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Toxicity of chromium and tin to *Anabaena doliolum*

Interaction with sulphur-containing amino acids and thiols

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Summary. Toxicity of chromium and tin on growth, heterocyst differentiation, nitrogenase activity and $^{14}CO_2$ uptake of *Anabaena doliolum* and its amelioration by sulphur-containing amino acids and thiols has been studied. The final growth yield was found to be approximately 51% and 58% of control at sublethal concentration of chromium and tin respectively. Among various amino acids tested, cysteine (0.05 mM) significantly restored growth, heterocyst differentiation, nitrogenase and $^{14}CO_2$ uptake of test alga. Dithiothreitol (1 mM) restored all the parameters and processes better than monothiol, mercaptoethanol. It is obvious from present investigation that sulphur-containing amino acids and thiols, viz. cysteine, methionine, cystine, mercaptoethanol and dithiothreitol, may appreciably alleviate the toxicity of heavy metals in N_2-fixing cyanobacteria if present in an aquatic ecosystem.

Key words: Heavy metal — Sulphur-containing amino acids — Thiols — Nitrogenase — $^{14}CO_2$ uptake

Introduction

The current rate of production of synthetic chemicals coupled with industrial development is adding a large array of hazardous substances to the aquatic environment. Various other sources of heavy metal input are fossil fuels, mining, smelting, industrial emissions, pesticides, sewage sludge, etc.

Despite the fact that many metals are essential for various physiological and biochemical processes of algae including cyanobacteria, many are known to affect the taxonomic diversity and productivity of algae (Whitton 1970, 1984; Rai et al. 1981). It has been shown in earlier studies that toxicity of heavy metals to the microbiota is governed by various intrinsic and extrinsic factors, viz. pH, redox potential, salinity, solubility, exchangeability of metals, presence of other cations, complexing inorganic and organic ligands, and organic acids. In addition to these factors, clay minerals, viz. montmorillonite and kaolinite, also influence the toxicity of heavy metals in terrestrial and aquatic communities either as a result of immobilization by adsorption to clay minerals and other particulates or by precipitation as phosphate, carbonate or sulphide salts (Babich and Stotzky 1980). Nevertheless, thiols also have enormous potential to mitigate metal toxicity either through binding (Nuzzi 1972; DeFilippis 1979b) or preventing the oxidation of $-SH$ groups by heavy metals (DeFilippis and Pallaghy 1976; Gould 1978; Rai 1979; Rai and Raizada 1987).

The role of sulphur-containing compounds and amino acids in alleviating heavy metal toxicity has not been studied extensively. Earlier studies have clearly revealed that sulphur-containing compounds act as reducing agents and decrease the toxicity of heavy metals (Rai 1979; Rai and Raizada 1987; Boney et al. 1959; Hill 1979; Singh and Pandey 1981; Wiegand et al. 1984). It is now understood that destruction and breakdown of the permeability of cells may depend upon the interaction of heavy metal ions with sulfhydryl groups. Earlier studies have confirmed that metal toxicity results as a consequence of binding of metal ions with $-SH$ groups of the enzyme (Porter and Sheridan 1981).

Ameliorative behaviour of sulphur-containing amino acids and thiols against metal toxicity has

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not yet been studied elaborately in N_2-fixing cyanobacteria with reference to heterocyst differentiation, nitrogen fixation and $^{14}CO_2$ uptake. Keeping in view the available information, the present investigation has been made to study the role of amino acids and thiols in amelioration of chromium and tin toxicity in a N_2-fixing cyanobacterium *Anabaena doliolum*.

Materials and methods

Organism and growth conditions. Anabaena doliolum Bharadwaja was grown axenically in modified Allen and Arnon's medium (1955) buffered with 4 mM Tris/HCl buffer (pH 7.5). The cultures were incubated in a 14-h light/10-h dark cycle at $26 \pm 2°$ C under 0.1 pW m^{-2} light intensity. Stock solutions of chromium trioxide, stannous chloride, methionine, cysteine, cystine, mercaptoethanol and dithiothreitol were prepared and filter-sterilized by passing through Millipore membrane filters (0.45 μm) before being added to the culture medium. Growth was measured in a Bausch and Lomb spectronic-20 spectrocolorimeter by recording the absorbance of acetone (80%) extracts of algae at 663 nm. The metal concentrations causing 50% mortality (LD_{50}) of algal cells were used for further studies.

