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Predicting copper speciation in estuarine waters – Is dissolved organic carbon a good proxy for the presence of organic ligands?

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Abstract

A new generation of speciation-based aquatic environmental quality standards (EQS) for metals have been developed using models to predict the free metal ion concentration, the most ecologically relevant form, to set site-specific values. Some countries such as the UK have moved towards this approach by setting a new estuarine and marine water EQS for copper, based on an empirical relationship between copper toxicity to mussels (*Mytilus* sp.) and ambient dissolved organic carbon (DOC) concentrations. This assumes an inverse relationship between DOC and free copper ion concentration owing to complexation by predominantly organic ligands. At low DOC concentrations the new EQS is more stringent, but above 162 µM DOC it is higher than the previous value. However, the relationship between DOC and copper speciation is poorly defined in estuarine waters. This research discusses the influence of DOC from different sources on copper speciation in estuaries and concludes that DOC is not necessarily an accurate predictor of copper speciation. Nevertheless, the determination of ligand strength and concentrations by Competitive Ligand Exchange Adsorptive Cathodic Stripping Voltammetry enabled the prediction of the free metal ion concentration within an order of magnitude for estuarine waters by using a readily available metal speciation model (Visual MINTEQ).

Keywords: Copper, speciation, estuarine waters, dissolved organic carbon, ligands, modelling

1. INTRODUCTION

The importance of chemical speciation is widely recognised in the scientific and regulatory community, where physico-chemical characteristics control solubility, reactivity, biological availability and toxicity in the environment.^{1,2} The most toxic form of copper (Cu) in natural waters is the free metal ion [Cu²⁺] which can also be incorporated into the “labile” fraction, consisting of the free hydrated Cu cation plus weakly bound (largely inorganically complexed) Cu.³ Water quality parameters, such as major ion concentrations, pH and organic chelating agents (ligands) associated with dissolved organic carbon (DOC) are recognised as quantifiable parameters that significantly influence metal toxicity, through competitive binding by other major cations at reactive sites,³ lowered ionic activity of the metal,⁴ or complexation of the metal by the organic ligands. Increasing DOC concentrations have been shown to mitigate the toxic effects of Cu in organisms.⁵

In the USA, speciation-based approaches to Cu regulation in fresh water have been established since 2003 using the Biotic Ligand Model (BLM).^{6,7} The BLM combines thermodynamic equilibrium calculations of free metal ion concentrations generated by development of the Free Ion Activity Model (FIAM) using information on ambient water quality including at its most basic level, calcium concentrations (hardness), pH and DOC, with ecotoxicological endpoints to predict toxicity to classes of organisms or to set site-specific water quality standards.^{6,7} The complex chemistry within estuarine systems and lack of research slowed the development of BLMs for estuarine and coastal waters. Recently, a number of studies have shown that Cu toxicity can be predicted: model development is underway predicting the fate of Cu in US harbours⁸ and a BLM has been developed for marine waters.⁹

Although some studies addressed the complexation of metals, such as Cu, in saline waters,^{10,11} data are limited and rarely combine other potentially important parameters such as DOC concentration and ligand characterisation with measurements of speciation. Currently no saline water BLM has been developed for the EU regulatory framework. The latest UK environmental quality standard (EQS) for Cu in saline waters has been revised under the Water Framework Directive (WFD) does, however recognise the bioavailability-controlling role of organic ligands present within the DOC pool.¹² The new Cu EQS is based on a species sensitivity distribution of toxicity data for predominantly marine organisms (salinity greater than 20). The generic EQS generated using this approach (applicable to all estuarine and coastal waters) is converted to a site-specific EQS based on the measured ambient DOC concentration as a surrogate for copper complexation (Figure S1 in the supporting information). The DOC correction factor developed for the new Cu EQS

applicable to estuaries and coastal waters across all salinities is based on observed mussel (*Mytilus galloprovincialis*) toxicity data across a range of DOC concentrations (Figure S2).¹² Up to a DOC concentration of 83 μM (1 mg C L^{-1}) the Cu EQS is fixed at 59 nM ($3.8 \mu\text{g Cu L}^{-1}$). The EQS increases linearly above DOC = 83 μM (1 mg C L^{-1}) to a point where, above DOC = 162 μM (1.95 mg C L^{-1}), the site specific Cu EQS is more relaxed than the previous fixed value of 78 nM Cu. If DOC concentrations reach 475 μM (5.71 mg C L^{-1}) the Cu EQS is twice the previous value.

The EQS derivation therefore relies on the assumption that DOC is 'protective' which is well established under laboratory conditions using DOC sources such as humic and fulvic acids.¹³ Figure S2 shows the relationship between DOC and EC50 (the concentration that negatively affects 50% of the tested population) for *Mytilus*. However, increased data scatter above 500 μM (6 mg L^{-1}) DOC is evident. For example, the EC50 varies by up to a factor of 5 at ca. 500 μM DOC, and DOC varies by up to a factor of 3 for a given EC50 of ca. 500 μM Cu. Although the EQS derivation uses the same experimental data expressed as EC10 rather than EC50, the same variability in DOC vs toxicity response is observed. This scatter originates in the variability of ecotoxicological data within and across experiments and the fact that not all DOC contributes to Cu complexation. The latter is reflected in existing computational models for Cu toxicity, where various correction factors are applied to observed DOC concentrations to account for the contribution of organic ligands to Cu complexation. This is described as 'active DOC' in the Windermere Humic Aqueous Model (WHAM) models,¹⁴ 'humic acid content' in BLMs³ and is converted into ligands via algorithms such as the NICA-Donnan, Gaussian or Stockholm Humic Models in Visual MINTEQ.¹⁵ The effectiveness of these assumptions in characterising the Cu complexation within saline waters is variable and often leads to over prediction of free Cu ion concentrations ($[\text{Cu}^{2+}]$), particularly at low Cu concentrations.¹⁴ Although this is conservative, a lack of agreement between predicted and actual values suggests improvements in the existing models are possible which would further strengthen the application of BLMs for saline waters BLMs. Studies generally show weak relationships between Cu complexation capacity ($[\text{L}_x]$) and DOC concentrations measured in saline waters (Figure 1),^{11,16-19} unless samples were taken across a limited range of salinities and/or restricted to a specific ligand strength, typically derived from a single source such as river humic and fulvic acids.^{16,18} It is important to note that in all cases the organic ligand complexation capacities are in the order of $1 \text{ to } 100 \times 10^{-9} \text{ M}$ of Cu compared with DOC concentrations in the $1 - 100 \times 10^{-6} \text{ M}$ range. In other words, the complexing ligands are present at one thousandth of the total DOC concentration.

