THE RELATIONSHIP BETWEEN CUPRIC ION ACTIVITY AND THE TOXICITY OF COPPER TO PHYTOPLANKTON

by

WILLIAM SUNDA

B.S., Lehigh University, Pennsylvania
(1968)

SUBMITTED IN PARTIAL FULFILLMENT OF THE
REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

at the

MASSACHUSETTS INSTITUTE OF TECHNOLOGY

and the

WOODS HOLE OCEANOGRAPHIC INSTITUTION

April, 1975

Signature of Author.....William B. Sunda.....
Joint Program in Oceanography, Massachusetts Institute of Technology - Woods Hole Oceanographic Institution, and Department of Earth and Planetary Sciences, and Department of Meteorology, Massachusetts Institute of Technology, April, 1975

Certified by.....Robert R. L. Guillard.....
Thesis Supervisor

Accepted by.....
Chairman, Joint Oceanography Committee in the Earth Sciences, Massachusetts Institute of Technology - Woods Hole Oceanographic Institution

ABSTRACT

THE RELATIONSHIP BETWEEN CUPRIC ION ACTIVITY AND THE TOXICITY OF COPPER TO PHYTOPLANKTON

WILLIAM SUNDA

Submitted to the Department of Earth and Planetary Sciences in April, 1975 in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

The purpose of this investigation is to quantify the relationship between cupric ion activity and the toxicity of copper to phytoplankton and further to study the effect on copper toxicity of naturally occurring organic ligands.

Culture experiments with an estuarine diatom Thalassiosira pseudonana (clone 3H) in highly chelated seawater media demonstrated that copper induced growth rate inhibition and 3 to 4 day cellular uptake of copper are both related to the calculated free cupric ion activity and are independent of the total copper concentration. Cupric ion activity and total copper concentration were independently altered through various combinations of chelator (trishydroxymethylamino methane) concentration, total copper concentration, and pH.

Cellular copper content, in moles per cell, followed a hyperbolic relationship

$$\text{Cu/cell} = \frac{4.8 \times 10^{-16}\, a_{Cu}}{a_{Cu} + 10^{-9.2}}$$

where a_{Cu} is the free cupric ion activity. The above relationship suggests a reversible binding of copper to a single set of cellular ligand sites having a total binding capacity of 4.8×10^{-16} moles per cell and an association constant for reaction with copper of $10^{9.2}$. For T. pseudonana (clone 3H) copper was inhibitory at pCu values below 10.7 (i.e. cupric ion activities above $10^{-10.7}$) with total growth inhibition occurring at pCu values below 8.3. The relationship between growth rate inhibition and cupric ion activity was not a simple hyperbolic relationship as was observed in the case of copper uptake. For an estuarine green alga Nannochloris atomus (clone GSB Nanno) and an open ocean strain of T. pseudonana (clone 13-1) partial growth rate inhibition occurred in the pCu ranges 10.3 to 8.4 and approximately 10 to 8, respectively.

Comparison of these growth inhibitory pCu levels with a calculated estimate of the pCu of seawater of pH 8.2 containing a typical total copper concentration of 0.012 μM and having no significant copper chelation, indicates that natural cupric ion activity levels in seawater may be inhibitory to these three clones.

Evidence was found for the complexation of copper by extracellular products of the alga T. pseudonana (clone 3H). Cupric ion selective electrode measurements of copper complexation in unused low salinity culture media and in identical media in which algae had been grown and from which they were subsequently filtered showed a higher degree of copper complexation in the used media. Parallel studies of copper toxicity and cellular copper uptake in an unused medium and in a culture filtrate demonstrated a lower copper toxicity and a decreased cellular copper uptake in the used medium.

Cupric ion-selective electrode measurements and bioassay experiments support the hypothesis that copper is complexed by organic ligands in at least some natural waters. Copper added to filtered untreated river water is more highly complexed than that added to river water that has been uv irradiated to remove some portion of the dissolved organic matter. Copper toxicity to N. atomus is significantly increased in seawater from Vineyard Sound and in salt marsh water subjected to prior ultraviolet irradiation.

Thesis Supervisor: Robert R. L. Guillard

Senior Scientist, Department of Biology, Woods Hole Oceanographic Institution, Woods Hole, Massachusetts

ACKNOWLEDGMENTS

I express my deepest appreciation to Robert Guillard, without whose advice, encouragement and guidance, this thesis would not have been possible.

I also thank Fred Sayles, Francois Morel, and John Farrington for critical advice on both experimental work and writing of this manuscript.

Many of the fundamental concepts dealt with in this thesis were influenced by a course I received in aquatic chemistry, taught by Werner Stumm. Discussions with Frank Manheim, Vaughan Bowen, and Paul Mangelsdorf were also helpful.

Assistance in computer programming by John Dunlap is appreciated.

This research was supported in part by Grant Number GB-33288 of the Biological Oceanography Section of the National Science Foundation, and in part by a Woods Hole Oceanograhic Institution Fellowship. The financial support of my parents is gratefully appreciated.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

I. INTRODUCTION

I-A. General

In recent years, much interest has been focused on the effect of trace metals and trace metal chelation on the productivity of phytoplankton in natural waters. Interest in this problem has been heightened by increasing pollution of many rivers, estuaries, and coastal waters by toxic trace metals (Hg, Cu, Cd, Pb, Zn, and others) and by man-made chelators such as NTA (nitrilotriacetic acid). Chelation is thought to affect the availability of trace metals to algae by altering both free metal ion activities and concentrations of soluble metal species.

This thesis investigates the relationship between the aqueous complexation chemistry of one toxic trace metal, copper, and the toxicity of copper to algae. An attempt is made to define copper toxicity quantitatively in terms of free cupric ion activities. Although copper is essential to all living cells, it can be toxic to at least some species of planktonic algae at extremely low concentrations (e.g. 1 ppb, Davey et al., 1973) and thus the toxicity of copper to algae is of apparent ecological significance (Steeman Nielsen and Wium-Anderson, 1970).

I-B. Background Literature

I-B-1. Biological and Chemical Evidence for Trace Metal Chelation in Natural Waters

Several phytoplankton productivity studies have suggested trace metal chelation as an important factor influencing phytoplankton growth in the

ocean. The addition of certain chelators, notably EDTA (ethylenediaminetetra-acetic acid), generally increased the ability of most seawater samples to support phytoplankton growth (Johnston, 1963, 1964). Johnston went so far as to say, "the supply of chelating substances is frequently the most critical aspect of phytoplankton nutrition in seawater".

In the upwelling system associated with the Cromwell current in the Equatorial Pacific, newly upwelled seawater was found to be a relatively poor medium for supporting phytoplankton growth; however, its productivity improved as it advected north and south along the surface, in spite of decreasing concentrations of the basic nutrients - nitrate, phosphate, and silicate (Barber and Ryther, 1969). Artificial enrichment of upwelled seawater with phosphate, nitrate, silicate, trace metals, and vitamins, either singly or in combination had little effect on algal growth. In contrast, the addition of an artificial chelator (EDTA) or an aqueous extract of homogenized zooplankton was markedly stimulatory, suggesting that a lack of chelators was a major factor limiting algal growth in the newly upwelled seawater. Barber and Ryther attributed the observed increase in the productivity of the upwelled seawater as it advected along the surface to an in situ production of natural chelators by algae and/or other organisms.

Still other studies implicate natural organic chelators as a factor influencing marine phytoplankton growth. Barber et al. (1971) encountered two basic types of surface seawater associated with the Peru coastal upwelling system. Growth of phytoplankton in seawater of the first type was not

stimulated by the addition of EDTA, was affected by inoculum size, and was significantly decreased by treatment of the seawater with activated charcoal. It was suggested that surface seawater of this first type contained sufficient natural organic chelators and thus phytoplankton growth was unaffected by the addition of the artificial chelator EDTA. Charcoal treatment removed some of the dissolved organic matter from this water and thus presumably decreased the amount of nutritionally important chelators. Growth of phytoplankton in surface seawater of the second type was stimulated by the addition of EDTA and was not significantly retarded by charcoal treatment. Surface seawater of type two apparently contained few chelating substances and therefore required the addition of an artificial chelator to obtain good algal growth.

Barber (1973) examined the effect of added chelator (EDTA) and of inoculum size on algal growth in ultraviolet irradiated and non-irradiated surface and deep (800 m) seawater. Algal growth in deep seawater was highly dependent on inoculum size, high density inocula having shorter lag periods and an increased growth rate. The addition of EDTA to low density inoculum cultures increased growth rate and decreased the lag period to about the values of the high density inoculum cultures. As in the other studies, this suggested an in situ production of an extracellular growth factor whose effect was similar to that of EDTA. Treating surface seawater with UV irradiation (known to destroy a significant portion of the dissolved organic

matter (Armstrong, 1966)), had a deleterious effect on algal growth, that could be completely reversed by the addition of EDTA. This was consistent with the hypothesized destruction of nutritionally beneficial organic chelators in the surface seawater by ultraviolet irradiation.

The above studies provide only circumstantial evidence for the chelation of trace metals by natural organic ligands. Furthermore, these studies provide no information concerning the extent to which specific trace metals are chelated.

To date, little chemical data are available that directly and unequivocally demonstrate the extent to which trace metals are chelated in natural waters. Anodic stripping voltametry, which measures free and weakly complexed metals, has been used recently to investigate natural trace metal speciation (Matson, 1968; Fitzgerald, 1970; Barsdate and Matson, 1966). Acidification or ultraviolet irradiation of seawater samples significantly increased the quantities of copper, lead, and cadmium measured in seawater by anodic stripping voltametry (Fitzgerald, 1970). Barsdate and Matson (1966) found no detectable lead, zinc, or copper in untreated water from an arctic lake containing a high concentration of organic matter, but after persulfate oxidation of the water, substantial quantities of these metals were measured. These results were interpreted as direct evidence for trace metal chelation. However, Hume and Carter (1972) and Stumm and Brauner (1973) have warned of major difficulties inherent in anodic stripping analysis

in natural waters. The adsorption of organic matter onto electrode surfaces may slow or completely inhibit metal plating or stripping steps. Changes in position and height of stripping peaks otherwise attributed to metal chelation may in fact be caused by adsorption of organic matter, thus making anodic stripping data extremely difficult to interpret.

Other chemical investigations provide evidence that copper may be organically complexed in seawater. Up to 50% of the copper in seawater can be extracted by chloroform, suggesting the presence of chloroform soluble complexes (Slowey, Jeffery and Hood, 1967). Chemical oxidation or photo-oxidation of organic matter in seawater generally increases the amount of copper measured by chelate extraction techniques (Corcoran and Alexander, 1964; Slowey and Hood, 1966; Williams, 1969a). Using ultraviolet irradiation to oxidize organic matter, Williams (1969a) estimated that up to 25% of the copper in the samples studied was organically bound. Such measurements, at best give only a lower estimate for organic complexation. For example, Williams' analyses could not detect any chelation of copper by 2 μM EDTA added to seawater even though copper was highly complexed.

A means of measuring trace metal complexation in natural waters directly and unambiguously is clearly needed.

I-B-2. Role of Chelators in Promoting Algal Growth in Nature and in Culture

Johnston (1964) claimed that the beneficial effect of added chelators was due to an increase of the availability of certain essential trace metal

nutrients - iron and possibly manganese - whose metal oxides or hydroxides are only sparingly soluble in seawater. This claim is supported by pure culture experiments with Chlorella in which iron chelated to EDTA is significantly more available to the cells than non-chelated iron (Walker, 1954). Further, it has been known for some time that chelating agents, such as EDTA, increase iron supply to higher plants (Wallace et al., 1957). Bacteria, yeasts, and other fungi are known to excrete large quantities of iron-specific chelating substances, hydroxamic acids, in response to iron limiting conditions (Neilands, 1957, 1967).

Other studies have indicated that the chelation of most metals generally renders them less "available" to algal cells, presumably by lowering the concentrations of free metal ions. For example, manganese, calcium, and zinc deficiencies can be induced by the addition of EDTA to growth media (Hutner et al., 1950; Walker, 1953 and 1954; Spencer, 1957).

Recently, Manahan and Smith (1973) have presented quantitative evidence that the copper growth requirements of two fresh water algal species (Chlorella vulgaris and Oocystis marssonii) are dependent on free copper ion activity. In their experiments, the yields of copper deficient cultures in chemically defined EDTA-metal buffered media were increased in a similar fashion either by the addition of higher concentrations of copper or by lower concentrations of EDTA. Algal growth inhibition induced by high concentrations of EDTA could be reversed by the addition of copper, indicating that the inhibitory effect of EDTA was due to a copper deficiency. Calcula-

tions from stability constants for metal-EDTA complexes showed that copper deficiency occurred at free metal ion activities below about 10^{-16}M.

Metal toxicity as well as nutritional supply is affected by the presence of chelators. Copper, which is toxic to Phaeodactylum tricornutum at concentrations in excess of 2 μM in the absence of chelators, is not toxic to this alga, when complexed to EDTA, at concentrations as high as 5 mM (Spencer, 1957). Copper growth inhibition of Thalassiosira pseudonana is reversed by NTA (nitrilotriacetic acid), a compound that strongly chelates copper (Erickson et al., 1970). In artificial seawater media containing the chelators EDTA or histidine, copper growth inhibiton curves for Thalassiosira pseudonana were found to resemble potentiometric copper titration curves, that is, a sharp inflection in the copper growth inhibition curves was observed at the point where the copper concentration exceeded that of the chelator (i.e. at the equivalence point).

The possibility thus exists that one stimulatory effect of EDTA in marine productivity studies results from a removal of trace metal inhibition (Steeman Nielsen and Wium-Anderson, 1970). This hypothesis assumes that one or more trace metals in seawater are inhibitory to phytoplankton and that the presence of chelators such as EDTA eliminates this inhibition by reducing the free ion activities of these toxic metals.

I-C. Experimental Objectives

The aforementioned experimental evidence suggests that metal toxicity is determined by free metal ion activity, and that chelators, whether natural

or artificial, affect toxicity by altering metal activities. However, at present, an actual functional relationship between metal toxicity to algae and free metal activity has not been demonstrated.

A major aim of this thesis is to test the hypothesis that copper toxicity to algae and copper uptake by algae are both functionally related to free cupric ion activity. This hypothesis is tested in highly chelated seawater cultures in which the free cupric ion activity is systematically varied though different combinations of total copper concentration, chelator (trishydroxymethylamino methane) concentration, and pH. Copper was chosen because of its extreme toxicity to algae (Steeman Nielsen and Wium-Anderson, 1970 and 1971; Erickson, 1972; and Davey et al., 1973) and thus its potential environmental significance. Tris was chosen because it is relatively non-toxic to algae (McLachlan, 1973) and simultaneously buffers both pCu and pH.

Barber and Ryther (1969) and Barber (1973) postulated the in situ production of extracellular trace metal chelators by algae. In the present study, culture experiments are performed to quantitatively measure, with a cupric ion-selective electrode, the extent to which copper is complexed by extracellular products of algae.

Finally, we wish to investigate the feasibility of using a cupric ion-selective electrode to measure complexation of copper in natural water samples.

II. MATERIALS AND METHODS

II-A. Introduction

The materials and methods section describes the experimental algae; culture conditions; the composition of experimental culture media; the determination of cell concentrations, growth rate, and cellular copper and potassium contents; and the ultraviolet irradiation technique used for the partial destruction of dissolved organic matter in natural water samples. The inhibition of algal growth rates and/or copper induced loss of cellular potassium were used as indices for copper toxicity to algae in culture experiments. Ultraviolet irradiation of natural water samples was used in an investigation of copper complexation by naturally occurring organic ligands.

The determination of pCu in culture media and in natural water samples is discussed in Section 3.

II-B. Algae

Two species of estuarine algae were obtained from the culture collection of R.R.L. Guillard, Woods Hole Oceanographic Institution, for use in this study: Thalassiosira pseudonana (Hustedt) Hasle and Heimdal (clone 3H), a diatom formerly called Cyclotella nana Hustedt, Hasle and Heimdal, 1970); and Nannochloris atomus Butcher (clone GSB Nanno), a green alga. A major reason for the choice of these two species is the widely varying chemical conditions under which they may be cultured. For example, both species may be cultured in media ranging in salinity from fresh water (less than

1 °/oo) to full strength seawater (35 °/oo) (Guillard and Ryther, 1962). Thalassiosira pseudonana has been grown at pH values ranging from 5.9 to above 9.0 (Degens et al., 1968).

Nannochloris atomus is roughly spherical in shape with a cell diameter of 1 to 2 μm. Thalassiosira pseudonana has a cylindrical shape and is somewhat larger with a diameter of approximately 4 μm and a length of 4 to 8 μm.

Thalassiosira pseudonana was isolated by R. Guillard on September 8, 1958 from the Forge River, Morichs Bay, which is adjacent to Great South Bay, Long Island, New York. Nannochloris atomus was isolated by John Ryther in 1952 from Great South Bay. Both species have bloomed periodically in Great South Bay (Ryther, 1954; Guillard and Ryther, 1962) and are of ecological importance in that estuary.

II-C. Culture Conditions

Experiments were carried out in batch cultures contained in 125-2000 ml borosilicate Erlynmeyer flasks pre-soaked with concentrated nitric acid to remove trace metal contaminants (Price and Quigley, 1966). Culture flasks were usually covered with borosilicate glass beakers allowing an air space for gas exchange.

For culture experiments in 0-5 mM tris-$CuSO_4$-seawater media (Section IV) and for most inoculation cultures for short term metal uptake and toxicity experiments, the cultures were aerated using nitric acid washed scintered

glass frits to control culture medium pH. Sterile air was first passed through an activated charcoal column to remove organic contaminants and then prebubbled through distilled water to minimize evaporation loss from the culture flasks. Aerated culture flasks were fitted with silicone stoppers.

Unless otherwise specified, experimental cultures were grown aseptically. Media were sterilized either by autoclaving for 15 minutes at 15 psi, or alternatively, where mentioned, by suction filtration through 0.2 μm membrane filters. High pH values encountered during autoclaving often caused the formation of alkaline precipitates. These were redissolved by aseptic bubbling with 5% CO_2 (to a pH of about 6.0) followed by aseptic aeration to re-equilibrate media with the atmosphere.

The algae were cultured at 20 ± 1°C in a temperature controlled culture room, with illumination provided on a 14/10 hour light-dark cycle by fluorescent lights (Sylvania Co., cool white). Light intensity from above was 4300 lux, from below 880 lux, and from the side 2000 lux as measured by a General Electric type 213 cosine corrected light meter.

Basic media used for culture experiments and stock culture maintenance are given in Tables (2-1 through 2-3). Experimental media were often modified, by the specified deletion of one or more components or by the addition of specified concentrations of the chelator trishydroxymethyl amino methane (tris). High purity, recrystallized, primary standard grade

Table (2-1) Composition of enriched seawater medium M-f/2*

Major nutrients	
$NaNO_3$	883 µM
$NaH_2PO_4 \cdot H_2O$	36.3µM
$Na_2SiO_3 \cdot 9H_2O$	100µM
Trace metals	
$FeCl_3 \cdot 6H_2O$	1.0 µM
$CuSO_4 \cdot 5H_2O$	0.04 µM
$ZnSO_4 \cdot 7H_2O$	0.08 µM
$CoCl_2 \cdot 6H_2O$	0.05 µM
$MnCl_2 \cdot 4H_2O$	0.9 µM
$Na_2MoO_4 \cdot 2H_2O$	0.03 µM
Vitamins	
Thiamin·HCl	0.1 mg/l
Biotin	0.5 µg/l
B_{12}	0.5 µg/l
Chelator: Na_2EDTA	1.0 µM

* Modified half strength medium f (Guillard and Ryther, 1962). Experimental media prepared from surface Sargasso seawater stored in 5 gallon borosilicate carboys. Trishydroxymethylamino methane was often added at specified concentrations (0.5-10 mM) as a pH and pCu buffer.

Table (2-2) Composition of artificial freshwater medium SWC*

Salts	Concentration in (mM)
$CaCl_2 \cdot 2H_2O$	0.10
$MgSO_4 \cdot 7H_2O$	0.10
$NaHCO_3$	1.0
K_2HPO_4	0.050
$NaNO_3$	1.0
$Na_2SiO_3 \cdot 9H_2O$	0.10

Trace metals, vitamins, and EDTA concentrations are the same as in medium M-f/2 (Table 2-1).

*Modification of medium WC (Guillard and Lorenzen, 1972).

Table (2-3) Composition of DSW medium (artificial enriched 1/10th strength seawater)

Salts	Concentration (mM)
NaCl	48
$MgSO_4 \cdot 7H_2O$	2.8
$MgCl_2 \cdot 6H_2O$	2.6
$CaCl_2 \cdot 2H_2O$	1.0
KCl	0.90
$NaHCO_3$	1.0
$NaNO_3$	0.88
K_2HPO_4	0.050

Media used to maintain algae and to grow experimental inoculum cultures also contained concentrations of trace metals, vitamins, and Na_2EDTA listed in Table (2-1). Experimental base medium contained 10 μM $MnSO_4$ in addition to the above salts.

tris was obtained from the Sigma Chemical Company. All other chemicals were standard reagent grade.

Seawater media (Table 2-1) were prepared using Sargasso Sea surface seawater collected in a plastic bucket and stored in acid cleaned five gallon borosilicate glass carboys. Artificial culture media and stock chemical solutions were made with water doubly or triply distilled from alkaline permanganate to minimize possible organic contamination.

For metal toxicity and algal metal uptake experiments employing low total metal concentrations (0.03 to 30 μM), metals were added aseptically from sterilized stock solutions immediately before culture inoculation. Stock metal salt solutions of molarity less than 1 mM were prepared fresh on the day they were added to the cultures by aseptic dilution of 1-100 mM solutions.

II-D. Measurement of Cell Concentrations and Specific Growth Rates

Cell concentrations were determined by counting in a Bright line haemacytometer (American Optical Co.). Whenever possible, at least 200 individuals were counted for each cell concentration determination. At the 95% confidence level, the accuracy is good to within 14% if 200 cells are counted (Guillard, 1973).

Specific growth rates were determined from log-linear portions of growth curves, that is where growth of the algae conforms to the equation

$$dN/dt = k\,N \qquad (2\text{-}1)$$

where N is the concentration of cells and k is a growth constant. Integration of this equation expressed as a log to the base two gives:

$$\log_2 N/N_o = \mu t \qquad (2\text{-}2)$$

where N_o is the concentration of cells at time zero and μ is the specific growth rate in divisions per day ($\mu = k/\ln 2$). Values for μ were obtained by a least squares linear regression of $\log_2 N$ vs. time.

II-E. Measurement of Cell Copper and Potassium Contents for T. pseudonana (Clone 3H)

The copper, and in some cases potassium, content of the cells were measured by atomic absorption spectrophotometry. In short term experiments, cells were harvested from log phase sterile aerated cultures by vacuum filtration (3 μ pore size, 47 mm diameter G. E. Nuclepore filters), washed with fresh medium, and then transferred to experimental media. Filtration caused no apparent damage to the cells; visual microscopic inspection revealed no obvious physical cell damage and transfer of filtered cells to fresh medium was followed by an immediate resumption of growth. In long term copper uptake experiments, cells were inoculated at low cell densities (1-2 x 10^4 cells/ml) and grown aseptically in the presence of copper for three or four days.

Cells exposed to copper were separated from the experimental media by vacuum filtration (again using Nuclepore filters), transferred along with the filters to 10 ml borosilicate glass vials and digested overnight in 1 ml of concentrated nitric acid. Five-fold dilutions of the acid digests were

then analyzed for copper and/or potassium content using a Perkin-Elmer model 403 atomic absorption spectrophotometer with an air-acetylene flame. Diluted digests were unfiltered and thus contained undigested diatom shells. Analysis of the digests for cells grown in media containing no added copper indicated that the presence of shells does not produce an appreciable background signal. Copper or potassium distilled water standards were prepared fresh before each analytical run by diluting 1 mM $CuSO_4$ or KCl standard stock solutions. For the determination of cell potassium, 10 mM NaCl was added to both samples and standards as an ionization depressant. Added NaCl showed no matrix interference in copper determinations.

Blanks were measured for portions of the experimental media containing no added cells and were processed in the same fashion as the samples. Blanks were subtracted from sample values to give corrected cell metal content. Usually, blank values were less than 10% of those of the samples. Additional blanks determined for diluted nitric acid (1/5) showed no detectable copper while those for nitric acid digests of Nuclepore filters gave values for acid extractable copper of 2-3 x 10^{-9} moles per filter, accounting for, in many cases, much of the blank values.

In two experiments, replicate copper analyses performed on 13 cell digests showed a mean difference of 2.8% and a maximum difference of 8.5%.

Cell metal content (in moles per cell) was calculated by dividing the "corrected" number of moles of metal in the acid cell digests by the number of cells filtered. Cell metal content values are estimated to be correct to

within ± 20% considering errors in cell counts (∿ 10%) and metal analysis (∿ 10%).

II-F. Photooxidation of Dissolved Organic Matter Using High Intensity Ultraviolet Irradiation

Ultraviolet irradiation was used in this study in an attempt to remove dissolved organic chelators from natural water samples. Armstrong et al., (1966) have shown that the oxidation of dissolved organic matter in seawater using ultraviolet irradiation from a high intensity mercury arc lamp is at least as effective as the widely used persulfate wet oxidation technique of Menzel and Vaccaro (1964). The following compounds were completely oxidized after two to three hours of ultraviolet irradiation: 2,2'-bipyridine, adenine, ethyl alcohol, methyl alcohol, glucose, glucosamine, casein, glycerol, acetic acid, oxalic acid, formic acid, palmitic acid, dimethylamine, phenyl-alanine, and "humic acid" (Armstrong et al., 1966). Urea on the other hand, was oxidized to the extent of 50% or less.

An additional comparison of high intensity ultraviolet irradiation with persulfate wet oxidation in 23 samples of seawater showed that irradiation was about 10% more effective in oxidizing dissolved carbon than was persulfate oxidation (Williams, 1969). Recent high temperature wet combustion analyses (Sharp, 1973) suggest that up to 50% of the dissolved organic carbon in seawater is not oxidized to CO_2 by persulfate oxidation. Thus, ultraviolet irradiation also may fail to oxidize a sizeable fraction of the dissolved organic matter in seawater and possibly in other natural water

samples as well. Ultraviolet irradiation, however, may be especially effective in removing dissolved organic chelators inasmuch as metal chelation markedly increases the rates of photooxidation of many organic ligands (Adamson, 1969; Trott et al., 1972).

The irradiation apparatus used in this study is essentially that described by Armstrong et al. (1966). Ultraviolet irradiation was provided by a 1200 watt mercury-arc lamp mounted axially in a cylindrical metal rack, 45 cm high, and 27 cm in diameter. Samples were contained in 100 ml fused quartz tubes, 30 cm long and 2.5 cm in diameter, arranged circularly around and parallel to the mercury lamp.

Armstrong et al. (1966) reported that no additional oxidation occurred after one hour of exposure in all seawater samples they tested. In the present study, samples were irradiated for a period of at least four hours to insure maximum reaction. One drop of 30% H_2O_2 was added to each 100 ml sample to insure more complete oxidizing conditions during irradiation. Samples often became quite hot (80-90^{o}C) resulting in evaporation; after irradiation, samples were restored to initial volume with distilled water. Subsequently, samples were aerated to re-equilibrate CO_2 with the atmosphere and to remove ozone produced during irradiation. Ozone is toxic to algae and thus its removal is essential for culture experiments using irradiated water.

III. DETERMINATION OF pCu

III-A. Introduction

Experiments were performed to determine the relationship between pCu (defined as the negative log of the copper activity) and copper induced inhibition of algal growth rate and between pCu and the cullular uptake of copper by algae. Other experiments include the measurement of copper complexation by extracellular ligands produced by algae and by natural organic ligands present in a highly colored river water sample.

In all of the above experiments, we need to determine pCu. Wherever possible, pCu was measured directly using a cupric ion-selective electrode (Orion Research Company, model # 9429). However, because of a chloride interference (Jasinski et al., 1974; see Appendix I), the cupric ion electrode could not be used to measure pCu in seawater. Therefore, for all culture experiments in seawater culture media, pCu was calculated both from existing thermodynamic data and from experimental data obtained with a cupric ion-selective electrode in solutions for which there was no interference. Table (3-1) lists the various experiments carried out in this present work along with a brief description of the experimental medium and the means employed to determine pCu.

III-B. Measurement of pCu with a Cupric Ion-Selective Electrode

III-B-1. Brief Electrode Description

Measurements of pCu were carried out using a cupric ion-selective electrode (Orion Research Company, model 9429) with a single junction Ag/AgCl

Table (3-1) Method of pCu determination in various experimental media

Experiments	Medium	Method of pCu determination
Determination of algal growth rate inhibition and cell copper uptake in highly chelated seawater media	M-f/2 (see table 2-1) Seawater media containing 0-10 mM tris, 1.0 μM EDTA and 4-1000 μM $CuSO_4$ Most culture flasks: pH 8.1-8.2 Three culture flasks: pH 7.7 or 8.7	Calculation from measured experimental data and known stability constants
Copper inhibition of algal growth rate in an artificial seawater medium (experimental data taken from Davey et. al., 1973)	Ultraviolet irradiated artificial 30 $^{o}/oo$ salinity seawater pH 8.2	Calculated from measured experimental data and literature data
Effect of a strong chelator EDTA on the uptake and toxicity of copper	Artificial enriched 1/10 strength seawater (DSW medium, see table 2-3) 0 or 2 μM EDTA 0 or 1 μM $CuSO_4$ pH 8.1-9.0	Measured directly in medium containing Cu and no EDTA Calculated from known stability constants for medium containing Cu plus EDTA
Production of extracellular complexing substances by algae	(1) Artificial enriched 1/10th strength seawater 1.0 μM $CuSO_4$ pH 8.1	Measured directly with a cupric ion-selective electrode
	(2) SWC medium (see table 2-2) 0.3 μM $CuSO_4$ pH 8.2	Measured directly with a cupric ion-selective electrode
Complexation of copper in untreated and ultraviolet irradiated filtered river water	Natural water sample from the Newport River in North Carolina	Measured directly with a cupric ion-selective electrode

reference electrode. The reference electrode contained an internal filling solution consisting of 1.7 M KNO_3, 0.64 M KCl, and 0.06 M NaCl saturated with AgCl (Orion filling solution # 90-01). This solution is equitransferent at 25°C (Orion Research Newsletter, 1964) which minimizes liquid junction potentials.

The cupric ion-selective electrode is a solid membrane type with the membrane consisting of a pellet containing two solid phases - CuS and Ag_2S. The membrane is permeable only to silver ions which travel through the Ag_2S crystal matrix as a lattice defect (Ross, 1969). The potential of the cupric ion-selective electrode/test solution/Ag/AgCl reference electrode cell is determined ultimately by the activity of silver ions in solution according to the Nernst equation

$$E = E' + \frac{2.3\ RT}{F} \log a_{Ag} \qquad (3\text{-}1)$$

where E, R, T, and F are respectively the cell potential, the gas constant, the temperature in °Kelvin, and the Faraday constant. The term, E', includes the activity of silver ions at an internal silver-wire membrane contact (which is constant) and the potential of the Ag/AgCl reference electrode (including liquid junction potential).

The electrode operates as a cupric ion sensing device through an equilibrium between CuS, Ag_2S and the test medium (Ross, 1969). This can be seen by considering the solubility products for the two sulfides:

$$a^2_{Ag}\, a_S = K_{Ag_2S} \qquad (3\text{-}2)$$

$$a_{Cu}\, a_S = K_{CuS} \qquad (3\text{-}3)$$

Solving these two equations for a_{Ag} in terms of a_{Cu}

$$a_{Ag} = \left(\frac{K_{Ag_2S}\ a_{Cu}}{K_{CuS}} \right)^{\frac{1}{2}} \qquad (3\text{-}4)$$

At equilibrium the activity of silver ion is a function of copper activity and thus, in a medium initially containing copper but no silver, electrode potential is determined by copper activity. Substituting equation (3-4) into equation (3-1) the Nernst equation for the cupric ion electrode-reference cell becomes

$$E = E' + \frac{2.3\ RT}{F} \log \left(\frac{a_{Cu}\ K_{Ag_2S}}{K_{CuS}} \right)^{\frac{1}{2}} \qquad (3\text{-}5)$$

and therefore

$$E = E_a + \frac{2.3\ RT}{2F} \log\ a_{Cu} \qquad (3\text{-}6)$$

where

$$E_a = E' + \frac{2.3\ RT}{2F} \log\ \frac{K_{Ag_2S}}{K_{CuS}} \qquad (3\text{-}7)$$

III-B-2. Measurement of pCu

The pCu of a test solution is calculated via the Nernst equation from the electrode potential difference between the test solution and a second solution of known copper activity (i.e., a pCu standard). The Nernst equation for the test solution is:

$$E_t = E_{a(t)} + \frac{2.3\ RT}{2F} \log a_{Cu(t)} \qquad (3\text{-}8)$$

and that for the standard is

$$E_s = E_{a(s)} + \frac{2.3\ RT}{2F} \log a_{Cu(s)} \qquad (3\text{-}9)$$

where the subscripts t and s denote test and standard solutions respectively. Subtracting equation (3-8) from equation (3-9) and rearranging terms we obtain an expression for the pCu of the test solution

$$pCu_t = (E_s - E_t) + (E_{a(t)} - E_{a(s)}) \frac{2F}{2.3\ RT} + pCu_s \qquad (3\text{-}10)$$

The term $(E_{a(s)} - E_{a(t)})$ arises from the difference in reference electrode liquid junction potential for the standard and test solutions.

In practice, the term $(E_{a(t)} - E_{a(s)})$, for which the actual value is unknown, is assumed to be small and is omitted from the pCu equation. Therefore, all pCu values are defined by the equation

$$pCu_t = \frac{2F\ (E_s - E_t)}{2.3\ RT} + pCu_s \qquad (3\text{-}11)$$

A 20 μM $CuSO_4$-distilled water standard was used for all copper potential measurements and pCu determinations. At this salt dilution activity coefficients differ negligibly from 1.0. The pH of standard solutions was 5.6 ± 0.1 and at this pH copper does not significantly complex with hydroxide ion (calculated using associated constant $K_{CuOH} = 10^{6.59}$ experimentally measured in Appendix 2). Thus, the activity of copper in the standard solution is simply equal to the concentration of $CuSO_4$. Standards were prepared fresh before each set of electrode measurements by dilution of a 1.0 mM $CuSO_4$ primary stock solution. Measurements in standard solutions were reproducible in most cases to within 0.01 pCu unit and in all cases to within 0.02 units.

III-B-3. Reference Electrode Liquid Junction Potentials in pCu and pH Measurements

Because of the omission of an unknown liquid junction potential term in determining pCu and pH from cupric ion-selective electrode and glass electrode measurements, experimentally determined pCu and pH values may not correspond exactly to the negative logs of cupric ion and hydrogen ion activities. So long as measurements are made in solutions of constant major ion composition liquid junction potentials are constant. In this case the presence of a residual liquid junction potential between test solutions and standards causes a small shift in the entire pCu or pH scale, but the measured pCu or pH values relative to one another are unaffected. Most of the experiments in this work are carried out in constant ionic media and are concerned with changes in pCu. In these experiments, the presence of an

unknown, but constant, liquid junction potential does not affect relative pCu and relative pH values.

The Henderson equation can be used to obtain a rough estimate of the liquid junction potential between solutions of different ionic composition (MacInnes, 1939) and thus an estimate of the uncertainty in absolute values of pCu and pH. This equation is given as follows:

$$E_L = \frac{RT}{F} \frac{\sum_i \lambda_i/z_i \, (C_{s(i)} - C_{r(i)})}{\sum_i \lambda_i \, (C_{s(i)} - C_{r(i)})} \log \frac{\sum_i \lambda_i \, C_{s(i)}}{\sum_i \lambda_i \, C_{r(i)}} \qquad (3\text{-}12)$$

where R, T, and F are respectively the gas constant, absolute temperature (^{o}K), and the Faraday constant; λ_i is the limiting equivalent conductance of the various ions in solution; and $C_{s(i)}$ and $C_{r(i)}$ are the concentrations of the various ions in the test solution and reference electrode filling solution respectively. Although many rough assumptions have been used in deriving this equation - for example, ion conductances and activity coefficients are assumed to be those at infinite dilution - fair agreement is often obtained between measured and calculated liquid junction potentials. Experimentally measured values and values calculated from the Henderson equation for the liquid junction potential between different equimolal (0.1 and 0.01 m) solutions of various univalent chloride salts agree in most cases to within 1 mv and in all cases to within 2 mv (MacInnes, 1939). The measured value (-3.2 mv) for the residual liquid junction potential be-

tween a 0.725 molal NaCl solution and a 0.05 molal phthlate pH standard buffer solution is in surprisingly close agreement with that calculated from the Henderson equation (-3.3 mv) (Hawley and Pytkowicz, 1973).

Calculated liquid junction potentials (equation 3-12) between various test solutions used in the present work and the internal filling solutions of the Ag/AgCl reference electrode and the saturated KCl calomel reference electrode are given in Table (3-2). (The saturated KCl calomel reference electrode was used in most glass electrode pH determinations). For the Ag/AgCl reference electrode, the absolute difference in calculated liquid junction potentials between test solutions and standards is in all cases less than 1.8 mv which corresponds to a maximum liquid junction pCu error of 0.04 pCu units. For the saturated KCl calomel reference electrode the maximum absolute difference between test solutions and dilute phosphate standards is approximately 4.5 mv corresponding to a maximum pH error of 0.08 pH units. Calculated values can be taken as only approximations; however, they do give us some handle on the relative magnitudes of liquid junction errors and suggest, that in most cases, these errors should be small.

Values in Table (3-2) suggest that the Ag/AgCl reference electrode containing the Orion (90-01) filling solution is especially suited for measurements in dilute solutions. In Appendix (2) pCu-pH measurements were performed in 0.5 mM, 1.0 mM, and 3.0 mM $NaHCO_3$ solutions, and in a 20 mM KNO_3 solution to determine association constants for copper-carbonate and copper-

Table (3-2) Liquid junction potentials, calculated from the Henderson equation, between test solutions and reference electrode internal filling solutions

Test solution	E_j for the Ag/AgCl reference electrode*		E_j for the saturated KCl calomel reference electrode	
	18°C	25°C	18°C	25°C
0.5 M KNO_3	0.1 mv	0.1 mv	0.9 mv	1.3 mv
0.02 M KNO_3	0.0	0.0		2.7
0.5 M $NaNO_3$		-1.7		
0.2 M KNO_3 0.07 M $Mg(NO_3)_2$ 0.015 M $Ca(NO_3)_2$	-1.4	-1.3		-2.6
seawater			-0.7	-0.5
3.0 mM $NaHCO_3$	0.0	0.0		3.8
1.0 mM $NaHCO_3$	0.0	0.0		4.3
0.5 mM $NaHCO_3$	0.0	0.0		4.6
20 μM $CuSO_4$	0.0	0.0		
0.025 M KH_2PO_4*** 0.025 M Na_2HPO_4				1.9***

E_j represents junction potential in millivolts calculated from the Henderson equation, using values for limiting equivalent conductances of individual ions taken from Robinson and Stokes (1959)

* Ag/AgCl reference electrode contains a filling solution consisting of 1.7 M KNO_3, 0.64 M KCl, and 0.06 M NaCl.

