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Copper Toxicity and Extracellular Release in *Selenastrum Capricornutum*

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COPPER TOXICITY AND EXTRACELLULAR RELEASE IN *Selenastrum capricornutum*

by

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Copper toxicity was investigated using the green alga, Selenastrum capricornutum. Two major areas were investigated: the forms of copper which are toxic to growth of the alga and factors that can change the concentration of free copper, $Cu^{2+}(aq)$, in the growth medium during the logarithmic growth of the organism.

This research has shown that S. capricornutum is sensitive to the free copper ion, $Cu^{2+}(aq)$, irrespective of the total copper concentration. Results indicate the following linear relationship:

$$\text{Growth rate constant} = (0.54\ \text{pCu}) - 3.6$$

A correlation coefficient of 0.92 was obtained within the pCu range of 10 to 7. The pCu in each culture was buffered with ethylenediaminetetraacetic acid (EDTA) or nitrilotriacetic acid (NTA). Some cultures showed a 1 day lag period regardless of the copper concentration. Growth inhibition due to copper was reversible at all copper concentrations tested.

Copper adaptation by the alga, changes in ionic strength during the growth of the alga in batch culture, and uptake of EDTA or copper by the alga were factors which were not accounted for in the calculation of free copper concentrations. All were found to have only a slight effect on this free copper concentration. However, extracellular release of organic products has a small but significant effect on ionic copper concentrations. The amount of carbon excreted was 5.5×10^{-14} ($\pm$ 3.1×10^{-14}) moles per cell. This is about two percent of the total amount of carbon fixed per cell. A 7 day culture of S. capricornutum, (about 1×10^{6} cells per ml) produced extracellular organic products that can complex 2×10^{-7} M copper as

determined from copper titrations using a copper selective electrode. This represents 1.2 times more copper complexed than that due to inorganic nutrients present in the medium. The conditional stability constant for these extracellular organic products is $10^{5.55}$ at pH 5.0. The equilibrium for this complexation is as follows:

$$Cu^{2+} + H_xL \rightleftarrows CuL + xH^+$$

This conditional stability constant is relatively weak when compared with that of copper-EDTA or copper-NTA complexes, but stronger than copper-fulvic acid complexes. Fulvic acid is native to natural waters. It was also apparent that the stoichiometry of complexation was one mole of metal ion complexed per mole of extracellular organic product. There was no increase in copper complexing capacity of products released by cells grown in the presence of copper concentrations that reduce the growth rate by one-half. Concentrated extracellular organic products were separated by thin layer chromatography. Eleven to thirteen products were recovered. The same products were isolated from cultures grown in the presence of an inhibiting amount of copper as were isolated from cultures grown with no excess copper. The copper complexing capacity of the separated fractions from the original medium, accounting for concentration effects, ranged from 1×10^{-10} to 2×10^{-8} molar.

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INTRODUCTION

I. THE AMOUNT OF COPPER PRESENT IN THE AQUATIC ENVIRONMENT

The widespread distribution of copper in natural waters is of particular importance because it is extremely toxic to phytoplankton. For example, copper naturally present in seawater can be toxic to some species of marine algae (Steemann-Nielsen and Wium-Andersen, 1970; Barber, 1973). Total copper concentrations between 0.4 and 12.3 µg per liter occur off the African coastline around the Canary Islands, (Riley and Taylor, 1971), while concentrations of 4 to 13 µg per liter occur off the coast of Florida (Alexander and Corcoran, 1967).

Copper mining has a pronounced effect on copper concentrations in nearby rivers and lakes. The copper released by mining may enter lakes and rivers in surface drainage or as atmospheric particulate fallout from smelters. Rainwater collected one mile from a smelter located in the Sudbury, Ontario mining area contained 122 µg per liter copper (Stokes and Hutchinson, 1976), even at a 12 mile distance there was 21 µg per liter copper in the rainwater. Numerous small "softwater" lakes exist around Sudbury in which copper concentrations up to 80 µg per liter have been reported. The River Hale which drains the abandoned copper mines in Cornwall, England contains a copper concentration of 120 µg per liter while upstream from this drainage site concentrations are less than 2 µg per liter (Foster, 1977).

Copper sulfate has been used worldwide as an algicide for several decades and applied in concentrations as high as 15 mg copper per liter. Its introduction into reservoirs, (Button and Hostetter, 1977), into seawater to control red tides, (Rounsefell and Evans, 1958), or into irrigation projects, (Bartley, 1976) has been commonplace.

II. THE FORM OF COPPER WHICH IS TOXIC TO PHYTOPLANKTON

Most studies of copper toxicity to phytoplankton have involved the comparison of relative copper sensitivity of several species of algae in one standard medium, (Thomas et al., 1977; Maloney and Palmer, 1956; Erickson, 1972). However, by the use of different defined media it has been shown that toxicity is not a function of total copper, (Fitzgerald and Faust, 1963; Fitzgerald, 1964; Steemann-Nielsen and Wium-Andersen, 1970; Stokes and Hutchinson, 1976). These authors concluded that organic complexing agents such as nitrilo-triacetic acid (NTA), ethylenediaminetetraacetic acid (EDTA), tris-(hydroxymethyl) aminomethane (TRIS) or citric acid, which are added to increase the iron solubility and complex copper diminish copper toxicity. Uncomplexed copper was hypothesized to be the toxic form to algae by Steemann-Nielsen and Wium-Andersen (1970) who noted that organic matter usually present in surface seawater is absent in deeper water. In the absence of organic matter, it would be expected that the ionic form of copper would be present in higher concentration. Thus, they concluded that phytoplankton cannot grow in freshly updwelled deep water, because this organic free water could contain ionic copper concentrations of 1 to 2 µg per liter copper which

is poisonous to many phytoplankton.

Ionic copper may be measured by anodic stripping voltametry, solvent and resin extraction, copper selective electrodes, or theoretical calculations using computer programs. Although anodic stripping voltametry has been used by several researchers in the measurement of ionic copper, the plating out of copper from the solution may perturb the weak copper complexes in the system. As yet there is no proven anodic stripping procedure to distinguish between ionic copper and soluble bound copper at concentrations present in natural waters (Gachter et al., 1973; Shuman and Woodward Jr., 1973).

Solvent extraction has been used to measure ionic copper concentrations (Kennish, 1978). The method extracts only a few percent of the bound copper thus perturbing the system very little. However, development of the best working conditions near neutral pH (as would occur in natural water samples) is still at the formative stage. At present only simple metal-ligand systems at an acidic pH have been successfully measured. Similar problems arise for methods which use ion exchange resins as an extraction procedure for copper. Again the best working conditions for natural waters have not been devised (Gamble et al., 1970).

Copper selective electrodes have been developed for measuring the activity of the ionic copper. The detection limit of copper selective electrodes is in the range of 10^{-8} to 10^{-9} M and linear response of potential in volts versus the log of ionic copper concentration is attained down to 3×10^{-7} M (Blaedel and Dinwiddie, 1974).

The ionic copper concentration in natural waters has been measured widely by this procedure (Stiff, 1971; Ramamoorthy and Kushner, 1975; Kimball, 1973; Cheam and Gamble, 1974). The most serious problem with this method is the detection limit, as many species of algae are inhibited at ionic copper concentrations below this detection limit.

At present, the only satisfactory method for estimating the concentration of low levels of ionic copper is an indirect calculation. Ionic copper concentrations can be calculated if all inorganic and organic anion and cation concentrations are known as well as all relevant acid dissociation constants, formation constants and solubility products. A data base is available in the following references (Sillén and Martell, 1964; 1971) and calculations for relatively simple systems which contain copper complexes provide useful information (Sylva, 1975; Sunda, 1975; Gardiner, 1975). In the last few years, computer programs have been devised which give a thermodynamic calculation of ionic copper in complex systems involving many ligands and metals. Two programs in wide use are MINEQL (Westall et al., 1976) and REDEQL2 (McDuff and Morel, 1973). Morel and Morgan (1972) explain the mechanics of calculating activities of chemical species from these equilibrium models. Calculated copper activity is referred to as pCu^{*} to distinguish it from experimentally determined pCu.

A calculated value of ionic copper has the primary advantage of estimating a concentration which may be far below the detection limit of analytical methods. These calculations provide a predictable

model for copper bioassays. The disadvantage with a computer calculation of ionic copper concentration is that the predictions are only as accurate as the assumed equilibrium constants used in the data base. There is often a fair disagreement in values between the same constant measured by different techniques. As discussed later in this dissertation, one or more of the acid dissociation constants for NTA used in MINEQL are in question. Other problems with a computer calculation of ionic copper concentration are the lack of kinetic considerations when ionic activities are calculated and the lack of applicability to undefined media such as natural aquatic systems.

In the last few years these computer programs or similar calculations have been employed to establish that ionic copper, rather than total copper, is the toxic form to aquatic organisms (Morel et al., 1978; Anderson and Morel, 1978; Andrew et al., 1976; Sunda and Guillard, 1976). Copper bioassays have been performed using several species of algae and a crustacean. The negative log of the copper ion concentration (pCu^*) was calculated for a defined medium (Morel et al., 1978) and a direct correlation between the growth rate and pCu^* was apparent. In addition, these results established that copper-TRIS, copper-EDTA or copper-NTA complexes are not toxic to algae (Anderson and Morel, 1978; Sunda and Guillard, 1976). Andrew et al. (1976) have also shown that soluble copper phosphate and copper carbonate complexes are not toxic to the crustacean, Daphnia magna.

III. RATIONALE FOR STUDYING THE EFFECT OF IONIC COPPER ON THE GROWTH OF *Selenastrum capricornutum*

The aim of this research was to examine factors (such as pH changes in the medium, total copper, EDTA or nutrient uptake by the algae, and extracellular release of algal products), which might change the ionic copper concentration during the period of logarithmic growth of the organism. The possibility of genetic mutation favoring copper tolerance was also investigated as another factor which could change copper toxicity to the organism. Other than changes in pH of the medium, these factors have not been extensively investigated in algae by other investigators. Copper bioassays were performed in order to test the hypothesis that this test organism is sensitive to ionic copper rather than other complexed forms of the metal and also to determine the range of ionic copper (Cu^{2+}) sensitivity.

Selenastrum capricornutum was selected as the bioassay organism because it is unicellular, nonmotile and quite small (an average of 5 to 10 microns in length). These properties facilitate reproducible viable counts. This organism also has been used widely in metal toxicity and nutrient deficiency studies (Clesceri et al., 1973; Miller et al., 1974; Bartlett et al., 1973; Miller et al., 1975; Weiss and Helms, 1971; Miller et al., 1976; Toerien and Huang, 1973; Goldman et al., 1974; Chiaudani and Vighi, 1977).

The major focus of this research was directed toward the study

of copper complexation on extracellular products released by S. capricornutum. Algae excrete a wide range of extracellular products (Hellebust, 1974; Fogg, 1971, 1966: 1962: Aaronson et al., 1971; Gocke, 1970; Weinmann, 1970) and there is some evidence that algal lysates can complex copper. Cell digests containing hydrolyzed amino acids from Anabaena cylindrica and Chlorella pyrenoidosa, a blue-green and green algae respectively, were found to reduce the copper toxicity to algae when added to a medium prior to inoculation (Fogg, 1955; Steemann-Nielsen and Kamp-Nielsen, 1969). Gibson (1972) also found that copper was less toxic for green and blue-green algae if copper was added to the medium after the initiation of algal growth. This indicated the possibility of extracellular excretion by the algae.

Several species of marine phytoplankton have been stressed with lethal and sublethal copper concentrations (Mandelli, 1969). Subsequent to copper stress there was an immediate copper absorption by the cells. It was demonstrated that after several minutes there was release of copper back into the medium. The excretion of organic compounds has been proposed by Mandelli (1969) as a mechanism to reduce the stress and Foster (1977) also found evidence of extracellular release. Copper bioassays were performed with two strains of Chlorella vulgaris, one of which is a copper tolerant variety. Both strains exhibited the same growth rate as a function of the amount of copper taken into the cell. Thus, both strains have physiologically the same tolerance to absorbed copper. However, the metal tolerant strain possessed larger growth rates than the nontolerant strain under the same conditions of metal stress. It appears that

the metal tolerant strain may have evolved the ability to produce an extracellular product which can lower the activity of the ionic copper or adaptation by the cell to regulate the uptake of copper against a high external copper gradient.

Copper complexation by algal exudates from several species of algae has been measured using copper selective electrodes (Swallow et al., 1978), since this method can provide direct evidence for excretion of algal products which complex copper. However, only one exudate from the species, Gleocystis gigas, showed any appreciable copper complexation. Filtrates from cultures of this alga were found to have a copper complexing capacity of 5×10^{-6} M with a conditional stability constant of 1.0 at pH 7.0 (the constant defined in the same manner as that of this dissertation). Copper complexation from the other algal filtrates was probably underestimated because the copper selective electrode was too insensitive to permit adequate measurement at low ligand concentrations. In addition, the reportedly lower copper complexation of these filtrates is also likely because added iron (at a concentration of 1×10^{-5} M) competed with copper for complexation sites.

IV. PROPOSED RESEARCH

In the present research, the copper complexing capacities were measured using copper selective electrodes on extracellular filtrates from S. capricornutum cultures. Filtrates from copper stressed and non-stressed cultures were compared in order to determine

if extracellular release was initiated at high copper concentrations. The following types of extracellular filtrates were measured: unconcentrated filtrates, desalted and concentrated filtrates, and 13 extracellular algal products which had been separated by thin layer chromatography. In order to show the copper affinity for these excreted products, conditional stability constants (from concentrated and desalted filtrates) were calculated from copper titration data obtained using a ion selective electrode. In addition, molecular weight and copper complexing capacities were also measured for these products.

An evaluation of all of these factors was made in order to assess whether or not the growth rate of S. capricornutum would be a good indicator for the concentration of ionic copper.

MATERIAL AND METHODS

I. THE TEST ORGANISM

An inoculum of Selenastrum capricornutum was obtained from the EPA (Environmental Protection Agency) at Corvallis, Oregon. Axenic cultures of the alga were prepared using a modified centrifugation and filtration technique (James, 1971). A sonication step was added between repeated washings. Conical centrifuge tubes (15 ml) containing 5 ml of algae were placed in a Branson 12 ultrasonic cleaner for 15 seconds. This presumably removes the bacteria from the cell wall of the alga. The final centrifuged algal syspension was streaked on Plate Count Agar (Taras, 1971) containing EPA medium, (Weiss and Helms, 1971). Colonies that were found to be axenic were transferred to 25 ml of EPA medium in 125 ml Erlenmeyer flasks. The cultures were transferred every 4 or 5 days to insure continuous log phase growth. All experimental cultures were inoculated with cells of S. capricornutum in the logarithmic phase of growth.

II. CULTURE MEDIUM AND PREPARATION OF THE STOCK SOLUTIONS

For most work, the standard EPA medium was modified to vary copper and EDTA as listed in Table 1. A stock solution of each nutrient was maintained at 1000-fold concentration and stored in 500 ml linear polyethylene bottles at room temperature. For testing the effect of complexing agents (or free metal concentrations) on the growth rate of S. capricornutum, four modifications of the

TABLE I

MODIFIED EPA MEDIUM

COMPONENT*	FINAL CONCENTRATION IN THE GROWTH MEDIUM (MOLAR)
$NaNO_3$	2.8×10^{-5}
K_2HPO_4	6.6×10^{-6}
$MgCl_2$	6.0×10^{-5}
$MgSO_4 \cdot 7H_2O$	5.9×10^{-5}
$CaCl_2 \cdot 2H_2O$	3.0×10^{-5}
$NaHCO_3$	1.7×10^{-4}
$FeCl_3$@	5.9×10^{-7}
H_3BO_3	2.2×10^{-6}
$MnCl_2$	2.1×10^{-6}
$ZnCl_2$	2.4×10^{-7}
$CoCl_2$	6.0×10^{-9}
$Na_2MoO_4 \cdot 2H_2O$	3.0×10^{-8}
EDTA or NTA	variable
$CuNO_3$	variable

*—Mallinckrodt, anayltical grade reagents were used except NTA which was obtained from Eastman Chem. Co. (Analytical grade) and $CuNO_3$ (Orion Ionanalyzer cupric Standard).

@— The ferric chloride solutions contained ethylenediaminetetraacetic acid (EDTA) or nitrilotriacetic acid (NTA) at various concentrations as indicated for a given experiment.

medium (shown in Table 1) were used: EDTA at 8.06×10^{-7} M, 5×10^{-6} M and 1×10^{-5} M or NTA at 1×10^{-6} M. The chelating agents were stored at 1000-fold concentrations in the $FeCl_3$ stock solutions. In this research these synthetic chelating agents are important in controlling the solubility of ferric iron and the ionic copper concentrations.

III. THE COPPER BIOASSAY

Culture medium was prepared from the stock solutions by diluting 1000 µl from each stock solution into approximately 500 ml of distilled water in a volumetric flask. The volume was adjusted with distilled water to one liter. After vigorous mixing, 25 ml of medium was transferred with a 25 ml automatic vacuum pipet to specially cleaned glassware (see subsection XIV. of this Material and Methods section for cleaning procedures). Ten µl of copper nitrate, initially diluted in distilled water, was transferred with a 10 ul pipet to each 25 ml of medium to yield a final concentration of 1.57 to 18.8 x 10^{-7} M copper. The Erlenmeyer flasks were closed with cotton stoppers and autoclaved for 20 minutes at 121 C. Flasks were placed in a dark cabinet at room temperature for 2 days to allow for the solutions to reach chemical equilibrium. At the start of the third day the axenic algal culture was transferred into each flask to give a final concentration of a thousand cells per ml. These inoculated flasks were then placed on a rotory shaker and oscillated at 120 revolutions

per minute. The shaker was positioned under a bank of Sylvania fluorescent life-line lights at 1.09×10^4 lux (measured at culture level). The temperature under the light bank varied from 20 to 25 C. Each 24 hours for 5 to 7 days, three parameters were measured in all cultures: (1) cell counts by a hemacytometer, (2) absorbance at 680 nm, and (3) pH of the medium. Cells were counted on an American Optical hemacytometer using a Zeiss phase contrast binocular microscope at 400 X. The optical density of cultures was measured using Perkin Elmer cylindrical 50 mm Spectrosil cuvettes in a Coleman-Hitachi 124, double beam spectrophotometer. The pH was measured with a Chemtrix combination pH electrode and Orion Research Ionanalyzer specific ion meter model 404.

Specific growth rates were calculated between days 1 and 2 using the general growth equation found in Appendix A. A plot of absorbance at 680 nm versus cell number showed a linear relationship in the absorbance range between 0.01 and 0.2 (Fig. 1). The linear relationship can be expressed as follows:

$$\text{Cell number} = 1.93 \times 10^6 \, (\text{Absorbance}_{680\ nm}) - 1.4 \times 10^4 \qquad (2)$$

From a comparison of 300 samples a correlation coefficient of 0.88 could be obtained.

IV. MEASUREMENT OF CARBON-14 UPTAKE AND RELEASE OF EXCRETED CARBON AS LABELED EXTRACELLULAR PRODUCTS

A volume of 300 ml of modified EPA medium containing 8.06×10^{-7} M EDTA (no copper) was placed in Kimax dissolved oxygen (D. O.) bottles and inoculated with a log phase culture of 1.5×10^7 cells

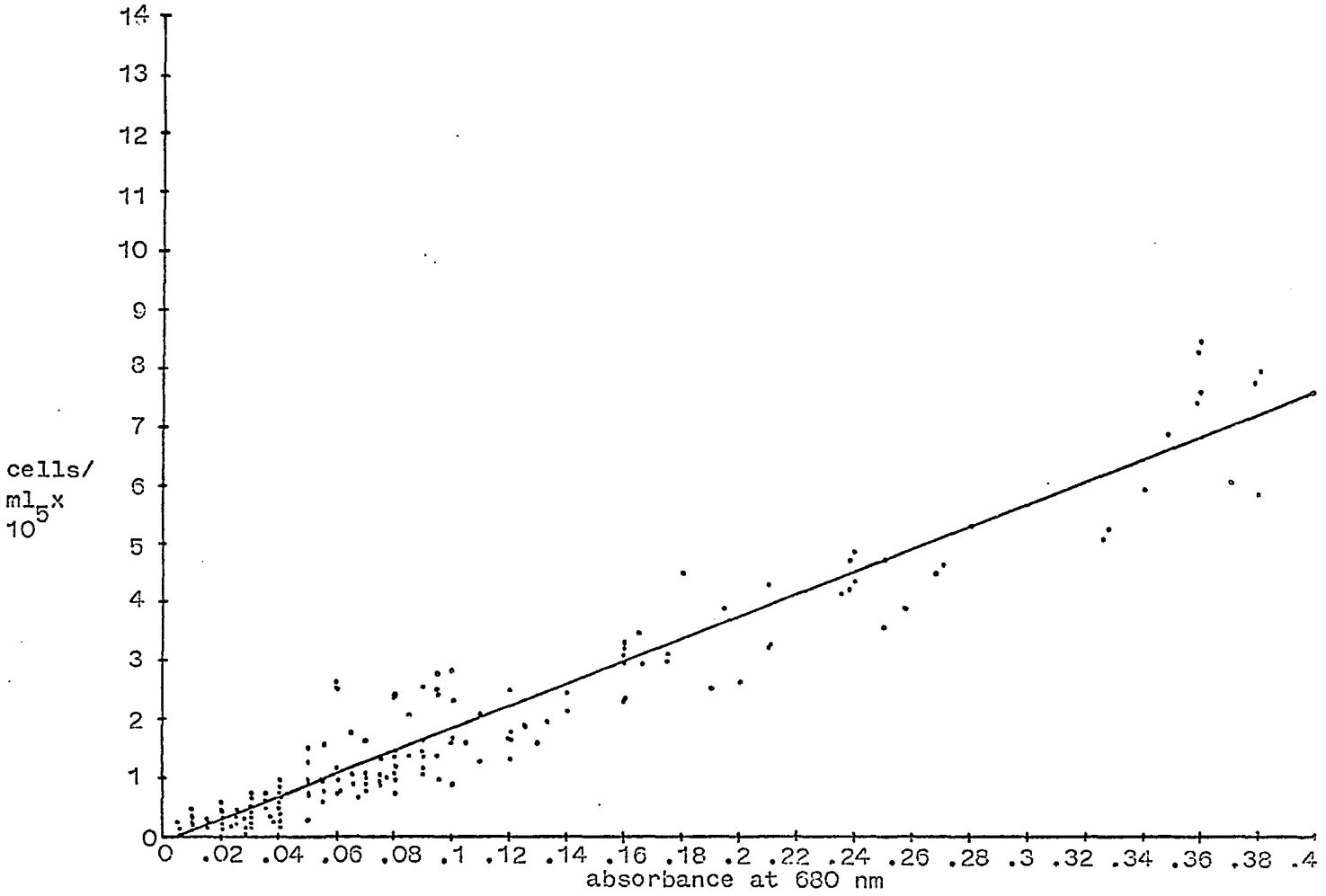

Fig. 1. A comparison between the 2 parameters of growth; hemacytometer cell counts and absorbance at 680 nm.

to give 5.0×10^4 cells per ml. The bottles were spiked with 5.0 uCi of $NaH^{14}CO_3$ and incubated in a water bath, 24 C, at 1×10^4 lux. In order to rule out bacterial contamination and fixation of $^{14}CO_2$ in the dark, D. O. bottles covered with black tape were used as controls and incubated as stated above. The total organic carbon fixed was measured in procedures described below. At various time intervals several bottles were removed and two, 30 ml volumes were collected from each bottle for analysis of carbon-14. The two volumes represent total organic carbon fixed and extracellular carbon released by algal cells. Procedures for both volumes were identical with the exception of an additional step required in the measurement of extracellular carbon. This extra step involved the removal of the algal pellet from the volume by centrifigation at room temperature in a Sorvall RC-B2 at 7500 X g for 10 minutes. Both volumes were acidified by the addition of 0.2 ml of 0.1 M HCL and aerated for 20 minutes as described by Schindler and Holmgren (1971). For determination of carbon-14 in all samples, 5 ml from each volume and 10 ml of Aquasol-2 (New England Nuclear) were added to scintillation vials. Radioactive disintegrations from these vials were counted in a Nuclear-Chiago Unilux-II liquid scintillation counter.

