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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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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 µM 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.^{1,2} The most toxic form of copper (Cu) in natural
41 waters is the free metal ion [Cu²⁺] 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.³ 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,³ lowered ionic activity of the metal,⁴ 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.⁵

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).^{6,7} 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.^{6,7} 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 harbours⁸ and a BLM has been developed for
59 marine waters.⁹

60 Although some studies addressed the complexation of metals, such as Cu, in saline
61 waters,^{10,11} 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.¹² 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).¹²
74 Up to a DOC concentration of 83 µM (1 mg C L⁻¹) the Cu EQS is fixed at 59 nM (3.8 µg Cu L⁻¹).
75 The EQS increases linearly above DOC = 83 µM (1 mg C L⁻¹) to a point where, above
76 DOC = 162 µM (1.95 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 µM (5.71 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.¹³ 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 µM (6 mg L⁻¹) DOC is evident. For example, the EC50 varies by up to a factor of
84 5 at ca. 500 µM DOC, and DOC varies by up to a factor of 3 for a given EC50 of ca. 500 µM
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,¹⁴ ‘humic acid content’ in BLMs³ and is converted into ligands via algorithms
93 such as the NICA-Donnan, Gaussian or Stockholm Humic Models in Visual MINTEQ.¹⁵ 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 ([Cu²⁺]),
96 particularly at low Cu concentrations.¹⁴ 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 ([L_x]) and
100 DOC concentrations measured in saline waters (Figure 1),^{11,16-19} 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.^{16,18} It is important to note
103 that in all cases the organic ligand complexation capacities are in the order of 1 to 100 × 10⁻⁹
104 M of Cu compared with DOC concentrations in the 1 – 100 × 10⁻⁶ M range. In other words,
105 the complexing ligands are present at one thousandth of the total DOC concentration.

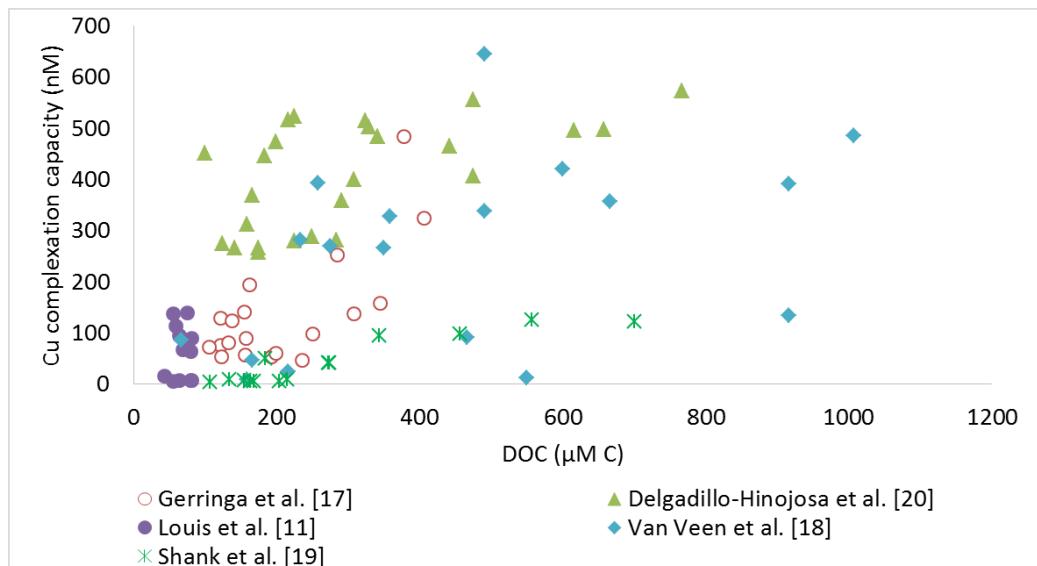


Figure 1. Complexation capacity for Cu vs. DOC (data from ^{11,17-20}).

