

# **Ability of 3 extraction methods (BCR, Tessier and Protease K) to estimate bioavailable metals in sediments from Huelva estuary (Southwestern Spain)**

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## **HIGHLIGHTS:**

- Protease K represents the bioavailable fraction in sediments most reliably.
- BCR and Tessier sequential extraction methods overestimate bioavailable fraction.
- BCR and Tessier methods have a similar ability to estimate bioavailable fraction.
- Bioavailable metals in sediment from Huelva estuary are related to total content.

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## **Abstract**

The bioavailable fraction of metals (Zn, Cu, Cd, Mn, Pb, Ni, Fe, and Cr) in sediments of the Huelva estuary and its littoral of influence has been estimated carrying out the most popular methods of sequential extraction (BCR and Tessier) and a biomimetic approach (protease K extraction). Results were compared to enrichments factors found in *Arenicola marina*. The linear correlation coefficients ( $R^2$ ) obtained between the fraction mobilized by the first step of the BCR sequential extraction, by the sum of the first and second steps of the Tessier sequential extraction, and by protease K, and enrichment factors in *Arenicola marina*, are at their highest for protease K extraction (0.709), followed by BCR first step (0.507) and the sum of the first and second steps of Tessier (0.465). This observation suggests that protease K represents the bioavailable fraction more reliably than traditional methods (BCR and Tessier), which have a similar ability.

## **Keywords**

Trace metals; sediment bioavailability; BCR Tessier sequential extraction; Protease K; biomimetic approach; Huelva estuary;

## 1 Introduction

Quite a few human activities produce waste, much of which is discharged in aquatic ecosystems (Salomons et al., 1987). Pollutants are present in these ecosystems in both water and sediment, where they accumulate over time as a result of various physical and chemical processes and can reach concentrations that are much higher than those in water (Ashokkumar et al., 2009). In addition, sediments are a food source for many species and any toxic substances contained in them can be transmitted to aquatic species and can affect humans through the food chain. For all these reasons, the presence of contaminants in the sediments of aquatic ecosystems has become a major environmental problem (Chapman and Wang, 2001; Ruiz et al., 2008; Sainz and Ruiz, 2006).

When a deposit-feeder organism ingests sediment, only a fraction of the total amount of contaminant present therein is absorbed and becomes part of its body, known as the bioavailable fraction. Therefore, methods to quantify the bioavailable fraction are needed. Sequential extractions are the most used methods to estimate the mobility of metals (Gleyzes et al., 2002; Izquierdo et al., 1997; Nieto et al., 2007), closely related to bioavailability. Among the various existing methods, the most widely used (Long et al., 2009; Rao et al., 2007; Yu et al., 2010) are BCR-701, developed by the Community Bureau of Reference (Pueyo et al., 2001), and the one proposed by Tessier et al. (1979). Many studies attest to the precision, accuracy and reproducibility (Sutherland, 2010; Tessier et al., 1979) of both. Furthermore, these methods meet all the necessary requirements for implementation over a wide area: simple, applicable to all kinds of sediments, low cost and easily understandable and comparable results.

However, sequential extractions use different chemical reagents to those found in the digestive tract of deposit-feeder organisms, where hydrolytic enzymes and chemicals derived from the pre-digestion abound (Mayer et al., 1997). Therefore, their use to quantify the bioavailable fraction is questioned (Turner, 2000).

There are methods specifically designed to estimate the bioavailability of metals in sediments, such as biomarkers or bioaccumulation studies. Biomarkers, also known as biochemical responses, are used as quick indicators of contamination in living organisms (Ellis and Schneider, 1997). However, many biomarkers respond to changes in environmental conditions (temperature, parasites, etc.), meaning that they are not reliable. Furthermore, a biomarker only responds to one or few contaminants, so this method is

complex and time-consuming to study a wide range of contaminants (Chen and Summons, 2001; Luca-Abbott, 2001; Viganò et al., 2001).

In bioaccumulation studies, pollutant concentrations are determined in tissues or fluids of the stomach and intestine of benthic organisms, such as oligochaetes, insects or mollusks (Chen and Chen, 1999; Chen and Mayer, 1999; Pempkowiak et al., 1999; Sanchiz et al., 2001; Usero, 1997). This is probably the most appropriate method to evaluate the bioavailable fraction of the pollutant in a particular species. However, extracting the required amount of fluid and thus obtaining a sufficient number of organisms, is time-consuming and is not suitable for analyzing a large number of samples (Turner, 2000).

Although biomarkers and bioaccumulation studies could provide a better estimate of the bioavailable fraction compared with sequential extractions, their implantation is complex over a large area because of the disadvantages outlined above.