Carbon fixation measurement. Carbon fixation was measured following the uptake of $^{14}CO_2$ from $NaH^{14}CO_3$ (specific activity 50 μCi) as described by Rai and Raizada (1985).

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.

Assay of nitrogenase. Nitrogenase activity was measured by an acetylene reduction technique (Stewart et al. 1968). The assay was performed in calibrated triplicate serum vials of about 7.5 ml capacity. The acetylene concentration was kept at 10% (by vol.) and 2 ml algal suspension was routinely injected into each vial. Reactions were run for 30 min at 28° C and 2500 lx light intensity. Reactions were terminated by injecting 0.3 ml 2 M NaOH. Ethylene produced in the reaction vessel was analysed in a CIC gas chromatograph fitted with a Porapak-R column and a hydrogen flame ionization detector. Nitrogenase activity was expressed in terms of nmol C_2H_4 (μg Chl $a)^{-1}$ h^{-1}.

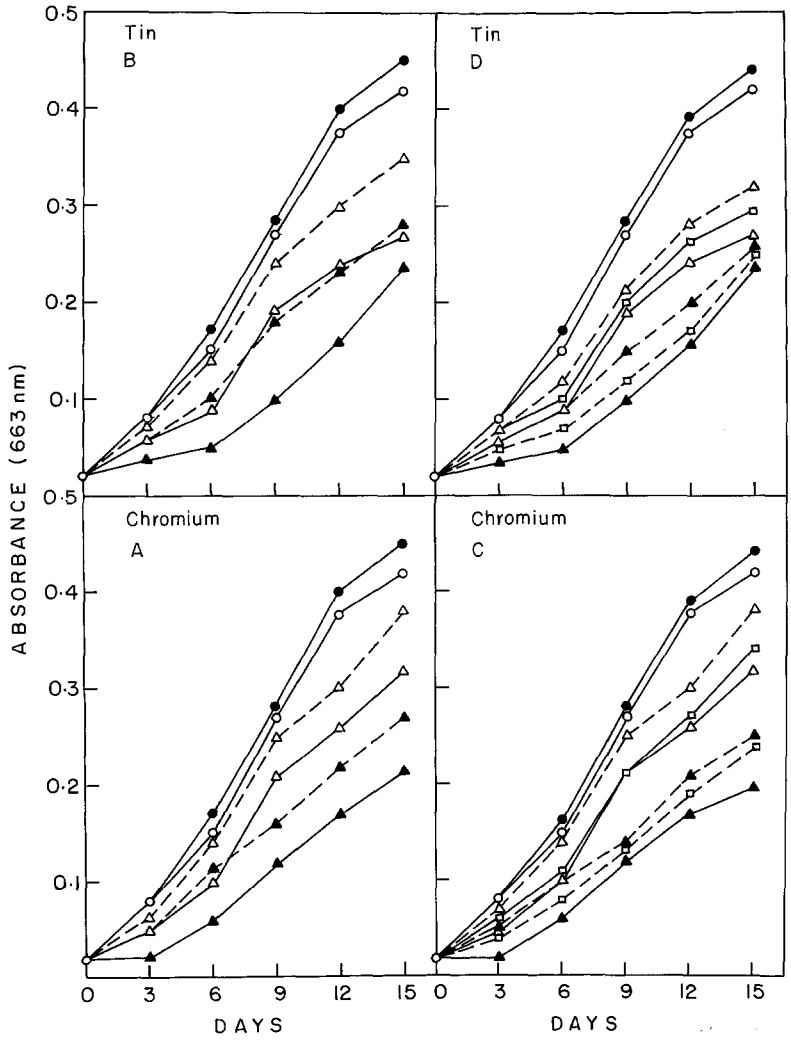

Fig. 1. Effect of chromium **(A, C)** and tin **(B, D)** on growth of *Anabaena doliolum* and amelioration of toxicity by sulphur-containing amino acids. **(A)** Control (○—○); control + cysteine (●—●); control + Cr 20 μg ml^{-1} (△—△); control + Cr 40 μg ml^{-1} (▲—▲); Cr 20 μg ml^{-1} + cysteine (△---△); Cr 40 μg ml^{-1} + cysteine (▲---▲). **(B)** Control (○—○); control + cysteine (●—●); control + Sn 30 μg ml^{-1} (△—△); control + Sn 50 μg ml^{-1} (▲—▲); Sn 30 μg ml^{-1} + cysteine (△---△); Sn 50 μg ml^{-1} + cysteine (▲---▲). **(C)** Control (○—○); control + methionine (●—●); control + Cr 20 μg ml^{-1} (△—△); Cr 20 μg ml^{-1} + methionine (△---△); control + Cr 40 μg ml^{-1} (▲—▲); Cr 40 μg ml^{-1} + methionine (▲---▲); Cr 20 μg ml^{-1} + cystine (□—□); Cr 40 μg ml^{-1} + cystine (□---□). **(D)** Control (○—○); control + methionine (●—●); control + Sn 30 μg ml^{-1} (△—△); control + Sn 50 μg ml^{-1} (▲—▲); Sn 30 μg ml^{-1} + methionine (△---△); Sn 50 μg ml^{-1} + methionine (▲---▲); Sn 30 μg ml^{-1} + cystine (□—□); Sn 50 μg ml^{-1} + cystine (□---□)

