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1   # Lability of copper bound to humic acid

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22   **HIGHLIGHTS:**

23   • Isotopic dilution was used to assess the lability of Cu bound to humic acids

24   • Time-dependent Cu fixation by humic acid was observed over 160 days

25   • Around 40% of Cu bound to HA may be non-labile.

26   • Non-labile Cu in humic acids may affect predicted speciation by geochemical models

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ABSTRACT

Geochemical speciation models generally include the assumption that all metal bound to humic acid and fulvic acid (HA, FA) is labile. However, in the current study, we determined the presence of a soluble 'non-labile' Cu fraction bound to HA extracted from grassland and peat soils. This was quantified by determining isotopically-exchangeable Cu (E-value) and EDTA-extraction of HA-bound Cu, separated by size-exclusion chromatography (SEC) and assayed by coupled ICP-MS. Evidence of time-dependent Cu fixation by HA was found during the course of an incubation study (160 days); up to 50% of dissolved HA-bound Cu was not isotopically exchangeable. This result was supported by extraction with EDTA where approximately 40% of Cu remained bound to HA despite dissolution in 0.05 M Na_2-EDTA. The presence of a substantial non-labile metal fraction held by HA challenges the assumption of wholly reversible equilibrium which is central to current geochemical models of metal binding to humic substances.

1. Introduction

Geochemical models, such as WHAM-VII (Lofts and Tipping, 2011; Tipping, 1998) and the NICA-Donnan Model (Kinniburgh et al., 1999), include the assumption that trace metals sorbed on humic substances are entirely labile – i.e. they participate in a reversible dynamic equilibrium with free metal ions. However, there is increasing evidence that a non-labile fraction may exist in sub-micron sized colloidal phases and bound to dissolved species. For example, in a study of 18 soils, Ma et al. (2006) found non-isotopically exchangeable Cu in the solution phase filtered through 0.2 μm cellulose acetate filters; they suggested the Cu was likely to be associated with dissolved organic matter. Similar suggestions have been made by the same research group (Lombi et al., 2003; Nolan et al., 2009) for Ni, Cu and Zn. Jackson et al. (2005) in a study of dissolved organic matter (DOM) in river water, measured Ni and U distribution in a colloidal phase separated by size exclusion chromatography (SEC) or flow field-flow fractionation (FIFFF) and concluded that Ni was 'more labile' than U because Ni^{2+} appeared to interact with the SEC column gel phase and the FIFFF membrane to a greater

60   degree. By contrast Laborda et al. (2011), used asymmetrical flow field flow fractionation

61   (AsFIFFF) to separate dispersed colloidal phases of isotopically spiked compost material and,

62   from measurements of the isotopic ratio within different size fractions including the free

63   inorganic phase, they concluded that all Cu and Pb present was isotopically exchangeable .

64   It is accepted that metals can be held by very strong binding sites on humic substances

65   through the formation of 'multidentate' complexes with carboxylic and phenolic groups or by

66   specific binding with N and S groups which are known to preferentially bind metals such as

67   Cr^{III}, Fe^{III}, Cu and Hg (Tipping, 2002). However, strong chemical bond formation does not

68   necessarily result in non-labile complex formation and there have been very few studies that

69   have attempted to determine the presence of such a metal fraction on dissolved humic

70   substances. It is well established that isotopic dilution (ID) approaches and some chemical

71   extractants (eg 0.05 M EDTA) can be used to estimate the labile fraction of trace metals in

72   soils (Degryse et al., 2009; Gabler et al., 2007). However, to measure metal lability on a

73   dissolved system containing a range of metal complex species requires a fractionation

74   technique to separate high MW DOM-bound metal from low MW organic and inorganic

75   complexes. This approach then also allows fractionation of spike isotopes introduced to

76   follow the kinetics of metal assimilation throughout the various species and quantification of

77   the isotopically exchangeable forms present. Size exclusion chromatography (SEC) coupled

78   with inductively coupled plasma mass spectrometry (ICP-MS) has been applied to the study

79   of trace element distributions within humic acids (Jackson et al., 2005; Kozai et al., 2013; Liu

80   and Cai, 2010; Liu et al., 2011; Schmitt et al., 2001).

81   The aim of this investigation was to determine the lability of Cu bound to dissolved humic

82   acid (HA) to test the hypothesis that Cu bound to dissolved HA is labile. To achieve this, two

83   popular techniques for measuring labile metals in soils were modified and applied to two

84   types of HAs fractionated using SEC-ICP-MS: extraction with EDTA and determination of

85   isotopic exchangeability. Dissolved HA was incubated with an enriched stable Cu isotope

86   and its subsequent extractability with EDTA determined from differences in isotopic

87   abundance between forms bound to HAs and to low MW complexes with EDTA. Humic

88   acid solutions were incubated with Cu (normal isotopic mix) for a period of time before they

89   were spiked with an enriched stable Cu isotope. The proportion of dissolved HA-bound metal

90   that was isotopically exchangeable (% E-values; Smolders et al., 1999; Young et al., 2000)

91   was then determined from changes in isotope distribution between Cu complexes with HAs

92   and the Tris buffer (Tris (hydroxymethyl) aminomethane) used as the chromatography eluent.

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2. Materials and Methods

2.1 Humic acid preparation and characterisation

Two humic acids were previously extracted and purified by Marshall et al. (1995) from a permanent grassland soil (G-HA; Nottinghamshire, UK) and Sphagnum moss peat (P-HA; North Yorkshire, UK). The ash content was measured by dry combustion at 500°C for 8 hours as 23.5 g kg^{-1} and 3.9 g kg^{-1} for G-HA and P-HA respectively. Total acid group concentrations determined by Marshall et al. (1995) were 5.50 mol$_c$ kg^{-1} for G-HA and 6.94 mol$_c$ kg^{-1} for P-HA using a modified Ba(OH)$_2$ method (Schnitzer and Khan, 1972) including a pre-dissolution step in 0.1 M NaOH to ensure complete hydration of the HA. In this study, the freeze dried HAs were dissolved in 0.5 M NaOH and pH adjusted to 7.0 with dilute nitric acid to provide working HA stock solutions of 16.0 g L^{-1}.

2.2 Size-exclusion chromatography (SEC-ICP-MS)

Fractionation of HA based on molecular size was undertaken using size-exclusion chromatography (SEC) with an ICS-3000 chromatography system (Thermo-Fisher Scientific) and a Superose 12 10/300 GL column (GE Healthcare) with a separation range from 1000 to 300,000 Da. A guard column (Hamilton, Peek PRP-X100) was installed prior to the SEC column. Humic acid samples of 100 μL were injected into an eluent comprising 0.1 M Tris buffer at pH 8.2 adjusted with nitric acid at a flow rate of 1 ml min^{-1}. The pH of the eluent was chosen to coincide broadly with the maximum buffer power of the Tris (pKa = 8.1) and the equivalence-point of carboxyl groups on the HA. The column outflow was connected directly to the nebuliser of an ICP-MS (XSeries^{II}; Thermo-Fisher Scientific) operating in 'collision cell with kinetic energy discrimination' mode (7% hydrogen in helium) to reduce polyatomic interferences (e.g. ^{35}Cl-^{35}Cl interference with ^{70}Zn). The minimum and maximum quadrupole settle times were adjusted to 1000 μs, to minimize the effects of plasma flicker, and Cu isotopes (^{63}Cu & ^{65}Cu) were measured with a quadrupole dwell time of 40 ms. The instrument 'dead time correction factor' was set to 37.2 ns after establishing the value which gave a zero slope in a plot of isotope ratio against concentration of Pb in solutions of the Pb isotope standard NIST-981. The ICP-MS was directly calibrated with both Cu isotopes, precluding the need for internal or external mass discrimination. For pragmatic reasons (nebuliser capacity) we did not include an internal standard line downstream of the SEC

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124   column. For discrete sample analysis which may vary in matrix composition this is vital but
125   considering (i) the use of standard additions for calibration (Section 2.5), (ii) prior knowledge
126   of the total Cu concentration in each sample and (iii) the constancy of the eluent matrix, we
127   did not consider that addition of an internal standard would improve reliability of data. In
128   practice, we found very high reproducibility of chromatograms without an internal standard.
129   A sample loop flushing step between each sample was standard on the SEC chromatography
130   program to avoid carryover and cross-contamination. Chromatographic data were collected
131   for 34 min but typically the data of interest were integrated between 5 and 25 min.

