Comparison and combined use of linear and non-linear fitting for the estimation of complexing parameters from metal titrations of estuarine samples by CLE/AdCSV

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ABSTRACT

Despite the need to determine the concentration and conditional stability constants (K′) of natural ligands, we are far from achieving a consensus about the mathematical procedure to use with metal titrations due to the complexity of the samples and the wide range of fitting procedures and problems associated with the selection of the sensitivity (S) of the method. Here, we used Competitive Ligand Exchange/Adsorptive Cathodic Stripping Voltammetry (CLE/AdCSV) empiric data from estuarine waters and computer generated titration sets to compare linear methods with iterative correction of S with non linear fitting adding S as a parameter. We demonstrate for the first time that, independent of the fitting procedure, S cannot be retrieved if all the ligands present in the sample are not included in the speciation model. We also investigated the variables, apart from analytical noise, that can cause flawed non linear fittings of titration data. Computer generated data under multiple combinations of analytical conditions showed that a long extension of the titration (at least twice the total ligand concentration for estuarine conditions) and an analytical window (as the side coefficient $\propto'$) centred below the complexing strength of the natural ligands are essential to produce reliable complexing parameters. We verified, using for the first time a combination of experimental and computer generated data, that faulty estimations of S and K' obtained in empiric titrations of estuarine samples were artifacts of non linear fitting. Non linear fitting flaws were caused by a combined effect of the analytical error, the analytical window and the ratio in between the copper concentration and the concentration of the strongest ligands. Here, we recommend for the study of estuarine waters to complement non linear fitting with iterative linear fitting in order to avoid severe overestimations of S and the conditional stability constant of strong metal ligands.

Introduction

Organic speciation is a key factor for the understanding of the biogeochemical cycle of trace metals in aquatic systems. For many metals (Cu, Pb, Fe, Zn, etc) the predominant species is that bound to the fraction of Dissolved Organic Matter (DOM) with high affinity for them (Boye et al., 2006; Bruland, 1989; Capodaglio et al., 1990; van den Berg and Donat, 1992). This fraction is called generically ligands and there is not yet a consensus about their nature and origin. Among the candidates we find cyanobacteria exudates (Mawji et al., 2011; Moffett and Brand, 1996), humic substances (Kogut and Voelker, 2001; Laglera and van den Berg, 2009), thiols (Laglera and van den Berg, 2003; Tang et al., 2005), exopolysaccharides (Hassler et al., 2011; Schreiber et al., 1990), etc.

Our knowledge on the binding properties of DOM has been mainly acquired via Competitive Ligand Equilibrium-Adsorptive Cathodic Stripping Voltammetry (CLE-AdCSV). The technique is based on the equilibration of the sample with a well defined artificial ligand (AL) that forms an electroactive adsorbable complex with a particular metal. The analysis is repeated in aliquots at increasing metal concentrations producing an array of metal concentrations vs analytical signals (i_p). After mathematical transformation the complexing capacity of the ligands for the metal titrated ([L]) and the conditional stability constant (K') of the metal-ligand complex (van den Berg, 1984) are estimated. Analyses at different [AL] or using a second AL of different affinity for the metal changes the competition of AL with the natural ligands for the metal. The interval of ligands that are determined at a specific [AL] as a function of their K´ is called analytical window and cannot be extended more than 3 orders of magnitude (Apte et al., 1988). In coastal and estuarine waters (high DOM) this concept is essential to interpret results due to the impossibility to include all ligands into a single analytical window (Buck and Bruland, 2005; van den Berg and Donat, 1992) .

One of the key features of the processing of titration data is the estimation of the analytical sensitivity (S). Habitually, S is obtained internally from the last few points of the titration curve where ligands are virtually saturated with the metal (S^{INT}). Turoczy and Sherwood proposed an iterative method to correct S (S^{ITE}) (Turoczy and Sherwood, 1997) that returned the real value of the sensitivity in simple ligand mixes (Wu and Jin, 2009). Other efforts to improve the estimation of S include the use of overloaded titrations (titrations at very high [AL]) with the raw sample and after UV digestion in order to estimate the S vs [AL] dependence (Hudson et al., 2003; Kogut and Voelker, 2001) and the simultaneous fitting of several titrations of the same sample obtained at different [AL] (Hudson et al., 2003; Sander et al., 2011). This last approach requires the use of overloaded titrations and the estimation of the S vs [AL] dependence. However, in the study of estuarine waters, overloaded titrations are not practical because S being a function of the ionic strength and the concentration of surfactants, it would be necessary to carry out at the very least 3 titrations per sample increasing exponentially the work of the analyst. A better option would be to fit S simultaneously with the complexing parameters (S^{FIT}) but fitting routines struggle to converge as the number of parameters increases. Probably due to its complexity and the difficulty in accurately determining the S vs [AL] relationship, multitration fitting has been used in few publications to date and without a consensus mathematical protocol (Buck and Bruland, 2005; Hudson et al., 2003; Moffett et al., 1997; Ndungu, 2012; Sander et al., 2011; van den Berg and Donat, 1992).

Complexing parameters are also dependent on the type of equation and fitting procedure selected (Omanovic et al., 2010). Titration data have been linearized by different methods in order to use simple fitting to one or two straight lines (Ruzic, 1982; Scatchard, 1949; Sposito, 1982; van den Berg, 1982). Despite the fact that more sophisticated methods have been developed that make use of non linear fitting of different transformations of the titration data

(Garnier et al., 2004; Omanovic et al., 2010; Voelker and Kogut, 2001), linearizing methods are still generally used.

This work is the result of investigating the problems found in obtaining the complexing parameters of ligands from copper titrations of samples from the Tagus estuary by CLE-AdCSV with salicylaldoxime (SA) as AL (Santos-Echeandia et al., 2013). The use of nonlinear fitting for two types of ligands adding S as a parameter led to estimates substantially different from the expected values. A failure to converge to a valid solution using non linear equations has been reported before (Hudson et al., 2003; Wu and Jin, 2009). However, to date there has not been a proper description of the outcome of failed convergences and systematic work on the causes (other than data noise) that could impede the accurate determination of complexing parameters from titration data sets is lacking. We have made use of computer generated titrations where extra heterogeneity over a two ligand system was introduced occasionally via the addition of a third ligand to study those parameters that, after nonlinear fitting, were more prone to accumulate deviations from the initial values. In addition, we have used this method to characterize the experimental settings that have greatest impact in impeding accurate estimations of the sensitivity and complexing parameters.

We found from computer titrations generated under different conditions that when the speciation model does not include all the types of ligands present in the sample there is no valid method to retrieve the real S including the Turoczy and Sherwood (1997) method to refine S and the non linear fitting of S as an extra parameter. We resolved that extending the titration to metal concentrations well over the total ligand concentration is essential for the estimation of S and the accuracy of the process ($>2x[L]_{TOTAL}$ in the studied conditions). We have tested the robustness of the use of non linear fitting to 5 parameters stretching the analytical conditions to find that biased solutions were caused by a combination of analytical

error, high analytical window and high copper concentration in the sample. For the first time problems found in empiric values were perfectly reproduced with computer generated data. We proved that the unrealistic estimations of S and the conditional stability constant of the stronger ligands that we obtained for the Tagus estuary samples were actually artifacts of the fitting process. Finally, in order to provide realistic estimates of S and the conditional stability constant for 2 types of ligands, we propose a protocol for the fitting of titrations from estuarine samples where the use of nonlinear fitting is complemented by linear fitting. This protocol freed the solution of flawed estimations even for the most sensitive complexing parameters.

THEORY AND METHODS

Metal titrations and complexing parameters.

The theory behind the determination of the complexing capacities for metals by CLE-AdCSV has been extensively described before (Campos and van den Berg, 1994; van den Berg, 1984). Here we present exclusively the concepts and equations necessary for the comprehension of the overall work. In this CLE-AdCSV study, samples were spiked with a buffer solution and AL (SA hereafter) and split into a series of aliquots that are analyzed after equilibration at increasing metal concentrations ($[Cu]_{TOT}$ hereafter) (Campos and van den Berg, 1994). The fraction labile corresponds to the concentration of $CuSA_x$ species (x=1 and 2). The different fractions are related through the following mass balance:

$$[Cu]_{TOT} = [Cu]_{lab} + \sum[CuL_i] \quad (1)$$

where CuL_i is the concentration of copper bound to the ligand L_i. To avoid confusion between those ligands preset arbitrarily to generate computer titrations and the solutions after data treatment we used the following tagging: types of ligands defined in order to generate

ideal titrations received the numeration 1 to n being n=2 or 3 (L_1/K'_{L1}/ L_2/K'_{L2}/ L_3/K'_{L3}) ; those ligands determined from fitting of titration data were either not labelled for a one ligand model (1LM), or received the subscripts S (strong) and W (weak) for a two ligand model (2LM) (L_S/K'_S/ L_W/K'_W).

