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Environmental Contamination and Toxicology

Effect of Copper on the Growth of *Anacystis nidulans*

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The cyanobacteria, previously known as blue green algae, are oxygenic, photosynthetic, autotrophic prokaryotes; some are nitrogen fixing. Anacystis nidulans, a rod shaped unicellular cyanobacter is found in fresh water. It has been suggested that these organisms would be useful as an indicator species of environmental pollution. Heavy metals are one type of pollution to which fresh water prokaryotes would likely be exposed. Mining and other forms of industrial waste (Griggs and Johnson,1978) are responsible for the release of large quantities of heavy metals into the environment. Many of these, such as iron, manganese, copper, molybdenum, zinc and cobalt are needed in trace quantities as nutrients, and only become toxic at higher concentrations. Others such as lead and mercury are toxic at low concentration and have no nutritional benefit (Snyder, 1982). The effects of several heavy metals on the growth of A. nidulans has previously been studied (Lee et al., 1991,1992a,b)

Copper is an essential trace element needed in small quantities by algae for plastocyanin production. However, at high concentrations it acts as an effective algacide (Kaplan et al., 1984). A high activity of free cupric ions causes greater lethality (Sunda and Guillard, 1976). The cupric ion activity is related not only to concentration of copper but also to pH and concentration of chelators (Hawkins and Griffiths,1982). When copper concentration is not lethal, it delays log phase of growth, slowing the rate of increased cell population density (Olafson,1986).

Acidic precipitation, usually in the form of nitrates and sulfates, contributes to eutrophication as well as acidification of lakes and streams. Acidic conditions in the environment, whatever the source, greatly increase the solubility of many toxic metals. While

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EDTA (Ethylene Diamine Tetraacetic Acid) acts to chelate the metal ions and reduce their toxicity (Lee et al., 1991). The effect of cupric ion on the growth of A. nidulans under laboratory conditions was studied using two different copper containing compounds at different concentrations.

MATERIALS AND METHODS

The culture of Anacystis nidulans 625 was obtained from Dr. McGowan, Brooklyn, NY. The cells were grown in sterile shake flasks containing 100 ml Mauro's Modified Medium (3M) (Kratz and Myers,1955). The cultures were grown under constant fluorescent light at ambient temperature with continuous slow agitation until stationary phase was achieved. The flasks were inoculated with approximately $1X10^7$ cells/ml of A. nidulans. For each experiment a control was prepared of untreated A. nidulans in 100 ml of 3M medium kept at the same conditions. Growth of the cultures was determined by two methods: 1. direct count using a Spencer hemocytometer or 2. indirect turbidometric reading using a Bausch and Lomb Spectronic 1001 spectrophotometer at 750 nm. Cultures were checked for contamination by plating on nutrient agar. PH readings were taken periodically.

Two stock solutions of 3M were prepared containing 20ppm (0.14mM) $CuSO_4.6H_2O$ or (0.06mM) $CuCl_2$ (1000X the normal concentration). One of these stock solutions contained the normal concentration of Na_4EDTA (0.213mM), and the other without Na_4EDTA. Stock solutions were diluted to 2ppm, 0.2 ppm with respect to $CuSO_4.6H_2O$ or $CuCl_2$. Dilutions were made using 3M containing no copper ion, either with or without Na_4EDTA, as appropriate. These media were adjusted to pH 7.9 with KOH before autoclaving.
Approximately 1 $X10^7$/ml of exponentially growing A. nidulans were used for inoculation in 100 ml of sterile test medium. Each flask was incubated on a gyratory shaker at room temperature, illuminated with cool white fluorescent light.

RESULTS AND DISCUSSION

A. nidulans was grown in cupric ion concentrations of 0, 0.2 (control), 2 and 20 ppm in standard 3M. The same series of cupric ion concentrations was prepared in 3M containing no EDTA. In these preparations, cupric ion was added as $CuSO_4.6H_2O$. Growth at 0.2 ppm was the same as the control. As seen in Figures 1 and 2, the growth of A. nidulans is severely affected by cupric ion concentration. At 2 ppm Cu^{+2}, there is a dramatic difference between growth in the presence of EDTA and in

