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Tolerance to mercury chloride in *Scenedesmus* strains

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Mercury chloride toxicity was investigated in two strains of *Chlorella* and in a strain of *Scenedesmus* isolated from polluted areas in Tuscany (Italy). No Hg resistance was found in the autotrophic microorganisms isolated, but *Scenedesmus* sp. strain AR-2489, isolated from the Arno river, was able to grow at concentrations of up to 5 μg ml^{-1} of Hg. This concentration was twice that which inhibited growth of the two *Chlorella* strains and *Scenedesmus acutus* 8M, the reference strain from a culture collection. Photosynthesizing cells of *Scenedesmus* sp. AR-2489 showed reduced Hg uptake, with the highest percentage of Hg removal from the medium. Loss of Hg was not due to Hg(0) volatilization, as shown by a comparison test with the broad-spectrum Hg-resistant *Pseudomonas putida* FB1. The metabolic differences between *Scenedesmus* sp. strain AR-2489 and *Siacatus* strain 8M were: (1) higher growth rate (doubling time of 6.0 h versus 10.6 h); (2) higher O_2 production rate (maximum 2 μmol h^{-1} mg^{-1} dry weight); and (3) higher intracellular pH during growth. The latter was imaged with a green fluorescence molecular probe (BCEFC–AM) and observed by scanning confocal laser microscopy (SCLM). The distribution of red-autofluorescence chlorophyll-a showed that strain AR-2489 had a rougher and hence more extended specific chloroplast surface than strain 8M. Hg tolerance in strain AR-2489 was related to the rapid increase in dissolved O_2 in the medium and in intracellular pH; this caused a loss of soluble mercury transformed to insoluble mercury hydroxide, which is thermodynamically more stable at alkaline pH in highly oxygenated systems.

Keywords: inorganic mercury, microalgae, pH molecular probe, photosynthesis rates, scanning confocal laser microscopy

Introduction

The mechanism of Hg resistance in bacteria is based on the reduction of ionic Hg to volatile elemental Hg(0), catalysed by an inducible enzyme, mercuric reductase. This enzyme has been cloned and sequenced (Robinson & Tuovinen 1984, Summer 1986, Foster 1987, Silver & Walderhaug 1992) and is responsible for environmental Hg detoxification. Hg volatilization has also been observed in the yeasts *Cryptococcus* spp., *Candida albicans* and *Saccharomyces cerevisiae* (Brunker & Bott 1974, Yannai *et al*. 1991) and in the algae *Chlamydomonas* sp. and *Chlorella pyrenoidosa* (Ben-Bassat & Mayer 1975, 1977). Ben-Bassat & Mayer (1977) observed Hg volatilization in *Chlorella pyrenoidosa*. Cultures of the microalga showed a lag phase of four days when spiked with 1.25 μg ml^{-1} $HgCl_2$ and volatilized Hg^{2+} to Hg(0) from the medium by means of an intracellular reducing factor of low molecular weight (less than 1200 Da) in an eight-day experiment. Complete Hg volatilization, which usually takes only a few minutes in bacteria, required some days in *C. pyrenoidosa*. Marine eukaryotic phytoplankton also reduce inorganic mercury to Hg(0) (Mason *et al*. 1995a). In *Thalassiosira weissflogii*, *Dunaliella tertiolecta* and *Pavlova lutheri* the mechanism of Hg volatilization is still unknown and the volatilization rate is lower than in marine cyanobacteria. In

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Synechococcus bacillaris the Hg volatilization rate has been estimated at 6.6 pmol μg^{-1} (chlorophyll-a) day^{-1}, which is about 30 times higher than in marine microalgae (Mason *et al.* 1995a). However, in eukaryotic microorganisms and cyanobacteria Hg volatilization does not seem to be based on the enzymatic reduction of Hg^{2+}. The aim of the present study was to isolate, from Hg-polluted soil and freshwater in Tuscany (Italy), photosynthetic microorganisms that volatilize Hg^{2+} to Hg(0), as reported for aerobic heterotrophic bacteria (Baldi *et al*. 1985, 1989), or that are endowed with other mechanisms of Hg resistance.

Materials and methods

Isolation of photosynthetic microorganisms

Water samples were taken axenically from Hg-polluted geothermal areas in Tuscany (Bagni San Filippo, Monte Amiata and Bagno Vignoni) and from polluted parts of the Arno and the Greve rivers. Subsamples were enriched with 1.5 g l^{-1} $NaNO_3$ and 0.04 g l^{-1} K_2HPO_4 and spiked with Hg^{2+} ($HgCl_2$) in order to reach Hg concentrations ranging from 0.1 $\mu g\ ml^{-1}$ to 10 mg ml^{-1}. The samples were incubated under dim light (30–40 $\mu mol\ photon\ m^{-2}\ s^{-1}$) at 30ºC. The phototrophic microorganisms which grew in concentrations up to 1 $\mu g\ ml^{-1}$ of Hg^{2+} as $HgCl_2$ were isolated by plating on BG-11 agar medium containing per litre: 1.5 g $NaNO_3$, 0.04 g $K_2HPO_4.3H_2O$, 0.075 g $MgSO_4.7H_2O$, 0.036 g $CaCl_2.2H_2O$, 0.02 g Na_2CO_3, 0.006 g $Fe^{3+}NH_4^+$citrate, 0.006 g citric acid, 0.001 g EDTA and 1 ml of trace element stock solution (0.0029 g H_3BO_3, 0.0018 g $MnCl_2$, 0.0004 g Na_2MoO_4, 0.0001 g $CuSO_4.5H_2O$ and 0.0001 g $CoCl_2.6H_2O$ per litre).

