Salt-marsh areas as copper complexing ligand sources to estuarine and coastal systems

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Abstract

Dissolved copper levels, copper complexing capacities and conditional stability constants have been determined in the Tagus estuarine waters and one of the saltmarshes located in this estuary, the Rosario saltmarsh. Tagus estuarine waters show a constant and around 20 nM copper concentration during the estuarine mixing. Most of this copper is organically complexed by a strong ligand (L_1) with a concentration that varies between 19-55 nM and a log K´ between 14.14-15.75. In addition L1/Cu ratios are quite constants and close to 1 all through the estuary, indicating the same source. A second and weakest ligand (L_2) was also detected in these waters in higher concentrations (36-368 nM) but with a lower log K´ that varies between 12.06-13.13. The present work has demonstrated that salt-marsh areas are important and continuous sources of copper complexing ligands to the Tagus estuary. Noticeable, tidal induced transport continuously feed these waters with copper and ligands, mainly with the strongest one. This continuous input, together with the high residence times of this system results in a quite constant concentration along the salinity gradient. This input represents 95% of the ligand present in the estuary.

Keywords: copper; ligands; speciation; estuary; salt-marsh; Tagus

1. INTRODUCTION

Salt-marshes are among the most common and extensive intertidal habitats along temperate coastlines. Several studies have shown that salt marshes incorporate large quantities of anthropogenic metals into the colonized sediments (Caçador et al., 1993; Sundby et al., 1998; Caetano et al., 2007). Root-sediment

interactions appear to contribute to the metal enrichment of colonized sediments and belowground biomass (Caçador et al., 1993) as well as porewaters (Santos-Echeandía et al., 2010).

Not only the concentrations, but also trace metals chemical forms in estuarine water (porewater and water column) may control their bioavailability and toxicity. In the water column, speciation of many biologically active trace metals is controlled by complexation with strong organic ligands (Bruland et al., 1991). Copper (Cu) is probably the most studied metal in terms of organic complexation in seawater and estuarine environments. This is because copper is a micronutrient, but it is also toxic, for example for microalgae, at relatively low concentration levels. In addition, Cu forms complexes of relatively high stability with various organic ligands. However, no many studies about copper complexation in salt-marsh areas (Mucha et al., 2008) and its influence on the ligand budget of an estuary have been published till these days.

Several sources of organic matter and ligands to estuarine and coastal areas have been pointed out in the last years determining trace metal distribution along the salinity gradient (Laglera and van den Berg, 2003; Santos-Echeandia et al., 2008). In this way, autochthonous organisms are major producers of the ligands that dominate the complexing capacity of open ocean and coastal productive waters (Croot et al., 2000; Gordon et al., 2000). Organisms such as coccolithophores (Leal et al., 1999), cyanobacteria, dinoflagellates (Croot et al., 2000) and heterotrophic bacteria (Gordon et al., 2000) have been observed to excrete strong Cu-complexing ligands, most likely as a defense mechanism against metal toxicity. The input of terrestrial humic substances through riverine waters has also been recognized as an important source of strong ligands to estuarine environments (Kogut and Voelker, 2001; Shank et al., 2004a; Laglera et al., 2009).

Recent studies have shown that estuarine sediments can also act as a significant source of Cu-complexing ligands to the overlying water, which may strongly influence the biogeochemistry and cycling of dissolved Cu by sediment/water exchange (Skrabal et al., 1997, 2000; Shank et al., 2004b, Chapman et al., 2009). This source of organic ligands would be enhanced if sediments are colonized by plants (Mucha et al., 2008). In the last years, several studies conducted in salt marsh areas, have shown that as roots die they supply important quantities of organic matter to the sediment (Caçador et al., 2004; Pereira et al., 2007). In addition, low molecular weight organic acids are exudated by salt-marsh

plant roots (Mucha et al., 2010) that can bind and redistribute metals (Mucha et al., 2008) due to their high affinity to organic matter.

As tidal water floods the salt marsh, large quantities of organic material, nutrients and trace metals accumulated in the area are exported to the estuary. On a semidiurnal tidal scale, advective fluxes are enhanced in salt marsh sediments (Santos-Echeandía et al., 2010) through a mosaic of small channels in the upper sediments. The relevance of the tidal induced transport on copper and complexing ligands may be crucial to understand the biogeochemical cycles of metals in coastal ecosystems where salt-marshes occupy large extensions.

Thus, the objectives of this work are: a) to measure dissolved copper and complexing ligand concentrations in the Tagus estuarine waters b) to determine dissolved copper and complexing ligand concentrations in the Rosário salt-marsh flooding and porewaters c) to estimate dissolved copper and ligand advective fluxes in the Rosário saltmarsh d) to ascertain the importance of this input over the Tagus estuarine waters.

2. MATERIAL AND METHODS

2.1. Study area

The 340 km^2 of the Tagus estuary represent one of the largest transitional systems in Europe. The estuary is composed by a large shallow inner bay and a deep straight and narrow inlet channel (Fig. 1). This channel reaches a depth of 40 m and constitutes the deepest part of the estuary. The bay has a complex bottom topography with channels, tidal flats and islands. The deepest channel, with a water depth of 5-10 m, is an extension of the inlet channel. The total amount of water in the estuary is around 1.9 km^3. The southern and eastern parts of the bay contain extensive inter-tidal mudflats; the northern part contains tidal flats, islands and several smaller channels. These channels merge upstream to a single narrow channel, marking the entrance of the Tagus River.

The Tagus River is the main source of freshwater to the estuary. The discharge usually shows a pronounced dry season/wet season as well as large inter-annual variation. The average annual discharge is ~400 $m^3\ s^{-1}$, but seasonally the average monthly discharge may vary from 1 $m^3\ s^{-1}$ to > 2200 $m^3\ s^{-1}$ (Loureiro, 1979). Consequently, the residence time of freshwater in the estuary is highly variable and

may range seasonally from 6 to 65 days (Braunschweig et al., 2003). The tides are semi-diurnal, with amplitudes at Lisbon ranging from ~ 1 m at neap tide to ~ 4 m at spring tide. The tidal effect reaches 80 km landward of the estuary mouth. Most pollutants are discharged from upper to the lower estuary (Fig. 1). Indeed, apart from being a major harbor, the fishing activities in the estuary are adversely affected by the inflow of effluents from about 3.5 million Greater Lisbon inhabitants, part of them untreated, coupled with industrial (chemicals and petrochemicals) and agricultural (fertilizers and pesticides) contributions.

Almost 40% of the estuary is composed by inter-tidal mudflats mainly in southern and eastern shores, containing extensive areas of salt marshes colonised mainly by *Sarcocornia fruticosa, Sarcocornia perennis, Halimione portulacoides* and *Spartina maritima* occupying an area around 20 km^2. The marsh selected for this study (Rosário) is located in the southern shoreline of the estuary (Fig.1). It covers an area of 2 km^2 (Crespo, 1993), being characterised by homogeneous stands of *S. maritima* as a pioneer species in the lower part, pure stands of *H. portulacoides* across the 20-50 cm elevation transect, and *S. fruticosa* and *S. perennis* in the higher salt marsh. The marsh is fully inundated twice a day by tidal action (2-4 m of tidal amplitude) through a highly branched system of channels that cross the elevation transect. The channels have 0.5-1.5 m depth promoting the inundation of the higher marsh even at low amplitude tides.

2.2. Sampling

A sampling cruise along the Tagus estuary was conducted on the 5th May 2010. A 93 m^3 s^{-1} flow was measured during that day what gives a residence time around 62 days for the water in the estuary (Braunschweig et al., 2003). Ten sub-surface water samples (TWB11-TWB410) were collected by hand in 1 L low density polyethylene (LDPE) bottles from a plastic boat (Fig. 1) during the low tide covering the salinity gradient of the estuary. In addition, flooding water during tidal inundation was sampled in two sites of the Rosário salt marsh (non-vegetated area and *S.maritima* colonised area and separated by less than 20 m). At low tide, when sediment was exposed to the atmosphere, two sediment cores (10 cm long) were collected at each site (vegetated and non-vegetated). The cores were sliced immediately after sampling in two 4 cm layers, prepared composite samples for each layer of the cores collected at each site, and material stored in acid pre-cleaned HDPE vials avoiding air presence inside. Sampling took

place in less than 3 min. Core material in the colonized area consisted of dense rooting sediments with no evidence of burrowing worms, crabs or bivalves. When tidal water starts to flood each site, flooding water was collected at each inundation time: 1, 5, 10, 15, 20 and 30 min for the non-vegetated area and 1, 5, 10, 15, 20, 30, 45 and 85 min for plant colonized area. Flooding water was sampled 1 cm above the sediment surface directly into acid pre-cleaned syringes. The water and sediment samples were kept in refrigerated boxes and immediately transported to the laboratory. A more detailed description of these sampling procedures can be found in previous works (Caetano et al., 2007).

2.3. Sample treatment

Estuarine and flooding water samples were filtered through 0.45 µm cellulose membranes placed in previously acid-cleaned filtration units (Nalgene). Filters were frozen and stored at -20°C pending analysis of particulate copper and aluminium. The main reason for measuring particulate aluminium is that it acts as a tracer of lithogenic or terrigenous material (Windom et al., 1989; Pohl et al., 2004) and will be useful for the interpretation of the various sources of material to estuarine waters (i.e. rivers or sediment resuspension) (Mota et al., 2005).

Pore waters were separated from the sediment layers by centrifugation at 10,160 rcfxg for 30 min at +4 °C and filtered through 0.45 µm cellulose acetate membranes. Filtration and subsequent manipulation of the samples were carried out in a glove box under argon atmosphere in order to avoid alteration of the initial conditions (Caetano et al., 2007; Santos-Echeandia et al., 2009). A portion of the filtered samples (around 200 mL) was frozen for speciation analysis while around 100 mL were acidified with suprapure HCl (pH < 2) and stored pending analysis of total dissolved copper.

2.4. Copper analysis in estuarine water

2.4.1. Total dissolved copper concentrations

Copper analyses were carried out using voltammetric equipment (Metrohm 797 VA Computrace) controlled by a computer (PC). The reference electrode was a double junction, Ag/AgCl, KCl (3 M), saturated AgCl, with a salt-bridge filled with 3 M KCl, and the counter electrode was a glassy carbon rod. A hanging mercury drop electrode (HMDE) was used as the working electrode. The copper concentration in the filtered samples was determined using a procedure similar to the one described

previously (Campos and van den Berg, 1994). Estuarine and flooding water was UV-digested (1 h) after acidification to pH 2.2 by the addition of 10 µL 6 M bidistilled HCl (AnalR BDH) per 10 mL of sample in acid-cleaned borosilicate glass tubes. A 10 mL sample aliquot was pipetted into the voltammetric cell and ammonia (AristarGrade Merck) was used to ascertain the approximate neutralization of the pH; also, HEPES buffer (BDH, final concentration 0.01 M) and Salycildoxime (SA, Sigma, final concentration of 25 µM SA) were added. The solution was deaerated by purging (5 min) with nitrogen. The voltammetric parameters were: deposition 60 s at -1.1 V whilst stirring, 8 s quiescence at -0.1 V, and a potential scan using the square-wave modulation: 10 Hz, step height 2.5 mV, pulse height 25 mV, from 0 to -0.8 V. The sensitivity was calibrated by incorporating standard copper additions (Spectrosol BDH) to each sample. Each sample was analyzed in triplicate. A blank with Milli-Q water (18.2 MΩ.cm) was made at intervals of every three samples and its concentration was subtracted in order to eliminate any copper contribution from reagents. Blank concentrations were around 0.27±0.06 nM.

2.4.2. Copper titrations

The copper complexing capacity of the waters of the Tagus estuary was determined by competing ligand exchange with adsorptive cathodic stripping voltammetry (CLE-AdCSV) of Cu-salycilaldoxime (SA) complexes according to previous works on copper complexation in estuarine waters (Campos and van den Berg, 1994; Laglera and Van Den Berg, 2003). Briefly, a 170-ml sample previously filtered by 0.45 µm was transferred to a polyethylene bottle (Nalgene), and spiked with HEPES/NH_4OH buffer (for a concentration of 0.01 M) and SA (mix concentration of 5 or 10 µM). After stirring, 10 mL aliquots were pipetted into 15 polystyrene vials (30 mL Bibby, Sterilin) that were previously spiked with copper to provide increasing concentrations in the range of 0–700 nM (actual range depending on the initial copper concentration and the ligand concentration to be determined). Prior to the first titration, the tubes were conditioned twice overnight with seawater containing the same range of copper concentrations in order to avoid copper loss in the tube walls. After that, aliquots of each sample were left to equilibrate overnight at room temperature due to the slow kinetics of the ligands and copper reactions (Campos and van den Berg, 1994). The equilibrium concentration of Cu-SA complexes was determined using the following parameters: adsorption potential of- 0.15 V, deposition time of 60 s, 8 s

quiescence, potential scan from 0 to -0.8 V using square-wave modulation at 10 Hz, step height 2.5mV and pulse height of 25 mV.

