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Effect of Organic Complexation on Copper Accumulation and Toxicity to the Estuarine Red Macroalga *Ceramium tenuicorne*: A Test of the Free Ion Activity Model

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Supporting Information

ABSTRACT: Current water quality criteria (WQC) regulations on copper toxicity to biota are still based on total dissolved (<0.4 μm membrane filter) copper concentrations with a hardness modification for freshwaters. There are however ongoing efforts to incorporate metal speciation in WQC and toxicity regulations (such as the biotic ligand model-BLM) for copper and other metals. Here, we show that copper accumulation and growth inhibition of the Baltic macroalga *Ceramium tenuicorne* exposed to copper in artificial seawater at typical coastal and estuarine DOC concentrations (similar to 2−4 mg/L-C as fulvic acid) are better correlated to weakly complexed and total dissolved copper concentrations rather than the free copper concentration [Cu^{2+}]. Our results using a combination of competitive ligand exchange-adsorptive cathodic stripping voltammetry (CLE-ACSV) measurements and model calculations (using visual MINTEQ incorporating the Stockholm Humic Model) show that copper accumulation in C. *tenuicorne* only correlates linearly well to [Cu^{2+}] at relatively high [Cu^{2+}] and in the absence of fulvic acid. Thus the FIAM fails to describe copper accumulation in C. *tenuicorne* at copper and DOC concentrations typical of most marine waters. These results seem to indicate that at ambient total dissolved copper concentration in coastal and estuarine waters, C. *tenuicorne* might be able to access a sizable fraction of organically complexed copper when free copper concentration to the cell membrane is diffusion limited.

INTRODUCTION

The bioavailability of copper in synthetic media appears to be controlled by the free metal ion concentration, as predicted by the free ion activity model (FIAM).[1,2] The FIAM follows from a simple premise: that equilibrium exists between the free metal ions in solution and the metal ions bound to transport enzymes (or other physiologically active sites) on algal cell membranes i.e. the biotic ligands.[3,4] Although current regulations for water quality criteria (WQC) and copper toxicity to biota are still based on total dissolved copper concentrations, there are ongoing efforts to incorporate metal speciation in WQC and toxicity regulations via the biotic ligand model (BLM).[3,5] The BLM is derived from the FIAM and takes into consideration the properties of natural water such as dissolved organic carbon (DOC), water hardness, and pH to account for the influence of competition between cations for the biotic ligand and the reduction of metal bioavailability by natural ligands (part of DOC).[3]

So far, BLMs have been developed for copper for selected biota in freshwaters,[4] but there is currently no BLM available for biota in marine waters. However, Arnold et al.[6] used a freshwater BLM to predict copper toxicity to the marine bivalve, *Mytilus* sp.[6] They found that the freshwater BLM predicted lower EC50s when measured copper EC50s were <160 nM.[6] Metal complexation with DOC components in the BLM is modeled using programs such as WHAM (Windermere Humic Aqueous Model).[7] Trace metal complexation data (stability constants) in WHAM and similar programs are usually based on data obtained at high metal and HS concentrations using ion selective electrodes (ISE) in freshwaters.[7] The concentrations of both trace metals and HS in marine waters are generally lower compared to freshwaters. In addition, the use of ISE for copper complexation measurements at ambient copper concentrations in marine waters is problematic due to its low sensitivity and the ISE's high susceptibility to interference by chloride ions,[8] although recent improvements on the technique has made it possible to use ISEs for [Cu^{2+}] measurements in coastal waters.[9] However, as Rivera-duarte and Zirino[9] noted, there are still significant problems associated with the use of the Cu-ISE: (i) the electrode may change its response slope (from that of a Cu(II) to that of a Cu(I) electrode) when exposed to greater than part-per-billion quantities of Cu(II)aq in the presence of light, and (ii) its response may differ from the expected in the presence of certain strong ligands. These shortcomings might explain the few reported application of ISEs for copper

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complexation by humic substances in marine waters (compared to freshwaters) and the conspicuous lack of a marine BLM so far. Thus, data on metal complexation at ambient marine water DOC and metal concentration are needed for future development of BLMs for copper to marine biota at ambient concentrations. Additionally, relationships between free metal ion activity and bioavailability remain to be demonstrated under natural conditions where the presence of fulvic and humic acids strongly influence metal speciation.[10]

The aim of this study was to investigate how copper speciation (due to organic complexation) affects its (copper) accumulation and toxicity to the red macroalga *Ceramium tenuicorne*, indigenous to the Baltic Sea. The Baltic Sea is one of the largest brackish water bodies in the world, and its ecosystem is particularly vulnerable to pollution due to its semienclosed character and hydrography.

We designed the laboratory copper uptake and toxicity experiments to simulate DOC variability in estuarine and coastal environments like the Baltic Sea. We used a Nordic reference fulvic acid (FA) with the assumption that a significant fraction of the NOM in estuarine and coastal environments is terrestrial FA.[11]

Theoretical chemodynamic studies on metal uptake by organisms[12,13] and a few preliminary studies seem to indicate that copper uptake and accumulation by periphyton[10,14] and algae [15,16] might be better related to the labile or weakly complexed copper fraction rather than the free copper concentration as predicted by FIAM. We therefore wanted to test the hypothesis that at $[Cu^{2+}]$ typical of estuarine and coastal waters (i.e., 10^{-12}–10^{-15} M),[17,18] the intracellular copper accumulation by *C. tenuicorne* would be better correlated to the concentration of weakly complexed copper concentration.