The isolated strains were rendered axenic by treatment with the antibiotics penicillin (1 g l^{-1}) and streptomycin (0.2 g l^{-1}), after which they were plated on solid medium and routinely cultured in liquid medium BG-11 at 30ºC and under dim light. The three isolates used in this study were identified as follows: *Chlorella* sp. strain AMI-5 (isolated from Monte Amiata), *Chlorella* sp. strain GR-1489 (isolated from the Greve River) and *Scenedesmus* sp. strain AR-2489 (isolated from the Arno River). *Scenedesmus acutus* strain 8M, from the Culture Collection of the Centro di Studio dei Microrganismi Autotrofi, Piazzale delle Cascine 27, I-50144 Florence, Italy, was used as reference strain.

Minimum inhibitory concentration (MIC) tests

The axenic microalgal cultures were diluted to a final cell concentration of 153 ± 2.1 mg l^{-1} (dry weight) for the two *Chlorella* strains and 56 ± 3.2 mg 1^{-1} (dry weight) for the two *Scenedesmus* strains. Aliquots of 5 ml were distributed in 10 ml test tubes and Hg (as $HgCl_2$) was added at different concentrations: 0.0, 0.5, 1.0, 2.5 and 5.0 $\mu g\ ml^{-1}$. The cultures were incubated at 30ºC and illuminated with 15 $\mu mol\ photon\ m^{-2}\ s^{-1}$ in a l6–8 h light–dark cycle. Growth was monitored every two days by measuring the optical density of the cultures at 640–700 nm with a Klett–Summersom photoelectric colorimeter. The MIC was defined as the minimum Hg concentration that inhibited cell growth. All experiments were performed in duplicate.

Accumulation of mercury by photosynthesizing, respiring and dead algal cells

Aliquots of 40 ml of *S. acutus* 8M and *Scenedesmus* sp. AR-2489 cultures were transferred to 250 ml Erlenmeyer flasks at an initial concentration of 265 ± 5.0 mg l^{-1} (dry weight). Some of the flasks were subjected to thermal treatment at 90ºC for 30 min in order to kill the cells; half of the remaining flasks (living cultures) were completely wrapped in aluminium foil in order to darken the suspension (respiring cultures), and the other half were exposed to light (photosynthesizing cultures). Additions of $HgCl_2$ were made in order to reach Hg concentrations of: 0.0, 0.5, 1.0, 2.5 and 5.0 $\mu g\ ml^{-1}$.

All flasks were incubated at 30ºC in a shaking chamber (100 rpm), in a CO_2 (3%) enriched atmosphere at a light intensity of 75 $\mu mol\ photon\ m^{-2}\ s^{-1}$. After 3 h of incubation the whole cell suspension of each flask was centrifuged and washed three times. The total mercury cell content of the pellets was determined by digestion with 3 ml concentrated HNO_3 in sealed vials at 60ºC for 3 h. The digested samples were made up to 10 ml with distilled water. Ionic Hg was atomized with 1 ml of a 10% $SnCl_2$ solution and total Hg determined by flameless atomic absorption spectrophotometer (Perkin Elmer model 300S). Standard additions of Hg as $HgCl_2$ were: 0.05, 0.1, 0.25, 0.5, 0.75, 1.0 and 2.5 $\mu g\ ml^{-1}$. All analyses were performed by additions of these Hg concentrations both on algal materials and in standard aqueous solutions to test likely Hg losses during digestion of samples. Five replicate analyses of every sample gave a coefficient of variation (CV) of 5.4%.

An aliquot of 3 ml supernatant from the first centrifugation was filtered through a nitrate cellulose filter with 0.2 μm pores (Sartorius, Göttingen, Germany) and treated as above for total Hg determination in the cell pellets. The Hg concentrations in algal cells ($\mu g\ mg^{-1}$ dry weight) and supernatants ($\mu g\ ml^{-1}$) were plotted versus the Hg^{2+} concentration ($\mu g\ ml^{-1}$) added to the cultures. The angular coefficients (b) and the relative correlation factors (R^2) for $n = 7$ data points from each linear regression were calculated by minimum squares. The angular coefficients obtained from plots of the two *Scenedesmus* cultures were used to calculate the relative accumulation factors (AF) from the rate b_1/b_2 of strain AR-2489 (b_1) with respect to the reference strain 8M (b_2)

Mercury volatilization test

Scenedesmus sp. AR-2489 and *S. acutus* 8M were compared with the broad-spectrum Hg-resistant *Pseudomonas*

putida strain FB1 for their capacity to volatilize ionic Hg to elemental Hg(0), as previously reported (Baldi *et al.* 1988). A 10 ml aliquot of each culture was incubated and then washed and resuspended in 1 ml of fresh medium BG-11.

P. putida FB1 was grown on Nelson medium (Nelson *et al.* 1973, Baldi *et al.* 1988). The three dense cultures (1 ml) were spiked with 1 μg of Hg as $HgCl_2$. At constant intervals, 1 ml of head space was sampled from tubes (18 ml) sealed with mininert vial cap (Supelco, Bellefonte, PA, USA) by means of a gas sampling syringe. The gas was injected into the atomizing cell of a flameless atomic absorption spectrophotometer. The calibration and the determination of gaseous elemental mercury were performed by injecting different head space volumes from a sealed vial containing 5.0 g elemental Hg. The Hg concentration in the head space was calculated from the partial pressure of Hg at temperatures measured during the analyses.

