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Assessment of Hg^{2+} toxicity to a N_2-fixing cyanobacterium in long- and short-term experiments

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Toxicological responses of the filamentous N_2-fixing cyanobacterium *Nostoc calcicola* Bréb. towards Hg^{2+} were studied to enumerate the decisive lethal events. In low-dose, long-term experiments (0.05–0.25 μM Hg^{2+}, 10 days), photoautotrophic growth was severely inhibited with concurrent loss of photosynthetic pigments (phycocyanin > chlorophyll *a* > carotenoids) and nucleic acids. The termination of growth after a day 4 exposure to 0.25 μM Hg^{2+} has been attributed to the complete inhibition of *in vivo* photosynthetic activity in the cyanobacterium (O_2 evolution > $^{14}CO_2$ incorporation). The elevated Hg^{2+} concentrations irreversibly damaged the cell membrane as observed under light microscopy, and as indicated by the leakage of intracellular electrolytes and phycocyanin. In high-dose, short-term experiments (0.5–20.0 μM Hg^{2+}, up to 6 h), the *in vivo* activities of selected enzymes (glutamine synthetase > nitrate reductase > nitrogenase) were less inhibited by Hg^{2+} than the uptake of nutrient ions ($NH_4^+ > NO_3^- > PO_4^{3-}$).

Keywords: Hg^{2+} toxicity, cyanobacterium, *Nostoc calcicola*, growth, photopigments, nucleic acids, photosynthesis, membrane integrity, nutrient uptake, enzymes

Introduction

Mercury, an extremely toxic pollutant in the biosphere, enters the hydrosphere from various natural as well as anthropogenic sources, and is recycled through geological and biological processes (Jeffries 1982, Ramamoorthy *et al.* 1982). It is also an important constituent of many commercial biocides (see Rai *et al.* 1981). Since algae are the primary producers of aquatic ecosystems, their susceptibility towards heavy metals has been the subject of numerous reports (Sorentino 1979, Rai *et al.* 1981, Duxbury 1985, Pandey *et al.* 1992). Ionic or organic forms of mercury are more toxic than metallic mercury and its toxicity is noticeable even in the concentration range of 1.84–184.15 nM (Knowles & Zingmark 1978). While elevated mercury concentrations are typically biocidal (Nuzzi 1972), the lower levels may be mutagenic or teratogenic (Leonard *et al.* 1983). At the same time, the algae could also be used as biomonitors and/or scavengers of heavy metals in freshwaters (Whitton 1983) and periphyton communities (Ramelow *et al.* 1987).

Cyanobacteria, being little explored in this context, have gradually become useful in the study of heavy metal induced physiological and biochemical aberrations (Rachlin *et al.* 1982, S. P. Singh *et al.* 1989, Asthana *et al.* 1990). However, comprehensive studies on Hg^{2+} toxicity to cyanobacteria, are fragmentary (Stratton *et al.* 1979, Singh *et al.* 1987, D.P. Singh *et al.* 1989, Singh & Singh 1992). Moreover, such reports are exclusively devoted to the action site(s) of mercury on physiological/biochemical details, and a holistic report on the identification and characterization of the main lethal events resulting due to Hg^{2+} toxicity is still missing. The present communication examines some decisive effects of dissolved inorganic mercury on photoautotrophic growth, pigment and nucleic acid contents, as well as nutrient uptake and various enzyme activities in the filamentous, heterocystous cyanobacterium *Nostoc calcicola* Bréb.

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Materials and methods

Organism and growth conditions

The diazotrophic cyanobacterium *N. calcicola* Bréb., a local isolate, was grown axenically in 500 ml Erlenmeyer flasks, containing 200 ml nitrogen-free liquid medium (Allen & Arnon 1955). The cultures were routinely maintained at 24 ± 1 °C in a culture room, illuminated with cool fluorescent light (intensity, 50 μmol photon m^{-2} s^{-1} on the surface of culture vessels) with a 18/6 h light/dark cycle.