V. CONCENTRATION OF EXTRACELLULAR PRODUCTS

Nine, 16-liter Pyrex carboys containing modified EPA medium with 8.06×10^{-7} M EDTA were inoculated with S. capricornutum at a final

concentration of a thousand cells per ml. Five carboys contained no copper and four carboys contained a concentration of 7.87×10^{-7} M total copper. This is enough total copper to depress the growth rate by one-half of the maximum. Once cultures reached the stationary phase of growth (3 to 5 x 10^6 cells per ml), they were centrifuged at 8000 X g at 5 C using a Syent-Gyorgyi and Blum KSB-R Continuous flow system connected to a Sorvall RC-2B refrigerated centrifuge. The algal pellets were freeze dried, weighed and stored at -- 20 C. The supernatant fluid was clarified by passage through a Millipore prefilter and 0.45 µM filter. The filtrates were acidified to pH 1.7 with nitric acid (Mallinckrodt AR).

Amberlite XAD-8 resin (Rohm and Haas) was washed on a filter with 3 to 4 bed volumes of distilled water to remove the sodium bicarbonate in which it was orginally packed. The resin was extracted for 24 hours with methanol (Mallinckrodt Spectr AR) in a Soxhlet extractor. Finally the resin (150 gm suspended in methanol) was packed in a 2 by 30 cm column.

The supernatant fluid from each set of carboys was washed through the column with a flow rate of 6 (liquid phase) bed volumes per hour. After all the supernatant fluid had been filtered through the column, the resin was eluted with 1 bed volume of 0.1 M KOH followed by several bed volumes of methanol. The 100 - 125 ml of elutant fraction was collected separately and stored in screw capped Erlenmeyer flasks. Ultimately, the eluate from the XAD-8 resin was concentrated by vacuum distillation at 40 C.

VI. TOTAL CAPACITY AND PERCENT EFFICIENCY OF XAD-8 RESIN OF EXTRACELLULAR PRODUCTS

Methanol extracted XAD-8 resin (15 gm) was packed into a 1.6 by 20 cm column. A 16 liter carboy of modified EPA medium (containing 8.06×10^{-7} M EDTA and spiked with 15 ml of 5 μCi per ml $NaH^{14}CO_3$) was inoculated to give 1000 cells per ml S. capricornutum and the culture was incubated at 7.6×10^3 lux at room temperature. After 7 days incubation, the supernatant fluid was obtained, acidified and aerated as previously described. Extracellular products in the supernatant fluid were concentrated by the XAD-8 column and the resulting effluent was collected in eleven, 1-liter fractions. In order to monitor the capacity of the column to concentrate extracellular products, 5 ml aliquots from each liter of effluent and 10 ml of Aquasol-2 were added to scintillation vials. Radioactivity was counted by the liquid scintillation method. The total capacity of the column was calculated from the change in activity from the monitored effluent as compared to the initial unfiltered supernatant fluid.

The column was eluted with one bed volume of 0.1 M KOH and several bed volumes of methanol. The methanol and KOH eluted fractions were evaporated and diluted to 5 mls with distilled water. The activity of these samples were measured by liquid scintillation counting. The percent efficiency of the resin was calculated either as the ratio of activity of the unfiltered supernatant fluid and effluent or as the ratio of the total activity from extracted fractions as compared

to the total calculated activity of unfiltered supernatant fluid.

VII. SEPARATION OF EXTRACELLULAR PRODUCTS

Products were separated by thin layer chromatography (TLC). Uncoated glass TLC plates were cleaned by immersion in saturated ethanol-KOH solution for one-half hour or longer, then thoroughly rinsed in distilled water in a TLC drying rack and dried in an oven at 105 C. The sorbent was silica gel G (E. M. Merk, EM Reagent). Silica gel G (30g) was added to 60 ml of distilled water in a 500 ml corked Erlenmeyer flask and shaken vigorously for 90 seconds (E. Stahl, 1969). The mixture was spread over four to five TLC plates (20 cm x 20 cm x 4.7 mm) using a Shandon Southern Unoplane spreader. Two sorbent thicknesses were selected: 0.25 mm for detection and 1 mm for preparative use.

Two solvent systems were ultimately selected for separation of the products; (1) ethyl acetate and (2) n-butanol, formic acid, and water (4:1:1 v/v/v).

For detection work, a two-dimensional chromatogram was determined using ethyl acetate in the first direction, and the n-butanol, formic acid, and water mixture 4:1:1 (v/v/v) in the second direction. The chromatography tank (25 x 27 x 7 cm) was lined with Whatman No. 1 filter paper to speed equilibration. The concentrated extracellular products (20 µl) were applied using a 10 µl micropipet and the samples were air dried on the TLC plates. When the solvent front had reached the top of the plate, the plates were removed from the tank and

air dried.

Detection of spots was accomplished by four methods (visible, fluoresence, iodine staining and ninhydrin reaction) and after detection the spots were traced onto overlays of plastic. Visible spots were detected by placing the plate on a 11 x 45 cm fluorescent light tray (Buchler Instruments). Spots which fluoresced under UV light were located by use of a Chromato-vue ultraviolet chamber having long wave UV at 365 nm and short wave UV at 254 nm. Compounds containing carbon-carbon double bonds were detected by incubation of the TLC plates in an enclosed chromatography tank containing iodine crystals (Mallinckrodt U. S. P.); after several minutes the yellow to brown spots were noted. Air was passed gently over the TLC plates for one-half to 1 hour in order to remove the iodine. The last detection system involved the spraying of TLC plates with a ninhydrin solution (0.3 g ninhydrin, Sigma, in 100 ml of n-butanol with 3 ml glacial acetic acid). Each plate was then placed in an oven at 105 C for 5 to 10 minutes for color development. Using plastic overlays obtained from the 4 detection systems, R_f values were calculated for the separated products.

TLC was also employed in the preparative separation of extracellular products. Concentrated eluates were applied onto silica gel G TLC plates one inch from the bottom using a 500 ul pipet and the streaks were air dried. Ethyl acetate was used to separate products initially. Products which did not move in the first solvent (ethyl acetate) were scraped from the orgin with a zone

collector and the products were extracted from the silica gel with methanol. This methanol extract was concentrated by evaporation of the methanol and then applied to a second plate in the manner mentioned previously. The 3 component solvent system (n-butanol, formic acid, and water 4:1:1, v/v/v) was used in this second separation. Finally all detected spots were recovered with the zone collector and the products stored in screw-capped Pyrex test tubes (9 x 150 mm) at 4 C.

VIII. POTENTIOMETRIC MEASUREMENT OF COPPER COMPLEXATION

The use of copper ion selective electrodes in determining the stability products for fulvic acid, humic materials, and algal products has been reported (Buffle et al., 1977; Cheam and Gamble, 1974; Cheam, 1973; Jasinski et al., 1974; Swallow et al., 1978). Copper ion selective electrodes (Orion model 94-29A) were used to determine copper complexing capacities, conditional stability constants and average molecular weights for extracellular products from S. capricornutum. Nitrilotriacetic acid was used as a standard ligand solution.

A Radiometer pH meter type pHM 26C with an Orion double junction reference electrode (model 90-02-00) was use to measure free copper. The pH was monitored simultaneously with the free copper concentration using an Orion digital 112 pH meter connected to a Sargent (S-30070-10) combination pH glass electrode. The following procedure was used for titrations. Samples containing 0.1 M KNO_3 to assure

constant ionic strength were contained in 100 ml polyethylene beakers. The solutions were placed on magnetic stirrers, and 10 µl volumes of titrant were added. The addition of the titrant did not appreciably change the total volume of the solution during the titration. The solutions were equilibrated for 10 minutes between each addition (Blaedel and Diwiddie, 1974).

Three types of titrations were performed: copper, ligand and acid-base titrations. Copper titrations were made in order to determine copper complexing capacities, conditional stability constants, and average molecular weights for ligand solutions at a given pH. Standard 10 µl aliquots of copper, prepared by dilution of the stock solution (0.1 M $CuNO_3$) from 1-10 to 1-10,000 with distilled water, were added to a solution at a constant pH and total ligand concentration. The copper complexing capacities of unconcentrated, XAD-8 concentrated algal filtrates, and purified extracellular products were determined. In addition, average molecular weights and conditional stability constants at pH 5.0 and 6.0 were determined (according to theory derived in Appendix B) for XAD-8 concentrated extracellular products.

Ligand titrations involved the addition of 10 µl of concentrated extracellular products to solutions at constant total copper concentrations. These titrations were maintained at constant pH and indicate the stoichiometry of copper ligand complexes (see Appendix B for calculations). Conditional stability constants also can be obtained by this method.

Once the concentration of ligand had been determined, acid-base titrations were made on NTA solutions and concentrated extracellular products in order to obtain conditional stability constants over the pH range, 2.0 to 7.0. These titrations involve addition of a strong acid (HNO_3) or strong base (KOH) to a solution containing both constant total ligand and copper concentrations.

Calibration curves were constructed as standards for pCu between the range 8.0 to 5.0. Solutions of 0.1 M KNO_3 were used as blanks in copper titrations. Total copper added is assumed to be uncomplexed and measured as free copper. According to the Nernst equation:

$$E = E_o + RT/\ nF\ \log(A_{cu} + A_x) \qquad (3)$$

where R equals the gas constant; T, the absolute temperature; F, the Faraday; E_o, the standard potential; E, the measured potential; A_{cu}, the activity of the ionic copper; A_x, the activity of the interfering ion x; n, the valence of the copper ion. Theoretically the value of RT/nF for a divalent cation is 29.6 millivolts at 25 C. Thus a 10-fold change in the activity of ionic copper produces a 29.6 millivolt change in potential. The experimental response of an electrode is usally less than the theoretical response as seen in Fig. 2. Near the detection limit of 10^{-7} M ionic copper, it is evident that the experimental slope drops off sharply.

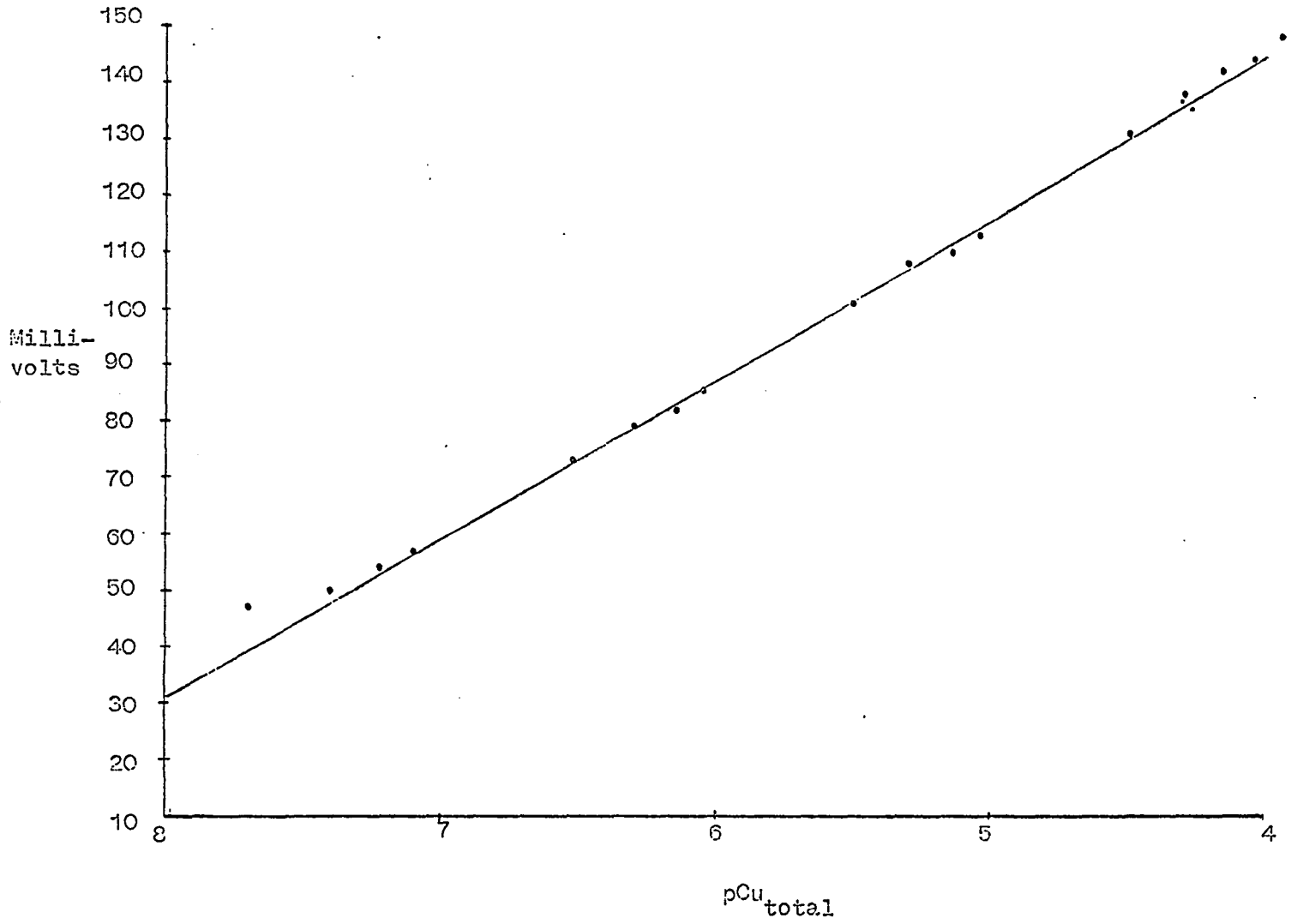

Fig. 2. A typical calibration curve for a copper sulfide electrode (Orion). at a pH of 5.0. Standard solutions of 10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4} M $CuNO_3$ were titrated in a 1 x 10^{-1} M KNO_3 solution (25 ml), using a 10 µl Eppendorf pipet.

IX. VIABILITY OF S. capricornutum FROM COPPER BIOASSAYS

Selenastrum capricornutum cells grown at copper concentrations between 1.57 to 18.8 x 10^{-7} M copper in modified EPA medium plus 8.06 x 10^{-7} M EDTA were sprayed onto petri dishes containing 15 g per liter Difco agar in modified EPA medium plus 8.06 x 10^{-7} M EDTA and no copper. The plates were counted for three days using a Zeiss phase contrast microscope at 400 magnification. Thirty random fields were counted for each culture and for each field the total number of cells and number of cells undergoing division were recorded. A ratio of dividing cells versus total cells was determined.

X. MEASUREMENT OF IONIC STRENGTH IN MODIFIED EPA MEDIUM PLUS 8.06 x 10^{-7} M EDTA DURING THE FOUR DAY GROWTH PERIOD OF S. capricornutum

Algal cells were grown in a 16-liter Pyrex carboy (containing modified EPA medium with 8.06 x 10^{-7} M EDTA without copper), at a final inoculum concentration of one thousand cells per ml. Each day, a one-liter sample was taken from the carboy and centrifuged at 8,000 X g for 10 minutes at 4 C, in a Sorvall RC-B2 refrigerated centrifuge. The supernatant fraction was concentrated 100-fold by vacuum distillation at 40 C and the concentration of major ions was determined. A Perkin-Elmer 305B atomic absorption spectrophotometer was used for the analysis of the major cations: K^{+}, Na^{++}, Mg^{++} and Ca^{++}. A barium precipitation method (Shakerin-Sweet, 1979) was

used for sulfate analysis. Chloride concentrations were measured by titration with mercuric nitrate (Taras, 1971). Nitrate was determined by a cadmium reduction method (Taras, 1971). The ionic strength of the medium (based on the total concentration of the major ions and a 1-100 concentration step) was calculated for each day.

XI. THE DETERMINATION OF GROWTH RATE VERSUS pCu DURING TRANSFER

Selenastrum capricornutum cells, grown for 7 days at total copper concentrations between 1.57 to 18.8 x 10^{-7} M copper, were transferred to fresh medium containing the same respective copper concentration. Growth rates were calculated from cell counts made at day 1 and 2; this is the same time period in which growth rates were initially calculated. A comparison of growth rates was made between the initial and transferred cultures grown at each concentration of copper.

XII. THE UPTAKE OF COPPER BY S. capricornutum

Flasks were inoculated with one thousand cells per ml of S. capricornutum which had been grown on modified EPA medium plus 8.06 x 10^{-7} M EDTA and either 6.29, 7.89, 9.44 or 11.1 x 10^{-7} M copper. The initial pCu was calculated to be 8.85, 7.94, 7.39, and 7.25. The flasks were incubated at 1.09 x 10^{4} lux for two days and harvested as previously described. The algal cells were lyophilized, weighed and stored in Pyrex bottles at -20 C. The

cells were thawed, resuspended in distilled water to 1/25 th of the orginal volume and the copper content of the cells was analyzed by flameless atomic absorption spectroscopy using an Instrumental Laboratory AA/AE spectrophotometer IL 551 with flameless atomizer 455.

XIII. GLASSWARE PREPARATION

All glassware was soaked in a saturated ethanol-KOH solution for one hour or longer, then thoroughly rinsed in distilled water and finally oven dried at 105 C. Pyrex flasks used in copper bioassay experiments were silanized to minimize copper adsorption onto inner glass surfaces. Dry Pyrex flasks used in the assay were filled with a 5 percent solution of dichlorodimethylsilane (Mallinckrodt OR) diluted in chloroform (Mallinckrodt AR) v/v, and soaked 30 minutes. All flasks were thoroughly rinsed with chloroform, distilled water and air dried.

RESULTS AND DISCUSSION

I. DEPENDENCE OF COPPER TOXICITY ON pCu

FORMER STUDIES PRIOR TO PRESENT RESEARCH

Steemann-Nielsen and Wium-Andersen (1970) proposed that algae are sensitive exclusively to the uncomplexed copper, $Cu^{2+}(aq)$. They proposed that the naturally occurring concentration of ionic copper in nutrient-depleted seawater was toxic to the phytoplankton community.

To date, several sutdies have indicated a linear relationship of growth rate to the negative log of the copper ion concentration (pCu) for a diatom Thalassiosira pseudonana (Sunda and Guillard, 1976), another diatom, Skeletonema costatum (Morel et al., 1978 a green alga, Nannochloris atomus, (Sunda and Guillard, 1976) and a Chrysophyte alga, Monochrysis lutheri (Sunda and Lewis, 1978). Andrew et al. (1976) extended this hypothesis to an invertabrate, the crustacean, Daphnia magna. Since most algae are sensitive to pCu far below the detection limit of most instrumental and chemical methods, most of the results were obtained using a calculated value of the negative log of the copper ion concentration (pCu^*). This calculated value (pCu^*) in a defined medium can be obtained by the use of computer programs such as MINEQL (Westall et al., 1976). Ionic copper has been found to be the toxic form of copper to all aquatic organisms examined to date.

Sunda and Guillard (1976) monitored the pH of their cultures during a copper bioassay experiment and recalculated pCu^* at these measured pH's in order to see if changes in pH had a significant effect on pCu^*. The pH of cultures showed a slight effect on pCu^*. In addition the copper content of the cells was analyzed to show copper uptake by cells and the affect on pCu^*. This affect was found to be insignificant.

Anderson and Morel (1978) found that chemical equilibrium between copper and EDTA was obtained only after 24 hours. Cultures inoculated before this equilibration time showed proportional increases in toxicity. On the other hand, copper-TRIS complexes formed almost immediately (Anderson and Morel, 1978).

Andrew et al. (1976) determined the concentration of copper required to kill one-half of the initial population of Daphnia magna in 24 hours. Their results indicated that while ionic copper is toxic to Daphnia magna, $CuCO_3(aq)$ and $Cu(PO_4)^-(aq)$ are not.

Sunda and Lewis (1978) used a copper ion selective electrode to measure $Cu^{2+}(aq)$ in the growth medium for the copper bioassay of M. lutheri and found a linear relationship between pCu and the growth rate. A second contribution from this paper is the finding that phytoplankton are sensitive to $Cu^{2+}(aq)$ in the presence of naturally occurring river water ligands. All previous studies not only employed calculated pCu^*, but used synthetic ligands to buffer $Cu^{2+}(aq)$. This work indicates (using a copper electrode) that

these natural copper-ligand complexes are not toxic to aquatic organisms. This paper claimed to obtain 99 percent of the theoretical slope for their calibration curves to a $Cu^{2+}(aq)$ concentration of 10^{-10} M. However, Jasinski et al. (1974) and Johansson and Edstrom (1972) reported a detection limit of 10^{-7} M using the same kind of electrode under the best conditions.

