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THE DIRECT EXAMINATION OF BIOLOGICALLY ACTIVE CU IN SEAWATER

by

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B.Sc., University of Victoria, Victoria, 1977

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY
in
THE FACULTY OF GRADUATE STUDIES
(Departments of Oceanography And Zoology)

We accept this thesis as conforming to the
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THE UNIVERSITY OF BRITISH COLUMBIA
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ABSTRACT

An analytical technique for the differentiation of biologically active copper (Cu) in seawater was developed. The procedure involves passing a seawater sample through an ion exchange resin of the sulponate type until complete breakthrough of metal ion is achieved. The sorbed Cu is then eluted and its total concentration is determined by anodic stripping voltammetry. Comparison with Cu adsorption from standard seawater samples of similar composition, pH, and ionic strength yields a Cu equivalent measurement that is related to the free cupric ion activity of the sample. Since the cupric ion is believed to be the toxic form of the metal, the Cu equivalent measurement can be related to the biologically active fraction of Cu.

The measurement of biologically active Cu by the resin technique was verified by comparing the analytical results with results from phytoplankton bioassays. Tests were first conducted in artificial seawater that had its chemistry well defined and where model organic ligand (EDTA, NTA, histidine and glutamic acid) were used to control the speciation of the metal. In the experiments using the organic ligands EDTA, NTA, or glutamic acid added to Cu spiked artificial seawater, a strong relationship between the Cu equivalent values and growth rates of the bioassay organism was found ($r=0.92$). However, in experiments with histidine, this relationship was much weaker and was attributed to the adsorption of positively charged Cu-histidine complexes onto the resin. The adsorption of these

complexes results in overestimating the amount of biologically active Cu present in the sample. The few studies on the electrochemical nature of organic complexing agents in seawater suggests, however, that most are negatively charged. Thus the technique would be suitable in many seawater systems.

The analytical and bioassay techniques were then applied to natural seawater samples collected from five depths in a local fjord. A discrepancy was found between some of the bioassay and resin test results. However, the discrepancy was attributed to a physiological Cu-Mn interaction in the bioassay organism and not to a problem with the resin technique.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ACKNOWLEDGEMENTS

I would sincerely like to thank my supervisor, Dr. A.G. Lewis, for his suggestions, advice and continued assistance throughout my studies. My deep appreciation is extended to Dr. E.V. Grill for his input and patience with my work.

I am grateful to my research committee members, Drs. Hall, Thompson, Gosline and Fletcher for their advice and suggestions for improving the manuscript. I wish to acknowledge my fellow graduate students, particularly Mark Wells, for their support and assistance.

Finally, I would especially like to thank my mother and father for their moral support throughout my education and Robin for her adherence.

I. INTRODUCTION

Copper (Cu) is an environmental constituent of considerable importance. Not only is it necessary for the proper functioning of many physiological processes in marine phytoplankton, it has also been found to be toxic to many species at the concentrations normally encountered in coastal seawater. The forms of Cu which can be utilized by phytoplankton are not definitely known but there is considerable evidence to suggest that its bioavailability is controlled by the activity of the free cupric ion and that both the inorganic and organic complexing agents that alter the ionic activity also affect the metal's biological availability. This has generated considerable interest in the question of the complexation of Cu by natural organics and the importance of this process in detoxifying heavy metals in natural systems. Thus the speciation of the metal, which is controlled by water chemistry, must be considered when assessing potential uptake and toxicity. Unfortunately, there are presently no analytical methods available that have demonstrated an ability to measure biologically active Cu in seawater and thus its environmental impact cannot be readily assessed except by tedious bioassay techniques. The major goal of this thesis was to develop a rapid analytical procedure that would be capable of estimating the concentration of biologically active Cu in natural seawater. Specifically, the hypothesis tested was that the response of a marine diatom to Cu was predictable from the amount of Cu adsorbed by a strongly acidic cation-exchange resin.

The effect of synthetic and natural chelators in reducing the toxicity of Cu to phytoplankton has been well documented (e.g., Spencer, 1957; Fitzgeral and Faust, 1963; Erickson et al., 1970; Morris and Russell, 1973; McKnight and Morel, 1980; Canterford and Canterford, 1980). Steeman Nielsen and Wium-Andersen (1970) suggested that the effect of organic chelators in stimulating the growth of algae, as was observed by Johnston (1963, 1964) and Barber and Ryther (1969), was due to the complexation of the cupric ion (Cu^{2+}) although such stimulation has sometimes been attributed to an increase in availability of trace metals (Johnston, 1964). Sunda and Guillard (1976) have provided direct evidence that correlates the growth of phytoplankton with the cupric ion activity (activity= concentration x activity coefficient) of the medium. Using an estuarine diatom and green alga in seawater in which the trace metals were chelated with trishydroxymethylaminomethane (TRIS), they demonstrated that growth rate inhibition and Cu content of cells was not related to the total Cu concentration but to the activity of the unchelated cupric ion. Since the chemistry of the medium was well defined, the cupric ion activity could be calculated by a computer equilibrium model. A similar study conducted by Anderson and Morel (1978) with the dinoflagellate Gonyaulax tamarensis demonstrated that the short term Cu sensitivity of the organism was a unique function of the cupric ion activity.

Davey et al. (1973) presented plots of growth inhibition of the diatom Thalassiosira pseudonana vs. Cu concentration in

media containing ethylenediaminetetraacetic acid (EDTA) or histidine (HIS), and found that the plots resembled potentiometric titration curves; i.e., a sharp inflection in the growth curve occurred at the point where the Cu concentration exceeded that of the chelator. A chemical speciation model was applied to Davey's results by Jackson and Morgan (1978) to compute the concentration of free cupric ion in their solutions. It was concluded that the free cupric ion was the toxic form because, even with different chelators present, identical calculated concentrations of cupric ion corresponded to identical growth rates.

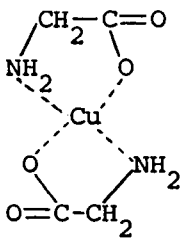
The studies above used synthetic chelators to control the concentration of the cupric ion in culture media. In applying these results to the natural environment it would seem reasonable to assume that phytoplankton will respond in the same manner to Cu chelated by natural organic matter. Sunda and Lewis (1978) studied the relationship between binding of Cu by organic matter present in river water and the toxicity of Cu to the algal species Monochrysis lutheri. Filtered river water containing high concentrations of organic matter was added in different proportions to their culture media to vary the level of Cu complexation. They found that an increase in the concentration of natural ligands was associated with a decrease in toxicity and that this decrease could be related to the cupric ion activity of the medium as was measured by an ion-selective electrode.

However, it may not only be the cupric ion that is toxic.

In studies with algae in freshwater, Wagemann and Barica (1979) found that the ability of Cu to act as an algicide was best explained by considering the toxic fraction to be comprised of Cu^{2+}, $CuOH^{+}$ and $Cu(OH)_2$. Unfortunately, data in the literature are too sparse to prove or disprove the toxicity of $CuOH^{+}$ or $Cu(OH)_2$. In the studies that have demonstrated the cupric ion to be the toxic form in seawater, the toxicity of the hydroxide species has not been considered.

The question of the toxic form of Cu cannot be examined without first reviewing the chemical forms of Cu that have been predicted to exist in seawater. Copper can theoretically appear in three phases which may or may not be separable; i.e., particulate, colloidal and soluble. In addition, these may be subdivided into organic and inorganic fractions (Fig. 1).

Due to interactions between the inorganic constituents in seawater, Cu will occur not only as the free hydrated ion but also in complexes formed with the anionic species such as OH^{-}, Cl^{-}, CO_3^{2-} and SO_4^{2-}. The concentration of these complexes, which includes species such as $[Cu^{2+}(H_2O)_6]^{2+}$, $CuOH^{+}$, $Cu(OH)_2$, $CuCO_3$, $Cu(CO_3)_2^{2-}$, $CuCl^{+}$, $CuCl_2$, and $CuSO_4$, has been estimated using chemical equilibrium models (Dyrssen and Wedborg, 1974; Stumm and Brauner, 1975). The predicted values differ considerably, however. For example, Zirino and Yamamoto (1972) predicted that, for seawater of 35 ppt salinity, 25°C and a pH of 8.0, the percentages of the major species were 90% $Cu(OH)_2$, 7.7% $CuCO_3$, and 1.0% $CuOH^{+}$. Morgan and Sibley (1975), on the other hand, predicted 95.5% $CuCl^{+}$, 3.5% $CuHCO_3^{+}$, and 3.5% $CuCO_2$

Simple hydrated metal	Simple inorganic complexes	Simple organic complexes	Stable organic complexes	Adsorbed onto inorganic and organic colloids	Particulate
$Cu(H_2O)_6^{2+}$	$CuOH^+$ $Cu(OH)_2$ $CuCO_3^0$ $Cu(CO_3)_2^{2-}$ $CuCl^+$ $CuSO_4$	Cu-SR* Cu-OOCR Cu-histidine		Cu^{2+}-Fe_2O_3 Cu^{2+} -MnO_2 Cu^{2+} -Humate	Remains of living organisms Organic particles Inorganic particles
Approximate diameter (nm)					
0.8	1	1-10	1-10	10-500	450

*R= Organic molecule

Figure 1. Possible chemical forms of Cu in seawater.

to be the percentage distribution. More recent calculations by a computer equilibrium model have predicted the major species to be $CuCO_3$ (Westall et al., 1976) The wide variation in these calculations (see Nordstrom et al. (1979) for a comparison of thirteen computer models) is the result of using different published values of the stability constants, particularly those of the hydroxo and carbonato species (Leckie and Davis, 1979).

In addition to inorganic complexation, the presence of Cu-organic complexes in seawater is highly likely because Cu has a great affinity for amino, imino, sulfhydryl, carboxyl and hydroxyl groups and thus bonds readily to organic molecules. But, as Pocklington (1977) pointed out, no one has yet isolated and characterized a Cu-organic complex from seawater. The presence of Cu associated with organics has been inferred from measurements of Cu before and after samples have undergone treatments designed to isolate or destroy the organic fraction of the sample. Slowey et al. (1967) found that when samples of filtered seawater were extracted with small portions of chloroform, 10-60% of the total Cu was transferred into the organic phase. According to Corcoran and Alexander (1964), Cu exists in seawater primarily in a soluble nonionic state and probably as an organic complex. They base their conclusion on ionic Cu analysis before and after digestion with perchloric acid; after digestion ionic Cu levels were greatly increased. In samples collected off the coast of Southern California by Williams (1969), from 5 to 28% of the total Cu was organically associated, based upon oxidation by ultraviolet (UV)

irradiation. Organically associated Cu has also been inferred from various toxicity and growth experiments (Johnston, 1964; Barber and Ryther, 1969; Steeman Nielsen and Wium Andersen, 1970; Lewis et al., 1973). A review of metallo-organic interactions in natural waters has been written by Mantoura (1981).

Copper may also be present adsorbed to colloidal or macroscopic sized particulate matter. Inorganic solids such as hydrous oxides of Mn and Fe have been shown to adsorb Cu (Vuceta and Morgan, 1978; Swallow et al., 1980) and such materials have been suggested to act as a possible environmental sink for heavy metals such as Cu (Jenne, 1968; Sholkovitz, 1978). A review on Cu in oceans and estuaries has been written by Lewis and Cave (1982).

II. ANALYTICAL METHODS FOR STUDYING CU SPECIATION

A. ELECTROCHEMICAL METHODS

There are many instrumental techniques capable of directly measuring dissolved metal levels in solution without initial chemical separation and/or preconcentration (e.g., neutron activation, atomic absorption, X-ray fluorescence). However, such methods give only the total metal concentration and provide no information on the particular forms of metal present. Voltammetry and specific ion potentiometry, by contrast, are techniques whose response is dependent on trace metal speciation although their application to seawater is fraught with problems.

1. Specific Ion Potentiometry

Ion-selective electrodes (ISE) develop a potential which is dependent solely on ionic activity. Thus, in a sense, they are capable of directly distinquishing between the free and bound metal ions. The cupric ion electrode has been used to determine the activity of the cupric ion in natural waters (Smith and Manahan, 1973; Stella and Ganzerii-Valentini, 1979) and seawater samples (Javinski et al, 1974; Williams and Baldwin, 1976). However, the use of these electrodes in seawater has been critised because of chloride interference (Midgley, 1976). Oglesby et al. (1977), using different background electrolytes, found that the response of the electrode was non-Nernstian in a chloride medium while the response was Nernstian in all the

other electrolytes studied. According to Westall et al. (1979), Cu(II) from the bulk solution is reduced at the electrode surface to Cu(I) which is stabilized by chloride complexation with subsequent oxidation of the mixed sulphide electrode material. This results in non-Nernstian and unpredictable behaviour. The behavior of the electrode has been suggested to resemble that of a polarized sensor rather that a standard ISE (Zirino and Seligman, 1981). Thus it is not possible to make absolute measurements of cupric ion in seawater with the cupric ion electrode although measurements of relative differences in the cupric ion activity of seawater samples has been attempted (Javinski et al, 1974; Williams and Baldwin, 1976).

2. Voltammetry

Voltammetric techniques that have been developed for the determination of metals in natural waters include pulse polarography (Fonds et al., 1964), differential pulse polarography (Barker and Gardiner, 1960), anodic stripping voltammetry (Christian, 1969) as well as many others. Anodic stripping voltammetry, because of its ability to preconcentrate the metal, is the most sensitive technique and is capable of directly measuring Cu concentrations at the levels encountered in natural seawater. There has been increasing use of this technique due to its ability to simultaneously determine several elements, nondestructively, at sub-ug l^{-1} concentrations and with relatively inexpensive instrumentation. Because of the

potential of ASV for studying Cu speciation and because it was used in the present study, a detailed description of the technique is given.

(a) Anodic stripping voltammetry

Anodic stripping voltammetry (ASV) is a two-step technique. The first step involves the cathodic deposition of a portion of the dissolved metal ion onto a suitable electrode. This working electrode is either a hanging mercury drop electrode (HMDE) or a thin mercury film electrode (TFE) deposited onto a substrate such as carbon (glassy carbon, wax impregnated graphite, carbon paste; Elving et al., 1964), Pt or Ag. This step preconcentrates the metal into a small volume. In the second step the metal is stripped out of the amalgam by the application of an anodic scan. Current and potential measurements are made during the second step and the position and height of the stripping peaks are characteristic of the type and concentration of metal ions originally in solution (Siegerman and O'Dom, 1972). The detection limit is dependent on the deposition time, the stirring rate and physical parameters of cell design used during the deposition step.

To increase the sensitivity of ASV a differential pulse wave-form can be applied during the stripping step (DPASV). In DPASV, the metals in the amalgam are reoxidized using a slow linear anodic potential ramp upon which are superimposed fixed height voltage pulses. Measurement of the resulting current increase is delayed until the latter stages of the pulse-life

when the capacitive component has largely decayed thereby increasing the signal to noise ratio. In the polarographic analyzer used in the present study, the pulse duration was 56.7 msec with the first 40 msec being used to allow the capacitive current to decay to a negligible value while the last 16.7 msec of the pulse essentially was used to measure the faradaic current. Current samples are taken just prior to the pulse application and again just before the pulse is completed. It is the difference between the two currents that is displayed as a peak shaped read-out. The overall sensitivity of the analysis is increased one to two orders of magnitude over conventional ASV by using the differential pulse wave-form (Siegerman and O'Dom, 1972).

(b) Chemical forms measured by ASV

ASV has been used extensively to study trace metals in natural waters (e.g., Allen et al., 1970; Abdullah and Royle, 1972; Duinker and Kramer, 1977; Florence and Batley, 1977a,b; Sugai and Healy, 1978; Nygaard and Hill, 1979; Figura and McDuffie, 1979). The technique is quite unique in that it can discriminate between 'labile' and 'bound' metal species (Chau, 1973; Chau et al., 1974a,b; Lazar et al., 1981; Plavšić et al., 1982); the labile fraction is measureable by ASV and this fraction includes the free hydrated ion, inorganic complexes such as chloro, hydroxo and carbonato species, and organic complexes that dissociate at the electrode in the time scale of the measurement. The experimental approach taken is to measure

the metal at the natural pH of the sample or after making it acidic (ca. pH 5) with CO_2 (Nygaard and Hill, 1979) or acetate buffer (Abdullah et al., 1976), and then remeasure it after acid digestion of the solution. The two measurements are then used to determine labile and bound metal (Allen et al., 1970; Gardiner and Stiff, 1975).

(c) Measurement of biologically active Cu

Since ASV is sensitive to the chemical form of the metal in solution, it has been suggested that the procedure might be able to identify the fraction that is biologically available (Mancy and Allen, 1977; Whitfield and Turner, 1979). A review of the literature, however, suggests that there is little hope of achieving this goal.

To successfully estimate the concentration of biologically active Cu, only those species that are available to the organism should contribute to the ASV signal. Figura and McDuffie (1979) measured the percent labile Cu in an acetate medium in the presence of EDTA, NTA, humic acids and glycine (all of which are known to form Cu complexes that are not biologically active). In the presence of 1×10^{-3}M glycine (K_1=8.1), Cu was found to be 100% labile. Even in the presence of EDTA, where the free Cu concentration was extremely low, some labile Cu was measured. Only with the addition of humic acids did Cu become entirely inactive. Gächter et al. (1973), using a complexing capacity technique developed by Chau et al. (1974b), performed ASV measurements on lake waters at pH 7.0 using a series of ligands

of different stability constants (tartrate, citrate, glycine, NTA, EDTA). They found that when the concentration of Cu was less than the concentration of total added ligand those complexes having a log conditional stability constant less that ca. 10 could be considered ASV labile; however, as Davison (1978) pointed out, this effective electroactivity of Cu complexes could be based on kinetic rather than thermodynamic considerations and, thus, that the stability constant may not be a good estimate of electroactivity. Since ASV has been shown to measure Cu in organic complexes which are known not to be biologically active with respect to phytoplankton (glycine, tartrate, EDTA, NTA), the ability of the ASV technique to measure biologically active Cu is in question.

The magnitude of the labile metal fraction also changes when the ASV parameters are changed. Deposition potential and deposition time have been used to discriminate between different species of Cu (Matson, 1968). Schonberger and Pickering (1980) demonstrated that, when determining Cu in solutions containing EDTA, the height of the current peak changed as the deposition potential was varied. In addition, since lability is dependent on kinetic as well as thermodynamic factors, it seems unlikely that there is a unique set of operating parameters for the ASV analysis suitable for measuring only biologically active Cu under all circumstances.

It is well known that the degree of ionization and the associated ability of many organics to form metal-ligand complexes are affected by pH. To change the pH of a sample from

its native value for analysis then may result in either increased or decreased ionization and metal binding capability. Therefore, to reduce the risk of altering the metal-organic association, the native pH of a sample must be used. However, this can pose a problem when ASV is to be used at the natural pH of seawater (ca. 8.2). In alkaline solutions, two Cu peaks are often observed in the analysis. It has been suggested that the more anodic peak is due to the formation of Cu(I) species at the surface during the oxidation of Cu(Hg) or an adsorption of Cu(II) hydroxy compounds (Sinko and Dolezal, 1970). Siebert and Hume (1981), using a mercury coated graphite electrode in raw seawater (pH 8.4), found that repeated cycles of plating and stripping caused a continual decrease in peak height with each cycle. This did not occur in acidified samples. They suggested that Cu(I) ions form an insoluble or highly adsorbable species which is electro-inactive and adheres to the electrode surface. Zirino and Kounaves (1980) suggested that the overall reduction of Cu(II) at the natural pH is kinetically hindered and thus is irreversible. With such interferences with the electrode, the calibration of Cu levels in a sample of pH 8.0 becomes difficult. Siebert and Hume (1981) concluded that, from a practical standpoint, the ASV determination of Cu in seawater at its natural pH should be avoided.

In conclusion, ASV is not suitable for studying Cu speciation in seawater particularly when trying to estimate biologically active metal. The technique, however, is a sensitive and reliable method for measuring total dissolved

metal concentrations in well defined and acidic solutions and, for this reason, ASV was used to measure total Cu concentrations in the artificial seawater used in the present study.

B. ANALYTICAL METHODS BASED ON PRELIMINARY CHEMICAL AND PHYSICAL SEPARATION

Trace metal speciation is normally studied using methods that involve a preliminary chemical or physical separation step whereby specific metal forms are isolated from the sample. The metal isolated is then quantified by an appropriate analytical technique, such as atomic absorption spectrophotometry. A comparison of levels before and after treatment is often used to separate several classes of metal species. A brief outline of the methods that have been used in natural waters and seawater samples will be given below. A brief summary of techniques used to study trace metal speciation in natural waters is presented in Table I.

1. Separation Based on Organic Solvent Solubility

Solvent extraction techniques have been used to distinquish between organic and inorganic bound Cu. The technique simply involves mixing a seawater sample with an organic solvent such as chloroform, hexanol or carbon tetrachloride. The amount of Cu in the solvent fraction is then equal to the Cu associated with organics. However, as Florence and Batley (1980) pointed out, the method probably gives low results because charged Cu complexes could not be extracted and Cu adsorbed onto organic colloidal matter may only be partially extracted. Solvent extraction has been applied to seawater (Slowey et al., 1967) to measure organically bound Cu.

Table I. Analytical methods for studying trace metal speciation in natural waters.

Electro-Chemical Methods	
Specific Ion Potentiometry:	e.g. Cupric ion electrode
Voltammetry:	Pulse Polarography Differential Pulse Polarography Differential Pulse Anodic Stripping Voltammetry
Methods Based on Preliminary Chemical and Physical Separation	
Organic-Inorganic Separation:	Solvent extraction Chelation-solvent extraction
Molecular Size Separation:	Membrane filtration Ultrafiltration Dialysis Gel chromatography Centrifugation
Separation based on charge:	Conventional ion-exchange resins Chelex-100
Calculation Methods	
Computer Models:	e.g. Comics Haltafall Mineql Redeql2

2. Molecular Size Separation

Techniques that fractionate trace metal species on the basis of size differences include filtration, gel filtration, dialysis and centrifugation. Ultrafiltration, dialysis and centrifugation generally are used to separate metal present in true solution from that associated with colloidal material.

Membrane filters with 0.45 μm pore diameter are normally used to distinguish between dissolved and particulate forms of metal. However, such a pore size is still large enough to allow colloidal matter to pass through and other procedures must be used to isolate the species in true solution. Ultrafiltration is simply a form of filtration in which the pore size of the filter is of the same magnitude as molecular or ionic species in true solution. Pore sizes can range from 1 to 10 nm, providing filters capable of rejecting molecules with molecular weights in the range of 500 to 300,000 (Schmidt, 1978). Generally the membranes have no net electrical charge although some have ionic sites. Ultrafiltration has been used in the investigation of trace metal forms in natural and model aquatic systems (Schindler, et al., 1972; Andren and Harris, 1975; Smith, 1976). Disadvantages of the technique include the cost, the time taken for filtration, the adsorption of metals from the solution onto the filters, and a problem of initial metal contamination of the filters (Hart and Davies, 1978).

In dialysis, the pore size of the membrane is such (1 nm) that only the free metal ion and the smallest complexed species are expected to diffuse through the membrane. It is thus used

to differentiate metal bound to colloidal matter and that in true solution (Hart and Davies, 1978). However, there are problems when dialysis membranes are used for metal speciation studies. The membranes usually have a negative charge associated with them and negative species experience a smaller effective pore size because of electrostatic repulsion and thus they require a long time to attain equilibrium (Benes and Steinnes, 1974). In addition, considerable dissociation of some metal complexes may occur at the membrane surface because of the negative charge (Guy and Chakrabarti, 1975). From a practical standpoint, dialysis is not convenient because of the time needed for the analysis (Benes and Steinnes, 1975) and because elimination of metal contamination from the dialysis membrane is extremely difficult.

Gel filtration chromatography is based upon the inclusion and subsequent elution of the solute through a stationary phase consisting of a heteroporous, cross-linked polymeric gel. Molecules exceeding the size of the exclusion limit of the gel are eluted initially while smaller molecules, which permeate the gel particles to varying degrees depending on their shape and size, are eluted from the gel column in order of decreasing molecular size.

Gel filtration has been used particularly to study metal-organic interactions in natural waters (Gjessing and Lee, 1967; Mantoura and Riley, 1975; Sugai and Healy, 1978). The disadvantages of the technique are that it is generally necessary to preconcentrate the sample, which may alter the

composition of the sample, and that the high ratio of solvent to sample volume can result in large blanks (Plumb and Lee, 1973; Benes et al, 1976).

3. Separation Based on Charge and Lability of Complexes

Chelating resins, such as Chelex-100, have been applied to the concentration and determination of trace metals in seawater (Riley and Taylor, 1968a,b; Riley and Taylor, 1972; Florence and Batley, 1975,1976). These resins show an unusually high preference for the transition metals over those of the alkali or alkaline earth metals. Figura and McDuffie (1980) developed an analytical scheme to differentiate trace metal species on their relative labilities with respect to Chelex-100 used in conjunction with ASV. Species were classified as 'very labile', 'moderately labile', 'slowly labile' or inert depending on the characteristic time scale of the measurement. Batley and Florence (1976a,b) also used Chelex-100 and ASV to quantitatively assay seven different heavy metal species in natural waters. In both studies, Chelex-100 was found to dissociate a larger percentage of the complexes in the samples than ASV; thus the most labile class of metal was that which was ASV labile. Since the ASV measurement cannot be used as a measure of biologically active Cu in seawater, the ion-exchange method, which dissociates even stronger complexes, would also not be expected to measure biologically active metal.

Conventional cation and anion exchange resins are capable of distinquishing particular metal species based upon their

charge. A few studies have used these ion-exchangers to study metal speciation in sewage (Cantwell et al., 1982), freshwaters (Filby et al., 1974; Benes and Steinnes, 1975; Benes et al., 1976; Shuman and Dempsey, 1977) and seawater samples (Marchand, 1974). The present study used a conventional cation exchange resin to study Cu speciation in seawater and a description of cation-exchange resins is given in Section IV.

III. THE MEASUREMENT OF BIOLOGICALLY ACTIVE CU BY A MARINE DIATOM

A. INTRODUCTION

Although Cu is a required trace element for phytoplankton, environmental concentrations as low as 1 ug l^{-1} can be toxic (Steeman Nielsen *et al.*, 1969; Mandelli, 1969; Martin and Olander, 1971; Erickson, 1972). The sensitivity to Cu can vary considerably, not only among algal groups but also among algal species of the same group (Erickson *et al.*, 1970). The dinoflagellates and cyanophytes seem to be the most sensitive while the green flagellates the most resistant (Mandelli, 1969; Erickson *et al.*, 1970). Diatoms, on the other hand, are found to be both quite sensitive and resistant to Cu. Steeman Nielsen and Wium-Andersen (1971) showed that as little as 1.2 ug l^{-1} Cu in synthetic seawater inhibited the growth of the diatom, *Nitzschia palea*, while Canterford *et al.* (1978) found that in enriched natural seawater Cu concentrations as high as 150 ug l^{-1} did not inhibit the growth of the diatom *Ditylum brightwelli*.

A wide range of morphological abnormalities are found when marine diatoms are exposed to toxic levels of Cu. The most prevalent effects are changes in cell size or external morphology, and reduced growth rates (Erickson, 1972; Fisher *et al.*, 1981; Thomas *et al.*, 1980). Other effects include a prolonged lag phase (Morel *et al.*, 1978) and changes in the internal appearance of the cell; when cells of *Thalassiosira*

aestivalis were exposed to high levels of Cu the cytoplasm became granular and yellowish in color, the chloroplast integrity was disrupted, and more delicate spines were extruded from the marginal processes (Thomas et al., 1980). Physiological processes shown to be inhibited by Cu have included nitrate uptake, photosynthetic carbon assimulation, and nitrate reductase synthesis (Harrison et al., 1977). In addition, silicic acid uptake has been shown to be reduced in the presence of Cu which was attributed to a hypothetical Cu-sensitive $Si(OH)_4$ transport site (Goering et al., 1977; Rueter et al., 1981).

It is believed that Cu produces its toxic effect by entering into strong complexes with organic ligands such as carboxyl, sulfhydryl, phosphatidic, amino and other groups present on the surface of the cell (Davies, 1978). At low activities Cu reacts primarily with the surface of the cell and disrupts membrane activities such as cell division and permeability (Erickson et al., 1970; Morel et al., 1978). Once Cu is within the cell it may inactivate numerous enzymes through displacement of the activating metal, or through binding to sulfhydryl groups (Rothstein, 1959) or other functional groups (Eichhorn, 1975). In freshwater algae, Cu has been shown to inactivate both the Hill reaction, a measure of photosystem II, and the modified Mehler reaction which measures photosystem I activity (Overnell, 1975). Fisher et al. (1981) found that when the diatom Asterionella japonica was treated with Cu it showed an above normal photosynthetic rate on a per cell basis

but the excretion of photosynthetically fixed carbon was depressed. This indicated an uncoupling of photosynthesis from cell division with the cells becoming enlarged when the fixed carbon could not be excreted or utilized in cell division.

In recent years numerous studies have demonstrated that the toxicity of Cu to phytoplankton is controlled by the cupric ion activity of the medium and not the total metal concentration (Sunda and Guillard, 1976; Anderson and Morel, 1978; Jackson and Morgan, 1978; Sunda and Lewis, 1978; Canterford and Canterford, 1980). This was demonstrated by experiments conducted in chemically well-defined media where the cupric ion activity was calculated by computer modelling techniques and model organic ligands were used to control the cupric ion activity. Bioassay tests showed that the growth rate of the phytoplankton organism was correlated to the calculated cupric ion activity but not to the total Cu concentration.

In the following section, data are presented on the growth of the phytoplankton organism, Thalassiosira pseudonana (WHOI, clone 3H), which was used to measure the amount of biologically active Cu in the artificial seawater medium Aquil (Morel et al., 1979). The organism was calibrated over a range of Cu concentrations and the chemical activity of the metal was, following the approach in the studies mentioned above, controlled with various model ligands. The ligands were chosen on the basis of the stability of their complexes with Cu and their effect on the activity of Cu was estimated using the computer equilibrium model MINEQL (Westall et al., 1976). The

purpose of these experiments was to determine the relationship between the growth rate of the assay organism and the cupric ion activity of the medium. These data will be subsequently used to test the analytical method developed for the measurement of biologically active Cu in seawater.

B. MATERIALS AND METHODS

1. Medium Preparation

Variations of the medium 'Aquil' (Morel et al., 1979) were used throughout the present study for both the stock and experimental cultures. Aquil was chosen because the trace metal speciation is well defined. Culture medium preparation involved the preparation of major salt, major nutrient, trace metal and vitamin solutions separately and then combining these just before use. All chemicals used in the medium preparation were of reagent grade. Any glassware used was initially rinsed 3X with glass distilled water (GDW), soaked in 6N HCL for 24 hr, and then rinsed 3X with GDW. The same glassware was used for a particular solution throughout the term of the study.

Major seawater salts (SOW): The major salts were prepared in 20 liter batches. The salts (Table II), excluding $MgCl_2$, were added to 18 l of GDW in a 20-l glass carboy and bubbled with acid cleaned (6N H_2SO_4), filtered (0.45 μm Nuclepore) air until the mixture was completely dissolved. $MgCl_2$ was then added to the salt solution which was brought up to 20 l with GDW ($MgCl_2$ was dryed for 2 days at 70°C before use because of its hygroscopic nature). The medium was then bubbled with acid cleaned, filtered air overnight to allow equilibration and to adjust the pH to 8.0±.05. The solution was then passed through an ion-exchange resin (Chelex-100, 100-200 mesh) and stored in acid cleaned 6-l borosilicate flasks until use. The preparation of the ion-exchange resin is described in Section III.B.5.

Background Cu levels in the chelex treated SOW were below detection limit (1.57×10^{-9}M Cu) as measured by anodic stripping voltammetry.

Nutrients: Phosphate, nitrate and silicate stocks were prepared separately at 1000X the final concentration. The stocks were prepared as given in Table II. Each solution was adjusted to approximately pH 8.0 and then passed through an ion-exchange resin (Chelex-100). To allow the use of the same ion-exchange column for all solutions, NaCl was added to the phosphate and silicate stocks to provide the same ionic strength in all stocks.

Vitamins: Stocks of biotin and B_{12} were prepared from crystalline compounds. These were then diluted and thiamine·HCl was added to give a vitamin mix at 5000X the final concentration (Table II). The final solution was frozen in 20 ml screw cap vials after heat sterilization (121°C, 15 min).

Trace Metals: Two separate stock solutions, were prepared from the salts given in Table II, the first consisting of Cu, Mo plus Co and the second of Mn plus Zn. Aliquots of each were then combined to give a trace metal mix at 1000X the final concentration. The Fe stock was prepared at 1000X the final concentration by adding $FeCl_3$ to GDW. The stock was made up fresh and then allowed to equilibrate for several hours before being added to the cultures. Special consideration was given to the method of Fe preparation and addition (see Section III.B.3).

Aquil preparation: Generally, 4 l of Aquil were needed for each bioassay. Aquil was prepared by adding 1 ml of the trace

Table II. Preparation of Aquil

SOW:

NaCl	490.6 g				
$MgCl_2.6H_2O$	222.0				
Na_2SO_4	81.9				
$CaCl_2.2H_2O$	30.8				
KCl	14.0	--->	20 l	---> Chelex	---> Storage
$NaHCO_3$	4.0				
KBr	2.0				
H_3BO_3	0.6				
$SrCl_2.6H_2O$	0.34				
NaF	0.06				

NUTRIENTS:

		pH adjusted to 8 NaOH			
NaH_2PO_4	1.38 g	-->	1 l	--> Chelex	--> Storage
NaCl	5.26				4°C
		NaOH			
$NaNO_3$	8.5	-->	1 l	--> Chelex	--> Storage 4°C
		HCL			
$NaSiO_3.9H_2O$	3.55	-->	1 l	--> Chelex	--> Storage
NaCl	4.38				4°C

TRACE METALS:

			1 ml		
$CuCl_2.5H_2O$	.249 g	-->	1 l	------->	
$(NH_4)_6Mo_7O_{24}.4H_2O$	.265		1 ml		
$CoCl_2.6H_2O$	.595	-->	1 l	------->	Mixed metal Stock 1 Liter
$MnCl_2.4H_2O$	.455		1 ml		
$ZnSO_4.7H_2O$	.115	-->	1 l	------->	
$FeCl_3.6H_2O$	.122	-->	1 l	------->	Fe Stock

VITAMIN MIX:

			.1 ml		
B_{12}	11.0 mg	-->.01	l	--------->	
			1ml		Vitamin
Biotin	10.0	--> .1	l	--------->	Stock 0.1 Liter
Thiamine	20.0	----------		---------->	

metal mix, 1 ml of each of the nutrient solutions, and 0.5 ml of vitamin mix to each 1 of SOW. The Aquil was then transfered to an acid cleaned 4-l Kimax aspirator bottle, bubbled with CO_2 to a pH of 5.7 and autoclaved (121°C) for 30 min (bubbling with CO_2 prevented precipitation during autoclaving). The medium was then cooled and, if necessary, bubbled with acid cleaned, filtered air until it had a pH of 8.0±.05. At this point, 1 ml of Fe stock was added to each 1 of Aquil to give a final concentration of 4.5×10^{-7}M. The Aquil was then ready to be used in the bioassays.

2. Bioassays

In preliminary experiments the sensitivity of the bioassay organism to Cu was determined in Aquil when no organic ligands were added. Three bioassays were performed using the Cu concentration ranges of 0.1-6.30 x 10^{-8}M at 1.57 x 10^{-8}M increments; 0.1 to 15.7 x10^{-8}M at 3.90 x 10^{-8}M increments; and 0.1-47.1 x 10^{-8}M at 15.7 x 10^{-8}M increments. The same bioassay procedure was followed as will be given below for the organic ligand bioassays.

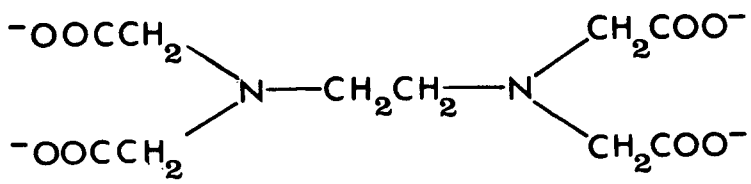
Bioassays were conducted with L-glutamic acid (GLU), L-histidine (HIS), nitrilotriacetic acid (NTA) and ethylenediaminetetraacetic acid (EDTA) (Fig. 2). These were used to change the concentration of the cupric ion while maintaining a constant concentration of total Cu. Ligand stocks were prepared at ca. 1000X the final concentration and stored in acid cleaned polypropylene bottles at 4°C in the dark. The

GLU stock was dissolved in GDW heated to 80°C.

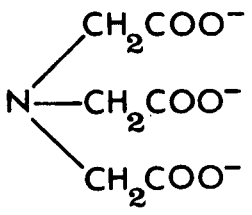
The bioassay procedure involved adding aliquots (250 ml) of the autoclaved Aquil to 15 previously autoclaved 500 ml polycarbonate erlenmeyer flasks. (Of the 15 flasks, 3 were used as controls while the remaining 12 were used to run 3 replicates at each of 4 different Cu concentrations). The appropriate ligand at the proper concentration was added to all flasks. Then, depending on the experiment, Cu was added in one of two concentration ranges. The values comprising these two ranges were 0.789, 1.57, 2.36, and 3.15 x 10^{-7}M Cu (i.e., 5, 10, 15, and 20 ug l^{-1} Cu) or 0.393, 0.789, 1.18, and 1.57 x 10^{-7}M Cu (i.e., 2.5, 5.0, 7.5, and 10 ug l^{-1} Cu). The medium was left covered for 2-3 hours before being innoculated with the test organism.