Growth parameters

For long-term experiments (up to 10 days), the exponentially growing *N. calcicola* cells were inoculated in a fresh, sterile medium (initial cell density, 35 μg protein ml culture^{-1}), supplemented with Hg^{2+} (as $HgCl_2$) to obtain 0.05–0.25 μM Hg^{2+} in the medium. Such suspensions were incubated for 10 days as routine cultures and 5.0 ml aliquots were withdrawn everyday, and analyzed in terms of absorption at 650 nm (Spectronic 20, Bausch & Lomb, USA) and protein content (Lowry *et al.* 1951; modified by Herbert *et al.* 1971). Specific growth constant (k) was calculated (h^{-1}) according to Kratz and Myers (1955). A V_0/V plot of 2 for k was used to calculate the 50% inhibitory initial lethal concentration (LCI_{50}, i.e. the particular concentration of Hg^{2+} which inhibited any process by 50% out of the Hg^{2+} concentration that was initially established in the medium) of Hg^{2+}, where V_0 was the value for control and V was the value for Hg^{2+}-treated cells.

Photosynthetic pigments

After 10 day exposures as above, 5.0 ml aliquots were centrifuged and the pellet suspended in 5.0 ml 80% acetone (v/v). Such suspensions were incubated at 4 °C for 24 h, followed by centrifugation and the supernatant fluid subjected to recording the absorption spectrum (400–700 nm) as well as determining the absorption at 663 nm to quantify chlorophyll *a* (Myers & Kratz 1955) and at 460 nm for carotenoids (Allen 1968) in a spectrophotometer (Lambda 3 UV/Visible, Perkin-Elmer, USA). Phycocyanin was extracted from the residue remaining after acetone treatment by suspending it in 5.0 ml distilled water, followed by repeated (5 times) freezing (4 °C), thawing (37 °C) and rigorous shaking. Such suspensions were centrifuged and the absorbance of the resultant supernatant fluid determined at 620 nm to quantify phycocyanin according to Brody & Brody (1961).

Nucleic acids

After 10 day exposure as above, 5.0 ml aliquots were used to determine the DNA and RNA contents (Herbert *et al.* 1971). The samples were centrifuged and pellet mixed with 5.0 ml ethanol:ether mixture (3:1, v/v). The pellet from such suspensions was again treated with the solvent mixture and finally suspended in 4.0 ml 3 N perchloric acid. This was repeated twice, followed by freezing (4 °C), thawing (37 °C) and shaking. The suspensions were centrifuged, and the pellet used for DNA determination and the supernatant fluid for RNA determination.

DNA determination. The pellet was resuspended in 3.0 ml 3 N perchloric acid and incubated at 80 °C for 30 min in a water bath, followed by centrifugation. The supernatant fluid was separated and the process repeated once more. A 5.0 ml aliquot of this fluid was mixed with 2.5 ml diphenylamine reagent and incubated at 100 °C for 30 min in a water bath. After cooling, the absorption of resultant solution was determined at 610 nm against a blank, and the DNA content quantified using a standard curve obtained with commercial DNA (product no. D 8259; Sigma, USA).

RNA determination. A 3.0 ml aliquot of the supernatant fluid was mixed with 2.5 ml orcinol reagent and incubated at 100 °C (30 min) in a water bath. After cooling, its absorption was colorimetrically determined at 610 nm against a reagent blank and the RNA content quantified by reference to a standard curve obtained with commercial RNA (product no. R 7125; Sigma, USA).

Photosynthetic O_2 evolution and $^{14}CO_2$ incorporation

Photosynthetic O_2 evolution and $^{14}CO_2$ incorporation in *N. calcicola* cells (100 μg protein ml culture^{-1}) treated with 0.05–0.25 μM Hg^{2+} for 96 h was determined in terms of O_2 evolved, using a Clark-type O_2 electrode connected to an Oxygraph (Model 5/6, Gilson, USA) and by measuring the incorporation of ^{14}C from $NaH^{14}CO_3$ (assay, 0.05 μCi ml^{-1}; BARC, India) in a liquid scintillation counter (Model LS 7000, Beckman, USA). The general methodology adopted was that of Singh & Singh (1987).