2.4.3. Evaluation of complexing capacities and conditional stability constants

Changes in the ratio between the concentrations of CuSA (labile to AdCSV) and CuL in the different aliquots permit the determination of the copper complexing capacity of the ligands present in solution (C_L) and their conditional stability constant to bind copper (K'_{CuL}). A more detailed explanation can be found elsewhere (Ruzic, 1982; van den Berg, 1982). The relationship between the different copper fractions is described by the following transformation of the Langmuir isotherm:

$$[Cu]_{TOT} = \Sigma (C_{Li} [Cu]_{lab}/K'_{CuLi\alpha'} + [Cu]_{lab}) + [Cu]_{lab} \quad (1)$$

L_i represents the variability of the nature of the different natural ligands. Those ligands are differentiated according to the stability of their complexes with copper. α' is the α-coefficient of Cu^{2+} with inorganic ligands and SA. $[Cu]_{TOT}$ and $[Cu]_{lab}$ are respectively the total and labile copper concentrations related by:

$$[Cu]_{TOT} = \Sigma[CuLi] + [Cu]_{lab} = \Sigma [CuLi] + [Cu(SA)] + [Cu(SA)_2] \quad (2)$$

The concentration of labile copper is calculated from:

$$[Cu]_{lab} = i_p/S \quad (3)$$

Where i_p is the voltammetric signal obtained from the reduction of the CuSA complexes adsorbed on the HMDE electrode and S is the ratio signal/concentration or sensitivity. S can be estimated from the slope of the last few points of the titration (S^{INT}) if the titration has been extended to copper concentrations enough to complete the saturation of the natural ligands.

As indicated in the previous section, samples were analyzed using two different SA concentrations (5 and 10 µM). Higher SA concentrations increase the competing ability of SA. In the presence of complex mixes of ligands characterized by the formation of complexes with copper in a wide range of stability constants, the effect of increasing the SA concentration is both, an overcompetition of those ligands of low affinity for copper and the equilibration of SA with ligands of very high stability constant. Therefore, in heterogeneous matrices, the shift of the SA concentration resolves a different fraction of ligands,

fraction that is defined as detection window. This is an important tool to estimate the heterogeneiety of the pool of copper ligands in the sample.

Values for the stability constants of the complexes CuSA and $Cu(SA)_2$ are necessary in order to obtain the value of α' at each salinity. Both stability constant were calculated using relationships obtained in a previous work (Campos and van den Berg, 1994):

$$\log K_{CuSA} = (10.12 \pm 0.03) - (0.37 \pm 0.02) \log \text{salinity} \quad (4)$$

$$\log \beta'_{Cu(SA)2} = (15.78 \pm 0.08) - (0.53 \pm 0.07) \log \text{salinity}. \quad (5)$$

A plot of $[Cu_{lab}]$ vs the ratio $[Cu]_{lab}/[CuL]$ is the most used procedure to solve C_L and K'_{CuL} from the slope and the Y-axis value respectively. When only one ligand is present, the data follow a straight line but if more than one type of ligands are present, the plot takes a curved shape. In this case C_L and K'_{CuL} can be calculated with accuracy for a maximum of two types of ligands.

The estimation of C_{L1}, $K'_{CuL1,}$ C_{L2} and K'_{CuL2} requires either an iterative refine procedure (Laglera-Baquer et al., 2001; van den Berg, 1982) or a nonlinear fitting (Gerringa et al., 1995). In the first case the solutions for C_{L1} and K'_{CuL1} are refined with estimations of C_{L2} and K'_{CuL2} from different linear sections of the plot until a convergent solution is obtained.

Complexing capacities, conditional stability constants and S can also be obtained fitting non-linearly the substitution of Eq(3) in Eq(1). Non-linear fitting prevents the uncertainty caused by the arbitrary necessity to split the titration data in two that iterative linear fitting requires.

In this work, we have used the fitting tool of a popular computer program, SigmaPlot (© Systat Software, Inc) to fit 3, 4 or 5 parameters. The program uses a Levenberg-Marquardt fitting algorithm. We first solved the titration data sets for the hypothesis of a single ligand model using non-linear fitting incorporating S as a parameter to C_L and K. For this purpose, a new equation was incorporated to the Regression Wizard of the software under the "User-Defined" category. The Code fed to the Regression Wizard is presented in Table S1.

Then, we prepared a new Equation for the case of two types of ligands, i.e. 5 parameters (C_{L1}, $K'_{CuL1,}$ C_{L2} and K'_{CuL2} and S) but the program struggled to offer realistic values in the form of very high S and K'_{CuL1} values. Removal of S from the list of parameters and use of S^{INT} did not solve problems with $K'_{CuL1}$for

some titrations. In those cases, K'_{CuL1} was obtained from the iterative linear routine and its value fixed in the *constrains* section of the code (Table S1) during the non-linear fitting of the rest of the complexing parameters.

2.4.4. Particulate copper and aluminum

In order to determine the amount of labile copper, filters obtained from filtered water were subject to digestion overnight with 0.1 M acetic acid in Teflon® vials (Savillex) at ambient temperature following the first step of the BCR extraction procedure (European Community Bureau of Reference; Quevauviller et al., 1997). The digest was then syringed-filtered (0.45 µm) using a Swinnex filtration unit and stored pending analysis; filters containing the remnant particles were microwave digested using a mixture of HF and HNO_3 (1:3) in order to analyze the total fraction (Biscombe, 2004).

Labile copper determination was carried out by means of cathodic stripping voltammetry as explained before (Campos and van den Berg, 1994) while total particulate copper and aluminum determination was carried out with the standard additions method. Concentrations of Al and Cu were determined using a quadrupole ICP-MS (Thermo Elemental, X-Series) equipped with a Peltier Impact bead spray chamber and a concentric Meinhard nebulizer. Coefficients of variation for metal counts (n = 5) varied between 0.5 and 2%. The precision and accuracy of each metal concentration measurements, determined through repeated analysis of certified reference material (PACS2), using Indium as internal standard, were 1–4% and 2–5%, respectively (data not shown). Procedural blanks always accounted for less than 1% of the total metal concentrations in samples.

2.5. Estimation of sediment-water interchange: tidal induced fluxes

As water floods over inter-tidal sediments the pore water solutes tend to be exported by advection and/or exchanged between mixing top sediments and tidal water. The associated pressure difference and tidal water movement are the driven mechanism for this transport. By using the temporal variation of copper and ligand concentrations in the flooding water the tidal induce transport (T) to the water column is calculated using the following expression (Caetano et al., 2007).

$$T = \Sigma\, [(C_{t+1} - C_t)/(2 - C_i)](h_{t+1} - h_t) \qquad (6)$$

where C_{t+1} and C_t are solute (copper and ligand) concentrations in the flooding water at times t+1 and t, C_i is the residual concentration (lowest value obtained for the flooding water) and ht and h_{t+1} are the water depth at the same times. It was observed that water depth during the field measurements increased on the average 0.5 and 0.8 cm per minute of inundation in *S. maritima* and non-vegetated areas, respectively. The tidal induced transport of trace metals is calculated for the first 85 and 30 min of inundation, respectively. Since inter-tidal sediments are inundated twice a day, the tidal induce transport (*T*) was multiplied by a factor of two to present values on a daily basis.

2.5. Statistics

The statistical analyses were performed using STATISTICA 6 (Statsoft).

3. RESULTS

3.1. Determination of the complexing parameters and the sensitivity by non-linear fitting

Table 1 shows the complexing parameters found for the estuarine water samples according to one and two ligands models. Table 2 shows those parameters for flooding waters and interstitial waters extracted from cores. For the one ligand model, S was added to the fitting procedure according to the code shown in Table S1. Values for S were in the range 94-113 % with respect to S^{INT} (average 98.9±4.1 %) indicating that any bias due to undersaturation of the ligands present in the sample caused by a too small copper concentration at the end of the titration was masked by the analytical error. For the sample collected at station 11, S was fitted at a very low value 90% of S^{INT} and the complexing parameters were recalculated using S^{INT}.

For the two ligands model, this approach was not possible as explained above. For some titration sets, S was estimated to be 103-114 % of S^{INT} but for many others this correction reached as much as 170 % which could be discarded simply by visual inspection. Values in Table 2 were obtained using S^{INT}. When the estimation of K'_{CuL1} produced a value over 20, the titration data set was solved by iterative linear fitting (Laglera-Baquer et al., 2001; Laglera and Van Den Berg, 2003) and the value of K'_{CuL1} fixed on the constrains section of the code. This led to a 3 parameters non-linear fitting. An extensive description of the routine to fit complexing parameters out of metal titration data sets used here is presented in a separate work (Laglera et al., 2012).

3.2. Dissolved copper and its organic speciation in the Tagus estuary

Dissolved copper concentrations along the salinity gradient in the Tagus estuary are plotted in Fig. 2a. With the exception of the station located upstream in the river (26.4 nM), values were quite constants and around 20 nM all over the estuary. The ligand concentration (L; Fig. 2a), for the 5µM SA detection window, showed higher values in riverine samples (399 nM) decreasing to 320-340 nM in the first 3 units of salinity. A new decay is found from salinity 5 to 25 (to 46 nM). This decreasing trend from fresh to marine waters has been observed before in other estuarine systems (Laglera et al., 2003; Plavsic et al., 2009). The conditional stability constant for these ligands (log K´) ranges between 12.20 and 13.26 (Table 1), and it presents a bowl-shape distribution along the salinity gradient, with lower values in the mid-estuary and maxima at marine (13.26) and riverine (13.11) end-members.

By fitting data to two types of ligands, a strong (L_1) and a weak (L_2) one can be observed in the Tagus estuary waters (Fig. 2a). Ligand L_1 varied between 14 and 55 nM being the highest concentration found in the two most riverine stations. Values decreased sharply to 15 nM at salinity 3 remaining almost constant until the marine end member (salinity 25). Otherwise, L_2 concentrations vary between 36 and 368 nM with a conservative distribution between salinities 5 and 25, but with lower values than expected for an ideal dilution line between salinities 0 and 5. The conditional stability constants for these ligands range between 14.10 and 15.75 for L_1 and between 12.06 and 13.13 for L_2 (Table1). The distribution along the salinity gradient is different among them; while log K_1' shows a saw tooth tendency with higher values in the riverine end-member, log K_2' distribution is comparable to log K´, with the lowest values in the mid estuary and increasing towards the river (13.13) or the sea (12.42) end members.

In order to detect stronger copper complexing ligands and check for homogeneity/heterogeneity of them in the estuary (van den Berg and Donat, 1992; Buck and Bruland., 2005; Santos-Echeandia et al., 2008), a detection window of 10 µM SA has been applied to some of the samples. Samples, fitted to one ligand are plotted in Fig. 2b. Ligand concentration increases between salinity 0 and 5 (from 121 to 142 nM) and progressively decreases to 30 nM at salinity 25 with a quite linear trend. The conditional stability constant (log K´) presented, again, lower values at mid salinities (13.11) increasing towards the riverine (14.52) and marine (13.76-14.15) end members (Table 1) as observed for the lower detection

window (5 µM SA). By fitting data to two types of ligands, the strongest one (L_1) concentrations ranged between 15 and 28 nM (Fig. 2b) with a quite irregular distribution. On the contrary, the weakest ligand of this detection window (L_2) shows a similar trend to L, with an initial increased from 145 to 174 nM decreasing progressively afterwards to 22 nM at salinity 25. The conditional stability constants, both log K_1' and log K_2' present higher values at low salinities (16.84 for K_1 and 13.87 for K_2) that sharply decreased at salinity 5 reaching 15.02 for log K_1' and 12.76 for log K_2' remaining relatively constant towards the marine end-member (Table 1).

3.3. Suspended Particulate Matter, particulate total and labile copper and aluminium concentrations in the Tagus estuary

Suspended particulate matter values oscillate between 4.8-14.7 mg L^{-1} in the estuary with an irregular distribution (Fig. S1a). The highest values where found at salinities 10-15 and the lowest between salinities 19-23. Particulate copper concentrations varied between 0.78 and 14.8 nM (Fig. S1b). The highest levels were found at mid-salinities (5-15 ups) while the lowest values measured at both end-members. The labile copper in the particulate matter accounts only for the 0.05-0.45 % of the total copper but it is noticeable that the lability was higher for the lowest salinities (Fig. S1c). Particulate aluminum concentrations ranged between 272 and 4930 nM with a similar distribution to particulate copper.