We wanted to fully characterize the copper speciation in the growth media and especially the capacity of the weak copper complexing ligand sites in the Nordic FA we used. We hypothesized that the concentration of these weak copper binding sites on FA would be important in regulating both the copper uptake and buffering the free copper concentration at increasing total dissolved copper concentrations. We chose a competitive ligand equilibration adsorptive cathodic stripping voltammetry (CLE-ACSV) method that utilizes salicylaldoxime (SA) as the added competitive ligand for its (CLE-ACSV) enhanced sensitivity over direct measurements of free copper concentration using either ISE or even anodic stripping voltammetry.[19−21]

MATERIALS AND METHODS

Ceramium Tenuicorne Growth Experiments. Alga test solutions were prepared by adding different concentrations of copper and FA to autoclaved (120 °C for 20 min) artificial seawater (ASW) prepared according to Spotte et al.,[22] with the addition of nitrogen (KNO_3; 3.46 mg N/L), phosphorus (KH_2PO_4; 0.78 mg P/L), iron ($FeCl_3x\ 6H_2O$; 0.10 mg Fe/L), and carbon ($NaHCO_3$; 16.5 mg C/L)[23] at different salinities (5, 10, and 15). The copper concentration in the test solutions after autoclaving was routinely monitored for copper contamination. All test solutions were allowed to equilibrate with the added copper and FA in darkness for at least 18 h before the start of the tests.

Copper toxicity tests were performed on a brackish clone of the filamentous red macroalga *C. tenuicorne* according to ISO 10710.[23] The toxicity data used in this study originates from previously published [25] and new data. A detailed description of the toxicity test is described elsewhere.[24] Briefly, tests were conducted in acid-cleaned and sterilized polystyrene Petri dishes. For each salinity tested, FA was added to a range of Cu solutions (0, 1, 2, 4, 8, 16, 32, and 64 μg Cu/L) to obtain two nominal FA concentrations (4 and 8 mg/L FA, corresponding to ca. 2 and 4 mg/L-C). For comparison, Cu solutions with no addition of FA were also prepared. Hence, for each salinity, three toxicity tests were prepared yielding nine toxicity tests in total. Five replicates were used per treatment with three pieces of alga in each 25 mL of test solution. The length of the alga was recorded at start (0 d) and end (7 d) of a test, and 50% inhibition of growth rate was calculated (EC50). The test was carried out at a temperature of 22 ± 2 °C, a light intensity of 70 ± 7 μmol m^{-2} s^{-1}, and at a daily rhythm of 14 h light and 10 h darkness. The pH of the test media was measured at the start (8 ± 0.2) and at the end (8 ± 0.1) of each test (see the Supporting Information). We observed a lower Cu total concentration at termination of each test compared with initiation of tests, especially for waters with no additions of FA. Therefore, experiments to test the loss of copper to the *C. tenuicorne* test container walls in ASW at pH 8 with no FA or algae added to the ASW test media were conducted. We found a loss of ca. 35% to the test container walls in 63 nM copper solutions. This loss decreased to <11% at copper concentrations above 250 nM.[25] Higher losses of Cu were observed in the *C. tenuicorne* toxicity experiments presumably due to Cu adsorption to the algal surface. Also the loss were substantially reduced (ca. 5 times) when FA was added to the system. We decided to use the data for Cu total concentrations analysis from test initiation since we do not know when, during the experimental time of 7 days, the loss was most substantial.

In order to obtain enough material for analysis, algae from different replicates were pooled. Extracellular copper was removed from the algae surfaces by washing with 1 mM EDTA (dissolved in ASW) followed by a rinse with ASW. The algae were then dried in an oven at ∼60 °C prior to nitric acid digestion and eventual copper determination of the HNO_3 digests by ICP-MS (RSD of measurements <5). EC-50 values were calculated using REGTOX 7.0.4 (http//eric.vindimian.9online.fr). The method calculates EC-50 values together with corresponding 95% limits by optimizing the curve fit with successive iterations.

Reagents, Sample Bottles, and Clean Facilities. All solutions were prepared with deionized water (18MΩcm) from a Milli-Q water purification system (Millipore). Nitric and hydrochloric acids and ammonia (Fisher) used for making buffer solution were all trace metal grade or in-house quartz-distilled equivalents (Q-HNO_3 or Q-HCl). Copper calibration standard solutions were prepared from commercial copper standard solution (Spex Plasma, Edison, NJ). Freeze-dried Nordic reference FA, isolated from a Nordic Lake, was obtained from the International Humic Substances Society. A 1 g-C/L (1.9 g/L FA) stock of this FA in Milli-Q water was made and kept in the dark at 4 °C.

All electrochemical measurements were performed in a HEPA-filtered air class 1000 lab, and all solutions and sample manipulations were performed under a HEPA-filtered air class 100 bench. Plastic-ware (polypropylene, Teflon, or LDPE) were acid cleaned. Test solutions for copper speciation were stored in 250 mL polypropylene (PP) bottles. The PP bottles were cleaned with Micro-90 liquid laboratory grade detergent (Cole-Parmer, Vernon Hills, IL) and deionized water when first used

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followed by soaking overnight in hot 12 M hydrochloric acid. The bottles were then thoroughly rinsed with Milli-Q water and dried under class 100 HEPA-filtered laminar flow air bench. All other plastic ware (polyethylene, LDPE, or Teflon) used for storing analytical solutions were cleaned using the same procedure, dried, capped, and stored under class 100 HEPA-filtered laminar flow air bench or double bagged in trace metal clean, self-locking (Zip loc) plastic bags. Both the ICP-MS used for total copper determinations and the μ-Autolab/VA-stand electrochemical system were placed in a class 1000 HEPA-filtered air room. The ICP-MS auto sampler was enclosed in a HEPA-filtered (Class 100) laminar flow canopy within a plastic enclosure. All sample manipulations were performed in a class 100 HEPA-filtered laminar flow air bench.

