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Impact of intracellular build-up of mercury on phycocyanin leakage in the planktonic cyanobacteria *Nostoc muscorum* and *Anabaena variabilis*

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Abstract
The intracellular accumulation of Hg^{2+} and subsequently the leakage of phycocyanin have been compared in two diazotrophic cyanobacteria *Nostoc muscorum* and *Anabaena variabilis* in the presence of natural as well as synthetic chelators. Based on our findings, it may be concluded that the uptake of Hg^{2+} by the cyanobacterium from the ambient medium having 0.05 – 1.0 µM Hg^{2+}, was active and very fast during first 30 min, followed by a slow rate in the next 30 min, which also accompanied the efflux of intracellularly accumulated Hg^{2+} ions during subsequent incubations; and the reduced rate of phycocyanin leakage in the presence of EDTA (10.0 µM) was the consequence of reduced mobility of resultant Hg^{2+}–EDTA-complex.

Key words: Cyanobacterium, *Nostoc muscorum*, *Anabaena variabilis*, Hg^{2+} uptake, Phycocyanin leakage, chelators.

Introduction

Heavy metal toxicity/uptake in cyanobacteria has been extensively reviewed (Whitton, 1970, Sorentino, 1979, Huntsman & Sunda, 1980, Rai *et al*. 1981, Singh, 1987 and Pant, 1993, 2000). Microalgae can efficiently sequester toxic heavy metals from aquatic environments. They may bind up to 10 % of their biomass as metals. To assess the potential biological impact of heavy metals in the environment, it is critical to know both the total and the biologically available levels of heavy metals.

The total heavy metal content is most frequently determined by atomic absorption or mass spectroscopy of acid solubilized samples and substrates. Bioavailable levels of heavy metals can indirectly be determined by similar analyses of the total metals present in an organism. These are bioassayed by the differential ability of different organisms to bioaccumulate metals intracelluralarly.

Mercury (Hg^{2+}) occupies the prime position in the list of toxic heavy metals (Rai *et al.* 1981). It is used in thermometers, barometers, dental fillings, batteries and fluorescent lamps. In addition, a number of organomercurials have also been deployed as effective fungicides. The comprehensive study on its interaction with the cyanobacterial cell (Stratton *et al*. 1979, Murthy *et al*. 1989, Singh & Singh, 1987, 1992a, 1992b, Singh *et al*. 1987) certifying the actual level of intracellular Hg^{2+} responsible for the observed effects (Wilkinson *et al*. 1989) as well as its transport across cyanobacterial cell membrane, is scant and is an active research area in the field of heavy metal phycology. Many prokaryotic and eukaryotic algae have been shown to accumulate organic mercury and inorganic salts as chlorides or sulphates, including lead acetate, tetraethyl lead and lead nitrate (DeFilippis & Pallaghy, 1976a, b; Mora & Fabregas, 1980, Hassett *et al*. 1981, Johnson & Shubert, 1986, Pant *et al.* 1992).

Cyanobacteria, a numerous and diverse group of photosynthetic prokaryotes largely responsible for global photosynthetic productivity (Ting *et al*. 2002), are excellent models of bioaccumulation studies (Arunakumara *et al*. 2008). The toxicity of heavy metals, however, may result in diverse effects, which depend on the type of algae, features and concentrations of the metal and the environmental conditions (Heng *et al*. 2004, Satoha *et al*. 2005, Greger *et al*. 2007). Growth inhibition and chlorosis are common symptoms of metal toxicity, in which photosynthesis is probably the most affected metabolic process (Ali *et al*. 2006). Alterations in morphology and ultrastructure have also been frequently reported (Choudhury & Panda 2005). Growth inhibition in micro algae is well known for metal toxicity and found to be related to the amount of metal bound to the algal cell surface in some cases, to the amount of intracellular metal (Franklin *et al*. 2000, 2001, Ma *et al*. 2003) and to the chemical nature of the metal (Tripathi & Gaur, 2006). Metal accumulation by algae is influenced by a number of abiotic (e.g. pH, chelating agents, redox potential, temperature, light) and biotic (e.g. cellular activity, algal biomass concentrations, extracellular products) factors (Fathi & Omair, 2006).

Cyanobacteria and algae possess a wide range of coloured components including carotenoids, chlorophylls and phycobiliproteins (Sarada *et al*. 1999). The principal phycobiliproteins are phycocyanin, allophycocyanin and

phycoerythrin. Phycocyanin is an important light harvesting photosynthetic pigment in cyanobacteria (Gantt, 1980, 1981). Phycocyanin is used as a colourant in food (chewing gums, dairy products, ice sherbaths, gellies etc.) and cosmetics such as eyeliners in Japan, Thailand and China (Muthulakshmi *et al*. 2012). It was also shown to have therapeutic value (immunomodulating activity and anticancer activity). Owing to its fluorescent properties it has gained importance in the development of phycoflour probes for immunodiagnostics (Kronik & Grossman, 1983). The prevailing environmental conditions such as temperature and ionic strength of the medium, considerably affect the aggregation and assembly of phycocyanin into phycobilisomes (Cohen-Bazire & Bryant, 1982, Almog & Berns, 1984). It has been a general observation that cyanobacteria extrude phycobiliproteins in the ambient medium when the cultures are sufficiently old, or even during log phase, if subjected to environmental stress (Allen, 1968).

Therefore, the present enquiry was aimed at determining the actual level of intracellularly accumulated Hg^{2+} responsible for damaging the cell membrane without cell lysis of the diazotrophic cyanobacterium *Nostoc muscorum* Breb. and *Anabaena variabilis* Breb., isolated from the ancient sacred ponds of the holy city of Kurukshetra, India (Biban & Singh, 2011, 2012). The responses of the mercury stress events were also examined in the presence of diluted spent medium (a natural chelator) and EDTA (a known synthetic chelator).

