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Uptake and excretion of total inorganic arsenic by the freshwater alga *Chlorella vulgaris*

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Arsenic-tolerant freshwater alga *Chlorella vulgaris* which had been collected from an arsenic-polluted environment were tested for uptake and excretion of inorganic arsenic.

Approximately half the quantity of arsenic taken up by *C. vulgaris* was estimated to be adhered to the extraneous coat (10 wt %) of the cell. The remainder was bioaccumulated by the cell. Both adhered and accumulated arsenic concentrations increased with an increase in arsenic(V) concentration of the aqueous phase.

Arsenic(V) accumulation was affected by the growth phase: arsenic was most actively accumulated when the cell was exposed to arsenic during the early exponential phase and then accumulation decreased with an increase in culture time exposed to arsenic.

The alga grew well in the modified Detmer (MD) medium containing 1 mg As(III) dm^{-3} and the growth curve was approximated by a 'logistic equation'. Arsenic(III) was accumulated up to the second day of the culture time and arsenic(III) accumulation decreased with an increase in the culture time after that.

Arsenic accumulation was also largely affected by various nutrients, especially by managanese, iron and phosphorus compounds. A modified MD medium with the three nutrients was proposed for the purpose of effective removal of arsenic from the aqueous phase.

Using radioactive arsenate ($Na_2H^{74}AsO_4$), the arsenic accumulated was found to be readily excreted under conditions which were unfavourable for the multiplication of *C. vulgaris*.

Keywords: Arsenic, excretion, accumulation, freshwater alga, *C. vulgaris*, culture conditions

INTRODUCTION

The following conclusions were drawn from our previous experimental results on the bioaccumulation of arsenic by the arsenic-tolerant freshwater alga *Chlorella vulgaris* Beijerinck var. *vulgaris*, which had been isolated from an arsenic-polluted environment[1–3]

(1) The growth of *C. vulgaris* increased with an increase in pentavalent inorganic arsenic(V) concentration in the medium up to 2000 mg dm^{-3}, but decreased in media containing trivalent arsenic(III) at concentrations higher than 10 mg dm^{-3}.
(2) The higher the arsenic(V) concentration of the culture medium, the higher was the arsenic accumulation by the cell.
(3) Heat-killed and ethanol-killed cells did not accumulate arsenic(V) *in vitro*.
(4) Living cells were hindered from arsenic(V) accumulation by dinitrophenol (respiration inhibitor), but not by sodium azide (NaN_3: photosynthesis inhibitor).[1,2]
(5) A model equation for the growth curve of *C. vulgaris*, arsenic accumulation and changes in arsenic concentrations both in the cell and medium during growth coincided with the experimental results.[3]
(6) The arsenic concentration in the cell reached a peak in the exponential growth phase and decreased with growth time after the exponential phase.[3] It was therefore supposed that the cells not only bioaccumulate arsenic but also excrete it.

These experimental results suggest that the uptake of arsenic by *C. vulgaris* is mediated by

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metabolic processes and the arsenic resistance is dependent on the ability for detoxification and excretion of arsenic taken up by the cell. Furthermore, the growth of the cell seems to be stimulated by arsenic.

Uptake, detoxification and excretion of arsenic by freshwater algae were assumed to be mediated by algal enzymes. However, very few papers[5,6] have been published on the uptake and excretion behavior of arsenic by the algae. This report describes some experimental results on the factors affecting the uptake and excretion of arsenic by *C. vulgaris*.

EXPERIMENTAL

General procedure of algal culture

Chlorella vulgaris Beijerinck var. *vulgaris* was stock-cultured on an agar plate culture of modified Detmer medium (KNO_3 1.0 g, $CaCl_2$ 0.1 g, $MgSO_4.7H_2O$ 0.25 g, NaCl 0.1 g, K_2HPO_4 0.25 g, $FeSO_4.7H_2O$ 0.02 g, H_3BO_3 2.86 mg, $MnCl_2.4H_2O$ 1.81 mg, $ZnSO_4.7H_2O$ 0.22 mg, $CuSO_4.5H_2O$ 0.08 mg, Na_2MoO_4 0.021 mg, pure water 1 dm^3, pH 8; 'MD medium') containing 100 mg dm^{-3} of arsenic [as elemental arsenic, using the appropriate concentration for Na_2HAsO_4, abbreviated as arsenic(V)]. A colony of the algae was placed in liquid MD medium (arsenic-free) and the culture was kept at 25–30 °C under constant aeration (2 dm^3 min^{-1}) and illumination (4000 lux; 12 h day^{-1}) for the set period of days under germ-free conditions. The cells were then harvested by centrifugation (3000*g*, 10 min) and washed three times with distilled water. The wet cells were heated at 60 °C for 24 h and then at 105 °C for 2 h. The dried cells were analyzed for arsenic.