The centric diatom Thalassiosira pseudonana (=Cyclotella nana Hustedt) Hasle and Heimdal (WHOI clone 3H) was used as the bioassay organism because of its sensitivity to Cu, its uniform size and shape (factors which makes it amenable to measurement by electronic particle counting), and its fast growth rate (up to 2.5 doublings day^{-1}). The organism varies from 2.5 to 10 μm in size and normally appears singly or in pairs in laboratory cultures.

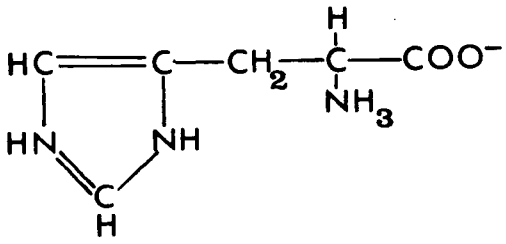
The experimental cultures were innoculated with enough stock culture (1-2 ml) to provide a cell concentration of 1000-2000 cell ml^{-1}. All cultures were grown at a temperature of 15±1°C at an in-flask light intensity of 95 μEin $m^{-2}s^{-1}$ on a 16:8 hour light-dark cycle. Cell concentrations were monitored



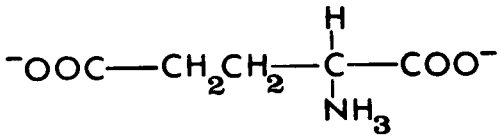
EDTA



NTA



HIS



GLU

Figure 2. The model organic ligands used in the bioassays.

daily over the test period with a Coulter Counter (model Zf). The pH was measured initially and on the third day of growth. All tests were run in triplicate unless otherwise stated.

Axenic stock cultures were initially obtained from the Northeast Pacific Culture Collection (University of B.C., Vancouver, B.C.) and maintained in Aquil prepared as above except that an Fe-EDTA mix (EDTA = 5.0×10^{-7}M) was added before autoclaving. Initially, EDTA was added because it was believed necessary to keep Fe soluble during the autoclaving step. It was later found that this addition was not necessary but the addition of EDTA was continued so as to maintain similar conditions in all bioassays.

All transfers and innoculations were conducted in a class 100 laminar flow hood in which all possible metal parts had been replaced by polypropylene. Precautions were taken to minimize bacterial and trace metal contamination.

3. Test of Mode of Fe Addition

Iron is very insoluble in seawater and in culturing procedures it is generally complexed with a complexing agent to keep it solubilized. Because the addition of complexing agents to the cultures for this purpose was not desirable bioassay tests were performed to determine (1) if freshly precipitated Fe could supply the organism with Fe as readily as a Fe-EDTA mix, (2) if autoclaving of the medium containing Fe or autoclaving of the Fe stocks could change the metal's availability, and (3) if ageing of the Fe stocks could change its availability. Two

bioassays were performed using the Aquil medium where the Fe was added in the manner outlined below. Fe was added to give a final concentration of 4.5×10^{-7}M in all flasks.

In the first test series, the methods of Fe addition employed were as follows: 1) Freshly prepared Fe was added before and after autoclaving of the medium; 2) The medium was autoclaved but there was no addition of Fe; 3) An Fe-EDTA mix (EDTA=5.0×10^{-7}M) was added to the medium before and after the medium was autoclaved; and 4) An Fe stock was autoclaved and added to autoclaved medium.

In the second test series, methods 1 and 2 were repeated together with the following additional methods: 5) A one week old Fe stock was added to an autoclaved medium; and 6) A three month old Fe stock was added to an autoclaved medium.

Freshly prepared Fe stock was added to the culture flasks at the end of the bioassay period to demonstrate that growth was limited by a Fe deficiency. The aged Fe stocks were stored in polypropylene bottles at room temperature and all stocks were stored at their hydrolysis pH. Innoculum cultures were grown in a medium to which no Fe was added so as to precondition the organism to Fe limitation and to reduce Fe carryover from the innoculum medium.

4. Growth Measurements

Cell number was used to express the amount of growth. Both cell yield and growth rate were used. Cell yield is simply the total population at the end of an experimental run minus that at

the start. It represents the total growth that occurred during the measurement period and fails to show what is happening at any particular time. Growth rate, on the other hand, reflects any changes occurring in the growth process at various times throughout the experiment. Therefore, depending on the sampling frequency, physiological changes in the population of different scale lengths can be measured. If growth rates are averaged over the entire experiment the information obtained is the same as that obtained from cell yield.

In the present study, growth rates were expressed using a 'logarithm-to-base-2' scale that indicates the number of divisions per day, K where:

$$K \text{ (divisions day}^{-1}) = \frac{\log (N_1 / N_0)}{t} \times 3.322$$

N_0 and N_1 were the cell concentrations at the beginning and end, respectively, of a period of time, t. Both daily growth rates and growth rates averaged over many days were used.

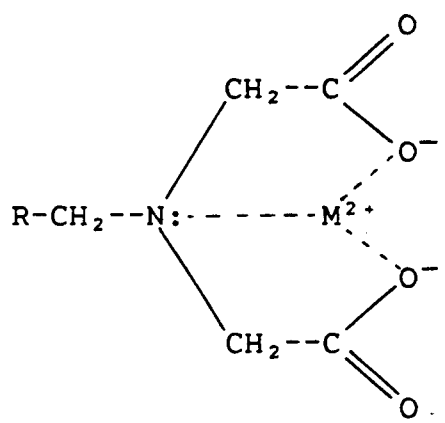
Relative growth rates, which express the amount of growth relative to that of the control cultures, were also used. This latter quantity is defined by the relationship:

$$K\ \% = \frac{K\ \text{test}}{K\ \text{control}} \times 100$$

where K % is the relative growth rate.

5. Chelex-100 as an Ion-Exchanger

Chelex-100 (Bio-Rad Laboratories, Richmond, California) was used to remove trace metal impurities in both the major salt solution and the nutrient stocks. It is a chelating cation exchanger comprised of a styrene divinylbenzene copolymer matrix containing iminodiacetate acid functional groups with cross-linkage of 1-2% and a pore size of approximately 25 Å. The selectivity of the resin for trace metals is based on chelate formation rather than on cation charge, size, or other physical characteristics and it is controlled by the properties of the resin's IDA groups; this functional group binds Cu and other heavy metals much more strongly than the alkaline earth and alkali metal cations.



(R represents the styrene-divinylbenzene copolymer matrix)

Chelex-100 preparation: Due to a low degree of cross linkage, Chelex was subject to large changes in volume as the cationic form of the resin was changed. Therefore, resin preparation and regeneration was performed in the batch mode prior to being added to a column. Chelex-100 was supplied in the Na^+ form and was converted to the ionic composition and pH of the solution to be purified so as to avoid any alkalinity changes that might occur in the medium if the resin gained or lost protons as the solution was passed through. The method of new resin preparation was different from the method used when the resin was to be regenerated.

New Chelex: A 40 g portion of the resin was placed in a 500 ml polypropylene disposable beaker (acid cleaned), rinsed with 100 ml of methanol to remove any residual organics, and rinsed 3X with 200 ml of GDW. The resin was then slurried in 300 ml of the solution to be cleaned and titrated with acid (6N HCl) to a pH of ca. 8.0. The solution was decanted, fresh solution added and the titration procedure repeated. Fresh solution was added four or five times or until the pH was stable after the addition of fresh solution. The resin was then ready to be poured into the column.

Regenerated Chelex: The columns were regenerated after ca. 60 l of SOW was purified. The top 5 cm of the resin was generally discolored and removed before regeneration. The resin was removed from the column, rinsed with 200 ml GDW, then slurried in 200 ml of 1N HCl and stirred for 30 min. The acid was then decanted and the resin was rinsed 3X with 200 ml of

GDW. To convert the resin to the Na^+ form, 200 ml of 0.5 NaOH was added, stirred for 30 min and rinsed 3X with GDW. The resin then underwent the same procedure as for new chelex.

The final step in the resin preparation was the preparation of the columns. A 5.0 x 60.0 cm glass column fitted with glass wool was filled with the solution to be cleaned. While the column was dripping, a slurry of the resin was poured into the column at a constant rate. A slurry was used so as to prevent any bubbles from being trapped as the resin was packed. A fluid head of 25 cm was maintained over the resin at all times.

C. COMPUTER MODELLING OF CU SPECIATION IN AQUIL

In the past, stability constants have been used singly or in pairs to calculate the equilibrium concentration of a particular metal-ligand species in aqueous systems. However, in a complex medium such as seawater many metals are capable of reacting with each ligand and many ligands can react with each metal. Thus to compute the correct equilibrium concentration of any species, all the competing equilibria must be taken into account simultaneously. Although the task is too laborious for manual calculation, computer programs have become available for solving such problems (e.g., Perrin, 1965; Perrin and Sayce, 1967; Morel and Morgan, 1972). More than a dozen programs applicable to natural water chemistry have been reviewed by Nordstrom et al. (1979). The machine computation efficiency of some of these programs is examined by Legget (1977).

One of the general methods used in solving the complex array of simultaneous equations that govern the species composition in seawater is termed the equilibrium constant approach. Accordingly to Nordstrom et al. (1979), this approach is "subject to the conditions of 1) mass balance and 2) chemical equilibrium. The mass balance conditions requires that the computed sum of the free and derived (complexes) species be equal to the given total concentration. Chemical equilibrium requires that the most stable arrangement for a given system be found, as defined by the equilibrium constants for all mass action expressions of the system."

In the present study, the distribution of Cu species in the

culture media was estimated using the computer program MINEQL (Westall et al., 1976). This model, which uses the equilibrium constant approach, is supplied with a data file consisting of equilibrium constants selected mainly from the tabulations of Sillen and Martell (1964,1971).

The model estimates the free metal ion concentration in solution when the total metal concentration and the thermodynamic constants for all the possible metal complexes are given. The stability constants for the more important Cu-complexes used in the computer model are given in Table III. Boron-Cu complexes were eliminated from the calculations because of the uncertainty of their stability constants in seawater.

The thermodynamic constants used in the model are first corrected to the appropriate ionic strength of the medium, in this case seawater, by means of activity coefficients estimated from the Davies approximation (Davies, 1962).

Ionic strength corrections: An activity coefficient, f, of an ion in a medium of ionic strength, I, can be estimated by:

$$\text{Log } f = Z^2 \epsilon T \left(\frac{I^{1/2}}{1 + I^{1/2}} - 0.2I \right) \qquad [1]$$

where: Z = ionic charge,

ϵ = dielectric constant of the solvent

T = temperature

Table III. Stability constants for the important Cu complexes used in the computer model.

Equilibrium	log K_1	log β_2 ($\beta_2 = K_1 \times K_2$)
Inorganic Complexes		
$Cu^{2+} + OH^- = Cu(OH)^+$	6.0	
$Cu^{2+} + 2OH^- = Cu(OH)_2^0$		16.68
$Cu^{2+} + CO_3^{2-} = CuCO_3^0$	6.70	
$Cu^{2+} + 2CO_3^{2-} = Cu(CO_3)_2^{2-}$		9.90
Organic Complexes		
Glutamic Acid	7.9	14.4
Histidine	11.1	19.40
NTA	14.5	17.10
EDTA	20.6	

I = ionic strength

Ionic strength is defined by:

$$I = 1/2 \sum Z_i^2 C_i \qquad i: \text{all species in solution}$$

where: Z_i = ionic charge of species i

C_i = concentration of species i

The thermodynamic equilibrium constant, K_1, for a reaction

$$M^{2+} + L^{2-} \rightleftharpoons ML$$

can be written:

$$K_1 = \frac{\{ ML \}}{\{ M \} \{ L \}} = \frac{[ML]}{[M] [L]} \cdot \frac{f_{ML}}{f_M \; f_L} \qquad [2]$$

where { } represents activity, [ ] represents concentration and f represents the activity coefficient. Charges are omitted for the sake of simplicity. Upon rearrangement:

$$[ML] = K_1 \frac{f_M \; f_L}{f_{ML}} \cdot [M] [L] \qquad [3]$$

The quantity

$$K' = \frac{f_M \, f_L}{f_{ML}} \cdot K_1 \qquad [4]$$

is termed an apparent equilibrium constant since it has a constant value in all solutions of given ionic strength.

D. RESULTS

1. Model Ligand Study

Table IV gives the experimental parameters and results of the bioassay tests. Included are one test run using the Aquil medium with various concentrations of Cu and six tests using Aquil with various ligand additions (EDTA, GLU, HIS, NTA), ligand concentrations and Cu concentrations.

Growth rates in the first 24 hours were generally lower than the maximum possible growth due to the organism becoming conditioned to the medium (see Appendix A) and, during this time period, the growth of the control cultures was usually identical to the test cultures except when high Cu levels were used. Because of this, the first 24 hours of growth was not included in the growth rate calculations and growth rates reported were averaged over the next 72 hours.

Test run 1 determined the effect of Cu on the growth rate of T. pseudonana when no organic ligands were added. The organism was found to be highly sensitive to Cu under these experimental conditions (Fig. 3). Cu began to inhibit growth when it was added in slight excess of 1.57×10^{-8}M Cu although a concentration of greater than 4.71×10^{-7}M was needed to obtain total growth inhibition. The growth response was not linear over the toxic range of the metal but appeared to be a 'two-step' response (Fig. 4). A rapid linear decrease in growth was seen initially with increasing Cu levels until growth rates decreased to just below 50% of the control. At this point, a

Table IV. Growth rate data from bioassays using the model organic ligands EDTA, NTA, HIS and GLU.

TEST	Ligand	Ligand Conc.(M)	Total Cu ($\times 10^{-8}$M)	Growth Rate (div day^{-1})	% of Control
1	NL[2]		0.1 control	1.49±.01[1]	
			15.7	0.49±.01	32±2[1]
			31.4	0.39±.01	26±3
			47.1	0.21±.01	14±1
			0.1 control	2.12±.01	
			3.9	1.59±.03	75±1
			7.9	1.01±.01	47±4
			11.8	1.01±.03	48±0
			15.7	0.92±.03	43±1
			0.1 control	2.02±.01	
			1.57	2.01±.02	100±1
			3.15	1.69±.11	84±5
			4.72	1.25±.02	62±2
			6.30	1.06±.02	52±1
2	EDTA	5.0×10^{-8}	0.1 control	1.52±.05	
			3.9	1.61±.03	100±2
			7.9	1.45±.10	95±7
			11.8	1.02±.06	67±4
			15.7	0.88±.03	58±2
		1.0×10^{-7}	0.1 control	1.80±.00	
			3.9	1.82±.03	101±1
			7.9	1.86±.01	104±1
			11.8	1.74±.04	97±2
			25.7	1.13±.05	63±2
3	GLU	1.0×10^{-5}	0.1 control	1.76±.03	
			7.9	1.37±.05	78±3
			15.7	0.99±.05	56±3
			23.6	0.88±.05	50±0
			31.5	0.81±.01	46±1
		2.5×10^{-5}	0.1 control	1.86±.09	
			7.9	1.79±.05	96±3
			15.7	1.29±.04	69±2
			23.6	1.03±.01	55±1
			31.5	0.93±05	50±3

Table IV. (con't)

TEST	Ligand	Ligand Conc.(M)	Total Cu ($\times 10^{-8}$M)		Growth Rate (div day^{-1})	% of Control
3	GLU	7.5×10^{-5}	0.1	control	1.98±.01	
			7.9		1.97±.02	100±1
			15.7		1.91±.09	97±5
			23.6		1.62±.10	82±5
			31.5		1.52±.05	77±2
		1.0×10^{-5}	0.1	control	1.93±.02	
			7.9		1.35±.07	70±3
			15.7		0.96±.00	50±0
		2.5×10^{-7}	7.9		1.93±.05	100±2
			15.7		1.32±.03	68±2
		5.0×10^{-5}	0.1	control	2.00±.04	
			7.9		2.02±.04	101±2
			15.7		1.75±.02	87±1
		7.5×10^{-5}	7.9		2.01±.01	100±1
			15.7		1.91±.02	95±1
4	NTA	1.0×10^{-7}	0.1	control	1.97±.01	
			7.9		1.17±.04	59±2
			15.7		0.87±.01	44±1
			23.6		0.86±.05	44±3
			31.5		0.70±.02	35±1
		2.5×10^{-7}	0.1	control	1.89±.02	
			7.9		1.50±.05	79±3
			15.7		1.05±.01	55±1
			23.6		0.88±.02	47±1
			31.5		0.84±.04	45±2
		5.0×10^{-7}	0.1	control	2.04±.03	
			7.9		1.89±.04	93±2
			15.7		1.31±.03	64±1
			23.6		1.09±.08	54±4
			31.5		1.07±.04	52±2
		7.5×10^{-7}	0.1	control	1.81±.04	
			7.9		1.84±.03	102±1
			15.7		1.40±.02	77±1
			23.6		1.13±.03	63±2
			31.5		0.97±.02	54±1

Table IV. (con't)

TEST	Ligand	Ligand Conc.(M)	Total Cu ($\times 10^{-8}$M)	Growth Rate (div day^{-1})	% of Control
5	HIS	1.0×10^{-7}	0.1 control	1.90±.01	
			7.9	1.87±.03	99±2
			15.7	0.89±.02	47±1
			23.6	0.52±.01	27±1
			31.5	0.27±.07	14±3
		2.5×10^{-7}	0.1 control	2.10±.01	
			7.9	2.11±.04	100±2
			15.7	2.14±.04	102±2
			23.6	1.47±.06	70±3
			31.6	1.02±.06	49±3
		5.0×10^{-6}	0.1 control	1.96±.01	
			15.7	1.98±.01	101±0
			31.5	2.05±.04	105±2
			47.2	1.98±.01	101±1
			63.0	1.93±.03	99±2

[1]Mean ±1 s.d. based on 2 replicates.
[2]NL No ligand addition.

plateau was seen in the curve and proportionately higher Cu levels were needed to reduce the growth rate further. A similar two-step process has been described for this clone of T. pseudonana by Gavis et al. (1981).

Growth rate measurements during the 24-48, 48-72 and 72-96 hour periods revealed a general response to Cu with respect to exposure time (Fig. 5). As exposure time increased the toxic effect of the metal was more pronounced and the lowest growth rates were observed after 72 hours. Such a delayed response has been previously reported for this organism by Sunda and Guillard (1976) (see also Gavis et al., 1981).

Test runs 2 through 5 examined the ability of specific organic ligands to reduce the toxicity of Cu. The growth patterns of the organism obtained in the presence of these ligands are shown in Figs. 6-9.

Although all four ligands tested reduced the toxicity of Cu, the concentration required to reduce the toxicity to a given level differed for each compound. The lowest concentration of each ligand that allowed full growth in the presence of 7.87×10^{-8}M Cu was: EDTA, 5.0×10^{-8}M; HIS, 1.0×10^{-7}M; NTA, 7.5×10^{-7}M; and GLU, 2.5×10^{-5}M. EDTA appeared to bind Cu in a 1:1 Cu:EDTA ratio as was suggested by a sharp decline in the growth rate when Cu was added in slightly greater concentrations than the ligand concentration (Table IV, Test 2).

The ability of HIS to reduce Cu toxicity was greater than that of NTA even though it has a lower stability constant for Cu. However, if one examines all the competing equilibria in

Figure 3. Growth of the bioassay organism in Aquil in the presence of Cu and with no organic ligands added. Symbols: □ Control; △ 1.57×10^{-8}M; ◇ 3.15×10^{-8}M; ▽ 4.72×10^{-8}M; ○ 6.29×10^{-8}M added Cu. Bars are ±1 s.d.

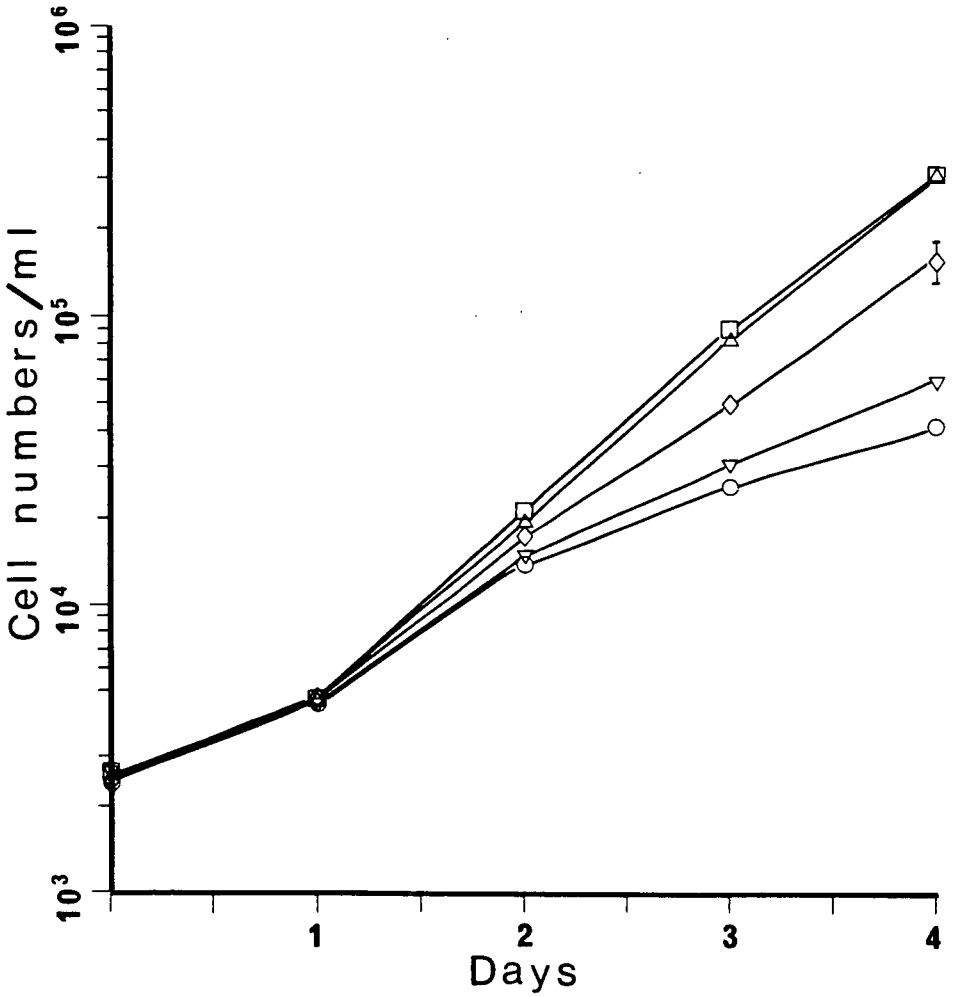


Figure 4. Growth rate (% of control) versus the -log of the added Cu concentration (Cu_T) in Aquil with no organics added. Bars are ±1 s.d.

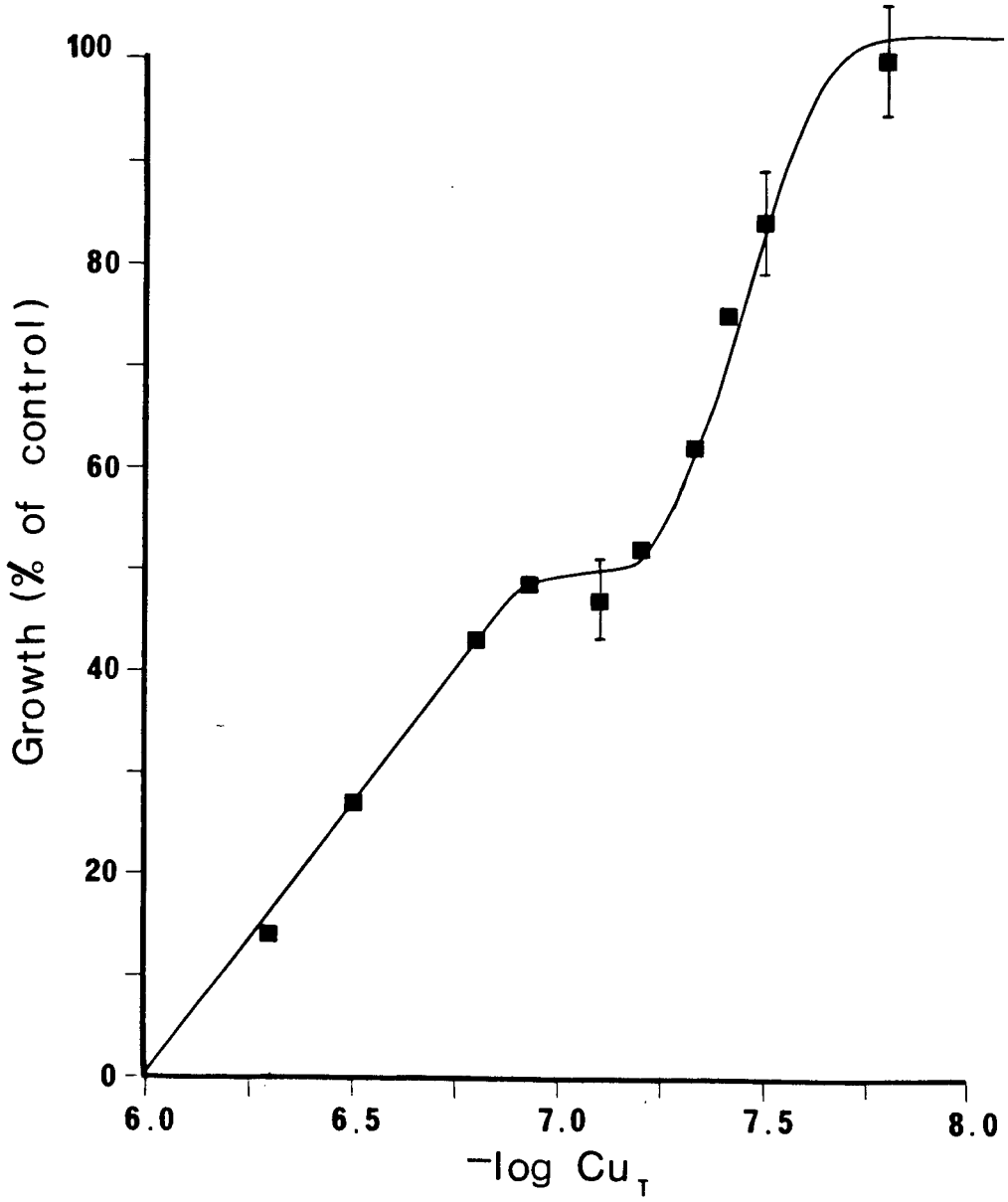


Figure 5. Effect of Cu upon growth (divs day^{-1}) during the 24-96 hr period. Symbols: ■ 24-48 hr.; ▲ 48-72hr.; ◆ 72-96 hr. period. Bars are ±1 s.d.

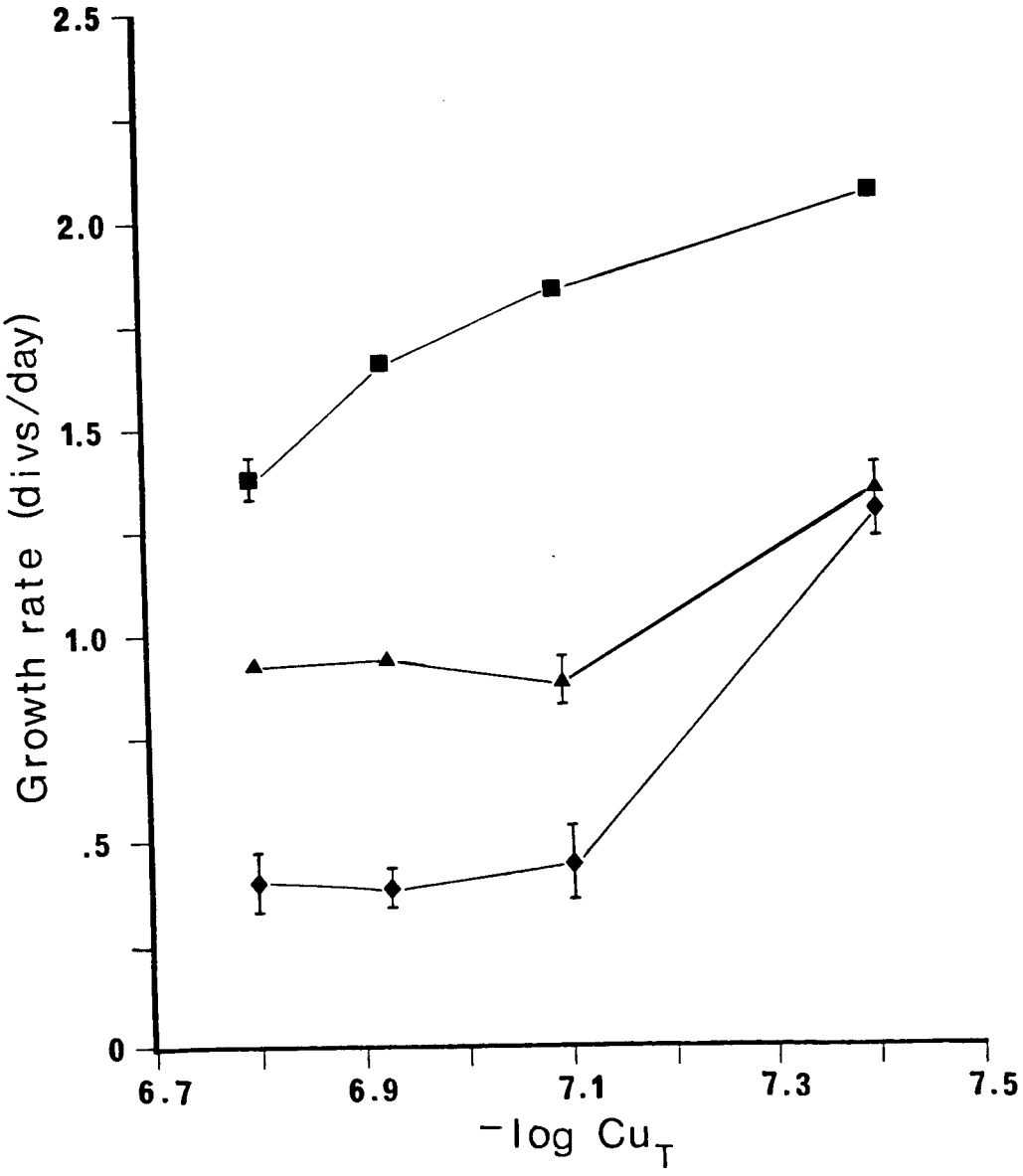


Figure 6. Growth rate (% of control) versus the -log of the Cu concentration (Cu_T) in the presence of GLU. Symbols: ■ $1.0x10^{-5}M$: ▲ $2.5x10^{-5}M$; and ◆ $7.5x10^{-5}M$ concentrations. Bars are ±1 s.d.

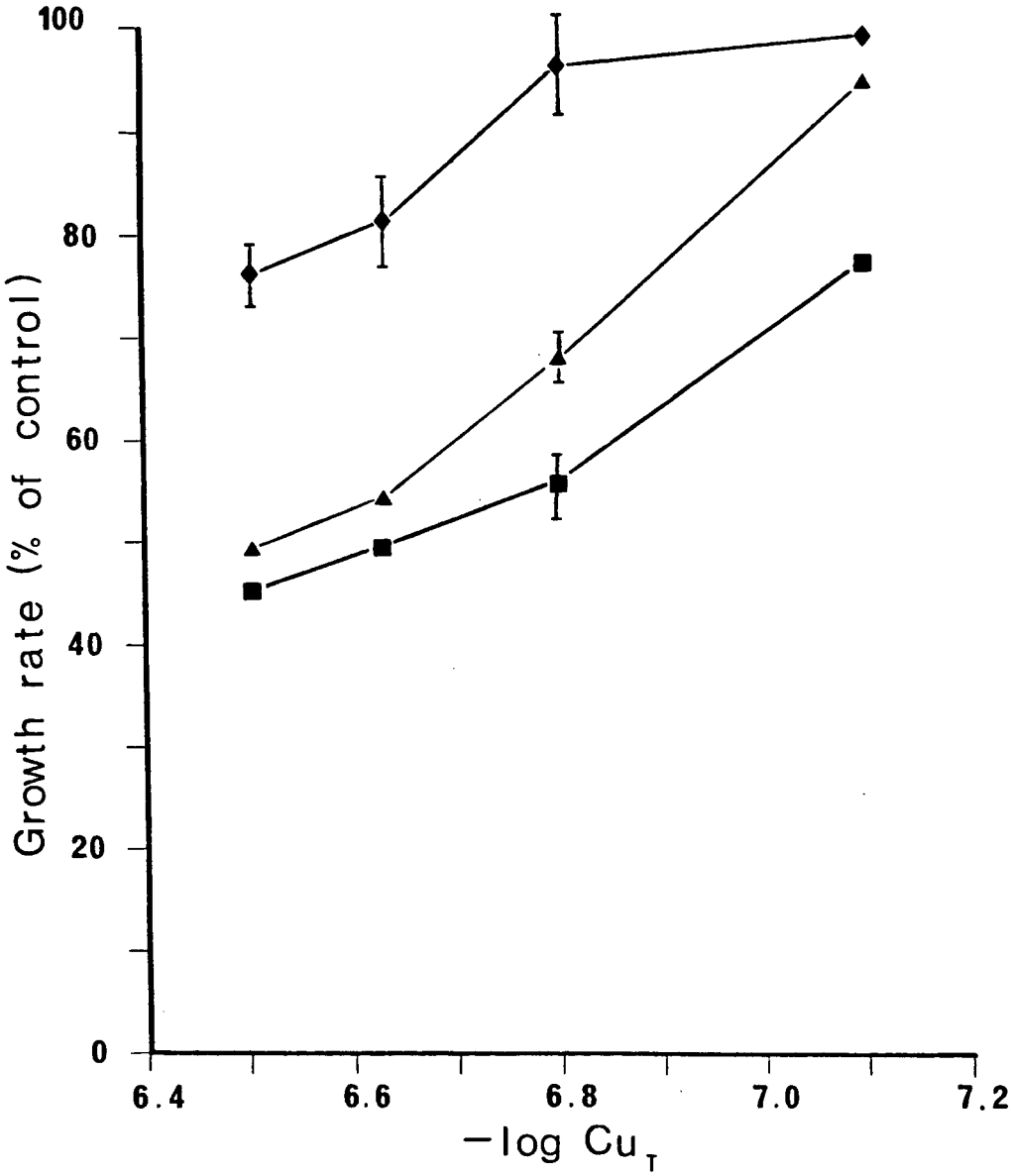


Figure 7. Growth rate (% of control) versus the -log of the Cu concentration (Cu_T) in the presence of HIS. Symbols: ■ 1.0×10^{-7}M; ▲ 2.5×10^{-7}M; and ◆ 5.0×10^{-7}M concentrations. Bars are ±1 s.d.

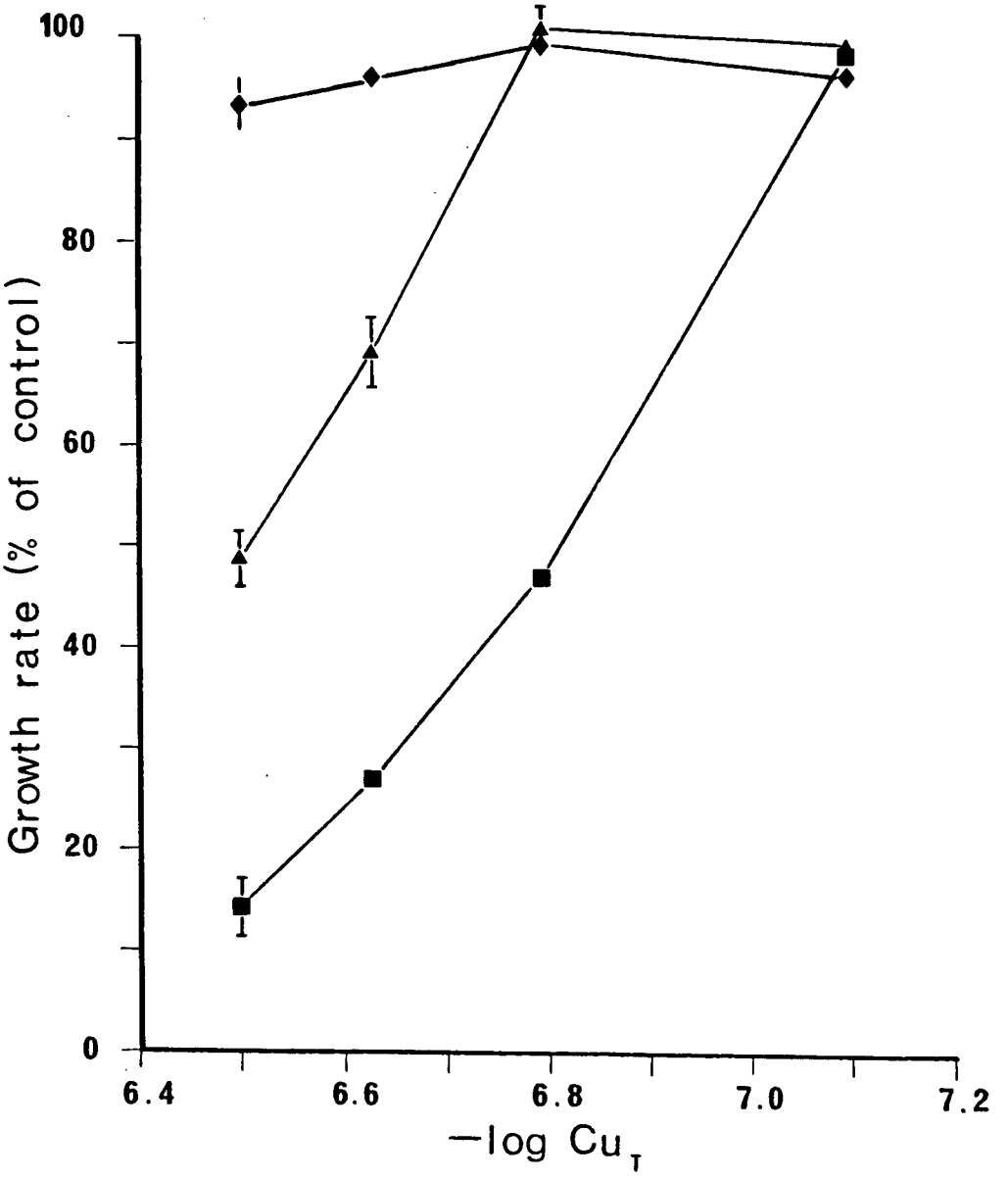


Figure 8. Growth rate (% of control) versus the -log of the Cu concentration (Cu_T) in the presence of NTA. Symbols: ■ $1.0x10^{-7}M$; ▲ $2.5x10^{-7}M$; ◆ $5.0x10^{-7}M$; and ● $7.5x10^{-7}M$ concentrations. Bars are ±1 s.d.

