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Comparative Baseline Levels of Heavy Metals and Histopathological Notes in Fish From two Coastal Ecosystems of South-West of Spain

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ABSTRACT: This work reports the comparative study of heavy metal water concentrations from two coastal ecosystems of Cadiz Gulf (SW Spain): Bay of Cadiz and Ria of Huelva estuary in samples taken on years 1999-2000. Sampling zones showed different heavy metal levels. Statistical analysis of dissolved trace metal concentrations (Cu, Zn, Cd and Pb) showed significant differences between Huelva and Cadiz zones based on the Cu level, with a marked seasonal factor and different metal sources (Industrial, geological, urban,...), observing a higher Cu concentration in Ria de Huelva respect to Bay of Cadiz. On the other hand, to evaluate the pollutant effects on aquatic biota, a histopathological analysis have been conducted in specimens of flat fish, *Solea senegalensis* from both zones. Lesions were more severe in fish from Ria of Huelva, being the most significant alterations: gill hyperplasia, fusion of secondary lamellae, disorganization of the hepatic parenchyma, cellular hypertrophy and vascular congestion in liver and tubular occlusion, loss of interstitial tissue and lipid-like vacuoles in kidney. Obtained results are useful as reference data for future environmental monitoring studies in these zones where to establish a heavy metal concentration temporal trend would be necessary.

Key words: Histopathology, Sole, Heavy metals, Seawater, Estuary

INTRODUCTION

The studied zones (on the north coast of Cadiz Gulf) are located in the confluence of Atlantic Ocean and Mediterranean Sea. The proximity of the Iberian Pyrite Belt and the influence of seawater inflows and outflows through the Gibraltar Strait have clearly influenced the heavy metal concentration and their distribution in these areas. The biochemical cycles of metals, as well as the chemical and physical processes, contribute to metal distribution within estuaries and coasts (Braungardt *et al.*, 1998; Vicente Martorell *et al.*, 2009). Bay of Cadiz receives regular discharges of untreated sewage from an urban area with a population of 300000 inhabitants, which increases during the summer because of tourism, a large saltmarsh area used to fish aquaculture, harbour facilities and industrial activities (naval, car and aircraft components), being the anthropogenic influence very high in this zone (Forja, Blasco & Gómez Parra, 1994; González Mazo, Pajares & Gómez Parra, 1998; Ligero *et al.*, 2002).

Ria of Huelva, receives acidic fluvial water discharges with high concentrations of sulphates and heavy metals from the natural sulphur deposits, the mining activities and the industrial facilities developed along the estuary (fertilizer factories, paper mills, copper foundries, oil refinery, etc) (Elbaz-Poulichet *et*

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*Corresponding author E-mail: milagrosa.oliva@uca.es *al.*, 1999; Davis *et al.*, 2000; Jiménez-Tenorio *et al.*, 2007; Oliva *et al.*, 2012). Fishing activities in Cadiz Gulf and the presence in the zone of the Doñana National Park (an important international protected natural area) indicate that the study of the pollutants impact on aquatic ecosystem is essential.

Seas and oceans are becoming pollution sinks due to the anthropogenic activities and the heavy metals levels are increasing progressively producing serious problems in the environment. Heavy metals in the marine environment promote adverse effects on the biota. Bioaccumulation process of these elements has a pernicious effect in the aquatic organisms. Due to these problems, researches on heavy metals in aquatic organisms are very important (Oliva *et al.*, 2012; Karan *et al.*, 1998; Arellano *et al.*, 1999, Ortiz *et al.*, 1999; Rashed, 2001; Oliva *et al.*, 2009).

Since the aquatic organisms present a dynamic relation with the environment, heavy metals are incorporated and excreted by the organisms until an osmotic balance. Histological changes produced by contaminants in organs/tissues can produce irreversible effects in the organisms-biota, histological methods can be used in combination with other parameters (metals quantification) and ecotoxicological biomarkers (glutathione, stress proteins, etc.) such as an early-warning system of great importance to the survival of the species and for the environmental protection (Van der Oost, Beyer & Vermeulen, 2003).

Solea senegalensis is a benthonic marine species living in sandy or muddy bottoms, off coastal areas up to 100 m depth, in brackish lakes and estuaries. Senegal sole feeds basically on benthonic invertebrate, such as larvae from polychaets, bivalve molluscs and small crustaceans. Sexual maturity is reached when size is 30 cm. Spawning happens between the months of March until June (Froese & Pauly, 2010). *Solea senegalensis* is a well adapted species to warm climates and is commonly exploited in extensive aquaculture production in Spain and Portugal (Drake, Arias & Rodríguez, 1984; Dinis, 1992; Sarasquete *et al.*, 1998). *S. senegalensis* have been used in field and laboratory toxicity assays being sensitive specie to pollutants (Olive *et al.*, 2009; Jiménez-Tenorio *et al.*, 2008; Costa *et al.*, 2009).

The present paper focuses on histopathological investigations which have been proved to be a sensitive tool to detect direct toxic effects of chemical compounds within target organs of fish in field investigation (Costa *et al.*, 2009; Schwaiger *et al.*, 1997; Stentiford *et al.*, 2003; Au, 2004). Histopathological responses were quantified to compare de severity of lesions in fish from different sampling sites. The aim of this paper was the study of the heavy metals concentrations in water and the histopathological alterations in *S. senegalensis* organs from Bay of Cadiz and Ria of Huelva to establish a historical reference for future studies of heavy metal pollution temporal trends in the zones.