** Saturated KCl is 4.2 M at 25°C and 4.0 M at 18°C

*** Approximate composition of pH standard buffer solution; junction potential value estimated from the Henderson equation by Bates (1964).

hydroxide complexes. To minimize liquid junction potentials between the various test solutions and hence errors in measured equilibrium constants, pH was measured with a glass electrode coupled to the Ag/AgCl reference electrode. Measurements of pH in 0.05 M KCl solutions (Section III-B-4) and in 20 μM $CuSO_4$ pCu standard solutions also were made using the Ag/AgCl reference electrode. For all other pH determinations, a saturated KCl calomel reference electrode was used.

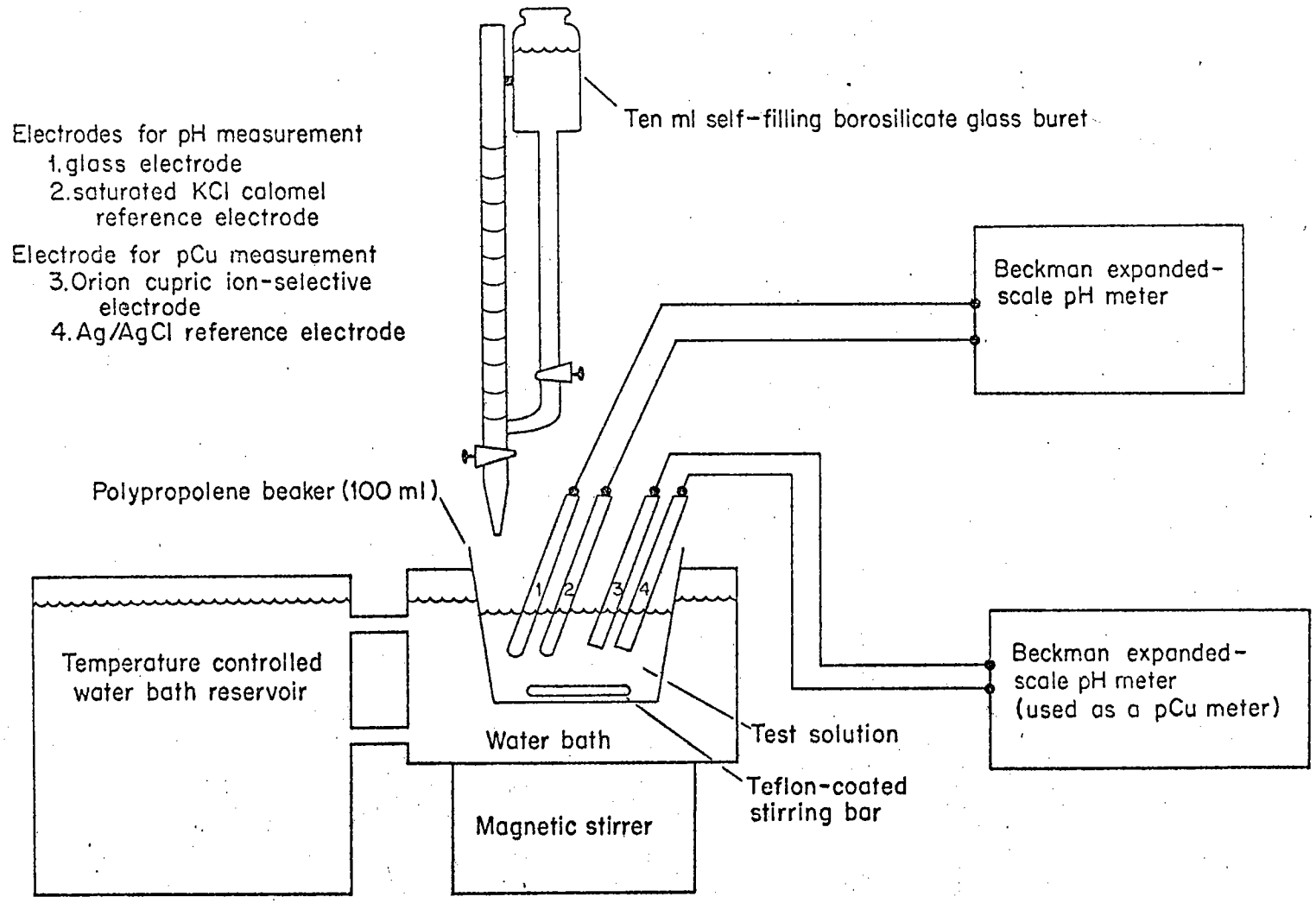
III-B-4. Potentiometric Titrations and pCu-pH Measurements

The extent to which copper is complexed in various media was determined by potentiometric titrations and simultaneous measurements of pCu and pH. The pCu of various test solutions was measured with the cupric ion-selective electrode as total copper concentration, tris concentration, pH, or P_{CO_2} was independently varied.

In both copper titrations and tris titrations (experimental apparatus, Figure 3-1), two solutions were prepared, a first containing a known concentration of copper or tris, and a second having a chemical composition identical to that of the first, but with tris or copper omitted. Cupric ion-selective electrode potential was measured as the first solution was titrated into the second. Because changes in pH can affect copper complexation, pH was measured several times during individual titrations to insure its constancy.

In many solutions, pCu and pH were measured simultaneously at known concentrations of $CuSO_4$ as acid or base was added. For pCu-pH measurements in dilute bicarbonate solutions, pH was varied by bubbling with a 5% CO_2-air

Figure (3-1) Schematic drawing of experimental apparatus used for pCu and pH measurements and for copper potentiometric titrations



mixture. In other solutions, pH was altered by addition of small quantities of strong acid (HCl or HNO_3), strong base (NaOH or KOH), or weak base ($NaHCO_3$). In all cases, the dilution from acid or base added was less than 0.5%.

For most measurements, temperature was controlled by a constant temperature water bath to within ± 0.3°C (Figure 3-1). Where a water bath was not used, temperature was measured with a mercury thermometer and remained constant to within ± 1°C.

For many pCu measurements in alkaline solutions (pH greater than ~ 6) containing low total copper concentrations (less than ~ 10 μM) adsorption loss of copper from the test solutions of up to 40% was found to occur. This loss was prevented and reproducible results obtained by presoaking the electrodes in the test solution for 30 minutes to one hour before making measurements in a fresh portion of test solution. During presoaking, adsorption was monitored by measuring the changes in pCu at constant pH.

III-B-5. Reproducibility and Nernstian Behavior

Measurements of pCu in 0.5 M KNO_3 solutions containing $CuSO_4$ in the concentration range 2×10^{-4} M to 2×10^{-7} M at 20°-21°C were performed on several occasions over a five month period (Figure 3-2). The solutions had pH values of 4.0 to 5.6 which is below the pH range where significant copper-hydroxide complexation occurs. A least squares linear regression of the experimental data yielded the relationship

$$pCu = -1.00 \pm 0.01 \log Cu_T + 0.58 \pm 0.04 \qquad (3\text{-}13)$$

where Cu_T is the added concentration of $CuSO_4$ and the error limits represent standard deviations. The data showed a standard error of 0.01. The one to one relationship between measured pCu (i.e. that calculated from measured potentials via the Nernst equation) and the negative log of the concentration of $CuSO_4$ is consistent with Nernstian response.

In section (V) of this thesis, pCu was measured with a cupric ion selective electrode in MWC culture media (artificial fresh water media, Table 2-2) containing 0.3 μM $CuSO_4$ at pH 8.2 and in DSW media (artificial 1/10th strength seawater, Table 2-3) containing 1 μM $CuSO_4$ at pH 8.1. The DSW medium contains an 0.05 M chloride concentration. Both media contain 1.0 mM $NaHCO_3$.

In Appendix (1), we demonstrated that chloride ion interferes with the cupric ion selective electrode at seawater chloride concentrations (i.e. approximately 0.5 M). Nerstian behavior was approached as the chloride ion concentration was decreased or as the degree to which copper was complexed was increased. The following pCu-pH measurements demonstrate that chloride ion interference does not occur in 0.05 M chloride solutions containing 1 mM $NaHCO_3$ in the pH range 7.5 to 8.2. Two solutions were tested; one containing 1 μM $CuSO_4$ and a second 0.2 μM $CuSO_4$ at 25°C. The pH was varied through changes in the test solution P_{CO_2}. Inasmuch as potassium and chloride ions are approximately equitransferent (Robinson and Stokes, 1959), the use of 0.05 M KCl should minimize liquid junction potentials.

Results of the measurements are presented in Figure (3-3). The solid curves in this figure represent computed pCu-pH curves which were calculated

Figure (3-2) Relationship between measured pCu and total copper concentrations in solutions containing 0.5 M KNO_3 and various concentrations of $CuSO_4$

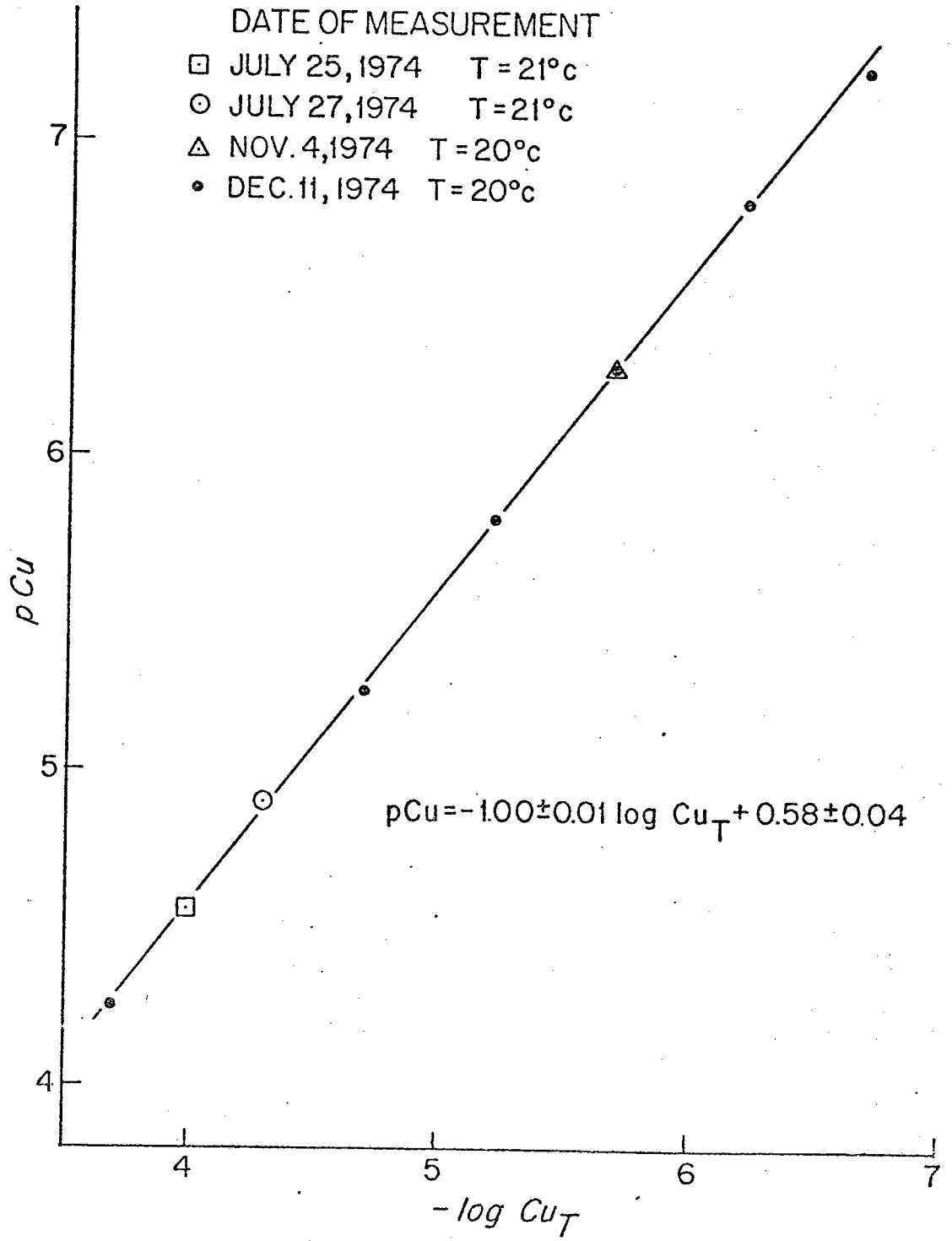
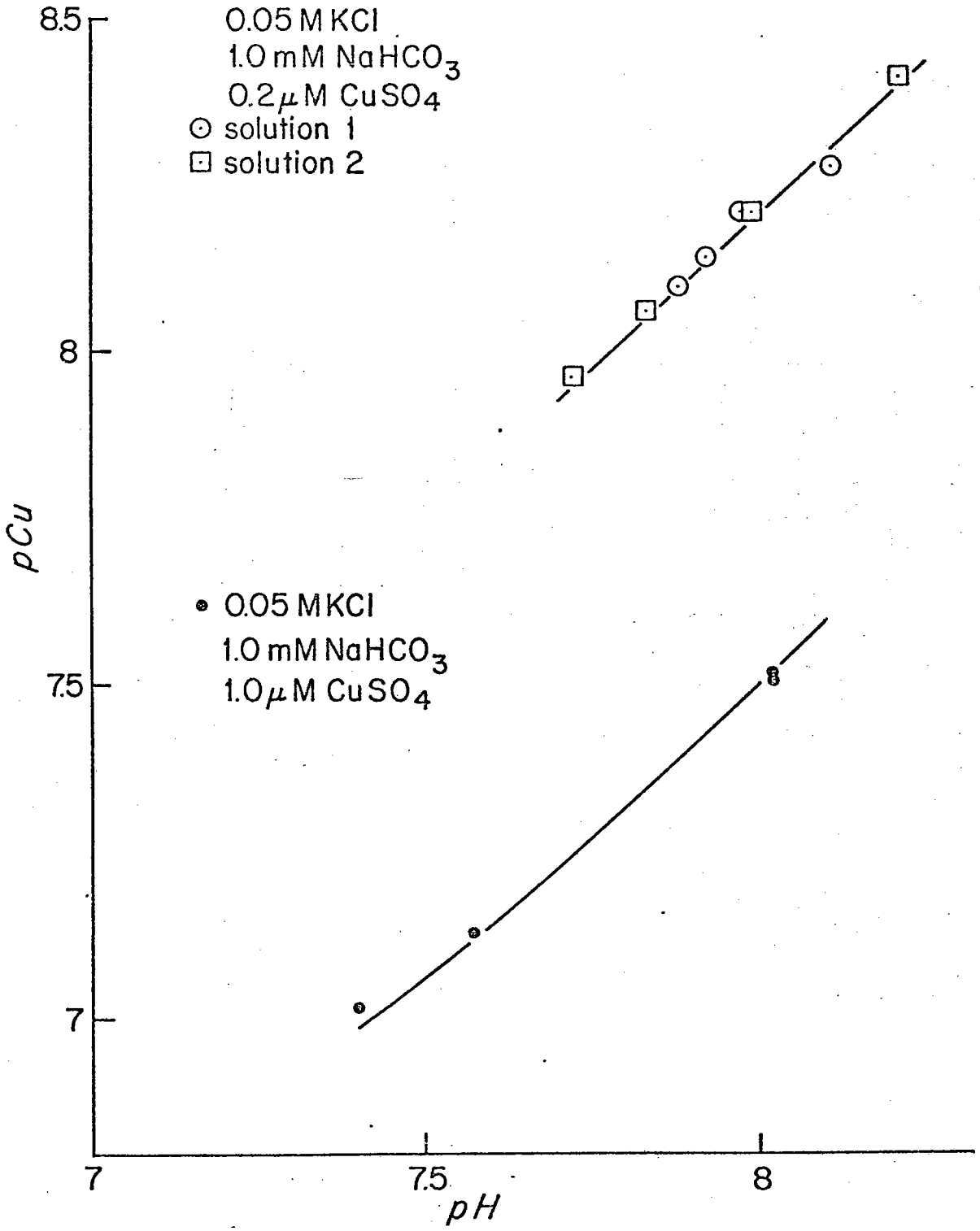


Figure (3-3) Comparison of measured and calculated pCu-pH values in solutions containing 0.05 M KCl, 1 mM $NaHCO_3$, and $CuSO_4$ at 25°C and variable P_{CO_2}.



from equation (A-2-8) in Appendix (2). The calculations take into account the formation of carbonate and hydroxide complex species $CuCO_3$, $Cu(CO_3)_2^{2-}$, and $CuOH^+$, whose association constants, as determined from pCu-pH measurements in dilute bicarbonate and hydroxide solutions (Appendix 2), are respectively $10^{6.80 \pm 0.01}$, $10^{10.40 \pm 0.04}$, $10^{6.59 \pm 0.03}$. Activity coefficients for the various copper species, which are also needed to compute copper complexation, were calculated from the Davies modification of the extended Debeye-Huckel equation (equation A-2-9 in Appendix 2).

The measured pCu-pH values in Figure (3-3) show good correlation with the calculated curves, again consistent with Nernstian response. Measured values in two separate solutions containing 0.2 µM $CuSO_4$ also show good reproducibility.

III-C. Calculation pCu in Seawater Culture Media

III-C-1. Introduction

Using known or experimentally measured equilibrium data, pCu was calculated for M-f/2 seawater-tris-$CuSO_4$ culture media used in copper growth inhibition and cellular copper uptake experiments. The pCu was also calculated for enriched artificial seawater media used in copper growth inhibition experiments by Davey et al. (1973) to compare their experimental results with ours.

The basic composition of seawater-tris-$CuSO_4$ media used in our experiments is given in Table (2-1). The media were prepared from surface Sargasso seawater (salinity ~ 35 $^{o}/_{oo}$) and contained 3-1000 µM $CuSO_4$, 0-10 mM of the chelator tris, and 1.0 µM of the chelator EDTA. For most experiments, the pH was

8.1-8.2; however, for one experiment with media containign 10 mM tris, the pH was varied in the range 7.7-8.7. Culture pCu is determined only for those media containing tris concentrations of 1 mM and above, i.e. only for those media that contain a large buffer capacity with respect to copper.

The culture medium used by Davey et al. (1973) was prepared according to the formulation of Kester et al. (1967), modified by the deletion of NaF, NaBr, and $SrCl_2$, addition of 3 μM NaH_2PO_4 and 30 μM $NaNO_3$ and the reduction of salinity to 30 °/oo. The experimental medium had a carbonate alkalinity of 2.03 mM, a pH of 8.2 ± 0.05, and a temperature of 20°C. Media were passed through a chelex resin column to remove possible trace metal contaminants and UV irradiated for three hours (using essentially the same method outlined in Section II-F) to minimize organic contaminants. Media prepared in this fashion contained less than 1 μgm Cu/l as determined by anodic stripping voltametry. Inasmuch as the seawater used in these experiments was prepared artificially and was treated with ultraviolet irradiation, we have assumed that the media were free from significant copper-organic complexation and thus have assumed that copper was only complexed by inorganic ligands.

III-C-2. General Equations

To calculated pCu all pertinent equilibria must be considered. The total concentration of copper in solution (Cu_T) is equal to the sum of the concentrations of the individual copper species

$$Cu_T = [Cu^{2+}] + \sum_i \sum_j [Cu(x_i)_j] \qquad (3\text{-}14)$$

where the square brackets denote molar concentrations of the enclosed species, x_i is a copper complexing ligand, and j is the number of ligands in an individual copper complex. Expressed in terms of activities equation (3-14) becomes

$$Cu_T = \frac{a_{Cu}}{\gamma_{Cu}} + \sum_i \sum_j \frac{a_{Cu(x_i)_j}}{\gamma_{Cu(x_i)_j}} \qquad (3\text{-}15)$$

At equilibrium

$$\frac{a_{Cu(x_i)_j}}{a_{Cu}\, a_{x_i}^{\,j}} = K_{Cu(x_i)_j} \qquad (3\text{-}16)$$

where $K_{Cu(x_i)_j}$ is an equilibrium association constant. Rearranging equation (3-16)

$$a_{Cu(x_i)_j} = a_{Cu}\, a_{x_i}^{\,j}\, K_{Cu(x_i)_j} \qquad (3\text{-}17)$$

and substituting this equation into equation (3-15)

$$Cu_T = a_{Cu}\left(\frac{1}{\gamma_{Cu}} + \sum_i \sum_j \frac{a_{x_i}^{\,j}\, K_{Cu(x_i)_j}}{\gamma_{Cu(x_i)_j}}\right) \qquad (3\text{-}18)$$

The pCu in culture media is obtained through the use of equation (3-18) or modifications thereof.

The pCu in the artificial seawater media of Davey et al. (1973) was determined from calculations and indirect measurements of the inorganic complexation of copper in seawater. For the calculation of pCu in Sargasso seawater-tris-$CuSO_4$ media used in our own experiments copper complexation by tris, EDTA, and inorganic ligands was considered. These calculations, which are presented in the following sections, show that in the latter media, copper is complexed predominantly by tris, to a much lesser extent by EDTA, and to a negligible extent by inorganic ligands. Because of the large concentrations of the chelator tris and total copper in these media, significant copper complexation by naturally occurring organic ligands is considered unlikely.

III-C-3. Inorganic Complexation of Copper in Seawater

The inorganic complexation of copper in seawater was computed by solving equation (3-18) in Section (III-C-2).

$$Cu_T = a_{Cu}\left(\frac{1}{\gamma_{Cu}} + \sum_i \sum_j \frac{a_{x_i}{}^j K_{Cu(x_i)_j}}{\gamma_{Cu(x_i)_j}}\right) \qquad (3\text{-}19)$$

where the individual terms in the double summation represent molar concentrations of the inorganic complexes $CuOH^+$, $CuCO_3$, $Cu(CO_3)_2^{2-}$, $CuCl^+$, $CuCl_2$, $CuCl_3^-$, $CuCl_4^{2-}$, and $CuSO_4$. Because of the lack of reliable association constants for copper-borate complexes, these cannot be considered in the present computations. The absence of significant borate complexation is demonstrated

by potentiometric measurement in Section (III-C-3-e).

To solve equation (3-19) we must obtain values for the various ligand activities, association constants, and activity coefficients.

Calculations were made for seawater at 25°C because pertinent thermodynamic data for the inorganic components of seawater are available at this temperature, but not at 20°C, the temperature of the culture media. Again, because of available data, calculations were performed for seawater of chlorinities 17 °/oo (salinity 31 °/oo) and 19 °/oo (salinity 35 °/oo).

III-C-3-a. Stability Constants

Association constants for the various copper complex species are listed in Table (3-3). Constants for the hydroxide and carbonate complexes $CuOH^+$, $CuCO_3$, and $Cu(CO_3)_2^{2-}$ were determined in the present work from potentiometric pCu-pH measurements (Appendix 2). The remaining were obtained from Sillen and Martel (1964).

III-C-3b. Activity Coefficients

Values for the activity coefficients for free copper ion and for the various copper complex species in seawater are not available in the literature and therefore must be estimated.

Seawater of chlorinity 19 °/oo has an ionic strength of 0.67 (Kester and Pytkowicz, 1967). At a chlorinity of 17 °/oo ionic strength would be approximately 0.6. A tabulation of mean activity coefficients ($\gamma \pm$) for several common electrolytes (Table 3-4) shows that activity coefficients are relatively

constant for the ionic strength range 0.5 to 0.9. By analogy we have assumed that activity coefficients for the various copper species are approximately the same for seawater of ionic strength 0.6 and 0.67.

If we make the assumption that activity coefficients are determined by the ionic strength and not the particular ionic composition of a solution (Garrels and Thompson, 1962) then the activity coefficient of cupric ion in seawater should be approximately equal to that in any other solution of the same ionic strength. Thus, a value of 0.26, measured with a cupric ion selective electrode in a 0.6 M KNO_3 solution, provides an estimate of the cupric ion activity coefficient in seawater.

Along with ionic strength, activity coefficients are primarily determined by the ion charge, and to a lesser extent by the ionic radius. Inasmuch as we have no way of knowing the ionic radii of the various copper complex species this factor cannot be considered and all species of the same ionic charge are assumed to have the same activity coefficient.

The activity coefficients for the divalent species $Cu(CO_3)_2^{2-}$ and $CuCl_4^{2-}$ are assigned a value of 0.26, the activity coefficient for the doubly charged cupric ion. Monovalent species $CuOH^+$, $CuCl^+$, and $CuCl_3^-$ are given activity coefficient values of 0.64 equal to that of singly charged chloride ion in salinity 35 $^o/oo$ seawater (Garrels and Thompson, 1962). Uncharged species $CuCO_3$, $CuCl_2$, and $CuSO_4$ are assumed to have activity coefficients equal to one.

III-C-3c. Activities of Inorganic Complexing Ligands

The activities of ligands CO_3^{2-}, Cl^-, and SO_4^{2-} were determined from either experimentally measured or calculated total ion activity coefficients (γ^*) from the literature. The total ion activity coefficient for an ion, for example $\gamma^*_{SO_4}$, is defined by the expression

$$\gamma^* SO_4 = \frac{a_{SO_4}}{(SO_4)_T} \qquad (3\text{-}20)$$

where $(SO_4)_T$ is the total concentration of sulfate including both "free" ions and ion-paired or complexed species.

Berner (1965) reported experimentally measured apparent total single ion activity coefficients for carbonate and bicarbonate ions in seawater of 17 $^o/oo$ and 19 $^o/oo$ chlorinity having respective carbonate alkalinities of 2.11 mM and 2.38 mM: At chlorinities of 17 $^o/oo$ and 19 $^o/oo$ respectively $\gamma^*_{HCO_3}$ = 0.56 ± 0.006 and 0.55 ± 0.007, and $\gamma^*_{CO_3}$ = 0.024 ± 0.04 and 0.020 ± 0.002.

The activity of carbonate ions in seawater of the above two chlorinities is calculated from a modification of equation (A2-6) in Appendix (2).

$$a_{CO_3} = \frac{\text{carbonate alkalinity}}{\frac{a_H \; 10^{10.33}}{\gamma^*_{HCO_3}} + \frac{2}{\gamma^*_{CO_3}}} \qquad (3\text{-}21)$$

where normal single ion activity coefficients, γ_{HCO_3} and γ_{CO_3}, have been re-

placed by total activity coefficients, $\gamma^*_{HCO_3}$ and $\gamma^*_{CO_3}$. A tabulation of carbonate activities as a function of pH is presented in Table (3-5).

From both measured and calculated sulfate total ion activity coefficients for seawater (Platford and Dafoe, 1965; Leyendekers, 1973; Whitfield, 1973)

$$\gamma^*_{SO_4} \quad (Cl = 19\ ^o/oo) = 0.12 \qquad (3\text{-}22)$$

$$\gamma^*_{SO_4} \quad (Cl = 17\ ^o/oo) = 0.13 \qquad (3\text{-}23)$$

For seawater at the above two chlorinities, sulfate concentrations are 0.028 and 0.025 and thus sulfate activities are calculated to be 0.0034 and 0.0033 M, respectively (equation 3-20).

Inasmuch as chloride ion is essentially uncomplexed in seawater (Garrels and Thompson, 1962), $\gamma^*_{Cl} = \gamma_{Cl}$. Chloride ion activities in seawater are calculated to be

$$0.55 \times 0.64 = 0.35M \quad (Cl = 19\ ^o/oo) \qquad (3\text{-}24)$$

$$0.49 \times 0.64 = 0.31M \quad (Cl = 17\ ^o/oo) \qquad (3\text{-}25)$$

where 0.55 M and 0.49 M are the chloride concentrations at the two chlorinities and 0.64 is the activity coefficient of chloride ion in seawater (Garrels and Thompson, 1962).

III-C-3-d. Calculation Results

The calculated speciation of copper in 17 $^o/oo$ and 19 $^o/oo$ chlorinity sea-

water along with values for log Cu_T/a_{Cu} are given in Table (3-6). Calculations show that higher order chloride complexes $CuCl_2$, $CuCl_3^-$, and $CuCl_4^{2-}$ are negligible and thus these complexes are not included in this table. Inorganic copper complexation for seawater of the two chlorinities (17 °/oo and 19 °/oo) is similar. At pH 8.2, the principal copper species are $CuCO_3$ (∿ 70% of the total copper), $CuOH^+$ (∿ 15%), $Cu(CO_3)_2^{2-}$ (∿ 7%), and Cu^{2+} (∿ 6%). At this pH chloride and sulfate species together account for less than 2% of the total copper. Because of the predominance of carbonate and hydroxide complexes, the inorganic copper complexation is highly pH dependent.

III-C-3-e. A Potentiometric Test of Calculated Inorganic Speciation

A test of the seawater calculations was provided by measurements of copper complexation in a solution of 0.65 M KNO_3, 4 μM $CuSO_4$, and $NaHCO_3$ (25°C) and a solution of 0.5 M KNO_3, 4 μM $CuSO_4$, and $NaHCO_3$ (20°C) equilibrated with atmospheric CO_2 ($P_{CO_2} \sim 10^{-3.5}$; Stumm and Morgan, 1970) at the equilibrium pH of seawater (8.2-8.3, Berner, 1965). Potassium nitrate was chosen to provide a background electrolyte of minimal junction potential (Table 3-2). The temperatures 20°C and 25°C are that of the culture experiments and that of the seawater calculations respectively.

Measurements in the above solutions take into account complexation to carbonate and hydroxide ions which our calculations showed accounted for ≥ 97% of the inorganic copper complexation at pH ≥ 8.1. Complexation by carbonate and hydroxide ions should be essentially the same in the KNO_3 solutions and

in seawater (in equilibrium with atmospheric CO_2 at the same pH) since the activities of these ligands and activity coefficients for various complex species should be approximately equal in both sets of solutions.

At constant pH, hydroxide ion activity is constant since

$$a_{OH^-} = K_W/a_{H^+} \tag{3-26}$$

where K_W is the dissociation constant for water.

At constant pH and P_{CO_2} the activity of carbonate ion is fixed at a constant value according to the expression

$$a_{CO_3^{2-}} = \frac{P_{CO_2}}{a_{H^+}^2 \, K_{H_2CO_3} \, K_{HCO_3^-} \, K_H} \tag{3-27}$$

where $K_{H_2CO_3}$ and $K_{HCO_3^-}$ are the hydrogen ion association constants for the formation of H_2CO_3 and HCO_3^- respectively and K_H is the equilibrium constant for the dissolution of CO_2 (equation 3-27 is modified from equation 8, page 126 in Stumm and Morgan, 1970).

At 25°C the equilibrium pH values for the chlorinity 17 °/oo and 19 °/oo seawater samples are respectively 8.25 ± 0.01 and 8.28 ± 0.02 (Berner, 1965). These values include a liquid junction error which is due to the difference in liquid junction potential of the saturated KCl/calomel reference electrode between seawater and dilute pH standard solutions. Because of junction po-

tential, the measured pH values do not correspond exactly with hydrogen ion activities. Hawley and Pytkowicz (1973) recently estimated that the liquid junction potential error for seawater is in the order of -0.05 pH units.

In our calculations, copper carbonate complexation was determined using apparent seawater total ion activity coefficients for carbonate and bicarbonate ions. Inasmuch as these values were determined from pH measurements and therefore also contain a liquid junction pH error, the present parameter of interest is measured pH and not hydrogen ion activity. A slight pH error will occur due to the difference in reference electrode liquid junction potential between seawater and concentrated KNO_3 solutions. Calculations using the Henderson equation (Table 3-2) estimate this error to be roughly 0.02 pH units.

The pCu was measured as a function of pH in the above KNO_3 - 4 μM $CuSO_4$ solutions as the alkalinity was varied through the addition of a concentrated solution of $NaHCO_3$ (Figure 3-4). After the addition of bicarbonate, the test solutions were atmospherically equilibrated by aeration through scintered glass frits. At the equilibrium pH for 17 $^{o}/_{oo}$ chlorinity seawater (8.25 $\pm$ 0.01) and 19 $^{o}/_{oo}$ chlorinity seawater (8.28 $\pm$ 0.02) the measured ratios for Cu_T/a_{Cu} (at 25°C) are respectively $10^{1.90 \pm 0.05}$ and $10^{1.96 \pm 0.08}$. These values agree well with calculated values: $10^{1.82}$ and $10^{1.86}$. The slight differences between measured and calculated values are probably accounted for by errors in the estimated activity coefficients for the various copper complex species and/or slight differences in the pH reference electrode junction potential between seawater and concentrated KNO_3 solutions.

For calculations of the inorganic speciation of copper in seawater, complexation to borate was assumed to be negligible and thus was not considered

in the computations. In the present set of measurements, the addition of a seawater concentration of H_3BO_3 (0.43 mM) to a solution containing 0.65 M KNO_3, 4 μM $CuSO_4$, and $NaHCO_3$ equilibrated with the atmosphere at pH 8.20 and 25°C, showed no measurable borate complexation, thus confirming the above assumption.

III-C-4. Determination of pCu in Artificial Seawater Culture Media of Davey et al. (1973)

For culture experiments of Davey et al. (1973) in artificial 30 °/00 seawater at 20°C and pH 8.2, pCu was determined using both the calculated values for inorganic complexation in 31 °/oo salinity seawater at pH 8.2 and 25°C and the measured value in the 0.5 KNO_3-$NaHCO_3$ solution in equilibrium with the atmosphere at pH 8.2 and 20°C. Calculated and measured values for the ratio of total copper concentration to copper ion activity (Cu_T/a_{Cu}) are respectively $10^{1.78}$ and $10^{1.74}$ yielding a mean value of $10^{1.76}$. Culture medium pCu is calculated from the equation

$$Cu_T = a_{Cu}\ 10^{1.76} \qquad (3\text{-}28)$$

where Cu_T is the added concentration of copper. Taking the log of equation (3-28)

$$pCu = -\log Cu_T + 1.76 \qquad (3\text{-}29)$$

III-C-5. Calculation of pCu in Seawater-Tris-$CuSO_4$ Culture Media

III-C-5-a. Determination of a pH Dependent Apparent Equilibrium Constant for Copper-Tris Complexation

The complexation of copper by tris in culture media was determined from an experimentally measured pH dependent apparent stability constant obtained

Table (3-3)

Association Constants for Inorganic Complexes and Ion Pairs of Copper in Seawater

Reaction	Log K
(1) $Cu^{2+} + OH^- = CuOH^+$	6.59 ± 0.03
(2) $Cu^{2+} + CO_3^{2-} = CuCO_3$	6.80 ± 0.01
(3) $Cu^{2+} + 2CO_3^{2-} = Cu(CO_3)_2^{2-}$	10.40 ± 0.04
(4) $Cu^{2+} + SO_4^{2-} = CuSO_4$	2.34
(5) $Cu^{2+} + Cl^- = CuCl^+$	0.08
(6) $Cu^{2+} + 2Cl^- = CuCl_2$	-0.7*
(7) $Cu^{2+} + 3Cl^- = CuCl_3^-$	-2.1*
(8) $Cu^{2+} + 4Cl^- = CuCl_4^{2-}$	-4.3*

*Constants at ~ 22°C; all other constants are for 25°C.
All association constants are at infinite dilution.

Table (3-4)

Mean Salt Activity Coefficients for Some Electrolytes at 25°C

Ionic Strength*	0.5	0.6	0.7	0.9
HCl	.76	0.76	0.77	0.79
HNO_3	.72	0.72	0.72	0.72
NaCl	.68	0.67	0.67	0.66
Na Acetate	.74	0.74	0.74	0.75
KCl	.65	0.64	0.63	0.61
K Acetate	.75	0.75	0.76	0.77
RbCl	.63	0.62	0.61	0.59
$MgCl_2$		0.49		0.48
$CaCl_2$		0.47		0.46
$Cu(NO_3)_2$		0.46		0.44

Values taken from Robinson and Stokes (1959).

*Ionic strength is defined by the equation

$$I = 1/2 \sum c_i z_i^2$$

where c_i and z_i are respectively the molal concentration and the charge of the individual ions.

Table (3-5)

Calculated Activity of Carbonate Ion in Seawater as a Function of pH

pH	a_{CO_3} ($\times 10^{-6}$) (seawater of chlorinity 19 °/oo and carbonate alkalinity 2.38 mM)	a_{CO_3} ($\times 10^{-6}$) (seawater of chlorinity 17 °/oo and carbonate alkalinity 2.11 mM)
7.7	2.7	2.5
7.8	3.3	3.0
7.9	4.0	3.7
8.0	4.8	4.5
8.1	5.9	5.5
8.2	6.9	6.5
8.3	8.1	7.7
8.4	9.4	9.0

Table (3-6)

Calculated Inorganic Speciation of Copper in Seawater as a Function of pH. Values for Copper Species are Given as the Percent of the Total Copper Present in an Inorganic Form.

Seawater of Chlorinity 19 $^{o}/_{oo}$ and Carbonate Alkalinity 2.38 mM

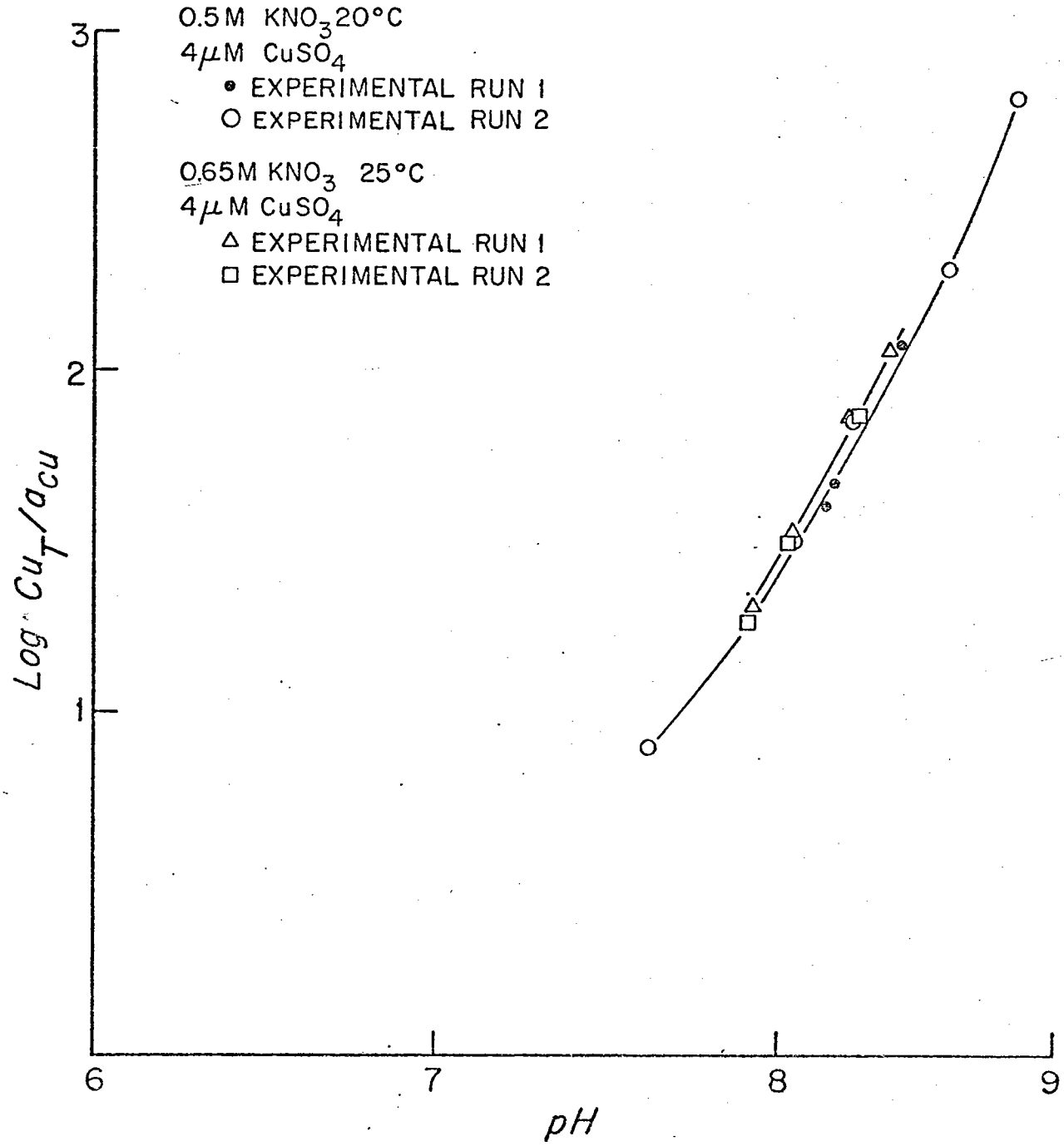
pH	7.7	7.8	7.9	8.0	8.1	8.2	8.3	8.4
Cu^{2+}	15	12	10	9	7	6	5	4
$CuCO_3$	65	67	69	69	70	69	68	67
$Cu(CO_3)_2^{2-}$	3	3	4	5	6	7	8	9
$CuOH^+$	12	12	13	14	14	15	16	17
$CuCl^+$	2	2	2	1	1	1	1	1
$CuSO_4$	3	2	2	2	1	1	1	1
log Cu_T/a_{Cu}	1.41	1.49	1.57	1.64	1.73	1.80	1.87	1.94

Seawater of Chlorinity 17 $^{o}/_{oo}$ and Carbonate Alkalinity 2.11 mM

pH	7.7	7.8	7.9	7.0	8.1	8.2	8.3	8.4
Cu^{2+}	16	13	11	9	8	7	6	5
$CuCO_3$	64	66	68	68	69	69	68	67
$Cu(CO_3)_2^{2-}$	2	3	4	5	6	7	8	9
$CuOH^+$	12	13	15	15	15	16	17	18
$CuCl^+$	2	2	2	1	1	1	1	1
$CuSO_4$	3	3	2	2	1	1	1	1
log Cu_T/a_{Cu}	1.39	1.46	1.54	1.62	1.70	1.78	1.86	1.93

Figure (3-4)

Measured copper complexation as a function of pH in solutions containing 0.5 M or 0.65 M KNO_3, 4 μM $CuSO_4$, and variable concentrations of $NaHCO_3$ in equilibrium with atmospheric CO_2. Log Cu_T/a_{Cu} = log Cu_T + pCu.



in solutions of 0.5 M KNO_3, tris, and $CuSO_4$. (Reliable equilibrium constants are not available in the literature.)

