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ECOLOGY OF SUBTROPICAL, SHALLOW WATER ENVIRONMENTS:
CHEMISTRY OF COPPER AND CHLORINE INTRODUCED INTO
MARINE SYSTEMS DURING ENERGY PRODUCTION

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DOE SUPPORTED PUBLICATIONS

Papers:

Carpenter, J. H., 1977. "Sunlight-induced bromate formation in chlorinated seawater", *Sci.*, 195(4284): 1335-1337 (with D. L. Macalady and C. A. Moore).

Carpenter, J. H., 1977. "Errors in determination of residual oxidants in chlorinated seawater", *Env. Sci. and Tech.*, 11: 992-994 (with C. A. Moore and D. L. Macalady).

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Abstracts:

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Abstracts and Reprints Removed

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1. BACKGROUND

For many years, only total trace metal concentrations were measured in environmental samples. However, scientists are now aware that the speciation of these metals is important for predicting their biochemical and geochemical reactions. In seawater copper is present in both organic and inorganic forms. The principal inorganic form is thought to be CuCO_3 with less than 1% of the total copper existing as the free Cu (II) ion (1). Very little is known about the organic copper species. This results from the lack of knowledge concerning the dissolved organic matter in seawater and from the limited information available concerning the biochemical cycle of copper in marine systems.

Copper is an essential element for life (2). Phytoplankton are sensitive to the activity of the free cupric ion (3,4). There exists a lower limit for the Cu (II) ion activity beyond which nutritional deficiency occurs, in addition to an upper limit beyond which toxic symptoms are produced. The chelating agent ethylene diaminetetraacetic acid is commonly added to culture media to chelate trace metal ions. Poor phytoplankton growth in upwelling areas is thought to arise due to insufficient concentrations of organic chelators (5). Phytoplankton growth was stimulated in this seawater after the addition of EDTA or a filtrate from a zooplankton homogenate but was not stimulated by the addition of trace metals, nutrients, vitamins, or amino acids. In some areas poor phytoplankton growth may instead be due to lack of utilizable trace metals with growth

being stimulated by the addition of trace metals (6).

In any case, chelation by organics will result in an equivalent decrease in the amount of inorganic complexation and in a further decrease in the level of the free copper (II) ion. Ideally one would like to know the activity of the cupric ion, however, there are presently no techniques by which this can be measured directly. The lack of information concerning the organic complexation equilibria prevents the calculation of this activity.

It is an extremely difficult task to determine the organic speciation of a trace element. Few instrumental techniques can be used in the ppb concentration range and general concentration measures will disturb the speciation equilibria. The methods utilized for determining the concentration of organic copper attempt to capitalize on the differences in chemical behavior between the inorganic and the organic species. Commonly, such a determination yields a measurement of labile and non-labile species. These species are characterized, respectively, by their reaction or lack of reaction in a given situation. Inorganic species are assumed to behave in a manner similar to the free copper (II) ion and are thereby included in the labile fraction. Organic species are then assumed not to be reactive without further treatment and thereby comprise the non-labile fraction.

Treatments such as acid digestion (7), UV oxidation (8), and chlorination (9) have been utilized for conversion of "organic" copper to a

reactive form. Since the reaction mechanisms for these processes differ, there exists the possibility of differential release of copper. There is no generally accepted method for the determination of total copper. Recently Florence and Batley (10) have refined these procedures to determine seven types of metal species depending on combinations of anodic stripping voltammetry, chelex chromatography and ultraviolet light irradiation.

Another technique for the determination of "organic" copper, involves the separation and concentration of this fraction by liquid-liquid extraction with chloroform. The "organic" copper may not truly be extracted by the chloroform but may instead accumulate in the interfacial layer (11). Since the interfacial layer is commonly drawn off along with the chloroform, it will still be measured as "organic" copper.

These measures, though crude, do serve to establish limits for the concentration of organic copper in seawater. Slowey et al. (12) determined that 8-56% of total copper in the Gulf of Mexico was in an organic form by chloroform extraction. Williams (13) found 0-28% of total copper in an organic copper form based on concentration changes resulting from UV oxidation.

In order to gain information concerning the amount of organic chelators that are present in a water sample, the chelating capacity can be measured. In this procedure the water sample is titrated with copper (II) ions. After each addition of titrant, labile copper is measured by anodic

stripping voltammetry (14) or free Cu (II) ion activity is measured with a copper ion selective electrode (15). The graph of measured copper versus titrant volume has two linear regions. In the first region with a smaller positive slope, much of the copper is reacting with organic chelators to form non-labile complexes. In the second region, the organic chelating capacity has been exceeded so that each additional increment of copper remains in a labile form thus resulting in a greater stripping response per incremental addition. At the intersection point of the two lines, the amount of copper added equals the amount of organic chelating agent present (assuming a 1:1 complex). According to Chau, only those complexing agents forming complexes having stability constants greater than 10^{13} will be measured by this titration. Surface active agents that adsorb onto the electrode surface will affect the electrode processes and may lead to significant error in the measurement of the complexing capacity by this technique (16, 17).