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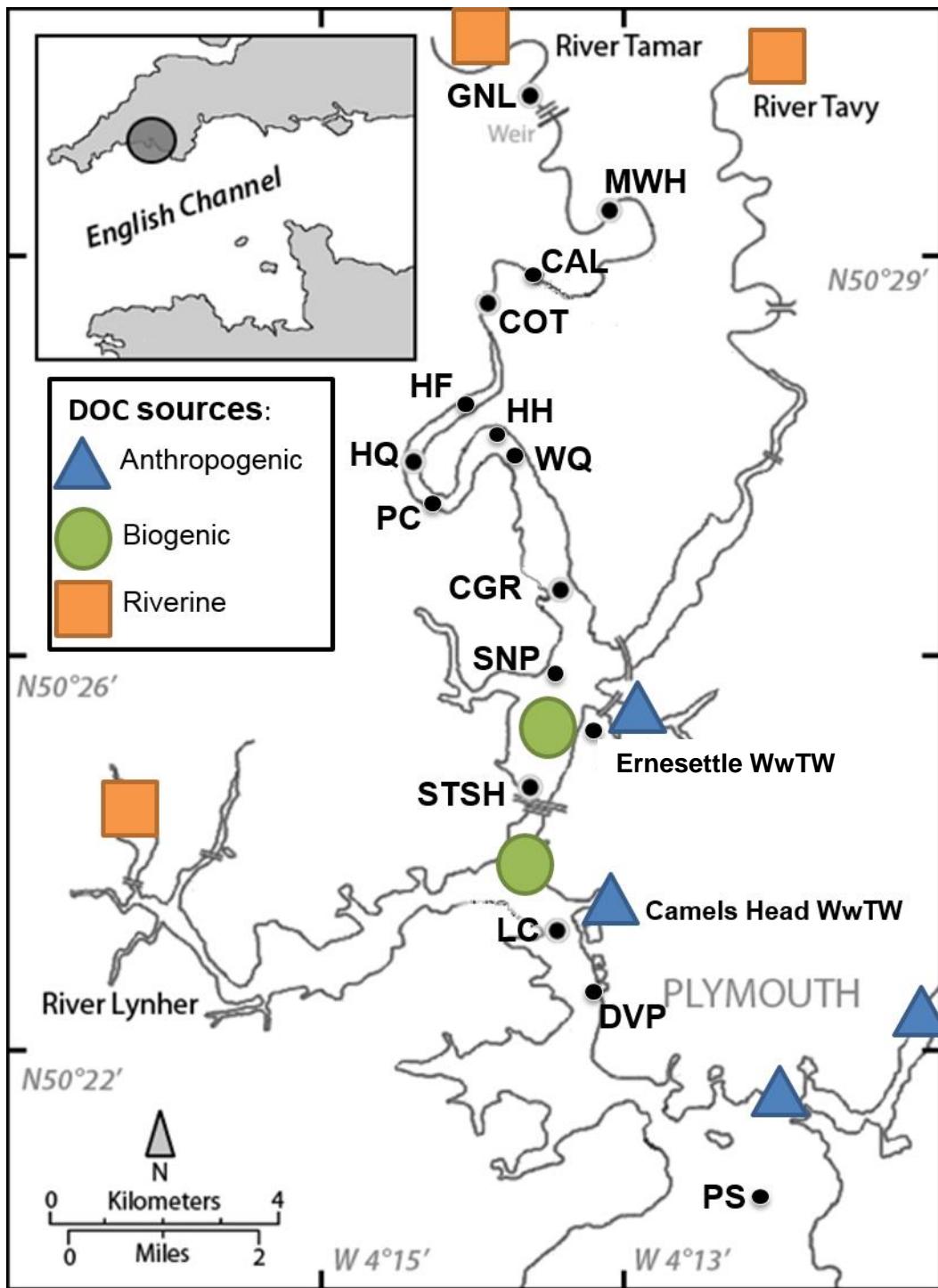
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109 Are there better ways to capture the effects of metal complexation by organic ligands in
110 estuarine waters than using DOC as a surrogate for bioavailability? Most existing research in
111 freshwater has focussed on the complexation of Cu by humic and fulvic acids, but these
112 materials, derived mainly from breakdown of terrestrially derived organic matter are, while
113 important,⁴ 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⁻¹)),²⁰ comprising natural and synthetic ligands capable of strongly complexing metals.^{17,21}
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.^{22,19}

