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Detoxification of Copper by *Nitzschia obtusa* Wm. Sm., a Pennate Diatom

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Phytoplankton have the ability to detoxify metals to survive in waters laden with high concentrations of heavy metals. Adaptations of algae to unfavorable metal environment include exclusion of heavy metals by binding metals outside the cell with extracellular materials such as polypeptides, polysaccharides and hydroxamates which have metal binding sites (Fogg 1966; Wang and Tischer 1973; Murphy et al. 1976). A change in metal's oxidation state alters the permeability of cells to metal ions (Davies 1976). Detoxification by forming metal binding proteins has been reported in Anacystis nidulans (Maclean et al. 1972); Scenedesmus sp. (Stokes et al. 1977); Euglena gracilis (Gingrich et al. 1986); Synechococcus sp. Bariaud et al. 1985) and Cyclotella meneghiniana (Rao et al. 1988). Shrift (1959) and Davies (1976) pointed out the significance of sulphydryl (-SH) groups in algae as binding sites for certain metals. Silverberg et al. (1976) reported that Cu formed intracellular complexes in Scenedesmus acutiformis B-4. Compartmentalization of heavy metals to sequester metals was observed in Chlorella saccharophila, Nitzschia closterium, Navicula incerta (Jensen et al. 1982) and Diatoma tenue (Sico - Goad and Stoermer 1979). In cyanophycean algae Cu caused an increase in bodies containing sulfoquinovocyl diglyceride to which Cu was bound (Wolk 1973). The present study is directed at finding out the possible detoxification mechanisms in Nitzschia obtusa Wm.Sm. to copper.

MATERIALS AND METHODS

Nitzschia obtusa Wm.Sm. (A 1271) obtained from the Culture Collection Centre, Centre of Advanced Study in Botany, University of Madras, Madras was made axenic and maintained in freshwater medium (Reimann et al 1963; p.76). For studies on Cu accumulation 10 mL of exponentially grown cells of N. obtusa was inoculated into basal media amended with 0.5 mg/L Cu and incubated for 4 d at 24 ± 1°C and 3000 lux light intensity in a 12 h: 12 h light/dark regime. At the end of incubation period Cu present in cells were ashed in a muffle furnace at 400°C for 12 hr, the ash dissolved in concentrated HNO_3 and analysed in an atomic absorption

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spectrophotometer (Varian Techtron AA1100, Australia). Copper ions adsorbed on the surface were analysed in cells washed with 10 mM EDTA. Cytosolic preparation was made with EDTA washed cells. Cu amended cells were suspended in 10 mL of 10 mM EDTA, sonicated for 30 min in an ultrasonic disintegrator and the clear supernatant was analysed for Cu. Cells washed with fresh medium without EDTA were also analysed.

After growing N. obtusa in 0.5 mg/L of Cu for 3 d the medium was replaced with fresh sterile Cu amended medium. On the 8th d cells were harvested from atleast 10 replicates, washed 3 times with fresh culture medium, suspended in 10 mL of 0.1 M cold Tris HCl buffer (pH 7.5) and ground with acid washed sand in a prechilled glass mortar for 10 min (keeping them in ice during operation). The slurry was spun at 5000 x g for 10 min in a refrigirated MSE centrifuge to remove cell debris. Cu binding proteins were isolated according to the following method of Stokes et al. (1977). The supernatant was centrifuged at $4^{o}C$ at 20,000 x g for 30 min, again centrifuged at 2,00,000 x g for 2 hr in a MSE superspeed 65 centrifuge and the high speed supernatant was dialysed and passed through sephadex G-75 (Pharmacia-superfine) column (1.75 x 45 cm). The standardized column was calibrated with known molecular weight marker proteins like lysozyme (MW 11,000), pepsin (MW 24,000), trypsin (MW 35,000) and ovalbumin (MW 45,000). The void volume was calculated as 30 mL. After checking the column with blue dextran 2000 the sample was applied to the column and operated at $4^{o}C$ with 0.1 M Tris HCl buffer (pH 7.5) at a flow rate of 0.75 mL/min. 5 mL fractions were collected and analysed for protein and Cu. Fractions containing Cu and protein were pooled, dialysed and concentrated by lyophilization for polyacrylamide gel electrophoresis according to the method of Davis (1964). The sample was also subjected to PAGE in the presence of sodium dodecyl sulphate along with the standard marker proteins following the method of Weber and Osborn (1969).

