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Title: The Copper Sensitivity of Oregon Coastal Phytoplankton Populations

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The copper sensitivity of natural populations of Oregon coastal phytoplankton was studied using both additions of ionic copper and Cu-TRIS free ion activity buffers in coastal seawater. Phytoplankton growth rate, taxonomic composition and copper content were examined in treatment additions.

Additions of 50 to 130 nM ionic copper and calculated copper free ion activities from 0.3 to 0.008 nM resulted in 50% growth rate inhibition of the natural populations. Assuming common models of inorganic speciation of copper in seawater, these results suggest the presence of organic complexing agents in coastal seawater. In four experiments with ionic copper additions the genus Thalassiosira increased in abundance relative to the genus Chaetoceros with increasing copper additions, up to some level where both genera were eliminated. In copper additions containing TRIS, however, the situation was reversed, with Chaetoceros becoming more abundant with increasing cupric free ion activity in seawater containing 5 mM TRIS,

and maintaining constant abundance in seawater containing 1 mM TRIS. At copper stresses in either ionic copper additions or in TRIS-free ion activity buffered seawater that eliminated the dominant planktonic diatom flora, cultures were often, but not always, found to allow growth of benthic pennate diatoms, or more rarely, flagellates.

The growth rate results suggested that the deficiency of another trace metal increased the apparent toxicity of copper to phytoplankton, especially in TRIS-free ion activity buffered seawater. Laboratory experiments with isolated coastal phytoplankton species indicated that manganese deficiency exacerbated copper toxicity, and that manganese deficiency was induced in TRIS buffered seawater by a TRIS-catalyzed oxidation of Mn. When manganese additions to natural populations were employed in conjunction with ionic copper additions and TRIS-free ion regulated seawater, they showed that ambient manganese concentrations were low enough to shift the onset of copper toxicity to lower copper concentrations.

The results suggest that while acute toxicity to phytoplankton by ambient concentrations of copper is unlikely, the interactions of copper and other metals, especially manganese, may influence natural coastal phytoplankton populations in more subtle ways, such as taxonomic composition.

The Copper Sensitivity of Oregon Coastal Phytoplankton

by

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I. INTRODUCTION

A. Research Objectives

The objective of this research has been to evaluate the role of copper, and the interactions of copper and other trace metals (most notably manganese) on phytoplankton in the Oregon coastal zone. Much work on the role of copper in phytoplankton ecology and physiology has been produced in the past. Most of such past work has been performed without reference to three important recent advances. The first advance is reliable measurement of trace metals in seawater, and derivatively, knowledge of their true levels and spatial distributions. The second advance is the development of quantitative models for the distributions of the various forms of trace metals in seawater. The third advance is a model for the bioavailability of various forms of trace metals.

The three new advances have resulted in general distrust of a large body of existing work. Most of this early work is either uninterpretable by virtue of contaminated trace metal analyses (Turekian, 1977), or can now be interpreted differently in light of the new advances. In this dissertation I hope to contribute to the current wave of insight in this field.

This dissertation also makes special reference to the coastal zone. Coastal zones are the sites of interaction of land and sea, and the primary area of man's interaction with the ocean. Coastal zones also have a distinct suite of biological, chemical and physical

properties. These properties need to be considered when evaluating the relevance of research performed in different provinces; i.e., when results from open ocean or lakes is applied to the coastal zone, or when research from the coastal zone is applied to the other provinces.

B. Specific Attributes Examined

The interaction of metals with phytoplankton could be studied in many ways, depending on the aspect of phytoplankton physiology or ecology of interest, and the metals of interest. I chose to investigate three important aspects of phytoplankton with respect to trace metals: growth rate, trace metal content, and species composition of the phytoplankton.

There are several reasons for narrowing the focus to these three parameters. Growth rate was selected because of its obvious importance to maintaining phytoplankton populations in environments where they experience large losses due to grazing and mixing (such as the coastal zone). Growth rate is also a sensitive indicator of sublethal effects of metals on a phytoplankton stock,since growth may be inhibited at stress levels that do not cause mortality (Davey et al., 1973; Sunda and Guillard, 1976). Further, growth rate is easily measured with a variety of techniques using small amounts of culture. The responses of phytoplankton to trace metals in previous studies have also frequently been expressed in terms of growth rate (Steemann Nielsen and Kamp-Nielsen, 1970; Steemann Nielsen and Wium-Anderson, 1970; Sunda and Guillard, 1976; Sunda et al., 1981).

Trace metal concentrations in phytoplankton are important for two

reasons. First, the responses of a cell to metal are ultimately due to the effects of that metal on the biochemistry of the cell. Such chemistry is determined by reactions whose rates and extents are controlled by concentration-dependent equilibria. The appropriate concentrations in these cases are clearly intracellular, not extracellular, concentrations. Second, concentrations of trace metals in phytoplankton from solutions of known composition (for example, heavily chelated media) may be used to compare with the trace metal levels of phytoplankton grown in less well characterized solutions.

Phytoplankton species composition is important because almost no phytoplankton assemblages are clonal, unialgal or otherwise homogeneous groups which respond to trace metals (or any other environmental feature) in exactly the same way. Presumably in the course of evolving to fit different environments, different phytoplankton taxa have also evolved to meet the different trace metal levels and ratios in these environments. Thus, phytoplankton-trace metal interactions may well be one set of mechanisms responsible for the maintenance of many species of organisms in an ocean realm which is relatively uniform and stable, in apparent violation of the competitive exclusion principle (Hardin, 1960, as noted by Hutchinson, 1961).

II. BACKGROUND

A. Introduction

In order to understand how given trace metal concentrations apply to environmental conditions it is important, but not sufficient, to know the levels and distributions of trace metals found in the ocean. Until recently, simply measuring the concentrations of trace metals in seawater has been an extremely difficult problem, and many old papers give concentrations which are questionable or clearly wrong. Since trace metal concentrations do appear to vary significantly within the marine provinces in the newer data, I will also discuss these variations, with particular reference to the Oregon coastal environment, when possible.

Also necessary to bridge the gap between measured environmental concentrations and biologically relevant trace metal concentrations for phytoplankton, is knowledge of the chemical states of various trace metals in seawater. It is clear that phytoplankton, and indeed all organisms, have different responses to various chemical states of a given trace metal.

The last areas I will review are the physiological and ecological effects of selected trace metals on phytoplankton, keeping in mind more recent developments in trace metal concentrations, distributions and speciation.

B. Trace Metal Concentrations and Distributions

1. Historical Perspective

In the words of Turekian (1977):

> "..... one has the feeling that the whole field of trace metal geochemistry would have been a completely dull one over the past fifty years if it weren't for analytical errors."

The state of knowledge of selected seawater trace metal concentrations at three different times is shown in Table 1. The first column is from Sverdrup et al. (1942), the second is from Goldberg (1963) and the third and fourth columns show a collection of more recent measurements of soluble trace metals for surface and deep ocean waters, respectively.

Table 1 clearly shows the effect of improved analytical technique on the perceived concentrations of most trace metals in the oceans. If one naively uses simple curve fitting to estimate the concentrations of trace metals in the ocean in, say 1492, one sees that Columbus could have dispensed with the Nina, Pinta, and Santa Maria, and simply driven wagons across the metallic Atlantic Ocean to discover the New World. Similarly, in the not too distant future one could expect to seek in vain for a single metal ion in solution in the world's oceans, despite any pollution. Obviously, such extrapolations are invalid, but they do serve to illustrate the extraordinary effect that improved analytical techniques have had on any aspect of science that needs knowledge of the concentrations of trace metals in seawater.

Table 1. Historical trends of measured trace metal concentrations in the oceans. All concentrations in nmol/kg.

			Present	
Metal	1942	1963	sfc.	deep
Al	18,500	370	37	20-40
Cr	-	0.8	1.5	3.0
Mn	400-4000	40	4	0.05
Ni	34	34	2	11
Cu	300-3000	50	1	8
Zn	1200	150	0.15	9.5
Se	50	50	0.35	1.6
Hg	0.2	0.2	0.015	0.025

2. Modern Values

How can we be certain that the trace metal concentrations currently being touted as the correct results will be proven to be correct in the long run? Two criteria for acceptance of measured trace metal concentrations in the oceans have been given by Boyle et al. (1977). The first criterion is reasonable agreement between two groups of analysts for an element in a similar environment. The second criterion is a demand for oceanographic and geochemical consistency for distributions of an element. Bruland et al. (1979) add a third criterion: that analyses performed on the same samples by at least two different techniques give similar results.

The first criterion serves to guard against reporting incorrect results due to hidden problems in a given analytical laboratory. The second criterion forces the analyst to make more measurements in a greater variety of cases, enabling comparisons with distributions of better known substances. The third criterion guards against subtle artifacts in single analytical techniques that give rise to erroneous values.

A few examples are needed to explain what is meant by consistent oceanographic distribution. One aspect of a consistent distribution often noted is a "nutrient-like" vertical concentration profile. Due to uptake by organisms (and possibly nonbiological adsorption of metals onto particles) in the surface waters, followed by sinking, dissolution and decay, nutrients and many metals typically show low concentrations in surface waters with graduation to higher concentrations in deep water (Turekian 1968; Broecker, 1974). The

exact form of such a vertical profile for a given element is variable depending on the location in the ocean, but profiles can generally be categorized as those showing steep gradients near the surface and little change in deep water, and those showing more consistent increase with depth. The first kind, typified by phosphorus, is caused by a high rate of regeneration in sinking particles. The second kind, typified by silicon, is due to more release of material deeper in the water column, plus release from the sediments.

Another aspect of consistent oceanographic distribution is the appearance of elemental concentration maxima or minima in horizontal or vertical concentration fields that correspond to known or suspected sources or sinks of an element. Sources include riverine input, subsurface hydrothermal vents, aeolian input, and pollution. Sinks are harder to define, but might include areas of biological uptake, sites of mineral precipitation, or sites of adsorption to particles.

A final aspect of consistent oceanographic distribution is that large scale horizontal distributions should show variations consistent with large scale physical circulation patterns in the ocean. For example, due to the production of deep water in the North Atlantic and Antarctic, concentrations of nutrients in deep water show a general increase in the series: N. Atlantic, S. Atlantic, Antarctic, Indian, S. Pacific, N. Pacific Oceans.

At present, at least eight elements fit one or more of our criteria for conditional acceptance of trace metal concentrations fairly well, at least well enough to seem reasonably secure in the future. These are Mn, Ni, Cu, Zn, Cr, Cd, Se, and Hg (Table 1). For this dissertation I will emphasize copper and manganese distributions

and concentrations, followed by some short discussion on other metals and the reasons they are less suitable for examining phytoplankton-trace metal interactions in the coastal zone.

a. Copper

Our knowledge of copper concentrations in the oceans appears to meet all the criteria for accepting their values. Similar concentrations have been measured by several investigators using different analytical methods, including atomic absorption spectrophotometry, anodic stripping voltammetry, and isotope dilution. Various investigators also give consistent oceanographic patterns for this element.

In a horizontal transect across the Antarctic Circumpolar Current near New Zealand, Boyle and Edmond (1975) measured an increase in surface Cu from 0.98 to 3.25 nmol/kg. This increase correlated quite well with a similar increase in nitrate concentration. In a vertical profile in the Sargasso Sea, Bender and Gagner (1976) found a slight increase in Cu toward the bottom, 1.9 to 3.1 nmol/kg from the surface to 4000 m. Moore and Burton (1976) found the same general pattern in a vertical profile off North Africa, with concentrations ranging from 1.4 to 3.5 nmol/kg. Boyle et al. (1977) presented vertical concentration profiles from three stations at widely separated sites in the Pacific, and concentrations of Cu ranged from 1.1 to 8.4 nmol/kg. Again Cu generally increased from surface to deep water, but often with elevated surface concentrations which were ascribed to aeolian input. Bruland et al. (1979) found from 0.54 to 5.34 nmol/kg in three vertical profiles from stations in the North Pacific. Values

increased rather consistently from surface to deep water, but in contrast to Boyle et al. (1977) no evidence of copper surface maxima was found. It was concluded that previously reported surface maxima were actually the result of ship-related contamination. The general increase of copper in the bottom water going from the Atlantic to the Pacific corresponds to the pattern noted earlier. Fig. 1 shows a vertical profile for Cu in the California Current, the body of water that dominates the Washington, Oregon and California coastline (Bruland, 1980).

An important question for my discussion is whether or not the concentration of Cu in the coastal zone is significantly different from that in the open sea. Regarding the overall distribution of Cu in coastal and estuarine areas, we are in a more uncertain position than in the open sea. Most of the analyses of Cu and other trace metals in coastal and estuarine environments were performed in the years just prior to the development of the ultra-clean sample handling techniques that have played such an important role in the recent improvement in the analyses of trace metals in seawater. Thus, when it appears that Cu and other trace metals are in higher concentration in coastal and estuarine waters, it is not entirely clear whether or not this is due to analytical problems of various sorts, or due to real differences in concentration due to pollution, natural runoff, or desorption from particles. An effort to produce new baseline estimates of trace metals in coastal and estuarine water now seems important in light of the new methodology. This may be of more value than continuing to measure Cu and other trace metals in the open oceans where concentrations and distributions now seem predictable.

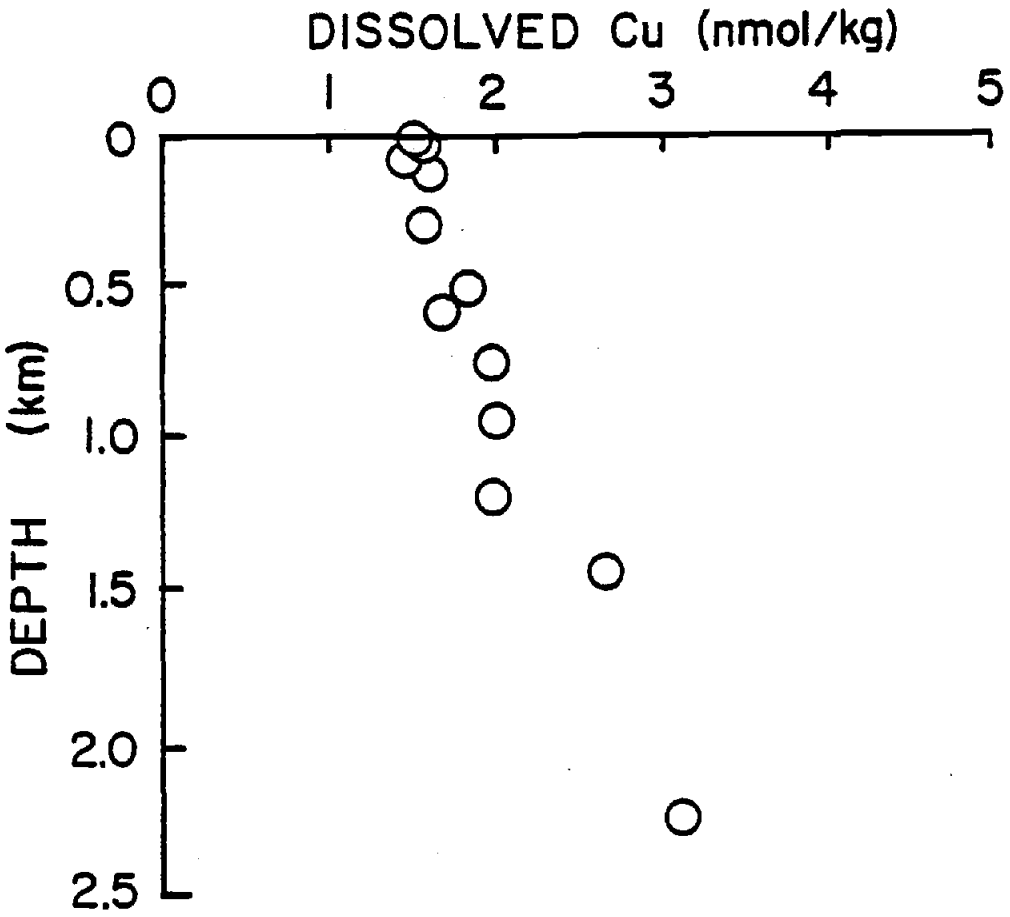

Fig. 1. The vertical distribution of Cu in the California current (Bruland, 1980).

Table 2 shows a compendium of Cu analyses for nearshore and estuarine waters, from the recent literature. The earliest analyses, such as those of Spencer and Brewer (1969) and Knauer and Martin (1973), are clearly in error, and in fact the latter authors have specifically retracted their reported values (see Bruland et al., 1979). The other papers show that copper concentrations in nearshore areas and estuaries frequently are measured within the concentration range of the deep sea. However, there does seem to be some elevation of Cu concentrations in certain cases, especially in estuaries. Klinkhammer and Bender (1981) show a particularly clear case in which pollution in the Hudson River estuary has raised the concentration of Cu in water of high salinity. Except near the mouth of heavily polluted estuaries, the concentration of copper in the coastal zone is not greatly elevated above that in the adjacent open ocean.

b. Manganese

As with Cu, we have recently achieved much in the knowledge of the distribution and abundance of Mn in the oceans. Bender et al. (1977) produced the first oceanographically consistent vertical concentration profile, from the Sargasso Sea. The water column yielded a rather constant 0.4 nmol/kg below a surface maximum of 2.4 nmol/kg. Since the Bender et al. report, a number of other studies have been completed with the following generalizations emerging. First, there is almost invariably a surface maximum in the Mn profiles, most certainly due to aeolian and riverine input (Klinkhammer and Bender, 1980; Landing and Bruland, 1980). Second, there is sometimes a deeper maximum, corresponding to the position of

Table 2. Concentrations of Cu in coastal and estuarine waters.

Author	Cu (nmol/kg)	Site
Spencer and Brewer (1969)	37.2	Atlantic slope water
Knauer and Martin (1973)	15.5	Monterey Bay, non-upwelling
Knauer and Martin (1973)	23.3	Monterey Bay, upwelling
Bewers, Sundby, and Yeats (1976)	8.7	Nova Scotia, surface shelf water
Bewers, Sundby and Yeats (1976)	6.0	Nova Scotia, deep shelf water
Bewers, Sundby and Yeats (1976)	3.7	Nova Scotia, Atlantic, surface water
Kremling and Peterson (1977)	7.8-23.3	Baltic Sea
Thomas and Grill (1977)	7.8-46.5	Strait of Georgia, Fraser River plume
Evans (1977)	<1.5-82.9	Newport River Estuary, N. Carolina
Evans (1977)	1.5-4.65	Atlantic shelf, N. Carolina
Batley and Gardner (1978)	2.6-24.8	Port Hacking Estuary, Australia
Klinkhammer and Bender (1981)	62.0-108	Hudson River Estuary, river end
Klinkhammer and Bender (1981)	~25.0	Hudson River Estuary, seaward end

the oxygen minimum zone in the Pacific, with which the Mn maximum has been tentatively linked (Klinkhammer and Bender, 1980; Landing and Bruland, 1980). Third, in the vicinity of deep ocean ridges there is often a large, deep, subsurface Mn maximum associated with hydrothermal vents (Klinkhammer et al., 1977; Klinkhammer, 1980; Klinkhammer and Bender, 1980; Landing and Bruland, 1980; Lupton et al., 1980).

In contrast to Cu, it seems clear that Mn in estuarine and coastal zones is usually in much higher concentration than in open ocean water. Manganese adsorption chemistry is such that Mn tends to be released from particles to which it is electrostatically adsorbed, when those particles are transferred from fresh to slightly saline waters (Murata, 1939; Fukai, 1966; Evans and Cutshall, 1973; Robertson et al., 1973; Gibbs, 1973; Lentsch et al., 1973; Wolfe et al., 1975). Manganese also has a unique redox chemistry that tends to enhance the concentration of dissolved Mn in estuaries. Mn(IV) is readily reduced to Mn(II) upon transition from highly oxidizing environments (such as most river waters and river sediments) to reducing environments (such as anoxic sediments present in many estuaries). Mn(IV) forms a number of highly insoluble oxides; however, Mn(II) is very soluble in both anoxic and oxygenated waters. Most transition metals form highly insoluble sulfides, but Mn does not. Thus, high levels of dissolved Mn are often found in anoxic estuarine sediments. In rivers with low pH (about 6.0), Mn(II) is stable in the presence of oxygen. The rate of oxidation of Mn(II) in natural waters is probably variable, depending on pH, oxygen concentrations, and the presence of catalytic surfaces (Stumm and Morgan, 1970), but it most likely occurs on the

order of days to weeks (Delfino and Lee, 1968; Fluorie, 1972; Riedel 1978; Santschi et al., 1980).

The chemical properties of Mn suggest a qualitative model of Mn behavior in a hypothetical estuary (Evans, 1977). Manganese enters the estuary in three forms: dissolved, adsorbed to particles (for this discussion particles can be either bedload or suspended particles), and as Mn in minerals. When particles with adsorbed Mn are first mixed with seawater in the estuary, Mn is desorbed through displacement by abundant cations in seawater, producing an initial rapid rise in the concentration of dissolved Mn. Particles often collect in mid-estuary (salinity 5-15 o/oo), where the sediments are often anoxic. Here Mn can be released from Mn oxides into the pore waters. Dissolved Mn in the pore waters is released to the overlying water column by diffusion, bioturbation, or tidal disturbance. In the zone down river from the anoxic sediments, Mn in the water column is progressively diluted by seawater. In highly oxygenated, high-pH seawater, Mn is also oxidized to Mn(IV), which precipitates as manganese oxide mineral phases. These Mn minerals aggregate with other particles and settle to the bottom in the lower estuary and coastal zone. These fine sediments are often transported back up the estuary by the currents bringing seawater into the estuary. They are then redeposited in the area of fine anoxic sediments, buried, reduced, and the Mn(II) freed for another cycle (Fig. 2).

How well is this model borne out in the real world? Fig. 3 shows dissolved Mn vs. salinity for four estuaries around the world. All resemble the model to a high degree, although there is some variation in the exact form of the curve. These variations are all probably due

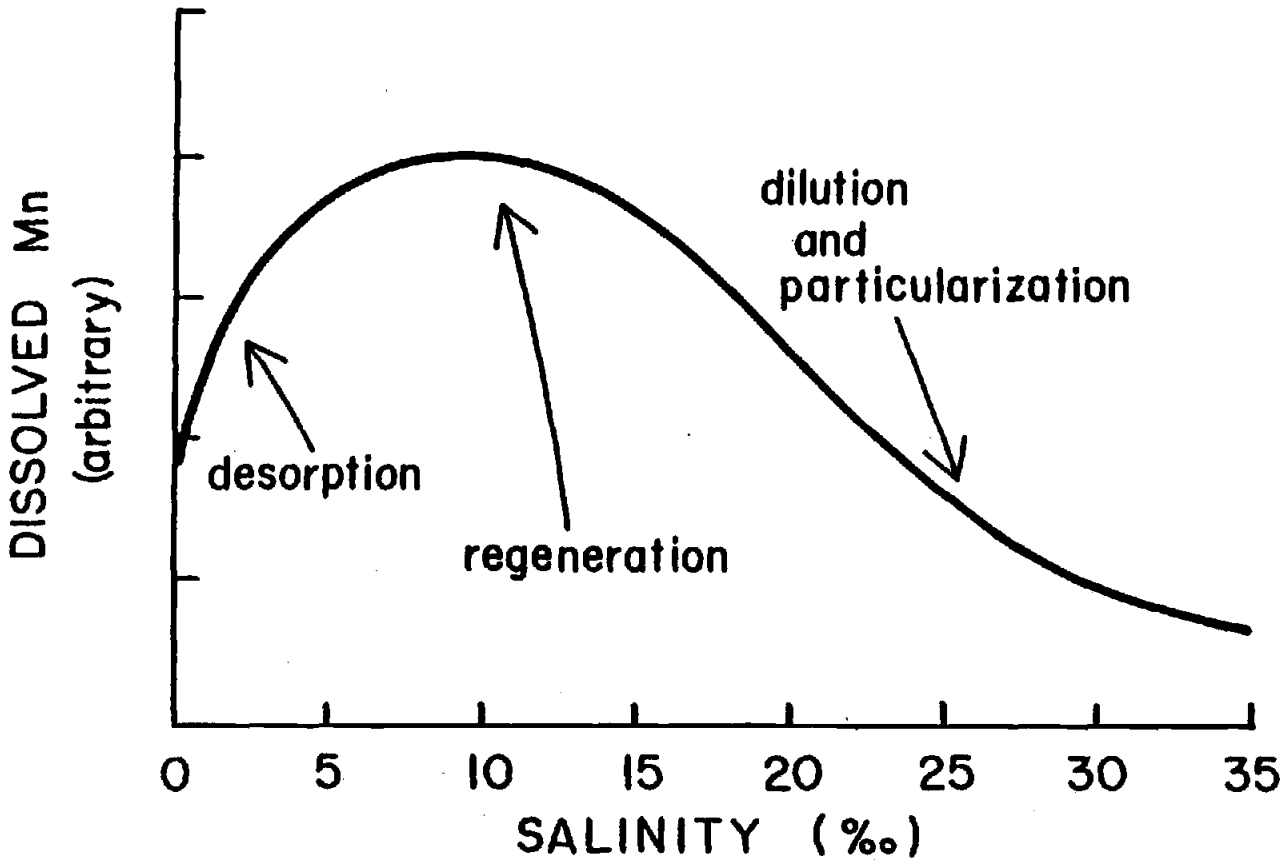

Fig. 2. The geochemical processes leading to the observed distribution of Mn in estuaries.

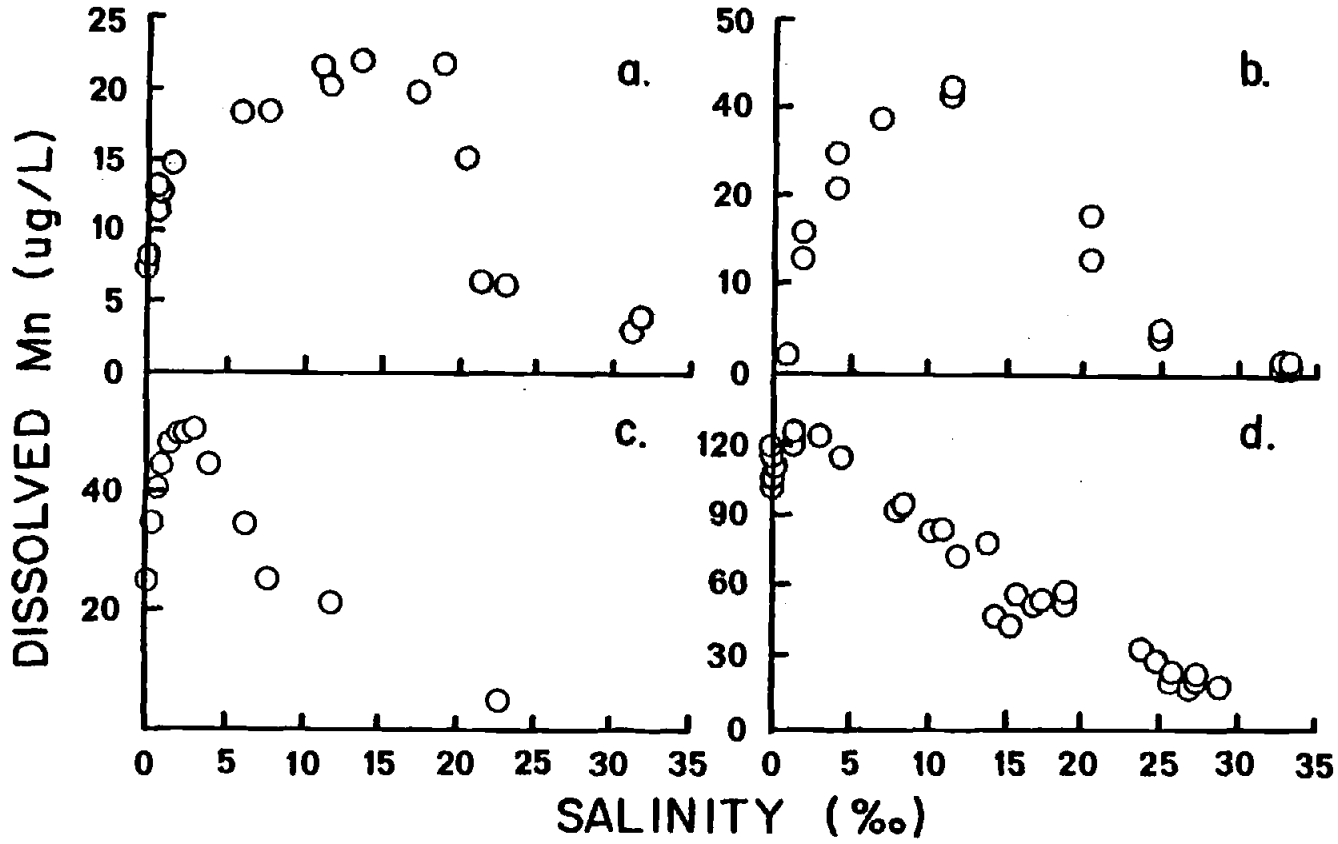

Fig. 3. The observed distribution of Mn in estuaries. a) Yaquina Bay, Oregon (Evans, unpublished manuscript). b) Newport River Estuary, North Carolina, (Evans et al., 1977). c) The Rhine Estuary (Duinker and Nolting, 1978). (d) Beaulieu Estuary, England (Holliday and Liss, 1976).

to variations in estuarine conditions such as the initial pH, the initial distribution of Mn forms, the river flow, the estuarine mixing regime, the sediment types, and many other things that vary from estuary to estuary. Nevertheless, the basic pattern of Mn behavior in estuaries seems to be sufficiently known.

Whether or not estuaries represent a net source of dissolved Mn to the ocean depends on whether the final loss of Mn to the sediments exceeds the sum of fresh water input of dissolved Mn, diagenesis, and desorption of Mn. From laboratory studies Sholkovitz (1976) has estimated that 25-45% of Mn is lost to the sediments during estuarine mixing, due to precipitation and flocculation.

In the Pacific, there appears to be substantially more Mn in the coastal zone than in the open ocean. Landing and Bruland (1980) produced a series of vertical profiles of Mn at stations from nearshore northern California to Hawaii. These showed strong surface maxima within the nearshore California Current, up to 11.9 nmol/kg. The intensity of the maxima decreased seaward to typical ocean values. Waters below 100 m had about 1.0 nmol/kg. I measured the concentration of Mn in a vertical profile at a station 13 miles from the Oregon coast (Fig. 4). It also shows a strong surface maximum of 14.2 nmol/kg, decreasing to 1.1 nmol/kg at 90 m.

It is interesting to note that summer upwelling off the Oregon and California coasts, while bringing nutrient rich water from about 100 m to the surface, is probably bringing extremely Mn-deficient water as well.