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 $[L_x]$), stability constants ($\log K$) of Cu-organic ligand complexes, $[\text{Cu}^{2+}]$ 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.

144



145
146 **Figure 2.** Map of the Tamar Estuary, UK, with sampling station locations. Abbrev:
147 PS Plymouth Sound; DVP Devonport; LC Lynher Confluence; STSH
148 Saltash; SNP South of Neal Point; CGR Cargreen; HF Haye Farm; WQ
149 Weir Quay; HH Holes Hole; PC Pentillie Castle; HQ Halton Quay; COT
150 Cotehele; CAL Calstock; MWH Morwellham Quay; GNL Gunnislake;
151 WwTW Waste water treatment works. Potential DOC sources are marked
152 with coloured symbols. Outline derived from Ordnance Survey (1:50000,
153 2001). Insert: Made with Natural Earth. Free vector and raster map data
154 @ 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 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

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

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 μm 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 μM 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.

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+} .

234

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).

242 2.5 Thermodynamic equilibrium speciation calculations

243 All calculations were undertaken using Visual MINTEQ version 3.1¹⁵, 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 model³¹ (see section S2.1) using the Gunnislake freshwater sample 250 composition from this survey and available Environment Agency data³² as well as reported 251 coastal water data³³ 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^+ , Sr^{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)³² and sea 257 water³³ 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 μM 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 salinity²³ and the extensive mudflats in mid-
280 estuary are contaminated as a result of the mining legacy within the catchment.²⁴
281 Both impact on overlying water chemistry and Cu speciation over the course of tidal
282 cycles.