The analytical signal, the free copper concentration and the $CuSA_x$ concentration are related via the equations:

$$[Cu]_{lab} = [CuSA_x] + [Cu'] = i_p/S \quad (2)$$

$$[Cu^{2+}] = i_p/S/\alpha' \quad (3)$$

α' being the side coefficient of all the labile species ($\alpha'=\alpha'_{CuSAx}+\alpha'_{Cu'}$). Its value as a function of the salinity has been published elsewhere (Campos and van den Berg, 1994). The relationship between the free copper ion concentrations and [L] and K' of the different ligands is expressed via the Langmuir isotherm:

$$\sum[CuL_j] = \sum \frac{[Cu^{2+}]\cdot[L_j]\cdot K'_j}{\left(1+[Cu^{2+}]\cdot K'_j\right)} \quad (4)$$

being j= 1 to 3.

Computer generation of titration data sets.

Titration data sets were generated from arbitrary complexing capacities and stability constants for 2 or 3 types of ligands following the procedure described before for one ligand (Apte et al., 1988) and detailed in the Supporting Information section. The method allows generation of i_p from preset $[Cu]_{TOT}$. The preset value of S was 0.5 nA nM^{-1} throughout the whole paper.

In some of the simulations we introduced experimental error with a random factor at a fixed percentage (in the range 3 to 6%) to confer a relative error to all the i_p data. Those are percentages close to values used in previous works (Miller and Bruland, 1997; Voelker and Kogut, 2001).

Iterative linear regression for two types of ligands.

Complexing parameters are usually estimated after linearization of Eq(4) according to the methods described before by Scatchard (plot [CuL]/[Cu^{2+}] vs [CuL]) (Scatchard, 1949) and simultaneously by Ruzic and van den Berg (plot [Cu^{2+}]/[CuL] vs [Cu^{2+}]) (Ruzic, 1982; van den Berg, 1982). When more than one ligand is present, the referred plots adopt a curved shape because those ligands with a higher stability (L_S) are titrated during the initial copper additions while those ligands of weaker complexing ability (L_W) are titrated at the final part of the titration. Extensive explanations and method comparison can be found elsewhere (Bruland et al., 2000; van den Berg, 1982). After splitting the titration data set into an initial and final quasi-linear sections, estimations of L_S and K'_S and L_W and K'_W can be obtained by any of the linear methods in the two sections. For an independent estimation, an iterative process is required where the contribution of L_S to the last segment and the contribution of L_W to the initial data are cleared via subtraction of the concentration of the metal bound by the ligand of no interest in that section of the titration. A detailed description of the equations used has been published before (van den Berg, 1984). The Scatchard linearization suffers the same problem; direct use of slopes and axis intercepts cannot give accurate estimations of the complexing parameters in samples with more than one ligand (Wu and Jin, 2009) and a similar iterative refinement is required (Laglera-Baquer et al., 2001). Quite often, the structure of the analytical error impedes the convergence of the fitting routine: the Ruzic/van den Berg linearization tends to give linear regressions with negative Y axis intercepts that make impossible the calculation of K'_S; on the other hand, the Scatchard linearization can

result in positive slopes with the same result on the estimation of K'_S. A detailed description of error distributions for the two linearizing plots and their implications can be found elsewhere (Miller and Bruland, 1994). A minimization of errors from parameter estimation and an improvement of the stability of the convergence can be obtained by using a combination of the Scatchard linearization for L_S and the van den Berg-Ruzic linearization for L_W (Laglera-Baquer et al., 2001). An extra advantage of mixing linearization methods is the use of the linearizing equation that minimizes the uncertainty of the estimation in that segment of the titration (Garnier et al., 2004; Laglera-Baquer et al., 2001). This method was used successfully before for the estimation of the binding properties for copper and lead of algae cell surfaces (González-Dávila et al., 2000; Santana-Casiano et al., 1999).

Here we used a home made spreadsheet where the analyst selects arbitrarily from the two quasi-linear sections of the linearizing plot the number of data used for the determination of $[L_S]$ and $\log K'_S$, and for $[L_W]$ and $\log K'_W$ respectively. Individual spreadsheets containing one iteration each, were set up to 34. For a level of tolerance (or the maximum correction to both complexing capacities that brings the iterative fitting routine to its end) of 10^{-3} ($[L_S]$ and $[L_W]$ expressed in nM) the process usually crashed or converged in less than 10 iterations.

Iterative correction of the sensitivity.

The mathematical background behind the iterative procedure to refine the value of sensitivity has been described elsewhere for solutions containing one (Turoczy and Sherwood, 1997) and two types of ligands (Wu and Jin, 2009). Here, we used a modified version for a 2LM that makes use of the iterative linear regression referred to above to estimate the complexing parameters for two types of ligands. Briefly, S^{INT} was used to obtain values for $[L_S]$, $[L_W]$, K'_S and K'_W via iterative linear fitting. With those parameters CuL was recalculated for all those data points used to obtain S^{INT} (Eq. (4)). New values of $[Cu]_{lab}$ and i_p were calculated

from Eq. (1) and (2) leading to a new estimation of S. The process was repeated until S converged to a stable value (S^{ITE}). In a few cases and for purposes of comparison we used this iterative process using non-linear fitting as described elsewhere (Wu and Jin, 2009). When S^{ITE} was obtained by both linear and non linear methods, S was branded $S^{ITE,lin}$ and $S^{ITE,non}$ respectively.

Non-linear fitting of titration data.

Titration data sets were fitted to obtain simultaneously the complexing parameters for a 2LM and S (S^{FIT}) using the "Regression wizard" tool available in the software package for scientific graphing and data analysis Sigma Plot Version 11.0 (© Systat Software, Inc.) where fitting is achieved by means of a Levenberg-Marquardt algorithm**.** The aim was to use a state of the art non-linear fitting tool, wide spread in the scientific community with a procedure that is user-friendly and easy to reproduce by any analyst. Details about equations and how to programme the routine are provided in the Supporting Information.

In order to differentiate the values of S^{FIT} obtained for 1LM and 2LM, the number of parameters fitted was added in between parenthesis ($S^{FIT(3)}$ and $S^{FIT(5)}$). A reduction of the number of floating parameters was sometimes necessary to facilitate the fitting process. When a parameter was fixed using the option "constraints", we indicated the reduction of floating parameters changing the superscript of S, i.e.: $S^{FIT(4)}$.

Determination of the concentration of the free copper ion concentration.

The presence of more than one type of ligand in the original sample increases the order of the equation to solve for the determination of $[Cu^{2+}]$ in the original sample (i.e.: before the addition of SA). The task is simplified using iterative procedures (Laglera and van den Berg, 2003). Details of the process are shown in the Supporting Information section.

Titration of samples from the Tagus estuary.

The findings obtained with computer generated titrations were put to test with copper titrations of waters of the Tagus estuary. A detailed description of the study area, the sampling procedure, analytical method and copper speciation results has been published elsewhere (Santos-Echeandia et al., 2013). Briefly, ten sub-surface samples were collected covering the salinity gradient of the estuary (0-25‰) and filtered through 0.22 µm. Titration data consisting of 17 to 20 data pairs were obtained by CLE-AdCSV of Cu-SA complexes according to previous works (Campos and van den Berg, 1994; Laglera and van den Berg, 2003).

Results and discussion

Correction of the sensitivity for a two ligand model. Effect of extra ligands.

In an ideal situation, the analyst should avoid the use of linearizing plots when more than one ligand is present. This is not just due to the slightly higher residuals that linear fitting produces (Garnier et al., 2004; Gerringa et al., 1995), but mainly to the fact that linearizing methods force the analyst to take an important arbitrary decision: the length of the two segments of the data array used for the calculation of $[L_S]$ and $\log K'_S$, and $[L_W]$ and $\log K'_W$ respectively (Fish et al., 1986). When data include analytical error, there are as many possible solutions as there are combinations of segment lengths.

It has been previously established that calculation of S from the slope of the last few data points of the titration (S^{INT}) can lead to underestimations of S (Kogut and Voelker, 2001) and therefore to biased values of [L] and $\log K'$. When S is refined by iteration (Turoczy and Sherwood, 1997) using error-added data, the solution is also dependent on the arbitrary number of data pairs used to estimate S^{INT} and after iteration, S^{ITE}. The ideal situation should

also include the addition of S as a parameter to the nonlinear fitting procedure (S^{FIT}). This method has only rarely been used for 2LMs due to the struggles reported to converge to realistic solutions as the number of parameters to be fitted increases from 3 to 4 or 5 (Wu and Jin, 2009).

The heterogeneity of the nature of metal ligands in natural waters has been extensively documented e.g. (Donat and van den Berg, 1992). However, the 2LM is the most complicated model available with common mathematical tools and the limited length of the titration. Since the presence of extra types of ligands is very possible, and almost certain in coastal and estuarine waters, we have investigated the effect of extra ligands on the performance of the 2LM by introducing the simplest case: the addition of a third ligand to the sample. .