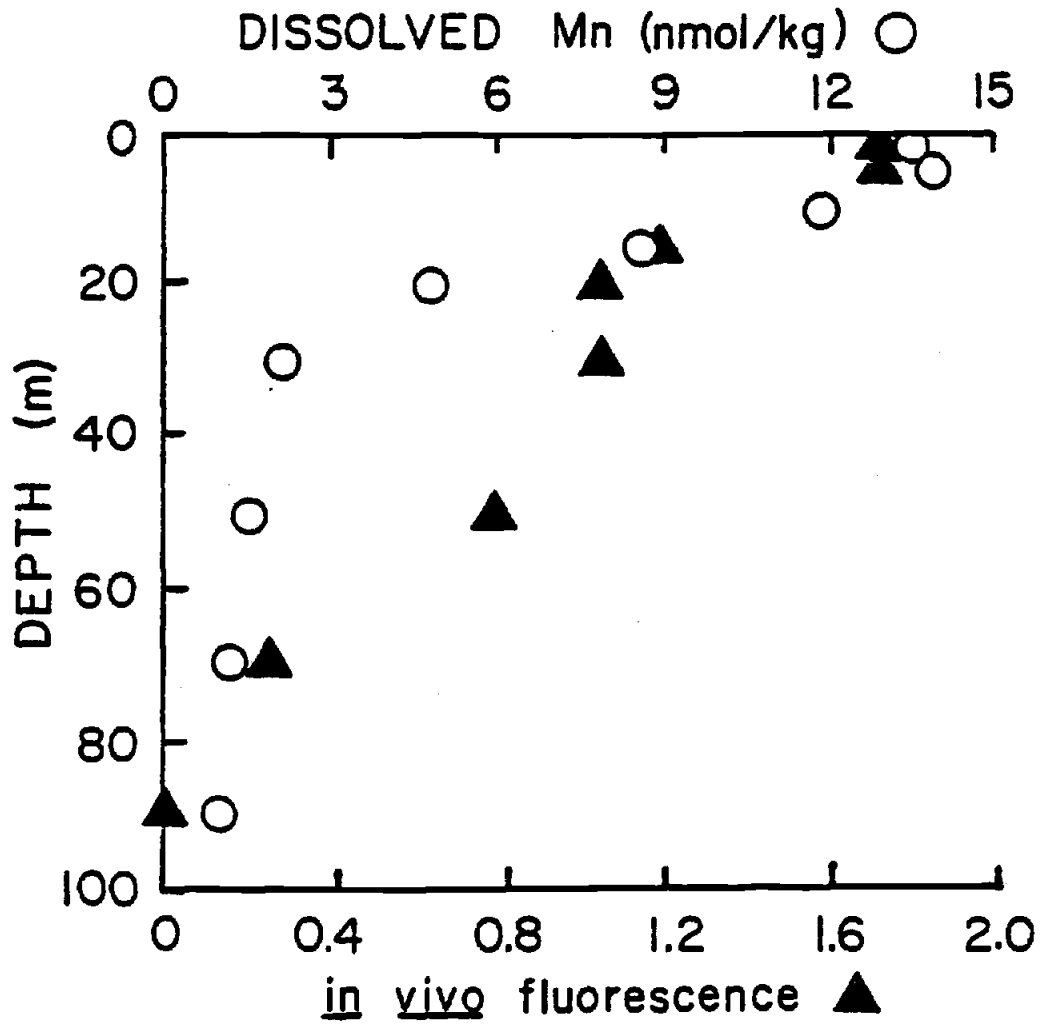

Fig. 4. The observed vertical distribution of Mn in the California Current at station SUN-13. (13 nautical miles seaward, 45°N)

c. Other Metals

Zinc has presented very severe contamination problems with regard to its analysis in seawater. However, some recent vertical concentration profiles in the North Pacific seem consistent (Bruland et al., 1978a; Bruland et al., 1979; Bruland, 1980). These profiles indicate that in open ocean water Zn concentrations correlate very well with silicon. Concentrations as low as 0.15 nmol/kg have been recorded in surface samples. Deep water, circa 3000 m, has as much as 9.5 nmol/kg. It is to be expected that upwelling or mixing could raise surface Zn concentrations to the same proportion that they raise surface Si concentrations.

Unfortunately, ultra-clean Zn analytical techniques have not yet been applied to coastal or estuarine waters. Therefore, when considering reports of high concentrations of Zn in coastal and estuarine waters, one is unsure whether or not the elevated values represent truth or contamination. However, it seems likely that rivers and estuaries that receive large amounts of metals from pollution would have zinc concentrations much higher than seawater, and that nearby coastal seawater would also have elevated concentrations. For example, the Beaulieu River has Zn concentrations up to 620 nmol/kg. The Zn declines nonconservatively in the estuary, indicating that the estuary is a sink for Zn from the river (Holliday and Liss, 1976). Whether or not rivers in general contribute a significant excess of Zn in coastal waters over oceanic waters is not yet known. Because of this and the extreme contamination problems in working with Zn I have only touched on the interactions of Zn and Cu.

Cadmium also shows a nutrient-like profile in the open ocean (Boyle et al., 1976; Bender and Gagner, 1976; Bruland et al., 1978b; Bruland et al., 1979; Bruland 1980; Knauer and Martin, 1981). The nutrient that Cd most resembles is phosphate. Like phosphate, Cd appears to recycle very rapidly in surface waters. As with Zn, we are rather unsure of the Cd concentration in coastal and estuarine waters. As contamination does not appear to be as severe a problem with Cd as with Zn, knowledge of the coastal distribution of Cd may be realized more easily in the future. Since Cd is not highly toxic to marine phytoplankton compared to Cu and is not thought to be an essential element, and because of the low concentrations of Cd in seawater, I did not seek to study Cu-Cd interactions.

From a biological viewpoint, iron is a very important element. Unfortunately, the chemistry of iron in seawater is quite unforgiving. In oxygenated seawater Fe (III) is expected to predominate over Fe (II) (Stumm and Morgan, 1970), in the absence of organic ligands which tend to stabilize Fe (II) (Theis and Singer, 1973). Fe (III) forms extremely insoluble hydroxides in alkaline water, so the calculated theoretical maximum concentrations of truly dissolved Fe, about 0.3 nmol/kg, is much lower than for other metals (Byrne and Kester, 1976). Actual "dissolved" Fe measurements in the ocean undoubtedly measure the sum of dissolved Fe and colloidal Fe which passes through a fine-pore filter. Colloidal Fe probably predominates over true dissolved Fe. For example, in test solutions, Byrne and Kester found that ten times as much Fe passed a 0.45 μm filter as passed a 0.05 μm filter. In measuring Fe in the ocean the problem is compounded by potential contamination from large steel ships and steel hydrowire.

It is clear that the Fe concentrations in the open ocean are very low. Most reported values are near or under the maximum soluble Fe concentration of about 20 nmol/kg predicted by Byrne and Kester (1976), but presently available Fe data in the open ocean do not meet any of the criteria previously discussed for tentative acceptance. In fresh water, lower pH values, higher levels of dissolved organics, and less competition for complexing sites by Ca and Mg allow much higher concentrations of soluble Fe than in seawater (Kester et al., 1975; Theis and Singer, 1973; Boyle, 1976). Loss of Fe from solution in estuaries beyond that of dilution is quite common when high-Fe river water mixes with low-Fe seawater. This can be shown both in real estuaries (Coonley et al., 1971; Boyle et al., 1974; Windom, 1975; Bewers et al., 1974; Harris et al., 1975; Holliday and Liss, 1976; Evans, 1977) and in laboratory mixing experiments (Sholkovitz, 1976; Eckert and Sholkovitz, 1976; Murray and Gill, 1978). Iron is clearly important to phytoplankton, but because of the uncertainties of Fe measurements and the form of Fe in seawater, I chose to avoid examination of any possible interactions of Cu and Fe.

Aluminum represents an unusual situation. There are currently two different sets of oceanographically consistent data showing different trends. Mackenzie et al. (1978) produced a vertical profile for Al in the Mediterranean. Aluminum covaried strongly with Si, with an approximately constant ratio. Caschetto and Wollast (1979) also obtained similar results in the Mediterranean. However, Hydes (1979) found that in vertical profiles in the Atlantic and Pacific, Al did not correlate very well with Si. In these oceans there was evidence of more bottom regeneration and deep water scavenging for Al than for

Si. Thus, it seems that processes which dominate the distribution of Al in one area of the ocean might not be important in another. Although not yet extensively studied, Al chemistry is so much like Fe in its precipitation and chelation chemistry that similar patterns of estuarine removal are to be expected (Hosokawa et al., 1970; Sholkovitz, 1976). With its minimal biological effect and the uncertainties in its concentration in coastal waters and estuaries, Al seems an unlikely element of interest to a study of phytoplankton-metal relationships.

Chromium has recently been reported to have an oceanographically consistent vertical distribution (Cranston and Murray, 1978). Chromium has two significant oxidation states in the environment, Cr (III) and Cr (VI). In seawater, Cr (VI) should predominate (Elderfield, 1970). Cranston and Murray (1978) found this to be true in the North Pacific, but a small mid-depth maximum of Cr (III) occurred at the primary nitrite maximum, which is also a zone of low oxygen content. Cr (VI) generally increased with depth in concert with Si, from 1.5 nmol/kg at the surface to 3.0 nmol/kg at 3,000 m. Cranston and Murray (1978) also measured Cr in the Columbia River and its estuary. Again, Cr (VI) predominated, although Cr (III) had a small increase in mid-estuary. Cr (VI) decreased from 3.3 nmol/kg in the river to 2.4 in the estuary. As Cr is without effect to phytoplankton at any realistic concentration (Frey et al. In Press) I have elected not to explore Cr-Cu interactions in this dissertation.

Like Cr, selenium also has two valence states of importance under natural conditions, Se (IV) and Se (VI). Thermodynamically, Se (VI) should predominate (Sillen, 1961). As with Al, two different groups

working in two different oceans have found slightly different trends: the Pacific Ocean has more Se in its deep water and a slightly higher proportion of Se (IV) than the Atlantic Ocean (Sugimura et al., 1976; Measures and Burton, 1980). There are also several features in common in the two oceans, e.g., Se (VI) predominates, both forms increase with depth, and Se (IV) persists despite unfavorable thermodynamics. Measures and Burton (1980) concluded that Se distributions were dominated by uptake at the surface, and by regeneration as Se (IV) in both shallow and deep water. Kinetic stability of Se (IV) is needed to account for its continued presence. Measures and Burton (1978) have also studied Se in a number of rivers and their estuaries. They found Se to be in higher concentration in rivers than in the ocean, and that Se (VI) predominated over Se (IV). They also noted that Se declined conservatively in the estuaries, so that the rivers are a source of Se to the ocean. Selenium chemistry and measurement may be sufficiently known to begin studies on Se-phytoplankton interactions; however, I did not examine possible Se-Cu interactions with phytoplankton studies.

Nickel has also been shown to have an oceanographically consistent vertical distribution. Bender and Gagner (1976) measured surface concentrations of about 2 nmol/kg, with increasing concentrations to 6.5 nmol/kg in deep water in the Sargasso Sea. Likewise, Sclater et al. (1976) reported values ranging from 3 to 11 nmol/kg, and they suggested both deep and shallow regeneration. Also, deep water concentrations were higher in the Pacific than in the Atlantic. Bruland (1980) found similar ranges and patterns in the Pacific. No comparable analyses of Ni in estuaries, rivers, or

coastal zones have yet been produced, so we have no knowledge of the concentrations and behavior of Ni in these areas. Nickel is also not known to have any substantial effect on phytoplankton, and therefore seems an unlikely to have important interactions with Cu in phytoplankton.

The last trace metal for which there is an oceanographically consistent pattern is mercury. Mukherji and Kester (1979) produced a vertical profile for Hg in the Gulf Stream. As is the case with several other trace metals, Hg correlated better with Si than with other nutrients, indicating deep-water regeneration. No reliable data are yet available for the behavior of Hg in estuaries or coastal waters. While Hg is extremely toxic to phytoplankton and other organisms, the lack of measurement in coastal and estuarine zones, the formidable analytical problems, and the relative lack of concentration variation in the open sea indicate that Hg is not likely to be a fruitful subject in a study of metal-phytoplankton interactions at this time.

C. Speciation of Trace Metals in Seawater

Trace metals in seawater can be associated with various other chemical species via complexation, adsorption and formation of solid phases. The distribution of a metal among such forms is an important factor in its interaction with biota. The speciation of metals in natural waters can be divided into two types, inorganic and organic. Inorganic speciation describes the distribution of metal ions in solution with inorganic ligands or solids. The association is usually in the form of ion pairs, complexes, colloids or particulate phases.

Similarly, organic speciation describes the distribution of metal ions with organic compounds. Here also, the nature of the relationship may include soluble complexes, colloids or particulates.

1. Inorganic Speciation of Trace Metals

The association of trace metals with inorganic species is relatively well understood (at least when compared to organic complexes), because the concentrations of inorganics in seawater is relatively constant and well known. In principal, one need only know the value of the association and/or solubility products for all the possible associations between all the metals and all the ligands in the system, plus their total concentrations, to compute the resultant concentrations for each species from a series of simultaneous equations (Denbigh, 1971). In practice, at least three major problems arise. First, not all of the appropriate equilibrium constants are known, and all of those "known" may not be right (Zirino and Yamamoto, 1972). Second, some reactions predicted from equilibrium constants are in reality so slow as to be unimportant. Third, for realistic assemblages of metals and ligands, the solutions for the equations are extremely complex, requiring large computer programs to handle the complex interactions. Recent computer models are capable of handling large numbers of metals and ligands, forming up to several thousand different species, including gases and solids (Morel and Morgan, 1972; Westall et al., 1967).

Table 3 shows the calculated inorganic speciation of Cu in ionic solution of typical seawater composition (Sunda, 1975). In this model, Cu is largely complexed by carbonate and hydroxide ion. The

Table 3. Calculated inorganic speciation of copper in seawater. Assumes chlorinity of 19 o/oo and carbonate alkalinity of 2.38 mM. Values given as the percent of the total copper present in a given inorganic species (from Sunda, 1975).

pH	7.7	7.8	7.9	8.0	8.1	8.2	8.3	8.4
Cu^{2+}	15	12	10	9	7	6	5	4
$CuCO_3$	65	67	69	69	70	69	68	67
$Cu(CO_3)_2^{2-}$	3	3	4	5	6	7	8	9
$CuOH^+$	12	12	12	14	14	15	16	17
$CuCl^+$	2	2	2	1	1	1	1	1
$CuSO_4$	3	2	2	2	1	1	1	1
% activity	3.9	3.5	2.9	2.4	2.0	1.7	1.4	1.1

equilibria are affected by pH within the range of normal seawater, with higher pH decreasing the amount of free Cu ion, but only slightly. Overall, Cu is approximately 95% complexed, and the activity of Cu is about 1.5% of the total Cu concentration due to the cupric ion activity coefficient.

2. Organic Speciation of Trace Metals

Ideally the numerical treatment accorded to inorganic ligands should also be applicable to the complexation of trace metals by organic compounds in seawater. Unfortunately, the nature of dissolved organic compounds in seawater is not simple. There are many such types, at this time largely unstudied. The concentration of dissolved organic matter in seawater ranges from about 0.4 to 4.0 mg/kg (Johnston, 1964), depending on site and season. The list of possible organic compounds is virtually endless:

> "To the list of those organic species known to be present in the oceans, every metabolite and its degradation products should be added." (Siegal, 1971)

Many organic compounds are known to have chelating or complexing abilities. Examples are proteins, amino acids, polysacharrides, fatty acids, and simple organic acids (Lindenbaum, 1973). The important question is whether or not the concentrations of potentially complexing organics, and their complexation strengths, are sufficient, in toto, to complex significant fractions of the available trace metals. In this regard, there have been two historical schools of thought. The first school has it that there is "convincing evidence for the existence of naturally occurring organic ligands that are

functional analogues of synthetic chelators such as EDTA" (Barber, 1973). The second school believes that "it is likely that chelates are of only minor importance in oceanic waters" (Williams, 1969). In their arguments, the first school relies heavily on 1) biological evidence suggesting that organisms respond to trace metals in seawater as if there were less metal available to organisms than that which was added or measured, and 2) analytical evidence showing that treatments which release metals from organic complexes often raise the yield of metal measured in seawater. The second school attacks these results as being artifacts of colloidal dispersions, ionic interactions, contaminated metal analyses, adsorption reactions or just plain "complexity of interpretation of data" (Stumm and Bilinski, 1973). The second school relies on the low concentrations of known chelators in seawater, the competition for ligands by abundant Ca and Mg ions, and relatively low specificity of most organic ligand functional groups. The first school would suggest that "If the organic solubilizers possessed chelating powers on the order of EDTA they need constitute only 0.01 to 0.1% of the naturally-occurring organic pool" (Johnston,1964). Since the dissolved organic pool in the ocean is not well characterized down to the last hundredths of percents, the existence of sufficient chelating is deemed likely by this school.

Without yet granting the importance of organic-metal complexation in seawater, what help does chemical theory give us regarding the general problem of complexation of metals with organic ligands? The Irving-Williams order gives a sequence of the relative binding strengths of any hypothetical ligand to members of the first row divalent transition elements, based on patterns of electronegativity

and ionic radius (Irving and Williams, 1953). This order is normally expressed as Mn<Fe<Co<Ni<Cu>Zn. Zn is not actually a transition metal, and so does not fit into the model perfectly, and does not, in general, have a fixed position in the order. Examples of the association constants of several ligands for metals in the Irving-Williams order (plus Ca, Mg and Zn) are shown in Fig. 5. Thus the Irving-Williams order predicts that, all other factors being equal, metals high on the order (e.g. Cu and Ni) should tend to be more heavily complexed in seawater than metals low on the order (e.g., Mn and Fe).

Given the debate over the importance of organic complexation of trace metals in seawater, it is appropriate to examine the analytical evidence. Data for trace metal-organic complexation has been accumulating for many years. Slowey et al. (1967), Corcoran and Alexander (1964), Slowey and Hood (1966), and Alexander and Corcoran (1967), all found evidence that Cu in seawater was bound to organics that either protected Cu from extraction unless it was destroyed, or allowed its direct extraction into chloroform. In a variation on a now familiar theme, however, values they found for Cu were far higher than those now believed correct. Thus it seems highly likely that many of these results are due to contamination. Similar problems have plagued the work of other investigators, and still do, even though a variety of analytical techniques have been employed to study several elements.

There appear to be several recent trustworthy indications of measured complexation in seawater. The most convincing of these are the results of Bruland et al. (1980). They used two different

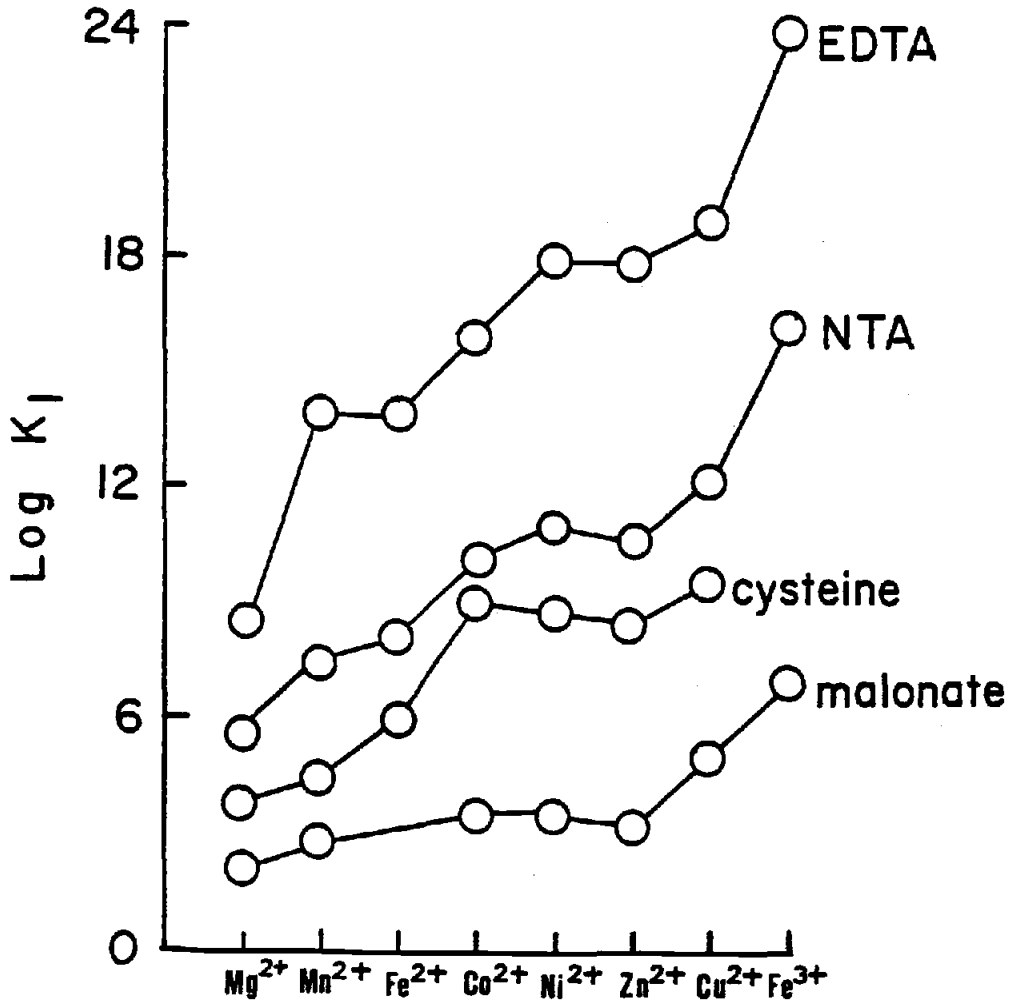

Fig. 5. The Irving-Williams order. Constants from Sillen and Martell (1964).

techniques to measure a variety of trace metal concentration profiles in the North Pacific. One technique was chelation with sodium diethyldithiocarbamate (NaDDC) and ammonium pyrrolidine dithiocarbamate (APDC) followed by solvent extraction of the chelated metals into chloroform, back extraction of the metals into acid, and analysis by flameless atomic absorption spectroscopy (FLAAS). The second was extraction on Chelex-100 resin, followed by acid elution and analysis by FLAAS. Both methods produced smooth, oceanographically consistent profiles, but for at least two elements, Cu and Ni, the two techniques produced different concentrations. The Chelex-100 technique yielded about 50% of the Cu and 80% of the Ni given by the solvent extraction technique. Since the extraction of Cu and Ni by Chelex-100 is known to be >99% efficient for purely inorganic Ni and Cu in seawater (Kingston et al., 1978), it seems likely that organics protected the Cu and Ni from extraction by Chelex-100. However, in the other technique, either the organically bound metal was directly extracted by chloroform or by competitive complexation by the NaDDC and APDC. The degree of apparent complexation of the two metals is consistent with the Irving-Williams order, in that Cu appeared more complexed than Ni.

Evans (1977) used Chelex-100 extraction and persulfate oxidation to show that most of the Fe and Cu, and about half of the Mn, in filtered Newport River (North Carolina) water was unavailable to Chelex-100 without prior digestion. Unfortunately, he did not examine the question of whether this complexation was important in portions of the estuary influenced by seawater.

Riedel (1978) used the same technique as Evans (1977), combined

with Zn-65 and Mn-54 in large volume, long-term marine phytoplankton cultures, to show that a large fraction (up to 70%) of the Zn was bound, but that no measureable fraction of Mn was organically bound. In that study, the high densities of phytoplankton may have excreted far more of the complexing agent than may be present in normal seawater.

Bately and Florence (1976) and Bately and Gardner (1978) developed a rather complex scheme using Chelex-100, anodic stripping voltammetry (ASV), and ultraviolet digestion to determine the organic and inorganic speciation for a number of metals. In applying this technique to the Port Hacking estuary (Australia) they found considerable fractions of the total Cu, Cd and Pb associated with organics. At least for Cu and Cd, their measured totals are close enough to the accepted values for seawater so as to have some credibility. However the distribution of all the metals among the different forms they found varies in a most disconcerting manner from time to time and site to site. The vagaries of the estuarine environment might be responsible.

Sunda and Lewis (1978) measured the division rate of the flagellate Pavlova (=Monochrysis) lutheri in mixtures of Newport River (North Carolina) water, distilled water and seawater, with tris(hydroxymethyl)amino methane (TRIS) and Cu additions. In the dilute seawater mixture, the free ion activity of Cu could be measured by Cu ion electrode. The growth rate was found to be a unique function of Cu ion activity. From the Cu activity and the concentrations of TRIS and inorganics, the extent of natural organic Cu complexing was found to be 98% in a solution of 90% riverwater, 5%

seawater and 5% distilled water in 1 mM TRIS. However, the Newport River drains a swampy area of the coastal plain, and contains a high concentration of dissloved organic carbon (22 mg C/liter). Much of the dissolved organic matter in Newport River water is believed to precipitate upon mixing with seawater, and so not reach the ocean (Evans, 1977).

The analytical evidence for soluble metal chelates in seawater is weak, at best. Most analytical evidence for chelates could also be evidence for organic colloids containing metal. Biologically speaking, there is probably little difference, at least for short term metal effects. Such evidence as does exist suggests that in open ocean water organic Cu complexation is at least 50%. Although the evidence is not as high a quality, a higher complexation percentage for Cu in estuarine and coastal water appears to exist. There is no evidence for substantial organic complexation for Mn in marine water, and given its low position in the Irving-Williams order and the competition from Ca and Mg, none is expected.

Very recently ligand exchange techniques have been used to estimate both the concentration and strength of binding ligands and the degree of organic Cu complexation in open-ocean surface seawater. Two such reports agree that Cu is greater than 95% complexed by organics, but disagree by about one order of magnitude on the amount and strength of the binding ligands (van der Berg, 1982; Hirose *et al.*, 1982).

D. The Influence of Trace Metals on Phytoplankton

Phytoplankton, as all organisms, respond to many trace metals either positively or negatively, depending on the metal concentration. This is, in reality, the two extremes of a continous response. At low concentrations certain elements are required for growth, and are called "essential." At high concentrations these and all other metals become inhibitory or "toxic." The accepted criteria for essentiality are:

> "An element is essential when a deficient intake consistently results in an impairment of a function from optimal to suboptimal and when supplementation with physiological levels of this element, but not others, prevents or cures this impairment" (Mertz, 1972).

To be generally acknowledged as essential an element should be demonstrated as essential in more than one species by more than one investigator (Mertz, 1981).

1. General Nature of Trace Metal Action

Most, if not all, of the effects of trace metals in organisms, including phytoplankton, are mediated through the activation of enzymes. Trace metals activate two kinds of enzymes: so-called metal-activated enzymes, and metallo-enzymes. The first term is used to describe a loose, kinetically labile association between enzyme and metal. Such enzymes may have activity without metals, and various different metals may activate them, causing different substrate affinities or reaction rates (Speck, 1949). Metallo-enzymes have metal ions as an integral part, and do not usually function without

that metal or when other metals are artificially emplaced.

Metals are small components of enzymes which, through catalytic action, affect large amounts of substrates. This catalytic function leads to considerable amplification of metal influence. In humans, for example, 1×10^{-11} g turnover of cobalt per day in vitamin B-12 ultimately allows 5 to 10 grams of protein synthesis per day (Mertz,1981). That metallo-enzymes are highly specific for certain metals, and regulate key points in metabolism, leads to high specificity for requirements for those elements. A deficiency of such an element cannot be substituted for by another chemically similar element. In fact, such substitution often exacerbates deficiency symptoms.

Toxic effects of trace metals, while heavily studied from the view of concentrations producing toxicity, are relatively unstudied in terms of the mechanism of toxic effect. As a general statement it appears likely that most toxic effects arise either when metals in excessive concentration occupy sites where some other metal ordinarily functions, or when by complexation equilibrium alone so much metal associates with some other organic component of the cell that it disturbs the function. This is supported by evidence of trace metals interactions in toxic and limiting concentrations, and by the observation that toxicity of trace metals in simple systems is a strong function of ligand strength for those metals (Williams, 1971; Fisher and Jones, 1981).

2. Relationships Between Copper and Marine Phytoplankton

In certain freshwater algae and higher plants, Cu is known to be an essential trace element. One essential function for Cu is in the functional group of plastaquinone, an electron carrier between photosystems I and II in photosynthesis. Copper is also known to be an essential activator for several other important enzymes such as cytochromes. Copper is no doubt essential to marine phytoplankton as well as freshwater algae, but no one to date has successfully depleted Cu in media to a level where marine phytoplankton growth is limited. When one considers the low concentrations of Cu in surface seawater and the relatively high levels of copper found in phytoplankton (Pequegnat, 1975; Martin and Knauer, 1973) it is obvious that marine phytoplankton can successfully extract needed Cu at low concentration.

Early in the study of oceanographic primary production, it was sometimes noted that water containing adequate major nutrients, particularly freshly upwelled water, would not support good phytoplankton growth. It was further found that good growth could be restored in several ways. One was by the addition of small amounts of chelated metals, with EDTA the usual chelating compound (Menzel et al., 1963; Barber, 1973). The second was by addition of either a chelator alone or an organic slurry potentially containing some unknown chelator (Johnston, 1964; Barber and Ryther, 1969). The third way was by direct addition of certain metals, notably Fe, Al and Mn. Additions of other metals alone were either ineffective or more inhibitory. Johnston (1964) concluded from his studies on phytoplankton growth that:

> "Assays and mixed culture experiments prove that the supply of chelating substances is frequently the most crucial aspect of phytoplankton nutrition in seawater. Experimental results, theoretical considerations and tentative calculations show that this supply is controlled by the stability of trace metal chelates and in the sea may be mediated by certain minor components of the dissolved organic matter."

Barber (1973) showed that ultraviolet treatment of seawater, to destroy organics such as the hypothetical chelators, reduced the growth potential of seawater. This effect could be reversed in part by the addition of EDTA, Fe and Mn, or by large inocula with untreated seawater or culture. These results were consistent with the ideas outlined by Johnston (1964), but at the same time it was realized the results were also consistent with another hypothesis, first proposed by Steemann Nielsen and Wium-Anderson (1970). This hypothesis held that Cu or some other metal in seawater might be toxic to phytoplankton, and that this toxicity might be ameliorated by organic complexation or hydroxide co-precipitation.

Most of the work on the effects of Cu on marine phytoplankton concern the toxic effects of added Cu, and the ameliorating effect of complexing agents. Early evidence for the extreme toxicity of Cu to phytoplankton in unchelated seawater came from research into apparent artifacts in the C-14 uptake technique of measuring primary productivity (Steemann Nielsen and Wium-andersen, 1970). It was found that C-14 solutions made with distilled water from commercial sources contained an average of 260 µg Cu/l. Steemann Nielsen and Wium-Andersen (1970) found that as little as 1 µg/l in simple media could substantially inhibit the C-14 uptake by phytoplankton. As C-14

solutions were normally added as a 1/100 dilution to seawater containing phytoplankton, it was clear that a Cu toxicity problem could result. Steemann Nielsen and Wium-Andersen (1970) also noted the amelioration of Cu toxicity by EDTA and citrate, by added protein in the media, and by adsorption onto colloidal $Fe(OH)_3$. It was also proposed that phytoplankton could detoxify a medium by excretion of organics, and that this could account for the apparent need for conditioning of freshly upwelled water.

Erickson (1972), examined growth depression after a series of Cu additions to natural seawater with a single clone of the diatom Thalassiosira pseudonana. He found spatial and temporal variations in the level of Cu that caused depression. In a subsequent study using the same diatom, Davey et al. (1973) showed that additions of strong chelators (EDTA and histidine) could suppress Cu toxicity up to their own concentration in seawater. They proposed this biological titration as a bioassay for the Cu complexation capacity of seawater.

