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Toxicity of chromium and tin to *Anabaena doliolum*

Interaction with bivalent cations

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Summary. The toxicity of chromium and tin on growth, photosynthetic carbon-fixation, oxygen evolution, heterocyst differentiation and nitrogenase activity of *Anabaena doliolum* and its interaction with bivalent cations has been studied. Some interacting cations, viz. Ca^{2+}, Mg^{2+} and Mn^{2+}, substantially antagonised the toxic effects of chromium and tin with reference to growth, heterocyst differentiation and nitrogenase activity in the following hierarchal sequence:
$Ca^{2+} > Mg^{2+} > Mn^{2+}$. However, the sequence of hierarchy was $Mg^{2+} > Ca^{2+} > Mn^{2+}$ for carbon fixation and $Mn^{2+} > Mg^{2+} > Ca^{2+}$ for photosynthetic oxygen evolution. Synergistically inhibitory patterns were noticed for all the parameters, viz. growth, $^{14}CO_2$ uptake, oxygen evolution, heterocyst differentiation and nitrogenase activity of *A. doliolum* when Ni^{2+}, Co^{2+} and Zn^{2+} were combined with the test metals in the growth medium. These cations followed the following sequence of synergistic inhibition: $Ni^{2+} > Co^{2+} > Zn^{2+}$. Among all the interacting cations, Ca^{2+}, Mg^{2+} and Mn^{2+} exhibited antagonistic effects which relieved the test cyanobacterium from metal toxicity. In contrast to this, Ni^{2+}, CO^{2+} and Zn^{2+} showed synergistic inhibition which potentiating the toxicity of test metals in the N_2-fixing cyanobacterium *A. doliolum*. It is evident from the present study that bivalent cations, viz. Ca^{2+}, Mg^{2+}, Mn^{2+}, Ni^{2+}, Co^{2+} and Zn^{2+}, may appreciably regulate the toxicity of heavy metals in N_2-fixing cyanobacteria if present in aquatic media.

Ke words: Heavy metal - Bivalent cations - Nitrogenase - $^{14}CO_2$ uptake - Synergistic - Antagonistic

Introduction

The toxicity of an environmental contaminant to the microbiota is influenced, in part, by the physico-chemical characteristics of the recipient environment. A large number of factors determine the toxicity: metal speciation, mobility, bioavailability, redox potential, pH, solubility, exchangeability of metals, presence of other cations, complexing inorganic and organic ligands and algal population density. Notwithstanding this, several inorganic substances, metal ligands, chelators and functional groups also determine the toxicity of metals in aquatic as well as laboratory microsoms. The metal-interaction experiments in environmental studies are vital because: (a) the algal nutrient media contain essential macro- and micro-nutrients, and (b) the natural environment never contains only one metal. Several investigations have revealed the co-existence of heavy metals in natural habitats (Whitton 1970; Rana and Kumar 1974; Henriksen and Wright 1978; Rai et al. 1981). Antagonism results from competition among cations for the common uptake sites on the target, while synergism involves the adsorption of both the cations on a common cell surface. The adsorption of one metal may increase the cell permeability to the second metal (Babich and Stotzky 1980). The effect of metal combinations on eucaryotic algae has been extensively studied (Whitton 1970; Rai et al. 1981; Stokes 1983; Starodub et al. 1987); however, only a few reports are available on cyanobacteria (Stratton and Corke 1979; Singh and Yadav 1984; Rai and Raizada 1985, 1987; Rai and Dubey 1989).