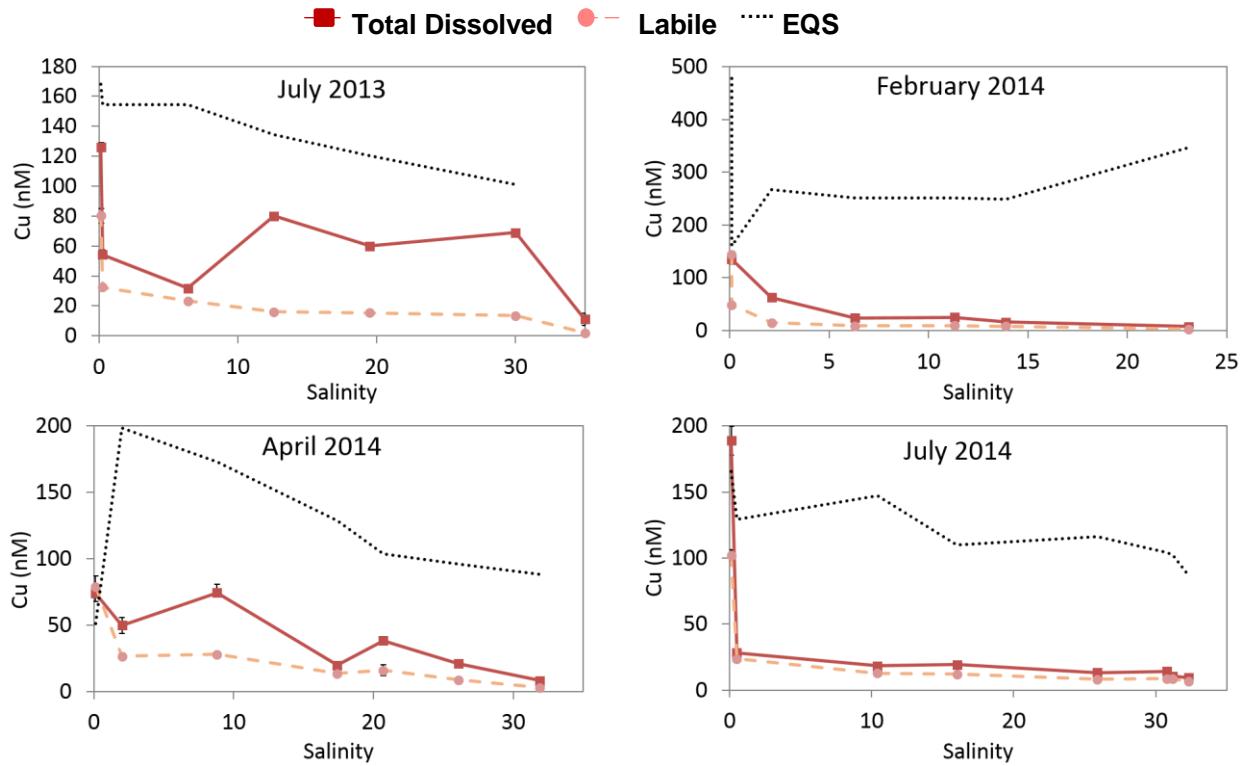
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284 The full set of $[\text{Cu}_{\text{TD}}]$ and $[\text{Cu}_{\text{LAB}}]$ for all the surveys are displayed in Table S
285 2 of the SI. The $[\text{Cu}_{\text{TD}}]$ ranged from 8 to 189 nM (Figure 3), with highest
286 concentrations invariably observed in the freshwater sample taken at Gunnislake,
287 reflecting the continuous contamination emanating from historic mine sites.²⁴ These
288 concentrations are comparable with those found in previous studies of the Tamar.³⁴ 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 TMZ³⁵ and/or (ii) precipitation reactions associated with amorphous Fe oxyhydroxides
293 and flocculation of colloidal material.³⁶

294

295 The increase of $[Cu_{TD}]$ 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)²⁴ or mobilisation of metal from bed sediment during tidal
298 pumping.³⁵ The combined effluent input to the lower estuary from Plymouth WwTW
299 works is approximately $72 \times 10^6 \text{ L d}^{-1}$, with median copper concentrations of $5.6 \mu\text{g L}^{-1}$
300 reported for English WwTW effluents²⁰. 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 $83 \mu\text{M}$ (1 mg C L^{-1}). 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.³⁶ 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.