Results

Effect of test metals on growth of A. doliolum: regulation of toxicity by S-containing amino acids and thiols

The final yields of test alga were 74.4% and 51.2% of control at 20 μg ml^{-1} and 40 μg ml^{-1} of chromium, respectively, and 62.8% and 58.1% at 30 μg ml^{-1} and 50 μg ml^{-1} tin. Growth was restored in cultures supplemented with cysteine, methionine and cystine even in the presence of sublethal concentrations of test metals. The final yield in cultures treated with chromium and then amino acids was restored by 11.6%, 6.9% and 4.6% for cysteine, methionine and cystine, respectively. However, the growth with these amino acids was restored only by 7.0%, 4.7% and 2.4%, respectively, in tin-treated cultures (Fig. 1). Similarly the final yield was restored 7% and 14% with mercaptoethanol and dithiothreitol in cultures treated with the LC_{50} of chromium. Growth was restored approximately 12% and 19% in tin-supplemented cultures following addition of the monothiol and dithiol, respectively (Fig. 2).

Effect of test metals on $^{14}CO_2$ uptake of A. doliolum: amelioration of toxicity by S-containing amino acids and thiols

Protection of $^{14}CO_2$ uptake was also observed following addition of amino acids. The protection was 36.8%, 26.3% and 13.1% in chromium-treated cultures supplemented with crysteine, methionine and cystine, respectively. Tin toxicity was reduced 39.5%, 28.9% and 23.7% by cysteine, methionine and cystine, respectively (Fig. 3). Dithiothreitol and mercaptoethanol restored $^{14}CO_2$ uptake by 36.8% and 23.6% in chromium-treated cultures and 33.5% and 26.3% in tin-treated cultures (Fig. 4).

Effect of test metals on nitrogenase activity and heterocyst differentiation of A. doliolum: amelioration of toxicity by S-containing amino acids and thiols

Nitrogenase activity and heterocyst frequency were also restored in the presence of the tested amino acids (Table 1). The order of protective efficiency of sulphur-containing amino acids was

