The relationship between cupric ion activity and the toxicity of copper to phytoplankton

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ABSTRACT

Culture experiments with the estuarine diatom *Thalassiosira pseudonana* (clone 3H) in highly chelated seawater media demonstrate that growth rate inhibition and copper content of cells are related to cupric ion activity, and not to total copper concentration. Cupric ion activity was altered independently of total copper concentration by varying the chelator concentration, and the pH. Cellular copper content (moles/cell) of 3 to 4 day old cultures followed a hyperbolic relation with cupric ion activity:

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

where a_{Cu} is the cupric ion activity. Copper inhibited growth rate at activities above 3×10^{-11} M and growth ceased at values above 5×10^{-9} M; however, the relation between growth rate inhibition and cupric ion activity was not a simple hyperbolic function. In experiments with the estuarine green alga *Nannochloris atomus* (clone GSB nanno), growth rate inhibition also was related to cupric ion activity with partial growth rate inhibition occuring in the activity range 4×10^{-11} to 2×10^{-9} M. Calculated estimates of cupric ion activity in seawater indicate that natural activity levels can be inhibitory to these phytoplankton depending on pH and the degree of copper complexation by natural organic ligands.

1. Introduction

Recently there has been much interest in the effect of trace metals and trace metal chelation on the growth of phytoplankton in natural waters. Productivity studies in seawater that demonstrate either a beneficial effect of artificially added chelators or a deleterious effect of organic matter removal suggest the importance of trace metal chelation in promoting healthy phytoplankton growth (Johnston, 1963, 1964; Barber and Ryther, 1969; Barber *et al.*, 1971; Barber, 1973). Concentrations of copper within the environmental range (i.e. as low as 1 ppb) can inhibit species of

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both marine and freshwater algae (Steeman Nielsen and Wium-Anderson, 1971; Davey *et al.*, 1973), suggesting that at least one of the beneficial effects of chelators to marine algae is the removal of natural copper toxicity (Steeman Nielsen and Wium-Anderson, 1970).

Numerous investigations have demonstrated that the chelation of most metals, including copper, lowers their availability to phytoplankton, presumably by lowering the concentrations of free metal ions. For example, manganese, calcium, and zinc deficiencies are induced by the addition of the chelator ethylenediaminetetraacetic acid (EDTA) to growth media (Hutner *et al.*, 1950; Walker, 1953, and 1954; Spencer, 1957). Manahan and Smith (1973) presented quantitative evidence that the growth of copper deficient cultures of fresh water algae (*Chlorella vulgaris* and *Oocystis marssonii*) is dependent on cupric ion activity. In their experiments, the cellular yields of cultures containing EDTA were increased in a similar fashion either by the addition of higher concentrations of copper or by lower concentrations of EDTA.

Metal toxicity as well as nutritional supply is affected by the presence of chelators. Copper is toxic to the diatom *Phaeodactylum tricornutum* at concentrations in excess of 2 μM in the absence of chelators, but is not toxic to this alga at concentrations as high as 5 mM when complexed to EDTA (Spencer, 1957). The chelation of copper by nitrilotriacetate or citrate also reduces copper toxicity to algae (Erickson *et al.*, 1970; Steemann Nielsen and Kamp-Nielsen, 1970). Plots of growth inhibition of *Thalassiosira pseudonana* (clone 13-1) vs copper concentration in media containing the chelators EDTA or histidine have been found to resemble potentiometric titration curves; that is, a sharp inflection in the curves occurred at the point where the copper concentration exceeded that of the chelator (Davey *et al.*, 1973). Finally, extracellular polypeptide chelators produced by *Anabaena cylindrica* protect this alga from copper toxicity (Fogg and Westlake, 1955).

The above evidence suggests that copper toxicity as well as nutritional supply is determined by the free cupric ion activity, and that chelators, whether natural or artificial, affect availability by altering free ion activities. Although the nutritional supply of copper to algae has been shown quantitatively to depend on cupric ion activity (Manahan and Smith, 1973), previous research has not demonstrated an actual functional relationship between copper toxicity and free metal activity.

The major aim of this investigation is to test the hypothesis that copper toxicity to algae and copper content of algal cells are functionally related to free cupric ion activity. This hypothesis is tested in highly chelated seawater cultures in which the free cupric ion activity is systematically varied through different combinations of total copper concentration, chelator (trishydroxymethylamino methane) concentration, and pH. Observations on cupric ion activity levels that inhibit the growth of marine phytoplankton are discussed relative to calculated estimates of cupric ion activity in seawater.

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

Major nutrients	
$NaNO_3$	883 μM
$NaH_2PO_4 \cdot H_2O$	36.3 μM
$Na_2SiO_3 \cdot 9H_2O$	100 μM
Trace metals	
$FeCl_3 \cdot 6H_2O$	1.0 μM
$CuSO_4 \cdot 5H_2O$	0.04 μM
$ZnSO_4 \cdot 7H_2O$	0.08 μM
$CoCl_2 \cdot 6H_2O$	0.05 μM
$MnCl_2 \cdot 4H_2O$	0.9 μM
$Na_2MoO_4 \cdot 2H_2O$	0.03 μM
Vitamins	
Thiamin • HCl	0.1 mg/1
Biotin	0.5 μg/1
B_{12}	0.5 μg/1
Chelator: Na_2EDTA	

* Modified half strength medium f (Guillard and Ryther, 1962). Experimental media prepared from filtered surface Sargasso seawater stored for several months in 5 gallon borosilicate carboys.

