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Bioaccumulation of Arsenic by Freshwater Algae and the Application to the Removal of Inorganic Arsenic from an Aqueous Phase. Part I. Screening of Freshwater Algae Having High Resistance to Inorganic Arsenic

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Abstract

Several freshwater alga having resistance to arsenic were screened from microorganisms which had been sampled at sites polluted with arsenic from a geothermal electric power plant and old mines and smelters of arsenic ores. The alga thus screened could grow in the liquid medium (Modified-Detmer culture medium) containing sodium arsenate at levels up to 2000 ppm as elemental arsenic concentration. Some mixed systems of alga grew rapidly in the media at the higher levels of arsenic ranging from 50 to 2000 ppm. The mixed systems of alga screened included predominantly blue-green algae, green algae, and diatom, and also included protozoa, rotifera, and bacteria as minor components. One pure algal culture was

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obtained by means of an agar plate culture, and the algae isolated was identified as *Chlorella vulgaris* Beijerinck var. *vulgaris*. The growth of *C. vulgaris* in a pure culture was unaffected by 100 ppm of arsenic.

INTRODUCTION

Since Chapman (*1*) first revealed the marine organisms such as shrimp contain arsenic at high levels, the arsenic content of many marine and terrestrial organisms have been recently determined by many investigators. The data obtained until 1977 were summarized in a paper (*2*) and a book (*3*). The most pronounced characteristics of these data are the great difference in the level of arsenic in marine and in terrestrial organisms. The level of arsenic in terrestrial organisms is seldom 1 ppm, whereas the corresponding values for marine organisms vary from several ppm to more than 100 ppm. It was also found that the lower members of the trophic level of marine ecosystems, such as algae, accumulate arsenic and alkylate the arsenic more efficiently than the higher members (*4, 5*). The highly alkylated arsenic compounds in the marine organisms, such as arsenobetain, seem to be nontoxic. Several years ago the author investigated the bioaccumulation of arsenic by some marine alga and the chemical form of the arsenic accumulated, and found that the marine alga tested had a resistance to 100 ppm of inorganic arsenic and had a great ability to accumulate the arsenic. The marine alga were also found to be able to biosynthesize organoarsenic compounds similar to arsenocholine (*6, 7*).

On the other hand, there were few papers on the bioaccumulation of arsenic by terrestrial organisms, but the experiments of Giddings (*8*) are very interesting. He investigated the fate of inorganic arsenic in 12 different freshwater microcosms and found that the organisms which could accumulate arsenic most effectively among the organisms living there were algae. This result suggests that the lower members of the trophic level may accumulate arsenic more efficiently than the higher members of the trophic level in a lake—as well as in a sea—ecosystem. If these organisms take up inorganic arsenic compounds efficiently in the aqueous phase and convert the arsenic into nontoxic organoarsenic compounds, these organisms might contribute to the reduction of terrestrial pollution by arsenic.

The above concept led the authors to investigate the possibility of removing inorganic arsenic compounds from freshwater by the aid of bioaccumulation. Among many terrestrial organisms, freshwater algae was estimated by the authors to have the highest resistance to arsenic impact and to accumulate arsenic most efficiently. Microorganism samples were collected from sites which had been polluted by arsenic for a long time.

This present paper reports the results of the screening of freshwater algae having a high resistance to arsenic impact.

EXPERIMENTAL

Sampling of Microorganisms

The sites shown in Fig. 1 and Table 1 were selected as sampling locations by reason of having been polluted with arsenic for a long time. The backgrounds of the sampling sites are also shown in Table 1. Toroku and Matsuo are particularly well known owing to arsenic poisoning (*9, 10*).