Total Dissolved Copper Determinations by ICP-MS. Total dissolved copper in the growth test ASW (5−15 salinity) was performed on a Thermo X-series ICP-MS. Both accuracy and precision of the measurements were evaluated via multiple analysis of the estuarine water certified reference material (CRM) SLEW-3 from The National Research Council of Canada. The RSD of multiple analyses of SLEW-3 CRM within an analytical session was consistently below 12%. And the mean copper concentration for a typical analytical run (X ± SD) was 24.9 ± 3.0 μg/L (n = 14), well within the certified value of 24.4 ± 1.9 μg/L.

Cu Speciation. The dissolved copper speciation was determined following a modified version of the competitive ligand exchange adsorptive cathodic stripping voltammetry (CLE-ACSV) method developed by Campos and Van den berg.[20] The voltammetric system used was a μ-Autolab potentiostat (Ecochemie, Netherlands), connected to a hanging mercury drop electrode stand (Metrohm model 663VA) with a glassy carbon rod counter electrode and a double junction, Ag/AgCl, reference electrode with a salt bridge filled with 3 M KCl. The instrument was controlled by GPES software. Solutions (10 mL) were placed in a quartz voltammetric cell, stirred by a rotating PTFE rod and oxygen was removed by purging with water-saturated argon. Although there was no signal caused by the reduction of FA complexes in samples with or without added ligand and copper at FA concentration below 1 mg/L FA, higher concentrations of the Nordic FA we used produced a broad peak around −200 mV that interfered with the $Cu(SA)_2$ (see Results and Discussion section below).

Competitive Ligand Exchange-Adsorptive Cathodic Stripping Voltammetry (CLE-ACSV). Details of determination for free cupric ion concentration and complexation parameters by CLE-ACSV with salicylaldoxime as the competing ligand have been described elsewhere.[19−21] Briefly, the method is based on ligand-exchange equilibrium between added SA and natural ligands in samples. The Cu complexes with SA are measured by adsorptive differential pulse cathodic stripping voltammetry (DPCSV). The sensitivity of the DPCSV method for Cu-SA complexes must be calibrated by Cu titration for each individual sample. It was determined from the slope of the titration curve at high Cu concentrations where essentially natural ligands were saturated, and the concentration of Cu-SA complexes was proportional to that of the added Cu concentration.

A 200-mL solution containing 1 mg/L FA solution and 10 mM EPPS buffer (pH 8) in 10 salinity ASW was prepared from the 1.9 g/L FA and 1 M buffer stocks respectively in 10 salinity artificial seawater. This solution was then aliquoted into a series of 12 clean voltammetric Teflon (FEP) cups. Dissolved copper was then added to all but two of the cups, with total added copper concentrations ranging from 0 to 300 nM (with more closely spaced additions at lower concentrations and more widely spaced additions at higher concentrations). The very high copper additions were used in an attempt to ensure saturation of any weak ligand sites on FA. The samples were allowed to equilibrate with the added copper for 2 h in their respective Teflon cups. This time has been shown to be sufficient to reach equilibrium between the added Cu and the FA in solution.[19] After the 2 h equilibration, the appropriate concentration of SA was added. The samples were subsequently left to equilibrate for 4−6 h or overnight. Following this second equilibration, each sample was purged for 3 min with water-saturated argon gas and analyzed using the CSV parameters detailed above.

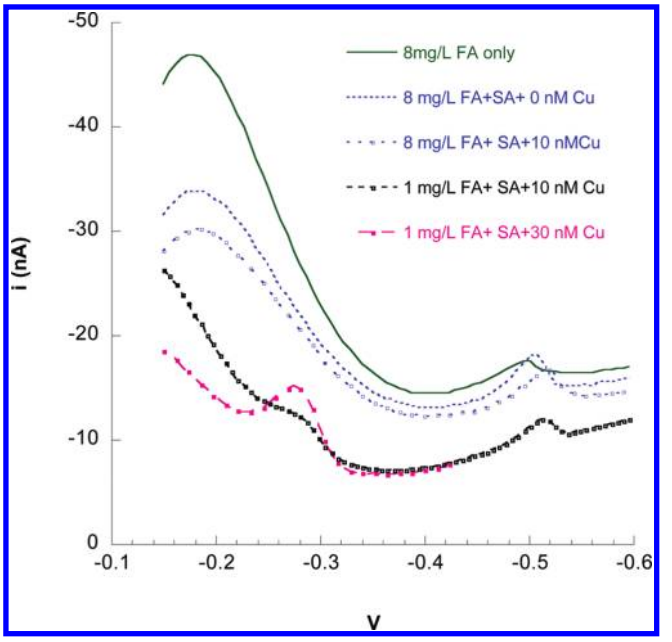

Figure 1. Interference from the Nordic fulvic acid (FA) on the analysis of copper by CLE-ACSV using SA as the competing ligand showing a broad FA peak at ca. −200 mV which masked the $Cu(SA)_2^0$ reduction peak at ca. −270 mV. The peak at ca. −500 mV is probably an Fe-FA peak[22] from the Fe added to the artificial seawater.

