



36 **1. Introduction**

37 Copper (Cu) is an essential micronutrient required in a number of cellular processes that are key to  
38 phytoplankton growth [1-4], It is also a well-known toxic element to phytoplankton and other living  
39 organisms and in most cases, the  $Cu_{free}$  metal ion is the bioavailable specie [5], although this is not  
40 always the case [6]. In algal cultures,  $Cu_{free}$  concentrations as low as 10 pM and 100 pM were found  
41 toxic to cyanobacteria and dinoflagellates, respectively [7]. These Cu toxicity thresholds are  
42 dependent on the species, the strains and the environment they live in [8, 9] but they are relatively  
43 low. Cu toxicity has been observed in waters impacted by atmospheric deposition [10-12], or in  
44 coastal waters [13, 14]. For accurate assessment of its potential toxicity to biota, the knowledge of  
45 the free ion concentration, which is considered as the key indicator [15, 16], is needed. In aqueous  
46 systems, Cu is present at nM levels but is extensively complexed to natural organic ligands, strongly  
47 reducing its free ion concentration [17, 18]. Presence of organic ligands is therefore of main  
48 significance in assessing Cu bioavailability [19-22].

49 Determination of the  $Cu_{free}$  ion concentration is thus challenging due to its low total concentration  
50 and extensive complexation by organic ligands. There is currently no simple, direct and sufficiently  
51 sensitive method to measure routinely  $[Cu_{free}]$  in marine systems. Determination cannot be  
52 achieved by standard dynamic techniques based on flux-based measurements (e.g. anodic stripping  
53 voltammetry ASV, diffusive gradients in thin-film gels DGT) because of the dissociation of labile  
54 compounds in the diffusion layer [23]. Free metal ion concentration can be estimated using  
55 equilibrium based methods such as CLE-CSV (Competitive Ligand Exchange – Cathodic Stripping  
56 Voltammetry) [24, 25], anodic stripping voltammetry (ASV) in equilibrium conditions [26], PLM  
57 (permeation liquid membrane) [27], ion-exchange column [28] or using a selective adsorption onto  
58 a chelating resin followed by medium exchange [29] and finally, by using an ion selective electrode  
59 (ISE). A new promising electroanalytical technique for measurement of free metal ion  
60 concentration called "absence of gradients and Nernstian equilibrium stripping" (AGNES) [30, 31]  
61 was recently used for the determination of free Cu by using gold vibrating electrode [26]. Due to its  
62 complexity, it is still not widely applied. Ion selective electrodes (ISE) are specifically designed to be  
63 sensitive to the free metal ion and they present several significant advantages: simple application,  
64 portability, fast response, robustness and low cost. There is a large number of ISEs for various  
65 cations and anions and the most known example is a Cu-ISE [32] based on jalpaite membrane. This

66 electrode has a low detection limit enabling not only free Cu ( $Cu_{free}$ ) determination in natural  
67 samples, but also total Cu ( $Cu_T$ ; in acidified and UV treated samples, corrected for the known  
68 inorganic side reaction) and the Cu complexing capacity (CuCC) [33-41]. However, their application  
69 in marine waters still face several challenges. First, the alteration of the electrode surface upon  
70 continuous interaction with sample matrix (e.g. electrode corrosion, electrode fouling by the  
71 chloride, hydroxide, organic ligands or other interferences, or deposition of copper sulphide/silver  
72 chloride film on the electrode surface [32, 42, 43]) is resulting in a drift of the electrode response,  
73 which can be minimized by using a preconditioning step in a sample with similar matrix [32, 43].  
74 Secondly, electrode dissolution (i.e. release of Cu from the electrode surface to the solution)  
75 restricts the analysis to solutions that have  $Cu_T$  below  $\sim 20$  nM [44, 45]. Solutions to minimize this  
76 dissolution problem include a flow-through system [40, 46] or the use of a strong hydrodynamic  
77 flow via high stirring, decreasing  $Cu_T$  down 0.1 nM [45-47]. If  $Cu_T$  is high enough at the electrode  
78 surface (i.e. in excess of the Cu levels originating from the dissolution of the electrode), very low  
79  $Cu_{free}$  concentrations can be measured (down to a reported  $10^{-19}$  M) [32, 40, 48, 49].

80 Under the assumption that the electrode response is caused by the Cu ion concentration at the  
81 electrode surface, controlled by rapid and reversible reactions at the electrode-solution interface  
82 (which do not change the composition in the solution), potential of the electrode can be related to  
83 the Cu ion activity (or  $Cu^{2+}$  concentration at constant ionic strength) (Eq. 1) [40, 50-52]:

$$84 \quad E = E^0 + S \times \log[Cu_{free}] \quad (1)$$

85 where  $E$  is the measured electrode potential,  $S$  is the slope and  $E^0$  the intercept or reference  
86 potential. Plotting  $E$  vs.  $\log[Cu_{free}]$  should give a Nernstian slope of nominally 29.6 mV/decade  
87 change in  $Cu_{free}$  ion concentration.

