Talanta 85 (2011) 1888–1896

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Electrochemical speciation of dissolved Cu, Pb and Zn in an estuarine ecosystem (Ria de Vigo, NW Spain): Comparison between data treatment methods

Iria Durán*, Óscar Nieto

Laboratorio de Ecoloxía Mariña (LEM). Departamento de Química Analítica e Alimentaria. Universidad de Vigo. 36310, Vigo, Spain

ARTICLE INFO

Article history:
Received 8 April 2011
Received in revised form 28 June 2011
Accepted 7 July 2011
Available online 18 July 2011

Keywords:
Chemical speciation
Ria de Vigo
Anodic stripping voltammetry
Cu Pb Zn
Speciation calculations comparison

ABSTRACT

The total concentration and chemical speciation of Cu, Pb and Zn were determined by square wave anodic stripping voltammetry (SWASV) in the Ría de Vigo, an estuarine area located in NW Spain. Surface and bottom waters from 6 locations were collected in two seasons during 3 years. The total Pb was below 1 nM, and Cu and Zn concentrations, ranged from 3 to 44 nM and from 9 to 300 nM respectively. A gradient from sampling points located in the port of Vigo to external areas was observed. The speciation of the metals (ligand concentration and apparent complex formation constant K') was calculated using several methods: The Langmuir and Scatchard linear fits for one and two ligands, the Lorenzo non-linear fit for one ligand and Langmuir non-linear fit for two ligands. The capability of the different methods to achieve reliable results have been discussed and Langmuir linear fit as well as Lorenzo non-linear fit are the most suitable. Cu presented the highest ligand concentrations, followed by Zn and Pb, while mean $\log K'$ values fell in the range 5–9 (±0.6) for all metals and samples. The adjustment of the data treatment methods used to calculate the speciation parameters was found to vary depending on the extent of complexation and on whether one or two ligands needed to be considered.

1. Introduction

Heavy metals are ubiquitous in the environment and their concentrations are being increased due to natural conditions and, more important than that, due to anthropogenic activities. This fact has already modified profoundly metal cycles on a regional and global basis [1]. Some of these metals such as copper or zinc are essential at certain concentrations for the organisms, while others, such as lead are non-essential and toxic even at low concentration. It is well known that metal bioavailability, and therefore toxicity, is not related to total metal concentration but to its chemical speciation. The presence of organic or inorganic compounds in water changes the free metal ion activity due to the formation of metal complexes decreasing [2] or increasing [3] the toxicity of the metal. Hence, it is important to study metal speciation in marine waters, especially in coastal areas where the human impact is concentrated because of high population, industrial activities or maritime traffic, and can potentially affect marine organisms.

In the case of the Galician Rias (North West Iberian Peninsula), they are highly productive estuaries with more than 250,000 metric tons of mussels being harvested among other species. The Ria de Vigo has also high port activity, including fishing, recreational, touristic and transport ports, contributing to the contamination of the area with heavy metals [4].

Among the suite of electrochemical methods, anodic stripping voltammetry (ASV) has one notable advantage, i.e. it allows the determination of the labile metal fraction in natural waters [5], even at low concentrations. Our research group has carried out some studies in which the labile metal fraction has been directly related to the bioavailable fraction of the metal using a combination of chemical analysis and toxicity bioassays with marine organisms [2,5].

Most of the studies where metal speciation has been carried out on the Galician coast have focused on regions receiving high inputs or fluxes of organic matter. Thus, Cu speciation has been determined in the inner part of the Ria de Vigo [6,7], or in sewage and river discharges ([8] and references therein). Cobelo and Prego [9] studied the speciation of Zn, Pb and Cu in only two samples and in another work they studied Pb speciation in the nearby Pontevedra Ria [10]. When trying to compare speciation results from different authors, the question of which part of the variability is due to different procedures (ASV, CSV...) remains unsolved, but even whit the same procedure, the use of different data treatment methods (Langmuir linearization [11,12], Scatchard linearization [13,14] or non-linear fitting methods [5,15] may cause differences in the results [16–19]. Few studies have compared various of these methods [16–22], most of them used modeled data or synthetic samples, and in our knowledge only three considered natural samples, but

---

* Corresponding author. Tel.: +34 986813815.
E-mail address: iduran@uvigo.es (I. Durán).

0039-9140/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.talanta.2011.07.025

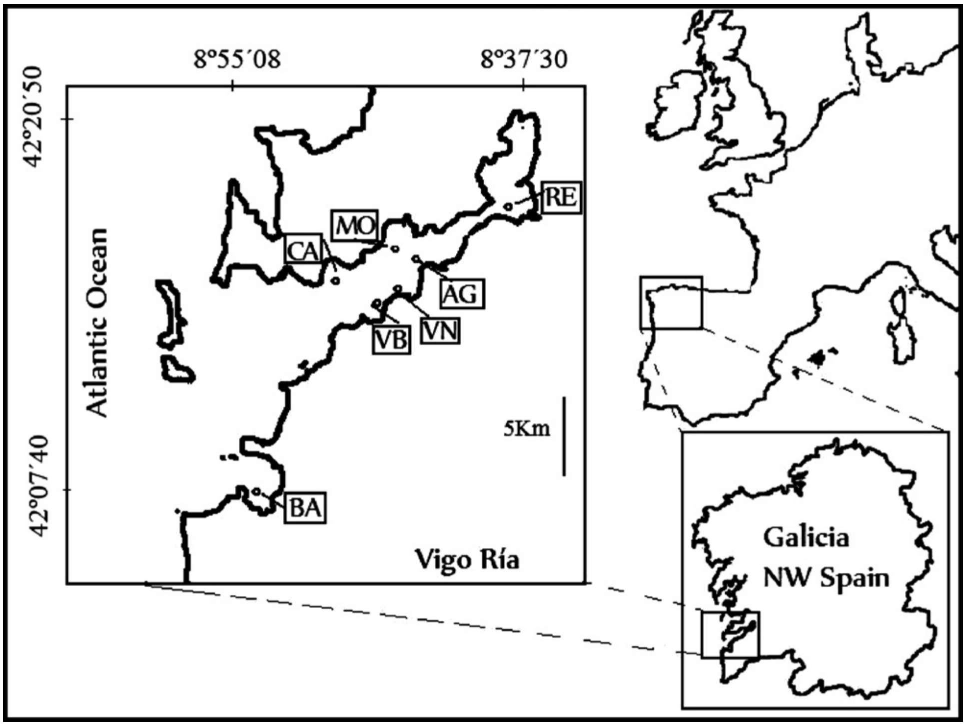

Fig. 1. Area of study and sampling locations.

studied only Cu speciation [19,21,22]. Therefore, further verification of the suitability and performance of the different titration data fitting procedures is called for.

In the present work the speciation parameters have been calculated with several methods of linearization and data adjustments and, subsequently, results have been compared between the different fitting procedures for Cu, Pb and Zn samples taken in a very industrialized area in the Ria de Vigo.

2. Material and methods

2.1. Survey and sampling area

The Ria de Vigo (Fig. 1) is located on the northwest coast of Spain between 42°09′ and 42°21′ North latitude, and 8°36′ and 8°54′ West longitude with an ENE–WSW orientation approximately from head to mouth. The Ria de Vigo is one of the biggest and the most meridional of the Rías Baixas, and an interesting area for the study of metals due to its high and diverse human influence, including shipyard industry, aquaculture, port activities, fishing or tourism. The Ria de Vigo can be divided into three zones according to the degree of continental or oceanic influence. The inner part is San Simon bay, with a typical estuarine circulation and the main riverine inputs to the Ria coming from Verdugo river. The middle zone (where most of our samples were collected) is under the influence of both continental and oceanic contributions. The Port of Vigo is located in this zone. The outer part is dominated by oceanic conditions.