In order to assess the bioavailability of metals in sediments by methods specifically designed for this purpose and which can be applied over large areas, some researchers developed biomimetic methods. These methods employ biological reagents located in the digestive system of deposit-feeder organisms (Turner, 2000) to reproduce in the laboratory the metal solubilization that occurs in the digestive tract of the deposit-feeder organism.

Gastrointestinal conditions can be simulated using digestive enzymes (Mayer et al., 1995), such as pepsin, trypsin and protease K (Ruby et al., 1996; Turner et al., 2001; Wróbel et al., 1999). Recent research (Bignasca et al., 2011; Ianni et al., 2010; Turner, 2006) demonstrates the potential of protease K to quantitatively imitate the interaction between metals and the digestive fluids of deposit-feeder organisms.

This study has been carried out in Huelva estuary, formed by the confluence of the rivers Tinto and Odiel, and consists of estuaries of both rivers and the Padre Santo Canal (Figure 1). The estuary of the Odiel has a secondary outlet to the sea known as Punta Umbria estuary.

Huelva estuary is highly polluted (Achterberg et al., 2003; Barba-Brioso et al., 2010; Ruiz et al., 2008) due to several contaminant sources in the area: metals from Tinto and Odiel rivers, industrial effluents from factories and sewage from the coastal towns. Both rivers pass through the Iberian Pyrite Belt, a region long known for its mining activities (Leblanc

et al., 2000), are acidic (the pH occasionally falls to 2 or 3) and contain large amounts of metal from erosion and mining (Nelson and Lamothe, 1993).

The main objective of this work is to estimate the bioavailable fraction of metals (Zn, Cu, Cd, Mn, Pb, Ni, Fe, and Cr) in sediments of the Huelva estuary and its littoral of influence by carrying out the most popular methods of sequential extraction (BCR and Tessier) and a biomimetic approach (extraction with protease K), compare the results obtained with enrichments factors found in *Arenicola marina* (Bignasca et al., 2011; Ianni et al., 2010; Turner, 2006) and clarify which method estimates the bioavailable fraction in the most reliable manner.

## 2 Materials and methods

### 2.1 Sampling and sample pre-treatment

16 sampling points were chosen to cover the Huelva estuary and its littoral of influence. 12 points were located in the Huelva estuary (see Table 1 and Figure 1): 5 in the Odiel River estuary (O1-O5), 4 in the Tinto River estuary (T1-T4) and 3 in the Padre Santo canal (C1-C3). Four points correspond to the coast (L1-L4).

Table 1. Coordinates of the sampling points.

Point	North	West
O1	37°22'50''	6°58'41''
O2	37°15'44''	6°57'55''
O3	37°14'45''	6°57'24''
O4	37°13'47''	6°57'19''
O5	37°12'35''	6°56'53''
T1	37°18'43''	6°49'17''
T2	37°14'41''	6°54'14''
T3	37°13'48''	6°55'12''
T4	37°12'50''	6°55'51''
C1	37°11'08''	6°55'47''
C2	37°10'06''	6°54'27''
C3	37°09'11''	6°52'58''
L1	37°11'41''	7°02'04''
L2	37°08'35''	6°54'19''
L3	37°07'01''	6°48'51''
L4	37°05'58''	6°46'44''

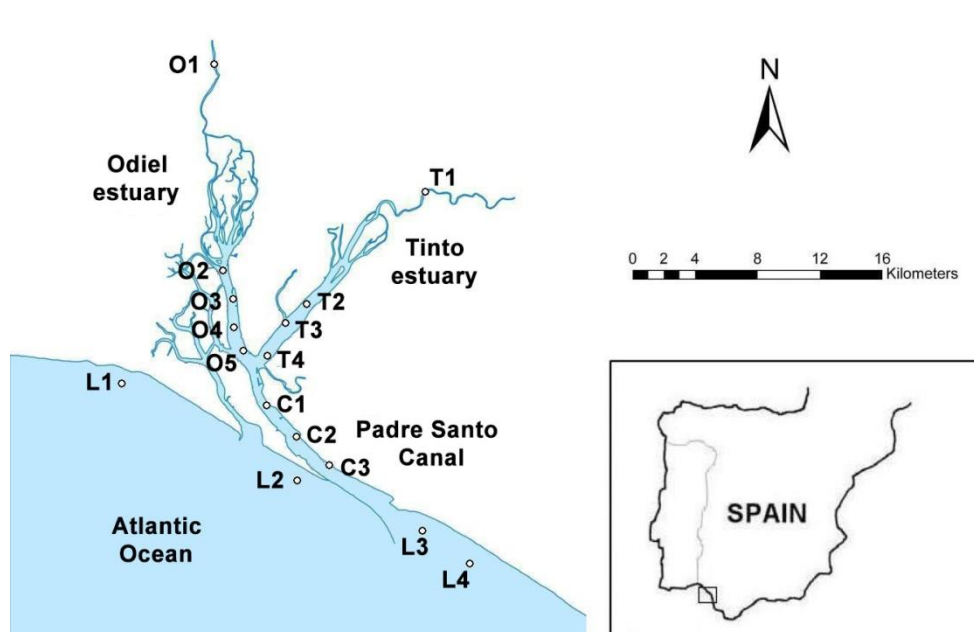


Figure 1. Location of the sampling points in the Huelva estuary.