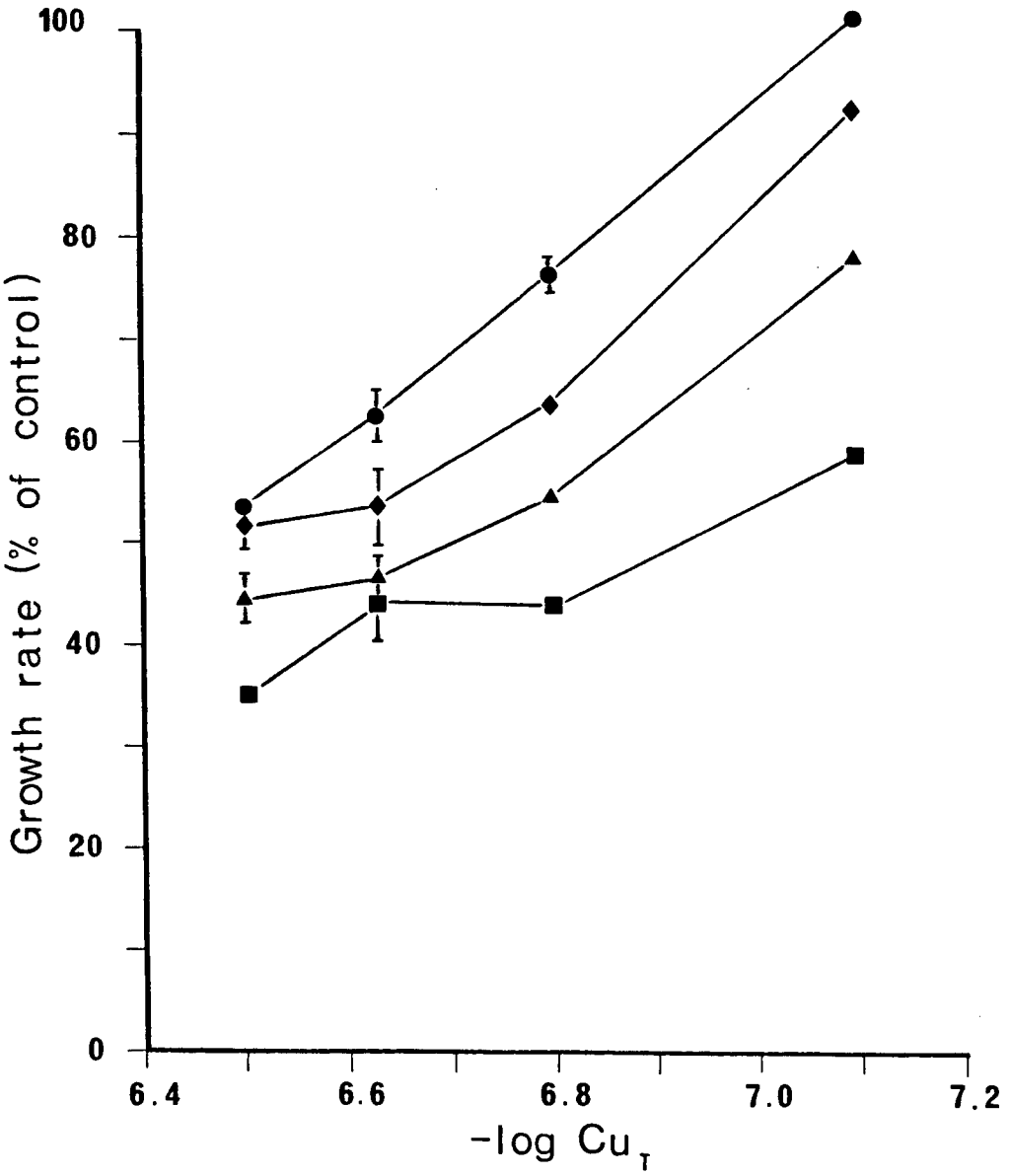
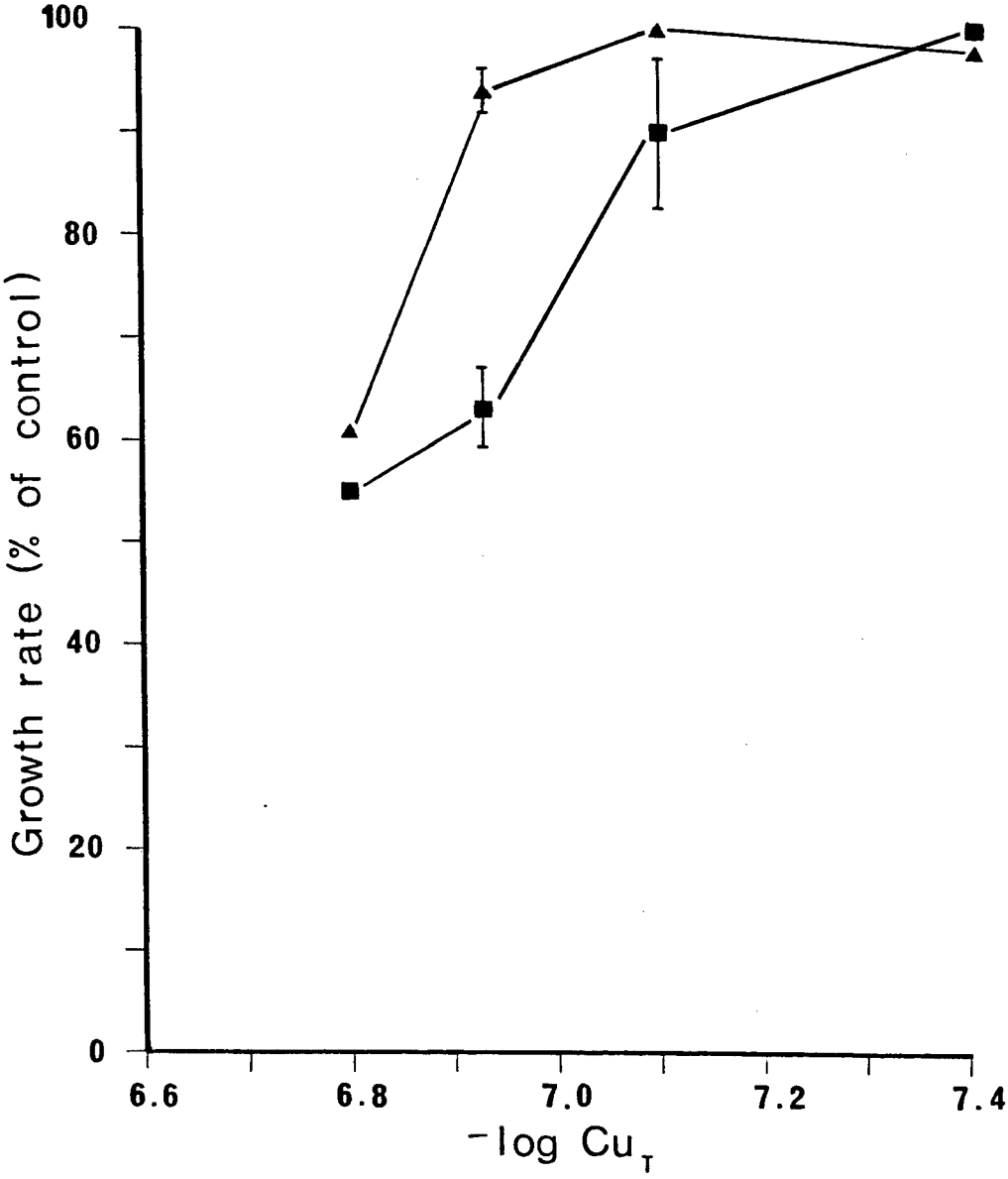


Figure 9. Growth rate (% of control) versus the -log of added Cu concentration (Cu_T) in the presence of EDTA. Symbols: ■ 5.0×10^{-8}M; and ▲ 1.0×10^{-7}M concentrations. Bars are ±1 s.d.



the artificial seawater by the computer model, it becomes apparent that a large percentage of NTA is bound in Ca and Mg complexes while HIS is not. Thus the amount of free ligand available for Cu complexation was greater for HIS than for NTA.

When EDTA was added in excess of Cu, slightly lower growth rates were found in the control cultures than in cultures containing the lower Cu concentrations (Table IV, Test 2). This effect was also seen in cultures that had high concentrations of HIS added (Table IV, Test 5). This may be due to these ligands binding many of the nutritional trace metals in the Aquil medium (Mn, Co, Mo, and Zn) which may cause some of these metals to become limiting. With the addition of Cu then, a proportion of the metals will be displaced from the ligand thus freeing them for uptake by the organism. A second possibility is that the addition of EDTA reduces the cupric ion concentration to a point where it is nutritionally deficient. With the addition of Cu the cupric ion activity simply increases to a point where it is no longer deficient.

The cultures whose growth rates were improved with the addition of low concentrations of Cu were believed to be more representative of a healthy population and were used as the control cultures for that particular test.

2. Growth Rate as a Function of Cupric Ion Activity

In Figs. 10-13, growth rates are plotted as a function of the negative log to the base 10 of the cupric ion activity (pCu* where * indicates it is a computed value). Cupric ion

concentrations were calculated using the computer model MINEQL and converted to activities by multiplication by an activity coefficient estimated from the Davies approximation (eqn. 1).

When examining each ligand separately, a strong linear relationship was seen between growth rate and pCu* levels of 8.4 and 10.0. Slight deviations from this linear relationship were seen in growth rates of cultures containing high concentrations of GLU (Fig. 10). Above a pCu* level of 10.0 cell growth was no longer inhibited while the plateau of the growth curve, described previously, was reached at a pCu* of approximately 8.4. The exception was that of HIS where the growth rates in the pCu* range of this plateau were generally lower than the other ligands.

The relationship between growth rate and pCu* was quite good when examining the ligands separately. However, when curves for each ligand were presented together (Fig. 14) the scatter in the curve was much greater although a linear relationship was still apparent. This scatter could be due to variability not taken into account in the bioassay technique, differences in biologically active Cu not associated with the cupric ion, or to errors in the calculation of pCu* by the computer model. The reason for the variability will be discussed in Section III.D.1.

In the computer model, it was calculated that the maximum pCu* possible before the precipitation of malachite ($Cu_2(OH)_2CO_3$) would occur was 8.13. With no organic ligands present, precipitation of malachite would occur in Aquil when a

Figure 10. Growth rate (% of control) versus the calculated pCu* in the presence of GLU. Symbols: ■ 1.0×10^{-5}M; ▲ 2.5×10^{-5}M; and ◆ 7.5×10^{-5}M added GLU. Bars are ±1 s.d.

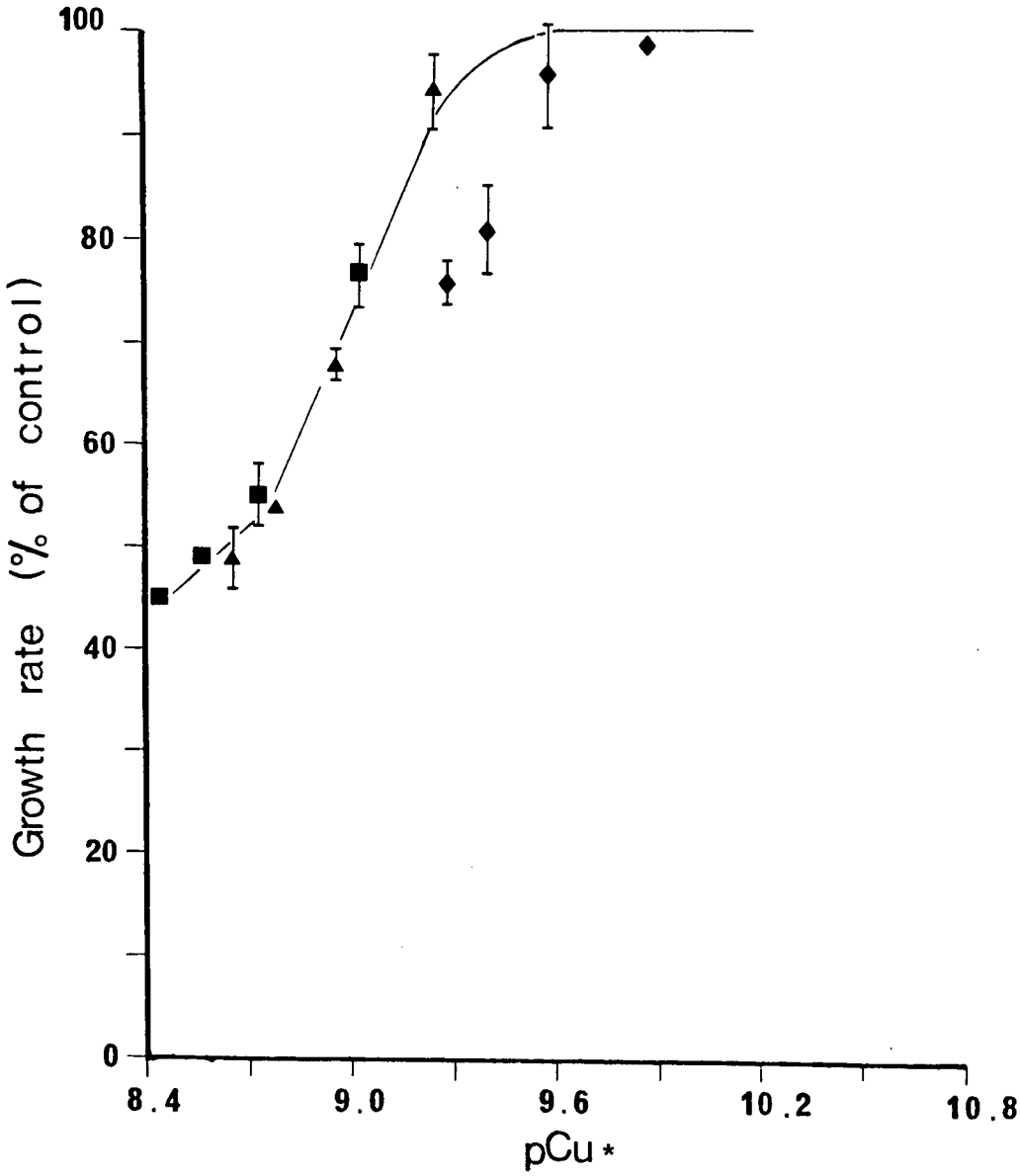


Figure 11. Growth rate (% of control) versus the calculated pCu* in the presence of HIS. Symbols: ■ 1.0×10^{-7}M; and ▲ 2.5×10^{-7}M added HIS. Bars are ±1 s.d.

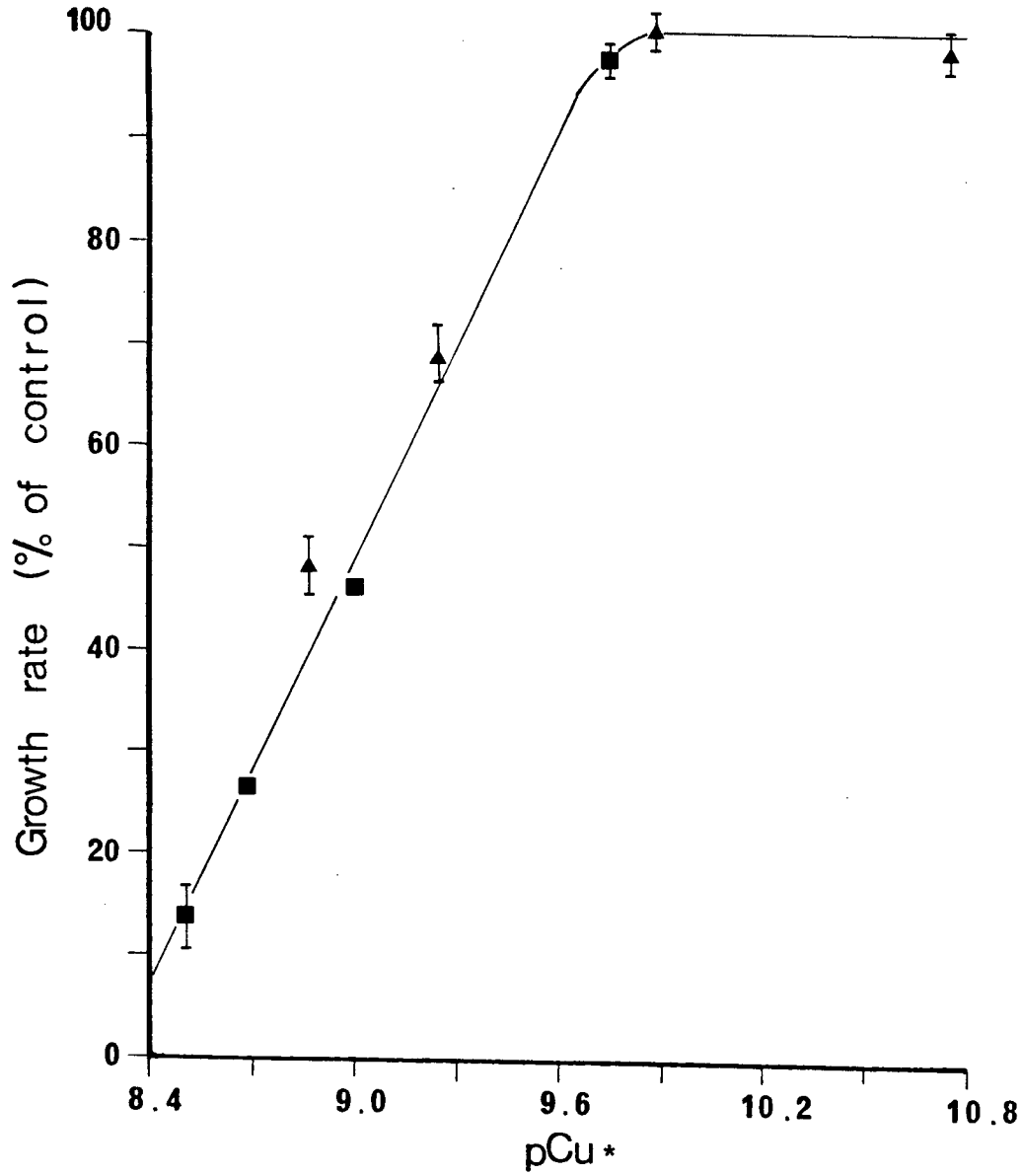


Figure 12. Growth rate (% of control) versus the calculated pCu* in the presence of NTA. Symbols: ■ 1.0×10^{-7}M; ▲ 2.5×10^{-7}M; ◆ 5.0×10^{-7}M; and ● 7.5×10^{-7}M added NTA. Bars are ±1 s.d.

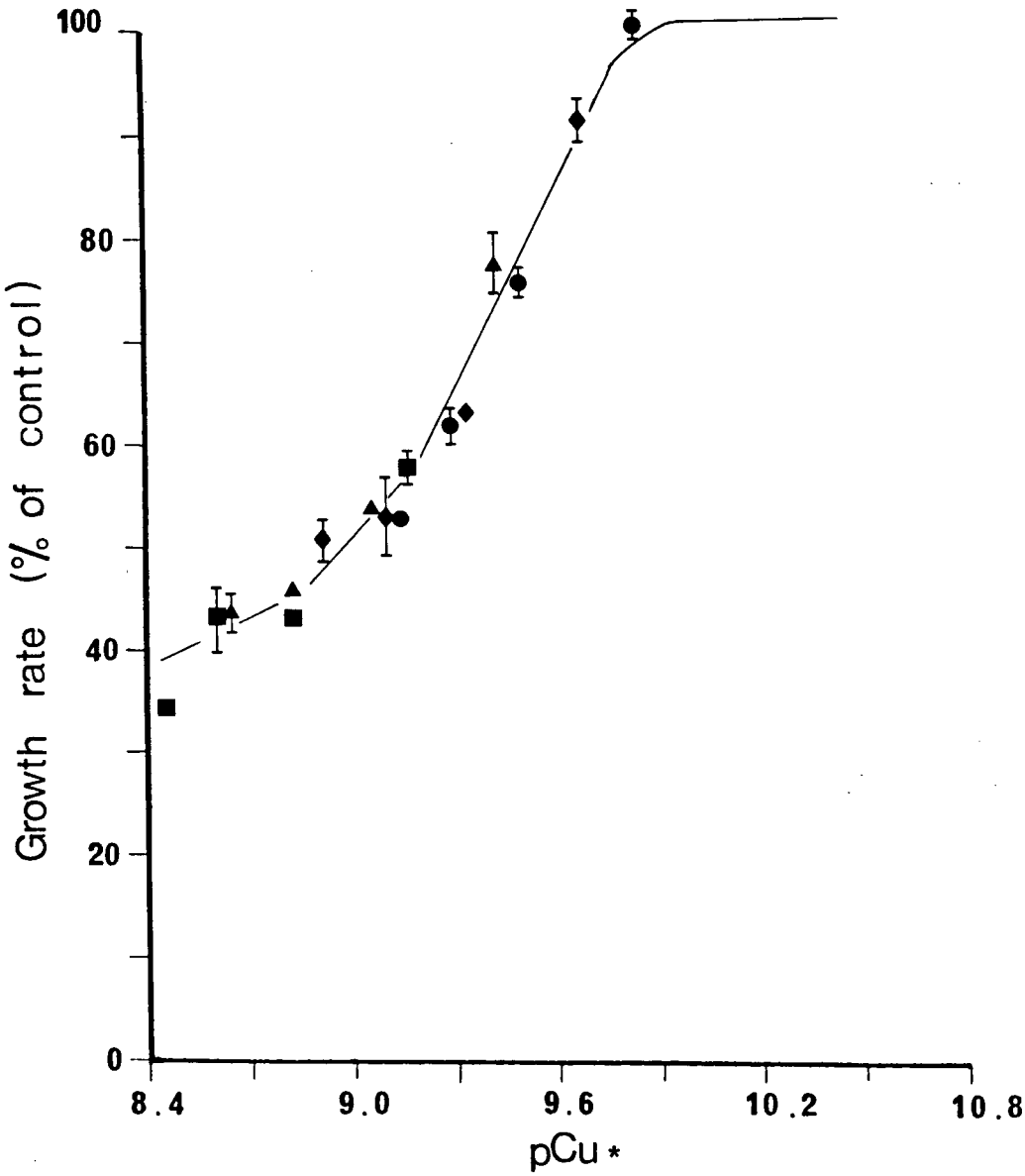


Figure 13. Growth rate (% of control) versus the calculated pCu* in the presence of EDTA. Symbols: ■ 5.0×10^{-8}M; and ▲ 1.0×10^{-7}M added EDTA. Bars are ±1 s.d.

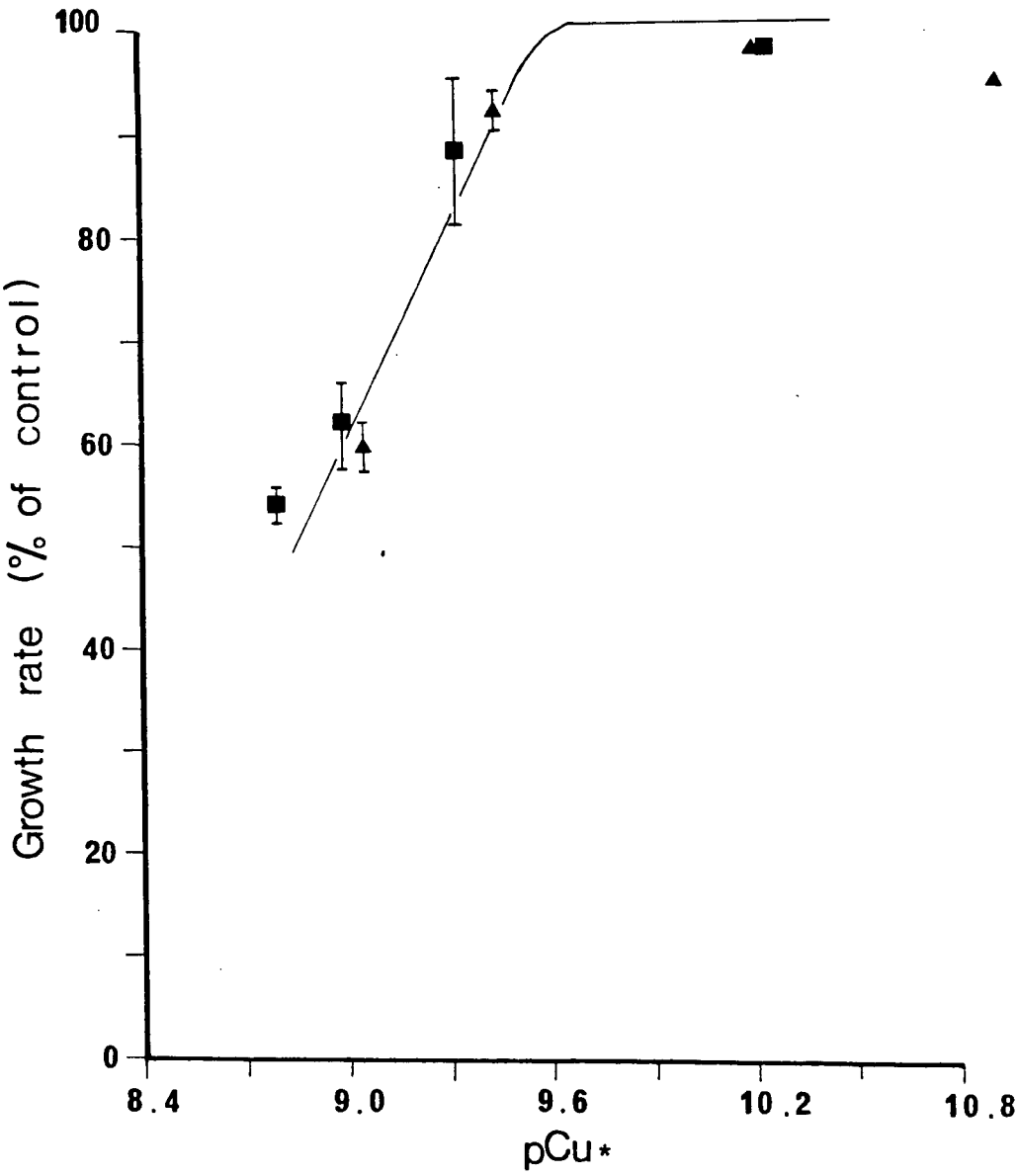
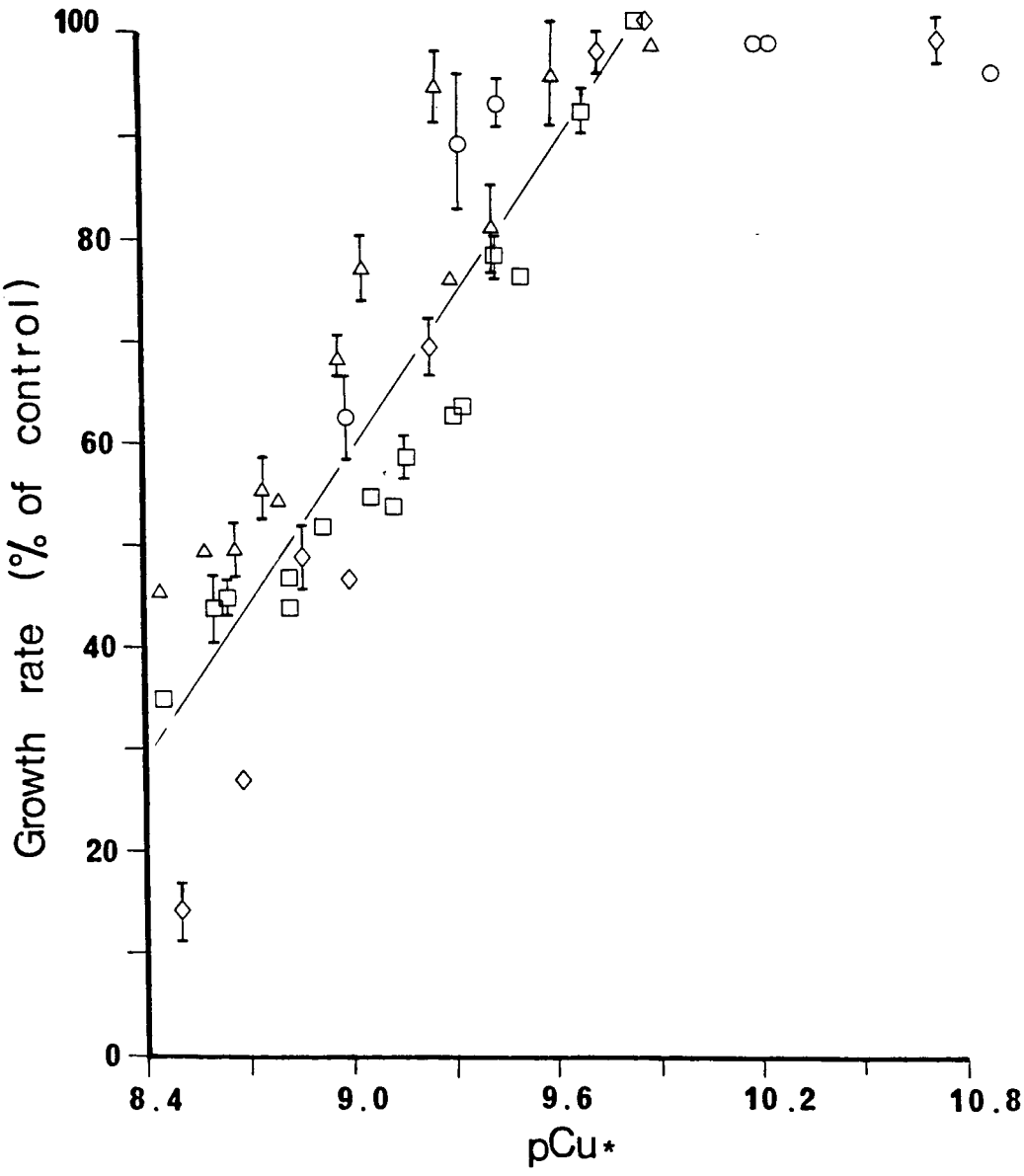


Figure 14. Growth rate (% of control) versus the calculated pCu* for all the bioassay results. Symbols: ○ EDTA; □ NTA; ◇ HIS; and △ GLU. Bars are ±1 s.d.



total Cu concentration of 11.9 x 10^{-7}M was exceeded. Since the organism should only respond to the cupric ion activity of the solution, no further decrease in growth should be seen when Cu is added above this solubility limit. However, this did not seem to be the case. Cu concentrations above 11.9 x 10^{-7}M did cause a further reduction in growth (Fig. 3). This phenomenon might be explained in two ways. First, it is possible to obtain a metastable state with respect to the free cupric ion whereby supersaturation of the solution does occur (E.V. Grill pers. comm.) or second, the computer model may overestimate the extent to which the carbonate ion complexes the cupric ion (error in the Cu-carbonate stability constants). In either case the cupric ion activity of the solution could then exceed the solubility predicted by the model. Because of this, precipitation was not considered when calculating pCu* levels in the culture medium.

3. Effect of pH

Since pH is an important factor when considering Cu chemistry, the pH changes that occur over the course of the bioassay were examined. The pH was monitored daily over a six-day period in cultures that had no Cu added and in cultures containing 1.57 x 10^{-7}M Cu. Copper was added to one set of cultures to limit growth to determine if a pH change was solely due to growth.

The pH in cultures containing 1.57 x 10^{-7}M Cu increased marginally over the six day period while the pH in cultures

without any Cu increased substantially in the last two days of the bioassay (Table V). The pH was related to the growth of the organism because of photosynthetic removal of CO_2; significant pH changes were seen only after cell numbers exceeded 50,000 cell ml^{-1}.

Table V. Variations in pH of cultures with and without the addition of Cu over a five day period

	No Cu added		1.57×10^{-7}M Cu added	
	pH	cells ml^{-1}	pH	cells ml^{-1}
Day 0	8.02±.05[1]	1791±166	8.02±.05	1620±104
Day 1	8.06±.03	5879±294	8.07±.02	3297±102
Day 2	8.08±.03	15127±792	8.07±.03	5475±82
Day 3	8.17±.02	47726±2254	8.09±.02	7727±188
Day 4	8.38±.03	124704±5519	8.11±.03	9045±307
Day 5	8.58±.07	238597±14488	8.11±.03	10156±467

[1]Mean ±1 s.d based on 3 replicates.

4. Fe Additions

Due to the low solubility of Fe in seawater, special consideration was given to the preparation and mode of Fe addition. EDTA has often been added to culture media to keep Fe solubilized (Droop, 1961; Johnston, 1964). This practice was not suitable for my application since EDTA has a strong affinity for Cu and would drastically alter the Cu speciation in the

experimental cultures. Therefore, tests were performed to determine the optimal method of Fe preparation and addition that would maximize the growth of the organism but eliminate the need for an Fe chelator.

In the first experiment, the beneficial effect of the Fe-EDTA mix was tested over that of a freshly prepared Fe stock. Cultures having an Fe-EDTA mix as an Fe source grew at the same rate and had the same cell yield as did cultures having only freshly precipitated ferric hydroxide added (Fig. 15). The freshly prepared ferric hydroxide stock could be added either before or after autoclaving with no change in the results.

Autoclaving of the Fe stock before its addition to the medium did, however, cause a substantial decrease in growth. Both the growth rate and final cell yield were considerably reduced as compared to the cultures with freshly prepared Fe and cultures having no Fe added (Fig. 16).

Ageing of the stocks had a similar effect as that of autoclaving. When a stock was aged for 3 months and added to the medium, both the growth rate and final cell yield of the organism was greatly reduced (Fig. 17). Ageing of the Fe stocks for one week was not sufficient to cause any reduction in the growth rate of the organism.

Because the organism grew for 3-4 days in test cultures containing no added Fe, residual Fe must have been present in the medium, even though the Fe level was below 1.79×10^{-8}M as measured by graphite furnace AAS. This Fe may be a result of carryover from the innoculum, Fe in the cell pool of the

organism, or Fe contamination in the sample. Further studies in this lab have been conducted on the chemistry of Fe in Aquil and its availability to phytoplankton (Wells *et al.*, in press).

Figure 15. Cell growth with fresh Fe stocks (□) and Fe-EDTA stocks added before (△) and after (◇) autoclaving.

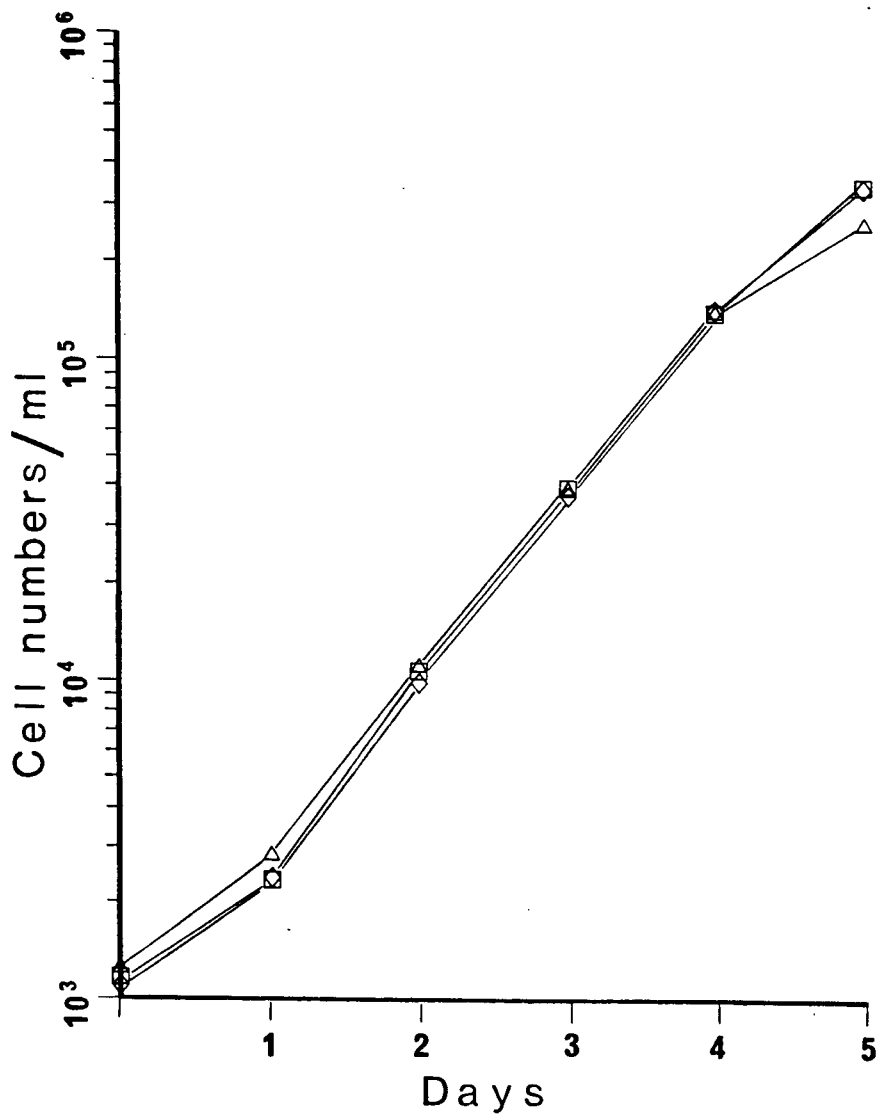


Figure 16. Cell growth with fresh Fe (△), no Fe (□) and autoclaved Fe stocks (◇) added to the culture medium.