3.4. Dissolved copper and its organic speciation in the Rosario Saltmarsh

3.4.1. Flooding waters

Dissolved copper concentrations in the flooding water in the two sites of the salt marsh (non-vegetated and plant colonized areas) are plotted in Fig. 3a. Copper levels in the non-vegetated zone increase 1 minute after arriving of tide water from 18.3 to 28.5 nM. In the following 5 minutes, levels decreased reaching 20.6 nM and remaining quite constant until 30 minutes after tidal inundation started, with the exception of minute 15 when a less pronounced increase (27.0 nM) was observed (Fig. 3a). In a similar way, dissolved copper levels increased in the colonized area 1 minute after the flood started reaching higher values (32.5 nM) than in the non-vegetated area. Afterwards, concentrations decreased to 17.7 nM and varied irregularly between 13.6 and 22.1 nM during the next 80 minutes of inundation.

325 The time course evolution of ligand concentration in the flooding waters is also plotted in Fig. 3a. A net
326 increase of its concentration (initial levels of 46 nM) was observed in both sampling areas but reaching
327 higher concentrations in the plant colonized (291 nM) than in the non-vegetated area (85 nM) during
328 the first minute of inundation. Afterwards levels in plant colonized sediments decreased sharply while in
329 non-vegetated area values diminished progressively reaching, at both areas similar values to those
330 found before inundation started. The conditional stability constants of ligand showed higher values in
331 the non-vegetated (13.73-14.04) than in the plant colonized area (13.11-13.89) (Table 2). The
332 significance for statistical analyses was $p<0.05$.

333 In addition, the time course evolution of log K´ values during the tidal inundation were different among
334 the two sites: while an increase of log K´ was found one minute after the inundation started in the non-
335 vegetated area, a progressive increase of log K´ with time was observed in the vegetated area during the
336 first 10 minutes.

337 The strongest ligand (L_1) concentrations vary between 46 and 61 nM in the non-vegetated area without
338 a trend all over the time (Fig. 3b). Nevertheless, L_1 concentrations in the colonized area are more
339 variable and ranged between 28 and 95 nM. The conditional stability constant of this ligand varied
340 between 14.12 and 14.79 in the non-vegetated area while in the colonized area a variation between
341 13.61 and 14.83 was found (Table 2). The weakest ligand concentrations were higher and varied
342 between 60 and 107 nM for the non-vegetated area and from 5.5 to 258 nM for the colonized area (Fig.
343 3b). It is noticeable the net increase in the colonized area in the first minutes of inundation that is not
344 observed in the non-vegetated area. Finally, conditionals stability constant for this weakest ligand
345 oscillate between 11.95-12.26 nM in the non-vegetated area and 11.87-13.05 in the colonized area
346 (Table 2).

347 Strongest ligands have been detected in the flooding waters moving the detection window to 10 µM SA.
348 Ligand concentration were lower (61-87 nM) in the non-vegetated area than in the colonized site (107-
349 137 nM) during the first 5 minutes of inundation (Fig. 3c). An increase was found in the first minute
350 comparing to the estuarine waters in the vicinity of the salt-marsh and concentrations slightly decrease
351 after 5 minutes. The respective conditional stability constants were similar in the non-vegetated area
352 (14.39-14.48 nM) and in the plant-colonized area (14.32-14.47 nM) (Table 2).

353 Two types of strong ligands were found in the colonized area with concentrations ranging between 58
354 and 82 nM for L_1 and from 59 to 138 nM for L_2 with a similar trend to the unique ligand (L). However, L_1
355 levels do not decrease after 5 minutes but continue to increase (Fig. 3c). Conditional stability constants
356 ranged in the following intervals: 14.63-15.69 for L_1; and 12.84-13.44 for L_2 (Table 2).

357 *3.4.2. Porewaters.*

358 Dissolved copper and ligand concentrations and their conditional stability constants in the porewaters of
359 the salt-marsh are shown in Table 3. Levels of dissolved copper in the non-vegetated area ranged from
360 45.3 to 53.9 nM with higher values in the upper layer than in the deeper one. However, higher ligand
361 concentrations were found in deeper porewaters (321 nM) than in surface ones (245 nM). The
362 conditional stability constants for these ligands varied between 13.24 for the deeper layer and 13.58 for
363 the upper one. Levels of the stronger ligand (L_1) ranged from 77 to 184 nM with higher values in the
364 upper layer. Nevertheless, the conditional stability constant was higher (14.77) in the deeper layer than
365 in the upper one (13.70). Concentrations of L_2 varied between 70 and 287 nM with increased values in
366 the deeper layer. The conditional stability constants of this weakest ligand were 12.68 for the deeper
367 layer and 13.09 for the upper one.

368 Higher dissolved copper concentrations in pore waters (71-142 nM) were found in the plant colonized
369 area (Table 3). Ligand concentrations vary between 767 nM in the deeper layer and 831 nM in the upper
370 one. Conditional stability constant for this ligand showed higher values in the upper layer. The stronger
371 ligand, L_1, levels varied between 115 and 203 nM with higher values in the upper layer and their
372 conditional stability constants were 15.17 for the upper layer and 14.66 for the deeper one.
373 Concentrations of L_2 were quite similar among layers (666 and 699 nM) as the conditional stability
374 constants (13.09 and 12.82).

375 Strongest ligands in pore waters were found by applying a higher detection window (10 µM) (Table 3).
376 Ligand concentration in the non-colonized area was 230 nM with a conditional stability constant of
377 14.28. A strong ligand with a log K_1' of 15.25 and a concentration of 67 nM and a weak ligand with a log
378 $K_2'$of 13.85 and a concentration of 182 nM result from the data fitting to two types of ligands. As found
379 with the 5µM SA detection window, higher values were observed in the vegetated area at 10µM
380 detection window. A ligand concentration of 419 nM with a log K´ of 13.95 was registered. In the data

fitting to two types of ligands, a strong ligand concentration of 148 nM with a log K_1' of 17.56 was recorded, while the weakest ligand showed a concentration of 846 with a log K_2' of 13.51.

3.5. Tidal induced transport of copper and organic ligands in the Rosario Saltmarsh

An estimation of the tidal induced transport of dissolved copper and ligands is shown in Table 4. A flux of 1.23 µmol m^{-2} d^{-1} of dissolved copper escapes from the sediment to the overlying waters in the non-vegetated area, while the ligand transport was 3.25 µmol m^{-2} d^{-1}. If the two ligand case is considered, an export of 2.16 µmol m^{-2} d^{-1} of the strongest ligand and 2.31 µmol m^{-2} d^{-1} of the weakest one was estimated. As expected from the results shown in previous sections calculated transports are higher in the plant colonized area, where a 2.43 µmol m^{-2} d^{-1} flux of dissolved copper is estimated. The ligand flux (48.2 µmol m^{-2} d^{-1}) was around fifteen times higher than in the non-vegetated area. Increased transport of both strong and weak ligand to the water column was also found with 11.5 µmol m^{-2} d^{-1} for L_1 and 64.7 µmol m^{-2} d^{-1} for L_2.

The tidal induced transport of ligands detected by the 10 µM SA detection window was similar in the non-vegetated area and lower in the colonized area. In this way, 4.25 µmol m^{-2} d^{-1} of ligands were exported in the non-colonized area while 6.87 µmol m^{-2} d^{-1} were exported in the plant colonized area. For the two ligand case, 3.80 µmol m^{-2} d^{-1} of the strongest ligand and 5.77 µmol m^{-2} d^{-1} of the weakest one were estimated.

4. DISCUSSION

4.1. Copper and organic ligands behavior during the estuarine mixing

Dissolved copper and complexing ligand behavior in the Tagus estuary can be clearly divided in two distinct chemical areas. The edge is marked by the salinity 5.6 sample. In the low salinity stretch, although dissolved copper values were only higher in the most riverine sample, the strong ligand values (L_1) were higher than in the rest of the estuary. As a consequence the L_1/Cu ratio is relatively constant and around 1. The only exceptions are the two most riverine stations (Fig. S2) where an excess of L_1, which may derived from the river end member was detected. This ratio is linear and progressively decreases with the increasing salinity if total ligand concentration is considered (Fig. S2). Moreover, the weakest ligand (L_2) also showed a different behavior in this zone of the estuary but with lower levels

than expected if the trend marked by the seven most saline stations is extended to the freshwater end-member.

The mixing of freshwater with the marine water dilutes L_1 ligand within the estuarine area. The long residence times (Braunschweig et al., 2003), confirmed by uniform suspended particulate matter content in a wide salinity range (Fig. S1a), allow the homogenization of the water body justifying the constancy of copper and strong ligand levels in the 6-25 salinity range. This pattern suggest that the tidal effect superimposes the river flow and no losses of copper or strong ligands occured during the estuarine mixing. Similar findings have been observed in the Scheldt estuary by Laglera and van den Berg (2003). A continuous lateral input, as detected in the Vigo Ria (Santos-Echeandía et al., 2008), in the Tagus estuary may also explain the constant values all over the salinity gradient. In addition, this area shows constant and close to the unit L_1/Cu ratios in a wide salinity range (Fig. S2). This means that copper and L_1 levels are similar probably because their source is the same. There is no evidence of copper transfer from the dissolved to the particulate fraction since values were quite low all through the estuary (Fig. S1b). However, L_2 experiments a progressive decrease in their levels towards the outer part of the estuary and no remarkable inputs of L_2 are observed at mid-high salinities as observed for L_1. It can be assumed that the river is not the major source of this weak ligand, but its origin is somewhere in between salinities 0-6, where although the mixing with seawater, its concentration remains constant or even increases. This increase is consistent with the trend observed at both detection windows for the weakest ligand (Fig. 2). The higher particulate aluminum concentrations at salinities 6-10 (Fig. S1c) and SPM at salinity 10 could reflect the maximum turbidity zone in this salinity gradient and at spring tides the bottom particle resuspension in this area may explain the release of the weakest ligand from the sediments (Shank et al., 2004; Santos-Echeandia et al., 2008). If copper variability during the estuarine mixing is associated to L_1, L behavior is ruled by L_2. This fact is supported by the spatial distribution of log K'_2, which is quite similar to log K´ (Table 1) at both detection windows (5 µM y 10 µM).

Regarding the nature of the ligands, by comparing L_1 with L_2 at both detection windows, the distribution of the stronger ligand (L_1) was homogeneous, mainly at high salinities, because the log $K'_{1,5\mu MSA}$ and log $K'_{1,10\mu MSA}$ were similar (Table 1) and, in general, their ratio was close to 1 (Fig.S3a). Moreover, ratios between L_1 measured at the low detection window and the L_1 observed at the high detection window

436 were closer to 1 (1.24±0.33) along the salinity gradient (Fig.S3b). Conversely, L_2 was very heterogeneous
437 and a shift to the higher detection window induces the decrease of the ligand concentrations. Thus, the
438 ratio between L_2 measured at low detection window and at high detection window is around 2
439 (2.09±0.48) (Fig. S3b). An increase of the log K´ values is also observed when moving the detection
440 window to 10 µM SA. As a consequence the ratios between log K´at high and low detection windows are
441 always well below 1 all over the salinity gradient (Fig. S3a).

442 *4.2. Saltmarsh inputs of copper and complexing ligands to the estuary*

443 Intertidal and saltmarsh areas constitute one of the possible sources of copper and complexing ligands
444 to estuarine waters. Mucha et al. (2008) demonstrated that salt-marsh plants are able to produce
445 relatively high amounts of strong Cu-complexing ligands in their root system and the present study
446 evidenced that these complexes are exported to estuarine waters through tidal induced transport (Table
447 4).

448 The constant dissolved copper and L_1 concentrations in a wide salinity gradient (6-25) is presumably
449 explained by the lateral input from the marsh areas along the gradient coupled with high residence time
450 of water in the estuary. Previous studies have showed that copper levels in the adjacent area to the
451 Tagus (S≥30) varied between 1.6 and 14.6, well below the values found at salinity 25 (Cotté-Krieff et al.,
452 2000; Santos-Echeandia et al., 2012). The correlation between Cu and L_1 ($r=0.67$, $p<0.05$) in the Tagus
453 estuarine waters suggest that the source of copper and ligands at mid-salinities is diluted at high
454 salinities. The inexistence of marshes downstream the inlet that connects the estuary to the sea
455 reinforces the hypothesis that salt marshes are the main source of copper and complexing ligands.
456 Moreover, the conditional stability constants for L_1 in the waters exported from the salt-marsh
457 (14.34±0.27 for the non-colonized area and 14.17±0.41 for the vegetated area) are comparable to the
458 ones present in the estuary (14.38±0.37).

459 Although the conditional stability constants for L_2 in the waters derived from salt-marshes (12.12±0.14
460 for the non-colonized area and 12.29±0.46 for the vegetated area) are similar to those present in the
461 estuary, the input through tidal cycles is not reflected in the estuarine distribution of the weak ligand. As
462 shown before, L_2 concentrations decrease towards higher salinities. A photo-degradation process during
463 the transportation of these ligands to estuarine water with low particulate matter concentrations (5-15

mg L^{-1} against 22-99 mg L^{-1} for the Scheldt estuary, Zwolsman and van Eck., 1999) is a possible explanation. The high residence time of the waters in the Tagus estuary would favor this degradation as shown in the Scheldt estuary (Laglera and van den Berg., 2006). According to these authors L_2 is more susceptible to photo-degradation than L_1, as the former is stabilized by its complexation with copper. In addition, due to the heterogeneous distribution of this ligand, it is possible that several sources (i.e. harbor, untreated effluents) contribute to the pool of weak ligands.

