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

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13

14 **Abstract**

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

33  
34 **Keywords:** Copper, speciation, estuarine waters, dissolved organic carbon, ligands,  
35 modelling



## 37 1. INTRODUCTION

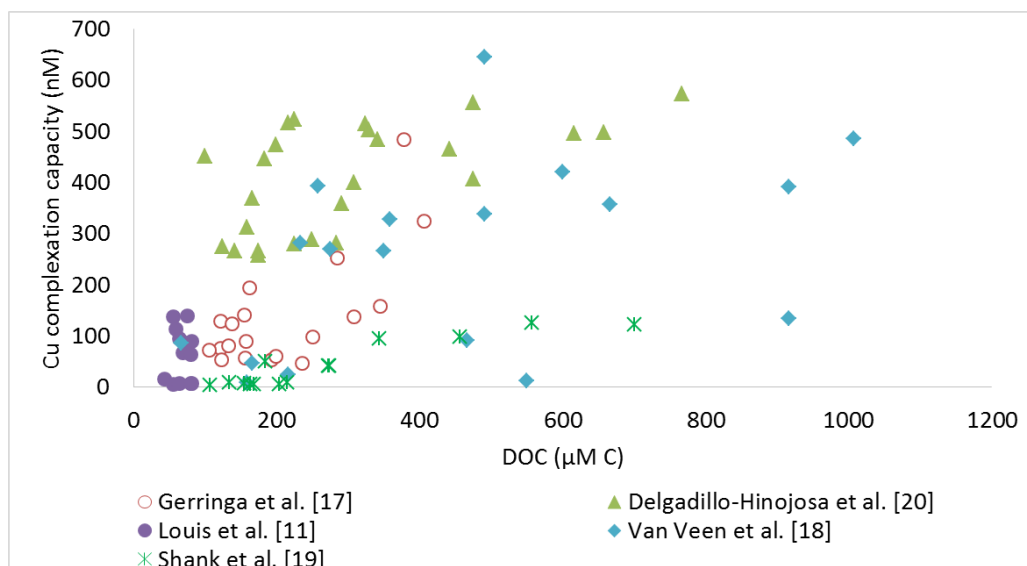
38 The importance of chemical speciation is widely recognised in the scientific and regulatory  
39 community, where physico-chemical characteristics control solubility, reactivity, biological  
40 availability and toxicity in the environment.<sup>1,2</sup> The most toxic form of copper (Cu) in natural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

49 In the USA, speciation-based approaches to Cu regulation in fresh water have been  
50 established since 2003 using the Biotic Ligand Model (BLM).<sup>6,7</sup> The BLM combines  
51 thermodynamic equilibrium calculations of free metal ion concentrations generated by  
52 development of the Free Ion Activity Model (FIAM) using information on ambient water  
53 quality including at its most basic level, calcium concentrations (hardness), pH and DOC,  
54 with ecotoxicological endpoints to predict toxicity to classes of organisms or to set site-  
55 specific water quality standards.<sup>6,7</sup> The complex chemistry within estuarine systems and lack  
56 of research slowed the development of BLMs for estuarine and coastal waters. Recently, a  
57 number of studies have shown that Cu toxicity can be predicted: model development is  
58 underway predicting the fate of Cu in US harbours<sup>8</sup> and a BLM has been developed for  
59 marine waters.<sup>9</sup>

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

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

79 The EQS derivation therefore relies on the assumption that DOC is 'protective' which is well  
80 established under laboratory conditions using DOC sources such as humic and fulvic  
81 acids.<sup>13</sup> Figure S2 shows the relationship between DOC and EC50 (the concentration that  
82 negatively affects 50% of the tested population) for *Mytilus*. However, increased data scatter  
83 above 500  $\mu\text{M}$  ( $6 \text{ mg L}^{-1}$ ) DOC is evident. For example, the EC50 varies by up to a factor of  
84 5 at ca. 500  $\mu\text{M}$  DOC, and DOC varies by up to a factor of 3 for a given EC50 of ca. 500  $\mu\text{M}$   
85 Cu. Although the EQS derivation uses the same experimental data expressed as EC10  
86 rather than EC50, the same variability in DOC vs toxicity response is observed. This scatter  
87 originates in the variability of ecotoxicological data within and across experiments and the  
88 fact that not all DOC contributes to Cu complexation. The latter is reflected in existing  
89 computational models for Cu toxicity, where various correction factors are applied to  
90 observed DOC concentrations to account for the contribution of organic ligands to Cu  
91 complexation. This is described as 'active DOC' in the Windermere Humic Aqueous Model  
92 (WHAM) models,<sup>14</sup> 'humic acid content' in BLMs<sup>3</sup> and is converted into ligands via algorithms  
93 such as the NICA-Donnan, Gaussian or Stockholm Humic Models in Visual MINTEQ.<sup>15</sup> The  
94 effectiveness of these assumptions in characterising the Cu complexation within saline  
95 waters is variable and often leads to over prediction of free Cu ion concentrations ( $[\text{Cu}^{2+}]$ ),  
96 particularly at low Cu concentrations.<sup>14</sup> Although this is conservative, a lack of agreement  
97 between predicted and actual values suggests improvements in the existing models are  
98 possible which would further strengthen the application of BLMs for saline waters BLMs.  
99 Studies generally show weak relationships between Cu complexation capacity ( $[\text{L}_x]$ ) and  
100 DOC concentrations measured in saline waters (Figure 1),<sup>11,16-19</sup> unless samples were taken  
101 across a limited range of salinities and/or restricted to a specific ligand strength, typically  
102 derived from a single source such as river humic and fulvic acids.<sup>16,18</sup> It is important to note  
103 that in all cases the organic ligand complexation capacities are in the order of  $1 \text{ to } 100 \times 10^{-9}$   
104 M of Cu compared with DOC concentrations in the  $1 - 100 \times 10^{-6}$  M range. In other words,  
105 the complexing ligands are present at one thousandth of the total DOC concentration.