Materials and methods

Organism and growth conditions The N_2-fixing cyanobacteria *Nostoc muscorum* Breb. and *Anabaena variabilis* Breb., isolated from local ancient sacred ponds of the holy city Kurukshetra, India, were axenically cultured and routinely grown in 500 ml Erlenmeyer flasks having 200 ml Allen & Arnon's nitrogen-deficient growth medium (Allen & Arnon, 1955), containing A_6 trace elements at 24 ± 1^0C under µmol photon/m^2/s light intensity (cool fluorescent light) on the surface of the culture vessels with a 18/6 h light/dark cycle. Protein was determined by the method of Lowry *et al*. (1951), modified by Herbert *et al*. (1971) using lysozyme as standard.

Leakage of phycocyanin The leakage of phycocyanin was estimated according to Singh & Singh (1992b). Exponentially growing *N. muscorum* and *A. variabilis* cells were harvested through centrifugation (3000 rpm, 4 min; Remi, India) and, after three times washing with sterile distilled water, inoculated in a fresh liquid medium, supplemented with Hg^{2+} (as $HgCl_2$) to establish its 0.05-1.0 µM strengths. Such sets were phototrophically incubated as routine culture for 16 h. 5.0 ml aliquots withdrawn at 2h interval, were centrifuged (3000 rpm, 4 min). The phycocyanin content was estimated as µg phycocyanin/mg protein (Fig. 3a and 3b) with the help of a spectrophotometer (Electronics, India).

Hg^{2+} *uptake* Hg^{2+} uptake by *N. muscorum* and *A. variabilis* was determined in a cold vapour atomic absorption spectrophotometer, Mercury Analyzer (Model, MA 5840, Electronics Corporation of India Limited, India; detection limit, 0.1 µg Hg^{2+}/l), in terms of quantifying the total intracellular built-up of Hg^{2+}. For this, the exponentially growing cyanobacterial cells were harvested as above, and inoculated in a fresh sterile medium, having 0.05-1.0 µM $HgCl_2$, as above. The 5.0 ml aliquots from these experimental suspensions withdrawn at desired intervals of 10 min. The cyanobacterial samples (pellets) and the supernatant spent medium were digested in HNO_3 as per the method described in Standard methods for Examination of Water and Waste Water by APHA (19^{th} Edition). The Hg^{2+} content of the acid digested samples was estimated as per the methodology described in the manual of Mercury Analyzer (MA 5840, ECIL, India). The Hg^{2+} content was quantified as µmol Hg^{2+} /mg protein by reference to a standard obtained with $HgCl_2$ in the methodology as above.

Experiments with Chelators To study the effect of EDTA, (a known synthetic chelator), exponentially growing *N. muscorum* and *A. variabilis* cells were inoculated in a fresh Allen & Arnon's medium, containing 10.0 µM EDTA plus 2.0µM Hg^{2+} in addition to its normal Fe-EDTA constituent. Similarly, the effect of 50 % diluted spent medium (a natural chelator) was also studied. The follow-up experimental design for the pigment leakage was the same as mentioned above.

Chemicals The chemicals used in the growth medium and the acids for digestion were purchased from HIMEDIA, India, respectively, while those for protein estimation and Hg^{2+} uptake were the products of Qualigens, India.

Stastical analysis Statistical analysis of the data was done as per the methodology dealt in detail (Singh & Singh 1990, 1992a, 1992b). All the data presented are the means of triplicate observations with standard errors shown in bars as applicable. The data were verified for significance at a particular probability level, and the variance ratio (F) was calculated as F= Treatment mean square/Residual mean square. The correlation coefficient (r) was also calculated.

Results

Fig. 1a shows the growth behaviour of the diazotrophic cyanobacterium, *Nostoc muscorum* in terms of protein content when exposed to inorganic mercury (0.05 – 1.0 µM) in the medium. The initial concentrations (0.05 to 0.1 µM) inhibited a little growth of the organism, whereas an exposure to higher levels (>0.1 µM Hg^{2+}) exerted much reduction in growth (r= 0.094, $F_{Hg^{2+}\,6,\,42}$ = 13.86, p< 0.01). An exposure of 0.5 µM Hg^{2+} ceased the algal growth upto 6 days of

incubation and showed a little increase in the growth yield during 8-12 days. At still higher strength of 1.0 µM Hg^{2+}, the organism died within 6 days ($F_{time\ 7,\ 42} = 2.67$, $p < 0.01$). Similarly the growth behaviour of the second experimental organism, *Anabaena variabilis* was studied (Fig.1b, $F_{Hg^{2+}\ 5,\ 35} = 10.98$, $p < 0.01$). On comparative grounds it was less than that of *N. muscorum* ($r = 0.886$, $p<0.01$).

An exposure to the same concentration range of Hg^{2+} (0.05-0.5 µM), the viability of *A. variabilis* lasted beyond 6 days, however, after 8 days the organism came to stand still. Like *N. muscorum*, Hg exerted almost toxic impacts on *A. variabilis* also. Whereas, an exposure to higher doses of Hg^{2+} (0.1 and 0.2 µM) in the medium greatly reduced its protein content.