132   **2.3   Determination of Cu extractable by EDTA from humic acid**

133   A stable isotope of Cu with enriched isotopic abundance (IA) was purchased from ISOFLEX
134   USA as a metal foil and dissolved in concentrated HNO_3. Dissolved humic acid, at
135   concentrations of 4.0 g L^{-1} (G-HA) or 4.4 g L^{-1} (P-HA), were incubated with the diluted stable
136   isotope solution: 168 µg L^{-1} ^{65}Cu (IA ≥ 99.0%). The Cu concentration was chosen to be high
137   enough to give a measurable ICP-MS signal after complex-formation. The concentration of
138   functional groups (total acidity) in the dissolved HA solutions (mol_c L^{-1}) was calculated from
139   the values provided by Marshall et al. (1995), as 0.022 mol_c L^{-1} for G-HA and 0.031 mol_c L^{-1}
140   for P-HA, while the spiked isotope solution contained 2.6×10^{-6} mol_c L^{-1} of Cu. After
141   equilibration for 40 or 160 days at 18°C the spiked solutions were diluted with 0.1 M Tris
142   buffer at pH 8.2 (to give final concentrations of 1.0 g L^{-1} for G-HA and 1.1 g L^{-1} for P-HA)
143   and equilibrated for 2 hours with a range of Na_2-EDTA concentrations (0, 0.0005, 0.0025,
144   0.01, 0.025 and 0.05 M). Samples were analysed by SEC-ICP-MS to determine the
145   concentrations of Cu complexes formed with EDTA, Tris and HA; all samples had Na_2-
146   EDTA added exactly 2 hours before the start of their chromatographic runs. The proportion
147   of EDTA-extractable Cu was calculated as the amount of ^{65}Cu removed from the HA
148   complexes by EDTA divided by the total ^{65}Cu added to the system. Estimation of Cu
149   recovery from the chromatographic process is discussed in Section 2.5 and the procedure for
150   chromatograph integration is discussed in Section 3.2.

151

152   **2.4   Isotopically exchangeable Cu**

153   Isotopic exchange kinetics and E-values for Cu (M_{E-HA}) were determined for G-HA (4.0 g L^{-1})
154   and P-HA (4.4 g L^{-1}) solutions previously incubated for 40 days with a spike prepared from a

155 Cu ICP-MS standard solution of 240 µg L^{-1} (natural isotopic abundance). Immediately
156 following further dilution with Tris buffer, the samples were spiked with sufficient enriched
157 ^{65}Cu solution to give measureable increases in ICP-MS signal. The final concentration of
158 added ^{65}Cu was 118.0 µg L^{-1}. This may seem quite a high spike level for measurement of E-
159 value but to measure the fate of the spike isotope against background, given the high isotopic
160 abundance of ^{65}Cu (31%), requires a higher spike level than would be necessary for elements
161 with more minor isotopes. Furthermore, the mole ratio of HA-carboxyl groups:Cu was still
162 approximately 1000:1 after spiking suggesting only minor disruption to the existing labile Cu
163 equilibrium. Finally, we also considered that the normal concerns associated with soil
164 materials, such as surface precipitation of spike metal, probably did not apply to dissolved
165 humic acid solutions. The spiked HA solutions were then analysed using SEC-ICP-MS
166 following isotopic equilibration times between 35 min and 3 days to monitor the progressive
167 mixing of the ^{65}Cu isotope with the pre-existing metal.

168 **2.5   Calibration and recovery of SEC-ICP-MS chromatograms**

169 Trace metal calibration in SEC-ICP-MS can present problems because it is unlikely that
170 inorganic salt standards would survive transition of the SEC column given the pH of the
171 mobile phase. A solution to this is to use HA as a carrier for the metal standards. Calibration
172 standards were therefore prepared by adding known concentrations of Cu （0, 30, 60 µg L^{-1}）
173 from ICP-MS standard solutions to a solution of G-HA (2 g L^{-1} HA).   The entire
174 chromatogram for the ^{63}Cu isotope was integrated and an integrated blank chromatogram (2 g
175 L^{-1} G-HA without added metal) subtracted to generate a calibration for Cu as (integrated)
176 counts per second (CPS) against metal concentration (R^2 ≥ 0.99). The total Cu concentration
177 in the incubated HA solutions (Section 2.4) was calculated using this 'standard addition'
178 calibration. An alternative measurement of the total metal concentration was also made on
179 dilutions of the HA solutions in a matrix of 0.005 M EDTA using 'direct aspiration' into the
180 ICP-MS. Recovery of metal through the SEC column was calculated by comparing the total
181 Cu concentration measured using these two approaches.

182

183 **2.6   Time-dependent Cu isotope adsorption by humic acid**

184 The extent of movement of spike isotope from the non-HA phase (mainly Tris complexes)
185 into HA-complexed forms, as a function of contact time, was modelled using an adapted

186 equation originally developed to describe the degree of mixing of radioactive isotopes with

187 total soil metal pools (Sinaj et al., 1999), Eq.1.

$$P_{(t)}=P_{(1)}\left[t+P_{(1)}^{1/n}\right]^{-n}+P_{(M)} \qquad (1)$$

189 In this application P is the proportion of spiked ^{65}Cu in the non-humic section of the

190 chromatogram (Tris complexes) at a specified time (t) or after 1 min (1), and n is an empirical

191 constant; $P_{(M)}$ is the value of $P_{(t)}$ at infinite time. The constants $P_{(1)}$, $P_{(M)}$ and n were

192 optimized by minimizing the residual standard deviation (RSD) between the measured and

193 modelled values of $P_{(t)}$ using the Solver function in Microsoft Excel.

194

195 **2.7   Calculation and prediction of Cu E-values**

196 The isotopic ratio of Tris-complexed Cu was used to calculate E-values in the presence of

197 humic acid (Cu_{E-HA}). The calculation was adapted from Gabler et al. (1999), and is shown in

198 Eq. 2 where ^{65}Cu is used as the spike isotope and ^{63}Cu is solely from native sources:

$$Cu_{E-HA}=\left(\frac{Cu_{HA}}{W}\right)\left(\frac{C_{spk}V_{spk}}{Cu_{spk}}\right)\left(\frac{^{65}IA_{spk}-{}^{63}IA_{spk}R_{ss}}{^{63}IA_{HA}R_{ss}-{}^{65}IA_{HA}}\right) \qquad (2)$$

200 In Eq. 2 Cu_{HA} and Cu_{spk} is the average atomic mass of Cu in the (unspiked) HA solution and

201 the enriched ^{65}Cu spike solution respectively, W is the weight of humic acid (kg), C_{spk} is the

202 gravimetric concentration of Cu in the spike solution, V_{spk} is the volume of spike added (L),

203 IA is the molar isotopic abundance of ^{65}Cu or ^{63}Cu in the spike solution or unspiked HA

204 solution, and R_{ss} is the isotopic ratio of ^{65}Cu to ^{63}Cu in the spiked HA solution measured in

205 the 'non-humic' section of the chromatogram.

