Biol Metals (1990) 2:229–234

BIOLOGY OF METALS

Effect of four heavy metals on the biology of *Nostoc muscorum*

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Received September 26, 1989

Summary. This study presents the effects of Cr, Pb, Ni and Ag on growth, pigments, protein, DNA, RNA, heterocyst frequency, uptake of NH_4^+ and NO_3^-, loss of electrolytes (Na^+ and K^+), nitrate reductase and glutamine synthetase activities of *Nostoc muscorum*. The statistical tests revealed a direct positive correlation between the metal concentration and inhibition of different processes. Ni was found to be more toxic against growth, pigments and heterocyst differentiation compared to the other metals. Inhibition of pigment showed the following trend: chlorophyll > phycocyanin > carotenoid. No generalized trend for inhibition of macromolecules was observed. The loss of K^+ and Na^+ as affected by Cr, Ni and Pb was similar but more pronounced for K^+ than Na^+. The inhibition of physiological variables depicted the following trend: Na^+ loss > K^+ loss > glutamine synthetase > NH_4^+ uptake > growth > NO_3^- uptake > nitrate reductase > heterocyst frequency. This study therefore suggests that loss of electrolytes can be used as a first signal of metal toxicity in cyanobacteria. However, further study is needed to confirm whether the abnormality induced by nickel (branch formation) is a physiological or genetic phenomenon.

Key words: Heavy metals - Efflux of Na^+ and K^+ - *Nostoc muscorum*

Introduction

Much information has been accumulated on the toxicity of heavy metals to different organisms including algae (Rai et al. 1981a; Stokes 1983; Whitton 1984). Although most heavy metals are toxic to microorganisms at higher concentrations, some are required in trace quantities by algae for various physiological and biochemical processes. Heavy metals are known to increase the generation time of algae and other microorganisms. De Filippis and Pallaghy (1976) and Rai et al. (1981b) noticed an increase in the carotenoid/chlorophyll ratio of *Chlorella* following treatment with mercury but this ratio decreases at higher concentrations of zinc. Conway (1978) found a significant lowering of pigment content after addition of cadmium in *Asterionella formosa* cultures.

Certain heavy metals, viz. Cd, Ni, Hg and Cr, are also known to inhibit growth, pigment synthesis, nutrient uptake, nitrogen fixation and photosynthesis in *Anabaena inaequalis, Anabaena doliolum* and *Nostoc muscorum* (Stratton et al. 1979; Rai and Raizada 1986). Heavy metals are also reported to interfere in the regulation of DNA synthesis by blocking the —SH groups or inhibition of DNA polymerase III activity (Vallee and Ulmer 1972; Bonaly et al. 1980).

The primary target of heavy metals is the cell membrane. A change in membrane potential of the slime mould *Physarum polycephalum* was observed following metal supplementation. Passow and Rothstein (1960) observed the loss of K^+ by Hg^{2+} ions in yeast cells. The loss of electrolytes has therefore, been used as a reliable criterion to ensure membrane damage caused by heavy metals. In addition to this, heavy metals are also known to induce formative changes and inhibit the vital metabolic processes.

It is worth mentioning here that all the above studies are mainly concerned with effects of one or two metals on specific metabolic processes of green algae. Though cyanobacteria are agriculturally most important, no worthwhile attempts have been made to study the heavy metal toxicity on their physiological processes. Keeping these considerations in view, we have studied the effect of Cr, Pb, Ni and Ag on growth, heterocyst differentiation, pigment content, macromolecules (protein, DNA, RNA), loss of K^+, Na^+, uptake of NO_3^-, NH_4^+ and the activities of nitrate reductase and glutamine synthetase. Attempts have been made to verify these results statistically.