106

107 **Figure 1. Complexation capacity for Cu vs. DOC (data from <sup>11,17-20</sup>).**

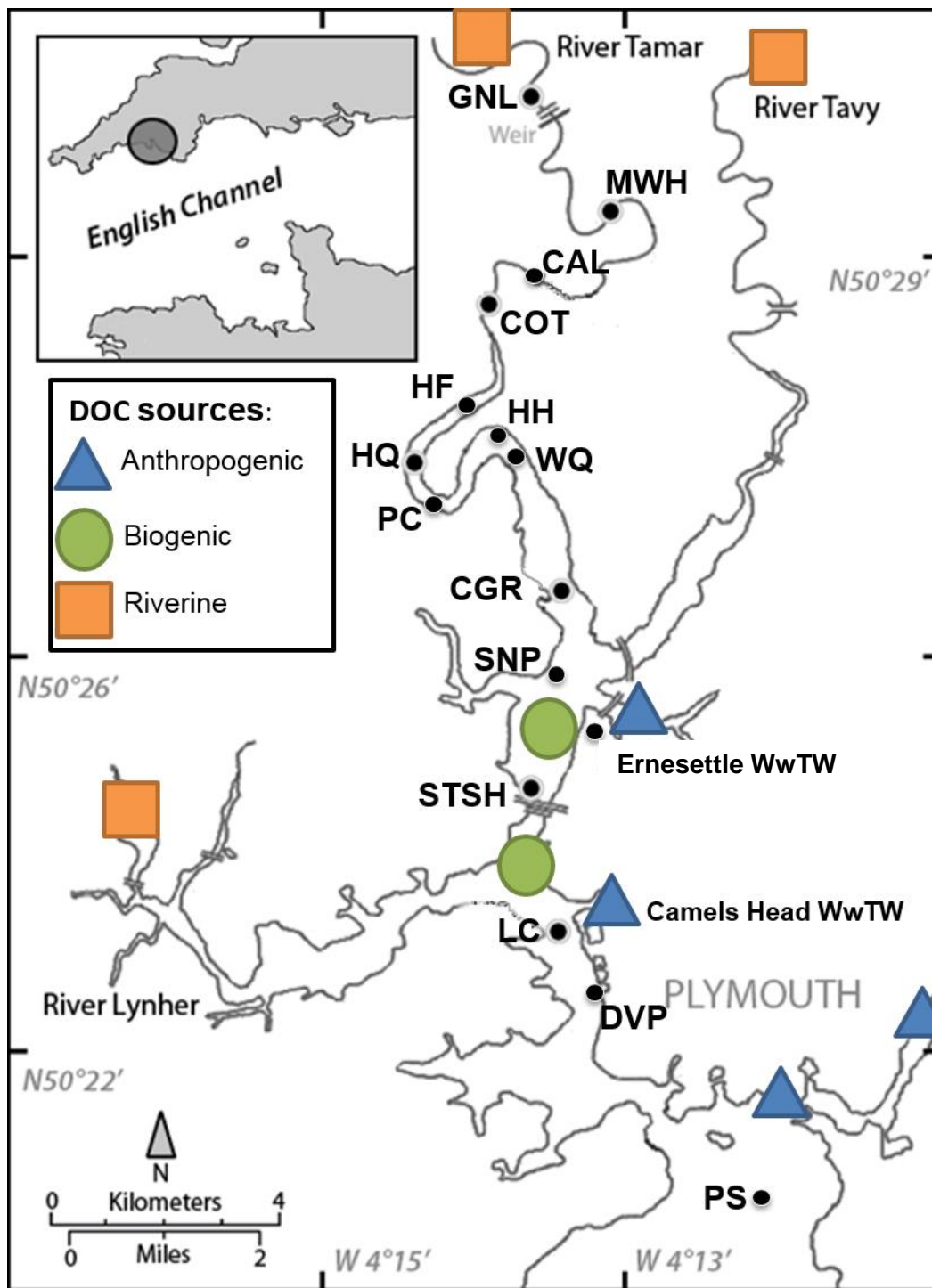
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109 Are there better ways to capture the effects of metal complexation by organic ligands in  
 110 estuarine waters than using DOC as a surrogate for bioavailability? Most existing research in  
 111 freshwater has focussed on the complexation of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

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

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

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**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](http://naturalearthdata.com).