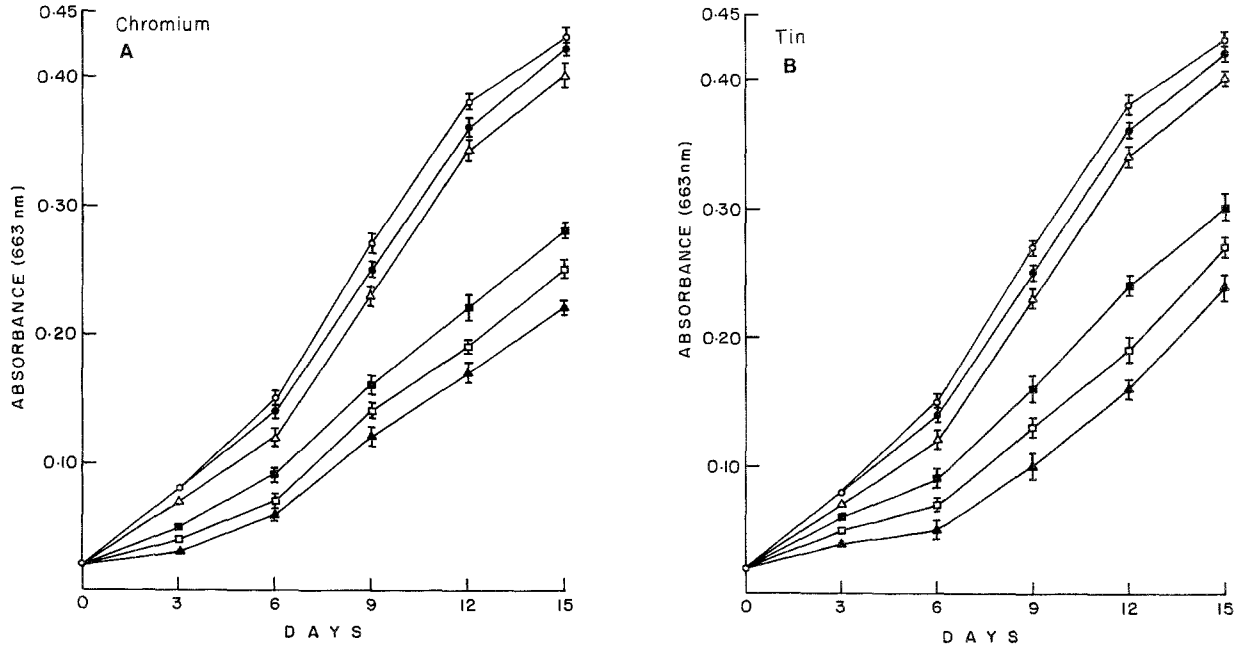

Fig. 2. Effect of chromium **(A)** and tin **(B)** on growth of *Anabaena doliolum:* mitigation of toxicity by thiols. **(A)** Control (○—○); control + mercaptoethanol (■—■); control + dithiothreitol (△—△); control + Cr 40 μg ml^{-1} (▲—▲); Cr 40 μg ml^{-1} + mercaptoethanol (□—□); Cr 40 μg ml^{-1} + dithiothreitol (■—■). **(B)** Control (○—○); control + mercaptoethanol (●—●); control + dithiothreitol (△—△); Sn 50 μg ml^{-1} (▲—▲); Sn 50 μg ml^{-1} + mercaptoethanol (□—□); Sn 50 μg ml^{-1} + dithiothreitol (■—■)