Perhaps the most significant single advance in Cu-phytoplankton relationships, and for that matter in all trace metal-phytoplankton relationships, is the work of Sunda and Guillard (1976). Using media heavily chelated with TRIS and EDTA, and using a cupric ion electrode to establish the cupric ion activity of chelated solutions resembling the culture media, they showed that toxicity of Cu to, and uptake of Cu by, two species of phytoplankton, T. pseudonana and Nanochloris atomis, were related solely to cupric ion activity, and not to total Cu, organically bound Cu, or inorganic species of Cu other than the free ion. This lead to the formulation of a model for the effect of different forms of metals on phytoplankton. Given certain conditions

as constant (light regime, temperature, nutrients, and other trace metals), the influence of a trace metal on the growth and intracellular concentration in phytoplankton is solely a function of that metal's free ion activity. All other forms contribute to the effect of that metal only insofar as they influence the free ion activity.

The free ion activity (of Cu, for example) is usually expressed in the form of its negative logarithm:

$$pCu = -\log a_{Cu}$$

where a_{Cu} = the thermodynamic activity of cupric ion. Translation of pCu units into equivalent total concentration units in seawater is dependent upon ionic strength, organic complexation and pH. From Table 3 we can see that the expected activity-to-concentration ratio for Cu in average seawater with only inorganic speciation is about 1.5×10^{-2}. Thus the expected range of pCu in the oceans, in the absence of organic complexation, is 11.0 to 9.8 (using the range of Cu in Table 1), with open-ocean surface values tending toward the high pCu (low Cu ion concentration) values.

Gavis et al. (1981) studied the Cu sensitivity of 24 algal clones using TRIS-buffered media, and reported that the threshold at which Cu inhibition began was between pCu 9.0 and 11.5 for all but four clones. These four clones grew well at pCu 8.5, the theoretical limiting value of Cu ion in seawater in equilibrium with malachite (the least soluble copper mineral in seawater solution). Gavis and co-workers also found that Cu toxicity was influenced by light level, such that more

favorable light conditions reduced the toxicity. They suggested that favorable culture conditions in general tend to reduce the apparent toxicity of Cu.

The biochemical nature of the toxic effect of Cu on phytoplankton is not known with certainty, and is probably variable from species to species given constant conditions, and within a given clone under different conditions. There is some evidence for a general Cu interference with the intracellular regulation of simple ions. For example, McBrien and Hassal (1965), Kamp-Nielsen (1971) and Sunda (1975) all found that Cu caused loss of intracellular potassium in algae. In addition, Sunda (1975) indicated that EDTA prevented this loss. Riisgard (1979) found that Cu inhibited volume regulation by the green flagellate Dunaliella marina. He suggested that Cu caused decreased cell membrane permeability to K^+ and/or Cl^- rather than inhibition of the Na^+/K^+ pump. This is not easily reconciled with the previous findings mentioned above. The different results may reflect alternate mechanisms underlying the toxicity for different species.

Copper toxicity for diatoms has been linked to their requirement for Si by a number of authors (Canterford, 1980; Thomas et al., 1980; Fisher et al., 1981). Canterford (1980) found that several metals, and especially Cu, caused elongation and abnormal morphology in cells of the diatom Ditylum brightwelli. EDTA was effective in preventing this effect. According to Thomas et al. (1980) Cu, Zn and Ge increased chain length and prevented Thalassiosira aestivalis from dividing, but did not distort the cells' normal morphology. Abnormal morphology, in addition to disrupted separation of daughter cells,

resulted from additions of Hg, Cd and Pb. Fisher et al. (1981) reported that Asterionella japonica continued photosynthesis after Cu inhibition, but Si uptake and DNA synthesis stopped. As a result, abnormally large, aberrant cells were formed after continued carbon fixation. He suggested that Cu inhibited Si uptake, which in turn inhibited synthesis of DNA. DNA synthesis and/or cell division in diatoms is known to be inhibited by Si deficiency (Werner, 1977).

Additional evidence for a Cu-Si metabolism link in diatoms comes from Morel et al. (1978). Lag-phase Skeletonema costatum responded to Cu addition by increasing its lag phase after transfer to fresh media. The length of the lag was related to both Cu concentrations added and Si concentration in the new media. Higher Si reduced the lag period. Rueter and Morel (cited in Rueter and Morel, 1981) demonstrated a competitive relationship between pCu and silicic acid concentration in T. pseudonana in terms of growth, silicic acid uptake and Cu uptake. Low pCu values (higher free ion concentrations) caused reduced silicic acid uptake, and high silicic acid concentrations in the media acted to reduce Cu uptake. Rueter and Morel (1981) later found that Zn ion also competitively reduced the Cu toxicity in this system. They proposed that silicic acid uptake was mediated by a Zn-activated enzyme, and that the enzyme was inhibited by displacement of the Zn by Cu at the active site on the enzyme.

Sunda et al. (1981) demonstrated that Cu ion toxicity for the diatom Chaetoceros socialis was increased by low levels of Mn ion in the media. Based on the necessity of Mn for the Hill reaction in photosynthesis, they proposed that Cu inhibited photosynthesis at that point. They also found that Cu toxicity of natural phytoplankton

populations was reduced by Mn additions. They proposed that Mn-Cu interactions in freshly upwelled seawater could possibly account for the "lack of conditioning" effect referred to previously by Barber (1973).

The toxic effects of Cu are not only evident in ion regulation and silicic acid metabolism. For example, the motility of the dinoflagellate Gonyaulax tamarensis was 50% inhibited (50% of the cells became non-motile) after a short exposure to pCu 10.4 (Anderson and Morel, 1978). Another aspect of the toxicity of Cu is the possible effect on the species composition in a phytoplankton assemblage. In experiments with very large enclosed seawater columns, Thomas and Seibert (1977) found that Cu additions of 75 nM caused the species composition to shift from primarily a mixture of centric diatoms and dinoflagellates to almost exclusively pennate diatoms. The overall species diversity decreased significantly. Bishop (1977) studied the effect of 31 nM Cu on growth and species succession in continuous cultures inoculated with a natural seawater sample. He found that the high Cu lowered the final cell density, but caused only minor differences in species composition. Sunda et al. (1981) examined the effects of added Cu, Mn, Fe, and EDTA to artificially upwelled seawater (water raised from 800 m off the North Carolina coast). Those Cu treatments that produced long lag phases caused shifts from the dominant diatom flora to small green flagellates.

E. Background Summary

Recent measurements of trace metals in seawater indicate that most have vertical distributions qualitatively like nutrients. Copper has a silica-like profile, ranging from 0.5 to 8.4 nM, with a relatively constant, increasing gradient from surface to bottom. In addition, soluble Cu concentrations in rivers and estuaries are not highly enriched with respect to seawater. Copper, because of its high binding position in the Irving-Williams order, is more likely to be chelated than other first-row transition elements. Therefore, even when Cu concentrations are enhanced in coastal and estuarine areas, the higher organic load may tend to offset its biological activity. Much biological and some analytical evidence suggests that Cu in seawater is, to some extent, complexed by unknown organics.

Manganese has a very different oceanographic distribution than Cu. Nearshore and estuarine waters are highly enriched relative to open ocean water. Open-ocean near-surface water is also enriched in Mn, when compared to open-ocean, deep water. Manganese is very low in the Irving-Williams order, and so is very unlikely to be significantly complexed by organic compounds in the marine environment. Concentration variations for dissolved Mn probably faithfully reflect variations in the biological availability of Mn to phytoplankton.

Manganese and copper, then, have opposite geographic trends in bioavailability in the marine environment. Copper is probably more available in low-organic, open-ocean or deep water than in more organically laden estuarine and coastal water. Manganese is likely much more available in estuarine and nearshore water. In the Pacific

Northwest there is also a strong vertical gradient of Mn in nearshore surface waters, with Mn declining rapidly with depth from the surface to about 100 m. No similar gradient is known or expected for Cu. Thus, mixing and upwelling which bring high-nutrient water into the euphotic zone near the coast also tend to bring water with low Mn/Cu ratios. While phytoplankton have a nutritional requirement for both Cu and Mn, the nutritional requirement for Cu is not thought to limit growth at any natural concentration. All available evidence suggests, if anything, that near-toxicity prevails at normal levels of Cu. Manganese, while less studied than Cu, is not normally associated with toxicity; rather, additions of Mn often stimulate phytoplankton growth. There is evidence of an interaction between Mn and Cu in some phytoplankton, with adequate Mn concentrations reducing Cu toxicity.

III. MATERIALS AND METHODS

A. Phytoplankton Growth Experiments

1. Laboratory Studies

a. Phytoplankton Culture Isolation and Maintenance

Several phytoplankton species were isolated into monoclonal culture for examination of copper toxicity. Seawater samples were collected at the Marine Science Center dock (Newport, Oregon), returned to the laboratory, enriched with f/20 levels of nutrients (Guillard and Ryther, 1962) and incubated at 15°C for several days in order to develop a rich bloom. Single cells or single short chains were picked out with a finely drawn Pasteur pipette and placed in 25 ml test tubes containing sterile f/20 medium. These were incubated at 15°C until the cultures reached moderate density. The cultures were then examined microscopically. Cultures without growth after several weeks were discarded, as were those with more than one species.

Cultures were maintained in 125 ml borosilicate glass Erlenmeyer flasks with 100 ml of sterile f/2 major nutrients with f/20 levels of trace metals. Transfers were made at 2-3 week intervals depending on the state of the cultures. The transfers were made into sterile medium, but were not sterile transfers, nor were the cultures axenic. Light intensity for the cultures was 120 μEinsteins/m^2-sec., as measured by a Li-Cor model 170 quantum meter. Water for culture maintainence was collected from the Marine Science Center dock at high tides and stored at 5°C in 13 gallon polyethylene carboys.

b. Experimental Medium Preparation

For most laboratory growth and Cu uptake experiments medium was prepared using TRIS as a cupric ion and pH buffer (Sunda and Guillard, 1976). Most experiments utilized media containing f/2 major nutrients, 1 μM FeEDTA, and f/20 metals except Cu, which was varied. Some experiments had other media to test metal interactions; these will be noted when the results of those experiments are reported. One experiment utilized AQUIL medium, a synthetic, totally defined artificial medium intended specifically for trace metal studies (Morel et al., 1978). TRIS concentrations were varied within the range of 1-10 mM to produce similar ranges of pCu with different levels of total Cu.

Metal stock solutions were prepared from appropriate metal salts diluted with glass-distilled water. Solutions of metals more dilute than 1 mM were prepared fresh daily from concentrated stock solutions.

c. Experimental Protocol

Seawater for growth experiments was filtered with 0.8 μm Millipore filters and autoclaved in polycarbonate flasks. TRIS, f/2 major nutrients and f/20 metals were added using a sterile 1.0 liter volumetric flask. The medium was then divided between the experimental flasks (100 ml each), and appropriate Cu concentrations were added using small volumes (0.1 to 1.0 ml). These flasks were then bubbled with filtered and humidified air, and the pH was measured daily. Media pH was adjusted with 12 N HCl or 1.0 N NaOH until stabilized near 8.1 (due to displacement of H^+ by Cu from TRIS,

different Cu treatments had differing initial pH). Bubbling was necessary to achieve equilibration with atmospheric air in a reasonable time.

Flasks for laboratory growth experiments were prepared by washing with laboratory detergent, giving copious hot tap-water rinses, rinsing five times with Milli-Q water, coating with Siliclad, soaking for 24 hr. in 3.0 N reagent-grade HCl, rinsing five more times with Milli-Q water, and then oven-drying them. The bubbling tubes (not autoclavable due to plastic parts) were sterilized with a 24 hr. soak in 6 N HCl.

Cultures for use as inoculum for laboratory experiments were prepared in the experimental medium without added Cu. These inoculum cultures were started with small volumes of stock culture and grown at the same light and temperature as the intended experiment. The inoculations into experimental flasks were made with small volumes (0.1 to 1.0 ml) of the inoculum culture in logarithmic growth, so that the final relative fluorescence in the experimental flasks was 0.03 to 0.3.

Cell growth was monitored using in vivo fluorescence (Thomas et al., 1974; Frey, 1977), which was measured with a Turner Designs fluorometer. The fluorometer was calibrated so that a solution of 0.05 µg/ml of coproporphyrin gave a reading of 10.0 in a 1 cm cuvette. Cultures were monitored for in vivo fluorescence twice daily if growing rapidly, or once daily if growing slowly or not at all. Five ml were removed from each culture each time it was measured, and the sample was not returned to the culture after measurement because of contamination possibilities. Sampling was held to a minimum

consistent with measurement of growth rates, minimize the opportunity for contamination and to prevent using up the cultures.

The pH of the cultures was measured daily during growth experiments with an Orion model 801 digital pH meter using a Corning 476050 glass/Ag/AgCl combination electrode. The pH was calibrated using pH 7.0 and 10.0 buffers. In TRIS-buffered media pCu is a strong function of pH, and rapidly growing phytoplankton at high cell densities tended to make the media more alkaline by depleting CO_2. Bubbling with air slowed, but did not stop, the pH shift. When the pH shifted more than 0.1 pH unit the culture was stopped, or at least the growth was no longer considered in the growth rate calculations. In some experiments the remaining culture was filtered to measure Cu in the phytoplankton, or was preserved for cell counts. Normally the pH became uncontrollable when the fluorescence reached about 20, but this varied with the growth rate and the strength of the TRIS buffer.

Due to rapid growth some cultures were not stopped until relative fluorescence reached 80 or more. As Cu effect usually lags behind growth for up to several days (Sunda, 1975; this study), and the effect of increased pH is to raise pCu (lower the ionic copper concentration) failure to stop rapidly growing cultures should not have substantially affected the growth rate results. Cell Cu content, however, is rapidly affected by pCu, so the harvesting of these cells after substantial shifts in pCu due to pH shifts may have been an important source of variation in the cellular Cu results.

2. Outdoor Enrichment Studies

For outdoor enrichment studies a large, fiber-glass-lined wooden tank was used as an incubator. The tank was open on the top, with sufficient heating and cooling capability to maintain a preset temperature in circulating water between 5 and 20°C (±1°C).

In preparation for an outdoor experiment seawater was collected either at high tide at the Marine Science Center dock, or at sea. Water from the dock was filtered through a spun polyethylene prefilter during collection, while water collected at sea was returned to Corvallis, then similarly filtered. Seawater was transported to the outdoor incubation tank site in acid-cleaned polyethylene carboys. The seawater was given a final 0.45 μm filtration using a Pall Ultipore AX filter as it was pumped into the growth containers.

The growth containers were polyethylene collapsing containers of various manufacture, ranging in size from 4-20 liters depending on the specific experiment. A commercially available plastic spigot was used to close the containers and to sample the contents. A short piece of Tygon tubing was affixed to the spigot, and by various means the containers were suspended in the temperature-controlled incubation tank.

Treatment additions were made to the growth containers prior to inoculation of cells, by using concentrated solutions to minimize volume changes. The growth containers were then allowed to come to temperature equilibrium prior to inoculation.

Seawater containing natural phytoplankton assemblages for inoculum was collected at the Marine Science Center dock on a high

tide and returned to Corvallis in a cooler. A volume of inoculum equal to either 1/1000 or 1/100 of the total volume of the experimental container was added to each container to initiate the growth experiments.

Samples were collected daily for determination of *in vivo* fluorescence. Growth containers were gently shaken to mix their contents thoroughly, and 50 ml samples were drawn from each container into a separate polyethylene bottle. The bottles were returned to the lab for determination of *in vivo* fluorescence. These samples were also used for pH monitoring for treatments with TRIS-buffered seawater.

At the conclusion of experiments, samples were preserved by addition of Lugol's solution. Phytoplankton cells were counted under a microscope using either a Fuchs-Rosenthal or Palmer counting cell, depending on the cell densities and sizes. At least 200 cells were counted in each sample. In order to facilitate processing, and because metal additions contort species morphological characteristics (Canterford, 1980; Thomas *et al.*, 1980; Fisher *et al.*, 1981), many taxa were identified only to genus level. By only determining taxonomic composition to genus level some information is lost, since it is certainly true that different species within the same genus may have different responses to certain conditions, however the same is true within clones isolated from the same species. On the other hand, by considering all species within a genus in results, variability in the results may be reduced to a manageable level, while preserving reasonable detail and applicability.

B. Trace Metal Analysis of Seawater

In some experiments, trace metals were measured in the seawater before and after growth experiments. For outdoor culture experiments samples were collected following the final filtration or after the experiment was terminated. For field samples, water samples were collected in acid washed 500 ml polyethylene bottles, returned to the laboratory packed in ice, then immediately filtered through acid-washed 0.4 μm Nuclepore filters, using N_2 gas pressure and in an all Teflon apparatus. The filtered samples were acidified with 1.0 ml of concentrated Baker Ultrex nitric acid and frozen at -20.0°C in polyethylene bottles until analysis (Pellenbarg and Church, 1978; Subramanian et al., 1980).

Preconcentration and matrix modification prior to analysis was accomplished using several modifications of the Chelex-100 method of Riley and Taylor (1968). The first modification involves the destruction of possible interfering organics by digestion with potassium persulfate (Evans et al., 1977; Riedel, 1978). This digestion frees Cu, Zn and Fe from complexing organics which may prevent concentration by Chelex-100, and is easily performed in the original sample bottle, with clean reagents. This rugged treatment was found to oxidize Mn^{2+} to MnO_2, which will not concentrate on Chelex-100 columns. To remedy this a subsequent reduction with hydroxylamine-HCl reduces MnO_2 quantitatively to Mn^{2+} and restores the yield of Mn to nearly 100% (Appendix). The final modification is the removal of Na, K, Mg and Ca from the Chelex-100 column prior to elution of the trace metals, using an ammonium acetate

buffer (Kingston et al., 1978). Blanks were prepared by adding back all the reagents to 100 ml of seawater previously stripped of trace metals by Chelex-100 extraction, and extracting these through Chelex-100 columns prepared for sample extraction. Normally four blanks were prepared with every group of sixteen or fewer samples. The exact steps in this procedure are detailed in Fig. 6.

Since seawater is very low in trace metals, samples were handled carefully to avoid contamination. To keep samples as free from contamination as reasonably possible, operations were performed under plastic dust covers, samples were handled in plasticware immediately after acid washing, reagents were kept free of trace metal contamination by batch mode Chelex-100 extraction whenever possible (basic and neutral reagents), and operations were performed with polyethylene gloves, in addition to other minor precautions. For the most part, these measures were successful (Table 4).

C. Trace Metal Analysis of Particulates and Phytoplankton

Particulate samples were taken on either 0.4 or 0.8 μm Nuclepore filters (for field and cultured phytoplankton, respectively). Samples were either collected with the all-Teflon-and-polycarbonate filtering apparatus (used when the water was to be saved for analysis), or with acid-washed Millipore filter holders with Teflon gaskets (used when the water was to be discarded). In either case the filter and filter holder were washed with large volumes of 4.0 N HNO_3 followed by volumes of distilled water prior to filtration.

After filtration the Nuclepore filters with the particulate material were folded carefully and placed in an acid-cleaned 13 ml

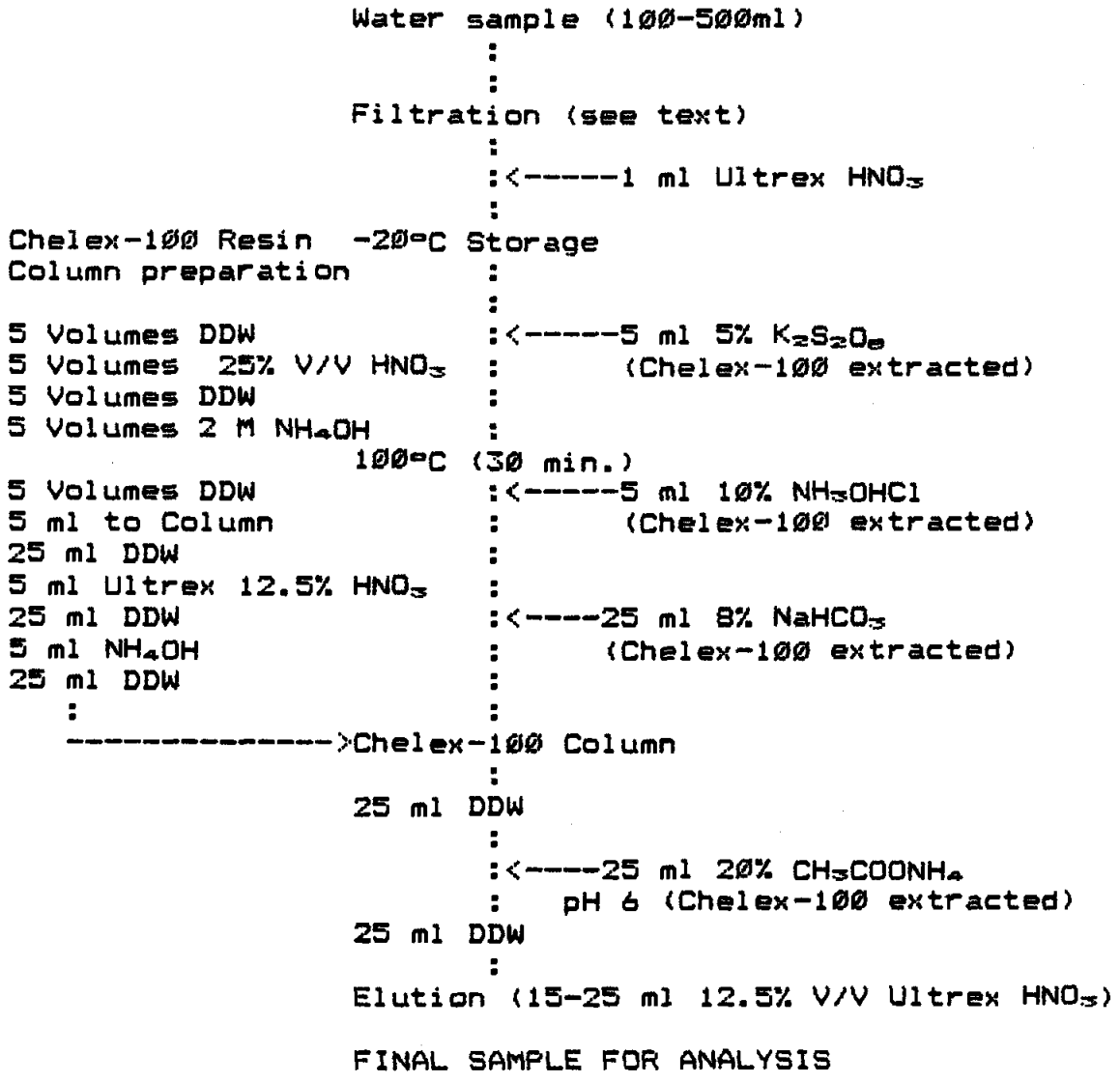

Fig. 6. Concentration and matrix modification of seawater samples for analysis of soluble trace metals.

TABLE 4. Typical blanks and lower limits of detections for trace metal analysis of marine samples. Assumes most commonly used detection method (FAA or FLAA), four blanks, replicate samples, 500 ml initial volumes and 25 ml volume for Chelex-100 extract and 5 ml final sample in particulate samples.

Element	Average Blank (1σ) (µg/l)	Lower Limit of Detection (µg/l)
Copper		
Soluble (FLAA)	0.5 (0.5)	0.06
Particulate (FLAA)	2.0 (1.0)	0.02
Iron		
Soluble (FAA)	23 (10)	1.1
Particulate (FAA)	34 (10)	0.20
Manganese		
Soluble (FAA)	5.2 (6.5)	0.66
Particulate (FAA)	1.5 (4.5)	0.10
Soluble (FLAA)	0.5 (0.5)	0.06
Particulate (FLAA)	0.5 (0.5)	0.01
Zinc		
Soluble (FAA)	12.3 (2.0)	0.20
Particulate (FAA)	1.0 (2.0)	0.05

[1]95% Confidence, one-tailed t-test.

polypropylene test tube. Field samples (which normally contained considerable clays) had 0.2 ml concentrated HNO_3 and 0.2 ml concentrated HF added. The test tubes were then tightly capped and heated to 60°C in an oven for 24 hours. Then the lids were partially opened and the samples taken to dryness. The samples were redissolved in 5.0 ml 12.5% V/V HNO_3. Blanks were prepared with filters taken through the entire procedure, except that in place of sample, 100 ml of "particle-free" seawater (seawater that had been previously drawn through a 0.2 μm Millipore filter) was filtered.

Trace metal analysis of these samples was performed on an AA5R Varian atomic absorption spectrometer. Depending on the concentration of a given trace metal in the sample, either flame atomic absorption spectrometry (FAAS) or flameless atomic absorption spectrometry (FLAAS) was employed. FAAS employed an air acetylene flame, using the manufacturer's recommended instrument settings. FLAAS was performed using a Varian CRA 63 carbon rod atomizer, with N_2 as sheathing gas. Standards for all analyses were series of mixed standards in 12.5% V/V HNO_3 made up from commercial standard solutions.

D. Determination of pCu in TRIS-Cupric Ion Buffered Media

To determine the effect of temperature on the pCu of TRIS-buffered media, buffers were made in 0.5 M KNO_3 with 0.06 M TRIS and 50 μM Cu. These buffers were cooled to the temperature desired, then adjusted to pH 8.10 with small amounts of concentrated HCl or NaOH. The pCu of the resulting solutions was determined with a cupric ion selective electrode as per Sunda (1975). The results are shown in Fig. 7. Assuming a stoichiometry of 2 TRIS molecules per

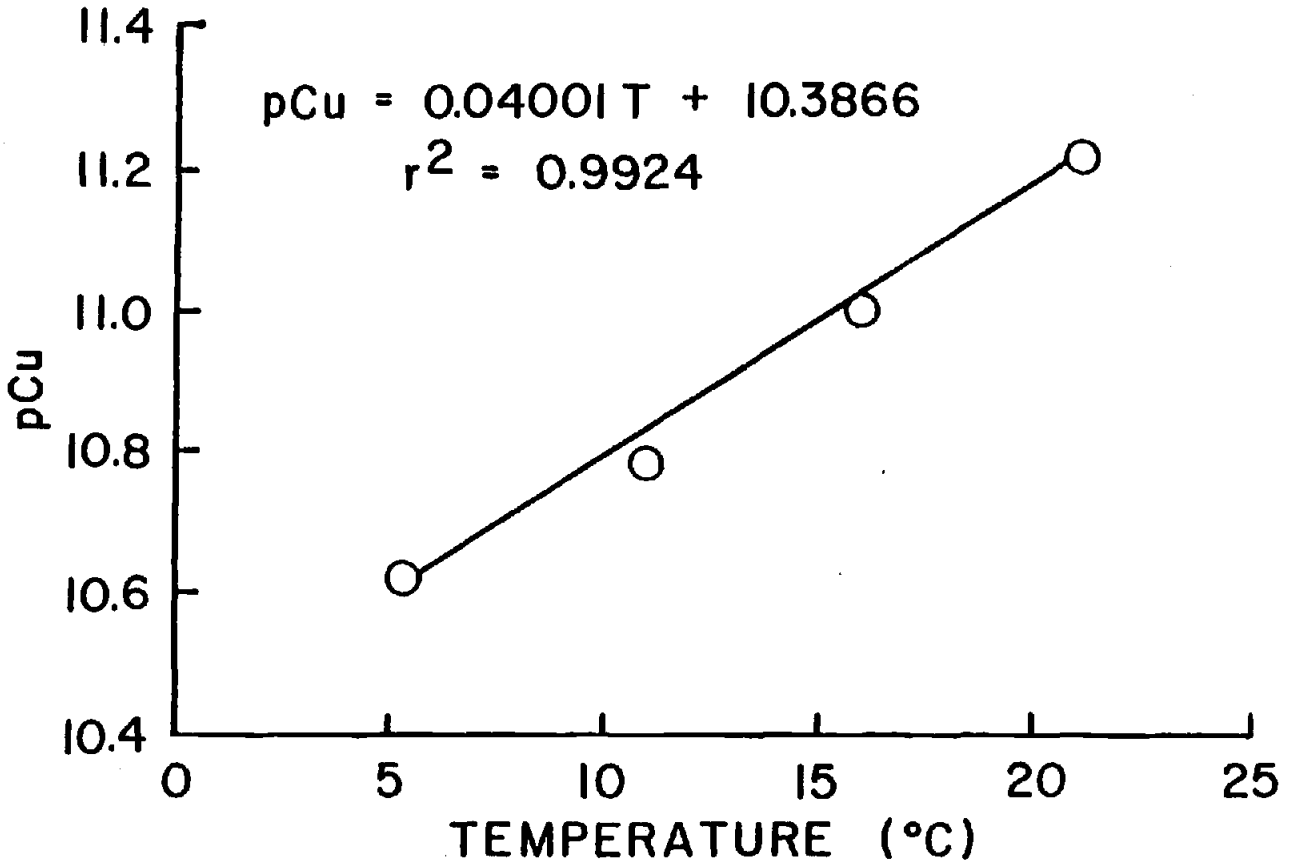

Fig. 7. The effect of temperature on the pCu of TRIS-Cu free ion activity buffers. Solutions contained 0.5 M KNO_3, 0.06 M TRIS and 50 µM total Cu at pH 8.1.

cupric ion and a pCu shift of +2.85 per unit pH (Sunda, 1975), this yields the apparent equilibrium constant:

$$\frac{[CuTRIS_2]}{a_{Cu}\,[TRIS]^2} = 10^{[(8.54 + (0.004t) + 2.85(pH - 8.1)]}$$

where [TRIS] = free TRIS concentration in a mixed protonated state, $[CuTRIS_2]$ = the sum of the concentrations of Cu-TRIS complexes, a_{Cu} = the thermodynamic activity of cupric ion and t = temperature in ^{o}C. This formulation gives slightly lower results (by 0.15 pCu units) for the same concentration buffers than the equation of Sunda (1975). This may be due to differences in the equipment used, such as liquid junction potentials in different types of pH electrodes.

For media containing EDTA, the influence of the additional chelator was accommodated by inclusion of the apparent equilibrium constant of Cu and EDTA in seawater, which includes the effect of complexation of Ca^{2+} and Mg^{2+} by EDTA (Sunda, 1975):

$$[CuEDTA] = \frac{EDTA_T\; a_{Cu}\; 10^{18.79}}{a_{Cu}\; 10^{18.79} + 10^{7.95}},$$

In addition, borate ion in seawater has some Cu complexing capacity not considered by Sunda (Anderson and Morel, 1978). The pertinant equilibria are (Sillen and Martel, 1964):

$$\frac{[B(OH)_3]}{a_{Cu}\;[B(OH)_4^-]} = 10^{-9.28}$$

and

$$\frac{[CuB(OH)_4^+]}{a_{Cu}\ [B(OH)_4^-]} = 10^{7.1}$$

These constants are for zero ionic strength, so to correct them to seawater ionic strength the following ion activity coefficients were used, $\gamma B(OH)_4^-$ = 0.365, $\gamma CuB(OH)_4^+$ = 0.68 and $\gamma B(OH)_3$ = 1.16 (Stumm and Morgan, 1970). Inclusion of borate complexation produced a small correction of the Cu-free-ion activity calculated for TRIS and TRIS-EDTA buffers, and was most significant at low pCu values in 1 mM TRIS solutions, where it raised the calculated pCu about 0.2 units.