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156

## 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 km<sup>2</sup> and its length is close to the average for U.K.  
163 estuaries.<sup>23</sup> 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.<sup>24</sup> 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.<sup>25</sup> 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.<sup>21</sup> 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.<sup>26</sup> Anthropogenically derived  
174 contamination originating from the naval dockyard and urbanised areas near the mouth of  
175 the estuary impacts on coastal water quality.<sup>27</sup> 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

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

### 193 2.3 Sampling and sample handling protocol

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

### 202 2.4 Analytical methods, procedures and calculations

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

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

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

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

234

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

## 242 2.5 Thermodynamic equilibrium speciation calculations

243 All calculations were undertaken using Visual MINTEQ version 3.1<sup>15</sup>, a freeware chemical  
244 equilibrium model for the calculation of metal speciation, solubility equilibria, sorption etc. for  
245 natural waters. It combines descriptions of sorption and complexation reactions on a  
246 Windows platform and has the capacity for users to input new ligands (in this case  $\log K'_{CuL_x}$   
247 values and ligand concentrations derived from the CCT field data at two separate artificial  
248 ligand strengths) into its database. Input cation and anion concentrations were generated  
249 using an ion-pairing model<sup>31</sup> (see section S2.1) using the Gunnislake freshwater sample  
250 composition from this survey and available Environment Agency data<sup>32</sup> as well as reported  
251 coastal water data<sup>33</sup> as endmembers.

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

- 254 1) Major ion concentrations ( $Na^+$ ,  $Mg^{2+}$ ,  $K^+$ ,  $H^+$ ,  $Si^{2+}$ ,  $Cl^-$ ,  $Br^-$ ,  $SO_4^{2-}$ ,  $F^-$ ) generated from  
255 conservative mixing of Tamar freshwater (mean concentrations from Gunnislake  
256 sample from present surveys and available Environment Agency data)<sup>32</sup> and sea  
257 water<sup>33</sup> end member data obtained from the ion-pairing seawater model (see section  
258 S2.1)
- 259 2)  $[Cu_{TD}]$  measured as part of this work
- 260 3) Ligand concentrations:

- 261 a. Measured as part of this work using 2 and 10  $\mu\text{M}$  SA with their accompanying  
262 conditional stability constants
- 263 b. Ligands generated within VM 3.1 using measured DOC concentrations from  
264 these surveys (Table S2) and the NICA-Donnan model for generating  
265 complexing ligands based on default assumptions that all active DOM is fulvic  
266 acid with no humic acid being 'active' in complexing Cu. Furthermore, 82.5%  
267 of DOC is assumed to be "generic fulvic acid" with a C content of 50%; giving  
268 a DOM to DOC ratio of 1.65 (see Section S2 of the SI)

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

274

## 275 3. RESULTS AND DISCUSSION

276

### 277 3.1 Measured Cu speciation within estuarine waters

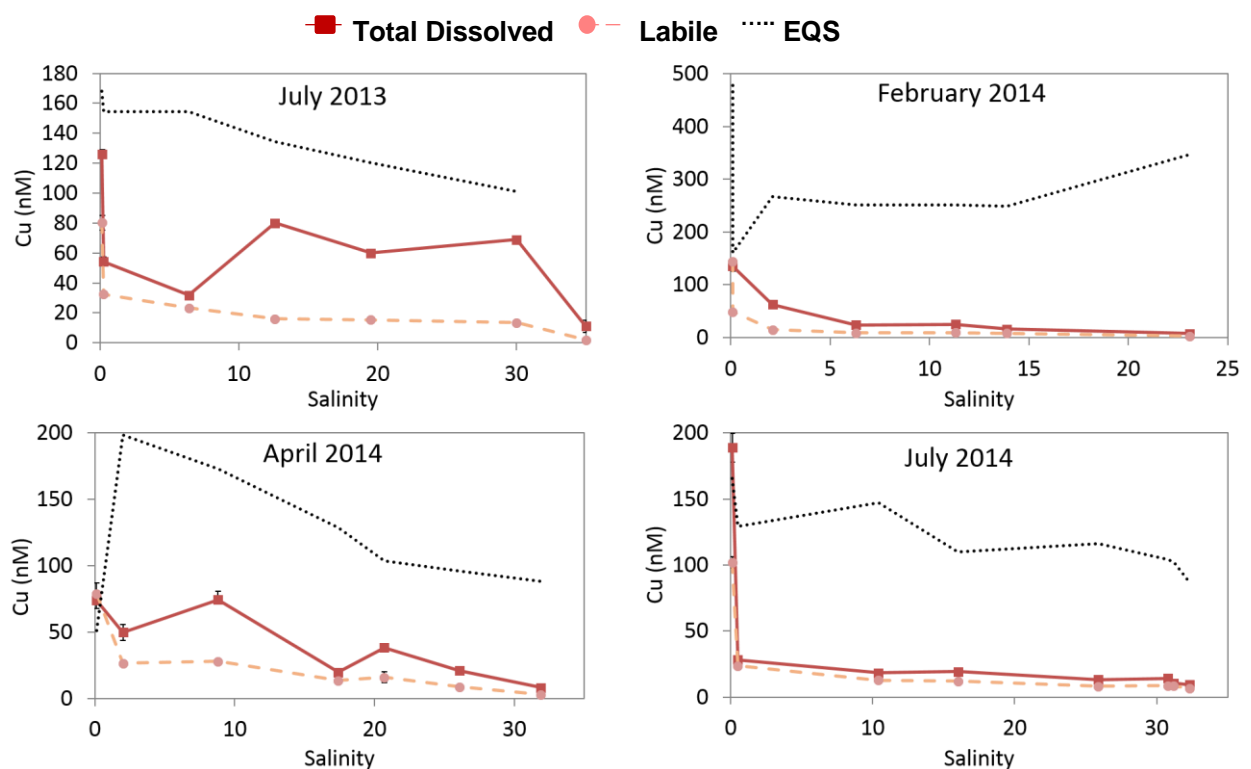
278 The Tamar estuary features a tidally induced, strong and well defined turbidity  
279 maximum zone (TMZ) between 0 and 10 salinity<sup>23</sup> and the extensive mudflats in mid-  
280 estuary are contaminated as a result of the mining legacy within the catchment.<sup>24</sup>  
281 Both impact on overlying water chemistry and Cu speciation over the course of tidal  
282 cycles.