Figure 1. Complexation capacity for Cu vs. DOC (data from ^{11,17-20}).

Are there better ways to capture the effects of metal complexation by organic ligands in estuarine waters than using DOC as a surrogate for bioavailability? Most existing research in freshwater has focussed on the complexation of Cu by humic and fulvic acids, but these materials, derived mainly from breakdown of terrestrially derived organic matter are, while important,⁴ not the only source of DOC to the estuarine environment. Wastewater Treatment Works (WwTW) effluent contains high concentrations of DOC (in excess of 833 μM (10 mg C L^{-1})),²⁰ comprising natural and synthetic ligands capable of strongly complexing metals.^{17,21} Estuaries are habitats of high primary productivity and ligand production through cell lysis after phytoplankton blooms often exceeding 1000's of cells per mL and proteinaceous exudates of macrophytes.^{22,19}

This study seeks relationships between the different forms of Cu within an estuarine environment subject to varying salinity, DOC concentration and potential organic ligand sources. A degree of *in situ* organic matter source characterisation was performed using established fluorimetric techniques to determine humification and biological indices (Section S2.5), to seek improved relationships between Cu speciation and potential organic ligands. This was achieved by collecting samples in different seasons between 2013 and 2015 from the Tamar; a temperate, macrotidal estuary in SW England. This estuary is subject to inputs of organic ligands from sources including WwTW effluent, natural breakdown of organic matter and primary production (Figure 2). Stripping voltammetry was used to determine operationally defined Cu-complexing organic ligand concentrations (complexation capacity, $[\text{L}_x]$), stability constants ($\text{Log } K$) of Cu-organic ligand complexes, $[\text{Cu}^{2+}]$ and labile Cu

concentrations. Copper speciation data have been interpreted in combination with measured DOC concentrations and characteristics (indices), which can indicate the presence of humic and fulvic acids and/or *in situ* biologically derived organic compounds. In order to establish if the toxic $[Cu^{2+}]$ fraction can be successfully predicted for the samples collected, a freely available program, Visual MINTEQ (VM) was used to calculate $[Cu^{2+}]$ in each sample in two modes: (i) using the “default” NICA-Donnan humic complexation model to convert measured DOC concentration into Cu-complexing organic ligand concentrations and (ii) using the measured $[L_x]$ and $\log K$ for two ligand strengths determined by stripping voltammetry with complexation capacity titrations. The results allowed an evaluation of the effectiveness of DOC as a proxy for predicting Cu speciation in saline waters and of the accuracy of in-built model algorithms for calculating the distribution of Cu species. The combination of measured environmental Cu speciation data, DOC characterisation and modelling carried out in this study provides vital data and critical analysis to support on-going BLM development for saline waters.

144

Figure 2. Map of the Tamar Estuary, UK, with sampling station locations. Abbrev: PS Plymouth Sound; DVP Devonport; LC Lynher Confluence; STSH Saltash; SNP South of Neal Point; CGR Cargreen; HF Haye Farm; WQ Weir Quay; HH Holes Hole; PC Pentillie Castle; HQ Halton Quay; COT Cotehele; CAL Calstock; MWH Morwellham Quay; GNL Gunnislake; WwTW Waste water treatment works. Potential DOC sources are marked with coloured symbols. Outline derived from Ordnance Survey (1:50000, 2001). Insert: Made with Natural Earth. Free vector and raster map data @ naturalearthdata.com.

157 2. EXPERIMENTAL

158

159 2.1 Tamar catchment and sampling sites

160 The Tamar Estuary extends 16 km from Gunnislake Weir to the English Channel via
161 Plymouth Sound, with the Lynher and Tavy rivers being significant tributaries near its mouth
162 (Figure 2). The catchment area is 1700 km² and its length is close to the average for U.K.
163 estuaries.²³ The upper estuary receives inputs of contaminants, such as As, Cu, Zn and Pb,
164 from unconstrained mining waste from historic mining activities, particularly in the vicinity of
165 Gunnislake, where the tidal influence terminates above a weir.²⁴ The DOC in the river inputs
166 from the Tamar, Lynher and Tavy are likely to be dominated by humic and fulvic acids from
167 degradation of vegetation and run-off from upstream agricultural and moor land.²⁵ Effluent
168 from WwTW at Ernesettle and Camels Head (Figure 2) with an approximate combined
169 discharge of 30 million litres per day also enters the estuary in the higher salinity region,
170 introducing metals, as well as natural and synthetic complexing ligands.²¹ From the
171 confluence with the Lynher (denoted as site LC in Figure 2), intense phytoplankton blooms
172 occur through the spring and summer months as visibly seen in the water column in the July
173 2013 and 2014 samples as part of this work and previously.²⁶ Anthropogenically derived
174 contamination originating from the naval dockyard and urbanised areas near the mouth of
175 the estuary impacts on coastal water quality.²⁷ Sampling sites (between 5 and 8 depending
176 on tidal regime) along the length of the estuary were chosen to provide a range of salinities
177 as well as a variety of known metal and ligand sources (Figure 2). Sampling was carried out
178 on five occasions between July 2013 and February 2015, covering different seasons with
179 variations in river flow, salinity, and the presence of phytoplankton blooms during the spring
180 and summer months.

181 2.2 Chemicals and reagents

182 All chemicals used were of analytical grade or higher, and ultrahigh purity (UHP) water (Elga
183 Process Water, resistivity = 18.2 MΩ cm) was used for all applications. Hydrochloric acid (6
184 M, ROMIL SpA) was used throughout. Element reference solutions (ROMIL PrimAg) were
185 used to prepare Cu standards to a concentration of 1 µM. A 1 M stock solution of HEPES
186 buffer was prepared from N-hydroxyethylpiperazine-N'-2'-ethanesulphonic acid (Biochemical
187 grade, BDH Laboratory Supplies). The pH of the HEPES buffer was adjusted to ~7.8 using
188 ammonium hydroxide solution (ROMIL SpA). A 0.05 M stock solution of salicylaldoxime (SA;
189 98% Acros Organics) was prepared by dissolving in 0.5 mL 6 M HCl and making up to 30
190 mL with UHP water. This was diluted daily to make a working stock solution of 0.01 M SA

when used for Cu complexation capacity titrations (CCT) at concentrations of 2 and 10 μM , and for determinations of $[\text{Cu}_{\text{LAB}}]$ and $[\text{Cu}_{\text{TD}}]$ at 25 μM .

