Bull. Environ. Contam. Toxicol. 30, 448–455 (1983)

An Algal Assay Method for Determination of Copper Complexation Capacities of Natural Waters

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It has become apparent that toxicity is independent of total metal concentration and that it is only the free metal ion which is toxic (PAGENKOPF et al. 1975, SUNDA & GUILLARD 1976, ANDERSON et al. 1978, SUNDA et al. 1978, ALLEN et al. 1980). Trace metal chelation has been suggested as one of the important factors influencing toxic response (STEEMAN NIELSEN & WIUM-ANDERSON 1970, BARBER 1973, JACKSON & MORGAN 1978).

Several authors have published data which indicate that naturally occurring organic materials can complex metals (CORCORAN & ALEXANDER 1974, SWALLOW et al. 1978, MCKNIGHT & MOREL 1979, SAAR & WEBER 1982). The importance of these naturally occuring materials has been indicated in several studies (PRAKASH & RASHID 1968, BARBER et al. 1971, PRAKASH et al. 1973) which show that their presence alters biological response.

Because complexation of metal reduces toxicity, the measurement of the complexing capacity of natural waters is important. Complexation capacity can be determined by following the course of a titration with a variety of methods such as anodic stripping voltammetry, ion selective electrodes, ion exchange equilibria, sorption by manganese dioxide, dialysis, fluorescence quenching or solubilization (NEUBECKER & ALLEN 1983).

Methods have also been developed for biological rather than chemical determination of complexation capacity. Bioassay methods have the advantage of being very sensitive to free metal ion and therefore they do not in general greatly alter the chemical environment of the system. DAVEY et al. (1973) measured the decrease in growth rate of a marine diatom in response to added copper in artificial seawater media containing known quantities of ligands and provided a semiquantitative estimate of the complexation capacity. GILLESPIE & VACCARO (1978) used a copper sensitive bacterium and measured ^{14}C-glucose assimilation as a function of added copper to determine complexation capacities for a variety of seawaters. SUNDA & GILLESPIE (1979) found that the inhibition of ^{14}C-glucose was related to cupric ion activity. This permitted estimation of cupric ion activity in natural water samples for which the bacterial uptake of ^{14}C-glucose was known. The cupric ion activity, alkalinity and pH were used to compute the concentration of inorganic copper species which was subtracted from the total copper concentration to give the concentration of organic bound copper. By evaluating the data for the titration of a sample, the complexation capacity could be evaluated.

We have, for a series of natural waters, compared the complexation capacity determined by the Davey method using a green alga to that determined by plotting the free copper ion concentration versus the growth rate.

MATERIALS AND METHODS

Stock cultures of the green alga Selanastrum capricornutum were obtained from the Environmental Protection Agency, Environmental Research Laboratory, Corvallis, Oregon. The cells were maintained in media made according to the EPA's Algal Assay Procedure Bottle Test (MILLER et al. 1978). Cells were aseptically transferred to fresh AAP media weekly to maintain the culture. Two to three days prior to innoculation, cells were transferred to new media to insure that the cells used were in the logarithmic growth phase.

A series of bioassays was conducted in AAP media for which the specified amount of EDTA was omitted and a desired amount of NTA or EDTA was added.

Complexation capacity was evaluated for eight natural water samples. Lake Michigan was sampled from Chicago's 31st Street pier in September, 1978. Three bodies of water in Forest Preserves in Cook County, IL (Hidden Pond, Maple Lake and Sagnashkee Slough) were sampled in August 1978 and another (Crawdad Slough) was sampled in October, 1978. Par and Beaver Ponds and Upper Three Runs River, located in Georgia, were sampled in April, 1979. The water samples were filtered through 0.45 μm Millipore Type HA filters upon their return to the laboratory and the samples were stored at $4^{o}C$.

Culture flasks were acid washed, 125 mL borosilicate erlenmeyer flasks into which 50 mL of media or sample water was placed. Copper was added from Eppendorf micropipets to give concentrations producing a range of algal responses ranging from no inhibition to total inhibition of growth. After allowing the copper to equilibrate in the sample water for one hour, an aliquot of the stock culture was added to give a innoculum of 5000 cells/mL. The flasks were placed on a 100 rpm oscillatory shaker with 500 $\pm$ 10% ft. candle, cool-white fluorescent illumination supplied from the bottom. Aliquots of the cells which had been grown at $25^{o}C$ were taken at 24 hour intervals for the 5-day duration of the test and the number of cells/mL was determined using a Coulter Electronic Particle Counter Model TA II.

The natural water samples were nutrient enriched by the addition of phosphate and nitrate at levels equivalent to those in AAP media. With the exception of $CuCl_2$, EDTA and $FeCl_3$ which were omitted, all other nutrients were added at 5% of AAP media levels. The Crawdad Slough water was diluted with a sodium bicarbonate solution of the same total alkalinity as the initial water sample to give samples containing 70, 40, 20 and 10% Crawdad Slough water. Nutrients were added to the diluted Crawdad Slough samples. The pH of all samples was maintained at 8.15 $\pm$ 0.15.