Leakage of phycocyanin and electrolytes

Log phase *N. calcicola* cells were inoculated in a fresh medium (500 μg protein ml culture^{-1}) containing 0.5–20.0 μM Hg^{2+}. Such sets were phototrophically incubated for 12 h, and 5.0 ml aliquots were taken every 2 h and centrifuged, and the absorption of the supernatant determined at 620 nm and quantified according to Brody & Brody (1961). Electrolyte leakage, under identical experimental conditions, was determined using a conductivity meter (sensitivity range, 0.02–200 mS mg protein^{-1}; Model 304, Systronics, India) following the methodology adopted by Singh & Singh (1990).

Uptake kinetics of NO_3^-, NH_4^+ and PO_4^{3-}

The *N. calcicola* cells were inoculated in a fresh medium (500 μg protein ml culture^{-1}), containing 0.5–5.0 μM Hg^{2+} and incubated phototrophically for 6 h. The uptake of NO_3^- (Nicholas & Nason 1957), NH_4^+ (Burris & Wilson 1957) and PO_4^{3-} (APHA 1989) by *N. calcicola* was determined by quantifying their disappearance from re-

spective ambient media containing either 20.0 mM NO_3^-, 1.0 mM NH_4^+ or 2.0 mM PO_4^{3-}. The aliquots withdrawn (5.0 ml each for NO_3^- and NH_4^+, and 1.0 ml for PO_4^{3-}) after a regular interval of 1 h were centrifuged and the supernatant fluid subjected to colorimetric determinations. The respective LCI_{50} Hg^{2+} concentrations for the uptake of NO_3^- (2.5 μM), NH_4^+ (1.5 μM) and PO_4^{3-} (3.5 μM) were calculated and the uptake kinetics studied by establishing them in the cyanobacterial suspensions having graded concentrations of the respective nutrient ions (NO_3^-, 1.25–20.0 mM; NH_4^+, 0.125–1.0 mM and PO_4^{3-}, 0.25–2.0 mM).

Glutamine synthetase, nitrate reductase and nitrogenase activity

Similar to nutrient uptake kinetics, high-dose short-term experiments (up to 3 h) were conducted on these enzymes to account for their respective *in vivo* activities in untreated and Hg^{2+}-treated (2.5–20.0 μM) *N. calcicola* cells (500 μg protein ml culture^{-1}). While glutamine synthetase (transferase) activity (Shapiro & Stadtman 1970) and nitrate reductase activity (Snell & Snell 1949) were estimated colorimetrically in the cell pellet obtained from 1.0 ml aliquots withdrawn every 30 min, nitrogenase activity (Stewart *et al.* 1968) was recorded in 2.0 ml of treated suspensions, using the acetylene reduction technique in a gas chromatograph (Model 540, Tracor, USA), fitted with Porapak R column and connected to an integrator (Hewlett-Packard, USA). The general methodology has been described elsewhere (Singh *et al.* 1987, Singh & Singh 1990).

Chemicals

Chemicals used in the growth medium, for the estimation of nucleic acids and to establish nutrient uptake kinetics were purchased from BDH, UK, while those for $^{14}CO_2$ incorporation and enzyme assays were the quality products of Sigma, USA.

Results and discussion

Effect of Hg^{2+} on photoautotrophic growth

In low-dose, long-term experiments on photoautotrophic growth, *N. calcicola* cells showed some tolerance up to 0.10 μM Hg^{2+} (≤ 50% inhibition), beyond which a sharp decline (> 50% inhibition) was evident (Figure 1a). The initial lag of 0–72 h for the Hg^{2+}-treated cyanobacterium possibly indicates its temporary metabolic adjustment phase against Hg^{2+} toxicity. Barring heterocysts, the presence of no intact cyanobacterial cell seen under light microscopy in the sets exposed to 0.25 μM Hg^{2+} for 96 h is suggestive of their enhanced Hg^{2+} tolerance over vegetative cells. The LCI_{50} Hg^{2+} concentration (0.10 μM) is based on a V_0/V plot as well as the