MATERIALS & METHODS

The present study was performed in two coastal systems from the Gulf of Cadiz (SW Spain): Bay of Cadiz and Ria of Huelva estuary (after the confluence of Tinto and Odiel Rivers) in years 1999-2000. Four areas (A, B, C and D) and three stations (Odiel (O: point 1), Tinto Rivers (T: point 2) and the end of the Ria of Huelva (E: point 18)) were sampled in the Huelva area (Fig. 1); and three areas (A, B and C) and two stations (the mouth of Guadalete river (G: point 18) and a closed point of San Fernando town, namely Zuazo Brigde (Z: point 17) in the Bay of Cadiz (Fig. 2). Stations were chosen in order to achieve a global description of both ecosystems.

Water samples were collected during two consecutive years, 1999 and 2000, in winter and summer seasons, aboard on a ship belonging to the Environmental Agency of the Government of the Andalusia Community. Water samples were collected using a 2 L Nansen sampling bottle with a remote system for sample collection at different depths: 1.0 m below the sea-surface, 1.0 above the sediment/water interface and intermediate depth between them. Integrated samples were obtained mixing the same volumes of samples from the three different depths of the water column in each station.

Salinity and pH were measured at the sampling sites using a Hanna Salinity Tester (Padova, Italy) and a Crison model 2001 pH-meter (Barcelona, Spain).
The samples were immediately filtered after collection (nylon filters of 0.45µm pore size, Osmonics, USA), acidified with 2 mL per litre of HNO_3 of Suprapur Merck grade (Darmstadt, Germany) and stored at 4ºC in polyethylene bottles.

The concentrations of Zn, Cd, Pb and Cu in the water were determined simultaneously by differential pulse anodic stripping voltammetry. The voltammetric system comprised a Metrohm Model 746 VA Trace Analyzer processor and a Metrohm 747 VA Electrode Stand with automatic hanging mercury drop electrode (HMDE), connected to a 717 Sample Changer Autosampler (Herisau, Switzerland). The reference electrode was Ag/AgCl, saturated AgCl, 3 mol/L KCl and the auxiliary electrode was a platinum wire. Metal standard additions for the internal calibration of each measurement were realised using metal ions standard

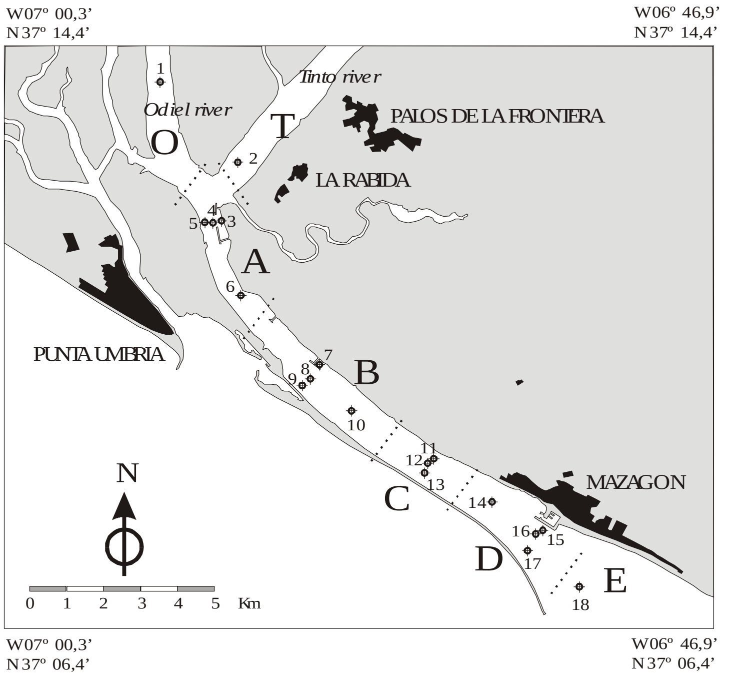


Fig. 1. Sampling stations and areas in the Ria of The Tinto and Odiel rivers estuary (south-west Spain)