Screening of Arsenic-Resistant Algae by Means of Liquid Culture Containing High Levels of Arsenic

Sixty-eight tall beakers (500 mL) equipped with air stones and watch dishes were each filled with 300 mL of Modified-Detmer culture medium (Table 2) and placed by a window with a southern or eastern exposure to

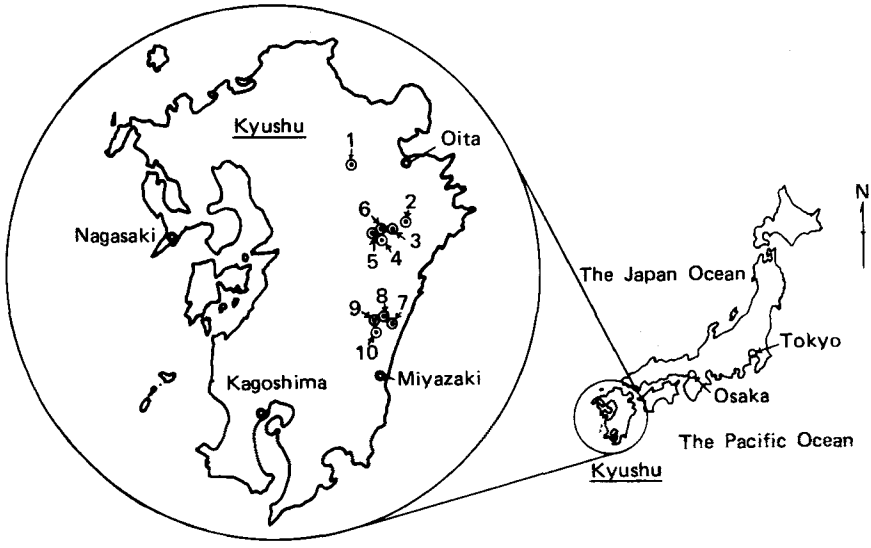

FIG. 1. Sampling sites. (Numbers refer the sample numbers shown in Table 1.)

TABLE 1
Microorganism Samples

Sample no.	Number of Samples	Sampling sites (abbreviation)	Location	Background (sampling point)
1	12	Kyushu Electric Power, Otake geothermal Power Plant (Otake)	Kokonoe, Kusu, Oita	Power capacity, 12500 kW; hot wastewater containing 3 ~ 5 ppm As is all reduced underground now (by As-removing test plant)
2	8	Kiura ex-mine (Kiura)	Ume, Minami-kaibe, Oita	Cassiterite and arsenopyrite were produced during 1955–1960 (by old dressing plant)
3	2	Mitate ex-mine and ex-smelter (Mitate)	Hinokage, Nishi-usuki, Miyazaki	Arsenopyrite and arsenic trioxide were produced during 1951–1970 (by old residence)
4	8	Tsuzura ex-mine (Tsuzura)	Takachiho, Nishi-usuki, Miyazaki	Copper pyrite and arsenopyrite were produced until 1950 (by old tunnel entrance)
5	2	Nakanouchi ex-mine (Nakanouchi)	Same as above	Tin stone was produced until World War II (by old tunnel entrance)
6	11	Toroku ex-mine and ex-smelter (Toroku)	Same as above	Arsenic trioxide was produced until 1973 (by smelter)
7	4	Ouchi ex-mine (Ouchi)	Togo, Higashi-usuki, Miyazaki	Copper pyrite was produced until 1971 (river sediments)
8	3	Taguchibaru ex-mine (Taguchibaru)	Same as above	Gold ore was produced until 1971 (by old tunnel entrance)
9	8	Osuzu ex-mine (Osuzu)	Same as above	Tin and gold ores were produced until several years ago (by settling basin)
10	9	Matsuo ex-mine and ex-smelter (Matsuo)	Kijo, Koyu, Miyazaki	Arsenic trioxide was produced until 1971 (by smelter)
Total	68	samples		

TABLE 2
Composition of Culture Medium (Modified Detmer)