283

284 The full set of  $[\text{Cu}_{\text{TD}}]$  and  $[\text{Cu}_{\text{LAB}}]$  for all the surveys are displayed in Table S  
285 2 of the SI. The  $[\text{Cu}_{\text{TD}}]$  ranged from 8 to 189 nM (Figure 3), with highest  
286 concentrations invariably observed in the freshwater sample taken at Gunnislake,  
287 reflecting the continuous contamination emanating from historic mine sites.<sup>24</sup> These  
288 concentrations are comparable with those found in previous studies of the Tamar.<sup>34</sup> A  
289 plot of  $[\text{Cu}_{\text{TD}}]$  versus salinity shows non-conservative behaviour as fresh and saline  
290 waters mix at low salinities. This was likely a result of (i) loss of metal from the  
291 dissolved phase owing to a combination of sorption to suspended solids within the  
292 TMZ<sup>35</sup> and/or (ii) precipitation reactions associated with amorphous Fe oxyhydroxides  
293 and flocculation of colloidal material.<sup>36</sup>

294

295 The increase of [Cu<sub>TD</sub>] at mid to high salinity in July 2013 and April 2014 is potentially  
 296 attributable to additional inputs from tributaries contaminated with historical mine  
 297 waste (Lynher, Tavy)<sup>24</sup> or mobilisation of metal from bed sediment during tidal  
 298 pumping.<sup>35</sup> The combined effluent input to the lower estuary from Plymouth WwTW  
 299 works is approximately 72 x 10<sup>6</sup> L d<sup>-1</sup>, with median copper concentrations of 5.6 µg L<sup>-1</sup>  
 300 reported for English WwTW effluents<sup>20</sup>. This equates to approximately 400 g of Cu  
 301 per day, which, with dilution, is unlikely to impact on observed concentrations.  
 302



303

304

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

310

311 In most cases, the site-specific Cu EQSs for estuarine waters were slightly higher  
 312 than the previous value (78 nM Cu), owing to the DOC concentrations determined in  
 313 the samples being significantly above 83 µM (1 mg C L<sup>-1</sup>). No exceedance of the EQS  
 314 was observed within the estuary for any survey, whether comparing with new or  
 315 previous standards. In terms of BLM development and further consideration of the

