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Silver toxicity in a nitrogen-fixing Cyanobacterium

Interaction with chromium, nickel and lead

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Summary. Exposure of *Nostoc muscorum* to combinations of Ag+Cr, Ag+Ni, Ag+PB and Ag, Cr, Ni and Cr alone brought about a complex pattern of inhibition of growth, NO_3, NH_4 and $^{14}CO_2$ uptake and nitrogenase activity. The sensitivity hierarchy of different parameters to metal combinations may be given as: (a) inhibition of NH_4 uptake and nitrogenase was almost identical for Ag+Ni, Ag+Cr and Ag+Pb; (b) the order of inhibition of growth and NO_3 uptake was Ag+Cr > Ag+Ni > Ag+Pb; (c) the combination of Ag+Pb was least toxic for growth, nitrogenase, NO_3 and NH_4 uptake, but most toxic for $^{14}CO_2$ uptake; (d) the toxicity hierarchy for all parameters together was $^{14}CO_2$ > nitrogenase > NH_4 uptake > NO_3 uptake > growth. Hence, carbon fixation may be employed for biological monitoring of metal toxicity in a laboratory microcosm and possibly in aquatic ecosystems.

Key words: Silver toxicity — *Nostoc muscorum* — Metal combinations — Carbon fixation

Introduction

A large number of heavy metals from natural and human sources are being progressively discharged into the aquatic ecosystem, many of them as a consequence of increasing human civilization. The wide-ranging occurrence of such metals both in marine and fresh waters has been authenticated by Whitton (1970) while studying the pollution in the Churnet and Cardiganshire rivers of Britain. Occurrence of metals in zinc smelters, mine wastes, and the river Ganges have also been reported from India (Rana and Kumar 1975; Mathur et al. 1987). Besides this, there is a body of information on the contamination of aquatic systems by metal combinations (Forstner and Wittmann 1979; Rai et al.1981; Whitton 1984).

Although considerable fluctuations in the concentrations of heavy metals, e.g. Al, Ag, Hg, Cd, Pb, Zn, Cu, Fe, As, Cr, etc., have been reported from freshwater and marine environments (Forstner and Wittmann 1979; Stokes 1983; Whitton 1984; Mathur et al. 1987), these concentrations mostly depend on the sources of input, nutrient status, pH, organic contents and other characteristics of the receiving water. The idea of selecting different concentrations of metals stems from the fact that such concentrations of metals are generally found in an aquatic environment. Therefore any investigation involving metals and cyanobacteria will be interesting and worthwhile if similar ranges of concentrations are selected for laboratory investigations. Three concentrations, viz. LC_{50}, one below and another above LC_{50}, were taken for each metal. Thus the results of laboratory observations are likely to provide information about the fate of cyanobacteria when exposed to varied concentrations of heavy metals; extrapolation of these findings to the level of the natural ecosystem could be more accurate than those based on only one concentration of all the test metals used.

While the impact of single metal ions on single algal species is worth studying, there is a great need to study how combinations of metal ions affect various metabolic processes of algae. The idea of employing algae for studying the combined effects of more than one metal is important because algae are primary producers of aquatic ecosystems where several metal ions may be found together. Some metal-interaction effects have been investigated with eucaryotic algae (Hutchinson and Stokes 1975) and bacteria (Ba-

bich and Stotzky 1978). However, with only a few exceptions (Stratton and Corke 1979), metal-interaction studies with nitrogen-fixing cyanobacteria seem to have been neglected. Considering the significant role of these microbes in the nitrogen economy and the lack of information on the effects of metal combinations, this study on how the toxicity of silver towards various parameters, such as growth, uptake of NO_3, NH_4, carbon fixation and nitrogenase activity of *Nostoc muscorum*, is affected by Cr, Ni and Pb was undertaken.

