The response of a marine bacterium to cupric ion and its use to estimate cupric ion activity in seawater

by William G. Sunda[1] and Paul A. Gillespie[2]

ABSTRACT

Experiments were conducted to determine the relationship between the response of a bacterial isolate to copper, as measured by cellular incorporation of ^{14}C-glucose, and the complexation of copper by organic ligands. Inhibition of glucose incorporation was dependent on the cupric ion activity and independent of the concentration of organic complexes of copper both in UV-treated seawater (36‰) containing different concentrations of a model chelator, nitrilotriacetate (NTA), and in low salinity media (1.8‰) containing varied amounts of commercially-prepared or river-borne humic compounds. The relationship between inhibition of glucose incorporation and cupric ion activity at the two salinities fit an equation derived from a molecular binding model:

$$I/I_{max} = \frac{1}{1 + A^2_{Cu}K^*}$$

where I is the rate of glucose incorporation in the presence of added copper, I_{max} is the rate in the absence of cupric ion inhibition, and A_{Cu} is the cupric ion activity. The value for K^*, a cellular inhibition site binding constant, was slightly higher at 1.8‰ salinity ($K^* = 10^{18.8}$) than at 36‰ salinity ($K^* = 10^{18.3}$).

An estimate of the relationship between cupric ion activity and total copper concentration in 35‰ estuarine seawater was obtained from a comparison of bacterial response to added copper in the natural seawater with the bacterial response-cupric ion activity relationship in Cu-NTA-seawater media. The data were consistent with the presence of a highly reactive ligand, probably a chelator, in the natural seawater at an equivalent concentration of 0.05 μM and with a conditional stability constant at pH 8.1 of $\geqslant 10^{10}$. Based on these values and an ambient copper concentration of 0.014 μM (0.9 ppb), cupric ion activity of the estuarine seawater is estimated to be $\leqslant 10^{-11}$M.

1. Introduction

Copper can be toxic to marine algae and bacteria at concentrations as low as 1 to 2 ppb (Davey *et al.*, 1973; Gillespie and Vaccaro, 1978), but is also an essential trace metal for growth and metabolism. Thus, either an oversupply or undersupply

1. National Marine Fisheries Service, NOAA, Southeast Fisheries Center, Beaufort Laboratory, Beaufort, North Carolina, 28516, U.S.A.
2. Cawthron Institute, Nelson, New Zealand.

of this element may limit photosynthesis and heterotrophic activity in natural waters.

For algae, cellular uptake, toxicity, and nutritional availability of copper have been shown to be related to cupric ion activity, but not the concentration of chelated copper (Manahan and Smith, 1973; Sunda and Guillard, 1976; Anderson and Morel, 1978; Sunda and Lewis, 1978; Jackson and Morgan, 1978). Despite its importance in determining the bioavailability of copper to algae and other organisms (Andrew *et al.*, 1977; Waiwood and Beamish, 1978; Chakoumakos *et al.*, 1979), there is considerable uncertainty concerning ambient cupric ion activities in seawater. This uncertainty results from variable, and as yet poorly quantified, levels of copper complexation to organic ligands.

There have been some attempts to estimate copper chelation in seawater using bioassays. Davey *et al.* (1973) measured the extent to which added copper inhibited the growth of a marine diatom in artificial seawater containing different concentrations of EDTA or histidine and in samples of coastal seawater. By comparing the toxicity of dissolved copper in the presence of known chelators with that in natural seawater, they obtained semiquantitative estimates of copper chelation capacity (i.e. chelator concentration) of natural seawater. Gillespie and Vaccaro (1978) used a similar technique with bacteria to estimate the chelation capacity of different marine waters. These experiments, although providing useful information concerning relative concentrations of copper binding ligands, did not quantify the extent of copper complexation in terms of the fraction of total copper present as free cupric ion. Knowledge of binding capacity alone is not sufficient to compute cupric ion activity; one also needs to know stability constants of the natural chelators as well as the concentration of total dissolved copper.

We conducted experiments with the isolate used by Gillespie and Vaccaro to investigate the use of bacterial bioassays to determine the extent of copper complexation by organic ligands in seawater. As a prerequisite for a quantitative bioassay of copper chelation, experiments were conducted to determine if copper toxicity to bacteria is indeed quantitatively related to cupric ion activity, and if so, what that relationship is. Complexation in these studies was varied by addition of different concentrations of synthetic or natural chelators. A comparison of the inhibition response of the bacteria to dissolved copper in defined seawater media in which cupric ion activity could be calculated with that in natural seawater was used to estimate cupric ion activity as a function of total copper concentration in estuarine seawater. From this data we estimated the equivalent concentration of organic ligands and a conditional stability constant for complex formation with copper.

2. Materials and methods

Bacterial isolate. A short, gram (–), euryhaline, motile rod isolated from Saanich Inlet, British Columbia was used in these experiments. Procedures and media used

for growing, harvesting, and washing of the bacteria are given by Gillespie and Vaccaro (1978).

Experimental media. Two different experiments were conducted: one in full strength seawater with and without different concentrations of a synthetic chelator, nitrilotriacetic acid (NTA), and a second in dilute (1.8‰ salinity) seawater containing different concentrations of natural organic ligands (humic substances). Offshore seawater (36‰) was collected from the Gulf Stream off the coast of North Carolina and estuarine seawater (35‰) was collected at high tide from the mouth of the Newport River estuary off the dock of the National Marine Fisheries Laboratory at Beaufort, N.C. Both samples were filtered through glass fiber filters (0.7 μm mean retention size, Sheldon, 1972) within several hours after collection. Offshore seawater was stored for 1 month and dock seawater for 1 day in polypropylene bottles at 4°C. The offshore seawater was exposed for 4 h to high intensity ultraviolet radiation from a 1200 watt mercury lamp (1 week before use) to photooxidize organic matter (Armstrong *et al.*, 1966; Williams, 1969). Seawater samples were refiltered (0.4 μm Nuclepore) several hours before the start of the experiment.