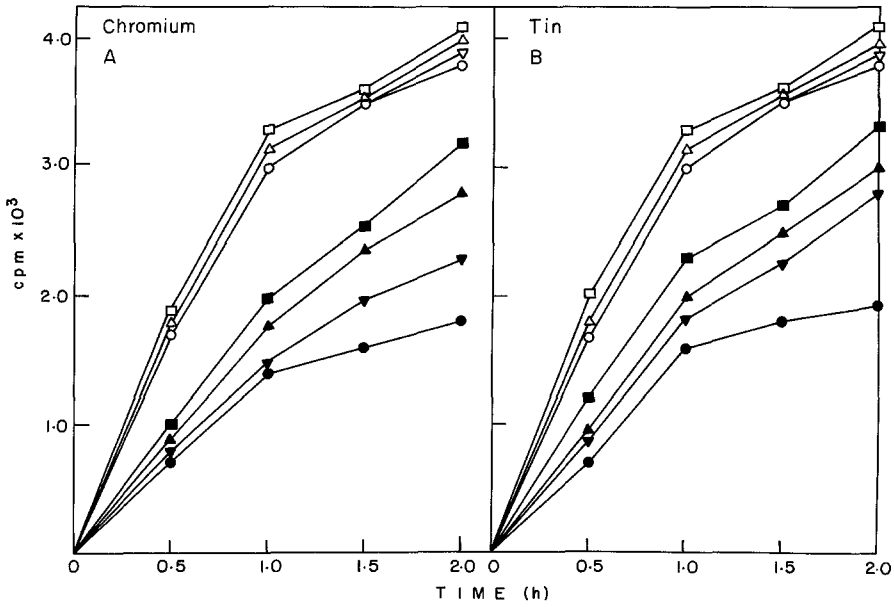

Fig. 3. Effect of chromium **(A)** and tin **(B)** on $^{14}CO_2$ uptake of *Anabaena doliolum:* amelioration of toxicity by sulphur-containing amino acids. **(A)** Control (○—○); control + cysteine (□—□); control + methionine (△—△); control + cystine ((▽—▽); control + Cr 40 μg ml^{-1} (●—●); Cr 40 μg ml^{-1} + cysteine (■—■); Cr 40 μg ml^{-1} + cystine (▼—▼); Cr 40 μg ml^{-1} + methionine (▲—▲). **(B)** As in A but with Sn in place of Cr

cysteine > methionine > cystine. Thiols have also been found to restore heterocyst differentiation and nitrogenase activity of *A. doliolum,* amounting to 25% and 17.3% for dithiothreitol and mercaptoethanol in chromium treated cultures and 21.3% and 15.4% for tin-treated cultures (Table 2).

Discussion

Heavy-metal toxicity is reduced in N_2-fixing cyanobacteria by various factors, viz. chelators, carbon sources, thiols, sulphur-free and sulphur-containing amino acids. Sulphur-containing amino acids, ascorbic acid, thiols and reduced glutathione were also found to alleviate metal toxicity in microorganisms and higher plants because they reduced the bioavailability of these metals (Fox et al. 1971).

In the present investigation sulphur-containing amino acids, viz. cysteine, methionine and cystine, and thiols, viz. mercaptoethanol and dithiothreitol, clearly reduced the toxicity of chromium and tin towards *A. doliolum.* Metal toxicity results from the binding of toxic cations with the sulfhydryl groups of enzymes (Porter and Sheridan 1981). We therefore attempted to protect the algae by exogenous addition of sulphur-contain-

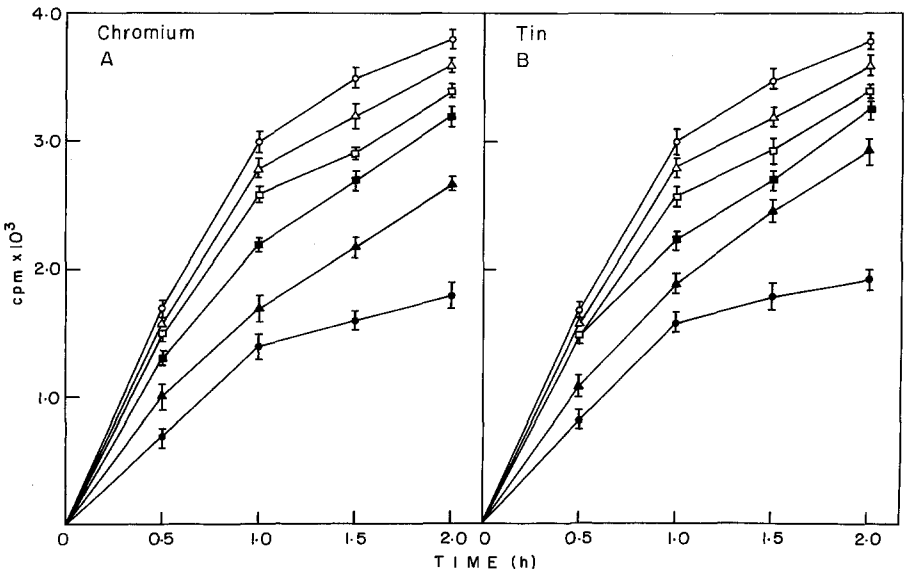

Fig. 4. Effect of chromium **(A)** and tin **(B)** on $^{14}CO_2$ uptake of *A. doliolum:* amelioration of toxicity by thiols. **(A, B)** Control (○—○); control + heavy metal (●—●); control + mercaptoethanol (△—△); control + dithiothreitol (□—□); heavy metal + mercaptoethanol (▲—▲); heavy metal + dithiothreitol (■—■)

