THE INTERACTION OF CHRONIC COPPER TOXICITY WITH NUTRIENT LIMITATION IN TWO CHLOROPHYTES

by

Julie Hall

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ABSTRACT

The interaction of N- and P-limitation with sublethal Cu toxicity was studied using batch and continuous culture techniques.

Batch culture experiments with Chlamydomonas sp. and Chlorella sp. were conducted with N and P limiting final yield. The sublethal effects of Cu toxicity included decreased growth rate and final yield; and increased cellular content of Cu and P. Final yield and growth rate were the most sensitive indicators of toxicity in P-limited and N-limited cultures respectively. Both Chlorella sp. and Chlamydomonas sp. were more sensitive to Cu toxicity in batch cultures under P-limitation than under N-limitation.

Experiments with chemostat cultures of Chlorella sp. were conducted with growth rate limited by either N or P. Sublethal effects of Cu toxicity included a decrease in cell numbers and an increase in cellular Cu content, although no changes in gross cell composition were observed. As in the batch culture experiments, Chlorella sp. was found to be more sensitive to Cu^{2+} toxicity under P-limitation than under N-limitation. Cells grown in chemostat cultures were more sensitive to Cu^{2+} activity by approximately 4 orders of magnitude when compared to batch cultures.

Results of these experiments have major ecological implications when nutrient limitation and chronic Cu toxicity in natural water bodies are considered.

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Chapter I

INTRODUCTION

1.1 DEFINITION OF "METALS"

The term "metals" as used in this thesis refers to the Borderline and Class B metals. The Borderline metals generally have biological roles but exhibit toxic action at elevated concentrations, for example, Cu^{2+}, Zn^{2+}, Ni^{2+}, and Cd^{2+} (Niebor and Richardson 1980). The Class B (sulphur seeking) metals are generally non-essential to plant growth and frequently highly toxic, for example, Hg^{2+}, Ag^{2+}, and Pb^{2+} (Niebor and Richardson 1980).

1.2 SOURCES OF METALS IN THE AQUATIC ENVIRONMENT

The four major sources of metals to the aquatic ecosystem are, geological weathering, industrial wastes particularly from the processing of metal ores, domestic sewage and stormwater runoff, and leachates from garbage and solid waste dumps.

1. Geological weathering of rock is the source of natural background metal concentrations, with elevated natural metal bearing rock formations are present.

2. Industrial wastes frequently have elevated metal concentrations (Moore and Ramamoorthy 1984). The prime source of metal contamination in industrial wastes is the processing of metal ores. Other industries with high concentrations of Cu in wastes are meat and fish processing, brewing, soft drink and flavouring manufacture, fur dressing and dyeing, and the laundry industry (Forstner and Wittman, 1983). The global refining of metals increased 16-57% between the decade 1961-1970 and 1971-1980. Ni refining increased 57%, Cr-46%, Zn-40%, Cu-26%, and Pb-16% (Moore and Ramamoorthy 1984). Such increased refining of metals indicates a potential for increased metal contamination of the environment. In fact approximately 85.8×10^3t of solid Cu wastes are discharged annually onto the earth's surface, with mine tailings and flyash accounting for approximately 75%. Approximately 17×10^3 t are deposited annually into the oceans (Nriagu 1979).

3. Domestic sewage and urban stormwater runoff are the largest sources of metal contamination to the aquatic environment (Forstner and Wittman 1983). Modern sewage systems allow the concentration of wastes in one area. For example, sewage sludge contains approximately 700 mg Cu kg^{-1} which is approximately 15 times the concentration found in uncontaminated areas of the earth's crust (Moore and Ramamoorthy 1984).

Urban stormwater runoff carries high concentrations of metals such as Pb, Zn and Cu (Whipple and Hunter 1977). By nature, stormwater runoff consists of pulse events which temporarily create high concentrations of metals. Such pulses can be more toxic to aquatic organisms than expected from mean levels.

4. Leachates from garbage and solid waste dumps frequently enter waterways via groundwater or overland runoff and can result in high levels of metal contamination. Recent dumping practices have reduced leaching from modern dump sites but in areas of old mine tailings, high concentrations of metals are still leached into natural water bodies (Forstner and Wittman 1983).

Point source inputs and dilution of contaminating metals result in concentrations ranging over more than four orders of magnitude with wide temporal and spatial fluctuations (Huntsman and Sunda 1980). In natural waters distant from sources of contamination the concentration of Cu in seawater is approximately 10^{-8}–10^{-9} M. Uncontaminated freshwater Cu concentrations are more varied with a mean of approximately 10^{-7} M (Boyle et al. 1977).

1.3 REMOBILIZATION OF METALS FROM SEDIMENTS

Metals, unlike many organic pollutants, do not degrade in the environment. The majority of metals enter the aquatic ecosystem in particulate form and are subsequently sedimented out. Gibbs (1977) estimates that approximately 3% of the metals transported in rivers are in the dissolved form (approximately 1% for Cu). Particulate metals in the sediments are relatively non-toxic but constitute a potential hazard if remobilized into the water column in a dissolved form. Remobilization can occur via a number of pathways (Forstner and Prosi 1979).

1. Physical disturbances of the sediments such as invertebrate feeding, erosion, and dredging result in resuspension of sedimented particulate metals, resulting in increases of dissolved metals in the water column.
2. Decreasing sediment pH can result in dissolution of hydroxy and carbonate complexes resulting in the remobilization of metals in a dissolved form.
3. Decreasing dissolved oxygen concentration at the sediment water interface can reduce the sediment redox potential leading to the dissolution of Mn and Fe oxides and associated metals such as Cu, Zn and Cd.
4. Microorganisms also remobilize metals from the sediments through the release of metal-containing organic complexes into the water column.

Such remobilization of metals from the sediments can create serious environmental problems in areas of heavily contaminated sediments. For example, the mobilization of As from sediments in Green Bay, Lake Michigan resulted in concentrations of As twice those permissible in drinking water. (Edgington and Callender 1970)

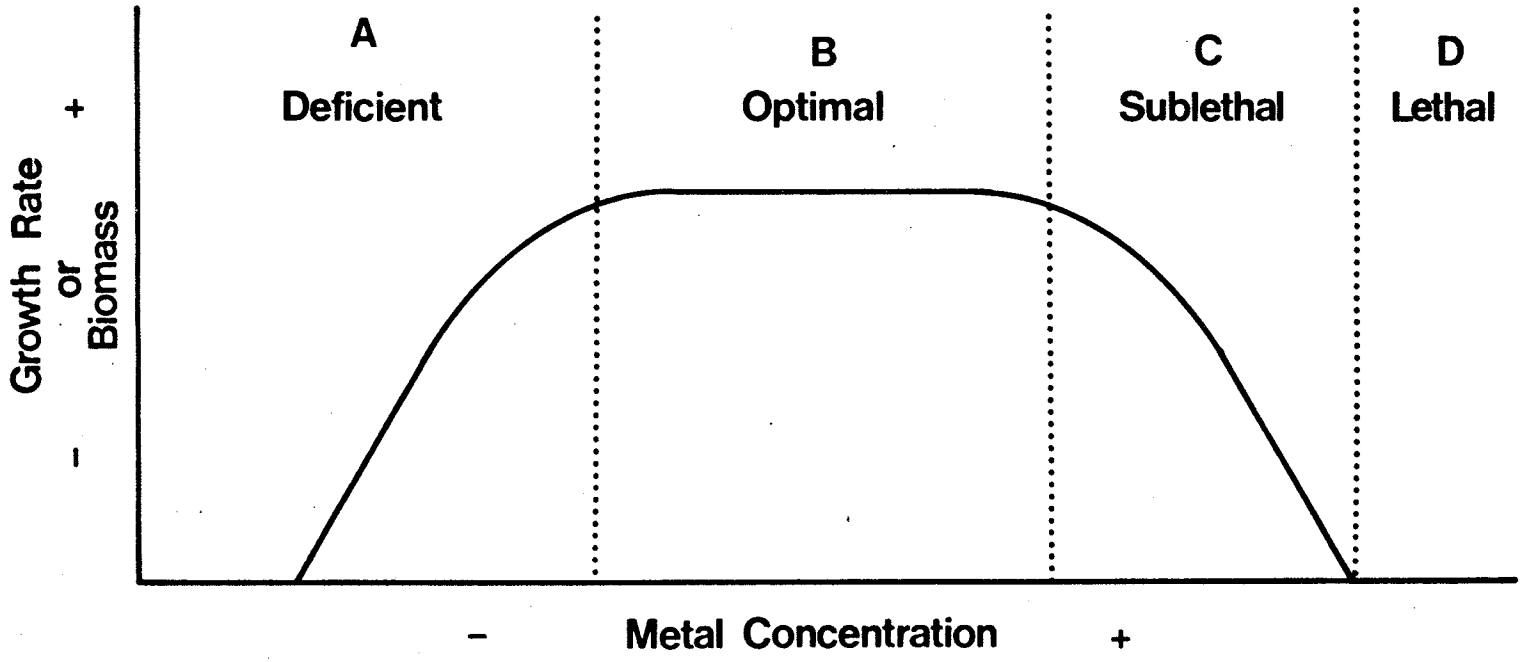
1.4 METALS AND CELL GROWTH

Metals essential for plant growth such as Cu, Zn, Co and Mn can have a range of influences on phytoplankton growth depending on the concentrations of the metal in the medium. At very low concentrations, metal deficiency can result in a reduction in cell growth and biomass (Fig. 1.1 A).

At concentrations above those which result in deficiency of the metal, cell growth is unrestricted by the metal (Fig. 1.1 B). This range of concentration is considered to be optimal for cell growth. Sublethal toxicity occurs when the concentration of the metal in solution exceeds that for optimal growth and results in a disruption of cell metabolism which is reflected in reduced growth rate and biomass (Fig. 1.1 C). Increasingly higher concentrations of the metal can be lethal (Fig. 1.1 D).

Cu is an essential metal which follows this pattern of deficiency, sufficiency and toxicity. The reduction of cell growth at very low concentrations of Cu has been reported. Sandman (1985) showed that a reduction in photosynthesis

Figure 1.1: The effect of increasing concentration of an essential metal on cell growth. (From Forstner and Wittman 1983).


A
Deficient
B
Optimal
C
Sublethal
D
Lethal
+
Growth Rate
or
Biomass
–
–
Metal Concentration
+

and respiration occurred in cultures of Dunaliella parva as a result of Cu deficiency. The reduction in photosynthesis may be explained by the requirement for Cu in the formation of plastocyanin. The reduction in respiration rate may result from the requirement for Cu in the formation of cytochrome oxidase (O'Kelley 1974). Other areas of cell metabolism may also be disrupted as Cu is required as a co-factor for several enzymes including alcohol dehydrogenase, carbonic anhydrase and carboxypeptidase. (Lehninger 1975). Chlorella vulgaris and Oocystis mansonii require $1.2x10^{-16}$ M and $1.6x10^{-16}$ M Cu^{2+} activity respectively for unrestricted growth (Manahan and Smith 1973).

The toxic effect of Cu at higher concentrations is well known with Cu being widely used as an algicide in the control of algal blooms in water reservoirs (Gibson 1972, Button et al. 1977, McKnight 1981, Raman 1985).

1.5 PARAMETERS USED TO QUANTIFY METAL TOXICITY

A number of parameters have been used to measure the toxicity of metals to algae in culture. These parameters include the growth rate, lag phase, and final yield of a culture. Growth rate estimates the ability of cells to divide and gives an overall measure of the metabolic state of the cells. Growth rate has been reported to decrease with increasing free metal ion activity (Erickson 1972, Canterford and Canterford 1980, Fisher et al. 1981, Gavis et al. 1981).

Lag phase, the period after initial cell inoculation and before exponential growth begins, is also frequently measured in conjunction with growth rates. It has been suggested that extension of the lag phase may enable cells to adapt to the medium or alter the medium to allow cell growth to occur. Morel et al. (1978) reported an increase in the length of lag phase in Skeletonema costatum with increasing Cu concentration. Following the extended lag phase, growth occurred at the same rate as in the control. Kawabara and Leland (1986) showed that the length of lag phase was the parameter most sensitive to Cu toxicity in Selenastrum capricornutum.

Final yield, which gives a measure of the cell number which can be supported by the medium, under the conditions of the culture, has been shown to decrease with increasing free metal ion activity in some studies (Jensen et al. 1974, Anderson, et al. 1978, Bates et al. 1983). Bates et al. (1983) showed that final yield was more sensitive to Zn toxicity than was growth rate, with a decline in final yield occurring at Zn activities below those which resulted in a reduction in growth rate.

Other parameters which give insight into the effects of metal toxicity on specific areas of cell metabolism and which elucidate factors which influence metal toxicity include, metal uptake (Parry and Hayward 1973, Bates et al. 1982, 1983, 1985, Harrison et al. 1986), nutrient uptake

(Rueter and Morel 1981, Rueter 1983, Peterson et al. 1984), carbon fixation (Steemann Nielsen and Wium-Andersen 1971, Thomas et al. 1977, Wurtsbough and Horne 1982), cell size (Erickson 1972, Davies 1974, Foster 1977, Fisher et al. 1981), nutrient content of cells (Thomas et al. 1977), chlorophyll a (Gross et al. 1970, De Filippis 1978, 1979a and b), and nitrogen fixation (Horne and Goldman 1974, Wurtsbough and Horne 1982).

1.6 MODES OF TOXIC ACTION OF COPPER

The toxic action of Cu to algal cells has been proposed to occur in two ways. At low Cu concentrations, binding of Cu to the cell membrane causes disruption of membrane function. At higher Cu concentrations, intracellular Cu increases and can result in the disruption of cellular metabolism (Huntsman and Sunda, 1980).

The uptake of metals also occurs in two stages, an initial rapid uptake within the first minutes of exposure to the metal, followed by a much slower uptake over an extended period. The initial uptake can be described by Langmuir isotherms suggesting the binding of metal ions to the cell membrane (Button and Hostetter 1977, Jennings and Rainbow 1979, Bates et al. 1982, Geisweid and Urbach 1983). Slower uptake has been suggested to reflect active uptake across the cell membrane (Button and Hostetter 1977, Gipps and Coller 1980, Geisweid and Urbach 1983).

The binding of Cu to sulphydryl groups of the cell membrane (Fisher et al. 1981) or acid groups may cause an impairment of membrane function, resulting in a loss of cytoplasmic components such as K^+ (Mierle and Stokes 1976, Gupta and Arora 1978, De Filippis 1979a and b). McBrien and Hassall (1965) noted that the ratio of Cu absorbed to K lost was not 1:1 suggesting that Cu disrupts membrane permeability. Alteration of the cell membrane permeability was confirmed using a methylene blue technique.

The transport of ions across the cell membrane has also been shown to be disrupted by Cu^{2+} (Gavis et al. 1981). In Scenedesmus quadricauda a reduction in P uptake as a result of Cu toxicity was shown to be highly pH dependent, possibly as a result of competition between H^+ and Cu^{2+} for binding sites on the cell membrane (Peterson et al. 1984). A reduction in $Si(OH)_4$ uptake has also been reported for several diatoms with increasing Cu concentration (Rueter and Morel 1981, Rueter 1983).

A disruption of membrane function may also result in a change in a cell's ability to divide. Fisher et al. (1981) observed the formation of two protoplasts within one frustule of Asterionella japonica probably resulting from a disruption of the link between cell growth and cell division. An uncoupling of cell growth and cell division has also been reported for Chlorella vulgaris (Foster 1977).

The disruption of cell metabolism can occur at higher Cu toxicity. For example Cu has been shown to inactivate the electron transport chain in photosystem II between the reaction center of photosystem II and the electron donating site of DPC (Shioi et al. 1978). Cu toxicity can also result in a reduction in the amount of chlorophyll either by inhibition of the reductive steps in chlorophyll biosynthesis (De Filippis 1979a), or by replacing Mg^{2+} in the chlorophyll a molecule causing a blue-spectral shift (De Filippis 1978) in conjunction with a reduction in photosynthesis (Gross et al. 1970).

1.7 FACTORS AFFECTING TOXICITY

The concentrations of metals in solution which have been reported to be toxic to phytoplankton are highly variable. This variability occurs between studies of the same species (Davies 1978) and also between species and clones within a study (Gavis et al. 1981).

A number of physical, chemical and biological factors have been shown to affect the toxicity of metals to algae and may explain at least some of the variations recorded.

1.7.1 Physical and Chemical Factors

1.7.1.1 Light

Kanazawa and Kanazawa (1969) showed that Cu toxicity was reduced when Chlorella ellipsoidea was kept in continuous light compared to a light/dark cycle of 17L:7D. Bates et al. (1985) reported the opposite effect with Chlamydomonas variabilis ; Zn toxicity under a 12L:12D cycle was reduced compared with continuous illumination. No resolution of these conflicting data is offered although they do highlight the need for controlled light regimes during metal toxicity studies.

1.7.1.2 Metal speciation

The toxicity of metals to phytoplankton has been shown to be dependent on the activity of the free metal ion in solution (the effective concentration of a chemical species which determines the rate and extent of any chemical reaction), rather than on the total dissolved metal in solution (Sunda and Guillard 1976, Sunda and Lewis 1978, Morel et al. 1978, Canterford and Canterford 1980, Allen et al. 1980). The activity of the free metal ions in solution can be measured using a selective ion electrode or can be calculated using chemical modelling solved using computer programs (Appendix B).

There are many factors such as pH, ionic strength, temperature, and chelating compounds which have been reported to affect the toxicity of a metal to phytoplankton. Many of these interactions can be explained by considering the effect of the factors on the activity of the free metal ion in

the medium. The activity of the free metal ion in solution is dependant on its' activity co-efficient in the solution and on the concentration of the free metal ion (Appendix B).

The activity coefficient of the free metal ion in solution is dependent on the ionic strength of the medium (Appendix B) and hence the activity of the free metal ion will decrease with increasing ionic strength. This may explain some variation between studies which use media of different ionic strengths.

Several factors, including pH, organic ligands, water hardness, salinity, and chemical composition of the medium can alter the concentration of the free metal ion in solution. The concentration of the free metal ion in the solution is pH dependent for some metals including Cu. As pH decreases the Cu^{2+} concentration increases, as does the solubility of total dissolved Cu.

Many organic ligands are able to bind metals and hence reduce the activity of the free metal ion in solution. Chelating substances such as EDTA and NTA are frequently added to growth media for algae and their presence must be taken into account when the toxicity of the metal is discussed. Steemann Nielsen and Laursen (1976) and Toledo et al. (1980) reported the reduction of Cu toxicity with the addition of humic acids to the growth medium. A similar result was observed with Chlorella pyrenoidosa when citric acid was

used in the growth medium (Steemann Nielsen and Kamp-Nielsen 1970). A reduction of Cu toxicity with the addition of organic ligands exuded by algae was reported by McKnight and Morel (1979) and Swallow et al. (1978). These compounds have been shown to bind Cu and reduce the Cu^{2+} concentration in solution (McKnight 1981, Zhou and Wangersky 1985).

The precipitation of metal compounds may also decrease the free metal ion concentration in solution. In hard waters metals can precipitate or co-precipitate in carbonate and hydroxide compounds of Ca and Mg and so reduce the free metal ion concentration. Fennikoh et al. (1978) and Say and Whitton (1977) and Wong (1980) demonstrated a reduction in the toxicity of Co and Zn in hard waters. Eisler and Gardner (1973) showed that increasing salinity also reduces Cu^{2+} concentration through the precipitation of Cu from the medium. This precipitation may explain the decreased Cu uptake with increasing salinity in several diatom and dinoflagellate species (Mandelli 1969). A similar result was observed with Cu toxicity on Chladophora (Betzer and Kott 1969). Zarnowski (1972) and Rai et al. (1981) have suggested that a reduction in metal toxicity with increased P in the medium may be due to the formation of insoluble metal phosphates which result in a reduction in metal ion concentration.

The large number of factors which may affect the activity of free metal ions in solution highlights the need for a

highly controlled environment and a knowledge of the activity of free metal ions in solution when toxicity studies are conducted.

1.7.2 Biological Factors

A large number of biological factors may also influence the toxic response of algae to metals.

1.7.2.1 Density of culture inoculum

Steemann Nielsen et al. (1969) found Cu toxicity to growth in batch cultures increased as inoculum density decreased. The uptake of Cu by Chlorella pyrenoidosa was also shown to be dependent on the initial cell density. Cu uptake per unit weight of culture was higher with a low density inoculum. The effect of inoculum density on subsequent toxicity was suggested to be due to increased Cu per unit area of cell membrane (Steemann Nielsen and Kamp-Nielsen 1970).

1.7.2.2 Age of cultures

The age of culture inoculum can influence the effect of toxic metals. Morel and Morel (1976) reported that the inoculation of stationary phase cells into Cu contaminated medium resulted in a prolonged lag phase before exponential growth commenced with the length of the lag phase depen-

dant on the Cu concentration. The lag phase was not extended when exponentially growing cells were inoculated into the same medium. An extended lag phase has been reported in several metal toxicity studies as a measure of toxicity (Morel et al. 1978, Kawabara and Leland 1986). This suggests that the extension of the lag phase may be dependant on the physiological state of the cells at the time of inoculation.

The age of cells has also been reported to influence metal uptake by algal cells. Harrison et al. (1986) and Bates et al. (1983) reported a decrease in the uptake of Zn by Chlamydomonas variabilis as culture age increased, possibly due to a reduction with age in the number of binding sites available per unit cell surface. Cain et al. (1980), however, reported the opposite effect with less cadmium uptake occurring in young, rapidly growing cells.

1.7.2.3 Nutrient interactions

Davies (1978) proposed that the toxicity of metals may be inversely proportional to the availability of macronutrients. Hannan and Patouillet (1972) found that increased concentrations of N and P in the medium decreased the toxicity of mercury to several algal species. They suggested that the reduction in mercury toxicity was due to physiological changes which occurred within the cells as a result of higher N and P concentration in the medium.

There are several reports of increased P concentrations in the medium resulting in a decrease in metal toxicity (Monahan 1973, Harding and Whitton 1977, Say and Whitton 1977, Li 1979). When the concentration of P in the medium is high the levels of cellular P are elevated with P in excess of immediate cell requirements being stored in polyphosphate bodies (Rhee 1972, Aitchison and Butt 1973, Rhee 1973). Polyphosphate bodies have been reported to act as a site for intracellular detoxification of metals (Sicko-Goad and Stoermer 1979, Jensen et al. 1982, Pettersson et al. 1985). When polyphosphate levels in the cells are enhanced, the cell's ability to detoxify metals may be increased resulting in lower metal toxicity.

Iron has been reported to reduce the toxicity of Cu to Nitzschia closterium through the formation of colloidal Fe-hydroxy complexes which bind to the cell membrane and reduce its ability to bind Cu (Stauber and Florence 1985).

Morel et al. (1978) showed that Cu toxicity was a function of silicic acid in the medium, indicating an interaction between Cu toxicity and Si metabolism. Silicic acid uptake was shown to be a function of the Cu^{2+} and Zn^{2+} activities in the medium, leading to the proposal of a Zn dependent uptake site for silicic acid (Rueter and Morel 1981). Rueter et al. (1981) suggested that Cu toxicity interferes with the silification process, as silicate was present in the cells although no cell division occurred.

1.7.2.4 pH

Rai et al. (1981) reported that metal toxicity increased with decreasing pH as a result of increasing Cu^{2+} activity. There is, however, a growing body of evidence suggesting that the opposite may be true. Steemann Nielsen and Kamp-Nielsen (1970) showed that Cu toxicity increased with increasing pH for Chlorella pyrenoidosa. A similar effect was shown for Zn and Cu toxicity in Hormidium rivulare (Hargreaves and Whitton 1976).

The reduction in the toxicity of metals with decreasing pH may result from decreased metal uptake at lower pH as demonstrated by Harrison et al. (1986), Parry and Hayward (1973) and Les and Walker (1983). Harrison et al. (1986) proposed that the reduction in uptake of metal ions with decreasing pH is due to competition between H^+ and free metal ions at the binding sites of the cell membrane. Peterson et al. (1984) proposed that competition between H^+ and Cu^{2+} or Cd^{2+} ions for binding sites resulted in a decrease Cu and Cd toxicity with decreasing pH.

1.7.2.5 Resistance and tolerance

A resistant species is one in which all populations are able to tolerate elevated metal concentrations even when found in non-contaminated environments. In some normally non-resistant species, tolerant populations may be isolated

from contaminated environments (Stokes 1983). In general, populations from unpolluted environments tend to be less tolerant of metal contamination than those from more polluted areas (Stokes 1983). Within a resistant species, isolates from highly contaminated areas may have greater resistance than those isolated from a less contaminated environment (Foster 1982b).

Several mechanisms have been proposed to explain the tolerance and resistance of algae to metal contamination.

The exclusion of Cu by Cu-tolerant Chlorella vulgaris cells was shown by Foster (1977). The tolerant strains contained less cellular Cu than the non-tolerant strains at the same external Cu concentrations. The two strains were, however, equally sensitive to intracellular Cu levels. Hall et al. (1979) reported similar findings for Cu tolerant strains of Ectocarpus siliculosus.

The excretion by algae of organic ligands capable of complexing metals has been reported in several studies (Swallow et al. 1978, McKnight and Morel 1979). McKnight and Morel (1979) found that although the 21 species of algae they tested excreted organic ligands capable of binding Cu, the ligands occurred in measurable concentrations only during the stationary growth phase of the culture. Recent work with the diatom Phaeodactylum tricornutum by Zhou and Wangersky (1985) using more sensitive analytical techniques has

shown that although the majority of organic ligands are released by cells at stationary phase, organic ligands capable of complexing Cu were released throughout growth. Butler et al. (1980) showed that the increase in tolerance in the Cu-tolerant strains of Chlorella vulgaris (Foster 1977) may result from higher metal binding capacity of the organic ligands excreted.

The detoxification of metals within the cell as a means of reducing metal toxicity can occur in several ways, including the sequestering of metals in polyphosphate bodies and intranuclear complexes, or by the binding of metals to proteins within the cell.

Jensen et al. (1982) showed that Cu, Co, Cd, Hg, Ni, Pb, Zn were all taken up by Plectonema boryanum and sequestered in polyphosphate bodies. Since polyphosphate bodies are osmotically inert, the sequestering reduces the toxicity of metals within the cell (Sicko-Goad and Stoermer 1979). Pettersson et al. (1985) showed a similar occurrence with Al sequestered in the polyphosphate bodies of Anabaena cylindrica. Polyphosphate levels within a cell are reduced with growth in the absence of external P, as P is remobilized from the polyphosphate bodies. This may result in a release of toxic metals within the cells and increased toxic effects (Bates et al. 1985). Daniel and Chamberlain (1981) suggested that detoxification within the polyphosphate bodies was due to Cu being bound on a 1:1 basis with sulphur in the

polyphosphate bodies which may produce a highly insoluble precipitate with the metal and thus results in detoxification of the metal.

A second mechanism of internal detoxification was reported by Silverberg et al. (1976), who showed that Cu was bound to protein ligands within intranuclear inclusions of the nucleoplasm in Scenedesmus sp. In tolerant cells the inclusions were excluded from the nucleolus. In non-tolerant cells however, inclusions were observed within the nucleolus and extensive membrane damage was observed.

A third possible mechanism of detoxification is the binding of metals by metallothioneins (metal binding proteins, rich in cysteinyl residues and of low molecular weight (Nakamura et al. 1981)). Metallothioneins are reported to occur widely in animals (Vallee 1979) and have also been reported in higher plants (Rauser 1981) and algae (Olafson 1979). Several functions have been proposed for these proteins including the detoxification of metals within the cell. Olafson (1979) reported the presence of Cd induced metal-binding protein in Synechococcus sp., although no direct link with detoxification of Cd was found. A peptide similar to the metallothioneins observed in animals, containing cysteine, glutamic acid, and glycine, has been identified in Cd stressed Rauolfia serpentina cells and has been suggested to be the principle metal binding peptide in these cells (Grill et al. 1985).

The absorption and subsequent release of metals back into the medium is also a possible mechanism for metal tolerance in algae. Bates et al. (1982) reported that a portion of the Zn adsorbed to the cell surface of Chlamydomonas variabilis was subsequently released into the medium. Butler et al. (1980) have suggested that the Zn may be combined with excreted organic matter by either initial binding to the ligand or complexing to the ligand in the medium. An alternative explanation is that Zn may be lost from the cell with portions of the cell walls shed into the medium (Bates et al. 1982).