GROWTH OF S. capricornutum ON COPPER BIOASSAY MEDIUM

In order to determine if ionic copper is toxic to Selenastrum capricornutum, copper bioassay experiments were performed using modified EPA medium (Table 1.). A typical example of a growth curve is represented in Fig. 3. From these results a number of observations can be made. First, the modified EPA medium without copper can support a final population of 4 to 7 x 10^6 cells per ml. Toerien and Huang (1973) have determined that phosphate is the limiting nutrient for S. capricornutum in modified EPA medium. Second, growth decreases with increasing total copper. Third, the variance in growth rate between replicates is larger at or near algistatic copper concentrations. Fourth, exponential growth usually ends by the third day with the highest growth rates between days 1 and 2. Although not shown in Fig. 3, some cultures display one day lag periods. It appears that the cells are easily shocked; but in no case was there longer lag periods at higher copper concentrations.

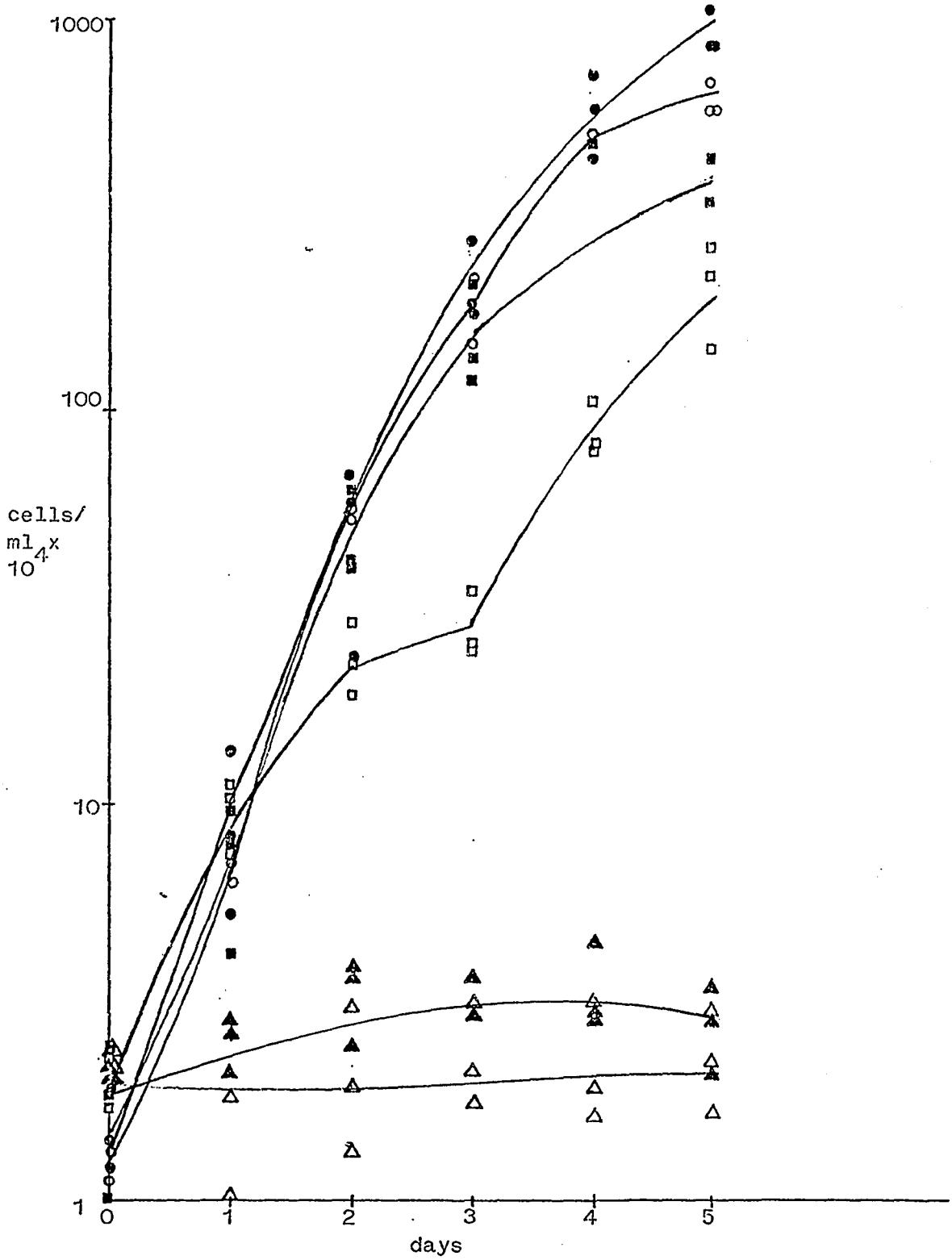

Fig. 3 . Copper bioassay growth curve for S. capricornutum cultured in E.P.A. medium with 5 x 10^{-6} M EDTA: hemacytometer count. Symbols represent the following: ● = 0 M total copper, ○ = 3 x 10^{-6} M, ■ = 4.2 x 10^{-6} M, □ = 4.7 x 10^{-6} M, ▲ = 6 x 10^{-6} M, △ = 2 x 10^{-5} M.

AFFECT OF COPPER CONCENTRATION ON GROWTH RATE

From the four copper bioassay experiment undertaken in this present research, the growth rates were plotted versus the negative logarithm of total copper concentration and displayed in Fig. 4. Vertical bars represent replicate cultures. The result from these data indicate that growth rate is not correlated directly to the total copper concentration. A family of four curves can be seen. Each curve represents a given chelator at varying concentration in the medium. Toxicity occurs, in the presence of such strong chelating agents as NTA or EDTA, at the point where most of the chelator is saturated with copper; this point is the region of greatest change in the growth rate per addition of total copper. Some research has shown that the number of possible ligand sites for copper (the copper complexing capacity of the medium) can be measured from this inflection point (Anderson and Morel, 1978; Davey et al., 1973; Sunda and Guillard, 1976; Sunda and Lewis, 1978; Gillespie and Vaccaro, 1978). Davey et al. (1973) used the copper bioassay method with T. pseudonana to measure the copper complexing capacity of artificial seawater, Charleston pond water and Brayton point water. Gillespie and Vaccaro (1978) used a naturally occurring bacterial population to do a copper bioassay in defined medium and natural water samples. Growth rate in their experiments was measured by the $^{14}CO_2$ uptake method. The experimental measurement of the copper complexing capacity of artificial media with different concentrations of

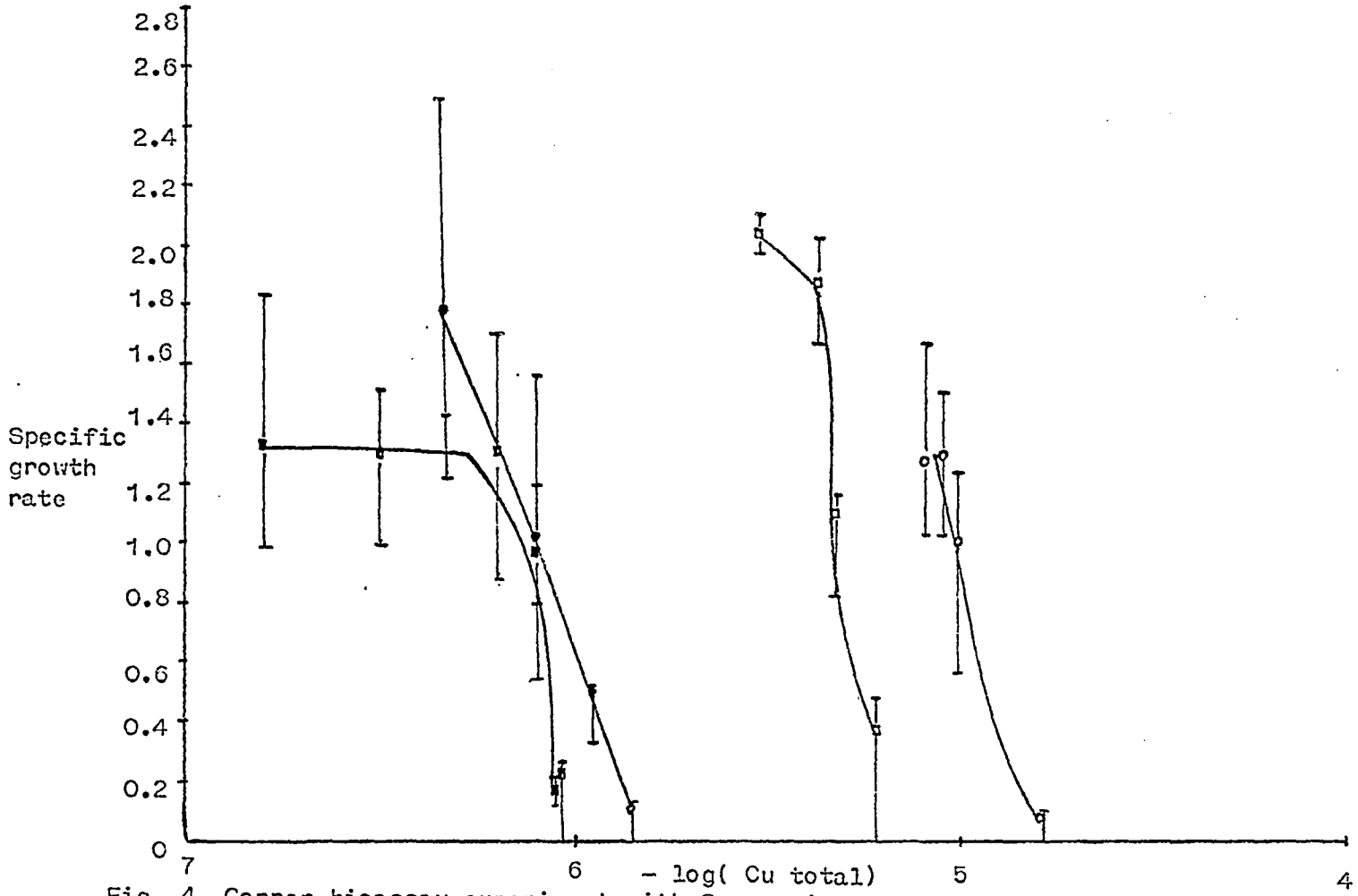

Fig. 4. Copper bioassay experiment with S. capricornutum: The specific growth rate versus the negative log of the total copper concentration. Vertical bars represent replicate cultures. Symbols represent the following: ■= 8.06 x 10^{-7} M EDTA, ●=1 x 10^{-6} M NTA, □= 5 x 10^{-6} M EDTA, ○= 1 x 10^{-5} M EDTA.

EDTA was found to be close to calculated values for copper complexation. Thus, it appears that copper bioassays can provide a reasonably good estimate of copper complexing capacities in natural waters. In this present research, the inflection point from each copper bioassay is near the chelator concentration; however, the method can only approximate the copper complexing capacity of a medium. A more precise method of calculating copper complexing capacities is discussed later in this section.

Although S. capricornutum is sensitive to total copper (Fig. 4), the actual linear relationship is between growth rate and pCu^* in the range 10.25 to 7.25 pCu^* units as seen in Fig. 5. This relationship can be expressed in the following manner:

$$\text{Growth rate} = 0.54\ pCu^* - 3.6 \qquad (4)$$

The correlation coefficient for these data is 0.92 for 300 samples. The vertical bars represent the range of growth rates within replicate cultures and the horozontial bars represent changes in pCu^* due to differences in pH within replicate cultures. Between days 1 and 2, the period of time in which the growth rate was calculated, the pH of the cultures changed by as much as 0.4 pH unit. This change in pH is primarily tied in with the carbonate buffering system in the medium. The aqueous CO_2 in the system is assumed to be in equilibrium with the air, and thus the total concentration of aqueous CO_2 is assumed to be constant at 1×10^{-5} M (see Appendix D for the calculation). Selenastrum capricornutum uses only aqueous CO_2 (Goldman et al., 1974; King and Novak, 1974). Since

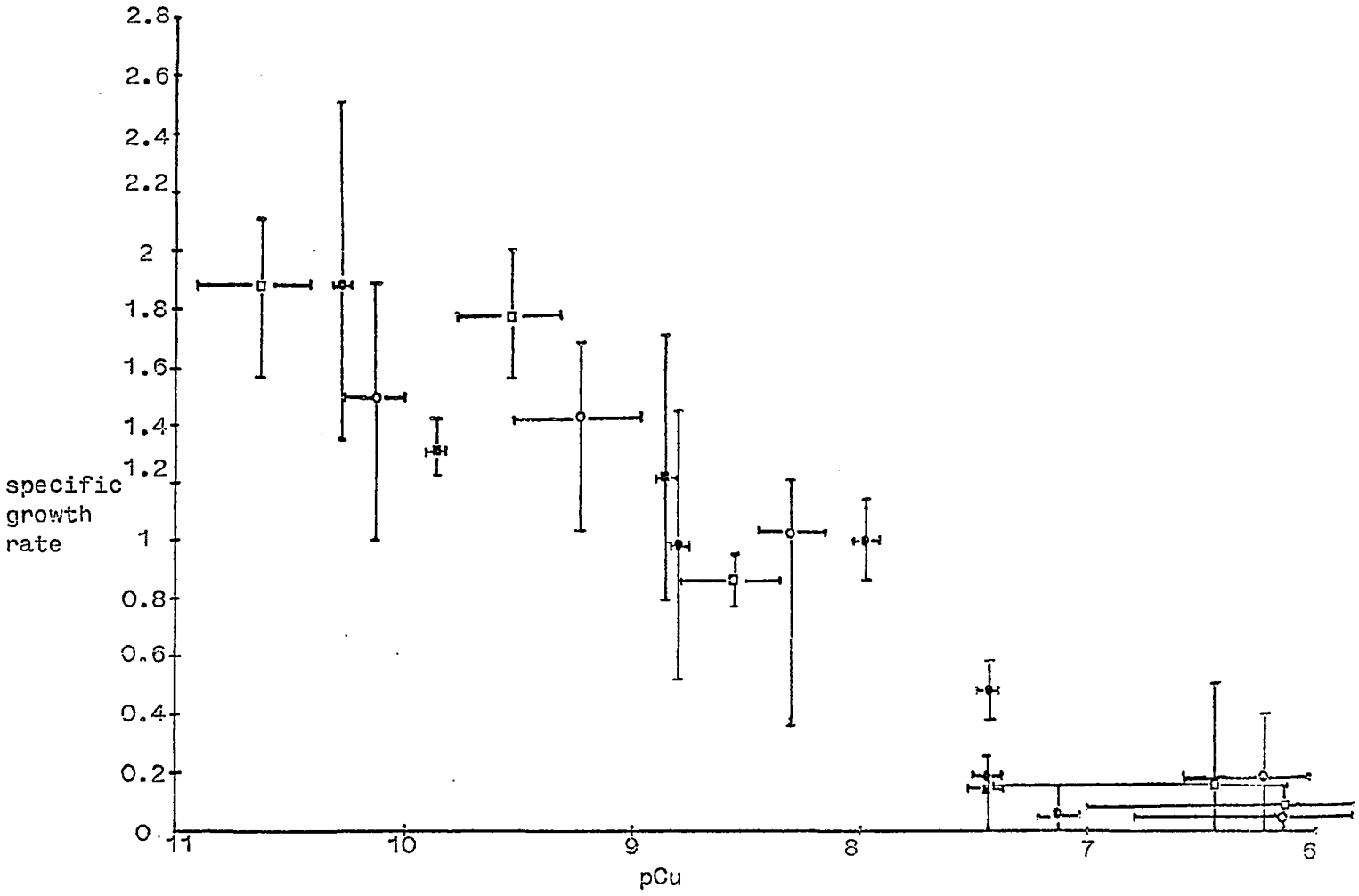

Fig. 5. Copper bioassay experiment with S. capricornutum: specific growth rate versus pCu. Vertical bars represent replicate cultures, horozontial bars represent changes in pCu due to changes in pH during the log phase growth of cultures. ■ = 8.06 x 10^{-7} M EDTA, ● = 1 x 10^{-6} M NTA, □ = 5 x 10^{-6} M EDTA, and ○ = 1 x 10^{-5} M EDTA.

the equilibration between aqueous CO_2 and CO_2 in the atmosphere is too slow a step to sustain growth, algae must get CO_2 from the soluble bicarbonate and carbonate species. The increase of pH is due to the release of hydroxide ion when bicarbonate and carbonate species are hydrolyzed to aqueous CO_2. Increases in pH also favor the shift from $Cu^{2+}(aq)$ to $CuOH^+$. The equilibrium constant for the hydrolysis of $Cu^{2+}(aq)$ is $10^{7.3}$, which is close to the initial pH of the medium. The pCu^* can be recalculated at the actual pH that has been measured in the culture by use of the MINEQL program. For most growth rates, pCu^* variability within replicate cultures appears to be no greater than 25 percent of the mean value. Other research also has established this linear relationship between pCu and growth rate for other organisms as shown in Table 2.

THE REVERSIBILITY OF COPPER TOXICITY

Selenastrum capricornutum cells from the copper bioassay study were recultured on agar containing modified EPA medium with 8.06×10^{-7} M EDTA. When cells grown for 6 days at various $Cu^{2+}(aq)$ concentrations were plated onto agar plates with no copper, the results of dividing versus total count (Table 3.) show that in 48 hours, all cells were viable. No algicidal conditions were found at any pCu^* used in the copper bioassay; thus under the bioassay conditions, copper was only algistatic. Bartlett et al. (1973) found that cultures of S. capricornutum grown in modified

TABLE II

RESEARCH ESTABLISHING A LINEAR RELATIONSHIP BETWEEN pCu AND THE GROWTH RATE

Research cited	Species	Method for determination of pCu	Correlation coefficient
Sunda and Lewis 1978	*Monochrysis lutheri*	Copper ion electrode	0.97
Anderson and Morel 1978	*Gonyaulax tamarensis*	calculated	0.98
Sunda and Guillard 1976	*Nannochloris atomos*	calculated	0.98
Sunda and Guillard 1976	*Thalassiosira pseudonana*	calculated	not linear

TABLE III

COPPER VIABILITY STUDY[+]

Total Copper concentration[@]	pCu[*]	The Percent cells undergoing division 6 hours	24 hours	48 hours
none added	—	36	61	100
none added	—	36	61	100
1.57	10.94	20	75	98
1.57	10.94	25	77	100
1.57	10.94	26	73	100
3.14	10.39	23	73	98
3.14	10.39	13	78	100
3.14	10.39	13	75	100
4.72	9.83	49	75	98
4.72	9.83	33	66	100
4.72	9.83	24	61	100
6.30	8.84	27	83	100
6.30	8.84	27	63	84
6.30	8.84	47	55	100
7.87	7.94	21	78	100
7.87	7.94	20	72	97
7.87	7.94	21	74	97
7.87	7.94	16	70	100
9.44	7.40	44	63	100
9.44	7.40	33	87	94
9.44	7.40	24	60	100
9.44	7.40	74	78	100
11.0	7.25	35	56	100
11.0	7.25	12	62	100
11.0	7.25	23	67	99
12.5	7.25	29	66	97
12.5	7.25	28	71	98
14.2	7.25	36	71	100
14.2	7.25	18	79	97
15.7	7.25	15	30	66
15.7	7.25	10	77	94
17.3	7.25	20	74	100
17.3	7.25	25	83	97
18.8	7.24	19	66	100
18.8	7.24	16	47	100

+— Cells were cultured on agar with Modified EPA medium (8.06×10^{-7} M EDTA without added copper)

@— The cells were initially grown in liquid culture in Modified EPA medium with 8.06×10^{-7} M EDTA. The units are $\times 10^{-7}$ M.

EPA medium at 8.06×10^{-7} M EDTA became algicidal at a total copper concentration of 4.72×10^{-6} M. This is more than twice the total copper concentration used in the present copper bioassay experiment for all sets of cultures. In modified EPA medium containing 8.06×10^{-7} M EDTA, the pCu, as well as all other soluble copper complexes, is buffered through malachite precipitation when the total copper concentration is 1×10^{-6} M or greater. The algistatic action of copper concentrations used in the present copper bioassays was reversible. Although not shown, algal cells will be killed after a considerable build up of malachite. Thus copper precipitation can affect algicidal conditions for S. capricornutum. This is also evidenced by the copper bioassay experiments of Morel et al. (1978) in which the diatom Skeletonema costatum showed a prolonged lag period when the malachite precipitation increased. Thus, the algicidal nature of metal is dependent not on $Cu^{2+}(aq)$, but on the precipitation of malachite in the medium.

A COMPARISON OF $Cu^{2+}(aq)$ SENSITIVITY OF 30 SPECIES OF ALGAE INCLUDING S. capricornutum

Maloney and Palmer (1956) grew 30 algal species on Gerloff's medium (Table 4.) in order to compare the growth rate of each species with the total copper concentration. Since growth rates of organisms grown on different media cannot be directly compared, as previously shown in Fig. 4., their results were used to calculate

TABLE IV

GERLOFF'S MEDIUM+

Component	Final Concentration molar
$Ca(NO_3)_2^+$	4.85×10^{-4}
K_2HPO_4	5.54×10^{-5}
$MgSO_4 \cdot 7H_2O$	1.1×10^{-4}
Na_2SiO_3	2.05×10^{-4}
Fe_3-citrate	2.40×10^{-5}
H_3-citrate	1.43×10^{-5}
$ZnSO_4 \cdot 7H_2O$	6.96×10^{-6}
$MnSO_4 \cdot 4H_2O$	1.59×10^{-5}
Na_2SiO_3	1.64×10^{-5}
$AlCl_3$	1.50×10^{-5}
H_3BO_3	3.23×10^{-5}
LiCl	2.36×10^{-5}
$CoCl_2 \cdot 6H_2O$	4.20×10^{-6}

+ -- No copper added and two times the NO_3 concentration

pCu^* from Gerloff's medium using the MINEQL program. The pCu^* values at two growth conditions were compared; one at the incipient condition and the other at the beginning of algistatic conditions for each organism tested (Table 5). The differences in $Cu^{2+}(aq)$ concentration between the most sensitive and least sensitive species shows a range of three orders of magnitude. Several reasons for this variability among species can be postulated: (1) internal cell physiological differences, (2) development of different active transport mechanisms, (3) cell wall modifications, or (4) extracellular release.