2. Materials and methods

Two species of estuarine algae were used in this study: a diatom, *Thalassiosira pseudonana* (*Hustedt*), *Hasle* and *Heimdal* (clone 3H), (formerly *Cyclotella nana Hustedt*), and a green alga, *Nannochloris atomus Butcher*. The algae were grown aseptically in batch cultures contained in 250-2000 ml borosilicate Erlynmeyer flasks at a temperature of 20 ± 1°C. Illumination was provided on a 14/10 hour light/dark cycle by fluorescent lights (Sylvania Co., cool white). Light intensity was 4300 lux from above, 880 lux from below, and 2000 lux from the side, as measured by a general electric type 213 cosine corrected light meter. Experimental cultures were inoculated from exponentially growing cultures in M-f/2 media (Table 1) containing no added tris.

The majority of experiments were performed in a modification of half strength medium f (Table 1) containing variable concentrations of $CuSO_4$ and trishydroxymethylamino methane (tris). Tris functioned in these media as both a cupric ion and hydrogen ion buffer. Media were sterilized by autoclaving for 15 minutes at 15 psi. High pH values encountered during the autoclaving of low tris culture media usually resulted in the formation of alkaline precipitates. These were redissolved by aseptic bubbling with 5% CO_2 to a pH of about 6, followed by aseptic aeration to equilibrate the media with the atmosphere.

In one experiment, copper inhibition of clone GSB nanno was investigated in ultraviolet light (uv) treated and untreated coastal seawater. Uv treatment was used to destroy a major portion of the dissolved organic matter present in the seawater

(Armstrong *et al.*, 1966; Williams, 1969). Coastal seawater was collected from the dock of the Woods Hole Oceanographic Institution and filtered immediately through a glass fiber filter (0.7 μm mean retention size, Sheldon, 1972). A portion of this water was exposed for 6 hours to high intensity ultraviolet radiation from a 1200 watt mercury arc tube (Armstrong *et al.*, 1966). After irradiation, the seawater was restored to initial volume with distilled water and aerated to equilibrate CO_2 with the atmosphere. Uv treated and untreated seawater was enriched with f/2 levels of phosphate, nitrate, and silicate (Table 1), and with 1 μM $FeCl_3$ to insure sufficient nutrients for algal growth. Enriched seawater was sterilized by suction filtration through 0.2 μm membrane filters. Additions of $CuSO_4$ were made aseptically just prior to culture inoculation.

All chemicals used in the preparation of culture media were of reagent grade. Culture flasks and glassware used in the preparation of culture media were presoaked in concentrated nitric acid to minimize trace metal contamination.

Cell concentrations were measured by counting in a Bright Line haemacytometer (American Optical Co.). Specific growth rates of cultures, used as an index of copper toxicity, were determined by a least squares linear regression of $\log_2$ cell concentration vs. time for exponential portions of growth curves.

Copper content of clone 3H was measured after 3 or 4 days exposure of the algae to copper-containing media. Cells were separated from culture media by vacuum filtration using 3 μm pore size, 47 mm diameter, G. E. nuclepore filters and digested along with the filters in 1 ml of concentrated nitric acid. Acid digests were diluted fivefold with distilled water and measured for copper content on a Perkin-Elmer model 403 atomic absorption spectrophotometer. Sample values were corrected for copper retention on blank filters, through which medium without cells had been passed. Cell copper content in moles per cell was calculated by dividing the "corrected" moles of copper in the cell digests by the number of cells filtered. Replicate measurements of copper concentration in 13 cell digests and of number of cells filtered from 6 cultures gave mean coefficients of variation of 2.9% and 5.2% respectively. Measurement of cell copper content in three cultures on day 3 and day 4 showed no trend between days and had a mean coefficient of variation within individual cultures of 7.6%.

The parameter of primary importance in our culture media is pCu, which, in an analogous fashion to pH, is defined as the negative logarithm of the cupric ion activity. pCu cannot be measured directly and therefore must be calculated. To calculate pCu one must know both the total copper concentration and the degree of copper complexation. This latter parameter is strongly influenced by pH.

In many of the cultures containing 3-50 μM $CuSO_4$ total copper concentration was measured directly by atomic absorption spectrophotometry (Table 2). These measurements, made in filtered media from 3 day old cultures, show generally good agreement with added concentration values (Table 2). Total copper concentration in

cultures in which this parameter was not measured is assumed to be equal to the concentration of added $CuSO_4$.

The degree of copper complexation in culture media was computed using experimentally measured stability constants (Sunda, 1975) and constants taken from the literature (Sillen and Martel, 1964). The somewhat lengthy and tedious details for the measurement of apparent stability constants for copper-tris complex formation and subsequent calculations of copper complexation in culture media are reported by Sunda (1975). Calculations for M-f/2 media containing 0.5-10 mM tris show that in all cases copper is complexed predominantly by tris, to a lesser to negligible extent by EDTA, and negligibly by inorganic ligands. Significant complexation by naturally occurring organic ligands in these Sargasso seawater based media is considered unlikely because of the large concentrations of added tris and copper. No attempt is made to calculate pCu values in M-f/2 media containing no added tris.

pCu value in uv treated coastal seawater was computed by considering the complexation of copper by inorganic ions CO_3^{2-}, OH^- SO_4^{2-}, Cl^--, and $B(OH)_4^-$ (Sunda, 1975). Any natural organic ligands that may complex significantly with copper are assumed to have been removed by uv treatment.

To maintain constant pCu, pH must remain constant. In cultures of clone 3H containing $\leq$ 5 mM tris, pH was controlled by the presence of tris and by aeration through sintered glass frits. Cultures containing higher tris concentrations were not aerated because in these, pH buffering by tris was sufficient to control pH. Due to photosynthetic removal of CO_2, the pH in cultures of clone 3H did not remain strictly constant, but increased slightly as the cultures grew (usually by less than 0.1 pH unit). Median pH values for 3H cultures are reported in Table 2 with error limits indicating the pH range of individual cultures.