RESULTS AND DISCUSSION

Copper Complexation by Fulvic Acid. We initially wanted to investigate copper complexation for the waters used (both for the start and end solutions) in the *C. tenuicorne* toxicity tests. However, we observed a broad interference peak at potentials (ca. −200−350 mV vs Ag/AgCl) close to the reduction of the electrochemically labile $Cu(SA)_2^0$ complex at FA concentration of 8 mg/L (Figure 1). This broad peak at around −200 mV (vs Ag/AgCl) interfered with the $Cu(SA)_2^0$ peak which occurred around 250−300 mV. We observed the peak with or without the addition of SA i.e. with FA dissolved in either 10 salinity ASW test solution or in Milli-Q water. These interferences have not been reported by previous workers using CLE-ACSV and SA as a competing ligand.[19,26,27] However, the highest FA concentration used in those studies was 1 mg/L FA (Suwannee River FA). We could not ascertain the source of the interfering peak, but we suspect it is from the Nordic reference FA since previous copper speciation measurements of water samples from the Baltic[17,18,27]

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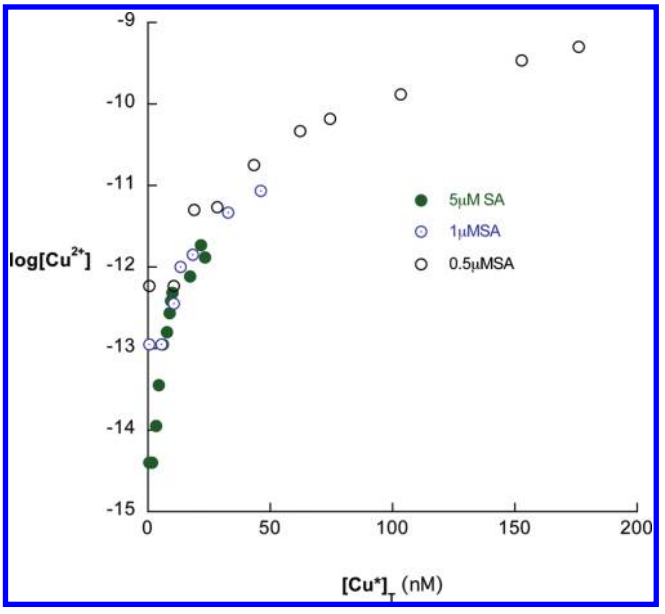

Figure 2. log $[Cu^{2+}]$ plotted as a function of $[Cu^*_T]$ for 1 mg/L FA solution in 10 salinity artificial seawater at three different added competing ligand (salicylaldoxime) concentrations.

and elsewhere with similar or higher natural DOC did not report these interferences. However, there was no interference peak at FA concentrations of 1 mg/L FA or less (red and black voltamograms in Figure 1). Humic substances and especially FA have and are still commonly used in CLE-ACSV copper complexation studies. It will therefore be necessary in the future to investigate whether this interference artifact is constrained to the Nordic FA we used in this study or if higher concentrations of the more widely used Suwannee River FA also interfere with Cu analysis when SA is used as a competing ligand in CLE-ACSV studies.

Copper Complexing Capacities of FA in Artificial Seawater (ASW). We designed the algae test experiments to simulate the organic carbon and salinity gradients typical of estuarine systems like the Baltic. Although not all the dissolved organic matter in these systems is composed of fulvic acids, we assumed that FA might constitute a significant fraction as recently proposed.[21,27] We therefore wanted to characterize the complexation capacities of the FA by using a Nordic FA solution in 10 salinity ASW. Figure 2 shows a plot of the free copper concentration $[Cu^{2+}]$, as a function of the total dissolved copper concentration, $[Cu^*_T]$. The total dissolved copper concentration, designated $[Cu^*_T]$, is the sum of all initial copper species (i.e., $[Cu^{2+}]$, copper complexed to inorganic ligands (such as $CuCO_3^0$ and copper complexed to FA) but excludes copper complexed to the added competing ligand SA (since SA is not a natural characteristic of this system).[18,19,21] At low total dissolved copper concentration, the free copper concentration is strongly buffered by the strong binding sites on FA as exemplified by the steep slope of the $\log[Cu^{2+}]$ vs $[Cu^*_{tot}]$ curve in Figure 2. As the strong metal binding sites are gradually saturated with copper, the $[Cu^{2+}]$ is controlled by the weaker binding sites. These sites (weak) though not as effective (as evidenced by the slope of the $\log[Cu^{2+}]$ vs $[Cu^*_{tot}]$ curve in Figure 2) are more abundant and become important when the ambient total dissolved copper concentration exceeds the concentration of the strong ligand sites.[18]

The ambient total dissolved copper concentrations in most natural (unpolluted or moderately impacted) waters including coastal estuarine systems like the Baltic are in the range 10 to 50 nM. The $[Cu^{2+}]$ in these waters is usually buffered by the strong ligand sites from picomolar to femtomolar (similar to 10^{-12}–10^{-15} M).[10,17,18] However, in highly impacted coastal environments (such as close to marinas) these (strong) ligand sites become saturated and $[Cu^{2+}]$ is then (albeit not as effectively) controlled by the more abundant but weaker ligand class.[18]

We titrated the ASW with the added FA (1 mg/L) with copper at different SA concentrations ranging from 0.5 to 5 μM SA. The use of multiple analytical windows (i.e., a range of SA concentrations) allowed a more comprehensive characterization of the spectrum of organically complexed copper by FA in the toxicity test solutions: from the strongly complexed copper to the weakly complexed copper.[19,28,29]

The copper complexing capacity at FA concentrations above 1 mg/L, can be obtained by normalizing the binding capacity to the organic carbon content of the FA (0.52 g-C/g-FA). We used the resulting linear ($R = 0.99$) log−log plot of $[Cu^{2+}]$ vs the FA-complexed copper to calculate the $[Cu^{2+}]$ at concentrations of 4 and 8 mg/L.[28] These FA concentrations would correspond to DOC concentrations of ca. 2 and 4 mg/L-C respectively, typical of the Baltic Proper estuarine gradient (assuming the DOC has similar binding characteristics to the FA). It is important to point out that natural DOC is likely to be more heterogeneous compared to the Nordic FA used in the ASW. However, most of the estuarine DOC is probably of terrestrial origin[21] and might be similar to the FA we used.