Since Ca^{2+}, Mg^{2+} and Mn^{2+} are required for various physiological and biochemical processes of living organisms, including algae, they are known to interact with toxic metals during uptake. Notwithstanding this, there are certain metal cations, viz. Ni^{2+}, Co^{2+}, Zn^{2+}, Cu^{2+} etc., which are required in trace amounts for various biochemical processes of mciroorganisms although they are toxic at higher concentrations. Since the aquatic environment possesses a large array of toxic and non-toxic cations, interaction of these cations with different processes of microorganisms including cyanobacteria is likely to be intricate and interesting from an ecological view point. Toxic cations, viz. Ni^{2+}, Hg^{2+}, Cd^{2+}, Zn^{2+} and Cu^{2+}, possibly interact with each other either in synergistic or antagonistic fashion de-

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Table 1. Effect of test metals on photosynthetic oxygen evolution, heterocyst differentiation and nitrogenase activity of *A. doliolum*: interaction with bivalent cations

Supplementation	Concentration (μg/ml)	O_2 evolved (ppm/10 min)	Heterocyst frequency (%)	Nitrogenase activity (nmol C_2H_4 μg Chl a^{-1} h^{-1})
None (control)	—	2.4	5.5	5.2±0.1
Cr	40	0.7	3.5	1.2±0.1
Sn	50	0.2	3.2	1.6±0.1
Cr+Mn	100	2.0	3.6	1.3±0.1
Cr+Mg	100	1.8	3.7	1.8±0.1
Cr+Ca	150	1.6	3.8	2.0±0.1
Cr+Ni	0.5	0.4	6.8	0.8±0.1
Cr+Co	5	0.5	6.4	0.9±0.1
Cr+Zn	2	0.6	6.0	1.0±0.1
SN+Mn	100	2.1	3.3	2.0±0.1
Sn+Mg	100	1.9	3.5	1.9±0.1
Sn+Ca	150	1.7	4.0	2.0±0.1
Sn+Ni	0.5	0.05	6.2	1.0±0.1
Sn+Co	5	0.10	6.0	1.3±0.1
Sn+Zn	2	0.15	5.7	1.5±0.1

The concentration of $CrCl_2$ was always 40 μg ml^{-1}, that of $SnCl_2$ always 50 μg ml^{-1}, the concentrations listed are those of the second chloride. Heterocyst frequency and nitrogenase activity were determined after 72 h of incubation

pending on the valency state, speciation, concentration and sequence of the cations used. It is well known that metal cations, viz. Mn^{2+}, Mg^{2+}, Ca^{2+}, Ni^{2+}, Co^{2+}, Zn^{2+}, Cu^{2+} etc., play an important role in the activation/biosynthesis of various macromolecules and enzymes of microorganisms but the interaction of these cations with chromium and tin has not been studied so far despite their significant role in the transport of nutrients, nitrogen-fixation, nitrate reduction, ammonia assimilation, chlorophyll biosynthesis and maintenance of integrity of cell envelopes.

Taking recourse to such considerations, it was proposed to study the interactive behaviour of bivalent cations, viz. Ca^{2+}, Mg^{2+}, Mn^{2+}, Ni^{2+}, Co^{2+} and Zn^{2+}, on the toxicity of chromium and tin in the N_2-fixing cyanobacterium *Anabaena doliolum* with reference to growth, carbon fication, O_2 evolution, heterocyst differentiation and nitrogenase activity.

Materials and methods

Organism and growth conditions. The test cyanobacterium *Anabaena doliolum* was grown axenically in a modified medium of Allen and Arnon (1955) buffered with Tris/HCl pH 7.5. The cultures were illuminated with fluorescent light of 2500 Lx intensity in a 14-h light and 10-h dark cycle at 25±1° C. These incubation conditions were standard for all growth, carbon fixation, O_2 evolution and nitrogenase activity experiments. Stock solutions of test metals (CrO_3, $SnCl_2$) and interactive metals ($CaCl_2$, $MgCl_2$, $MnCl_2$, $NiCl_2$, $CoCl_2$ and $ZnCl_2$) were prepared separately and sterilized by passing through Millipore membrane filters (0.45 μm) before adding them to algal cultures. The percentage survival was scored as given by Dubey et al. (1986). Sublethal concentrations of chromium chloride (40 μg ml^{-1}) and tin chloride (50 μg ml^{-1}) were used in metal interaction experiments. Chlorides of interacting cations, viz. $CaCl_2$, $MnCl_2$, $MgCl_2$, $CoCl_2$, $NiCl_2$ and $ZnCl_2$, were used at their non-inhibitory concentrations (see Table 1). Growth of the alga was recorded by measuring absorbance at 663 nm in a Bausch and Lomb Spectronic-20 spectrocolorimeter and chlorophyll *a* content as by Mackinney (1941).