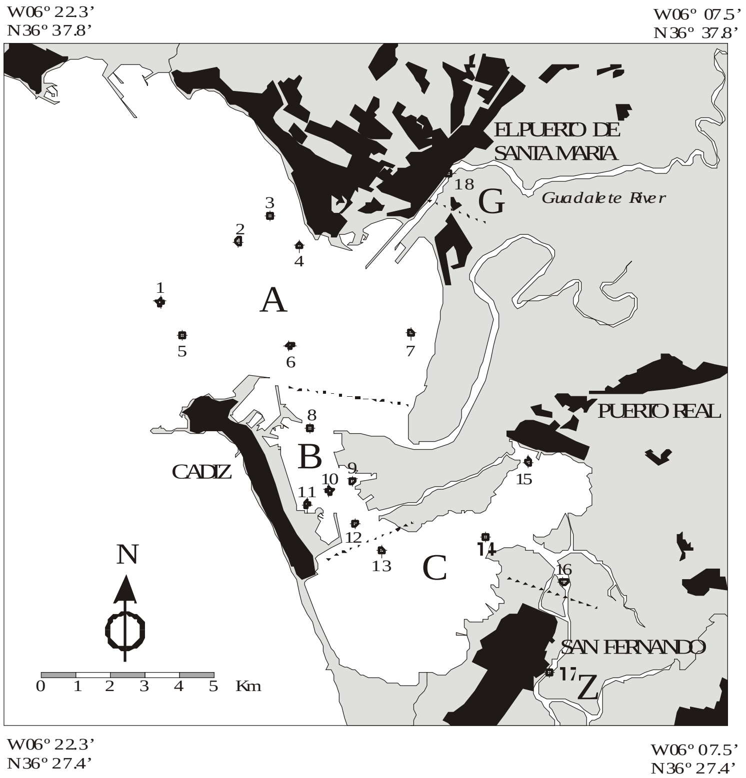


Fig. 2. Sampling stations and areas in the Bay of Cadiz (south-west Spain)

solutions using AAS Merck standard solutions of 1000 mg/L (Darmstadt, Germany).

The accuracy of the heavy metal analytical procedure was assessed using certified reference material (water SRM 1643 d, NIST, USA). Good recoveries (>90%) were obtained for all metals.

To avoid the contamination of water samples during analysis, the voltmeter system was placed into a clean-hood and all sample containers and laboratory materials was cleaned with detergent, water, 2 mol/L of HNO_3 and finally rinses with Milli-Q grade water. Suprapur

Merck grade reagents (Darmstadt, Germany) and deionised Milli-Q water (Millipore, USA) were used. The biological material of this study was composed by adult specimens (20–30 cm length, 350-400 g weight) of Senegal sole, *Solea senegalensis*, captured in the Ria of Huelva and Bay of Cadiz. Fifteen specimens for each sampling zone were transported in aerated tanks to laboratory. Fish were dissected and samples of liver and gills of each fish were taken to histopathological analysis. The tissue samples for the histological exam were fixed in formalin (10%), washed in running tap water, dehydrated in alcohol and acetone, cleared in xilene and embedded in paraffin wax. Sections (6 µm) were cut and mounted on gelatinized slides using a rotary microtome. Sections were rehydrated in distilled water and stained with hematoxylin/eosin (H/E) and hematoxylin/VOF (H/V) (light green, orange-G and acid fuchsine). Pathology criteria were derived from several tissue alterations specified by the International Council for the Exploration of the Sea (ICES) and additional pathology observed in the sampled specimens (Anon, 1999). Histopathological sections were read after the course of each sampling period. Alterations were evaluated quantitatively by ranking the severity of the alteration in the tissue. Ranking was: grade 0 (no alterations), grade 1 (focal mild alteration), grade 2 (moderate alteration) and grade 3 (extended severe alteration). This ranking was used to establish an overall assessment value of the histopathological lesions for each organ of individual fish. Finally, statistical techniques to interpret the variability observed in the obtained data were used. The Statgraphics Plus Package 5.0 (Statgraphics, 2000) was used for the multivariate analyses.

RESULTS & DISCUSSION

Table 1 and 2 show some of the statistics derived from the univariate analysis. Ria of Huelva showed dissolved metal concentrations higher than Bay of Cadiz. In the Ria of Huelva, metal contents decreased gradually and significantly from the rivers to E station because of the precipitation of the dissolved metals being less in the Odiel River. The mixture of sea water and river water causes the metal precipitation, producing high concentrations of these metals in estuary sediments, although the increase of pH and salinity along Ria of Huelva is soft and typically marine (Grande, Borrego & Morales, 2000). However, the industrial activities can modify the physicochemical conditions and favour the releasing in solution of metals which were previously immobilized in the sediments increasing metal concentrations in water. So, there was a few stations where the metal concentrations was higher than expected values, as Pb and Cu in some points of B area (max_{Pb} = 122.10 nmol/L, $mean_{Pb}$ = 42.86 nmol/L, sd_{Pb} = 27.01; max_{Cu} = 1510.62 nmol/L; $mean_{Cu}$ = 630.27 nmol/L, sd_{Cu} = 314.97), indicating a clear influence of industrial activities in outstanding sampling points. In Bay of Cadiz, metals come from the Guadalete River, with high metal content of Zn, Pb and Cu and from sewage discharges of cities of Puerto of Santa María and San Fernando (A and Z areas, respectively) with higher concentrations of Zn and Cu. Nevertheless, the values were not high in comparison with other ones reported (Crompton, 1998). A comparison of the results was made with ranges of metal concentrations found in estuary, bay and coastal waters reported in Table 3. It can be observed that regarding with the Ria of Huelva, metal concentrations were higher than the reported values inthis table: Cu in Tinto, Odiel, A, B and C areas, Pb in Tinto, Odiel, A and B areas, and Zn in Tinto and Odiel points. In the Bay of Cadiz, the values were within the concentration range found in coastal waters. Also, it was interesting to compare the results with the concentrations of metal that cause adverse effects on marine life (Crompton, 1998). The Table 4 shows the metal composition of three known types of coastal waters and reports the effects on sea organisms in order to compare our metal concentrations with those which showed adverse effects. From Ria of Huelva data, it would be expected severe effects by Cu (in Odiel and Tinto Rivers), Zn (in Odiel, Tinto, A and B areas) and Cd (in Odiel, Tinto and A area), and few occasional adverse effects by Zn (in C area), Cd (in B area), Pb (in all points) and Cu (in A, B, C, D and E areas). In Cadiz, the bay waters seemed to be quite clean and it only would cause occasional adverse effects in very few stations by Zn (G point), Pb (G and Z points) and Cu (in G and Z points). Table 5 summarizes the main histopathological disorders in specimens of *Solea senegalensis* in Bay of Cadiz and Ria of Huelva. Systematically, the histopathological disorders were more frequently recognized in the second marine environment. The gills of *Solea senegalensis* specimen present the same histological characteristic as other fish species. The gills are formed by four pairs of brachial arches with two lines of filaments in each arch separated by the gill septum; each filament supports the respiratory lamellae (Fig. 1A). The filaments are covered by epithelial tissue being the most characteristic cell types the chloride cells and the calceiform cells. The respiratory lamellae are formed by pillar cells which limit the vascular spaces. The most significant histopathological observed alterations were gill hyperplasia, fusion of adjacent lamellae with obliteration of the interlamellar spaces and epithelial lifting (Fig. 1B). The *Solea senegalensis* liver structure presents the same characteristics than the ones described for other teleostean species. The hepatocytes are polygonal-shaped cells with a central or eccentric nucleus and a clear nucleolus. In addition to these parenchymatous cells, there are connective tissue fibbers that provide support and a vascular system with a clear endothelium (Fig. 1C).

