Lability of copper bound to humic acid

Lingchen MAO, Scott D. YOUNG* & Elizabeth H. BAILEY

Affiliation address: Division of Agricultural and Environmental Sciences, School of Biosciences, University of Nottingham, Sutton Bonington Campus, Loughborough, Leicestershire LE12 5RD, United Kingdom

**Corresponding Author*

Scott Young: scott.young@nottingham.ac.uk; Tel. +44(0)115 9516256

Postal Address: Gateway Building, Sutton Bonington Campus, Loughborough, Leicestershire, LE12 5RD, United Kingdom

Lingchen Mao: lingchen_mao@163.com

Liz Bailey: liz.bailey@nottingham.ac.uk

Mao, L., Young, S.D. and Bailey, E.H. 2015. Lability of copper bound to humic acid. Chemosphere, 131, 201 - 208.
doi:10.1016/j.chemosphere.2015.03.035

HIGHLIGHTS:

- Isotopic dilution was used to assess the lability of Cu bound to humic acids
- Time-dependent Cu fixation by humic acid was observed over 160 days
- Around 40% of Cu bound to HA may be non-labile.
- Non-labile Cu in humic acids may affect predicted speciation by geochemical models

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.

Keywords

Humic acid, Non-labile metal, WHAM model, Isotopic dilution, EDTA extraction, SEC-ICP-MS

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 degree. By contrast Laborda et al. (2011), used asymmetrical flow field flow fractionation (AsFIFFF) to separate dispersed colloidal phases of isotopically spiked compost material and, from measurements of the isotopic ratio within different size fractions including the free inorganic phase, they concluded that all Cu and Pb present was isotopically exchangeable .

It is accepted that metals can be held by very strong binding sites on humic substances through the formation of ‘multidentate’ complexes with carboxylic and phenolic groups or by specific binding with N and S groups which are known to preferentially bind metals such as Cr^{III}, Fe^{III}, Cu and Hg (Tipping, 2002). However, strong chemical bond formation does not necessarily result in non-labile complex formation and there have been very few studies that have attempted to determine the presence of such a metal fraction on dissolved humic substances. It is well established that isotopic dilution (ID) approaches and some chemical extractants (eg 0.05 M EDTA) can be used to estimate the labile fraction of trace metals in soils (Degryse et al., 2009; Gabler et al., 2007). However, to measure metal lability on a dissolved system containing a range of metal complex species requires a fractionation technique to separate high MW DOM-bound metal from low MW organic and inorganic complexes. This approach then also allows fractionation of spike isotopes introduced to follow the kinetics of metal assimilation throughout the various species and quantification of the isotopically exchangeable forms present. Size exclusion chromatography (SEC) coupled with inductively coupled plasma mass spectrometry (ICP-MS) has been applied to the study of trace element distributions within humic acids (Jackson et al., 2005; Kozai et al., 2013; Liu and Cai, 2010; Liu et al., 2011; Schmitt et al., 2001).

The aim of this investigation was to determine the lability of Cu bound to dissolved humic acid (HA) to test the hypothesis that Cu bound to dissolved HA is labile. To achieve this, two popular techniques for measuring labile metals in soils were modified and applied to two types of HAs fractionated using SEC-ICP-MS: extraction with EDTA and determination of isotopic exchangeability. Dissolved HA was incubated with an enriched stable Cu isotope and its subsequent extractability with EDTA determined from differences in isotopic abundance between forms bound to HAs and to low MW complexes with EDTA. Humic acid solutions were incubated with Cu (normal isotopic mix) for a period of time before they were spiked with an enriched stable Cu isotope. The proportion of dissolved HA-bound metal that was isotopically exchangeable (% *E-values*; Smolders et al., 1999; Young et al., 2000) was then determined from changes in isotope distribution between Cu complexes with HAs and the Tris buffer (Tris (hydroxymethyl) aminomethane) used as the chromatography eluent.

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

2.3 Determination of Cu extractable by EDTA from humic acid

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

2.4 Isotopically exchangeable Cu

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

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

2.5 Calibration and recovery of SEC-ICP-MS chromatograms

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

2.6 Time-dependent Cu isotope adsorption by humic acid

The extent of movement of spike isotope from the non-HA phase (mainly Tris complexes) into HA-complexed forms, as a function of contact time, was modelled using an adapted equation originally developed to describe the degree of mixing of radioactive isotopes with total soil metal pools (Sinaj et al., 1999), Eq.1.

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

In this application P is the proportion of spiked ^{65}Cu in the non-humic section of the chromatogram (Tris complexes) at a specified time (t) or after 1 min (1), and n is an empirical constant; $P_{(M)}$ is the value of $P_{(t)}$ at infinite time. The constants $P_{(1)}$, $P_{(M)}$ and n were optimized by minimizing the residual standard deviation (RSD) between the measured and modelled values of $P_{(t)}$ using the *Solver* function in *Microsoft Excel.*

2.7 Calculation and prediction of Cu E-values

The isotopic ratio of Tris-complexed Cu was used to calculate E-values in the presence of humic acid ($Cu_{E\text{-}HA}$). The calculation was adapted from Gabler et al. (1999), and is shown in Eq. 2 where ^{65}Cu is used as the spike isotope and ^{63}Cu is solely from native sources:

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

In Eq. 2 Cu_{HA} and Cu_{spk} is the average atomic mass of Cu in the (unspiked) HA solution and the enriched ^{65}Cu spike solution respectively, W is the weight of humic acid (kg), C_{spk} is the gravimetric concentration of Cu in the spike solution, V_{spk} is the volume of spike added (L), IA is the molar isotopic abundance of ^{65}Cu or ^{63}Cu in the spike solution or unspiked HA solution, and R_{ss} is the isotopic ratio of ^{65}Cu to ^{63}Cu in the spiked HA solution measured in the ‘non-humic’ section of the chromatogram.