Effect of Copper Speciation on Growth of C. tenuicorne. *Total Dissolved Copper.* The primary aim of our study was to investigate how organic complexation affects copper toxicity to the macroalga *C. tenuicorne*. We monitored *C. tenuicorne*s growth rate at three salinities and two DOC concentrations, including a hypothetical situation where no organic carbon (no FA added) was added to the ASW. In the absence of FA, the EC-50 increased from a total dissolved copper concentration of ca. 70 nM copper at a salinity of 5 to ca. 110 nM when the salinity was tripled to 15 (Figure 3A). This is probably due to increased competition from Ca^{2+} and Mg^{2+} for binding sites on the cell membrane at higher salinities.[4] However, at FA concentrations above ca. 4 mg/L FA (corresponding to ca. 2 mg/L-C), the effect of salinity is much less and copper toxicity to *C. tenuicorne* appears to be controlled more by organic complexation of copper. For example, the EC-50 total copper concentration is 2.5 times higher (120 nM to 280 nM total copper) when the FA concentration is doubled to 8 mg/L in the salinity 5 ASW, while at salinities of 10 the EC-50 total copper concentration increased by 20% (from 200 to 240 nM).

As expected the EC-50 based on total copper concentration increases with increasing FA concentration. This increase in copper concentration needed to elicit the same effect on growth is probably due to increased competition (for metal binding sites on the alga cell membrane i.e. the biotic ligand) by ligand sites on the added FA and hence a reduction on the fraction of copper available to bind to the biotic ligand.[3] According to the BLM, copper must first bind to the biotic ligands in order to be transported into the cell and thus elicit its toxic effects.[3,5] The added FA therefore binds some of the copper strongly enough to render it unavailable to the biotic ligand.

Free Copper and Weakly Complexed Copper. Both the BLM[3,5] and its predecessor FIAM[2] are based on the assumption that copper toxicity to algae and other phytoplankton can be

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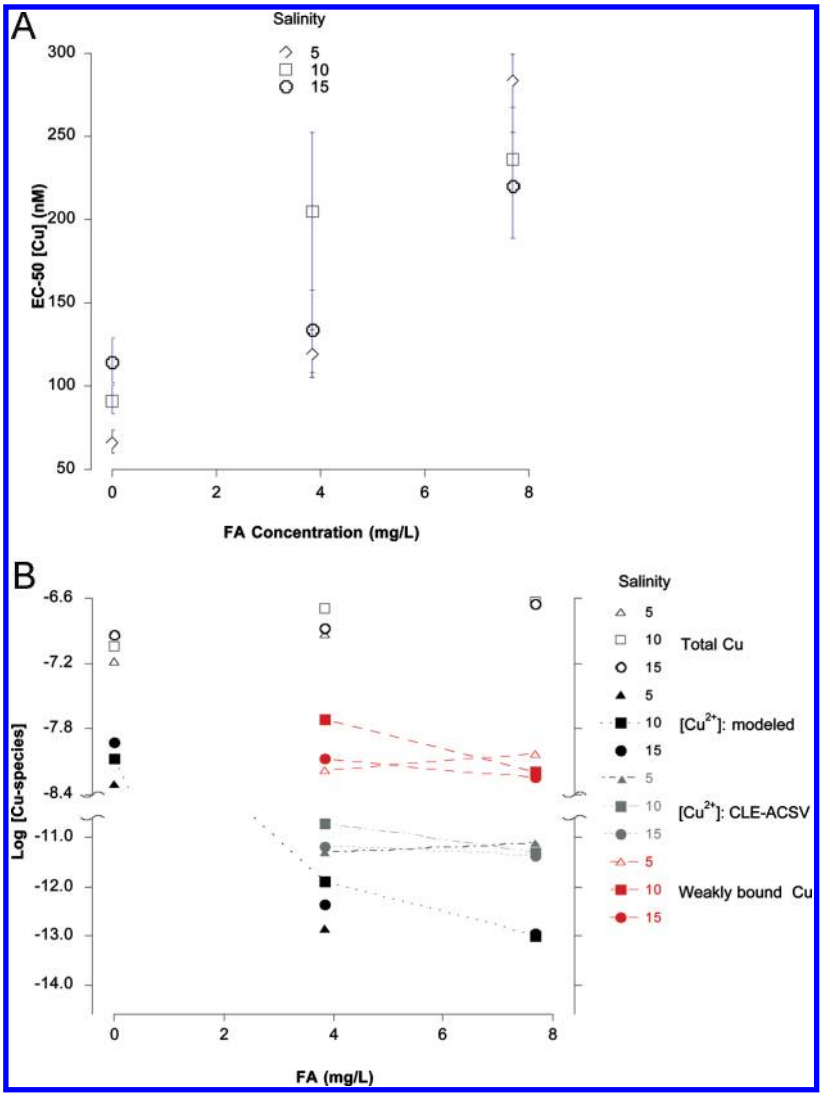

Figure 3. Macroalgae EC-50 concentration as a function of copper speciation (as influenced by binding to fulvic acid) in artificial seawater (ASW) at different salinities. A: Growth inhibition EC-50 expressed as total dissolved Cu concentration. B: Log EC-50 as a function of; total dissolved Cu concentration (open symbols), free Cu concentration i.e. $[Cu^{2+}]$ (as modeled by Visual MINTEQ (black), $[Cu^{2+}]$ as measured by CLE-ACSV (gray) and weakly bound Cu i.e. exchangeable with 1 uM SA in CLE-ACSV (red), plotted against fulvic acid concentration in ASW at salinities of 5 (triangles), 10 (squares), and 15 (circles).