Estuarine areas (18) and lake waters (14) have been found to have similar complexing capacities of 0.1 - 0.6 $\mu\text{mole/l}$. This contrasts greatly with a heavily polluted river sample which possessed a chelating capacity of 25.6 $\mu\text{mole/l}$ (15). The complexing capacity of the river water which flows into the Georgia estuary was spread evenly over the whole molecular weight range (18). In the estuary, as the salinity increased, the chelating capacity was derived to a greater extent by lower molecular weight compounds.

Another method for the study of copper-organic interactions involves the use of model compounds. The choice of a suitable model compound is, however, fraught with problems. The majority of the organic matter in sea water is uncharacterized and is generally known as humic substances. This is a carryover of a name from soil organic work and does not necessarily indicate any structural similarity. The category humic substances is further subdivided into humic and fubic acids.

Humic acids are defined as that fraction of the organic matter that is soluble in basic but not in acidic solutions (19). Fulvic acids differ in that they are soluble in both acidic and basic solutions and generally possess lower molecular weights. These definitions, thus, key on general solubility characteristics of substances rather than on specific structural properties. These generic classes thus divulge very little structural or compositional information.

Fulvic acids from surface Sargasso sea water differed markedly in molecular weight distribution and in chromophore content from coastal water and soil fulvic acids (20). Thus, the use of reagent grade humic acid, which is of terrestrial origin, is not justified for sea water model experiments. The use of humic compounds collected from the marine environment presents another problem, that due to chemical alteration of the substances during the concentration and separation procedures. Commonly the humic compounds are collected on XAD-2 resin from acidified sea water. The substances are eluted with strong ammonia solutions and

the water removed by freeze drying or by using a rotary evaporator (21). These procedures may markedly change the chemical properties of the humic substances.

Despite these problems, several investigators have used humic substances as model compounds. By titrating a solution containing free Cu (II) ion with a reagent grade humic acid solution, O'Shea et al. (22) were able to demonstrate the formation of non-labile Cu-humate complexes. A dynamic equilibrium binding gel filtration procedure utilizing soil, peat, fresh water, marine and sediment humic compounds was used by Mantoura and Riley (23, 24) to demonstrate the formation of copper complexes. The fresh water humic compounds were found to contain approximately 1.5 times more complexing sites and result in the formation of stronger complexes than the soil humic compounds. Sea water humic substances formed complexes with stabilities equal to those from fresh waters. Equilibrium calculations using this data predict that as much as 80% of the copper is in an organic form. This prediction may be low since the speciation is calculated using overall complex stability constants. A small increase of the copper complex stability relative to the calcium and magnesium complex stabilities in a small percentage of the total chelating sites will increase the calculated concentration of "organic" copper. In many coastal areas there may be essentially no inorganic copper.

There is, however, no direct link between copper complexing substances

and classical humic type compounds. Humic type compounds also form complexes with Mg and Ca as well so that few sites would remain available for copper chelation in sea water. Thus the stability constant determined from a model system may not give us all the information needed to assess the speciation of a metal. It is important that the stability constant of the copper complex be large, but of crucial importance is the selectivity for copper. Without this selectivity concentrations of free metals determine the extent to which each metal is complexed by the organic ligands. In this case copper would lose out to metals in higher abundance such as Ca, Mg, Fe and Al. Only by invoking some chelation specificity for copper, can organic chelation of copper be explained in the absence of biota.

Living organisms can greatly alter the speciation of an element. Since copper is an essential element like iron, copper sequestering agents may be secreted by phytoplankton resulting in the formation of a copper complex that can be assimilated by the phytoplankton. These compounds would thus be analogous to the iron sequestering siderophiles. Alternately excess siderophiles may act as copper chelating agents (25). Phytoplankton may also act to detoxify ionic copper by exuding complexing agents. Excess intracellular copper may be converted to an organic water soluble form and excreted. These complexes may be kinetically stable in sea water and exist as dissolved copper species.

Thus, it is difficult to speculate as to the type of organics

responsible for the chelation of copper. Humic acids seem a poor choice because of their general non-specific complexing capability. Peptides are a more likely source. Peptides and proteins possess structures that commonly produce high substrate specificities. Hydroxamic acids have been identified that are very specific iron chelators; perhaps there exist copper analogues. Due to the vast number of compounds synthesized by organisms there is a strong possibility that none of the above classes are at all important. If this is true, model experiments using these compounds are doomed to failure. More must be known concerning the actual chelators present in sea water before models can be useful.

2. INTRODUCTION

During the last three contract years, we have been involved in the study of the chemistry of the copper binding compounds occurring in coastal seawater. Initially our efforts were oriented towards the study of the complexing capacity of waters collected at various locations in the Miami, Florida area. Our study then shifted towards the concentration and the elucidation of these chelators.