Samples were taken in spring and autumn from year 2004 to 2006. Fig. 1 shows the location of sampling sites where surface and bottom water samples were taken. One sample point was located in the inner part of the Ría, Redondela (RE), and the rest are distributed along the middle part. Those points are distributed along the north coast: Cangas (CA) and Moaña (MO); and the south coast: A Guía (AG). Finally, two samples in the port area were selected: Vigo Bouzas (VB) located in a shipyard and Vigo Nautico (VN) in a recreational port. Only one sample was taken in the outer zone of the Ria, Baiona (BA), in March 2004.

Temperature, dissolved oxygen and salinity were measured *in situ*. Physico-chemical parameters, location and sampling dates are listed in Table 1.

Water samples were collected from the research vessel Navaz (Instituto Español de Oceanografía) using Go-Flo bottles at two different depths: one in surface (S) waters (1 m depth) and the other at 1 m above the bottom (B). The first RE sample taken on March 2004 was collected using a Niskin bottle. All material was acid washed in HNO_3 (5%) and rinsed with ultrapure water before use. Subsequently, water samples were filtered with 0.45 μm PES filters (hydrophilic polyethersulfone, Pall Co. MI) and maintained frozen until analysis. A laminar flow chamber was used during all the analytical steps in order to avoid contamination while clean procedures were carried out for measurements and manipulation of all samples.

2.2. Total metal concentration

Total dissolved Cu, Pb and Zn were determined by square wave anodic stripping voltammetry (SWASV) after digestion of organic matter with UV light (UV digestor Metrohm model 705, Switzerland) assisted with HNO_3 (pH 2) and 0.15% H_2O_2. Nitrites produced during mineralization were neutralized by addition of sulphamic acid up to a concentration of 1 μM which did not increase the blank signals. A 663 VA polarographic stand (Metrohm, Switzerland) containing a PTFE cell is coupled to an Eco-Chemie AutoLab PGSTAT10 (Echochemie, The Netherlands) for recording the voltammograms. The working electrode was used in the hanging mercury drop mode and the reference and auxiliary electrodes were of Ag|AgCl|3 M KCl and platinum respectively. The experimental conditions were the following: accumulation potential of −1.1 V for 120 s; equilibration time of 20 s; scan range from −1.1 to 0.1 V at a frequency of 25 Hz, amplitude of 25 mV and scan increment of 2 mV. Stirring was maintained at the maximum rate allowed by the equipment during accumulation only (3,000 rpm). Quantification of the total metal concentration was performed using the standard addition method.

Table 1

Sampling dates and locations. The first value of each date is the surface sample (S) and the second one is the bottom sample (*B*) (n.m: not measured).

		Depth m	Surface (S)/Bottom (*B*)	Date	Temperature °C	Salinity ‰	O_2 mg/L
Baiona (BA)	42°07.33′N-8°50.41′W	9	S	12-3-04	12.9	35	9.9
			B		n.m.	n.m.	10.1
A Guía (AG)	42°17.35′N-8°42.63′W	21	S	11-3-04	13.5	34.2	9.5
			B		n.m.	n.m.	9.6
			S	28-10-04	15.7	33.1	n.m.
			B		16.3	34.6	n.m.
			S	3-3-05	11	35.7	6.86
			B		11.1	35.9	7.37
			S	26-10-05	16.3	36.3	5.27
			B		16.4	36.6	6.27
			S	6-4-06	15.2	31.9	7.47
			B		15.1	33.8	7.55
Cangas (CA)	42°14.58′N-8°46.95′W	28	S	29-10-04	16	34.1	n.m.
			B		16.3	34.8	n.m.
			S	4-3-05	10.7	36	8.08
			B		10.9	35.7	8.72
			S	26-10-05	16.7	36.5	5.65
			B		16.7	36.6	5.12
			S	6-4-06	14.6	31.7	7.35
			B		14.5	34	7.5
Moaña (MO)	42°16.08′N-8°44.38′W	16	S	11-3-04	13.8	34.6	9.2
			B		n.m.	n.m.	8.9
			S	28-10-04	16.2	34.1	n.m.
			B		16.8	34.1	n.m.
			S	3-3-05	10.9	35.5	7.2
			B		10.8	35.4	7.33
			S	26-10-05	16.6	35.4	5.32
			B		16.3	36.5	5.08
			S	6-4-06	14.8	27.5	7.4
			B		14.3	33.7	6.6
Redondela (RE)	42°17.35′N-8°38.08′W	6	S	11-3-04	12.8	35	9.5
			S	28-10-04	15.9	32	n.m.
			S	3-3-05	9.8	35.1	6.56
			S	26-10-05	16.4	35.1	4.85
			S	6-4-06	14.6	29.1	6.54
Vigo Bouzas (VB)	42°13.85′N-8°44.50′W		S	29-10-04	16	34.1	n.m.
			B		16	n.m.	n.m.
			S	4-3-05	10.7	36	8.36
			B		10.8	35.9	8.56
			S	26-10-05	16.8	36.5	4.38
			B		16.6	36.5	4.03
			S	6-4-06	14.8	32.6	7.14
			B		14.7	34.1	6.64
Vigo Náutico (VN)	42°14.492′N-8°43.386′W	3.7	S	4-3-05	11.3	35.5	6.84
			S	26-10-05	16.4	35.9	4.27
			S	6-4-06	15.3	31.9	6.86

The quality of results has been assured by the use of CRM 403 (Community Bureau of Reference) and CASS-4 (National Research council Canada) reference materials. Detection limits were calculated as $3S_{y/x}$/slope [23] with artificial sea water (prepared as described in [24]) and resulted in 0.6 nM Cu, 0.2 nM Pb and 3.9 nM Zn. For determination of labile metal, the detection limits were established in 1.3 nM for Cu, 0.3 nM for Pb and 3.8 nM for Zn.

2.3. Metal speciation

For metal speciation analysis, several aliquots (from 15 to 20) of 20 mL of the samples were spiked with one metal to make concentrations from 0 to at least 724 nM Pb, 2360 nM Cu and 2294 nM Zn and allowed to equilibrate for 24 h [2]. With the aim of obtaining more points in the initial part of the titration curve, where organic matter has not already been saturated by metal [17], additions of 1 nM Pb and 3 nM Cu and Zn were made initially, and they were progressively increased up to additions of 144 nM Pb, 470 nM Cu and 470 nM Zn. Then, the first 10 mL of the aliquot were used for conditioning the polarographic cell for 5 min and the voltammetric scan was recorded in the other 10 mL. The measurements were carried out with the same equipment. The same experimental conditions were used as for total metal determinations but the deposition potentials were of −1.1 V for Zn, and −0.8 V for Cu and Pb.

2.4. Reagents

Ultrapure HNO_3 69% for trace analysis (Scharlau, Spain), H_2O_2 30% pro analysis (Merk, Germany) were used for UV digestion of organic matter and Cu, Pb and Zn 1 g/L standard solutions (Panreac, Spain) were used for metal additions. Standard solutions of 100 μg/L, 1 mg/L and 10 mg/L were prepared daily with ultrapure water. Sulphamic acid analytical grade (Panreac, Spain) was used.

2.5. Calculations

The labile fraction of metal $[M']$ is defined as the concentration of metal that can be detected by the mercury electrode when organic matter is present in the seawater sample. For the proposed methods, once the voltammograms have been recorded, peak current (i_p) is represented *vs.* the total concentration of metal $[M_T]$ (the sum of initial plus added metal).

