

Bioavailability of heavy metals monitoring water, sediments and fish species from a polluted estuary

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ABSTRACT

Concentrations of heavy metals (Cu, Zn, Cd, Pb and As) were measured in water, sediment and two fish species, *Sparus aurata* and *Solea senegalensis*, from the estuary of Tinto and Odiel rivers in Huelva (Spain), one of the most metallic polluted estuaries in Europe.

As a forward step to understand metal bioavailability and assess the potential impact on aquatic biota, a study of heavy metal speciation of sediments and water were achieved. High levels of total and dissolved Zn and Cu were found in water and high pollution of Zn, Pb, As and Cu were found in sediments. Availability of metals was established as following ranking: Cd > Zn > Cu > Pb in both water and sediment. In addition, the effect of this pollution was studied by evaluation of metal bioaccumulations and correlations obtained between metal levels in fractions of water and sediment and metal levels in fish tissues. High Cu and Zn levels were observed in liver tissue of both species, in according with higher total content and more available metals in water and sediment. Correlations among metal content in tissues and different fractions of metal in water for *S. aurata* and sediment for *S. senegalensis* were found.

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1. Introduction

The Tinto and Odiel rivers cross the province of Huelva, in southwest of Spain, from north to south. Both rivers flow into Atlantic Ocean, forming a common estuary called the “Ría de Huelva” (outer estuary) extending over approximately 15 km.

This has been world-famous as one of the most heavy metal contaminated estuaries in the world [1–4]. The contaminant load transported by Tinto and Odiel rivers to the Ría de Huelva and industrial activities are the main causes of contamination [5].

The Tinto and Odiel rivers drain the central and eastern part of the Iberian Pyrite Belt (IPB), one of the most famous sulphide-mining regions in the world, which contained original reserves of the order of 1700 million t divided in more than 50 massive sulphide deposits [6]. This predominantly Zn–Cu–Pb mineralization has been mined continuously since the Phoenician and Romans era, although the large-scale exploitation of these deposits was stopped until the second half of 19th century [4,7,8]. The legacy of this long time mining activity has resulted in a total of 57

abandoned waste piles (with a total volume of 107 Hm³) and 10 tailings dumps (42 Hm³) in the province of Huelva alone [9], which represents one of the world’s largest accumulations of pyritic mine waste where acid mine drainage (AMD) happens. Sulphides are stables and very insoluble in redox condition, but mining activity exposes the minerals to atmospheric condition, then sulphide oxidation takes place generating acidity, sulphates and liberating Fe and accessory metals and metalloids [10].

Furthermore, in the 1960s a large chemical industry area was installed in the Ría de Huelva, the largest in Andalusia and one the largest in Spain. Its industries discharged into the estuary a yearly input of 50,000 t of equivalent sulphuric acid, 700,000 t of sulphates and some 12,000 t of heavy metals in 1987, before the local government elaborated an effluent remediation plan in order to mitigate environmental impacts in the Ría de Huelva [5]. These industrial discharges aggravated the pollution problem in the estuary. In this sense, Tinto and Odiel rivers flow into the Ría de Huelva where many of the transported metals precipitate due to the pH increase caused by the mixture with the seawater and owing to that, sediments from the Ría de Huelva present very high metal concentrations [2].

There are many studies of the dissolved heavy metal load transported by the Tinto and Odiel rivers that show the enormous amount of heavy metals discharge into the Ría de Huelva and they

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found that loads show important variation associated to variation in the flow regimen [3,4,8,11]. However, trace metals global assessment including bioaccumulation studies and availability of metal contamination as function of their speciation in water and sediment are scarce.

The aim of this work was to study the distribution of heavy metal in different compartments of aquatic environment in the Ría de Huelva and to assess bioavailability and potential impact on aquatic biota. For this purpose, concentrations of heavy metals (Cu, Zn, Cd, Pb and As) were measured in tissues (gill, liver and muscle) of two fish species (*Sparus aurata* and *Solea senegalensis* with pelagic and benthic behaviours, respectively) and the study of heavy metal speciation of water and sediments along the Ría de Huelva were carried out. Analyses of metal concentrations in tissues were performed by inductively coupled plasma-mass spectrometry (ICP-MS) or atomic emission spectroscopy (ICP-AES) after lyophilization and acid digestion. A modification of schemes of speciation Fischer et al. [12] and Batley [13], based on metal fractionation to different pH, was applied to the study of metallic distribution in water. Metal concentrations in water were determined by differential pulse anodic stripping voltammetry (DPASV) obtaining the following different metal fractions: labile and moderately labile forms, both organic and inorganic, and non-labile. Speciation analyses of arsenic were performed by hydride generated coupled atomic absorption spectroscopy (HGAAS), with an operational discrimination among particulate and organic and inorganic dissolved arsenic species. Sediments speciation using the BCR sequential extraction [14] (exchangeable + weak acid soluble, reducible, oxidisable and non-extractable metal fractions) was carried out and analyses were performed by ICP-AES and atomic absorption spectroscopy (AAS).

2. Materials and methods

2.1. Sampling sites

The Tinto river spring in the mountain of La Granada and has its mouth in San Juan del Puerto, after running along 83 km and developing 720 km² watersheds. The Odiel river spring in the mountains of Sierra of Aracena and has its mouth in Gibráleon, with 140 km in length and about 2330 km² watershed. The mean water discharges of the Odiel and the Tinto rivers are 15 and 3 m³ s⁻¹, respectively, with seasonal variations yielding high discharges during winter after important rain events and low discharges during the driest months. Both rivers flow together from Punta del Sebo into the Atlantic Ocean forming a common canal known as Canal del Padre Santo belonging to the Ría de Huelva. The area has a dry Mediterranean climate with an average pluviometry varying between 500 and 800 mm.

Three sampling sites were selected in this area of the south-west coastal of Spain (Fig. 1). The first one was located near Odiel river's mouth in front of the factory Atlantic Copper; the second one was located near Tinto river's mouth in front of the dock of La Rábida near phosphogypsum deposits; and the last one was located at Palos's industrial park in front of the Cepsa oil refinery of the Rábida (Huelva), being in the common estuary formed by Odiel and Tinto rivers.

Four samplings were made from October 2004 to May 2006, two of them were realized at autumn (October 2004 and October 2005) and the others were realized at spring (April 2005 and May 2006). So that it allowed to study the seasonal variability related with fish life cycles.

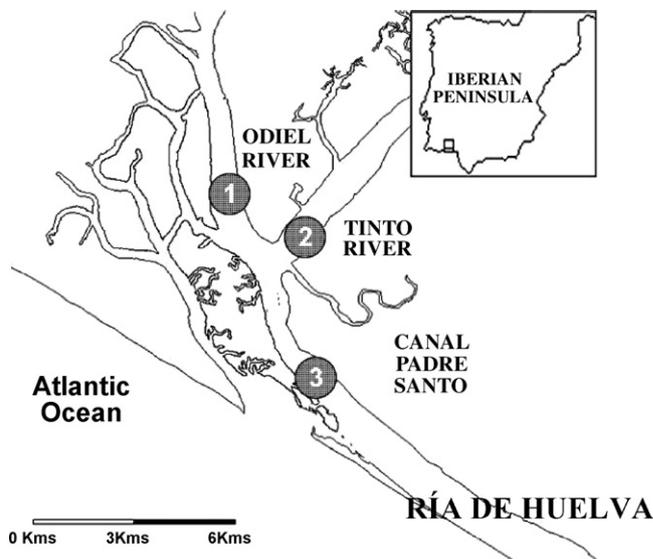


Fig. 1. Location map of sampling sites in Huelva (Spain): (1) Odiel river; (2) Tinto river; (3) Canal del Padre Santo.

2.2. Water collection and analysis

Samples collection was carried through on board a recreational boat in the sampling sites. Surface water samples were collected using a peristaltic pump (Masterflex 7521-00, poppet head 07518-10, Cole-Parmer Instrument Co., IL, USA), rigid Teflon pipes (FEP A-06450-07) and flexible Tygon tubing (6424-71). Water samples were picked up into LDPE bottles, which were pre-cleaned with 3 mol L⁻¹ HCl, rinsed with 10% HNO₃ and Milli-Q deionised water (Millipore, USA) six times, and stored in polyethylene bags until required. For dissolved metals analyses, they were filtered *in situ* using 0.45 μm Calyx Capsule MSI filter (3/8" barb, 1/PK, polypropylene; Osmonics, USA) connected on line with the Tygon tubes. In addition, no filtered samples were collected for total metal analyses. Several water samples were collected in each site in order to study distribution of heavy metal in water, based on metal fractionation to different pH and speciation analysis of arsenic.

Samples (250 mL) for total metal and total dissolved metal determination were acidified with 2 mL L⁻¹ HNO₃ of Suprapur grade, kept to room temperature during 1 week, and after were stored at -18 °C until analysis. In order to evaluate the different dissolved metal fractions according to fractionation scheme (Fig. 2), a filtered sample (250 mL) at natural pH for determining labile fractions was stored at -18 °C immediately after sampling and other filtered sample (250 mL) was conditioned at pH 4.7 for moderately labile fractions determination and stored at -18 °C as soon as possible. For that, addition of 10 mL of 3 mol L⁻¹ acetate buffer (pH 4.7) to 250 mL filtered water was carried out. Sub-samples (250 mL) used to speciation analysis of arsenic were acidified with 2 mL L⁻¹ HCl Suprapur and stored at -18 °C until analysis.