3. COMPLEXING CAPACITY

3.1 Assessment of the complexing capacity of local waters.

The complexing capacity is thought to represent the concentration of organic ligands that are able to complex copper in a seawater solution. Thus, the complexing capacity curve can be likened to a titration curve. This curve is derived by measuring the copper by anodic stripping voltametry at successively increasing levels of added copper to the sample, as seen in Figure 1. Ideally, the chelation curve consists of two linear portions. The initial portion of the curve has a lesser slope than the latter portion. The slope of the initial curve is controlled by the organic complexes of copper which give copper up to the electrode at a lesser rate than the inorganic complexes. The final linear portion has a slope which is determined by the response of the electrode to the inorganic complexes. The intersection of the two lines is taken as the complexing capacity.

Figure 1 is a "typical" titration plot for a sample of water from the western (landward) area of Biscayne Bay that is representative of the

Figure 1. Copper Titration: Matheson Hammock Water

cooling waters used at the Cutler Ridge and Turkey Point generating stations of the Florida Power and Light Company. This water was a mixture of ca. 20% upland drainage and 80% seawater; i.e., salinity/29 parts per thousand. The apparent copper complexing capacity was 12 ppb.

Samples collected along a transect running across Biscayne Bay out through the coastal water to the Gulf Stream showed decreasing copper complexing capacity. The Gulf Stream samples had a copper complexing capacity of only 0.2 ppb, so that the southeast Florida environment appears to have a range of nearly two orders of magnitude with respect to the potential capacity to detoxify added copper. Ecological effects of added copper can be expected to be quite site specific. The low copper complexing capacity of the Gulf Stream waters may need to be considered in evaluating possible environmental effect of OTEC operations.

3.2 The effects of chlorination on the complexing capacity

Not only is the copper complexing capacity quite site specific, but it is also modified by chlorination at doses that are currently used at power plants in the United States. An additional sample of the waters that produced the results shown in figure 1 was chlorinated with a dosage to 1.5 ppm Cl_2 . The dosage level does not produce a measurable "residual oxidant" concentration after thirty minutes of reaction. The results of titrating this chlorinated sample are shown in figure 2. The copper complexing capacity was reduced to ca. 6 ppb. Also the nature of the complexing species appears to have been changed, as shown by the increased slope of the curve

Figure 2. Copper Titration: Matheson Hammock Water Chlorinated to 1.5 ppm Cl_2

before the end-point was reached indicating that the copper complex was more electrochemically "labile." The biological significance of this feature needs to be evaluated using bioassay observations with good characterizations of the copper species during the experiments.

Figure 3 shows the results of copper titration of a sample collected in the eastern (seaward) area of Biscayne Bay, which had a copper complexing capacity of 6 ppb. An aliquot of this sample was chlorinated (5 ppm Cl_2 added), which did not produce a measurable, persistent residual oxidant concentration. As shown in figure 4, this addition of chlorine removed all the measurable copper complexing capacity from this water.

An obvious inference from these results is that chlorination of seawater may produce toxicity and growth reduction through the indirect mechanism of modifying the copper complexing capacity of the treated waters.

3.3 Measurement of copper demand

Thus far, we have examined the chelating capacity as if it involved a simple metal complex association reaction that could be easily followed by means of an amperometric titration. In nature, however, there are many chelators possessing a wide range of chelation capabilities. The various complexes formed will differ in their ability to give copper up to the electrode. Due to this, the initial portion of a complexing capacity plot will commonly not be linear but curved with a constantly increasing slope. In this case it becomes difficult to determine the slope of the initial portion of the curve. One could standardize the technique and

Figure 3. Copper Titration: Safety Valve Water

Figure 4. Copper Titration: Safety Valve Water Chlorinated to 5 ppm Cl_2

define the initial slope as the change in response between the original solution and the solution plus one copper addition. However, one is doomed to failure with this since then the endpoint location becomes a function of the first addition and also of the range of chelators involved; the chelation capacities are then no longer additive.

In order to circumvent this problem, a new property has been defined - copper demand. This property is defined as that total amount of the added copper that behaves as a nonlabile copper complex. The copper demand point is located at the intersection of the final response line and a horizontal line drawn from the initial sample point as seen in figure 5.

Copper demand determined for Biscayne Bay waters varied from 1.0 $\mu\text{g}/\text{kg}$ to 8.0 $\mu\text{g}/\text{kg}$ copper equivalents. The copper demand varies inversely with the height of the tide. The inshore waters having high copper demands are diluted by the slope water which has a lower copper demand. Copper demands of up to 0.8 $\mu\text{g}/\text{kg}$ have been measured in offshore waters.