The optical density (at 640 nm) of the living cell suspension was found to be proportional to the cell concentration, so growth of the cell (g dry weight cell dm^{-3} medium) was obtained by determination of the optical density of the culture.

Determination of total arsenic

The dried-powdered cells (10–20 mg) were mixed with 50% magnesium nitrate solution (2 cm^3), and the mixture was dried and mineralized by heating at 550 °C for 6 h. The mineralized samples were dissolved with 10 mol dm^{-3} hydrochloric acid (10 cm^3), 40% potassium iodide solution (1 cm^3) was added, the solution was extracted twice with chloroform (5 cm^3) and the chloroform phase was then back-extracted with 0.025% magnesium nitrate solution (2 cm^3). Total arsenic was determined in the water phase by graphite furnace atomic absorption spectroscopy.

Excretion experiment using radioactive arsenic

Experiments of the excretion of arsenic were also performed by use of radioactive arsenic as $Na_2H^{74}AsO_4$. The radioactive arsenate solution (1 mCi, 0.25 mol dm^{-3}, 1 cm^3) was purchased from Amersham Japan Co. Ltd and used after appropriate dilution for excretion experiments.

Chlorella cells were inoculated in MD medium (200 cm^3) containing the radioactive arsenate and cultured under germ-free conditions by shaking (100 times per minute) for one week with illumination (4000 lux). The cells were collected by centrifugation and washed three times with pure water. Five arsenic-accumulated algal cell samples (70 mg each, on dry base) were put into five arsenic-free media (200 cm^3 each) and incubated under germ-free conditions by shaking for set times. The cell suspension which had been incubated for a set time was at once centrifuged, the radioactivity (cpm, 550–680 keV) of the supernatant (3 cm^3) was determined with a gamma counter (Packard Autogamma 500) and the arsenic excreted into the water phase was calculated from the result. For arsenic remaining in the cell, the whole-cell suspension was centrifuged, the wet cells were rinsed three times with pure water and resuspended in pure water (3 cm^3) and the cell suspension was analyzed for radioactivity.

RESULTS AND DISCUSSION

Adhesion of arsenic(V) on the extraneous coat of *C. vulgaris* cell

Living cells which had been pre-cultured in arsenic-free MD medium (500 cm^3) were inoculated in MD media containing 1, 10, 100 and 1000 mg As(V) dm^{-3}, and cultured for seven days under the conditions described above as general procedure.

One of the cell suspensions was centrifuged and a hard-packed cell mass was obtained. The hard-

Table 1 Arsenic taken up by *C. vulgaris* cultured for seven days in MD medium containing four different levels of arsenate

Arsenic(V) in medium (mg dm^{-3})	Cell[a] dry wt (g dm^{-3})	Arsenic					
		Total		Adhered		Accumulated	
		(μg)	(μg g^{-1})[b]	(μg)	(%)	(μg)	(%)
1	0.284	41.3	145[b]	9.3	23	32	77
10	0.362	135	373	47	35	88	65
100	0.214	810	3790	260	32	550	68
1000	0.222	2500	11 300	1300	52	1200	48

[a] Cell weight after washing with water.
[b] Total arsenic concentration (μg g^{-1}) adhered and accumulated by living cells.

packed cell mass was re-suspended in pure water (25 cm^3), the suspension was centrifuged, and the cells and supernatant were separated. The cells were washed with pure water nine times by repeating this procedure. The nine washings were analyzed for arsenic. No arsenic was detected in the washings after the fourth washing: therefore, the other cell samples were washed only three times with water.