Tables 1 and 2 show the results obtained by different methods to solve error-free titrations generated from a mix of three ligands (titration data in Table S-3). Those methods were: iterative linear calculation for 2LM using S^{INT} (van den Berg, 1984), iterative linear calculation for 2LM with iterative correction of S ($S^{ITE,lin}$) (Laglera and van den Berg, 2006; Turoczy and Sherwood, 1997), non-linear fitting for 2LM with iterative correction of S ($S^{ITE,non}$) (Wu and Jin, 2009) and non-linear fitting of Eq(1) for 1LM and 2LM adding S as parameter ($S^{FIT(3)}$, $S^{FIT(5)}$). Table 1 corresponds to the presence of 3 ligands on the upper end of the analytical window (centred in both cases at $\propto'_{CuSAx}$=40,000): $[L_1]$=10 nM, (log K'_{L1}=16), $[L_2]$=40 nM, (log K'_{L2}=14) and $[L_3]$=150 nM, (log K'_{L3}=12). Table 2 instead, shows the result of applying the same routine to a mix of ligands placed on the lower end of the analytical window: $[L_1]$=10 nM, (log K'_{L1}=14), $[L_2]$=40 nM, (log K'_{L2}=12) and $[L_3]$=150 nM, (log K'_{L3}=10). Those values in Table 2 are similar to the values found for natural copper ligands in estuarine and coastal waters (Buck and Bruland, 2005; Laglera and van den Berg, 2003).

The extension of the titrations to 2-3 times the total ligand concentration (Table 1 and 2) brought S^{INT} to values within 5% short of the real S in both cases. The presence of a third ligand (L_3) caused S^{ITE} and S^{FIT} to be underestimations of the preset S. For S^{ITE}, this shortcoming was independent of the use of linear or non linear fitting. $S^{ITE,lin}$ and $S^{ITE,non}$ were slightly different for the case given in Table 1, showing that they provide different solutions. For the ligands preset in Tables 1 and 2, the 2LM gave S^{ITE} and S^{FIT} underestimations of less than 1% and 3% of the real S respectively compared to 5% and 4% using S^{INT}. It is obvious that those differences would have been increased by higher concentrations of L_3 or a fourth ligand. Fitting of S using a 1LM returned $S^{FIT(3)}$ values even lower than S^{INT}. This surprising result was also observed during the analysis of natural samples (see below). Another unexpected result is that S^{ITE} was slightly better than $S^{FIT(5)}$. From the results in Tables 1 and 2 we could infer that in the presence of a third ligand, the 2LM could not retrieve the real S even for error-free titrations. Thus, when the number of ligands contemplated in the model is lower than the number of types of ligands present in the sample, S cannot be retrieved by any fitting routine including iterative correction or simultaneous nonlinear fitting.

Depending on the characteristics of the ligands we observed that linear and non linear fitting gave significantly different solutions when very strong ligands were present (Table 1). Linear fitting gave estimations of $[L_S]$ and $\log K'_S$ much closer to $[L_1]$ and $\log K'_{L1}$ ($[L_S]=1.7x[L_1]$) than non-linear fitting for both $S^{ITE,non}$ and $S^{FIT(5)}$ (where $[L_S]\sim4x[L_1]$). This is due to the limited use for the calculation of $[L_S]$ and K'_S, of those initial data in which the metal is mostly complexed by L_1 as opposed to the combined use of all data during non-linear fitting. As a result, linear fitting better resolved those ligands with very high stability constants getting $[L_S]$ and K'_S values closer to $[L_1]$ and K'_{L1} and what it is more important, a better pCu (99.8% compared to 97.9%). Surprisingly, feeding of the non linear routine with the preset S did not improve the estimation of pCu. Non-linear fitting gave estimations of L_W and K'_W

closer to L_3 and K'_{L3}. That difference could suggest that the definition of the upper limit of the analytical window could also depend on the fitting method selected and not just on the accuracy of the analytical procedure as pointed out before (Apte et al., 1988). This possible redefinition should be addressed in future work as it would change our understanding of the analytical window. The fixing of S to its real value during non linear fitting gave results that did not differ significantly from those found using $S^{ITE,non}$ or $S^{FIT(5)}$.

When weaker ligands were used to generate the titration (Table 2), linear and non linear methods gave close solutions. Both gave good estimates of the stronger ligands (L_S~L_1 and L_W~L_2) and barely sensed L_3 due to out competition by SA. Here, the use of the real S produced a solution where the weight of L_3 is slightly increased affecting $[L_S]$ and K'_S and $[L_W]$ and K'_W but not improving significantly the estimation of pCu, of which closer estimation is obtained again using $S^{ITE,lin}$.

Effect of the range of metal additions on the precision of the estimation of complexing parameters.

Titrations used to generate Tables 1 and 2 were extended well beyond the combined concentration of all ligands. The logical prolongation of the last section would be to study the effect of $[L_3]$. However, for a fixed array of $[Cu]_{TOT}$ we would move from a situation of nearly titrated ligands to insufficient copper additions. To avoid this artifact, we decided to study the performance of different fitting methods as a function of the extent of the titration when a third ligand is present. We studied the whole range of circumstances, from subsaturation to over titration of the ligands by stretching the $[Cu]_{MAX}/[L]_{TOT}$ ratio from 0.2 to 3. Figure 1 shows the effect on error-free titration data generated from the following conditions: $[L_1]$=20 nM (log K'_{L1}=14), $[L_2]$=100 nM (log K'_{L2}=12), $[L_3]$= 200 nM (log K'_{L3}=11); $\propto'_{CuSA}$=15,000; Cu_{TOT}=10 nM(pCu=14.085). In this case, we selected a difference

in between K'_{L2} and K'_{L3} of only 1 log units to maximize the effect of L_3. For the sake of clarity and due to the similarities explained above, only iterative linear fitting using both S^{INT} and $S^{ITE,lin}$ and non-linear fitting of 5 parameters were plotted. Arrows in Fig. 1 indicate the preset conditions. Figure 1 shows again how the presence of ligands not included in the model impeded the calculation of the real S independently of the method used to refine S. The correction of S was substantial (S^{INT} 20-60% of the preset value) when $[Cu]_{MAX}/[L]_{TOT}$ was less than one. However, at low $[Cu]_{MAX}/[L]_{TOT}$ iterative correction or non linear fittingof S also offered a poor approximation to the real S (about 50-70%). In titrations extended to $[Cu]_{MAX}/[L]_{TOT}$~3 differences in between S^{INT}, corrected S and the real S were of the same magnitude as the common analytical error (<6%). When facing the analysis of natural samples, it is impossible to predict the number of ligands present and how far the metal additions go into ligand saturation. However, the good performance of S^{INT} at high $[Cu]_{MAX}$ must be taken into account by the analyst and thus our recommendation is to try to stretch the titration as much as permitted by the analytical conditions. This good performance will be shown to be important due to the uncertainties we found determining S^{ITE} and S^{FIT} in natural samples (below). As a guideline, total ligand concentrations reported before by CLE-AdCSV in estuarine waters lied in the range 20-300 nM (Buck and Bruland, 2005; Dryden et al., 2007; Kozelka and Bruland, 1998; Laglera and van den Berg, 2006; Ndungu, 2012; Santos-Echeandia et al., 2008) with higher values up to 400-600 nM when the freshwater end was analysed (Gerringa et al., 1996; Laglera and van den Berg, 2003; Santos-Echeandia et al., 2013). Accordingly, to be on the safe side copper titrations should be stretched at least to 600-800 nM for estuarine samples containing high DOM concentrations and significantly further for the freshwater end samples. Micromolar copper additions are incompatible with the analytical method due to two different causes: loss of linearity caused by the saturation of the electrode surface at such $CuSA_x$ concentrations and the absence in those conditions of one

of the requirements of the method, [AL]>>Σ[L]. In that situation we recommend sample dilution using a volume of the same sample previously UV digested to avoid variations of the sample matrix.

With respect to the complexing parameters, further titration of L_3 increased its weight in the solution taking $[L_S]$ and K'_S and $[L_W]$ and K'_W away from $[L_1]$ and K'_{L1} and $[L_2]$ and K'_{L2}. In agreement with previous findings, Fig. 1 shows that linear fitting with $S^{ITE,lin}$ gave $[L_S]$ and K'_S closer to $[L_1]$ and K'_{L1} than non-linear fitting as $S^{FIT(5)}$ gave $[L_W]$ and K'_W estimates closer to a combination of $[L_2]$ and K'_{L2} and $[L_3]$ and K'_{L3}. Surprisingly, the use of S^{INT} reduced this effect and better estimates of $[L_S]$ and K'_S and pCu were obtained. The consequence is that, for extended titrations, pCu in the original sample was approached better via linear fitting (S^{INT} or $S^{ITE,lin}$) than using non-linear fitting.

The Sigma Plot fitting routine was also set to solve 3 types of ligands but for 7 parameters the convergence gave flawed estimations of S and the complexing parameters of L_3.

Consequently, in multiligand solutions the limitation of the model to a 2LM is a harder restraint than the use of a correction for S if the titration is extended appropriately. Although simultaneous non-linear fitting of the complexing parameters and S did not produce the best estimations of pCu, we must consider that, in the case of these error-free titrations, solutions by linear fitting benefited from being independent of the number of points selected for both L_S and L_W, which is never the case in empiric situations.