Measure of photosynthetic oxygen evolution, pH and biomass concentrations

Scenedesmus sp. AR-2489 and *S. acutus* 8M culture suspensions were diluted to obtain an initial concentration of 180 mg l^{-1} (dry weight) and a pH of 7. Cultures were incubated at 30ºC in 50 ml Erlenmeyer flasks for 20 h in a shaking incubator at 100 rpm with a light intensity of 75 μmol photon m^{-2} s^{-1} and a CO_2 enriched atmosphere (3%). Biomass concentration, oxygen evolution and pH were determined at different intervals. Biomass was determined as cell dry weight by filtering 10 ml of algal suspension through a nitrate cellulose filter with 5 μm pores (Sartorius) and drying at 70ºC to constant weight. Oxygen evolution was monitored with a Clark-type electrode (YSI Biological Oxygen Monitor model 5300, Yellow Spring, OH, USA). The pH was measured with a pH meter (Crison Instruments, model 2000, Alella, Spain) calibrated against standard solutions at neutral and alkaline pH values.

Scanning confocal laser microscopy with the fluorescence probe BCECF–AM

S. acutus 8M and *Scenedesmus* sp. AR-2489 were grown in BG-11 medium and incubated overnight at 30ºC with a light intensity of 75 μmol photon m^{-2} s^{-1}. The cultures were then transferred to fresh medium and 1 ml of each cell suspension at the exponential phase was incubated for 10 min with 10 μl (1 mM solution) of the molecular probe 2′-7′-bis(2-carboxyethyl)-5-(and 6)-carboxyfluorescein acetoxymethyl ester (BCECF–AM) in order to observe pH distribution in living cells. This probe is freely distributed, crossing cell membranes. Once inside the cell, the non-fluorescent BCECF–AM is hydrolysed by intracellular esterases to release BCECF, which is retained by the cell and emits light (range 530–540 nm) proportionally to pH increases (Haugland 1992).

The fluorescence distribution was imaged by a scanning confocal laser (MRC-600, Bio-Rad Microscience Division) mounted on a Nikon Microphot microscope (SCLM). This instrument was equipped with a 1.4 numerical aperture (NA), oil immersion × 60 lens (Nikon Corp.) and was used in line with SCLM to produce images with sub-200 nm horizontal resolution in the *xy* axis and with reduced defocused information from the *xz* plane. Optical thin sections in the *xy* plane, as well as *xz* sagittal images, were collected. A krypton–argon laser with maximum emission lines at 488 nm and 568 nm was used to distinguish the excitation source for the fluorescent compounds from the green fluorescent BCECF and the red-autofluorescent photosynthetic pigments (chlorophyll-a), respectively, of the *Scenedesmus* strains.

The scanning control and the image processor were housed in an IBM-PC/AT-compatible desktop computer. Intervals between optical thin sections (*xy* section separation) and the position of sagittal sections (*xz* position) were defined by Bio-Rad interactive software in conjunction with a computer-controlled motor-driven focusing system connected to the Nikon photo microscope. Images were collected with a Bio-Rad photomultiplier pickup device and were integrated and digitized with a Kalman true-running-average filter. The recorded video images (512 × 768 pixels) were displayed on a flat 7-inch screen 16 Mhz black-and-white, high-resolution, video (VM 1710, H. Lucius & Baer, Geretsried, Germany) and photographed with a Kodak F-301 camera equipped with a 105 mm lens. Photographs were taken with Tmax 100 ASA film (Kodak, Rochester, NY, USA).

Analysis of pixel intensity on photographed sections of intracellular pH distribution in *Scenedemsus* sp. AR-2489 was carried out with Comos software (Bio-Rad Laboratories, Hertfordshire, UK) and then processed with the NIH Image 1.49 program on Power Macintosh.

Results

The growth curves of microalgae exposed to different concentrations of $HgCl_2$ are shown in Figures 1A, B, 2A, B. In the two *Chlorella* strains (Figure 1A and B), a significant reduction in growth was observed even at the lowest $HgCl_2$ concentration (0.5 μg ml^{-1}). At Hg concentrations above 1.0 μg ml^{-1}, growth was completely inhibited. An initial decrease in absorbance due to the bleaching of pigments was followed by complete lysis which was detected by optical microscope after eight days of incubation.

S. acutus 8M (Figure 2A) showed a similar inhibition pathway, its growth ceasing completely at concentrations above 1 μg ml^{-1} of Hg^{2+}. In this strain, however, bleaching was observed only at

