Uptake of Dissolved Organic Carbon-Complexed Cu-65 by the Green Mussel Perna viridis

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# Uptake of Dissolved Organic Carbon-Complexed [65]Cu by the Green Mussel *Perna viridis*

Huan Zhong,[†,‡] Douglas Evans,[†] and Wen-Xiong Wang*[,‡]

[†]Environmental and Resource Studies Program (ERS), Trent University, Peterborough, Ontario, Canada
[‡]Division of Life Science, The Hong Kong University of Science and Technology (HKUST), Clearwater Bay, Kowloon, Hong Kong

S *Supporting Information*

ABSTRACT: Stable Cu isotope ([65]Cu) was complexed with various representative dissolved organic carbon (DOC) types, including coastal seawater DOC, fulvic acid (FA), cyanobacteria spirulina (SP) DOC, histidine (His), cysteine (Cys), and lipophilic diethyl dithiocarbamate (DDC) at different concentrations. The uptake of these dissolved Cu species by the coastal green mussel *Perna viridis* was quantified for the first time. Copper complexed with different DOC types were taken up in some measure by mussels, depending on the DOC types. However, complexation generally reduced Cu uptake as compared to that of inorganic Cu species, and DOC type-specific negative relationships were found between DOC levels and Cu uptake. Strong Cu binding sites (including His and organic sulfur functional groups) within DOC appeared to control the inhibitory effects of DOC on Cu uptake, possibly due to the competitive binding of Cu between the dissolved phase and biological membranes. Therefore, differences in strong Cu binding site levels may explain the differences in bioavailability of Cu complexed with different types of DOC. At the same time, the variations in Cu–DOC uptake may also be partly attributed to the absorption of Cu–DOC complexes, especially for the small Cu–DOC complexes (e.g., Cu–Cys, Cu–His, or Cu–DDC). Our study highlights the importance of considering the specificity of Cu–DOC complexes when assessing biological exposure to dissolved Cu in natural waters, especially during events, such as phytoplankton bloom periods, that could modify DOC composition and concentrations.
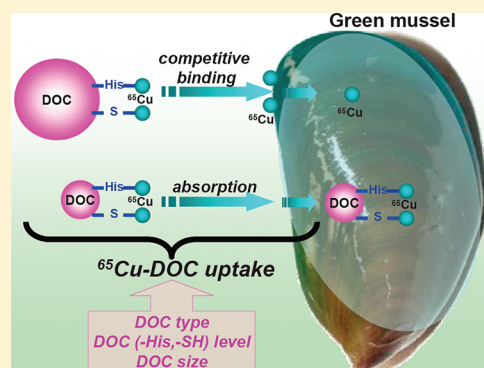
INTRODUCTION

The speciation of metals can affect their bioavailability, but no consensus has been reached as to whether the dissolved metal complexes are bioavailable, especially for marine bivalves.[1] Copper (Cu) is a micronutrient in aquatic systems but can be toxic at higher concentrations. The free Cu ions are believed to be the most bioavailable, and therefore in some studies, biological uptake as well as toxicity of Cu can be explained by free Cu ion concentrations.[2−6] Nevertheless, considering that the majority of dissolved Cu is complexed by organic ligands (especially humic substances) in marine environments due to their strong binding affinity,[2,6−8] the bioavailability of organic-complexed Cu should also be examined. Organic complexation was generally found to decrease the Cu bioavailability,[9−12] which could possibly be explained by the biotic ligand model (BLM).[13]

Dissolved organic carbon in seawater is heterogeneous. Most marine DOC originates from phytoplankton production and degradation[14,15] including both protein-like materials (e.g., amino acids, peptides, and proteins,[16]) and humic substances (e.g., fulvic and humic acids, by humification due to photochemical and bacterial respiration,[17,18]). Synthetic chelating ligands used in industry, for example ethylenediaminetetraacetic acid (EDTA), and diethyl dithiocarbamate (DDC), also constitute part of DOC in some ecosystems and can strongly

complex with metals.[19] Thus, the complexation of Cu by different types of DOC may modify Cu bioavailability and toxicity in natural waters.[6]

Because Cu is a micronutrient with relatively high and variable body concentrations in organisms, it is difficult to accurately quantify small changes in tissue Cu concentration after moderate exposure to stable Cu. Copper radiotracers with a suitable half-life (to the longest lived radioisotope being 61.83 h for [67]Cu) also are lacking. These two factors have impeded study of uptake of Cu−DOC complexes by aquatic organisms.[5,20] With the help of commercially available enriched Cu stable isotopes and inductively coupled plasma mass spectrometry (ICP-MS), which can determine the different Cu stable isotopes simultaneously and with extremely low background detection limits (ng/L), it is possible to trace spiked, enriched Cu isotopes and accurately quantify biological uptake of Cu.[20−23]