The effect of cell metabolism on pH was not a critical factor for cultures of GSB nanno because of a combination of small cell size (1-2 μm) and relatively slow growth rate. Cultures of GSB nanno in M-f/2 media containing no tris show no detectable influence on pH at cell concentrations $\leq 1 \times 10^6$ cells/ml at a growth rate of about 0.8 divisions/day. In all GSB nanno cultures for which pCu is calculated, both growth rates and cell concentrations are less than the above values and thus the presence of the cells should not have altered pH. pH in these cultures (Table 2) was measured immediately before culture inoculation.

3. Results and discussion

Table 2 gives a compilation of parameters and results of culture experiments. Included in this table are 6 experiments with clone 3H in M-f/2 seawater media containing different concentrations of copper and tris, an experiment with clone GSB-nanno in M-f/2 media containing 10 mM tris and variable copper concentrations, and an experiment in uv-treated and untreated coastal seawater with different additions of copper. In most experiments with both clones 3H and GSB nanno,

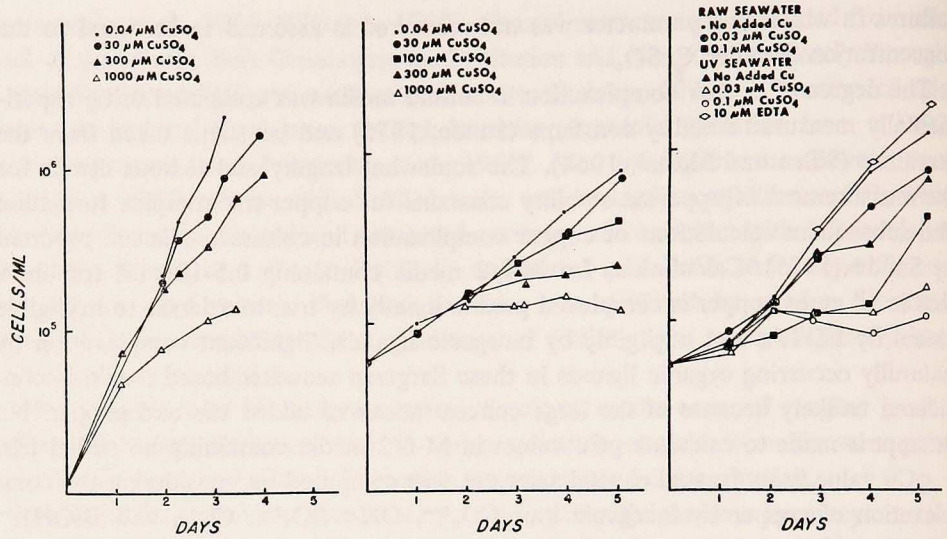

Figure 1. Growth curves: (A) clone 3H in M-f/2 medium plus 10 mM tris; (B) clone GSB nanno in M-f/2 medium plus 10 mM tris; (C) clone GSB nanno in uv treated and untreated coastal seawater.

copper caused little or no inhibition of cell divisions until 1.5-2 days after culture inoculation (Fig. 1). Because of this delayed effect, growth rates were determined for the time period ~ 2-4 days (clone 3H) or ~ 2-5 days (clone GSB nanno) after initiation of the cultures.

Experiment 1 (Table 2) tested the effect of tris alone on the growth rate of clone 3H in M-f/2 medium. The addition of 1, 5, or 10 mM tris had little effect on growth rate; however, 20 mM tris was noticeably inhibitory. In all subsequent experiments with clone 3H, tris concentrations do not exceed 10 mM and thus, tris should not be directly inhibitory.

The presence of 10 mM tris in experiment 7 (Table 2) also was not toxic to clone GSB nanno. A culture in M-f/2 medium containing 10 mM tris had a growth rate of 0.84 divisions/day which is in good agreement with those in M-f/2 media containing no tris (0.78, 0.76, and 0.81 divisions/day).

Relation between growth rate inhibition and pCu for clone 3H. The relation between growth rate inhibition and total copper concentration is strongly influenced by tris concentration and by pH (Fig. 2). At constant copper concentration and pH, an increase in tris concentration decreases growth rate inhibition. Likewise at constant copper and tris concentrations, an increase in pH also decreases inhibition. Because of hydrogen ion competition, the chelation of copper by tris is a strong increasing function of pH (Sunda, 1975; Bai and Martell, 1969). Thus an increase in tris concentration or in pH causes an increase in copper chelation which in turn is related with decreased copper toxicity.