In order to learn more about this copper demand, this property was determined for a sample of Biscayne Bay water just after collection, after two weeks storage, after filtration through 0.2 μ nucleopore filters, and after filtration through both 0.2 and 0.15 μ nucleopore filters. The results are seen in Table I. In this sample, the copper demand increased with time. If one looks carefully at the titration curves, a basic difference appears. The initial portion of the fresh sample titration curve approximates a straight line with a slope of 33 units/ $\mu\text{g}/\text{kg}$, and there is an abrupt change at the end point. In the stored sample there is much curvature

Figure 5

Table I

Copper Demand

1A Water* Collected 2-14-79

2-15-79	1A Water	1.3 $\mu\text{g/kg}$
2-28-79	1A Water	2.5 $\mu\text{g/kg}$
3-01-79	1A Water thru 0.2 filter	2.1 $\mu\text{g/kg}$
3-02-79	1A Water thru 0.2 and 0.015 Filters	2.7 $\mu\text{g/kg}$

* Biscayne Bay 25° 37'N 81° 11'W

in the initial portion whose influence extends out to larger copper added values. The slope of the first two points is, however, similar to that observed with the fresh water. Thus, upon storage there is an increase in total chelators. The chelators produced during storage are more labile than those initially present, and there is no loss of the original chelators present in the sample over time. No significant differences were noted after either of the filtrations except that these samples aged in a similar fashion as the stored sample. Thus, particulates and colloids with sizes $>.015\mu$ appear not to influence the chelation capacity of this water. It is, however, possible that some of the colloids had broken down into dissolved components prior to filtration and may in this manner be involved in the aging phenomena seen.

4. CONCENTRATION OF THE NATURAL OCCURRING ORGANIC MATTER

4.1 Solvent extraction of copper complexes

Based on reports in the literature (Slowey and Hood, 1971²⁶ and Slowey, et al., 1967¹²) that a substantial fraction (20 to 40 percent) of the copper in Gulf of Mexico seawaters can be extracted with organic solvents, we had proposed that work on this fraction be carried out early during the contract period. It has been a surprise that we can not duplicate the literature observations of substantial copper concentrations that are soluble in organic solvents.

Two one liter portions of the water spiked to 10 ppb copper were extracted, one with 50 ml of chloroform and the other with 100 ml of isobutanol. The copper content of the organic solvents was then deter-

mined. If any of the copper complexes were extracted by the solvent, the copper content of the solvent would be increased. No increase in copper content was seen in either case. Thus, the organic extractable complexes present were below the detection limits of the system which were 0.25 $\mu\text{g/kg}$ copper equivalents for isobutanol and 0.12 $\mu\text{g/kg}$ for chloroform. These observations are in agreement with recent work of Moore and Burton²⁷, who found that the extractable copper amounted to 10 percent or less in a series of samples from the North Atlantic ocean. Ion-pair extractions were also performed in a manner similar to the solvent extractions. In this case, the extractant was a 10% solution of n-trioctylamine in chloroform. No detectable copper ($<0.12 \mu\text{g/kgCu}$) was seen in the organic portion after extraction.

The difference between our results and earlier reports may lie in the details of the extraction procedure carried out. In this study the aim was to assess the amount of copper complexes truly soluble in the organic solvent utilized. For that reason the interfacial layer was not included in the solvent fraction drawn off for analysis. In addition, the solvent was analyzed directly for copper content. In some earlier studies¹¹ a different procedure was utilized. The interfacial layer was drawn off along with the solvent. The liquid was evaporated off and the residue redissolved for analysis. The mysterious soluble copper probably accumulates in the interfacial layer. By the latter procedure it would have been measured as organic soluble copper even though it was not ever dissolved in the organic solvent.

4.2 Solid support concentration techniques.

The next phase of the work concerns the determination of the capabilities of two solid supports to concentrate the chelating organics. The resins involved were XAD-2 and acetylated polyamide 6. The procedure used for assessing the efficiency of the resin concentration techniques involved monitoring the effluent for organic chelators. In order for this to work there must be no significant bleed of complexing substances from the resin itself. There also must not be bleed of substances that interfere with the anodic stripping voltammetric assessment of the chelator content. After extensive washings with both acidic and basic solutions, neither of the two resins met the above bleed conditions. The effluent from XAD-2 resin had a background copper demand of 0.7 µg/kg copper equivalents. The acetylated polyamide effluent contained organic compounds that gave interfering peaks in anodic stripping voltametry. For these reasons, further resin work at this stage was abandoned.

4.3 Ultrafiltration

The last preconcentration technique studied was ultrafiltration. The ultrafilter utilized was the Amicon UM05 ultrafilter which has a nominal molecular weight cutoff of 500. Compounds smaller than 500 molecular weight are only partially retained by this ultrafilter. The plastic sleeve of the cell and the red silicone O-rings produced a high metal blank for the cell. This blank was reduced by copious washing but was still considerable. By replacing the sleeve with a siliconized quartz

cylinder and by wrapping the O-rings with acid washed teflon tape, the contamination was brought under control to below 1.5 $\mu\text{g/kg}$ for Cu and below 0.5 ppb for Pb and Cd.