2.3 Sampling and sample handling protocol

Section A1 (SI) details the sampling protocol. All sampling and filtration equipment was acid washed and rinsed with UHP water prior to use. Samples for dissolved Cu were filtered through acid washed track-edged 47mm $<0.45 \mu\text{m}$ Nuclepore (Whatman) membrane filters using acid washed Nalgene filtration units. Samples for DOC were filtered within 24 h of collection, acidified to ca. pH 2, and refrigerated in glass vials. Samples for the determination of total dissolved ($[\text{Cu}_{\text{TD}}]$) and labile ($[\text{Cu}_{\text{LAB}}]$) Cu concentrations were refrigerated and analysed within 48 h at ambient room temperature. Samples for the determination of metal complexation capacity were frozen for later analysis.

2.4 Analytical methods, procedures and calculations

All dissolved Cu analysis was undertaken by competitive ligand exchange adsorptive cathodic stripping voltammetry (CLE-AdCSV, Section S2, SI). Labile Cu is operationally defined as the fraction of Cu that will form a complex with the added ligand salicylaldoxime (SA) at a set concentration. This includes $[\text{Cu}^{2+}]$ plus Cu held in weak complexes (e.g. bound by inorganic and weak organic ligands) that are substituted by the SA ligand within the timeframe of the determination. $[\text{Cu}_{\text{LAB}}]$ was calculated from triplicate voltammetric scans ($\text{RSD} \leq 5\%$) conducted on duplicate aliquots of sample ($\text{RSD} \leq 10\%$).

Prior to $[\text{Cu}_{\text{TD}}]$ determinations, acidified samples were UV irradiated in the presence of hydrogen peroxide (final concentration of 15 mM). Sample pH was raised to ca. 6 with ammonia solution (SpA, ROMIL) prior to adding HEPES and SA for analysis using the same voltammetric procedure as for labile Cu. Certified reference materials (CRMs) were included with each batch of samples. Recoveries were between 89 and 112%, with a typical precision of $\leq 10\%$ RSD. The limit of detection (LOD) was typically 0.33 nM Cu using maximum drop size, stirring speed and 60 s deposition.

Complexation capacity titrations (CCT) were performed at a fixed pH of 7.8 using HEPES buffer (at a final concentration of 10 mM in the 10 mL sample) at two competitive ligand strengths, using SA at 2 and 10 μM to provide detection windows ($\log \alpha_{\text{CuSA}} = 3.01 - 4.59$ and $3.03 - 5.29$, respectively), consistent with Cu ligand strengths previously investigated in the estuarine environment. Aliquots of sample were spiked with 10 incremental additions of Cu to a final concentration of ca 1.5 orders of magnitude greater than $[\text{Cu}_{\text{TD}}]$ in the sample and allowed to equilibrate overnight. Each sample aliquot was determined by CLE-AdCSV using analytical parameters provided in Section A2. Peaks for each voltammetric sweep were typically in the 5 to 100 nA range.

Duplicate titrations were carried out on all samples at each SA concentration, with triplicate voltammetric sweeps carried out on each aliquot to generate a mean peak height for each Cu addition. Data were transformed using a method reported by van den Berg/Ruzic^{28, 29} to quantify the $[L_x]$ (in nM), the conditional stability constant ($\log K'_{CuL}$) of the Cu-natural ligand complexes and $[Cu^{2+}]$. Owing to the very low concentration of $[Cu^{2+}]$ in natural waters (typically 10^{-13} to 10^{-11} M), for the purpose of displaying the data and showing trends, the negative logarithm of the Cu concentration has been taken and concentrations (rather than activity) shown as pCu^{2+} .

DOC was determined using high temperature catalytic combustion (Shimadzu TOC V analyser).³⁰ A marine water CRM (Florida Strait 700 m depth) was determined with each batch of samples and the LOD for DOC determination was 4 μ M. Characterisation of the DOC was undertaken using 3-D fluorimetry with a Hitachi F-4500 FL spectrophotometer. Calibration standards were diluted into UHP using Sigma Aldrich humic acid (55.1% C; Sigma Aldrich, UK) and Nordic aquatic fulvic acid reference material supplied by the International Humic Substances Society (45% C).

2.5 Thermodynamic equilibrium speciation calculations

All calculations were undertaken using Visual MINTEQ version 3.1¹⁵, a freeware chemical equilibrium model for the calculation of metal speciation, solubility equilibria, sorption etc. for natural waters. It combines descriptions of sorption and complexation reactions on a Windows platform and has the capacity for users to input new ligands (in this case $\log K'_{CuL_x}$ values and ligand concentrations derived from the CCT field data at two separate artificial ligand strengths) into its database. Input cation and anion concentrations were generated using an ion-pairing model³¹ (see section S2.1) using the Gunnislake freshwater sample composition from this survey and available Environment Agency data³² as well as reported coastal water data³³ as endmembers.

VM was used to predict $[Cu^{2+}]$ using the following input values (further default parameters in section S3):

- 1) Major ion concentrations (Na^+ , Mg^{2+} , K^+ , H^+ , Sr^{2+} , Cl^- , Br^- , SO_4^{2-} , F^-) generated from conservative mixing of Tamar freshwater (mean concentrations from Gunnislake sample from present surveys and available Environment Agency data)³² and sea water³³ end member data obtained from the ion-pairing seawater model (see section S2.1)
- 2) $[Cu_{TD}]$ measured as part of this work
- 3) Ligand concentrations:

- a. Measured as part of this work using 2 and 10 μM SA with their accompanying conditional stability constants
- b. Ligands generated within VM 3.1 using measured DOC concentrations from these surveys (Table S2) and the NICA-Donnan model for generating complexing ligands based on default assumptions that all active DOM is fulvic acid with no humic acid being ‘active’ in complexing Cu. Furthermore, 82.5% of DOC is assumed to be “generic fulvic acid” with a C content of 50%; giving a DOM to DOC ratio of 1.65 (see Section S2 of the SI)

The calculations were run at pH 7.8 and the default temperature of 15 °C, using Davies method for activity correction, with no other organic ligands present and assuming thermodynamic equilibrium. Sensitivity analyses (results not shown) performed by running VM calculations at the natural sample pH and temperature showed negligible difference in calculated speciation using either approach.

3. RESULTS AND DISCUSSION

3.1 Measured Cu speciation within estuarine waters

The Tamar estuary features a tidally induced, strong and well defined turbidity maximum zone (TMZ) between 0 and 10 salinity²³ and the extensive mudflats in mid-estuary are contaminated as a result of the mining legacy within the catchment.²⁴ Both impact on overlying water chemistry and Cu speciation over the course of tidal cycles.