Background concentrations of copper in the refiltered dock seawater and UV-treated seawater were measured by flameless atomic absorption spectrophotometry (Perkin Elmer model 403). Samples were analyzed by direct injection using a standard additions technique. One-half millimole of NH_4NO_3 was added per milliliter of seawater to reduce matrix interferences caused by sea salt (Ediger *et al.*, 1974).

The second experiment was conducted in low salinity media containing 5% by volume UV-treated seawater and different concentrations of natural organic ligands and $CuSO_4$. Cupric ion activities were measured directly with a cupric ion-selective electrode. The low salinity of these media was required to avoid chloride interference of the electrode (Sunda, 1975). Two of these media contained different amounts (21.2 and 4.2 mg $\cdot$ l^{-1} on an ash free basis) of commercially prepared humic acid (ICN Pharma.). Two others contained 95% and 20% by volume of Newport River water. The river water contained a high concentration of dissolved organic matter (22 mg C $\cdot$ l^{-1}) that apparently is composed primarily of humic and fulvic acids. Organic ligands in this water form stable complexes with copper (Sunda and Hanson, 1979). Additional details concerning the chemical composition of the river water are given by Sunda and Lewis (1978). The river water was filtered (glass fiber) within 1 day after collection and stored in a borosilicate glass carboy at 4°C for 2 months before use. Spectrophotometric measurements of light absorption in the wavelength range 700-250 nm indicated little detectable change in the concentration or composition of the organic matter during storage. Also measurements with a cupric ion-selective electrode at 1 week and 2 months showed that storage caused no demonstrable change in the complexing characteristics of the organic

matter. One millimole $NaHCO_3$ was added per liter of dilute seawater media to adjust pH to ~ 8. Media were filtered through 0.4 μm Nuclepore filters before use.

Experimental procedure. Experiment 1. Ten-milliliter portions of UV-treated or dock seawater were dispensed in triplicate into 15 ml screw cap vials that had been coated with silicone to minimize adsorptive loss of copper (Erickson *et al.*, 1970). Different quantities of $CuSO_4$ and NTA were added to the seawater after which the test media were equilibrated for 2 h. Washed bacteria suspended in filtered seawater (Gillespie and Vaccaro, 1978) were then inoculated into the test media at a concentration of ~ 2×10^6 cells • ml^{-1} followed by a preincubation period of 2 h. After preincubation, 0.2 ml of a 0.5 μCi • ml^{-1} solution of glucose (New England Nuclear, specific activity ~ 200 Ci • mol^{-1}) was added to each tube. The bacteria were incubated for 0.5 h after which glucose uptake was terminated by adding 0.5 ml of a 10% formaldehyde solution. The bacteria were then filtered onto 0.45-μm Millipore filters and the glucose incorporated by the bacteria was measured using standard liquid scintillation counting techniques. Incorporation values were corrected for filter blanks of UV-treated or dock seawater containing no added bacteria. All values are reported in counts per minute (cpm ± SD) which are proportional to disintegrations per minute since quench errors were constant. Three replicates were run per treatment.

The procedure for experiment 2 was the same as that in experiment 1 except for a change in the order in which the media were dispensed and copper was added. In experiment 2 dilute seawater media were divided into 100-ml portions and placed in silicone-coated 125-ml borosilicate flasks. After additions of $CuSO_4$, 10-ml portions were dispensed in triplicate into silicone-coated vials and the remaining 70 ml was retained for measurements of cupric ion and pH. Both experiments were conducted at 20°C.

Determination of pCu. Cupric ion activities in UV-treated seawater media containing 0, 1.0, or 10 μM NTA and 0.02 to 9 μM dissolved copper were determined from thermodynamic calculations similar to those described by Sunda (1975) and Sunda *et al.* (1978). The equation used in these calculations was:

$$Cu_T = \frac{A_{Cu}}{R} + \frac{A_{Cu}NTA_T\, 10^{12.96}}{A_{Cu}\, 10^{12.96} + A_{Ca}\, 10^{6.41} + A_{Mg}\, 10^{5.41}} \quad (1)$$

where Cu_T and NTA_T are the total dissolved copper and NTA concentrations. A_{Cu}, A_{Ca}, and A_{Mg} are the activities of the subscripted metal ions. The two terms on the right-hand side of Eq. (1) represent the total concentration of inorganic species of copper (primarily Cu^{2+} and carbonato and hydroxo complexes) and the concentration of Cu-NTA complex. The symbol R is the ratio of cupric ion activity to the concentration of dissolved inorganic copper species and is independent of the total dissolved copper concentration. The value for R ($10^{-1.80}$) for the UV-treated sea-

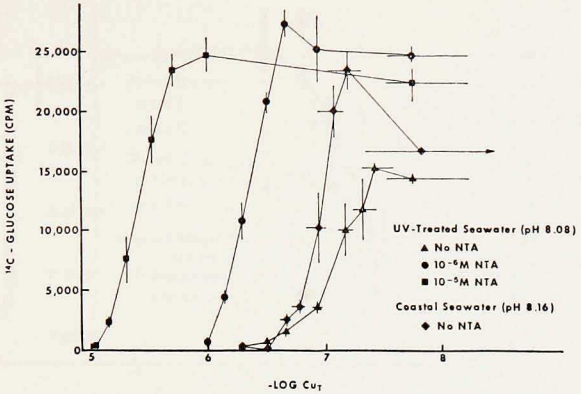

Figure 1. Bacterial incorporation of ^{14}C-glucose in seawater media containing different concentrations of dissolved copper (Cu_T) and NTA. Dissolved copper is the sum of the background copper plus added $CuSO_4$. Horizontal error bars arise from the 95% confidence limits for the analysis of background copper concentrations. Vertical error bars represent ± SD for 3 replicates.

water at pH 8.10 was estimated from complexation data given by Sunda (1975) and from ion-selective electrode measurements of copper complexation in a synthetic seawater medium in which chloride was replaced by nitrate or perchlorate ion (Sunda, unpublished data).