88 The applicability of Cu-ISE in seawater has been tested in a number of studies which all concluded  
89 that if the electrode is calibrated in standard  $Cu_{free}$  buffer (e.g. Cu-EN solution), it can be used for  
90  $Cu_{free}$  measurement in spite of a high chloride content [37, 43, 47, 53]. In this work, we re-examine  
91 the applicability of Cu-ISE (based on jalpaite membrane) for the measurement of  $Cu_{free}$  in seawater.  
92 Cu-ISE methodology is commonly based on the use of single calibration approach which consists in  
93 taking a solution with a similar matrix as the sample, adding a very high level of Cu (typically 0.2 –  
94 1 mM), adding a Cu-ligand of known stability constant (typically ethylenediamine; EN) and varying

95 [Cu<sub>free</sub>] by controlling the pH [37, 40, 46, 53]. We show here that this approach is flawed because  
96 the Nernstian behavior predicted by Eq. 1 is not observed at lower [Cu<sub>T</sub>] (15 nM - 100 μM). To  
97 overcome this problem, we applied a meta-calibration approach i.e. set of calibrations at various  
98 Cu<sub>T</sub> (15 nM – 1 mM) using EN (5 μM - 15 mM) to buffer Cu<sub>free</sub>. The set of calibration parameters  
99 (slope and intercept) were then fitted using the Gompertz function, allowing the choice of  
100 optimized values at any Cu<sub>T</sub> concentration. Our new meta-calibration approach was tested in UV  
101 digested seawater in presence and absence of organic ligands (EN or humic acid) and in a natural  
102 estuarine sample) for the determination of complexing parameters (ligand concentrations and  
103 stability constants). For each of those tests, we compared the two approaches (single and meta-  
104 calibration) to predictions from a modelling software Visual MINTEQ [54]. To our knowledge, this  
105 is the first time that a meta-calibration approach is used for speciation of Cu using Cu-ISE.

106

## 107 **2. Material and methods**

108 All measuring solutions were prepared using ultrapure water (18.2 MΩ, Synthesis, Millipore, USA;  
109 referred thereafter as Milli-Q). Copper stock solutions were prepared by appropriate dilutions of  
110 an atomic absorption spectrometry standard solution (1 g dm<sup>-3</sup>, pH = 2; *TraceCERT*, Fluka). pH  
111 control was achieved by addition of a borate buffer containing 1 M boric acid (H<sub>3</sub>BO<sub>3</sub>; *Suprapur*,  
112 Sigma-Aldrich) and 0.6 M sodium hydroxide (NaOH; *suprapur*, Merck). Acidification and  
113 neutralization were obtained via addition of dilute hydrochloric acid (HCl; *suprapur*, Merck) or  
114 sodium hydroxide (NaOH; *suprapur*, Merck). Ethylenediamine (EN) (puriss. p.a., ≥99.5%, Sigma-  
115 Aldrich) stock solutions were prepared at concentrations of 1 and 10 mM. A humic acid stock  
116 solution (HA; 600-1000 Da, Sigma-Aldrich), was prepared by dissolution in 1 mM NaOH. Prepared  
117 solution of 2 mg dm<sup>-3</sup> HA contains ~ 1 mg dm<sup>-3</sup> dissolved organic carbon (DOC). Experiments were  
118 performed in synthetic solutions using sodium nitrate or sodium chloride (both *suprapur*, Merck)  
119 to regulate the ionic strength, or in organic matter free seawater (UVSW). UVSW was UV-oxidized  
120 using a homebuilt system (250 W high-pressure mercury vapor lamp), for 24 h to decompose  
121 natural organic matter; it was then purified using MnO<sub>2</sub> suspension for 24 h at room temperature  
122 before filtration through pre-cleaned 0.22 μm CA filters (Sartorius) [55].

123 The free ion copper concentration was determined using an Orion Cu-ISE (Model 9429BN) having  
124 a jalpaite Ag<sub>1.5</sub>Cu<sub>0.5</sub>S membrane. Potentials were recorded relative to a double junction

125 Ag|AgCl|sat. NaCl reference electrode (model 6.0728.120+6.1245.010, Metrohm, Switzerland)  
126 containing purified UVSW as the outer filling solution in the bridge. The pH was simultaneously  
127 recorded during each experiment using a double junction pH electrode calibrated against NIST  
128 (*National Institute of Standards and Technology*) traceable pH buffer solutions (Merck). Each  
129 electrode was connected to a dedicated potentiometer (Orion research, Expandable ion Analyzer  
130 EA 920). The voltage outputs of both potentiometers were connected to the high-resolution data  
131 acquisition USB datalogger ADC-20 (Pico Technology, Cambridge, UK) which was used to convert  
132 analog signal to digital form. A homebuilt software was developed for data collection/recording,  
133 graphical presentation and treatment.