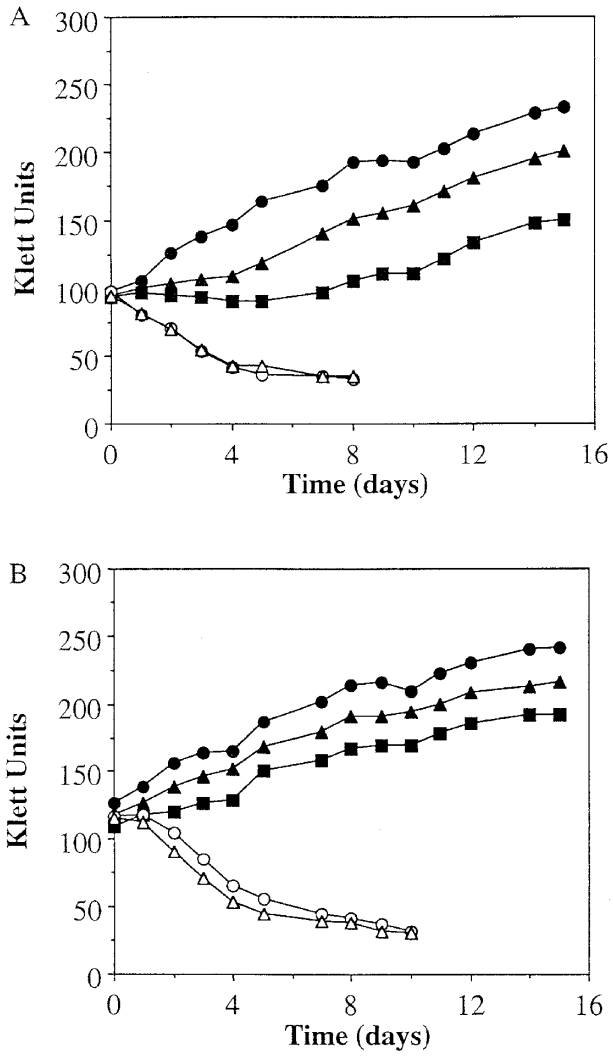

Figure 1. Inhibitory concentration curves of the growth of (A) *Chlorella* sp. strain AMI-5 and (B) *Chlorella* sp. strain GR-1489 with 0(●), 0.5(▲), 1.0(■), 2.5(○) and 5.0(△) μg ml^{-1} Hg as $HgCl_2$.

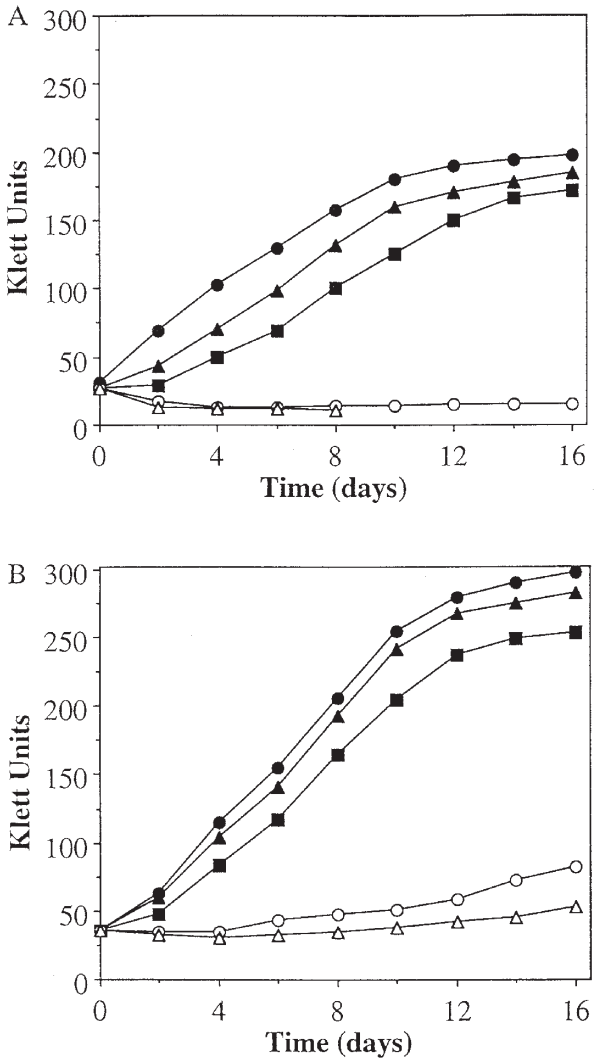

Figure 2. Inhibitory concentration curves of the growth of (A) *Scenedesmus acutus* 8M and (B) *Scenedesmus* sp. AR-2489 with 0(●), 0.5(▲), 1.0(■), 2.5(○) and 5.0(△) μg ml^{-1} Hg as $HgCl_2$.

5 μg ml^{-1}. In *Scenedesmus* sp. AR-2489, no bleaching occurred, and after a lag phase of four days, growth started at 2.5 and 5 μg ml^{-1} Hg as $HgCl_2$ (Figure 2B).

In order to elucidate the mechanism of Hg tolerance in *Scenedesmus* sp. AR-2489, Hg uptake experiments were performed with photosynthesizing, respiring and dead cells of both *Scenedesmus* strains. The total Hg content of the biomass and supernatant was determined after 3 h incubation.

Photosynthesizing cells of *Scenedesmus* sp. AR-2489 showed a lower relative accumulation factor (AF) than *S. acutus* 8M. The AF factor was calculated from the ratio of angular coefficients of the two linear regressions (0.549/0.884 = 0.62) (Table 1). In cells kept in the dark (respiring cells), this coefficient was higher (AF = 0.82), showing a better uptake; however, this accumulation factor was still lower for strain AR-2489 (0.82). Dead cells of both strains had a similar uptake (AF = 1.09).

During the Hg uptake by both *Scenedesmus* strains, a fraction of soluble Hg remained in the medium. The percentage of soluble Hg in the medium of photosynthesizing cells of *Scenedesmus* sp. AR-2489 was surprisingly lower (47%) (Figure 3A), than for strain 8M (68%). By contrast, dead cells of both strains removed the same amount of Hg (~30%) from the medium (Figure 3B).