1.7.3 Synergistic and Antagonistic Interactions of Metals

Although most studies consider the toxicity of only one metal, this is a rare occurrence in natural water bodies, where generally two or more metals are present together (Rai et al. 1981). There are several reports of both antagonistic and synergistic interactions of various metal combinations in metal toxicity studies with phytoplankton (Rai et al. 1981). Antagonism between two metals results in a decrease in toxicity whereas synergism results in an increase in toxicity.

The type of interaction is dependent on both the metals involved, the algal species and possibly the parameter used to measure toxicity. Braek and Jensen (1976) showed three of four diatom species tested to have a synergistic response

to Cu and Zn toxicity. The fourth species showed an antagonistic response. In the same study a synergistic action of Cd and Zn toxicity was shown in two marine diatoms, and an antagonistic reaction was reported for two other species. Stratton and Corke (1979) showed that Mg and Cd ions were antagonistic when final cell yield was measured and synergistic when photosynthesis was measured in Anabaena inaequalis. Adsorption studies (Braek et al. 1980) indicated that the antagonistic reactions of Zn and Cd may have been a result of the two metal ions competing for the same uptake sites on the cell membrane.

1.7.4 Co-tolerance

Co-tolerance of different metals has been reported to be quite common in algae isolated from the Hayle and Gannel rivers (Foster 1982b). Stokes (1981) showed that a Cu tolerant species of Scenedesmus sp. was also tolerant to Ni but not Co or Zn; however, Cu tolerant Ectocarpus siliculosus was found to be Co and Zn tolerant as well (Hall et al. 1979). The co-tolerance of algae to Zn and Cd has been demonstrated in many species (Say et al. 1977, Foster 1982b). This co-tolerance is not surprising since the two metals generally occur together geologically and are chemically similar (Foster 1982b). Cadmium is known to replace Zn in enzyme systems (Vallee and Ulmer 1972). Opposite results have been shown by Shehata and Whitton (1982); laboratory

cultures of Anacystis nidulans selected for Co, Ni, Cu and Cd resistance showed little evidence of co-tolerance.

Stokes (1983) has proposed that differences in detoxification mechanisms may influence a species' ability to be co-tolerant to a number of metals. This proposal is supported by data from Foster (1982b) where Cu resistant algae isolated from highly Cu polluted sites were also significantly resistant to Pb. Species isolated from highly Pb polluted sites however, were Pb resistant but sensitivity to Cu was enhanced. Two mechanisms of Pb resistance have been suggested to occur, one which specifically detoxifies Pb and does not confer Cu tolerance, and a second which is a 'by-product' of the cells' resistance to Cu which also provides the cells with Pb resistance (Foster 1982b).

1.8 ECOLOGY

The variability in the sensitivity of species to toxic metals suggests that the major effect of metal toxicity at the community level should be changes in species composition (Huntsman and Sunda 1980). Tolerant species have been shown to replace more sensitive species when metal contamination occurs. For example, McKnight (1981) reported that the use of Cu as an algicide in Mill Pond reservoir to control blooms of Ceratium hirundinella resulted in two green algae, Nannochloris sp. and Ourococcus sp. replacing the more sensitive species, Ceratium hirundinella. In the Cefri Reser-

voir in North Wales, the algicidal use of Cu resulted in Anabaena flos-aqua being quickly replaced by Scenedesmus quadricauda (Gibson 1972). Steemann Nielsen and Laursen (1976) reported that the toxic action of Cu resulted in changes in the species composition in three natural phytoplankton assemblages.

Enclosure studies with natural marine populations showed large changes in species composition when Cu was added; dinoflagellates disappeared and pennate diatoms replaced centric forms (Thomas and Seibert 1977). Ibragim and Patin (1976) also reported a replacement of centric by pennate diatoms in phytoplankton assemblages from the Mediterranean Sea when exposed to Hg, Cu, Cd, and Pb. Dinoflagellates, blue-green algae and diatoms appear to be more sensitive to metals than are green algae. Wide variability in data shows that generalizations on the effects of metal toxicity on algal species or on species composition in natural communities should, however, not be made (Davies 1983).

Decreased species diversity has also been reported with metal contamination in natural communities. Foster (1982a) observed a decrease in species diversity at sites of high Cu and Pb contamination. In Cu contaminated enclosures in the CEPEX experiments diversity was also shown to decrease as sensitive species were replaced by resistant ones (Thomas and Seibert 1977).

Algal biomass has been shown to change with metal contamination in enclosure studies (Thomas and Seibert 1977). This variation in response may result from the relative importance of the indirect effect of reduced photosynthesis and decreased grazing pressure. Gachter and Mares (1979) showed a depression of algal carbon fixation but higher phytoplankton biomass in metal contaminated enclosures. The increase in biomass was related to a decrease in grazing pressure due to a reduction in zooplankton populations.

Changes in species composition or biomass in natural phytoplankton populations can have repercussions at higher trophic levels of the aquatic ecosystem (Rai et al. 1981 and Davies 1983). Metal toxicity may create a shortage of food for filter feeding zooplankton and fish due to a reduction in phytoplankton biomass and a possible reduction in edible algal species as a result of community change. Coleman et al. (1971) reported several algal species were capable of bioaccumulating Co and Zn to an extent that may be toxic to higher levels of the food chain. The problem of bioaccumulation may not be as severe with Cu contamination, because the concentration factor of $1x10^4$ to $1x10^5$ for Cu in algae is lower than that for most other metals (Moore and Ramamoorthy 1984). Field studies have revealed that there is little bioaccumulation of Cu in the aquatic food chain. Wilber (1969) showed that concentration of Cu occurred between phytoplankton and zooplankton but not at higher trophic levels.

Chapter II

PAPER 1: THE INTERACTION OF CHRONIC COPPER TOXICITY WITH NUTRIENT LIMITATION IN BATCH CULTURE EXPERIMENTS WITH TWO CHLOROPHYTES

2.1 INTRODUCTION

Copper is an essential micronutrient for algal growth being required for both photosynthesis and respiration (Sandman 1985). At concentrations above those required for optimal growth Cu is known to be toxic.

Several parameters of algal batch culture, including growth rate, final yield and cell size have been used to measure Cu toxicity. Growth rate decreases with increasing cupric ion activity (Sunda and Guillard 1976, Anderson and Morel 1978, Gavis et al. 1981, Fisher et al. 1981), although Cu^{2+} activities which cause a decrease in growth rate vary widely both between species and clones (Gavis et al. 1981). Final yield decreases with increasing Cu^{2+} (Jensen et al. 1974, Mierle and Stokes 1976) and Zn^{2+} activities (Bates et al 1985). Increasing Cu^{2+} activity can cause increased cell size (Stokes 1975, Foster 1977, Gupta and Arora 1978). This may be due to disruption of the link between growth and cell division (Fisher et al. 1981), possibly mediated via a change in membrane function (Huntsman and Sunda 1980).

The toxicity of Cu has been reported to be more directly related to the Cu^{2+} activity in solution than to the total dissolved Cu concentration (Sunda and Guillard 1976, Anderson and Morel 1978, Morel et al. 1978, Sunda and Lewis 1978, Canterford and Canterford 1980, Allen et al. 1980). Intracellular Cu levels (Sunda and Guillard 1976) and uptake of Cu are dependent on the Cu^{2+} activity in solution and not on total dissolved copper (Hardstedt-Romeo and Gnassia-Barelli 1980). Similar results have been reported for Zn uptake (Bates et al. 1982, 1983).

The Cu^{2+} activities which have been reported to be toxic to algae vary widely. This can be partially attributed to differences in experimental conditions but may also reflect a large amount of interspecific and clonal variation of Cu tolerance amongst algae. Interspecific variation can be high; for example, Gavis et al. (1981) in a study of 10 species reported variation of more than 2 orders of magnitude in the Cu^{2+} activity required to reduce growth rate to 50% of the controls. They also showed that the Cu^{2+} activity lethal to clones of Thalassiosira pseudonana varied by more than one order of magnitude. Foster (1982b) reported several species of Chlamydomonas and Chlorella to be resistant to Cu, with increased tolerance occurring in clones isolated from highly Cu contaminated areas. Stokes et al. (1973) demonstrated Scenedesmus acutiformis and Chlorella fusca, isolated from contaminated lakes, to be more tolerant

to Cu than the commonly used laboratory species Scenedesmus accuminatus and Chlorella vulgaris.

Several mechanisms of detoxification have been proposed that could confer resistance or tolerance on an algal species. They include exclusion of Cu from the cell (Foster 1977, Hall et al. 1979); detoxification of Cu within the cell in polyphosphate bodies (Sicko-Goad and Stoermer 1979, Jensen et al. 1982, Pettersson et al. 1985), or in intranuclear complexes (Silverberg et al. 1976); and the complexing of Cu by excreted organic ligands (Swallow et al. 1978, McKnight and Morel 1979, McKnight 1981, Zhou and Wangersky, 1985).

The variation in Cu sensitivity between species and clones found in laboratory experiments may help to account for the changes observed in species composition when natural water bodies are stressed with toxic levels of Cu (Gibson 1972, Thomas and Seibert 1977, McKnight 1981, Raman 1985, Oliveira 1985). These changes in taxonomic composition and diversity may have repercussions at higher trophic levels of the food chain (Davies 1983, Rai et al. 1981).

Bates et al. (1985) have suggested that macronutrients may play an important role in the regulation of metal toxicity to phytoplankton. Increased P concentrations in the medium has been demonstrated to reduce Cu toxicity to Chlamydomonas sp. (Meijer 1972). Similar effects with Zn toxicity

have also been reported (Zarnowski, 1972, Harding and Whitton 1977, Say and Whitton 1977, Say et al. 1977, Shehata and Whitton 1982). Increased nitrate concentrations in the medium have been similarly shown to reduce Cd toxicity to Thalassiosira fluvatilis (Li 1979); however, nitrate had no effect on Zn toxicity to Chlorella vulgaris or Plectonema boryanum (Rana and Kumar 1974).

Since P and N are frequently the nutrients limiting natural phytoplankton populations (Wetzel 1983), any interaction between N or P and Cu toxicity may have far reaching ecological implications in terms of the Cu concentrations which are toxic to algae.

This study was initiated to investigate possible differences in the toxicity of Cu to cultures of Chlorella sp. and Chlamydomonas sp. limited by either N or P.

2.2 METHODS

Batch culture experiments were conducted with Chlorella sp. and Chlamydomonas sp. isolated from the Cu and Zn contaminated Schist Lake in the Flin Flon region of northern Manitoba, Canada (54°45'N 101°50'W).

Both species were grown under final yield limiting concentrations of N and of P (Appendix A), with 3 replicate experiments being conducted for each limiting nutrient. Each experiment consisted of 10 Cu concentrations ranging from

0.04 to 45 uM total dissolved Cu with 3 replicate cultures at each concentration.

All experiments were conducted using WC medium (Guillard and Lorenzen 1972) with the following modifications. The Na_2EDTA(11.7 uM) was replaced by 23.4 uM Na_2NTA to allow calculation of the chemical speciation of Cu in the medium. 5mM MES was added to maintain a stable pH of 6.3 throughout the experiments. Neither Na_2NTA nor MES showed any phytotoxicity at the concentrations used (Appendix A). In N-limited experiments, $NaNO_3$ was reduced from 1000 uM to 5 uM for Chlorella sp. and to 100 uM for Chlamydomonas sp. In P-limited experiments, K_2HPO_4 was reduced from 50 uM to 5 uM for Chlorella sp. and to 20 uM for Chlamydomonas sp. Stock solutions were added to the medium before autoclaving with the exception of the NTA-Fe and vitamin solutions. These were filter-sterilized (0.2 uM membrane filter) and added after autoclaving. All chemicals used in these experiments were reagent grade or better and stock solutions were stored in polyethylene bottles. All glassware and storage containers were presoaked for 48 hours in 3N HNO_3 and rinsed thoroughly with distilled deionised water. The deionised water used in making medium and rinsing glassware was frequently tested for Cu contamination. Cu concentrations were either negligible or below detection.

For each experiment 800 mL of medium was dispensed into each of 30 1-L sterile pyrex flasks with silicone stoppers.

A filter sterilized Cu solution was added to each flask to give the required Cu concentrations. The medium was then allowed to stand for at least 18 hours for chemical equilibrium to be achieved (Anderson and Morel 1978). Exponentially growing cells were inoculated into each flask to provide initial cell densities of approximately 8,000 cells mL^{-1} for Chlorella sp. and approximately 3,000 cells mL^{-1} for Chlamydomonas sp.

The flasks were placed in a controlled environment chamber at 20°C under continuous illumination of 150 $uE \cdot m^{-2} \cdot s^{-1}$ (Li Cor Model L1-185A) from fluorescent Cool White VHO tubes (Sylvania). The cultures were bubbled vigorously with air which had been passed through 2 N H_2SO_4, deionised water, and a sterile 0.45 uM membrane filter.

5ml samples for cell counts were removed from each flask 3-4 times per day and counted using a Model B Coulter Counter. Cell counts were used to calculate growth rate and final yield. Growth rate was calculated by plotting the natural log of cell numbers against time and calculating a linear regression for the exponential growth phase (8-12 points). Final yield was calculated by taking the mean of the first 3 cell counts after stationary phase was reached (increase in cell numbers not exceeding 10% over 36 hours)

At stationary phase, samples were removed from each flask for filtration onto Whatman GF/A filters for analysis of

particulate C, P, Cu, and N. Filters used to collect cells for C, N, and P analysis were combusted at 550°C for 20 hours prior to use (Stainton et al. 1977). Those for Cu analysis were soaked in 3 N HNO_3 for 24 hours and rinsed thoroughly with deionised water before use.

Particulate C for the P-limited experiments was analysed using wet dichromate oxidation (Strickland and Parsons 1968). For the N-limited experiments particulate C and N analyses were conducted using a Perkin Elmer 240 Elemental Analyser (Stainton et al. 1977). Particulate Cu analyses were conducted by placing filters in 50 mL teflon beakers which had been soap washed, soaked in 30% HNO_3 for 48 hours and rinsed with deionised water. 0.5 mL of concentrated HNO_3 and 5 drops HF were added to the beakers which were then placed on a hot plate and the acids allowed to evaporate. The beakers were then cooled and 0.5 mL of HNO_3 and 9.5 mL deionised water added. The samples were subsequently analysed directly by Graphite Furnace Atomic Absorption Spectrophotometer (detection limit of $0.5ug \cdot L^{-1}$) for low Cu concentrations and Flame Atomic Absorption Spectrophotometer (detection limit of $10ug \cdot L^{-1}$) for higher Cu concentrations, standardized with Fisher Certified Reference Standards.

The concentration of free cupric ion, Cu^{2+}, present in the medium was computed using the chemical equilibrium model MACS80 (Appendix B). MACS80 is a Fortran-based program which gives similar results to other models when tested with

a common data set (Peterson et al. 1984). The MACS80 program calculations did not take into account the boron, vitamin or MES present in the medium. The boron and vitamins are known to complex Cu but were present at very low concentrations. The MES was present at a higher concentration, but has been reported to have negligible binding for Cu (Good et al. 1966). These factors would influence the Cu speciation slightly, but in a similar fashion for all the experiments conducted. The calculated activity of Cu^{2+} will be referred to as pCu (pCu = -log Cu^{2+} activity), following Anderson and Morel (1978).

2.3 RESULTS

2.3.1 Cellular Copper

Chlorella sp.

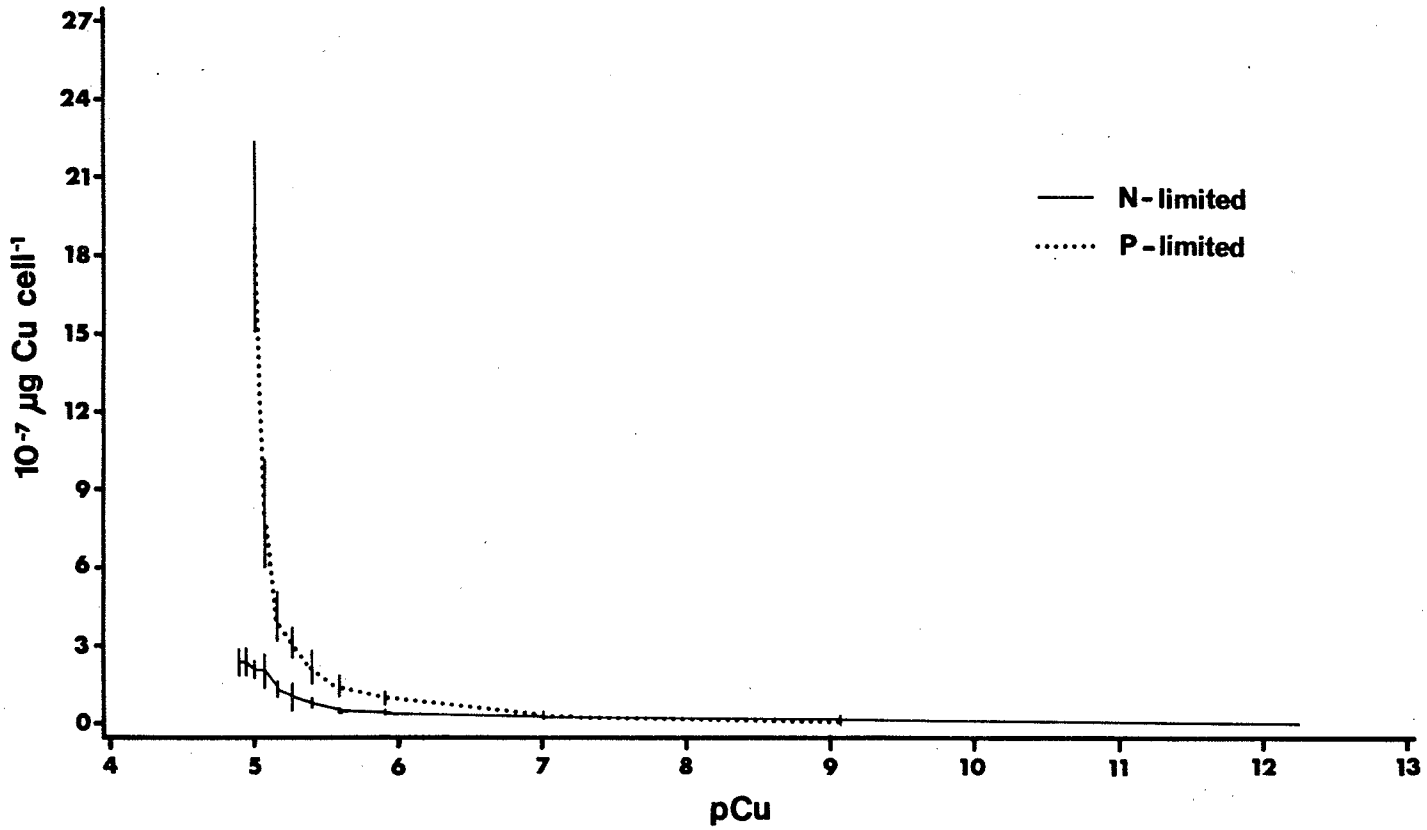
Cellular Cu, the total of intracellular and adsorbed Cu per cell, increased significantly with decreasing pCu in both P-limited and N-limited cultures (Table 2.1, Fig. 2.1). Under P-limitation cellular Cu increased from 0.048×10^{-7} to 18.5×10^{7} ug Cu $cell^{-1}$ as pCu decreased from 9.1 to 5.0. In N-limited cultures however, the increase in cellular Cu was much smaller (from 0.025×10^{-7} to 2.40×10^{-7} ug Cu $cell^{-1}$ over the pCu range 12.2 to 4.9). The lower Cu content in the N-limited cells may be a result of the higher final yield of the N-limited cultures compared to the P-limited cultures, as the total particulate Cu mL^{-1} of culture was higher under

Table 2.1: Summary of ANOVA results for individual parameters in batch cultures with decreasing pCu. Data for all parameters were log transformed as indicated by Taylor's Power Law. For each parameter, Cu^{2+} activities in which all 3 replicates were missing for one experiment (empty cell) were deleted from the analysis (Mount and McClure, pers. comm.).

Figure 2.1: Cellular Cu content (adsorbed and intracellular Cu) of N- and P-limited Chlorella sp. cells, harvested at stationary phase, as a function of Cu^{2+} activity. Vertical bars: ±2 standard errors.

Variable	N limited		P limited	
	Probability	Significance	Probability	Significance
Chlorella sp.				
Final Yield	.68	*	.0001	***
Growth Rate	.05	**	.0001	***
Cellular Carbon	.96	*	.31	*
Cellular Phosphorus	.95	*	.0009	***
Cellular Copper	.0001	***	.0001	***
Chlamydomonas sp.				
Final Yield	.43	*	.0001	***
Growth Rate	.05	**	.02	**
Cellular Carbon	.96	*	.26	*
Cellular Phosphorus	.66	*	.001	**
Cellular Copper	.0001	***	.0001	***

significant at $\alpha = .001$ ***
significant at $\alpha = .05$ **
no signific. diff = *


27
24
21
18
15
12
9
6
3
0
10^{-7} µg Cu cell^{-1}
4
5
6
7
8
9
10
11
12
13
pCu
N-limited
P-limited

N-limitation (0.48 ug Cu mL^{-1} compared to 0.24 ug Cu mL^{-1} in P-limited cultures at pCu 5.0).

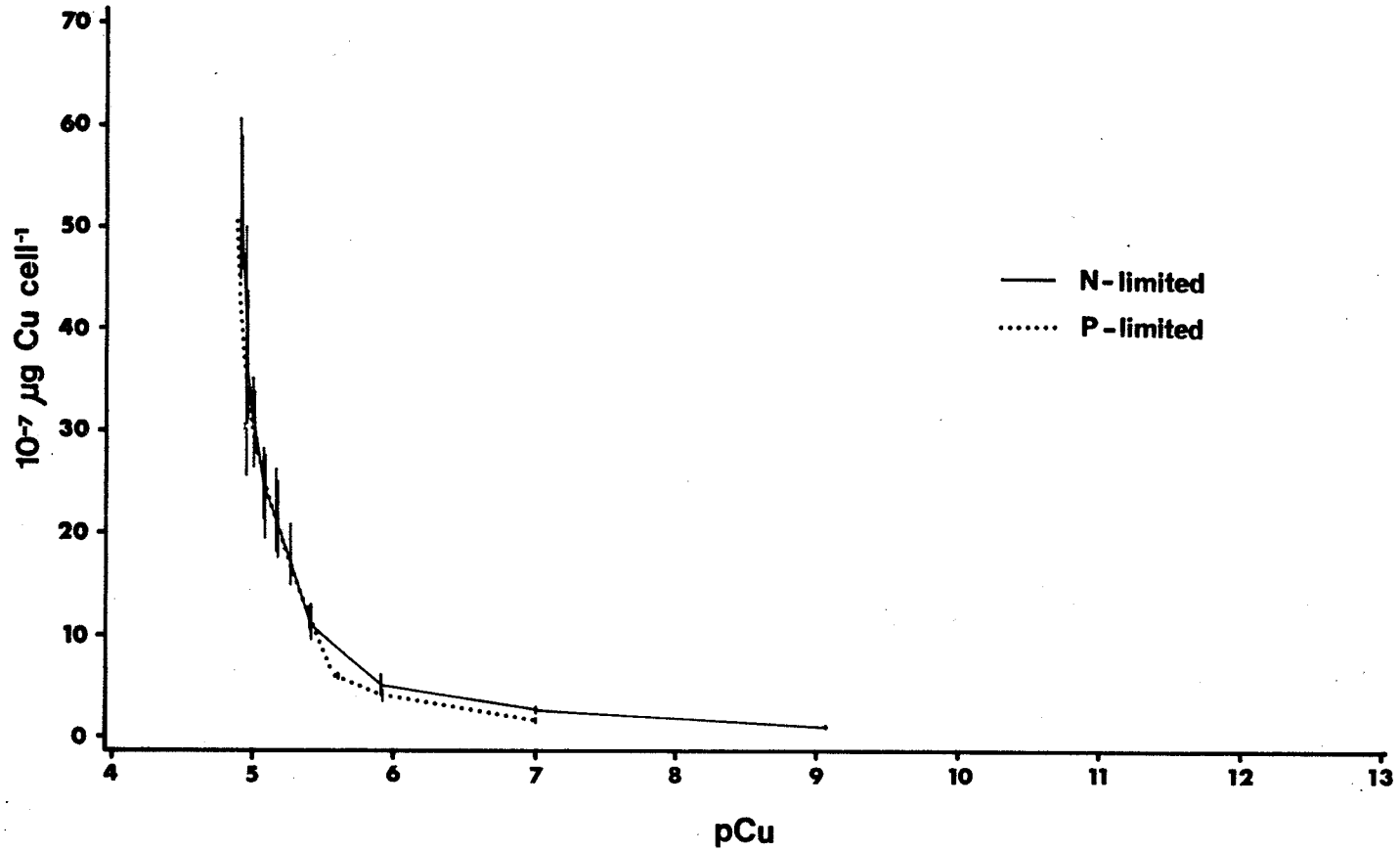
The range of cellular Cu:C ratios (based on weight and used to estimate cellular Cu concentration) was approximately 0-8.2 in P-limited cultures and 0-3.4 in N-limited cultures. The Cu:C ratio increased with decreasing pCu similar to Cu $cell^{-1}$ with higher values in the P-limited than in the N-limited cultures.

Chlamydomonas sp.

Cellular Cu increased with decreasing pCu from 1.68×10^{-7}ug Cu $cell^{-1}$ to a maximum of 52.7×10^{-7}ug Cu $cell^{-1}$ at pCu 4.9 in the P-limited cultures. (Table 2.1, Fig. 2.2). The N-limited cultures had a similar increase in cellular Cu with a maximum of 51.2×10^{-7}ug Cu $cell^{-1}$ at pCu 4.9 (Fig. 2.2).

The Cu:C ratio ranged between approximately 0-2.0 and 0-6.3 in P-limited and N-limited cultures respectively. The Cu:C ratio increased with decreasing pCu similar to cellular Cu.

Figure 2.2: Cellular Cu content (adsorbed and intracellular Cu) of N- and P-limited Chlamydomonas sp. cells, harvested at stationary phase, as a function of Cu^{2+} activity. Vertical bars: ±2 standard errors.


70
60
50
40
30
20
10
0
10⁻⁷ µg Cu cell⁻¹
4
5
6
7
8
9
10
11
12
13
pCu
N-limited
P-limited

2.3.2 Growth Rate

Chlorella sp.

In P-limited cultures division rate decreased steeply between pCu 5.26 and 5.0 from 2.24 divisions d^{-1} to 0.55 divisions d^{-1} (Table 2.1, Fig. 2.3) A similar trend but of smaller magnitude was seen in the N-limited cultures which decreased from 2.4 to 1.62 divisions d^{-1} between pCu 5.07 and 4.9. (Table 2.1, Fig. 2.3). The overall decrease in growth rate was much greater in P-limited cultures with an 80% decrease between pCu 12.2 and 5.0 compared with a 40% decrease in N-limited cultures over the same range of pCu.

In Figure 2.4 growth rate has been expressed as a percentage of the control to allow direct comparison between the two limiting nutrients. A moving average of 5 points was calculated and overlayed on the plot. Over the Cu:C range of 0-3.0 there was a slight decrease in growth rate in both N-limited and P-limited cultures with increasing Cu:C. Above Cu:C of 3.0, the growth rate in the P-limited culteased steeply. There were no corresponding data for N-limited cultures as the N-limited cultures did not reach these high Cu:C ratios.

Chlamydomonas sp.

Growth rate decreased from 2.07 to 1.21 divisions d^{-1} at pCu levels below 5.2 in P-limited cultures and from 2.29 to 1.66 divisions d^{-1} for N-limited cultures (Table 2.1, Fig. 2.5). The overall decrease in growth rate with decreasing pCu was

Figure 2.3: Growth rate of N- and P-limited Chlorella sp. as a function of Cu^{2+} activity. Vertical bars: ±2 standard errors.