206 The change in $\%Cu_{E-HA}$ with time, following addition of the spike, was described by an

207 empirical equation (Eq. 3) adapted from Eq.1.

$$\%Cu_{E-HA}=100\left[1-K\left(t+K^{\frac{1}{n}}\right)^{-n}\right] \qquad (3)$$

209 In Eq. 3, K and n are empirical constants that determine the shape of the relationship. The

210 values of K and n were optimized using the Solver function in Microsoft Excel to minimize

211 the residual standard deviation (RSD) between the modelled and measured values of %Cu$_{E-}$

212 HA. The main feature of this equation is that it successfully describes an initially steep
213 increase in the value of %Cu_{E-HA}, from zero, followed by sharp change in slope and a very
214 slow continuous increase with time (t) thereafter, reaching a final asymptote of 100% at
215 infinite time (100% to 6 decimal places at 10^{200} min).

3. Results and discussion

3.1 General characterization of Cu on dissolved humic acid

218 Chromatograms of the two non-humic complexes of Cu (EDTA and Tris) used in this study
219 are shown in Fig. 1. The EDTA complex emerged at about 16 min and consisted of a single
220 sharp peak with minor shoulders suggesting minimal Cu re-distribution due to Tris-Cu
221 complex formation in the eluent. The Tris-Cu complex peak emerged at about 19 min and,
222 by contrast, was broad with a long tail, suggesting some Cu re-distribution during transition
223 through the column, possibly due to Tris (0.1 M) in the flowing eluent.

224

225 Chromatograms showing both HAs (G-HA and P-HA) incubated with Cu for 40 days, eluted
226 in a Tris buffer, are shown in Fig. 2. The distribution of Cu between HA and Tris complexes
227 is reasonably clear, with larger organic species having a shorter residence time. The first
228 sharp peak (c. 7 min) is the excluded fraction. Although this peak probably consists mainly
229 of HA-Cu complexes larger than 300,000 Da it may also include inorganic nanoparticles; this
230 is discussed further in section 3.4. Subsequently, a broad peak (7.5 - 13 min) was seen in all
231 chromatograms reflecting the distribution of molecular masses of the HA but also probably
232 arising from various forms of HA interaction with the column matrix, either involving
233 hydrophobic aromatic functional groups (Asakawa et al., 2011) or charged surfaces, leading
234 to changes in retention time (Chin and Gschwend, 1991; Jackson et al., 2005; Pelekani et al.,
235 1999). Copper complexes were eluted over 20 mins with a diffuse peak at 16-19 mins
236 possibly a result of active Cu-Tris complex formation during elution. A comparison with Fig.
237 1 suggests that Cu-Tris complexes were eluted earlier than was seen in Fig. 1 – although still
238 mainly after the Cu-HA complex elution. This is not unexpected because the origin of Cu-
239 Tris complexes in Fig. 2 must have been labile Cu stripped out from the HA during its
240 comparatively rapid transit through the SEC column due to its greater molecular size.
241 McPhail and Goodman (1984) studied a Cu-enzyme-Tris system, analysing the spectra of Cu

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complexes with bovine superoxide dismutase and Cu-Tris complexes. Their results confirmed the presence of Tris-Cu complexes at pH 8.0, causing the authors to advise caution when using Tris as an eluent in chromatographic work. In our application, however, Tris complex formation with Cu is actually 'required' as it enables determination of the isotopic ratio of the labile metal fraction (see Section 3.4). Total Cu recovery was reasonably close to 100% for both G-HA (103%) and P-HA (94%).

3.2   Integration of Cu chromatograms

As shown in Fig. 3, the distinction between HA- and Tris-bonded forms of Cu was quite poor. To calculate the E-value for Cu (Cu_{E-HA}) on HA it was necessary to integrate the ^{65}Cu and ^{63}Cu peaks representing the Cu-Tris complex; from this a labile $^{65}Cu/^{63}Cu$ isotopic ratio could then be calculated to compare with the HA-bound Cu isotopes. To avoid using an arbitrary division between HA-Cu and Tris-Cu a test was undertaken to establish the effect of progressively integrating from both ends of the chromatogram. The G-HA solution was spiked with a ^{65}Cu enriched solution and equilibrated for 72 hours before analysis (Fig. 3). Ten points were selected, spanning the chromatogram between 7 and 23 min. Cumulative integration of the ^{65}Cu and ^{63}Cu signals was undertaken in both directions along the time axis, i.e. from high molecular weight (MW) to low MW (5 min to 25 min), and vice versa, and the isotope ratio $^{65}Cu/^{63}Cu$ for the cumulative signal was calculated. Complete isotopic mixing of the ^{65}Cu spike within 72 h would have produced a $^{65}Cu/^{63}Cu$ ratio of 2.181 (solid horizontal line in Fig. 3). However, when integrating from the high MW end (the start) of the chromatogram isotopic ratios before 13.3 min (mainly HA-bound Cu) were much lower than 2.181. Similarly, when integrating backwards, from the end of the chromatogram, the $^{65}Cu/^{63}Cu$ ratio (Tris complexed forms) was greater than 2.181. There was, therefore, incomplete mixing of the ^{65}Cu spike with Cu held by the dissolved HA which appears to prove the existence of non-labile forms of Cu bound to dissolved humic acid. Thus, only isotopic ratios calculated after 16.3 min, associated with labile Tris-Cu complexes (Rss in Eq. 2), were used in the calculation of Cu_{E-HA} in HA.

3.3 EDTA extraction of Cu on humic acid

Concentrations of weak acid functional groups on HA were 5.5×10^{-3} M and 7.7×10^{-3} M for the G-HA and P-HA solutions respectively calculated from the total acidity as measured by Marshall et al. (1995). Concentrations of EDTA carboxyl groups in the extracting solutions ranged from 2×10^{-3} M (in 0.0005 M EDTA) to 0.2 M (in 0.05 M EDTA). Therefore, it is reasonable to expect that the highest concentrations of EDTA should have enabled complex formation with all labile Cu whereas at the two lowest EDTA concentrations HA should have been a strong competitor for Cu binding. Figure 4 shows ^{63}Cu and ^{65}Cu chromatograms for P-HA in 0.0005 M and 0.05 M EDTA. It should be noted that the (native) ^{63}Cu present originates solely from residual Cu in the 'purified' HA which has either resisted acid dialysis during the preparation of the HA or been adsorbed from distilled water during the extended dialysis against water used to remove residual acid (Marshall et al., 1995). The ^{65}Cu was equilibrated in the HA solutions for 40 days prior to extraction for 2 h with EDTA. A decrease in ICP signal was observed for both isotopes bound to HA, with an increase in the EDTA-bound metal peak, as the EDTA concentration was increased. However, even though it might be expected that 0.05 M EDTA would be able to complex all Cu in the system, a significant proportion of Cu (> 40%), for both residual (e.g. ^{63}Cu) and spiked (^{65}Cu) isotopes, remained bound to both HAs. This provides further evidence of non-labile Cu bound to humic acid. The diffuse peak representing Tris-Cu complexes (c. 15 – 19 min) also diminished, as the EDTA concentration increased. The EDTA-Cu peak shifted to a longer elution time at higher EDTA concentrations which may indicate changes in speciation during elution within the SEC column.