It can also be seen that different strains of the same species show differences in $Cu^{2+}(aq)$ sensitivity. Two strains of Ankistrodesmus falcatus differ by one-hundred fold in sensitivity to $Cu^{2+}(aq)$ at algistatic growth conditions. Foster (1977) has also shown differences in $Cu^{2+}(aq)$ sensitivity between two strains of Chlorella vulgaris; one strain grew much better in stressed copper medium, but both strains were equally sensitive to the same concentrations of intracellular copper. Thus, it can be concluded that physiological behavior of both strains is the same, but the tolerant strain must either lower the $Cu^{2+}(aq)$ concentration by producing extracellular products or possess the ability to prohibit transport of copper inside the cells or to transport copper outside the cell.

Looking at copper sensitivity in terms of groups or phyla of algae, it is worth noting that diatoms are probably the most sensitive of all algal groups. Differences in copper toxicity between the diatom, Nizschia palea, and a green alga, Chlorella

TABLE V

A COMPARISON OF pCu^{*} SENSITIVITY IN 30 SPECIES OF ALGAE

ALGAL SPECIES tested	pCu^{*} obtained at: INCIPIENT[©] GROWTH	ALGISTATIC[¢] GROWTH
Blue-green algae		
Calthrix braunii	-	8.15
Cylindrospermum licheniforme	11.03	10.35
Microcystis aeruginosa	-	11.03
Nostoc muscorum	10.35	9.92
Phormidium tenue	11.03	10.70
Plectonema mostocorum	-	11.03
Symploca erecta	9.92	8.15
Green algae		
Ankistrodesmus falcatus	10.70	9.92
Ankistrodesmus falcatus	10.35	8.15
Chlamydomonas communis	10.35	9.92
Chlamydomonas paradoxa	10.70	10.35
Chlorella variegata	-	10.70
Chlorococcum botryoides	-	9.92
Chlorococcum humicola	11.03	10.70
Coccomyxa simplex	11.34	10.70
Coelastrum proboscideum	10.35	9.92
Gloecystis grevillei	11.03	10.70
Mestaenium caldariorum	10.35	9.92
Oocystis marsonii lacustris	10.35	9.92
Oocystis marsonii	10.35	9.92
Scenedesmus basilensis	10.35	9.92
Scenedesmus obliquus	8.15	8.04
Selenastrum capricornutum	10.4	7.95
Sphaerella lacustris	-	10.70
Stigeoclonium nanum	11.03	9.92
Nannochloris atomus	10.40	8.70
Diatoms		
Achnathes linearis	10.70	10.35
Glomphonems paruulum	11.34	11.03
Nitzschia palea strain 1	-	11.03
Nitzschia palea strain 2	-	10.35
Nitzschia palea strain 3	11.03	10.70
Thalassiosira pseudonana	10.40	8.70

©— The pCu^{*} at which the growth rate is distinguishably lower than the growth rate of control cultures.

¢— The pCu^{*} at which the growth rate is close to 0.

pyrendoidosa, were compared (Steemann-Nielsen and Wium-Andersen, 1970). Copper toxicity was first expressed in terms of lag periods in the green alga at high pCu^{*}; however, inhibition of photosynthesis was the first sign of toxicity in the diatom. They concluded that copper in the green alga first adheres to the cell wall interfering with cell division while in the diatom, copper penetrates the cell more easily and blocks the photosynthetic pathway. In addition the membranes of the diatom were leaky with the loss of organic matter.

Selenastrum capricornutum is a fairly copper resistant algal species compared to 30 other species tested. Scenedesmus obliquus and Ankistrodesmus falcatus; green algae and Calothrix braunii and Syploca etecta; blue-green algae are other examples of $Cu^{2+}(aq)$ resistant algae.

II. PRODUCTION OF EXTRACELLULAR CARBON

Total carbon fixed and carbon excreted, in the absence of added copper, were experimentally measured in several $^{14}CO_2$ uptake experiments. The algae were grown in modified EPA medium as described in Materials and Methods. Samples were taken over a 48 hour growth period. The results of $^{14}CO_2$ uptake are shown in Fig. 6. Dark bottle counts (the $^{14}CO_2$ uptake under dark conditions) have been subtracted from the results. Bottles incubated in the dark showed very little $^{14}CO_2$ uptake and the amount of $^{14}CO_2$ uptake did not

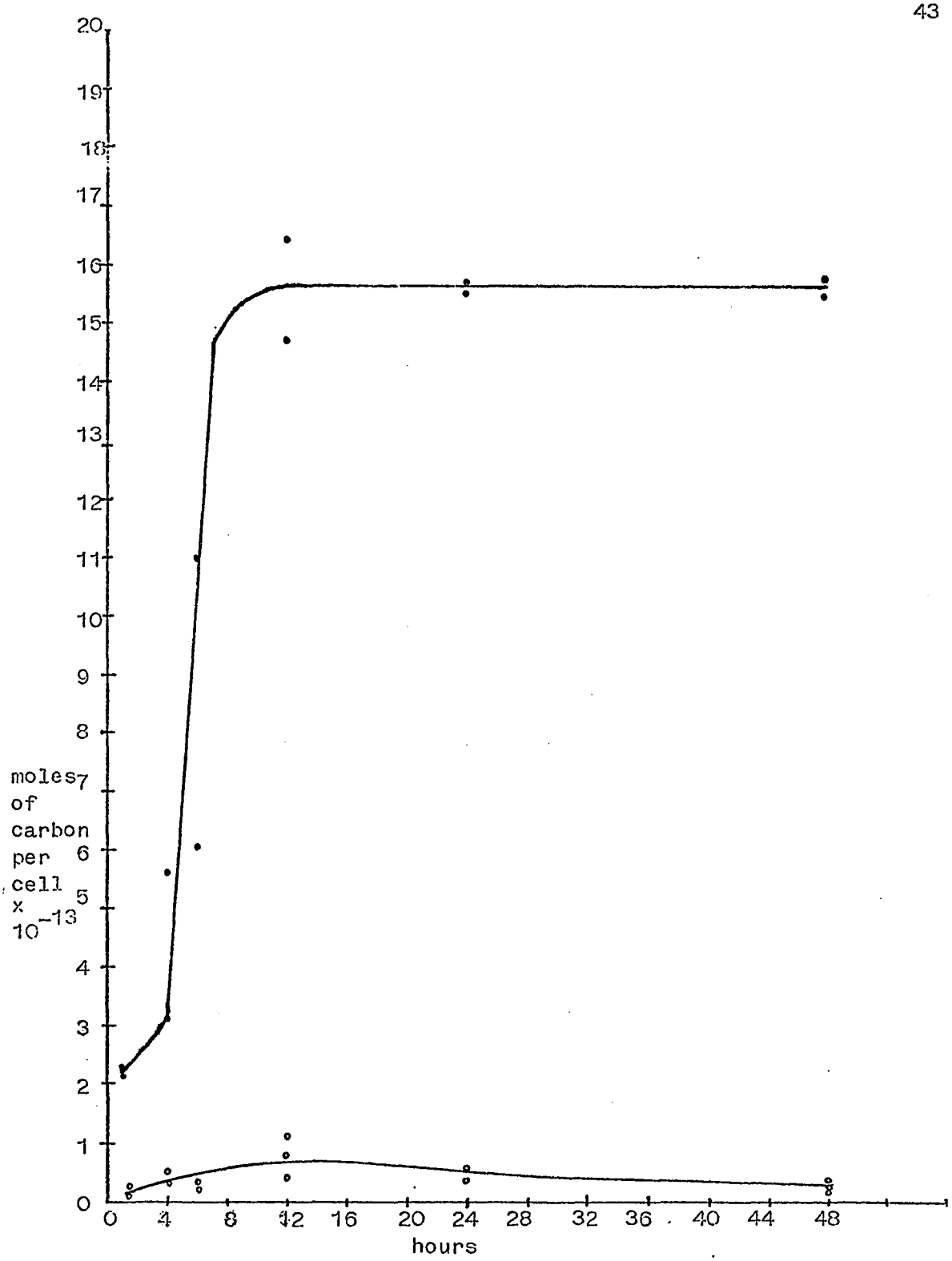

Fig. 6. Carbon 14 uptake by S. capricornutum. Symbols represent the following: • = the total carbon uptake by the cell and o = the extracellular carbon excreted.

increase with time. For an inital algal population of 5×10^{4} cells per ml, 1.2×10^{-14} ($\pm$ 4×10^{-15}) moles of carbon per cell was fixed under dark conditions. The uptake in the dark was one percent or less of the uptake in the light. As seen in Fig. 6., there is a pronounced increase in the total carbon fixed by the cells during the first 12 hours. This rapid increase of $^{14}CO_2$ is due to the difference in specific activity of carbon (the ratio of $^{14}CO_2$ to $^{12}CO_2$) inside the cell compared with that of the medium. Noting that cell division occurs once every 9.25 hours, nonsynchronously, a cell uptake rate for carbon can be calculated after this 12 hour equilibration period. The total organic carbon uptake was found to be 1.6×10^{-12} ($\pm$ 2×10^{-13}) moles per cell. The value for $^{14}CO_2$ uptake found in this study is similar to the value of 1.25×10^{-12} moles per cell calculated from the percentage of carbon (Goldman et al., 1974) and the dry weight of S. capricornutum (Toerien and Huang, 1973).

The amount of carbon excreted was found to be 5×10^{-14} ($\pm$ 3×10^{-14}) moles of carbon per cell. Since the values were obtained using cells cultured in modified EPA medium at 8.06×10^{-7} M EDTA without copper, it is evident that S. capricornutum produced extracellular products in the absence of toxic concentrations of copper. The amount of carbon excreted is about 3 percent of the total amount fixed by the cell.

As seen in Table 6., much higher amounts of excreted carbon

TABLE VI

EXTRACELLULAR RELEASE IN ALGAE AS A PERCENT OF TOTAL ASSIMILATED CARBON

Author	Species	Percent excreted+
Lewin 1956	Chlamydomonas spp.	15% - 57%
Jones 1962	Porphydridium cruentum	15%
Prager et al. 1959	Katodinium dorsalisulcum	90%
Moore and Tischer 1965	Anabaena flos-aquae	28%
Guillard and Hellebust 1971	Phaeocystis pouchetti	26% - 36.8%
Fogg 1965	naturally occurring phytoplankton	7% - 50%
Watt and Fogg 1966	naturally occurring phytoplankton	13% - 92%

+ Percent is expressed as the ratio of excreted carbon products to total carbon fixed by the cell.

have been reported. Smaller values of excretion, between 0 to 4 percent, have been reported by Majak et al. (1966) and Craigie et al. (1966) for a red and also a green alga. Many of the papers which report high values for extracellular excretion used the $^{14}CO_2$ uptake method. The reason for the high values of extracellular excretion reported by many papers may be found in a paper entitled, "Excretion of Organic Matter by Marine Phytoplankton: Do healthy cells do it?" (Sharp, 1977). Sharp (1977) states that the amount of excretion by phytoplankton may be an artifact, i.e. filtration or centrifugation may cause cell damage which would give an anomaly. This anomaly would make it appear that extracellular products had been released by the cells during growth. Sharp also states the need for controls which would eliminate problems with residual inorganic carbon and cell density effects on excretion. Fogg (1978) lists six possible sources of error from measured excretion by $^{14}CO_2$ uptake mathods. The error found most frequently is damage to the cells by (1) filtration, (2) centrifugation, and (3) changes in the ionic strength of the medium. Errors less commonly found are: (1) inadequate removal of inorganic $^{14}CO_2$ from the samples, (2) organic contamination from bicarbonate carbon-14 ampules, (3) loss during the storage and transport of field samples, (4) incubation times, and (5) errors introduced by the use of small volumes of filtrate in carbon-14 analysis. All of these errors would lead to high excretion values which are artifacts. Sharp (1978) states that algae probably

actively excrete materials, but at low levels. The present research attempted to minimize these errors which have been shown to create artificial excretion products.

III. THE CONCENTRATION OF EXTRACELLULAR PRODUCTS BY USE OF AMBERLITE XAD-8 RESIN

The algal filtrates require a concentration step in the analysis of copper complexation. This step eliminates cations and inorganic molecules, removes chelating agent i.e. EDTA or NTA, and concentrates extracellular products. Concentrations of extracellular products greater than 1×10^{-6} M are needed for detection of copper complexation using a copper ion electrode. Swallow et al. (1978) using the same methods as employed in this research for determination of copper complexation found little copper complexing capacity in unconcentrated algal filtrates. This may be due to the low concentration of extracellular products present in the medium.

In the concentration of organic molecules, there are only a few techniques which are efficient and convenient. Rohm and Haas Company (Anonymous , 1970) has produced several non-ionic exchange resins which are useful; included are XAD-2 (a polystyrene resin), XAD-7 and XAD-8 resins (acrylic ester polymer). In the present research, extracellular products from S. capricornutum were concentrated on XAD-8 resin.

Charged complexes and small ions of substantial net charge will not concentrate on XAD resin. Thus, by adjusting the pH of the material to be concentrated, inorganic ions and chelating agents can be eliminated during this concentration step.

Mantoura and Riley (1975b) concentrated decayed products from two species of algae, Hemiselmis referscens and Dunaliella primolecta, on XAD-2 resin. The filtrates were acidified to pH 2.2 prior to the concentration step. Adsorption efficiencies of 92 percent and 86 percent respectively, were obtained for these two species. The percent recovery from the elution of the products with methanol and 2 M ammonium hydroxide (1:1, v/v) was measured at 85 percent and 82 percent respectively. This result suggests that adsorption efficiencies for algal products are high on XAD resin and that a majority of these algal products are moderately basic.

Table 7. shows the resin efficiencies for the extracellular products from S. capricornutum. Samples were acidified to pH 1.7 prior to the concentration step. This pH was chosen because high recovery was obtained and because natural humic substances have been reported to show maximum retention by the column near this pH (Mantoura and Riley, 1975b). Labeled carbon-14 extracellular products from S. capricornutum were concentrated on an XAD-8 column. The percentage of extracellular products retained by the resin was calculated to be 95 percent and 96.5 percent for two separate and identical experiments. In addition, the recovery of materials from the resin by methanol elution was calculated

TABLE VII

THE EFFICIENCY OF XAD-8 RESIN

A. THE EFFICIENCY BASED ON THE ACTIVITY DIFFERENCES BETWEEN LABELED $^{14}CO_2$ EXTRACELLULAR PRODUCTS BEFORE AND AFTER FILTRATION THROUGH XAD-8 RESIN.

Experiment	Activity+	Activity#	Percent Efficiency
1	915.2 ± 41.73	44.62 ± 15.25	95.13
2	12,705	435 ± 185.8	96.57

B. EFFICIENCY BASED ON THE TOTAL ACTIVITY OF METHANOL ELUTION AND THE TOTAL ACTIVITY OF THE ELUATE

Experiment	Total Activity*	Total Activity@	Percent Efficiency
3	5.15×10^5	5.04×10^5	97.9

+-- The activity before filtration in countes per 10 minutes per 5 ml filtrates is calculated in experiments 1 and 2.

#-- The activity after filtration in counts per 10 minutes per 5 ml filtrates is calculated in experiments 1 and 2.

*-- Total activity was calculated from the radioactivity concentrated by XAD-8 resin in experiment shown above.

@-- Total activity was calculated from counts per 10 minutes per 5 ml sample corrected to base of the total volume of methanol-eluate.

to be 97 percent (Table 6., experiment 3.). From the high efficiency obtained, it appears that XAD-8 resin is effective in concentrating the extracellular products at pH 1.7.

The holding capacity of the XAD-8 resin was determined. This capacity is defined in terms of milliliters of filtrate that can be filtered through a gram of resin before exhaustion of the resin, as outlined in Method and Materials (subsection VI.). Algal filtrate, initially containing a final yield of 6×10^6 cells per ml (grown in modified EPA medium with 8.06×10^{-7} M EDTA without copper) was concentrated on a column containing 11 grams of resin. Fig. 7. shows a plot of carbon-14 activity versus liters of algal filtrate passed through the column. This figure can be restated as a holding capacity of 4.5 liters per gm of resin at pH 1.7. For this research no more than 20 percent of the holding capacity was used.

IV. THIN LAYER CHROMATOGRAPHIC (TLC) SEPARATION OF CONCENTRATED PRODUCTS

Filtrates from 16-liter cultures of S. capricornutum were concentrated on XAD-8 resin. The concentrated extracellular products were eluted with methanol and the products were separated by TLC as described in Materials and Methods (subsection VII.). Two types of filtrates were used. One of the filtrates was obtained from cells grown in a medium containing a copper concentration large enough to reduce the growth rate by one-half, while the second

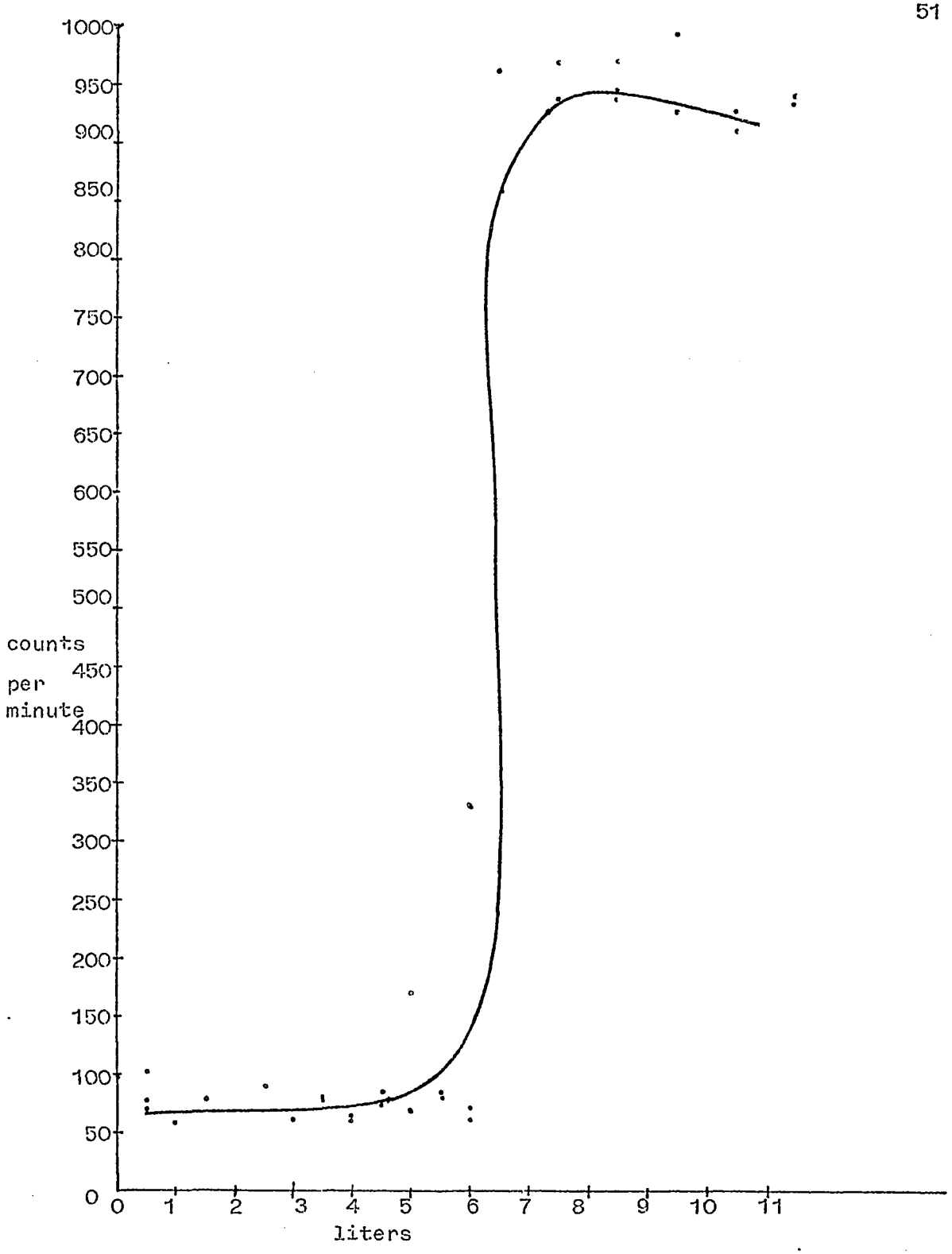

Fig. 7. The holding capacity of XAD-8 resin for carbon 14 labeled extracellular products from S. capricornutum at a pH of 1.7.

filtrate was obtained from cells grown in a medium containing no added copper. The concentrated extracellular products from the medium containing no added copper were separated on a two-dimensional TLC plate (Fig. 8.). Visualization of the products by ninhydrin, iodine staining or fluorescence showed at least 11 products. The concentrated extracellular products from the medium containing near toxic levels of copper were separated in a similar manner and the results are shown in Fig. 9. Visualization of the products showed at least 13 products. There is a great deal of variability in the migration of the products. However, in the concentrate from the medium containing copper, one additional product (which can be detected by its yellow color and by iodine staining) was always found to separate during chromatography. This product is shown by the arrow in Fig. 9. As shown later, the copper complexing capacity of this product is small and could be due to the absorption of copper to a ligand which may not be totally removed during the acidification and concentration steps. Preparative TLC plates were run in duplicate. One plate (to which reduced amounts of extracellular products were applied) was developed and the products were detected by the various methods: i.e. color, iodine staining, fluorescence or ninhydrin staining. Separate fractions were isolated from the other preparative plate by the procedure described in Materials and Methods (subsection VII.). A comparison between products obtained from the copper-free and

Fig. 8. Two-dimensional (TLC) chromatogram of concentrated Algal products from S. capricornutum, grown w/o Cu. The first separation is in the vertical direction with ethyl acetate, second separation, horozontial direction from left to right with n-butanol, formic acid and water 4:1:1 v/v/v. Symbols represent the following: ||| = ninhydrin positive reaction, ≡ = fluorescent activity, /// = visible, \\\ = iodine staining.