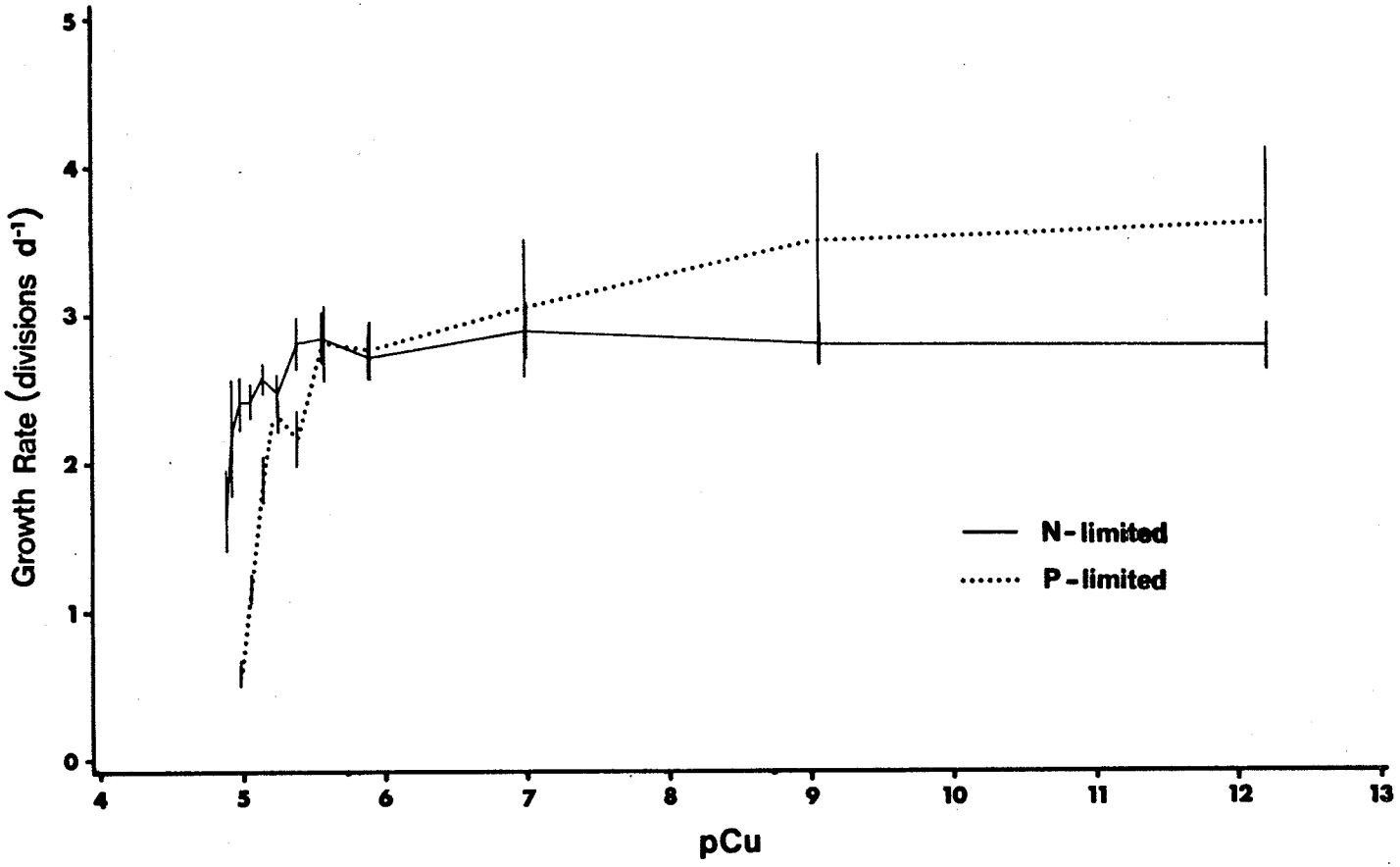

Growth Rate (divisions d-1)
0
1
2
3
4
5
4
5
6
7
8
9
10
11
12
13
pCu
N-limited
P-limited

Figure 2.4: Growth rate of N- and P-limited Chlorella sp. as a function of cellular Cu concentration at stationary phase relative to control growth rate. Lines connect a moving average calculated over 5 points.

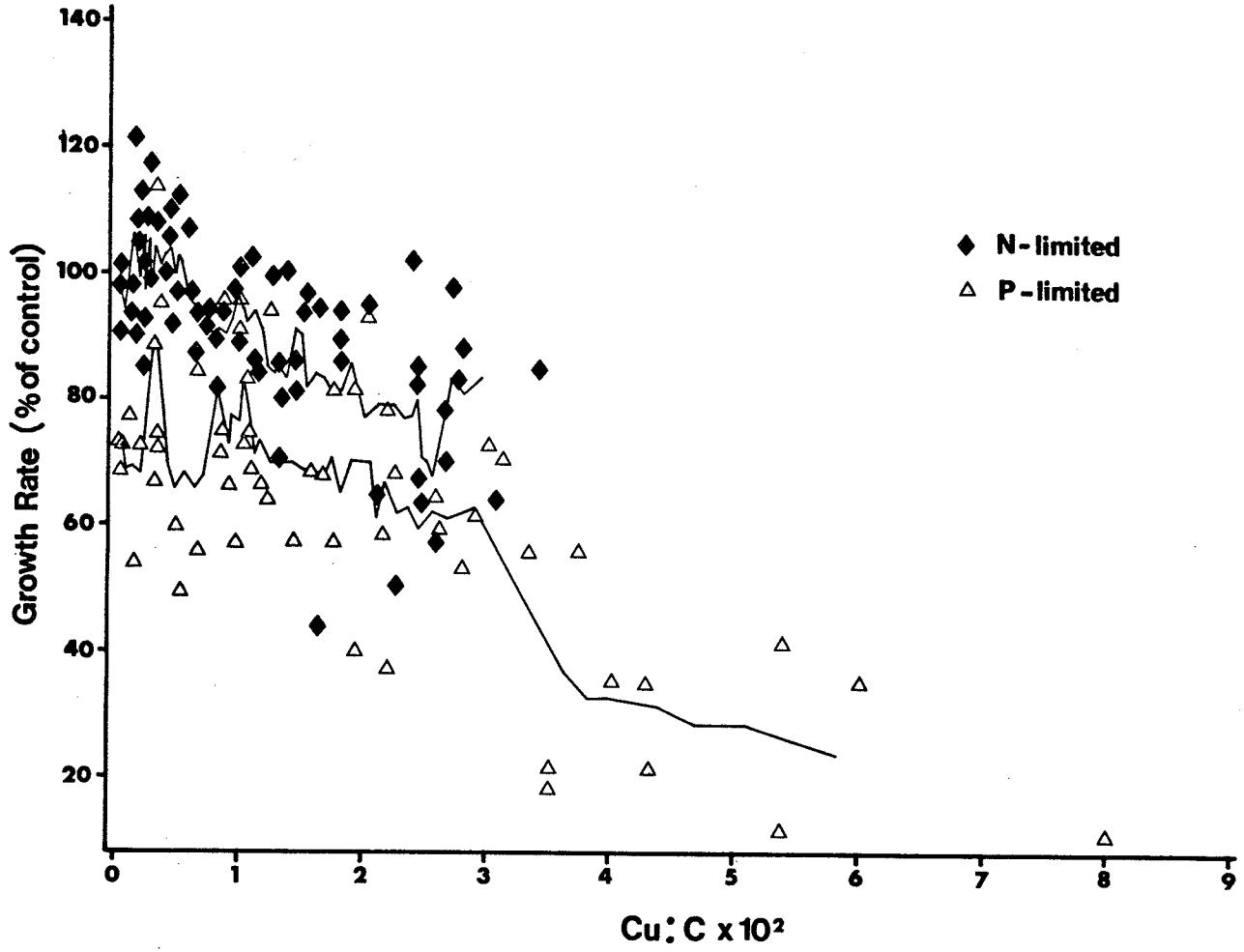
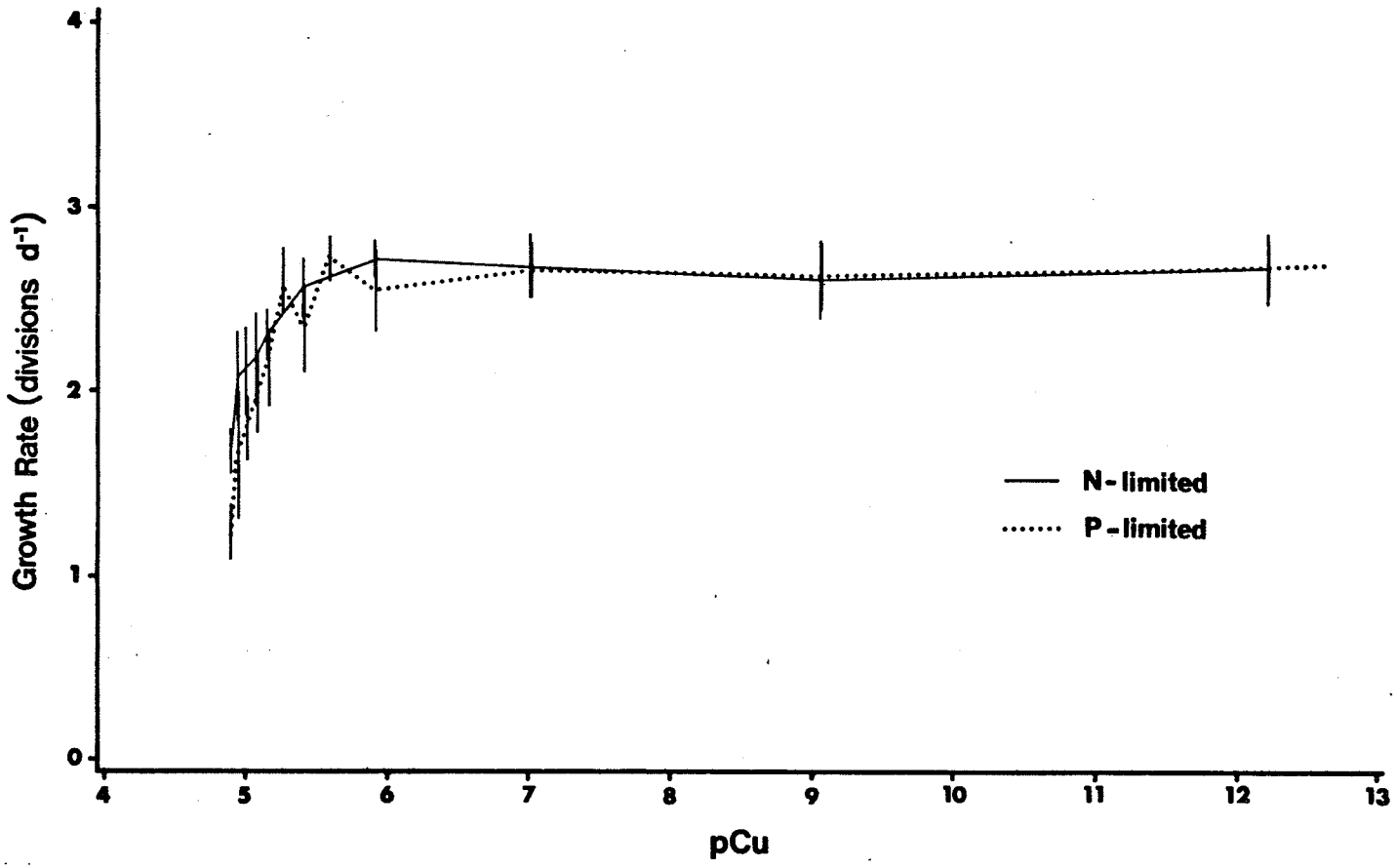

140
120
100
80
60
40
20
Growth Rate (% of control)
0
1
2
3
4
5
6
7
8
9
Cu: C x 10²
N-limited
P-limited

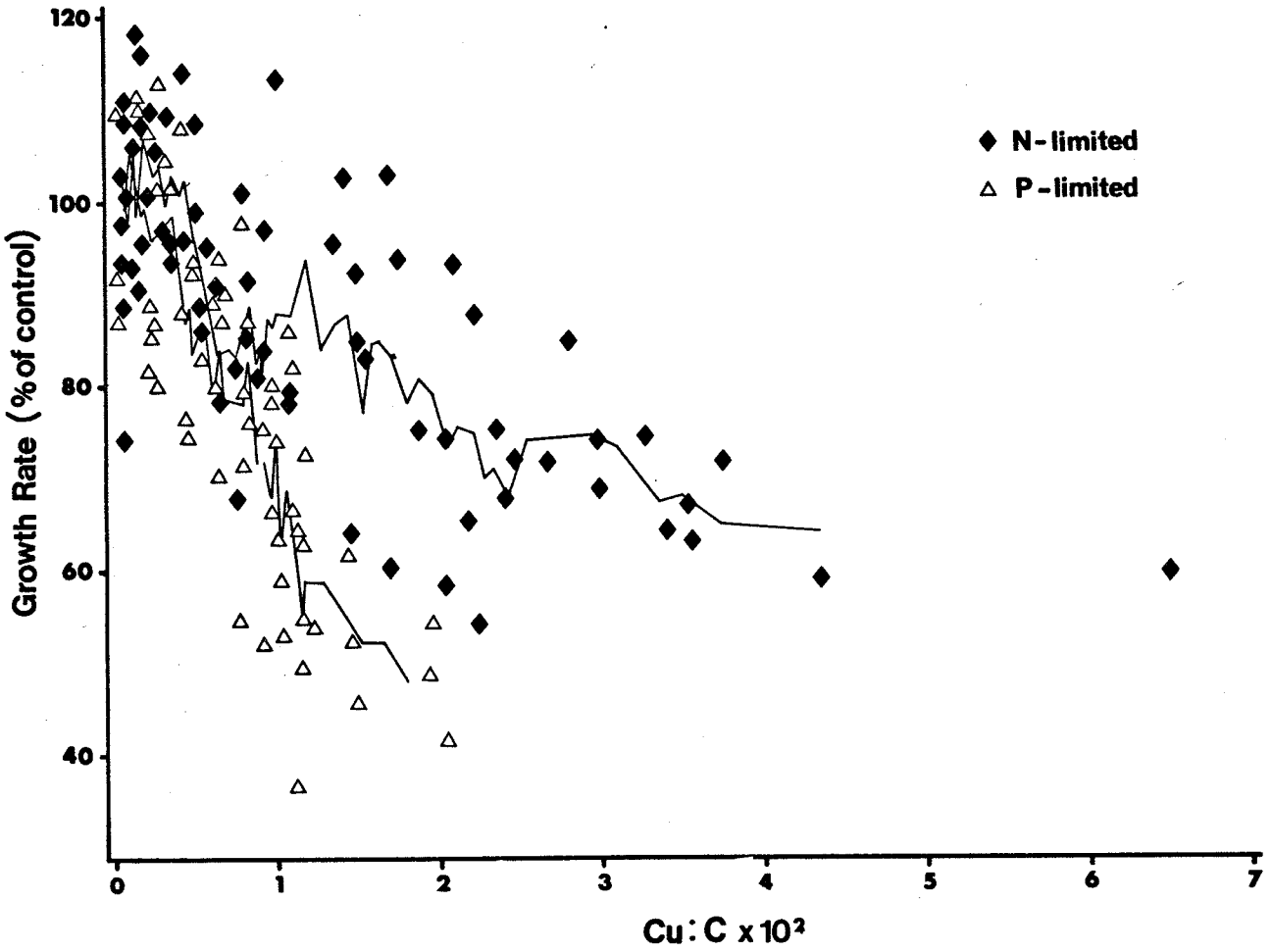
Figure 2.5: Growth rate of N- and P-limited Chlamydomonas sp. as a function of Cu^{2+} activity. Vertical bars: ±2 standard errors.


Growth Rate (divisions d-1)
4
3
2
1
0
4
5
6
7
8
9
10
11
12
13
pCu
N-limited
P-limited

greater under P-limitation, showing a decrease of 86% compared to 38% in N-limited cultures.

Growth rate decreased to approximately 80% of the control in both N-limited and P-limited cultures at a Cu:C ratio of approximately 0.8. At higher Cu:C ratios the decrease in growth rate was much greater under P-limitation, decreasing to approximately 46% of the control compared to approximately 77% in the N-limited cultures at a Cu:C ratio of 2.0 (Fig. 2.6).

Figure 2.6: Growth rate of N- and P-limited Chlamydomonas sp. as a function of cellular Cu concentration at stationary phase relative to control growth rate. Lines connect a moving average calculated over 5 points.


120
100
80
60
40
Growth Rate (% of control)
0
1
2
3
4
5
6
7
Cu : C x 10²
N-limited
P-limited

2.3.3 Final Yield

Chlorella sp.

The response of final yield to decreasing pCu differed with limiting nutrient. N-limited cultures showed no significant trend with decreasing pCu while the final yield of P-limited cultures decreased significantly with decreasing pCu (Table 2.1, Fig. 2.7). At pCu 12.2 the final yield was similar (approximately 2.5×10^{-6} cells mL^{-1}) in both N-limited and P-limited cultures. Below pCu 9.0 final yield in the P-limited cultures decreased steadily to a low of approximately 1.42×10^{5} cells mL^{-1} at pCu 5.0 (Fig. 2.7).

No trend in final yield was observed with increasing Cu:C ratio in N-limited cultures (Fig. 2.8). In P-limited cultures there was an initially steep decline in final yield followed by a more steady gradual decline with increasing Cu:C ratio to a low of approximately 10% of control at a Cu:C of 8.0. In the range of overlapping Cu:C ratios the final yield of P-limited cultures was much lower than the final yield of N-limited cultures (20% vs. 90% of control).

Chlamydomonas sp.

Final yield responses of Chlamydomonas sp. were similar to those of Chlorella sp. (Table 2.1, Fig. 2.9). Final yield decreased from approximately 3.5×10^{5} cells mL^{-1} in the P-limited control cultures to approximately 1.1×10^{5} at pCu 4.9. This represents a decrease in final yield of approxi-

Figure 2.7: Final yield of N- and P-limited Chlorella sp. as a function of Cu^{2+} activity. Vertical bars: ±2 standard errors.

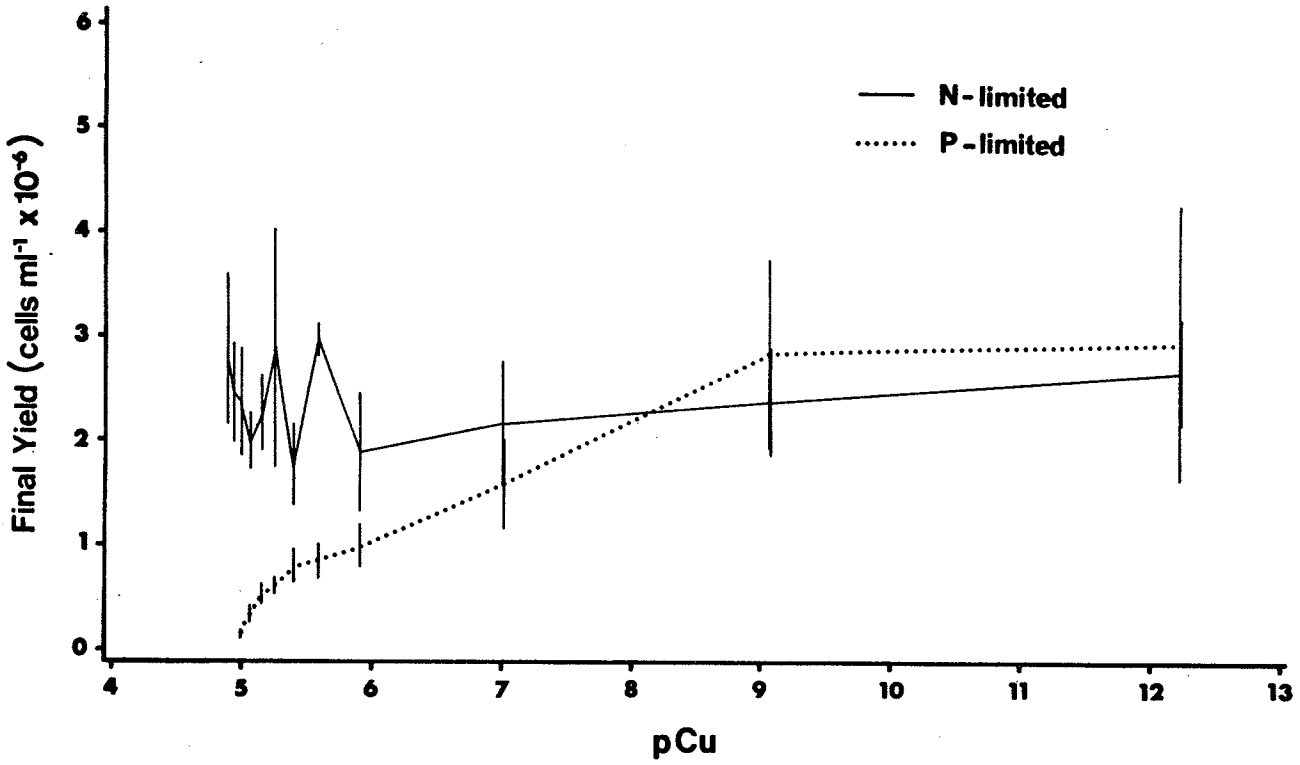

N-limited
P-limited
Final Yield (cells ml⁻¹ x 10⁻⁶)
0
1
2
3
4
5
6
4
5
6
7
8
9
10
11
12
13
pCu

Figure 2.8: Relative final yield of N- and P-limited Chlorella sp. as a function of cellular Cu concentration at stationary phase. Lines connect a moving average calculated over 5 points.

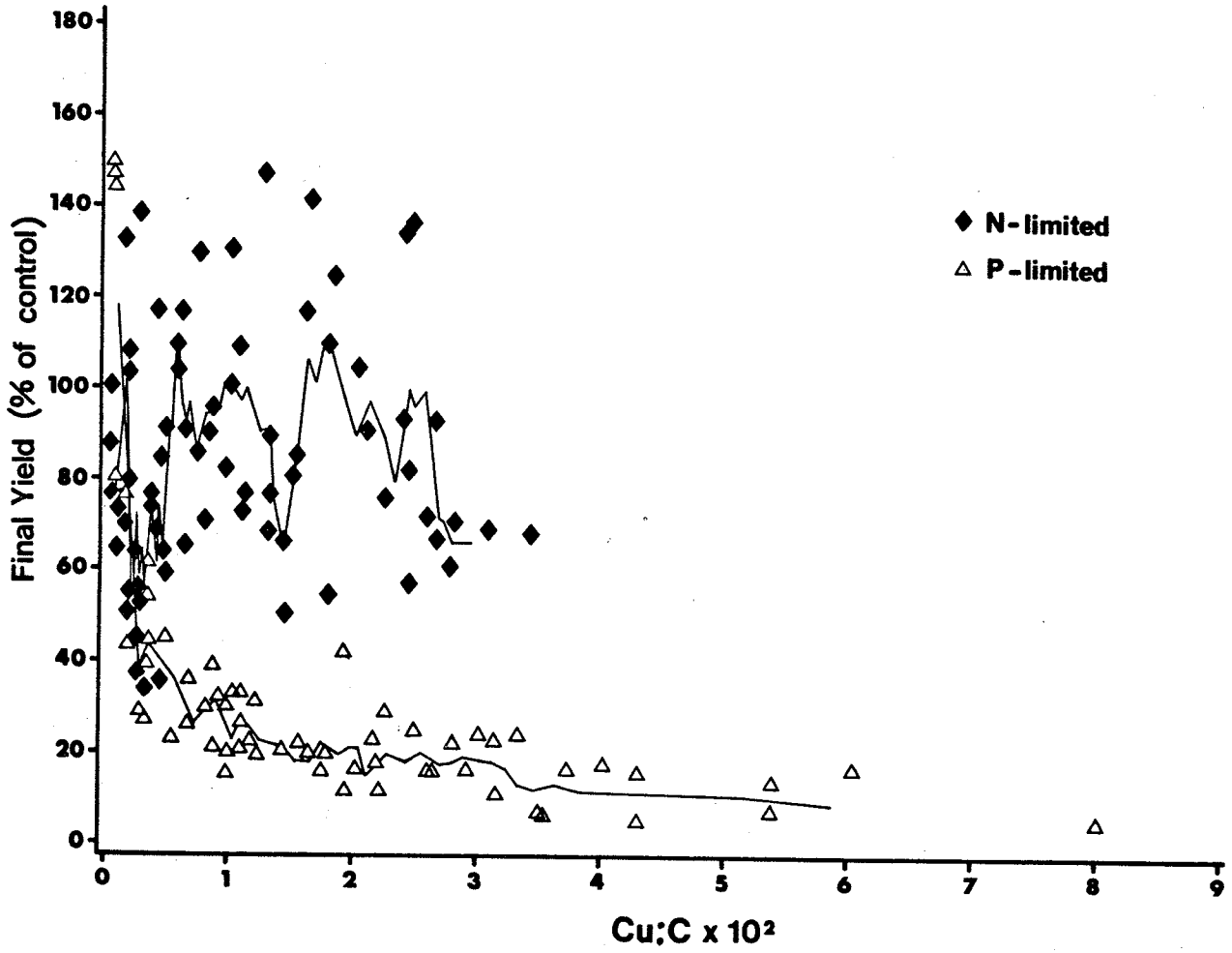
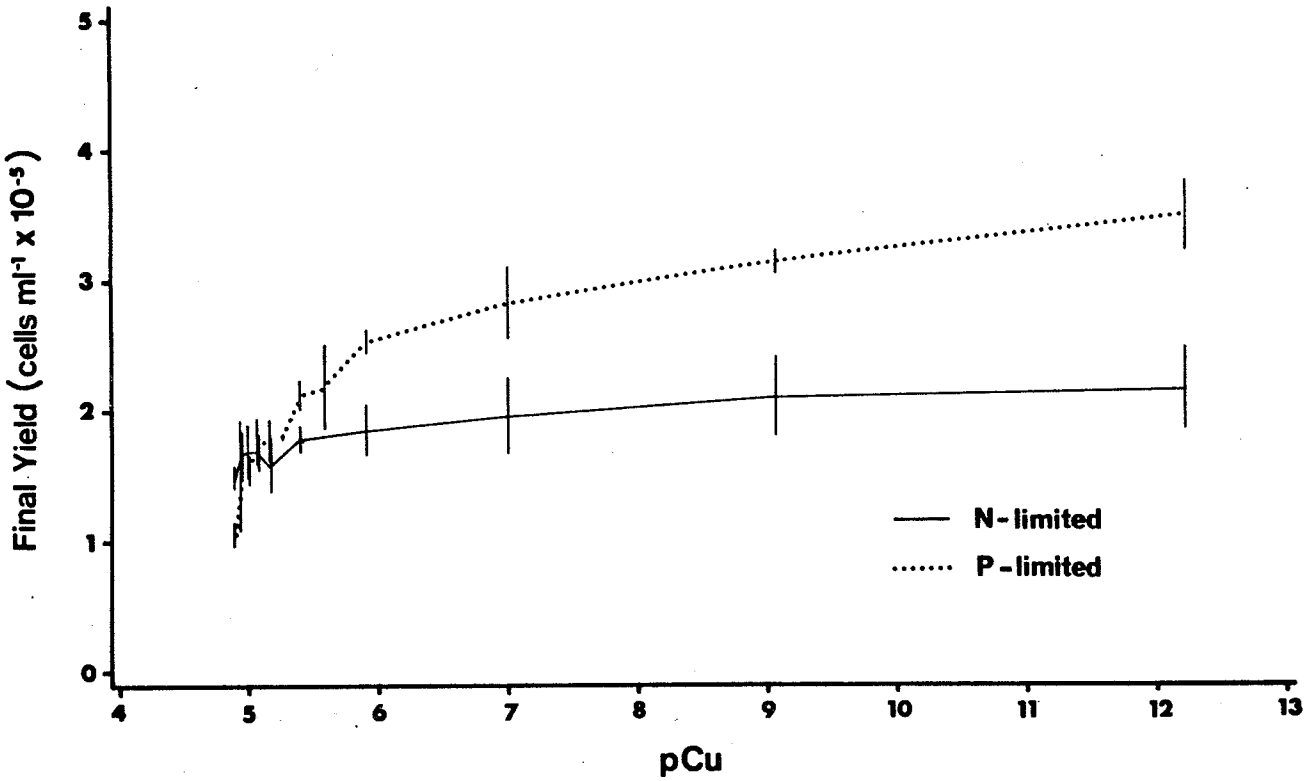

Final Yield (% of control)
180
160
140
120
100
80
60
40
20
0
0
1
2
3
4
5
6
7
8
9
Cu:C x 10²
N-limited
P-limited

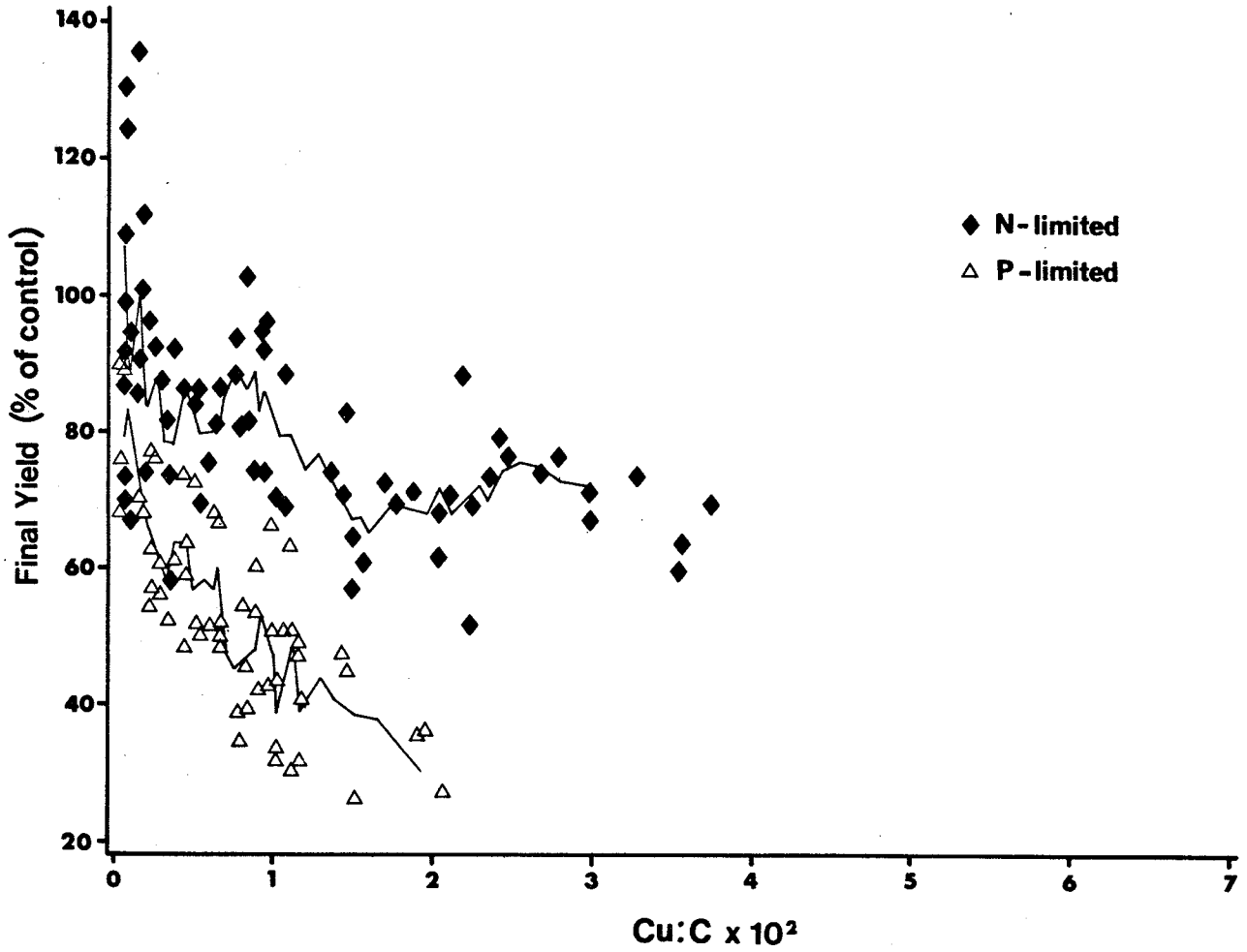
Figure 2.9: Final yield of N- and P-limited Chlamydomonas sp. as a function of Cu^{2+} activity. Vertical bars: ±2 standard errors.