Total metal and total dissolved metal fraction were analysed by DPASV after acid digestion and slight neutralization with NaOH. With this aim, 45 mL of sample added with 0.2 mL of 65% HNO₃ and 0.125 mL of 70% HClO₄ (Suprapur grade) was digested in a closed Teflon reactor (PTFE, 100 mL, BRAND, 1305 38, Weirtheim, Germany) for 8 h at 120 °C, cooled and diluted up to 50 mL in a volumetric flask.

Labile (using filtered water at natural pH) (F1) and moderately labile (using filtered water at pH 4.7) (F3) inorganic dissolved

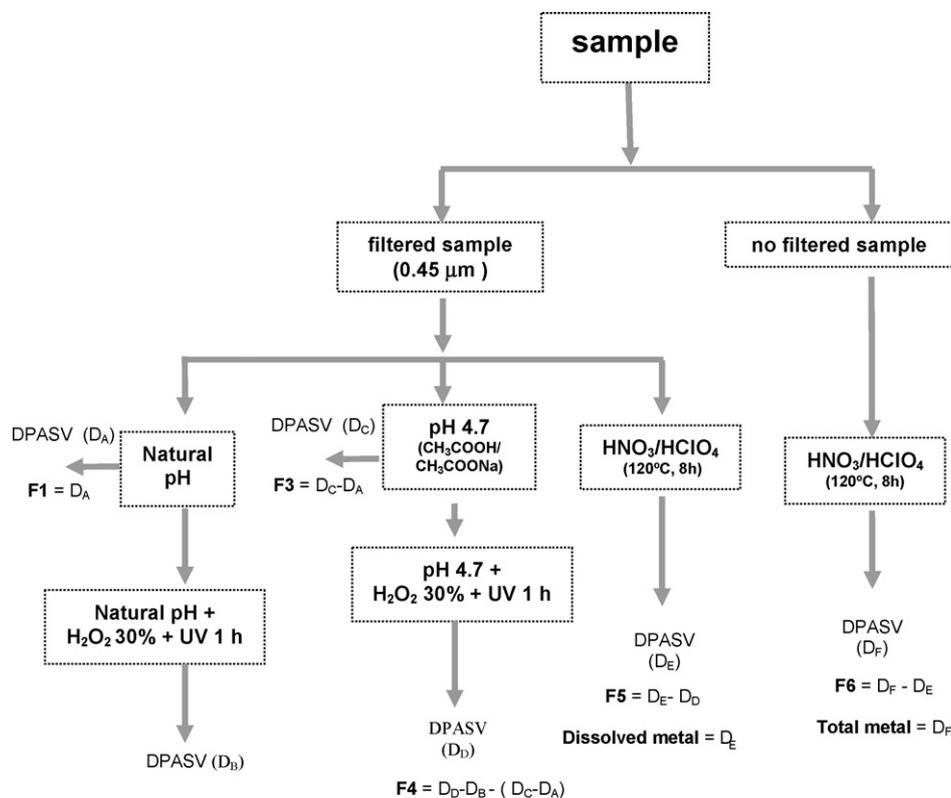


Fig. 2. Scheme of chemical fractionation of metal in water based on change of pH.

fractions were analysed directly by DPASV. Labile and moderately labile total dissolved fractions of samples were analysed by DAPS after UV digestion procedure. For that, 50 μL of 30% H_2O_2 (Suprapur grade) were added to 15 mL of sample and it was digested for 2 h using a UV digester. The contents of labile (F2) and moderately labile (F4) organic dissolved metal were determined by difference between both results, i.e. labile and moderately labile inorganic fraction from labile and moderately labile total fraction leaves organic fractions.

Speciation analyses of arsenic were performed by hydride generated coupled atomic absorption spectroscopy, with an operational discrimination among particulate and organic and inorganic dissolved arsenic species [15]. For that, two different acid digestions were accomplished. Inorganic dissolved arsenic species were analysed after reducing sample solution with HCl (1%, v/v) in a microwave heating. Total and dissolved arsenic was analysed by HGAAS after acid digestion of samples using HNO_3 (0.1%, v/v) and HCl (1%, v/v) heated in a microwave heating and using no filtered and filtered water, respectively. Particulate contents were determined by difference between total and dissolved

contents and organic dissolved contents were determined by difference between total dissolved and inorganic dissolved arsenic species.

In all cases, blanks were performed using ultrapure water (Milli-Q) as samples following the same protocol for each phase of speciation scheme and taken account for results. Sample handling and preparation of solutions were carried out using acid cleaned material, wearing polyethylene gloves and under laminar flow cabinet.

The quality of metals analysis methods in water was checked by using a certified reference water of the Community Bureau of Reference (BCR): CRM 505 (trace elements in estuarine water) with good satisfactory results (Table 1).

Temperature, pH, salinity and dissolved oxygen of water were measured *in situ* with an electrochemical portable device. Suspended solids (SS) were gravimetrically quantified by using 0.45 μm nylon filters (Osmonics, USA) and dissolved organic carbon was evaluated in different samples that those for metal analysis and using TOC analyzer.

Table 1

Analysis of trace metals from reference materials by DPASV (water), ICP-AES (sediment) and ICP-MS (As, Pb, Cd)/ICP-AES (Zn, Cu) (fish tissue)

Metal	CRM 505 ($\mu\text{g L}^{-1}$)		1649 a (mg kg^{-1})		DOLT-3 ($\mu\text{g g}^{-1}$)		DORM-2 ($\mu\text{g g}^{-1}$)	
	Certified	Found	Certified	Found	Certified	Found	Certified	Found
Zn	11.24 \pm 0.719	9.29 \pm 1.55	48.9 \pm 1.6	31.94	86.6 \pm 2.4	80.28 \pm 6.26	25.6 \pm 2.3	24.66 \pm 1.08
Cd	0.09 \pm 0.005	0.08 \pm 0.01	0.148 \pm 0.007	0.15	19.4 \pm 0.6	18.66 \pm 1.04	0.043 \pm 0.01	0.041 \pm 0.01
Pb	0.05 \pm 0.03	0.09 \pm 0.01	11.7 \pm 1.2	9.41	0.319 \pm 0.05	0.30 \pm 0.03	0.065 \pm 0.01	0.047 \pm 0.02
Cu	1.87 \pm 0.095	2.00 \pm 0.13	10.01 \pm 0.34	9.74	31.2 \pm 1.00	24.18 \pm 3.79	2.34 \pm 0.16	2.00 \pm 0.42
As	–	–	6.23 \pm 0.21	5.93	10.2 \pm 0.5	9.79 \pm 0.57	18 \pm 1.10	19.50 \pm 2.05

2.3. Sediments collection and analysis

Sediment samples (1000 mg) were collected from the surface sediment (2–20 cm) using an Eckman–Birdge dredge, immediately placed in polyethylene bags and transported to the laboratory where were stored at -4°C until analysis. Samples were dried at 105°C during 24 h, grinded using an agate mortar, sieved through a stainless steel mesh in order to obtain fine particle-size fractions and stored in polyethylene bottles until sequential extraction analysis.

The sequential extractions were performed by means of the three-step BCR extraction procedure described by Davidson et al. [16]. In the first step, 1 g of sediment was mechanically shaken for 16 h (frequency 150/min) with 40 mL 0.11 mol L^{-1} acetic acid and the extract separated by centrifugation. The residue, in the second step, was shaken for 16 h with 40 mL 0.1 mol L^{-1} $\text{NH}_2\text{OH}\cdot\text{HCl}$ adjusted to pH 2 with HNO_3 and the extract separated by centrifugation. In the step 3, the residue was twice heated at 85°C with 10 mL of 8.8 mol L^{-1} hydrogen peroxide (Suprapur), then extracted with 50 mL 1.0 mol L^{-1} $\text{NH}_4\text{CH}_3\text{COO}$ and separated by centrifugation. The residual fraction was twice heated by IR lamp with 5 mL HF 48% (Suprapur) in a Teflon Petri dish until dryness. This procedure was repeated twice with 5 mL HNO_3 65% (Suprapur) and the residue was lixiviated with 20 mL of 3.86 mol L^{-1} HCl by heating and magnetic agitation during 1 h.

Total acid digestions of sediments were performed using the same procedure employed with the residual fraction. The extracts collected in sequential extraction steps and in the total acid digestions were made up to 50 and 25 mL, respectively. All of them were stored at -4°C in acid washed polyethylene bottle until were analysed by ICP-AES or AAS. In all cases, respective blank samples were prepared and taken account.

The quality of total acid digestion of the sediment was checked by using a certified reference material of the National Institute of Standard & Technology (NIST) 1649a (estuarine sediment), yielded good accuracy of analysis (Table 1).