134 If not used for longer period, the sensor membrane (jalpaite) was protected with the plastic cover  
135 cap provided by the producer. After prolonged period of continuous use (~ 1 week), storage (>1  
136 month) or decrease of the electrode response, the electrode is polished by using the polishing kit  
137 supplied by the producer. Between measurements, the Cu-ISE was stored in slightly acidified Milli-  
138 Q water (pH ~ 5) in the dark, because it was shown that storage in darkness minimizes the  
139 undesirable photooxidation of the  $\text{Ag}_{1.5}\text{Cu}_{0.5}\text{S}$  membrane [47, 56]. Before the measurements, it was  
140 rinsed with Milli-Q water and conditioned for at least one hour in an identical solution as the one  
141 to be measured, until a steady potential value had been obtained. All measurements were made in  
142 20 mL solutions, at room temperature (~ 24 °C) under constant stirring (magnetic stirrer, 950 rpm).  
143 Solutions for electrode calibration were prepared in 4 different media (0.1 and 0.5 M NaCl, UVSW  
144 and 0.5 M  $\text{NaNO}_3$ ) at various  $\text{Cu}_T$  concentrations (1 mM – 15 nM) in presence of EN. Concentration  
145 of EN at each  $\text{Cu}_T$  concentration was chosen to maintain  $[\text{Cu}_{\text{free}}]$  below 0.1 pM ( $\log[\text{Cu}_{\text{free}}] < -13$ ) at  
146 the highest pH value. For the electrode calibrations, the  $\text{Cu}_{\text{free}}$  concentration in the solution was  
147 varied by adjusting the pH. At the beginning of the acid titration, pH of the solution was set above  
148 8.5 using borate buffer (0.02 M) and gradual additions of the dilute HCl solution were used to lower  
149 the pH down to ~ 3 (there is no complexation of Cu by EN at this low pH, only inorganic  
150 complexation occur) [43]. The cell potential was recorded upon stabilization after each acid  
151 addition, using a stability criterion of 0.15 mV min<sup>-1</sup>. The electrode slopes (potential vs  $\log[\text{Cu}_{\text{free}}]$ )  
152 were calculated by linear regression and were reproducible to within  $\pm 2.4$  mV/decade over a one-  
153 year period.

154 Equilibrium speciation calculations of  $Cu_{free}$  were performed using Visual MINTEQ (*MINeral Thermal*  
155 *Equilibrium model*) [54].  $Cu_T$  and EN concentrations were corrected for the dilution factor due to  
156 the addition of acid. This was done automatically by Visual MINTEQ using a prepared Excel file. A  
157 seawater composition of salinity 38 (Table S1) was used to setup Visual MINTEQ in order to  
158 calculate  $Cu_{free}$  needed in experiments performed in UVSW. The modeling of Cu interaction with  
159 model humic acid (HA) organic matter was performed by using default setup provided in Visual  
160 MINTEQ. For modeling of natural organic matter representing estuarine sample, a default model  
161 setup comprising 100% fulvic acid (FA) was used.

162 Cu titrations (addition of an increasing concentration of  $Cu_T$ ) were performed in: (1) model solutions  
163 (0.5 M  $NaNO_3$ , 0.1 and 0.5 M  $NaCl$  and UVSW) without organic ligands at pH 3 and 8.5; (2) model  
164 solutions (0.5 M  $NaCl$  and UVSW) with addition of EN or HA as organic Cu-ligands and (3) in a natural  
165 estuarine water sample (collected in the Krka River estuary, Croatia, in July 2019 (GPS coordinates:  
166  $43^{\circ}44'07.92$  N,  $15^{\circ}52'39.61$  E) at 0.5 m depth,  $S = 28$ ). The latter sample was filtered through 0.22  
167  $\mu m$  CA filters (Sartorius) by using pre-cleaned syringe (5% v/v  $HNO_3$ , rinsed 3 times with Milli-Q  
168 water) and stored at 4 °C in pre-cleaned (1% v/v  $HCl$ , rinsed 3 times with Milli-Q water) FEP  
169 (Nalgene) bottle until analysis. Total dissolved Cu was measured by means of standard addition  
170 method using differential pulse anodic stripping voltammetry (DPASV) in an acidified (pH 2) UV  
171 digested sample [57]. Measurements were carried out using an PGSTAT128N potentiostat  
172 (Metrohm-Autolab, Utrecht, The Netherlands) controlled by GPES 4.9 software in a three-electrode  
173 cell (663 VA Stand, Metrohm). DOC concentration was determined by high temperature catalytic  
174 oxidation using a Shimadzu TOC-VCSN carbon analyzer [58]. Cu titrations were performed by  
175 increasing the Cu concentration with 11-15 additions equally distributed in logarithmic scale, i.e.  
176 similar increments in  $\log[Cu]_T$  [17, 59]. The potential was measured by Cu-ISE after every Cu  
177 addition and converted to  $Cu_{free}$  by both the single and the new proposed meta-calibration  
178 approaches. By plotting the dependance of  $Cu_{free}$  on increasing  $Cu_T$  concentrations, the titration  
179 curves were constructed. Treatment of the titration curve obtained in natural estuarine sample was  
180 performed using the ProMCC software which provided an estimation of complexation parameters  
181 (concentration of Cu-complexing ligands [ $L_i$ ] and conditional stability constants;  $K'_{CuLi}$ ) [60].