The effect of the analytical window and the initial metal concentration on the non linear fitting of titration data sets.

In this section we present the effect of other conditions that interfere with the fitting of titration data: the centre of the analytical window and the initial copper concentration. Higher

[SA] (an analytical window shifted to higher K′) would be less prone to be affected by complexation by weak ligands and would give better estimates of S. Higher $[Cu]_{INI}$ could fully saturate L_1 before the beginning of the titration and therefore change the estimation of $[L_S]$ and K'_S. In Fig. 2 we show the results obtained by non-linear fitting from error-free titrations that start at different $[Cu]_{INI}$ (range 1 to 50) but end at the same $[Cu]_{MAX}$ of 400 nM ($[Cu]_{MAX}/[L]_{TOT}$ =2). All titrations were generated using the following two ligands $[L_1]$=20 nM (log K'_{L1}=14), $[L_2]$=80 nM (log K'_{L2}=12). $[L_3]$ was 100 nM but in two cases log K'_{L3} was preset at 10 and in the other two at 11. The effect of [SA] was studied using α'_{CuSA}=15,000 in two studies and α'_{CuSA}=86,000 in the other two. Three clear effects were observed in Fig. 2:

As expected, higher α'_{CuSA} facilitated the estimation of S by overcompetition of L_3. Better $S^{FIT(5)}$ estimations brought $[L_W]$, K'_W, $[L_S]$ and K'_S closer to L_1 and L_2 complexing parameters (arrows in Fig.2). Second, the effect of increasing K'_{L3} (and therefore its power to compete with L_1, L_2 and SA) was a higher weight of L_3 on the fitting process. The stronger the competition caused by ligands not included in the speciation model, the more weight they have in the estimation of the complexing parameters of L_S and L_W and thus increasing the underestimation of $S^{FIT(5)}$. Finally, $[Cu]_{INI}$ had an important effect on the estimation of S and the complexing parameters. This effect was clearly more important for $[L_S]$ and K'_S (up to $[L_S]$~2.5x$[L_1]$ and a decrease of 1.2 log units for log K'_S).

The accuracy for the measurement of $[Cu^{2+}]$ is shown in Fig. 2 as the ratio pCu (from ligands 1 to 3) / pCu (from ligands S and W); this ratio showed different trends as a function of the combinations of α'_{CuSA} and the complexing strength of L_3. Higher $[Cu]_{INI}$ translated into higher deviations from the real pCu except for one case: low α'_{CuSA} and log K'_{L3}=11. Although in those circumstances, increasing $[Cu]_{INI}$ forced $[L_S]$ and K'_S significantly away from $[L_1]$ and K'_{L1}, surprisingly the estimations of S and pCu improved significantly.

The use of non linear fitting for the titration of natural estuarine samples.

Motivated by the excellent performance that non linear fitting showed in previous sections, we tested its performance to determine the complexing parameters of the natural copper ligands present in samples from the Tagus estuary (results shown in Table S3). Here we present the problems found using 5-parameter non linear fitting, their causes and the solutions proposed to fix them.

In order to get a general picture of the characteristics of the ligands and despite obtaining clearly curved Scatchard plots, we determined the general complexation trends by performing non-linear fitting of all data sets for a 1LM. $S^{FIT(3)}$ was not significantly different with respect to S^{INT} as seen before for 3L computer generated titrations. Average of $S^{FIT(3)}$ as a percentage of S^{INT} was found to be 101.0% ± 6.0% (ranges 0.251 to 0.400 and 0.267 to 0.353 for S^{INT} and $S^{FIT(3)}$ respectively). $S^{FIT(3)}$ values below S^{INT} were in line with previous findings (Tables 1 and 2). When the model was extended to two types of ligands, S received different corrections for different samples: $S^{FIT(5)}$ ranged from 103.7% to 170.3% with respect to S^{INT} (range 0.325 – 0.519). Because L_W and K'_W are more directly linked to the sensitivity (Miller and Bruland, 1997), highly increased S translated into important increments of total ligand concentrations (L_S+L_W) with respect to the concentrations obtained with the 1LM (up to 166%). [L_S] spanned in a range ~30 nM wide but K'_S strikingly ranged by 8 orders of magnitude (log K'_S 14.3 to 22.4).

The effect of the analytical window was studied by repetition of some analyses at twice the [SA] (Table S3). For many samples S^{INT} did not increase significantly, indicating that probably [SA] of 5-10 μM were into the saturation range for this specific type of samples and electrode size. One of the fittings produced a faulty result in the form of a impossible [L_W] caused by an extremely large $S^{FIT(5)}$ ($S^{FIT(5)}/S^{INT}$=422) (TW11, 10μM [SA]). TW13 also

produced a suspicious $S^{FIT(5)}$ more than twice S^{INT} that translated in a illogical huge increment of $[L_W]$ with respect to the 5 μM [SA] sample. For the rest, again K'_S was spread in a range 8 orders of magnitude wide.

Some of the $S^{FIT(5)}$ and K'_S values raised our suspicions as they were off the main trends shown by the majority of the data. log K'_S values fell in the ranges 14-17 and 21-24 but never in between (Table S3). We decided to make use of different fitting methods for comparison purposes. Table 3 and Fig S1 show the results obtained for 2 selected titrations characterized by very different responses to variation of the fitting procedure. TW49 is a sample close to the river end member of the estuary diluted x2 in UV digested estuarine water of the same salinity (0.4 ‰). TW25 corresponds to a sample from the central part of the estuary (sal 15‰). TW25 is an example of the problems found in fitting some titrations.

For station TW49, corrections of S showed a simple pattern consistent with previous observations from computer generated titrations. $S^{FIT(3)}$ and S^{INT} were not significantly different (0.3034 ± 0.0097 and 0.2972 ± 0.0020 respectively). Iterative correction of the sensitivity produced $S^{ITE,lin}$ and $S^{ITE,non}$ in excellent agreement (0.3285 and 0.3254) that were significantly higher than S^{INT} (8-10%). $S^{FIT(5)}$ (0.3302) was close to $S^{ITE,lin}$ and $S^{ITE,non}$. Complexing parameters obtained with any of the corrected S were in excellent agreement (see Table 3). Even non linear fitting using S^{INT} gave complexing parameters not significantly different (except for $[L_W]$). This case is clearly similar to those presented in Tables 1 and 2. The titration of Station TW25 on the other hand, gave results strongly dependent on the fitting methodology. $S^{FIT(3)}$ was close to S^{INT} but for the 2LM, the iterative correction of S differed significantly depending on the fitting procedure: $S^{ITE,lin}$ and $S^{ITE,non}$ were 0.4510 and 0.3526 respectively. $S^{ITE,lin}$ was plotted in Fig S1 to emphasize its detachment from the data trend. $S^{FIT(5)}$ (0.3503) was close to $S^{ITE,non}$. Differences in S brought significant differences in

[L_W]. As opposed to results shown in Tables 1 and 2, non linear fitting gave lower [L_S] and higher K'_S than iterative linear fitting. Non linear fitting gave log K'_S values around 22 whereas linear fitting returned values around 14, more in agreement with the results from the majority of the other titrations (Table S3). With respect to pCu here estimations for 2LM were spread more than one unit, which was not the case for TW 49.

The analyst could feel tempted to accept the results and consider that some samples required huge corrections of S and that pseudo-inert ligands ($\log K'_S > 20$) were present in some samples. We discarded this scenario for all the following reasons: the first one relates to the area of study: samples were collected in an estuary that does not present diversity of characteristics or strong side inputs (Santos-Echeandia et al., 2013); there was no indication that inert ligands could be patched in some areas of the estuary. In our study, all titrations were extended to similar copper concentrations, well beyond the ligand concentrations obtained (TW25 titration was extended to 471 nM, 1.8-2.5 times [L_S]+[L_W], Table 3). Moreover, S^{INT} showed an excellent similarity among titrations of different samples. It was improbable that S^{INT} required strong corrections only in some cases. Change of 1LM to 2LM (with $S^{FIT(5)}$) forced a transformation of the ligands behaviour across the estuary. Whereas the use of the 1LM or S^{INT} for 2LM gave a perfect conservative behaviour (r^2=0.96; Figure S2), this character was lost after nonlinear fitting to 5 parameters (r^2=0.77). Another indicator was that all $\log K' > 20$ values were coupled to standard errors ~8 logarithmic units. Results from titrations repeated at a higher analytical window (10 μM [SA]) did not always support those $\log K' > 20$. An increase of [SA] improves the conditions for the estimation of [L_S] and K'_S; in that condition K'_S must be equal or higher depending on the heterogeneity of the ligands. Table 3 shows the results for TW13. Non linear fitting of the titration to 5 parameters gave log K'_S values of 22.2 and 14.3 for 5 and 10 μM [SA] respectively, supporting the hypothesis that the first was an artifact.