Materials and methods

Growth of algae. Nostoc muscorum was grown in modified Chu-10 medium (Gerloff et al. 1950) as described by Rai and Raizada (1985). In order to avoid any change in pH, the culture medium was buffered to pH 7.5 with Tris (Sigma grade). To study the effect of Cr, Ni, Pb and Ag and the interaction of Ag with Cr, Pb and Ni, stock solutions of test metals were prepared separately and sterilized by passing through Millipore membrane filters before introduction into the culture medium. Besides Ag, the effects of the other three metals on all the parameters were also investigated separately. For studying the effects of metal combinations, exponentially growing *Nostoc* cells were harvested, washed, centrifuged and transferred to sterile medium containing different concentrations of test metals (Cr: 96, 192 and 288 μM; Pb: 48, 96 and 144 μM; Ni: 8, 17 and 20 μM; Ag: 18, 36 and 72 nM). Keeping the concentrations of Ag constant, the concentrations of the other metals were varied. Different combinations of two metals (only Ag with Cr, Ni and Pb) were taken. While the effect of different concentrations of all the metal combinations was studied on growth, only sublethal concentrations of these metals were used for studying their effects on NO_3^-, NH_4^+, $^{14}CO_2$ uptakes and nitrogenase activity of test cyanobacterium. For studying the effect of metal combinations on NO_3^- and NH_4^+ uptake by *Nostoc*, cultures grown in nitrogen-free medium were incubated in 5 mM KNO_3 and 1 mM NH_4Cl, respectively. Uptake was estimated every 24 h up to 76 h.

Assays. Uptake of ammonia was estimated colorimetrically using Nessler's reagent (Sigma grade). The culture filtrate after two or three centrifugations was used for ammonia estimation. First an optimum amount of reagent was determined and then a standard curve (absorbance at 420 nm vs different concentrations of ammonia) was obtained; measurements were made within 10 min of the reagent addition. Nitrate in the culture medium was estimated by the brucine/sulphuric acid method of Nicholas and Nason (1957). Uptake of $^{14}CO_2$ by cyanobacterial suspensions supplied with 50 μl hydrogen [^{14}C]carbonate to give a final activity of 1.0 kBq ml^{-1} was measured in an LS 7000 liquid scintillation counter as described by Rai and Raizada (1987). Nitrogenase activity was measured by the acetylene reduction technique of 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 cell suspension was routinely injected into each vial. Reactions were terminated by injecting 0.8 ml 15% (mass/vol.) trichloroacetic acid. The ethylene formed was measured on a CIC gas chromatograph equipped with a Porapak R column and flame ionization detector.

Statistical analysis. The analysis of variance (F) for various metal treatments and exposure time with respect to uptake of nitrate and ammonium, carbon fixation and nitrogenase activity was calculated according to the following formula: F=treatment mean square/residual mean square.

Results

The effect of silver on the growth behaviour of *Nostoc muscorum*, expressed in terms of specific growth rate and final yield, clearly indicates the toxic potential of this metal (Table 1). Although the actual toxic potential of the test metal is not clear from the data of specific growth rate, it is quite distinct from the growth yield.

Effect of metal combination on growth

Silver and chromium. The interactive effect of chromium and silver on the growth behaviour of the test cyanobacterium is given in Fig. 1. The combination of chromium and silver shows additive inhibition of growth which was noticed from the 3rd day until termination (15th day) of the experiment. Changes in concentration of chromium did not seem to affect the inhibition pattern much. However, the level of inhibition was found to increase with time, for others more clearly for this interaction effect than for others.

Silver and lead. The response of *N. muscorum* to lead and silver alone as well as in combination is

Table 1. Effect of different concentrations of silver on growth of *N. muscorum*

[Ag] (nM)	Specific growth rate (ΔA_{663})		Final yield attained on 15th day	
	Mean ± SD	Inhibition (%)	Mean ± SD	Inhibition (%)
(Control)	0.42 ± 0.007	—	0.27 ± 0.001	—
18	0.40 ± 0.001	4.76	0.15 ± 0.001	31.57
36	0.37 ± 0.002	11.36	0.12 ± 0.002	42.86
72	0.31 ± 0.001	25.00	0.04 ± 0.001	85.20