Final Yield (cells ml-1 x 10-3)
5
4
3
2
1
0
4
5
6
7
8
9
10
11
12
13
pCu
N-limited
P-limited

mately 70% under P-limitation compared with no significant changes from the control under N-limitation (Table 2.1, Fig. 2.9).

In P-limited cultures of Chlamydomonas sp., the final yiels as percentage of control decreased steadily with increasing Cu:C to a low of approximately 35% of the controls at Cu:C of 2 (Fig. 2.10). Under N-limitation there was a small initial decline in final yield to approximately 75% of control cultures followed by little change with increasing Cu:C ratios (Fig. 2.10).

Figure 2.10: Relative final yield of N- and P-limited Chlamydomonas sp. as a function of cellular Cu concentration at stationary phase. Lines connect a moving average calculated over 5 points.


140
120
100
80
60
40
20
Final Yield (% of control)
0
1
2
3
4
5
6
7
Cu:C x 10²
N-limited
P-limited

2.3.4 Cellular Carbon

Chlorella sp.

Cellular C levels were approximately the same ($1.0x10^{-5}$ C $cell^{-1}$) in the control cultures at pCu 12.2 under both N and P limitation (Fig. 2.11). No significant difference was found in cellular C with decreasing pCu under either limiting nutrient (Table 2.1). While not significant over the range of pCu tested, there was a trend of increasing cellular C with decreasing pCu in P-limited cultures.

Chlamydomonas sp.

The response of cellular C to decreasing pCu (Fig. 2.12) was similar to that shown with Chlorella sp. (Fig. 2.11). Cellular C levels were similar (approximately $1.8x10^{-5}$ ug C $cell^{-1}$) at pCu 12.2 under both limiting nutrients and no significant changes were observed with decreasing pCu (Table 2.1, Fig. 2.12), though there was a non-significant trend of increasing cellular C with decreasing pCu under P-limitation.

Total C mL^{-1} can be used as a measure of biomass. Since no significant changes were observed in cellular C, the decreases in final yield in the P-limited cultures of both species also reflect a decrease in total biomass.

Figure 2.11: Cellular C content at stationary phase of N- and P-limited Chlorella sp. as a function of Cu^{2+} activity. Vertical bars: ±2 standard errors.

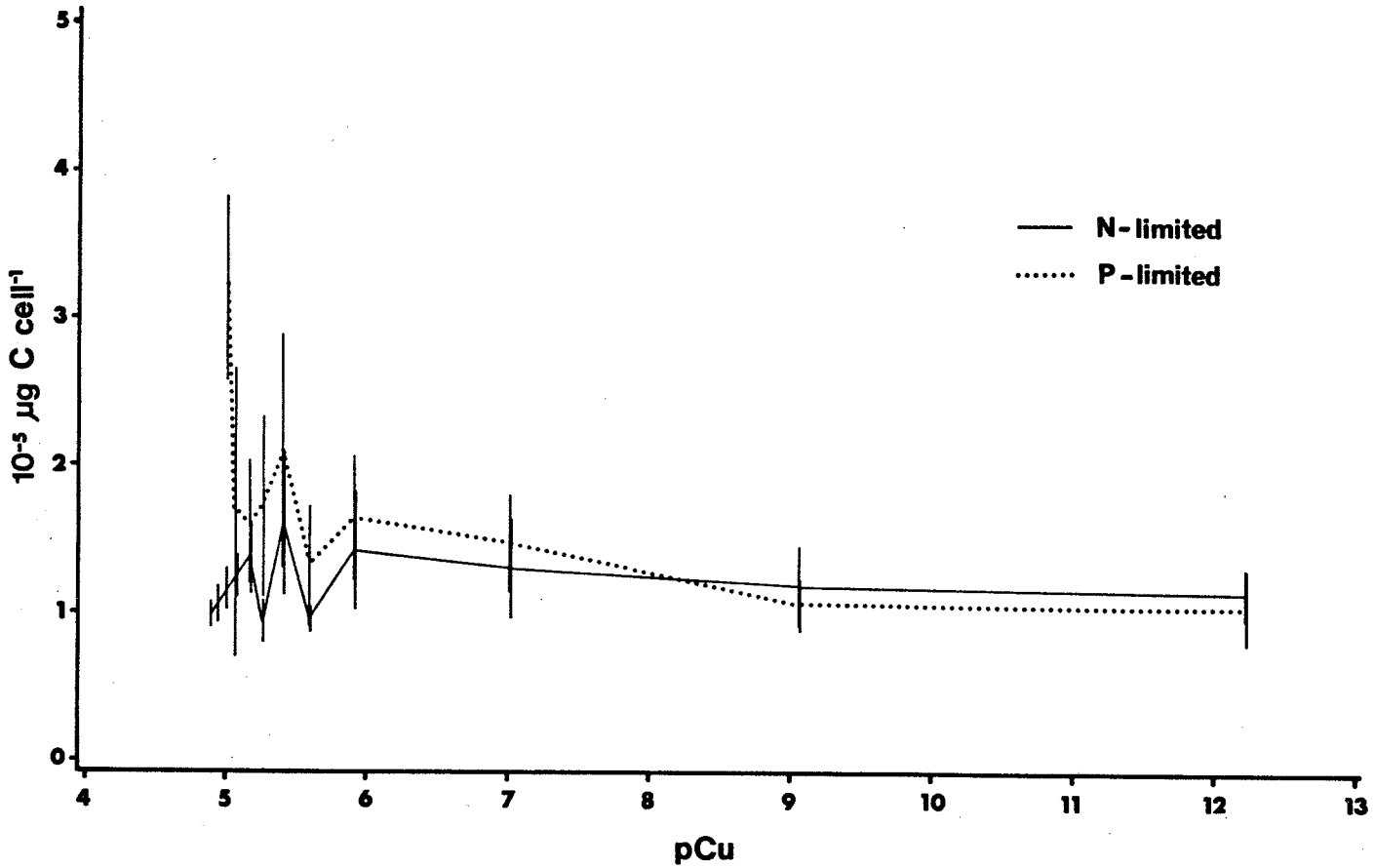
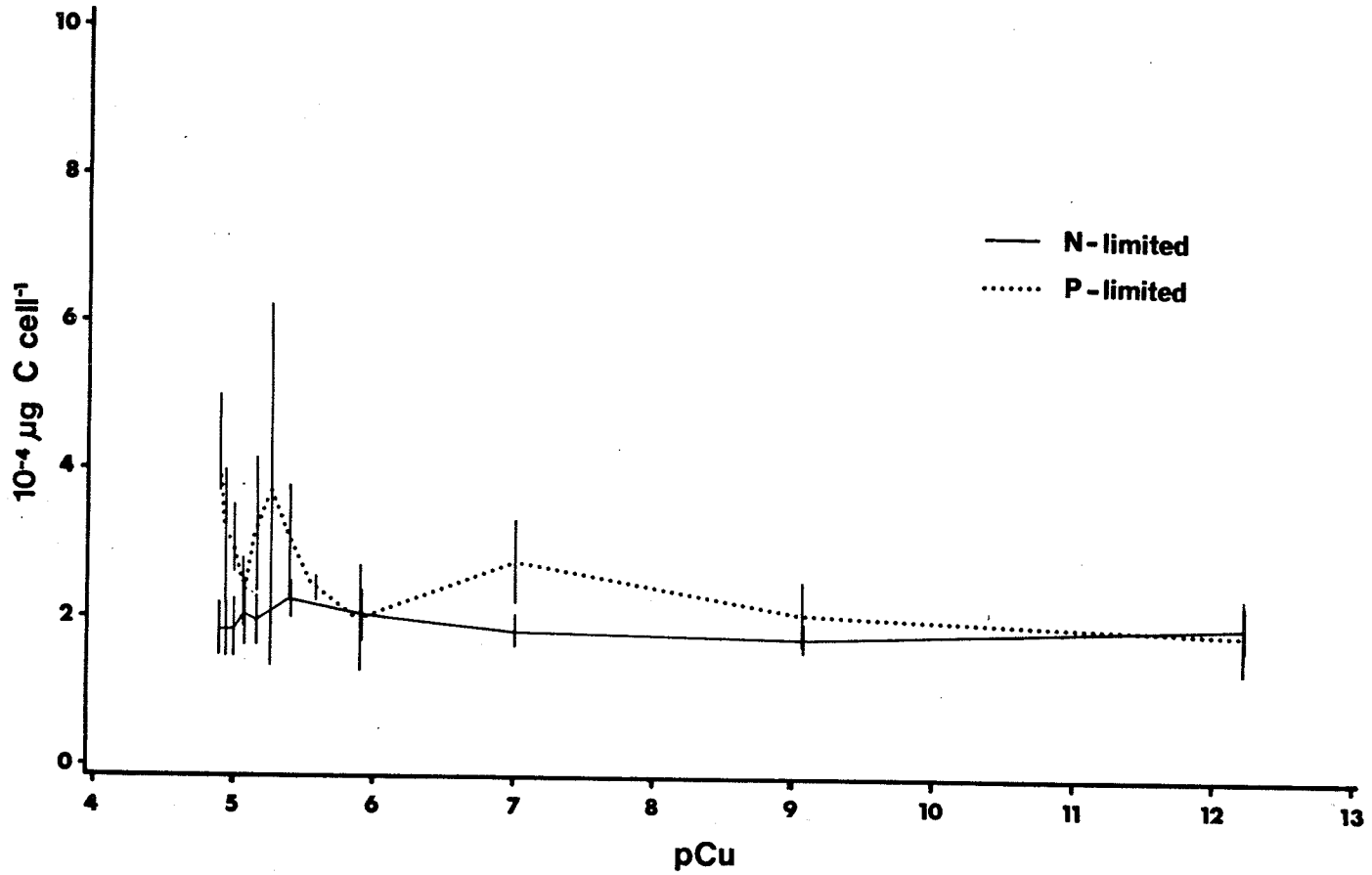

N-limited
P-limited
10^{-5} µg C cell^{-1}
0
1
2
3
4
5
4
5
6
7
8
9
10
11
12
13
pCu

Figure 2.12: Cellular C content at stationary phase of N- and P-limited Chlamydomonas sp. as a function of Cu^{2+} activity. Vertical bars: ±2 standard errors.


10
8
6
4
2
0
10^{-4} µg C cell^{-1}
N-limited
P-limited
4
5
6
7
8
9
10
11
12
13
pCu

2.3.5 Cellular Phosphorous

Chlorella sp.

Cellular P in P-limited cultures increased significantly with decreasing pCu from 0.27×10^{-7} ug P $cell^{-1}$ at pCu 12.2 to 4.1×10^{-7} ug P $cell^{-1}$ at pCu 5.0 (Table 2.1, Fig. 2.13). This was similar to the trend observed with cellular Cu (Fig. 2.1) which increased rapidly at pCu levels below 7.0 (Fig. 2.13).

In N-limited cultures there was no significant trend of cellular P with decreasing pCu (Table 2.1, Fig. 2.13).

Chlamydomonas sp.

Cellular P increased significantly in the P-limited cultures with decreasing pCu (Table 2.1, Fig. 2.14) from 1.55×10^{-6} ug P $cell^{-1}$ at pCu 12.2 to 4.9×10^{-6} ug P $cell^{-1}$ at pCu 4.9. The largest increase in cellular P occurred at pCu levels below 5.5. Under N-limitation, cellular P did not change significantly with decreasing pCu (Table 2.1, Fig. 2.14).

Figure 2.13: Cellular P content at stationary phase of N- and P-limited Chlorella sp. as a function of Cu^{2+} activity. Vertical bars: ±2 standard errors.

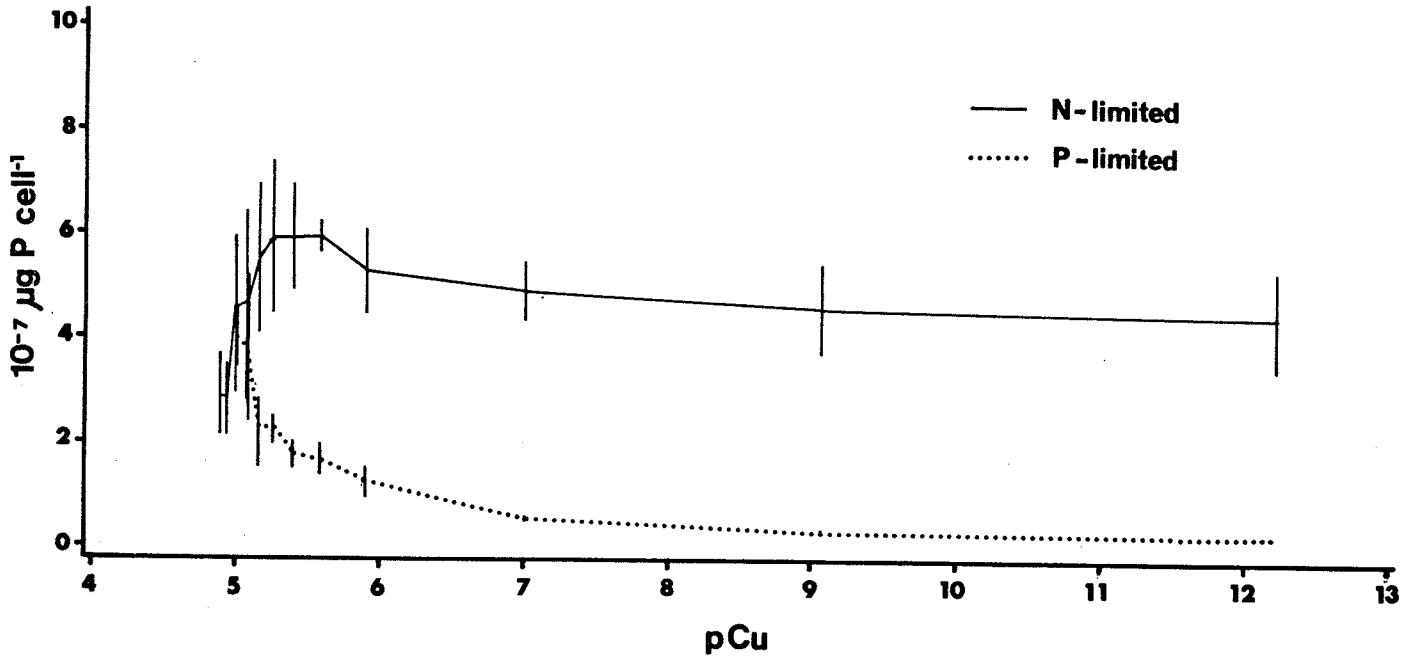
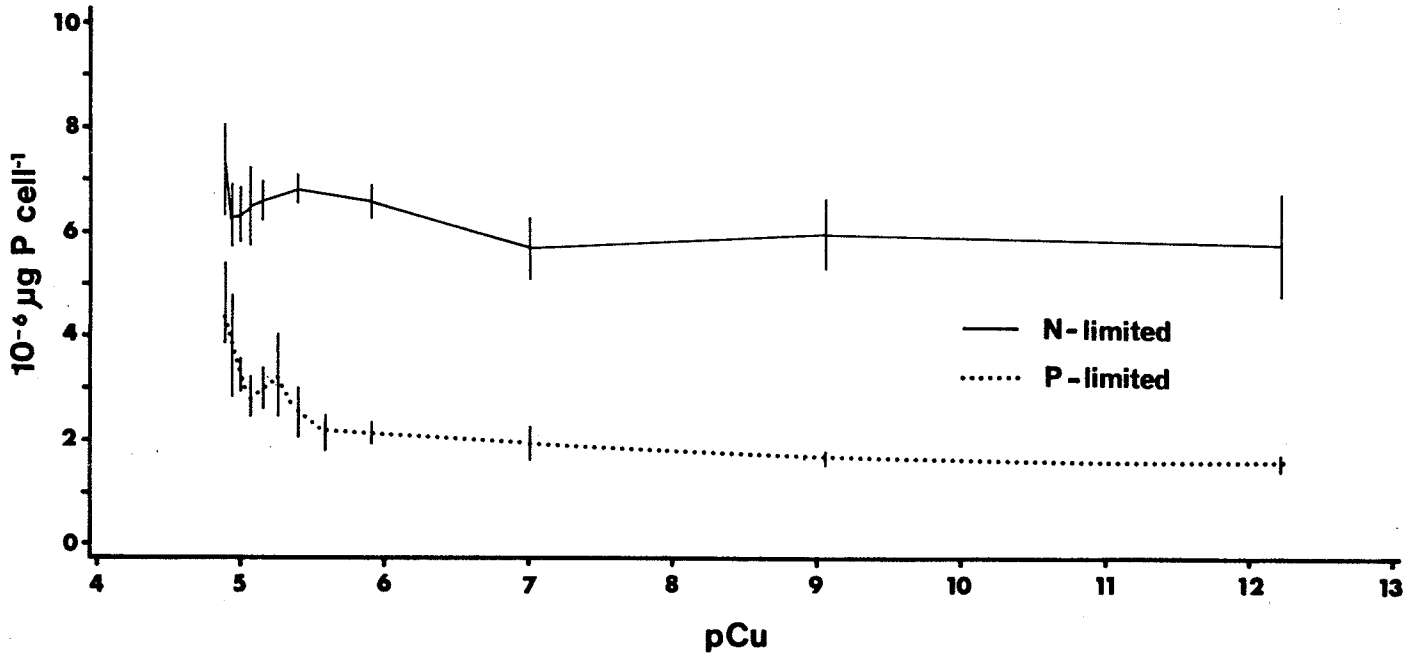

N-limited
P-limited
10
8
6
4
2
0
10^{-7} µg P cell^{-1}
4
5
6
7
8
9
10
11
12
13
pCu

Figure 2.14: Cellular P content at stationary phase of N- and P-limited Chlamydomonas sp. as a function of Cu^{2+} activity. Vertical bars: ±2 standard errors.


10
8
6
4
2
0
10^{-6} µg P $cell^{-1}$
4
5
6
7
8
9
10
11
12
13
pCu
N-limited
P-limited

2.4 DISCUSSION

The observed decline of growth rate in response to decreasing pCu (Figs. 2.3 and 2.5) has been widely reported (Sunda and Guillard 1976, Saifullah 1978, Canterford and Canterford 1980, Gavis et al. 1981). Gavis et al. (1981) reported a 50% reduction in the growth rate of several marine algal species at pCu levels between 10.5 and 8.5. In the experiments reported here, 50% reduction in growth rate occurred at higher Cu^{2+} activities of approximately pCu 5.0 for both Chlorella sp. and Chlamydomonas sp. indicating greater tolerance than that observed by Gavis et al. (1981). These tolerance levels are similar to those reported by Stokes et al. (1973) for Scenedesmus acutiformis and Chlorella fusca which had also been isolated from Cu contaminated lakes. Foster (1982b) reported that several species of Chlorella and Chlamydomonas were possibly naturally resistant to Cu and that such resistance can be expected to be elevated in isolates from areas of high Cu contamination.

The response of final yield to increasing Cu^{2+} activity has been reported to vary widely. Stokes et al. (1973), Jensen et al. (1974), and Anderson et al. (1978) have found decreasing final yield with decreasing pCu; whereas Kawabara and Leland 1986, have reported finding no changes in final yield. This variation may result from differences in the limiting nutrient as shown in this study where the final yield of P-limited cultures declined greatly with de-

creasing pCu and N-limited cultures showed no significant changes in final yield.

Under P-limitation final yield was found to be a more sensitive indicator of Cu toxicity than growth rate in both species tested (Figs. 2.3, 2.5, 2.7, and 2.9), as it was for P-limited Chlamydomonas variabilis with Zn^{2+} toxicity (Bates et al. 1983). In N-limited cultures however, growth rate was found to be a more sensitive indicator of Cu toxicity (Figs. 2.3, 2.5, 2.7, and 2.9). The growth rate of T. weissflogii under Si limitation was also more sensitive than final yield (Anderson et al. 1978). This suggests a need for careful consideration of the parameters used to measure Cu toxicity, and the nutrient limiting the final yield of the batch cultures.

Increased cellular Cu was found in both species under both nutrient regimes with decreasing pCu (Figs. 2.1 and 2.2). This is consistent with the results of Bates et al. (1982) who showed with Chlamydomonas variabilis that both adsorbed and transported Zn were a function of Zn^{2+} activity in short term uptake experiments and long term growth experiments. The cellular Cu content of Chlamydomonas sp. was similar under both N and P limitation (Fig. 2.2). In cultures of Chlorella sp., however, the cellular Cu content was much higher in P-limited cultures (Fig. 2.1). These elevated levels may be attributed to the observed reduction in final yield (Figs. 2.7 and 2.9) or to an increase in cell

permeability under P-limitation. The data do not permit the two possibilities to be distinguished.

The difference in sensitivity of final yield of N- and P-limited cultures must reflect a difference in response to cellular Cu, since cell growth has been suggested to be directly related to intracellular levels of the factor which controls growth (Davies and Sleep 1980, Davies 1983). To correct for differences in cell size between the two species and any changes in cell size that might occur with Cu treatment, the ratio of cellular Cu:cellular C can be used as an estimate of cellular Cu concentration. The final yield of Chlorella sp. under N-limitation was insensitive to increasing cellular Cu concentration when compared to the decrease in final yield of P-limited cultures (Fig. 2.8). Chlamydomonas sp. showed a response similar to Chlorella sp. with P-limited cultures having substantially lower final yields when compared with N-limited cultures at the same cellular Cu concentrations (Fig. 2.10).

The trend of decreasing growth rate and final yield of Chlamydomonas sp. and final yield of Chlorella sp. with increasing cellular Cu concentration was much more pronounced in P-limited cultures than in N-limited cultures at similar cellular Cu concentrations (Fig. 2.6). In Chlorella sp., however, the greater sensitivity of growth rate in the P-limited cultures may be attributable to the higher cellular Cu concentrations in P-limited cultures (Fig. 2.4). This

may be an artifact of cellular Cu concentrations being measured at stationary phase; whereas, growth rate is a parameter of exponential growth prior to stationary phase.

The data from this study support the hypothesis of Bates et al. (1985) that an interaction occurs between P and metal toxicity. A possible explanation for the increased sensitivity to cellular Cu concentration of P-limited cultures compared with N-limited cultures may lie in a mechanism for detoxification of metals within the cell proposed by Sicko-Goad and Stoermer (1979), Jensen et al. (1982), and Pettersson et al. (1985). They proposed that metals may be complexed by polyphosphates within the osmotically inert polyphosphate bodies and so be detoxified. Plectonema boryanum has been shown to accumulate Cu, Cd, Co, Hg, Ni, Pb and Zn in polyphosphate bodies (Jensen et al. 1982). Pettersson et al. (1985) showed a similar effect for Al in Anabaena cylindrica as did Sicko-Goad and Stoermer (1979) for Pb in Diatoma tenue. Since the formation of polyphosphate bodies is dependent on P availability (Rhee 1973), the availability of P may influence a cell's ability to detoxify Cu, and hence result in a greater sensitivity to Cu in P-limited cultures.

Cellular P was found to increase in P-limited cultures with increasing Cu^{2+} activity since all available P was removed from the medium. The increase in cellular P may simply result from a decrease in final yield leading to an in-

crease in stored P. Alternatively, a change in cell metabolism may increase cellular demand for P. This increase in demand may result from the complexing of Cu with polyphosphates which may limit the reactivation of P from polyphosphate bodies and hence reduce P availability and limit final yield.

As well as explaining some of the variablity in the Cu^{2+} activities which have been reported in the literature to be toxic to algae in culture, the differences in Cu sensitivity between N- and P-limited cultures also have ecological implications, particularly since P is frequently the nutrient which limits phytoplankton growth in freshwater ecosystems (Wetzel 1983). For example, the interaction of nutrient limitation and Cu toxicity may influence the outcome of species competition. Rhee and Gotham (1980) showed that the optimum N:P ratios (the ratio at which a species changes from N-limitation to P-limitation or vice versa) are highly variable between species (ranging from 7-30). Niche separation through the limitation of different species by different nutrients has been proposed by Tilman (1982) in his resource based competition theory. If two or more species are limited by different nutrients (e.g., N and P), when Cu is introduced to the system, the eventual species composition may well be determined by the interaction of Cu toxicity and the growth limiting nutrient.

The greater sensitivity of P-limited algae to Cu toxicity also suggests that Cu toxicity may be greater in natural water bodies than would be predicted from many batch culture studies which do not have P as the limiting nutrient.