Main element			Trace element (A_5)		
KNO_3	1.0	g	H_3BO_3	2.86	g
$CaCl_2$	0.1	g	$MnCl_2 \cdot 4H_2O$	1.81	g
$MgSO_4 \cdot 7H_2O$	0.25	g	$ZnSO_4 \cdot 7H_2O$	0.22	g
NaCl	0.1	g	$CuSO_4 \cdot 5H_2O$	0.08	g
$K_2HPO_4 \cdot 7H_2O$	0.25	g	Na_2MoO_4	0.021	g
$FeSO_4 \cdot 7H_2O$	0.02	g	Pure water	1000	mL
Trace element (A_5)	1.0	mL	Conc H_2SO_4	1 drop	
Pure water	1000	mL			
pH	8.0				

indirect sunlight. Sodium arsenate (Na_2HAsO_4) aqueous solution was added and adjusted to 1 ppm of elemental arsenic concentration in each culture medium. About 1 g of each of the microorganisms sampled (soils or water) was added to these culture media. The sixty-eight cultures were then started simultaneously with air-bubbling.

The optical density of the culture solution was measured at 640 nm as a measure of the growth of planktonic alga at constant intervals, and microscopic observation was also made at the stationary growth phase. The water level of the culture solution was maintained constant by the addition of pure water during the entire culture period.

Thirty-four microorganism samples were screened out after 2 weeks because of poor growth. Thirty-four inocula (3 mL each) were taken from the remaining thirty-four cultures after the culture had been homogenously suspended. They were inoculated into thirty-four new Modified-Detmer media containing 10 ppm of arsenic.

The cultures of microorganisms with good growth were screened at the stationary growth phase, and the suspended culture solutions (3 mL each) were inoculated successively into new media containing higher levels of arsenic.

The process of screening arsenic-resistant algae is summarized in Table 3. As can be seen, four samples (Otake, Tsuzura, Toroku, and Matsuo) were screened at the fifth step. In these four screened culture solutions, planktonic unicellular alga (blue-green algae, green algae, and diatoms) and attached alga (blue-green algae and green algae) were the predominant organisms, and protozoa, rotifera, and bacteria were minor organisms. Each culture solution screened included several species of alga. The whole sum of algal species observed in these four samples was about 10 species. These four culture solutions were again inoculated into media containing 1000 ppm of arsenic

TABLE 3
Screening of Arsenic-Resistant Alga (Modified-Detmer culture medium)

Culture	Step	As(V) added (mg/L)	Cultivating period	Samples screened
Liquid culture	I	1	2 weeks	68 samples
	II	10	2 weeks	34 samples
	III	100	3 weeks	10 samples
	IV	100	1 week, 6 times	10 samples
	V	500	2 weeks	4 samples:
	VI	1000	10 days	Otake
				Tsuzura
				Toroku
				Matsuo
Agar plate culture	VII	500 (UV irradiated)	2 weeks, 2 times	2 samples: Otake, Matsuo
	VIII	500	3 weeks	1 sample: Matsuo

for the purpose of further screening, but no variation in the number of algal species was observed. It was thought that further screening of arsenic-resistant alga by means of the liquid culture would yield no new results. Therefore, screening by means of agar culture was tried for the purpose of isolating planktonic green algae which was commonly present in all four culture solutions.

Isolation of a Planktonic Green Algae by Means of Agar Culture

Agar powder (2% w/v) was dissolved in a Modified-Detmer culture medium containing 500 ppm of arsenic, and the solution was poured into Petri dishes, sterilized in an autoclave, and allowed to stand at room temperature to be used as the plate culture of solid agar. Four inocula from the four microorganism samples screened were sterilized by UV radiation (15 W UV-1amp at 50 cm distance for 3 min) and inoculated separately on the agar culture by use of a platinum loop or a bent glass rod. The Petri dishes were sealed with tape, placed in the same place as mentioned before, and allowed to stand for 2 weeks at room temperature.

Colonies were found on the agar cultures of Otake and Matsuo which had been inoculated by use of a platinum loop. One green algae was isolated from both samples after repeated inoculation on the agar culture medium containing 500 ppm of arsenic.