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

318

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

336

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

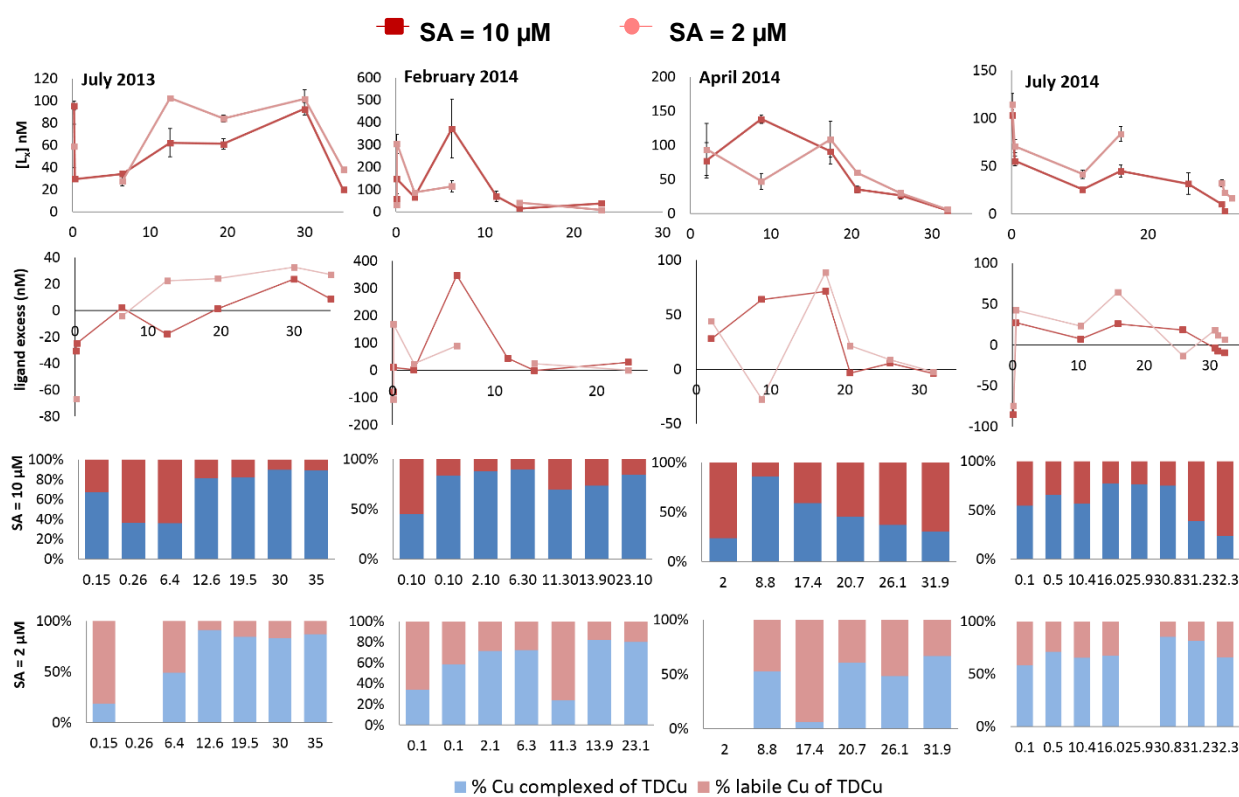
347

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

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

360

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



363

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

371

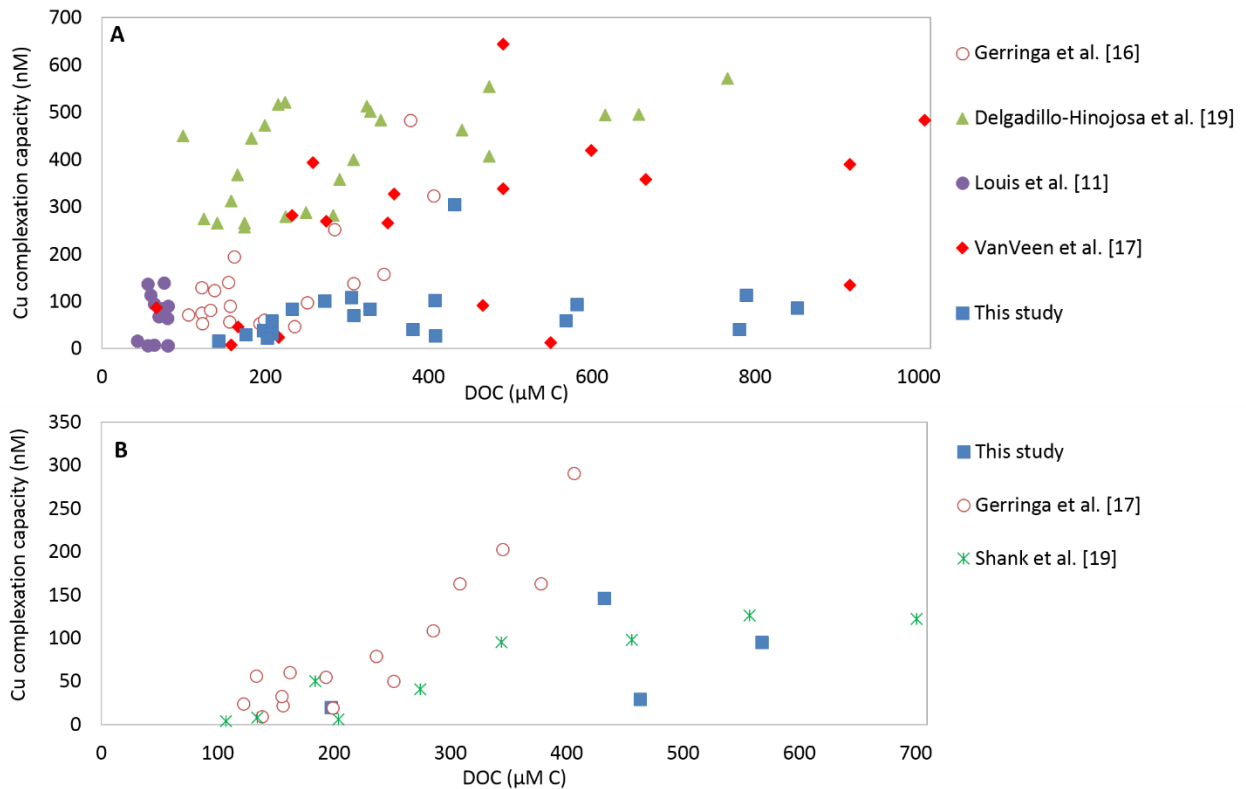
372

### 373 3.2 DOC characterisation and influence on copper speciation

374 A comparison of the data from this study in a plot of Cu complexing capacity ( $[L_x]$ ) versus  
375 [DOC] reported in previous studies of other saline waters shows no obvious correlation (data  
376 not shown), nor is this the case when separating out weaker ligands ( $\text{Log } K < 13$ ) likely to be  
377 derived from a mixture of autochthonous and allochthonous sources (Figure 5A). However,  
378 trends emerge for two of the datasets<sup>16,18</sup> when isolating the stronger ligands with a  $\text{Log } K >$   
379 13 (Figure 5B) that are potentially derived from terrestrial humic sources.<sup>18</sup> A study on the  
380 river Scheldt<sup>16</sup> showed that the ligand concentration can be predicted based on measured  
381 DOC and salinity because the source of organic ligands was dominated by a single riverine  
382 source. This indicates that for estuaries that feature a strong negative correlation between  
383 salinity and DOC, the assumption is reasonable that the DOC is dominated by a riverine  
384 source and may therefore be associated with humic and fulvic acids known to complex  