EVALUATION OF COMPLEXATION CAPACITY

The biological response used to evaluate the effect of added copper was the specific growth rate coefficient. The growth rate is a direct measure of the rate of total metabolism leading to cell synthesis (MEYERS 1962) and can provide a criterion for assessing adequacy of the nutrient medium. The specific growth rate coefficient is determined from the following equation in which common logarithms have been used:

$$k' = \frac{\log N_t - \log N_o}{t}$$

where N_t = number of cells at time t, N_o = number of cells at time zero, t = time in days and k' = specific growth rate coefficient in reciprocal days.

To express these rate coefficients in a manner in which all the data would be comparable, each rate coefficient was normalized as a percentage of the maximum growth, and is designated k. A typical bioassay curve, for the Crawdad Slough water, in which the decrease in k is plotted as a function of increasing added concentration of copper, is shown in Figure 1. For each bioassay the concentration of copper required to inhibit the growth rate to a value 50% of the maximum rate was determined as a reference value to indicate the ability of a sample to prevent toxicity due to added copper. The concentration of copper causing growth to be inhibited by 50% is called the IC_{50} value.

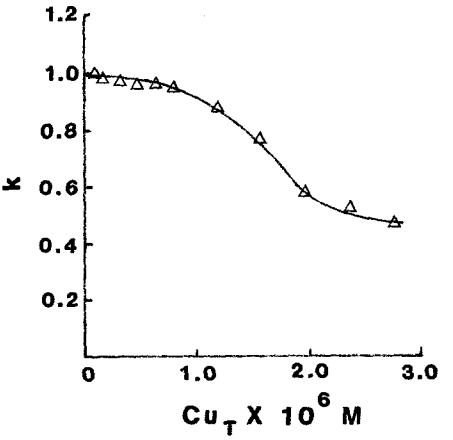

Figure 1. Response of Selanastrum capricornutum to copper added to Crawdad Slough water.

Complexation capacities were determined from the bioassay curves using the method first described by Davey et al. (1973). The endpoint of the titration is considered to be the point of maximum inflection of the sigmoid shaped bioassay curve. The inflection point was determined by plotting the first derivative (dk/dCu_T) as a function of the average added copper concentrations. The maximum occurs at the point where the effective ligand concentration is equivalent to the concentration of added copper. After this point the additional added copper exists primarily as the free ion and the decrease in cell growth rate is rapid.

We also assessed the complexation capacity by a method we call the biological titration method which is based on the relationship between free copper ion and the algal growth rate.

The free copper ion concentration is determined from a calibration curve relating k to the free copper ion concentration in much the same manner as the calibration curve used by SUNDA & GILLESPIE (1979). This calibration curve is determined from the results of the bioassays in defined media containing known amounts of synthetic ligands. In such media, at each level of added copper, the speciation of the copper can be calculated from known equilibria and stability constants. Thus, a calibration curve (Figure 2) can be obtained which relates k, in the region of partial growth inhibition, to the free copper ion concentration. The equation for the relationship is $k = 0.39 \log [Cu^{2+}] - 2.73$.

For bioassays performed in natural waters, the concentration of free copper ion at each level of total added copper can be evaluated from the calibration curve and the experimental value of k. The free copper ion concentration is plotted as a function of total added copper (Figure 3). The break in the titration curve corresponds to the complexation capacity, at which point the concentration of added metal is equivalent to the effective ligand concentration. After this point the concentration of the free copper ion increases rapidly with the addition of more copper.

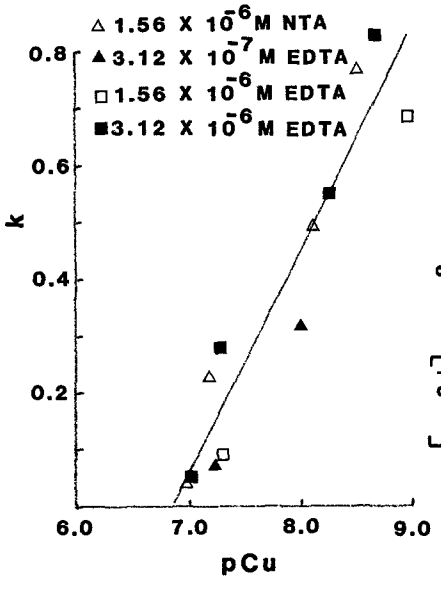

Figure 2. Relationship between normalized growth rate of Selenastrum capricornutum and computed concentration of free copper ion for media containing NTA of EDTA.