The 0.2 and 0.015 μ water was ultrafiltered by filling the 400 ml cell up to the 350 ml mark and refilling the cell when the total volume reached 50 ml. The pressure on the cell was set at 60 psi. This ultrafiltration process was very slow, in total requiring ten days to concentrate 4 liters to a final volume of 50 ml. The efficiency of the ultrafiltration process was determined in two ways. The first method was to spike a portion of irradiated seawater with the ultrafiltrand so that the ultrafiltrand was diluted back to a concentration factor of 1 as compared to the original solution. The copper demand of this solution was measured. The second technique was to measure the copper demands of successive one liter portions of the ultrafiltrate. The results are shown in Table II. A considerable portion of the chelators passed through the ultrafiltration membrane. As indicated by the initial portion of the ASV curve, these chelators are quite labile and are similar to the chelators gained during the aging of the water sample. The diluted ultrafiltrand shows a much lower lability in the initial portion of the ASV curve and an abrupt change in response at the end point. This curve is very similar to that obtained with the fresh water sample. Perhaps the original chelators are being concentrated by the ultrafilter and the chelators arising from aging pass through the

Table II

Copper Demand
1A Water* Collected 2-14-79

3-07-79	Ultrafiltrate 1	0.8 µg/kg
3-08-79	Ultrafiltrate 2	1.3 µg/kg
3-09-79	Ultrafiltrate 3	0.9 µg/kg
3-12-79	Irradiated SW spiked with Ultrafiltrate	1.1 µg/kg

* Biscayne Bay 25° 37'N 81° 11'W

membrane. This would tend to indicate that the chelators produced by the aging of the water are lower in molecular weight than the original chelators. The ultrafiltrand plus the ultrafiltrates account for about 78% of the chelators present in the 0.2 and 0.015 filtered seawater. The ultrafiltrand itself accounts for 85% of the original chelators in the sample. The collection losses are probably due to adsorption on the ultrafiltration cell walls and/or the membrane itself.

5. SIZE EXCLUSION CHROMATOGRAPHY OF METAL COMPLEXES

This area of research concerns the development and utilization of a chromatographic procedure for separating metal complexes by molecular weight. One criterion for such a procedure is that the technique be able to utilize seawater as the eluent. This allows metal chelation to be studied directly in the presence of calcium, magnesium and the general ionic background of seawater. The second criterion is that the effluent be able to be monitored for both organic content by UV absorption at 254 nm and metal content by atomic absorption spectrometry. The third criterion is that the eluent be spiked with a given amount of copper and the column be equilibrated with this eluent. The chelators will equilibrate with this background copper during their transit through the column. The presence of chelating organics will be indicated by a rise in total copper in that portion of the effluent.

5.1 The selection of the size exclusion packing

The first chromatographic support examined was sephadex G-10. Much of the organic material in the ultrafiltrand solution eluted from this

column near the void volume signifying that the material was excluded by the pores of the gel. Within this excluded portion, there is no separation of the organic matter. In addition there was a problem that concerned the affinity of the gel for copper. The column had to be equilibrated with a copper spiked seawater solution for several days before it was equilibrated.

The next support studied was glycophas CPG-40. This support consists of porous silica beads having a pore size of 40 A. The surface of the silica is covered with a hydrophilic sugar phase so that it resembles sephadex. This material is supposed to be more physically stable than sephadex, not be subject to microbial attack, and have an exclusion limit of about 8000 daltons. The pore volume of this material is only about 0.1 ml per gram support. The ultrafiltrand eluted well after the void volume and produced a nice bell shaped curve. The total width of the curve was around 10 ml. This did not allow for much distinction in the metal trace since the automatic sampler for the atomic absorption spectrometer presently requires a 0.7 ml sample. The one copper trace obtained showed that the copper distribution followed the distribution of the UV absorbing organics quite well.

The last support utilized was sephadex G-25. This support still needed to be equilibrated with copper but had an exclusion limit of 5000 daltons. The organic concentrate separates out over a wide range of elution volumes with only a small percentage of the material eluting near the void volume. The column utilized consists of a teflon tube 0.9 cm x 100 cm. The samples are injected using a sample loop injection valve. A peristaltic pump

using silicone tubing was used to pump the eluent through the column. The eluent was pumped continuously through the column in order that it remain in equilibrium with the copper spiked eluent solution. During the chromatographic run the effluent passed through a UV flow cell and then directly into the AA sample cups. Increasing the sample size to 2 ml did not affect the final organic distribution. The larger sample size resulted in a copper curve in which the chelators are cleanly resolved from the background level of copper, normally 5 $\mu\text{g/kg}$. The overall column has a volume of 60 ml. The void volume is 18 ml and the total pore volume is about 24 ml.