As filter-feeding bivalves, mussels are exposed to dissolved metals including Cu, because they pump large volumes of water for respiration or feeding. Thus, mussels are an ideal organism

to assess the biological availability of dissolved metals, as well as to monitor the metal pollution in aquatic systems, partly due to their relative immobility through their life span. In this study, we investigated the effects of different types and levels of DOC on bioavailability of dissolved Cu to a coastal green mussel, *Perna viridis*. We complexed stable ^{65}Cu isotope at environmentally realistic concentrations with representative DOC, including coastal seawater DOC, free amino acids (histidine or His and cysteine or Cys), fulvic acid (FA), a lipophilic organic ligand (diethyl dithiocarbamate), and biogenic (cyanobacteria spirulina, SP) DOC and then quantified the uptake of ^{65}Cu. The controls of DOC on bioavailability of Cu were then examined as a function of DOC type and concentration, as well as the key functional groups within DOC.

■ MATERIALS AND METHODS

Organisms and Chemicals. Coastal green mussels, *Perna viridis* (3−4 cm), were collected from Ma Liu Shui, Tolo Harbour, Hong Kong. After being brought back to the laboratory, mussels were acclimated in seawater (Clearwater Bay, Hong Kong) at room temperature for 7 days and fed a pure powder of cyanobacteria spirulina *Arthrospira platensis* (Puritan's Pride, New York, NY). Ten mussels were removed to measure background Cu concentrations both before and after the acclimation period. Stable ^{65}Cu isotope [as Cu(NO$_3$)$_2$] was purchased from Trace Sciences International, Richmond Hill, Ontario, Canada. Fulvic acid (2S101F, Suwannee River Fulvic Acid Standard II) was obtained from the International Humic Substance Society, Minnesota, USA, and L-amino acids histidine (His), cysteine (Cys), and lipophilic organic sodium diethyl dithiocarbamate (DDC) from Sigma-Aldrich (St. Louis, MO). All seawater collected from Clearwater Bay (CW, DOC: 76 μM, chloride: 0.52 M), Hong Kong, was filtered through 0.22 μm nitrocellulose membranes (Millipore) before use and was considered to be pristine from anthropogenic activity. A portion of seawater was photo-oxidized (ACE UV lamp, model 7480) for 48 h to remove DOC, after which all DOC was eliminated, i.e., the DOC concentration was not detectable. The concentration of DOC was measured by a Shimadzu-5000A TOC analyzer.

Dissolved Uptake of DOC-Complexed ^{65}Cu. The stable isotope ^{65}Cu was used in this study to trace uptake of Cu. Two sets of experiments were conducted (Table 1): (1) DOC type experiments where mussels were exposed to ^{65}Cu complexed with different representative forms of DOC (natural CW DOC, humic substance FA, biogenic SP DOC, amino acids His and Cys, and lipophilic DDC) in seawater with or without CW DOC (by photo-oxidation); (2) DOC concentration experiments where mussels were exposed to ^{65}Cu complexed with different concentrations of DOC (CW, FA, and SP DOC). Concentrations of organics listed below are expressed as concentrations of DOC unless otherwise specified. The unit μM was used for concentrations of all organics. For CW and SP DOC, the DOC concentrations (μM) were calculated by dividing mass-based DOC concentrations (mg C/L, by measurement) by carbon molecular weight (12 μg/μmol). For FA, DDC, His, and Cys, these were calculated by multiplying concentrations of organics (mg/L, by weighing) with % (weight) of carbon in the organic molecule (based on product information sheet) and then dividing by the molecular weight of carbon (12 g/mol). For example in DOC type experiments, DOC concentrations in Clearwater Bay seawater (CW DOC) treatment or FA treatment were calculated as:

Table 1. Treatments Used in the ^{65}Cu Uptake Experiments^a

treatment	CW DOC (μM)	added DOC (μM)	Cys/Org-S+His (nM/μM DOC)^b
DOC Type			
UV-CW	0	0	0.00
CW	76	0	1.25
UV-FA	0	100	4.13
CW+FA	76	100	2.89
UV-Spirulina (SP)	0	100	8.93
CW+Spirulina (SP)	76	100	5.62
UV-DDC	0	78	400
CW+DDC	76	78	203
UV-His	0	94	166
CW+His	76	94	92.5
UV-Cys	0	47	332
CW+Cys	76	47	128
DOC Quantity			
CW-L	0.1563	0	1.25
CW-M	15.63	0	1.25
UV-FA-1	0	0.1563	4.13
UV-FA-2	0	15.63	4.13
UV-FA-3	0	1563	4.13
UV-Spirulina (SP)-1	0	0.1563	8.93
UV-Spirulina (SP)-2	0	15.63	8.93
UV-Spirulina (SP)-3	0	1563	8.93

^{a}UV represents Clearwater (CW) Bay seawater without DOC (i.e., after photo-oxidation). ^{b}Summed concentrations of strong Cu binding sites: cysteine (Cys) or organic sulfur (S) and histidine (His), in different treatments were estimated based on measurement or references (see Materials and Methods for details in DOC).

