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Accumulation of Arsenic by Rhaphydophyceae *Chattonella antiqua* (Hada) Ono

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Rhaphydophyceae *Chattonella antiqua* (Hada) Ono was grown in seawater containing an arsenic concentration up to 50 mg dm^{-3}, and survived even at 200 mg dm^{-3}. The arsenic content increased with an increase of the surrounding arsenic, iron and manganese concentrations. However, arsenic accumulation was unaffected by phosphorus concentration. Also, arsenic content in *C. antiqua* decreased at a selenium concentration of up 20 mg dm^{-3}, and was reduced by the addition of antimony. In the living cells, about 52% of the arsenic which accumulated in each cell was found in the intracellular fraction, 27% in the lipid fraction, and 21% in the cell wall fraction.

Keywords: arsenic; accumulation; *Chattonella antiqua*; microalgae

INTRODUCTION

Microalgae are native to a vast array of freshwater and marine environments, and can be grown in large quantities with relative ease. Some marine algae can accumulate large amounts of arsenic from their surroundings.[1,2] This phenomenon has important implications in wastewater treatment; Maeda *et al.*[3] have pointed out that microorganisms could be used to remove arsenic from wastewater and in the mining industry. More importantly, the technique of arsenic removal by the cell walls of either living or dead organisms has the ability to reduce arsenic concentrations in aqueous solutions to 1 mg dm^{-3} or less. Several investigators[4–8] have worked on arsenic accumulation using microalgae. However, so far no Rhaphydophyceae have been tested. *Chattonella antiqua* (Hada) Ono, Rhahidophyceae, are the most noxious red-tide flagellates, particularly for cultured yellowtail, in Japanese coastal waters.[9] Because of the serious damage to fish farming caused by *C. antiqua* red tides, intensive studies have been conducted on the mechanisms of the blooms. There has been no report on the accumulation of arsenic using *C. antiqua*.

This report describes the effects of various elements (nitrogen, phosphorus, iron, manganese, selenium and antimony) on arsenic accumulation, and on growth inhibition of *C. antiqua* in coexistence with arsenic and these elements.

MATERIALS AND METHODS

Microalgae

The green alga *Dunaliella* sp. and Raphydophyceae *Chattonella antiqua* (Hada) Ono were obtained from the Hiroshima Prefecture Fisheries Laboratory, and the green alga *Dunaliella saline* 19/30 from the Culture of Algae and Protozoa (University of Cambridge, UK).

Culture of algae

The composition of an Iwasaki SW-2 medium[10] for the culture of *C. antiqua* was as follows: KNO_3 72 mg; KH_2PO_4 4.5 mg; Fe–EDTA 1 mg; and natural seawater 1 dm^3, which was collected from inshore and filtered (0.22 μm) to remove particulate materials. Stock cultures were maintained in the stationary growth phase in 500 cm^3 of the appropriate medium in stoppered 1 dm^3 Erlenmeyer flasks. Stock and test cultures of *C. antiqua* were incubated in a growth chamber, illuminated with Toshiba cool white fluorescent lamps. The mean light intensity around the flasks was determined to be 4500 lx. The temperature was maintained at 23°C. The pH of each sample

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was adjusted by addition of dilute hydrochloric acid or sodium hydroxide to pH 8.2. The cells in the linear growth phase were collected by centrifugation at 2500 rpm for 10 min, washed three times with seawater, and used for the arsenic accumulation experiments.

The intensity of fluorescence of living cell suspensions was found to be proportional to the cell density, so measurement of the growth of the cells (g dry weight of cells per dm^3 of medium) was obtained by determination of the intensity of fluorescence of the culture.

Accumulation of arsenic by *C. antiqua* cells

In the arsenic accumulation experiments, preculture algal cells (1 mg dry weight basis) were suspended in a 1 dm^3 Erlenmeyer flask with seawater containing the desired amount of arsenic (0, 1, 10, 50, 100, 200 mg As dm^{-3}). Arsenic was added as Na_2HAsO_4 and elements (manganese, iron, selenium, antimony) were added with concentration ranges of 0–50 mg compound per dm^3 to the arsenic-containing medium. The compounds used were $MnCl_2$, Fe–EDTA, Na_2SeO_3 and $SbCl_3$.

The arsenic accumulation experiments were carried out in light (4500 lx) with sterile air at 23 °C and pH 8.2. After an appropriate time, the cells were collected by centrifugation at 3000 rpm for 5 min, washed three times with deionized water, and freeze-dried.

