A study of the toxic effects and binding capacity for the heavy metals cadmium, copper, and zinc by the blue-green alga Chroococcus paris.

A STUDY OF THE TOXIC EFFECTS AND BINDING CAPACITY FOR THE HEAVY METALS CADMIUM, COPPER, AND ZINC BY THE BLUE-GREEN ALGA *CHROOCOCCUS PARIS*

A Thesis Presented

By

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To my wife, Cathy

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ABSTRACT

A Study of the Toxic Effects and Binding Capacity for the Heavy Metals Cadmium, Copper, and Zinc by the Blue-green Alga Chroococcus paris

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The heavy metals cadmium, copper, and zinc were bound rapidly by the blue-green alga, Chroococcus paris, with approximately 90% of the total amount of added metal being bound within one minute. Metal binding capacity of C. paris was determined to be 53, 120, and 65 mg/g dry algal weight for cadmium, copper, and zinc, respectively. Metal binding increased with increasing ambient metal concentrations. Binding curves for the three metals followed the Langmuir adsorption isotherm model.

The amount of metal bound increased with increasing ambient pH values. Metal binding increased as much as 46% when the ambient pH was increased from pH 4 to pH 7.

It is suggested that initial, short-term heavy metal binding occurs at binding sites located on or within the

capsular matrix since bound metals were found to be EDTA extractable. The order of metal toxicity to C. paris cultures was Cu > Zn > Cd as determined by growth inhibition studies. All concentrations of copper and zinc studied induced some degree of growth inhibition. Copper and zinc concentrations of 1.0 and 5.0 mg/l, respectively, resulted in complete suppression of algal growth. A cadmium concentration of 0.4 mg/l caused no significant reduction in algal growth rate, while concentrations of 0.1 and 0.2 mg Cd^{2+}/l stimulated growth to ca. 20% above that of the control.

It is possible that a correlation exists between the order of metal toxicity and the affinity of cellular binding sites for specific metal ions and the resultant formation of metal-ligand complexes. In both cases this order was Cu > Zn > Cd.

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INTRODUCTION

The greatly increased use of heavy metals and, alarmingly, their widespread distribution in the environment has recently stimulated numerous studies on metal toxicity and bioaccumulation by aquatic biota. There, however, remains a singular lack of detailed knowledge concerning the effects of metals on phytoplanktonic growth and their bioconcentrating ability. Such knowledge is important when attempting to assess and understand the various impacts of heavy metals on the aquatic eco-system.

The cyanophyta, a representative of which is the subject of this study, occupy a broad range of habitats in nature and can be considered ubiquitous. Natural aquatic populations consisting largely of unicellular blue-green algae may often increase greatly in numbers and form blooms in lentic waters. As such, these algae become dominant factors in minor element metabolism and function in the micro-element dynamics of the aquatic system (Boyd and Lawrence, 1966).

Since phytoplankton form the base of the ecological food chain, consideration must be given to the effects of heavy metals accumulation and transfer to higher trophic

levels in the system. This consideration can only be realized by understanding the extent to which the primary producers can accumulate such metals, distribute them to higher orders, and tolerate metal concentrations before becoming affected themselves.

The accumulation of heavy metals by cultured phytoplankton depends upon many different factors. Such factors include species composition, culture composition and conditions, metal concentration, and chemical state of the element (either free ions, inorganically complexed, or organically complexed or chelated). The chemical behavior of trace elements often mimic essential elements biologically. Cadmium, for instance, may replace zinc in metalloenzymes and alter normal cellular activity. Even essential metals such as copper, zinc, and iron, if in sufficiently high concentrations, will be detrimental to the living cell.

The accumulation of metallic ions by microorganisms is, therefore, by no means a revelation. Many chemical compounds associated with biological systems are known to contain trace elements. Metalloenzymes and other cellular materials such as peptides, proteins, and polysaccharides are known to complex and/or chelate trace metals. The chlorophyll molecule is an example of one such organometallic chelate. The ability to accumulate elements,

therefore, makes it possible for organisms to concentrate metal ions from their surrounding environment, thus fulfilling cellular requirements.

Most phytoplankton have the ability to concentrate heavy metals intracellularly and extracellularly far in excess of the concentrations found in the ambient waters. The blue-green algae, an important component of the phytoplanktonic community, produce copious amounts of extracellular products (slime, sheath or soluble extracellular material) of known metal complexing capacity. Very little research has been done to determine the role of these organisms in heavy metal bioaccumulation. Because of this lack of knowledge, a member of this group, Chroococcus paris, has been selected upon which to base these studies.

C H A P T E R I I

LITERATURE REVIEW

As a result of several of man's activities such as mining, industry, and agriculture, there is an alarmingly increasing amount of cadmium being introduced into the environment (Friberg et al., 1974). Increasingly high levels of heavy metals have been found in many reservoirs and rivers whose waters have been contaminated with mine drainage wastes, industrial effluents, and power generation station wastewater (Filip and Lynn, 1972). The release of quantities of heavy metals such as cadmium, copper, and zinc as well as many other elements from stationary conventional combustion processes adds substantially to the total burden placed on the environment (Edwards et al., 1981). The abundance of trace elements in the environment, their distribution, production, and consumption is well documented (Jenkins, 1981; Hem, 1972).

Even though excessive concentrations of heavy metals exist in contaminated watercourses, these waters may still support a variety of phytoplankton (Trollope and Evans, 1976). Certain algae are able to accumulate heavy metals

either within their cellular matrix or on the surface of the cell wall or envelope (Filip and Lynn, 1972; Trollope and Evans, 1976; Hart and Scaife, 1977; Reiniger, 1977; Laube et al., 1980). The binding capacity for copper, mercury, lead, and cadmium has been shown to be a function of algal species composition rather than mere biomass or physicochemical parameters of the water (Briand, Trucco, and Ramamoorthy, 1978).

The blue-green algae are often considered excellent candidates for metal binding due to their ability to produce extracellular envelopes and/or soluble products of high binding ability (Fogg, 1966; Lange,1973; Kohn, 1975). An example of a strong, soluble metal-complexing agent is the polyhydroxamate siderachrome schizokinen, produced by the blue-green alga Anabaena sp. (Simpson and Neilands, 1976). Although peptides and amino acids were found to be involved in metal chelation (Fogg, 1966), other work showed that the algal polysaccharides should not be excluded from consideration as prime complexing agents (O'Colla, 1962; Moore and Tischer, 1965; Wang and Tischer, 1973). Moore and Tischer (1965) isolated the sugars glucose, xylose, ribose, and uronic acids from Anabaena flos-aquae extracellular polysaccharide material.

Studies of heavy metal binding and toxicological effects on freshwater diatoms indicated that sorption of arsenic was a function of ambient concentrations and

reached concentrations greater than 130 ug As/g, most of which was found associated with the organic layer around the frustule (Conway,1977). Cadmium, in this instance, was found associated with intracellular materials more so than with the outer organic layer. Similar investigations conducted on marine diatoms showed cadmium uptake to be greatest at the exponential phase of growth (Cossa, 1976). The binding of lead and cadmium to marine algal polysaccharides of Eucheuma striatum and E. spinosum was found to be significant with 147 mg Cd/gram carrageenan and 284 mg Pb/gram carrageenan being bound (Veroy et al., 1980).

Many bacterial species, both capsulated and non-capsulated, also possess a capacity to bind heavy metals. Khazaelli and Mitra (1981) isolated a cadmium-binding protein component of accomodated Escherichia coli which bound a total of 0.7 ug Cd^{2+}/mg of cellular protein. Ubiquitous film-forming marine bacteria possessing extra-cellular polymeric materials are able to bind and precipitate copper sulfate and other copper salts in solution (Corpe, 1972). Cells of Saccharomyces cerevisiae and Pseudomonas aeruginosa were able to accumulate uranium from solution extracellularly and intracellularly respectively (Strandsberg et al., 1981). It is of interest to note that intracellular binding of uranium

in P. aeruginosa occurred very rapidly (less than 10 seconds). Cell bound material (metal), however, was found to occur in only 32% of the S. cerevisiae cells and in 44% of the P. aeruginosa cells. This raises the question of whether genetic or environmental conditions govern uranium uptake in these bacteria. Zajic and Chiu (1972), also working with uranium, found the mold Penicillium able to uptake 70% of a 100 mg/l uranium containing medium. Friedman and Dugan (1968) as well as Sterritt (1981) demonstrated the capacity of zoogleal material from zooglea-forming bacteria to bind several heavy metals. Uptake of metal ions by zooglea formers was approximately twice that accumulated by non-zoogleal strains. Nonetheless, non-zoogleal organisms accumulated 34% of their total weight as copper ions (Cu^{2+}) and approximately 25% as cobalt ions. Total uptake, therefore, appears to be due to a combination of both cellular and surrounding matrix accumulation.

The toxicity of several heavy metals to freshwater algae is well documented (Hart and Scaife, 1977; Kallqvist and Meadows, 1978; Wong et al., 1978; Laube, 1980). Questions concerning the specific nature and mechanisms of metal toxicity, however, remain only partially answered.

Kallqvist and Meadows (1978) investigated the effects of copper released from a copperoxychloride production

plant on Spirulina platensis, a filamentous blue-green alga. The addition of 0.2 mg Cu^{2+}/l to an S. platensis culture resulted in a 50% reduction in chlorophyll production, while a concentration of only 0.02 mg Cu^{2+}/l inhibited growth. A concentration of 10^{-7}M to 10^{-5}M Cu^{2+} extended the lag phase of Anabaena while 10^{-4} to 10^{-3}M completely inhibited growth (Laube et al., 1980). On the other hand, Tkachenko et al., (1974) noted a growth stimulation of some marine algae by low levels of cadmium while higher concentrations inhibited growth. The growth of three freshwater green algae, Ankistrodesmus falcatus, Scenedesmus obliqus, and Chlorococcum sp. was noted to be stimulated by cadmium concentrations of 0.1 mg/l to as high as 1.0 mg/l (Devi Prasad, 1981). When Anabaena sp. was exposed to a medium containing ca. 1.1 mg Cd^{2+}/l, approximately half of the cadmium was cell associated and the rest remained in solution in a non-ionic form (Laube et al., 1980). Cadmium bound in greatest concentrations at the beginning of the growth cycle. Cadmium binding then decreased later during the stationary phase of growth. The same observations were made by other investigators (Cossa, 1976; Conway, 1978).

The release of extracellular chelating substances, whether metabolic intermediates or metabolic end products (Fogg, 1966), may present an explanation for this binding

phenomenon. The type I, intermediate metabolites, are usually of low molecular weight whereas the type II, end product metabolites, are of high molecular weights. Lewin (1956) demonstrated that the amount of polysaccharide material released by algae was proportional to the amount of cellular material in the cultures. Furthermore, it was shown that the soluble polysaccharides released from capsule-forming algae were similar to the capsular material. Thus, the presumption that those polysaccharides released were derived from solution or hydrolysis of the capsular material can be made.

Toxicity and bioaccumulation of cadmium in a green alga, Chlorella pyrenoidosa, was examined by Hart and Scaife, (1977). Raising the pH of the growth medium from pH 7 to pH 8 caused a reduction in toxicity at all concentration levels studied (0.0, 0.25, 0.50, and 1.0 mg Cd^{2+}/l). These results are in contrast with those of Kallqvist (1978) who suggested that competition between hydrogen ions and metal ions exists when these ions compete for binding sites on cell walls and membranes. Accordingly, an increase in pH from pH 6 to pH 7 should give copper ions the competitive edge over H^{+} during any competition for binding sites. It must be remembered, however, that the water chemistry of the solution greatly influences the activity and availability of metal ions and

that competition between ion species is much more complex than previously stated.

Both the total amount of trace metal present and the chemical form of the metal are important when assessing the effects of trace elements on growth rates of phytoplankton (Davey et al., 1973). Organic compounds may play a significant role in changing metal speciation by complexing or chelating metal ions. Florence (1977) has shown that in freshwaters, copper is predominantly complexed to organic matter, cadmium is present as the free divalent ion, and zinc is present as both inorganic colloidal and free ionic forms. The biological availability of a trace element, therefore, is highly dependent upon its chemical form (Barber, 1973).

Copper is highly toxic to phytoplankton at very low concentrations (Laube et al., 1980). Sundra and Guillard (1976) showed copper toxicity to be directly proportional to free cupric ion activity. Also observed was the decrease in total copper accumulated by Nannachloris atomus upon addition of the chelator trishydroxymethylamino methane to the culture medium.

Cossa (1976) found an insignificant uptake of cadmium by Phaeodactylum tricornutum when cadmium was chelated by EDTA (ethylenediaminetetraacetic acid). Algal exudates from several algal species was found to reduce copper

toxicity by forming complexed species (Van den Berg and Chau, 1979). Detoxification of the trace metal copper by chelation to ligands in seawater media has been shown by Jackson and Morgan (1978). The toxicity of cadmium, in both soluble ionic and particulate forms, is dependent upon the degree of chelation of the metal (Houba and Remacle, 1982). The less complexed forms accumulated in greater concentrations and proved to be the most toxic.

The nature of bonding between metals and organic ligands, especially polysaccharides, has become an increasingly popular but still complex subject. Many factors including linear charge density, cation effective charge, donor group basicity, chelation, and intermolecular binding contribute to the formation and stability of metal polysaccharide complexes (Kohn, 1975; Rendleman, 1977). Potential sites for binding on the anionic ligands include hydroxyl, carboxyl, and free oxygen groups. Sulfate groups on polysaccharides such as the carrageenans are strongly involved in metal binding of some marine algal polysaccharides (Veroy et al., 1980). Most bonds of this nature are largely ionic with some of covalent character (Rendleman, 1977). Where ligand polysaccharides are uncharged, the characteristic attraction between metal and ligand is mainly covalent. Neutral polysaccharides tend to form weak complexes with cations in neutral or alkaline

solution. Anionic ligands, under similar neutral or alkaline conditions, have strong affinities for cations (Rendleman, 1977).

Crist et al. (1981) studied the nature of binding between metal ions and algal cell wall fragments from Vaucheria sp. Using a pH titration method, they demonstrated an increase in cell wall bound strontium with increasing pH from pH 3.5 to pH 6.5. Copper ions at pH 4.5 were adsorbed by algal wall fragments to the extent of 600 umol Cu^{2+}/gram wall material. It should be noted, however, that fragmented cell walls were first washed with an acidified deionized water. All extraneous matter including mucilage and exchangeable metallic ions were presumed absent. Cell wall fragments compared to intact cells exhibited a 25% increase in bound copper ions. Adsorption of metal ions followed the Langmuir adsorption isotherm. Similar adsorption data was obtained with manganese using colonies of Nostoc commune (Pollard and Smith, 1951).