Carbon fixation measurement. Carbon fixation by *A. doliolum* was measured following uptake of $^{14}CO_2$ from $NaH^{14}CO_3$ (50 μCi), as described by Rai and Raizada (1985); 0.2 ml dilute $NaH^{14}CO_3$ was added to 1.0 ml algal suspension and incubated for known time periods. The reaction was terminated by adding 0.2 ml 50% acetic acid. The resulting suspension, after bubbling with air for 4 min, was spiked with 5 ml scintillation cocktail. A Beckman liquid scintillation counter LS-7000 was employed to measure the radioactivity (as counts/minute).

Measurement of oxygen evolution. Photosynthetic O_2 evolution was measured using an oxyen electrode. Approximately 15 ml algal suspension ($A_{663}=0.2$) was taken in a cuvette. The temperature of the cuvette was controlled by a thermocirculator. Oxyen evolution was recorded (as ppm/min) with an O_2 analyser (Biochem. 76T model).

Heterocyst frequency. Heterocyst frequency was determined by counting the number of heterocysts/100 vegetative cells in at least 25–30 filaments of approximately equal length.

Nitrogenase activity measurement. Nitrogenase activity was measured by acetylene reduction (Stewart et al. 1968). Activity was measured in triplicate in serum vials of about 7.5-ml capacity. The acetylene concentration was kept at 10% (by vol.) and 2 ml algal suspension was injected into each vial. Reactions were terminated following injection of 0.8 ml 15% (mass/vol.) trichloroacetic acid. Ethylene formed was measured in a CIC gas chromatograph (Baroda, India) equipped with a Porapak-R column and a hydrogen flame ionization detector.

Chemicals. $NaH^{14}CO_3$ was obtained from BARC, Bombay, other chemicals from BDH, India.

Results

Effect of chromium and tin on growth: interaction with metal cations

It is evident from Fig. 1 that the final growth yield of *A. doliolum* exposed to the LD_{50} of chromium and tin was 51.2% and 55.8% of the control, respectively. Substantial recovery (74% in chromium and 79% in tin-spiked cultures) in the final yield was recorded following supplementation by calcium. Similar recovery patterns in growth were noticed for chromium (65%) and tin (72%) after supplementation of 100 μg ml^{-1} $MgCl_2$. The final yield was however, found to be 58.1% and 65.1% of control in cultures treated with bimetallic combinations of $Cr^{2+}+Mn^{2+}$ and $Sn^{2+}+Mn^{2+}$, respectively (Fig. 1). Nickel, cobalt and zinc supplementation with sublethal concentrations of chromium produced significant inhibitory patterns of interaction giving final yields of 23.3%, 30.2% and 37.2% of control for cultures grown in metal combinations $Cr^{2+}+Ni^{2+}$, $Cr^{2+}+Co^{2+}$ and $Cr^{2+}+Zn^{2+}$, respectively. Almost similar interaction patterns were observed when these cations were combined with the LD_{50} of tin. The final yield recorded was 32.6% for $Sn^{2+}+Ni^{2+}$, 37.2% for $Sn^{2+}+Co^{2+}$ and 48.8% for $Sn^{2+}+Zn^{2+}$ combinations (Fig. 2). It is interesting to note that nickel, cobalt and zinc were non-inhibitory to test algae at the concentrations used individually but showed synergistic inhibition of growth when mixed with the LD_{50} of chromium and tin. Nickel addition proved to be more toxic to *A. doliolum* than cobalt and zinc.