There was a clear increase in %EDTA extractable Cu when EDTA concentration was above 0.01 M (Fig. 5). A significant change in %EDTA-extractable Cu with HA-Cu equilibration time (40 or 160 days) was observed while residual Cu, present within the HA, was least extractable. This indicates that time-dependent fixation of the added metal occurred during incubation (40 and 160 days) and that the residual metal (present in the purified HA) was held in the most stable form. An ageing effect for metals bound to humic substances has also been reported in other studies (Burba, 1994; Rate et al., 1993) and two hypotheses have been proposed to explain the phenomenon. Choppin and Clark (1991) suggested that there was movement of metals from weak binding sites to strong sites when the macromolecular

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304 structure of HA opens as a result of charge repulsion between ionized acidic functional

305 groups enabling the aqueous solute to access otherwise hydrophobic domains within the

306 DOM (McCarthy et al., 1989). An alternative theory, known as the 'thermodynamic'

307 hypothesis (Cabaniss, 1990; Rate et al., 1993) is that it simply takes time for metals such as

308 Cu, Zn and Pb to migrate to the strongest binding sites while being effectively impeded by

309 (temporary) binding to weaker sites.

310 For both types of HA, Cu was strongly bound, with only 60% extracted in 0.05 M EDTA. It

311 has been suggested that Cu binding to soil HA is stronger than to peat HA (Sahu and

312 Banerjee, 1990), but the results in Fig. 5 suggest the reverse. However, this may be a

313 consequence of the lower concentrations of residual Cu in P-HA (21.6 mg kg^{-1}) than that in

314 G-HA (91.0 mg kg^{-1}), perhaps resulting in greater availability of strong binding sites in P-HA.

315

316 **3.4   Isotopically exchangeable Cu on humic acid**

317 Chromatograms of ^{63}Cu and ^{65}Cu in G-HA and P-HA solutions are shown in Fig. 6a; the

318 ^{63}Cu-enriched HA solutions were incubated for 40 days before spiking with ^{65}Cu and

319 equilibrating for a further 3 days. For both HAs the distribution of the spiked ^{65}Cu appeared

320 to coincide with that of the ^{63}Cu.   Closer investigation by comparing the isotopic ratio

321 (^{65}Cu/^{63}Cu) in three regions of the HA chromatograms that were free from the influence of

322 Tris complexes (6 - 7.5 min; 7.5 - 8.6 min and 8.6 - 10.1 min) confirmed that there was no

323 significant difference between the three fractions (P > 0.1). Comparison of isotopic ratios in

324 the same three sections of chromatograms for non-spiked samples also showed no change.

325 This suggests that (i) added Cu was distributed across the HA molecular size range in the

326 same proportion as native Cu and (ii) there was no confounding effect of metal spiking such

327 as conformational changes in the humic structure. One of the regions within the HA section

328 of the chromatogram (6 − 7.5 min) includes the excluded fraction (first sharp peak in Fig. 6a)

329 which may include inorganic nanoparticles along with large MW HA molecules. We tested

330 this by comparing the Cu isotopic ratio ^{65}Cu/^{63}Cu and found that it was very similar in both

331 the excluded and non-excluded sections of the chromatograph. However, we also measured

332 Pb and Zn (data not shown) and found that there was a significant change in the elemental

333 ratios (Pb/Cu and Zn/Cu) between the excluded and non-excluded sections suggesting metal

334 binding particles other than HA. Thus it appears that while there may be inorganic

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335   nanoparticles (containing Zn and Pb) in the excluded fraction there is also HA and the Cu is
336   predominantly held in the latter.

337

338   For both G-HA and P-HA, Eq. 1 described the distribution of the ^{65}Cu spike between HA and
339   Tris complexes very closely (Fig. 6b). The optimized constants in Eq. 1 for G-HA were P(1)
340   = 0.86, P(M) = 0.48, n = 0.29 and for P-HA they were P(1) = 0.99, P(M) = 0.21, n = 0.17.
341   Changes in the Cu isotopic ratio of Tris complexes and HAs, as a function of time after
342   spiking with ^{65}Cu, are shown in Fig. 6c. The difference between the ratio of ^{65}Cu/^{63}Cu in Tris
343   complexes and that in the HA phase of the chromatogram implies the existence of non-labile
344   Cu in HA. The flat trends of both curves after 24 hr suggest a progressively slower approach
345   to an eventual equilibrium. Isotopic ratios for Tris-complexed Cu were used to calculate $Cu_{E\text{-}}$
346    $_{HA}$ according to Eq. 2. Lability (%$Cu_{E\text{-}HA}$) was then calculated by dividing $Cu_{E\text{-}HA}$ (mg kg^{-1})
347   by total Cu concentration measured after SEC elution. Values of %$Cu_{E\text{-}HA}$ after 2-3 days (48-
348   72 hours) were very close to the value of %EDTA extractable measured in 0.05 M EDTA
349   extracts of the HAs spiked with ^{65}Cu and incubated for 40 days (Fig. 5). The lability of Cu in
350   G-HA was slightly greater than that in P-HA. Values of %$Cu_{E\text{-}HA}$ increased with time (Fig. 7)
351   suggesting the spike isotope gradually accessed more binding sites within the HA structure.
352   The increase in %$Cu_{E\text{-}HA}$ in the first hour was approximately 20% for G-HA and 10% for P-
353   HA. There was then a gradual increase in the apparent E-value, implying a slow reaction with
354   stronger binding sites, tending eventually towards 100% at infinite time when theoretically all
355   the spiked isotopes were fully mixed with the incubated metals. A visual appraisal of Fig. 7
356   suggests a reasonably stable value of %$Cu_{E\text{-}HA}$ was obtained >24 hr after spiking. Although
357   the fitted trend line (Eq. 3) eventually reaches an asymptote at 100%, the data suggests a clear
358   distinction between labile and non-labile forms of HA-bound Cu. Therefore, to avoid fixation
359   of isotopes on HAs, whilst allowing sufficient time for metal to access all isotopically
360   exchangeable binding sites, it appears prudent to use an equilibrium time of 24 to 48 hr to
361   determine apparent $Cu_{E\text{-}HA}$. The optimized values of K and n in Eq. 3 are presented in Table 1.

362   It is reasonable to ask whether Cu-Tris complex formation might involve loss of 'non-labile'
363   Cu from the HA - rather than only labile forms, on which the analysis and interpretation
364   presented depends. A comparison with EDTA is useful in this context. A study by Nagaj et
365   al., (2013) cites a range of published (log$_{10}$) stability complexes for the 1:1 Cu-Tris complex,
366   ranging from 3.8 – 5.8. These contrast markedly with the (much larger) Cu-EDTA stability

367 constant ($\log_{10}K = 18.8$) and Fig. 1 shows little or no evidence of Cu-EDTA dissociation by
368 Tris in the eluent – ie there is a single sharp Cu-EDTA peak. That suggests that Tris cannot
369 extract Cu from the EDTA complex. Turning to EDTA extraction of Cu from HA (Fig. 5)
370 there was a remarkably flat response in %Cu extracted with increase in EDTA concentration -
371 even up to an EDTA concentration of 0.05 M. That suggests that even EDTA cannot easily
372 extract non-labile Cu from HA – and so we suggest that Tris is very unlikely to have much
373 impact on non-labile HA-Cu complexes. We think this provides some evidence that the non-
374 labile Cu phase in the HA was largely intact in the presence of Tris and this validates the
375 calculation of E-values.