Considering the water volume of the Tagus estuary ($1.9x10^9$ m^3), the residence time during the sampling conditions (60 days), the area covered by the intertidal non-vegetated zones (128 km^2) and salt marshes (20 km^2) and the tidal induce transport of copper and ligands from the sediment to the water column in the Rosário salt-marsh (Table 4), a contribution of around 7 nM of copper, 16 nM of L_1 and 52 nM of L_2 to the Tagus estuarine waters has been estimated. This account for 35% of Cu, 94% of L_1 and 45% of L_2 present in the estuary (S≥6).

This continuous input of copper complexing ligands is beneficial for the health of the estuary. Several inputs of copper coming from a variety of sources, especially in such an industrialized and populated area as it is the Tagus estuary can be neutralized by its binding to natural ligands. This would decrease its toxicity to living organisms when an extra input of copper occurs (Louis et al., 2009).

5. CONCLUSIONS

Tagus estuarine waters show a relatively constant copper concentration during the estuarine mixing. Most of this copper is organically complexed by a strong ligand (L_1) all through the estuary. A second and weakest ligand (L_2) was also detected in these waters in higher concentrations. Salt-marsh areas are the major sources of copper complexing ligands to the Tagus estuary. Noticeable, tidal induced transport continuously feed estuarine waters with copper and ligands, mainly with the strongest one. Its input can represent 95% of the ligand present in the estuary. Salt-marsh input of copper complexing ligands determines copper distribution along the salinity gradient of the Tagus estuary.

Although future research should be performed to compare the importance of this source of ligands with other sources like urban effluents, rivers or benthic inputs, the present work has demonstrated that salt marsh areas are the main inputs of copper complexing ligands to the Tagus estuary.

491 This type of fluxes should be taken into account when assessing trace metal contamination problems
492 and remediation in estuarine and coastal areas. Furthermore, one may speculate that inundation of
493 coastal areas associated with the climatic changes, either to increase of water levels or floods will result
494 in additional fluxes of trace elements and copper complexing ligands from inter-tidal areas to estuarine
495 and coastal waters. The relevance of the tidal induced transport on copper and complexing ligands
496 coming from salt marsh areas may be crucial to understand the biogeochemical cycles of metals in
497 coastal ecosystems all over the world where salt-marshes occupy large extensions.

498

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Salt-marsh areas as copper complexing ligand sources to estuarine and coastal systems

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Abstract

Dissolved copper levels, copper complexing capacities and conditional stability constants have been determined in the Tagus estuarine waters and one of the saltmarshes located in this estuary, the Rosario saltmarsh. Tagus estuarine waters show a constant and around 20 nM copper concentration during the estuarine mixing. Most of this copper is organically complexed by a strong ligand (L_1) with a concentration that varies between 19-55 nM and a log K´ between 14.14-15.75. In addition L1/Cu ratios are quite constants and close to 1 all through the estuary, indicating the same source. A second and weakest ligand (L_2) was also detected in these waters in higher concentrations (36-368 nM) but with a lower log K´ that varies between 12.06-13.13. The present work has demonstrated that salt-marsh areas are important and continuous sources of copper complexing ligands to the Tagus estuary. Noticeable, tidal induced transport continuously feed these waters with copper and ligands, mainly with the strongest one. This continuous input, together with the high residence times of this system results in a quite constant concentration along the salinity gradient. This input represents 95% of the ligand present in the estuary.

Keywords: copper; ligands; speciation; estuary; salt-marsh; Tagus

1. INTRODUCTION

Salt-marshes are among the most common and extensive intertidal habitats along temperate coastlines. Several studies have shown that salt marshes incorporate large quantities of anthropogenic metals into the colonized sediments (Caçador et al., 1993; Sundby et al., 1998; Caetano et al., 2007). Root-sediment

interactions appear to contribute to the metal enrichment of colonized sediments and belowground biomass (Caçador et al., 1993) as well as porewaters (Santos-Echeandía et al., 2010).

Not only the concentrations, but also trace metals chemical forms in estuarine water (porewater and water column) may control their bioavailability and toxicity. In the water column, speciation of many biologically active trace metals is controlled by complexation with strong organic ligands (Bruland et al., 1991). Copper (Cu) is probably the most studied metal in terms of organic complexation in seawater and estuarine environments. This is because copper is a micronutrient, but it is also toxic, for example for microalgae, at relatively low concentration levels. In addition, Cu forms complexes of relatively high stability with various organic ligands. However, no many studies about copper complexation in salt-marsh areas (Mucha et al., 2008) and its influence on the ligand budget of an estuary have been published till these days.

Several sources of organic matter and ligands to estuarine and coastal areas have been pointed out in the last years determining trace metal distribution along the salinity gradient (Laglera and van den Berg, 2003; Santos-Echeandia et al., 2008). In this way, autochthonous organisms are major producers of the ligands that dominate the complexing capacity of open ocean and coastal productive waters (Croot et al., 2000; Gordon et al., 2000). Organisms such as coccolithophores (Leal et al., 1999), cyanobacteria, dinoflagellates (Croot et al., 2000) and heterotrophic bacteria (Gordon et al., 2000) have been observed to excrete strong Cu-complexing ligands, most likely as a defense mechanism against metal toxicity. The input of terrestrial humic substances through riverine waters has also been recognized as an important source of strong ligands to estuarine environments (Kogut and Voelker, 2001; Shank et al., 2004a; Laglera et al., 2009).

Recent studies have shown that estuarine sediments can also act as a significant source of Cu-complexing ligands to the overlying water, which may strongly influence the biogeochemistry and cycling of dissolved Cu by sediment/water exchange (Skrabal et al., 1997, 2000; Shank et al., 2004b, Chapman et al., 2009). This source of organic ligands would be enhanced if sediments are colonized by plants (Mucha et al., 2008). In the last years, several studies conducted in salt marsh areas, have shown that as roots die they supply important quantities of organic matter to the sediment (Caçador et al., 2004; Pereira et al., 2007). In addition, low molecular weight organic acids are exudated by salt-marsh

54 plant roots (Mucha et al., 2010) that can bind and redistribute metals (Mucha et al., 2008) due to their
55 high affinity to organic matter.

56 As tidal water floods the salt marsh, large quantities of organic material, nutrients and trace metals
57 accumulated in the area are exported to the estuary. On a semidiurnal tidal scale, advective fluxes are
58 enhanced in salt marsh sediments (Santos-Echeandía et al., 2010) through a mosaic of small channels in
59 the upper sediments. The relevance of the tidal induced transport on copper and complexing ligands
60 may be crucial to understand the biogeochemical cycles of metals in coastal ecosystems where salt-
61 marshes occupy large extensions.

62 Thus, the objectives of this work are: a) to measure dissolved copper and complexing ligand
63 concentrations in the Tagus estuarine waters b) to determine dissolved copper and complexing ligand
64 concentrations in the Rosário salt-marsh flooding and porewaters c) to estimate dissolved copper and
65 ligand advective fluxes in the Rosário saltmarsh d) to ascertain the importance of this input over the
66 Tagus estuarine waters.

67 **2. MATERIAL AND METHODS**

68 *2.1. Study area*

69 The 340 km^2 of the Tagus estuary represent one of the largest transitional systems in Europe. The
70 estuary is composed by a large shallow inner bay and a deep straight and narrow inlet channel (Fig. 1).
71 This channel reaches a depth of 40 m and constitutes the deepest part of the estuary. The bay has a
72 complex bottom topography with channels, tidal flats and islands. The deepest channel, with a water
73 depth of 5-10 m, is an extension of the inlet channel. The total amount of water in the estuary is around
74 1.9 km^3. The southern and eastern parts of the bay contain extensive inter-tidal mudflats; the northern
75 part contains tidal flats, islands and several smaller channels. These channels merge upstream to a single
76 narrow channel, marking the entrance of the Tagus River.

77 The Tagus River is the main source of freshwater to the estuary. The discharge usually shows a
78 pronounced dry season/wet season as well as large inter-annual variation. The average annual discharge
79 is ~400 $m^3\ s^{-1}$, but seasonally the average monthly discharge may vary from 1 $m^3\ s^{-1}$ to > 2200 $m^3\ s^{-1}$
80 (Loureiro, 1979). Consequently, the residence time of freshwater in the estuary is highly variable and

may range seasonally from 6 to 65 days (Braunschweig et al., 2003). The tides are semi-diurnal, with amplitudes at Lisbon ranging from ~ 1 m at neap tide to ~ 4 m at spring tide. The tidal effect reaches 80 km landward of the estuary mouth. Most pollutants are discharged from upper to the lower estuary (Fig. 1). Indeed, apart from being a major harbor, the fishing activities in the estuary are adversely affected by the inflow of effluents from about 3.5 million Greater Lisbon inhabitants, part of them untreated, coupled with industrial (chemicals and petrochemicals) and agricultural (fertilizers and pesticides) contributions.

Almost 40% of the estuary is composed by inter-tidal mudflats mainly in southern and eastern shores, containing extensive areas of salt marshes colonised mainly by *Sarcocornia fruticosa, Sarcocornia perennis, Halimione portulacoides* and *Spartina maritima* occupying an area around 20 km^2. The marsh selected for this study (Rosário) is located in the southern shoreline of the estuary (Fig.1). It covers an area of 2 km^2 (Crespo, 1993), being characterised by homogeneous stands of *S. maritima* as a pioneer species in the lower part, pure stands of *H. portulacoides* across the 20-50 cm elevation transect, and *S. fruticosa* and *S. perennis* in the higher salt marsh. The marsh is fully inundated twice a day by tidal action (2-4 m of tidal amplitude) through a highly branched system of channels that cross the elevation transect. The channels have 0.5-1.5 m depth promoting the inundation of the higher marsh even at low amplitude tides.

2.2. Sampling

A sampling cruise along the Tagus estuary was conducted on the 5th May 2010. A 93 m^3 s^{-1} flow was measured during that day what gives a residence time around 62 days for the water in the estuary (Braunschweig et al., 2003). Ten sub-surface water samples (TWB11-TWB410) were collected by hand in 1 L low density polyethylene (LDPE) bottles from a plastic boat (Fig. 1) during the low tide covering the salinity gradient of the estuary. In addition, flooding water during tidal inundation was sampled in two sites of the Rosário salt marsh (non-vegetated area and *S.maritima* colonised area and separated by less than 20 m). At low tide, when sediment was exposed to the atmosphere, two sediment cores (10 cm long) were collected at each site (vegetated and non-vegetated). The cores were sliced immediately after sampling in two 4 cm layers, prepared composite samples for each layer of the cores collected at each site, and material stored in acid pre-cleaned HDPE vials avoiding air presence inside. Sampling took

place in less than 3 min. Core material in the colonized area consisted of dense rooting sediments with no evidence of burrowing worms, crabs or bivalves. When tidal water starts to flood each site, flooding water was collected at each inundation time: 1, 5, 10, 15, 20 and 30 min for the non-vegetated area and 1, 5, 10, 15, 20, 30, 45 and 85 min for plant colonized area. Flooding water was sampled 1 cm above the sediment surface directly into acid pre-cleaned syringes. The water and sediment samples were kept in refrigerated boxes and immediately transported to the laboratory. A more detailed description of these sampling procedures can be found in previous works (Caetano et al., 2007).

2.3. Sample treatment

Estuarine and flooding water samples were filtered through 0.45 µm cellulose membranes placed in previously acid-cleaned filtration units (Nalgene). Filters were frozen and stored at -20°C pending analysis of particulate copper and aluminium. The main reason for measuring particulate aluminium is that it acts as a tracer of lithogenic or terrigenous material (Windom et al., 1989; Pohl et al., 2004) and will be useful for the interpretation of the various sources of material to estuarine waters (i.e. rivers or sediment resuspension) (Mota et al., 2005).

Pore waters were separated from the sediment layers by centrifugation at 10,160 rcfxg for 30 min at +4 °C and filtered through 0.45 µm cellulose acetate membranes. Filtration and subsequent manipulation of the samples were carried out in a glove box under argon atmosphere in order to avoid alteration of the initial conditions (Caetano et al., 2007; Santos-Echeandia et al., 2009). A portion of the filtered samples (around 200 mL) was frozen for speciation analysis while around 100 mL were acidified with suprapure HCl (pH < 2) and stored pending analysis of total dissolved copper.