described by the equilibrium concentration of the free copper $[Cu^{2+}]$, species. Slaveykova and Wilkinson[30] recently examined the underlying equilibrium assumptions in the BLM and identified the conditions that are appropriate for its application and several documented examples of failures of the BLM.[30] To test the FIAM hypothesis on Cu uptake, we compared the EC-50 values with respect to $[Cu^{2+}]$ measured by CLE-ACSV and theoretical values calculated using Visual MINTEQ incorporating the Stockholm Humic Model[31] (Figure 3B). We also compared the concentration of weakly bound copper, defined as copper species exchangeable with a concentration of 1 μM of salicylaldoxime, added to the ASW to compete for copper binding with binding sites on FA in CLE-ACSV measurements (Figure 3B). There is good agreement (within an order of magnitude) between CLE-ACSV and Visual MINTEQ calculated $[Cu^{2+}]$ in test ASW solutions containing FA. The change in salinity, as discussed above, also seems to exert little effect on toxicity with added FA. However, the EC-50 expressed as $[Cu^{2+}]$ is not independent of FA concentration, as the FIAM would predict. Although the EC-50 as $[Cu^{2+}]$ does seem to be independent of FA in test solutions with 4 and 8 mg/L FA, the EC-50 value is considerably higher in the test with no FA present (Figure 3). The concentration of copper species shown in Figure 3 A and B are for samples at the start of growth inhibition experiments.

We propose three possible explanations to explain the higher EC-50 as $[Cu^{2+}]$ in ASW test with no organic carbon present compared to the FA amended water: (1) That the macroalgae produced copper-complexing ligands at high $[Cu^{2+}]$ in the absence of added FA, to complex the excess $[Cu^{2+}]$, (2) That some of the copper was lost to the test container walls or via nonspecific adsorption on the algae and (3) That the algae can access some of the organically bound copper (weakly complexed copper).

Gledhill et al.[32] reported release of copper-complexing ligands by the brown alga *Fucus vesiculosus* (Phaeophyceae) in response

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Table 1. Diffusive Fluxes, Uptake Fluxes, and Copper Accumulation by *Ceramium tenuicorne* as a Function of Copper Speciation in 10 Salinity Artificial Seawater

FA (mg/L)	total Cu (nM)	weakly bound Cu (nM)	[a]$[Cu^{2+}]$ (pM)	intracellular Cu (μmol/g alga-dw)	w/s (g alg-dw cm^{-2})	uptake flux J_u (mol min^{-1} cm^{-2})	diffusive flux $J_{Cu\text{-}diff}$: (mol min^{-1}cm^{-2})	diffusive flux $J_{wk\text{-}diff}$ (mol min^{-1}cm^{-2})
0	16		1500	0.9	1.7x-10^{-4}	2×10^{-14}	1×10^{-14}	
0	25		3000	1.5	1.7x-10^{-4}	3×10^{-14}	3×10^{-14}	
0	61		5900	3.1	1.7x-10^{-4}	5×10^{-14}	5×10^{-14}	
4	16	0.1	0.1	0.6	1.7x-10^{-4}	1×10^{-14}	8×10^{-19}	8×10^{-16}
4	27	0.3	0.2	0.9	1.7x-10^{-4}	2×10^{-14}	2×10^{-18}	3×10^{-15}
4	53	1.4	0.8	1.4	1.7x-10^{-4}	2×10^{-14}	7×10^{-18}	1×10^{-14}
8	24	0.1	0.1	0.9	1.7x-10^{-4}	2×10^{-14}	8×10^{-19}	8×10^{-16}
8	47	0.3	0.1	2.1	1.7x-10^{-4}	4×10^{-14}	8×10^{-19}	3×10^{-15}
8	104	1.3	0.8	2.8	1.7x-10^{-4}	5×10^{-14}	7×10^{-18}	1×10^{-14}

[a] $[Cu^{2+}]$ in artificial seawater test solutions without added FA were calculated from Visual MINTEQ program.

to increasing total copper levels.[32] To test the possibility that the algae were producing copper-complexing ligands at high unchelated copper concentration, we carried out CLE-ACSV copper titrations on samples where the macro alga was exposed to copper without added FA. These samples were taken at the end of the exposure (7d) experiments at varying total dissolved copper concentrations. We could not detect the presence of any copper complexing ligands in solution in exposure test solutions with copper concentrations ranging from 0 to 260 nM. There is however a possibility that any such ligands produced by the algae might have been saturated by the high concentrations of copper (260 nM) in the test water thus making their (ligands) detection by CLE-ACSV difficult with the competing ligand used (salicylaldoxime).

Copper Accumulation by C. tenuicorne. We investigated the possibility that the algae could access some of the organically complexed copper by measuring the intracellular copper concentration in macroalgae exposed to ASW at different total copper (0 to 120 nM) and FA (0, 4, and 8 mg/L) concentration in 10 salinity ASW. For the test water without added FA, we used the speciation program Visual MINTEQ to calculate the $[Cu^{2+}]$.[31] Table 1 summarizes the macroalga's intracellular copper concentration as a function of its (copper) speciation.

The free copper concentrations in the ASW test media containing 4 and 8 mg/L FA ranged from less than 0.1 pM to about 1 pM. The corresponding free copper concentrations, in the absence of FA, were considerably higher i.e. 1.5 to 5.9 nM (modeled by visual MINTEQ). The concentration of the weakly complexed copper (i.e., copper species exchangeable with 1 μM of the CLE-ACSV competing ligand, salicylaldoxime) in the water containing FA was an order of magnitude lower (Table 1) to that without added FA, i.e. ca. 1% of the total copper concentration (16 to 104 nM). The intracellular copper concentration in the algae exposed to this water ranged from 0.9 to 3.1 μmol/g dw. The corresponding intracellular copper concentration in the macroalgae exposed to ASW containing 4 and 8 mg/L of FA was similar and ranged from 0.6 to 2.1 μmol/g dw. This similarity in the copper concentration accumulated by the macroalgae is despite the fact that the free copper concentration in the waters the latter algae were exposed to was 4−5 orders of magnitude lower.

