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Methyl mercury uptake by free and immobilized cyanobacterium

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Methyl mercury uptake in free cells and different immobilizates of the cyanobacterium *Nostoc calcicola* has been examined. The general growth of the immobilized cyanobacterial cells could be negatively correlated with methyl mercury uptake. Alginate spheres proved most efficient in terms of uptake rate (0.48 nmol mg protein^{-1} min^{-1}, 10 min) and total bioaccumulation (10.71 nmol mg protein^{-1}, 1 h) with a bioconcentration factor of 3.3×10^3. Alginate biofilms showed a faster methyl mercury accumulation rate (0.83 nmol mg protein^{-1} min^{-1}, 10 min) with a saturation of 10.28 nmol mg protein^{-1} reached within only 30 min (bioconcentration factor, 3.1×10^3). Foam preparations with a slow initial uptake approximated biofilms but were characterized by a lower bioconcentration factor (2.8×10^3). Free cells, in comparison, maintained the initial slow rate of uptake (0.62 nmol mg protein^{-1} min^{-1}, 10 min), saturating at 30 min (8.81 nmol mg protein^{-1}), and the resultant lowest bioconcentration factor (2.7×10^3). Cell ageing (30 days) brought a drastic reduction (3-fold) in organomercury uptake by free cells while alginate spheres maintained the same potential. Foam preparations of the same age showed a significant improvement in methyl mercury uptake followed by only a marginal decline in alginate biofilms. Data are discussed in the light of the physiological efficiency and longevity of immobilized cells.

Keywords: biofilm, cell aging, cyanobacterium foam, immobilization, *Nostoc calcicola*, spheres

Introduction

Mercury remains highly mobile in the environment, forming species like metallic mercury, inorganic mercury and organic mercury. Previous studies have provided evidence for the metabolism of mercury in phytoplankton (Havlik *et al.* 1979), including its biotransformation by bacterial (Ramamoorthy *et al.* 1983) and algal cells (Wilkinson *et al.* 1989). The latter investigators also deployed immobilized cells of *Chlorella emersonii* for efficient accumulation and volatilization of mercury in long-term experiments. Similar immobilizates from cyanobacteria resulted in efficient copper uptake (Singh *et al.* 1989); improved production of ammonia (Jeanfils & Loudeche 1986), hydrogen (Muallem *et al.* 1983) and protein synthesis (Potts 1985); and glutamate biosynthesis (Matsunaga *et al.* 1991). However, such enhanced activities were negatively correlated with the general growth of immobilized cyanobacterial cells (Gendel & Nöhr 1989). There are reports that organic mercury is highly toxic over its inorganic counterpart (Röderer 1983, Singh & Singh 1992). Also, the former is accumulated very rapidly by algae like *Chlorella, Scenedesmus* and *Microcystis*, and transferred to inorganic mercury following 48 h of uptake (Havlik *et al.* 1979). As there are no previous reports, the present communication describes uptake of methyl mercury by cells of a diazotrophic cyanobacterium, *Nostoc calcicola*, as alginate biofilms, spheres and immobilized in polyurethane foams in the exponential and late stationary growth phase, along with a comparison with free cells.

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Materials and methods

Organisms and growth conditions

The target organism was the diazotrophic cyanobacterium *Nostoc calcicola*, Bréb, a local isolate maintained in liquid growth medium (Allen & Arnon 1955) with A6 trace

elements at 24 ± 1 °C under cool white fluorescent illumination (14.4 watt m^{-2}) with a 18/6 h light/dark cycle.

Immobilization

Alginate spheres. Log phase (6 days) cyanobacterial cells were concentrated through centrifugation and repeated washings (triple glass distilled water) to obtain the desired cell protein value in 2% sodium alginate (w/v; Fluka AG, Switzerland). The suspension was mixed thoroughly and suspended dropwise in cool calcium chloride solution (0.2 M) to give alginate spheres with an average diameter of 5 mm and then allowed to harden for 2 h. This was followed by repeated washings with sterile water before inoculation into fresh growth medium. Methyl mercury uptake experiments utilized 6 or 30 day old spheres.