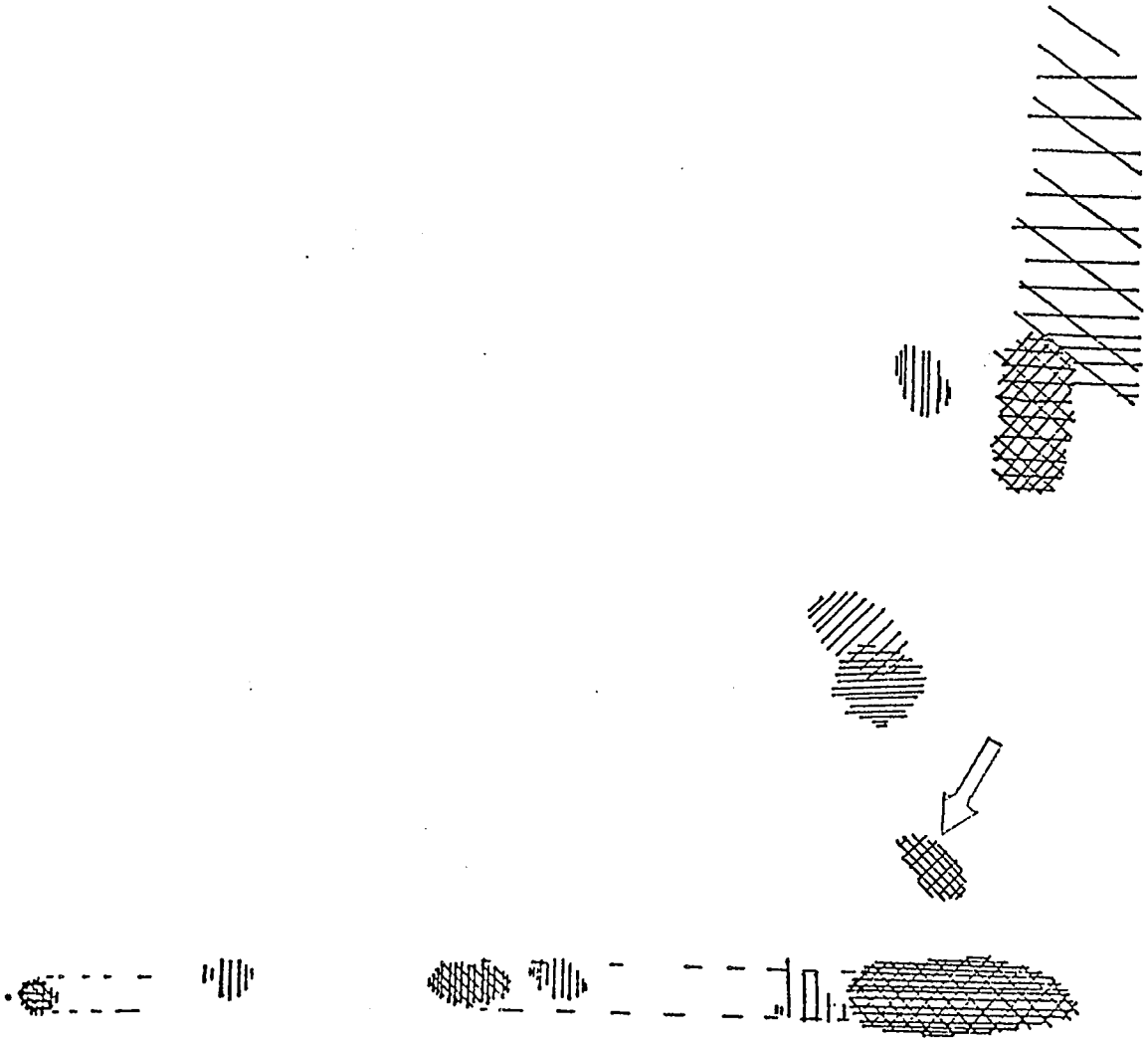

Fig. 9. Two-dimensional (TLC) chromatogram of concentrated algal products from S. capricornutum, grown with copper. The first separation is in the vertical direction with ethyl acetate as the solvent. The second separation is in the horozontial direction from left to right with n-butanol, formic acid and water 4:1:1 v/v/v. Symbols represent the following: ||| = ninhydrin positive reaction, ≡ = fluorescent activity, /// = visible, \\\ = iodine staining.

copper containing media is shown in Table 8. Essentially, both TLC plates represents the same mixture of products.

V. COPPER COMPLEXING CAPACITIES OF ALGAL FILTRATES AND CONTROL SOLUTIONS

DEFINITION

"Copper complexing capacity" of a solution is defined as the number of copper binding sites in moles per liter. The capacity is the number of copper binding sites, but not a measure of the types of copper-ligand complexes present.

METHOD AND THEORY

The copper complexing capacity was measured with a copper selective electrode, as outlined in Methods and Materials (subsection VIII.). Copper complexing capacities have been measured for various natural water samples using this potentiometric method (Ramamoorthy and Kushner,1975; Sunda and Hanson, 1978; Swallow et al., 1978; Sunda and Lewis, 1978).

Copper complexing capacities were based on the following theory. Under excess copper conditions the concentration of bound metal is equal to the total ligand concentration. Thus, the following relationship is established for mononuclear complexes:

$$\Sigma_i(CuL_i) = L_t \qquad (5)$$

where L_t is the total ligand concentration and $\Sigma_i(CuL_i)$ is the sum of all metal-ligand complexes, and i is the type of ligand. Assuming only one metal-ligand stoichiometry, the mass balance of

TABLE VIII

DETECTION OF SEPARATED FRACTIONS OF EXTRACELLULAR PRODUCTS

Fraction number	R_f	Color	Fluor-esence	Iodine stain	Ninhydrin stain
1a*+	0.09	+	–	–	–
1b@	0.09	++	–	–	–
2a	0.22	+	–	–	–
2b	0.17	+	–	–	–
3a	0.38	+	–	+	–
3b	0.30	+	–	+	–
4a	0.52	–	++	–	–
4b	0.56	–	++	–	–
5a	0.64	+++	–	–	–
5b	0.62	+++	–	–	–
6a	0.87	++	–	+	+
6b	0.95	++	–	+	+
7a	0.96	–	+	–	–
7b	0.97	–	–	–	–
8a#	0.11	–	+	+	–
8b	0.09	–	+	+	–
9a	0.31	–	+	++	++
9b	0.23	–	+	++	++
10a	0.45	–	+	–	++
10b	0.46	–	+	–	++
11a	0.63	–	+	–	+
11b	0.78	–	+	–	+
12a	0.86	+	+	+	–
12b	0.92	+	+	+	–
13a	0.96	+	++	+	–
13b	0.95	+	++	+	–

*— Separated fractions, algal cells were initially grown without added copper.

@— Separated fractions, algal cells were initially grown at 50 µg/l copper in Modified EPA medium with 8.06×10^{-7} M EDTA.

+— Numbers 1–7 were separated on TLC with ethyl acetate.

#— Numbers 8–13 were separated on TLC with n-butanol, formic acid and water 4:1:1 (v/v/v).

metal in the system can be expressed as:

$$Cu_t = Cu^{2+}z + \Sigma_i(CuL_i) \quad (6)$$

where Cu_t is the total metal in the system and $Cu^{2+}z$ is the sum of all copper-carbonate and copper-hydroxide and ionic copper concentrations. The z term is defined in Appendix D. Substituting equation (5) into equation (6), the following equation can be derived for the determination of the copper complexing capacity of a solution:

$$Cu_t = Cu^{2+}z + L_t \quad (7)$$

At any stated pH, under excess copper conditions, all ligand sites are bound to copper, thus any change in the total copper concentration yields an equal and proportional increase in free copper, copper-hydroxide, and copper-carbonate species. As shown in Appendix D, if the ionic copper is measured at an acidic pH, less than 5.0, the amount of copper-hydroxide and copper-carbonate species approaches zero and can be neglected, hence z equals unity. A plot of Cu_t versus Cu^{2+}(aq) gives a slope of 1 with an x-intercept equat to L_t, the copper complexing capacity of the solution. Fig. 10. shows a representative plot for a solution of NTA (1.13×10^{-4} M). A similar plot for an algal filtrate is shown in Fig. 11. Both experiments were performed at pH 4.0.

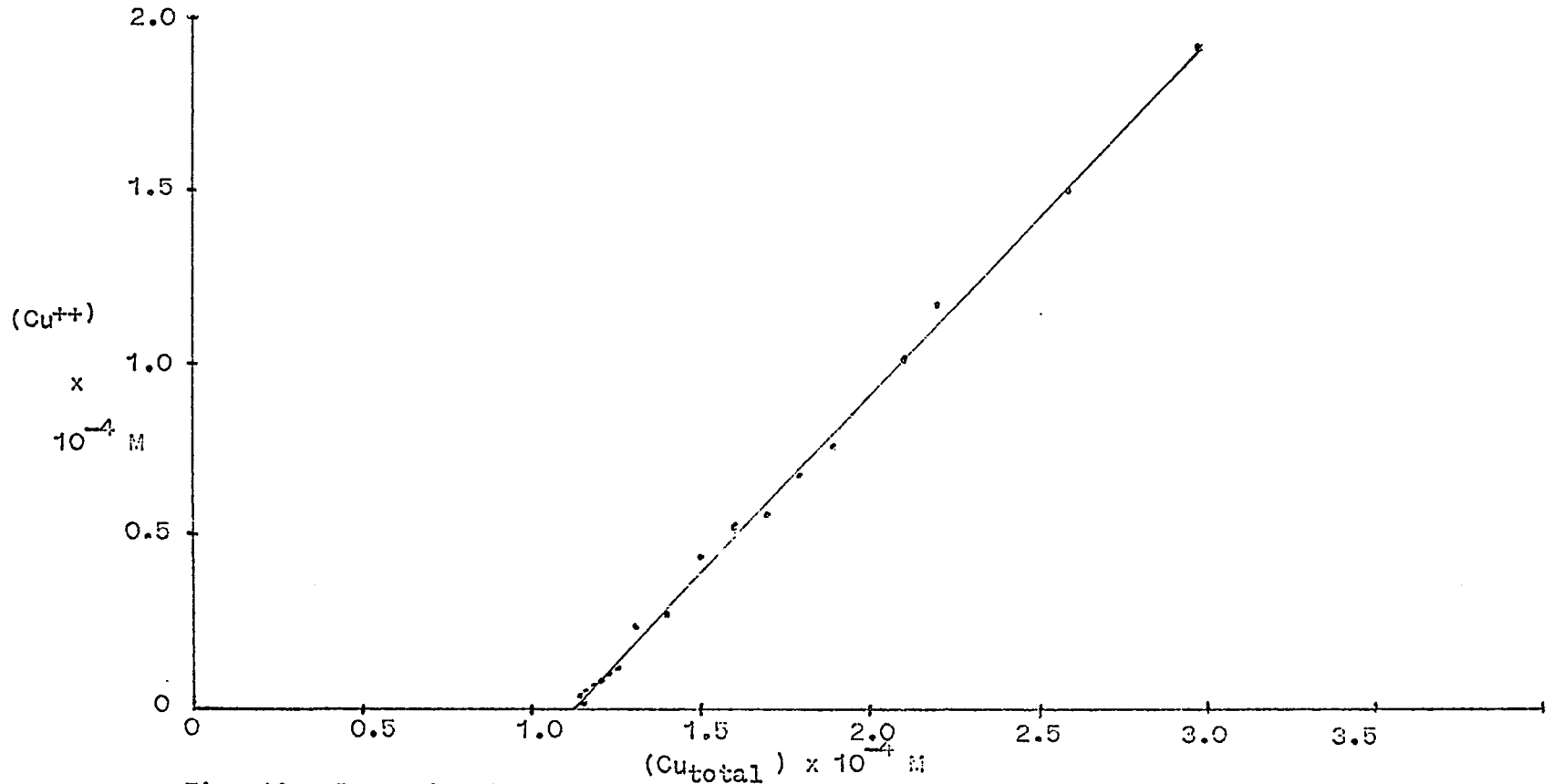

Fig. 10. Determination of the copper complexing capacity from a copper titration of 1.13×10^{-4} M NTA solution, pH=4.0 in 1×10^{-1} M KNO_3.

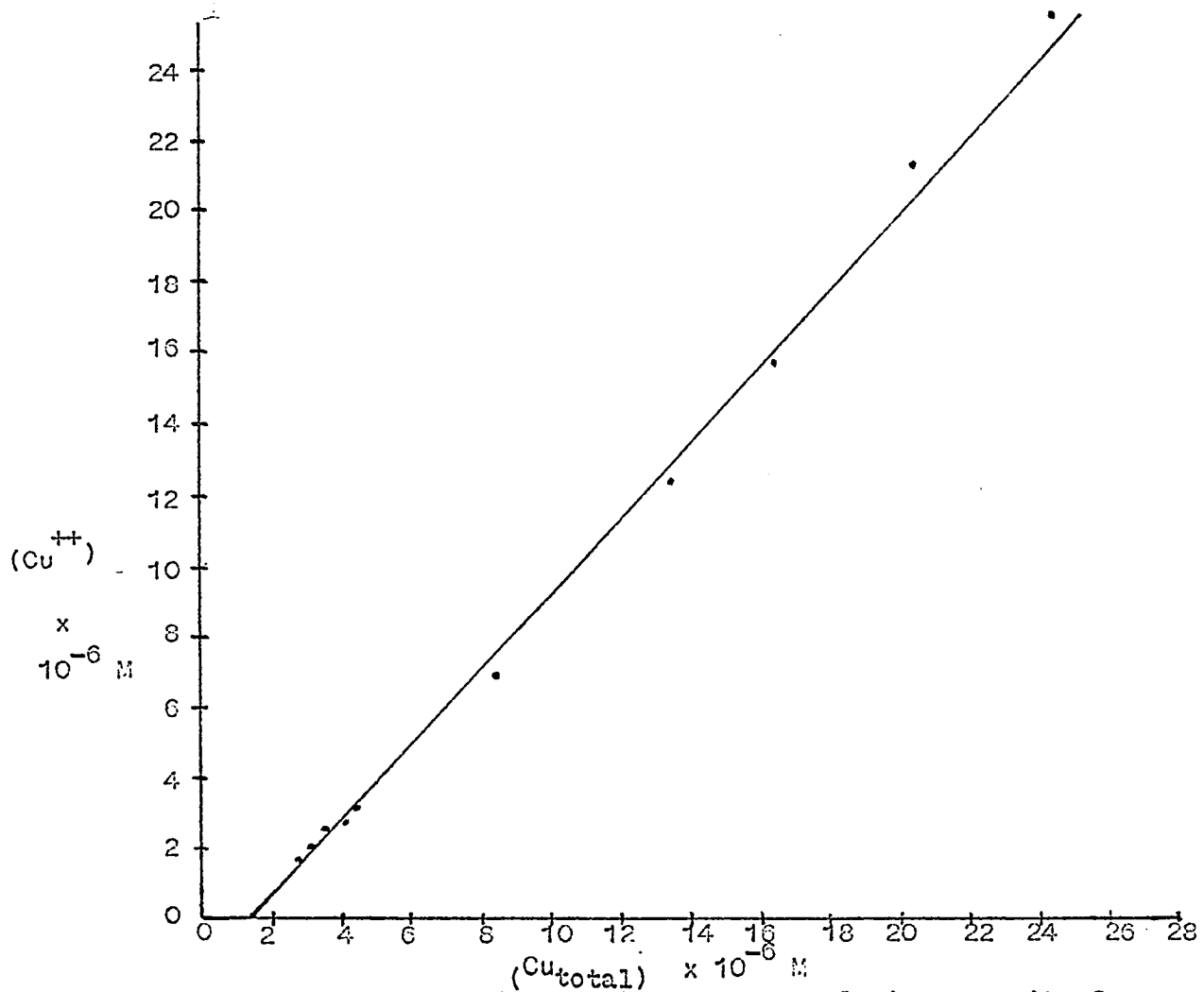

Fig. 11. Determination of the copper complexing capacity from a copper titration of a solution containing extracellular products from S. capricornutum, pH=4.0 in 1 x 10^{-1} M KNO_3.

THE COPPER COMPLEXING CAPACITY OF EXTRACELLULAR PRODUCTS FROM UNCONCENTRATED ALGAL FILTRATES

The copper complexing capacity of 7 day algal filtrates initially containing 3×10^{-6} M EDTA was measured. Complexation due to inorganic nutrients and EDTA were subtracted from the total copper complexing capacity in order to determine the capacity of the extracellular products alone. This extracellular copper complexing capacity was experimentally determined at 1.8×10^{-7} M ($\pm$ 5.7×10^{-8} M), at pH 5.0. These results indicate that the release of extracellular products might have a marginal effect on the ionic copper concentration in weakly chelated laboratory medium, but in natural waters where concentrations of complexing agents are low, the effect of these extracellular products may be quite significant in regulating $Cu^{2+}(aq)$.

The copper complexing capacity of several control solutions was considered. Experimental copper complexing capacities using copper selective electrodes were compared with theoretical values for different control solutions (Table 9.). The results show good correlation between measured and calculated values (copper complexing capacities).

In order to check if XAD-8 resin had any affinity for EDTA in solution at pH 1.7, another control was devised. The copper complexing capacities of solutions containing EDTA were determined

TABLE IX

THE COPPER COMPLEXING CAPACITY OF MODIFIED EPA MEDIUM CONTAINING VARIOUS EDTA CONCENTRATIONS

	COMPLEXING CAPACITY	
EDTA CONCENTRATION	CALCULATED VALUE@	MEASURED VALUE*
0	1.65×10^{-7}	2.04×10^{-7}
5×10^{-6}	5.17×10^{-6}	5.10×10^{-6}
3×10^{-6}	3.17×10^{-6}	3.08×10^{-6}

@— Calculated using the MINEQL program in molar concentration.

*— Measured using the copper ion selective electrode method in molar concentration.

before and after passing a solution through XAD-8 resin. In duplicate experiments the copper complexing capacity before filtering was 6.55×10^{-6} M and 7.04×10^{-6} M. After passage through the column, complexing capacities of 6.61×10^{-6} M and 7.09×10^{-6} M were obtained. It is apparent that there is little difference between the copper complexing capacity of the effluent and influent for either solution. Thus, it can be said that XAD-8 resin does not concentrate EDTA under the experimental conditions used for this work.

A control experiment was also devised in order to determine the extent of uptake of EDTA by S. capricornutum, since EDTA is the primary copper buffering agent used in modified EPA medium. Seven day algal filtrates, cultured in modified EPA medium with 3.0×10^{-6} M EDTA (without copper), were concentrated on XAD-8 resin. A comparison between the copper complexing capacity of this eluate and that of fresh modified EPA medium with 3.0×10^{-6} M EDTA was made. The copper complexing capacity of the nutrient medium was calculated to be 3.16×10^{-6} M while direct measurements gave a mean value of 3.08×10^{-6} M. The algal filtrate from XAD-8 resin had a measured copper complexing capacity of 3.11×10^{-6} M. These results indicate that there is little difference between uninoculated modified EPA medium and the algal filtrate after passage through XAD-8 resin. Since EDTA is not concentrated by the resin, most of the inorganics and all of the EDTA must be

in the effluent. These results suggest that S. capricornutum did not concentrate EDTA inside the cell. Since the cells were filtered from the medium at physiological pH, these results also indicate that EDTA was not concentrated on the cell surface.

THE COPPER COMPLEXING CAPACITY OF CONCENTRATED EXTRACELLULAR PRODUCTS

As shown in Table 10., copper titrations were performed at pH 5.0 and 6.0 for concentrated filtrates in which cells were either grown without copper or at a copper concentration high enough to reduce the growth rate by one-half. The copper complexing capacity of concentrated products obtained from filtrates of cells grown without copper is 2.0×10^{-7} M ($\pm$ 7×10^{-8} M). Even though there is a large variability in copper complexing capacity, the results are comparable to those calculated from unconcentrated algal filtrates. The concentrated fraction obtained from filtrates of cells grown with copper had a copper complexing capacity of 1.8×10^{-7} M ($\pm$ 6×10^{-8}M). The copper complexing capacities of both concentrated algal filtrates were calculated back to the capacity of the unconcentrated nutrient medium. A one-tailed t-test was performed in order to determine if there was a significant difference between the two sets of copper complexing capacities for concentrated filtrates. The results indicate, at a 95 percent confidence level, that there is no difference between the two

TABLE X

THE COPPER COMPLEXING CAPACITIES, CONDITIONAL STABILITY CONSTANTS AND AVERAGE MOLECULAR WEIGHTS OF CONCENTRATED EXTRACELLULAR PRODUCTS FROM Selenastrum capricornutum

Copper titration experiment	pH	Copper[@] complexing capacity	Log K_c^*	Molecular weight (x 10^4)	Correlation coefficient
1A[+]	5.0	1.41	5.67	11.0	0.977
2A	5.0	1.17	5.77	13.2	0.990
3A	6.0	3.01	5.39	5.14	0.980
4A	6.0	2.61	5.41	5.91	0.987
1B[#]	5.0	1.95	5.55	7.92	0.997
2B	5.0	2.49	5.45	5.42	0.996
3B	5.0	1.12	5.80	11.5	0.997
4B	6.0	2.15	5.53	6.28	0.981
5B	6.0	2.09	5.80	6.46	0.870

@— The units are in moles per gram x 10^{-6}.
*— K_c is the conditional stability constant.
+ — A^e represents concentrated filtrates in which cells were initially grown in the absence of added copper.
#— B represents concentrated filtrates in which cells were initially grown in 50 µg / l copper in modified EPA medium at 8.06 x 10^{-7} EDTA.

groups. It appears that S. capricornutum grown at near toxic copper concentrations does not produce products which specifically complex copper. As discussed later, average conditional stability constants and average molecular weights for these two types of concentrated XAD-8 materials furthur support this conclusion.