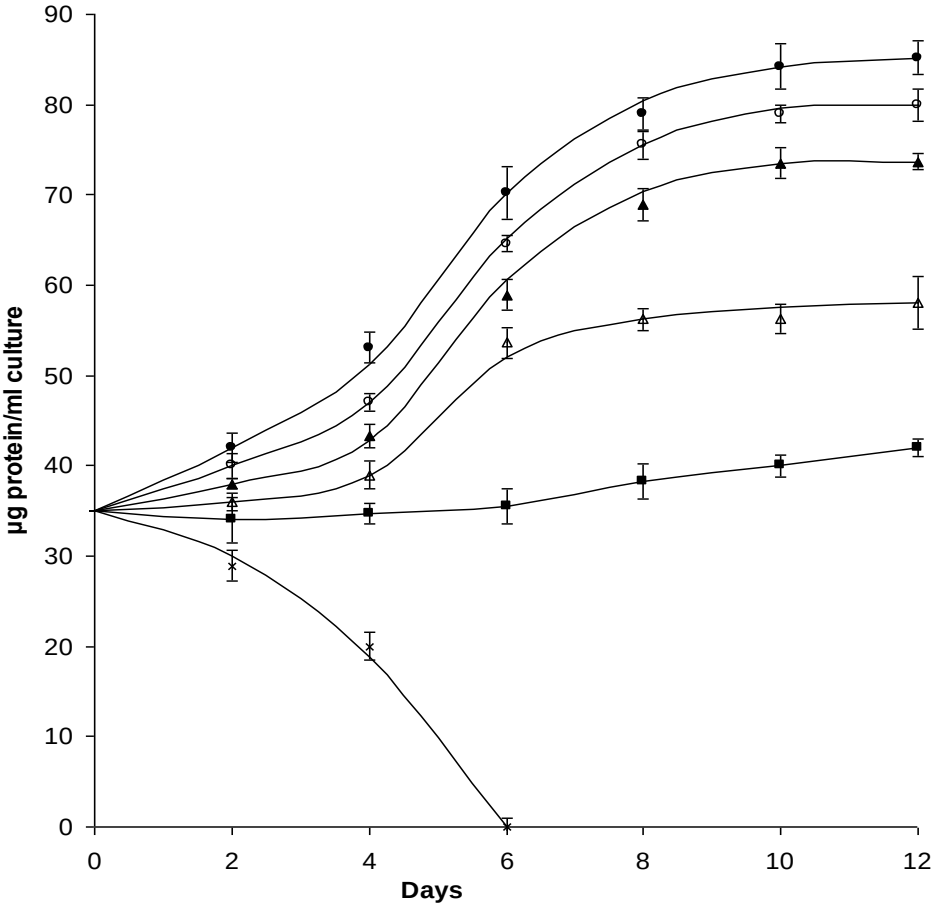

Fig. 1a: Protein content of *Nostoc muscorum* cells exposed to graded concentrations of Hg^{2+} during 2-12 days incubations: Hg^{2+}-less control (●), 0.05 µM (O), 0.10 µM (▲), 0.20 µM (Δ), 0.5 µM (■) and 1.0 µM (x). Values are mean ±3 SE.

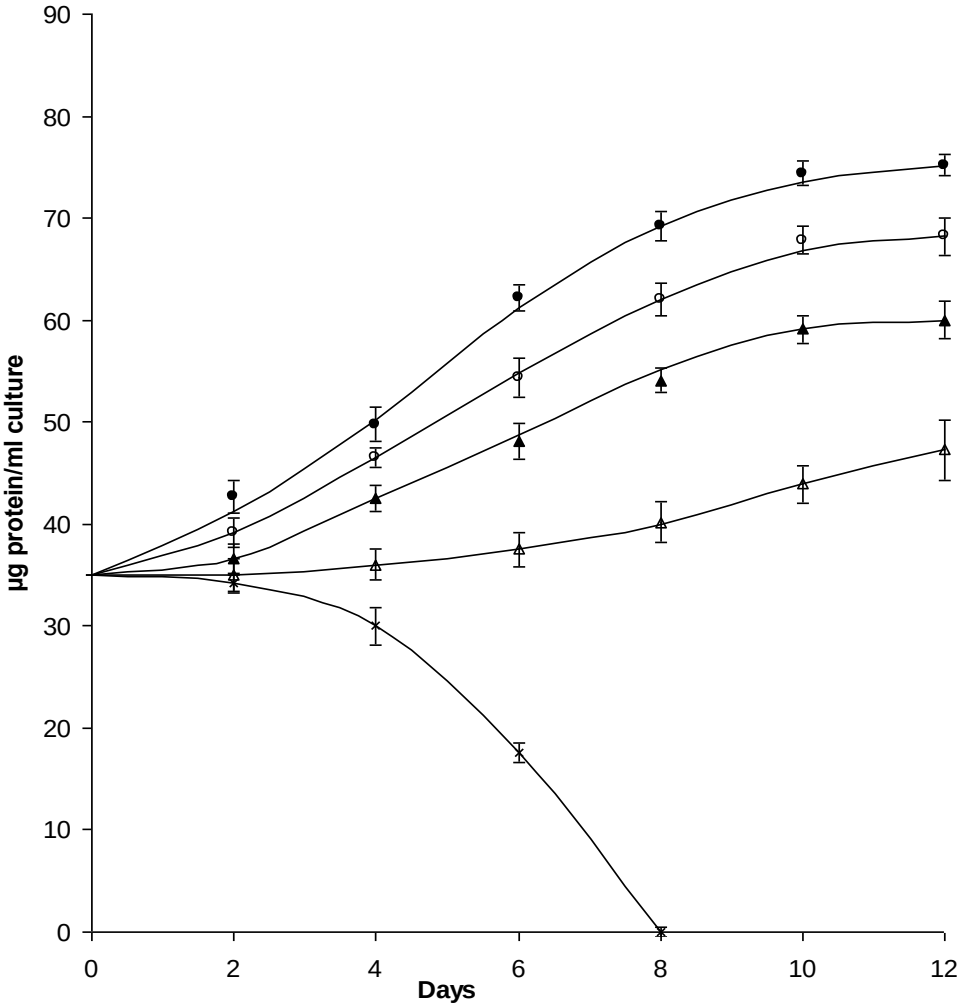

Fig. 1b: Protein content of *Anabaena variabilis* cells exposed to graded concentrations of Hg^{2+} during 2-12 days incubations: Hg^{2+}-less control (●), 0.05 µM (O), 0.10 µM (▲), 0.20 µM (Δ) and 0.5 µM (x). Values are mean ±3 SE.