OBJECTIVES

The objectives of this research study are as follows:

1. To determine the concentrations of cadmium, copper, and zinc which affect the growth of Chroococcus paris.

2. To determine the binding capacity of C. paris for the heavy metals cadmium, copper, and zinc.

3. To determine the binding rate of the three metals to C. paris.

4. To determine competitive binding by different metals to C. paris cells.

5. To determine the effects of pH on metal binding capabilities.

6. To determine whether short term binding involves internal cellular components, soluble extracellular products, or extracellular sheath material.

C H A P T E R I I I

MATERIALS AND METHODS

Chemicals and Glassware. The chemicals used in making standard metal solutions were as follows: $Cd(NO_3)_2 \cdot 4H_2O$, $Cu(NO_3)_2 \cdot 3H_2O$, and $ZnSO_4 \cdot 7H_2O$. All chemical compounds used for metal standards and culture media were of A.C.S. reagent grade. All water used in nutrient media and metal solutions was distilled and deionized (Millipore Milli-Q System) to laboratory reagent grade.

Stock metal standards were stored in polyethylene bottles and acidified to approximately pH 2-3 using concentrated nitric acid. Nutrient media was prepared fresh before sterilization and algal inoculation.

All glassware was washed using a detergent, rinsed under tap water, soaked in 50% (v/v) nitric acid, and rinsed thoroughly with distilled deionized water.

Culture Techniques.

Organism. The organism chosen for this study was Chroococcus paris, a unicellular, sheath forming blue-green alga. An axenic culture of C. paris was graciously donated by Dr. Mary M. Allen, Wellesley College, for the

purpose of this study.

Growth of stock cultures. C. paris, as well as most strains of unicellular blue-green algae, can be grown in a mineral medium. The medium selected for culture was Medium BG-11 (see Appendix) (Allen, 1968). Medium BG-11 is neutral after sterilization and will support growth of algae in vessels aerated using air or air enriched with carbon dioxide. Stock cultures were maintained in test tubes without aeration at a temperature of 26°C and at a constant illumination of 50 foot candles. Illumination was provided by 20 watt cool-white fluorescent lamps positioned above the cultures (Stanier et al., 1971).

Growth of experimental cultures. Experimental cultures were grown in Erlenmeyer flasks of various size volumes ranging from 125 ml to 500 ml as needed. Large cultures were grown in 2.8 liter Fernbach flasks. All cultures were maintained at a temperature of 26°C and at a constant illumination of ca. 100 foot candles. Illumination was provided by a similar source used for stock cultures. Aeration and mixing were provided by shaking on a New Brunswick gyrotory shaker at a constant rotation rate of 115 rotations per minute.

Larger cultures up to 15 liters in volume were grown in 20 liter Pyrex carboys gassed with filtered air

(Lewin, 1955) below the medium surface. Temperature and illumination were the same as for shaker grown cultures.

All culture media was sterilized immediately after preparation in accordance with Standard Methods (American Public Health Association, 1981). Culture inoculations and transfers were completed under aseptic conditions. Sterility determinations were made after culture sample inoculation onto nutrient agar maintained at 26°C.

Growth Determinations. Biomass determinations of C. paris cultures using simple counting methods results in highly inaccurate data. Since reproduction of C. paris is by binary fission in three planes (Rippka et al., 1979), daughter cells resulting from a single binary fission are contained within a common (original) mucilaginous sheath as well as within individual sheaths. Each successive fission results in more cells which remain enclosed in the common sheath.

The resultant pallmeloid colonies may contain as many as 32 cells before colony separation occurs. Accurately counting individual cells is difficult. Alternatively, counting colonies alone is more practical but results in erroneous biomass data due to the fact that all colonies do not contain equivalent numbers of algal cells.

As a result, the method chosen to measure biomass in this study involves analysis of total biomass expressed on a dry weight basis. Dry weight determinations were made by first filtering the algal culture or an aliquot thereof through a Gelman glass fiber filter. Subsequently, the tared filter and algae were dried to constancy in an air convection oven. Drying for one hour at 70°C was found to be sufficient. The filter and algal sample were then weighed using an analytical grade balance.

In order to reduce time required and to further simplify such determinations, optical densities of algal cultures were analyzed at 450 nm using a Perkin-Elmer Hitachi 200 double-beam spectrophotometer. Optical density may then be related to biomass (dry weight) using correlation data (Figure 1). A correlation coefficient of 0.999 was obtained for this data.

General Methods for Heavy Metal Binding Determinations. All metal binding determinations involved the following general experimental outline:

1. Cells were washed free of BG-11 medium twice using deionized water and slow speed centrifugation at 3000 x g

2. Approximately 15 mg dry wt. of washed cells were added to a 125 ml Erlenmeyer flask containing ca. 45 ml

Fig. 1. Biomass measurement plot showing correlation between culture optical density @ 450nm and culture dry weight concentration (mg dry weight/l).

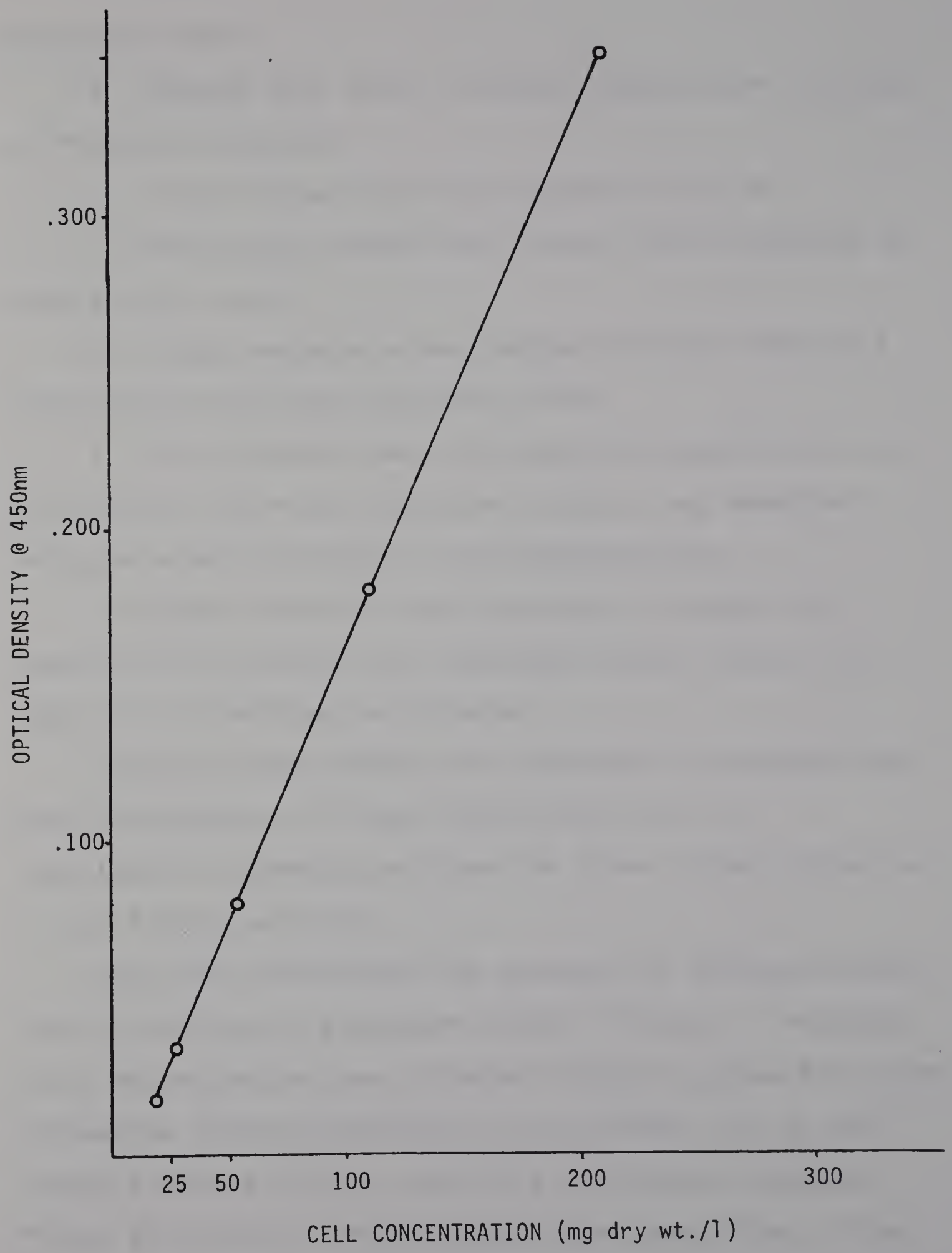
.300
.200
.100
OPTICAL DENSITY @ 450nm
25
50
100
200
300
CELL CONCENTRATION (mg dry wt./l)

FIGURE 1

deionized water.

3. Metals were added to algal suspensions via micro or volumetric pipette.

4. Total volume was then adjusted to 50 ml

5. Metal-algae system was stirred for 15 minutes to ensure good contact

6. Algal suspension was vacuum filtered through a 0.45 micron Millipore membrane filter

7. The filtrate was collected and transferred to a 30 ml screw cap vial, acidified to pH 2, and analyzed using an atomic absorption spectrophotometer.

In studies where it was necessary to adjust the sample pH, titrations were performed using either 0.1N HNO_3 or 0.1N NaOH and a pH meter.

Separate experiments were conducted to resolve that metal binding was to algal cells alone; not to experimental apparatus such as the glass filter system or to the filter material.

The first experiment was designed to determine metal loss by binding to glassware and/or filters. A standard metal concentration was filtered through a glass Millipore filtration system containing (1) no filter, (2) a 0.45 micron membrane filter, and (3) a 0.45 micron membrane filter along with a Gelman glass fiber pre-filter. The use of a pre-filter would enable greater filtration rate

and volume by decreasing membrane filter clogging.

The second experiment intended to prove how measurement of filtrate metal concentrations, after metal addition to an algal suspension, was a viable means of determining metal binding by algae. Metal concentrations were added to separate algal suspensions in 25 ml of distilled, deionized water. Algal suspensions were then filtered after a 15 minute metal-algae contact time. The filtrates were collected, acidified to pH 2, and quantitatively analyzed by atomic absorption spectroscopy. The filtered algae were placed in a 50 ml beaker and digested on a hot plate with 10 ml of concentrated nitric acid. Digestion was continued until approximately 1 ml of digest remained. The digest was then transferred to a 25 ml volumetric flask and brought up to volume with deionized water, acidified, and analyzed as before.

Since the metal concentration remaining in the filtrate represents the unbound portion of metal added, the digest should contain a concentration of metal equal to the difference between metal concentration added and metal concentration found in the original filtrate. Thus, analysis of the filtrate metal concentration would yield an efficient and viable means of quantitatively determining metal binding by algae.

Determination of Intra- or Extracellular Binding of Heavy Metals by C. paris. Since C. paris produces an extracellular sheath material, it was suggested that the metal binding mechanism may have involved metal adsorption to available binding sites on the sheath. The following study was performed to determine whether short-term metal binding takes place intracellularly or extracellularly.

Metal concentrations of 1.0, 2.0, and 5.0 mg/l were added to two sets of flasks containing 50 ml of a 650 mg/l algal suspension. Triplicate samples were used for each metal concentration. Suspensions from one set were filtered after a 15 minute contact time between metal and algae. The filtrate was collected, acidified to pH 2, and analyzed via atomic absorption spectroscopy.

Ethylenediaminetetraacetic acid (EDTA) in a 2:1 mole ratio (EDTA:metal) was added to the remaining set of algal suspensions immediately following the 15 minute contact time. These samples were filtered through membrane filters after 10 minutes. The filtrate was treated as before. Metal concentrations of both sets, i.e. with and without EDTA addition, were compared.

Growth Inhibition Determinations. Stock cultures of C. paris were inoculated into 2.8 liter Fernbach flasks

containing two liters of BG-11 medium and placed onto a gyrotory shaker under conditions previously stated. Optical density at 450nm of these cultures was monitored until cultures reached an O.D. reading of ca. 0.017-0.018. This corresponded to an algal cell concentration of 15-16 mg/l on a dry weight basis. These cultures were then aseptically transferred into sterile 125 ml flasks in 50 ml volumes. Heavy metal concentrations (0.0, 0.1, 0.2, 0.4, 1.0, 2.0, 5.0 mg/l) were added to cultures at this time. The experimental cultures were then placed onto a shaker where they were maintained at 26°C under constant 100 foot candle illumination.

Each metal concentration was run in triplicate. Culture optical density was analyzed on consecutive even days for a total of 10 days. Positions of cultures on the shaker were rotated daily so as to reduce bias from possible localization of light intensity. Representative cultures from each metal concentration group were microscopically examined at the end of the study period.

C H A P T E R I V

RESULTS

Binding Rates of the Heavy Metals, Cadmium, Copper and Zinc by Chroococcus paris. The results of the metal rate binding study demonstrate that all three metals were rapidly bound by C. paris cell cultures. The results from these experiments also provide a foundation, vis a vis metal-algae contact time, upon which further studies were based.

Approximately ninety percent of all three heavy metals were bound within one minute after metal addition to the algal culture suspensions (Figure 2). No significant differences in binding rate were observed among the three metals. Metal binding of copper, zinc, and cadmium increased by 5, 6, and 8%, respectively, after an additional nine minute contact time.(Table 1). All metals showed a similar pattern of binding in that a period of rapid binding was followed by one of slower binding rate.

All further experiments, therefore, involving heavy metal binding were performed using a standard contact time of 15 minutes between metals and algae. This time

Fig. 2. Plot showing cadmium, copper, and zinc metal binding by C. paris vs. time; 0.1 mg metal was added to a 15 mg dry weight suspension of algae in 50 ml of deionized water and stirred.

