 mg cm^{-3}, 5000 lux, 23 °C)

Arsenic (mg dm^{-3})	Lutein[a] (μg mg^{-1})	Chlorophyll *a*[a] (μg mg^{-1})	β-Carotene[a] (μg mg^{-1})
0	5.1	4.2	54.2
1	6.5	4.9	59.5
10	4.9	5.7	75.6
50	6.0	4.7	74.4
100	7.6	5.5	117.9
250	10.0	7.1	150.7
500	9.2	7.3	132.5

[a] Dry algae.

the medium containing arsenic than in the arsenic-free medium. Similar results were reported in a previous paper: the growth of *D.sp.*[2,7] increased with an increase in arsenic concentration in the medium up to 2000 mg dm^{-3}, with cells surviving even at 5000 mg dm^{-3}. One reason why the algae had a tolerance for higher arsenic levels seems to be that the algae have an ability to prevent inorganic arsenic from reacting with the —SH group of an enzyme, thus maintaining the activity of the enzyme even if inorganic arsenic had entered the cell through the cell membrane.[8] Table 1 shows the relation between β-carotene accumulation in the cells and arsenic levels in the medium. β-Carotene accumulation increased with an increase in the surrounding arsenic concentration up to 250 mg dm^{-3}. The amount of β-carotene accumulation decreased with an increase in the surrounding arsenic content from 250 top 500 mg dm^{-3}. The concentration of chlorophyll *a* and lutein in *D. salina* was

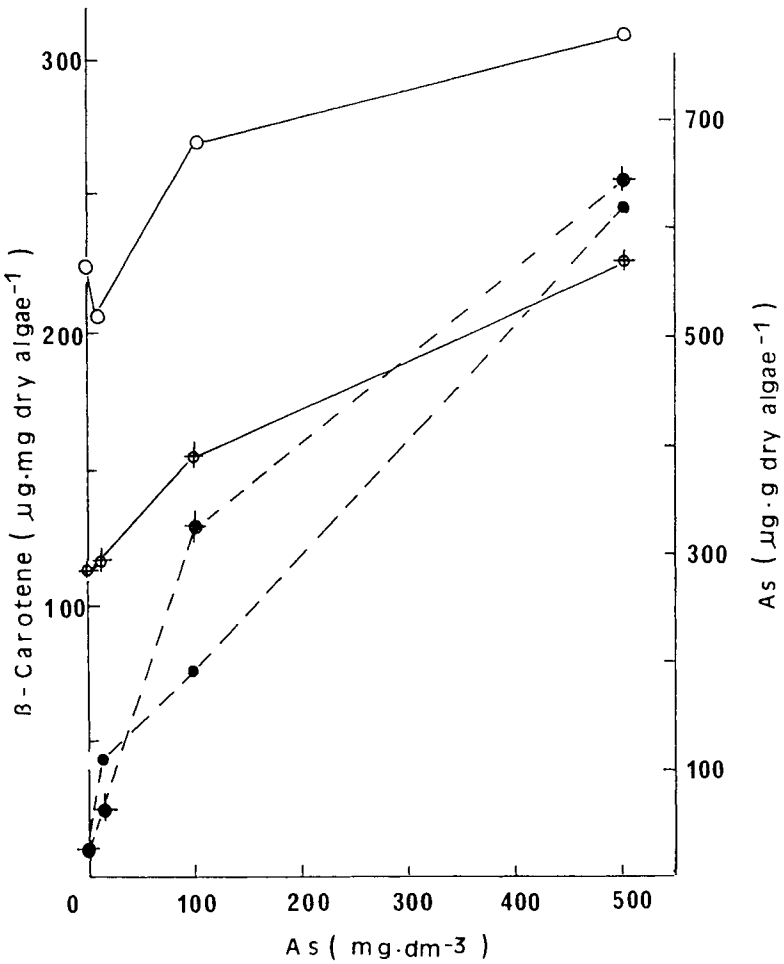

Figure 2 Effect of arsenic concentration in the medium on the β-carotene and arsenic accumulation by *Dunaliella salina*. Conditions: KH_2PO_4, 4.5 mg dm^{-3}; Fe-EDTA, 1 mg dm^{-3}; in aged seawater; 23 °C, 10 000 lux, 14 days. -o-: β-carotene (KNO_3, 4.5 mg dm^{-3}); -⌖-: β-carotene (KNO_3, 72 mg dm^{-3}); --•--: arsenic (KNO_3, 4.5 mg dm^{-3}); --⌖--: arsenic (KNO_3, 72 mg dm^{-3}).