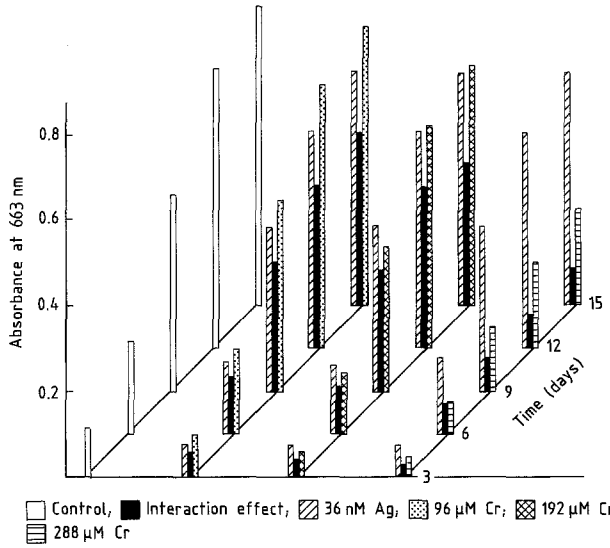

Fig. 1. Effect of bimetallic combination of silver and chromium on growth of *N. muscorum*

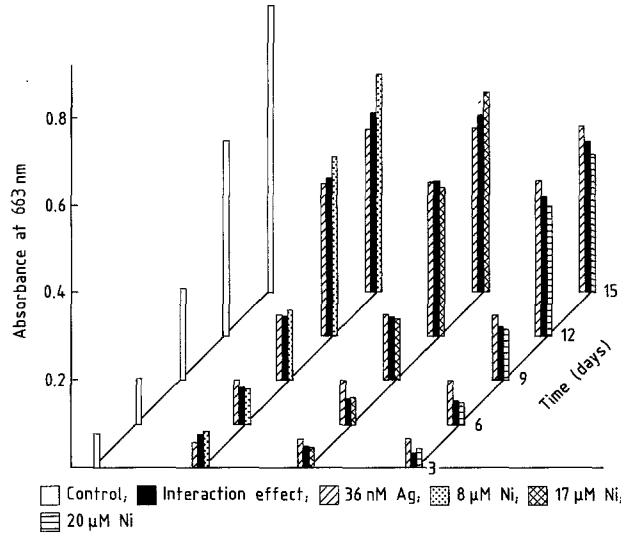

Fig. 3. Influence of silver and nickel ion combination on growth of *N. muscorum*

depicted in Fig. 2. It is clear from this figure that when 36 nM Ag was spiked with 48 and 96 µM Pb separately, better growth of test cyanobacterium was noticed in combination than when treated with Ag alone. Thus lead seems to counteract the toxicity of silver. However, when 38 nM Ag was combined with 144 µM Pb, a mixed response was visible. This combination does not show any definite trend. It is worth montioning that silver alone was always more toxic than lead at the concentrations used except for 144 µM lead on the 15th day

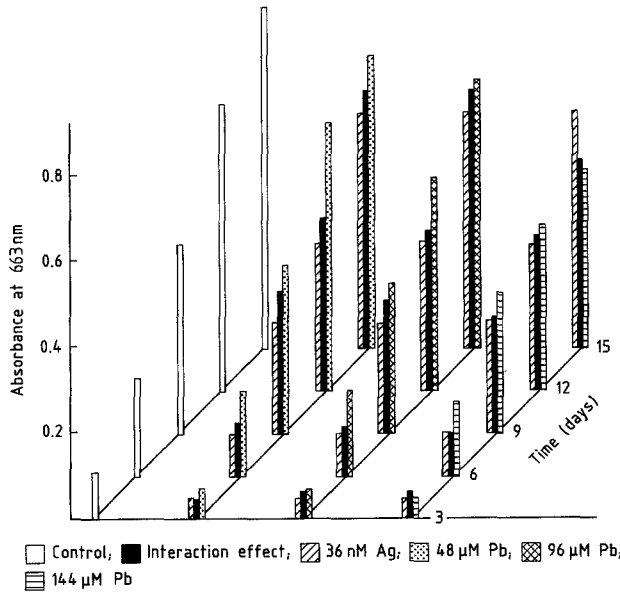

Fig. 2. Growth of *N. muscorum* as influenced by silver and lead in combination

of the experiment (Fig. 2). It is seen from this graph that, while keeping the concentration of Ag unchanged, any increase in lead concentration brings about further reduction in growth.