Finally, inorganic Cu (other than borate-complexed Cu) was assumed to be negligible compared to TRIS and EDTA-complexed Cu, in a mass balance equation for Cu:

$$\text{Total Cu} = [CuTRIS_2] + [CuEDTA] + [CuB(OH)_4^+]$$

The above simultaneous equations were solved iteratively using a computer program written in APPLESOFT BASIC on an APPLE II+ microcomputer.

IV RESULTS

A. Introduction

This research was performed in two basic modes, field and laboratory. First, a series of outdoor enrichemennt experiments was performed. These experiments were designed to determine the sensitivity of natural populations of phytoplankton to Cu, using the TRIS-pCu buffers first developed by Sunda (1975). These buffers had been applied by Sunda to two only species in the laboratory. After several of these experiments had been performed, it became clear that complications had arisen in the transfer of this laboratory-developed technique to the study of field populations. At that point, it was necessary to perform some laboratory experiments to determine the source of the differences between the original work on laboratory clones, and these results for field cultures. The laboratory experiments provided a partial answer to the questions raised by the field experiments. Finally, the implications derived from the laboratory experiments were tested in a final outdoor experiment.

As a result of this experimental development, the general scheme of experiments ran Field-Laboratory-Field; however, to simplify the presentation, the results of all the field experiments have been presented in one section, and the results of all the laboratory experiments in another.

B. Effect of Copper on Natural Phytoplankton Assemblages

1. Experimental Conditions

The incubation temperature, salinity and important dates concerning the start of the outdoor experiments are given in Table 5. Initial nutrient and trace metal concentrations for the source seawater are shown in Table 6 (metal additions, if any, are not shown). The types of treatments included are shown in Table 7.

2. Effect of Copper on Growth Rates

a. General

One obvious difference between the experiments is the range of maximum growth rate for untreated cultures (Fig 8). Maximum growth rates ranged from 0.85 doublings/day in Experiment II (Fig. 8b) to 4.0 in Experiment I (Fig. 8a). This difference was caused primarily by differences in incubation temperature and ambient light intensity. For example, Exp. II, in which the lowest maximum growth rate was observed, took place at the lowest temperature, ($5^{o}C$), during February, a month of short and often heavily overcast days.

A convenient way to compare overall Cu toxicity from one series of treatments to the next is the EC50 test, the Estimated Concentration that causes 50% growth rate inhibition (Canterford and Canterford, 1980). In some cases of high Cu stress, no growth rates could be determined because the treatment produced a flora dominated by a distinctive medium sized pennate diatom, possibly of genus Amphiprora (pennate form #1), whose tendency to stick to the culture

Table 5. Physical parameters and dates concerning outdoor enrichment experiments.

Experiment	T (^{o}C)	S (o/oo)	Water Collection	Inoculum Collection	Inoculation
I	10.9	31.3	4/8/78	4/18/78	4/19/78
II	5.4	30.3	1/26/79	1/26/79	1/26/79
III	12.4	29.4	4/18/79	4/26/79	4/27/79
IV	20.0	32.6	6/9/79	6/13/79	6/14/79
V	20.0	32.6	2/20/81	3/23/81	3/24/81

Table 6. Ambient major nutrient and trace metal concentrations for outdoor enrichment experiments.

Experiment	NO_2+NO_3 (µM)	PO_4 (µM)	$Si(OH)_4$ (µM)	Cu (nM)	Mn (nM)	Fe (nM)
I	9.5	1.2	22.6	3.2	46.8	9.7
II	20.1	1.5	44.1	3.3	69.2	80.5
III	8.2	0.7	-----	3.6	72.9	23.3
IV	24.1	3.0	55.1	3.0	125	125
V	54.7	4.8	19.9	4.8	11.1	----

Table 7. Treatment additions, calculated pCu values and growth rates for outdoor experiments I-V. The mean pH value accompanying treatments containing TRIS was used to calculate pCu. Starred zero growth rates indicate growth of pennate diatoms.

	Culture #	Cu (M)	TRIS (mM)	FeEDTA (μM)	pH	Mn (M)	pCu	μ (div./day)
Exp. I								
	1	-	-	-	-	-	-	3.49
	2	-	-	-	-	-	-	3.82
	3	7.87E-9	-	-	-	-	-	3.63
	4	7.87E-9	-	-	-	-	-	4.05
	5	1.57E-8	-	-	-	-	-	1.41
	6	1.57E-8	-	-	-	-	-	1.73
	7	1.57E-8	-	-	-	-	-	1.20
	8	7.87E-8	-	-	-	-	-	1.92
	9	7.87E-8	-	-	-	-	-	0.87
	10	7.87E-8	-	-	-	-	-	1.31
Exp. II								
	1	-	-	-	-	-	-	0.82
	2	-	-	-	-	-	-	0.89
	3	1.0E-8	-	-	-	-	-	0.86
	4	1.0E-8	-	-	-	-	-	0.83
	5	3.0E-8	-	-	-	-	-	0.68
	6	3.0E-8	-	-	-	-	-	0.80
	7	1.0E-7	-	-	-	-	-	0*
	8	1.0E-7	-	-	-	-	-	0*
	9	3.0E-7	-	-	-	-	-	0*
	10	3.0E-7	-	-	-	-	-	0*
	11	1.0E-6	-	-	-	-	-	0
	12	1.0E-6	-	-	-	-	-	0
	13	-	5	-	8.77	-	14+	0.84
	14	1.0E-7	5	-	8.79	-	13.1	0.92
	15	3.0E-7	5	-	8.78	-	12.6	0.82
	16	3.0E-7	5	-	8.76	-	12.6	0.87
	17	1.0E-6	5	-	8.78	-	12.1	0.80
	18	1.0E-6	5	-	8.73	-	11.9	0.74
	19	3.0E-6	5	-	8.74	-	11.5	0.69
	20	3.0E-6	5	-	8.75	-	11.5	0.70
	21	1.0E-5	5	-	8.74	-	11.0	0.69
	22	1.0E-5	5	-	8.76	-	11.0	0.70
	23	3.0E-5	5	-	8.75	-	10.5	0.47
	24	3.0E-5	5	-	8.73	-	10.5	0.20
	25	1.0E-4	5	-	8.70	-	9.8	0
	26	3.0E-4	5	-	8.70	-	9.2	0

Table 7 continued

	Culture #	Cu (M)	TRIS (mM)	FeEDTA (μM)	pH	Mn (M)	pCu	μ (div./day)
Exp III								
	1	-	-	-	-	-	-	1.72
	2	-	-	-	-	-	-	1.62
	3	1.0E-8	-	-	-	-	-	1.70
	4	1.0E-8	-	-	-	-	-	1.70
	5	3.0E-8	-	-	-	-	-	1.67
	6	3.0E-8	-	-	-	-	-	1.62
	7	1.0E-7	-	-	-	-	-	1.17
	8	1.0E-7	-	-	-	-	-	1.21
	9	3.0E-7	-	-	-	-	-	0
	10	3.0E-7	-	-	-	-	-	0
	11	1.0E-6	-	-	-	-	-	0
	12	1.0E-6	-	-	-	-	-	0
	13	-	1	-	8.59	-	14+	1.69
	14	-	1	-	8.57	-	14+	1.68
	15	3.75E-9	1	-	8.57	-	12.8	1.73
	16	3.75E-9	1	-	8.57	-	12.8	1.61
	17	1.25E-8	1	-	8.62	-	12.4	1.66
	18	1.25E-8	1	-	8.57	-	12.3	1.67
	19	3.75E-8	1	-	8.58	-	11.8	1.72
	20	3.75E-8	1	-	8.61	-	11.9	1.73
	21	1.25E-7	1	-	8.54	-	11.2	1.50
	22	1.25E-7	1	-	8.56	-	11.3	1.51
	23	3.75E-7	1	-	8.39	-	10.3	1.15
	24	3.75E-7	1	-	8.39	-	10.3	1.12
	25	1.25E-6	1	-	8.37	-	9.7	0
	26	1.25E-6	1	-	8.35	-	9.7	0
	27	3.75E-6	1	-	8.36	-	9.2	0
	28	3.75E-6	1	-	8.35	-	9.2	0
	29	-	5	-	8.41	-	14+	1.69
	30	-	5	-	8.40	-	14+	1.75
	31	2.50E-8	5	-	8.38	-	12.9	1.68
	32	2.50E-8	5	-	8.42	-	12.8	1.64
	33	6.25E-8	5	-	8.37	-	12.5	1.66
	34	6.25E-8	5	-	8.41	-	12.4	1.69
	35	2.50E-7	5	-	8.41	-	11.9	1.50
	36	2.50E-7	5	-	8.32	-	11.6	1.50
	37	6.25E-7	5	-	8.28	-	11.1	1.22
	38	6.25E-7	5	-	8.28	-	11.1	1.19
	39	2.50E-6	5	-	8.28	-	10.5	0.79
	40	2.50E-6	5	-	8.25	-	10.4	0.77
	41	6.25E-6	5	-	8.38	-	10.2	0
	42	6.25E-6	5	-	8.32	-	10.2	0
	43	2.50E-5	5	-	8.29	-	9.6	0
	44	6.25E-5	5	-	8.29	-	9.2	0
	45	2.50E-4	5	-	8.29	-	8.5	0

Table 7 continued

	Culture #	Cu (M)	TRIS (mM)	FeEDTA (μM)	pH	Mn (M)	pCu	μ (div./day)
Exp. IV								
	1	-	-	-	-	-	-	2.14
	2	-	-	-	-	-	-	2.60
	3	1.00E-8	-	-	-	-	-	2.16
	4	1.00E-8	-	-	-	-	-	2.51
	5	3.00E-8	-	-	-	-	-	2.18
	6	3.00E-8	-	-	-	-	-	2.61
	7	1.00E-7	-	-	-	-	-	1.72
	8	1.00E-7	-	-	-	-	-	1.53
	9	3.00E-7	-	-	-	-	-	0.30
	10	3.00E-7	-	-	-	-	-	0.25
	11	1.00E-6	-	-	-	-	-	0*
	12	1.00E-6	-	-	-	-	-	0*

Table 7 continued

	Culture #	Cu (M)	TRIS (mM)	FeEDTA (μM)	pH	Mn (M)	pCu	μ (div./day)
Exp. V								
	1	-	-	-	-	-	-	2.25
	2	-	-	-	-	-	-	2.25
	3	1.00E-8	-	-	-	-	-	1.87
	4	1.00E-8	-	-	-	-	-	1.91
	5	3.00E-8	-	-	-	-	-	1.25
	6	3.00E-8	-	-	-	-	-	1.48
	7	1.00E-7	-	-	-	-	-	0.91
	8	1.00E-7	-	-	-	-	-	1.22
	9	3.00E-7	-	-	-	-	-	0
	10	3.00E-7	-	-	-	-	-	0
	11	1.00E-6	-	-	-	-	-	0
	12	1.00E-6	-	-	-	-	-	0
	13	-	-	-	-	1.00E-6	-	2.31
	14	-	-	-	-	1.00E-6	-	2.80
	15	1.00E-8	-	-	-	1.00E-6	-	2.31
	16	1.00E-8	-	-	-	1.00E-6	-	2.57
	17	3.00E-8	-	-	-	1.00E-6	-	2.25
	18	3.00E-8	-	-	-	1.00E-6	-	2.52
	19	1.00E-7	-	-	-	1.00E-6	-	1.02
	20	1.00E-7	-	-	-	1.00E-6	-	1.28
	21	3.00E-7	-	-	-	1.00E-6	-	0*
	22	3.00E-7	-	-	-	1.00E-6	-	0*
	23	1.00E-6	-	-	-	1.00E-6	-	0*
	24	1.00E-6	-	-	-	1.00E-6	-	0*
	25	4.00E-7	1	1	8.09	-	11.1	1.61
	26	4.00E-7	1	1	8.07	-	11.0	1.22
	27	7.50E-7	1	1	8.02	-	10.5	1.01
	28	7.50E-7	1	1	7.99	-	10.4	0.87
	29	1.00E-6	1	1	8.07	-	10.1	1.03
	30	-	1	1	8.10	-	13.1	1.99
	31	1.50E-6	1	1	8.02	-	9.4	0*
	32	-	1	1	8.16	-	13.1	2.00
	33	3.00E-6	1	1	7.94	-	8.7	0
	34	3.00E-6	1	1	8.02	-	8.9	0
	35	5.00E-6	1	1	7.92	-	8.3	0
	36	5.00E-6	1	1	7.94	-	8.4	0

Table 7 continued

Culture #	Cu (M)	TRIS (mM)	FeEDTA (μM)	pH	Mn (M)	pCu	μ (div./day)
37	7.50E-7	3	1	8.14	-	10.9	1.31
38	7.50E-7	3	1	8.16	-	11.0	1.30
39	1.50E-6	3	1	8.13	-	10.5	1.27
40	-	3	1	8.14	-	13.3	1.93
41	3.00E-6	3	1	8.12	-	10.0	0.94
42	-	3	1	8.12	-	13.3	1.97
43	6.00E-6	3	1	8.12	-	9.6	0*
44	-	3	1	8.14	-	13.3	2.13
45	1.50E-5	3	1	8.11	-	9.2	0
46	1.50E-5	3	1	8.10	-	9.1	0
47	3.50E-5	3	1	8.07	-	8.7	0
48	3.50E-5	3	1	7.99	-	8.5	0
49	3.00E-6	10	1	8.16	-	11.1	1.47
50	3.00E-6	10	1	8.09	-	10.9	1.10
51	1.00E-5	10	1	8.15	-	10.5	0*
52	1.00E-5	10	1	8.12	-	10.4	0*
53	3.00E-5	10	1	8.12	-	9.9	0
54	3.00E-5	10	1	8.08	-	9.8	0
55	7.50E-5	10	1	8.05	-	9.3	0
56	7.50E-5	10	1	8.00	-	9.2	0
57	1.50E-4	10	1	8.05	-	9.0	0
58	1.50E-4	10	1	8.02	-	8.9	0
59	4.00E-4	10	1	8.01	-	8.4	0
60	4.00E-4	10	1	8.03	-	8.4	0
61	4.00E-7	1	1	8.02	1.00E-6	11.0	2.04
62	4.00E-7	1	1	8.07	1.00E-6	11.0	1.90
63	7.50E-7	1	1	8.17	1.00E-6	10.6	1.53
64	7.50E-7	1	1	7.98	1.00E-6	10.4	1.74
65	1.00E-6	1	1	8.12	1.00E-6	10.2	1.74
66	1.00E-6	1	1	8.08	1.00E-6	10.1	1.84
67	1.50E-6	1	1	8.07	1.00E-6	9.5	1.34
68	1.50E-6	1	1	8.08	1.00E-6	9.6	1.24
69	3.00E-6	1	1	8.06	1.00E-6	9.0	0*
70	3.00E-6	1	1	7.85	1.00E-6	8.5	0*
71	5.00E-6	1	1	8.05	1.00E-6	8.7	0*
72	5.00E-6	1	1	8.06	1.00E-6	8.7	0*

Table 7 continued

Culture #	Cu (M)	TRIS (mM)	FeEDTA (μM)	pH	Mn (M)	pCu	μ (div./day)
73	7.50E-7	3	1	8.16	1.00E-6	11.0	1.66
74	7.50E-7	3	1	8.12	1.00E-6	10.9	1.98
75	1.50E-6	3	1	8.18	1.00E-6	10.6	1.40
76	1.50E-6	3	1	8.11	1.00E-6	10.4	1.67
77	3.00E-6	3	1	8.15	1.00E-6	10.1	1.16
78	3.00E-6	3	1	8.11	1.00E-6	10.0	1.34
79	6.00E-6	3	1	8.12	1.00E-6	9.6	0*
80	6.00E-6	3	1	8.12	1.00E-6	9.6	0*
81	1.50E-5	3	1	8.10	1.00E-6	9.0	0*
82	1.50E-6	3	1	8.07	1.00E-6	9.1	0*
83	3.50E-6	3	1	7.96	1.00E-6	8.3	0*
84	3.50E-6	3	1	7.93	1.00E-6	8.2	0*
85	3.00E-6	10	1	8.16	1.00E-6	11.1	1.40
86	3.00E-6	10	1	8.13	1.00E-6	11.0	1.40
87	1.00E-5	10	1	8.15	1.00E-6	10.5	0*
88	1.00E-5	10	1	8.11	1.00E-6	10.4	0*
89	3.00E-5	10	1	8.10	1.00E-6	9.8	0*
90	3.00E-5	10	1	8.08	1.00E-6	9.8	0*
91	7.50E-5	10	1	8.02	1.00E-6	9.2	0
92	7.50E-5	10	1	8.02	1.00E-6	9.2	0
93	1.50E-4	10	1	8.02	1.00E-6	9.0	0
94	1.50E-4	10	1	8.05	1.00E-6	8.9	0
95	4.00E-4	10	1	8.01	1.00E-6	8.6	0
96	4.00E-4	10	1	8.08	1.00E-6	8.4	0

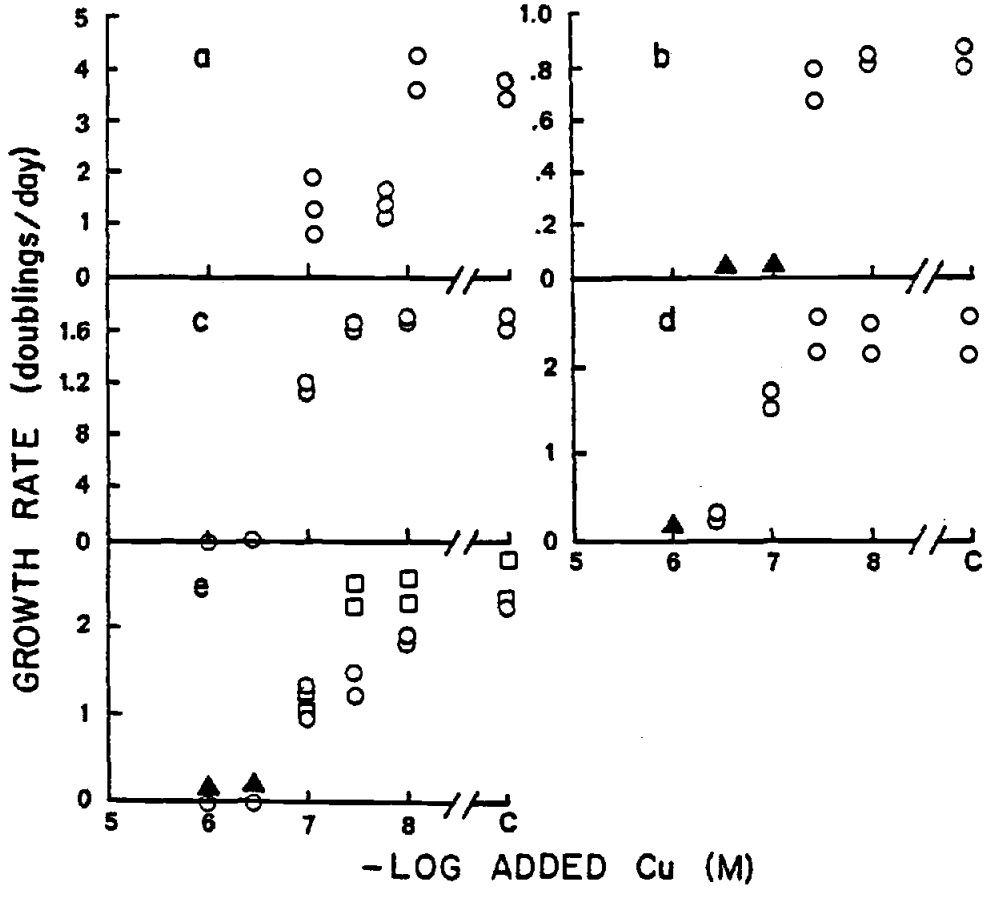

Fig. 8. The effect of ionic Cu additions on the growth rate of natural phytoplankton populations. a) Exp. I, b) Exp. II, c) Exp. III, d) Exp. IV, e) Exp V. ○ - no added Mn, □ - +1000nM Mn. ▲ - Cultures with benthic algae, growth rates not calculated. C marks the no copper added control cultures.

vessel walls prevented satisfactory daily sampling. These points are noted in Fig. 8b,d,e and 10a-e. In these cases, the EC50 value represents only estimates of the value for the phytoplankton.

b. Ionic Copper Additions

The EC50 values for Cu in two of the five field experiments without Mn addition were approximately 50 nM (Table 8). The other three were about 130 nM. In Exp. I, the form of the response was unlike that in any other experiment, in that growth rate showed an initial drop at low Cu additions, followed by a subsequent plateau at higher Cu additions (Fig. 8). The EC50 value of 130 nM was chosen since cultures grew at high rates (compared to other experiments) up to 100 nM, the highest level of Cu in this experiment. This value cannot be very reliable. The EC50 value after 1000 nM Mn addition in Exp. V was about 100 nM. The cultures without Mn in Exp. V had lower growth rates at all added Cu concentrations except the 10^{-7} M additions, when compared to cultures with 1000 nM Mn added (Fig. 8e). At 1 x 10^{-7} M of added Cu, the growth rates were approximately equal. In the 10^{-7} M Cu addition without added Mn, green flagellates made up the entire assemblage of phytoplankton and so determined the growth rate. In the 10^{-7} M additions with added Mn, the diatom Chaetoceros dominated the assemblage. Chaetoceros was the dominant taxon under low Cu stress throughout Exp. V. If only the growth rates of cultures in which Chaetoceros was dominant are considered (see species composition results, later), the EC50 in the series without added Mn is about 50 nM (Table 8).

Table 8. The estimated concentration of added ionic Cu causing 50% growth rate inhibition of the dominant phytoplankton population in the outdoor experiments. EC50 values interpolated from growth rates and Cu additions listed in Table 7.

Exp. #		EC50 (nM)
I		130
II		56
III		130
IV		130
V	no added Mn	50
V	+1000 nM Mn	100

Table 9. The estimated Cu free ion activity causing 50% growth rate inhibition of the dominant phytoplankton population in the outdoor experiments. EC50 values interpolated from growth rates and calculated pCu values listed in Table 7.

Exp. #	TRIS (mM)		EC50 (pCu)
II	5		11.1
III	1		10.1
III	5		10.6
V	1	no added Mn	10.0
V	3	no added Mn	10.2
V	10	no added Mn	10.9
V	1	+1000 nM Mn	9.5
V	3	+1000 nM Mn	9.8
V	10	+1000 nM Mn	11.0

c. TRIS-Cupric Ion Activity Buffers

Growth rates have been plotted as a function of calculated pCu for treatments from Exps. II and III (Fig 9). Experiment II was the first experiment in which TRIS-cupric ion activity buffers were included, and only one series was made, using 5 mM TRIS (Fig. 9a). The EC50 was estimated to be pCu = 11.1, an activity of 7.9 x 10^{-12} M, equivalent to approximately 0.4 nM total Cu in seawater at pH 8.1 with only inorganic speciation considered. In Experiment III, two series of TRIS-cupric ion activity buffers were made, using 1 and 5 mM TRIS (Fig. 9b). The EC50 for the 1mM TRIS series occurred at pCu 10.1, while for the 5 mM series it was at pCu 10.6, a greater difference than could reasonably be accounted for by pH measurement error and growth rate differences. The phytoplankton thus showed a different toxic response to pCu apparently depending on the TRIS concentration. However when plotted versus total Cu (essentially equal to the concentration of the Cu-TRIS complex in media where TRIS is the only chelator), the two series did not have equal EC50 values either (Fig. 9c). This suggested that the toxicity of the Cu-TRIS complex was not controling growth rate, but that a combination of Cu ion toxicity and Cu-TRIS toxicity might, or that some other aspect of TRIS was responsible.

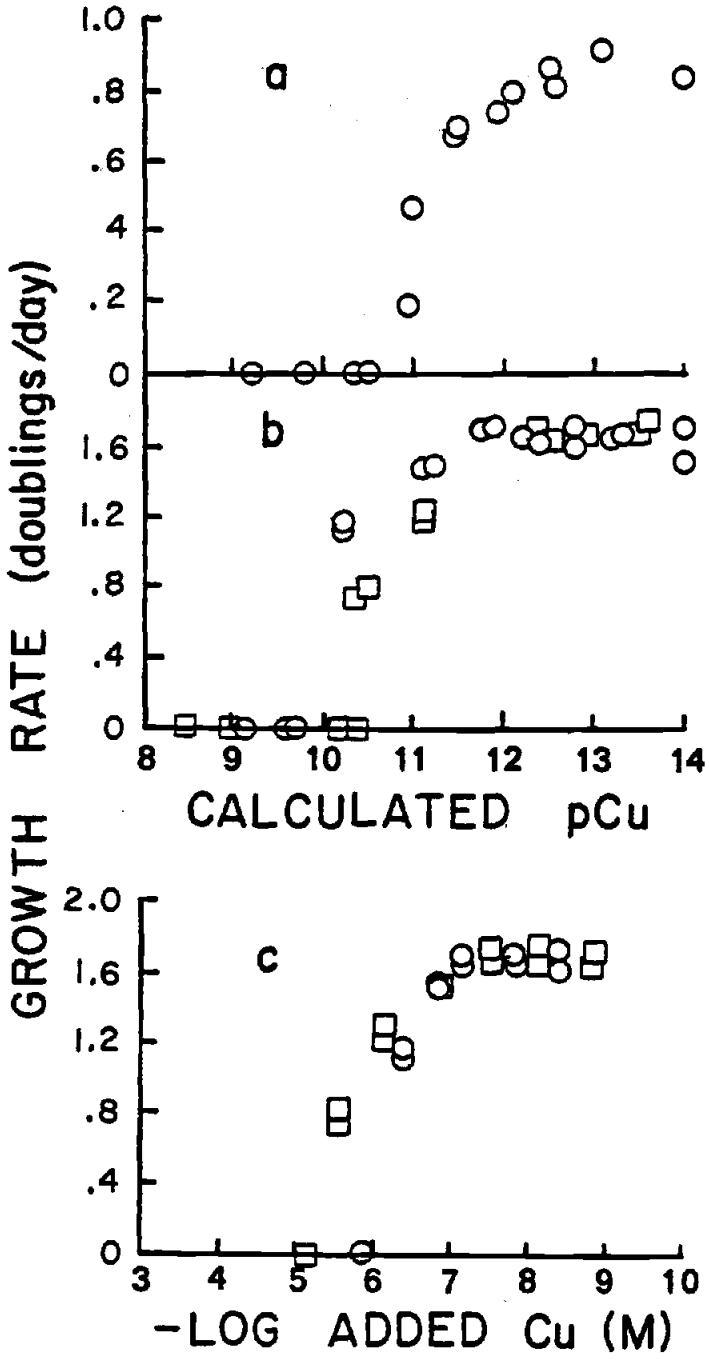

Fig. 9. The effect of pCu on the growth rate of natural phytoplankton populations in TRIS buffered seawater. a) Exp. II, 5 mM TRIS. b) Exp. III c) Exp. III plotted as a function of total Cu. ○ - 1 mM TRIS, □ - 5 mM TRIS.

d. The Relationship of Manganese to Copper Toxicity

As detailed in a later section, the laboratory experiments following Exp. IV led to the hypothesis that a deficiency of manganese in conjunction with high TRIS concentrations could cause Cu to become toxic at higher pCu values (lower Cu concentrations) than might occur at low TRIS or high Mn. Exp. V was set up to test the importance of this phenomenon in the outdoor enrichment experiments. Several changes were made from the procedures of Exps. II, III, and IV. One change was to collect the water for Exp. V farther at sea, in an attempt to lower the ambient Mn concentration. This was successful (Table 6). Another change was to duplicate all treatments, with one set receiving 1000 nM added Mn. Based on the preceeding laboratory experiments this was far more than enough Mn to offset any deficiency. A third change was that TRIS-cupric ion activity buffers were made up with 1, 3, and 10 mM TRIS, not just 1 and 5 mM as before. The final change was that all TRIS treatments had 1 μM of FeEDTA added, to avoid possible iron deficiency due to the expected low Fe content of offshore seawater. The laboratory experiments had demonstrated that after inclusion of EDTA in pCu calculations, there was no negative effect of EDTA on phytoplankton growth.

The results of Experiment V showed that added Mn had a very significant effect on the level at which Cu ion became toxic to the growth of natural phytoplankton, especially at the lower TRIS concentrations (Fig. 10). The EC50 for the 1 mM TRIS series without added Mn was pCu = 10.0, while that for the 1 mM TRIS series with added Mn was pCu = 9.5. In the 3 mM TRIS series the EC50 without

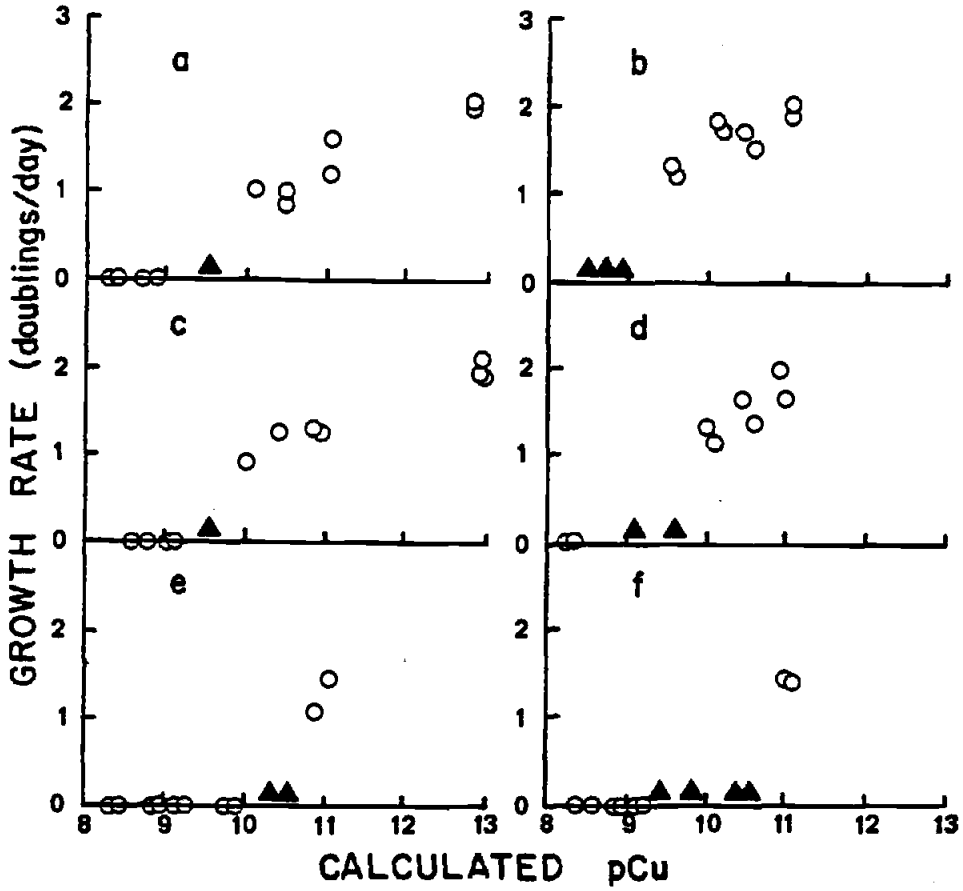

Fig. 10. The effect of pCu on the growth rate of natural phytoplankton populations in TRIS-EDTA buffered seawater. Exp. V. a) 1mM TRIS, no added Mn, b) 1mM TRIS, 1000 nM Mn added, c) 3mM TRIS, no added Mn, d) 3mM TRIS, 1000 nM Mn added, e) 10 mM TRIS, no added Mn, f) 10 mM TRIS, 1000 nM Mn added.
▲- Cultures with benthic diatoms, no growth rates calculated.

added Mn was pCu = 10.2. With Mn the EC50 was pCu = 9.8. In the 10 mM series, the maximum growth rate had to be inferred from experiments with lower TRIS concentrations and non-TRIS controls, because the range of pCu values did not extend into non-inhibitory ranges. The estimated EC50 values were pCu = 10.9 and 11.0, with and without Mn addition. However, when we examine the results for containers in which growth rate of the phytoplankton could not be calculated due to growth of benthic diatoms (see later), it will be noted that the Mn additions extended the Cu concentrations over which the benthic diatoms would grow, even in the 10 mM TRIS series.