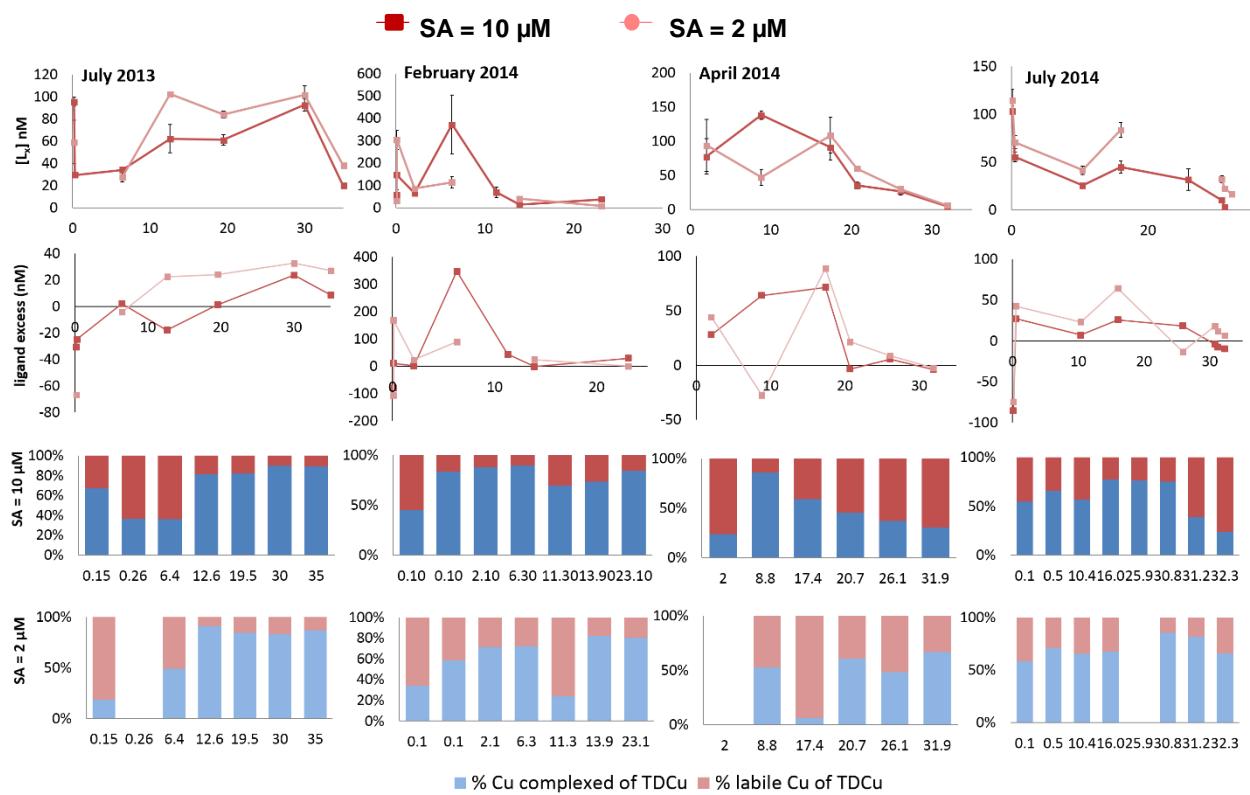
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348 In this work, $[Cu^{2+}]$ is a more defined sub-set of Cu speciation than $[Cu_{LAB}]$ (see
349 equations 1 and 2 in Section S2.1) as determination is undertaken at a predefined
350 competitive ligand strength and reflects only the free metal ion, 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.^{8,18,37} 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).³⁸ 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

