CHEMICAL AND PHYSIOLOGICAL INVESTIGATIONS

ON

HEAVY METAL - PHYTOPLANKTON INTERACTIONS

By

© JAMES STEPHEN GOUDEY

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HEAVY METAL - PHYTOPLANKTON INTERACTIONS

For My Wife, Encouragement And Affection Not Forgotten

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AUTHOR: James Stephen Goudey, B.Sc. (Acadia University)

SUPERVISOR: Dr. G. P. Harris

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ABSTRACT

Binding of zinc-65 to the surface of Chlamydomonas was reduced by 60-80% under suboptimal physiological conditions of light, temperature, and pH. Zinc bound to the surface was composed of a labile fraction and a non-exchangeable fraction. The non-exchangeable fraction corresponded to the quantity bound under suboptimal conditions. Maximum surface binding capacities for zinc in 7 species of phytoplankton were compared and support metal ion exclusion as a potential tolerance mechanism. Factorial experiments were conducted to assess the effects of Cu, Sr, Ni, Cd, Mn, Ca, Mg, and Co (singly and in all combinations) on zinc uptake, fluorescence, and photosynthesis in 5 species of phytoplankton. The results provide valuable insights into the nature of interaction between metals and the observed response (antagonistic, synergistic, or independent).

The effects of metals on the growth of Chlamydomonas were as follows; Hg>Cu>Cd>Ni=Zn>Co>Cr>Fe=Be=Sr. Growth of laboratory strains and field isolates exposed to metals could be classified either by an extended lag phase or a gradual reduction in cell numbers over time. The nature of the response was related to changes in the available metal concentration over time, kinetics of binding and uptake, and the mode of toxic action (surface or internal sites).

Field studies were conducted on the fates of Ni, Cd, Cu, Pb, Zn, Co, and Sr added to closed, 8000 L limnocorals located in Hamilton Harbour, Hamilton, Ontario. Sedimentation was the principal mechanism regulating the availability of heavy metals in situ with rates

dependent upon; 1. the reactivity of individual metals (affinity for particulates), 2. biological factors (contributions to particulate phase), and 3. physical stability and chemical composition of the water column.

Important kinetic interactions between variables and processes regulating the binding of metal to the cell surface, uptake, and the observed toxic response were incorporated into a dynamic model to simulate the effects of metal stress on phytoplankton growth and metal uptake.

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I. INTRODUCTION

The increasing use of natural waters as receiving bodies for industrial and domestic wastes is a problem of environmental concern. Of the various classes of pollutants dumped into the environment, trace metals in particular can directly endanger human life and have a lasting effect on aquatic systems. Metals tend to accumulate within inorganic and organic substances and are not easily removed by natural processes once introduced into the system. Some metals can be transformed into more toxic compounds, such as methyl-mercury, and can be remobilized from the sediments by a number of biological and chemical processes. The effects of acid precipitation on the bio-geochemical cycling of metals in soft water lakes and surrounding watersheds have also received considerable attention in recent years. Possible consequences of metal contamination can be devastating, especially when the metal is accumulated within the food chain. Isolated outbreaks of chronic mercury poisoning, also known as Minimata Disease, focused public attention on the potentially, devastating effects of metal pollution. Reliable methods must be developed to assess the current extent and future impact of metal contamination on aquatic systems. Current standards for water quality criteria are, in some instances, arbitrarily established without a scientific basis because the quantitative and qualitative nature of the effects of some metals on man are unknown.

Phytoplankton are a vital component of the food chain in

aquatic systems and they are sensitive to small changes in the physical and chemical environment. Phytoplankton are known to accumulate a a variety of toxic substances and are an important factor regulating the cycling and distribution of metals in natural waters. The use of phytoplankton to monitor changes in water quality and for screening toxic substances has great potential. Phytoplankton are also considered one of the more visible consequences of nutrient pollution and chlorophyll levels of roughly 10-20 µg/L can impart a greenish tinge to the water. Consequently, phytoplankton have been the subject of numerous developmental, biochemical, and physiological studies. The effects of metals on the growth and physiology of phytoplankton are of primary concern and have been reviewed periodically in the literature (Whitton, 1970a; Davies, 1978; Eisler, 1979; Prosi, 1979; Hunstman and Sunda, 1980; Rai et al., 1981).

Although we have accumulated a large body of knowledge on the effects of metals on the growth and physiology of phytoplankton, factors regulating the available metal concentration in solution, binding to the cell surface, and modes of toxic action are poorly understood. These areas formed the basis of my research objectives. Interactions between important chemical, physical, and biological factors and the kinetics of metal binding to the cell surface were related to the nature of the toxic response. Results from field and laboratory studies were then incorporated into a mathematical model to simulate the effects of metal stress on the growth of phytoplankton and metal uptake.

The introduction is divided into three sections. In the first

section I define important environmental variables regulating the availability of metals in solution and metal binding to the cell surface. A classification, based on kinetic interactions between these variables, is developed and extended to identify major factors and processes regulating the toxicity of metals towards phytoplankton. I also emphasize the necessity and importance for interpreting results from toxicological investigations in terms of processes operating over cellular scales. In the second section, short term physiological responses and long term changes in population abundance and community diversity under metal stress are examined. These effects are then interpreted in the context of a time dependent, dose/response relationship. Selection of a response variable and the magnitude of the dose over time, relative to the level at which the metal interacts with the cell (membrane or internal sites of toxic action), are critical for interpreting results from toxicological investigations. The integrated dose becomes a complex function of perturbations at the cell/solution interface and is dependent upon the net rate of accumulation and cell growth. Specific objectives of my research are outlined in the last section.

I.I. Factors Regulating the Availability of Heavy Metals in Solution

Important biological, chemical, and physical factors that can directly or indirectly regulate the availability of metals in solution, binding to the cell surface, and uptake are presented in Table I (structured after Bryan 1976). The number of possible

Table I. Factors Regulating the Availability of Heavy Metals in Solution

Biological, chemical, and physical factors that can directly, or indirectly, regulate the availability of metals in solution, binding to the cell surface, and uptake are presented.

CHEMICAL: Speciation
- free ion
- hydrated ion
- complexed ion
  - inorganic
  - organic ligands
    - particles (clay)
    - colloids
    - flocculates
    - precipitates
    - humic/fulvic acids

Properties
- complexation, exchange, and redox reactions
- solubility

Other
- solution composition/concentration
- presence of binding agents
- DO, pH, and pE

PHYSICAL:
- temperature, light, ionic strength, turbulence

BIOLOGICAL:
- stage in life cycle (ability to form resting spores/cysts)
- physiological condition
- size (surface to volume ratio)
- motility/buoyancy regulation (behavioral response-avoidance)
- adaptation or tolerance; modify surface environment by reducing the number of binding sites or excretion of organic material which can bind metals, and/or sequester metals in cell
- presence of other cells may result in a localized reduction of the available metal concentration

---

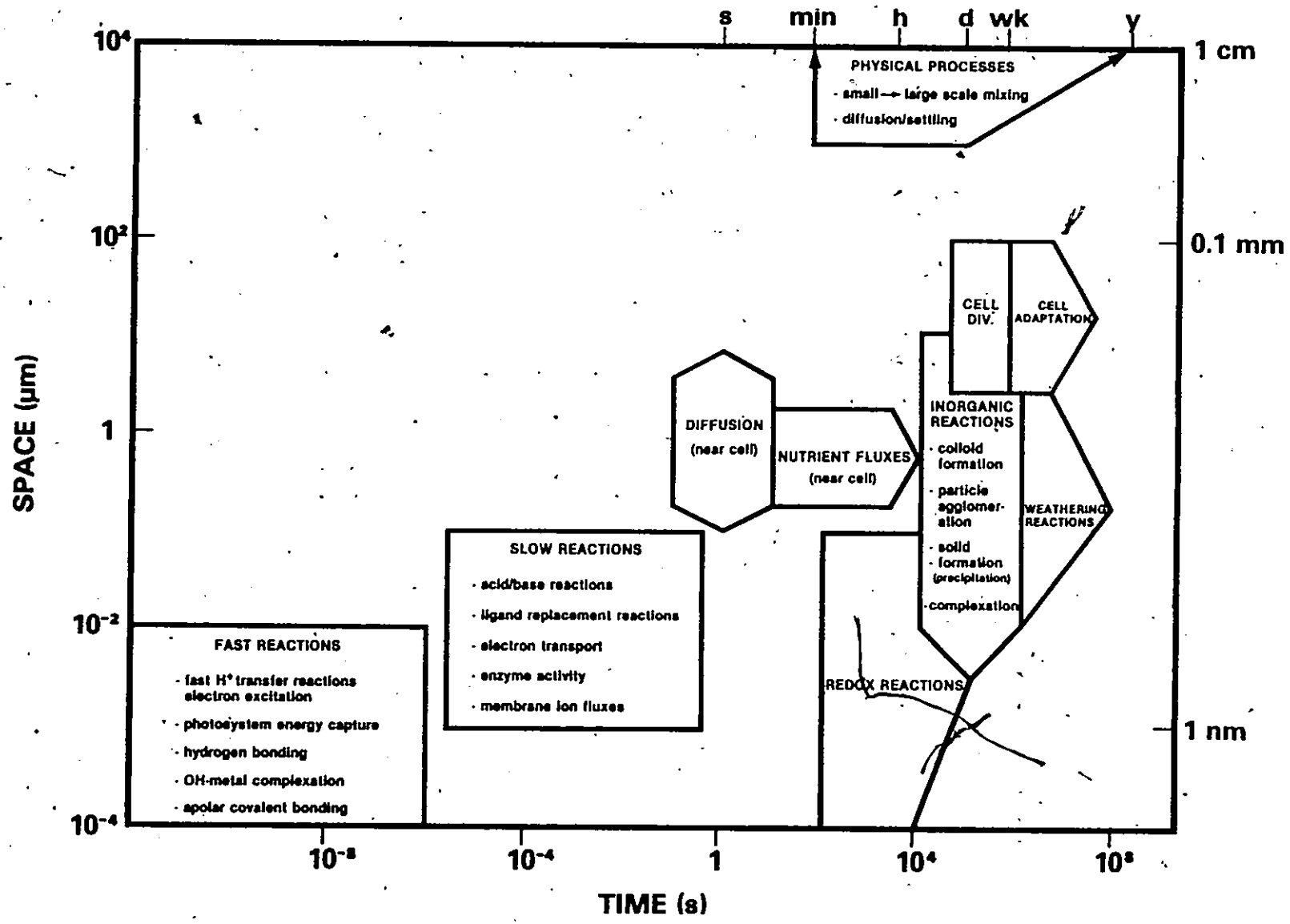
interactions between these variables are overwhelming if one considers that each variable may change with time and in space (distribution) with a concomitant variation in the level or degree of interaction. An infinite number of graded interactions are possible over the continuum delimited by no interaction and a theoretical maximum level of interaction. Temporal and spatial dimensions, however, can be used to classify and reduce these components into a hierarchy of interactions relevant to some measurable or observable phenomenon. These relationships are illustrated in Figure I.

Chemical reactions in solution and those mediated by biological systems overlap and have been collectively classified into fast and slow components. These processes are restricted in space to physical scales approaching molecular dimensions, especially in the case of diffusion and convection. Other processes operating over longer spatial and temporal scales, such as mixing or prolonged exposure to elevated metal concentrations, do not directly influence individual cells within the generation time and are not considered. The cell is assumed fixed in space and is exposed to both periodic and random fluctuations within the surrounding physico-chemical environment, including other cells. Consequently, spatial scales are of minor importance from a cellular perspective, except for diffusive processes and chemical reactions at the cell surface/solution interface.

The integrated effects of individual processes are manifested into higher order processes which operate over correspondingly longer scales. For example, small molecules synthesized by the cell can be incorporated into larger, more complex structures. Similarly,

Fig. I. Time and Space Relationships for Important Physical, Chemical, and Biological Processes.

The cell is assumed fixed in space and exposed to random and periodic fluctuations within the surrounding physico-chemical environment. Phytoplankton cannot predict, only compensate for these fluctuations and, consequently, it is essential that processes operating over relevant cellular scales be separated and examined in detail. Time can be used to separate and identify important variables and processes that regulate the toxicity of heavy metals towards phytoplankton. Spatial scales greater than 10^2 μm (1 mm) are of minor importance from a cellular perspective.


s
min
h
d
wk
y
10^4
10^2
1
10^{-2}
10^{-4}
1 cm
0.1 mm
1 nm
SPACE (μm)
PHYSICAL PROCESSES
- small → large scale mixing
- diffusion/settling
CELL DIV.
CELL ADAPTATION
DIFFUSION (near cell)
NUTRIENT FLUXES (near cell)
INORGANIC REACTIONS
- colloid formation
- particle agglomeration
- solid formation (precipitation)
- complexation
WEATHERING REACTIONS
SLOW REACTIONS
- acid/base reactions
- ligand replacement reactions
- electron transport
- enzyme activity
- membrane ion fluxes
FAST REACTIONS
- fast H^+ transfer reactions electron excitation
- photosystem energy capture
- hydrogen bonding
- OH-metal complexation
- apolar covalent bonding
REDOX REACTIONS
10^{-8}
10^{-4}
1
10^4
10^8
TIME (s)

reactions at the solid/solution interface, collectively over time, result in the process of weathering. This is analagous to the interpretation by Harris (1980a) of algal responses to fluctuating environmental conditions. He used the hierarchy theory of Simon (1973) to illustrate that lower level (or higher frequency) events "collapse into higher level responses" (photosynthesis -> nutrient uptake -> growth -> seasonal community changes -> annual changes).

The relative temporal scales, over which variables within the system operate, determine which processes are time invariant. Reactions either proceed at a constant rate in one direction (steady state) or with equivalent rates in both directions. The latter are considered at equilibrium and also at steady state (refer to Lerman, 1971, for a discussion on time and steady state for chemical processes in aquatic systems). The existence of either condition depends upon how the system is perceived and defined (discussed in Allen, 1977 and Harris, 1980a). The heterogeneity associated with all ecosystems, however, ensures that true equilibrium or steady state conditions are never completely attained over all scales.

Numerical models, based on mass balance principles and known physico-chemical processes, have been used to simulate factors regulating the composition of natural waters. Random climatological events and the heterogeneity characteristic to all ecosystems limit their utility to scales which minimize high frequency fluctuations. Both determinsitic and stochastic models cannot account for all aspects of environmental variability. Averaging to satisfy steady state and equilibrium conditions may remove important physiological

components of environmental heterogeneity, especially processes operating over cellular scales. Mixing zones near discharges of metal laden wastes and physical transport of the organism through the water column can result in small scale perturbations in the chemical environment surrounding the cell. These fluctuations could influence the composition and physiological activity of planktonic organisms, also dependent upon short term changes in the physical regime (light and temperature for example, Frempong, 1981). The effects of small scale, high frequency fluctuations in water quality on planktonic organisms have been largely ignored and are examined in later sections.

These concepts of time and steady state on cellular scales are applied to the variables presented in Table I to identify dominant factors regulating the availability, binding, and uptake of metals by phytoplankton. Important aspects of solution chemistry are presented first, followed by biological variables, and finally, interactions between biological and chemical variables. Physical variables are inherently interrelated to biological and chemical processes and, consequently, have been incorporated into these sections. Spatial scales, apart from those involved with diffusion, are of minor importance and are largely ignored.

I.I.I. Chemical Variables

Chemical reactions in solution, relative to processes associated with metal uptake and cell growth, are either; 1. instantaneous, or 2. proceed at a constant rate. The relative

concentrations and physico-chemical characteristics of individual metals and ligands present in solution determine the degree and nature of solute interactions and the ultimate equilibrium or steady state distribution. The influence of physical factors such as light, ionic strength, and temperature on rates of reactions are insignificant in regulating the availability of metals to phytoplankton within the generation time, but are important over longer temporal scales (also includes redox processes). These arguments are justified on theoretical grounds and supported by data from the literature.

The importance of metal speciation in regulating metal toxicity is widely accepted but the relative availability of complexed or chelated ions is poorly understood. Computer programs, such as REDEQL 2 (McDuff and Morel, 1973), can be used to compute the equilibrium distributions of simple metal/ligand systems. The results, however, are dependent on how well the system is defined (including all possible species) and limited by the accuracy and availability of the stability constants incorporated into the program. Ligand exchange and complexation reactions, along with solid formation, coagulation, and redox processes, can operate over a broad range of temporal scales not compensated for by theoretical equilibrium calculations.

The temporal components associated with exchange reactions and complexation with simple inorganic and organic ligands are instantaneous relative to processes regulating uptake by phytoplankton (Fig. I). Bates et al. (1982) found that zinc assimilation in a chemically well defined medium was rate limited by biological rather

than chemical processes (uptake was a linear function of the free zinc ion concentration and not the total zinc concentration). Formation of organic/metal complexes or polynuclear and multidentate inorganic associations, however, can be relatively slow (Garrels, 1959). Complete equilibrium between aqueous species of aluminium hydroxy complexes (Smith, 1971) and hafnium (Bilinski and Tyree, 1971), for example, can take months to years. Jonasson (1981) found that aluminum-lumogallion complexation was composed of fast and slow components. The kinetic differences resulted from the degree of hydrolysis of aluminum, simple monomeric species reacting much faster than rates of hydroxypolymeric conversion. Bernhard and Zattera (1969) found that Phaeodactylum tricornutum differentially accumulated stable and isotopic zinc. The radioisotope added to the seawater did not fully equilibrate with the stable zinc initially present, even after one year (Piro et al., 1973).

Precipitation of metal oxides, hydroxides, carbonates, or phosphates from standard algal growth media during the course of an experiment can have profound effects on results obtained from batch culture bioassays (Lee, 1973). Iron precipitates, for example, can coprecipitate and adsorb other metals in the culture solution reducing their availability over time (Krauskopf, 1956). Steemann-Nielsen and Kamp-Nielsen (1970) reported that concentrations of 6 µg/L Fe reduced the effects of copper on growth of Chlorella. They attributed these results to the adsorption of copper onto the colloidal precipitate $Fe(OH)_3$ (or possibly FeOOH), formed under the alkaline growth conditions.

Since rates of interconversion between complexes and free ions cannot be determined, the binding of complexed or chelated ions to the cell surface cannot be unequivocally disputed based on the equilibrium distributions. Consequently, there is considerable debate as to the relative toxicity and availability of individual metal/ligand complexes. The mechanism(s) by which the addition of artificial or natural chelators enhance growth, either by increasing the availability of individual metals or reducing the toxicity of other trace elements, has not been resolved. Davis and Leckie (1978) examined the adsorption of complexing inorganic and organic ligands onto hydrous oxide surfaces. They found that metal ligand complexes could be adsorbed or, alternatively, metals are complexed by ligands adsorbed to the surface. Uptake of complex organic molecules by phytoplankton provides a mechanism through which complexed species could be bound to the cell surface and transported across the membrane. Uptake of ferro-enterochelins by bacteria (Weinberg, 1978) and the increased toxicity of Cu, Pb, and Cd towards Anabaena in the presence of the chelating agent NTA (Laube et al., 1980) provides additional evidence for the binding and transport of complexed metals across the cell wall/membrane interface. Also, Rathsac and Lohs (1969) reported that complexed copper reduced the growth of Chlorella and was accumulated to a greater extent than labile copper. Jackson and Morgan (1978) incorporated equilibrium and transport models in the vicinity of the cell surface to interpret trace metal/chelator interactions. Although they concluded that free cupric ion was the most toxic species they also stated the importance of other labile forms of the

metal. More importantly, they were the first to recognize the need to combine equilibrium models with chemical kinetics and transport rates to separate biological and chemical components.

The toxicity of copper has received considerable attention but only qualitative conclusions have been made regarding the toxicity of individual inorganic copper complexes (Pagenkof et al., 1974; Shaw and Brown, 1974; Howarth and Sprague, 1978; Andrew et al., 1977; Chakommakas et al., 1979). Magnuson et al. (1979) used factor analysis to delineate between toxic and nontoxic species, providing a more rigorous method for interpreting data from toxicity bioassays. All these results, however, were obtained by correlating the observed toxic response to computed concentrations of copper complexes without considering rates of interconversion or uptake of complexed species. Since the concentrations of all complexes are proportionally interrelated, correlations between toxicity and individual complexes are not surprising.

Clearly, little is known about rates of interconversion and how to define the available fraction of the total metal concentration in aquatic systems. The important temporal scales regulating the availability of metals in solution are assumed to be instantaneous relative to uptake and cell growth. This includes inorganic complexation and ligand exchange reactions both in solution and at surfaces of cells, particles, adsorbates, and precipitates. Diffusion of metals into the crystal lattice of clay particles or metals bound in the internal regions of complex organic and inorganic associations (humic acids, colloids, and precipitates for example) are assumed

unreactive and not readily exchangeable. The availability of these metals is limited by diffusion and redox processes, generally much slower in comparison to uptake and cell growth (Fig. I).

I.I.II. Biological Factors

Biological factors can directly or indirectly modify the availability of metals in solution or binding to the cell surface. Changes in the physiological condition of the cell arising from alterations in the physical environment, or during cell growth, can indirectly influence the quantity of metal bound to the cell surface. (The cell surface is considered as a three dimensional structure extending from and including the surface of the plasmalemma to the external solution.) Excretion of organic substances in the presence of a metal can directly control the available fraction of the total metal concentration in solution.

The effects of temperature, light, dissolved oxygen, and the physiological condition of the population on the binding and uptake of metals are presented in this section. Results on metal - phytoplankton interactions under suboptimal physiological conditions, however, must be treated with caution because the effects exerted by a metal can be confounded by other sources of physiological stress. The results are also dependent upon the experimental conditions, including pretreatment of the inoculum. Proposed tolerance mechanisms under physiological control are also included, followed by the direct influences of biological variables (exudates) on the availability of

metals in culture and *in situ*.

Guillard and Ryther (1962) found that *Skeletonema costatum* was more tolerant to copper at higher temperatures (30°C) even though total copper uptake in this species was found to increase with temperature (Mandelli, 1969). Measurable temperature effects (e.g. greater than the experimental error) were observed for Ca, K, and Na binding to barley roots (Baker, 1978) along with Rb (Stassart *et al.*, 1981) and for a number of metals binding to the surfaces of phytoplankton (Findenegg *et al.*, 1971; Sheih and Barber, 1973; De Filippis and Pallaghy, 1976a; Sakaguchi *et al.*, 1977; Whilidal and Broda, 1977; Huisman *et al.*, 1980). These results are difficult to interpret, however, since no physiological measurements were made. Temperature differences of roughly 10-20°C frequently resulted in a 10 to 60% change in metal uptake and were, in some cases, highly variable. Processes regulating metal uptake in yeast are also temperature sensitive (Somers, 1963; Fuhrmann and Rothstein, 1968; Failla *et al.*, 1976). The fluid nature of the plasmalemma, along with synthesis of membrane components, are temperature dependent which may account for observed differences in rates of metal uptake. At low temperatures, the membrane lipids can crystallize (phase change) and could, theoretically, form an impermeable shell encasing the cell. The actual mechanisms responsible for these effects are, however, poorly understood and could, possibly, involve active uptake systems.

A light sensitive component for metal binding to the cell surface has been reported for a number of species. Gutnetch (1965) found that zinc uptake by *Fucus* increased in the dark but zinc and

copper uptake in Ulva was reduced under the same conditions. Uptake of ^{109}Cd by Porphyra was reduced in the dark (McLean and Williamson, 1977), along with zinc uptake in Laminaria (Bryan, 1969). A light sensitive component in Chlorella was observed for zinc (Matzku and Broda, 1970; Findenegg et al., 1971), copper (McBrien and Hassall, 1965; Findenegg et al., 1971; Sakaguchi et al., 1977), cadmium (Hart and Scaife, 1976), cobalt and lead (Findenegg et al., 1971), and to a lesser extent uranium and manganese (Nakajima et al., 1979c; Horikoshi et al., 1979a). Enhanced binding in the light was also observed in Scenedesmus for copper (Mierle, 1981), and in Monochyrsis and Phaeodactylum for ^{237}Pu uptake (Yen, 1981). Arsenic uptake in Chlorella was not reduced under dark conditions (Bottino et al., 1978). Arsenic is a competitive inhibitior of phosphate uptake (Blum, 1966; Planas and Healey, 1978; Klumpp, 1980) which may account for the lack of a light sensitive component, since phosphate uptake is only partially light dependent (MacRobbie 1974). Phosphate uptake is also dependent upon the nutritional history of the cell. However, Klumpp (1980) found that uptake of arsenic by Fucus spiralis in the presence of photosynthetic inhibitors (DCMU and CMU) was greater in the dark, providing evidence for the existence of an energy dependent active uptake process (or alternate pathway into the cell).

Increased rates of copper uptake in Chlorella during short term incubations under anaerobic conditions have been observed (Hassall, 1963; McBrien and Hassall, 1967; Mierle, 1981). A reducing environment could enhance the availability of sulfhydryl moieties on the cell surface which are unreactive under normal conditions. In Gracilaria,

however, the absence of air and light both reduced the rate of ^{137}Cs exchange (Gutnetch, 1965). Macka et al. (1979) did not observe any difference between uptake of mercury and cadmium under dark/anaerobic or light/aerobic conditions in Chlorella cultures.

Clearly there is some physiological control over metal binding to the cell surface and uptake, but the actual mechanism(s) involved have not been resolved. Any inconsistencies in results from the literature may be methodological since different growth media and assay conditions were used. Interspecific and intraspecific differences are well documented and the interactions of all metals may not be similar due to differences in their physico-chemical properties and reactivity.

The importance of the physiological condition of the cell in regulating metal uptake is evident from studies using synchronous cultures. In synchronous populations of Chlorella, the effects of copper (Steemann-Nielsen and Wium-Andersen, 1971) and cadmium (Lue-Kim et al., 1980) were significantly reduced during periods of cell division. Similar results were observed for arsenic uptake in Tetraselmis and Hymemomonas (Bottino et al., 1978) along with the effects of Cu, Cd, and Pb on Ankistrodesmus and Anabaena (Laube et al., 1980). Exponentially growing cultures of diatoms were also found to take up copper (Morel et al., 1978), cadmium (Cossa, 1976), and mercury (Fujita and Hashizume, 1975) at greater rates than cells in the stationary growth phase. Metabolic activity of the cell changes dramatically between different phases of the life cycle. Photosynthetic activity is lowest prior to division (50 % of the

maximum rate, Sorokin, 1957) along with changes in nitrogen and phosphorus uptake (Schmidt, 1961) and the accumulation of K, Mg, and Fe (Kanazawa, 1958). Unfortunately, the time required to observe a response in synchronous populations exposed to elevated metal concentrations may also coincide with large changes in the chemical composition and physiological condition of the cells progressing through the various phases of the life cycle.

McBrien and Hassall (1965) used vital dyes to demonstrate the variable effects of copper on asynchronously growing populations of Chlorella. They observed that under all conditions only a fraction of the population was affected by copper during short term exposures, which can be explained on the basis of reduced uptake during division. Gross and Dugger (1968), in studies with Chlorella, also demonstrated that only a small proportion of the population remained viable and developed resistence to D-mannose. All individuals in populations of Chlorella vulgaris, however, adapted to the antimetabolite selenomethionine (Shrift et al., 1959a). The nature of interaction between individual toxicants and the cell will determine the ultimate response only when the capacity of the cell to compensate for the imposed stress is not exceeded.

Variations in the rate of metal uptake as a result of the age structure within asynchronous populations may account for the plateau effect observed in short term toxicity experiments (Steemann-Nielsen et al., 1969; Steemann-Nielsen and Wium-Andersen, 1971; Overnell, 1975a,1976). Results from these investigations are similar in that complete inhibition of photosynthesis was rarely observed (except for

mercury). The maximum degree of inhibition, or plateau, was independent of the total metal concentration. The time required to reach this plateau, however, was a function of the external metal concentration. This result could be attributable to the variable age structure of the population and the kinetics of binding and transport to the site(s) of toxic action. The fraction of the population undergoing division have reduced rates of metal uptake and are not immediately influenced by the metal. Clearly there is some physiological control over metal binding and uptake, which could be explained by: 1. specific, active uptake processes; 2. alternate, active uptake processes for individual metals interacting with solute transport systems in the membrane (arsenic and phosphate for example); 3. indirect modification of the chemical properties of the cell surface resulting from a general stimulation of metabolic activity and solute fluxes across the cell membrane (sub-optimal versus optimal physiological conditions).

Resistant strains of phytoplankton have been successfully isolated from waters containing elevated metal concentrations (Russell and Morris, 1970; Stokes et al., 1973a,b; Goodman et al., 1976; Harding and Whitton, 1976; Hargreaves and Whitton, 1976; Foster, 1977,1982b; Say and Whitton, 1977; Hall et al., 1979). Subculturing in progressively higher metal concentrations has also been used to select for tolerant strains (De Filippis and Pallaghy, 1976b; Li, 1979; Shehata and Whitton, 1982). Exclusion and/or internal detoxicification are believed to be the principle mechanisms confering tolerance (Russell and Morris, 1970; Stokes et al., 1973a,b; Davies, 1976;

De Filippis and Pallaghy, 1976b; Mierle and Stokes, 1976; Foster, 1977,1982b; Butler et al., 1980; Stokes, 1981). Differential tolerance results from epigenetic and physiological variation within a population and is not likely an induced genetic response. Rates of spontaneous, or random genetic mutation are of minor importance and cannot account for the variable growth responses observed in cultures under heavy metal stress. Selection of genetic mutants in the strictest sense is unlikely, but may be related to metabolic changes within individual cells phased with cell division. A physiological readjustment, possibly involving stimulation of enzyme systems normally inactive, or alternate systems, could account for the observed reversibility of metal tolerance (Shrift et al., 1959a,b; De Filippis and Pallaghy, 1976b; Li, 1979; Stokes and Dreier, 1981). Genetic selection of tolerant species would not require traces of the inducing agent to maintain tolerance. Shrift et al. (1959b) reported the adaptation of all individuals in populations of Chlorella to the antimetabolite selenomethionine. Tolerance to this substance was maintained in the absence of the compound and transfered between generations. The actual mechanism is unknown, possibly involving induction of higher level enzyme systems, and tolerance could only be reversed by specific physiological treatments (Shrift et al., 1959b).

Studies on the effects of metals on individual cells are prerequisite for understanding the actual tolerance mechanism(s) involved. Co-tolerance to a number of metals is rarely observed, indicating the existence of a variety of specific mechanisms, or options, available to the cell. Controlled laboratory conditions,

however, remove aspects of environmental heterogeneity which may be important factors in regulating and maintaining tolerance. Artificial growth media, incubation conditions, problems associated with cloning strains of organisms, and other unknown, additional sources of physiological stress could confound the results obtained from these investigation.

Formation of innocuous precipitates or binding of metal to organic compounds within the cell wall/membrane can indirectly modify the algicidal properties of individual metals. Metal binding to proteins in the cell wall may lead to irreversible conformational changes and the physical structure and co-ordination of surface ligands could significantly influence complex stability and reactivity. Little is known about the chemical environment within this region, but the formation of metal precipitates in the walls of algae (silicon and calcium carbonate), yeast cells (Ashida et al., 1962) and roots of higher plants (DeFilippis and Pallaghy, 1975; Baker, 1978) reinforce the need to pursue alternative possibilities. Complexation or precipitation reactions near the membrane surface may facilitate transport into the cell over temporal scales consistent with lags in the growth response (days). High levels of heavy metals have also been detected in dinoflagellate chromosomes (Kearns and Sigee, 1979), polyphosphate bodies of diatoms (Sicko-Goad and Stoermer, 1979), and the mitochondria of various green algae (Silverberg, 1976). The actual mechanisms responsible for producing these deposits are poorly understood, but could involve the transport of complexed inorganic metal across the plasmalemma.

Organics excreted by algae, also associated with the cell wall and/or mucilage sheaths, have been shown to complex metals (Stolzberg and Rosin, 1977; Gnassia-Barelli et al., 1978; Swallow et al., 1978; McKnight and Morel, 1979; van den Berg et al., 1979; Butler et al., 1980; Sueur et al., 1982). Alterations in membrane permeability may result in losses of organic compounds that could complex metals, but no evidence has been presented for an inducible excretory mechanism. Fibrillar colloids were observed associated with natural phytoplankton and bacterial populations (Leppard et al., 1977; Massalski and Leppard, 1979a,b). These fibrils may promote physical associations between cells and particles (Massalski and Leppard, 1979b) and more importantly serve as a mechanism to accumulate cations essential for growth (Leppard et al., 1977). The origin and function of these structures are at present not well known.

Isolated cell wall fragments appear to have a greater total binding capacity for metals than intact cells (Hassall, 1963; Gutnetch, 1965; Cushing and Watson, 1968; Glooschenko, 1969; Matzku and Broda, 1970; Button and Hostetter, 1977; McLean and Williamson, 1977; Horikoshi et al., 1979a for algal cell walls and Somers, 1963; Ponta and Broda, 1976 for yeast fragments). This is probably due to a greater exposed negative surface area, possibly enhanced during isolation of the cell walls.

Investigations by Davies and Sleep (1979), Hollibaugh et al. (1980), and Johnston (1964) have provided insights into the effects of metal stress on natural populations but more studies using natural populations and combinations of species are required. Dayton and Lewin

(1975) found that growth of a flagellate incubated with deleterious lead concentrations was enhanced in the presence of a diatom. Extracellular products released by the diatom were believed to reduce the available metal concentration. Interspecific growth regulation in species succession through competition for vitiman B_{12} was observed by Messina and Baker (1982). The beneficial effects of different species may, in part, explain observed differences in the planktonic flora *in situ* of areas enriched with heavy metals.

The cycling of lead in a lake receiving discharges and run off from mine tailings was reported by Denny and Welch (1979). Phytoplankton concentrated lead in excess of 10^5 times without any apparent algicidal effects and lead was also found to be transfered and accumulated within the zooplankton communitty. During bloom formation, phytoplankton contributed significantly to the cycling of this metal within the water column and transport to the sediments. Forstner and Wittmann (1979) and Rai *et al*. (1981) have reviewed metal pollution in aquatic environments and clearly illustrate the importance of biological variables in regulating the distribution and cycling of metals in aqueous systems and sediments.

Biological factors controlling the availability of heavy metals can be exceedingly complex and involve a number of passive and/or active interactions. The nutritional status of the cell, stage in the life cycle, indirect and direct influences of other cells or species, and the physical environment are all important variables which must be considered. Interactions between these variables provide numerous feedback mechanisms which may also be influenced by any antagonistic

action exerted by the metal on the cell. Fluctuations in the chemical environment and processes regulating metal binding to the cell surface and uptake are manifested, over time, into a response. The response is dependent upon the magnitude and duration of the dose and the ability of the cell to compensate for the stress over time. The nature of these interactions are defined in the next section and the temporal components are examined in the section dealing with dose/response relationships.

I.I.III. Interactions between Biological, Chemical, and Physical Variables

The degree and nature of competition between metals for available ligands in solution and on the cell surface is determined by their relative abundance and physico-chemical properties. Differential affinities of metals for ligands are clearly established by the relative magnitudes of stability constants for individual complexes. The Irving-Williams order (Irving and Williams, 1953) is a classical and well referenced example which can also be explained on the basis of relative crystal field stabilization energies (Stumm and Morgan, 1981). In general, the stability of metal ligand complexes increases in the series: Mn < Fe < Co < Ni < Cu > Zn. Manganese and zinc have no crystal field stabilization energy, but the sequence is valid for almost every ligand (Stumm and Morgan, 1981). Results from studies on metal binding to humic and fulvic acids found in natural waters are consistent with this order (Mantoura and Riley, 1975; Mantoura *et al.*,

1978; Kerndorff and Schnitzer, 1980). Other classifications based on the relative electron mobility (polarizability) and electronegativity of individual metals have been proposed (Pearson, 1968a,b). Differences in the stability of metal ligand complexes are explained in terms of hard and soft electron acceptors and donors or hard and soft Lewis acids and bases (HSAB). Nieboer and Richardson (1980) developed a classification based on the work of Arhland et al. (1958), in which metals are separated according to their affinities for oxygen, nitrogen, and sulfur containing ligands. This classification incorporates physico-chemical properties of individual metals in relation to the solution chemistry and reactivity in biological systems.

Interactions between biological and chemical variables are determined by the nature and reactivity of the cell surface which has never been rigorously characterized or defined. The toxicity sequences reported in the literature are frequently based on single metal batch culture bioassays in which the growth rate or cellular metal content at the termination of the experiment were used. These results do not provide any information on metal interactions with surface ligands and comparisons with the Irving-Williams order, which at times are consistent, are not valid. The cell surface may contain a variety of potential ligands with distinctly different physico-chemical properties and cannot be functionally related to a single metal/ligand system. Only combinations of metals can provide reliable information on metal/ligand interactions at the cell surface.

Crist et al. (1981) titrated cell wall fragments previously

loaded with other metals and established the following sequence for displacement: Cu > Sr > Zn > Mg > Ba > Na. Calcium has been shown to reduce copper uptake in Scenedesmus (Mierle, 1981) and zinc uptake in Chlorella (Matzku and Broda, 1970). Copper also inhibits silicon uptake (Morel et al., 1978; Reuter et al., 1981) and phosphate and carbonate reduce uranium binding to the surface of Chlorella (Nakajima et al., 1979a). High concentrations of potassium can reduce copper binding by up to 50 % (Steemann-Nielsen et al., 1969; Shioi et al., 1978; Mierle, 1981). Arsenic was found to inhibit phosphate uptake and reductions in the rate of zinc accumulation in Laminaria by Cu, Cd, and Mn was observed by Bryan (1969). The experiments with Laminaria were conducted over relatively long time scales (days) and the results may have been confounded by changes in the available metal concentrations and growth of the alga. The antagonistic action of similar metals, such as K and Rb, Sr and Ca, Ge and Si, are also widely known and a number of studies on the interactions between cations and uptake in higher plants have also been reported (Baker, 1978; Coughtrey and Martin, 1979; Stassart et al., 1981). The ubiquitous involvement of hydrogen ions in biological systems and solution chemistry has a profound influence on the toxicity of other metals. Increasing pH minimizes the competition of H^+ for available ligands (including OH^-) which promotes formation of other metal/ligand complexes. Sunda and Guillard (1976) and Kanazawa and Kanazawa (1969) found that growth rate inhibition was correlated with the free copper ion concentration when regulated by changes in pH. Singh and Pandey (1981) found a similar relationship for cadmium toxicity in Nostoc

calciola.

The combined effects of heavy metals under different physical conditions have been largely ignored due to the vast number of permutations involved. Results from studies on the combined effects of two metals on phytoplankton growth (Hutchinson, 1973; Bartlett et al., 1974; Braek et al., 1976; Christensen et al., 1979; Stratton and Corke, 1979) and the antagonistic action between metals (Section I.I.II.) reinforce the need to critically re-evaluate current experimental designs which are oversimplified simulations of in situ conditions.

Styron et al. (1976) found no clear relationship between temperature, salinity, and growth of six marine phytoplankton on the uptake of cesium and zinc. This particular study, however, emphasizes the importance of examining the entire range of environmental parameters encountered in situ which are normally overlooked in simple, highly regimented experimental designs. Current methods are inadequate to understand the nature and significance of interactions between physical and chemical variables and biological responses. Factorially designed experiments can, however, provide insights into important variables and interactions not afforded by more conventional methods. I have used this approach to characterize the nature and reactivity of the cell surface and obtain information on interactions between nine metals on zinc binding and the physiology of freshwater phytoplankton.

I.II. Concepts of Dose and Response in Relation to Time

The level at which a metal interacts and disrupts normal cell function determines the temporal characteristics associated with the observed response. The reactivity of individual metals, concentration, and the fraction of the total metal concentration available to the cell are also important factors of consideration. These factors determine the magnitude of the imposed stress relative to the level at which individual metals interact with the cell. The temporal sequence of events culminating in the ultimate, or observed, response include the binding of metal to the cell surface, transport to sensitive site(s), followed by an indirect or direct effect on cellular metabolic activity. The response is also dependent on the ability of the organism to compensate for any physiological imbalance created by the antagonistic action of the metal on normal cell function. Consequently, short and long term responses cannot be directly compared without prior knowledge on the temporal components of processes regulating the toxicity of heavy metals towards phytoplankton.

Temporal components and processes that define the dose/response relationship are presented first using available information on short term physiological effects of metals on phytoplankton. This relationship is extended and interpreted in consideration of fluctuating environmental conditions encountered <u>in situ</u>, specifically in the case of a variable dose. Long term responses, or the integrated effects of metals on growth, are examined in the next section.

Characteristic growth responses of phytoplankton populations under metal stress are identified and explained using information presented in previous sections on the interactions between physico-chemical and biological variables over time. Finally, the prolonged effects of elevated metal concentrations on community structure and processes are reviewed.

I.II.I. Time Dependent, Dose Response Relationships and Short Term Physiological Effects

The magnitude of the dose and the temporal characteristics of the reponse are primarily dependent upon the level of interaction between the metal and the cell. Alteration of membrane function, for example, would produce a corresponding response over shorter time scales than a direct effect of the metal on internal systems. Transport of the metal into the cell and to the site of toxic action involve a number of time dependent processes and result in apparent lags between the time of addition of the metal and the observed response. These factors are incorporated into a time dependent, dose/response relationship based on results from short term physiological experiments reported in the literature. This relationship is then extended to non-steady state conditions involving a variable dose.

Metal binding to the cell surface follows simple hyperbolic saturation kinetics. Equilibrium between metal bound and in solution is usually complete after one to two hours (McBrien and Hassall, 1965;

Matzku and Broda, 1970; Findenegg et al., 1971; Davies, 1973,1974,1976; Skarr et al., 1974; Cossa, 1976; Sakaguchi et al., 1977; Whilidal and Broda, 1977; Conway, 1978; Horikoshi et al., 1979a,b; Macka et al., 1979; Nakajima et al., 1979a,b,c,1981; Sakaguchi et al., 1979; Mierle, 1981; Yen, 1981). Additional uptake beyond this time is due to transport of the metal into the cell with a rate proportional to the total external metal concentration and quantity of bound metal. Other fluctuations in the cellular metal content over time are related to population changes modified by the influences of the metal on the cell. Steemann-Nielsen and Wium-Andersen (1971) reported that inhibition of photosynthesis in diatoms by copper required at least two hours before any effects could be observed. They also found that this lag was directly proportional to the external metal concentration. Similar lags were observed for the effects of K and Na (Pratt, 1943) and copper (Greenfield, 1942; Steemann-Nielsen et al., 1969) on photosynthesis in Chlorella along with copper uptake and potassium loss in Scenedesmus (McBrien and Hassall, 1967). Short term effects of copper on oxygen evolution in Elodea were proportional to the total metal concentration in solution and time (Brown and Rattigan, 1979). Mierle (1981) reported that recovery of Scenedesmus was dependent upon the concentration and period of exposure to copper. These results can be explained by changes in the age structure of asynchronous populations and the sensitivity of individual cells to copper. Mercury completely inhibited ^{14}C uptake in Attheya decora (Overnell, 1976) and, consequently, must react with the cell surface at rates exceeding

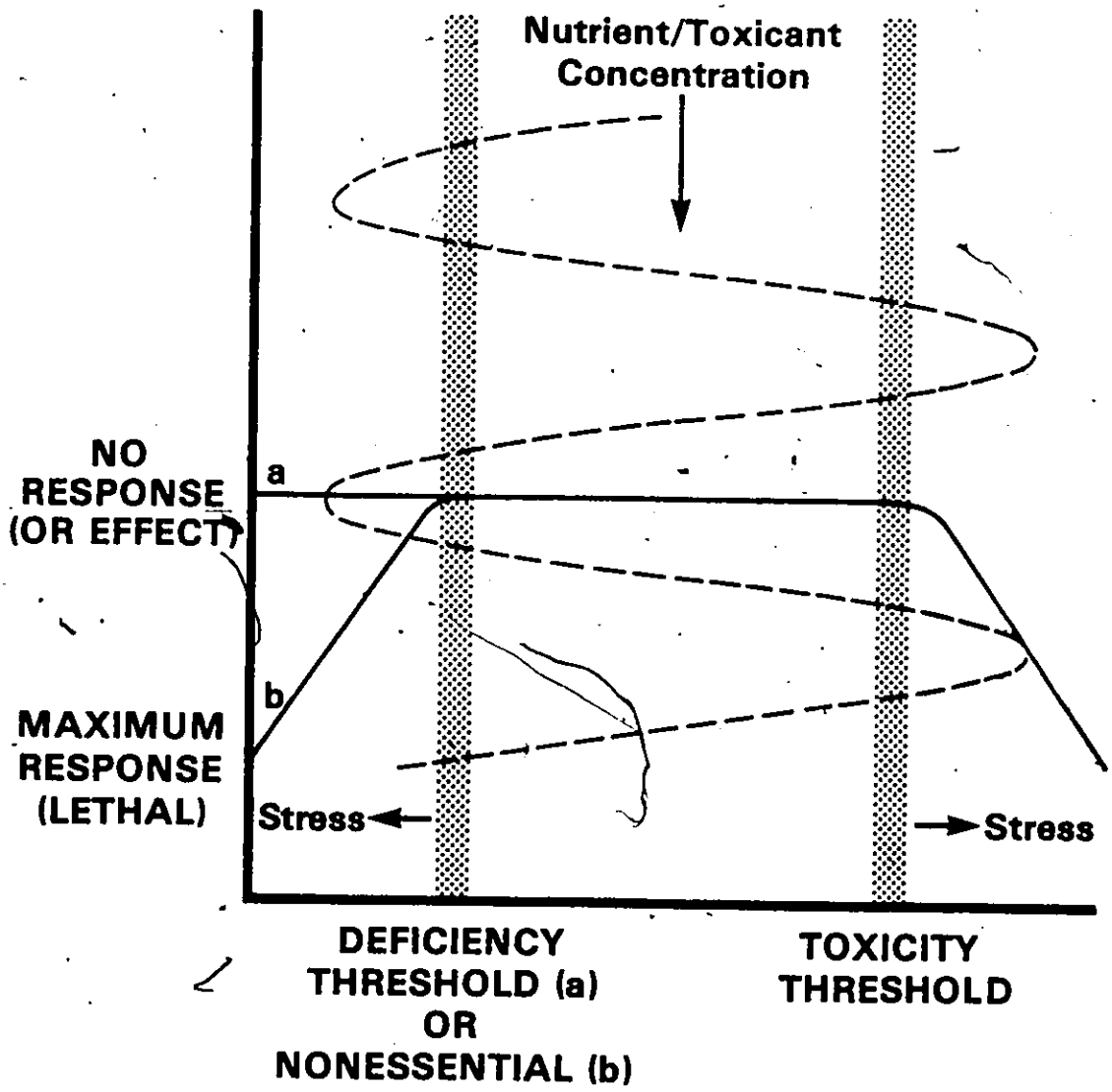
carbon assimilation (seconds). In summary, metal toxicity is a function of the available metal concentration relative to the level at which the metal interacts with the cell (magnitude of the stress). The ultimate response is a complex function of the integrated dose over time, or the duration of the imposed stress.

The dose/response relationship for a fluctuating external toxicant concentration and a generalized response (relative to some control condition) is illustrated in Figure II. The classical dose/response curve is represented as a solid line and the toxicity and deficiency thresholds as shaded areas. For essential elements, if the available concentration falls below the deficiency threshold a corresponding increase in the response (effect relative to a control) is observed. Deficient conditions for non-essential elements are never attained and the absence of these metals will not alter the response. When the concentration of the element increases, a tolerance plateau is reached over which the response is unaffected. The extent of this plateau is determined by the level at which the metal interacts with the cell and the ability of the cell to compensate (tolerate) for the imposed stress and may vary between species for different elements. At the point where the magnitude of the dose exceeds the toxicity threshold, an increase in the response (effect) is observed leading to complete inhibition at progressively higher concentrations.

This relationship becomes more complex when the magnitude of the dose varies over time (e.g. duration). Fluctuations in the available metal concentration, or dose, is represented by the sine curve. In this example, variations in the dose are of uniform

Fig. II. Dose Response Relationship Involving a Fluctuating External Nutrient/Element Concentration.

The ultimate response is a complex function of the magnitude and duration of the stress, or the period when the available nutrient/toxicant concentration exceeds the toxicity or deficiency thresholds. The response (solid line) must be interpreted as a continuum extending from no effect to a theoretical maximum effect of the metal on the cell relative to a normal, homeostatic condition (the effect depends upon the level of interaction between the metal and the cell, period and magnitude of the stress). The response is unaffected in the absence of non-essential elements (curve a) but increases with decreasing concentrations of essential elements (deficient conditions, curve b). The toxicty and deficiency thresholds are represented as shaded areas because of the variable ability of phytoplankton to compensate for (tolerate) minor stressful perturbations (refer to Section I.I.II). If a metal disrupts membrane function then the duration of the stress (time the available metal concentration exceeds the toxicity threshold) required to elicit a response would be considerably less than for a metal that exerts a toxic influence on processes inside the cell. In the later, rates for mechanisms regulating transport into the cell and to the site(s) of toxic action determine the kinetics of the response (also determined by the duration and magnitude of the stress). Increased stress (deficient or toxic regions) for prolonged periods may result in an induced adaptive response, selection, or cellular death.


Nutrient/Toxicant Concentration
NO RESPONSE (OR EFFECT)
a
b
MAXIMUM RESPONSE (LETHAL)
Stress
Stress
DEFICIENCY THRESHOLD (a) OR NONESSENTIAL (b)
TOXICITY THRESHOLD

magnitude and periodicity which is never observed in culture or *in situ*. Harris (1980a) provided an identical relationship for the effects of a fluctuating resource on cell performance. Changes in the available metal concentrations *in vitro* may arise through the establishment of depletion zones around the cells when uptake exceeds diffusion. As a result, growth on container walls, in clumps, or the lack of sufficient turbulence could significantly alter the chemical environment near the cell (uptake exceeds diffusion). The presence of other cells or debris along with fluctuations in the light climate may also influence the nature and composition of this microenvironment. Fluctuating metal concentrations *in situ* may arise through: 1. active transport of the organism via turbulent physical motion, 2. active or passive transport by the organism (swimming or through bouyancy control and settling), 3. presence or absence of strong chelating agents (including surfaces of other organisms) or change in the redox potential, pH, ionic strength and composition, and other metals, 4. formation of depletion zones immediately adjacent to the cell when uptake exceeds diffusion (especially during bloom formation). Clearly, the number of potential mechanims that can effectively alter the available metal concentration, or dose, are great, even under the most rigidly controlled experimental conditions. There is, however, an important relationship between the magnitude of the dose and time.

I mentioned earlier that the response is determined by the magnitude of the dose and the level at which the metal interacts with the cell. The temporal components associated with metals that disrupt membrane function, indirectly altering cell metabolism, are

instantaneous and the observed response is independent of any short term (high frequency) fluctuations in the available concentration. The magnitude of the dose, however, must at some point in time exceed the tolerance plateau for the organism to produce an effect. Survival of the organism, in turn, is dependent upon the reversibility of reactions at the cell/solution interface and the ability of the cell to compensate for the imposed stress. The frequency and duration of any dose that exceeds the tolerance plateau are, also, important factors in determining the ultimate response. For example, assume that the available metal concentration is represented by the sine curve in Figure II and the metal is reversibly bound to the cell surface (kinetics of binding and release are assumed instantaneous). The time required for a metal to exert a toxic effect must be less than the period of time over which the available concentration exceeds the tolerance plateau to induce a response. Under any other conditions, no effect will be observed if the metal is reversibly bound. Specific and non-exchangeable binding of metals to the cell surface will dampen small scale perturbations in the available metal concentration and enhance the potential algicidal properties of the metal. Under these conditions the dose (metal at the cell surface) remains relatively constant and only increases in relation to the available metal concentration until all surface sites are saturated. Attempts to rigorously define the extent of the tolerance plateau are confounded by the variable response of organisms under heavy metal stress. The toxicity and deficiency thresholds are represented as shaded areas in Figure II for this reason. These variable responses are evident in

long term growth experiments and are examined in a later section.

Kinetic interactions between physical and chemical variables, and physiological processes can directly, or indirectly, alter the availability and binding of metals to the cell surface. These interactions may also influence nutrient uptake in phytoplankton. A theoretical analysis of the role of microlayers in controlling primary productivity can be found in Olofsson (1980). Falco et al. (1975) used mass transport theory to interpret nutrient phytoplankton dynamics. They stressed the potential importance of physical processes (diffusion and turbulence) in regulating nutrient availability at the cell surface/solution interface. The magnitude of the external stress or dose may be uncorrelated to the observed response due to the dynamic nature of competing equilibria within this interfacial region and surrounding medium. Knowledge of this interfacial environment is essential to comprehend observed cellular responses to and interactions with the external medium. The nature and reactivity of the cell surface is, at present, poorly understood and will be rigorously examined in this investigation.

I.II.II. Long Term Growth Responses

This section is primarily concerned with the long term effects of metals on the growth and physiology of phytoplankton in culture and in situ. A brief review of culture methods, assumptions, and limitations are presented first because the experimental design can significantly influence the results and lead to erroneous

interpretations. Growth responses of cultures exposed to metals are then categorized based on the information presented in previous sections and observed changes in the population over time are examined relative to the effects of metals on individual cells. Investigations documenting the long term effects of elevated metal concentrations on community structure in situ are reviewed in the next section.

Fundamental differences in the concepts of steady state and equilibrium under culture conditions have not been resolved and the results are usually accepted de facto. Relationships between temporal components associated with biological, chemical, and physical factors regulating the availability of metals are normally overlooked in the interpretation of the data. The observed response is only a measure of the average effect exerted by a toxicant on a population and not for individual cells. The biological factors discussed in the previous sections ensure that the response of individuals within a population will not be uniform. A degree of variability is to be expected, which is dependent upon the physiological condition of individual cells and the experimental design.