Alginate biofilms. Cyanobacterial cells mixed with a lower concentration of alginate (0.25%, w/v) were slowly spread over calcium chloride solution (0.2 M) to a thickness that allowed the formation of floating films containing algal cells (average thickness 1 mm). Precautions were taken during repeated washings of such biofilms because of the problem of breakage. Such preparations were allowed to grow in diazotrophic medium as before for 6 or 30 days.

Polyurethane foams. Polyurethane foams were cut to a size of 5 mm and pre-sterilized before transfer to cyanobacterial cultures. Visual observations indicated that 4–6 days were required for establishment of cyanobacterial cells in the foam matrices. Since a minor fraction of the cell population leaked out of the foam preparations, growth and methyl mercury uptake experiments were based on the amount of cyanobacterial cells retained in the foam cubes. While free cell suspensions underwent repeated shakings, alginate biofilms, spheres and foam preparations remained unshaken during growth.

Methyl mercury uptake experiments

Stock solutions of methyl mercuric chloride (Wilson Ltd, India) were prepared in sterile water (w/v) and the desired molarity (1.25 μM, saturating concentration for uptake) was obtained in HEPES–NaOH buffer (10 mM, pH 7.5). The final cell mass for free cells, alginate biofilms, spheres and polyurethane foams in methyl mercury uptake experiments was adjusted to a protein value of 300 μg ml^{-1}. Foam cubes containing cyanobacterial cells were directly suspended in the uptake medium. The desired cell mass of free cells, biofilms, spheres and cells established in foams was removed at intervals (0–60 min) and centrifuged to procure cell pellets for the estimation of cellular methyl mercury. The supernatant was used to record the extracellular level.

EDTA (10 mM) was used to account for the amount of methyl mercury adsorbed in different preparations. Adsorption experiments also included alginate spheres, biofilms and foams lacking cyanobacterial cells. Methyl mercury was estimated using an MA 5800 E Mercury Analyzer (ECIL, India) based on cold vapor atomic absorption spectrophotometry, with a detection limit of 0.1 μg $Hg\,l^{-1}$. Uptake values are expressed in terms of nanomoles of mercury per unit protein. The cell pellet and supernatant were subjected to acid hydrolysis with a mixture of nitric acid (10%) and stannous chloride (20%) as specified in the analytical procedure.

Growth estimations

While free cell suspensions were directly taken out for recording absorbance changes (0–8 days), biofilms and alginate spheres were dissolved in sodium citrate (0.1%) to yield a homogenous suspension, and foams were squeezed to release the cells for recording whole cell absorbance at 650 nm in a Bausch and Lomb spectronic 20 colorimeter.

Protein estimations

Protein was estimated by the method of Lowry *et al.* (1951) with the modifications of Herbert *et al.* (1971).

Heterocyst frequency

Such estimates are based on the microscopic examination of cyanobacterial filaments from liquid cultures, alginate biofilms, spheres and those removed from foam preparations (heterocyst frequency is expressed as percentage).

Analysis

All data presented are the means of triplicate observations with standard errors shown as bars as applicable. The data for CH_3Hg^+ uptake for cells in different states and exposure time were verified for the significance at a particular probability level, and the variance ratio (F) calculated as F = treatment mean square/residual mean square.

Results and discussion

The immobilized cyanobacteria have previously been investigated with reference to ammonia production (Musgrave *et al.* 1982, Jeanfils & Loudeche 1986), photoproduction of H_2 and $NADPH_2$ (Mullem *et al.* 1983), protein synthesis and proteolysis (Potts 1985), biochemical physiological and morphological features (Shi *et al.* 1987), stability of permeaplast (Papageorgiou *et al.* 1988), long-term stability of photosynthetic electron transport (Affolter & Hall 1986), ammonia and H_2 production (Brouers & Hall 1986), glutamate production (Matsunaga *et al.* 1991), and heavy metal uptake (Singh *et al.* 1989). The capability of cyanobacteria to undergo immobilization on the exterior of fibrous substrates would, to a major extent, improve the ease of manipulation of cells, control of growth and product recovery, and seems superior over the complicated immobilization by entrapment described above (Gendel & Nöhr 1989).