2.4. Fish collection and analysis

The samples of mature *S. aurata* (gilthead) and *S. senegalensis* (sole) were collected at sampling sites and were transported in aerated tanks to Mazagon's port (Huelva). Fishes were dissected and tissue sub-samples were taken out quickly from liver, gills and muscle. A total of 40 and 230 tissue samples of gilthead and sole, respectively, were obtained. Fish tissues were transported to the laboratory into nitrogen liquid and stored at -80°C .

Samples were freeze-dried and digested by microwave heating. Gills and muscle samples (0.2 g) were digested with 7 mL HNO_3 65%, and liver samples (0.2 g), containing higher organic matter, were digested with 4 mL of 65% HNO_3 (Suprapur) and 2 mL of 30% H_2O_2 (Suprapur). The following microwave program was applied: 4 min for 200 W, 5 min for 400 W and as last step 10 min for 600 W. After microwave digestion, samples were diluted up to 25 mL with Milli-Q deionised water. Metal concentrations of digested tissue samples were analysed by ICP-MS and ICP-AES. Blanks were also performed and measured for all metals following the same procedure.

The accuracy of the analytical methodology applied was satisfactory evaluated through the analysis of two certified reference biological materials of the National Research Council Canada (NRCC) DOLT-3 (dogfish liver) and DORM-2 (dogfish muscle) (Table 1).

2.5. Instruments and reagents

Differential pulse anodic stripping voltammetric measurements were obtained with a Metrohm 757 VA Computrace Stand (Metrohm, Switzerland) with automated hanging mercury drop electrode as working electrode. The cell also included an Ag/AgCl reference electrode (saturated with 3 M KCl) and a platinum wire as auxiliary electrode. The stand was controlled by PC software VA Computrace 2.0 installed in a personal computer using a 5326 VA Computrace Interface (Metrohm, Switzerland). A Metrohm model 746 VA Trace Analyzer processor with a Metrohm 747 VA Electrode Stand (Metrohm, Switzerland) was also used.

Atomic spectroscopy measurements were achieved by using following equipments: an atomic absorption Solar M Series spectrometer (AAS) (Thermo Elemental, UK), an inductively coupled plasma emission Iris Intrepid spectrometer (ICP-AES) (Thermo Elemental, UK) and X7 Series plasma scan sequential inductively coupled mass spectrometer (ICP-MS) (Thermo Elemental, UK). Hydride generated coupled atomic absorption measurements were carried out by AAS equipment coupled a HG accessory Unicam VP90 (Thermo Elemental, UK).

Organic matter was removed for real water samples by UV irradiation with a Metrohm model 705 UV Digester (Metrohm, Switzerland) in quartz tubes. Dissolved organic carbon was evaluated using Shimadzu TOC Analyzer model 5050 (Shimadzu, USA). Microwave assisted digestions were made by an Ethos 1600 microwave heating (Milestone, Italy). Promax 2020 shaker (reciprocal motion) (Heidolph, Germany) was used during lixiviation processes and the extracts separated by centrifugation using 4K10 Sigma Centrifuge (Oesterode, Germany). Fish tissues were freeze-dried using an Heto CT60e equipment (Allerd, Denmark).

Temperature, pH, salinity and dissolved oxygen of water were measured *in situ* with an electrochemical portable device (Sension 156, Hach Co., USA). A model 2001 pH-meter equipped with 52-02 combined glass-Ag/AgCl electrode (Crison, Spain) was used for lab pH measurements. Sample handling and preparation of solutions were performed using polyethylene gloves and under a class 100 laminar flow hood cabinet Crusair model 9005-FL (Cruma, Spain). Water was purified by reverse osmosis with an Elix 3 (Milli-RO) system followed by ion exchange with an 18 M Ω cm $^{-1}$ deionised Milli-Q 50 System (Millipore, USA).

All reagents for treatment of samples during trace analysis were Suprapur or Ultrapure grade, supplied by Merck (Darmstadt, Germany) or Sigma–Aldrich (Steinheim, Germany). All solutions were prepared using Milli-Q deionised water. High purity gases were used during analysis purchased by Air Liquid (Spain).

Statistical treatments were performed using the statistical software package STATISTICA (data analysis software system) Version 7 (2004, Statsoft Inc., USA).

3. Results and discussions

3.1. Metal concentrations in water

3.1.1. Total metal

Physical–chemical parameters and total metal concentrations of water samples evaluated in all samplings and mean values of each sampling sites are shown in Tables 2 and 3, respectively. Total metals were ranging over following intervals: Zn: 49.8–381.6 $\mu\text{g L}^{-1}$; Cd: 0.7–8.9 $\mu\text{g L}^{-1}$; Pb: 2.6–17.8 $\mu\text{g L}^{-1}$; Cu: 20.9–72.4 $\mu\text{g L}^{-1}$; As: 2.6–9.3 $\mu\text{g L}^{-1}$, showing a high metal enrichment comparing with other European estuaries. These results were in good agreement with previous studies carried out by Morillo et

Table 2
Physical–chemical parameters and total metal concentrations in water

Date	Sampling station	pH	Dissolved oxygen (mg L ⁻¹)	T (°C)	Salinity (g L ⁻¹)	Suspended solids (mg L ⁻¹)	DOC ^a (mg L ⁻¹)	Zn (μg L ⁻¹)	Cd (μg L ⁻¹)	Pb (μg L ⁻¹)	Cu (μg L ⁻¹)	As (μg L ⁻¹)
October 2004	1	7.85	6.30	24.0	35.8	12.4	3.67	80.6	1.3	3.7	34.5	4.7
	2	7.82	6.40	23.9	36.1	55.5	3.84	324.8	1.3	5.7	37.1	3.0
	3	8.01	6.74	23.9	35.2	23.4	4.11	121.5	0.8	2.6	20.9	2.8
April 2005	1	8.14	11.31	18.0	32.4	35.3	1.94	191.2	3.1	6.1	57.2	4.1
	2	8.21	7.45	18.7	32.4	34.8	2.22	198.3	4.5	7.4	68.0	6.0
	3	8.37	9.25	17.3	33.9	45.3	0.87	52.8	0.8	5.2	27.8	3.2
October 2005	1	7.87	8.40	20.0	34.7	38.3	2.81	124.9	1.6	17.8	56.7	9.3
	2	7.86	7.45	20.8	34.6	32.2	2.33	119.8	2.2	3.5	47.3	7.5
	3	8.15	13.60	21.1	35.2	51.1	1.48	49.8	0.7	5.7	22.2	6.2
May 2006	1	7.74	13.32	22.1	30.2	52.2	7.72	381.6	8.9	6.2	54.7	2.9
	2	7.84	13.3	21.7	29.5	55.0	7.4	252.0	6.8	5.0	72.4	5.3
	3	7.92	9.40	19.1	33.9	70.1	6.4	108.7	1.7	6.2	57.7	2.6

Sampling sites: (1) Odiel river; (2) Tinto river; (3) Canal del Padre Santo.

^a DOC: dissolved organic carbon.

al. [17] in 2001 and 2002 (Zn: 40–350 μg L⁻¹; Cd: 0.7–3.6 μg L⁻¹; Cu: 9–82 μg L⁻¹; As: 7–15 μg L⁻¹). Generally, content of metal in Tinto and Odiel sites were higher than content of metal in the Canal del Padre Santo site. Previous reports have described that the acid-rich waters from the Tinto and Odiel rivers mix with seawater and undergo a notable increase in pH, which causes a large amount of metal precipitation and diminishing dissolved metal. Otherwise, slight higher mean values were found for Zn and Cu in Tinto water comparing with Odiel samples. Conversely, higher mean values were found for Pb in Odiel water. As and Cd showed similar mean concentrations in both sites.

Linear correlations (Pearson's) between parameters analysed ($p < 0.05$) showed the following relationships: negative correlation for Cd ($R(x,y) = -0.919$) and Cu ($R(x,y) = -0.714$) with salinity was found showing a conservative behaviour of these metals in the estuary [18]; also, significant seasonal variability for these metals was found, being higher their concentrations during spring. Besides, Zn and Cd contents were positively correlated with DOC. Outstanding correlations between suspended matter, pH, T and dissolved oxygen with total metal concentrations were not found.

Comparing total metal concentrations, Cd showed a positive correlation with Cu and Zn, and Pb and As, with different behaviour than other metals, showed a positive correlation between them, probably owing to a similar source in the ecosystem.

The metal with the highest concentration was Zn and Cu. They can be arranged as follows (higher content to lesser content): Zn > Cu > Pb ≈ As > Cd and grouped depending on their behaviour in two sets: Zn–Cu–Cd and Pb–As. Pb and As average concentrations were similar in Tinto sampling site.

3.1.2. Dissolved and particulate metal distribution

The scheme of metal speciation in water applied in this work allowed to perform a first fractionation between dissolved and particulate metal, considering the last fraction as less available. Braungardt et al. [18] reported that metal distribution between liquid and solid phases in the Ría de Huelva was largely dominated by mixing behaviour up to high salinities, being this distribution of fundamental significance to geochemical modelling and pollution impact assessment.