The three washings for each cell sample were gathered and analyzed for arsenic. The cells washed with water were heated to dryness and analyzed for arsenic.

The experimental results are shown in Table 1.

About 10% of the cell weight was lost during the three washing stages. The washed-out arsenic was thought to have been adhered to the extraneous coat of the *Chlorella* cells. The retained arsenic after the washing should have been bioaccumulated by the cells. The former is defined here as adhered arsenic and the latter as accumulated arsenic.

From the experimental results shown in Table 1, it was found that both the quantities of arsenic adhered and accumulated increased with an increase in arsenic levels of the culture medium. The former is assumed to be adsorbed physicochemically to the extraneous coat of the cells and to be washed out with this extraneous coat. The latter should be biologically accumulated, probably by mediation of metabolic processes, because the accumulation occurred only in living cells.[1,2]

The data in Table 1 show that the quantity of arsenic adhered was smaller than or comparable with that accumulated. The relative quantity of adhered arsenic tended to increase with an increase in arsenic concentration of the medium. For the purpose of removal of arsenic by algae from aqueous phases, not only accumulation but also adhesion of arsenic is a significant factor.

Effect of growth phase on arsenic(V) accumulation

Four living *C. vulgaris* cell samples (6 mg each, on a dry weight basis) were inoculated into four arsenic-free MD media (1 dm^3 each) and cultured under the general culture conditions described above. The cells were exposed to arsenic(V) at a level of 10 mg dm^{-3} at times when the growth reached the lag phase and early, middle and late log phases, as shown in Fig. 1. All the cells were then continuously cultured until reaching their stationary phase, when they were harvested and analyzed for arsenic. The experimental data are shown in Table 2.

As shown in Table 2, the earlier time of exposure to arsenic in the growth phase of *C. vulgaris*, the higher was the arsenic accumulation. This finding is in harmony with previous experimental results that arsenic accumulation by *C. vulgaris* increased with an increase in culture time, reaching a peak at the exponential growth phase and then decreasing.[3]

Decrease in arsenic accumulation after the later exponential growth phase was considered to be caused by a decrease in the arsenic demand of the cells or by a decrease in the arsenic detoxification ability of the cells, or by an acceleration of the excretion of accumulated arsenic.

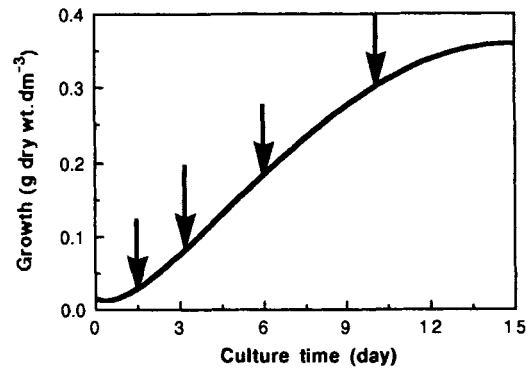

Figure 1 Effect of growth phase on arsenic accumulation by *C. vulgaris*. The cells were exposed to 10 mg As(V) dm^{-3} at four different growth phases (see text and Table 2) indicated by arrows, and cultured until they reached the stationary phase.

Table 2 Effect of growth phase on arsenic accumulation by *C. vulgaris*

Growth phase in which arsenic was added	Growth ($g\ dm^{-3}$)	Arsenic accumulation ($\mu g\ As\ g^{-1}$ dry wt)
Lag phase	0.02	354
Log phase		
Early	0.09	343
Middle	0.17	176
Late	0.31	48

Effect of arsenic state on the growth curve and arsenic(III) accumulation

As described before,[2] the growth of *C. vulgaris* increased with an increase in arsenic(V) levels in the medium up to 2000 mg dm^{-3}, but it decreased in media containing arsenic(III) at levels higher than 10 mg dm^{-3}. The cells were killed by cytolysis at arsenic(III) levels higher than 80 mg dm^{-3}, but the cells multiplied even at arsenic(V) levels of 10 000 mg dm^{-3}. These experimental results meant that arsenic(III) was found to be a hundred times more toxic to *C. vulgaris* than arsenic(V). There was a great difference in the effects of arsenic oxidation state on the growth. However, the growth curve of *C. vulgaris* in the presence of arsenic(III) and the pattern of arsenic accumulation had not been investigated, so the following experiment was carried out.