RESULTS AND DISCUSSION

Analyses of whole cells without EDTA washing showed 0.13 µ g of $Cu/10^{6}$ cells. Approximately 30% of Cu that disappeared from the medium was adsorbed on the surface of cells. The reduction in the amount of metal in cytosolic fraction of cells may be due to binding of Cu to the membranes of intracellular inclusions (Table 1). For purification of Cu binding protein a method proposed by Stokes et al. (1977) was adopted. Recovery of protein and Cu by this method was 10.9 and 11.1%, respectively (Table 2). Protein preparations from cells of N. obtusa grown in culture media lacking even a micronutrient level of Cu were eluted and the material was resolved into two peaks (Fig.1). Fractions from control culture did not show any trace of Cu. Fractions from cells grown in Cu-amended medium on gel filtration resolved into three peaks (Fig. 2). The first high molecular weight protein was in the void volume of the column. Protein preparations (fractions I, II and III)

for polyacrylamide gel electrophoresis (PAGE) showed three coomassie brilliant blue stained protein bands. Another protein sample was subjected to SDS-PAGE analyses and the molecular weights of protein bands formed were 41, 21 and 11 kilo daltons, respectively.

Table 1. Uptake and accumulation of Cu in N. obtusa amended with 0.5 mg/L of Cu in 10 mL of culture medium.

Source	Accumulation of Cu (μ g/10^6 cells)
Whole cells washed without EDTA and ashed	0.13
Whole cells washed with EDTA and ashed	0.09
Adsorbed metals removed by EDTA washing	0.03
Cytosolic fraction	0.05

Table 2. Purification and recovery of Cu and protein in N. obtusa grown in 0.5 mg/L of Cu amended culture medium.

Purification Steps	Control				Treated			
	Total		Recovery		Total		Recovery	
	*P (mg)	Cu (μ g)	P (%)	Cu (%)	P (mg)	Cu (μ g)	P (%)	Cu (%)
Crude extract	105	1.25	100	100	110	225	100	100
Low speed supernatant	60	trace	57.1	-	65	120	59	53.3
High speed supernatant	53	trace	33.3	-	46	65	36.3	28.8
Sephadex G-75 column gelfitration	9	-	8.6	-	12	25	10.9	11.1

*Protein

A schematic representation of our current knowledge on uptake of heavy metals and the mechanisms of tolerance in algae is given in Fig. 3. The scheme represents the route of uptake and identifies nine possible mechanisms or sites where tolerance mechanisms can operate in algal cells. Metal ions present in free and bound states

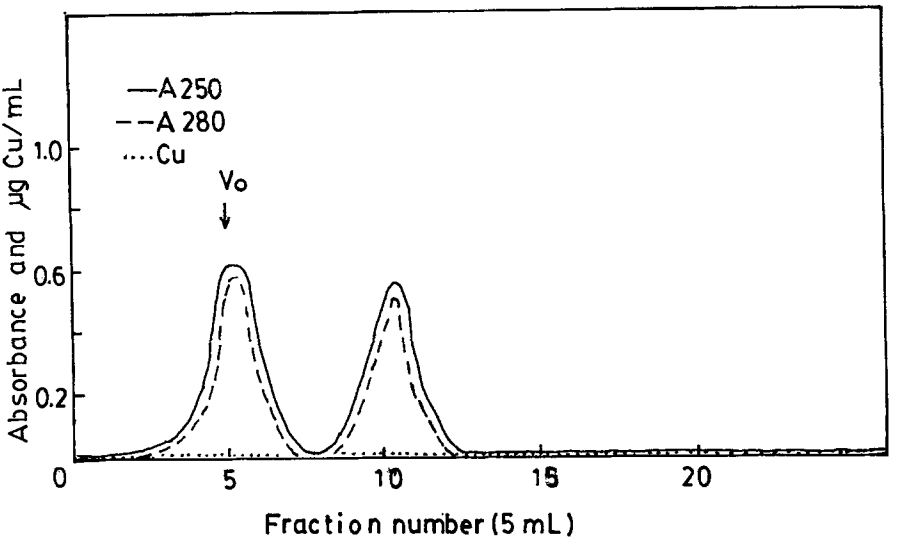

Figure 1. Sephadex G-75 elution profile of the supernatant from N. obtusa grown in medium without Cu.