The EC50 values estimated for Cu in terms of pCu, with and without Mn additions are summarized in Table 9.

3. Effect of Copper on Phytoplankton Species Composition

a. General Considerations

The enrichment culturing experiments reported here frequently show low diversity in the phytoplankton assemblage compared to natural samples, probably because they encourage the growth of a few species well adapted to precisely the conditions provided, and by not providing a variety of conditions spatiotemporally that allow other species to compete. In addition, the small size and low cell density of the inoculum also decrease the probability of rare species occurring. It should be noted, however, that even nature frequently produces nearly unialgal blooms.

A problem inherent in the study of the effects of metals on species assemblages is the effect of metals on the morphology of

phytoplankton (Canterford, 1980; Thomas et al., 1980; and Fisher et al., 1981). Examples of the effect of Cu on the morphology of Thalassiosira taken from my experiments are shown in Fig. 11. This problem compounds the difficulty in correctly identifying algae to the species level. Thus, phytoplankton have been identified only to genus. In a few cases where very severe Cu toxicity produced a community of very small, difficult-to-identify pennate diatoms or green flagellates, no further identification was attempted. Taxa which had less than 5% representation in at least one treatment of a related series have not been considered.

b. Ionic Copper Additions

The relative frequencies of the important taxa as a function of ionic Cu additions are shown in Fig. 12. These taxonomic data belie the apparent simplicity of the growth rate results. For example, in all four cases where Thalassiosira and Chaetoceros co-occurred (Experiments I-IV), Fig. 12 a-d), increasing Cu levels tended to enhance the fraction of Thalassiosira at the expense of Chaetoceros. Chaetoceros was always virtually eliminated at an added ionic Cu concentration of 100 nM, (-log added Cu = 7) and showed decreases relative to Thalassiosira at between 10 and 30 nM. In Exp. I,(Fig. 12a) the treatments were not carried out to Cu levels high enough to prevent the growth of Thalassiosira and Skeletonema. In Exp. IV, however, mixed pennate diatoms were found at an added ionic Cu concentration of 1000 nM (-log added Cu = 6), essentially replacing Thalassiosira at this high concentration (Fig. 12d). The pennate diatom Nitzschia also outcompeted Thalassiosira at 100 nM Cu levels

Fig. 11. An example of the effect of Cu on the morphology of diatoms from the outdoor enrichment experiments. a) Thalassiosira sp. from no added Cu treatment of Exp. IV. Note even dispersion of chloroplasts. b) Thalassiosira sp. from 300 nM Cu added treatment from Exp. IV. Note the compact central chloroplasts. Cells in water mount, stained with acid Lugol's solution, magnification 1000x, oil immersion phase contrast.

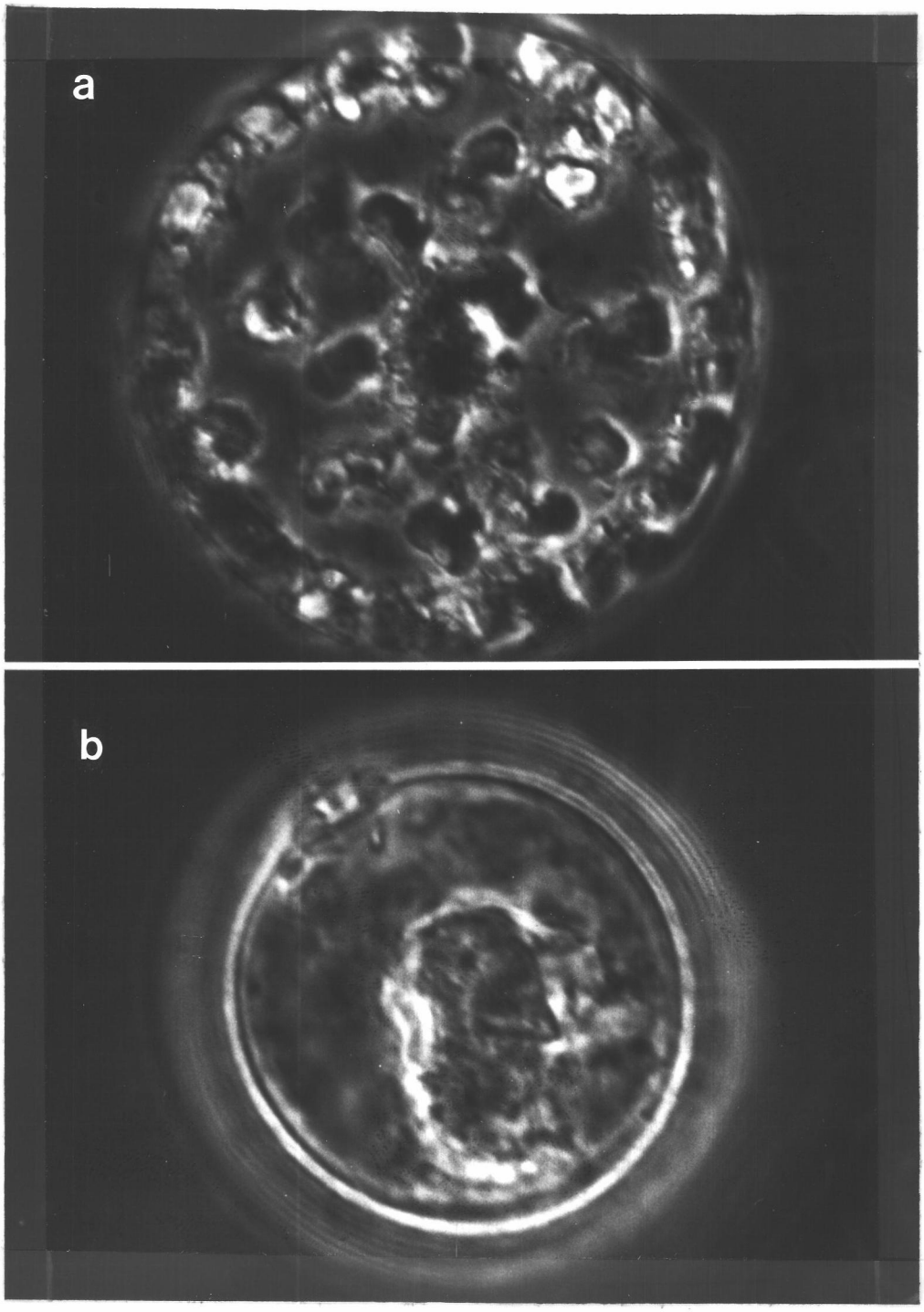

Fig. 11

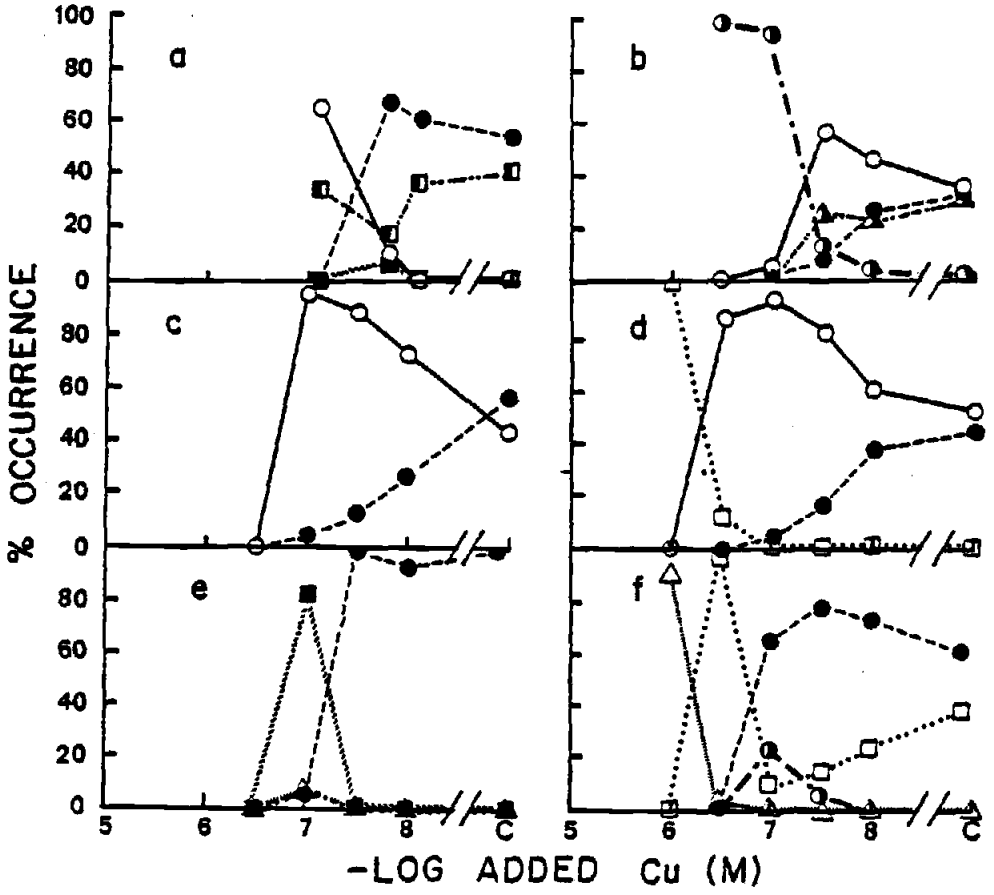

Fig. 12. The effects of ionic Cu additions on the taxonomic composition of natural phytoplankton populations. a) Exp. I, b) Exp. II, c) Exp. III, d) Exp. IV, e) Exp. V, no added Mn, f) Exp. V, 1000 nM Mn added.

Thalassiosira — Chaetoceros
pennate form #1 — Asterionella
Nitzschia — Skeletonema
flagellates — small mixed pennates

and higher in Exp. II (Fig. 12b). It should be noted here that the great difference in relative abundances may not reflect great differences in the Cu tolerances of the diatoms. Given enough generations, in this case on the order of ten, a small difference in the growth rate between any two species could lead to a large difference in relative abundance. In the cases where pennate diatoms predominated in treatments of high Cu stress, long lags before growth indicated that more common centric diatoms were probably completely inhibited.

In Exp. V (Fig. 12e,f), Chaetoceros was a predominant genus; however, no appreciable numbers of Thalassiosira grew in any of the treatments, with or without Mn additions. The reason is not clear, however, it may be as simple as an overwhelming abundance of Chaetoceros in the inoculum. Small green flagellates predominated at 100 nM added Cu in the absence of added Mn (Fig. 12e), but they were replaced by Chaetoceros when Mn was present (Fig. 12f).

c. TRIS-Cupric Ion Activity Buffers

The species composition patterns in treatment series in which TRIS-cupric ion activity buffers were used in Exps. II and III (Fig. 13), were entirely different from those patterns in Exps. II and III deriving from additions of ionic Cu only (Fig. 12b,c). The most striking difference was the Thalassiosira-Chaetoceros relationship in Exp. III without TRIS (Fig. 12c) and with 5 mM added TRIS (Fig. 13b). With the 5 mM TRIS treatments, Chaetoceros, rather than Thalassiosira, became dominant as pCu was lowered from about 14 to 10. The same general relationship was found in the 5 mM TRIS addition in Exp. II

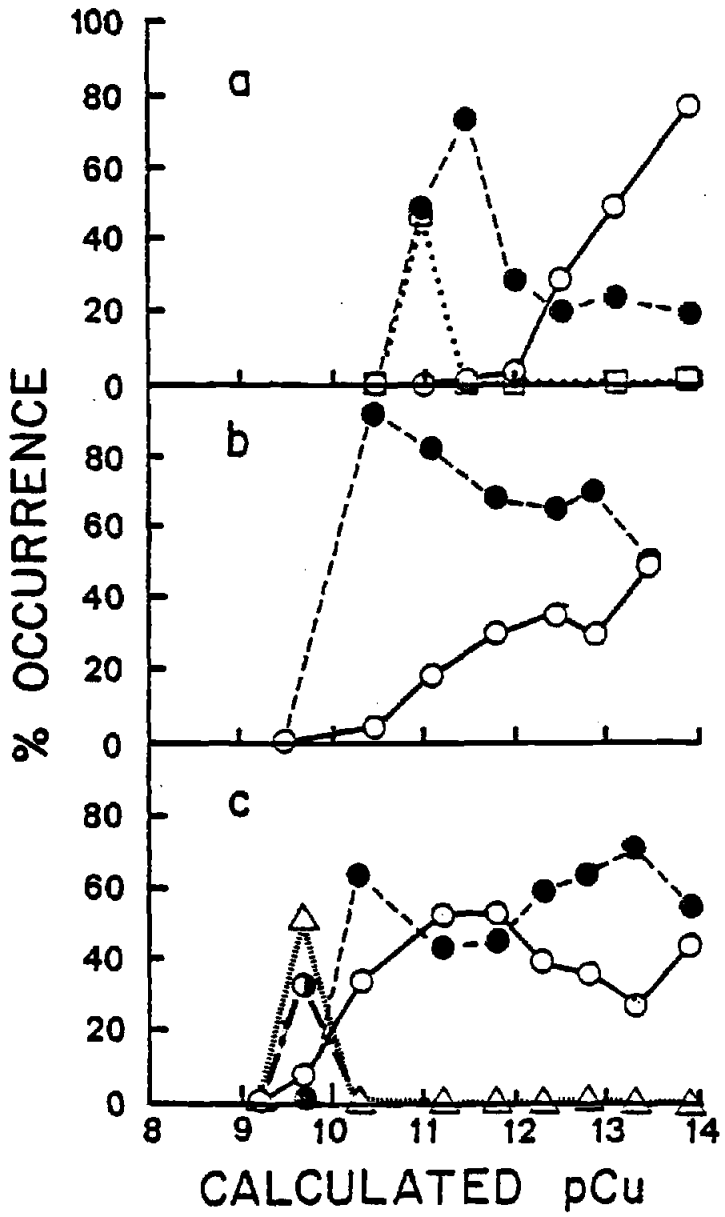

Fig. 13. The effects of pCu on the taxonomic composition of natural phytoplankton populations in TRIS buffered seawater. a) Exp. II, 5 mM TRIS, b) Exp. III, 1mM TRIS, c) Exp. III, 5 mM TRIS.

Thalassiosira — Chaetoceros
pennate form #1 — Asterionella
Nitzschia — Skeletonema
flagellates — small mixed pennates

(Fig. 13a) except the tolerance of both Thalassiosira and Chaetoceros to increasing Cu concentrations was not as great as in Exp. III. In the 1 mM TRIS treatments of Exp. III (Fig. 13c), Thalassiosira and Chaetoceros maintained approximately equal abundance until high copper stress eliminated them in favor of pennate form #1 and Nitzschia. Thus, there was a trend in which Thalassiosira increased its predominance relative to Chaetoceros under increasing ionic Cu conditions (Fig. 12a-d), but this trend was reversed as the TRIS concentrations in the water were raised from 1 to 5mM (Fig. 13).

In Exp. V, with water taken from well offshore, Thalassiosira was not present in the resultant phytoplankton assemblage (Fig. 12e,f and Fig. 14). Comparing results in 1 mM TRIS additions in Exps. III (Fig. 13c) and V (Fig. 14a), shows that Chaetoceros in the absence of Thalassiosira absolutely dominated the phytoplankton assemblage at pCu values higher than 10. Large pennate diatoms and, in one instance small flagellates, predominated at pCu values less than 10. The same situation held for 3 mM TRIS additions (Fig. 14c). The same relationship was also noted at 10 mM TRIS additions (Fig. 14e) except the whole relationship was displaced toward higher pCu values (lower Cu concentrations); thus, Chaetoceros absolutely dominated the assemblages at pCu values of 11 and greater, with a pennate (pennate form #1 in this case) dominating at pCu values below 10.5, and no growth of anything at pCu values lower than about 10.

With respect to the benthic diatom community in general (the pennate diatoms, including pennate form #1 and Nitzschia), high Cu activities (low pCu values) became more inhibitory as the TRIS concentration increased. For example, the 5 mM TRIS concentration in

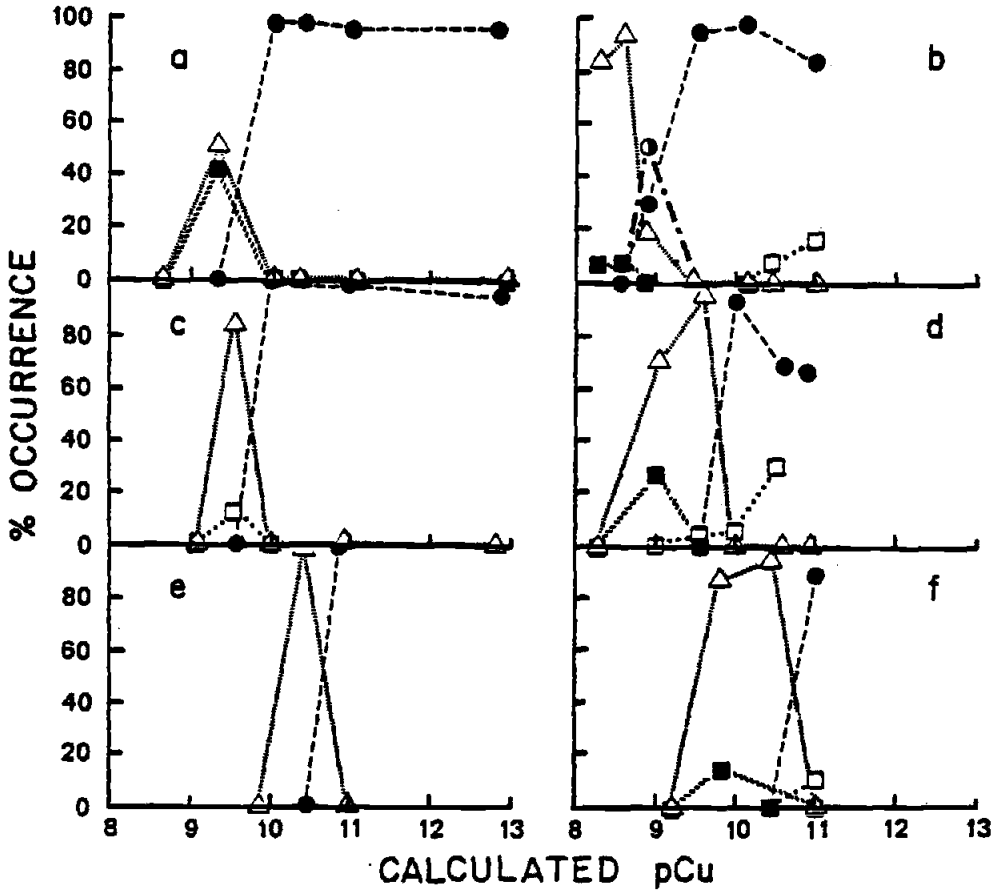

Fig. 14. The effects of pCu on the taxonomic composition of natural phytoplankton populations in TRIS-EDTA buffered seawater. Exp. V. a) 1mM TRIS, no added Mn, b) 1mM TRIS, 1000 nM Mn added, c) 3mM TRIS, no added Mn, d) 3mM TRIS, 1000 nM Mn added, e) 10 mM TRIS, no added Mn, f) 10 mM TRIS, 1000 nM Mn added.

Chaetoceros — pennate form #1

Nitzschia — flagellates

Exp. II (Fig. 13a) apparently restricted pennate diatom growth below pCu = 10.5, while the 1 mM TRIS concentration in Exp. III, (Fig. 13b) allowed pennates to grow at pCu values below 10. In Exp. V, the 10 mM TRIS concentration (without added Mn) restricted pennate form #1 to pCu values above about 10 (Fig. 14e), the 3 mM TRIS concentration restricted pennate form #1 to pCu values greater than 9 and less than 10 (Fig. 14c), and the 1 mM TRIS concentration allowed pennate form #1 to grow at pCu values less than 9. In all cases, the benthic community tolerated higher Cu stress than did the phytoplankton.

The diatom Asterionella was abundant in certain treatments in Exp. II, but in no other experiments. Its sensitivity in 5 mM TRIS was apparently midway between that of Thalassiosira and Chaetoceros (Fig. 13a). Skeletonema was an important taxon in Exp. I and was not seen in any other experiment. Small green flagellates appeared at low pCu values, concurrent with pennate form #1, in Exp. V in the 1 mM TRIS series without added Mn (Fig. 14a). Whether or not a TRIS effect restricts flagellate occurrence cannot be shown with the data in hand; however no flagellates appeared in the 3 and 10 mM TRIS series at high Cu stress, although pennate form #1 continued to appear (Fig. 14c,e).

d. The Effect of Added Manganese

In Exp. V the effect of added Mn on the occurrence of different algal taxa was studied. Mn additions allowed Chaetoceros to grow at higher Cu concentrations when TRIS concentrations were 1 mM or less. For example, with no TRIS in the water, no Chaetoceros occurred when ionic Cu additions were greater than 10^{-7} M in the absence of added Mn (Fig. 12e); however, on the addition of 1000 nM Mn

Chaetoceros represented over 60% of the algal cells at a Cu addition of 10^{-7} M (Fig. 12f). Similarly, with 1 mM TRIS in the water, at least 30% occurrence of Chaetoceros was observed at pCu = 9 when 1000 nM Mn was added (Fig. 14b), but Chaetoceros was not present at pCu = 9 without the Mn addition (Fig. 14a). At 3 and 10 mM TRIS, Mn additions had little effect on the occurrence of Chaetoceros (Fig. 14c-f): Chaetoceros disappeared at about pCu = 9.5 at 3 mM TRIS, with or without added Mn, and it disappeared at about pCu = 10.5 at 10 mM TRIS, with or without added Mn.

With the benthic pennate diatom pennate form #1, Mn additions improved percent occurrence and the ability to withstand Cu stress at all concentrations of TRIS. For example, pennate form #1 comprised less than 10% of the cell population at a 10^{-7} M Cu addition (with no TRIS or Mn additions), and was not observed at all at $10^{-6.5}$ M ionic Cu (Fig. 12e). On addition of 1000 nM Mn, pennate form #1 represented 90% of the cell population at the highest added Cu concentration, 10^{-6} M (Fig. 12f). It should be noted that pennate form #1 was not found at 10^{-7} M added Cu when Mn was added, but apparently was outcompeted under those situations by Chaetoceros and other pennates. Similarly, at 1 mM TRIS and with no Mn addition, pennate form #1 was not found at pCu values below 8.5, and represented only about 50% of the cell population at its peak abundance at a pCu somewhat greater than 9 (Fig. 14a). With 1000 nM added Mn, pennate form #1 showed peak occurrence (95%) at the lowest pCu value tested, 8.2 (Fig. 14b), Chaetoceros and pennates (including Nitzschia) apparently outcompeted pennate form #1 at about pCu 9 in treatments with 1000 nM added Mn. Similar patterns were observed at TRIS

concentrations of 3 and 10 mM, except that with the addition of Mn Chaetoceros and small pennates did not increase their ability to tolerate lower pCu values, but pennate form #1 did (Fig. 14c-f). This broadened the pCu range over which pennate form #1 was the dominant taxon.

It is difficult to evaluate the effect of Mn on the occurrence of green flagellates, since they were only rarely abundant, and the species in different treatments were clearly different.

C. Copper Content of the Experimental Phytoplankton Assemblages

1. General

In attempting to estimate the trace metal content of phytoplankton, some estimate of biomass must be used to normalize the results. Fortunately, when cells are in logarithmic growth phase, the various components of phytoplankton maintain rather constant relative composition, and most algal biomass measures can be interconverted to others using simple, if somwhat approximate, factors. In my experiments, Cu content has been expressed on the basis of in vivo fluorescence (f)(i.e. mol Cu-liter^{-1}(fluor. unit)$^{-1}$, because in vivo fluorescence is a sensitive measure of phytoplankton biomass and growth (Thomas et al., 1974), and it was measured on all the samples at the time of filtration.

2. Ionic Copper Additions

The copper in phytoplankton from experimental treatments with ionic Cu additions was measured in Experiments IV and V (Fig. 15a).

In Experiment IV (Fig. 15a), the samples were taken about one day after growth had ceased because of nutrient limitation. A very good log-log relationship was found between the soluble Cu in the water and Cu/f in the phytoplankton, for soluble Cu concentrations between 10 and 100 nM (-log soluble [Cu] = 7 to 8):

$$-\log Cu/f = 1.508(-\log [Cu]) + 1.385,$$

where [Cu] is the measured soluble Cu in mol/liter and Cu/f is in units of mol Cu-liter^{-1}(fluor. unit)$^{-1}$. The simple correlation coefficient (r) is 0.993, and the relationship is significant at the $p < 0.01$ level. Between the control (~3 nM ambient Cu, -log [Cu] = 8.5) and the 10 nM addition (-log [Cu] = 8.0), Cu/f shows only a slight increase.

In Experiment V (Fig. 15b) ionic Cu additions were made with and without 1000 nM manganese additions. The addition of Mn did not significantly change the relationship between Cu/f and [Cu]. Two values at low soluble Cu appear to have been contaminated. With these values eliminated the relationship is:

$$-\log Cu/f = 1.753(-\log [Cu]) - 0.977$$

and the regression simple coefficient (r) = 0.977. Unlike Exp. IV, the cells in this experiment were harvested during log phase, so that pooling the results of these experiments is not useful. It is of interest that the slope of the lines were in both cases greater than one, which may either indicate that the binding sites for cellular Cu

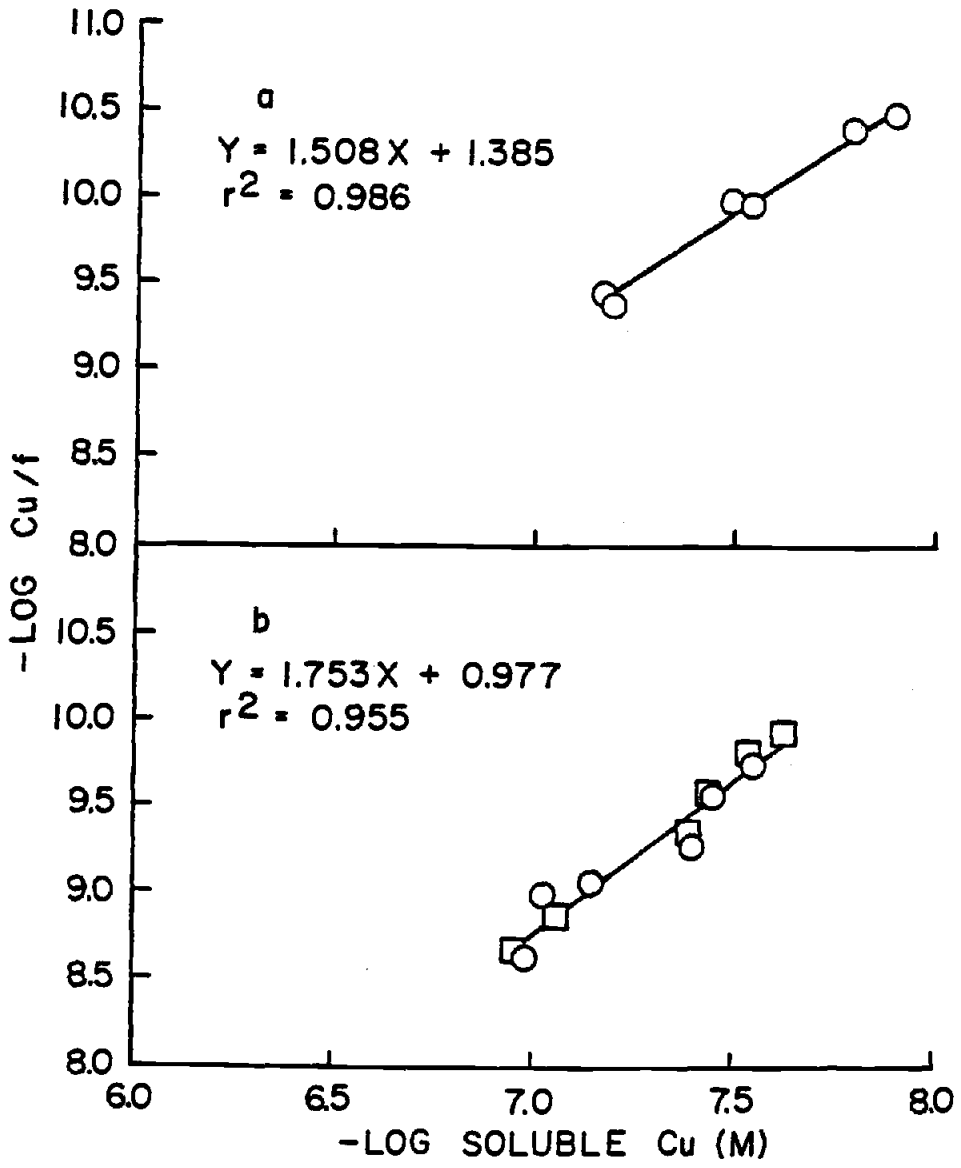

Fig. 15. Cell Cu content versus soluble Cu. a) Exp. IV, b) Exp. V.
○ - no added Mn, □ - 1000nM Mn added.

may bind more than one Cu per site site, or that the degree of complexation of Cu is decreasing as Cu concentration increases.

3. TRIS-Cupric Ion Activity Buffers

Cu/f was measured in the pCu series of Experiment V (Fig. 16). The overall relationship fitted by least squares regression for Cu/f on pCu was:

$$-\log Cu/f = 0.331\ pCu + 5.515,$$

with $r = 0.864$. The relationship was significant at the $p < 0.01$ level. There was no significant effect of TRIS concentration or Mn addition on this relationship ($p > .10$).

C. Laboratory Experiments

1. Effect of pCu on Isolated Species in Complete Media

The series of laboratory experiments addressed from two basic questions which arose from results of the outdoor enrichment experiments. The first question regarded the pCu that was found to be toxic to the natural populations of phytoplankton. In Exps. II and III the pCu values at which EC50 was reached for the natural phytoplankton assemblages ranged from 10.1 to 11.1 (Table 9). These levels contrasted to pCu = 8.4 for the diatom Thalassiosira pseudonana (3H) for which the first Cu toxicity study employing TRIS-cupric ion activity buffers was conducted (Sunda and Guillard, 1976). Is T.