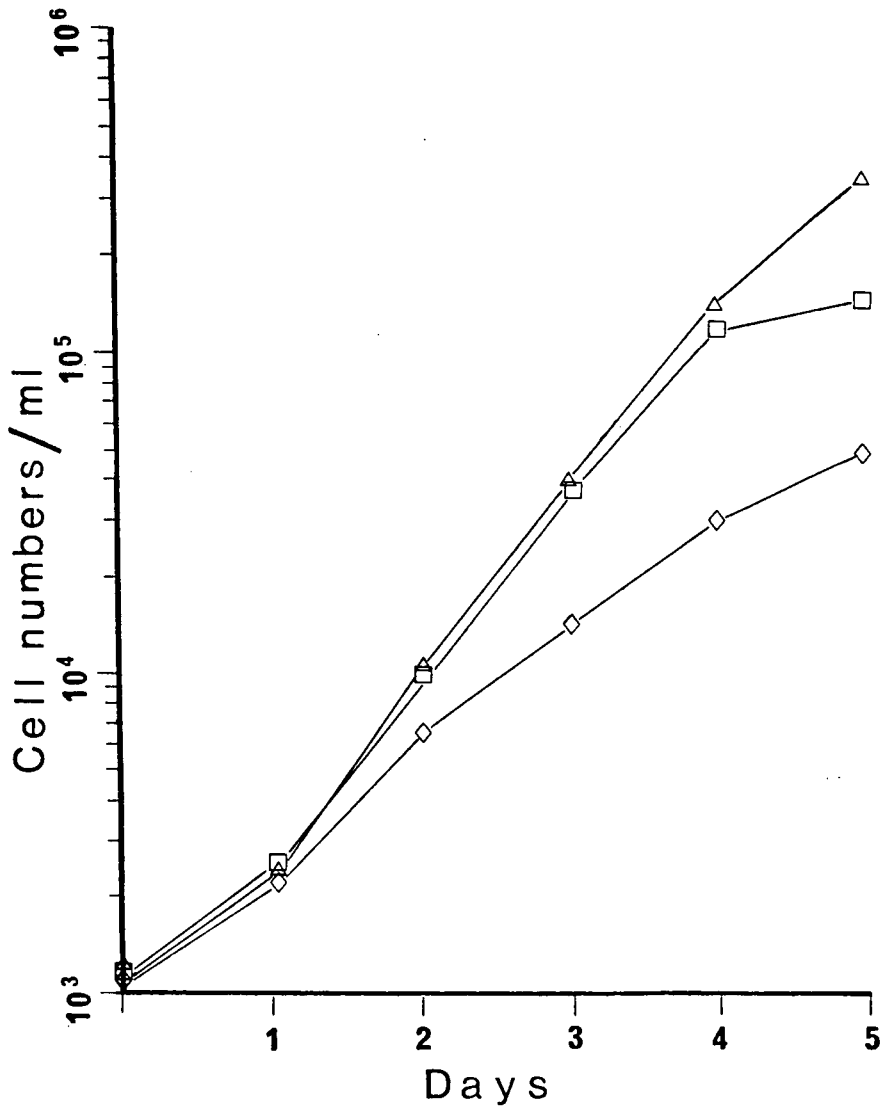
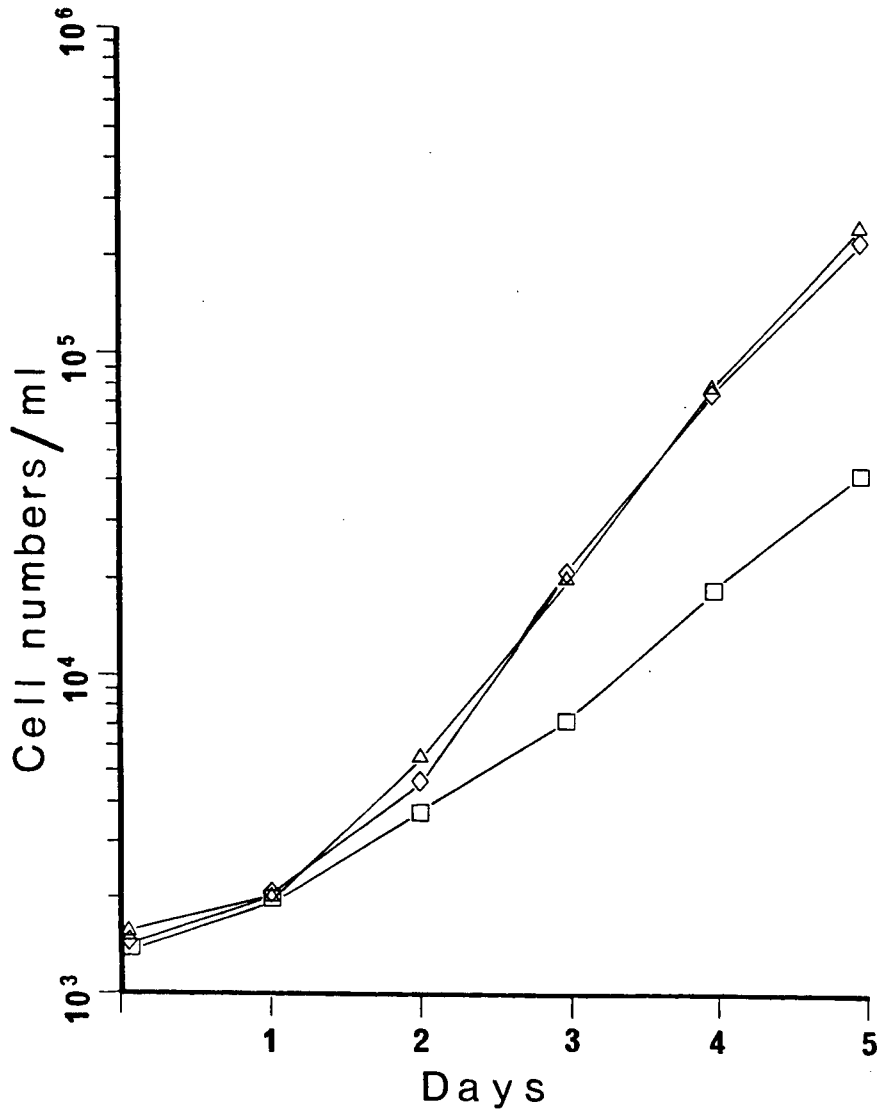


Figure 17. Cell growth with fresh Fe stocks (△) and Fe stocks aged for one week (◇) and three months (□) added to the culture medium.



D. DISCUSSION

1. Growth Rate as a Function of pCu*

In cultures containing no organic ligands, growth inhibition of T. pseudonana was found when Cu exceeded a concentration of 1.57×10^{-8}M. However, upon the addition of EDTA, GLU, HIS, or NTA, much higher Cu levels could be added with no deleterious effect. This indicated a reduction in biologically active Cu in the presence of these complexing agents. The decrease in toxicity was attributed to a decrease in the cupric ion activity of the medium by complexation of the cupric ion. Other workers have presented evidence that the effect of Cu on the growth of T. pseudonana (Davey, 1975; Sunda and Guillard, 1976; Gavis et al., 1981) as well as other phytoplankton species (Anderson and Morel, 1978; Canterford and Canterford, 1980; Jackson and Morgan, 1978) can be correlated better with the cupric ion concentration than with the total Cu concentration.

An estimation of the pCu* in the medium was made using the computer model MINEQL. From these results, growth inhibition of T. pseudonana was seen below a pCu* of 10.0 while total growth inhibition occurred at a pCu* below 8.0. Sunda and Guillard (1976) found similar results; i.e., growth inhibition of T. pseudonana was found below a pCu* of 10.5 and total growth inhibition was seen below 8.3. They calculated pCu* values in the cultures of Davey et al. (1973) and found that total growth inhibition of their test organism also occurred at a pCu* below

8.0.

The calculation of pCu* in seawater has its limitations. Variations in published stability constant data and, to a lesser extent, problems with activity coefficient corrections seriously compromise the accuracy with which pCu* can be computed. Most stability constants are derived in solutions unlike that of seawater and are determined at a different ionic strength. The extrapolation of these stability constants to a seawater matrix and the concurrent ionic strength corrections needed may lead to inaccurate results.

The relationship between the calculated pCu* and growth rate was quite strong when any one ligand was examined (Figs. 10-13). However, when the pCu* values for all the ligands and ligand concentrations were combined, the relationship was not nearly as strong (Fig. 14) and this weakness was attributed primarily to inaccuracies generated in the computer model. When considering each ligand, even though inaccurate thermodynamic data may be used to determine the pCu* of the medium, the bias in the calculations would be consistent over all the Cu and ligand concentrations used. However, the bias in the calculations will differ for each ligand. The result will then be a greater variability in the estimation of pCu* in a multi-ligand plot with a subsequent weaker relationship between pCu* and growth rate.

The growth rate data will be used for comparison purposes with an analytical technique designed to estimate biologically active metal. Presumably growth rate is an absolute measure of

pCu even though the latter value cannot be calculated precisely in the culture medium.

2. Experimental Considerations

The bioassay technique was used to measure biologically active Cu in a culture medium containing a particular combination of ligand and Cu concentrations. Many factors must be considered when using this technique. The important considerations will be discussed below.

Changes in Organism Response to Cupric Ion Activities: While the difficulties in accurately calculating pCu* have been discussed, the reproducibility of the growth rate of the organism at a given pCu has not been considered. To use growth rate to estimate pCu, it must be assumed that the organism's response to a specific pCu value does not change with the addition of any other component in the medium. However, recent studies have indicated that the addition of $Si(OH)_4$ (Rueter et al., 1981) and Mn^{2+} (Sunda et al., 1981) can increase the growth of phytoplankton while a given pCu level is maintained in the medium.

Rueter et al. (1981) hypothesized that Cu inhibits the functioning of a $Si(OH)_4$ transport site on the cell and that both are transported into the cell at the same site. In addition, he suggested that the pCu in the medium controls the rate of Cu uptake and the concentration of intracellular Cu, which in turn controls the growth rate. Therefore, increasing the concentration of $Si(OH)_4$ allows it to compete more

successfully for this common uptake site and reduces Cu uptake.

To explain the Mn effect, Sunda et al. (1981) proposed a simple cellular binding model in which Cu competes with Mn at an internal binding site, such as an enzyme. When the enzyme is bound to Cu it would be inactivated and growth would be decreased. An increase in the Mn concentration allows Mn to displace Cu from this site and lesser amounts of the enzyme will be deactivated. This Mn-Cu interaction will be discussed in more detail in Section V.

In the case of Mn, the concentration added to the culture medium was much lower than the concentration of Cu needed to cause toxicity. At such a ratio it was assumed that the competition of Mn was at a minimum. Therefore, little or no manganese interference was expected.

Changes in pH over the term of the bioassay: In seawater, the distribution of cupric ion and its complexes varies with pH (Zirino and Yamamoto, 1972). In the presence of only inorganic ligands, cupric ion is thought to be mainly complexed by OH^- and CO_3^{2-} ions in seawater at a pH of 8.0 (Florence and Batley, 1976; Kester et al., 1975; Zirino and Yamamoto, 1972). An increase in pH results in increasing the concentration of these ligands and hence an increase in the complexation of Cu and a decrease in the free cupric ion concentration. In the presence of organic ligands, their ability to complex Cu is dependent on their inherent stability constant and the pH of the solution. As the pH increases there is less proton competition for the ligand and more free ligand is available for complexation. (The

effect of pH is dependent on the pK of the functional group that complexes the metal). Thus an increase in pH will not only increase the concentration of both OH^- and CO_3^{2-} ions, but also may increase the concentration of the active ligand concentration.

For these reasons, it is important to maintain a steady pH throughout the period of the experiment. Buffers are a way of accomplishing this but were not considered because such agents generally complex metal ions. In the pH experiment, major pH changes were seen in rapidly growing cultures at the end of the experimental period (Table V) and the pH was related to the concentration of cells in the medium and not to the time span of the experiment. An increase in pH became apparent when the medium contained 50,000 cells ml^{-1} or greater. However, in the bioassay experiments, cultures inhibited by Cu infrequently reached the cell concentrations that were found to affect pH. In some instances though, higher cell concentrations were obtained but only on the last day of the test. Because of this, the pH changes that did occur in the cultures were not believed to have a significant effect. Although there was a larger increase in pH in the control cultures, a higher pH did not affect growth in such cultures.

Bacterial Considerations: The presence of bacteria could affect the results of the bioassay by possible modification of the culture medium. Although precautions were taken to eliminate bacteria from the culture medium, possible contamination could result from the unsterilized trace metal

stocks added to the medium. Erickson (1972) examined the effect of bacteria on Cu toxicity to T. pseudonana and found that the growth response to Cu was the same in both bacteria free and bacteria containing seawater. It was noted that if the seawater he employed had only a small amount of organic substrate present then large numbers of bacteria were probably not present. Because of Erickson's work, the precautions taken to reduce bacteria, and the low amounts of organic material present in the Aquil medium, bacteria were not believed to affect the results in a significant manner.

3. Environmental Consideration

To determine the relevance of the pCu* values found to be toxic in this study comparisons were made with pCu* values calculated for natural waters. Sunda (1975) obtained a ratio for the activity of cupric ion to the total Cu concentration of $10^{-1.8}$ in seawater free of organic ligands having a chlorinity of 19 ppt, a temperature of 25°C and a pH of 8.2. Sunda and Guillard (1976), using Sunda's ratio and Chester and Stoner's (1974) estimations of Cu levels in open ocean waters (2-60 x 10^{-9}M), calculated a seawater pCu* range of 10.5-9.0 with a mean pCu* of 9.7. This range overlaps the pCu* range found to be inhibitory to T. pseudonana. This implies that natural Cu levels could be toxic to this phytoplankton species in seawater containing little or no organic complexing agents.

Binding of Cu to organic molecules has been proposed as a means of reducing Cu toxicity in natural waters (Steeman Nielsen

and Wium-Andersen, 1970). Studies involving Cu titrations of river water, lake water or coastal seawater utilizing ion-selective electrodes (Stiff, 1971; Sunda and Hansen, 1979), toxicity bioassays (Gächter et al., 1978; Gillespie and Vaccaro, 1978) and Cu adsorption by MnO_2 (van den Berg and Kramer, 1979a,b) all predicted that dissolved Cu is primarily bound by organics. If a phytoplankton organism only responds to free Cu and not to organically bound Cu, as indicated by the present study, then to measure the total concentration of the metal in natural waters as an estimate of toxicity is meaningless.

IV. THE MEASUREMENT OF BIOLOGICALLY ACTIVE CU BY A STRONGLY ACIDIC CATION EXCHANGER

A. INTRODUCTION

None of the analytical methods developed to date have been shown to measure the level of biologically active Cu in seawater. However, certain analytical methods have the potential for measuring the chemical (and hence the biological) activity of metals in solution. One of these methods is ion-exchange (Allen et al, 1975; Mancy and Allen, 1977; Treit et al., 1983).

Ion-exchange can be used to separate cations or anions using, respectively, strongly acidic or strongly basic resins. Filby et al. (1974) passed filtered river water through both an anion-exchange and a cation-exchange resin and divided the dissolved metals into anionic and cationic adsorbed species. Neutral species did not adsorb on either resin. In the three rivers studied, Zn was found to be mostly cation-exchangeable.

Shuman and Dempsey (1977) also used a strongly acidic cation-exchange resin to pre-concentrate cationic metal species in river waters before the metals were determined by atomic absorbtion spectrophotometry. Cationic species of Cd, Cr, Cu, Pb and Zn were observed with some anionic species of Cr, Cu and Zn also being present. Using natural seawater, Marchand (1974) determined various physico-chemical forms of five radioactive metal isotopes by cation-exchange chromatography. An aliquot of seawater was spiked with the appropriate radionuclide and added

to the top of a cation-exchange column. The column was then eluted with seawater. Anionic, cationic, neutral and colloidal species were estimated from the rate of metal elution. The metal species that rapidly eluted were grouped as neutral and negatively charged and the slowly eluted species as positively charged.

In this section, the potential of a strongly acidic cation-exchange resin to measure the concentration of the free cationic species of Cu in seawater was examined. An equilibrium approach using a column procedure was utilized. The model organic ligands used in the bioassays were also used to control the chemistry of Cu in the resin experiments. To determine if the resin measurement could be used as an estimate of biologically active metal, growth rates from the bioassays were compared to the results of the resin analysis.

B. THEORETICAL CONSIDERATIONS

1. Introduction to Ion-Exchange Resins

The most widely used ion-exchangers are synthetic organic resins. Adams and Holmes (1935) originally described the preparation of synthetic ion-exchange resins. Synthetic resins offer advantages over natural ion-exchangers in that (1) synthetic resins have greater physical and chemical stability, (2) they can be prepared as spheres of uniform size, (3) they have much greater exchange capacities, (4) the rate of exchange is faster, and (5) the resin can be made-to-order for various needs (Rieman and Walton, 1970).

Modern cation-exchangers are considered to be porous salts containing an insoluble anion and mobile cations that can be exchanged for ions of equal charge from the surrounding medium. The resulting ion-exchange reaction is reversible, stoichiometric and follows the law of mass action. Their exchange characteristics are mainly determined by the acidic groups attached to the organic matrix such as phenolate, sulphonate or carboxylate groups (Schubert, 1948).

Many factors influence the differential uptake of ions onto a cation-exchange resin. The adsorption of ions is influenced by their size and charge, the intrinsic properties of the resin (such as mesh size, capacity, degree of cross-linkage), the relative concentrations of the ions capable of exchange, and the reaction time (Dorfner, 1972). Rules indicating how each of these factors affects selectivity have been found empirically

and these can be found in Helfferich (1962) and Dorfner (1972).

Conventional cation-exchange resins have been used to distinquish free metal ions from those bound to organic ligands for many decades. The measurement of the concentration of free metal in equilibrium with a cation-exchange resin has been used to examine stability constants for different metal-organic complexes in soils (Schnitzer and Skinner, 1966; Matsuda and Ito, 1970; Ardakani and Stevenson, 1972) and in freshwater (Allen et al, 1975).

2. Theory

If a cation-exchange resin of the strong acid type that is loaded with sodium ions, Na^+, is brought in contact with a solution containing a divalent metal ion, M^{2+}, the exchange reaction can be expressed by the equation

$$M^{2+} + 2Na^+R^- = M^{2+}(R^-)_2 + 2Na^+ \qquad [5]$$

where R^- represents a negative site in the ion-exchange matrix. Thus applying the law of mass action one obtains the following expression

$$K_1 = \frac{\{ MR \} \{ Na \}^2}{\{ M \} \{ NaR \}^2} \qquad [6]$$

where { } denotes activity, K_1 is the thermodynamic equilibrium

constant and ionic charges are omitted for the sake of simplicity. Rewritting [6] in terms of concentrations and appropriate activity coefficients we obtain

$$K_1 = \frac{f_{MR}\ f_{Na}}{f_M\ f_{NaR}} \cdot \frac{[\ MR\ ]\ [\ Na\ ]^2}{[\ M\ ]\ [\ NaR\ ]^2} \qquad [7]$$

where the activity coefficients are denoted by f and [ ] denotes the metal concentration in the solution and on the resin. Although the activity coefficient for an ion in the solution outside the resin bead can be estimated (e.g., using the Davies approximation; see eqn. 1, Section III.B.C.), that of the ions inside the beads cannot be determined easily. Because of this, K_1 is usually combined with the activity coefficients to obtain an apparent equilibrium constant

$$K' = \frac{[\ MR\ ]\ [\ Na\ ]^2}{[\ M\ ]\ [\ NaR\ ]^2} = K_1 \frac{f_M\ f_{NaR}}{f_{MR}\ f_{Na}} \qquad [8]$$

which has a value entirely dependent on analytically measureable quantities. It must be kept in mind that, unlike a thermodynamic constant, K' varies with the nature and concentration of the electrolyte; i.e., K' will remain constant only as long as the activity coefficient ratios of the species undergoing exchange remain constant.

When the metal ion is of a considerably lower concentration than the cation of the background electrolyte ([Na] >> [M]), any ion-exchange reaction involving the metal ion will not have a significant effect on the composition of the solution or resin phases. As a result, the activity coefficient ratios of the ions in the resin and solution phases will remain essentially constant. Moreover, since the concentration of Na^+ in both the solution and resin phases undergoes only a negligible change, equation [8] can be rewritten in the form

$$\lambda = \frac{[MR]}{[M]} = K_1 \frac{[NaR]^2}{[Na]^2} \qquad [9]$$

where λ is termed the distribution coefficient for M. The value of the distribution coefficient, λ, is clearly dependent on the characteristics of the metal and solution for which it is measured.

Schubert's Approach: As demonstrated by Schubert (1948), ion-exchange can be used to estimate stability constants of metal complexes by measuring their adsorptions onto a cation-exchange resin. That is, by first measuring the distribution coefficient, λ, in the absence of a complexing agent and then determining the amount of metal on the resin in the presence of a complexing agent, it is possible to estimate the amount of metal bound by the complexing agent. This can be done because, even with the complexing agent present (compare Section

III.B.C.), the resin is still in equilibrium with the free metal ion as expressed by equation [9]; thus the concentration of the free metal ion in solution is given by

$$[M] = \frac{[MR]}{\lambda}, \qquad [10]$$

and that contained in the complex is just the difference between the total and free metal concentrations.

3. Application of the Ion-Exchange Procedure to the Determination of Cationic Cu Species in Seawater

In complex media such as seawater, where Cu occurs not only as the free ion but also in various cationic complexes such as $CuCl^+$ and $Cu(OH)^+$, the amount of Cu that binds to a cation-exchange resin will be determined by the concentration and distribution coefficient of all such species. However, as long as the various solution parameters (i.e., salinity, pH, alkalinity) remain constant, then, due to equilibria that exist between the various species, their concentrations are all proportional to the activity of the free Cu ions. The total amount of Cu adsorbed by the resin is therefore a fixed function of the activity and concentration of free Cu and, moreover, of the total Cu present in dissolved inorganic species. Therefore, equation [9] can be rewritten using the total inorganic Cu concentration instead of the free cupric ion:

$$\lambda_1 = \frac{[MR]}{[M\ inorg]} \qquad [11]$$

where λ_1 is a conditional distribution coefficient defined in terms of [M inorg], the total amount of Cu contained in inorganic species.

As a consequence of the above relationship, if seawater is passed through a column of cation-exchange resin until the latter has achieved equilibrium with the feed, the amount of Cu adsorbed will be directly related to the total inorganic Cu concentration and , hence, the pCu of the sample. Before these values can be determined, however, it is first necessary to calibrate the resin. This can be done by determining how the amount of Cu adsorbed from seawater free of organic ligands and having a given salinity and pH varies as a function of the total Cu concentration. The distribution coefficient, λ_1, which is given by the slope of this relationship, can then be used to calculate the concentration of inorganic Cu from values of adsorbed Cu observed for seawater samples containing various organic ligands (providing that the complexes formed with the latter are neutral or negatively charged).

To further estimate the pCu of seawater, all the complexing ligands, their concentrations, and the total Cu concentration must be known. With these, the concentration of the free cupric ion can be calculated by computer modelling techniques (e.g.,

Westall et al. 1976; MINEQL). An activity coefficient, estimated by the Davies approximation, can then be applied to this free metal ion concentration to obtain the pCu for that particular total Cu concentration. Once a ratio between total inorganic Cu and pCu is established this can be applied to other total inorganic Cu concentrations to determine their appropriate pCu. (This ratio is constant as long as the precipitation of Cu does not occur, the concentration of the inorganic ligands does not change, and the salinity and pH remain constant.) Since the ratio of the cupric ion activity to the total inorganic concentration as calculated by MINEQL at an ionic strength of 0.7, is $10^{-1.83}$, multiplication of [M inorg] by this factor provides an estimate of the pCu of the solution. Thus one can write an equation of the form

$$\lambda_2 = \frac{[MR]}{[M\ inorg] \times 10^{-1.83}} = \frac{[MR]}{\{ Cu \}} \qquad [12]$$

where λ_2 represents a distribution coefficient defined in terms of pCu. Thus to estimate the pCu of an unknown sample, it is passed through the resin column until equilibrium is achieved with the feed solution, and then the concentration of Cu bound to the resin is measured and the pCu of the sample evaluated by dividing the concentration of adsorbed Cu by λ_2.

It should be noted that for the purpose of estimating trace metal activities in seawater, the above method will only work if

the resin has similar selectivities for alkali, alkaline earth and transition metals. If a resin that is highly selective for transition metals, such as Chelex-100, is used, the amount of Cu adsorbed will be affected by competition for sites between the different transition metals in the sample. Consequently, the amount of Cu adsorbed would be a function of the trace metal composition which, in contrast to the major seawater cations, is highly variable and not related to the salinity.

C. MATERIALS AND METHODS

1. Column Preparation

(a) Materials

Artificial seawater (SOW) was used as the experimental medium in all tests. Its preparation is described in Section III.B.1. The SOW was passed through a Chelex-100 column before use to remove trace metal contaminants. For each batch of SOW, the pH was measured and, if necessary, adjusted to pH 8.0±.05. Copper stocks were prepared daily from a 1000 mg l^{-1} standard of $CuCl_2$ made up in nitric acid. To obtain ultrapure acid, 12N HCl was isothermally distilled to give a final concentration of purified 5N HCL. Acidified SOW was used in the elution of the resin columns. This solution was prepared by adding 2 ml of 5N HCL to each liter of SOW.

Resin-column experiments were performed with 1.0 cm (i.d.) x 5.0 cm Econocolumns® (Bio-Rad laboratories, Richmond, CA. U.S.A.) which consisted of a glass tube, a polyethylene bed support, and a polypropylene reservoir and column tip; both ends of the column were equipped with easy connector luer fittings (Fig. 18). Particles up to 35 μm were retained by the bed support. Precise flow rates in the columns were maintained by a Technicon® autoanalyzer pump used in conjunction with Lancer manifold pump tubing (Lancer, St. Louis, Mo. U.S.A.). The flow rates were determined by the diameter of the tube used. Polypropylene microtubing (i.d. 1.19 mm) was used to connect the

pump tubes to both the columns and sample bottles. Eppendorf pipette tips (clear, 10-100 µl) were used as connectors between the microtubing, columns and pump tubing.

Strongly acidic Dowex-50 cation-exchange resin was used (Bio-Rad Laboratories AG 50W-X12). The sulphonic acid resin was obtained in the hydrogen form as 200-400 mesh beads: Batch No. 10572. The resin, which is composed of sulphonic acid groups attached to a styrene divinylbenzene polymer lattice ($R-SO_3^-$), had an exchange capacity of 5.0 meq g^{-1} (dry wt.), 12% crosslinkage and an apparent pK < 1 (Helfferich, 1962, p.86).

Various sizes of Nalgene polypropylene bottles were used throughout the study. To reduce trace metal contamination, the bottles were soaked in 6N HCL for 3 days, rinsed 3X with GDW, and then soaked in 0.5N HCL for one week. Finally, they were rinsed, filled with GDW, and stored until use.

(b) Preparation of and column procedure

All column manipulation was carried out in the Laminar flow hood described earlier. One gram of resin (direct from the bottle) was weighed out, slurried in 4-5 ml of GDW and poured into a column. The resin was washed with 50 ml of GDW and then allowed to equilibrate with the GDW for 1-2 days prior to use. A soluble red compound was evident in the initial wash water and was assumed to be an organic by-product of the manufacturing process. Initially, methanol was used to remove any residual organics present in the resin; however, there did not seem to be any beneficial effect of the methanol rinse over that of simply

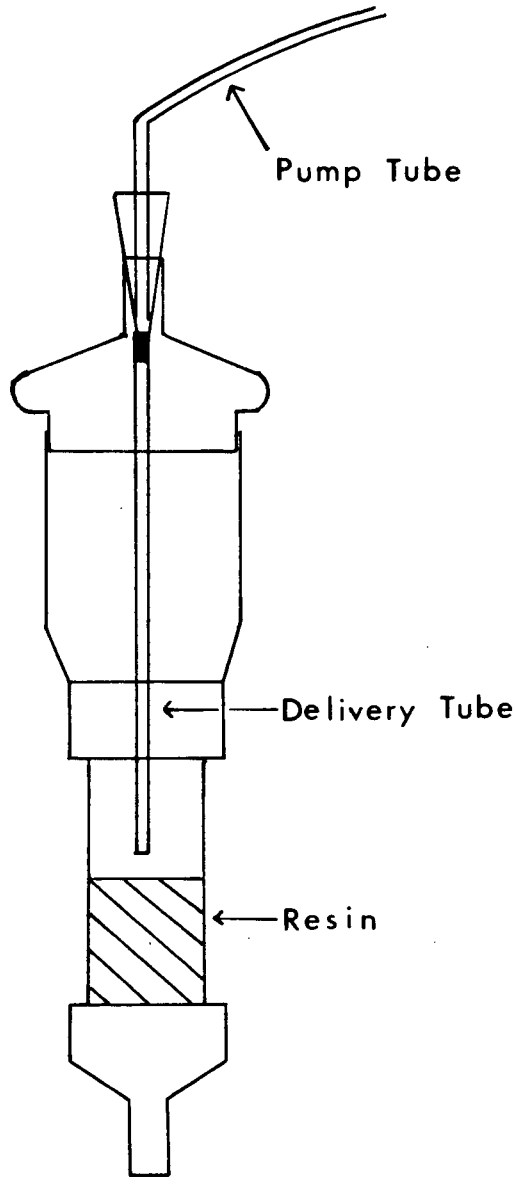


Figure 18. Econocolumn.

washing with GDW so this step was eliminated. One to ten columns were prepared at any one time.

To remove any trace metal contaminants present, the resin was washed with 50 ml of acidified SOW (pH ca. 1.0). It was then converted to the appropriate ionic composition and pH by reacting it with 50 ml of SOW at pH 8.0±.05. During the conversion step, the pH of the effluent began to rise sharply after only 10-15 ml of SOW and was the same as the influent after 35-40 ml. A fluid head of 1 cm was kept above the resin at all times.

Due to the high degree of resin cross-linkage, there was only a small amount of shrinking or swelling with a change in ionic form. Also, a slight darkening of the resin was apparent as the resin lost H^+ and began to equilibrate with the SOW.

At this point the appropriate solution was ready to be passed through the column. The test solution was pumped via the autoanalyzer pump apparatus to the econocolumn at a constant flow rate. The solution was delivered to just above the resin bed by a polypropylene microtube (see Fig. 18).

After the samples had passed through the resin, any sample present in the interstial spaces of the resin or in the luer fitting of the column was removed before the elution step was begun. This was accomplished by allowing a slight positive pressure to build up in the columns by continuing the pumping action of the flow system after the delivery tubes had run dry. (Approximately 0.3 ml of residual solution was expelled.)

Because the transfer tubes for feeding the sample solution

were also used for the eluent, the tubes were cleaned before the eluent was passed through. This involved disconnecting the tubes from the columns, flushing them with GDW, and with acidified SOW. Finally, the tubes were reconnected to the columns and the elution procedure was begun.

(c) Procedure for elution of column prior to ASV

To determine the amount of Cu bound to the resin at the end of an analysis, the column was eluted with acidified SOW (pH ca. 1.0) and the Cu concentration in the eluate determined by ASV. The first 25 ml of the eluate was collected in 50 ml polycarbonate centrifuge tubes containing polypropylene caps. Preliminary studies indicated that most of the Cu was removed with the first 15 ml of the eluate. However, 25 ml was collected to ensure maximum Cu recovery at all Cu concentrations. The same apparatus used to pump the sample through the column was also used to pump the eluent.

When the elution procedure was completed the resin was immediately regenerated by passing a further 15 ml of acidified SOW through the resin and then reacting it with 50 ml of SOW at pH 8.0±.05. The resin was stirred with a glass rod to remove any bubbles trapped in the resin during the elution procedure. The columns were stored in this ionic form until future use.

2. ASV Procedure for Measuring Total Cu

(a) Equipment

The current-voltage measurements were made with a Princeton Applied Research Corporation (PAR) model 374 polarographic analyser. The electrolysis cell consisted of a PAR K66 cell top and a PAR K60 borosilicate glass cell bottom. The glass cells were coated with a 0.5% chlorosilane solution to reduce Cu adsorption onto the wall of the vessel. Sample solutions were stirred with an 8 x 13 mm teflon-covered stirring bar coupled to a constant speed magnetic stirrer. A PAR K77 saturated calomel reference electrode (SCE) was placed in a PAR K65 reference electrode bridge tube with a vycor tip to form a double-junction reference electrode. The outer junction was filled with artificial sea water (SOW). A bare platinum wire served as the auxiliary electrode.

Glassy carbon electrodes were prepared by sealing wafers of 0.63 cm diameter glassy carbon (Beckwith Carbon Corp.) into a 112 cm length of Pyrex glass tubing with epoxy resin. The glassy carbon was then polished metallographically with a 0.05 μm alumina slurry being used in the final polishing step to attain a mirror-like finish. A small quantity of mercury was placed inside the glass tube to make an electrical contact between a platinum wire and the glassy carbon disc. Wax was poured into the tube to prevent the loss of mercury. Finally, the electrode was soaked in dilute HCL for 24 hr to remove contaminants from the polishing process. Polishing of the

electrode was necessary intermittently and this simply involved a light scrubbing of the electrode with a 0.05 μm alumina slurry on a piece of felt.

(b) Pre-plating and pre-conditioning of the electrode

A separate electrolysis cell and ASV unit was used in the pre-plating of the electrode. A PAR model 174A polarographic analyser was used in conjunction with a 200 ml teflon electrolysis cell machined from a teflon rod. Stirring was by a teflon stirring bar used in conjunction with a magnetic stirrer.

Before beginning any analyses, the electrode was pre-plated to produce a mercury film. This step involved placing the electrode in a solution of 500 mg l^{-1} $HgCl_2$, made slightly acidic with nitric acid. After purging with N_2 for 10 min, a potential of -0.2 V vs SCE was applied to the electrode for 50 seconds. The current to the electrode was kept between 0.2 and 0.3 ma by adjustment of the stirring rate. The Hg film thickness was controlled by the stirring rate and the deposition time.

Once the plating step had been completed, the electrode was transfered to the electrolysis cell of the model 374 polarographic analyser where it was then placed in a solution of acidified SOW and subjected to 3-4 plating-stripping sequences under the same operating conditions used in the subsequent analysis. The electrode was prevented from drying during its transfer by keeping a large drop of solution at the end of the electrode.

The thickness of the Hg film is an important parameter. The solubility of Cu in Hg is reported to be only 0.002% at 20°C (Stephen and Stephen, 1963). Therefore, if the film is too thin the Hg can become supersaturated with Cu at low solution concentrations, a phenomenon which will result in a change of slope in the response curve. Thick films were also not desirable because there is a broadening of the Cu peak, presumably due to a longer time required for diffusion of the Cu out of the Hg film.

(c) General procedure

Once the electrode was pre-plated and pre-conditioned a sample was placed in the electrolysis cell. Initially, the sample was purged with N_2 for 5 min to remove all the O_2 from the sample (the N_2 was deoxygenated by passing it through a vanadous chloride solution as described by PAR application note No. 108). After purging, a potential of -0.7 V vs SCE was applied to the electrode for a preselected time to concentrate the metal into the mercury film. During this time the solution was stirred at a constant rate and a N_2 atmosphere was maintained in the cell. After plating, stirring was ceased and the solution was allowed to come to rest for 15 sec while maintaining the applied potential. Finally, an anodic scan from -0.7 V to -0.15 V was made using the differential pulse mode (pulse height, 50 mv) at 5 mv s^{-1} to strip the metal out of the mercury film. The deposition time was dependent upon the

concentration of Cu in the sample.

In preliminary studies, it was found that the height of the current peak for Cu in the first plating-stripping sequence was always lower than the peak height of the second and further sequences. Therefore, two plating-stripping sequences were performed on the sample with the first sequence used to condition the electrode and the second sequence used to quantify the metal.