376

377 **3.5   Implications for geochemical modelling of humic binding of Cu**

378 Geochemical models, such as WHAM VII and NICA-Donnan, describe cation and proton
379 binding to humic substances. They are parameterized from large datasets (248 for Model VII,
380 Tipping et al., 2011). Most of these data originate from potentiometric studies of humic and
381 fulvic acids, titrated over quite short periods of time with metal salts; typically less than 1
382 hour is allowed for equilibration. Thus the formation of non-labile metal complexes is not
383 generally considered (e.g. Benedetti et al., 1996; Christl and Krnetzschmar, 2001; Fitch et al.,
384 1986). A model parameterised in this way may reliably predict HA-metal binding over the
385 short term but is at risk of underestimating the degree of organic complex formation in field-
386 collected samples of river water or soil solutions, in which non-labile humic-bound metal is
387 likely to be present. In this context, the models may over-predict the free ion activity and
388 under-estimate the organic bound fraction. Only a few studies have investigated how the
389 presence of a non-labile humic-bound fraction may affect model predictions of speciation in
390 natural water. Weng et al. (2002) applied WHAM Model VI to predict metal complexation
391 with DOM from total trace metal concentration in soil solution. They observed
392 overestimation of free metal ion activity and this was more significant for Cu and Pb than for
393 Ni, Zn and Cd. By contrast, discrepancies are not likely to be observed when measuring and
394 modelling a system in which organic matter has been purified and trace metals freshly added.
395 For example, Cheng and Allen (2006) titrated 'purified' natural organic matter from lake and
396 river water with Zn, in which the residual trace metals were negligible compared to the
397 amount that was added, and found good agreement between the measured and modelled
398 (WHAM VI) estimates of free Zn ion activity in pH neutral samples.

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399 It is difficult to assess the extent to which a non-labile humic-bound metal fraction would

400 affect model prediction generally as other factors are likely to influence the prediction of free

401 metal ion activity.   There is still uncertainty in measuring free ion activity at low

402 concentrations by Donnan or resin exchange techniques (Degryse et al., 2009) or using ISEs

403 (Fish and Brassard, 1997; Westall et al., 1979). There is also likely to be an effect of pH on

404 the proportions of labile and non-labile metal, as widely found for soils, and the proportion of

405 metal fixed will probably depend on its level of occupancy of available HA groups. WHAM

406 VII allows for a small number of very strongly bonding tri-dentate group configurations

407 which are required to explain strong metal binding observed at low concentrations. Tipping

408 (1998) estimated that the proportion of groups on HA forming tridentate complexes was only

409 0.065 and within the model only 0.9% of these form the strongest category of complexes with

410 metals. With a default (WHAM VII) site density on HA of 3.4×10^{-3} mol g^{-1}, the number of

411 tridentate sites forming the strongest bonds is therefore estimated as 2×10^{-6} mol g^{-1}. The

412 concentrations of non-labile Cu measured were 1.1×10^{-6} and 0.6×10^{-6} mol g^{-1} for G-HA

413 and P-HA respectively. Of course, it is not possible to demonstrate a causal association

414 between these measured values and what is a fitted model binding capacity term. It must also

415 be remembered that the high affinity tridentate sites postulated within the WHAM VII model

416 are also implicitly regarded as reversible (labile).

417 **4. Conclusions**

418 Time-dependent non-labile Cu binding to dissolved humic acids from grassland and peat soils

419 was observed using EDTA extraction and isotopic dilution following speciation by LC-ICP-

420 MS. Results indicated that the proportion of non-labile Cu on HAs can be 40-50% within the

421 range of metal amendments used. It is difficult to draw general conclusions regarding the

422 likely impact of non-labile metal on geochemical speciation model predictions. Deviations

423 from model predictions due solely to this factor will depend critically on the history of HA-

424 metal contact, speciation-controlling factors such as pH and the ratio of metal to humic

425 binding capacity in the system under study. However, it is suggested that the assumption

426 made in geochemical speciation models that all metals bound to dissolved humic acid are

427 labile (reversibly bound), needs refinement and testing. It would also be interesting to extend

428 the approach used here to include fulvic acid which is probably more active in determining

429 trace metal mobility but may differ from HA in its capacity to bind Cu in a non-labile form.

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431 **ACKNOWLEDGEMENTS:**

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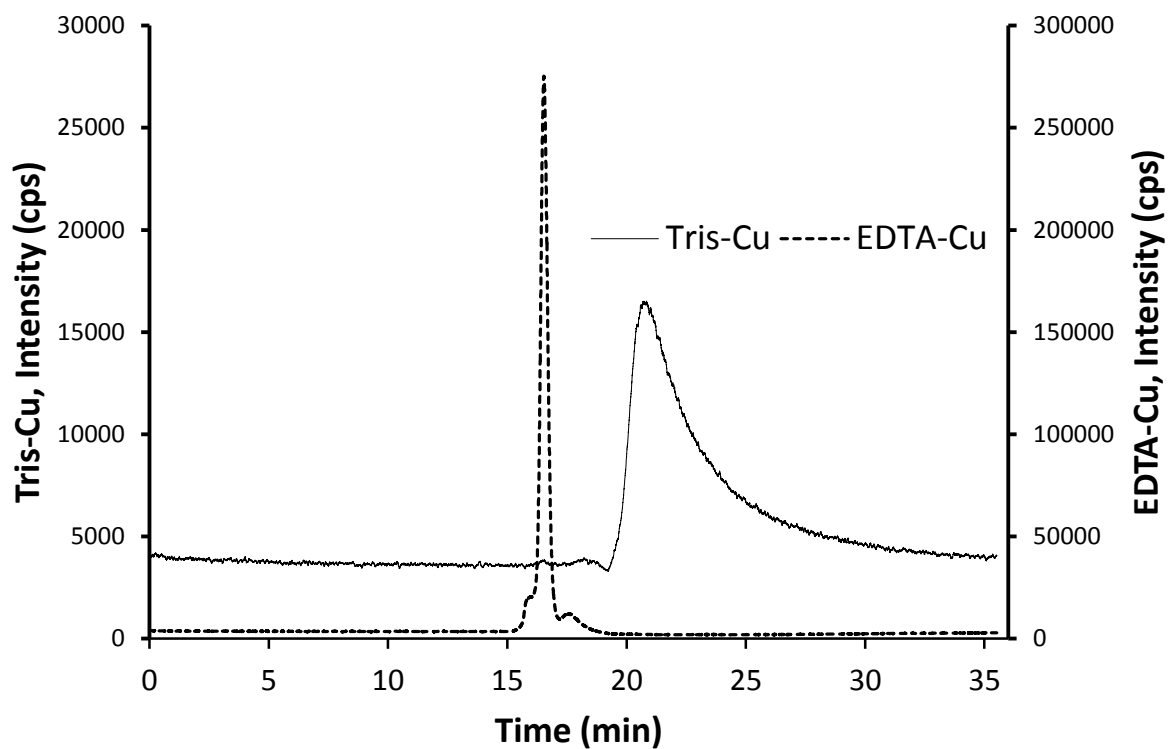539 **Table 1:** Values of the parameterized model constants and RSD for Equations 1 and 3