The values $10^{12.96}$, $10^{6.41}$, and $10^{5.41}$ are stability constants for NTA complex formation with Cu^{2+}, Ca^{2+}, and Mg^{2+} respectively (Sillen and Martell, 1964). The activities of calcium and mangesium ions in seawater are 0.0021 and 0.014 M (Sunda, 1975).

Cupric ion activities in dilute seawater media were measured directly with a cupric ion-selective electrode (Orion model 92-29) using procedures outlined by Sunda and Lewis (1978). Measurements of cupric ion activity and pH were made 3-6 h after addition of $CuSO_4$.

All glassware and plasticware used in these experiments were pre-rinsed with 2 N HCl to minimize trace metal contamination.

3. Results and discussion

Relationships between glucose incorporation and cupric ion activity. Bacterial incorporation of glucose at a given concentration of dissolved copper varied markedly among the different seawater-NTA media (Fig. 1). Dissolved copper in UV-treated seawater was least toxic in the presence of 10^{-5}M NTA and most toxic in the absence of NTA. Copper was less toxic in the dock seawater than in the UV-treated seawater without NTA.

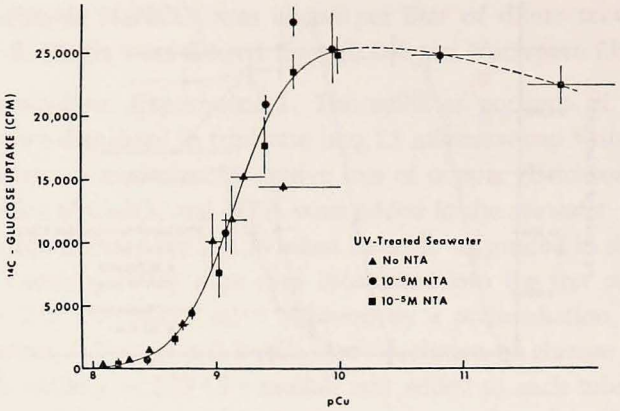

Figure 2. Bacterial incorporation of ^{14}C-glucose as a function of pCu (−log cupric ion activity) in uv-treated seawater containing 0, 10^{-6}, 10^{-5}M NTA. Vertical and horizontal error bars are the same as defined in Figure 1. Solid curve is a semi-log plot of the equation

$$\text{cmp} = \frac{25800}{1 + A_{Cu^2}\, 10^{18.3}} \cdot$$

The incorporation of glucose was depressed (14,400 cpm) in the UV-treated seawater containing no added NTA or copper (background copper = 0.018 μM), but at NTA concentrations of 1.0 and 10 μM, incorporation increased to values of 24,700 and 22,300 cpm (Fig. 1). Labeled glucose incorporation also was depressed (16,600 cpm) in the dock seawater containing no added copper (background copper = 0.014 μM). Increasing the total dissolved copper concentration in this medium to 0.065 μM increased the incorporation of labeled glucose to 23,400 cpm, which is not significantly different from maximum values observed in the seawater-NTA media.

Bacterial incorporation in the UV-treated seawater containing different concentrations of NTA and $CuSO_4$ was directly related to the calculated cupric ion activity (Fig. 2). Thus, essentially all of the variability in glucose uptake with changing dissolved copper and NTA concentrations was accounted for by a single parameter: cupric ion activity. The relationship betwen inhibition of glucose incorporation and cupric ion activity was independent of the total copper concentration in the range 0.02 to 9 μM and the concentration of NTA. Reductions in ambient ionic activities of zinc, cobalt, and nickel resulting from chelation by NTA also had no effect on the sensitivity of the bacteria to cupric ion activity.

Glucose incorporation was maximal at pCu values above ~10. (pCu is defined as the negative log of cupric ion activity). Fifty percent inhibition of glucose incorporation occurred at a pCu of 9.1 with total inhibition at values below 8.3.

In experiments with natural organic matter at 1.8‰ salinity, complexation of copper by organic ligands also decreased the toxicity of added $CuSO_4$ (Fig. 3). The ratio of cupric ion activity to total copper concentration in these media varied

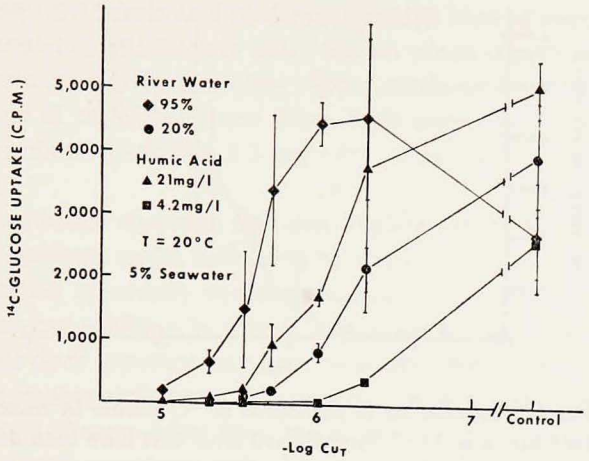

Figure 3. Incorporation of ^{14}C-glucose by bacteria as a function of pCu in 1.8‰ salinity media containing different concentrations of natural organic matter at 20°C and pH 7.8 to 8.1. Total dissolved copper (Cu_T) is assumed to equal the concentration of added $CuSO_4$. Control cultures contain no $CuSO_4$. Vertical error bars give ± SD for 3 replicates.