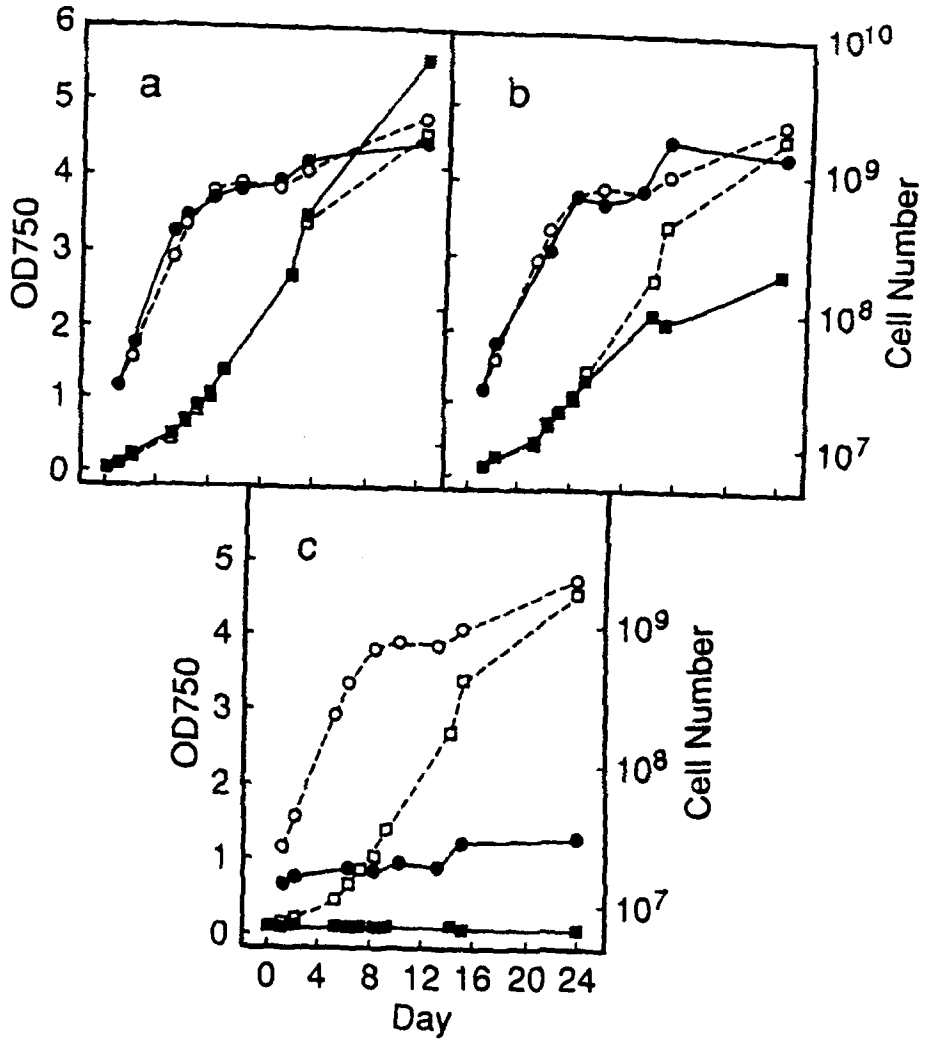

Figure 1. Growth of A. nidulans in 100 ml of 3M medium containing cupric sulfate at 0(a), 2(b), 20(c) ppm with EDTA. Growth was measured by optical density readings at 750 nm (■ --■)treated (□ --□)control, and by direct count (● -- ●)treated (o -- o)control.

its absence. Without EDTA, cell growth is almost completely inhibited. A slight rise in cell number during the first week is probably due to cell division without concomitant increase in biomass. A decrease in cell size was observed under the microscope during this period. At 2 ppm Cu^{+2} and standard EDTA concentration, cell growth is comparable the control until approximately day 15 when optical density levels off, though cell densities still correlate well. In this culture, cell size was also observed to decrease at about the time that optical density stopped increasing. At 20 ppm Cu^{+2} growth is inhibited in 3M with or