An extent of intracellular build-up of Hg^{2+} in the concentration range of 0.05-1.0 µM during its 0-60 min exposure was studied in *N. muscorum* (Fig.2a) and *A. variabilis* (Fig. 2b) both. The two factor ANOVA showed that Hg uptake was found in a time dependent ($F_{time\ 7,\ 35} = 42.29$, $p < 0.025$) as well as concentration dependent fashion ($F_{Hg^{2+}\ 5,\ 35} = 31.39$, $p < 0.025$). However, intracellular Hg^{2+} build-up was found little faster in *N. muscorum* than *A. variabilis* ($F_{Hg^{2+}\ 4,\ 28} = 31.39$, $p < 0.005$). At 0.05 µM exogenous Hg^{2+}, the actual intracellular build-up was 19.5 µmolHg^{2+} /mg protein within 20 min of exposure, which was raised to 23.1 µmolHg^{2+}/mg protein after 60 min. A significant correlation was found in the exogenous concentration and intracellular build up of Hg^{2+} in both the organisms ($r = 0.877$, $p < 0.025$). However, the pattern appeared to be more tolerant in case of *N. muscorum* than *A. variabilis*. The two organisms revealed similar faster rate of Hg^{2+} accumulation up to 30 min exposure, beyond which it appeared diminished at higher levels (0.2 to 1.0 µM Hg^{2+}). The maximum of Hg accumulation was at 1.0 µM for initial 30 min for both the organisms, after which no change was observed even after increasing the incubation period. In this way, 1.0 µM Hg^{2+} exposure to *N. muscorum* appeared to be the saturating concentration, while it was 0.5 µM for *A. variabilis*.

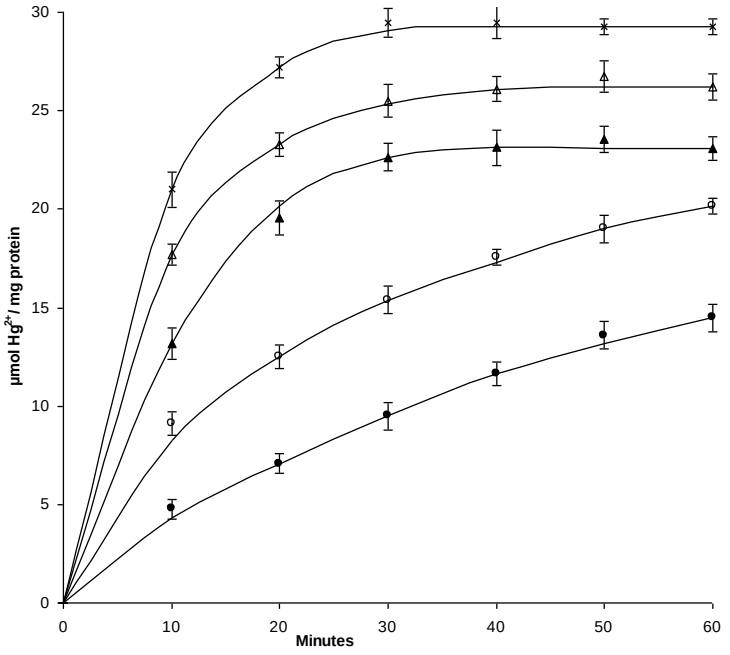

Fig.2a Extent of total intracellular build-up of Hg^{2+} in *Nostoc muscorum* cells during 0-60 min incubations at graded concentrations of $HgCl_2$: 0.05 µM (●), 0.1 µM (O), 0.2 µM (▲), 0.5 µM (Δ) and 1.0 µM (x). Values are mean ±3 SE.

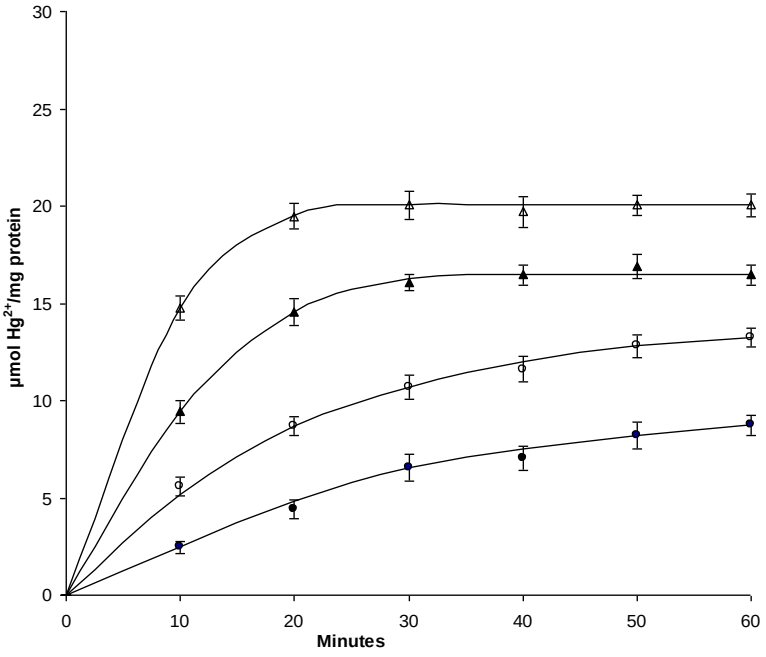

Fig. 2b: Extent of total intracellular build-up of Hg^{2+} in *Anabaena variabilis* cells during 0-60 min incubations at graded concentrations of $HgCl_2$: 0.05 µM (●), 0.1 µM (O), 0.2 µM (▲) and 0.5 µM (Δ). Values are mean ±3 SE.