385 strongly with Cu.<sup>39</sup> For the Tamar, no obvious trends were observed between DOC and  
386 either  $[\text{Cu}^{2+}]$  or salinity (Figure S4), confirming the complex nature of the interactions  
387 between Cu, ligands, sources and concentrations, set against a background of varying  
388 physico-chemical conditions within this estuary. Furthermore, DOC concentrations in  
389 estuarine waters are typically between 100 and 1000  $\mu\text{M}$ ,<sup>17</sup> whereas the reported  
390 concentrations of copper complexing ligands are typically in the 4 to 400 nM range,<sup>12</sup> i.e. up  
391 to 3 orders of magnitude lower, which may explain the observed apparent threshold of 100  
392  $\mu\text{M}$  DOC before significant, detectable complexation capacity is measured (Figure 5 A and  
393 B).

394





396

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

400

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

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

414 to a predominantly autochthonous origin of DOC from recent aquatic and bacterial  
415 activity freshly released into water.<sup>41</sup>

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

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

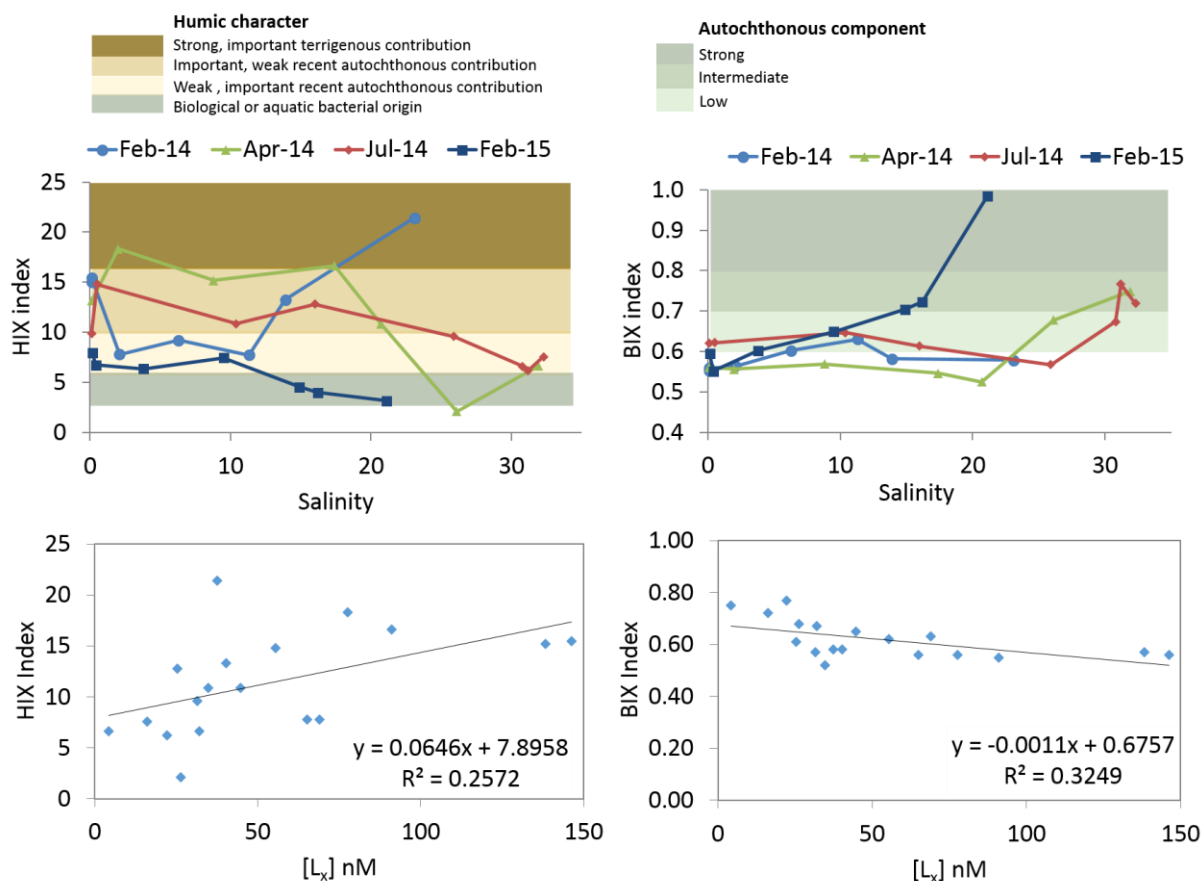
431 Data from February 2014 appeared to show extreme DOC characteristics within the  
432 estuary. Sampling followed a prolonged period of intense rainfall across the SW of  
433 England, resulting in widespread flooding. River flows into the Tamar estuary were  
434 high enough to suppress salinity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