Analysis of arsenic and heavy metals

The freeze-dried cells containing arsenic and other elements (zinc, iron, manganese, selenium, antimony) were digested with a mixed solution containing 3 cm^3 of concentrated nitric acid, 1 cm^3 of concentrated sulphuric acid and 1 cm^3 of 60% perchloric acid. The amounts of arsenic, selenium and antimony were determined by means of a hydride-generation–atomic absorption spectrophotometer (AA) system and those of zinc, iron and manganese were determined using a flame atomic absorption spectrophotometer[11] (Jurrell Ash Co., Model AA-1 MK 2). The amount of potassium in the algal cells was determined by X-ray fluorescence spectrometry (Kevex Co., Model EDAX-771).[12, 13]

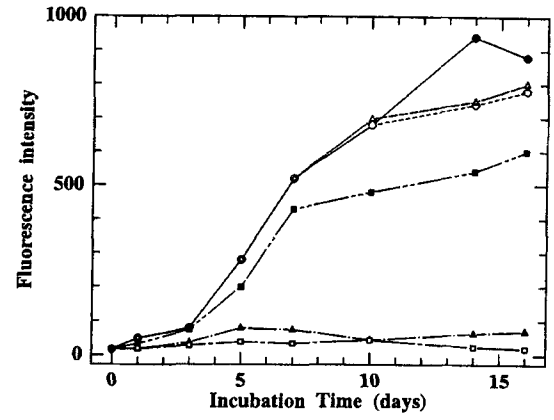

Figure 1 Growth curves of *C. antiqua* cultured in medium containing arsenic. Growth was monitored by measuring *in vivo* fluoresence: there is a close correlation between the relative intensity of fluorescence and the biomass of *C. antiqua*. ●, 10; ○, 1; △, 0; ■, 50; ▲, 100; □, 200 mg As(V) dm^{-3}.

RESULTS AND DISCUSSION

Effects of arsenic on the growth of *C. antiqua*

The effects of arsenic on the growth of *C. antiqua* were examined in the arsenic concentration range from 0 to 200 mg dm^{-3}, of which the results were shown in Fig. 1. It was found that this organism could survive concentrations of arsenate as high as 200 mg dm^{-3} when the arsenate concentration of the medium was increased stepwise to allow for adaptation. Cells also survived when they were added without adaptation to a medium containing 200 mg dm^{-3}. The growth of *C. antiqua* seemed to be unaffected by arsenic at levels ranging from 0 to 10 mg dm^{-3} during 14 days, although in the 50 mg dm^{-3} arsenic-medium cell-growth experiment, the total yield of cells was greatly reduced relative to those in the arsenic-free medium. The motion of algal cells when viewed with a microscope was quite erratic compared with the movement of cells in the arsenic-free medium. This result suggests that the growth was inhibited to some degree by arsenic at arsenic levels higher than 10 mg dm^{-3}. On the other hand, the arsenic content of *C. antiqua* gradually rose with an increase in the arsenic level in the medium (Table 1). Iron and potassium contents in *C. antiqua* were unaffected, but zinc contents in *C. antiqua* were affected by an increase of arsenic concentration in the medium. This value of the

Table 1 Arsenic accumulation by living cells of *Chattonella antiqua* in a medium containing various concentrations of arsenic[a]

Arsenate in medium (mg As(V) dm^{-3})	Algal biomass (mg dm^{-3})	Element accumulated in cells (mg dm^{-3} dry cell) As	Fe	Zn	K
0	11	14	191	120	2998
1	10	107	193	516	2525
10	13	173	223	370	2660
50	7	182	185	420	2903
100	4	287	187	468	2978
200	1	326	196	270	3869

[a] For analytical method, see Ref. 12. The medium contained: KNO_3, 72 mg dm^{-3}; KH_2PO_4, 4.5 mg dm^{-3}; Fe–EDTA, 1 mg dm^{-3}. The precultured living cells (dry weight: 1 mg) were suspended in 500 cm^3 of the medium containing the indicated amounts of arsenate for 14 days.

arsenic content in *C. antiqua* is about 1/10 lower than the arsenic content in *Dunaliella*.[14] Yamaoka *et al.*[13] reported that zinc concentrations in *Dunaliella* cells were greatly affected by addition of arsenic. The results suggest that the ability to accumulate arsenic differs with different species of microalgae and is affected by the content of zinc in the cells.