Measurements were made in 0.5 M KNO_3 (I = 0.5) to provide an ionic medium of minimal reference electrode junction potential with an ionic strength close to that of seawater (I = 0.67). Activity coefficients for various chemical species involved in copper-tris complexation should be similar for seawater and for 0.5 M KNO_3 (see Table 3-4).

The apparent constant for copper-tris complexation was determined by measuring pCu as a function of tris concentration in a solution of 0.5 M KNO_3 and 0.05 mM $CuSO_4$ at pH 8.10 and 21°C (Figure 3-5), and by measuring pCu as a function of pH in 0.5 M KNO_3 solutions containing 28.6 mM tris plus 0.05 mM $CuSO_4$ at 21°C; 10 mM tris plus 0.1 mM $CuSO_4$ at 21°C; and 2.0 mM tris plus 0.02 mM $CuSO_4$ at 20°C (Figure 3-6).

The measured relationship between pCu and tris concentration at pH 8.10 (Figure 3-5) was consistent with the expression

$$pCu = -\log Cu_T + 2.00 \pm 0.01 \log [tris^*] + 9.52 \pm 0.03 \quad (3\text{-}30)$$

where

$$[tris^*] = Tris_T - 2\,Cu_T \quad (3\text{-}31)$$

Constants plus error limits were obtained from a least squares linear regression of pCu vs. $[tris^*]$. The slope of two in equation (3-30) indicates the presence of 2:1 tris-copper complexes (see Appendix 3). The term $[tris^*]$ represents the concentration of uncomplexed tris since essentially all of

the copper in solution is complexed by tris and each copper ion binds with two tris molecules.

Measurements of pCu as a function of pH in solutions containing 2, 10, and 28.6 mM tris (Figure 3-6) show that complexation of copper by tris is highly pH dependent. In the experimental pH range of the culture experiments, 7.7-8.7, the measured pCu-pH curves are linear and parallel to one another with a slope of 2.85 ± 0.05.

All of the experimental pCu-pH data taken together for the pH range 7.5 to 8.7 which include tris concentrations from 1 to 29 mM are consistent (± 0.03 pCu) with the equation

$$pCu = -\log Cu_T + 2 \log [tris^*] + 9.51 + 2.85\,(pH - 8.1) \qquad (3\text{-}32)$$

(see Table 3-7).

Taking the antilog of equation (3-32), we derive the expression

$$\frac{Cu_T}{a_{Cu}\,[tris^*]^2} = 10^{[9.51 + 2.85\,(pH-8.1)]} \qquad (3\text{-}33)$$

Since the copper in solution is almost totally present as 2:1 tris copper complexes

$$Cu_T \doteq \sum_{i=o}^{2} [CuH_i(tris)_2] \qquad (3\text{-}34)$$

and therefore combining equations (3-40) and (3-41) we obtain an expression for the total concentration of copper-tris complexes in solution

$$\sum_{i=o}^{2} [CuH_i(tris)_2] = a_{Cu} [tris^*]^2 \; 10^{(9.51 + 2.85\ (pH-8.1))} \qquad (3\text{-}35)$$

Seawater contains relatively high concentrations of sodium (0.46 M for 35 $^{o}/_{oo}$ seawater), calcium (0.010 M), and magnesium (0.056 M). Any complexation of these ions by tris, would lower the concentration of free tris ions in solution and thus decrease the amount of tris available for copper complexation.

Good et al. (1966) report that tris does not complex appreciably with either calcium or magnesium ions. Measurements of pCu as a function of pH in a solution containing 0.2 M KNO_3, 0.07 M $Mg(NO_3)_2$ and 0.015 M $Ca(NO_3)$ (ionic strength 0.46) at 20^{o}C are consistent with this (Figure 3-7, Table 3-8). Similar measurements in a solution of 0.5 M $NaNO_3$, 1.0 mM tris, and 0.01 mM $CuSO_4$ agree with equation (3-32) to within 0.01 pCu unit indicating that sodium ion does not appreciably affect copper-tris complexation (Figure 3-7, Table 3-8).

III-C-5-b. Determination of pCu

The pCu in seawater-tris-$CuSO_4$ culture media is calculated from the equation

$$Cu_T = a_{Cu} [tris^*]^2 \; 10^{(9.51 + 2.85\ (pH - 8.1))} + \frac{a_{Cu}\ EDTA_T\ 10^{18.79}}{a_{Cu}\ 10^{18.79} + 10^{7.95}} \qquad (3\text{-}36)$$

Figure (3-5)

Relationship between pCu and "free" tris concentration in a solution containing 0.5 M KNO_3 and 0.05 mM $CuSO_4$ at 21°C. The solid line, which has a slope of 2.00 ± 0.01, represents a least squares linear regression of pCu vs. -log [tris*]. The term [tris*] represents the concentration of tris in the solution that has not reacted with copper. $[tris^*] = tris_T - 2\ Cu_T$

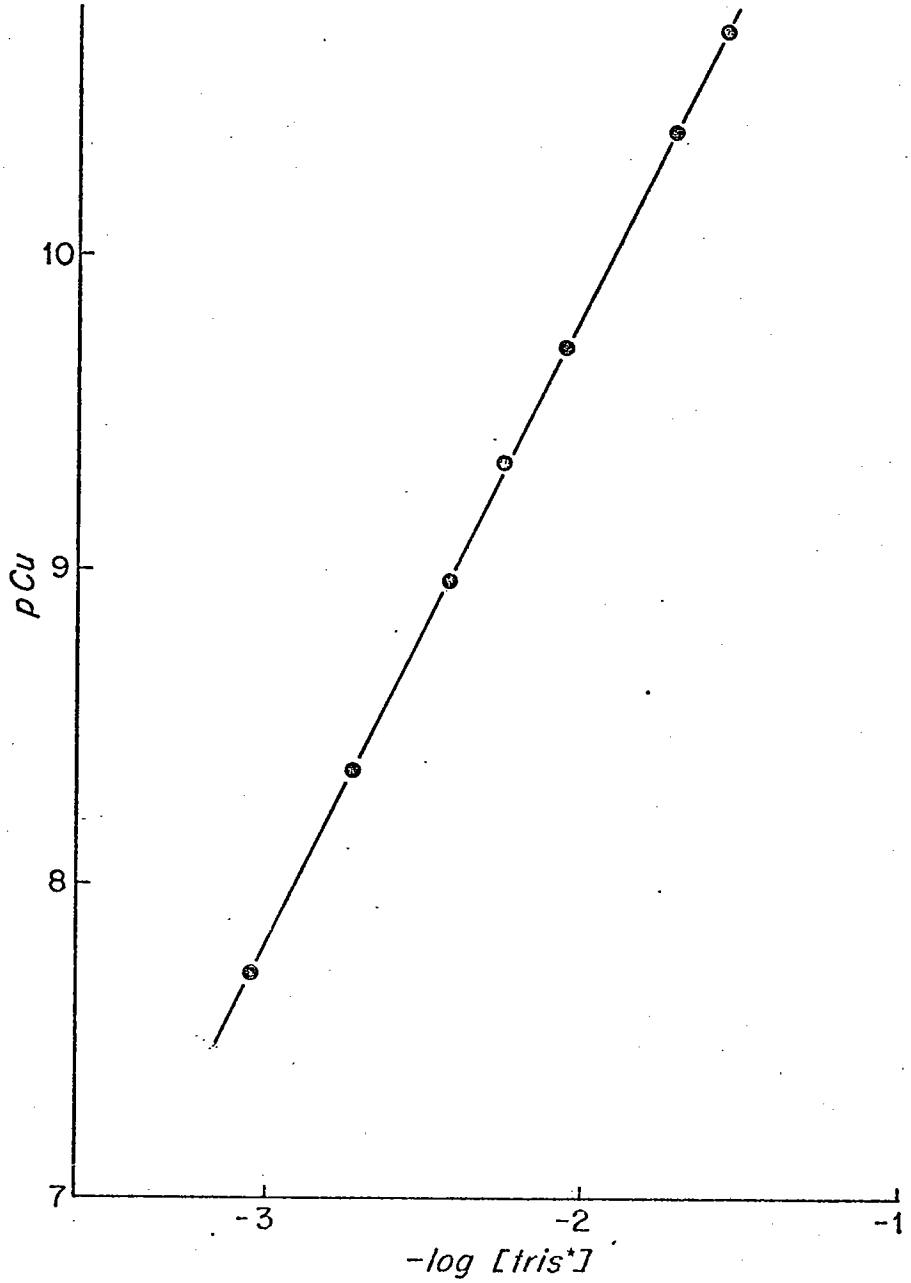


Figure (3-6)

Measured relationship between pCu and pH in solutions containing 0.5 M KNO_3 and specified concentrations of tris and $CuSO_4$

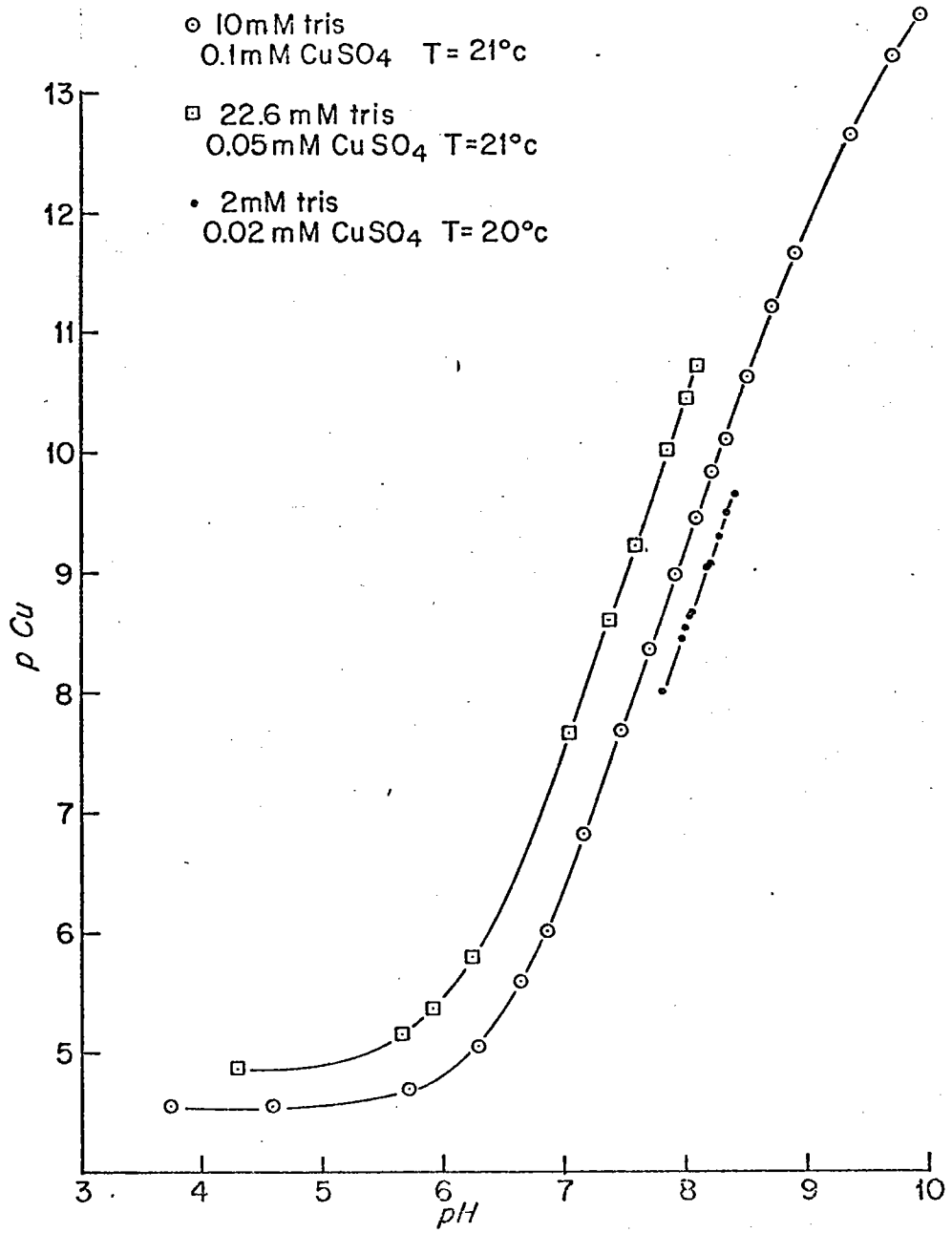


Figure (3-7)

Measured pCu-pH relationships in $CuSO_4$-tris solutions at 20°C: effect of sodium, magnesium, and calcium ions.

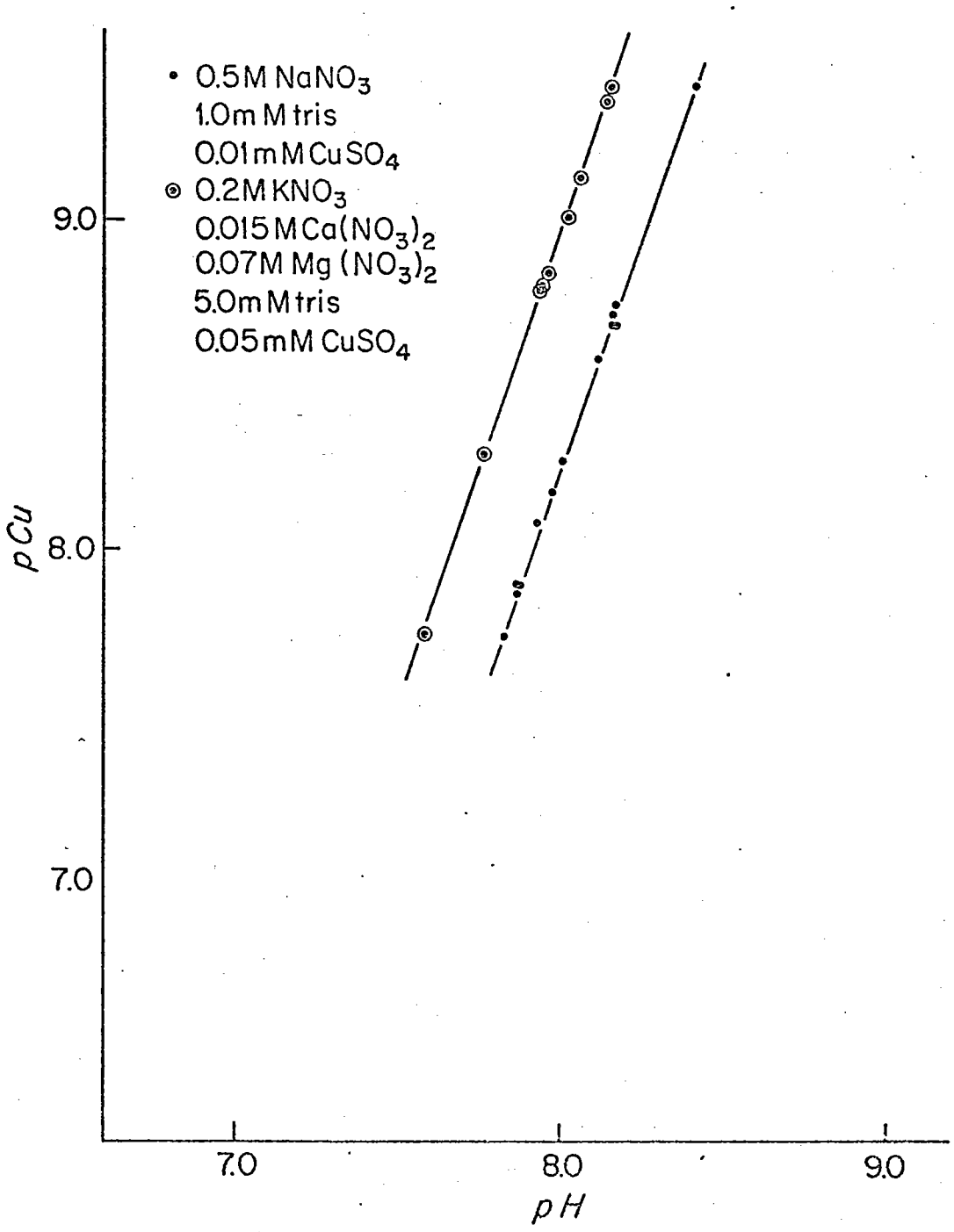


Table (3-7)

Comparison of measured and calculated pCu as a function of pH and tris concentration in media containing 0.5 M KNO_3, tris, and $CuSO_4$.

Test Solution No.	Temperature (°C)	$Tris_T$ (mM)	$CuSO_4$ (mM)	pH	pCu (measured)	pCu (calculated*)	Difference
1	21	0.99	0.05	8.10	7.71	7.71	0.00
		1.96		8.10	8.35	8.35	0.00
		3.85		8.10	8.97	8.96	0.01
		5.66		8.10	9.34	9.30	0.04
		9.09		8.10	9.71	9.72	-0.01
		19.4		8.10	10.39	10.38	0.01
		28.6		8.10	10.72	10.72	0.00
				8.01	10.45	10.46	-0.01
				7.86	10.01	10.04	-0.03
2	21	10.0	0.10	8.08	9.44	9.44	0.00
				8.22	9.83	9.83	0.00
				8.35	10.11	10.20	-0.09
				8.49	10.62	10.60	0.02
				8.72	11.20	11.26	-0.06
				8.20	9.82	9.78	0.04
				7.93	8.98	9.01	-0.03
				7.70	8.35	8.35	0.00
				7.47	7.68	7.70	0.02
3	20	10.0	0.10	8.10	9.52	9.49	0.03
4	20	2.0	0.02	8.05	8.65	8.65	0.00
				8.03	8.62	8.60	0.02
				8.17	9.03	8.99	0.04
				8.27	9.29	9.30	-0.01
				7.82	8.01	8.00	0.01
				7.97	8.46	8.42	0.04
				8.06	8.67	8.68	-0.01
				8.40	9.65	9.65	0.00
5	20	2.0	0.02	8.00	8.54	8.51	0.03
				8.20	9.06	9.08	-0.02
				8.34	9.48	9.48	0.00

*$pCu = -\log Cu_T + 2 \log [tris^*] + 2.85 (pH - 8.1) + 9.51$

Mean difference for measured minus calculated pCu values is 0.00.

Standard deviation is 0.03.

Table (3-8)

pCu as a function of pH in solutions containing tris, $CuSO_4$, and high concentrations of $NaNO_3$ or $Mg(NO_3)_2$ and $Ca(NO_3)_2$ at 20°C.

Background Medium (M)	$Tris_T$	Cu_T	pH	pCu (measured)	pCu (calculated*)	Difference
1) 0.5 M $NaNO_3$	1.0	0.01	7.88	7.89	7.87	0.02
			7.87	7.86	7.84	0.02
			8.18	8.68	8.72	-0.04
			8.42	9.41	9.44	-0.03
			7.98	8.18	8.15	0.03
			7.83	7.73	7.77	-0.04
			7.93	8.08	8.01	0.07
			8.02	8.27	8.26	0.01
			8.17	8.68	8.69	-0.01
			7.87	7.90	7.84	0.06
			8.12	8.58	8.55	0.03
			8.18	8.75	8.72	0.03
			8.17	8.71	8.69	0.02
2) 0.07 M $Mg(NO_3)_2$ 0.015 M $Ca(NO_3)_2$ 0.2 M KNO_3	5.0	0.05	7.58	7.74	7.69	0.05
			7.93	8.79	8.69	0.10
			7.94	8.80	8.72	0.08
			8.15	9.40	9.31	0.09
			7.76	8.29	8.20	0.09
			7.96	8.84	8.77	0.07
			8.06	9.13	9.06	0.07
			8.14	9.36	8.29	0.07
			8.02	9.01	8.94	0.07

Calculated from equation (3-32) $pCu = -\log Cu_T + 2 \log [tris^*] + 2.85\ (pH\ 8.1) + 9.51$.

Mean difference of measured vs. calculated pCu:

Solution (1) 0.01

Solution (2) 0.08

where the first term on the right hand side of the equation is the concentration of copper-tris complexes and the second term is the concentration of $CuEDTA^{2-}$ (derivation of this second term is given in Appendix 4-A). The concentration of tris that is not complexed to copper, $[tris^*]$, is calculated from the following equations

$$[tris^*] = [H_2tris^+] + [Htris] \quad (3\text{-}37)$$

$$Cu_T = \sum_{i=o}^{2} [CuH_i(tris)_2] + [CuEDTA^{2-}] \quad (3\text{-}38)$$

$$Tris_T = [H_2tris^+] + [Htris] + 2\sum_{i=0}^{2} [CuH_i(tris)_2] \quad (3\text{-}39)$$

Therefore

$$[Tris^*] = tris_T - 2(Cu_T - [CuEDTA^{2-}]) \quad (3\text{-}40)$$

The pCu in culture media is determined by solving equations (3-36) and (3-40) simultaneously.

The ratio Cu_T/a_{Cu} calculated from this equation is in all cases $\geq 10^{3.6}$ and therefore the presence of inorganic copper complexes has a negligible effect on culture pCu.

Calculated pCu values for the various experimental cultures, along with values for added tris ($Tris_T$), added $CuSO_4$ (Cu_A), measured total copper concentration (Cu_M), $[CuEDTA^{2-}]$, and pH are reported in Table (3-9).

Table (3-9)

Calculated pCu for culture experiments in seawater-tris-1.0 μM EDTA-$CuSO_4$ media.

Experimental Run	$Tris_T$ (mM)	Cu_A (μM)	Cu_M (μM)	$CuEDTA^{2-}$ (μM)	pH	pCu
A - T. pseudonana	10	3		0.3 ± 0.1	8.16 ± 0.05	11.25 ± 0.14
	10	5		0.4 ± 0.1	8.15 ± 0.05	10.99 ± 0.14
	10	10		0.6 ± 0.1	8.16 ± 0.06	10.71 ± 0.17
	10	20		0.7 ± 0.1	8.16 ± 0.06	10.40 ± 0.17
	10	30		0.8 ± 0.1	8.16 ± 0.06	10.22 ± 0.17
	10	100		0.9	8.14 ± 0.04	9.61 ± 0.11
	10	200		1.0	8.15 ± 0.05	9.32 ± 0.14
	10	500		1.0	8.15 ± 0.06	8.86 ± 0.17
	10	1000		1.0	8.14 ± 0.04	8.43 ± 0.11
B - T. pseudonana	3	30		1.0	8.16 ± 0.04	9.16 ± 0.11
	3	100		1.0	8.13 ± 0.03	8.49 ± 0.09
	10	30		0.8 ± 0.1	8.16 ± 0.06	10.22 ± 0.17
	10	100		0.9	8.15 ± 0.05	9.64 ± 0.14
	10	300		1.0	8.15 ± 0.04	9.12 ± 0.11
	10	1000		1.0	8.14 ± 0.04	8.43 ± 0.11
C - T. pseudonana	1	5	5.2	1.0	8.13 ± 0.03	8.96 ± 0.09
	1	10	10.1	1.0	8.13 ± 0.03	8.61 ± 0.09
	2	10	10.4	1.0	8.12 ± 0.03	9.19 ± 0.09
	2	20	20.3	1.0	8.11 ± 0.02	8.84 ± 0.06
	2	50	48.3	1.0	8.12 ± 0.02	8.45 ± 0.06
	5	30	31.3	1.0	8.10 ± 0.01	9.42 ± 0.03
	5	80		1.0	8.10 ± 0.02	8.98 ± 0.06
D - T. pseudonana	2	4	3.2	0.9	8.15 ± 0.04	9.89 ± 0.11
	3	10	9.9	0.9	8.13 ± 0.02	9.59 ± 0.06
	3	20	19.9	1.0	8.14 ± 0.03	9.29 ± 0.09
	5	4	3.1	0.6	8.12 ± 0.02	10.57 ± 0.06
	5	10	10.1	0.9	8.10 ± 0.01	9.94 ± 0.03
	5	20	20.1	0.9	8.10 ± 0.01	9.62 ± 0.03
	5	50	49.4	1.0	8.09 ± 0.01	9.18 ± 0.03
	5	400		1.0	8.10 ± 0.01	8.15 ± 0.03

Table (3-9) (Continued)

Experimental Run	$Tris_T$ (mM)	Cu_A (μM)	Cu_M (μM)	$CuEDTA^{2-}$ (μM)	pH	pCu
E - T. pseudonana	10	10		0.09	7.73 ± 0.04	9.50 ± 0.11
	10	100		0.10	7.71 ± 0.03	8.38 ± 0.09
	10	100		0.09	8.17 ± 0.05	9.69 ± 0.14
	10	100		0.03	8.70 ± 0.03	11.20 ± 0.09
F - N. atomus	10	0.04		0.007	8.10 ± 0.02	13.00 ± 0.06
	10	30		0.09	8.10 ± 0.02	10.05 ± 0.06
	10	100		0.09	8.10 ± 0.02	9.49 ± 0.06
	10	300		1.0	8.10 ± 0.02	8.98 ± 0.06
	10	1000		1.0	8.10 ± 0.02	8.32 ± 0.06

Definition of Terms

Cu_A is the concentration of added $CuSO_4$

Cu_M is the measured concentration of copper in experimental media

$Tris_T$ is the concentration of added tris

pH values represent mean culture values - error limits correspond to changes in culture pH during experimental runs

pCu values are mean culture values with error limits corresponding to culture pCu variation due to changes in pH

In roughly one-third of the cultures, including most of the cultures for which cell copper content was determined, the total concentration of copper in the culture medium was measured directly, using atomic absorption spectrophotometry, for culture filtrates taken three days after culture initiation.

For pCu calculations, the total copper concentration was set equal to the measured concentration in all cases where this parameter was measured. In all other cases, Cu_T was given a value equal to the added concentration. Good agreement was generally obtained between measured and added concentration values (see Table 3-9).

In all tris containing cultures for which cell copper contents were determined, cell copper represented less than 1/10,000 of the added copper concentration. Thus, cell copper uptake did not significantly alter the concentration of copper in the culture media.

In order to maintain a relatively constant pCu, culture medium pH must be controlled. In cultures containing $\leq$ 5 mM tris, pH was controlled by the presence of tris, which acts as both a pCu and pH buffer, and by aeration through scintered glass frits. In experimental cultures containing 10 mM tris, the cultures were not aerated and pH was controlled just by the buffering action of tris.

Due to photosynthetic removal of CO_2, the pH in cultures of T. pseudonana (clone 3H) did not remain strictly constant, but increased slightly as the

cultures grew (usually by less than 0.1 pH unit). Mean pH values for culture experiments with T. pseudonana are reported in Table (3-9) with error limits indicating the pH change that occurred in the individual flasks. The reported error limits for pCu (Table 3-9) result from the slight changes in culture pH.

For the experiment F - N. atomus the time dependent change in culture medium pH was not measured. However, for cultures in identical media containing no tris, and thus having a significantly lower buffer capacity, no pH increase was detected for cell concentrations less than or equal to 1.1 x 10^6 cells/ml. In the present seawater-tris cultures, cell concentrations were in all cases less than this amount and thus the presence of the cells should not have altered culture medium pH significantly. The pH values for these cultures reported in Table (3-9) were measured immediately before cell inoculation.

IV. RELATIONSHIP BETWEEN pCu AND CELL COPPER CONTENT AND BETWEEN pCu AND COPPER INDUCED GROWTH INHIBITION

Culture experiments were performed to test the hypothesis that cellular copper content and copper induced growth inhibition are determined by the free cupric ion activity and not by the total concentration of copper present. The experiments were carried out in M-f/2 seawater media (Table 2-1) containing (zero - 10 mM) concentrations of the copper chelator tris. The activity of copper in these media was systematically varied by using different combinations of tris concentration, copper concentration, and pH.

IV - A. Copper Induced Growth Inhibition to T. pseudonana (Clone 3H) in Seawater-tris-$CuSO_4$ Culture Media

Growth rates of copper inhibited cultures are plotted both as a function of the total concentration of copper in solution (Figure 4-1) and as a function of the calculated pCu (Figure 4-2). Growth rates were determined for the period 2 to 4 days after initial cell inoculation. During this period copper growth inhibition was maximal and the growth rates for the individual cultures was relatively constant (Figure 4-3).

Culture experiments were also performed to determine the effect of tris alone on algal growth rate. The cultures contained an f/2 concentration of copper (i.e. 0.04 μM) which was added to prevent the occurrence of copper deficiency. Variations in tris concentration in the range 0-10 mM had no effect on culture growth to within experimental counting error (Figure 4-4). However, at 20 mM tris culture growth rate was inhibited by 20% relative to that at lower tris concentrations. In the present set of experi-

ments tris concentrations were limited to the noninhibitory range of 0-10 mM.

A plot of culture growth rate as a function of the total concentration of copper at tris concentrations of 0, 0.5, 1, 2, 3, 5, and 10 mM at pH 8.1-8.3 (Figure 4-1) shows that the inhibitory effect of copper is decreased as the concentration of tris in the medium is increased, i.e. tris has a protective effect on copper growth inhibition. For example, in the absence of tris, growth rate is inhibited by 70% in the presence of 3 μM $CuSO_4$, whereas in cultures containing 10 mM tris, 300 times this amount of copper (9×10^{-4} M) must be added to obtain a similar growth inhibition.

We also observe that a constant copper and tris concentration (0.1 mM and 10 mM respectively) growth inhibition is markedly reduced by increasing culture medium pH. At pH 7.7 and 8.2 growth rate is inhibited by 25% and 60% respectively, whereas at pH 8.7 no growth inhibition is observed (Figure 4-1). Because of hydrogen ion competition, increasing pH markedly increases the chelation of copper by tris (Figure 3-6, Section III). Thus, an increase in the complexation of copper, whether brought about by an increase in tris concentration at constant pH or by an increase in pH at constant tris concentration correlates with a decrease in copper toxicity.

Culture growth rate plotted as a function of calculated pCu in media containing 1 to 10 mM tris (Figure 4-2), falls on a single curve confirming our main hypothesis that copper growth inhibition is functionally related to pCu irrespective of the total concentration of copper present in the culture media. The data show that the relationship between growth rate inhibition

Figure (4-1)

Growth rate of T. pseudonana (clone 3H) plotted as a function of the negative log of the total copper concentration in M-f/2 seawater media containing 0-10 mM tris at pH 7.7 to 8.7. Results are for five separate experiments. Error bars represent the standard deviation for least squares linear regression of growth curves.

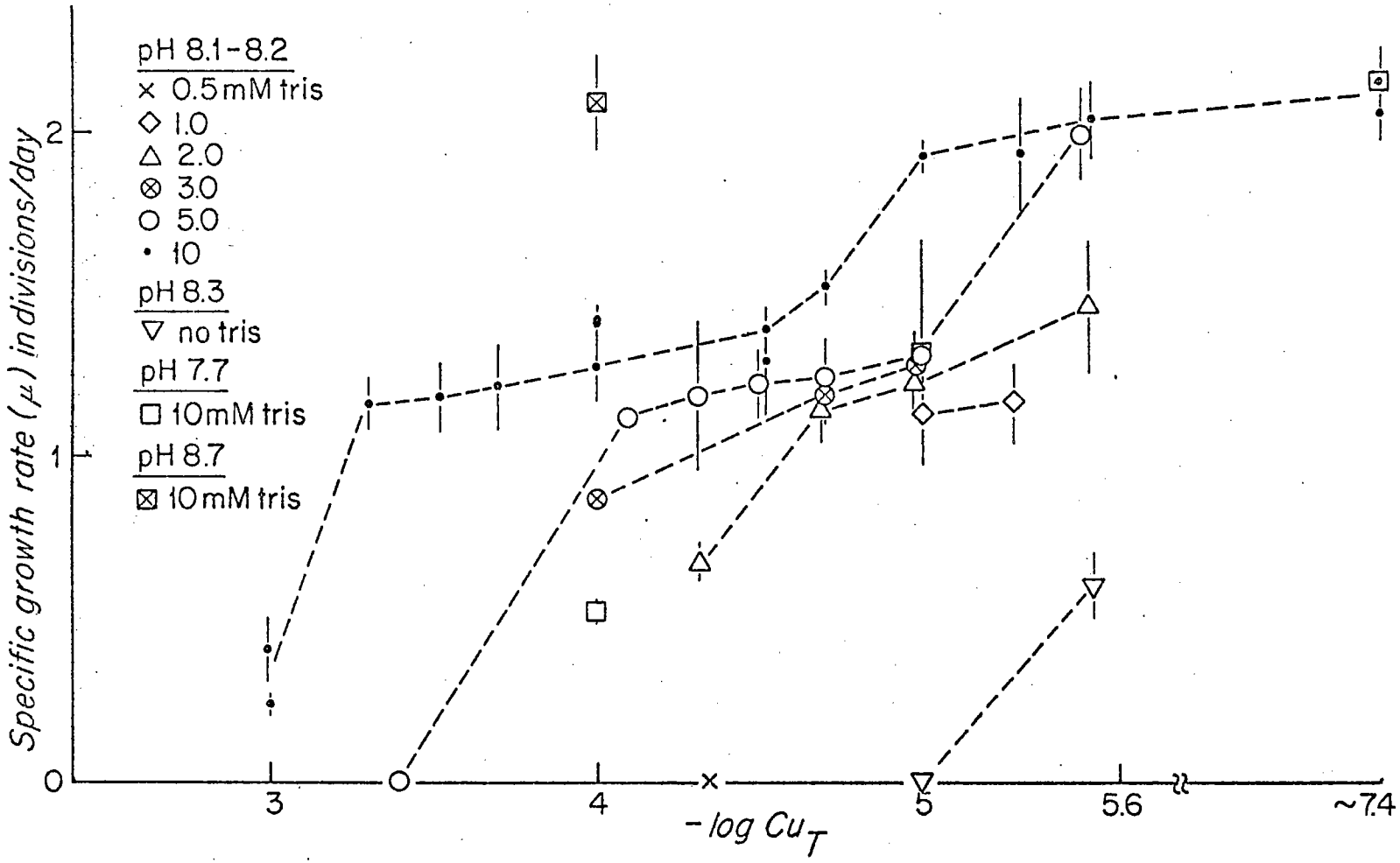


Figure (4-2)

Growth rate of T. pseudonana plotted as a function of calculated pCu in M-f/2 seawater culture media containing 1-10 mM tris for the pH range 7.7 to 8.7. Individual points are from 5 separate experiments. The pCu error bars represent variation in pCu caused by changes in culture pH.

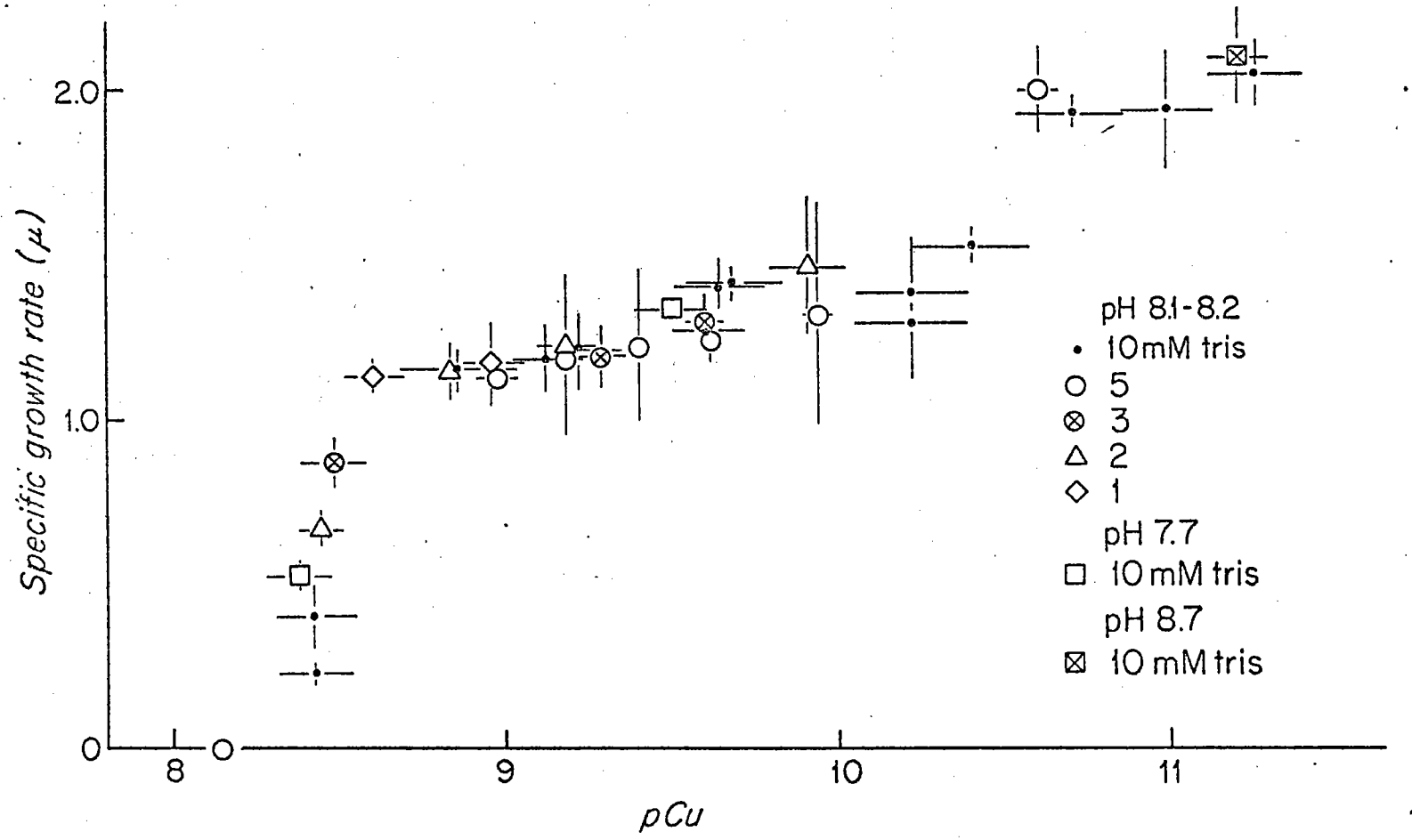


Figure (4-3)

Copper induced growth inhibition of T. pseudonana (clone 3H) in M-f/2 seawater media containing 10 mM tris at pH 8.1-8.2.