THE COPPER COMPLEXING CAPACITY OF SEPARATED PRODUCTS

The copper complexing capacity of 13 concentrated and TLC separated fractions are listed in Table 11. It can be seen that the slope for most plots of total copper concentration versus $Cu^{2+}(aq)$ concentration is approximately 1 with greater than 99 percent correlation. Although not shown, only one-third of the total copper complexing capacity was recover by TLC from the concentrated filtrates. This low recovery may be accounted for by the incomplete elution of the products from silica gel G.

As seen in Table 11, no individual fraction could account for more than 35 percent of the total recovered copper complexing capacity when cells were grown in the absence of copper (fraction 3a contained 32 percent and fraction 9a contained 24 percent of the total recovered capacity). The majority of the fractions complexed less than 5 percent of the total complexing capacity. However, fraction 2b recovered from the filtrate of cell grown in the presence of copper, complexes 40 percent of the total copper complexing capacity recovered. Again, for filtrates

TABLE XI

THE COPPER COMPLEXING CAPACITY OF SEPARATED FRACTIONS

Fraction number	Complexing capacity+	% Total recovery	Slope	Correlation coefficient
1a*	35.6	5.28	1.01	0.9998
1b@	77.0	14.86	0.96	0.9995
2a	95.8	14.23	1.05	0.9989
2b	189.0	36.48	1.40	0.9977
3a	216.6	32.18	1.32	0.9998
3b	30.0	5.79	0.91	0.9996
4a	20.0	2.96	1.08	0.9996
4b	18.8	3.63	0.88	0.9997
5a	82.7	12.29	1.27	0.9981
5b	19.2	3.70	1.03	0.9999
6a	5.0	0.75	1.14	0.9996
6b	1.5	0.23	1.04	0.9979
7a	6.2	0.93	1.18	0.9985
7b	1.3	0.26	1.10	0.9994
8a	1.6	0.25	1.07	0.9999
8b	14.4	2.78	1.02	0.9989
9a	164	24.37	0.99	0.9998
9b	7.3	1.42	0.97	0.9996
10a	7.6	1.13	0.98	0.9995
10b	63.8	13.1	1.10	0.9997
11a				
11b	51.1	9.86	1.03	0.9957
12a	37.8	5.62	1.00	0.9984
12b	8.2	1.58	1.26	0.9996
13a	14.3	2.76	0.98	0.9995
13b	17.0	3.28	0.95	0.9994

*— Separated fractions, algal cells were grown without copper.
@— Separated fractions, algal cells were grown at 50 µg/l copper in modified EPA medium with 8.06×10^{-7} M copper.
+— The complexing capacity is in 10^{-10} M units.

whose cells were initially grown with copper, a majority of fractions complexed less than 5 percent of the total capacity. It can be concluded from these results that the copper complexing capacities from the two sets of separated products, whose cells were grown initially with and without copper, are similar.

VI. DETERMINATION OF CONDITIONAL STABILITY CONSTANTS FOR CONCENTRATED EXTRACELLULAR PRODUCTS

The stoichiometry of metal-ligand complexes is of critical importance in the calculation of conditional stability constants. This stoichiometry can be determined from ligand titrations (where metal is titrated with ligand) as seen in Appendix B. Methods for ligand titrations are found in Methods and Materials (subsection VIII.).

The conditional stability constants and average molecular weights for concentrated extracellular products were determined from Scatchard plots. Data needed for Scatchard plots are obtained from copper titrations (ligand titrated with metal) where the ionic copper activity is measured at constant pH and constant total ligand concentration. A derivation of a Scatchard equation for a 1:1 metal-ligand system is given in Appendix C. The molecular weight of the ligand and the conditional stability constant of the copper-ligand complex can be obtained from the slope and y-intercept of Scatchard plots. Scatchard plots have been used to calculate

stability constants for copper-ligand complexes associated with natural waters (Mantoura and Riley, 1975a;Sunda and Hanson, 1978). The calculation of concentrations of soluble copper-hydroxides and copper-carbonates, as a function of pH, is found in Appendix D. Copper-hydroxide and copper-carbonate species have been eliminated from stability constants. The constants reflect only the extracellular product complexation.

To test the Scatchard method for determining copper complexation of solutions, 1×10^{-4} M NTA samples were titrated with copper at pH 4.0. From a linear regression of a Scatchard plot for this titration, correlation coefficient of 0.9986 was obtained, as seen in Fig. 12. From the slope and y-intercept, a molecular weight of 190.8 and a conditional stability constant of $10^{7.39}$ was calculated. The atomic weight of NTA is 190 and at pH 4.0 the calculated conditional stability constant is $10^{7.48}$ based on constants given in Irving et al. (1967). Thus it is apparent that experimental values close to theoretical can be obtained.

For a mixture of ligands, a conditional stability constant may depend upon the concentration of total ligand and copper. Cheam (1973) and Ramamoorthy and Kushner (1975) used copper ion selective electrode to measure stability constants for mixtures of ligands. In each case, calculated values of the conditional stability constants changed during the titration. MacCarthy (1977; 1979) has determined specific limiting conditions where conditional stability constants for mixtures of ligands do not

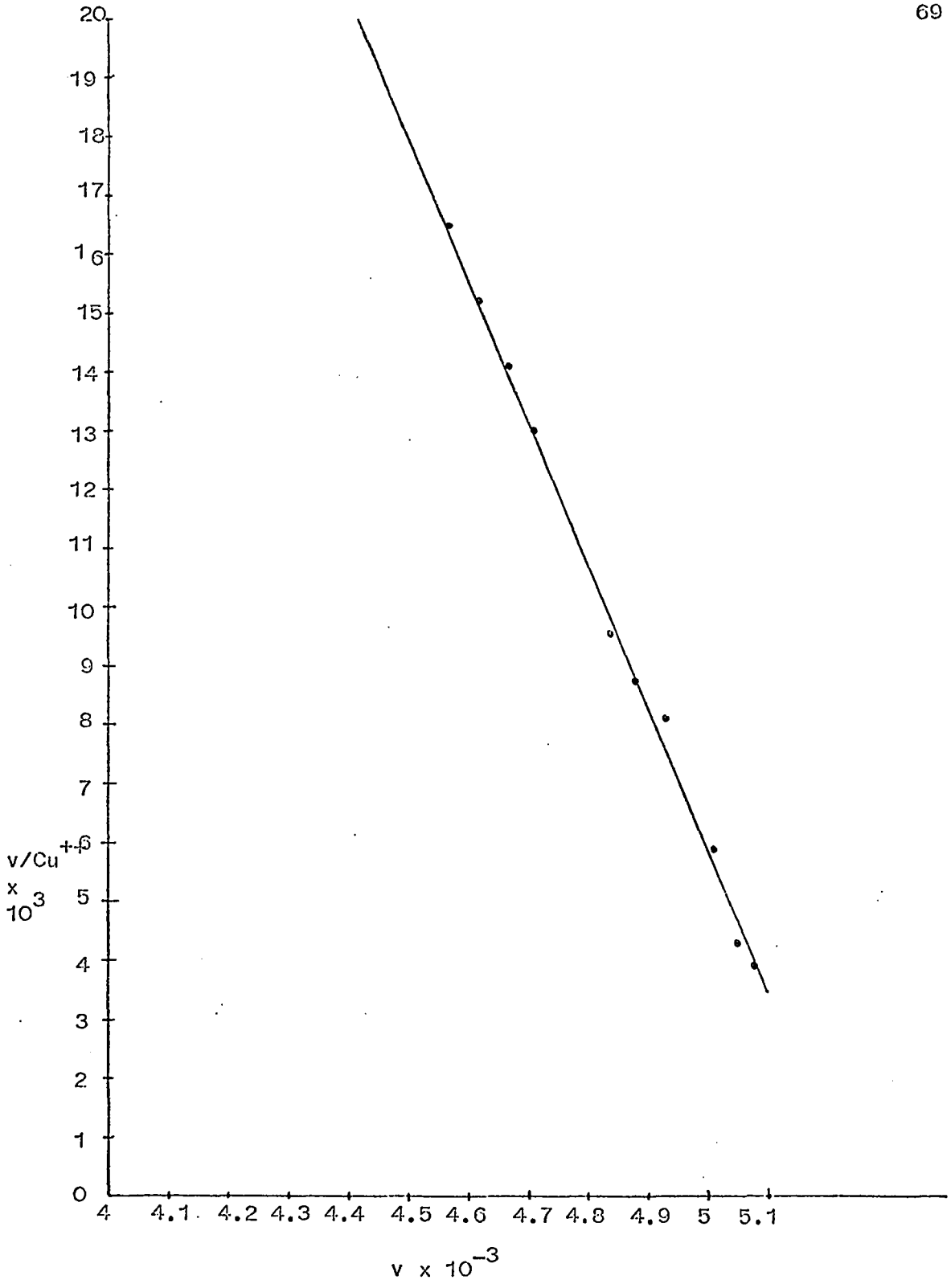

Fig. 12. A typical Scatchard plot from a solution containing 1×10^{-4} M NTA, pH= 4.0, 1×10^{-1} M KNO_3 at pH 4.0.

change during a copper or ligand titration. The constants at limiting conditions are called conditional limiting average stability products (C.L.A.S.P.). Two limiting conditions are specified; (1) Shubert's condition where there is very little metal present relative to the amount of ligand and, (2) the conditions in which excess metal is present. Both conditions minimize ligand competition for the metal. MacCarthy et al. (1979) provide mathematical validity for C.L.A.S.P. values in which multiple complexes of different stoichiometries may be involved; however, at the time of this writing only mononuclear complexes are involved in the calculations. Experimentally, Cheam (1973) obtained unchanged stability constants (C.L.A.S.P. values) from a copper titration of fulvic acid when the mole fraction of copper was greater than 0.3

Concentrated extracellular products from S. capricornutum were titrated with copper. Scatchard plots of these copper titrations produced linear functions over three orders of total copper concentration indicating that the conditional stability constant has reached C.L.A.S.P. values (Fig. 13.). A ratio of 1:1 copper-ligand was determined using titration data with theoretical calculations listed in Appendices B and C. From the concentrated filtrates of cells grown in the absence of copper, an average conditional stability constant of 3.8×10^5 ($\pm$ 1×10^5) was obtained with an average molecular weight of 8.8×10^4 ($\pm$ 3×10^4). The following equilibrium is specified for the average conditional

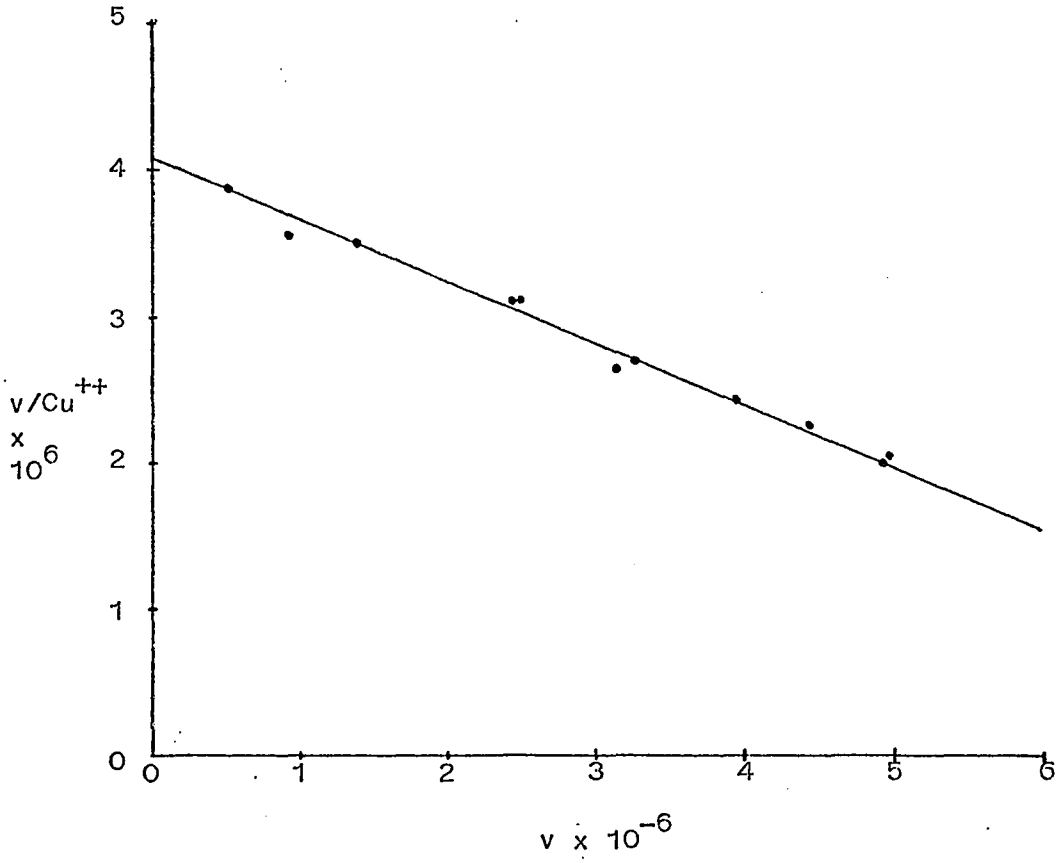

Fig. 13. A Scatchard plot of a solution containing extracellular products from S. capricornutum, pH= 5.0, 1×10^{-1} M KNO_3 at pH 5.0.

stability constant:

$$Cu^{2+}(aq) + H_xL \rightleftarrows CuL + xH^+ \qquad (8)$$

where H_xL is the protonated ligand(s) and CuL is the copper ligand complex(es). The conditional stability constant is defined as follows:

$$K_e = \frac{\beta^*(1-\alpha)}{(H^+)^x} = \frac{(CuL)}{(H_xL)(Cu^{2+})} \qquad (9)$$

where K_e equals the conditional stability constant and $1-\alpha$ is the protonated fraction for the ligand(s). The conditional stability constants for concentrated filtrates from cells grown in the presence of copper as previously described were measured at 4.7×10^5 ($\pm 1 \times 10^5$) with an average molecular weight of 7.5×10^5 ($\pm 2 \times 10^4$). A one-tailed t-test was performed on the two groups of filtrates to see if there was any statistical difference between the two. At the 95 percent confidence level the two sets were the same.

The conditional stability constants for NTA were calculated between pH 2.0 and 4.0 (Fig. 14.) from acid-base titrations in which $Cu^{2+}(aq)$ was measured with a copper selective electrode at constant total copper and ligand concentrations. The measurement of the conditional stability constant is based on the pH, $Cu^{2+}(aq)$, and total ligand and copper concentrations as shown in Appendix E. Methods for acid-base titrations are found in Methods and Materials (subsection VIII). In addition, theoretical

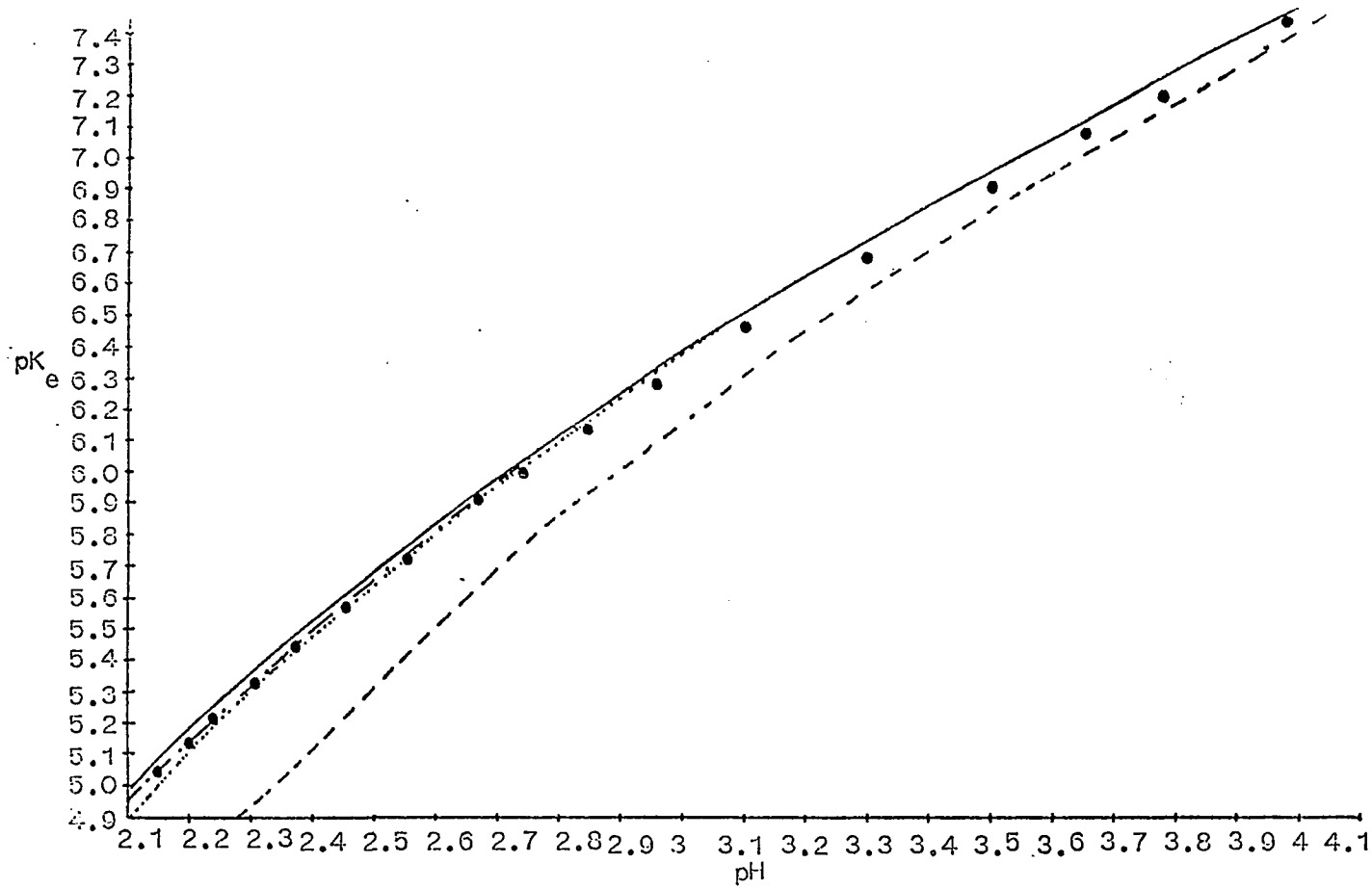

Fig. 14. The conditional stability constants for NTA as a function of pH. Solid and dotted lines represent theoretical caluclations of conditional stability constants where……= pK_1=1.89, pK_2=2.49, pK_3=9.73, pK_{CuNTA}=13.23;-·-·= pK_1=1.71, pK_2=2.47, pK_3=9.71, pK_{CuNTA}=13.21; —= pK_1=1.71, pK_2=2.47, pK_3=9.73, pK_{CuNTA}=13.23, pK_{CuHNTA}= 14.46;---=pK_1= 1.98, pK_2= 2.84, pK_3= 9.76, pK_{CuNTA}=13.18, MINEQL constants. The solid dots represent experimental values from the copper electrode data in this research and in McBryde et al. (1973), $Cu(NO_3)_2$ = 1.05 x 10^{-3} M and NTA = 2.11 x 10^{-3} M.

values for the conditional stability constants of NTA can be calculated using pK_a's and copper-NTA stability constants found in the literature (Irving et al., 1967; Schwarzenbach et al., 1949; Westall et al., 1976; McBryde et al., 1973) The derivation of the equation required to calculate conditional stability constants is in Appendix F. Experimental and theoretical values for the conditional stability constants for NTA versus pH is given in Fig. 14. Results indicate a close correlation between the experimental and theoretical values of conditional stability constants calculated from the pK_a's determined by Irving et al. (1967) and Schwarzenbach (1949) and copper-NTA stability constants from McBryde et al. (1973). However, calculated values based on constants from the data base in the MINEQL program (Westall et al., 1976) were lower than the experimental results. Over the entire range of pH, the calculated values underestimated the conditional stability products. From these results it can be seen that a theoretical calculation of a conditional stability constant is only as accurate as the metal-ligand constants used to calculate it.

Acid-base titrations were also performed on the concentrated extracellular filtrates from S. capricornutum. The total concentration of copper was 4.0×10^{-5} M and the total concentration of ligand was 4.4×10^{-5} M. As shown in Fig. 15., the conditional stability constant remains unchanged over a pH range

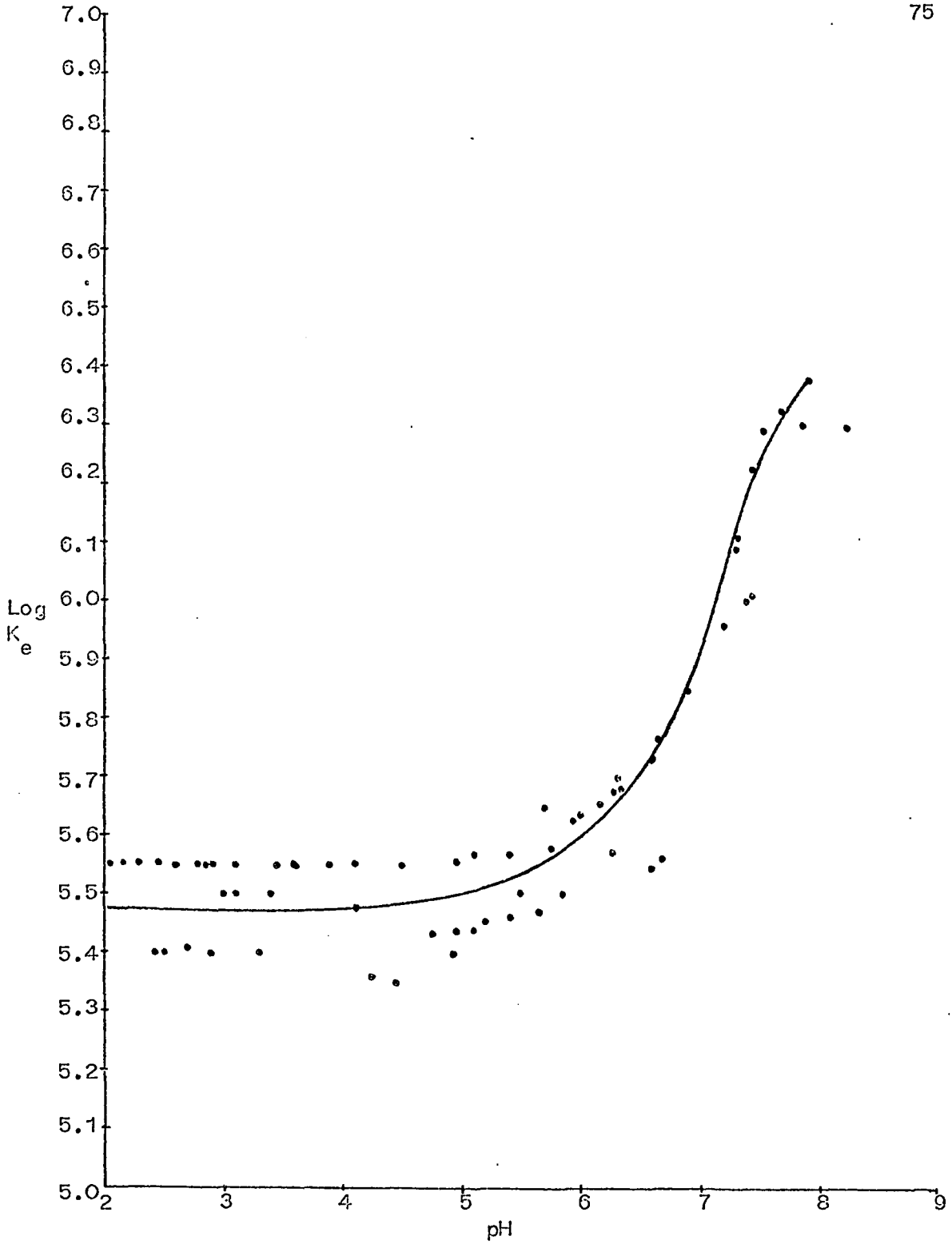

Fig. 15. The conditional stability constants from concentrated extracellular products from S. capricornutum as a function of pH.

from 2.0 to 6.0 and then starts to climb due to decrease in proton competition for the ligand sites.