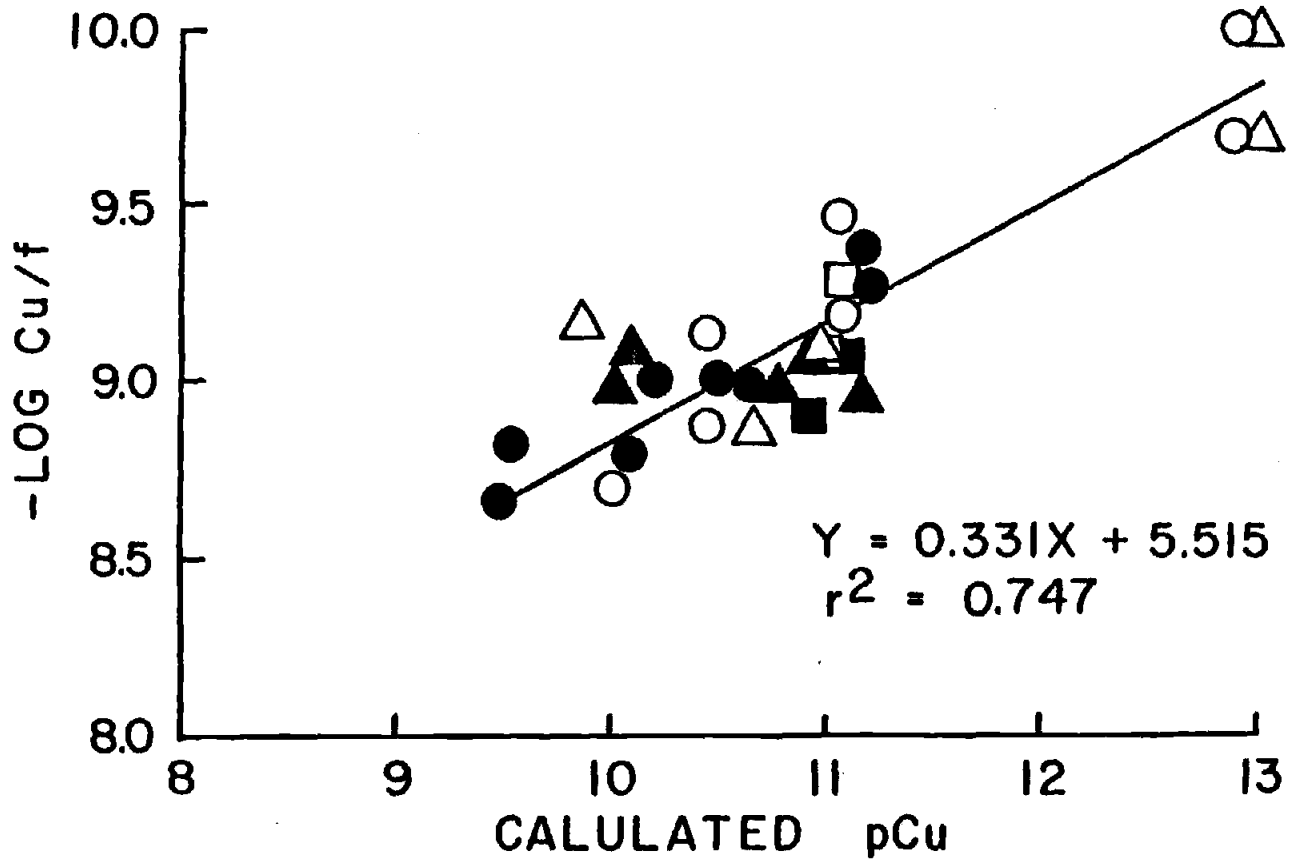

Fig. 16. Cell Cu content versus pCu. Exp. V.
○ - 1 mM TRIS, no added Mn, ● - 1 mM TRIS, 1000 nM added Mn
□ - 3 mM TRIS, no added Mn, ■ - 3 mM TRIS, 1000 nM added Mn
△ - 10 mM TRIS, no added Mn, ▲ - 10 mM TRIS, 1000 nM added Mn

pseudonana (3H) exceptionally resistant to Cu toxicity compared to most of the phytoplankton found in coastal seawater, or does the apparent difference in pCu values represent an artifact in either the laboratory or the outdoor experimental procedures? The second question was whether the majority of the natural phytoplankton responded to Cu solely as a function of pCu in Cu-TRIS buffered media, or whether the Cu-TRIS complex itself had a toxic effect. This latter possibility may have been indicated in Exp. III by the apparently greater toxicity of a constant pCu in higher TRIS medium (Fig. 9b,c). Stated more explicitly, I needed to determine: 1) Whether under laboratory conditions similar to those of Sunda and Guillard (1976), diatoms exhibit toxicity to Cu as a function by pCu alone, or as a function of the Cu-TRIS complex; and 2) If toxicity is a function of pCu alone, does the pCu value found to be toxic most approximate the levels found in the outdoor enrichment experiments, or the laboratory experiments of Sunda and Guillard (1976) using T. pseudonana (3H).

To answer these questions, diatoms reflecting the usual composition of the outdoor experiments were isolated into clonal cultures, and were subjected to growth rate experiments in various enriched media designed to test specific hypotheses concerning these questions.

Growth rate experiments similar to those of Sunda and Guillard, (1976), in which different TRIS concentrations were used to make up an overlapping series of pCu values, were carried out on three clones, Thalassiosira sp. (T-1), Chaetoceros constrictus (CHS-1), and Chaetoceros sp. (AS-1A). The first clone (T-1) appeared identical to many of the larger Thalassiosira in the outdoor Exps. I-IV. It was

about 40 µm in diameter and 10-15 µm in width, and formed chains connected by a mucous thread. Chaetoceros constrictus (CHS-1) was a typical medium-sized, chain-forming species, with each cell about 25 µm wide by 10-20 um long. Chaetoceros sp. (AS-1A) was a small (10 µm long by 5 um wide) non-chain-forming Chaetoceros, of a type often found in the outdoor experiments (though they never made up a large fraction of the total Chaetoceros abundance).

a. Effect of pCu on Growth Rates

The growth response of Thalassiosira sp. (T-1) to pCu in modified f/2 media made up at different TRIS and FeEDTA concentrations was examined in three experiments (Fig. 17). In all of these experiments the pCu value at which EC50 was reached was 9.7 $\pm$ 0.1, with no apparent alteration due to different concentrations of TRIS within the range 1-10 mM, or FeEDTA within the range 1.1-10 µM. Similarly, clones Chaetoceros constrictus (CHS-1) and Chaetoceros sp. (AS-1A) showed EC50 pCu values of 9.7 and 9.6, respectively, with no apparent shift at different concentrations of TRIS for clone CHS-1 (Fig. 18a) and only a slight suggestion of a shift in EC50 with TRIS concentration for clone AS-1A (Fig. 18b). This suggestion of a difference is less than the difference that would be caused by direct toxicity of the Cu-TRIS complex since calculated Cu-Tris concentrations at the EC50 in the three different TRIS series vary over a factor of one hundred, while the activity of cupric ion may vary by a factor of three.

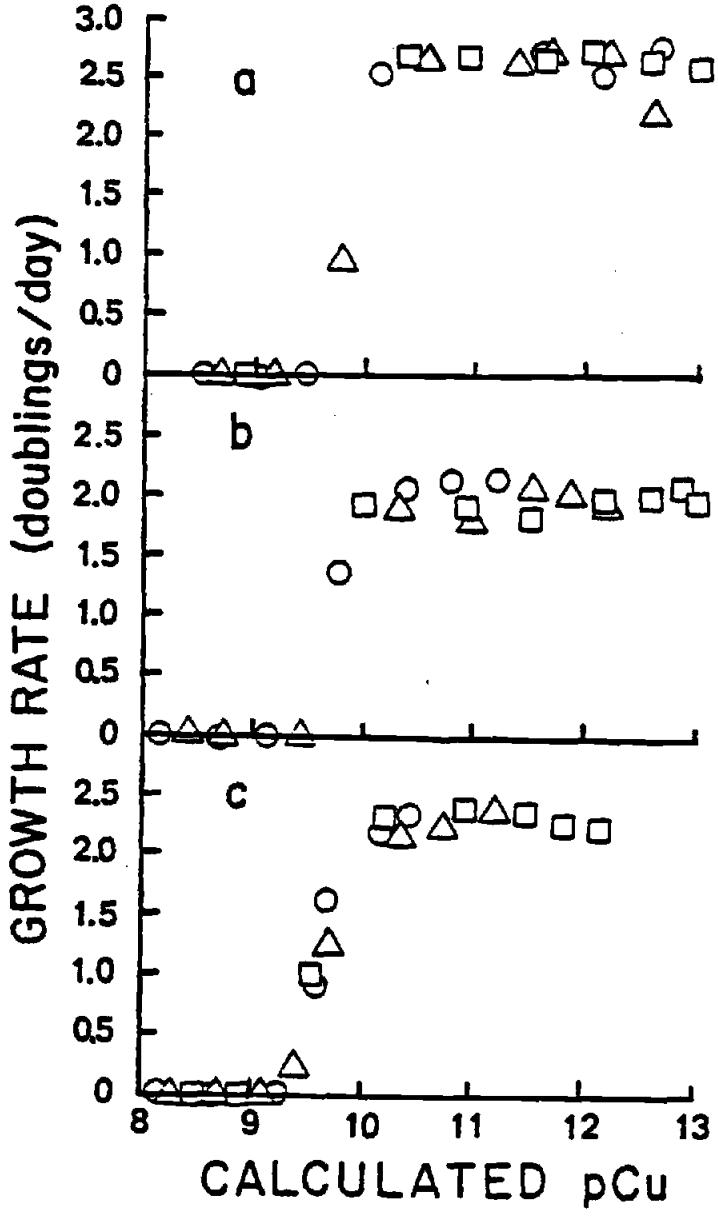

Fig. 17. The effect of pCu on the growth rate of Thalassiosira sp. (T-1) in various combinations of TRIS-EDTA buffered media.
A) O - 1 mM TRIS, □ - 5 mM TRIS, △ - 10 mM TRIS; all 1.1 µM FeEDTA
B) O - 1 mM TRIS, □ - 5 mM TRIS, △ - 10 mM TRIS; all 5.5 µM FeEDTA
C) O - 1 mM TRIS, □ - 3 mM TRIS, △ - 10 mM TRIS; all 10 µM FeEDTA

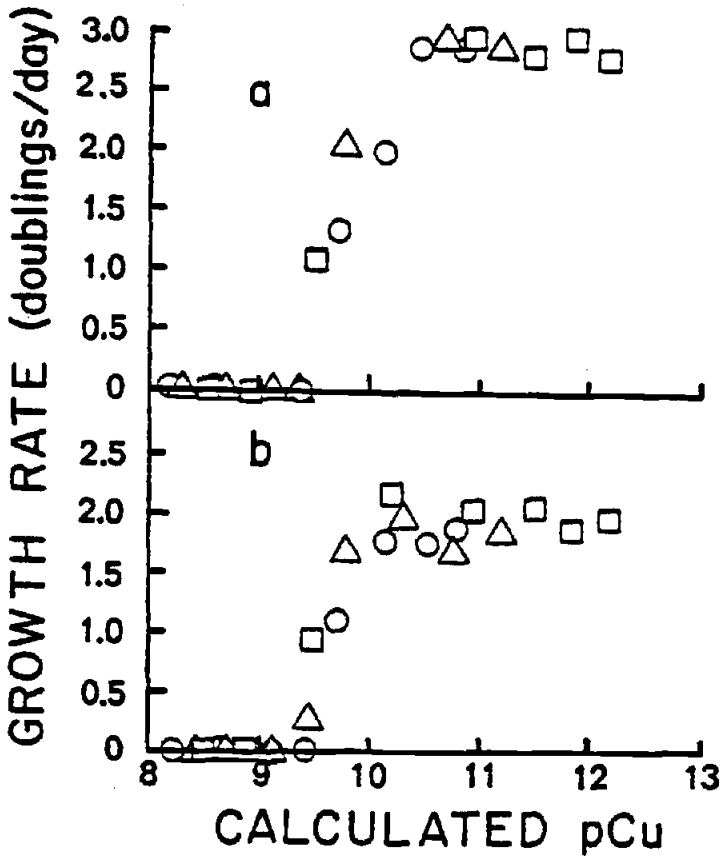

Fig. 18 The effect of pCu on growth rate of Chaetoceros constrictus (CHS-1) (a) and Chaetoceros sp. (AS-1A) (b).
○ - 1 mM TRIS, □ - 3 mM TRIS, △ - 10 mM TRIS; all 1.0 μM FeEDTA

b. Effect of pCu on Cell Copper Concentrations

Samples for measuring Cu in the cells were taken in the final Thalassiosira sp. (T-1) experiment, and in the experiment with Chaetoceros sp. (AS-1A). Because of the small culture containers and the loss of volume due to sampling, and because of the relatively high blanks for Cu in Nuclepore filters, particulate Cu values could not be determined in some of the highest pCu treatments. In addition, in cultures where the growth rate was substantially reduced by Cu stress, Cu/f did not follow the essentially linear relationship between Cu/f and pCu in the non-inhibited cultures. For plotting and statistical purposes, the pCu corresponding to each Cu/f value has been calculated with the pH measured in that culture on the day of harvesting, since the relatively high biomass had often significantly raised the pH from its initial value by depletion of CO_2.

For Thalassiosira sp. (T-1), the relationship between pCu and Cu/f was log-linear within the range of pCu values from 9.7 to 11.3 (Fig. 19a). Within this range the relationship was:

$$-\log Cu/f = 1.022\ pCu - 1.8426$$

The correlation coefficient was 0.996. Furthermore, cultures with different TRIS concentrations were not significantly different. Chaetoceros sp. (AS-1A) did not produce exactly the same result (Fig. 19b). While there was an increase of Cu/f with decreasing pCu values (i.e. an increase in cellular Cu with increasing Cu ion activities), the higher TRIS concentrations had significantly higher Cu/f values at

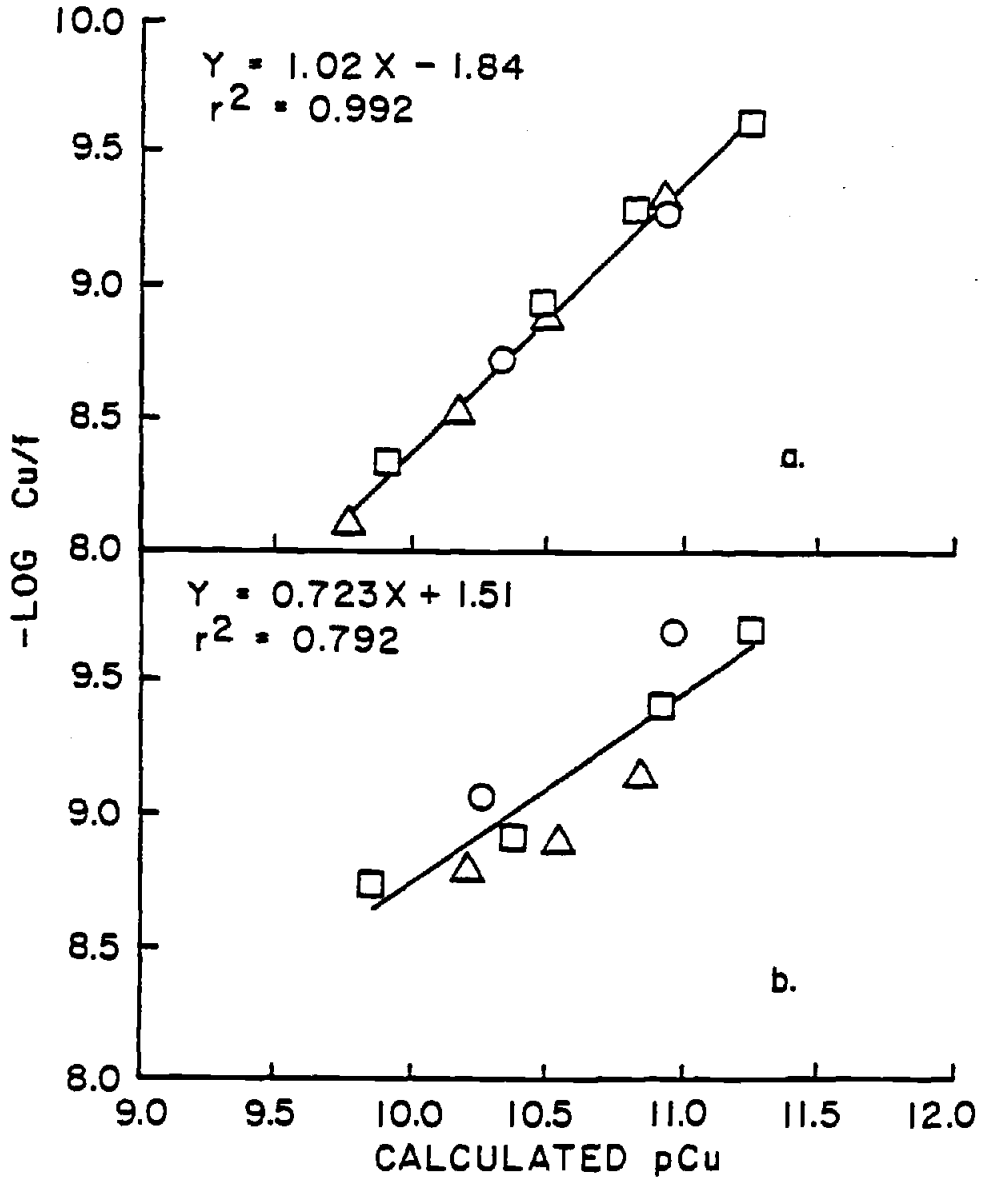

Fig. 19. The effect of pCu on cellular Cu. a) Thalassiosira sp. (T-1). b) Chaetoceros sp. (AS-1A).
O - 1 mM TRIS, □ - 3 mM TRIS, △ - 10 mM TRIS

comparable pCu values ($p < 0.01$). For a fixed pCu, Cu/f varied by only a factor of three, while the calculated concentration of the Cu-TRIS complex varied by a factor of over one hundred (i.e. about 100 times as much Cu is required to produce the same pCu in 10 mM TRIs medium as in 1 mM TRIS medium). It will be remembered that the growth rate results for clone AS-1A also showed a slight trend toward higher toxicity in treatments of higher TRIS (Fig. 18b). Both growth rate and Cu/f results in this experiment may reflect the presence of a small concentration of a chelator unaccounted for in the calculation of pCu.

c. Summary

The above results for both growth rate and cell Cu content appeared to answer the questions raised at the beginning of this section. First, in these enriched cultures, Cu toxicity was not related to the concentrations of the Cu-TRIS complex to any extent, but was rather closely related to calculated pCu. Given that all species chosen to reflect the typical species found in the outdoor experiments were found to respond in this way, it seemed quite unlikely that the majority of the phytoplankton in the outdoor enrichments responded to the Cu-TRIS complex as if it were toxic.

On the other hand, the laboratory experiments did tend to indicate that the typical Cu resistance of coastal diatoms was considerably less than that of T. pseudonana (3H). The pCu-EC50 values found for Thalassiosira sp. (T-1), Chaetoceros constrictus (CHS-1) and Chaetoceros sp. (AS-1A) were, in fact, rather similar to the values found in the 1mM TRIS series of Exp. III (pCu =10.1)

(Fig. 9c) and in the 1 and 3 mM TRIS series of Exp. V, with added Mn (pCu = 9.5 and 9.8, respectively) (Fig. 14b,d)

2. Effect of Trace Metal Limitation on Copper Toxicity.

Although it seemed likely that the toxicity of Cu to most coastal diatoms was regulated by pCu and not total Cu or Cu-TRIS, the results of Exp. III had indicated that higher TRIS concentrations produced EC50 at higher pCu values (Fig. 9b,c). One difference between the outdoor enrichment cultures and the laboratory cultures was in the trace metal levels other than Cu. No metals were added in the outdoor experiments until Exp. V, but an f/10 concentration of metals was added to the laboratory cultures. Thus it was reasonable to hypothesize that TRIS in the outdoor experiments reduced the availability of other required trace metals to the point that Cu ion began to act more toxic at a given pCu. Higher levels of TRIS would lead to greater sequestering of the nutritive metal, and lead to greater apparent toxicity of Cu.

Accordingly, an experiment was performed to test the above hypothesis. Seawater was stripped of its ambient trace metals with Chelex-100 resin, and only 1 µM Fe was added back. The necessity for Fe is well established, and failure to include it would certainly have resulted in little or no growth. Iron additions between 1 and 10 uM were already shown to have no effect on the pCu-EC50 values at different TRIS concentrations for Thalassiosira sp. (T-1) (Fig. 17). Thus, Thalassiosira sp. (T-1) was used as the assay organism in the next experiment, and its growth rate was examined under different pCu and TRIS levels. This experiment showed what was anticipated; that in

very low concentrations of trace metals, increasingly higher EC50-pCu values resulted from increasingly higher concentrations of TRIS (Fig. 20). In 1 mM TRIS, the EC50 value was about 10.3, in 3 mM TRIS, the value was about 10.6, and in 10 mM TRIS, the value was about 11.6. On this basis it was determined that some trace element removable by Chelex-100 was responsible for the effect of TRIS concentration observed in this experiment, and most probably in outdoor Exp. III as well. It is important to note that the low metal concentration was not, by itself, a growth-limiting effect, because at the highest pCu levels in this experiment Thalassiosira sp. (T-1) grew about as well as it had in any experiment with metals added. Therefore, the relationship between the unknown metal(s), Cu and TRIS could more properly be called an interaction: higher trace metal levels (within a certain range) combined with lower TRIS concentrations, raise the concentration of ionic Cu necessary for Cu toxicity to be shown, and vice versa.

3. Identification of the Interacting Element

To determine which metal (or metals) was responsible, in its absence, for the increased sensitivity of Thalassiosira sp. (T-1) to Cu, another experiment was performed. Metal-free medium was made as before, Fe was re-added, and a series of 10 mM TRIS-Cu ion activity buffers was made in the medium. This series was then split into four series of flasks and given different trace metal treatments. One treatment consisted of no addition, to test the efficacy of the metal-stripping procedure. Two other treatments were the addition of f/10 levels of Mn and Zn (182 and 1.4 nM, respectively). The fourth

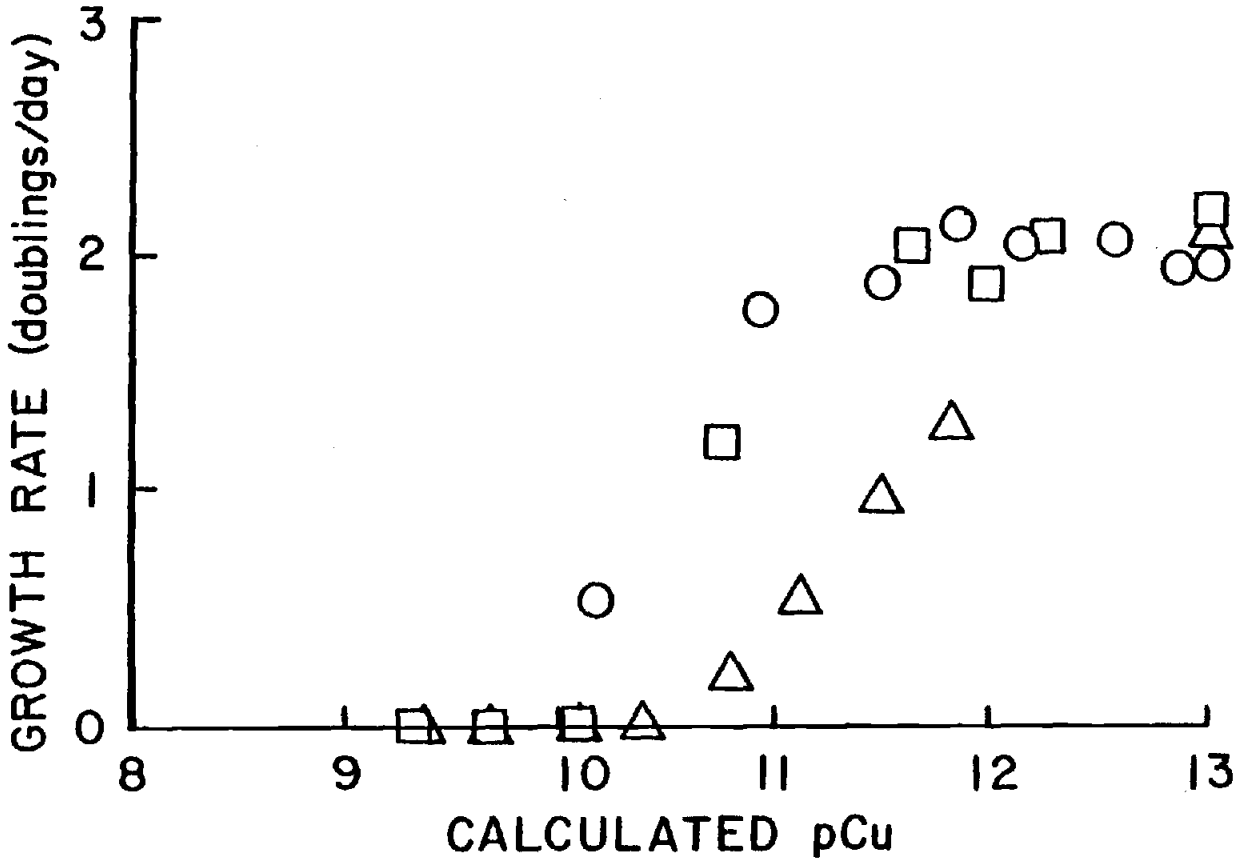

Fig. 20. The effect of metal deficiency on the growth rate of Thalassiosira sp. (T-1) as a function of pCu and TRIS concentration. ○ - 1 mM TRIS, □ - 3 mM TRIS, △- 10 mM TRIS

treatment was the addition of the complete f/10 metal suite, to verify the restoration of "normal" Cu sensitivity. These flasks were inoculated with Thalassiosira sp. (T-1). The complete experiment was repeated, with slightly altered pCu levels.

Manganese and zinc were singled out for study on the following basis. Both are cationic in seawater, and easily removed by Chelex-100 (unlike molybdenum, for example). Cobalt is also partially cationic in seawater, and extractable by Chelex-100, but many phytoplankton readily utilize Co in the form of vitamin B-12, which is not extractable by Chelex-100, and is added in large amounts in the f/10 vitamin mix. Other extractable metals (e.g., Ni, Cd and Pb) are not thought to be essential micronutrients for phytoplankton, and thus were not considered likely candidates for strong interactive effects with Cu at very low concentrations.

The experiments showed that Mn was the primary element in the restoration of "normal" Cu sensitivity (Fig. 21). In the first experiment (Fig. 21a) the Mn addition caused a shift in the EC50-pCu from about 10.7 in the no-metal-added control to 9.9, almost that of the EC50 value in the treatment with complete f/10 metals (EC50 value at pCu 9.6) (Fig. 17a). Previously determined EC50-pCu values for this diatom in fully enriched media averaged 9.7 $\pm$ 0.1 (Fig. 17a,b,c). The addition of Zn by itself appeared actually to increase the Cu sensitivity of the cells (EC50-pCu = 11.5), so that the growth rate at any given pCu level was actually less than that in the "stripped" water with no metal additions (EC50-pCu = 10.7). In the repeat of this experiment (Fig. 21b), Mn was again the most restorative single treatment (EC50-pCu = 10.2), though apparently not to the same degree

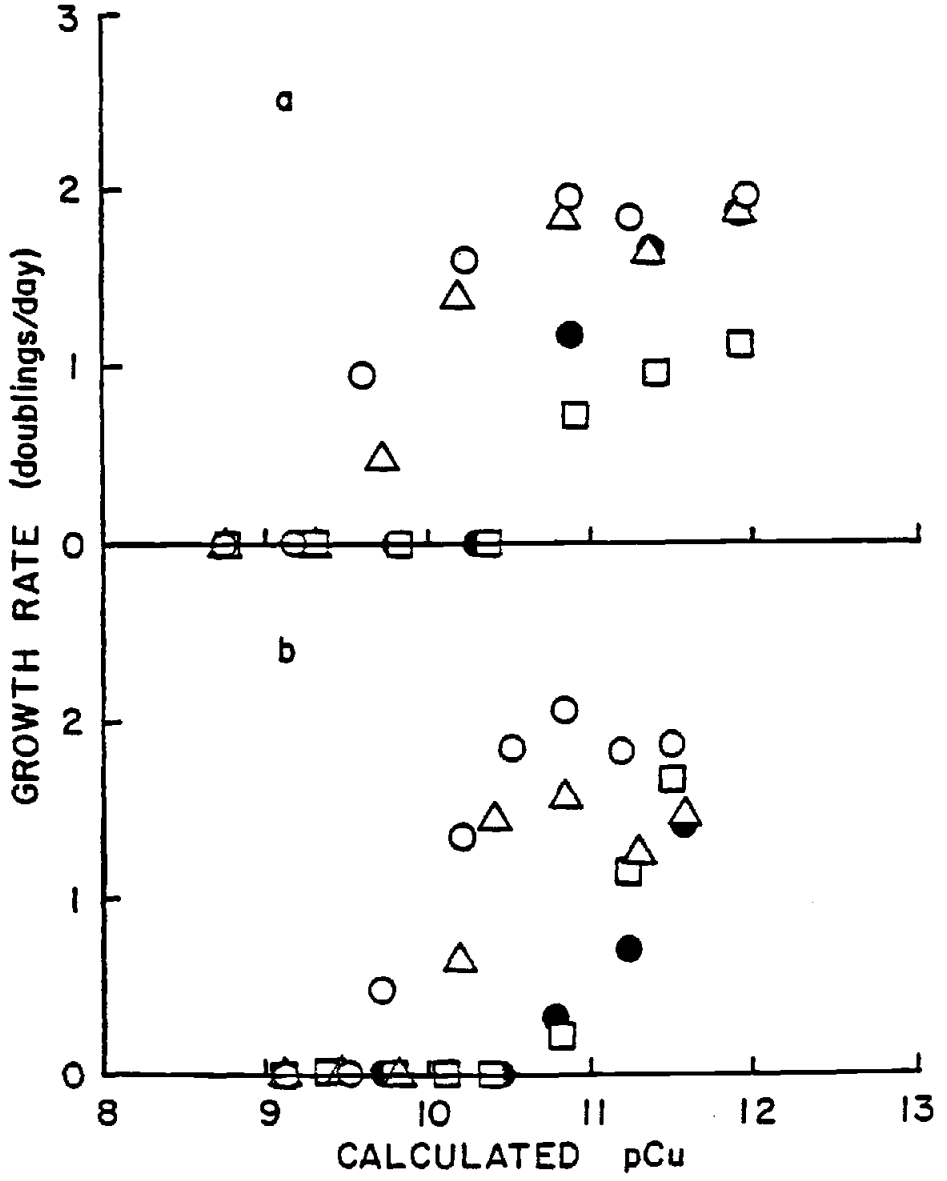

Fig. 21. The effect of metal additions to growth rate of Thalassiosira sp. (T-1) as a function of pCu in metal deficient medium. All 10 mM TRIS - 1 μM FeEDTA.
● - no metal addition, □ - f/10 Zn addition
△ - f/10 Mn addition, ○ - f/10 Mn, Zn, Co and Mo addition

as in the first experiment. The EC50-pCu value of the full f/10 addition in the repeat experiment was 9.9. Zinc had little or no restorative effect in the repeat experiment (EC50-pCu = 11.3). The treatment without metal additions also had an EC50-pCu value of 11.3.