Sediment samples were taken in October 2013 with a Van Veen grab and in duplicate at each point. Only grabs that achieved adequate penetration ( $2/3$  of total volume) to collect the first 5 cm of the sediment and that showed no evidence of leakage or surface disturbance were retained, transferred and stored in a dark cooler at 4 °C.

In the laboratory, a representative sample from each station was prepared by mixing and homogenizing the two samples taken at each sampling point.

The samples were dried at 60 °C in an oven, were disaggregated with an agate mortar and sieved to obtain a fraction with a particle size  $<63 \mu\text{m}$ , as recommended by many researchers (Jones and Turki, 1997; Ouyang et al., 2002) to minimize the effect of difference grain sizes in the samples.

## 2.2 Total metal content

To determine total metal content, sediment underwent the method proposed by the BCR (Pueyo et al., 2001), in which 3 g of every sample are digested with 28 ml of aqua regia (commercial nitric and hydrochloric acids, 1:3 by volume) in a reaction flask with a reflux condenser and a sand bath. Then, the extract was centrifuged 20 min at 4,200 rpm (3,000 g) to separate the solid and liquid phases.

The supernatant was used to determine Zn, Cu, Cd, Mn, Pb, Ni, Fe and Cr by atomic absorption spectrophotometry (AAS) using a double-beam Perkin–Elmer 2380 AAS with deuterium background correction and, in the case of low concentrations, by graphite furnace atomic absorption spectrometry (GFAAS) using a Varian spectra 220 with Zeeman-effect background correction.

Total metal content determination was tested using the reference material, BCR-701. The concentrations obtained for each of the elements analyzed demonstrated recoveries greater than 85%, which can be considered satisfactory for this type of analysis (Pueyo et al., 2001).

### 2.3 Extraction procedures

The first steps of two sequential extraction procedures were carried out in sediments to obtain the acid extractable fraction of BCR procedure (Pueyo et al., 2001) and the exchangeable and bound to carbonates fractions of Tessier sequential extraction (Tessier et al., 1979). Conditions defined by both procedures are given in Table 2.

Table 2. Comparison of extraction procedures for BCR, Tessier and protease K (Pueyo et al., 2001; Tessier et al., 1979; Ianni et al., 2010; Turner, 2006).

Protocol	Fraction	Solute	Concentration	pH	Time
BCR	Acid extractable	Acetic acid	0.11 M	-	16 h
Tessier	Exchangeable	MgCl <sub>2</sub>	1 M	7	1 h
	Bound to carbonates	Sodium acetate	1 M	5	5 h
Protease K	-	Protease K	0.4 mg/ml	7.4	3 h

To obtain the acid extractable fraction following the BCR procedure (Pueyo et al., 2001), 1 g of every sample were digested with 40 ml of acetic acid. After the extraction, the suspension was centrifuged for 20 min at 4,200 rpm (3,000 g). The supernatant was used to determine Zn, Cu, Cd, Mn, Pb, Ni, Fe and Cr as described for the total metal content.

As in the case of total metal content, the first step of BCR sequential extraction was tested using the reference material BCR-701, achieving recoveries greater than 85% for each element.

Tessier's exchangeable fraction (F1) (Tessier et al., 1979) required digesting 3 g of every sample with 24 ml of the  $MgCl_2$  solution. After the extraction, the suspension was centrifuged for 20 min at 4,200 rpm (3,000 g), the extract was stored at 4 °C and the solid residue was washed with distilled water and centrifuged again. To extract the bound to carbonates fraction (F2), solid residue from the previous step was treated with 24 ml of the sodium acetate solution and centrifuged for 20 min at 4,200 rpm (3,000 g) after the extraction. Then, Zn, Cu, Cd, Mn, Pb, Ni, Fe and Cr were analyzed in the remaining solution as described for the total metal content.

