Assessment of heavy metals bioavailability and toxicity toward Vibrio fischeri in sediment of the Huelva estuary

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HIGHLIGHTS:

- Metals are an important source of toxicity in sediments of the Huelva estuary.
- Bioavailable metals show a strong correlation with toxicity.
- All the samples located in Huelva estuary are considered toxic.
- Highest average toxicity was found in sediments of the Tinto estuary.

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Abstract

Relationship between toxicity and bioavailable metals in sediments from the Huelva estuary and its littoral of influence was analyzed. Toxicity was assessed with Microtox® bioassay using a marine luminescent bacterium: *Vibrio fischeri*. Bioavailable metals were considered as both, acid extractable fraction of BCR procedure and the sum of exchangeable and bound to carbonates fractions of Tessier sequential extraction. A bioavailable metals index was calculated to integrate results in a single figure.

Toxicity and bioavailable metals showed a similar pattern. Higher levels were found in the estuary than in the littoral (140 TU/g). In Huelva estuary, highest levels were found in the Tinto estuary (5,725 TU/g), followed by the Odiel estuary (5,100 TU/g) and the Padre Santo Canal (2,500 TU/g). Results in this area were well over than those in nearby estuaries. Furthermore, they are similar to or even higher than those in other polluted sediments around the world.

Bioavailable metal index showed a stronger correlation with acid extractable fraction of BCR ($R^2 = 0.704$) than that for the sum of exchangeable and bound to carbonates fractions of Tessier ($R^2 = 0.661$). These results suggest that bioavailable metals are an important source of sediment toxicity in the Huelva estuary and its littoral of influence, an area with one of the highest mortality risks of Spain.

Keywords

Sediment; metals; bioavailability; BCR; toxicity; Huelva estuary;

1 Introduction

Sediments are the main sink for various kinds of hazardous substances dissolved in water. They may reach concentrations many times higher than those in the water column. Accumulation of pollutants in sediments can lead to pollution peaks in water and bioconcentration (Izquierdo et al., 1997; Zoumis et al., 2001) in the event of a change in environmental conditions (pH, redox potential, etc.). Sediments can also be ingested and pollutants bioabsorbed initiating biomagnification trough the food chain (Sijm et al., 2007). The latter is especially important because human beings usually bear the highest concentrations. In addition, ingestion of mother's milk by nursing infants is a potential source of exposure to toxic substances (Van Engelen et al., 2007).

Chemical composition assessments can determine the concentrations of the contaminants in the sediment. However, given the vast range of different substances, it is difficult to analyze all of them. Toxicity analyses are able to intuitively reflect the detrimental effects of contaminants on organisms (Li et al., 2013a, 2013b). The application of luminescent bacteria to toxicity assays captured researchers' attention and was quickly developed (Jennings et al., 2001; Ma et al., 2014; Silva et al., 2004).

The Microtox solid phase test (MSPT) is one of the most popular tests for sediment toxicity assessments due to its ecological relevance and sensitivity (Burga Pérez et al., 2012; Burton et al., 1996; Ghirardini et al., 2009; Libralato et al., 2008; Losso et al., 2004; Van Beelen, 2003). It has been reported that the test correlated well with other toxicity tests, such as seed germination-root elongation in *Cucumis sativus* (Burton et al., 1996) and Luminotox solid phase assay (Dellamatrice et al., 2006; Jennings et al., 2001). It has been demonstrated that the test is easy, quick and reproducible (Parvez et al., 2006; Stronkhorst et al., 2004). The key factor in this test is that the bioluminescent bacteria (*Vibrio fischeri,* formerly known as *Photobacterium phosphoreum*) can contact toxicants in both the particulate and dissolved phases. Briefly, bacteria are exposed to serial dilutions of sediments, and after a few minutes of contact, disposable filters are inserted into the test tubes in order to separate the liquid phase containing the bacteria from the sediment. Light output is then measured after 5, 10, 15 or 30 min with the bacteria contained in the liquid phase.

Huelva estuary is a highly polluted zone (Achterberg et al., 2003; Barba-Brioso et al., 2010; Ruiz et al., 2008) due to several contaminant sources in the area: metals from the Tinto and Odiel rivers, industrial effluents from factories and sewage from the coastal towns. Metals

are specially relevant in the area and a source of toxicity. Tinto and Odiel 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).

Sequential extraction procedures have been successfully applied to divide metals in sediments into different binding forms: exchangeable, carbonate-bound, Fe-Mn oxide-bound, organic matter/sulfide-bound, and residual. Metals in first fractions are easier mobilizable from the sediment matrix than those in the latest. Thus, first fractions are also more bioavailable and have a higher impact in toxicity.