The system used to examine the influences of heavy metals on phytoplankton can produce subtle differences in the observed effects and, consequently, interpretation of the results. Harvey and Patrick (1967) found significant differences between batch and continuous flow systems for metal uptake and growth of diatoms, green, and bluegreen algae. Similar differences were also observed for the effects of cadmium (Li, 1979) along with mercury (Cloutier-Mantha and Harrison, 1980), and arsenic (Conway, 1977). Cellular metal levels were found to

be relatively constant over long time periods in continuous flow through systems unlike variations observed in batch culture. Fluctuations in the available metal concentration can be minimized in continuous systems and the long term effects of sublethal heavy metal stress can be examined. These systems provide the closest available simulation of steady state and equilibrium conditions which are seldom, if ever, attained in batch culture. Jensen et al. (1974) used a combined method in which the cultures were grown in dialysis bags suspended in a flow through system. Growth of the population within the dialysis bags, however, is similar to batch culture conditions except under the influences of a constant external metal concentration. The available concentration may be controlled by using metal ion buffers (Huisman et al., 1980) but this method is limited by the kinetics of complexation and exchange reactions between components of the system.

Investigations in batch culture have been exploited for obvious reasons of simplicity and economy. Advances in our understanding of solution equilibria has added a degree of sophistication to these studies, but in theory and practice they have remained relatively unchanged. Experiments are typically conducted in defined media under constant physical conditions of light and temperature, and the effects of one metal on growth or some other physiological parameter determined. A batch culture contains a finite total metal concentration, which in conjunction with the size and physiological condition of the inoculum, determines the level of metal stress on individual cells. The initial cell burden is functionally related to

the available metal concentration, speciation in solution, and uptake by cells and debris (reviewed in Davies, 1978). Also, epigenetic variation within the population and the dependence of metal uptake on the physiological condition of the cell (refer to Section I.I.II.) will produce a variety of reponses. A chemical steady state in solution exists only when relative changes in metal speciation remain constant. Equilibrium conditions, however, are never completely attained since changes in the population and the physiological activity of the cells will produce small perturbations in the composition of the medium. Binding of metal to cell debris or exudates, formation of precipitates, reactions at the container surface, and other biological and chemical factors discussed in previous sections, can also influence the level of metal stress.

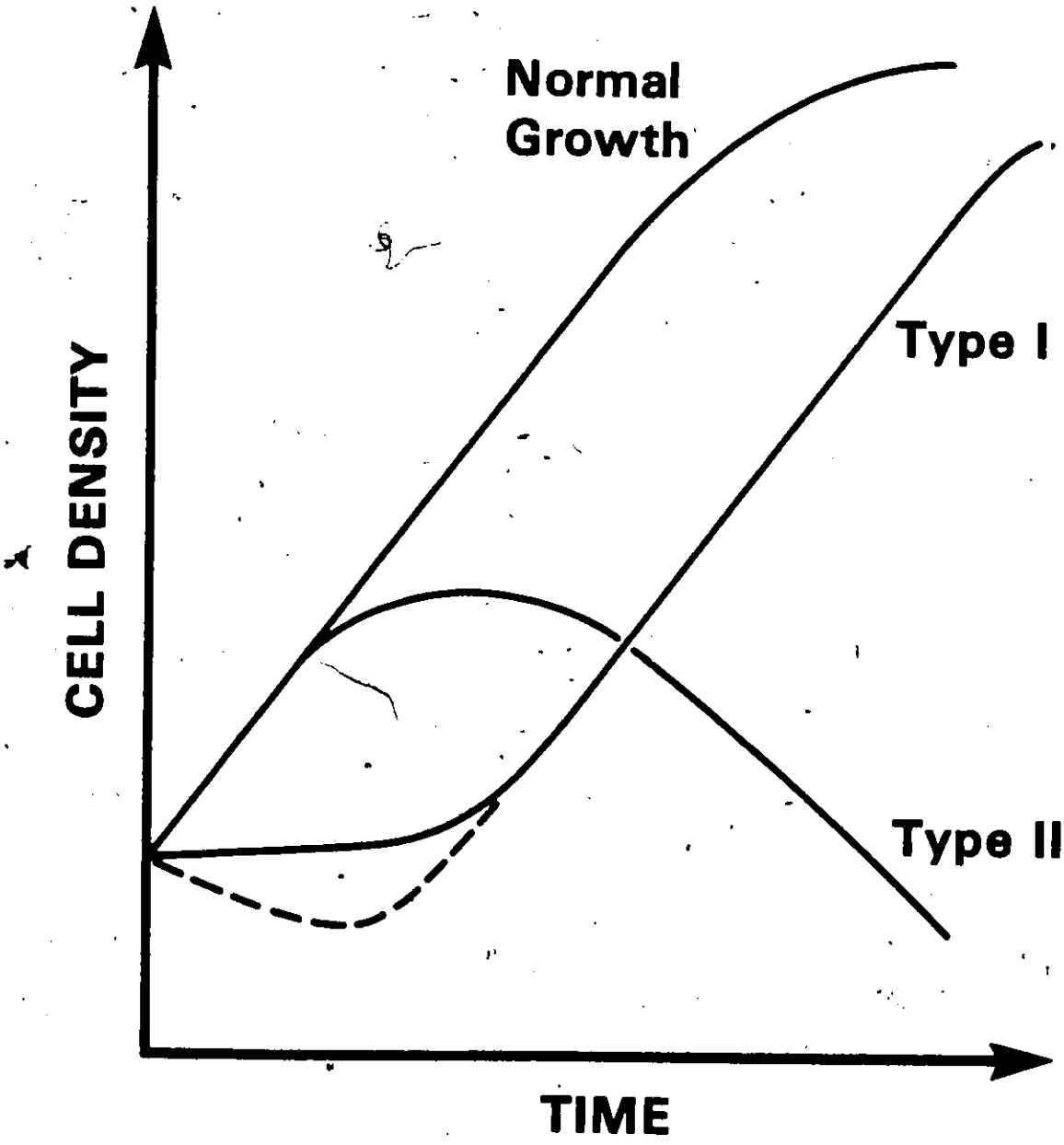
The importance of these factors are clearly illustrated in the variable growth repsonses observed for phytoplankton under the influences of heavy metals. Observed changes in cell number under heavy metal stress can be interpreted as either: all cells growing at a maximum rate out of phase (asynchronously) with only a fraction of the population succumbing to the influence of the metal, or the growth rates of individual cells are uniformly reduced (reduced slope in the exponential region of the growth curve), or a combination of both. It is assumed that phytoplankton are opportunistic in that all cellular activity is directed towards maximizing growth, even in the presence of deleterious metal concentrations (in toxicity bioassays under nutrient sufficient conditions). Chlorella continued to divide in the presence of cadmium even though abnormal daughter cells resulting

from unequal division, were produced (Lue-Kim et al., 1980). Similar observations for marine phytoplankton exposed to high copper concentrations were reported by Bentley-Mowat and Reid (1977). Changes in chemical composition of cells exposed to heavy metals may not necessarily be a direct response but a manifestation of an indirect effect (Fisher et al., 1981). The influence exerted by the metal could be expressed as a general reduction in the overall level of cellular metabolic activity which is, subsequently, translated into a lowered rate of growth.

All reported observations on the growth of phytoplankton populations under metal stress can be categorized according to the presence of an extended lag phase or as a gradual reduction in the observed growth rate over time (Type I and Type II responses respectively, Fig. III). Temporal scales associated with the growth rate and metal uptake, both of which are interrelated, determine which response will be observed. Uptake is dependent upon the available metal concentration (stress) which can be influenced by other physico-chemical and biological factors, all of which may vary during the incubation period. An extended lag phase (resurgence of growth or Type I response) has been observed for a number of species treated with different metals (Steemann-Nielsen and Kamp-Nielsen, 1970; Bentley-Mowat and Reid, 1977; Berland et al., 1976; Bottino et al., 1978; Gnassia-Barelli et al., 1978; Morel et al., 1978; Stratton and Corke, 1979; Butler et al., 1980; Cloutier-Mantha and Harrison, 1980; Laube et al., 1980; Mierle, 1980; Singh and Pandey, 1981) and pesticides (Mahanty and Gresshoff, 1978; Walsh and Alexander, 1980).

Fig. III. Characteristic Growth Response Curves

Growth responses (changes in cell density, or cells per unit volume, over time) for populations under various degrees of metal stress are characterized by either an extended lag phase (Type I), or a gradual reduction in cell numbers over time (Type II). This classification can be extended to interpret the effects of any toxicant on the development of phytoplankton populations in batch culture over time.


Normal
Growth
Type I
Type II
CELL DENSITY
TIME

The duration of this lag phase is proportional to the level of metal stress and length of exposure. Changes in the available metal concentration, mediated by other chemical or biological processes, may account for the renewed growth. Davies (1974,1976) incorporated these factors into a quantitative relationship describing the effects of mercury on the growth of phytoplankton. The available mercury concentration was reduced over time (volatilized by the cells) which resulted in a recommencement of growth.

Associated with a growing population is a corresponding increase in the level of cellular debris which can also bind significant quantities of metal. The presence of cell debris, which may be introduced with the inoculum, can effectively reduce the level of metal stress or cellular burden. Consequently, the initial conditions of the experiment are decisive in determining the ultimate observed response of a population to heavy metal stress. The lag phase may be explained as either a result of complete growth inhibition or the point where new growth is offset by cell death (lysis). The latter seems more reasonable since metal uptake is regulated, in part, by the physiological condition and age of the cell. Also, the presence of organics released during cell lysis could reduce the available metal concentration to a point favoring a net increase in the population. The second growth response (Type II) provides additional evidence for the antagonistic action between growth and cell lysis (Fig. III).

This response is characterized by an initial increase in the population followed, over time, by a gradual decrease in the growth rate. Type II growth has been observed for a number of species treated

with different metals (Jensen et al., 1974,1976; Berland et al., 1976; Sunda and Guillard, 1976; Braek et al., 1976; Bentley-Mowat and Reid, 1977; Gnassia-Barelli et al., 1978; Canterford, 1979; Rivkin, 1979; Stratton and Corke, 1979; Canterford and Canterford, 1980; Fisher et al., 1981) and is also dependent upon the experimental conditions and, more importantly, time. Consequently, the point at which the experiment is terminated can have a significant influence on the results. Sublethal effects of metal stress and the adaptation, or selection of tolerant cells cannot be adequately assessed in short term experiments because the response and dose may change with time.

Published effects of Cu, Cd, Zn, and Hg on phytoplankton growth in batch culture will now be compared to demonstrate the importance of time and the level at which a metal interacts with the cell in determining the ultimate response. If the metal exerts an immediate toxic effect on the cell, or the available metal concentration (dose) is time invariant, then the observed long term response is directly related to the initial cell burden and experimental conditions. In both situations, the average response of the population is assumed to reflect the effect of each metal on individual cells. Linear regression analysis of the calculated initial cell burden against the observed growth rate produced highly significant correlation coefficients ($r = 0.90$ to 1.00, Table XXIV of the Appendix). Only data over the range of metal concentrations that influenced growth (reduction in final cell numbers) at the termination of each experiment were used in this analysis. These results are surprising because the data was obtained from studies involving a diverse array

of experimental conditions. This finding does, however, indicate that for any given experimental design a common mechanism or process regulates the magnitude of the dose over time. The slope provides information on the width of the range over which the metal influences growth, analagous to the toxicity threshold. High slopes reflect a narrow range and low slopes correspond to a broad range. The availability and level at which each metal interacts with the cell also determines the extent or width of the toxicity threshold.

Concentrations of each metal that reduced the observed growth rate by 50% for each species (LC_{50}) were calculated from the regression equation and plotted against the slope of the line (Fig. IV). These plots provide a number of interesting insights into the effects of different metals on phytoplankton growth. The length of each line in Figure IV is indicative of tolerance differences between species and among genera towards each metal and reflect the variable nature and extent of the toxicity threshold described earlier (Section I.II.I.). The time required for each metal to elicit a toxic response also determines, to a degree, the spread in the LC_{50} data (length of line), along with the available metal concentration, or speciation, under the culture conditions employed in each experiment.

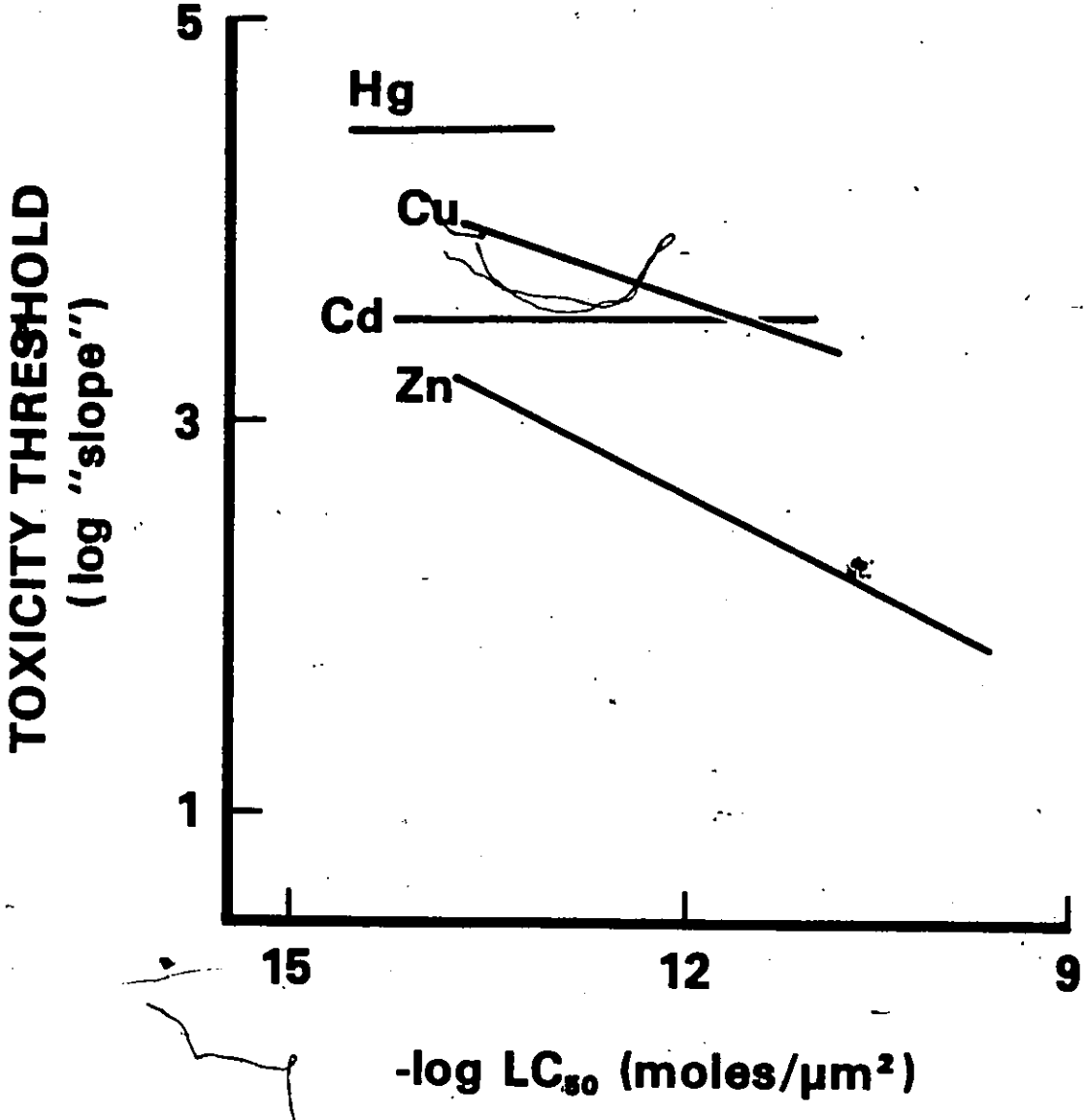
Mercury and cadmium were not significantly correlated (at the 0.10 level) to the slopes over the range of metal concentrations that influenced growth. I stated earlier that the effects of mercury were relatively fast compared to other metals, possibly attributable to a direct effect on membrane function rather than internal sites. Consequently, the ultimate response of phytoplankton exposed to this

Fig. IV. Relationship between Metal Toxicity and the Long Term Growth Response

This figure qualitatively illustrates the relationship between the level at which a metal interacts with the cell, changes in the available metal concentration (duration and magnitude of the stress), and time. The final response is represented as the initial cell burden reducing the growth rate by 50 % (LC_{50}) at the termination of the experiment. The slope of the line over the region where the metal influences growth provides a measure for the extent, or width, of the toxicity threshold (plot of initial cell burden versus the relative growth rate as a percent of the controls). A high slope signifies a narrow toxicity threshold, or a narrow region over which the metal influenced growth. Conversely, a low slope indicates a broad range. The length of each line also represent the theoretical limits for the toxicity threshold between species and among genera.

Regression Coefficients (plot of log(slope) versus $-\log(LC_{50})$, "slope" is from plot of growth rate versus metal concentration over the range influencing growth, data in Table XXV)

Metal	Sample Size	Corr.Coeff. (r)	Slope	Intercept	Level of Sig.
Cd	21	0.032	0.0159	5.34	ns(0.10)
Hg	19	0.054	-0.0167	6.94	ns(0.10)
Cu	60	0.395	0.277	2.67	0.01
Zn	18	0.809	0.519	-0.944	0.001


5
3
1
Hg
Cu
Cd
Zn
TOXICITY THRESHOLD
(log "slope")
15
12
9
-log LC50 (moles/μm²)

metal is determined by the intial experimental conditions independent of time. Cadmium is not highly toxic indicating that other processes similar to the effects of mercury, and not previously observed, are involved. Cadmium in solution, however, is relatively unreactive (low stability constants for inorganic complexes) and the available concentration should not appreciably change over time. As a result, the long term effects can be directly compared to the initial conditions (dose constant).

If the dose or response varies over time then the LC_{50} (representing the ultimate or observed response) is a function of the slope, as the range over which the initial metal concentration influnces growth increases. Slopes for copper and zinc were negatively correlated with thee LC_{50} data. As the LC_{50} decreased the slope increased, or the range over which each metal influenced growth narrowed. Growth of the population and the available metal concentration are interdependent; as the level of cell debris increases the available metal concentration decreases, in turn promoting growth. Consequently, at the termination of the experiment the final yield may appear lower even though the population has resumed normal growth. Non-steady state conditions, reflected by the variable growth responses presented earlier, result in a correlation between the LC_{50} data and the slope of the regression line over the range of metal concentrations influencing growth. For this reason, the long term response of the populations under the influence of copper of zinc are not related to the initial conditions.

Irrespective of the assumptions and simplifications made in the

preceeding analysis, the results clearly illustrate the importance of time, relative to the mode of toxic action (internal or external sites) and processes regulating the available metal concentration, in determining the long term growth response of phytoplankton under metal stress. Short term physiological effects can provide information on the temporal characteristics on the dose/response relationship if the chemical properties of the assay medium are rigorously defined. Long term changes in the magnitude of the dose (level of stress) can only be assessed through precise determination and definition of the available metal concentration in solution (free metal ion and labile fractions). The nature and reactivity of the cell surface and the influence of cell debris and exudates on the available metal concentration are, also, important factors of consideration. My research specifically deals with the short term physiological effects, including reactions at the cell surface/solution interface, and the interpretation of the observed, long term growth responses of populations under metal stress. Binding studies must be combined with physiological investigations in order to fully understand the time dependent, dose/response relationship.

Differences in the physiological condition of the population at any point in time cannot be unequivocally ascribed to the antagonistic action of the metal. In any population, a degree of physiological and morphological variability is to be expected and must be considered in the interpretation of the results along with how one perceives the nature of the growth response (Section I.II.II.). Results from simple physiological measurements, such as photosynthesis, may be indicative

of only a fraction of the population which is not affected by the toxicant. Analysis of metal uptake data must also be interpreted with caution since metal associated with cell debris cannot be distinguished from metal bound to intact cells. This is clearly illustrated in the work of Li (1980) on the cellular accumulation and distribution of cadmium in Isochrysis. Changes in cell volume (Davies, 1974, 1976; De Filippis and Pallaghy, 1976a; De Filippis, 1979; Li, 1979) or morphology (Lue-Kim et al., 1980; Thomas et al., 1980; Fischer et al., 1981) will produce a corresponding variation in dry and wet weight measurements which could significantly influence interpretation of the results. Little is known about processes operating over cellular scales and the effects of metal stress on individual cells relative to the observed, averaged effects on the population as a whole.

The sensitvity of phytoplankton to toxic substances can be used to monitor water quality and establish suitable management criteria. Attempts to develop bioassays with protozoans (Carter and Cameron, 1973), zooplankton (Kaiser, 1980; Borgmann, 1981), phytoplankton (Fritzgerald and Faust, 1963; Hannan and Patouillet, 1972; Rachlin and Farran, 1974; Guy and Kean, 1980; Walsh and Alexander, 1980), and bacteria (Gillespie and Vaccaro, 1978) however, are limited by assumptions and restrictions imposed on batch culture techniques (the variable nature of the long term growth response, for example). In order to resolve the impact of elevated metal concentrations on the biota, events such as uptake, excretion, and metabolism must be evaluated at the appropriate scales. Information on short term

physiological responses can be effectively and efficiently obtained using conventional and well defined culture techniques.

I.II.III. Long Term Effects of Metals *In Situ*

The influence of elevated levels of heavy metals on phytoplankton communities *in situ* are briefly reviewed. Methods and problems in assessing and quantifying the long term effects of metals on aquatic systems are also presented, including application of biotic indices to assess and monitor changes in water quality.

Field studies have concentrated on the prolonged effects of metal stress on community processes and structure, along with shorter term exposures in isolated systems enriched with metals. Prolonged metal stress is usually accompanied by a corresponding decrease in species abundance and diversity (Whitton, 1970b; Prosi, 1979; Evenson *et al.*, 1981; Rushford *et al.*, 1981; Foster, 1982a). Intraspecific and interspecific tolerance differences confer selective advantages on individual cells that can proliferate under these adverse conditions (Foster, 1982a,b). Growth of tolerant species may be enhanced due to the absence of competition for available resources. Differential tolerance of individual species towards various pollutants can provide a sensitive method for monitioring water quality. Co-tolerance of species to other metals is rarely observed and the use of indicator organisms or changes in the phytoplankton community in assessing the extent and quality of contamination has great potential. Lange-Bertalot and Lorbach (1979) were able to categorize diatom

species based on similar tolerances to waste water loadings in the river Main. They found good correlations between gradual changes within the diatom communities and water quality, even over seasonal time scales. Rushford et al. (1981) also reported correlations between seasonal differences in the diatom flora and fluctuations in ambient heavy metal concentrations.

Studies on the ultimate environmental impact of pollutants do not provide information for the effects of small scale perturbations in water quality on planktonic organisms. This variability, discussed in previous sections, can limit the reliability of using the metal content of natual populations as indices for the degree of metal contamination. Invertebrates or attached macrophytes can concentrate metals and may integrate fluctuations in the chemical environment over time and provide a relative measure of the extent of metal pollution. Serious difficulties may be encountered, however, in interpreting seasonal influences, translocation, or release of metals by these organisms. Interactions between variables regulating the magnitude of the stress, period of exposure, and growth of the organism must all be considered when using biological indices to monitor water quality.

Assessing the effects of metals on community processes and structure in open systems complicates complete separation of important biological, chemical, and physical variables. Whole lakes, large volume enclosures, and small chambers have been used to examine the relati[illegible]rt term (days to months) effects of heavy metal enrichm[illegible]Knight (1981) treated a small pond with copper to control a nuissance bloom of Ceratium. Although a 90% decrease in the

Ceratium population was observed one day after addition of the metal, this species persisted throughout the study period. Reduced grazing pressures following a decrease in the zooplankton abundance, promoted the growth of smaller more tolerant green algae (Nanochloris and Ourococcus). A corresponding decrease in primary productivity was also noted which returned to pretreatment levels in ten days and a two month period was required before copper was uniformly distributed throughout the water column. This investigation clearly demonstrates the importance of physico-chemical and biological interactions which operate over distinctly different temporal scales. Losses of individual complexed metal species however, cannot be accurately determined, complicating attempts to quantify processes regulating the distribution and cycling of metals within the water column.

De Noyelles et al. (1980) examined the influences of light, phosphate, and cadmium on natural phytoplankton communities in situ using large volume enclosures and small flow through chambers (5 L) which were also incubated under laboratory conditions. Comparable results were found for changes in community structure and composition in the small chambers, in situ and in the laboratory, and for responses to perturbations in light, phosphate, and cadmium between the small chambers and large volume enclosures. Eppley et al. (1978) found that copper additions to large volume enclosures reduced zooplankton standing stocks by only 0.5% and tended to stimulate growth, as reflected by increases in species diversity and levels of chlorophyll a.

Isolation of natural phytoplankton populations in all

situations however, removes important components of enviornmental heterogeneity regulating the cycling and distribution of heavy metals and community processes (such as turbulence and the light climate). Temporal scales for processes under investigation will determine which methods are employed. Short term uptake kinetics and interactions between chemical variables can be successfully simulated in relatively small chambers. Recycle mechanisms and changes in species composition and abundance require larger enclosures. Containment effects may areis through stimulation of bacterial growth on the sides of the enclosure, which may influence nutrient availability, and the volume of the enclosure must be balanced against the duration of the study to prevent nutrient limitation. Analysis of these effects and circulation within large volume enclosures have been reported in the literature (Lund, 1972; Lack and Lund, 1974; Steele et al., 1977; Eppley et al., 1978; Round and Chapman, 1982).

I.III. Objectives

The greatest short term impact of metal contamination in aquatic systems will be in the upper surface waters corresponding to the region of maximum biological production. The availability of heavy metals within this region is regulated by biological, chemical, and physical variables interacting over a spectrum of spatial and temporal scales. These variables must be defined and interactions ranked relative to processes controlling the biologically available fraction of the total metal concentration in solution. Spatial dimensions are of minor importance relative to processes operating over cellular scales. Temporal scales, however, associated with uptake and cell growth (doubling times) are critical for understanding the potential lethal-sublethal effects of short term (hours to days) exposures to elevated concentrations of heavy metals. I believe that the complex nature and reactivity of the cell surface, regulated in part by the physiological condition of the organism, can provide a homeostatic mechanism sufficient to dampen small scale environmental perturbations in solution equilibria (short term effects at otherwise long term lethal exposures). This includes metal binding to the cell surface, transport across the cell wall/membrane interface, and the time required to exceed the cell's capacity to compensate for the stress. The temporal sequence of events required to elicit a toxic response can also be modified by fluctuations in the external environment and the physiological condition of the cell. In other words, the heterogeneity characteristic of all ecosystems can modify the duration

and magnitude of the stress imposed on the cell by individual metals.

The main objective of my research was to characterize the nature and reactivity of the cell surface and to identify and define, in terms of temporal interactions and variability, processes regulating the availability of heavy metals in aquatic systems. A nontoxic chemically well defined inorganic medium was developed to obtain reliable information on metal binding to the cell surface and short term physiological effects. Factorial experiments were implemented to provide insights into the nature of interaction between metals for available sites on the cell surface and uptake under different physical conditions (light and temperature). Reductions in the amount of zinc bound to the cell surface provided a relative index for the level of competition between individual metals for available sites. Information on the binding of metals and short term physiological effects were then related to the long term changes in growth of phytoplankton populations under metal stress.

Zinc has a number of desirable properties compatible with my research objectives. Zinc is ubiquitous in nature and is an essential micronutrient for growth and metabolism of various algae and, consequently, has been extensively studied. Most algae can tolerate relatively high concentrations of zinc that can be determined using conventional analytical techniques. Zinc forms stable complexes with oxygen, nitrogen, and sulfur containing ligands encountered in biological systems with an overall reactivity intermediate to other, potentially toxic metals (classified as "borderline" by Nieboer and Richardson, 1980). The nature and reactivity of the cell

surface can be characterized by the extent of exchange reactions between zinc and other metals. Also, the radioactive zinc isotope has a long half life (256 d), is economically and readily available, and can be detected using conventional methods.

Field investigations were conducted using large volume enclosures to identify factors regulating the cycling and distribution of metals in situ following short term, impulse loadings. These studies were designed to provide information on kinetic interactions between important chemical and physical variables and biological processes regulating the available metal concentration in aquatic systems.

Results from the field and culture investigations were then incorporated into a mathematical model to simulate the effects of metal stress on phytoplankton growth and metal uptake. Important biological processes were combined with chemical kinetics and transport rates to provide new insights into the nature of the time dependent, dose/response relationship.

II. MATERIALS AND METHODS

Culture and field investigations are treated in separate sections.

II.I. Culture Investigations

Experiments with radioactive zinc are treated separately because they involve specialized techniques and include the factorial designed experiments. This is the first attempt to systematically examine the effects of combinations of metals on phytoplankton and, consequently, the methods, assumptions, and limitations are presented in detail in Section II.I.III.

II.I.I Media and Culture Material

Bristol's solution modified by Bold (Nichols and Bold, 1965) was selected as the standard growth medium (BBM, Table II). All chemicals used were reagent grade or better and double distilled water was used throughout. To avoid the use of chelators such as EDTA, trace elements including $FeCl_3$ were supplied at concentrations 1/100 th the recommended or less. Trace elements were not provided to cultures incubated for experiments. Contamination from the distilled water and chemicals, and carry over from the inoculum was believed sufficient to satisfy any physiological requirements during the experiment (roughly

Table II Composition of Bold's Basal Medium (BBM) and the Modified Bold's Medium (MB) used in Zinc Uptake Experiments

Nutrient	Final Concentration of Added Nutrients (mM) BBM	MB
Ca^{2+}	0.17	-
Mg^{2+}	0.30	-
Na^{+}	3.37	3.40
K^{+}	2.12	8.0-13.5
$PO_4^{=}$	1.72	6.75
$SO_4^{=}$	0.30	-
HCO_3^{-}	-	1.00
NO_3^{-}	2.94	2.00
Cl^{-}	0.77	0.40

The chloride and nitrate salts of the metals examined in all experiments did not contribute significantly to the concentration of each species in the MB test medium. The pH was adjusted by varying the ratio of K_2HPO_4 to KH_2PO_4 (total $PO_4^{=}$ = 6.75 mM), hence the range of K levels. A diluted Woods Hole trace element solution was added to stock cultures (minus EDTA) in BBM.

2 weeks). No significant reductions in growth for species subcultured in BBM minus trace elements were observed in preliminary experiments.

A nontoxic, well defined inorganic medium (MB) was developed for use in metal binding and short term physiological experiments (Table II). Other complete growth media were unsuitable and provided no control over the formation of precipitates and adsorbates, or precise definition of the chemical speciation between components. Speciation in MB was obtained using the computer program REDEQL 2 (McDuff and Morel, 1973). An example of the calculated equilibirum distributions for the major ions of interest (Ca, Cd, Co, Cu, Mg, Mn, Ni, Sr, and Zn), also included in the factorial experiments, at pH 6.2, 7.2, and 8.2 are presented in Table III. The total metal concentrations were set at 5 µM, except zinc at 0.86 µM, in the assay medium. Nitrate, potassium, and sodium were omitted from the compilation because they do not form any important aqueous complexes. Stability constants for metal complexes with the chelator tris(hydroxymethyl)aminomethane (THAM) were not included in REDEQL 2 (pH 8.2-P, Table III).

The large phosphate concentration in MB was required to minimize changes in pH during metal additions and over the course of an experiment, particularly at pH 6.2 and 8.2 (maximum buffer capacity for the phosphate system in MB is around pH 7.2 equal to the second acid dissocation constant, pK_{a2}, for H_3PO_4). In one factorial experiment, the phosphate buffer was replaced with THAM to examine the influences of large changes in speciation on zinc uptake and fluorescence (pH 8.2-P, Table III). This medium did not influence

Table III Speciation in MB Medium from Equilibrium Calculations using the Computer Program REDEQL2

Free Metal and Total Complex Concentrations (-log)

		METAL										
pH	LIGAND	Ca	Cd	Co	Cu	Mg	Mn	Ni	Sr	Zn	H	FREE LIG
6.2	-	5.32	5.32	5.30	5.93	5.34	5.65	5.30	5.30	6.21	6.20	-
7.2		5.38	5.32	5.30	6.72	5.50	6.22	5.31	5.33	6.60	7.20	-
8.2		5.45	5.38	5.34	7.98	5.64	6.50	5.40	5.41	6.84	8.20	-
8.2-P		5.31	5.38	5.34	7.99	5.31	5.33	5.41	5.30	6.09	8.20	-
6.2	$PO_4^=$	6.83	11.27	-	5.45	6.36	5.57	-	7.32	6.62	2.17	9.30
7.2		6.12	9.50	-	5.46	5.74	5.36	-	6.51	6.22	2.17	7.56
8.2		5.87	8.25	-	6.41	5.57	5.33	-	5.95	6.16	2.17	6.29
6.2	$CO_3^=$	7.41	9.64	-	6.75	7.44	7.25	-	-	-	3.00	7.16
7.2		7.18	8.37	-	6.26	7.29	7.54	-	-	-	3.00	5.89
8.2		7.08	7.38	-	6.47	7.21	7.77	-	-	-	3.01	4.87
8.2-P		6.89	7.33	-	6.43	6.83	6.55	-	-	-	3.01	4.90
6.2	Cl^-	-	6.71	8.38	8.91	-	8.14	8.38	-	10.28	-	3.40
7.2		-	6.70	8.37	9.69	-	8.70	8.38	-	10.65	-	3.40
8.2		-	6.75	8.40	10.94	-	8.97	8.46	-	10.87	-	3.40
8.2-P		-	6.71	8.36	10.91	-	7.76	8.43	-	10.91	-	3.40
6.2	OH^-	11.41	8.21	8.39	7.18	11.41	9.64	7.56	11.79	9.61	-	7.56
7.2		9.58	7.20	7.38	6.10	10.46	9.20	6.90	10.81	8.99	-	6.73
8.2		9.52	6.24	6.36	5.37	9.52	8.47	5.98	9.88	8.20	-	5.74
8.2-P		9.35	6.22	6.33	5.33	8.35	7.27	5.96	9.74	7.42	-	5.74

Speciation of the principle metal ions investigated in MB medium are presented. Total metal concentrations were set equal to 5.0 uM with a total zinc concentration of 0.86 uM. No complexes involving these metals and NO_3^- (2.0 mM) were included in the computer program REDQEL2. The pH "8.2-P" represents speciation in the absence of phosphate (THAM buffer system).

fluorescence, photosynthetic activity (^{14}C uptake), or the motility of Chlamydomonas after a 10 h incubation. Nutritional requirements of Chlamydomonas for long term growth experiments were not met by MB so BBM minus trace elements was used in these investigations. All MB medium was filtered through 0.45 µm membrane filters after phosphate additions and before use.

The pH of the culture medium (6, 7, or 8 $\pm$ 0.2 pH units) was adjusted by varying the ratio of $H_2PO_4^-$ to $HPO_4^=$. All conductivity and pH measurements were made using a Radiometer Conductivity Meter and a Radiometer Model 26 pH Meter with an expanded scale connected to a Model GK 2401 B Radiometer combination glass electrode. No significant changes in pH were observed in the short term experiments. Long term growth experiments were all conducted at pH 7.2 and terminated before any changes in pH were detected (7 days). The chemical properties of the medium may be altered during extended incubation periods due to the uptake and release of solutes by the test population. Over a 7 day period and employing low initial cell densities these effects were minimized.

All metal stock solutions (25 mM) and nutrient solutions were replaced every one and two months respectively and filtered through 0.45 µm membrane filters after preparation. Glassware was routinely washed with phosphate free detergent, soaked in 1% HNO_3 overnight, rinsed with distilled water and soaked a further 12 hours in distilled water. Glassware and media (minus phosphate) were autoclaved at 130°C under approximately 20 lbs pressure. Phosphate additions were made via sterile filtration after autoclaving when the media had cooled.

Representative genera of the Chlorophyta, orders Chlorococcales (Coelastrum, Chlorella, and Scenedesmus) and Volvocales (Chlamydomonas), were selected for this work because they are ubiquitous associates of most freshwater phytoplankton communities and have been extensively used for toxicological, biochemical, and developmental studies. General characteristics include; formation of non-filamentous unicellular clusters or colonies with no vegetative cellular division, chlorophylls a and b predominate, and cells posses one or more pyrenoids surrounded by a starch sheath (Prescott, 1968). Members of the order Volvocales, however, undergo vegetative cellular division and are motile in the vegetative state, posessing two or more flagella. A more thorough description of individual species characteristics can be obtained from Prescott (1968), Bold and Wynne (1978), or other keys to the freshwater phytoplankton. The relatively simple geometries of these species are advantageous for modelling processes at the cell surface/solution interface. A list of the phytoplankton strains and sources used in this investigation is presented in Table IV.

Stock cultures were maintained for extended periods of time on agar slants containing the BBM plus trace elements and supplemented with proteose peptone (15 g Bacto-agar, 1 g Bacto-peptone (Difco) per liter of medium). Material was transfered to fresh agar every four months except for cultures which exhibited obvious fungal or bacterial contamination. Stock cultures were maintained under conditions of continuous low light provided by cool-white fluorescent tubes (20-30 $\mu Em^{-2}s^{-1}$) at 20°C after an initial period of elevated illumination

Table IV A Listing of Phytoplankton Species used in this Investigation

CULTURE	IDENTIFICATION		CODE
Chlorella pyrenoidosa Chick	UTEX 29	CCAP 211/8a	UT
Coelastrum microporum Näg.	UTEX 280	CCAP 217/1b	UT
Coelastrum microporum Näg.	-	-	HH
Scenedesmus sp.	-	-	HH
Scenedesmus quadricauda (Turp.) Brèb	UTEX 76	CCAP 276/4b	UT
Chlamydomonas sp.	-	-	-
Chlamydomonas reinhardtii	-	-	W

The identification numbers used by the University of Texas and the origin of the culture (CCAP = Culture Centre for Algae and Protozoa Cambridge, England) are presented. Species notation is as follows: UT, cultures obtained from the University of Texas at Austin; HH, cultures isolated from Hamilton Harbour; W, wall-less mutant obtained from R. Wright, Carnegie-Mellon University, Pittsburg, Pennsylvania. The other species of Chlamydomonas was obtained from the Ward's Natural Science Establishment, Rochester, New York.

(5-7 days at 130-150 $\mu Em^{-2}s^{-1}$) to stimulate growth. Liquid cultures were also maintained under the same conditions except for shorter periods of time.

Prior to each experiment culture material was transferred from solid to liquid BBM without trace elements. Cultures were maintained in 4 L Erlenmeyer flasks containing 3 L of media under the growth conditions required for each experimental run, agitation provided using magnetic stirrers and followers (conditions listed in Table V). Semi-continuous cultures were maintained in the mid to late exponential growth phase by monitoring growth and diluting with fresh BBM.

The concentration of cellular debris in the inoculum is frequently ignored in studies on metal - phytoplankton interactions. This source of experimental error was reduced by repeated washings, followed with centrifugation, of the inoculum in all cases except for Chlamydomonas. The phototatic response of Chlamydomonas was used to isolate healthy, motile cells with similar physiological responses and with a minimum of debris (confirmed using phase contrast microscopy). Both methods provided cells of relatively uniform size.

II.I.II. Experiments with Non-Radioactive Metals

Chlorophyll a was determined using either a modified acetone extraction method of Strickland and Parsons (1968) or a hot methanol method. In the first method samples were filtered onto glass fiber filters, macerated in a tissue grinder with 100% acetone until

Table V Incubation Conditions for the Culture Experiments

Experiment	Vol.(mls) Flask(Medium)	Medium	pH	Temp. (^{o}C)	Light ($\mu Em^{-2}s^{-1}$)	Time (h)
Long Term Growth	250(100)	BBM	7.1	20	100-110	170
Short Term						
^{14}C Uptake	250(100)	BBM	6.2-8.2	10-30	0-500	3-4
^{65}Zn Uptake	1000(500)	MB/BBM	6.2-8.2	10-30	0-300	0-10
Factorials	250(100)	MB	6.2-8.2	20	0,110	3,10

Notes:

1. A 16:8 h light/dark photoperiod was used in the growth experiments.
2. Agitation was provided using a rotary shaker in all cases except for the ^{65}Zn uptake experiments in which magnetic stirrers and followers were used.
3. Trace elememts were not included in the BBM or MB used in these experiments.
4. All lighting was provided by cool-white fluorescent tubes.

homogenized, extracted overnight at 5°C, and then centrifuged. Absorbances at 750 and 665 nm before and after acidification with one drop of 5 % HCl were then measured. After appropriate corrections for blanks, chlorophyll concentrations were calculated using the equations of Strickland and Parsons (1968). Hot methanol extractions were carried out using samples filtered onto 0.45 μm membrane filters and chlorophyll concentrations determined from fluorescence measurements. The equation used to calculate levels of cellular chlorophyll is as follows:

$$\mu g \text{ Chl a / cell} = (X * F * v) / CD$$

where F is the fluorescence, v the volume of methanol used in the extraction, CD is the cell density (cells/ml), and X is a constant derived from similar extractions using the acetone method for each species.

Fluorescence was measured with a Turner Designs Model 10 Fluorometer using the method described by Harris (1979). In vivo fluorescence (IVF) measurements were made on an 8 to 10 ml sample volume after a one hour incubation period under ambient light (roughly 10-13 $\mu E\ m^{-2} s^{-1}$) while methanol extracts were read directly. Steady state fluorescence was obtained in less than one minute after insertion of the cuvette into the fluorometer, and values were recorded in arbitrary units. In some cases 0.05 ml of the herbicide 3-(3,4-dichlorophenyl)-1,1-dimethyl urea (DCMU) was added to a final concentration of roughly 1 μM, the cuvette shaken and replaced into

the fluorometer, and values recorded as before. Ratios of DCMU induced fluorescence to IVF were then calculated.

The influence of temperature, light, and other metals on photosynthesis was measured as the uptake of $H^{14}CO_3^-$ under the conditions outlined in Table V. Prior to addition of the metals and labelled bicarbonate, the flasks containing cells were preincubated for 100 min under the appropriate conditions of light, temperature, and pH. Metal solutions and 100 µl of $NaH^{14}CO_3$ (obtained from New England Nuclear with a specific activity of 0.5 µCi / 50 µg in 1 ml) were then added at time zero and samples removed periodically, or after a 200 min incubation period. The 200 min incubation period was selected to minimize spurious effects attributable to increases in the population and such that uptake of ^{14}C was linear over the entire interval. This time was also consistent with the short term zinc uptake experiments. Experiments in which ^{14}C uptake was followed over time were carried out using 8 replicate flasks for each condition (2 filtered off after 0.5, 1, 2, and 4 hours). Sufficient levels of activity could only be obtained using the entire contents of one flask due to the low cell densities. Cells were separated from the medium through filtration onto 0.45 µm membrane filters under low vacuum (< '15 lbs/in^2) followed by a wash with a 0.1% HCl solution to remove adsorbed label. The filter was then placed into a 20 ml scintillation vial containing 10 ml of ACS scintillation cocktail (Amersham Corp.) and counted on a Beckman Model LS 250 Scintillation System. Counting efficiencies, determined by the channel ratios method, were relatively constant and quenching from the photopigments was not found to be

significant with such a low total cell concentration.

Measurements of cell densities were obtained using a Coulter Counter Model F equipped with a 140 µl aperture and a 0.5 ml manometer. The instrument was calibrated from manual counts using an Ultraplane Fuchs Rosenthal Haemocytometer and from measurements of cell diameters. (A Zeiss MOP-3 Digitizer arranged in combination with a camera lucida was also used to acquire data on cell dimensions.) The concentration of cells was adjusted in 0.5% NaCl to yield a count of less than 30,000 per 0.5 ml (< 60,000 cells/ml) and corrected for coincidence using the chart supplied by the manufacturer. The Coulter Counter provided a precision of greater than $\pm$ 0.1%. Manual cell counts were repeated until a precision of $\pm$ 5.0% was achieved. Optical density measurements were also made at 750 nm using a Pye Unicam SP 1800 Ultraviolet Double Beam Spectrophotometer but were more variable. All subsequent references to absorbance measurements, unless noted, were obtained using this instrument.

The initial conditions for the long term growth experiments, including treatment of media and the inoculum, and preparation of samples for energy dispersive x-ray (EDX) analysis are now presented. All media was filtered through 0.45 µm membrane filters before autoclaving to remove any residual microscopic contamination. Metal solutions were made up to volume such that 100 µl added to 100 ml of medium would yeild the desired total metal concentration. Culture material was concentrated and washed twice with fresh filtered medium under gentle centrifugation to remove cellular debris and dissolved organic matter, and made up to volume to provide an initial cell

density of 2000-3000 cells/ml in 100 mls using a 500 µl inoculum. Low initial cell densities are a prerequisite in batch culture investigations. Each experiment was carried out using 250 Erlenmeyer flasks containing 100 ml of filtered BBM, plugged with cotton bungs and capped with aluminum foil. Metal solutions were added to the corresponding flasks using a 100 µl Eppendorf pipette prior to addition of the culture material with a 500 µl Eppendorf pipette. Sterilized, cleaned pipette tips were used throughout and the absolute volume delivered by each Eppendorf was confirmed gravimetrically (the coefficient of variation did not exceed $\pm$ 0.5% of the expected volume). Conditions for incubation are outlined in Table V. Aliquots were removed periodically to monitor growth, fluorescence and chlorophyll a in some instances, and did not exceed 10 mls over the duration of the experiment. Conductivity and pH measurements were also made at the begining and termination of each experiment.

At the termination of selected growth experiments cell material was concentrated by centrifugation and the supernatant removed. The cell material was then freeze dried by immersing the tubes into an isopentane solution cooled in liquid nitrogen and left under vacuum (10^{-3} torr) overnight. Subsamples of the dried cell material were placed onto formvar covered nickel grids and carbon coated for energy dispersive x-ray (EDX) analysis. EDX was performed on 50 cells and debris selected at random. Background x-ray scatter from the grid and column was minized by analyzing cells centrally located between the grid bars. The spot size was adjusted to accommodate one cell and was kept constant for all analyses. Results were scored according to the

presence or absence of the selected element. Although quantitative methods exist, the objectives of these experiments did not merit their application.

II.I.III. Isotope Experiments with ^{65}Zn

Radioactive zinc was manufactured in the McMaster University nuclear reactor. Aliquots of $Zn(NO_3)_2$ (1-3 mg total zinc) were placed into quartz vials (30 x 8 mm, I.D. 4 mm), dried overnight, capped and wrapped with aluminum foil. The period of irradiation was adjusted (depending upon the position in the reactor) to yield a specific activity of 80-100 μCi per mg of metal. After irradiation the samples were transfered to glass scintillation vials containing 10 mls of double distilled water and 0.40 mls of concentrated nitric acid. Subsamples were then taken for zinc analysis using atomic absorption spectrophotometry and 0.20 mls of 20% NaOH was then added (total volume = 10.5 ml). The solution had to be partially neutralized (final pH between 2.5-3.5) to avoid altering the pH of the test medium when large amounts of isotope were added. The ^{65}Zn produced was routinely examined for impurities by Dr. C. Weber (Nuclear Medicine, McMaster University) and for determination of the specific activity using cobalt standards. No significant quantities of contamination were evident in all batches of isotope. The stock isotope solution was later diluted to provide the desired zinc concentraton without requiring dilution with stable zinc.

Decay of ^{65}Zn occurs primarily by orbital electron capture

(98.5%) along with positron emission (1.5%). Both modes result in the loss of a proton and gain of one neutron per nucleus with the excess energy emitted as gamma radiation (Wang et al., 1975). Although a copper isotope results from the decay of radioactive zinc the total concentration of copper from this source of contamination is negligible. Isotope discrimination or the timing of decay modes and emission of gamma radiation is not known and assumed not to be significant in the analysis of the results.

Partitioning of zinc into adsorbed and free (in solution) fractions was obtained from measurements of the activity in an unfiltered sample and in a sample concentrated onto a 0.45 µm membrane filter. The sample was passed through two filters and the bottom filter used to correct for the level of activity associated with the filter. Sample size, inoculum, or the amount of isotope added were adjusted to yield about 10,000 CPM with a background correction < 10%. Under all conditions the total zinc bound to the cells was < 1.0% of the total zinc present in solution. No significant losses of zinc from solution were observed and all isotope added to the test flasks could be accounted for (± 2%). Prior filtration of the medium along with washing the inoculum removes debris and other contaminants which would otherwise bind zinc and be retained upon the filter. Results from preliminary experiments demonstrated that this source of error is variable and can be significant. All filters were sucked completely dry to minimize the amount of residual solution present on and within the filter matrix. Filters were not rinsed after filtration to avoid disrupting the more labile fraction of isotopic zinc on the cell

surface.

Samples were placed into 25 ml disposable plastic scintillation vials containing ACS scintillation cocktail (Amersham Corp.) and counted on a Beckman Model LS 250 Scintillation System. Liquid samples (1-2 ml) and membrane filters were counted in 10 and 20 mls of scintillation cocktail respectively. Quench standards for membrane filters, chlorophyll, and water were also run for each experiment. Although employing conventional liquid scintillation methods to detect ^{65}Zn introduces more sources of error, they are outweighed by the increase in the detection limits (> 350%) and availability of instrumentation. The error components associated with this experimental design are listed in Table VI and the observed sample standard deviation among replicates did not exceed $\pm$ 10%.

Similar experiments to those described in the previous section were conducted with ^{65}Zn in lieu of ^{14}C using the MB test medium (refer to Table V for incubation conditions). Equilibrium between the metal bound onto the cell surface and in solution reached a steady state after 100-150 min. A constant gradual increase beyond this time was attributed to the transport of metal into the cell (refer to Fig. V). Subsequently, a 200 min incubation period with the isotope was selected. The zinc isotope was added with the metal solutions (when required) to the test flasks and allowed to equilibrate for 100 min before the inoculum was introduced to ensure that all metals were present in the same physico-chemical form. IVF and pH were measured at the termination of each experiment and the cultures were fractionated and counted using the method previously described.

Table VI Error Components of Zinc Uptake Experiments

Source	Maximum Relative Error Encountered (± %)
Counting Errors (^{65}Zn):	
Precision:	
Standards	
Zinc	0.2
Membrane Filter Quenching	0.2
Chlorophyll Quenching	0.2
Samples	
Bottom Filter	5.0
Top Filter with Cells	1.0
Sample Variation:	
Standards	
Zinc	0.1
Membrane Filter Quenching	3.0
Chlorophyll Quenching	3.0
Samples	
Bottom Filters	5.0
Top Filters with Cells	5.0
Other Error:	
- addition of metals/volume adjustments	5.0
- Coulter Counter	0.1
- conversion of counts using the Coulter Counter to cells/ml	5.0

Estimate of total relative variance ($V_T = \Sigma$ (Rel.Err.)^2) = 144 %
with a maximum relative standard deviation of ± 12 %.
The observed sample standard deviation among replicates < ± 10 %.

II.I.IV. Factorial Experiments

An efficient and logical method for examining the influence of a number of metals on zinc uptake is through the use of factorial experiments. Factorial designs, especially two level, are frequently implemented in areas where information on the effects of one or more variables on a response is required. The nature of this approach minimizes the number of runs required to resolve major trends or factors which singly or in combination exert the greatest influence on the measured response. They do not provide complete coverage of all n-dimensional factor space but can resolve between how the variables interact (additively or nonadditively) and provide an estimate of the degree of interaction. The theoretical basis for factorially designed experiments and industrial applications are well documented in the literature including textbooks specifically dealing with the subject. A major portion of the information presented below was obtained from Statistics for Experimenters (Box et al., 1978) and Applications of Statistics in Industrial Experiments (Daniel, 1976). This is the first attempt to systematically investigate the effects of combinations of more than two metals on phytoplankton. Consequently, the theory, assumptions, and limitations of factorially designed experiments, as applied to multiple metals and phytoplankton, are presented in detail.

The variables examined in this investigation include; H, Cd, Cu, Ni, Sr, Co, Mg, Ca, and Mn. The effects of these metals on zinc uptake, fluoresence, and in some instances ^{14}C uptake were measured. Each variable was assigned two levels (+/-) set according to

preliminary runs on the influence of each metal on short term rates of ^{14}C assimilation. The upper level (+) was established as the concentration which significantly lowered the relative rate of photosynthesis. For metals which did not influence rates of photosynthesis the +/- levels were determined from either long term growth experiments (reduction in final yield), set according to I.J.C. water quality standards (IJC, 1977. New and revised Great Lake water quality objectives, Vol. 1. International Joint Commission), or arbitrarily adjusted according to the average concentration found in natural waters. The metal was omitted for the lower setting "-" to provide greater resolution between the effects of individual metals.