Table 2. Parameters and Results of Culture Experiments

Experiment	Seawater medium	algae	Tris (mM)	Cu_A* (μM)	Cu_M** (μM)	pH†	pCu††	growth rate‡ (div/day)	Cu/cell (10^{-17} moles/cell)
1	M-f/2	clone 3H	0	0.04		8.07 ± 0.01		1.87 ± 0.07	
			1	0.04		8.07 ± 0.00	12.3	1.93 ± 0.05	
			5	0.04		8.16 ± 0.10	12.7 ± 0.2	1.81 ± 0.06	
			10	0.04		8.15 ± 0.07	13.1 ± 0.2	2.02 ± 0.04	
			20	0.04		8.14 ± 0.06	13.7 ± 0.2	1.28 ± 0.10	
2	M-f/2	clone 3H	10	0.04		8.16 ± 0.06	13.1 ± 0.2	2.06 ± 0.07	
			10	3		8.16 ± 0.05	11.25 ± 0.14	2.04 ± 0.11	
			10	5		8.15 ± 0.05	10.99 ± 0.14	1.93 ± 0.17	
			10	10		8.16 ± 0.06	10.71 ± 0.17	1.92 ± 0.04	
			10	20		8.16 ± 0.06	10.40 ± 0.17	1.52 ± 0.05	
			10	30		8.16 ± 0.06	10.22 ± 0.17	1.29 ± 0.17	
			10	100		8.14 ± 0.04	9.61 ± 0.11	1.27 ± 0.10	
			10	200		8.15 ± 0.05	9.32 ± 0.14	1.21 ± 0.12	
			10	500		8.15 ± 0.06	8.86 ± 0.17	1.15 ± 0.06	
			10	1000		8.14 ± 0.04	8.43 ± 0.11	0.23 ± 0.03	
3	M-f/2	clone 3H	0	10		8.23 ± 0.03		0.0 ± 0.0	
			3	30		8.16 ± 0.04	9.16 ± 0.11		23
			3	100		8.13 ± 0.03	8.49 ± 0.09	0.86 ± 0.07	85
			10	0.04		8.15 ± 0.05	13.1 ± 0.1	2.16 ± 0.10	
			10	30		8.16 ± 0.04	10.22 ± 0.17	1.38 ± 0.12	
			10	100		8.15 ± 0.05	9.64 ± 0.14	1.40 ± 0.06	
			10	300		8.15 ± 0.04	9.12 ± 0.11	1.18 ± 0.10	
			10	1000		8.14 ± 0.04	8.43 ± 0.11	0.40 ± 0.11	

Table 2 (continued).

Experiment	Seawater medium	algae	Tris (mM)	Cu_A* (μM)	Cu_M** (μM)	pH†	pCu††	growth rate‡ (div/day)	Cu/cell (10^{-17} moles/cell)
4	M-f/2	clone 3H	1	5	5.2	8.13 ± 0.03	8.96 ± 0.09	1.17 ± 0.12	30
			1	10	10.1	8.13 ± 0.03	8.61 ± 0.09	1.13 ± 0.05	40
			2	10	10.4	8.12 ± 0.03	9.19 ± 0.09	1.22 ± 0.10	26
			2	20	20.3	8.11 ± 0.02	8.84 ± 0.06	1.14 ± 0.09	32
			2	50	48.3	8.12 ± 0.02	8.45 ± 0.06	0.66 ± 0.05	
			5	30	31.3	8.10 ± 0.01	9.42 ± 0.03	1.22 ± 0.10	19.2
			5	80		8.10 ± 0.02	8.98 ± 0.06	1.12 ± 0.01	32
5	M-f/2	clone 3H	0.5	50	44.9	8.10 ± 0.01	7.28 ± 0.03	0.04	
			2	4	3.2	8.15 ± 0.04	9.89 ± 0.11	1.46 ± 0.21	7.0
			3	10	9.9	8.13 ± 0.02	9.59 ± 0.06	1.29 ± 0.09	13.1
			3	20	19.9	8.14 ± 0.03	9.29 ± 0.09	1.19 ± 0.09	19.4
			5	4	3.1	8.12 ± 0.02	10.57 ± 0.06	1.99 ± 0.13	2.1
			5	10	10.1	8.10 ± 0.01	9.94 ± 0.03	1.31 ± 0.34	7.2
			5	20	20.1	8.10 ± 0.01	9.62 ± 0.03	1.24 ± 0.12	13.2
			5	50	49.4	8.09 ± 0.01	9.18 ± 0.03	1.18 ± 0.23	23.8
			5	400		8.10 ± 0.01	8.15 ± 0.03	—0.04 ± 0.01	

6	M-f/2	clone 3H	10	0.04	7.74 ± 0.00	12.4	2.16 ± 0.10
			10	10	7.73 ± 0.04	9.50 ± 0.11	1.33 ± 0.01
			10	100	7.71 ± 0.03	8.38 ± 0.09	0.52 ± 0.03
			10	0.04	8.21 ± 0.04	13.3 ± 0.1	2.19 ± 0.11
			10	100	8.17 ± 0.05	9.69 ± 0.14	1.41 ± 0.03
			10	0.04	8.70	14.6	1.32 ± 0.03
			10	100	8.70 ± 0.03	11.20 ± 0.09	2.09 ± 0.15
7	M-f/2	clone GSB nanno	10	0.04	8.10	13.0	0.84 ± 0.04
			10	30	8.10	10.05	0.73 ± 0.06
			10	100	8.10	9.49	0.47 ± 0.06
			10	300	8.10	8.98	0.16 ± 0.05
			10	1000	8.10	8.32	—0.02 ± 0.05
8	Raw Coastal Seawater	clone GSB nanno	0	0	8.2		1.04 ± 0.12
			0	0.03	8.2		0.83 ± 0.06
			0	0.10	8.2		0.70 ± 0.20
	UV Treated Coastal Seawater		0	0	8.1		0.72 ± 0.13
			0	0.03	8.1	9.2	0.24 ± 0.13
			0	0.10	8.1	8.7	—0.06 ± 0.09
	UV Seawater plus 10 μM EDTA		0	0	8.1		1.06 ± 0.06

* Concentration of added $CuSO_4$
** Measured concentration of copper
† Median culture pH — error limits give the range in culture pH during experimental runs
†† Median culture pCu — error limits indicate the range due to variation in pH
‡ Growth rate error limits represent ± 1 standard deviation