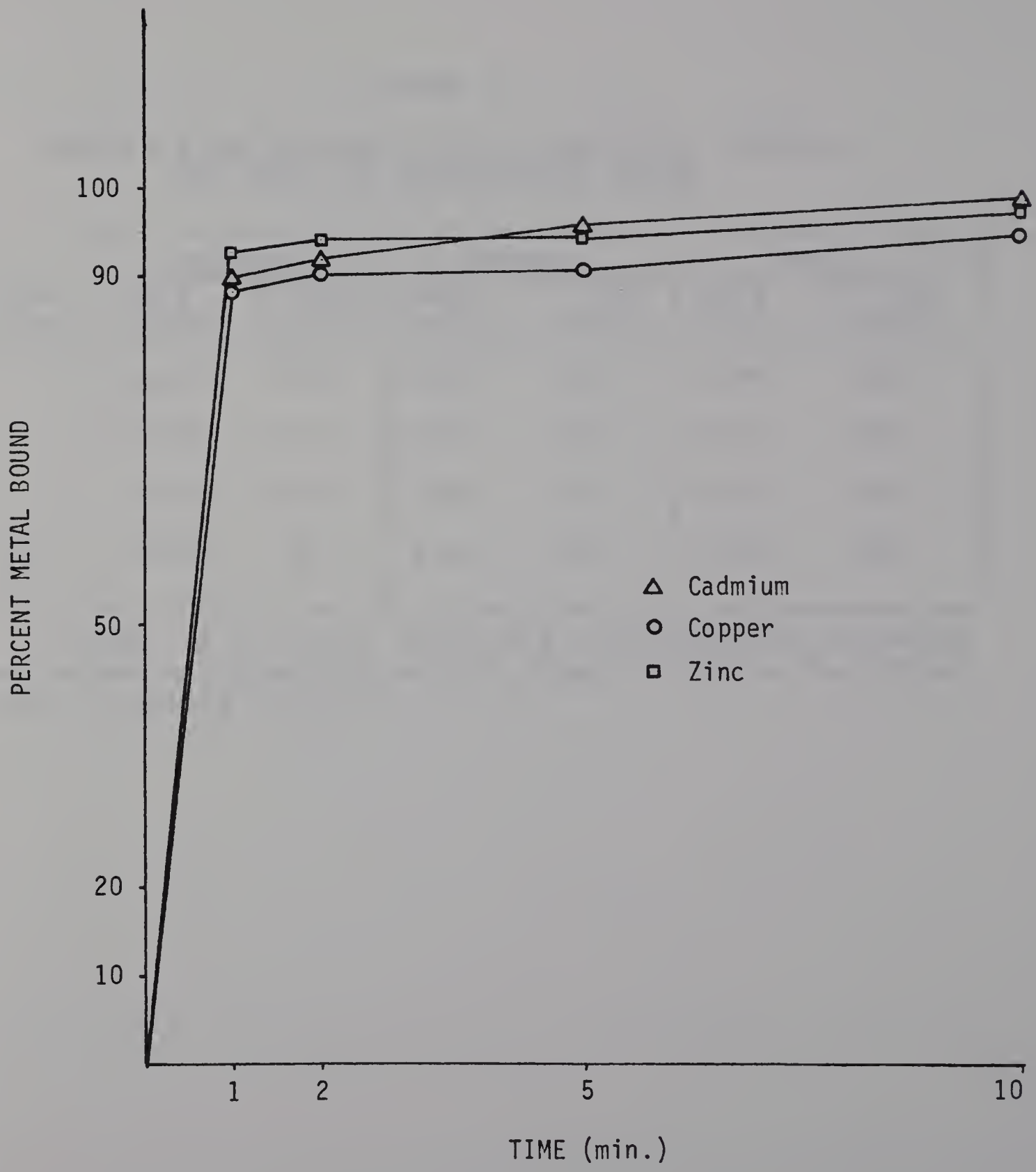
PERCENT METAL BOUND
100
90
50
20
10
1
2
5
10
TIME (min.)
Cadmium
Copper
Zinc

Figure 2

TABLE 1

BINDING RATE OF HEAVY METALS CADMIUM, COPPER, AND ZINC BY CHROOCOCCUS PARIS

Time (min.)	Cadmium		Copper		Zinc	
	mg/g bound	% total bound	mg/g bound	% total bound	mg/g bound	% total bound
1	2.81	90	2.78	89	2.88	92
2	2.84	91	2.81	90	2.91	93
5	2.97	95	2.81	90	2.94	94
10	3.06	98	2.94	94	3.06	98

NOTE: A 1.0 mg/l (0.05 mg) concentration of metal was added to 32 mg dry weight algae in 100 ml deionized water adjusted to pH 7.

frame takes into account not only that amount of metal rapidly bound but the significant amount which binds afterwards.

Metal Binding to Algae - Control Study.

Heavy metal binding to experimental filter apparatus.

Metal solutions which were filtered through the glass filter system and filter system with a 0.45 micron Millipore membrane filter were found to be unaltered in concentration. A one percent average reduction in the 5.0 mg/l metal concentration was noted after metal filtration through the 0.45 micron membrane filter. This amount, however, falls within experimental error.

Metal solutions filtered through a 0.45 micron membrane filter and glass fiber pre-filter did show significant decreases in concentration after filtration (Table 2). An average reduction in metal concentration of 45% was recorded after filtration of a 1.0 mg/l metal solution. All metal binding experiments were, therefore, performed using only the membrane filters. Sufficient filtration rates were obtained by decreasing the vacuum to 3 - 5 psi. during filtration.

Cellular binding conformation. Metal concentrations in algal digestions corresponded closely to predicted

TABLE 2

CONTROL STUDY OF HEAVY METAL BINDING TO FILTRATION APPARATUS AND FILTERS

Metal added (mg/l)	mg/l		% Bound of Control
	0.45micron filter	0.45micron + glass fiber	
1.0	1.0*	0.55*	45*
2.0	2.0	1.30	35
5.0	4.95	3.95	21

*NOTE: Values represent averages for cadmium, copper, and zinc metals studied. Respective concentrations were added to ca. 15 mg dry weight algae in 50 ml deionized water at pH 7.

values (Table 3). These predictions were made on the basis of metal concentrations found in the respective algal sample filtrates. Measurement of metal binding ability by C. paris was therefore determined by filtration and subsequent filtrate analysis throughout this study.

Heavy Metal Binding Capacity of C. paris. The heavy metals, cadmium, copper, and zinc were bound by C. paris cell cultures in a manner which followed the Langmuir adsorption isotherm (Figures 3,4 and 5). Cadmium, copper, and zinc were accumulated in concentrations reaching 53, 122, and 66 mg/l dry weight algae, respectively (Table 4). The short-term accumulation maxima were reached at different ambient metal concentrations which were added to the algal cultures. These values were approximately 70, 150, and 120 mg/l for cadmium, copper, and zinc, respectively.

When comparing the metal uptake at various ambient metal concentrations, apparent differences were observed in trace metal accumulation. At ambient metal ion concentrations below 20 mg/l, the order of metals bound in greatest amount was Cd > Cu > Zn. The order of metals bound was Cu > Cd > Zn at ambient metal concentrations ranging from 20-60 mg/l and Cu > Zn > Cd at ambient concentrations greater than 60 mg/l (Figure 6).

TABLE 3

CONTROL STUDY OF CADMIUM, COPPER, AND ZINC BINDING TO CHROOCOCCUS PARIS CELLULAR MATERIAL

Metal added (mg)	Cadmium			Copper		
	f	d	total	f	d	total
0.25	.045	.20	.245	.060	.18	.240
0.50	.175	.32	.495	.195	.29	.485
1.00	.470	.54	1.010	.510	.45	.960

Metal added (mg)	Zinc		
	f	d	total
0.25	.065	.17	.235
0.50	.205	.29	.495
1.00	.510	.46	.970

NOTE: Respective metal concentrations added to algal suspensions and filtered. Algae digested using conc. HNO_3. f = filtrate; d = digest; total = f + d where all values are in milligrams.

Fig. 3. Metal uptake of cadmium by C. paris vs. metal ion concentration. Cadmium concentrations were added to 16 mg dry wt. algae in 50 ml deionized water and stirred. Algal suspension maintained at pH 7. Increased metal uptake occurs with increasing metal concentration until saturation level is reached. Curve follows Langmuir isotherm.

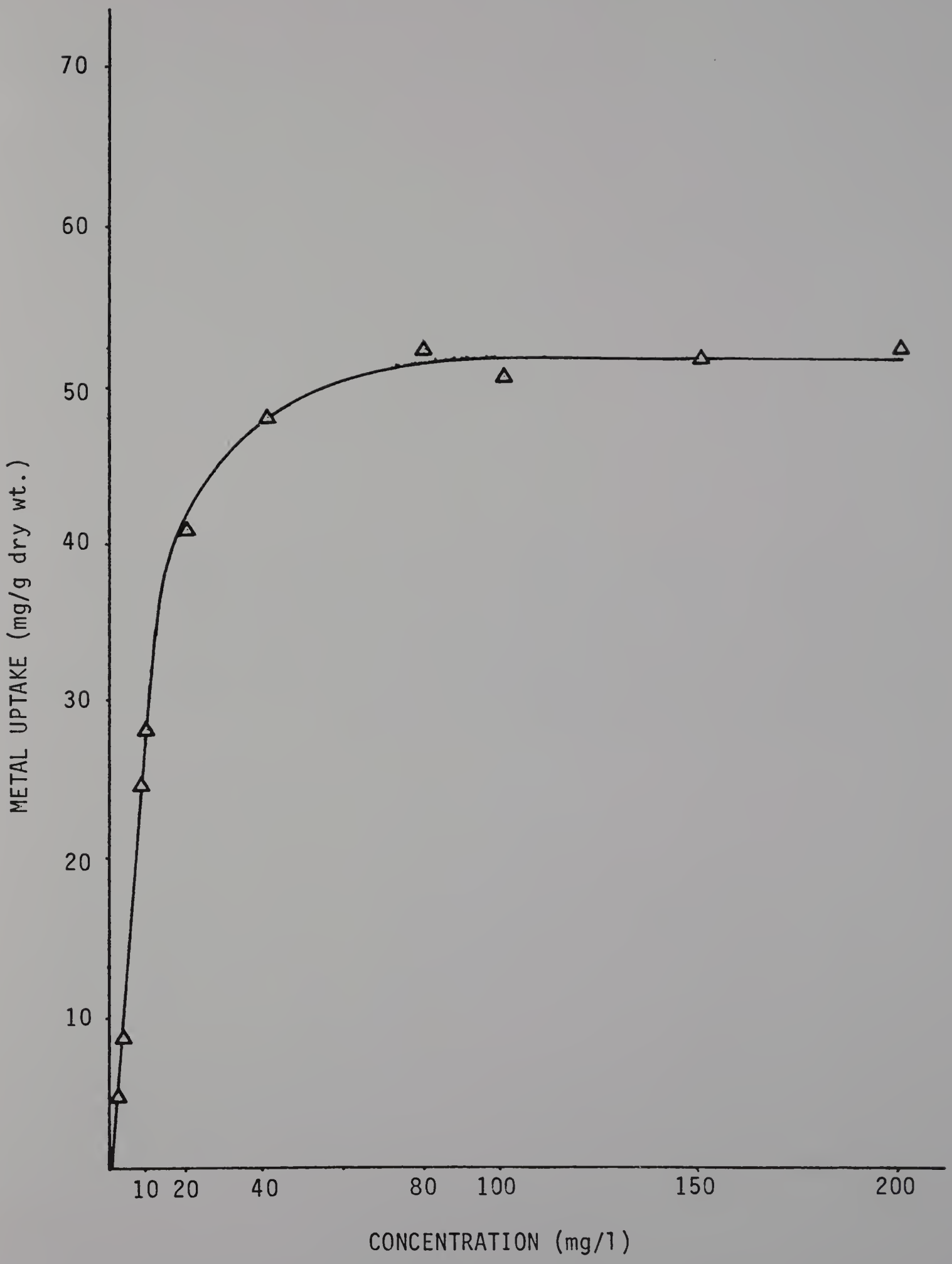
70
60
50
40
30
20
10
METAL UPTAKE (mg/g dry wt.)
10 20 40 80 100 150 200
CONCENTRATION (mg/l)

Figure 3

Fig. 4. Metal uptake of copper by C. paris vs. metal concentration. Copper concentrations were added to 16 mg dry wt. algae in 50 ml deionized water and stirred. Algal suspension maintained at pH 7. Increased metal uptake occurs with increasing metal concentration until saturation level is reached. Curve follows Langmuir adsorption isotherm.