In recent years, people's awareness of the potential adverse health effects of pollution has increased due to the identification of the Huelva province as one of the areas with the highest mortality risks in Spain (Benach et al., 2004; Bleda and Tobías, 2002; López-Abente et al., 2006).

The aims of this study were: (1) to use Microtox® bioassay to evaluate the toxicity of sediments collected from the Huelva estuary and its littoral of influence (2), to assess the bioavailability forms of metals in sediments; (3) to analyze a possible relationship between the observed toxicity and bioavailability of metals.

2 Materials and methods

2.1 Sampling and sample pre-treatment

This study has been carried out in Huelva estuary, formed by the confluence of the Tinto and Odiel rivers and consisting 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.

16 sampling points were chosen to cover the Huelva estuary and its area of influence (the Huelva littoral). According to these guidelines, 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 littoral (L1-L4). The reference point (L1) was placed west of the estuary to avoid the influence of prevailing west to east water currents, known as longshore drift (Morillo et al., 2004).

Table 1. Coordinates of the sampling points.

Point	North	West
T1	37º18´43´´	6º49´17´´
T2	37º14´41´´	6º54´14´´
T3	37º13´48´´	6º55´12´´
T4	37º12´50´´	6º55´51´´
O1	37º22´50´´	6º58´41´´
O2	37º15´44´´	6º57´55´´
O3	37º14´45´´	6º57´24´´
O4	37º13´47´´	6º57´19´´
O5	37º12´35´´	6º56´53´´
C1	37º11´08´´	6º55´47´´
C2	37º10´06´´	6º54´27´´
C3	37º09´11´´	6º52´58´´
L1	37º11´41´´	7º02´04´´
L2	37º08´35´´	6º54´19´´
L3	37º07´01´´	6º48´51´´
L4	37º05´58´´	6º46´44´´

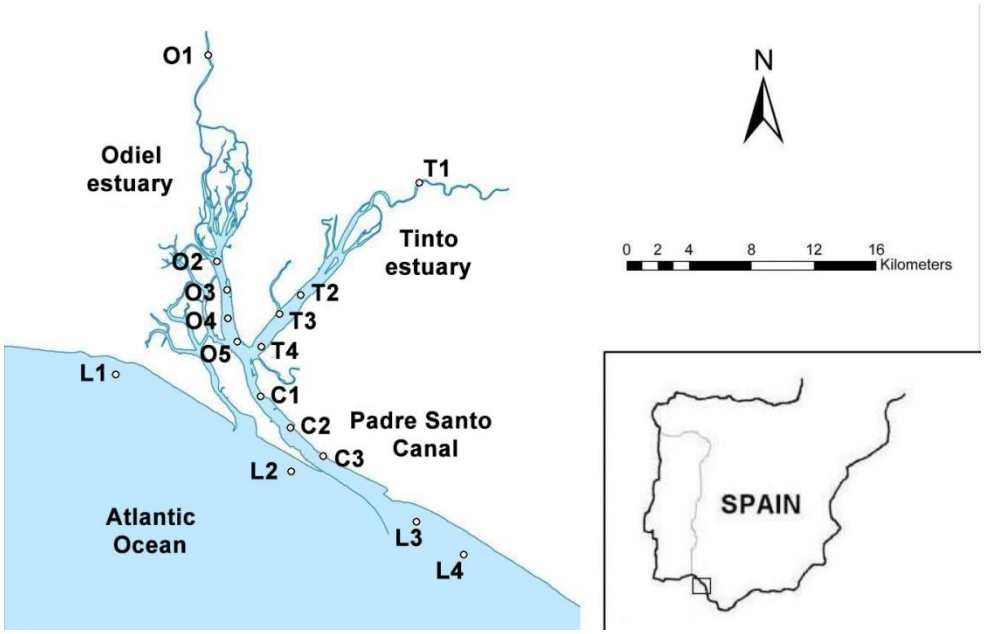

Figure 1. Location of the sampling points in the Huelva estuary and its littoral of influence.

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 μm as recommended by many researchers (Förstner, 1989; Jones and Turki, 1997; Ouyang et al., 2002) to minimize the effect of difference grain size in the samples.

2.2 Toxicity assay

Toxicity was determined by using a marine luminescent bacterium, *Vibrio fischeri*, naturally adapted to a saline environment. The acute toxicity tests on the sediments were performed according to the procedure described by Svenson et al. (1996), in which a preassay allows to estimate the toxicity prior to the main assessment. A suspension of 3 g of the sediment was prepared in 30 ml of a 2% NaCl solution, in which pH was adjusted to 7.3 with NaOH or HCl and was magnetically stirred for 10 min; then a series of dilutions were made and bacteria were exposed to these dilutions and to a blank (2% NaCl solution). Bioluminescence at 15 ^{o}C was measured with a Microtox luminescence meter (Microbics Corp., Carlsbad, CA) after 30 min of incubation. Inhibition was initially calculated as the concentration of sediment (mg/L) that caused a 50% reduction in the light emitted by the bacteria (EC_{50}).