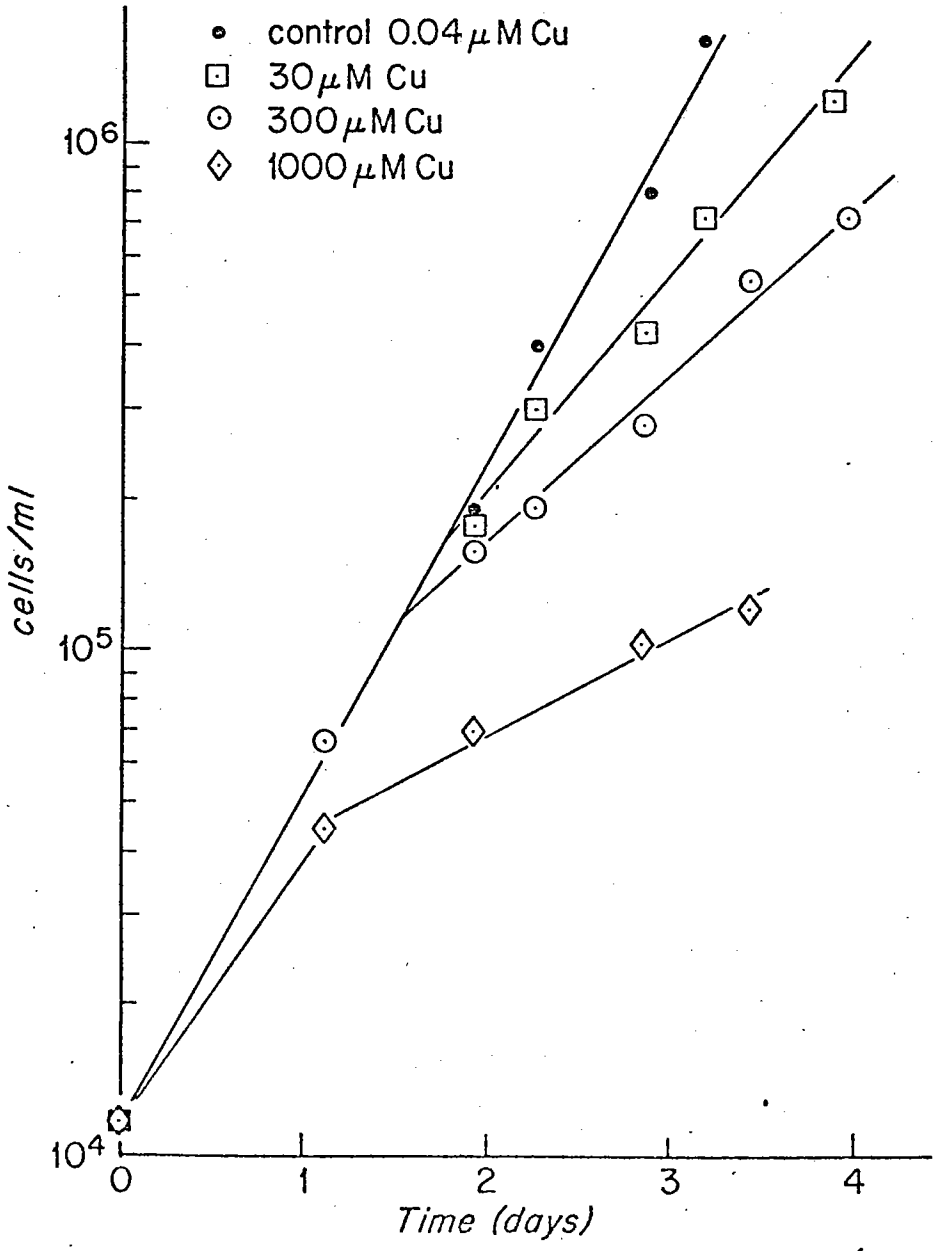
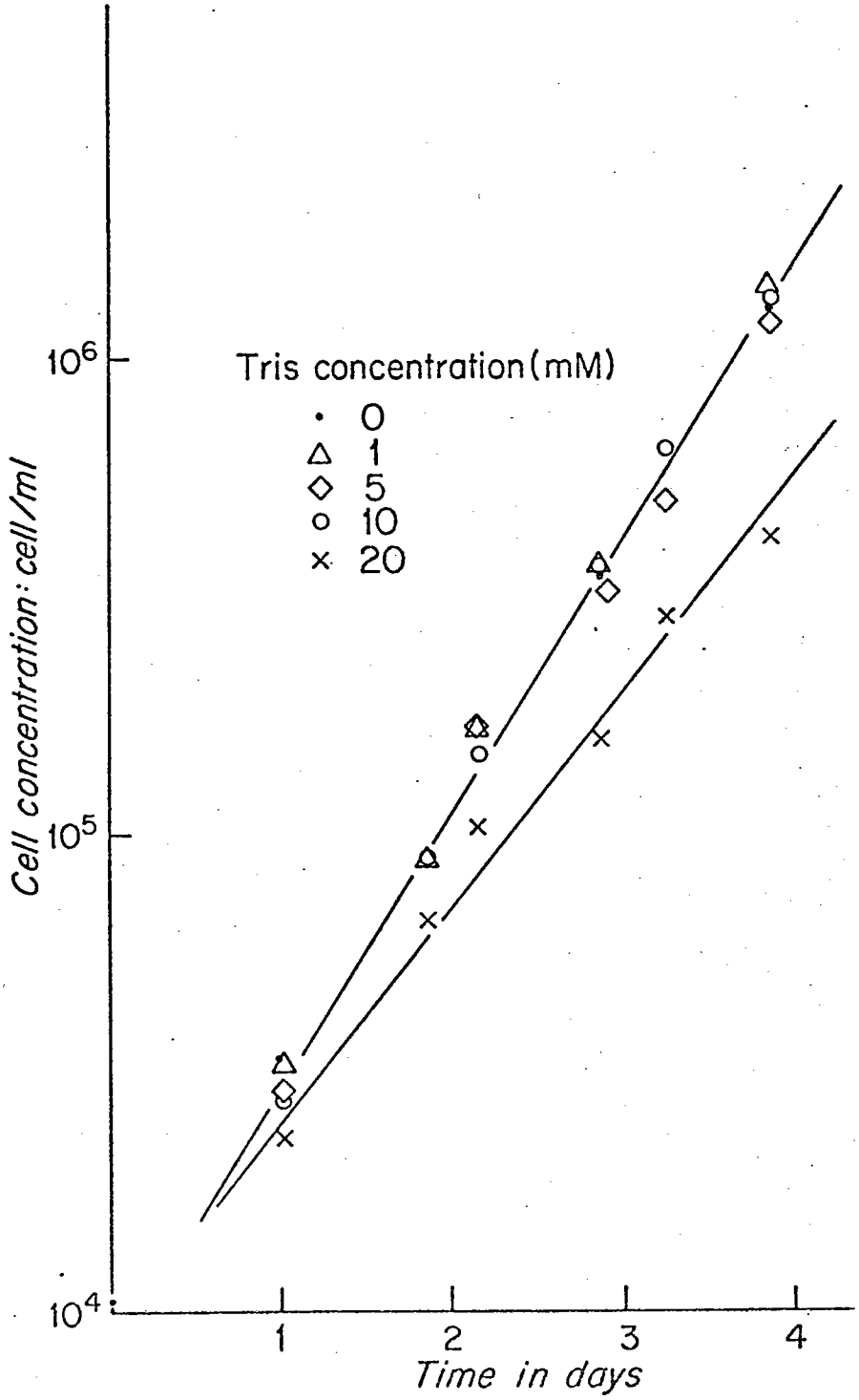


Figure (4-4)

Effect of tris concentration on cell growth for M-f/2 seawater cultures of clone 3H at pH 8.1. Cultures contain 0.04 μM $CuSO_4$.



and pCu is independent of pH in the range 7.7 to 8.7. The decrease in copper toxicity brought about either by an increase in tris concentration or by an increase in pH at the same total copper concentration can thus be accounted for totally by the resulting change in pCu.

Copper growth inhibition is a two-stepped function of pCu possessing three inflections at pCu values of approximately 8.4, 9.4, and 10.5. Copper inhibits the growth of clone 3H at pCu values below approximately 10.7 with total growth inhibition occurring at pCu values below 8.3.

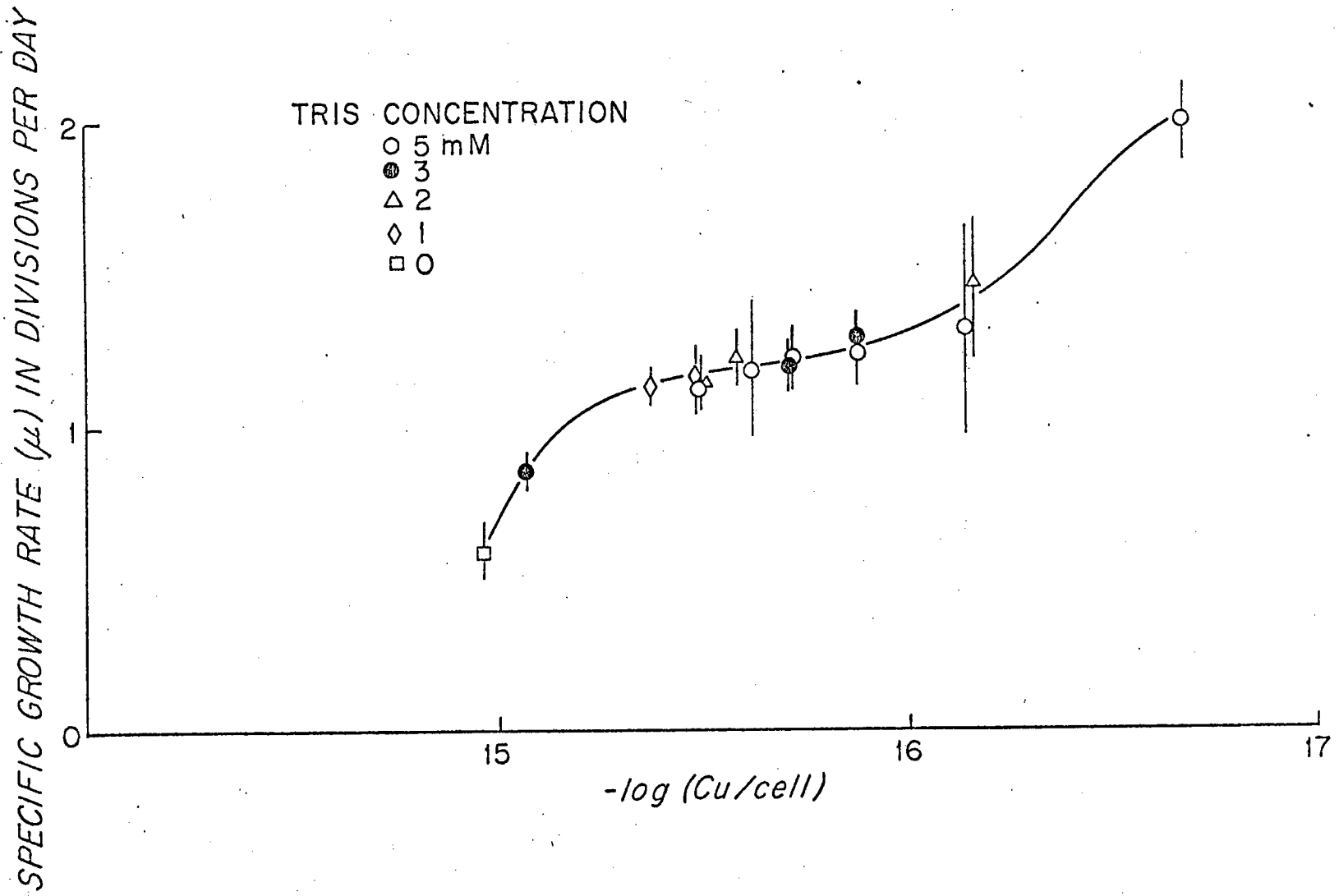
Other characteristics of copper inhibition correlate well with the two-stepped nature of the growth inhibition curve. For the step in the inhibition curve occurring in the narrow pCu range 8.6 to 8.3, copper inhibition is associated with cellular elongation and morphological distortion. For copper growth inhibition above this pCu range (i.e. for pCu 8.6 to 10.7) cell morphology and cell size remain unchanged.

IV-B. Relationship Between Growth Rate Inhibition and Cell Copper Content for Clone 3H

Figure (4-5) shows growth rate of copper inhibited cultures as a function of the negative log of cell copper content for cultures containing 0-5 mM tris at pH 8.1-8.3. Cell copper was determined after either 3 or 4 days exposure of the cells to copper containing media. No apparent change in cell copper occurred between days 3 and 4: in three cultures containing 2 mM tris plus 20 μM $CuSO_4$, 5 mM tris plus 30 μM $CuSO_4$, and 5 mM tris plus 80 μM $CuSO_4$,

Figure (4-5)

The relationship between the growth rate of T. pseudonana (clone 3H) and the negative log of the cell copper content (in moles/cell) in M-f/2 seawater media containing 0-5 mM tris at pH 8.1 to 8.3. Results are taken from three separate experiments.



measurements of cell copper content on days 3 and 4, had respective values of 2.9 and 3.2, 1.8 and 1.9, and 3.8 and 3.2 x 10^{-16} moles/cell showing no significant difference to within the estimated experimental error ($\sim$20%).

Copper induced growth inhibition correlates well with cell copper content. Like the curve for growth rate as a function of pCu, that for growth rate as a function of -log (Cu/cell) also exhibits two individual steps.

IV-C. Relationship Between Cell Copper Content and pCu for Clone 3H

Plots of 3 or 4 day cell copper content as functions of total copper concentration and calculated pCu are given in Figures (4-6 and 4-7) for cultures containing 1-5 mM tris at pH 8.1-8.2. In the pCu range of these experiments (8.6-10.6) cell morphology and cell size are unaffected by copper. Figure (4-6) shows that increasing the concentration of tris in the culture medium from 1 to 5 mM markedly decreases cell copper content at equivalent total concentrations of copper.

Cell copper content plotted as a function of pCu shows good correlation with a single hyperbolic function

$$\text{Cu/cell} = \frac{4.8 \times 10^{-16}\, a_{Cu}}{a_{Cu} + 10^{-9.2}} \quad \text{(moles/cell)} \qquad (4\text{-}1)$$

The constants 4.8 x 10^{-16} and $10^{-9.2}$ were obtained from a standard Lineweaver-Burke plot (i.e. a linear fit to a plot of 1/Cu(per cell) vs. $1/a_{Cu}$). The

Figure (4-6)

Cell copper content of clone 3H as a function of the negative log of the total copper concentration for cultures containing 1, 2, 3 and 5 mM tris at pH 8.1-8.2. Measurements of cell copper made after 3 or 4 days exposure of the cells to copper.

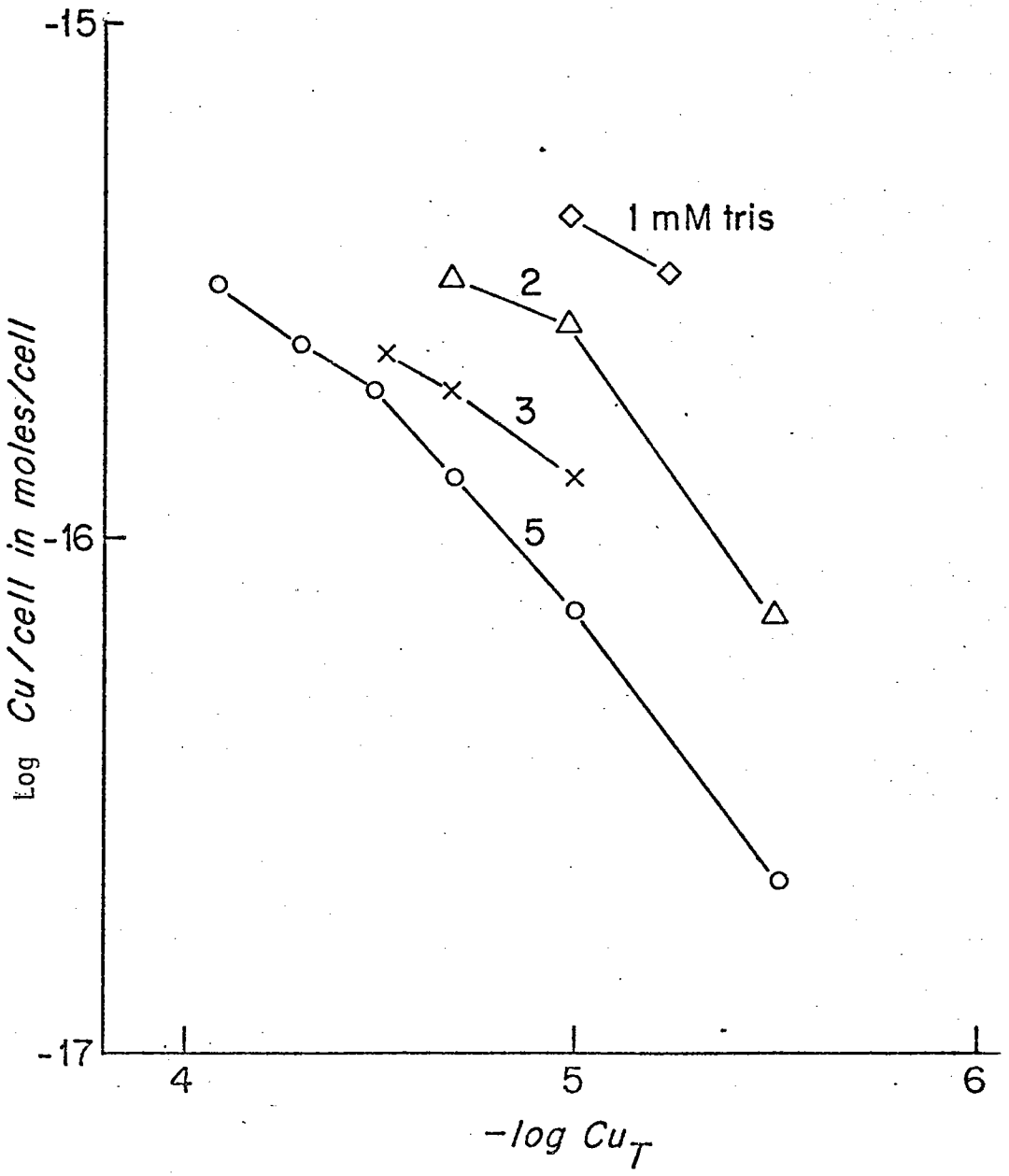
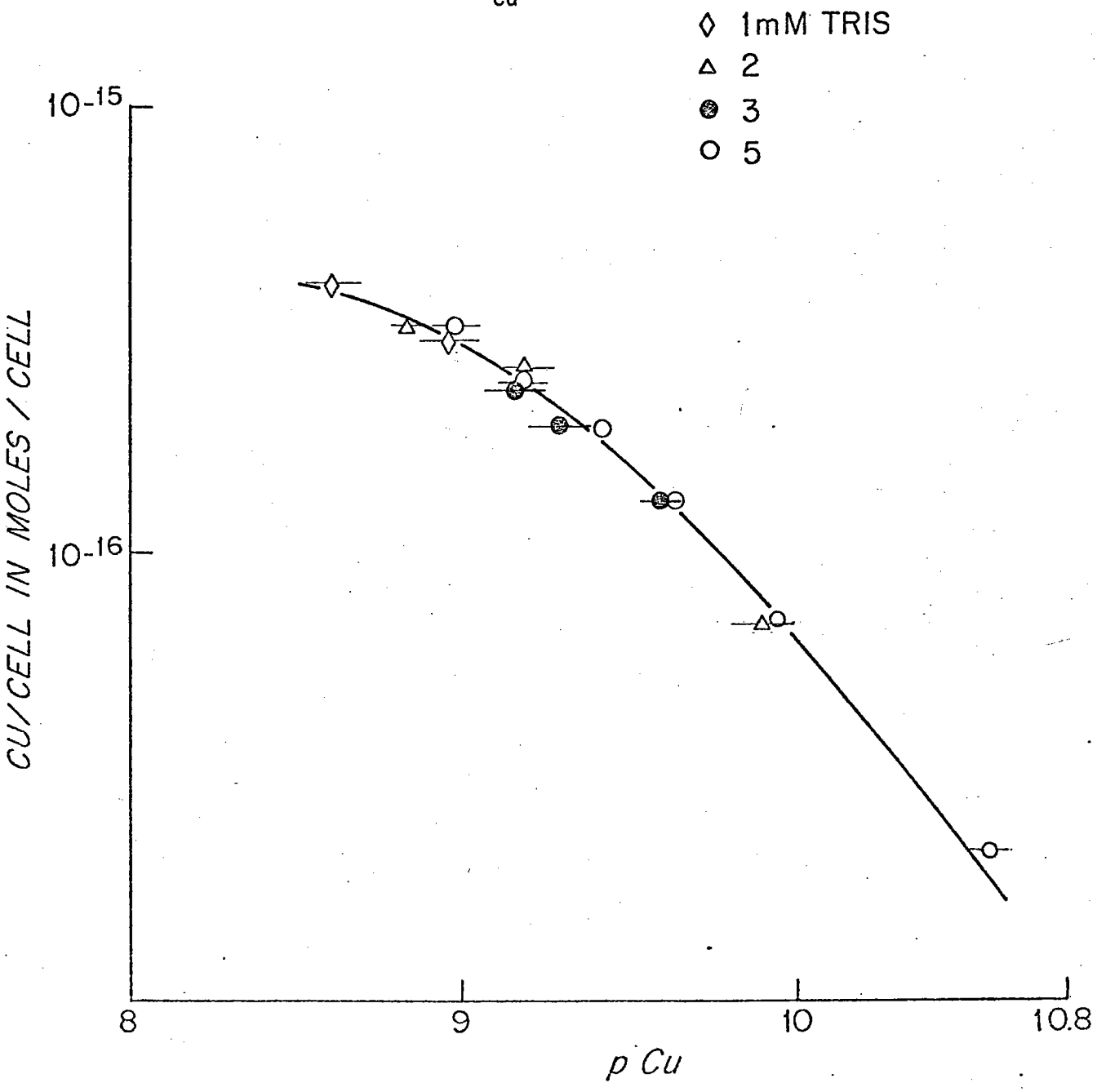


Figure (4-7)

Cell copper content of T. pseudonana (clone 3H) plotted as a function of calculated pCu in M-f/2 seawater culture media containing 1-5 mM tris and 3-80 μM $CuSO_4$ at pH 8.1-8.2. Data are for 3 separate experiments. Error bars represent variation in pCu due to changes in culture pH. The solid curve is a log-log plot of the hyperbolic function:

$$\text{Cu/cell} = \frac{4.8 \times 10^{-16}\, a_{Cu}}{a_{Cu} + 10^{-9.2}}$$



results of the copper uptake study confirm the hypothesis that copper uptake by algae is functionally related to the pCu of the culture medium and is independent of the total concentration of copper present.

IV-D. Copper Growth Inhibition of Nannochloris atomus in 10 mM tris-Seawater Media at pH 8.1

As was the case with T. pseudonana, copper had very little inhibitory influence on cell growth rate of N. atomus until approximately 1.5 to 2 days after exposure to copper (Figure 4-8). Because of this, culture growth rates were determined for the time period 2-5 days after culture inoculation. One exception was the culture containing 30 μM Cu where no cell density determinations were made for days 2 and 3. To obtain enough data points for a growth rate calculation the entire five day growth curve was used.

A control culture containing 0.04 μM added copper and 10 mM tris exhibited a growth rate of 0.78 cell divisions per day. Three cultures in identical media with tris deleted had growth rates of 0.78, 0.76, and 0.81 divisions per day indicating that the presence of tris was not toxic to the algae.

The growth of N. atomus in 10 mM tris-seawater media was inhibited at pCu values below approximately 10.4 (Figure 4-9); total growth rate inhibition occurred at a pCu value of 8.4. The pCu range for partial growth rate inhibition of N. atomus ($\sim$10.4 to 8.4) is thus similar to that for T. pseudonana (clone 3H) (10.7 to 8.3). Unlike the growth inhibition curve for

Figure (4-8)

Effect of copper on the growth of N. atomus in M-f/2 seawater-tris culture media at pH 8.1.

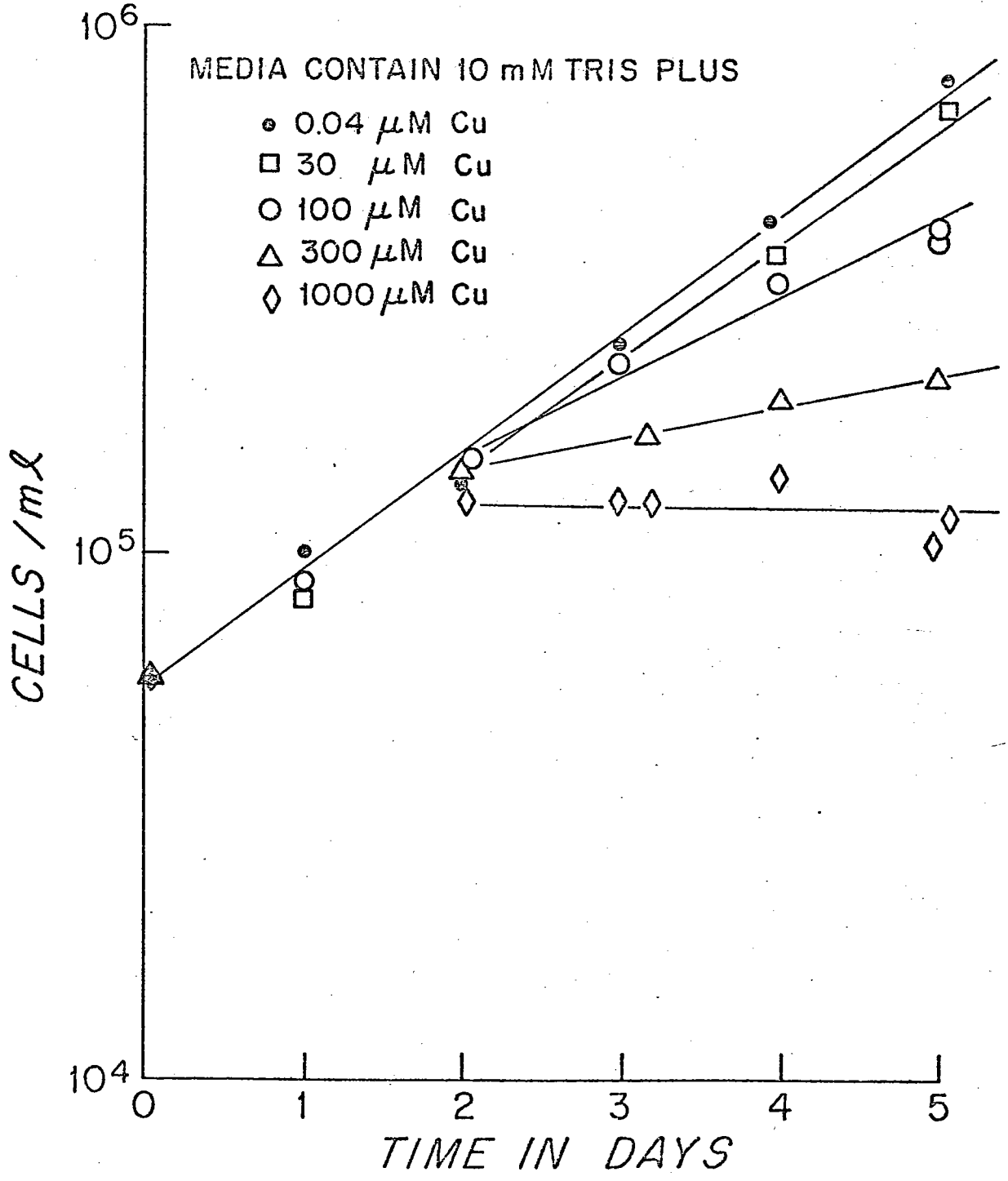
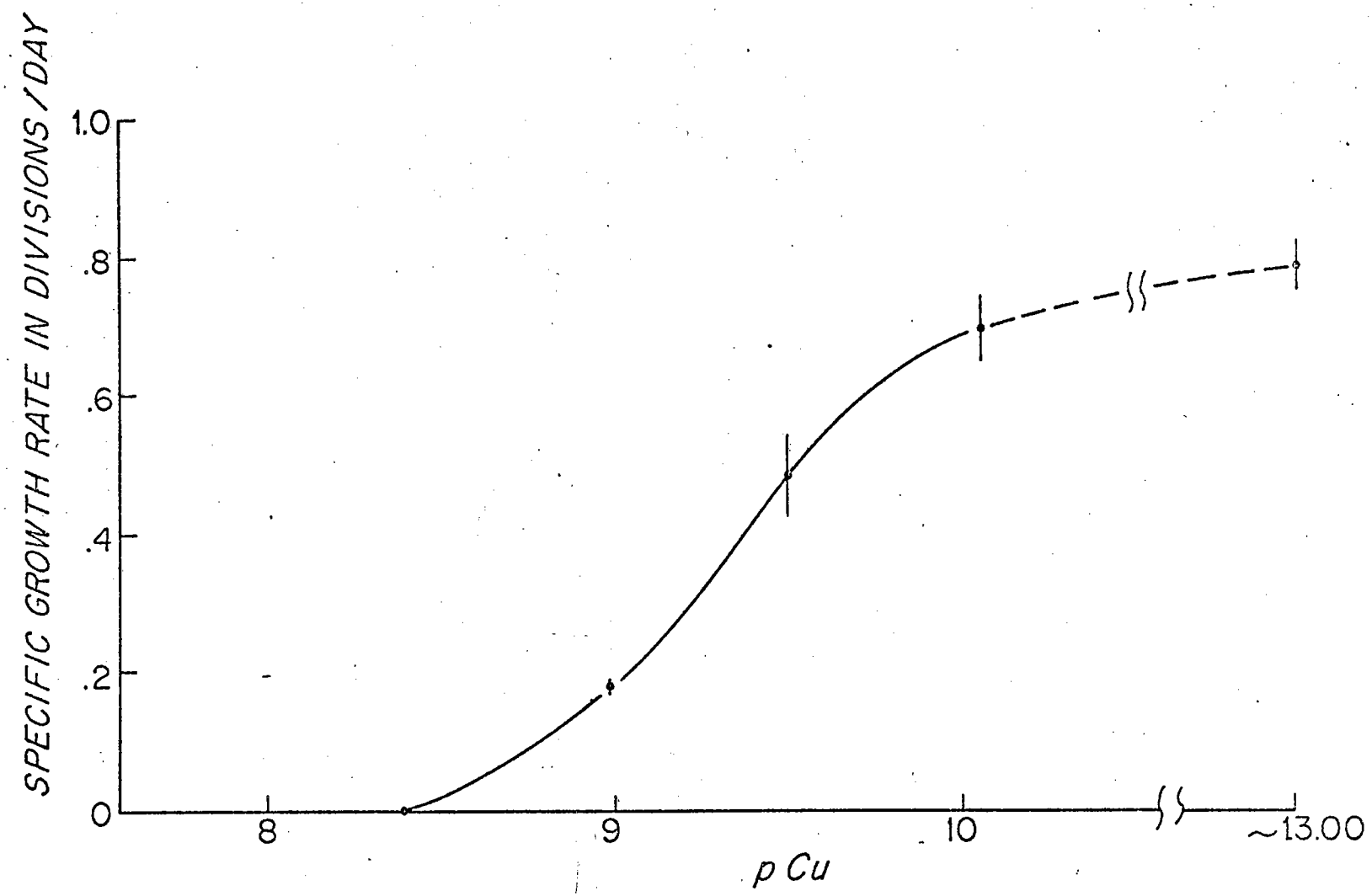


Figure (4-9)

Growth rate of N. atomus as a function of calculated pCu in M-f/2 seawater media containing 10 mM tris at pH 8.1.



T. pseudonana, the inhibition curve for N. atomus has a smooth sigmoidal shape with a single inflection at the 50% growth rate inhibition pCu value of 9.3.

IV-E. Copper Growth Inhibition of T. pseudonana (Clone 13-1) in Artificial Seawater

Davey et al. (1973) investigated copper growth inhibition of T. pseudonana (clone 13-1) in chemically well defined 30 °/oo artificial seawater at 20°C and pH 8.2. This study reported time dependent growth curves as a function of added copper concentration (these curves are shown in Figure 4-10). Clone 13-1 is an open ocean strain of T. pseudonana isolated from the Sargasso Sea.

We wish to compare the results of Davey et al. (1973) with our own copper growth inhibition experiments with T. pseudonana (clone 3H) and N. atomus in highly chelated seawater-tris-$CuSO_4$ culture media. To do this, growth rates for the copper treated cultures of clone 13-1 were calculated from the growth curves in Figure (4-10) for the time period 18-44 hours after the start of the cultures. Artificial seawater culture media pCu values were determined from calculations and indirect measurements of the inorganic complexation of copper in seawater. Details for this are given in Section (III - C-4). The exact composition of the artificial seawater culture medium and method of preparation are given in Section (III - C-1).

The growth rate of copper treated cultures of 13-1 relative to the control culture containing no added copper is plotted as a function of pCu in Figure (4-11). The curve shows a half inhibition pCu value of about 9.3 with a pCu range for partial growth rate inhibition of approximately 10-8.

Figure (4-10)

Effect of copper on the growth of T. pseudonana (clone 13-1) in 30 °/oo salinity artificial seawater culture media at pH 8.2. Media contain no added chelators. Data taken from Davey et al. (1973).

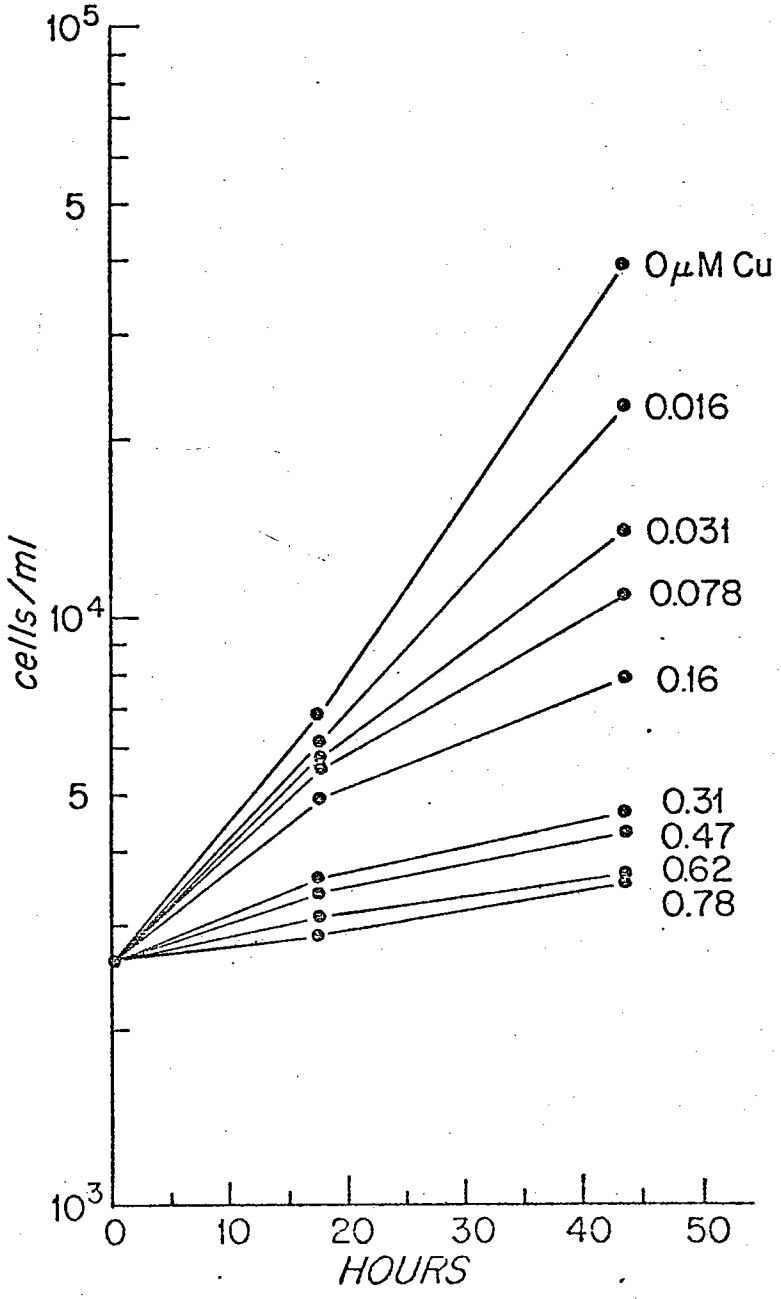
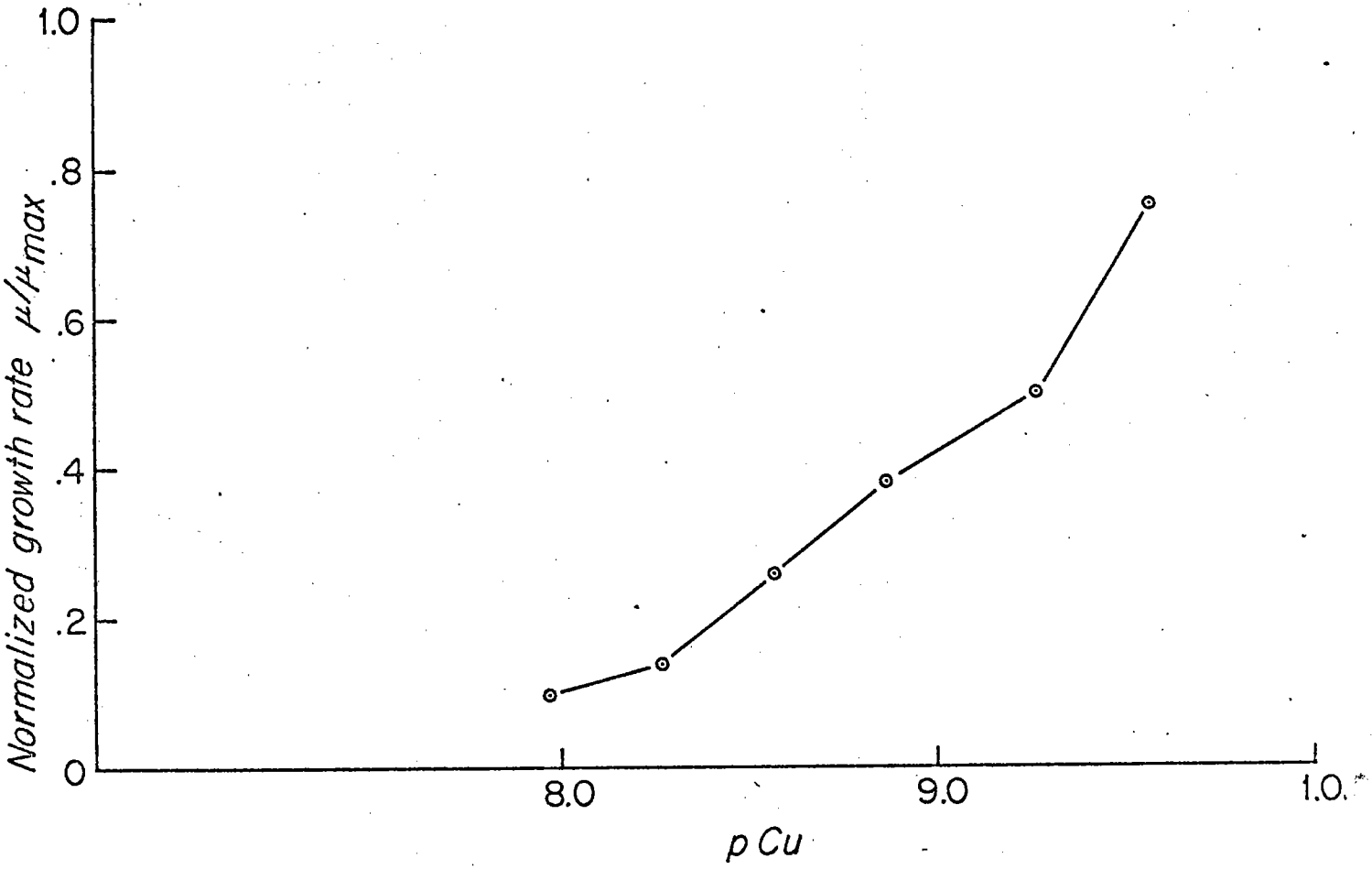


Figure (4-11)

Normalized growth rate of T. pseudonana (clone 13-1) as a function of calculated pCu in 30 o/oo salinity artificial seawater at pH 8.2.