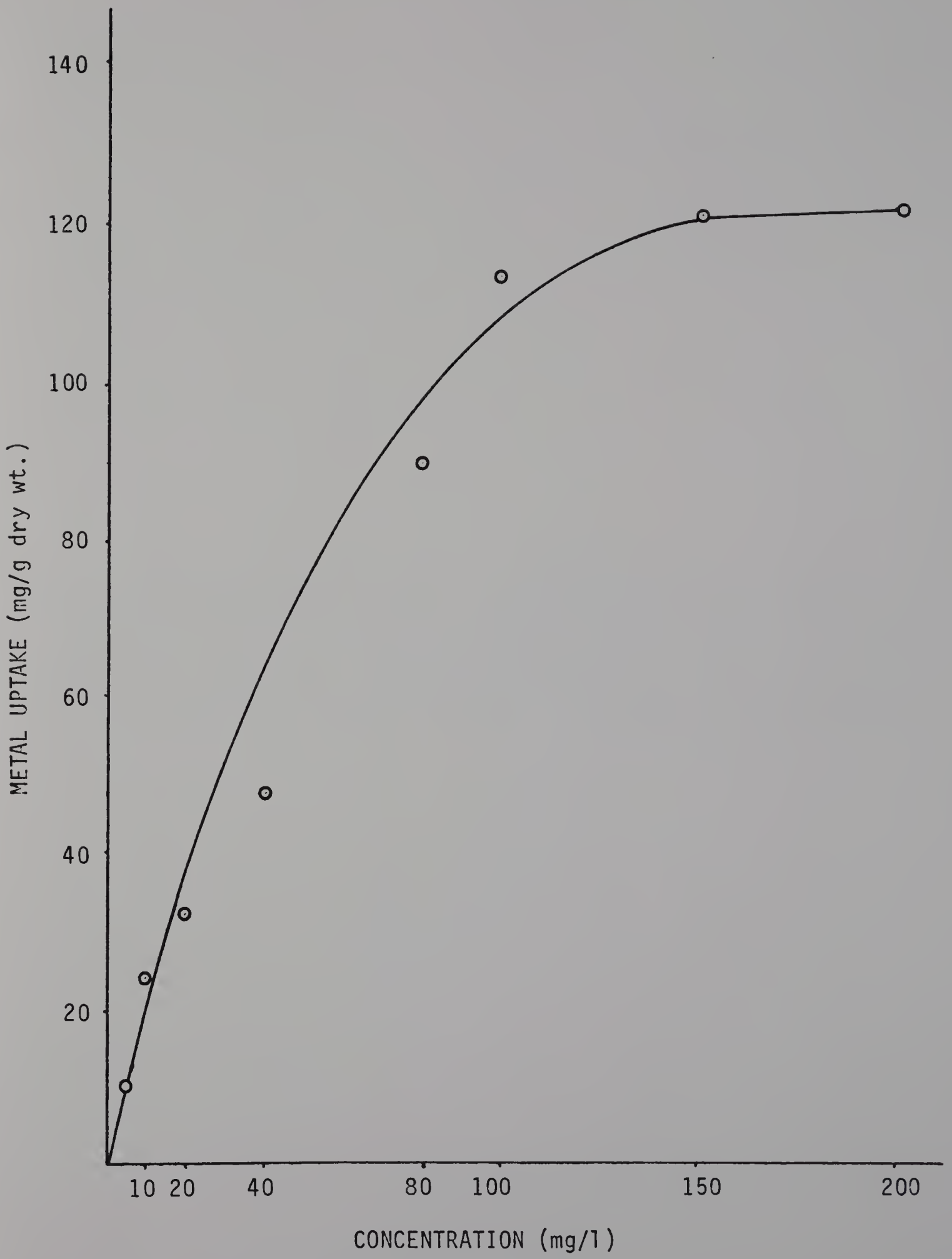
140
120
100
80
60
40
20
METAL UPTAKE (mg/g dry wt.)
10 20 40 80 100 150 200
CONCENTRATION (mg/l)

Figure 4

Fig. 5. Metal uptake of zinc by C. paris cells vs. metal ion concentration. Metal concentrations were added to 16 mg dry wt. algae in 50 ml deionized water. Algal suspension maintained at pH 7. Increased metal uptake occurs with increasing metal concentration until saturation is reached. Curve follows the Langmuir adsorption isotherm.

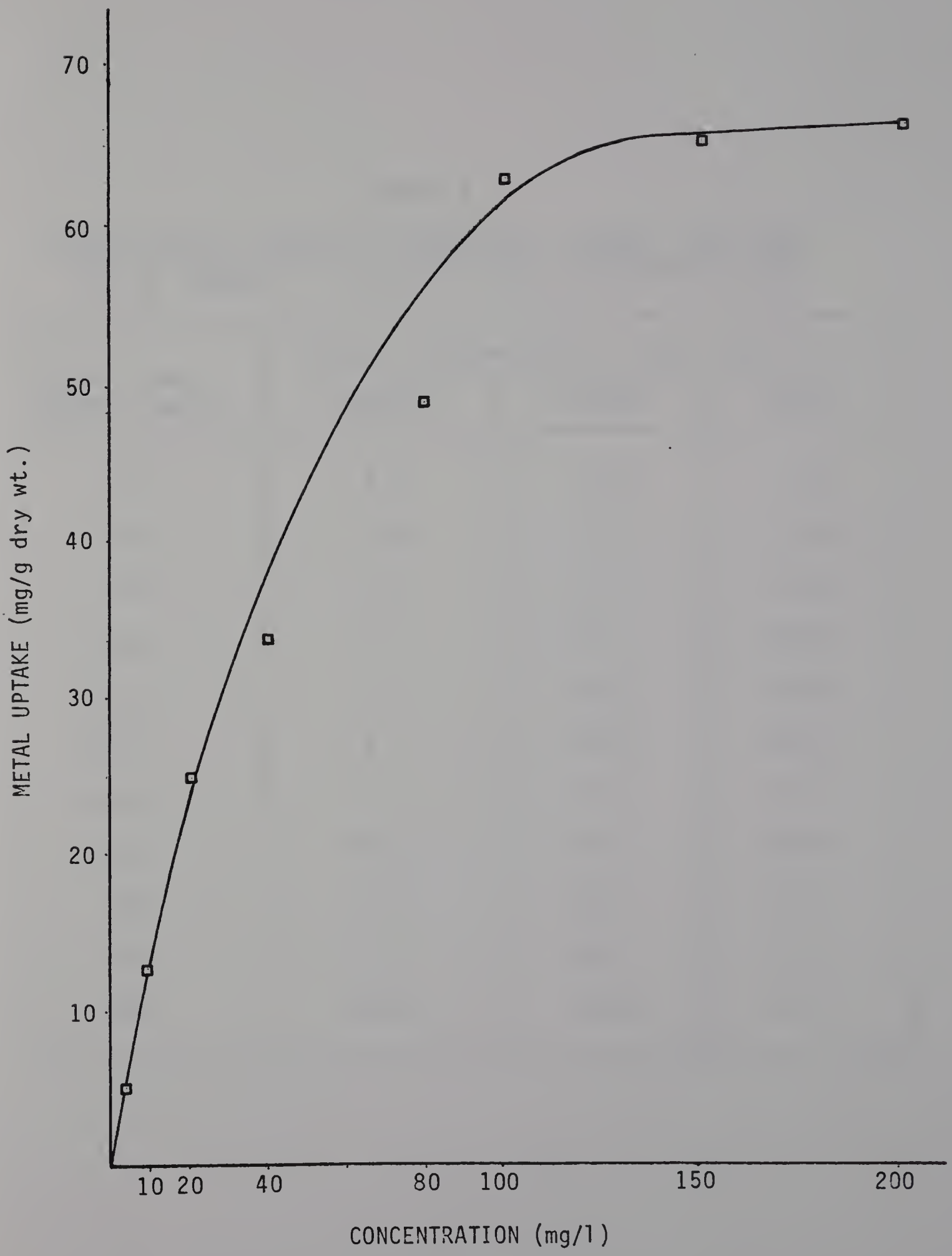
70
60
50
40
30
20
10
METAL UPTAKE (mg/g dry wt.)
10 20 40 80 100 150 200
CONCENTRATION (mg/l)

Figure 5

TABLE 4

HEAVY METAL UPTAKE OF CADMIUM, COPPER, AND ZINC BY *C. PARIS* VS. AMBIENT METAL CONCENTRATIONS

Metal conc. added (mg/l)	Metal Accumulation (mg/g dry wt.)		
	Cadmium	Copper	Zinc
1.0	4.0	2.5	1.5
2.0	8.0	5.1	2.5
4.0	16	11	5.0
8.0	25	21	10.5
10.0	28	24.5	12.5
20.0	41	32.5	25.5
40.0	49	47	33
80.0	52.5	90	48.5
100	51	113	63
150	52	122	65
200	53.5	122.5	66

Fig. 6. Metal uptake of cadmium, copper, and zinc by C. paris vs. metal ion concentration. Metal concentrations shown were added to 16 mg dry wt. algae in 50 ml deionized water and stirred. Metal-algal system maintained at pH 7. Increased metal uptake occurs with increasing metal concentration until saturation level is reached.

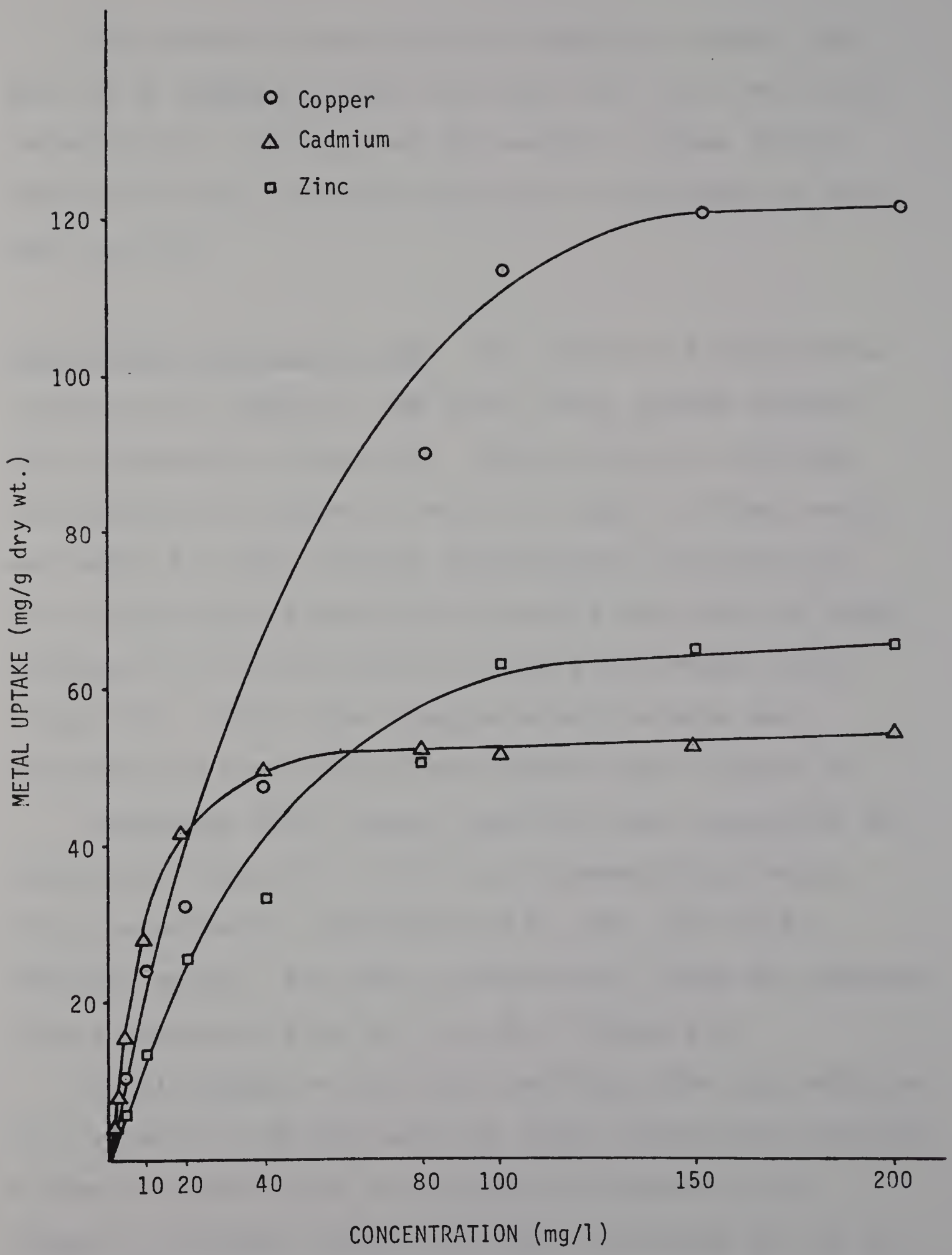
Copper
Cadmium
Zinc
120
100
80
60
40
20
METAL UPTAKE (mg/g dry wt.)
10 20 40 80 100 150 200
CONCENTRATION (mg/l)

Figure 6

The maximum accumulation of cadmium, copper, and zinc by C. paris cultures was only 5.3, 12.2, and 6.6%, respectively, of their own dry weight. These values deserve further attention and will be discussed in the next section.

Heavy Metal Binding vs. pH. The results of heavy metal binding by C. paris vs. pH shows metal uptake increase with increasing culture pH. Metal uptake vs. pH data resulting from studies in which 5.0 mg/l (0.25mg) metal was added to algal culture suspensions containing ca. 300 mg dry weigh algae/liter revealed the order of metal binding to be Cu >Zn >Cd on a umol/g dry weight basis (Figure 7). This order changes when the data was presented on a mg metal/g dry weight basis (Figure 8).

Increased metal uptake resulted from increasing the ambient pH from pH 4 to pH 7 in incremental pH units. Metal accumulation increased by 41, 28, and 11% for cadmium, copper, and zinc, respectively, when the ambient pH was increased from pH 4 to pH 7.(Table 5).

Metal uptake vs. pH data resulting from the addition of 10.0 mg/l (0.50 mg) metal to algal suspensions yielded a metal binding order of Cu >Zn >Cd (Figures 9,10). Uptake of cadmium, copper, and zinc increased by 15, 16, and 8%, respectively, as a result of increasing the pH

Fig. 7. Effect of pH on metal binding capacity by C. paris cells. Four separate 15 mg dry wt. algal suspensions were titrated using 0.1N HNO_3 to the pH values shown. A total of 0.25 mg metal was added to the suspension and stirred. See Methods and Materials.