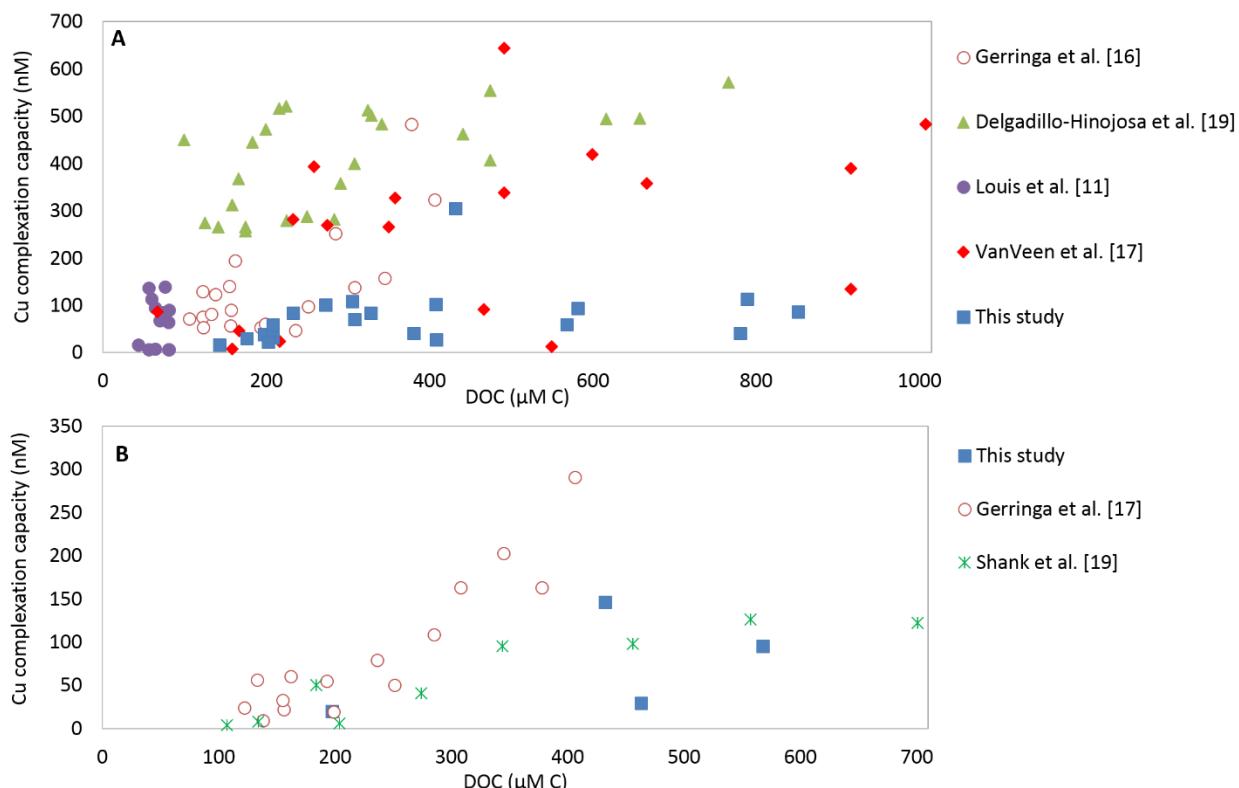
364 **Figure 4. Ligand concentrations ($[L_x]$), Ligand excess ($[L_x] - [Cu_{TD}]$), and labile and
 365 (organically) complexed Cu as a percentage of total dissolved Cu, for each sampling
 366 occasion. Dark red lines represent concentrations determined using 10 μM SA, pale
 367 red lines 2 μM SA. 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^{16,18} when isolating the stronger ligands with a $\text{Log } K >$
379 13 (Figure 5B) that are potentially derived from terrestrial humic sources.¹⁸ A study on the
380 river Scheldt¹⁶ 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.³⁹ 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 μM ,¹⁷ whereas the reported
390 concentrations of copper complexing ligands are typically in the 4 to 400 nM range,¹² i.e. up
391 to 3 orders of magnitude lower, which may explain the observed apparent threshold of 100
392 μM DOC before significant, detectable complexation capacity is measured (Figure 5 A and
393 B).

394



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]: $\log K > 13.0$.**

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 (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 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 ($\text{Ex}260-\text{Em}320$)/($\text{Ex}260-\text{Em}460$) < 4 suggest
411 biological or aquatic bacterial origin, while increasing humic character up to ratios > 16
412 show progressively stronger humic character (Figure 6).⁴⁰ For BIX ($\text{Ex}310-$
413 $\text{Em}380$)/($\text{Ex}310-\text{Em}430$) 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.⁴¹

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 72×10^6
423 L d⁻¹, with median DOC concentrations of 12 mg L⁻¹ reported for English effluents,²⁰ 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.