The two factor ANOVA showed that *N. muscorum* showed marked decrease in its phycocyanin content (from 70.9 to 30.0 µg/ml culture) with increasing concentration of Hg^{2+} (Hg^{2+}-less culture to 0.5 µM) as shown in Fig. 3a ($F_{Hg^{2+}\ 6,\ 42}$ = 12.87, $p< 0.01$, r= 0.94). An over concentration of 1.0 µM was lethal to the organism leading to total loss of phycocyanin just in 6 h of exposure ($F_{time7,\ 42}$ = 5.95, $p< 0.01$). Similar toxicological effect of Hg^{2+} was seen in case of *A. variabilis* (Fig. 3b; r= 0.985, $p< 0.05$) showing reduced level of intracellular phycocyanin (70.0 to 19.7 µg/ml culture) with increased toxicity of Hg^{2+} (from Hg^{2+}-less culture to 0.2 µM; $F_{Hg^{2+}\ 5,\ 35}$ = 12.4, $p< 0.05$). Further exposure to 0.5 µM did not show any positive sign of growth and killed the organism in just a few hours.

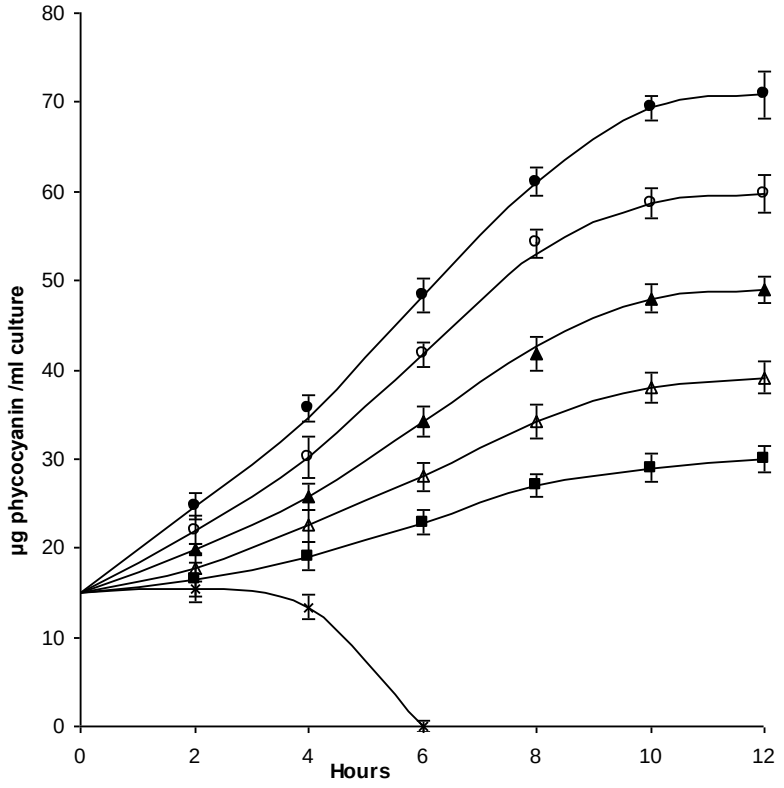

Fig. 3a: Intracellular levels of phycocyanin in *Nostoc muscorum* at different concentrations of Hg^{2+} during 2-12 hours incubations: Hg^{2+}-less control (●), 0.05 µM (O), 0.10 µM (▲), 0.20 µM (Δ), 0.5 µM (■) and 1.0 µM (x). Values are mean ±3 SE.

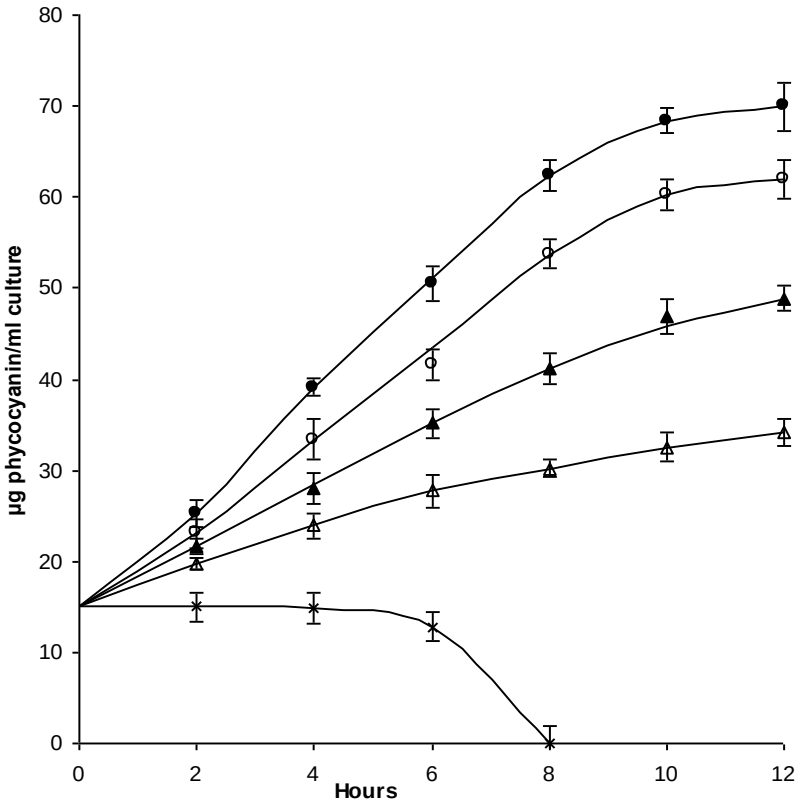

Fig. 3b: Intracellular levels of phycocyanin in *Anabaena variabilis* at different concentrations of Hg^{2+} during 2-12 hours incubations: Hg^{2+}-less control (●), 0.05 µM (O), 0.10 µM (▲), 0.20 µM (Δ) and 0.5 µM (x). Values are mean ±3 SE.