For the determination of dose–response curve and the EC_{50} value the following equation was used:

$$\Gamma = \frac{I_c}{I_s} - 1$$

Γ is the light lost at the end of exposure time for a given sample concentration; I_c and I_s are the intensity of bioluminescence of the control and a given sample concentration, respectively. Data of concentrations and their associated Γ values of the two dilution series are transformed in log form and are used all together for linear regression analysis. EC_{50} value is calculated from the intercept. The test result was accepted when R^2>0.95.

Inhibition was expressed as toxicity units per g dry soil (TU_{50}), which is calculated as following:

$$TU_{50} = \frac{1}{EC_{50}} \times 100$$

Toxicity units provide a comparative scale on which the higher the value of TU_{50} the higher the toxicity, contrary to EC_{50} values, which decrease as toxicity increases (Van den Brink and Kater, 2006).

2.3 Bioavailable metal assessment

The first steps of two sequential extraction procedures were carried out in sediments to obtain the most bioavailable metal fractions: 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). Within this study, the term bioavailable fraction was applied to both: the acid extractable fraction of BCR procedure and the sum of exchangeable and bound to carbonates fractions of Tessier sequential extraction. Conditions defined by both procedures are given in Table 2.

Table 2. Comparison of BCR and Tessier extraction procedures (Pueyo et al., 2001; Tessier et al., 1979).

Protocol	Fraction	Solute	Concentration	pH	Time
BCR	Acid extractable	Acetic acid	0.11 M	-	16 h
Tessier	Exchangeable	$MgCl_2$	1 M	7	1 h
	Bound to carbonates	Sodium acetate	1 M	5	5 h

To obtain the acid extractable fraction following the BCR procedure (Pueyo et al., 2001), 1 g of every sample was 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 analysis of Zn, Cu, Cd, Mn, Pb, Ni, Fe, and Cr in the supernatant was carried out 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.

All reagents were Merck analytical grade or extrapure quality. Stock solutions (Merck) of 1,000 mg/l with certificates of analysis traceable to NIST of the different elements analyzed were used to prepare the calibration standards. The water used was purified using a Milli-Q water-purification system (Millipore, Bedford, MA, USA).

All the analyses were performed within the laboratory's updated rigorous quality control system (International Standard Organization ISO/IEC 17025, 1999), which guarantees the reliability of all the results. In order to check the accuracy of the analytical procedures for BCR first step extraction, reference material BCR-701 was used.

Replicate analysis of BCR-701 showed good accuracy, with recovery rates between 89 and 107% (Zn, 95%; Cu, 107%; Cd, 99%; Mn, 99%; Pb, 89%; Ni, 101%; Fe, 102%; Cr, 98%).

Tessier's exchangeable fraction (F1) (Tessier et al., 1979) required digesting 3 g of every sample with 24 ml of the $MgCl_2$ solution (Table 2). After the extraction, the suspension was centrifuged for 20 min at 4,200 rpm (3,000 g), the extract was stored at 4 ^{o}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 (Table 2) 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 acid extractable fraction following the BCR procedure.

Bioavailable metals concentrations were integrated in the bioavailable metals index. To determine the cited index, first, normalized values (NVs) related to the reference station (L1) were calculated with the following equation:

$$NV_i = \frac{P_{iE}}{P_{iL1}}$$

NV_i, P_{iE} and P_{iL1} respectively refer to the normalized value of the metal i, bioavailable concentration of the metal i in the sampling station E and the bioavailable concentration of the metal i in the reference station L1. In the case of Tessier extraction procedure, P_{iE} was calculated as the sum of the two fractions obtained (exchangeable and bound to carbonates). Then, 16 NVs were calculated in every sampling point, 1 for every protocol and metal assessed.

Next, two bioavailable metals indeces were calculated in each sampling point (1 for every protocol) as the NVs geometric mean of the eight metals assessed according to the expression:

$$\text{Bioavailable metals index} = \sqrt[n]{NV_1 \times NV_2 \times \ldots \times NV_n}$$

2.4 Statistical analysis

All differences in toxicity and bioavailable metals in sediments were analyzed by t-test and one-way ANOVA. A linear regression model was used to test the relations between toxicity and bioavailable metals in the sediments. Statistical analysis of the results was done with SPSS software.