The hepatic histological alterations were more significant in specimens from Tinto and Odiel Rivers than the collected in Bay of Cadiz (Fig.3). In general, a disorganization of the hepatic parenchyma, cytoplasmatic vacuolisation, cellular hypertrophy and vascular congestion were observed (Fig. 1D). Excretory portion of kidney from control specimens contains renal corpuscle and renal tubules immersed in lymphoid tissue. The renal corpuscle is composed of a glomerulus and its capsule and the renal tubules consists of a single layer of prismatic epithelia cells (Fig. 1E). Nuclear pycnosis, tubular occlusions and decreasing of haematopoietic tissue were detected (Fig. 1F).

Table 1. Mean, minima, maxima and standard deviations of heavy metals water concentrations (nmol/L), pH and salinity from the Ria of Huelva sampling sites

Parameter	Zone	Cases	Mean	Min.	Max.	SD
Zn	O	3	3644.8	1166.8	7963.0	3753.2
	T	3	5071.1	1258.6	9871.5	4390.6
	A	15	2649.2	564.3	8005.8	2138.7
	B	15	1772.4	414.4	4580.2	1399.2
	C	12	851.2	360.9	1995.7	597.7
	D	15	457.9	194.2	1165.3	283.8
	E	3	286.0	163.6	417.5	127.2
Cd	O	3	28.85	17.70	35.76	9.74
	T	3	29.83	21.88	36.92	7.56
	A	15	18.82	7.38	33.27	7.38
	B	15	12.14	6.14	22.51	4.65
	C	12	5.06	3.29	7.74	1.55
	D	15	2.37	0.76	7.03	1.61
	E	3	1.28	0.80	2.05	0.67
Pb	O	3	36.31	21.23	60.33	21.03
	T	3	46.2	27.75	79.48	28.88
	A	15	38.70	22.39	54.53	10.77
	B	15	42.86	15.88	122.10	27.01
	C	12	25.63	17.33	49.61	9.51
	D	15	19.04	10.47	33.64	8.03
	E	3	9.65	7.82	11.01	1.64
Cu	O	3	795.18	602.68	974.04	186.06
	T	3	1100.44	756.88	1427.22	335.49
	A	15	717.97	385.52	1038.55	198.70
	B	15	630.27	267.51	1510.62	314.97
	C	12	366.64	243.90	646.73	116.61
	D	15	253.94	139.58	546.03	115.31
	E	3	139.73	116.44	177.81	33.25
pH	O	3	7.83	7.64	8.06	0.21
	T	3	7.80	7.61	8.07	0.24
	A	15	7.85	7.59	8.22	0.20
	B	15	7.96	7.76	8.30	0.16
	C	12	8.07	7.95	8.31	0.13
	D	15	8.13	7.85	8.39	0.16
	E	3	8.09	7.99	8.18	0.09
Salinity	O	3	36.7	36.0	37.0	0.6
	T	3	36.5	36.0	37.0	0.5
	A	15	36.3	34.0	38.0	1.2
	B	15	36.2	32.0	38.0	1.5
	C	12	37.0	35.0	39.0	1.6
	D	15	37.2	35.0	40.0	1.5
	E	3	37.0	35.0	39.0	2.0

Table 2. Mean, minima, maxima and standard deviations of heavy metals water concentrations (nmol/L), pH and salinity from the sampling zones and points from the Bay of Cadiz