5.2 Sephadex G-25 results

The injection of a typical ultrafiltrand on the column produced a smooth UV absorption curve as seen in Figure 6. The peak maximum occurs at 33 ml and there is a slight shoulder at 38 ml. The copper curve shows a very different distribution (Figure 7). The copper maximum lags the organic maximum, occurring at 36 ml. Thus, the properties of copper chelation and UV absorbance do not correlate in this organic concentrate sample. The average UV absorbing organic molecule appears to be of larger size than the average copper chelator. The copper curve increases abruptly in the region near the copper maximum. This may represent a specific fraction of chelators that has fairly homogeneous properties. The UV and the copper curves are quite reproducible. There is some variation in the high molecular weight region from run to run. When there is UV absorbing material present near the void volume, there is

Figure 6. Ultraviolet absorbance λ 254 nm: Sephadex G-25 Chromatography of Biscayne Bay water ultrafiltrand 2-14-79.

Figure 7. Copper content of fractions from sephadex G-25 chromatography of Biscayne Bay water ultrafiltrand 2-14-79.

a corresponding rise in copper content. Rechromatographing individual fractions results in curves with peak maxima occurring at the same position as the original fraction. Thus, the wide bell shaped distribution is actually representative of the separation of the organics and not just produced by peak broadening. When the ultrafiltrand solution was spiked before injection with 300 µg/kg copper and was let equilibrate for an hour, there was an increase in the copper peak especially in the region of the peak maximum. Free copper is eluted at about 42 ml on this column.

Several samples from both nearshore and offshore locations in Biscayne Bay have been concentrated and chromatographed in this manner. The ratio of peak UV absorbance to peak of copper concentration varies by a factor of 2. In all the striking result is that the distributions are so similar. One feature that does differ in these samples is the amount of material eluting at the void volume. Both a rise in UV and in total copper occurs there in chromatograms of certain samples. From this we can conclude that the organic matter is generally similar from one sample to another. However, the amount of high molecular weight material present in the organic matter appears to be very variable.

5.3 Testing of preconcentration techniques

The chromatographic procedure developed was then used to test possible preconcentration procedures. This allows the preconcentration techniques to be tested directly using the ultrafiltrand thereby eliminating the resin blank problems. For tests at pH 8, the trap containing the adsorbent was put in line at the end of the sample loop. The sample solution then would

pass through the trap and then onto the column. This allows the organics that are not trapped by the adsorbent to be detected directly in the column effluent. Three adsorbents were tested: XAD-2, acetylated polyamide 6, and SEPPAK C-18. The results of the XAD-2 run show a 14% reduction in the UV absorption peak height but an overall distribution similar to those obtained with the ultrafiltrand directly. The copper curve of the 300 $\mu\text{g/kg}$ equilibrated sample shows general attenuation after passage through the trap but otherwise resembles closely the original copper curve of the unspiked ultrafiltrand. The run involving the acetylated polyamide 6 shows a similar UV curve with a 16% reduction peak height and the addition of a contaminant at an elution volume of 46 ml. There also was a loss of absorbance in the high molecular weight range. The copper results are similar to that of XAD-2. The SEPPAK C-18 UV results closely resemble those obtained using the ultrafiltrand itself except that there is no rise in the UV near the void volume. The copper curve also resembles the original curve except for a loss of copper in the high molecular weight range. Thus, none of these three adsorbents collect much of the organic matter. The SEPPAK C-18 appears to have collected specifically the high molecular weight material, some of which is also capable of chelating copper.

5.4 The study of copper complex lability

In order to study the lability of the complexes formed between copper and these natural organic ligands, aliquots of the concentrates were

chromatographed on sephadex G-25 columns that had not been equilibrated with copper. In this case the columns had a considerable affinity for copper. The concentrate was prespiked so as to preform the copper complexes. Any copper above the background level passing through the column indicates the presence of complexes that are kinetically stable during the time scale of the column transit of about 1.5 hours. Any complex dissociating during transit would lose its copper to the column packing. The result was that there was a 41% recovery of complexed copper. The pattern of complexed copper was similar to the equilibrated result except that there was no large spike in the 38 ml region. Thus both non-labile and labile organic complexes of copper must be considered as integral components of the species of copper present in natural waters.

5.5 Distilled water used as eluent

By switching to distilled water as the eluent information is gained concerning the large number of general metal chelation sites. In this case the activity of free copper ions with which the chelating components equilibrate with is up to 400 times greater at the same total copper than with seawater. This arises because in distilled water there are much lower concentrations of inorganic complex forming ligands and because in distilled water the activity coefficient for free copper ions is nearly 1. In addition to this there is a large decrease in the concentrations of the competing ions Mg and Ca.