Table 3
Mean values of physical–chemical parameters and metal concentrations in water for each sampling sites: (1) Odiel river; (2) Tinto river; (3) Canal del Padre Santo

Sampling station	Sampling site 1		Sampling site 2		Sampling site 3	
	Mean	S.D.	Mean	S.D.	Mean	S.D.
pH	7.9	0.2	7.9	0.2	8.1	0.2
Dissolved oxygen (mg L ⁻¹)	9.83	3.10	8.65	3.14	9.75	2.84
T (°C)	21.03	2.60	21.28	2.15	20.35	2.83
Salinity (g L ⁻¹)	33.28	2.49	33.15	2.87	34.55	0.75
Suspended solids (mg L ⁻¹)	34.55	16.50	44.38	12.60	47.48	19.23
DOC ^a (mg L ⁻¹)	4.04	2.56	3.95	2.42	3.22	2.55
Zn (μg L ⁻¹)	194.59	132.68	223.71	86.54	83.21	37.24
Cd (μg L ⁻¹)	3.73	3.55	3.70	2.49	0.99	0.45
Pb (μg L ⁻¹)	8.47	6.36	5.41	1.62	4.92	1.63
Cu (μg L ⁻¹)	50.80	10.91	56.19	16.81	32.16	17.30
As (μg L ⁻¹)	5.23	2.78	5.45	1.86	3.70	1.66
Dissolved Zn (μg L ⁻¹)	107.65	30.30	109.75	20.35	52.39	34.20
Particulate Zn (μg L ⁻¹)	86.95	113.07	113.96	77.44	30.82	23.10
Dissolved Cd (μg L ⁻¹)	1.81	0.78	2.18	0.96	0.64	0.20
Particulate Cd (μg L ⁻¹)	1.91	3.11	1.52	1.67	0.35	0.25
Dissolved Pb (μg L ⁻¹)	2.89	1.64	1.90	0.54	1.87	1.35
Particulate Pb (μg L ⁻¹)	5.58	5.20	3.51	1.80	3.05	2.05
Dissolved Cu (μg L ⁻¹)	20.38	7.64	23.11	15.96	11.00	1.79
Particulate Cu (μg L ⁻¹)	30.42	9.51	33.08	14.11	21.16	17.88
Dissolved As (μg L ⁻¹)	4.68	2.55	4.96	1.78	3.52	1.67
Particulate As (μg L ⁻¹)	0.56	0.30	0.49	0.22	0.17	0.18

^a DOC: dissolved organic carbon.

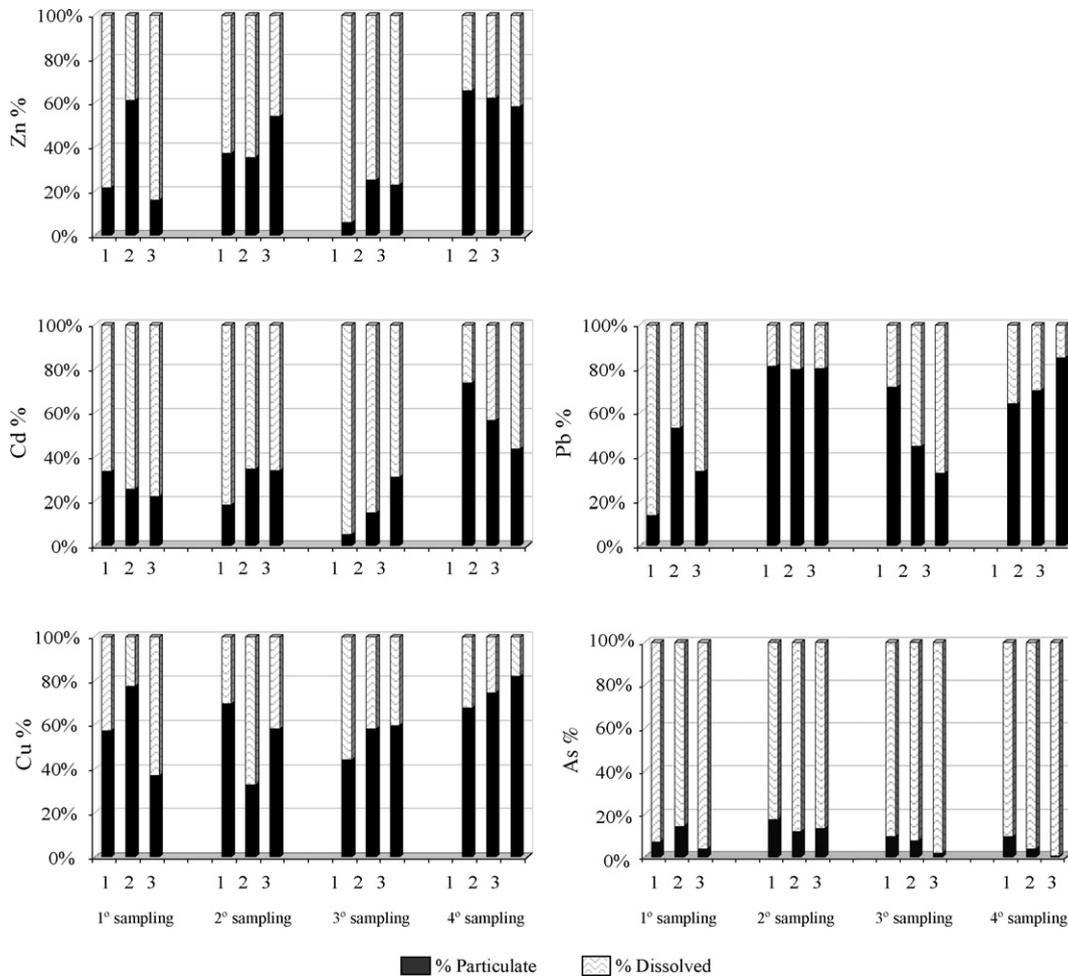


Fig. 3. Distribution of dissolved and particulate metal in samples of Ría de Huelva. Sampling sites: (1) Odiel river; (2) Tinto river; (3) Canal del Padre Santo.

Distributions of dissolved and particulate metals in percentage, obtained in this study, are shown in Fig. 3 and average values for each sampling site are listed in Table 3. Spatial variation was found for dissolved and particulate metal mean concentrations, being higher concentrations in Tinto and Odiel sampling sites, except for Pb with similar values between Tinto and Canal del Padre Santo and lower than those founded in Odiel point.

Otherwise, high percentages of particulate metal were obtained for Pb (13.9–85.1%) with a seasonal variation, increasing in spring samples. Cu, with percentages ranging from 32.7% to 88.2%, did not show clear variability with surveys. Zn particulate fraction increased in spring samples and sometimes constituted 54–66% of the total metal in water. Cd appeared mainly in dissolved fraction, although particulate fraction increased 43.7–73.6% during the last sampling. As in water was found principally as dissolved metal

Table 4
Background level, nature concentration and guidelines levels of metals in water described in the literature

	Reference	Zn ($\mu\text{g L}^{-1}$)	Cd ($\mu\text{g L}^{-1}$)	Pb ($\mu\text{g L}^{-1}$)	Cu ($\mu\text{g L}^{-1}$)	As ($\mu\text{g L}^{-1}$)
Background level	[20]	0.01	0.01	0.005–0.015	0.04–0.1	2.1
Nature concentration	[21]	<0.6	<0.025	<0.02	0.01	1.3–2.5
NOAA–EPA						
CMC ^a	[22]	90	40	210	4.8	69
CCC ^b		81	8.8	8.1	3.1	36
Quality of water (IV ^c)						
Limited water	[23]	80	5	20	40	50
No limited water		60	2.5	10	20	25
Total metal	Present study	49.8–381.6	0.7–8.9	2.6–17.8	20.9–72.4	2.6–9.2
Dissolved metal	Present study	24.3–130.3	0.48–3	0.9–5	8.4–45.8	2.6–8.4

^a CMC: criteria maximum concentration (dissolved metal).

^b CCC: criteria continuous concentration (dissolved metal).

^c IV: imperative values (total metal) proposed by Andalusia Government (Spain).