Chapter III

PAPER 2: THE INTERACTION OF CHRONIC COPPER TOXICITY WITH NUTRIENT LIMITATION IN CHEMOSTAT CULTURE EXPERIMENTS WITH CHLORELLA SP

3.1 INTRODUCTION

The toxic properties of Cu in the aquatic environment are well known since Cu has been widely used as an algicide for many years (Fitzgerald and Faust 1963, Gibson 1972, McKnight 1981, Raman 1985). Increasingly however, natural water bodies are being exposed to chronic, elevated Cu concentrations through man's increased industrial activities.

The impact of Cu toxicity on algae has generally been investigated using batch culture experiments, although such a system suffers several shortcomings. In natural water bodies phytoplankton are generally exposed to Cu contamination on a long term basis with continual input of Cu into the water body. Algae in batch culture systems, on the other hand, are exposed to a single pulse of toxic metal at the beginning of the experiment (Rice et al. 1973). The toxicity of this single pulse of toxic metal may be influenced by initial cell numbers (Steemann Nielsen et al. 1969, Steemann Nielsen and Kamp-Nielsen 1970, Gipps and Coller 1980), age of the inoculum culture (Morel and Morel 1976, Morel et al. 1978, Kawabara and Leland 1986). As well the nutrient

status of cells in batch cultures changes over time with cells being nutrient sufficient at the beginning of each experiment, unlike most natural populations which are generally nutrient limited.

The interaction of macronutrients and metal toxicity was demonstrated in Chapter 2 and has been shown elsewhere (Meijer 1972, Zarnowski 1972, Harding and Whitton 1977, Say and Whitton 1977, Say et al. 1977, Shehata and Whitton 1982, Bates et al. 1985). The decreased toxicity of metals with increased available P has been related to the detoxification of metals within the polyphosphate bodies (Sicko-Goad and Stoermer 1979, Jensen et al. 1982, Pettersson et al. 1985). Bates et al. (1985) have proposed that macronutrients may play a major role in the regulation of metal toxicity, highlighting the need for consideration of nutrient limitation in metal toxicity studies.

An alternative method to the batch culture system is the continuous culture which more closely approximates the natural environment. Two types of continuous culture systems have been used in toxicity studies: the turbidostat in which cells grow in a nutrient sufficient medium with the dilution rate corresponding to the growth rate of the culture (Kayser 1976, Lederman and Rhee 1982), and the chemostat in which growth rate is determined by dilution rate and controlled by a known limiting nutrient (Lederman and Rhee 1982).

Continuous culture systems allow study of long term exposure to a toxic metal (Rice et al. 1973, Kayser 1976), and in the case of the chemostat, allow for the control of a limiting nutrient. A third advantage of continuous cultures is the ability to control gross cell composition. It has been proposed that gross cell composition may be a sensitive indicator of metal toxicity reflecting changes in cell metabolism (Rice et al. 1973, Li 1979, Lederman and Rhee 1982). Changes in gross cell composition have been reported in conjunction with several types of stress: temperature (Goldman 1977a, 1977b), light (Davis 1976), nutrient limitation (Healey and Hendzel 1975, Rhee 1978) and Cd (Li 1979).

The few studies which have been conducted to compare metal toxicity in batch and continuous culture have produced contradictory results. Bentley-Mowatt and Reid (1977) showed that four species of marine phytoplankton had similar sensitivity to Cu in batch and continuous culture, although, in the continuous culture experiments, only one pulse of Cu was added. A similar result was obtained by Kayser (1976) with mercuric acetate toxicity to three marine dinoflagellates in batch and turbidostat culture. Saifullah (1978) observed the opposite effect with the toxicity of Cu to three marine dinoflagellates being higher in semicontinuous culture than in batch culture.

The study reported here investigates the toxic effects of long term exposure of Chlorella sp. to Cu under N- and P-limitation.

3.2 METHODS

A description of the Chlorella sp. isolate used in these experiments can be found in Chapter 2.

The chemostat culture apparatus was constructed and operated following Healey and Hendzel (1975), with silicone stoppers being used to close the tubes. The tubes were assembled and linked with silicone tubing to the medium reservoirs before autoclaving. All glassware used in the experiments was prepared for use as described in Chapter 2.

The medium used was a modified WC medium (Chapter 2) with either 5.0 uM P or 5.0 uM N as the limiting nutrient concentrations. These concentrations were shown to limit the cell numbers of the cultures. After autoclaving, the tubes were placed in a controlled environment chamber at 20°C with continuous illumination of 150 $uEm^{-2}s^{-1}$ from fluorescent Cool White V.H.O. (Sylvania) lights on one side of the culture tubes.

For each experiment, each tube was inoculated with 3 mL of axenic, exponentially growing culture via the inoculation port into approximately 100 mL of medium. The sterility of the cultures was checked visually throughout the experiments, any contaminated cultures were removed and restarted.

A Manostat Cassette Pump was used to deliver the medium from 4 L pyrex reservoirs at a dilution rate of 0.25 d^{-1}

into a culture volume of 500 mL. The cultures were first allowed to grow undisturbed for approximately 48 hours prior to commencing pumping. Air used to agitate the cultures was scrubbed with 3N H_2SO_4 to remove any NH_3 contamination and passed through a 0.45 uM filter.

Experimental treatments began when individual cultures reached steady state. At that time approximately 75 mL of culture was removed from each tube to be used for the day 0 analyses; sterile Cu stock was then added via the inoculation port to bring the Cu concentration in the culture to that required in the experiment. The medium reservoir was then replaced with one containing medium of the required Cu concentration for the experiment. Cu levels ranged from the controls at pCu 12.2 to pCu 10.45 in P-limited cultures and pCu 10.3 in N-limited cultures. Three replicate cultures at each Cu^{2+} activity were set up using a common medium reservoir.

On day 0 and every 3 or 4 days until day 20, 75 mL was removed from each culture and filtered onto Whatman GF/A filters which were then analysed for particulate C, P, and N. Filter preparation and analyses were conducted following the procedures outlined in Chapter 2.

Total Cu per mL of culture was analysed as follows: 20 mL of sample was placed in an acid cleaned polyethylene bottle and acidified to 0.5% with concentrated Baker Instra-A-

nalysed HNO_3. The samples were then stored at 4°C until direct analysis by Graphite Furnace Atomic Absorption Spectrophotometer standardized using Fisher Certified Standards. A complete digestion of several samples (evaporation and residue digestion with nitric acid) confirmed that the non-digestion technique was measuring total Cu in the sample.

For particulate Cu analysis 24 mL of culture was centrifuged in polypropylene tubes (washed with soap, soaked in 3N HNO_3 for 48 hours, and thoroughly rinsed) and the supernatant removed by suction and discarded. Samples were stored frozen until analysis when samples were oven dried at 80°C to remove residual moisture, and digested with 5.0 mL concentrated HNO_3 at 90°C for 1 hour. The digest was adjusted to a volume of 5.0 mL with deionised water and analysed following the method for total Cu.

Alkaline phosphatase activity was also determined on each sampling day using the 0-methyl-fluorescein phosphate method (Healey and Hendzel 1979). Measurements were conducted at pH 8.0 using 10 mM HEPES (N-2-hydroxyethylpeperazine-N'-2-ethane sulfonic acid) to buffer pH. The resuspension medium used was the same as that used in the experiments except for the replacement of K_2HPO_4 by equimolar KCl, and the replacement of 5mM MES by 5mM HEPES.

3.3 RESULTS

3.3.1 Cell Numbers Over Time

In P-limited cultures the steady state cell number of the control was approximately 3.1×10^6 cells mL^{-1}. In cultures at pCu 11.4 and 11.1 cell numbers initially decreased but returned almost to the control level by day 20 (Fig. 3.1). At pCu 10.8 there was no noticeable effect of the Cu addition on cell numbers. At pCu 10.5 cell numbers initially declined rapidly followed by a slower but continuous decline to day 20 (Fig. 3.1). In N-limited cultures the steady state cell number of the control cultures was approximately 4.3×10^6 cells mL^{-1}. At pCu levels below 10.6 cell numbers decreased steadily over the 20 day period (Fig. 3.2).

Because of the lack of re-establishment of a steady state (steady state = cell numbers ± 5% over 2 generations) at the higher pCu levels, it was decided to compare data from the different pCu levels after two time periods: on day 16, 4 generations after Cu addition, and day 20, 5 generations after Cu addition. These times were chosen to allow the effects of long term exposure to Cu to be assessed.

Figure 3.1: Mean cell numbers over time of P-limited *Chlorella* sp. grown in chemostat culture at 5 Cu^{2+} activities. Cu added to cultures on day 0.

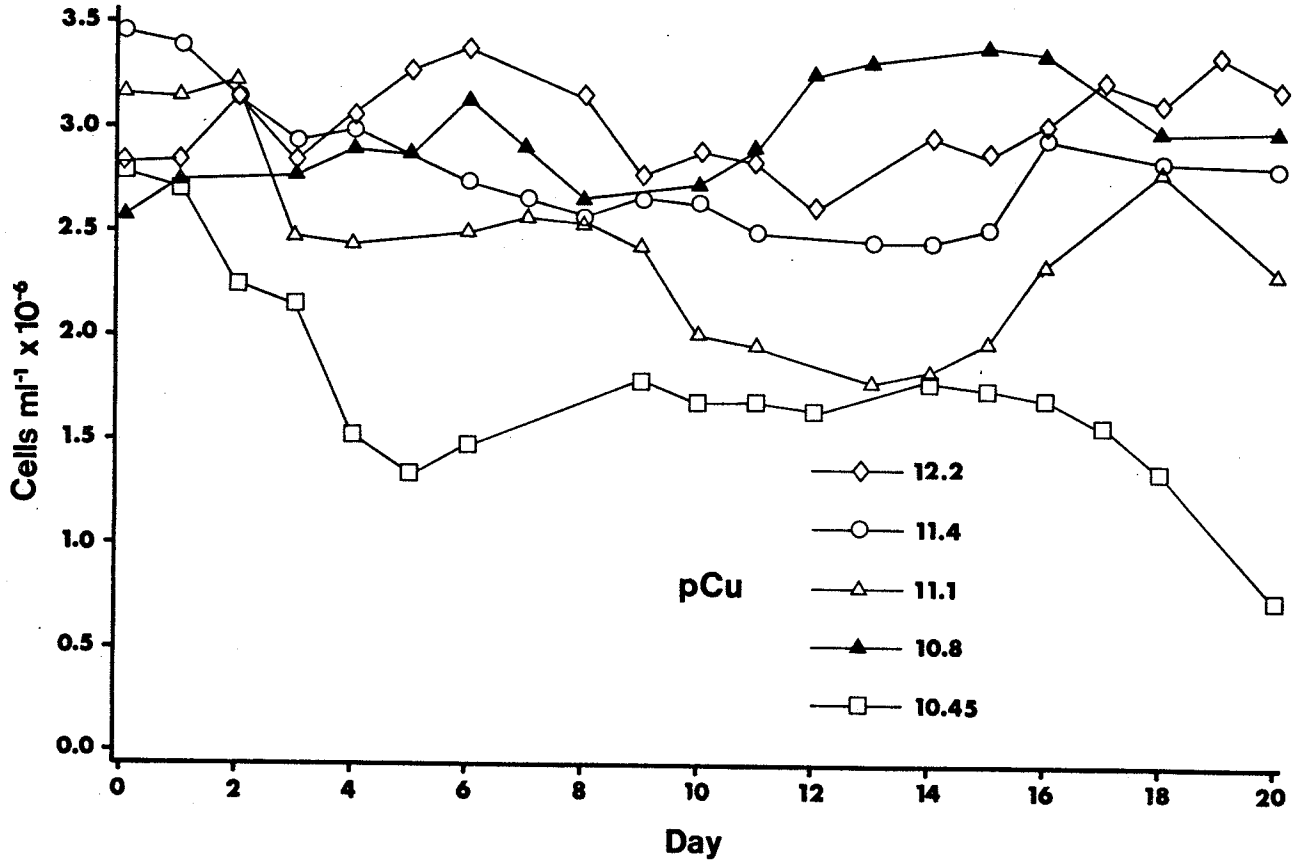
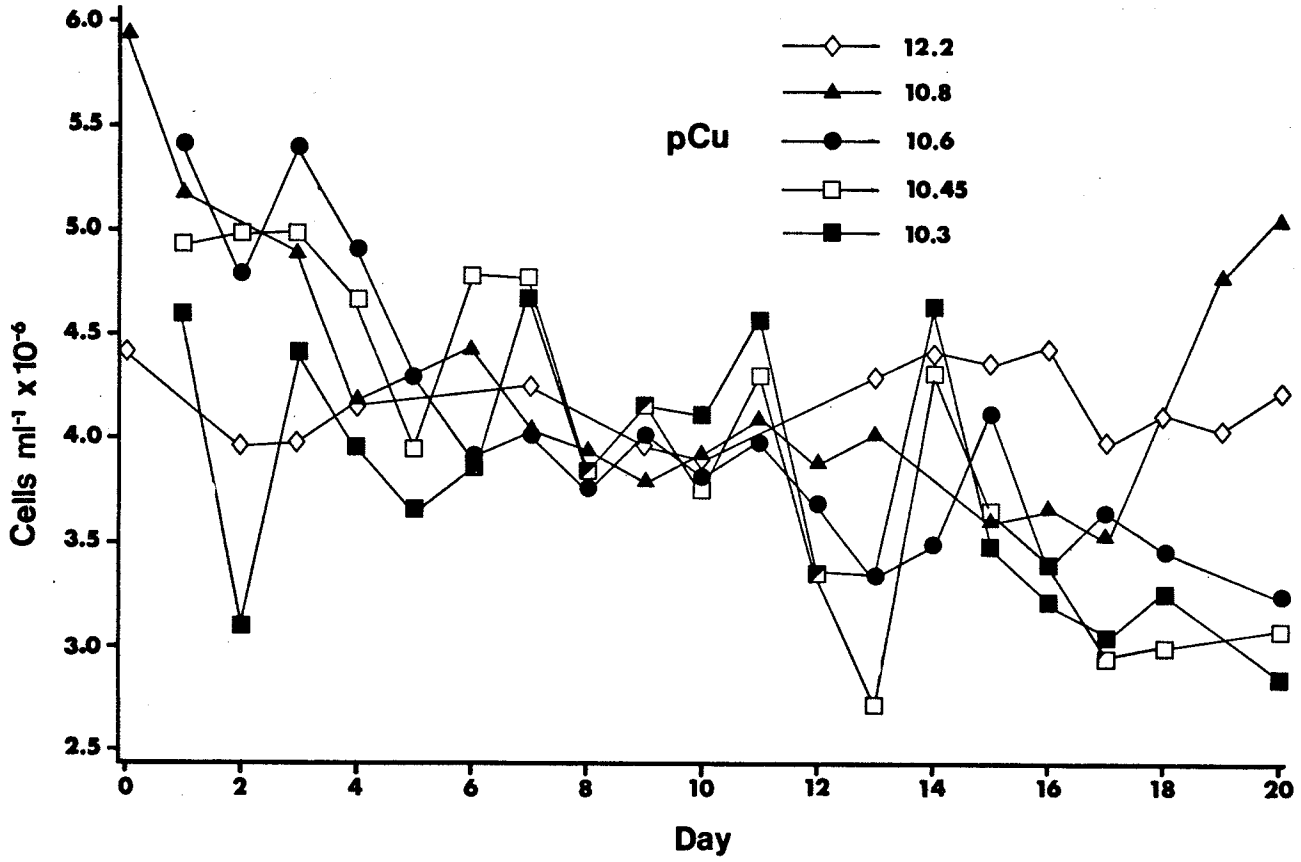

Cells ml⁻¹ x 10⁻⁶
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
2
4
6
8
10
12
14
16
18
20
Day
pCu
12.2
11.4
11.1
10.8
10.45

Figure 3.2: Mean cell numbers over time of N-limited *Chlorella* sp. grown in chemostat culture at 5 Cu^{2+} activities. Cu added to cultures on day 0.


pCu
12.2
10.8
10.6
10.45
10.3
Cells ml⁻¹ x 10⁻⁶
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
0
2
4
6
8
10
12
14
16
18
20
Day

3.3.2 Cellular Copper

Cellular Cu (intracellular and adsorbed) levels were similar to the controls ($0.6x10^{-8}$ ng $cell^{-1}$) above pCu 10.8 in both P-limited and N-limited cultures (Fig. 3.3). Below pCu 10.8 the cellular Cu levels increased significantly to a high of $2.2x10^{-8}$ ng $cell^{-1}$ in the P-limited cultures and $6.0x10^{-8}$ ng $cell^{-1}$ for N-limited cultures at pCu 10.45 and 10.3 respectively (Table 3.1). The trend was similar on both days in both the N-limited and P-limited cultures (Fig. 3.3), although the trend was not significant in the P-limited cultures on day 20 (Table 3.1).

The cellular Cu:C ratio was used as an estimate of the Cu concentration per cell, independent of changes in cell size. In P-limited cultures on day 16 the Cu:C ratio increased significantly with decreasing pCu to a high of $1.4x10^{-6}$; however on day 20, the Cu:C ratio increase was not significant with decreasing pCu (Fig. 3.4,Table 3.1). In the N-limited cultures the Cu:C ratio increased significantly to $3.7x10^{-6}$ at pCu 10.45 on day 16, and increased to $3.9x10^{-6}$ at pCu 10.3. on day 20 (Fig. 3.4, Table 3.1).

Figure 3.3: Cellular Cu content of N- and P-limited *Chlorella* sp. as a function of Cu^{2+} activity. Vertical bars: ±1 standard error.

N_{16} and N_{20} represent N-limited cultures at days 16 and 20 respectively.
P_{16} and P_{20} represent P-limited cultures at days 16 and 20 respectively.

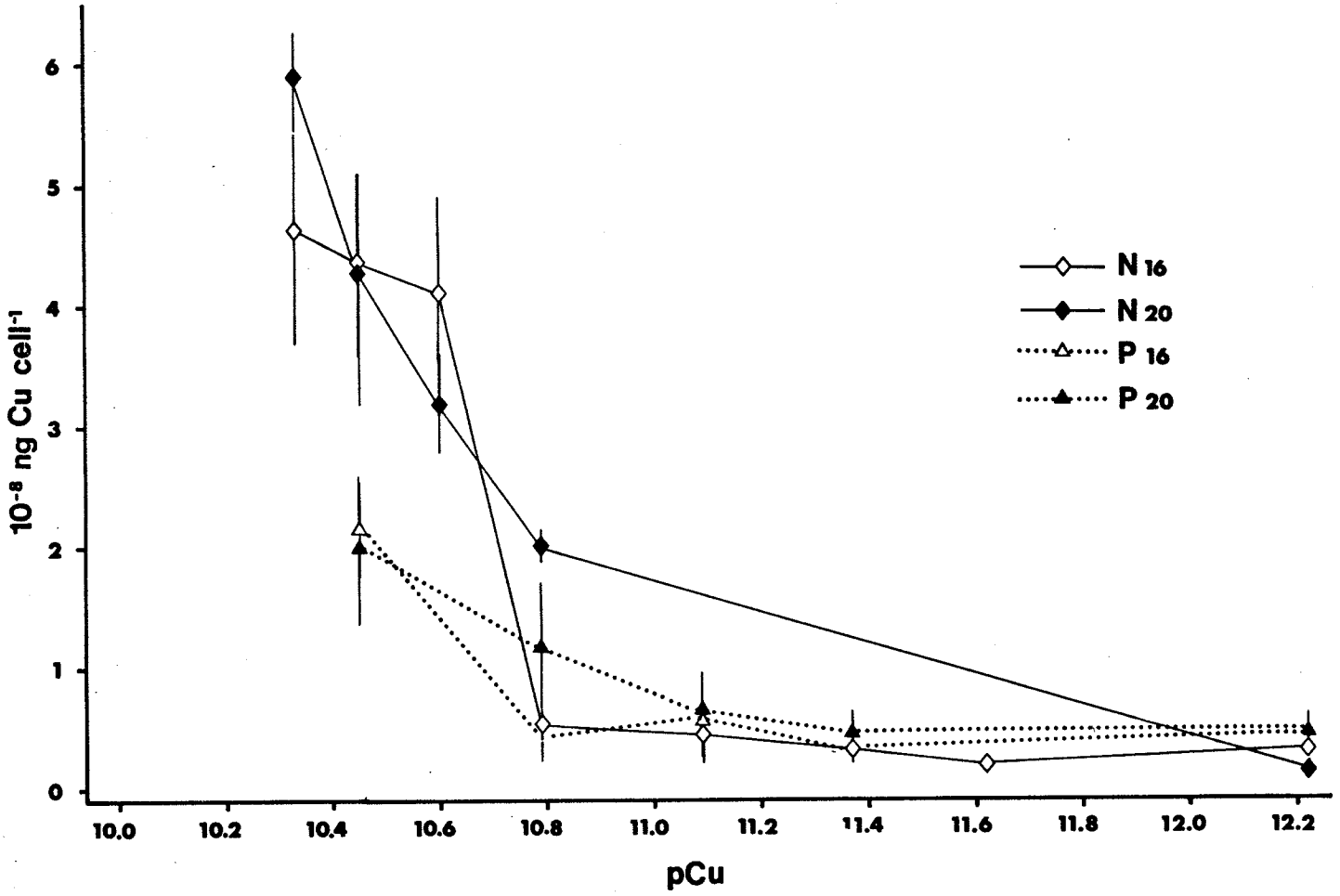

6
5
4
3
2
1
0
10-8 ng Cu cell-1
10.0
10.2
10.4
10.6
10.8
11.0
11.2
11.4
11.6
11.8
12.0
12.2
pCu
N 16
N 20
P 16
P 20

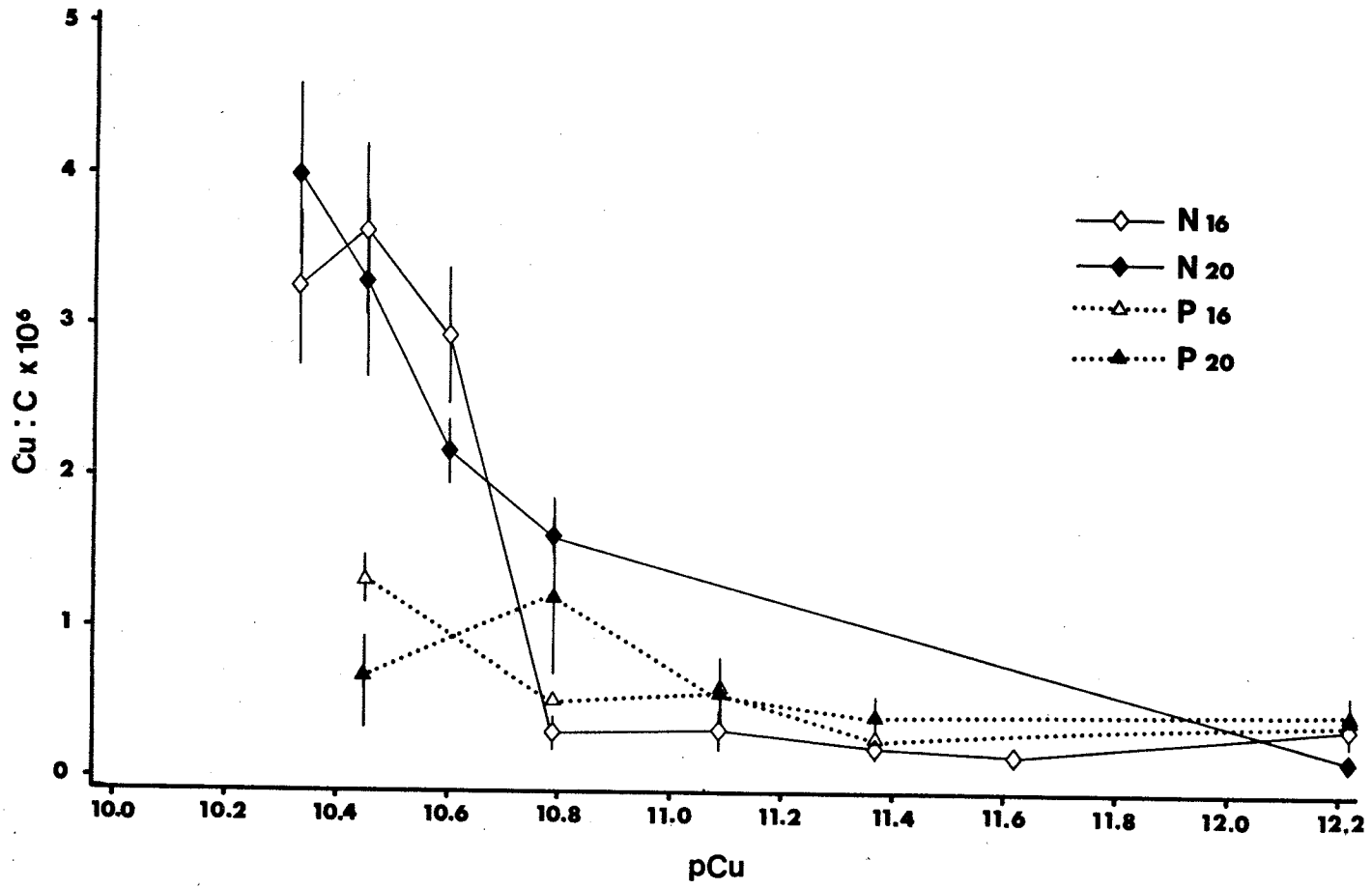
Table 3.1: Summary of ANOVA results testing for differences in individual parameters (log transformed) with decreasing pCu in chemostat cultures.

Variable	Day 16 Probability	Day 16 Significance	Day 20 Probability	Day 20 Significance
N-Limited Cultures				
Cellular Cu	.0001	***	.0001	***
Cellular Cu Concentration	.0001	***	.0001	***
Cell numbers	.0046	**	.0047	**
Biomass	.0003	***	.195	*
Cellular C	.0001	***	.05	**
C:N Ratio	.0002	***	.02	*
C:P Ratio	.27	*	.22	*
P-limited Culture				
Cellular Cu	.0002	***	.15	*
Cellular Cu Concentration	.0002	***	.47	*
Ce-1 numbers	.025	*	.0023	**
Biomass	.14	*	.20	*
Cellular C	.06	*	.0001	*
C:N Ratio	.05	**	.33	*
C:P Ratio	.65	*	.69	*
Alkaline phosphatase activity:				
Cellular C	.06	*	.08	*

significant at = .001 ***
significant at = .05 **
no signif. diff. = *

Figure 3.4: Cellular Cu concentration of N- and P-limited *Chlorella* sp. as a function of Cu^{2+} activity. Vertical bars: ±1 standard error.

N_{16} and N_{20} represent N-limited cultures at days 16 and 20 respectively.
P_{16} and P_{20} represent P-limited cultures at days 16 and 20 respectively.


Cu : C x 10⁶
5
4
3
2
1
0
10.0
10.2
10.4
10.6
10.8
11.0
11.2
11.4
11.6
11.8
12.0
12.2
pCu
N 16
N 20
P 16
P 20

3.3.3 Cell Numbers and Biomass

Cell numbers decreased significantly in the P-limited cultures to a low of approximately 55% and 20% of the controls on days 16 and 20 respectively at pCu 10.45 (Fig. 3.5, Table 3.1). The decrease in cell numbers under N-limitation was significant but smaller, dropping to a low of approximately 70% and 64% of the controls on days 16 and 20 respectively at pCu 10.3 (Fig. 3.5, Table 3.1).

Cell numbers can be considered in relation to Cu:C ratio. In the N-limited cultures there was a trend of decreasing cell numbers with increasing Cu:C ratio to a low of approximately 60% of the controls on both days (Fig. 3.6). On day 16 the P-limited cultures showed a decrease in cell numbers with increasing Cu:C ratio (Fig. 3.6). This trend was not clear on day 20; however, the trend may have been obscured by the decrease in Cu:C ratio on day 20 at pCu 10.45 (Fig. 3.4).

The P-limited cultures showed a trend of decreasing biomass (C mL^{-1}) with decreasing pCu to a low of approximately 70% and 62% of the controls on days 16 and 20 respectively (Fig. 3.7), although the trend was not statistically significant (Table 3.1). Biomass increased significantly at intermediate pCu levels in N-limited cultures on day 16, then declined to a biomass similar to the controls at pCu 10.3. A similar but non-significant trend was seen on day 20 (Fig. 3.7, Table 3.1).