The full set of $[\text{Cu}_{\text{TD}}]$ and $[\text{Cu}_{\text{LAB}}]$ for all the surveys are displayed in Table S 2 of the SI. The $[\text{Cu}_{\text{TD}}]$ ranged from 8 to 189 nM (Figure 3), with highest concentrations invariably observed in the freshwater sample taken at Gunnislake, reflecting the continuous contamination emanating from historic mine sites.²⁴ These concentrations are comparable with those found in previous studies of the Tamar.³⁴ A plot of $[\text{Cu}_{\text{TD}}]$ versus salinity shows non-conservative behaviour as fresh and saline waters mix at low salinities. This was likely a result of (i) loss of metal from the dissolved phase owing to a combination of sorption to suspended solids within the TMZ³⁵ and/or (ii) precipitation reactions associated with amorphous Fe oxyhydroxides and flocculation of colloidal material.³⁶

The increase of $[Cu_{TD}]$ at mid to high salinity in July 2013 and April 2014 is potentially attributable to additional inputs from tributaries contaminated with historical mine waste (Lynher, Tavy)²⁴ or mobilisation of metal from bed sediment during tidal pumping.³⁵ The combined effluent input to the lower estuary from Plymouth WwTW works is approximately $72 \times 10^6 \text{ L d}^{-1}$, with median copper concentrations of $5.6 \mu\text{g L}^{-1}$ reported for English WwTW effluents²⁰. This equates to approximately 400 g of Cu per day, which, with dilution, is unlikely to impact on observed concentrations.

Figure 3. Total dissolved (dark lines) and labile Cu (light lines) and new EQS (dotted lines) concentration profiles plotted against salinity observed during the seasonal Tamar transects (note the different scale for February 2014). Error bars represent the range about the mean of duplicate aliquots.

In most cases, the site-specific Cu EQSs for estuarine waters were slightly higher than the previous value (78 nM Cu), owing to the DOC concentrations determined in the samples being significantly above $83 \mu\text{M}$ (1 mg C L^{-1}). No exceedance of the EQS was observed within the estuary for any survey, whether comparing with new or previous standards. In terms of BLM development and further consideration of the

bioavailability of the metals however, it is necessary to consider the speciation of Cu in more detail.

Compared with total dissolved concentrations, $[Cu_{LAB}]$ can better reflect the likely bioavailability of the metal.³⁶ Labile Cu is relatively simple to measure and if it can be shown to provide useable relationships with DOC or other measures of metal complexing ligands then it could have a place within a regulatory framework. Overall, proportions of labile and complexed Cu determined using the two competitive ligand strengths showed similar patterns along the estuary (Figure 4). There were, however, no strong trends in the % labile Cu present across the estuary transects, reflecting the complex physico-chemical nature of this estuarine system. This, combined with the presence of varying organic and inorganic ligand concentrations and strength (potentially linked to source), means that strong trends in observed Cu speciation along the length of an estuary are unlikely. However, some broad observations are possible. Firstly, both the lowest $[Cu_{TD}]$ and $[L_x]$ are recorded at the seawater end member, reflecting high dilution of any riverine sources and a lack of other significant inputs. As a result, a significant proportion of the Cu is complexed at the mouth of the estuary. The proportion, however, varies between 20% and 90% depending on the survey and ligand strength detected. At the highest salinities, in almost all cases there is only marginal organic ligand excess over $[Cu_{TD}]$.

It is also clear from the data that the Tamar is not always the main source of organic ligands within the Tamar Estuary. The $[Cu_{TD}]$ is generally high in the river water end member and can exceed the $[L_x]$, resulting in increased $[Cu_{LAB}]$. Furthermore, there is an obvious input of ligands within the estuary at salinities between 5 and 25 for all surveys. This drives a ligand excess in mid-estuary, leading to percentages of complexed Cu being greater than 70% of the total present for all but the April 2014 survey. The HIX and BIX ratios suggest an important humic character of the DOC present in this region (Figure 5), possibly originating from tidal resuspension from the mudflats rich in organic matter, and/or potential inputs from the Tavy and Lynher rivers.

In this work, $[Cu^{2+}]$ is a more defined sub-set of Cu speciation than $[Cu_{LAB}]$ (see equations 1 and 2 in Section S2.1) as determination is undertaken at a predefined competitive ligand strength and reflects only the free metal ion, rather than additional 'labile' forms. Free Cu^{2+} concentrations in the region of 10^{-12} M were determined (Table S2, Figure S3, not shown owing to the scale), which are typical of those

measured in other estuaries.^{8,18,37} The $[\text{Cu}^{2+}]$ determined with any of the three ligand strengths fluctuated throughout the estuary, with no obvious relationship with salinity (contrary to the trend of increasing $[\text{Cu}^{2+}]$ with salinity reported elsewhere).³⁸ This highlights the fact that the complex nature and diverse sources of organic ligands makes it difficult to predict their binding capacity, and thus the potential $[\text{Cu}^{2+}]$ in the water column, without some form of direct measurement, even when organic carbon is characterised and quantified.

The next question is, therefore, how influential is DOC and its measurement on the observed Cu speciation?

Figure 4. Ligand concentrations ($[\text{L}_x]$), Ligand excess ($[\text{L}_x] - [\text{Cu}_{\text{TD}}]$), and labile and (organically) complexed Cu as a percentage of total dissolved Cu, for each sampling occasion. Dark red lines represent concentrations determined using 10 μM SA, pale red lines 2 μM SA. The x-axes represent salinity in all cases. Error bars on $[\text{L}_x]$ plots represent upper and lower confidence limits calculated from the standard error of the slope of the transformed data. NB. $[\text{Cu}^{2+}]$ concentrations are < 1 % of $[\text{Cu}_{\text{TD}}]$ for all samples.

3.2 DOC characterisation and influence on copper speciation

A comparison of the data from this study in a plot of Cu complexing capacity ($[L_x]$) versus [DOC] reported in previous studies of other saline waters shows no obvious correlation (data not shown), nor is this the case when separating out weaker ligands ($\text{Log } K < 13$) likely to be derived from a mixture of autochthonous and allochthonous sources (Figure 5A). However, trends emerge for two of the datasets^{16,18} when isolating the stronger ligands with a $\text{Log } K > 13$ (Figure 5B) that are potentially derived from terrestrial humic sources.¹⁸ A study on the river Scheldt¹⁶ showed that the ligand concentration can be predicted based on measured DOC and salinity because the source of organic ligands was dominated by a single riverine source. This indicates that for estuaries that feature a strong negative correlation between salinity and DOC, the assumption is reasonable that the DOC is dominated by a riverine source and may therefore be associated with humic and fulvic acids known to complex strongly with Cu.³⁹ For the Tamar, no obvious trends were observed between DOC and either $[\text{Cu}^{2+}]$ or salinity (Figure S4), confirming the complex nature of the interactions between Cu, ligands, sources and concentrations, set against a background of varying physico-chemical conditions within this estuary. Furthermore, DOC concentrations in estuarine waters are typically between 100 and 1000 μM ,¹⁷ whereas the reported concentrations of copper complexing ligands are typically in the 4 to 400 nM range,¹² i.e. up to 3 orders of magnitude lower, which may explain the observed apparent threshold of 100 μM DOC before significant, detectable complexation capacity is measured (Figure 5 A and B).