(0.91 mg C/L)/(12 g/mol) = 76 μmol/L or (2.3 mg/L × 52.34% C)/(12 μg/μmol) = 100 μmol/L. A concentrated stock of spirulina (SP) DOC was freshly prepared by mixing 0.11 g of spirulina powder with 30 mL of Milli-Q, followed by filtration through 0.22 μm membranes. The measured SP DOC concentration in the filtrate was 33 mM .

To prepare the uptake media, DOC of different concentrations and ^{65}Cu (2 μg/L) were spiked into seawater with or without photo-oxidation and equilibrated at 18 °C for 2 h before use. Six mussels were used for each treatment; two mussels were placed in a Teflon beaker containing 800 mL uptake medium. The uptake medium was renewed after 4 h to sustain a relatively constant dissolved ^{65}Cu level. After 8 h of exposure, the mussels were removed and placed into beakers with nonspiked seawater for 1 min to remove the weakly bound Cu. After that, the animals were rinsed with Milli-Q water and dissected. Shells were cut open and soft tissue was wiped dry and rinsed with Milli-Q water to remove the residual ^{65}Cu not related to biological uptake. The whole mussel tissue was dried in an oven at 80 °C to constant weight. A time series (2, 4, 6, and 8 h) uptake experiment in ^{65}Cu spiked (2 μg/L) CW seawater was also conducted to examine whether the Cu uptake increased linearly with exposure time. All containers were acid-washed and rinsed with Milli-Q water before use.

Determination of ^{65}Cu and Amino Acid Concentrations. Dry mussel tissue, as well as certified reference materials (TORT-2, National Research Council Canada, certified total

[Cu] = 106 ± 10 μg/g), were digested with double distilled nitric acid (Cu < 0.05 μg/L) in capped Teflon tubes at 220 °C and 100 bar for 20 min in a high pressure microwave digestion system (UltraCLAVE III, Milestone, Shelton, CT). Cu isotope (^{63}Cu and ^{65}Cu) concentrations were determined by ICP-MS (820-MS, Varian, Palo Alto, CA). The recovery rate of Cu in the reference material was always higher than 90% (>100.2 μg/g).

Intensities of both ^{63}Cu and ^{65}Cu were measured, after which concentrations of the individual ^{63}Cu or ^{65}Cu isotopes in each digested tissue sample were calculated from isotope-specific calibration curves. Background concentrations of ^{65}Cu in the tissue (Tissue (^{65}Cu)$_{background}$) representing ^{65}Cu not due to the uptake of spiked ^{65}Cu (most of which was present at $T = 0$) were calculated from the measured concentrations of the nonspiked isotope ^{63}Cu (tissue (^{63}Cu) $_{measured}$) and the natural isotope ratios (^{63}Cu: 69.15%, ^{65}Cu: 30.85%), where:

$$\text{tissue}(^{65}\text{Cu})_{background}$$
$$= (30.85/69.15) \times \text{tissue}(^{63}\text{Cu})_{measured} \quad (1)$$

Similarly, background concentration of total Cu in the tissue was calculated as:

$$\text{tissue Cu}_{background}$$
$$= (100/69.15) \times \text{tissue}(^{63}\text{Cu})_{measured} \quad (2)$$

The net ^{65}Cu uptake was calculated by subtracting the background tissue ^{65}Cu concentrations from measured ^{65}Cu concentrations:

$$\text{Tissue}(^{65}\text{Cu})_{accumulated}$$
$$= \text{Tissue}(^{65}\text{Cu})_{measured} - \text{Tissue}^{65}\text{Cu})_{background}$$
$$(3)$$

This approach to determination of the background concentrations was verified by spiking different amounts of ^{65}Cu into the certified reference material NIST 2976 mussel tissue (total [Cu] = 4.02 ± 0.33 μg/g). Spiked ^{65}Cu concentrations were 0.5, 1, and 2 μg/g (each concentration with five replicates). Using eqs 1 and 2, the nonspiked total Cu concentrations were in the range of 3.71−4.07 μg/g, within the range of the certified value. The ^{65}Cu concentrations determined using eq 1−3 were 0.484 ± 0.007, 0.986 ± 0.009, and 1.966 ± 0.010 μg/g, respectively, i.e., recovery rates of added ^{65}Cu were higher than 95%.