When the metal added has saturated the organic matter binding sites, the subsequent additions give a linear response with the peak current. The slope of the last points of the representation, which presented a linear shape, is the sensitivity of the method (S) and, therefore, the labile fraction of metal $[M']$ for each addition can be calculated as

$$[M'] = \frac{i_p}{S} \tag{1}$$

If we consider each functional group of organic matter as one single ligand, binding one metal ion, the following equations for a 1:1 stoichiometry complex can be used:

$$K' \cdot [M'] \cdot [L'] = [ML] \tag{2}$$

$$[M_T] = [M'] + [ML] \tag{3}$$

$$C_L = [L'] + [ML] \tag{4}$$

where K' is the conditional stability constant; $[L']$ is the apparent concentration of the ligands not bound to metals; $[ML]$ is the concentrations of the metal complex and C_L is the total concentration of ligands in the sample. The concentration of ligands is expressed as moles equivalents to metal.

In order to compare the results, speciation parameters were calculated using two linearization methods (*a* and *b*) and two non-linear adjustments (*c* and *d*). For linear fits Microsoft Excel and SPSS v.17 (SPSS Inc) were used while SigmaPlot v.10 (SPSS Inc) was chosen for non linear fits.

2.5.1. *Langmuir linearization (a) [11,12]*

This transformation is based on the following equation:

$$\frac{[M']}{[M_T] - [M']} = \frac{[M']}{C_L} + \frac{1}{K'C_L} \tag{5}$$

The conditional stability constant and the ligand concentration are calculated from the values of the slope and the intercept of the linear equation.

2.5.2. *Scatchard linearization (b) [13,14]*

Based on the linear adjustment of the equation:

$$\frac{[M_T] - [M']}{[M']} = -K'[ML] + K'C_L \tag{6}$$

Scatchard linearization in the presence of two ligands can be adjusted to two different straight lines: complexation with the strongest ligand (L_1) is found in the very first part of the titration curve (and commonly reported to be present at a lower concentration) while complexation reactions with the weak ligand (L_2) dominate at higher concentrations [11,14].

The deviations in Langmuir and Scatchard speciation parameters were calculated by error propagation.

2.5.3. *Lorenzo non-linear fit (c) [5,15]*

This non-linear adjustment method was carried out as described in a previous study [5]. Assuming again, only one type of ligand and a 1:1 stoichiometry, the following equation can be used:

$$[M'] = \frac{-\left([M_T] + C_L + {}^{1}\!/_{K'}\right) + \sqrt{\left([M_T] + C_L + {}^{1}\!/_{K'}\right)^2 + 4\left({}^{[M_T]}\!/_{K'}\right)}}{2} \tag{7}$$

By means of a non-linear fit software the values of C_L and K' as well as their corresponding deviations were calculated.

2.5.4. *Non-linear fit for Langmuir equation for two ligands (d) [25]*

In case of more than one type of ligand are present in the sample, Langmuir method shows a deviation at low concentrations of the labile fraction of metal. Hence, calculations for two ligands with Langmuir equation are performed with a non-linear fit software for the Langmuir equation adjusted for two ligands. Values of C_L and K' as well as their corresponding deviations were calculated with the software.

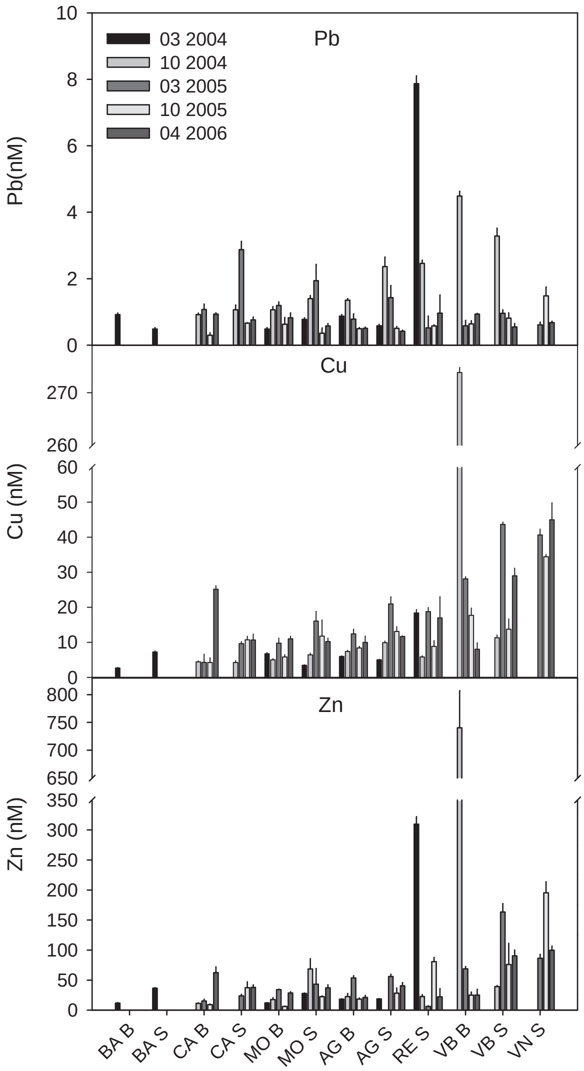

Fig. 2. Total metal concentration in samples (S: surface water; B: bottom water; BA: Baiona, AG: A Guía, CA: Cangas, MO: Moaña, RE: Redondela, VB Vigo Bouzas, VN: Vigo Náutico).

In order to quantify the quality of the results of the different fitting methods, an error fitting parameter (e_r) was calculated for each titration curve. This was calculated as the mean of all the relative errors for the points in each curve. This relative errors were calculated considering the measured *y* value ($[M']/([M_T] - [M'])$ for Langmuir, $([M_T] - [M'])/[M']$ for Scatchard and $[M']$ for Lorenzo) and the theoretical one calculated with Eqs. (5), (6) or (7) and C_L and Log K' results. This e_r is an estimation of the goodness of fit of the measured values and the theoretical model, and can be used to compare the quality of the different models fits regardless of the different scales in each one.

3. Results and discussion

3.1. *Total metal concentration*

The values of the total concentrations of Zn, Pb and Cu in sampling sites are shown in Fig. 2. As a general view, the lowest concentrations are found in the sampling sites located in the outer part of the Ria de Vigo (BA, CA and MO). In spite of BA is very close to the mouth of the Ria and open oceanic waters are characteristic of this area, the concentration of metals is similar to CA and MO because the influence of the recreational ship ports in the bay. The highest concentrations are found at the sampling site in the Port of Vigo (VB and VN), which are influenced by severe anthropogenic impact. The high concentrations observed at Redondela (RE) for Zn and Pb in the first sampling campaign may be attributed to contamination of the sample because it was collected by a Niskin bottle [26]. Otherwise, surface waters usually showed slightly higher concentrations than bottom samples. This fact can be due to an anthropogenic source of heavy metals through rivers, rainfall or other surface inputs [27].

The total concentrations found for lead were usually below 1 nM (Fig. 2) and only few samples presented Pb levels above this value, especially during October 2004 and March 2005 cruises. Although lead concentrations were usually lower than Cu and Zn, they were still elevated compared to oceanic waters [28].

In the case of Cu, Fig. 2 shows also a gradient of concentrations from the port samples to the points located in the central part of the Ria. Cu levels ranged from 2.7 to 43.7 nM, although a significant higher value of 274 nM was detected at VB in October 2004 (Fig. 2). Total Cu concentrations found in most of the samples in this study were slightly higher than the values observed in other studies carried out in the inner part of the Ria [29], as well as in those studies performed in oceanic waters from the Galician continental shelf [27]. This increase in concentration can be due to possible Cu inputs in sewages or to the influence of the port [6,7]. Cu levels found in this study were near the thresholds that may cause toxicity to several marine organisms (3–250 nM) [30–33].