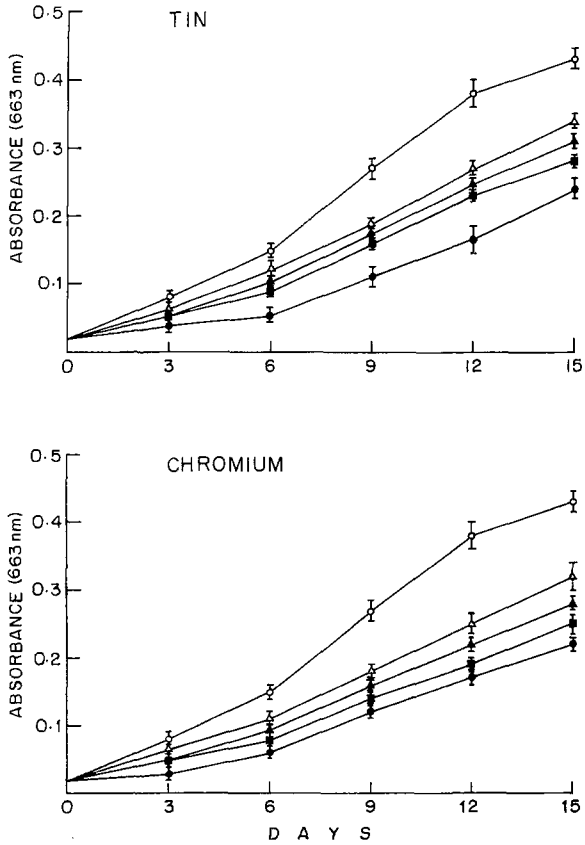

Fig. 1. Effect of sublethal concentrations of **(A)** chromium and **(B)** tin on growth behaviour of *A. doliolum:* interaction with metal cations. **(A)** Control (○); Cr^{2+} (●); $Cr^{2+}+Ca^{2+}$ (△); $Cr^{2+}+Mg^{2+}$ (▲); $Cr^{2+}+Mn^{2+}$ (■). **(B)** Control (○); Sn^{2+} (●); $Sn^{2+}+Ca^{2+}$ (△); $Sn^{2+}+Mg^{2+}$ (▲); $Sn^{2+}+Mn^{2+}$ (■)

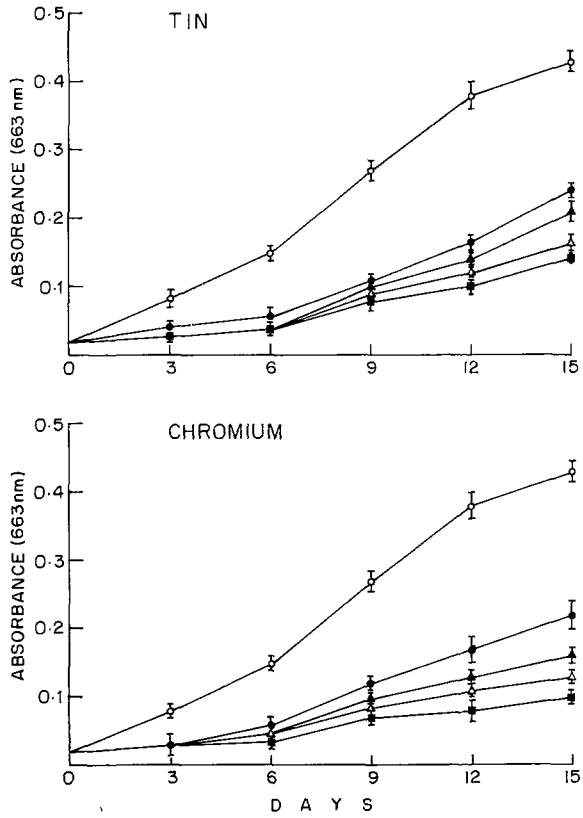

Fig. 2. Effect of sublethal concentrations of **(A)** chromium and **(B)** tin on growth of *Anabaena doliolum*:interaction with metal cations. **(A)** Control (○); Cr^{2} (●); $Cr^{2+}+Zn^{2+}$ (▲); $Cr^{2+}+Co^{2+}$ (△); $Cr^{2+}+Ni^{2+}$ (■). **(B)** Control (○); Sn^{2+} (●); $Sn^{2+}+Zn^{2+}$ (▲); $Sn^{2+}+Co^{2+}$ (△); $Sn^{2+}+Ni^{2+}$ (■)