We studied the effect of fixing the value of some parameters before proceeding with non linear fitting (underlined in Table 3). In this case we tested the result of setting the value of S to $S^{FIT(5)}$ and S^{INT} for sample TW25 in order to decrease the number of floating parameters and facilitate the work of the fitting routine. $[L_W]$ and $\log K'_W$ were clearly a function of S. Again, non linear fitting with reduction of the number of floating parameter generated $\log K'_S > 20$. When we fixed $\log K'_S$ to the value obtained with linear fitting (14.10), $S^{FIT(4)}$ was close to the values of $S^{FIT(5)}$ and $S^{ITE,non}$; $[L_W]$, K'_W and $[L_S]$ were very close to those obtained with $S^{FIT(5)}$ and $S^{ITE,non}$ at the expense of increased standard errors. If both S and K'_S were fixed with S^{INT} and K'_S from the linear method, $[L_W]$, K'_W and $[L_S]$ were close to those obtained with linear fitting and S^{INT}. Summarizing, non linear fitting always produced $\log K' > 20$ that were not produced by linear fitting. On the other hand $S^{ITE,lin}$ seemed overcorrections of S.

Below, we tried to verify using computer generated titrations that faulty overestimations of $S^{FIT(5)}$ and K'_S found in empiric titrations can be created as an artifact of non linear fitting.

Conditions that create failed nonlinear fittings: Analytical window, initial copper concentration and analytical error.

We generated error-free titrations from two types of ligands: $[L_1]$=20 nM ($\log K'_{L1}$=14) and $[L_2]$=80 nM ($\log K'_{L2}$=12), switching $\log \alpha'_{CuSAx}$ 5 units (2-7). This is the equivalent to moving from bent titrations to almost perfect straight lines for the same sample. The strong complexation by L_1 and L_2 ($\log \alpha'_{\Sigma CuL}$=6.32) allowed the study in higher detail of the lower end of the analytical window. Figure 3 shows that non linear fitting gave faulty results at $\alpha'_{CuSAx} >= 5$ fold $\alpha'_{\Sigma CuL}$ returning wrong estimations of $[L_2]$ and K'_{L2} (as much as 1.5 orders of magnitude) and overestimations of S^{FIT}. On the other end, titrations generated using α'_{CuSA} as low as 100 (10^4 smaller than $\alpha'_{\Sigma CuL}$) were accurately resolved. When the linear iterative

procedure was fed with the same titrations the right values were obtained in the whole α'_{CuSAx} range. Therefore, under the $\alpha'_{CuSAx} >> \alpha'_{\Sigma CuL}$ condition not only the analysis cannot be carried out due to the proximity in between the labile and total copper fractions that leads to attempts to determine minute Σ[CuL] close to or below the limit of detection (see Eq.1) (van den Berg and Donat, 1992), but as we have shown above, nonlinear fitting on Eq (S2) becomes inoperable even in the absence of analytical error. The experiment was repeated using weaker complexation: log K'_{L1}=12 and log K'_{L2}=10 (log $\alpha'_{\Sigma CuL}$=4.32) and log α'_{CuSAx} spanning from 1 to 6 with identical results at both ends of the α'_{CuSAx} range (data not shown).

Samples from the Tagus estuary were analyzed using α'_{CuSA} in the range 4.3 to 6.2 (function mainly of the salinity and to a lesser extent of [SA]) and resulted in $\alpha'_{\Sigma CuL}$ in the range 7 to 7.6. Those values imply that our experimental conditions were kept at the $\alpha'_{CuSAx} < \alpha'_{\Sigma CuL}$ condition.

Next, we incorporated analytical error into computer generated data. In Table 4 we present the combined effects of the analytical error, the analytical window and $[Cu]_{INI}$. For this purpose, we generated data from only 2 ligands, $[L_1]$=20 nM (log K'_{L1}=14), $[L_2]$=100 nM (log K'_{L2}=12) with $[Cu]_{MAX}$=240, a series of 20 titrations, repeating the process at increasing analytical errors (3 to 6%). The effect of α'_{CuSAx} and $[Cu]_{INI}$ was studied replicating the process at two analytical windows (α'_{CuSAx}= 15,000 and 86,000) and two different $[Cu]_{INI}/[L_1]$ ratios (0.5 and 1). Results for the complexing parameters and $S^{FIT(5)}$ are shown as averages (n=20) plus the range of minimum and maximum values obtained (Table 4).

Careful observation of Table 4 discloses a series of trends:

At a constant α'_{CuSAx}, high $[Cu]_{INI}$ led to faulty estimations of K'_S. At log α'_{CuSA}= 15,000, the increase of $[Cu]_{INI}$ from 10 to 20 nM, broadened minimum/maximum log K'_S ranges

significantly. For instance, at a error of 4%, log K'_S range was 13.9-14.1 ($[Cu]_{INI}$=10 nM) that widened to 13.7-14.6 if $[Cu]_{INI}$=20 nM. We also found one very high log K'_S (22.7; n=80). Except for this specific K'_S outlier, the complexing parameters of L_W and L_S were estimated accurately. At $\propto'_{CuSAx}$=86,000, $[L_W]$, K'_W and $S^{FIT(5)}$ were poorly estimated in all cases (in an effect we will address below). log K'_S at $[Cu]_{INI}$=10 nM was included in a range 1 to 1.5 units around the value of K'_{L1} at the different error levels. For $[Cu]_{INI}$ = 20 nM the incidence of log K'_S >20 values was of 9 in 80.

At a constant $[Cu]_{INI}$ high $\propto'_{CuSA}$ impeded the estimation of $S^{FIT(5)}$. Whereas at $[Cu]_{INI}$=10 nM and $\propto'_{CuSAx}$=15,000 all the parameters were well approached, the increase of $\propto'_{CuSAx}$ to 86,000 brought $S^{FIT(5)}$ values up to 11 $nAnM^{-1}$ (2,200% preset S) that did not translate into high errors for $[L_S]$ and K'_S but impeded the determination of $[L_W]$ (values up to 1,400 nM). As observed for K'_S, $S^{FIT(5)}$ took values in an asymmetric range with some extraordinarily high values. At $[Cu]_{INI}$=20 nM we found a similar situation. $S^{FIT(5)}$ at $\propto'_{CuSAx}$=15,000 was constrained in a symmetric range of 0.2 $nAnM^{-1}$ (40% of real S), whereas at $\propto'_{CuSAx}$=86,000 the range was stretched from 0.38 to 10 $nAnM^{-1}$. This translated into $[L_W]$ up to 85 fold $[L_2]$. With respect to the analytical error, there was proportionality among the percentage of analytical error and the uncertainty added to the estimation of parameters. Higher percentages widened max/min ranges for all parameters although the width of those ranges did not show linear correlation with the percentage of analytical error.

We investigated the potential utility of the "constraints" options implemented in the software to sort out the inconsistencies found by fixing those parameters with a greater tendency to accumulate fitting errors: K'_S and S. For the most negative combination of $[Cu]_{INI}/[L_S]$ and $\propto'_{CuSAx}$ seen above, the effect of fixing K'_S and $S^{FIT(5)}$ to K'_{L1} and real S is shown in Table S5:

Fixing of S to 0.5 ($S^{FIT(4)}$) did not prevent the appearance of K'_S>20 although it lowered satisfactorily their incidence. The estimation of $[L_W]$ and K'_W was improved but at high analytical error values spread in a wide range (81-570 nM for $[L_W]$ and 10.8-12.3 for log K'_W). On the other hand, fixing of log K'_S to 14.0 did not prevent the appearance of huge $S^{FIT(4)}$ estimations and a new problem appeared in the form of one infinitesimal $[L_W]$. Because fixing of log K'_S at an error of 3% was inadequate, we did not investigate further at higher errors.

The deviations found for estimations of $S^{FIT(5)}$ and K'_S were significantly different. When all the data presented in Tables 4 and S4 were plotted together (n=420) we observed in excellent agreement with findings from natural samples (Figure S3) that solutions for log K'_S were found either in the range 13.5-16 corresponding to good estimates or in the range 21-26, flawed values that can only be ascribed to artifacts of the fitting procedure. This facilitates extraordinarily the detection of faulty K'_S estimations. On the other hand, S^{FIT} was distributed in a continuous range up to 12 (240% the preset value). In this case it would be more difficult to recognize many of the flawed overestimations (Figure S3).

When S and K'_S were both fixed to arbitrary values simultaneously (bringing the number of parameters down to 3) titrations were always resolved satisfactorily. Use of iterative correction of S with linear fitting could not solve the problem because whereas this method never gave log K'_S >20, $S^{ITE,lin}$ often gave faulty overestimations (as it was the case for TW25). Testing of iterative linear fitting with $S^{ITE,lin}$ under different analytical conditions requires the generation of a whole new set of model titrations and would extend the work well beyond the limit of an article. The problem will be addressed in a future work.

Protocol adopted to calculate the complexing parameters of natural ligands from estuarine samples.

All the deviations for $S^{FIT(5)}$ and K'_S dscribed above for computer generated titrations were identical to those found for the Tagus samples. In our samples $[Cu]_{INI}/[L_S]$ ratios were close to 1 (Table S5), which is associated with the risk to produce K'_S outliers by non linear fitting. Similar ratios have been reported before for other estuarine waters (Buck and Bruland, 2005; Laglera and van den Berg, 2003).