The bioavailable fraction was also estimated (Table 2) using Protease K solutions (0.4 mg/ml) following a procedure based on those proposed previously (Ianni et al., 2010; Turner, 2006). Protease K was purchased lyophilized from Sigma-Aldrich (P-8044) and solved in pH 7.4 standard phosphate buffer solution prepared from solid salts: 1.179 g of potassium dihydrogen phosphate ( $KH_2PO_4$ ) and 4.300 g of disodium hydrogen phosphate ( $Na_2HPO_4$ ) in 1 L of distilled water.

Protease K extractable fraction was obtained by digesting 0.5 g of every sample with 25 ml of the Protease K solution for 3 h at room temperature with magnetic stirring (250 rpm). Then the suspensions were centrifuged for 20 min at 4,200 rpm (3,000 g). The supernatant was used to determine Zn, Cu, Cd, Mn, Pb, Ni, Fe and Cr as described for the total metal content.

### **3 Results and discussion**

#### **3.1 Total and bioavailable metal content**

Metal concentrations found in the sediments of the Huelva estuary (Table 3) are generally very high compared to background levels and quality criteria (Bray, 2008; Crommentuijn et al., 2000; DelValls et al., 2004; Long et al., 1995; MacDonald et al., 2000; Turekian and Wedepohl, 1961), especially considering metals of pyritic origin (Zn, Cu, Cd, Pb, Fe, As and Se). The waters of the Tinto and Odiel rivers are enriched in these metals (Morillo and Usero, 2008; Morillo et al., 2005) in the upper zones of the basins when they flow through the "Iberian Pyrite Belt" (Leblanc et al., 2000; Sánchez España et al., 2005). Downstream, much of the metals dissolved precipitate in the estuaries of both rivers and the Padre Santo Canal due to the strong increase in pH, that changes from remarkably acid in the upper and



middle basins to close to 8 near the mouth (Galán et al., 2003; Morillo et al., 2008, 2002; Ruiz, 2001; Sainz and Ruiz, 2006; Santos Bermejo et al., 2003; Usero et al., 2008, 2005).

Massive metal precipitation in the Huelva estuary means that most metals concentrations in sediments can only be compared to those found in polluted areas. In the case of Zn and Pb, the values found in the Huelva estuary are comparable to those found in the Urumea river (Sánchez et al., 1998), affected by mining activities of Zn and Pb. Cu concentrations are only comparable to those obtained in areas of high pollution, as in the Tigris river (Varol, 2011), which receives discharges from Cu mines. Cd levels are similar to those found by Li et al. (2007) in sediments of the estuary of the Pearl River (China), with a strong anthropogenic pollution. The Fe concentration in sediments is similar (Padre Santo Canal and Odiel estuary) or higher (Tinto estuary) to those found in contaminated sediments of the estuary of Marabasco, Mexico (Marmolejo-Rodríguez et al., 2007), although lower than those obtained in the Mandovi estuary, Indian River (Alagarsamy, 2006), influenced by the discharge of existing Fe mines in the basin. As, often associated in nature with various metal sulfides, particularly arsenopyrite (FeAsS), presents similar concentrations (Canal and Odiel) or higher (Tinto) to those found in sediments of d'Alene river, northern Idaho, USA, (Chamberlain et al., 1995), heavily polluted by mining activities. The presence of Se is comparable to that of contaminated sediments from the Sado estuary, Portugal (Costa et al., 2011), and Yealm, Plym, Looe, Fal estuaries in southwest England (Turner, 2013). The Hg content exceeds levels obtained in polluted sediments of Minamata Bay, Japan (Nakata et al., 2008), although they are well below those found in the estuary of Patos, Brazil (Mirlean et al., 2003). Unlike the metals described above, the concentrations of Mn, Ni and Cr in sediments are in the range of background levels, or even lower.

The spatial distribution of total metal content in the 4 areas studied, estuaries of the Tinto and Odiel rivers, Padre Santo Canal and Huelva littoral (Table 3), shows that most metals have similar trends (especially those of pyritic origin) characterized by the following. In the estuary of the Tinto river, metal concentrations in sediments increase seawards and at the end of the estuary (T4) the highest concentrations of all samples studied in several metals (Zn, Cu, Cd, Pb, Fe, Hg, Se and As) are reached, especially those of a pyritic origin. The Tinto River is more affected by the Iberian Pyrite Belt than the Odiel and, therefore, the waters of the Tinto River have higher concentrations of metals and lower pH levels than those found in the Odiel River. In the estuary of the Odiel, the trend is not as significant as in the Tinto, since the metal concentrations in the waters of the Odiel River

are lower and pH is higher. In the case of Zn and Cu, total concentrations in sediments increase until the middle of the estuary (O3), from where they start to fall. Pb, As, Se and Hg show higher concentrations in point O1 and lower in O2, from where they increase towards the sea. Cd shows a similar behaviour in both estuaries, being less concentrated in the sediments of the upper area of the estuary and more concentrated downstream. In the other elements, no notable trends are seen. In the Padre Santo Canal a general decline in the concentrations of metals takes place seawards.