3 Results and discussion

3.1 Toxicity in Huelva littoral

Toxicity results (TU/g) obtained in this study are shown in Figure 2. Among the four areas studied (Huelva littoral, Odiel estuary, Tinto estuary and Padre Santo Canal) the lowest average toxicity is in the littoral (140 TU/g; $p<0.05$). In Huelva littoral, minimum value of 54 TU/g is located at point L1 (farthest from the mouth of the Huelva estuary), followed by point L2 (affected by Punta Umbría estuary, but free of the influence of the Padre Santo Canal). Toxicity reaches its maximum value (230 TU/g) in the littoral at point L3 (closest to the mouth of the Padre Santo Canal), followed by L4 (210 TU/g).

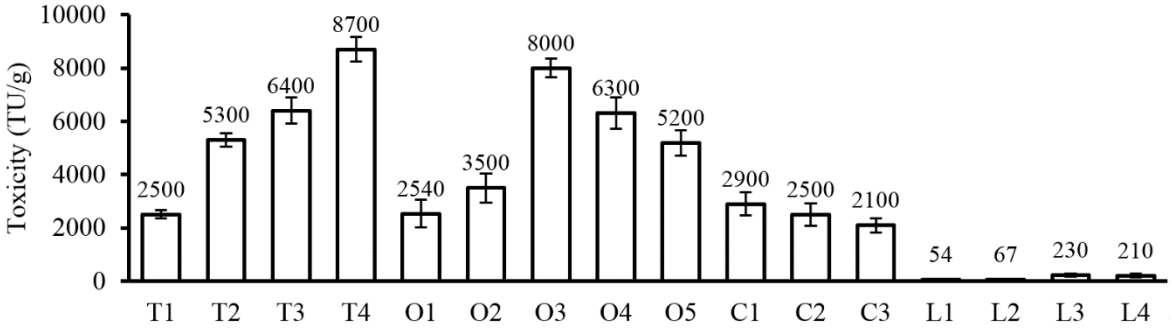

Figure 2. Toxicity in sediments from the Huelva estuary and its littoral of influence (TU/g) and standard error.

The slight increase in toxicity between points L1 and L2 ($p<0.05$) could be explained by the effluents from the Huelva estuary through the estuary of Punta Umbria. However, results in both points are similar to those found in other nearby coastal sediments free of human influence (Table 3): the coast of Huelva (Usero et al., 2008) and the Bay of Cádiz (Morales-Caselles et al., 2007; Riba et al., 2004a, 2004b). They are also similar to sediments in unpolloted littorals around the world: the Bay of Santander (Coz et al., 2008) and Corme-Laxe estuary (Morales-Caselles et al., 2008). In addition, they are considered no toxic samples following the toxicity criteria of Environment Canada (2002), set in TU/g>100

(EC_{50}<1,000 mg/L), and this of some Spanish authors (Casado-Martínez et al., 2006; DelValls et al., 2004; Morales-Caselles et al., 2008) that increase the toxicity threshold to TU/g>133 (EC_{50}<750 mg/L).

Toxicity in points L3 and L4 exceeds the values found in other nearby coastal areas free of human influence (Morales-Caselles et al., 2007; Riba et al., 2004a, 2004b; Usero et al., 2008). They are in the same order of magnitude as those found by Martínez-Lladó et al. (2007) in polluted sediments from the port of Barcelona (Spain) and by Morales-Caselles et al. (2007) in the sediments of the Cies islands and the island of Ons (Galicia, Spain) shortly after the oil spill caused by the breaking up and sinking of the tanker Prestige (November 2002). In addition, they are considered toxic following the criteria explained above: Environment Canada (2002) and this of some Spanish authors (Casado-Martínez et al., 2006; DelValls et al., 2004; Morales-Caselles et al., 2008).

Significant difference between average toxicity in sampling points situated west to the Padre Santo Canal (L1-L2) and east (L3-L4) was found ($p<0.05$). Considering that the predominant currents in the area are from west to east (longshore drift), this result suggests that the effluent from the Huelva estuary contributes to increase toxicity.

Table 3. Toxicity in sediments from the Huelva littoral and other nearby and distant coastal areas (TU/g).