Since there were 9 variables, 2^9 or 512 runs were required to resolve all the individual effects and interactions between variables. Some variables were combined, based on preliminary short term experiments, to reduce the total number of runs to 64 or 6 variables (Ca/Mg and Sr/Co). The effects of hydrogen ion concentration was examined at three levels (pH 6, 7, and 8) and provided a reliable method to block the entire factorial array into a manageable number of runs and samples to process. Changes in pH had the greatest aggregrate effect and confining treatment comparisons (other metals) within blocks of constant pH provided greater precision and resolution of all effects. In order to establish a sequence for the variable settings a coded matrix of 6 variables by 64 runs consisting of +/- signs was constructed. The first column consisted of alternating minus and positive signs, the second column 2 minus followed by 2 positive signs, and the n^{th} column had 2^{n-1} minus signs followed by 2^{n-1}

Table VII. Factorial Design Matrix

Run#	Variables	Variable Settings: Cu 1	Ca/Mg 2	Sr/Co 3	Ni 4	Cd 5	Mn 6
1	-	-	-	-	-	-	-
2	1	+	-	-	-	-	-
3	2	-	+	-	-	-	-
4	12	+	+	-	-	-	-
5	3	-	-	+	-	-	-
6	13	+	-	+	-	-	-
7	23	-	+	+	-	-	-
8	123	+	+	+	-	-	-
9	4	-	-	-	+	-	-
10	14	+	-	-	+	-	-
11	24	-	+	-	+	-	-
12	124	+	+	-	+	-	-
13	34	-	-	+	+	-	-
14	134	+	-	+	+	-	-
15	234	-	+	+	+	-	-
16	1234	+	+	+	+	-	-
17	5	-	-	-	-	+	-
18	15	+	-	-	-	+	-
19	25	-	+	-	-	+	-
20	125	+	+	-	-	+	-
21	35	-	-	+	-	+	-
22	135	+	-	+	-	+	-
23	235	-	+	+	-	+	-
24	1235	+	+	+	-	+	-
25	45	-	-	-	+	+	-
26	145	+	-	-	+	+	-
27	245	-	+	-	+	+	-
28	1245	+	+	-	+	+	-
29	345	-	-	+	+	+	-
30	1345	+	-	+	+	+	-
31	2345	-	+	+	+	+	-
32	12345	+	+	+	+	+	-
33	6	-	-	-	-	-	+
34	16	+	-	-	-	-	+
35	26	-	+	-	-	-	+
36	126	+	+	-	-	-	+
37	36	-	-	+	-	-	+
38	136	+	-	+	-	-	+
39	236	-	+	+	-	-	+
40	1236	+	+	+	-	-	+
41	46	-	-	-	+	-	+
42	146	+	-	-	+	-	+
43	246	-	+	-	+	-	+
44	1246	+	+	-	+	-	+
45	346	-	-	+	+	-	+
46	1346	+	-	+	+	-	+
47	2346	-	+	+	+	-	+
48	12346	+	+	+	+	-	+
49	56	-	-	-	-	+	+
50	156	+	-	-	-	+	+
51	256	-	+	-	-	+	+
52	1256	+	+	-	-	+	+
53	356	-	-	+	-	+	+
54	1356	+	-	+	-	+	+
55	2356	-	+	+	-	+	+
56	12356	+	+	+	-	+	+
57	456	-	-	-	+	+	+
58	1456	+	-	-	+	+	+
59	2456	-	+	-	+	+	+
60	12456	+	+	-	+	+	+
61	3456	-	-	+	+	+	+
62	13456	+	-	+	+	+	+
63	23456	-	+	+	+	+	+
64	123456	+	+	+	+	+	+

The numbers in the variable column represent the high setting for the respective variables (metals) present in each run ("+"/"-" correspond to high (5.0 uM) and low (omitted) settings for the variables in each run). Run number "64" for example, all metals are added to the flask (variable settings at "+" level), and the respective numerical code in the variable column is "123456". All experiments were carried out at 20°C in MB with constant agitation provided by a rotary shaker (refer to Tables V and VIII for incubation conditions and initial conditions for each experiment).

positive signs. The amount of each metal added to a flask was determined from the "+" or "-" setting in the corresponding row (Table VII). The "+" settings for metals present within each flask are represented by a numerical code for easier visual interpretation (1=Cu, 2=Ca/Mg, 3=Sr/Co, 4=Ni, 5=Cd, 6=Mn). For example, copper and nickel added to flask # 10 (run #10 Table VII) are represented in the variable column as "14".

The main effect of each variable, or change from a "-" to "+" level, is provided in 2^{n-1} observations (2^{n-1} "+" or 2^{n-1} "-") or with a precision of a 2^{n-1} fold replicated difference using all observations. For the response y for each run n, the main effect of a variable (denoted as V_i, for variable i) would be:

$$V_i = \frac{\Sigma y_i\ (\text{"+"})}{2^{(n-1)}} - \frac{\Sigma y_i\ (\text{"-"})}{2^{(n-1)}} \qquad (3)$$

or the difference between two averages. A measure of the interaction between variables is obtained from a difference between the average effects of each variable for both levels of the remaining variables taken individually. A two factor interaction for example, for the variables V_1 and V_2 is calculated from the difference of the average effects (V_a) of V_1 at both levels of V_2.

$$(V_2\ \text{"-"}) \quad V_a = \frac{\Sigma V_1\ (\text{"+"})}{2^{(n-2)}} - \frac{\Sigma V_1\ (\text{"-"})}{2^{(n-2)}} \qquad (4a)$$

$$(V_2\ \text{"+"})\quad V_a = \frac{\Sigma V_1\ (\text{"+"})}{2^{(n-2)}} - \frac{\Sigma V_1\ (\text{"-"})}{2^{(n-2)}} \qquad (4b)$$

where n is the total number of runs. At each level of V_2 there are $2^{(n-2)}$ runs for both levels of V_1. Half the difference is termed variable one by variable two interaction by convention (Box et al., 1978), or $V_1 \times V_2$ interaction.

$$V_1 \times V_2 \text{ Interaction} = \frac{V_a\ (\text{at } V_2\ \text{"-"}) - V_a\ (\text{at } V_2\ \text{"+"})}{2} \qquad (5)$$

Higher order interactions in the case of a continuous response function tend to average out the effects of individual variables and can provide a measure of the experimental error.

The design matrix consisting of plus and minus signs can be used to calculate main effects and interactions. Each effect can be obtained by multiplying the signs of the respective variables with the response. The sum of the responses divided by $2^{(n-1)}$ provides the estimate for each effect. Yates algorithm, however, provides an efficient method for computing all main effects and interactions, especially for more than 3 variables. Application of this method to a design matrix is discussed fully in most textbooks on factorial design and will not be expanded upon (Box et al., 1978). A program was developed on an Apple II microprocessor for analyzing the experimental results using Yates algorithim and provides numerous subroutines for data manipulation. A reverse Yates algorithim was also incorporated

into the program to obtain estimates of the results ($\hat{y}$) and verify which effects arose primarily from noise. The estimates ($\hat{y}$) were used to calculate residuals ($y-\hat{y}$) providing an additional diagnostic check through normal plots of the data. The calculated effects supply information on the influence of other metals present in solution both singly and in combination on zinc uptake, fluorescence, and rates of photosynthesis.

It must be noted that each effect is a measure of the average response between the high and low settings of the variable. Consequently, the nature of interaction between variables (additive or otherwise) will determine the overall magnitude and sign of each effect relative to the average response for all variable settings.

Occasional experiments were replicated, along with controls in each series, to obtain estimates of the standard errors associated with each effect and experimental error. Individual variances (S_i) for N=2 replicates at each of the g sets of conditions is given by,

$$S_i^2 = d_i^2 / 2 \qquad (6)$$

with only one degree of freedom, and d_i represents the difference between duplicate observations of the i^{th} set of conditions. The total variance (S) is estimated from

$$S^2 = \Sigma d_i^2 / 2g \qquad (7)$$

where g equals the number of replicated runs. Main effects and

interactions are all averages of 2^{n-1} observations (statistic in the form of $\bar{y}$ (+) - $\bar{y}$ (-)). The variance of each effect with the assumption of independent errors is,

$$V_i(\text{effect}) = V_i(\bar{y}\ (+) - \bar{y}\ (-)) = (1/g + 1/g)\sigma^2 = \sigma^2/2\ g \qquad (8)$$

The standard error of an effect is obtained by substituting Equation 6 into Equation 7 for $S^2 = \sigma^2$ (the variance S^2 as an estimate for σ^2) and is given by,

$$SE = \pm\ (\Sigma\ d_i^2\ /\ 4\ g^2)^{1/2} \qquad (9)$$

The estimated standard error was used to resolve between the effects of various metals (singly and in combination) which could not be attributed to random error (noise).

The initial conditions for each factorial experiment are presented in Table VIII. The previously described MB test medium was used in all experiments except one series in which the phosphate buffer was replaced with 6.0 mM tris(hydroxymethyl)aminomethane (THAM) at pH 8.2 (denoted as PH8TH). In order to evaluate the influence of these metals on photosynthesis (uptake of ^{14}C), stable zinc was substituted for the radiaoactive isotope. Glassware was treated as described in Section II.I.I. and all samples were processed according to the methods outlined in Sections II.I.II. and II.I.III. for ^{14}C and ^{65}Zn respectively. Variance associated with the experimental conditons (sampling and analytical variance) was minimized by following the same

Table VIII Initial Conditions for the Factorial Experiments

Series	Species	pH	Cell/ml (10^4)	SA[1] (μm^2)	Time (min)	Zinc Uptake[2] (10^{-16} moles/cell) Controls	Avg
PH6L	Chlamydomonas	6.18	4.31	180(53)	200-230	3.22	1.58
PH6L	"	6.20	3.01	162(47)	200-230	3.25	1.62
PH7L	"	7.18	3.00	170(61)	200-230	1.10	.65
PH7D	"	7.17	4.09	164(52)	220-240	.49	.41
PH7C	"	7.18	3.68	134(44)	220-250	-	-
PH7t	"	7.18	3.68	148(60)	620-640	1.13	1.00
PH8L	"	8.21	4.59	130(41)	210-240	.39	.24
PH8TH	"	8.12	4.36	166(44)	220-240	1.44	1.91
PH7L	Chlorella UT	7.18	9.54	25(12)	200-230	.44	.32
PH7L	Coelastrum UT	7.18	6.94	26(11)	200-230	.21	.10
PH7L	Scenedesmus UT	7.17	3.96	56(18)	210-240	.60	.27
PH7L	Scenedesmus HH	7.18	1.02	212(76)	200-230	3.62	1.24

Notes 1. Mean surface area and standard deviation (in brackets) of population prior to each experimental series. Measurements were made on 200 individuals assuming a spherical geometry.

2. Control values are based on 6 replicates in the absence of other divalent cations. The average zinc uptake represents the grand average of all flasks under all conditions for each series.

procedure for each run (preparation of metal solutions, washing glassware, inoculation, etc.). All flasks were renumbered at random prior to each experiment to avoid complications which may result from residual metal adsorbed onto the container walls not removed by the acid treatment. Flasks were also randomly distributed on the table of the rotary shaker to avoid introducing additional experimental bias.

II.II. Large Volume Enclosures

This section is subdivided into three parts including; 1. a description of the study site and the large volume enclosures, 2. sample collection and storage, 3. analysis of chemical and biological parameters.

II.II.I. Description and Location

Hamilton Harbour is a receiving body for domestic and industrial wastes containing elevated levels of heavy metals including copper, cadmium, manganese, and zinc. The biology, chemistry, and physics of this water body have been extensively documented and will not be reviewed here. All field studies were conducted in limnocorals anchored in Hamilton Harbour in a lagoon west of the Lax property (Lat: 79° 52' 35" W. / Long; 43° 16' 20" N.) over a two month period from July 5 to September 11, 1981. The site was approximately 1 km southeast of the Desjardins Canal which joins the harbour to Cootes Paradise.

Three triangular enclosures were constructed out of blue vinyl coated nylon mesh (Felcoat # 18985 (644g), registered trademark of Fell-Fab Products, Hamilton, Ontario) approximately 2.1 m on 3 sides by 8 m deep and closed at the bottom (surface area = 1.1 m^2, volume = 8.8 m^3). Ethafoam inserts at the top provided sufficient flotation to raise the lip of each bag 8-12 cm above the the water surface. Concrete weights were attached to an open sleeve on the bottom hem of each bag. The tops of all three bags were moored to a wooden frame anchored in three points to concrete blocks. All bags were filled on the same day with surface water by first towing behind a boat, positioning in the frame, and then topping off with a pump. Mixing of the bags was accomplished using a modified airlift constructed out of 8 inch diameter flu pipe. The bags had to be aerated periodically due to the tremendous oxygen demand of the water column. After a five day preincubation period metal solutions were slowly added to Bags 1 and 2 during mixing (Bag 3 was mixed and untreated). The metals used in this investigation include; Cd, Co, Cu, Ni, Pb, Sr, and Zn. The amounts of each metal added to Bag 1 were calculated (using a volume of 8000 L) to yield a final concentration of 0.2 mg/L for Cu, Cd, and Pb and 2.0 mg/L for Co, Ni, Sr, and Zn. Half these amounts were added to Bag 2.

Three panels approximately 1 m^2 were suspended in the middle of each bag at 0.5, 3.0, 7.0 m depths. One side of the panel was scraped halfway through each experiment and both sides at the termination of the experiment. These samples were frozen intact after a subsample had been removed and preserved with Lugol's iodide solution.

II.II.II. Sample Collection and Storage

All bags were sampled on a daily basis. Temperature and oxygen profiles were obtained using a YSI Model 57 dissolved oxygen meter equipped with a thermistor. Water samples were collected in an opaque 2 L Van Dorn from 0.5, 4, and 8 m depths along with an integrated sample taken with an 8 m length of Tygon tubing (ID = 0.5 in) and transported back to the lab in precleaned (1% HNO_3 wash followed with a distilled water rinse) 2 L polyethylene bottles.

Samples for metal analysis were partitioned into particulate and dissolved fractions by gentle filtration (vacuum did not exceed 15 lbs/in^2) through 4.25 cm Whatman glass fiber filters. Duplicate aliquots of the dissolved fraction (about 20 ml) were placed into 20 ml polyethylene scintillation vials (Fisher Scientific Company) after the vials were rinsed with the supernatant, acidified with 1 ml of concentrated nitric acid, and frozen solid until analyzed. Periodically, blanks containing 20 ml of 0.5% nitric acid in double distilled water were prepared and frozen to check for any contamination arising from the scintillation vials, sample treatment, or reagents. Duplicate filters were also stored frozen until analyzed. Volumes of water filtered were adjusted (250 or 500 ml) such that the surface of the filter was heavily coated with particulate matter.

Duplicate samples for particulate carbon analysis were filtered off onto 2.4 cm Whatman glass fiber filters and frozen. Volumes filtered were identical to those used in the samples for metal analysis.

Nutrient samples were also stored frozen in 20 ml scintillation vials after processing in the following manner; total phosphorus (TP) unfiltered, soluble reactive phosphorus (SRP) and soluble silicate (Si) filtered plus 2 drops concentrated HCl, nitrogen (nitrate, nitrite, and ammonium) filtered plus two drops of chloroform. Additional unfiltered samples were preserved with Lugol's iodide solution for cell counts. Zooplankton samples were collected every three days using a plexiglass Schindler trap from three depths in each bag.

Particle size distributions were obtained on all samples using a Coulter Counter Model F (described in Section II.I.II.) by varying the threshold level (2, 5, 10, 15, 30, 50, and 75%).

Chlorophyll a determinations were carried out on samples filtered onto 0.45 μm membrane filters using the hot methanol method described in Section II.I.II. All determinations were carried out in duplicate with 50 or 100 ml sample volumes on the same sampling day.

Alkalinity was measured every third day on the integrated samples using a modified Gran titration. All titrations were carried out at 20°C using an automatic pipette (Metrohm Hersiau Multi-Dosimat E415) and a combination glass electrode connected to a Radiometer 26 pH meter with an expanded scale.

Conductivity and pH were measured daily on all samples using the instrumentation described in Section II.I.I.

Attempts to measure the light climate using a Lambda/Licor underwater quantum sensor were complicated by shading from the sides of the bags and did not provide any useful information. Continuous

fluorescence profiles were also attempted but scattering due to elevated concentrations of particulate matter produced variable results.

II.II.III. Analysis of Chemical and Biological Parameters

Nutrients were determined using a Technicon Autoanalzer II according to the following methods; TP (after persulphate digestion) and SRP with the stannous chloride/ammonium molybdate method of Kramer et al. (1972), Si by the silicon-moybdate-molybdeum blue reaction (Technicon Corp. 1973), nitrite and nitrate (after reduction to nitrite using a copper cadmium reductionn column) by the Technicon method (Technicon Corp. 1971a), and ammonium using phenoxide, sodium hypochlorite, and potassium tartrate reaction (Technicon Corp. 1971b).

Metal analysis was performed on a Perkin Elmer Model 603 atomic absorption spectrophotometer using both the flame mode and graphite furnace (HGA-2100). Operating conditions for each element are summarized in Table IX along with detection limits and standards. Hollow cathode lamps were used for all elements except cadmium (electrodeless discharge lamp) and operated at the recommended current. Elevated concentrations of certain metals, beyond the linear working range of the flame mode, were determined by rotating the burner head, adjusting the slit width, and/or reducing the flow through the atomizer. Sample and standard volumes for the furnace mode were selected according to the level of metal present in the samples. The internal gas flow was set at 35 ml/min and both the dry and char

Table IX Operating Conditions for the Perkin Elmer Model 603 Atomic Absorption Spectrophotometer used for the Metal Analysis

FLAME MODE

Element	Standard	Line[1] (nm)	Slit (nm)	Flame[2] (ox/rd)	Sens[3] (mg/L)	Standard[4] (mg/L/ABS)	LR[5] (mg/L)
Cd	Cd(s)	228.8	1.4	ox	0.025	2.0/0.35	2
Ca	$CaCO_3$	422.7	1.4	ox	0.08	4.0/0.20	5
Co	$Co(NO_3)_2$	240.7	0.2	ox	0.15	5.0/0.15	5
Cr	K_2CrO_4	357.9	0.7	rd	0.1	2.0/0.10	5
Cu	Cu(s)	324.8	0.7	ox	0.09	5.0/0.25	5
Fe	Fe(s)	248.3	0.2	ox	0.12	5.0/0.18	5
K	KCl	766.5	1.4	ox	0.04	2.0/0.22	2
Mg	Mg(s)	285.2	0.7	ox	0.007	0.3/0.19	0.5
Mn	Mn(s)	279.5	0.2	ox	0.055	2.0/0.16	3
Na	NaCl	589.6	1.4	ox	0.015	0.8/0.23	1
Ni	$Ni(NO_3)_2$	232.0	0.2	ox	0.15	5.0/0.15	5
Pb	$Pb(NO_3)_2$	283.3	0.7	ox	0.5	20.0/0.18	20
Sr	$Sr(NO_3)_2$	460.7	1.4	rd	0.12	5.0/0.18	5
Zn	Zn(s)	213.9	0.7	ox	0.18	0.5/0.12	1

GRAPHITE FURNACE

Element	Dry		Char		Atomize	
	Time (s)	Temp. (°C)	Time (s)	Temp. (°C)	Time (s)	Temp. (°C)
Cd	60	120	60	400	10	2300
Co	60	120	60	500	10	2700
Cu	40	130	40	300	8	2700
Ni	60	130	60	500	8	2700
Pb	60	130	60	500	10	2600
Sr	60	130	60	500	10	2700

This information was obtained from the handbook on Analytical Methods for Atomic Absorption Spectrophotometry supplied by Perkin Elmer. The sensitivity, standard absorptions, and linear working range are provided only as a guide for instrument operation. The absorptions of standard solutions used for metal analysis were 60-80 % of the above values. Notation is as follows: 1, wavelength; 2, oxidizing or reducing flame; 3, sensitivity reported as concentration giving 1% absorption; 4, absorption (ABS) of standard solution with concentration in mg/L; 5, upper limit to linear working range.

steps were ramped for 20 and 30 seconds respectively. Cycle times and temperatures were optimized using the method outlined in the manual supplied by the manufacturer. A deuterium arc background corrector was routinely employed except where noted.

Particulate samples were combusted in a muffle furnace (500-530°C, higher temperatures result in scintering of the glass fiber filters) for at least 20 h and placed into scintillation vials containing 20 ml of 0.5% nitric acid in double distilled water. Panel samples were dried overnight (65°C) in preweighed dry crucibles, ashed, reweighed, then transfered to scintillation vials using the 20 ml of 0.5% nitric acid. Loss of metals during combustion was not checked but blank filters were included in all analyses. All samples from one bag were combusted at the same time to ensure that the results were comparable at least within each bag. The analysis of each metal was verified by the method of standard additions using Hamilton Harbour water treated in an identical fashion (filtered and frozen).

Particulate carbon was determined by a modified wet dichromate oxidation method of Strickland and Parsons (1968). Addition of phosphoric acid was omitted due to the low chloride concentrations and oxidation of the samples was carried out using an autoclave (20 lbs/in^2 at 220°C). After autoclaving for 60 min, the samples were cooled, shaken, and then centrifuged. The method was linear up to 1500 µg C (glucose standards) with a coefficient of variation <10%. All samples analyzed contained between 400 and 1200 µg C as glucose. Aliquots of the panel samples were dried overnight prior to the oxidation.

Phytoplankton in the integrated samples were enumerated using a Zeiss UPL inverted microscope on 10 ml aliquots of the preserved samples previously sedimented overnight in settling chambers. All zooplankton present in the sample were counted uder low power and 15 random fields were counted under high power (500X). Panel samples were examined in a similar fashion except that only the presence of dominant algal species were recorded without any attempts at quantifying the results.

III. RESULTS

Results from the culture and field investigations are treated separately.

III.I. Culture Investigations

Results from culture investigations are presented in two parts; 1. physiological responses, and 2. binding studies. The first section includes the short term effects of heavy metals on photosynthesis and fluorescence and the long term influences on growth of phytoplankton in batch culture. Adsorption of zinc to the cell surface, including the effects of light, temperature, and other metals, are presented in the second section. Results from the factorial experiments are presented in a separate section.

III.I.I. Physiological Responses

The age structure and physiological condition of the test population can significantly influence results obtained from toxicity bioassays (Section I.I.II.). The technique used to isolate Chlamydomonas provided cells with similar physiological responses (phototaxis) and in short term studies cell cycle differences within the population were assumed insignificant.

The short term effects of 8 metals (Cd, Cu, Co, Hg, Ni, Pb, Sr,

Zn) on photosynthesis in Chlamydomonas at pH 6.2, 7.2, and 8.2 are presented in Table X. (These experiments were carried out during the initial stages of my research and I used metal concentrations in units of mg/L, but later adopted the more rigorous convention of moles/L.) Of all metals examined, only Cu and Hg inhibited photosynthesis at concentrations greater than 0.01 and 0.1 mg/L, respectively. The effects of both metals decreased with increasing pH due to a corresponding decrease in the available metal concentrations. The pH dependence of photosynthesis in Chlamydomonas may also account for this result (Fig. XIa). Cellular requirements for carbonic anhydrase to facilitate bicarbonate uptake are largely determined by the pH of the growth medium and species of alga (Raven, 1974). The slightly acidic pH of my standard growth medium did not necessitate synthesis of this enzyme for uptake of inorganic carbon to satisfy the metabolic requirements of Chlamydomonas. Since rates of photosynthesis decreased with increasing pH, the degree of inhibition will also decrease and, possibly, be obscured by experimental error.

The effects of copper were both time and concentration dependent at concentrations between 0.01 and 0.05 mg/L (Fig. V). Mercury, however, completely inhibited photosynthesis at concentrations greater than 0.10 mg/L (pH 6.2). One temporal component associated with the toxic action of Hg must be < 5 min, the difference in time between addition of the metal and the ^{14}C solutions. Concentrations of mercury between 0.05 and 0.10 mg/L stimulated photosynthesis at pH 7.2, but the nature of this effect was unknown.

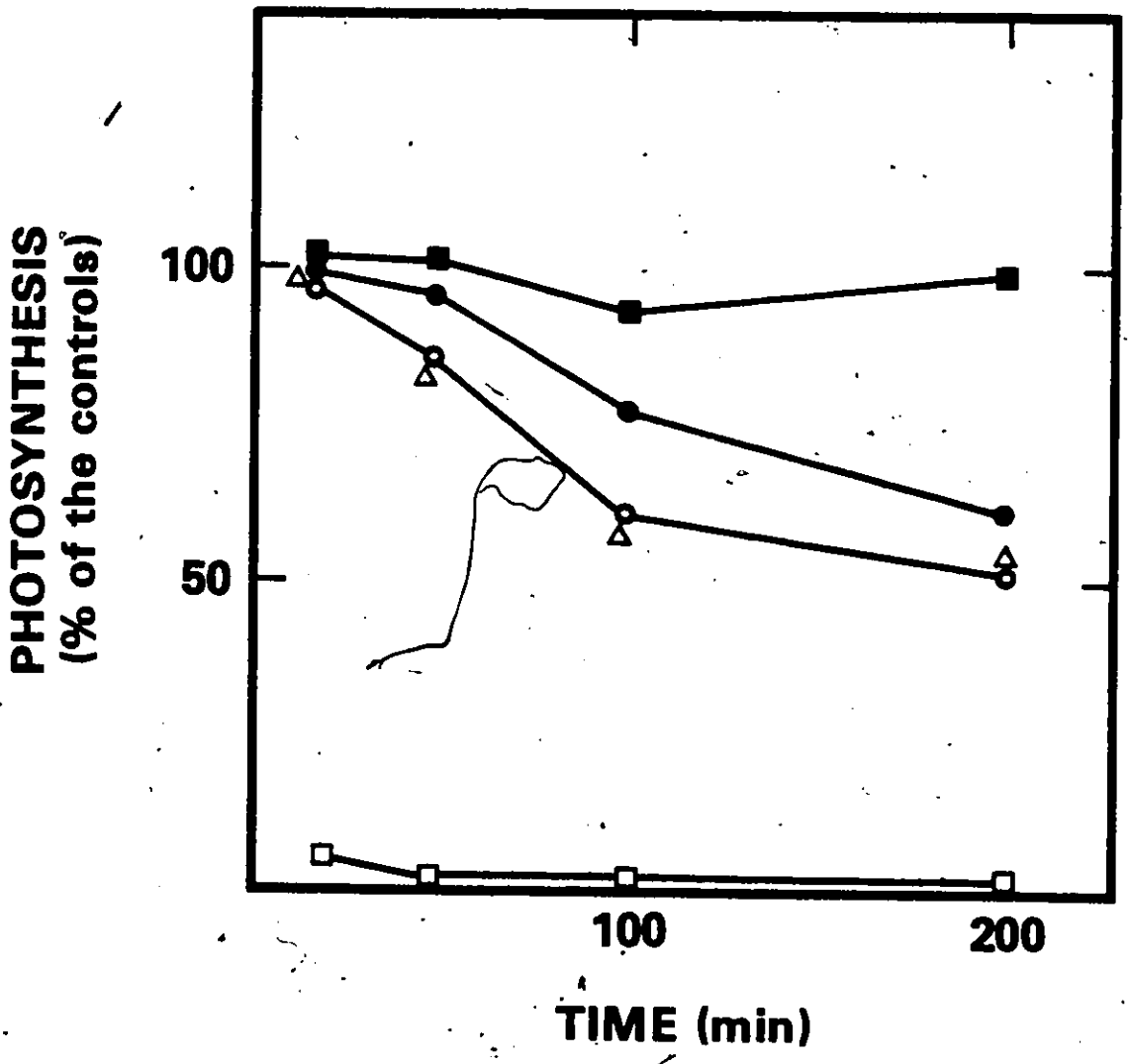
Table X. Short Term Effects of Various Metals at pH 6.2, 7.2, and 8.2 on Photosynthesis (^{14}C Uptake) and Fluorescence in Chlamydomonas

	PHOTOSYNTHESIS					FLUORESCENCE				
	Metal Concentration (mg/L)					Metal Concentration (mg/L)				
Metal	0.01	0.05	0.10	0.50	1.00	0.01	0.05	0.10	0.50	1.00
pH 6.2										
Hg	116	105	114	4	1	96	122	133	52	55
Pb	86	88	89	83	87	87	84	80	87	83
Cu	71	52	52	55	55					
Ni	104	84	103	80	93	80	95	98	93	96
Cd	103	100	91	96	96	89	85	90	85	94
Zn	106	101	96	102	101	96	101	105	104	99
Co	98	89	134	122	103					
Sr	114	107	93	109	85	100	82	95	110	116
pH 7.2										
Hg	121	152	135	45	3	101	136	97	73	69
Pb	108	103	86	97	107	90	78	88	87	91
Cu	67	54	47	55	69					
Ni	93	92	94	92	91	109	104	107	100	99
Cd	98	109	105	95	102	79	98	106	90	87
Zn	105	103	102	96	115	89	94	94	94	94
Co	89	86	102	93	86					
Sr	110	111	100	103	80	119	115	119	118	117
pH 8.2										
Hg	112	109	110	83	22	92	115	116	71	67
Pb	98	101	96	113	92	90	93	98	105	108
Cu	118	85	99	102	96					
Ni	105	114	116	114	108	90	97	104	102	103
Cd	102	91	110	104	115	102	104	99	96	101
Zn	115	122	106	120	118	99	99	89	95	98
Co	112	104	95	107	103					
Sr	87	95	97	85	82	93	85	108	100	92

All results are reported as a percent of the controls with a relative sample standard deviation < ± 15 % (photosynthesis data) and < ± 20 % (fluorescence data).

Fig. V. Time Course for Inhibition of Photosynthesis by Cu, Hg, and Zn

Rates of photosynthesis expressed as a percent of the controls. Zinc did not influence short term rates of photosynthesis ("■" = 1.0 mg/L Zn). Mercury concentrations in excess of 0.5 mg/L completely inhibited photosynthesis ("□" = 0.5 mg/L Hg). The effects of copper are both time and concentration dependent ("●", "○", and "Δ" represent 0.01, 0.05, and 0.1 mg/L Cu respectively), reaching a maximum or plateau at the end of the incubation (200 h). All measurements were obtained in BBM, pH 6.2, at 20°C with an overhead irradiance of 100-120 $\mu Em^{-2}s^{-1}$.

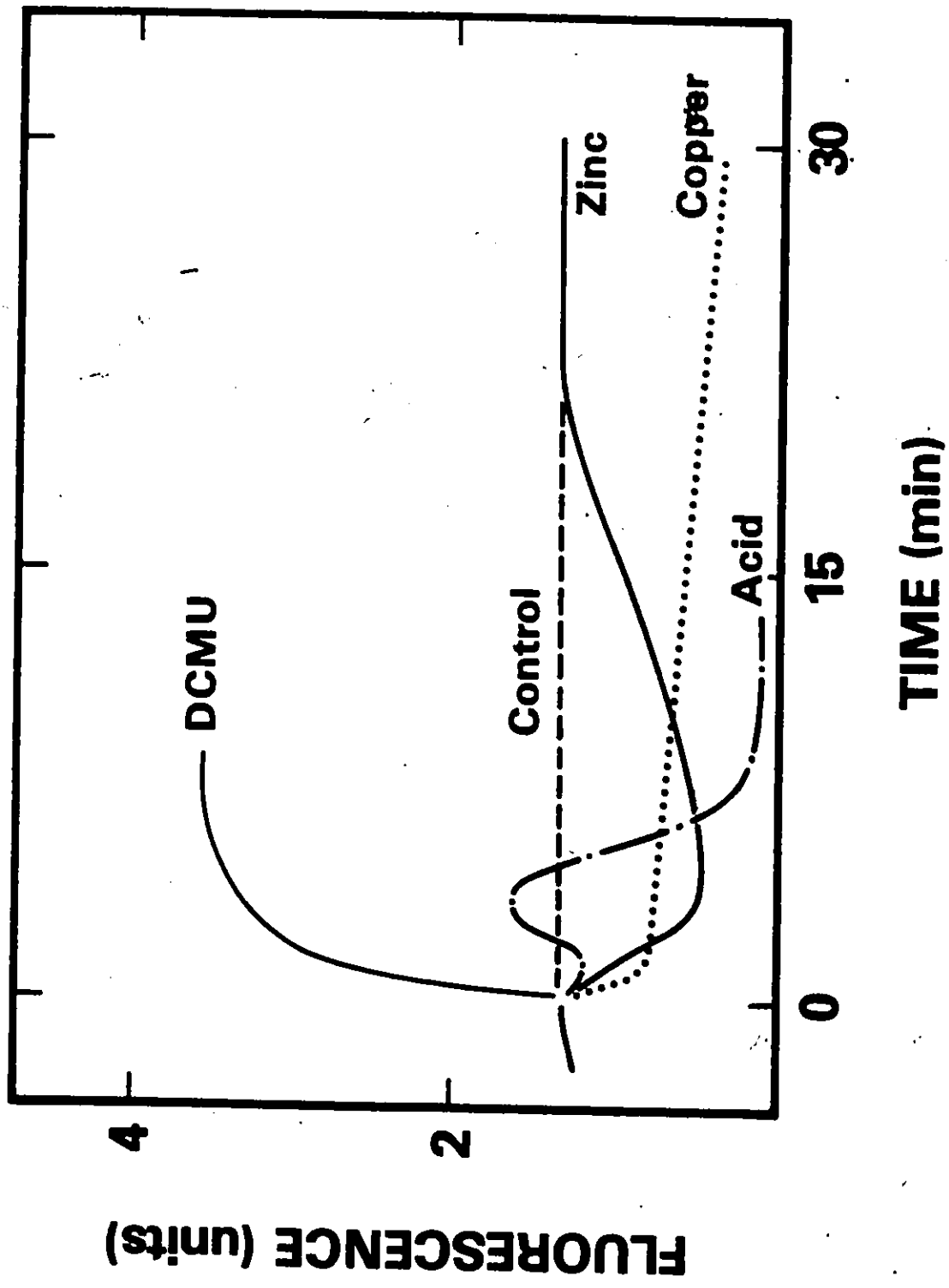

PHOTOSYNTHESIS
(% of the controls)
100
50
100
200
TIME (min)

Fluorescence data was variable and results for cells treated with Cu and Co were not obtained. No significant reductions in fluorescence were detected for all the remaining metals except mercury (Table X). Reductions of between 30 to 60% were observed for mercury concentrations corresponding to those inhibiting photosynthesis.

Fluorescence transients were observed in preliminary experiments with zinc and copper. Additions of 5.0 mg/L zinc resulted in a rapid decrease in IVF which gradually increased back to the original level after 20-30 minutes (Fig. VI). Clearly, the cells respond to minor perturbations in solution because the amount of zinc added does not significantly influence the physico-chemical properties of the assay medium (pH and ionic strength for example). The kinetics of zinc binding and transport into the cell (presented in the next section) cannot account for these observations, especially alterations at the level of chloroplast. The rapid decrease in fluorescence may have resulted from an ionic imbalance across the membrane, indirectly influencing the chloroplast. Rapid changes in the K, Na, Ca, and Mg content of Scenedesmus during copper treatment (Mierle, 1981) support this hypothesis and additional studies are required to fully resolve the nature of this effect. The gradual increase in fluorescence over time, in turn, reflects the ability of the cells to compensate for the initial imbalance. Periodic agitation of the cuvette (roughly 10 min intervals) to maintain the cells in suspension and exposure to low light levels ($< 10\ \mu Em^{-2}s^{-1}$) did not influence the results. Copper additions of 0.2 mg/L resulted in a rapid decrease in fluorescence similar to that observed for zinc, but was followed by a prolonged,

Fig. VI. Transient Fluorescence Response Characteristics

Representative traces from short term fluorescence measurements (IVF). Addition of the herbicide DCMU, blocks non-cyclic electron transport increasing IVF. Acid treatment rapidly impairs normal cell function along with reactions in the chloroplast. Additions of 5 mg/L zinc resulted in a rapid decrease in IVF followed by a gradual increase back to the original level over time. An initial rapid decrease was observed for copper (0.2 mg/L), but was followed by a gradual reduction in IVF over time. The initial effects of both Zn and Cu on IVF were proportional to the concentration of each metal added. The cuvette was removed at 15 min intervals and shaken under ambient light levels to maintain the cells in suspension.


FLUORESCENCE (units)
4
2
0
15
30
TIME (min)
DCMU
Control
Zinc
Copper
Acid

gradual decrease in fluorescence over time. In this case, the cells were not able to compensate for the initial imbalance, possibly resulting from a direct inhibition of membrane bound Na^+/K^+ ATPase.

The effects of heavy metals on the growth of five species of phytoplankton are presented in Table XI. The growth rates (doublings per day) were based on the net increase in cell numbers at the termination of each experiment (N_o and N_t, the initial and final (at t days) cell concentration per ml, respectively) and were obtained from the logistic equation, $r = \log_2(N_t/N_o)/t$. The effects of chloride, nitrate, or sulphate salts of copper and zinc were not significantly different from each other. Species of Scenedesmus and Coelastrum, isolated from Hamilton Harbour, were more tolerant to these metals than similar laboratory strains. The relatively high initial cell densities used in these experiments were necessary to obtain different growth responses (presented below) and the observations are strictly qualitative.

Results from experiments in which the effects of copper and zinc on the growth of Scenedesmus and Coelastrum were followed over time (Fig. VII) were consistent with the generalized growth responses presented in the Introduction (Section I.II.II.). No aberrant morphological changes were detected for either species under all conditions. Increases in the populations of cultures treated with zinc were identical to the controls over the first two days, then decreased in proportion to the total metal concentration added (Type II response). Populations treated with copper were either inhibited after the first day or unaffected. A resurgence of growth was observed in

Table XI Long Term Effects of Various Metals on the Growth of Phytoplankton in Batch Culture

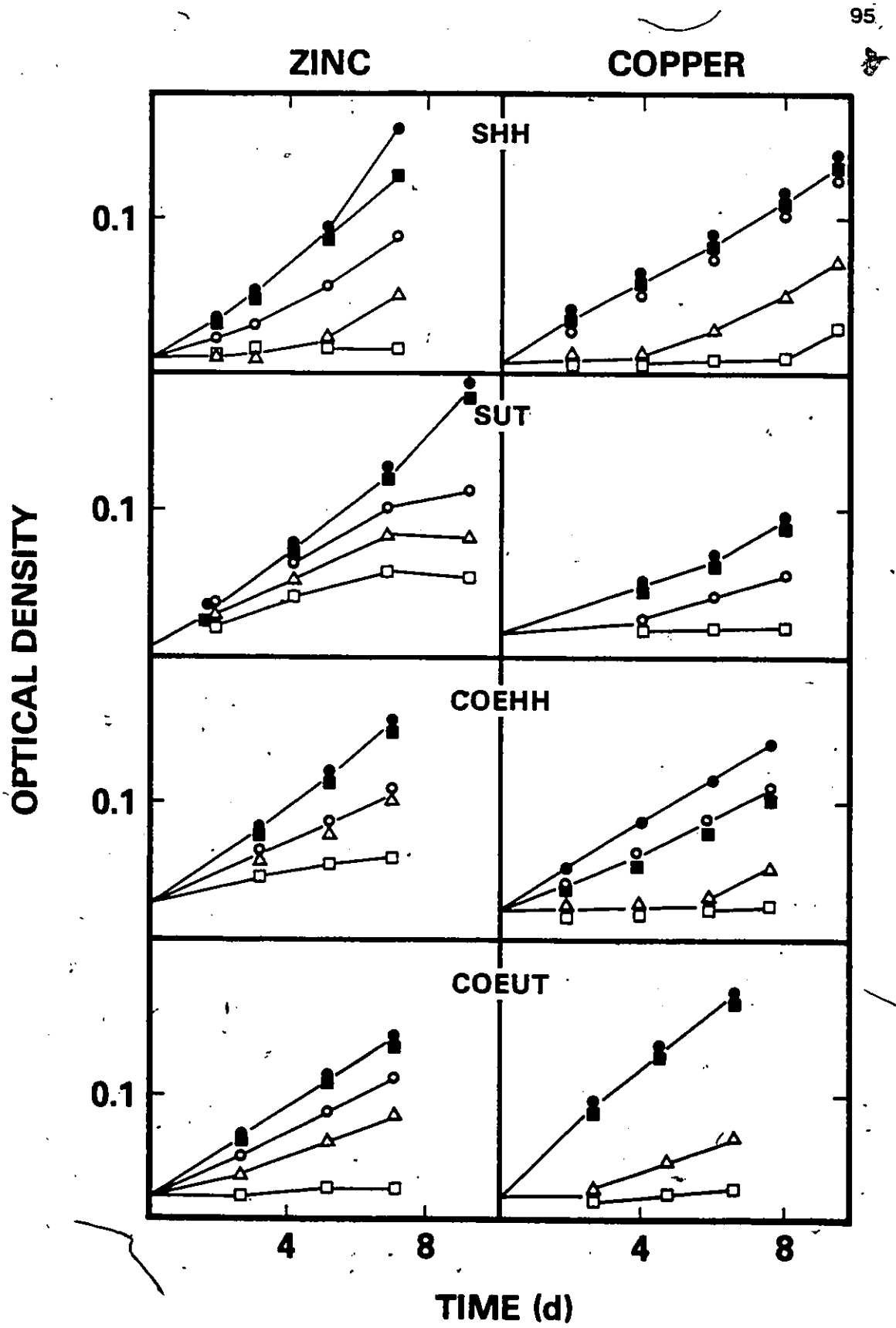
			GROWTH RATE (dbl/day) Metal Concentration (μM)							
Species	Metal	N_o	0.00	0.10	0.20	1.0	2/5	10	20	100
CHL	$HgSO_4$	3.4	1.23	1.22	0.59	–	–	–		
	$HgCl_2$	3.4	1.23	1.12	0.47	0.13	–	–		
	$Cu(NO_3)_2$	3.7	1.20		1.14	0.28	0.09	0.06	0.21	
	$NiCl_2$	4.4	1.19		1.19	1.14	1.14	1.16	0.34	
	$CdCl_2$	3.7	1.22	1.37	1.22	0.88	0.30	0.06		
	$ZnCl_2$	4.4	1.19		1.22	1.19	1.14	0.99	0.45	
	$Zn(NO_3)_2$	2.9	1.18		1.28	1.27	1.23	1.05	0.37	
	$Co(NO_3)_2$	2.9	1.18		1.22	1.28	1.19	1.00	0.66	
	$SrCl_2$	4.4	1.19		1.17	1.16	1.17	1.15	1.20	
	CrO_3	3.7	1.22		1.19	1.21	1.25	1.21	1.05	
	$FeCl_3$	3.7	1.22		1.34	1.38	1.28	1.26	1.33	
	$BeSO_4$	3.4	1.23			1.24	1.17	1.25	1.10	1.14
SCENHH	$Cu(NO_3)_2$	30	0.70	0.67		0.62	0.31	0.12	–	–
	$ZnCl_2$	30	0.74			0.74	0.02	–	–	–
	$Zn(NO_3)_2$	30	0.74			0.70	–	–	–	–
	$ZnSO_4$	151	0.72			0.55	0.50	0.34	0.17	–
SCENUT	$CuCl_2$	100	0.26	0.26		0.17	–	–		–
	$Cu(NO_3)_2$	76	0.26	0.26		0.14	0.05	–		–
	$CuSO_4$	100	0.31	0.33		0.34	0.04	–		–
	$ZnCl_2$	76	0.31			0.30	0.20	0.19		0.14
	$Zn(NO_3)_2$	100	0.26			0.24	0.24	0.22		0.12
	$ZnSO_4$	76	0.31			0.34	0.20	0.17		0.11
COEHH	$CuSO_4$	54	0.83	0.72		0.71	0.18	–		–
	$ZnSO_4$	54	0.83			0.75	0.68	0.46		0.23
COEUT	$CuSO_4$	50	0.63	0.60		0.28	–	–		–
	$ZnSO_4$	50	0.63			0.62	0.56	0.45		–

Growth rate calculations are based on the increase in cell number after a fixed incubation period. Cases in which a net decrease in cell number was observed are represented by "–". N_o represents the cell density (1000/ml) at the start of each experiment. Notation for species; CHL = Chlamydomonas, SCENHH and COEHH refer to Scenedesmus and Coelastrum from Hamilton Harbour, SCENUT and COEUT refer to Scenedesmus and Coelastrum from the University of Texas. Column headed "2/5" uM; 2 uM concentrations for CHL and 5 μM for the other species.

Fig. VII. Growth Curve Characteristics Observed for Phytoplankton under Various Degrees of Heavy Metal Stress

Representative growth curves for phytoplankton populations under various degrees of heavy metal stress. Type I response is characterized by a period of reduced growth, or extended lag phase, followed by normal growth (note growth curves for SHH at 5 and 10 μM Cu). Type II response is represented by a gradual decrease in the growth rate over time (note growth curves for SUT at 5, 10, 100 μM Zn). The time at which the experiment is terminated determines, to a degree, characteristics of the observed response.

Notation: SHH, Scenedesmus from Hamilton Harbour; SUT, Scenedesmus from the University of Texas; COEHH, Coelastrum from Hamilton Harbour; COEUT, Coelastrum from the University of Texas; t, time in days; OD, optical density at 750 nm; Metal concentrations; "■", "○", "△", "□", 1, 5, 10, 100 μM Zn and 0.1, 1, 5, 10 μM Cu respectively ("●" = controls) for all species except SHH (zinc concentrations of 1, 10, 20, and 100 μM were used for SHH).


ZINC
COPPER
SHH
SUT
COEHH
COEUT
0.1
0.1
0.1
0.1
OPTICAL DENSITY
4
8
4
8
TIME (d)

75% of the cultures incubated with 0.01 µM copper after 3 to 5 days (Type I response, extended lag phase). Lower total copper concentrations were not toxic.

The effects of 10 metals on the growth of Chlamydomonas were consistent with the Irving-Williams series and comparable to results from other investigations with different species of phytoplankton. The order, based on the metal concentration (µM) that resulted in a 50% reduction in the calculated growth rate, was as follows: Hg > Cu > Cd > Zn = Ni > Co > Cr > Fe = Be = Sr. Under all conditions intact cells and a net increase in the population, except at elevated Hg and Cu concentrations, were observed at the termination of each experiment. Fluorescence data was variable but on a per cell basis clearly indicated that the cells present at the termination of each experiment, under all conditions except for 20 µM Cu, possessed functional chloroplasts (Table XIII). The implications of this finding on the interpretation of results obtained after long term exposures are great, especially in relating the influence of metals on a population to individual cells. This observation provides additional evidence for differential sensitivity, or tolerance, of individuals within a population to metals and provides some explaination for the Type I growth response presented in Section I.II.II. The viability of these cells, however, was not determined by subculturing in media minus metals.

At the termination of the growth experiments with Coelastrum from Hamilton Harbour, cultures exposed to elevated levels of copper and zinc (10 and 100 µM, respectively) were subjected to energy

Table XII Long Term Effects of Various Metals On Fluorescence

Species	Metal	N_o	FLUORESCENCE (units/cell) Metal Concentration (μM) 0.00	0.10	0.20	1.0	2/5	10	20	100
CHL	$HgSO_4$	3.4	40	60	139	77	91	40		
	$HgCl_2$	3.4	40	47	101	18	200	77		
	$Cu(NO_3)_2$	3.7	81		61	288	125	41	0	
	$NiCl_2$	4.4	65		58	67	70	69	65	
	$CdCl_2$	3.7	48	20	44	89	73	149		
	$ZnCl_2$	4.4	65		54	60	76	88	250	
	$Zn(NO_3)_2$	2.9	64		57	48	64	108	264	
	$Co(NO_3)_2$	2.9	64		58	66	74	86	119	
	$SrCl_2$	4.4	65		74	65	60	59	60	
	CrO_3	3.7	48		53	40	47	50	50	
	$FeCl_3$	3.7	48		33	32	34	44	38	
	$BeSO_4$	3.4	40			30	26	37	26	36
SCENUT	$CuCl_2$	100	13	16		10	ND	7		10
	$Cu(NO_3)_2$	76	13	16		11	ND	5		10
	$CuSO_4$	100	18	15		32	ND	4		10
	$ZnCl_2$	76	18			32	33	32		9
	$Zn(NO_3)_2$	100	13			14	13	14		3
	$ZnSO_4$	76	18			30	38	30		0
COEHH	$CuSO_4$	54	7	11		7	ND	2		0
	$ZnSO_4$	54	7			8	13	ND		17
COEUT	$CuSO_4$	50	17	19		30	ND	10		10
	$ZnSO_4$	50	17			18	ND	32		62

Fluorescence was measured at the termination of the growth experiments and recorded in arbitrary units (units/cell, ND = not determined). N_o represents the cell density (1000/ml) at the start of each experiment. Species notation as in Table XI. Column headed "2/5" μM; 2 μM concentrations for CHL and 5 μM for the other species. Sample standard deviation was $< \pm 25\%$ (3 determinations).

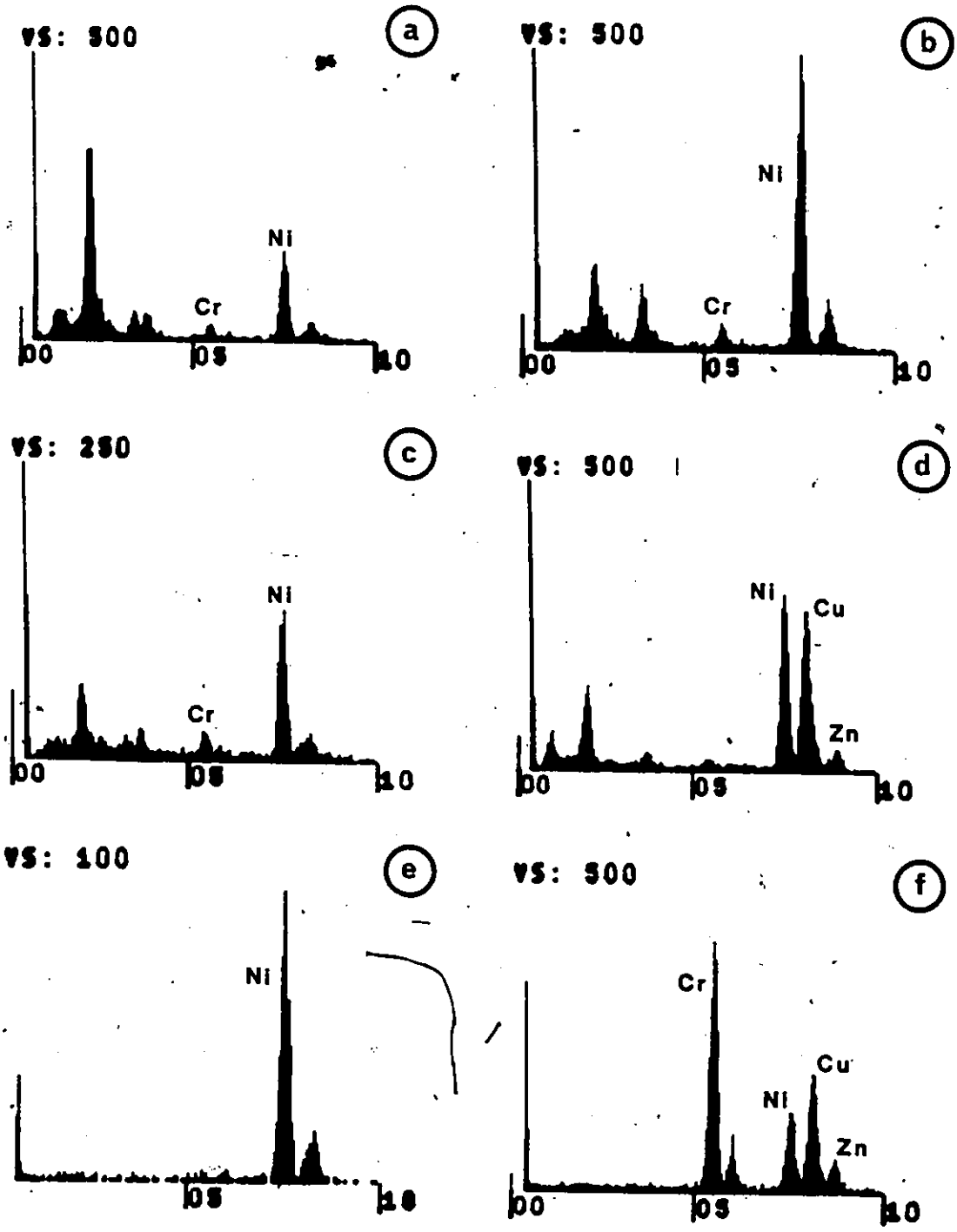
dispersive x-ray analysis (EDX, Fig. VIII). Copper was only detected in cell debris and not in intact cells. Maximum zinc binding capacities for individual cells (presented in the next section) are roughly two orders of magnitude less than the sensitivity of EDX analysis and, consequently, this metal was not detected in the debris or intact cells. The presence of significant copper concentrations associated with the debris, however, indicates that the maximum binding capacity of the cell surface for copper is much greater than for zinc. The degree of complexation of these metals by dissolved organic matter released during cell lysis could not be ascertained.

Results from the short and long term physiological experiments provide a number of insights into factors regulating heavy metal - phytoplankton interactions in culture. The nature of the cell surface and interactions of the metal with other metals/ligands in solution all influence the available metal concentration, hence binding and uptake. Phytoplankton respond to changes in the physical and chemical environment over relatively short time scales (s to min) compared to the binding of metal to the cell surface (h, presented in the next section) and the generation time (d). A lethal response is observed when the integrated inward flux of metal exceeds losses from dilution of cell metal through growth and the capacity of the cell to compensate for the stress over time. Loss of metal from solution, in the form of precipitates or the non-exchangeable binding to cell debris and walls of the experimental flask, may reduce the level of stress (cell burden) over time. A significant reduction in the available metal concentration, in turn, may result in the resumption

Fig. VIII. Repesentative EDX Analysis Spectra for Intact Cells and Debris from Long Term Growth Experiments

Samples from experiments on the effects of 100 μM zinc and 10 μM copper on the growth of Coelastrum were subjected to EDX analysis. All spectra were obtained on intact cells and debris (spot size held constant) using an accelerating voltage of 80 kV with an analysis time of 60 s except for the grid blank (figure e, analysis time 30 s). The objective of this exercise was to confirm the importance of debris in sequestering metals during the course of a batch culture experiment. In this respect, the presence or absence of other elements are not considered and only major peaks for the metals of interest (Cr, Ni, Zn, and Cu) are indentified on the spectra. Copper was only detected in the debris and not intact cells and zinc was not detected in either fraction. Copper peaks may orginate from the column (along with Cr and Zn), but by comparing the spectra with obvious contamination (blanks, figures e and f) the presence of copper associated with the debris can be resolved (Cr peak absent). Principal emmission lines (values in keV) for elements of interest include: Cu 8.04, $K_{\alpha 1,2}$; Zn 8.63, $K_{\alpha 1}$; Ni 7.48, $K_{\alpha 1}$; Cr 5.41, $K_{\alpha 1,2}$. The $K_{\beta 2}$ peak for Ni (8.33 keV, 20 % $K_{\alpha 1}$ peak) was obscured by Cu and is not identified.