Metal concentrations found in sediments of the Huelva littoral are significantly lower than those of the Huelva estuary and, in many cases, are even below background levels (Turekian and Wedepohl, 1961). The majority of the metals present in the waters of the Tinto and Odiel rivers precipitate in the estuary before reaching the mouth (Galán et al., 2003; Morillo et al., 2008, 2002; Ruiz, 2001; Sainz and Ruiz, 2006; Santos Bermejo et al., 2003; Usero et al., 2008, 2005). Huelva littoral generally presents little difference between the values found in the 4 sampling points. However, point L3 (closest to the mouth of Huelva estuary) usually has the highest concentrations of metals.

Overall, the bioavailability of the metals that precipitate in the sediment is higher than those whose source is a longer-term process. Therefore, heavier precipitation implies that total and bioavailable amounts of metal in the sediment are greater. Thus, total metal concentrations in sediments are related to those obtained in the first step of the BCR, the sum of the first and the second step of extraction by Tessier and protease K (bioavailable fractions). Concentrations of Zn show that the bioavailable fractions are closely related to the total metal content, reproducing the rising values towards the sea in the Tinto estuary, the rise in the estuary of the Odiel up to point O3 and descent downstream from this point, the decline in the Padre Santo Canal and the variations along the littoral. The rest of the metals assessed reproduce the spatial distribution of the total metal content in bioavailable concentrations. However, Cu, Cd, Fe, Mn and Ni concentrations reach higher bioavailable concentrations than expected for point T1(Tinto estuary).

Ability of 3 extraction methods (BCR, Tessier and Protease K) to estimate bioavailable metals in sediments from Huelva estuary (Southwestern Spain)

Table 3. Total metal content and labile fractions (BCR first step, Tessier first+second step and protease K) in sediments from the Huelva estuary and its littoral of influence.

	T1	T2	T3	T4	O1	O2	O3	O4	O5	C1	C2	C3	L1	L2	L3	L4
<b>Total metal content (mg/kg)</b>																
Zn	968	1420	1640	2920	200	1050	2750	1910	1770	2170	2110	1800	88	171	243	212
Cu	1880	1930	1940	2420	363	1310	2190	1790	1820	2020	1740	1480	18	46	106	89