Effect of chromium and tin on carbon fixation: interaction with metal cations

The influence of sublethal concentrations of test metals in combination with metal cations on carbon fixation of *A. doliolum* is given in Figs. 3 and 4. Restoration of carbon fixation by Ca^{2+}, Mg^{2+} and Mn^{2+} was 22%, 31% and 14% respectively in chromium-spiked cultures. $^{14}CO_2$ uptake of test alga as influenced by bimetallic combinations of $Sn^{2+}+Ca^{2+}$, $Sn^{2+}+Mg^{2+}$, $Sn^{2+}+Mn^{2+}$ was 83.3%, 88.8% and 77.7% of control, respectively, thereby showing protection of carbon fixation against metal toxicity (Fig. 3). However, combination of chromium with nickel, cobalt and zinc resulted in the synergistic inhibition of $^{14}CO_2$ uptake as the recorded uptake was 30.5%, 36.1% and 41.6%, respectively. Likewise interaction of Sn^{2+} with Ni^{2+}, Co^{2+} and Zn^{2+} also showed synergistic inhibition of $^{14}CO_2$ uptake. It is interesting to note that interaction of nickel with test

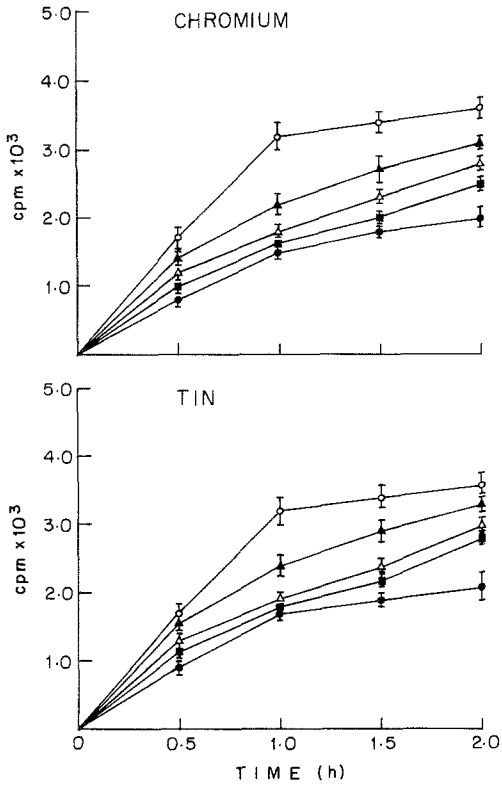

Fig. 3. $^{14}CO_2$ uptake of *A. doliolum* at sublethal concentrations of **(A)** chromium and **(B)** tin: interaction with metal cations. **(A)** Control (○); Cr^{2+} (●); $Cr^{2+}+Ca^{2+}$ (△); $Cr^{2+}+Mg^{2+}$ (▲); $Cr^{2+}+Mn^{2+}$ (■). **(B)** Control (○); Sn^{2+} (●); $Sn^{2+}+Ca^{2+}$ (△); $Sn^{2+}+Mg^{2+}$ (▲); $Sn^{2+}+Mn^{2+}$ (■)