Figure 3.5: Relative cell numbers of N- and P-limited *Chlorella* sp. as a function of Cu^{2+} activity. Vertical bars: ±1 standard error.

N_{16} and N_{20} represent N-limited cultures at days 16 and 20 respectively.
P_{16} and P_{20} represent P-limited cultures at days 16 and 20 respectively.

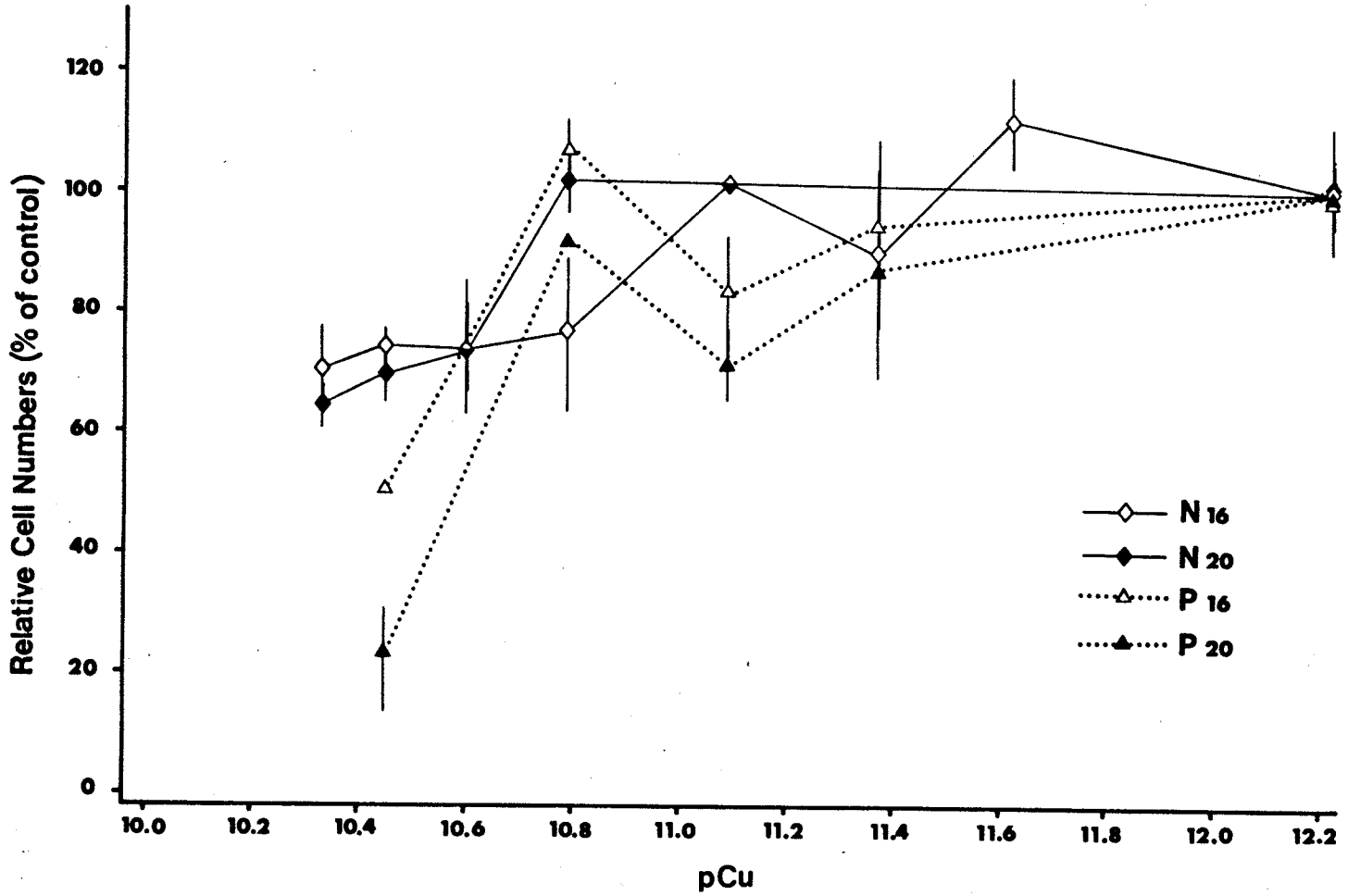

Relative Cell Numbers (% of control)
120
100
80
60
40
20
0
10.0
10.2
10.4
10.6
10.8
11.0
11.2
11.4
11.6
11.8
12.0
12.2
pCu
N 16
N 20
P 16
P 20

Figure 3.6: Relative cell numbers of N- and P-limited *Chlorella* sp. as a function of Cu concentration. Lines connect a moving average of 3 points.

N_{16} and N_{20} represent N-limited cultures at days 16 and 20 respectively.
P_{16} and P_{20} represent P-limited cultures at days 16 and 20 respectively.

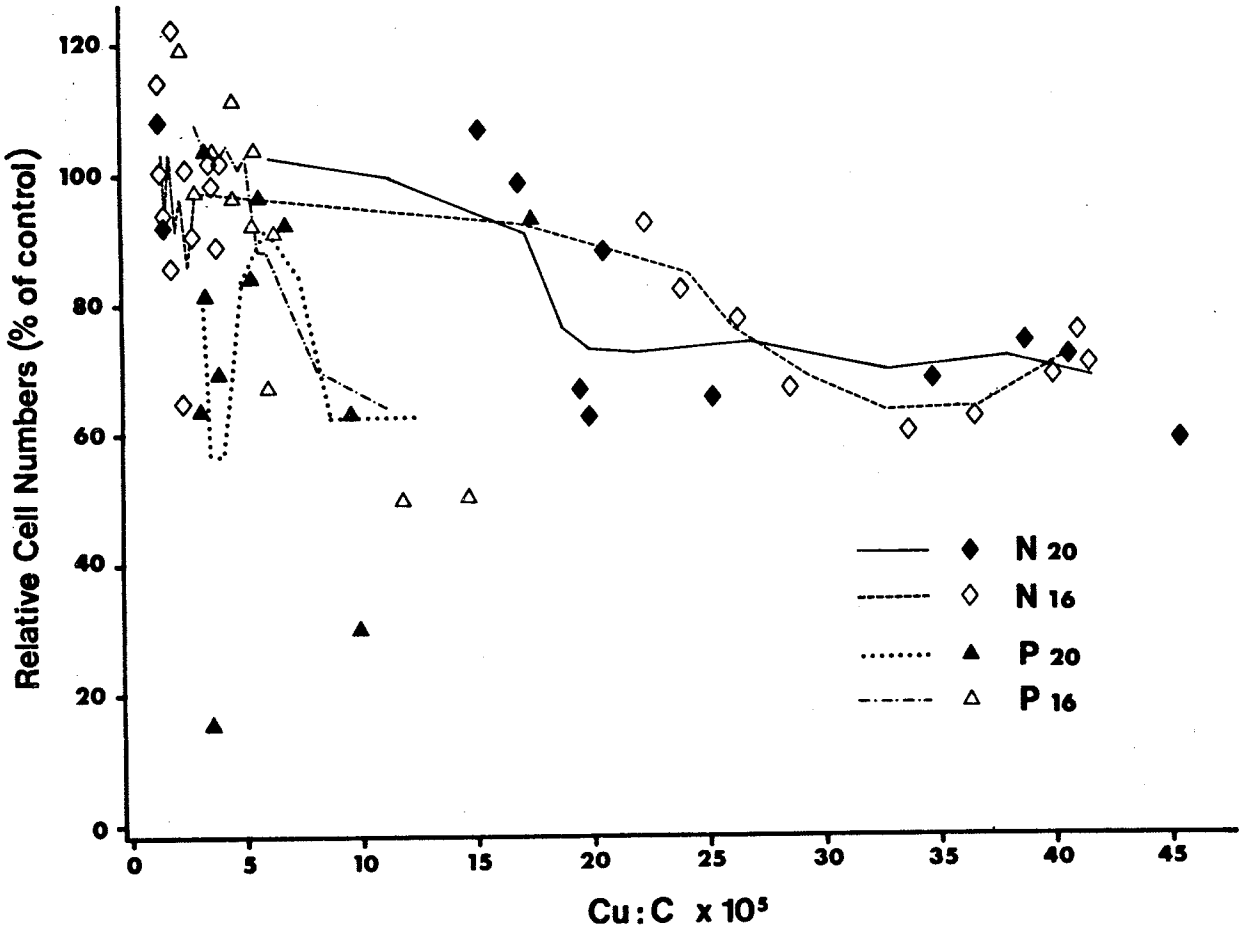
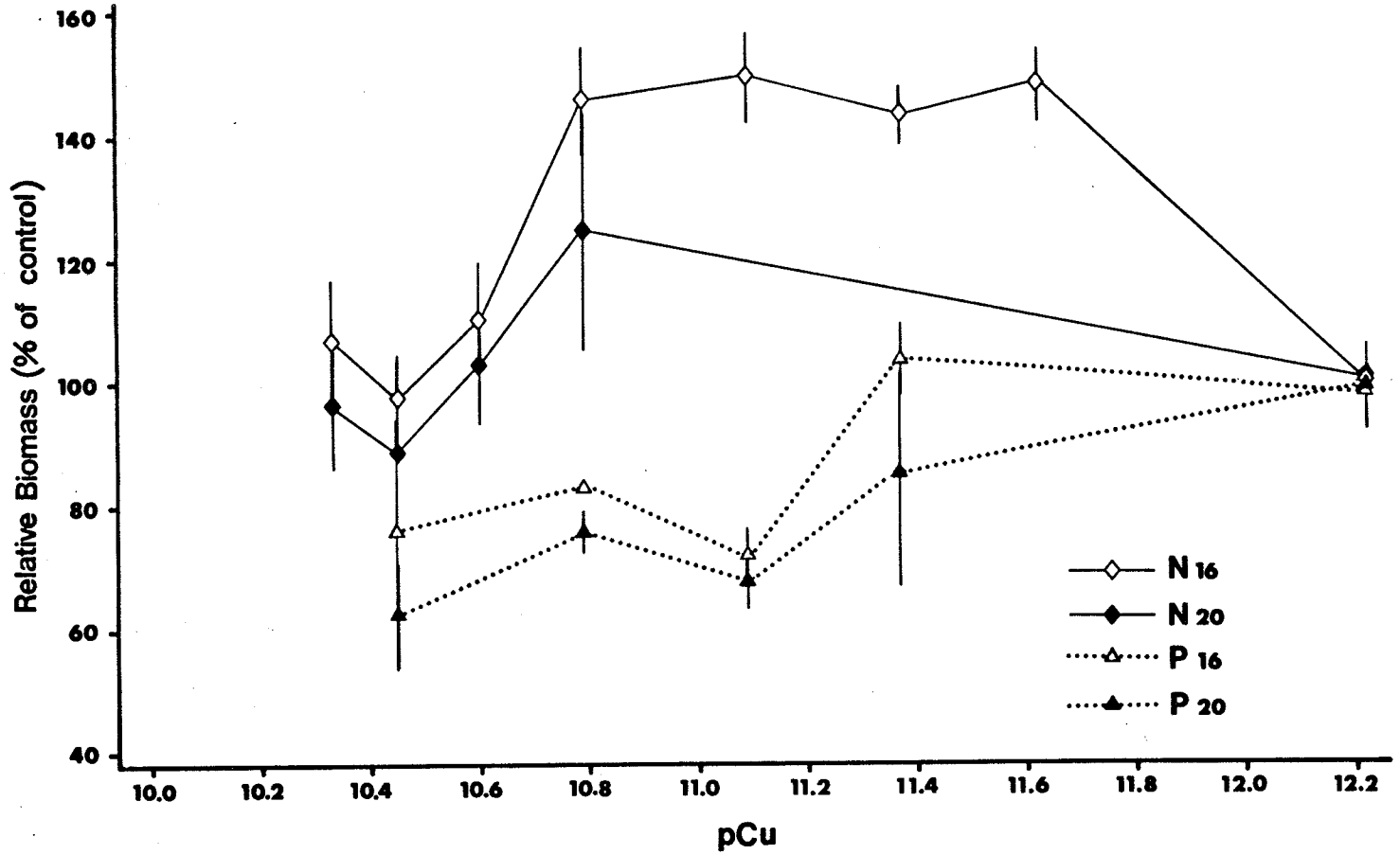

Relative Cell Numbers (% of control)
120
100
80
60
40
20
0
0
5
10
15
20
25
30
35
40
45
Cu : C x 10⁵
N 20
N 16
P 20
P 16

Figure 3.7: Relative biomass of N- and P-limited Chlorella sp. as a function of Cu^{2+} activity. Vertical bars: ±1 standard error.

N_{16} and N_{20} represent N-limited cultures at days 16 and 20 respectively.
P_{16} and P_{20} represent P-limited cultures at days 16 and 20 respectively.


Relative Biomass (% of control)
160
140
120
100
80
60
40
10.0
10.2
10.4
10.6
10.8
11.0
11.2
11.4
11.6
11.8
12.0
12.2
pCu
N 16
N 20
P 16
P 20

3.3.4 Cellular Composition

Cellular C increased significantly with decreasing pCu to a high of 285% of the control on day 20 in P-limited cultures. A similar but non-significant trend was observed on day 16 (Fig. 3.8, Table 3.1). In the N-limited cultures on day 16 cellular C increased significantly to a high of 180% of the controls at pCu 10.8. On day 20 cellular C increased significantly to a high of 150% of the controls at pCu 10.3 (Fig. 3.8, Table 3.1).

In P-limited cultures there was no significant change in the C:N ratio (by weight) with decreasing pCu from the control value of 9.7 (Fig. 3.9, Table 3.1). Under N-limitation the C:N ratio increased significantly from the controls of 19 to 25 at pCu 10.45 on day 16. However on day 20 the C:N ratio in N-limited cultures was not significantly different from the controls (Fig. 3.9, Table 3.1).

In both the N- and P-limited cultures the C:P ratio (by weight) was very stable with no significant difference from the control value of 270 in P-limited cultures and 60 in N-limited cultures with decreasing pCu (Fig. 3.10, Table 3.1).

Alkaline phosphatase has been considered on a per C basis to remove any effect of cell size. The decline in alkaline phosphatase activity from the control value of 15 on day 16 and 11 on day 20, was not significant (Fig. 3.11, Table 3.1).

Figure 3.8: Relative cellular C content of N- and P-limited *Chlorella* sp. as a function of Cu^{2+} activity. Vertical bars: ±1 standard error.

N_{16} and N_{20} represent N-limited cultures at days 16 and 20 respectively.
P_{16} and P_{20} represent P-limited cultures at days 16 and 20 respectively.

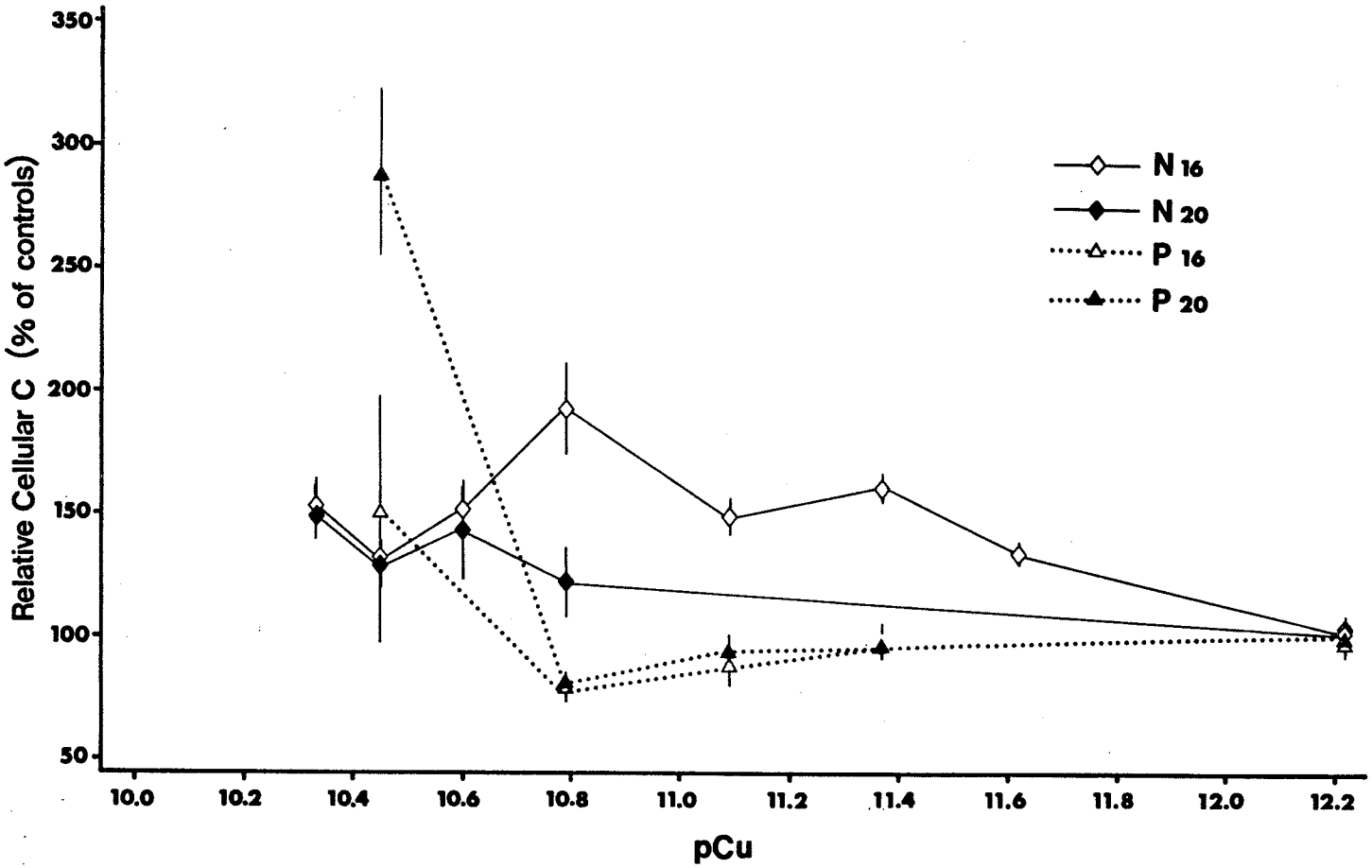

Relative Cellular C (% of controls)
350
300
250
200
150
100
50
10.0
10.2
10.4
10.6
10.8
11.0
11.2
11.4
11.6
11.8
12.0
12.2
pCu
N 16
N 20
P 16
P 20

Figure 3.9: C:N ratio of N- and P-limited Chlorella sp. as a function of Cu^{2+} activity. Vertical bars: ±1 standard error.

N_{16} and N_{20} represent N-limited cultures at days 16 and 20 respectively.
P_{16} and P_{20} represent P-limited cultures at days 16 and 20 respectively.

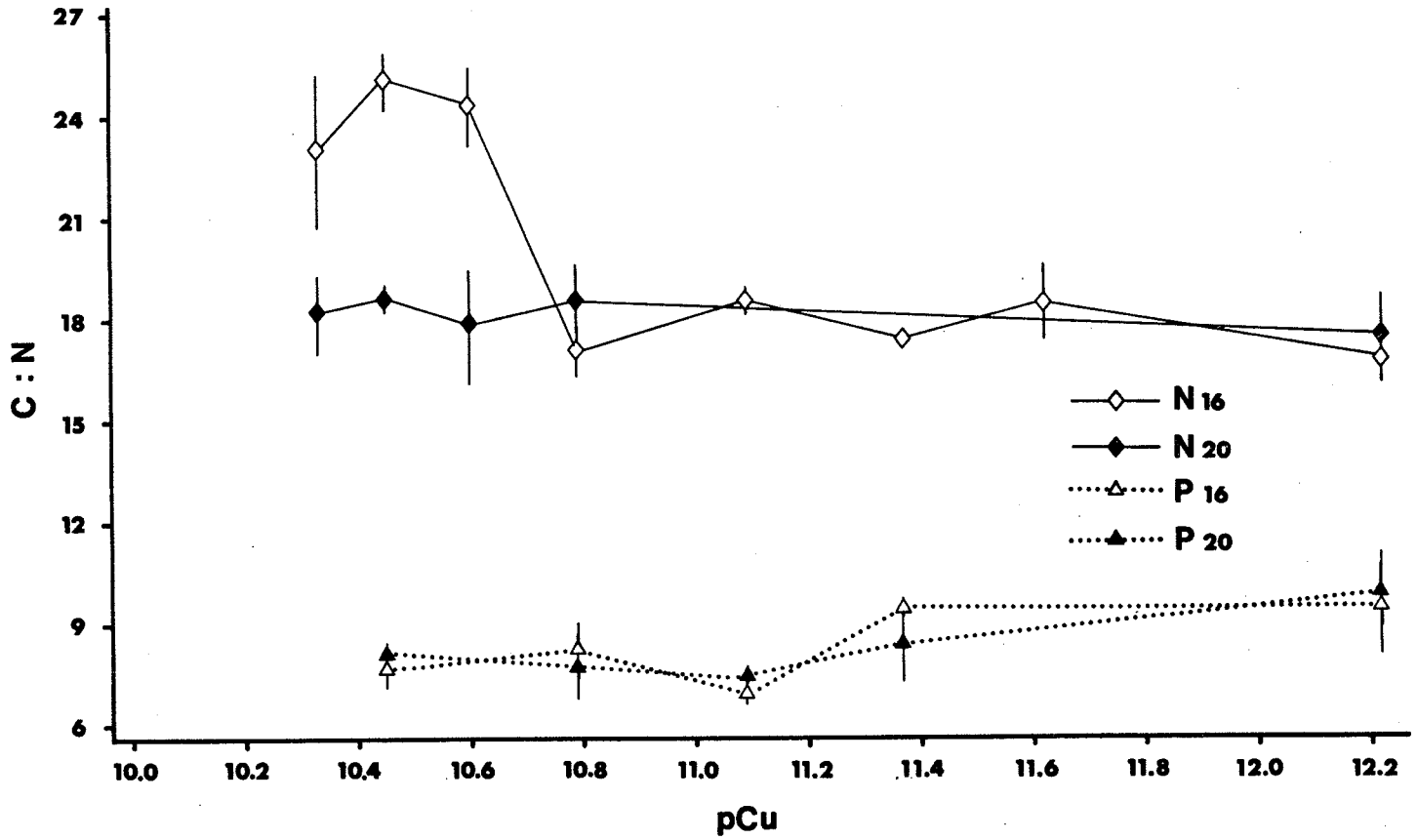

27
24
21
18
15
12
9
6
C : N
10.0
10.2
10.4
10.6
10.8
11.0
11.2
11.4
11.6
11.8
12.0
12.2
pCu
N 16
N 20
P 16
P 20

Figure 3.10: C:P ratio of N- and P-limited Chlorella sp. as a function of Cu^{2+} activity. Vertical bars: ±1 standard error.

N_{16} and N_{20} represent N-limited cultures at days 16 and 20 respectively.
P_{16} and P_{20} represent P-limited cultures at days 16 and 20 respectively.

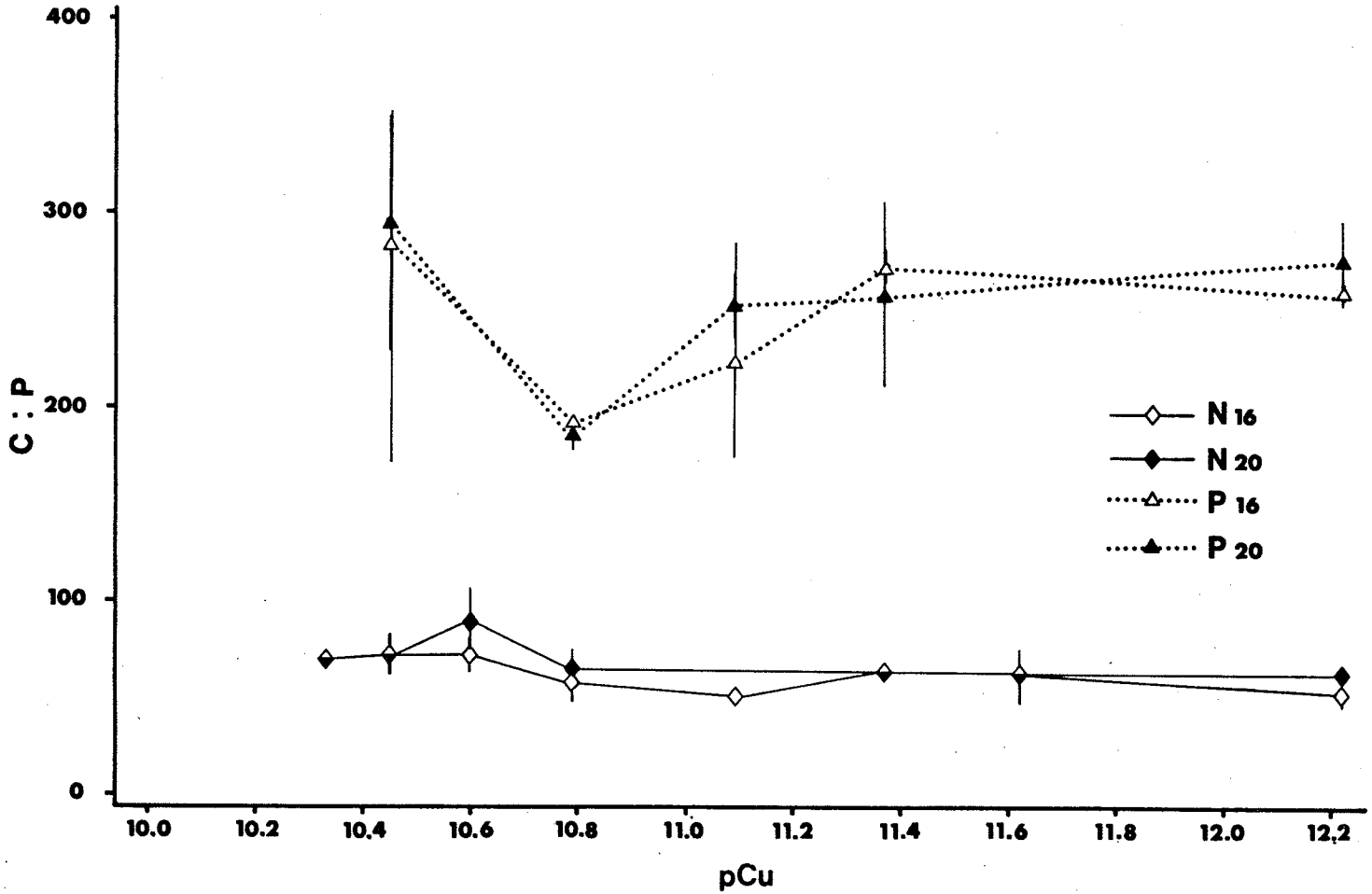
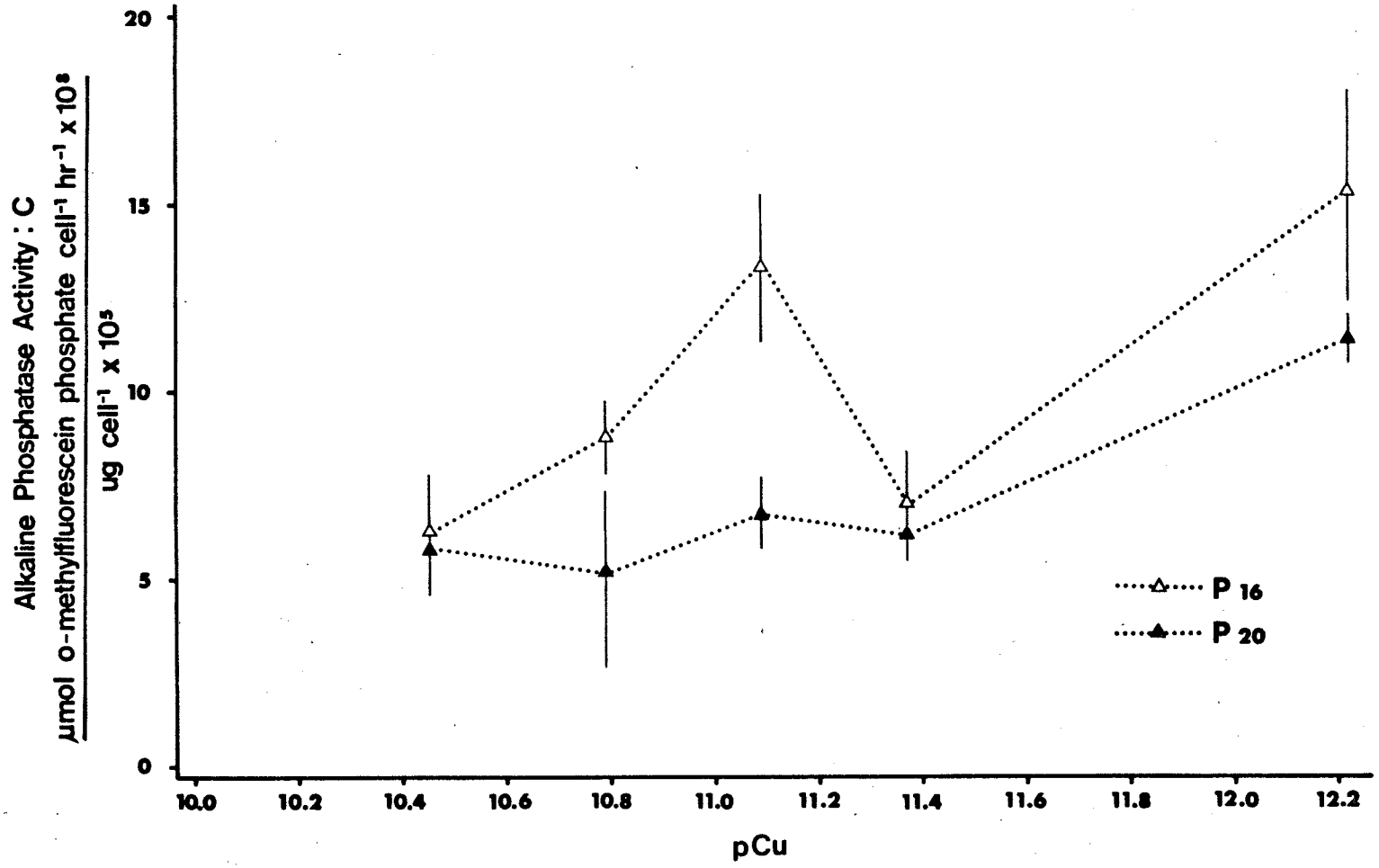

400
300
200
100
0
C : P
10.0
10.2
10.4
10.6
10.8
11.0
11.2
11.4
11.6
11.8
12.0
12.2
pCu
N 16
N 20
P 16
P 20

Figure 3.11: Ratio of alkaline phosphatase activity (umol O-methyl-fluorescein phosphate $cell^{-1}$ h^{-1} x 10^8) to cellular C (ug C $cell^{-1}$ x 10^{-5}) as a function of Cu^{2+} activity

P_{16} and P_{20} represent P-limited cultures at days 16 and 20 respectively.