Zn levels in samples were also higher than oceanic concentrations [28] and varied between 9 and 740 nM. Total Cu and Zn concentrations showed a similar pattern between samples and both metals had a significant correlation ($r^2 = 0.85$, $p < 0.001$), which could point to common sources. Cotté-Krief et al. [34] also found this correlation between Cu and Zn in Portuguese coastal waters and was attributed to a common origin for both metals.

When our results for total metal are compared with those obtained in the axial section of the Ria [27], it is suggested that Cu, Zn and, to a smaller extent Pb have a diffuse input towards the Ria through the coast, and a decreasing gradient of concentrations can be observed from the cities and ports to the mouth of the Ria. Considering a temporal trend for the total metal concentrations, no evident conclusions could be derived from the data, since no clear increase or decrease in total metal concentrations was observed.

3.2. *Metal speciation*

Fig. 3 shows two representative titration curves from our data set. The first section of each curve shows a low response due to the complexation of the added metal with free ligands present in the sample. When organic matter is saturated with metal, a linear increase is obtained between i_p and metal concentration. The slope of this linear section was used to calculate labile metal concentration, as described in the methods section. Some studies emphasize that saturation of organic matter may not be achieved in spite of obtaining a linear response between i_p and $[M_T]$; therefore, a series of methods to calibrate the value of S [35,36] were suggested. When applying this iterative method for a series of samples, the small variations of S do not cause significant variations in the values of $\log K'$ and C_L. This could be explained because titrations in the present study reached metal concentrations at least 3 times above ligand concentrations for all samples. In fact, according to the relationship between K',$\cdot C_L$ and the amount of metal that should be added to saturate the organic material described in Scarponi et al. [37], this saturation has been reached in all samples but in four of them (from the initial values K' and C_L obtained by the method of Langmuir). These samples are marked in bold in Tables 2 and 3.

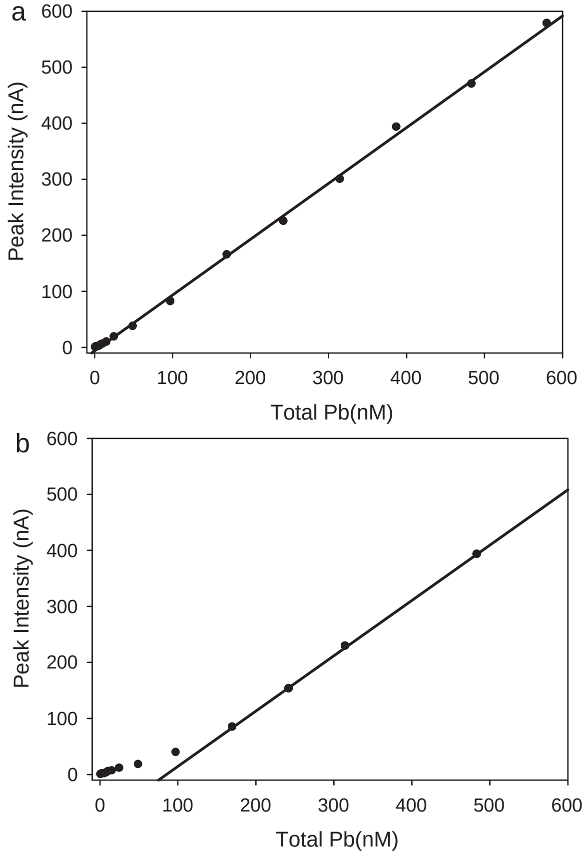

Fig. 3. Titration curves showing two frequently shapes in our samples. a) No initial curvature present. b) Curved initial part of the graph.

The iterative process used for calibration of S, was applied to the four samples allegedly unsaturated and no significant variations from the values of C_L and $\log K'$ were obtained in three of these samples either. In the other sample, the wide dispersion of data points was the main limitation of the iterative process, producing changes in the values of C_L and $\log K'$ of 14 and 3% respectively.

Cu and Pb speciation were studied in all samples, but Zn speciation was only calculated in some representative samples for each cruise. Labile Cu′ and Pb′ were only detected in some samples because these metals were totally complexed with organic matter. In fact, Pb′ was only detected in MO S (10 2004) and CA S (04 2006), and in both cases labile lead accounted for a 20% of its total concentration. Cu’ was detected only in VN (10 2005) and VB (03 2005) accounting for 30% of the total Cu concentration. On the other hand, Zn showed a very different trend, since the labile form was detected in all studied samples. Most of samples had 70–80%, of metal in its labile form, two samples (RE S and VN S in 2006) were reaching 100%, and the rest were above 40%.

Speciation results (C_L and $\log K'$) are shown in Tables 2–4 for all methods and all sampling dates except for the first cruise, where Langmuir linearization was only performed and no deviations were calculated. The concentration of ligands bound to Pb (C_L) was below 50 nM eq. of Pb in most of the cases. However, in some samples collected during the autumn period (excluding the points located in the port of Vigo), C_L increased up to 250 nM eq. of Pb. Complexation of Pb with two ligands was observed more frequently in the spring samples (03 03 2005 and 05 04 2006) than in the October samples. This difference between seasons was not observed for Cu ligand concentrations. Higher concentrations of Cu-binding than Pb-binding ligands were present (between 50 and 200 nM in most of the cases) but neither a clear spatial nor temporal tendency was

Table 2
Speciation results of Pb for Langmuir and Scatchard linear fits and Lorenzo non linear fit. Concentrations are shown in nM eq. of metal. Samples which may be non-saturated (according text) are marked in bold.