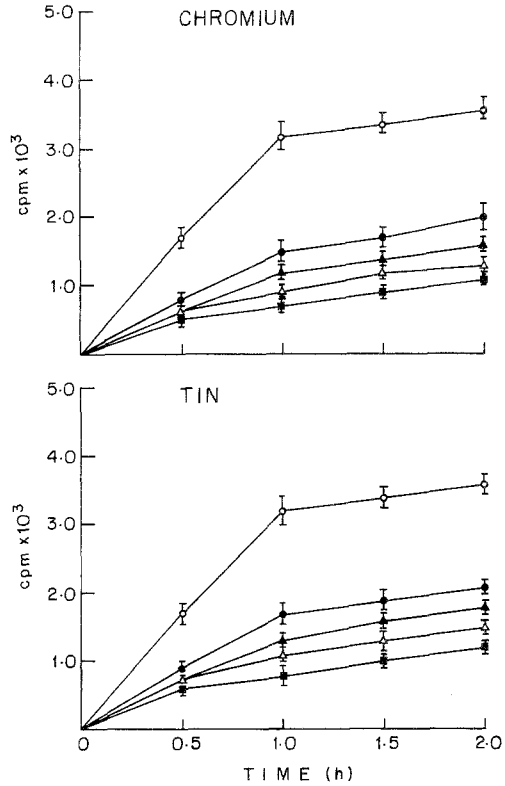

Fig. 4. $^{14}CO_2$ uptake of *A. doliolum* at sublethal concentrations of **(A)** chromium and **(B)** tin: interaction with metal cations. **(A)** Control (○); Cr^{2+} (●); $Cr^{2+}+Zn^{2+}$ (▲); $Cr^{2+}+Co^{2+}$ (△); $Cr^{2+}+Ni^{2+}$ (■). **(B)** Control (○); Sn^{2+} (●); $Sn^{2+}+Zn^{2+}$ (▲); $Sn^{2+}+Co^{2+}$ (△); $Sn^{2+}+Ni^{2+}$ (■)

metals depicted the most pronounced synergistic inhibition of carbon fixation (Fig. 4).

Effect of test metals on oxygen evolution: interaction with metal cations

Photosynthetic oxygen evolution by *A. doliolum* in metal-spiked cultures is shown in Table 1. It is interesting to note that Mn^{2+} and Mg^{2+} stimulated O_2 evolution while Ca^{2+} was slightly inhibitory. Supplementation with Ni^{2+}, Co^{2+} and Zn^{2+} separately inhibited the O_2 evolution differentially. Approximately 2.5% inhibition of O_2 evolution was observed for nickel-supplemented cultures and this was followed by Zn^{2+} (20.8%) and Co^{2+} (21%). Cultures treated with sublethal concentrations of Cr^{2+} and Sn^{2+} exhibited 29.0% and 8.0% O_2 evolution of the control, respectively. It is evident from Table 1 that oxygen evolution was restored in cultures treated with Cr^{2+} and Sn^{2+} and amended with Ca^{2+}, Mg^{2+} and Mn^{2+}. The restoration pattern of O_2 evolution by bivalent cations appears to be in the following sequence: $Mn^{2+}>Mg^{2+}>Ca^{2+}$. Approximately 54.0% restoration of O_2 evolution was recorded in the presence of Mn^{2+}. Bimetallic combinations, viz. $Cr^{2+}+Ni^{2+}$, $Cr^{2+}+Co^{2+}$, $Cr^{2+}+Zn^{2+}$, $Sn^{2+}+Ni^{2+}$, $Sn^{2+}+Co^{2+}$ and $Sn^{2+}+Zn^{2+}$, exibited synergistic inhibition of photosynthetic oxygen evolution.

Effect of chromium and tin on nitrogenase activity and heterocyst differentiation: interaction with metal cations

Approximately 23% and 31% inhibition of nitrogenase activity was noticed at sublethal concentrations of chromium and tin, respectively (Table 1). It is also clear from Table 1 that calcium, magnesium and manganese showed antagonistic interaction with test metals. Their protective efficiency may be expressed in the following hierarchial sequence: $Ca^{2+}>Mg^{2+}>Mn^{2+}$. Calcium restored the nitrogenase activity of *A. doliolum* comparatively better than magnesium and manganese in metal-spiked conditions. The levels of restoration of nitrogenase activity by Ca^{2+}, Mg^{2+} and Mn^{2+} were 15.5%, 11.6% and 2.0% for chromium-supplemented cultures and 11.5%, 7.7% and 5.7% for tin-supplemented test algae. However nickel, cobalt and zinc inhibited the nitrogenase activity synergistically in the following hierarchial sequence: $Ni^{2+}>Co^{2+}>Zn^{2+}$, when supplemented with test metals. A slight induction in heterocyst frequency (from 5.5% to 5.8%) was noticed by calcium supplementation while Mg^{2+} and Mn^{2+} proved ineffective for heterocyst induction. Maximum induction of heterocyst frequency was observed for nickel (from 5.5% to 8.0%), followed by cobalt (5.5% to 7.7%) and zinc (from 5.5% to 6.0%). Combinations of chromium and tin with Ca^{2+}, Mg^{2+} and Mn^{2+} caused restoration of heterocyst differentiation in the following se-