Unlike copper growth inhibition to the estuarine clone of the same species (clone 3H) the copper growth inhibition curve for clone 13-1 does not exhibit two distinct individual steps, however, the inhibitory pCu range for the two clones is similar. That for clone 3H is roughly pCu 10.7 to 8.3 (Figure 4-4).

Results in Figure (4-11) also agree fairly closely with copper growth inhibition to N. atomus in 10 mM tris-$CuSO_4$-seawater media (Figure 4-9). For N. atomus partial growth inhibition occurs over the pCu range 10.4 to 8.4 with a half inhibition value of 9.3 identical to that for clone 13-1 in artificial seawater.

IV-F. Discussion

IV-F-1. Cell Copper Content

Cellular copper content for T. pseudonana (clone 3H) plotted as a function of pCu was consistent with a hyperbolic relationship

$$\text{Cu/cell} = \frac{4.8 \times 10^{-16}\, a_{Cu}}{a_{Cu} + 10^{-9.2}} \quad \text{(moles/cell)} \qquad (4\text{-}2)$$

where $a_{Cu} = 10^{-pCu}$ (see Figure 4-7). This relationship suggests an equilibrium binding of copper to a single ligand site or to several sites having similar binding constants for copper. This can be seen from the following.

The reaction of copper with a cellular ligand site can be written as

$$Cu + X = CuX \qquad (4\text{-}3)$$

At equilibrium the following mixed dissociation expression would apply

$$\frac{a_{Cu}[X]}{[CuX]} = K_d \qquad (4\text{-}4)$$

where the square brackets denote molar concentrations of the enclosed species and K_d is an apparent dissociation constant for the reaction. Rearranging equation (4-4)

$$[CuX] = \frac{a_{Cu}\,[X]}{K_d} \qquad (4\text{-}5)$$

The mass balance relationship for the total concentration of X is

$$X_T = [CuX] + [X] \qquad (4\text{-}6)$$

Using this relationship

$$\frac{[CuX]}{X_T} = \frac{[CuX]}{[CuX] + [X]} \qquad (4\text{-}7)$$

and substituting equation (4-5) into equation (4-7)

$$\frac{[CuX]}{X_T} = \frac{a_{Cu}\,[X]/K_d}{a_{Cu}\,[X]/K_d + [X]} = \frac{a_{Cu}}{a_{Cu} + K_d} \qquad (4\text{-}8)$$

If we assume that the total concentration of copper in the cells (in moles per liter) is equal to [CuX] and divide the numerator and denomenator of the left hand side of equation (4-8) by the number of cells per liter then

$$Cu(\text{per cell}) = \frac{X_T(\text{per cell})\, a_{Cu}}{a_{Cu} + K_d} \qquad (4\text{-}9)$$

Assuming this simple equilibrium binding model for the cellular copper content of T. pseudonana (clone 3H), the total amount of binding site X per cell would be about 5×10^{-16} moles/cell and the apparent association constant for the reaction of copper with cellular ligand would be $10^{9.2}$ ($K_{association} = 1/K_d$).

IV-F-2. Comparison of Copper Uptake and Copper Growth Rate Inhibition

Copper growth rate inhibition for T. pseudonana (clones 3H and 13-1) and for N. atomus (clone GSB Nanno) were all found to occur in a similar pCu range. The three clones had respective pCu ranges for partial growth rate inhibition to total inhibition of 10.7-8.3, approximately 10-8, and approximately 10.4-8.4.

Clones (3H) and (13-1) are respectively estuarine (Great South Bay, Long Island) and oceanic (Sargasso Sea) isolates of the same diatom species T. pseudonana. Nannochloris atomus (clone GSB nanno) was also isolated from Great South Bay. Our results show no major difference in copper sensitivity between oceanic and estuarine isolates.

Half saturation for cellular copper content of (3H) occurred at a pCu of 9.2, which falls in the middle of the pCu range for partial growth rate inhibition. One possible explanation for this is that the same cellular copper binding site is responsible for both cellular copper uptake and growth inhibition. In such a case copper inhibition would be functionally related to the fraction of major cellular binding site complexed by copper

$$\text{Inhibition} = f\left(\frac{[CuX]}{X_T}\right) \qquad (4\text{-}10)$$

From our hypothesized reversible binding site model for copper uptake by 3H

$$\frac{[CuX]}{X_T} = \frac{Cu/cell}{4.8 \times 10^{-16}} = \frac{a_{Cu}}{a_{Cu} + 10^{-9.2}} \qquad (4\text{-}11)$$

Combining equations (4-10) and (4-11)

$$\text{Inhibition} = f\left(\frac{a_{Cu}}{a_{Cu} + 10^{-9.2}}\right) \qquad (4\text{-}12)$$

The term in the brackets takes on values from 0 to 1. At pCu values much less than 9.2 (i.e. at sufficiently high copper activities) this term is equal to one, meaning that essentially all of the cellular site is complexed by copper, and thus cellular inhibition is maximal. For pCu values much greater than 9.2 the term would be equal to zero and no cellular inhibition would occur.

Figure (4-12) presents a plot of $\frac{a_{Cu}}{a_{Cu} + 10^{-9.2}}$ as a function of pCu together with normalized growth rate inhibition curves for clones 3H, 13-1, and GSB Nanno. Normalized growth rate inhibition is computed from the equation

$$\text{Normalized inhibition} = 1 - \mu/\mu_{max} \qquad (4\text{-}13)$$

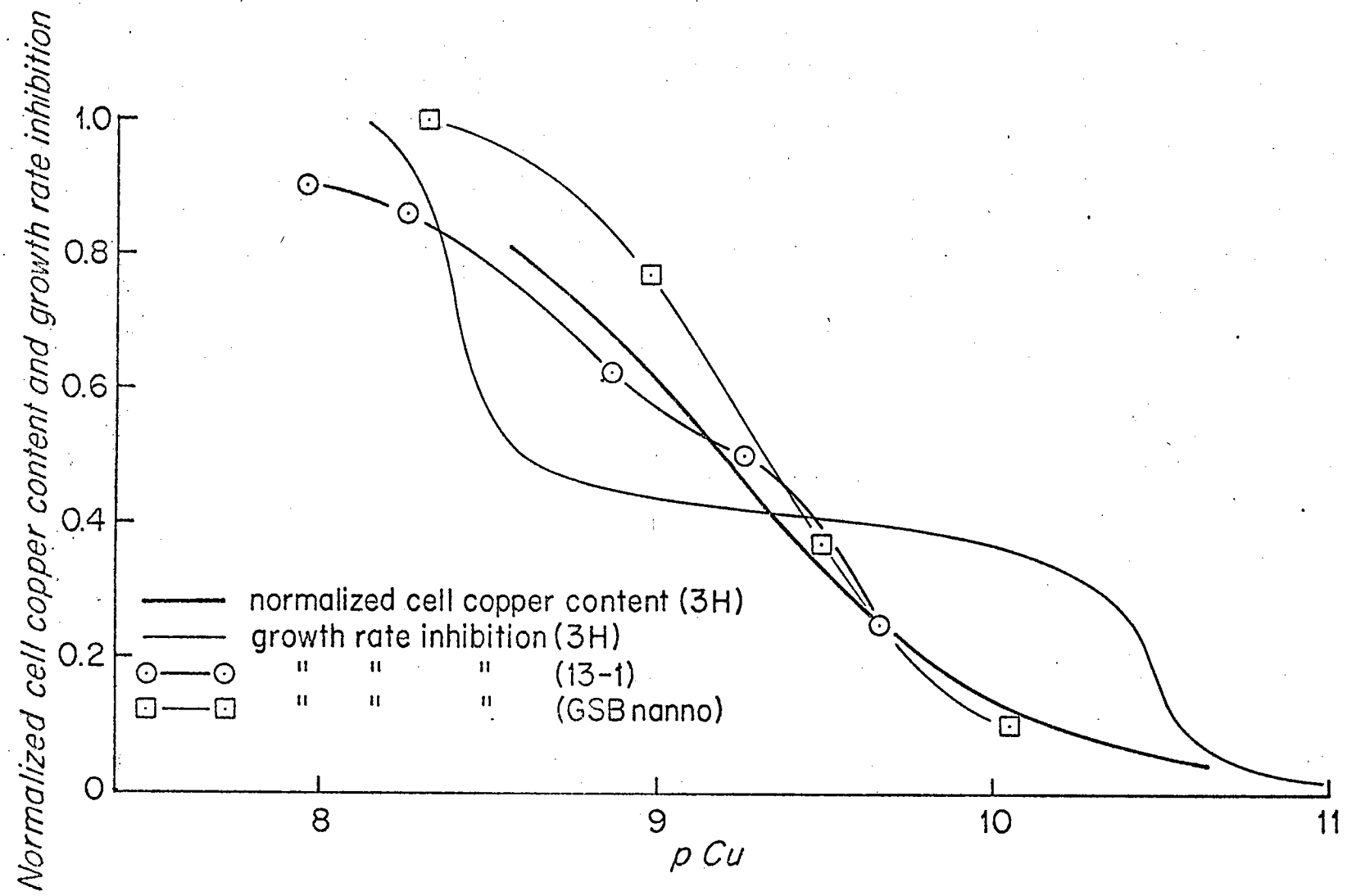
where μ is the specific growth rate and μ_{max} is the maximum specific growth rate obtained at low copper activities (i.e. in the absence of copper inhi-

bition). Normalized growth rate inhibition takes on values from 0 to 1 corresponding respectively to no inhibition to total inhibition. For clones 3H and GSB Nanno, μ_{max} is the specific growth rate for pCu approximately 12-14 and for 13-1 μ_{max} is the specific growth rate in an artificial seawater medium containing no added copper. The artificial seawater medium was treated with chelex resin and contained less than 0.015 μM copper (Davey et al., 1973). This means that the pCu of the culture containing no added copper had an unknown value greater than 9.7. Inasmuch as the pCu of this zero added Cu culture was unknown we cannot be sure that the culture was indeed free from copper inhibition.

The curves in Figure (4-12) are all comparable with respect to their positions on the pCu axis, however, they all have somewhat different shapes. The normalized cellular copper content curve for 3H is a smooth sigmoidal function of pCu with a single inflection (point of maximum negative slope) at pCu 9.2. On the other hand, the normalized growth rate inhibition curve for this clone has two rather steep sigmoidal sections with inflections at pCu 8.4 and 10.5. Although we cannot rule out the possibility that copper inhibition and cellular copper uptake for 3H involved the same cellular site(s) the marked difference in the shapes of the cell copper content and growth rate inhibition curves suggests that this is not the case. The two-stepped nature of the growth inhibition curve for 3H instead suggests the presence of at least two individual copper inhibition sites that bind successively with copper.

Figure (4-12)

Normalized cell copper content $\frac{a_{Cu}}{a_{Cu} + 10^{-9,2}}$ for T. pseudonana (clone 3H) and normalized growth rate inhibition ($1 - \mu/\mu_{max}$) for T. pseudonana (clones 3H and 13-1) and for N. atomus as functions of pCu.



IV-F-3. Copper Inhibition of Algae in Natural Seawater

Copper induced growth inhibition has been defined in terms of pCu for both an estuarine clone (3H) and open ocean clone (13-1) of the diatom Thalassiora pseudonana and for the green alga Nannochloris atomus (Figure 4-12). We now compare pCu levels that are inhibitory to these three clones with estimated pCu values for seawater.

As a first approximation we shall assume that copper is not significantly complexed by organic ligands in seawater and thus that copper speciation is determined by complexation to inorganic ligands. (This gives a minimum estimate of copper complexation and thus a maximum estimate for toxicity.) According to calculations of inorganic copper complexation for surface seawater of chlorinity 19 $^{o}/_{oo}$, temperature 25^{o}C, and pH 8.2 (Park, 1966), the ratio a_{Cu}/Cu_T has a value of about $10^{-1.8}$ (Section III. C-3). To estimate an activity of copper in seawater we need an estimate of the total copper concentration.

Chester and Stoner (1974) reported copper concentrations for 38 open ocean seawater samples falling in the range 0.002 to 0.06 μM with a mean concentration of 0.012 μM. Form the same study the mean copper concentration for 51 coastal seawater samples (Northeastern Atlantic, South African Coast, Inland Sea (Japan), South Japan Coast, Java Sea, Malacca Straits, Sea of Japan, and China Sea) was 0.014 μM, close to that for open ocean samples. Thus at pH 8.2, with $a_{Cu}/Cu_T = 10^{-1.8}$ and Cu_T = 0.012 μM, the pCu of the

seawater would be 9.7. At this pCu, copper inhibited the growth rates of T. pseudonana (clone 3H), T. pseudonana (clone 13-1) and N. atomus by 40%, 20%, and 20% respectively (Figure 4-12). These results indicate that in the absence of organic complexation, natural levels of copper present in the ocean should be inhibitory to at least some species of marine phytoplankton.

Our calculations and indirect measurements of the inorganic complexation of copper in seawater indicate that pCu is dependent on pH. Experiments with 3H showed that at constant total copper and chelator concentrations, pH induced changes in pCu significantly altered copper toxicity (Figure 4-1). The pH of surface seawater may vary, especially in regions where deep seawater having relatively low pH values is brought to the surface by upwelling. Changes in surface seawater pH associated with upwelling off the Oregon Coast during the summer of 1964 have been described by Park (1968). During the fall and winter when upwelling did not occur, seawater pH was vertically stratified with a pH maximum of 8.1 to 8.3 at the surface, decreasing to a pH minimum of 7.7 at 200-300 meters depth. During the summer, upwelling along the coast advected pH 7.7 seawater to the surface and created a sharp surface pH gradient (7.7 to 8.2) perpendicular to the shore. The salinity of the upwelled surface water was 32-34 $^{o}/_{oo}$. If we ignore possible organic complexation, assume a temperature of 25^{o}C (the temperature of our seawater calculations for copper complexation), a salinity of 33 $^{o}/_{oo}$, and a copper concentration of 0.014 μM (the mean

coastal seawater value of Chester and Stoner, 1974) then from calculations of the inorganic complexation of copper in seawater (Table 3-6), the pCu of the seawater would be 9.2 at pH 7.7 and 9.6 at pH 8.2.

What effect might such a pH induced pCu change have on copper toxicity? For clone 3H the functional relationship between growth rate inhibition and pCu was independent of pH for the pH range 7.7 to 8.2 (Figure 4-2). For this clone a pH induced pCu change from 9.6 to 9.2 would cause only a small increase in growth inhibition (approximately 3%, see Figure 4-12). Assuming that the relationship between growth inhibition and pCu is also pH independent for N. atomus (clone GSB Nanno) as for clone 3H, the above pH induced pCu change would cause growth rate inhibition for this clone to increase from 20% at pH 8.2 to 60% at pH 7.7 (see Figure 4-12).

Changes in surface water pH such as those that occur in regions of active upwelling therefore may be a significant factor affecting seawater pCu, and thus the toxicity of copper to phytoplankton.

V. EFFECT OF EDTA AND OF EXTRACELLULAR LIGANDS IN SHORT TERM CULTURE EXPERIMENTS

V - A. Effect of EDTA on Cell Copper Uptake and Toxicity for T. pseudonana

In tris-$CuSO_4$-seawater culture experiments, EDTA was present as a minor copper complexing ligand. The present experiment investigates whether the CuEDTA complex is toxic to T. pseudonana or is taken up by the cells to any appreciable extent.

Thalassiosira pseudonana cells were grown in an artificial 1/10th strength seawater meduim (DSW medium, Table 2-3) to a cell concentration of 9.1×10^5 cells per ml, filtered, washed with fresh medium, and then transferred into experimental media having the following compositions:

medium 1 - DSW, no addition

medium 2 - DSW, plus 1 μM $CuSO_4$

medium 3 - DSW plus 1 μM $CuSO_4$ and 2 μM EDTA

The experimental cell concentration was 4.1×10^5 cells per ml.

In medium 2, the initial pCu at pH 8.1 was 7.7 as measured with the cupric ion-selective electrode. The pCu value calculated from equation (A-2-8) in Appendix 2, which takes into account copper-hydroxide and copper-carbonate complex formation, is 7.6 in good agreement with the measured value.

The pCu in medium 3, containing copper plus EDTA, is 10.5 as calculated from known stability constants for $CuEDTA^{2-}$, $CaEDTA^{2-}$, and $MnEDTA^{2-}$ reported in Sillen and Martel (1964). The details for the calculation of pCu in this medium are given in Appendix (4-B). The pCu in this medium is independent of

pH in the experimental pH range 8.1 to 9.1 (Figure 5-3).

Figure (5-1) shows that the presence of EDTA almost completely prevents cell copper uptake. In the presence of 1 μM copper and no EDTA, the cells absorbed 8.2×10^{-16} moles Cu/cell after four hours. The addition of 2 μM EDTA at the same total copper concentration reduced cell copper uptake to 3.4×10^{-17} moles/cell, a drop of over an order of magnitude.

At a pCu of 10.6 in a tris-$CuSO_4$-seawater medium (experiments in Section III) copper cell content after 3 days exposure was 2.2×10^{-17} moles/cell. By comparison at a slightly lower calculated pCu of 10.5 (i.e. higher activity) in the copper plus EDTA medium, 3.4×10^{-17} moles/copper/cell was taken up by the algae, consistent with the hypothesis of control by pCu.

One toxic effect of copper to algae is the loss of normal cell potassium content (McBrien and Hassall, 1965; Kamp-Nielsen, 1971). The presence of EDTA prevents copper induced cell potassium loss (Figure 5-2). In the medium containing no copper or EDTA and in the medium with copper plus EDTA, cell potassium content remains essentially constant at $9.5 \pm 0.4 \times 10^{-15}$ moles/cell during the course of the experiment. Only in the culture containing copper alone was potassium lost from the cells.

The results of this experiment indicate that the copper-EDTA complex is not appreciably taken up by the cells and that it is not toxic to the algae.

V - B. Algal Induced Changes in Culture Medium pCu

In the culture containing 1 μM $CuSO_4$ and no EDTA, the pCu measured in culture filtrates increased markedly during the 4-hour course of the experiment, from an initial value of 7.7 to a final one of 9.1 (Figure 5-4). Possible

causes for this observed increase in pCu are: (1) the adsorption of copper onto the walls of the culture vessel, (2) uptake of copper by the cells, (3) an increase in the inorganic complexation of copper due to a photosynthetically induced increased in culture pH (Figure 5-3), and (4) the production of extracellular complexing ligands by the algae.

In a control medium, to which algae were not added, no change in pCu was measured after either 4 or 6 hours indicating no adsorption of copper onto the walls of the culture vessel, and implying that similar adsorption did not occur in the experimental culture.

The total content of copper in the cells after four hours was 3.4×10^{-7} moles per liter of culture medium. Uptake by the cells thus accounts for a decrease in the log of the culture medium total copper concentration of 0.2, far too little to account for the observed 1.4 unit increase in pCu.

The pH of the culture filtrates increased from an initial value of 8.1 to a final value of 8.8 (Figure 5-4) due to the photosynthetic removal of CO_2 by the cells. To determine the effect of this pH increase on pCu values, filtered portions of the culture medium, sampled at 40, 125, and 243 minutes after culture initiation, were briefly bubbled with 5% CO_2 to adjust both P_{CO_2} and pH to the initial levels (i.e. that before the start of the experiment, pH 8.1). The pCu was then measured (Figure 5-4). The pCu values for pH adjusted culture filtrates taken at times 0, 40, 125, and 243 minutes after culture initiation were respectively 7.7, 8.0, 8.1, and 8.3. Thus a large portion (but not all) of the increase in culture medium pCu during the experiment can be accounted for by the photosynthetically induced increase in pH.

Figure (5-1)

Effect of EDTA on the uptake of copper by T. pseudonana (clone 3H) in DSW media

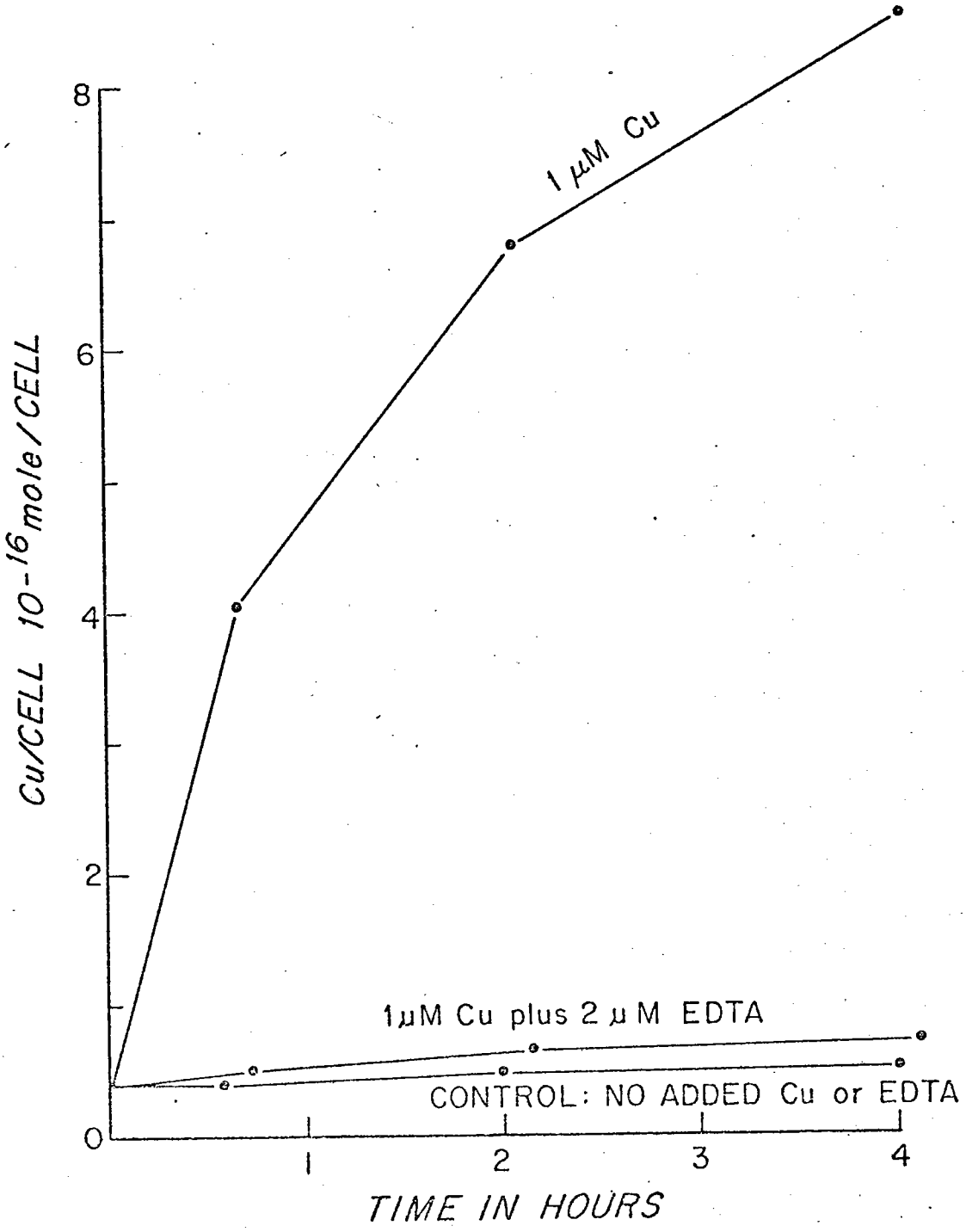


Figure (5-2)

Effect of EDTA on copper induced loss of cell potassium for T. pseudonana (clone 3H) in DSW media

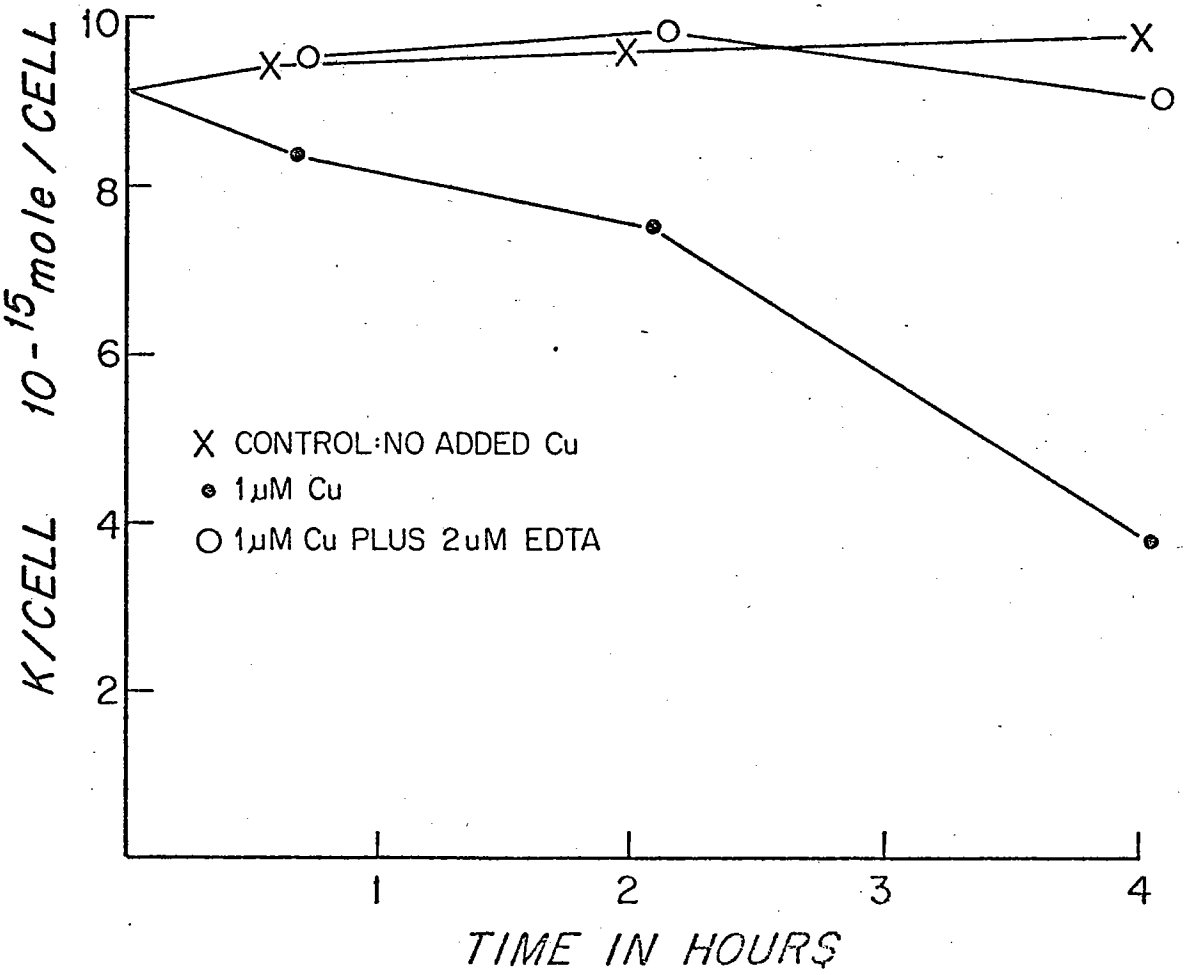


Figure (5-3)

Photosynthetically induced changes in culture pH. Measurements made in filtered culture media.

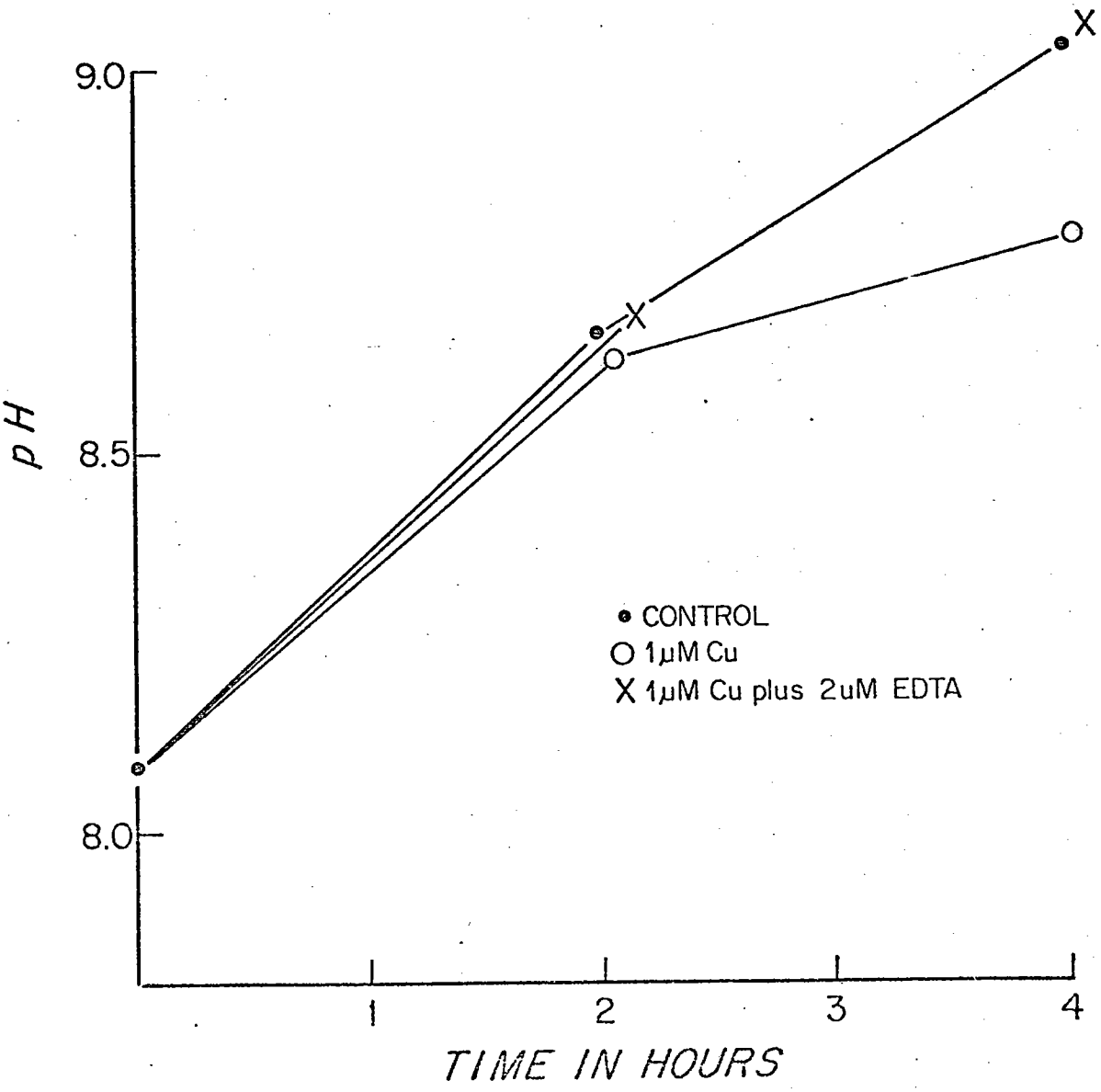
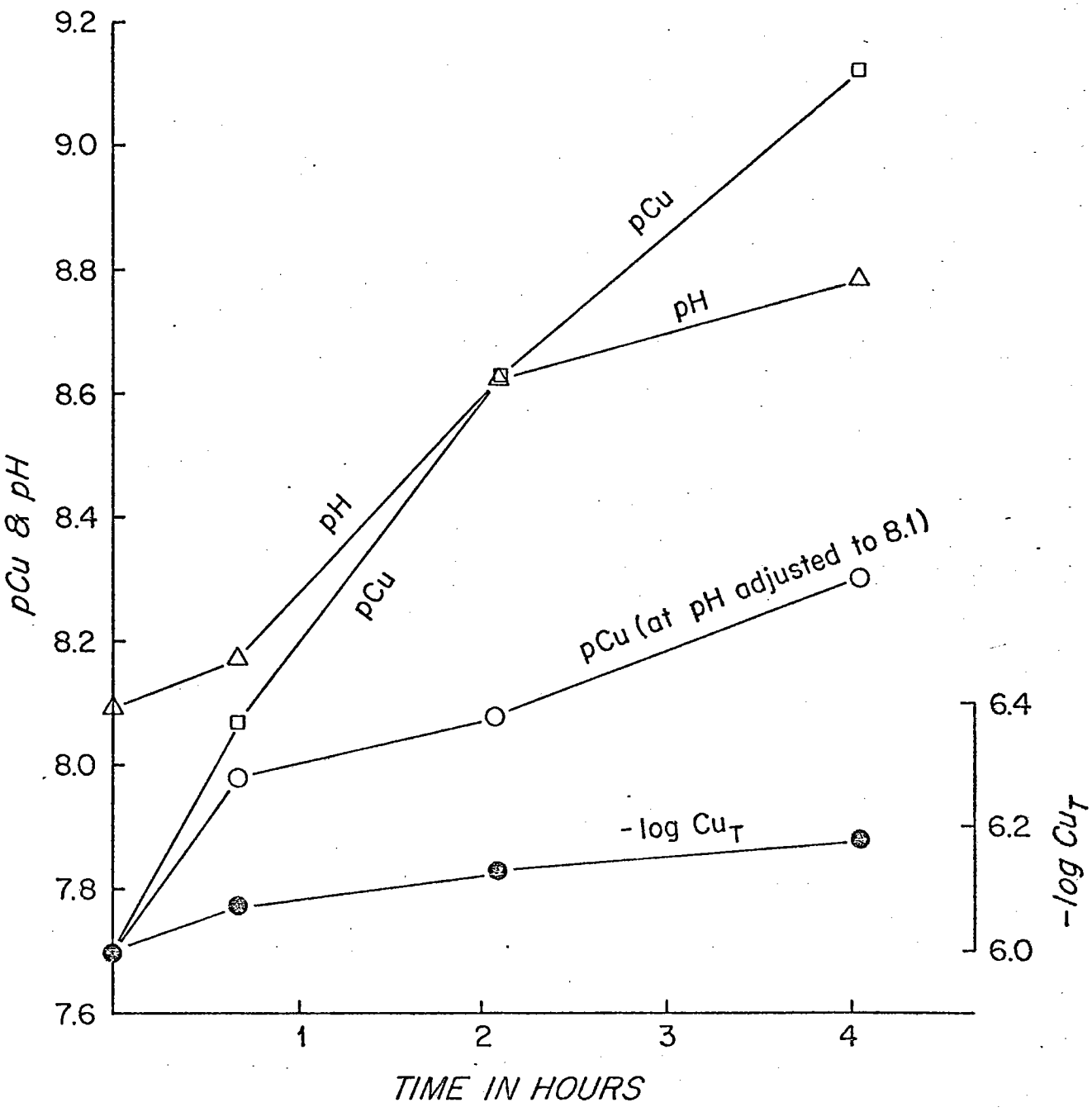


Figure (5-4)

Changes in pCu, pH, and -log Cu_T with time for a culture of T. pseudonana (clone 3H) in DSW medium containing 1μM $CuSO_4$. pCu, pH, and pCu (at pH adjusted to 8.1) were measured in culture filtrates.



The ratio of the activity of copper to the total copper concentration in solution (a_{Cu}/Cu_T) can be used as a complexation index. The higher the degree of copper complexation, the smaller this ratio will be. If we take Cu_T to be equal to the initial copper concentration minus that taken up by the cells, then this ratio has values of $10^{-1.7}$, $10^{-1.9}$, $10^{-1.9}$, and $10^{-2.1}$ respectively for culture filtrates sampled at times 0, 40, 125 and 243 minutes. These values are consistent with an increased copper complexation in the culture medium apparently due to the presence of extracellular complexing ligands.

The present experiment thus demonstrates two mechanisms, and suggests a third, by which the presence of the cells in the culture medium decreases the activity of copper. These are the photosynthetically induced increase in pH, uptake of copper by the cells, and the introduction of extracellular complexing ligands. All three represent potential feedback mechanisms through which the algae may reduce the cupric ion activity and thus copper toxicity.

V - C. Additional Evidence for the Production of Extracellular Complexing Ligands by Clone 3H

Results of the previous experiment with T. pseudonana in a 1 μM $CuSO_4$-DSW medium suggest that cells excrete and/or leak out extracellular copper complexing ligands into the external growth medium. The following experiment further investigates this phenomenon.

The experimental procedure was as follows. A fresh water SWC medium (Table 2-2) having the following alterations - deletion of EDTA, addition of trace metals (except Fe) at only one-fifth their normal concentrations, and addition of 2 μM $FeCl_3$ - was made up and autoclaved. Cells were then grown

aseptically in a portion of this medium to a concentration of 5.4×10^5 cells/ml, filtered, and washed with fresh medium. The culture filtrate was aerated to adjust its pH to 8.2 (the value of the unused portion of the culture medium). Copper sulfate was added to the culture filtrate and to the unused portion of culture medium to give a concentration of 0.3 μM in both media. The washed cells were reinoculated at a cell concentration of 2.5×10^5 cells/ml into the copper spiked new and used media and cell copper uptake, potassium loss, and growth were measured.