Pb		Langmuir		Scatchard				Lorenzo	
		C_L	Log K'	C_{L1}	Log K'_1	C_{L2}	Log K'_2	C_L	Log K'
AG B	22/11/2004	36 ± 2	7.1 ± 0.1	6 ± 6	7.8 ± 0.4	22 ± 10	6.3 ± 0.4		
AG S		41 ± 3	7.3 ± 0.1	7 ± 3	8.3 ± 0.2	29 ± 11	7.4 ± 0.1	24 ± 6	8.0 ± 0.7
CA B		138 ± 2	9 ± 4	133 ± 21	9.2 ± 0.1				
CA S		255 ± 32	8.0 ± 0.3	271 ± 112	8.2 ± 0.2				
MO B		22 ± 1	7.8 ± 0.3	4 ± 1	8.7 ± 0.1	2 ± 9	6.7 ± 0.3	18 ± 4	8.3 ± 0.8
MO S		4.9 ± 0.3	8.4 ± 0.1	4 ± 2	8.6 ± 0.2				
RE S		137 ± 1	8.4 ± 0.6	156 ± 82	8.2 ± 0.2				
VIB B		14.1 ± 0.8	9.0 ± 0.4	16 ± 13	9.2 ± 0.3				
VIB S									
VIN S									
AG B	03/03/2005	34 ± 2	7.6 ± 0.1	13 ± 7	8.3 ± 0.2	19 ± 12	7.6 ± 0.3		
AG S		32 ± 3	7.6 ± 0.2	5 ± 1	8.8 ± 0.1	23 ± 33	6.8 ± 0.6		
CA B		14 ± 2	7.4 ± 0.1	3,6 ± 0,8	8.2 ± 0.1	12 ± 4	7.0 ± 0.2	7 ± 3	8.1 ± 0.8
CA S		26.0 ± 0.9	7.9 ± 0.3	26 ± 12	7.9 ± 0.2				
MO B		65 ± 7	7.6 ± 0.2	8 ± 8	8.2 ± 0.4	40 ± 19	6.8 ± 0.2	111 ± 12	7.2 ± 0.
MO S		15.5 ± 0.7	8.1 ± 0.2	5 ± 4	8.8 ± 0.3	6 ± 1	8.4 ± 0.1	8 ± 2	8.7 ± 0.
RE S		31 ± 1	7.7 ± 0.2	11 ± 3	8.0 ± 0.1	13 ± 10	7.6 ± 0.3	15 ± 7	8 ± 2
VIB B		13.0 ± 02	7.79 ± 0.03	4 ± 3	8.9 ± 0.3	15 ± 12	7.0 ± 0.3		
VIB S		65 ± 10	6.9 ± 0.1	64 ± 13	6.9 ± 0.1	21 ± 19	8 ± 2		
VIN S		15.8 ± 0.8	7.9 ± 0.1	15 ± 9	8.0 ± 0.3	59 ± 46	6.9 ± 0.3	7 ± 3	9 ± 2
AG B	27/10/2005	183 ± 16	7.4 ± 0.2						
AG S		47 ± 1	8 ± 1	40	7.6	89 ± 30	6.8 ± 0.1	61 ± 8	8.0 ± 0.7
CA B		227 ± 36	7.9 ± 0.9						
CA S		15 ± 2	7.9 ± 0.1	10 ± 5	8.1 ± 0.2	45 ± 22	6.7 ± 0.2		
MO B		94 ± 5	9 ± 1						
MO S		127 ± 5	9 ± 4	194 ± 346	7.0 ± 0.8				
RE S		92 ± 3	8.0 ± 0.3						
VIB B		44 ± 3	7.2 ± 0.1	14 ± 4	8.1 ± 0.1				
VIB S		35 ± 1	7.7 ± 0.1	40 ± 6	7.6 ± 0.1			38 ± 2	7.7 ± 0.1
VIN S		14.5 ± 0.2	8.0 ± 0.1	4.0 ± 0.9	9.0 ± 0.1	6 ± 4	8.1 ± 0.3		
AG B	05/04/2006	10.2 ± 0.3	7.7 ± 0.1	7 ± 2	8.0 ± 0.1	19 ± 13	6.6 ± 0.3	21 ± 5	7.1 ± 0.4
AG S		23.2 ± 0.6	7.6 ± 0.1	8 ± 1	8.2 ± 0.1	18 ± 5	7.3 ± 0.1	14 ± 2	8.2 ± 0.4
CA B		47 ± 2	7.8 ± 0.2	11	8.4	31 ± 150	6.8 ± 2.1	40 ± 3	7.9 ± 0.3
CA S		13.8 ± 0.4	9 ± 1	8 ± 1	8.2 ± 0.1	18 ± 9	7.4 ± 0.2	16 ± 3	8.2 ± 0.6
MO B		27.0 ± 0.8	7.9 ± 0.2	10 ± 13	8.5 ± 0.6	14 ± 20	6.7 ± 0.6	15 ± 5	8 ± 2
MO S		41 ± 1	7.5 ± 0.1	11 ± 3	8.0 ± 0.1	22 ± 6	7.0 ± 0.1	43 ± 2	7.4 ± 0.1
RE S		60 ± 1	7.6 ± 0.1	24 ± 10	7.9 ± 0.2	49 ± 14	7.3 ± 0.1	55 ± 4	7.7 ± 0.2
VIB B		12.7 ± 0.8	8.0 ± 0.1	7 ± 3	8.4 ± 0.2	15 ± 7	7.3 ± 0.2	7 ± 3	8.7 ± 1.4
VIB S		23.5 ± 0.8	7.7 ± 0.1	6 ± 4	8.0 ± 0.3	9 ± 1	7.6 ± 0.1	20 ± 3	7.9 ± 0.4
VIN S		27.8 ± 0.9	7.5 ± 0.1	8 ± 10	8.1 ± 0.6	23 ± 8	7.1 ± 0.1	15 ± 8	5 ± 1

observed. Finally, the few values of C_L that were determined for Zn samples were intermediate between those of the other two metals (between 3 and 96 nM eq. Zn).

Mean log K' values (±standard deviation) determined by Langmuir linearization were of 7.9 (±0.5) for Pb, 7.8 (±0.7) for Zn, and 7.5 (±0.5) for Cu, and were very similar to those obtained for Lorenzo non-linear fit: 7.9 (±0.9) for Pb and 7.7 (±0.8) for Cu (Tables 2–4). The close similarity between the conditional stability constants for each metal cannot be explained solely by the role of the analytical detection window. Similar values of conditional stability constants for the three metals have been reported before in other coastal areas [20]. This observation may reflect the presence of relatively non-specific, low-affinity ligands in these waters, which could be integrated by both terrestrial and marine origin humic substances and also by protein-like substances [38].

From a total of 43 samples, two different ligands were detected by the Scatchard method in half the samples for Pb, and Cu and only one sample for Zn. The mean values of log K' for the first and the second ligands were 8.2 (±0.5) and 7.1 (±0.5) for Pb; and 7.2 (±0.5) and 6.2 (±0.5) for Cu and 8.2 (±0.1) and 7.4 for Zn. As can be seen all methods resulted in very similar log K' values. In spite of the similar log K' values obtained for the divalent metals studied, different ligand concentrations were found because the binding sites of the organic matter pool do not have to be the same for each metal [2,5]. We must bear in mind that the ligand concentrations here reported are expressed as equivalents of metal bound to those binding sites of the organic matter mixture.

Lower conditional stability constants and, higher C_L values have been obtained in this study if compared to studies performed by cathodic stripping voltammetry (CSV) [6,7] in the coastal region of freshwater influence. The authors of these studies suggest that forward titration procedures detect ligands of different characteristics, and generally weaker, than those detected by reverse titrations. Consistent with this interpretation, another ligand which appears to be of terrestrial origin has been systematically detected in those studies. Also another explanation could be that CSV is not influenced by dissociation of some complexes during the measurements [39] while ASV does. However, the dissociation of those complexes can be avoided by maintaining the stirring rate of the equipment to the maximum [25,40]. Any comparisons must therefore consider data obtained with the same detection window [41]. This window is determined not only by the technique used (ASV *vs.* CSV) but also its operation parameters. For example, ASV as used in a competitive ligand equilibrium mode [42] can successfully detect the presence and characteristics of high-affinity Cu-binding ligands [43]. Previous studies performed in the Galician Rias by using ASV [9,10] have generated similar values to those found in the present study. These lower K' and higher C_L values are attributed to the higher metal concentrations found in these waters which cause the stronger ligands to be saturated.

Table 3
Speciation results of Cu for Langmuir and Scatchard linear fits and Lorenzo non linear fit. Concentrations are shown in nM eq. of metal. Samples which may be non saturated (according text) are marked in bold.