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Materials and methods

The test system

Survival of the test alga was scored by the plate/colony count method (Raizada and Rai 1985), selecting 10–40 μg ml^{-1} Cr and Pb, 0.5–1.5 μg ml^{-1} Ni and 0.002–0.01 μg ml^{-1} Ag. To determine the effect of different doses of test metals (Cr, Pb, Ni and Ag) on growth, the experimental medium was supplemented with (10, 20, 30 μg ml^{-1} Cr and Pb, 0.5–1.2 μg ml^{-1} Ni and 0.002–0.008 μg ml^{-1} Ag) taking different amounts of reagent-grade $K_2Cr_2O_7$, $PbCl_2$, $NiCl_2 \cdot 6H_2O$ and $AgCl_2$. Stock solutions of test metals and other chemical were prepared in sterilized double-distilled water. The pH of the growth medium was maintained by Tris/HCl pH 7.5. The final yield and pigment content were estimated as described by Rai et al. (1981b). The protein and nucleic acid contents were estimated by the method of Lowry et al. (1951) and Herbert et al. (1971), respectively. In order to study heterocyst frequency the method of Rai and Raizada (1986) was followed. Uptake of NO_3^- and NH_4^+ was measured by determining their depletion from the external growth medium by the methods of Nicholas and Nason (1957) and Herbert et al. (1971), respectively. The loss of K^+ and Na^+ contents from algal cells exposed to metals was measured as given by Rai and Raizada (1987). Nitrate reductase and glutamine synthetase activities in vivo were estimated by the methods of Camm and Stein (1974) and Stacey et al. (1977), as described by Dubey and Rai (1987).

Statistical analysis. Correlation coefficients (r) were calculated for metal concentration and percentage inhibition of different processes according to the following equation:

$$r = \frac{n\Sigma XY - (\Sigma X)(\Sigma Y)}{\sqrt{n\Sigma X^2 - (\Sigma X)^2}\sqrt{n\Sigma Y^2 - (\Sigma Y)^2}}$$

where, X represents the metal concentration, Y represents the percentage inhibition of any process and n is the number of observations.

Results

The LC_{50} values for four test metals against *Nostoc* were 20, 20, 1.0 and 0.004 μg ml^{-1} for chromium, lead, nickel and silver, respectively. Growth response of test algae to different concentrations of metals used is given in Table 1. With increase in metal concentrations, there was a gradual decline in final yield (negative and significant correlation $r = -0.876$, $df = 11$, $P < 0.001$). At higher concentrations, however, a severe reduction in growth was noticed only for silver (14.8%). It can be inferred from Table 1 that silver is more toxic than other metals. Table 2 showed the effect of different concentrations of test metals on chlorophyll, carotenoid and phycocyanin contents of *Nostoc muscorum* attained on 15th day of experiment. Maximum inhibition of chlorophyll by all the test metals was noticed ($r = -0.748$, $P < 0.01$). The test metals caused a concentration-dependent decrease in pigment contents. The

Table 1. Effect of different concentrations of test metals on growth and heterocyst frequency of *N. muscorum*

Metal	Concentration (μg/ml)	Final yield attained on 15th day ($A_{663\,nm}$)	Heterocyst frequency (%)
None	(control)	0.27 ± 0.0001 (−)	4.94 (−)
Cr	10	0.22 ± 0.001 (18.5)	4.60 (6.8)
	20	0.14 ± 0.005 (48.2)	3.50 (29.5)
	30	0.05 ± 0.001 (81.5)	3.20 (35.5)
Pb	10	0.20 ± 0.001 (19.1)	4.40 (10.9)
	20	0.10 ± 0.002 (54.8)	4.00 (19.0)
	30	0.05 ± 0.004 (79.7)	3.50 (29.1)
Ni	0.5	0.19 ± 0.002 (26.6)	4.88 (1.20)
	1.0	0.11 ± 0.003 (53.4)	3.90 (21.0)
	1.2	0.10 ± 0.001 (56.7)	3.41 (30.9)
Ag	0.002	0.15 ± 0.001 (31.5)	4.00 (19.0)
	0.004	0.12 ± 0.002 (42.9)	3.50 (29.1)
	0.008	0.04 ± 0.001 (85.2)	3.00 (39.2)

Data in parentheses denote percentage inhibition

Table 2. Effect of chromium, lead, nickel and silver on chlorophyll, carotenoid and phycocyanin contents of *N. muscorum* (after 15th day)