Figure 5. Comparison of data from this study with previously reported DOC and Cu complexation capacity in saline waters for ligands with [A]: Log K < 13.0 and [B]: Log K > 13.0.

From a modelling perspective the use of DOC alone as a proxy for Cu complexation and hence speciation and, more importantly, as a predictor of the most toxic (Cu^{2+}) form, is therefore unlikely to generate sufficiently accurate estimates. The ability to predict Cu speciation is considered in more detail in the next section.

It is possible to further characterise DOC in water using established 3-D fluorimetry methods which can also help to imply sources of the DOC present and this was explored as a another option for potentially predicting Cu speciation. The ratios of observed fluorescence peaks can be used to categorise the organic carbon as humic and fulvic, terrestrial or *in situ* generated material using the humification (HIX) and biological (BIX) indices (A2.5). HIX ratios ($\text{Ex260-Em320}/(\text{Ex260-Em460}) < 4$ suggest biological or aquatic bacterial origin, while increasing humic character up to ratios > 16 show progressively stronger humic character (Figure 6).⁴⁰ For BIX ($\text{Ex310-Em380}/(\text{Ex310-Em430})$ it has been observed that high ratios (0.8–1.0) corresponded

to a predominantly autochthonous origin of DOC from recent aquatic and bacterial activity freshly released into water.⁴¹

Fluorescence data for the Tamar surveys showed HIX values generally decreased towards the sea water end member (Figure 6, no data for July 2013). Important humic character was indicated for the fresh water endmember ($\text{HIX} > 10$), while DOC generated by *in situ* biological processes increased in importance towards the sea water end member ($\text{BIX} > 0.7$). It is likely that the DOC in the lower estuary was at least partially derived from phytoplanktonic activity and/or WwTW effluents. The combined effluent input to the lower estuary from Plymouth works is approximately $72 \times 10^6 \text{ L d}^{-1}$, with median DOC concentrations of 12 mg L^{-1} reported for English effluents,²⁰ thus equating to an input of ca. 864 kg of DOC per day.

Upper estuary and riverine HIX indices were > 10 during three surveys, supporting the hypothesis that the DOC was of mainly terrestrial origin, comprising mostly humic and fulvic acids. The BIX index corroborated this, with values increasing towards the sea water end member, demonstrating the autochthonous origin of the DOC present likely to be from primary production during summer months when this signal was strongest at salinities greater than 20.

Data from February 2014 appeared to show extreme DOC characteristics within the estuary. Sampling followed a prolonged period of intense rainfall across the SW of England, resulting in widespread flooding. River flows into the Tamar estuary were high enough to suppress salinity at its mouth in Plymouth Sound to 20, where the HIX index of > 20 indicated the strong terrigenous humic character of the DOC. These unusual estuarine conditions are also reflected in the Cu speciation discussed above (Figures 3 and 4).

The data presented above shows that $[\text{L}_x]$ was higher at salinities below 20 than towards the seawater end member, reflecting dilution with seawater containing low concentrations of DOC (Figure 4). Concurrently, mostly higher HIX and lower BIX values were observed at salinity < 20 , suggesting the DOC present was derived from riverine sources and largely humic in character (Figure 5).

A plot of $[\text{L}_x]$ against HIX and BIX shows weak positive and negative correlations, respectively (Figure 6); albeit it should be noted that the range of BIX indices is low for the Tamar Estuary (typically 0.6 to 0.8). Highest HIX values were associated with higher complexation capacities, which reflects the strong affinity of Cu for humic and fulvic acids,^{18,42} which dominate the lower salinity regions in the upper estuary.

However, this is not necessarily reflected in the observed Cu speciation (Figures 3 and 4), which would be expected to give rise to less $[Cu_{LAB}]$ and/or $[Cu^{2+}]$ in this region of the estuary owing to complexation (Figures 3, 4 and A3). The presence of a turbidity maximum with commensurate resuspension of contaminated bottom sediment and mine water discharges in the upper estuary containing labile or free metal ion, is therefore likely to be a complicating factor, potentially masking the expectation of lower labile Cu concentration occurring concomitant with a higher HIX index. Based on this dataset, although the indices provide useful information regarding possible sources of ligands, it appears that correlations are not sufficiently robust to improve greatly on the existing use of DOC concentration as a surrogate for Cu complexation characterisation.

Figure 6. The humification (HIX) and biological (BIX) indices for each seasonal transect against salinity (top) and complexation capacity, $[L_x]$ (bottom). Note dissolved organic carbon characterisation was not carried out for the July 2013 survey.

3.3 Predicting Cu speciation and implications for regulation

Models such as the BLM⁴³ and Gill Surface Interaction Model (GSIM)⁴³ combine predictions of metal ion activity and organism response to generate site-specific water quality standards taking account of ambient conditions. It should be noted that these models use metal ion activity, not concentration, for calculations. In general, the activity depends on any factor that alters the chemical potential, including concentration, temperature, pressure, interactions between chemical species and electric fields. For saline waters ionic strength can be a significant factor in determining model accuracy⁴⁴ and models typically use the Debye–Hückel or Davies equations to predict species activity.⁴³

In addition, any chemical speciation model within a BLM needs to incorporate thermodynamic data for organic ligands that are known to be capable of complexing metals in natural waters. Although metal interactions with individual synthetic and some natural organic ligands are well defined, metal – organic ligand interactions in natural waters are complex, variable and more poorly characterised. Given the analytical complexity and time required to determine the complexation capacity and strength of natural organic ligand mixtures, most models apply an algorithm to the DOC concentration to parameterise metal - natural organic ligand interactions. These algorithms are based on the assumption that humic and fulvic acids are the dominant metal complexing ligands in natural waters and estimate their concentrations. Model codes that use such algorithms for DOC corrections, and for which predicted and observed organically bound and/or free metal ion concentrations have been compared, include WHAM VII,⁴⁵ FIAM,⁴⁶ and VM⁴⁷. However, Figure 1 demonstrates that DOC is often a poor predictor of $[L_x]$ and possible explanations for this include the assumption of chemical equilibrium in thermodynamic speciation models (unlikely within an estuarine environment) and the very small proportion of metal complexing ligands in the overall DOC pool in natural waters. Some of the challenges associated with predicting free metal ions in saline waters were recently highlighted using the WHAM VII model, which assumes a fixed proportion of measured DOC actively complexes Cu.⁴⁵ The authors showed that approximately 59% of predictions were within one order of magnitude of the data determined using competitive ligand methods, although the agreement was much better at higher free ion concentrations ($>10^{-13}$ M), which are more typically observed in estuarine waters.