The change in $\%Cu_{E\text{-}HA}$ with time, following addition of the spike, was described by an empirical equation (Eq. 3) adapted from Eq.1.

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

In Eq. 3, K and n are empirical constants that determine the shape of the relationship. The values of K and n were optimized using the *Solver* function in *Microsoft Excel* to minimize the residual standard deviation (RSD) between the modelled and measured values of $\%Cu_{E\text{-}}$

$_{HA}$. The main feature of this equation is that it successfully describes an initially steep increase in the value of %$Cu_{E\text{-}HA}$, from zero, followed by sharp change in slope and a very slow continuous increase with time (t) thereafter, reaching a final asymptote of 100% at 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

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

Chromatograms showing both HAs (G-HA and P-HA) incubated with Cu for 40 days, eluted in a Tris buffer, are shown in Fig. 2. The distribution of Cu between HA and Tris complexes is reasonably clear, with larger organic species having a shorter residence time. The first sharp peak (c. 7 min) is the excluded fraction. Although this peak probably consists mainly of HA-Cu complexes larger than 300,000 Da it may also include inorganic nanoparticles; this is discussed further in section 3.4. Subsequently, a broad peak (7.5 - 13 min) was seen in all chromatograms reflecting the distribution of molecular masses of the HA but also probably arising from various forms of HA interaction with the column matrix, either involving hydrophobic aromatic functional groups (Asakawa et al., 2011) or charged surfaces, leading to changes in retention time (Chin and Gschwend, 1991; Jackson et al., 2005; Pelekani et al., 1999). Copper complexes were eluted over 20 mins with a diffuse peak at 16-19 mins possibly a result of active Cu-Tris complex formation during elution. A comparison with Fig. 1 suggests that Cu-Tris complexes were eluted earlier than was seen in Fig. 1 – although still mainly after the Cu-HA complex elution. This is not unexpected because the origin of Cu-Tris complexes in Fig. 2 must have been labile Cu stripped out from the HA during its comparatively rapid transit through the SEC column due to its greater molecular size. McPhail and Goodman (1984) studied a Cu-enzyme-Tris system, analysing the spectra of Cu 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 ($Cu_{E\text{-}HA}$) 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 $Cu_{E\text{-}HA}$ 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 structure of HA opens as a result of charge repulsion between ionized acidic functional groups enabling the aqueous solute to access otherwise hydrophobic domains within the DOM (McCarthy et al., 1989). An alternative theory, known as the ‘thermodynamic’ hypothesis (Cabaniss, 1990; Rate et al., 1993) is that it simply takes time for metals such as Cu, Zn and Pb to migrate to the strongest binding sites while being effectively impeded by (temporary) binding to weaker sites.

For both types of HA, Cu was strongly bound, with only 60% extracted in 0.05 M EDTA. It has been suggested that Cu binding to soil HA is stronger than to peat HA (Sahu and Banerjee, 1990), but the results in Fig. 5 suggest the reverse. However, this may be a consequence of the lower concentrations of residual Cu in P-HA (21.6 mg kg^{-1}) than that in G-HA (91.0 mg kg^{-1}), perhaps resulting in greater availability of strong binding sites in P-HA.

3.4 Isotopically exchangeable Cu on humic acid

Chromatograms of ^{63}Cu and ^{65}Cu in G-HA and P-HA solutions are shown in Fig. 6a; the ^{63}Cu-enriched HA solutions were incubated for 40 days before spiking with ^{65}Cu and equilibrating for a further 3 days. For both HAs the distribution of the spiked ^{65}Cu appeared to coincide with that of the ^{63}Cu. Closer investigation by comparing the isotopic ratio (^{65}Cu/^{63}Cu) in three regions of the HA chromatograms that were free from the influence of Tris complexes (6 - 7.5 min; 7.5 - 8.6 min and 8.6 - 10.1 min) confirmed that there was no significant difference between the three fractions ($P > 0.1$). Comparison of isotopic ratios in the same three sections of chromatograms for non-spiked samples also showed no change. This suggests that (i) added Cu was distributed across the HA molecular size range in the same proportion as native Cu and (ii) there was no confounding effect of metal spiking such as conformational changes in the humic structure. One of the regions within the HA section of the chromatogram (6 – 7.5 min) includes the excluded fraction (first sharp peak in Fig. 6a) which may include inorganic nanoparticles along with large MW HA molecules. We tested this by comparing the Cu isotopic ratio ^{65}Cu/^{63}Cu and found that it was very similar in both the excluded and non-excluded sections of the chromatograph. However, we also measured Pb and Zn (data not shown) and found that there was a significant change in the elemental ratios (Pb/Cu and Zn/Cu) between the excluded and non-excluded sections suggesting metal binding particles other than HA. Thus it appears that while there may be inorganic nanoparticles (containing Zn and Pb) in the excluded fraction there is also HA and the Cu is predominantly held in the latter.

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

It is reasonable to ask whether Cu-Tris complex formation might involve loss of ‘non-labile’ Cu from the HA - rather than only labile forms, on which the analysis and interpretation presented depends. A comparison with EDTA is useful in this context. A study by Nagaj et al., (2013) cites a range of published ($\log_{10}$) stability complexes for the 1:1 Cu-Tris complex, ranging from 3.8 – 5.8. These contrast markedly with the (much larger) Cu-EDTA stability constant ($\log_{10}K = 18.8$) and Fig. 1 shows little or no evidence of Cu-EDTA dissociation by Tris in the eluent – ie there is a single sharp Cu-EDTA peak. That suggests that Tris cannot extract Cu from the EDTA complex. Turning to EDTA extraction of Cu from HA (Fig. 5) there was a remarkably flat response in %Cu extracted with increase in EDTA concentration - even up to an EDTA concentration of 0.05 M. That suggests that even EDTA cannot easily extract non-labile Cu from HA – and so we suggest that Tris is very unlikely to have much impact on non-labile HA-Cu complexes. We think this provides some evidence that the non-labile Cu phase in the HA was largely intact in the presence of Tris and this validates the calculation of E-values.