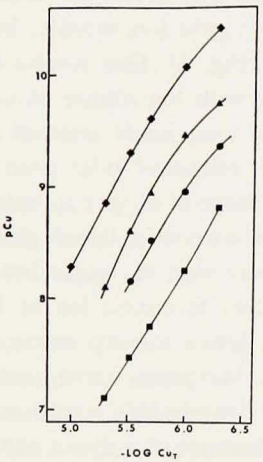

Figure 4. Relations between measured pCu (−log cupric ion activity) and −log Cu_T for 1.8‰ salinity media containing different concentrations of dissolved organic matter. Total dissolved copper (Cu_T) is assumed to equal the added concentration of $CuSO_4$. Increases in pCu at constant Cu_T indicate increased complexation of copper. Complexation increases with increased concentration of dissolved organic carbon (DOC). Symbols: ◆ 95% river water (21 mg DOC • l^{-1}) at pH 7.80 ± 0.05 (± SD); ● 20% river water (4.4 mg DOC • l^{-1}) at pH 7.89 ± 0.03; ▲ 21 mg • l^{-1} humic acid (10 mg • l^{-1} DOC) at pH 8.04 ± 0.01; ■ 4.2 mg • l^{-1} humic acid (2.0 mg • l^{-1} DOC) at pH 8.08 + 0.04.

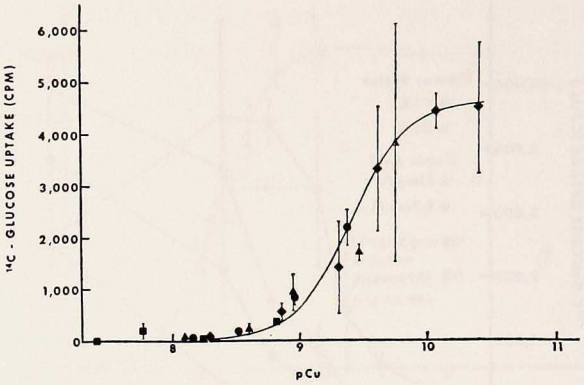

Figure 5. The relationship between the incorporation of ^{14}C-glucose by bacteria and measured *p*Cu in 1.8‰ salinity media at 20°C. Symbols and error bars have been defined in Figure 4. Solid curve is a semi-log plot of the equation:

$$\text{cmp} = \frac{4600}{1 + A_{Cu}{}^2\, 10^{18.8}}$$

from $10^{-1.4}$ to $10^{-4.1}$, a 2.7 order of magnitude change in copper complexation (Fig. 4). Complexation increased with increasing concentration of river water organic matter or humic acid and decreasing concentrations of total dissolved copper. As with the previous experiment with NTA, the deleterious effect of dissolved copper was related exclusively to cupric ion activity, but not to the concentration of organic complexes of copper (Fig. 5). Our results demonstrate that sensitivity to cupric ion activity is invariant with the source of the natural organic ligands; i.e. commercially prepared humic compounds isolated from soils or organic ligands present in river water. Also the relationship between cupric ion activity and copper toxicity holds true over a wide range of copper complexation.

In the 1.8‰ salinity media copper inhibited glucose incorporation below *p*Cu values of 10.1 with total suppression at values below 8.5 (Fig. 5). The bacteria were only slightly more sensitive to cupric ion at 1.8‰ salinity than at 36‰ as fifty percent inhibition at the lower salinity occurred at *p*Cu ~ 9.4 and that in undiluted seawater at ~ 9.1. Maximum incorporation of glucose in the diluted seawater (~ 4,600 cpm) was considerably less than that in full strength seawater (~ 25,000 cpm), presumably because of a direct adverse effect of reduced salinity.

Reduced incorporation of glucose (~ 2,600 cpm) was observed in the two control media with the highest (95% Newport River water) and lowest (4.2 mg • l^{-1} humic acid) level of copper complexation (Figs. 3, 4). The addition of $CuSO_4$ at concentrations of 0.5 and 1.0 μM had opposite effects in these two media. For the medium containing 95% river water, the addition of copper at these levels caused an increase in glucose incorporation (from 2,600 to 4,500 cpm) whereas the addition of the same concentrations of copper caused almost a complete blockage of incorpora-

tion in the 4.2 mg • l^{-1} humic acid medium. The high level of copper complexation in the medium containing 95% river water and no added copper may have resulted in too low a cupric ion activity rendering copper nutritionally deficient. By contrast, background levels of copper or some other toxic trace metal may have been inhibitory in the medium containing 4.2 mg • l^{-1} humic acid due to too low a level of complexation.

Excess and insufficient chelation also may explain variations in bacterial glucose incorporation in seawater media containing no added copper or NTA (Fig. 1). Thus, glucose incorporation apparently was depressed in the UV-treated seawater because of a lack of chelators resulting in a toxic cupric ion activity (Fig. 2), whereas incorporation in the dock seawater may have been depressed because of excess chelation resulting in copper deficiency. Alternatively, it is possible that overchelation caused a deficiency of some other trace metal and that a portion of this metal was displaced from organic complexes with addition of copper.

We also examined the Cu-bacterial inhibition data of Gillespie and Vaccaro (1978) in UV-treated Nantucket seawater (32.5‰) containing 0, 0.2, 0.4, 1, 2 and 4 μM EDTA and 0.03 to 5 μM added copper using procedures analogous to those described in this paper. Cupric ion activity in their media was computed from an equation similar to Eq. (1) using stability constants of $10^{18.79}$, $10^{10.59}$, and $10^{8.69}$ respectively for the formation of copper, calcium and magnesium complexes of EDTA (Sillen and Martell, 1964). As with results in our Cu-NTA-seawater media, the incorporation of glucose by bacteria in their Cu-EDTA media was related to the calculated *p*Cu. Half inhibition occurred at a *p*Cu of $\sim$ 9.1, the same value we obtained in NTA-seawater media. These results strengthen our earlier conclusion that the relationship between copper toxicity and *p*Cu is invariant with the types or concentrations of chelators or with reductions in the activities of other trace metals that form stable complexes with NTA or EDTA. In addition, Albright *et al.* (1972) report that copper inhibition of glucose incorporation by mixed populations of bacteria is noncompetitive which means that the relative extent of copper inhibition is independent of glucose concentration.