Once we have established that some of our flagged K'_S and $S^{FIT(5)}$ are likely faulty solutions, we created a protocol in order to treat all our titrations uniformly:

K'_S: we took the value produced by the non-linear fitting of the data set in all those cases where log K'_S < 20. For higher values we ran in parallel iterative linear fitting of the data with iterative correction of the sensitivity ($S^{ITE,lin}$) and used the K'_S value produced (always in the range 14-16) to constrain its value during non-linear fitting.

S: because overestimations of S are impossible to detect and due to the confidence that our copper addition titrated completely those ligands ruling the speciation of copper, we decided to use the higher of S^{INT} or $S^{FIT(3)}$. According to this premise S could be underestimated perhaps by up to 10%, which is the maximum correction introduced by $S^{FIT(5)}$ in those cases where the fitting routine converged without problems (as TW49). However, we did not reach proper $S^{FIT(5)}$ for some titrations even after fixing of K'_S. To avoid double standards and after consideration of findings shown in Fig 1, we adopted this decision except for the case of TW410 (10 μM SA) where the use of S^{INT} produced a log K'_S< 20. In some cases, use of S^{INT} produced log K'_S< 20 that forced the fixing of K'_S.

Thus the number of parameters fitted was 3 or 4 depending on the estimation of K'_S. The resulting study about copper speciation and the origin of natural ligands in the estuary has been published elsewhere (Santos-Echeandia et al., 2013) and the result of applying the protocol is presented in Table S5 for comparison. Those values constrained were underlined.

Under those conditions, the concentration of total ligands recovered the conservative behaviour observed with the 1LM (r^2=0.954).

Conclusions

We have programmed a new user friendly fitting tool in a wide spread statistical package for the non linear simultaneous fitting of S and the complexing parameters of metal titrations for a two ligand model. We have tested its fitting power with CLE-AdCSV data from the analysis of copper complexation in estuarine waters and computer generated data in order to keep control of the number of ligands present. Using both, non linear and linear fitting methods, we demonstrated that full correction of S is impossible if our binding model does not include all the ligands present in the sample. This includes iterative correction of S and simultaneous non linear fitting of S. A long extension of the titration and meeting the condition $\propto'_{CuSA} <= \propto'_{\Sigma CuL}$ are essential in order to produce reliable complexing parameters.

We proved that despite the promising results that non-linear fitting for 5 parameters gave for error-free multiligand solutions, when challenged by error–added titrations (computer generated and empirical), biased solutions are obtained. We established via computer generation of titrations using different conditions (heterogeneity, ligand binding strength, initial metal concentration and titration extension) that the instability concentrates in two of the parameters: S and K'_S and that it is caused by a combined effect of the analytical error, the analytical windows and the $[Cu]_{INI}/[L_S]$ ratio. Results in computer generated titrations mimicked perfectly the problems found with titration of natural samples.

In this work we recommend the combined use of linear and non linear fitting methods in order to avoid overestimations of S and K'_S. Linear methods, despite relying on arbitrary decisions, did not produce faulty K'_S. We demonstrated that at properly extended titrations, the error introduced by trying to correct or fit S could be much higher than the

underestimation introduced by using S^{INT}, dramatically affecting the estimation of the complexing parameters.

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Method	$[L_S]$ (nM)	$\log K'_S$	$[L_W]$ (nM)	$\log K'_W$	S ($nAnM^{-1}$)	pCu
Linear, S^{INT}	16.8 ± 0.2	15.47 ± 0.06	156.9 ± 1.9	12.37 ± 0.07	0.4760	15.366
Linear, $S^{ITE,lin}$	17.2 ± 0.2	15.47 ± 0.06	178.4 ± 0.2	12.16 ± 0.01	0.4971	15.391
Non-linear, $S^{FIT(3)}$	-	-	151.1 ± 11.7	12.82 ± 0.13	0.4522	13.995
Non-linear, $S^{ITE,non}$	42.1 ± 4.1	14.55 ± 0.12	154.6 ± 4.1	12.06 ± 0.05	0.4979	15.096
Non-linear, $S^{FIT(5)}$	39.0 ± 5.1	14.61 ± 0.11	147.2 ± 10.0	12.15 ± 0.10	0.4871	15.113
Non-linear, S=0.5	42.6 ± 5.0	14.54 ± 0.10	156.2 ± 13.0	12.05 ± 0.09	0.5000	15.092

Table 1. Complexing parameters, sensitivity and free copper ion concentration from applying different fitting methods to an error-free titration. Generated from the following characteristics: $[L_1]$= 10 nM, ($\log K'_1$= 16), $[L_2]$=40 nM, ($\log K'_2$=14) and $[L_3]$=150 nM, ($\log K'_3$=12). $\propto'_{SACu}$=40,000; S=0.5 nA nM^{-1}; $[Cu]_{TOT}$= 9.48 nM; $[Cu]_{MAX}$=586 nM; pCu=15.417. Linear solutions from 4 (L_S) and 6 (L_W) pairs of data.

Fitting method	$[L_S]$ (nM)	log K'_S	$[L_W]$ (nM)	log K'_W	S ($nAnM^{-1}$)	pCu
Linear, S^{INT}	8.0 ± 2.1	14.42 ± 0.13	36.6 ± 0.51	12.22 ± 0.06	0.4784	13.232
Linear, $S^{ITE,lin}$	10.2 ± 0.1	13.97 ± 0.01	41.5 ± 0.2	11.96 ± 0.01	0.4850	13.225
Non-linear, $S^{FIT(3)}$	-	-	37.7 ± 2.7	12.62 ± 0.10	0.4708	13.019
Non-linear, $S^{ITE,non}$	10.3 ± 0.1	13.93 ± 0.02	41.3 ± 0.1	11.96 ± 0.01	0.4850	13.222
Non-linear, $S^{FIT(5)}$	10.2 ± 0.1	13.96 ± 0.02	41.0 ± 0.1	11.97 ± 0.01	0.4846	13.227
Non-linear, S=0.5	15.1 ± 1.5	13.49 ± 0.11	54.8 ± 1.3	11.61 ± 0.05	0.5000	13.255

Table 2. Complexing parameters, sensitivity and free copper ion concentration from applying different fitting methods to a error-free titration. Generated from the following characteristics: $[L_1]$= 10 nM, (log K'_1= 14), $[L_2]$=40 nM, (log K'_2= 12) and $[L_3]$=150 nM, (log K'_3= 10). $\propto'_{SACu}$=40,000; S=0.5 nA nM^{-1}; $[Cu]_{TOT}$= 10.76 nM. $[Cu]_{MAX}$= 464 nM; pCu=13.243. Linear solutions from 3 (L_S) and 7 (L_W) pairs of data.

Fitting method	S (s.e.)	$[L_S]$ (s.e.)	log K'_S (s.e.)	$[L_W]$ (s.e.)	log K'_W (s.e.)	pCu
TW49 (diluted 1:1 with UV digested TW49)						
non linear 1LM $S^{FIT(3)}$	0.3034 (0.0097)	-	-	166.4 (14.8)	13.26 (0.09)	14.45
non lin 2LM S^{INT}	0.2972 (0.0020)	27.4 (10.3)	15.10 (0.35)	143.5 (9.3)	13.13 (0.10)	15.38
non lin 2LM $S^{ITE,non}$	0.3254	27.9 (6.5)	14.66 (0.16)	182.1 (5.5)	12.91 (0.05)	15.00
lin 2LM $S^{ITE,lin}$	0.3285	32.6 (0.4)	14.72 (0.19)	184.4 (2.0)	12.85 (0.02)	15.12
non lin 2LM $S^{FIT(5)}$	0.3302 (0.0106)	29.6 (8.4)	14.63 (0.17)	188.9 (15.4)	12.87 (0.11)	15.00
TW25						
non linear 1LM $S^{FIT(3)}$	0.2898 (0.0249)	-	-	178.1 (28.9)	12.26 (0.11)	13.13
linear 2LM S^{INT}	0.2944 (0.0207)	25.1 (1.6)	14.10 (0.35)	182.4 (15.7)	11.89 (0.09)	13.67
non lin 2LM S^{INT}	<u>0.2944</u>	15.1 (5.7)	21.98 (7.85)	178.6 (6.1)	12.08 (0.07)	13.53
non lin 2LM $S^{ITE,non}$	0.3526	16.6 (4.3)	22.50 (8.1)	246.5 (6.5)	12.03 (0.05)	13.75
lin 2LM $S^{ITE,lin}$	0.4510	24.4 (1.1)	14.39 (0.78)	375.2 (6.1)	12.77 (0.06)	14.38
non lin 2LM $S^{FIT(5)}$	0.3503 (0.0388)	16.5 (5.2)	22.31 (8.02)	243.9 (42.9)	11.97 (0.09)	13.68
non lin 2LM $S^{FIT(4)}$, S=0.3503	<u>0.3503</u>	16.5 (4.3)	23.00 (8.65)	244.0 (6.5)	11.97 (0.05)	13.68
non lin 2LM $S^{FIT(4)}$, logK_S=14.10	0.3557 (0.0613)	24.0 (11.2)	<u>14.10</u> (0.33)	248.3 (70.4)	12.10 (0.17)	13.78
non lin 2LM $S^{FIT(3)}$, S=0.3503, logK_S=14.10	<u>0.2944</u>	19.0 (9.2)	<u>14.10</u> (0.46)	176.0 (7.9)	12.06 (0.10)	13.51
TW13						
non lin $S^{FIT(5)}$ 5 μM SA	0.3760 (0.0155)	14.4 (6.3)	22.22 (8.14)	48.5 (8.7)	12.37 (0.50)	13.42
non lin $S^{FIT(5)}$ 10 μM SA	0.3738 (0.0099)	15.9 (4.9)	14.56 (0.41)	22.6 (4.2)	12.76 (0.27)	13.66