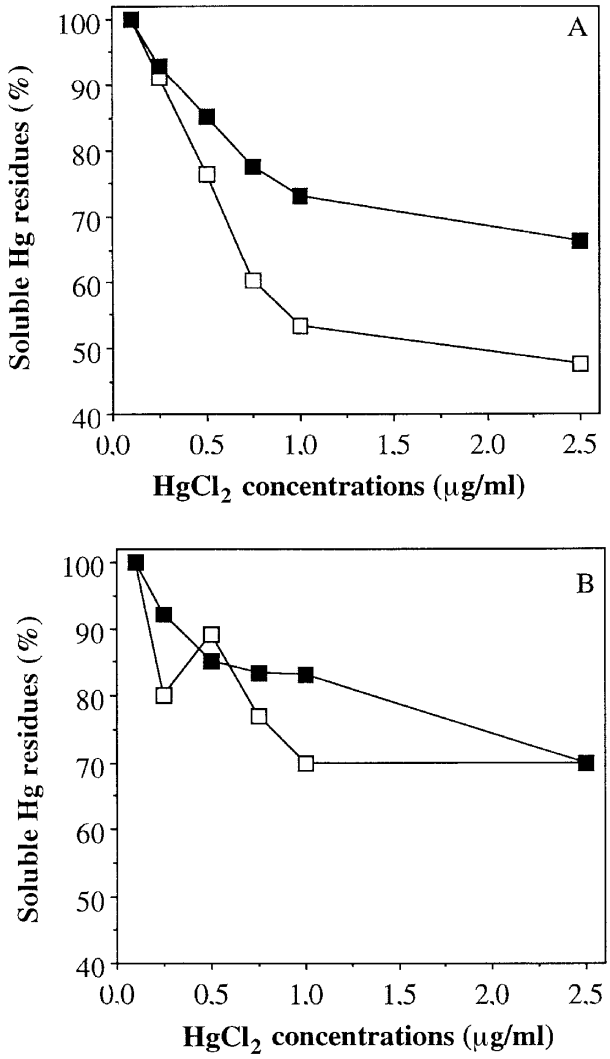

Figure 3. Soluble Hg left in the medium after centrifugation and filtration of (A) photosynthesizing cultures and (B) dead cells of *Scenedesmus* sp. AR-2489 (□) and *S. acutus* strain 8M (■) spiked with different concentrations of $HgCl_2$.

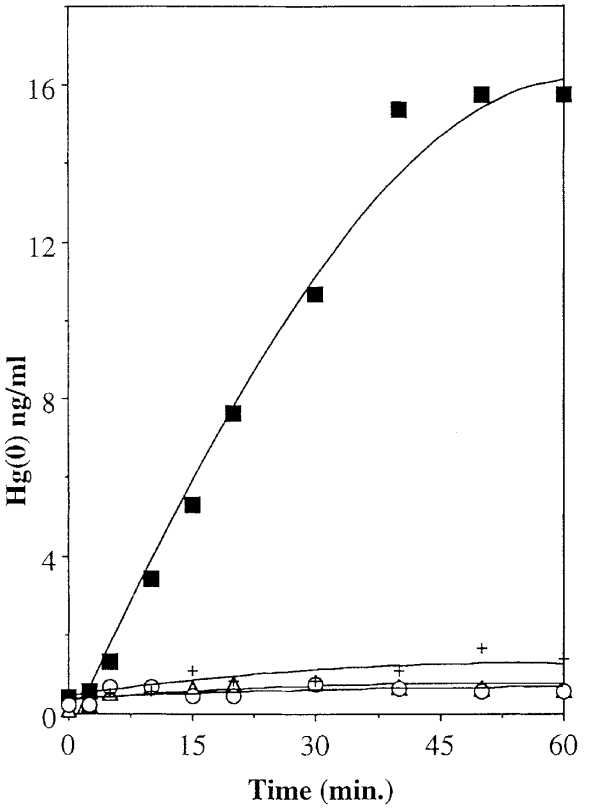

Figure 4. Mercury volatilization test: formation of Hg(0) (ng ml^{-1}) from 1 μg of Hg as $HgCl_2$ by a reference strain of *Pseudomonas putida* FB1 (■), by *Scenedesmus* sp. strain AR-2489 (○), by *S. acutus* strain 8M (△) and by an uninoculated sample (+).

Table 1. Different accumulation modes of mercury by two strains of *Scenedesmus* incubated for 3 h at $HgCl_2$ concentrations (coefficient of variance = 5.4) from 0.1 μg ml^{-1} to 5.0 μg ml^{-1}, exposed to light, in the dark and with dead cells

	Scenedesmus sp. strain AR-2489	*S. acutus* strain 8M	
	Angular coefficients [$b_{(1 \text{ or } 2)}$]; (R^2); for n = 7		
	b_1	b_2	*AF
Light	0.549 (0.998)	0.884 (0.986)	0.62
Dark	0.844 (0.986)	1.018 (0.997)	0.82
Dead cells	1.324 (0.991)	1.210 (0.993)	1.09

*AF = Hg accumulation factor from ratio of b_1/b_2, where b_1 and b_2 are the relative angular coefficients of the following linear regression; y [$HgCl_2$ (μg ml^{-1}) spiked in the medium] = $b_{(1 \text{ or } 2)}$ × [total Hg in cells (μg mg^{-1} dry weight)].