Metal	Concentration (μg/ml)	Chlorophyll (mg/l)	Carotenoid (mg/l)	Phycocyanin (mg/l)
None (control)		24.54 ± 0.460 (−)	83.33 ± 0.054 (−)	0.182 ± 0.004 (−)
Chromium	10	17.87 ± 0.062 (27.1)	73.33 ± 0.009 (12.0)	0.147 ± 0.011 (19.2)
	20	11.47 ± 0.011 (53.2)	57.33 ± 0.001 (31.2)	0.095 ± 0.004 (47.8)
	30	05.00 ± 0.001 (79.6)	14.00 ± 0.110 (83.2)	no growth
Lead	10	20.14 ± 0.004 (17.9)	81.00 ± 0.001 (2.79)	0.180 ± 0.001 (1.10)
	20	11.84 ± 0.002 (51.7)	69.00 ± 0.001 (17.1)	0.120 ± 0.007 (34.0)
	30	02.00 ± 0.140 (91.8)	08.33 ± 0.011 (90.0)	no growth
Nickel	0.5	15.95 ± 0.005 (35.0)	76.00 ± 0.030 (8.7)	0.140 ± 0.011 (23.0)
	1.0	10.80 ± 0.007 (55.99)	42.13 ± 0.001 (49.4)	0.090 ± 0.011 (50.50)
	1.2	09.29 ± 0.001 (62.1)	38.31 ± 0.011 (54.0)	0.060 ± 0.003 (67.0)
Silver	0.002	16.75 ± 0.260 (31.7)	56.81 ± 0.001 (31.8)	0.170 ± 0.007 (6.5)
	0.004	14.00 ± 0.041 (42.9)	49.23 ± 0.011 (40.9)	0.120 ± 0.001 (34.0)
	0.008	02.81 ± 0.001 (88.5)	10.41 ± 0.002 (87.5)	0.09 ± 0.011 (50.5)

Data in parentheses denote percentage inhibition

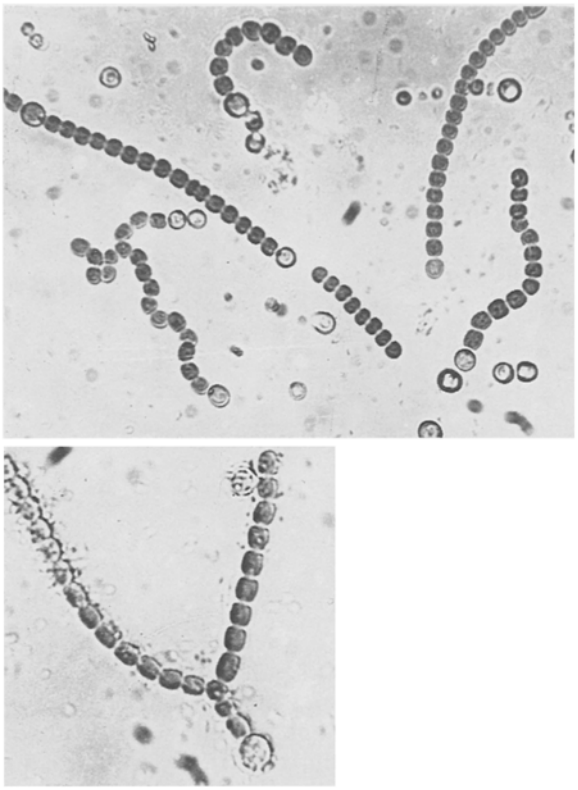

Fig. 1. Photomicrograph of *N. muscorum* showing irregular arrangement of cells and branch formation

inhibition of pigments follows the following trend: chlorophyll > phycocyanin > carotenoid.

Microscopic observations revealed cell enlargement at 20 μg ml^{-1} each of Cr and Pb and irregular arrangement of cells in the filaments in case of 1.0 μg ml^{-1} of Ni after one week of treatment (Fig. 1). Reduction in heterocyst frequency was concentration-dependent (Table 1) and followed the course of growth. Changes in the cell constituents, viz. protein, DNA, and RNA at different levels of metals, are given in Fig. 2. Maximum inhibition was found in the case of silver: at sublethal concentrations, inhibitions of 57%, 50% and 57% were observed for protein, RNA and DNA, respectively.

Table 3 summarizes the effect of different concentrations of test metals on cellular K^+ and Na^+ in long-term experiments (after 48 h). The extent of loss of Na^+ and K^+ from cells as induced by Cr, Pb and Ni was similar. However, the loss of K^+ from the cells was more pronounced as compared to Na^+. Maximum loss of K^+ and Na^+ from the silver-treated algae further confirms the greater toxic potential of this metal as compared to others.