Table 3. Complexing parameters, sensitivity and free copper ion concentration from applying different fitting methods to copper titration data obtained by CLE-AdCSV of different samples collected at the Tagus estuary. All ligand concentrations in nM, sensitivities in $nAnM^{-1}$ (t_{dep} = 60 s). Underlined values: values fixed using the constraints option

Conditions	Error	$[L_S]$ [min-max]	log K'_S [min-max]	$[L_W]$ [min-max]	log K'_W [min-max]	$S^{FIT(5)}$ [min-max]	# logK'_S > 20
$[Cu]_{INI}$=10 nM; $\propto'_{CuSA}$=15,000	3%	20.3 [17.3-22.9]	14.0 [13.9-14.1]	100 [88-114]	12.0 [11.9-12.1]	0.51 [0.45-0.59]	0
	4%	19.9 [17.7-23.5]	14.0 [13.9-14.1]	102 [89-111]	12.0 [11.9-12.1]	0.50 [0.46-0.55]	0
	5%	20.1 [17.4-23.9]	14.0 [13.9-14.1]	102 [89-124]	12.0 [11.9-12.1]	0.50 [0.44-0.61]	0
	6%	21.4 [17.7-28.5]	14.0 [13.9-14.1]	111 [86-151]	12.0 [11.8-12.1]	0.55 [0.44-0.73]	0
$[Cu]_{INI}$ =20 nM; $\propto'_{CuSA}$=15,000	3%	19.8 [16.1-23.8]	14.1 [13.8-14.7]	99 [92-108]	12.0 [11.9-12.1]	0.50 [0.46-0.54]	0
	4%	21.0 [16.5-25.0]	14.0 [13.7-14.6]	102 [92-117]	12.0 [11.9-12.1]	0.51 [0.46-0.58]	0
	5%	20.2 [15.1-27.4]	14.5 [13.7-**22.7**]	102 [87-129]	12.0 [11.8-12.1]	0.51 [0.44-0.63]	1
	6%	20.6 [15.7-33.5]	14.1 [13.5-15.5]	104 [93-124]	12.0 [11.7-12.1]	0.52 [0.47-0.61]	0
$[Cu]_{INI}$ =10 nM; $\propto'_{CuSA}$=86,000	3%	20.9 [15.6-24.3]	14.2 [13.9-14.9]	251 [49-772]	12.0 [11.8-12.4]	1.31 [0.42-5.62]	0
	4%	19.2 [13.4-23.4]	14.2 [13.9-15.1]	183 [37-848]	12.2 [11.8-12.6]	1.26 [0.40-8.44]	0
	5%	20.2 [16.4-24.4]	14.4 [14.0-15.4]	364 [40-955]	12.1 [11.7-12.8]	2.79 [0.40-11.49]	0
	6%	20.0 [11.9-32.3]	14.2 [13.6-15.1]	310 [36-1357]	12.1 [11.1-12.7]	1.37 [0.38-8.23]	0
$[Cu]_{INI}$=20 nM; $\propto'_{CuSA}$=86,000	3%	21.0 [14.4-28.4]	14.2 [14.0-14.9]	304 [48-916]	12.0 [11.8-12.4]	1.50 [0.41-5.99]	0
	4%	21.7 [12.1-29.3]	14.6 [13.9-**22.5**]	484 [36-1007]	12.2 [11.8-12.6]	4.12 [0.39-9.29]	2
	5%	19.4 [12.0-29.9]	15.8 [14.0-**23.5**]	427 [40-1225]	12.3 [11.9-12.6]	2.68 [0.41-7.77]	3

	6%	21.1 [11.5-43.3]	16.3 [13.7-**25.0**]	1122 [40-8470]	12.2 [10.4-12.8]	3.58 [0.39-9.91]	4

Table 4. Complexing parameters and sensitivity from applying non linear fitting of 5 parameters. Four groups of 20 titrations were generated at increasing error percentages and solved at every combination of $[Cu]_{INI}$ and $\propto'_{CuSAx}$. All ligand concentrations in nM, sensitivities in $nAnM^{-1}$. Computer titrations generated from the following characteristics: $[L_1]$=20 nM (log K'_{L1}=14), $[L_2]$=100 nM (log K'_{L2}=12) with $[Cu]_{MAX}$=240. log K'_S values flagged as sure artifacts in bold, their frequency over the 20 titration series is shown in the last column.

FIGURE CAPTIONS

Figure 1. Ligand concentrations (A and C), conditional stability constants (B and D), sensitivity (E) and free copper ion concentrations (F) obtained for a two ligands model following three different methods: green triangles: iterative linear fitting with S^{INT}; red squares: iterative linear fitting with $S^{ITE,lin}$, blue diamonds: non linear fitting obtaining $S^{FIT(5)}$. All data generated from the following characteristics: $[L_1]$= 20 nM (log K'_{L1}=14), $[L_2]$=100 nM (log K'_{L2}=12), $[L_3]$= 200 nM (log K'_{L3}=11); α'_{CuSA}=15,000; $[Cu]_{INI}$=10 nM; pCu= 14.085; S=0.5 nA nM^{-1}. Arrows indicate preset values. Determination repeated stretching the titration data range from $[Cu]_{MAX}$ =80 to 1000 nM.

Figure 2. Ligand concentrations (A and C), conditional stability constants (B and D), sensitivity ($S^{FIT(5)}$, E) and free copper ion concentrations (F) (as the ratio in between pCu for L_S and L_W with respect to L_1, L_2 and L_3) obtained for a two ligand model by non linear fitting of error-free titration data generated from the following parameters: $[L_1]$= 20 nM (log K'_{L1}= 14), $[L_2]$=80 nM (log K'_{L2}= 12) and $[L_3]$=200 nM with S =0.5 nA nM^{-1}. Blue solid diamonds: α'_{CuSAx}=15,000, logK'_{L3}=10; red solid squares α'_{CuSAx}=86,000, logK'_{L3}=10; blue empty diamonds: α'_{CuSAx}=15,000, logK'_{L3}=11; red empty squares: α'_{CuSAx}=86.000, log K'_{L3} =11. Arrows indicate preset values

Figure 3. Sensitivity ($S^{FIT(5)}$, left panel) and complexing parameters for L_W (right panel) obtained for a two ligands model by non linear fitting of error-free titration data generated from the following parameters: $[L_1]$=20 nM (log K'_{L1}=14), $[L_2]$=80 nM (log K'_{L2}=12). Data generation was repeated in a α'_{CuSA} range 5 orders of magnitude wide (10^2-10^7). Lines show the preset values used for the generation of the different titrations and red arrows points to the value of log $\alpha'_{\Sigma CuL}$ (6.32).