The extracellular extrusion of phycocyanin was compared in the two diazotrophic cyanobacteria, *N. muscorum* and *A. variabilis* (Fig 4a & 4b). The minimum concentration of 0.05 µM Hg^{2+} resulted in 14.0 µg (*N. muscorum*) and 18.2 µg (*A. variabilis*) phycocyanin leaked per ml of medium just in 2 h of exposure. *N. muscorum* showed a maximum leakage of 50.8 µg phycocyanin on exposing to 1.0 µM Hg^{2+} ($F_{Hg^{2+}\ 5,\ 35} = 26.41$, $p < 0.025$) whereas *A. variabilis* showed maximum leakage of 50.4 µg phycocyanin on exposing to 0.5 µM after 12 h of incubation ($F_{Hg^{2+}\ 4,26} = 25.53$, $p < 0.025$). On comparison, it was concluded that *A. variabilis* is more sensitive to Hg^{2+} and showed higher phycocyanin leakage (Fig. 4b; $r = 0.94$, $p < 0.025$) than *N. muscorum* (Fig. 4a; $r = 0.965$, $p < 0.025$). On increasing toxicity of metal and exposure time, the intracellular phycocyanin began to leak out and accumulate into the ambient medium, which may further be purified. The stability of extruded phycocyanin (if kept in a refrigerator) ranged for several days depending upon the experimental organism.

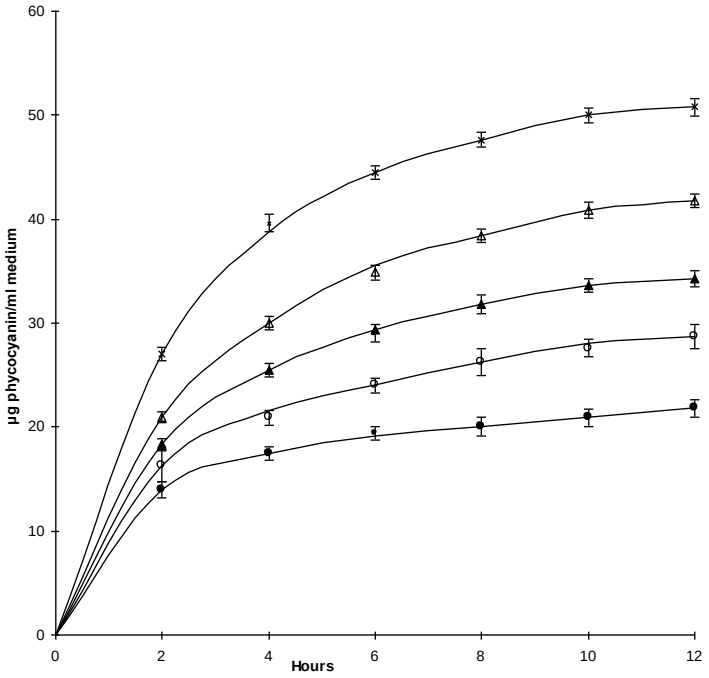

Fig. 4a: A comparison of the phycocyanin extruded out in the ambient medium from *Nostoc muscorum* treated with Hg^{2+} during 2-12 days of incubation: 0.05 µM (●), 0.1 µM (O), 0.2 µM (▲), 0.5 µM (Δ) and 1.0 µM (x). Values are mean ±3 SE.

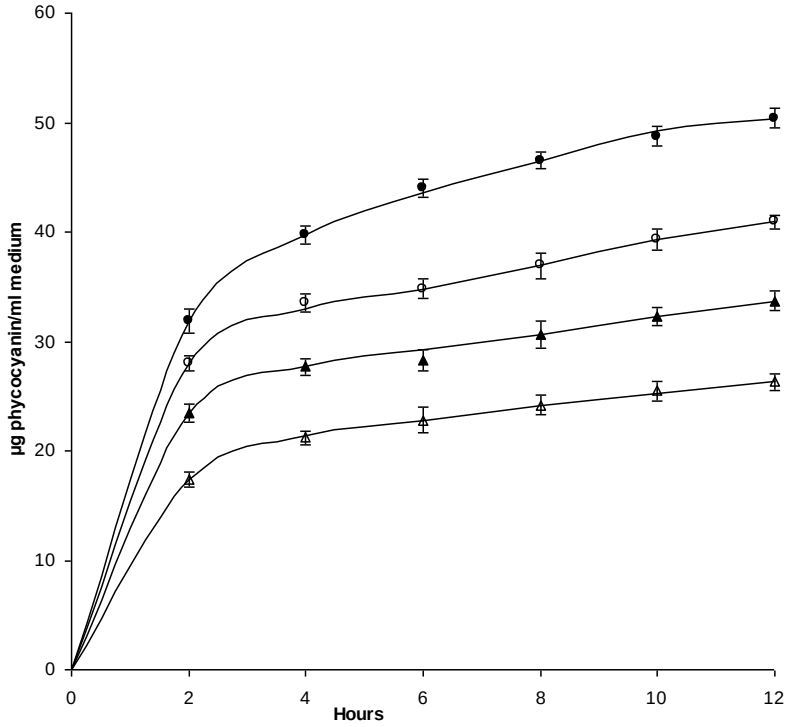

Fig. 4b: A comparison of the phycocyanin extruded out in the ambient medium from *Anabaena variabilis* treated with Hg^{2+} during 2-12 days of incubation: 0.05 µM (Δ), 0.1 µM (▲), 0.2 µM (O) and 0.5 µM (●). Values are mean ±3 SE.

