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BIOLOGY OF METALS

Impact of bimetallic combinations of Cu, Ni and Fe on growth rate, uptake of nitrate and ammonium, $^{14}CO_2$ fixation, nitrate reductase and urease activity of *Chlorella vulgaris*

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Summary. The toxicity of Cu, Ni and Fe individually, as well as in combination (Cu + Ni, Cu + Fe, Ni + Fe), on growth-rate depression, uptake of NO_3^- and NH_4^+, photosynthesis, nitrate reductase and urease activity of *Chlorella vulgaris* has been studied. All the test metals when used individually showed pronounced toxicity on all the parameters studied. However, their interactive effect was mostly antagonistic except for Cu + Ni (synergism). Pre-addition of Fe offered more protection to the cells against copper and nickel toxicity. The data of statistical analysis reconfirmed that $^{14}CO_2$ uptake is the most sensitive parameter (significant at $P < 0.005$, both for time and treatment) than others in metal toxicity assessment. However, these results suggest further that exposure time and sequence of metal addition are very important in biomonitoring of heavy metal toxicity.

Key words: Heavy metal — *Chlorella vulgaris* — ATP — $^{14}CO_2$ Uptake

Introduction

Biological monitoring of heavy metal toxicity using algae as a tool has been much appreciated during the last few years. Most earlier laboratory toxicity tests are based on the effect of a particular metal on various metabolic processes of the test organism (Stratton and Corke 1979; Rai et al. 1981; Whitton 1984; Rai and Raizada 1986, 1987, 1989; Dubey and Rai 1987). Since in natural aquatic ecosystems, metal ions always occur in association and not in isolation, studies on the interaction of metal combinations should be ecologically more significant.

Despite the fact that metals generally occur in combination in natural ecosystems (Rai et al. 1981; Whitton 1984; Morel 1986; Wong 1987), very few attempts seem to have been made to study the metal-combination effects (Hutchinson and Stokes 1975; Stratton and Corke 1979; Prasad and Prasad 1982). The study of Stratton and Corke on *Anabaena inaqualis* is mainly concerned with the reduction in growth of alga. Though the use of growth in biological monitoring of heavy metal toxicity cannot be denigrated, there is a pressing need for some physiological and biochemical processes that could be both sensitive and quick. The major objective of our laboratory has been to develop a suitable algal test system which could be successfully employed in the laboratory and possibly in the field for assessment of heavy metal pollution. Considering the wide gap in our knowledge of multimetallic toxicity, it was decided to study the toxicity of Cu, Ni and Fe alone as well as in combination using such parameters as growth-rate depression, nutrient (NO_3^-, NH_4^+) uptake, photosynthesis, nitrate reductase and urease activity of a freshwater green alga *Chlorella vulgaris*. The effect of the metal addition sequence on the toxicity of the metals towards the test alga has also been assessed.

Materials and methods

Test system. The unicellular green alga *Chlorella vulgaris* was grown axenically in modified Chu-10 medium (Gerloff et al. 1950) buffered with 4 mM Tris/HCl (pH 6.5) under 14.4 $W\,m^{-2}$ of light intensity and a 14/10-h photoperiod at 26° C ± 2° C. Cultures from the logarithmic phase were used for toxicity tests. Stock solutions of $CuCl_2 \cdot 2H_2O$, $NiCl_2 \cdot 6H_2O$ and $FeCl_3$ were filter-sterilized by passing through Millipore

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membrane filters (0.45 μm) before addition to the culture medium. Metals were added to the experimental systems in the following ways: (a) both the metals were added simultaneously or (b) one metal was added 12 h and 1 h period to addition of the other metal respectively for growth and other physiological experiments. The actual metal concentrations used were the EC_{50} values determined previously for individual metals by the plate/colony count method. However, the growth behaviour of *Chlorella* was studied at three different concentrations viz (a) EC_{50}, (b) one concentration below EC_{50} and (c) one concentration above EC_{50} for all the test metals used.

Growth measurement. Growth of the organism was measured with the help of a Bausch and Lomb spectronic-20 spectrocolorimeter by recording the absorbance at 663 nm. The percentage of growth-rate depression, i, was calculated by using the following equation (Beg et al. 1982):

$$i = \left(1 - \frac{\mu_i}{\mu_C}\right) \times 100$$

where μ_C is the specific growth rate of the control culture and μ_i is the specific growth rate of the treated culture. The specific growth rate (μ) was calculated by

$$\mu = \frac{\ln(n_2/n_1)}{t_2 - t_1}$$

where n_2 and n_1 are the absorbances of the culture suspension at time intervals t_1 and t_2. Interactive effects between two heavy metals were defined as follows:

synergistic if $i_{1,2} > i_1 + i_2$;
additive if $i_{1,2} = i_1 + i_2$;
antagonistic if $i_{1,2} < i_1$ or i_2

where, $i_{1,2}$ is the percentage of growth-rate inhibition induced by interaction between two different metals; i_1 and i_2 represent the percentage of growth-rate inhibition induced by metals 1 and 2 respectively.