Measured pCu values for the new and used culture media before cell inoculation were respectively 8.5 and 8.8, consistent with an increased degree of copper complexation in the used medium. This correlates well with cell copper uptake data (Figure 4-6): the measured initial copper activity in the used medium is half that in the new medium and roughly half as much copper it taken up by the cells after 3 hours in the used medium compared with that taken up in the new. Cell potassium loss and growth (Figures 6-6 and 6-7) show a decreased deleterious effect of added copper in the used medium compared with that in the new.

These results provide strong additional evidence for the production of extracellular substances that complex with copper, reducing both its uptake by the cells and its inhibiting effects.

V - D. Discussion - Extracellular Chelators

Considerable evidence now exists that algae "excrete" extracellular metabolites in quantities that in some cases account for a sizeable fraction of the total fixed carbon (Hellebust, 1967; Sieburth, 1969). Fogg (1966),

for example, has reported that in some instances extracellular organics comprise as much as 50% of the total fixed carbon. Many of these compounds (amino acids, peptides, polysacharides) are metal chelators and may be expected under appropriate conditions to complex with heavy metal ions.

Fogg and Westlake (1955) concentrated partially purified extracellular polypeptide from the fresh water blue-green alga Anabena cylindrica and demonstrated that this material formed complexes with various trace metals including copper, iron and zinc. They further showed that addition of this material back into fresh cultures of Anabena containing added copper sulfate detoxified the copper. The authors postulated that heavy metal detoxification may be an important physiological function of extracellular algal products.

Figure (5-5)

Uptake of copper and loss of cell potassium for T. pseudonana (clone 3H) in new and used SWC culture media containing 0.3 μM $CuSO_4$.

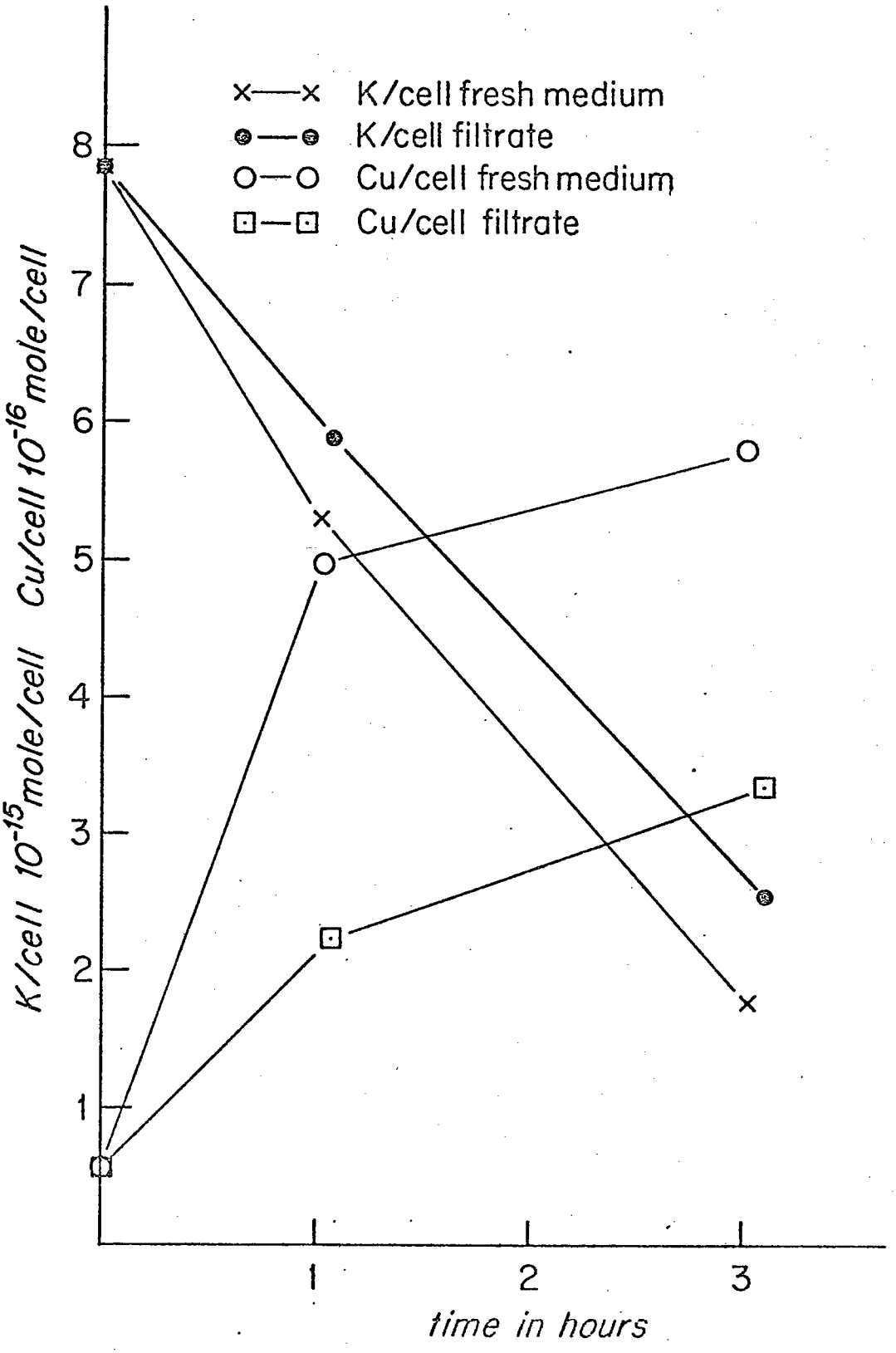
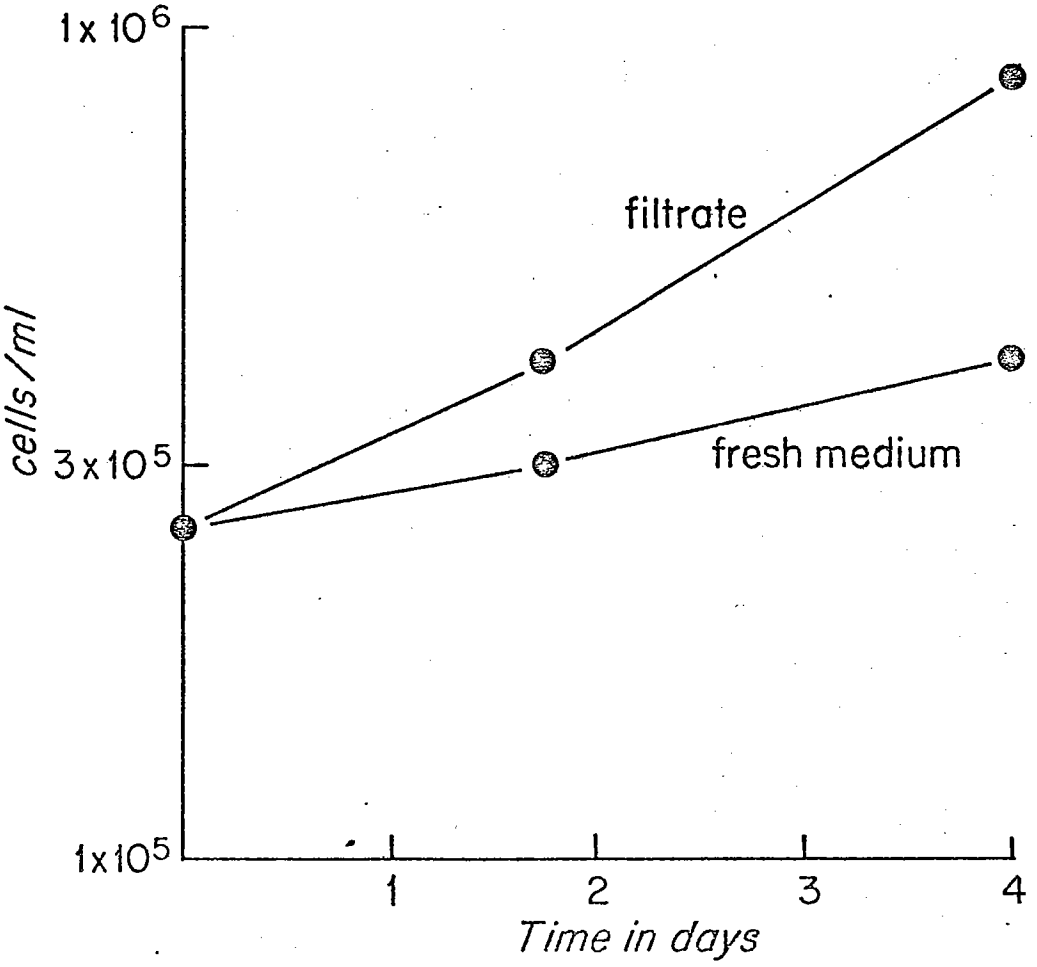


Figure (5-6)

Growth of T. pseudonana (clone 3H) in new and used SWC culture media containing 0.3 µM $CuSO_4$.



VI. STUDIES WITH NATURAL WATER SAMPLES

VI - A. Introduction

Barber (1973) found that the growth of phytoplankton in ultraviolet irradiated surface seawater was significantly reduced relative to that in untreated seawater, an effect that could be completely reversed by the addition of the artificial chelator EDTA to the irradiated seawater. It was postulated that ultraviolet irradiation destroyed naturally occurring organic chelators, whose presence either detoxified certain trace metals or increased the nutritional supply of iron to the algae. Steeman Nielsen and Wium-Anderson (1970) have suggested that the beneficial effect of added chelators in marine productivity studies is due to a removal of copper toxicity. Marked differences in the toxicity of added copper in different coastal seawater samples have been attributed to a variable complexation of copper by naturally occurring organic ligands (Davey et. al., 1973).

The present set of experiments are designed (1) to directly measure (using a cupric ion-selective electrode) the complexation of added copper in an uv irradiated and untreated river water sample to determine whether ultraviolet irradiation removes naturally occurring copper complexing ligands, and (2) to determine whether uv irradiation of coastal seawater or water from a salt marsh pond increases the toxicity of copper to algae grown in this water.

VI - B. Complexation of Copper in a Highly Colored River Water

Highly colored water was collected from the Newport River, near Beaufort, North Carolina, a river that is relatively unpolluted from industrial activity.

Within several hours after collection, the sample was filtered through a glass fiber filter (pore size ~ 1 μm) to remove particulate matter and organisms, and subsequently stored for two days in 500 ml borosilicate bottles before analysis.

Salinity of the water, as measured with a refractometer, was less than $0.5^{o}/oo$. The river water contained less than 0.3 μM copper as measured by atomic absorption spectrophotometry.

A portion of the river water was ultraviolet irradiated for a period of six hours (see Section II-F). Before irradiation, the water was the color of strong tea, apparently due to the presence of colored organic materials (i.e. gelbstoff). Spectrophotometric measurements (Figure 6-1) showed that ultraviolet irradiation removed essentially all of these colored materials from the water.

VI - B - 1. Copper Potentiometric Titrations

Potentiometric copper titrations were performed on both the raw filtered and ultraviolet irradiated samples (Figure 6-2). Prior to the titrations, the samples were equilibriated with the atmosphere by aeration using scintered glass frits. The pH of the samples was measured several times during the titrations and was constant to within ± 0.02 pH units.

Results of the titrations are given in Figure (6-2). The titration curve for the ultraviolet irradiated sample is linear throughout the titration range, with a slope of 31.5 millivolts/decade change in copper concentration, close to the Nernst slope of 29.7 millivolts/decade at $26^{o}C$. The titration curve for the raw filtered sample, on the other hand, is shifted to significantly

lower potentials. This curve is roughly linear in the concentration range 5.7-44 μM added copper with a slope of 60 millivolts/decade change in copper concentration. Below this range, the curve decreases in slope and assymtotically approaches a constant potential at concentrations of added copper below 1 μM.

For the raw sample, the marked increase in titration curve slope relative to the Nernst value (29.7) is consistent with a progressive saturation of ligands with copper (causing a decrease in free ligand activities) as more and more copper is added to the sample. The absence of any sharp breaks (i.e. end points) suggests that copper is complexed by a variety of different ligands having different associations constants for reaction with copper. Both the shift in titration curve to higher potentials and the decrease in curve slope for the ultraviolet irradiated sample are consistent with at least a partial destruction of organic complexing ligands by ultraviolet irradiation.

The leveling of the titration curve for the raw sample at submicromolar added copper concentrations is apparently due to the sensitivity limit of the cupric ion-selective electrode at combined low copper activity and low total copper concentration (Ross, 1969; Blaedel and Dinwiddie, 1974).

VI - B - 2. pCu - pH Measurements

Simultaneous measurements of pCu and pH were performed in an ultraviolet irradiated sample containing 5.7 μM $CuSO_4$ and in two raw samples containing 10 and 44 μM $CuSO_4$. During the measurements, the samples were aerated to maintain equilibrium with the atmosphere. For the ultraviolet irradiated

sample and for the raw sample containing 10 μM $CuSO_4$ measurements were first made as pH was decreased to values of 3-4 and again as pH was increased. For the raw sample containing 44 μM $CuSO_4$, measurements were made only for decreasing pH.

Results (Figure 6-3) show that the pCu-pH measurements are reproducible and reversible; that is, measurements made at decreasing pH agree well with those at increasing pH. The pCu is highly pH dependent in both the spiked raw filtered and ultraviolet irradiated samples. As pH is decreased, copper ions are apparently displaced from coordinating ligands resulting in an increase in the concentration of free copper ions (and thus a decrease in pCu).

The log of the ratio Cu_T/a_{Cu} can be used as an index for the degree of copper complexation with large ratios indicating high degrees of complexation. Plots of the log of this ratio as a function of pH (Figure 6-4) are roughly parallel for raw filtered and ultraviolet irradiated samples in the pH range 7.0 to 8.0, but are displaced from one another on the log Cu_T/a_{Cu} axis. At pH 7.7, values for log Cu_T/a_{Cu} for the raw samples containing 10 μM and 44 μM $CuSO_4$ and for the ultraviolet irradiated sample containing 5.7 μM $CuSO_4$ are respectively 3.4, 2.6, and 1.2. This shows a significantly higher degree of copper complexation in the raw samples and indicates that a major portion of the copper complexing ligands present in the river water was removed by ultraviolet irradiation. For the raw samples, the lower degree of copper complexation in the sample containing the higher $CuSO_4$ concentration is consistent once again with a larger degree of ligand saturation by copper ions as the total concentration of copper is increased.

Figure (6-1)

Light absorbance for raw filtered and ultraviolet irradiated river water.

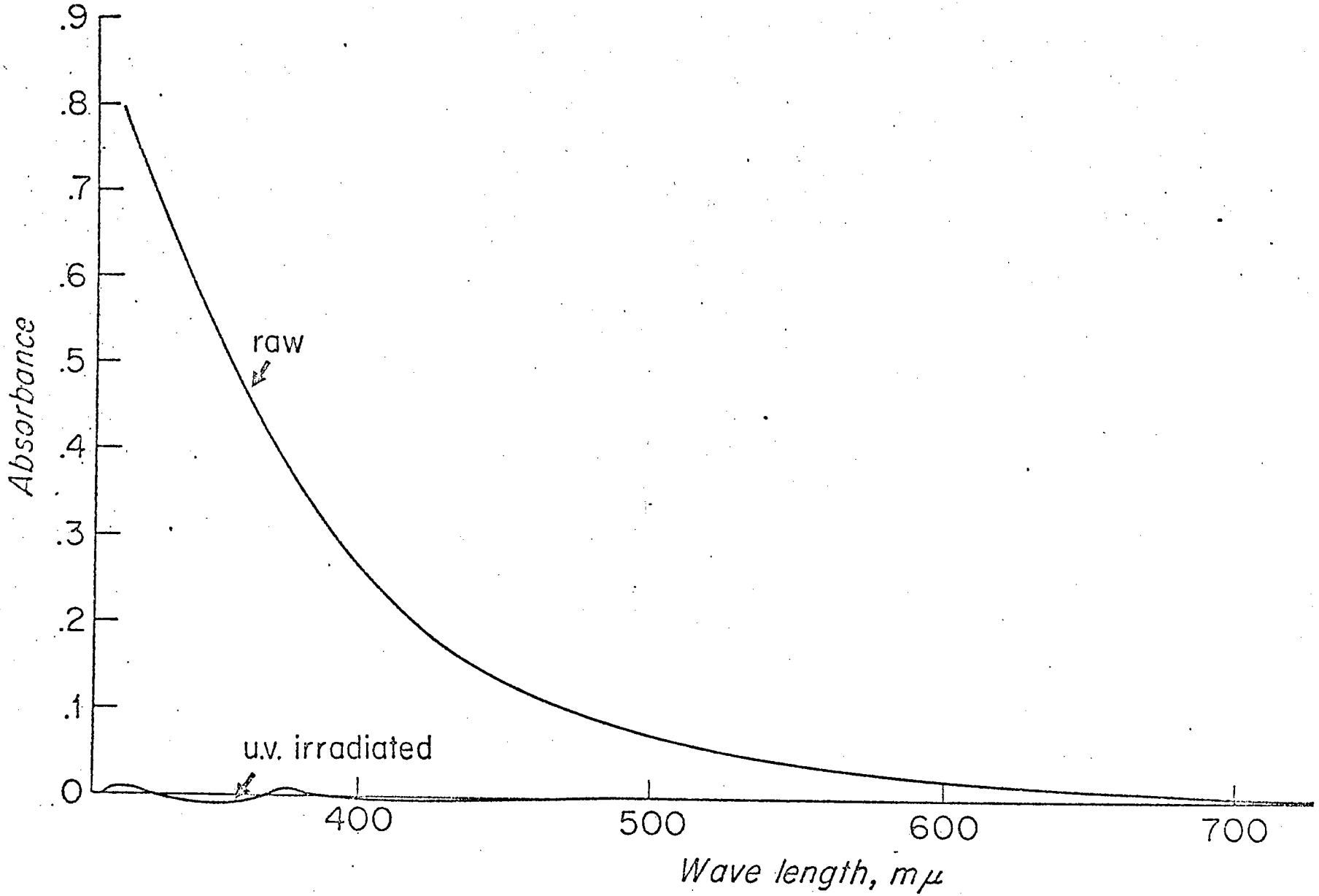


Figure (6-2)

Copper potentiometric titrations of raw filtered and ultraviolet irradiated river water samples at 26°C.

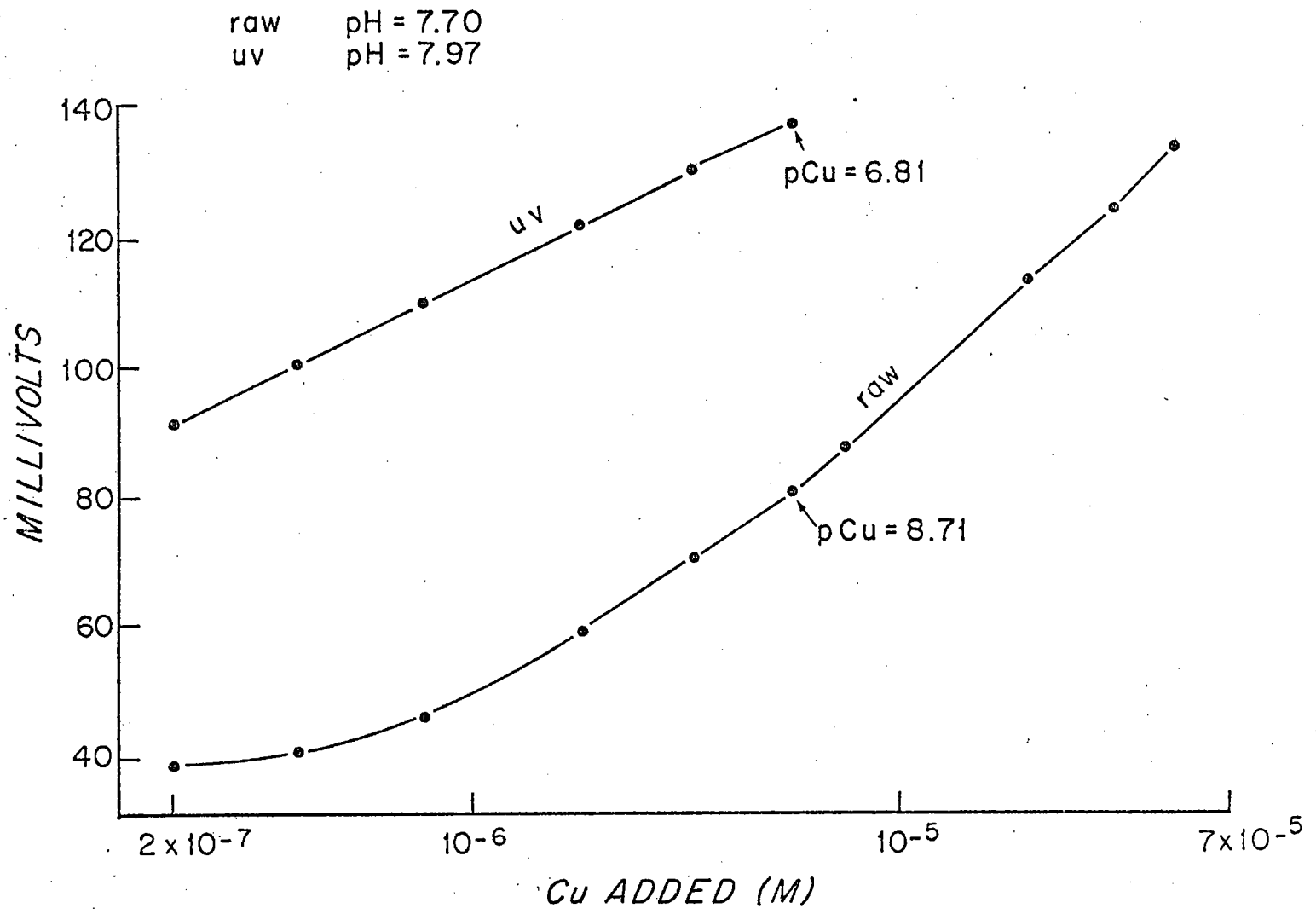


Figure (6-3)

Measured relationships between pCu and pH in raw filtered and uv irradiated river water samples containing various specified concentrations of $CuSO_4$ $T = 26^{o}C$.

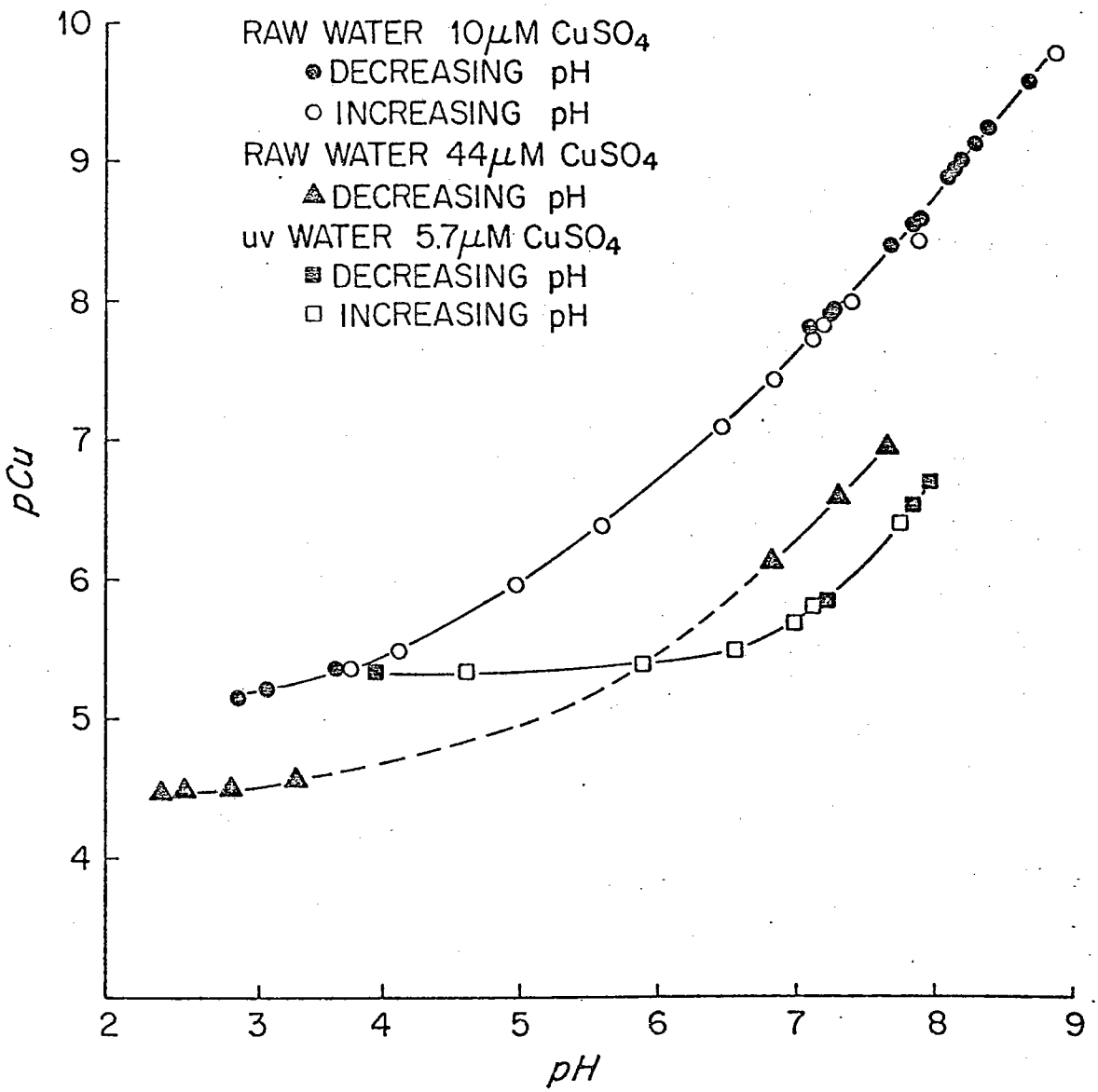
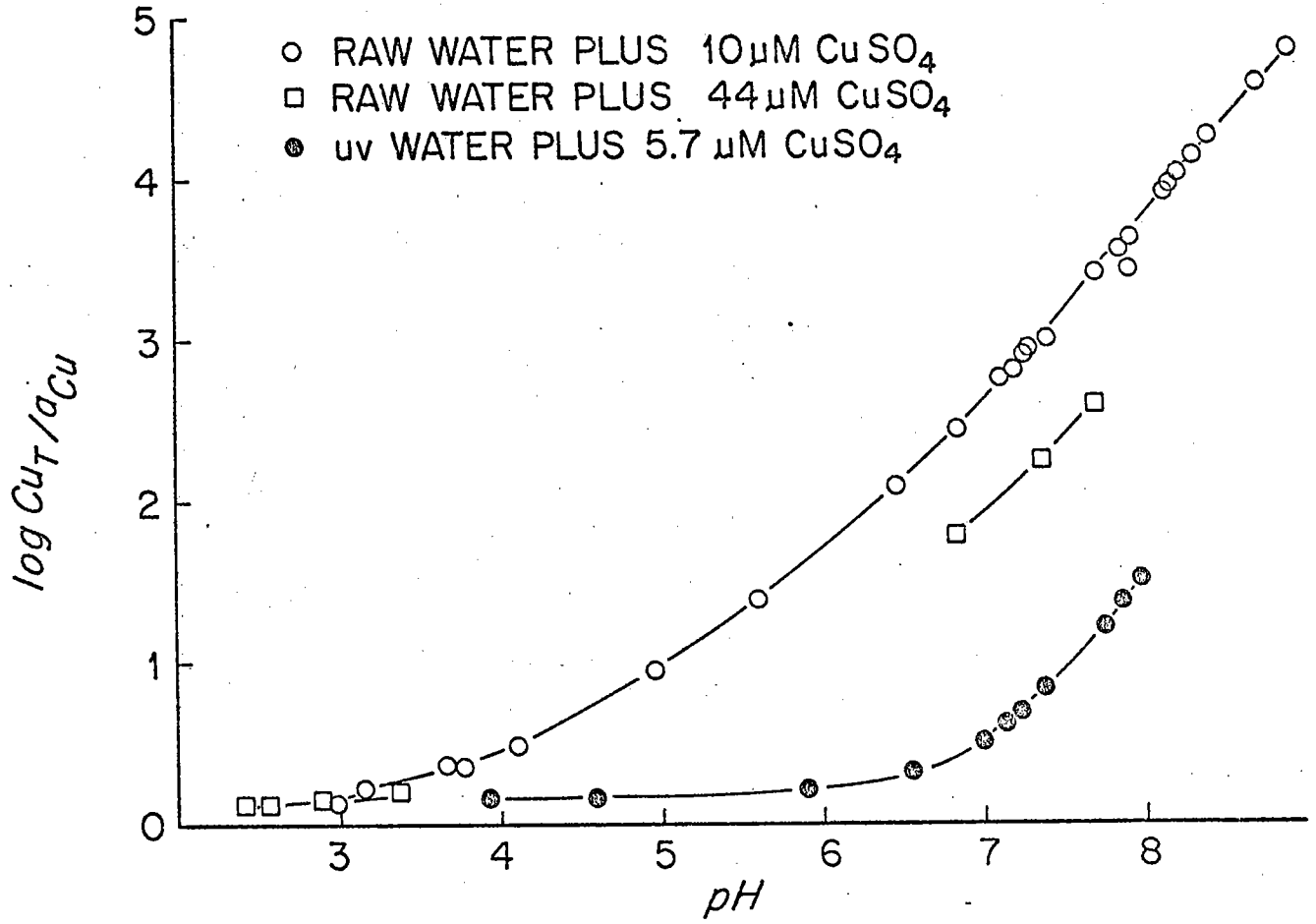


Figure (6-4)

Log Cu_T/a_{Cu} plotted as functions of pH for raw and uv irradiated river water samples containing various specified concentrations of $CuSO_4$. Cu_T is assumed to be equal to the added concentration of $CuSO_4$.



VI - C. Copper Toxicity in UV Irradiated and Raw Filtered Coastal Seawater and Marsh Water

In section (6-A), it was demonstrated that ultraviolet irradiation could be used to remove essentially all colored organic compounds and a large portion of copper complexing ligands from a highly colored river water. In the present section, the effect of prior ultraviolet irradiation of coastal seawater and salt marsh water samples on the subsequent toxicity of copper to algae is investigated.

VI - C - 1. Experimental

The species Nannochloris atomus (clone GSB Nanno) was chosen for this study because of its small size (1-2μ) and because of its previously determined low iron requirement (unpublished data). Since we wish to investigate the effect of removing organic chelators on the toxicity of copper to algae, we should therefore like to minimize the effect which chelator removal might have on iron supply.

The small size and relatively slow growth rate of N. atomus (i.e. relative to the other test alga 3H) also minimizes the effect of algal growth on the pH of the culture media. In separate experiments performed in M-f/2 medium, healthy log phase cultures of N. atomus with a growth rate of 0.75 divisions per day exerted no influence on culture medium pH at cell densities equal to or less than 1.1×10^6 cells per ml. In the present culture experiments cell densities were less than 10^6 cells per ml and thus algal

growth should have little effect on pH.

Vineyard Sound seawater was collected from the Woods Hole dock on February 15, 1971. The sample had a salinity of 32 $\pm$ 1 $^{o}/_{oo}$ and a pH of 8.2. Marsh water was collected from a pond of approximately one meter depth in Little Sippewissett Salt Marsh on September 22, 1972. The marsh water had a salinity of 20 $^{o}/_{oo}$ and a pH when equilibrated with the atmosphere of 8.1.

Portions of glass fiber ($\sim$ 1 µm pore size) filtered Vineyard Sound water and marsh pond water were ultraviolet irradiated for periods of 6 and 9 hours, respectively (see section II-F). Filtered marsh water had a pale yellow color before irradiation, but no visible color after irradiation, suggesting the removal of colored organic materials.

Culture experiments in Vineyard Sound water were performed aseptically (sterile filtration) whereas those in marsh pond water were not. Vineyard Sound and marsh pond water cultures were enriched with nutrients to insure adequate algal growth: the former with f/2 levels of nitrate, phosphate, and silicate and the latter with f/4 nitrate and phosphate (Table 1-1). One µM $FeCl_3$ was also added.

VI - C - 2. Results of Copper Toxicity Assays

Growth curves for the copper toxicity assays are shown in Figures (6-5 through 6-7). Ultraviolet irradiation of Vineyard Sound seawater caused a small decrease in the growth rate of the control cultures containing no added copper; however, this effect was completely reversed by the addition of the artificial chelator, EDTA. For cultures in marsh pond water, growth of ultraviolet treated and untreated controls was essentially the same

indicating that the irradiation process did not produce any toxic compounds in the water.

In all cases, added copper has a significantly increased inhibitory effect in the ultraviolet irradiated water. In raw filtered marsh pond water, the addition of 0.3 μM, copper has no inhibitory effect on algal growth whereas in the ultraviolet irradiated water added concentrations of 0.1 and 0.3 μM $CuSO_4$ inhibit growth rate by 34% and 71%, respectively. In untreated Vineyard Sound seawater, 0.03 and 0.1 μM copper inhibit growth rate by 18% and 39% compared with inhibitions of 59% and 76% for equivalent concentrations of copper in the ultraviolet irradiated seawater.

The results are consistent with the hypothesis that both marsh water and Vineyard Sound seawater contained organic substances that complex with and thus detoxify copper ions. The removal of some portion of these organic copper complexing substances would increase the fraction of copper present as free copper ion and thus increase the toxicity of added copper.

VI - C - 3. Copper Potentiometric Titrations of Raw Filtered and uv Irradiated Marsh Pond Water

The above hypothesis concerning the destruction of copper complexing organic substances by ultraviolet irradiation is supported by the results of copper titrations of raw filtered and ultraviolet irradiated river water (section VI-B) and the results of similar titrations in raw filtered and ultraviolet irradiated marsh pond water (Figure 6-8). The copper titration curve for raw filtered marsh water is noticably sigmoid in shape with a maximum slope of 74 millivolts per decade change in added copper concentration in the range

0.4 to 0.6 μM $CuSO_4$. The titration curve for the ultraviolet irradiated sample is shifted to higher potentials with a significantly decreased slope; average slope for the added copper concentration range 0.2 to 2.0 μM is 64 millivolts/decade for the titration curve of the raw filtered water as compared with 41 millivolts/decade for that of the ultraviolet irradiated water. As copper concentration is increased, the two titration curves approach one another, which is consistent with a saturation of chelation sites by copper. The non-Nernstian slope for the titration curve of the ultraviolet irradiated 20^{o}/oo salinity marsh pond water is apparently caused mostly by a chloride interference of the cupric ion-selective electrode (see appendix 1). Some portion of this increase slope may also be due to an incomplete photo-oxidation of organic ligands.

It should be noted here that copper titrations could be used to detect the addition of 2 μM of the chelator nitrilotriacetic acid (NTA) to Sargasso seawater (appendix 1, figure A1-3) even though the electrode was not responding in an ideal Nernstian fashion due to a chloride interference. The addition of NTA caused a significant decrease in cupric ion selective electrode potentials at equivalent concentrations of added $CuSO_4$ and an increase in titration curve slope which is exactly opposite to the observed effect of ultraviolet irradiation of the marsh pond water sample.

Figure (6-5)

Effect of added copper or added EDTA on the growth of N. atomus in raw and uv irradiated Vineyard Sound seawater. Control cultures contain no added copper or EDTA.

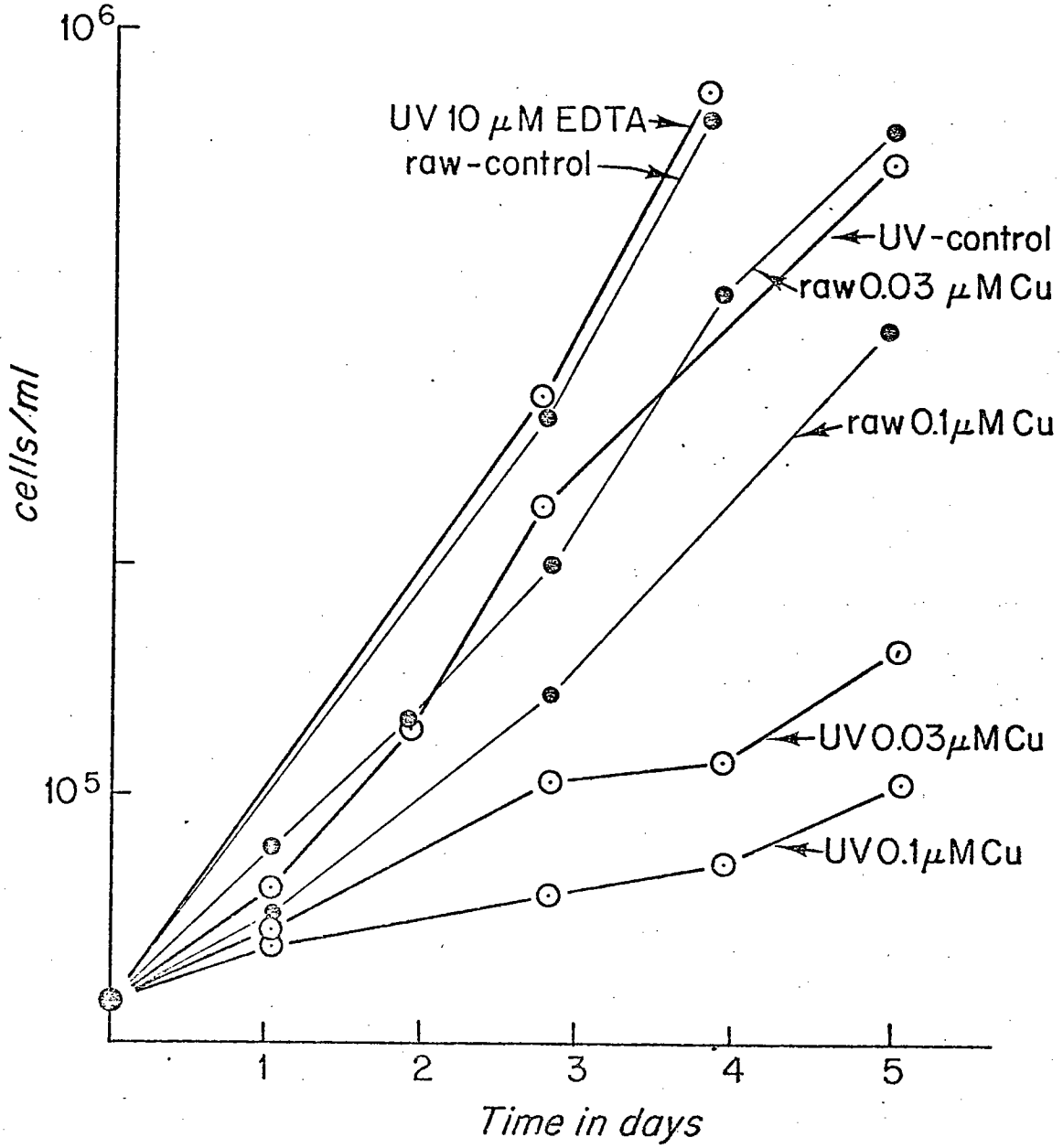


Figure (6-6)

Effect of added copper on the growth of N. atomus in raw and uv irradiated marsh pond water.