Although there were significant regressions in both cases, the plot of ^{14}C-glucose incorporation vs *p*Cu for the Cu-EDTA media of Gillespie and Vaccaro showed more scatter about a single curve ($R^2 = 0.48$; $n = 15$) than that for our Cu-NTA media ($R^2 = 0.88$; $n = 12$). These linear regressions were conducted in the *p*Cu range 8.6 to 9.6 in which ^{14}C-glucose incorporation is an approximate linear function of *p*Cu (Fig. 2). The increased scatter of the data for the EDTA media relative to that with NTA appears to result at least in part from differences in the complexing affinities of the two chelators, EDTA being a much stronger chelator. The following illustrates this point. In Cu-NTA media, inhibition of glucose incorporation varied from 95% to 5% in the *p*Cu range 8.5 to 9.8 (Fig. 2). If we had a 1 μM concentration of chelator in seawater, then the range in total copper concentration

necessary to achieve this *p*Cu range would be 0.15 to 0.96 μM for NTA and 0.93 to 1.19 μM for EDTA. Thus, for the Cu-EDTA system small errors in the addition of copper or EDTA to experimental media would result in relatively large errors in computed *p*Cu and, as a consequence, sizable errors in quantifying the relationship between inhibition and *p*Cu. This is because the end point of a copper titration of EDTA falls within the *p*Cu range of biological interest. Because of such considerations we chose NTA rather than EDTA to control *p*Cu in the present experiments.

Molecular models for cupric ion inhibition. Data relating the inhibition of glucose incorporation to the measured or calculated cupric ion activity fit a second order molecular inhibition model (Figs. 2, 5). The mathematical expression for this model is:

$$\frac{\text{cpm}}{\text{cpm}_{\max}} = I/I_{\max} = \frac{1}{1 + A_{\text{Cu}^2}K^*} \tag{2}$$

where K^* is a constant and I and $I_{\max}$ are rates of glucose incorporation in the presence and absence of cupric ion inhibition. cpm and $\text{cpm}_{\max}$ are defined similarly. Equation 2 was derived from the following chemical relationships and assumptions:

Assume that the rate of glucose incorporation by bacteria is directly related to the biological activity of a critical biomolecule, E, perhaps an enzyme. Assume that some number of cupric ions (n) react chemically with this molecule;

$$n\text{Cu} + E \rightleftarrows \text{Cu}_nE \tag{3}$$

resulting in a biochemical deactivation. If reaction 6 is reversible, then at equilibrium the relationship among the concentrations of free molecule and copper molecular complex, and the activity of cupric ion will be described by a mass action equation:

$$\frac{[\text{Cu}_nE]}{A_{\text{Cu}^n}[E]} = K^* \tag{4}$$

where K^* is a conditional stability constant. The rate of glucose uptake will be proportional to the fraction of molecule E in its active uncomplexed form:

$$I/I_{\max} = \frac{[E]}{E_T} = \frac{[E]}{[E] + [\text{Cu}_nE]} \tag{5}$$

where E_T is the total concentration of E (i.e. "free" plus complexed). By combining Eqs. 4 and 5 we obtain the relationship:

$$\frac{\text{cpm}}{\text{cpm}_{\max}} = I/I_{\max} = \frac{[E]}{[E] + A_{\text{Cu}^n}[E]K^*} = \frac{1}{1 + A_{\text{Cu}^n}K^*} \tag{6}$$

Equation 6 was solved for n and K^* by regression analysis using a log-linear transformation:

$$\log (\text{cpm}_{max}/\text{cpm} - 1) = n \log A_{Cu} + \log K^* \quad (7)$$

To solve Eq. 7 for n and log K^* in UV-treated seawater, we first estimated a value for cpm_{max} (25,000), the maximum observed incorporation in the pCu range 10-11. Values for log (25,000/cpm −1) were then regressed against log A_{Cu} for each data set in the pCu range 8.7 to 9.6 excluding that for UV-treated seawater containing no added Cu or NTA because of the large error in the calculated pCu. The slope of this regression was 2.1 ± 0.1 (± *SD*) indicating that a molecular model in which 2 cupric ions react at the inhibition site would be appropriate. Using a value of n = 2, Eq. 6 was next rearranged into a second linear form:

$$\frac{1}{\text{cpm}} = \frac{1}{\text{cpm}_{max}} + \frac{A_{Cu}^2 K^*}{\text{cpm}_{max}} \quad (8)$$

From the slope and y intercept of the regression of cpm^{-1} vs A_{Cu}^2 we obtained values of cpm_{max} (25,800) and K^* ($10^{18.3}$). Data for cupric ion inhibition of glucose uptake in 1.8‰ seawater were analyzed similarly. In each case the equations derived from the second-order binding model agreed well with the experimental data (Figs. 2, 5).

McBrien and Hassall (1967) have suggested that copper poisoning of *Chlorella* is caused by copper-induced splitting of protein disulfide bonds. A second order inhibition reaction would be consistent with this hypothesis in that the overall reaction would probably involve two copper ions:

$$2\,Cu^{2+} + -S\text{–}S- + 2e^- \rightleftarrows 2\,CuS^{\pm}$$

where $-S\text{–}S-$ is a disulfide bond and e^- is an electron. That copper-induced splitting of disulfide bonds is indeed a second order reaction has been demonstrated by Klotz and Campbell (1962) for the reaction of copper with the model disulfide compound 2,2-(2-hydroxy-6-sulfonaphthyl-l-azo) diphenyl disulfide.