Estimation of protein. Protein was estimated by the Folin phenol reagent (Lowry et al. 1951) using bovine serum albumin as standard. The absorbance of the blue colour developed was measured at 700 nm in a colorimeter. The amount of protein was estimated from the standard curve.

Estimation of NH_4^+ and NO_3^- uptake. For studying the effect of metal combinations on NH_4^+ and NO_3^- uptake, cultures were incubated in 5 mM KNO_3 and 1 mM NH_4Cl respectively. Uptake of NH_4^+ was estimated colorimetrically using Nessler's reagent (Herbert et al. 1971). The resulting orange-red colour was measured at 420 nm. NO_3^- was estimated by the brucine/sulphuric acid method of Nicholas and Nason (1957) by recording the absorbance at 410 nm.

$^{14}CO_2$ uptake. Photosynthesis was assayed as a function of uptake of $^{14}CO_2$ from $NaH^{14}CO_3$. 1.0 ml exponentially grown culture of *C. vulgaris* (100 μg protein ml^{-1}) was transferred to scintillation vials (Beckman, USA) supplemented with test metals and 50 μl of $NaH^{14}CO_3$ (specific activity 1.85 Bq). The uptake was measured in a liquid scintillation counter after 1 h of treatment as described by Rai and Raizada (1986). The values are expressed (counts/minute).

Measurement of nitrate reductase activity. Enzyme activity was estimated by method of Camm and Stein (1974). Incubated samples were withdrawn at intervals and nitrite formed was measured by the diazo-coupling method of Lowe and Evans (1964).

Urease activity. This was estimated by measuring the formation of NH_4^+ by breakdown of urea in the medium.

Table 1. Effect of metal combinations on growth behaviour of *Chlorella vulgaris*

Concentration (μg ml^{-1})	Growth rate depression, i (%)	Metal combinations	$i_{1,2}$ (%)	$i_1 + i_2$ (%)	Remarks
		Cu 2.0+ 1.0 Ni	100	78.7	S
Control	—	Cu 2.0+ 2.0 Ni	100	142.2	—
Cu 1.0	10.7	Cu 2.0+ 3.0 Ni	100	168.2	—
Cu 2.0	74.2	Cu 2.0+ 5.0 Fe	34.7	86.2	A
Cu 3.0	NG	Cu 2.0+10.0 Fe	59.0	126.9	A
		Cu 2.0+15.0 Fe	100	144.5	—
		Ni 2.0+ 1.0 Cu	100	82.0	S
Ni 1.0	7.5	Ni 2.0+ 2.0 Cu	100	142.2	—
Ni 2.0	68.1	Ni 2.0+ 3.0 Cu	100	153.1	—
Ni 3.0	93.0	Ni 2.0+ 5.0 Fe	39.2	92.3	A
		Ni 2.0+10.0 Fe	69.9	133.0	A
		Ni 2.0+15.0 Fe	92.7	150.6	A
		Fe 10.0+1.0 Cu	31.1	69.5	A
		Fe 10.0+2.0 Cu	69.9	133.0	A
Fe 5.0	11.7	Fe 10.0+3.0 Cu	89.3	158.3	A
Fe 10.0	58.8	Fe 10.0+1.0 Ni	30.3	66.3	A
Fe 15.0	88.1	Fe 10.0+2.0 Ni	59.0	126.0	A
		Fe 10.0+3.0 Ni	87.5	137.5	A

NG = no growth; S = synergistic; A = antagonistic

NH_4^+ formed was measured by Nessler's reagent (Herbert et al. 1971).

Analysis of variance (ANOVA). The variance ratio (F) for different parameters was calculated by following equation: F=treatment mean square/residual mean square

Results

Effect of metal interaction on growth

The growth behaviour of *Chlorella vulgaris* as influenced by individual metal ions as well as their combinations has been studied and the observed percentage depression in growth rate ($i_{1,2}$), along with its expected values (i_1+i_2), are given in Table 1. The observed values of growth-rate depression for Cu and Ni combination was found to be higher than the expected values. This combination showed a very clear case of synergistic interaction at lower concentrations (expected inhibition=79%, observed inhibition=100%). The mode of interaction did not change even at higher concentrations of Ni. All the combinations of Cu-Fe and Ni-Fe were found to interact antagonistically. However, any change in the concentration of either metal of this combination produced a pronounced growth-rate inhibition (Table 1).