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

443 A plot of  $[L_x]$  against HIX and BIX shows weak positive and negative correlations,  
444 respectively (Figure 6); albeit it should be noted that the range of BIX indices is low  
445 for the Tamar Estuary (typically 0.6 to 0.8). Highest HIX values were associated with  
446 higher complexation capacities, which reflects the strong affinity of Cu for humic and  
447 fulvic acids,<sup>18,42</sup> which dominate the lower salinity regions in the upper estuary.

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

459



460

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

465

### 466 3.3 Predicting Cu speciation and implications for regulation

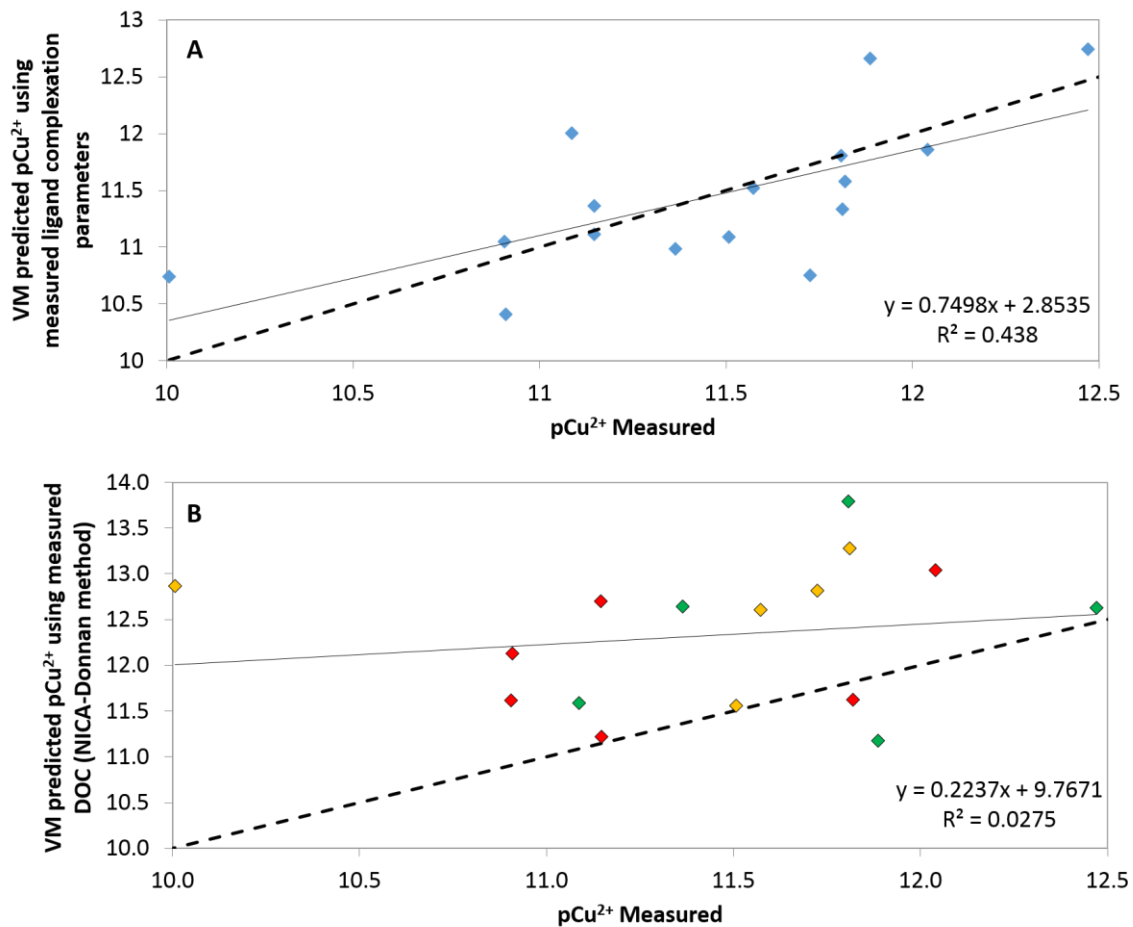
467 Models such as the BLM<sup>43</sup> and Gill Surface Interaction Model (GSIM)<sup>43</sup> combine  
468 predictions of metal ion activity and organism response to generate site-specific water  
469 quality standards taking account of ambient conditions. It should be noted that these  
470 models use metal ion activity, not concentration, for calculations. In general, the  
471 activity depends on any factor that alters the chemical potential, including  
472 concentration, temperature, pressure, interactions between chemical species and  
473 electric fields. For saline waters ionic strength can be a significant factor in  
474 determining model accuracy<sup>44</sup> and models typically use the Debye–Hückel or Davies  
475 equations to predict species activity.<sup>43</sup>