Distribution of arsenic accumulation by *C. antiqua*

To investigate the distribution of arsenic in *C. antiqua* cell fractions, the cells which accumulated arsenic were fractionated as described previously.[12] The wet living cells (1.2 g, on a dry weight basis) were homogenized with chloroform/methanol (2:1) using a Teflon homogenizer, the slurry was filtered under reduced pressure through a Millipore filter (0.22 μm), and the residue was washed with the mixed solvent until the filtrate became colourless. The filtrate was combined with the washings and shaken with one-quarter of their total volume of water, the mixture was allowed to stand at room temperature overnight, and the upper phase (water-soluble) and the lower (lipid-soluble) were separated and evaporated to dryness. The lipid-soluble (lipid), water-soluble (intracellular) and residue (cell-wall) fractions were digested with a mixed solution (nitric acid/sulphuric acid/60% perchloric acid = 3:1:1) and analysed by hydride-generation–AA methods.[11] As shown in Table 2, in the living cells about 52% of arsenic accumulated in the cells was found in the intracellular fraction, 27% in the lipid fraction and 21% in the cell-wall fraction. As described in previous paper,[15, 16] the arsenic in *Dunaliella* sp. cells was in the forms arsenate, arsenite and organic arsenic, and most of it was arsenate. Also, Yamaoka *et al.*[6] have reported that lipid-fraction arsenic in *Dunaliella* sp. occupied 3.8% of the entire accumulation and was present to a small extent. From the difference between the two results, we presume that the form of arsenic accumulation varied in conformity with the kind of algae. Arsenic content in the cell-wall fraction in *C. antiqua* was found to be higher than that in the cell-wall fraction of *Dunaliella* sp. This difference in the arsenic content in the cell-wall fraction is probably caused by differences in the organic component of the cell wall[16] and in physical adsorption or ion exchange at the cell surface. Details of the distribution of arsenic within the algal cells will be studied further in the future.

Table 2 Distribution of arsenic accumulated in living cells of *Chattonella antiqua*

Alga	Fraction	Arsenic accumulated in cells (μg cm^3)	Distribution ratio (%)
Chattonella antiqua	Lipid	32	27
	Intracellular	62	52
	Cell-wall	25	21
Dunaliella sp.	Lipid	12	4
	Intracellular	283	94
	Cell-wall	7	2

Effect of phosphorus and nitrogen concentrations on arsenic accumulation

In order to demonstrate the effect of nitrogen and phosphorus on arsenic accumulation, *C. antiqua* was cultured in a medium containing various levels of nitrogen (KNO_3), phosphorus (KH_2PO_4) and 10 mg dm^{-3} of Na_2HAsO_4, and growth and arsenic accumulation were measured (Table 3). The growth of *C. antiqua* became greater with an increase in nitrogen and phosphorus concentrations in the medium, and reached a maximum at 72 mg dm^{-3} nitrogen and 9 mg dm^{-3} phosphorus, respectively. The arsenic and potassium content in *C. antiqua* increased when the phosphate concentration was raised from 2.25 to 4.5 mg dm^{-3}, but the iron content in

Table 3 Effect of nitrogen and phosphorus on the growth and accumulation of arsenic by *Chattonella antiqua*, after 14 days' culture[a]

Element concn in medium (mg dm^{-3})	Algal biomass (mg dm^{-3})	As accumulated in cells (mg dm^{-3} dry cell)	Element accumulated in cells (mg dm^{-3} dry cell) Fe	K
Phosphorus (KH_2PO_4)[b]				
2.25	9.4	56	209	1333
4.5	11	104	188	2076
9	12	82	197	2243
18	11	80	339	815
Nitrogen(KNO_3)[c]				
18	9.7	205	267	1057
36	12	164	336	1076
72	17	112	190	1976
144	12	125	102	1989
288	13	89	130	2056

[a] For analytical method, see Ref. 12. [b,c] The medium contained: [b]KNO_3, 72 mg; Fe–EDTA, 1 mg dm^{-3}, or [c]KH_2PO_4, 4.5 mg Fe–EDTA, 1 mg dm^{-3}. The precultured living cells (dry weight: 2 mg) were suspended in 500 cm^3 of medium containing 10 mg dm^{-3} of Na_2HAsO_4 for 14 days.

the cells was unchanged. Generally, arsenic and phosphate compete for accumulation by algal cells.[7] However, the dependence of arsenic accumulation in *C. antiqua* on the phosphate concentration did not show a simple competitive inhibitory pattern. On the other hand, the arsenic and iron content in *C. antiqua* gradually decreased and the potassium content rose with increase in the nitrogen level in the medium. This result suggests that arsenic accumulation was inhibited to some degree by nitrogen at nitrogen levels higher than 18 mg dm^{-3}.