Effect of sequential addition of metals

Copper and nickel. The interactive effect of copper and nickel on the growth behaviour of the test organism with respect to its pre- and post-addition effects is presented in Fig. 1. Simultaneous addition in this case was found to be more toxic. No significant difference in the growth performance of test organisms was noticed by changing the sequence of metal addition (either copper first or nickel).

Copper and iron. Figure 2 summarizes the interactive effect of copper and iron on growth behaviour of *C. vulgaris*. When both the metals were added to the medium simultaneously, 66.7% inhibition of final yield was noticed. Addition of iron prior to copper produced a considerable increase in the growth (Fig. 2). However, pre-addition of copper resulted in greater inhibition of growth than either the addition of Fe first or both the metals simultaneously.

Nickel and iron. Iron and nickel were found to behave in the same way as Cu+Fe (Fig. 3). The pre-addition of nickel was found to be more toxic than pre- or simultaneous addition of iron.

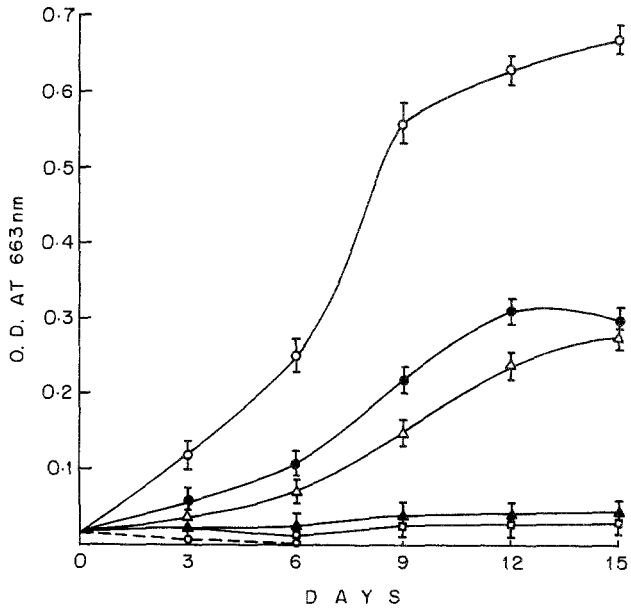

Fig. 1. Interactive effect of Cu and Ni on growth performance of *C. vulgaris*. Control (O—O); Cu alone (●—●); Ni alone (△—△); Cu (pre-addition)+Ni (▲—▲); Ni (pre-addition)+Cu (□—□); simultaneous (O---O)

Effect of metal combination on NO_3^-, NH_4^+ and $^{14}CO_2$ uptake

The effect of sublethal concentrations of test metals alone, as well as in combination, on NO_3^-, NH_4^+ and $^{14}CO_2$ uptake of the test alga is

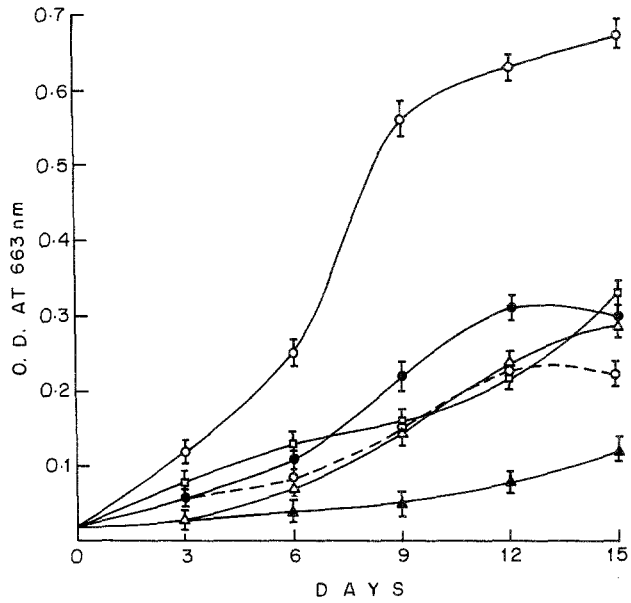

Fig. 2. Interactive effect of Cu and Fe on growth performance of *C. vulgaris*. Control (O—O); Cu alone (●—●); Fe alone (△—△); Cu (pre-addition)+Fe (▲—▲); Fe (pre-addition)+Cu (□—□); simultaneous (O---O)