The conditional stability constants for three copper ligand complexes are compared with concentrated extracellular filtrates from S. capricornutum over a pH range from 2.0 to 7.0 (Fig. 16.). It appears that the products in the concentrated extracellular filtrate have less affinity for copper than does NTA, but more than fulvic acid, a class of ligands prevelant in natural waters. This indicates that the conditional stability constants of the organic matter of extracellular filtrates from S. capricornutum are of sufficient strength to effectively compete for copper with many organic and inorganic ligands present in natural waters.

VII. EFFECT OF COPPER UPTAKE ON $Cu^{2+}(aq)$

Uptake of copper by the bioassay organism may have an affect on $Cu^{2+}(aq)$ if the alga is cultured in a weakly metal buffered medium. Sunda and Guillard (1976) measured the amount of copper taken up by the diatom Thalassiosira pseudonana after three days of growth in a defined seawater medium. At near toxic copper concentrations, the highest measured copper content in T. pseudonana was 3×10^{-15} moles of copper per cell at a pCu of 8.5. This accounts for an uptake of 3×10^{-7} moles of copper per liter for cultures containing 1×10^{5} cells per ml.

The copper content of S. capricornutum cells was analyzed at four copper concentrations in modified EPA medium with

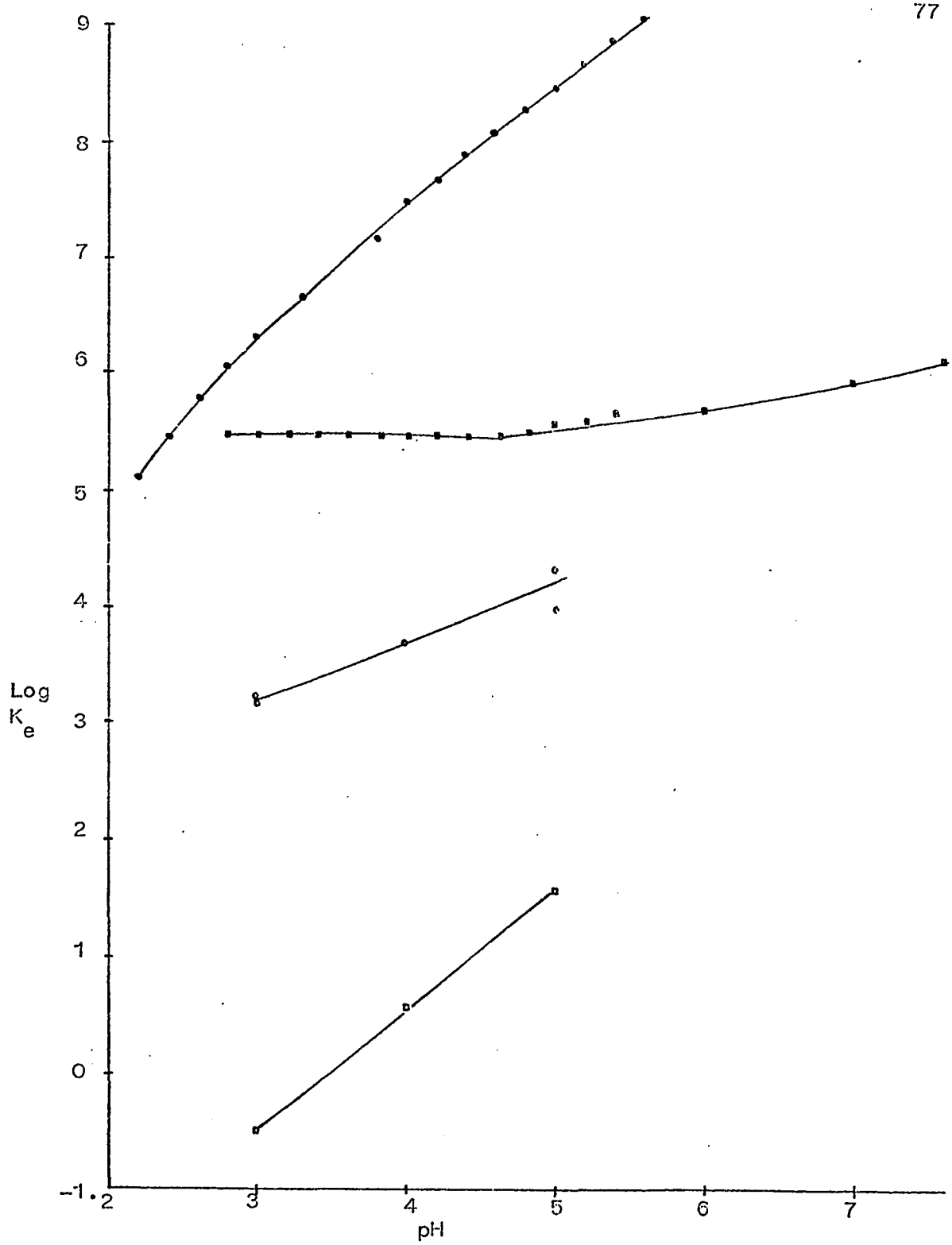

Fig. 10. A comparison of the conditional stability constants for several copper ligand complexes. Symbols represent the following: □ = copper salicylic acid, ○ = copper fulvic acid, ● = copper NTA, ■ = copper products from S. capricornutum, □ and ○ in Cheam and Gamble (1974).

8.06×10^{-7} M EDTA. Two pCu^{2+} values for the medium were decidedly toxic (7.25 and 7.39), one marginally algistatic (7.94) and the fourth (8.85) close to the incipient copper concentration. Algal cells were centrifuged on the second day of the growth period and the copper content of the cells analyzed using flameless atomic absorption. The percent uptake of total copper in the medium as well as the change in pCu^{*} at the four copper concentrations are listed in Table 12. The change in pCu^{*} was calculated using the MINEQL program (Westall et al., 1976).

The maximum concentration of copper taken into S. capricornutum cells was 5×10^{-8} moles of copper per liter when 1×10^{5} cells per ml were present. As seen in Table 12., this is only a fraction of the total copper present in the medium. This result shows substantially less copper uptake by S. capricornutum than that reported by Sunda and Guillard (1976) for T. pseudonana. Results indicate that even at algistatic copper concentrations the amount of copper taken into cells is less than a few percent of the total copper concentration in the medium and thus the effect on pCu is minimal.

VIII. THE EFFECT OF SUBCULTURE AND NUTRIENT UPTAKE ON COPPER TOLERANCE IN S. capricornutum

Copper resistance has been demonstrated in Chlorella vulgaris var vulgaris (Foster, 1977) and Scenedesmus sp. (Stokes and Hutchinson, 1976). Both algae were collected in close proximity to copper mining areas and the concentrations of copper

TABLE XII

THE EFFECT OF COPPER UPTAKE ON pCu^{*} AFTER 3 DAYS OF GROWTH IN MODIFIED EPA MEDIUM WITH 8.06×10^{-7} M EDTA

Total Copper Concentration (molar)	Percent Copper uptake	pCu^{*} before inoculation	pCu^{*} after cell growth
6.29×10^{-7}	1.48	8.85	8.91
6.29×10^{-7}	1.70	8.85	8.92
6.29×10^{-7}	1.10	8.85	8.89
7.89×10^{-7}	1.62	7.94	7.99
7.89×10^{-7}	1.27	7.94	7.99
7.89×10^{-7}	0.75	7.94	7.97
9.44×10^{-7}	2.28	7.39	7.44
9.44×10^{-7}	2.03	7.39	7.43
9.44×10^{-7}	1.31	7.39	7.42
1.10×10^{-6}	1.53	7.25	7.25
1.10×10^{-6}	4.60	7.25	7.25

found in these waters were much higher than an average natural water. Chlorella vulgaris was isolated from the River Hayle which drains abandoned copper mines in Cornwall, England. Scenedesmus sp. was isolated in Boucher Lake which is close to the Sudbury, Ontario smelters. The whole region is a mining and smelting region for nickel and copper. Stokes and Hutchinson (1976) and Foster (1977) have shown that these algae are able to tolerate more copper than their equivalent laboratory strains. The copper resistance in these species strongly indicates genetic adaptation. Comparing copper content of algal pellets from C. vulgaris with its equivalent laboratory strain at one growth rate, Foster (1977) found the same amount of intracellular copper. This indicates, for C. vulgaris, that copper tolerance is due either to the production of some extracellular products or the development of some copper exclusion mechanism. The release of metabolites which accumulate in algal cells may be due to mutation by the loss of a control mechanism in a specific metabolic pathway (Hellebust, 1974). It is possible that selection for this mutation occurs under high metal stress conditions.

To test for the selection of a genetic change in copper tolerance, S. capricornutum was examined by subculturing six day old cells from the copper bioassay experiment into fresh modified EPA medium at the same pCu and chelator concentrations. Growth rates were compared to values obtained on the previous medium (Fig. 17.). Results show that the growth rate of transferred cultures were

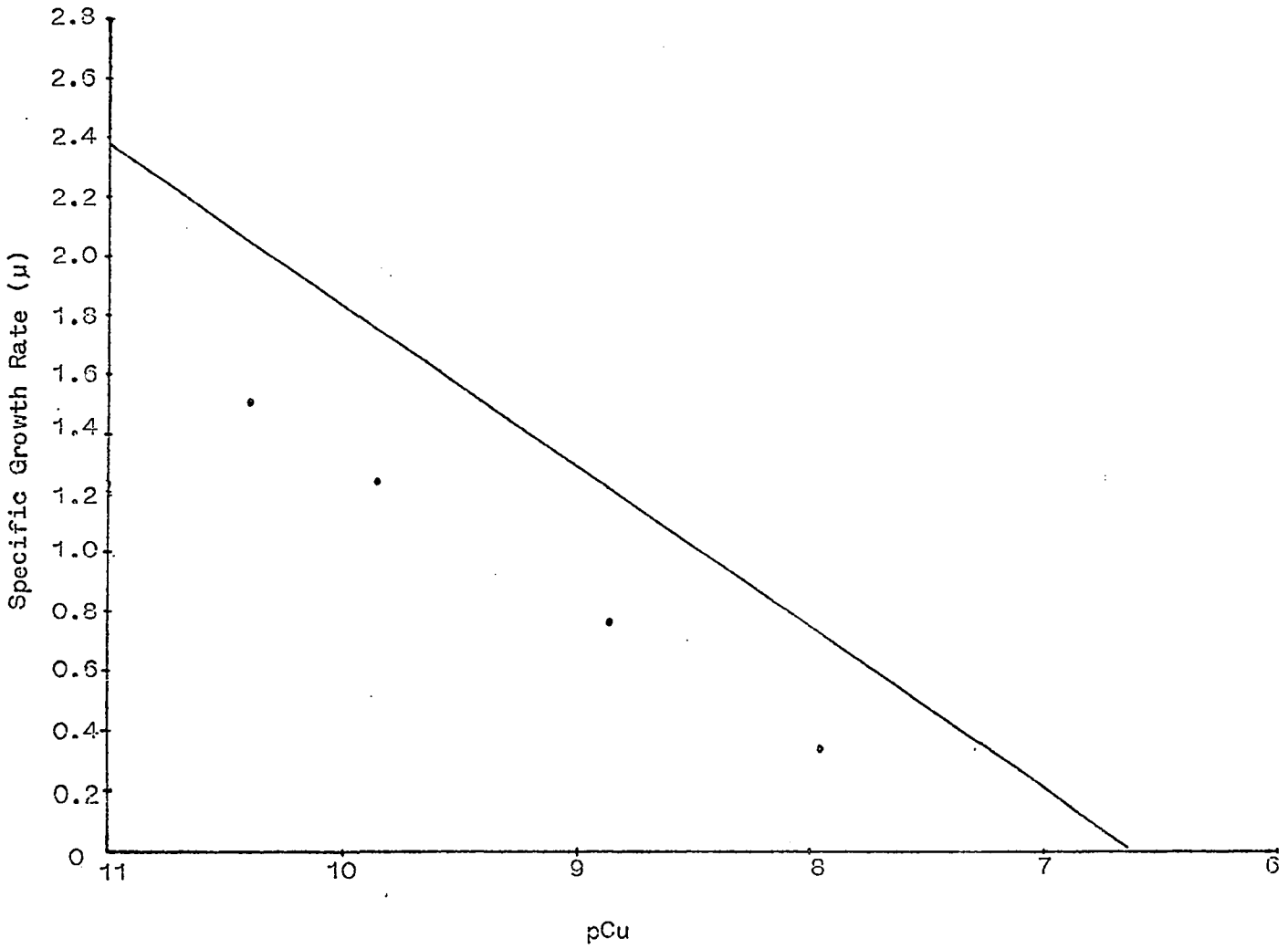

Figure 17. The effect of subculturing on μ ; the solid line represents the linear relationship established between growth rate and pCu for S. capricornutum from data obtained in the copper bioassay experiment. The dots represent recultured cells from that experiment.

lower than those initially grown. The lower growth rate for sub-cultured cells may be due to an increase in internal copper concentration or the accumulation of copper on the outside of the cell wall preventing cell division (Steemann-Nielsen and Wium-Anderson, 1970). Stokes and Hutchinson (1976) and Hassal (1963) have demonstrated that algae accumulate copper internally over time at high pCu. In conclusion, the hypothesis of copper adaptation has not been confirmed for this species. In other research, evidence suggests that the length of exposure to pCu is another factor in determining toxicity.

Selenastrum capricornutum is sensitive to copper ion activity based on the evidence from the copper bioassay experiment. Since a change in ionic strength can affect ion activity (Debye-Huckel) it became important to investigate nutrient uptake during the growth of S. capricornutum in batch culture. The major ions responsible for contribution to ionic strength were measured on each of four days according to procedures in Method and Materials (subsection XIII.). The ionic strength of the complete medium and the concentration of the various anions and cations versus the days of growth are listed in Tables 13 and 14. The major ions are completely ionized in the modified EPA medium as indicated from the MINEQL program (Westall et al., 1976). Thus the calculation of ionic strength was based on the total ion concentrations. The results shown in Tables 13 and 14 indicate that the ionic strength

TABLE XIII

NUTRIENT UPTAKE EXPERIMENT: THE VARIATION OF CATIONS AND IONIC STRENGTH OVER A FOUR DAY GROWTH PERIOD.

Time days	potassium x 10^{-5} M	sodium x 10^{-4} M	magnesium x 10^{-5} M	calcium x 10^{-5} M	Ionic strength x 10^{-4} M
0	1.68	4.89	1.48	2.09	9.44
0.04	1.33	3.84	1.27	1.80	6.15
1	1.12	4.05	1.48	1.60	5.20
2	1.30	6.65	1.36	2.14	6.50
3	1.12	3.44	1.48	2.03	5.50
4	1.40	3.44	1.44	2.29	5.75

TABLE XIV

NUTRIENT UPTAKE EXPERIMENT: THE VARIATION OF ANIONS OVER A FOUR DAY GROWTH PERIOD.

Time days	sulfate x 10^{-5}M	carbonate x 10^{-7}M	nitrate x 10^{-5}M	chloride x 10^{-4}M	bicarbonate x 10^{-4}M
0	22.0	2.08	6.85	2.16	1.40
0.04	7.23	2.08	8.80	1.95	1.40
1	3.10	2.08	3.65	2.01	1.40
2	4.69	2.08	2.10	1.50	1.40
3	3.98	5.22	1.65	2.04	2.22
4	3.59	8.28	1.11	2.07	2.80

did not change enough to affect the copper ion activity coefficient during the growth period. Even if the ionic strength has been substantially reduced due to nutrient uptake, there would be only a 12 percent increase in the copper activity coefficient based on data given in Kielland (1937).

SUMMARY AND CONCLUSIONS

Copper toxicity to the green alga, Selenastrum capricornutum was investigated. Two general areas of inquiry have been studied. The first question addressed was---What form of copper is toxic to the alga? Results indicate that ionic copper is the toxic form. The ionic copper concentration $Cu^{2+}(aq)$ in each medium used was buffered by EDTA or NTA. Growth rates were calculated between the second and third days of growth. The ionic copper concentration in each medium used was calculated using the computer program, MINEQL. A linear relationship was established between growth rate and the log of the calculated ionic copper concentration (pCu^{*}), but no relationship between the log of the total copper concentration and growth rate was found. This result shows that it is the ionic form of copper that is toxic to Selenastrum capricornutum. In comparison with 30 other algal species, S. capricornutum was found to be a copper resistant species.

The second question addressed was---Does the concentration of ionic copper, $Cu^{2+}(aq)$, change during log phase growth of S. capricornutum? A $^{14}CO_2$ uptake experiment showed that S. capricornutum produced extracellular organic products in log phase of growth which amount to three percent of the total fixed carbon. Extracellular organic products were concentrated on XAD-8 Amberlite (a macroreticular non-ionic exchange resin). Two filtrates were concentrated: one filtrate was obtained from algae which were

cultured in the absence of added copper and the second filtrate was obtained from algae which were cultured at near toxic copper concentrations. The copper complexing capacity, conditional stability constants and average molecular weights for these concentrated organic products were calculated from data obtained by use of a copper ion selective electrode. In addition, the concentrated organic products from the two algal filtrates were separated by thin layer chromotography into 11 to 13 fractions and the copper complexing capacity of each individual fraction was determined. Results from this study indicated that S. capricornutum cannot be induced to produce a new product or to increase its copper complexing capacity by culturing cells in the presence of increasing concentrations of copper. The copper complexing capacity of the extracellular products in concentrated filtrates was 2.1×10^{-7} ($\pm$ 8×10^{-8}) M. The conditional stability constant was found to be $10^{5.55}$ between pH 2.0 to 6.0. An average molecular weight of 8.8×10^{4} ($\pm$ 3.4×10^{4}) was obtained for these products.

The pH is another factor which could change pCu during the log phase growth of S. capricornutum. The pH of some cultures in the copper bioassay experiment may increase as much as 0.4 pH units by the second or third day of growth. This is due in part to carbonate and bicarbonate ion uptake by the algae. This pH change affects the ionic copper concentration through the conversion of ionic copper to copper hydroxide. A calculation of ionic copper concentration can be obtained at any pH in a

defined medium with the MINEQL computer program. The pH of each culture was experimentally measured and at the experimental pH of the culture, ionic copper concentrations were calculated. Between most replicate cultures, the variation in pH caused less than a two-fold change in $Cu^{2+}(aq)$ concentration.

Copper uptake by the alga during growth in batch culture had a marginal effect on pCu. A copper bioassay experiment was duplicated using modified EPA medium with 8.06×10^{-7} M EDTA. Selenastrum capricornutum cells were collected on the third day of the growth period. This growth period was the interval of time over which the growth rate was calculated. Total copper content of the cells was determined using flameless atomic absorption spectroscopy. At toxic copper concentrations, 1.75 percent of the total copper was found in the cells. This uptake of copper changed pCu^{*} from 7.38 to 7.43 ($\pm$ 0.01). Ethylenediaminetetraacetic acid uptake, previous growth of cells in the presence of copper, and nutrient uptake by S. capricornutum had little or no affect on pCu during the log phase growth period.

The algistatic affects of copper are totally reversible at all copper concentrations used in these copper bioassays. Cells were taken from 7 day cultures which had been grown at various pCu's in the copper bioassay experiments. Each set of cells was sprayed onto agar containing modified EPA medium with 8.06×10^{-7} M EDTA (but no added copper) and the percent cells undergoing division were recorded at 24 hour intervals for three days. Results show that all cells were undergoing division by the second or third day

regardless of the concentration of copper present in the previous growth medium. It is evident that a pCu level can be used to predict the growth rate of an alga.

Since the pCu-dependent growth rate of S. capricornutum is relatively unaffected by other factors in the growth medium, this organism would be particularly effective as a test organism for monitoring pCu. In addition, a copper bioassay, similar to those described in this research, could be performed on any natural water using the natural phytoplankton from that water. A copper complexing capacity for that natural water could be obtained. If the primary productivity of an eutrophic natural water needed to be reduced with the standard treatment of copper sulfate, the quantity needed to effect a change could be based on this copper complexing capacity. Thus, copper bioassays could predict the best concentration of copper in controlling the algal growth in natural waters.