To investigate the controls of DOC functional groups on the uptake of Cu, total hydrolyzed amino acid (AA) concentrations in spirulina and seawater DOC were determined using a Hitachi 835-50 Amino Acid Analyzer (Tokyo, Japan).

■ RESULTS AND DISCUSSION

Background and Accumulated Cu in Mussels. In this study, we quantified the uptake of Cu at an environmentally realistic concentration (2 μg ^{65}Cu/L). Background tissue concentrations of Cu (shown in Supporting Information Figure S1), representing Cu in tissue that was not due to the uptake of spiked ^{65}Cu, did not change significantly (one-way ANOVA, $p < 0.05$) after laboratory acclimation and dissolved uptake experiments (except for Cu−DDC, which increased by 13%, because DDC inherently contained Cu). In contrast, the ^{65}Cu accumulated in mussels after the uptake experiments was 0.5−

1.8 μg/g (DOC type averaged), corresponding to 9.2−30.7% of the background tissue concentration of Cu (Supporting Information Figure 1), which would not be possible to accurately quantify without using isotope tracing.

Influences of DOC Type and Levels on ^{65}Cu Uptake. Few studies report the uptake rate of dissolved Cu−DOC complexes, partly because of the relatively high background concentrations of stable Cu in organisms and the lack of suitable radiotracers.[5] With the help of ^{65}Cu, uptake of ^{65}Cu complexed by different types of DOC was quantified in this study. Uptake of ^{65}Cu complexed with CW DOC increased linearly with time within 8 h (Supporting Information Figure S2). In an earlier study, the influx rates of Cu in green mussels were linearly related to dissolved Cu concentrations.[24] Thus, the uptake rate constant (k_u, L/g tissue/d) of ^{65}Cu for different treatments was calculated by dividing the accumulated tissue ^{65}Cu (μg/g) by the exposure time and the exposure ^{65}Cu concentrations (μg/L) (Figure 1). Copper in the presence of all
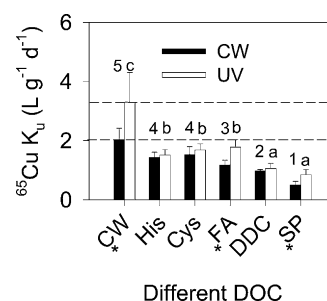

Figure 1. Calculated uptake rates of different ^{65}Cu−DOC complexes (DOC type experiments) by the green mussel, *Perna viridis*, in Clearwater (CW) Bay seawater before or after UV oxidation (UV). Asterisks indicate significant difference between CW and UV treatments for certain DOC type, numbers indicate significant difference among different DOC types in CW seawater, and letters indicate significant difference among different DOC types in UV-CW seawater ($p < 0.05$, one-way ANOVA). The treatment abbreviations are defined in Table 1. Uptake rate of ^{65}Cu in CW or UV-CW treatment is indicated by a dashed line. Mean ± SD ($n = 6$).

DOC types investigated in this study could be absorbed by mussels (absorption of Cu mentioned below referred to the accumulation of Cu in mussel soft tissue from uptake medium), while uptake rates depended on DOC types. However, complexation of Cu with DOC generally inhibited Cu uptake. Inorganic Cu species in UV-CW treatment had the highest uptake rate (3.30 L/g/d). In contrast, addition of SP DOC (UV-SP) and DDC (UV-DDC) into UVCW inhibited Cu uptake the most, followed by FA (UV-FA) and free amino acids (UV-His and UV-Cys). Similarly, addition of those DOC types into CW seawater reduced Cu uptake as well, further demonstrating the inhibitory effects of DOC on Cu bioavailability. Our results are in agreement with previous studies where DOC buffered the biological exposure of Cu,[2−5,9−12] considering that the highest uptake rate of Cu was in the UV-CW treatment. In such a seawater system without organic ligands, the dominant Cu species should be $CuCO_3^0$, with approximately 7% of the dissolved Cu existing as the free hydrated Cu ion.[6] To our knowledge, this is the first study that has systematically investigated uptake of different Cu−DOC complexes by marine animals.