Place		Toxicity (TU/g)			Source
		Average	Minimum	Maximum	
Study area	Huelva littoral (Spain)	140	54	230	This study
Nearby places	Coast of Huelva (Spain)	30	19	58	Usero et al. (2008)
	Bay of Cádiz (Spain)	5	5	5	Riba et al., (2004a, 2004b)
		17	17	17	Morales-Caselles et al. (2007)
Rest of the world	Bay of Santander (Spain)	18	1	38	Coz et al. (2008)
	Corme-Laxe estuary (Spain)	16	5	22	Morales-Caselles et al. (2008)
	Ons and Cies Islands (Spain)	178	60	279	Morales-Caselles et al. (2007)
	Port of Barcelona (Spain)	240	23	645	Martínez-Lladó et al. (2007)

3.2 Toxicity in Huelva estuary

Toxicity in the Huelva estuary has significant differences ($p<0.05$) among its 3 areas (Figure 2) according to ANOVA test. Following the littoral, the area with the lowest toxicity average in sediments is the Padre Santo Canal (2,500 TU/g), where toxicity decreases ($p<0.05$) along the canal towards the sea (from 2,900 TU/g at point C1 to 2,100 TU/g at C3). The areas with the highest average toxicities are the estuaries of the Tinto (5,725 TU/g) and Odiel (5,100 TU/g). In the case of the Tinto, toxicity increases downstream ($p<0.05$), culminating at point T4 (8,700 TU/g), where the maximum value of all surveyed points is reached. In the estuary of the Odiel, toxicity increases ($p<0.05$) from O1 (2,540 TU/g) to O3 (8,000 TU/g), from where it starts to decrease until O5 (5,200 TU/g).

Toxicity found in the Huelva estuary is much higher than that found by various researchers in nearby estuaries (Table 4). This is the case of Riba et al. (2004a, 2004b) in the estuary of the Guadalquivir River, Morales-Caselles et al. (2007) in Guadarranque River estuary (Bay of Algeciras) and Serafim et al. (2013) in the estuary of the Guadiana River. This suggests that there are sources of toxicity in the Huelva estuary that are not present in the nearby estuaries.

The toxicity values of the Huelva estuary can only be compared to those found in polluted areas. These are the cases of Mar Piccolo (Taranto, Italy) where toxicity in sediment reaches 3,693 TU/g with an average of 2,194 TU/g (Calace et al., 2005), Kafue River (Zambia), where toxicity ranges from 1,200 TU/g to 13,300 TU/g with an average of 4,882 TU/g (Mwase et al., 1998), and the sediments of the inner canals of the city of Venice (Italy) that reach up to 3,067 TU/g, with an average of 1,890 TU/g (Salizzato et al., 1998). In addition, they are considered contaminated toxic following the criteria previously mentioned in Huelva littoral results section: Environment Canada (2002) and this of some Spanish authors (Casado-Martínez et al., 2006; DelValls et al., 2004; Morales-Caselles et al., 2008).

Table 4. Toxicity in sediments from the Huelva estuary and other nearby and distant areas (TU/g).

Place		Toxicity (TU/g)			Source
		Average	Minimum	Maximum	
Study area	Tinto estuary (Spain)	5,725	2,500	8,700	This study
	Odiel estuary (Spain)	5,108	2,540	8,000	
	Padre Santo Canal (Spain)	2,500	2,100	2,900	
Nearby places	Guadalquivir estuary (Spain)	115	42	187	Riba et al. (2004a, 2004b)
	Guadarranque estuary (Spain)	340	192	426	Morales-Caselles et al. (2007)
	Guadiana estuary (Spain)	20	9	30	Serafim et al. (2013)
Rest of the world	Mar Piccolo (Italy)	2,194	1,305	3,693	Calace et al. (2005)
	Kafue River (Zambia)	4,882	145	13,300	Mwase et al. (1998)
	Venice canals (Italy)	1,890	700	3,067	Salizzato et al. (1998)

3.3 Bioavailable metal concentrations and correlation to toxicity

Bioavailable metal concentrations found in sediments (Table 5) and bioavailable metals index (Figure 3) replicate the trends showed in toxicity. Bioavailable metal concentrations in the Huelva littoral are significantly lower than those of the Huelva estuary ($p<0.05$). This is undoubtedly because the vast majority of the metals present in the waters of the Tinto and Odiel rivers precipitate in the estuary, before reaching the mouth.