Cu		Langmuir		Scatchard				Lorenzo	
		C_{L}	Log K'	C_{L1}	Log K'_1	C_{L2}	Log K'_2	C_{L}	Log K'
AG B	22/11/2004	300 ± 7	6.9 ± 0.1	27 ± 7	8.2 ± 0.1	116 ± 76	6.0 ± 0.3	278 ± 48	6.4 ± 0.3
AG S		95 ± 3	7.4 ± 0.1	38 ± 23	7.0 ± 0.3	143 ± 38	6.1 ± 0.1	96 ± 7	7.4 ± 0.2
CA B		200 ± 3	7.16 ± 0.04	202 ± 30	7.0 ± 0.1			148 ± 19	7.5 ± 0.6
CA S		118 ± 3	7.3 ± 0.1	119 ± 36	7.2 ± 0.1			79 ± 14	7.8 ± 0.8
MO B		76 ± 4	7.9 ± 0.2	166 ± 65	7.0 ± 0.2			62 ± 9	7.8 ± 0.4
MO S		211 ± 18	6.3 ± 0.1	248 ± 136	6.1 ± 0.2				
RE S		477 ± 32	6.54 ± 0.05	458 ± 60	6.6 ± 0.1			275 ± 25	7.1 ± 0.3
VIB B									
VIB S		442 ± 13	6.60 ± 0.03	141 ± 66	7.1 ± 0.2	392 ± 172	5.8 ± 0.2	339 ± 36	6.8 ± 0.2
VIN S									
AG B	03/03/2005	118 ± 16	7.5 ± 1.1	137 ± 178	6.9 ± 0.6	530 ± 511	5.9 ± 0.4	116 ± 15	8.1 ± 0.8
AG S		64 ± 2	7.7 ± 0.4					73 ± 17	8 ± 1
CA B		270 ± 37	7.2 ± 0.4	340 ± 156	6.7 ± 0.2				
CA S		149 ± 11	7.1 ± 0.2	236 ± 270	6.9 ± 0.5	90 ± 13	8.5 ± 1.5		
MO B		105 ± 5	7.6 ± 0.1	63 ± 9	7.5 ± 0.1	82 ± 23	7.2 ± 0.1	105 ± 5	7.6 ± 0.1
MO S		417 ± 30	6.1 ± 0.1	139 ± 43	7.0 ± 0.1	358 ± 76	6.2 ± 0.1	165 ± 15	7.0 ± 0.2
RE S		125 ± 3	7.5 ± 0.2	58	7.1	119 ± 28	6.5 ± 0.1		
VIB B		72.5 ± 0,2	8.4 ± 0.1	72 ± 8	7.90 ± 0.05	125 ± 64	7.0 ± 0.2	81 ± 7	8.1 ± 0.2
VIB S		46.5 ± 0,5	8.6 ± 0.9	147 ± 302	6.4 ± 0.9				
VIN S		109 ± 3	7.3 ± 0.2	52 ± 5	7.62 ± 0.05	99 ± 27	6.3 ± 0.1	81 ± 6	8.0 ± 0.3
AG B	27/10/2005	64 ± 2	7.8 ± 0.3	56 ± 41	7.4 ± 0.3				
AG S		88 ± 4	7.9 ± 0.1	100 ± 31	7.5 ± 0.1			31 ± 18	7 ± 1
CA B		50 ± 3	9.0 ± 3.0	57 ± 19	7.3 ± 0.1	81 ± 55	6.3 ± 0.3		
CA S		179 ± 5	8.2 ± 1.6	293 ± 107	7.3 ± 0.2	194 ± 38	7.1 ± 0.6		
MO B		170 ± 5	7.6 ± 0.1	142 ± 32	7.4 ± 0.1	361 ± 274	6.3 ± 0.3	132 ± 7	8.1 ± 0.4
MO S		182 ± 3	7.8 ± 0.2	219 ± 64	7.2 ± 0.1	129 ± 17	8 ± 1		
RE S		122 ± 4	7.7 ± 0.6	78 ± 43	6.8 ± 0.2	113 ± 19	6.1 ± 0.1	120 ± 17	7.5 ± 0.6
VIB B		156 ± 1	7.8 ± 0.1	152 ± 3	7.2 ± 0.0	703 ± 214	5.3 ± 0.1	193 ± 67	7 ± 1
VIB S		185 ± 1	7.51 ± 0.03	228 ± 55	6.7 ± 0.1	132 ± 17	7.7 ± 0.7		
VIN S		69 ± 2	7.4 ± 0.1	79 ± 33	7.2 ± 0.2	36 ± 7	8.2 ± 0.8		
AG B	05/04/2006	76 ± 2	7.3 ± 0.2	97 ± 36	7.0 ± 0.2				
AG S		200 ± 4	7.8 ± 0.4	203 ± 111	7.7 ± 0.2				
CA B		256 ± 6	7.8 ± 0.2	257 ± 33	7.7 ± 0.1			200 ± 24	9 ± 2
CA S		159 ± 3	7.5 ± 0.3	156 ± 138	7.5 ± 0.4				
MO B		63 ± 2	8.0 ± 0.8	90	8.0	247 ± 188	6.0 ± 0.3		
MO S		124 ± 7	8.1 ± 1.5	275 ± 131	7.0 ± 0.2			118 ± 9	9 ± 4
RE S		175 ± 9	7.1 ± 0.2	226 ± 58	6.4 ± 0.1	360 ± 291	5.7 ± 0.4	182 ± 21	8 ± 1
VIB B		71 ± 2	7.5 ± 0.3	67 ± 31	7.9 ± 0.2	86 ± 66	6.7 ± 0.3		
VIB S		101 ± 2	7.6 ± 0.3	97 ± 59	7.7 ± 0.3				
VIN S		71 ± 3	7.7 ± 0.4	76 ± 15	8.1 ± 0.1	323 ± 262	6.0 ± 0.4		

3.3. *Comparison between fitting methods*

As it is shown in Tables 2–4, Langmuir linearization for one ligand (method *a*) was the only method that could produce meaningful complexation parameters for all samples and metals with low deviation in results, presenting mean coefficient of variation (CV) of 4 and 5% for C_{L} and $\log K'$ respectively. On the contrary, Langmuir non-linear fit for two ligands (method *d*) was the one which presented more difficulties; because most data did not fit the equation or the results were unreliable because the deviations were always greater than the calculated values. Scatchard linear fit (method *b*) was valid for most of the samples when one ligand was present and yielded higher deviations than *a* for C_{L} (40% CV) but lower for $\log K'$ (2%). Both Scatchard for two ligands and Lorenzo non-linear fit (method *c*) methods were able to describe the titration data for most samples of Cu and only spring samples of Pb. In addition, higher deviations of results than Langmuir are obtained: Scatchard yields CVs for C_{L} of 66% and for $\log K'$ of 4% as well as CVs of 21% and 11% of C_{L} and $\log K'$, respectively, for Lorenzo's method. In the case of Zn speciation, no samples could be modeled with *c* and only RE S March 2005 was modeled with *b* for two ligands because the overall degree of organic complexation of Zn was very low.

To compare the C_{L} values obtained with *a* or *c* methods with those from *b*, we must consider the sum of both ligands detected with Scatchard as equivalent to C_{L} in the other two methods.

Table 4
Speciation results of Zn for Langmuir and Scatchard linear fits and Lorenzo non linear fit. Concentrations are shown in nM eq. of metal.

	Zn	Langmuir			Scatchard		
		C_{L}	Log K'	C_{L1}	Log K'_1	C_{L2}	Log K'_2
MO S	22/11/2004	2.7 ± 0.1	8.9 ± 0.1				
MO S	03/03/2005	36.5 ± 0.6	8.3 ± 0.6				
RE S		23.5 ± 0.8	7.3 ± 0.1	7 ± 4	9.1 ± 0.3	11 ± 6	7.4 ± 0.2
VIB S		74 ± 3	6.79 ± 0.05				
VIB S	27/10/2005	61 ± 2	8 ± 2	60 ± 14	8.2 ± 0.1		
VIN S		96 ± 3	7.5 ± 0.3				
MO S	05/04/2006	38 ± 1	7.32 ± 0.04	36 ± 5	7.4 ± 0.1		

Table 5
Error fitting parameter (e_r) results: median, standard deviation and 90th percentile for Langmuir, Scatchard and Lorenzo methods.

	Langmuir	Scatchard		Lorenzo
		1 ligand	2 ligands	
Median	0.3	6.7	1.4	0.2
Standard deviation	0.2	27	17	0.2
90th percentile	0.6	61	37	0.5

Therefore, the C_L results observed in *b* method are similar to that in *a*, but with higher deviations in the former one. This finding was reported in a previous work [22] where the authors explained the higher dispersion in Scatchard results because this method depends on a few number of points with low $([M_T] - [M'])/[M']$ values, making Scatchard linear fit less reliable than Langmuir.