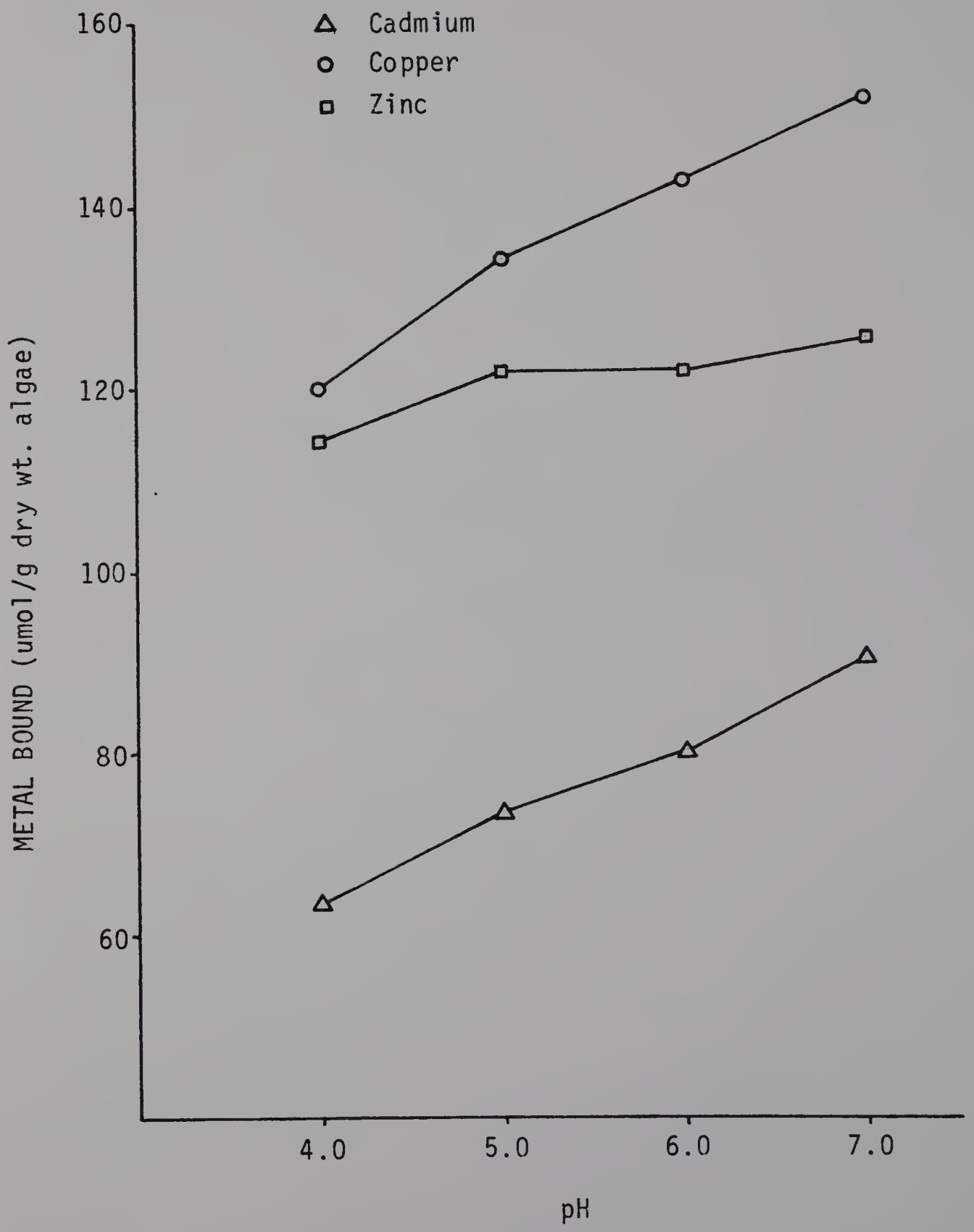
Cadmium
Copper
Zinc
160
140
120
100
80
60
METAL BOUND (umol/g dry wt. algae)
4.0
5.0
6.0
7.0
pH

Figure 7

Fig. 8. Effect of pH on metal binding capacity by C. paris. Metal accumulation values expressed as mg/g dry weight algae. Four separate 15 mg dry wt. algal suspensions were titrated using 0.1N HNO_3 to the pH shown. A total of 0.25mg metal was added to the suspensions and stirred.

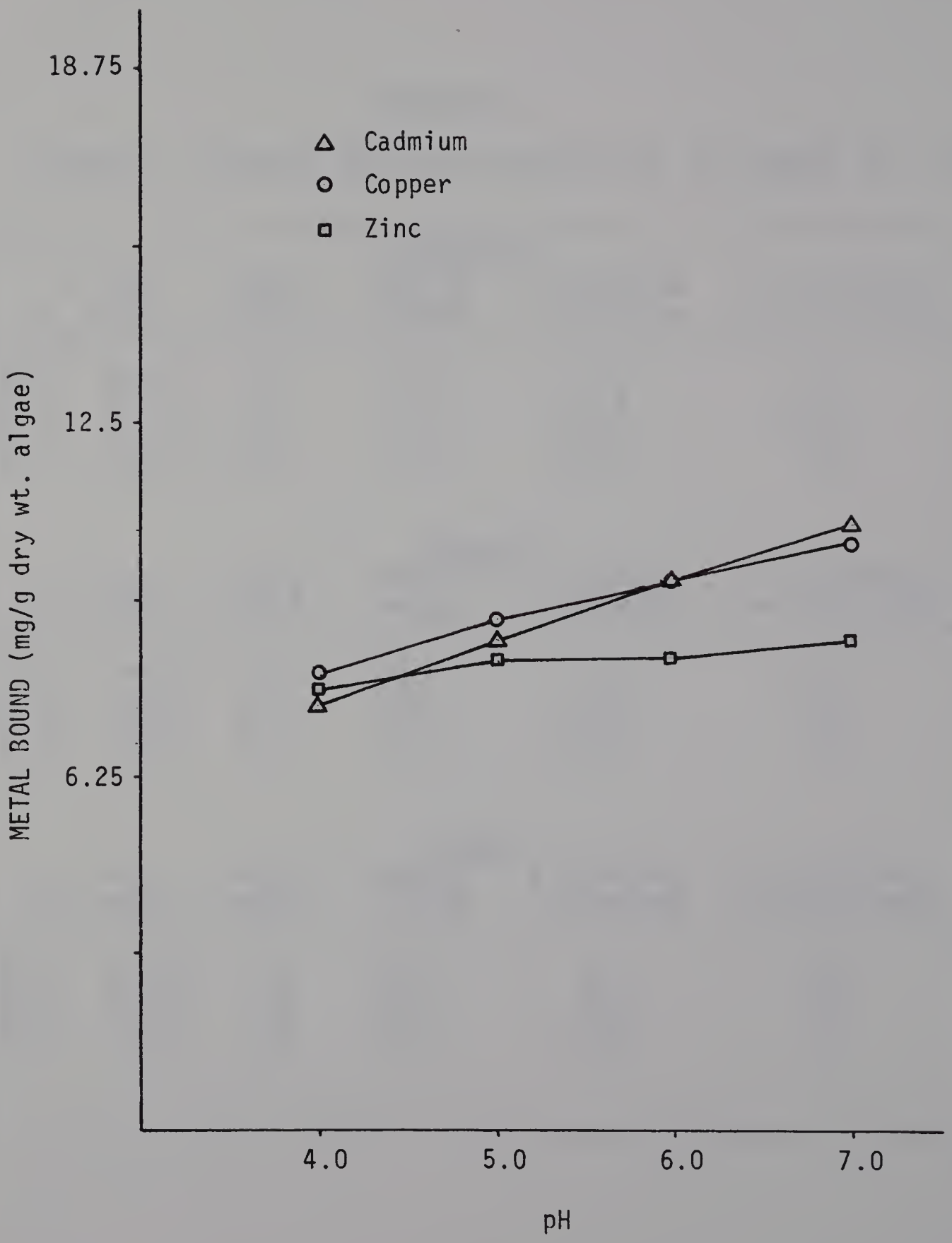
18.75
12.5
6.25
METAL BOUND (mg/g dry wt. algae)
Cadmium
Copper
Zinc
4.0
5.0
6.0
7.0
pH

FIGURE 8

TABLE 5

CADMIUM, COPPER, AND ZINC UPTAKE BY C. PARIS VS. PH

pH	mg added	mg/g bound	Cadmium umol/g bound	% binding increase	% increase per pH unit
4.0	0.25	7.1	63	---	---
5.0	0.25	8.2	73	13.4	13.4
6.0	0.25	9.1	81	22.0	10.6
7.0	0.25	10.0	89	29.0	7.0

pH	mg added	mg/g bound	Copper umol/g bound	% binding increase	% increase per pH unit
4.0	0.25	7.6	120	---	---
5.0	0.25	8.5	134	11.8	11.8
6.0	0.25	9.1	143	19.7	7.9
7.0	0.25	9.7	153	27.6	7.9

pH	mg added	mg/g bound	Zinc umol/g bound	% binding increase	% increase per pH unit
4.0	0.25	7.4	113	---	---
5.0	0.25	7.9	121	6.8	6.8
6.0	0.25	7.9	121	6.8	0
7.0	0.25	8.2	125	10.8	4.0

Fig. 9. Effect of pH on metal binding capacity by C. paris cells. Four separate 15 mg dry wt. algal suspensions were titrated using 0.1N HNO_3 to the pH values shown. A total of 0.50 mg metal was added to the suspension and stirred. See Materials and Methods.

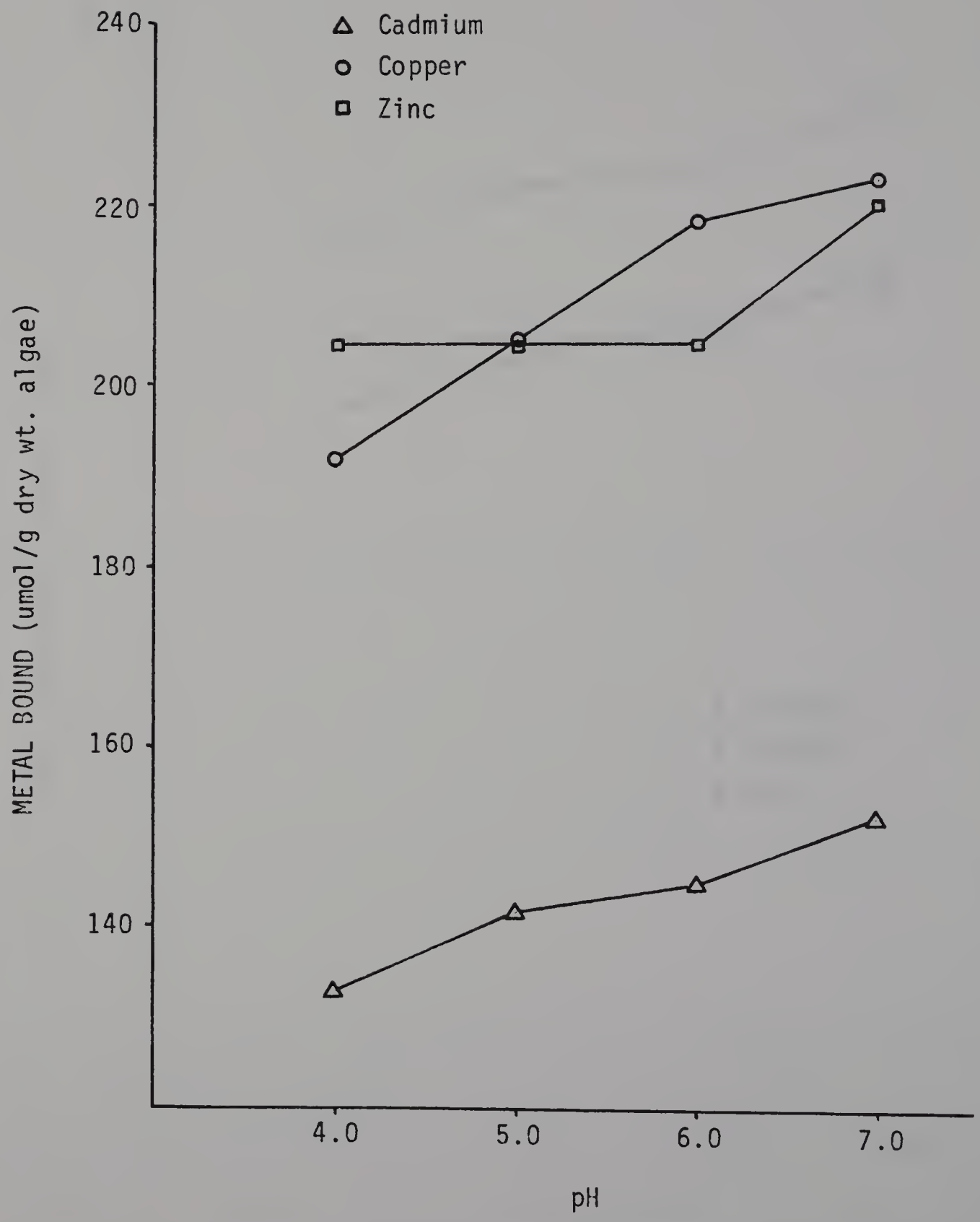
Cadmium
Copper
Zinc
240
220
200
180
160
140
METAL BOUND (umol/g dry wt. algae)
4.0
5.0
6.0
7.0
pH

Figure 9

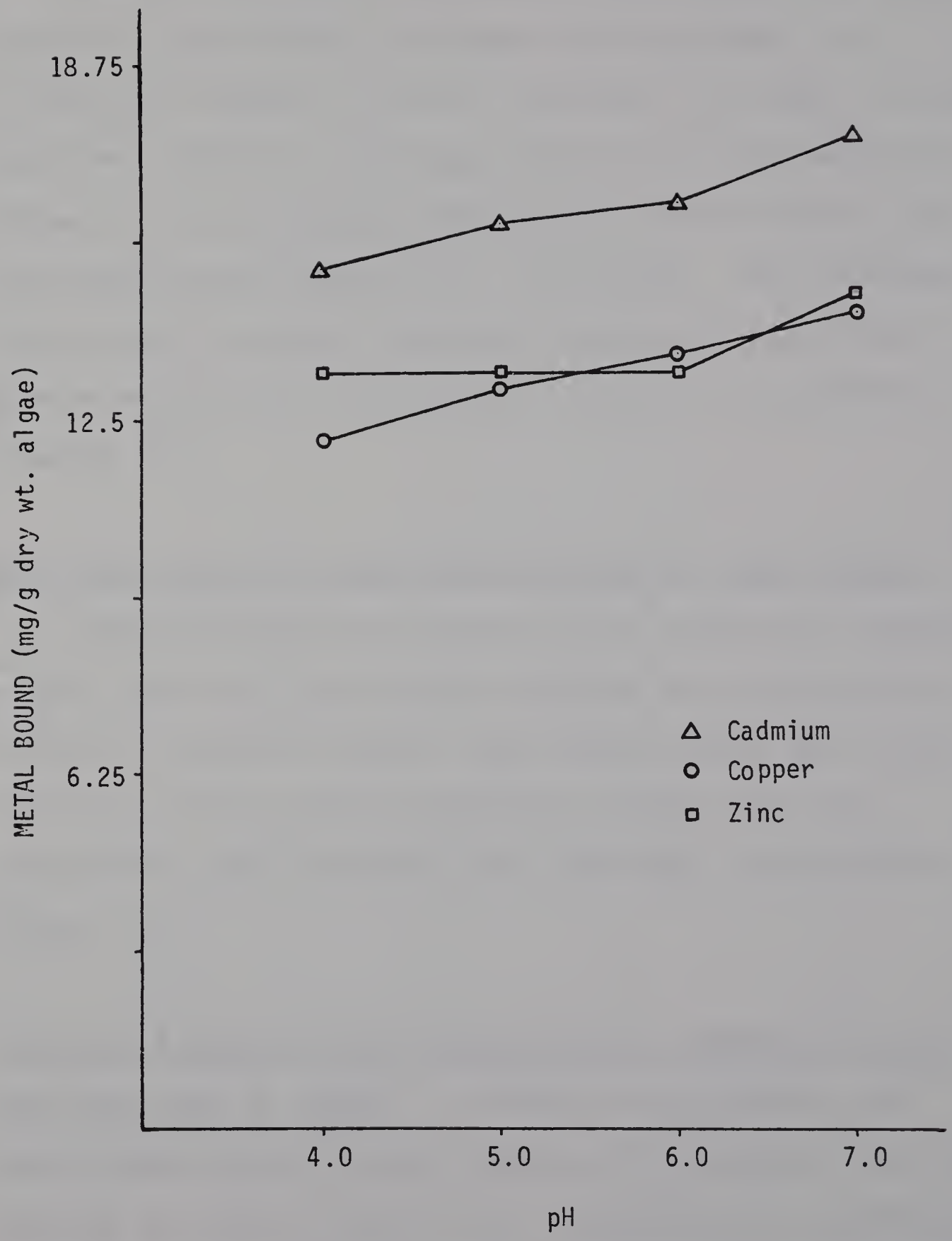

Fig. 10. Effect of pH on metal binding capacity by C. paris. A total of 0.50 mg metal was added to the suspensions and stirred. Metal uptake values in mg/g dry weight algae.

from pH 4 to pH 7 (Table 6).