3.5 Implications for geochemical modelling of humic binding of Cu

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

It is difficult to assess the extent to which a non-labile humic-bound metal fraction would affect model prediction generally as other factors are likely to influence the prediction of free metal ion activity. There is still uncertainty in measuring free ion activity at low concentrations by Donnan or resin exchange techniques (Degryse et al., 2009) or using ISEs (Fish and Brassard, 1997; Westall et al., 1979). There is also likely to be an effect of pH on the proportions of labile and non-labile metal, as widely found for soils, and the proportion of metal fixed will probably depend on its level of occupancy of available HA groups. WHAM VII allows for a small number of very strongly bonding tri-dentate group configurations which are required to explain strong metal binding observed at low concentrations. Tipping (1998) estimated that the proportion of groups on HA forming tridentate complexes was only 0.065 and within the model only 0.9% of these form the strongest category of complexes with metals. With a default (WHAM VII) site density on HA of 3.4×10^{-3} mol g^{-1}, the number of tridentate sites forming the strongest bonds is therefore estimated as 2×10^{-6} mol g^{-1}. The concentrations of non-labile Cu measured were 1.1×10^{-6} and 0.6×10^{-6} mol g^{-1} for G-HA and P-HA respectively. Of course, it is not possible to demonstrate a causal association between these measured values and what is a fitted model binding capacity term. It must also be remembered that the high affinity tridentate sites postulated within the WHAM VII model are also implicitly regarded as reversible (labile).

4. Conclusions

Time-dependent non-labile Cu binding to dissolved humic acids from grassland and peat soils was observed using EDTA extraction and isotopic dilution following speciation by LC-ICP-MS. Results indicated that the proportion of non-labile Cu on HAs can be 40-50% within the range of metal amendments used. It is difficult to draw general conclusions regarding the likely impact of non-labile metal on geochemical speciation model predictions. Deviations from model predictions due solely to this factor will depend critically on the history of HA-metal contact, speciation-controlling factors such as pH and the ratio of metal to humic binding capacity in the system under study. However, it is suggested that the assumption made in geochemical speciation models that all metals bound to dissolved humic acid are labile (reversibly bound), needs refinement and testing. It would also be interesting to extend the approach used here to include fulvic acid which is probably more active in determining trace metal mobility but may differ from HA in its capacity to bind Cu in a non-labile form.

ACKNOWLEDGEMENTS:

The authors thank the University of Nottingham for providing research funding.

REFERENCES:

Asakawa, D., Iimura, Y., Kiyota, T., Yanagi, Y., Fujitake, N., 2011. Molecular size fractionation of soil humic acids using preparative high performance size-exclusion chromatography. Journal of Chromatography A 1218, 6448-6453.

Benedetti, M.F., VanRiemsdijk, W.H., Koopal, L.K., Kinniburgh, D.G., Gooddy, D.C., Milne, C.J., 1996. Metal ion binding by natural organic matter: From the model to the field. Geochim. Cosmochim. Acta 60, 2503-2513.

Burba, P., 1994. Labile/inert metal species in aquatic humic substances - an ion-exchange study. Fresenius J. Anal. Chem. 348, 301-311.

Cabaniss, S.E., 1990. pH and ionic-strength effects on nickel fulvic-acid dissociation kinetics. Environ. Sci. Technol. 24, 583-588.

Cheng, T., Allen, H.E., 2006. Comparison of zinc complexation properties of dissolved natural organic matter from different surface waters. Journal of Environmental Management 80, 222-229.

Chin, Y.P., Gschwend, P.M., 1991. The abundance, distribution, and configuration of porewater organic colloids in recent sediments. Geochimica Et Cosmochimica Acta 55, 1309-1317.

Choppin, G.R., Clark, S.B., 1991. The kinetic interactions of metal-ions with humic acids. Mar. Chem. 36, 27-38.

Christla, I., Kretzschmar, R., 2001. Interaction of copper and fulvic acid at the hematite-water interface. Geochim. Cosmochim. Acta 65, 3435-3442.

Degryse, F., Smolders, E., Parker, D.R., 2009. Partitioning of metals (Cd, Co, Cu, Ni, Pb, Zn) in soils: concepts, methodologies, prediction and applications - a review. Eur. J. Soil Sci. 60, 590-612.

Fish, S.J., Brassard, P., 1997. Dialysis Membrane to Prevent Cadmium Ion Specific Electrode fouling. Talanta 44, 939-945.

Fitch, A., Stevenson, F.J., Chen, Y., 1986. Complexation of Cu(II) with a soil humic acid: Response characteristics of the Cu(II) ion-selective electrode and ligand concentration effects. Organic Geochemistry 9, 109-116.

Gabler, H.E., Bahr, A., Heidkamp, A., Utermann, J., 2007. Enriched stable isotopes for determining the isotopically exchangeable element content in soils. Eur. J. Soil Sci. 58, 746-757.

Gabler, H.E., Bahr, A., Mieke, B., 1999. Determination of the interchangeable heavy-metal fraction in soils by isotope dilution mass spectrometry. Fresenius J. Anal. Chem. 365, 409-414.

Jackson, B.P., Ranville, J.F., Bertsch, P.M., Sowder, A.G., 2005. Characterization of colloidal and humic-bound Ni and U in the "dissolved" fraction of contaminated sediment extracts. Environ. Sci. Technol. 39, 2478-2485.

Kinniburgh, D.G., van Riemsdijk, W.H., Koopal, L.K., Borkovec, M., Benedetti, M.F., Avena, M.J., 1999. Ion binding to natural organic matter: competition, heterogeneity, stoichiometry and thermodynamic consistency. Colloids and Surfaces a-Physicochemical and Engineering Aspects 151, 147-166.

Kozai, N., Ohnuki, T., Iwatsuki, T., 2013. Characterization of saline groundwater at Horonobe, Hokkaido, Japan by SEC-UV-ICP-MS: Speciation of uranium and iodine. Water Res. 47, 1570-1584.