4. Effect of Manganese Concentrations on Copper Sensitivity

In the experiments described above, the effects of Mn on Cu sensitivity were determined in response to the absence of Mn (less than 1 nM) and the presence of Mn at f/10 levels (182 nM). The concentrations actually required to restore "normal" Cu sensitivity could have fallen anywhere within this range. Further, it was anticipated that at low pCu values, and/or at higher TRIS concentrations higher Mn concentrations might be required for the restorative effect. A manganese-addition experiment was undertaken to investigate more fully the effect of Mn on Cu sensitivity of Thalassiosira sp. clone (T-1).

To simplify the experiment, I decided to work with only 10 mM TRIS buffered medium, since the effect of low Mn on Cu sensitivity was most extreme at high TRIS concentrations. Two different pCu values were chosen at which to examine the effect of added Mn on growth rate on Thalassiosira sp. (T-1). The first pCu was 9.9. Previous experiments had shown consistently that this pCu was close to the point at which Cu sensitivity began for clone (T-1) enriched to f/10 metal concentrations. The second pCu value chosen for examination was 10.6. At this pCu Thalassiosira sp. (T-1) had consistently shown good growth in 10 mM TRIS with f/10 metals, but poor or no growth when Mn had been left out of the medium.

The experiment was performed in modified AQUIL medium (Morel et al., 1978), so that equilibrium calculations of the Cu and Mn speciation could be carried out via a computer model; however, as shall be detailed later, equilibrium modeling proved to be fruitless, due to an unforseen chemical reaction. The manganese additions used in the experiment were 3, 10, 30, 100, 300 and 1000 nM, which covered the range of Mn available in all the previous outdoor experiments (Table 6).

In the series with pCu = 10.6, growth was good and about equal, in both 1000 and 300nM Mn additions; however, only one of the cultures with the 100 nM additions grew (Fig. 22). The 1000 and 300 nM additions in the pCu = 9.9 series allowed growth at lower rates than in the pCu = 10.6 series, and there was no growth at 100 nM Mn. At pCu = 9.9 some inhibition was expected even at high Mn enrichments, based on previous experiments. From Fig. 22 it can be seen that the f/10 (182 nM) Mn addition in previous experiments was apparently just sufficient to produce "normal" Cu sensitivity in Thalassiosira sp. (T-1), at the highest TRIS concentration tested, 10 mM.

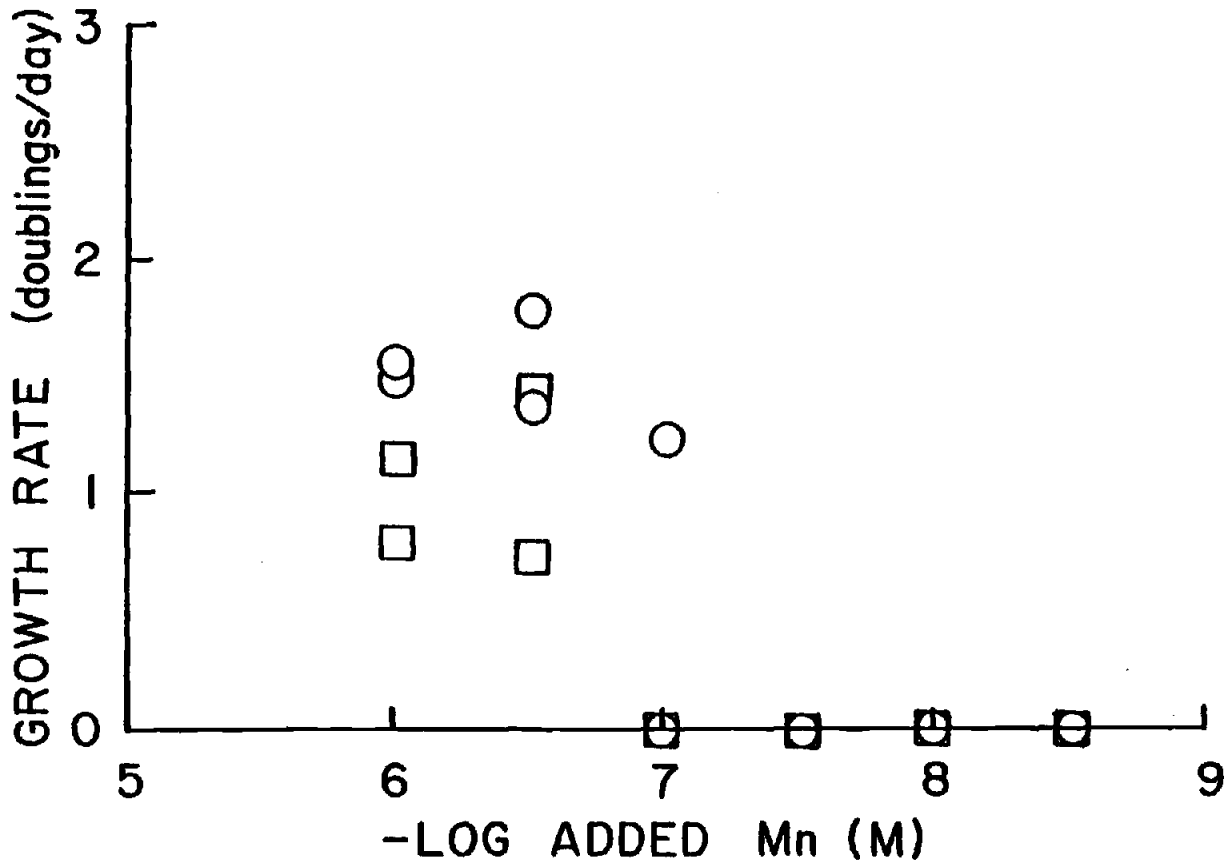

Fig. 22. The effect of Mn concentration on growth rate of Thalassiosira sp. (T-1) at two potentially inhibitory pCu values in modified AQUIL containing 10 mM TRIS.
○ - pCu 10.6, □ - pCu 9.9

V. DISCUSSION

A. Growth Rate Inhibition of Phytoplankton by Copper

1. Inhibition as a Function of pCu

The inhibition of natural coastal phytoplanktonic assemblages by Cu, in the presence of normal values of other trace metals, was usually severe at pCu values less than 9.5, based on outdoor enrichment experiments, and supported by the laboratory measurements on individual clones of representative diatoms. This pCu corresponds to a concentration of approximately 15 nM total Cu, assuming only inorganic speciation of Cu (Table 3). Some series within experiments showed EC50 at pCu values as high as 10.5, but these higher values were at least partially explicable as a result of an Mn-Cu-TRIS interaction; that is, when the Mn concentration was low and the TRIS concentration high, the pCu at which toxicity became apparent was high.

Because of the Mn-Cu-TRIS interaction, the EC50 values of 9.5 to 10 at the lower TRIS concentrations of Exps. III and V are better estimates of the EC50 value for Cu ion activity in a "natural" setting; i.e. seawater without heavy concentrations of TRIS buffer. The EC50-pCu values for these mixed-phytoplankton experiments agreed well with the EC50 values for selected species in enriched media in a wide range of TRIS concentrations. Outdoor experiments with TRIS concentrations higher than 1 mM showed higher EC50-pCu values. In Exp. V, the addition of Mn lowered the EC50-pCu value even in the 1 mM TRIS treatment; however, the seawater for this experiment had been

especially collected farther at sea so as to be of lower initial Mn (Table 6), and thus increase the magnitude of the Mn-Cu-TRIS interaction. In outdoor Exps. I, II and III, the Mn initially present was less than the concentration required (>100 nM Mn approximately) to fully reverse the Mn-Cu interaction in the laboratory experiments with Thalassiosira sp. (T-1) in 10 mM TRIS, however, reversal was probably largely complete at the lower TRIS levels. The high levels of Mn added to half of the treatments of Exp. V should have completely restored the Mn sufficient EC50-pCu value.

The results of previous studies on the toxicity of Cu ion to phytoplankton have shown a wide range of Cu susceptibilities. The initial Cu-TRIS pCu results of Sunda and Guillard (1976) showed that Thalassiosira pseudonana (3H) had essentially a two-step pCu inhibition curve; that is, its growth rate was reduced by 25% at about pCu 10.5, but growth continued up to 50% of the maximum rate at pCu 8.5. Therefore, in terms of retention of a species in a high Cu stress environment (given adequate Mn), Thalassiosira pseudonana (3H) has a considerable advantage over the natural populations I studied. Morel et al. (1978) showed that Skeletonema costatum (Skel) would also grow at pCu values as low as 8.5, given optimal conditions (in this case silicic acid concentrations lower than 12.5 uM led to Cu inhibition at higher pCu values). Gavis et al. (1981) examined the response of 24 clones of 11 species of algae, mostly diatoms, versus pCu in 10 mM TRIS buffers, and found that only four could survive at pCu = 8.5. The four exceptional clones were T. pseudonana (3H), Skeletonema costatum (Skel), Phaeodactylum tricornutum (Phaeo) and Nannochloris atomis (GSB Nanno). These clones are often used for

phytoplankton physiological research, probably due to their tolerance to a wide variety of conditions. All had been isolated from chronically polluted environments. With these four clones excepted, the modal value for EC50 of the remaining 20 clones was pCu = 9.7, with a range from 8.9 to 10.4. The results of my experiments, both field and laboratory, agreed with these results.

Reuter et al. (1979) used 1 mM TRIS buffered artificial seawater to determine the effect of varying Cu ion activities on C-14 fixation of freshly collected colonies of Oscillatoria theibautii, an oceanic cyanobacterium. Healthy subsurface colonies were 50% inhibited at pCu = 9.3, while less healthy surface colonies were 50% inhibited at pCu = 10.0. The results of short-term C-14 incubation experiments may not reflect population growth or maintainence over longer time scales. For example, in many of my laboratory experiments, I observed that growth continued for one or more days at reduced rates before growth ceased, especially at the more marginal toxic levels.

Because of the failure of "mixed" phytoplanktonic algae to grow in pCu values lower than 9.5 in my outdoor experiments, any resistant phytoplankton (such as the four clones of Gavis et al., 1981) must be extremely rare in Oregon coastal waters. If any significant fraction of resistant cells had been present in the inocula of my experiments, there would have been growth at lower pCu values. Adaptations that make cells Cu resistant may also tend to make them less competitive under less stressful conditions. It is also possible, given the relatively pristine nature of the Oregon coastal zone, that there simply are fewer pollution sources to initiate development of resistant strains. Copper toxicity bioassays in a more industrialized

area, such as the east coast of the United States, might show greater incidence of Cu resistant phytoplankton.

2. Inhibition as a Function of Added Total Copper

Reviewing the results of the ionic Cu additions in Exps. I - V (Fig. 8), the concentrations of uncomplexed Cu required to reach EC50 for the phytoplanktonic community were varied between 50-130 nM (Table 8). In Exp. V the addition of 1000 nM Mn to relatively low-Mn water raised the amount of Cu necessary to reach EC50 for the dominant community from about 50 to 100 nM.

Only a few other studies have examined the effects of added Cu on natural marine phytoplankton assemblages. The culture conditions used and the criteria for Cu toxicity vary considerably, so that direct comparisons between different investigators must be made with care. One of the earliest and most extensive studies of the effects of Cu on natural phytoplankton was the Controlled Ecosystem Pollution Experiment (CEPEX). Two experiments were performed in which large natural water columns were enclosed, copper at various levels was added, and various biological parameters were monitored for 28 days. For the CEPEX Cu-addition experiments 38 m^3 water columns were enclosed and 0, 5, and 10 µg/l Cu (0, 78.7, and 158 nM Cu) were added in one experiment, and 0, 10, and 50 µg/l Cu (0, 158 and 787 nM Cu) were added in the second experiment. Cu concentrations were maintained in the CEPEX by measurement and addition of more Cu, except for the 50 µg/l treatment in the second experiment. This treatment declined to 23 µg/l by the end of the experiment. The water columns already had well developed phytoplankton populations at the time of

enclosure, and both controls (no Cu additions) and Cu treatments showed declines in biomass right after enclosure. Thus, the effect of Cu on growth rates could not be determined in these experiments. The effect of Cu on the mixed assemblages was judged by the extent of the biomass decline, by the lag before biomass increase resumed, and by the standing stock finally attained. The 78.7 and 158 nM Cu additions had relatively little initial effect, with somewhat longer times required to restore the biomass to previous levels. The final standing stocks were actually increased in the Cu treatments (Thomas et al., 1977a). The 354 and 787 nM Cu treatments in the second experiment showed greater initial biomass declines, long lag phases, slow recovery, and decreases in the final standing stock (Thomas et al., 1977b).

One basic difference in concept between the CEPEX experiments and my own concerns the initial conditions of the phytoplankton. In the CEPEX experiments the water columns already had well developed phytoplankton and zooplankton populations at the time of enclosure. The CEPEX enclosures themselves appear to have exerted an initial negative effect on the phytoplankton populations enclosed. The effect of Cu on these populations was observable as an additional burden on an already declining community. In my experiments, small inocula of phytoplankton were placed in water free of other phytoplankton and grazers. While an "enclosure effect" may have existed, eliminating species not suited for the enclosure, the predominant effect of enclosure in my experiments was, in essence, to free the phytoplankton from the effects of competition and grazing and allow growth to proceed at some rate allowed by the combined effects of the ambient

light-temperature-nutrient regime, and the Cu treatments.

In my experiments, Cu additions of up to 30 nM allowed normal growth rates and biomass of the total phytoplankton assemblage (Fig. 8). In the CEPEX studies, all the treatments other than no Cu additions were higher than this, and all resulted in faster biomass declines than in the controls. Thus, the results of the CEPEX experiments and my own experiments are essentially in agreement to the extent that they can be compared.

At the time of the CEPEX experiments the effects of Mn and other trace metals on Cu toxicity were not appreciated, and little consideration was given to the concentration of other trace elements in the enclosures. Therefore, we do not know how trace metal-trace metal interactions may have influenced the CEPEX results. Since Saanich Inlet, British Columbia (where the CEPEX Cu experiments were carried out) is a coastal inlet in the Pacific Northwest, with rather heavy freshwater influence in its surface waters, a reasonable guess is that Mn, Fe and Zn concentrations were relatively high, and that the Cu experiments probably showed results reflective of overall trace metal sufficiency.

Sunda et al. (1981) performed long term Cu bioassays on systems designed to represent upwelled water (water from deeper than 800 m was inoculated with surface water bearing phytoplankton populations). The water was such that phytoplankton growth rates could be substantially enhanced initially by additions of 10 nM Mn, 1 μM EDTA, or 10 μM $FeCl_3$. Addition of 10 nM Cu caused growth rate inhibition only in the absence of Mn, Fe, or EDTA additions. At 50 nM Cu, inhibition was only partly reversed by 100 nM Mn, and at 100 nM Cu, the inhibition

was even more marked for all but the 10 uM $FeCl_3$ and 1 uM EDTA treatments. These results are also in general accord with my experiments.

Rueter et al. (1979), in addition to the experiments with TRIS buffered artificial seawater discussed in the previous section, also tested the toxicity of added unchelated Cu to Oscillatoria theibauti in natural seawater. Concentrations of added Cu from 10 to 100 nM caused slight but significant lowering of C-14 fixation to about 80% of the controls. This result was not appreciably different from mine in which mixed populations were assesed by long-term growth response. However 50% inhibition occurred at about 300 nM added Cu, yielding an EC50 (determined using C-14 uptake) 2-6 times higher Cu concentrations than in my outdoor experiments (Table 8). Fitzwater et al. (1982) studied the effect of Cu additions on C-14 uptake to phytoplankton populations from oceanic environments. Concentrations of Cu as low as 10 ng/l (0.158 nM) showed noticeable inhibition, with 50% inhibition at about 100 ng/l (1.58 nM). The low concentrations at which Cu was found to be inhibitory in these oceanic environments may reflect the species composition of the phytoplankton, the low concentrations of required trace metals, and other nutrients in the open ocean, or problems of comparing short-term C-14 uptake experiments to long-term growth experiments.

In both of the previous two examples, Cu toxicity has been evaluated using short-term C-14 uptake. This method is inherently different from using the response determined by growth rate experiments over longer periods. First, Cu toxicity may take effect over longer times than the incubation times of C-14 experiments, as is

almost certainly indicated by the continuation of growth of cells for up to 3 days after transfer to slightly toxic Cu-TRIS buffered (Sunda, 1975; and personal observation). Second, while Cu may inhibit a large fraction of the total phytoplankton in a short term C-14 experiment, the surviving resistant phytoplankton may soon restore the biomass through rapid growth, for example when green flagellates replaced Chaetoceros in my Exp. V cultures containing 10 mM TRIS and no added Mn. Finally, additions of unchelated Cu to seawater with kinetically slow chelators may result in a rapid initial Cu uptake and inhibition of phytoplankton before an equilibrium is established between the chelator and Cu. Such an effect has been noted, for example, between the artificial chelator EDTA, Cu and the dinoflagellate Gonyaulax tamarensis (Anderson et al., 1978).

B. Bioassay Estimates of Cupric Free Ion Activity in Seawater

A traditional problem in trace metal-phytoplankton research has been that some unknown fraction of the total analytical metal is biologically active. While certain analytical techniques can determine that some fraction of a metal is bound, no practical technique has yet been devised which can certainly separate all inorganic forms of a metal from all organic forms. Furthermore, no analytical technique has been devised which can determine if a given form, inorganic or organic, is biologically active.

The use of heavily chelated media with known ion activity has demonstrated, at least for a few anthropogenic organic compounds, that the responses of phytoplankton to metals are functions of the free ion activities of the metals. Unfortunately, analytical chemists have

been unable to determine the free ion activity of Cu or most trace metals in natural seawater, given the analytical concentration of the metal. Presently, the most that analytical chemistry can do is estimate an upper limit on the free ion activity, based on analytical concentrations, calculated inorganic speciation models, and perhaps measured concentrations of certain types of metal-organic complexes.

The analytical chemistry problem suggests the use of phytoplankton (or other biota) in a method to estimate the free ion activity of natural seawater. By comparing an effect of a metal at known free ion activities to the effect produced by metal additions to otherwise untreated seawater, the metal additions can be tentatively assigned free ion activities. Two biological attributes that would seem appropriate for such an assay of effects with Cu, are phytoplankton growth rate inhibition and cell Cu concentration.

This proposed technique is not without theoretical and practical pitfalls. Theoretically, and in a few proven instances in real life, metal-organics may be biologically active (Guy et al., 1979). Such metal complexes, if present in natural seawater, would be determined as free ion activity by the bioassay technique above. Possibly more importantly, it has never been prove that in seawater without both added chelators and higher-than-natural metal concentrations, biological attributes are uniquely affected by free ion activity in the same way that they are in media with high concentrations of metals and artificial chelators. For example, it is possible 1) that in metal buffered media, organically complexed metals (which are in high concentration) enter cells readily, allowing the inside of the cell to take on the free ion activity of the external medium; and 2) that the

effect of Cu in that cell is determined by the free ion activity inside the cell. By contrast, in a natural seawater solution, the low concentration of metal-organic complex might not support the rate of diffusion of metal into the cell necessary to produce the same free ion activity in the cell's interior as in the exterior seawater, particularly when cellular "dilution" of the metal is occurring as a result of cell growth and division. In this model, which is plausible but not proven, the free ion activity as estimated by the proposed bioassay could underestimate the true "thermodynamic" free ion activity and give rise to false ideas of the extent of complexation.

From a practical point of view, one problem with this proposed bioassay is the uncertainty that the metal added without heavy chelation remains in near-constant concentration. Possible contamination, adsorption to container surfaces, adsorption to non-biological suspended particulates, and biological uptake could all alter the concentration of metal. However, these problems can be ameliorated by clean techniques, selection of less adsorptive containers, containers with a large surface area to volume ratio, filtration of seawater to eliminate endogenous suspended materials and selection of media to minimize inorganic precipitation of new particulates, synoptic sampling of the seawater for soluble trace metals, and sampling at low biomass.

With these caveats aside, we can now proceed to consider a biological measurement which might be called "Biological Equivalent Metal Ion Activity" (BEMIA). This measurement is the numerical free metal ion activity in complexed solutions that produce equivalent biological effects as the seawater in question.

This approach was first used by Rueter et al. (1979) in their study of Oscillatoria theibautii. Noting that the first detectable growth rate decline (of 15%) below control levels occurred at pCu 12, they concluded that the maximum background activity of Cu in Sargasso Sea surface water was less than 10^{-12} M. Using 2 nM as the value for total Cu in surface Sargasso seawater (Bender and Gagner, 1976), the activity-to-concentration ratio for Sargasso seawater was less than 5×10^{-4} at ambient Cu concentrations. Since inorganic models for the complexation of Cu in seawater predict that the ratio of activity to concentration in the absence of organics should be about 1.5×10^{-2}, considerable complexation of Cu is implied. However, the reported 15% growth rate reduction overlapped the controls, and was not statistically significant at the 95% level; and hence, the calculation should not be regarded as very precise.

However, the data of Rueter et al. (1979) offer another opportunity for a calculation of the activity of Cu, using a much more distinctive point of equality of effect between the effects of pCu in TRIS buffers and those with added uncomplexed Cu. Addition of about 300 nM Cu to untreated seawater, and pCu = 9.3 in 1mM TRIS buffer both resulted in 50% inhibition of photosynthetic oxygen production by Oscillatoria theibautii. Assuming that the two treatments have the same pCu implies an activity-to-concentration ratio of 1.6×10^{-3}, about tenfold below that predicted for seawater with only inorganic complexation. The 1.6×10^{-3} ratio is greater than, but fairly close to the estimate using the 15% growth rate reduction (5×10^{-4}). However, it is different in the direction expected upon raising the total Cu levels in the presence of small concentrations of

chelators.

In my study, Exps. II, III, and V offer similar opportunities to estimate the activity-to-concentration ratios at the point where both the TRIS buffered pCu series and unchelated Cu additions inhibit the phytoplankton growth rate 50%. Two additional problems arise in these comparisons. First, treatment series with and without TRIS buffers frequently showed different patterns of taxonomic composition at the points of comparison (e.g. Exp II and III, Fig. 12 and Fig. 13), and thus the populations whose response are being equated are not strictly comparable. Second, the exacerbation of Mn limitation by TRIS may alter the effect of Cu-free ion activity to the TRIS treatment series but not the ionic additions series. In Exp. V, however, both problems are minimized, the first by the fortunate (for this comparison) dominance of the entire experiment by Chaetoceros, and the second by the addition of high concentrations of Mn.

In Exp. II, the EC50-pCu in the 5 mM TRIS-buffered series was 11.1 (Fig. 9a), while the EC50 for added unchelated Cu was about 5.6×10^{-8} M total Cu (Fig. 8b). This yields an activity to concentration ratio of 1.4×10^{-4}. However, as previously discussed, the use of 5 mM TRIS buffers without Mn additions may lead to an overestimate of the pCu i.e., an underestimate of the Cu free ion activity at which phytoplankton would respond to Cu in the water as toxic, compared to a non-TRIS treatment. Thus, an overestimate of the amount of complexation is possible.

In Exp. III, two strengths of TRIS buffer were used, 1 and 5 mM. These yielded EC50 values of pCu 10.1 and 10.6, respectively (Fig. 9b). In the series with uncomplexed Cu additions, EC50 occurred at

about 1.3×10^{-7} M (Fig. 8c). Activity-to-concentration ratios of 6.3×10^{-4} and 2.0×10^{-4} were calculated from the 1 and 5 mM TRIS series, respectively. As discussed previously, the Cu-Mn-TRIS interaction may affect both results, but the 5 mM series would be more severly affected than the 1 mM series. The difference between the activity-to-concentration ratio for the 1 and 5 mM TRIS series is an indication of the sensitivity of the BEMIA technique to any artifacts which may affect the response of the phytoplankton to Cu free ion activity in highly chelated media.

Exp. V had two components (one group of treatments with, and one without, 1000 nM added Mn) to examine the effect of the Cu-Mn-TRIS interaction. For this discussion I will consider only those treatments with Mn added since the addition of Mn did act to restore both the EC50-pCu and the EC50-added Cu relationships to Mn saturation, and thus makes them more comparable. The EC50 for added Cu with added Mn was approximately 10^{-7} M (Fig. 8e). For the 1 mM TRIS series with added Mn, the EC50 was at pCu = 9.5 (Fig. 10b); and in the 3 mM series with added Mn at pCu = 9.8 (Fig. 10d). These results were close to the resolution of EC50 given the treatment spacing and thus average, yielding an overall EC50-pCu of 9.65. This implies an activity to concentration ratio of between 1.6 and 3.1×10^{-3}. This is somewhat less than either Exps. II or III. Part of the difference was undoubtedly the result of eliminating the Mn-Cu-TRIS interaction with a Mn addition, but part might result from the fact that the water for this experiment was collected farther at sea and could well have had a lower Cu complexing capacity to begin with. In fact, the results from this experiment are more similar to

the results of Rueter et al. (1979) in the Sargasso Sea, than to my own previous experiments using water from Yaquina Bay.

An inherent limitation of the BEMIA technique, as others and I have applied it so far, is that it can only be used at Cu concentrations and pCu values that cause significant decreases of phytoplankton growth rates or photosynthesis. As we have seen, however, Cu additions of up to 10 times ambient concentrations may cause insignificant effects. To determine the activity of Cu in seawater at Cu concentrations lower than those which give rise to inhibitory effects, we need another biological feature closely correlated with activity in solutions of known activity.

The indicator for activity that I propose is the cell Cu content. Sunda (1975) and this study have both shown that the cell Cu content was almost totally a linear function of pCu, even at activities where the effects of Cu on growth rate were insignificant or nonexistent. Likewise in this study, uncomplexed Cu added to seawater caused increases in the cell Cu content when no change in growth rate was seen.

In Exp. V, relationships between Cu/f and both pCu produced by TRIS-EDTA buffers (Fig. 16) and measured soluble Cu in otherwise unchelated systems (Fig. 15b) were determined. By equating the two relationships through their common term, (-log Cu/f), a single equation relating pCu to measured soluble Cu can be derived:

$$pCu = 4.72(-\log[Cu]) - 22.62$$

Using this equation, we can enter a pCu of interest, and estimate the

soluble Cu necessary to achieve this pCu; or enter a soluble Cu concentration and determine the resulting pCu. For example, using the pCu = 9.65 (the composite EC50 of Exp. V at 1 and 3 mM TRIS and 1000 nM added Mn), the soluble Cu necessary to reach this pCu was $10^{-6.84}$ M (compared to the experimentaly determined 10^{-7} M, Table 8). The activity-to-concentration ratio is thus 1.5×10^{-3}, close to the lower bound of the range determined using the growth inhibition results above, indicating about 10 fold complexation over that predicted by inorganic speciation models alone.

Using the equation to estimate the apparent pCu experienced by the phytoplankton in Exp. V at 10^{-7}, $10^{-7.5}$, 10^{-8}, and $10^{-8.5}$ M soluble Cu, we find pCu values of 10.4, 12.7, 15.1 and 17.5, respectively. This implies activity-to-concentration ratios of 4×10^{-4}, 6×10^{-6}, 8×10^{-8}, and 1×10^{-9} respectively. This dramatic increase in the apparent extent of Cu complexation at low Cu concentrations could be interpreted as being the result of a powerful chelator at sub-micromolar concentrations. Powerful chelators have been inferred in seawater by a number of authors, based on biological phenomena (Johnston, 1964; Barber and Ryther, 1969; Barber, 1973; Rueter et al., 1979). The presence of such chelators is still a matter of controversy to marine chemists, but very recent papers indicate that chelators about as strong as EDTA may be widespread in seawater at concentrations of hundreds of nanomolar (van der Berg, 1982; Hirose et al., 1982). Relatively high densities of phytoplankton in my cultures may have resulted in the production of unusual quantities of chelators being produced in the water. Production of poweful chelating organics by a few freshwater blue

green algae has been reported previously in (Simpson and Nielands, 1976; Murphy et al., 1976), and recently in an estuarine dinoflagellate (Trick et al., 1983). Few attempts have been made to find strong chelators released by diatoms (the dominant flora in the treatments under consideration) and they have been largely negative (Sunda, 1975; McKnight and Morel, 1979).

The dramamtic increase in calculated pCu with decreased total Cu results from the low slope of the -log Cu/f versus pCu for this experiment (.33), implying only a third of an order of magnitude change in Cu/f for an order of magnitude increase in Cu activity, and that increasing total soluble Cu by one order of magnitude resulted in greater than one order of magnitude increase in Cu/f. The low slope of the -log Cu/f vs pCu curve was not typical of either my own laboratory experiments (Fig. 19) or those of Sunda (1975). If some analytical problem, such as adsorption onto particles, caused a false low slope, and the slope was in reality closer to 1, the increase in chelation at low soluble Cu would be much less dramatic.

Another explanation for the relationship between apparent pCu and soluble Cu should be reiterated: phytoplankton may not respond to low concentrations of total Cu as a function of free ion activity as they do in heavily metal buffered solutions. This should be especially considered in light of the low slope for the relationship between pCu and -log Cu/f for the TRIS treatments in Exp. V. A test of this hypothesis would require a reliable analytical method to determine the activity of Cu in seawater.

C. The Effect of Copper on the Species Composition of Enrichment Cultures

Of necessity, any discussion of the effect of Cu on the detailed species composition in experiments with "natural populations" of phytoplankton must be largely anecdotal; the realm of possible combinations of species is simply too vast. Additionally, many of the results of the outdoor experiment were influenced by factors beyond reasonable control, such as the species composition of the inoculum, initial water chemistry, daily and seasonal changes in temperature and light, etc. Because of this inherent complexity, only generalities were discussed in the species composition results of the outdoor experiments. Therefore, only general discussion of the results can be attempted.

Most intriguing, and quite clear-cut, were the opposite relationships between Thalassiosira and Chaetoceros under conditions of added Cu in unchelated form versus added Cu as TRIS-Cu ion buffers. In each case where unchelated Cu was added as the source of Cu, the fraction of Thalassiosira increased with increasing Cu at the expense of Chaetoceros, up to some level where Thalassiosira was itself eliminated (Fig. 12a-d). In contrast, in Exp. II, where a pCu series was constructed using 5 mM TRIS (Fig. 13a), and in Exp. III, where one of two pCu series was constructed with 5 mM TRIS (Fig. 13b), increasing Cu stress apparently caused an increase in the relative abundance of Chaetoceros and a concomitant decrease of Thalassiosira. In the 1 mM TRIS pCu series in Exp. III (Fig. 13c), Thalassiosira and Chaetoceros both remained approximately equal fractions of the population, until they were both eliminated at low pCu values. The

trend in which Thalassiosira was favored by unchelated Cu additions, but Chaetoceros was favored when Cu was added in the form of a TRIS buffer, has not been previously reported.