The amount of chelators determined in this manner from the chromatographic run is about 100 x greater than if seawater was used as the eluent. This may be in fact equal to the total number of available metal sites. The number of sites when calculated back to the original seawater sample amounts to 1-2 μ moles per milligram organic carbon. This compares with a value of 11 μ moles per milligram organic carbon²⁸ of weak acid sites as determined by acid-base titration. Since one would expect copper complexes to adopt a coordination numbers of 4 when possible, the two studies agree quite well. An important point is that the number of sites examined in our seawater studies is only ~1% of this total. This small portion is quickly lost when faced by examination of the whole. This fact quickly points up the importance of using seawater as the eluent in such studies. Another result is that in the distilled water run the copper peak is coincident with the UV absorption peak. The chelators of lower molecular weight seen in the seawater runs have been overshadowed by the general metal chelation sites present in the bulk of the organic matter.

5.6 Biogel P-2

So far the assumption has been made that the sephadex G-25 column packing separates the organic components purely by molecular size. There may, however, be chemical interactions between a component and the gel that produce separation of the organics in addition to separation processes that

act based on molecular size alone. These chemical effects serve to retard the elution of the interacting component. The net result is that the component appears to be smaller than it actually is.

If gel permeation chromatography media separates purely by size then two different packings with similar pore size and resolution characteristics will yield similar chromatographs. The sephadex G-25 packing was compared to biogel P-2. The results are seen in figures 8-11. The UV results for biogel P-2 is similar to that for sephadex G-25. The peak obtained with the biogel P-2 packing occurs at a volume closer to the void volume since the exclusion characteristics of the packings do differ. The exclusion limit for biogel P-2 is 1800 daltons whereas that for sephadex G-25 is 5000 daltons. The total integrated UV absorbance from the biogel P-2 run indicates a loss of about 20% as compared to elution on sephadex G-25. In agreement with the UV runs there are 20% fewer copper chelators in the biogel P-2 profile. The copper profiles for the two columns are widely different. Thus, the apparent molecular weight distributions for the majority of the organic components are consistent and probably represent a true molecular weight distribution. The copper complexing components important in a seawater matrix are a completely different story. With biogel P-2 the copper peak is coincident with the UV peak. There is, in addition, a large rise in copper just after the void volume. This would indicate that a large portion of the chelating substances are of higher molecular weight than the average organic component. The biogel profile probably represents the profile

Figure 8. Ultraviolet absorbance λ 254 nm: Sephadex G-25 chromatography of Biscayne Bay water ultrafiltrand 1-10-80.

Figure 9. Ultraviolet absorbance λ 254 nm: biogel P-2 chromatography of Biscayne Bay water ultrafiltrand 1-10-80.

Figure 10. Copper content of fractions from sephadex G-25 chromatography of Biscayne Bay water ultrafiltrand 1-10-80.

Figure 11. Copper contents of fractions from biogel P-2 chromatography of Biscayne Bay water ultrafiltrand 1-10-80.

closer to the real distribution. Column effects normally retard the elution of a component, not accelerate it. This does not totally invalidate the sephadex profiles. These profile are probably not purely based on molecular size, but on some combination of molecular size and sephadex affinity. Such a distribution will still be consistant from run to run as long as the same packing material is used. Thus, the average copper chelator seems to interact more strongly with the sephadex matrix than the average UV absorbing component.

5.7 Degradation studies

In an effort to determine the nature of the copper complexing sites, degradation experiments were carried out. The concentrate was subjected to acid hydrolysis. The sample was diluted 1:1 with concentrated HCl, and the vessel was evacuated and capped. The reaction vessel was kept at 110° for 24 hours. Such a hydrolysis should result in the rupture of all peptide bonds. Such a hydrolysis also results in complete destruction of tryptophan. Typical results can be seen in figures 12-15. The hydrolysate was neutralized with NaOH as soon as it cooled down. The profiles for the hydrolysate have been corrected for dilution and for the reagent blank. The large UV peak occdrring at 47 ml in the hydrolysate is a variable component from the reagents which has not been accounted for in the reagent blank. The most obvious results are the loss of UV signal and copper content in the

Figure 12. UV absorbance λ 254 nm: Sephadex G-25 chromatography of RSMAS dock water ultrafiltrand 9-30-79.

Figure 13. UV absorbance λ 254 nm corrected for dilution and reagent blank: Sephadex G-25 chromatography of acid hydrolysate of RSMAS dock water ultrafiltrand 9-30-79.

Figure 14. Copper content of fractions from sephadex G-25 chromatography of RSMAS dock water ultrafiltrand 9-30-79.

Figure 15. Copper content corrected for dilution and reagent blank of fractions from sephadex G-25 chromatography of acid hydrolysate of RSMAS dock water ultrafiltrand 9-30-79.

region of the void volume and the general lower amount of copper in the hydrolysate profiles. The UV absorbance from the hydrolysate has decreased only 33% as compared to the original concentrate. Thus, the backbone of the organic matter is definitely not proteinaceous. Since this decrease occurs over the entire molecular weight range, the proteinaceous components may have existed as side chains on a wide variety of molecules.