The uptake of ammonium, as influenced by different doses of metals, is given in Fig. 3. It can be inferred from this graph that the extent of inhibition of ammonium uptake by Cr, Pb and Ag followed almost the same course at sublethal as well as higher concentrations of these metals. The situation was different for nickel, however. At sublethal and highest concentrations of nickel used, the uptake of ammonium was twofold higher than for other metals. Similar results were also found for NO_3^- uptake (Fig. 4), the uptake of which was higher (59% and 40%) for nickel concentrations of 1.0 and 1.2 μg ml^{-1}, respectively.

Sublethal concentrations of Cr, Pb, Ni and Ag were found to inhibit nitrate reductase activity by 78%, 70%, 70% and 78%, respectively (Fig. 5). Inhibition of glutamine synthetase activity was also concentration-dependent ($r = -0.721$, $P < 0.001$) with inhibitions of approximately 53%, 44%, 61% and 62%, respectively at LC_{50} levels of test metals. Like other parameters, glu-

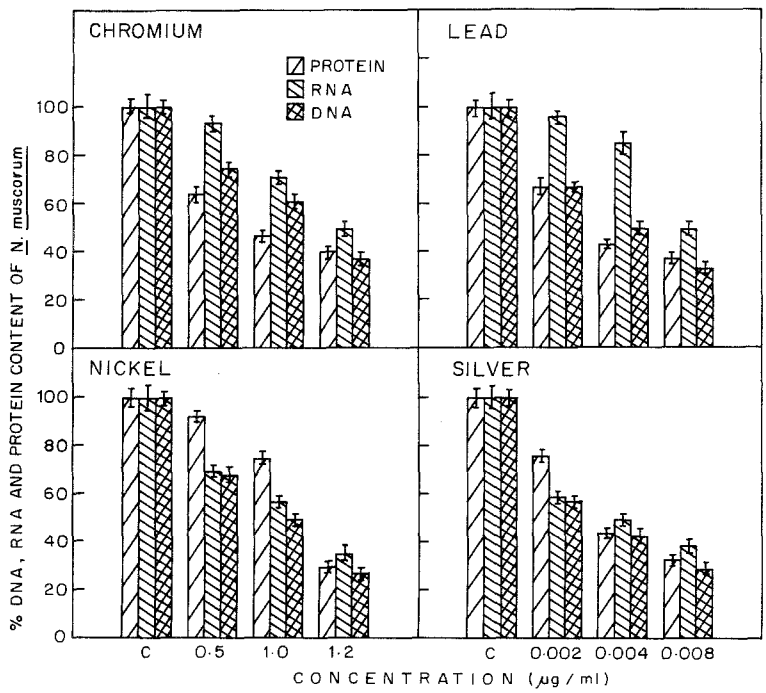

Fig. 2. Effect of different concentrations of Cr, Pb, Ni and Ag on protein, DNA and RNA content of *N. muscorum*

Table 3. Loss of K^+ and Na^+ from *N. muscorum* cells exposed to test metals (after 48 h)

Metal	Concentration (μg/ml)	K^+ (μg/mg protein)	Loss of K^+ (%)	Na^+ (μg/mg protein)	Loss of Na^+ (%)
None (control)		60±0.002	—	15±0.003	—
Chromium	10	30±0.001	50	9±0.011	40
	20	15±0.004	75	3±0.002	80
	30	10±0.001	84	2±0.006	83
Lead	10	36±0.002	40	9±0.003	40
	20	18±0.003	70	4±0.004	74
	30	15±0.001	75	3±0.004	80
Nickel	0.5	25±0.003	59	8±0.005	47
	1.0	15±0.001	75	3±0.001	80
	1.5	08±0.004	83	2±0.003	83
Silver	0.002	24±0.011	60	10±0.001	34
	0.004	15±0.001	75	7±0.004	53
	0.008	06±0.002	90	4±0.003	73

tamine synthetase also displayed highest sensitivity for silver.