Effect of iron and manganese concentrations on arsenic accumulation

Iron and manganese are essential trace elements for the growth of *C. antiqua*.[12] In order to demonstrate the effect of iron and manganese on

Table 4 Effect of iron and manganese on the growth and accumulation of arsenic by *Chattonella antiqua*[a]

Elemental concn in medium (mg dm^{-3})	Algal biomass (mg dm^{-3})	As accumulated in cells (mg dm^{-3} dry cell)	Element accumulated in cells (mg dm^{-3} dry cell)
Iron			
0	20	55	161
1	51	130	412
10	72	3056	2373
20	51	4740	2415
Manganese			
0	19	109	0.5
1	81	161	74
10	64	145	161

[a] For analytical method, see Ref. 12. The medium contained: KNO_3, 72 mg dm^{-3}; KH_2PO_4, 5 mg dm^{-3}. The precultured living cells (dry weight: 2 mg) were suspended in 500 cm^3 of medium containing 10 mg dm^{-3} of Na_2HAsO_4 for 14 days.

Table 5 Effect of selenium (Na_2SeO_3) and antimony on the growth and accumulation of arsenic by *Chattonella antiqua*[a]

Elemental concn in medium (mg dm^{-3})	Algal biomass (mg dm^{-3})	As accumulated in cells (mg dm^{-3} dry cell)	Element accumulated in cell (mg dm^{-3} dry cell)
Selenium			
0	14	107	0.8
1	16	165	22
10	20	136	134
20	18	109	185
50	9	67	311
Antimony			
0	13	115	0
1	22	106	813
10	20	52	2343
50	16	51	4156

[a] For analytical method, see Ref. 12. The medium contained: KNO_3, 72 mg dm^{-3}; KH_2PO_4, 4.5 mg dm^{-3}; Fe–EDTA, 1 mg dm^{-3}. The precultured living cells (dry weight: 2 mg) were suspended in 500 cm^3 of medium containing 10 mg dm^{-3} of Na_2HAsO_4 for 14 days.

arsenic accumulation, *C. antiqua* was cultured in 500 cm^3 of a medium containing various levels of iron (0–20 mg dm^{-3}), and manganese (0–10 mg dm^{-3}), 10 mg dm^{-3} of Na_2HAsO_4, 72 mg dm^{-3} of KNO_3 and 4.5 mg dm^{-3} of KH_2PO_4 for 14 days. Table 4 shows that the growth of *C. antiqua* tended to increase with addition of iron and manganese, but decreased at concentrations higher than 10 mg dm^{-3} (Mn) and 1 mg dm^{-3} (Fe), and that the arsenic, iron and manganese contents in *C. antiqua* increased with increase in the manganese and iron concentrations in the medium. Maeda *et al.*[4] have recognized, for *Chlorella*, a tendency similar to our experimental result. Also, adsorption of arsenic occurs on iron oxyhydroxides formed diagenetically in natural water.[17] These results suggested that arsenic in algal cells, coupled with intracellular iron or manganese oxyhydroxides, accumulated arsenic by the normal biological process.

Effect of selenium and antimony concentrations on arsenic accumulation

Like most trace elements the metalloids selenium, antimony and arsenic can be both toxic and essential to marine organisms. The growth, and the selenium and antimony contents, in *C. antiqua* in a medium containing various levels of selenium and antimony are shown in Table 5 With an increase of selenium(IV) and antimony concentration in the medium, the selenium and antimony content in *C. antiqua* increased, while the arsenic content in the cells reached a maximum at a selenium level of 1 to 10 mg dm^{-3} and decreased at concentrations higher than 20 mg dm^{-3}. Also, the arsenic content in *C. antiqua* was inhibited with an increase of antimony concentration in the medium. These results suggest that the growth and arsenic accumulation of *C. antiqua* are affected by selenium and antimony. From the above experimental results, the medium containing 1 mg dm^{-3} selenium was recommended for the purpose of the removal of arsenic by *C. antiqua* from the aqueous phase, as this medium accumulates 1.5 times more arsenic than the selenium-free medium.

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