In the case of Pb speciation, the results obtained with *b* linearization and *c* non-linear fit had a similar tendency, although C_L concentrations were lower in the Lorenzo method. The similarities of both C_L values are in agreement with a previous study performed by Lund et al. [16], where they made the comparison of speciation methods with synthetic aqueous solutions containing humic substances at different concentrations.

If we consider that the K' values obtained by methods *a* or *c* represent averages between the two values of K' determined by *b*, it follows that Lorenzo method results in slightly higher K' values than *a* or *b*. This was already described in another work [21], which reported lower $\log K'$ values obtained from linear methods compared to non-linear fitting procedures. These authors concluded that it was preferable to use a non-linear fit procedure, especially in estuarine waters with high organic matter content, because linear fits modify to a large extent the distribution of residues.

It was observed that for Lorenzo non-linear fit, reliable parameter estimates were obtained for synthetic samples or natural samples with higher metal or organic matter concentrations [3,5]. In natural samples, however, where both the labile metal and ligand concentrations were low, method *c* could not cope with all the data sets generated here. Gerringa et al. [21] stated that, even though linear fits were more sensitive to scatter in the data, all methods produced similar values if data followed the Langmuir isotherm model. Moreover, they had problems fitting their data with linear procedures because they obtained negative intercepts for samples with high noise, which was not our case. This could be explained in part by the addition pattern. As demonstrated with simulated titration curves, logarithmic additions improve the results for linear fits [17], by increasing the number of points in the lower part of the titration curve, as it was performed in this study.

Fig. 4 shows an example of the curves obtained with Langmuir, Scatchard and Lorenzo models and the theoretical values calculated with Eqs. (5), (6) and (7), using the corresponding values of C_L and K'. Langmuir linear method (Fig. 4a) typically had a homogeneous distribution of the relative errors along the curve. On the contrary, the Scatchard method (Fig. 4b), showed higher deviations in the points of the curve with low and high $[ML]$ and, in some cases, one point on the curve presented large deviations with respect to the theoretical value, increasing the e_r. The Lorenzo method presented major differences in points of the curve with lower $[M_T]$, but these differences scarcely affected the value of e_r. The results obtained with the nonlinear method *d* are not shown because they were incoherent.

As seen in Table 5, the methods of Langmuir and Lorenzo (*a* and *c*, respectively) gave values of e_r of the same order, as expected with the good fits obtained. Taking this into account and also the similar results and deviations yielded for both methods, the major difference between these methods is the limitation of the method of Lorenzo to converge in all samples. On the other hand, the method of Scatchard has higher e_r than previous ones due to the major differences found between theoretical and experimental values. However, it should be highlighted the better e_r values obtained when applying this method to samples with two types of ligands. This fact lead us to the conclusion that Scatchard method should only be used when two ligands are detected in the titration data, while in the presence of only one ligand, another more precise method (Langmuir or Lorenzo) is better recommended.

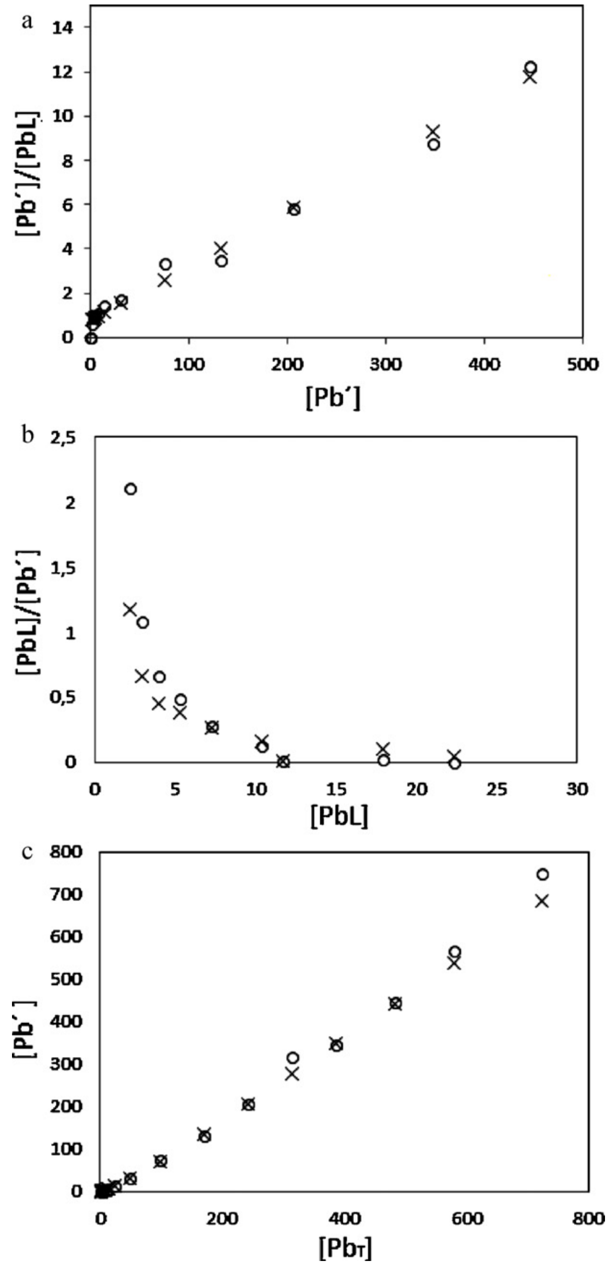

Fig. 4. Titration curves showing the measured (o) and theoretical (*x*) values for Langmuir (*a*), Scatchard (*b*) and Lorenzo (*c*) methods. Error fitting parameter (e_r) of 0.1, 0.5 and 0.06 for a), b) and c) respectively.

In this study, the main limitation of non-linear methods was their capability to provide a precise estimation of the parameters, while linear fit was possible in all cases.

Most of the studies concluding that better results were obtained with non-linear procedures have been carried out with theoretical titrations curves, simulated data [17,18] organic-rich estuarine and laboratory cultures [21]. However, it has also been reported that linear or non-linear methods give similar values when implementing under the proper conditions, such as logarithmic additions, high ligand to metal ratios and good data agreement with theoretical models [19,21].

In the case of two ligands, however, the only option was the Scatchard linear fit because non-linear fitting method *d* was unable to uniquely identify the two sets of complexation parameters. This is in accordance with other works [18,19], where the authors concluded that non-linear fit for two ligands were not valid when data has much scatter, as frequently occur in natural samples.

The outcome of the different data treatment methods studied can be also predicted from the shape of the titration curves. When the titration curve has a near-linear shape as the one shown in Fig. 3a, the data can only be analysed by Langmuir linearization because non-noticeable curvature is present in the lower range of metal additions. This makes it difficult for the Scatchard method as well as the Lorenzo non-linear method, to provide an unique fit to the data. This was the case of most of Pb titrations (most of autumn samples and some of spring ones). On the other hand, when the titration curve presented a more curved shape, or two clearly differentiated linear zones (Fig. 3b), two ligands can be detected most of times with both the Scatchard and also the data were satisfactory explained by the Lorenzo approach.

4. Conclusions

Total metal concentrations in the Ria de Vigo presented a gradient from the harbour to the outer zone of the estuary. Pb levels were generally low while Cu and Zn were higher with a possible common source. Cu was the metal with the narrower interval between environmental concentrations and toxic levels, and therefore is the metal of more environmental concern in the Ria de Vigo.

Ligand concentrations were higher for Cu than for Pb or Zn while the conditional stability constants were similar for each metal in all the samples and can be attributed to complexation by a relatively unselective ligand occurring in the organic matter pool. These values of K' and C_L differ from those found in other estuarine areas but seem to be characteristic of coastal zones with high concentrations of dissolved metals.