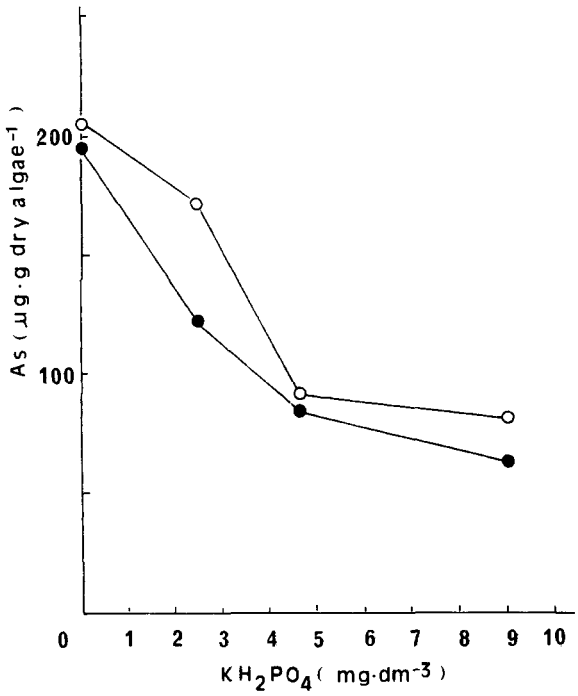

Figure 3 Effect of the concentration of phosphorus on the uptake of arsenic by *Dunaliella salina*. Conditions: containing 10 mg dm^{-3} of arsenic(V) (as Na_2HAsO_4), Fe-EDTA, 1 mg dm^{-3}; in aged seawater; 23 °C, 10 000 lux, 14 days. ●, KNO_3, 4.5 mg dm^{-3}; ○, KNO_3, 72 mg dm^{-3}.

slightly affected by an increase in the arsenic level in the medium. The accumulation of β-carotene by the cell is thought to be one toleration process for inorganic arsenic in the interior of the cell.

Figure 2 shows the relation between arsenic accumulation and β-carotene accumulation by *D. salina*. The optimum illumination (15 000 lux)[4] for β-carotene accumulation by *D. salina* was used in the following experiments. The arsenic concentration of cells also increased with increase of arsenic content in the medium. β-Carotene accumulation by *D. salina* was unaffected by 10 mg dm^{-3} of arsenic. However, an increasing tendency towards β-carotene accumulation was recognized when the arsenic concentration increased to 100 mg dm^{-3}. More β-carotene was accumulated in the cells when the surrounding nitrogen (KNO_3) concentration was 4.5 mg dm^{-3} compared with 72 mg dm^{-3} in the medium, while more arsenic in the cells was accumulated when the surrounding nitrogen concentration was 72 mg dm^{-3} compared with a 4.5 mg dm^{-3} medium. Arsenic accumulation by *D. salina* increased at a nitrogen concentration of 72 mg dm^{-3}, as compared with a nitrogen concentration of 4.5 mg dm^{-3} in the medium. This result suggests that arsenic concentration in algal cells increases if *D. salina* is grown in a medium of high nitrogen concentration.

Effect of phosphorus on growth and arsenic accumulation by *D. salina*

Figure 3 shows the relationship between arsenic accumulation by *D. salina* and phosphorus (KH_2PO_4) concentration in the medium. The accumulation of arsenic by *D. salina* decreased with increase of phosphorus concentration in the medium. Several researchers have recognized, for *Chlorella vulgaris*[8,9] and *D. sp.*[7], a tendency similar to our experimental result and it is often

Table 2 Yields of carbohydrates in fractionation of *Dunaliella salina* (Fe 1 mg dm^{-3}, 15 000 lux, 23 °C)

Medium (mg dm^{-3})			Carbohydrate fractions (%)		
KNO_3	KH_2PO_4	Arsenate	Water extract	Residue	Total carbohydrate[a]
4.5	4.5	0	26.2	73.8	32.7
4.5	4.5	10	28.8	71.2	38.3
4.5	4.5	100	28.5	71.5	42.8
4.5	4.5	500	29.5	70.5	30.0
72	4.5	0	23.8	76.2	24.0
72	4.5	10	28.4	71.6	23.0
72	4.5	100	25.2	74.8	24.0
72	4.5	500	25.1	74.9	19.0

[a] (Total carbohydrates/dry algae) × 100.