Thus the intracellular copper concentration seems to be better related to both the total copper concentration and also to the weakly complexed copper, rather than the free copper concentration $[Cu^{2+}]$. This observation becomes clearer when the intracellular copper concentration is expressed as a function of the concentration of the three main copper species in the test ASW i.e. the free copper, the weakly complexed copper, and the total copper concentrations (Figure 4). The slopes of the intracellular copper to total copper concentration in the ASW are similar and relatively independent of the DOC (0, 4, and 8 mg/L FA) concentration (Figure 4A). The total dissolved copper concentration is also well linearly correlated to the intracellular copper concentration. The weakly complexed copper species could only be measured in test water with added FA (Figure 4B). The intracellular copper concentration also shows good linear correlation with this fraction, although not as good as that for the total copper. The slope of the intracellular copper to weakly complexed copper is within an order of magnitude that of the total dissolved copper and also relatively independent of the FA concentration. It is important to add that the fraction of the weakly complexed copper as defined here, would be higher if a lower concentration of the added competing ligand in the CLE-ACSV titration (in this case SA) was used instead, or if a competing ligand with a lower copper binding strength (e.g., catechol[14] or benzoylacetone[18]) replaced SA. The relationship between the free copper concentration (unlike the total and weakly complexed copper concentrations) and copper accumulated in the algae cells differ widely and are dependent on the FA concentration (Figure 4C). *In toto* these results seem to suggest that the macroalgae can access organically complexed copper when the free copper concentration becomes limiting.

Calculations of Copper Uptake Kinetics and Diffusion Fluxes. From the above observation, it appears that at low $[Cu^{2+}]$, copper uptake by C. *tenuicorne* might be limited by the diffusive flux of the free Cu^{2+} to the cell membrane.[12] Such a situation would be characterized by an uptake flux that exceeds the diffusive flux of the free Cu^{2+}. The corresponding gradient in the diffusion layer would then lead to dissociation of sufficiently labile complex species which then effectively contribute to the supply of Cu^{2+} to the biointerface.[12,13]

To test this hypothesis, we calculated the diffusive flux of the free Cu^{2+} and the weakly bound copper species and compared them to the uptake flux. The transport of Cu^{2+} and the weakly bound copper species to the membrane i.e. the diffusion flux and the measured internalization or uptake rate are shown in the last three

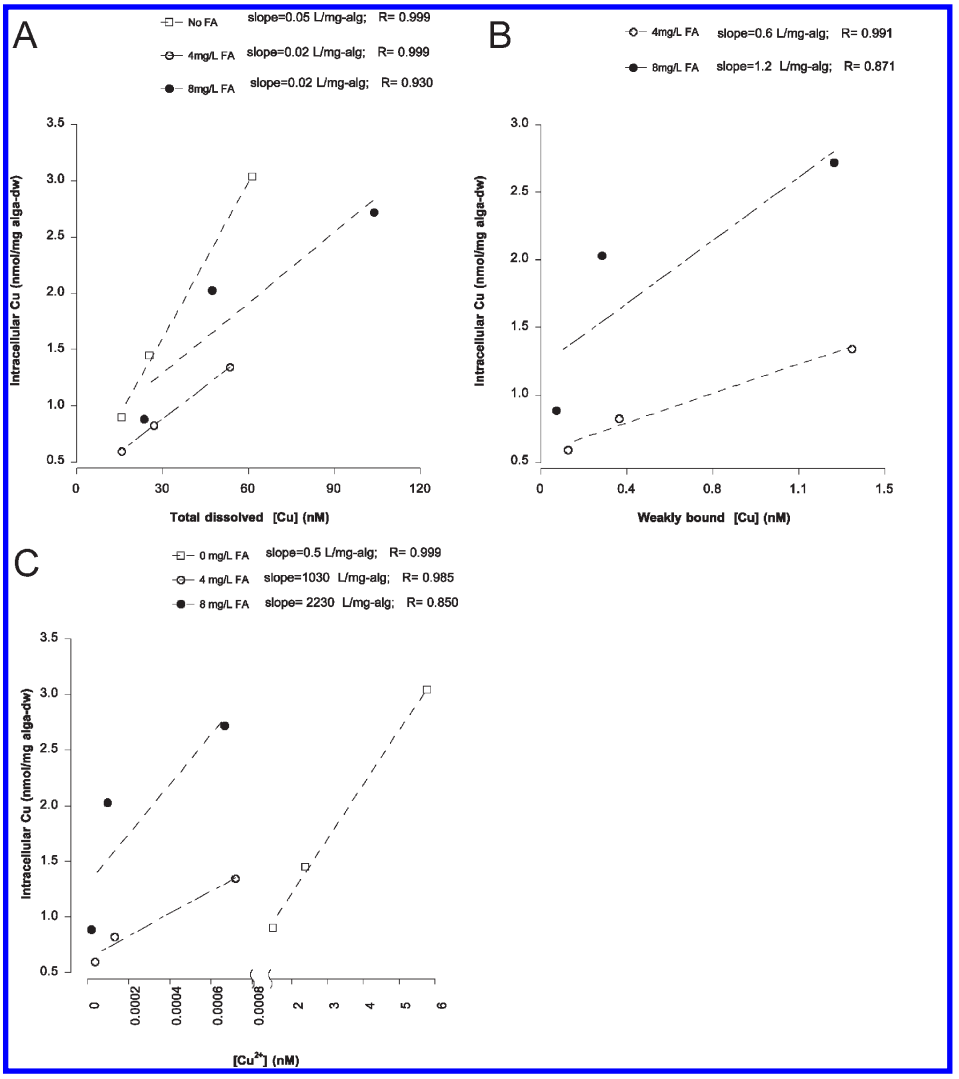

Figure 4. Relationship between intracellular copper in the macroalga *Ceramium tenuicorne* (10 salinity) and (a) total copper, (b) weakly complexed copper, and (c) free copper concentrations in the artificial seawater test media.