That the presence and strength of TRIS as a chelator appeared to cause the different species compositions in Figs. 12 and 13 cannot be proved unquestionably. However, laboratory experiments showed that high TRIS concentrations in metal-free water led to greater Cu inhibition of Thalassiosira than low TRIS concentrations (Fig. 20) and that the effect could be reversed by adding Mn (Fig. 21), suggests that, on the average, species of the genus Thalassiosira have lower tolerances for Cu than Chaetoceros at comparable suboptimal Mn concentrations. On the other hand, Thalassiosira must be the superior competitor at adequate Mn concentrations. One of the intentions in the selection of treatments in Exp. V was to provide an opportunity to test this hypothesis by having TRIS-pCu series with and without added Mn. Unfortunately, the cultures developed only with Chaetoceros, and no Thalassiosira. This may simply have been due to a low number of Thalassiosira in the phytoplankton when the inoculum was collected.

Are there any known general differences between the genera Thalassiosira and Chaetoceros that might be related to the difference between the way the two genera respond to chelated versus unchelated Cu? Both genera are described by Cupp (1943) as predominantly neritic. In the marine waters of Yaquina Bay, both genera are abundant during the same general seasons, with simultaneous abundance peaks in February through April and staggered peaks of abundance in summer through fall, with various Chaetoceros species in highest abundance from May through October, and two species of Thalassiosira

with abundance peaks in August (Karentz, 1975). This in itself may indicate that the genus Chaetoceros has a broader range of environmental tolerances than genus Thalassiosira, but gives no hint of the origin.

Frey (1977) studied the effects of various major and minor nutrient enrichments on phytoplankton species composition in Yaquina Bay, through all seasons. Both Thalassiosira and Chaetoceros were common species in his experiments with Thalassiosira the predominant taxon in untreated water in spring, and Chaetoceros the predominant taxon in summer. He found that Thalassiosira was enhanced by additions of a trace metal mix (which included the chelator EDTA at 232 nM), or by Fe as FeEDTA. EDTA additions of 232 nM without metals were either of no benefit or decreased the abundance of Thalassiosira. Chaetoceros was either not affected or stimulated (relative to other phytoplankton) by additions of EDTA alone. Together, these results indicate that Chaetoceros species are more tolerant of low nutrient metal ion activities than Thalassiosira.

While the evidence is at present somewhat nebulous, it would seem likely that Thalassiosira species in general are "more neritic" than Chaetoceros in that 1) they apparently have a higher requirement for metals in high concentration in continental runoff, such as Fe and Mn (Frey, 1977), and/or 2) they have a lower tolerance for Cu (which is not especially enriched in freshwater runoff compared to Fe or Mn), when Mn is in low concentration.

Another effect of extremely high Cu stress common to both TRIS-chelated and unchelated treatments was the development of an entirely different set of dominant species than the usual

Thalassiosira - *Chaetoceros* mixture. In most cases this population consisted of one or two species of benthic pennate diatoms, such as pennate form #1 or *Nitzschia*, although in a few cases green flagellates were dominant. The dominance of pennate diatoms after Cu treatment has also been reported by Thomas and Seibert (1977) and Sunda *et al*. (1981). No reason has yet been proposed for a generally higher level of resistance for benthic pennate diatoms as opposed to planktonic diatoms. In the process of diagenesis in oxygenated sediments, organic substrates with bound Cu are oxidized, with concomitant release of the Cu, which diffuses into the overlying water (Boyle *et al*., 1977). Thus it might be expected that the pore waters in such cases would have higher Cu ion activity than the overlying water. Diatoms adapted for living in or near pore waters might be expected to tolerate relatively high Cu ion concentrations. Manganese is also in very high concentrations in pore waters (Evans, 1977), and the effect of Mn in increasing the tolerance of the pennate diatoms to Cu, either in TRIS-complexed or uncomplexed media, is readily apparent (Fig. 12e,f; Fig. 14a-f).

The appearance of green flagellates as an important taxonomic component of high-Cu-stress treatments has also been previously reported by Thomas *et al* (1977) and Sunda *et al* (1981). In my experiments flagellates appeared more infrequently than pennate diatoms, and were only really significant in Exp. V with low initial Mn concentrations. In Exp. V flagellates ("species #1") were predominant in the treatment in which 10^{-7} M Cu, but no Mn, was added (Fig. 12e). These flagellates were not common in the unchelated treatments in which Mn was added, and growth of *Chaetoceros* was still

good at 10^{-7} M, and pennate form #1 grew to 10^{-6} M with the Mn addition (Fig. 12e). Possibly the lack of Mn caused Cu inhibition of all the diatoms, but the flagellates, insensitive to the Cu-Mn effect, were allowed to express themselves.

In the TRIS-buffered treatments of Exp. V (Fig. 14) a different species of flagellates ("species #2") appeared, although it was never the most abundant taxon, and always co-occurred with pennate form #1 when it was present at all. At the lowest TRIS level (1 mM) this flagellate (sp. 2) was more abundant in water without added Mn (Fig. 14a,b), but at the 3 and 10 mM TRIS levels this flagellate was more abundant in water without added Mn (Fig. 14c-f). In contrast to the results in the unchelated Cu series with flagellate (sp. 1), Mn additions favored the growth of flagellate (sp. 2) in TRIS buffered media. Because Mn additions also favored pennate form #1 growth at low pCu values and at all TRIS levels, the percent occurrence of the flagellate (sp. 2) was never very high. Pennate form #1 always appeared to be the better competitor.

D. The Interaction of Copper Inhibition and Manganese Limitation

1. TRIS-Cupric Free Ion Activity Buffers, and the Role of Manganese

Ideally, given the total concentrations of all components of experimental seawater, and the association constants for their complexes, the total free ion concentrations (or activities) could be determined. It was my intention to model the increase of Cu toxicity caused by Mn deficiency in the various TRIS-EDTA, buffers and determine quantitatively how low Mn activity increased Cu inhibition,

as was recently reported by Sunda et al. (1981), using seawater chelated with EDTA and NTA (nitrilo-acetic acid).

There are no association constants reported in the literature for any TRIS-Mn complexes. Hanlon et al., (1966) reported an attempt to determine these association constants, but could not detect any association below pH 8.8. Above pH 8.8 a precipitate formed, which was ascribed to hydrolysis by the Mn^{2+} ions. This situation would be ideal for seawater media, as the lack of Mn-TRIS complexation at normal seawater pH values would simplify equilibrium calculations of Mn activity. However, Hobey and Prybyla (1978) reported that at pH values above 8.3 TRIS catalyzed the oxidation of Mn^{2+} to Mn^{3+} by O_2. This was deduced from the simple observation that a buffer solution containing large amounts of TRIS and Mn was found to darken to a straw color. Elimination of TRIS, Mn, or oxygen eliminated the darkening. In the absence of a chelating agent Mn^{3+} is unstable in solution, and disproportes to Mn^{2+} and MnO_2:

$$2Mn^{3+} + 2H_2O \rightleftharpoons Mn^{2+} + MnO_2(s) + 4H^+;\ K = 10^9$$

However, Mn^{3+} can be stabilized in solution by complexation. As Mn^{3+} has much stronger affinity for ligands than Mn^{2+}, even TRIS may serve as a stabilizing agent. I also tested solutions of TRIS and Mn^{2+}, at pH 8.0 in 1M TRIS and 0.01M $MnCl_2$ (the high concentrations speed up the reaction and allow it to be visibly detected), and noted that the straw color began to develop within a few minutes and continued to darken. After 18 hours a large amount of black solid, presumably MnO_2, was formed.

It therefore appeared likely that the Mn^{2+} free ion activities in the various TRIS containing treatments in my experiments were controlled not by complexation equilibrium but rather by the slow kinetics of TRIS catalyzed oxidation of Mn^{2+} to Mn^{3+}, with subsequent chelation to prevent disproportionation back to Mn^{2+} and MnO_2. In a simple model of this system, Mn^{2+} is oxidized to Mn^{3+} by O_2 at a rate proportional to TRIS, Mn^{2+}, and O_2 concentrations (as O_2 concentrations are fixed by atmospheric pressure, they are presumed to be constant). The Mn^{3+} produced is then stabilized either by complexation with TRIS or EDTA (again no TRIS-Mn^{3+} complex is known, but EDTA has a high affinity for Mn^{3+}, with pK = 24.8. This affinity is sufficient to effectively chelate all the Mn in experiments in which total Mn is less than the total EDTA, if total Mn were all converted to Mn^{3+}. Thus if (or when) Mn^{2+} is produced by TRIS catalyzed oxidation in experimental media, it is probably rapidly chelated by EDTA and/or TRIS and protected from disproportionation. If the reaction is first order in all components (Mn^{2+}, TRIS, and O_2) and initial Mn^{2+} is assumed equal, the reaction would proceed at one tenth the rate in 1mM TRIS as in 10mM TRIS. Actually, since TRIS often forms complexes involving two or more TRIS molecules per metal ion (Hanlon et al., 1966) it is likely that the reaction is of higher order with respect to TRIS, and the effect of TRIS concentration on the rate would be correspondingly greater.

To determine whether or not the Mn^{2+} added to the media in my experiments remained soluble I tested solutions of 1.0 µM Mn plus 1.0 µM Fe-EDTA plus either 1, 3 or 10 mM TRIS, all at pH 8.0 and 8.5 in

sterile seawater. These six bottles were incubated at conditions similar to those of experimental phytoplankton cultures (20°C in the light), and each bottle was sampled once per week for three weeks. The samples were filtered through a 0.45 μm membrane filter and extracted with Chelex-100 for Mn. No persulfate oxidation was used to destroy organics. All the added Mn was found to be extractable in all the samples. However, a straw colored area (the color similar to that formed in the preliminary experiment with very high concentrations of TRIS and Mn) was visible at the top of the resin columns. It was particularly noticeable in the high-pH series, and became progressively darker with increasing TRIS concentrations and with longer times. This result supported the reactions outlined above. Also, because the Mn was extractable with Chelex-100, it further suggests that the Mn^{3+} formed may have been present as a TRIS complex rather than as an EDTA chelate (since EDTA complexes are rather slow to dissociate and not readily available for chelation during passage through the resin).

Based on the above scheme, the probable fate of the Mn added to, or initially present in, the media in all my experiemnts with TRIS was as follows:

1. Initially Mn^{2+} was largely unchelated by either TRIS or EDTA, and the free-ion activity was controlled by chloride complexation.
2. Free Mn^{2+} was oxidized by O_2 at a rate which was some function of Mn^{2+} concentration, O_2 pressure (constant), and pH (approximately constant within any single experiment).
3. Free Mn^{3+} created by the reaction above was chelated by EDTA and/or TRIS to a very low activity.

4. Free Mn^{3+} disproportionated at some very low rate (due to the low Mn^{3+} free-ion activity) to form MnO_2 and Mn^{2+}.

From the observations of the color in the Chelex-100 columns it appeared that over three weeks the reaction had gone a long way toward completion (to predominantly chelated Mn^{3+}) in 10 mM TRIS (deepest observable color). In the 1 mM TRIS solutions, the reaction had not proceeded very far at all in three weeks (much less color than in the 10 mM TRIS). With regard to my results on phytoplankton growth and species composition in outdoor experiments employing Cu-TRIS buffers, it appeared that most of the Mn added to 1 mM TRIS systems would be available to the cells as Mn^{2+} at the conclusion of the experiments. Thus, these systems behaved very much like systems with no TRIS, and phytoplankton were subject to "normal" Cu-Mn effects (with ambient Mn^{2+} reducing the apparent Cu toxicity to the cells, as in Figs. 8e and 12e,f). In the 10 mM TRIS treatments, however, only a small fraction (less than 10%) of the Mn would be available as Mn^{2+}, assuming 2-3 weeks from the introduction of TRIS and Mn until the conclusions of the experiments. Such treatments would appear Mn-deficient systems to the phytoplankton, with exacerbated Cu toxicity due to low Mn^{2+} activity. As a great many of the kinetic parameters presented here are still only qualitatively known, and some of the reactions are still speculative, modeling the Cu-Mn-TRIS system quantitatively to determine free Mn ion activity through time in the various experiments is fruitless.

Another possible problem relating to the use of TRIS in phytoplankton growth media can be found in the biochemical literature concerning photosynthesis. A large body of literature concerns the

use of TRIS washing of isolated chloroplasts to inhibit the Hill reaction in photosystem II (Yamashita and Butler, 1968). The concentration of TRIS used to inhibit photosystem II, however, is quite high (0.8 M), and lower concentrations (0.05 M, or five times the highest concentration used in my experiments) are used in the isolation of chloroplasts without causing inhibition (Cheniae and Martin, 1970). The most interesting feature of the mechanism by which TRIS washing inhibits the Hill reaction concerns manganese. It has been shown that the TRIS washing of chloroplasts frees about 60% of the Mn bound in the chloroplast, and releases it into the thylakoid space, where it is measurable by Electron Paramagnetic Resonance spectroscopy (EPR) as free Mn^{2+}. The Mn so released remains localized within the thylakoid space and can be reassociated with the active site, restoring Hill reaction activity (Blankenship and Sauer, 1974).

It seems unlikely that the effect of TRIS on the Hill reaction in isolated chloroplasts is related to the effect of Mn deficiency on the inhibition of growth rate in whole cells by Cu, for several reasons. First, the effect in chloroplasts occurs only at much higher TRIS concentrations than used in phytoplankton cultures (eighty times my highest concentration). Second, TRIS would have to pass the cell wall and cell membrane in addition to the chloroplast membrane in order to affect the chloroplast in intact cells. Third, cells in treatments of my experiments containing high TRIS but low Cu free ion activities were not inhibited. There is no indication in the biochemical literature that Cu is required for inhibition of chloroplasts by TRIS. Fourth, in Exp. V, Mn additions appear to have ameliorated Cu toxicity

in naturally Mn deficient seawater, with no TRIS present.

Surprisingly, the two interactions of TRIS with Mn, one an inorganic oxidation and the other an effect on chloroplasts, apparently have never been associated with one another in the literature. It is interesting to speculate that the TRIS-chloroplast effect arises due to the oxidation of bound Mn in the chloroplast, causing its release from the Mn^{2+}-Hill reaction site (a subsequent reduction would be necessary to account for EPR evidence of free Mn^{2+}).

The problems associated with the uses of TRIS buffer in trace metal experiments perhaps serve best as a caution regarding the use of artificial complexing agents in conjunction with organisms. Presently hundreds of artificial complexing agents are known, and while a vast literature exists on their complexation chemistry in the absence of organisms, relatively little is known of other aspects of their chemistry such as kinetics, catalysis, and their effects on cellular chemistry. At the same time it is important to remember that artificial complexation agents have been crucial to the advance of our knowledge of phytoplankton-trace metal relationships.

2. Unchelated Natural Seawater

In the final outdoor enrichment experiment (Exp. V), seawater with initially dissolved Mn (11 nM) was tested for growth of natural phytoplankton populations with and without 1000 nM of added Mn. As discussed previously, the addition of 1000 nM Mn raised the EC50 for Cu inhibition for the predominant Chaetoceros population from about

5×10^{-8} to 1×10^{-7} M. In addition, at added Cu concentrations of 3×10^{-7} M, 1×10^{-7} M and 0 M the added Mn raised the growth rates 74, 30 and 13%, respectively. The 13% increase in the face of no added Cu was noteworthy, if not statistically significant. The coastal phytoplankton population grown in this oceanic water appeared to be slightly Mn deficient, and that deficiency was aggravated by added Cu.

The initial Mn concentration of the seawater used in Exp. V was low compared to the water near the mouth of the Yaquina River estuary (Table 6), but it was higher than what might be expected at the surface in water upwelled from 100 m (This study, Fig. 4; Landing and Bruland, 1980). Such water could be as low as 1.0 nM Mn. Upwelling of 100 m water is a common summer event along the Oregon coast (Small and Menzies, 1981). The lowest Cu addition in my experiments (1×10^{-8} M) was about ten times the expected Cu concentrations of recently upwelled water (Fig. 1). Thus, if the toxic effect of Cu in this system is largely a function of the Cu/Mn ratio, as suggested by Sunda et al., 1981, the natural Cu/Mn ratios in upwelled waters off Oregon could be close to the threshold of significant inhibition for the dominant Chaetoceros cells. Furthermore, previous experiments (Exps. II and III) suggested that Chaetoceros was less susceptible to Mn deficiency than another common coastal genus, Thalassiosira. Subtle shifts in Cu/Mn ratios within the ranges allowed by the observed distributions of these two elements in the field may alter the delicate balance of fitness between different phytoplankton taxa. In the field, however, such effects would be extremely difficult to discriminate from many other variables causing differential growth and

mortality of species, e.g. nutrient concentrations, vitamins, light levels, temperature, grazing and turbulence. Nevertheless, trace metals, and trace metal interactions with one another, may play a significant role in the maintenance of the diversity of phytoplankton populations.

E. The Modes of Copper Toxicity and the Prospect of Environmentally Significant Copper Inhibition

The results of the laboratory experiments reported here, and those of Sunda et al. (1981) suggest that two modes of Cu toxicity probably exist for many marine phytoplankton: one mode is insensitive to increased Mn concentrations (free ion activities) above some saturating Mn concentration (Fig. 22); and the second is expressed at lower concentrations of both elements, resulting in the Cu-Mn effect reported herein.

Some previous experiments tend to indicate that the mechanism for the Mn-independent mode of Cu toxicity may relate to the loss of potassium from the cell, possibly as a result of damage to the cell membrane proteins that transport potassium (McBrien and Hassal, 1965; Kamp-Nielsen, 1971; Sunda, 1975). Another possibility raised by several investigators relates Cu toxicity to silicon uptake (Morel et al., 1978; Reuter and Morel, 1981; Fisher et al., 1981), possibly another example of an effect on the cell membrane, in this case damage to the sites responsible for Si uptake. No work has been done to determine the exact site of the Mn-dependent Cu inhibition. However, keeping in mind the essential role of Mn in the Hill reaction in photosynthesis, and the evidence that excessive Cu in higher plants

inhibits the Hill reaction (Habermann, 1969), the photosynthetic mechanism would be an excellent starting place for initial experiments.

Sunda et al. (1981) has quantitatively evaluated the interaction of Cu and Mn for growth rate of the diatom Chaetoceros socialis in terms of an equilibrium chemical model, and determined that the data fit reasonably well to a two-site model. One site is assumed to be a Mn-dependent site (S1) to which Cu is a competetive inhibitor [Mn^{2+} + S1 $\rightleftharpoons$ MnS1, Cu^{2+} + S1 $\rightleftharpoons$ CuS1; K(Mn) = $10^{10.9}$, K(Cu) = $10^{11.5}$], while the second site is a Mn-independent site (S2) of Cu toxicity, [$2Cu^{2+}$ + S2 $\rightleftharpoons$ Cu_2S2, K = $10^{17.6}$]. The second site would only be appreciably inhibited by Cu activities above $10^{-9.5}$ M, above the Cu activity expected in any unpolluted seawater, while the first site could produce reduced growth rates at Cu free ion activities much lower than $10^{-9.5}$ M, depending on the Mn free ion activity. If the estimated Mn concentration at 100 m in the northeast Pacific is 1 x 10^{-9} M, the activity of Mn would be estimated at 1 x $10^{-9.7}$ M (Mantoura et al., 1978), assuming no organic Mn complexation. Using the relationship developed by Sunda et al. (1981) the EC50 for Chaetoceros socialis would be shifted to pCu = 10.3, compared to pCu = 8.9 in Mn saturating conditions. Thus, if Cu inhibition does occur in Pacific Northwest upwelling waters, it will most likely be due to the Mn dependent mode. If the copper concentration in Pacific Northwest upwelling is 1 x 10^{-9} M, and assuming only inorganic speciation of Cu, the pCu would be about 10.7 (Table 3). According to the relationship found by Sunda et al. (1981), this Cu/Mn combination would result in about 30% growth rate

inhibition of Chaetoceros socialis in the hypothetical upwelled seawater.

An important assumption in this scenario is the lack of inorganic complexation of Cu. In Exp. V however, the concentration of Cu in phytoplankton just at the onset of obvious growth inhibition by Cu in both chelated and natural seawater indicated that Cu activity in the natural seawater was only about 10% of that predicted by inorganic speciation alone. At lower concentrations of added Cu, Cu activity may have been even more reduced. Such reduced activity would eliminate the likelihood of a strong growth rate inhibition due to a high Cu/Mn ratio in freshly upwelled seawater. Only a very low concentration of organic chelators in the 100 m seawater would prevent such a conclusion.

Analytical measurements of complexation generally agree that at a minimum, about 50% of the total analytical copper is complexed in seawater (Batley and Gardner, 1978; Bruland et al., 1979). Very recent work (van der Berg, 1982; Hirose et al., 1982) has indicated that Cu is even more highly complexed and probably only about 5% of the total analytical Cu is in inorganic forms, thus vindicating biological oceanographers who have been insisting that it must be the case. An ironic aspect of attempts to show Cu inhibition of phytoplankton by "environmentally reasonable" Cu concentrations, is that while biological oceanographers have been consistently pushing down the levels of Cu that might prove toxic, chemical oceanographers have, at the same time, been pushing down just as fast,the concentration of Cu available to cause the inhibition.

Estuaries and very nearshore zones are pelagic waters in that

they usually have high concentrations of Mn. Therefore, any Cu inhibition by phytoplankton in estuaries is likely to be of the Mn-independent mode; i.e., generally higher Cu activities would be required for inhibition (even assuming the phytoplankton are not more resistant). Furthermore, estuarine and coastal pollution is not normally a single substance, and many urban and industrial waste waters contain high concentrations of relatively strong Cu complexing agents such as NTA and EDTA. Thus, the prospects of Cu toxicity having a significant impact in estuaries are also reduced. Of course, a single strong source of unchelated copper could have severe local consequences, regardless of the chemical make-up of the receiving waters.

VI. CONCLUSIONS

In this study the inhibition of Oregon coastal marine phytoplankton populations was examined from two points of view. First, the amount of inorganic Cu required to severly inhibit growth was sought, as Cu is frequently cited as a possible pollutant and is known to be highly toxic to algae. Second, Cu inhibition was examined in terms of the Cu free ion activity fixed by TRIS-Cu or TRIS-EDTA-Cu mixtures. It had been shown that phytoplankton respond to Cu as a function of free ion activity rather than to total analytical Cu, and that naturally high Cu free ion activity in some seawater had been proposed to cause inhibition of natural phytoplankton populations at certain places in the oceans.

The growth rate of natural coastal marine phytoplankton populations was reduced to 50% of normal by added inorganic Cu in natural seawater at about 1×10^{-7} M in three experiments (in seawater collected very nearshore), and at 5×10^{-8} M in one experiment (with seawater collected further offshore). In TRIS-Cu ion activity buffered seawater, the free ion activity that caused 50% growth rate inhibition ranged from about 3×10^{-10} to 8×10^{-12} M. Assuming that phytoplankton in the natural seawater respond to Cu as a function of its free ion activity, and assuming standard models of inorganic Cu speciation, these two results indicate that Cu in normal coastal seawater is 90%+ complexed by organics.

Two common genera of planktonic diatoms Thalassiosira and Chaetoceros were abundant in most of the experiments. Thalassiosira was found to increase relative to Chaetoceros with increasing Cu

additions in treatments without TRIS, while Chaetoceros increased relative to Thalassiosira with decreasing pCu in TRIS-buffered seawater. In cultures in which either added inorganic Cu or Cu free ion activities sufficient to completely eliminate the dominant phytoplankton (centric diatoms), benthic pennate diatoms, (or in a few cases, green flagellates) were found. These benthic forms apparently were tolerant to higher Cu stress than the usual dominant phytoplankton.

Manganese deficiency was found to exacerbate Cu growth rate inhibition, at least in diatoms. The range of Mn possible in the Oregon coastal zone includes concentrations both high enough to completely satisfy Mn requirements, and low enough to produce Mn-dependent Cu inhibition, given sufficient Cu free ion activities. Local coastal upwelling in particular may bring up seawater with especially low Mn, somewhat higher Cu concentrations, and lower dissolved organics believed important in detoxifying inorganic Cu. Under marginally inhibitory Cu-Mn regimes, the effects of Mn-dependent Cu inhibition are more likely to be expressed by changes in species composition determined by relative fitness to a whole suite of conditions (including the Cu-Mn regime), rather than by gross Cu inhibition in surface waters.

The concentrations of Cu in phytoplankton grown in waters with added Cu compared to the concentrations of Cu in populations grown in TRIS-Cu free-ion activity buffers, as well as the differences between the growth rate inhibition in TRIS-buffered seawater and unamended seawater, indicate a degree of complexation in natural seawater which would preclude significant gross Cu inhibition of natural populations

at any natural Cu concentrations.

Results of this study, and several cited within, have ramifications that depend upon certain conditions or facts which are as yet not fully known. High on the list of required knowledge is the true Cu free ion activity in seawater (or really, a method which can determine it in many cases). Clearly, a whole host of biological and chemical observations point to the inadequacy of considering only inorganic speciation. Yet at present we can only measure total analytical Cu, apply inorganic speciation models to the results, and perhaps find an estimate of the fraction of Cu bound to organics (which will no doubt disagree strongly with someone else's estimate). All of the proposals for Cu being inhibitory to phytoplankton in nature depend on whether or not the free ion concentrations are close to inorganic speciation predictions or are much lower. In my work in Oregon coastal waters, they appear to be much lower. Once this question is truly settled, biological research using Cu free ion activity can be put into its proper perspective.

At a more basic analytical level, the distributions of trace elements in marine waters recently produced with improved techniques, are known from an alarmingly few studies, and many of these have been taken far at sea so as to be representative only of the open ocean. A few have been developed in estuaries. The coastal zone, which can be expected to be far more spatiotemporally heterogeneous with regard to trace element distributions than the open sea, has been, in a sense, largely passed over. To some extent this is because the open ocean with the lowest concentrations, is the greatest challenge; and partly because the homogeneity of the distributions there serves to validate

the measurements. That the coastal zone has been slighted is unfortunate, considering that the primary site of man's interaction with marine waters is in the coastal zone. Ryther (1969) stated that virtually all of man's fishing catch occured in the 10% of the ocean surface area that is coastal zone and equatorial upwelling area.

The primary responsibility I see ahead for biological oceanographers in the field of phytoplankton-metal interactions is not to be solved by a single improvement in methodology. Likely no single advance over methods now available will be of great benefit. The problem for the biologist is the great diversity of organisms, each species (and perhaps each clone) different in some particular from the others. To determine the overall importance of Cu-Mn-organic matter interactions will require evaluation of many species from a wide variety of environments and taxa. Only such a survey, carried out with knowledge of the various local distributions of Cu and other trace elements, will prove the significance or insignificance of Cu to the regulation of phytoplankton growth and species composition on a global scale.

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APPENDIX

Modification of the Persulfate Oxidation by Subsequent Reduction With Hydroxylamine-HCl to Restore Mn and Fe Extraction Efficiency

Destruction of organic complexing compounds in estuarine and seawater is often necessary to achieve extraction of organically bound metals with Chelex-100 (Evans, 1977; Riedel, 1978; Bately and Gardner, 1978). Oxidation with potassium persulfate has long been used to destroy such organics (Slowey and Hood, 1966), and is, in fact, used in the determination of total dissolved organic carbon by wet digestion and measurement as CO_2. In my own experience (Riedel, 1978) and that of Evans (1977) no problem was found with regard to efficiency with Mn or Fe extraction efficiency after digestion in estuarine water or seawater from large-scale phytoplankton cultures. Upon starting trace element analysis in water from the Yaquina River estuary, it soon became evident that Mn in persulfate digested samples was not being efficiently extracted. The possibility that Mn^{2+} was becoming oxidized to MnO_2 by the harsh oxidation, and then remaining in the digestion vessel, or settling in the Chelex-100 column and not dissolving in the eluting acid was immediately suggested.

The following experiment was carried out to investigate and solve this problem. Nine 125 ml polyethylene bottles with 100 ml Artificial Seawater (ASW) were prepared with 1 mg/l each of Mn, Fe, Cu, and Zn. These were split into three groups of three and the following changes from the Methods outlined in Fig. 6 were applied:

A. No acid added, no persulfate digestion, no bicarbonate neutralization

B. Persulfate oxidation, no hydroxylamine reduction

C. As per Fig. 6

After the digestion it was noted that the digested samples had formed a dark brown precipitate, presumably MnO_2 and/or $Fe(OH)_3$. The samples treated with hydroxylamine-HCl cleared immediately. Upon loading the Chelex-100 column a small fraction of the brown precipitate adhered to the polyethylene bottle it was digested in, but most went with the solution, and that portion visibly became trapped in the top 1 cm of the resin column. This precipitate remained visible after elution of the columns with 2N HNO_3. One of these columns was then eluted with 2N HNO_3 containing 5% W/V hydroxylamine-HCl. This column cleared immediately. This extract was also analyzed and is designated treatment "D". Table 10 shows the percent recovery of the added metal from the various treatments as measured by FAA.

The digestion under these conditions had apparently oxidized virtually all of the Mn to an unextractable form (almost surely MnO_2). About half the Fe had also become available for extraction, presumably as $Fe(OH)_3$, and also a smaller fraction of Cu, probably adsorbed to the precipitate. The efficient Mn extractions previously found by Evans (1977) and myself after persulfate oxidation are not entirely explicable. For my own work, the previous samples had been much lower in Mn. Possibly, the autocatalytic oxidation of Mn^{2+} by MnO_2 increases the rate at higher Mn concentrations. In Evans case, the conditions of the oxidation were somewhat different, being longer times at lower temperature. Perhaps that combination of conditions does not lead to the same degree of oxidation of Mn. One factor both my previous

Table 10. Effect of persulfate oxidation and hydroxylamine reduction on the extraction of trace metals from artificial seawater with Chelex-100 . A - no treatment. B - persulfate oxidation and bicarbonate neutralization only. C - full treatment as per Fig. 6. D - Hydroxylamine-HNO_3 extract from column remaining from treatment B. Percent of the added metal extracted and 1 standard deviation.

Treatment	Mn	Fe	Cu	Zn
A	94.0 (1.7)	100.0 (0.0)	95.6 (1.2)	99.5 (1.3)
B	6.8 (0.3)	54.3 (2.3)	93.0 (1.7)	99.7 (2.9)
C	95.8 (4.0)	92.9 (5.0)	95.3 (3.5)	99.0 (5.6)
D	83.7	49.9	6.6	2.1

oxidations and Evans had in common different from the new procedure was the use of HCl to preserve the sample instead of HNO_3. It is also possible that nitrate, also a strong oxidant, serves to couple the oxidation of Mn^{2+} by persulfate. The reason for an effect of the digestion on Fe extraction is not clear either. In any case, the Fe remained as Fe III throughout the treatments, having been added as $FeCl_3$. However, the oxidation appears to have accelerated the formation of a relatively inert (aged) iron hydroxide phase.

The hydroxylamine-HCl reduction restored the Mn extraction to its pre-digestion efficiency, and the Fe extraction to nearly its pre-digestion efficiency. The missing Mn, Fe and Cu was largely extracted from the Chelex-100 resin by the HNO_3-hydroxylamine-HCl elution of treatment "D".

Once it was shown that hydroxylamine reduction solved the problems of poor extraction of Mn and Fe in the water samples, I tested whether or not the hydroxylamine-HCl solution could be adequately cleaned by Chelex-100 for use in very low level trace metal analysis. This being shown, the reduction step was permanently incorporated into my scheme for the analysis of trace metals dissolved in seawater.