Figure 1 shows a comparison of the photoautotrophic growth of *N. calcicola* as free cells and those entrapped in alginate spheres, biofilms and polyurethane foam cubes. The uppermost curve characterizes liquid growth behavior of free cyanobacterial cells with initiation of multiplication even within 24 h that attained a peak on day 4 ($k = 0.046$). Further incubations up to 12 days showed insignificant enhancement in growth yield. Similar cells entrapped in the alginate biofilm showed growth improvement with a slower pace compared with their free cell counterparts, leaving a significant margin on days 2 and 4 of incubation in fresh growth medium ($k = 0.0054$); the ever increasing trend in absorbance values also showed that cells in this state never attained the stationary phase of growth. Microscopic examination of cells revealed (i) enhanced hormogone and pigment production, and (ii) heterocyst frequency of 21% compared with only 7.5% in free cells. Cyanobacterial cells established at the inner matrices of polyurethane foams (within 4–5 days of inoculation) were also characterized by a slow growth ($k = 0.057$) during the same time course under comparison (0–12 days); however, the heterocyst frequency increased to nearly 27% (a 6% enhancement over alginate biofilms). Cyanobacterial cells in alginate spheres were also characterized by a slow pace of growth ($k = 0.024$), but the heterocyst frequency (31%) was the highest of all sets compared. Slow growth of immobilized cyanobacteria has been attributed to photonic and diffusional barriers as, in most cases, the immobilized cells operate at 28% of the efficiency of free cells (Papageorgiou *et al.* 1988). Light penetration of free cells confined within alginate is thought to be at par with light penetration within liquid cultures at low light intensities (Matsunaga *et al.* 1991). In a similar comparison, Gendel & Nöhr (1989) observed a low growth rate and high specific activity of nitrogenase in *N. muscorum* cells immobilized on the exterior of a variety of fibrous substrata.

The differential behavior of *N. calcicola* immobilizates tempted us to look into the rates and extent of methyl mercury uptake keeping free cells as control for comparison. Methyl mercury uptake was invariably very fast within 5 min of initial uptake, thus suggesting high mobility of the organomercurial. Since the EDTA-washable fraction (adsorption) constituted around 2.5% of the total methyl mercury amount added, it is apparent that the organomercurial is taken in very fast with the high cellular intake. Free cells recorded an initial jump in the uptake rate (0.62 nmol mg protein^{-1} min^{-1}, 10 min), saturating within 30 min with a bioconcentration factor of 2.7×10^3; a value which was the lowest of all the immobilizates (Figure 2). Alginate spheres

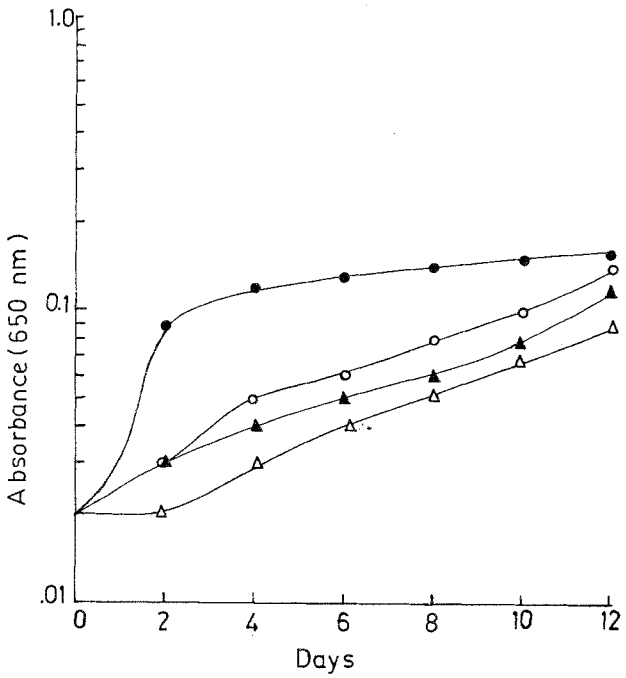

Figure 1. Growth behavior of *N. calcicola* as free cells (●), and in biofilms (○), foams (▲) and alginate spheres (△). $F_{days}(7, 21) = 1.867$, $P < 0.25$; $F_{states}(3, 21) = 20.16$, $P < 0.025$.