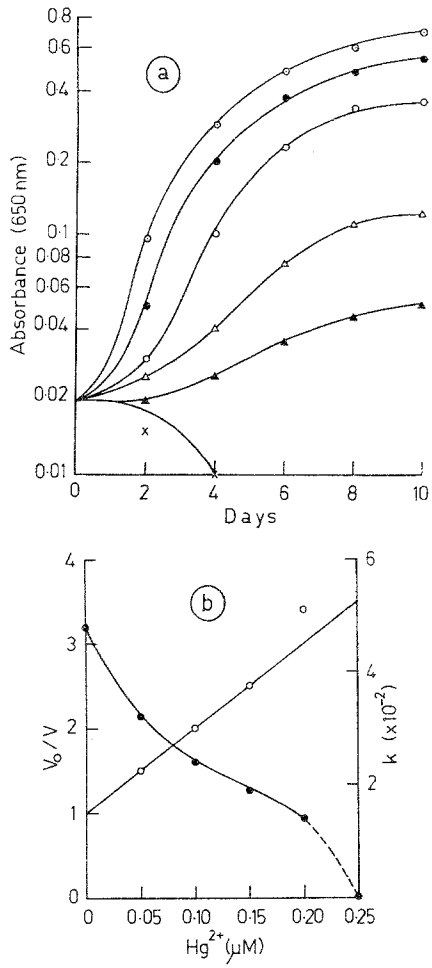

Figure 1. (**a**) Photoautotrophic growth of Hg^{2+}-treated *N. calcicola*: Untreated control (⊙), 0.05 μM (●), 0.10 μM (○), 0.15 μM (△), 0.20 μM (▲)and 0.25 μM (×); $F_{\text{days } 4,16} = 8.31$ and $F_{\text{concentrations } 4,16} = 13.86$, $P < 0.005$. (**b**) Specific growth rate k (●) of Hg^{2+}-treated *N. calcicola* in relation to V_0/V plot (○).

Hg^{2+} concentration corresponding to $k = 0.024$, which is half the value obtained for untreated cyanobacterium ($k = 0.048$; Figure 1b). In a similar study, the photoautotrophic growth of the planktonic cyanobacterium *Anabaena inaequalis* was found to be considerably reduced in the presence of 7.37 nM Hg^{2+} and completely inhibited at 29.46 nM (Stratton *et al.* 1979). Such Hg^{2+}-induced growth inhibition is suggested to result as a consequence of severe inhibition of *in vivo* photosynthetic activity, as observed for *A. inaequalis* (Stratton *et al.* 1979), *N. calcicola* (Singh & Singh 1987) and *Cylindrospermum* sp. (D.P. Singh *et al.* 1989).

Effect of Hg^{2+} on photosynthetic pigments

The absorption spectra for the acetone-soluble photopigments revealed only quantitative reductions

(without qualitative changes) in the peak heights corresponding to the increasing Hg^{2+} concentrations (Figure 2a). Figure 2(b) shows a comparative account of the total quantitative loss of photosynthetic pigments after a 10 day exposure to a series of Hg^{2+} concentrations (0.05–0.25 μM); dotted lines show cell lysis. It is evident that, compared with chlorophyll *a* and phycocyanin, the carotenoids are somewhat more resistant against all Hg^{2+} concentrations tested. Phycocyanin and chlorophyll *a*, on the other hand, were almost equally sensitive to Hg^{2+}. Chlorophyll *a* and its derivatives generally interfere in the absorbance at 460 nm and the loss of absorbance for degrading chlorophyll at 663 nm is much faster than the loss at 460 nm (see Murthy *et al.* 1989; Murthy *et al.* 1990). Therefore, the carotenoids seem to be more resistant than chlorophyll *a*. Based on such findings, as well as on the basis of the drop in the total pigment contents at the LCI_{50} Hg^{2+} concentration (0.10 μM), the Hg^{2+}