Laborda, F., Ruiz-Begueria, S., Bolea, E., Castillo, J.R., 2011. Study of the size-based environmental availability of metals associated to natural organic matter by stable isotope exchange and quadrupole inductively coupled plasma mass spectrometry coupled to asymmetrical flow field flow fractionation. J. Chromatogr. A 1218, 4199-4205.

Liu, G.L., Cai, Y., 2010. Complexation of arsenite with dissolved organic matter Conditional distribution coefficients and apparent stability constants. Chemosphere 81, 890-896.

Liu, G.L., Fernandez, A., Cai, Y., 2011. Complexation of Arsenite with Humic Acid in the Presence of Ferric Iron. Environ. Sci. Technol. 45, 3210-3216.

Lofts, S., Tipping, E., 2011. Assessing WHAM/Model VII against field measurements of free metal ion concentrations: model performance and the role of uncertainty in parameters and inputs. Environ. Chem. 8, 501-516.

Lombi, E., Hamon, R.E., McGrath, S.P., McLaughlin, M.J., 2003. Lability of Cd, Cu, and Zn in polluted soils treated with lime, beringite, and red mud and identification of a non-labile colloidal fraction of metals using isotopic techniques. Environ. Sci. Technol. 37, 979-984.

Ma, Y.B., Lombi, E., Nolan, A.L., McLaughlin, M.J., 2006. Determination of labile Cu in soils and isotopic exchangeability of colloidal Cu complexes. Eur. J. Soil Sci. 57, 147-153.

Marshall, S.J., Young, S.D., Gregson, K., 1995. Humic-acid proton equilibria - a comparison of two models and assessment of titration error. Eur. J. Soil Sci. 46, 471-480.

McCarthy, J.F., Roberson, L.E., Burrus, L.W., 1989. Association of benzo(a)pyrene with dissolved organic matter: Prediction of Kdom from structural and chemical properties of the organic matter. Chemosphere 19, 1911-1920.

McPhail, D.B., Goodman, B.A., 1984. Tris buffer--a case for caution in its use in copper-containing systems. Biochemical Journal 221, 559-560.

Nagaj, J., Stokowa-Sołtys, K., Kurowska, E., Frączyk, T., Jeżowska-Bojczuk, M., Bal, W. 2013. Revised coordination model and stability constants of Cu(II) complexes of Tris buffer. Inorg. Chem. 52, 13927–13933

Nolan, A.L., Ma, Y.B., Lombi, E., McLaughlin, M.J., 2009. Speciation and Isotopic Exchangeability of Nickel in Soil Solution. J. Environ. Qual. 38, 485-492.

Pelekani, C., Newcombe, G., Snoeyink, V.L., Hepplewhite, C., Assemi, S., Beckett, R., 1999. Characterization of natural organic matter using high performance size exclusion chromatography. Environ. Sci. Technol. 33, 2807-2813.

Rate, A.W., McLaren, R.G., Swift, R.S., 1993. Response of copper (II) humic-acid dissociation kinetics to factors influencing complex stability and macromolecular conformation. Environ. Sci. Technol. 27, 1408-1414.

Sahu, S., Banerjee, D.K., 1990. Complexation Properties of Typical Soil and Peat Humic Acids with Copper(II) and Cadmium(II). Int. J. Environ. Anal. Chem. 42, 35-44.

Schmitt, D., Muller, M.B., Frimmel, F.H., 2001. Metal distribution in different size fractions of natural organic matter. Acta Hydrochim. Hydrobiol. 28, 400-410.

Schnitzer, M., Khan, S.U., 1972. Humic substances in the environment. M. Dekker, New York.

Sinaj, S., Machler, F., Frossard, E., 1999. Assessment of isotopically exchangeable zinc in polluted and nonpolluted soils. Soil Sci. Soc. Am. J. 63, 1618-1625.

Smolders, E., Brans, K., Foldi, A., Merckx, R., 1999. Cadmium fixation in soils measured by isotopic dilution. Soil Sci. Soc. Am. J. 63, 78-85.

Tipping, E., 1998. Humic ion-binding model VI: An improved description of the interactions of protons and metal ions with humic substances. Aquat. Geochem. 4, 3-48.

Tipping, E., 2002. Cation binding by humic substances. Cambridge, Cambridge.

Tipping, E., Lofts, S., Sonke, J.E., 2011. Humic Ion-Binding Model VII: a revised parameterisation of cation binding by humic substances. Environ. Chem. 8, 225-235.

Weng, L.P., Temminghoff, E.J.M., Lofts, S., Tipping, E., Van Riemsdijk, W.H., 2002. Complexation with dissolved organic matter and solubility control of heavy metals in a sandy soil. Environ. Sci. Technol. 36, 4804-4810.

Westall, J.C., Morel, F.M.M., Hume, D.N., 1979. Chloride Interference in Cupric Ion Selective Electrode Measurements. Anal. Chem. 51, 1792-1798.

Young, S.D., Tye, A., Carstensen, A., Resende, L., Crout, N., 2000. Methods for determining labile cadmium and zinc in soil. Eur. J. Soil Sci. 51, 129-136.