C. vulgaris (16 mg, on a dry weight basis) which had been pre-cultured in an arsenic-free MD medium was inoculated in an MD medium containing 1 mg As(III) dm^{-3} (as $NaAsO_2$) and cultured for two weeks under the standard conditions. The daily algal growth and arsenic accumulation at 2, 4, 8 and 14 days' culture were determined. The experimental results are shown in Fig. 2.

In Fig. 2, the solid circles indicate the algal growths observed and the solid curve is calculated by the following logistic equation:

$$y = \frac{CM}{1 + (M-1)\exp[-K(x-W)]} \qquad [1]$$

where y is algal growth (g dry wt dm^{-3} medium), x is culture time (days), C is initial algal concentration (16 mg dm^{-3}, on a dry weight basis), M is the multiplication factor of the cell (the cell ratio of final algal concentration to initial algal concentration) and K and W are the growth parameters. K and W were chosen so as to minimize the deviation between the observed data and the calculated curve from Eqn [1] above.

It was found that algal growth data observed in the presence of arsenic(III) were approximated well by the logistic curve in the same manner as the growth of *C. vulgaris*[3] and *Phormidium* sp.[4] with arsenic(V). Arsenic(III) accumulation was highest on the second day of culture and then decreased. As described in the previous paper,[3] arsenic(V) accumulation increased with an increase in the culture time and reached a peak at the late exponential growth phase and then decreased. The peak of arsenic(III) accumulation was found to be observed earlier than that of arsenic(V) accumulation. This difference in the growth phase of maximum arsenic accumulation is probably caused by the difference in the toxicity of the arsenicals.

Arsenic(III) accumulation and algal growth at 2, 4, 8 and 14 days' culture were 79, 22, 13 and 7 $\mu g\ As\ g^{-1}$ dry weight, and 31, 100, 253 and 456 mg dry weight dm^{-3} medium, respectively. The ratios of the algal growths at 4, 8 and 14 days' culture to the growth on the second day are approximately 3, 8 and 15. If no arsenic(III) accumulation occurred after the second day of culture, the average arsenic concentration in the algal cells should decrease in inverse proportion to the ratios of the growth. If this assumption is correct, the arsenic(III) accumulation at 4, 8 and 14 days' culture should decrease to 1/3, 1/8 and 1/15 times that of the second days' (79 $\mu g\ As\ g^{-1}$ dry weight), corresponding to 26, 10 and

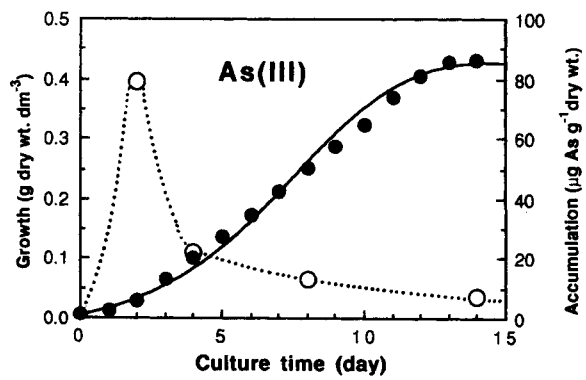

Figure 2 The growth curve (●) of *C. vulgaris* and arsenic accumulation (○) in the MD medium containing 1 mg As(III) dm^{-3}. The solid growth curve was calculated from a 'logistic equation', Eqn [1]. Initial algal concentration, C, was 16 mg cells dm^{-3} on a dry weight basis and multiplication, M, was 27. The parameters K and W were 0.46 and 0, respectively.

5 μg As g^{-1}, respectively. These arsenic(III) accumulation levels calculated by the assumption are approximated by the experimental results. This means that the decrease of arsenic(III) accumulation from the second day of culture time was probably caused by a maintenance of the arsenic(III) accumulation but dilution on increasing the algal growth after the second day of culture time.