Silver and nickel. The effect of silver and nickel alone, as well as in combination, on test cyanobacterium is summarized in Fig. 3. Combination of silver with nickel does not produce any definite trend. When 8 µM Ni was spiked with silver (36 nM), better growth was observed in combination than with silver alone on days 3, 12 and 15 of the experiment. Growth inhibition was, however, more pronounced in the beginning with 17 µM Ni and a sublethal concentration of silver. With further increase in incubation time, growth resumed and a better performance was noticed in combination than with silver alone on the 15th day. Any increase in nickel concentration was found to decrease growth. Thus, when 20 µM Ni was combined with 36 nM silver, growth was always less than with silver alone.

Effect of metal combinations on nitrate uptake

The effect of sublethal concentration of silver, nickel, chromium and lead alone as well as silver in combination with Cr, Ni and Pb on NO_3 uptake by test cyanobacterium is given in Table 2. The inhibition of nitrate uptake by silver showed an increase with increase in time up to 72 h, after which it declined. Response of *Nostoc* to the combined effect of Cr and Ag showed mild antagonism

Table 2. Effect of silver, nickel, chromium and lead on NO_3 uptake of *N. muscorum:* interaction of Ag with metals (Me) Cr, Pb and Ni

Concentrations		NO_3/chlorophyll *a* (by mass) and inhibition (%) at							
[Ag] (nM)	[Me] (μM)	24 h		48 h		72 h		96 h	
0	0	0.42±0.110	—	0.50±0.110	—	0.80±0.112	—	0.90±0.110	—
0	192 (Cr)	0.35±0.120	16.67	0.45±0.001	10.10	0.27±0.110	66.25	0.20±0.003	77.78
0	96 (Pb)	0.15±0.140	64.29	0.35±0.003	30.00	0.50±0.002	37.50	0.55±0.005	38.89
0	17 (Ni)	0.25±0.011	40.48	0.30±0.002	40.00	0.35±0.110	56.25	0.43±0.011	52.22
36	0	0.20±0.013	52.38	0.20±0.002	60.00	0.25±0.110	68.75	0.43±0.110	52.22
36	192 (Cr)	0.20±0.110	52.38	0.30±0.002	40.00	0.20±0.002	75.00	0.20±0.011	77.78
36	96 (Pb)	0.15±0.003	64.30	0.20±0.110	60.00	0.30±0.001	62.50	0.50±0.110	44.44
36	17 (Ni)	0.15±0.001	64.30	0.18±0.011	65.00	0.18±0.001	78.12	0.23±0.004	75.00

$F_{\text{exposure time } 3,21} = 4.12$, $P < 0.25$
$F_{\text{metal combination } 7,21} = 6.37$, $P < 0.025$

after 48 h and thereafter it declined. However, a mixed response of *Nostoc* to the lead and silver combination is evident from data of Table 2. The inhibition of NO_3^- uptake was more after 24 h and 72 h as compared to 48 h and 96 h. It is evident from Table 2 that the combination of Ni and Ag inhibited NO_3^- uptake maximally, irrespective of incubation time: an increase in toxicity with the Ag+Ni combination was evident for all incubation times except 48 h were no appreciable change in percentage inhibition was noticed. This metal combination did not present any specific type of interaction.

Effect of metal combinations on ammonium uptake

The effect of sublethal concentrations of Ni, Cr, Pb and Ag and the interaction of Ag with Cr, Pb and Ni is summarized in Table 3. Maximum inhibition (35%) of NH_4 uptake by Ag was noticed in the first 24 h, following which the inhibition level decreased. Nickel also followed the pattern of silver in inhibiting NH_4^+ uptake. Of all the four test metals, Pb was the most toxic towards ammonium uptake. The combination of Ag with Cr, Pb, and Ni gives very interesting results: the Ag+Cr combination shows increased inhibition of NH_4^+ uptake after 24 h of incubation, following which the inhibition becomes synergistic in nature. In contrast to this, the Ag+Pb combination showed mild antagonism after 48 h and 72 h of incubation, thereafter no change was noticed. The Ag+Ni interaction followed a trend almost similar to Ag+Cr: a decrease in Ni toxicity by Ag was evident after 24 h; the mode of interaction then changed completely to synergism after 48 h and continued up to 96 h (Table 3).