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

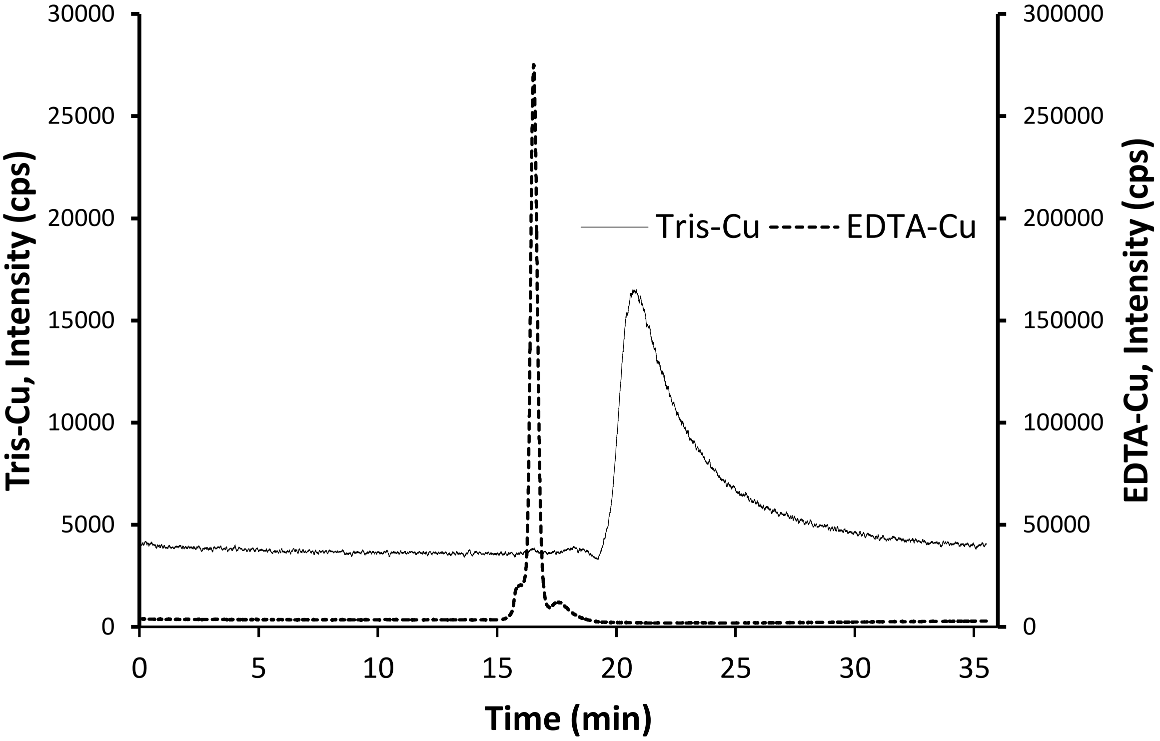


Figure 1. SEC-ICP-MS chromatograms of Tris-Cu (solid line) and EDTA-Cu (broken line) complexes (0.1 M Tris eluent at pH 8.2). Samples containing Cu (25 µg L^{-1}) in 0.075 M Tris or 0.0125 M $(NH_4)_2$-EDTA were equilibrated for 72 hr prior to analysis.

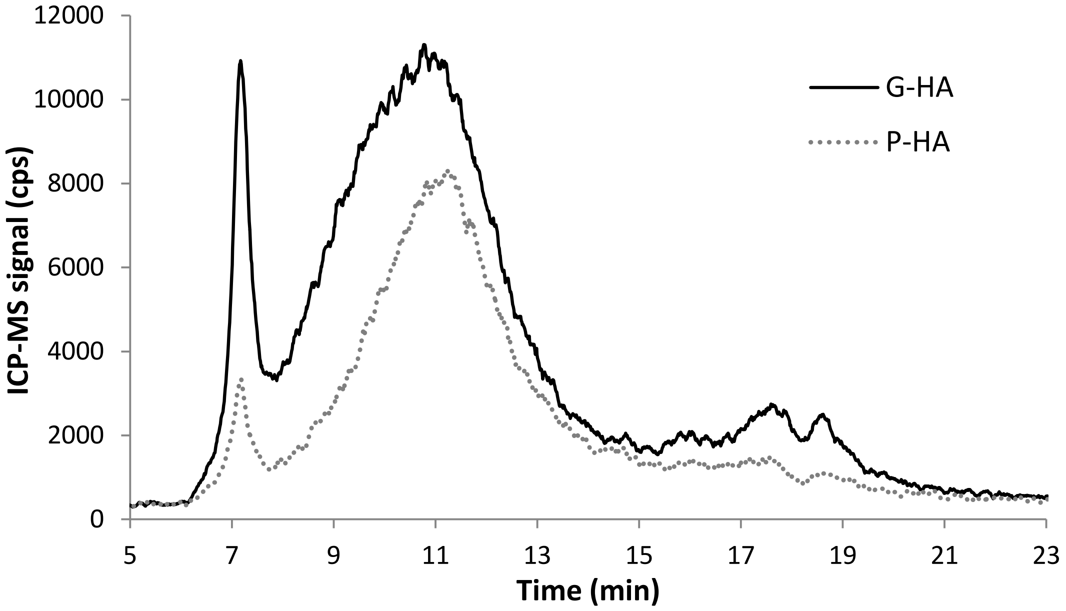


Figure 2: SEC-ICP-MS chromatograms of ^{63}Cu showing complex formation with grassland soil humic acid (G-HA; 1.0 g L^{-1}) and peat humic acid (P-HA; 1.1 g L^{-1}) at pH 7.0. Both humic acids were incubated with Cu (natural isotopic abundance) for 40 days. The final Cu concentrations were 151 mg kg^{-1}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