Parameter	Zone	Cases	Mean	Min.	Max.	SD
Zn	A	28	111.76	9.79	539.84	113.38
	B	18	58.29	5.51	152.93	34.70
	C	14	57.69	14.53	153.85	34.54
	G	4	192.58	62.70	402.2	147.07
	Z	2	102.39	88.55	116.23	19.57
Cd	A	28	1.73	0.18	18.06	3.30
	B	18	4.58	0.27	66.45	15.46
	C	14	1.08	0.27	3.68	0.99
	G	4	0.81	0.09	1.6	0.62
	Z	2	2.81	0.82	4.8	2.81
Pb	A	28	7.42	2.36	28.43	5.87
	B	18	7.70	1.94	55.02	11.97
	C	14	7.49	3.16	13.71	3.05
	G	4	23.82	7.05	63.08	26.43
	Z	2	10.14	8.83	11.44	1.85
Cu	A	28	48.81	29.11	119.28	20.33
	B	18	33.15	19.31	71.13	12.06
	C	14	39.42	19.35	61.05	11.80
	G	4	72.23	34.62	112.82	33.72
	Z	2	78.44	44.06	112.82	48.62
pH	A	27	8.11	7.95	8.18	-----
	B	18	8.13	7.94	8.25	0.05
	C	14	8.21	7.96	8.6	0.17
	G	4	8.03	7.8	8.2	0.16
	Z	2	7.99	7.96	8.02	0.04
Salinity	A	27	37.13	35.0	40.0	1.37
	B	18	37.19	36.0	39.0	0.75
	C	14	37.54	37.0	39.0	0.60
	G	4	38.12	37.5	39.0	0.63
	Z	2	35.0	33.0	37.0	2.82

Table 3. Ranges of metal concentrations found in coastal waters (surface estuary, bay and coastal waters) (Crompton, 1998)

Metal	Concentration range (nmol/L)
Zn	0.11-3058.6
Cd	0.13-44.48
Pb	0.18-35.91
Cu	1.09-314.7

Table 4. Adverse effects of metals on marine life in estuary, coastal and seawaters (Crompton, 1998)

Sampling area	Metal concentration (nmol/L)			
	Zn	**Cd**	**Pb** [d]	**Cu**
Coastal water adjacent to sewage discharge [a]	1728.1-1758.7	13.3-22.2	144.8-149.6	764.7-778.9
Humber Estuary [b]	30.6-764.6	1.8-6.2	2.4-4.8	1.6-125.9
Severn Estuary [c]	168.2-336.4	2.8-13.2	7.2-19.8	34.6-66.1

[a] Adverse effects expected: adult bivalve molluscs (Cd, Cu and Zn), mollusc larvae (Cu and Zn), adult hydrozoans (Cd, Cu), adult annelids (Cu), annelid larvae (Cu, Zn), adult echinoderms (Cu), adult gastropods (Cu), adult crustaceans and crustacean larvae (Cu and Zn) and adult fish and fish larvae (Cu).

[b] Adverse effects expected: bivalve molluscs and their larvae (Zn and Cu). These waters support fisheries.

[c] Adverse effects expected: bivalve molluscs and hydrozoan adults (Cd), bivalve molluscs adults and larvae (Cu and Zn). These waters support some fisheries like salmon, eel and shellfisheries.

[d] Adverse effects expected: 6.2 nmol/L should not exceed to avoid them on mussels and on human who eat them.

Table 5. Frequency of histopathological alterations in specimens of *Solea senegalensis* from sample zones

Tissue	Histopathology	Percentage of affected organisms [a]	
		Huelva	**Cádiz**
Gill	Lamellar fusion	+++	+
	Hyperplasia	++	+
	Vascular dilatation	++	+
	Epithelial lifting	+	+
	Aneurysms	+	+
	Oedemas	++/+++	+
Liver	Lipid-like vacuoles	+++	+
	Hyperplasia/hypertrophy	++	+
	Blood stagnation	++	+
	Vascular dilatation	+	+
	Pycnosis	+	+
Kidney	Tubular occlusion	+++	+
	Pycnosis	++	+
	Tubular disintegration	++	+
	Tubular dilatation	+	+
	Hypertrophy/vacuolisation	+	+
	Loss of interstitial tissue	+++	+

[a] Percentage: +, no frequent (0-25 %); ++, frequent (25-75%); +++, very frequent; in practically all studied organisms (75-100%).

First, a discriminant analysis was applied to the set of all cases (n=132). The model was used to discriminate among studied coastal systems (Bay of Cadiz and Ria of Huelva). The predictor variables were pH, salinity and four heavy metals (Cu, Pb, Cd and Zn). Using a discriminating function statistically significant at the 95% confidence level, 87.02% of observations were correctly classified, being 100% of Cadiz and 74.24% of Huelva. The wrong observations were observed in D and E zones, where the metal concentration decreased with the marine water influence. So, the cases were classified as Cadiz with fewer sources of metals than Huelva. The coefficients of the functions used to classify observations and the standardized discriminating functions are in Table 6. The relative magnitude of the coefficients in the last function showed how the independent variables were being used to discriminate among the groups, being Cu, and in less extend Zn, discriminant variables between samples of Cadiz and Huelva.