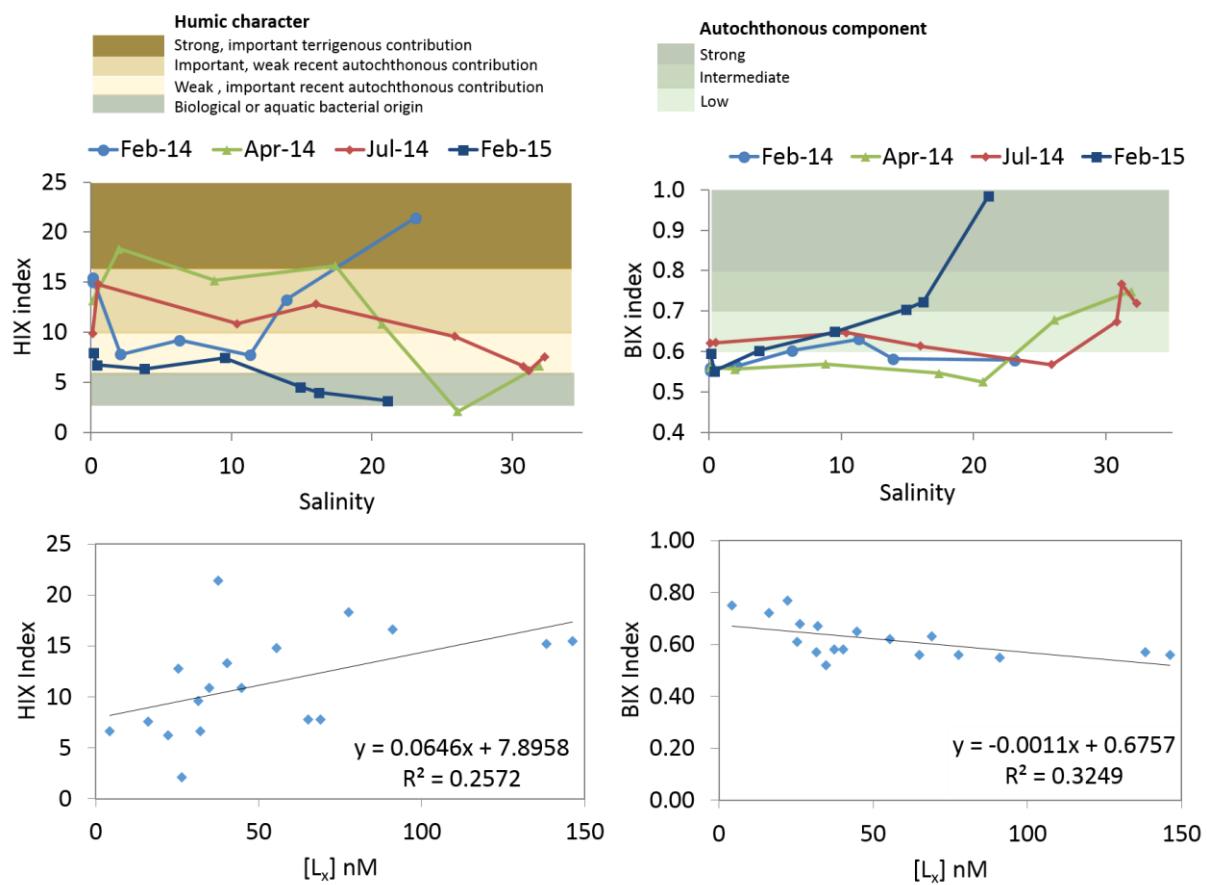
431 Data from February 2014 appeared to show extreme DOC characteristics within the
432 estuary. Sampling followed a prolonged period of intense rainfall across the SW of
433 England, resulting in widespread flooding. River flows into the Tamar estuary were
434 high enough to suppress salinity at its 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,^{18,42} 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