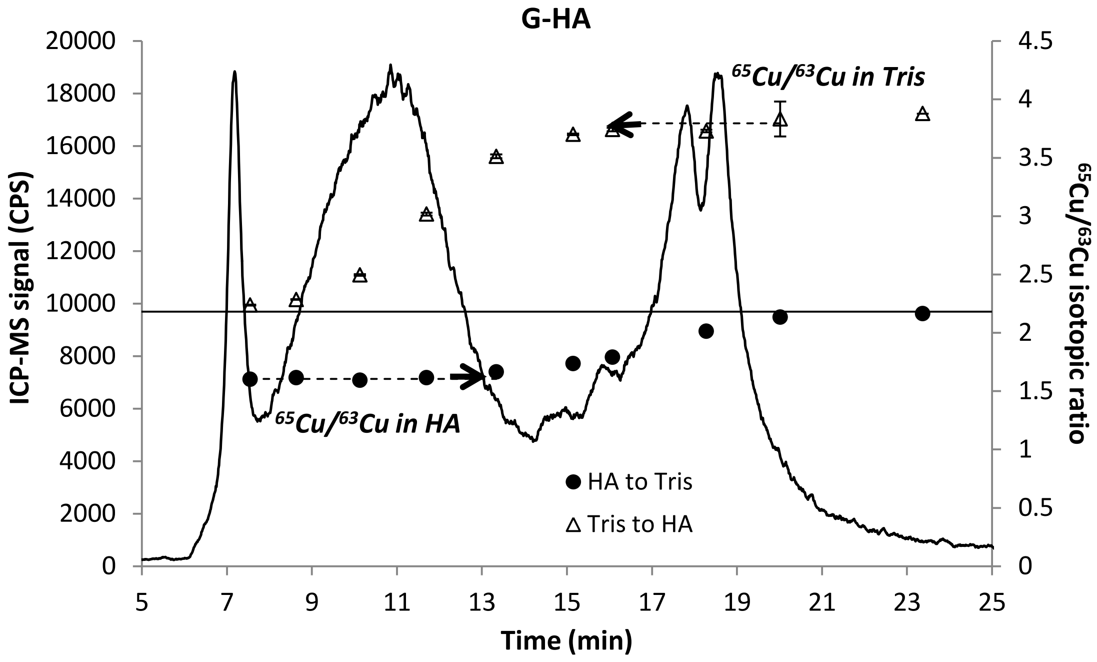


Figure 3: A ^{65}Cu chromatogram of G-HA solution (pH 7.0) 72 hours after spiking with ^{65}Cu (solid line). The isotopic ratio ^{65}Cu/^{63}Cu measured using different integration times is shown where chromatograms were progressively integrated from left (solely HA) to right (●), or from the right hand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

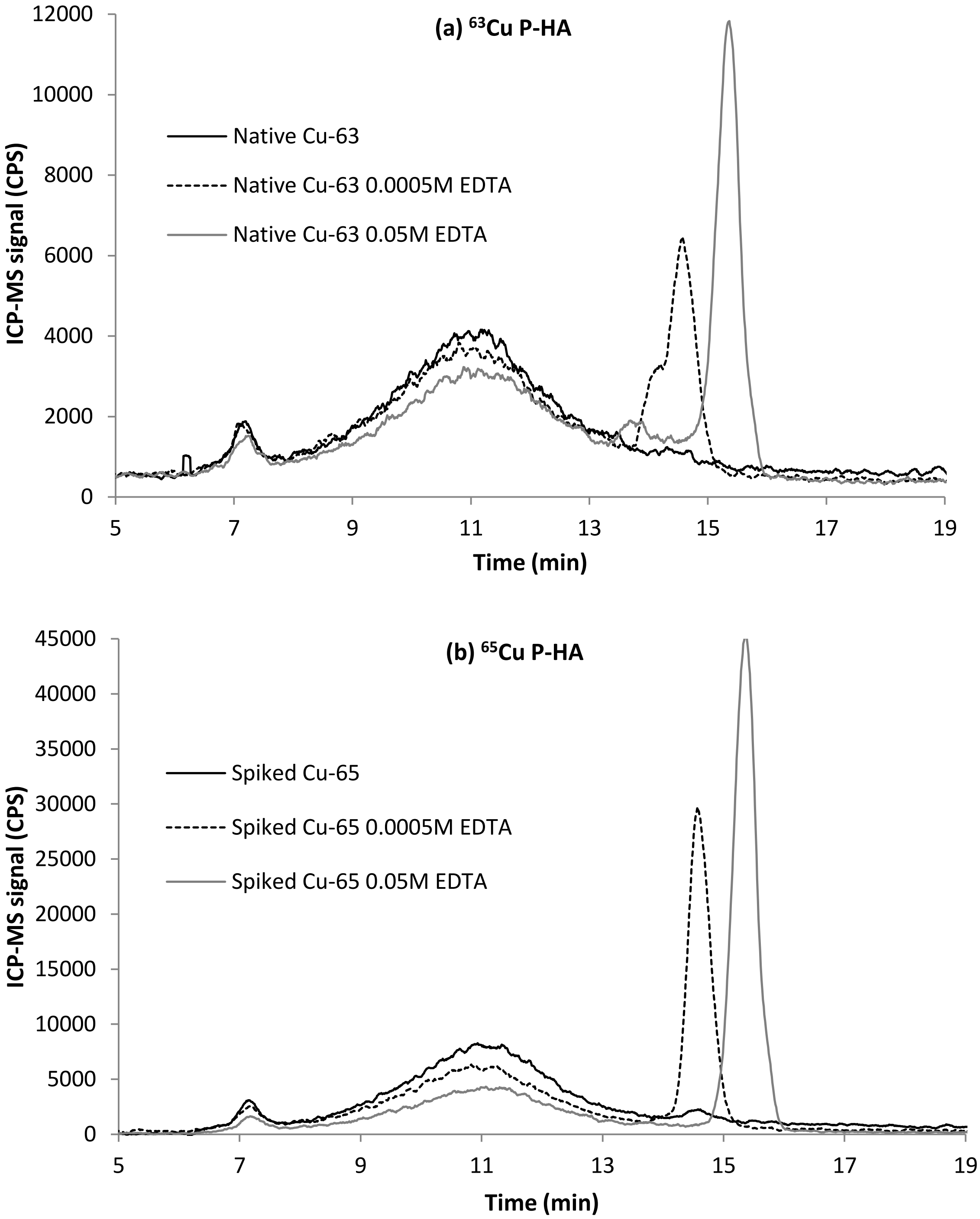
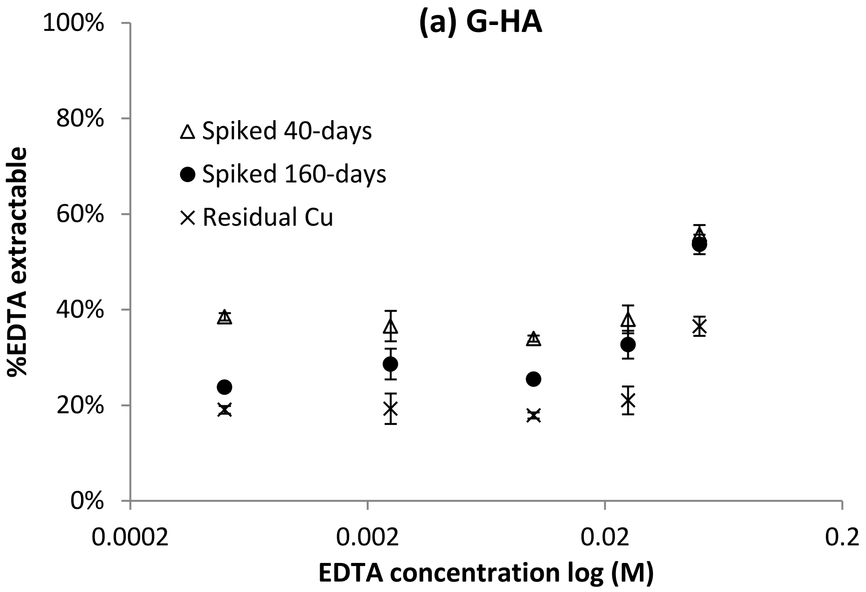