Figure notation is as follows: a, intact cell from flasks treated with 100 μM zinc centrally located on grid; b, intact cell from flasks treated with 10 μM copper near grid edge; c, debris in flasks treated with 100 μM zinc (zinc absent); d, debris in flasks treated with 10 μM copper (copper detected, chromium absent); e. blank near grid bar (Ni peak from grid); f. blank near grid edge (Ni, Cu, Zn, and Cr peaks from grid and column).


VS: 500
a
Ni
Cr
00
05
10
VS: 500
b
Ni
Cr
00
05
10
VS: 250
c
Ni
Cr
00
05
10
VS: 500
d
Ni
Cu
Zn
00
05
10
VS: 100
e
Ni
05
10
VS: 500
f
Cr
Cu
Ni
Zn
00
05
10

of normal growth (Type I response, extended lag). If the cell burden is not significantly reduced over time, then changes in cell numbers will depend on rates of uptake, dilution of cell metal through growth, and the ability of cells to compensate for the influx of metal (Type II response, gradual reduction in cell numbers over time). Results from the EDX analysis demonstrate the potential importance of cell debris in regulating the available metal concentration in solution. Random genetic mutations cannot account for the renewed growth after an extended lag phase. Differential tolerance of individuals within a population (epigenetic variablity) may, however, be an important factor determining the nature of the observed response.

III.I.II. Binding Studies

Levels of debris in the distilled water and from other sources of contamination can significantly influence the results in quantitative metal uptake experiments. This source of error was reduced by filtering the media through precleaned 0.45 um membrane filters before use. Lead and iron formed innocuous precipitates in the modified test medium that were retained on the filter and that adsorbed zinc (confirmed by x-ray fluorescence analysis, note the increased binding of zinc in the presence of both metals in Table XVI). Parks (1975) discussed the negative surface charge of iron oxide in relation to cation adsorption but the contribution of the cell surface interacting with these metals in solution is not known. Consequently, the influences of lead and iron on zinc uptake were not

included in the multiple metal competition experiments.

The irradiation periods used for the production of ^{65}Zn were adjusted (position and time within the reactor) so that further dilution with stable isotope was not necessary in all applications. This procedure eliminated dilution effects and insured that all zinc was present in the same physiochemical form for experimental use. A one hour preincubation period of the flasks containing metals, or EDTA, and ^{65}Zn (minus cells) was assumed sufficient to attain a complete equilibrium between all components in solution. No significant losses of zinc from the MB medium were observed and all isotope added to the test flasks could be accounted for ($\pm$ 2%).

Under all conditions, adsorption of zinc onto to the cell surface followed simple hyperbolic saturation kinetics reaching an apparent equilibrium (plateau) after 50-75 min. Estimates for the binding capacities and stability constants for the surface site-zinc complex were obtained by fitting the uptake data, over a range of available zinc concentrations, to the Langmuir and Freundlich adsorption isotherms. The simplicity of these functions has led to their extensive use (and abuse) in describing processes of metal adsorption onto cell surfaces and particulates (O'Connor and Mueller, 1980; Stumm and Morgan, 1981). Although both relationships fit the data reasonably well, the theoretical basis for these models cannot be extended to interpret binding of metals onto to the surfaces of phytoplankton at present time. Knowledge on the nature and spacing of surface sites and the influence of bound metal on the local charge distribution is required for a more rigorous, theoretical analysis.

Consequently, only qualitative statements are made for comparative purposes.

The Langmuir isotherm can be represented by the following equation for a reversible equilibrium,

$$Z_c = (Z_m Z_f) / (1/K + Z_f) \quad (1)$$

where Z_c is the concentration of metal bound to the surface (moles/μm^2) with a maximum binding capacity, Z_m, and the conditional stability constant, K, for the surface ligand-metal complex (M units). Z_f is the free metal ion concentration (M) obtained from the computer program REDEQL 2 (McDuff and Morel, 1973). Estimates for Z_m and K were obtained from a least squares analysis of Z_f/Z_c versus Z_f with a slope of $1/Z_m$ and intercept = $1/(K Z_m)$. All regressions were significant at the $p = 0.01$ with 5 degrees of freedom.

Calculated stability constants for the surface ligand-zinc complex increased on average of one log unit from pH 6.2 to 8.2 for all 7 species of phytoplankton examined (Table XIII). Maximum surface binding capacities (Z_m) decreased with increasing pH contrary to previous beliefs on the competition of hydrogen ion and metals for available surface sites. Even though intraspecific and interspecific differences in M_m were observed, the calculated stability constants for the surface site-zinc complexes were similar. This result indicates that the nature of zinc binding between and among genera is similar and dependent upon the relative concentration of surface ligands (Table XIII). Binding capacities for both species of

Table XIII Constants for Zinc Adsorption Obtained from the Langmuir Isotherm and Scatchard Plots of the Data

Species	LANGMUIR		SCATCHARD PLOT					
	Z_m	logK	$n_o = (n_1 + n_2)$			$\log K_o = (\log K_1 + \log K_2)/2$		
pH 6.2								
Chlorella UT	.53	5.0	.68	.14	.54	6.0	5.3	6.7
Coelastrum HH	.03	5.5	.016	.007	.009	5.6	5.4	5.8
Coelastrum UT	.37	5.5	.25	.09	.16	5.7	5.3	6.2
Scenedesmus HH	.39	5.6	.28	.11	.17	5.6	5.4	6.0
Scenedesmus UT	.19	5.6	.28	.06	.22	6.3	5.5	7.2
Chlamydomonas W	.09	6.1	.21	.08	.14	6.1	5.8	6.4
Chlamydomonas MB	.30	5.1	.087	.036	.051	5.4	5.0	5.9
Chlamydomonas BBM	.096	5.4	.050	.022	.028	5.5	5.3	5.7
pH 7.2								
Chlorella UT	.076	6.0	.26	.06	.20	6.4	5.8	7.1
Coelastrum HH	.005	6.3	.020	.006	.014	6.3	5.9	6.8
Coelastrum UT	.047	6.1	.16	.04	.12	6.4	5.9	6.9
Scenedesmus HH	.11	5.8	.16	.07	.09	6.0	5.8	6.3
Scenedesmus UT	.12	5.8	.17	.06	.11	6.1	5.6	6.6
Chlamydomonas W	.033	6.1	.21	.03	.18	6.6	5.9	7.4
Chlamydomonas MB	.10	5.7	.12	.05	.07	5.8	5.5	6.2
Chlamydomonas BBM	.097	5.5	.076	.029	.049	5.9	5.4	6.5
pH 8.2								
Chlorella UT	.045	6.4	.39	.09	.31	6.8	6.2	7.4
Coelastrum HH	.003	6.6	.020	.005	.015	6.1	5.4	6.9
Coelastrum UT	.021	6.6	.19	.08	.12	6.7	6.6	6.9
Scenedesmus HH	.053	6.1	.24	.05	.19	6.6	5.8	7.4
Scenedesmus UT	.060	6.2	.23	.07	.16	6.5	6.0	7.0
Chlamydomonas W	.017	6.6	.25	.05	.20	7.0	6.5	7.5
Chlamydomonas MB	.054	6.1	.16	.06	.10	6.2	6.0	6.5
Chlamydomonas BBM	.070	5.9	.12	.05	.07	6.1	5.8	6.5

Maximum binding capacities (Z_m) and the number of binding sites (n_1 and n_2 for each linear segment of the curve and the respective total, n_o) are in units of 10^{-16} moles/μm^2. Stability constants for the surface site-zinc complex (K, $K_{0,1,2}$) are in units of M. Species notation: UT, University of Texas; HH, Hamilton Harbour; W, wall-less mutant; MB, assay medium; BBM, standard growth medium.

Scenedesmus were similar, but the Z_m for the laboratory strain of Coelastrum was an order of magnitude greater than for the species isolated from Hamilton Harbour. This confirms results obtained in preliminary long term experiments and metal ion exclusion as a likely tolerance mechanism.

Nothing can be concluded about the spacing of the surface binding sites or the influence of bound metal on surface charge. However, if one assumes that only unhydrated zinc binds to the surface, the theoretical amount of zinc required to form a monolayer is 1.5×10^{-18} moles/μm^2 (calculated using a crystalline ionic radius of 0.6 nm, Stumm and Morgan, 1981). Maximum zinc binding capacities obtained for the surfaces of different species of phytoplankton ranged from 0.3 to 50×10^{-18} moles/μm^2 (Table XIII). When the thickness of the cell wall is considered (100-300 nm) the possibility of establishing a monlayer resulting in a condition favoring charge reversal is remote. Also, an unrealistically high available zinc concentration in solution would be required to fully saturate the surface (> 100 µM for 99.99% saturation). These results indicate, however, that the number of sites involved in metal binding is in reasonable accord with the heterogeneous composition of the cell wall and membrane.

The maximum zinc binding capacities for the wall-less mutant of Chlamydomonas (reduced wall not completely absent) were roughly 30 to 50% of Z_m estimates for the normal strain (Table XIII). Clearly the majority of metal binding sites are associated with the cell wall. However, values for Z_m were unrelated to surface area or surface area

to volume ratios. The differences in the binding capacities, in turn, may be attributable to not only the composition but the thickness of the cell wall, or the physical limits to the boundary zone surrounding and influenced by the cell.

Various forms of the Freundlich isotherm have been successfully incorporated into partition models describing the distribution of metals between a particulate phase and in solution (O'Connor and Mueller, 1980; Stumm and Morgan, 1981). The relationship for this isotherm is given by,

$$Z_c = K_f Z_f^m \qquad (2)$$

where Z_c is the metal bound to the cells with a distribution coefficient equal to K_f. Z_f is the calculated free zinc ion concentration and m is an empirical constant less than unity. Linear regression analysis following a log transformation of Equation 2 yields a straight line with a slope = m and intercept = K_f. Significant correlation coefficients at the p = 0.001 level were obtained using all the data from pH 6.2 to 8.2 (15 degrees of freedom). Intraspecific and interspecific differences in the relationships between K_f and m were consistent to those found for the Langmuir isotherm (compare Tables XIII and XIV). A log transformation of the data reduces minor differences between observations producing highly correlated results.

The predictive capabilities of both isotherms in describing zinc binding to the surface of Chlamydomonas at pH 6.2 to 8.2 are

Table XIV Constants for Zinc Adsorption from the Freundlich Isotherm

Species	m	pK_f	Corr.Coeff.(r)
Chlorella UT	.67	13.22	0.876
Coelastrum HH	.70	14.22	0.974
Coelastrum UT	.71	13.21	0.902
Scenedesmus HH	.79	12.59	0.947
Scenedesmus UT	.69	13.28	0.992
Chlamydomonas W	.57	14.27	0.892
Chlamydomonas MB	.73	13.11	0.992
Chlamydomonas BBM	.76	13.10	0.983

The Freundlich isotherm is represented by $Z_c = K_f Z_f^m$ and a plot of log Z_c (moles/μm^2) versus log Z_f (M) yields a straight line with a slope=m and the intercept = K_f. K_f ($L/\mu m^2$) is the distribution coefficient and m is an empirical constant. Correlation coefficients for all regressions were significant at the 0.001 level or better (15 DOF). All data from pH 6.2, 7.2, and 8.2 were combined. Other notation as in Table XIII.

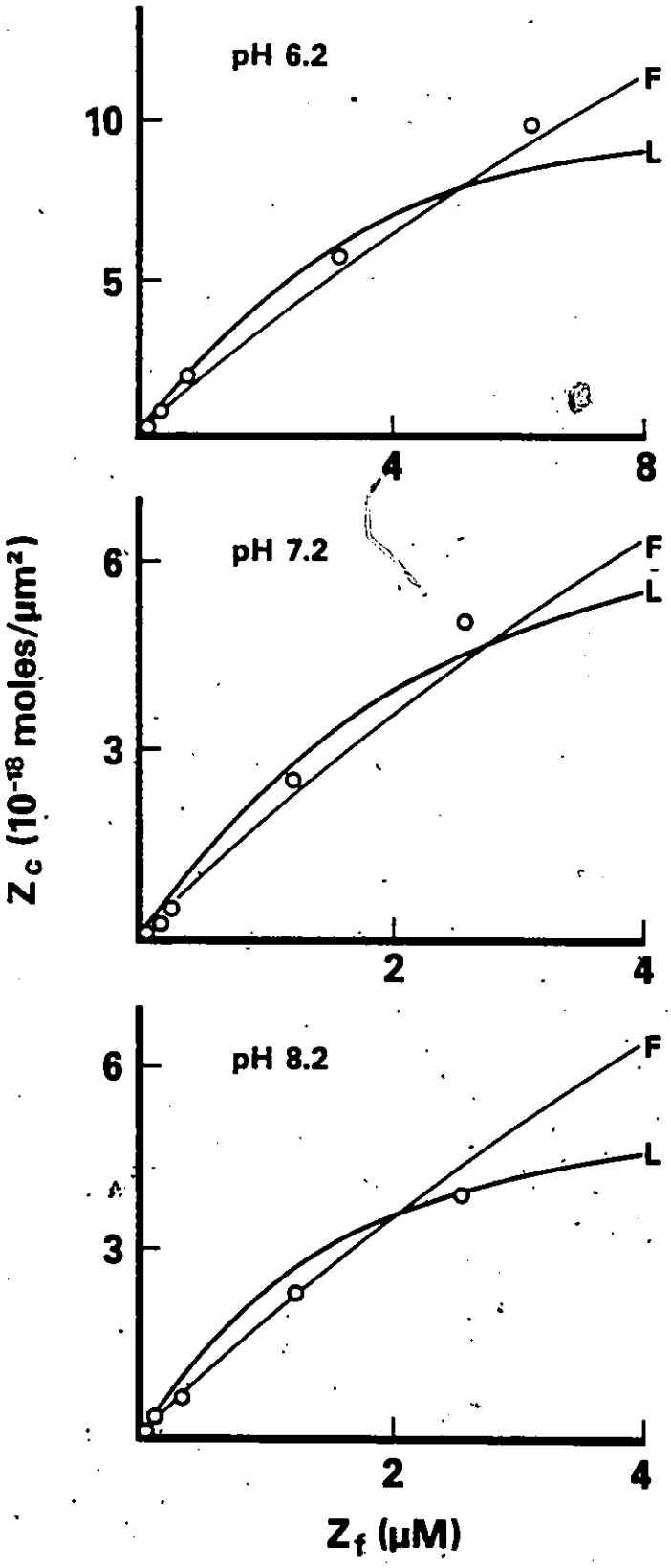
clearly illustrated in Figure IX. Although both functions are statistically significant they do not adequately describe the results. Physiological control over the chemical environment of the cell wall/membrane region may violate the assumptions on which these relationships are based. These functions must be qualified in context to their application and at present are only descriptive analogs of the observations.

Scatchard plots of the data (Z_c/Z_f versus Z_c) were constructed to further demonstrate that metal binding to the cell surface cannot be adequately described by simple adsorption isotherms. These plots were distinctly curved which suggests; 1. the presence of multiple independent binding sites, 2. active uptake mechanism(s) operating, 3. unknown equilibrium shift affecting the free zinc ion concentration not included in the equilibrium calculations or that a true thermodynamic equilibrium was not attained. The two linear components of the plots were analyzed separately and the averaged results (conditional stability constant, K_0, and binding capacity, n_0) were consistent with those obtained from the Langmuir isotherm. Each linear segment has a slope equal to the negative value of the stability constant for the surface site/zinc complex ($-K_1$ and $-K_2$ for the respective segments) with an intercept on the Z_e axis equal to the total number of binding sites (n_1 and n_2, also equal to the maximum surface binding capacity for each site). Although only two components (linear portions of the curve) could be resolved due to the lack of data, one component (denoted as n_2) was more sensitive to pH and characterized by an overall lower stability constant (Table XIII).

Fig. IX. Langmuir and Freundlich Adsorption Isotherms for Zinc Binding to Chlamydomonas at pH 6.2, 7.2, and 8.2

Data from the zinc uptake experiments ("o") with Chlamydomonas are plotted, along with the curves generated from the Langmuir (L) and Freundlich (F) isotherms. Although both functions are statistically significant, they do not adequately describe the results.

Zinc bound (Z_c) is in units of 10^{-18} moles/um^2, plotted against the calculated free zinc ion concentration in solution (Z_f in μM). All experiments were conducted in MB at 20°C under 100-110 $\mu Em^{-2}s^{-1}$ ph.a.r. for 200-230 min. Curves for each isotherm were generated using the coefficients listed in Tables XIII and XIV.


pH 6.2
F
L
10
5
4
8
pH 7.2
F
L
6
3
2
4
pH 8.2
F
L
6
3
2
4
Z_c (10^{-18} moles/μm²)
Z_f (μM)

Zinc binding involves a variety of ligands which cannot be completely characterized by conventional means due to the similarity of stability constants. The variation observed in all binding constants can be explained by the relative available concentration of each ligand type on the cell surface.

Zinc binding to the surface of Chlamydomonas was composed of a passive and a light/temperature sensitive component (Fig. X and Table XV). The binding of zinc under all conditions, however, followed hyperbolic saturation kinetics and the light/temperature sensitive component was not likely an active transport mechanism (compare Fig. X and XIa,b with the maximum binding capacities, Z_m, in Table XIII). The pH, light, and temperature dependence of photosynthesis closely paralleled zinc uptake under the same conditions. Fluctuations in the H^+ concentration from carbonic anhydrase activity ($HCO_3^- \leftrightarrows CO_2 + OH^-$) could be responsible for the enhanced uptake (e.g. indirect modification of surface reactivity). Uptake of bicarbonate and excretion of OH^- in the light would reduce the H^+ concentration in the area immediately adjacent to the cell. Reducing the competition of H^+ for available ligands may, in turn, promote the dissociation of more basic functional groups (sulhydryl, amino, and phenolic hydroxyl groups).

Zinc adsorption was also independent of cell density when the total metal bound was significantly less than the calculated free metal ion concentration available in solution (compare the Cell Range and Uptake columns in Table XV). Under all conditions, the amount of zinc bound to the cells did not exceed 1% of the available zinc

Fig. X. Influence of Temperature and Light on Zinc Uptake over Time.

Equilibration between zinc in solution and bound to the cell surface is complete after 60 to 100 min under both sub-optimal (dark uptake at 20°C, "■", and light uptake at 7°C, "○") and optimal conditions (light uptake at 20°C, "●"). Zinc bound under sub-optimal conditions accounts for only 25-40% of the binding capacity of the cell surface under optimal conditions. A general increase in the level of cell metabolism (in turn fluxes across the membrane) indirectly modifies surface reactivity and accounts for the enhanced uptake under optimum conditions of light and temperature.

Data for Chlamydomonas in MB (pH 7.2) with a total zinc concentration of 0.86 µM. All cultures were incubated at the predetermined conditions of light (dark or under 100-110 $\mu Em^{-2}s^{-1}$ ph.a.r.) and temperature (20°C or 7°C) for 1 h prior to metal additions. Sample standard deviation did not exceed ± 12% and all experiments were carried out in duplicate. The curves are only approximations of the hyberbolic relationship between uptake and time.

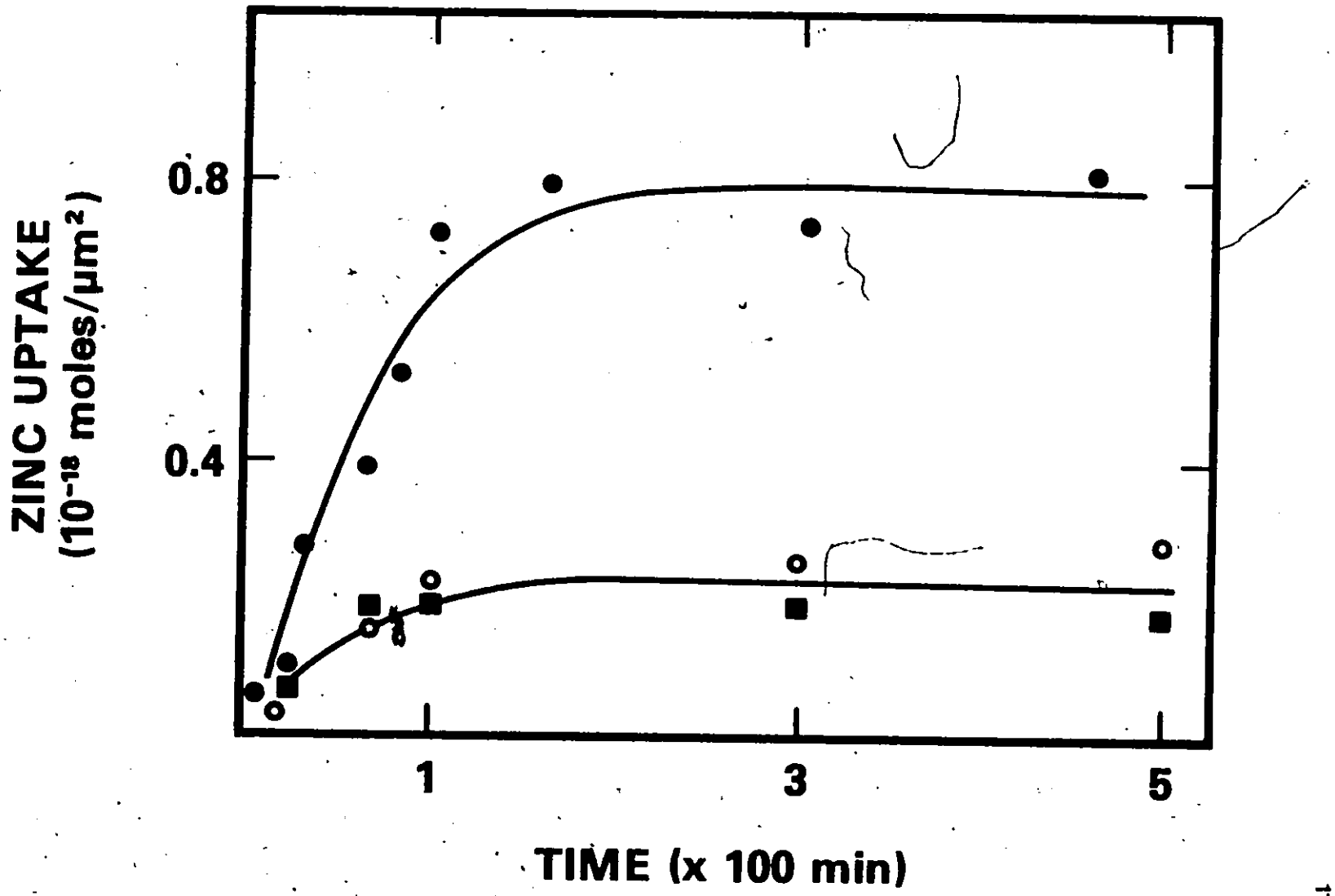

ZINC UPTAKE
(10⁻¹⁸ moles/μm²)
0.8
0.4
1
3
5
TIME (x 100 min)

Table XV The Influence of Cell Density, Temperature, and Light on Zinc Uptake

CELL DENSITY[1]

pH	Cell Range ($\times 10^4$ cells/ml)	Uptake (10^{-18} moles/μm^2) Mean	± SD	n
6.2	1.2 - 12.3	1.7	0.4	14
7.2	1.3 - 12.3	0.84	0.13	9
8.2	1.1 - 10.7	0.74	0.16	15

LIGHT AND TEMPERATURE[2]

Temp. (°C)	Light ($\mu E/m^2s$)	Uptake (10^{-18} moles/μm^2) Mean	± SD
20	0	0.18	0.03
	10-20	0.36	0.06
	50-60	0.65	0.06
	100-110	0.74	0.16
	200-230	1.13	0.20
5	0	0.10	0.03
	100-110	0.11	0.03

1. Cultures incubated under a light intensity of 100-110 $\mu Em^{-2}s^{-1}$ for 200-240 min with constant agitation provided by a rotary shaker. Total zinc concentration in all cultures was 0.858 µM. Results are compiled from control flasks in "n" experiments carried out over a four month period with 2 or more control flasks sampled in triplicate for each experiment.

2. Cultures incubated with 0.858 µM total zinc (pH 8.2) for 230 min.

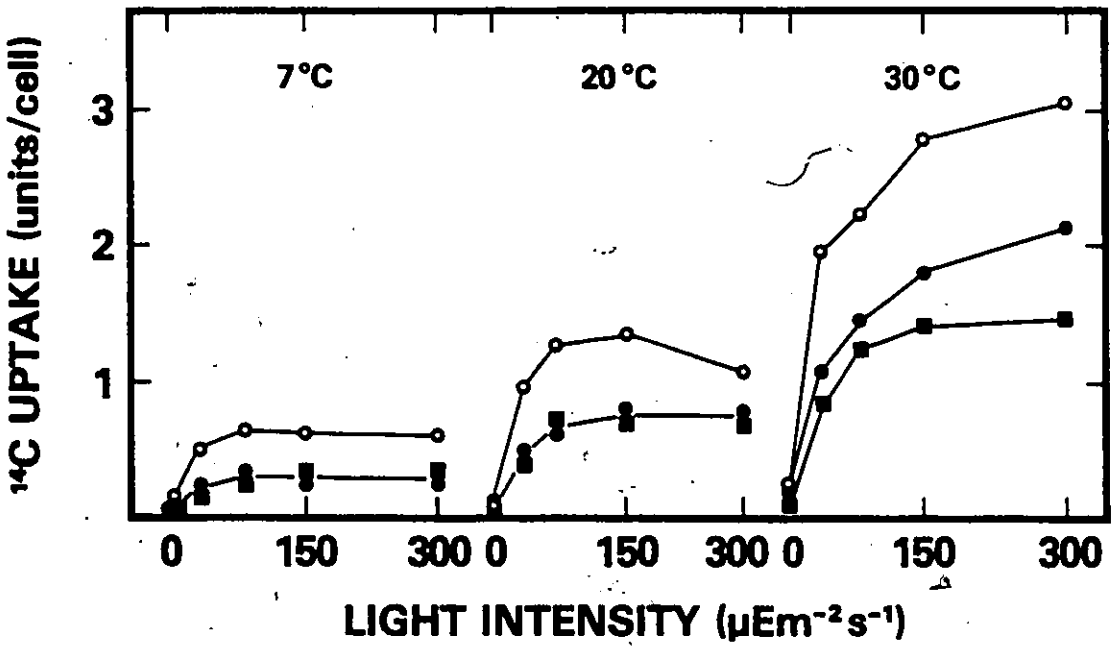
Fig. XIa. Effects of Light, Temperature, and pH on Photosynthesis (Incorporation of ^{14}C) in Chlamydomonas

The relationship between light, temperature, and pH on photosynthesis in Chlamydomonas. Rates of carbon assimilation increase with decreasing pH and increasing temperature. The pH dependence of photosynthesis is due to this species temporary inability to utilize bicarbonate (pH of growth BBM slightly acidic). Zinc binding to the surface of Chlamydomonas closely parallels changes in photosynthetic activity (compare pH dependence of the maximum binding capacities in Table XIII and the light/temperature sensitivity illustrated in Figure X). All assays were carried out in BBM within the following range of temperatures: 7°C = 5-10°C, 20°C = 19-21°C, 30°C = 28-30°C. Notation is as follows: "○" = pH 6.2; "●" = pH 7.2; "■" = pH 8.2; rates of carbon assimilation are in units of CPM/(1000 cells). Sample standard deviations were < ± 20%.

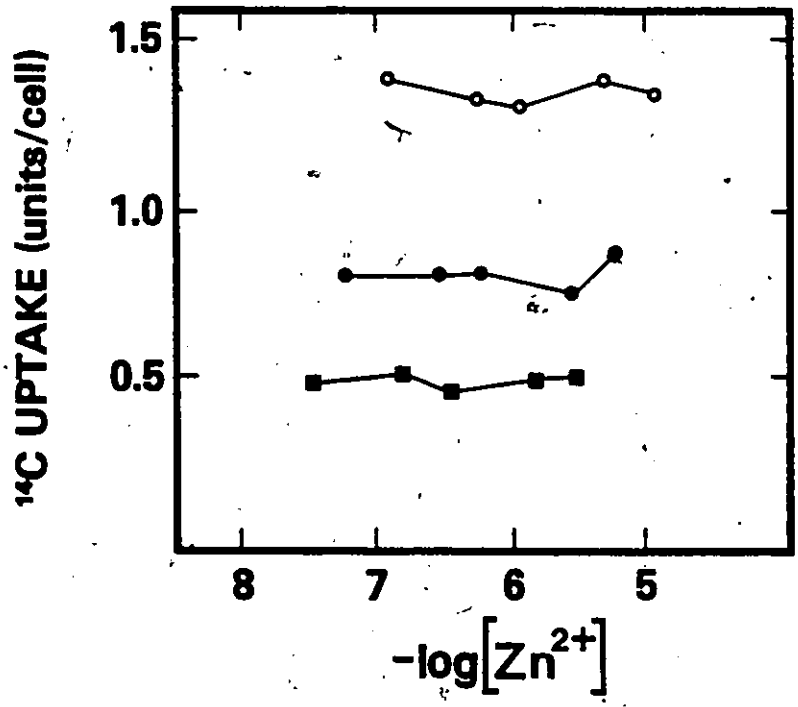
Fig. XIb. Effects of Zinc and pH on Photosynthesis (Incorporation of ^{14}C) in Chlamydomonas

Calculated free zinc ion concentrations (plotted as the negative logarithm) of less than 10 μM did not influence rates of carbon assimilation. Notation as in Figure XIa and all determinations were carried out at 20°C in BBM under 130-150 $\mu Em^{-2}s^{-1}$ ph.a.r.

a


7°C
20°C
30°C
^{14}C UPTAKE (units/cell)
3
2
1
0
150
300
LIGHT INTENSITY (μEm^{-2}s^{-1})

b


^{14}C UPTAKE (units/cell)
1.5
1.0
0.5
8
7
6
5
-log[Zn^{2+}]

concentration in solution. Higher cell densities, or lower metal concentrations, could significantly influence the cell burden (available metal per cell) and produce variable results (Davies, 1978). A constant available metal concentration, or cell burden, is essential to obtain reliable and accurate results in binding studies.

The influence of other transition metals on zinc binding follows the Irving-Williams series, based on the stability of individual metal-ligand complexes. The total zinc added to all flasks was 0.858 uM and the concentration of each metal was varied between 0.1 and 10.0 uM. This concentration of zinc lies within the steepest portion of the adsorption isotherm and was selected to optimize detection of the effects of other metals on zinc binding. The order based on the reduction in zinc uptake at pH 6.2 was; Cu > Ni > Co > Mn > Cd > Ca, Mg, Sr (Table XVI). This order was also consistent with the relative ion exchange constants calculated for each metal (Table XVI). Ion exchange constants were calculated from extrapolated values for the reduction in zinc bound in the presence of calculated, equivalent free ion concentrations. For example, 18 % of the zinc bound at pH 6.2 was displaced by nickel at equivalent, calculated free ion concentrations. At pH 8.2 only 1 % of the zinc was displaced by nickel. Increasing IEC reflect the ability of individual metals to displace zinc. The effects of all metals on zinc binding were reduced under more alkaline conditions due to an overall reduction in the calculated, free metal ion components. Since the quantity of zinc bound decreased with increasing pH, the degree of displacement of zinc by other metals will also decrease and, possibly, be obscured by

Table XVIa Reduction in Zinc Uptake by Other Cations and Ion Exchange Constants (IEC)

	Reduction in Zinc Uptake (% of the Controls) Concentration of Added Cation (μM)					IEC pH	
Cation	0.1	0.5	2.0	5.0	10.0	6.2	8.2
Cu	83	[illegible]	48	36 (72)	–	0.67	>1.0
Ni	83	84	62	63 (81)	–	0.18	0.01
Cd	101	101	87	81 (87)	–	0.0	0.0
Co	93	94	67	65 (86)	59	0.08	0.0
Mn	107	104	86	72 (86)	–	0.06	0.06
Ca	94	102	92	88 –	55	0.0	0.0
Sr	94	99	96	73 (121)	49	0.01	0.01
Mg	111	112	91	82 (86)	86	0.0	0.01
Be	–	–	–	99 (77)	–	0.0	0.0
Fe	95	637	–	– –	722	–	–
Pb	125	114	236	340 (137)	–	–	–
EDTA	103	57	34	29 (60)	25	0.45	–

Table XVIb Effects of Additions of Ca/Mg and Sr/Co in Various Combinations on Zinc Uptake

Mg^{2+} (μM)	Ca^{2+} (μM) .01	1.0	10.0	Sr^{2+} (μM)	Co^{2+} (μM) .01	1.0	10.0
.01	96	86	55	.01	90	90	59
1.0	87	83	67	1.0	91	70	58
10.0	86	84	52	10.0	49	49	47

All values except the IEC are expressed as a percent of the controls. Ion exchange constants were calculated from extrapolated values for the reduction in zinc bound in the presence of equivalent free ion concentrations, obtained from REDEQL 2. The total zinc concentration was 0.858 μM in MB medium under the standard assay conditions (bracketed values obtained at pH 8.2 in BBM - $MgSO_4$). Sample standard deviation for all results < ± 10%

experimental error.

Elevated levels of each metal and EDTA (concentration 500 x greater than the total zinc concentration) however, did not completely inhibit zinc binding. This provides additional evidence for the existence of multiple classes of binding sites on the cell surface. The possibility that a true equilibrium was not attained after the one hour preincubation period (minus cells) or that computed free metal concentrations were inaccurate (unknown complexes or erroneous stability constants) cannot be unequivocally disputed. Exchange reactions involving certain surface and solution ligands must also be considered along with the binding of complex and chelated ions.

Magnesium has little effect on zinc binding but 10 uM calcium reduced uptake by 50 %. The effects of Sr and Co were not additive and both metals reduced zinc binding by 50 % when added separately (Table XVI).

III.I.III. Factorial Experiments

Results from the factorial experiments are presented in Table XXVI of the Appendix. Factorial experiments using Chlamydomonas were carried out under the following conditions: 1. pH 6.2, 7.2, and 8.2; 2. light and dark (pH 7.2); 3. change in speciation (THAM buffer substituted for the phosphate system at pH 8.2); 4. time (standard assay time of 200 min versus an extended incubation of 600 min at pH 7.2). An additional factorial experiment was conducted with unlabelled zinc to examine the effects of all metals on photosynthetic activity

(^{14}C incorporation at pH 7.2). Fluorescence was measured at the termination of each experiment to provide additional information on the physiological condition of the cells. Factorial experiments were also carried out at pH 7.2 using laboratory strains of Chlorella, Coelastrum, and Scenedesmus along with the Hamilton Harbour isolate of Scenedesmus.

Effects, calculated using Yates algorithm (Section II.I.IV.), of each metal and combinations of metals on zinc binding are presented first, followed by the effects on fluorescence and photosynthesis. Regression analysis of the factorial data is presented in the next section. An "effect" is a measure of the average response between the high and low settings of the one variable ("+" or "-" levels). "Interaction" refers to the effect of more than one variable. The nature of interaction between variables (additive or otherwise) determines the overall magnitude and sign of each effect relative to the average response for all variables and not the controls (refer to Section II.I.IV.). A "variable" refers to the high setting ("+") of each metal present within the flask during the experiment, coded as follows; 1 = Cu, 2 = Ca/Mg, 3 = Sr/Co, 4 = Ni, 5 = Cd, 6 = Mn. Interactions are represented by the numerical code for each metal(s). (For example, the interaction between Cu ("1") and Ca/Mg ("2") is represented as "12".) All data is presented in Table XXVI and the calculated effects using Yates algorithm in Table XXVII of the Appendix. Results from Yates algorithm have been ordered relative to the magnitude of each effect (greatest effect at top) and coded according to variable interactions (Table XVII and Table XXVIII for

$^{65}Zn/^{14}C$ and fluorescence results respectively). Ordering the coded effects (numerical representations) in this fashion eliminates the need to normalize the results relative to the controls (zinc only) and is easier to interpret.

The calculated average effect for all treatments within each experiment were significantly less than the controls, confirming that all treatments resulted in a significant or measurable reduction in zinc uptake (refer to the controls, Table VIII in Section II.I.IV.). Extending the incubation period from 200 to 600 min (pH 7.2) reduced the average effect of all metals on the binding of zinc but did not increase the extent of zinc adsorption in the control flasks. This result indicates that zinc bound and the available metal in solution equilibrated within the standard assay time of 200 min. The decrease in the average response during the extended incubation period must result from either changes in the solution speciation or enhanced uptake of zinc as an indirect result of the effects of other metals on the cells. Alterations in membrane permeability, for example, could enhance the binding and uptake of other metals.

The average effects of all metals on photosynthesis were not significantly different from the controls (1.16 compared to 1.01 counts per cell, arbitrary units). This result was expected because the concetrations of each metal, apart form copper, did not reduce rates of photosynthesis in previous experiments (Section III.I.I.). Substitution of THAM for the phosphate buffer increased the extent of zinc adsorption due to an increase in the available zinc concentration in solution (absence of the phosphate ligand), or possibly binding of

complexed zinc. Under these conditions, the average effect was greater than the controls.

Due to the amount of data, only the significant main effects and interactions are considered. Bracketed interactions (Table XVII, XXVIII) are not significantly different from the average response for all variable settings (or each other) based on estimates of the standard error associated with the effects. Normal plots of the effects can also be used to resolve significant interactions. Random variation about a fixed mean (normally distributed) would yield a straight line. The effects were analyzed in this fashion and compared to results obtained using random data sets. Estimates of the standard errors for the effects, based on replicated experiments and controls (9 control flasks were run in each experiment), however, provided a more accurate criterion for identifying significant effects. The results were confirmed (significant effects not explained as chance occurences) using a reverse Yates algorithm. Effects less than the standard error were set equal to zero and estimates for each repsonse obtained by reversing Yates algorithim. This analysis is restricted by the number of effects set = 0 and becomes less precise as the number of values eliminated approaches the total number of runs. Normal plots of the residuals (estimate - observation) confirmed that the effects of the interactions bracketed in Tables XVII and XXVIII are not significantly different from each other or the experimental error associated with the average response.

The effects of individual metals were not additive which confirms the presence of multiple binding sites on the cell surface

Table XVII. Factorial Data - Ordered Effects on Zinc Uptake And Photosynthesis Data

			Chlamydomonas						Chlorella UT	Coelastrum UT	Scenedesmus III	Scenedesmus UT
RUN	PH6L	PH6L	PH7L	PH7D	PH7C	PH7t	PH8L	PH8TH	PH7L	PH7L	PH7L	PH7L
1	2	2	6	6	23	1	2	25	6	5	5	5
2	3	3	1	5	1	4	1	2	5	6	1	6
3	4	4	5	3	5	12	3	24	1	4	4	4
4	6	6	2346	2345	2346	256	456	356	4	1	456	3
5	12	156	1345	2346	26	2456	5	245	3	3	3	1
6	134	124	4	25	12345	1346	56	13456	2	135	2	456
7	145	135	1235	345	4	23	26	1235	234	1236	135	345
8	16	145	13	123	124	123456	46	134	12345	345	156	13456
9	124	13	123456	1	16	15	4	36	156	2	245	123
10	345	16	156	4	25	6	35	16	145	1234	123	156
11	1236	456	1234	235	234	135	16	23456	23456	136	145	135
12	1256	235	2345	234	1345	126	136	2346	12356	456	356	125
13	46	12	12456	134	123456	12356	125	12	124	256	345	12456
14	2456	1246	146	135	1235	1456	135	145	346	246	1256	2
15	135	1235	12	23	13	2345	123	234	356	245	12345	126
16	3456	12345	1256	12456	1256	1245	6	156	12346	13456	246	356
17	235	1236	23	2	12	124	12345	345	134	1246	1346	346
18	12345	134	3	156	2345	1235	12356	123	135	123456	235	145
19	2346	356	134	1256	1356	145	13456	12456	136	134	23456	245
20	1356	12456	16	456	6	136	245	136	12456	234	1246	134
21	2356	13456	235	35	12456	5	2346	1346	125	12456	346	146
22	56	23456	1236	1345	56	1345	124	1246	256	124	1234	124
23	245	12356	126	346	1234	345	1245	12356	245	346	1236	234
24	156	345	25	245	1246	16	134	246	146	1235	16	12345
25	236	234	1246	12	126	125	236	135	235	156	234	136
26	356	246	34	126	12356	12346	1356	456	345	356	1245	235
27	234	146	245	12345	146	1236	156	34	123	23456	26	256
28	12346	1346	123	123456	12346	1356	235	2345	126	2346	1235	12346
29	1235	46	12356	1236	1256	2356	1256	1456	13456	2356	6	12356
30	13456	256	2356	2356	235	56	12346	1345	3456	1456	124	246
31	1245	245	1346	356	135	356	145	125	1245	126	1456	236
32	256	346	1456	34	246	1234	13	346	246	236	25	2456
33	346	2345	136	24	136	13	356	1236	2356	1256	134	1245
34	146	2456	1245	16	1245	234	346	15	1356	23	12356	1256
35	1246	236	135	1246	134	1246	234	1	1235	235	256	23456
36	456	25	23456	1234	13456	246	1246	146	1236	2345	12	1235
37	123456	45	346	1356	1456	12456	146	124	23	16	126	1345
38	136	2346	24	26	24	2346	1456	1245	1346	12346	123456	123456
39	12356	12346	1356	23456	125	156	2345	26	236	12	136	2346
40	12456	3456	356	124	156	236	1346	14	1456	14	46	1346
41	1346	125	3456	14	34	346	14	1356	456	128	13456	2356
42	2345	1245	456	13456	356	3456	25	12345	2345	2456	146	1246
43	1456	56	12346	46	346	146	3456	1256	24	26	2346	26
44	45	126	12345	145	145	13456	12456	2456	1246	145	2356	3456
45	123	123	35	146	15	25	45	3456	26	1245	12346	1236
46	36	1345	125	1235	2356	26	23456	1234	1256	1356	23	1456
47	1	1234	234	1346	14	12345	24	126	2346	13	1345	2345
48	23456	123456	2456	2456	236	46	1236	46	2456	146	12456	25
49	125	35	46	1456	2456	24	34	35	12	1346	36	1356
50	13	34	14	125	3456	456	1235	256	36	12356	2345	1234
51	246	1456	256	246	45	36	123456	23	1245	1345	125	12
52	26	136	236	15	46	235	2456	123456	1234	123	1356	16
53	5	1256	15	12356	345	45	12	12346	45	35	3456	15
54	126	23	345	1245	456	1256	256	13	13	3456	24	23
55	25	26	145	136	123	23456	2356	3	16	36	13	24
56	24	2356	26	3456	245	245	1345	2356	46	25	2456	36
57	34	1356	124	56	23456	123	126	235	35	12345	236	45
58	1345	36	13456	256	256	34	15	236	25	15	35	13
59	1234	1	246	36	1346	134	345	56	123456	24	56	35
60	35	24	45	236	35	35	1234	4	34	34	15	46
61	23	5	2	13	36	3	246	45	56	46	14	14
62	14	14	56	12346	3	14	23	6	14	45	34	34
63	15	15	36	45	2	2	36	5	15	56	45	56
64	avg	avg	avg	avg	avg	avg	avg	avg	avg	avg	avg	avg

Effects ordered relative to the degree of inhibition (variables at top of table had the greatest effect) of zinc uptake, or rate of carbon assimilation, compared to the overall average effect (average of all values). Bracketed effects in each column are not significantly different from the average effect (less than the standard error of an effect). The numerical codes for each effect represent the high setting for the respective variables (metals) present in each run ("+"/"-" correspond to high (5.0 uM) and low (omitted) settings for the variables in each run). Run number "64" for example, all metals are added to the flask (variable settings at "+" level), and the respective numerical code for the effect is "123456". The effect is also a measure of the degree of interaction between variables. All experiments were carried out at 20°C in MB with constant agitation provided by a rotary shaker (refer to Tables V and VIII for incubation conditions and initial conditions for each factorial experiment). Notation for column headings is as follows: PH6, PH7, PH8 refer to experiments carried out at pH 6.2, pH 7.2, and pH 8.2 (± 0.05 units) respectively; L, light intensity of 100-110 uEm⁻² s⁻¹ ph.a.r.; D, dark experiment; t, incubation period of 600 min (3 times the standard assay period); C, photosynthesis experiment with stable zinc; TH, 0.006 M THAM buffer system substituted for phosphate (pH 8.2); HH, Hamilton Harbour species; UT, University of Texas species.

with different affinities for each metal. If all metals interacted additively, then the effects would be ordered relative to the total metal concentration present within each test flask. Information on the indirect effects between other metals on zinc uptake (competition for the same site) can be obtained from the results with the assumption that there exists a finite number of independent binding sites on the cell surface. This is explained in detail in the discussion and only significant results will be pointed out here.

The main effects associated with each variable changed with pH. The main effects of copper and cadmium increased from pH 6.2 to 8.2 while the main effects for the remaining metals were reduced under more alkaline conditions (Table XVII). Shifts in solution equilibria and the available metal ion concentrations are primarily responsible for these results, particularly in the case for Mn but not for Cu. Metals such as Sr/Co, Ca/Mg, Mn, and Ni, however, appear to displace the labile zinc from the cell surface and do not directly compete for specific zinc binding sites (non-exchangeable fraction). This competitive interaction accounts for the relative increase in the main effect of Cu with increasing pH even though the calculated cupric ion concentration decreased from 23 to < 0.1% of the total metal added from pH 6.2 to 8.2, respectively. The calculated free cadmium concentration did not appreciably change with pH which explains the increase in the main effect of this metal under more alkaline conditions. However, since the quantity of zinc bound decreases with increasing pH, the degree of displacement of zinc will also decrease and, possibly, be obscured by experimental error.

Since each effect is a measure of the average response under all conditions, the low main effect of Cu at pH 6.2 indicates that the influences of this metal on zinc binding is reduced in the presence of other metals. Copper in combination with Ni or Cd, however, reduced the observed individual effects of these metals on zinc binding (e.g. increased the quantity of zinc bound, Table XVII). The effects of Cu were reduced in the dark but increased during the extended incubation period. The influences of changes in solution speciation are clearly illustrated in the factorial experiments at pH 8.2 with the phosphate and THAM buffer systems. THAM complexes with a number of metals, as evidenced by changes in the ordering of the effects (Table XVII).

The effects of all metals were relatively consistent for all species examined except Chlamydomonas and Scenedesmus HH in which Mn exhibited a significantly lower effect. Zinc uptake in Chlamydomonas was also less influenced by Ca/Mg and Sr/Co. Irrespective of these differences, the results indicate that reactions at algal surfaces, between species and among genera, must involve similar processes and binding sites. Changes in the observed binding characteristics, or interactions between different metals, may arise from the presence or absence of various classes of surface ligands. Additonal studies are required to fully classify and quantitate surface binding sites, especially between tolerant and non-tolerant species.

The effects of other metals on zinc binding were different from the effects on photosynthesis and fluorescence. Fluorescence results were less variable than for ^{14}C incorporation and zinc binding, but the calculated effects were not as significant. The transient nature

of the fluorescence response in phytoplankton populations exposed to heavy metals may account for these results (refer to Section II.I.I.). Copper had the greatest overall effect on fluorescence under all conditions (Table XXVIII). The effects of Cd decreased with increasing pH and were substantially reduced in the presence of copper.

III.I.IV. Regression Analysis of Factorial Data

Main effects and interactions in factorial experiments can also be calculated using a least squares analysis (Box et al., 1978). Least squares estimates must be multiplied by a factor of 2 in order to obtain main effects since the input variable changes by two units (-1 to +1) whereas the slope (coefficient) measures the change in a response by only one unit (0 to +1). This analysis was carried out for the following reasons: 1. to verify the results obtained from Yates algorithm; 2. to compensate for differences in the calculated free metal ion concentrations (total metal concentrations were 5.0 μM); 3. to obtain relationships between the individual metals, based on the pattern of zinc binding, that can be used to further characterize the nature and reactivity of the cell surface.

A first order additive model and a multiplicative model, linear and nonlinear in parameters respectively, were analyzed in detail. Exponential and reciprocal models, along with various transformations of the response variable, provided similar statistical results and overall predictive capability and, consequently, were not included.

The two models are represented by the following equations:

Additive: $$Y = b_o + b_1M_1 + \ldots + b_nM_n + E \quad (10)$$

Multiplicative: $$Y = a_o M_1^{a_1} M_2^{a_2} \ldots M_n^{a_n} E \quad (11)$$

Transformed: $$\log Y = \log a_o + a_1 \log M_1 \ldots + a_n \log M_n + \log E \quad (12)$$

The additive and transformed multiplicative models are linear in coefficients a_n/b_n respectively. Values for the independent variables ($M_{n=1,2,3\ldots}$) are established by the design matrix and set equal to the total metal added or the computed free metal ion concentration (uM). A value of 10^{-6} µM was assigned to cases in which the metal was excluded ("-" level). The response variable, Y, was the amount of zinc bound to the cells and E was an error term. Data from the repeated factorial (PH6L) and the combined results from pH 6.2, 7.2, and 8.2 were analyzed using each model.

Both models were statistically significant with multiple correlation coefficients of > p = 0.001 and F-ratios (mean sum of squares due to the regression/residual sum of squares, MS_r/S_r^2) exceeding tabulated critical F values at the 0.001 % level in all cases (F(6,121,0.999) = 4.04 and F(8,247,0.999) = 3.55 compare to the observed ratio of 21.66(23.64) and 109.76(207.26) for the replicated PH6L data and the combined results (additive(multiplicative), respectively, Table XX). The high number of degrees of freedom (DOF) heavily influences these results and closer examination reveals the

Table XVIII Analysis of Variance Summary for Multiplicative and Additive Models

ADDITIVE MODEL

Data	Source	DOF	SS	MS	R^2	F-Ratio
PH6-R	Total	127	44.515		0.5178	
	Regression	6	23.016	3.815		21.66
	Residuals	121	21.468	0.1774		
	LOF	57	18.112	0.3178		6.06
	PE	64	3.356	0.0542		
PH6,7,8	Total	255	137.377		0.7804	
	Regression	8	107.213	13.402		109.76
	Residuals	247	30.164	0.1221		
	LOF	183	26.808	0.1465		2.79
	PE	64	3.356	0.0524		

MULTIPLICATIVE MODEL

Data	Source	DOF	SS	MS	R^2	F-Ratio
PH6-R	Total	127	2.9372		0.5400	
	Regression	6	1.5862	0.2644		23.64
	Residuals	121	1.3510	0.0112		
	LOF	57	1.1613	0.0204		6.87
	PE	64	0.1897	0.00296		
PH6,7,8	Total	255	39.8163		0.8704	
	Regression	8	34.6545	4.3318		207.26
	Residuals	247	5.1619	0.0209		
	LOF	183	4.9722	0.0272		9.17
	PE	64	0.1897	0.00296		

Notation is as follows: DOF, degrees of freedom; SS, sum of squares; MS, mean sum of squares; R, multiple correlation coefficient; LOF, lack of fit; PE, pure error; PH6-R = data from replicated factorial at pH 6.2; PH6,7,8 = data from factorials at pH 6.2 (both sets), 7.2, and 8.2; MS for residuals represents the variance associated with the regression ($^{4}S_r^2$).

existence of significant lack of fit (LOF). The residual variance was broken down into two components, LOF and pure error (PE). Estimates of PE were obtained from the replicated factorial at pH 6 and LOF from subtraction. F-ratios (MS_{LOF}/S^2_{PE}) were signifcant at $\sigma = 0.01$ or greater in all situations. Comparing the standard deviation of the residuals (S_r) and the mean response (a_o/b_o) indicates the existence of a considerable amount of unexplained variation (12-168%).

Coefficients for Cd and Cu were not significantly different from zero at the 0.05 level (90%) for all cases. This result implies that the influences of these metals are insignificant (account for only a small fraction of the total variance) which is peculiar since both metals significantly influnced zinc binding. However, this can be explained by the fact that physiological effects (which may be modified by the presence of other metals) are not incorporated into either model which may also account for the unexplained variation.

Residual plots of the data provided a partial explanation for the degree of LOF. Variance in the additive model was greatest at both high and low metal concentrations (plot of residuals versus the independent variables were hourglass shaped). The multiplicative model tended to normalize this observed variance providing a more random distribution of the residuals when plotted against the independent variables. Plots of the residuals against the measured response were sigmoidal and versus pH were slightly curved. Some, but not all of the variation may be attributed to experimental error, particularly for data obtained at pH 8.2 (e.g. the quantity of zinc bound decreased with increasing pH resulting in correspondingly lower effects). These

Table XIX Coefficients for Regression Models

		ADDITIVE MODEL		MULTIPLICATIVE MODEL	
Coeff.	Var.	PH6	PH6,7,8	PH6	PH6,7,8
a_0/b_0	Const.	2.29	-0.207*	0.745	-1.16*
a_1/b_1	Zn	-	5.39	-	-0.794
a_2/b_2	Cu	0.8027*	0.0742*	0.0031*	-0.0051*
a_3/b_3	Ca/Mg	-0.0565	-0.0405	-0.0203	-0.0228
a_4/b_4	Sr/Co	-0.0463	-0.0270	-0.0168	-0.0123
a_5/b_5	Ni	-0.0747	-0.0443	-0.0144	-0.0088
a_6/b_6	Cd	0.0286*	0.0096*	0.0073@	-0.0016*
a_7/b_7	Mn	-0.1080	-0.1110	-0.0095	-0.0109
a_8/b_8	pH	-	-1.02	-	0.688

Notes:

1. The signficance of each variable was determined using a two sided t-test (t-ratio or coefficient/standard deviation). All coefficients except where noted are significantly different from 0 at the 0.0005 level or better.
2. "*" not significantly different from 0 at 0.05 level (90%)
   "@" not significantly different from 0 at 0.005 level (99%)
3. The variables pH and Zn were not included in the regressions using PH6 data (replicated factorial at pH 6.2) since they are constant (the calcualted free zinc concentration and pH constant for any pH).
4. The coefficients a and b for each variable correspond to the additive and multiplicative models, respectively.

results, however, suggest that linear or quadratic terms in pH should be incorporated into the model since no other trends were detected with the other variables.