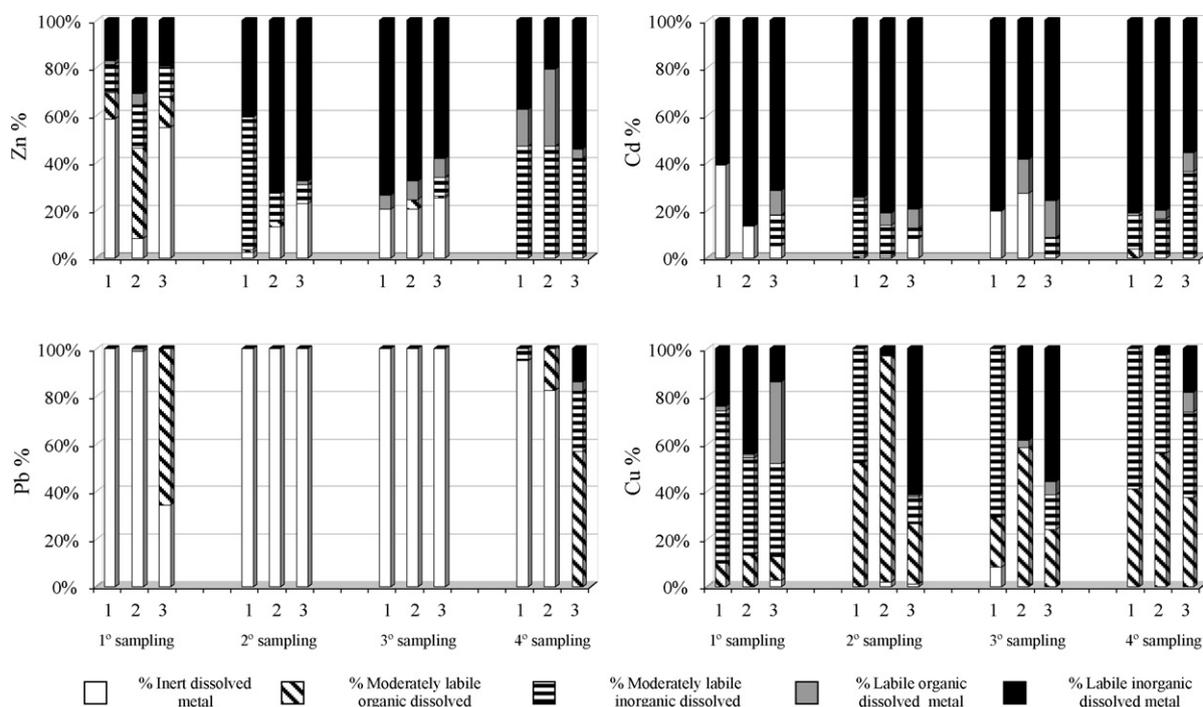


Fig. 4. Speciation of dissolved metal in water from sampling sites: (1) Odiel river; (2) Tinto river; (3) Canal del Padre Santo; for 1–4 surveys (in percentage).

(82.6–99.5%) in all sampling, being more available as this fraction. So, metals arranged by higher percentages of dissolved (related with more availability) were: As > Cd > Zn > Pb > Cu with following average percentages, 91.5%, 67.3%, 61.1%, 40.7% and 40.1%, respectively.

Partition coefficient (K_d (mL g^{-1}) = $[M_{\text{particulate}}] / [M_{\text{dissolved}}]$) were obtained and they were relatively low comparing with those usually found in estuaries and similar than those obtained by Achterberg et al. [19]. They ranged from 0.1 to 6.1 for As, 9.5–124 for Pb, 1.4–53.4 for Cd, 13.9–109.1 for Cu and 1.7–36.9 for Zn. So, higher K_d were obtained for Pb and Cu, indicating affinity of metal to be associated and transported with the solid phase. Partition coefficients showed no trend for Cu, Cd and Pb as a function of suspended matter. However, a decrease in K_d for As was

observed in association with the addition of dissolved As to the water column; and a increase in K_d for Zn was obtained linking to permanently suspended material transported through the waters.

In order to assess the potential hazardous impacts, the heavy metal levels in water have been compared with reported reference values, background and nature concentration, and also compared with quality guidelines for protection of aquatic life proposed by several organizations guidelines and imperative values of regional government (Table 4). Metal concentrations studied were considerably higher than background values and nature concentrations in water. However, concentration values of As, Cd and Pb were lower than those proposed by EPA criteria of maximum concentration (CMC) and continuous concentration (CCC), both

Table 5
Total and average metal concentration in sediments for each sampling sites: (1) Odiel river; (2) Tinto river; (3) Canal del Padre Santo

Date	Sampling station	Zn (mg kg^{-1})	Cd (mg kg^{-1})	Pb (mg kg^{-1})	Cu (mg kg^{-1})	As (mg kg^{-1})
October 2004	1	602.94	3.43	136.76	1115.70	85.15
	2	1506.68	4.84	246.15	2099.49	198.79
	3	1327.12	3.87	343.25	2325.75	237.74
April 2005	1	1736.95	33.20	1166.67	3333.63	615.43
	2	738.41	5.11	358.38	1470.11	284.29
	3	1489.18	8.93	879.06	2469.38	599.81
October 2005	1	1308.01	15.99	817.35	2157.68	375.03
	2	1161.15	18.30	974.95	1513.77	530.39
	3	1569.12	14.04	1028.15	2652.79	486.77
May 2006	1	966.92	5.08	238.87	1829.17	246.24
	2	1054.66	5.41	404.33	2505.34	343.86
	3	1337.25	1.50	269.64	1412.51	211.98
Mean value	1	1153.71	14.43	589.91	2109.05	330.46
	2	1115.23	8.42	495.95	1897.18	339.33
	3	1430.67	7.09	630.03	2215.11	384.08
S.D.	1	483.81	13.70	487.50	925.03	223.95
	2	316.76	6.59	326.17	496.72	140.60
	3	118.38	5.58	379.75	551.55	189.84

established to prevent from acute and chronic toxicity to aquatic organisms, respectively.

Special attention should be care on dissolved Cu concentrations because their concentrations were higher (mean of five times) than those recommended by CMC ($4.8 \mu\text{g L}^{-1}$) and CCC ($3.1 \mu\text{g L}^{-1}$) levels. In addition, imperative values of regional government were exceeded in all sample sites except for first sampling, considering Tinto and Odiel point as limited area and Canal del Padre Santo as no limited area (in accordance with Andalusia Government instructions). Zn concentrations exceeded EPA criteria and imperative values in water of Tinto and Odiel and sometimes in the Canal del Padre Santo. Cd only exceeded imperative values for fourth sampling in Tinto and Odiel sites.

3.1.3. Speciation of dissolved Zn, Cd, Cu and Pb

Five metal fractions associated to dissolved metal (Zn, Cd, Cu and Pb) were obtained following the scheme of speciation based on metal fractionation to different pH (Fig. 2). They were labile inorganic metal (F1), labile organic metal (F2), moderately labile inorganic metal (F3), moderately labile organic metal (F4) and inert fraction metal (F5). It is important to emphasize that labile fractions (inorganic and organic) are the most available. The results obtained from metal speciation (Fig. 4) suggested the following behaviours of metal in the studied area:

- Zn appeared associated to inert fraction and organic matter being scarce available during first sampling, but after labile dissolved Zn increased showing a maximum of 74% labile inorganic dissolved fraction in Odiel site during the third sampling when availability of this metal was maximum. During last survey, Zn appeared associated to moderately labile inorganic fraction (40–47%).
- Cd was one of the most available metals in this study, appearing in all cases mainly associated to labile inorganic dissolved fraction (55–86%) and slightly associated to inert dissolved fraction in Odiel and Tinto sites at autumn samples and to moderately labile inorganic dissolved fraction in spring samples.
- Conversely, Pb was the less available metal, usually associated to the inert fraction principally during second and third sampling being the unique fraction, although moderately labile organic dissolved fraction was slightly significant in Canal Padre Santo water during first and last sampling (65% and 57%, respectively).

Table 6

Guidelines levels of metals in marine sediment described in the literature and metals levels in Ría de Huelva

Metal level	Reference	Zn (mg kg^{-1})	Cd (mg kg^{-1})	Pb (mg kg^{-1})	Cu (mg kg^{-1})	As (mg kg^{-1})
Canadian SQG						
ISQG ^a	[27]	124	0.7	30.2	18.7	7.24
PEL ^b		271	4.2	112	108	41.6
NOAA (NS&T)						
ERL ^c	[28,29]	150	1.2	47	34	8.2
PEL ^b		271	4.21	112.2	108.2	41.6
ERM ^d		410	9.6	218	270	70
ERL ^c	[30]	120	0.6	31	16	6
ERM ^d		820	10	250	110	33
PEC ^e	[31]	459	5	128	149	33
Ría de Huelva	[17,24,25]	1000–3000 840–7460 1800–3150	3–12 1.4–10.2 0.8–70	1000–2400 260–1660 300–900	2000–2700 480–2700 200–2500	100–1090
	Present study	602–1737	1.5–33.2	137–1167	1116–3334	85–615

^a ISQG: interim marine sediment quality guidelines.

^b PEL: probable effects levels.

^c ERL: effects range low.

^d ERM: effects range medium.

^e PEC: probable effect concentration.

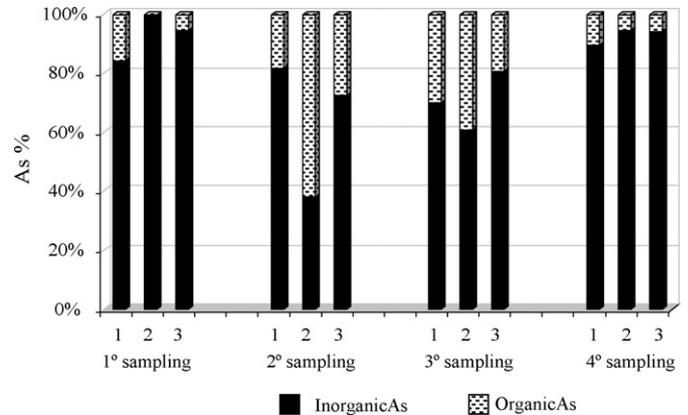


Fig. 5. Distribution of inorganic and organic dissolved As in water of Ría de Huelva. Sampling sites: (1) Odiel river; (2) Tinto river; (3) Canal del Padre Santo; for 1–4 surveys (in percentage).