(d) Calibration

There are at least two methods of relating the voltammetric response to the metal concentration in the sample. These are through the use of a working curve obtained from analysis of standard solutions and through the method of standard additions.

Calibration using a working curve, whereby the voltammetric responses from a set of standards are compared to the voltammetric response from the sample in question, is the simplest method. However, care must be taken to simulate the same conditions in the working standards as those found in the solution of interest as peak height can be dependent on factors in the solutions other than the concentration of the metal being measured. Such factors include pH, the presence of surface active agents, the ionic strength, and the nature of the supporting electrolyte.

To compensate for the effect of the sample matrix the method of standard additions is often used. It involves first an initial determination of the height of the current peak of

the metal in solution and then, after adding a spike of the same metal, determining the increase in height of the current peak. If the added metal proportions itself amongst all the forms of the metal in the sample the standard addition method measures the total metal content of the sample. If, however, the standard only proportions itself amongst the ASV labile species, then the analysis gives only the labile metal concentration. Interpretation of the ASV results from the standard addition method is thus difficult when the distribution of the added metal is uncertain.

For ASV to unambiguously determine the 'total' metal in a sample, all the metal must be converted to ASV labile forms. In the case of total dissolved metal in seawater, the sample is usually first filtered through a conventional 0.45 μm filter and then acidified to ca. pH 2 with HCL or HNO_3. Under the acid conditions, the metal is believed to be quantitatively converted to labile forms. Other more severe treatments such as ultraviolet irradiation or nitric acid digestion are sometimes used to attempt complete conversion (Florence and Batley, 1977a).

Throughout the present study, only measurements of total Cu were made. Thus all samples were acidified with isothermally distilled HCL to attain a pH of ca. 2.0. Both the working curve and standard addition methods were used to calibrate the metal level in a sample. In most cases the working curve was used when measuring Cu levels in a chemically well defined solution such as SOW while the standard addition method was used in the

analysis of natural seawater samples so as to take into account any matrix interferences.

(e) Procedure for measuring total Cu in the eluates of the resin analysis

In the resin technique, Cu was eluted from the resin using 25 ml of acidified SOW and the total Cu concentration in the eluates was determined by ASV using the working curve method.

The general procedure involved (1) preparing and pre-conditioning an electrode, (2) generating two voltammetric traces for each sample, and (3) generating a standard curve. The same operating conditions and procedures as outlined in Section IV.C.2.c were used when analyzing these samples. Generally, an aliquot of 10 ml was necessary for each ASV measurement.

Standard curves were generated from the acidified SOW that was used to elute the columns. An aliquot (10 ml) of this solution was added to the electrolysis cell and subsequently spiked with increasing Cu concentrations. The voltammetric response for each Cu spike was then measured. The height of the voltammetric current peak for Cu of the samples was then compared to that of the standards to estimate the Cu concentration in the samples. The concentration range of the standard curve was determined by the concentration range of the samples under study.

The sensitivity of the electrode would infrequently change over the course of the analysis. When this occurred, standard

curves were run during the sample analysis which reduced the effect of any sensitivity changes.

An experiment to determine the precision of the ASV procedure was conducted. A 500 ml solution of acidified SOW (pH ca. 2.0) was spiked with Cu to give a total Cu concentration of 2.36×10^{-7}M. The sample was allowed to equilibrate for 2 hr before aliquots (25 ml) were transferred to 10 acid cleaned 50 ml polycarbonate tubes. These samples then underwent the same analysis procedure used to estimate the Cu levels in the eluates of the resin analysis.

3. Characterization of the Resin AG 50W-X12

(a) Column equilibration

To determine the volume of sample needed to attain column equilibration, SOW containing a known concentration of Cu was passed through the resin and the Cu in the influent and effluent was measured.

One l of SOW was spiked with Cu to give a concentration of 7.89×10^{-8}M and left for 2 hr to allow equilibration. Two columns were prepared and 300 ml of the sample was passed through each column at a flow rate of 1.7±0.1 ml min^{-1}. The effluent was collected in 50 ml aliquots and both the influent and effluent was measured for total Cu by ASV.

The effect of flow rate on the equilibration of the resin was studied. Copper was added to three 500 ml portions of SOW to give a concentration of 7.89×10^{-8}M and left for 12 hr prior

to use. Six columns were prepared and 250 ml of SOW were passed through the appropriate columns at 0.8 ml min^{-1}, 2.0 ml min^{-1} or 2.9 ml min^{-1}. Each flow rate was run in duplicate. When the solutions had passed through the resin the columns were eluted and the eluates measured for Cu by ASV.

(b) Precision

Precision was determined by replicate analysis of a sample of SOW containing 7.89 x 10^{-8}M Cu. Two aliquots of resin were tested under different operating and chemical conditions. For the first aliquot, eight columns were prepared and 250 ml of SOW containing 7.89 x 10^{-8}M Cu was passed through each column at a flow rate of 1.7 ml min^{-1}. The eight columns were then eluted and the eluates analysed for Cu by ASV. In the second aliquot, ten columns were prepared and 250 ml of SOW containing 7.89 x 10^{-8}M Cu was passed through each column at a flow rate of 2.9 ml min^{-1}. The columns were eluted and the eluates analysed for Cu by ASV. In this experiment, Fe was added to all test solutions at a concentration of 4.5 x 10^{-7}M to determine the effect of Fe on the precision.

(c) Effect of pH on the adsorption of Cu by the resin

Ten columns were prepared for this experiment. Five 500 ml aliquots of SOW containing 7.89 x 10^{-8}M Cu were adjusted to different pH values (7.53, 7.96, 8.13, 8.15, 8.42) by the addition of 0.1N HCL or 0.1N NaOH. These samples were then

passed through the appropriate column at a flow rate of 1.7 ml min^{-1}. All the columns were eluted and the eluates analysed for total Cu by ASV. Samples for each pH were run in duplicate.

(d) Effect of salinity on the adsorption of Cu by the resin

Four 500 ml aliquots of SOW of 5, 15, 25 and 35 ppt salinity were prepared by dilution of SOW at 35 ppt with GDW. Copper was added to all samples to give a final concentration of 7.89×10^{-8}M. The pH of the samples was measured and, if necessary, adjusted to ca. pH 8.0 with the addition of 0.1N NaOH.

Ten columns were prepared and 250 ml of the various salinity samples were passed through eight columns at a flow rate of 2.9 ml min^{-1}. Two additional columns had 250 ml of SOW (35 ppt), without added Cu, passed through them. All tests were run in duplicate. All columns were eluted and the eluates analysed for Cu by ASV.

(e) Effects of nutrients on adsorption of Cu by the resin

In the first experiment, two 500 ml samples of SOW were prepared. In the first sample, Cu was added to give a concentration of 7.89×10^{-8}M while in the second, Cu was added in the same concentration but, in addition, all the Aquil nutrients and trace metals were added at their Aquil concentrations. Then 250 ml of both samples were passed through separate columns at a flow rate of 1.7 ml min^{-1}. Duplicates of

each sample were run. The columns were eluted and the eluates analysed for Cu by ASV.

In the second experiment, six 500 ml samples were prepared as follows: 1) SOW containing 7.89 x 10^{-8}M Cu; 2) SOW containing 7.89 x 10^{-8}M Cu plus the Aquil nutrients and trace metals except for Fe; 3) SOW containing 7.89 x 10^{-8}M Cu with Fe at the Aquil concentration (4.5 x 10^{-7}M); 4) same as 3 but 2x the Fe concentration; 5) same as 3 but 3x the Fe concentration; 6) same as 3 but 4x the Fe concentration. The samples were left for 12 hr to equilibrate after which 250 ml of each sample was passed through the appropriate columns at a flow rate of 2.9 ml min^{-1}. The columns were then eluted and the eluates analysed for Cu by ASV. In addition, the total Cu concentration in all samples was determined by ASV.

In the third and final experiment, Fe stocks were prepared in different manners. Three Fe stocks were prepared at their Aquil concentration as follows: 1) $FeCl_3$ was added to GDW and left for 5 min before its addition; 2) as in 1 but the Fe stock was allowed to precipitate and age for 6 hr; 3) as in 2 but the Fe stock was aged for 72 hr. The Cu and Fe stocks were then added to SOW to give solutions having 1) only 7.89 x 10^{-8}M Cu; 2) only a 5 min aged Fe stock; 3) 7.89x 10^{-8}M Cu plus a 5 min aged Fe stock; 4) 7.89 x 10^{-8}M Cu plus a 6 hr aged Fe stock; and 5) 7.87 x 10^{-8}M Cu and a 72 hr aged Fe stock. The solutions were allowed to equilibrate for 12 hr and then 250 ml of each sample was passed through the appropriate column at a flow rate of 2.9 ml min^{-1}. The columns were eluted and the eluates

analysed for Cu by ASV.

(f) Adsorption curves for Cu in SOW with no organic ligands present

Adsorption curves for Cu were generated under different flow rates, with and without the addition of Fe. In the first experiment, eight columns were prepared and 250 ml portions of SOW containing 3.93, 7.87, 11.9 or 15.7 x 10^{-8}M Cu were passed through the appropriate column at a flow rate of 1.7 ml min^{-1}. Each Cu concentration was run in duplicate. The columns were eluted and the eluate analysed for Cu by ASV. In the second test, nine columns were prepared and 250 ml portions of SOW containing Cu from 0 to 31.5 x 10^{-8}M Cu at 3.93 x 10^{-8}M increments were passed through the appropriate column at 2.9 ml min^{-1}. Iron was added to all of these samples at a concentration of 4.5 x 10^{-7}M. Once again the columns were eluted and the eluates analysed for Cu by ASV.

4. Model Ligand Study

The same ligands used in the bioassays (EDTA, GLU, HIS, and NTA) were used in these resin experiments. Ligand stocks were prepared from reagent grade chemicals and were made up at ca. 1000X the final ligand concentration. The ligand and Cu concentrations used are shown in Table VI. Two series of experiments were performed using the same ligands and Cu concentrations except that some operational parameters were

changed. All Fe stocks were prepared fresh and allowed to equilibrate for several hours before use.

Table VI. The ligands, ligand concentrations and Cu concentrations studied (+).

Ligand	Ligand Addition ($\times 10^{-7}$M)	Cu Addition ($\times 10^{-8}$M) 3.93	7.89	11.8	15.7
EDTA	0.5	+	+	+	+
	1.0	+	+	+	+
	2.5		+		+
	5.0		+		+
GLU	100.		+		+
	250.		+		+
	500.		+		+
	750.		+		+
HIS	1.0		+		
	2.5	+	+	+	+
	5.0		+		
NTA	1.0		+		+
	2.5		+		+
	5.0		+		+
	7.5		+		+
	10.0		+		+

In the first series of experiments, eight columns were prepared and were run simultaneously. Of these eight, six columns were used for the test solutions and two columns were used for standard Cu solutions. The test solutions were made up in SOW by adding a concentration of Cu (either 7.89 x 10^{-8}M or 15.7 x 10^{-8}M Cu) and the appropriate concentration of ligand. The standard Cu solutions were made up in SOW by adding either 7.89 x 10^{-8}M or 15.7 x 10^{-8}M Cu, with no ligands added. Three concentrations of the same ligand and one standard were all run

at the same time.

Both the test and standard Cu solutions were made up in 500 ml portions 12 hr prior to use to allow equilibration of the ligand with Cu. Once the samples had equilibrated, 250 ml of the standard and each of the test solutions were passed through the appropriate column at a flow rate of 1.7 ml min^{-1}. Duplicates of both the test and standard solutions were run. The columns were then eluted and the eluates analysed for Cu by ASV. The Cu concentration bound to the resin from the standard Cu solution was used to calibrate the amount of Cu bound to the resin from the test samples.

Except for EDTA, the same ligand concentrations and Cu concentrations were studied in the second series as in the first but some operational and chemical conditions were changed. The flow rate was changed from 1.7 ml min^{-1} to 2.9 ml min^{-1} and Fe was added to all the samples to give a final concentration of 4.5×10^{-7}M. Ten columns were prepared and run simultaneously without duplication to allow two concentrations of Cu (7.89×10^{-8}M or 15.7×10^{-8}M), four concentrations of one ligand, and two standard solution (either 7.89×10^{-8}M or 15.7×10^{-8}M Cu) to be analysed at the same time. Samples were prepared in 250 ml portions and left to equilibrate for 12 hr. The samples were then passed through the appropriate column. Finally, the columns were eluted and the eluates analysed for Cu by ASV. With EDTA, four concentrations of Cu (3.93, 7.89, 11.8 and 15.7×10^{-8}M) and two ligand concentrations (5.0×10^{-8}M and 10.0×10^{-8}M) were used at one time.

D. RESULTS

1. Characterization of the Resin in Artificial Seawater

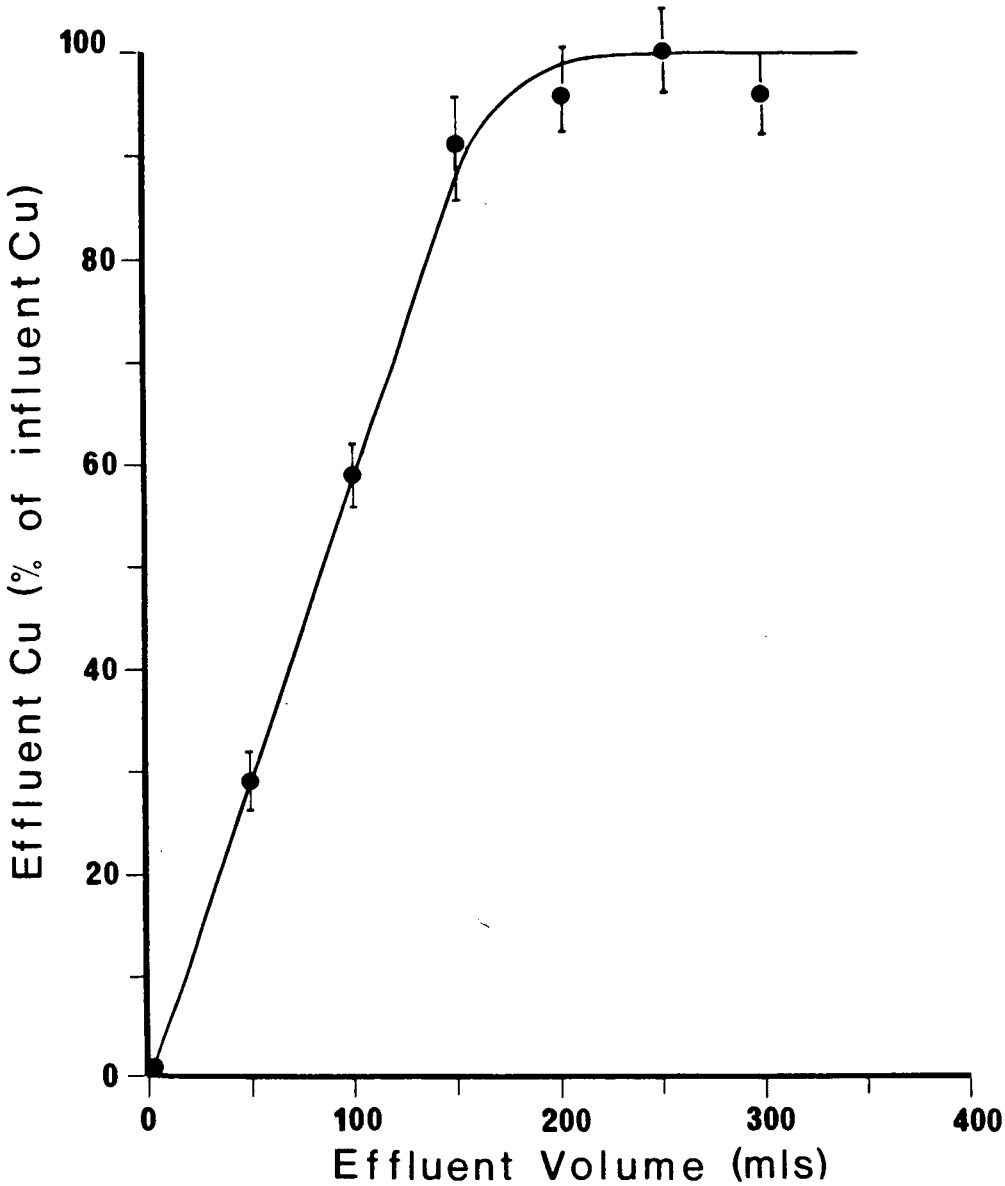
(a) Equilibration of the resin to Cu

It is important that the ion-exchanger attain complete equilibrium with the input solution before determining the amount of Cu bound to the resin. To determine the volume of sample needed to equilibrate one gram of the resin, the column effluent was monitored for Cu as 300 ml of SOW containing 7.87 x 10^{-8}M Cu was passed through it. Fig. 19 shows the effluent Cu concentration calculated as a percentage of the influent plotted against the volume of effluent. A large percentage of the Cu in solution was removed by the resin from the first 200 ml of sample. Beyond 200 ml, however, the difference between the influent and effluent concentrations was less than the standard deviation of the ASV technique. Consequently, a sample volume of 250 ml was used throughout the resin experiments.

The volume of sample needed to equilibrate the resin potentially changes with variations in the flow rate. As the flow rate increases the time that a unit volume of solution is in contact with the resin decreases leaving less time for equilibration. On the other hand, increasing the flow rate decreases the film diffusion layer around the resin bead, possibly resulting in faster equilibration times (Dorfner, 1972).

In order to test the effect of flow rate on column

Figure 19. Effluent Cu (% of influent) versus the effluent volume. Values are a mean of 2 replicates ±1 s.d.



equilibration, SOW containing 7.87 x 10^{-8}M Cu was passed through the resin at 0.8, 2.0 and 2.9 ml min^{-1}. While less Cu was adsorbed by the resin at the lowest flow rate (0.8 ml min^{-1}), above 2.0 ml min^{-1} the difference was not significant (Table VII). Thus, it appears that flow rates equal to or exceeding 2.0 ml min^{-1} do not have a significant influence on the volume needed for equilibration. The observed difference at the lowest flow rate may, in part, be due to pressure effects caused by pumping the samples through the columns.

Flow rates much above 2.9 ml min^{-1} were not possible because of the back pressure developed, while very low flow rates were not practical because of the time needed for the analysis.

Table VII. The effect of flow rate on the adsorption of Cu by the resin. SOW containing 7.87 x 10^{-8}M Cu was passed through the resin at three flow rates.

Flow rate	Eluate Cu (x10^{-9}mol g^{-1})
0.8 ml min^{-1}	5.04±0.31[1]
2.0 ml min^{-1}	6.14±0.00
2.9 ml min^{-1}	5.98±0.47

[1]Mean ±1 s.d. based on 2 replicates

(b) Precision of the ASV and column adsorption technique

The precision of the adsorption technique was determined using two separate batches of resin. SOW containing 7.87 x 10^{-8}M Cu was passed through a series of columns and the amount of Cu adsorbed by the resin then measured. The relative standard deviation of the analysis, which averaged 8% (Table VIII), was similar in both cases. Although, the mean Cu concentration bound to the resin was different in the two tests, these differences are attributable to the different batches of resin used and variations arising from their preparation.

However, the precision of the column method includes the variability of the ASV technique used to quantify the Cu in the column eluates. To separate the variability of the ASV technique from that of the variability due to the resin, the precision of the ASV procedure was determined. Ten aliquots (25 ml) of SOW, having 2.36 x 10^{-7}M Cu added, were transfered to the polycarbonate tubes used to collect the column eluates. The Cu levels in these tube were then determined by using the normal ASV procedure.

The precision of the ASV procedure was the same as reported for the overall column method (RSD 8%, Table IX). Thus it was concluded that the variability between the resin columns was, at least, within the variability of the ASV procedure.

Table VIII. Precision of the resin analysis determined by replicate analysis of SOW containing 7.87 x 10^{-8}M Cu. A and B are analyses on two separate batches of resin.

Column	Cu adsorbed to resin ($x10^{-9}$mol g^{-1}) A	B
1	5.51	4.41
2	6.14	4.41
3	5.82	4.41
4	5.98	4.72
5	5.82	4.56
6	5.82	4.25
7	5.19	4.41
8	4.88	4.72
9		5.04
10		4.41
Mean	5.67	4.56
Standard Deviation	0.47	0.32
Relative Standard Deviation	8 %	7 %

Table IX. Precision of the ASV procedure. Determined by replicate analysis of SOW containing 2.36 x 10^{-7}M Cu.

Tube Number	Cu Conc. ($\times 10^{-7}$M)
1	2.64
2	2.68
3	2.47
4	2.29
5	2.27
6	2.20
7	2.38
8	2.25
9	2.24
10	2.15
Mean	2.36
Standard Deviation	.183
Relative Standard Deviation	8 %

(c) Effect of pH

The response of the resin was tested over the entire pH range that would be encountered in these experiments. There were no significant differences in the uptake of Cu by the resin between pH values of 7.57 and 8.15 (Table X). However, an increase of pH to 8.42, did cause a reduction in the amount of Cu bound to the resin.

Table X. The effect of pH on the adsorption of Cu by the resin. SOW containing 7.87 x 10^{-8}M Cu was passed through the resin at various pH values.

pH	Eluate Cu ($x10^{-9}$mol g^{-1})
7.53	6.77±0.02[1]
7.96	7.08±0.11
8.13	7.08±0.26
8.15	6.92±n.a.[2]
8.42	5.51±0.33

[1]Mean ±1 s.d. based on 2 replicates.
[2]n.a. not available.

(d) Nutrient effects

The Aquil nutrients and trace metal mix were added to SOW to determine if their presence affected the adsorption of Cu by the resin. A preliminary study indicated that their addition

increased the amount of Cu bound to the resin as compared to when they were not present (Table XI). Further tests were performed to determine which nutrients caused this response.

Table XI. The effect of the Aquil nutrients and trace metals on the adsorption of Cu by the resin. SOW containing 7.87×10^{-8}M Cu with and without the addition of Aquil nutrients and trace metals was passed through the resin.

	Eluate Cu ($\times 10^{-9}$mol g^{-1})
SOW alone	6.30±0.35[1]
SOW + Nutrients + Trace Metals	7.71±0.06

[1]Mean ±1 s.d. based on 2 replicates

Adsorption of Cu by ferric hydroxide is well documented (Davis and Leckie, 1978; Swallow et al., 1980), and its addition to SOW might therefore be expected to effect the response of the resin. Consequently, Fe was the first nutrient tested.

In the first experiment, the adsorption of Cu in the presence of Fe alone was compared with that in the presence of all the other nutrients except Fe. SOW without nutrients or Fe addition was used as a control. It was found that similar amounts of Cu were bound to the resin from both the control samples and the samples with the Aquil nutrients minus Fe (Table XII). When Fe was added to SOW, however, the amount of Cu bound to the resin increased, although the addition of up to 4X the initial Fe concentration caused no further increase.

Increased Cu uptake in the presence of Fe was not due Cu contamination from the Fe stock. There was no increase in the total Cu concentrations of the samples upon the addition of the Fe stock, as measured by ASV (Table XII) and , increasing the Fe concentration up to 4X did not increase the Cu uptake significantly as would be expected if the Fe stock was contaminating the sample.

Table XII. The effect of Fe on the adsorption of Cu. SOW containing 7.87 x 10^{-8}M Cu with various Aquil nutrient and Fe additions passed through the resin. Also the total Cu levels in the samples before analysis are presented.

Treatment	Eluate Cu ($x10^{-9}$mol g^{-1})	Sample Cu ($x10^{-8}$M)
SOW alone	6.45	7.08
SOW + Nutrients -FE	6.92	7.08
SOW + Fe	8.18	6.92
SOW + 2X Fe	8.50	8.03
SOW + 3X Fe	8.81	6.92
SOW + 4X Fe	8.50	6.92

To further test the effect of Fe on the resin, Fe stocks aged for different lengths of time were added to SOW containing 7.87 x 10^{-8}M Cu. Fe stocks freshly prepared (added before any precipitate was evident), aged for 6 hr and aged for 72 hr were added to the SOW and passed through the resin. The highest Cu concentrations bound to the resin were with the SOW containing

the freshly prepared Fe stock (Table XIII). The 6 hr and 72 hr Fe samples had less adsorption of Cu than did the 5 min Fe stock sample but they still had higher levels than the SOW having no Fe added.

When freshly prepared Fe stock was added to SOW containing no Cu and passed through the resin, there were non-detectable levels of Cu bound to the resin. This was a further indication that there was no Cu contamination from the Fe stocks. This suggests that the increased adsorption of Cu by the resin was correlated with an adsorption of colloidal ferric hydroxide.

Table XIII. The effect of aged Fe stocks on the adsorption of Cu. SOW containing 7.87 x 10^{-9}M Cu with the addition of Fe stocks aged for 5 min, 6 hr and 72 hr was passed through the resin.

	Eluate Cu ($x10^{-9}$mol g^{-1})
No Cu-5 min Fe	n.d.[2]
No Fe (control)	5.82±0.63[1]
Cu-5 min Fe	11.80±0.47
Cu-6 hr Fe	8.97±0.47
Cu-72 hr Fe	8.81±0.31

[1]Mean ±1 s.d. based on 2 replicates
[2]n.d. non-detectable

A mechanism that might account for adsorption of hydrous ferric oxides by the resin is suggested by the observation of Parfitt and Smart (1978). They found a strong adsoption of

sulphate ions by Fe oxides which they attributed to a displacement of surface hydroxyl groups and coordination of the adsorbed SO_4^{2-} ion by surface Fe^{3+} ions. However, according to the site-binding model of Davis and Leckie (1978) on adsorption onto Fe oxide surfaces, the sulphate ion is bound at protonated surface sites forming $Fe-OH_2-SO_4^-$ or $Fe-OH_2-HSO_4$ groups, a model also applied by Balistrieri and Murray (1981) to describe the adsorption of sulphate by goethite in seawater. Thus the adsorption of Fe-oxide by the ion-exchange resin might be explained by a similar interaction between the sulphonate groups of the resin and surface $FeOH^{2+}$ groups.

The population of protonated $FeOH_2^+$ surface sites, and hence the adsorption of sulfate ions, is normally only significant at pH values below 8. However, under weakly basic conditions, a coadsorption of Cu and Fe oxides by the resin might occur as a result of a mechanism similar to that observed by Tipping (1981), who found that negatively charged humates were adsorbed onto negatively charged iron oxide surfaces in solutions containing divalent cations. Electrophoretic measurements indicated that there was a coadsorption of divalent cations that presumably decreased the electrostatic repulsion between the adsorbent and adsorbate. Thus, the adsorption of negatively charged Fe-oxides by the resin might be brought about by a similar coadsorption of divalent cations including Cu^{2+}.

Another possible explanation is that there may be trapping of colloidal Fe hydroxide by the resin. However, the aged Fe stocks showed the least activity and they were expected to have

the largest colloidal particles. Also, because of the pore size of the resin beads, colloidal particles are believed to be excluded from the resin. Florence (1977) showed that solutions of colloidal hydrated ferric oxide and large organic dyes were quantitatively rejected by an ion-exchange resin (Chelex-100).

(e) Ionic strength effects

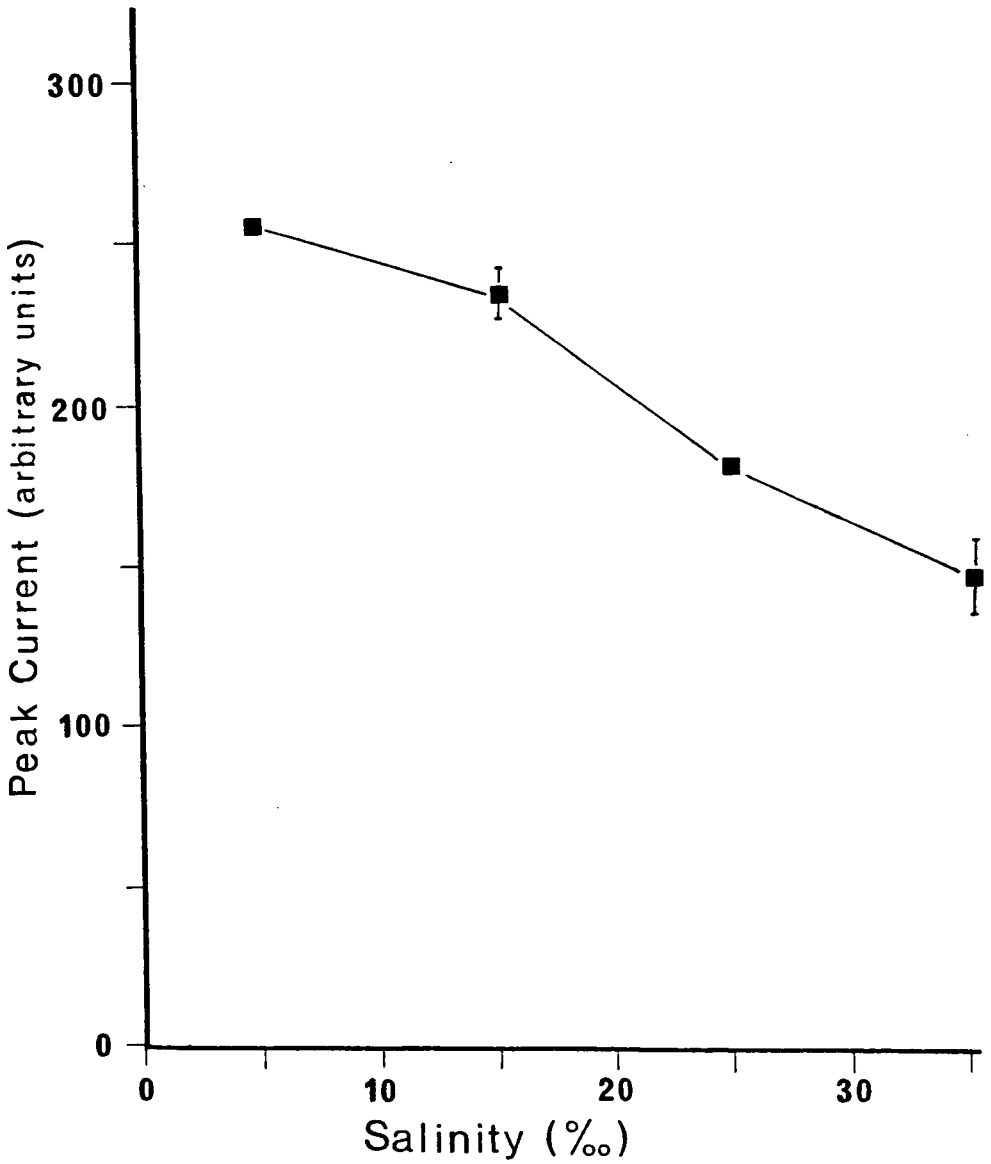
The salinity of the SOW used in the laboratory study was 35 ppt. However, as this technique might be used in future studies at other salinities, it was desirable to determine how the adsorption of Cu varies with salinity.

As the salinity of a sample decreases the concentration of cations competing with Cu for binding sites on the resin also decreases and, hence, the total amount of Cu taken up by the resin should increase. This expectation is confirmed by Fig. 20, which shows that there was almost a doubling of the amount of Cu adsorbed from 5 ppt SOW as compared to 35 ppt SOW. It is evident from these results that calibration of the resin must be made at each salinity studied.

(f) Adsorption curves for Cu

The resin was to be calibrated in the model ligand study by comparing the amount of Cu adsorbed to the resin from the test solutions with that adsorbed from standard Cu solutions containing no organic ligands. To minimize the number of standard Cu solutions needed for calibration a linear

Figure 20. Change in the adsorption of Cu with salinity. Values are a mean of 2 replicates ±1 s.d.



relationship between the amount of Cu bound to the resin and the total Cu concentration in the standard solution was desired.

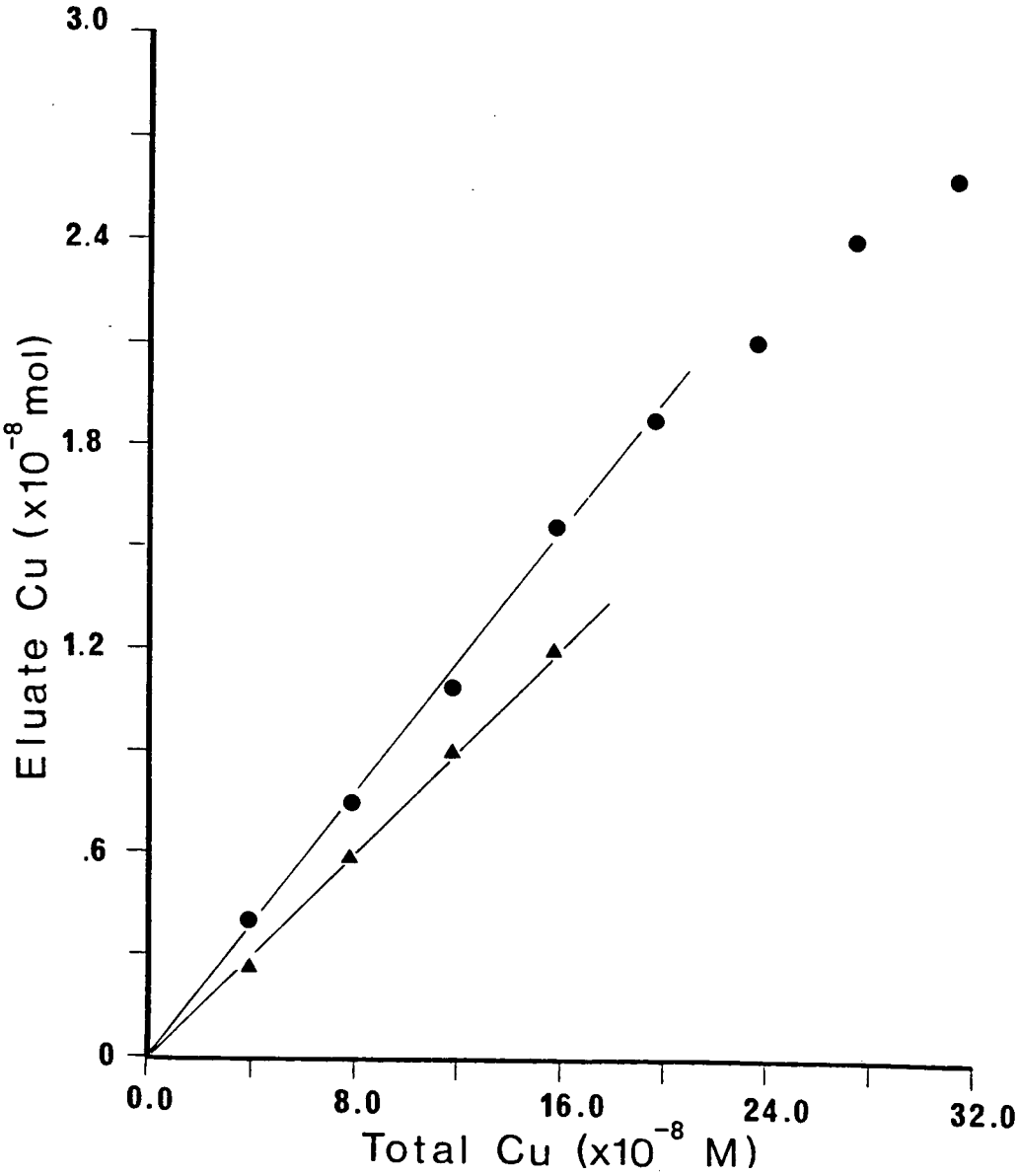
The relationship between the amount of Cu bound by the resin and its concentration in solution between 0 and 15.7 x 10^{-8}M (at 3.93 x 10^{-8}M increments) was found to be linear (Fig. 21). Between 0 and 31.5 x 10^{-8}M (at 3.93 x 10^{-8}M increments), on the other hand, the relationship was only linear up to a concentration of approximately 19.67 x 10^{-8}M Cu (Fig. 21) but, at higher Cu concentrations, the slope of the adsorption curve began to decline.

The slope of the adsorption curves differed between the two tests. An increased adsorption of Cu was seen in the second test due to the presence of Fe in these samples. The increased adsorption due to Fe has been previously discussed (Section IV.D.1.d).

In theory, the adsorption of Cu by the resin is dependent on the cupric ion activity of the sample. Therefore, a linear calibration relationship can be assumed in the case of samples having a total metal concentration in the range of non-linearity as long as their cupric ion activities are in the linear range. Cupric ion activity can be decreased while maintaining a high total Cu concentration by the presence of organic complexing agents.

It has been shown that the calibration relationship is linear within the limits indicated above; however, the slopes of this relationship may differ with different operating conditions. Also, there was some slight variability from one

Figure 21. Adsorption curves for Cu in SOW with (●) and without (▲) the addition of Fe.



series of tests to the next even under apparently identical operating conditions. Hence, standard solutions were always run at the same time and under the same conditions as the sample solution.

3. Model Ligand Study

The ability of the resin technique to measure the complexation of Cu by EDTA, GLU, HIS and NTA was examined. The results of these experiments were reported as 'Cu equivalents' (Cu equiv) which represents the concentration of Cu that, when present in organic ligand free SOW (S=35 ppt, pH=8.0), results in the adsorption of an amount of Cu equal to that adsorbed from the test solution; e.g., a Cu equiv value of 2.5 x 10^{-8}M means that the test solution had adsorbed the same amount of Cu onto the resin as would be adsorbed from a 2.5 x 10^{-8}M standard Cu solution. A Cu equiv is a value proportional to the pCu of a sample, a quantity which can be estimated by the method described in Section IV.B.3. However, the accuracy of the pCu estimate is dependent on the accuracy of calculating the pCu of the standard Cu solutions used to calibrate the resin. Unfortunately, the calculation of pCu, even in well defined artificial seawater, is questionable.