Cd	2.30	2.40	2.60	5.50	0.60	0.60	2.00	2.40	2.80	1.60	1.40	1.30	0.06	0.09	0.13	0.10
Pb	788	1370	1370	2420	610	290	454	475	548	584	538	533	19	24	57	37
Fe ***	154	120	120	158	98	86	77	71	101	76	82	75	14	17	18	16
As	578	794	857	1310	503	202	350	297	702	440	368	358	12	18	39	29
Se	4.2	6.4	5.8	13.4	3.2	2.5	3.2	3.7	3.9	3.1	2.7	3.0	0.1	0.1	0.1	0.1
Hg	1.8	3.2	3.5	8.3	4.2	2.5	2.4	3.4	4.6	3.4	3.1	2.9	0.2	0.4	0.7	0.6
Mn	121	180	247	274	215	341	275	301	296	416	396	352	190	237	220	178
Ni	18	33	30	27	17	25	33	40	31	40	39	31	8	10	14	9
Cr	85	105	118	117	74	72	99	96	106	98	84	86	24	28	29	24
<b>BCR first step (mg/kg)</b>																
Zn	466	524	492	1020	52	327	1010	747	914	770	760	673	41	71	111	110
Cu	622	416	514	163	66	179	184	162	107	104	92	84	6.8	21	66	58
Cd	1.91	1.33	1.88	2.51	0.42	0.54	1.67	1.67	1.35	1.05	0.92	0.83	0.05	0.07	0.10	0.07
Pb	11	17	55	27	7.5	20	19	19	12	8.7	9.6	9.1	3.3	3.3	7.0	6.7
Fe	1670	1070	1020	1690	680	1670	1570	1410	935	1740	1840	1520	293	377	423	467
Mn	41	22	31	65	53	55	63	63	88	163	178	166	100	84	75	87
Ni	2.5	2.1	2.1	3.3	0.8	0.8	3.3	2.5	3.4	4.1	3.3	2.5	1.2	0.8	1.4	1.1
Cr	1.2	1.7	3.3	2.1	0.8	1.7	2.9	2.1	1.1	2.1	1.7	1.7	0.3	0.4	0.4	0.3
<b>Tessier 1+2 steps (mg/kg)</b>																
Zn	302	289	182	651	69	136	596	487	633	566	562	511	26.6	47.1	76.1	71.4
Cu	273	172	107	130	41	61	106	74	63	25	22	17	8	20	42	37
Cd	1.80	1.26	1.56	2.62	0.42	0.50	1.26	1.26	0.92	0.92	0.59	0.56	0.05	0.07	0.11	0.08
Pb	48	70	128	109	7.7	39	53	64	37	43	39	39	4.5	8.2	13.3	13.7
Fe	1140	838	951	935	774	1240	758	883	875	1470	1860	1540	138	187	236	245
Mn	25	13	13	33	37	29	34	31	47	71	87	80	62	52	34	53
Ni	1.1	1.0	1.0	1.6	0.9	1.0	1.5	1.3	1.8	1.8	1.8	1.6	0.5	0.4	1.4	1.0
Cr	1.5	2.3	3.1	4.4	1.2	1.5	3.1	2.9	3.2	3.3	3.8	4.3	0.6	1.1	0.8	0.7
<b>Protease K extraction (mg/kg)</b>																
Zn	4.29	5.28	4.09	10.53	3.94	4.19	9.99	7.35	6.38	7.82	8.39	6.25	2.87	3.13	4.17	3.65
Cu	120	81	87	55	26	47	77	70	73	67	71	72	6	15	22	21
Cd	0.10	0.03	0.03	0.11	0.01	0.03	0.03	0.04	0.05	0.02	0.03	0.03	0.01	0.01	0.01	0.01
Pb	5.07	8.89	7.86	10.00	3.16	3.40	3.68	3.15	3.66	4.69	4.45	4.53	1.04	1.56	2.08	2.60
Fe	275	151	117	184	254	114	118	131	149	105	102	112	41	22	34	51
Mn	1.57	1.05	1.62	2.63	2.63	4.19	2.63	2.73	2.51	4.17	5.77	4.43	3.13	2.76	1.56	3.23
Ni	0.34	0.52	0.93	0.77	1.33	0.79	1.00	1.23	1.01	0.63	0.58	0.52	0.26	0.29	0.42	0.20
Cr	0.07	0.09	0.08	0.10	0.06	0.06	0.09	0.09	0.10	0.09	0.08	0.08	0.03	0.03	0.04	0.03