182  
183

### 184 3. Results and discussion

#### 185 3.1. Standard single Cu-ISE calibrations

186 Calibration curves (Fig. 1) of electrode potential vs  $\log[\text{Cu}_{\text{free}}]$  obtained in 0.5 M  $\text{NaNO}_3$ , 0.1 M NaCl,  
187 0.5 M NaCl or UVSW in presence of a high concentration of copper ( $300 \mu\text{M Cu}_T$ ) and 1 mM EN had  
188 regression lines with (near)Nernstian slopes of 30.2, 29.7, 27.7 and 24.9 mV/decade, respectively.  
189 However, while the linearity in 0.5 M  $\text{NaNO}_3$  and 0.1 M NaCl was good along the entire range ( $-13$   
190  $< \log[\text{Cu}_T] < -3$ ) with  $R^2$  of 0.9989 and 0.9999, good linearity in UVSW and 0.5 M NaCl was only  
191 obtained when  $[\text{Cu}_{\text{free}}] < \sim 1 \mu\text{M}$ . At higher concentrations, the pH is such that Cu-buffering by EN  
192 is low and chloride interference occurs [61]. Chloride interference was already observed, at  $[\text{Cu}_{\text{free}}]$   
193  $> 10^{-8} \text{ M}$ , due to a formation of Cu(I)-chloro complexes in the electrode diffusion layer at high  $[\text{Cu}_{\text{free}}]$   
194 [50, 56, 61]. However, for  $[\text{Cu}_{\text{free}}] < \sim 1 \mu\text{M}$ , (near)Nernstian slopes were also obtained (28.8 and  
195 27.1 mV/decade for 0.5 NaCl and UVSW respectively). These slopes are in agreement with those  
196 reported in the literature (Table 1).

197 We repeated these calibration curves in the same solutions but with a lower  $\text{Cu}_T$  concentration of  
198 300 nM. Linear relationships were still obtained but the slopes of the regression lines (Table 1) were  
199 all significantly lower (21.6, 14.8 and 12.5 mV/decade in 0.5 M  $\text{NaNO}_3$ , 0.5 M NaCl and UVSW  
200 respectively). Decrease of slopes between high and low  $\text{Cu}_T$  was more pronounced in chloride  
201 containing solutions (47% and 52% loss in 0.5 NaCl and UVSW respectively) than in chloride free  
202 solution (28% loss in 0.5 M  $\text{NaNO}_3$ ). These results are generally consistent with those found by  
203 Avdeef et al. (1983) [48] who reported a 10% decrease of slope in 10 mM  $\text{KNO}_3$  when passing from  
204 1 mM  $\text{Cu}_T$  down to 200 nM  $\text{Cu}_T$ .

205 It thus appears that the electrode response is dependent on the total Cu concentration. To test this  
206 hypothesis, we carried out meta-calibrations at different  $\text{Cu}_T$  concentrations.

207

#### 208 3.2. Cu-ISE meta-calibrations

209 To estimate the dependence of the Cu-ISE calibration parameters on  $[\text{Cu}_T]$ , calibrations were  
210 performed at various concentrations of  $\text{Cu}_T$  (in the range 1 mM – 15 nM) and EN (15 mM - 5  $\mu\text{M}$ ) in  
211 UVSW (Fig. 2), 0.5 M NaCl (Fig. S1) and 0.5 M  $\text{NaNO}_3$  (Fig. S2). All calibrations produced linear  
212 response down to pM/fM level of  $\text{Cu}_{\text{free}}$  and slopes (mV per decade of  $\text{Cu}_{\text{free}}$ ) were found to  
213 decrease with decreasing  $\text{Cu}_T$ .