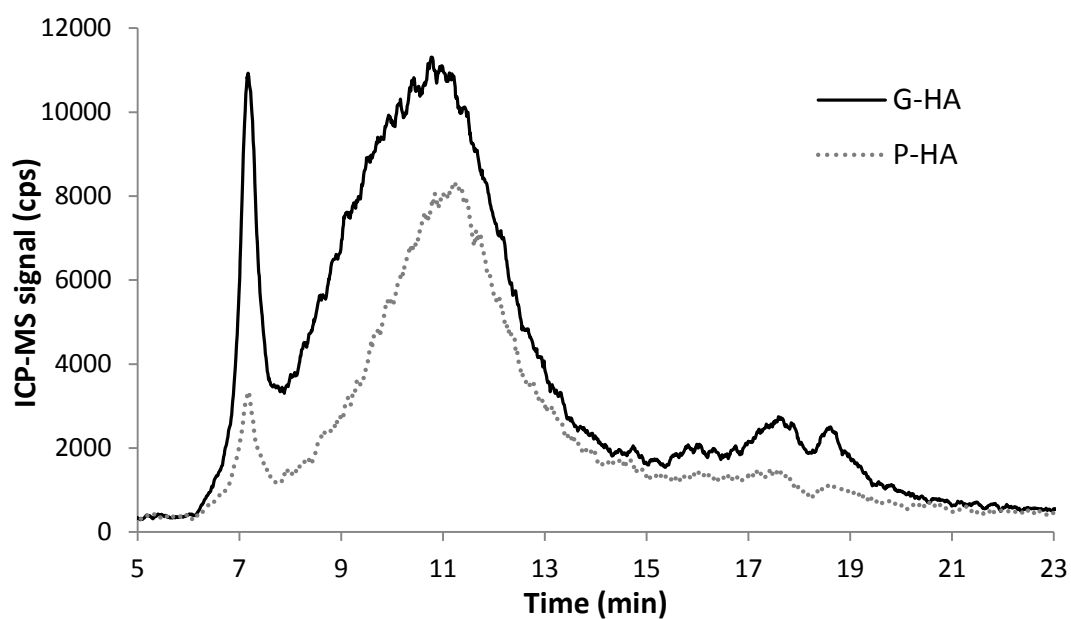| Eq. 1	G-HA	P-HA	Eq. 3	G-HA	P-HA
$P_{(1)}$	0.86	0.99	K	0.762	0.987
$P_{(M)}$	0.48	0.21	n	0.073	0.093
n	0.29	0.17			
RSD(prop.)	0.009	0.005	RSD(%)	4.83	1.52

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547 Figure 1. SEC-ICP-MS chromatograms of Tris-Cu (solid line) and EDTA-Cu (broken line)

548 complexes (0.1 M Tris eluent at pH 8.2). Samples containing Cu (25 µg L^{-1}) in 0.075 M Tris

549 or 0.0125 M $(NH_4)_2$-EDTA were equilibrated for 72 hr prior to analysis.

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553 **Figure 2**: SEC-ICP-MS chromatograms of ^{63}Cu showing complex formation with grassland
554 soil humic acid (G-HA; 1.0 g L^{-1}) and peat humic acid (P-HA; 1.1 g L^{-1}) at pH 7.0. Both
555 humic acids were incubated with Cu (natural isotopic abundance) for 40 days. The final Cu
556 concentrations were 151 mg kg^{-1} and 81.6 mg kg^{-1} for G-HA and P-HA respectively.

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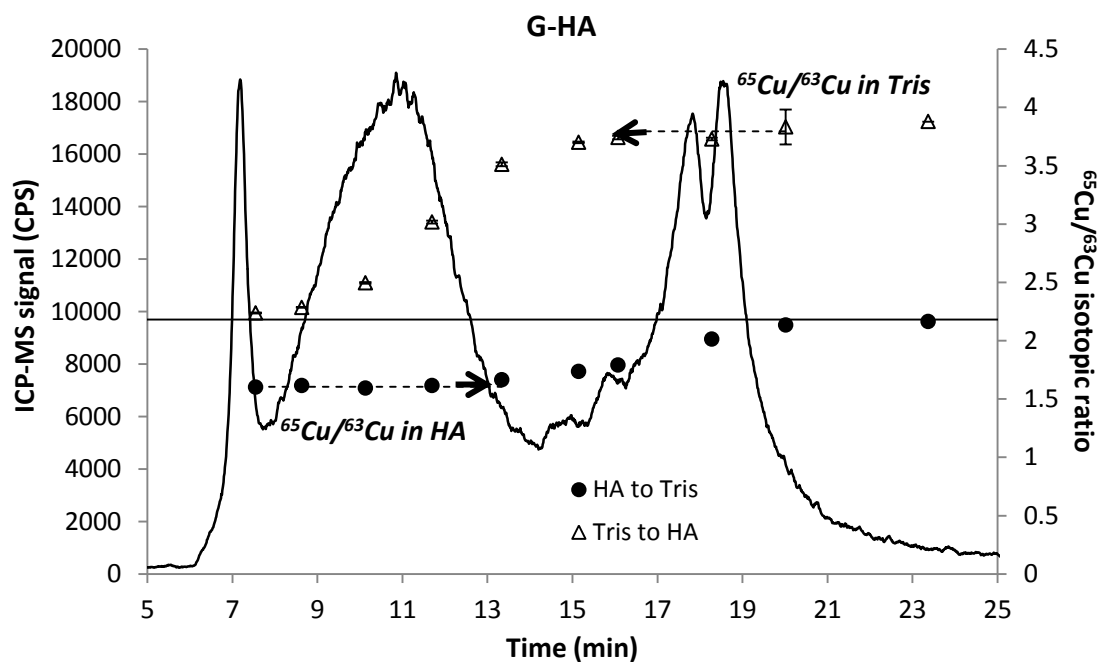
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561 **Figure 3**: A ^{65}Cu chromatogram of G-HA solution (pH 7.0) 72 hours after spiking with ^{65}Cu
562 (solid line). The isotopic ratio ^{65}Cu/^{63}Cu measured using different integration times is shown
563 where chromatograms were progressively integrated from left (solely HA) to right (●), or
564 from the right hand side (solely Tris-Cu complexes) to left (Δ); arrows indicate the direction
565 of integration. The broken lines represent the isotopic ratios for the HA-Cu and Tris-Cu
566 complexes. Error bars are the standard error of duplicates.