The use of biological response to estimate cupric ion activity in seawater. The inhibition of glucose incorporation was related to cupric ion activity in all media in which this parameter was either calculated or measured. From the quantification of this relationship in seawater (Fig. 2), we can estimate the activity of cupric ion in the copper amended dock seawater, in which there is an unknown level of natural complexation. The solid line in Figure 2 is essentialy a calibration curve relating the amount of glucose incorporated by the bacteria in seawater to pCu values in the region of cupric ion inhibition. Thus, if we know the amount of glucose incorporated, we know the cupric ion activity, and vice versa. We measured glucose incorporation at different concentrations of copper in dock seawater (Fig. 1) and from Eq. 2 computed the corresponding cupric ion activities. Note that this can only be done in the pCu range ~ 8.5 to 9.7 because above this range, glucose incorpora-

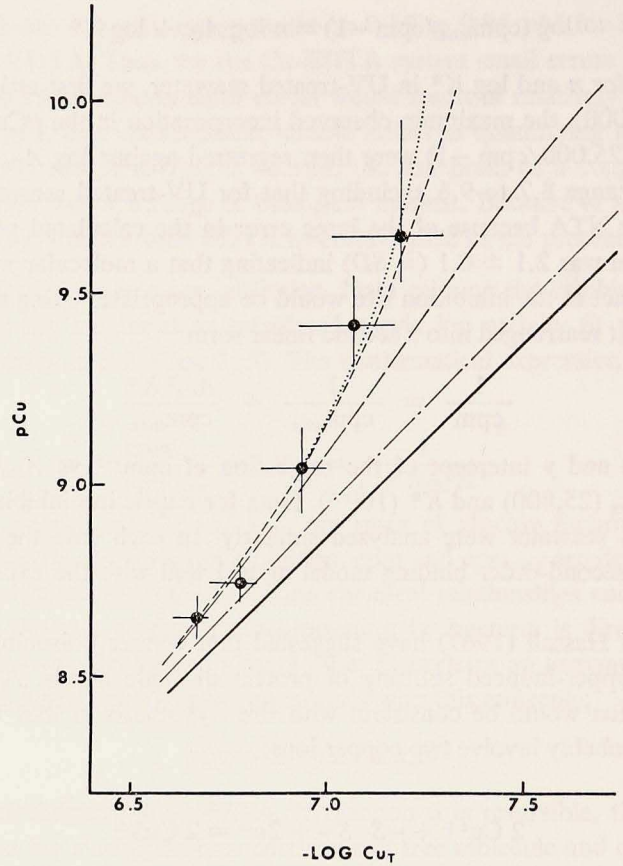

Figure 6. Relationship between the *p*Cu obtained from bacterial assay and the negative log of the total dissolved copper concentration (Cu_T) in filtered coastal sea water at pH 8.16 and 20°C. Cu_T = background copper plus added $CuSO_4$. Error bars for $-\log Cu_T$ are calculated on the basis of the 95% confidence limit for the determination of background copper. Vertical error bars are based on the standard deviation for the measured uptake of ^{14}C-glucose in filtered natural seawater. Curves in the figure are calculated from the equation:

$$Cu_T = A_{Cu}\, 10^{1.86} + \frac{L_T\, A_{Cu} K^*}{1 + A_{Cu} K^*}$$

where L_T is the total concentration (0.05 μM) of an organic complexing ligand L. The first term on the right-hand side of the equation equals the concentration of dissolved inorganic species of copper. The second represents the concentration of CuL complex where at equilibrium

$$\frac{[CuL]}{[Cu^{2+}]\,[L]} = K$$

$K^* = K \cdot \gamma_{Cu}^{-1}$ where γ_{Cu} is the activity coefficient of cupric ion in seawater and is equal to 0.26. The curves have been calculated for different values of K:
$K = 0$ (i.e., only inorganic complexation) ————— ;
$K = 10^8$ —— • ——— ; $K = 10^9$ ——— ——— ——— ——— ; $K = 10^{10}$ — — — — — ;
$K = 10^{11}$ ····· . The computed curves fit the bacterial assay data for apparent stability constants, $K \geqslant 10^{10}$.

Table 1. Computed speciation of copper in filtered estuarine seawater based on assayed values of cupric ion activity.

Cu_T (μM)	Cu-inorganic* (μM)	Cu-organic complex** (μM)	% Cu_T
0.21	0.16	0.05	24
0.17	0.13	0.04	24
0.11	0.066	0.048	43
0.084	0.028	0.056	67
0.064	0.017	0.048	75

* Calculated from the equation

[Cu-inorganic] = $A_{Cu}\ 10^{1.86}$

A_{Cu} was determined by bacterial assay

** Computed from the equation

[Cu-organic complex] = Cu_T − [Cu-inorganic]

tion approaches a constant value, cpm_{max}, and below this range it approaches zero (Fig. 2). Also we have assumed that the value of cpm_{max} (25,800) is the same in both UV-treated seawater and dock seawater in computing *p*Cu in the natural seawater. This assumption is supported by data shown in Figure 1.

Figure 6 shows the relationship between cupric ion activity and the dissolved copper concentration in dock seawater as determined from the bacterial assay. Using a value of 0.26 for the activity coefficient of cupric ion in seawater (Sunda, 1975), we converted cupric ion activities to cupric ion concentrations. In the dissolved copper range of 0.064 to 0.21 μM, cupric ion concentrations thus obtained ranged from 0.0008 to 0.008 μM or 4 to 1.3% of the total copper. The decrease in the fraction of dissolved copper present as cupric ion with decreasing concentration is apparently due to complexation by natural organic ligands. Evidence supporting this hypothesis is given by Gillespie and Vaccaro (1978), who showed that prior photooxidation of organic matter in coastal seawater from different locales appreciably increased cupric ion inhibition of bacterial incorporation of glucose at a given concentration of copper. Similarly, added copper was found to be more toxic to phytoplankton in photooxidized coastal seawater than in natural seawater (Sunda and Guillard, 1976).

The solid line in Figure 6 gives the computed relationship between *p*Cu and the negative log of the concentration of inorganic copper species for seawater at pH 8.16 (estimated from calculations and measurements reported by Sunda, 1975). This relationship is given by the equation: $pCu = -\log$ [Cu-inorganic] + 1.86, and was used to calculate the total concentration of inorganic copper species for a given activity of cupric ion (Table 1). By subtracting the calculated concentration of inorganic copper species from the total concentration of dissolved copper, we arrived at estimates of the concentration of organically complexed copper (Table 1).

(We cannot, however, exclude the possibility that some of the copper is bound by colloidal particles). The computed concentration of copper bound by such ligands (0.05 ± 0.01 μM) is independent of the total dissolved copper concentration although the fraction of the dissolved copper present as these complexes increases from 24% at $Cu_T = 0.2$ μM to 75% at $Cu_T = 0.06$ μM.