214 Calibration parameters (i.e. slope,  $S$  and the reference potential,  $E^0$ ) at a given  $Cu_T$  were calculated  
215 by fitting all the experimental points. They are shown against  $\log[Cu_T]$  in bottom graphs of Figs. 2,  
216 S1 and S2. The regression slopes decreased from a (near)Nernstian values (at  $Cu_T$  above  $\sim 50 \mu M$ )  
217 down to 7.3 and 9.4 mV/decade in UVSW and 0.5 M NaCl (at 15 nM  $Cu_T$ ), respectively and down to  
218 21.9 mV/decade in 0.5 M  $NaNO_3$  (at 35 nM  $Cu_T$ ); the intercept decreased from 234 mV to 45 mV in  
219 UVSW, 264 mV to 85 mV in 0.5 M NaCl and 323 mV to 255 mV in 0.5 M  $NaNO_3$  (at referred  $Cu_T$   
220 concentrations).

221 Both calibration parameters followed a sigmoidal relationship with  $Cu_T$ , the greatest variation being  
222 between 35 nM and 10  $\mu M$   $Cu_T$  ( $-7.0 < \log[Cu_T] < -5.0$ ) in all three media (Figs. 2, S2 and S3). At high  
223 Cu levels ( $Cu_T$  above  $\sim 50 \mu M$ ), both  $S$  and  $E^0$  were relatively constant. With decreasing  
224 concentrations,  $S$  decreased showing a loss of sensitivity towards  $Cu_{free}$ . The lowest reachable  
225 potential at  $Cu_T$  below 0.3  $\mu M$  was  $\sim -60$  mV while, for the same  $\log[Cu_{free}]$ , potential was  $\sim -120$  mV  
226 at  $Cu_T > 10 \mu M$ . At  $Cu_T$  below 20 nM,  $S$  and  $E^0$  both reach a constant value, irrespective of  $Cu_T$ . This  
227 is due to the dissolution of the electrode membrane which sets up the detection limit of the ISE  
228 [45].

229 Several attempts have been made to explain the chloride interference on Cu-ISE measurements:  
230 some of them considered the exchange reactions at the electrode surface and other redox  
231 reactions with membrane material [52, 56, 61, 62]. Lewenstam et al. (1985) [52] provided a model  
232 which describes how the presence of halide ions affects the exchange reactions at the electrode-  
233 solution interface by forming amorphous sulphur. According to these authors, this reaction  
234 mechanically blocks the electrode surface and causes irreversible reactions, which may be the  
235 explanation of the more prominent loss of sensitivity towards  $Cu_{free}$  in the high chloride media  
236 observed here.

237 Whatever the reasons, we can conclude that this significant change of calibration parameters ( $S$   
238 and  $E^0$ ) at varying  $Cu_T$  concentration simply prevents the use of a single calibration approach. For  
239 reliable measurements, potentials have to be correlated to the appropriate calibration curve, which  
240 is dependent on the  $Cu_T$  concentration. To predict the correct  $S$  and  $E^0$  at any  $[Cu_T]$ , we tried to fit  
241 experimental results on various sigmoidal functions, among which the Gompertz function (Eq. 2)  
242 showed the best matching:



243 
$$f(x) = y_0 + ae^{-e^{\frac{(x-x_0)}{b}}} \quad (2)$$

244 where  $y_0$  is the base value,  $x_0$  is the  $\text{Cu}_T$  at mid-slope value, while  $a$  and  $b$  are fitting parameters,  
245 not having any chemical meaning. The fitted equations for  $S$  and  $E^0$  in UVSW are given in Fig. 2 (see  
246 Fig. S1 for 0.5 M NaCl). Note that "S"-shaped Gompertz function is used here due to the wide range  
247 of the examined  $\text{Cu}_T$ . However, if the calibrations are performed in the narrower range of  $\text{Cu}_T$  (e.g.  
248 up to 10  $\mu\text{M}$ ), the obtained relationships might not be fully sigmoidal, and as such, the other  
249 empirical functions could also be used (e.g. polynomial or other sigmoidal functions), as long as the  
250 fitting of the datasets is satisfactory.

251 The proposed meta-calibration approach for determination of  $\text{Cu}_{\text{free}}$  concentration comprises the  
252 two prerequisites: (1) the known concentration of  $\text{Cu}_T$  in the sample being analyzed and (2) the two  
253 valid Gompertz (or other) functions needed to create calibration line (slope + intercept) at any  
254 concentration of  $\text{Cu}_T$  (they are electrode dependent). For measurements at lower  $\text{Cu}_T$   
255 concentrations ( $\sim <1 \mu\text{M}$ ), it is suggested that the electrode is conditioned by the sample being  
256 analyzed for at least 30 minutes, after which the new fresh sample is taken, and the potential  
257 reading taken upon stabilization.