The analysis for each system was performed in the same way based on the following levels: area, tide, season (winter, summer), year (1999, 2000) and depths in the sampling sites (<6, 6-12, >12 m). The best model to discriminate among the studied levels was the discriminating function by season, where for Cadiz amongst the 65 observations used to fit the model, 84.61% were correctly classified with salinity and Zn with the highest relative magnitude of the coefficients used to discriminate. For Huelva, we could discriminate cases between dry/wet season with a 98.48% of cases correctly classified, with only 1 observations incorrectly classified. In this system, the most influential variables in the discriminating function were Zn, and in less extend Cu. In both cases, Zn concentrations are clearly influenced by seasons: in Huelva, Zn seems to have a natural origin because of the influence of rainfall that favours the lixiviation processes in winter. In Cadiz, the seasonal variability controlled by Zn can be explained with an urban source of this metal, because during the summer, the tourist population increases the waste water inflows. Using these discriminant functions to classify observations, additional observations can be predicted and they can be used as guide of seasonal variability of metal concentrations. The “nearest neighbour” clustering method (distance metric: Euclidean) was performed to data of Huelva and Cadiz, in order to know which sample stations had a similar behaviour. The resulting dendograms are represented in Figures 4 and 5. From the Cadiz data, there was a group of observations with similar characteristics and distance between them lower than 2. The rest of the cases, four samples of summer (12 during 1999 and G, 11 and Z during 2000) and winter (1 and Z during 1999 and 4 and 5 during 2000) had a higher distance and corresponding to few different samples, pointed out the existence of polluted spots. In Huelva, the samples had metal contents more similar during the summer, and in winter, when the industrial activities are more important, there was a progressive increase of the distance between the observations, being higher the difference of the