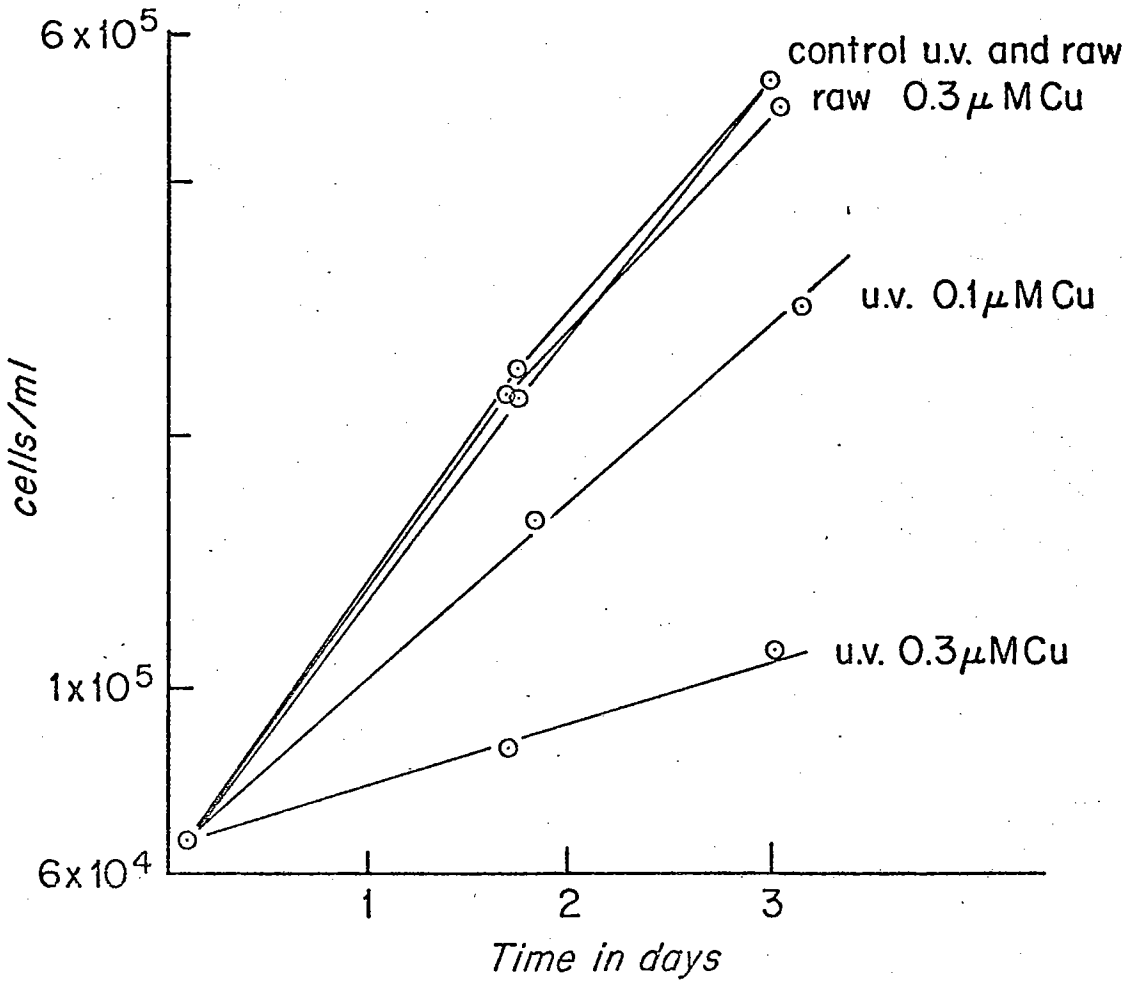


Figure (6-7)

Effect of added copper on the growth of N. atomus in raw marsh pond water.

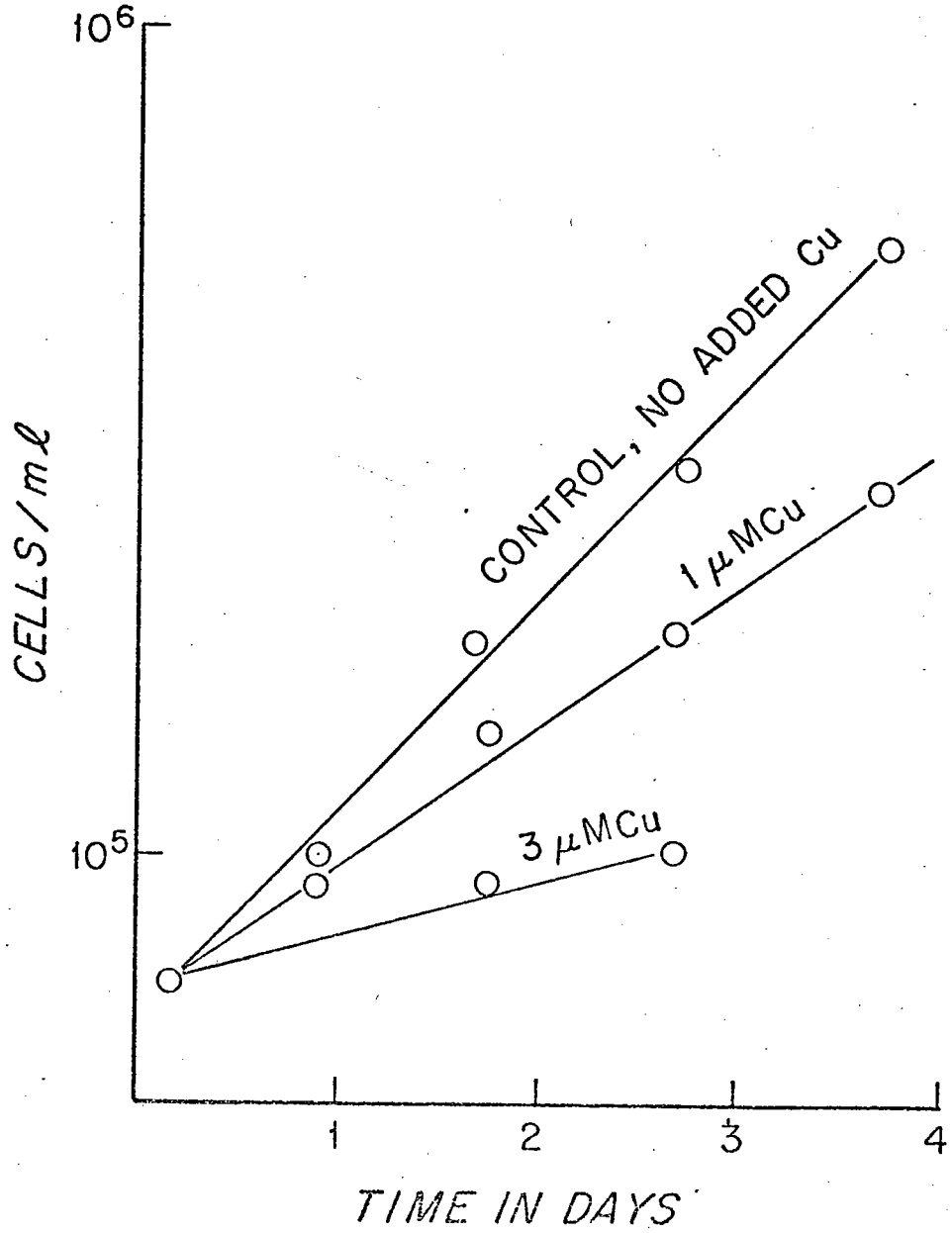
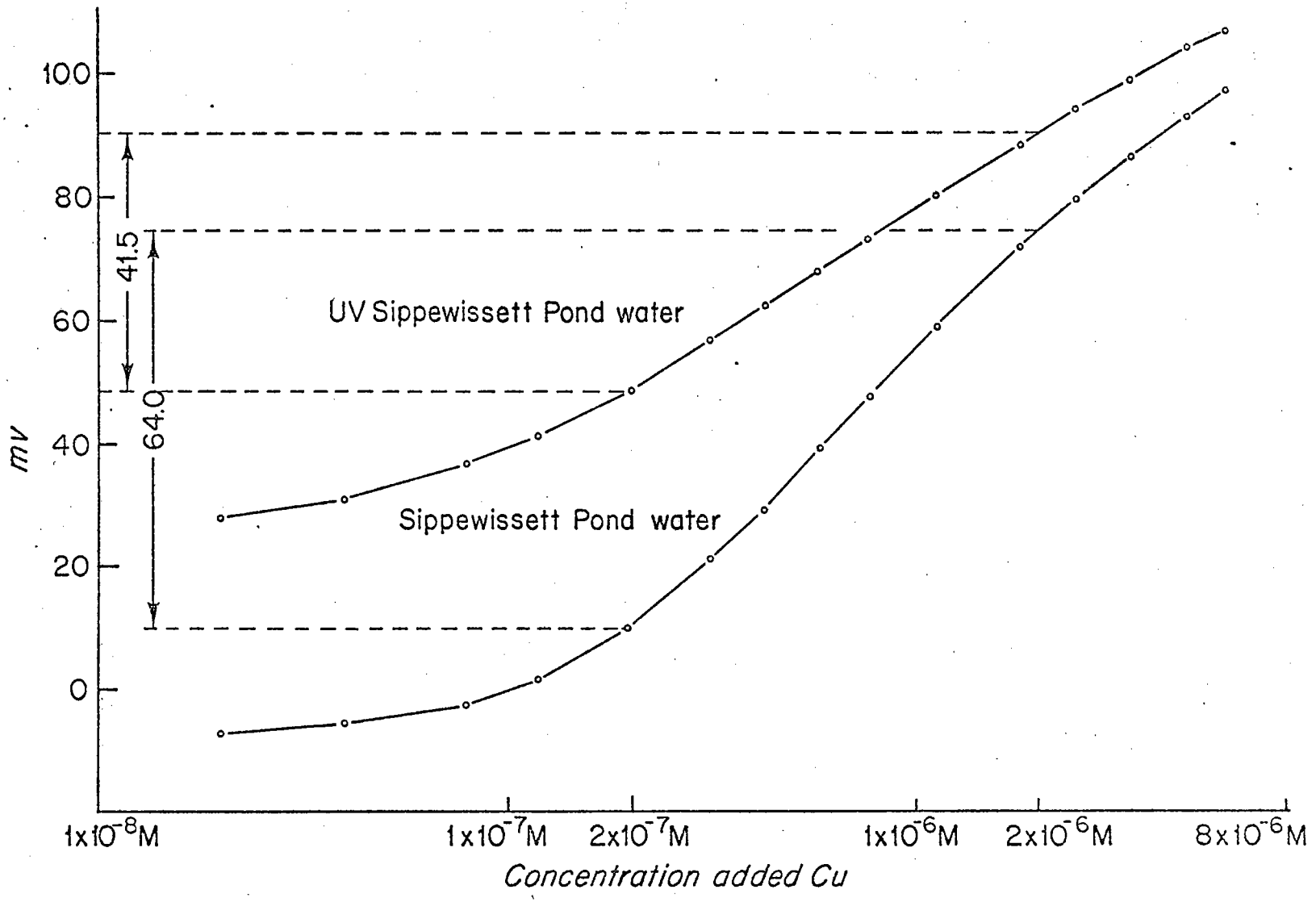


Figure (6-8)

Copper potentiometric titrations of raw and ultraviolet irradiated 20 °/oo salinity marsh pond water at 25°C and pH 8.10.



VII CONCLUSIONS AND PROSPECTUS

Experiments in tris-buffered seawater culture media confirm the hypothesis that copper inhibition to algae and cell copper uptake are related to the cupric ion activity and are independent of total copper concentration. This hypothesis is not new,but our experiments represent the first direct demonstration that such is the case. In view of our results, it becomes important that copper toxicity to algae be defined in terms of pCu, just as the effect of hydrogen ions on algae and other organisms is defined in terms of pH. Environmental water quality standards that consider only the concentration of toxic metals, and ignore the degree of metal complexation, are clearly inadequate.

Inasmuch as toxicity and cell copper uptake are related to cupric ion activities, it is important that we determine pCu levels in natural waters, either by direct measurement or by calculations that take into account the degree to which copper is complexed by natural ligands. At present, we are a long way from fulfilling this goal. As demonstrated in section (VI-B), the Orion cupric ion-selective electrode is an extremely promising tool for direct quantitative measurement of copper complexation in natural waters. This electrode, however, is no panacea, having several major shortcomings. The first of these is a chloride interference, which prevents the quantitative measurement of copper complexation in seawater. The sensitivity limit of the electrode should also prevent, in most cases, the direct measurement of naturally occurring cupric ion activities or of copper complexation at naturally occurring copper concentrations.

An approach to determining cupric ion activity in seawater and other natural waters is direct calculation. Because of our present meager knowledge of the composition of organic matter in natural waters, we cannot directly compute complexation by organic ligands and thus we can only consider inorganic complexation. Bioassays in coastal seawater and marsh water and cupric ion-selective electrode measurements in river water (section VI) strongly suggest that copper is significantly complexed by organic ligands in at least some natural waters. Calculations of pCu which consider only inorganic ligands may markedly underestimate total complexation and thus pCu. Such inorganic calculations of pCu in seawater (section III-C) may best apply to regions of low organic matter such as the Sargasso Sea or to newly upwelled seawater.

A comparison of the pCu sensitivity levels for clones 3H, 13-1, and GSB Nanno with calculated estimates of pCu in seawater indicates that in the absence of organic complexation, natural pCu levels are inhibitory to these three clones.

Results in section (V) provide evidence for three feedback mechanisms through which algae may reduce cupric ion activity in their growth medium and thus reduce copper toxicity. These are (1) the uptake of copper by cells (i.e. the reaction of copper with cellular ligands, (2) increased copper complexation caused by a photosynthetically induced increase in pH, and (3) the complexation of copper by extracellular ligands produced by the algae. All of these are of likely significance in natural waters; however, it is difficult to extrapolate quantitatively from short term experiments (lasting several hours to several days) made with single clone cultures at relatively high cell concentrations (10^5 to 10^6 cells per ml), to natural environments

where time scales are much longer, cell concentrations are typically much smaller (less than 10^4 cells per ml), and a variety of different species are present.

The present work is in many respects of a preliminary nature. Future research should be carried out to determine to what extent different species of phytoplankton differ in sensitivity to free cupric ion activity. Differential sensitivity might markedly affect species composition in natural waters in which copper toxicity occurs. Synergisms and antagonisms between copper and other metals are undoubtedly also of considerable importance and should be investigated. We should like to determine not only the extent to which species of algae other than T. pseudonana produce extracellular complexing ligands, but the conditions that affect the production of these materials and ultimately the identity of specific extracellular ligands. Finally, cupric ion-selective electrode measurements of copper complexation in a variety of natural waters of low salinity (such as that studied in section VI-B) should appreciably extend our present limited knowledge of copper speciation in the aquatic environment. Copper speciation is of importance not only to copper toxicity, but also to metal transport processes and geochemical reactions such as adsorption, precipitation, and solid solution.

VIII SUMMARY AND CONCLUSIONS

(1) Culture experiments with T. pseudonana (clone 3H) carried out in $CuSO_4$-trishydroxymethylamino methane metal buffered seawater culture media, in which the chelator concentration, the total copper concentration, and the pH are systematically varied, demonstrate that cell copper content and copper induced growth rate inhibition are both related to pCu and are independent of the total copper concentration.

(2) Cellular copper content for clone 3H was found to be a hyperbolic function of cupric ion activity for the pCu range (8.6-10.5)

$$\text{Cu/cell} = \frac{4.8 \times 10^{-16} \text{ (moles/cell)} \; a_{Cu}}{a_{Cu} + 10^{-9.2}}$$

where a_{Cu} represents the cupric ion activity. The above relationship suggests the binding of copper to a single set of cellular ligand sites having a total binding capacity of 4.8×10^{-16} moles/cell and an apparent association constant for reaction with copper of $10^{-9.2}$.

(3) The use of copper-chelate metal buffered culture media is an excellent technique for quantitatively measuring the relationship between the toxicity of copper to algae and pCu. In experiments carried out in such media - i.e. seawater-$CuSO_4$-tris - copper inhibited the growth rate of the estuarine diatom T. pseudonana (clone 3H) at pCu values below 10.7 with total growth rate inhibition occurring at pCu values below 8.3. For an estuarine green alga N. atomus the pCu range for partial to total growth rate inhibition was approximately 10.3 to 8.4. Calculation of culture pCu values for

experiments with an open ocean isolate of T. pseudonana (clone 13-1) in artificial seawater media containing no added chelators (experiments of Davey et. al., 1973) yielded a growth rate inhibitory pCu range for this clone similar to those for T. pseudonana (clone 3H) and N. atomus in the highly chelated seawater-tris culture media.

(4) A comparison of the pCu levels which inhibit the growth of the above three isolates with an estimate of the natural pCu levels for seawater indicates that in seawater containing no significant organic chelation, natural levels of copper should be inhibitory to these algae. Changes in pCu due to pH variation in seawater and other natural waters may be an important factor affecting copper toxicity to algae.

(5) Quantitative evidence was found for the complexation of copper in culture media by extracellular ligands produced by algae. The growth of clone 3H in low salinity culture media resulted in an increase in copper complexation in the culture media. This correlated well with a decrease in copper toxicity and a decrease in copper uptake by cells in culture media in which algae had been previously grown. The detoxification of trace metals may represent an important physiological function of extracellular algal products.

(6) The results of potentiometric measurements and bioassays in several untreated and ultraviolet irradiated natural water samples are consistent with the Hypothesis that copper is significantly complexed by organic ligands in at least some natural waters, and that such complexation decreases the toxicity of copper to algae. Ultraviolet irradiation of a highly colored river water sample resulted in a large decrease in the

complexation of added copper, which is consistent with at least a partial photooxidation of naturally occurring organic ligands by ultraviolet irradiation. Bioassays with N. atomus in Vinyeard Sound coastal seawater and in water collected from a salt marsh pond showed a significantly higher copper toxicity to algae in water samples previously treated with ultraviolet irradiation than in untreated water samples.

(7) The Orion cupric ion-selective electrode is a promising tool for the investigation of copper complexation in culture media and natural waters containing low chloride concentrations (i.e. $\leq$ 0.05M).

BIBLIOGRAPHY

1. Adamson, A. W. (1969) Photochemistry of transition metal coordination compounds, Pure and Applied Chem. 20, 25-52.

2. Armstrong, F. A. J., P. M. Williams, and J. H. D. Strickland (1966) Photooxidation of organic matter in seawater by ultraviolet radiation, analytical and other applications, Nature 211, 481-487.

3. Barber, R. T., and J. H. Ryther (1969) Organic chelators: factors affecting primary production in the Cromwell Current upwelling, J. exp. mar. Biol. Ecol. 3, 191-199.

4. Barber, R. T., R. C. Dugdale, J. J. MacIsaac, and R. L. Smith (1971) Variations in phytoplankton growth associated with the source and conditioning of upwelling water, Inv. Pesq. 35, 171-193.

5. Barber, R. T. (1973) Organic ligands and phytoplankton growth in nutrient-rich seawater, in Trace Metals and Metal Organic Interactions in Natural Waters, P. C. Singer, ed., Ann Arbor Science, Ann Arbor, Mich.

6. Barsdate, R. J., and W. R. Matson (1966) Trace metals in arctic and sub-arctic lakes with reference to the organic complexes of metals, in Radioecological Concentrations Processes, B. Aberg and F. Hungate, eds., Pergamon Press, Oxford, pp. 711-719.

7. Bates, R. G. (1964) Determination of pH - Theory and Practice, Wiley, New York, N. Y., 435 pp.

8. Berner, R. A. (1965) Activity coefficients of bicarbonate, carbonate, and calcium ions in seawater, Geochim. Cosmochim. Acta 29, 947-965.

9. Berner, R. A. (1971) Principles of Chemical Sedimentology, McGraw-Hill, New York, pp. 44-48.

10. Blaedel, W. J. and D. E. Dinwiddie (1974) Study of the behavior of copper ion-selectrodes at submicromolar concentration levels, Analyt. Chem. 46, 873-877.

11. Chester, R., and J. H. Stoner (1974) The distribution of zinc, nickel, manganese, cadmium, copper and iron in some surface waters from the world ocean, Marine Chemistry 2, 17-32.

12. Corcoran, E. F. and J. E. Alexander (1964) The distribution of certain trace elements in tropical seawater and their biological significance, Bull. Marine Sci. Gulf and Caribbean 14, 594.

13. Davey, E. W., M. J. Morgan, and S. J. Erickson (1973) A biological measurement of copper complexation capacity of seawater, Limnol. and Oceanogr. 18, 993-997.

14. Degens, E. T., R. R. L. Guillard, W. M. Sackett, and J. A. Hellebust (1968) Metabolic fractionation of carbon isotopes in marine plankton - I. Temperature and respiration experiments, Deep Sea Res. 15, 1-9.

15. Erickson, S. J. (1972) Toxicity of copper to a marine diatom in unenriched inshore seawater, J. Phycol. 8, 318-323.

16. Erickson, S. J., T. E. Maloney, and J. H. Gentile (1970) Effect of nitrilotriacetic acid on the growth and metabolism of estuarine phytoplankton, J. Water Pollution Control 42, 329-335.

17. Fitzgerald, W. F. (1970) A study of certain trace metals in seawater using anodic stripping voltametry, Thesis, M. I. T.

18. Fogg, G. E. (1966) The extracellular products of algae, Oceanogr. Mar. Biol. Annu. Rev. 4, 195-212.

19. Fogg, G. E., and P. F. Westlake (1955) The importance of extracellular products of algae in fresh water, Verhandel. intern. Ver. Limnol. 12, 219-232.

20. Garrels, R. M., and M. E. Thompson (1962) A chemical model for seawater at 25°C and one atmosphere total pressure, Am. J. Sci. 260, 57-66.

21. Goldberg, E. W. (1965) Minor elements in seawater, in Chemical Oceanography, Vol. I, J. P. Riley and G. Skirrow, eds., Academic Press, London and New York, pp. 163-196.

22. Good, N. E., G. D. Winget, W. Winter, T. N. Connolly, S. Izawa, and R. M. M. Singh (1966) Hydrogen ion buffers for biological research, Biochemistry 5, 467-477.

23. Guillard, R. R. L., and J. H. Ryther (1962) Studies of marine planktonic diatoms. I. Cyclotella nana Hustedt and Detonula confervacea (cleve) gran, Canad. J. of Microbio. 8, 229-238.

24. Guillard, R. R. L., and C. J. Lorenzen (1972) Yellow-green algae with chlorophyllide C, J. Phycol. 8, 318-323.

25. Guillard, R. R. L. (1973) Division rates, in Handbook of Phycological Methods - Culture Methods and Growth Measurements, J. R. Stein, ed., Cambridge University Press, pp. 290-311.

26. Hall, J. L., J. A. Swisher, D. G. Brannon, and T. L. Liden (1962) Copper (II) and nickel (II) complexes with 2-amino-2-(hydroxy methyl)-1,3-propanediol. Reactions with solium hydroxide, Inorg. Chem. 1, 409-413.

27. Hasle, G. R., and B. R. Heimdal (1970) Some species of the centric diatom genus Thalassiosira studied in the light and electron microscope, Nova Hedwigia 31, 559-581.

28. Hawley, J. E., and R. M. Pytkowicz (1973) Interpretation of pH measurements in concentrated electrolyte solutions, Mar. Chem. 1, 245-250.

29. Hellebust, J. A. (1967) Excretion of organic compounds by cultured and natural populations of marine phytoplankton, in Estuaries: Nutrients and Biological Production, Amer. Ass. for the Adv. of Sci., 361-366.

30. Hume, D. N., and J. N. Carter (1972) Characteristics of the mercury coated graphite electrode in anodic stripping voltametry: application to the study of trace metals in environmental water systems, Chemia Analityczna 17, 747-759.

31. Hutner, S. H., L. Provasoli, A. Schatz, and C. P. Haskins (1950) Some approaches to the study of the role of metals in the metabolism of microorganissm, Proc. of the Amer. Philosoph. Soc., 94, 152-170.

32. Jasinski, R., I. Trachtenberg, and D. Andrychuk (1974) Potentiometric measurements of copper in seawater with ion-selective electrodes, Analyt. Chem. 46, 364-369.

33. Johnston, R. (1963) Seawater, the natural medium of phytoplankton. I. General features, J. mar. biol. Ass. U. K. 43, 427-456.

34. Johnston, R. (1964) Seawater the natural medium of phytoplankton. II. Trace metals and chelation, and general discussion, J. mar. biol. Ass. U. K. 44, 87-109.

35. Kamp-Neilsen, L. (1971) The effect of deleterious concentrations of mercury on photosynthesis and growth of Chlorella pyrenoidosa, Physiol. Plant. 24, 556-561.

36. Kester, D. R., and R. M. Pytkowicz (1967) Determination of the apparent dissociation constants of phosphoric acid, Limnol. and Oceanogr. 12, 243-252.

37. Kester, D. R., I. W. Duedall, D. N. Connors, and R. M. Pytkowicz (1967) Preparation of artificial seawater, Limnol. and Oceanogr. 12, 176-179.

38. Leyendekkers, J. V. (1973) The chemical potentials of seawater components, Mar. Chem. 1, 75-88.

39. MacInnes, D. A. (1939) The Principles of Electrochemistry, International Textbook Press, Scranton, Pa., 478 pp.

40. Manahan, S. E. and M. J. Smith (1973) Copper micronutrient requirement for algae, Environ. Sci. and Tech. 7, 829-833.

41. Matson, W. R. (1968) Trace metals, equilibrium and kinetics of trace metal complexes in natural media, Thesis, M. I. T.

42. McBrien, D. and K. A. Hassall (1965) Loss of cell postassium by Chlorella vulgaris after contact with toxic amounts of copper sulfate, Physiol. Plant. 18, 1059-1069.

43. McLachlan, J. (1973) Growth media - marine, in Handbook of Phycological Methods, Culture Methods and Growth Measurements, J. R. Stein, ed., Cambridge University Press, pp. 25-51.

44. Menzel, D. W., and R. F. Vaccaro (1964) The measurement of dissolved organic and particulate carbon in seawater, Limnol. and Oceanogr. 9, 138-142.

45. Neilands, J. B. (1957) Some aspects of microbial iron metabolism, Bacteriol. Rev. 21, 101-111.

46. Neilands, J. B. (1967) Hydroxamic acids in nature, Science 156, 1443-1447.

47. Orion Research Newsletter (1969) Orion finds great "potential" in liquid junctions, Orion Research, Inc., Cambridge, Mass.

48. Park, K. (1966) Deep-sea pH, Science 154, 1540-1541.

49. Park, K. (1968) Alkalinity and pH off the coast of Oregon, Deep-Sea Res. 15, 171-183.

50. Platford, R. F. and T. Dafoe (1965) The activity coefficient of sodium sulfate in seawater, J. Mar. Res. 23, 63-68.

51. Platford, R. F. (1965) The activity coefficient of sodium chloride in seawater, J. Mar. Res. 23, 55-62.

52. Price, C. A., and J. W. Quigley (1966) A method for determining quantitative zinc requirements for growth, Soil Sci. 101, 11-16.

53. Robinson, R. A., and R. H. Stokes (1959) Electrolyte Solutions, Butterworth and Co., Ltd., London, 571 pp.

54. Ross, J. W. (1969) Solid-state and liquid membrane ion-selective electrodes, in Ion-Selective Electrodes, National Bureau of Standards Spec. Pub. 314, U. S. Gov. Print. Off., Washington, D. C.

55. Ryther, J. H. (1954) The ecology of phytoplankton blooms in Moriches Bay and Great South Bay, Long Island, New York, Biol. Bull. 106, 198-209.

56. Schlindler, P. W. (1967) Heterogeneous equilibria involving oxides, hydroxides, carbonates, and hydroxide-carbonates, in Equilibrium Concepts in Natural Water Systems, R. F. Gould, ed., Adv. Chem. Ser. 67, Amer. Chem. Soc., Washington, D. C., pp. 196-221.

57. Sieburth, J. McN. (1969) Studies of algal substances in the sea. III, The Production of extracellular organic matter by littoral marine algae, J. exp. mar. Bio. Ecol. 3, 290-309.

58. Sillen, L. G., and A. E. Martell (1964, 1971) Stability Constants, Special Pub. no. 17 and no. 25, The Chem. Soc. of London.

59. Slowey, J. F., and D. W. Hood (1966) The chemistry and analysis of trace metals in seawater, Ann. Rept. A.E.C.-Contract No. AT-(40-1)-2799, Texas A. and M. Report 66-2F.

60. Slowey, J. F., L. M. Jeffrey, and D. W. Hood (1967) Evidence for organic complexed copper in seawater, Nature 214, 377-378.

61. Sharp, J. H. (1973) Total organic carbon in seawater - comparison of measurements using persulfate oxidation and high temperature combustion, Mar. Chem. 1, 211-229.

62. Spencer, C. P. (1957) Utilization of trace elements by marine unicellular algae, J. gen. Microbiol. 16, 282-285.

63. Steeman Nielsen, E., and S. Wium-Anderson (1970) Copper ions as poison in the sea and freshwater, Marine Biology 6, 93-97.

64. Steeman Nielsen and S. Wium-Anderson (1971) The influence of copper on photosynthesis and growth in diatoms, Physiol. Plant. 24, 480-484.

65. Stumm, W. and J. J. Morgan (1970) Aquatic Chemistry, Wiley Interscience, New York.

66. Stumm, W. and P. A. Brauner (1973) Chemical speciation, in Chemical Oceanography, 2nd edition, J. P. Riley and G. Skirrow, eds., Academic Press.

67. Thompson, M. E. (1966) Magnesium in seawater: an electrode measurement, Science 153, 866-867.

68. Thompson, M. E., and J. W. Ross (1966) Calcium in seawater by electrode measurement, Science 154, 1643-1644.

69. Van Breemen, N. (1973) Calculation of ionic activities in natural waters, Geochim. Cosmochim. Acta 37, 101-107.

70. Walker, J. B. (1953) Inorganic micronutrient requirements of Chlorella, I. Requirements for calcium (or strontium), copper, and molybdenum, Arch. Biochem. Biophys. 46, 1-11.

71. Walker, J. B. (1954) Inorganic micronutrient requirements of Chlorella, II. Quantitative requirements for iron, manganese, and zinc, Arch. Biochem. Biophys. 53, 1-8.

72. Wallace, A., L. M. Shannon, O. R. Lunt, and R. L. Impey (1957) Some aspects of the use of metal chelates as micronutrient fertilizer sources, Soil Sci. 84, 27-41.

73. Whitfield, M. (1973) A chemical model for the major electrolyte components of seawater based on the Brønsted-Guggenheim hypothesis, Mar. Chem. 1, 251-266.

74. Trott, T., W. Henwood, and C. H. Langford (1972) Sunlight photochemistry of ferric nitrilotiacetate complexes, Environ. Sci. and Tech. 6, 367-368.

75. Williams, P. M., (1969a) The association of copper with dissolved organic matter in seawater, Limnol. and Oceanogr. 14, 156.

76. Williams, P. M., (1969b) The determination of dissolved organic carbon in seawater: a comparison of two methods, Limnol. and Oceanogr. 14, 297-298.

APPENDIX 1: CUPRIC ION-SELECTIVE ELECTRODE MEASUREMENTS IN SEAWATER: EFFECT OF CHLORIDE ION

Jasinski et. al. (1974) investigated the behavior of the Orion cupric ion-selective electrode in various seawater samples. Replicate calibration curves in Gulf of Mexico open ocean seawater showed a linear electrode response with a slope of 52±2 mv/decade [added copper]. A comparison of calibration curves in seawater with those in 1 M KCl at pH 2 and 0.5 M NaCl adjusted with base to pH 8 suggested that the increase in the calibration curve slope in open ocean seawater relative to the electrode Nernst slope (∿ 30) was caused by a chloride interference.

In our present study, a comparison of copper potentiometric titrations in distilled water, 0.1 M NaCl, 0.5 M NaCl, 1.25 M NaCl, and 0.5 M NaCl plus 2 mM $NaHCO_3$ solutions at 25°C (Figure A1-1) also indicate the presence of a chloride interference that causes an increase in the electrode response slope from that predicted by the Nernst equation for a divalent ion. The titration curve for distilled water is linear throughout the titration range of 0.2 to 5.7 μM $CuSO_4$ with a slope of 29.3 mv/decade [$CuSO_4$], in good agreement with the expected Nernst slope (29.6). By contrast, the curves for 0.1, 0.5, and 1.25 M NaCl have linear slopes (for the upper portions of the titrations curves) of 35.5, 51.2, and 57.2. According to ideal electrode behavior, a titration curve slope equal to the Nernst value would be expected in the NaCl solutions since, for any single titration, the ionic strength and the degree to which copper is complexed to chloride ion are constant.

The titration curve for the solution containing 0.5 M NaCl and 2 mM $NaHCO_3$ is shifted downward on the potential axis and has a slightly decreased linear slope of 48.5 relative to the curve for 0.5 M NaCl alone. The shift in the curve to more negative potentials is consistent with the increased complexation of copper by carbonate and hydroxide ions (see sections 3-C and appendix 2).

For solutions containing 0.5 M NaCl, 0.5 M NaCl plus 2 mM $NaHCO_3$, and 1.25 M NaCl the titration curves deviate from linearity with decreasing slopes at added copper concentrations below 0.4, 0.4, and 1.0 μM, respectively. This departure from linearity is possibly caused by either the presence of trace contaminants - initial copper, silver, or mercury - possibly introduced as impurities in the reagent grade NaCl used to prepare the solutions (Jasinski et. al., 1974) or by the sensitivity of the electrode at combined low free ion activities and total concentrations of copper (Blaedel and Dinwiddie, 1974; Ross, 1969).

Cupric ion-selective electrode measurements were also made in solutions containing 10 μM $CuSO_4$ as functions of NaCl concentration or $NaNO_3$ concentration at 25°C (Figure A1-2). The measured curves are identical for concentrations of NaCl and $NaNO_3 \leq 0.05$ M indicating that chloride interference does not occur below this concentration. As the concentrations of NaCl and $NaNO_3$ are increased above 0.1 M, the two curves depart markedly from one another due mostly to the chloride interference and to a much lesser extent chloride complexation in the solution containing added NaCl.

I - A. Copper Potentiometric Titration Curves of Sargasso Seawater With and Without Added Chelators

Copper potentiometric titrations were carried out in surface Sargasso

Figure (A1-1)

Cupric ion-selective electrode potentiometric titrations in distilled water, 0.1 M NaCl, 0.5 M NaCl, 1.25 M NaCl, and 0.5 M NaCl plus 2 mM $NaHCO_3$ (pH 8.17) at 25°C. For these titrations and those in figures (A1-2 and A1-3) the electrode potential for a 20 μM $CuSO_4$ distilled water solution has been artitrarily set at 200 millivolts (the upper limit of the Beckman pH meter's millivolt scale). All other measured potentials are relative to this arbitrarily set value.

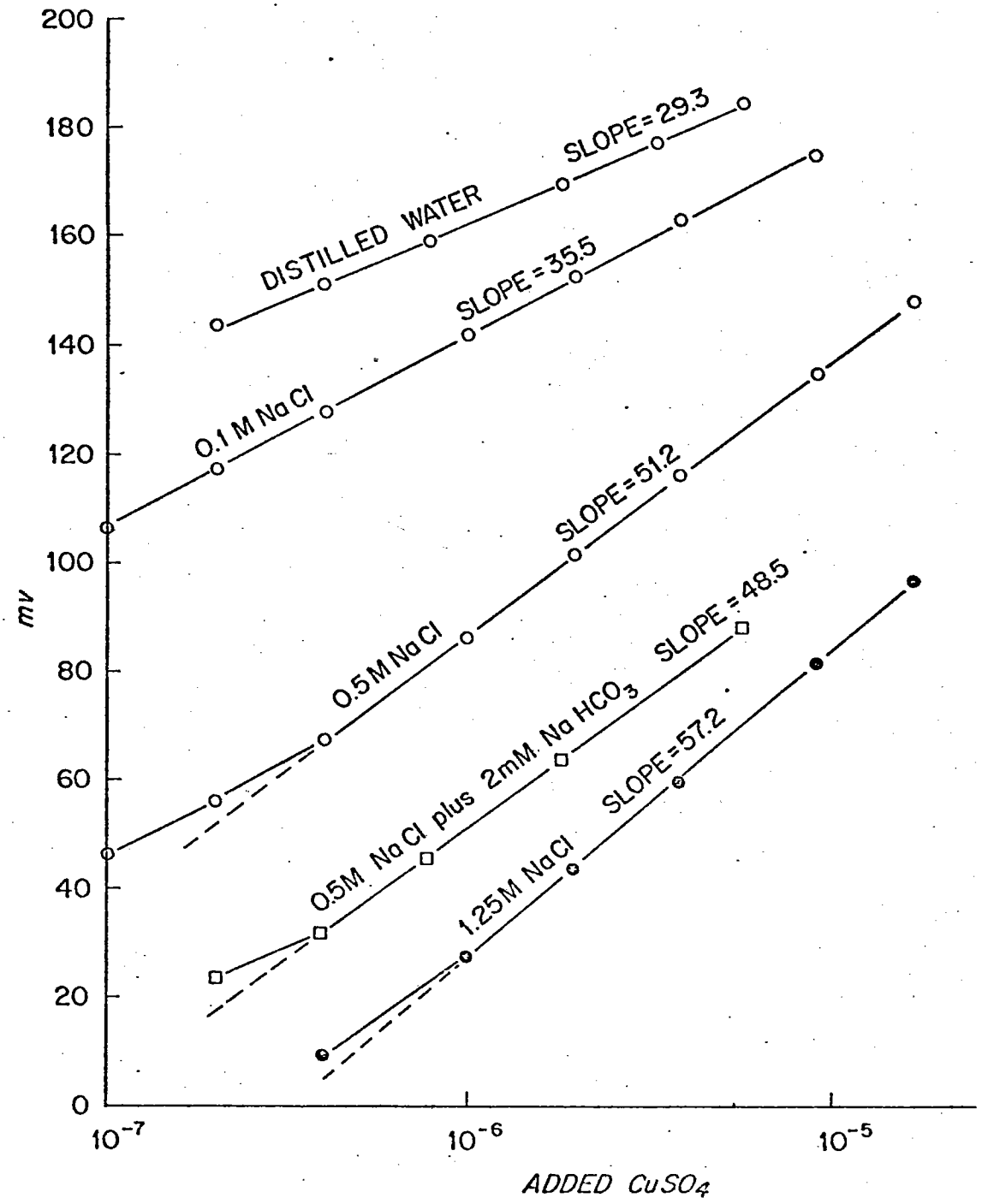
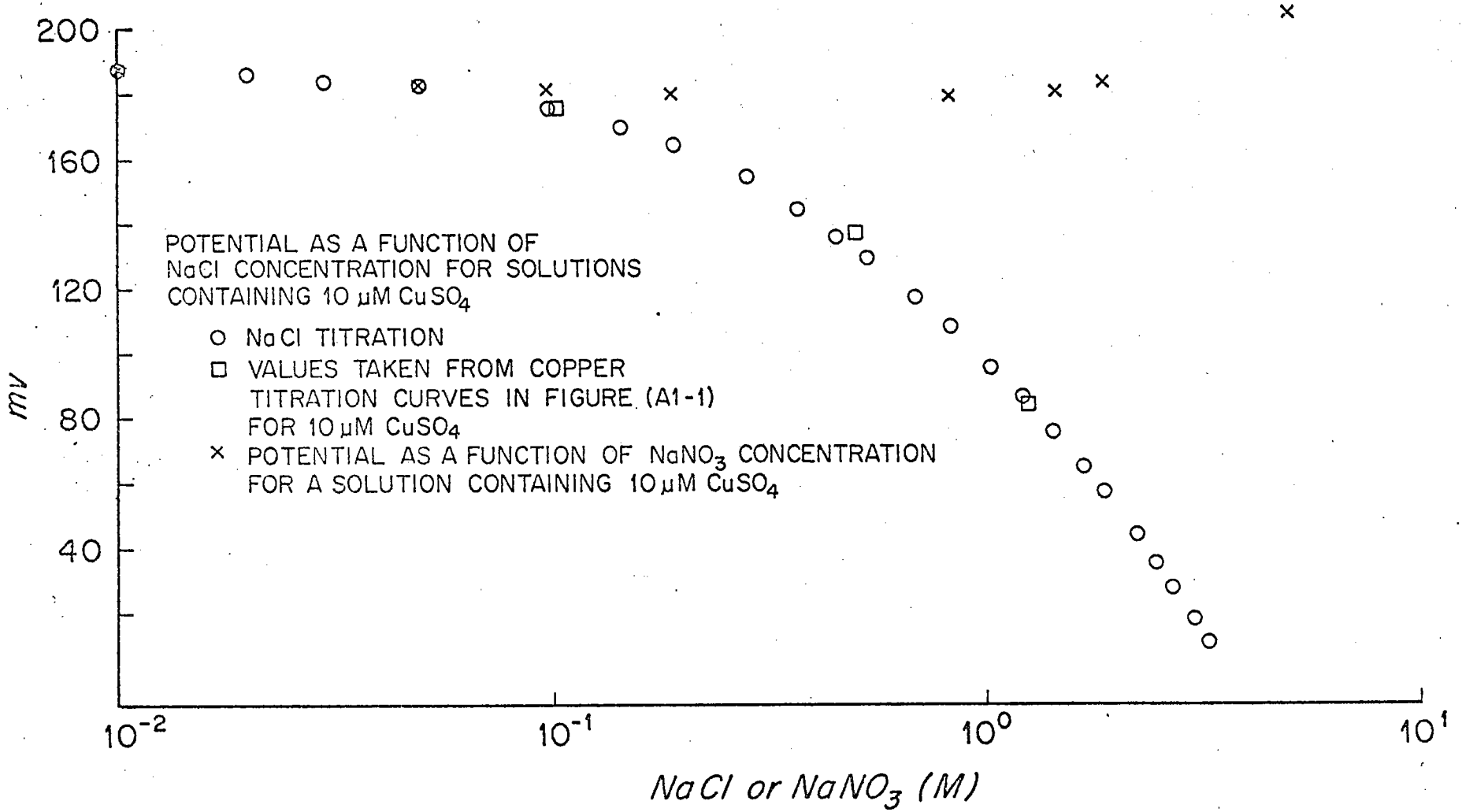


Figure (A1-2)

Effect of NaCl and $NaNO_3$ concentration on the potential of the cupric ion-selective electrode in solutions containing 10 μM $CuSO_4$ at 25°C.



seawater (which had been stored in a 5 gallon borosilicate glass carboy for several months), Sargasso seawater plus 2 μM nitrilotriacetic acid (NTA), and Sargasso seawater plus 10 mM tris at pH 8.23 and 25°C to determine the feasibility of using the cupric ion-selective electrode to detect the chelation of copper in seawater and possibly other solutions of high chloride content (Figure A1-3).