Discussion

Though some heavy metals are needed by living organisms for various metabolic processes, the physiological and/or metabolic requirements of such metals as chromium, lead, nickel, and silver are not properly understood. The immediate effect of an increased supply of the heavy metals seems to result in inhibition of general growth of *N. muscorum*. The observed changes in pigment contents of the cyanobacterium suggest similar responses of chlorophyll and carotenoid to increasing concentrations of the test metals. The level of phycocyanin, however, declined markedly with increasing concentrations of metals. Such a decline in phycocyanin level might be attributable to its possible degradation, thus leading to nitrogen starvation (Allen

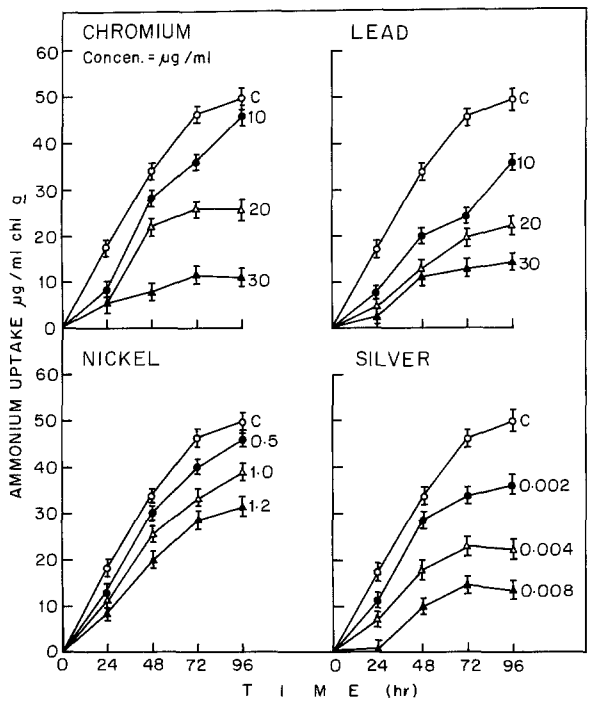

Fig. 3. Inhibition of ammonium uptake by different concentrations of Cr, Pb, Ni and Ag

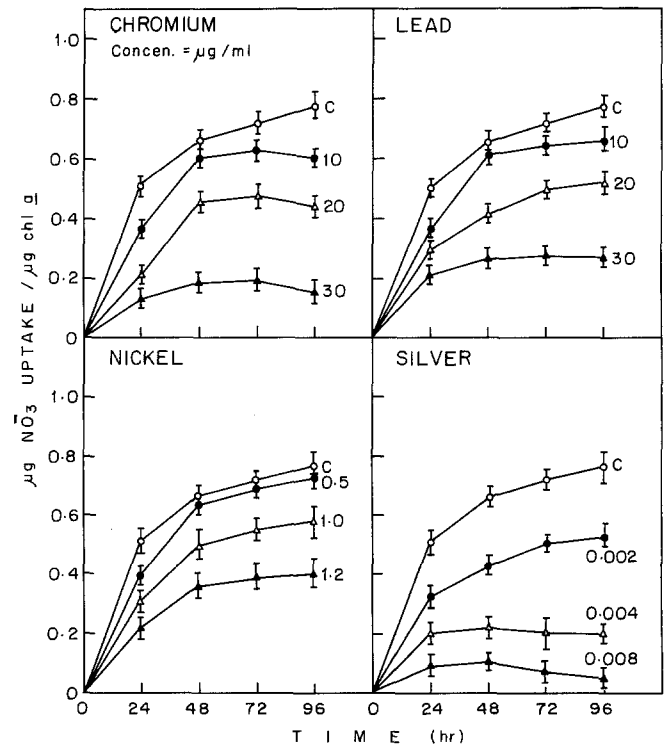

Fig. 4. Inhibition of nitrate uptake by different concentrations of Cr, Pb, Ni and Ag