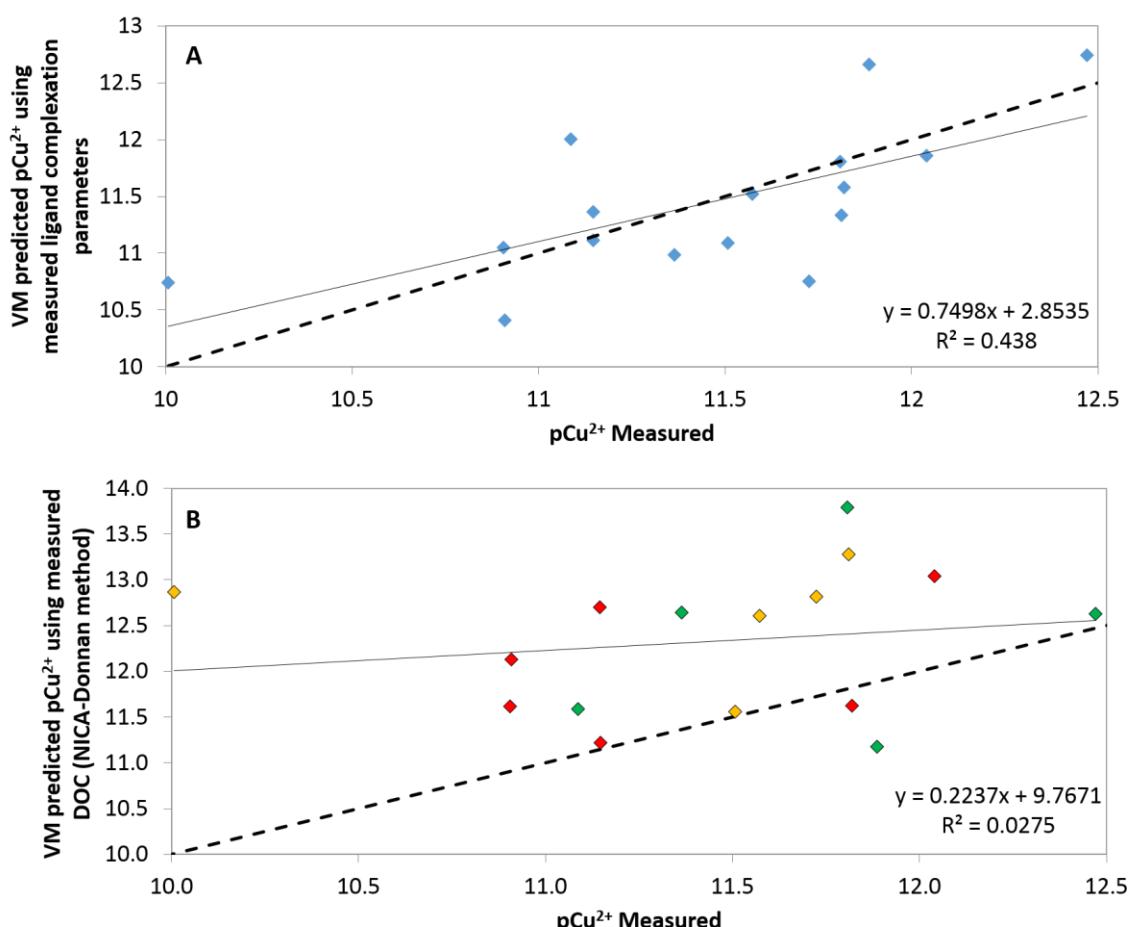
467 Models such as the BLM⁴³ and Gill Surface Interaction Model (GSIM)⁴³ combine
468 predictions of metal ion activity and organism response to generate site-specific water
469 quality standards taking account of ambient conditions. It should be noted that these
470 models use metal ion activity, not 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 accuracy⁴⁴ and models typically use the Debye–Hückel or Davies
475 equations to predict species activity.⁴³

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,⁴⁵ FIAM,⁴⁶ and VM⁴⁷. 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.⁴⁵ 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 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.



523 **Figure 7.** Measured versus predicted (using Visual Minteq) [Cu²⁺] concentration
 524 plotted as pCu²⁺ (-log₁₀ of the molar concentration) with 1:1 line included
 525 (dashed line). Solid lines represent the linear least squares regression of
 526 the data. [A] using ligands measured as part of this work and [B] using VM
 527 ligands generated using measured DOC and the Nica-Donnan method
 528 (Green, amber and red symbols = salinity <10; 10-20 and >20 respectively).

530 The under-prediction of [Cu²⁺] 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 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 acids⁴⁸ 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.

575

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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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