Due to the lack of equilibrium constants for some organic complex species, we did not calculate the Cu speciation in

different uptake media. Similarly, it is difficult to discriminate free Cu ions and various inorganic or organic Cu species by dialysis or ultrafiltration, in view of the large variation in the size of different Cu complexes.[2] Consequently, it was difficult to conclude whether the observed biological uptake of Cu was due to absorption of Cu−DOC complexes or inorganic Cu species, especially free Cu ions that dissociate from the complexes during the experiments. On one hand, Cu should be mostly complexed by DOC in most treatments (except the UV-CW treatment where there is no DOC), considering the high molar ratios between DOC and Cu (1500−5700) as well as those between strong Cu binding sites and Cu,[7,25] e.g., strong binding sites:Cu molar ratio is >10 in all treatments except the CW (where it is three) or UV-CW (calculation of strong Cu binding sites levels was discussed below). Moreover, it has been reported that free Cu ions and other inorganic Cu species are insignificant at high dissolved organic carbon levels (DOC > 1 mg/L),[26] further suggesting that the majority of the Cu will be present as Cu−DOC complexes in most uptake media. This may suggest that the variable uptake rates of Cu in presence of various DOC types could be explained by the bioavailability of different Cu−DOC complexes. On the other hand, the observed differences in Cu uptake may possibly be due to the competitive binding of Cu ions between the dissolved phase (e.g., with different DOC) and biological membranes, and interpreted by the biotic ligand model (BLM). In this case, the Cu uptake could be controlled by binding affinity of Cu to DOC, especially the strong Cu binding sites within DOC. Both mechanisms may account for the observed Cu−DOC uptake and are further discussed below.

To further examine the effects of DOC levels on Cu uptake, we used different concentrations of natural seawater CW DOC as well as fulvic acid (FA) and spirulina (SP) DOC, representing the two most common components of coastal seawater DOC (humic-like and protein-like DOC). With an increase in DOC concentration, Cu uptake decreased in all three types of DOC treatments, in particular the SP treatment (Figure 2, upper right panels), further demonstrating the inhibitory effects of DOC on Cu bioavailability. At extremely high DOC concentrations (1.56 mM), Cu uptake decreased to zero. Considering all DOC types, there was a general trend with Cu uptake decreasing with increased DOC levels (Figure 2, upper left panels). Such inhibitory effects could be explained by the competitive binding of Cu with DOC in the uptake medium and binding sites on the biological membranes. However, the uptake of Cu−SP DOC was always lower than that of Cu−CW or Cu−FA at comparable DOC levels (Figure 2, upper left panel and Supporting Information Figure S3). In addition, the uptake rates of Cu did not change significantly when the DOC levels increased in the His, Cys, and DDC treatments by adding CW DOC (Figure 1). Therefore, DOC concentrations cannot fully explain the variation in Cu uptake observed in this study.

The wide range in DOC concentrations (0−76 μM for CW, 0.16−1600 μM for FA and SP) examined in this study cover the common DOC levels found in coastal seawater, suggesting that the enhanced DOC levels in natural coastal environments could greatly protect aquatic life from Cu exposure. It has been reported that DOC concentrations can reach 500 μM after the decay of a simulated bloom of marine phytoplankton[16] and 500−583 μM during an estuarine algal bloom.[27] Similar to our results, Sanders and Riedel[27] demonstrated that the percentage of organic-complexed Cu increased with the development of
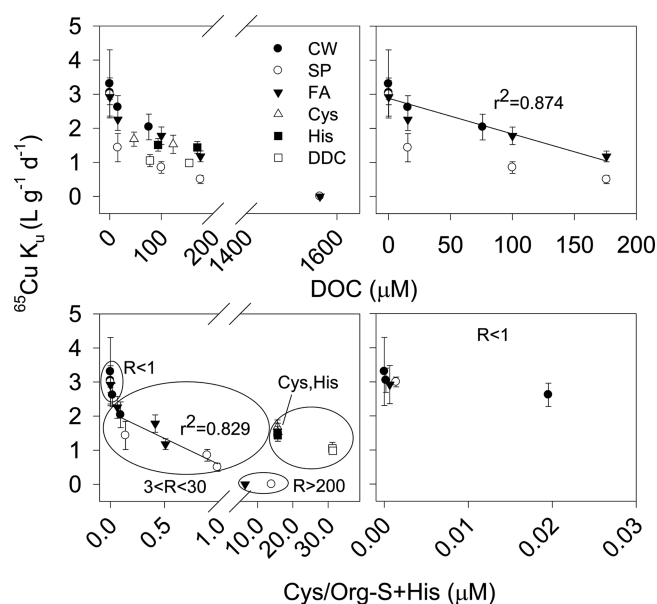

Figure 2. Relationship between total DOC concentrations (CW DOC and added DOC) and calculated uptake rates of different ^{65}Cu−DOC complexes (all treatments in DOC type and DOC quantity experiments depicted in upper left panel; treatments with lower DOC levels in DOC quantity experiment, i.e., CW, FA, and SP, depicted in upper right panel); relationship between estimated concentrations of strong Cu binding sites (Cys/Org-S+His) and calculated uptake rates of different ^{65}Cu−DOC complexes (all treatments in DOC type and DOC quantity experiments depicted in lower left panel; treatments with lower Cys/Org-S+His levels, in DOC quantity experiment, i.e., CW, FA, and SP, depicted in lower right panel). *R* indicates ratio of Cys/Org-S+His to ^{65}Cu. The treatment abbreviations are defined in Table 1. Mean ± SD (*n* = 6).

the estuarine algal bloom, transforming Cu into less toxic forms.