Table 1. Effect of sublethal concentration of test metals on heterocyst frequency and nitrogenase activity of *A. doliolum:* interaction with sulphur-containing amino acids

Supplementation	Heterocyst frequency (%)	Nitrogenase activity (nmol C_2H_4 μg Chl a^{-1} h^{-1})
Control (C)	5.5	5.20±0.10 (100%)
+cysteine (0.05 mM)	6.5	5.09±0.10 (97.80%)
+methionine (0.05 mM)	6.7	4.91±0.15 (94.40)
+cystine (0.05 mM)	6.9	4.80±0.10 (92.30)
Cr (40 μg ml^{-1})	3.5	1.20±0.10 (23.08)
+cysteine (0.05 mM)	4.0	4.60±0.10 (88.47)
+methionine (0.05 mM)	4.2	4.30±0.10 (82.70)
+cystine (0.05 mM)	4.6	3.60±0.10 (69.23)
Sn 50 μg ml^{-1}	3.2	1.60±0.10 (30.77)
+cysteine (0.05 mM)	4.3	4.90±0.10 (94.23)
+methionine (0.05 mM)	4.6	4.60±0.10 (88.87)
+cystine (0.05 mM)	4.9	4.00±0.10 (76.93)

Heterocyst frequency and nitrogenase activity was determined after 72 h of incubation. Data in parentheses denote percentage of control

ing amino acids and thiols. It is evident from the results that dithiols were more protective than monothiols and cysteine, while methionine was better than cystine with reference to various important processes of the test alga (Figs. 1–4). Our findings are in agreement with the findings of Smarelli and Campbell (1983) who emphasized protection of nitrate reductase activity of cucurbits by exogenous supplementation of thiols in the medium. The toxicity of heavy metals is also reduced by formation of complexes with S^- and SO_4^- ions which are unable to cross the interior of biomembranes because they are too large.

The better protective efficiency of dithiothreitol and mercaptoethanol could be due to restoration of proton transfer across the biomembrane (Gould 1978). Like reduced glutathione, thiols and sulphur-containing amino acids, viz. L-methionine and L-cysteine, are also known to reduce toxicity of mercury and zinc in *Chlorella* (DeFilippis 1979). It has been shown in various earlier investigations that destruction and breakdown of the permeability barrier of cells may depend upon the interaction of heavy metal ions with sulfhydryl groups.

In the present investigation cysteine and methionine protected the algae more efficiently than cystine. This may be due to the –SH groups present in amino acids which provide reducing power and protect the cyanobacterial membrane from oxidation. The more sulfhydryl groups available in the system, the greater is the metal binding, ev-

Table 2. Effect of subletal concentration of test metals on heterocyst frequency and nitrogenase activity of *A. doliolum:* interaction with thiols

Supplementation	Heterocyst frequency (%)	Nitrogenase activity (nmol C_2H_4 μg Chl a^{-1} h^{-1})
Control (C)	5.5	5.20±0.10 (100%)
+mercaptoethanol (1 mM)	7.0	4.50±0.10 (86.40)
+dithiothreitol (1 mM)	6.8	3.75±0.12 (72.00)
Cr+(40 μg ml^{-1})	3.5	1.20±0.15 (23.08)
+mercaptoethanol	4.8	2.10±0.15 (40.39)
+dithiothreitol	4.0	2.50±0.10 (48.08)
Sn (50 μg ml^{-1})	3.2	1.60±0.10 (30.77)
+mercaptoethanol	5.1	2.40±0.10 (46.16)
+dithiothreitol	4.6	2.70±0.10 (51.93)

Heterocyst frequency and nitrogenase activity were determined after 72 h of incubation. Data in parentheses denote percentage of control

entually leading to amelioration of its toxicity. It may be concluded from the present investigation that these compounds, if present in the natural ecosystem, may appreciably ameliorate the toxicity of heavy metals. Thus bioproductivity and nitrogen-fixing potential of cyanobacteria and other nitrogen-fixing microbes may be ameliorated by these organic compounds and ligands in aquatic as well as terrestrial eco-systems.

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