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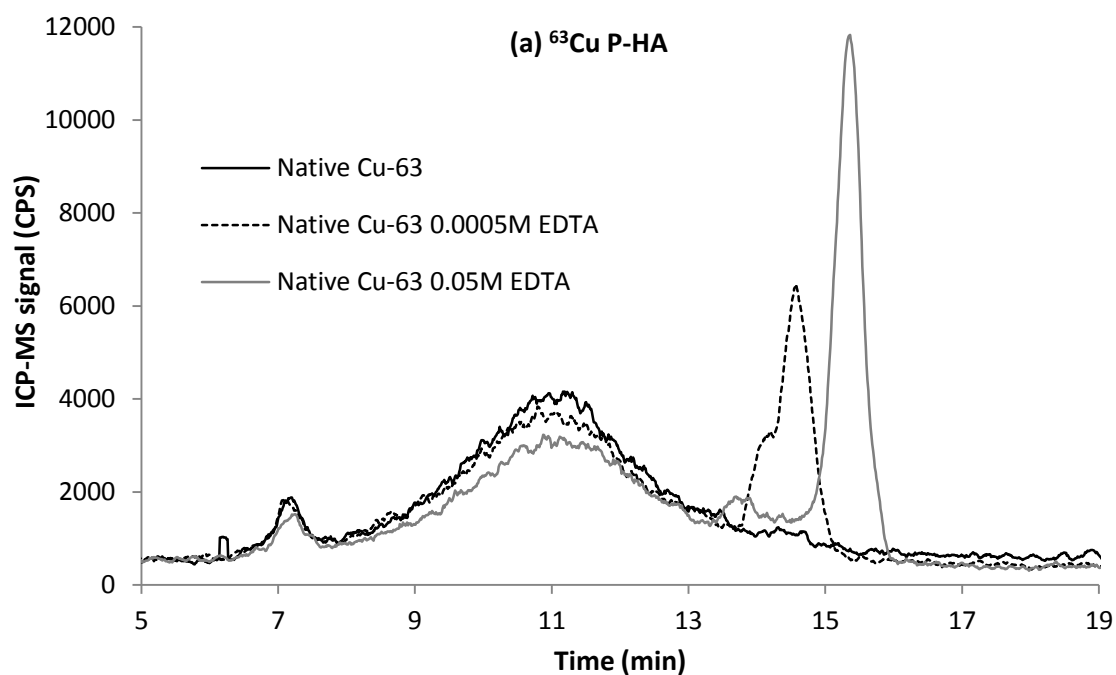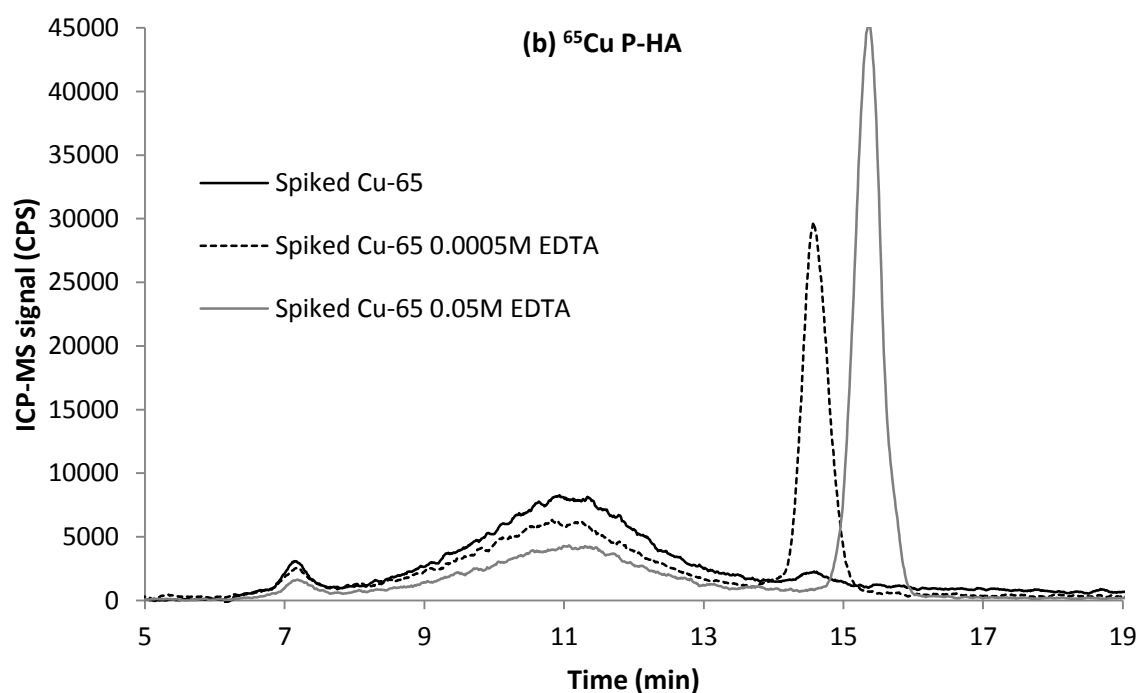
Figure 4: SEC-ICP-MS chromatograms of residual ^{63}Cu (a) and spiked ^{65}Cu (b) in peat humic acid (P-HA; 1.1 g L^{-1}) solutions (pH 7.0) at three EDTA concentrations (0.0, 5x10^{-4}, 5x10^{-2} M). The HA was incubated with an enriched ^{65}Cu spike for 40 days before EDTA was added. Chromatograms were run two hours after EDTA addition.

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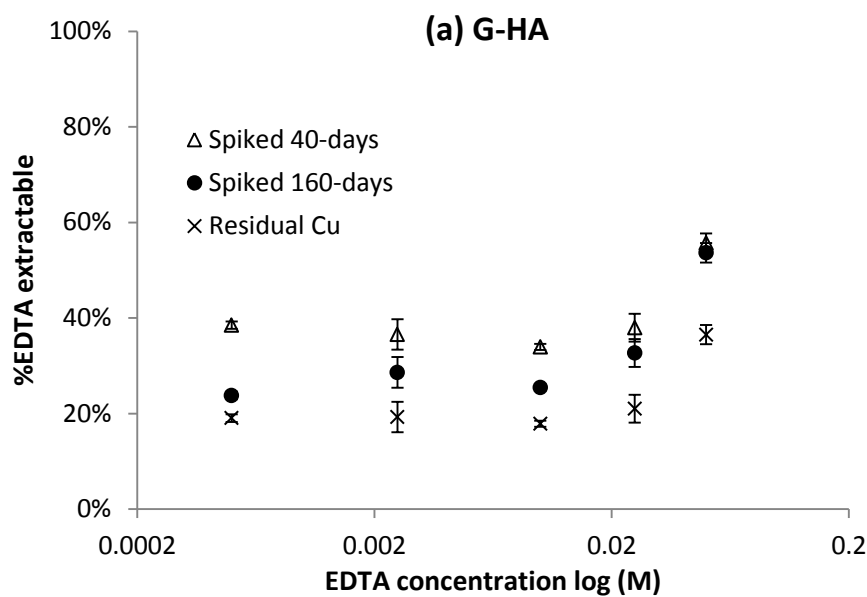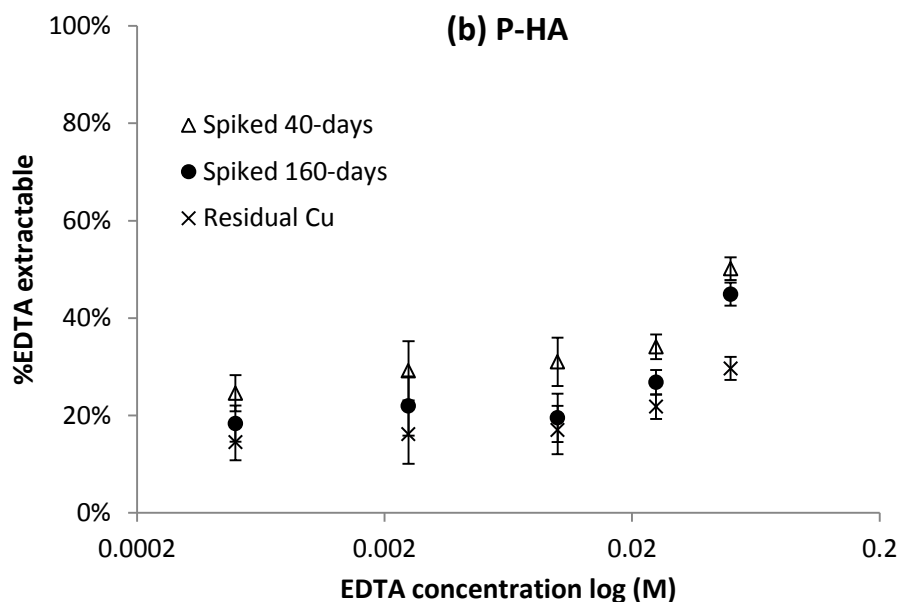
Figure 5: Proportion of Cu extracted by EDTA from (a) G-HA and (b) P-HA humic acids as a function of EDTA concentration (M; $\log_{10}$ scale). Data is shown for 'residual' metal (x) present in the purified HA and spiked treatment (^{65}Cu) incubated with HA for 40 d ($\triangle$) and 160 d ($\bullet$) (Error bars are the estimated standard error between duplicates for the native ^{63}Cu isotope measured in spiked 40-days and 160-days samples.