DOC Functional Groups and Uptake of ^{65}Cu. Little is known about the important functional groups involved in DOC binding with Cu and the subsequent effects on biological uptake.[6] Normally, Cu-binding ligands are characterized as strong (L$_1$) and weak (L$_2$) ligand classes, depending on their conditional stability constants: $K_{L1} = 10^{11-14}$, $K_{L2} = 10^{8-10.2}$. The Cu−L$_1$ complexes are relatively less bioavailable (e.g., to phytoplankton[28]). The source of L$_1$ ligands may be biological, e.g., exudates from phytoplankton (especially cyanobacteria) and decomposition of dead organisms, while L$_2$ ligands could be contained within humic substances.[2,6,25,29] However, there are inconsistent observations on the characteristics of these ligands, e.g., functional groups for Cu binding, and sources of L$_1$ and L$_2$ ligands.[30−32] Furthermore, these ligand classes have not been related to biological uptake of Cu.

Both His and Cys, which contain the strong donor groups imidazoles and thiols, respectively, in their side chains, could efficiently complex metals. In fact, His can bind strongly with Cu; reported Cu−His binding constants range from $10^{10.2}$ to $10^{18.1}$ depending on the Cu:His ratios,[33] consistent with that of strong ligands (L$_1$). Previous studies have indicated that both natural Cu-binding organic ligands and those from microorganisms contain organic sulfur (e.g., thiol) groups, which could be the key Cu-binding sites.[2,30,34] Furthermore, Cys and His were suspected to be the main amino acids involved in Cu binding in protein-like DOC originating from phytoplankton.[16] Both His and organic sulfur are abundant in many DOC

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types,[34,35] and could be the key strong Cu binding sites in DOC. However, it is still unknown whether Cys/organic S+His levels represent the commonly recognized strong Cu binding sites of DOC or L_1 ligands (stability constants = 10^{11-14}).

To investigate the controls of those strong Cu binding sites on Cu bioavailability, we assessed the Cys/organic S+His levels in different types of DOC based on measurement or reference in this study. The total hydrolyzed His content (free His and its residuals) in Clearwater Bay seawater was too low to be detected, 0.000459 μM in 100 μM FA solution (International Humic Substances Society Web site), and 0.521 μM in 100 μM spirulina solution. In view of the complexity of organic sulfur (e.g., free Cys, Cys residues, and other thiol groups), we can only estimate its level in the DOC. For spirulina DOC, the organic sulfur level was estimated based on the measured total hydrolyzed Cys content (0.372 μM in 100 μM spirulina solution), considering that it is mainly composed of amino acids and proteins. For FA (Suwannee river), the organic sulfur content was inferred from Leenheer et al.[36] (Suwannee River fulvic acid, 0.413 μM in 100 μM FA solution). Total organic sulfur levels in natural waters are rarely reported.[37−39] Because dissolved organic sulfur content is related to DOC levels,[39] the organic sulfur concentration in the CW DOC was estimated to be 95 nM, based on the average organic sulfur concentration in Chesapeake Bay seawater[38] as well as their respective DOC levels. This level is comparable to the strong Cu binding ligands level (23−103 nM) reported for San Francisco Bay.[7] For DDC, each molecule contains two thiol groups but no His residue. The summed cysteine or organic sulfur and histidine (Cys/Org S+His) levels in different treatments are listed in Table 1.