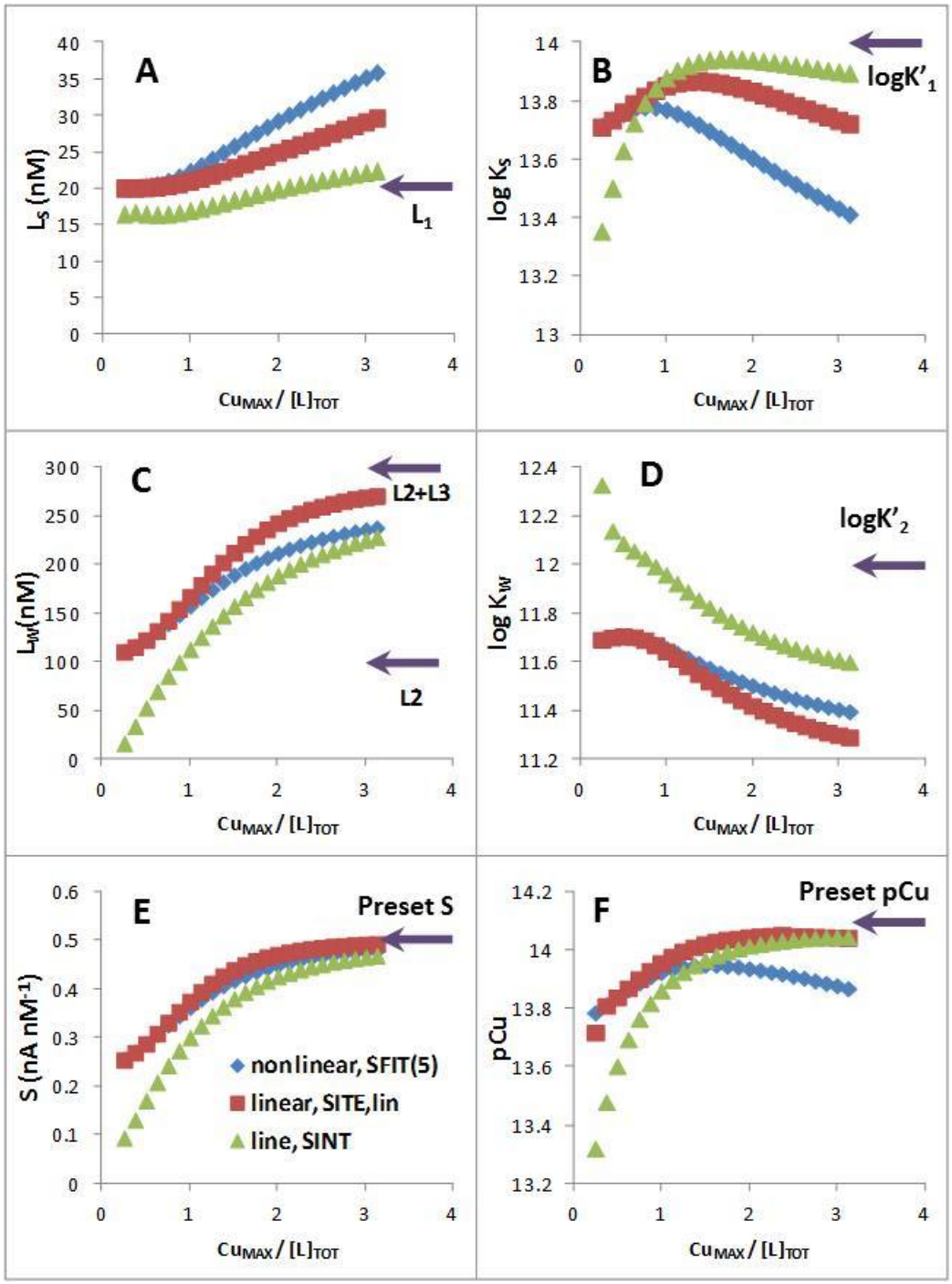


Figure 1

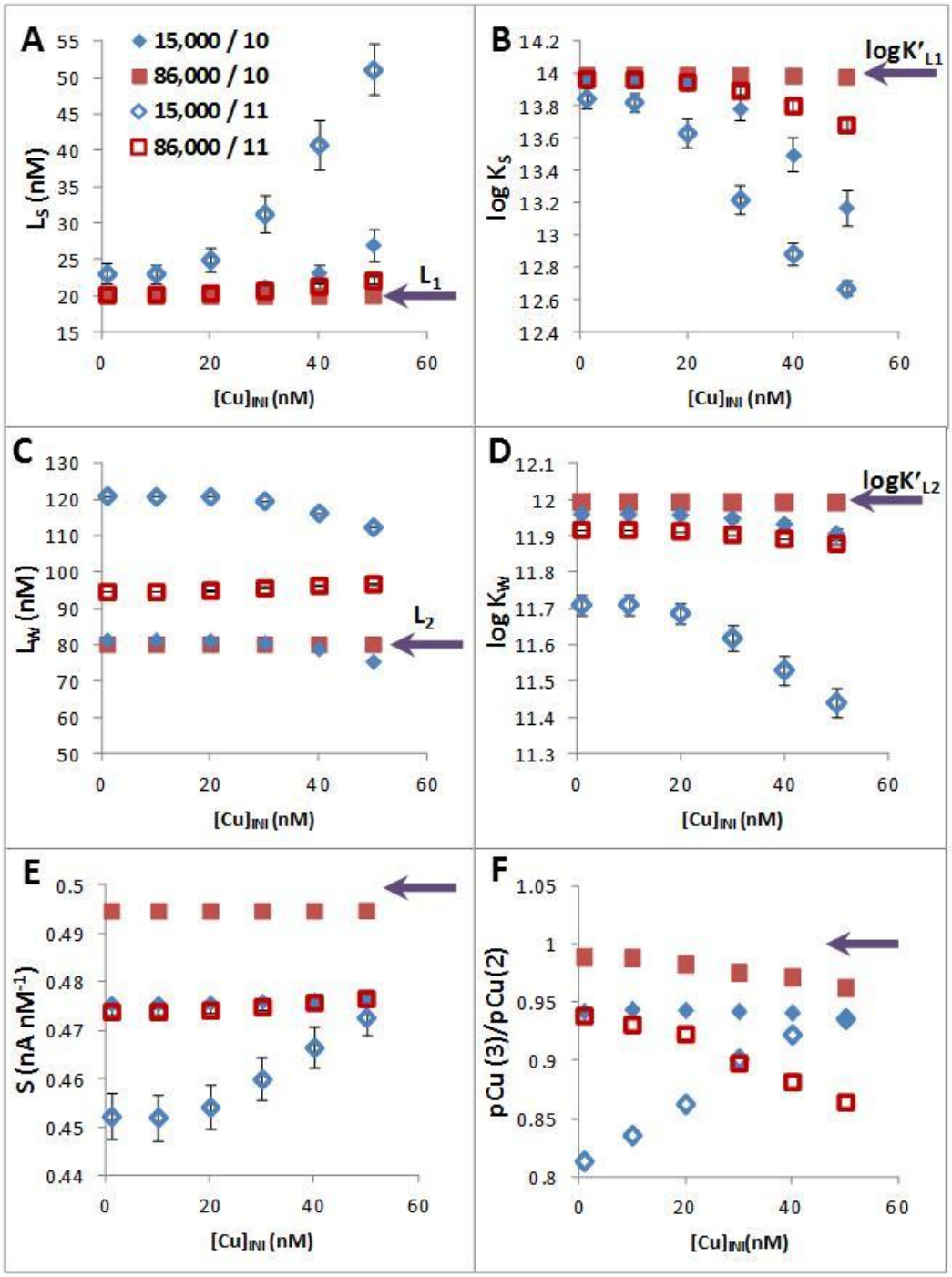


Figure 2

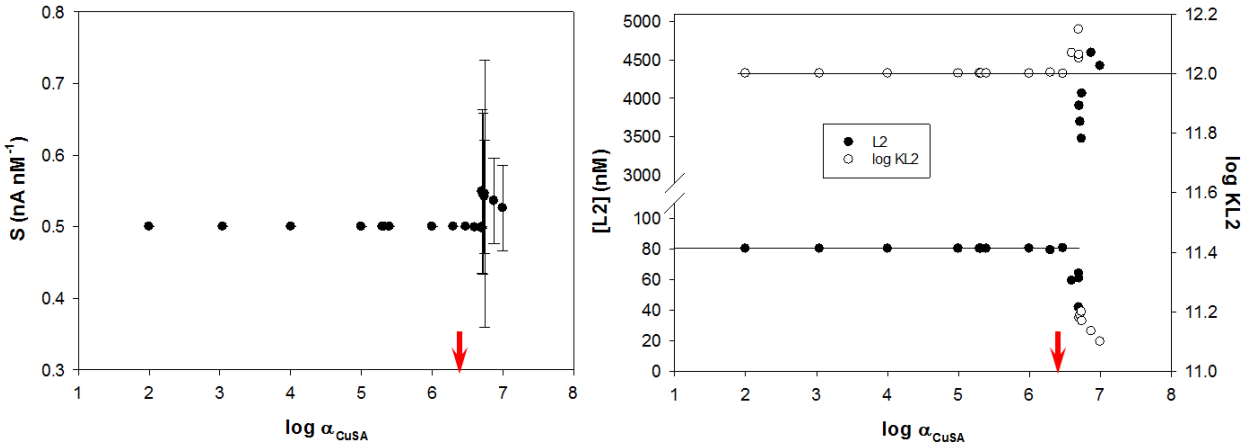
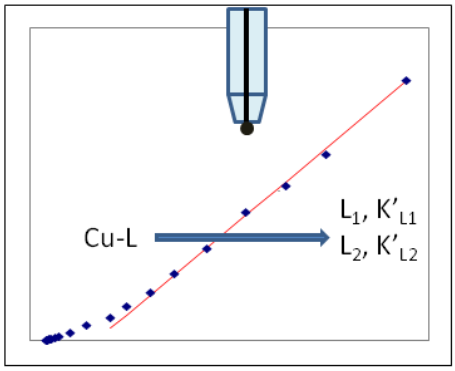


Figure 3



Graphical abstract

Highlights

Sensitivity of metal titration underestimated if model ligands less than sample ligands

Nonlinear fitting (Levenberg-Marquardt algorithm) gives flawed complexing parameters

Flawed values from balance of error, analytical window and total metal concentration

Bias accumulates in sensitivity and stability constant of strongest ligands

Assessment improved by combining linear and nonlinear fitting