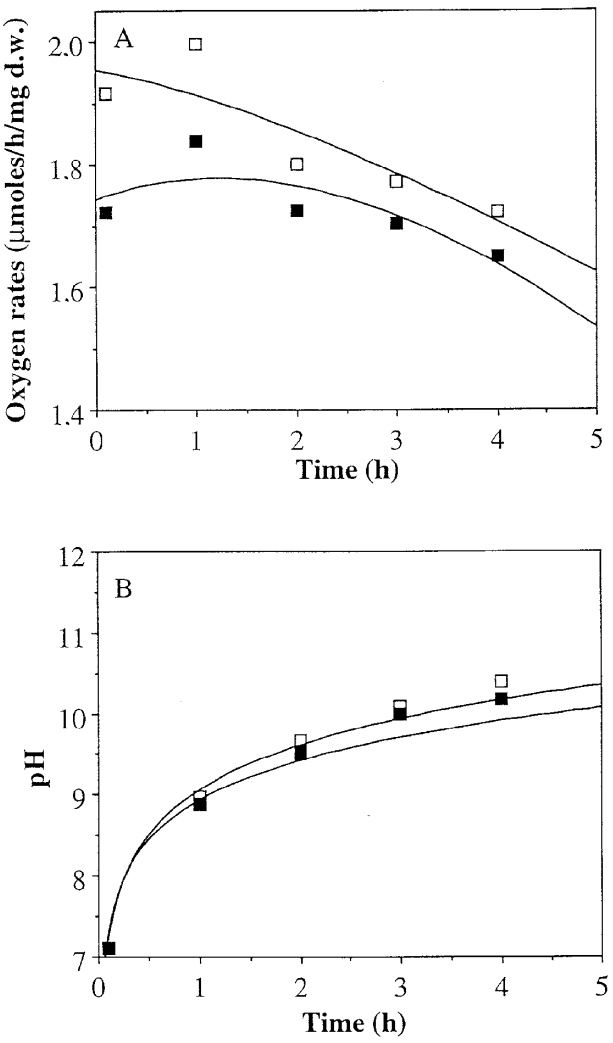

Figure 5. (A) Oxygen evolution rates of cultures of *Scenedesmus* sp. strain AR-2489 (□) and (B) increase in pH of medium inoculated with *S. acutus* strain 8M (■) without additions of $HgCl_2$.

Since *Scenedesmus* sp. AR-2489 showed a reduced accumulation of Hg, it was expected to find more soluble Hg residues in its own medium; conversely, less soluble Hg was found. It was hypothesized that Hg was lost by volatilization. A test was performed to compare the Hg volatilizing activities of the two *Scenedesmus* strains and the broad-spectrum mercury-resistant *Pseudomonas putida* FB1 (Baldi *et al.* 1988). No Hg(0) was observed in the head space of microalgal cultures (Figure 4).

The growth curves of the Hg-tolerant and the Hg-sensitive strain in the absence of Hg clearly showed that *Scenedesmus* sp. AR-2489 had a significantly higher growth rate (doubling time = 6.0 h) than *S. acutus* 8M (10.6 h). During the 4 h of photosynthesis, the specific rate of O_2 evolution (μmoles h^{-1} mg^{-1}) was always higher in *Scenedesmus* sp. AR-2489 than in *S. acutus* 8M cultures (Figure 5A). The pH of either medium increased from 7 to 10 in 4 h with slight differences between the two cultures (Figure 5B). This parameter, being measured by means of a logarithm scale is less sensitive than the measurement of oxygen production. To confirm these increases in both strains, the pH distribution was investigated at the cellular level using BCEFC–AM.

During the growth cycle the two *Scenedesmus* species produced a polysaccharide wall which harboured four cells (coenobium). When the two strains were incubated with BCEFC–AM, the coenobium showed intense green fluorescence (518 nm) in both strains, related to higher alkalinity as shown in *Scenedesmus* sp. AR-2489 (Figure 6a). The green fluorescence is the result of intracellular cleavage of the molecular probe BCEFC–AM to BCEFC and is significantly correlated with pH increases above neutrality (Tsujimoto *et al.* 1988, Efiok & Webster 1990, Haugland 1992).

The photosynthesis apparatus was easily revealed because chlorophyll-a emits a strong red fluorescence (568 nm). Its size was measured with an electronic ruler on the computer screen by Comos image analysis. This method gave similar dimensions for both strains: 7.64 μm ± 0.67 (standard deviation) long × 3.15 μm ± 0.48 wide for *Scenedesmus acutus* 8M, and 7.41 μm ± 0.79 × 3.05 μm ± 0.59 for *Scenedesmus* sp. AR-2489.

Differences in the surface of the photosynthesis apparatus were found in strain AR-2489; it was irregular (Figures 6b and 6d), especially when the four cells were harboured in the coenobium (Figure 6b); in *S. acutus* 8M the distribution of chlorophyll-a was more regular (Figure 6f). Another difference between the two strains was observed when the coenobium naturally broke up and released the free living cells into the medium. The cells of strain AR-2489 showed an alkaline intracellular pH (Figure 6c), whereas *S. acutus* 8M did not show any fluorescence (Figure 6e), except for some residues of coenobium. To demonstrate that the intracellular fluorescence due to higher pH in strain AR-2489 cells was significant, we quantified the pixel intensities. A single cell (see arrow Figure 6c) was chosen and framed in a virtual rectangle produced by image analysis software. A pixel (image unit) was equivalent to 0.238 μm in this image. This information was automatically obtained from Comos software. The rectangle measured 8 μm × 4μm and therefore contained 568 pixels. This number in the chosen