columns of Table 1. The copper uptake flux J_u (mol min^{-1} cm^{-2}) and the diffusive fluxes of both Cu^{2+}, $J_{diff\text{-}Cu}$, and the weakly bound copper, $J_{wk\text{-}Cu}$ (mol min^{-1} cm^{-2}), were evaluated for this study using the following expressions[14]

$$J_u = U_{Cu}(w/s)$$

$$J_{diff} = C_m D_m/\delta$$

where U_{Cu} (mol min^{-1} g^{-1}dw) is the uptake rate, w/s is a coefficient (g dw cm^{-2}) relating the macroalga dry weight to the its surface, C_m (mol cm^{-3}) is the metal concentration in the bulk solution, D_m (cm^2 min^{-1}) is the diffusion coefficient, and δ (cm) is the diffusive boundary layer.[14] The copper uptake rates U_{Cu}, data were calculated from macroalgae exposed for seven days to ASW amended with different concentrations of both FA (i.e., 0, 4, and 8 mg/L, Table 1) and copper. The macroalga *C. tenuicorne* exhibits an even dichotomous growth pattern which greatly facilitates length measurements.[24] The average (one dimension) length of macroalgae exposed to 10 salinity ASW for 7 d was about 7 mm, while the total two-dimensional (i.e., including all the branches) length was 125 mm per macroalga. The dry weight per alga was about 75 μg (average of 40−50 pooled algae). Diffusion coefficient for Cu^{2+} was assumed to be about 4.3 × 10^{-4} cm^2 min^{-1},[33,34] while the diffusive boundary layer in still water was assumed to be between 0.5 and 1 mm.[33,34] The diffusive fluxes for Cu^{2+} ($J_{diff\ Cu}$) and the weakly bound copper species ($J_{wk\text{-}Cu}$) were calculated from ACSV copper speciation and Visual MINTEQ (when no FA was added)[31] measurements.

The weakly bound copper concentration is defined here as copper species exchangeable with a concentration of 1 μM of salicylaldoxime, added to the ASW to compete for copper binding with binding sites on FA in CLE-ACSV measurements (Figure 3B). The copper species accumulations versus time are shown in Figure 4. The transport of Cu^{2+} to the cell ($J_{Cu\text{-}diff}$, 1 × 10^{-18} to 7 × 10^{-18} mol min^{-1} cm^{-2}) appeared to be 3−4 orders of magnitude smaller than the uptake of copper (J_{Cu}, 1 × 10^{-14} to 5 × 10^{-14} mol min^{-1} cm^{-2}) in ASW with added FA (4−8 mg/L, Table 1). This indicates that, even under the most favorable conditions (small boundary layer and high diffusion coefficient), the bulk solution might not supply enough free

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copper ions to the algal cells. The consequence is that labile or weakly bound copper in solution provides the metal to the cell wall. For the range of weakly bound copper concentrations encountered in this study (0.1−1.4 nM), the estimated diffusion flux ranges from about 1×10^{-15} to 1×10^{-14} mol min^{-1} cm^{-2} (Table 1), which is comparable to the estimated uptake flux of copper (1×10^{-14} to 5×10^{-14} mol min^{-1} cm^{-2}).

These results are in agreement with the few reported studies on copper uptake by periphyton, using natural ligands.[10,14] They also support theoretical predictions on metal uptake based on chemodynamics as recently reviewed in refs 12 and 35 where metal uptake and accumulation has been shown to be better related to the labile copper (weakly complexed), rather than the free copper concentration. In addition to the weakly bound copper species, uptake of lipophilic copper complexes by several algal species have also been shown to be important in controlling the bioaccumulation of copper in natural waters.[36,37]

Environmental Significance. The results of this study underscore the major influence of aqueous metal speciation in the accumulation and toxicity of copper to macroalgae. While these findings further reinforce the ongoing efforts (such as the BLM) aimed at incorporating metal speciation into toxicity and WQC regulations, they also show that the application of the BLM will not be straightforward. FA contains a continuum of metal binding sites of varying strength and concentration probably with a low concentration of high affinity sites and a much higher concentration of low affinity sites. The concentration of the strong binding sites is usually around or slightly more than the ambient total dissolved copper concentration in unpolluted estuarine and coastal waters.[17,18] At these copper levels, the copper speciation will most likely be controlled by the concentration of these strong binding sites in FA (or HA) at ambient copper concentrations. When the total copper concentration exceeds the concentration of the strong binding sites, copper speciation will then be controlled by the weaker ligands whose concentration is much higher.

Growth inhibition of the Baltic macroalgae in this study occurred at relatively high free copper concentration (EC-50 of ca. 10 nM (modeled), in ASW without added FA), in agreement with previous toxicity studies on other algae.[38,39] It is thus likely that copper toxicity to *C. tenuicorne* and other algae does not occur before diffusion limitation is reduced,[10,14] although toxicity inside the cell might still be controlled by the free copper concentration.[10,14] Thus although equilibrium free copper concentration from model calculations or actual measurements of metal speciation in coastal marine waters (having similar dissolved organic matter to the ones in this study) might show very low free copper concentrations, the intracellular and hence copper toxicity, might not be correlated to this concentration. Rather, the sum of this (free copper concentration) and some of the weakly complexed copper might determine copper accumulation and probably its eventual toxicity to macroalgae in marine waters.

■ ASSOCIATED CONTENT

Supporting Information. Table listing the input species data used to model copper speciation with the Visual MINTEQ program and a table with detailed pH data for the macroalga test solutions. This material is available free of charge via the Internet at http://pubs.acs.org.

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