Effect of metal combinations on $^{14}CO_2$ uptake

The effects of the four test metals individually

Table 3. Effect of silver, chromium, nickel and lead on ammonium uptake: interaction of Ag with metals (Me), Cr, Ni and Pb

Concentrations		NH_4 uptake/chlorophyll *a* (by mass) and inhibition (%) at							
[Ag] (nM)	[Me] (μM)	24 h		48 h		72 h		96 h	
0	0	27.45±0.110	—	42.76±0.011	—	52.63±0.140	—	50.00±0.101	—
0	192 (Cr)	11.76±0.110	57.16	22.55±0.120	27.26	44.00±0.004	16.40	43.00±0.005	14.00
0	96 (Pb)	5.92±0.010	72.43	7.86±0.012	81.62	20.31±0.013	61.41	37.06±0.021	25.88
0	17 (Ni)	15.26±0.110	44.41	40.53±0.011	5.22	50.38±0.004	4.28	50.00±0.004	—
36	0	17.84±0.140	35.01	32.00±0.101	25.05	40.00±0.011	24.00	35.00±0.004	30.00
36	192 (Cr)	4.50±0.101	83.61	17.50±0.002	59.07	20.00±0.110	62.00	10.00±0.004	80.00
36	96 (Pb)	7.84±0.002	71.44	12.00±0.011	71.94	25.00±0.004	52.50	34.38±0.005	31.24
36	17 (Ni)	20.00±0.110	27.14	21.25±0.003	50.77	31.58±0.001	40.00	0.80±0.004	84.00

$F_{\text{exposure time } 3,21} = 8.935$, $P < 0.1$
$F_{\text{metal combinations } 7,21} = 4.838$, $P < 0.025$

and of silver in combination with Cr, Ni and Pb on $^{14}CO_2$ uptake of *N. muscorum* are summarized in Table 4. Of all the test metals used, Ag proved to be the most toxic to $^{14}CO_2$ fixation. Maximum inhibition of carbon fixation by Ag occurred up to 60 min, thereafter showing a recovery of the process. It is interesting to note that, after supplementation of Cr into Ag-containing medium, the toxicity was completely ameliorated up to 30 min, following which the toxicity increased until 1 h and then decreased after 2 h. In contrast to this, the interaction effect of Pb + Ag was more inhibitory than the other two combinations studied, showing an increasing trend in toxicity and culminating finally in synergistic inhibition of carbon fixation. However, when Ni and Ag were used together, antagonism was found all through the experiment, being very mild up to 60 min and thereafter becoming more prominent.

Effect of metal interaction on nitrogenase activity

The response of nitrogenase to Cr, Ni, Pb and Ag and the interaction of Ag with Cr, Ni and Pb has been summarised in Table 5. Maximum inhibition of the enzyme activity by all the test metals used individually was noticed after 48 h of treatment. The combination of Cr with Ag brings about synergistic inhibition of nitrogenase throughout the experiment. Inhibition of nitrogenase for this metal combination after 48 h of incubation parallels closely the inhibition generated by the same metals when used separately. However, the combination of Ag + Pb does not show any appreciable change until 24 h, following which toxicity increased and reached the level of synergism after 72 h. It can be inferred from this that toxicity increases with increasing incubation time. The interaction of Ni and Ag as listed in Table 5 shows

Table 4. Effect of silver, chromium, nickel and lead on $^{14}CO_2$ uptake of *N. muscorum:* interaction of Ag with metals (Me) Cr, Ni and Pb