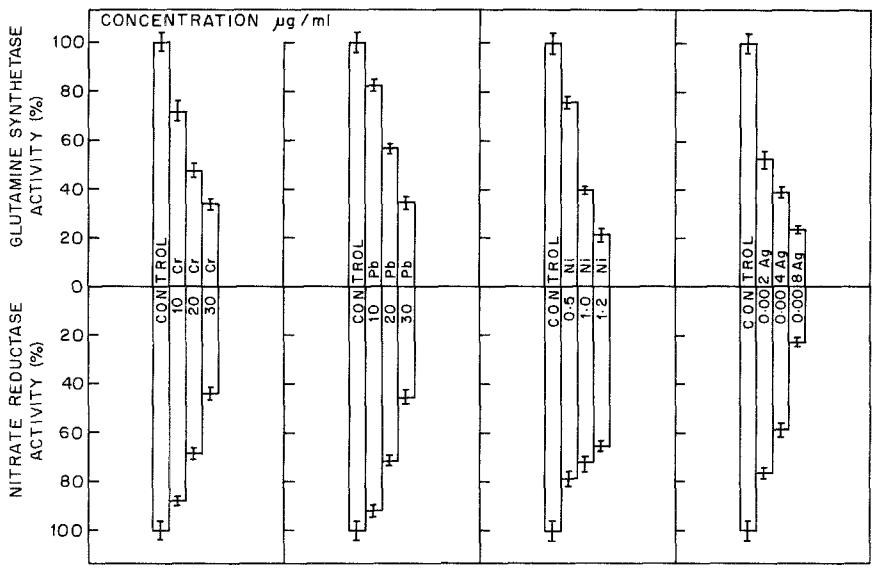

Fig. 5. Inhibition of nitrate reductase and glutamine synthetase activities of *N. muscorum* at different concentrations of Cr, Pb, Ni and Ag

and Smith 1969), as phycocyanins are major reserves for nitrogen (Cohen-Bazire and Bryant 1982) in cyanobacteria. It is quite clear from our observations that the test metals affect the synthesis of light-harvesting pigments in *N. muscorum*. Decline in pigment contents may be due to lysis of the cell wall and disruption of the thylakoid membrane as known for *Anabaena flos-aquae* (Rai et al. 1989). Present observations on cell abnormality are in agreement with Stratton et al. (1979) in *Anabaena inaequalis*. Cell abnormality, as suggested by these workers, might result from uptake of metal ions rather than adsorption onto the cell surface. Though not very frequent, nickel (1.0 µg ml^{-1}) was found to induce formation of abnormal cells and branch-like structures in *N. muscorum*. Thus this study supports the earlier findings of Pandey (1981) where irregular arrangement of cells and branch formation was noticed in *Nostoc calcicola* as a result of cadmium toxicity.

The data on macromolecule synthesis indicate that protein content was affected more by all the test metals than were DNA and RNA. Inhibition of DNA content as observed for *N. muscorum* agrees with that for *Euglena gracilis* (Bonaly et al. 1980). This reduction might be due to blocking of —SH groups (Vallee and Ulmer 1972) or inhibition of DNA polymerase III activity by test metals. The reduction in protein content could also be due to inhibiton of membrane protein (Brown and Beckett 1984).

Thomas and Apte (1984) explicitly demonstrated the crucial involvement of sodium for cyanobacterial nitrogen metabolism. A concentration-dependent loss of Na^+ and K^+ ions as observed in the present study could be attributed to increased cell permeability, thus allowing the ions to be released from the cyanobacterial cells. The increased cell permeability may make the cells more prone to metals, thus resulting in their death at high concentration.

Cyanobacteria have many alternative ways to meet their nitrogen demand, either through biological nitrogen fixation or utilization of NO_2^-, NO_3^- or NH_4^+ which are finally converted into organic nitrogen. The inhibition of NH_4^+ and NO_3^- uptake may be due either to competition of heavy metals with these nutrients (Vallee and Ulmer 1972), inactivation of the enzyme complexes by binding with their sulfhydryl groups (Porter and Sheridan 1981) or exhaustion of energy-yielding substrates (Norris and Kelly 1977) which are required for the active uptake. The inhibition of nitrate reductase and glutamine synthetase by the test metals can be attributed either to exhaustion of energy-yielding substrates or direct inactivation of enzyme complexes.

These observations suggest that heavy metals exert differential toxicity on different parameters of cyanobacteria. On the basis of the present observations, the order of metal toxicity to *N. muscorum* can be given as Ag > Ni > Cr > Pb.

Acknowledgements. This work was supported in part by a grant from Department of Environment and Forest, and Career Award Project of U.G.C. to L. C. Rai.

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