To verify the cyanobacterial ability to withstand the toxicological effects of Hg^{2+}, the intracellular levels of phycocyanin of *N. muscorum* and *A. variabilis* (both treated with 0.2 µM Hg^{2+}) were compared in the light of a natural chelator (spent medium) and a synthetic chelator (EDTA). As shown in Fig. 5a & 5b, the algal cells retained their phycocyanin for a longer extent of time in the presence of chelators. After 2 h of incubation, the intracellular level of phycocyanin was 19.7 µg (in Hg^{2+}-treated cultures) which was raised to 24.8 µg (in Hg^{2+}-treated cultures supplemented with EDTA) which was further raised to 31.5 µg (in Hg^{2+}-treated cultures supplemented with spent medium) in *A. variabilis* (Fig. 5b; $F_{\text{treatment } 3,\ 21} = 28.89$; $p < 0.025$) which was raised to a maximum of 46.5 µg (in Hg^{2+}-treated cultures supplemented with spent medium) after 12 h of exposure. Similarly, *N. muscorum*, after 2 h of incubation, showed an intracellular phycocyanin content of 17.8 µg (in Hg^{2+}-treated cultures), which was raised to 22.4 µg (in Hg^{2+}-treated cultures supplemented with EDTA) and 28.1 µg (in Hg^{2+}-treated cultures supplemented with spent medium) (Fig. 5a; $F_{\text{treatment } 3,\ 21} = 17.82$; $p<0.025$). On comparing the individual effect of both the chelators, it may be concluded that the diluted spent medium was more effective than EDTA in controlling the phycocyanin leakage in the cyanobacterial cultures.

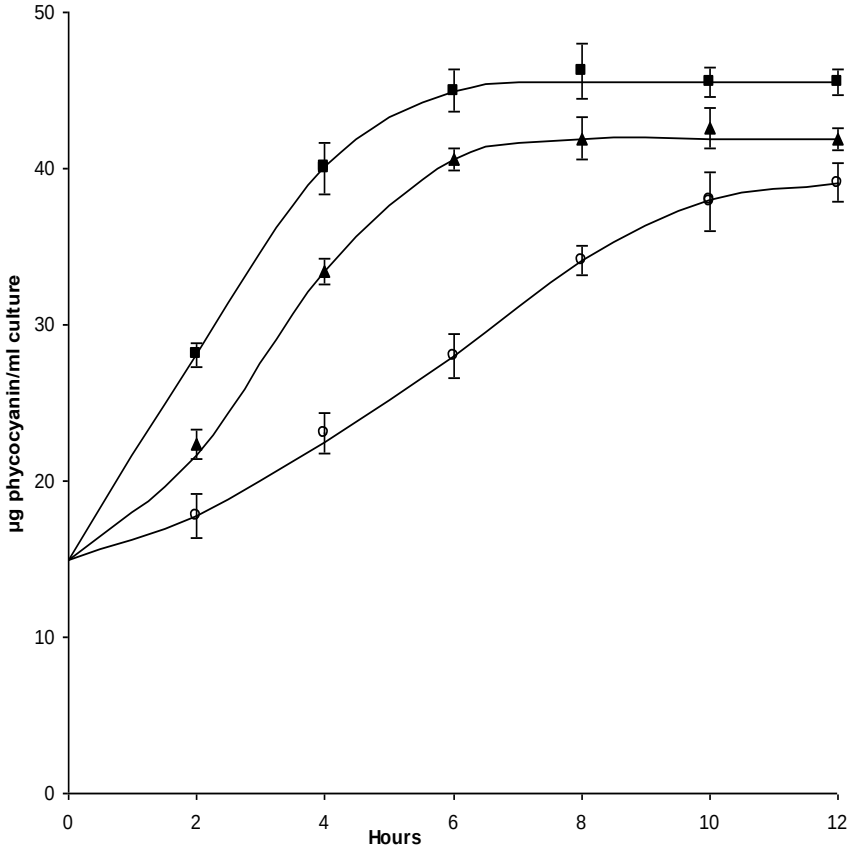

Fig. 5a: Effect of natural and synthetic chelators on phycocyanin content of *Nostoc muscorum* cells treated with 2.0 µM Hg^{2+} during 2-12 hour incubations: Hg^{2+} alone (O), Hg^{2+} + EDTA (▲) and Hg^{2+} + Spent medium (■); values are mean ±3 SE.