Effect of concentration of three inorganic nutrients (phosphorus, managanese and iron) on arsenic accumulation

Preliminary experiments using radioactive arsenic compounds ($Na_2H^{74}AsO_4$) revealed that concentrations of phosphorus, manganese and iron nutrients had an effect on arsenic accumulation greater than those of the other MD nutrients (nitrogen, boron, copper and zinc) tested. On the basis of the preliminary experimental results, the following experiment was performed to determine the detailed effect of these three nutrients on arsenic(V) accumulation.

C. vulgaris cells (38 mg dry weight) which had been pre-cultured in an arsenic-free MD medium were inoculated in modified MD medium (as described below; 250 cm^3; 152 mg dry wt cell dm^{-3}) and cultured for seven days with illumination (24 h day^{-1}). Six different modified MD media commonly containing both 10 mg As(V) dm^{-3} and 0.1 mol dm^{-3} Bicine (pH buffer reagent) were prepared for the nutrient to be tested, in which the nutrient concentration to be tested in the MD medium was varied to 0, 0.1, 0.5, 1, 10 and 100 times the normal concentration in MD medium.

The growth of *C. vulgaris* and arsenic accumulation were determined after seven days' culture. The experimental results are shown in Figs 3–5.

Figure 3(a) shows that growth of *C. vulgaris* tended to increase with an increase in manganese concentration, but decreased remarkably at concentrations higher than 5 mg dm^{-3}, and that arsenic accumulation increased with an increase in manganese concentration up to 50 mg dm^{-3}. For the purpose of removal of arsenic from an aqueous phase, the higher the levels of both arsenic accumulation and algal growth, the better is the efficiency of arsenic removal by use of the alga. From this viewpoint, the manganese concentration in the medium leading to the largest

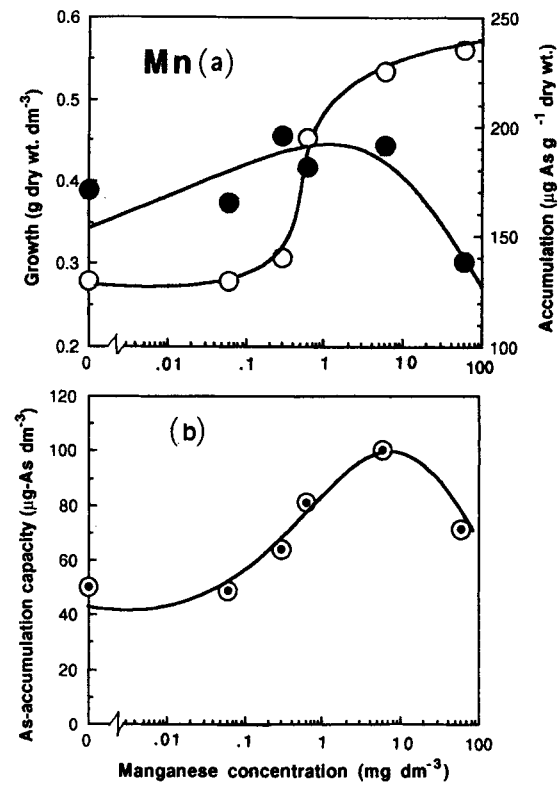

Figure 3 Effect of manganese concentration in MD medium containing 10 mg As(V) dm^{-3} (a) on the growth (●) and arsenic accumulation (○) and (b) on the arsenic-accumulation capacity (product of growth multiplied by arsenic accumulation) of *C. vulgaris*.

product of arsenic accumulation (μg As g^{-1} dry wt cell) multiplied by algal growth (g dry wt cell dm^{-3} medium) would be recommended, the product being defined here as an arsenic-accumulation capacity (μg As dm^{-3} medium) of *C. vulgaris*. Figure 3(b) shows the arsenic-accumulation capacity versus manganese concentration in the MD medium. From Fig. 3(b), 5 mg Mn dm^{-3} MD medium was recommended for the purpose of removal of arsenic by *C. vulgaris* from a water phase which is ten times higher than the normal manganese concentration in the MD medium (0.5 mg Mn dm^{-3}).