If the Cu equiv value of a test sample was different from its total Cu concentration this indicated that the complexation of Cu in this sample was greater than in a Cu standard of the same total Cu concentration. Since Cu is already complexed by the CO_3^{2-} and OH^- ions in the standards, reduced Cu equiv values

indicated complexation by ligands other than these inorganic ligands. Cu equiv values can therefore be used to indicate the complexation of Cu by the model organic ligands.

In the first series of experiments, the addition of EDTA, GLU or NTA was found to reduce the Cu equiv values of the test solutions (Tables XIV, XV) thus indicating the resin's ability to detect complexation of Cu by these ligands. As the ligand's concentration increased the Cu equiv value of the sample decreased and the extent of reduction was related to the ligand's stability constant for Cu. EDTA, with the highest stability constant (log K_1=20.6), reduced the Cu equiv values markedly at approximately the same concentration as the total Cu concentration levels of the solution. Glutamic acid (log k_1=7.87), on the other hand, had to be added in concentrations two orders of magnitude higher than EDTA to give a similar reduction in the Cu equiv value.

In a second series of experiments the same ligands, ligand concentrations and Cu concentrations were studied but the procedure and some operational conditions were modified. The main procedural change was that all test samples for a particular ligand were used in one experimental run. This approach was taken to eliminate inter-test variations by using the same Cu standards to calibrate all the samples. Two operational conditions were also modified. First, a higher flow rate was used (2.9 ml min^{-1} as opposed to 1.7 ml min^{-1}) to reduce the analysis time. Second, Fe was added to all samples at the Aquil concentration to simulate conditions in the culture

Table XIV. Cu equiv values in SOW in the presence of EDTA GLU and NTA for Series I. Values are Cu equiv ($x10^{-8}M$)

Ligand	Ligand Conc. ($x10^{-7}M$)	Cu Addition $7.87x10^{-8}M$	$15.7x10^{-8}M$
EDTA	0.5	3.93±0.32[1]	10.90±0.00
	1.0	2.52±0.32	6.30±0.15
	2.5	1.26±0.47	0.47±0.02
	5.0	1.10±0.47	n.a[2]
GLU	100.	6.77±0.16	10.54±0.00
	250.	5.19±0.06	7.87±0.47
	500.	3.30±0.47	5.04±0.47
	750.	n.a	3.93±0.32
NTA	1.0	5.19±0.16	11.49±0.15
	2.5	4.56±0.16	7.24±0.47
	5.0	3.93±0.47	5.67±0.15
	7.5	3.46±0.00	4.72±0.47
	10.0	3.46±0.63	n.a.

[1]Mean ±1 s.d. based on 2 replicates.
[2]n.a. not available.

media used in the bioassays.

Once again the addition of EDTA, GLU and NTA reduced the Cu equiv values over that of the total Cu concentration but there were some difference in the absolute values as compared to series one. When using NTA, the Cu equiv values were all higher than the estimates in series one; although the largest difference was only 23%. When using GLU the opposite was true, the results in series two were, on the average, 15% lower than series one with a maximum difference of 32% found in the highest concentration of GLU used. For EDTA, the results between the series were very similar and any differences were within the range of experimental error.

The second series of experiments were believed to be more representitive of the conditions found in the bioassay culture medium because of the presence of Fe. Because of this, the results of series two were used for comparison with the bioassay results.

The addition of HIS produced results very different from that of the other ligands. When HIS was added to SOW (containing 7.87×10^{-8}M Cu), at concentrations high enough to complex a substantial fraction of the Cu, there was a greater amount of Cu adsorbed to the resin than when using HIS-free SOW (Table XVI). Only at the highest ligand concentration did the amount of Cu on the resin begin to decrease.

The experiment with HIS was then repeated using one ligand concentration (2.5×10^{-7}M) and four Cu concentrations (3.93, 7.87, 11.8, 15.7×10^{-8}M). Once again, there was a greater

Table XV. Cu equiv values in SOW in the presence of EDTA, GLU and NTA for Series II. Values are Cu equiv ($x10^{-8}M$).

Ligand	Ligand Conc. ($x\ 10^{-7}M$)	Cu Addition ($x10^{-8}M$) 3.93	7.87	11.9	15.7
EDTA	0.5	2.52	4.09	8.03	12.27
	1.0	1.73	2.83	3.93	6.45
NTA	1.0		6.61		14.16
	2.5		5.51		9.29
	5.0		3.78		6.14
	7.5		3.62		5.51
GLU	100.		6.14		10.70
	250.		4.41		5.67
	500.		3.15		4.41
	750.		2.68		2.68

Table XVI. The adsorption of Cu in the presence of HIS. Solutions of SOW containing 7.87 x 10^{-8}M Cu and various concentration of HIS were passed through the resin.

Ligand Conc. ($x10^{-7}$M)	Eluate Cu ($x10^{-9}$mol g^{-1})
0.0	4.72±0.16[1]
1.0	5.19±0.16
2.5	5.35±0.16
5.0	3.78±0.47

[1]Mean ±1 s.d. based on 2 replicates

concentration of Cu adsorbed up by the resin when HIS was present (Table XVII). Increased Cu levels on the resin, as compared to the standards, are reflected in Cu equiv values being greater than the total Cu concentration present in the sample. These higher levels could not be attributed to contamination from the HIS stock as there was no measurable increase in the Cu concentration of the sample upon the addition of HIS as measured by ASV.

These results can be explained by the uptake of positively charged Cu-HIS complexes by the resin. At the pH of seawater HIS exists mainly as neutral molecules with a small percentage existing as positively charged species (pK's are given in Table XVIII). Complexation of the ligand with Cu will therefore result in complexes with a charge of +2 or +3 which, because of their charge, should be adsorbed by the resin.

Affinity of this type of resin for charged organics has been reported in the literature. Moore and Stein (1951) found a

Table XVII. Cu equiv values from SOW in the presence of 2.5 x 10^{-7}M HIS.

Cu Conc. in SOW ($x10^{-8}$M)	Eluate Cu ($x10^{-9}$mol g^{-1})	Cu equiv. ($x10^{-8}$M)
3.93	3.78±0.16[1]	5.04±0.16[1]
7.87	6.61±0.16	8.66±0.16
11.8	9.13±0.16	12.12±0.32
15.7	11.65±01.2	15.42±01.4
NO HIS (standard)		
7.87	5.98±0.03	

[1]Mean ±1 s.d. based on 2 replicates

strong affinity for HIS by polysulphonated polystyrene resins when examining the separation of amino acids. They found reduced recoveries of basic amino acids, such as HIS, from the resin as compared with the other eleven amino acids tested. Adsorption of positively charged organics have also been shown for other resins. Pakalns et al. (1978) found that 13% of the total exchange capacity of Chelex-100 resin was used in the adsorption of cationic detergents where only 2 and 3% of the total exchange capacity was used for anionic and non-ionic detergents, respectively.

The uptake of the Cu-HIS complex could not only explain the resin's lack of response to the complexation of Cu by the ligand, but also the increased adsorption of Cu by the resin. An increase in adsorption could occur because of the resin responding to both the activity of the inorganic Cu species as well as the activity of the Cu-HIS complex.

Table XVIII. pK's of Histidine

pK of		
H_3L^{2+}	H_2L^{+}	HL
1.82	6.05	9.17

Taken from Sillen and Martel (1971).

4. Comparison of the Chemical Assay and Bioassay Results

The growth rate of the bioassay organism for each combination of ligand and Cu concentration analysed was compared to 1) the Cu equiv values determined by the resin technique, 2) the estimate of pCu calculated from the resin results using the method described in Section IV.B.3 (eqn. 12) and 3) the estimate of pCu* as calculated with the computer model MINEQL. Growth rates and both estimates of pCu are presented in Table XIX.

In Fig. 22, growth rates are plotted as a function of the negative log of the Cu equiv values. There was a strong correlation between the -log Cu equiv estimated by the resin analysis and the growth rate of the organism. A linear relationship was seen between the values of 7.5 and 6.85 which corresponded to concentrations of 3.16×10^{-8}M and 14.1×10^{-8}M Cu, respectively. A plateau in the growth curve was apparent at -log Cu equiv values less than 7.15.

Growth rates were also plotted as a function of pCu as estimated by the resin analysis (Fig. 23). A strong linear relationship was seen between a pCu of 8.7 and 9.3 while, above 9.3, there was no further inhibition of growth. A correlation

Table XIX. Growth rates, and pCu as estimated by the resin analysis and by calculation.

Ligand	Ligand Conc.(M)	Total Cu ($\times 10^{-8}$M)	Resin pCu	Mineql pCu*	Growth rate (% Control)
EDTA	5.0×10^{-8}	3.9	9.44	10.25	100
		7.9	9.21	9.33	90±7[1]
		11.8	8.92	8.99	63±4
		15.7	8.74	8.79	55±2
	1.0×10^{-7}	3.9	9.61	10.91	98±1
		7.9	9.39	10.21	100±1
		11.8	9.23	9.44	94±1
		15.7	9.02	9.05	61±2
GLU	1.0×10^{-5}	7.9	9.04	9.04	78±3
		15.7	8.80	8.74	56±3
	2.5×10^{-5}	7.9	9.19	9.26	96±3
		15.7	9.08	8.97	69±2
	5.0×10^{-5}	7.9	9.34	9.63	101±2
		15.7	9.19	9.33	87±1
	7.5×10^{-5}	7.9	9.42	9.90	100±1
		15.7	9.36	9.60	97±5
NTA	1.0×10^{-7}	7.9	9.01	9.17	59±2
		15.7	8.68	8.82	44±1
	2.5×10^{-7}	7.9	9.09	9.43	79±3
		15.7	8.86	9.06	55±1
	5.0×10^{-7}	7.9	9.25	9.69	93±2
		15.7	9.04	9.34	64±1
	7.5×10^{-7}	7.9	9.28	9.85	102±1
		15.7	9.09	9.51	77±1

[1]Mean ±1 s.d. based on 3 replicates.

coefficient (r) for the curve, excluding points where the growth rates were 100% of the controls, was 0.92.

When the same growth data were plotted as a function of pCu* as estimated by the computer model (Fig. 24), the correlation was not as strong. The correlation coefficient (r) for this curve was 0.77. This suggests that there may have been either interactions in the media affecting the speciation of Cu which the model fails to account for or, as previously discussed, the use of inaccurate stability constants.

Figure 22. Growth rate (% of control) versus the negative log of the Cu equiv values. Data from tests using □ EDTA, △ GLU and ◇ NTA as the model organic ligands.

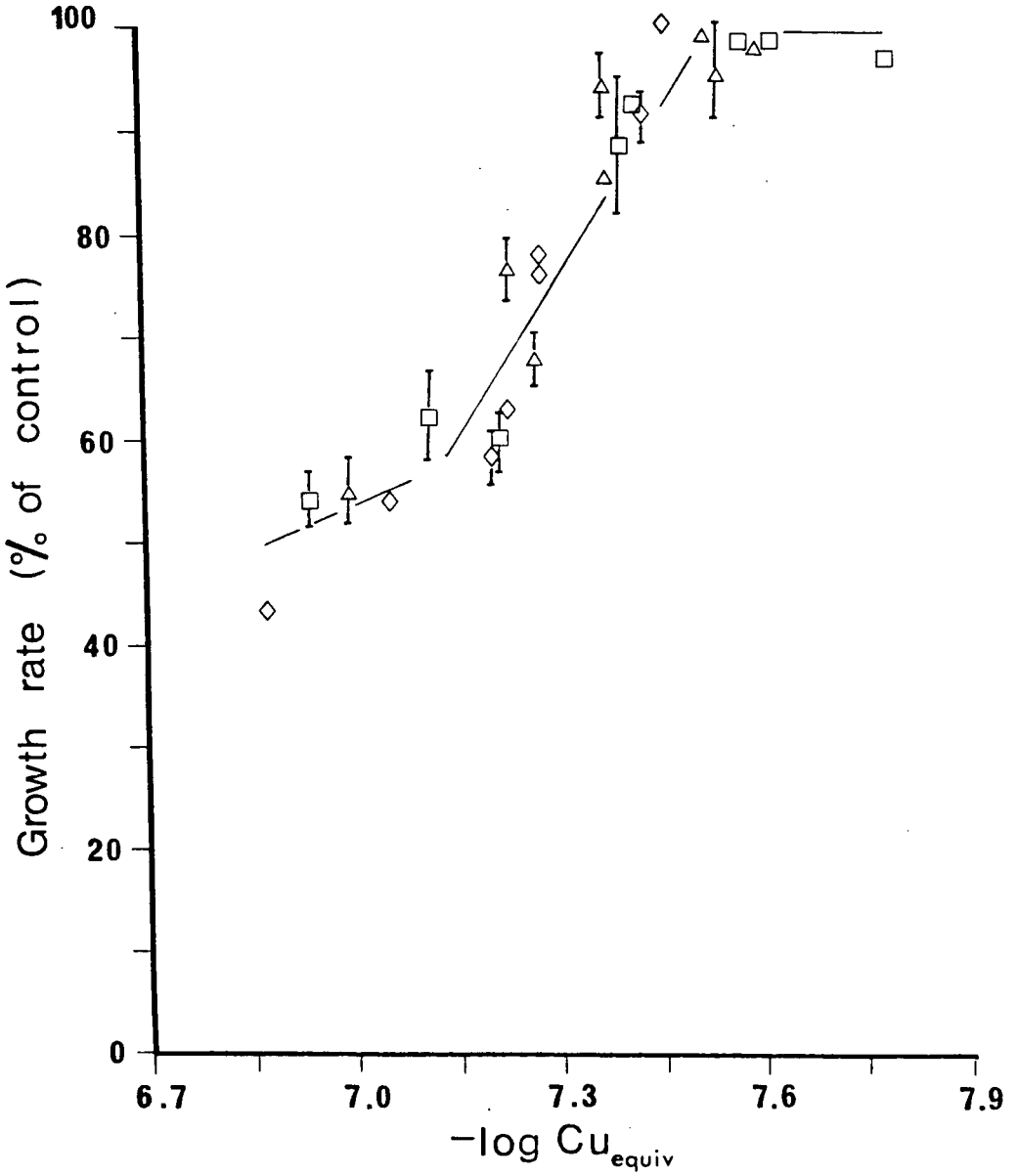


Figure 23. Growth rate (% of control) versus pCu estimated from the resin results. Data from tests using □ EDTA, △ GLU and ◇ NTA as the model organic ligands. Bars are ±1 s.d.

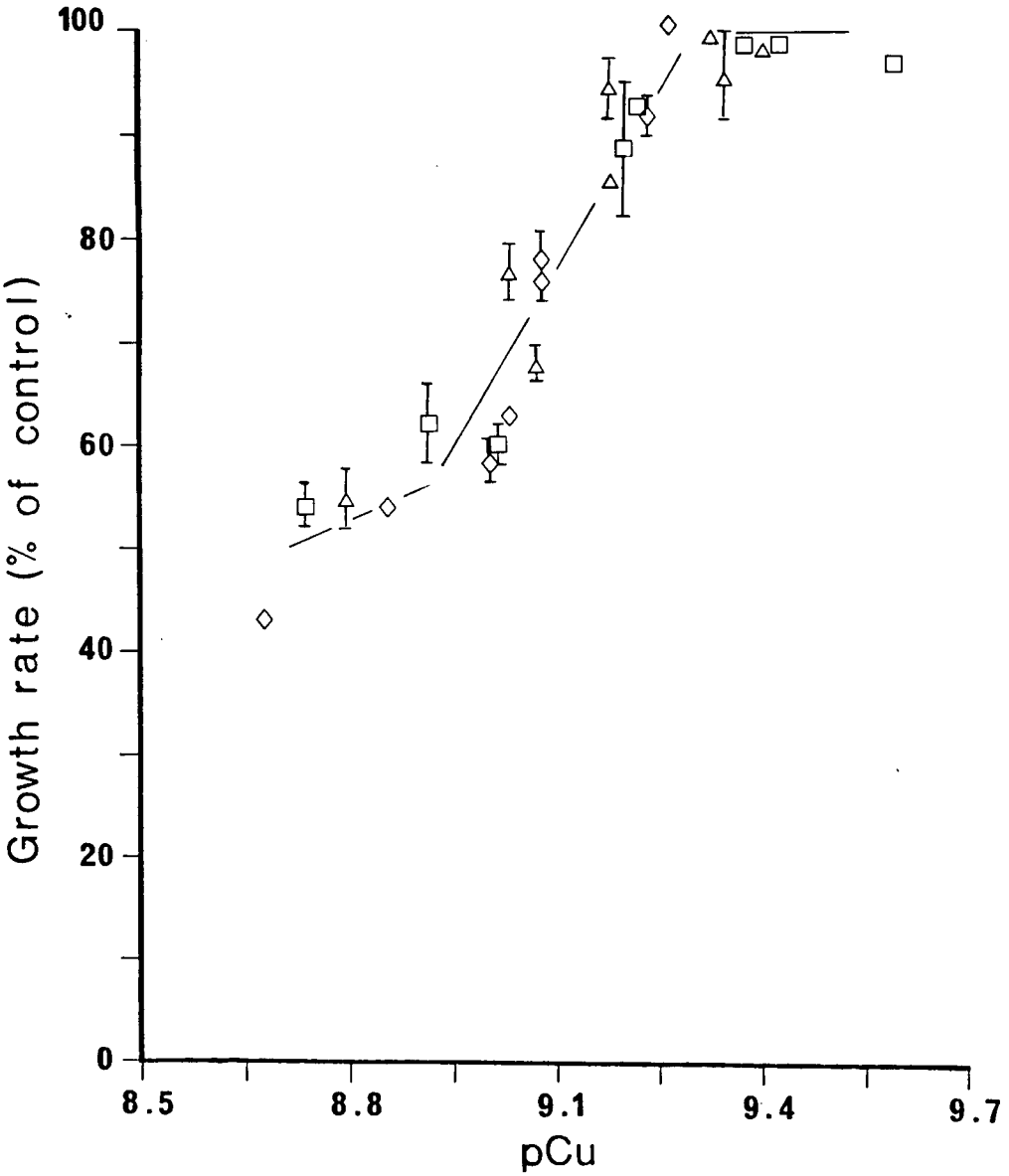
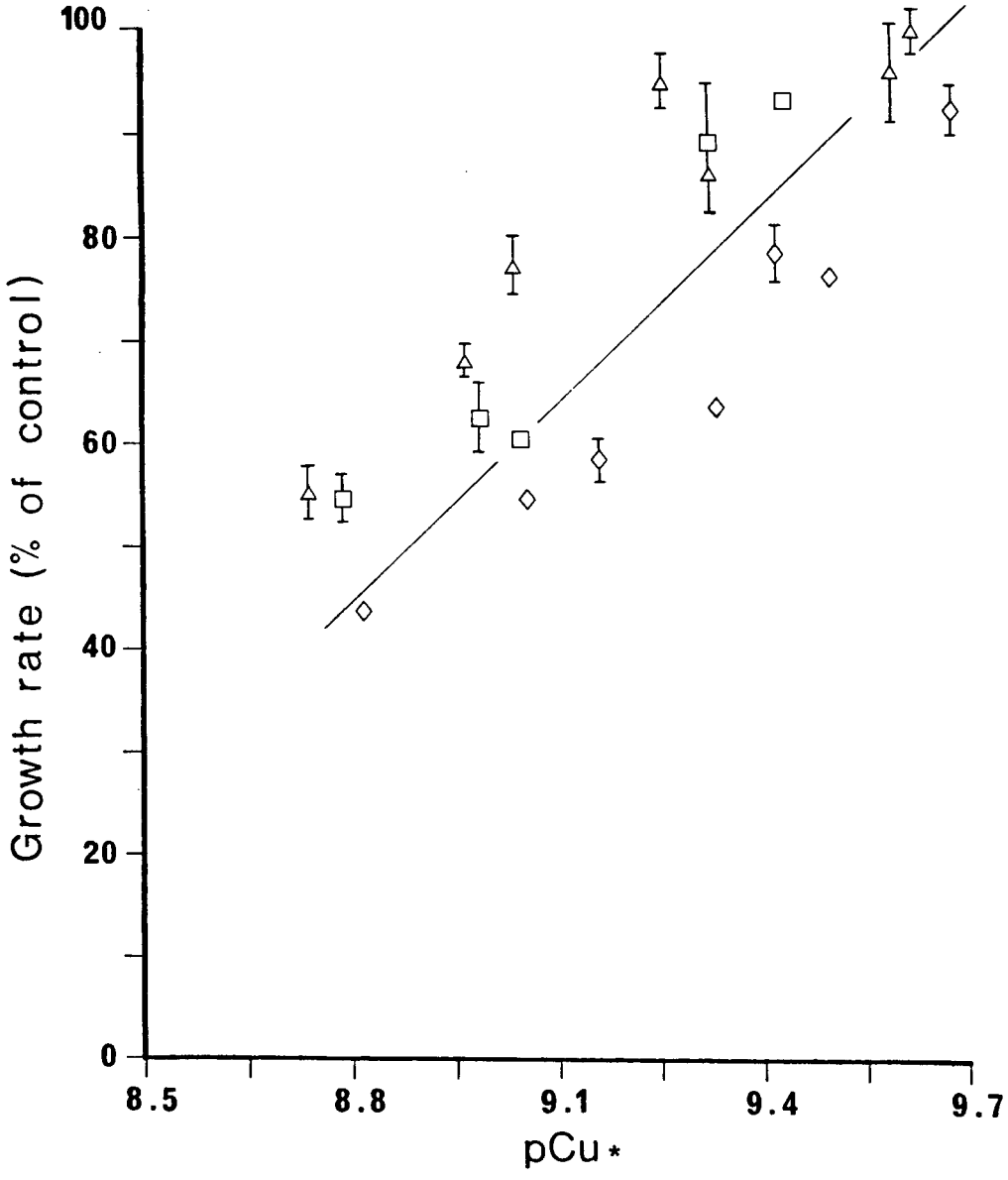


Figure 24. Growth rate (% of control) versus pCu* calculated by MINEQL. Data from tests using □ EDTA, △ GLU and ◇ NTA as the model organic ligands. Bars are ±1 s.d.



E. DISCUSSION

In the preliminary experiments in which the adsorption of Cu by the resin was studied in organic ligand free artificial seawater (SOW) of well-defined composition, the overall precision of the technique, indicated by the relative standard deviation, was found to be 8% which includes the error in the ASV analysis. The sensitivity of the technique was shown to be limited only by the quantity of resin used (ca. 3.1×10^{-9} mol g^{-1} of resin); however, as the quantity of resin determines the volume of sample needed for the analysis the latter may in fact be the limiting factor. The adsorption of Cu by the resin was found to be linear up to a solution Cu concentration of approximately 19.7×10^{-8}M. Above this the resin still adsorbed Cu but the slope of the adsorption curve was reduced. However, as the region of linear response encompassed the concentration range that caused sublethal effects in the bioassay organism and the range of concentrations that could be expected in coastal marine waters (see Forstner and Wittman, 1979, p.83), this departure from linearity is of little practical significance.

At a constant Cu concentration, the addition of the model ligands EDTA, GLU and NTA reduced the Cu equiv levels of the samples reflecting a reduction in the adsorption of Cu by the resin in their presence. The extent of the reduction was related to the ligand concentration and the stability of its complexes with Cu. As the concentration of the ligand or its stability constant increased, the Cu equiv value of the sample decreased. From this it was evident that adsorption by the

resin was controlled not by the total Cu concentration but by the fraction of the total level that was not complexed by the organic ligands.

To determine if the adsorption of Cu by the resin could be related to the response of the bioassay test organism, growth rates of the organism were plotted as a function of the negative log of the Cu equiv values as determined by the resin technique. For all the combinations of Cu and ligand concentrations studied, there was a strong relationship between the Cu equiv values and growth rates. This indicates that the resin technique can be used to estimate the toxicity of Cu in these test solutions.

Since the cupric ion has been shown to be the principal toxic form of the metal for many phytoplankton species (Sunda and Guillard, 1976; Anderson and Morel, 1978; Jackson and Morgan, 1978; Gavis et al., 1981), a strong relationship between growth rate and the results of the resin technique would infer that the response of the resin was to the activity of the cupric ion, although the activity of the other positively charged Cu species found in seawater ($CuCl^+$, $CuOH^+$) may also contribute to the amount of Cu adsorbed onto the resin. However, at a given pH and salinity, the concentration of $CuCl^+$, $CuOH^+$ and all other Cu complexes formed with major constituents is directly proportional to the activity of Cu^{2+}, and thus the resin is still responding to the cupric ion activity.

Growth rates were also plotted as a function of the pCu* determined by calculation and pCu as estimated from the resin

analysis. The relationship was much stronger with the pCu estimated by the resin analysis than with pCu* calculated by the computer model. The weak relationship between growth rates and calculated pCu* values can be attributed to calculation inaccuracies generated by errors in the thermodynamic data or else to undocumented interactions.

The resin did not always respond properly to the complexation of Cu by the addition of an organic complexing agent. Upon the addition of HIS to a sample, at concentrations known to complex Cu, the amount of Cu bound to the resin was greater than with a similar sample that lacked HIS, a result apparently due the adsorption of cationic Cu-HIS complexes.

This adsorption of positively charged Cu-complexes indicates a potential limitation to the technique's ability to measure biologically active metal in natural waters. The existence of such a limitation depends on the presence of these types of complexes in the water body under study. It is thus useful to examine the type of Cu-complexes that may be encountered in natural waters.

Many natural waters can partially mask the presence of metal ions by rendering them unavailable for measurement by conventional chemical (Chau et al., 1974a,b) and biological (Davey et al., 1973; Gillespie and Vaccaro, 1978) metal sensing techniques; evidence supports the concept that this masking is due to organic complexation of these metals (Duinker and Kramer, 1977). The reported distribution of organically associated Cu in marine and freshwater has ranged from 0-100% (e.g., Slowey et

al., 1967; Williams, 1969; Batley and Gardner, 1978). However, Mantoura (1981) reports that a consistent average of ca. 20% of the total Cu in seawater appears to be in an organic form although the number of metallo-organic complexes actually identified is very small. Therefore, the presence of metallo-organic complexes is generally inferred from the presence of organic material that has the apparent potential to complex metals.

Many researchers have implicated intermediate and high molecular weight organic compounds in metallo-organic interactions (Ramamoorthy and Kushner, 1975a,b; Benes et al., 1976; Gillespie and Vaccaro, 1978); with humic compounds being most frequently indicated because of their abundance in natural waters (Schnitzer and Khan, 1972). Low molecular weight organic ligands (<200 AMU) are potentially capable of binding metals (Sillen and Martel, 1971) but the concentration of these compounds is believed to be very low making it unlikely that they would complex metals to any great degree (Duursma, 1970; Stumm and Morgan, 1970). In addition, the stability constants of these compounds for Cu (e.g., amino acids) are not particularly large.

The major factor determining the adsorbability of Cu-organic complexes is the type and amount of electrical charge carried by the organic ligand because this will generally determine the overall charge of the metallo-organic complex. In the few reports that are available the dissolved organic matter (DOM) in natural water has been generally found to have a net

negative charge (Packlam, 1964). For example, Neihof and Loeb (1974) found that when test particles of quartz, germanium and an anion-exchange resin were exposed to natural seawater, they all displayed negative surface charges. When these particles were immersed in the same natural seawater following its exposure to ultra violet irradiation to photo-oxidize organic matter, the surface charges were nearly the same as when they were exposed to artificial seawater containing no organic matter. They attributed this negative charge to adsorbed organic constituents and listed proteins, humic acids and other substances derived from the degradation of natural products of marine and terrestrial origin as possible agents because they are surface-active polymers that carry a net negative charge at the pH of seawater (from Duursma, 1965).

Rashid (1971) found that when both divalent and trivalent metals reacted with humic acids of marine origin the metals appeared to lose their ionic characteristics. When using a cation exchange resin, Pillai et al. (1971) found that the major fraction of Al, Fe, Cu and Zn in humic acid extracts of marine sediments escaped through the resin although some small fraction of Cu did undergo some exchange. Since the Cu-humate complex was not adsorbed by the resin this indicated that the complex did not carry a net positive charge.

The data available suggest that much or most of the metallo-organic complexes in natural waters bear a negative charge. The presence of positively charged Cu-complexes is, nevertheless, still possible and, thus, must be considered when

applying the resin technique to natural waters.

Another potential problem when applying the resin technique to natural water samples is with respect to Fe. The addition of Fe to samples increased the uptake of Cu by the resin although all other facets of the analysis appeared to be unaffected. It was also found that freshly prepared Fe stocks were more reactive than aged Fe stocks. If Fe is present in a natural seawater sample then it should be added to the Cu standards so the conditions of adsorption will be the same in both the sample and the standard. However, if the Fe in the sample is unreactive with respect to the resin, the addition of freshly prepared Fe to the standards, which is reactive, would bias the results. Obviously, this problem can be resolved only by further study of the nature of the Fe in natural seawater and its reactivity with respect to the resin.

V. APPLICATION OF THE RESIN TECHNIQUE TO NATURAL SEAWATER

A. INTRODUCTION

Many studies have examined the complexing capacity of natural waters (e.g., Chau et al., 1974a,b; Duinker and Kramer, 1977; Gurtisen et al., 1977). This 'capacity' represents the extent to which the organic or inorganic constituents of natural waters can bind a metal ion by complexation or adsorption (Chau and Wong, 1976). The chemical structures of the natural compounds responsible for the strong interactions with metal ions have not yet been elucidated (Srna et al., 1980), although recent work (Trick et al., 1983) suggests that phytoplankton metabolites may play a role in the complexing capacity of natural waters. A number of biological (Davey et al., 1973; Gillespie and Vaccaro, 1978) and chemical techniques (Chau et al., 1974a; Shuman and Woodward, 1977; Stolzberg and Rosin, 1977; Sugai and Healy, 1978) have been developed to quantify the complexing capacity of natural waters. The values vary considerably but Mantoura (1981) estimates an average of 0.27 μM Cu l^{-1} for estuarine and coastal marine waters.

It has been suggested that the observed variations in Cu toxicity (Davey et al., 1973; Anderson and Morel, 1978) and succession of phytoplankton species (Gächter et al., 1973) could be due to differences in the complexation capacities of natural waters. Sunda and Lewis (1978) found that by increasing the quantity of river water, which contained a high concentration of organic matter, to their culture medium, the toxicity of Cu was

progressively reduced. Srna et al. (1980) demonstrated a strong negative correlation between complexation capacity and Cu toxicity for various bodies of water that included a freshwater lake, open ocean waters, surf zones, bays and coastal lagoons.

In the present study, a local marine inlet was chosen as a sampling site for seawater collection because previous reports indicated that it had a measurable complexing capacity. Erickson (1973) inferred from ASV measurements that the Cu in the inlet was partially complexed by naturally occurring dissolved organic solutes. Quantitative information on the complexing capacity of the inlet was presented by Whitfield and Lewis (1976). They reported complexing capacities as EDTA equivalent values and found values of up to 5.6×10^{-7}M. They concluded that the inlet's complexing ability varied through the year and, subsequent to winter intrusions, there was an increase in the complexation capacity due to the intrusion of organic material.

To test the ability of the resin technique to measure the complexation of Cu by naturally occurring complexing agents, seawater with a complexing capacity was necessary. Seawater from Indian Arm was collected from a series of depths in the expectation that it had a complexing ability and that this ability would change with depth (Whitfield and Lewis, 1976). In addition, to verify that the resin was responding to biologically active metal, bioassays with T. pseudonana were conducted on the same water samples. Assays were also carried out using seawater soluble complexing agents extracted from

marine sediments. These agents had previously been shown to reduce Cu toxicity (Lewis et al., 1973).

B. COLLECTION OF NATURAL SEAWATER

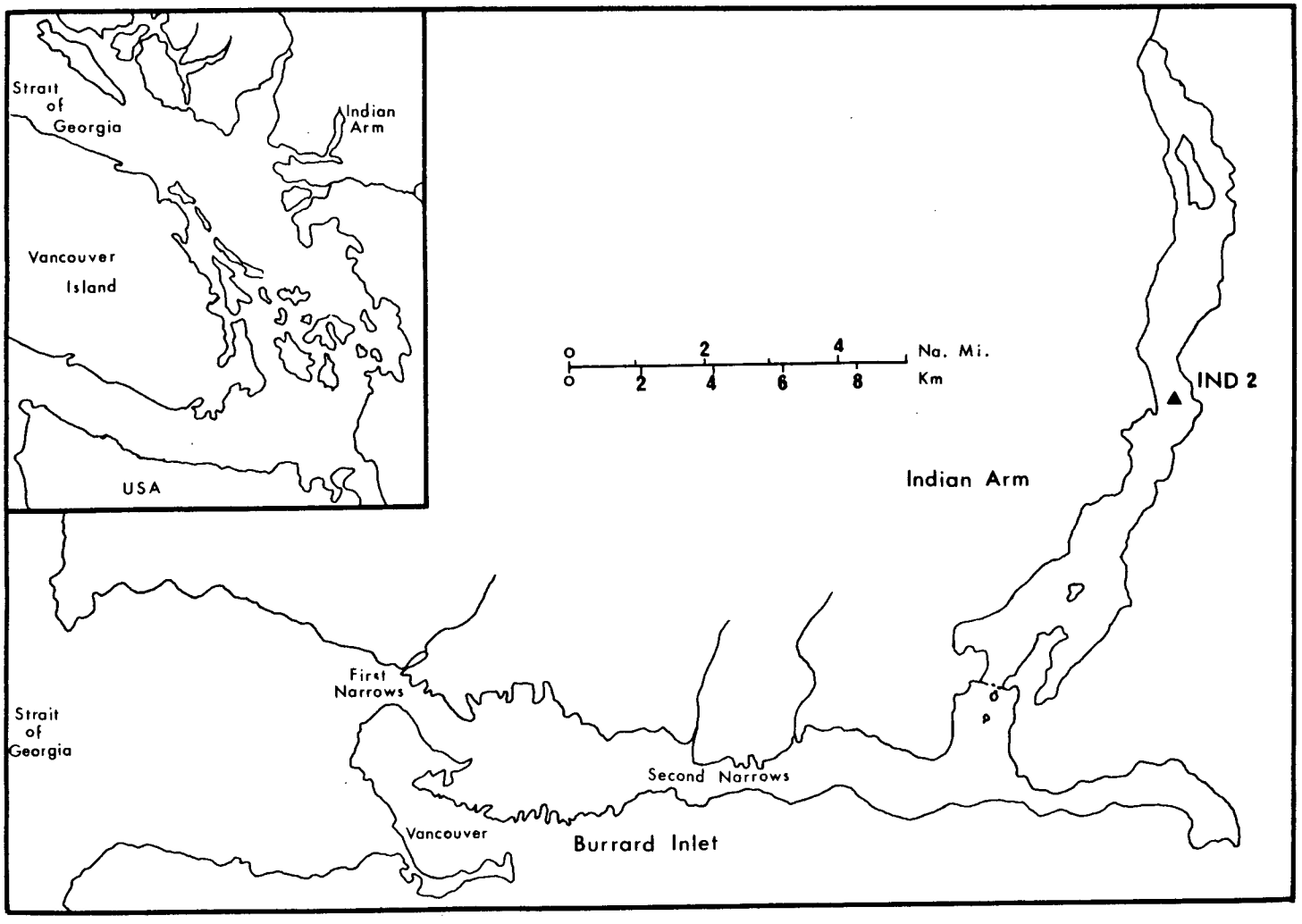
Seawater was collected in Indian Arm, a shallow silled fjord near Vancouver, B.C. (Fig. 25), which is connected to the Strait of Georgia through Burrard Inlet and the port of Vancouver. The sill at the entrance of the Inlet is only 26 meters (m) deep while the depth of the central basin is over 200 m. There is a characteristic two layer estuarine circulation with a thin layer of relatively brackish water flowing out at the surface and a compensating inflow of denser, more saline water below this (Gilmartin, 1962). Significant exchange and overturn of the deep water occurs only intermittently with the exchange being partially controlled by the volume of fresh water runoff, tidal mixing and density of the Strait of Georgia water (Davidson, 1979). The physical characteristics of the Inlet and hydrographic data are given in great detail by Gilmartin (1962) and Davidson (1979).

Water samples from five depths were taken at station IND-2 (49°23.5'N, 122°52.5'W) which is located at the southern end of the deepest portion of the basin. Forty liters of seawater from each depth were collected with a 96-l fiberglass and lucite sampler (Whitfield and Lewis, 1976). The samples were filtered within 6 hr of collection through a Gelmann 293 mm, 0.45 μm filter. Five liters of sample were passed through the filter as a rinse which was discarded before each sample was filtered.

The filtered samples were stored in 23-l polyethylene containers at 4°C in the dark. Prior to use, the storage carboys were rinsed with 6N HCL, soaked in 0.5N HCL for 3 days, rinsed 3X with GDW and finally, filled with GDW.

Hydrographic data were collected at the specific depths using NIO sampling bottles (National Institute of Oceanography, Wormley, U.K.). Temperatures were determined with reversing thermometers (±0.01°C) and water samples were drawn for salinity determinations. Oxygen determinations were done on board ship using a modified Winkler technique (Carritt and Carpenter, 1966). In addition, a continuous profile of temperature and conductivity was completed using a CTD.