### 3.2 Percentages of extraction

Figure 2 shows that the metal extracted at the highest percentage, i.e. the most bioavailable, is Cd, followed by Zn, Mn, Cu, Pb, Ni, Cr and Fe. Figure 2 also shows that the first step of BCR extracts the highest amount ( $p < 0.05$ ) of Zn, Cu, Cd, Mn, Ni and Fe, followed by the sum of the first and second steps of Tessier and protease K. These results agree with those found by Turner (2006) and Ianni et al. (2010), who pointed out that the traditional methods of sequential extraction of metals (BCR and Tessier) overestimate the most labile fractions. In the case of Cu, the difference between the traditional methods and the protease K is relatively small. This result is similar to those obtained by Turner (2006) and Ianni et al. (2010) who agreed that the fraction extracted by protease K and the first stage of the BCR method resemble each other more for copper than for other metals. Unlike the previously mentioned, the sum of the first and second steps of Tessier extracted more Pb and Cr ( $p < 0.05$ ) than the first step of BCR and protease K.

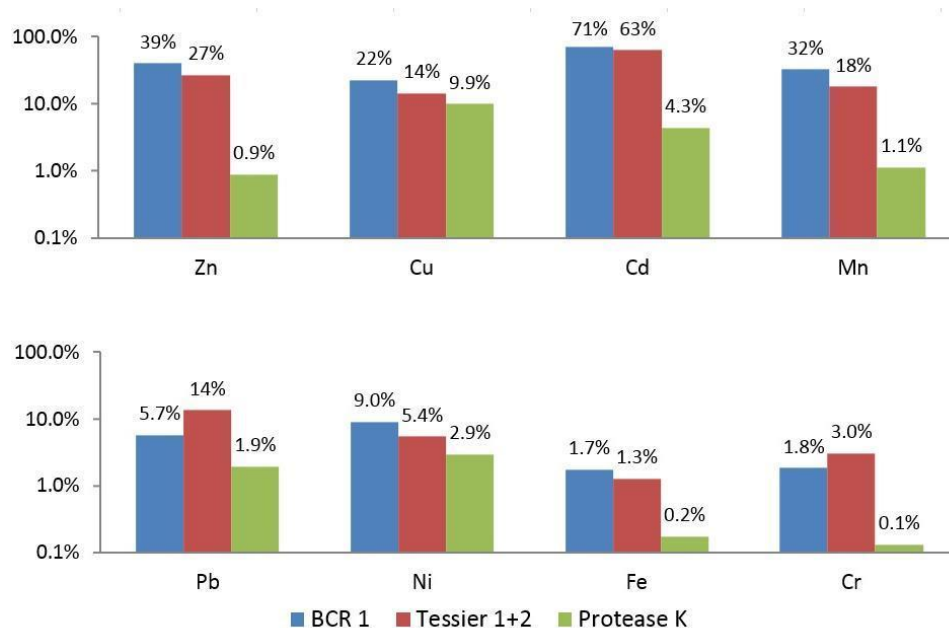


Figure 2. Mean percentages of each metal extraction in the first step of BCR, sum of first and second steps of Tessier and Protease K.

### 3.3 Enrichment factors and correlations

Following the method proposed by Turner (2006) and Ianni et al. (2010), the bioavailable metal ratios ( $f_x$ ) were calculated as the mean in the 17 sediment samples of the concentration mobilized for each metal by the first step of the BCR sequential extraction ( $f_b$ ), by the sum of first and second steps of the Tessier sequential extraction ( $f_t$ ), and by the

protease K ( $f_p$ ) divided by the total metal concentration (Table 4). Logarithms of bioavailable metal ratios were correlated with logarithms of enrichment factors (EF), defined as metal concentrations in gut fluids of *Arenicola marina* relative to metal concentrations in continental rock (Chen et al., 2000), in order to elucidate what method showed the best fit and better represents the bioavailable fraction.

Table 4. Fractions mobilized by first step of the BCR sequential extraction ( $f_b$ ), sum of first and second steps of the Tessier sequential extraction ( $f_t$ ) and protease K ( $f_p$ ) and enrichment factors (EF) in *Arenicola marina*.

	<b>Zn</b>	<b>Cu</b>	<b>Cd</b>	<b>Mn</b>	<b>Pb</b>	<b>Ni</b>	<b>Fe</b>	<b>Cr</b>
$f_b$	0.393	0.231	0.702	0.312	0.058	0.092	0.017	0.018
$f_t$	0.262	0.147	0.632	0.169	0.132	0.054	0.012	0.030
$f_p$	0.009	0.100	0.045	0.011	0.020	0.029	0.002	0.001
EF	3950	5580	16200	85.2	808	3880	100	22.9

As shown in Figure 3, the linear correlation coefficients ( $R^2$ ) between metal extracted ratio ( $f_b$ ,  $f_t$  and  $f_p$ ) and enrichment factors in *Arenicola marina* shows its highest values for protease K extraction (0.709), followed by BCR first step (0.507) and the sum of the first and second steps of Tessier (0.465). As previously underlined by Turner (2006) and Ianni et al. (2010), this observation suggests that protease K represents the bioavailable fraction more reliably than traditional methods (BCR and Tessier). It further indicates that the first step of BCR and the sum of the first and second steps of Tessier overestimate bioavailable fraction compared to protease K and that their ability to estimate the bioavailable fraction is similar.