501 To test the NICA-Donnan approach for converting DOC into active Cu binding ligands, the
502 freeware metal speciation programme Visual MINTEQ¹⁵ was applied to the dataset obtained
503 from the Tamar Estuary and model outputs compared with observed Cu speciation. Using
504 default parameters (Section S3), concentrations for 16 samples from this study across the
505 full salinity range (Table S3) were entered into VM. Two scenarios were run, (i) $[L_x]$ and
506 $\log K_{CuL_x}$ obtained via CLE-AdCSV at two detection windows were entered to calculate $[Cu^{2+}]$
507 (Figure 7A) and (ii) DOC concentrations were entered to calculate $[Cu^{2+}]$ using the NICA-
508 Donnan humic complexation model approach (Figure 6B). The results showed a significant
509 difference in the outputs. Predicted versus measured $[pCu^{2+}]$ for scenario (i) showed that the
510 16 data points were relatively evenly distributed ($r^2 = 0.44$) along the 1:1 line, with up to an
511 order of magnitude variation either side and no obvious bias (Figure 7A). In contrast,
512 scenario (ii) resulted in a biased prediction of $[Cu^{2+}]$, generally under-predicting by as much
513 as two orders of magnitude (Figure 7B), clearly showing that not all of the DOC was active in
514 Cu complexation. Insufficient HIX data did not permit examination as to whether the bias
515 was directly associated with humic character. However, as higher HIX ratios would be
516 expected at low salinity (Figure 5) and there was no systematic variance with salinity
517 (coloured symbols), this estuary is unlikely to show a relationship between humic acids and
518 speciation. Because predictions were generally closer to observations at higher $[Cu^{2+}]$, either
519 modelling approach can more accurately predict $[Cu^{2+}]$ when the risk of toxic effects
520 occurring is greater.

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Figure 7. Measured versus predicted (using Visual Minteq) $[Cu^{2+}]$ concentration plotted as pCu^{2+} ($-\log_{10}$ of the molar concentration) with 1:1 line included (dashed line). Solid lines represent the linear least squares regression of the data. [A] using ligands measured as part of this work and [B] using VM ligands generated using measured DOC and the Nica-Donnan method (Green, amber and red symbols = salinity <10 ; $10-20$ and >20 respectively).

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The under-prediction of $[Cu^{2+}]$ present in estuarine waters would be a concern, as regulators and practitioners rely on models to provide conservative estimates to ensure environmental protection. Under-predicting the most toxic fraction of Cu cannot be considered precautionary and may not provide adequate protection to vulnerable aquatic species.

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These results show that VM or similar equilibrium speciation codes can be used to predict free metal ion concentrations with adequate certainty, given sufficient Cu-ligand data. Obtained results can be used in conjunction with data on ecotoxicological endpoints to assist in developing BLMs. This approach may help to eliminate the uncertainties observed in EC_{50} values plotted against DOC (Figure S2). The priority

is to characterise a set of organic ligands within individual estuarine scenarios where the sources are potentially very varied and quite different from riverine situations.

From a regulatory perspective, estuarine dissolved Cu concentrations were lower than the new DOC-corrected Cu EQS; however the relevance of DOC as an accurate proxy for Cu speciation (and hence potential toxicity) throughout an estuarine salinity profile is questionable, as discussed in section 3.3. The Tamar data shows no direct relationship between complexation capacity or Cu speciation and DOC concentration, and metal complexation trends are not related to the source and characteristics of the Cu complexing ligands. The lack of relationships between these parameters in the Tamar Estuary is probably the result of a number of factors including ligand source and strength, resuspension and particulate sorption chemistry, chemical precipitation reactions and colloidal interactions. The Tamar Estuary is not likely to be unique in this complexity and in addition, many estuaries contain a continuum of ligands with strengths that vary from very weak to very strong, at variable concentrations and which also exhibit temporal variability. Consequently, attributing a single factor to explain observed metal speciation may not be adequate to provide regulators with sufficiently accurate speciation predictions.

Even with this degree of complex physico-chemical interactions, and accepting that speciation models are based on an assumption of chemical equilibrium, this research has shown that it is possible to predict free metal concentrations within an order of magnitude of measured concentrations by using freely available chemical speciation software and inputting determined major ion concentrations and Cu ligand data (concentration and $\log K_{CuL_x}$ values). In contrast, using the NICA-Donnan complexation model to predict Cu speciation based on inputting DOC concentrations generated a bias in $[Cu^{2+}]$ predictions which overestimated the free metal ion concentration for 14 out of 16 samples across all salinities by up to almost two orders of magnitude. It should be noted that as part of the Nica-Donnan assumptions, the DOM:DOC ratio is generated from Swedish lakes and rivers samples dominated by humic and fulvic acids⁴⁸ which may not be reflective of sources of DOC in estuaries for the reasons discussed above. The data presented here show that, although DOC is broadly indicative of Cu complexation, it cannot be relied on as a basis for predicting free Cu concentrations in estuarine waters. Consequently, the use of DOC concentration in setting EQS should be considered as an interim step, with future BLM development for estuarine waters needing to take into account more detailed Cu speciation. Further data are required regarding the characterisation of organic ligands and their sources, matched to observed ecotoxicological outcomes, to generate a robust BLM upon which future environmental legislation and robust environmental quality standards can be based.

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583 Supporting Information.