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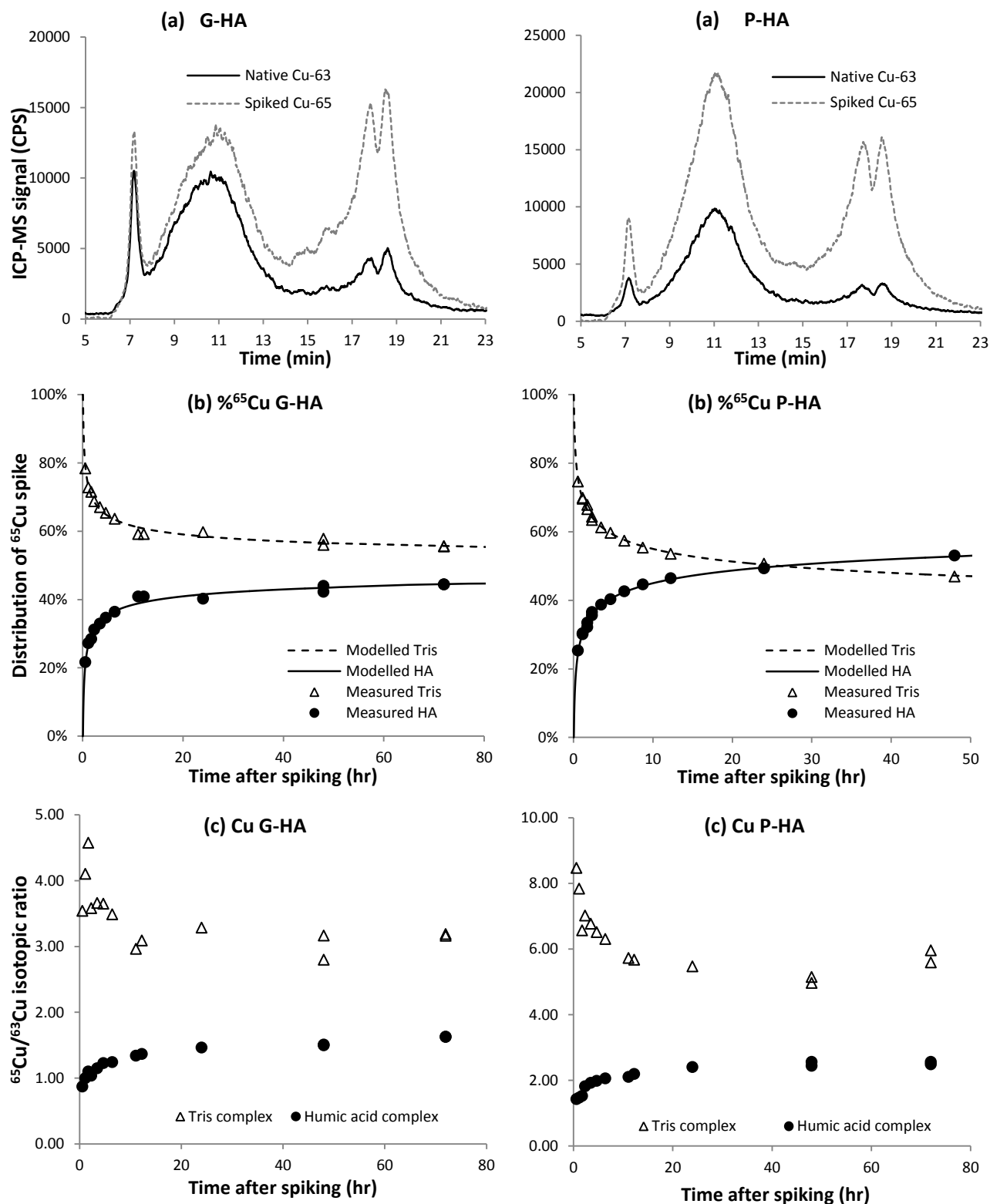
586 **Figure 6**: (a) SEC-ICP-MS chromatograms of spiked [65]Cu and incubated [63]Cu on grassland
587 soil humic acid (G-HA; 72 hours after spiking) and for peat humic acid (P-HA; 48 hours after
588 spiking). The humic acids were previously incubated with mixed metal standards for 40 days.
589 (b) Proportions of [65]Cu-humic acid (●) and Tris (Δ) complexes in whole [65]Cu chromatogram
590 as a function of time after spiking; model lines are from Equation 1. (c) Isotopic ratio of
591 [65]Cu/[63]Cu on humic acid (●) and Tris (Δ) complexes as a function of time after spiking.

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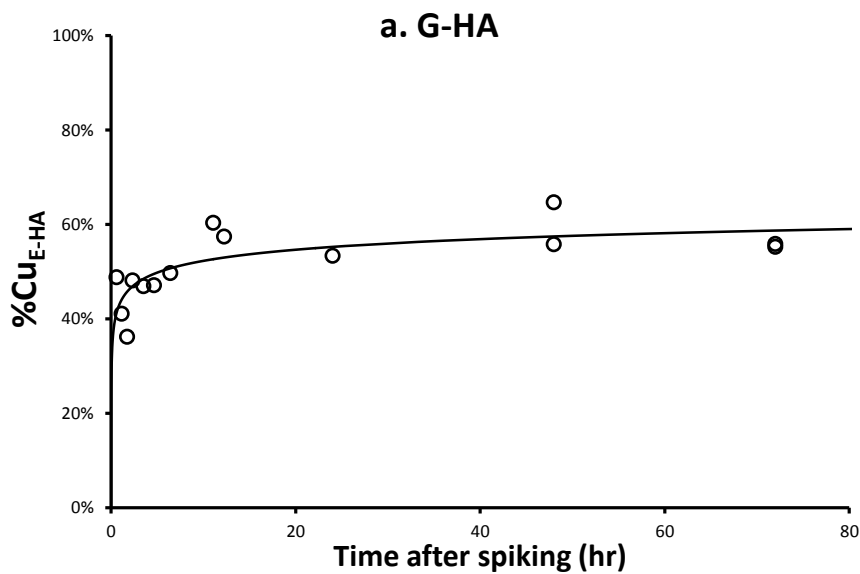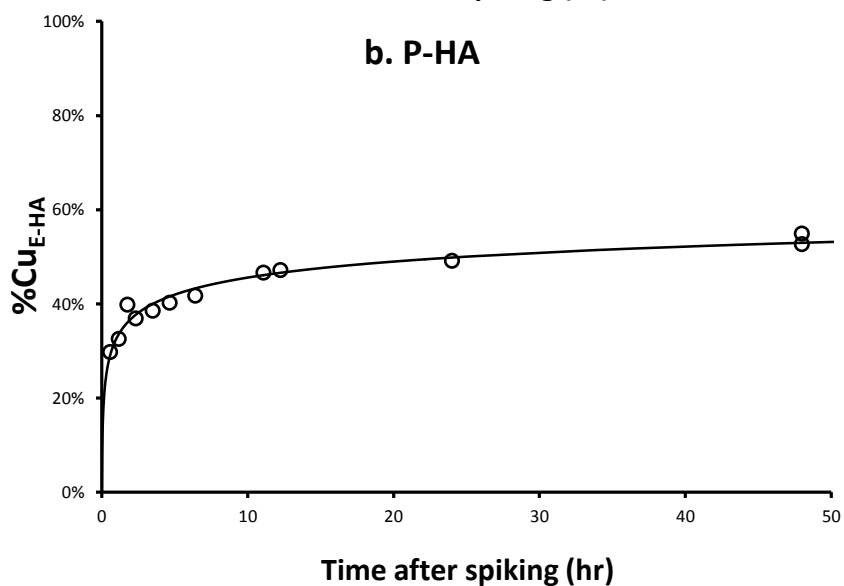
Figure 7: Values of $\%Cu_{E-HA}$ on (a) grassland soil (G-HA) and (b) peat (P-HA) HAs as a function of spike isotope equilibration time. The solid lines are modelled values of $\%Cu_{E-HA}$, predicted from a parameterized version of Eq. 3, as a function of time. For G-HA, K = 0.762, n = 0.073 (RSD = 4.8%); for P-HA, K = 0.987, n = 0.093 (RSD = 1.52%).

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