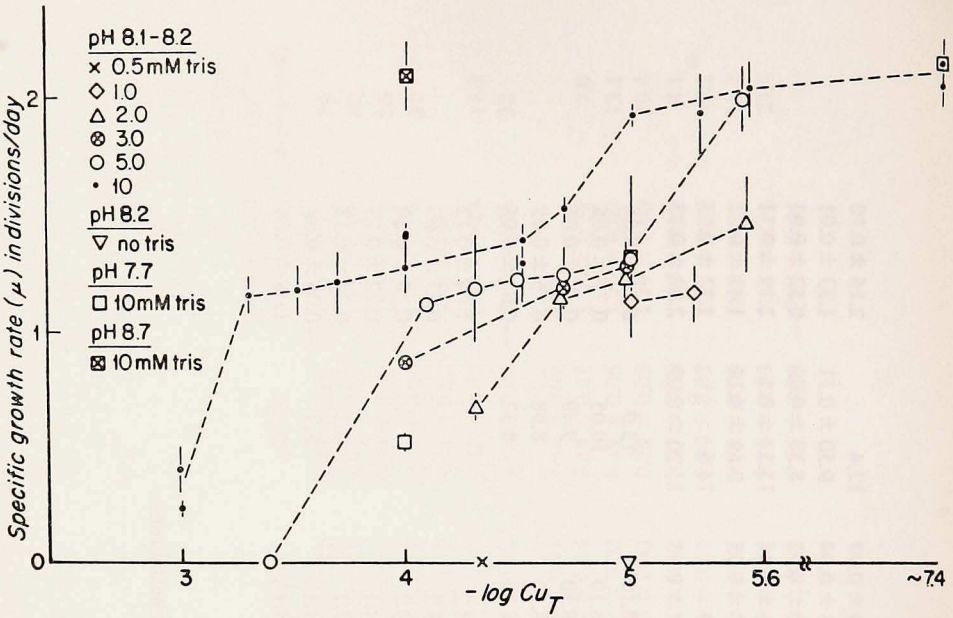

Figure 2. Growth rate of clone 3H vs the negative log of the total copper concentration in M-f/2 seawater media containing 0.10 mM tris at pH 7.7 to 8.7. Results are from experiments 1-5. Error bars represent the standard deviation for least squares linear regression of growth curves.

Culture growth rate plotted as a function of calculated pCu in media containing 0.5 to 10 mM tris (Fig. 3), falls on a single curve confirming the hypothesis that growth inhibition by copper is functionally related to pCu irrespective of the total copper concentration in the culture medium. The relationship between growth rate inhibition and pCu is independent of pH in the range 7.7 to 8.7. Thus the decrease in copper toxicity brought about either by increased tris concentration or by increased pH can be accounted for totally by the resulting change in pCu.

Copper growth inhibition is a two-stepped function of pCu possessing three inflections at pCu values of approximately 8.4, 9.4, and 10.5. Copper inhibits the growth of clone 3H at pCu values below approximately 10.6 with total growth inhibition occurring at pCu values below 8.3.

Other characteristics of copper inhibition correlate well with the two-stepped nature of the growth inhibition curve. For the step in the inhibition curve occurring in the narrow pCu range 8.6 to 8.3, copper inhibition is associated with cellular elongation and morphological distortion. Above this range, at pCu values 8.6 to 10.6, copper causes a delayed inhibition of growth, but does not cause a change in cell morphology or size. Below pCu 8.2 growth rate inhibition occurs immediately and there is no increase in cell size.

In the pCu range 13.3 to 10.6, growth rate is maximal at about 2 divisions/day

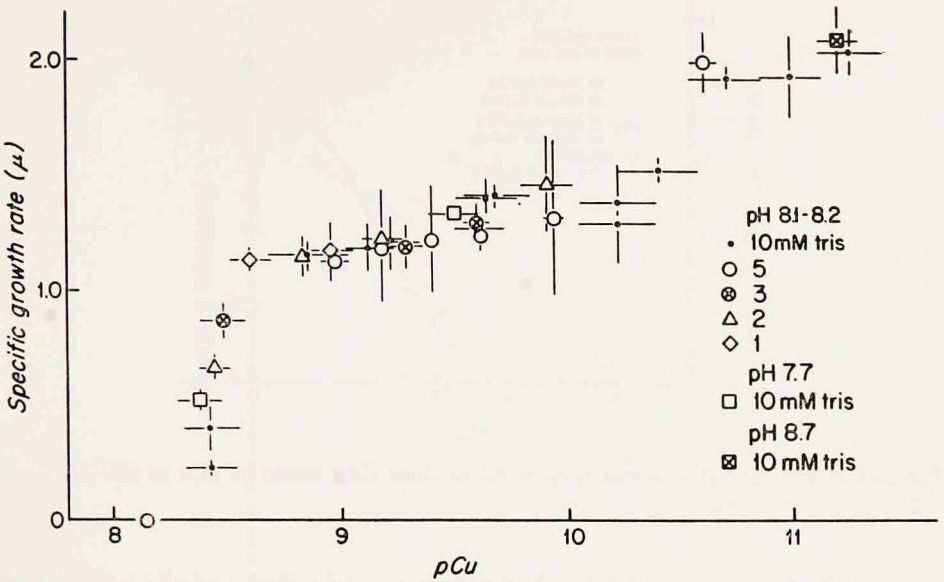

Figure 3. Growth rate of clone 3H vs pCu in M-f/2 seawater culture media containing 1-10 mM tris for the pH range 7.7 to 8.7. Individual points are from experiments 1-5. The pCu error bars represent variation in pCu caused by changes in culture pH.

and is independent of cupric ion activity. A nutritional deficiency resulting in 40% reduction of growth rate appears to occur at a pCu of 14.6 in a culture containing 0.04 μM $CuSO_4$, 10 mM tris, at pH 8.7 (exp. 6, Table 2). In a similar medium containing 100 μM $CuSO_4$ (pH 8.7 and pCu 11.2) maximal growth rate is observed indicating that the low growth rate at pCu 14.6 was due to copper deficiency and not to high pH.