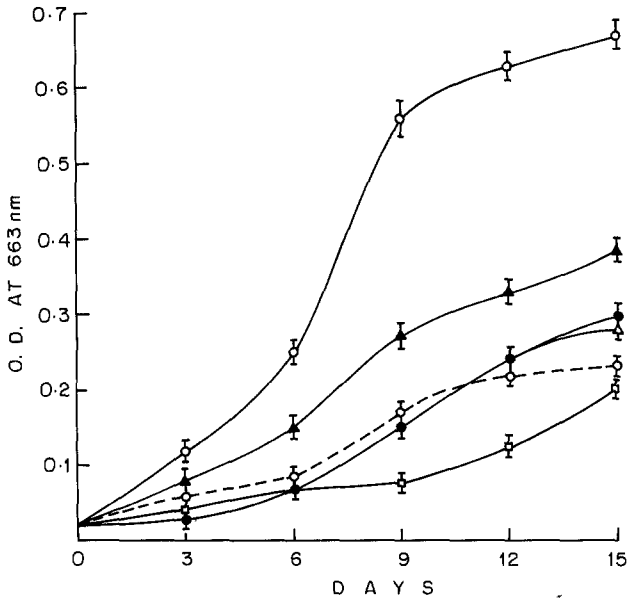

Fig. 3. Interactive effect of Ni and Fe on growth performance of *C. vulgaris*. Control (O—O); Ni alone (Δ—Δ); Fe alone (●—●); Ni (pre-addition)+Fe (□—□); Fe (pre-addition)+Ni (▲—▲); simultaneous (O---O)

Table 3. Effect of metal combinations on nitrate reductase and urease activity after 72 h of treatment

Concentration ($\mu g\ ml^{-1}$)	Activity (inhibition, %) of	
	Nitrate reductase ($\mu g\ NO_2^- \cdot \mu g$ protein^{-1})	Urease ($\mu g\ NH_4^+ \cdot \mu g$ protein^{-1})
Control	0.129 (−)	1.087 (−)
2.0 Cu	0.031 (76.0)	0.490 (55.1)
2.0 Ni	0.043 (66.7)	0.378 (65.3)
10.0 Fe	0.006 (95.4)	0.841 (32.8)
a. Cu+Ni	0.023 (82.2)	0.199 (81.8)
b. Ni+Cu	0.029 (77.6)	0.160 (86.3)
c. Cu+Ni	0.013 (90.0)	0.139 (88.8)
a. Cu+Fe	0.015 (87.4)	0.291 (73.3)
b. Fe+Cu	0.006 (95.4)	0.471 (51.7)
c. Cu+Fe	0.008 (93.8)	0.301 (72.4)
a. Ni+Fe	0.019 (85.3)	0.288 (73.6)
b. Fe+Ni	0.006 (95.4)	0.485 (55.4)
c. Ni+Fe	0.011 (91.5)	0.299 (72.5)

a, b and c are the same as in Table 2. Data in parentheses denote percentage inhibition. ANOVA $F_{12,24}$ (treatment) value is significant at $P<0.005$ for all results

given in Table 2. The sequential addition of Cu or Ni showed lower inhibitory effect on NO_3^- uptake than simultaneous addition. But as in the case of Cu+Fe and Fe+Ni combinations, the uptake of NO_3^- was least inhibited by pre-addition of iron. For ammonium uptake the combinations of Cu+Ni, Cu+Fe and Ni+Fe interacted in the same way as noted for NO_3^- uptake. Maximum inhibition (94%) of $^{14}CO_2$ uptake was noted following a 1-h exposure of algal cells in iron-containing medium. However, on spiking Fe with Cu and Ni the percentage of inhibition decreased

Table 2. Effect of metal combinations on nutrient uptake (NO_3^- and NH_4^+) and carbon fixation following treatment for 72 h and 1 h, respectively