Results from the competitive binding of the three metals vs. pH showed a similar binding order, i.e. Cu > Zn > Cd (Figures 11,12). Although 10.0 mg/l of each metal was added to the algal suspensions, accumulation values at all pH levels were reduced below values seen for single metal binding vs. pH studies. The increase in total metal bound and binding increase of individual metals relative to total bound vs. pH is illustrated (Figure 13).

EDTA Extraction of Bound Metals from C. paris Cells.

Ethylenediaminetetraacetic acid extracted cadmium, copper, and zinc previously bound by the algal cells. After a 10 minute contact time between EDTA and bound metal (2:1 mole ratio),nearly all bound metal was solubilized and recovered from the algal suspensions (Table 7).

Toxicity Studies of the Heavy Metals, Cadmium, Copper, and Zinc upon C. paris. A direct relationship was established between algal biomass (dry weight) and optical density at 450nm (Figure 14). A correlation coefficient of 0.999 was obtained. Measurement of algal culture optical density was used, therefore, as an efficient and

TABLE 6

CADMIUM, COPPER, AND ZINC UPTAKE BY C. PARIS VS. PH

pH	mg added	mg/g bound	Cadmium umol/g bound	% binding increase	% increase per pH unit
4.0	0.50	15.0	133	---	---
5.0	0.50	15.9	141	6.3	6.3
6.0	0.50	16.3	145	8.3	2.0
7.0	0.50	17.2	153	14.6	6.3

pH	mg added	mg/g bound	Copper umol/g bound	% binding increase	% increase per pH unit
4.0	0.50	12.2	192	---	---
5.0	0.50	13.1	206	7.7	7.7
6.0	0.50	13.8	217	12.8	5.1
7.0	0.50	14.1	222	15.4	2.6

pH	mg added	mg/g bound	Zinc umol/g bound	% binding increase	% increase per pH unit
4.0	0.50	13.4	205	---	---
5.0	0.50	13.4	205	0	0
6.0	0.50	13.4	205	0	0
7.0	0.50	14.4	220	7.0	7.0

Fig. 11. Competitive metal binding by C. paris vs. pH ; 0.5 mg of each metal (cadmium, copper, and zinc) was added to pH adjusted, 16 mg dry wt. suspensions of algal cells and stirred. See Materials and Methods.

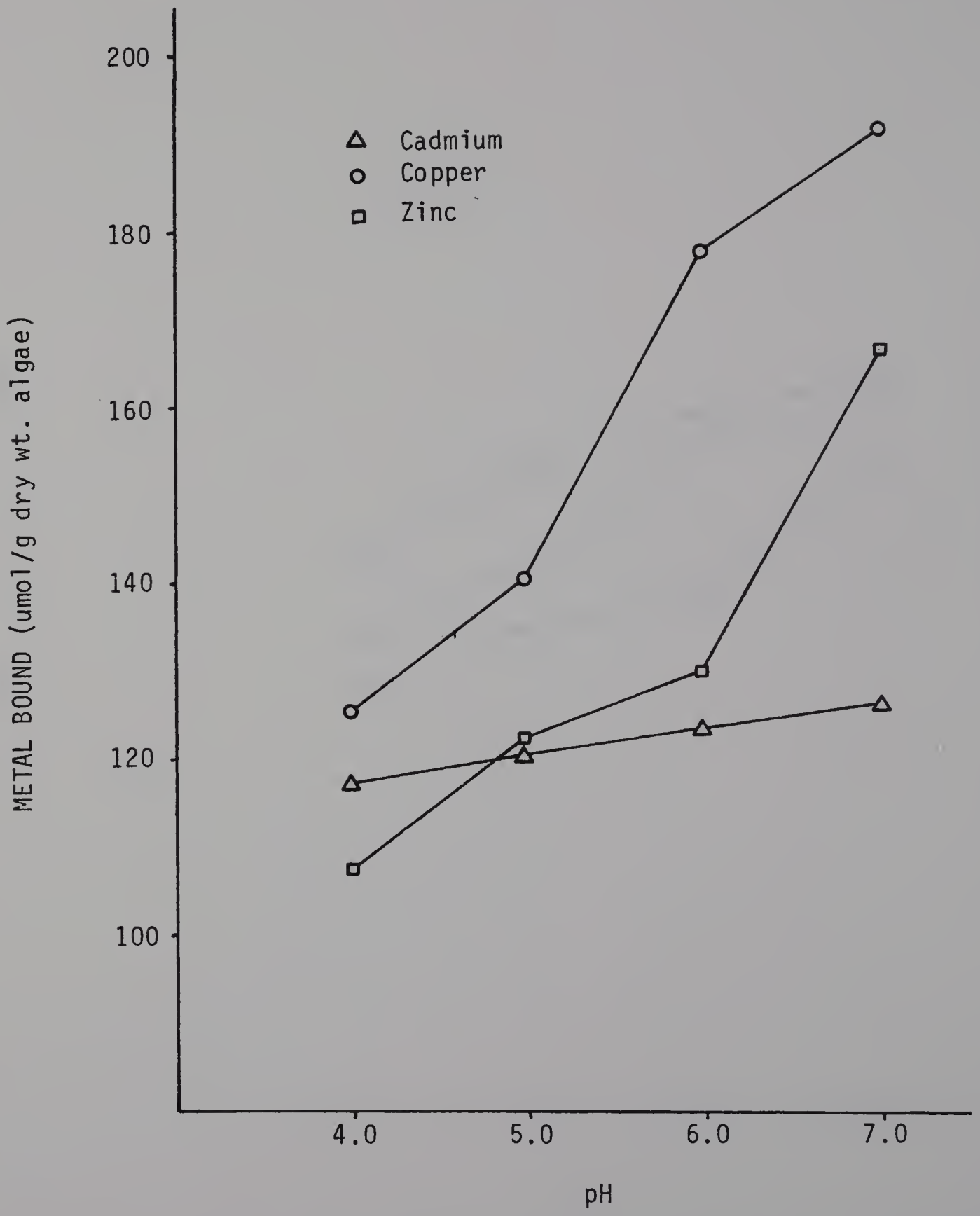
200
180
160
140
120
100
METAL BOUND (umol/g dry wt. algae)
Cadmium
Copper
Zinc
4.0
5.0
6.0
7.0
pH

Figure 11

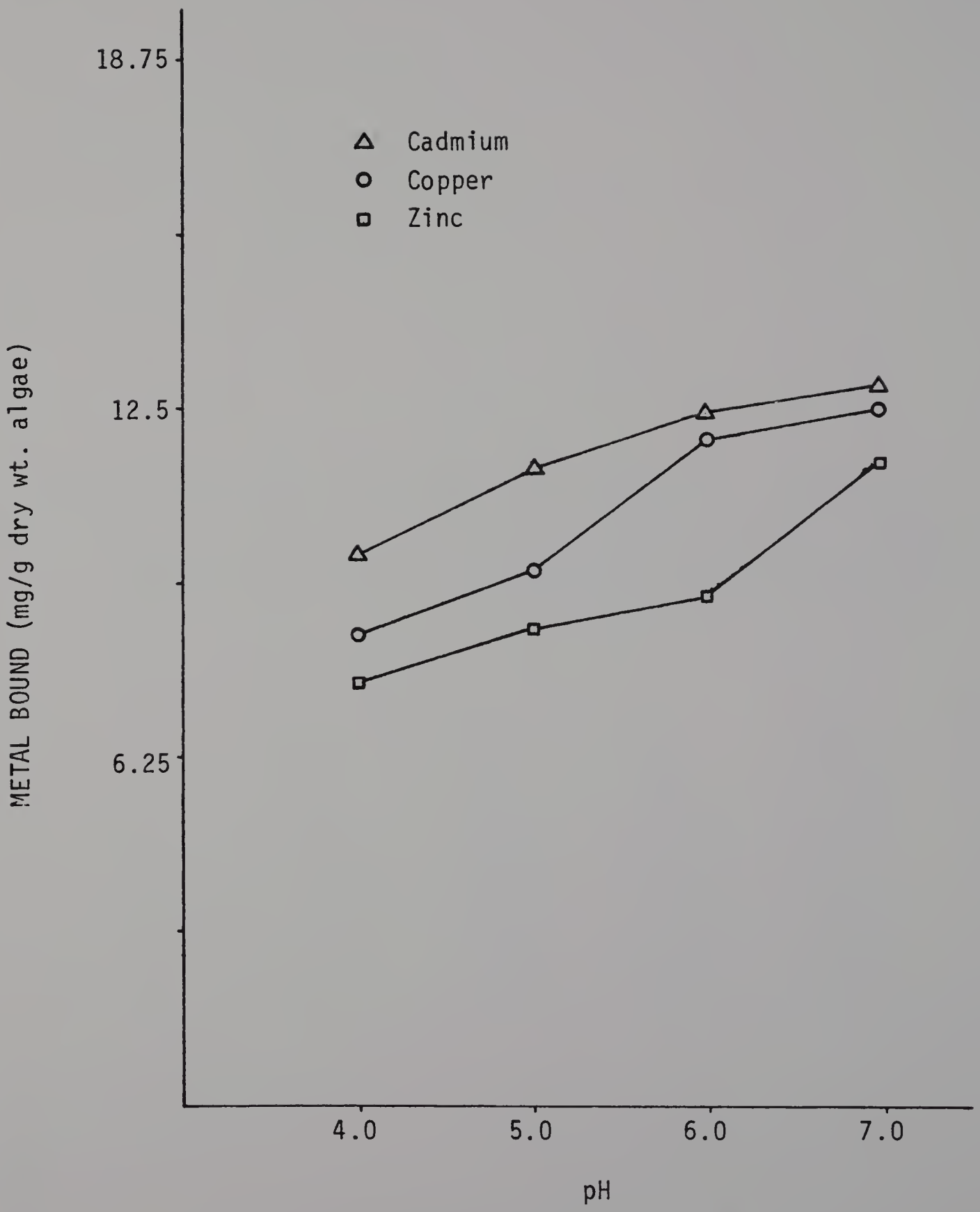

Figure 12. Competitive metal binding by C. paris vs. pH; 0.5 mg of each metal was added to cells and stirred. Accumulation values expressed as mg/g.

Fig.13. Comparison of individual amounts of metals bound by C. paris cells to total metals bound (cadmium + copper + zinc) with respect to pH; 0.5 mg of each metal was added to a pH adjusted, 16 mg dry wt. algal suspension in 50 ml deionized water and stirred.

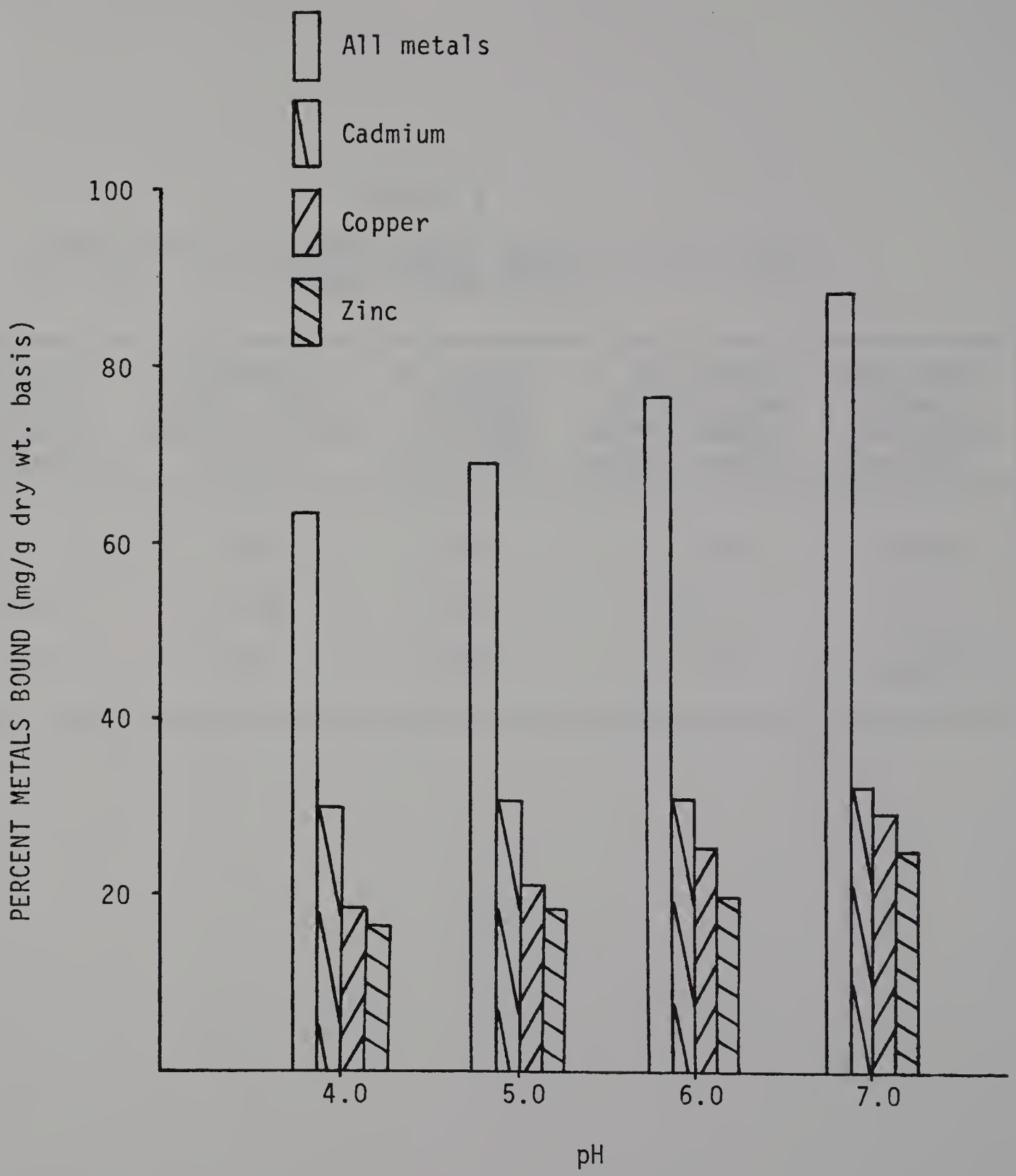
All metals
Cadmium
Copper
Zinc
100
80
60
40
20
PERCENT METALS BOUND (mg/g dry wt. basis)
4.0
5.0
6.0
7.0
pH

Figure 13

TABLE 7

EXTRACTION OF BOUND HEAVY METALS FROM ALGAL CELLS USING EDTA

Metal conc. added (mg/l)	Filtrate metal conc. after 15 min (mg/l)	mg metal bound to cells w/o EDTA	Filtrate metal conc. after EDTA add. (mg/l)	mg metal bound to cells w/EDTA
1.0	0.09	0.05	0.98	<0.01
2.0	0.16	0.09	2.0	0
5.0	0.5	0.23	4.95	<0.01

Fig. 14. Biomass measurement plot showing the correlation between algal culture optical density at 450nm and culture dry weight concentration (mg/l).