Figure 4: SEC-ICP-MS chromatograms of residual ^{63}Cu (a) and spiked ^{65}Cu (b) in peat humic acid (P-HA; 1.1 g L^{-1}) solutions (pH 7.0) at three EDTA concentrations (0.0, $5x10^{-4}$, $5x10^{-2}$ M). The HA was incubated with an enriched ^{65}Cu spike for 40 days before EDTA was added. Chromatograms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



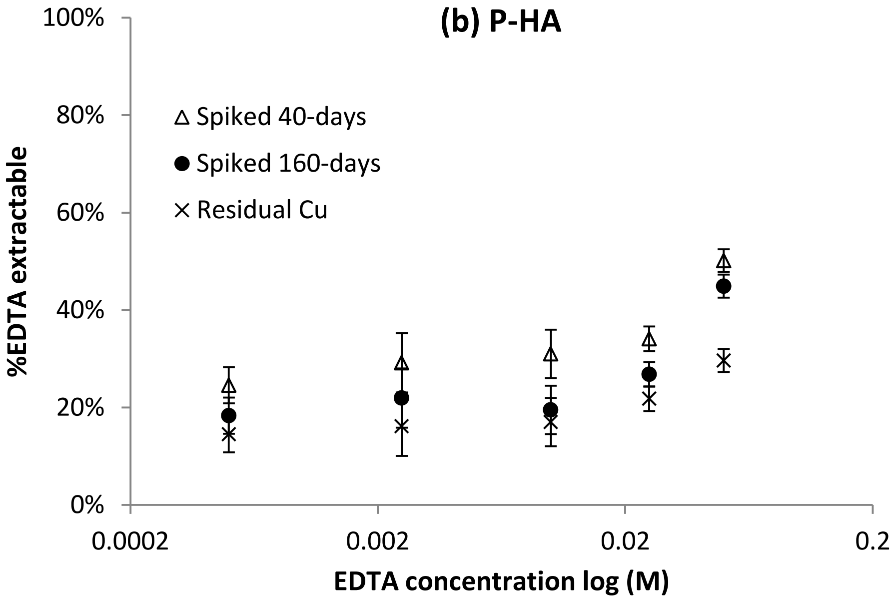


Figure 5: Proportion of Cu extracted by EDTA from (a) G-HA and (b) P-HA humic acids as a function of EDTA concentration (M; $\log_{10}$ scale). Data is shown for 'residual' metal (X) present in the purified HA and spiked treatment (^{65}Cu) incubated with HA for 40 d (△) and 160 d (●) (Error bars are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