2.4. Copper analysis in estuarine water

2.4.1. Total dissolved copper concentrations

Copper analyses were carried out using voltammetric equipment (Metrohm 797 VA Computrace) controlled by a computer (PC). The reference electrode was a double junction, Ag/AgCl, KCl (3 M), saturated AgCl, with a salt-bridge filled with 3 M KCl, and the counter electrode was a glassy carbon rod. A hanging mercury drop electrode (HMDE) was used as the working electrode. The copper concentration in the filtered samples was determined using a procedure similar to the one described

previously (Campos and van den Berg, 1994). Estuarine and flooding water was UV-digested (1 h) after acidification to pH 2.2 by the addition of 10 µL 6 M bidistilled HCl (AnalR BDH) per 10 mL of sample in acid-cleaned borosilicate glass tubes. A 10 mL sample aliquot was pipetted into the voltammetric cell and ammonia (AristarGrade Merck) was used to ascertain the approximate neutralization of the pH; also, HEPES buffer (BDH, final concentration 0.01 M) and Salycildoxime (SA, Sigma, final concentration of 25 µM SA) were added. The solution was deaerated by purging (5 min) with nitrogen. The voltammetric parameters were: deposition 60 s at -1.1 V whilst stirring, 8 s quiescence at -0.1 V, and a potential scan using the square-wave modulation: 10 Hz, step height 2.5 mV, pulse height 25 mV, from 0 to -0.8 V. The sensitivity was calibrated by incorporating standard copper additions (Spectrosol BDH) to each sample. Each sample was analyzed in triplicate. A blank with Milli-Q water (18.2 MΩ.cm) was made at intervals of every three samples and its concentration was subtracted in order to eliminate any copper contribution from reagents. Blank concentrations were around 0.27±0.06 nM.

2.4.2. Copper titrations

The copper complexing capacity of the waters of the Tagus estuary was determined by competing ligand exchange with adsorptive cathodic stripping voltammetry (CLE-AdCSV) of Cu-salycilaldoxime (SA) complexes according to previous works on copper complexation in estuarine waters (Campos and van den Berg, 1994; Laglera and Van Den Berg, 2003). Briefly, a 170-ml sample previously filtered by 0.45 µm was transferred to a polyethylene bottle (Nalgene), and spiked with HEPES/NH_4OH buffer (for a concentration of 0.01 M) and SA (mix concentration of 5 or 10 µM). After stirring, 10 mL aliquots were pipetted into 15 polystyrene vials (30 mL Bibby, Sterilin) that were previously spiked with copper to provide increasing concentrations in the range of 0–700 nM (actual range depending on the initial copper concentration and the ligand concentration to be determined). Prior to the first titration, the tubes were conditioned twice overnight with seawater containing the same range of copper concentrations in order to avoid copper loss in the tube walls. After that, aliquots of each sample were left to equilibrate overnight at room temperature due to the slow kinetics of the ligands and copper reactions (Campos and van den Berg, 1994). The equilibrium concentration of Cu-SA complexes was determined using the following parameters: adsorption potential of- 0.15 V, deposition time of 60 s, 8 s

164 quiescence, potential scan from 0 to -0.8 V using square-wave modulation at 10 Hz, step height 2.5mV
165 and pulse height of 25 mV.

166 *2.4.3. Evaluation of complexing capacities and conditional stability constants*

167 Changes in the ratio between the concentrations of CuSA (labile to AdCSV) and CuL in the different
168 aliquots permit the determination of the copper complexing capacity of the ligands present in solution
169 (C_L) and their conditional stability constant to bind copper (K'_{CuL}). A more detailed explanation can be
170 found elsewhere (Ruzic, 1982; van den Berg, 1982). The relationship between the different copper
171 fractions is described by the following transformation of the Langmuir isotherm:

172 $$[Cu]_{TOT} = \Sigma\ (C_{Li}\ [Cu]_{lab}/K'_{CuLi\alpha'} + [Cu]_{lab}) + [Cu]_{lab} \quad (1)$$

173 L_i represents the variability of the nature of the different natural ligands. Those ligands are
174 differentiated according to the stability of their complexes with copper. α' is the α-coefficient of Cu^{2+}
175 with inorganic ligands and SA. $[Cu]_{TOT}$ and $[Cu]_{lab}$ are respectively the total and labile copper
176 concentrations related by:

177 $$[Cu]_{TOT} = \Sigma[CuLi] + [Cu]_{lab} = \Sigma\ [CuLi] + [Cu(SA)] + [Cu(SA)_2] \quad (2)$$

178 The concentration of labile copper is calculated from:

179 $$[Cu]_{lab} = i_p/S \quad (3)$$

180 Where i_p is the voltammetric signal obtained from the reduction of the CuSA complexes adsorbed on the
181 HMDE electrode and S is the ratio signal/concentration or sensitivity. S can be estimated from the slope
182 of the last few points of the titration (S^{INT}) if the titration has been extended to copper concentrations
183 enough to complete the saturation of the natural ligands.

184 As indicated in the previous section, samples were analyzed using two different SA concentrations (5
185 and 10 µM). Higher SA concentrations increase the competing ability of SA. In the presence of complex
186 mixes of ligands characterized by the formation of complexes with copper in a wide range of stability
187 constants, the effect of increasing the SA concentration is both, an overcompetition of those ligands of
188 low affinity for copper and the equilibration of SA with ligands of very high stability constant. Therefore,
189 in heterogeneous matrices, the shift of the SA concentration resolves a different fraction of ligands,

fraction that is defined as detection window. This is an important tool to estimate the heterogeneiety of the pool of copper ligands in the sample.

Values for the stability constants of the complexes CuSA and $Cu(SA)_2$ are necessary in order to obtain the value of α' at each salinity. Both stability constant were calculated using relationships obtained in a previous work (Campos and van den Berg, 1994):

$$\log K_{CuSA} = (10.12 \pm 0.03) - (0.37 \pm 0.02) \log \text{salinity} \quad (4)$$

$$\log \beta'_{Cu(SA)2} = (15.78 \pm 0.08) - (0.53 \pm 0.07) \log \text{salinity}. \quad (5)$$

A plot of $[Cu_{lab}]$ vs the ratio $[Cu]_{lab}/[CuL]$ is the most used procedure to solve C_L and K'_{CuL} from the slope and the Y-axis value respectively. When only one ligand is present, the data follow a straight line but if more than one type of ligands are present, the plot takes a curved shape. In this case C_L and K'_{CuL} can be calculated with accuracy for a maximum of two types of ligands.

The estimation of C_{L1}, $K'_{CuL1,}$ C_{L2} and K'_{CuL2} requires either an iterative refine procedure (Laglera-Baquer et al., 2001; van den Berg, 1982) or a nonlinear fitting (Gerringa et al., 1995). In the first case the solutions for C_{L1} and K'_{CuL1} are refined with estimations of C_{L2} and K'_{CuL2} from different linear sections of the plot until a convergent solution is obtained.

Complexing capacities, conditional stability constants and S can also be obtained fitting non-linearly the substitution of Eq(3) in Eq(1). Non-linear fitting prevents the uncertainty caused by the arbitrary necessity to split the titration data in two that iterative linear fitting requires.

In this work, we have used the fitting tool of a popular computer program, SigmaPlot (© Systat Software, Inc) to fit 3, 4 or 5 parameters. The program uses a Levenberg-Marquardt fitting algorithm. We first solved the titration data sets for the hypothesis of a single ligand model using non-linear fitting incorporating S as a parameter to C_L and K. For this purpose, a new equation was incorporated to the Regression Wizard of the software under the "User-Defined" category. The Code fed to the Regression Wizard is presented in Table S1.

Then, we prepared a new Equation for the case of two types of ligands, i.e. 5 parameters (C_{L1}, $K'_{CuL1,}$ C_{L2} and K'_{CuL2} and S) but the program struggled to offer realistic values in the form of very high S and K'_{CuL1} values. Removal of S from the list of parameters and use of S^{INT} did not solve problems with $K'_{CuL1}$for

some titrations. In those cases, K'_{CuL1} was obtained from the iterative linear routine and its value fixed in the *constrains* section of the code (Table S1) during the non-linear fitting of the rest of the complexing parameters.

2.4.4. Particulate copper and aluminum

In order to determine the amount of labile copper, filters obtained from filtered water were subject to digestion overnight with 0.1 M acetic acid in Teflon® vials (Savillex) at ambient temperature following the first step of the BCR extraction procedure (European Community Bureau of Reference; Quevauviller et al., 1997). The digest was then syringed-filtered (0.45 µm) using a Swinnex filtration unit and stored pending analysis; filters containing the remnant particles were microwave digested using a mixture of HF and HNO_3 (1:3) in order to analyze the total fraction (Biscombe, 2004).

Labile copper determination was carried out by means of cathodic stripping voltammetry as explained before (Campos and van den Berg, 1994) while total particulate copper and aluminum determination was carried out with the standard additions method. Concentrations of Al and Cu were determined using a quadrupole ICP-MS (Thermo Elemental, X-Series) equipped with a Peltier Impact bead spray chamber and a concentric Meinhard nebulizer. Coefficients of variation for metal counts (n = 5) varied between 0.5 and 2%. The precision and accuracy of each metal concentration measurements, determined through repeated analysis of certified reference material (PACS2), using Indium as internal standard, were 1–4% and 2–5%, respectively (data not shown). Procedural blanks always accounted for less than 1% of the total metal concentrations in samples.

2.5. Estimation of sediment-water interchange: tidal induced fluxes

As water floods over inter-tidal sediments the pore water solutes tend to be exported by advection and/or exchanged between mixing top sediments and tidal water. The associated pressure difference and tidal water movement are the driven mechanism for this transport. By using the temporal variation of copper and ligand concentrations in the flooding water the tidal induce transport (T) to the water column is calculated using the following expression (Caetano et al., 2007).

$$T = \Sigma\, [(C_{t+1} - C_t)/(2 - C_i)](h_{t+1} - h_t) \qquad (6)$$

where C_{t+1} and C_t are solute (copper and ligand) concentrations in the flooding water at times t+1 and t, C_i is the residual concentration (lowest value obtained for the flooding water) and ht and h_{t+1} are the water depth at the same times. It was observed that water depth during the field measurements increased on the average 0.5 and 0.8 cm per minute of inundation in *S. maritima* and non-vegetated areas, respectively. The tidal induced transport of trace metals is calculated for the first 85 and 30 min of inundation, respectively. Since inter-tidal sediments are inundated twice a day, the tidal induce transport (*T*) was multiplied by a factor of two to present values on a daily basis.

2.5. Statistics

The statistical analyses were performed using STATISTICA 6 (Statsoft).

3. RESULTS

3.1. Determination of the complexing parameters and the sensitivity by non-linear fitting

Table 1 shows the complexing parameters found for the estuarine water samples according to one and two ligands models. Table 2 shows those parameters for flooding waters and interstitial waters extracted from cores. For the one ligand model, S was added to the fitting procedure according to the code shown in Table S1. Values for S were in the range 94-113 % with respect to S^{INT} (average 98.9±4.1 %) indicating that any bias due to undersaturation of the ligands present in the sample caused by a too small copper concentration at the end of the titration was masked by the analytical error. For the sample collected at station 11, S was fitted at a very low value 90% of S^{INT} and the complexing parameters were recalculated using S^{INT}.

For the two ligands model, this approach was not possible as explained above. For some titration sets, S was estimated to be 103-114 % of S^{INT} but for many others this correction reached as much as 170 % which could be discarded simply by visual inspection. Values in Table 2 were obtained using S^{INT}. When the estimation of K'_{CuL1} produced a value over 20, the titration data set was solved by iterative linear fitting (Laglera-Baquer et al., 2001; Laglera and Van Den Berg, 2003) and the value of K'_{CuL1} fixed on the constrains section of the code. This led to a 3 parameters non-linear fitting. An extensive description of the routine to fit complexing parameters out of metal titration data sets used here is presented in a separate work (Laglera et al., 2012).

3.2. Dissolved copper and its organic speciation in the Tagus estuary

Dissolved copper concentrations along the salinity gradient in the Tagus estuary are plotted in Fig. 2a. With the exception of the station located upstream in the river (26.4 nM), values were quite constants and around 20 nM all over the estuary. The ligand concentration (L; Fig. 2a), for the 5µM SA detection window, showed higher values in riverine samples (399 nM) decreasing to 320-340 nM in the first 3 units of salinity. A new decay is found from salinity 5 to 25 (to 46 nM). This decreasing trend from fresh to marine waters has been observed before in other estuarine systems (Laglera et al., 2003; Plavsic et al., 2009). The conditional stability constant for these ligands (log K´) ranges between 12.20 and 13.26 (Table 1), and it presents a bowl-shape distribution along the salinity gradient, with lower values in the mid-estuary and maxima at marine (13.26) and riverine (13.11) end-members.