- Cu appeared associated to moderately labile inorganic and organic dissolved fraction with an important percentage of labile metal during autumn samplings, mainly in Tinto and Canal Padre Santo sites, being more available. Otherwise, it has been observed a increase of labile inorganic dissolved fraction of Cu in Canal Padre Santo water during samplings of 2005 (56–61%). As above mentioned, dissolved concentration of this metal were high and now, this study showed also its availability.

Based on these fractionation studies of dissolved metal, the element under study can be ordered by ranking of availability as follows $\text{Cd} > \text{Zn} > \text{Cu} > \text{Pb}$.

3.1.4. Speciation of dissolved As

The speciation of As applied in this study distinguishes between inorganic and organic dissolved As, being inorganic fraction the more toxic of them. The results obtained are shown in Fig. 5. Inorganic As was the majority fraction in Ría de Huelva even with a 95% of dissolved metal, although an increase of organic As was observed during 2005, principally in Tinto river (62% in the second sampling). It is important outstanding that dissolved fraction was very higher than particulate fraction, increasing the availability of this metal.

Table 7

Average metal concentrations in tissue of *Sparus aurata* (gilthead) and *Solea senegalensis* (sole) fishes from Ría de Huelva (mg kg⁻¹ dry weight)

Fish	Tissue	Zn (mg kg ⁻¹)	Cd (mg kg ⁻¹)	Pb (mg kg ⁻¹)	Cu (mg kg ⁻¹)	As (mg kg ⁻¹)
Gilthead	Gill	97.52	0.10	3.68	9.96	11.17
	Liver	230.45	4.24	1.58	394.26	7.13
	Muscle	44.86	0.01	0.32	1.87	13.58
Sole	Gill	73.70	0.56	4.52	11.90	13.82
	Liver	88.20	1.82	1.46	433.45	14.56
	Muscle	23.68	0.01	0.40	1.41	10.61

3.2. Metal concentrations in sediment

Estuaries are zones of complex interaction between fluvial and marine process that act as geochemical trap for heavy metals bonded in the fine-grained sediments; also, estuarine mixing leads to a depositional acceleration of clay mineral due to salinity change, favouring the entrapment and accumulation of metal adsorbed on clay particles [1]. It has been reported that heavy metals carried by Tinto and Odiel rivers display a great affinity for clay fraction. Due to this process of co-precipitation that happen when metal-rich waters of the rivers mix with seawater and undergo a notable increase of pH, the content of metal in sediment of estuary are higher than the content in the rivers as many author has described [24–26].

3.2.1. Total metal

The total metal concentrations and average values for each sampling sites found in sediment of Ría de Huelva in this study are shown in Table 5. Metal contents were ranging over following intervals: Zn: 602.94–1736.9 mg kg⁻¹; Cd: 1.5–33.2 mg kg⁻¹; Pb: 136.8–1166.7 mg kg⁻¹; Cu: 1115.7–3333.6 mg kg⁻¹; As: 85.1–615.4 mg kg⁻¹. Mean contents of the ecosystem studied were: Zn: 1233.20 mg kg⁻¹; Cd: 9.98 mg kg⁻¹; Pb: 571.96 mg kg⁻¹; Cu: 2073.78 mg kg⁻¹; As: 351.29 mg kg⁻¹, allowing to arrange the metals from higher to lower mean content in this estuary as: Cu > Zn > Pb > As > Cd. A comparison of this ranking with that obtained for dissolved metal content, it can be seen that metals with higher percentages of particulate fraction (Cu, Pb and Zn) have now higher content in the sediment. Higher values were

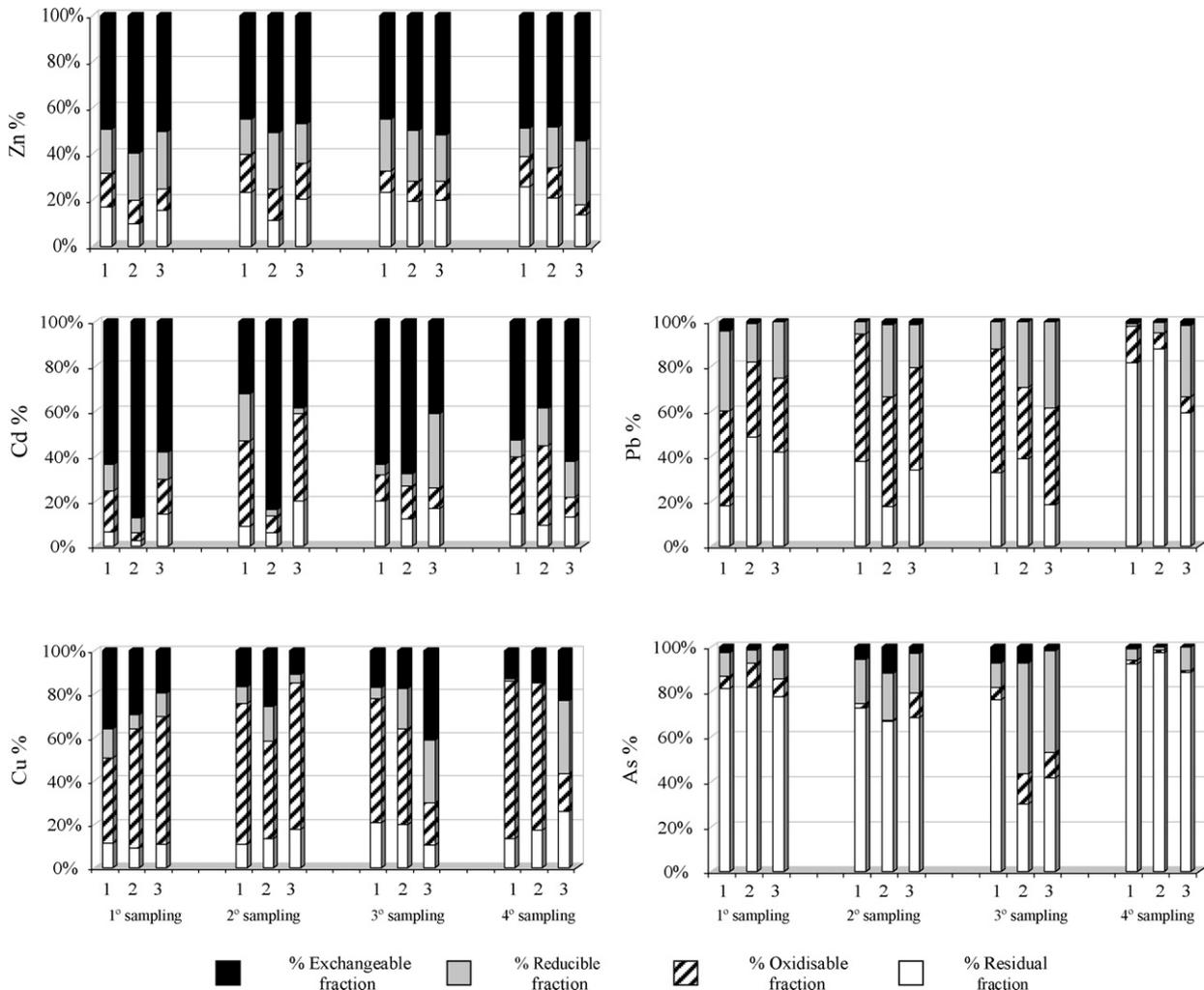


Fig. 6. Distribution of heavy metals in sediment from Ría de Huelva as determined by sequential chemical extraction (BCR procedure). Sampling sites: (1) Odiel river; (2) Tinto river; (3) Canal del Padre Santo. Values for 1–4 surveys (in percentage).

found in site 1 during April 2005 sampling and conversely, lower values were found in the same site during October 2004. It is noteworthy that higher mean values were found in the last point (sampling site 3) near the mouth of the Ría, according to process of metal deposition above mentioned. Only Cd has higher mean values in Odiel site.

The results were compared with guidelines levels of the impact on biological activity by means of established levels for the protection of aquatic life proposed by the US National Oceanic and Atmospheric Administration (NOAA (NS&T: National State and Trend program)) and Canadian sediment quality guidelines for the protection of aquatic life proposed by the Canadian Council of Ministers of Environment. These values are included in Table 6. Other reference levels previously reported and, in addition, metal concentrations from sediment of Ría de Huelva evaluated by others authors have been included in the same table.

Concentrations of all metals studied in sediment were higher than guidelines values recommended by Canadian government (ISQG and PEL) in all samples collected. Cd always reached the level of effects range low (ERL) and only sometimes reached the

level of effects range medium (ERM). The rest of metals under study use to overcome ERM values. In the same sense that happened with dissolved Cu in water, special attention should be care on Cu concentrations because their concentrations were too much high.

Otherwise, these results were in agreement with the studies of other authors and demonstrated the high risk for aquatic life due to the high metallic pollution.

3.2.2. Sequential extraction of metal

The distribution of metals in sediment (in percentage) following BCR procedure is shown in Fig. 6. This fractionation remained practically homogeneous during this study for all metals and significant differences were not observed between sampling.