Figure 25. Location of sample collection.


Strait of Georgia
Indian Arm
Vancouver Island
USA
0 2 4 Na. Mi.
0 2 4 6 8 Km
IND 2
Indian Arm
First Narrows
Strait of Georgia
Second Narrows
Vancouver
Burrard Inlet

C. MATERIALS AND METHODS

1. Preliminary Sample Preparation

For the resin and bioassay tests conducted on each depth, a 10-l aliquot of sample was removed from the storage carboy and put in a 12-l polypropylene carboy. To reduce trace metal contamination, all bottles and sample carboys went through the same cleaning procedure as described in the previous section. Once transfered to the 12-l carboy the pH of the sample was measured and then adjusted to 8.05±.05 by bubbling the sample with acid cleaned (H_2SO_4), filtered (0.45 μm) air. All samples were below pH 8.05 initially and bubbling times ranged from 6-12 hours depending on the sample. After bubbling, the 10-l sample was passed through a Sartorius membrane filter (0.45 μm) to remove any particulate matter formed during the bubbling procedure or during the storage period (algal growth). The filtrate was collected in a 12-l polycarbonate carboy. The first liter of filtrate was discarded to reduce contamination from the filter. The total dissolved Cu concentration in the sample was determined by ASV. The sample was then ready to be used in both the bioassay tests and the resin analysis.

2. Bioassays

The bioassay procedure followed that used in Section III, with the exception that the autoclaving procedure was eliminated because of the effect heat may have on the organics present in

the water. An outline of the bioassay procedure is given below.

For each bioassay, 4 l of a prepared sample was transferred to a 4-l Pyrex aspirator bottle. To ensure adequate nutrient concentrations over the term of the bioassay, the Aquil nutrients and trace metals were added at their Aquil concentrations with the Fe stock being freshly prepared and allowed to precipitate for several hours before its addition. Aliquots (250 ml) of the sample were then transferred to 15 previously autoclaved 500 ml polycarbonate Erylenmeyer flasks and Cu was added at concentrations of 0.0, 3.93, 7.87, 11.8 or 15.7 x 10^{-8}M. The samples were left for three hours to equilibrate. (Each Cu concentration was run in triplicate.) Finally, the medium was innoculated with enough test organism to obtain an initial cell number between 1000-2000 cell ml^{-1}. Cell numbers were monitored over a five day period by a Coulter Counter

In addition to the bioassays with the natural water samples, a bioassay using low salinity SOW was performed. GDW was added to SOW until the salinity was the same as the Indian Arm 200 m sample (S=27.5 ppt). The pH was measured and adjusted to pH 8.0±.05. The same bioassay procedure was then followed as given above.

3. Calibration of the Resin in Low Salinity SOW

Since salinity has been previously shown to influence the resin analysis, the resin was characterized in artificial seawater (SOW) that had been diluted to the salinity of the

natural water samples. Three tests were conducted. The first test was to determine the volume needed to bring the resin into equilibrium with the sample. The second was to test if the adsorption of Cu by the resin was still linear at these lower salinities. The final test examined the adsorption of Cu by the resin in the presence of the Aquil nutrients and trace metals. Column preparation, column procedure, and the ASV technique used to quantify the Cu in the eluate were all the same as described in Section IV.B. Except that, a flow rate of 2.9 ml min^{-1} was used.

In a resin equilibrium experiment, SOW at a salinity of 27.5 ppt was prepared by diluting SOW (35 ppt) with GDW. The pH was measured and adjusted to 8.05±.05 with 0.1N NaOH. Copper was then added to a 2-l aliquot to give a final Cu concentration of 7.87 x 10^{-8}M. Ten columns were prepared and 50, 100, 150, 200, 250 or 300 ml was passed through the appropriate column with the columns being run in duplicate. Finally, the columns were eluted with acidified SOW (pH ca. 1.0) and the eluates analysed for Cu by ASV.

In a salinity experiment, three adsorption curves were generated using SOW at 24.0, 25.5 and 27.5 ppt to test the linearity of adsorption at the approximate salinities of the natural water samples. Samples were prepared by diluting SOW with GDW. SOW of each salinity was run in a separate experiment. For each experiment, five 500 ml samples were prepared in 500 ml polypropylene bottles. To these samples, the Aquil nutrients and trace metals including Fe were added at

their Aquil concentration. Each sample was then spiked with 0.0, 3.93, 7.87, 11.8, or 15.7 x 10^{-8}M Cu and left for two hours to equilibrate. Finally, 250 ml of each sample were passed through the appropriate column with each sample being run in duplicate. The columns were eluted with acidified SOW (pH ca. 1.0) and the eluates analysed for Cu by ASV. In addition, the experiment using the 25.5 ppt SOW was repeated except that the Aquil nutrients and trace metals were not added.

4. Application of the Ion-Exchange Method to Natural Seawater

In most cases, the resin and bioassay tests were conducted on the same batch of prepared seawater. For the resin analysis, four 500 ml aliquots of a prepared seawater sample were transfered to 500 ml polypropylene bottles. The Aquil nutrients and trace metals were added at their Aquil concentrations to all samples. The Fe stock was freshly prepared and allowed to precipitate for several hours before use. Each sample was then spiked with 3.93, 7.87, 11.8 or 15.7 x 10^{-8}M Cu. The samples were left for 12 hr to allow equilibration between Cu and the solution. Eight resin columns were prepared and 250 ml of each of the four solutions were run through the appropriate column with each sample being run in duplicate. The columns were eluted with acidified SOW and the eluates analysed for Cu by ASV.

To calibrate the sample columns, a standard SOW solution was prepared at the same time, in the same manner, and then run through a set of columns under exactly the same conditions used

for the sample columns. The standard solutions were made up in SOW (35 ppt) diluted with GDW to the same salinity as the sample under study. The same nutrients and trace metals added to the the natural water samples were added to the standards. If necessary, the pH of the standard was adjusted to the pH of the samples. In most tests Cu was added to give a final concentration of 7.87×10^{-8}M in the standard solution.

In another experiment, the resin analysis of the 50 m seawater sample was repeated without nutrient or trace metal addition. The results of the analysis with and without nutrients were then compared to indicate the effect of the Aquil nutrients. The standards used for calibration of the columns in this experiment were made up in SOW diluted to the salinity of the 50 m sample, without the addition of nutrients, but with the addition of 7.87×10^{-8}M Cu.

5. Manganese-Copper Interaction

Since dissolved Mn concentrations are high in Indian Arm waters (Whitfield, 1974), the Mn concentrations in the water samples were determined by direct injection into the graphite furnace of an atomic absorption spectrophotometer. Injection volumes were 50 μl. A 1000 mg l^{-1} Mn stock was prepared by the addition of $MnCl_2 \cdot 4H_2O$ to GDW. Mn standards were made up from this stock in SOW diluted to a salinity of 25 ppt with GDW. The standards were acidified with 0.05 ml of isothermally distilled 5N HCL. The samples to be analysed were acidified to pH 2 by the addition of 5N HCl and left for two days. A standard curve

was generated and the Mn concentrations of the samples were determined by interpolation from this graph.

To test the effect of Mn on the response of the organism to Cu, two bioassays were run simultaneously using the seawater from the 50 m depth, where the ambient Mn concentration was lowest. The bioassay procedure was the same as described for the other natural water samples except that, in one of the bioassays, 0.05 ml of a 1000 mg l^{-1} Mn stock was added, which gave a final Mn concentration of 3.64 x 10^{-6}M. In the second of the two bioassays Mn was only present in the Aquil concentration plus the background concentration (3.9 x 10^{-8}M). The bioassays were run for five days and the growth of the organism was monitored daily with a Coulter Counter.

6. Natural Seawater Soluble Agents Extracted from Sediments

The resin and bioassay techniques were also used to detect the complexation of Cu by natural organics extracted from sediments. Sediment (1000 g) from Indian Arm was added to 8 l of SOW (35 ppt) in a 10-l round bottom flask. The mixture was stirred for two days using a large teflon coated stirring bar in combination with a magnetic stirrer and then allowed to settle. The SOW was pumped out of the flask with a peristaltic pump and passed through a 192 mm Sartorius membrane filter (0.45 μm) to remove particulate matter. The final filtrate was collected in a 12-l polycarbonate carboy. The pH of SOW did not change with addition of the sediment and the total Cu concentration in the sample was below the ASV detection limit of 1.57 x 10^{-9}M.

Before the bioassay or resin analyses were conducted the filtrate was diluted with GDW to a salinity of 27.5 ppt. Four liters were then used in the bioassay and 2.5 liters in the resin analyses. The pH was measured but no adjustment was necessary.

In the bioassay, four Cu concentrations (3.93, 7.87, 11.8 and 15.7 x 10^{-8}M) were used. The same bioassay procedure was followed as in Section V.B.2. Cell numbers were monitored over a five day period by a Coulter Counter.

For the resin analysis, five 500 ml aliquots of the prepared sample were transferred to 500 ml polypropylene bottles to which the Aquil nutrients and trace metals were then added at their Aquil concentration. The samples were spiked with 3.93, 7.87, 11.8 or 15.7 x 10^{-8}M Cu. The samples were allowed to equilibrate for 12 hr before 250 ml of each was passed through the appropriate column. Each sample was run in duplicate. The columns were then eluted with acidified SOW and the eluates analysed for Cu by ASV.

Two standard columns were used to calibrate the sample columns. The standard solution was made up in SOW diluted to the same salinity and with the same nutrient addition as the samples. Cu was added to give a concentration of 7.87 x 10^{-8}M.

D. RESULTS

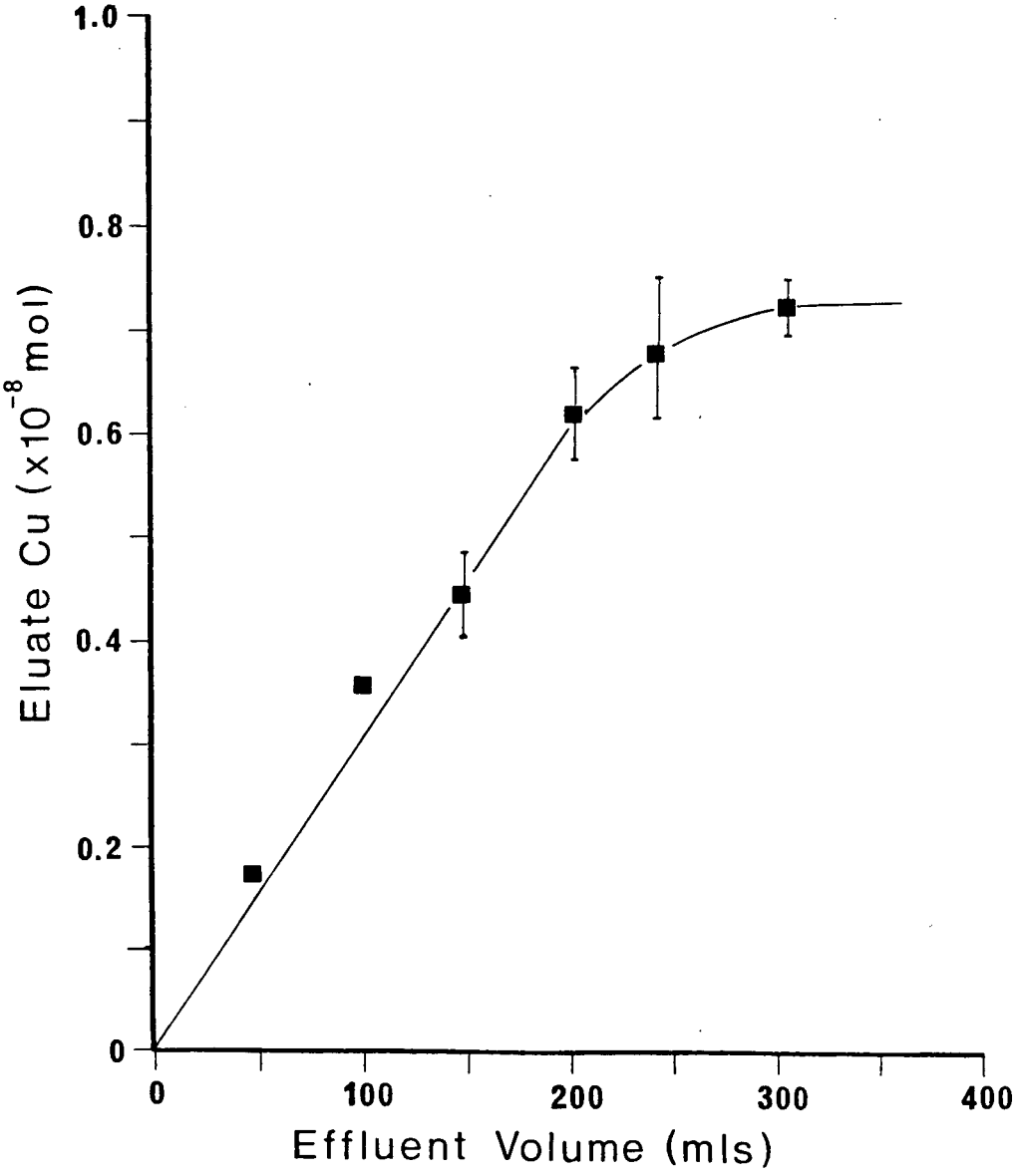
1. Preliminary Tests

The salinity of the Indian Arm samples was lower than that of the medium used to characterize the resin in Section IV. Since salinity has been shown to affect the resin, preliminary tests were conducted to characterize the resin at the salinity of the natural samples.

In the first experiment, the volume of sample needed to achieve column equilibration was determined. As in SOW of salinity 35 ppt, a sample volume of 250 ml appeared to be the minimum volume necessary to bring the resin into equilibrium with the solution (Fig. 26). The amount of Cu bound to the resin after 300 ml was slightly higher than after 250 ml, but the difference was within the experimental error of the technique. Once again, 250 ml was chosen as the sample volume for all experiments in this section.

In the second experiment, three Cu adsorption curves were generated using SOW diluted to 24.0, 25.5 and 27.5 ppt to determine if the adsorption of Cu by the resin was linear at these lower salinities. Fig. 27 shows a linear adsorption curve at these salinities although the line of best fit for all curves does not go through the origin. This is attributed to the adsorption of Cu by the resin being less at a low solution Cu concentration (3.93×10^{-8}M) than at higher Cu levels. For example, at a salinity of 25.5 ppt the addition of 3.93×10^{-8}M Cu gives an adsorption of 2.97×10^{-9}mol Cu per gram of resin.

Figure 26. Eluate Cu versus the effluent volume for the equilibrium experiment. Values are a mean of 2 replicates ±1 s.d.



However, with an additional 3.93 x 10^{-8}M spike of Cu to SOW, the amount of Cu bound to the resin is 6.88 x 10^{-9}mol which corresponds to a 3.91 x 10^{-9}mol increase. This is an increase of approximately 1.0 x 10^{-9}mol Cu over that of the first spike and such an increase accounts for the change in slope of the adsorption curves.

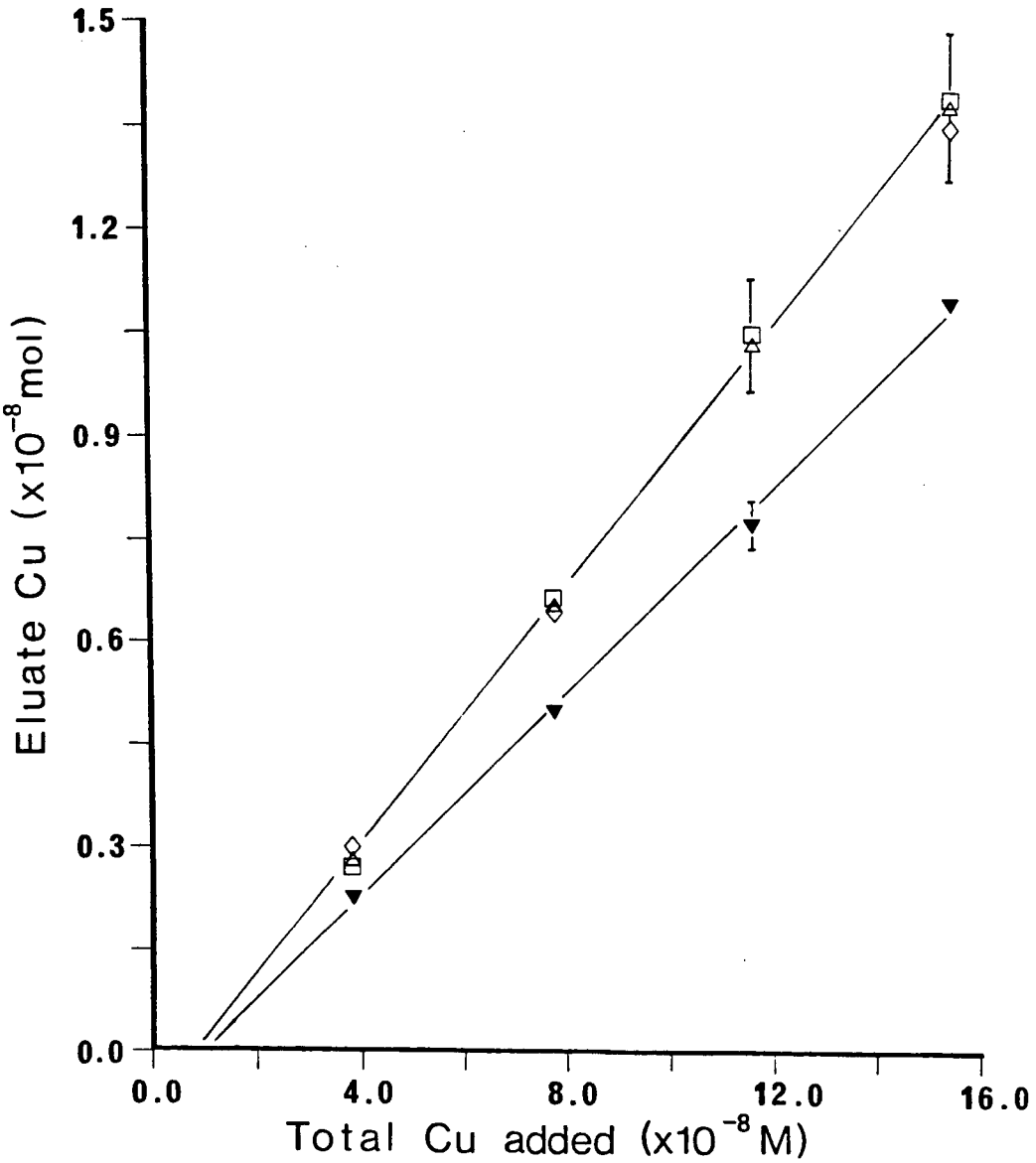
These results suggest that possibly not all Cu was being eluted and that a constant amount was retained (or lost in some other fashion) by each column. The difference in the adsorption of Cu at low and high solution Cu concentrations is not great but indicates the advisability of using standards that closely resemble the Cu levels in the samples to be analysed.

In the experiment to determine the adsorption of Cu in the presence of the Aquil nutrients and trace metals, adsorption curves were generated in 25.5 ppt salinity SOW with and without the addition of nutrients or trace metals. From Fig. 27, it is clear that the slope of the curve increases when all the stocks were added. The increase in slope was expected and was attributed to the Fe addition; the effect of which has been previously determined in SOW at 35 ppt (Fig. 21).

2. Bioassays

At all the added Cu levels, the samples from the 100 and 200 m depths supported better growth than did the samples from 10 or 50 m. The greatest difference in growth was seen between the 50 and 200 m samples (Table XX). For example, when Cu was added to the 50 m water at 11.8 x 10^{-8}M the growth rate of the

Figure 27. Adsorption curves for Cu using low salinity SOW with and without the addition of Fe. Symbols: □ 24 ppt; △ 25.5 ppt; and ◇ 27.5 ppt SOW. ▼ 25.5 ppt SOW with no Fe addition. Values are a mean of 2 replicates ±1 s.d.



organism was reduced by 66% but, with the same addition of Cu to the 200 m water, the growth rate was reduced by only 13%. The 75 m water also supported better growth than did the shallower waters but not to the same extent as the deeper waters. The differences found in the growth rates were initially interpreted as differences in the concentration of natural complexing agents in the samples. It first appeared that the complexation of Cu by natural complexing agents was much greater in the deep waters of 100 and 200 m than in the shallower waters of 10, 50 and 75 m.

The growth patterns found in these bioassays were then compared to the growth pattern obtained in a bioassay using SOW that was diluted to the salinity of the natural water samples (Fig. 28). The growth pattern of the 10 and 50 m seawater most closely resembled the pattern found in the diluted SOW, although better growth was still seen at the Cu levels in the natural waters. This was an indication that there was still some reduction in the biologically active Cu by natural complexing agents or some other mechanism in the shallower water samples.

3. Application of the Resin Technique to Natural Seawater

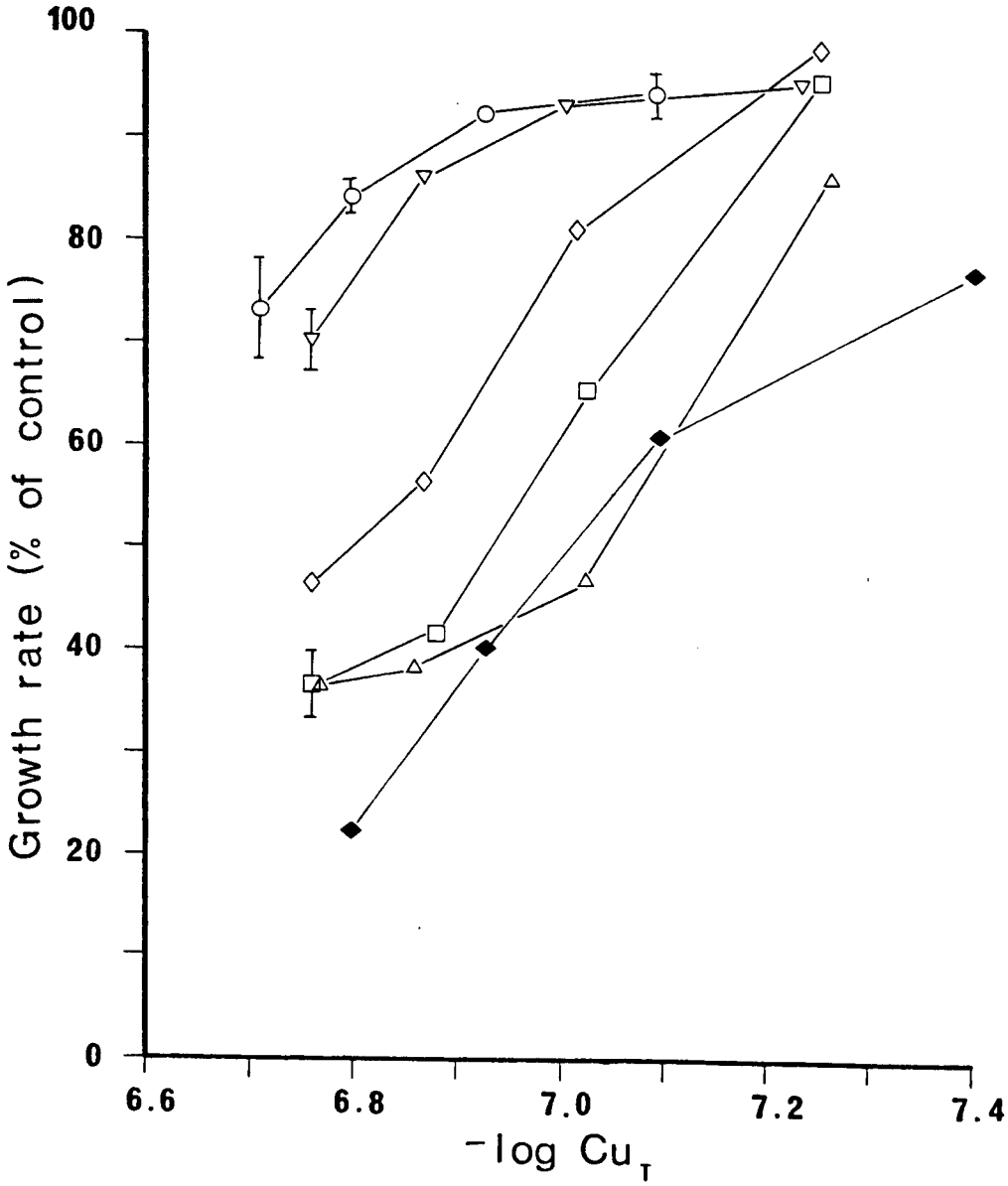
The results of the resin analysis are reported as Cu equiv as described in Section IV.D.2. To reiterate, the Cu equiv value of a sample is dependent on both the total Cu concentration and the extent to which free Cu is complexed by the ligands in solution. This value will be equivalent to the total Cu concentration of the sample only when the ligands

Table XX. Growth rates of the bioassay organism in seawater taken from five depths in Indian Arm. Also growth rates in SOW at 27.5 ppt are presented.

Depth m	Cu added ($\times 10^{-8}$M)	Growth Rate (div day^{-1})	% of Control
SOW (27.5 ppt)	0.1	1.75±0.03[1]	
	3.9	1.34±0.05	77±3[1]
	7.9	1.07±0.06	61±4
	11.8	0.70±0.03	40±2
	15.7	0.39±0.03	22±2
10	0.1	2.33±0.03	
	3.9	2.23±0.07	96±3
	7.9	1.55±0.02	66±1
	11.8	0.98±0.03	42±1
	15.7	0.86±0.07	37±3
50	0.1	2.07±0.04	
	3.9	1.82±0.02	88±1
	7.9	0.86±0.02	42±1
	11.8	0.77±0.03	34±1
	15.7	0.71±0.03	34±1
75	0.1	2.36±0.01	
	3.9	2.34±0.02	99±1
	7.9	1.95±0.01	82±0
	11.8	1.34±0.02	57±1
	15.7	1.10±0.02	47±1
100	0.1	2.07±0.02	
	3.9	1.97±0.04	95±2
	7.9	1.93±0.01	93±0
	11.8	1.75±0.02	85±1
	15.7	1.53±0.10	74±5
200	0.1	2.42±0.01	
	3.9	2.33±0.02	96±1
	7.9	2.27±0.00	94±0
	11.8	2.11±0.01	87±1
	15.7	1.73±0.06	71±3

[1]Mean ±1 s.d. based on 3 replicates.

Figure 28. Growth rate (% of control) versus the total Cu_T concentration in the natural water samples. Symbols: □ 10 meter; △ 50 meter; ◇ 75 meter; ○ 100 meter; and ▽ 200 meter samples; ◆ SOW at 27.5 ppt. Bars are ±1 s.d.



present in the sample are the same as those present in the standard SOW used for the calibration of the sample. In SOW, the hydroxide and carbonate ions are the only major ions thought to affect Cu speciation. Therefore, a difference between the Cu equiv value and the total Cu concentration will give an indication of the extent of Cu complexation by ligands other than the hydroxide and carbonate ions.

The Cu equiv values for the various samples are presented in Table XXI. The highest values are found in the 100 m seawater. In fact, these values are higher than the concentration of Cu added to the samples. However, this is due to the high background concentration of Cu in the water from this depth (3.95×10^{-8}M), and when the Cu equiv level is compared to the total Cu concentration the values are actually found to be lower (Table XXI). The 75 m water has the lowest values but the differences between it and the 10, 50 and 200 m samples are marginal.

The difference between the total Cu concentration and the Cu equiv values of a sample can be used to indicate the extent of Cu complexation by naturally occurring complexing agents. The Cu equiv values for all depths were lower than the total Cu concentration present in the samples. Also from the Cu equiv values it was inferred that the 100 m water had the least complexing ability while the 75 m had the highest. The differences found between the 10, 50 and 200 m water were well within the experimental error of the technique.

Table XXI. Results of resin analysis conducted on the natural water samples.

Depth (m)	Cu added ($x10^{-8}M$)	Total Cu ($x10^{-8}M$)	Cu equiv ($x10^{-8}M$)	% Active[2]
10	3.9	5.46	3.01±.25[1]	55
	7.9	9.40	5.82±.41	62
	11.8	13.33	9.73±.13	73
	15.7	17.23	12.92±.17	75
50	3.9	5.36	2.60±.01	49
	7.9	9.30	6.12±.13	66
	11.8	13.23	9.66±.25	73
	15.7	17.13	14.70±.01	86
75	3.9	5.54	2.31±.06	42
	7.9	9.48	5.49±.27	58
	11.8	13.41	8.23±.43	61
	15.7	17.31	12.38±.61	72
100	3.9	7.88	5.63±.35	71
	7.9	11.82	9.08±.06	77
	11.8	15.75	13.01±.30	66
	15.7	19.65	17.04±.00	87
200	3.9	5.74	3.21±.33	56
	7.9	9.68	6.22±.22	64
	11.8	13.61	9.99±.01	73
	15.7	17.51	13.79±.14	79

[1]Mean ±1 s.d. based on 2 replicates.
[2]Cu equiv/Total Cu conc. x 100.

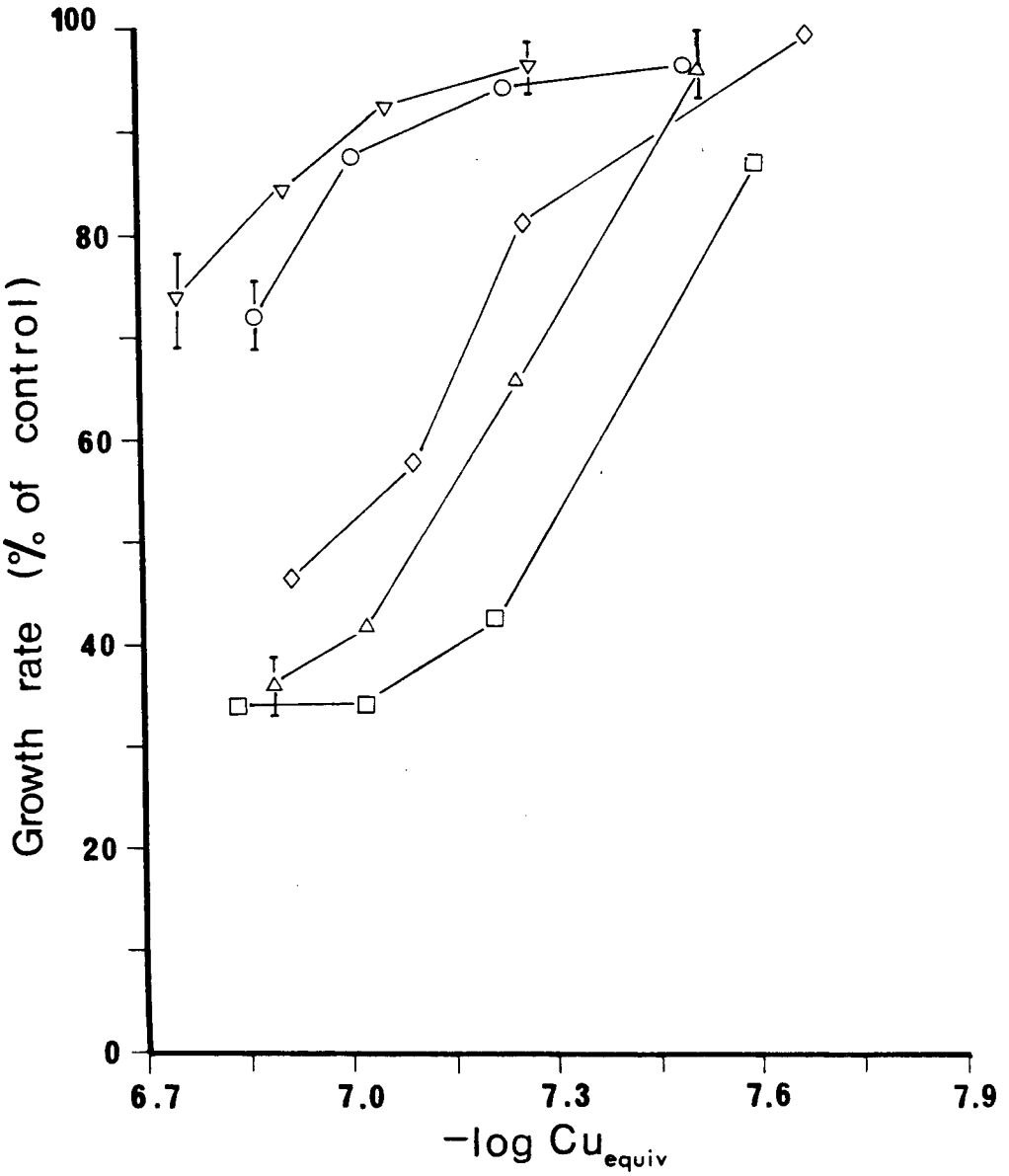
4. Comparison of the Resin and Bioassay Results

The results of the resin analysis were compared to the results of the bioassays conducted on the same samples to determine its ability to estimate biologically active Cu in natural seawater. In Fig. 29 the growth rates of the bioassay organism are plotted as a function of the -log of the Cu equiv values as estimated by the resin analysis. If the adsorption of Cu by the resin was related to the same fraction of metal species that were toxic to the organism then a single curve would describe both sets of data. From Fig. 29 it is evident that there is only a weak association between growth rate and the Cu equiv values. It also appears that the relationship is dependent on the depth from which the water sample was obtained. Generally, as the depth increases the Cu equiv value needed to cause a specific decrease in growth also increases. The plot, in fact, closely resembles the pattern found when growth rates were plotted as a function of the total Cu concentration (Fig. 28).

It is apparent from these results that the resin analysis cannot be used to estimate the toxicity of this particular set of natural water samples. With the success of the technique under laboratory conditions, however, other possible reasons to explain these results were examined.

In the resin technique it is assumed that the resin responds to the free cupric ion activity or that the response is proportional to this value as long as positively charged Cu-organic complexes, such as HIS, are not present. If positively

Figure 29. Growth rate (% of control) versus the -log of the Cu equiv values. Symbols: △ 10 meter; □ 50 meter; ◇ 75 meter; ▽ 100 meter; and ○ 200 meter samples. Bars are ±1 s.d.



charged Cu-complexes are present, the resin is likely to adsorb these complexes which would result in an overestimation of the cupric ion activity (higher Cu equiv values) of the sample. The resin analysis would then indicate the medium to be more toxic than it actually is and the growth rates found in this medium would be higher than expected from the resin analysis.

This is a possible explanation for the results obtained in the natural samples. If positively charged organics were present in the 100 and 200 m samples then the resin analysis would predict these samples to be much more toxic than the bioassays would show and a poor relationship between Cu equiv values and growth rate would be found.

Another explanation for the discrepancy between the resin and bioassay techniques concerns the assumptions made in the bioassay technique. The principal assumption is that the response of the organism is constant for a specific cupric ion activity. However, a physiological interaction between Cu and Mn has recently been described (Sunda et al., 1981) which indicates that with increasing Mn, the toxicity of Cu decreases. If this occurred in the Indian Arm samples, the change in toxicity could have been due to changes in the Mn concentration and not to any complexation reactions affecting the Cu chemistry.

Whitfield (1974) found that Mn increased considerably in the deeper waters of Indian Arm, at the same station where the water samples were collected for the present study (IND-2). He found Mn levels 6 to 10 times greater in the deep water than in

the surface water.

Because of this the Mn concentrations in the Indian Arm water samples were determined. A dramatic difference was seen in the Mn concentration between the surface and deep waters (Table XXII). The 200 m water had a Mn concentration approximately two orders of magnitude greater than did the 10, 50 and 75 m waters; the 200 m water had a Mn concentration of 3.64×10^{-6}M while the lowest Mn level was found in the seawater from 50 m at 1.63×10^{-8}M.

Table XXII. Hydrographic and trace metal data from water samples collected from five depths at station IND-2.

Depth (M)	Temp. (°C)	Salinity (ppt)	Oxygen (ml l^{-1})	Total Cu ($\times 10^{-8}$M)	Total Mn ($\times 10^{-7}$M)	pH
10	11.89	23.432	5.81	1.53±.16[1]	0.60±.06[1]	7.96
50	9.24	25.961	4.60	1.43±.08	0.16±.01	7.67
75	8.28	26.886	2.88	1.61±.11	1.30±.11	7.65
100	8.10	27.200	2.28	3.95±.22	5.49±.44	7.39
200	8.05	27.503	0.70	1.81±.07	36.40±1.5	7.58

[1]Mean ±1 s.d. based on 3 replicates

Since a large difference was found in the Mn concentrations of the natural samples, the effect of Mn on the growth response of the bioassay organism to Cu was tested. Two bioassays were performed on the water collected from the 50 m depth where the Mn level was lowest. In the first bioassay, Mn was added to the

sample at the Mn concentration of the 200 m seawater (3.64×10^{-6}M), while in the second bioassay, Mn was added only in the Aquil concentration. Both bioassays were run simultaneously.