quence: $Ca^{2+} > Mg^{2+} > Mn^{2+}$. Stimulation of heterocyst differentiation, on the one hand, and inhibition of nitrogenase activity, on the other, critically suggested a dual role for nickel, cobalt and zinc in *A. doliolum*.

Discussion

Heavy metal toxicological studies with reference to algae have assumed considerable attention with the result that a large number of research papers and reviews dealing with various aspects of metal toxicity have been published (Rai et al. 1981; Stokes 1983; Raizada and Rai 1985; Rai and Raizada 1987). Reduction in growth and content of macromolecules of test alga at increasing concentrations of test metals confirms their toxic characteristics. Toxicity may be manifested either by disruption of the integrity of cell membranes or by inhibition of photosynthetic pigments and key enzymes of nitrogen metabolism, viz. nitrogenase, nitrate reductase and glutamine synthetase of cyanobacteria (Dubey et al. 1986; Dubey and Rai 1987; Rai and Dubey 1989). Heavy metal toxicity is regulated by such physico-chemical factors as pH, redox potential, salinity, alkalinity, availability of nutrients, population density, extracellular metabolites, chelating agents and organic acids. Micronutrients may influence the bio-availability and uptake of heavy metals to the microbiota as the aquatic environment is comprised of several toxic and non-toxic metallic ions and their inorganic/organic complexes which regulate the bioavailability of metals to the microorganisms exposed to these metals (Steemann-Nielsen et al. 1969). The exact mechanism of metal interaction in the natural environment has not yet been clearly explored as the toxicity of heavy metals is governed by several factors acting together at one time. The non-toxic cations present in the aquatic environs, viz. Ca^{2+}, Mg^{2+}, Mn^{2+}, Na^{+}, K^{+} etc., exert protective effects against toxic metals towards microorganisms including cyanobacteria. Amelioration of test metal toxicity by Ca^{2+}, Mg^{2+} and Mn^{2+} may be due to the antagonistic interaction of these nutrients with toxic heavy metals. It results from competition between non-toxic cations for common sites on the surface of target cells, thus the more efficient competitor prevents the uptake of other toxic cations. The synergistic interaction of the test metal cations Cr^{2+} and Sn^{2+} with Ni^{2+}, Co^{2+} and Zn^{2+} possibly resulted from the adsorption of both cations on the surface of cyanobacterial cells. Calcium is known to regulate the toxicity of Cd^{2+}, Zn^{2+}, Pb^{2+}, Ni^{2+} and Ag^{2+} in green algae and cyanobacteria (Say and Whitton 1977; Fennikoh et al. 1978; Rai et al. 1981; Rai and Raizada 1985; Raizada and Rai 1985). In the present study the toxicity of chromium and tin was ameliorated not only by Ca^{2+} but also by Mn^{2+} and Mg^{2+}, especially with reference to growth, nitrogenase, oxygen evolution and carbon fixation of *A. doliolum*. The suggested mechanisms for the ameliorative behaviour of Ca^{2+} ions may be (a) competition for transport across the biomembranes, and (b) protection of target sites from toxic metals at the cell interior (Gipps and Coller 1980).

This study has demonstrated that metal toxicity can be regulated by different metal cations in the laboratory microcosm. However, such interaction in field conditions remains to be explored.

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