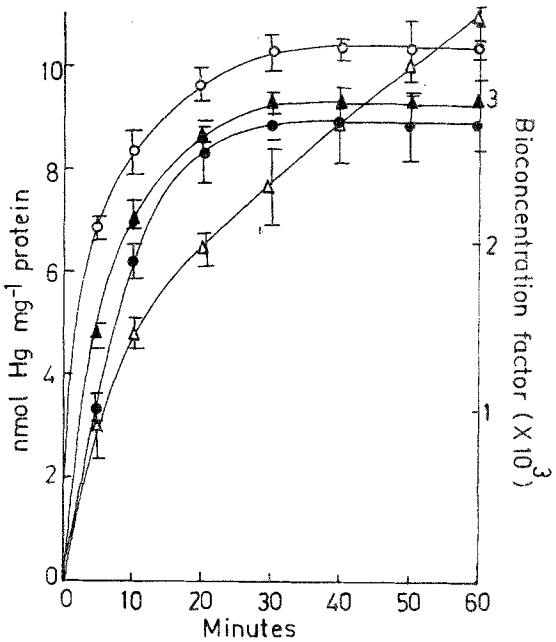

Figure 2. Methyl mercury uptake pattern in *N. calcicola* as free cells (●), and in biofilms (○), foam (▲) and alginate spheres (△) (6 day old cells). $F_{min}(6, 18) = 32.11$, $P < 0.005$; $F_{states}(3, 18) = 7.22$, $P < 0.10$.

showed a sluggish start with regard to methyl mercury uptake (0.48 nmol mg protein^{-1} min^{-1}, 10 min); however, the total uptake (10.71 nmol mg protein^{1}, 1 h), as well as the bioconcentration factor (3.3×10^3), remained the highest among the immobilizates compared. In a previous report, Singh *et al.* (1989) observed that immobilized *N. calcicola* cells showed enhanced copper uptake over free cells by a factor of 2, thus emphasizing the metabolic efficiency of the former. In long-term (12 day) experiments the immobilized cells of *Chlorella emersonii* removed 99% of added mercury compared with 92% in free cells (Wilkinson *et al.* 1989).

The use of biofilms on inert matrices is a further means of immobilization, and *Citrobacter* cells showed uranium and lead uptake capacities comparable to those of gel immobilized cells under such conditions (Macaskie & Dean 1987). Similarly, simultaneous denitrification and metal removal could also be achieved using mixed bacterial cells as a film on anthracite, coal particles or immobilized on polyvinyl chloride or polypropylene webs (Shumate & Strandberg 1985). Microbial biofilms also act as biosorbents of metals (Hutchins *et al.* 1986). *N. calcicola* cells entrapped as alginate biofilms proved most efficient in terms of initial uptake rate as well as the final cellular build-up of methyl mercury as the uptake rate for such preparations was 0.83 nmol mg protein^{-1} min^{-1} (10 min); a value almost twice that of the alginate spheres described previously. Also, subsequent methyl mercury uptake recorded a rising trend, saturating at 10.28 nmol mg protein^{-1} min^{-1} (30 min) with a bioconcentration factor of 3.1×10^3. *Anabaena azollae* cells immobilized in synthetic polymer foams also showed stable photosynthetic oxygen evolution activity and MSX-induced ammonia production over 1 week (Park *et al.* 1991). Similar establishment of *N. calcicola* cells in polyurethane reflected a slow initial start of methyl mercury uptake but certainly approximated those of biofilms in terms of uptake rate (0.83 nmol mg protein^{-1} min, 10 min). Nevertheless, uptake efficiency was reduced with the passage of time, as evident from the corresponding bioconcentration factor (2.3×10^3). The overall efficiency of methyl mercury uptake can be arranged as: spheres > alginate biofilms > foam > free cells.

Immobilization of cyanobacteria enhanced nitrogen fixation activity, hydrogen evolution and ammonia production (Vincenzini *et al.* 1981, Brouers & Hall, 1986), and it seemed imperative to compare methyl mercury uptake in 30 day old immobilizates (as above) with those of free cyanobacterial cells of the same age (Figure 3). The methyl mercury uptake