Cd was the most mobile metal mainly bound to weak acid and carbonates (32–90%); a similar distribution of Cd was found by others researches previous studies about marine sediments from the southwest coast of Spain [25,32]. Related to that, Cd in water showed seasonal behaviour with higher values during spring and dissolved Cd was the most available metal in water although exceeded guide values in few sampling. As conclusion, a similar pat-

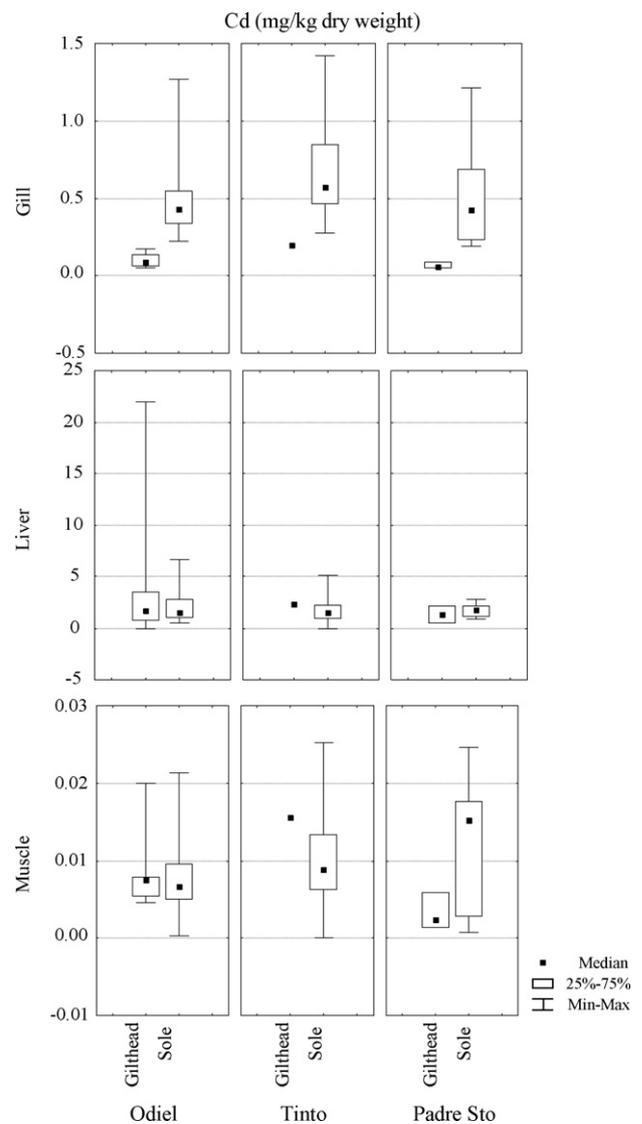
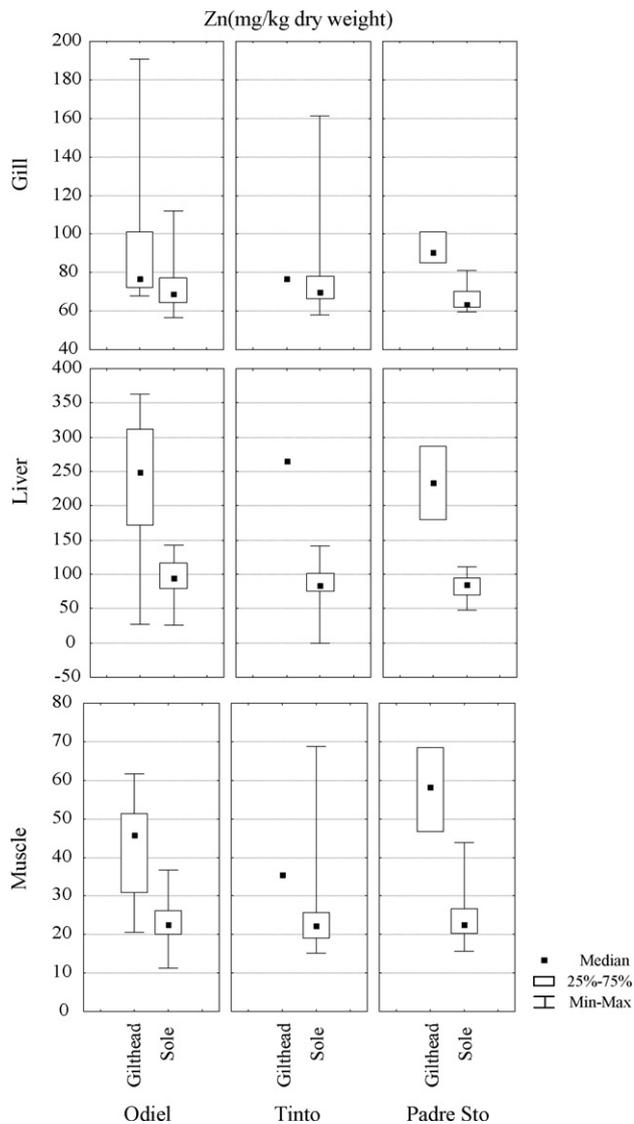


Fig. 7. Box-Whisker graphics of Zn in fish tissues (gill, liver and muscle) of Ría de Huelva.

Fig. 8. Box-Whisker graphics of Cd in fish tissues (gill, liver and muscle) of Ría de Huelva.

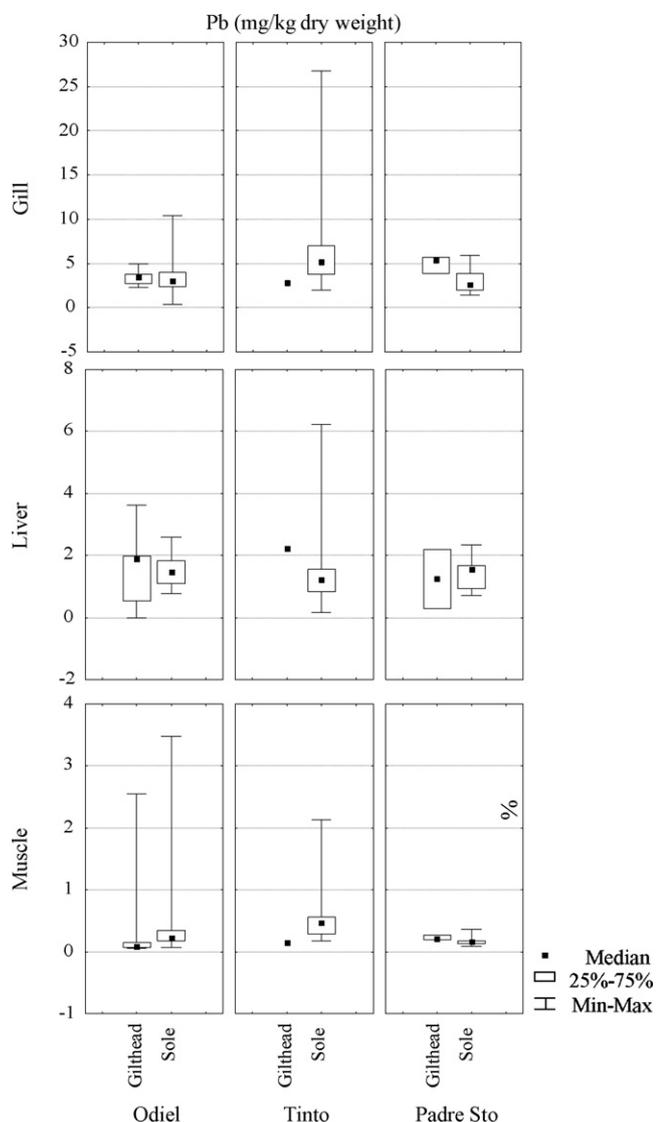


Fig. 9. Box-Whisker graphics of Pb in fish tissues (gill, liver and muscle) of Ría de Huelva.

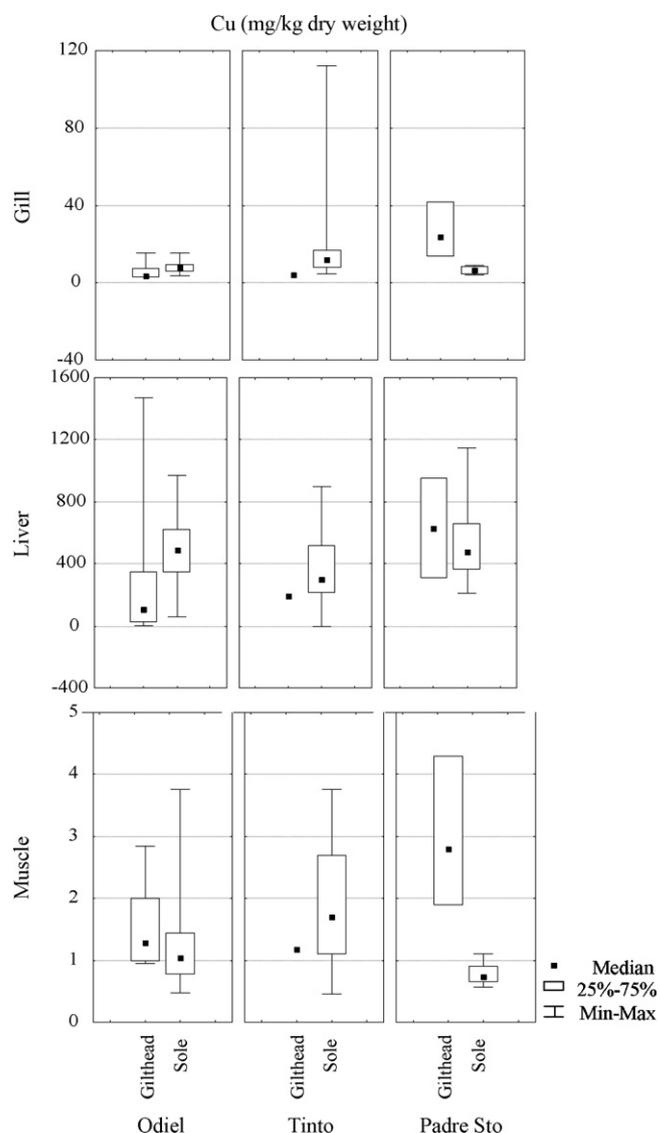


Fig. 10. Box-Whisker graphics of Cu in fish tissues (gill, liver and muscle) of Ría de Huelva.

tern of Cd in both compartments of the aquatic system was found in this ecosystem.