Figure 4 shows a summary of the experimental results on the effects of iron concentration on growth, arsenic accumulation and arsenic-accumulation capacity. Growth of *C. vulgaris* decreased steadily with an increased in iron concentration, and arsenic accumulation increased with an increase in iron concentration up to 40 mg dm^{-3}. The arsenic-accumulation capacity

[Fig. 4(b)] was the highest at an iron concentration of 40 mg dm^{-3}. From the same viewpoint as above, 40 mg Fe dm^{-3} medium was recommended for arsenic removal, which is ten times the normal iron concentration in MD medium (4 mg dm^{-3}).

Figure 5 shows that growth of *C. vulgaris* increased but that arsenic accumulation decreased with an increase in phosphorus concentration in the MD medium, and that the arsenic-accumulation capacity decreased with an increase in phosphorus concentration in the medium. Figure 5(b) shows that the lower the phosphorus concentration in the medium, the greater is the arsenic-accumulation capacity. For the purpose for arsenic removal, this experimental result seems to recommend a phosphorus-free medium. However, it was found that the pH of normal MD medium without a pH buffer (Bicine) was not maintained at neutrality at phosphorus concentrations less than 22.5 mg dm^{-3}, because the phosphorus nutrient (K_2HPO_4) plays an important role as a buffering agent in MD nutrient media. Also, the algal growth was found to decrease at phosphorus concentrations less than 22.5 mg P dm^{-3} when the initial cell concentration was of the

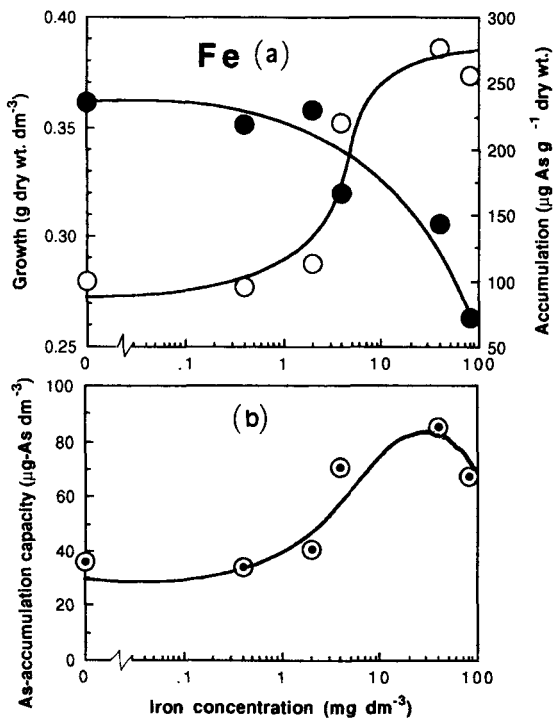

Figure 4 Effect of iron concentration in MD medium containing 10 mg As(V) dm^{-3} (a) on the growth (●) and arsenic accumulation (○) and (b) on the arsenic-accumulation capacity of *C. vulgaris*.

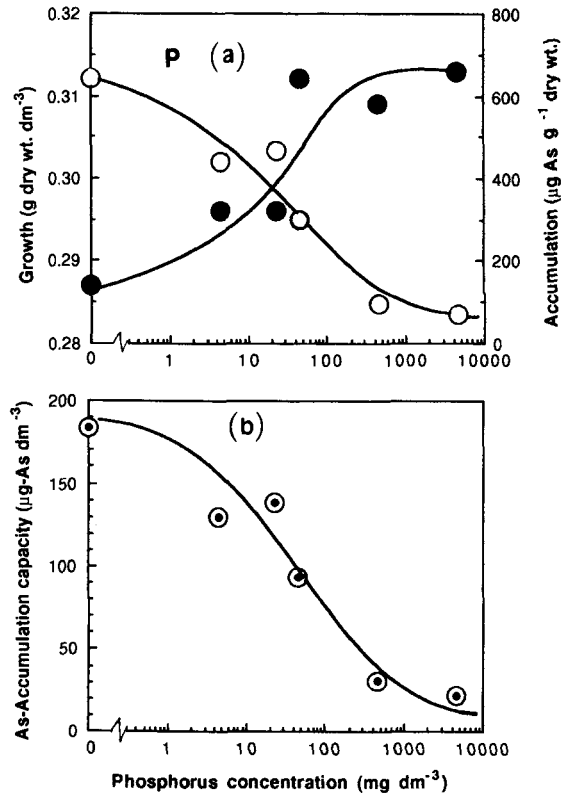

Figure 5 Effect of phosphorus concentration in MD medium containing 10 mg As(V) dm^{-3} (a) on the growth (●) and arsenic accumulation (○) and (b) on the arsenic-accumulation capacity of *C. vulgaris*.

order of a ten milligrams dry mass cell per dm^3 medium (ordinary cell concentration for inoculation). For these two reasons, the phosphorus concentration was required to be 22.5 mg dm^{-3}.