Despite the fact that the calculation methods used in this study provided similar results, it was not always possible to use all of them in every case because of the nature of the experimental data sets. All titrations sets could be fitted using the Langmuir linear fit for one ligand with the smaller deviation in the results. There must be taken into account that Langmuir linear fit is used in great part of the literature reporting speciation of metals, and therefore this is the most recommended fit because the values are easily comparable with other studies.

Both Langmuir and Lorenzo non-linear fit methods have a good agreement with theoretical values but Lorenzo fit presented slightly higher deviations in the results than Langmuir fit. On the contrary, Lorenzo fit allows an easy and rapid calculation of deviation parameters (calculated by the non-linear fit software) without modifying the distribution of the residues, as it occurs with the other non-linear methods. Nevertheless, only some samples could be described successfully by this non-linear fit. It is worth stressing that both total metal and organic ligand concentrations were low in our samples than in the samples used previously to validate Lorenzo non-linear fitting techniques.

Taking into account the Scatchard linear fit, only this method produced satisfactory results in samples containing two ligands even though it presented greater deviations than the other compared methods, and should be avoided for samples with one ligand.

Acknowledgements

This study was founded by research project REN2003-00958. We thank the crew of the research vessel Navaz (Instituto Español de Oceanografía). I. Durán was supported with an FPU fellowship from the Spanish Ministry of Education and Science. We acknowledge the technical support of M. Espiñeira and M. López during the course of this study. We acknowledge the comments of two anonymous reviewers who helped improving the manuscript.

References

[1] J.J. Morgan, W. Stumm, in: E. Merian (Ed.), Metals and Their compounds in the Environment. Occurrence, analysis and biological relevance, VCH publishers, Inc., New York (USA), 1991, pp. 67–103.
[2] J.I. Lorenzo, O. Nieto, R. Beiras, Environ. Toxicol. Chem. 25 (2006) 36–44.
[3] P. Sánchez-Marín, J. Santos-Echeandía, M. Nieto-Cid, X.A. Álvarez-Salgado, R. Beiras, Aquat. Toxicol. 96 (2010) 90–102.
[4] J. Bellas, N. Fernández, I. Lorenzo, R. Beiras, Chemosphere 72 (2008) 826–835.
[5] J.I. Lorenzo, O. Nieto, R. Beiras, Aquat. Toxicol. 58 (2002) 27–41.
[6] J. Santos-Echeandía, L.M. Laglera, R. Prego, C.M.G. van den Berg, Mar. Chem. 108 (2008) 148–158.
[7] J. Santos-Echeandía, L.M. Laglera, R. Prego, C.M.G. van den Berg, Estuar. Coast. Shelf Sci. 76 (2008) 447–453.
[8] J. Santos-Echeandía, L.M. Laglera, R. Prego, C.M.G. van den Berg, Mar. Pollut. Bull. 56 (2008) 308–317.
[9] A. Cobelo-García, R. Prego, Anal. Chim. Acta 524 (2004) 109–114.
[10] A. Cobelo-García, R. Prego, O. Nieto, Ciencias Marinas 29 (2003) 377–388.
[11] I. Ružić, Anal. Chim. Acta 140 (1982) 99–113.
[12] C.M.G. van den Berg, Mar. Chem. 11 (1982) 307–342.
[13] G. Scatchard, Ann. N.Y. Acad. 51 (1949) 660–672.
[14] R.F.C. Mantoura, J.P. Riley, Anal. Chim. Acta 78 (1975) 193–200.
[15] J.I. Lorenzo, M. Nieto-Cid, X.A. Álvarez-Salgado, P. Pérez, R. Beiras, Mar. Chem. 103 (2007) 61–75.
[16] W. Lund, I.A. Helbak, H.M. Seip, Sci. Total Environ. 92 (1990) 269–281.
[17] C. Garnier, I. Pižeta, S. Mounier, J.Y. Benaïm, M. Branica, Anal. Chim. Acta 505 (2004) 263–275.
[18] L.A. Miller, K.W. Bruland, Anal. Chim. Acta 343 (1997) 161–181.
[19] F.L.L. Muller, S. Jacquet, W.H. Wilson, Limnol. Oceanogr. 48 (2003) 2289–2302.
[20] P.B. Kozelka, K.W. Bruland, Mar. Chem. 60 (1998) 267–282.
[21] L.J.A. Gerringa, P.M.J. Herman, T.C.W. Poortvliet, Mar. Chem. 48 (1995) 131–142.
[22] C.J.M. Kramer, Mar. Chem. 18 (1986) 335–349.
[23] J.N. Miller, J.C. Miller, Statistics and Chemometrics for Analytical Chemistry , 4th ed., Prentice-Hall, Harlow, England, 2000.
[24] G.E. Zaroogian, G. Pesch, G. Morrison, Am. Zool. 9 (1969) 1144 (Abstr. No. 549).
[25] G. Capodaglio, G. Scarponi, G. Toscano, C. Barbante, P. Pescon, Fres. J. Anal. Chem. 351 (1995) 386–392.
[26] L. Brügmann, K. Kremlin, in: K. Grashoff, K. Kremling, M. Ehrhardt (Eds.), Methods of Seawater Analysis, Wiley-VCH, Weinheim, 1999, pp. 12–13.
[27] R. Prego, M.H. Cotté, A. Cobelo-García, J.M. Martin, Estuar. Coast. Shelf. Sci. 68 (2006) 289–296.
[28] J. Santos-Echeandía, R. Prego, A. Cobelo-García, Mar. Pollut. Bull. 58 (2009) 299–304.
[29] R. Beiras, J. Bellas, N. Fernández, J.I. Lorenzo, A. Cobelo-García, Mar. Environ. Res. 56 (2003) 531–553.
[30] J.C. Mariño-Balsa, E. Poza, E. Vázquez, R. Beiras, Arch. Environ. Contam. Toxicol. 39 (2000) 345–351.
[31] N. Fernández, R. Beiras, Ecotoxicology 10 (2001) 263–271.
[32] R. Beiras, M. Albentosa, Aquaculture 230 (2004) 205–213.
[33] J.L. Levy, J.L. Stauber, D.F. Jolley, Sci. Total Environ. 387 (2007) 141–154.
[34] M.H. Cotté-Krief, C. Guieu, A.J. Thomas, J.M. Martin, Mar. Chem. 71 (2000) 199–214.
[35] R.M.J. Hudson, E.L. Rue, K.W. Bruland, Environ. Sci. Technol. 37 (2003) 1553–1562.
[36] C.M.G. van den Berg, Anal. Chem. 78 (2006) 156–163.
[37] G. Scarponi, G. Capodaglio, C. Barbante, P. Cescon, in: S. Caroli (Ed.), Element speciation in Bioinorganic Chemistry, John Wiley and Sons, Inc., New York (USA), 1996, pp. 363–418.
[38] M. Nieto-Cid, X.A. Álvarez-Salgado, J. Gago, F.F. Pérez, Mar. Ecol. Prog. Ser. 297 (2005) 33–50.
[39] C.M.G. van Den Berg, Mar. Chem. 15 (1984) 1–18.
[40] G. Capodaglio, K.H. Coale, K.W. Bruland, Mar. Chem. 29 (1990) 221–233.
[41] C.M.G. van den Berg, M. Nimmo, P. Daly, D.R. Turner, Anal. Chim. Acta 232 (1990) 149–159.
[42] G. Scarano, E. Bramanti, A. Zirino, Anal. Chim. Acta 264 (1992) 153–162.
[43] F.L.L. Muller, S.B. Gulin, A. Kalvoy, Mar. Chem. 76 (2001) 233–251.