258 Based on the results presented above, we suggest the following analytical protocol for the  
259 determination of  $\text{Cu}_{\text{free}}$  in chloride containing media:

- 260 1. determine the Gompertz functions for both the slope and intercept; ideally, this should be  
261 obtained in the expected range of  $\text{Cu}_T$  and at salinity close to the sample of interest,  
262 2. determine the dissolved  $\text{Cu}_T$  concentrations of the samples of interest,  
263 3. measure the potential ( $E$ ) using Cu-ISE electrode,  
264 4. determine the  $\text{Cu}_{\text{free}}$  concentration based on equation (1) using the appropriate calibration  
265 parameters ( $S$  and  $E^0$ ) extracted by using the Gompertz functions (step 1) for the known  
266 concentration of dissolved  $\text{Cu}_T$  (step 2).

267

### 268 3.3. Applicability of the meta-calibration approach

#### 269 3.3.1. Model solution without organic ligands

270 The response of the electrode was first tested in absence of organic ligands at pH of 3 and 8.5 for  
271 each of the following solution: 0.5 M  $\text{NaNO}_3$ , UVSW, 0.1 and 0.5 M NaCl. Cu levels were increased

272 from 15 nM to 110  $\mu$ M and  $[Cu_{free}]$  were obtained from Visual MINTEQ predictions. At pH 3, the  
273 expected theoretical slope was again obtained in 0.5 M  $NaNO_3$  and 0.1 M  $NaCl$  (28.9 and 29.5  
274 mV/ $\log[Cu_{free}]$ , respectively), whereas a "super-Nernstian" response was obtained in UVSW and 0.5  
275 M  $NaCl$  at pH 3 (38.0 and 41.3 mV/ $\log[Cu_{free}]$ , respectively), and at pH 8.5 (38.1 and 38.3  
276 mV/decade, respectively) (Figs. 3, 4 and S3). All experiments were repeated several times over a  
277 period of one year and they gave similar results. At pH 3, the electrode response to  $Cu_T$  additions  
278 in UVSW and 0.5 M  $NaCl$  was linear down to  $\sim 25$  nM  $Cu_{free}$ , whereas it was linear down to  $\sim 1$  nM  
279  $Cu_{free}$  at pH 8.5 (Figs. 4A/B and S3A/B) as a result of buffering effect of carbonate and hydroxide  
280 present in the solution (Table S2). This is consistent with the previous observation that, in the  
281 absence of any organic ligand, buffering effect of hydroxy and carbonate complexes is enough to  
282 allow reliable measurements of  $Cu_{free} < 20$  nM [49]. In the absence of organic ligands (Figs. 4A/B  
283 and S3A/B), the proportionality between  $Cu_T$  and  $Cu_{free}$  is given by the inorganic side reaction  
284 coefficient,  $\alpha ([Cu_T]/[Cu_{free}])$  [62]. At pH 3,  $\alpha \sim 1.5$  due to Cu complexation with chloride and sulfate  
285 while at pH 8.5,  $\alpha \sim 33$  due to complexation with carbonate and hydroxide. The observed shifts  
286 between  $[Cu_{free}]$  and  $[Cu_T]$  along the X-axis in Figs. 4A/B correspond to  $\alpha$ -factors at two pH values.

287 The applicability of our meta-calibration approach was first checked in UVSW at both pH 3 (Fig. 4C)  
288 and 8.5 (Fig. 4D) by plotting  $Cu_{free}$  as a function of  $Cu_T$  using the single calibration approach (Fig. 1),  
289 the meta-calibration approach (i.e. using the empirical equations given in Fig. 2) or Visual MINTEQ  
290 predictions. Similar graphs are shown in Fig. S3C/D for 0.5 M  $NaCl$ . At both pH, the single calibration  
291 approach displayed a sigmoidal shape similar to what is usually obtained in the presence of organic  
292 ligands in solution: a weak curvature at the lowest Cu levels followed by a linear increase in  $Cu_{free}$   
293 in response to higher Cu additions, analogous to ligand saturation. This response has been  
294 previously reported and explained by the lack of sensitivity of the Cu-ISE at the initial concentration  
295 level [37, 53]. However, when applying our meta-calibration approach, our calculated  $Cu_{free}$   
296 concentrations are in much better agreement with modeled data than the single calibration  
297 approach. This is particularly true at pH 3. At pH 8.5, at  $Cu_T$  concentration above 10  $\mu$ M, Cu  
298 precipitation of Cu hydroxide species is predicted, which may explain the plateau observed at these  
299 high Cu levels (top empty circles).