An identical analysis was carried out using randomly distributed values, with equal probability, between two points (set equal to the minimum and maximum of the results) along with a Poisson distribution using the mean and standard deviation associated with the observations. Residual plots were uniformly distributed against all independent variables. Low and high values were under and over estimated respectively, which was expected. Multiple correlation coefficients were not significant at the $p=0.1$ level and the calculated F ratios were $<< 0.01$.

These results demonstrate the inadequacy of applying simple deterministic models, which can be extended to include models based on physico-chemical properties of individual metals, in describing metal - phytoplankton interactions. The additive and multiplicative models, in their present form, cannot accurately predict the influence of other metals on zinc binding. These results may be attributable to direct or indirect physiological control over the chemical properties of the cell surface, possibly altered in the presence of individual metals or combinations of metals. Consequently, processes regulating the binding of metals to the cell surface cannot be directly compared to similar studies with inert systems.

III.II. Large Volume Enclosures

The objective of this study was to examine the short term effects (days to weeks) of metals on natural populations and obtain information on factors regulating the availability of metals in situ. The greatest short term impact will be in the upper surface waters associated with the region of maximum biological production. The enclosures were designed to simulate conditions within this region.

Weather over the experimental period was characterized by relatively cool and windy conditions, punctuated by four heavy rain storms. Dilution of the bags by rainfall and wave action during storm events was observed on 2 occasions. Following one storm (August 27-29) chlorophyll levels doubled and the average DO increased by 50-500% in bags 2&3 and bag 1, respectively. The particulate metal concentrations were reduced, but the dissolved metal concentrations were unaffected. Unfortunately, this storm coincided with aeration of the bags (August 27) and the effects of both perturbations were unclear.

Considerable difficulty was encountered in keeping waterfowl away from the bags during the later half of the incubation period. The contribution from this source to particulate, total carbon, and chlorophyll levels could not be quantitatively assessed but the nutrient stocks in each bag were unaffected. The fecal material rapidly settled out of the water column, collecting in the bottom of each bag. Samples which contained obvious concentrations of this material (usually large particulates) were marked accordingly and passed through a 153 μm Nitex mesh prior to particulate

determinations. Similarly treated samples were also analyzed for total carbon and chlorophyll and compared with untreated samples. Significant increases in both total carbon and chlorophyll (10 to 60%) were observed with a decrease in precision for the untreated samples. Even though the bags were well mixed the fecal material was associated with only one or two samples taken from the different depths. Comparing results obtained with samples at these depths with other depths provided a means to isolate unusual observations.

III.II.I. Nutrient Stocks and the Physical Regime.

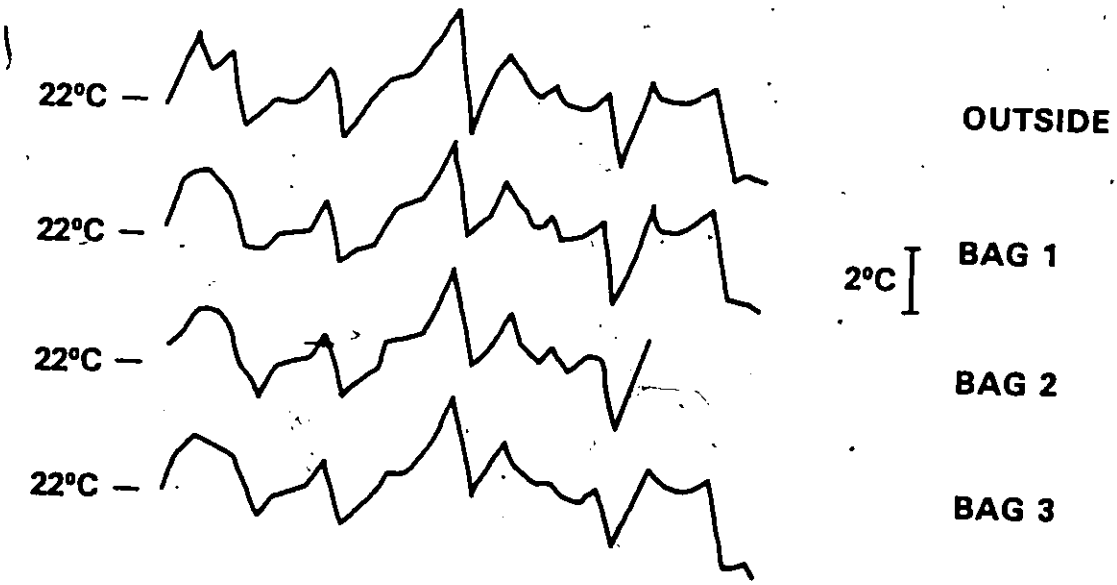
The thermal structure within each bag closely followed that of the surrounding waters (Fig. XII). Formation of a distinct thermocline or prolonged thermal gradient was not observed. The temperature ranged from a high of 24°C to a low of 19°C. The uniform distribution of metals, particulates, cells, and other biological and chemical parameters may have resulted from the fluctuating thermal regime of the surrounding waters. The relative differences in temperature (hence density) inducing thermohaline circulation, or convection, also enhanced by diel temperature changes (nocturnal cooling). Wave action and subsequent movement of the bag walls also contributed to the turbulence regime inside the bags along with slight mixing during sampling. Direct wind effects were, however, negligible due to the limited fetch or exposed surface area of each bag.

Water column oxygen demand was high (1.1-1.5 mg O_2/L/d) and complicated attempts to prevent the bags from going anoxic (Fig.

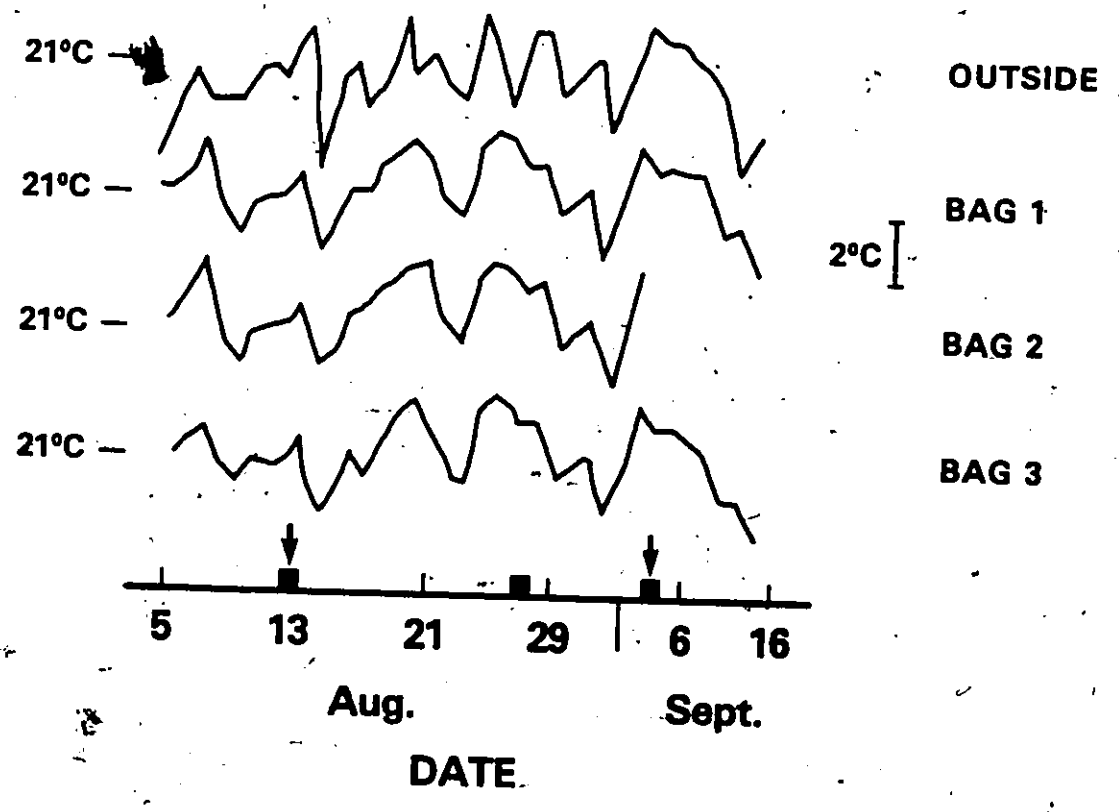
Fig. XII. Average Surface (0-2 m) and Bottom (6-8 m) Temperatures (^{o}C) within each Bag and Surrounding Waters over the Duration of the Study Period

Fluctuations in the thermal regime within each bag closely followed that of the surrounding water. No prolonged thermal gradients were observed throughout the study period. The arrows indicate the time of metal additions to bags 1 and 2, bag 3 was the control. One half the metal concentrations added to bag 1 were added to bag 2. The bags were aerated on Aug. 13 and 27, and Sept. 2-4 (represented by the blackened areas) and the period of incubation was from Aug. 5 to Sept. 11, 1981. All sampling was carried out on a daily basis between 10:00 and 12:00 AM.

SURFACE TEMP. (0-2m)



BOTTOM TEMP. (6-8 m)



XIII). Surface values fluctuated considerably depending on the direction of wave action and the extent of dilution. The dissolved oxygen concentration in the bottoms of each bag reached levels < 0.1 mg/L between periods of mixing. Average DO values in the bags over the study period, however, were around 2 mg/L.

No consistent relationships were detected in the nutrient stocks and the variance between replicate samples was at times highly significant (up to ± 75%). The observed ranges for each nutrient in all bags during the study period were as follows: SRP, 5-50 µg/L (TP, 60-160 µg/L); Si, 0.0-5.0 mg/L; NH_3, 0.1-1.7 mg/L; NO_2^- & NO_3^-, 0.8-4.0 mg/L. The degree (if any) of nutrient limitation could not be ascertained with the available data.

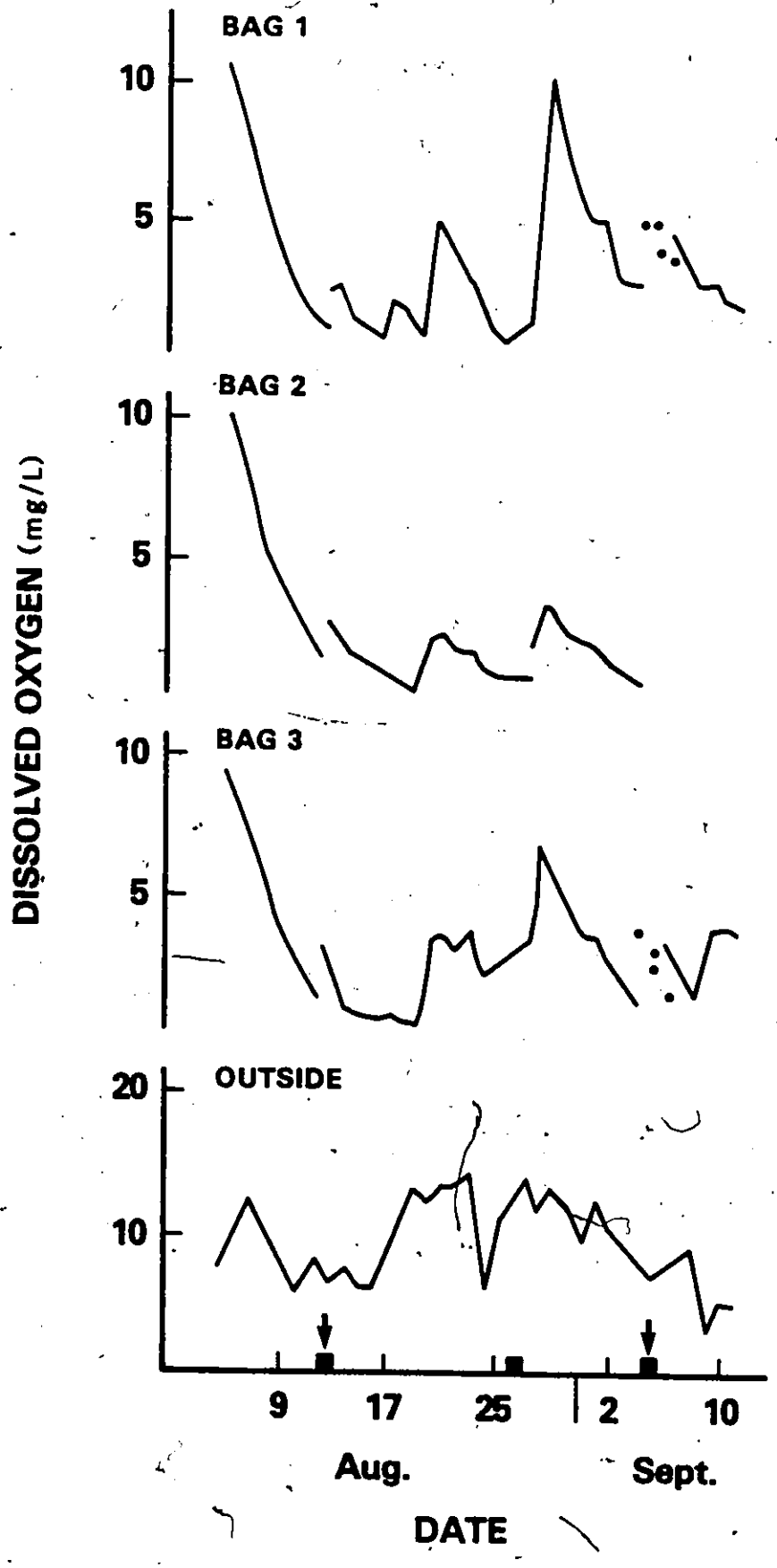
No apparent trends were detected in conductivity, pH, and alkalinity determinations with depth or time. Conductivity ranged from 460 to 500 µmhos and total alkalinity varied between 1.1 and 1.8 meq/L. Data obtained using one particular pH electrode had to be omitted because of an unusually sluggish and variable response. The pH ranged from 7.6 to 8.6 and no significant differences with depth or between bags were observed. The highest pH was measured when the bags were initially filled but later fell to within pH 7.9 - 8.2 on day 5 (metals were added on day 7).

III.II.II. Biomass - Carbon, Chlorophyll, and Species Distributions

Due to the well mixed nature of the bags no distinct vertical trends were observed. Consequently, all data was combined from the 3

Fig. XIII. Average Daily Dissolved Oxygen Concentration (mg/L) within each Bag and Surrounding Waters over the Duration of the Study Period

The oxygen demand of the water column is clearly illustrated in the exponential decline in DO between Aug. 5 and Aug. 11, the time between filling and metal additions. Fluctuations in the average DO concentration beyond this time resulted from dilution effects through wave action and rainfall. The large increase in DO on Aug. 27 was a result of both aeration and a series of thunderstorms. Only the average DO concentration within each bag is presented. Other notation as in Figure XII.


BAG 1
10
5
BAG 2
10
5
BAG 3
10
5
OUTSIDE
20
10
DISSOLVED OXYGEN (mg/L)
9
17
25
2
10
Aug.
Sept.
DATE

depths (0.5, 3-4, and 6-7 m in duplicate), averaged, and then combined with results obtained from the integrated samples (each in duplicate). Species enumeration and cell counts, however, were carried out using only the integrated samples.

Variations in the chlorophyll levels in all bags were similar over time (Fig. XIV). Chlorophyll levels decreased over the 7 day preincubation period, but later increased after the metal solutions were added and the bags mixed. Chlorophyll levels peaked around August 29 and declined slightly during the last 2 weeks of the experiment. Similar results were obtained for total carbon, and the particle distributions (Fig. XV). The increase in total carbon was greatest in Bag 1 (highest added metal concentrations) but the chlorophyll levels were similar to the control (Bag 3). Chlorophyll levels in Bag 2 (one half the metal concentrations added to Bag 1) were less than within the other bags.

The phytoplankton populations in all bags were dominated by both large and small species with a variable standing zooplankton crop. The increase in phytoplankton abundance was not a result of reduced grazing pressures but likely due to the relative stability of the water column. Total phytoplankton biomass decreased in all bags during the first week, followed by an increase after metal additions and mixing of the bags (Fig. XIV). Maximum levels were obtained on days 28-29 (20-30,000 cells/ml) and declined slightly towards the end of the study period. The population was dominated throughout the study period by Chlamydomonas sphagnicola, Oocystis, three species of small cells, Cryptomonas & Rhodomonas presented in the relative order of

Fig. XIV. . Changes in the Average Chlorophyll Concentration over the Duration of the Study Period

Chlorophyll levels (µg/L) initially decreased, then increased after metal additions (Aug. 11), and declined towards the end of the study period. The increase in chlorophyll was partly due to the relative stability of the water column within each bag over the duration of the experiment. The dramatic increase in the average chlorophyll concentration around Aug. 27 was a result of induced mixing (aeration of the bags) which coincided with a series of thunderstorms. The effects of either perturbation on ambient chlorophyll levels could not be fully resolved. Other notation as in Figure XII.

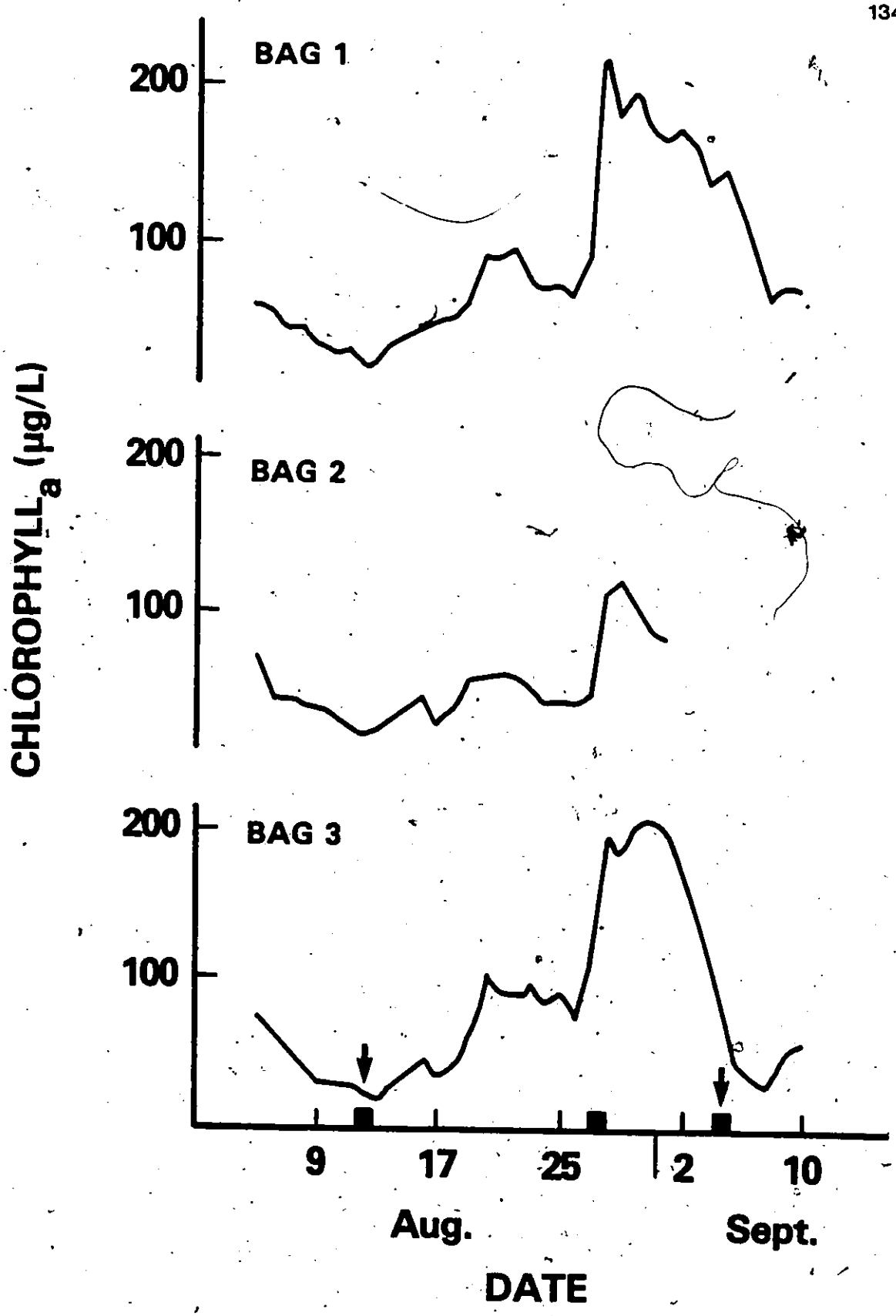
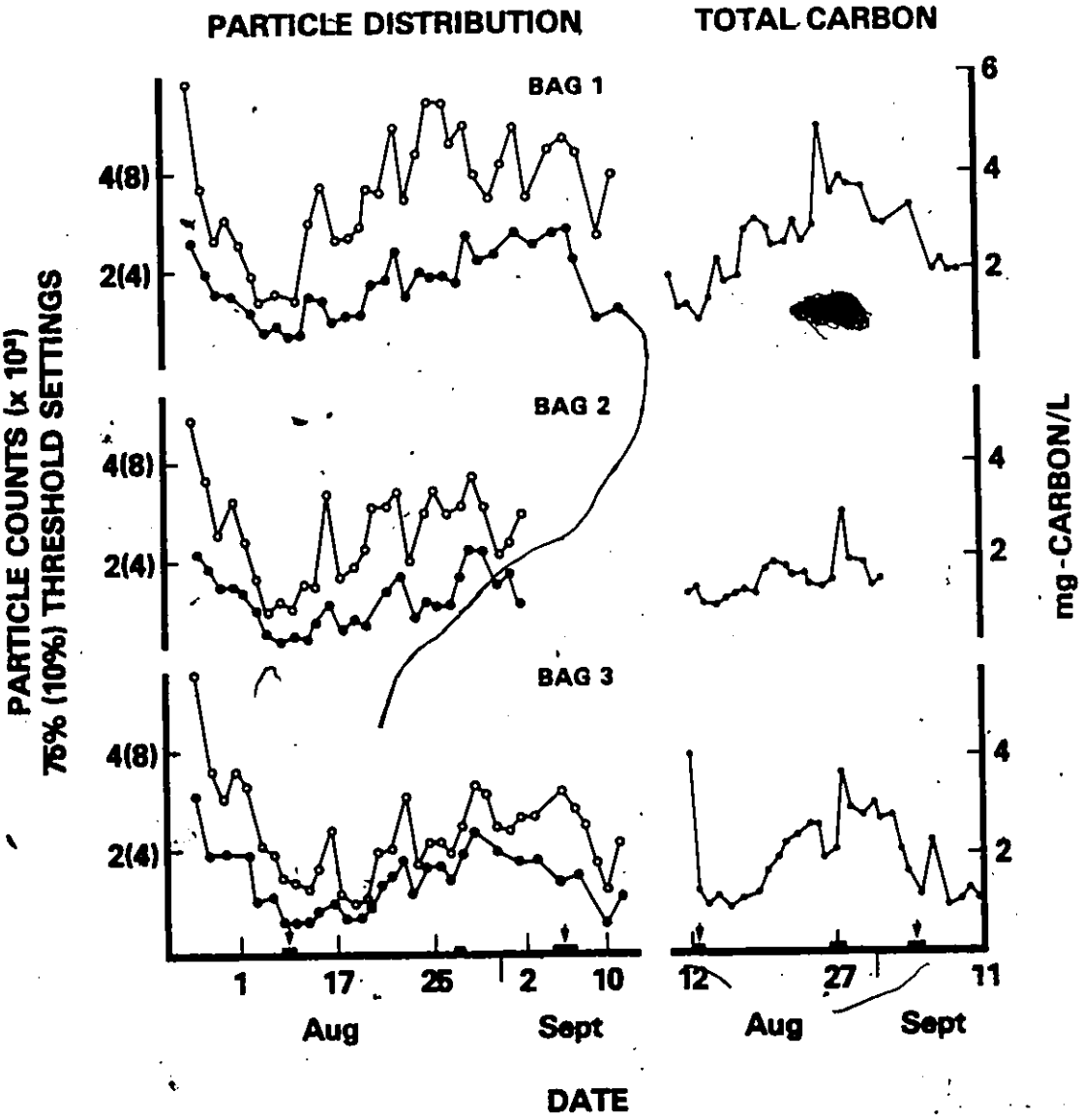

BAG 1
200
100
BAG 2
200
100
BAG 3
200
100
CHLOROPHYLL$_a$ (µg/L)
9
17
25
2
10
Aug.
Sept.
DATE

Fig. XV. Relative Particle Size Distributions and Total Carbon Concentrations within each Bag over the Duration of the Study Period

Particle distributions within each bag closely followed changes in the particulate carbon concentrations. Carbon was determined using a wet dichromate oxidation and is presented in units of mg carbon as glucose per L filtered. Particle counts were obtained using a Coulter Counter Model F. The threshold setting was varied to provide some indication of the particle size distribution (10 % setting for all particles greater than 20 μm^3 and the 75 % setting for particles exceeding 100 μm^3). Variations in both size fractions over time were correlated, indicating a common mechanism regulating particulate distributions (particles greater than 20 μm^3) within each bag.

Notation is as follows: "●" = particle counts (10^3/ml) at the 75 % threshold setting; "○" = particle counts at the 10 % threshold setting (bracketed values).


PARTICLE DISTRIBUTION
TOTAL CARBON
BAG 1
BAG 2
BAG 3
4(8)
2(4)
6
4
2
PARTICLE COUNTS (x 10³)
75% (10%) THRESHOLD SETTINGS
mg-CARBON/L
1
17
25
2
10
12
27
11
Aug
Sept
Aug
Sept
DATE

abundance. The small cell fraction was composed of the bluegreen Chroococus, a chrysophyte classified as Ochromonas, and the green alga Chlorella. This small cell fraction was combined due to the uncertainty in correctly identifying each species under 500 x magnification. Distinctly ovoid cells with obvious subcellular organization were counted as Chlorella. Small cells without any apparent subcellular organization were classified according to the presence or absence of flagella (Ochromonas and Chroococus respectively).

Zooplankton biomass was variable and high with a mean value of 2000 individuals per liter. Species of Brachionus and Keratella, and Bosmina longirostris were predominant in all samples. The observed species abundance ranged from 14-32 and 0-11 for phytoplankton and zooplankton respectively (numbers of species encountered based on the method of enumeration). Phytoplankton abundance was determined only by the species counted in 15 random fields which accounts for the large variation. A large number of unidentified zooplankton and/or insect larvae were also observed but not included in the results.

Fluctuations in phytoplankton biomass were consistent with changes in chlorophyll levels, total carbon, and relative particle distributions. Metal additions did not influence community structure or composition. Growth on the panels, however, was reduced in bags treated with metals. Copper was believed responsible for inhibiting growth of attached populations (Section III.II.IV.).

III.II.III. Relative Growth Rates

Growth rates were estimated for the total biomass, Oocystis, and the small cell fraction by fitting the results to a logistic equation (presented in Section III.I.I.). Only data from the period over which the bags were relatively undisturbed by storm related events (August 13-27) were used in these calculations. Growth rates (time to complete one doubling) for the total biomass, Oocystis, and small cells were 6.8, 3.6-8.3, and 5.8 days respectively. All regressions were significant at the 0.01 level or better. Cell counts for Chlamydomonas were too variable to provide a reliable growth rate estimate. Longer doubling times were expected because shading from the sides of the enclosures significantly reduced the available light (possibly, to limiting levels) and losses through grazing and settling were not considered.

Similar calculations were performed on the chlorophyll, carbon, and particulate data over this time interval. The calculated ranges for these doubling times were 5.0-6.0, 5.0-5.8, and 7.4-9.3 days respectively and all highly significant at the 0.001 level. One unusually high value was obtained for Bag 2 - total carbon (17 days) which has been omitted due to the relatively low values of carbon measured in this bag.

III.II.IV. Cycling and Distributions of Heavy Metals

Data for the particulate and dissolved metal concentrations from the different depths were combined as described earlier because no distinct vertical trends were observed within each bag during the study period.

An exponential loss of the dissolved metal fractions (not retained on a glass fiber filter) from the water column in each bag were observed. Residence times (time required to reduce ambient concentrations by 50%) were obtained by fitting an exponential equation to the data over the periods August 23 to 27, and September 5 to 11 (Bag 1 only). All regressions were highly significant at the 0.001 level. Residence times for Cd and Cu were the lowest, 3.8 $\pm$ 0.8 and 3.5 $\pm$ 2.0 days. Ni was retained the longest (7.3 $\pm$ 2.2 days), followed by Sr, Co, and Zn (6.1 $\pm$ 1.1, 6.1 $\pm$ 2.0, and 5.2 $\pm$ 2.3 days respectively. Lead precipitated during addition and was not detected in any of the dissolved or particulate metal samples throughout the study period. The reported values represent the pooled mean and standard deviation of all residence times calculated for each metal (Table XX).

Calculated enrichment factors (EF) for metals bound to the walls of the bags were highly variable (Tables XXI and XXV). The EF is represented as the ratio of metal in the panel scrapings to the average dissolved metal concentration over the period in which the panels were in place. Slow colonization of the panels prohibited frequent sampling and the high loss rate of dissolved metal introduced

Table XX Rentention Times (R) for Metals in the Dissolved Fraction

Retention Time, R, is the time required to reduce the initial metal concentration, M_o, by 50 %. Estimates for the loss constant, P, and retention times are presented along with the correlation coefficent (r^2) for the regression lines ($M = M_o \exp(P\,t)$, M = ambient metal concentration - daily average for all depths within each bag)

Sample	r^2	M_o	P	R	r^2	M_o	P	R
	Sr				Co			
Bag 1 A	0.863	1.09	-0.103	6.8	0.858	0.99	-0.181	3.8
B	0.923	0.97	-0.103	6.7	0.923	0.73	-0.094	7.4
2 A	0.772	0.90	-0.143	4.8	0.907	0.53	-0.099	7.0
	Zn				Ni			
Bag 1 A	0.785	0.84	-0.163	4.3	0.774	1.61	-0.130	5.3
B	0.945	0.96	-0.206	3.4	0.955	1.50	-0.101	6.9
2 A	0.889	0.59	-0.089	7.8	0.833	1.13	-0.071	9.7
	Cu				Cd			
Bag 1 A	0.909	0.017	-0.241	2.9	0.763	0.014	-0.319	2.2
B	0.956	0.022	-0.156	4.4	0.932	0.008	-0.117	5.9
2 A	0.846	0.010	-0.171	4.1	0.878	0.008	-0.304	2.3

SUMMARY

Metal	Loss Constant (mean ± SD)	Retention Time (days)
Cu	-0.189 (0.046)	3.8 (0.8)
Ni	-0.152 (0.059)	7.3 (2.2)
Cd	-0.247 (0.112)	3.5 (2.0)
Zn	-0.101 (0.029)	5.2 (2.3)
Co	-0.126 (0.050)	6.1 (2.0)
Sr	-0.116 (0.023)	6.1 (1.1)

Sample notation is as follows: A, data taken from August 13 to 26; B, data taken from September 5-11 2. All regressions were highly significant at the 0.001 level and bracketed values (summary section) represent standard deviations for the combined results. No estimates for the retention times for lead were obtained.

Table XXI Enrichment Factors for Metals in the Panel Scrapings

Sample	Time	METAL ENRICHMENT FACTORS									
		Cu	Ni	Cd	Zn	Co	Sr	Mg	Ca	K	Na
Bag 1	A	285	2.2	44	26	3.3	-	2.7	1.0	3.3	0.5
	B	241	4.2	61	24	6.0	-	2.4	0.7	3.2	0.7
	C	288	5.5	203	30	7.0	-	2.5	0.9	3.5	0.7
Bag 2	A	100	4.3	38	24	4.4	-	3.1	2.8	2.6	0.4
	B	206	6.3	148	75	8.6	1	3.7	1.9	3.1	0.6
	C	1772	8.5	420	100	1.4	-	3.6	2.0	2.8	0.7
Bag 3	A	-	-	-	-	-	-	2.5	3.1	2.3	0.2
	B	-	-	-	23	-	2	1.6	0.3	2.2	0.1
	C	-	-	-	-	-	-	1.5	1.6	2.5	0.2

The enrichment factor is defined as the ratio of metal bound to material on the walls of each bag (panels) to the average concentration within the water column over the time period in which the panel was in place. Other notation is as follows: A, one side scraped 10 days after metal additions (day 16 of the experiment, Aug. 22, 1981); B, side (previously scraped on day 16) scraped on day 37 (Sept. 11, 1981), except in Bag 2 which was terminated on day 27 (Sept. 2, 1981); C, unscraped side scraped at the termination of the study period.

a relatively large standard deviation in the calculation of each EF. Enrichment factors for the metals in Bags 1 & 2, presented in decreasing order, were as follows: Cu > Cd > Zn > Co > Ni > Sr. These results were consistent with the retention times of the respective metals within the dissolved phase (Table XX).

Particulate and dissolved metal concentrations were uncorrelated (Fig. XXIV). Fluctuations in either fraction as a result of induced mixing or dilution by wave action, did not produce any concomittant variations in the other fraction. This indicates that metal bound to large particulates was essentially unavailable and the equilibrium kinetics (release of particulate metal) were too slow to contribute significantly to the dissolved metal fraction. The dissolved metal fraction, on the other hand, includes metals bound to inorganic and organic ligands not retained on glass fiber filters (small molecules to humic substances). Clearly this fraction requires further separation to resolve important components involved in regulating available metal concentrations. Also this fraction is retained within the water column for extended periods of time, relative to the larger particulate phase, and potential recycle mechanisms (bacterial degradation etc.) cannot be ignored.

Variations in the particulate manganese concentrations paralleled changes in chlorophyll levels over the duration of the experiment (Fig. XIV, XXIV). Manganese is an essential micronutrient for a number of phytoplankton (Huntsman and Sunda 1980) and the reduction of manganese (IV to II) under low dissolved oxygen concentrations and in the presence of catalytic organic surfaces at

near neutral pH (Stumm and Morgan 1981) may have enhanced the availability of this metal. Particulate iron concentrations, however, remained relatively constant throughout the study period.

Daily variations in conservative metals (K, Na, Ca, and Mg) were significant and, as expected, highly correlated (Fig. XXV). Dilution of the bags by wave action or rainfall account for most of the variation even though K, Mg, and Ca adsorbed onto the walls of the bags with enrichment factors of roughly 2 (Table XXI). Desorption and adsorption of these metals, combined with analytical error and averaging of the data from all depths, could potentially dampen the magnitude of the observed fluctuations within the water column. Variations in the concentrations of these elements in Hamilton Harbour surface waters over the same period were, however, significantly greater than within the bags (Fig. XXV). This variability resulted from rainfall, differential exchange of water from Cootes Paradise at the west end and Lake Ontario at the east end, and the complex physical regime within Hamilton Harbour.

Fluctuations in the Ca, Mg, K, and Na concentrations are not sufficient to directly modify metal binding to surface of phytoplankton or dissolved and particulate matter. Changes in the ambient concentrations of these elements may, however, influence cellular physiological activity, indirectly altering the uptake of metals. Additional binding and physiological studies are required to understand interactions between phytoplankton and a variable environment. Clearly processes involved with surface mixing and suspension of phytoplankton in the euphotic zone can result in a high

degree of spatial and temporal variability. The magnitude and frequency of these fluctuations can significantly alter the ability of phytoplankton to efficiently utilize available resources and confound attempts to identify direct cause and effect relationships between important environmental variables and a biological response.

IV. DISCUSSION

The distribution and cycling of metals in aquatic systems is regulated by biological, physical, and chemical variables interacting over a broad spectrum of spatial and temporal scales. Spatial scales, however, are of minor importance on a cellular level. In this perspective, the organism is assumed fixed in space and is exposed to random and periodic fluctuations in the physico-chemical environment, including toxic substances, nutrients, and other cells. The magnitude and duration of heavy metal stress (toxic or deficient levels) can significantly alter the ability of aquatic organisms to integrate fluctuations in available resources into growth, or maintenance of basal metabolic processes. Prolonged stress usually results in a decrease in species abundance and diversity, and favors selection of more tolerant strains (Whitton, 1970a; Forstner and Wittmann, 1979; Lange-Bertalot and Lorbach, 1979; Evenson et al. 1981; Foster, 1982a,b). Short term exposures, however, involve processes operating over distinctly different temporal scales. Factors regulating the availability of metals in solution, binding to the cell wall, and transport into the cell must be considered under these conditions. The ultimate response is dependent upon the net flux of metal into the cell relative to losses through growth and dilution, including the ability of individual cells to tolerate elevated external or internal metal concentrations. Survival of the organism is determined by changes in the available metal concentration in solution when the

internal metal concentration exerts a deleterious influence on cellular metabolic processes and the capacity of the cell to compensate for the imposed stress. Understanding the kinetic interactions between these variables is essential to comprehend observed cellular responses to perturbations in the external physical and chemical environment. These concepts can also provide an effective means to interpret and compare results from culture and in situ investigations.

The discussion is subdivided into two sections concerned with; 1. the nature and reactivity of the cell surface, and 2. kinetic interactions between variables and processes regulating the toxicity of heavy metals towards phytoplankton. In the first section, the chemical reactivity and basic structure of algal cell surfaces are discussed, along with mechanisms for transport of metals across the cell membrane. Properties of the surface regulate the extent and rates of reaction with available metals in solution. The quantity of surface bound metal, in turn, determines the kinetics of transport into the cell and the observed toxic response. Dynamic interactions between these variables, processes, and modifying factors are discussed in the second section.

IV.I. The Nature and Reactivity of the Cell Surface

The cell surface extends from and includes the surface of the plasmalemma to the external solution. Metal binding to the cell surface and uptake is a dynamic transport phenomenon (active or

passive movement of ions in space) which involves a number of important physico-chemical and physiological variables. The cell wall, including mucilage sheaths, and plasmalemma are physical barriers with distinctly different chemical properties. The nature and reactivity of these structures regulate solute movement around the cell and incorporation into the cell. The surface must be considered in a dynamic state of flux because cell wall and membrane components are continually synthesized during growth. Direct or indirect influences of cellular metabolic activity on and within this region, possibly altered in the presence of a metal, must also be considered. Each of these factors are discussed in the following sections in the process of developing a conceptual model for the cell surface compatible with my results and with those reported in the literature.

IV.I.I. Structural Aspects of Algal Cell Walls

Algal cell walls can be exceedingly complex structures composed mainly of polysaccharides and proteins with mineralized deposits (silicon and carbonate based) or specialized organic compounds (such as sporopollenin) characteristic to individual species (Mackie and Preston, 1974). A complete review of the diversity of algal cell wall ultrastructure is beyond the scope of my work, but certain generalizations can be made. The cell wall is a highly organized, three dimensional structure permeable to various solutes. Movement of ions across this barrier is regulated by chemical and physical interactions between the solute and cell wall components. Compounds

actively or passively secreted by the cell may be physically confined within the internal regions of the cell wall. Functional groups associated with the amino acid and sugar residues of cell wall components, along with reactive groups on exudates, (primarily oxygen, sulfur, and nitrogen containing ligands) are known to bind metallic ions and participate in proton transfer reactions (Stolzberg and Rosin, 1977; Gnassia-Barelli et al., 1978; Swallow et al., 1978; McKnight and Morel, 1978; van den Berg et al., 1979; Butler et al., 1980; Crist et al., 1981; Sueur et al., 1982). These groups and other small molecules which permeate the cell wall impart a net negative surface charge at near neutral pH values as indicated by a negative electrophoretic mobility (zero point charge for algae is around pH 3, Stumm and Morgan, 1981). Repulsive electrostatic forces may inhibit formation of algal-organic associations and account for the clean surfaces of healthy phytoplankton growing in situ.

Synthesis of individual cell wall components occurs asynchrously and may extend over the duration of the cell cycle (Mahler and Cordes, 1971; Atkinson et al., 1972). The influence of these processes and the indirect effects of cellular metabolic activity on the chemical reactivity of the cell surface are not fully understood. However, differences in the maximum binding capacities for isolated cell walls, and between the two strains of Chlamydomonas used in this investigation, confirm the importance of this region in regulating the extent of metal adsorption.

Maximum binding capacities of isolated cell walls, for a variety of metals, are significantly greater than for walls of intact

cells (Hassall, 1963; Gutnetch, 1965; Glooschenko, 1969; Matzku and Broda, 1970; Button and Hostetter, 1977; McLean and Williamson, 1977; Horikoshi et al., 1979b). Alteration of the physico-chemical properties of the cell wall during mechanical or chemical separation and the greater exposed negative surface area may account for this difference. The total zinc binding capacities for the wall-less mutant of Chlamydomonas used in this study, however, were roughly 30-70 % of estimates obtained for the wild strain (Table XIII). The cell wall of the wild strain is composed of seven layers which may be reduced to three or five layers in the mutant (Roberts et al., 1972). Binding of zinc to the surface of the wall-less mutant was not examined under suboptimal conditions (low temperature and light), but the results indicate some relationship between the binding capacity and thickness of the cell wall, or the physical limits to the boundary zone surrounding and influenced by the cell. Both species were grown and separated for experimental use (refer to Section II.I.I.) in an identical fashion to minimize physiological differences between the populations. Differences in cell wall volume may confer selective advantages on individual cells for tolerating variable degrees of heavy metal stress and provide a passive mechanism for buffering small scale fluctuations in available resources.

IV.I.II. The Reactivity of Algal Cell Walls: Physiological Considerations

The actual mechanism for adsorption of metallic ions onto the surfaces of phytoplankton are not known, but may involve both covalent and ionic charge bonding. The reactivity and availability of binding sites may also be influenced by the cell, along with the experimental conditions (pretreatment of the inoculum and medium composition). Ion fluxes across the membrane and the release of organic substances can significantly alter the chemical properties and reactivity of the cell surface, hence the binding of metals. The most convincing evidence for these effects is provided by studies on the binding of metals under suboptimal and optimal physiological conditions of pH, light, and temperature. In these studies, an increase in the extent of metal adsorption is frequently observed under optimal physiological conditions. Based on my results, I believe that the increase in zinc binding under optimal physiological conditions results from indirect effects of metabolic processes on surface reactivity (including synthesis of membrane and cell wall components, or new binding sites) and not active uptake. Consequently, these effects are separated from active uptake and presented in this section because the cell wall defines the physical limits to the boundary zone surrounding and influenced by the cell. Although a number of alternative pathways sensitive to changes in the physico-chemical environment are possible (specific interactions between individual metals and solute transport systems confined in the membrane), this hypothesis may also apply to

the enhanced binding of other metals under optimal physiological conditions.

Differences in the surface binding of metals under suboptimal and optimal conditions reported in the literature can be confounded by the design of the experiment and assay conditions. Medium composition and concentration (discussed in the next section), along with the age structure and physiological condition of the inoculum (including level of cellular debris) can significantly influence the results (refer to Section I.I.II.). The results may also be confounded by additional stress imposed on the cells under suboptimal physiological conditions.

The quantity of zinc bound to the surface of Chlamydomonas decreased with temperature and in the dark (Table XV, Fig. X). Similar observations for the binding of other metals to the surfaces of different species of phytoplankton have been reported (Gutnetch, 1965; McBrien and Hassall, 1965; Bryan, 1969; Matzku and Broda, 1970; Findenegg et al., 1971; Hart and Scaife, 1976; McLean and Williamson, 1977; Mierle, 1981; Yen, 1981). These effects are believed a result of active uptake, even though there is no real physiological requirement for synthesis of systems to selectively accumulate metals. Light sensitive adsorption to the surface of Chlorella was reported for copper (McBrien and Hassall, 1965; Matzku and Broda, 1970; Nakajima et al., 1979b), cadmium (Hart and Scaife, 1977), zinc and cobalt (Findenegg et al., 1971), and to a lesser extent manganese and uranium (Nakajima et al., 1979a,c). The presence of either; 1. a common uptake mechanism, or 2. specific uptake systems is unlikely, if one considers the different physico-chemical properties (reactivity) of these

metals. It is interesting to note that seasonal patterns of metal uptake in marine macrophytes were also consistent with changes in the level of cellular metabolic activity dependent upon light and temperature (Eide et al., 1980).

Under all conditions, adsorption of zinc onto the surface of Chlamydomonas followed simple hyperbolic saturation kinetics with identical temporal characteristics (Fig. X). Attainment of different, but coincidental upper asymptotes in time, under suboptimal and optimal conditions would not be observed for an energy dependent active uptake process. If an active uptake process were involved then a common maximum, or a single upper asymptote, under all conditions would result, with a rate (time to reach the upper asymptote) dependent upon the degree of inhibition (reduction in activity). Also, the large differences in the extent of adsorption observed in my investigation (3 to 4 fold difference) cannot be explained on the basis of active uptake, especially since an upper maximum (saturation level) was attained under all conditions. If 50 % of the metal bound to the cell surface was actively incorporated, then the observed rate of uptake must exceed 10^{-16} moles/cell/h. Maximum binding capacities of the cell surface ranged from 0.2 to 5.0 x 10^{-17} moles/μm^2 (Table XIII) with surface areas of 50 to 100 μm^2/cell. The plateau, in turn, would result from either; 1. inhibition of the active uptake system by the metal, or 2. active excretion at a level equal to uptake.

Methylation, reduction, and incorporation of arsenic into lipids reached equilibrium levels within a 45 minute period in the marine chlorophyte Dunaliella tertiolecta (Wrench and Addison, 1981).

Competitive interactions between arsenic and phosphorus may account for this particular result (alternate pathway into the cell). Either possibility, however, is unlikely for zinc if one compares the cell metal concentration that reduces growth with the proposed uptake coefficient (inhibiting concentrations range from 0.1 to 6.0 x 10^{-15} moles/cell, Table XXIII). The relatively high uptake rate combined with a low inhibiting cell metal content would be instanteously lethal to the cell, even in the presence of low available metal concentrations. Also, active uptake cannot account for the different growth responses observed for phytoplankton populations under metal stress (extended lag phase or gradual reduction in cell numbers over time, Type I and II responses, respectively).

All these results, however, can be explained by changes in the chemical properties of the cell surface indirectly under physiological control. The similarity between the pH, light, and temperature dependence of photosynthesis and zinc adsorption provides additional evidence for this argument (Table XV and Fig. XIa,b). Photosynthesis can be a reliable indicator for the general level of cellular metabolic activity under nutrient sufficient conditions. Consequently, pH differences between the assay and standard growth media could influence the results. Hart and Scaife (1977) found a 30 to 50% reduction in cadmium binding at pH 8 compared to pH 7 and all stock cultures were maintained at the lower pH. Similar results were observed for uranium absortpion in the same species (Nakajima et al., 1979a). Zinc binding to cell wall fragments of Vaucheria, however, peaked at pH 6 and decreased in higher and lower pH regions (Crist et

al., 1981). These results are difficult to interpret since field samples were used and methods for isolating the cell walls were not fully described. The correlation between zinc binding and photosynthetic activity, however, implicates a light dependent process in regulating the reactivity of the cell surface. Also, the quantity of exchangeable zinc bound to the cell surface roughly corresponds to the degree of enhanced binding under optimal physiological conditions (discussed in the next section). Clearly, the labile fraction is not transported into the cell.

Accumulation of metals beyond the apparent saturation of surface sites (also includes metal within the intracellular wall spaces) results from either transport of metal into the cell, or synthesis of new sites. Since the extent of binding to the cell surface is dependent upon the stage of life cycle (Steemann-Nielsen and Wium-Andersen, 1971; Fujita and Hashizume, 1975; Cossa, 1976; Bottino et al., 1978; Morel et al., 1978; Laube et al., 1980. Lue-Kim et al., 1981), cells entering the growth phase will correspondingly bind greater quantities of metal. Changes in the age structure of asynchronously growing populations, hence physiological activity, may account for this residual uptake (asymptotic).

Continual synthesis of cell wall and membrane components may also create additional binding sites. I believe this mechanism is primarily responsible for the prolonged binding of zinc (asymptotic) to the surface of Chlamydomonas observed in the short term experiments. The phototactic response of this species was used to obtain a physiologically homogeneous population with a narrow age

structure, based on a uniform and consistent cell size. Physical isolation of populations of uniform cell size, either using mechanical methods or phototatic responses (Wetherell, 1958; Bernstein, 1960), are the most reliable methods to provide starting material for synchronized cultures (Tamiya et al., 1961). Inducing synchrony in randomly growing cultures using single environmental factors (light, temperature, and starvation) can lead to technical problems in the phasing of physiological and genetic processes (Tamiya et al., 1961). Concentrations of zinc used in all uptake experiments did not inhibit the growth or motility of Chlamydomonas and all results are indicative of the extremely slow rates of zinc transport into the cell (e.g. not active uptake). Low net rates of zinc accumulation, balanced against losses through dilution, are substantiated by the time lags I observed in the growth responses of Chlamydomonas, Scenedesmus, Coelastrum, and Chlorella exposed to deleterious zinc concentrations.

The importance of physiological control over surface reactivity cannot be overlooked and at present is poorly understood. Solute fluxes across the membrane may alter the chemical properties of the cell surface and is the most likely mechanism responsible for enhanced uptake of zinc under optimal conditions of pH, light, and temperature. In summary, the stimulatory effects of light and temperature cannot be ascribed to specific, active uptake systems for the following reasons: 1. the magnitude of the uptake coefficient would result in an instantaneously lethal influx of metal into the cell; 2. the diversity of systems required to accumulate metals with distinctly different physico-chemical properties is unlikely; 3. binding kinetics and the

attainment of different upper asymptotes under all conditions cannot be attributed to active uptake systems; 4. the fraction of zinc bound under optimal conditions is easily displaced and not transported into the cell. Additional studies are required to resolve the nature of control over surface reactivity in regulating heavy metal - phytoplankton interactions, especially under non-steady state conditions encountered in situ.

IV.I.III. The Reactivity of Algal Cell Walls: Binding of Metals

The heterogeneous composition of the cell wall ensures the presence of different reactive groups with varying affinities for individual metal ions and complexes. Based on my results, these sites can be classified according to their affinity for zinc. One class of sites specifically and irreversibly binds zinc and is not significantly influenced by solute fluxes across the membrane (e.g. relative level of cell metabolism). The other class of surface ligands readily exchanges zinc for other cations and is sensitive to changes in the physico-chemical properties of the cell surface, indirectly influenced by the cell (solute fluxes across the membrane). Evidence for the existence of both classes of binding sites is presented and discussed in light of the results from my studies and from those reported in the literature.

Nonlinear Scatchard plots of the zinc uptake data confirm the presence of multiple binding sites on the surface of all species examined (Table XIII). Residual binding of zinc beyond saturation of

the initially available surface sites (< 5% of the total metal bound) cannot account for the nonlinear nature of these plots. Results from the displacement studies with zinc and other metals and EDTA (Table XVI) substantiates the existence of multiple binding sites on the cell surface. Concentrations of the chelator EDTA in excess of 500 times the available zinc concentration did not completely inhibit binding of zinc to the surface of Chlamydomonas. In previous studies, artificial chelators were used to obtain estimates of the quantity of metal assimilated by the cell (Steemann-Nielsen et al., 1969; Davies, 1973; Button and Hostetter, 1977; Horikoshi et al., 1979a; Li, 1979; Nakajima et al., 1979a,b; Sakaguchi et al., 1979; Bates et al., 1982). The procedure involves addition of a chelator to subsamples of the test population and the decrease in cellular metal followed over time. In all cases, only a portion of the total metal was removed. Adding cells to a previously equilibrated metal/chelator mixture (2 h in MB assay medium) resulted in a net binding of zinc to the cell surface roughly equivalent to this non-extractable fraction (20-50 % the total metal bound). Binding of EDTA-zinc complexes cannot account for these observations since additions of other transition cations produced similar results (Table XVI). In other studies, excess concentrations of EDTA in the presence of iron did not completely block cadmium uptake in Chlorella (Hart and Scaife, 1977).

My results can be explained by the presence of a class of ligands on the cell surface that specifically and irreversibly binds zinc, rather than an active uptake process. Nonexchangeable, specific binding has been reported for a number of metals to the surfaces of

different species of phytoplankton (Hassall, 1963; Steemann-Nielsen et al., 1969; Davies, 1973; Hart and Scaife, 1976; Button and Hostetter, 1977; Horikoshi et al., 1979a; Nakajima et al., 1979a,b; Sakaguchi et al., 1979; Li, 1979; Yen, 1981; Bates et al., 1982). The concentration of surface ligands that preferentially bind zinc roughly corresponds to the maximum quantity of zinc bound under suboptimal physiological conditions, providing additional evidence for the indirect influences of metabolic processes on surface reactivity.

The specific and non-exchangeable binding of different metals may involve one or a number of ligand types which can only be resolved by simultaneous exposure of the surface to combinations of metals. Spacing of sites and the influence of bound metals on local charge distributions are not known but could significantly modify the mobility of individual metals within the cell wall/membrane region. Specific sites of acid and base secretion were observed in Nitella (Spear et al., 1969) and Chara (Smith, 1970). Localized regions of base secretion on Characean cells are believed to be responsible for the production of banded calcium carbonate deposits. Consequently, clustering of reactive sites on the cell surface and interactions between these sites must also be considered.