BCR first step (mg/kg)	**Zn**	**Error**	**Cu**	**Error**	**Cd**	**Error**	**Pb**	**Error**	**Fe**	**Error**	**Mn**	**Error**	**Ni**	**Error**	**Cr**	**Error**
T1	470	60	620	50	1.9	0.3	11.0	0.5	1700	100	41	6	2.5	0.2	1.2	0.2
T2	520	60	420	90	1.33	0.08	17	2	1070	60	22	4	2.1	0.2	1.7	0.2
T3	490	50	510	50	1.9	0.2	55	5	1020	40	31	3	2.1	0.5	3.3	0.2
T4	1000	100	160	20	2.5	0.3	27	6	1690	70	70	10	3.3	0.5	2.1	0.2
O1	52	6	70	10	0.42	0.04	7.5	0.5	680	40	53	5	0.8	0.1	0.8	0.2
O2	330	30	180	30	0.54	0.05	20	3	1700	100	55	1	0.8	0.1	1.7	0.1
O3	1000	200	180	30	1.7	0.1	19	1	1570	90	63	4	3.3	0.2	2.9	0.1
O4	750	60	160	20	1.7	0.1	19	4	1410	90	63	3	2.5	0.5	2.1	0.2
O5	900	100	110	20	1.4	0.2	12.0	0.8	900	200	88	7	3.4	0.6	1.1	0.1
C1	770	40	104	4	1.1	0.1	8.7	0.2	1700	300	160	30	4.1	0.5	2.1	0.1
C2	760	40	90	20	0.92	0.07	10	1	1800	300	178	9	3.3	0.6	1.7	0.4
C3	670	40	80	10	0.83	0.08	9.1	0.5	1500	100	166	3	2.5	0.1	1.7	0.1
L1	41	8	7	1	0.05	0.01	3.3	0.5	290	20	100	4	1.2	0.2	0.30	0.04
L2	71	2	21	1	0.07	0.01	3.3	0.2	380	20	80	10	0.8	0.1	0.40	0.01
L3	110	10	66	4	0.10	0.01	7.0	0.3	420	50	80	10	1.4	0.2	0.40	0.05
L4	110	20	58	2	0.07	0.01	6.7	0.4	467	5	87	6	1.1	0.1	0.30	0.04
Tessier 1+2 steps (mg/kg)	Zn	Error	Cu	Error	Cd	Error	Pb	Error	Fe	Error	Mn	Error	Ni	Error	Cr	Error
T1	300	70	270	60	1.8	0.3	48	6	1100	100	25	9	1.1	0.1	1.5	0.2
T2	290	80	170	20	1.3	0.3	70	5	840	60	13	1	1.0	0.1	2.3	0.2
T3	180	40	107	9	1.6	0.3	130	10	950	40	13	4	1.0	0.1	3.1	0.2
T4	650	40	130	10	2.6	0.8	110	10	940	70	33	4	1.6	0.2	4.4	0.2
O1	69	3	41	4	0.42	0.05	8	1	770	40	37	4	0.9	0.3	1.2	0.2
O2	140	20	60	10	0.50	0.06	39	5	1200	100	29	6	1.0	0.1	1.5	0.1
O3	600	200	110	30	1.3	0.2	50	10	760	90	34	4	1.5	0.1	3.1	0.1
O4	500	100	70	10	1.3	0.1	64	5	880	90	30	10	1.3	0.1	2.9	0.2
O5	630	30	63	7	0.9	0.1	37	4	900	200	47	8	1.8	0.5	3.2	0.1
C1	570	50	25	7	0.9	0.1	43	9	1500	300	70	10	1.8	0.2	3.3	0.1
C2	600	100	22	7	0.59	0.01	39	6	1900	300	90	10	1.8	0.1	3.8	0.4

C3	500	100	17	4	0.56	0.07	39	6	1500	100	80	20	1.6	0.1	4.3	0.1
L1	27	1	8	2	0.05	0.01	5	1	140	20	62	7	0.5	0.1	0.60	0.04
L2	47	5	20	1	0.07	0.01	8	2	190	20	52	1	0.4	0.1	1.10	0.01
L3	76	6	42	1	0.11	0.01	13	2	240	50	34	1	1.4	0.1	0.80	0.05
L4	70	20	37	6	0.08	0.01	14	4	245	5	53	6	1.0	0.1	0.70	0.04

Table 5. Bioavailable metal concentrations in sediments from the Huelva estuary and its littoral of influence (mg/kg).