From the above experimental results, the following modified MD medium was recommended for arsenic removal from aqueous phase by the freshwater alga *C. vulgaris*: Mn, 0.6, Fe 40, and P 22.5 mg dm^{-3}; the other nutrient concentrations were the same as those in the MD medium without Bicine.

In order to examine the effect of modification of the MD medium, *C. vulgaris* was cultured for 10 days under the general conditions in both the modified MD and normal MD media containing 10 mg As(V) dm^{-3} and both arsenic accumulations were compared. The arsenic accumulations were 2130 and 1390 µg As g^{-1} dry wt by the algae cultured in the modified MD and normal MD media, respectively, i.e. the former was about two-fold larger than the latter. It was found that the modification of the MD medium

with respect to manganese, iron and phosphorus nutrients was quite effective for arsenic accumulation by *C. vulgaris*.

Excretion behavior of radioactive arsenic ($Na_2H^{74}AsO_4$)

As described in the previous paper,[3] arsenic accumulation by *C. vulgaris* varied with the growth phase, reaching a peak at the exponential phase and then decreasing after the late exponential phase. A few experiments on the uptake of arsenic have been conducted, but few data on factors concerning the excretion of arsenic by algae have been reported.[6] The authors investigated the excretion behavior of arsenic by *C. vulgaris* by using radioactive arsenate ($Na_2H^{74}AsO_4$).

C. vulgaris was inoculated and cultured for six days in an MD medium containing the radioactive arsenate, the algal cells accumulating the radioactive arsenic were separated and washed three times with pure water by using a centrifuge. A portion of the cells (70 mg, on a dry weight basis) was put into arsenic-free MD medium or pure water (200 cm^3 each) and incubated for two days under illumination or in the dark. A small portion of the cell suspension was drawn at set intervals and the radioactivities of the cells and aqueous phase were determined.

Changes in the radioactivities of the cells and water phase during the initial 6 h incubation are plotted in Fig. 6. Little change in the radioactivity was observed during further incubation.

The experimental results shown in Fig. 6 lead to the following conclusions:

(1) *C. vulgaris* excreted 24–41% radioactive accumulated arsenic into the aqueous phase in 6 h.
(2) The largest part of the arsenic excretion was carried out in the initial 15–45 min.
(3) *C. vulgaris* excreted arsenic preferably into pure water rather than into the MD medium, and preferably in the dark rather than under illumination.

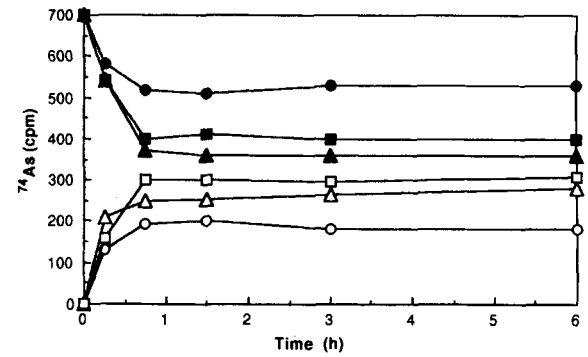

Figure 6 Effect of culture conditions on excretion of arsenic (^{74}As) by *C. vulgaris*. The alga, cultured for seven days in MD medium containing $Na_2H^{74}AsO_4$, was incubated in pure water in the dark (△, ▲), or in arsenic-free MD medium with illumination (○, ●) or in the dark (□, ■). Open and solid symbols refer to radioactivities of the water phase and cells, respectively.

These experimental results suggested that arsenic was readily excreted under conditions which were unfavourable for multiplication of *C. vulgaris*.

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