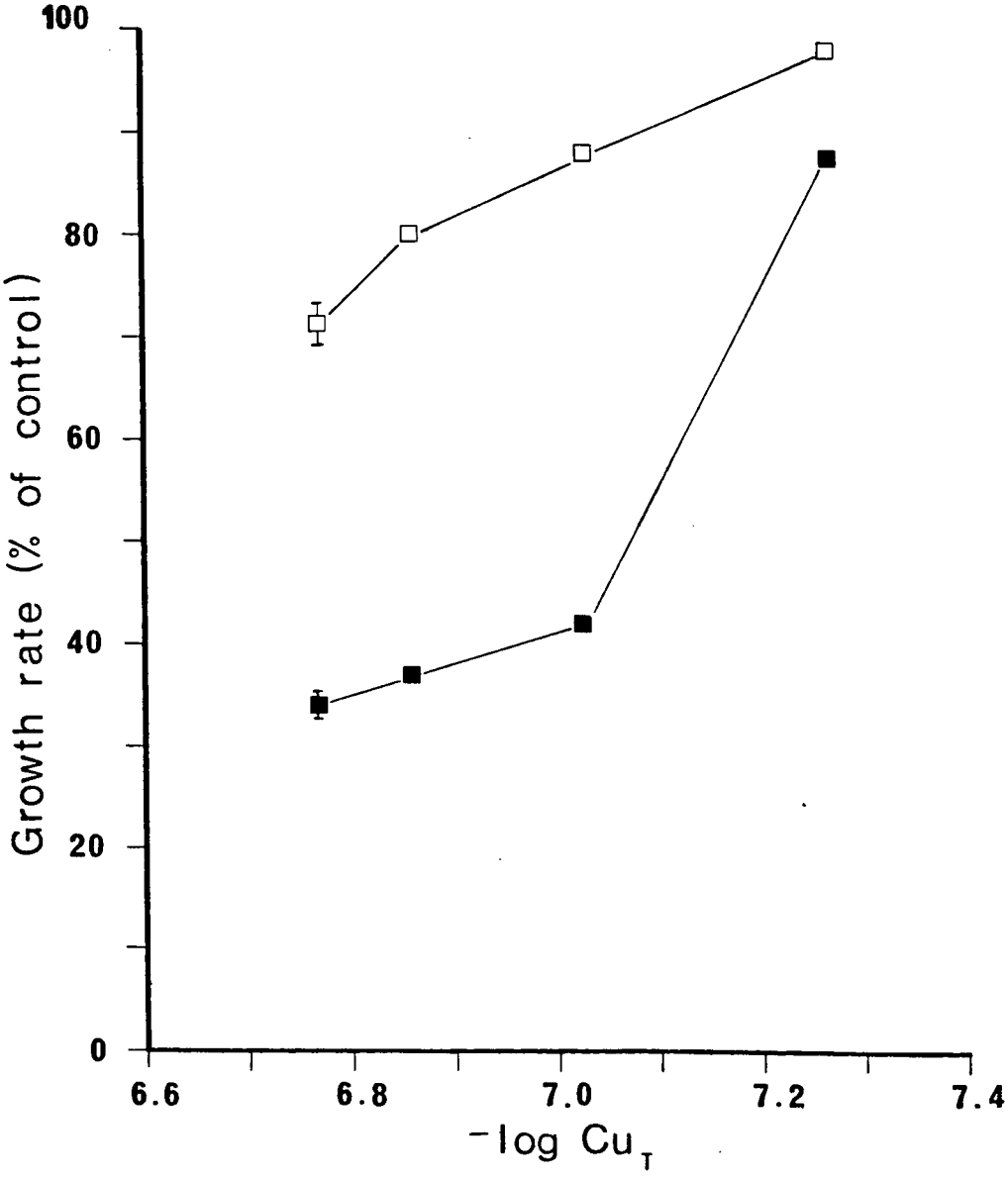
The growth patterns of the organism obtained in the bioassays with and without the addition of Mn are presented in Fig. 30. It is evident that the addition of Mn has an impact on the growth rate of the organism at all the Cu concentrations studied. The medium with the high concentration of Mn had much higher growth rates than did the medium having low Mn. In fact, the growth pattern observed in the medium containing high Mn concentrations closely resembles the growth pattern observed in the natural water samples from 100 and 200 m, both of which had high Mn concentrations.

It appears that the response of the organism to Cu changes with the Mn concentration of the sample. In the natural water samples then, increased growth rates are expected in the 100 and 200 m samples as compared to the shallower waters because of the high Mn concentration present even though the resin analysis estimated the concentration of biologically active metal in the deep waters to be greater than in the shallower waters.

4. Study of Water Soluble Agents from Sediments

The ability of the resin analysis to detect any complexation of Cu by high levels of natural complexing agents was tested. Natural seawater soluble agents were extracted from a sediment taken from Indian Arm by mixing the sediment with artificial seawater (SOW) for a specified period of time. There

Figure 30. The effect of Mn on reducing the toxicity of Cu. Symbols: ■ 50 meter sample with no Mn added; □ 50 meter sample with 3.64 x 10^{-6}M Mn added. Bars are ±1 s.d.



was no attempt to identify the type or determine the quantity of any organic agents present. The sample was titrated with Cu and the amount of resin active metal at each Cu concentration was measured. A bioassay was then conducted on the sample using the same Cu concentrations as the resin analysis and the amount of biologically active metal was estimated. The results of the sediment study are presented in Table XXIII.

Table XXIII. Resin and bioassay results from the sediment study.

Cu added ($\times 10^{-8}$M)	Cu equiv ($\times 10^{-8}$M)	% Active[3]	Growth rate (divs day^{-1})	% of Control
0.1	n.d.		1.79±0.02[1]	
3.93	1.29±0.04[2]	32	1.75±0.06	98
7.87	2.31±0.01	29	1.85±0.02	102
11.8	3.30±0.08	28	1.85±0.02	104
15.7	4.66±0.11	30	1.89±0.01	106

[1]Mean ±1 s.d. based on 3 replicates
[2]Mean ±1 s.d. based on 2 replicates
[3]Cu equiv/Total Cu conc. x 100

In the bioassay tests it was found that Cu could be added in concentrations of up to 15.7 x 10^{-8}M with no deleterious effect. High growth rates were seen in cultures at all Cu concentration throughout the four days of growth; after the fourth day all cultures had reached scenescence. The high growth rates indicated that there was considerable complexation of Cu in this medium such that the toxic form of the metal was

reduced to below the concentration that affects the organism.

The growth rate in the cultures having higher Cu levels were slightly greater than for low Cu concentrations. The explanation for this may be the same as that given for the results obtained when using high EDTA or HIS concentrations (see Section III.D.1).

The resin analysis, conducted on similar samples as the bioassay, also indicated strong complexation of Cu in this medium. The Cu equiv values determined for each Cu concentration was at most 32% of the total Cu concentration as represented by the % active value in Table XXIII. Also, the values were below the levels that would cause a toxic response in the bioassay organism except for the sample having the largest Cu spike (15.7×10^{-8}M) where a Cu eqiv value of 4.66×10^{-8}M Cu was found in the sample.

As compared to the experiments with model organic ligands in Section III, there should be a reduction in growth rate at the Cu equiv level found in the sample with the largest Cu spike but, as was indicated by the bioassay, there was no toxic response. This could be explained, once again, by the presence of high Mn concentrations found in the samples; levels above 1 mg l^{-1} were found in the samples as measured by graphite furnace AAS.

E. DISCUSSION

In Section IV, it was demonstrated that the ion-exchange equilibrium method could be used to estimate biologically active Cu in artificial seawater when the speciation of Cu was affected by various organic ligands. In this section, a similar experimental approach was taken except that natural seawater containing natural complexing agents was used to test the ion-exchange technique. Seawater was collected at various depths from a local fjord whose waters had previously been shown to have a complexing capacity and then tested by the proposed bioassay and resin techniques.

In the bioassays, the growth patterns obtained for the range of Cu concentrations used were different for each natural water sample although the 100 and 200 m samples were very similar. The 100 and 200 m samples appeared to support much better growth than did the samples from the 10, 50 or 75 m depths (Fig. 28). This was initially interpreted as an ability of the deep waters to sequester Cu due to the presence of natural complexing agents. In addition, it appeared that the shallower waters sequestered Cu to a limited degree as was indicated by these waters supporting better growth than did artificial seawater (SOW) having no organic ligands present.

Complexation of Cu in the natural water samples was also indicated by the resin analysis. This was shown by the Cu equiv values of all samples being lower than the total Cu concentration. The lowest percentage of complexed metal was seen in the 100 m seawater (high % active values) while similar

complexation was found in the 10, 50, 75 and 200 m.

Although the Cu equiv values of the natural water samples were lower than their corresponding total Cu concentration, these values were such that a toxic response in the organism was expected. From these values it appeared that the 100 m sample should have the lowest growth rates while better growth was expected in the 10, 50, 75 and 200 m samples. However, these conclusions were contradicted by the actual bioassay data. The 100 and 200 m samples had the highest growth rates while the 10 and 50 m waters were found to have the lowest. The weak relationship between the resin analysis and the bioassays was best illustrated when the -log of the Cu equiv values were plotted as a function of the growth rates (Fig. 29).

This weak relationship was not attributed to an error in the resin analysis but to a physiological Cu-Mn interaction found in the bioassay organism. Such an interaction has been recently discussed in detail first by Sunda et al. (1981) and then Sunda and Huntsman (in prep.). Sunda et al. (1981) found that the addition of Mn to their cultures of the diatom Chaetoceros socialis could reverse the toxic effect of Cu. They discussed the possibility of Cu adsorption to manganese oxides formed by the oxidation of Mn(II) to explain this antagonism. The adsorption of Cu would reduce the cupric ion activity of the medium and subsequently reduce its toxicity. However, they presented evidence to show that the kinetics of oxidation of Mn(II) to manganese oxides are exceedingly slow at the pH of seawater. They added 2×10^{-5}M $MnCl_2$ to seawater collected at

800 m and then stored the solution at room temperature for seven years. At the end of this period, they concluded that the manganese was all in the soluble manganous form and that no detectable oxidation had occurred. To further support their assumption they found that exceedingly low concentrations of manganese were required to reverse the toxic nature of Cu to their test organism. They doubted that at such low Mn concentrations, even if all the Mn(II) was oxidized to manganese oxides, enough Cu could be absorbed to reverse Cu toxicity to the extent found in their tests.

The presence of reduced Mn is also likely in natural seawater. Sunda et al. (1983) presented evidence that photoreduction of manganese oxides by dissolved organic substances occurs in seawater. They stated that such reactions appeared to be important in maintaining manganese in a dissolved reduced form in photic waters.

With the adsorption of Cu by Mn unlikely, Sunda et al. (1981) proposed that the growth stimulation by Mn must be physiological. The ability of Mn to reverse Cu toxicity was explained by a competitive interaction between the two metals at cellular sites involved in manganese nutrition. The sites could be either surface or intracellular sites. Further, they described a cellular model to explain the relationship between the growth rate of their test organism and the ionic activities of Mn and Cu. A two-site cellular binding model was developed in which Cu competes with Mn at one of the sites but only Cu reacts at the second. A two site model was developed because

they found that growth rate was dependent on the ratio of manganous ion activity to cupric ion activity at low cupric ion activities but, at higher cupric ion activities, the dependence of Cu toxicity on manganous activity was not nearly as pronounced. The exact mechanism of the Cu-Mn growth rate antagonism has not yet been clearly elucidated.

In the present study the competition between Cu and Mn for nutritional sites on/in the cell could explain the weak relationship between the Cu equiv values and growth rates. With toxic levels of Cu, growth rates would increase as the Mn concentration increased even though the cupric ion activity of the solution remained constant. This type of response was found in the natural water samples of Indian Arm. As the Mn concentration increased in the samples their toxicity decreased even though the resin analysis estimated the samples to have similar biologically active Cu concentrations.

Further evidence to support the Cu-Mn antagonism was found in the bioassay experiment using Cu spiked 50 m seawater samples with and without the addition of Mn. When Mn was added to the 50 m water at the same Mn concentration found in the 200 m seawater (3.64×10^{-6}M), the growth rates were higher, at all the Cu concentrations used, than that of the 50 m water having Mn added at the Aquil concentration. In fact, the growth pattern found at high Mn levels closely resembled the growth pattern found in the bioassays of the 100 and 200 m seawater which also had high Mn levels.

Because of this Mn interference, the bioassay technique

could not be used to estimate the biological activity of the cupric ion in samples enriched with Mn unless the relationship between Mn and Cu was known and subsequently the measurement of biologically active metal by the resin could not be verified. Examination of the Cu-Mn interaction was beyond the scope of the present study but further work is strongly suggested as this is such an important consideration in toxicity studies. However, the measurements from the resin analysis did indicate complexation of Cu by complexing agents in the samples and therefore the technique showed a potential for measuring the complexing capacity of natural waters.

To further test the potential of the resin for studying complexing capacity, the adsorption curve for Cu in the presence of complexing agents extracted from sediments was examined in low salinity SOW. On average, only 30% of each of the spiked Cu concentrations was resin active, thus suggesting considerable complexation of the metal. The bioassay performed on the same sample also indicated strong complexation of Cu. Growth rates were at their maximum for all the Cu concentrations used. However, the Cu equiv value in the sample having the largest Cu spike (15.7×10^{-8}M) was at a level that was expected to cause toxicity. This discrepancy can, at least partly, be explained by the presence of high background Mn concentrations found in the samples thereby reducing the toxicity of Cu to the organism.

In conclusion, it appears that complexation of Cu by natural seawater soluble agents did occur, as demonstrated by the results of the resin analysis and measurement of

biologically active Cu by the bioassay technique. The interpretation of the results, however, has some limitations because of the Cu-Mn interference.

VI. GENERAL CONCLUSIONS

The adsorption of Cu onto a strongly acidic cation-exchange resin of the sulphonate type (AG 50W X12) can be used to differentiate Cu in cationic species from that of anionic or uncharged species present in seawater. The procedure involves bringing the resin into equilibrium with a seawater sample and then measuring the concentration of adsorbed Cu. Comparison with Cu adsorption from standard Cu seawater solutions of similar composition, pH, and ionic strength yields a Cu equivalent measurement that can be related to the concentration (or activity) of the free cupric ion. A Cu equivalent value represents the concentration of Cu that, when present in organic ligand free artificial seawater, results in the adsorption of an amount of Cu equal to that adsorbed from the test solution.

In artificial seawater (SOW), the addition of model organic ligands (EDTA, NTA, GLU) decreases the adsorption of Cu by the resin and the extent of this decrease is related to the concentration and the stability of the Cu-ligand complex. Not all Cu complexing agents shows such a response, however. When HIS is added to SOW, Cu adsorption is actually higher than in similar samples lacking HIS. This increase is attributed to the adsorption of the positively charged Cu-HIS complexes. From this it is evident that care must be taken when studying natural water samples that may contain positively charged organic complexing agents.

A bioassay procedure was developed that measured the biologically active fraction of Cu in seawater. Bioassays were

conducted on similar artificial seawater samples to those used in the resin analysis. A strong relationship between the growth rates and the Cu equivalent values was found thus indicating the ability of the ion-exchange technique to measure biologically active Cu.

The bioassay and resin techniques were then used on natural seawater samples collected from a local fjord. In the bioassays, growth rates were higher in the samples from the 100 and 200 meter depths than in the shallower water samples. However, as concluded from the Cu equivalent values, the biologically active fraction of Cu in the water samples was similar or even higher in the deep waters (the 100 meter water had the highest Cu equiv values). Plotting growth rates as a function of the -log of the Cu equiv values demonstrated the weak relationship between the results of the resin and bioassay procedures. However, the weak relationship is due to a physiological Cu-Mn interaction that affects the bioassay organism and not to a problem with the resin technique. Bioassays in the 50 meter water with and without the addition of Mn at the concentration found in the 200 meter water (3.64×10^{-6}M), showed a decrease in Cu toxicity with the addition of the Mn. In fact, the growth found in the 50 meter water plus Mn closely resembled the growth found in the 200 meter sample.

Although the measurement of biologically active Cu by the resin technique could not be verified by the bioassay tests, it was evident that the technique was responding to complexation of Cu by natural complexing agents. This was indicated by the Cu

equiv values of all the natural water samples being lower than their total Cu concentration. Furthermore, a high level of complexation was indicated by the resin analysis of SOW that had been mixed with a sediment previously shown to contain seawater soluble complexing agents.

The potential of the ion-exchange method for studying Cu speciation in seawater has been demonstrated. The analytical scheme can be applied to the direct measurement of biologically active Cu or may be incorporated into other types of measurement, such as the determination of the complexing capacity of natural or polluted waters. The technique is not only limited to Cu but is applicable to many of the other trace metals present in seawater. Practical advantages include low equipment costs, relatively fast analysis time, good sensitivity and being conducive to automation.

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APENNDIX A. BIOASSAY DATA

Histidine

L-Histidine (1.0×10^{-7}M) (Sep 7/81)

	Cu Concentration ($\times 10^{-8}$M)				
Days	Control	7.9	15.7	23.6	31.5
0-1	1.00(.40)	1.07(.18)	0.96(.06)	0.31(.10)	0.17(.12)
1-2	2.10(.03)	1.97(.08)	1.29(.07)	0.58(.05)	0.08(.06)
2-3	2.04(.03)	1.74(.02)	0.76(.05)	0.57(.07)	0.53(.38)
3-4	1.56(.01)	1.91(.02)	0.63(.01)	0.40(.05)	0.19(.18)
$\bar{X}$ 1-4	1.90(.01)	1.87(.03)	0.89(.02)	0.52(.01)	0.27(.07)
% of Control		99 (2)	47 (1)	27 (1)	14 (3)

L-Histidine (2.5×10^{-7}M) (Aug 17/81)

	Cu Concentration ($\times 10^{-8}$M)				
Days	Control	7.9	15.7	23.6	31.5
0-1	0.24(.04)	0.30(.05)	0.27(.07)	0.18(.06)	0.30(.11)
1-2	2.45(.04)	2.37(.08)	2.46(.07)	2.08(.04)	1.72(.11)
2-3	1.98(.01)	2.06(.03)	2.08(.05)	1.19(.04)	0.79(.05)
3-4	1.86(.03)	1.92(.09)	1.87(.06)	1.16(.11)	0.56(.10)
$\bar{X}$ 1-4	2.10(.01)	2.11(.04)	2.14(.04)	1.47(.06)	1.02(.06)
% of Control		100 (2)	102 (2)	70 (3)	49 (3)

[1]Mean of K ±1 s.d. based on 3 replicates.

Histidine (con't)

L-Histidine (5.0×10^{-6}M) (Jul 27/81)

Cu Concentration ($\times 10^{-8}$M)

Days	Control	7.9	15.7	23.6	31.5
0-1	0.18(.04)	0.23(.06)	0.16(.07)	0.29(.07)	0.30(.04)
1-2	1.84(.03)	1.88(.02)	1.89(.02)	1.86(.03)	1.84(.02)
2-3	2.08(.04)	2.08(.04)	2.19(.01)	2.10(.04)	2.00(.13)
3-4	1.96(.04)	1.98(.03)	2.06(.14)	1.99(.01)	1.96(.03)
$\bar{X}$ 1-4	1.96(.01)	1.98(.01)	2.05(.04)	1.98(.01)	1.93(.03)
% of Control					

NTA

NTA (1.0×10^{-7}M) (Sep 28/82)

Cu Concentration ($\times 10^{-8}$M)

Days	Control	7.9	15.7	23.6	31.5
0-1	0.83(.02)	0.93(.25)	0.68(.03)	0.40(.11)	0.37(.13)
1-2	2.55(.02)	1.84(.04)	1.65(.07)	1.68(.18)	1.14(.03)
2-3	2.13(.06)	0.88(.04)	0.62(.05)	0.61(.04)	0.69(.02)
3-4	1.22(.07)	0.78(.09)	0.35(.05)	0.31(.01)	0.26(.01)
$\bar{X}$ 1-4	1.97(.01)	1.17(.04)	0.87(.01)	0.86(.05)	0.7 (.02)
% of Control		59 (2)	44 (1)	44 (3)	35 (1)

NTA (2.5×10^{-7}M) (Oct 5/82)

Cu Concentration ($\times 10^{-8}$M)

Days	Control	7.9	15.7	23.6	31.5
0-1	1.25(.14)	1.32(.03)	1.28(.06)	1.26(.12)	1.10(.07)
1-2	2.42(.14)	2.11(.05)	1.84(.06)	1.67(.06)	1.78(.00)
2-3	2.09(.14)	1.24(.06)	0.74(.05)	0.58(.07)	0.46(.06)
3-4	1.16(.05)	1.17(.04)	0.56(.03)	0.40(.06)	0.29(.04)
$\bar{X}$ 1-4	1.89(.02)	1.5 (.05)	1.05(.01)	0.88(.02)	0.84(.04)
% of Control		79 (3)	55 (1)	47 (1)	45 (2)

NTA (con't)

NTA (5.0×10^{-7}M) (Oct 19/82)

Cu Concentration ($\times 10^{-8}$M)

Days	Control	7.9	15.7	23.6	31.5
0-1	1.01(.07)	0.90(.05)	0.98(.14)	0.95(.15)	1.01(.10)
1-2	2.22(.06)	2.32(1.73	2.07(.13)	1.85(.08)	1.87(.13)
2-3	1.97(.03)	1.73(.02)	1.01(.03)	0.89(.05)	0.81(.09)
3-4	1.92(.01)	1.61(.05)	0.86(.02)	0.65(.05)	0.52(.13)
$\bar{X}$ 1-4	2.04(.03)	1.89(.04)	1.31(.03)	1.09(.08)	1.07(.04)
% of Control		93 (2)	64 (1)	54 (4)	52 (2)

NTA (7.5×10^{-7}M) (Jan 11/82)

Cu Concentration ($\times 10^{-8}$M)

Days	Control	7.9	15.7	23.6	31.5
0-1	2.43(.37)	2.47(.16)	2.13(.22)	2.71(.24)	2.56(.27)
1-2	1.80(.07)	1.90(.05)	1.68(.04)	1.62(.11)	1.55(.06)
2-3	1.82(.05)	1.83(.09)	1.37(.02)	1.01(.05)	0.81(.05)
3-4	1.80(.01)	1.80(.12)	1.13(.01)	0.76(.05)	0.56(.07)
$\bar{X}$ 1-4	1.81(.04)	1.84(.03)	1.40(.02)	1.13(.03)	0.97(.02)
% of Control		102 (1)	77 (1)	63 (2)	54 (1)

Glutamic Acid

L-Glutamic Acid (1.0×10^{-5}M) (Mar 21/82)

	Cu Concentration ($\times 10^{-8}$M)				
Days	Control	7.9	15.7	23.6	31.5
0-1	1.86(.07)	1.84(.12)	1.76(.15)	1.63(.08)	1.63(.05)
1-2	2.32(.09)	2.11(.06)	1.69(.07)	1.65(.05)	1.48(.03)
2-3	1.53(.04)	1.23(.06)	0.89(.05)	0.72(.03)	0.68(.03)
3-4	1.44(.05)	0.76(0.03	0.39(.03)	0.27(.05)	0.27(.07)
$\bar{X}$ 1-4	1.76(.03)	1.37(.05)	0.99(.05)	0.88(.01)	0.81(.02)
% of Control		78 (3)	56 (3)	50 (0)	46 (1)

L-Glutamic Acid (2.5×10^{-5}M) (Mar 15/82)

	Cu Concentration ($\times 10^{-8}$M)				
Days	Control	7.9	15.7	23.6	31.5
0-1	1.60(.23)	1.64(.10)	1.55(.18)	1.33(.15)	1.20(.09)
1-2	2.52(.13)	2.42(.14)	2.04(.08)	1.80(.05)	1.66(.04)
2-3	1.43(.02)	1.42(.12)	1.01(.05)	0.79(.06)	0.75(.01)
3-4	1.64(.17)	1.55(.16)	0.81(.06)	0.48(.04)	0.40(.16)
$\bar{X}$ 1-4	1.86(.09)	1.79(.05)	1.29(.04)	1.03(.01)	0.93(.05)
% of Control		96 (3)	69 (2)	55 (1)	50 (3)

Glutamic Acid (con't)

L-Glutamic Acid (7.5×10^{-5}M) (Sep 14/82)

Cu Concentration ($\times 10^{-8}$M)

Days	Control	7.9	15.7	23.6	31.5
0-1	0.92(.05)	0.88(.06)	0.89(.08)	0.89(.13)	0.87(.04)
1-2	2.32(.03)	2.30(.09)	2.15(.12)	2.02(.08)	1.95(.08)
2-3	2.06(.02)	2.08(.04)	1.73(.08)	1.50(.11)	1.34(.05)
3-4	1.54(.02)	1.53(.05)	1.82(.08)	1.35(.11)	1.29(.07)
$\bar{X}$ 1-4	1.98(.01)	1.97(.02)	1.91(.09)	1.62(.10)	1.52(.05)
% of Control		100 (1)	97 (5)	82 (5)	77 (2)

L-Glutamic Acid (1.0×10^{-5}M and 2.5×10^{-5}M) (Jun 7/82)

Cu Concentration ($\times 10^{-8}$M)

		1.0×10^{-5}		2.5×10^{-5}M	
Days	Control	7.9	15.7	7.9	15.7
0-1	1.71(.25)	1.49(.07)	1.75(.09)	1.89(.13)	1.99(.17)
1-2	2.21(.05)	1.89(.07)	1.62(.03)	2.37(.06)	1.84(.04)
2-3	1.81(.04)	1.22(.09)	0.84(.05)	1.79(.03)	1.21(.05)
3-4	1.76(.04)	0.95(.06)	0.43(.03)	1.62(.08)	0.91(.03)
$\bar{X}$ 1-4	1.93(.02)	1.35(.07)	0.96(.00)	1.93(.05)	1.32(.03)
% of Control		70 (3)	50 (0)	100 (2)	68 (2)

Glutamic Acid (con't)

L-Glutamic Acid (5.0×10^{-5}M and 7.5×10^{-5}M) (Jun 14/82)

Cu Concentration ($\times 10^{-8}$M)

Days	Control	5.0×10^{-5}M 7.9	5.0×10^{-5}M 15.7	7.5×10^{-5}M 7.9	7.5×10^{-5}M 15.7
0-1	1.18(.12)	1.30(.04)	1.35(.08)	1.34(.19)	1.32(.03)
1-2	2.21(.06)	2.32(.12)	2.16(.04)	2.15(.11)	2.28(.21)
2-3	1.78(.03)	1.79(.05)	1.68(.09)	1.93(.10)	1.53(.23)
3-4	2.03(.04)	1.94(.04)	1.41(.03)	1.94(.07)	1.91(.03)
$\bar{X}$ 1-4	2.00(.04)	2.02(.04)	1.75(.02)	2.01(.01)	1.91(.02)
% of Control		101 (2)	87 (1)	100 (1)	95 (1)

EDTA

EDTA (5.0×10^{-8}M) (May 24/82)

Days	Cu Concentration ($\times 10^{-8}$M) Control	3.9	7.9	11.8	15.7
0-1	0.54(.02)	0.66(.08)	0.64(.13)	0.54(.18)	0.52(.05)
1-2	1.58(.13)	1.58(.06)	1.54(.08)	1.31(.13)	1.21(.07)
2-3	1.56(.06)	1.71(.06)	1.48(.11)	1.06(.02)	0.91(.03)
3-4	1.41(.09)	1.55(.09)	1.32(.14)	0.69(.10)	0.53(.02)
$\bar{X}$ 1-4	1.52(.05)	1.61(.03)	1.45(.10)	1.02(.06)	0.88(.03)
% of Control		106 (2)	95 (7)	67 (4)	58 (2)

EDTA (1.0×10^{-7}M) (May 31/82)

Days	Cu Concentration ($\times 10^{-8}$M) Control	3.9	7.9	11.8	15.7
0-1	1.72(.25)	2.00(.04)	2.04(.2)	1.97(.04)	1.86(.33)
1-2	1.78(.01)	1.85(.03)	1.83(.03)	1.74(.02)	1.49(.05)
2-3	1.90(.00)	1.93(.01)	1.93(.01)	1.80(.01)	1.02(.13)
3-4	1.72(.01)	1.69(.13)	1.83(.04)	1.68(.11)	0.88(.07)
$\bar{X}$ 1-4	1.80(.00)	1.82(.03)	1.86(.01)	1.74(.04)	1.13(.05)
% of Control		101 (1)	104 (1)	97 (2)	63 (2)

No Ligand Addition

No Addition (Low Salinity) (Jul 27/82)

Cu Concentration ($x10^{-8}M$)

Days	Control	3.9	7.9	11.8	15.7
0-1	1.40(.05)	1.40(.21)	0.99(.05)	0.23(.04)	0.02(.05)
1-2	2.15(.01)	1.94(.04)	1.75(.19)	0.89(.05)	0.42(.32)
2-3	2.07(.05)	1.09(.07)	0.94(.05)	0.80(.15)	0.28(.09)
3-4	1.03(.02)	1.00(.08)	0.51(.03)	0.42(.04)	0.16(.26)
4-5	0.05(.03)	0.83(.01)	0.37(.06)	0.23(.07)	-.25(.26)
$\bar{X}$ 1-4	1.75(.03)	1.34(.05)	1.07(.06)	0.70(.03)	0.39(.03)
% of Control		77 (3)	61 (4)	40 (2)	22 (2)

No Addition (Jun 21/82)

Cu Concentration ($x10^{-8}M$)

Days	Control	3.9	7.9	11.8	15.7
0-1	1.68(.14)	1.85(.08)	1.54(.04)	1.18(.10)	1.03(.13)
1-2	2.38(.02)	2.09(.03)	1.86(.03)	1.68(.03)	1.40(.05)
2-3	2.16(.03)	1.37(.06)	0.90(.04)	0.96(.02)	0.95(.03)
3-4	1.82(.01)	1.31(.05)	0.20(.35)	0.39(.04)	0.41(.07)
$\bar{X}$ 1-4	2.12(.01)	1.59(.03)	1.01(.01)	1.01(.03)	0.92(.03)
% of Control		75 (1)	47 (4)	48 (0)	43 (1)

No Ligand Addition (con't)

No Addition

	Cu Concentration ($x10^{-8}M$)				
Days	Control	1.0	2.0	3.0	4.0
0-1	0.86(.01)	0.93(.03)	0.92(.07)	0.87(.07)	0.90(.10)
1-2	2.16(.04)	2.03(.04)	1.90(.14)	1.69(.04)	1.58(.05)
2-3	2.09(.03)	2.12(.05)	1.54(.06)	1.07(.05)	0.89(.07)
3-4	1.80(.03)	1.88(.04)	1.63(.17)	0.98(.08)	0.71(.02)
$\bar{X}$ 1-4	2.02(.01)	2.01(.02)	1.69(.11)	1.25(.02)	1.06(.02)
% of Control		100 (1)	84 (5)	62 (1)	52 (1)

Natural Water Samples

10 meters (Jul 7/82)

Cu Concentration ($x10^{-8}M$)

Days	Control	3.89	7.87	11.8	15.7
0-1	0.74(.02)	0.64(.10)	0.69(.14)	0.61(.12)	0.48(.13)
1-2	2.40(.05)	2.38(.18)	1.92(.21)	1.76(.13)	1.64(.23)
2-3	2.27(.08)	1.88(.15)	1.24(.09)	0.75(.05)	0.73(.08)
3-4	2.31(.08)	2.42(.05)	1.50(.09)	0.44(.07)	0.20(.03)
4-5	0.04(.01)	0.43(.10)	2.13(.13)	0.37(.08)	0.15(.05)
$\bar{X}$ 1-4	2.33(.03)	2.23(.07)	1.55(.02)	0.98(.03)	0.86(.07)
% of Control		96 (3)	66 (1)	42 (1)	37 (3)

50 Meters (Jul 12/82)

Cu Concentration ($x10^{-8}M$)

Days	Control	7.9	15.7	23.6	31.5
0-1	1.59(.14)	1.87(.11)	1.70(.04)	1.50(.09)	1.24(.12)
1-2	2.46(.15)	1.99(.12)	1.71(.08)	1.42(.07)	1.23(.06)
2-3	2.51(.08)	1.64(.04)	1.08(.24)	0.71(.05)	0.91(.14)
3-4	1.88(.21)	2.36(.20)	1.30(.53)	0.57(.04)	0.38(.17)
4-5	0.03(.07)	0.75(.33)	1.34(.81)	0.08(.01)	0.12(.04)
$\bar{X}$ 1-4	2.29(.05)	1.99(.11)	1.36(.28)	0.90(.01)	0.84(.03)
% of control		87 (5)	59 (12)	39 (0)	37 (1)

Natural Water Samples (con't)

75 Meters (Jul 14/82)

Cu Concentration ($x10^{-8}M$)

Days	Control	3.9	7.9	11.8	15.7
0-1	2.00(.14)	2.11(.05)	2.16(.08)	1.97(.16)	1.51(.08)
1-2	2.65(.03)	2.54(.05)	2.14(.08)	1.80(.02)	1.55(.07)
2-3	2.47(.07)	2.26(.05)	1.68(.06)	1.14(.05)	1.09(.02)
3-4	1.96(.06)	2.21(.03)	2.03(.06)	1.08(.02)	0.65(.04)
4-5	0.17(.05)	0.31(.09)	1.38(.04)	1.41(.00)	0.52(.04)
$\bar{X}$ 1-4	2.36(.01)	2.34(.02)	1.95(.01)	1.34(.02)	1.10(.02)
% of Control		99 (1)	82 (0)	57 (1)	47 (1)

200 Meters (Jul 19/82)

Cu Concentration ($x10^{-8}M$)

Days	Control	3.9	7.9	11.8	15.7
0-1	2.13(.04)	2.12(.05)	2.03(.02)	1.84(.02)	1.57(.02)
1-2	2.69(.06)	2.46(.04)	2.36(.05)	2.09(.07)	1.88(.02)
2-3	2.50(.05)	2.50(.01)	2.52(.05)	2.35(.03)	2.13(.05)
3-4	1.62(.06)	1.81(.10)	1.90(.01)	2.07(.03)	2.05(.03)
4-5	0.03(.02)	0.01(.07)	0.12(.12)	0.54(.12)	1.35(.09)
$\bar{X}$ 1-4	2.27(.03)	2.26(.03)	2.26(.03)	2.17(.02)	2.02(.02)
% of Control		100 (1)	100 (1)	96 (1)	89 (1)

Natural Water Samples (con't)
Mn Experiment

50 Meters (repeat)
without Mn addition (Aug 16/82)

Cu Concentration ($x10^{-8}M$)

Days	Control	3.9	7.9	11.8	15.7
0-1	1.53(.24)	1.65(.06)	1.51(.13)	1.39(.02)	1.33(.06)
1-2	2.38(.10)	2.12(.03)	1.89(.00)	1.71(.06)	1.54(.09)
2-3	2.04(.02)	1.60(.04)	0.63(.05)	0.60(.11)	0.55(.04)
3-4	1.79(.04)	1.74(.04)	0.02(.13)	0.02(.02)	-.02(.09)
4-5	-.19(.17)	0.38(.18)	-.03(.07)	-.14(.04)	0.00(.19)
$\bar{X}$ 1-4	2.07(.04)	1.82(.02)	0.86(.02)	0.77(.02)	0.71(.03)
% of Control		88 (1)	42 (1)	37 (1)	34 (1)

50 Meters (with $3.64x10^{-6}M$ Mn) (Aug 16/82)

Cu Concentration ($x10^{-8}M$)

Days	Control	3.9	7.9	11.8	15.7
0-1	1.91(.05)	1.84(.11)	1.79(.01)	1.75(.20)	1.37(.09)
1-2	2.20(.09)	2.16(.01)	1.99(.07)	1.66(.10)	1.49(.05)
2-3	2.15(.02)	2.13(.02)	1.88(.05)	1.82(.05)	1.56(.08)
3-4	1.91(.07)	1.89(.04)	1.70(.07)	1.58(.07)	1.45(.06)
4-5	0.01(.03)	0.03(.02)	0.36(.13)	0.65(12)	1.47(.12)
$\bar{X}$ 1-4	2.08(.02)	2.06(.02)	1.86(.03)	1.69(.02)	1.50(.04)
% of Control		99 (1)	89 (1)	81 (1)	72 (2)

Natural Water Samples (con't)

200 Meters (repeat) (Jul 28/82)

Cu Concentration ($x10^{-8}M$)

Days	Control	3.9	7.9	11.8	15.7
0-1	1.16(.04)	1.31(.11)	1.15(.07)	0.87(.13)	0.74(.13)
1-2	2.26(.01)	2.17(.01)	2.10(.06)	1.77(.05)	1.13(.04)
2-3	2.57(.05)	2.48(.04)	2.45(.02)	2.25(.04)	1.86(.08)
3-4	2.42(.01)	2.34(.01)	2.27(.04)	2.29(.03)	2.20(.06)
4-5	0.71(.06)	1.02(.08)	1.20(.01)	1.77(.08)	2.11(.06)
$\bar{X}$ 1-4	2.42(.01)	2.33(.02)	2.27(.00)	2.11(.01)	1.73(.06)
% of Control		97 (1)	94 (0)	88 (1)	72 (2)

Sediment Study

Mud Sample (Jul 26/82)

Cu Concentration ($x10^{-8}M$)

Days	Control	3.9	7.9	11.8	15.7
0-1	2.03(.16)	2.22(.17)	2.12(.07)	2.08(.05)	2.07(.01)
1-2	1.60(.06)	1.54(.10)	1.66(.09)	1.63(.05)	1.59(.05)
2-3	2.35(.04)	2.39(.04)	2.35(.01)	2.36(.01)	2.33(.06)
3-4	1.42(.13)	1.33(.04)	1.48(.06)	1.56(.07)	1.74(.07)
4-5	-.04(.00)	-.07(.03)	-.02(.01)	-.01(.02)	-.04(.03)
$\bar{X}$ 1-4	1.79(.02)	1.75(.06)	1.83(.01)	1.85(.02)	1.89(.01)
% of Control		98 (3)	102 (1)	104 (1)	106 (0)