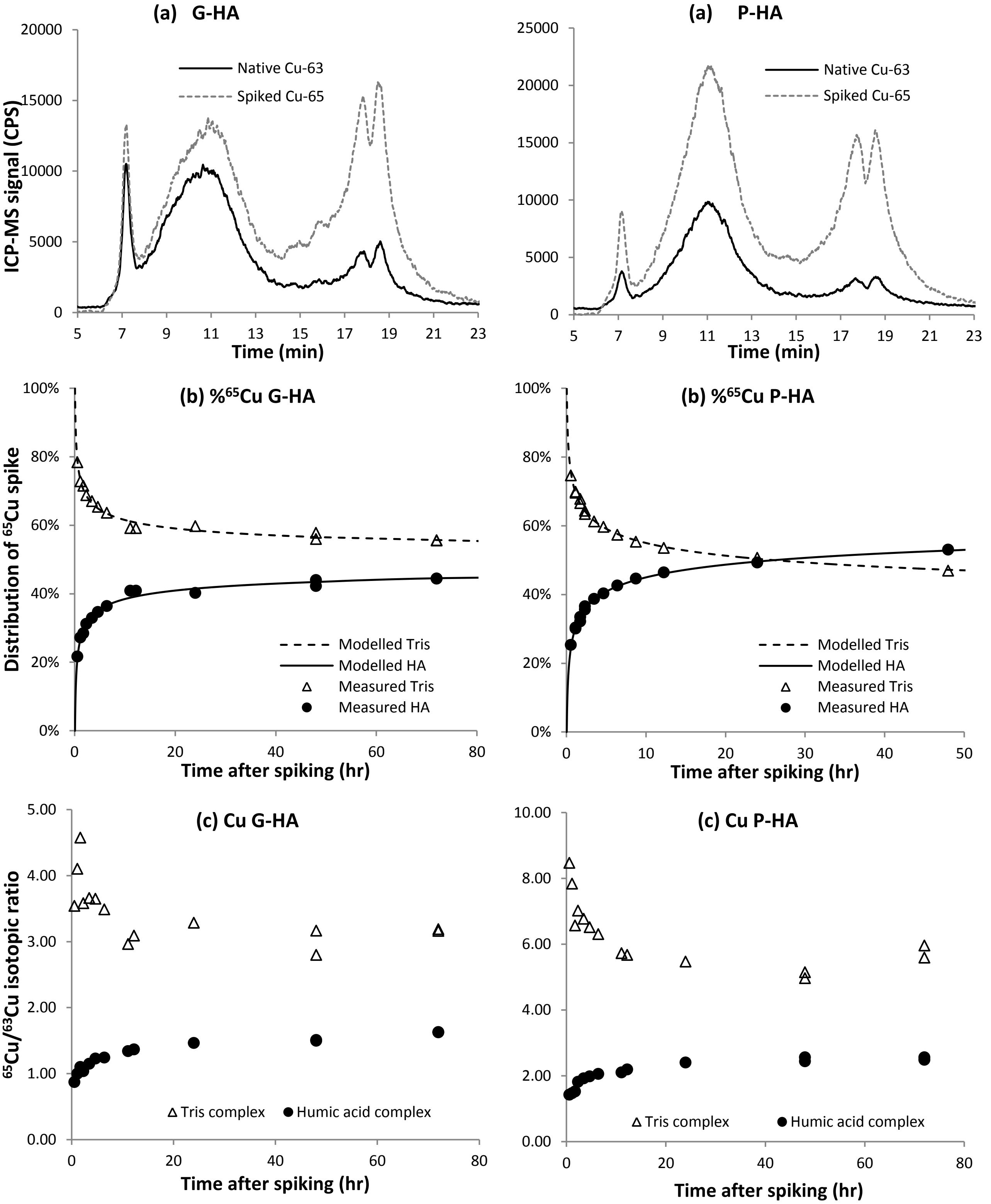


Figure 6: (a) SEC-ICP-MS chromatograms of spiked ^{65}Cu and incubated ^{63}Cu on grassland soil humic acid (G-HA; 72 hours after spiking) and for peat humic acid (P-HA; 48 hours after spiking). The humic acids were previously incubated with mixed metal standards for 40 days. (b) Proportions of ^{65}Cu-humic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

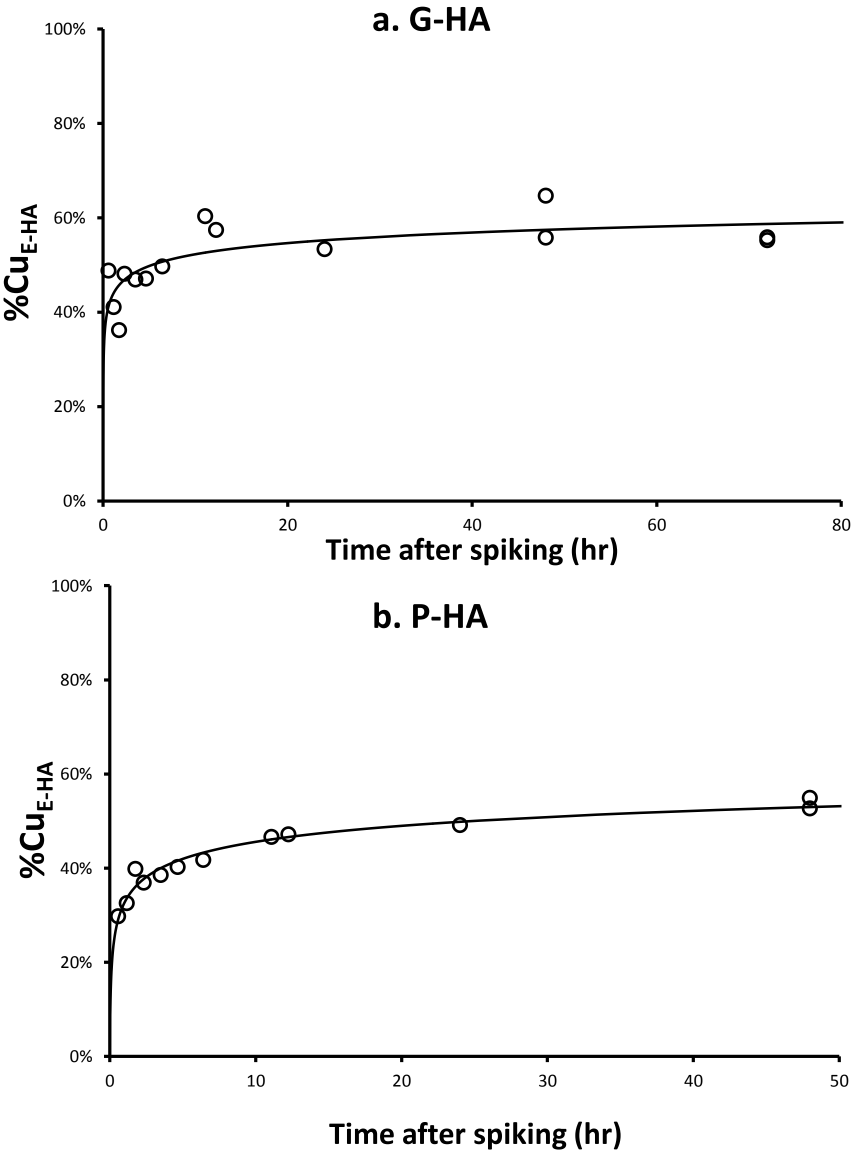


Figure 7: Values of %Cu$_{E\text{-}HA}$ on (a) grassland soil (G-HA) and (b) peat (P-HA) HAs as a function of spike isotope equilibration time. The solid lines are modelled values of %Cu$_{E\text{-}HA}$, predicted from a parameterized version of Eq. 3, as a function of time. For G-HA, K = 0.762, n =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