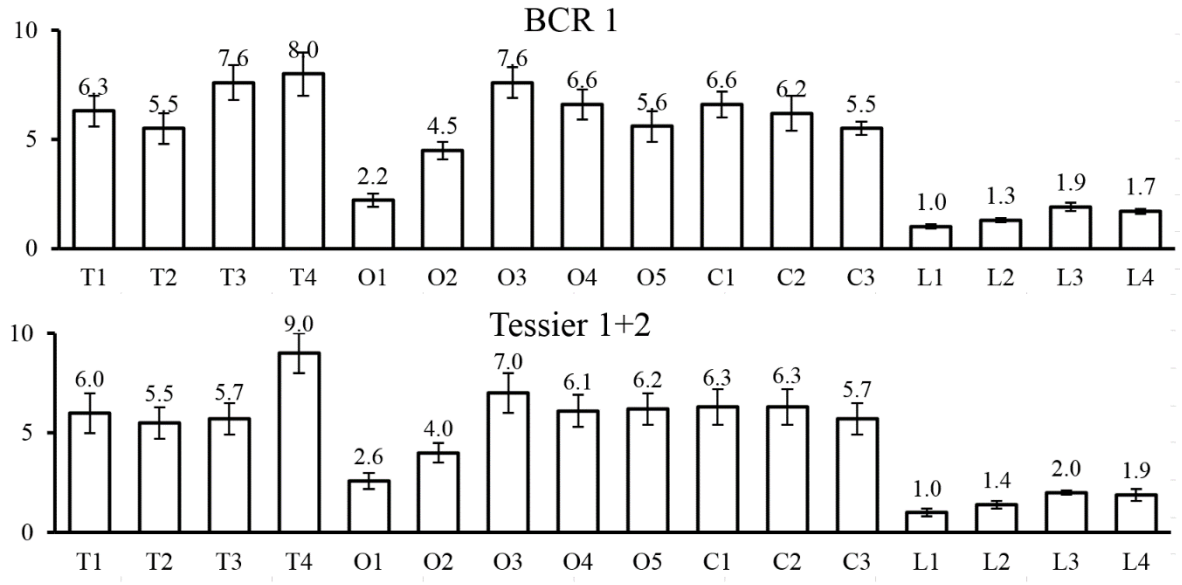

Figure 3. Bioavailable metal index according to BCR first step and the Tessier 1+2 steps in Huelva estuary and its littoral of influence.

In the littoral, bioavailable metal concentrations are significantly lower ($p<0.05$) west to the Padre Santo Canal (L1 and L2) than east (L3 and L4). The result suggests that the effluent from the Huelva estuary contributed to increase toxicity. The influence of the Huelva estuary on the coast is not equal in all directions. The western coastal area is less affected than the eastern area, due to the presence of longshore drift, the left-to-right current that flows along the coast (Morillo et al., 2004).

Bioavailable metal concentrations in the sediments of the Huelva estuary are very high, particularly in the case of pyritic metals. The highest concentrations of bioavailable metals of the Huelva estuary are located in the Tinto and Odiel estuaries ($p<0.05$), especially the Tinto, where they increase towards the sea ($p<0.05$). This result makes sense considering that mining activity has been more intense in the Tinto River catchment (Braungardt et al., 2003). The opposite happens in the Padre Santo Canal, where there is a great decrease in the bioavailable metal concentrations towards the sea ($p<0.05$).

Waters of the Tinto and Odiel rivers are acidic and have very high metal concentrations compared to other European estuaries (Morillo and Usero, 2008; Morillo et al., 2005), especially of pyritic metals (Zn, Cu, Cd, Pb and Fe). Mining activities throughout historical times and a catchment rich in metals (part of the the Iberian Pirite Belt) are the main reasons

behind this high levels (Leblanc et al., 2000; Sánchez España et al., 2005) (VanGeen et al., 1997).

Once the waters of the Tinto and Odiel rivers reach the zone of tidal influence (estuaries of both rivers and Padre Santo Canal), pH and salinity starts to increase due to mixing with seawater. The pH change is particularly strong in Huelva estuary compared to other estuaries in the world. The pH of the water in both rivers is remarkably acid (reaches 2-3) and increases to 8 along the Huelva estuary (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), leading to the precipitation of large amounts of metals into the sediment (Morillo and Usero, 2008; Morillo et al., 2005).

Metals precipitated after the contact of acidic river water with seawater are weakly bound to the sediments and easily bioavailable and, consequently, their toxicity is higher and can cause greater damage to macrobenthic communities.

Bioavailable metals index was correlated to toxicity in sediments (Figure 4), showing a strong correlation to BCR first step ($R^2 = 0.704$) and Tessier's 1+2 steps ($R^2 = 0.661$). These results suggest that bioavailable metals an important source of sediment toxicity in the Huelva estuary and its littoral of influence.