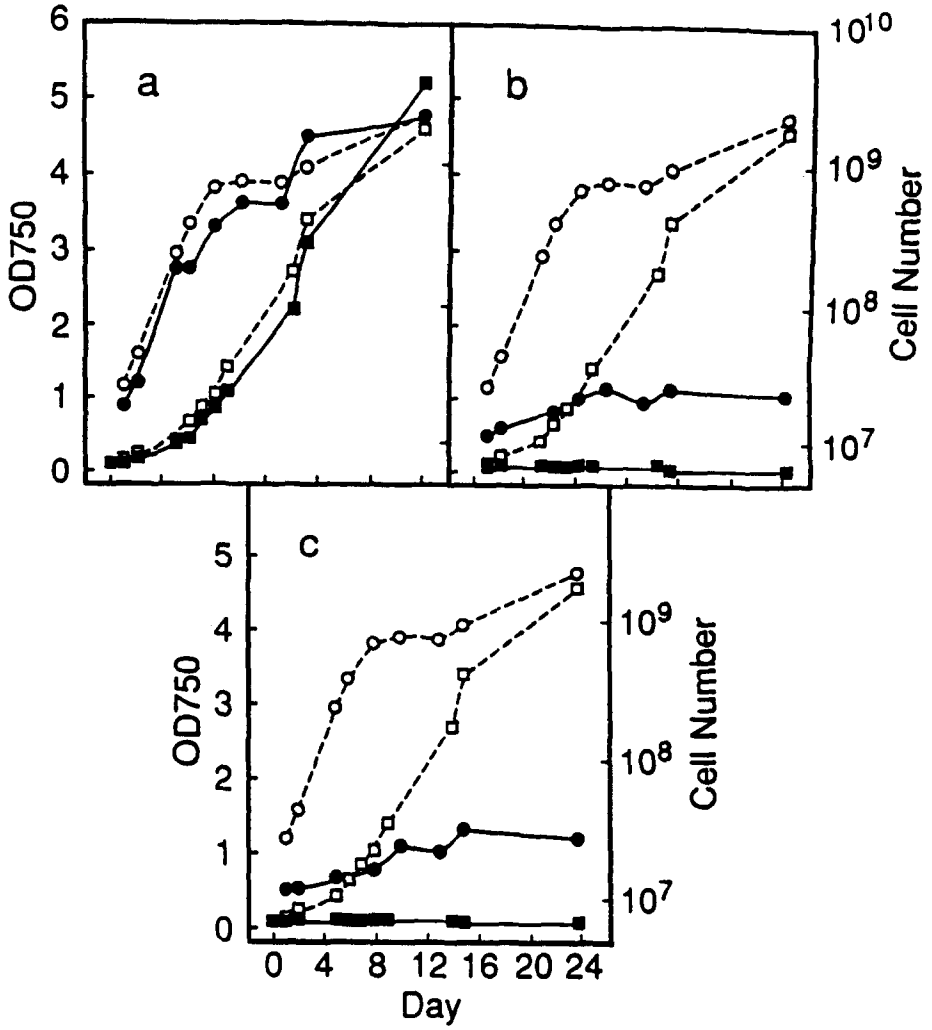

Figure 2. Growth of A. nidulans in 100 ml of 3M medium containing cupric sulfate at 0 (a), 2 (b), 20 (c) ppm without EDTA. Growth was measured by optical density readings at 750 nm (■ --■)treated (□ --□)control, and by direct count(● --●)treated (○ --○)control.

without standard concentrations of EDTA. Again, a slight increase in cell density, not in turbidity, occurs as cells were observed to decrease in size. Periodic pH measurements of the cultures revealed that, in general, pH increased during normal cell growth. In the three cultures exhibiting no growth (2 ppm Cu^{+2} without EDTA, and 20 ppm Cu^{+2}, with/without EDTA) pH remained close to neutral throughout the experiment. Each of the cultures without EDTA showed a slower increase in pH than did its counterpart with the same Cu^{+2} concentration but standard EDTA concentration. At Cu^{+2} concentrations of 0 ppm, this was the case even