A general pattern emerges pointing out the dual nature of copper as both a cell nutrient and toxin. At an intermediate pCu range of 10.6 to at least 13.3, cupric ion activity is nonlimiting to cell growth. Nutritional deficiency limits growth rate at pCu values above this optimal range. At pCu values below this range, copper toxicity occurs.

Relationship between the growth rate inhibition and pCu for clone GSB nanno. In experiment 8 (Table 2, Fig. 1C), treatment of coastal seawater with ultraviolet light resulted in a 30% reduction in the growth rate of clone GSB nanno, an effect that was completely reversed by the addition of 10 μM EDTA. These results suggest that the deleterious effect of uv treatment was caused by a removal of naturally occurring organic ligands (Barber, 1973). Copper added at concentrations of 0.03 and 0.1 μM is significantly more toxic in the uv treated water than in untreated water consistent with a higher degree of copper complexation in the untreated water, and again suggesting the removal of organic ligands by uv treatment.

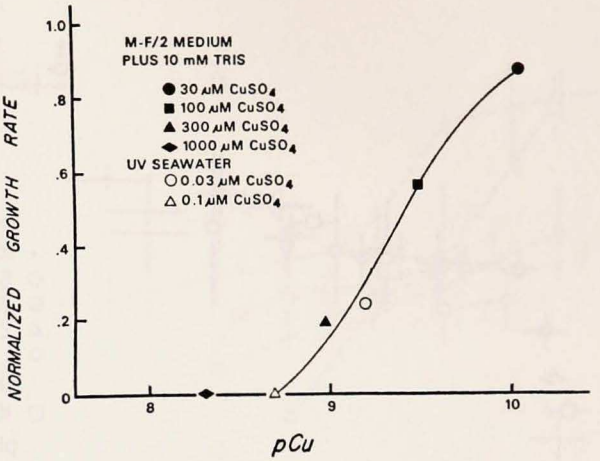

Figure 4. Normalized growth rate (μ/μ max) of clone GSB nanno vs pCu at pH 8.1. Results from experiments 7 and 8.

Normalized growth rate (μ/μ_{max}) of copper treated cultures of clone GSB nanno in M-f/2 medium containing 10 mM tris and in uv treated coastal seawater correlates with calculated pCu despite 4 orders of magnitude differences in total copper concentration (Fig. 4). In M-f/2 medium containing 10 mM tris, μ_{max} is the growth rate of a culture containing 0.04 μM $CuSO_4$(pCu ~ 13.0). In uv treated seawater, μ_{max} is the growth rate of a culture containing 10 μM EDTA and no added copper (estimated pCu > 13 assuming a background copper concentration of < 0.03 μM). Results in Fig. 4 indicate that relationships between cell inhibition and cupric ion activity obtained in highly buffered media such as those containing high tris and copper concentrations also apply in poorly buffered media such as seawater containing little or no natural organic ligands.

Growth rate of clone GSB nanno is inhibited at pCu values below ~ 10.4 with total growth rate inhibition occurring at values below 8.7. The pCu range for partial growth rate inhibition of clone GSB nanno (~ 10.4 to 8.7) falls within that for clone 3H (10.6 to 8.3). Unlike the growth inhibition curve for clone 3H, the inhibition curve for clone GSB nanno does not show a distinct two-stepped behavior.

Relationship between cell copper content and pCu for clone 3H. Cellular copper content of clone 3H is plotted as a function of total copper concentration (Fig. 5) and pCu (Fig. 6) for cultures containing 1-5 mM tris at pH 8.1-8.2 (exp. 3, 4, and 5; Table 2). In the pCu range of the culture media (8.6 to 10.6), cell morphology and cell size are unaffected by copper, and thus mean cell surface area and mean cell volume are constant. Cell copper contents of cultures in the pCu range 8.3-8.6 are excluded from these figures because of an unmeasured increase in cell size.

Increasing the concentration of tris in the medium from 1 to 5 mM markedly

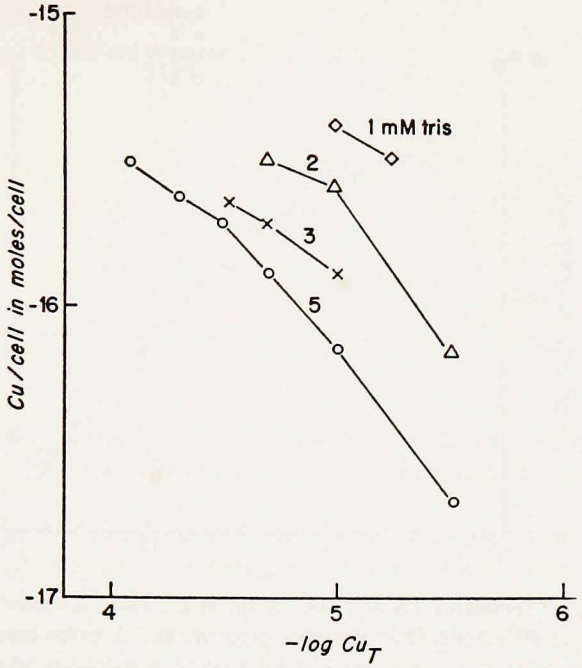

Figure 5. Cell copper content of clone 3H vs negative log of the total copper concentration for cultures containing 1, 2, 3 and 5 mM tris at pH 8.1-8.2. Results from experiments 3-5.

decreases cellular copper content at equivalent total concentrations of copper (Fig. 5). Copper content shows good correlation with pCu (Fig. 6). Thus the decrease in copper content of cells brought about by increased tris concentration is accounted for totally by an increase in copper complexation. The results confirm the hypothesis that copper uptake by algae is related to the free cupric ion activity and is independent of the total copper concentration.