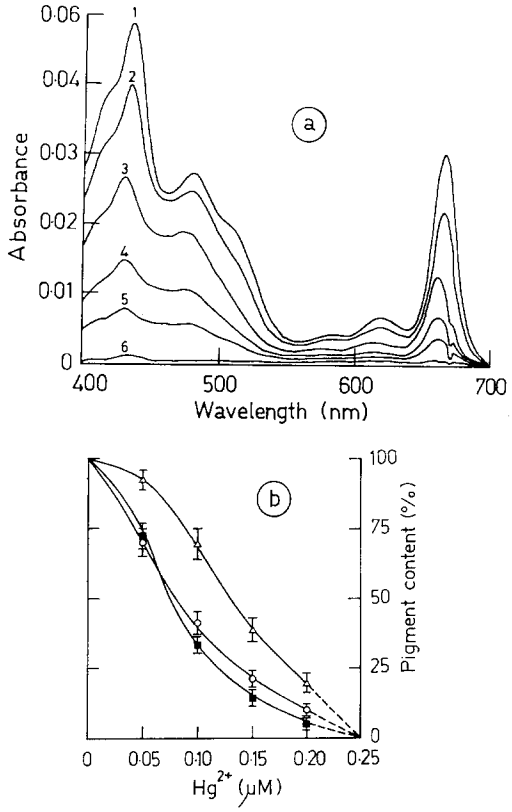

Figure 2. (**b**) Absorption spectra for acetone-soluble photopigments in *N. calcicola* exposed to Hg^{2+} for 10 days: untreated control (1), 0.05 μM (2), 0.10 μM (3), 0.15 μM (4), 0.20 μM (5) and 0.25 μM (6). (**b**) Chlorophyll *a* (○), carotenoids (△) and phycocyanin (■) contents in *N. calcicola* exposed to Hg^{2+} for 10 days. Values represent the mean ± 3SE; $F_{\text{photopigments } 2,10} = 7.13$, $P < 0.25$ and $F_{\text{concentrations } 5,10} = 80.22$, $P < 0.005$.

sensitivity of photosynthetic pigments in the cyanobacterium has been arranged as: phycocyanin > chlorophyll *a* > carotenoids.

Effect of Hg^{2+} on nucleic acids

From Table 1 it is evident that the Hg^{2+}-induced inhibition of photoautotrophic growth of *N. calcicola* was not *at par* with that of the drop in total DNA and RNA contents determined after a 10 day exposure to Hg^{2+}. Though concentrations up to 0.10 μM Hg^{2+} reduced the cyanobacterial growth by less than 50%, not more than 23.8% decline could be recorded for the nucleic acids, and concentrations exceeding 0.15 μM Hg^{2+} were required for a comparable extent of decline. Both the nucleic acids, however, responded almost similarly towards Hg^{2+} toxicity at all concentrations tested (0.05–0.25 μM) and remained strongly correlated with each other ($r = 0.995$, $P < 0.01$) in terms of Hg^{2+} inhibition. On the basis of such findings, it is difficult to conclude whether the degradation of nucleic acids is also being affected, although the data on the 10 day exposure of cells to Hg^{2+} suggest that their biosynthesis is adversely affected; such inhibitions may not be the only cause of inhibited photoautotrophic growth.

Effect of Hg^{2+} on photosynthesis

Such findings tempted us to correlate the photoautotrophic growth with *in vivo* photosynthetic activity in the Hg^{2+}-treated *N. calcicola* under identical experimental conditions (Table 2). Exposure to 0.10 μM Hg^{2+} for 96 h reduced the cyanobacterial growth by 65.5% and O_2 evolution by 62.8%, but more than 0.10 μM Hg^{2+} was required to similarly inhibit $^{14}CO_2$ incorporation. However, the three parameters shared an equal extent of 100% inhibition at 0.25 μM Hg^{2+}. Such variations in the extent of inhibitions with respect to the increase in Hg^{2+} concentrations from 0.05 to 0.25 μM, were significant at $P < 0.5\%$. The comparatively low correlation coefficient (r) value (0.915) between growth and $^{14}CO_2$ incorporation than that of the growth versus O_2 evolution ($r = 0.998$) suggests that Hg^{2+} inhibition of O_2 evolution was the prime cause of growth inhibition. The inhibition of *in vivo* photosynthetic activities by dissolved inorganic mercury has also been considered as one of the key metabolic events leading to inhibition of photoautotrophic growth of other cyanobacteria (Stratton *et al.* 1979, D.P. Singh *et al.* 1989).