Alkaline Phosphatase Activity : C
µmol o-methylfluorescein phosphate cell⁻¹ hr⁻¹ x 10⁸
ug cell⁻¹ x 10⁵
20
15
10
5
0
10.0
10.2
10.4
10.6
10.8
11.0
11.2
11.4
11.6
11.8
12.0
12.2
pCu
P 16
P 20

3.4 DISCUSSION

3.4.1 Cell Composition

The increase observed in cellular Cu content with increasing Cu^{2+} activity (Fig. 3.3) is consistent with the batch culture studies of Chapter 2. Bates et al. (1982) found similar results for Zn uptake in batch culture and short term Zn uptake experiments with Chlamydomonas variabilis.

Cellular Cu levels were higher in N-limited than P-limited cultures at pCu levels less than 10.8. Under both nutrient limitations however, there was no increase in cellular Cu over time with levels being similar on days 16-20 (Fig. 3.3). These results are similar to that of Conway (1978) for cellular As in Asterionella formosa but contrary to cellular Cd which increased with time of exposure in continuous culture in the same study.

The cellular Cu concentration, represented by the cellular Cu:C ratio, did not follow a consistently increasing trend with higher Cu^{2+} activity as observed in the batch culture experiments of Chapter 2. Since cellular Cu increased consistently with increasing Cu^{2+} activity (Fig. 3.3) the inconsistency of Cu:C may be due to a change in Cu uptake with increasing cell size. Cellular C increased with higher Cu^{2+} activities in both N-limited and P-limited cultures (Fig. 3.8). This is consistent with batch culture

studies where increased cell size has been commonly reported at higher metal activities (Stokes et al. 1973, Foster 1977, Gupta and Arora 1978). Foster (1977) and Fisher et al. (1981) proposed that an increase in cell size is due to an uncoupling of cell growth and cell division as a result of Cu toxicity.

Rice et al. (1973) have suggested that changes in cell metabolism as a result of metal toxicity may cause changes in gross cell composition. Changes in cell composition have been shown to result from other stresses (Healey and Hendzel 1975, Davis 1976, Goldman 1977a and b, Rhee 1978). The observed increase in cellular C with decreasing pCu was accompanied by a proportionate increase in cellular P which is reflected in the stability of C:P ratios (Fig. 3.10). Cellular N also increased proportionately with cellular C (Fig. 3.9) except in N-limited cultures on day 16 when the C:N ratio increased at the higher Cu^{2+} activities. This effect was not evident on day 20, perhaps reflecting the dynamic nature of the cultures. The lack of change in gross cell composition is contrary to Li (1979) who showed an increase in cellular N as a result of increased cell protein in Cd stressed Thalassiosira weissflogii in continuous culture. The data were however, consistent with observations of natural phytoplankton populations in the CEPEX experiments where no significant differences were found in the C:N or C:P ratio between the control and Cu treated enclosures (Thomas et al. 1977).

Alkaline phosphatase activity can be used as an indicator of the degree to which cells are P deficient, with activity increasing with increasing deficiency (Healey and Hendzel 1975). Alkaline phosphatase activity showed no change in the Cu^{2+} activity range in which Cu toxicity occurred (Fig. 3.11). This suggests that Cu toxicity was not sufficient to overcome P-limitation. This finding is also supported by the stability of the C:P ratio.

3.4.2 Comparison of Toxicity in N and P-Limited Cultures

The decrease in cell numbers (Fig. 3.5) indicates that Cu toxicity occurred at pCu 10.45 in P-limited cultures. The increasing rate of decline in cell numbers between days 16 and 20 at pCu 10.45 indicates increasing toxicity with time despite the the absence of a corresponding increase in cellular Cu concentration. This suggests that toxicity may not only be a function of cellular Cu but also of the time of exposure to Cu.

Under N-limitation cell numbers decreased at pCu levels below 10.55 but unlike P-limited cultures (Fig. 3.5) little difference was observed between days 16 and 20. Although cell numbers decreased, no significant decrease in biomass was observed in either the N- or P-limited cultures. The difference in the effect of Cu toxicity on cell numbers and biomass in both N- and P-limited cultures reflects an increase in cell size with increased Cu toxicity and possibly

an uncoupling of cell growth and cell division (Foster 1977, Fisher et al. 1981). The decrease in cell numbers in P-limited cultures was much greater than the decrease in cell numbers in N-limited cultures at the same Cu^{2+} activity (Fig. 3.5).

In Chapter 2 the increasing toxicity of Cu in the P-limited cultures was proposed to be a result of increased sensitivity to cellular Cu concentration under P-limitation. The results of these chemostat culture experiments support this proposal with cell numbers being lower in P-limited cultures than N-limited cultures at similar Cu:C ratios (Fig. 3.6). The difference in sensity to cellular Cu concentration between N and P limited chemostat cultures may possibly be explained as it was in batch culture experiments. In P-limited cultures cells contain few polyphosphate bodies whereas in N-limited cultures much higher numbers of polyphosphate bodies may be present (Rhee 1973). It has been proposed that metals may be detoxified within polyphosphate bodies in the cell (Sicko-Goad and Stoermer 1979, Jensen et al. 1982, Pettersson 1985) hence N-limited cells would have a greater ability to detoxify the Cu.

3.4.3 Comparison of Cu^{2+} Activities Which Are Toxic In Batch and Chemostat Cultures

The Cu^{2+} activities that were found to be toxic in chemostat cultures were approximately 4 orders of magnitude

less than those observed in batch culture. The toxic effect of Cu in batch culture to Chlorella sp. occurred in the range pCu 5.0-6.0 (Chapter 2) in comparison to approximately pCu 10.3-10.8 in chemostat cultures. The cellular Cu concentrations found to be toxic also differed between batch and chemostat culture. Cu toxicity occurred at a Cu:C ratio of approximately $1\text{-}3\text{x}10^{2}$ in batch culture (Chapter 2) compared to $2\text{-}15\text{x}10^{5}$ in chemostat culture (Fig. 3.6), suggesting that the increased sensitivity in the chemostat cultures was not due to accumulation of cellular Cu as a result of long term exposure to Cu. These results are consistent with those of Saifullah (1978) who showed Cu toxicity to be greater to marine dinoflagellates in semi-continuous culture than in batch culture, but were contrary to those of Kayser (1976) where mercuric acetate had similar toxicity in both batch and continuous culture. In the latter study the continuous culture was a turbidostat, and hence cell growth was nutrient sufficient at all times.

In batch culture cells were exposed to a single pulse of Cu while in a nutrient sufficient state. In chemostat culture, copper was continuously added to cells that were stressed by nutrient limitation and so were possibly less able to detoxify the Cu. It appears that nutrient limitation and Cu toxicity may interact synergistically resulting in higher Cu toxicity in nutrient limited cultures. There is a need for further investigation of Cu toxicity under

varying degrees of nutrient limitation to clarify this relationship.

The observation that Cu is substantially more toxic in chemostat culture brings into question the applicability to the natural environment of Cu^{2+} activities reported to be toxic to algae in batch culture. Furthermore, the Chlorella sp. used in these experiments is highly tolerant to Cu toxicity (Chapter 2). If less tolerant species were to exhibit a similar decrease in tolerance when grown in nutrient limited chemostat cultures as that demonstrated here, it may be expected that the Cu^{2+} activity tolerated by algae in P-limited natural water bodies may be extremely low.

Chapter IV

CONCLUSIONS

In batch culture experiments under both N and P-limitation, sublethal effects of Cu toxicity included a decrease in growth rate and an increase in cellular C content. P-limited cultures in addition showed a decrease in final yield and an increase in cellular P content.

Both Chlorella sp. and Chlamydomonas sp. were more sensitive to Cu toxicity under P-limitation compared to N-limitation. This decreased tolerance to Cu toxicity appeared to be a result of increased sensitivity to cellular Cu concentration in the P-limited cultures.

In chemostat cultures of Chlorella sp. sublethal Cu toxicity resulted in an increase in cellular Cu content and a decrease in cell numbers. This decrease was greater under P-limitation than under N-limitation. The increased Cu toxicity in the P-limited chemostat cultures was again shown to be a result of an increased sensitivity to cellular Cu concentration. These results were consistent with those of the batch culture.

Chemostat cultures of Chlorella sp. were approximately 4 orders of magnitude more sensitive to Cu than were batch

cultures. Similarly, sensitivity to cellular Cu concentration was approximately 3 orders of magnitude greater in the chemostat cultures.

The possible synergistic interaction of Cu toxicity with nutrient limitation has implications for the ecology of natural water bodies. For example, the interaction of nutrient limitation and Cu toxicity may influence the outcome of species competition. Rhee and Gotham (1980) showed that the optimum N:P ratios (the ratio at which a species changes from N-limitation to P-limitation or vice versa) are highly variable between species (ranging from 7-30). Niche separation through the limitation of different species by different nutrients has been proposed by Tilman (1982) in his resource based competition theory. If two or more species are limited by different nutrients (e.g., N and P), when Cu is introduced to the system, the eventual species composition may well be determined by the interaction of Cu toxicity and the growth limiting nutrient.

The greater sensitivity to Cu toxicity of chemostat cultures in comparison with batch cultures suggests that Cu toxicity in nature may have been previously under-estimated using batch culture results.

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Appendix A

DEVELOPMENT OF MEDIUM

The basic medium used in this study was the WC medium developed by Guillard and Lorenzen (1972) (Table A.1). There were several problems with the WC medium in relation to metal toxicity studies and hence a need to modify the medium. Several recent studies have indicated that the toxicity of Cu to algae is not directly related to the concentration of total dissolved Cu but to the activity of the free cupric ion Cu^{2+} (Sunda and Guillard 1976, Allen et al. 1980, Canterford and Canterford 1980). It is therefore important to know not only the concentration of total dissolved Cu in the medium but also the chemical speciation of the Cu. The activity of Cu^{2+} can be neither easily nor accurately measured at low levels. It can however be calculated using relatively complex calculations that are generally carried out using computer programs written for this purpose. In order to accurately calculate the speciation of Cu in the medium the program requires equilibrium constants for each chemical interaction in the medium and the concentration of each anion and cation in solution.

For the studies reported in this thesis, the program MACS80 (a metal speciation computer program developed by Dr.

Table A.1: Composition of standard WC medium (Guillard and Lorenzen 1972).

Compound	Concentration in Medium
$CaCl_2.H_2O$	250 µM
$MgSO_4.7H_2O$	150 µM
$NaHCO_3$	150 µM
K_2HPO_4	50 µM
$NaNO_3$	1000 µM
$Na_2SiO_3.9H_2O$	100 µM
Na_2EDTA	11.7 µM
$FeCl_3.6H_2O$	11.7 µM
$CuSO_4.5H_2O$	0.04 µM
$ZnSO_4.7H_2O$	0.08 µM
$CoCl_2.6H_2O$	0.05 µM
$MnCl_2.4H_2O$	0.9 µM
$Na_2MoO_4.2H_2O$	0.03 µM
H_3BO_3	16 µM
Vitamins	
Thiamin.HCl	0.1 mg/l
Biotin	0.5 µg/l
B_{12}	0.5 µg/l
Buffers	
Glycyglcine	3.79 mM
Tris	4.1 mM

pH 7 - 8

R. Wagemann, Freshwater Institute, Winnipeg) was used to calculate speciation of the Cu in the medium. Certain limitations of this program necessitated modifications to the WC medium in order to calculate metal speciation. The nutrient requirements and Cu tolerance of the algal species used in the study also led to modifications of the WC medium.

A.1 IRON CHELATION

Lewin and Chen (1973) have reported that algae require a chelated form of iron for growth. Na_2EDTA is the chelator used in the WC medium (Table A.1). In these studies there were two problems with the use of Na_2EDTA as the iron chelator. Firstly, the equilibrium constants for Na_2EDTA required to calculate Cu speciation were not included in the MACS80 program. As iron chelators play a major role in the speciation of metals in solution it is important that the interaction between the iron chelator and Cu be included in the speciation calculations (Anderson and Morel 1978). The second problem was that Na_2EDTA has been reported to break down under prolonged exposure to light (Healey, pers. comm.) leading to changes in the speciation of Cu in the medium over time. These problems were solved by using the chelator Na_2NTA since the data required were included in the MACS80 program and Na_2NTA is not as prone to photo-breakdown (Healey, pers. comm.).

Na_2EDTA could not be replaced mole for mole with Na_2NTA since the metal binding capacity of Na_2NTA is approximately half that of Na_2EDTA (Wagemann, pers. comm.). Experiments testing for precipitation of iron showed that a concentration of 23.4 uM Na_2NTA would maintain the 11.7 uM Fe required by the WC medium in solution.

Chlorella sp. was used to test for toxic effects of Na_2NTA at 23.4 uM. There was no evidence of toxicity due to the Na_2NTA as estimated from growth rate and final yield.

A.2 PH BUFFERING AND CU CONCENTRATION

The speciation of Cu is very pH dependent with small changes in pH having a significant affect on the activity of Cu^{2+} present in the medium (Table A.2). Due to the importance of the concentration of the Cu^{2+} ion in Cu toxicity studies it was necessary to have a specified and stable pH. WC medium specifies two possible buffers:

1. glycylglycine at 3.79 mM or
2. Tris(hydroxymethyl)aminomethane (Tris) at 4.1 mM,

neither these nor any other pH buffers had equilibrium constants in the MACS80 program. It was therefore important to choose a pH buffer which was known to have little or no binding affinity for metals. Telvin (1978) reported that the pH buffer HEPES (N-2-hydroxyethylpeperazine-N'-2-ethane sulfonic acid) does not bind metals and is not toxic to al-

Table A.2: The effect of pH on the Cu^{2+} concentration in WC medium as calculated by the MACS80 program.

pH	Cu^{2+}
6.00	$1.0836\text{-}10^{-8}$ M
6.10	$8.4248\text{-}10^{-9}$ M
6.20	$6.5383\text{-}10^{-9}$ M
6.30	$5.1685\text{-}10^{-9}$ M
6.40	$4.0741\text{-}10\text{-}^{9}$ M
6.50	$3.2237\text{-}10^{-9}$ M
6.60	$2.5597\text{-}10^{-9}$ M
6.70	$2.0396\text{-}10^{-9}$ M
6.80	$1.6308\text{-}10\text{-}^{9}$ M
6.90	$1.3086\text{-}10^{-9}$ M
7.00	$1.0540\text{-}10\text{-}^{9}$ M

gae. Experiments with Chlorella sp. confirmed that HEPES was not toxic in terms of growth rate or final yield at concentrations up to 9mM. Experiments also showed that a concentration of 5 mM HEPES would maintain a pH of 6.8 with negligible drift.

Initial experiments with Chlamydomonas sp. showed that at pH 6.8 the medium could not maintain sufficient Cu^{2+} in solution to induce significant toxicity, as only approximately 25uM of total dissolved Cu could be maintained in the medium without precipitation (as calculated by MACS80).

Decreasing the pH of the medium allowed a greater concentration of total dissolved Cu to be maintained in the medium, but necessitated changing pH buffers. The buffer 2(N-morpholino)ethane sulfonic acid (MES) was reported by Good (1966) to have a low binding affinity for metals and a pH range of 5.5-6.7. Experiments with Chlorella sp and Chlamydomonas sp showed that MES was not toxic up to at least 10 mM in terms of growth rate and final yield (Table A.3). A concentration of 5 mM MES was sufficient to maintain a stable pH of 6.3 with negligible drift.

Table A.3: Data from experiments testing for MES toxicity in *Chlorella* sp. and *Chlamydomonas* sp.

Species	mM MES	Generation Time in hours $\bar{X}$(n=3)± STD Error	Final Yield Cells/Ml $\bar{X}$ (n=3)± STD Error
CHORELLA Sp	5	7.9 ± .05	11 482 935 ± 1 095 113
	7	8.2 ± .03	9 679 577 ± 154 686
	10	8.2 ± .10	10 963 436 ± 202 354
	12	8.1 ± .06	10 095 915 ± 326 885
	15	8.1 ± .27	9 221 544 ± 1 599 133
CHLAMYDOMONAS Sp	5	8.1 ± .14	542 995 ± 6 234
	7	8.3 ± .13	525 334 ± 6 040
	10	7.9 ± .36	520 457 ± 25 168

A.3 SILICON CONCENTRATION

Initial experiments with the diatom Synedra sp. showed a very restricted growth in WC medium. Silicon in the WC medium has been reported to limit final yield of other diatom species severely (Goldsborough, pers. comm.). The silicon concentration was, therefore, increased to 819 uM, although no data on Synedra sp. are presented in this thesis.

A.4 PHOSPHORUS AND NITROGEN CONCENTRATIONS

In order to study the interaction between P or N limitation and Cu toxicity the concentration of N or P which limited final yield was determined. Batch cultures were grown at different concentrations of N or P, and concentrations which limited final yield and that had little or no effect on growth rate were chosen for both Chlorella sp. and Chlamydomonas sp. (Tables A.4 and A.5 respectively).

Table A.4: Data from experiments used to establish N concentrations limiting final yield but not growth rate of *Chlorella* sp. and *Chlamydomonas* sp.

Species	uM N	Generation Time in hours $\bar{X}$ (n=3) ± STD Error	Final Yield Cells/Ml $\bar{x}$ (n=3) ± Error
CHORELLA Sp.	*50	11.0 ± .62	5 849 495 ± 704 318
	75	10.1 ± .14	7 235 412 ± 273 636
	100	9.9 ± .11	7 545 371 ± 259 357
	500	9.8 ± .08	8 290 850 ±1 562 239
CHLAMYDOMONAS Sp.	*100	11.1 ± .44	172 704 ± 9 470
	200	10.3 ± .50	283 853 ± 14 919
	300	10.5 ± .39	335 538 ± 11 856
	400	10.4 ± .42	360 368 ± 3 368
	500	10.3 ± .06	560 524 ± 25 759

* Nitrogen concentration chosen for nitrogen limited cultures.

Table A.5: Data from experiments used to establish P concentrations limiting final yield but not growth rate of *Chlorella* sp. and *Chlamydomonas* sp.

Species	µM PO_4	Generation Time in hours $\bar{X}$ (n=3) ± STD Error	Final Cells Cells/Ml $\bar{X}$ (n=3) ± STD Error
CHORELLA Sp.	2	11.6 ± 1.89	2 679 500 ± 44 561
	*5	8.4 ± .25	6 444 231 ± 337 580
	10	8.2 ± .07	11 878 536 ± 598 808
	15	8.1 ± .20	10 612 060 ± 63 367
	25	8.0 ± .08	11 482 935 ± 1 095 113
	30	7.8 ± .27	11 061 081 ± 775 889
CHLAMYDOMONAS Sp.	2	17.9 ± 1.01	24 603 ± 991
	5	11.8 ± .44	67 447 ± 1 906
	7.5	10.7 ± .61	107 603 ± 15 499
	10	9.3 ± .36	164 106 ± 3 830
	15	9.6 ± .53	232 237 ± 13 446
	*20	8.8 ± .19	381 762 ± 35 851
	25	7.7 ± .63	540 979 ± 14 982
	50	8.1 ± .14	542 995 ± 3 234

* Denotes concentration of PO_4 used in Phosphorous limited experiments.

Appendix B

THE MEASUREMENT AND CALCULATION OF COPPER SPECIATION

Recent studies have indicated that the toxicity of Cu to aquatic organisms is related to the activity of the free metal ion Cu^{2+} in the medium (Sunda and Guillard 1976, Sunda and Lewis 1978, Morel et al. 1978, Allen et al. 1980, Canterford and Canterford 1980), and not necessarily to the total dissolved Cu concentration. It is therefore important to know the activity of the Cu^{2+} ion in solution. The Cu^{2+} activity in solution can be measured using a selective ion electrode or it can be calculated through a computerized speciation program. There are restrictions with both methods which will be outlined in the following. As well, the Cu^{2+} activities and concentrations of the medium used in the batch culture experiments (Chapter 2) are determined with both methods and the results compared.

B.1 DIRECT MEASUREMENT OF CU^{2+} ACTIVITY

Solid state cupric ion specific electrodes can be used to measure the activity of Cu^{2+} ions in solution with a number of associated advantages. Electrodes can be used with coloured or turbid samples where colorimetric methods cannot, and measurements can be recorded on a continuous basis al-

lowing monitoring of rapidly changing activity levels. Additionally, in natural water bodies it may be difficult to calculate the Cu^{2+} activity with chemical models due to the presence of unknown organic ligands which are capable of binding the Cu^{2+} ions and hence reducing Cu^{2+} activity (Moody and Thomas 1978).

There are however, several possible sources of error in the case of Cu^{2+} selective electrodes. The electrode has generally been assumed to respond only to Cu^{2+} activity, whereas Wagemann (1980), has shown that this assumption can cause a substantial error in measurements conducted in alkaline solutions. Above pH 7.0 the electrode is also sensitive to other inorganic Cu complexes such as $CuOH^{+}$, $Cu(OH)_2^{2+}$, $CuHCO_3^{+}$. The electrode's response to these can result in a major error (10 x at pH 8.3 to 40 x at pH 9.0), if the change in electrode sensitivity is not considered (Wagemann 1980).

A number of ions have also been reported to interfere with Cu^{2+} activity measurements. Hg^{+} and Ag^{+} can "poison" the electrode sensing element, thus eliminating the use of the electrode in solutions where either Hg or Ag are present (Moody and Thomas 1978). Ferric ions can "poison" the membrane surface but only if the ferric ion concentration is greater than 1/10 of the Cu^{2+} concentration (Moody and Thomas 1978). Cl^{-} and Br^{-} have been shown to cause electrode malfunction but only at elevated concentrations:

$(Cu^{2+})(Cl^{-2}) > 1.6x10^{-6}M$, $(Cu^{2+})(Br^{-2}) > 1.3x10^{-12}M$)(Orion 1973).

Errors may also be introduced if the standards and the samples to be measured do not have the same solution matrix, which is frequently the case. This source of error can be reduced by using standards that are of the same ionic strength as the solutions to be measured (Wagemann, pers. comm.).

Electrode drift can also cause errors. The major cause of this drift is changing temperature of the sample. This source of error can be reduced if care is taken to avoid changes in the temperature of the samples.

The reference electrode is a frequent source of error due to problems created at the liquid junction of the electrode and the sample. The membrane is prone to blockage by precipitation of inorganic complexes at the surface or the deposition of organic matter such as proteins on the membrane. Care must be taken to ensure the membrane is clear of any blockage.

B.2 CALCULATING CU^{2+} ACTIVITY

B.2.1 Theoretical Problems

To calculate the free metal ion activity in a solution an aqueous chemical model must be used (Nordstrom et al. 1978). These models calculate the chemical interactions be-

tween the components of the solution and predict the concentration or activity of each chemical form for the solution at equilibrium. These models are based on "ion association theories" and use either an Equilibrium Constant or Gibbs Free Energy approach.

The Equilibrium Constant approach is more widely used due to a larger and more reliable data base (Nordstrom et al. 1978) and will be discussed here. In this approach, the most stable arrangement of ions in solution is predicted using chemical equilibrium equations with all possible ionic interactions in the solution being modelled. These equilibrium equations are of the following form:

$$K = \frac{[A]\ [B]}{[C]}$$

where K = chemical equilibrium constant

[ ] = concentration of the ligand

A,B,C = ions or complexes being considered

These equations are used in conjunction with mass balance equations for each ion;

Total X = X complexes + free ion

where; X = element under consideration

to define the equilibrium state of the solution. The equations for all chemical forms in solution make up a set of non linear equations which can be solved simultaneously to calculate the concentration of each chemical species in solution.

There are several problems in the chemical theory of these models which will be discussed below.

B.2.1.1 Equilibrium equations

The equilibrium equation (see above) describes the equilibrium of an ideal solution at zero ionic strength. In a real solution, zero ionic strength is never achieved so this equation must be modified to account for non-ideal behaviour of aqueous electrolytes by the inclusion of activity coefficients;

$$K = \frac{\gamma a[A]\ \gamma b[B]}{\gamma c\ [C]}$$

where;

K = chemical equilibrium constant

a,b,c = activity coefficient of the ligand

[A] = concentration of the ligand

A,B,C = ions or complexes being considered

This can be expressed as

$$K = \frac{[A]\ [B]}{[C]}$$

where;

$$\alpha = \frac{\gamma a\ \gamma b}{\gamma c}$$

For example,

$$\alpha 1 \frac{[HCO_3^-]\ [H^+]}{[H_2CO_3]} = K_1$$

$$\alpha 6 \frac{[Ca^{2+}]\ [CO_3^{2-}]}{[CaCO_3]_5} = K_6$$

$$\alpha 75 \frac{[Ca^{2+}]^5\ [PO_4^{3-}]\ [OH^-]}{Ca_5\ (PO_4)_3(OH)\,solid} = K_{75}$$

The activity coefficient is a measure of the "effective concentration" of an ion or complex in solution and determines the rate and extent of any chemical reactions. The activity coefficient for each ion or ion complex is determined by the temperature, pressure and ionic strength of the solution. The activity coefficient must be calculated for each chemical species in the solution.

B.2.1.2 Activity coefficients

There are several different equations (Garrels and Crist 1965) (Table B.1) that may be used to calculate these coefficients. The existence of several semi-empirical equations indicates uncertainty in the calculation of single ion activity coefficients particularly at ionic strength greater than 0.1M (Garrels and Crist 1965). Many models incorporate several equations, with equation selection depending on the ionic strength of the solution; for example, Geochem (Mattigod and Sposito 1978). The Extended Debye-Huckel equation with an added linear term is widely used when ionic strength

Table B.1: Equations used to calculate activity coefficients of an ion (from Garrels and Crist 1965).