The hydrolysate has lost about 52% of its complexing capability. The percentage decrease is lowest at the copper peak maximum and greatest at lower elution volumes. Thus, it would appear that the high molecular weight complexes are more proteinaceous than those of lower molecular weight and that those components with molecular weights approaching or exceeding the exclusion of molecular weight are completely proteinaceous. This latter conclusion agrees with the results of Tuschall and Brezonik²⁹ which indicate that the high molecular weight organics are predominately proteinaceous.

6. SEPARATION OF THE ORGANICS

From the preceeding studies it has been shown that the copper chelating substances are heterogeneous in all characteristics so far determined. This greatly increases the difficulty involved in their elucidation. Once again, the natural world has been shown to be a

complex situation free of quick and easy solutions to problems.

In order to progress further with this study, it will be necessary to separate out the copper chelators from the remainder of the organic matter with its general metal sites. Two techniques have been studied for this separation capability. In the previous year a C-18 bonded phase mini-liquid chromatography trap (Waters SEP-PAK C-18) was studied for organic concentration capability at natural pH. This medium was found to retain only a small percentage of the organic matter.

6.1 SEP-PAK C-18

This year the organic retention experiment was repeated except that the pH of the sample was decreased to 2. At this pH about 44% of the organic eluted from the trap passed right through the trap. Another 44% were released when the pH of the eluent seawater was changed to its natural pH. A second switch of eluent to 10% MeOH in seawater released 13% more of the organic matter. The fraction that marches right through the trap accounts for 70% of the chelators present in the concentrate aliquot. The ratio of copper content to UV absorbance in this fraction is increased by a factor of 3 over the original sample. The distribution of UV and copper content from a sephadex G-25 run of this fraction is similar to the original sample. The UV shows a more distinct shoulder in the 36-38 ml region. The copper profile also shows an increase in this region. Since the desired fraction is not being held on the column, this method is not

suitable for the concentration of the chelating organics. Even though the separation is far away from the desired 1:100 separation it comes a long way with a relatively crude technique and will be useful as a quick enrichment technique.

6.2 Cu-chelex.

The second method involves the use of a copper chelex column similar to those used for concentration amino acids from seawater. The idea is that the chelators will be retained as copper chelates by the column. The remaining components will pass unhindered right through the column. The chelators would then be released like the amino acids by 2M NH_4OH . This works for amino acids so by analogy it should work for our chelators. There are two important considerations to be made concerning this process. The first is that the column may retain many more components than those sought since the high content of copper in the column may result in the retention of components whose copper complex seawater association constants are much lower than those that we desire to study. The second consideration is that the strong chelators may not be satisfied with the 1-2 coordination sites available at the resin attached copper. This may result in the removal of copper from its resin site. The complex would then pass right through the remainder of the column.

The first column chosen was 5 x 1 cm and included a plug of chelex in the Na^+ form at the end to remove copper eluted by the ammonia. Without this there was a considerable background UV absorbance from the copper ammonia complex. The ultrafiltrand sample passed right through this column. It

would appear that our second consideration is valid in this case.

In order to increase the ability of the column to retain its copper, a packing that was only 50% saturated was obtained by mixing equal portions of saturated Cu-chelex with Na^+ -chelex. The column size was increased to 2.2 x 30 cm. The end of the column was again filled with Na^+ -chelex. The elution of a 5 ml aliquot of a concentrate is shown in figure 16. The first peak at 75 ml represents components passing right through the column. The second peak at 192 ml represents organics retained by the column and eluted with 0.008N NH_4OH . Overall there was a 75% recovery in UV absorbance from the original sample. The integrated UV absorbance contained in the two peaks correspond to a 1:1 ratio. The dilution of the components eluting as the first peak made this fraction unsuitable for further study. The second fraction corresponds to 80% of the chelators present in the original aliquot. The Cu/UV ratio has been enriched by a factor of 1.7.

This technique does not separate out our chelator fraction as well as the C-18 trap. In this case, however, the chelator fraction is retained by the column. This technique thus may be better suited for use as a general concentration procedure. The ultrafiltration technique used so far is fine with Biscayne Bay samples when a concentration factor of 100 is needed. The resin column may allow us to study other waters having lower concentrations of chelating organics.

So far the door to the elucidation of the chemical nature of the copper complexing organics has been pushed open a bit. The complex nature of these components results in small hard won accomplishments. These compounds

Figure 16. UV absorbance λ 254 nm: copper-chelex chromatography of Biscayne Bay water ultrafiltrand 1-10-80.

have been shown to possess a wide range of molecular weights. Some of these complexes are labile and others are non-labile in a 1-2 hour time scale. Approximately half of the copper chelation sites are predominantly proteinaceous. Further study is needed to determine the complete nature of these chelating organics.

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