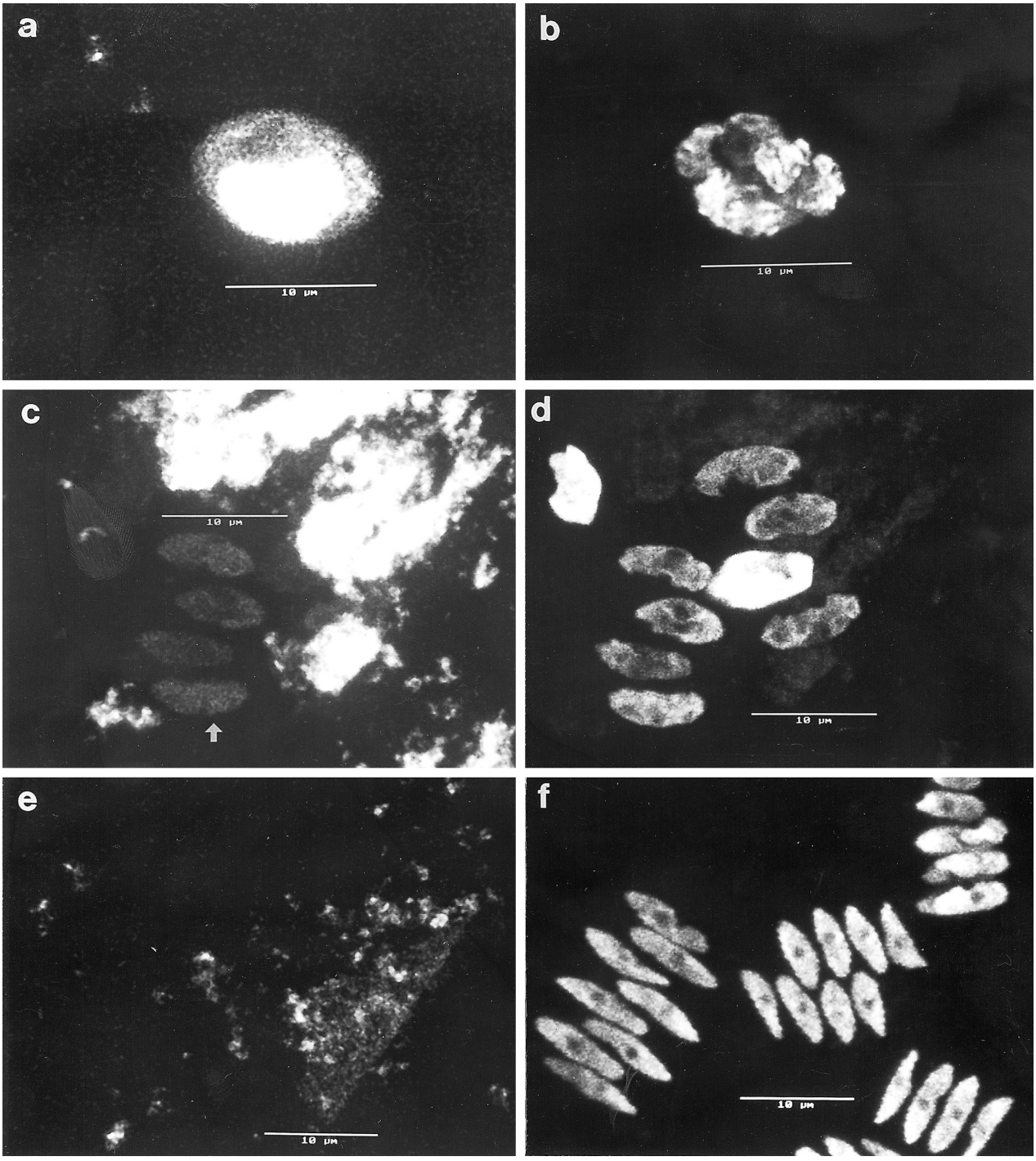

Figure 6. (a) Fluorescence distribution (SCLM green-channel) of the molecular probe BCEFC–AM in the coenobium of *Scenedesmus* sp. AR-2489 containing four cells. The intensity of the fluorescence is proportional to higher pH. (b) The same specimen as in (a) – autofluorescence of chlorophyll-a in the chloroplasts of the four cells harboured in the coenobium (SCLM red-channel). Notice the irregular surface of the photosynthesis apparatus. (c) Green fluorescence of the BCEFC probe in the coenobium debris and single *Scenedesmus* sp. AR-2489 cells. The intensity of the fluorescence is enhanced by high intracellular pH. The fluorescence of a single cell (see arrow) was processed by image analysis (Figure 7). (d) The same specimen as in (c) – (SCLM red-channel) autofluorescence of chlorophyll-a showing irregular surface of photosynthesis apparatus in free-living cells of strain AR-2489. (e) Green fluorescence of BCEFC distribution in coenobium debris of the reference strain *Scenedesmus acutus.* (f) Red autoflorescence of the photosynthesis apparatus of *S. acutus* strain 8M cells showing regular distribution of chlorophyll-a.

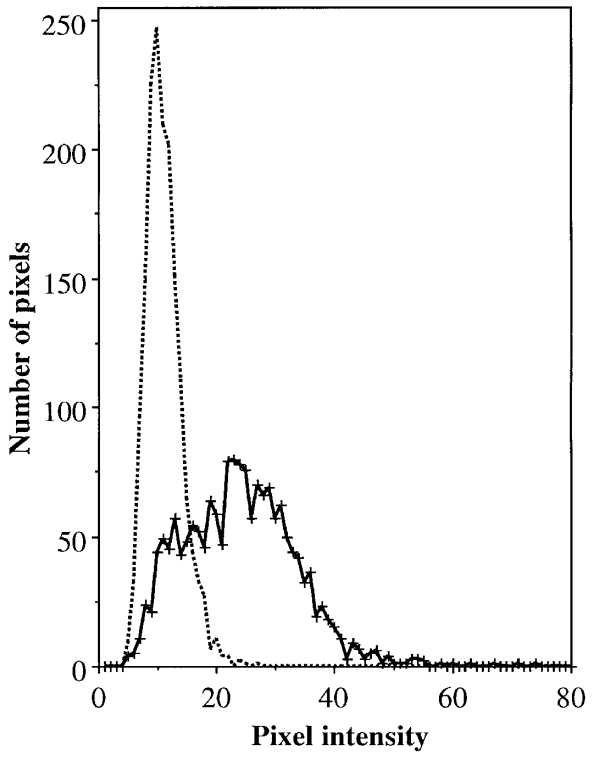

Figure 7. Distribution of pixel numbers versus pixel intensities of green fluorescence, produced by cleavage of the BCEFC–AM probe to BCEFC, in a virtual rectangle (8 μm × 4 μm containing 568 pixels; 1 pixel equals 0.238 μm) superimposed on one cell of *Scenedesmus* sp. AR-2489 (continuous line) (arrow in Figure 6c) and on an equal rectangle of background (dotted line). The pixel intensity is shifted to higher values due to increasing intracellular pH.

rectangle was constant, but the pixel intensity changed and shifted to the higher values (*x* axis) up to 55 (Figure 7). This shift is due to a significant fluorescence related to a higher intracellular pH.