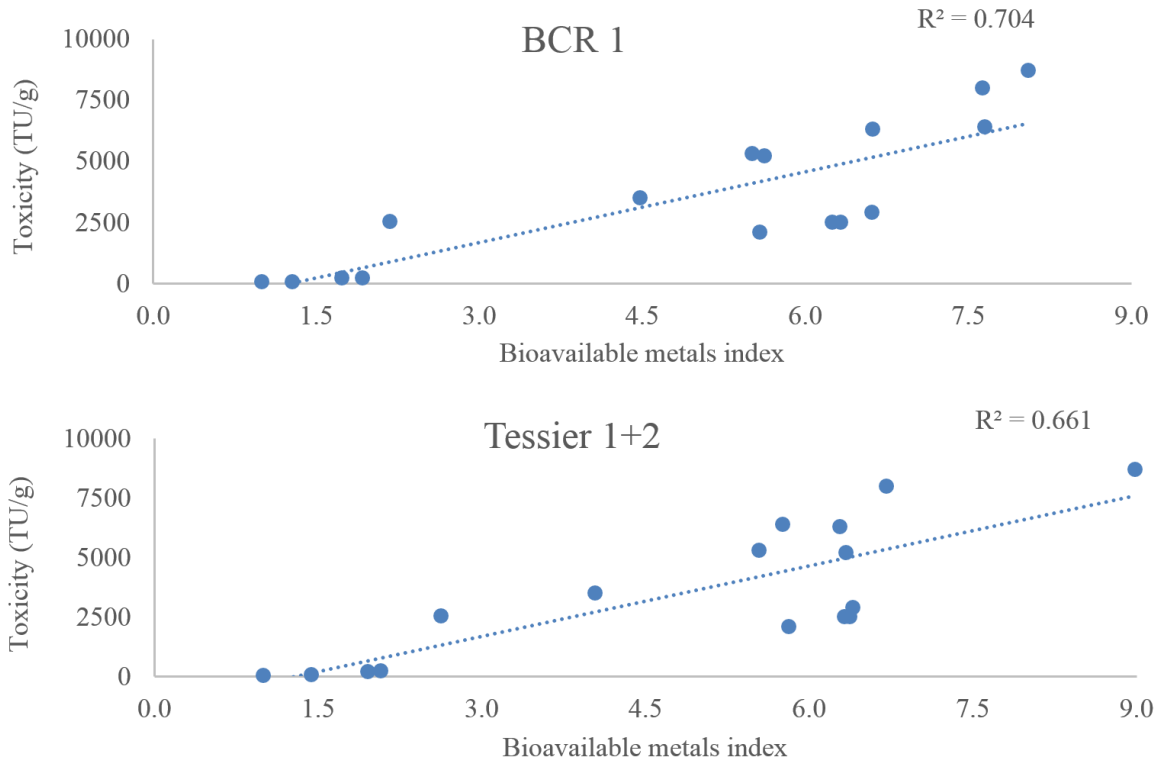

Figure 4. Correlation between toxicity (TU/g) and bioavailable metal index of 2 extraction procedures (BCR first step and Tessier first and second steps).

Bioavailable metal high values are a key factor in Huelva estuary and they are linked to the biota (Usero et al., 2005; Vicente-Martorell et al., 2009). Morillo and Usero (2008) and Morillo et al. (2005) found that metal concentrations in clam tissues (*Balanus amphitrite* and *Balanus balanoides*) from the Huelva estuary behave similarly to those in sediments. They showed the highest values in the two estuaries, especially the Tinto, and a decrease as they flow downstream, through the Padre Santo Canal and toward the sea. Furthermore, Usero et al. (2004) concluded that the metal concentrations of Cu in the liver of three fish species (*Solea vulgaris*, *Anguilla anguilla* and *Liza aurata*) were above the Spanish legal limits, in a region identificated as one of the areas with the highest mortality risks in Spain (Benach et al., 2004; Bleda and Tobías, 2002; López-Abente et al., 2006).

4 Conclusions

Microtox acute toxicity tests have been carried out on sediment samples of Huelva estuary and its littoral of influence, finding higher toxicity in the estuary than in the littoral. Littoral samples located west of the estuary showed lower toxicity compared to those located east of

the estuary, suggesting Huelva estuary influence over toxicity samples because of predominant west to east current (longshore drift).

Samples located west of the estuary showed toxicity similar to those found in free pollution sediments in nearby areas and the rest of the world and comply with the toxicity standards suggested by Environment Canada (2002) and certain Spanish authors (Casado-Martínez et al., 2006; DelValls et al., 2004; Morales-Caselles et al., 2008). However, samples located east of the estuary showed toxicity levels comparable to those of polluted places like the port of Barcelona and exceed toxicity thresholds suggested by Environment Canada and Spanish authors.

Highest average toxicity in Huelva estuary sediments was found in the Tinto estuary, followed by the Odiel estuary and the Padre Santo Canal. In the case of the Tinto, toxicity increases downstream, up to the area in which the waters of the two rivers merge, where the maximum value of all surveyed points is reached. In the estuary of the Odiel, toxicity increases downwards up to approximately the middle of the estuary, where the toxicity begins to decrease. In the Padre Santo Canal the toxicity decreases along the canal towards the sea.

All the samples located in Huelva estuary showed a toxicity well over the toxicities found by other researchers in nearby estuaries, suggesting that peculiar phenomena take place in Huelva estuary. Toxicity values are similar to or even higher than those in other polluted sediments around the world and all samples analyzed in the Huelva estuary far exceed the threshold to be considered toxic, in the worst cases, by more than 50 times.

The analysis carried out in this study found a strong correlation between concentrations of bioavailable metals in sediment and toxicity in a region identificated as one of the areas with the highest mortality risks in Spain.

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