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APPENDIX A

GENERATION TIME AND DOUBLINGS PER UNIT TIME AS A FUNCTION OF THE SPECIFIC GROWTH RATE CONSTANT

The specific growth rate constant is defined as follows:

$$\mu = \frac{1}{t_2 - t_1}\left\{\ln\left\{\frac{N_2}{N_1}\right\}\right\} \tag{1}$$

where N_1 and N_2 are the population sizes, in cells per ml, at time t_1 and t_2 respectively, where $t_2 > t_1$. The generation time, g, in terms of the specific growth rate constant is defined as:

$$g = \frac{\ln 2}{\mu} = \frac{0.693}{\mu} \tag{2}$$

Doublings per unit time, k, in terms of the specific growth rate constant is defined as follows:

$$k = \frac{\mu}{\ln 2} = \frac{\mu}{0.693} \tag{3}$$

APPENDIX B

THE DETERMINATION OF STOICHIOMETRIES FOR MONONUCLEAR METAL-LIGAND COMPLEXES

a. 1:n complexes only; parenthesises indicate molar concentrations.

The formation of a 1:n metal-ligand complex occurs as follows:

$$M^{++} + nH_xL \rightleftarrows ML_n + nxH^+ \quad (1)$$

with the conditional stability constant defined as follows:

$$K_e = \frac{{}^{*}K\,(1-\alpha)}{(H^+)^{nx}} = (ML_n) \,/\, ((M^{++})((H_xL)+(H_{x-1}L)+\ldots+(L))) \quad (2)$$

where n is the number of ligands associated during the formation of the metal-ligand complex, x is the number of protons fully associated to the complex, $(1-\alpha)$ is the unionized fraction of the ligand, (M^{++}) is the free metal, and ML_n is the metal-ligand complex of stoichiometry n. The mass balance equation for metal is as follows:

$$M_t = (M^{++})\, z + (ML_n) \quad (3)$$

where $(M^{++})\, z$ is defined as the sum of all inorganic metal complexes and z is defined in Appendix D. Dividing equation 3 by (M^{++}), we have the following relationship:

$$M_t \,/\, (M^{++}) = z + ((ML_n) \,/\, (M^{++})) \quad (4)$$

Substituting equation 2 for metal-ligand complex, (ML_n), we have the following relationship:

$$M_t / (M^{++}) = z + K_e ((H_xL) + (H_{x-1}L) +...+ (L)) \quad (5)$$

Rearranging equation 5, complexed metal divided by free metal is defined as follows:

$$(M_t - (M^{++}) z) / (M^{++}) = K_e ((H_xL) + (H_{x-1}L) +...+ (L)) \quad (6)$$

Under Shubert's conditions, where there is very little metal present in relation to ligand, unbound ligand is about equal to the total ligand concentration in the system:

$$L_t^n = ((H_xL) + (H_{x-1}L) +...+ (L)) \quad (7)$$

Thus under Shubert's condition the conditional stability constant is equal to the following relationship:

$$K_e = (M_t - (M^{++}) z) / (M^{++}) L_t^n \quad (8)$$

K_e is equal to S_n as defined in Appendix E, for multiligand systems. Thus, equation 8 transforms into the following relationship:

$$(M_t - (M^{++}) z) / (M^{++}) = S_n L_t^n \quad (9)$$

If a plot of $M_t - (M^{++}) z / (M^{++})$ versus L_t^n yields a linear relationship, then 1:n complexes are established for the solution, where the slope is equal to S_n and the y-intercept is 0.

b. 1:n and 1:p metal-ligand complexes mononuclear system), assuming p > n.

The following metal-ligand equilibria with their formation constants are given as follows:

$$M^{++} + nH_xL \rightleftarrows ML_n + nxH^+ \quad (10)$$

$$K_n = \frac{{}^{*}K_n\,(1-\alpha)}{(H^+)^{nx}} = \frac{(ML_n)}{(M^{++})((H_xL) + (H_{n-1}L) + \ldots + (L))^n} \tag{11}$$

$$M^{++} + pH_xL \rightleftarrows ML_p + pxH^+ \tag{12}$$

$$K_p = \frac{{}^{*}K_p(1-\alpha)}{(H^+)^{px}} = \frac{(ML_p)}{(M^{++})((H_xL) + (H_{n-1}L) + \ldots + (L))^p} \tag{13}$$

where n and p are the number of ligands associated to the metal, x is the number of protons fully associated to the ligand, K_n and K_p are the conditional stability products for the formation of (ML_n) and (ML_p) complexes. The mass balance for total metal is as follows:

$$M_t = (M^{++})\ z + (ML_n) + (ML_p) \tag{14}$$

Dividing equation 14 by (M^{++}) we have the following relationship:

$$M_t\ /\ (M^{++}) = z + ((ML_n)\ /\ (M^{++})) + ((ML_p)\ /\ (M^{++})) \tag{15}$$

Substituting equations 11 and 13 for (ML_n) and (ML_p) and assuming Shubert's condition so that unbound ligand equals the total ligand concentration, as seen in equation 7, the following relationship is established:

$$M_t\ /\ (M^{++}) = z + K_n\ (L_t)^n + K_p\ (L_t)^p \tag{16}$$

Rearranging equation 16 we have the following relationship:

$$(M_t - (M^{++})\ z)\ /\ (M^{++})(L_t)^n = K_n + K_p\ (L_t)^{p-n} \tag{17}$$

As defined in Appendix E, K_n and K_p are equal to S_n and S_p, thus equation 17 transforms into the following equation:

$$(M_t - (M^{++})_z)\ /((M^{++})(L_t)^n) = S_n + S_p\ (L_t)^{p-n} \tag{18}$$

A plot of $(M_t - (M^{++})\ z)/\ (M^{++})(L_t)^n$ versus $(L_t)^{p-n}$ will establish 1:n and 1:p complexes in the system. The true stoichiometries are established if a linear relationship exists. The slope equals S_p and the y-intercept S_n.

APPENDIX C

DERIVATION OF A SCATCHARD PLOT: A COMPLEX WITH ONE METAL AND ONE LIGAND

The data for these plots are obtained from copper titrations at a fixed pH and total ligand concentration. The association of a copper-ligand complex is as follows:

$$Cu^{++} + H_xL \rightleftarrows CuL + xH^+ \qquad (1)$$

where the conditional stability constant is defined as follows:

$$K_e = \frac{{}^{*}K(1-\alpha)}{(H^+)^x} = (CuL) \,/\, ((Cu^{++})((H_xL) + (H_{x-1}L) + \ldots + (L))) \qquad (2)$$

where n is the number of ligands associated during the formation of the copper-ligand complex, x is the number of protons fully associated to the complex, $(1-\alpha)$ is the unionized fraction of the ligand, (Cu^{++}) is the free copper concentration, and (CuL) is the copper-ligand complex. The mass balance equation for total ligand is as follows:

$$\{L_t\}/\,MW = (H_xL) + (H_{x-1}L) + \ldots + (L) + (CuL) \qquad (3)$$

$\{L_t\}$ is the total ligand concentration in grams per liter and MW is the molecular weight of the ligand. Substituting equation 3 into 2 for (CuL) we have the following relationship:

$$K_e = \frac{(CuL)}{(Cu^{++})(\{L_t\}/\,MW - (CuL))} \qquad (4)$$

The mass balance for total copper in the system is:

$$Cu_t = (Cu^{++})\ z + (CuL) \qquad (5)$$

where $(Cu^{++})\ z$ is defined as the sum of all inorganic metal complexes and z is defined in Appendix D. Cu_t is the total copper concentration. Substituting equation 6 into equation 4 we have the following relationship.

$$K_e = (Cu_t - (Cu^{++})\ z) \ / \ ((Cu^{++}) \frac{\{L_t\}}{MW} - (Cu^{++})\ z) \qquad (6)$$

Rearranging equation 6 the following relationship is derived:

$$\frac{(Cu_t - (Cu^{++})\ z)}{(Cu^{++})} = K_e\left\{\frac{\{L_t\}}{MW} - (Cu_t - (Cu^{++})\ z)\right\} \qquad (7)$$

Dividing both sides of the equation by (L_t), the total ligand concentration, the Scatchard form of the equation is derived:

$$\frac{(Cu_t - (Cu^{++})\ z)}{(Cu^{++})\{L_t\}} = K_e \frac{1}{MW} - \frac{(Cu_t - (Cu^{++})\ z)}{\{L_t\}} \qquad (8)$$

$(Cu_t - (Cu^{++})\ z) \ / \ (Cu^{++})\{L_t\}$ is plotted against $(Cu_t - (Cu^{++})\ z)/\{L_t\}$. If a linear relationship can be obtained from this plot then, the absolute value of the slope is the conditional stability constant for (CuL) and the y-intercept is the value of the conditional stability constant divided by the average molecular weight of the ligand.

APPENDIX D

THE CALCULATION OF COPPER HYDROXIDE AND COPPER CARBONATE CONCENTRATIONS AS A FUNCTION OF pH AND pCu

a. COPPER HYDROXIDE COMPLEXES

Copper hydrolysis equilibria at 25 C (I=0) with the appropiate constants are listed as follows:

$$Cu^{++} + H_2O \rightleftarrows CuOH^{+} + H^{+} \quad (1)$$

$$^{*}K_1 = (CuOH^{+})(H^{+}) \,/\, (Cu^{++}) = 10^{-7.3} \quad (2)$$

$$2Cu^{++} + 2H_2O \rightleftarrows Cu_2(OH)_2^{2+} + 2H^{+} \quad (3)$$

$$^{*}\beta_{22} = (Cu_2(OH)_2^{2+})(H^{+}) \,/\, (Cu^{++})^2 = 10^{-10.95} \quad (4)$$

$$Cu^{++} + 2H_2O \rightleftarrows Cu(OH)_2(aq) + 2H^{+} \quad (5)$$

$$^{*}\beta_2 = Cu(OH)_2(aq)\,(H^{+})^2 \,/\, (Cu^{++}) = 10^{-15.2} \quad (6)$$

$$Cu^{++} + 3H_2O \rightleftarrows Cu(OH)_3^{-} + 3H^{+} \quad (7)$$

$$^{*}\beta_3 = (Cu(OH)_3^{-})(H^{+})^3 \,/\, (Cu^{++}) = 10^{-26.3} \quad (8)$$

$$Cu^{++} + 4H_2O \rightleftarrows Cu(OH)_4^{2-} + 4H^{+} \quad (9)$$

$$^{*}\beta_4 = (Cu(OH)_4^{2-})(H^{+})^4 \,/\, (Cu^{++}) = 10^{-39.4} \quad (10)$$

Values for copper hydrolysis constants are in Sillen and Martell (1964). All hydroxide concentrations are a function of pH and (Cu^{++}). Rearranging equations 2,4,6,8,10 we have the following relationships:

$$(CuOH)^{+} = (10^{-7.3})(Cu^{++}) \,/\, (H^{+}) \quad (11)$$

$$(Cu_2(OH)_2^{2+}(aq)) = (10^{-10.95})(Cu^{++})^2 / (H^{+})^2 \quad (12)$$

$$(Cu(OH)_2\,(aq)) = (10^{-15.2})(Cu^{++}) \,/\, (H^{+})^2 \quad (13)$$

$$(Cu(OH)_3^-) = (10^{-26.3})\ (Cu^{++})\ /\ (H^+)^3 \quad (14)$$

$$(Cu(OH)_4^{2-}) = (10^{-39.4})(Cu^{++})\ /\ (H^+)^4 \quad (15)$$

b. COPPER CARBONATE COMPLEXES

In a system open to the atmosphere, the concentration of copper-carbonate complexes are based on the unbound copper concentration, (Cu^{++}), and the avaiable free carbonate concentration. The concentration of carbonate and bicarbonate ions are defined as follows:

$$(CO^{2-}) = K_1K_2K_h pCO_2\ /\ (H^+)^2 = 10^{-21.55 + 2pH} \quad (16)$$

$$(HCO_3^-) = K_1K_h pCO_2\ /\ (H^+) = 10^{-11.3 + pH} \quad (17)$$

where the following constants are defined as:

$$K_1 = (H^+)(HCO_3^-)\ /\ (H_2CO_3) = 10^{-6.3} \quad (18)$$

$$K_2 = (H^+)(CO_3^{2-})\ /\ (HCO_3^-) = 10^{-10.25} \quad (19)$$

$$K_h = CO_2(aq)\ /\ CO_2\ (g)\ /\ RT = 10^{-1.5} \quad (20)$$

$$pCO_2 = 10^{-3.5} \quad (21)$$

where R is the gas constant, T is the absolute temperature, and pCO_2 is the partial pressure of CO_2 in the atmosphere. The formation of $Cu(CO_3)$ occurs as follows:

$$Cu^{++} + CO_3^{2-} \rightleftarrows CuCO_3(aq) \quad (22)$$

The copper carbonate formation constant is defined as follows:

$$K_{CuCO_3} = (CuCO_3)(aq)\ /\ (Cu^{++})(CO_3^{2-}) = 10^{6.77} \quad (23)$$

Thus, the concentration of $(CuCO_3)(aq)$ is defined as follows:

$$(CuCO_3)(aq) = K_{CuCO_3}\ (Cu^{++})(CO_3^{2-}) \quad (24)$$

Substituting equation 16 for the carbonate concentration into equation 24, we have the following relationship:

$$(CuCO_3^{2-}) = K_{CuCO_3}(Cu^{++})K_1K_2K_h pCO_2 / (H^+)^2 \quad (25)$$

Thus the copper carbonate concentration is equal to the following:

$$(CuCO_3)(aq) = 10^{-14.78 + 2pH} \quad (26)$$

The formation of $Cu(CO_3)_2$ occurs as follows:

$$Cu^{++} + 2CO_3^{2-} \rightleftarrows Cu(CO_3)_2(aq) \quad (27)$$

with the copper carbonate formation constant defined as follows:

$$K_{Cu(CO_3)_2} = (Cu(CO_3)_2\ (aq))/\ (Cu^{++})(CO_3^{2-})^2 = 10^{10.01} \quad (28)$$

Thus, the concentration of this complex is equal to:

$$(Cu(CO_3)_2(aq) = (Cu^{++})\ K_{Cu(CO_3)_2}(CO_3^{2-})^2 \quad (29)$$

Substituting equation 16 for the carbonate ion concentration into equation 29, we have the following relationship:

$$Cu(CO_3)_2(aq) = \frac{K_{Cu(CO_3)_2}(Cu^{++})(K_1K_2K_h pCO_2)^2}{(H^+)^2} \quad (30)$$

Thus this complex is equal to:

$$Cu(CO_3)_2(aq) = 10^{-33.09 + 4\ pH} \quad (31)$$

The constants for copper carbonate complexes are in Sillén and Martell (1964).

c. DEFINITION OF z

Throughout all calculations involving conditional stability constants, copper hydroxide and copper carbonate complexation has been accounted for in calculations so that organic complexation

represents the only complexed copper in the system. This complexed copper is defined as follows:

$$Cu(L1) + Cu(L2) + \ldots + Cu(LN) = Cu_t - (Cu^{++})\, z \qquad (32)$$

where z is defined throughout the text as follows:

$$z = \frac{1 + (CuOH^{+}) + (Cu_2(OH)_2^{2+}) + (Cu(OH)_2(aq)) + (Cu(OH)_3^{-}) +}{(Cu^{++})}$$

$$\frac{(Cu(OH)_4^{2-}) + (CuCO_3^{+}) + (Cu(CO_3)_2)}{(Cu^{++})} \qquad (33)$$

As a function of pH and (Cu^{++}), z is defined in the following relationship employing equations 11,12,13,14,15,26,31:

$$z = 1 + (10^{-7.3})/(H^{+}) + (10^{-10.95})(Cu^{++})/(H^{+})^2 + (10^{-15.2})/(H^{+})^2 + (10^{-26.3})/(H^{+})^3 + (10^{-39.4})/(H^{+})^4 + (10^{-14.87 + 2pH}) + (10^{-33.1 + 4pH}) \qquad (34)$$

APPENDIX E

THE CALCULATION OF A CONDITIONAL STABILITY CONSTANT AS A FUNCTION OF pH for 1:n METAL-LIGAND COMPLEXES

Derived in MacCarthy and Smith (1979), the conditional stability function, S_n, is defined as follows for a mixture of ligands:

$$S_n = \frac{\sum^{TN}(M(L1)_{b1}(L2)_{b2}\cdots(LN)_{bN})}{(M^{++})(\sum_i^N (L_i)^n} \qquad (1)$$

where TN is the total number of complexes of stoichiometry 1:N, (M^{++}) is the unbound metal species, N is the number of ligand species in the system, n is the stoichiometry of the complexes, bi is the number of times Li appears in the complex. S_n equals K_e, the conditional stability constant, when there is minimum competition for ligands or metal (C.L.A.S.P. values). See the discussion section on conditional stability constants for a more detailed discussion of this topic. C.L.A.S.P. values are reached under two conditions, Shubert conditions where there is very little metal in the system, and excess metal conditions.

Under Shubert conditions, the total ligand concentration is about equal to the total concentration of unbound ligand in the system:

$$L_t = \sum_{i=1}^{N} (L_i) \qquad (2)$$

All metal-ligand complexes are of very low molarity compared to the unbound ligand species. Under Shubert conditions, S_n, is modified in the form below for a mixture of ligands of 1:n metal-ligand stoichiometries:

$$S_n = \sum^{TN} (M^{++}(L1)_{b1}(L2)_{b2}\ldots(LN)_{bn}) / ((M^{++})(L_t)^n \quad (3)$$

The total metal complexed in the system is as follows:

$$\sum^{TN} (M^{++}(L1)_{b1}(L2)_{b2}\ldots(LN)_{bn}) = (M_t - (M^{++})) \quad (4)$$

If the metal hydroxide and metal carbonate complexes are excluded from S_n then equation 4 is modified to the form:

$$\sum^{TN} (M^{++}(L1)_{b1}(L2)_{b2}\ldots(LN)_{bn}) = (M_t - (M^{++}) \cdot z) \quad (5)$$

where z is defined in Appendix D. Substituting equation 5 in this Appendix into equation 3, S_n is defined under Shubert conditions for a mixture of lignads:

$$S_n = (M_t - (M^{++})\ z) \ / \ ((M^{++})(L_t)^n) \quad (6)$$

Thus this C.L.A.S.P. value, S_n, is obtained from: (1) the total metal in the system, M_t, (2) the free metal obtained from a metal selective electrode, (3) the pH, and (4) the total concentration of ligand, L_t, obtained from the copper complexing capacity experiment. See the discussion section for this calculation.

For a mixture of ligands containing 1:1 metal-ligand complexes only, S_n in equation 6 reduces to the following equation under Shubert conditions:

$$S_n = (M_t - (M^{++})\ z) \ / \ ((M^{++})(L_t)) \quad (7)$$

Under all total metal and total ligand conditions, the total unbound ligand species is defined as follows:

$$\sum_{i=1}^{N} (L_i)^n = L_t - n\sum^{TN}((M^{++})(L1)_{b1}(L2)_{b2}\ldots(LN)_{bn}) \quad (8)$$

Substituting equation 8 into equation 1, S_n is defined as follows:

$$S_n = \frac{\sum^{TN} ((M^{++})(L1)_{b1}(L2)_{b2}\ldots(LN)_{bn})}{(M^{++})(L_t - n\sum^{TN} ((M^{++})(L1)_{b1}(L2)_{b2}\ldots(LN)_{bn})^n} \quad (9)$$

From the substitution of equation 4 into equation 9 we have the following relationship:

$$S_n = (M_t - (M^{++})/((L_t - n\,(M_t - (M^{++}))^n\,(M^{++})) \quad (10)$$

Excluding metal-hydroxide and metal-carbonate complexes, S_n, is defined as follows over a wide range of total metal and ligand concentrations:

$$S_n = (M_t - (M^{++})z/((L_t - n\,(M_t - (M^{++}))^n\,(M^{++})) \quad (11)$$

APPENDIX F

DETERMINATION OF CONDITIONAL STABILITY CONSTANTS FOR NTA AS A FUNCTION OF pH

Copper-NTA complex is formed by the following association:

$$Cu^{++} + NTA^{3-} \rightleftarrows CuNTA^{-} \quad (1)$$

where β_{cuNTA} for the copper-NTA complex is defined as:

$$\beta_{cuNTA} = (CuNTA^{-}) / ((Cu^{++})(NTA^{3-})) \quad (2)$$

NTA is also found in different protonated forms, $HNTA^{2-}$, H_2NTA^{-}, H_3NTA with the proportion of each based on pH. The fraction of NTA is the non-protonated state is defined as follows:

$$\alpha = \frac{(NTA^{3-})}{((H_3NTA)(H_2NTA^{-})(HNTA^{2-})(NTA^{3-}))} \quad (3)$$

The fraction can be calculated from the acid dissocaition constants of NTA and pH in the following relationship:

$$\alpha = (1 + K_3^{-1}(H^{+}) + K_3^{-1}K_2^{-1}(H^{+})^2 + K_3^{-1}K_2^{-1}K_1^{-1}(H^{+})^3)^{-1} \quad (4)$$

where the acid dissociation constants for NTA are as follows:

$$K_1 = (H^{+})(H_2NTA^{-})/(H_3NTA) \quad (5)$$

$$K_2 = (H^{+})(HNTA^{2-})/(H_2NTA^{-}) \quad (6)$$

$$K_3 = (H^{+})(NTA^{3-})/(HNTA^{2-}) \quad (7)$$

The conditional stability constant for NTA is defined as follows:

$$K_e = (CuNTA^{-})/ (Cu^{++})((H_3NTA)+(H_2NTA^{-})+(HNTA^{2-})+(NTA^{3-})) \quad (8)$$

The conditional stability constant, K_e, relates to β_{cuNTA} in the

following relationship:

$$K_e = \beta_{cuNTA}\alpha \tag{9}$$

If an additional protonated form of the copper-ligand complex, (CuHNTA) is included in the calculation of the conditional stability constant, then the constant is defined as follows:

$$K_e = \beta_{cuNTA}\ \alpha + \beta_{cuHNTA}(H^+)\ \alpha \tag{10}$$

where β_{cuHNTA} for the formation of copper protonated NTA complex is defined as follows:

$$\beta_{cuHNTA} = (CuHNTA) \ / \ ((Cu^{++})(H^+)(NTA^{3-})) \tag{11}$$