The exchangeable or labile fraction of the total zinc bound to the cell surface constitutes the second class of surface ligands. Skaar et al. (1974) reported that a portion of the nickel bound to the surface of Phaeodactylum was rapidly lost when the cells were separated and resuspended in nickel free medium. Plutonium, however, was not easily desorbed from the surface of this species (Yen, 1981).

The reversible nature of metal uptake indicates that a portion of the metal associated with the cell wall may be ionically bound or free within the intracellular wall spaces. This accounts for the displacement of zinc by Mg and Ca (Table XVI), and exchange reactions with other metals for different species of phytoplankton (Hassall, 1963; Bryan, 1969; Steemann-Nielsen et al., 1969; Matzku and Broda, 1970; Shioi et al., 1978; Huismann et al., 1980; Crist et al., 1981; Mierle, 1981).

The quantity of metal bound to both classes of surface binding sites could be entirely obscured by the composition of the assay medium. The presence of other metals, such as Ca and Mg, could displace the labile, or loosely bound metal and result in lower estimates for the maximum binding capacities. Similar results could arise from trace metal contamination in the growth and assay media. Non-exchangeable binding of metals (contamination) to surface ligands will render these sites unavailable. Stratton and Corke (1979) in studies on multiple metal interactions found that the observed response was dependent upon the order of metal additions. Similar results were found for the effects of mercury on growth of Chlorella and pretreatment of cells with selenium (DeFilippis, 1979).

Clearly, the cell surface contains a number of binding sites with varying affinities for individual metals. One class of sites specifically and irreversibly binds zinc. Zinc bound to the other surface sites is readily exchangeable and dependent upon changes in the physiological condition of the cell. Results from the factorially designed, multiple metal competition experiments in which

physiological responses were measured are presented to illustrate the complex nature and reactivity of the cell surface.

IV.I.IV. Multiple Metal Competition Experiments

Interactions between nine metals, present singly and in all combinations, for available ligands on the cell surface and uptake were examined using factorial experiments. Reductions in the amount of zinc bound to the cell surface provided a relative index for the level of competition between individual metals for available binding sites. A measure of metal transported into the cell was provided by changes in fluorescence (IVF) and rates of photosynthesis. (Uptake is loosely defined by the physiological effects of metals on the cell, including indirect effects at the cell surface.) Although these parameters do not provide information on the lethal-sublethal effects of metals on the cells, they are sensitive indicators of chloroplast function and the general physiological condition of the test population. Studies on the interactions between more than three metals have not been reported and special methods of analysis are not available. The number of chemical and biological parameters involved in multivariate experiments severely restricts conclusive statements to qualitative trends in the measured response. Factorial experiments, however, can provide information on important variables and interactions not easily detected in rigidly controlled experiments (e.g. one variable at a time).

Transient fluorescence (IVF) responses were observed in

preliminary short term studies. Following addition of the metal, IVF rapidly decreased then gradually increased over time back to the orginal level (Fig. VI). Consequently, fluorescence measurements at the termination of each factorial experiment did not provide a reliable measure of the effects of different metals on the cells. However, the significant IVF results that were obtained, along with the effects on photosynthesis, contrasted with the observed reductions in zinc binding. These findings can be explained in terms of preferential binding of individual metals and the number of available sites on the cell surface.

The results clearly illustrate the complexity of interactions between different metals for available ligands and uptake. Copper, for example, reduced zinc binding by 60% in preliminary two metal experiments (Table XVI), but the average effect of this metal was significantly reduced in the presence of other divalent cations (each effect is a measure of the average response under all conditions, refer to Section II.I.IV.). Interactions between copper and other cations modified the influence of copper on photosynthesis and IVF, in turn zinc uptake. Specific binding of individual metals to available ligands may enhance movement of other metals within the cell wall/membrane region (e.g. synergistic interactions). The influence of cadmium was similar to copper except equivalent cadmium concentrations did not alone inhibit ^{14}C assimilation in preliminary studies (Table X). The deleterious effect of cadmium on photosynthesis in the presence of other metals, however, provides some evidence for enhanced movement arising from preferential binding of other metals.

The interdependence of metabolic activity and zinc binding previously discussed, may also account for the reduced effects of cadmium on zinc binding in the presence of other metals. This is especially evident under optimal conditions of temperature, light, and pH at which the potential magnitude of the observed effect is the greatest. At pH 7.2 for example, rates of ^{14}C assimilation by Chlamydomonas are considerably less than at pH 6.2. Any inhibition of photosynthetic activity at the higher pH will be proportionately lower and possibly, the difference obscured by experimental error. These factors, along with changes in solution equilibria (different levels of pH) and time, are primarily responsible for the poor resolution between variable interactions under the different physical conditions examined in the factorial array.

Combinations of two or more metals (two factor and higher level interactions) provide a number of insights into the competition of individual metals for available ligands on the cell surface. Some effects may be indirect results of the deleterious influence exerted by one metal on cell metabolism, hence the binding of zinc. Nickel significantly reduced zinc uptake at pH 6.2, but in the presence of copper the effect of nickel was considerably less. This observation can be explained by the interactions between both metals and the effects of copper on the cell. Similar results were obtained for Cu and Cd, Sr/Co, and Ni, along with Cd and Sr/Co, Ca/Mg, Ni, and Mn. Ca/Mg alone had the greatest effect of zinc binding a pH 6.2 which was not significantly altered in the presence of copper (Table XVII). The effects of manganese alone and with copper were also similar.

Competition between Mn and Ca/Mg in the presence of copper for available sites could be attributed to; 1. enhanced uptake of Cu, in turn influencing cell metabolism and binding of zinc, or 2. a preferential antagonism between Zn, Mn, and Ca/Mg not influenced by copper. However, Ca/Mg and Mn together have little effect on zinc binding implicating copper in modifying zinc binding. Singularly, Mn and Cd significantly reduced zinc binding under all factorial conditions, but the overall joint effect was reduced. Competition between Mn and Cd was observed in studies with Chlorella (Hart and Scaife, 1977), and both metals reduced zinc adsorption by 20 to 30% (Table XVI) but did not individually inhibit short term rates of ^{14}C incorporation (Table X). Cadmium, however, inhibited IVF and photosynthesis in the factorial experiments (Tables XVII and XXVIII, each effect is a measure of the average response under all conditions, refer to Section II.I.IV.) which may explain the variable effects and interactions between Mn and Cd. Cadmium exchanges with zinc, but manganese displaces cadmium modifying the toxicity of cadmium, in turn enhancing the relative total quantity of metal bound (specifically zinc but may also include all metal ions). Clearly, interactions between all variables are exceedingly complex, especially when metals exert deleterious effects on cellular metabolic processes.

The effects of other metals on zinc adsorption between species and among genera were relatively consistent, reflecting the universal nature and reactivity of algal cell walls. This result is of particular interest in modelling heavy metal - phytoplankton interactions. The influences of manganese on zinc binding to the

surface of Scenedesmus isolated from Hamilton Harbour were significantly less than for the laboratory strain (Table XVI). Competitive interactions between manganese and zinc are consistent with observed effects of this metal on zinc binding under all experimental conditions. Differences in the effects of manganese on zinc binding between both species of Scenedesmus may be related to the tolerance of each species towards zinc (Table XI).

Results from the factorial experiments were consistent with interactions between different metals on the growth of various phytoplankton. The influences of combinations of copper, zinc, and cadmium on the growth of Selenastrum capricorutum were identical to equivalent zinc concentrations (Bartlett et al., 1979). Hutchinson (1973) reported synergistic interactions between copper and nickel and antagonistic effects between selenium and cadmium in four species of freshwater algae. Similar responses for copper and nickel were reported for other phytoplankton (Hutchinson, 1973; Stokes, 1981). Joint effects of copper and zinc on marine phytoplankton were considerably more complex and species dependent (Braek et al., 1976). Antagonistic effects of copper and cadmium (Bartlett et al., 1974), nickel and cadmium (Stratton and Corke, 1979), and the synergistic effects of zinc and cadmium (Say and Whitton, 1977), manganese and copper (Christensen et al., 1979) on cell growth have also been observed. These results and the influence of various metals on the growth of tolerant and non-tolerant species of phytoplankton (Jenson et al., 1974; Stokes, 1981; Stokes and Dreier, 1981; Shehata and Whitton, 1982; Foster, 1982b) can all be explained by classes of sites

on the cell surface specific for similar metals. A reduction in the number of available sites specific for individual metals confer tolerance and produce marked changes in the observed binding characteristics for other metals. The apparent lack of co-tolerance of species for all metals substantiates the presence of specific classes of binding sites under a common control mechanism.

Results from the factorial experiments also confirm the existence of multiple binding sites on the cell surface with varying affinities for individual metals. The nonadditive nature of interactions between metals is clearly illustrated in the ordering of the effects (Table XVII). If all metals compete equally (additively) for available ligands the effects would be ordered according to the total concentration of metal present within each test flask. Significant reductions in zinc binding by individual metals (main effects) and lower order interactions, are clearly nonadditive.

Results from the factorial experiments were also analyzed using simple nonlinear and linear models to confirm the existence of multiple binding sites on the cell surface (Section III.I.IV.). Correlations between estimates of the coefficients for each model and known stability constants for metal ligand complexes could not be obtained. The heterogeneous and variable composition of surface ligands, partly under physiological control, cannot be rigorously characterized using conventional means. The uncertainty associated with both regressions using all the data is a direct result of the antagonistic action of individual metals on cellular metabolic processes, hence modifying uptake. These results clearly demonstrate

the inadequacy of applying simple regression models to simulate and predict metal - phytoplankton interactions.

Physiological influences on the binding of metals and inherent feedback mechanisms resulting from any deleterious effects exerted by the metal also, violate the assumptions around which simple adsorption isotherms are formulated. It is essential that physiological effects of the metal be rigorously evaluated under the conditions used in metal uptake experiments. Only when the biological interactions are fully understood can adsorption isotherms be applied to the data. The transient nature of metal uptake and apparent, short term, desorption (biphasic uptake kinetics) reported in the literature result from changes in the physiological condition of the population under metal stress.

Considerable caution is required in comparing results from short term uptake and long term growth experiments. Physiological readjustment or adaptation to the imposed stress, and changes in the available metal concentration over time may confound observed changes in the growth repsonse and levels of metal accumulation (also influenced by the level of cell debris) and lead to erroneous interpretations and conclusions. Physiological stress during short term investigations may also significantly alter the results. Copper and iron, for example, interacted synergistically on short term rates of respiration in Chlorella (Hassall, 1963) but were antagonistic towards growth of the same species (Steemann-Nielsen and Kamp-Nielsen, 1970). The observed individual and combined effects of cadmium, nickel, and mercury on Anabaena were dependent upon the order of

addition, response measured, and the relative concentrations (Stratton and Corke, 1979). The experimental design must be compatable with research objectives, both of which must be clearly stated (see Stockner and Antia, 1976).

IV.I.V. Membrane Structure and Transport Processes

It is evident from the preceeding discussion that the cell surface contains a variety of binding sites with varying affinities for individual metals. I have intentionally ascribed reactions at the cell surface to properties of the cell wall without direct reference to interactions at and within the membrane. Proteins bound to the external regions of the plasmalemma and within the membrane matrix, and reactive groups associated with the lipids, can participate in complexation reactions. These reactions, however, cannot be separated from interactions with cell wall components. My research did not directly concentrate on membrane reactivity and transport processes, but knowledge of the structural and functional relationships of membrane components is essential in the interpretation of the results from toxicological investigations. These relationships, including transport processes, are discussed in this section. Basic principles of diffusion are reviewed and applied to observations on heavy metal - phytoplankton interactions in the process of characterizing mechanism(s) regulating transport of metals into the cell.

Algal cell membranes are primarily composed of lipids and proteins with smaller quantities of carbohydrates and RNA (Mahler and

Cordes, 1971). The structural orientation and properties of membrane lipids provide a permeability barrier selective to various solutes. Membranes also contain numerous enzymes and active uptake systems for a number of nutrients which, with other membrane components, can be uniformly distributed or occur in patches on the cell surface. Clustering of components may promote highly co-operative structural and conformational changes affecting membrane function. The multidimensional anisotropic characteristic of membranes is evident from studies on acid/base secretion in Characean cells (Spear et al., 1969; Smith, 1970), coccolith formation, silicification, and calcification (Darley, 1974). The heterogeneous composition and reactivity of the plasmalemma arises from the diversity of transport functions and the continuous turnover of membrane components during growth.

Mechanisms that have been proposed for transport of solutes across the cell membrane involve either passive or active processes. Passive transport includes both simple diffusion and mediated transport driven by diffusive forces (Wheeler, 1981). Simple diffusion is dependent upon the solubility of the solute and concentration gradient across the membrane. Selective pores within the membrane (negatively charged or neutral) may also participate in simple diffusion (Mahler and Cordes, 1971). Carrier molecules that are confined to the membrane and can translocate from side to side complexed with the solute or unbound (polar or neutral carriers respectively) mediate passive transport driven by the same forces as simple diffusion (Wheeler, 1981). In principle, active transport

across this barrier requires energy and may be facilitated by specific transmembrane bound systems and involve carrier molecules (Wheeler, 1981). Although formation of micropinocytotic vesicles in algae has never been reported, an endocytotic mechanism for incorporation of metals (free, complexed, or precipitated) could provide an additional transport process. Metals binding to specific regions of the membrane may result in conformational changes and structural reorientation promoting endocytosis.

Transport of metals into the cell may be facilitated by a number of mechanisms operating simultaneously, all of which are not fully understood. Metals may disrupt membrane function, directly interfering with nutrient transport systems or by altering the structure and permeability of the membrane. The primary modes of toxic action for mercury and copper are believed to operate at the membrane level. Mercury completely inhibited short term rates of ^{14}C uptake in Chlamydomonas (Fig. V), also observed by Overnell (1975a) in the same species, and in Atheya decora (Overnell, 1976). Mercury must exert a deleterious influence on membrane function to account for the rapid and complete inhibition of photosynthesis. Competitive interactions between arsenic and phosphorus (Blumm, 1966; Planas and Healey, 1978; Klumpp, 1980), selenium and sulfate (Shrift, 1954; Wheeler et al., 1982), along with copper and silicon (Morel et al., 1978; Fisher et al., 1981; Reuter et al., 1981) provides additional evidence for the effects of some metals on membrane function and possible transport mechanisms for individual metals across the plasmalemma.

Previous investigators concluded that alterations in the

structure and permeability of algal membranes resulting in cation loss was a principal mode of toxic action for various metals (Hassall, 1963; Kamp-Nielsen, 1971; Sheih and Barber, 1973; Overnell, 1975b; Nakajima et al., 1979b; Mierle, 1981). Davies (1976) however, did not find significant losses of intracellular potassium in Isochrysis and Duneliella incubated with mercury and ascribed previous observations of cation loss to severe experimental conditions required to cause the effect (high cell density and metal concentrations). Potassium loss in Chlorella exposed to twelve different metals, including organic derivatives, was minimal and the observed toxicity (or resistance) did not result from gross changes in cellular potassium (De Filippis, 1979). Sheih and Barber (1973) reported similar observations for the effects of mercury on potassium exchange in Chlorella. Copper induced potassium and magnesium loss in Scenedesmus was reduced in the presence of calcium which also enhanced sodium uptake (Mierle, 1981). Metal induced cation losses may involve a number of different processes and the results can be heavily dependent upon the composition and concentration of the assay medium and physiological condition of the test population.

Transport of metals into the cell is believed a result of processes driven by diffusive forces. Metal salts are, in general, insoluble in nonpolar solvents and carrier mediated transport or the formation of lipid soluble complexes (inorganic or organic) are the most probable uptake mechanisms. Solubility of the metal within the membrane lipid phase, or lipophilicity, is important in regulating transport into the cell and possibly, in the bioaccumulation of

various metals. Organo-metallic derivatives of tin (Wong et al., 1982) and mercury (Overnell, 1975a,b) are relatively more toxic than inorganic species. These processes are dependent upon forces regulating simple diffusion and are consistent with previously reported observations on metal uptake kinetics. Davies (1976) incorporated aspects of these arguments to explain mercury tolerance in Dunaliella. He postulated that tolerance in this species was due to reduced rates of transport into the cell coupled to a cellular detoxification mechanism, possibly precipitation in the membrane. Complexation or precipitation reactions near the membrane surface may facilitate transport into the cell over temporal scales consistent with lags in the growth response (d). Clearly, factors regulating diffusion of metal into the cell must be examined in greater detail. Simple calculations, based on the principles of diffusion, are presented in the following discussion to illustrate the potential importance of this process in regulating metal transport into the cell.

A concentration gradient across the membrane must be established to facilitate diffusion. Effects of individual metals on sensitive metabolic systems within the cytoplasm, including organelles, can indirectly disrupt the concentration gradient by reducing the total quantity of metal bound (e.g. physiological control). Assuming a steady state, average cellular concentration for a metal essential for growth of roughly 10^{-19} moles (rough estimate for illustrative purposes) and a mean cell volume of 100 μm^3, the equivalent volumetric concentration is 1.0 μM. Consequently, the

available metal concentration at the cell surface must exceed 1.0 μM to establish a diffusion gradient. The surface area of a cell with a volume of 100 μm^3 is approximately 100 μm^2, and with an average cell wall thickness of 0.1 μm the required surface concentration is 10^{-20} moles/cell (10^{-6} moles/L X 10^{-15} L/μm^3 X 10 μm^3/cell, the volume of the cell wall). Maximum surface binding capacities for zinc ranged from 0.1 to 2 X 10^{-15} moles/cell (Table XIII) or roughly five orders of magnitude greater than the surface concentration required to establish a concentration gradient under these conditions. Preferential and non-exchangeable binding of metals to the fraction of specific sites on the cell surface, even under low ambient metal concentrations, could also create and maintain a significant gradient. These calculations demonstrate in a qualitative fashion the existence of a gradient at concentrations of essential trace metals normally encountered in natural waters (nM or μg/L range). Consequently, processes driven by diffusive forces may be the principal mechanisms regulating metal uptake in phytoplankton. Metal uptake may also be enhanced by interactions with membrane bound systems involved in nutrient and ion transport (alternative, as opposed to specific, active uptake systems), all under various degrees of physiological control.

There is, however, some question as to the validity of applying concepts of diffusion de facto. Possible mechanisms driven by diffusive forces and compatible with the observed temporal kinetics of uptake include; 1. carrier mediated transport by systems confined within the membrane, 2. formation of lipid soluble complexes

(inorganic or organic) within the cell wall/membrane region, and 3. specialized pores in the membrane. If the diffusive flux is determined by the concentration gradient across the membrane then a constant uptake rate dependent upon the magnitude of the external surface concentration will be observed. The internal free metal concentration is assumed negligible until all sites are saturated, most likely at the point of cell lysis or death. A diffusive flux resulting from a charge imbalance across the membrane can, to a degree, be compensated for by the cell (cation pumps) but requires 1. high transport rates across the membrane, and 2. significant changes in the ionic composition of the cell. Solubility of the metal within the plasmalemma limits transport into the cell. Also, the cell can easily accomodate for any charge imbalance if one considers the binding of metal to cell components that are continually synthesized during growth (cell proteins and low molecular weight compounds) in conjunction with the net influx of metal. A more intensive theoretical analysis and experimentation are required to understand metal - phytoplankton interactions at the cell surface/solution interface and processes regulating uptake.

IV.I.VI. A Conceptual Model of the Cell Surface

In previous sections I discussed the reactivity of the cell surface and factors that directly, or indirectly, influence the availability of metals towards phytoplankton. These concepts are outlined in Figure XVI. The available metal concentration in solution

(M_a) is dependent upon the presence or absence of other metals/ligands and rates of interconversion between complexed species. Metal bound to the cell surface is composed of two fractions involving different classes of ligands. One fraction, M_i, specifically and irreversibly binds individual metals. The other fraction, M_e, is readily exchangeable with other cations in solution (M) and is more sensitive to changes in surface reactivity, indirectly under physiological control (e.g. solute fluxes across the membrane).

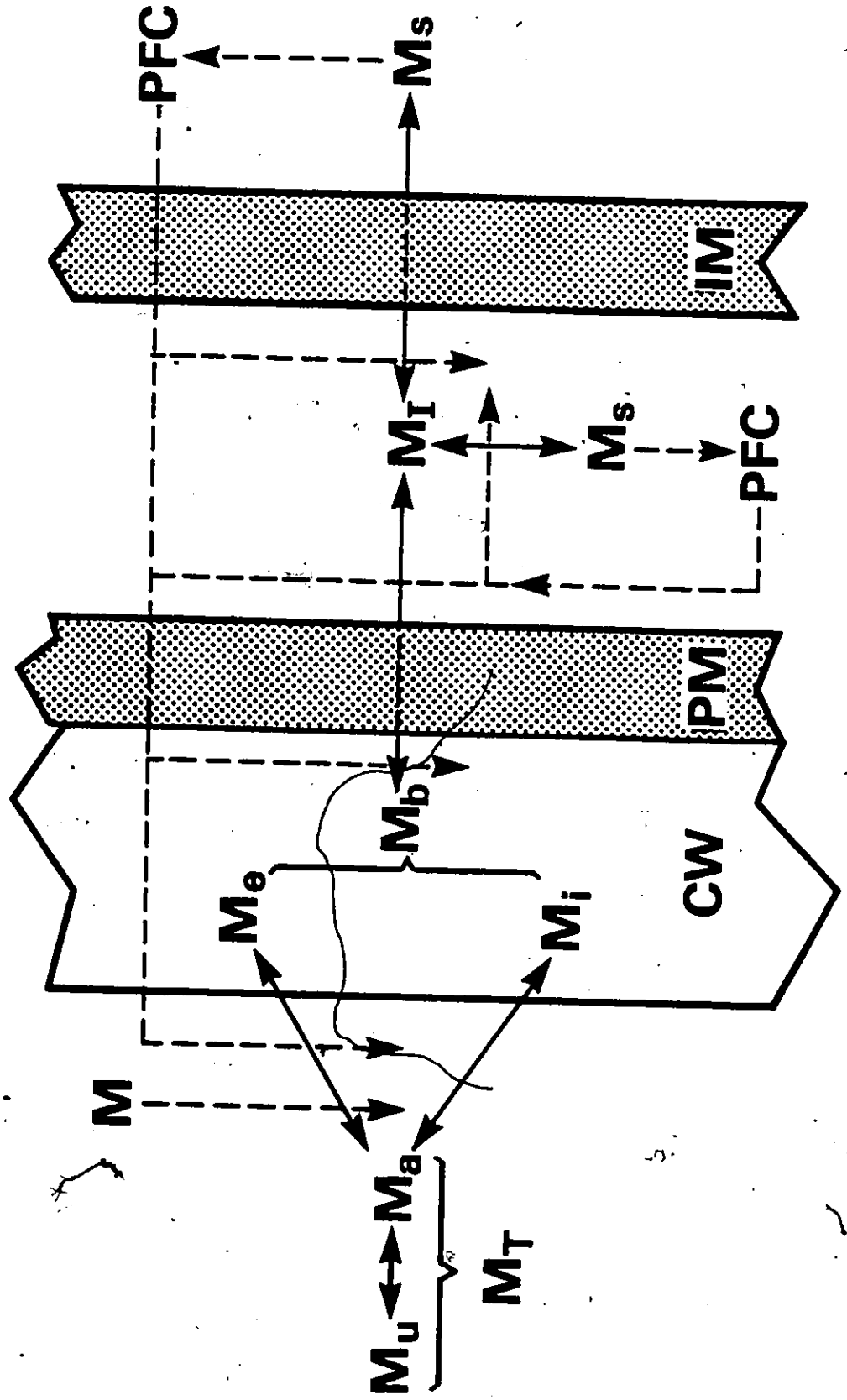
Transport of metal into the cell may be facilitated by a carrier mediated system driven by diffusive forces and may also involve direct intervention with specific nutrient uptake systems. Both fractions of the total metal bound maybe transported into the cell. Non-exchangeable binding to perhipheral proteins adjacent to the external surface of the membrane, for example, could promote conformational changes resulting in greater solubility within the membrane and uptake of the metal.

Possible feedback mechanisms influencing binding and transport of the metal are also included (dashed lines Fig. XVI). The physical regime (light and temperature for example), or the antagonistic action of the metal on the cell (site of action M_s), can influence the physiological condition of the organism, in turn, binding of metal to the cell surface. Other metals in solution can also reduce the extent of binding through competition for available surface sites.

Primary modes of toxic action (M_s) exerted by any metal on a cell cannot be resolved because of the complex interdependent interactions between the metal and the cell. Indirect effects arising

Fig. XVI. Conceptual Model for the Cell Surface

A conceptual model for the cell surface is presented, compatible with my results and with those reported in the literature. The total metal concentration near the cell (M_T) is composed of two fractions; 1. complexed and unavailable (M_u), and 2. available metal (M_a). Metal associated with the cell surface is composed of a loosely bound, or exchangeable fraction (M_e) and a non-exchangeable, specifically bound fraction (Mi). Bound metal ($M_b = M_i + M_e$) is transported into the cell and to the site of toxic action (M_I represents the internal metal and M_s is the metal at the site of toxic action). Physiological control (PFC or physiological feedback control mechanism) over surface reactivity determines the extent of binding (M_e fraction) along with the presence of other metals (M) in solution competing for available surface sites. The antagonistic action exerted by the metal may also influence the extent of binding to the cell surface. These interactions are represented as broken lines. Other notation is as follows: CW, cell wall; PM, plasmalemma; IM, internal membrane system.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from the cell attempting to compensate for the imposed stress may produce other chemical or physiological imbalances creating additional stress. Changes in the chemical composition and metabolic activity are manifested over time into an observed response and, consequently, obscure resolution of the principal site of toxic action. Until specific sites are identified, the influences of toxic substances on phytoplankton can only be interpreted as a general decrease in the level of metabolic efficiency, or the cell's ability to translate available resources into growth. Conferring on an organism the ability to synthesize specific metal uptake systems or internal detoxification mechanisms is unrealistic, especially from results obtained under severely perturbed conditions. Drastic changes in the physico-chemical environment arising from addition of a metal or even transfer of cells to fresh media cannot be predicted by the organism, only compensated for.

This model is compatible with the information presented in previous sections and establishes a theoretical framework for identifying areas requiring additional investigation. In the following section, kinetic interactions between variables and processes outlined in the model are discussed.

IV.II. Temporal Aspects of Heavy Metal - Phytoplankton Interactions

Phytoplankton cannot predict, only compensate for fluctuations in the external physico-chemical environment. This was clearly demonstrated in recent time series analyses of phytoplankton

population dynamics (see references by Harris cited in Harris, 1980a). Physiological readjustment to changing environmental conditions produced characteristic lags in the observed species abundance dependent upon the magnitude and frequency of the perturbation (c.f. Harris, 1980a). Consequently, reproductive success (or survival) is dependent upon the cell's ability to integrate available resources into growth and maintenance of basal metabolic processes. Any factors that directly, or indirectly, alter the ability of the cell to utilize available resources is manifested over time into a lower growth rate. The growth rate, in turn, provides a functional time frame on which to identify and relate important factors regulating the availability of metals in solution, binding to the cell surface, and uptake in culture and in situ.

Growth rates for monospecific populations in culture range from 0.10 to 4.0 doublings per day (Table XI, Jorgensen, 1979). These are average values for the development of entire populations and provide no information on life cycle times for individual cells. Epigenetic variability and differences in the age structure of a population could lead to significant asynchrony in life cycle times, even within synchronous populations (Campbell, 1957; Tamiya et al., 1961; Hoogenhout, 1963). Estimates for in situ growth rates are complicated by the temporal and spatial heterogeneity characteristic to all ecosystems. Sampling and enumeration errors (Butterwick et al., 1982) and losses through sinking or grazing further confound attempts to obtain accurate estimates for in situ growth rates. Literature values for a number of species range from 0.01 to 1.0 doublings per day

(Jorgensen, 1979; Round and Chapman, 1982). Calculated growth rates for phytoplankton in the large volume enclosures ranged from 0.1 to 0.3 doublings per day (Section III.II.III.). Similar values were obtained from the chlorophyll, carbon, and particulate data. The uncertainty associated with in situ growth rate estimates may also, reflect the ability of individual species to utilize available resources in a non-steady state, variable environment.

Zinc bound to the surface of Chlamydomonas and available zinc in solution equilibrated within a 1 to 2 h period (Fig. X). Similar results have been reported for other metals and species of phytoplankton (Section I.I.II.). Consequently, binding of metals to the cell surface (including surfaces of solids and semi-solids, and complexation reactions with other ligands in solution) can be considered instantaneous relative to the growth rate. The magnitude of the stress is determined by the surface concentration (functionally related to the available metal concentration), the level at which the metal disrupts normal cell function (membrane or internal sites of toxic action), and the ability of the cell to compensate for the imposed stress over time. Any factors that directly, or indirectly, alter the magnitude of the imposed stress within the generation time can also influence the nature of the observed response. For example, the formation of cell walls and membranes in the course of cell division, and loss of the old mother cell wall, can directly alter the level of metal stress at the cell surface. The quantity of metal bound to the cell surface can also be reduced in the presence of other metals/ligands, or through reductions in the available metal

concentration in solution over time.

Factors regulating the available metal concentration, or cell burden, in culture and in situ involve similar processes but operate over different temporal scales. The effects of a changing available metal concentration and the ability of cells to compensate for the stress over time were clearly illustrated in the different growth responses for phytoplankton populations in culture (Type I and II, extended lag phase and a gradual reduction in cell numbers over time respectively, Section I.II.II.). The relatively high growth rates for phytoplankton in culture; in turn, can significantly alter the available metal concentration over short time periods (d). Under these conditions, binding of metal to newly formed cells and debris (old cell walls and organic matter from cell lysis) are the principle mechanisms regulating the magnitude of the stress (cell burden) over time. Metabolic transformation of the metal into less toxic derivatives (mercury volatilization for example) may also be an important factor of consideration for individual metals. Losses of metal from solution (formation of precipitates or binding to the walls of the experimental flask) can also reduce the available metal concentration, particularly in poorly buffered systems.

Phytoplankton growing in situ can be exposed to large fluctuations in the surrounding physico-chemical environment within the generation time (1 to 10 d). Diel changes in the chemical composition and physiological activity of phytoplankton are well documented (refer to Frempong, 1981) along with short term light dependent variations in chloroplast function and structure. Due to the

interdependence of interactions between all physical, chemical, and biological factors in aquatic systems, the magnitude and duration of the imposed stress cannot be fully resolved. Consequently, direct cause and effect relationships for the level of metal contamination (or any toxicant) on biological processes are obscured by the variable nature of aquatic systems. Factors regulating the cycling and distribution of metals in situ must be examined and related to processes operating over relevant cellular scales. The greatest short term impact (within the generation time) for non-steady loadings of metals will be in the upper surface waters associated with the region of maximum biological production. My large volume enclosures were designed to simulate conditions encountered within the upper surface waters or epilimnion.

IV.II.I. Cycling and Distribution of Metals *In Situ*

The capacity of any aquatic system to assimilate inputs of substances from anthropogenic sources is heavily dependent upon the total level of biological production. Hamilton Harbour is a hyper-eutrophic body of water with chlorophyll levels frequently exceeding 20 µg/L during bloom formation (Harris *et al.*, 1980b). The high level of biological activity in this system, combined with a high concentration of suspended solids and a variable physical regime, may significantly reduce the potential toxic properties of individual metals (magnitude and duration of the stress). Changes in the dissolved and particulate metal concentrations over time provide

insights into the relationship between the assimilative capacity of natural waters and the importance of biological processes.

Sedimentation and growth of populations suspended in the water column were the principal mechanisms regulating the cycling and distribution of the heavy metals added to the enclosures. Losses of dissolved metal resulted from either adsorption onto larger particles or through the formation of precipitates, aggregrates, or dispersed colloidal systems that could be retained on a glass fiber filter. Direct sedimentation of dissolved metal was unlikely due to the well mixed nature of the enclosures and the absence of distinct vertical gradients in all biological and chemical parameters measured. Increases in the particulate Cd, Co, and Ni concentrations over time provided some indication for the degree of vertical turbulence within the enclosures. Adsorption of metals onto the walls of the enclosures may have been an important sink for Cu, and to a lesser extent Cd and Zn, but not Co, Ni, and Sr. Also, the average total concentration of each metal bound to the walls of the enclosures did not exceed 1% (5% for Cu) of the maximum total metal concentration in the water column.

The relative changes in the level of biological production and both fractions of each metal suspended within the water column over time provided some evidence for the direct sedimentation of a portion of the total particulate metal. Particle counts, cell counts, total carbon, and chlorophyll levels roughly doubled during the first two weeks of the experiment. A similar increase was not observed for the particulate concentrations of all metals, except Co. These results can be explained by the dilution of the particulate phase through

biological growth and the sedimentation of metal bound to particulates. Changes in the particulate Ni and Cd concentrations over time supported this hypothesis. The significant increase in particulate metals following aeration of the enclosures and mixing from the storm on August 27-29 confirmed that particulate metal had sedimented out of the water column. No changes in the dissolved metal concentrations were observed during this period. The importance of biological production and sedimentation in regulating the particulate metal concentrations, however, could not be fully separated with the available data.

Particulate zinc concentrations were relatively constant throughout the study period and were not correlated to changes in the fraction of dissolved zinc. The relatively constant particulate zinc concentration could have resulted from the non-exchangeable binding of this metal to a fraction of the particulate matter retained within the water column. Zinc was classified as "borderline" by Nieboer and Richardson (1980), with an affinity for oxygen, sulfur, and nitrogen containing ligands intermediate to other transition metals. Clearly, this metal may associate with a wide variety of organic and inorganic binding agents. Binding and release of zinc was unlikely because the dissolved zinc concentrations decreased steadily over time. Any recycle mechanism for zinc must involve bound metal, possibly associated with a smaller particle size with a low settling velocity, but large enough to be retained on a glass fiber filter. Also, particulate zinc concentrations were not influenced by aeration of the enclosures and mixing during the storm on August 27-29. This result

confirmed that particulate zinc was not sedimented out during the experimental period. Clearly, mechanisms regulating the cycling and distribution of zinc within the water column require further investigation.

Residence times (time to reduce ambient disolved metal concentrations by 50%) for metals in the dissolved fraction were consistent between both enclosures treated with metals and containment effects were assumed, at least, reproducible. Although all metals were added simulataneously, the calculated residence times for individual metals were significantly different. The order of increasing residence times was consistent with the Irving-Williams series (Irving and Williams, 1953) along with results from investigations on the complexation of metals with organic substances in natural waters (Mantoura and Riley, 1975; Mantoura et al., 1978; Vuceta and Morgan, 1978; Kerndorff and Schnitzer, 1980; Nriagu and Coker, 1980). A similar relationship between residence times and various physico-chemical properties of different metals was reported by Jackson et al. (1980).

Hesslein et al. (1980) reported residence times of 25 and 20 days for zinc and cobalt in an oligotrophic lake (Lake 224, ELA in NW Ontario) dominated by Chrysophytes with an average summer chlorophyll concentration of roughly 1.5 µg/L. In a related study on soft water lakes (Lakes 303 and 223, ELA in NW Ontario) using 1 and 25 m^3 limnocorals, residence times of 6.5 days for cobalt and 8.5 days for zinc were observed (Jackson et al., 1980). Lowering the pH from 6.8 to 5.1 reduced rates of metal adsorption to organic matter in the

sediments, increasing the residence times for cobalt and zinc in the water column on the order of 1.1 and 1.3 times, respectively (Schindler et al., 1980). Residence times for copper in shallow eutrophic prairie "potholes" ranged from 1 to 7 days (Wagemann and Barcia, 1979) and in a mesotrophic reservoir 50 % of the copper added was removed in roughly 12 days (McKnight, 1981). Chlorophyll concentrations in the reservoir study ranged from 4 to 20 µg/L and roughly two months were required to obtain a homogeneous vertical distribution of copper.

The large differences in reported residence times resulted from the methods used to separate particulate and soluble metal fractions, enclosure effects, and the relative concentration of particulate matter in the water column. Large organics, including fulvic and humic acids, that pass through glass fiber filters could be retained on cellulose acetate filters with a more defined pore size. Pretreatment of the filter could also influence the results. The glass fiber filters enabled me to separate relatively large sample volumes and provided a highly concentrated particulate fraction for metal and carbon determinations. Higher concentrations of suspended solids in Hamilton Harbour waters resulted in lower residence times compared to data from low alkalinity, oligotrophic lakes. The relative order of residence times for individual metals in all systems, however, indicated a common removal mechanism dependent upon the reactivity of each metal (affinity for particulates). The principal removal mechanism for metals in lakes involves sedimentation and the rate is dependent upon particle size and composition, and the degree of

vertical turbulence, or stability of the water column.

Irrespective of the variability in the data, the results clearly illustrated the importance of biological factors in regulating the distribution and cycling of metals in situ. De Haan et al. (1981) reported that the metal binding capacity was greatly affected by population changes in abundant bluegreen algal species. Significant correlations between the binding of some toxic metals and the occurrence of certain species of green algae were observed by Briand et al. (1978). These species, however, only accounted for a fraction of the total algal volume and the dependence of metal binding on the periodicity of abundant algae requires further investigation.

The initial distribution of dissolved and particulate complexing agents and planktonic organisms determines the magnitude (partitioning and cell burden) of metal stress. Removal of metal from the water column is dependent upon sedimentation and dilution through growth of planktonic organisms. Consequently, the duration of the stress is determined by sedimentary processes and contributions from the biota to the particulate phase. These factors also influence the magnitude of the stress, which decrease with time following short term, impulse loadings. The affinities of metals for particulates (also related to the toxicity) determines the residence time in the water column. The ability of individual cells to compensate for relatively short term exposures to elevated metal concentrations (time of addition) will depend upon the level at which the metal interacts with the cell (membrane or internal sites of toxic action).

The increase in particulate metal over time resulted from the

adsorption of dissolved metal onto particles or through the formation of substances large enough to be retained on a glass fiber filter. Metal bound to large particulates was essentially removed from the water column (unreactive) because large changes in particulate metal during mixing did not result in a corresponding increase in the dissolved metal concentration. The biologically available fraction of the total metal concentration in solution could not be fully resolved, but was most likely associated with the dissolved phase.

Growth and processes regulating transport of phytoplankton may provide important dispersal mechanisms for contaminants in aquatic systems, particularly in the case for zinc. The non-homogeneous distribution of biological variables determines, in part, the heterogeneous distribution of PM and FM concentrations and the impact of short term exposures to elevated metal concentrations (initial partitioning). Due to the importance of biological variables in the bio-geochemistry and cycling of metals, simple partition, mass balance models based solely on the relative distributions between particulate and dissolved fractions are inadequate. A dynamic model, incorporating biological processes combined with chemical kinetics and transport rates, has been developed to simulate the effects of metal stress on phytoplankton growth and metal uptake, and is presented in the next section. The theoretical basis for the model was derived from concepts developed in the preceeding sections and provides new insights in the time dependent, dose/response relationship under culture and <u>in situ</u> conditions.

V. GROWTH MODEL

Toxicological research is primarily descriptive and few attempts have been made to develop quantitative models of heavy metal - phytoplankton interactions. This deficiency is surprising considering the wealth of available information and the abundance of mathematical models simulating nutrient phytoplankton dynamics and growth. In previous sections I demonstrated the inadequacy of applying simple regression models and the inconsistencies associated with correlating various degrees of observed "toxicity" to physico-chemical properties and reactivities of individual metals. Kaiser (1980) used multiple linear regressions incorporating ion specific physical and chemical parameters for predicting metal toxicity to aquatic biota. Fisher and Jones (1981) provided a similar relationship based on the solubility products of metal sulfides. Other investigators have used more empirical methods to relate total metal or pesticide concentration, or free metal ion concentration to the level of growth inhibition or some other physiological parameter (Fitzgerald and Faust, 1963; Erickson et al., 1970; Carter and Cameron, 1973; Gillespie and Vaccaro, 1978; Sunda and Gillespie, 1979; Walsh and Alexander, 1980; Borgmann, 1981). All these relationships are based on results obtained from single metal batch culture bioassays over a predetermined time interval and cannot be universally applied without understanding the temporal sequence of events and processes responsible for producing the effect. In previous sections, I

discussed the complexity of interactions between different metals and the physiological condition of the cell in determining the kinetic characteristics of the dose/response relationship. The ability of cells to compensate for perturbations in the physico-chemical environment, severely restricts the predictive capacity and utility of these time invariant regression models, or the empirical approach in general.

In the process of developing quantitative mathematical models one is required to critically examine and formally define all potential mechanisms and interactions operating within the system. Known physico-chemical properties and biological processes can provide the framework for model construction, particularly for defining kinetic interactions between system compartments. A theoretical approach may not be entirely realistic but can provide insights into dominant system interactions. The objectives must be clearly stated and the utility of any model depends heavily upon how the system is defined (perceived), including variable interactions. Calibration and verification of the model's response can provide, through an iterative process, a critical assessment and validation of the assumptions and conceptual framework inherent to the design of the model and form a basis for additional experimentation.

Davies (1973,1974), Bates et al. (1982), and Jackson and Morgan (1978) have made significant contributions in developing quantitative models of metal - phytoplankton interactions. Different interpretations of the structure and reactivity of the cell surface prevent direct comparisons between these models and the one presented

here even though similar relationships are used to describe binding of metal to the cell surface, uptake, and cell growth. In the light of my results previous assumptions are not entirely valid. My primary objective in developing the model is to provide a formal, mathematical representation of important processes regulating metal - phytoplankton interactions compatable with results from my work and reported in the literature. The model in its present form can be incorporated into more complex models to simulate the effects of one or more metals on different populations, in turn community structure, or further refined to include aspects of solution chemistry (speciation and competition for available sites on the cell surface).

Construction of the model, including variable interactions and formulation of model components, is presented in the next section. Results from the sensitivity analysis and the general response characteristics of the model are discussed in the second section. Absolute verification of the model is, at present, not possible due to the lack of available and reliable data. In previous sections I discussed the importance of the generation time in controlling observed phytoplankton population dynamics, along with kinetic interactions between variables regulating the availability of metals in solution. All interactions incorporated into the model are grouped into one of three time frames including; 1. instantaneous processes, 2. intermediate processes and time to elicit a response, and 3. long term or slow processes. The temporal limit of resolution imposed on the model is one hour (intermediate time frame) in line with the time required to reach an equilibrium between the metal in solution and

bound to the cell surface. Complexation reactions involving simple inorganic ligands and binding to the cell surface are assumed instantaneous relative to this time scale. High frequency environmental fluctuations (< 1 s in duration) are dampened by feedback systems and the interdependence of cellular physiological processes. This buffering mechanism also provides a lower limit to the kinetic components of the response. On the other hand, reductions in the available metal concentration resulting from precipitation reactions or adsorption of metal onto the walls of an experimental flask operate over much longer temporal scales. These processes are represented by a linear removal mechanism (constant loss rate) and are not significant over a one hour period.

V.I. Model Formulation

The two most important factors which determine the ultimate response of phytoplankton to heavy metal stress are the generation time and net flux of metal into the cell. The rate of incorporation is dependent upon the concentration at the cell membrane. This concentration is regulated by the binding of metal to the cell wall and subsequent diffusive flux through the cell wall matrix (including organo-metallic complexes, hydrated ions, etc.). The nature of the cell wall surface and associated structures, and interactions between the metal and other metals/ligands in solution can all influence binding and uptake. The metal becomes lethal when the diffusive inward flux exerts a deleterious influence upon the cell, culminating in a

net increase in the doubling time or cellular death. This antagonism along with other equilibrium shifts in solution yields a variety of growth (yield) responses (presented in Section I.II.II.). Specific modes of toxic action are excluded from the model, along with changes in cell volume resulting from the uncoupling of growth and division, direct pathological influence on cell morphology, or through alterations in the chemical composition of the cell. Volume changes have been reported for a number of phytoplankton exposed to various degrees of metal stress (Shrift et al., 1959a,b; Erickson, 1972; Davies, 1974,1976; DeFilippis and Pallaghy, 1976a; Bentley-Mowat and Reid, 1977; Berland et al., 1977; Christensen et al., 1979; Li, 1979; Rivkin, 1979) and the increase in surface area could significantly modify the extent of adsorption. No information is available, however, on the physiological condition of these cells, or the actual mechanisms involved. Significantly differences in weight, volume, sulfur and nitrogen content, and endogenous respiration were initially observed in cultures of Chlorella exposed to selenomethionine (Shrift et al., 1959a,b). Over time, however, the cells resumed normal growth and the composition and physiological condition of the populations were indistinguishable from the controls. This work of Shrift et al. (1959a,b) provides additional evidence for cells directing all metabolic activity towards growth and emphasizes the necessity to delineate between long and short term algicidal effects. Due to the interdependence of binding and the level of cell metabolism (e.g. membrane fluxes influencing the physico-chemical properties of the cell surface), aberrant morphologies are assumed insignificant in the

observed growth response.

Model compartments and system interactions are illustrated in Figure XVII and all notation is defined in Table XXII. The size of each compartment may vary over time or remain constant depending upon the settings for individual parameters. A number of processes regulating the availability and uptake of metals have been intentionally simplified to eliminate partial redundancy and minimize the total level of uncertainty. Uptake, for example, is described as a linear first order process in line with a one hour time step and includes metal interactions at the cell solution interface. More elaborate expressions for metal speciation in solution, competition between metals for surface sites, and diffusion into the cell can be included. However, the lack of information on the nature and reactivity of the cell surface and mechanisms regulating transport into the cell equally justifies the more simplified and empirical approach.

Growth of the test population is described by the following logistic equation:

$$dN/dt = r\ N\ (1 - N/P)\ (1 - M_c/M_m) \qquad (13)$$

N represents the cell density with a growth rate r (doublings/hour) which is influenced by both the internal cellular metal concentration (M_c/M_m where M_c = the internal cellular metal concentration and M_m = the internal cellular metal concentration at which growth is inhibited) and a density dependent factor, P. Davies (1973,1974) used

Fig. XVII. Schematic Diagram of Model Compartments and System Interactions

This diagram outlines the basic processes and interactions incorporated into the model. Metal was partitioned into three compartments, normalized to a standard volume of 1 ml, and represented by: 1. available metal (M_f); 2. metal associated with the cells ($N M_c$, cell density times the average cell metal content); 3. metal bound to debris (determined by $K_d D M_f$, the distribution coefficient times the debris concentration and the available metal concentration). All possible interactions were included in the model, along with mechanisms that could either directly or indirectly modify each process. For example, the physical environment influences cell metabolism and indirectly alters the extent of metal bound to the cell surface and growth. Reduced growth also results in a lower net rate of increase in the particulate concentration (cell debris). Partitioning of metal between all compartments was instantaneous (complexation and exchange reactions were assumed complete over a one hour time step) and reversible. The size of each compartment, or the quantity of metal associated with cells, debris, or available in solution may change with time.

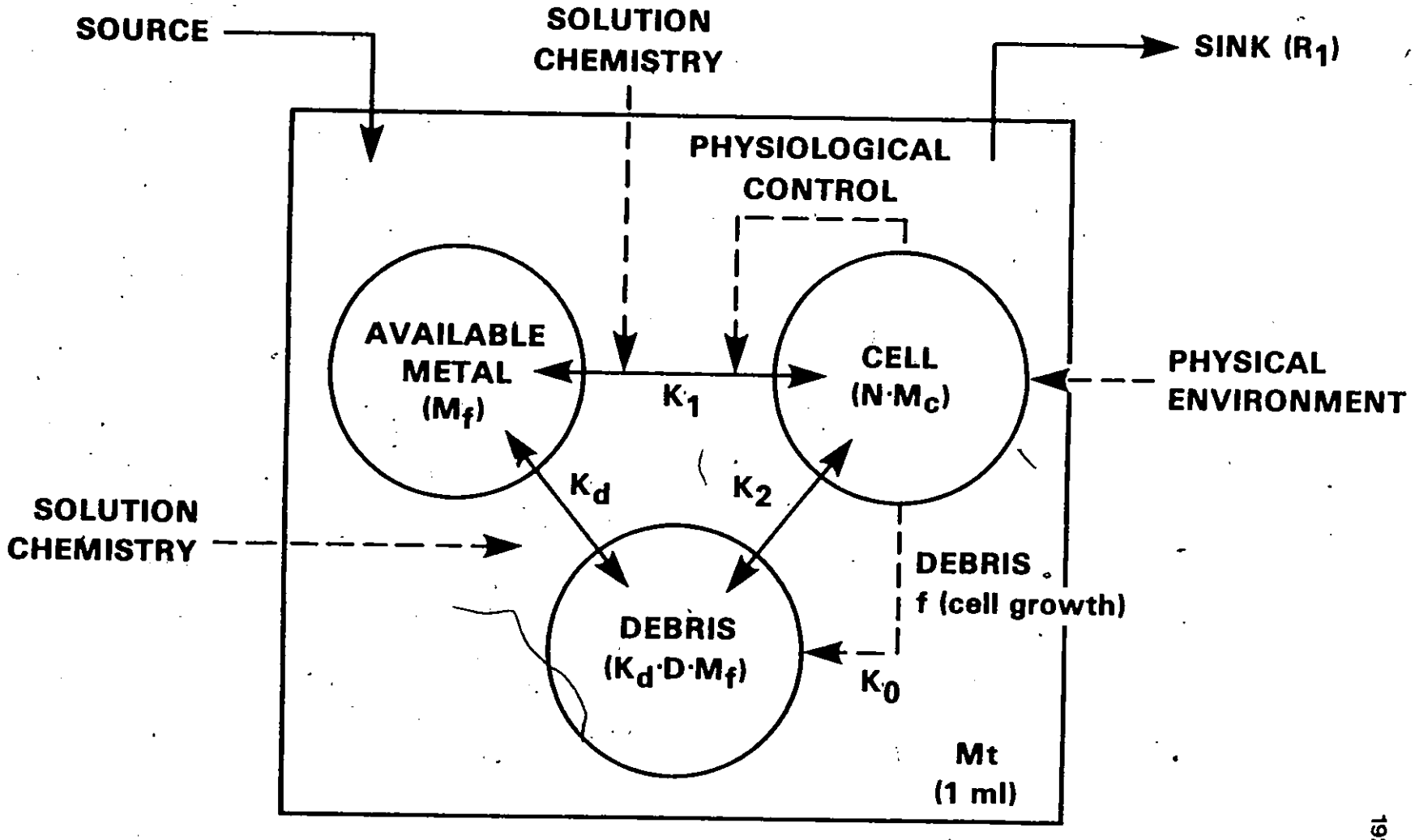

SOURCE
SOLUTION CHEMISTRY
SINK (R_1)
PHYSIOLOGICAL CONTROL
AVAILABLE METAL (M_f)
K_1
CELL ($N \cdot M_c$)
PHYSICAL ENVIRONMENT
K_d
K_2
SOLUTION CHEMISTRY
DEBRIS f (cell growth)
DEBRIS ($K_d \cdot D \cdot M_f$)
K_0
Mt (1 ml)

Table XXII Notation, Definition, and Initial Values for Model Coefficients

Symbol	Definition	Range	Units
N	cell concentration	10^3 (t=0)	cells/ml
P	carrying capacity	10^6	cells/ml
R	growth rate	0.005 - 0.1	dbl/h
t	time increment	1	h
D	debris concentration	0 (t=0)	μm^2/ml
K_0	conversion factor cells to debris	25 - 100	μm^2/cell
K_d	distribution coefficient for metal associated with cellular debris, (metal/debris)/(metal in solution)	10^{-15}	ml/μm^2
K_1	uptake rate	10^{-7} - 10^{-16}	ml/h/cell
K_2	loss constant (dilution)	1 - 2	-
Mc	average cell metal content	10^{-21} (t=0)	moles/cell
M_s	maximum cell metal content	10^{-15}	moles/cell
M_m	toxic cell metal content	0.1-0.001 * M_s	moles/cell
M_t	total metal concentration	10^{-7}-10^{-10}	moles/ml
M_f	available metal concentration	-	moles/ml
R_1	loss constant total metal	0.5-4.75 * 10^{-3}	1/h

a similar equation but omitted a density dependent factor restricting growth and metal associated with the cell was partitioned into two compartments (surface bound and internal). The growth rate (r) was varied between 0.1 to 0.005 dbl/h, corresponding to doubling times in the order of 0.5 to 8 days. These values were obtained from culture investigations and estimates of *in situ* growth rates from the bag experiments and cover literature values under similar conditions.

Values for the initial population and P used in the model were set equal to 10^3 and 10^6 cells/ml respectively. These parameters were not varied because an increase in the inoculum results in a corresponding decrease in the effect exerted by the metal, or a change in the available metal concentration (initial partitioning of the metal or cell burden). The importance of using low initial cell densities is widely accepted for this reason and to avoid complications arising from changing available metal concentrations with time.

Changes in the cellular metal content, M_c, is represented by the following equation,

$$dM_c/dt = M_f K_1 (1 - M_c/M_s) - M_c K_2 |dN/(N\,dt)| \qquad (14)$$

where,

$$M_f = (M_t (1 - R_1 t) - M_c N)/(1 + K_d D) \qquad (15)$$

and,

$$dD/dt = K_0 \, |dN/dt| \qquad (16)$$

Important features of Equation 14 are incorporated into the factors regulating cellular metal levels coupled to the available metal concentration and the relative increase in the population. A decrease in the available metal concentration resulting from uptake by the cells and debris (Equation 15) along with other losses from solution (adsorption onto container walls etc.) reduces the net uptake of metal by the cells. The influence of cellular debris, or exudates, on the available metal concentration has not been quantified but results from other experiments (discussed in Sections I.I.II. and III.I.I.) clearly demonstrated the importance of this compartment.