By fitting data to two types of ligands, a strong (L_1) and a weak (L_2) one can be observed in the Tagus estuary waters (Fig. 2a). Ligand L_1 varied between 14 and 55 nM being the highest concentration found in the two most riverine stations. Values decreased sharply to 15 nM at salinity 3 remaining almost constant until the marine end member (salinity 25). Otherwise, L_2 concentrations vary between 36 and 368 nM with a conservative distribution between salinities 5 and 25, but with lower values than expected for an ideal dilution line between salinities 0 and 5. The conditional stability constants for these ligands range between 14.10 and 15.75 for L_1 and between 12.06 and 13.13 for L_2 (Table1). The distribution along the salinity gradient is different among them; while log K_1´ shows a saw tooth tendency with higher values in the riverine end-member, log K_2´ distribution is comparable to log K´, with the lowest values in the mid estuary and increasing towards the river (13.13) or the sea (12.42) end members.

In order to detect stronger copper complexing ligands and check for homogeneity/heterogeneity of them in the estuary (van den Berg and Donat, 1992; Buck and Bruland., 2005; Santos-Echeandia et al., 2008), a detection window of 10 µM SA has been applied to some of the samples. Samples, fitted to one ligand are plotted in Fig. 2b. Ligand concentration increases between salinity 0 and 5 (from 121 to 142 nM) and progressively decreases to 30 nM at salinity 25 with a quite linear trend. The conditional stability constant (log K´) presented, again, lower values at mid salinities (13.11) increasing towards the riverine (14.52) and marine (13.76-14.15) end members (Table 1) as observed for the lower detection

298 window (5 µM SA). By fitting data to two types of ligands, the strongest one (L_1) concentrations ranged
299 between 15 and 28 nM (Fig. 2b) with a quite irregular distribution. On the contrary, the weakest ligand
300 of this detection window (L_2) shows a similar trend to L, with an initial increased from 145 to 174 nM
301 decreasing progressively afterwards to 22 nM at salinity 25. The conditional stability constants, both log
302 K_1' and log K_2' present higher values at low salinities (16.84 for K_1 and 13.87 for K_2) that sharply
303 decreased at salinity 5 reaching 15.02 for log K_1' and 12.76 for log K_2' remaining relatively constant
304 towards the marine end-member (Table 1).

305 *3.3. Suspended Particulate Matter, particulate total and labile copper and aluminium concentrations in*
306 *the Tagus estuary*

307 Suspended particulate matter values oscillate between 4.8-14.7 mg L^{-1} in the estuary with an irregular
308 distribution (Fig. S1a). The highest values where found at salinities 10-15 and the lowest between
309 salinities 19-23. Particulate copper concentrations varied between 0.78 and 14.8 nM (Fig. S1b). The
310 highest levels were found at mid-salinities (5-15 ups) while the lowest values measured at both end-
311 members. The labile copper in the particulate matter accounts only for the 0.05-0.45 % of the total
312 copper but it is noticeable that the lability was higher for the lowest salinities (Fig. S1c). Particulate
313 aluminum concentrations ranged between 272 and 4930 nM with a similar distribution to particulate
314 copper.

315 *3.4. Dissolved copper and its organic speciation in the Rosario Saltmarsh*

316 *3.4.1. Flooding waters*

317 Dissolved copper concentrations in the flooding water in the two sites of the salt marsh (non-vegetated
318 and plant colonized areas) are plotted in Fig. 3a. Copper levels in the non-vegetated zone increase 1
319 minute after arriving of tide water from 18.3 to 28.5 nM. In the following 5 minutes, levels decreased
320 reaching 20.6 nM and remaining quite constant until 30 minutes after tidal inundation started, with the
321 exception of minute 15 when a less pronounced increase (27.0 nM) was observed (Fig. 3a). In a similar
322 way, dissolved copper levels increased in the colonized area 1 minute after the flood started reaching
323 higher values (32.5 nM) than in the non-vegetated area. Afterwards, concentrations decreased to 17.7
324 nM and varied irregularly between 13.6 and 22.1 nM during the next 80 minutes of inundation.

325 The time course evolution of ligand concentration in the flooding waters is also plotted in Fig. 3a. A net
326 increase of its concentration (initial levels of 46 nM) was observed in both sampling areas but reaching
327 higher concentrations in the plant colonized (291 nM) than in the non-vegetated area (85 nM) during
328 the first minute of inundation. Afterwards levels in plant colonized sediments decreased sharply while in
329 non-vegetated area values diminished progressively reaching, at both areas similar values to those
330 found before inundation started. The conditional stability constants of ligand showed higher values in
331 the non-vegetated (13.73-14.04) than in the plant colonized area (13.11-13.89) (Table 2). The
332 significance for statistical analyses was $p<0.05$.

333 In addition, the time course evolution of log K´ values during the tidal inundation were different among
334 the two sites: while an increase of log K´ was found one minute after the inundation started in the non-
335 vegetated area, a progressive increase of log K´ with time was observed in the vegetated area during the
336 first 10 minutes.

337 The strongest ligand (L_1) concentrations vary between 46 and 61 nM in the non-vegetated area without
338 a trend all over the time (Fig. 3b). Nevertheless, L_1 concentrations in the colonized area are more
339 variable and ranged between 28 and 95 nM. The conditional stability constant of this ligand varied
340 between 14.12 and 14.79 in the non-vegetated area while in the colonized area a variation between
341 13.61 and 14.83 was found (Table 2). The weakest ligand concentrations were higher and varied
342 between 60 and 107 nM for the non-vegetated area and from 5.5 to 258 nM for the colonized area (Fig.
343 3b). It is noticeable the net increase in the colonized area in the first minutes of inundation that is not
344 observed in the non-vegetated area. Finally, conditionals stability constant for this weakest ligand
345 oscillate between 11.95-12.26 nM in the non-vegetated area and 11.87-13.05 in the colonized area
346 (Table 2).

347 Strongest ligands have been detected in the flooding waters moving the detection window to 10 µM SA.
348 Ligand concentration were lower (61-87 nM) in the non-vegetated area than in the colonized site (107-
349 137 nM) during the first 5 minutes of inundation (Fig. 3c). An increase was found in the first minute
350 comparing to the estuarine waters in the vicinity of the salt-marsh and concentrations slightly decrease
351 after 5 minutes. The respective conditional stability constants were similar in the non-vegetated area
352 (14.39-14.48 nM) and in the plant-colonized area (14.32-14.47 nM) (Table 2).

353 Two types of strong ligands were found in the colonized area with concentrations ranging between 58
354 and 82 nM for L_1 and from 59 to 138 nM for L_2 with a similar trend to the unique ligand (L). However, L_1
355 levels do not decrease after 5 minutes but continue to increase (Fig. 3c). Conditional stability constants
356 ranged in the following intervals: 14.63-15.69 for L_1; and 12.84-13.44 for L_2 (Table 2).

357 *3.4.2. Porewaters.*

358 Dissolved copper and ligand concentrations and their conditional stability constants in the porewaters of
359 the salt-marsh are shown in Table 3. Levels of dissolved copper in the non-vegetated area ranged from
360 45.3 to 53.9 nM with higher values in the upper layer than in the deeper one. However, higher ligand
361 concentrations were found in deeper porewaters (321 nM) than in surface ones (245 nM). The
362 conditional stability constants for these ligands varied between 13.24 for the deeper layer and 13.58 for
363 the upper one. Levels of the stronger ligand (L_1) ranged from 77 to 184 nM with higher values in the
364 upper layer. Nevertheless, the conditional stability constant was higher (14.77) in the deeper layer than
365 in the upper one (13.70). Concentrations of L_2 varied between 70 and 287 nM with increased values in
366 the deeper layer. The conditional stability constants of this weakest ligand were 12.68 for the deeper
367 layer and 13.09 for the upper one.

368 Higher dissolved copper concentrations in pore waters (71-142 nM) were found in the plant colonized
369 area (Table 3). Ligand concentrations vary between 767 nM in the deeper layer and 831 nM in the upper
370 one. Conditional stability constant for this ligand showed higher values in the upper layer. The stronger
371 ligand, L_1, levels varied between 115 and 203 nM with higher values in the upper layer and their
372 conditional stability constants were 15.17 for the upper layer and 14.66 for the deeper one.
373 Concentrations of L_2 were quite similar among layers (666 and 699 nM) as the conditional stability
374 constants (13.09 and 12.82).

375 Strongest ligands in pore waters were found by applying a higher detection window (10 µM) (Table 3).
376 Ligand concentration in the non-colonized area was 230 nM with a conditional stability constant of
377 14.28. A strong ligand with a log K_1' of 15.25 and a concentration of 67 nM and a weak ligand with a log
378 $K_2'$of 13.85 and a concentration of 182 nM result from the data fitting to two types of ligands. As found
379 with the 5µM SA detection window, higher values were observed in the vegetated area at 10µM
380 detection window. A ligand concentration of 419 nM with a log K´ of 13.95 was registered. In the data

fitting to two types of ligands, a strong ligand concentration of 148 nM with a log K_1' of 17.56 was recorded, while the weakest ligand showed a concentration of 846 with a log K_2' of 13.51.

3.5. Tidal induced transport of copper and organic ligands in the Rosario Saltmarsh

An estimation of the tidal induced transport of dissolved copper and ligands is shown in Table 4. A flux of 1.23 µmol m^{-2} d^{-1} of dissolved copper escapes from the sediment to the overlying waters in the non-vegetated area, while the ligand transport was 3.25 µmol m^{-2} d^{-1}. If the two ligand case is considered, an export of 2.16 µmol m^{-2} d^{-1} of the strongest ligand and 2.31 µmol m^{-2} d^{-1} of the weakest one was estimated. As expected from the results shown in previous sections calculated transports are higher in the plant colonized area, where a 2.43 µmol m^{-2} d^{-1} flux of dissolved copper is estimated. The ligand flux (48.2 µmol m^{-2} d^{-1}) was around fifteen times higher than in the non-vegetated area. Increased transport of both strong and weak ligand to the water column was also found with 11.5 µmol m^{-2} d^{-1} for L_1 and 64.7 µmol m^{-2} d^{-1} for L_2.

The tidal induced transport of ligands detected by the 10 µM SA detection window was similar in the non-vegetated area and lower in the colonized area. In this way, 4.25 µmol m^{-2} d^{-1} of ligands were exported in the non-colonized area while 6.87 µmol m^{-2} d^{-1} were exported in the plant colonized area. For the two ligand case, 3.80 µmol m^{-2} d^{-1} of the strongest ligand and 5.77 µmol m^{-2} d^{-1} of the weakest one were estimated.

4. DISCUSSION

4.1. Copper and organic ligands behavior during the estuarine mixing

Dissolved copper and complexing ligand behavior in the Tagus estuary can be clearly divided in two distinct chemical areas. The edge is marked by the salinity 5.6 sample. In the low salinity stretch, although dissolved copper values were only higher in the most riverine sample, the strong ligand values (L_1) were higher than in the rest of the estuary. As a consequence the L_1/Cu ratio is relatively constant and around 1. The only exceptions are the two most riverine stations (Fig. S2) where an excess of L_1, which may derived from the river end member was detected. This ratio is linear and progressively decreases with the increasing salinity if total ligand concentration is considered (Fig. S2). Moreover, the weakest ligand (L_2) also showed a different behavior in this zone of the estuary but with lower levels

than expected if the trend marked by the seven most saline stations is extended to the freshwater end-member.

The mixing of freshwater with the marine water dilutes L_1 ligand within the estuarine area. The long residence times (Braunschweig et al., 2003), confirmed by uniform suspended particulate matter content in a wide salinity range (Fig. S1a), allow the homogenization of the water body justifying the constancy of copper and strong ligand levels in the 6-25 salinity range. This pattern suggest that the tidal effect superimposes the river flow and no losses of copper or strong ligands occured during the estuarine mixing. Similar findings have been observed in the Scheldt estuary by Laglera and van den Berg (2003). A continuous lateral input, as detected in the Vigo Ria (Santos-Echeandía et al., 2008), in the Tagus estuary may also explain the constant values all over the salinity gradient. In addition, this area shows constant and close to the unit L_1/Cu ratios in a wide salinity range (Fig. S2). This means that copper and L_1 levels are similar probably because their source is the same. There is no evidence of copper transfer from the dissolved to the particulate fraction since values were quite low all through the estuary (Fig. S1b). However, L_2 experiments a progressive decrease in their levels towards the outer part of the estuary and no remarkable inputs of L_2 are observed at mid-high salinities as observed for L_1. It can be assumed that the river is not the major source of this weak ligand, but its origin is somewhere in between salinities 0-6, where although the mixing with seawater, its concentration remains constant or even increases. This increase is consistent with the trend observed at both detection windows for the weakest ligand (Fig. 2). The higher particulate aluminum concentrations at salinities 6-10 (Fig. S1c) and SPM at salinity 10 could reflect the maximum turbidity zone in this salinity gradient and at spring tides the bottom particle resuspension in this area may explain the release of the weakest ligand from the sediments (Shank et al., 2004; Santos-Echeandia et al., 2008). If copper variability during the estuarine mixing is associated to L_1, L behavior is ruled by L_2. This fact is supported by the spatial distribution of log K'_2, which is quite similar to log K´ (Table 1) at both detection windows (5 µM y 10 µM).