The titration curve for surface Sargasso seawater is approximately linear for the concentration range 0.2 to 5.7 μM added $CuSO_4$ with a slope of 55 mv/decade [added copper]. This slope is similar to that observed by Jasinski et. al. (1974) for surface Gulf of Mexico seawater (i.e. 52±2 mv). The increase in curve slope over the theoretical Nernst value of 29.6 is apparently mostly accounted for by chloride interference though we cannot rule out the possibility that some of the observed increase may in fact be caused by the chelation of copper by trace concentrations of organic ligands.

In contrast to the titration curve for Sargasso seawater alone, that for Sargasso seawater containing 2 μM NTA is sigmoidal in shape with a maximum slope of 129 mv/decade [added copper] and is significantly shifted to lower potentials. The titration curves for Sargasso seawater with and without NTA approach one another as the concentration of copper exceeds that of the added NTA. These results indicate that cupric ion-selective electrode potentiometric titrations can be used in a qualitative fashion to detect relatively low concentrations of strong chelators in seawater in spite of the apparent non-Nernstian response caused by the presence of chloride ion.

The titration curve for Sargasso seawater plus 10 mM tris (pH 8.23)

is also shifted to significantly lower potentials from that for Sargasso seawater alone. This shift is consistent with the expected high degree of copper chelation by added tris (see section III-C-5). In the concentration range of 2-57 μM added copper, the curve is linear with a slope of 30.2 which is close to the Nernst slope of 29.6. For this titration, the ratio between cupric ion activity and the total copper concentration should be constant since (1) copper is essentially totally complexed by tris (see section III-C-5) and (2) the total concentration of copper is in all cases less than 0.004 that of tris and thus changes in copper concentration do not appreciably alter the free ion activities of various tris species which chelate with copper. The close agreement between the observed linear slope and the Nernst slope indicates the absence, or near absence, of chloride interference for seawater in which there is a high degree of copper-tris complexation.

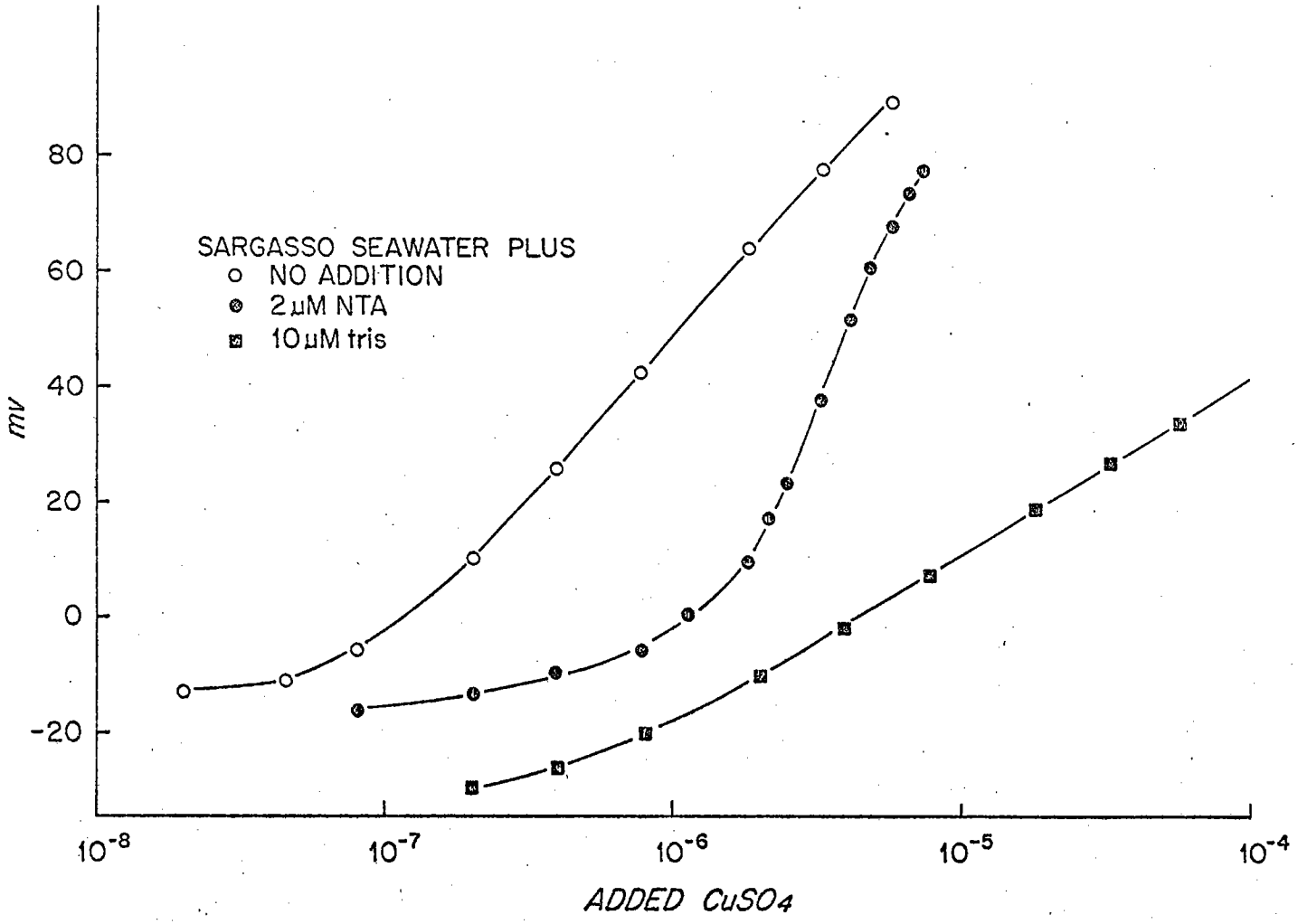
A comparison of "measured" pCu for the solution containing seawater, 10 mM tris, and 10 μM $CuSO_4$ (taken from the titration curve in Figure A1-3) shows good agreement with a calculated pCu value obtained from an apparent stability constant for copper-tris complexation measured in 0.5 M KNO_3 solutions at 20-21°C (see section III-C-5). Measured pCu values obtained from the Nernst equation using either the ideal slope (29.6) or the measured slope (30.2) are 11.08 and 10.93, respectively. The calculated pCu is 10.90 in good agreement with the above two measured values.

In conclusion, our results indicate the presence of a chloride interference which causes an increase in the cupric ion-selective electrode response slope and which occurs, in the absence of additional copper complexing agents,

at chloride concentrations above 0.05 M. This chloride interference appears to be reduced in the presence of a high degree of copper complexation. The mechanistic nature of the chloride interference is not understood at the present time.

Figure (A1-3)

Copper potentiometric titrations of surface Sargasso seawater with and without the addition of NTA or tris at pH 8.23 and 25°C.



APPENDIX 2: DETERMINATION OF STABILITY CONSTANTS FOR THE COMPLEX SPECIES $CuCO_3$, $Cu(CO_3)_2^{2-}$, and $CuOH^+$ AT 25° C

Simultaneous measurements of pCu and pH were made in $CuSO_4$-$NaHCO_3$ solutions and in a solution containing 4 μM $CuSO_4$, 20 mM KNO_3 and small amounts of KOH to determine stability constants for the complexation of copper by carbonate and hydroxide ions. These measurements were consistent with the presence of three complex species: $CuCO_3$, $Cu(CO_3)_2^{2-}$, and $CuOH^+$.

For sodium bicarbonate solutions, measurements were made at constant alkalinity, but variable P_{CO_2}. In these solutions, the P_{CO_2} and thus the pH was varied by bubbling the test solutions for short periods of time with a gas mixture containing 5% CO_2 and 95% air.

From measurements in the test solution containing 20 mM KNO_3 and 4 μM $CuSO_4$ we wished to determine copper-hydroxide complexation in the absence of carbonate complexation. To minimize dissolved CO_2, which would react with hydroxide ions to form carbonate ion, the test solution was bubbled continuously with nitrogen. The pH of this solution was varied through the addition of small quantities (some fraction of a drop) of concentrated KOH or HNO_3 solutions.

Measured pCu-pH curves for solutions containing 3 mM $NaHCO_3$-4 μM $CuSO_4$, 1 mM $NaHCO_3$-2 μM $CuSO_4$, 0.5 mM $NaHCO_3$-4 μM $CuSO_4$, 0.5 mM $NaHCO_3$-1.5 μM $CuSO_4$, and 20 mM KNO_3-4 μM $CuSO_4$ are presented in figure (A2-1).

Stability constants for copper carbonate and copper hydroxide complexes were determined from a multiple linear regression fit of experimental data to a theoretical equation derived from a mass balance equation and from equilibrium mass action expressions. The pertinent equilibrium

expressions are given in Table (A 2-1). The mass balance equation written in terms of the activities of the individual copper species is

$$Cu_T = \frac{a_{Cu^{2+}}}{\gamma_{Cu^{2+}}} + \frac{a_{CuOH^+}}{\gamma_{CuOH^+}} + \frac{a_{CuCO_3}}{\gamma_{CuCO_3}} + \frac{a_{Cu(CO_3)_2^{2-}}}{\gamma_{Cu(CO_3)_2^{2-}}} \qquad \text{(A 2-1)}$$

where Cu_T is the concentration of added copper sulfate and γ is the activity coefficient of the subscripted species. Substituting equilibrium relationships 1-3 from Table (A 2-1) into equation (A 2-6) we obtain

$$Cu_T = a_{Cu^{2+}} \left(\frac{1}{\gamma_{Cu^{2+}}} + \frac{a_{OH^-} K_{CuOH^+}}{\gamma_{CuOH^+}} + \frac{a_{CO_3^{2-}} K_{CuCO_3}}{\gamma_{CuCO_3}} + \frac{(a_{CO_3^{2-}})^2 K_{Cu(CO_3)_2^{2-}}}{\gamma_{Cu(CO_3)_2^{2-}}} \right) \qquad \text{(A 2-2)}$$

An expression for the carbonate ion activity in the sodium bicarbonate test solutions is derived from the carbonate alkalinity equation and from the hydrogen ion association equilibrium for carbonate ion (equation 4, Table A2-1). Carbonate alkalinity is defined as

$$Alk = [HCO_3^-] + 2[CO_3^{2-}] + [OH^-] - [H^+] \qquad \text{(A 2-3)}$$

For sodium bicarbonate solutions in the experimental pH range 5.8-8.4 the concentration of hydrogen and hydroxyl ions is negligible compared to that of carbonate and bicarbonate ions and therefore equation (A 2-3) becomes

$$Alk = [HCO_3^-] + 2[CO_3^{2-}] = \frac{a_{HCO_3^-}}{\gamma_{HCO_3^-}} + \frac{2\, a_{CO_3^{2-}}}{\gamma_{CO_3^{2-}}} \qquad \text{(A 2-4)}$$

From the equilibrium expression (4) in Table (A 2-1)

$$a_{HCO_3^-} = a_{H^+}\, a_{CO_3^{2-}}\, 10^{10.33} \qquad \text{(A 2-5)}$$

Combining equations (A 2-4) and (A 2-5)

$$a_{CO_3^{2-}} = \frac{Alk}{\frac{a_{H^+}\, 10^{10.33}}{\gamma_{HCO_3^-}} + \frac{2}{\gamma_{CO_3^{2-}}}} \qquad \text{(A 2-6)}$$

For $NaHCO_3$-$CuSO_4$ solutions the alkalinity is simply equal to the concentration of sodium bicarbonate. Inasmuch as the 20 mM KNO_3-4.0 μM $CuSO_4$ solution contained no added sodium bicarbonate and was bubbled with nitrogen gas to minimize dissolved CO_2, the carbonate ion activity in this solution can be considered to be essentially zero. From the acid base association equilibrium for water (equation 5, Table A 2-1)

$$a_{OH^-} = 10^{-14.00}/a_{H^+} \qquad \text{(A 2-7)}$$

Eliminating carbonate ion and hydroxide ion activities from equation (A 2-2) using equations (A 2-6) and (A 2-7) (A 2-8)

$$Cu_T = a_{Cu^{2+}}\left(\frac{1}{\gamma_{Cu^{2+}}} + \frac{(Alk)K_{CuCO_3}}{\left(\frac{a_{H^+}10^{10.33}}{\gamma_{HCO_3^-}} + \frac{2}{\gamma_{CO_3^{2-}}}\right)\gamma_{CuCO_3}} + \frac{(Alk)^2 K_{Cu(CO_3)_2^{2-}}}{\left(\frac{a_{H^+}10^{10.33}}{\gamma_{HCO_3^-}} + \frac{2}{\gamma_{CO_3^{2-}}}\right)^2 \gamma_{Cu(CO_3)_2^{2-}}} + \frac{10^{-14.00}K_{CuOH^+}}{a_{H^+}\,\gamma_{CuOH^+}}\right)$$

Values for the activity coefficients in equation (A 2-8) were calculated from the Davies modification of the Debeye-Huckel equation

$$\log = -A\, Z^2\left(\frac{\sqrt{I}}{1 + \sqrt{I}} - 0.3\, I\right) \qquad \text{(A 2-9)}$$

where Z is the ionic charge; I is the ionic strength of the medium; and A is a constant equal to 0.51 for water at 25°C. Ionic strength of the test media is calculated from the formula

$$I = 1/2 \sum_i C_i Z_i^2 \qquad \text{(A 2-10)}$$

where C_i is the molar concentration of the individual ions in solution.

Equation (A 2-8) was solved for the constants K_{CuOH^+}, K_{CuCO_3}, and $K_{Cu(CO_3)_2^{2-}}$ by multiple linear regression analysis using a Wang Model 462-1 advanced statistical calculator which is preprogrammed to carry out multiple linear regression calculations. To perform the regression, it was necessary first to transform equation (A 2-8) to a standard linear form

$$y = A_o + A_1 x_1 + a_2 x_2 \qquad \text{(A 2-11)}$$

where

$$y = (Cu_T/a_{Cu^{2+}} - 1/\gamma_{Cu^{2+}})\ \gamma_{CuOH^+}\ a_{H^+} \qquad \text{(A 2-12)}$$

$$x_1 = \frac{A\ \gamma_{CuOH^+}\ a_{H^+}}{\gamma_{CuCO_3}\left(\frac{a_{H^+}\ 10^{10.33}}{\gamma_{HCO_3^-}} + \frac{2}{\gamma_{CO_3^{2-}}}\right)} \qquad \text{(A 2-13)}$$

$$x_2 = \frac{A^2\ \gamma_{CuOH^+}\ a_{H^+}}{\gamma_{Cu(CO_3)_2^{2-}}\left(\frac{a_{H^+}\ 10^{10.33}}{\gamma_{HCO_3^-}} + \frac{2}{\gamma_{CO_3^{2-}}}\right)^2} \qquad \text{(A 2-14)}$$

$$A_o = K_{CuOH^+};\ A_1 = K_{CuCO_3};\ A_2 = K_{Cu(CO_3)_2^{2-}} \qquad \text{(A 2-15)}$$

The calculations of the variables y, x_1, and x_2 from the experimental pCu and pH values were carried out by computer.

The multiple linear regression of y as a function of x_1 and x_2 yielded a multiple correlation coefficient of 0.9992 and an F-value of 14059. Computed values for K_{CuOH^+}, K_{CuCO_3}, and $K_{Cu(CO_3)_2^{2-}}$ (including standard errors) are respectively $10^{6.59\pm0.03}$, $10^{6.80\pm0.01}$, and $10^{10.40\pm0.04}$. These values agree favorably with stability constants reported in Sillen and Martel (1964) (Table A 2-2).

Table (A 2-1)

Equilibria at 25°C and pH 5.8 to 8.4 for the experimental test solutions:

3.0 mM $NaHCO_3$ plus 4.0 μM $CuSO_4$

1.0 mM $NaHCO_3$ plus 2.0 μM $CuSO_4$

0.5 mM $NaHCO_3$ plus 1.5 μM $CuSO_4$

0.5 mM $NaHCO_3$ plus 4.0 μM $CuSO_4$

20 mM KNO_3 plus 4.0 μM $CuSO_4$

Reaction	Equilibrium Equation	Log K
(1) $Cu^{2+} + OH^- = CuOH^+$	$\frac{a_{CuOH^+}}{a_{Cu^{2+}}\, a_{OH^-}} = K_{CuOH^+}$	To be determined
(2) $Cu^{2+} + CO_3^{2-} = CuCO_3$	$\frac{a_{CuCO_3}}{a_{Cu^{2+}}\quad a_{CO_3^{2-}}} = K_{CuCO_3}$	To be determined
(3) $Cu^{2+} + 2\ CO_3^{2-} = Cu(CO_3)_2^{2-}$	$\frac{a_{Cu(CO_3)_2^{2-}}}{a_{Cu^{2+}}(a_{CO_3^{2-}})^2} = K_{Cu(CO_3)_2^{2-}}$	To be determined
(4) $H^+ + CO_3^{2-} = HCO_3^-$	$\frac{a_{HCO_3^-}}{a_{H^+}\, a_{CO_3^{2-}}} = K_{HCO_3^-}$	10.33*
(5) $H^+ + OH^- = H_2O$	$a_{H^+}\, a_{OH^-} = K_{H_2O}$	-14.0*

*Constants taken from Schlindler (1967).

Figure (A-2-1)

Measured relationships between pCu and pH for solutions of the following compositions:

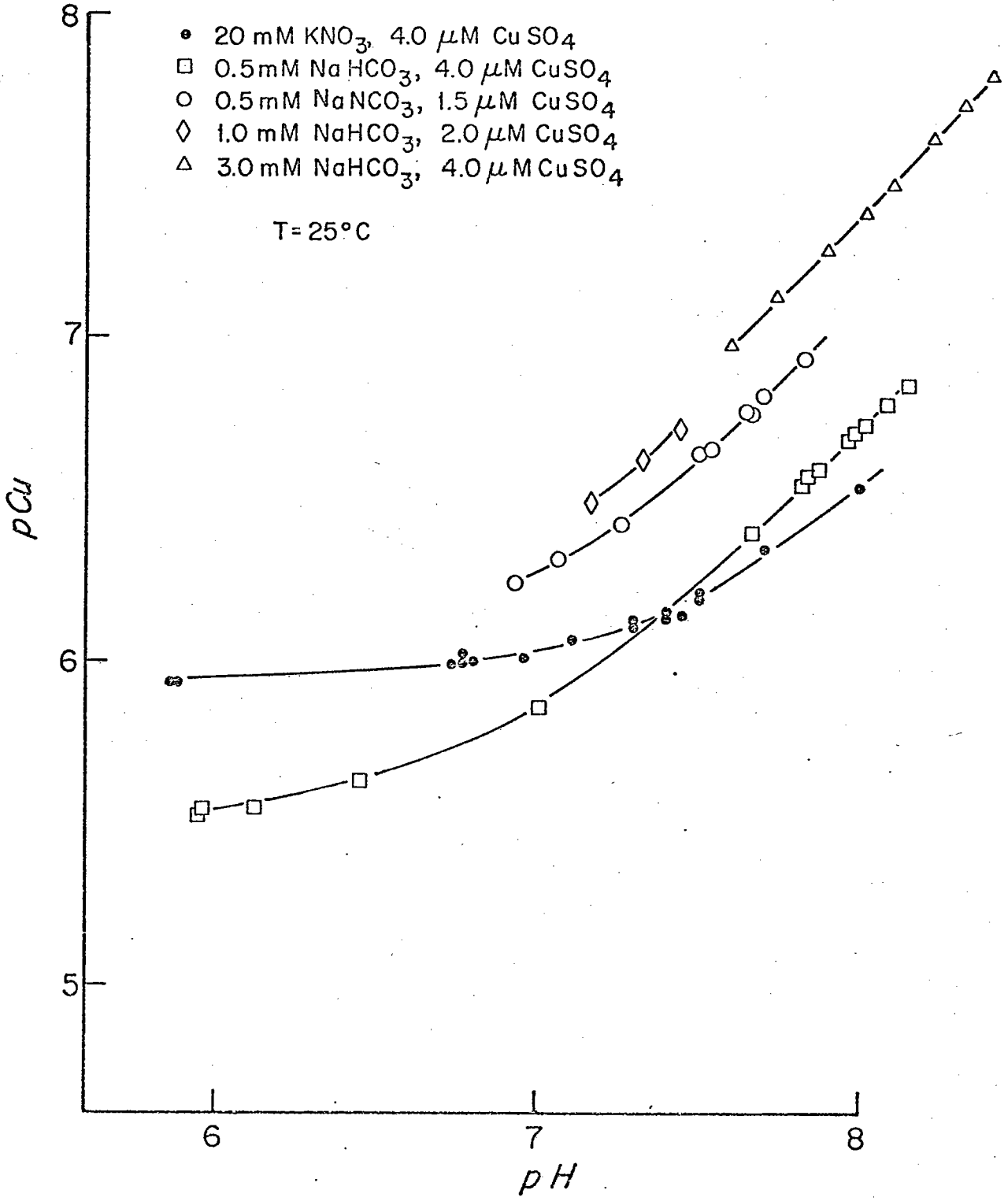


Table (A 2-2)

Comparison of association constants from this work with values reported in Sillen and Martel (1964) for the copper complex species $CuCO_3$, $Cu(CO_3)_2^{2-}$, and $CuOH^+$.

Complex	Temperature (°C)	Medium	Log K	Reference
$CuCO_3^o$	25	0 corr.**	6.80 ± 0.01	This work
	25	0 corr.	6.77	SM*
	25	0 corr.	6.34	SM
$Cu(CO_3)_2^{2-}$	25	0 corr.	10.40 ± 0.04	This work
	25	0 corr.	10.01	SM
	18	1.7 KNO_3	8.6	SM
$CuOH^+$	25	0 corr.	6.59 ± 0.03	This work
	100	dilute	7.77	SM
	100	dilute	6.60	SM
	20	$CuAc_2$ (variable)	7.22	SM
	18	0.02 Cu	7.53	SM
	25	0 corr.	6.47	SM
	25	0 corr.	6.27	SM
	?	variable	7.5	SM
	18	0	6.03	SM
	18	0	6.0	SM
	30	0.1 KCl	7.2	SM
	25	0 corr.	6.66	SM

*Sillen and Martel (1964)

**Corrected to zero ionic strength.

APPENDIX 3: CALCULATIONS OF COPPER-TRIS COMPLEXATION FOR THE SOLUTION 50μM $CuSO_4$-(1-30) mM TRIS - 0.5 M KNO_3 AT 21°C

Consider the following four reactions and mixed associated equilibrium expressions

$$Cu^{2+} + 2\ Htris = Cu(Htris)_2^{2+} \quad \text{(A 3-1)}$$

$$\frac{[Cu(Htris)_2^{2+}]}{a_{Cu^{2+}}\,[Htris]^2} = K_{Cu(Htris)} \quad \text{(A 3-2)}$$

$$Cu(Htris)_2^{2+} = Cu(tris)(Htris)^{+} + H^{+} \quad \text{(A 3-3)}$$

$$\frac{[Cu(Htris)(tris)^{+}]\ a_{H^+}}{[Cu(Htris)_2^{2+}]} = K_1^* \quad \text{(A 3-4)}$$

$$Cu(tris)(Htris)^{+} = Cu(tris)_2 + H^{+} \quad \text{(A 3-5)}$$

$$\frac{[Cu(tris)_2]\ a_{H^+}}{[Cu(Htris)(tris)^{+}]} = K_2^* \quad \text{(A 3-6)}$$

$$Htris + H^{+} = H_2tris^{+} \quad \text{(A 3-7)}$$

$$\frac{[H_2tris^{+}]}{[Htris]\ a_{H^+}} = K_{H_2tris} \quad \text{(A 3-8)}$$

where Htris is the neutrally charged tris species with the amine group in its basic form and $tris^-$ represents a tris species with a hydrogen ion removed from

one of the three hydroxyl groups. The square brackets indicate concentrations of enclosed species. Reactions (A 3-1, 3 and 5) are taken from Hall et al. (1962).

A mass balance equation for copper may be written

$$Cu_T = [Cu(Htris)_2^{2+}] + [Cu(Htris)(tris)^+] + [Cu(tris)_2] \qquad (A\ 3\text{-}9)$$

ignoring the inorganic species Cu^{2+}, $CuCO_3^o$, and $CuOH^+$ whose concentrations are negligible compared to that of tris-copper complexes.

From equations (A 3-2, A 3-4, and A 3-6)

$$[Cu(Htris)_2^{2+}] = a_{Cu^{2+}} [Htris]^2 K_{Cu(Htris)_2} \qquad (A\ 3\text{-}10)$$

$$[Cu(Htris)(tris)^+] = \frac{[Cu(Htris)_2^{2+}] K_1^*}{a_{H^+}} \qquad (A\ 3\text{-}11)$$

$$= \frac{a_{Cu^{2+}} [Htris]^2 K_{Cu(Htris)_2} K_1^*}{a_{H^+}} \qquad (A\ 3\text{-}12)$$

$$[Cu(tris)_2] = \frac{[Cu(Htris)(tris)^+] K_2^*}{a_{H^+}} \qquad (A\ 3\text{-}13)$$

$$[Cu(tris)_2] = \frac{a_{Cu^{2+}} [Htris]^2 K_{Cu(Htris)_2} K_1^* K_2^*}{(a_{H^+})^2} \qquad (A\ 3\text{-}14)$$

Solving the mass balance equation using equations (A 3-10, A 3-12, and A 3-14)

$$Cu_T = a_{Cu^{2+}} [Htris]^2 K_{Cu(Htris)_2} (1 + K_1^*/a_{H^+} + K_1^* K_2^*/(a_{H^+})^2) \qquad (A\ 3\text{-}15)$$

We now need to express [Htris] in terms of the total concentration of tris not complexed by copper, i.e. [tris*]. From the definition

$$[\text{tris}^*] = [H_2\text{tris}^+] + [\text{Htris}] \qquad \text{(A 3-16)}$$

and using equations (A 3-16 and A 3-8)

$$\frac{[\text{Htris}]}{[\text{tris}^*]} = \frac{[\text{Htris}]}{[H_2\text{tris}^+] + [\text{Htris}]} = \frac{[\text{Htris}]}{a_{H^+}\,[\text{Htris}]\,K_{H_2\text{tris}} + [\text{Htris}]} \qquad \text{(A 3-17)}$$

Thus

$$[\text{Htris}] = \frac{[\text{tris}^*]}{1 + a_{H^+}\,K_{H_2\text{tris}}} \qquad \text{(A 3-18)}$$

Combining equations (A 3-15) and (A 3-18)

$$Cu_T = \frac{a_{Cu^{2+}}\,[\text{tris}^*]^2\,K_{Cu(\text{Htris})_2}\,(1 + K_1^*/a_{H^+} + K_1^*\,K_2^*/(a_{H^+})^2)}{(1 + a_{H^+}\,K_{H_2\text{tris}})^2} \qquad \text{(A 3-19)}$$

Taking the log of the above expression and rearranging terms

(A 3-20)

$$pCu = 2\log[\text{tris}^*] - \log Cu_T + \log \frac{(1 + K_1^*/a_{H^+} + K_1^*\,K_2^*/(a_{H^+})^2)\,K_{Cu(\text{Htris})_2}}{(1 + a_{H^+}\,K_{H_2\text{tris}})^2}$$

Equation (A 3-20) agrees with experimentally measured equation (3-30) with

$$\log \frac{(1 + K_1^*/a_{H^+} + K_1^*\,K_2^*/(a_{H^+})^2)\,K_{Cu(\text{Htris})_2}}{(1 + a_{H^+}\,K_{H_2\text{tris}})^2} = 9.52 \qquad \text{(A 3-21)}$$

at pH = 8.10, T = 21°C and ionic strength = 0.5.

APPENDIX 4: CALCULATION OF $[CuEDTA^{2-}]$

4-A. Calculation of $[CuEDTA^{2-}]$ in Seawater Culture Media

For 1.0 μM EDTA in tris-$CuSO_4$-seawater culture media, EDTA should be principally complexed to Cu^{2+}, Ca^{2+}, and Mg^{2+}. Because of the insolubility of $Fe(OH)_3$ at pH values around 8.0 we can ignore EDTA compelxation with iron (Stumm and Morgan, 1970). Also, because of the large degree of complexation to divalent metals acidic EDTA species - $HEDTA^{3-}$, H_2EDTA^{2-}, H_3EDTA^{-}, and H_4EDTA may similarly be ignored. Therefore, the total concentration of EDTA in solution is equal to the sum of the concentrations of the individual divalent metal complexes:

$$EDTA_T = [CuEDTA^{2-}] + [CaEDTA^{2-}] + [MgEDTA^{2-}] \qquad (A\ 4\text{-}1)$$

The fraction of the total concentration of EDTA present as $CuEDTA^{2-}$ is

$$\frac{[CuEDTA^{2-}]}{EDTA_T} = \frac{{}^{a}CuEDTA/\gamma CuEDTA}{{}^{a}CuEDTA/\gamma CuEDTA + {}^{a}CaEDTA/\gamma CaEDTA + {}^{a}MgEDTA/\gamma MgEDTA} \qquad (A\ 4\text{-}2)$$

where the activity of the individual complex species divided by the activity coefficient (γ) is equal to the molar concentration. We may assume that the activity coefficients for the various divalent metal-EDTA complexes are approximately equal and therefore can be cancelled out of equation (A 4-2):

$$[CuEDTA^{2-}] = \frac{EDTA_T\ {}^{a}CuEDTA}{{}^{a}CuEDTA + {}^{a}CaEDTA + {}^{a}MgEDTA} \qquad (A\ 4\text{-}3)$$

To solve equation (A 4-3) we need equilibrium expressions for the three divalent metal-EDTA complexes:

$$\frac{[CuEDTA^{2-}]}{[Cu^{2+}][EDTA^{4-}]} = 10^{18.79} \qquad \text{(A 4-4)}$$

$$\frac{[CaEDTA^{2-}]}{[Ca^{2+}][EDTA^{4-}]} = 10^{10.59} \qquad \text{(A 4-5)}$$

$$\frac{[MgEDTA]}{[Mg^{2+}][EDTA^{4-}]} = 10^{8.69} \qquad \text{(A 4-6)}$$

where $10^{18.79}$, $10^{10.59}$, and $10^{8.69}$ are apparent association constants for the ionic medium 0.1 M KNO_3 (I = 0.1) at a temperature of 20°C (Sillen and Martel, 1964). Equations (A 4-4 through A 4-6) can be expressed in terms of activities by dividing the concentrations of the individual species by appropriate activity coefficients. For example, equation (A 4-4) may be written as

$$\frac{a_{CuEDTA}}{a_{Cu}\, a_{EDTA}} \cdot \frac{\gamma_{Cu(0.1)}\, \gamma_{EDTA(0.1)}}{\gamma_{CuEDTA(0.1)}} = 10^{18.79} \qquad \text{(A 4-7)}$$

where the subscript (0.1) denotes the ionic strength at which the activity coefficients apply. Rearranging equation (A 4-7)

$$a_{CuEDTA} = \frac{a_{Cu}\, a_{EDTA}\, \gamma_{CuEDTA(0.1)}\, 10^{18.79}}{\gamma_{Cu(0.1)}\, \gamma_{EDTA(0.1)}} \qquad \text{(A 4-8)}$$

Substitution equation (A 4-8) and similar equations for the activities of $CaEDTA^{2-}$ and $MgEDTA^{2-}$ into equation (A 4-3)

$$[CuEDTA^{2-}] = \frac{\dfrac{EDTA_T a_{Cu} a_{EDTA} \gamma_{CuEDTA(0.1)} 10^{18.79}}{\gamma_{Cu(0.1)} \gamma_{EDTA(0.1)}}}{\dfrac{a_{Cu} a_{EDTA} \gamma_{CuEDTA(0.1)} 10^{18.79}}{\gamma_{Cu(0.1)} \gamma_{EDTA(0.1)}} + \dfrac{a_{Ca} a_{EDTA} \gamma_{CaEDTA(0.1)} 10^{10.59}}{\gamma_{Ca(0.1)} \gamma_{EDTA(0.1)}} + \dfrac{a_{Mg} a_{EDTA} \gamma_{MgEDTA(.1)}}{\gamma_{Mg(0.1)} \gamma_{EDTA(0.} 1}} \qquad \text{(A 4-9)}$$

Since the ionic radii of the three divalent metals are similar (Stumm and Morgan, 1970)

$$\gamma_{Cu(0.1)} \simeq \gamma_{Ca(0.1)} \simeq \gamma_{Mg(0.1)} \qquad \text{(A 4-10)}$$

and likewise

$$\gamma_{CuEDTA(0.1)} \simeq \gamma_{CaEDTA(0.1)} \simeq \gamma_{MgEDTA(0.1)} \qquad \text{(A 4-11)}$$

Therefore the term $\dfrac{a_{EDTA}\ \gamma_{MeEDTA(0.1)}}{\gamma_{Me(0.1)}\ \gamma_{EDTA(0.1)}}$ (A 4-12)

(where Me represents Cu, Mg, and Ca) can be factored out of equation (A 4-9) giving

$$[CuEDTA^{2-}] = \frac{EDTA_T\ a_{Cu}\ 10^{18.79}}{a_{Cu}\ 10^{18.79} + a_{Ca}\ 10^{10.59} + a_{Mg}\ 10^{8.69}} \qquad \text{(A 4-13)}$$

where $EDTA_T = 10^{-6.0}$ M.

The activities of calcium and magnesium in seawater were calculated from total ion activity coefficients for seawater γ^*_{Ca} and γ^*_{Mg}: $\gamma^*_{Ca} = 0.21 \pm 0.01$ (Leyendekkers, 1973; Whitfield, 1973; Thompson and Ross, 1966; Berner, 1965) and $\gamma^*_{Mg} = 0.26 \pm 0.01$ (Van Breeman, 1973; Jones and Truesdell, 1973; Berner 1971; Thompson, 1966).

$$a_{Ca} = \gamma^*_{Ca}\, Ca_T = 0.21 \times 0.010 = 0.0021 \qquad \text{(A 4-14)}$$

$$a_{Mg} = \gamma^*_{Mg}\, Mg_T = 0.26 \times 0.056 = 0.014 \qquad \text{(A 4-15)}$$

Values for the total concentrations of Ca and Mg in seawater were taken from Goldberg (1965).

Combining equations (A 4-13 through A 4-15)

$$[CuEDTA^{2-}] = \frac{EDTA_T\, a_{Cu}\, 10^{18.79}}{a_{Cu}\, 10^{18.79} + 10^{7.95}} \qquad \text{(A 4-16)}$$

4-B. Calculation of Copper Activity in the Medium DSW-1.0 μM $CuSO_4$-2.0 μM EDTA

In this medium EDTA is principally complexed to Ca^{2+}, Mn^{2+}, and Cu^{2+} whose respective total concentrations are $10^{-3.0}$, $10^{-5.0}$, and $10^{-6.0}$ M. From similar computations to those used to calculate $CuEDTA^{2-}$ in seawater-$CuSO_4$- tris media, the following equation was derived for copper-EDTA complexation in the DSW-1.0μM EDTA medium:

$$[CuEDTA^{2-}] = \frac{EDTA_T \, a_{Cu} \, 10^{18.79}}{a_{Cu}10^{18.79} + a_{Ca}10^{10.59} + a_{Mn}10^{13.58}} \quad \text{(A 4-17)}$$

where $10^{13.58}$ is the apparent association constant for $MnEDTA^{2-}$ at 20°C and an ionic strength of 0.1 (Sillen and Martell, 1964). Essentially, all of the copper in solution should be complexed by EDTA and thus

$$[CuEDTA^{2-}] = Cu_T = 1.0 \times 10^{-6} \text{ M.} \quad \text{(A 4-18)}$$

To solve equation (A 4-17) we also need values for the activities of Mn^{2+} and Ca^{2+}. If we assume negligible inorganic complexation of these two metals then

$$a_{Ca} = \gamma_{Ca}[Ca^{2+}] \simeq \gamma_{Ca} \, Ca_T \quad \text{(A 4-19)}$$

$$a_{Mn} = \gamma_{Mn}[Mn^{2+}] \quad \text{(A 4-20)}$$

where

$$[Mn^{2+}] \simeq Mn_T - [MnEDTA^{2-}] \quad \text{(A 4-21)}$$

Using the Davies modification of the Debeye-Huckel equation (equation A 2-21,

Appendix 2) at a calculated ionic strength of 0.07 (equation A 2-9), Appendix 2), the activity coefficient both for calcium and manganese ions is 0.45. Solving equations (A 4-17 to A 4-21), pCu is calculated to be 10.55. In the experimental pH range of 8-9, pCu is independent of pH.