A linear first order uptake process has been included in the model (K_1). More complicated equations describing diffusion across the cell wall have been used (Davies, 1973,1974,1976; Jackson and Morgan, 1978) but for a one hour time step this relationship is sufficient and in line with temporal components associated with metal binding to the cell surface (assumed instantaneous relative to uptake). Estimates of long term uptake rates are confounded by changes in the population and available metal concentration in solution. For this reason no previous attempts have been made to define or determine the magnitude of this coefficient. Studies on nutrient uptake kinetics provide some information on rate constants associated with facilitated transport of solutes across the membrane. Uptake rates for ammonium and nitrate

ranged from 10^{-14} to 10^{-16} moles/h/cell and for silicon from 10^{-13} to 10^{-16} moles/h/cell but all involve energy dependent active uptake processes (MacRobbie, 1974). Binding of metal to the cell surface, or the fraction associated with the cell, is linearly related to available metal concentration in solution. This result was clearly illustrated for cadmium uptake in continuous cultures (Li, 1980) and is evident from the success in applying equations for hyperbolic saturation kinetics to metal uptake data (the Freundlich isotherm in particular). Consequently, K_1 was varied between 10^{-7} and 10^{-16} ml/h/cell and is interpreted as the fractional uptake of the total metal bound per cell over a one hour increment, directly proportional to the external metal concentration. If, for example, $K_1 = 10^{-7}$ ml/h/cell and the available metal concentration, M_f, equals 10^{-7} moles/ml (1 μM) then the uptake rate equals, $M_f K_1$ or 10^{-16} moles/h/cell. Consequently, uptake is a linear function of the available metal concentration. The upper limit to K_1 is determined by mass balance constraints and values $< 10^{-16}$ ml/h/cell did not effectively reduce growth but lowered the average cellular metal content since dilution exceeded uptake. This parameter was intentionally varied over a broad range to define the limits, or magnitude of this term, and the importance of long term uptake in regulating metal - phytoplankton interactions.

Uptake is restricted by a factor M_s which represents the maximum cellular concentration (complete saturation of all internal sites). There are physical restraints on the magnitude of this value since the total theoretical amount of metal which could fill a volume

of 200 μm^3 (cell volume) is roughly 10^{-12} moles, equivalent to a 5 M solution (calculated using an average crystalline ionic radius of 1.0 nm). Consequently, M_s was set equal to 10^{-15} moles per cell based on the relative chemical composition of the cell (80 to 95% water) and solubility of the metal. The uptake coefficient (K_1) must be less than M_s/M_f because uptake cannot exceed the maximum binding capacity of the cell. The internal metal concentration which reduces growth, M_m (lethal level), is set equal to and varied between 0.1 to 0.001 M_s since uptake can occur when growth has been inhibited. Literature values for the cellular metal concentration inhibiting growth range from 10^{-16} to 10^{-14} moles/cell for a variety of metals and species of phytoplankton (Table XXIII). The uncertainty associated with the level of debris present in the assay medium and experimental conditions under which this information was obtained were minimized by employing low average cell weights and volumes in converting the data. The range over which M_m was varied, however, is in reasonable accord with results from previous investigations.

The available metal concentration (M_f), which in part regulates uptake, is dependent on changes in the total metal concentration arising from adsorption onto cells and debris, along with other losses from solution over time. Under equlibrium conditions the available metal concentration is proportionally related to the total metal concentration according to Equation 15. Complexation by other ligands present in solution are not considered in this model but the appropriate relationships can be incorporated into this equation. Losses of metal as a result of adsorption onto container walls or

Table XXIII Estimates for the Level of Cellular Metal Inhibiting Growth in Various Phytoplankton

(M_m in units of 10^{-15} moles/cell)

Species	Metal	M_m	Comment	Ref.
Scenedesmus	Cu	1-6	40-100% growth inhibition (tolerant/nontolerant strains)	Stokes, 1981
	Ni	1-5	20-100% " "	
	Cd	0.2-5.0	20-100% " "	
	Co	1-50	60-100% " "	
Asterionella	As	> 0.08	no inhibition < 160 PPB	Conway, 1978
	Cd	0.7-1.6	growth rate < dilution rate	
Ditylum	Cu	0.1	decrease in final yield	Canterford et al., 1978
	Zn	0.2	no effect	
	Cd	0.2-0.4	decrease in final yield	
	Pb	0.07-0.2	" " " "	
Phaeodactylum	Cd	> 10	no inhibition < 10 PPB	Cossa, 1976
	Ni	> 0.5	" " < 1 PPM	Skarr et al., 1974
Chlorella	Cd	5.6	inhibition of division	Lue-Kim et al., 1980
Chlorella	Cu	> 2	temporary inhibition (tolerant/nontolerant strains)	Butler et al., 1980
Isochrysis	Hg	2	temporary inhibition	Davies, 1974
	Cd	0.1	" "	Li, 1980
Dunaliella	Hg	2	" "	Davies, 1976
Thalassiosira	Cd	0.2	" "	Li, 1979

All estimates of M_m were calculated using a mean cell weight of 10^{-10} g and volume of 200 μm^3 where applicable.

through some other removal mechanism is represented by R_1. This coefficient is dependent upon the total metal concentration, composition of the solution, pH, and the nature of the container surface. In preliminary experiments using ^{65}Zn the net loss of metal over a 10 day period in both BBM and MB media (1 μM zinc total, pH 8.2, 20°C, continuous light and agitation, without cells) did not exceed 10 ± 3.0 % or roughly 0.0005/h. Studies on the preservation of trace metal samples from natural waters provide additional information on the magnitude of this loss term. Massee et al. (1981) observed losses of 0.01-0.04/day and 0-0.01/day for silver and cadmium at pH 4 and 8.5 respectively (distilled water, total metal concentration = 0.1 uM) during storage in borosilicate containers. No losses of zinc from solution were reported. Other comparable data can be obtained from the literature but the results are dependent upon the factors previously mentioned. This term was varied between 0.0005 to 0.005 per hour which represent the maximum loss rates observed in culture and calcuated retention times for individual metals in situ, respectively. Increasing the time over which the model is run or the value of R_1 can result in significant reductions in M_t.

Reduction in the internal metal concentration arising from dilution during division or through cell lysis is represented by the term $M_c K_2 |dN/(Ndt)|$. This expression corrects the average cell metal content for changes in the population, compensating for the fractional increase or decrease in cell numbers over time (hence absolute value). The constant K_2 was varied bewteen 1 and 2 and corrects for the disproportionately higher metal concentration associated with the cell

wall that is sloughed off during division or cellular metal associated with cells removed from the population. Cell lysis occurs when $M_c > M_m$ and, consequently, this fraction of the population contains a relatively higher metal content. Epigenetic variation within an asynchronously growing population, however, results in differential uptake of metal because binding is dependent upon the physiological condition of the cell (refer to Sections I.I.II and IV.I.). The population considered in this model is assumed to be totally asynchronous and the effects of a metal on individual cells cannot be resolved, which is reflected in the limited range over which K_2 was varied.

Metal associated with the particulates is regulated by the partition coefficient K_d (μm^2/mole) and the available metal concentration. The maximum particulate concentration that can be attained is K_0 P, the conversion factor from cells to debris multiplied by the maximum population density. The conversion factor K_0 was varied between 25 and 100 μm^2/cell based on an average surface area per cell wall of 200 μm^2 (surface area of the cell (100 μm^2) multiplied by a factor of 2 since both sides are exposed when the cell wall is sloughed off) with the formation of 2 to 8 daughter cells per division respectively. The area of the old mother cell wall (debris) in relation to the number of daughter cells produced determines the value for K_0 (if 2 cells are produced, then K_0 = 200 μm^2/(2 cells), or 100 μm^2/cell). The maximum concentration of metal associated with the debris must be less than the total metal concentration minus the maximum cellular metal concentration per unit volume ($K_d P K_0 < M_t - M_s P$)

due to mass balance constraints. The total metal concentration (M_t) was varied between 10^{-10} and 10^{-7} moles/ml (0.1 to 100 μM). Higher concentrations are both unrealistic and limited by the solubility of the metal in solution. The upper limit for K_d is 10^{-15} μm^2/mole based on the maximum settings for the values P, K_0, M_t, and M_s (10^6 cells/ml, 100 μm^2/cell, 10^{-7} moles/ml, and 10^{-17} moles/cell respectively). This upper limit is in reasonable accord with the calculated coefficients for the Freundlich isotherms obtained from the zinc uptake experiments (Table XIV) and these values were used as initial estimates for K_d (10^{-15} - 10^{-17} μm^2/mole in one ml). Since the particulate metal concentration was uncorrelated with the concentration of metal in the dissolved phase no in situ estimates for K_d could be obtained. Based on the results from the bag experiments, literature values for K_d are suspect and are also in units that are not compatable with the model (the error associated with converting from weight or carbon to surface area is prohibitive). Oxidation of organic matter or degradation of debris is not considered in the model since the temporal scales associated with these processes are not significant relative to the period over which the model is run. The influences of organics excreted by the cells are not considered but can be included in the conversion factor K_0, or in the loss term, R_1, if the metal is irreversibly bound (unavailable).

Cell debris has a higher total binding capacity for metals than intact cells, resulting from a greater exposed, negative surface area that may be enhanced by the relaxation of the cell wall matrix when sloughed off during division (refer to Section I.I.II.). Crist et al.

(1981) did not observe any significant differences between the adsorption of metallic ions on intact cells and cell fragments of Vaucheria. This result cannot be fully interpreted because they omitted methods for culturing and pretreatment of the cell material. Their values for metallic ion adsorption ranged from 10^{-15} to 10^{-16} mole/μm^2 (based on a mean cell weight of 10^{-10}g and a cell area of 100 μm^2). These values are estimates for the maximum binding capacilities of all cellular components since the cell walls were not completely separated from the homogenized material. Also, field samples were analyzed and the level of contamination was not determined. Reductions in the available metal concentration resulting from metal bound onto debris and associated with the cells are interdependent due to the feeback nature inherent within the model. Cellular metal contents that inhibited growth minimized the influence of both terms on the available metal concentration. However, when the population declines the level of debris continues to increase (absolute change in the population with time, Equation 16) due to cell lysis. When growth, hence dilution, exceeds uptake there is a concomittant decrease in the available metal concentration but as N approaches P uptake continues and may result in cell death.

Cellular metal is also proportionally related to changes in the cell density. Increasing N results in a corresponding increase in the average cellular metal content based on rates of uptake and dilution (partitioning). In an asynchronously growing population variations in the cellular metal content are expected since uptake is dependent upon the the physiological condition of the cell. The light/temperature

dependent components associated with metal uptake provides an additional measure of the variability which could be expected in the extent of metal adsorption. A reduction in N will decrease the average cell metal content through cell lysis, since this fraction of the population has a characteristically higher cell metal content. Selective removal of individuals from a population ($M_c > M_m$) may also influence the result but has been averaged into the response of the entire population. This interaction can be interpreted as follows; 1. that when M_c is $> M_m$ all cellular division is inhibited, or 2. a fraction of the population continues to divide but the net losses exceed increases in the population.

Variations in the division rate or uptake/loss (light/dark dependence) along with oscillations in the available metal concentration can be simulated by randomizing the associated parameters. Either ramdon variation about a mean with a specified standard deviation or between limits with equal probability can be imposed. Cell size differences can be incorporated into the variable uptake/loss rates or into the conversion factor K_0, relating debris to cell density.

Computations were carried out using simple finite difference equations and other numerical methods for approximating solutions are not presented. No oscillations were observed in the solutions and numerical stability was confirmed by comparing the stepwise changes in the magnitude of each approximation. All simulations were run for a 10 day period (hourly increments), sufficient to yield 1 to 20 doublings of the population over the range of growth rates examined. This time

frame was sufficiently broad to encompass important processes and variable interactions encountered in culture and aquatic systems.

V.II. Model Simulations of Metal - Phytoplankton Interactions

I am primarily concerned with developing a quantitative model that; 1. incorporates well known concepts, 2. is consistent with observations on heavy metal - phytoplankton interactions, and 3. can be applied to specific systems and extended to explore regions of theoretical importance not possible by conventional techniques. Previous models are dedicated to single metals under well defined conditions and cannot be extended to other systems. Data available in the literature is usually incomplete and often obtained under widely different conditions. Consequently, a rigorous and independent verification of the model is not possible at present time and only the general response characteristics of model simulations are examined in detail. Also, the vast number of permutations involved over the range of parameter settings investigated are overwhelming and cannot be adequately presented in the context of this work. This restricted approach does, however, provide valuable insights into heavy metal - phytoplankton interactions and a foundation on which to interpret results from toxicological investigations in culture and in situ. Basic concepts incorporated into the model are substantiated by the general response characteristics of model output for a broad range of parameter settings. These characteristics are discussed in light of the observed growth responses for phytoplankton exposed to various

degrees of metal stress. The model is then extended to simulate in situ conditions involving random fluctuations in the available metal concentration, changing growth rate, and uptake coefficient.

In formulating the model I assumed that the growth rate was related to the overall level of cellular metabolic activity which, in turn, determines the extent of metal binding to the cell surface and uptake. Rapid growth (short generation time) results in a corresponding increase in solute transport across the membrane to satisfy metabolic demands, altering the reactivity of the cell surface and the extent of metal adsorption. The rate cells are removed from the population (lysis when $M_c > M_m$) is, in part, regulated by the growth rate. A decrease in the final yield results from a slow decline in cell numbers proportional to the growth rate and cannot be resolved in batch culture bioassays that are terminated after a fixed time. Population changes must be followed over time to observe this response. No attempt was made to standardize losses from the population as a function of only M_c and M_m (selective removal of a fraction of the population with a higher cell metal content). Lowering the internal cellular metal concentration inhibiting growth (M_m) from 10^{-16} to 10^{-18} moles/cell, or increasing the uptake coefficient by two orders of magnitude, produced identical 240 h cellular metal contents (M_c) and final yields (Figs. XVIII and XIX). In order to interpret results obtained after a fixed incubation period, model output at 240 hours, for a variety of parameter settings, are compared. These results are then interpreted in light of changes in cell metal content and growth of the population over time.

The response surfaces generated for different values of K_1 (Figs. XVIII and XIX) are comparable with previously reported observations on population growth under various degrees of metal stress. Increasing the internal cellular metal concentration inhibiting growth (M_m), the uptake coefficient (K_1), or external metal concentration (M_t) all result in lower final yields as expected (Fig. XVIII). Cellular metal concentrations (M_c) at 240 h are linearly proportional to the available metal concentration for a constant growth rate when the net inward flux of metal (determined by the magnitude of K_1 balanced against losses through dilution) does not exceed M_m (Fig. XIX). When $M_c > M_m$ cell lysis occurs and the decrease in cell numbers with time is related to the growth rate and $1-M_c/M_m$, producing a nonlinear profile for any given K_1 and growth rate (r). This nonlinear relationship for either M_c and N, occasionally refered to as "biphasic", is frequently observed in results from single metal batch culture bioassays. (Citing all examples is prohibitive because of the frequency with which this relationship is observed in the data.) These results are usually attributed to an exclusion mechanism or differential tolerance of individuals towards the metal; or in many instances, simply presented and not interpreted. Model simulations, however, indicate that all these observations are a result of the experimental conditions, obviating the need to postulate hypothetical tolerance mechanisms.

High total metal concentrations (M_t) and uptake rates (R_1) combined with a low cellular metal concentration inhibiting growth (M_m) resulted in a rapid increase in M_c (cell metal content) and

Fig. XVIII. Relationship between Final Yield (% Maximum Attainable), Growth Rate (r), Total Metal Concentration (M_t), and the Uptake Coefficient (K_1)

This figure illustrates model response (final yield or cell density) for a broad range of parameter settings and can be used to interpret results obtained from batch culture bioassays terminated after a fixed time period. Final yields (N at t=240 h) as a function of the uptake coefficient (K_1 set equal to 10^{-7}, 10^{-9}, 10^{-11}, and 10^{-13} ml/(h cell)), available metal concentration (M_f set equal to 10^{-7}, 10^{-8}, 10^{-9}, and 10^{-10} moles/ml), and the growth rate (r) are presented for different values of the cell metal content inhibitng growth (M_m = 10^{-16} and 10^{-18} moles/cell). An important aspect of this figure is the nonlinear relationship between final yield and growth rate over the range of available metal concentrations examined. This nonlinear relationship has been observed in batch culture bioassays and results from the antagonistic action exerted by the metal on the cell (uptake approaches M_m influencing growth) balanced against dilution of cell metal through growth. If a constant growth rate is assumed then rough estimates for the uptake coefficient can be obtained by superimposing the final results (cell densities) onto this figure. The situation becomes more complex for a variable growth rate. The effects of a metal on individual cells, however, cannot be directly inferred from the response of the population (including growth rate) and this exercise would not provide any meaningful information at present time.

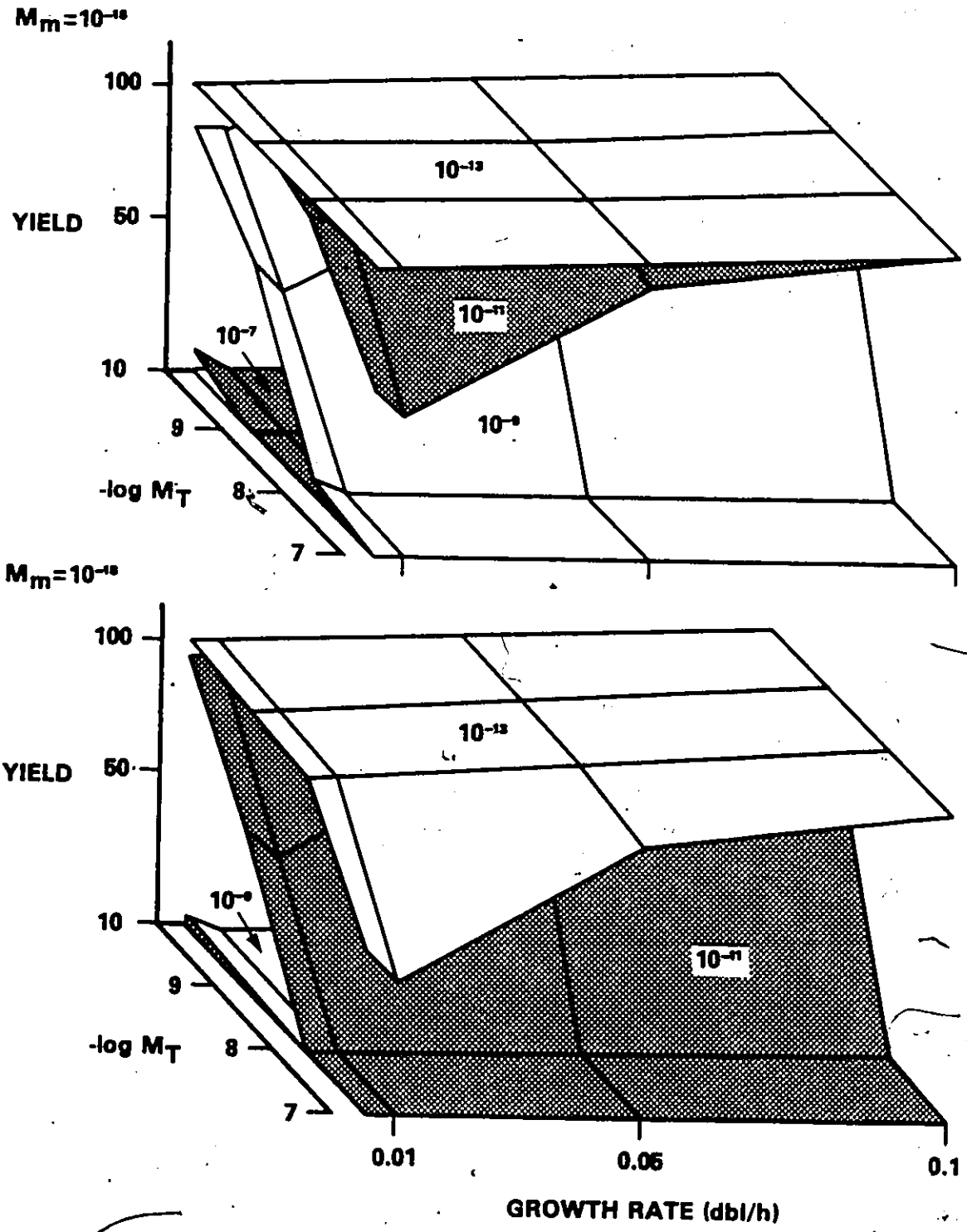
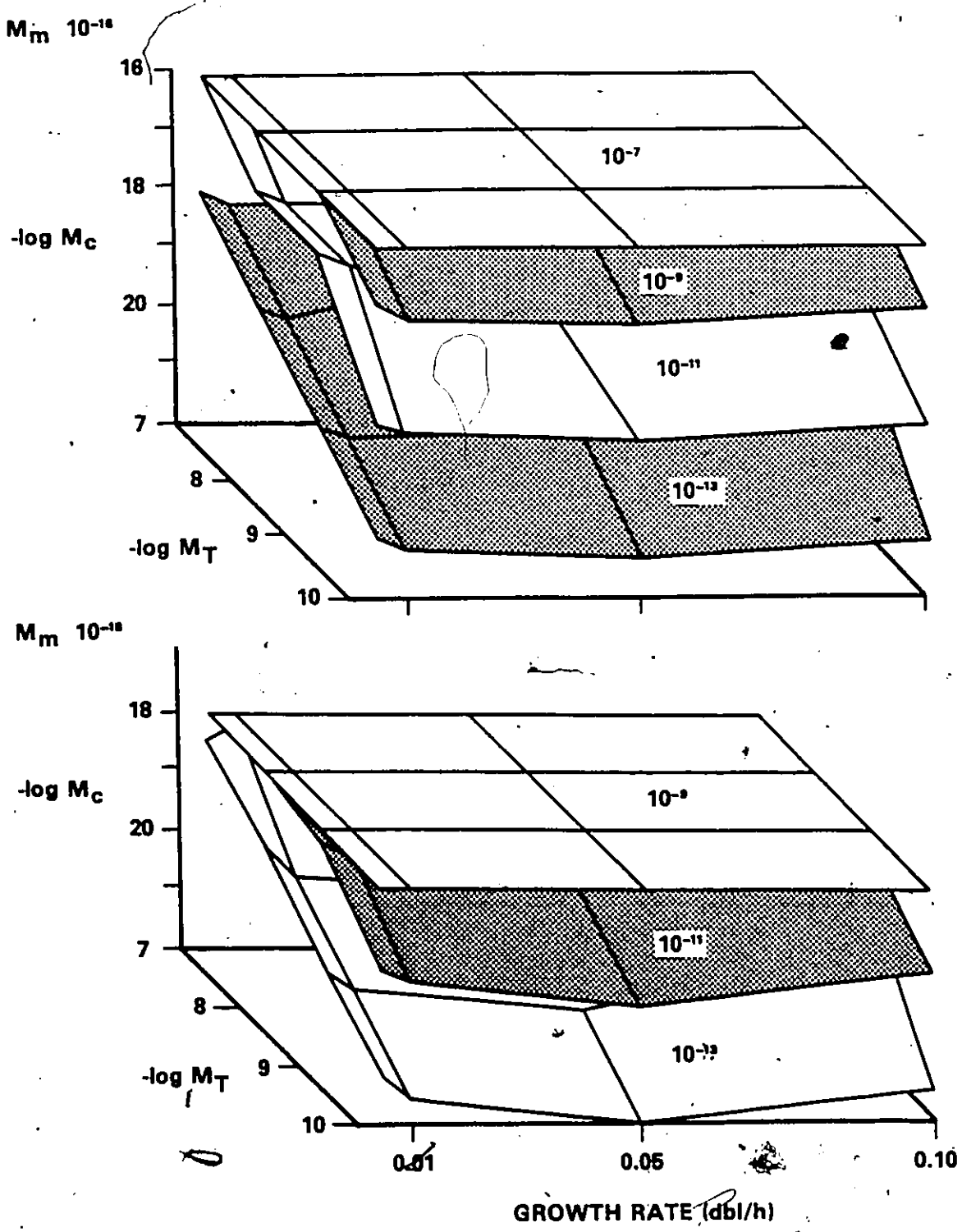

$M_m=10^{-16}$
100
YIELD
50
10
9
-log M_T
8
7
10^{-13}
10^{-11}
10^{-7}
10^{-9}
$M_m=10^{-16}$
100
YIELD
50
10
9
-log M_T
8
7
10^{-13}
10^{-9}
10^{-11}
0.01
0.05
0.1
GROWTH RATE (dbl/h)

Fig. XIX. Relationship between the Total Cellular Metal Content (M_c), Growth Rate (r), Total Metal Concentration (M_t), and the Uptake Coefficient (K_1)

Final cell metal contents at 240 h associated with the final yields in Figure XVIII are presented in this figure. Cellular metal concentrations that did not inhibit growth ($M_c < M_m$) were linearly related to the total metal concentration. When growth was inhibited, changes M_c were dependent upon the uptake coefficient and dilution through growth (also influenced by decreases in the available metal concentration over time). Similar arguments to those presented with Figure XVIII apply when comparing the final cell metal content with results obtained from batch culture bioassays.


M_m 10^{-16}
16
18
20
-log M_c
10^{-7}
10^{-9}
10^{-11}
10^{-13}
7
8
9
10
-log M_T
M_m 10^{-16}
18
20
-log M_c
10^{-9}
10^{-11}
10^{-13}
7
8
9
10
-log M_T
0.01
0.05
0.10
GROWTH RATE (dbl/h)

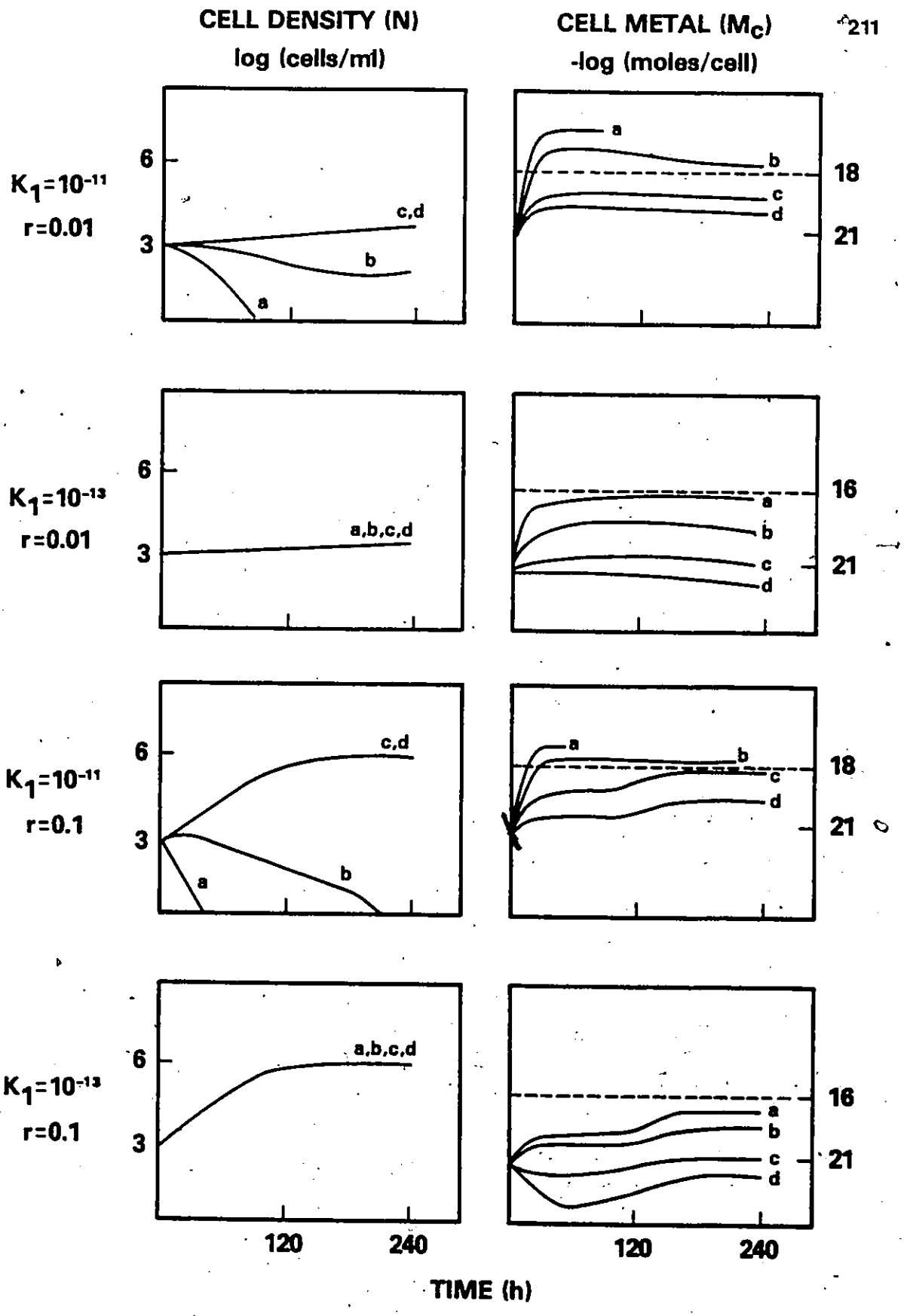
growth was arrested. Frequently, results are presented as a decrease in final yield, or transformed into relative growth rates, and are often erroneously interpreted. Relative growth rates provide no information on changes in the population over time which may be increasing or decreasing when the experiment is terminated. This measure also implies the existence of a constant life cycle time for all individuals in the population (out of phase or asynchronous) that is altered uniformly in proportion to the level of the imposed stress. Alternatively, if all cellular metabolic activity is directed towards maximizing growth, the growth rate remains constant and the observed changes in cell number is a function of losses from the population through cell lysis. Both interpretations are compatable with the results but are not important factors in determining the response characteristics of populations under metal stress simulated by the model. A random growth rate with equal probability between the specified limits for r (0.005 to 0.1 dbl/h) at one hour time steps did not significantly alter the observed response (compare Fig. XX and XXII). This situation is never encountered and unrealistic but the results emphasize the importance of the uptake and loss coefficients (K_1 and K_2 respectively, Equation 14) in determining the ultimate response of the population. Additional studies, however, are required to determine whether life cycle times remain constant or vary in relation to the magnitude and duration of an imposed stress. Similar arguments can be extended to explain variations in the uptake and loss coefficients over time, and both require delineation between the effects of metals on individual cells and the observed changes in the

population (refer to Sections I.I.II. and I.II.). Variations in K_1 with time, however, are negligible because physiological control over metal binding to the cell surface (labile fraction) is insignificant compared to the total binding capacity (30 to 70% of the maximum binding capacity). The exchangeable quantity of metal bound (fraction influenced by changes in the physiological condition of the cell) is significantly reduced in the presence of other cations, especially at concentrations encountered in situ. Also, the absolute change in the magnitude of K_1 required to produce a corresponding change in the response is much greater than the extent of physiological control over binding.

Closer examination of the changes in cell metal content and the population over time provides insights into the interpretation of results obtained after a fixed incubation period. The antagonism between uptake and dilution with time is illustrated in Figure XX. Equilibration between the metal associated with the cells and in solution is essentially complete after the first few hours (increments) due to the nature of the uptake process. During the period of exponential growth, the cellular metal concentration (M_c) remains constant and increases as N approaches P. This initial plateau is only observed when the internal cellular metal concentration does not inhibit growth ($M_c < M_m$) and represents the region over which uptake equals the rate of dilution through growth. When dilution exceeds uptake during the initial stages of growth, a concomitant reduction in M_c to a level at which uptake equals dilution is observed. As the population enters a stationary phase, growth is

Fig. XX. The Relationship between Changes in the Population (N) and Average Cellular Metal Content (M_c) as a Function of Time and the Total Metal Concentration (M_t)

Model simulations of changes in the population and the average cell metal content over time for different growth rates (r), uptake coefficients (K_1), and concentrations of cell metal inhibiting growth (M_m) are presented. This figure illustrates the relationship between uptake and dilution through growth modified by the antagonistic action exerted by the metal on the cell. Cell metal rapidly equilibrated with the available metal concentration in solution at a level where uptake was balanced against losses through growth. Once growth was influenced by the density dependent function, P, (similar to a stationary phase) uptake increased as the rate of dilution through growth decreased. The effects of the loss constant, R_1, on the total metal concentration, hence the partitioning of metal between compartments, does not significantly influence the results. Losses of metal were, however, reflected over longer time scales by the gradual decrease in M_c towards the end of the simulation run. When the cell metal concentration exceeded M_m growth was inhibited and the population decreased with time. The different values for M_m are represented by planes in the figures for M_c plotted against time. Once $M_c > M_m$, M_c exceeded the level delineated by the plane, a lethal response was observed. When the population was eliminated (N=0), the stepwise integration was terminated producing incomplete response surfaces for M_c versus time.


CELL DENSITY (N)
log (cells/ml)
CELL METAL (M_C)
-log (moles/cell)
$K_1=10^{-11}$
r=0.01
$K_1=10^{-13}$
r=0.01
$K_1=10^{-11}$
r=0.1
$K_1=10^{-13}$
r=0.1
6
3
18
21
16
21
18
21
16
21
a
b
c
d
c,d
a,b,c,d
120
240
TIME (h)

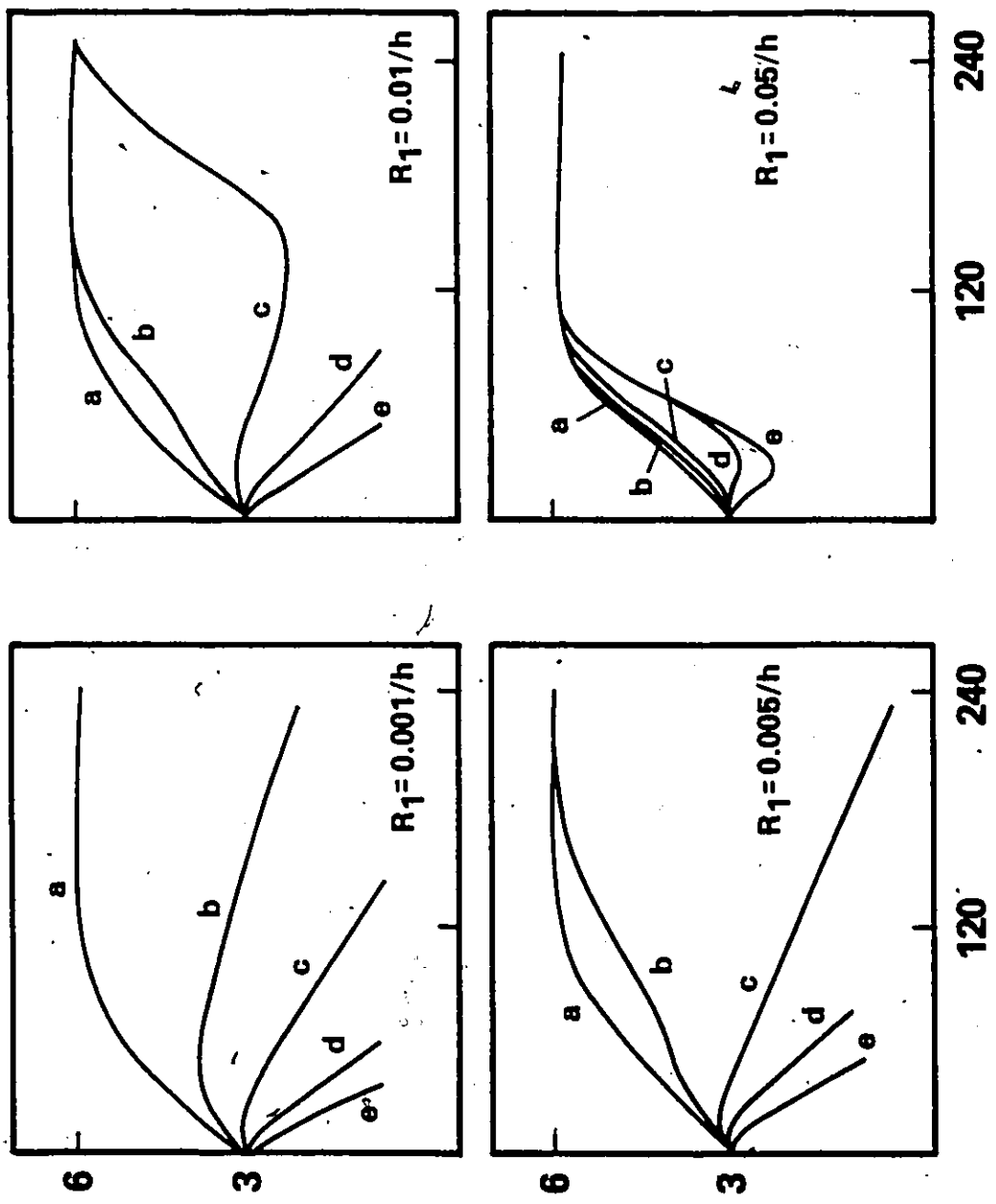
reduced along with losses of cellular metal, resulting in a net increase in M_c. Cell metal is directly proportional to the available metal concentration and the growth rate when the experiment is terminated during the period of exponential growth. Comparing results from stationary and exponentially growing cultures will produce the nonlinear relationship discussed previously. This variable uptake has only been observed by Li (1980) for cadmium accumulation in cultures of Isochrysis and is usually obscured by variations in the available metal concentration (influenced in part by the level of cell debris) normally encountered in batch culture bioassays. In these other studies, the available metal concentration was reduced to a level where dilution exceeded uptake and a net decrease in cellular metal, after an initial increase, was observed over time (Davies, 1973,1974,1976; Skarr et al., 1974; Bentley-Mowat and Reid, 1977; Butler et al., 1980; Yen, 1981; Bates et al., 1982). Increasing the magnitude of the loss term (R_1) or the conversion factor from cells to debris (K_0) balanced against the appropriate total metal concentration will produce similar results.

The importance of the loss term (R_1) in determining the observed growth response is illustrated in Figure XXI. This loss rate incorporates precipitation reactions, non-exchangeable binding of metal to cell walls and debris, biochemical transformations (volatilization for example), and other irreversible reactions. The loss term, R_1, was varied between 0.02 to 0.0005 h^{-1} and the initial settings for the other parameters were held constant. Model output under these conditions reproduced the two growth responses

Fig. XXI. Characteristics Growth Response Curves Generated by the Model

The characteristic growth response curves for phytoplankton populations exposed to varying degrees of metal stress were presented in the introduction (Section I.II.II.) and include: Type I response, extended lag phase; Type II response, initial increase in cell density followed by a gradual decrease in cell numbers over time. Both responses are dependent upon the net rate of metal uptake balanced against losses from dilution through growth, and modified by the antagonistic action exerted by the metal on the cell. Uptake is dependent upon the available metal concentration, which also determines the magnitude of the stress. Consequently, different loss rates were incoporated into the model to simulate changing available metal concentrations and duplicate the characteristic growth response curves. The rate metal was removed from solution determined the nature of the response curve. Compare these results with Figure III.

Initial conditions for each run were as follows: $M_m = 10^{-18}$ moles/cell; $K_1 = 10^{-11}$ ml/(cell h); r = 0.1 dbl/h. Each series of curves represent different initial total metal concentration (M_t) coded as follows: a, 1 µM; b, 5 µM; c, 10 µM; d, 25 µM; e, 50 µM.


log (cells/ml)
6
3
120
240
TIME (h)
R_1=0.001/h
R_1=0.005/h
R_1=0.01/h
R_1=0.05/h
a
b
c
d
e

characterized in the introduction (Section I.II.II.). When uptake exceeded dilution through cell growth a lethal response was observed. The time required to produce this response was dependent upon the magnitude of the uptake coefficient and the available metal concentration. For a constant uptake coefficient, the available metal concentration either inhibited growth or resulted in an initial period of unaltered growth that gradually decreased with time in proportion to the level of metal stress (Type II response). A growth resurgence was observed when the cellular metal content decreased ($M_c < M_m$) as a result of losses in the total metal concentration over time and dilution of M_c through growth (Type I response). These arguments can be extended to interpret all reported observations on the growth of phytoplankton exposed to varying degrees of heavy metal stress.

The available metal concentration, or level of metal stress, had to be significantly reduced over relatively short time scales to produce these variable responses. Binding of metal to cell wall fragments cannot account for all losses and other processes not explicity incorporated into the model must be involved. The distribution coefficient (K_d) for metal bound to debris cannot be increased due to mass balance constraints and the magnitude of K_0 (cell wall to surface area conversion factor) is in reasonable accord with the potential surface area associated with the cell wall sloughed off during division. Sequestering of metal by algal exudates, or debris from cell lysis, can effectively lower the total metal concentration over relatively short periods of time (function of the cell density) in line with the results. Cell mediated transformations

of the metal into volatile complexes or less toxic derivatives may also operate over similar temporal scales (Davies, 1974; Ben-Bassat and Mayer, 1977; De Filippis and Pallaghy, 1976b; Wrench and Addison, 1980). Reported observations on large changes in the cellular metal content over time are primarily a result of carefully selected experimental conditions and reinforce the need to critically re-evaluate current methods and interpretations of data obtained from batch culture bioassays. My results, however, provide some insights into relationships between uptake and dilution of cellular metal through growth validating basic concepts incorporated into the model. These relationships were further evaluated by extending the model to simulate the effects of random fluctuations in the physico-chemical environment on the growth of a population and metal uptake.

Variations in the growth rate, partition coefficient (K_d), along with the influences of cell debris, did not significantly affect model output over the range of values examined. The growth rate determines the rate of dilution of cellular metal (M_c) during division when the cell wall is sloughed off and during the growth phase of the cell cycle when cell volume increases. Loss rates (R'_1) resulting in a significant decrease in the available metal concentration after 240 h (> 10%), along with variations in the uptake coefficient (K_1) coupled to the total metal concentration in solution, did, however, influence model output. The loss rate and uptake coefficient are both dependent upon the available metal concentration. Consequently, the effects of a variable available metal concentration was examined in greater detail.

In order to assess the effects of a fluctuating available metal

concentration on the population, the expression for the available metal concentration was replaced with a random function. Values of equal probability were generated between 10^{-10} and 10^{-8} moles/ml, or 0.1 and 10 μM, over a range of temporal periodicities (e.g. newly generated values were held constant for times of 1, 10, and 100 h or increments). Fluctuations over the shortest period examined did not significantly alter model response, even when a random function was substituted in for the growth rate (Fig. XXII). A lethal response, however, was observed for values of K_1 greater than 10^{-12} and 10^{-13} ml/h/cell for each of the respective conditions. This descrepancy may have resulted from the initial values assigned to each variable and randomization of the available metal concentration. Relationships between uptake and the available metal concentration require further investigation in order to define the significance of each.

Desorption of metal from the cell surface resulting from changes in the chemical environment surrounding the cell was not explicitly included in the model, but incorporated into the uptake coefficient. I demonstrated that a portion of the zinc adsorbed to the cell surface is readily exchangeable with relatively non-toxic cations, Ca and Mg in particular. Consequently, changes in the bulk solution supporting the cell can result in a significant loss of metal. Concentrations of conservative ions <u>in situ</u> (Ca, K, Na, and Mg for example) will, however, minimize this type of binding in turn limit the extent of ion exchange reactions. Since cellular metal can only be reduced through growth of the organism, decreases in M_c lag behind fluctuations in the external metal concentration balanced

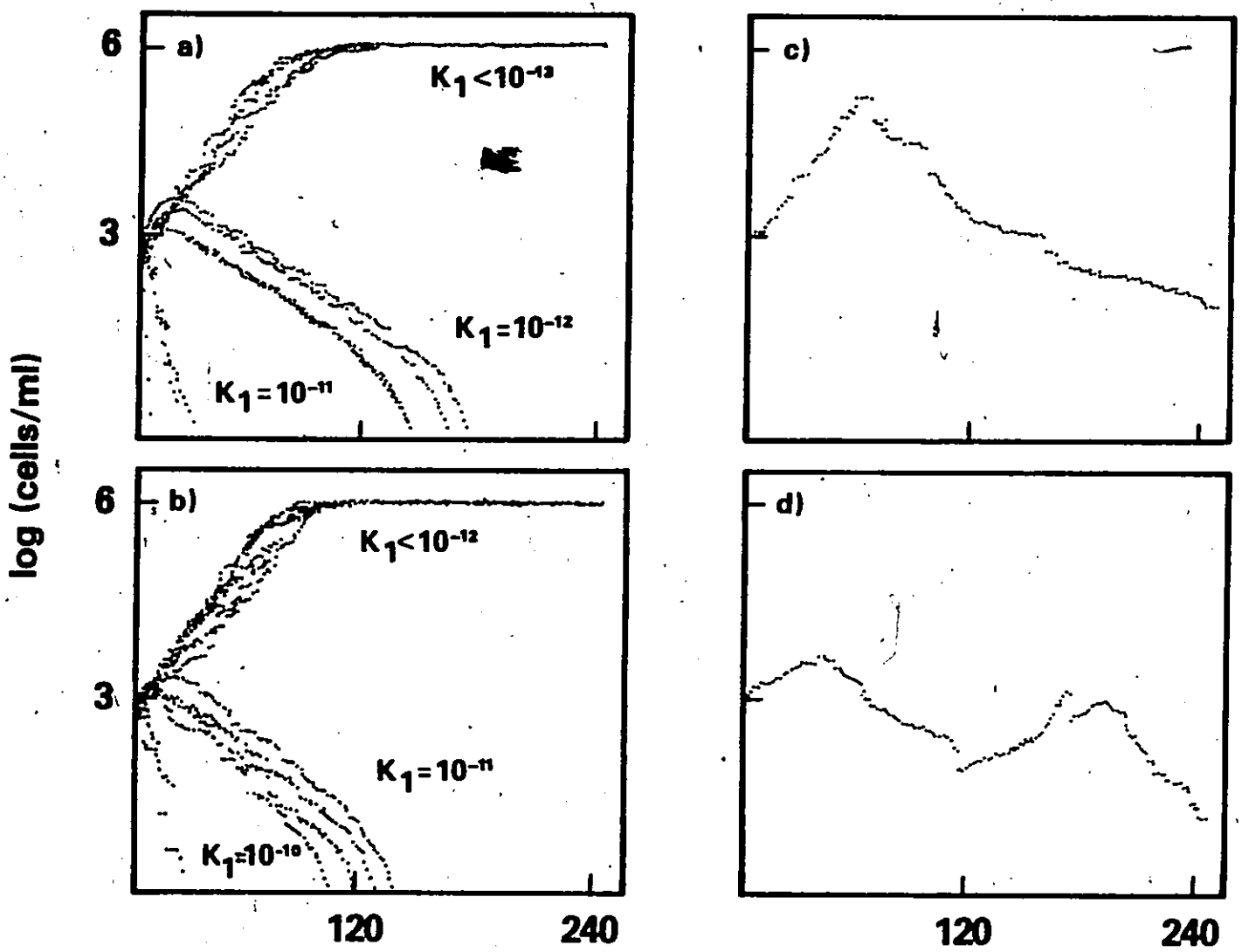
Fig. XXII. Model Response to Variations in Growth Rate (r), Available Metal Concentration (M_f), and the Uptake Coefficient (K_1)

Random functions were substituted into the model for the growth rate, available metal concentration, and uptake coefficient. Variations in the growth rate (Fig. XXIIa) and the available metal concentration (Fig. XXIIb) did not significantly alter model response. Simultaneous variations in all three prarmeters, however, resulted in either; 1. elimination of the population, or 2. a response dependent upon the magnitude and duration of the imposed stress (Fig. XXIIc and XXIId, respectively). When growth was inhibited by the density dependent factor, P, uptake exceeded dilution and the ability of the cell to compensate for the influx of metal. Consequently, a lethal response was observed independent of changes in the other parameters over time. In the second response, growth was only influenced by the cell metal content and not the carrying capacity (P), and changes in the population were regulated, primarily, by variations in the available metal concentration coupled to the uptake coefficient. Ranges over which each parameter was randomly varied with equal probability between the specified limits include: growth rate, r, 0.001-0.1 dbl/h; uptake coefficient, K_1, 0.1-5 x 10^{-11} ml/(cell h); available metal concentration, M_f, .1-10 μM. M_m was set equal to 10^{-18} moles/cell, and the loss rate R_1 set equal to 0.001/h.

Fig. XXIIa. random growth rate, M_t = 10 μM, K_1 set equal to 10^{-11}, 10^{-12}, and 10^{-13} ml/(cell h)

Fig. XXIIb. random growth rate and available metal concentration, K_1 set equal to 10^{-10}, 10^{-11}, and 10^{-12} ml/(cell h)

Fig. XXIIc,d random growth rate, available metal concentration, and uptake coefficient


a)
$K_1 < 10^{-13}$
$K_1 = 10^{-12}$
$K_1 = 10^{-11}$
b)
$K_1 < 10^{-12}$
$K_1 = 10^{-11}$
$K_1 = 10^{-10}$
c)
d)
6
3
120
240
log (cells/ml)
TIME (h)

against uptake. Uptake coefficients in excess of 10^{-12} ml/h/cell produced a lethal response, eliminating the entire population, and lower values of K_1 did not influence the results. This value corresponds to uptake rates of between 10^{-22} and 10^{-20} moles/h/cell for available metal concentrations ranging from 10^{-10} to 10^{-8} moles/ml, or 0.1 to 10 μM (uptake rate = K_1 M_f). These rates are roughly 4 to 6 orders of magnitude less than facilitated transport (active uptake) of solutes across the membrane (refer to Section IV.I.V.). Based on this result, metal uptake appears to involve processes driven by diffusive forces. Processes regulating the uptake of metals are critical in determining the response of phytoplankton populations under heavy metal stress.

The uptake coefficient in this model incorporates a number of factors and is a major simplification but can be justified on both theoretical and empirical grounds. Uptake of metal into the cell is assumed constant and proportional to the external metal concentration and results in rapid equlibration between metal associated with the cell and in solution. Consequently, the effects of a variable uptake coefficient on model output is a function of the available metal concentration independent of the time period over which K_1 is held constant. Additional evidence justifying a constant uptake rate is apparent from my results on the specific binding of various metals and exchangeable fraction of the total metal adsorbed to the cell surface. The quantity of nonexchangeable zinc bound to the cell surface roughly corresponds to the maximum binding capacities obtained under sub-optimal conditions. The presence of surface ligands specific for

individual metals will tend to minimize variations in the external metal concentration and maintain a relatively constant surface concentration. Also, the labile fraction of the total metal bound to the cell surface in situ is assumed insignificant in the presence of elevated concentrations of other cations. These results indicate that any variations in the uptake coefficient are of minor importance compared to fluctuations in bulk metal concentrations, especially since model output is relatively insensitive to large changes in K_1 alone. For comparative purposes, however, K_1 was randomized over the range most critical to model output (10^{-10} to 10^{-12} ml/h/cell). Although variations in the uptake coefficient spanning two orders of magnitude are highly unlikely, the generated responses produced some interesting results.

The growth rate and K_1 were randomized prior to each time increment and the available metal concentration was held constant for a 12 h period, roughly equivalent to large scale mixing processes or vertical transport of the organism through the water column. Three simulations were carried out and a variety of growth responses were produced. When growth of the population was inhibited by the carrying capacity (P), the cell metal content was dependent upon variations in the uptake coefficient and the available metal concentration (Fig. XXI). In this situation, dilution of cell metal through growth was reduced and uptake eventually exceeded the capacity of the cell to compensate for the influx of metal, eliminating the population. Growth of populations not influenced by P, rapidly responded, or compensated for variations in K_1 and the available metal concentration (Fig.

XXII). These results illustrate the complex interactions between uptake, dilution, and the available metal concentration. Other factors that influence growth, such as the carrying capacity or the physical regime, also influence the capacity of the cell to compensate for varying degrees of heavy metal stress. The integrated effects could lead to subtle long term changes within populations dependent upon the ability of individual cells to compensate for the stress. If the cells cannot compensate for the stress then the population will decrease over time irrespective of fluctuations in the surrounding environment. Lags in the dose response relationship are primarily responsible for this result since phytoplankton cannot predict, only compensate for perturbations in the physico-chemical environment.

Additional culture investigations are required to verify or refute model assumptions. Understanding the role of mass transport processes in the suspending medium and the reactivity of algal surfaces in regulating metal uptake is prerequisite to determine the nature of metal - phytoplankton interactions and assess the impact of contaminants on aquatic systems. The extent of desorption and adsorption of metals onto the cell surface under fluctuating physical and chemical conditions, along with physiological control over the chemical environment within the cell wall/membrane region, must be rigorously evaluated. Short and long term variability in aquatic environments and the concomittant effects on biological activity has recieved little emphasis in ecological studies even though the heterogeneous nature of aquatic systems is primarily responsible for most of the observed changes in species abundance and community

structure. Short term or high frequency fluctuations in available resources must be integrated over time by the cell into growth and maintenance of basal metabolic processes. Perturbations in the physico-chemical environment that alter the ability of the cell to utilize available resources are manifested over time into a reduced growth rate. Cause and effect relationships cannot be directly resolved in non-steady state systems but the potential importance of individual processes in regulating metal (or nutrient) phytoplankton dynamics can be systematically and rigorously evaluated. The model can be used to identify key factors and interactions, providing the basis for additional experimentation and subsequent analysis of model formulation and assumptions.