This green algae was identified as *Chlorella vulgaris* Beijerinck var. *vulgaris* (*11*) by Shin Watanabe and Isamu Umezaki.

A stock culture of the isolated algae was preserved on a slant agar medium in a refrigerator. *Chlorella vulgaris* has a globular form about 3 μm in a diameter as shown in Fig. 2.

Isolation of the other alga present in the four microorganism samples screened is in progress.

RESULTS AND DISCUSSION

Effects of Arsenic Impact on the Growth of Mixed Systems of Alga

Four mixed microorganisms which were survivors at the fifth step of screening were inoculated into the liquid media with five different levels of

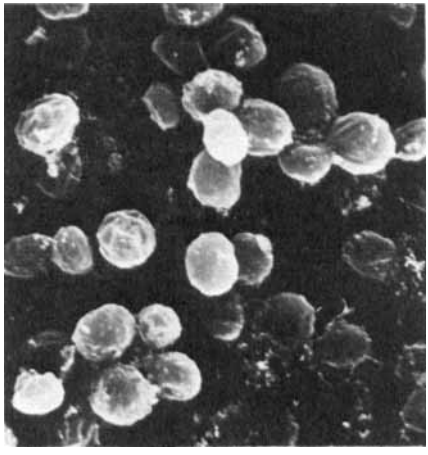

FIG. 2. Scanning electron micrograph of isolated *Chlorella vulgaris*.

arsenic and cultured by the same method as mentioned above. The growth of the mixed alga at various levels of arsenic is plotted against time in Fig. 3. As can be seen, no lag phase was observed in the growth curves even at the highest level of arsenic (2000 ppm). The growth of alga in the mixed systems seemed to be unaffected by arsenic at levels ranging from 50 to 2000 ppm. On comparison of the algal growths at 50 and 2000 ppm, the planktonic alga of Tsuzura grew rapidly at 2000 ppm rather than at 50 ppm. Such planktonic alga might prefer a higher level of arsenic medium than one of lower level.

Since the growth of attached alga did not reflect the absorbance of culture solution in Fig. 3, total algal concentration was obtained by means of centrifuging the whole culture solution at the stationary growth phase. The data are shown in Table 4.

With cell concentration based on absorbance (Fig. 3), the maximum appears at 2000 ppm in three samples (Otake, Tsuzura, and Matsuo), but with cell concentration based on the total algal concentration, the maximum appears at 1000 ppm in three samples (Otake, Toroku, and Matsuo). The differences were attributable to the coexistence of attached alga in the culture solutions. From a comparison of the results of Fig. 3 with those of Table 4, and from macroscopic observatioan of the culture solutions, the planktonic

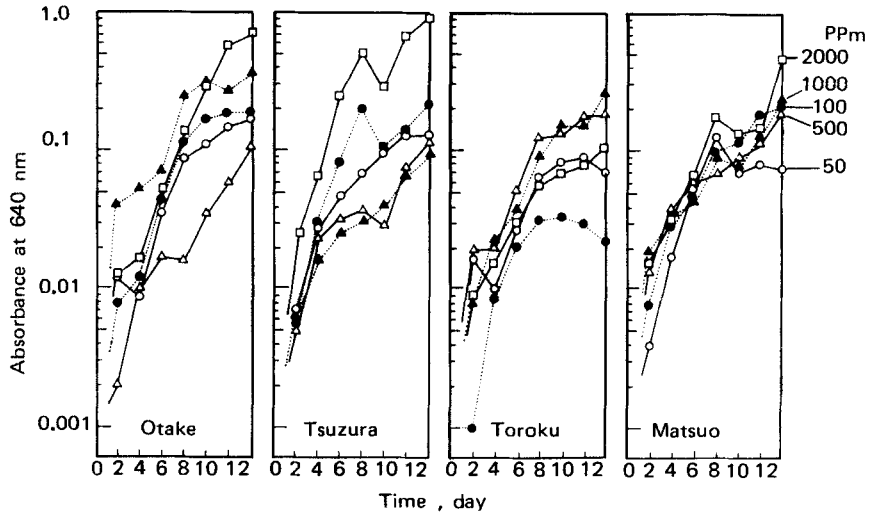

FIG. 3. Arsenic impact on the growth curve of planktonic alga in the four samples screened from sixty-eight microorganism samples. Culture medium: Modified-Detmer culture medium containing 50 (○), 100 (●), 500 (△), 1000 (▲), and 2000 (□) ppm of arsenic.