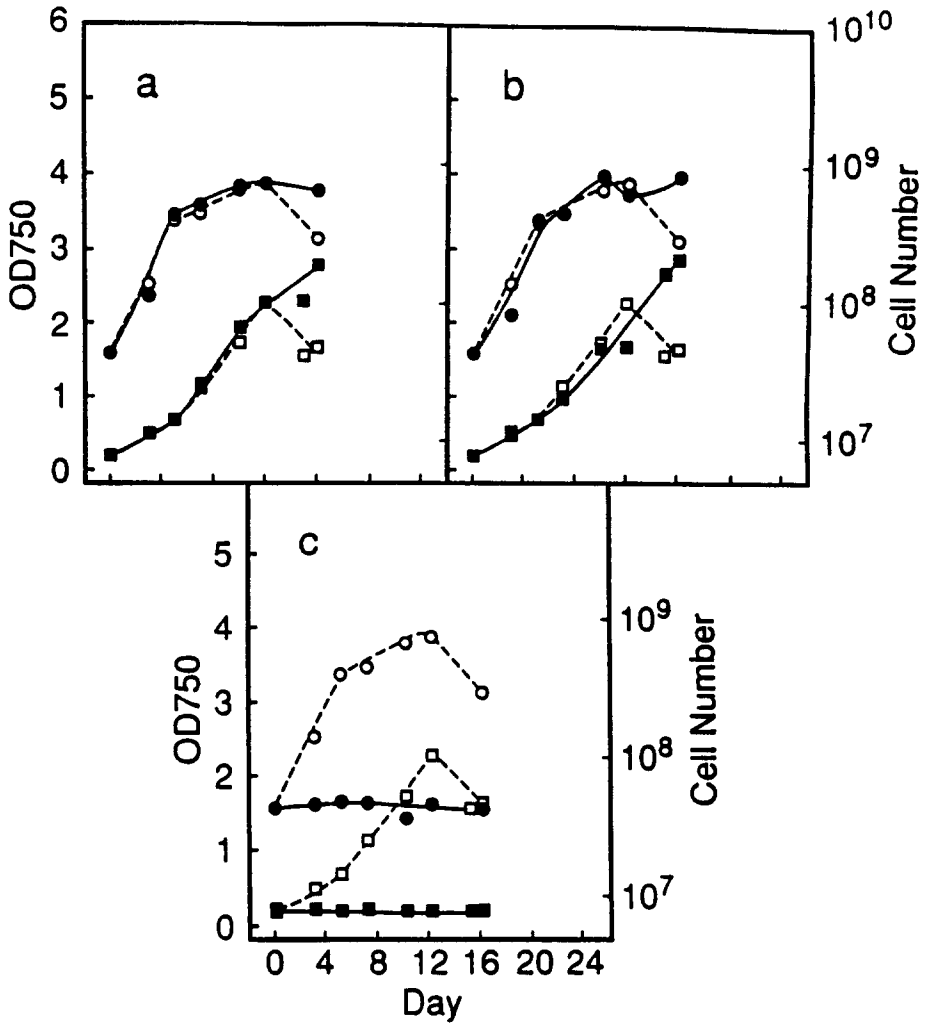

Figure 3. Growth of A. nidulans in 100 ml of 3M medium containing cupric chloride at 0 (a), 2 (b), 20 (c) ppm with EDTA. Growth was measured by optical density readings at 750 nm (■ --■)treated (□ --□)control, and by direct count(● --●)treated (○ --○)control.

though little or no difference was observed between growth curves in the presence or absence of EDTA. At 0 ppm Cu_{+2}, visual observation of the culture flasks revealed no variations among the cultures. 2.0 ppm with EDTA was indistinguishable from controls. At 20ppm the lack of cell growth revealed a bluish cast to the medium due directly to the high concentration of Cu_{+2} in solution. Growth of A. nidulans was clearly inhibited by higher concentrations of cupric sulfate. In order to due to an be sure that the observed effects on growth rate were increase in the concentration of copper alone, rather than due to an increase in the concentration of