VI. SUMMARY AND CONCLUSIONS

Investigations have been conducted on the long and short term effects of heavy metals on phytoplankton. The ultimate response was dependent upon the magnitude of the imposed stress, time, and the level at which the metal disrupts normal cell function (membrane or internal sites of toxic action). Culture investigations were directed towards understanding the nature of the response and factors that could singly, or in combination, significantly modify the availability and binding of metals to algal cell surfaces (light, temperature, and other metals). Studies on the cycling and distribution of metals *in situ* demonstrated that similar processes regulating the availability of metals in culture were involved but operated over different temporal scales. The potential algicidal properties of individual metals could be effectively reduced by the hierarchy of temporal interactions between biological, chemical, and physical variables in aquatic systems. The complex nature and reactivity of algal cell surfaces, partly under physiological control, may provide a homoestatic mechanism sufficient to dampen small scale environmental perturbations in solution equilibria.

Binding of zinc-65 to the surface of *Chlamydomonas* was examined under optimal and sub-optimal conditions of light and temperature and in the presence of 12 other metals. (Cu, Sr, Co, Ni, Cd, Mn, Ca, Mg, Be, Fe, Pb, and H). Roughly 20-40% of the zinc bound to the cell surface was non-exchangeable and could not be displaced by excess

concentrations of other cations or EDTA. The remaining 60-80% of the zinc associated with the cell surface was easily displaced by other cations and was sensitive to changes in light, temperature, and pH. I believe that the effects of light, temperature, and pH were an indirect result of cell metabolism on surface reactivity, possibly due to the differential exchange of solutes across the cell membrane under sub-optimal versus optimal physiological conditions. The quantity of exchangeable zinc associated with the cell surface corresponded to the degree of enhanced binding under optimal physiological conditions. Based on my results, zinc uptake appears to involve processes driven by diffusive forces.

Significant differences in the total metal binding capacity of the cell surface were observed between species and among genera. Intra-specific differences between isolates from Hamilton Harbour and laboratory clones suggested that metal exclusion (reduction in the total metal binding capacity of the cell surface) was the principal tolerance mechanism. Inter-specific differences could impart selective advantages to indivdual species and all results could be explained by the relative available concentrations of each ligand type on the cell surface. The results indicated that zinc binding to the surfaces of different species of phytoplankton involved a common mechanism. Metal binding capacities were also two orders of magnitude less than the theoretical amount of metal required to form a monolayer.

The effects of these metals on photosynthesis, fluorescence, and growth were also examined. Only concentrations of Cu and Hg, greater than 0.01 and 0.1 mg/L respectively, inhibited fluorescence

and photosynthesis over relatively short time scales. The effects of heavy metals on the growth of phytoplankton were classified according to the presence of an extended lag phase or a gradual reduction in cell numbers over time. The magnitude of dose and changes in the available metal concentration over time determined the nature of the response. Cellular fluorescence was not significantly altered at the termination of all experiments, even when growth was inhibited.

Factorial experiments were conducted to assess the effects of Cu, Co, Ca, Mg, Sr, Cd, Ni, Mn, and H, present singly and in all combinations, on the adsorption of ^{65}Zn, fluorescence, and photosynthesis in five species of phytoplankton. Changes in solution composition, light, and time were also examined. The results provided numerous insights into the nature of competition between metals for available binding sites and the observed toxic response (antagonistic, synergistic, or independent).

Estimates for the degree of environmental variability and temporal components associated with the cycling of Zn, Cd, Cu, Ni, Sr, and Co in situ were obtained using large volume enclosures. Mechanisms regulating the cycling of these metals operated over relatively short time scales compared to processes influencing community structure and composition. Sedimentation was the principal removal mechanism, dependent upon the reactivity of each metal (affinity for particulate matter) and the particulate matter concentration. Variations in the dissolved phase (filtered through 4.25 cm GF/C) were uncorrelated to particulate metal concentrations over short and long temporal scales (1-30 days). Metal bound to the particulate fraction was essentially

removed from the water column contrary to previous assumptions on the reactivity of this phase. Variability associated with the chemical and physical regimes over time was sufficient to effectively reduce the availability of individual metals within the water column. The initial distribution of particulate matter will, however, determine the relative magnitude of heavy metal stress exerted on planktonic organisms. The greatest effect will be observed when the metals are initially introduced (minutes to hours) and dependent upon the inital partitioning of the metal between all available ligands in solution. Colonization on the surfaces of the bags treated with metals was inhibited by the enrichment of copper within this phase. Growth of populations within the large volume enclosures significantly increased the level of particulate matter, in turn, enhanced dilution and removal of metal from the water column.

The influence of biological variables in regulating the distribution and cycling of metals <u>in situ</u> must be incorporated into models simulating the short term impact of elevated metal concentrations on aquatic systems. Short term perturbations in solution equilibria, arising from atmospheric deposition or impulse loadings, will exert an effect on community processes within the water column proportional to the magnitude of the stress (initial partitioning), but the greatest long term impact will be on sessile or benthic organisms. Recycle mechanisms at the sediment water interface must also be examined in order to manage discharges of toxic wastes in natural waters. Time and space can be used to classify and reduce all processes operating within a system into a hierarchy of interactions

compatible with research objectives. It is important that future studies on heavy metal - phytoplankton interactions include a broad range of environmental parameters encompassing conditions normally encountered in aquatic ecosystems.

A mathematical model was developed to simulate the effects of heavy metal stress on algal growth and metal uptake. Fluctuations in the available metal concentration, cell size, and variations in the growth rate observed in culture and in situ can also be incorporated into the model. The nature of the cell wall surface and associated matrix, and interactions of the metal with other metals/ligands in solution all influence metal uptake. A lethal response is observed when the integrated inward flux of metal exceeds losses from dilution (cell growth and division) or through shifts in solution equilibria. Specific sites of toxic action could not be identified and a general reduction in cellular metabolic activity, culminating in a net increase in the doubling time, occurs as a direct result of heavy metal stress. The formulation of model compartments, interactions, and parameter settings were verified using data from the literature and from experimental results. In rapidly growing phytoplankton populations, cellular metal concentrations are dependent upon the rate of uptake balanced against losses through dilution. Cell metal contents for phytoplankton with longer generation times, commonly encountered in situ, are primarily determined by fluctuations in the available metal concentration.

Processes regulating the cycling and distribution of metals in aquatic systems are sufficiently rapid, compared to the doubling time,

to reduce the level of metal stress imposed on the cell. This model effectively incorporates important temporal components for processes regulating heavy metal - phytoplankton interactions and provides new insights into the interpretation of results from toxicological investigations. Current concepts must be redefined in light of these results and greater emphasis must be placed on understanding the nature and reactivity of the cell surface.

Phytoplankton can only compensate for fluctuations in the physical and chemical environment surrounding the cell. Consequently, results from studies on heavy metal - phytoplankton interactions must be related to processes operating over cellular scales. Only by adopting this perspective can we interpret observed cellular responses to (and interactions with) the external medium. In order to develop our understanding of heavy metal - phytoplankton interactions, future studies should be directed towards:

1. defining long term rates of metal uptake under constant and fluctuating external metal concentrations;
2. determine the extent of physiological control over the binding of metal to the cell surface under a variable physical regime;
3. verify or refute the assumption of a constant life cycle time that is uniformly influenced in proportion to the level of stress imposed on the cell, as opposed to selective removal of individual cells from the population, in explaining the observed changes in cell density with time;

4. refined estimates for the internal cellular metal concentration inhibiting growth (minimum and maximum levels tolerated by individual species);
5. acquisition of additional data on the variability of the chemical environment in situ, including the effects on metal binding to the cell surface and level of physiological activity;
6. application of numerical techniques to estimate model parameters using the equations and literature data for growth of phytoplankton under various degrees of metal stress, assuming both a variable and constant growth rate;
7. multiple metal uptake experiments to determine the number of classes of specific metal binding sites on the cell surface, especially for tolerant and nontolerant species;
8. simultaneous metal uptake and physiological experiments;
9. develop methods to separate cell debris from intact cells and standarization of results from toxicological experiments;
10. continuous and synchronous cultures exposed to various degrees of heavy metal stress (sublethal), single and multiple metal additions;
11. autoradiographic studies (labelled nutrients or radioactive metal isotopes) to determine the degree of variability for metal binding to the cell surface within a population - including single cell fluorescence characteristics under various degrees of metal stress;
12. subcellular investigations on the structure and composition of cell walls in tolerant and nontolerant species;

13. adaptation of alternate methods to determine relative stability constants for reactive groups on the cell surface (possibly nonaqueous titrations);
14. studies directed towards characterizing the distribution of binding sites on the cell surface, or areas of acid/base secretion (in clusters or homogeneously distributed over the surface of the cell), especially between tolerant and non-tolerant species of phytoplankton.

VII. APPENDIX

Fig. XXIII. The Influence of pH and Free Zinc Ion Concentration on Zinc Uptake for Seven Species of Phytoplankton

Plots of zinc uptake data for seven species of phytoplankton at pH 6.2, 7.2, and 8.2. These curves illustrate the degree of variation in metal uptake observed between species and among genera.

Zinc uptake in units of 10^{-18} moles/μm^2 plotted against the computed free zinc ion concentration (μM). All experiments were conducted at 20°C under 100-110 $\mu Em^{-2}s^{-1}$ ph.a.r. in MB except where noted. Notation for individual species is as follows: "1", Chlorella pyrenoidosa; "2", Scenedesmus UT; "3", Scenedesmus HH; "4", Coelastrum UT; "5", Coelastrum HH; "6", Chlamydomonas W; "7", Chlamydomonas; "8", Chlamydomonas in BBM.

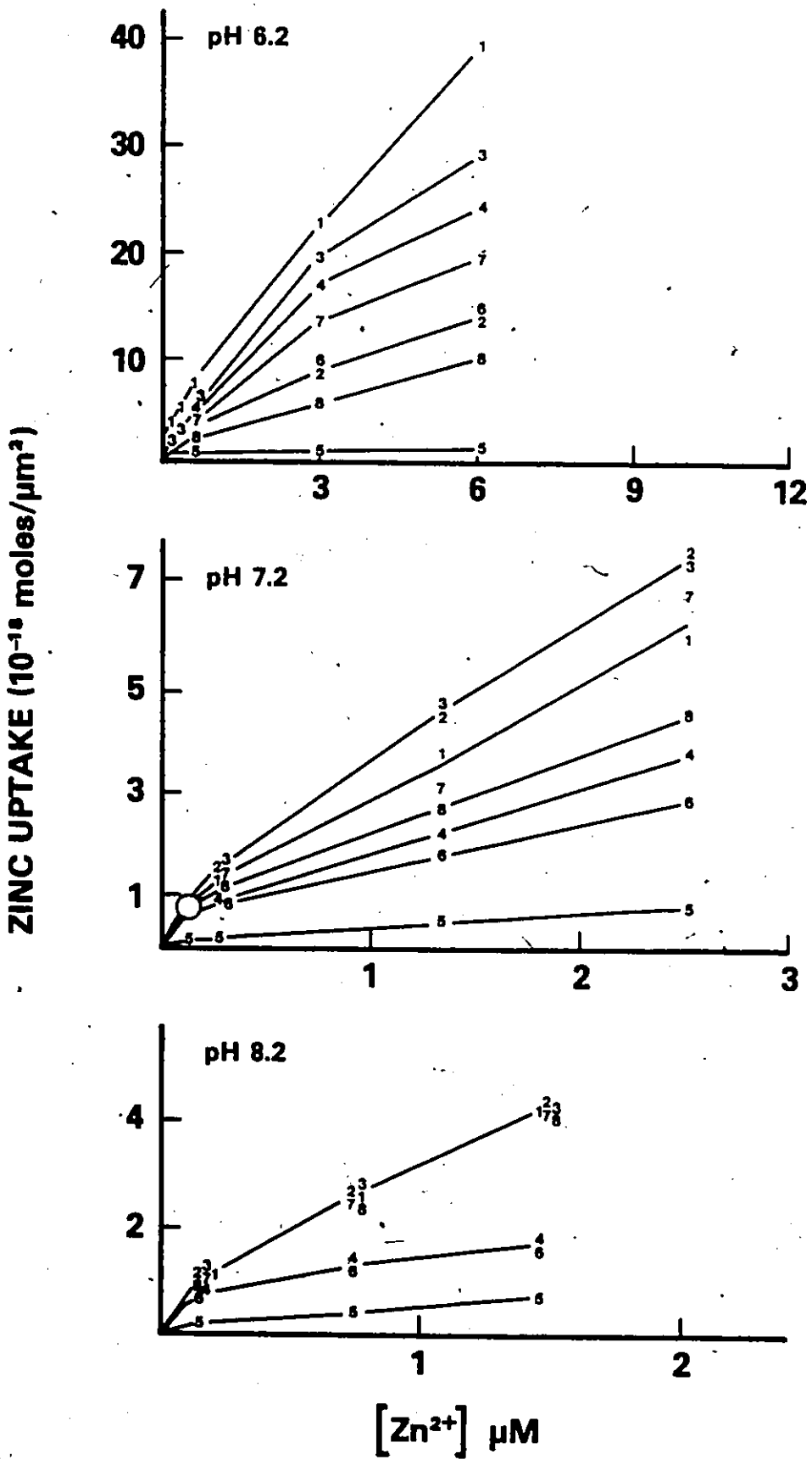

pH 6.2
40
30
20
10
3
6
9
12
pH 7.2
7
5
3
1
1
2
3
pH 8.2
4
2
1
2
ZINC UPTAKE (10^{-18} moles/μm^2)
$[Zn^{2+}]$ μM

Fig. XXIV. Average Particulate and Dissolved Metal Concentrations wthin each Bag over the Duration of the Study Period

Dissolved (FM) and particulate (PM) metal concentrations are in units of mg/L, left and right axis, respectively. Losses of both fractions from the water column over time are illustrated. Variations in PM Cu, Sr, and Zn were uncorrelated to changes in the dissolved fraction. PM Cd, Co, and Ni increased as the respective FM fractions decreased.

Notation, unless otherwise noted, is as follows: "○" PM metal concentration; "●" FM concentration. Other notation as in Figure XII.

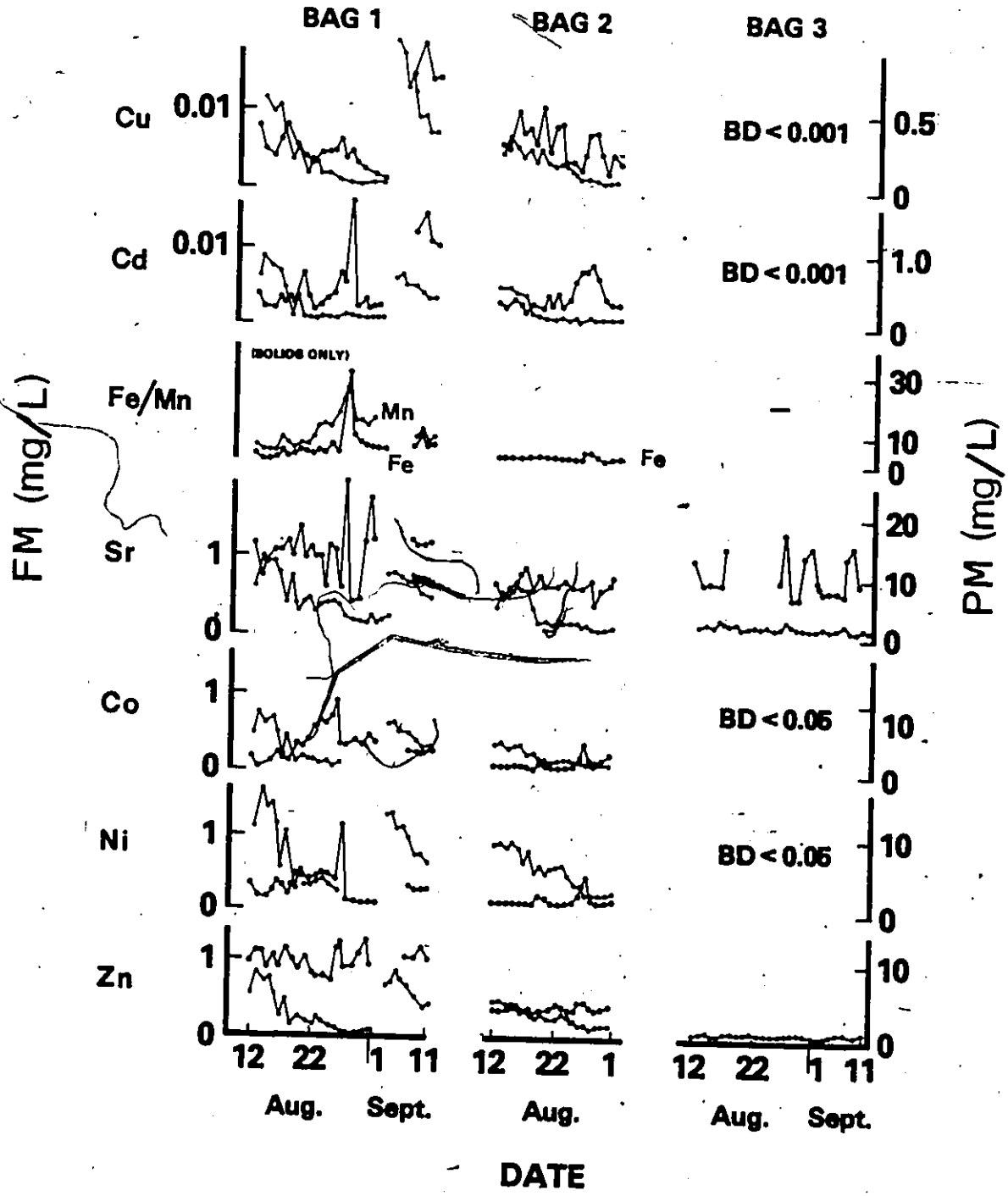

BAG 1
BAG 2
BAG 3
Cu
0.01
BD < 0.001
0.5
0
Cd
0.01
BD < 0.001
1.0
0
(SOLIDS ONLY)
Fe/Mn
Mn
Fe
Fe
30
10
0
FM (mg/L)
Sr
1
0
20
10
0
PM (mg/L)
Co
1
0
BD < 0.05
10
0
Ni
1
0
BD < 0.05
10
0
Zn
1
0
10
0
12 22 1 11
Aug. Sept.
12 22 1
Aug.
12 22 1 11
Aug. Sept.
DATE

Fig. XXV. Average Daily Variations in K^+, Ca^{2+}, Mg^{2+}, and Na^+ Concentrations within each Bag and in Hamilton Harbour

The variable nature of the chemical environment in natural waters is illustrated by the temporal fluctuations in conservative ions observed in the bags and Hamilton Harbour during the study period. These variations may not directly alter the availability of metals towards phytoplankton, but could influence the cell's ability to utilize available resources. All concentrations in mg/L.

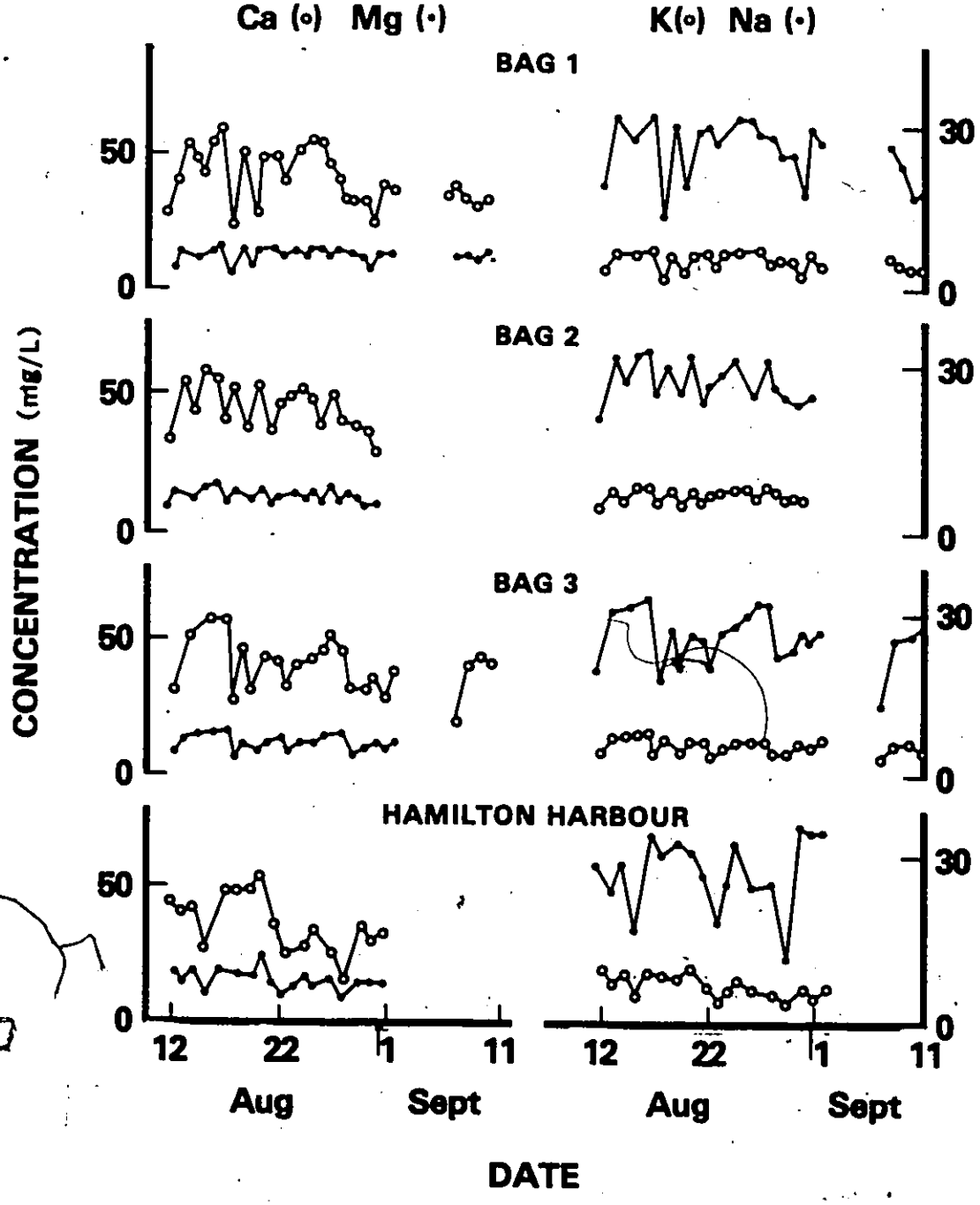

Ca (o) Mg (•)
K(o) Na (•)
BAG 1
BAG 2
BAG 3
HAMILTON HARBOUR
CONCENTRATION (mg/L)
50
0
30
0
12
22
1
11
Aug
Sept
DATE

Table XXIV. Summary of Data from Studies on the Effects of Cu, Cd, Hg, and Zn on Phytoplankton Growth in Batch Culture

SPECIES (SURFACE AREA um^2)	COPPER SLOPE	COPPER LC_{50}	COPPER REF	ZINC SLOPE	ZINC LC_{50}	ZINC REF	MERCURY SLOPE	MERCURY LC_{50}	MERCURY REF	CADMIUM SLOPE	CADMIUM LC_{50}	CADMIUM REF
Amphidinium carteri (400)	4.6	334	1	0.57	3003	1	44.6	56	2	2.2	430	2
	3.5	118	2									
	15.9	1033	3									
	31.8	668	3									
	7.0	2350	3									
Chaetoceros didymus (840)	24.1	40	2				59.8	40	2	20.0	67	2
Chlamydomonas (190)	7.9	493	2				35.7	23	2	1.05	275	2
Chlorella (350)	3.2	238	4	0.042	1232	5				0.35	4	2
	12.1	13	4							0.30	5	2
Coelastrum microporum (350)	4.3	21	7	0.135	32340	7						
Coelastrum pseudomicroporum (350)	2.4	111	7	0.212	19142	7						
Cricosphaera elongata (170)	21.6	2400	8									
Cryptomonas pseudobaltica (270)	3.5	2735	2				59.8	97	2	1.05	815	2
Dunaliella tertiolecta (661)	2.1	1400	3									
	1.7	1479	3									
	0.8	3291	3									
	159.0	127	2									
Exuviaella marine-lebourine (400)							89.3	72	2	2.5	610	2
Fragilaria pinnata (240)	2.1	1400	2				35.7	16	2	2.5	105	2
Heterothrix (100)	3.2	14200	2				59.8	186	2	0.7	3720	2
Isochrysis galbana (980)	8.7	366	3				59.8	4	9			
	7.6	29	3									
	1.9	165	3									
Lauderia borealis (4400)	7.0	493	3				89.3	10	2	2.5	170	2
Monallantus salina (150)	1.7	2274	2				44.6	35	2	2.5	195	2
Monochrysis lutheri (50)	3.5	8840	2				44.6	271	2	1.1	2780	2
	5.1	19430	10									
Nitzschia closterium (190)	3.5	4643	2				89.3	110	2	1.1	115	2
Olisthodiscus luteus (770)	15.9	22	3									
	15.9	27	3									
	4.9	84	3									
Pavlova pinguis (700)	3.2	1303	2				44.6	14	2	2.5	80	2
Phaeodactylum tricornutum (330)	1.7	445	1	0.024	34912	1	35.7	5	2	0.55	140	2
	3.2	604	2	0.032	724	12						
Porphyridium marinum (1000)	39.7	16	2				44.6	5	2	1.0	45	2
Prasinocladus marinus (530)	7.9	175	2				19.6	113	2	1.0	615	2
Scenedesmus sp. (190)	1.5	795	4									
	3.7	191	4									
Scenedesmus sp. (200)	2.1	8983	7	0.077	158805	7						
				0.176	22700	7						
				0.014	106383	7						
Scenedesmus quadricauda (200)	0.2	270	7	0.045	5190	7						
	2.1	922	7	0.068	2279	7						
	0.2	413	7									
Selenastrum capricornutum (55)	4.3	11	13	11.5	6	13				3.7	4	13
	39.7	11	13									
Skeletonema costatum (600)	8.3	81	1	2.04	20	12	59.8	9	2	2.0	240	2
	3.5	557	2	1.17	20	12						
	10.0	183	3	2.75	246	1						
	10.3	286	3	3.49	185	1						
	19.9	1510	6									
	7.6	1622	6									
	9.7	238	1									
Tetraselmis striata (240)	7.9	397	2				25.9	121	2	1.0	1580	2
Thalassiosira pseudonana (70)	9.1	668	1	1.07	539	12	89.3	255	2	1.0	3645	2
	6.7	5899	2	0.024	163101	1						
	15.9	1129	3									
	9.9	1463	3									
	2.5	12370	3									
	20.2	175	11									
	37.5	81	11									
	39.1	43	11									

Bracketed values next to species represents the value for the surface area used in the calculations (SA). Values for the slope (units in ml/mole) were obtained from a regression analysis of the relative growth rate (as a percent of the controls) and the total metal concentration over the range of metal concentrations influencing growth (positive slopes obtained from plot of growth rate versus decreasing metal concentration). Estimates for the metal concentration resulting in a 50 % decrease in the final growth rate were then obtained. These values are presented in the LC_{50} column and are in units of metal per unit surface area (10^{-17} moles/um^2). Correlation coefficients (r) for all regressions were greater than 0.90. References (REF): 1, Braek et al., 1976; 2, Berland et al., 1976; 3, Erickson et al., 1970; 4, Stokes et al., 1973; 5, Rachlin and Farran, 1974; 6, Morel et al., 1975; 7, this study; 8, Gnassia-Barelli et al., 1978; 9, Davies, 1974; 10, Sunda and Lewis, 1978; 11, Erickson, 1972; 12, Jensen et al., 1974; 13, Bartlett et al., 1974.

Table XXV Metal Analysis of Panel Scrapings (ug/g ash weight)

Bag	Sample[1]	Weight[2] Dry	Ash	Element Na	K	Mg	Ca	Fe	Mn	Cr	Sr	Co	Ni	Zn	Cd	Cu	Pb
1	A 0.5	13	19	13	20	37	54	65	3.4	-	-	1.9	2.3	13.1	0.7	4.1	0.4
	3.0	15	18	12	18	24	24	26	1.6	-	-	0.9	1.2	7.5	0.3	3.7	0.3
	6.0	21	18	10	15	35	41	54	1.5	-	-	1.2	2.3	11.1	0.6	7.9	0.5
	B 0.5	36	17	16	17	31	28	29	2.0	-	-	3.9	5.5	10.9	1.2	9.8	0.3
	3.0	31	17	13	16	25	34	24	2.7	-	-	1.3	1.7	7.8	2.5	14.4	1.2
	6.0	28	14	21	18	26	22	36	0.8	-	-	0.9	2.1	7.2	2.4	20.7	0.3
	C 0.5	37	19	14	20	29	49	27	4.2	-	0.6	3.4	4.4	10.0	0.9	7.3	0.1
	3.0	20	14	28	23	30	26	24	2.6	-	-	1.5	2.2	7.9	1.5	15.5	0.6
	6.0	33	23	11	14	27	30	34	1.2	0.2	-	0.8	1.8	5.4	0.7	15.6	0.6
2	A 0.5	13	28	9	18	31	48	29	2.4	-	-	2.0	1.3	7.1	0.3	3.2	0.2
	3.0	13	28	13	14	31	35	35	2.0	-	-	0.8	1.1	7.0	0.5	5.1	0.2
	6.0	23	31	12	13	33	89	8	1.9	-	0.3	1.0	1.7	8.6	1.5	4.6	-
	B 0.5	23	19	13	13	41	158	1	3.7	-	0.5	3.9	4.3	16.1	0.6	3.2	-
	3.0	15	15	22	28	51	46	1	1.4	-	-	1.2	2.6	19.5	3.5	7.8	-
	6.0	20	24	10	14	41	52	1	1.0	-	-	1.1	1.8	15.3	2.2	4.5	-
	C 0.5	22	16	17	14	38	140	2	2.9	-	0.4	3.2	4.1	14.9	0.8	3.9	-
	3.0	15	14	30	19	49	73	1	1.8	-	-	1.4	2.8	18.8	3.5	6.6	-
	6.0	10	22	20	16	42	62	1	1.3	-	-	1.1	2.4	15.8	1.9	3.7	-
3	A 0.5	57	34	3	9	20	307	10	2.1	0.1	0.4	0.1	0.1	1.1	-	0.3	-
	3.0	24	21	9	15	31	31	56	10.3	0.3	-	-	0.2	4.9	0.1	0.9	-
	6.0	25	33	5	12	38	37	32	2.7	0.3	-	-	0.1	4.7	0.2	0.5	0.1
	B 0.5	119	48	3	11	18	12	16	6.9	0.1	-	0.1	0.1	1.6	0.1	0.1	-
	3.0	83	51	2	9	21	22	22	1.2	0.2	-	-	0.1	2.3	-	0.1	-
	6.0	36	28	5	15	20	8	31	9.6	0.3	-	0.1	0.2	2.6	-	0.3	-
	C 0.5	232	44	2	9	13	166	2	1.7	0.1	0.3	0.1	0.1	0.6	-	0.1	-
	3.0	35	30	9	16	22	12	27	3.4	0.2	-	-	0.2	3.1	-	0.6	-
	6.0	33	32	8	15	19	8	23	8.7	0.2	-	-	0.1	2.2	0.1	0.4	-

Notes: 1. Sample Date/Depth:

A = one side scraped on day 16 after metals added (Aug. 22, 1981)

B = side (previously scraped on day 16) scraped on day 29 (Sept. 11, 1981)

C = side (unscraped) scraped on day 29 (Sept. 11, 1981)

(0.5, 3.0, 6.0 refer to depth at which each panel was suspended)

2. dry weight in g/m^2, ash weight expressed as a percent of the dry weight

3. sample standard deviations did not exceed $\pm$ 15 % and all determinations were carried out in triplicate

Table XXVI Photosynthesis and Zinc Binding Data from the Factorial Designed Experiments

		Chlamydomonas								Chlorella UT	Coelastrum UT	Scenedesmus UT	Scenedesmus HH
RUN	VAR	PH6L	PH6L	PH7L	PH7D	PH7C	PH7t	PH8L	PH8TH	PH7L	PH7L	PH7L	PH7L
1	-	3.25	3.41	1.08	.59	.101	1.08	.54	1.41	.763	.403	1.13	3.56
2	1	2.22	2.29	.70	.53	.092	.87	.28	1.14	.391	.341	.49	2.61
3	2	2.54	2.19	.82	.66	.151	1.45	.21	1.57	.492	.383	.71	2.99
4	12	.96	1.25	.86	.59	.138	1.05	.18	1.16	.398	.156	.51	1.58
5	3	1.63	2.15	.95	.52	.128	1.24	.26	1.07	.483	.282	.46	1.94
6	13	1.59	1.43	.63	.51	.113	.79	.36	1.37	.359	.215	.34	1.57
7	23	1.54	1.60	.88	.49	.130	1.30	.22	1.11	.455	.094	.39	1.96
8	123	.70	1.03	.70	.49	.127	.91	.12	1.36	.314	.226	.33	.89
9	4	1.65	1.70	.88	.51	.096	1.06	.39	.94	.440	.184	.43	1.21
10	14	3.06	2.15	.73	.47	.109	.69	.33	1.10	.380	.101	.30	.47
11	24	1.47	1.66	.83	.52	.137	1.20	.16	1.32	.472	.205	.37	1.61
12	124	1.04	1.22	.69	.47	.122	.81	.15	1.25	.339	.096	.30	.82
13	34	1.33	1.35	.64	.44	.113	.98	.27	1.08	.447	.181	.29	.96
14	134	1.40	1.22	.61	.48	.098	.86	.19	1.22	.365	.124	.25	.95
15	234	1.11	1.11	.78	.55	.134	1.10	.18	1.18	.350	.168	.28	1.07
16	1234	.80	.95	.65	.46	.126	.91	.14	1.23	.313	.100	.28	.91
17	5	2.30	1.72	.67	.48	.107	1.15	.35	1.76	.391	.106	.40	.97
18	15	2.84	3.25	.54	.46	.096	.80	.34	2.12	.328	.110	.25	.87
19	25	1.46	1.38	.86	.48	.134	1.34	.20	1.46	.408	.113	.30	.91
20	125	1.91	2.32	.67	.45	.113	.89	.22	1.43	.304	.079	.27	1.00
21	35	1.48	1.96	.56	.45	.132	1.22	.23	2.26	.333	.100	.23	1.23
22	135	2.24	2.40	.61	.48	.119	.80	.20	2.71	.285	.061	.17	.45
23	235	1.06	1.09	.65	.40	.118	1.49	.09	1.92	.321	.095	.25	.51
24	1235	1.89	1.56	.54	.42	.124	.94	.08	1.75	.291	.063	.15	.36
25	45	1.48	1.33	.67	.53	.084	.81	.43	3.02	.298	.068	.24	1.36
26	145	2.84	2.72	.72	.49	.093	.67	.43	2.83	.271	.057	.16	.87
27	245	1.19	1.20	.53	.45	.120	1.36	.24	1.69	.282	.086	.21	.77
28	1245	1.58	2.36	.80	.56	.123	.82	.16	1.82	.262	.074	.21	1.14
29	345	1.21	1.28	.73	.51	.123	.98	.31	2.94	.312	.065	.17	1.23
30	1345	2.03	2.06	.55	.46	.114	.72	.36	3.39	.290	.010	.17	1.23
31	2345	1.11	.97	.59	.38	.119	1.26	.16	1.99	.273	.041	.18	.99
32	12345	1.58	1.45	.55	.33	.110	.94	.12	1.99	.249	.067	.17	.70
33	6	3.68	2.34	.57	.45	.112	1.09	.52	1.26	.432	.188	.49	2.87
34	16	1.96	1.71	.63	.34	.104	.80	.33	1.25	.305	.109	.33	1.38
35	26	2.09	1.69	.68	.35	.126	1.51	.13	1.53	.375	.140	.33	2.05
36	126	.80	1.17	.55	.34	.119	.88	.11	1.26	.265	.080	.25	1.22
37	36	1.60	1.91	.67	.28	.135	1.15	.48	1.47	.365	.129	.24	1.65
38	136	1.29	1.32	.62	.39	.111	.79	.34	1.32	.253	.059	.21	1.18
39	236	1.41	1.34	.78	.40	.149	1.41	.19	1.56	.321	.097	.25	1.61
40	1236	.71	.88	.63	.34	.128	.90	.13	1.50	.251	.063	.16	1.02
41	46	1.39	1.31	.53	.37	.106	.78	.42	1.27	.325	.094	.27	1.58
42	146	1.78	1.76	.42	.31	.098	.71	.32	1.47	.280	.023	.17	1.32
43	246	1.27	1.40	.66	.36	.141	1.25	.20	1.47	.289	.086	.26	1.12
44	1246	.94	.89	.57	.31	.105	.83	.15	1.32	.230	.047	.23	1.00
45	346	1.12	1.19	.61	.30	.136	.92	.44	1.49	.295	.053	.21	1.30
46	1346	1.14	1.17	.37	.32	.123	.78	.29	1.57	.254	.070	.20	1.24
47	2346	1.07	1.14	.69	.33	.113	1.20	.14	1.33	.272	.097	.19	1.33
48	12346	.77	.86	.56	.32	.108	.93	.12	1.55	.242	.013	.28	1.08
49	56	2.01	2.43	.61	.39	.099	1.04	.44	3.39	.316	.088	.32	2.04
50	156	2.17	2.27	.51	.31	.096	.72	.38	2.60	.237	.039	.15	1.20
51	256	1.47	1.30	.63	.32	.111	1.32	.15	1.88	.253	.039	.26	1.57
52	1256	1.63	2.02	.45	.26	.100	.86	.16	2.03	.216	.046	.15	1.02
53	356	1.50	1.76	.60	.28	.132	1.17	.35	2.09	.253	.075	.17	1.31
54	1356	1.91	1.93	.51	.30	.101	.74	.24	2.62	.212	.013	.18	.78
55	2356	1.28	1.25	.72	.33	.123	1.39	.15	2.57	.252	.059	.20	1.23
56	12356	1.34	1.62	.53	.28	.113	.86	.02	2.44	.200	.011	.16	.66
57	456	1.25	1.27	.51	.31	.088	.89	.26	3.62	.246	.012	.16	1.34
58	1456	2.18	2.00	.38	.31	.081	.67	.26	3.72	.222	.021	.18	.70
59	2456	1.04	1.11	.69	.38	.117	1.18	.06	2.46	.240	.015	.18	.74
60	12456	1.39	1.63	.68	.37	.110	.80	.05	2.49	.236	.032	.11	.53
61	3456	.94	.99	.63	.32	.122	1.01	.26	3.56	.250	.040	.12	.88
62	13456	1.55	1.60	.60	.34	.131	.79	.16	3.20	.212	.025	.14	.82
63	23456	.82	1.09	.85	.25	.146	1.38	.13	2.73	.227	.041	.22	1.05
64	123456	1.13	1.73	.61	.22	.116	.93	.11	3.59	.200	.052	.16	.75

The numbers in the variable column (VAR) represent the high setting for the respective variables (metals) present in each run ("+"/"-" correspond to high (5.0 uM) and low (omitted) settings for the variables in each run). Run number "64" for example, all metals are added to the flask (variable settings at "+" level), and the respective numerical code in the variable column is "123456". All experiments were carried out at 20°C in MB with constant agitation provided by a rotary shaker (refer to Tables V and VIII for incubation conditions and intial conditions for each factorial experiment). Notation for column headings is as follows: PH6, PH7, PH8 refer to experiments carried out at pH 6.2, pH 7.2, and pH 8.2 (± 0.1 units) respectively; L, light intensity of 100-110 uEin m⁻² sec⁻¹ p.a.r.; D, dark experiment; t, incubation period of 600 min (3 times the standard assay period); C, photosynthesis experiment with stable zinc; TH, 0.006 M TRAM buffer system substituted for phosphate (pH 8.2); HH, Hamilton Harbour species; UT, University of Texas species. All valued are in units of 10^{-15} moles zinc/cell except data in column PH7C which are in units of 0.1 DPM/cell (^{65}Zn uptake experiment).

Table XXVII. Effects Calculated using Yates Algorithm on Photosynthesis and Zinc Binding Data

		Chlamydomonas								Chlorella UT	Coelastrum UT	Scenedesmus HH	Scenedesmus UT
RUN	INT	PH6L	PH6L	PH7L	PH7D	PH7C	PH7E	PH8L	PH8TH	PH7L	PH7L	PH7L	PH7L
1	avg	1.580	1.623	.651	.408	11.64	1.003	.240	1.910	.315	.0995	.271	1.238
2	1	.052	.145	-.097	-.020	-1.00	-.351	-.054	.044	-.071	-.0403	-.078	-.459
3	2	-.590	-.468	.067	-.012	1.43	.207	-.191	-.323	-.031	-.0135	-.022	-.217
4	12	-.223	-.055	-.018	-.007	-0.17	-.079	.017	-.020	.010	.0056	.025	.029
5	3	-.518	-.408	-.017	-.043	1.22	.044	-.053	.127	-.037	-.0273	-.091	-.303
6	13	.064	-.080	-.010	.016	-0.19	.001	-.001	.110	.014	.0097	.047	.101
7	23	.241	.088	-.017	-.013	-1.10	-.025	.026	.099	.004	.0031	.029	.051
8	123	.048	.052	.001	-.020	0.35	.027	-.015	-.007	-.004	.0128	-.026	-.100
9	4	-.369	-.378	-.034	-.020	-0.37	-.115	-.019	.297	-.045	-.0534	-.099	-.407
10	14	.309	.211	.015	.003	0.21	.070	.009	.060	.029	.0063	.049	.207
11	24	.089	.196	.005	-.002	0.02	.018	.013	-.115	.006	.0206	.033	.101
12	124	-.118	-.123	.038	.003	-0.36	-.010	-.007	.050	-.010	-.0039	-.014	.003
13	34	.116	.061	-.001	-.002	0.04	.029	.014	.012	.021	.0226	.062	.295
14	134	-.214	-.034	-.013	-.014	-0.01	.034	-.005	-.033	-.009	-.0063	-.018	.009
15	234	-.026	-.013	.012	-.016	-0.21	.002	.003	-.020	-.016	-.0062	-.013	-.026
16	1234	.125	.055	-.027	.000	-0.12	-.001	.023	.079	.012	-.0128	.021	-.040
17	5	.079	.196	-.071	-.046	-0.68	-.001	-.037	1.190	-.089	-.0890	-.143	-.525
18	15	.488	.530	.020	.007	0.17	-.024	.022	.044	.031	.0196	.028	.144
19	25	.088	.036	-.008	-.022	-0.31	.016	.010	-.040	.016	.0179	.020	.007
20	125	.063	.043	.011	.006	0.03	-.004	-.017	.037	-.008	.0066	-.024	.085
21	35	.163	.060	.010	-.008	0.49	.039	-.019	.086	.015	.0141	.047	.124
22	135	-.070	-.100	.004	-.014	-0.07	-.022	-.015	.008	-.009	-.0216	-.024	-.121
23	235	-.067	-.061	-.013	-.020	-0.07	.021	-.003	.151	-.006	.0034	-.009	-.051
24	1235	-.002	-.046	-.033	.005	-0.20	-.010	.014	-.076	.004	-.0035	.006	-.008
25	45	.046	.036	.061	.027	0.24	.022	.012	.330	.013	.0294	.047	.345
26	145	-.194	-.096	.024	.004	0.11	-.010	-.002	-.020	-.012	.0079	-.020	-.096
27	245	-.041	.021	.000	-.007	0.42	.026	-.010	-.099	-.007	-.0086	-.019	-.112
28	1245	.004	.046	.004	.009	-0.04	-.012	-.006	.060	.012	.0083	.004	-.022
29	345	-.084	-.018	.023	-.021	0.31	-.007	.023	-.009	-.004	-.0149	-.032	-.093
30	1345	.116	.052	-.044	-.007	-0.21	-.009	.018	.026	.000	.0111	.007	.051
31	2345	.038	.027	-.026	-.023	-0.16	-.012	.008	.013	.005	.0038	.014	.079
32	12345	-.058	-.042	.008	-.005	-0.45	.017	-.013	.070	-.014	.1840	-.012	-.081
33	6	-.247	-.240	-.112	-.160	-0.13	-.024	-.014	.407	-.098	-.0781	-.108	-.002
34	16	-.128	-.080	-.013	-.002	-0.33	-.054	-.017	-.028	.015	.0043	.027	-.028
35	26	.073	.100	.027	.001	-0.49	.016	-.027	.060	.007	.0078	.014	-.009
36	126	.084	.050	-.009	-.006	-0.10	-.022	.019	.084	-.003	.0016	-.022	.030
37	36	.051	.125	.073	.013	0.51	.021	.030	-.032	.011	.0157	.044	.063
38	136	.025	.069	.003	.010	-0.05	-.009	-.017	-.001	-.009	-.0124	-.010	.031
39	236	-.036	.032	.016	.015	0.23	.009	-.005	.154	.004	.0021	-.001	.121
40	1236	-.078	-.034	-.012	-.005	-0.19	-.004	.013	.040	.004	-.0199	.014	-.031
41	46	-.074	.016	.015	.004	0.27	.017	-.027	.084	.015	.0228	.049	.033
42	146	.016	-.009	-.024	.004	-0.09	.014	.006	.048	-.007	.0104	-.016	.041
43	246	.066	-.012	.057	.006	-0.06	.003	.024	.007	.000	-.0091	-.001	-.073
44	1246	.018	-.047	-.006	.000	-0.14	.002	.004	.001	.006	-.0077	.012	-.044
45	346	.015	.022	.004	-.007	0.10	.011	.002	.040	-.010	-.0036	-.020	-.043
46	1346	.037	.015	.003	.005	0.48	-.025	.009	.001	.004	.0108	.009	-.070
47	2346	-.053	.040	-.046	-.023	-0.54	.006	-.008	-.022	.009	-.0008	.008	.044
48	12346	-.010	.043	.008	.018	-0.09	-.004	-.002	.109	-.009	.0055	-.005	.048
49	56	-.042	.050	.067	.013	-0.12	-.003	-.032	.212	.027	.0412	.067	.129
50	156	-.036	-.146	-.027	-.008	0.03	.006	-.003	-.010	-.013	-.0031	-.026	-.120
51	256	.005	.016	.015	.013	0.44	-.029	.018	.088	-.007	-.0110	-.009	.028
52	1256	-.077	.074	-.018	-.008	-0.09	.024	-.002	.076	.008	.0022	.004	-.089
53	356	-.029	-.034	.006	-.003	0.08	-.002	.001	-.107	-.010	-.0025	-.021	.095
54	1356	-.047	.107	.006	.001	-0.13	-.004	-.003	.062	.003	.0090	.020	.087
55	2356	-.043	.106	.003	-.004	0.17	-.003	.018	.134	.001	-.0005	.012	.044
56	12356	.026	-.027	.002	.008	-0.10	-.017	-.013	.002	-.011	.0109	-.003	.015
57	456	.019	-.069	.008	-.008	0.32	.020	-.040	.009	.005	-.0120	-.038	-.344
58	1456	.043	.068	.003	.006	0.01	-.016	.006	.022	.004	.0005	.014	.007
59	2456	-.072	.031	.013	.006	0.23	-.026	.017	.076	.010	.0066	.002	.105
60	12456	.010	-.033	-.025	-.012	-0.13	.004	.011	-.002	-.008	-.0045	-.023	.056
61	3456	-.067	.043	.008	.011	0.23	.013	.011	.079	.000	.0154	.014	.097
62	13456	-.002	-.030	.038	.003	-0.01	.015	-.011	-.080	-.002	-.0077	-.027	.036
63	23456	.062	-.028	.004	.001	0.44	.025	.012	-.025	-.012	-.0011	.005	-.045
64	123456	.020	.056	-.029	-.005	-0.20	-.025	.014	.105	.017	-.0069	.008	.031
SE	(±)	0.048	0.048	0.020	0.013	0.36	0.031	0.010	0.060	0.010	0.0031	0.009	0.039

The calculate standard error of an effect is represented by SE. The numbers in the INT (interaction) column represent the high setting for the respective variables (metals) present in each run ("+"/"-" correspond to high (5.0 uM) and low (omitted) settings for the variables in each run). Run number "64" for example, all metals are added to the flask (variable settings at "+" level), and the respective numerical code in the variable column is "123456". All experiments were carried out at 20°C in MA with constant agitation provided by a rotary shaker (refer to Tables V and VIII for incubation conditions and initial conditions for each factorial experiment). Notation for column headings is as follows: PH6, PH7, PH8 refer to experiments carried out at pH 6.2, pH 7.2, and pH 8.2 (± 0.05 units) respectively; L, light intensity of 100-110 uEm⁻²s⁻¹ ph.s.r.; D, dark experiment; t, incubation period of 600 min (3 times the standard assay period); C, photosynthesis experiment with stable zinc; TH, 0.006 M TRAM buffer system substituted for phosphate (pH 8.2); HH, Hamilton Harbour species; UT, University of Texas species.

Table XXVIII Ordered Effects of Metals on Fluorescence

	Chalmydomonas					Chlorella UT	Coelastrum UT	Scenedesmus HH	Scenedesmus UT
RUN	PH6L	PH6L	PH7C	PH8L	PHRTH	PH7L	PH7L	PH7L	PH7L
1	1	1	2	1	15	1	3456	3	3
2	15	15	56	23	1	5	5	124	1
3	5	5	16	46	345	2346	246	236	2
4	36	356	234	2456	45	56	145	246	34
5	12456	36	1236	1235	123	23456	35	34	23456
6	23456	345	12356	246	136	124	2356	146	56
7	25	1256	1256	56	1456	14	345	356	36
8	346	3456	2456	1356	4	345	1256	135	124
9	135	25	135	136	12356	1345	23	12456	26
10	3456	125	134	456	14	3456	1236	2	1245
11	34	12345	136	1245	1235	1245	12356	1346	1346
12	26	136	1345	16	1246	46	34	45	1246
13	46	346	346	123	23	346	1346	36	156
14	125	13456	1235	14	36	3	56	1456	15
15	356	1236	26	24	1345	12	124	145	256
16	23	256	1346	1346	456	134	235	4	12456
17	1236	12346	13	245	12346	125	26	35	46
18	1256	135	14	236	234	1235	14	12346	2456
19	24	236	12456	3456	13456	34	123456	245	2346
20	1345	1245	1356	256	13	23	156	2356	145
21	56	26	2345	2356	1234	1456	256	345	345
22	256	2456	126	356	1346	2	245	456	12356
23	2345	16	12	135	2346	1236	125	146	13
24	16	1356	125	2345	34	12356	1	346	5
25	1456	24	2346	36	256	126	3	1356	134
26	14	124	356	146	1245	2456	135	23456	16
27	345	1346	23	346	1236	235	1345	5	126
28	126	56	145	12456	2456	145	36	12345	14
29	146	12456	24	25	236	1246	13	16	2345
30	123	2345	5	125	23456	25	24	134	1236
31	2456	235	15	1256	126	156	1235	15	24
32	124	126	25	1234	12456	1356	356	136	1235
33	12345	46	235	2346	24	13456	25	13456	2356
34	1246	146	3456	12356	246	245	134	1246	3456
35	123456	245	2356	234	146	146	2456	46	234
36	243	134	1246	1246	346	234	123	1256	125
37	245	1234	123456	12345	1256	26	45	123456	4
38	1245	156	123	23456	3456	13	15	235	1345
39	156	6	1245	13	26	2345	126	25	12345
40	235	23456	35	15	134	12346	12	126	236
41	1235	45	45	45	16	1346	1245	12	346
42	1356	1246	146	145	2356	2356	2	1345	356
43	13456	1456	13456	1236	124	45	16	1236	246
44	1234	1235	245	12346	156	16	12345	23	456
45	2346	14	1456	156	2	4	46	13	1234
46	12346	1345	236	123456	123456	35	1246	256	146
47	35	35	345	124	46	12456	346	123	12346
48	246	2356	256	1345	6	15	12456	2345	25
49	236	123	46	1456	125	123	234	12	45
50	2356	456	23456	13456	135	24	146	125	35
51	1346	23	1234	34	56	1256	12346	234	136
52	12356	246	12345	235	145	456	1456	1235	1456
53	45	34	4	134	2345	12345	1234	24	12
54	134	2346	246	126	1356	1234	13456	26	123456
55	456	12356	156	5	356	135	136	12356	135
56	12	145	34	35	12345	36	1356	2346	13456
57	3	234	36	345	12	256	23456	3456	235
58	145	123456	124	26	235	236	236	6	6
59	136	13	456	4	245	6	456	56	1356
60	13	3	12346	3	3	136	4	2456	1256
61	4	4	1	12	35	356	2346	1245	123
62	2	12	3	6	5	246	2345	1234	23
63	6	2	6	2	25	123456	6	1	245
64	avg	avg	avg	avg	avg	avg	avg	avg	avg
Mean	22	28	115	150	147	178	153	209	90
±X	2	2	5	8	8	9	8	11	5

Average Fluorescence (Arbitrary Units) for all Settings and Standard Error Estimates from Replicated Controls (±)

Effects ordered relative to the overall average fluorescence response (greatest effect at top of table) and bracketed effects are not significantly different from the average response. Numerical codes for each effect represent the high setting for the respective variables (metals) present in each run ("+"/"-" correspond to high (5.0 uM) and low (omitted) settings for the variables in each run). Run number "64" for example, all metals are added to the flask (variable settings at "+" level), and the respective numerical code for the effect is "123456". All experiments were carried out at 20°C in MB with constant agitation provided by a rotary shaker (refer to Tables V and VIII for incubation conditions and initial conditions for each factorial experiment). Notation for column headings is as follows: PH6, PH7, PH8 refer to experiments carried out at pH 6.2, pH 7.2, and pH 8.2 (± 0.1 units) respectively; L, light intensity of 100-110 uEm⁻² s⁻¹ ph.a.r.; D, dark experiment; t, incubation period of 600 min (3 times the standard assay period); C, photosynthesis experiment with stable zinc; TH, 0.006 M TRIS buffer system substituted for phosphate (pH 8.2); HH, Hamilton Harbour species; UT, Uncontrolled

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