Regarding the nature of the ligands, by comparing L_1 with L_2 at both detection windows, the distribution of the stronger ligand (L_1) was homogeneous, mainly at high salinities, because the log $K'_{1,5\mu MSA}$ and log $K'_{1,10\mu MSA}$ were similar (Table 1) and, in general, their ratio was close to 1 (Fig.S3a). Moreover, ratios between L_1 measured at the low detection window and the L_1 observed at the high detection window

436 were closer to 1 (1.24±0.33) along the salinity gradient (Fig.S3b). Conversely, L_2 was very heterogeneous
437 and a shift to the higher detection window induces the decrease of the ligand concentrations. Thus, the
438 ratio between L_2 measured at low detection window and at high detection window is around 2
439 (2.09±0.48) (Fig. S3b). An increase of the log K´ values is also observed when moving the detection
440 window to 10 µM SA. As a consequence the ratios between log K´at high and low detection windows are
441 always well below 1 all over the salinity gradient (Fig. S3a).

442 *4.2. Saltmarsh inputs of copper and complexing ligands to the estuary*

443 Intertidal and saltmarsh areas constitute one of the possible sources of copper and complexing ligands
444 to estuarine waters. Mucha et al. (2008) demonstrated that salt-marsh plants are able to produce
445 relatively high amounts of strong Cu-complexing ligands in their root system and the present study
446 evidenced that these complexes are exported to estuarine waters through tidal induced transport (Table
447 4).

448 The constant dissolved copper and L_1 concentrations in a wide salinity gradient (6-25) is presumably
449 explained by the lateral input from the marsh areas along the gradient coupled with high residence time
450 of water in the estuary. Previous studies have showed that copper levels in the adjacent area to the
451 Tagus (S≥30) varied between 1.6 and 14.6, well below the values found at salinity 25 (Cotté-Krieff et al.,
452 2000; Santos-Echeandia et al., 2012). The correlation between Cu and L_1 ($r=0.67$, $p<0.05$) in the Tagus
453 estuarine waters suggest that the source of copper and ligands at mid-salinities is diluted at high
454 salinities. The inexistence of marshes downstream the inlet that connects the estuary to the sea
455 reinforces the hypothesis that salt marshes are the main source of copper and complexing ligands.
456 Moreover, the conditional stability constants for L_1 in the waters exported from the salt-marsh
457 (14.34±0.27 for the non-colonized area and 14.17±0.41 for the vegetated area) are comparable to the
458 ones present in the estuary (14.38±0.37).

459 Although the conditional stability constants for L_2 in the waters derived from salt-marshes (12.12±0.14
460 for the non-colonized area and 12.29±0.46 for the vegetated area) are similar to those present in the
461 estuary, the input through tidal cycles is not reflected in the estuarine distribution of the weak ligand. As
462 shown before, L_2 concentrations decrease towards higher salinities. A photo-degradation process during
463 the transportation of these ligands to estuarine water with low particulate matter concentrations (5-15

mg L^{-1} against 22-99 mg L^{-1} for the Scheldt estuary, Zwolsman and van Eck., 1999) is a possible explanation. The high residence time of the waters in the Tagus estuary would favor this degradation as shown in the Scheldt estuary (Laglera and van den Berg., 2006). According to these authors L_2 is more susceptible to photo-degradation than L_1, as the former is stabilized by its complexation with copper. In addition, due to the heterogeneous distribution of this ligand, it is possible that several sources (i.e. harbor, untreated effluents) contribute to the pool of weak ligands.

Considering the water volume of the Tagus estuary ($1.9x10^9$ m^3), the residence time during the sampling conditions (60 days), the area covered by the intertidal non-vegetated zones (128 km^2) and salt marshes (20 km^2) and the tidal induce transport of copper and ligands from the sediment to the water column in the Rosário salt-marsh (Table 4), a contribution of around 7 nM of copper, 16 nM of L_1 and 52 nM of L_2 to the Tagus estuarine waters has been estimated. This account for 35% of Cu, 94% of L_1 and 45% of L_2 present in the estuary ($S \geq 6$).

This continuous input of copper complexing ligands is beneficial for the health of the estuary. Several inputs of copper coming from a variety of sources, especially in such an industrialized and populated area as it is the Tagus estuary can be neutralized by its binding to natural ligands. This would decrease its toxicity to living organisms when an extra input of copper occurs (Louis et al., 2009).

5. CONCLUSIONS

Tagus estuarine waters show a relatively constant copper concentration during the estuarine mixing. Most of this copper is organically complexed by a strong ligand (L_1) all through the estuary. A second and weakest ligand (L_2) was also detected in these waters in higher concentrations. Salt-marsh areas are the major sources of copper complexing ligands to the Tagus estuary. Noticeable, tidal induced transport continuously feed estuarine waters with copper and ligands, mainly with the strongest one. Its input can represent 95% of the ligand present in the estuary. Salt-marsh input of copper complexing ligands determines copper distribution along the salinity gradient of the Tagus estuary.

Although future research should be performed to compare the importance of this source of ligands with other sources like urban effluents, rivers or benthic inputs, the present work has demonstrated that salt marsh areas are the main inputs of copper complexing ligands to the Tagus estuary.

491 This type of fluxes should be taken into account when assessing trace metal contamination problems
492 and remediation in estuarine and coastal areas. Furthermore, one may speculate that inundation of
493 coastal areas associated with the climatic changes, either to increase of water levels or floods will result
494 in additional fluxes of trace elements and copper complexing ligands from inter-tidal areas to estuarine
495 and coastal waters. The relevance of the tidal induced transport on copper and complexing ligands
496 coming from salt marsh areas may be crucial to understand the biogeochemical cycles of metals in
497 coastal ecosystems all over the world where salt-marshes occupy large extensions.

498

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Figure captions.

Figure 1. Map of the Tagus estuary with the estuarine sampling stations and the Rosário salt-marsh (a) and schematic illustration of the sampling procedure in the salt-marsh

Figure 2. Dissolved copper and copper complexing ligands distribution during the Tagus estuarine mixing (a) 5 µM SA and (b) 10 µM SA detection windows. Values presented are the mean of three replicates.

Figure 3. Time course evolution of (a) copper and total ligand concentrations in the non-vegetated area (*SP*) and in the colonized area (*CP*) for the 5 µM detection window, (b) strong (L_1) and weak (L_2) ligand for the 5 µM detection window and (c) ligands (total, strong and weak) for the 10 µM detection window in flooding waters. Values presented are the mean of three replicates.

Supplementary material

Figure S1. Suspended particulate matter levels (a), particulate copper and aluminium concentrations (b), and labile copper concentrations (c) in the Tagus estuarine waters. Values presented are the mean of three replicates.

Figure S2. Ligand to copper ratios for the total ligand (L) and strong ligand (L_1) along the salinity gradient of the Tagus estuary.

Figure S3. (a) Low to high detection window ratios for the strong (L_1) and weak (L_2) ligand in the Tagus estuarine waters. (b) High to low detection window ratios for the strong (L_1) and weak (L_2) conditional stability constants.

Figure

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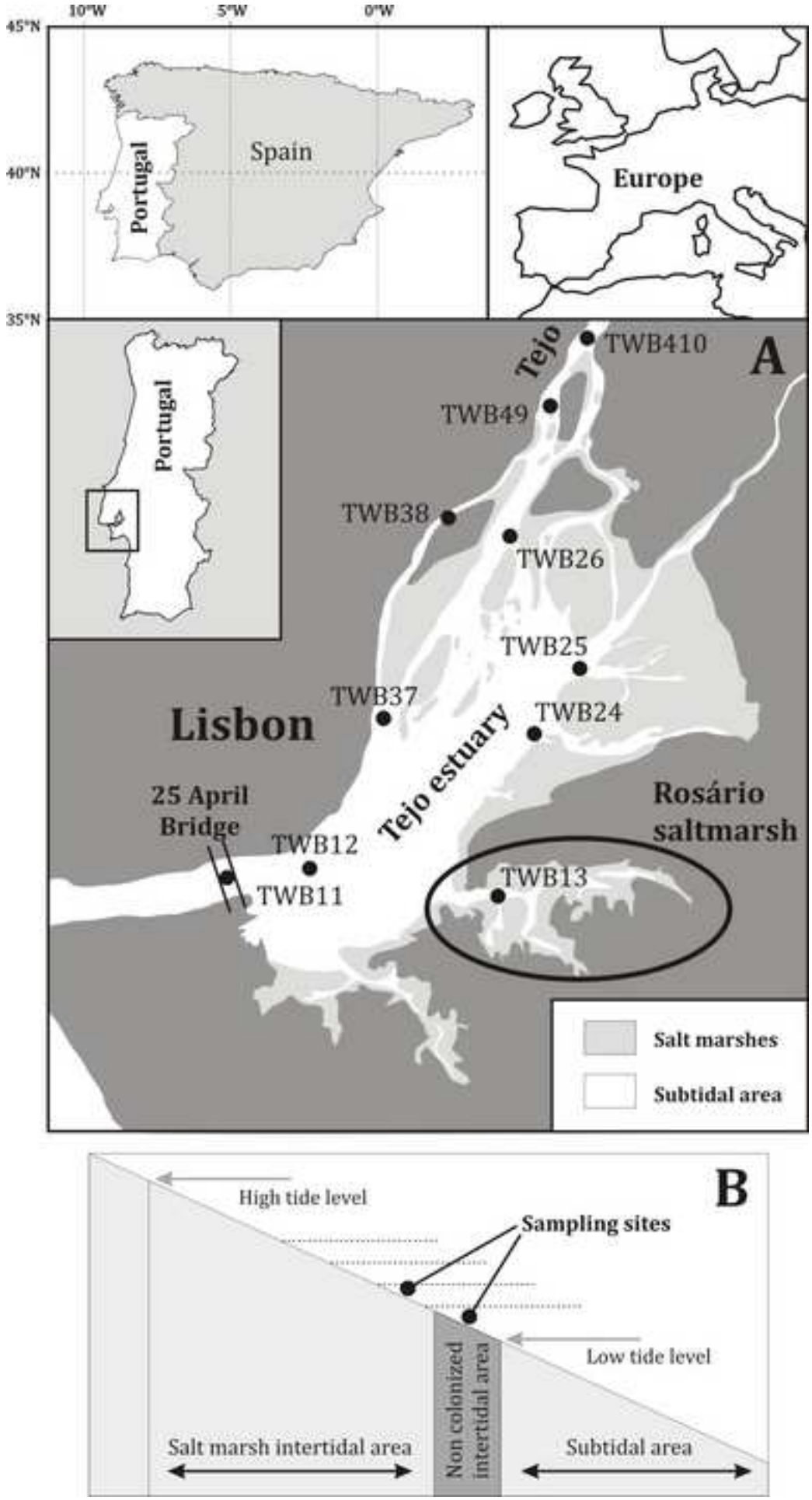
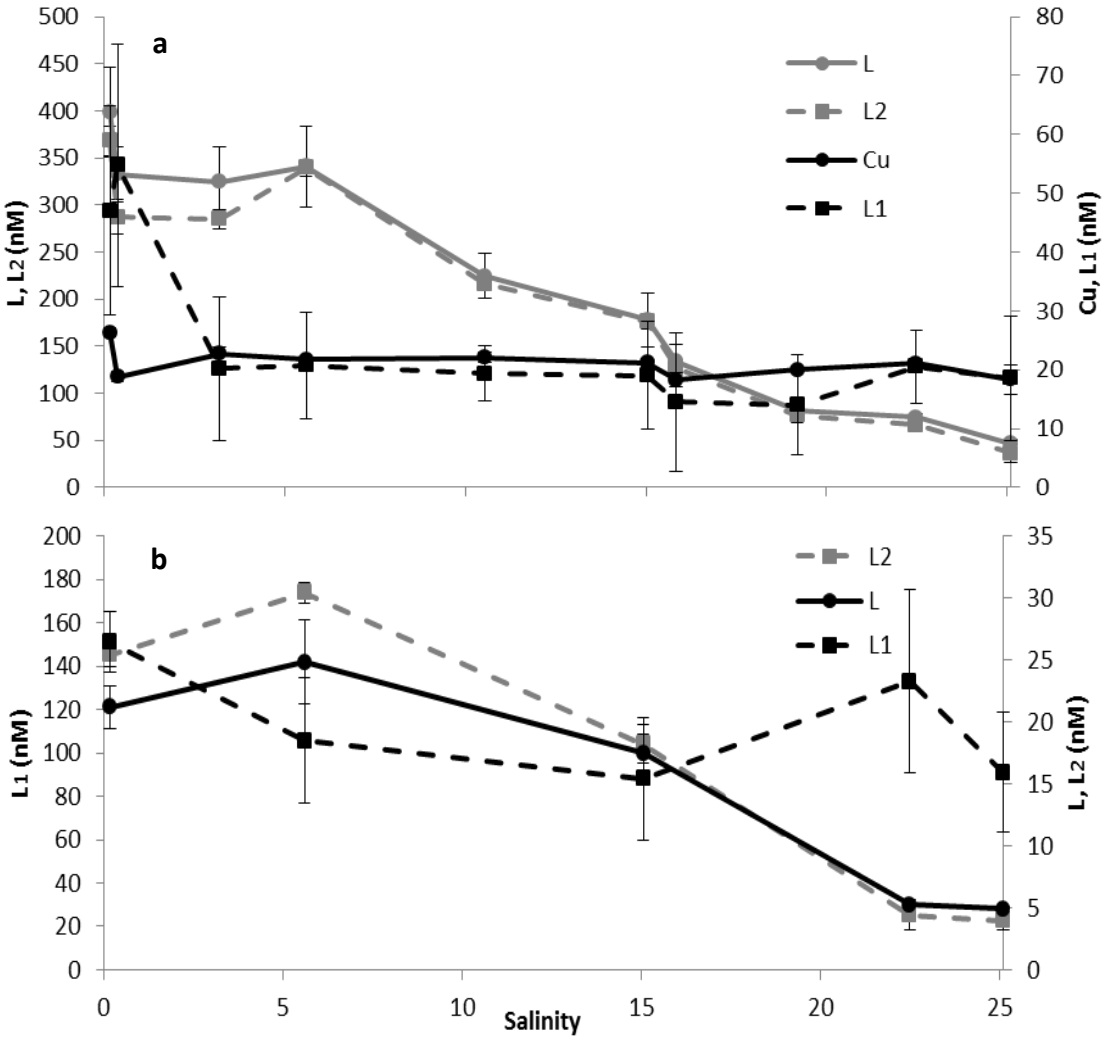


Figure

Figure 2.