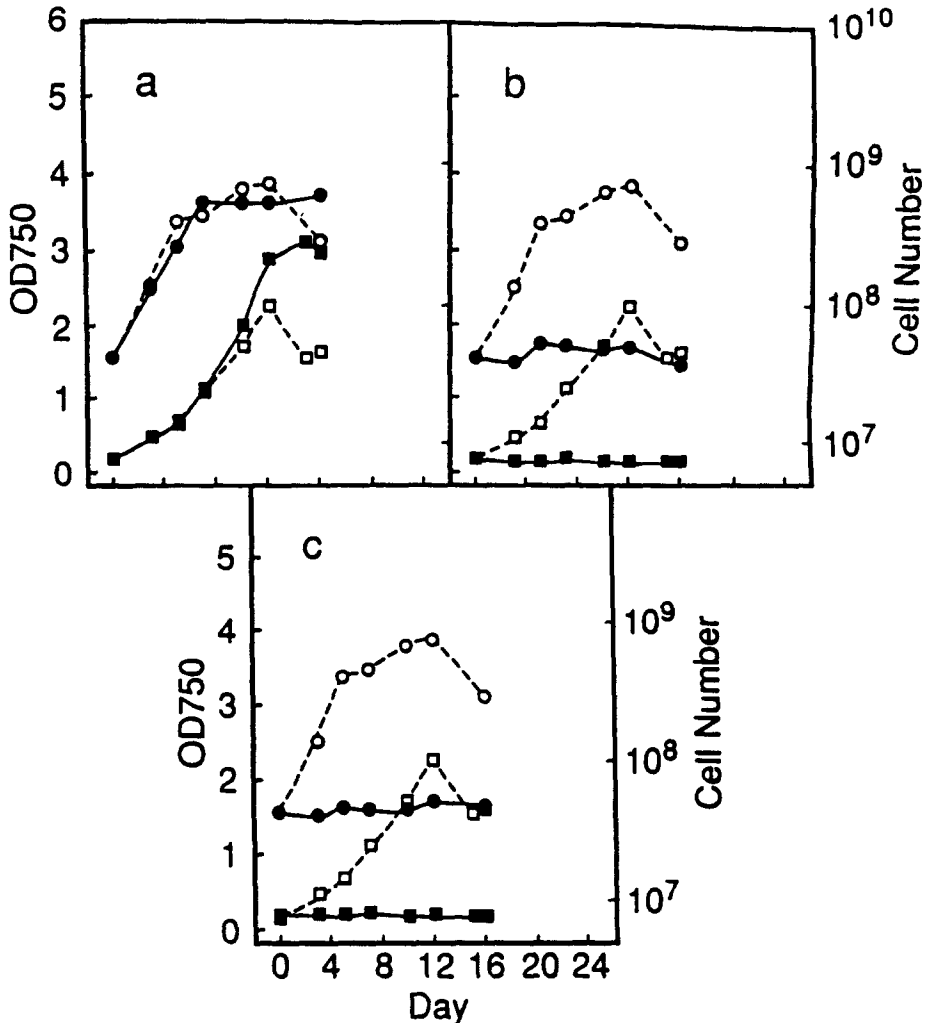

Figure 4. Growth of A. nidulans in 100 ml of 3M medium containing cupric chloride at 0 (a), 2 (b), 20 (c) ppm without EDTA. Growth was measured by optical density readings at 750 nm (■ --■)treated (□ --□)control, and by direct count(● --●)treated (○ -- ○)control.

the accompanying anion or a combination of both, additional experiments were performed. In this series, the effects of copper as cupric chloride was tested. Cupric ion concentrations were the same in both series of experiments. Results from this series of experiments parallel results from the cupric sulfate series. At 0 and 0.2 ppm Cu^{+2}, the growth curves for cupric chloride and for cupric sulfate are the same and are similar to the control . At 2.0 ppm, growth is not distinguishable from that of the control when standard EDTA concentration is used (Figures 3&4). In the absence of EDTA, however, the cultures show no growth at all. At 20 ppm, there is no growth with or without

EDTA. While the molarities of $CuCl_2$ and $CuSO_4$ are different, the resulting available cupric ion concentration is such that the results obtained with cupric chloride are identical to those obtained for cupric sulfate. The pH histories of the cupric chloride test cultures also parallel the results for the cupric sulfate.

In both cases, the cupric form of copper was tested and the ppm concentrations were the same. The difference between the two was the partner anion and the results were very similar. Inhibition of growth, therefore, appears to be the effect of cupric ion concentration alone. The concomitant increase in chloride ion or sulfate ion concentration has little or no effect on growth. Clearly, the effect of chelating agents such as EDTA are important. Chelators, such as EDTA act to sequester metallic ions, such as iron and copper. Higher concentrations of cupric ions exceed the chelating constant of EDTA. Therefore the available free Cu_{+2} concentration becomes toxic to the cyanobacteria. In the absence of added EDTA, inhibition of growth occurs at a concentration somewhat below 2.0 ppm Cu_{+2}. While in the presence of EDTA in 3M, inhibition of growth begins somewhere between 2.0 ppm and 20.0 ppm Cu_{+2}. It is likely that this phenomenon is due to the fact that chelated ions are sequestered and not available to affect the growing culture, as dissolved free ions would be. In other words, the effective concentration of Cu_{+2} is lowered when EDTA is added.

The lethality of copper is related to its binding to the cell surface and alteration of transport systems (Gavis et al (1981). Thus when EDTA is not present in the medium, and copper is made available to the cell, this active ion can affect membrane transport mechanisms as well as the photosynthetic apparatus that is present within the cell.

It is known that many compounds dissolved in fresh water by natural phenomena will act as natural chelators (Raizada, 1988). For example, previous studies have shown that release of schizokinen,(a siderophore) by Anabaena sp will act as a chelator for heavy metals (Clarke et al., 1987). It will be necessary to take the naturally occurring chelating capacity of a lake or stream into account when discussing the toxicity of heavy metals in the natural environment. A secondary or indirect effect of dissolved Cu_{+2} in the natural environment is that a drop in pH is often the cause of increased copper concentration. The combined effects of heavy metals and lowered pH therefore may be additive or synergistic.

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