TABLE 4
Arsenic Impact on the Total Growth of Alga Including both Planktonic and Attached Alga in Four Microorganism Samples Screened (Modified-Detmer liquid culture)

Arsenic level (ppm)	Cell concentration (mg dry cell/mL culture solution)			
	Otake	Tsuzura	Toroku	Matsuo
50	0.26	0.40	0.19	0.44
100	0.37	0.53	0.20	0.40
500	0.33	0.32	0.36	0.39
1000	0.72	0.33	0.38	0.46
2000	0.65	0.66	0.22	0.29

alga were found to be more resistant to arsenic impact than the attached alga.

Effects of Arsenic Impact on the Growth of *C. vulgaris*

The inoculum (30 mL) was taken from the stock culture in its second day of logarithmic growth and inoculated into a Modified-Detmer culture medium (2000 mL) containing 100 ppm of arsenic. During the entire period of the culture, germ-free air was bubbled and all operations were done under germ-free conditions. Light radiation (12 h/d) at about 6000 lux was obtained from 2 banks of 20 W cool-white and 2 banks of 20 W fish-lux (wavelength, 650 nm) fluorescent lights. Culture solution (100 mL) was drawn at regular intervals, and the absorbance at 640 nm, cell number, and dry cell weight in the culture solution drawn were determined. The results are shown in Fig. 4.

There is good correlation of the absorbance at 640 nm with the cell number or the dry well weight of the culture solution in the case of the pure culture of planktonic unicellular algae: *C. vulgaris*.

Figure 4 shows that the growth of *C. vulgaris* seemed not to be prevented by 100 ppm of arsenic because of the absence of a lag phase in the growth curve. The growth reached stationary phase on the seventh day when the cell concentration was 0.3 mg dry cell/mL-culture solution.

The effects of arsenic levels, temperature, light intensity, and components of culture medium on the growth of *C. vulgaris*, and the relationship between the growth and the bioaccumulation of arsenic should be considered; these are now under investigation and will be reported soon.

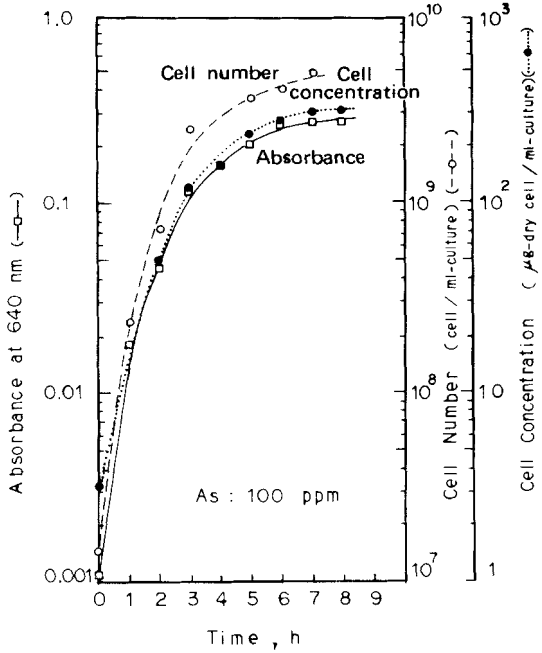

FIG. 4. Arsenic impact on growth curve of *C. vulgaris*. Culture medium: Modified-Detmer culture medium. Light: 6000 lux UV-lump, 12 h/d at room temperature.

Acknowledgments

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