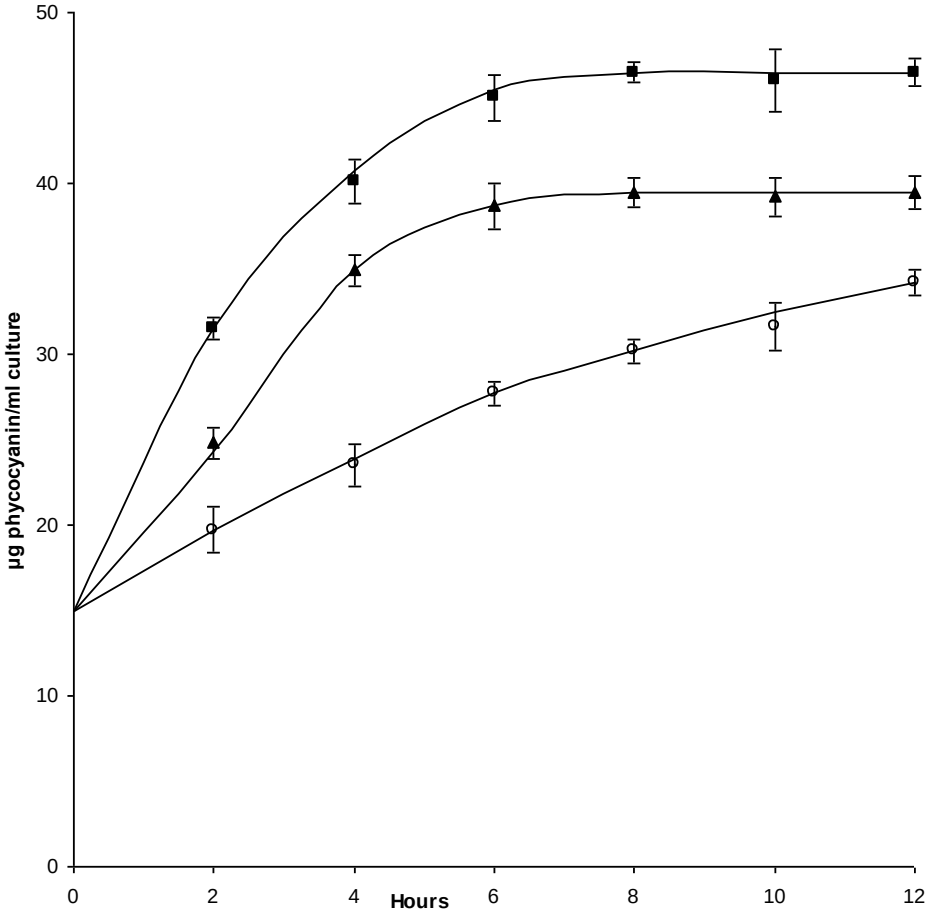

Fig. 5b: Effect of natural and synthetic chelators on phycocyanin content of *Anabaena variabilis* cells treated with 1.0 µM Hg^{2+} during 2-12 hour incubations: Hg^{2+} alone (O), Hg^{2+} + EDTA (▲) and Hg^{2+} + Spent medium (■); values are mean ±3 SE.

Discussion

The uptake and toxicity of heavy metals by the target cells of microorganisms, including cyanobacteria, is regulated by a complex mechanism conforming to physiological status of cells, prevailing environmental conditions and chemical forms of the metal present (Drbal *et al*. 1985, Pettersson *et al*. 1985); once taken in, such cations adversely affect the cyanobacterial physiology and biochemistry (Asthana *et al*. 1990, 1992, Pandey *et al*. 1992). The general growth of the cyanobacterial cells could be negatively correlated with mecury uptake. Here the data are discussed in the light of the physiological efficiency. The viability of the cyanobacterial cells was of prime concern during exposure to the elevated Hg^{2+} levels (Fig. 1a & 1b). The 2-way ANOVA (analysis of variance) suggested that the leakages were more respondent to exposure time than the Hg^{2+} [Figs. 2(a,b) & 3(a,b)]. Leaked phycocyanin was not detected in the ambient medium even after 4 h exposure to the highest tested Hg^{2+} concentration (1.0 µM), and its highest amount was obtained after 24 h exposure to 1.0 µM Hg^{2+}. Such observations suggest that the association of phycocyanin with lamellar proteins was so strong that it took a minimum of 4 h for Hg^{2+} ions to disrupt its association as well as assembly into phycobilisomes, and to make it free to move. That's why; even if the cell membrane was disrupted already, leaked phycocyanin could not be detected within first 4 h of photoincubation.

The rapidness in the uptake of Hg^{2+} ions by *N. calcicola* during the first 30 min exposure to all Hg^{2+} concentrations indicates towards the energy-dependent nature of the event, as reported for Cu^{2+} uptake by some cyanobacterium (Verma & Singh, 1990) and Cd^{2+} uptake by *Anacystis nidulans* (Singh & Yadava, 1985). The

consistent influx of Hg^{2+} ions during this period enhanced the intracellular Hg^{2+} build-up within first 30 min in a concentration dependent manner. It also permeabilized the cell membrane and enhanced membrane potential, as observed in *Cylindrospermum* (Singh *et al*. 1989). As a consequence of this, the cell membrane was disrupted and the intracellularly accumulated Hg^{2+} ions were also released into the ambient medium during >30 min exposures, and their follow-up uptake took place passively. This active plus passive uptake of Hg^{2+} was collectively responsible for the observed leakages. A corollary of these findings is that the extent of intracellular Hg^{2+} build-up achieved within first 30 min incubation is the ultimate crucial moiety responsible for making it unviable to retain the free phycocyanin and accumulated Hg^{2+} ions intracellularly.

There are reports that organic mercury is highly toxic over its inorganic counterpart (Röderer, 1983, Singh & Singh, 1992a). Also the former (organic) is accumulated very rapidly by algae like *Chlorella*, *Scendesmus* and *Microcystis*, and transferred to inorganic mercury following 48 h of uptake (Havlik *et al*. 1979). 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 & Yadava, 1985), copper (Singh, 1985), aluminium (Pettersson *et al*. 1986) nickel (Campbell & Smith, 1986) and zinc (McHardy & George, 1990).

The observed reductions in the effectiveness of Hg^{2+} ions to induce the leakage of phycocyanin and electrolytes from *N. calcicola* cells in the presence of EDTA (Figs. 5a, b), have been attributed to the lesser bioavailability of Hg^{2+} ions due to the formation of less mobile Hg^{2+}-EDTA-complex, imparting less chances for the Hg^{2+} ions to get adsorbed on the cell surface for its follow-up uptake. This interpretation is based on the fact that the chelated toxic metal ions become less toxic than the corresponding free metal ions as a consequence of reduced mobility of metal-chelate-complex, resulted due to metal-chelate interactions (Hart, 1981, Rai & Raizada, 1985), which in turn is governed by the bonding preferences of metal ion (the donor preference) and the chelating agent (acceptor preference; Jones, 1984). It has also been reported that the metal uptake capacities of certain algae are much higher than activated carbon, natural zeolite and synthetic ion exchange resins. The work of Volesky (Volesky, 1992, 2001, Volesky & Holan, 1995) has demonstrated the use of marine algae to take metal ions. Algae, a renewable natural biomass exhibit different affinities towards different metals and therefore, are a very important candidate to be employed as a biosorbent material. The use of both marine and river algae for adsorption and elution of gold, silver and cobalt has been reported.

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