Equilibrium calculations (after Stumm and Morgan, 1970) show that the biologically assayed relationship between cupric ion activity and total dissolved copper in dock seawater is consistent with the binding of copper with one or more ligands (presumably chelators) present at an equivalent concentration of 0.05 μM with a fairly high stability constant ($\geqslant 10^{10}$) for reaction with copper (Fig. 6). The high stability constant further supports the hypothesis that these ligands are chelators.

Previous ion-selective electrode measurements (Sunda and Hanson, 1979) and those in this study (Fig. 4) indicate that copper is highly bound by natural organic ligands in Newport River water. An analysis of potentiometric titration data (Sunda and Hanson, 1979) was consistent with binding of ambient copper in the river predominantly by ligands present at an equivalent concentration of $\sim$ 1 μM with a conditional stability constant of $10^{10.9}$ at pH 8. The dock seawater was collected near the mouth of the Newport River estuary and thus it is quite possible that organic ligands in this seawater are of riverine origin.

Gillespie and Vaccaro (1978) determined relationships between inhibition of glucose incorporation and added copper concentration for samples of coastal seawater using the same bacterial isolate and experimental methods used in this study. We analyzed their data for ligand concentrations and stability constants by the methods described in this paper using the quantified relationship between inhibition and cupric ion activity (Fig. 2). From this analysis we estimated conditional stability constants of $10^{9.0}$ and $10^{9.6}$ and equivalent ligand concentrations of 0.16 and 0.20 μM for filtered (0.4μ) seawater from Nantucket Sound (salinity 32.5‰, pH 7.9) and Saanich Inlet (29‰, pH 8.0) at 20°C. These stability constants along with that for Beaufort dock water ($\geqslant 10^{10}$) are consistent with constants determined by gel chromatography for copper complexation to organic ligands isolated from the Irish Sea ($10^{8.9}$ and $10^{9.7}$), Loch Etioc ($10^{8.9}$ and $10^{10.2}$), and marine sediments ($10^{9.9}$ to $10^{11.3}$) (Mantoura *et al.*, 1978).

In previous copper chelation bioassays with a marine diatom (Davey *et al.*, 1973) and with the present strain of bacteria (Gillespie and Vaccaro, 1978), the authors assumed that the chelation capacity was equal to the concentration of copper at the steepest portion or inflection of the curve for biological response vs Cu addition. This inflection usually occurred in the range of 30 to 50% inhibition. This criterion for chelation capacity is not strictly valid and can give erroneous estimates of the equivalent concentration of organic complexing ligands. For example, we observed maximum slope at copper concentrations of $\sim$ 0.4 and 4μM in UV-treated seawater containing 1.0 and 10μM NTA (Fig. 1). Thus, if we had used the maximum slope

criterion, we would have underestimated the chelation capacity of these media by about 60%. The relationships between glucose incorporation and computed *p*Cu in UV-treated seawater with and without NTA are consistent with there being no residual chelation of copper in the UV-treated Gulf Stream seawater (Fig. 2): i.e. a chelation capacity of zero. Maximum slope for the copper inhibition curve (at 50% inhibition) in this seawater occurred at a copper concentration of ~ 0.05 μM and thus, in this case, the use of a maximum slope criterion would result in a sizable overestimation of chelation capacity.

Effect of chelators on the bioavailability of copper in marine waters. The presence of chelators, which strongly bind copper, has important implications concerning the toxicity of copper to marine organisms. Anderson and Morel (1978) have shown that cupric ion is extremely toxic to the red tide dinoflagellate, *Gonyaulax tamarensis*, causing 50% loss of cell motility at a *p*Cu of 10.4. This *p*Cu corresponds to a dissolved copper concentration of about 0.0025 μM assuming only inorganic complexation in seawater at pH 8.1. Since copper concentrations in coastal seawater are often higher than this (Chester and Stoner, 1974), Anderson and Morel concluded that copper may be toxic to *Gonyaulax* under natural conditions. For estuarine seawater containing a strong chelator ($\log K \geqslant 10$) present at a concentration of 0.05 μM, however, a *p*Cu of 10.4 corresponds to a dissolved copper concentration $\geqslant 0.02$ μM. Such concentrations are at the upper end of or above the natural range (Chester and Stoner, 1974; Boyle *et al.*, 1977), and thus we would conclude that natural levels of copper in this seawater would not usually inhibit cell motility of *Gonyaulax*. We measured a copper concentration of 0.014 μM in the Beaufort dock estuarine seawater which falls within the range of values (0.002 to 0.016 μM) previously measured for seawater sampled from our dock (Evans, 1977). From our measured copper concentration and estimated organic ligand concentration and stability constant, we calculated cupric ion activity to be $\leqslant 10^{-11}$M in the dock seawater, a level that should not be toxic to most phytoplankton, including *Gonyaulax* (Sunda and Guillard, 1976; Anderson and Morel, 1978; Morel *et al.*, 1978).

4. Conclusions

Our results demonstrate that the toxicity of copper to a marine bacterial isolate is related to cupric ion activity and not total dissolved copper concentration. In this respect, experiments with varied copper chelation to synthetic chelators (EDTA and NTA) and different natural ligands show consistent results.

We have shown that the toxicity of copper can be modeled on the basis of a reversible chemical reaction in which 2 cupric ions combine with a critical, but unidentified, cellular molecule, possibly an enzyme. Such models are of interest because cellular toxicity ultimately must be based on the reaction of copper with molecular components of cells.

Since the bioavailability of copper to many aquatic organisms is determined by cupric ion activity, this parameter is extremely important in assessing the interaction of copper with organisms in the aquatic environment; particularly those which do not ingest food and thus, whose sole source of metal is from solution. Our results show a direct relationship between toxic response of bacteria to copper and the voltage response of a cupric ion-selective electrode, which demonstrates the potential utility of these electrodes in predicting the bioavailability of copper in non-saline waters. Also we have shown that one can use bacterial response to cupric ion to estimate cupric ion activity in marine waters, where direct potentiometric measurements are not possible. Such bioassay techniques, when combined with measurements of total metal concentration, should be useful tools for the investigation of the chemical speciation of copper and other toxic trace metals in natural waters.