Table 3 Effect of arsenic on the fatty-acid composition of *Dunaliella salina* (15 000 lux, 23 °C)

Medium (mg dm^{-3})			Fatty acid contents (% total)								Total fatty acids
KNO_3	KH_2PO_4	As	16:0	16:1	18:0	18:1	18:2	18:2	18:3	Other	(% dry algae)
4.5	4.5	0	30.5	1.6	0.6	19.2	9.8	10.7	17.4	10.2	12.0
4.5	4.5	10	30.6	1.1	0.4	19.7	8.0	10.7	18.4	11.1	9.4
4.5	4.5	100	31.7	1.8	0.6	19.6	8.2	12.2	17.4	8.5	10.2
4.5	4.5	500	31.6	1.4	0.8	18.9	7.1	11.7	17.6	10.9	10.4
72	4.5	0	29.0	1.7	0.8	4.6	14.1	9.2	26.6	14.0	7.6
72	4.5	10	26.6	2.1	0.8	6.1	14.0	8.6	28.9	12.9	5.2
72	4.5	100	26.2	1.3	0.6	7.1	15.4	8.7	29.6	11.1	7.6
72	4.5	500	26.0	0.6	0.4	6.4	14.4	8.8.	28.4	15.0	9.8

believed that phosphorus competes with arsenic. It is more likely that arsenic is taken up directly as arsenic(V) anyway, since the most common biological uptake pathway for arsenic is via the phosphate active transport system.[10] At a nitrogen concentration of 4.5 mg dm^{-3}, the β-carotene accumulation increased and arsenic accumulation slightly increased, while β-carotene accumulation decreased at a nitrogen concentration of 72 mg dm^{-3}. This result suggests that arsenic and phosphorus in the medium might affect metabolic processes and β-carotene accumulation by *D. salina*.

Effect of arsenic on carbohydrates of *D. salina*

Table 2 shows the relationship between carbohydrate from *D. salina* and arsenic concentration in the medium. In the comparison between an arsenic-containing medium and an arsenic-free medium, either on equivalent carbohydrate level or a slightly higher level in the arsenic-containing medium was recognized. In the case of an arsenic concentration of 500 mg dm^{-3}, carbohydrate in the cell decreased. Total carbohydrate for *D. salina* was greater with a nitrogen concentration of 4.5 mg dm^{-3} in the medium than at a nitrogen concentration of 72 mg dm^{-3}. In order to estimate the total carbohydrate content in *D. salina*, carbohydrate was divided into cell-wall carbohydrate (residue) and intracellular carbohydrate (water-extractable). The cell-wall carbohydrate occupied 71–76% of total carbohydrate (residue) in *D. salina*. Water-extractable carbohydrate increased with increase of arsenic concentration in the medium. It is suggested that *D. salina* controls the balance of arsenic with metabolic carbohydrate.

Effect of arsenic on fatty acid composition of *D. salina*

Table 3 shows the relationship between arsenic in the medium and fatty-acid composition of *D. salina*. The content of fatty acid in cells was lower with a nitrogen concentration of 72 mg dm^{-3} than with a nitrogen concentration of 4.5 mg dm^{-3}. At a nitrogen concentration of 4.5 mg dm^{-3}, the fatty-acid content in the cells decreased with the addition of arsenic in the medium. However, the fatty-acid content in cells was unchanged with an increase of arsenic concentration in the medium, while at a nitrogen concentration of 72 mg dm^{-3} the fatty-acid content in the cells increased with increase of arsenic concentration in the medium. This result means that the fatty-acid content in the cell was affected by increase of arsenic concentration in the medium. At a nitrogen concentration of 4.5 mg dm^{-3} in the medium, the fatty-acid composition in the cells was unchanged with increase of arsenic concentration in the medium. At a nitrogen concentration of 72 mg dm^{-3}, saturated acid (C_{16}:O) decreased with increase of arsenic, while unsaturated acid ($C_{18:1}$, $C_{18:3}$) increased. This result suggests that *D. salina* probably possesses resistance by utilizing an increase of its unsaturability to the added arsenic.

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