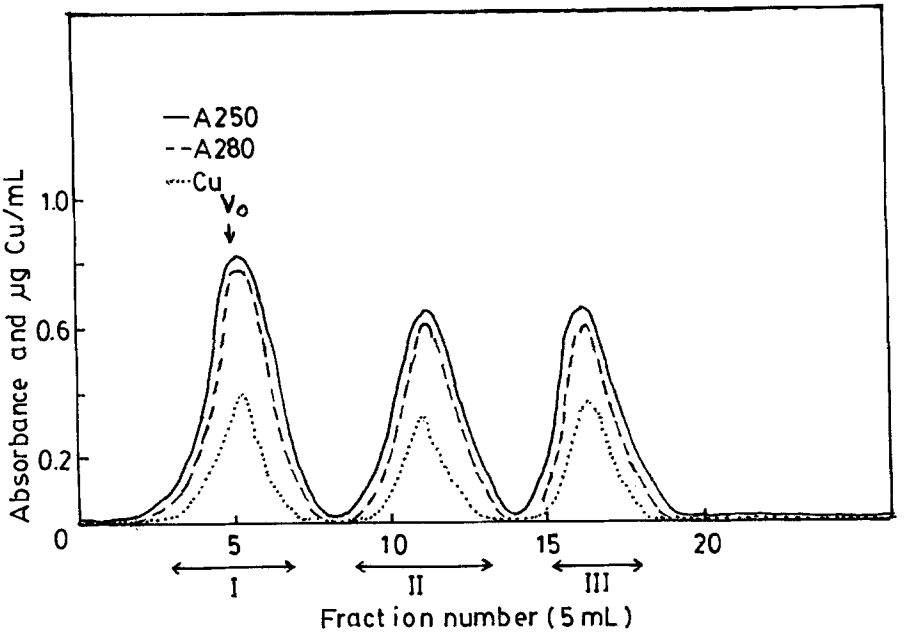

Figure 2. Sephadex G-75 elution profile of the supernatant fraction from N. obtusa grown in medium amended with 0.5 mg/L of Cu.

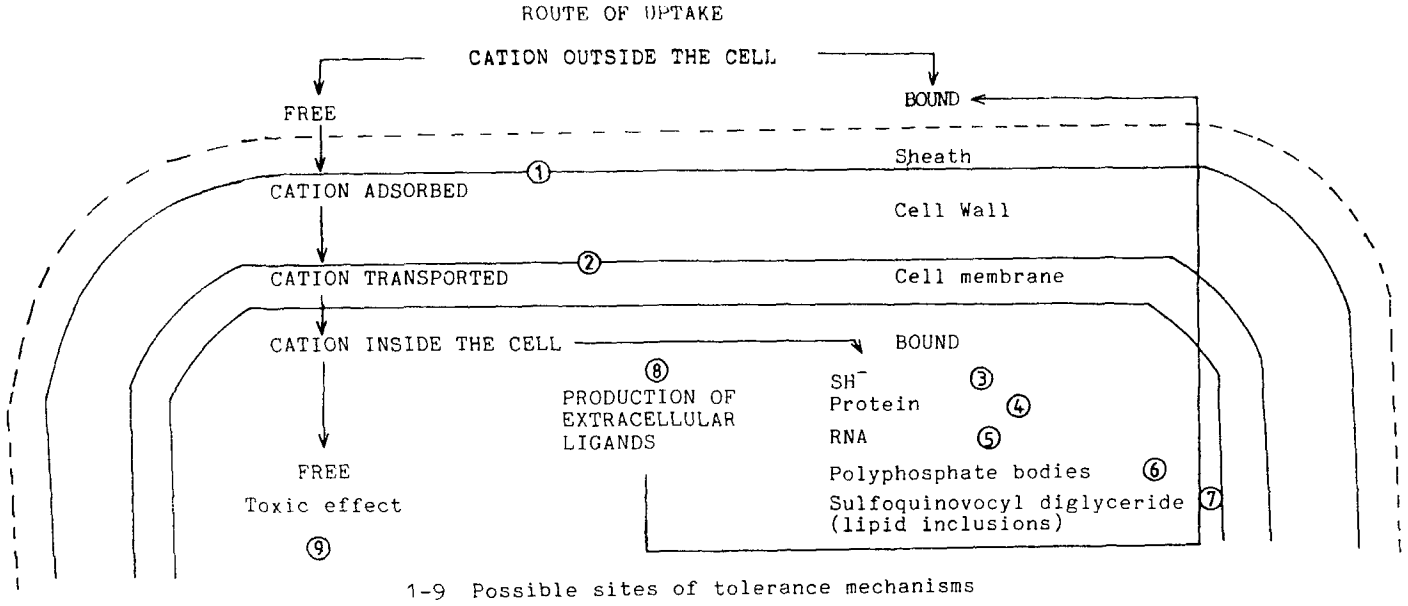

Figure 3. Schematic representation of the uptake of metals and possible mechanisms of tolerance in algae (modified from Stokes 1983).

have to either pass through or become adsorbed on the cell wall. Exclusion can operate at this point if the cell wall is able to take up the metal ion and thus prevent its encounter with the plasmalemma (1). Free ions will pass though the membrane with the help of carriers and permeability can be controlled so that ions may not pass through or, if they do, they do so only slowly (2). Beyond this, mechanisms like binding of metals with sulphydryl groups (3), protein (4), RNA (5), polyphosphate bodies (6) and sulfoquinovocyl diglyceride bodies (7) may operate. Another mechanism (8) may operate which requires production of extracellular ligands which either transport the metals outside or more probably bind them externally and prevent their further entry. The free ions may cause the toxic effect (9). In the present study two mechanisms of tolerance were encountered (1) adsorption of cations on the cell surface and (2) formation of metallothionein-like protein complex in the cytoplasm of cells. More studies on the detoxification mechanisms exhibited by diatoms in response to various metals may help us to evolve a biological treatment system for industrial effluents containing high amounts of heavy metals.

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