Concentrations		$^{14}CO_2$ uptake (dpm) and inhibition (%) at						
[Ag] (nM)	[Me] (μM)	0 h	0.5 h		1 h		2 h	
0	0	39 ± 12	12180 ± 12	—	19750 ± 16	—	23575 ± 14	—
0	192 (Cr)	39 ± 12	6700 ± 12	45.00	11922 ± 11	39.64	17500 ± 14	25.77
0	96 (Pb)	39 ± 12	4880 ± 11	59.94	14111 ± 10	29.56	21544 ± 16	8.62
0	17 (Ni)	39 ± 12	6688 ± 10	45.10	7966 ± 9	59.67	20077 ± 13	14.84
36	0	39 ± 12	4300 ± 11	64.70	4933 ± 13	75.03	11505 ± 12	51.20
36	192 (Cr)	39 ± 12	13475 ± 7	−10.63[a]	1804 ± 11	90.66	13230 ± 12	43.89
36	96 (Pb)	39 ± 12	1084 ± 6	91.10	88 ± 12	99.56	2268 ± 8	90.38
36	17 (Ni)	39 ± 12	5755 ± 7	52.76	8810 ± 12	55.40	15093 ± 6	35.98

$F_{\text{exposure time } 2,14} = 11.65$, $P < 0.1$
$F_{\text{metal combinations } 7,14} = 5.25$, $P < 0.1$

Table 5. Toxicity of silver, chromium, nickel and lead on nitrogenase: interaction of Ag with metals (Me) Cr, Ni and Pb

Concentrations		C_2H_2 nmol μg protein^{-1} h^{-1} and inhibition (%)						
[Ag] (nM)	[Me] (μM)	0 h	24 h		48 h		72 h	
0	0	0.08 ± 0.02	0.28 ± 0.022	—	0.55 ± 0.045	—	0.64 ± 0.030	—
0	192 (Cr)	0.08 ± 0.02	0.19 ± 0.030	32.14	0.24 ± 0.040	56.36	0.44 ± 0.020	31.25
0	96 (Pb)	0.08 ± 0.02	0.17 ± 0.040	39.29	0.21 ± 0.020	61.82	0.55 ± 0.030	14.06
0	17 (Ni)	0.08 ± 0.02	0.24 ± 0.020	13.57	0.48 ± 0.030	11.82	0.57 ± 0.020	11.56
36	0	0.08 ± 0.02	0.22 ± 0.030	21.43	0.40 ± 0.040	27.27	0.49 ± 0.035	23.44
36	192 (Cr)	0.08 ± 0.02	0.11 ± 0.040	60.71	0.08 ± 0.120	86.00	0.23 ± 0.011	64.06
36	96 (Pb)	0.08 ± 0.02	0.17 ± 0.011	39.29	0.20 ± 0.011	63.44	0.25 ± 0.230	60.94
36	17 (Ni)	0.08 ± 0.02	0.31 ± 0.110	−11.00[a]	0.17 ± 0.020	69.09	0.08 ± 0.022	87.50

$F_{\text{exposure time } 2,14} = 5.52$, $P < 0.25$
$F_{\text{metal combinations } 7,14} = 3.24$, $P < 0.01$

[a] Negative sign shows stimulation over control

strong antagonism only after 24 h of experiment; this antagonism brings about an 11% increase in nitrogenase activity compared to the control. A contrasting situation with regard to the effect of these metal ions after 48 h of treatment was observed where the synergistic inhibition of enzyme activity was found to increase until 72 h of experiment.

Discussion

Of the free-living nitrogen-fixing procaryotes, cyanobacteria assume special significance not only because of their morphological characters but also due to their wide ecological adaptation with regard to distribution in diverse environments. While growing in an aquatic system, these microbes are likely to be influenced by various physico-chemical factors including heavy metals present in the surrounding medium. In such situations they are, therefore, left with no alternative but to suffer the toxicity caused by heavy metals.

Physiological studies involving metal combinations and such parameters as growth (Prasad and Prasad 1982), cell volume, photosynthesis (De Filippis and Pallaghy 1976) and respiration of eucaryotic algae are quite numerous. Though considerably interesting, metal-interaction studies using nitrogen-fixing cyanobacteria have been completely ignored but for the sole report of Stratton and Corke (1979). The data on the effects of these metal combinations on growth present a case either of mild antagonism (Ag + Pb), mixed effect (Ag + Ni) or additive interaction (Ag + Cr).