In the same way, Zn showed a high mobility appearing mainly bound to most labile fractions (exchangeable fraction ranging over 46–64%), probably due to the incorporation into these phase of Zn that precipitates as coating onto amorphous or poorly crystallized components of the sediments. This aspect was appointed when partitioning coefficient were calculated for this metal, when an increase in K_d was observed in association with increase of suspended matter.

Cu showed the highest percentage of oxidisable fraction (ranging from 18% to 70%), probably due to Cu forms easily complexes with organic compounds and the estuary receives sewage from city of Huelva, who carries a large content of organic matter associated to oxidisable fraction. The organic fraction released in the oxidisable step is not considered very available because it is thought to be associated with stable high-molecular weight humic substances that release small amounts of metal slowly [25]. Therefore, Cu mobility was lower than Cd and Zn availability from sediment as in water samples happened.

Pb and As yielded the least mobility, being mainly Pb associated to reducible, oxidisable and residual fractions and As associated to residual fraction which represents metals largely embedded in the crystal lattice of the sediment fraction and should not be available for remobilization except under extreme conditions.

According the results, the availability patterns of metals were similar in water and sediment samples, being the ranking of metal availability in sediment as follows: Cd > Zn > Cu > Pb > As.

3.3. Metal concentrations in tissues of fish

The metal concentrations in muscle, gill and liver of *S. aurata* (gilthead) and *S. senegalensis* (sole) fishes from Ría de Huelva were measured from 40 and 230 specimens of gilthead and sole, respectively. Average metal concentrations in different tissues are shown in Table 7 and Figs. 7–11 shows Box-Whisker graphics for metal concentrations. This box plot describes the central tendency of the variable in terms of the median of the values, the variability represented by the quartiles (the 25th and 75th percentiles) and the minimum and maximum values of the variable. High Cu and Zn levels were observed in liver tissue in according with higher

Table 8
Maximum and standards levels in mg kg^{-1} (wet weight) of metals in fish described in the literature and range of concentrations found in muscle of fishes from Huelva

	Reference	Zn	Cd	Pb	Cu	As
Maximum levels	[33]		0.05 ^a /0.10 ^b /0.30 ^c	0.3		
International standard						
New Zealand	[34]	40	1	2	30	1
Hong Kong			2	6		1.4
Zambia		100		10	100	5
Turkish guidelines	[35]	50	0.1	1	20	
Tolerance level in fish (FAO/WHO, 1989; ITS, 2000)		50	0.1	0.5		
Gilthead	Present study ^d	4.72–15.75	0.0003–0.0046	0.012–0.586	0.218–0.988	1.15–7.46
Sole		2.61–15.80	nd ^e –0.006	0.018–0.801	0.106–0.866	0.47–14.04

^a Muscle metal of fish excluding species listed in (b) and (c).

^b Muscle metal of following fish: anchovy, bonito, common two-banded seabream, eel, grey mullet, horse mackerel or scad, louver or luvar, sardine, sadinops, tuna and wedge sole.

^c Muscle meat of swordfish.

^d Muscle of gilthead and sole.

^e No detected.

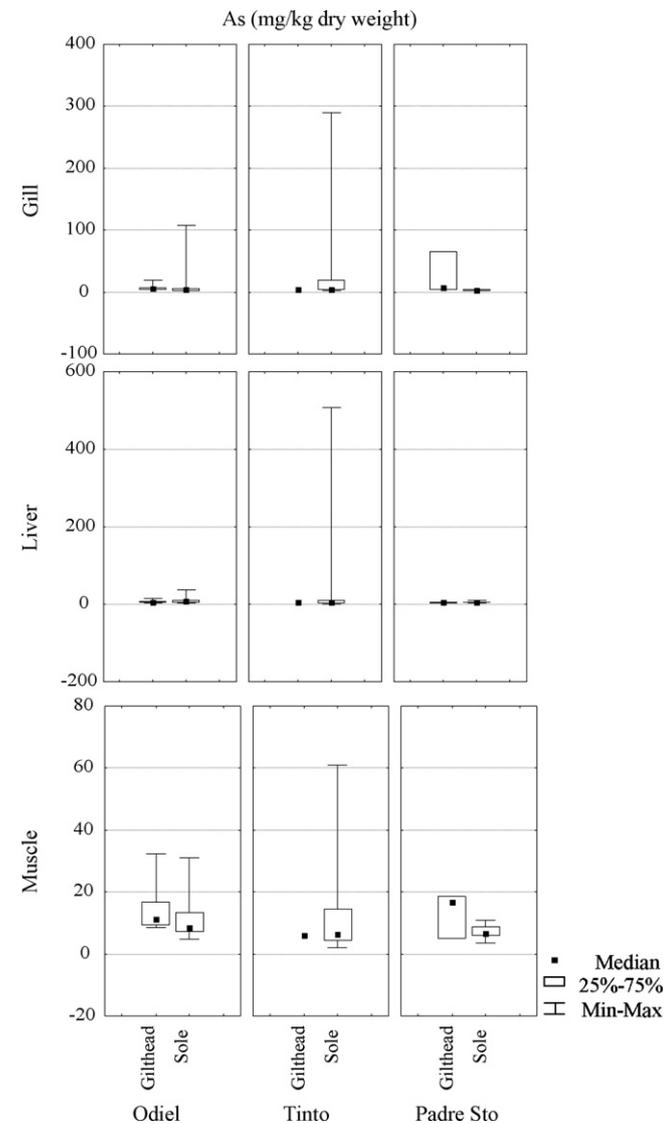


Fig. 11. Box-Whisker graphics of As in fish tissues (gill, liver and muscle) of Ría de Huelva.

total content and more available metals both water and sediment. Comparing fishes, gilthead had the higher mean contents of Zn, Cd and Cu in liver and Pb and As in gill. For sole, the higher mean contents of Zn, Cd, Cu and As were found in liver and of Pb in gill. Comparing mean concentration in tissues, gill and muscle showed the following accumulation ranking: $\text{Zn} > \text{As} > \text{Cu} > \text{Pb} > \text{Cd}$ while liver had the following one: $\text{Cu} > \text{Zn} > \text{As} > \text{Cd} > \text{Pb}$. Therefore, differences between fish species and tissues were observed. Conversely, significant differences among sampling sites were not found.

The results obtained for muscle samples were compared with limit values and guidelines found in the bibliography (Table 8) using wet weights. These values usually are referred to muscle or pool content. The levels of metals in muscle were usually less than maximum levels and guidelines values for two fish species studied. However, several muscle tissues had higher concentrations of As than standard levels described in the literature. So, mean values of 4.57 mg kg^{-1} (wet weight) for muscle of *S. aurata* and 3.29 mg kg^{-1} (wet weight) for muscle of *S. senegalensis* were found for As and they were higher than some guidelines levels. Generally, the levels of metal in liver samples were higher than FAO/WHO and CE limits values but they probably are not comparables with those usually referred to muscle or fish pool.

3.4. Bioavailability of metals in fish species of Ría de Huelva

The Pearson's lineal correlation was obtained between metal levels in fractions of water and sediment and metal levels in fish tissues in order to study bioavailability of heavy metal. Significant correlations ($p < 0.1$ and $p < 0.05$) between metal levels in fishes and speciation phases of water and sediment are shown in Table 9.

It is remarkable that positive correlations were found between metals in tissues and water, being more important for *S. aurata*, a pelagic fish more related to water column. Correlations with total Zn and Cd concentrations in water can be based on correlations with labile forms, labile organic and moderately labile in the case of Zn and labile inorganic and labile and moderately labile organic in the case of Cd, meaning the "bio" availability of these metals in this phase. On the other hand, *S. senegalensis* showed positive correlations for no labile fraction of Zn in water. Also positive correlations among moderately labile organic fraction of Cd and Pb were found.

Correlations with sediment fractions were scarce, outstanding the association between content of Cd in sole with oxidisable fraction of sediment. This fish has a benthic behaviour and could be more influenced by sediment. Therefore, the "bio" availability of this metal would depend on Cd bound to organic matter instead of

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