Table 1. Photoautotrophic growth, DNA and RNA contents of *N. calcicola* exposed to Hg^{2+} for 10 days (relative values)

Hg^{2+} concentrations (μM)	Photoautotrophic growth		DNA content		RNA content	
	Absorbance at 650 nm	%[a]	μg DNA mg protein^{-1}	%[a]	μg RNA mg protein^{-1}	%[a]
Control	0.70	100.0	4.8	100.0	21.0	100.0
0.05	0.58	82.9	4.4	91.7	19.0	90.5
0.10	0.36	51.4	3.8	79.2	16.0	76.2
0.15	0.12	17.1	2.6	54.2	11.5	54.8
0.20	0.045	6.4	1.25	26.0	7.5	35.7
0.25	0.0	0.0	0.0	0.0	0.0	0.0

Statistical analysis:

(i) ANOVA: $F_{\text{parameters } 2,8} = 7.66$, $P < 0.25$.
$F_{Hg^{2+} \text{ concentrations } 4,8} = 37.98$, $P < 0.005$.

(ii) Correlation coefficient (r) between DNA and growth in terms of Hg^{2+} inhibition: 0.955, d.f. 3, $P < 0.01$.
Correlation coefficient (r) between RNA and growth in terms of Hg^{2+} inhibition: 0.980, d.f. 3, $P < 0.01$.
Correlation coefficient (r) between DNA and RNA in terms of Hg^{2+} inhibition: 0.994, d.f. 3, $P < 0.01$.

[a]Values used for statistical calculations.

Leakage of electrolytes and phycocyanin under Hg^{2+} stress

Little is known about Hg^{2+} concentrations damaging the cell membrane without cell lysis and leading to the uncontrolled leakage of metabolites (Shieh & Barber 1973, Simkiss 1979). From Table 3 it is evident that, unlike phycocyanin, negligible efflux of electrolytes also occurred in the untreated *N. calcicola* cells. The leakage of phycocyanin and electrolytes from Hg^{2+}-treated cyanobacterial cells was directly proportional to the exposure time (0–12 h) in the concentration range of 0.5–20.0 μM. While both the responses were almost parallel to the increase in Hg^{2+} stress up to 10.0 μM, the net percent leakage of phycocyanin after 12 h exposure to below 10.0 μM Hg^{2+} remained invariably higher over that of electrolytes. It is clear that the pronounced effects of Hg^{2+} on both leakages could be

Table 2. Photoautotrophic growth of *N. calcicola* relative to that of photosynthetic O_2 evolution and $^{14}CO_2$ incorporation after 96 h exposure to Hg^{2+}

Hg^{2+} concentrations (μM)	Photoautotrophic growth		O_2 evolution		$^{14}CO_2$ incorporation	
	Absorbance at 650 nm	%[a]	μmol O_2 mg protein h^{-1}	%[a]	dpm mg protein^{-1}	%[a]
Control	0.29	100.0	54.6	100.0	745238	100.0
0.05	0.20	69.0	40.8	74.7	650593	87.3
0.10	0.10	34.5	20.3	37.2	541788	72.7
0.15	0.04	13.8	7.1	13.0	257107	34.5
0.20	0.025	8.6	3.7	6.8	73033	9.8
0.25	0.0	0.0	0.0	0.0	0	0.0

Statistical analysis:

(i) ANOVA: $F_{\text{parameters } 2,8} = 4.90$, $P < 0.25$.
$F_{Hg^{2+} \text{ concentrations } 4,8} = 56.22$, $P < 0.005$.

(ii) Correlation coefficient (r) between O_2 evolution and growth in terms of Hg^{2+} inhibition: 0.998, d.f. 3, $P < 0.01$.
Correlation coefficient (r) between $^{14}CO_2$ incorporation and growth in terms of Hg^{2+} inhibition: 0.915, d.f. 3, $P < 0.05$.
Correlation coefficient (r) between O_2 evolution and $^{14}CO_2$ incorporation in terms of Hg^{2+} inhibition: 0.933, d.f. 3, $P < 0.05$.