A critical analysis of the effects of Ag + Pb and Ag + Ni (Figs. 2 and 3) suggests that toxicity always increases on changing the concentration of either metal of these combinations. A close similarity in toxicity regulation by Pb and Ag in the Ag + Pb combination suggests that the metals in this combination are either bivalent or monovalent cations. Since monovalent and bivalent cations are likely to occupy different binding sites, it is likely that any increase or decrease in the concentration of either metal will affect the toxicity. Interaction of Ag + Cr suggests that toxicity is regulated mostly by Ag. A mixed effect on growth, as noticed in Fig. 3, by Ag + Ni may possibly be explained in the light of the stimulatory effect of Ni as observed on different processes of *Nostoc* (Rai and Raizada 1986). Nickel is also known to be required for growth of the cyanobacterium *Oscillatoria* 3NT (Van Baalen and O'Donnel 1978).

The effects of metal combination on nitrate uptake as summarized in Table 1 do not show any definite trend. Mild antagonism for Ag + Cr and Ag + Pb was nocited after 48 and 96 h, respectively. It is further inferred from this table that the combination of Ag + Ni causes a higher percentage inhibition of nitrate uptake; this could be due to inhibition of the enzyme nitrate reductase (unpublished data).

Unlike nitrate uptake, the results of ammonium uptake show a mixed interaction, resulting either in antagonism, synergism or no change. Inhibition of NH_4^+ uptake as noticed here could simply be due either to growth inhibition (see Figs. 1-3) or to inhibition of the enzyme glutamine synthetase, responsible for ammonia assimilation in cyanobacteria (unpublished data). It may be mentioned here that these metal combinations inhibit $^{14}CO_2$ fixation most severely (Table 4). Since, in cyanobacteria, carbon fixation is responsible for the generation of ATP and NADPH, which are ultimately required by such enzymes as nitrogenase, glutamine synthetase and others, this unavailability of energy and reducing power might also lead to inhibition of NH_4^+ uptake. All the metal combinations used (except for Ag + Cr up to 30 min) were found to inhibit the $^{14}CO_2$ uptake most severely. Thus our findings are in accordance with those of Stratton and Corke (1979) who noticed an antagonistic effect of Hg + Cd on growth and a synergistic effect on $^{14}CO_2$ uptake. Statistical treatment of the data (Table 4) suggests that variations in $^{14}CO_2$ uptake observed with respect to exposure time ($F_{2,14} = 11.65$) are more significant than that of metal combinations ($F_{7,14} = 5.25$) and that results are significant at a probability level ($P < 0.1$). A decreased toxicity in the Ag + Cr combination (Table 4) could possibly be due to a requirement for chromium in some metabolic processes of test cyanobacterium as Cr has already been suggested to be useful for eucaryotic algae (Round 1973). However, an increase in the level of inhibition after 1 h of incubation could presumably be due to the fact that, following Ag binding, there may be a change in cell permeability thus allowing more Cr to enter into the cell membrane.

The response of nitrogenase to the Ag + Pb combination initially shows no appreciable change, following which toxicity increased and reached the level of synergistic inhibition (Table 5). Since metals in this combination are monovalent and divalent cations, they share different sites for attachment, thus leading to increased toxicity. The synergistic inhibition of nitrogenase by Ag + Cr closely parallels the results of Stratton

and Corke (1979). That Ag is more toxic than Hg has been confirmed many times (Rai et al. 1981). Besides this, Hg has also been found to be directly involved in the lysis of *Anabaena* cells (Stratton and Corke 1979). Therefore the possibility of lysis of *Nostoc* by Ag cannot be ruled out. Under such circumstances, the nitrogenase would be exposed not only to Ag but also to oxygen present in the surrounding medium, thus becoming increasingly inhibited by the latter. The antagonistic interation by Ag + Ni is due to a requirement for Ni by nitrogenase (Rai and Raizada 1986). However, toxicity due to this metal combination after 48 h could possibly be due to Ag toxicity.

This study therefore suggests that metal combinations produce very unpredictable results. As these laboratory results are so complex, behaviour in the aquatic system is likely to be even more complex since physico-chemical parameters will keep on changing. Considering the sensitivity of $^{14}CO_2$ fixation towards metals, it is suggested that carbon fixation may be employed as a tool for the bioassay of metal toxicity in the laboratory and possibly also in the aquatic environments.

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