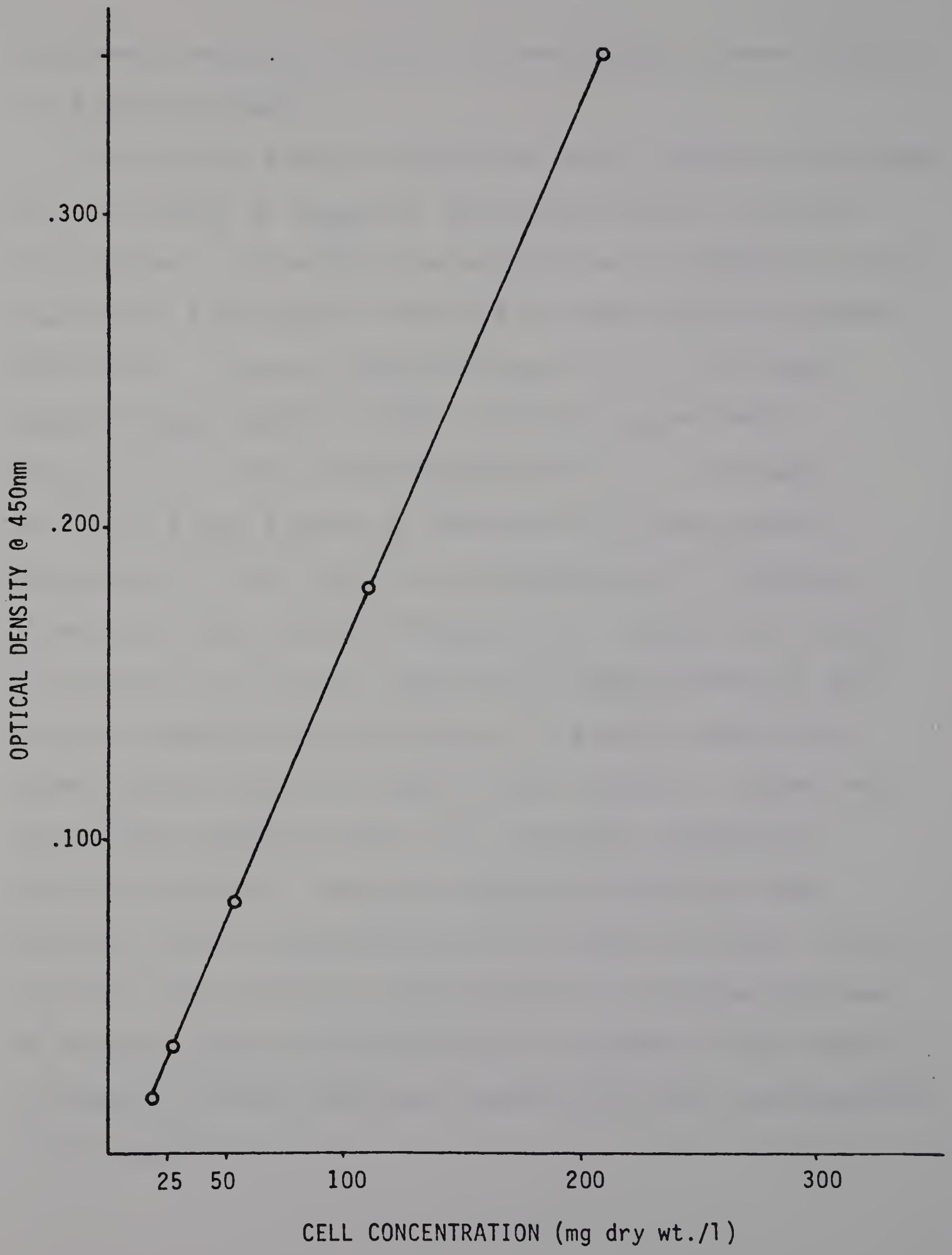
OPTICAL DENSITY @ 450nm
.300
.200
.100
25
50
100
200
300
CELL CONCENTRATION (mg dry wt./l)

Figure 14

relatively sensitive method for measuring biomass during the toxicity study.

All metals studied exhibited toxic, growth inhibiting effects toward C. paris at concentrations of 1.0 mg/l and greater. All metal concentrations of copper and zinc studied (0.1-5.0 mg/l) resulted in some degree of growth inhibition. Copper concentrations of 0.1 - 0.2 mg/l reduced algal growth by 56% and 80%, respectively (Figure 15). Zinc concentrations of 0.1 - 0.2 mg/l inhibited algal growth by 10% and 15%, respectively, (Figure 16), while similar concentrations of cadmium stimulated algal growth (Figure 17). Copper, at a concentration of 0.4 mg/l, reduced C. paris growth by 95% while a cadmium concentration of 0.4 mg/l resulted in growth nearly equal to that of the control. Copper concentrations ranging from 1.0 - 5.0 mg/l completely inhibited growth. Similar complete inhibition was recorded for a concentration of 5.0 mg/l of zinc. Table 7 provides data relating algal growth in cultures exposed to various metal concentrations to growth of the control in terms of final standing biomass after the experimental 10 day period.

Fig. 15. Growth curves showing growth inhibition of C. paris cells after addition of copper concentrations (shown) to algal cultures. Initial optical density of 0.018 corresponds to 15 mg dry wt. algae/liter. Copper concentrations of 1.0, 2.0, and 5.0 mg/l completely inhibited growth.

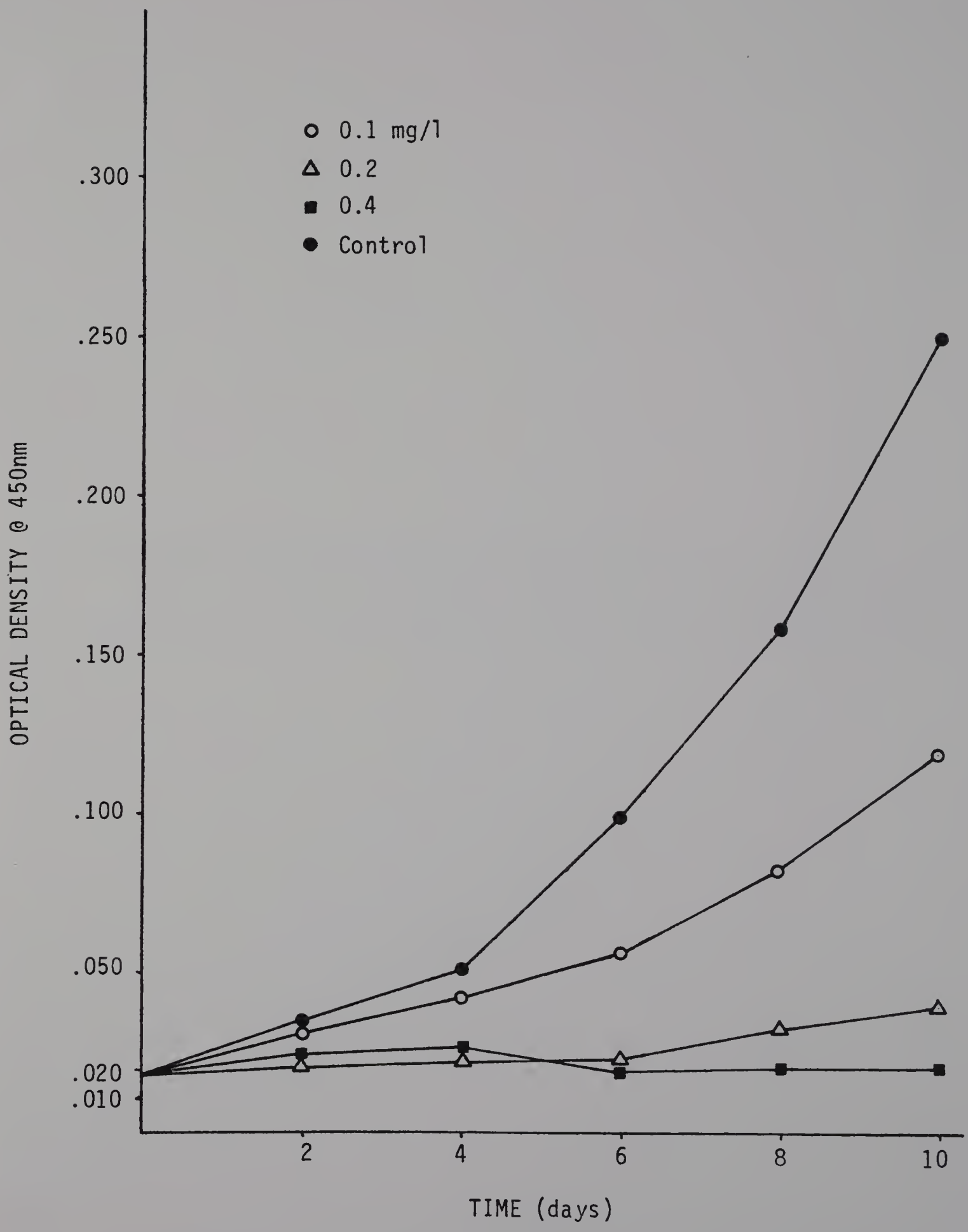
0.1 mg/l
0.2
0.4
Control
.300
.250
.200
.150
.100
.050
.020
.010
OPTICAL DENSITY @ 450nm
2
4
6
8
10
TIME (days)

Figure 15

Fig. 16. Growth curves showing growth inhibition of C. paris after addition of zinc concentrations (shown) to algal cultures. Initial optical density of 0.018 corresponds to 15 mg dry wt. of algae/liter.

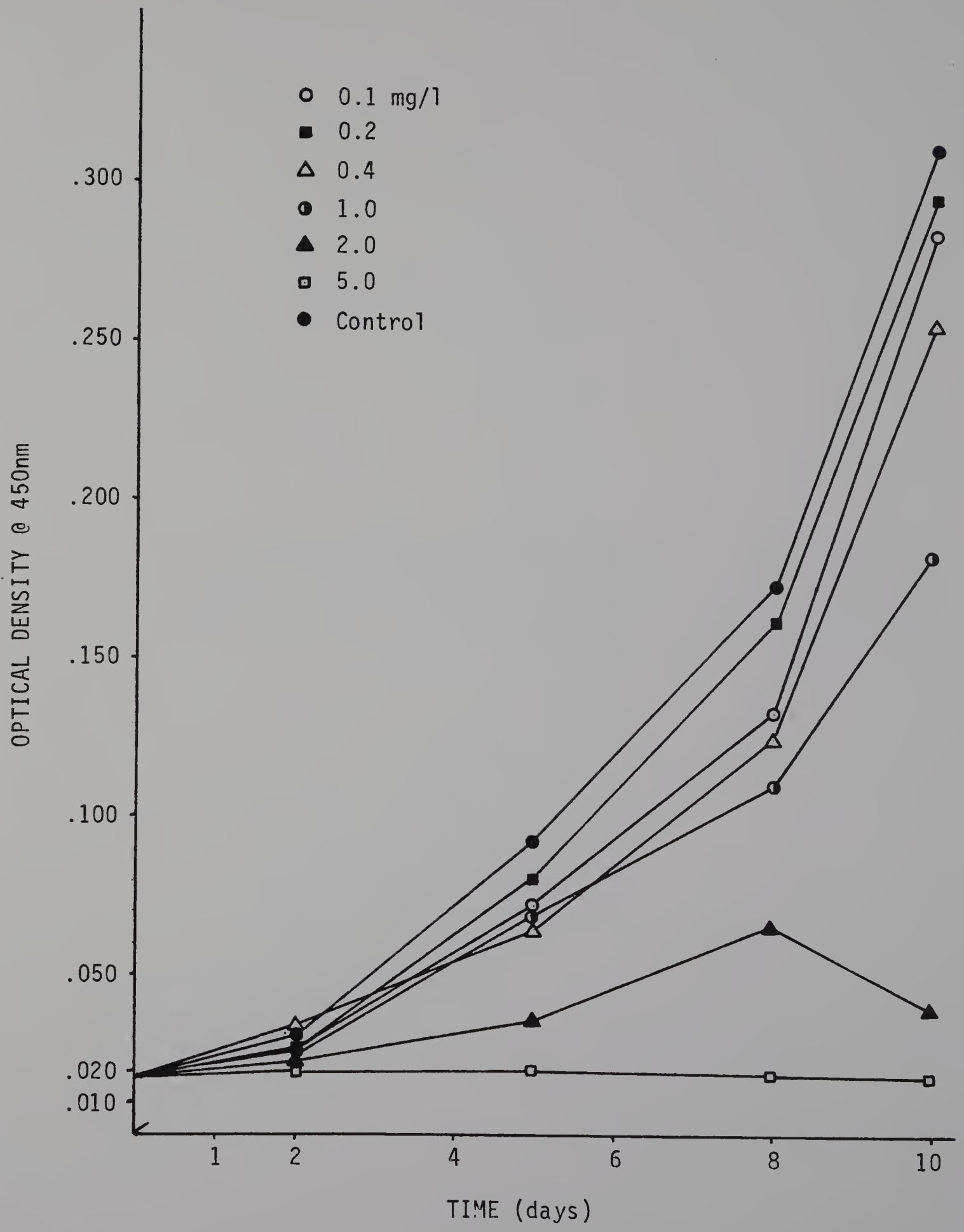
0.1 mg/l
0.2
0.4
1.0
2.0
5.0
Control
.300
.250
.200
.150
.100
.050
.020
.010
OPTICAL DENSITY @ 450nm
1
2
4
6
8
10
TIME (days)

Figure 16

Fig. 17. Growth curves showing growth rate inhibition of C. paris after addition of cadmium concentrations (0.1 mg/l to 5.0 mg/l) to algal cultures. Initial optical density of 0.018 corresponds to 15 mg dry weight of algae/liter.