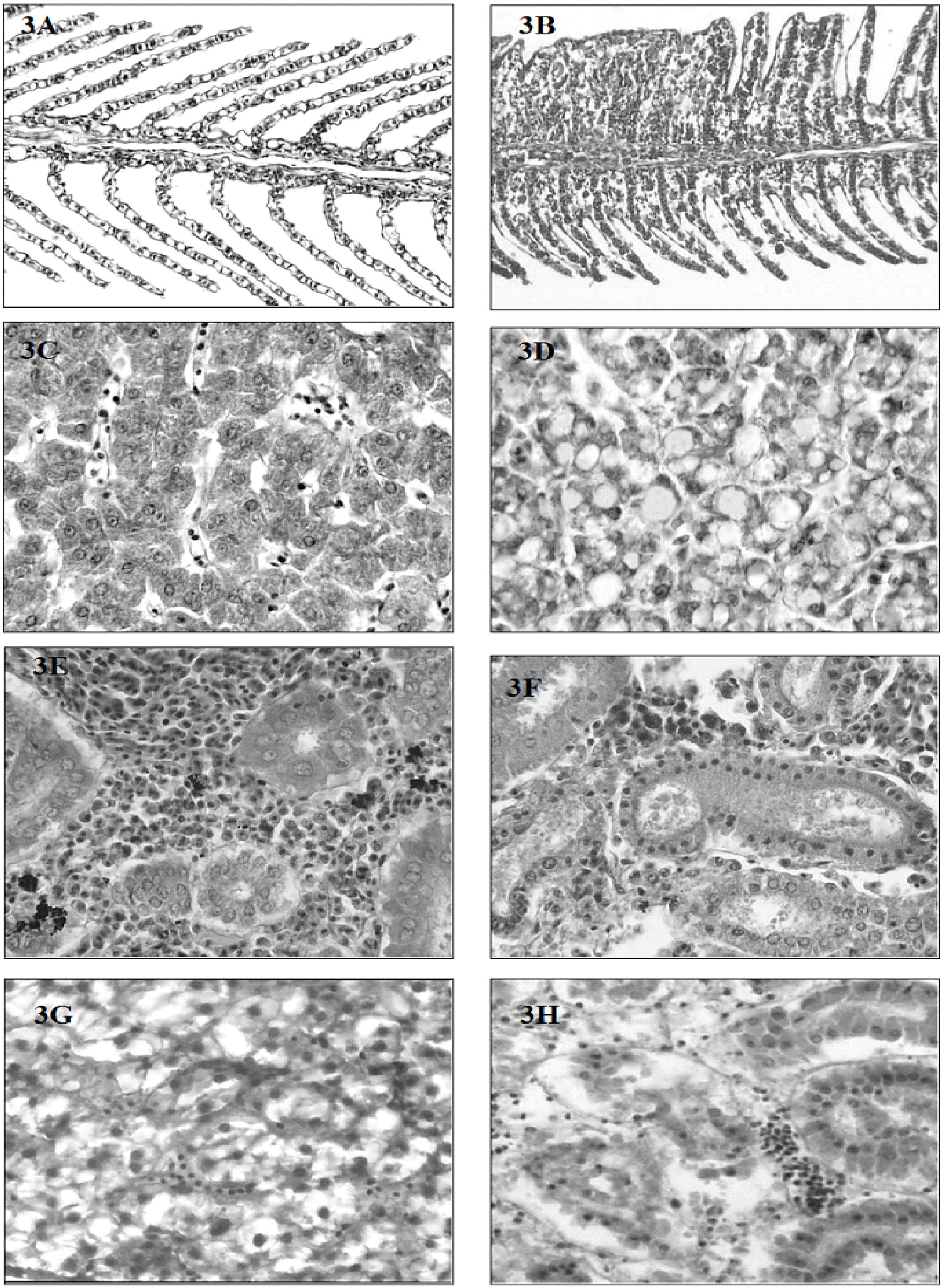


Fig. 3. (**A**) Gill histological section of Cadiz Bay *Solea senegalensis*, showing the respiratory lamellas with the chloride and calyciforms cells (x 20 H/VOF); **(B)** Gills of specimens of Huelva estuary. Gill hyperplasia with fusion of adjacent lamellas (x 20 H/VOF); **(C)** Liver of specimens of Cadiz Zone, showing the hepatocytes disposition in cords around the sinusoids (x 40 H/E); **(D)** Cytoplasmic vacuolisation and cellular hypertrophy in sections of Huelva *Solea senegalensis* (x 40 H/E); **(E)** Posterior portion of kidney of Huelva Zone specimens (x 40 H/E); **(F)** Alterations in collector tubules and occlusion in the lumen of cavity (x 40 H/E); **(G)** Liver exposed for 15 days to 1573,56 nmol/L of Cu^{2+}, showing great vacuolization of the parenchyma(X40 H/E); **(H)** Kidney exposed for 15 days to 100 µm/L of Cu^{2+}, showing tubular epithelial necrosis

Table 6. Discriminate analysis for Huelva and Cadiz coastal systems ($p<0.05$)

Classification Variable	Cases well classified	Function	Coefficients						
			Zn	Cd	Pb	Cu	pH	SAL	Constant
Coastal systems n = 131	Huelva 74.24 % Cadiz 100 %	Classification for Huelva	0.012	1.077	0.083	0.026	383.23	2.331	-1597.98
		Classification for Cadiz	0.013	1.107	0.113	0.009	384.89	2.447	-1612.81
		Standardized Discrimination	-0.647	-0.117	-0.198	1.562	-0.114	-0.065	
Season for Huelva Data n = 66	Winter 100 % Summer 97.22 %	Classification for Winter	0.065	1.671	2.272	-0.211	566.02	21.87	-2767.95
		Classification for Summer	0.061	1.703	2.097	-0.196	549.24	19.65	-2548.13
		Standardized Discrimination	2.019	-0.086	0.921	-1.315	0.879	0.598	
Season for Cadiz Data n = 65	Winter 86.67 % Summer 82.86 %	Classification for Winter	-0.044	0.733	-0.561	1.401	773.21	49.65	-122.20
		Classification for Summer	-0.057	0.739	-0.520	1.426	766.95	48.31	-021.80
		Standardized Discrimination	-0.669	0.028	0.243	0.310	-0.404	-0.777	

behaviour among them. Five observations had a distance equal or higher than 2, corresponding to sampling points of winter (7 during 1999 and T during 2000) and summer (9 during 1999 and O and T during 2000), near of industrial sources. Factor analysis was applied to each ecosystem to determine the factors that control their behavior and compare them. The values of the variables were autoscaled prior to analysis. The eigenvalues and the variance accumulative percentages corresponding to the principal components with varimax rotation were obtained using the Kaiser criterion Kaiser, 1960). Two factors were retained for data from Huelva and three ones for data from Cadiz, explaining 77.3% and 72.0% of total variance, respectively.

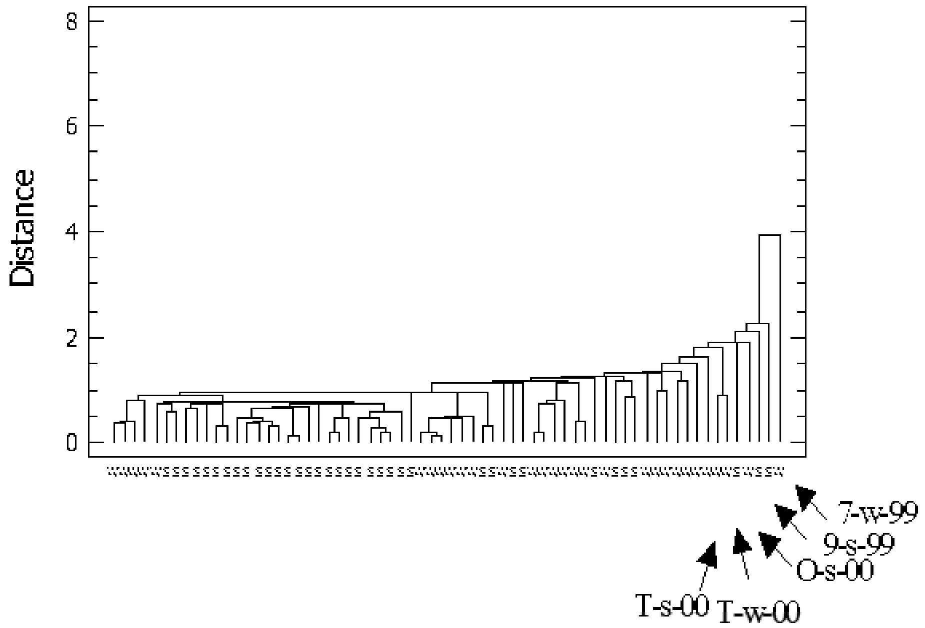


Fig. 4. Dendogram of Cluster Analysis of Data of Huelva (samples codes: a) sampling site (number: 1-18; O: point 1; and T: point 2); b) winter (w) or summer (s) campaigns; and c) year (year 1999: 99; year 2000: 00)