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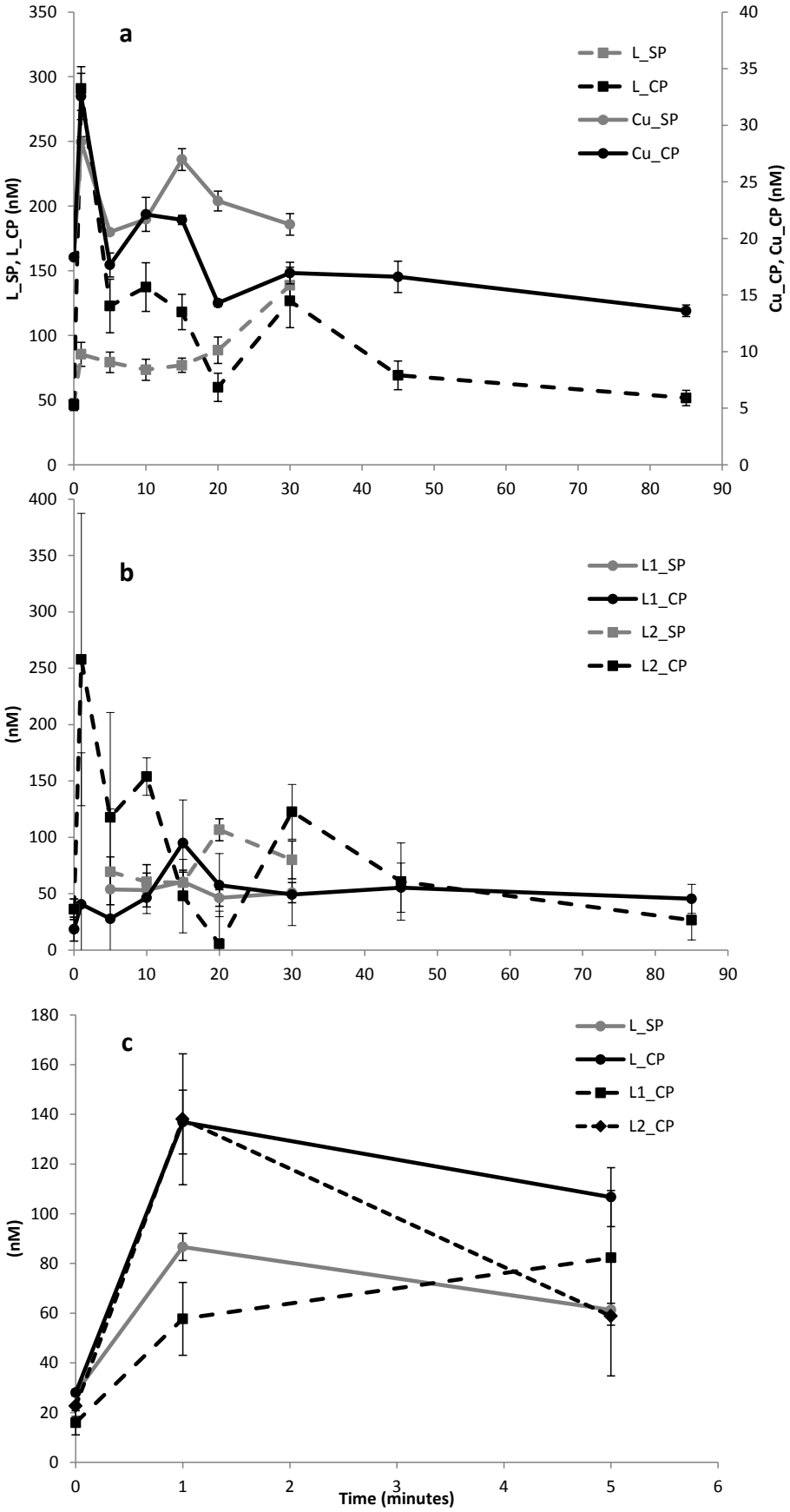

Figure 3.

Table

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Table 1. Dissolved ligand concentrations (nM) and conditional stability constants at two different detection windows (5 µM SA and 10 µM SA) in the Tagus estuarine waters. * sample 11, non linear fitting underestimated S, S^{INT} was used. Values underlined were obtained from an iterative linear fitting and used to obtain the rest of the parameters by nonlinear fitting. Values presented are the mean of three replicates.

		5 µM SA						10 µM SA					
Station	Salinity	L (nM)	Log K´	L_1 (nM)	Log K_1´	L_2 (nM)	Log K_2´	L (nM)	Log K´	L_1 (nM)	Log K_1´	L_2 (nM)	Log K_2´
TWB 410	0.2	399±48	13.15±0.11	47.0±17.8	15.09±0.09	368±16	12.96±0.09	121±10	14.52±0.11	26.4±2.5	16.84±0.50	145±6	13.87±0.05
TWB 49	0.4	333±30	13.26±0.09	54.8±20.6	15.10±0.35	287±19	13.13±0.10						
TWB 38	3.2	325±37	12.45±0.07	20.2±12.3	15.75±1.53	285±11	12.38±0.08						
TWB 26	5.6	341±43	12.21±0.06	20.7±9.0	*14.16±0.41*	339±9	12.08±0.06	142±19	13.13±0.11	18.5±5.1	*15.02±0.44*	174±5	12.76±0.05
TWB 37	10.5	225±24	12.25±0.08	19.4±4.7	*14.79±0.54*	217±5	12.09±0.04						
TWB 25	15.1	178±29	12.26±0.11	19.0±9.2	*14.10±0.46*	176±8	12.06±0.10	100±9	13.11±0.09	15.4±5.0	14.71±0.37	104±9	12.76±0.10
TWB 24	15.8	134±18	12.27±0.12	14.5±11.8	*14.14±0.71*	126±10	12.08±0.16						
TWB 12	19.2	82.4±5.5	12.75±0.08	14.0±8.5	*14.86±1.14*	76.9±7.5	12.37±0.16						
TWB 11*	22.5	74.6±4.0	12.93±0.11	20.6±6.2	*14.37±0.42*	66.5±5.4	12.29±0.19	30.0±2.2	14.15±0.19	23.3±7.4	14.59±0.45	25.1±6.5	12.50±0.34
TWB 13	25.1	46.5±4.3	13.11±0.13	18.6±10.6	*14.14±0.43*	36.2±9.2	12.42±0.59	28.0±1.3	13.76±0.07	15.9±4.9	14.56±0.41	22.6±4.2	12.76±0.27

Table

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Table 2. Dissolved ligand concentrations and conditional stability constants at two different detection windows (5 µM SA and 10 µM SA) in the flooding waters for the non-vegetated and vegetated areas of the Rosário salt-marsh. Values presented are the mean of three replicates.

	Non-vegetated area						Vegetated area					
					5µM SA							
Time (minutes)	L (nM)	Log K´	L_1 (nM)	Log K_1´	L_2 (nM)	Log K_2´	L (nM)	Log K´	L_1 (nM)	Log K_1´	L_2 (nM)	Log K_2´
0	46.5±4.4	13.11±0.10	18.6±10.6	14.00±0.43	36.2±9.2	12.42±0.59	46.5±4.4	13.11±0.10	18.6±11	14.00±0.43	36.2±9.2	12.42±0.59
1	85.4±9.3	14.04±0.26					291±17	13.11±0.09	40.5±134	14.08±0.81	258±130	12.91±0.27
5	79.2±7.9	13.73±0.19	53.8±13.6	14.17±0.52	69.3±13.3	12.18±0.29	123±21	13.55±0.63	27.7±97	14.83±1.13	118±93	13.05±0.50
10	73.5±8.1	13.94±0.25	53.2±15.0	14.32±0.79	60.6±15.2	12.22±0.35	137±19	13.22±0.26	46.5±14	14.71±0.36	154±17	12.27±0.18
15	77.0±5.5	13.84±0.13	60.6±8.5	14.12±0.24	59.7±11.2	12.01±0.26	118±14	13.40±0.19	95.0±38	13.61±0.25	47.9±33	11.96±0.62
20	88.6±10.3	13.77±0.26	46.3±7.4	14.79±0.54	107±9.8	12.26±0.16	59.9±11	13.88±0.63	57.6±28	13.92±0.36	5.5±29	11.96±1.38
30	139±14	13.91±0.23	50.9±8.9	14.31±0.41	70.1±16.8	11.95±0.25	127±21	13.22±5.13	49.2±28	14.14±0.39	123±24	12.30±0.31
45							69.2±11	13.76±5.15	55.4±22	14.00±0.33	60.8±34	11.87±0.50
85							51.7±5.9	13.89±5.07	45.5±13	14.03±0.25	26.5±18	11.99±0.59
					10µM SA							
0	28.0±1.3	13.76±0.07	15.9±4.9	14.56±0.41	22.6±4.2	12.76±0.27	28.0±1.3	13.76±0.07	15.9±4.9	14.56±0.41	22.6±4.2	12.76±0.27
1	86.7±5.4	14.48±0.10					137±13	14.47±0.17	57.7±15	15.69±0.39	138±26	13.44±0.21
5	61.4±2.6	14.39±0.06					107±12	14.32±0.20	82.3±27	14.63±0.26	58.8±24	12.84±0.49

Table

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Table 3. Dissolved copper and ligand concentrations and conditional stability constants at two different detection windows (5 µM SA and 10 µM SA) in the porewaters of the Rosário salt-marsh for the non-vegetated and vegetated areas. Values presented are the mean of three replicates.

ROSARIO SALT-MARSH (5µM SA)														
	Non-vegetated area							Vegetated area						
Depth (cm)	Cu (nM)	L (nM)	Log K´	L_1 (nM)	Log K_1´	L_2	Log K_2´	Cu (nM)	L (nM)	Log K´	L_1 (nM)	Log K_1´	L_2	Log K_2´
2	53.9±5.4	245±32	13.58±0.28	184±24	13.70±1.03	70.1±24	13.09±1.42	142±8	831±80	13.55±0.19	203±187	15.17±0.82	666±176	13.09±0.27
6	45.3±1.0	321±34	13.24±0.17	77.2±43	14.77±0.58	287±39	12.68±0.21	70.7±2.0	767±36	12.88±0.07	115±61	14.66±0.44	699±57	12.82±0.10
ROSARIO SALT-MARSH (10µM SA)														
	Non-vegetated área							Vegetated área						
2	53.9±5.4	230±18	14.28±0.10	66.8±94	15.25±0.71	182±89	13.85±0.29	142±8	419±13	13.95±0.14	148±55	17.56±2.44	846±65	13.51±0.22

Table

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Table 4. Estimated tidal induced transport of dissolved copper and complexing ligands in Rosário sediments for the non-vegetated and colonized areas.

		Tidal induced transport (µmol m^{-2} d^{-1})	
		Non-vegetated	Colonized
5µM SA			
	Cu	1.23	2.43
	L	3.25	48.2
	L_1	2.16	11.5
	L_2	2.31	64.7
10µM SA			
	L	4.25	6.87
	L_1	-	3.80
	L_2	-	5.77

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