Discussion

Of the microalgae isolated from polluted areas in Tuscany, *Scenedesmus* strains were found to have a higher tolerance to Hg. The growth of this organism is affected by the presence of Hg, even at lower concentrations. *Scenedesmus* sp. AR-2489 should not be considered a strictly Hg-resistant strain but rather a Hg-tolerant one. Differences between tolerance and resistance to Hg have already been presented (Baldi 1997). Metal tolerance is a non-specific response to the toxicant due to an independent mechanism not induced by the metal.

A further reason for considering *Scenedesmus* sp. AR-2489 as much more Hg tolerant than *Chlorella* strains (Ben-Bassat & Mayer 1977) was that the initial biomass of the two *Scenedesmus* strains in the minimum Hg inhibitory concentration tests was one third that of the *Chlorella* spp. The Hg tolerance of *Scenedesmus* spp. with respect to *Chlorella* spp. could be due to the production of coenobium, which might protect the cells from Hg stress.

Among the three microalgal strains isolated, only *Scenedesmus* sp. strain AR-2489 grew up to 5 μg ml^{-1} Hg (25 mM). This concentration is generally the Hg threshold used to isolate Hg-resistant bacteria from the environment. All bacteria which grow on these enriched media usually transform ionic Hg to elemental Hg enzymatically (Baldi *et al.* 1985, 1992, 1995).

The inhibitory threshold concentration of 5 μg ml^{-1} is several orders of magnitude higher than the minimum inhibitory concentrations reported for cyanobacteria, which are among the most sensitive prokaryotes to inorganic mercury (Fiore & Trevors 1994). Toxicity studies with microalgae are few and mostly concern the capacity of dead microalgal cells to bind trace elements (sorption activity) (Crist *et al.* 1981, Collins & Stotzky 1989).

In our experiments Hg tolerance was due to reduced Hg accumulation. At first glance, Hg-reduced transport into the *Scenedesmus* cells seemed to be energy dependent because photosynthesizing cells, which are more efficient in terms of energy production, accumulated less Hg than cells kept in the dark (respiration). Conversely, when the cells were dead, they accumulated the highest amounts of Hg and there were no differences in Hg uptake between the two *Scenedesmus* strains.

The removal of soluble Hg from the medium was at variance with its accumulation in the cells. Light exposed cells of *Scenedesmus* sp. strain AR-2489 showed a reduced Hg accumulation, with the highest Hg removal from the medium, and this was not due to Hg volatilization. The latter was sometimes observed in preliminary experiments with non-axenic cultures after several hours of incubation. In the end, Hg volatilization was discovered to be due to Hg-resistant bacteria (as contaminants) associated with the microalga. This microbial association probably protects the microalgae from Hg stress.

The active photosynthesis, leading to high O_2 evolution rates and inter- and extra-cellular pH increases, suggests that soluble $HgCl_2$ underwent chemical conversion. The well known Eh/pH diagrams for mercury species (Hahne & Kroontje

1973, Andersson 1979) show that in alkaline environments and with a high positive redox potential (oxygenated environments), the domain stability of ionic Hg is as the insoluble mercury hydroxide as follows:

$$HgCl_2 + HO^- = Hg(OH)_2 + Cl^-$$

Without chloride additions to the medium, the $HgCl_2$ was converted faster to the insoluble $Hg(OH)_2$ by strain AR-2489 due to its higher constitutional rate of photosynthesis, giving it a metabolic advantage and resulting in a higher Hg tolerance than for strain 8M. The higher constitutional rate of O_2 evolution by strain AR-2489; was probably due to a larger photosynthetic surface area due to its roughness observed by confocal microscopy. These constitutional features were probably related to the higher internal pH determined by the BCEFC–AM probe in strain AR-2489; by contrast, differences in the pH of the medium were less evident, since this is a logarithmic measurement. The high pH and O_2 evolution facilitated the conversion of $HgCl_2$ to $Hg(OH)_2$ by both *Scenedesmus* strains, and especially in strain AR-2489. In conclusion, the Hg tolerance of these microalgae may be ascribed to a chemical conversion, which presumably occurs intracellularly, and produces a Hg species with less reactivity in the cell metabolism. Moreover, extracellular $Hg(OH)_2$ is less available to the cells and probably co-precipitates as colloidal particles associated with other mineral hydroxides in the medium. This could explain why the soluble Hg residues in the medium were found to be significantly lower in *Scenedesmus* sp. AR-2489 than in *S. acutus* 8M.

There is as yet no direct way to determine mercury hydroxide at ppm concentrations, but its stability can be predicted indirectly from pH/Eh diagrams. Mercury hydroxide has been presumed to form in superficial sea water in the colloidal fraction associated with photosynthetic pigments (Mason *et al*. 1995b). Under laboratory conditions *Scenedesmus obliquus* cultures induce the formation of colloidal ferric hydroxides, phosphates and carbonates which co-precipitate ionic Cu and Cd (Drbal *et al*. 1985). The importance of the formation of colloidal metal hydroxides induced by photosynthetic organisms has also been described by Bruland *et al*. (1991), and it is known that mercury hydroxide is one of the most common species in freshwater aquatic environments (Robinson & Tuovinen 1984).

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