Acknowledgments. We thank Dr. Peter Hanson of the NMFS Beaufort Laboratory for measurements of background copper concentrations in seawater samples. The technical assistance of Jo Ann Lewis is also gratefully acknowledged.

This research was supported by a cooperative agreement between the Department of Energy and the National Marine Fisheries Service and by National Science Foundation (IDOE) grant ID073-9541. Contribution Number 97-B32 from the Southeast Fisheries Center, National Marine Fisheries Service, NOAA.

REFERENCES

Albright, L. J., J. W. Wentworth, and E. M. Wilson. 1972. Technique for measuring metallic salt effects upon the indigenous heterotrophic microflora of a natural water. Wat. Res., *6,* 1589–1596.

Anderson, D. M., and F. M. M. Morel. 1978. Copper sensitivity of *Gonyaulax tamarensis.* Limnol. Oceanogr., *23,* 283–295.

Andrew, R. W., K. E. Biesinger, and G. E. Glass. 1977. Effects of inorganic complexing on the toxicity of copper to *Daphnia magn.* Wat. Res., *11,* 309–315.

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

Boyle, E. A., F. R. Schlater, and J. M. Edmond. 1977. The distribution of dissolved copper in the Pacific. Earth Planet. Sci. Lett., *37,* 38–54.

Chakoumakos, C., Russo, R. C., and R. V. Thurston. 1979. Toxicity of copper to cutthroat trout *Salmo clarki* under different conditions of alkalinity, pH, and hardness. Environ. Sci. Tech., *13,* 213 p.

Chester, R., and J. H. Stoner. 1974. The distribution of zinc, nickel, manganese, cadmium, copper and iron in some surface waters from the world ocean. Mar. Chem., *2,* 17–32.

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

Ediger, R. D., G. E. Peterson, and J. D. Kerber. 1974. Application of the graphite furnace to saline water analysis. Atomic Absorption Newsletter, *13,* 61–64.

Erickson, S. F., T. E. Maloney, and J. H. Gentile. 1970. Effect of nitrilotriacetic acid on the growth and metabolism of estuarine phytoplankton. J. Water Pollut. Contr. Fed., *42,* 329–335.

Evans, D. W. 1977. Exchange of manganese, iron, copper, and zinc between dissolved and particulate forms in the Newport River estuary, North Carolina. Ph.D. thesis, Oregon State University. 218 p.

Gillespie, P. A. and R. F. Vaccaro. 1978. A bacterial bioassay for measuring the copper-chelation capacity of seawater. Limnol. Oceanogr., *23,* 543–548.

Jackson, G. A., and J. J. Morgan. 1978. Trace metal-chelator interactions in seawater media: theoretical analysis and comparison with reported observations. Limnol. Oceanogr., *23,* 268–282.

Klotz, I. M. and B. J. Campbell. 1962. Copper-induced hydrolysis of the disulfide bond. Arch. Biochem. Biophys., *96,* 92–99.

Manahan, S. E., and M. J. Smith. 1973. Copper micronutrient requirement for algae. Environ. Sci. Tech., *7,* 829–833.

Mantoura, R. F. C., A. Dickson, and J. P. Riley. 1978. The complexation of metals with humic materials in natural waters. Est. Coast. Mar. Sci., *6,* 387–408.

McBrien, D. C. H. and K. A. Hassall. 1967. The effect of toxic doses of copper upon respiration, photosynthesis and growth of *Chlorella vulgaris*. Physiol. Plant., *20,* 113–117.

Morel, N. M. L., J. G. Reuter, and F. M. M. Morel. 1978. Copper toxicity to *Skeletonema costatum*. J. Phycol., *14,* 43–48.

Sheldon, R. W. 1972. Size separation of marine seston by membrane and glass fiber filters. Limnol. Oceanogr., *17,* 494–498.

Sillen, L. G., and A. E. Martell. 1964. Stability constants of metal-ion complexes. Chem. Soc. Lond. Spec. Publ. 17. 754 p.

Stumm, W. and J. J. Morgan. 1970. Aquatic chemistry. Wiley Interscience, N.Y. 583 p.

Sunda, W. G. 1975. Relationship between cupric ion activity and the toxicity of copper to phytoplankton. Ph.D. thesis, Massachusetts Institute of Technology, Cambridge. 168 p.

Sunda, W. G., and R. R. L. Guillard. 1976. The relationship between cupric ion activity and the toxicity of copper to phytoplankton. J. Mar. Res., *34,* 511–529.

Sunda, W. G., and J. M. Lewis. 1978. Effect of complexation by natural organic ligands on the toxicity of copper to a unicellular alga, *Monochrysis lutheri*. Limnol. Oceanogr., *23,* 870–876.

Sunda, W. G., D. W. Engel, and R. M. Thuotte. 1978. Effect of chemical speciation on toxicity of cadmium to grass shrimp, *Palaemonetes pugio*: Importance of free cadmium ion. Environ. Sci. Technol., *12,* 409–413.

Sunda, W. G., and P. J. Hanson. 1979. Chemical speciation of copper in river water: Effect of total copper, pH, carbonate, and dissolved organic matter, *in* E. A. Jenne, ed., Chemical Modeling-Speciation, Sorption, Solubility and Kinetics in Aqueous Systems. Am. Chem. Soc. Symposium Series, *93,* 147–180.

Waiwood, K. G., and F. W. H. Beamish. 1978. Effects of copper, pH and hardness on the critical swimming performance of rainbow trout (*Salmo gairdneri* Richardson) Wat. Res., *12,* 611–619.

Williams, P. M. 1969. The determination of dissolved organic carbon in seawater: a comparison of two methods. Limnol. Oceanogr., *14,* 297–298.

Received: *4 June, 1969;* revised: *9 September, 1979.*