[a]Values used for statistical calculations.

Table 3. Leakage of intracellular electrolytes and phycocyanin from *N. calcicola* cells to Hg^{2+} for 12 h

Hg^{2+} concentrations (μM)	Electrolyte leakage			Phycocyanin leakage	
	Rate (μS mg protein^{-1}h^{-1})	Total leakage (%)	Net leakage (%)[a]	Rate (μg mg protein^{-1}h^{-1})	Total leakage (%)[a]
Control	0.75	6.1	0.00	0.00	0.0
0.5	3.25	20.6	14.5	1.25	26.3
1.25	7.56	47.7	41.6	2.38	50.0
2.5	11.38	72.9	66.8	3.25	68.4
5.0	13.00	85.1	79.0	3.81	80.3
10.0	13.50	97.2	91.1	4.75	100.0
15.0	13.63	98.1	92.0	4.00	90.8
20.0	13.88	100.0	93.9	3.83	80.3

Statistical analysis:
(i) ANOVA $F_{\text{parameters } 1,7} = 0.57$, $P > 0.25$.
$F_{Hg^{2+} \text{ concentrations } 7,7} = 78.90$, $P < 0.005$.
(ii) Correlation coefficient (r) between the leakage of electrolytes and phycocyanin: 0.977, d.f. 6, $P < 0.01$.

[a]Values used for statistical calculations.

arranged into two main groups: (i) Hg^{2+}, less than 5.0 μM, permeabilized the cell membrane for uncontrolled leakage of electrolytes and phycocyanin, and (ii) concentrations exceeding 5.0 μM caused cell lysis. Such mercury–membrane interactions are reported to alter the membrane potential in *Cylindrospermum* sp. (D.P. Singh *et al.* 1989). Therefore, it is suggested that the mode of Hg^{2+} toxicity to *N. calcicola* in the present case is mainly with respect to the cell membrane.

Effect of Hg^{2+} on nutrient uptake kinetics

A comparison of the uptake rates (1.0, 0.038 and 0.31 μmol nutrient μg protein^{-1} h^{-1}, respectively) in untreated *N. calcicola* cells at 20.0 mM NO_3^-, 1.0 mM NH_4^+ and 2.0 mM PO_4^{3-} indicates that the rate of NO_3^- uptake was fastest, followed by PO_4^{3-}; NH_4^+ uptake being the slowest (Figure 3a–c). At such nutrient concentrations 2.5, 1.5 and 3.5 μM Hg^{2+} were, respectively, needed to inhibit NO_3^-, NH_4^+ and PO_4^{3-} uptake by 50%. On the basis of such observations, the Hg^{2+} sensitivity sequence of nutrient uptake in the cyanobacterium has been arranged as: $NH_4^+ > NO_3^- > PO_4^{3-}$. The double reciprocal plots show that the K_m values for each nutrient uptake remained constant in the presence of such 50% inhibitory Hg^{2+} concentrations (i.e. 3.57 mM for NO_3^-, 0.285 mM for NH_4^+ and 1.25 mM for PO_4^{3-} uptake) and only the V_{max} values declined compared with untreated controls (0.667 and 1.25 for NO_3^-, 0.0227 and 0.0435 for NH_4^+ and 0.25 and 0.50 μmol substrate μg protein^{-1} h^{-1} for PO_4^{3-} uptake). The

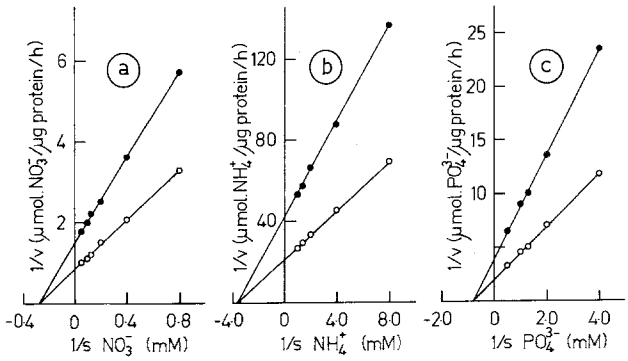

Figure 3. Double reciprocal plots for the uptake of NO_3^- (**a**), NH_4^+ (**b**) and PO_4^{3-} (**c**) in untreated (○) and Hg^{2+}-treated (●) *N. calcicola*.