584 Provides details:

585 New vs previous EQS for Cu in estuarine and coastal waters

586 Mussel toxicity data vs DOC

587 Free Cu figures vs salinity and vs DOC for two different ligand strengths

588 Details of sampling protocol and detailed analytical methodology.

589 Further details of the VM model

590 Tabulated raw data for the Tamar surveys and tabulated VM input data

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593 References

- 594 1. Reeder, R. J.; Schoonen, M. A.; Lanzirrotti, A., Metal speciation and its role in
595 bioaccessibility and bioavailability. *Rev. Mineral. and Geochem.* **2006**, *64*, (1), 59-113.
- 596 2. Caussy, D.; Gochfeld, M.; Gurzau, E.; Neagu, C.; Ruedel, H., Lessons from case
597 studies of metals: investigating exposure, bioavailability, and risk. *Ecotox. and Environ.*
598 *Safety* **2003**, *56*, (1), 45-51.
- 599 3. Di Toro, D. M.; Allen, H. E.; Bergman, H. L.; Meyer, J. S.; Paquin, P. R.; Santore,
600 R. C., Biotic ligand model of the acute toxicity of metals. 1. Technical Basis. *Environ.*
601 *Toxicol. and Chem.* **2001**, *20*, (10), 2383-2396.
- 602 4. Heijerick, D. G.; De Schamphelaere, K. A. C.; Janssen, C. R., Predicting acute
603 zinc toxicity for *Daphnia magna* as a function of key water chemistry characteristics:
604 Development and validation of a biotic ligand model. *Environ. Toxicol. and Chem.* **2002**,
605 *21*, (6), 1309-1315.
- 606 5. Gillis, P. L.; McGeer, J. C.; Mackie, G. L.; Wilkie, M. P.; Ackerman, J. D., The
607 effect of natural dissolved organic carbon on the acute toxicity of copper to larval
608 freshwater mussels (glochidia). *Environ. Toxicol. and Chem.* **2010**, *29*, (11), 2519-2528.
- 609 6. USEPA *Draft Update of Ambient Water Quality Criteria for Copper*, 2003.
- 610 7. USEPA *United States Environmental Protection Agency, National*
611 *Recommended Water Quality Criteria - Aquatic Life Criteria Table*;
612 [https://www.epa.gov/wqc/national-recommended-water-quality-criteria-aquatic-life-](https://www.epa.gov/wqc/national-recommended-water-quality-criteria-aquatic-life-criteria-table)
613 [criteria-table](https://www.epa.gov/wqc/national-recommended-water-quality-criteria-aquatic-life-criteria-table), 2016.
- 614 8. Chadwick, D. B.; Rivera Duarte I.; Rosen G., Wang P.F., Santore R.C., Ryan
615 A.C., Paquin P.R., Hafner S.D. and Choi W. *Demonstration of an integrated compliance*
616 *model for predicting copper fate and effects in DoD harbors*; Space And Naval Warfare
617 Systems Center Pacific: San Diego CA, 2008.
- 618 9. USEPA *United States Environmental Protection Agency, National*
619 *Recommended Water Quality Criteria - Aquatic Life Criteria Table*;
620 [https://www.epa.gov/wqc/national-recommended-water-quality-criteria-aquatic-](https://www.epa.gov/wqc/national-recommended-water-quality-criteria-aquatic-life-criteria-table)
621 [life-criteria-table](https://www.epa.gov/wqc/national-recommended-water-quality-criteria-aquatic-life-criteria-table), 2016.
- 622 10. Louis, Y.; Garnier, C.; Lenoble, V.; Mounier, S.; Cukrov, N.; Omanović, D.;
623 Pižeta, I., Kinetic and equilibrium studies of copper-dissolved organic matter

complexation in water column of the stratified Krka River estuary (Croatia). *Mar. Chem.* **2009**, *114*, (3–4), 110-119.

11. Van Veen, E.; Gardner, M. J.; Comber, S. D. W., Temporal variation of copper and zinc complexation capacity in the Humber estuary. *J. Environ. Mon.* **2001**, *3*, (3), 322-323.

12. Maycock, D.; Graham; Peters, A. *Proposed EQS for Water Framework Directive Annex VIII substances: copper (saltwater) (For consultation)*; Edinburgh, 2012.

13. Arnold, W. R.; Cotsifas, J. S.; Ogle, R. S.; DePalma, S. G. S.; Smith, D. S., A comparison of the copper sensitivity of six invertebrate species in ambient salt water of varying dissolved organic matter concentrations. *Environ. Toxicol. and Chem.* **2010**, *29*, (2), 311-319.

14. Sarathy, V.; Allen, H. E., Copper complexation by dissolved organic matter from surface water and wastewater effluent. *Ecotox. and Environ. Safety* **2005**, *61*, (3), 337-344.

15. Gustafsson, J., Visual MINTEQ version 3.1. *Department of Land and Water Resources Engineering, Royal Institute of Technology, Stockholm, Sweden* **2013**.

16. Gerringa, L. J. A.; Hummel, H.; Moerdijk-Poortvliet, T. C. W., Relations between free copper and salinity, dissolved and particulate organic carbon in the Oosterschelde and Westerschelde, Netherlands. *J. of Sea Res.* **1998**, *40*, (3–4), 193-203.

17. Van Veen, E.; Burton, N.; Comber, S.; Gardner, M., Speciation of copper in sewage effluents and its toxicity to *Daphnia magna*. *Environ. Toxicol. and Chem.* **2002**, *21*, (2), 275-280.

18. Shank, G. C.; Skrabal, S. A.; Whitehead, R. F.; Avery, G. B.; Kieber, R. J., River discharge of strong Cu-complexing ligands to South Atlantic Bight waters. *Mar. Chem.* **2004**, *88*, (1–2), 41-51.

19. Delgadillo-Hinojosa, F.; Zirino, A.; Nasci, C., Copper complexation capacity in surface waters of the Venice Lagoon. *Mar. Environ. Res.* **2008**, *66*, (4), 404-411.

20. Gardner, M.; Comber, S.; Scrimshaw, M. D.; Cartmell, E.; Lester, J.; Ellor, B., The significance of hazardous chemicals in wastewater treatment works effluents. *Sci. Tot. Environ.* **2012**, *437*, (0), 363-372.

21. Constantino, C. The effect of sewage effluent on trace metal speciation: Implications for the biotic ligand model approach. Brunel University, Uxbridge, 2012.

22. Andrade, S.; Pulido, M. J.; Correa, J. A., The effect of organic ligands exuded by intertidal seaweeds on copper complexation. *Chemosphere* **2010**, *78*, (4), 397-401.

23. Langston, W. J.; Chesman, B. S.; Burt, G. R.; Hawkins, S. J.; Readman, J.; Worsfold, P., Characterisation of the South West European Marine Sites. *Plymouth Sound and Estuaries cSAC, SPA. Occasional Publications.* **2003**, *Mar. Biol. Assoc. UK* (9) 202p.

24. Mighanetara, K.; Braungardt, C. B.; Rieuwerts, J. S.; Azizi, F., Contaminant fluxes from point and diffuse sources from abandoned mines in the River Tamar catchment, UK. *J. Geochem. Explor.* **2009**, *100*, (2–3), 116-124.

25. Miller, A. E. J., Seasonal Investigations of Dissolved Organic Carbon Dynamics in the Tamar Estuary, U.K. *Est. Coast. Shelf Sci.* **1999**, *49*, (6), 891-908.

26. Trigueros, J. M.; Orive, E., Tidally driven distribution of phytoplankton blooms in a shallow, macrotidal estuary. *J. Plank. Res.* **2000**, *22*, (5), 969-986.