476

477 In addition, any chemical speciation model within a BLM needs to incorporate  
478 thermodynamic data for organic ligands that are known to be capable of complexing  
479 metals in natural waters. Although metal interactions with individual synthetic and  
480 some natural organic ligands are well defined, metal – organic ligand interactions in  
481 natural waters are complex, variable and more poorly characterised. Given the  
482 analytical complexity and time required to determine the complexation capacity and  
483 strength of natural organic ligand mixtures, most models apply an algorithm to the  
484 DOC concentration to parameterise metal - natural organic ligand interactions. These  
485 algorithms are based on the assumption that humic and fulvic acids are the dominant  
486 metal complexing ligands in natural waters and estimate their concentrations. Model  
487 codes that use such algorithms for DOC corrections, and for which predicted and  
488 observed organically bound and/or free metal ion concentrations have been  
489 compared, include WHAM VII,<sup>45</sup> FIAM,<sup>46</sup> and VM<sup>47</sup>. However, Figure 1 demonstrates  
490 that DOC is often a poor predictor of  $[L_x]$  and possible explanations for this include  
491 the assumption of chemical equilibrium in thermodynamic speciation models (unlikely  
492 within an estuarine environment) and the very small proportion of metal complexing  
493 ligands in the overall DOC pool in natural waters. Some of the challenges associated  
494 with predicting free metal ions in saline waters were recently highlighted using the  
495 WHAM VII model, which assumes a fixed proportion of measured DOC actively  
496 complexes Cu.<sup>45</sup> The authors showed that approximately 59% of predictions were  
497 within one order of magnitude of the data determined using competitive ligand  
498 methods, although the agreement was much better at higher free ion concentrations  
499 ( $>10^{-13}$  M), which are more typically observed in estuarine waters.

500

501 To test the NICA-Donnan approach for converting DOC into active Cu binding ligands, the  
502 freeware metal speciation programme Visual MINTEQ<sup>15</sup> 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.



522

523 **Figure 7. Measured versus predicted (using Visual Minteq)  $[Cu^{2+}]$  concentration**  
 524 **plotted as  $pCu^{2+}$  ( $-\log_{10}$  of the molar concentration) with 1:1 line included**  
 525 **(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**  
 526 **ligands generated using measured DOC and the Nica-Donnan method (Green, amber and red symbols = salinity <10; 10-20 and >20 respectively).**  
 527  
 528

529

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

534 These results show that VM or similar equilibrium speciation codes can be used to  
 535 predict free metal ion concentrations with adequate certainty, given sufficient Cu-  
 536 ligand data. Obtained results can be used in conjunction with data on ecotoxicological  
 537 endpoints to assist in developing BLMs. This approach may help to eliminate the  
 538 uncertainties observed in EC50 values plotted against DOC (Figure S2). The priority

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

541

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

556 Even with this degree of complex physico-chemical interactions, and accepting that  
557 speciation models are based on an assumption of chemical equilibrium, this research has  
558 shown that it is possible to predict free metal concentrations within an order of magnitude of  
559 measured concentrations by using freely available chemical speciation software and  
560 inputting determined major ion concentrations and Cu ligand data (concentration and  
561  $\log K_{CuL_x}$  values). In contrast, using the NICA-Donnan complexation model to predict Cu  
562 speciation based on inputting DOC concentrations generated a bias in  $[Cu^{2+}]$  predictions  
563 which overestimated the free metal ion concentration for 14 out of 16 samples across all  
564 salinities by up to almost two orders of magnitude. It should be noted that as part of the  
565 Nica-Donnan assumptions, the DOM:DOC ratio is generated from Swedish lakes and rivers  
566 samples dominated by humic and fulvic acids<sup>48</sup> which may not be reflective of sources of  
567 DOC in estuaries for the reasons discussed above. The data presented here show that,  
568 although DOC is broadly indicative of Cu complexation, it cannot be relied on as a basis for  
569 predicting free Cu concentrations in estuarine waters. Consequently, the use of DOC  
570 concentration in setting EQS should be considered as an interim step, with future BLM  
571 development for estuarine waters needing to take into account more detailed Cu speciation.  
572 Further data are required regarding the characterisation of organic ligands and their sources,  
573 matched to observed ecotoxicological outcomes, to generate a robust BLM upon which  
574 future environmental legislation and robust environmental quality standards can be based.

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581 assistance in the laboratory and Dr. Alan Tappin for DOC analysis.

582

## 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

591

592

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