300 Super-Nernstian response to increasing Cu concentration was already observed in other studies  
301 and attributed to the presence of chloride ions [52, 62]. Using a Orion Cu-ISE, Jasinski et al. (1974)  
302 [62] observed a Nernstian slope in nitrate and sulfate media and a super-Nernstian slope in 1 M KCl  
303 at pH 2 and in 0.5 M NaCl at pH 8. They suggested that this anomalous response was due to both  
304 the electrode material and the high chloride ion concentration rather than the presence of small  
305 quantities of chelating agents in the solution. Belli and Zirino (1993) [53] reported super-Nernstian  
306 response in high-chloride media, but only in alkaline conditions. They assumed that the matrix binds  
307 different fraction of Cu, depending on the Cu concentration, in artificial seawater at pH 8 and that  
308 there are neglected Cu species in the model. We obtained slightly different slopes at pH 8.5 (Fig. 4)  
309 between  $E$  vs  $\log[\text{Cu}_T]$  (slope of 38.8 mV/decade) and  $E$  vs  $\log[\text{Cu}_{\text{free}}]$  (38.1 mV/decade) as a result  
310 of slight change in inorganic side reaction coefficient at increasing Cu concentration, mostly coming  
311 from hydroxide ions. Notwithstanding, this difference is quite negligible and is probably not the  
312 reason for super Nernstian response during Cu titration, as suggested by Belli and Zirino (1993)  
313 [53]. Moreover, the same response was also observed here at pH 3 where the inorganic side  
314 reaction coefficient is constant. Super-Nernstian response most likely occurs due to the gradual  
315 shift in sensitivity during increasing  $\text{Cu}_T$  concentration. Finally, if we take only the two last points  
316 from the Cu titration at pH = 3 (Fig. 4A), where  $[\text{Cu}_T]$  is high enough (Fig. 2), we obtain a Nernstian  
317 slope of 28.89 mV/ $-\log[\text{Cu}]$ .

318 The decrease of slope with lowering  $\text{Cu}_T$  concentration might explain the strong disagreement of  
319 experimental results with the predicted ones in the presence of synthetic ligands (EN and the  
320 polyaminocarboxylic acids EDTA, CDTA and NTA) at lower  $\text{Cu}_T$  concentration ( $< 1 \mu\text{M}$ ) obtained by  
321 Rivera-Duarte and Zirino (2004) [37], which was also specifically pointed out by Sánchez-Marín  
322 (2020) [39]. The leveling of pCu they observed below 10 nM of  $\text{Cu}_T$  is related to the detection limit  
323 of Cu-ISE electrode caused by the dissolution of the electrode membrane, maintaining the relatively  
324 high  $\text{Cu}_T$  in the vicinity of the electrode surface [45]. Furthermore, in their experiment in the  
325 absence of organic matter (Fig. 2 in [37]), a disagreement between modeled and measured  $[\text{Cu}_{\text{free}}]$   
326 are in agreement with our results when using the single calibration approach (Fig. 4C/D). As shown  
327 here in Fig. 4C/D, using proposed meta-calibration approach the agreement with modeled data for  
328 the same experiment type was much better, signifying the benefit of our calibration approach for  
329 the measurements of Cu speciation in natural waters.

330 *3.3.2. Model solutions with known concentrations of organic ligands*

331 The validity of our meta-calibration approach was also tested in UVSW (pH = 8.2) in presence of  
332 organic ligands, either 5  $\mu\text{M}$  EN or 2  $\text{mg dm}^{-3}$  HA. Cu titrations were achieved in both solutions and  
333 concentrations of  $\text{Cu}_{\text{free}}$  were calculated at each step using the single and meta-calibration  
334 approaches and compared to Visual MINTEQ predictions (Fig. 5).

335 In both cases, the meta-calibration approach provided a much better agreement with the modeled  
336 values than the single calibration approach. In presence of EN (Fig. 5A/B), both methods displayed  
337 a good agreement with Visual MINTEQ at  $\text{Cu}_{\text{T}}$  levels above 10  $\mu\text{M}$  (because of similar calibration  
338 parameters in these conditions; Fig. 2) but the single calibration approach significantly  
339 overestimated  $\text{Cu}_{\text{free}}$  at  $\text{Cu}_{\text{T}}$  levels below  $\sim 1 \mu\text{M}$ . In presence of HA, the single calibration approach  
340 strongly underestimated  $\text{Cu}_{\text{free}}$  at higher Cu levels and strongly overestimated them at lower. At Cu  
341 levels below  $\sim 30 \text{ nM}$  ( $\log[\text{Cu}_{\text{T}}] < -7.5$ ), a plateau value limit was reached confirming that Cu-ISE is  
342 not suitable for measurements of lower levels in our cell configuration. This would prevent the  
343 analysis of open ocean or open coastal seas that contain low nM levels of Cu [63], but it can allow  
344 Cu speciation (i.e. measurements of  $\text{Cu}_{\text{T}}$ ,  $\text{Cu}_{\text{free}}$  and Cu-binding organic ligands) in coastal areas with  
345 strong anthropogenic influence [64].