Concentration ($\mu g\ ml^{-1}$)	Uptake (inhibition, %) of		
	NO_3^- ($\mu g\ NO_3^- \cdot \mu g$ protein^{-1})	NH_4^+ ($\mu g\ NH_4^+ \cdot \mu g$ protein^{-1})	$^{14}CO_2$ (cpm $\times 10^{-3}$)
Control	0.70 (−)	1.15 (−)	7.505 (−)
2.0 Cu	0.29 (59.6)	0.68 (40.8)	2.300 (70.3)
2.0 Ni	0.32 (47.2)	0.92 (20.6)	4.939 (34.2)
10.0 Fe	0.37 (47.2)	0.87 (24.1)	1.195 (94.0)
a. Cu+Ni	0.07 (90.0)	0.44 (61.7)	1.710 (77.1)
b. Ni+Cu	0.09 (87.9)	0.37 (67.8)	2.109 (71.9)
c. Cu+Fe	0.02 (97.8)	0.31 (73.1)	1.855 (75.4)
a. Cu+Fe	0.09 (87.9)	0.54 (53.1)	1.979 (73.7)
b. Fe+Cu	0.19 (72.9)	0.63 (45.3)	0.868 (88.4)
c. Cu+Fe	0.13 (81.5)	0.55 (52.2)	0.915 (87.8)
a. Ni+Fe	0.12 (82.9)	0.53 (53.9)	1.991 (73.5)
b. Fe+Ni	0.25 (64.3)	0.61 (46.9)	1.092 (85.4)
c. Ni+Fe	0.15 (77.6)	0.55 (52.2)	1.627 (79.3)

In (a) and (b) the second metal was added 1 h after the addition of the first metal; (c) addition was simultaneous.
Data in parentheses denote inhibition (%). For NO_3^- and NH_4^+, ANOVA $F_{12,24}$ (treatment) value is significant at $P<0.005$; for $^{14}CO_2$, ANOVA $F_{12,24}$ (treatment) and $F_{2,24}$ (time) are significant at $P<0.005$

(Table 2), thus depicting a clear case of antagonism. Interestingly however, the combination of Cu and Ni interacted additively on this process.

Effect of metal combinations on nitrate reductase and urease activities

Response of nitrate reductase and urease to metal combinations is summarized in Table 3. Nitrate reductase was maximally inhibited by Cu + Fe and Ni + Fe combinations. In Cu + Ni combination pre-addition of Cu was found to be more toxic than either adding Ni first or both simultaneously. The interactive effect of test metals on urease was similar to nitrate reductase. However, the pre-addition of Fe proved less inhibitory for urease activity.

Dicussion

The significant difference between observed and expected toxicity values of metal combinations showed a complex interactive behaviour of metal mixtures. A critical analysis of the effect of Ni + Cu combination reveals a clear synergism on the growth behaviour of *C. vulgaris*. This synergistic effect of Cu + Ni combination parallels the findings of Hutchinson (1973), Stokes (1975) and Hutchinson and Stokes (1975). However, in the Cu + Fe and Ni + Fe combination antagonistic interaction has been observed (actual percentage of growth-rate depression was less than the expected values). The reduction in Cu toxicity following Fe addition may be due to reduced Cu uptake by *Chlorella* in the presence of Fe (De Filipps and Pallaghy 1976). Since Ni has the same valency state as Cu, the antagonistic interaction of Ni + Fe might be explained in the same way as for Cu + Fe.

The sequence of metal addition seems to have considerable bearing on the metal toxicity. Our results clearly indicated that pre-addition of Fe decreased the toxicity. Similarly, in the case of Cu + Ni combination, the toxicity was reduced a little when Cu and Ni were added in a sequential fashion as compared to their simultaneous addition. The protection offered by one metal against another can be explained in the light of reduction in available cellular binding sites as they are occupied by the metal added first.

We did not notice any deviation in the results dealing with effects of metals on uptake of NO_3^-, NH_4^+ and $^{14}CO_2$, or in nitrate reductase and urease activities. Thus reduction in toxicity following addition of Fe are the artifact of some interactive strategy of metals. As nitrate reductase and $^{14}CO_2$ uptake were found to be inhibited maximally by Fe alone, this continued even in combination with other metals.

The response from photosynthetic experiments offers further support to the above findings (clear antagonism in Cu + Fe and Ni + Fe combination). However, in the Cu + Ni combination, additive interaction has been observed for this process. It has been reported earlier that, when copper and nickel are present together, the nickel initially accumulate on the cell wall while the copper moves into the cytoplasm. The increased internal concentration of copper influences the permeability of the plasmalemma which allows nickel to flow into the cell, thus resulting in synergistic inhibition (Stokes 1975). The shifting of synergism to additive interaction may be due to insufficient exposure time, which is necessary for mass flow of nickel into the cell.

Based on the present study the following important conclusions can be made: (a) a generalized trend in assessing toxicity cannot be drawn from single-metal-supplemented data; (b) the interaction of metals not only depends on the order of metal addition but also on the test criterion chosen; (c) $^{14}CO_2$ uptake is the most sensitive parameter in toxicity evaluation and, hence, it should be employed as a tool for the bioassay of metal toxicity in the laboratory and, possibly, in the natural ecosystem.

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