27. Rule, K. L.; Comber, S. D. W.; Ross, D.; Thornton, A.; Makropoulos, C. K.; Rautiu, R., Sources of priority substances entering an urban wastewater catchment—trace organic chemicals. *Chemosphere* **2006**, *63*, (4), 581-591.

28. Ružić, I., Theoretical aspects of the direct titration of natural waters and its information yield for trace metal speciation. *Anal. Chim. Acta* **1982**, *140*, (1), 99-113.

29. van den Berg, C. M. G., Determination of copper complexation with natural organic ligands in seawater by equilibration with MnO₂. Theory. *Mar. Chem.* **1982**, *11*, (4), 307-322.

30. Badr, E.-S. A.; Achterberg, E. P.; Tappin, A. D.; Hill, S. J.; Braungardt, C. B., Determination of dissolved organic nitrogen in natural waters using high-temperature catalytic oxidation. *TrAC Trends in Anal. Chem.* **2003**, *22*, (11), 819-827.

31. van den Berg, C. M. G.; Kramer, J. R., Determination of complexing capacities of ligands in natural waters and conditional stability constants of the copper complexes by means of manganese dioxide. *Anal. Chim. Acta* **1979**, *106*, (1), 113-120.
32. Environment Agency (2016) Environmental Agency Water Quality archive (WIMS) available at <http://environment.data.gov.uk/water-quality/view/landing>; accessed 18 March 2016.
33. Stumm, W.; Morgan, J. J., Aquatic chemistry: chemical equilibria and rates in natural waters. 3rd ed.; John Wiley and Sons: 1995; Vol. 126.
34. Howell, K. A.; Achterberg, E. P.; Tappin, A. D.; Worsfold, P. J., Colloidal Metals in the Tamar Estuary and their Influence on Metal Fractionation by Membrane Filtration. *Environ. Chem.* **2006**, *3*, (3), 199-207.
35. Grabemann, I.; Uncles, R. J.; Krause, G.; Stephens, J. A., Behaviour of Turbidity Maxima in the Tamar (U.K.) and Weser (F.R.G.) Estuaries. *Est. Coast. Shelf Sci.* **1997**, *45*, (2), 235-246.
36. Howard, A. G.; Apte, S. C.; Comber, S. D. W.; Morris, R. J., Biogeochemical control of the summer distribution and speciation of arsenic in the Tamar estuary. *Est. Coast. Shelf Sci.* **1988**, *27*, (4), 427-443.
35. van den Berg, C. M. G.; Khan, S. H.; Daly, P. J.; Riley, J. P.; Turner, D. R., An electrochemical study of Ni, Sb, Se, Sn, U and V in the estuary of the Tamar. *Est. Coast. Shelf Sci.* **1991**, *33*, (3), 309-322.
36. Stauber, J. L.; Andrade, S.; Ramirez, M.; Adams, M.; Correa, J. A., Copper bioavailability in a coastal environment of Northern Chile: Comparison of bioassay and analytical speciation approaches. *Mar. Poll. Bull.* **2005**, *50*, (11), 1363-1372.
37. Tang, D.; Warnken, K. W. and Santschi, P. H., T, Organic complexation of copper in surface waters of Galveston Bay. *Limnol Oceanogr* 2001, (46), 321-330.
38. van den Berg, C. M. G.; Nimmo, M.; Daly, P.; Turner, D. R., Effects of the detection window on the determination of organic copper speciation in estuarine waters. *Anal. Chim. Acta* **1990**, *232*, (0), 149-159.
39. Paquin, P.R.; Gorsuch, J.W.; Apte, S.; Batley, G.E.; Bowles, K.C.; Campbell, P.G.C.; Delos, C.G.; Di Toro, D.M.; Dwyer, R.L.; Galvez, F.; Gensemer, R.W.; Goss, G.G.; Hogstrand, C.; Janssen, C.R.; McGeer, J.C.; Naddy, R.B.; Playle, R.C.; Santore, R.C.; Schneider, U.; Stubblefield, W.A.; Wood, C.M.; Wu, K.B., The biotic ligand model: a historical overview. *Comp. Biochem. & Physiol. Part C: Toxicol. & Pharmacol.* **2002**, *133*, (1-2), 3-35.
40. Zsolnay, Á., Dissolved organic matter: artefacts, definitions, and functions. *Geoderma* **2003**, *113*, (3-4), 187-209.
41. Huguet, A.; Vacher, L.; Relexans, S.; Saubusse, S.; Froidefond, J. M.; Parlanti, E., Properties of fluorescent dissolved organic matter in the Gironde Estuary. *Org. Geochem.* **2009**, *40*, (6), 706-719.
42. Abualhaija, M. M.; Whitby, H.; van den Berg, C. M. G., Competition between copper and iron for humic ligands in estuarine waters. *Mar. Chem.* **2015**, *172*, 46-56.
43. Pagenkopf, G. K., Gill surface interaction model for trace-metal toxicity to fishes: role of complexation, pH, and water hardness. *Environ. Sci. & Technol.* **1983**, *17*, (6), 342-347.
44. Jardim, W.F. and Allen, H.E., 1984. Measurement of copper complexation by naturally occurring ligands. In: C.J.M. Kramer and J.C. Duinker (Editors), Complexation of Trace Metals in Natural Waters. Nijhoff/Junk, The Hague, The Netherlands, pp. 1-15.
45. Stockdale, A.; Tipping, E.; Lofts, S., Dissolved trace metal speciation in estuarine and coastal waters: Comparison of WHAM/Model VII predictions with analytical results. *Environ. Toxicol. and Chem.* **2015**, *34*, (1), 53-63.
46. Hudson, R. J. M., Trace metal uptake, natural organic matter, and the free-ion model. *J. Phycology* **2005**, *41*, (1), 1-4.
47. Unsworth, E. R.; Warnken, K. W.; Zhang, H.; Davison, W.; Black, F.; Buffle, J.; Cao, J.; Cleven, R.; Galceran, J.; Gunkel, P.; Kalis, E.; Kistler, D.; van Leeuwen, H. P.; Martin, M.; Noel, S.; Nur, Y.; Odzak, N.; Puy, J.; van Riemsdijk, W. H.; Sigg, L.; Temminghoff, E.; Tercier-Waeber, M. L.; Toepperwien, S.; Town, R. M.; Weng, L. P.; Xue, H. B., Model predictions of metal speciation in freshwaters compared to measurements by in situ techniques. *Environ. Sci. & Technol.* **2006**, *40*, (6), 1942-1949.

738 48 Sjöstedt, C.S., Gustafsson, J.P., Köhler, S.J., Chemical equilibrium modeling of
739 organic acids, pH, aluminum, and iron in Swedish surface waters. Environ. Sci. Technol.
740 2010, 44, 8587-8593.
741
742
743
744