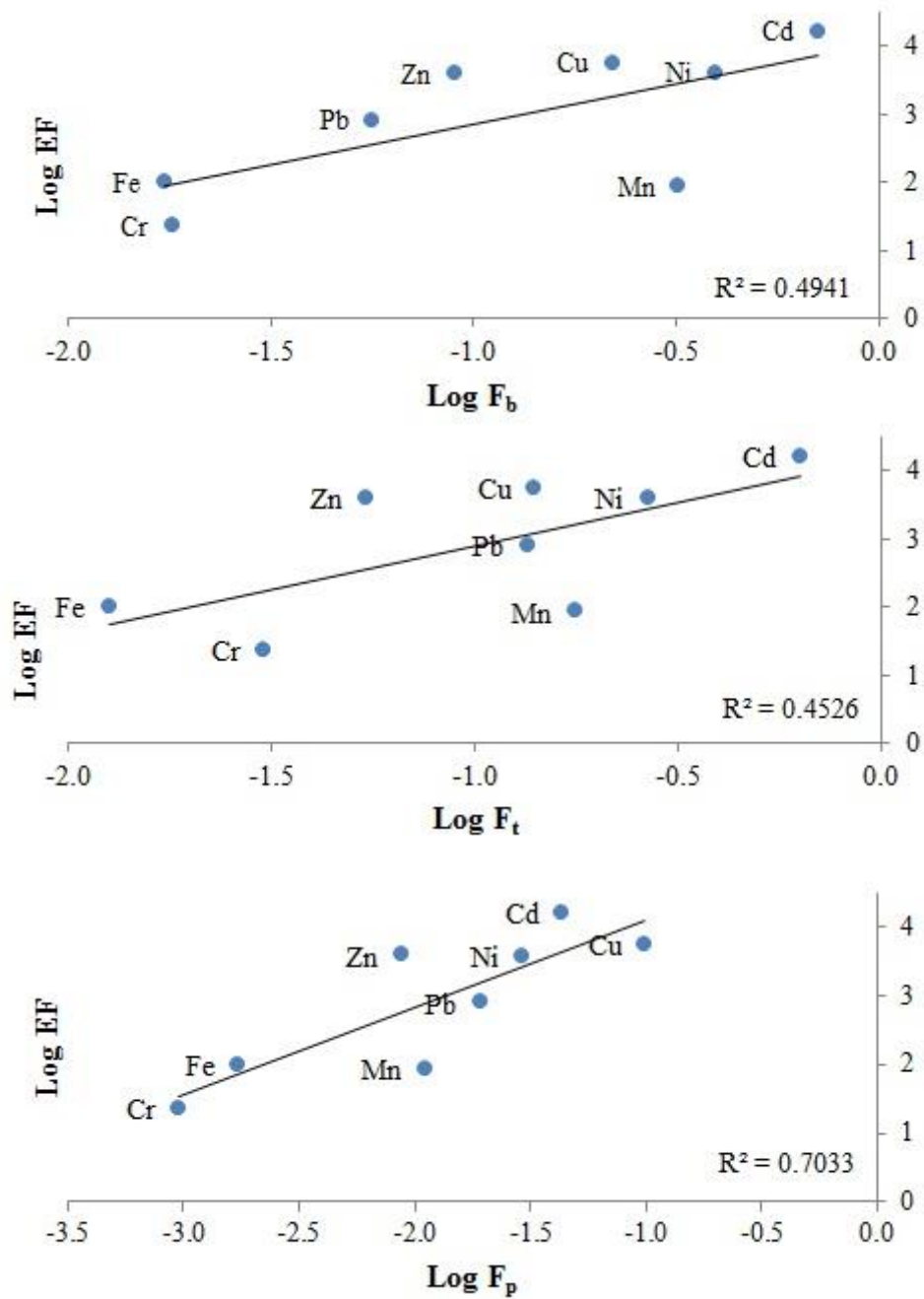


Figure 3. Correlations between fractions mobilized by first step of the BCR ( $f_b$ ), sum of first and second steps of Tessier ( $f_t$ ) and protease K ( $f_p$ ) and enrichment factors (EF).

## 4 Conclusions

A total metal content assay as well as two bioavailable metal extractions procedures based on traditional chemical sequential extraction methods (first step of BCR and first and second steps Tessier) and one based on a biomimetic approach (Protease K) have been carried out on sediment samples of Huelva estuary and its littoral of influence.

Total metal content found in the sediments of the Huelva estuary are generally very high compared to background levels and quality criteria and are in the range of those found in polluted areas, especially considering the metals of pyritic origin (Zn, Cu, Cd, Pb, Fe, As and Se). However, total metal content found in sediments of the Huelva littoral are significantly lower than those of the Huelva estuary and, in many cases, are even below background levels.

Spatial distribution of total metal content in the 4 areas studied (estuaries of the Tinto and Odiel rivers, Padre Santo Canal and Huelva littoral) shows that metals of pyritic origin have similar trends, which are reproduced in the concentrations of bioavailable fractions obtained by sequential extraction procedures (BCR and Tessier) and biomimetic approach (protease K). This trend is characterized by an increase in the concentration of metals in sediments of Tinto estuary seawards, reaching the highest concentrations where Tinto and Odiel estuaries merge.

The first phase of BCR extracted the highest amount ( $p < 0.05$ ) of six metals (Zn, Cu, Cd, Mn, Ni and Fe), followed by the sum of the first and second steps of Tessier and protease K. In the case of Cu, the difference between the traditional methods (BCR and Tessier) and the protease K is reduced to be almost equal in all three cases. The sum of the first and second steps of Tessier extracted higher amounts of Pb and Cr ( $p < 0.05$ ) than the first step of BCR and protease K.

The linear correlation coefficients ( $R^2$ ) between metal extracted ratio, defined as the fraction mobilized by the first step of the BCR sequential extraction, by the sum of first and second steps of the Tessier sequential extraction, and by protease K, and enrichment factors (ER) in *Arenicola marina*, shows its highest value for protease K extraction (0.709), followed by BCR first step (0.507) and the sum of the first and second steps of Tessier (0.465). This observation suggests that protease K represents the bioavailable fraction more reliably than traditional methods (BCR and Tessier), which overestimate bioavailable fraction and have a similar ability to estimate the bioavailable fraction.

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