The relationship between Cys/org S+His levels and Cu uptake rates is plotted in Figure 2, lower panels. Compared to DOC levels, the concentrations of Cys/org S+His appear to better explain the variation of Cu uptake in different treatments (Figure 2). The much lower Cys/org S+His levels in CW DOC (0.095 μM/76 μM DOC) explain why the addition of CW DOC did not further inhibit Cu uptake in the His, Cys, and DDC treatments (Figure 1). Generally speaking, the lower Cu uptake in SP DOC (Figure 2, upper right panel) could be explained by the relatively higher Cys/org S+His levels in SP DOC. For DOC with larger sizes (CW DOC, SP DOC, FA), the effects of Cys/org S+His on Cu uptake depend on the ratio between Cys/org S+His and Cu (R). When the ratio is low (R < 1), there is no obvious effect of Cys/org S+His (Figure 2, lower panels). This may be due to the fact that a significant amount of Cu was in an inorganic form (e.g., free Cu ions), which controlled the Cu bioavailability. But when the ratio was higher ($3 < R < 30$), Cu uptake decreased linearly with Cys/org S+His levels (Figure 2, lower left panel). At extremely high ratios ($R > 200$), the Cu uptake decreased to zero. All the above results suggest that His and organic sulfur have some control on Cu bioavailability. The reason for this control could be the strong binding between Cu and His or organic sulfur groups (e.g., thiol groups) in the uptake medium (e.g., with DOC), leading to a decrease in Cu association with binding sites on the biological membrane and subsequently decreased biological uptake of Cu. However, for those smaller organic molecules (free His, Cys, and DDC), their Cys/org S+His related Cu uptake rates are relatively higher than the other DOC types, and Cu could still be taken up by mussels even at high Cys/org S+His levels (Figure 2, lower left panel). This could possibly be explained by their smaller sizes and thus the direct absorption by the mussels (discussed below). In all, the strong Cu binding

sites within DOC and thus the Cu−DOC binding have a great control on the biological uptake of Cu by mussels, and competitive binding of Cu between DOC and biological membrane may explain most of the variation in Cu−DOC uptake observed in this study.

The strong Cu-binding functional groups (e.g., His, organic sulfur groups) within DOC could be key factors in controlling the speciation and bioavailability of dissolved Cu in aquatic systems. Thus, the previously reported difference in the bioavailability of the two ligand classes (L_1 and L_2 ligands) may be explained by the different strong functional group levels. Biogenic dissolved organics (e.g., L_1 ligands) considered to be relatively nonbioavailable could be rich in strong Cu-binding ligands especially organic sulfur.[2,16,30,34] In contrast, humic substances including FA (e.g., L_2 ligands) normally contain fewer amino groups including His and Cys[40] and may bind more loosely with Cu, leading to higher Cu uptake. In view of the complexity of composition and functional groups of DOC, it is more probable that there are no specific DOC sources for the defined strong (L_1) and weak (L_2) ligand classes. For example, humic substances could contain a series of Cu binding sites with different affinities, thus the stability constants of Cu−Humic complexes depend on the ratio between Cu and humic concentrations. Similarly, Town and Fiella[29] reviewed the conditional stability (K) constants and complexation capabilities of different organics over 34 years and found a range in Cu binding sites with different stabilities in both humic and biotic organics. They proposed that it was not necessary to separate ligands into classes (e.g., L_1 and L_2 ligands) based on their stability constants (K); rather the Cu complexing ligands in natural waters were actually a mixture of both terrestrial (e.g., FA) and biogenic organics.

In this study, we also found that spirulina DOC possessed Cu binding sites (His and organic sulfur groups) stronger than those of FA, while CW DOC had an amount of strong Cu binding sites comparable to those of FA (Table 1). This may explain why a linear relationship was found between Cu uptake and DOC levels in the CW, FA, and CW+FA treatments while Cu uptake in the SP or SP+CW treatments was always below the regression line (Figure 2 upper right panel). This observation could possibly suggest that humic-like DOC with less strong Cu binding sites instead of biogenic DOC accounts for more Cu binding in CW seawater. The low productivity in the CW seawater, indicated by the relatively low Chl a levels (average 1.8 μg/L[41]), may result in lower protein-like DOC levels.

Absorption of ^{65}Cu−DOC Complexes. Many exceptions to the free-ion activity model (FIAM, explaining the correlation between free ion concentrations and biological effects) have been reported.[42] For example, Cd and Zn complexed with small organic ligands (e.g., EDTA, NTA, diethyldithiocarbamate) were partially bioavailable to green mussel *Perna viridis*,[1] possibly due to the transport of metal−organic complexes across the plasmalemma of the cells. For Cu, some recent studies have proposed the possibility of absorption of complexed Cu species, e.g., Cu−DOC complexes. In a recent study, Hoang and Rand[43] suggest that the poor relationship between dissolved free Cu ion concentrations and Florida apple snail Cu tissue concentrations may be due to the uptake of Cu−DOC complexes and other Cu species. Using bioluminescent bacterial reporter strains, Brandt et al.[26] found that bioavailability of Cu (indicated by inducing bacteria gene expression but not assuming intracellular uptake) in manure-

amended soil was not related to free Cu ion activity, implying that Cu−DOC (possibly Cu−citrate) could be taken up by bacteria. Using the same biosensor, it was reported that Cu could form both bioavailable and unavailable complexes with soil-extracted natural dissolved organic matter, while the availability of specific Cu−DOC complexes (except EDTA and citrate) was still unknown.[44] Thus, quantification of uptake of Cu complexed with DOC may help explain poor relationships between total dissolved Cu or free Cu ion concentration and Cu bioaccumulation or toxicity.[26,45]