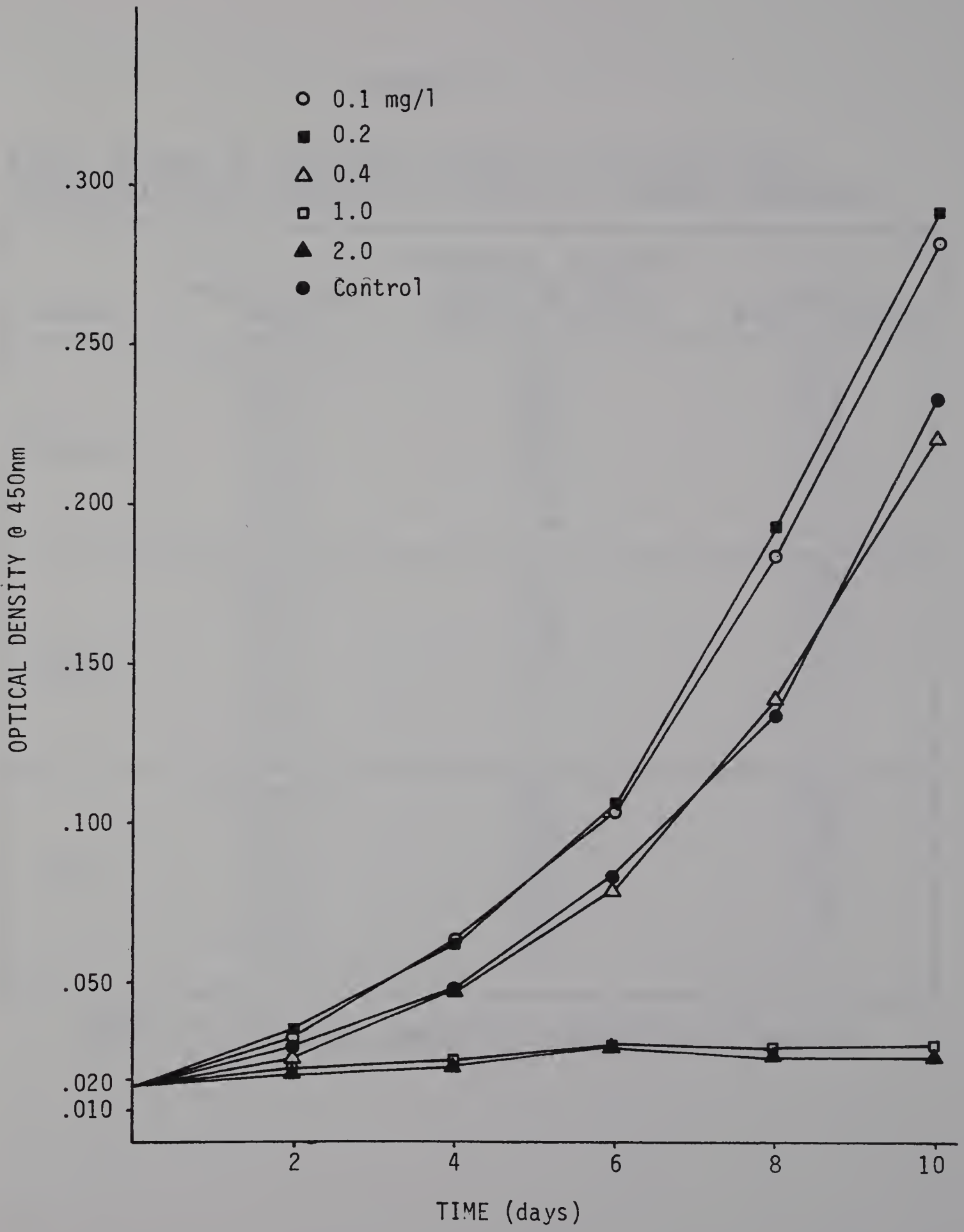
0.1 mg/l
0.2
0.4
1.0
2.0
Control
.300
.250
.200
.150
.100
.050
.020
.010
OPTICAL DENSITY @ 450nm
2
4
6
8
10
TIME (days)

Figure 17

TABLE 7

ALGAL GROWTH IN CULTURES EXPOSED TO VARIOUS METAL CONCENTRATIONS COMPARED TO GROWTH OF CONTROL CULTURES

Metal	Metal conc. (mg/l)	Standing biomass after 10 days (mg dry wt./l)	Growth % of control
Cadmium	Control	193	100
	0.1	225	117
	0.2	230	119
	0.4	187	97
	1.0	27	14
	2.0	25	13
	5.0	-*	-
Copper	Control	155	100
	0.1	77	44
	0.2	32	12
	0.4	22	5
	1.0	-	-
	2.0	-	-
	5.0	-	-
Zinc	Control	188	100
	0.1	168	89
	0.2	175	93
	0.4	153	81
	1.0	110	53
	2.0	35	9
	5.0	15	-

NOTE: * - denotes complete inhibition of growth

C H A P T E R V

DISCUSSION

The initial rapid uptake of the heavy metals cadmium, copper, and zinc by exponentially growing C. paris cultures demonstrated the presence of cellular binding sites with high complex/chelate forming ability. Since this alga is enclosed by a capsular sheath, the initial contact between metal and ligand probably occurs at the outer surface of the sheath material. The few studies heretofore performed on blue-green extracellular polysaccharides (Fogg, 1966; Bishop et al., 1970; Wang and Tischer, 1973) suggest that this substance is non-homogeneous in nature. Therefore, it is likely that metals were bound to more than one class of ligand upon complex - chelate formation. The characteristics of the ligand extant in these binding reactions, therefore, play a highly significant role in the type and stability of complex formed (Rendleman, 1981).

In an entirely polysaccharide type capsular matrix, one would expect to find two major ligand types: hydroxyl and carboxyl. The latter would be expected if uronic acid monomers were present in the sheath material. Each ligand

also forms complexes which has a particular degree of stability. This stability, however, is also a function of the metal involved in the complex formation.

Initially, ligands which have high affinities for metals, forming strong, stable complexes bind metals rapidly until becoming saturated with metal ions. Metals may then bind to ligands having lower affinities for comples formation, but at a slower rate of binding. This may explain, in part, the observed initial rapid metal accumulation followed by a slower binding rate.

A second explanation of these results involves simple mass action effects. As more metal ions are bound, less become available for further complexation. Similarly, as more binding sites become occupied by metal ions, fewer become available for complexing metal ions.

Since metal binding capacities for cadmium, copper, and zinc were previously shown to be approximately 50, 120, and 65 mg/g dry weight algae, respectively, the former explanation appears unlikely. Because the metal binding capacity far exceeds the total metal accumulation (3-15 mg/g dry weight) seen in the binding rate study, a surplus of binding sites existed.

Careful consideration should be given to the type of filter used when metal binding determinations are performed via filtrate analysis as demonstrated in this

study. Millipore 0.45 micron membrane filters did not accumulate metals during the filtration of metal-algal suspensions while the Gelman glass fiber prefilters bound metals to a significant extent.

The amount of metal bound by C. paris was demonstrated to increase with increasing ambient metal concentration. The binding curves developed for all three metals followed the Langmuir adsorption isotherm model. This investigation has demonstrated that of the metals studied, copper was bound in greatest amount. Algal binding capacities for cadmium and zinc were approximately 50% that of the binding capacity for copper.

C. paris bound the metals cadmium, copper, and zinc to maxima of approximately 5, 12, and 7% of their own dry weight. These values, however, may not truly represent the maximum metal binding capacity of these algae. Cultures of C. paris used in the binding studies were microscopically examined and were found to contain palmelloid colonies, most of which held between four and sixteen cells/colony. This resulted in algal cultures which had relatively low surface area to biomass ratios. It is therefore speculated that greater metal binding capacity might have resulted if algal cultures, containing the same amount of biomass, possessed a greater overall number of smaller, fewer celled colonies, and single cells.

This would increase the surface area to biomass ratio and subsequently increase the number of available binding sites.

The order of metals binding in greatest amount vs. ambient metal concentrations were seen to change at different ambient metal concentrations. At concentrations below 20 mg/l the order of metals was Cd > Cu > Zn while an order of Cu > Zn > Cd was observed at concentrations above 60 mg/l.

Cadmium, copper, and zinc accumulation was influenced by the pH of the algal culture suspensions. Washed algal cultures at pH 7 exposed to 10.0 mg/l cadmium, copper, and zinc concentrations accumulated 14.6, 15.4, and 7.0% more metal, respectively, than did cultures maintained at pH 4.0. Similar results which showed increased metal binding with increased pH were observed by other investigators (Hart and Scaife, 1977; Crist et al., 1980). These data suggest that greater cation exchange takes place as the pH is increased. Kallqvist and Meadows (1978) believed that competition existed between H^+ and M^{n+} (metal ion) for binding sites on organic cellular matter. As pH is increased, greater displacement of H^+ from algal organic material occurs. Simultaneously, metal ions present form complexes at the active binding sites where protons have been displaced (Kohn, 1981).

When metal bound algal cultures were extracted with ethylenediaminetetraacetic acid, greater than 98% of the bound metals were recovered in the filtrate after algal suspensions were filtered through a 0.45 micron membrane filter. Since it has been shown that ethylene-diaminetetraacetic acid does not permeate through the algal cell membrane (Jackson and Morgan, 1978), it is suggested that any metals extracted by EDTA were those bound extracellularly to cell wall or capsular sheath material.

This research has demonstrated the toxic effects of various concentrations of cadmium, copper, and zinc upon the growth rate of exponentially growing C. paris cell cultures. All concentrations of copper and zinc studied (0.1-5.0 mg/l) exhibited growth inhibiting effects. Studies on the toxic effect of copper on Spirulina platensis, a filamentous blue-green alga, demonstrated similar growth inhibiting results even at a concentration of 0.02 mg/l (Kallqvist and Meadows, 1978). Various other investigators have shown copper to be algicidal at levels ranging from 0.06 - 0.3 mg/l (Bartlett and Rabe, 1974; Rosko, 1977; Laube et al., 1980). Although no speculation as to the mechanism of copper toxicity will be mentioned here, some observations on the mechanism of copper damage in Chlorella have been documented (Gross et al., 1970).

Zinc concentrations of 0.1-0.2 mg/l reduced the growth rate to 80% that of the control. Similar cadmium concentrations were observed to stimulate C. paris growth to 17% above that of the control. Other investigators have also documented stimulatory effects of low cadmium concentrations (Devi Prassad and Devi prassad, 1981). A copper concentration of 0.4 mg/l reduced the growth rate by 95% while cadmium, at that same concentration, showed no growth reduction when compared to the control.

The order of toxicity based upon these growth rate data shows Cu > Zn > Cd. A general pattern of toxicity to aquatic organisms has been developed by Silva (1976) and is as follows: Hg(II) > Cu(II) > Pb(II) > Cd(II) Cr(III) > Zn(II) > Ni(II) > Al(III). The exact ordering, however, will vary among organisms.

It appears that two generalizations, based on coordination chemistry principles, are relavent to heavy metal toxicity. The first generalization specifies that the greater the degree of Class b characteristics (large ionic size, large degree of polarizability, low oxidation state and electronegativity, and high affinity for sulfide ions) the more deleterious a trace metal will be to an organism (Nieboer and Richardson, 1980).

The second generalization, which was mentioned earlier in this work, relates toxicity of metals to their

chemical state or speciation as they exist in a water body. The emergent pattern is one in which strongly complexed trace metals are less toxic than weakly complexed or ionic forms (Steeman Nielsen - Wium Andersen, 1971; Pagenkopf,1974; Florence,1970).

It was interesting to note that the order of metal toxicity was the same as the order of metal binding, i.e. Cu>Zn>Cd on a molecular scale. It might be suggested that a relationship exists between the amount of metal bound to the surficial binding sites and the degree of metal toxicity. This, however, also raises the question concerning the mechanism of cation transport across the algal cell membranes, cell wall, and sheath material. Further work in these areas is essential for a more complete understanding of metal-algal interactions.

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APPENDIX

BG-11 Medium: Allen, M.M., J. Phycol. 4:1(1968)

$NaNO_3$	1.5 g/l
HPO_4 as K_2HPO_4	0.04
$MgSO_4 \cdot 7H_2O$	0.075
$Na_2SiO_3 \cdot 9H_2O$	0.058
Na_2CO_3	0.02
$CaCl_2$	0.027
EDTA	0.001
Citric acid	0.006
Ferric citrate	0.006
Trace metal sol'n *	1.0 ml
Distilled H_2O	make to 1 liter

pH 7.1 - 7.4 after sterilization

*	H_3BO_3	2.86 g/l
	$MnSO_4 \cdot H_2O$	1.54
	$ZnSO_4 \cdot 7H_2O$	0.222
	$Na_2MoO_4 \cdot 2H_2O$	0.39
	$CuSO_4 \cdot 5H_2O$	0.079
	$Co(NO_3)_2 \cdot 6H_2O$	0.0494
	Distilled H_2O	to 1 liter