The relationship between cellular copper content and cupric ion activity shows good agreement with the hyperbolic function:

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

where a_{Cu} is the cupric ion activity in the culture medium (Fig. 6).

Comparison of cellular copper content and growth rate inhibition for clone 3H. Growth rate of copper inhibited cultures of clone 3H correlates with cellular copper content (Fig. 7). This does not necessarily imply a causal relationship between copper uptake and cell inhibition, i.e. the major copper uptake site may not be the site of copper inhibition. Comparative plots of cellular copper content vs. pCu and growth rate inhibition vs. pCu (Fig. 8) show that the maximum slope in the cell

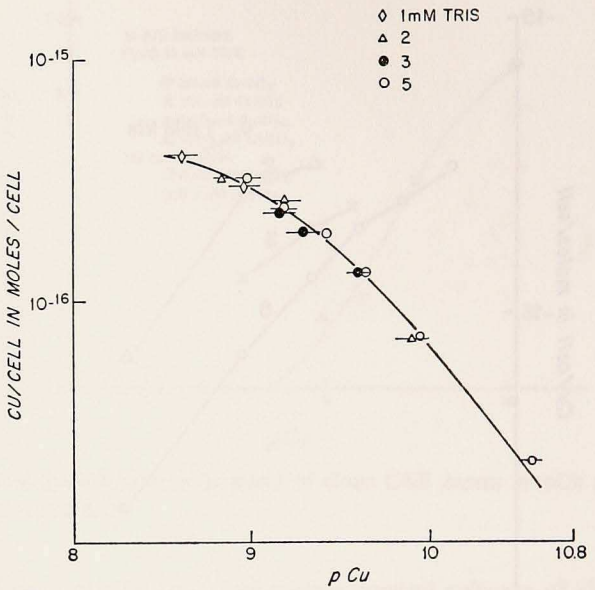

Figure 6. Cell copper content of clone 3H vs pCu in M-f/2 media containing 1-5 mM tris and 3-80 μM $CuSO_4$ at pH 8.1-8.2. Data are from experiments 3-5. Error bars represent variation in pCu due to changes in culture pH. The solid curve is a log-log plot of the hyperbolic function: $\text{Cu/cell} = \dfrac{4.8 \times 10^{-16}\, a_{Cu}}{a_{Cu} + 10^{-9.2}}$

copper content curve (at pCu 9.2) occurs in the range of minimum change in growth inhibition. The marked difference between the shapes of the copper content and growth inhibition curves suggests different cellular sites for copper uptake and inhibition. This difference further implies that the major pool of copper in the cells does not affect cell division.

The two-stepped nature of the inhibition curve and associated changes in cell size and shape characteristics suggest that copper inhibition involves at least two separate inhibition sites: a first that is affected by copper at pCu values below 10.6 and a second that is affected by pCu values below 8.6.

The hyperbolic relationship between cell copper content and cupric ion activity is consistent with the reversible binding of copper to a single cellular ligand site or to several similar sites with a mean maximum binding capacity of 5×10^{-16} moles/cell and an apparent association constant of $10^{9.2}$. However, other mechanisms, such as site or carrier limited membrane transport, also predict hyperbolic relationships.

Copper toxicity in seawater. Our experiments demonstrate that growth inhibition of clones 3H and GSB nanno is dependent on pCu. The pCu levels that inhibit these clones are now compared with an estimated pCu range for seawater.

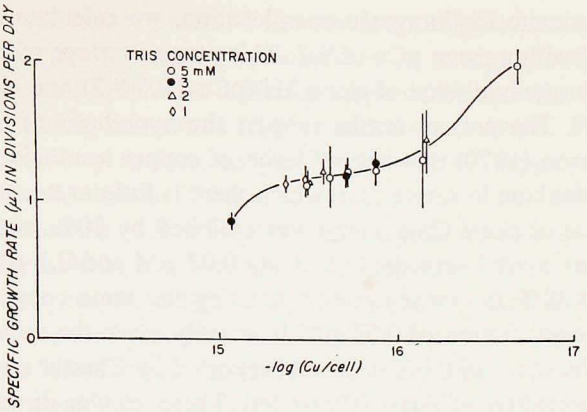

Figure 7. The relationship between the growth rate of clone 3H and the negative log of the cell copper content (in moles/cell) at pH 8.1-8.2. Results are from experiments 3-5.

To estimate seawater pCu, we need to know both total copper complexation and total copper concentration. As a first approximation we shall assume that copper is not complexed significantly by organic ligands and thus assume that copper speciation is determined only by complexation to inorganic ligands. This gives a minimum estimate of copper complexation and thus a maximum estimate of cupric ion activity. Calculations of inorganic complexation in seawater (chlorinity 19‰, temperature 25°C, and pH 8.2) yields a ratio for the activity of cupric ion to the total copper concentration of $10^{-1.8}$ (Sunda, 1975). Chester and Stoner (1974) report copper concentrations in the range 0.002 to 0.06 μM with a mean value of 0.012 μM for 38 samples of open ocean seawater. Using these concentration values

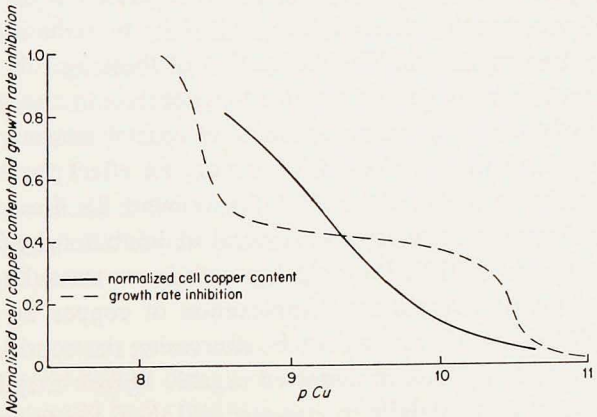

Figure 8. Normalized cell copper content $\frac{a_{Cu}}{a_{Cu} + 10^{-9.2}}$ and normalized growth rate inhibition $(1 - \mu/\mu_{max})$ vs pCu for clone 3H. μ_{max} is the mean growth rate of clone 3H in the pCu range 11-13.