According to the Irving-Williams series (relative stabilities of complexes formed by a metal ion, Mn(II) < Fe(II) < Co(II) < Ni(II) < Cu(II) > Zn(II)), copper may have the highest affinity to amino acids among transition metals. Free amino acids, as small organic molecules, can be taken up by mussels,[46] and thus Cu complexed with His and Cys may be cotransported through biological membranes of mussels. At the same time, low molecular weight, lipophilic organic matter, including DDC, could be taken up by organisms by passive diffusion across the cell membrane.[19] So it is quite possible that both free Cu ions and Cu complexed with free Cys, His, and DDC were adsorbed by mussels. This may explain why, for those smaller organic molecules (free His, Cys, and DDC), their Cys/org S+His related Cu uptake rates are relatively higher than the other DOC types (Figure 2, lower left panel).

Spirulina DOC, with 65% protein (product introduction) including some large DOC molecules (e.g., large structural proteins), may complex Cu strongly (e.g., by His or organic sulfur groups) and thus inhibit the diffusion of Cu through the cell membrane. However at the same time, a proportion of Cu could be complexed with small organic molecules (e.g., free amino acids, around 8% of total hydrolyzed amino acids in SP DOC according to our measurement) within SP DOC and taken up by mussels. Similarly, although the size of FA molecules are relatively large (weight-averaged molecular weight of 1910 Da for Suwannee fulvic acid),[47] we cannot exclude the possibility that some smaller Cu−FA complexes were taken up in the digestive tract, considering the relatively wide molecular weight distribution of FA used in the experiment.[47] Futhermore, Pan and Wang[48] demonstrated that macromolecules could be accumulated directly in the digestive glands of green mussels.

Therefore, besides competitive binding of Cu between the dissolved phase and biological membranes, which could be mainly controlled by the key Cu binding site (e.g., His and organic sulfur functional groups) levels as we discussed above, absorption of small Cu−DOC complexes may also contribute to the observed Cu uptake by mussels. Further investigations on the concentrations of small Cu−DOC complexes in natural waters and their contribution to Cu uptake relative to that of inorganic Cu species (e.g., free Cu ions) are warranted.

To conclude, common seawater Cu−DOC complexes could also contribute to the biological uptake of Cu by mussels and Cu complexed with various DOC types has different uptake rates. To our knowledge, this is the first time that the uptake of Cu complexed by different types of DOC by aquatic animals has been reported and quantified. However the complexation of Cu with DOC will decrease Cu bioavailability relative to inorganic Cu species (e.g., free Cu ions), depending on DOC types and levels. Such inhibitory effects could possibly be explained by the competitive binding of Cu between DOC in dissolved phase and biological membranes of mussels and largely controlled by strong Cu binding groups (including His

and organic sulfur groups) of DOC. Therefore, the variations in the uptake of different Cu−DOC complexes at various concentrations could possibly be explained by the different His and organic sulfur group levels within DOC as well as DOC molecular size, considering that some small Cu−DOC complexes (e.g., Cu−Cys, Cu−His, or Cu−DDC) may be taken up by mussels. In natural waters, DOC composition and events that could affect its composition (e.g., phytoplankton blooms that enhance biogenic DOC levels) could greatly influence the speciation of dissolved Cu and its bioavailability. Because we could not directly calculate or determine the dissolved Cu speciation in this study, contribution of inorganic Cu species including free Cu ions and Cu−DOC complexes (especially those small complexes) to Cu uptake in different treatments were not quantified. Thus, the mechanism of Cu−DOC uptake is not fully elucidated in this study, especially with respect to the uptake routes of specific Cu−DOC species. Direct comparison of bioavailability of inorganic and organic Cu species using stable isotope should be carried in the future.

Stable isotopes offer many advantages over metal or radioactive isotopes when investigating metal bioavailability. One can discriminate background from added tracer concentrations by monitoring influx and efflux simultaneously using two isotopes, and there are low detection limits.[21] In particular, the application of stable isotope tracers has greatly facilitated studies on metal biodynamics,[21−23] especially for metals without appropriate radioactive isotopes such as Cu. With the more widely available laboratory use of ICP-MS, stable isotopes should be a welcome alternative to other tracers in metal bioavailability research.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Additional information as noted in the text. This material is available free of charge via the Internet at http://pubs.acs.org/.

■ AUTHOR INFORMATION

Corresponding Author

*Phone: (852) 23587346; fax: (852) 23581559; e-mail: wwang@ust.hk.

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