Equation	Valid Range of Ionic Strengths
Debye-Huckel equation $\log f = -Az^2\sqrt{I}$	$10^{-2.3}$ M
Extended Debye-Huckel equation $\log f = \frac{-Az^2\sqrt{I}}{1+B\,a\sqrt{I}}$	10^{-1} M
Gunteberg equation $\log f = \frac{-Az^2\sqrt{I}}{1+\sqrt{I}}$	10^{-1} M
Davies equation $\log f = \frac{-Az^2\sqrt{I}}{1+\sqrt{I}} + 0.2\,Az^2I$	0.5 M

where; log f = activity of the ion

A & B = constants characteristic of the solvent at specified temperature and pressure

z = change of i^{th} ion in solution

a = species dependent "effective diameter" of an ion in solution

I = ionic strength, $I = 1/2\ Mz^2$ where M = molarity

is greater than 0.1 M.; for example, Equil (I and Nancollas 1972) and WateQ2 (Ball et al. 1978).

B.2.1.3 Electrically neutral complexes

A serious problem in the chemical modelling of metal speciation occurs when activity coefficients of electrically neutral complexes are considered, as there are few reliable data on the activity of these complexes. The activity coefficients of electrically neutral complexes are generally set at 1.0. In the case of many organic complexes this estimate may not be particularly accurate (Wagemann, pers. comm.). Neutral complexes often dominate aqueous solutions which can create an error in the calculation of metal speciation (Nordstrom et al. 1978).

B.2.2 Calculation Problems

The chemical models discussed so far are solved using computer programs such as Equil (I and Nancollas 1972), WateQ2 (Ball et al. 1978), and Geochem (Mattigod and Sposito 1978). The program MACS80 (developed by Dr. R. Wagemann, F.W.I. Winnipeg) was used in this study to calculate the Cu^{2+} activity in the experimental medium. The program design and the databases used also introduce errors into the calculation of metal speciation.

The largest single source of variability in the calculation of metal speciation is the variation in the thermodynamic data used. Equilibrium constants (K), on which calculations are based can vary by up to 3 orders of magnitude (Nordstrom et al. 1978). The effects of temperature on speciation should also be taken into account. Most equilibrium constants are reported for standard conditions of 25°C but frequently the medium is not at 25°C. A temperature correction factor can be used, if available, to adjust the K values, but not all programs carry out this correction (Nordstrom et al. 1978). In the MACS80 program temperature is adjusted for if data are available. Activity coefficients are also temperature dependent since the constants A and B are temperature dependent (Table B.2). Adjustments for temperature changes should also be made at this level.

The total number of aqueous species considered in a program is important and varies widely. Geochem (Mattigod and Sposito 1978) considers 2000 species, whereas WateQ2 (Ball et al. 1978) considers 220 species, MACS80 considers 300 species. The degree of error caused by the omission of a species is dependent on the chemical complexing ability of that species and its concentration in solution. If an ion complexes a metal very weakly its omission is unlikely to affect speciation of the metal unduly. If a strong metal complexing ion is omitted errors can be large, especially as its concentration increases.

To summarize: Calculation of metal speciation involves a chemical model based on "ion association theory" which uses equilibrium and mass balance equations. The solution of these simultaneous equations yields the speciation of the metals in solution. To account for non-ideal behaviour of aqueous electrolytes, activity coefficients must be included in the equilibrium equations. There are several sources of potential variation in programs written to calculate metal speciation. The major source of error is the thermodynamic data base (K values) used in the equilibrium equations. Other sources involve temperature correction and the number of chemical species considered by the model. These sources of variation between programs generally do not have a large effect on calculations involving major constituents of a solution. Calculation of metal speciation at trace levels of a metal however, are sensitive to variations in equilibrium constants, pH and temperature.

B.3 MEASUREMENT OF Cu^{2+} ACTIVITY IN MEDIUM USED IN BATCH CULTURE EXPERIMENTS

The Cu^{2+} activities in the P-limited medium used in the experiments of Chapter 2 were measured using an Orion Cupric Ion Activity Electrode, Model 94-92 in combination with an Orion Single Junction Reference Electrode, Model 90-01 filled with Orion 90-00-01 internal filling solution.

The electrode was standardized using a range of 1.0×10^{-3} to 5.0×10^{-9} M Cu solutions made from a stock solution of 1.0^{-1} M Cu diluted with 1.0^{-2} M KNO_3 to bring the standards to the same ionic strength as the medium. The stock solution was produced by dissolving Cu metal in 30 mL of concentrated Instra-Analyzed HNO_3 and making up to volume with distilled deionised water. The calculation of the activity of Cu^{2+} ion in the standard solutions required the calculation of the activity coefficient of Cu^{2+} in the standard solutions using:

$$A = Cf$$

Where A = activity of Cu^{2+} ion

α = activity coefficient

Cf = concentration of Cu^{2+} ion

The activity coefficient of Cu^{2+} was calculated using the Extended Debye-Huckel Equation:

$$-\log \alpha = \frac{-Az^2 \sqrt{I}}{1 + B\, a_i \sqrt{I}}$$

a_i = species dependent "effective diameter" of an ion in solution.

= 6

A + B = constants characteristic of the solvent at specified temperature and pressure.

A = .511

B = .329

Z = charge of i^{th} ion in solution

= 2

I = ionic strength

$I = .01$

$\alpha = 0.592$

The electrode was standardized four times with fresh standards ranging from pCu 3.23 to 8.53 and the electrode response was found to be very stable (Fig. B.1). Below pCu 6.23 however the response of the electrode was non-linear suggesting it was unreliable below this activity (the maximum sensitivity of the electrode was quoted as pCu 8.0 (Orion 1973)). Three separate batches of medium were made using the procedure as previously outlined (Chapter 2) and the Cu^{2+} activity measured at 11 concentrations of total dissolved Cu ranging from 0.04 to 45 uM (Table B.2).

Figure B.1: Standard curve for the Cupric Ion Selective Electrode. This curve is average of 4 standard curves. Vertical bars: ±1 standard error.

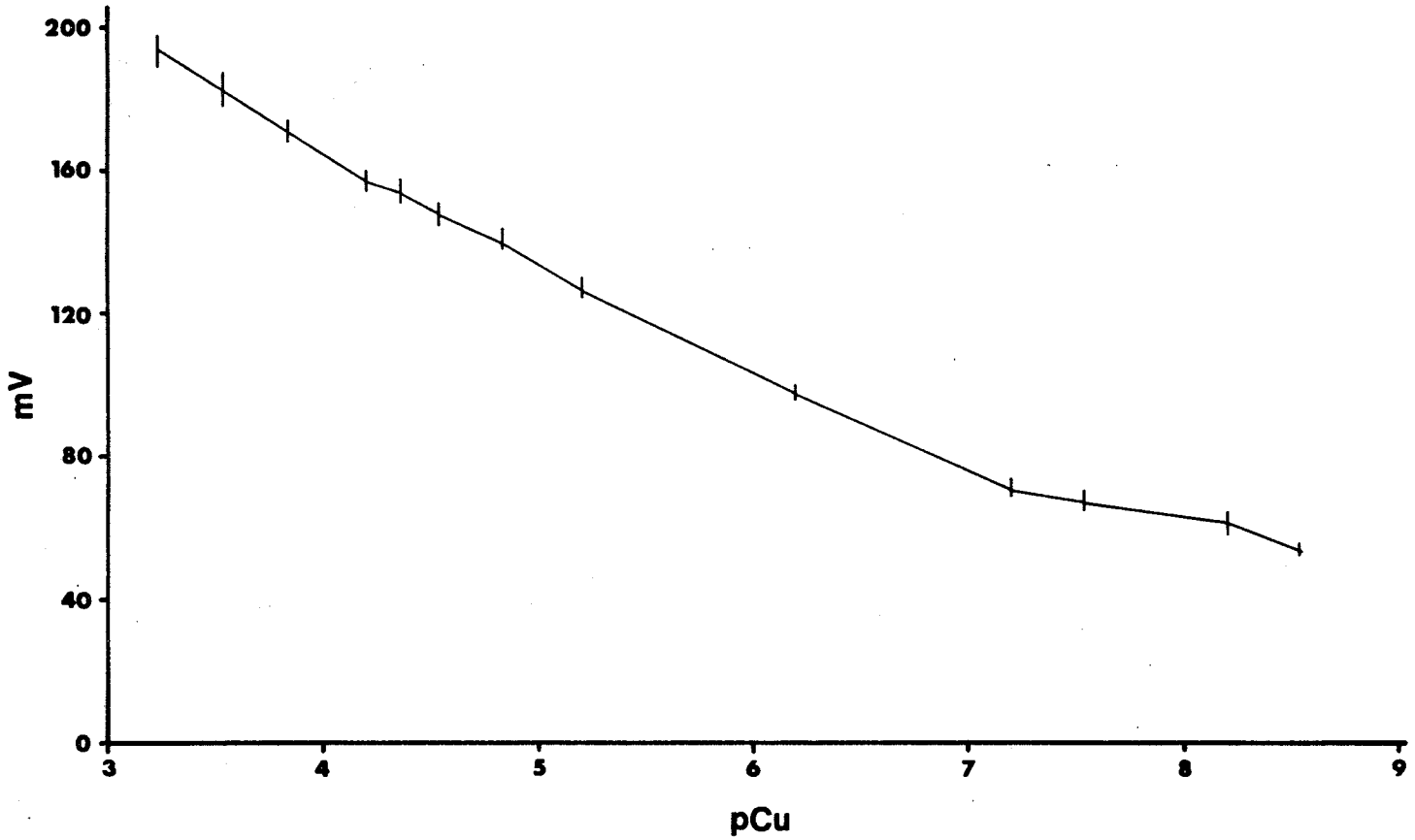

200
160
120
80
40
0
mV
3
4
5
6
7
8
9
pCu

Table B.2: Cu^{2+} activities measured in 3 separate batches of medium. (pCu=-log Cu^{2+} activity. TDCU is total dissolved Cu concentration in the medium.)

TDCU μM	Medium 1 pCu	Medium 2 pCu	Medium 3 pCu	$\bar{x}$	SE
.04	8.7	8.30	8.21	8.40	.153
10	8.61	7.96	8.11	8.23	.200
20	8.30	7.48	7.76	7.85	.245
25	7.69	6.75	6.88	7.11	.30
27.5	7.20	6.30	6.83	6.78	.26
30	6.69	5.81	6.44	6.31	.26
32.5	6.55	5.64	6.23	6.14	.27
35	6.35	5.42	6.09	5.95	.28
40	5.96	5.27	5.85	5.69	.21
42.5	5.78	5.23	5.76	5.59	.18
45	5.75	5.10	5.38	5.41	.19

B.4 CALCULATION OF Cu^{2+} ACTIVITY IN MEDIUM USED IN BATCH CULTURE EXPERIMENTS

The MACS80 program was used to calculate the Cu^{2+} concentrations and activities in the medium. The input data to the MACS80 program for P-limited medium with a total dissolved Cu concentration of 40 uM are shown in Table B.3. The output data from MACS80 give the concentrations of each Cu species in solution including Cu^{2+} (Table B.4). MACS80 also calculates the activity coefficient for each ion in solution including Cu^{2+} using the Extended Debye-Huckel equation allowing calculation of Cu^{2+} activity using:

$$A = [Cu^{2+}]$$

$$= .675 \times 1.92 \times 10^{-5} \text{ at pH } 6.3$$

$$= 1.30 \times 10^{-5}$$

where; $\alpha = .675$ (as calculated by MACS80).

The MACS80 program was used to calculate the Cu^{2+} activity for all the total dissolved Cu concentrations (Table B.5). It should be noted that the Cu^{2+} concentration and Cu^{2+} activity coefficient varied between N- and P-limited medium, but when Cu^{2+} activity was calculated the differences cancelled out to give the same Cu^{2+} activity in both N- and P-limited medium at the same total dissolved Cu concentrations.

Table B.3: Input data used with the MACS80 program for P-limited modified WC medium.

DEPARTMENT OF FISHERIES & OCEANS - WESTERN REGION
CHEMICAL METHODOLOGY - R. WAGEMANN

TESTER

TEMPERATURE ..... 20.000000 DEG C

PCO2 ..... 0.000350 ATM. CONTROLS T.D. IC = F

IONIC STRENGTH ..... 0.010200 MOLES/L

CONDUCTIVITY ..... 0.000000 UMHOS/CM

pH RANGE:
MINIMUM 6.00
MAXIMUM 7.00
INCREMENT 0.10

pH SPECIFIC ..... NONE

OUTPUT OPTIONS:

UNITS:
MOLES/L ..... T
MG/L ..... F

TABLES:
DEFINITIONS ..... T

TESTER

TOTAL DISSOLVED VALUES:

	MOLES/L	MG/L	SOLID NUMBER	SPECIES REPORT
SULFATE	1.500000D-04	4.809860D 00	0	F
EDTA	0.000000D-01	0.000000D-01	0	F
NTA	2.340000D-05	1.685370 00	0	F
INORGANIC CARBON	1.500000D-04	1.801650 00	0	F
CHLORIDE	5.358000D-04	1.903120 01	0	F
ORGANIC CARBON	0.000000D-01	0.000000D-01	0	F
SELENITE	0.000000D-01	0.000000D-01	0	F
SILICATE	8.190000D-04	2.300240 01	0	F
AMMONIA	0.000000D-01	0.000000D-01	0	F
ORTHO-PHOSPHATE	5.000000D-06	1.548700-01	0	F
ACETATE	0.000000D-01	0.000000D-01	0	F
SULFIDE	0.000000D-01	0.000000D-01	0	F
SODIUM	1.845000D-03	4.241850 01	0	F
MAGNESIUM	1.500000D-04	3.646800 00	0	F
CALCIUM	2.500000D-04	1.002000 01	0	F
IRON(III)	1.170000D-05	6.534100-01	0	F
MERCURY(I)	0.000000D-01	0.000000D-01	0	F
MERCURY(II)	0.000000D-01	0.000000D-01	0	F
CADMIUM	0.000000D-01	0.000000D-01	0	F
COPPER	4.500000D-06	2.859300 00	0	T
IRON(II)	0.000000D-01	0.000000D-01	0	F
MANGANESE(II)	9.000000D-07	4.944420-02	0	F
ZINC	8.000000D-08	5.230400-03	0	F
POTASSIUM	4.000000D-06	1.563920-01	0	F
COBALT	5.000000D-08	2.946650-03	0	F

Table B.4: Output data from the MACS80 program generated from the input data of Table B.3.

TESTER

COPPER SYSTEM IN MOLES/L IN TERMS OF SPECIES

pH	Cu(+2)	CuOH(+)	Cu(OH)2(0)	Cu(OH)3(-)	Cu(OH)4(-2)	Cu2(OH)2(+2)	CuSO4(0)	CuEDTA(-2)	CuHEDTA(-)	CuOHEDTA(-3
6.00	2.0584E-05	3.1105E-07	2.9053E-07	2.4446E-14	5.2738E-21	7.8999E-09	2.8740E-07	0.0000E-01	0.0000E-01	0.0000E-01
6.10	2.0263E-05	3.8529E-07	4.5305E-07	4.7892E-14	1.3034E-20	1.1814E-08	2.8263E-07	0.0000E-01	0.0000E-01	0.0000E-01
6.20	1.9816E-05	4.7438E-07	7.0223E-07	9.3648E-14	3.2018E-20	1.7808E-08	2.8520E-07	0.0000E-01	0.0000E-01	0.0000E-01
6.30	1.9204E-05	5.7872E-07	1.0785E-06	1.8107E-13	7.7842E-20	2.8854E-08	2.7737E-07	0.0000E-01	0.0000E-01	0.0000E-01
6.40	1.8372E-05	6.8701E-07	1.6353E-06	3.4564E-13	1.8730E-19	3.8563E-08	2.6538E-07	0.0000E-01	0.0000E-01	0.0000E-01
6.50	1.7255E-05	8.2461E-07	2.4356E-06	6.4809E-13	4.4213E-19	5.4116E-08	2.4942E-07	0.0000E-01	0.0000E-01	0.0000E-01
6.60	1.5840E-05	9.5247E-07	3.5417E-06	1.1864E-12	1.0188E-18	7.2188E-08	2.2887E-07	0.0000E-01	0.0000E-01	0.0000E-01
6.70	1.4093E-05	1.0668E-06	4.9939E-06	2.1060E-12	2.2771E-18	9.0569E-08	2.0365E-07	0.0000E-01	0.0000E-01	0.0000E-01
6.80	1.2077E-05	1.1509E-06	6.7826E-06	3.5009E-12	4.8015E-18	1.0541E-07	1.7456E-07	0.0000E-01	0.0000E-01	0.0000E-01
6.90	9.8188E-06	1.1898E-06	8.8272E-06	5.0999E-12	1.0110E-17	1.1285E-07	1.4337E-07	0.0000E-01	0.0000E-01	0.0000E-01
7.00	7.7842E-06	1.1757E-06	1.0882E-05	9.2403E-12	1.8934E-17	1.1001E-07	1.1258E-07	0.0000E-01	0.0000E-01	0.0000E-01

pH	CuNTA(-)	Cu(NTA)2(-4)	CuOHNTA(-2)	CuCO3(0)	Cu(CO3)2(-2)	CuHCO3(+)	CuCl(+)	CuCl2(0)	CuA2	CuNH3(+2)
6.00	2.3221E-05	1.6458E-14	6.6992E-08	1.3235E-07	6.6833E-13	5.4258E-08	1.8884E-08	4.1991E-12	0.0000E-01	0.0000E-01
6.10	2.3227E-05	1.8737E-14	8.4360E-08	1.9077E-07	1.4112E-12	6.2118E-08	1.8383E-08	4.1315E-12	0.0000E-01	0.0000E-01
6.20	2.3223E-05	1.7108E-14	1.0818E-07	2.8994E-07	2.6891E-12	6.9819E-08	1.7978E-08	4.0405E-12	0.0000E-01	0.0000E-01
6.30	2.3209E-05	1.7833E-14	1.3360E-07	3.7357E-07	5.7087E-12	7.6750E-08	1.7422E-08	3.9156E-12	0.0000E-01	0.0000E-01
6.40	2.3185E-05	1.8383E-14	1.6801E-07	5.0359E-07	1.0848E-11	8.2185E-08	1.6668E-08	3.7480E-12	0.0000E-01	0.0000E-01
6.50	2.3149E-05	1.9512E-14	2.1119E-07	6.5803E-07	1.9706E-11	8.5302E-08	1.5663E-08	3.5203E-12	0.0000E-01	0.0000E-01
6.60	2.3101E-05	2.1177E-14	2.6531E-07	8.2873E-07	3.4066E-11	8.5335E-08	1.4371E-08	3.2299E-12	0.0000E-01	0.0000E-01
6.70	2.3037E-05	2.3672E-14	3.3308E-07	9.9988E-07	5.5740E-11	8.1783E-08	1.2785E-08	2.8735E-12	0.0000E-01	0.0000E-01
6.80	2.2954E-05	2.7428E-14	4.1782E-07	1.1483E-06	8.5931E-11	7.4867E-08	1.0956E-08	2.4625E-12	0.0000E-01	0.0000E-01
6.90	2.2849E-05	3.3084E-14	5.2360E-07	1.2534E-06	1.2448E-10	6.4686E-08	8.9970E-09	2.0221E-12	0.0000E-01	0.0000E-01
7.00	2.2717E-05	4.1873E-14	6.5536E-07	1.2956E-06	1.6943E-10	5.3111E-08	7.0823E-09	1.5873E-12	0.0000E-01	0.0000E-01

pH	Cu(NH3)2(+2)	Cu(NH3)3(+2)	CuAc(+)	CuAc2(0)	T.D.Cu
6.00	0.0000E-01	0.0000E-01	0.0000E-01	0.0000E-01	4.5002E-05
6.10	0.0000E-01	0.0000E-01	0.0000E-01	0.0000E-01	4.5000E-05
6.20	0.0000E-01	0.0000E-01	0.0000E-01	0.0000E-01	4.5002E-05
6.30	0.0000E-01	0.0000E-01	0.0000E-01	0.0000E-01	4.5002E-05
6.40	0.0000E-01	0.0000E-01	0.0000E-01	0.0000E-01	4.5002E-05
6.50	0.0000E-01	0.0000E-01	0.0000E-01	0.0000E-01	4.5002E-05
6.60	0.0000E-01	0.0000E-01	0.0000E-01	0.0000E-01	4.5002E-05
6.70	0.0000E-01	0.0000E-01	0.0000E-01	0.0000E-01	4.5002E-05
6.80	0.0000E-01	0.0000E-01	0.0000E-01	0.0000E-01	4.5002E-05
6.90	0.0000E-01	0.0000E-01	0.0000E-01	0.0000E-01	4.5002E-05
7.00	0.0000E-01	0.0000E-01	0.0000E-01	0.0000E-01	4.5002E-05

Table B.5: Calculated Cu^{2+} concentration and Cu^{2+} activity for the medium used in the P-limited batch culture experiments. (pCu=-log Cu^{2+} activity. TDCU is total dissolved Cu concentration in the medium.)

TDCU μM	$[Cu^{2+}]$ M	pCu M
0.04	8.79×10^{-13}	12.22
10	1.26×10^{-9}	9.07
20	1.43×10^{-7}	7.01
25	1.81×10^{-6}	5.91
27.5	3.83×10^{-6}	5.59
30	5.97×10^{-6}	5.40
32.5	8.15×10^{-6}	5.26
35	1.03×10^{-5}	5.16
37.5	1.25×10^{-5}	5.07
40	1.47×10^{-5}	5.00
42.5	1.69×10^{-5}	4.94
45	1.91×10^{-5}	4.89

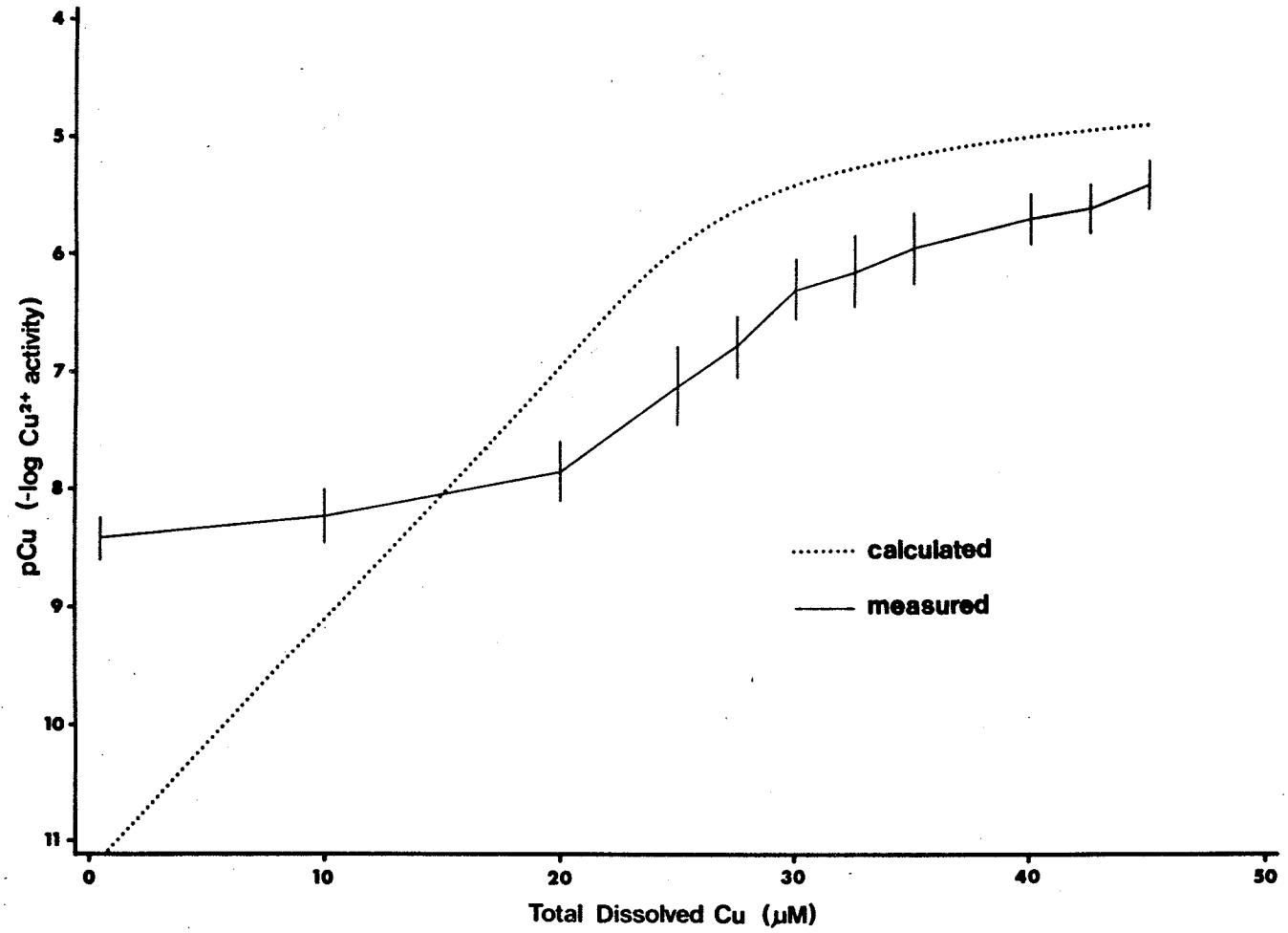
B.5 COMPARISON OF MEASURED AND CALCULATED Cu^{2+} ACTIVITIES IN THE MEDIUM USED IN BATCH CULTURE EXPERIMENTS

The results of the calculated and measured activities (Fig. B.2) show the measured values to be consistently lower than those calculated until the detection limit of the electrode was reached. The consistent difference between the two data sets shows that the factors not taken into account in the MACS80 calculations have a similar influence over the range of total dissolved Cu concentrations where the two methods can be compared.

The calculations of Cu speciation by MACS80 used in this study did not take into account the possible interaction between Cu^{2+} ions and the pH buffer MES in the medium. This interaction should be minimal as the binding capacity of MES has been reported to be negligible (Good 1966). Two other possible interactions are not considered, the binding of Cu^{2+} ions by the vitamins in solution and the interaction of Cu^{2+} ions with boron. The interactions of boron with Cu^{2+} have been shown to be minimal. These three interactions which are not considered by the MACS80 program would reduce the Cu^{2+} activity in the medium and hence the levels of Cu^{2+} activity measured using the Cupric Ion Selective Electrode would be expected to be less than the calculated value.

As the detection limits of the Cupric Ion Selective Electrode exclude approximately half the range of Cu concentrations used in the batch culture experiments (Chapter 2), the

Figure B.2: Calculated and measured Cu^{2+} activities as a function of total dissolved Cu concentration. The measured values are a mean of 3 replicates. Vertical bars: ±1 standard error.


4
5
6
7
8
9
10
11
pCu (-log Cu²⁺ activity)
0
10
20
30
40
50
Total Dissolved Cu (μM)
calculated
measured

calculated values of Cu^{2+} activity were used throughout the experiments with the knowledge that the Cu^{2+} activity levels are a slight overestimation (in the order of 5 to 10 times) that which is present.

Calculations using the MACS80 chemical speciation program were used to estimate the pCu levels in natural water bodies. Chemical composition data from control lakes in the Experimental Lakes Area (ELA) in northwestern Ontario were used in conjunction with Cu concentrations recorded in Cu contaminated lakes in the Flin Flon area. Both sets of lakes are typical of the Canadian Shield. The levels calculated for Cu contaminated lakes were pCu 5.71-6.78. A pCu of 8.60 was calculated for background Cu levels in the non-contaminated lakes. The calculated pCu of the control lakes appears to be high in light of the chemostat studies which showed Cu toxicity to occur in the range 10.3-10.7 pCu. The calculated pCu values will over-estimate the actual Cu^{2+} activity as many factors which influence Cu^{2+} activity in natural waters are not taken into account in the calculations. The major factors not considered are the varied binding capacities of dissolved organic materials and the binding of Cu to particulate matter. These omissions make it unrealistic to calculate pCu accurately for natural waters with the information available. However, even if the calculated pCu values are over-estimated, it appears that pCu levels in contaminated lakes may well be within the range shown to be toxic in the chemostat experiments.