346

347 *3.3.3. Natural estuarine sample*

348 The efficiency of the meta-calibration method in the determination of  $[\text{Cu}_{\text{free}}]$ , as well as in the  
349 determination of concentration and strength of natural organic ligands, was evaluated here by Cu  
350 titration on an estuarine sample collected from the Krka River estuary (pH = 8.2); this sample had a  
351 total Cu concentration of 20 nM and contained 1.5  $\text{mg dm}^{-3}$  DOC.  $\text{Cu}_{\text{free}}$  concentrations obtained  
352 using the single and meta-calibration approaches, were compared to the modeled data obtained  
353 by Visual MINTEQ (Fig. 6).

354 Very good agreement was obtained between the meta-calibration approach and Visual MINTEQ  
355 predictions in the linear part of the titration curve (i.e. at  $[\text{Cu}_{\text{T}}] > 3 \mu\text{M}$ ) while the single calibration  
356 approach strongly underestimated  $[\text{Cu}_{\text{free}}]$  in that region, similar to with HA (Fig. 5C/D). At the lower  
357 end of  $[\text{Cu}_{\text{T}}]$ , the single calibration approach predicts a much higher  $[\text{Cu}_{\text{free}}]$  (30 times higher, similar  
358 to results obtained in presence of HA), whereas much closer values to those predicted by Visual

359 MINTEQ were obtained using meta-calibration approach. Although the general trend of measured  
360  $[Cu_{free}]$  agree well with the predicted values along the full titration range, slightly higher values at  
361 the concentration range below 1  $\mu M$  of  $Cu_T$  could be explained by the difference in the ligand  
362 characteristics of estuarine natural organic matter from the one used in Visual MINTEQ modeling  
363 (fulvic acid).

364 Ligand concentrations and conditional stability constants were calculated from each titration curve  
365 using ProMCC [60] and are compared in Fig. 7. Good agreement was obtained between Visual  
366 MINTEQ and the meta-calibration approach in terms of number of ligand classes (represented here  
367 as  $L_1$ ,  $L_2$  and  $L_3$ ), their concentrations and associated stability constants. However, the strongest  
368 class of ligands  $L_1$  was not identified when the single calibration approach was applied, which leads  
369 to  $\sim 50\times$  overestimation of  $[Cu_{free}]$  calculated based on the derived complexation parameters (Fig.  
370 7A). As this class of ligands ( $\log K_1$ ) is the most important for the complexation of Cu at its low  
371 ambient concentration, the single calibration approach would therefore tend to highly  
372 overestimate the Cu toxicity of the sample.

373

#### 374 **4. Conclusions**

375 This work demonstrates that at  $Cu_T$  concentrations below 100  $\mu M$ , the Nernstian slope is decreasing  
376 with decreasing  $Cu_T$  for the jalpaite electrodes. Although Cu concentrations can reach high levels in  
377 highly polluted coastal areas [19, 65, 66], they are almost never higher than 30  $\mu M$  where effective  
378 calibration parameters begin to deviate from the theoretical values (Figs. 2 and S1). In natural  
379 waters, commonly occurring  $Cu_T$  concentrations are much lower and closer to the detection limit  
380 of Cu-ISE, preventing the use of the standard single calibration approach. We show here that a  
381 meta-calibration approach can be successfully used instead, by applying optimized calibration  
382 parameters at appropriate  $Cu_T$  level. The results obtained in synthetic solutions as well as in  
383 seawater are in good agreement with modeled predictions, preventing a high overestimation of  
384  $Cu_{free}$  that is observed through the single calibration approach. This new analytical procedure is  
385 simple and could be used to enable Cu speciation studies in natural and synthetic samples,  
386 measurement of  $Cu_{free}$  in toxicological experiments and in a number of other studies. The  
387 sensitivity, the ease of use, the rapid response time and the robustness of the electrode over a long  
388 period of time are all assets to this new analytical procedure that can be used in natural waters,

389 including marine. The use of flow-through cell [40], rotating electrode [45] or highly efficient wall-  
390 jet system [67] is expected to decrease the detection limit and/or associated problems of the  
391 electrode dissolution. This is our next objective.

392

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