and the above estimate for inorganic complexation, we calculate a seawater pCu range of 10.5-9.0 with a mean pCu of 9.7. This range overlaps with the pCu range for partial growth rate inhibition of clone 3H (pCu 10.6-8.3) and clone GSB nanno (pCu ~ 10.4-8.7). The present results support the hypothesis of Steeman Nielsen and Wium-Anderson (1970) that natural levels of copper are toxic to at least some species of phytoplankton in seawater in which there is little or no organic chelation.

The growth rate of clone GSB nanno was inhibited by 80% and 100% respectively in uv treated coastal seawater containing 0.03 μM and 0.1 μM added $CuSO_4$ and by 20% and 30% in raw seawater containing the same copper additions (experiment 8). A concentration of 0.03 μM is roughly twice the mean of 0.014 μM and falls within the range of 0.005-0.06 μM reported by Chester and Stoner (1974) for copper concentration in coastal seawater. These results directly support the hypothesis that natural levels of copper in seawater can be toxic to phytoplankton.

Experiments with *T. pseudonana* (clone 13-1) in uv treated artificial seawater (Davey *et al.*, 1973) show a sensitivity of this clone to trace levels of copper. Artificial seawater, used in these experiments, was passed through a chelex resin column which reduced background copper to less than 0.016 μM. Growth rate of cultures containing 0.016 and 0.03 μM added Cu were inhibited by 25% and 50% respectively after 18-44 hours exposure relative to the growth rate of a control culture containing no added copper. By computing copper complexation by inorganic ligands in the artificial seawater (Sunda, 1975), we estimate pCu values of 9.6 and 9.3 for the above copper concentrations. These pCu values (corresponding to 25% and 50% growth rate inhibition) fall within the range for partial growth rate inhibition of clone 3H (pCu 10.6-8.3) and of clone GSB nanno (pCu ~ 10.4-8.7).

The increased toxicity of added copper in uv treated seawater relative to that in raw seawater (experiment 8) is consistent with the presence of natural organic ligands in coastal seawater that decrease copper toxicity by reducing the fraction of copper present as free cupric ion. The destruction of these ligands by uv radiation would cause an increase in copper toxicity due to a decrease in copper complexation.

In the absence of added copper, uv treatment of coastal seawater caused a 30% reduction in the growth rate of clone GSB nanno, an effect that was completely reversed by the addition of 10 μM EDTA (experiment 8). Growth inhibition of algae in uv treated surface seawater and reversal of inhibition by EDTA has also been reported by Barber (1973). Poor algal growth in uv treated seawater may be caused by copper toxicity. Again the complexation of copper by natural organic ligands or by EDTA would reduce toxicity by decreasing the cupric ion activity.

Spatial and temporal variation in dissolved organic ligands may be an important factor controlling cupric ion activity in seawater and thus, the toxicity of copper to phytoplankton. Davey *et al.* (1973) attributed differences in the toxicity of added copper in different samples of coastal seawater to variation in copper complexation by organic ligands. Compared to open ocean seawater, coastal seawater may have

a relatively high content of natural chelators due to high concentrations of organic matter. Poor growth of natural phytoplankton in newly upwelled seawater, and stimulation of growth by EDTA addition, suggest that upwelled seawater contains relatively few chelating substances (Barber and Ryther, 1969). Reduced growth in upwelled seawater may at least in part be caused by copper toxicity (Steeman Nielsen and Wium-Anderson, 1970).

Variations in seawater pH may also affect copper complexation. Complexation of copper by inorganic ligands increases with increasing pH due to a predominance of carbonate and hydroxide complexes (Schindler, 1967; Sunda, 1975). In addition, the chelation of copper by most organic ligands is also dependent on pH. Experiments with clone 3H showed that at constant total copper and tris concentrations, changes in pCu caused by variations in pH significantly altered copper toxicity (Fig. 2).

Changes in surface seawater pH associated with upwelling off the Oregon coast have been described by Park (1968). During the fall and winter, when upwelling did not occur, seawater pH was vertically stratified with a maximum of 8.1 to 8.3 at the surface, decreasing to a pH minimum of 7.7 at 200-300 meters depth. During the summer, upwelling along the coast advected pH 7.7 seawater to the surface and created a sharp pH gradiant (7.7 to 8.2) perpendicular to the shore. Again, if we assume only inorganic complexation of copper in the upwelled seawater and assume a copper concentration of 0.014 μM (a mean coastal value; Chester and Stoner, 1974), then we estimate a seawater pCu of 9.2 at pH 7.7 and 9.6 at pH 8.2 (Sunda, 1975).

What effect might such a pH induced pCu change have on copper toxicity? For clone 3H the relation between growth rate inhibition and pCu was independent of pH in the pH range 7.7 to 8.2. For this clone a pH induced pCu change from 9.6 to 9.2 would cause only a small increase in growth inhibition (approximately 3%). Assuming that the relationship between growth inhibition and pCu is also independent of pH for clone GSB nanno, the above pH induced pCu change would cause growth rate inhibition for this clone to increase from 35% at pH 8.2 to 70% at pH 7.7 (see Fig. 11).

Along with variations in the concentrations of natural organic ligands, changes in seawater pH should be an important factor controlling cupric ion activity, and therefore, copper toxicity to phytoplankton.

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