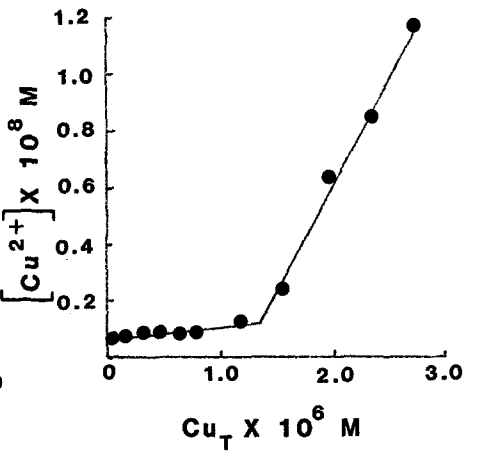

Figure 3. Biological titration curve for Crawdad Slough water. The concentration of free copper is determined from the relationship in Figure 2.

RESULTS AND DISCUSSION

The results of the bioassays conducted in defined media are presented in Table 1. With defined systems, in which the stoichiometry of the copper-ligand reaction and the concentration of the ligand are known, the value of the biologically determined complexation capacity can be compared to the known ligand concentration. Table 1 lists the true ligand concentration, the IC_{50}, the ligand concentration determined by the Davey method and the value of the equivalent toxic response.

The equivalent toxic response represents the percentage of growth inhibition which would be found for a specified level of copper. For instance, when 3.12 µM EDTA is present in the medium, the equivalence point occurs when 3.12 µM copper has been added. This point on the bioassay curve corresponds to a value of k of 0.843. The growth of the cells was inhibited by 15.7% at the equivalence point and the equivalent toxic response to this true ligand concentration is the $IC_{15.7}$. The mean equivalent toxic response of the ligand concentrations determined by the Davey method was the IC_{55} and the standard deviation was 11. The ligand concentrations determined by the Davey method were compared to the concentrations which had been added. Using a paired-observation t-test there was no significant difference between the concentration ($P<0.05$).

Table 1. Complexing Capacities and Equivalent Toxic Response in Defined Media

Ligand	Concentration (µM)	IC_{50} (µM)	Equivalent Toxic Response of Added Ligand (%)	Ligand Concentration by Davey Method (µM)	Equivalent Toxic Response to Ligand Concentration Determined by Davey Method (%)
EDTA	0.31	0.30	52	0.24	68
	1.56	1.61	28	1.96	59
	3.12	3.36	16	2.94	43
NTA	1.56	1.76	49	1.77	49

Table 2. Complexing Capacities and Equivalent Toxic Responses In Natural Waters

Sampling Location	Natural Water* (%)	IC_{50} (μM)	Biological Titration		Davey Method	
			Complexation Capacity (μM)	Equivalent Toxic Response (%)	Complexation Capacity (μM)	Equivalent Toxic Response (%)
Lake Michigan	100	0.18	0.12	30	0.12	30
Par Pond	100	0.25	0.24	44	0.20	30
Maple Lake	100	0.58	0.42	22	0.59	53
Upper Three Runs River	100	0.79	0.40	25	0.51	35
Hidden Pond	100	0.86	0.62	32	0.51	24
Sagnashkee Slough	100	0.88	0.66	26	0.71	31
Crawdad Slough	100	2.26	1.34	17	1.77	32
Crawdad Slough	70	1.39	0.85	22	1.20	38
Crawdad Slough	40	1.02	0.49	19	0.65	36
Crawdad Slough	20	0.47	0.31	36	0.39	44
Crawdad Slough	10	0.33	0.16	22	0.12	18
Beaver Pond	100	2.50	2.00	28	1.60	24

* Dilution with bicarbonate solution.

For the bioassays conducted in the eight natural water samples and the four dilutions of the Crawdad Slough water (Table 2), a paired-observation t-test indicated that there was no statistically significant difference ($P<0.05$) between the complexation capacity determined by the biological titration and the Davey methods. The mean equivalent toxic response of the complexation capacities determined by biological titration (IC_{27}) and by Davey's method (IC_{33}) are less than the mean equivalent toxic response determined for the known ligands (IC_{36}), but these differences are not significantly different ($P<0.05$).

Summary and conclusion

The complexation capacities determined for natural waters by the Davey and the biological titration methods were statistically the same. However, these two methods differ in an important way. For Davey's method only those data in the vicinity of the equivalence point are used. For the biological titration all data can be used if the complex is strong. For weaker complexes, the data for total metal concentrations similar to that of the complexation capacity will deviate from linear behavior. In these cases the data which deviate can be disregarded. The biological titration method is easier to use because it does not require that a significant number of samples have metal concentrations similar to the value of the complexation capacity.

ACKNOWLEDGEMENT

Support provided by the U.S. Department of Energy through Argonne National Laboratory project number 31-109-38-3660 and by National Oceanic and Atmospheric Administration project number NOAA-022-44030. Thomas D. Brisbin was supported, in part, by a Laboratory Graduate Participant Award at the Argonne National Laboratory.

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Accepted January 24, 1983