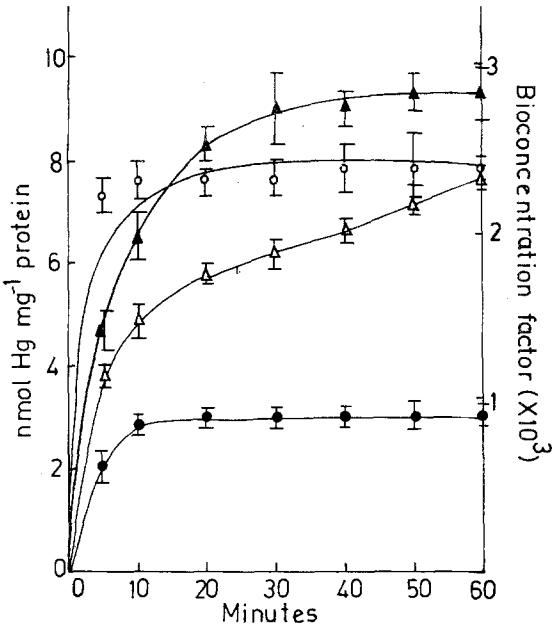

Figure 3. Methyl mercury uptake pattern in 30 day old *N. calcicola* as free cells (●), and in biofilms (○), foam (▲) and alginate spheres (△). $F_{min}(6, 18) = 6.62$, $P < 0.025$; $F_{states}(3, 18) = 70.60$, $P < 0.005$.

pattern showed a drastic reduction for 30 day old free cells in terms of (i) 0.29 nmol mg protein^{-1} min at 10 min, thus amounting to an almost 2-fold decline over 6 day old free cells (compare Figures 2 & 3), (ii) reduction in saturation time (20 min prior to that of log-phase cells) and (iii) a 3-fold decline in methyl mercury saturating concentration (3.04 nmol mg protein^{-1}) over free cells (8.81 nmol mg protein^{-1}) accompanied by reduction in the corresponding bioconcentration factor (0.92×10^3). In contrast, alginate biofilms of the same age showed a marginal decline in the initial rate of methyl mercury uptake (0.76 nmol mg protein^{-1} min^{-1}, 10 min) and a shortening of the saturating time to 10 min compared with 30 min for 6 day old preparations. The corresponding bioconcentration factor (2.4×10^3) also reflected this marginal difference. Alginate spheres, whether 6 or 30 days old, maintained almost the same profile of methyl mercury bioconcentration characterized by a slow and steady start and an ever increasing trend lasting for 60 min. The physiological longevity of gel spheres was also apparent in terms of an insignificant change even in the initial uptake rate (0.50 nmol mg protein^{-1} min^{-1}, 10 min) over the log-phase spheres (0.48 nmol mg protein^{-1} min). Such spheres showed significant methyl mercury uptake up to 1 h and the values approximated alginate biofilms of the same age (7.65 nmol mg protein^{-1}). Cell ageing was also

evident in such preparations; however, methyl mercury uptake was only 1.4-fold lower over the log phase spheres. The corresponding bioconcentration factors for 30 (2.3×10^3) and 6 day old (2.8×10^3) spheres also reflected the insignificant difference. Foam preparations, being inferior to either alginate spheres or biofilms at 6 days (see Figure 2), proved their overall superiority in terms of (i) maintenance of the same initial methyl mercury accumulation rate (0.83 nmol mg protein^{-1} min, 10 min), (ii) initiation of saturation time (30 min) as well as the saturating concentration (9.3 nmol mg protein^{-1}) and (iii) the same bioconcentration factor of 2.8×10^3 as applicable to 6 day old foam preparations.

Cyanobacterial cells, in line with eukaryotic algae, accumulate heavy metals through a faster initial reaction (adsorption), followed in sequence by relatively slower, metabolism-dependent intracellular cation uptake, as observed for zinc (Shehata & Whitton 1982), cadmium, copper and zinc (Les & Walker 1984), cadmium (Singh and Yadava 1985), copper (Singh 1985), aluminium (Pettersson *et al.* 1986), nickel (Campbell & Smith 1986), and zinc (McHardy & George 1990). Likewise, many prokaryotic and eukaryotic algae have been shown to accumulate organic mercury and inorganic salts as chlorides or sulfates, including lead acetate, tetraethyl lead and lead nitrate (DeFilippis & Pallaghy 1976a, b, Mora & Fabregas 1980, Röderer 1980, 1983, Hassett *et al.* 1981, Johnson & Shubert 1986). The present observations also conform with these reports and, in addition, present evidence that immobilization offers a convenient and efficient method to use microbe-based technologies to remove heavy metals.

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