lowest K_m for NH_4^+ uptake points towards the comparatively high affinity of this nitrogen source for uptake. The unchanged K_m and concurrent reduction in the V_{max} values indicate non-competitive interaction between Hg^{2+} and these nutrient ions for cellular uptake site(s). However, in a similar study, the non-competitive Cd^{2+} inhibition of NH_4^+ and PO_4^{3-} uptake in the unicellular cyanobacterium *Anacystis nidulans* has been suggested to arise mainly due to metal-induced depletion of ATP and reductants (Singh & Yadava 1984).

Effect of Hg^{2+} on glutamine synthetase, nitrate reductase and nitrogenase activities

Compared with the parameters above, the *in vivo* glutamine synthetase, nitrate reductase and nitro-

genase activities showed high Hg^{2+} tolerance during high-dose (2.5–20.0 μM) short-time (3 h) experiments, because very high concentrations (above 2.5 μM Hg^{2+}) were needed to reveal any noticeable extent of inhibition (12.9%) in the enzyme activities (Table 4). Their tolerant nature was also evident from a considerable percentage of enzyme activity (33.3%) still remaining even at the highest Hg^{2+} concentration used (20.0 μM). However, on the basis of respective 50% inhibitory Hg^{2+} concentrations for glutamine synthetase, nitrate reductase and nitrogenase (10.0, 12.5 and 15.0 μM), their Hg^{2+} sensitivity may be arranged as: glutamine synthetase > nitrate reductase > nitrogenase. Though a 3 h exposure to such elevated Hg^{2+} concentrations is only 50% inhibitory in dense cyanobacterial cultures (500 μg protein ml culture^{-1}), this is too high compared with that adopted for growth inhibition studies. Therefore, keeping all the parameters of the present investigation in view, it is difficult to infer whether this was caused by general cell damage or by the specific inhibitions of the enzymes compared (Singh *et al.* 1987).

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Table 4. *In vivo* enzyme activities of glutamine synthetase, nitrate reductase and nitrogenase in *N. calcicola* cells exposed to Hg^{2+} for 3 h

Hg^{2+} concentrations (μM)	Glutamine synthetase		Nitrate reductase		Nitrogenase	
	μmol γ-glutamylhydroxamate mg protein^{-1} min^{-1}	%[a]	μmol NO_2^- mg protein^{-1} min^{-1}	%[a]	nmol C_2H_4 mg protein^{-1} min^{-1}	%[a]
Control	1.98	100.0	151.11	100.0	5.4	100.0
2.5	1.59	80.3	126.63	83.8	4.7	87.1
5.0	1.27	64.1	107.89	71.4	4.3	79.6
10.0	1.00	50.5	84.77	56.1	3.5	64.8
15.0	0.81	40.9	68.30	45.2	2.7	50.0
20.0	0.66	33.3	55.46	36.7	2.4	44.4

Statistical analysis:
(i) ANOVA: $F_{\text{parameters } 2,10} = 16.42$, $P < 0.10$.
$F_{Hg^{2+} \text{ concentrations } 5,10} = 202.56$, $P < 0.005$.

[a] Values used for statistical calculations.

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Appendix 1: statistical analysis

Analysis of variance (ANOVA)

The data for various Hg^{2+} treatments and exposure time were verified for their significance at a particular probability level, and the variance ratio (F) calculated as:

$$F = \frac{\text{treatment mean square}}{\text{residual mean square}}$$

Correlation coefficient (r)

The correlation coefficient (r) was calculated to account for the extent of correlation between two particular variables (X and Y), or to establish the correlation of one parameter (e.g. growth) with two varying parameters (e.g. O_2 evolution and $^{14}CO_2$ incorporation). The following formula was employed for the purpose:

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

Note: d.f. for r is $(n - 2)$ and for others $(n - 1)$.