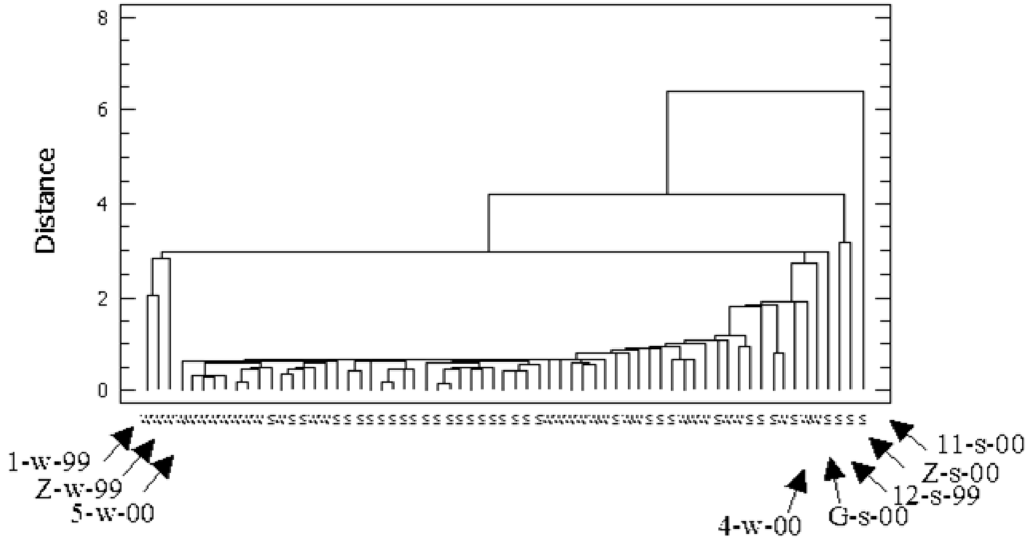


Fig. 5. Dendogram of Cluster Analysis of Data of Cádiz (samples codes: a) sampling site (number: 1-18; Z: point 17; and G: point 18); b) winter (w) or summer (s) campaigns; and c) year (year 1999: 99; year 2000: 00)

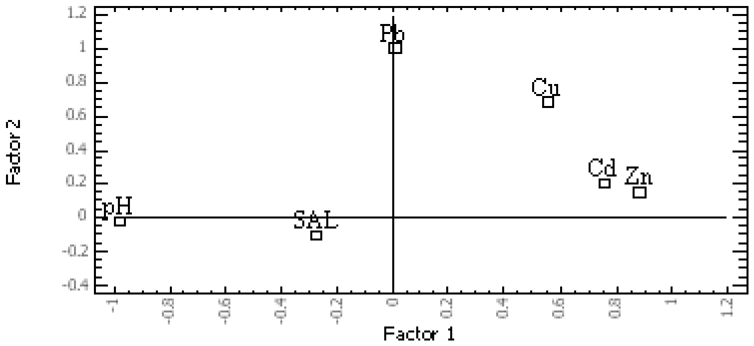
According to the first one, two principal factors were obtained in Huelva, where Cd, Cu and Zn were correlated and pH was negatively correlated with them, as an indicator of fluvial origin (Factor 1: eigenvalue, 3.21; percentage of total variance, 53.55 %; Cd factor loading, 0.93; Zn factor loading, 0.87; Cu factor loading, 0,72; pH factor loading, -0.35). The second factor (Factor 2: eigenvalue, 1.43; percentage of total variance, 23.75 %; Pb factor loading, 0.93; Cu factor loading, 0.60) was related to the Pb, a toxic metal and with an industrial origin; in addition, Cu was included in this factor too, showing two different sources because it was correlated with both factors with similar factor loading (0.72 and 0.60). Salinity did not belong to any factor, describing the Ria of Huelva as a channel of seawater. In Cadiz, each factor was related to a parameter: Factor 1 related to urban source (eigenvalue, 2.12; percentage of total variance, 35.58 %) including Zn (factor loading, 0.94), Factor 2 related to industrial source (Eigen value, 1.12; percentage of total variance, 18.39 %) including Pb (factor loading, 0.96) (industrial source) and Factor 3 as a hydrochemical component (Eigen value, 1.08; percentage of total variance, 18.03 %) with the pH variable (factor loading, 0.99). These different factors could be showing uncommon source of the studied metals. However, Cu was associated with both F1 and F2 factors as in Huelva, but with factor loadings 0.39 and 0.31 respectively, meaning two different sources for this metal.

Factor analysis (Table 7) of the metal concentrations performed with winter and summer samples, respectively, showed the following results. In Huelva, the Cu source was more associated to Zn and Cd origins (fluvial) during summer, on the other hand, in winter, this metal showed an industrial origin with Pb (factor loading 2 was increased and factor loading 1 was decreased for this metal) (Fig. 6). Also, in the scatterplot (Fig. 7) the samples showed higher variability with F1 during winter, because the fluvial influence was most important. Some samples outstood with a higher factor score of F2 (i.e. point 7 in winter-99) near of industrial facilities. In summer the trend of the values of scatterplot was more linear.

Table 7. Factor analysis with varimax rotation

Ria of Huelva			
Factor Number	Eigenvalue (>1)	Cumulative Percentage	Factor Loading
1 *(fluvial)*	3.213	53.55 %	0.93 (Cd), 0.87 (Zn), -0.35 (pH)
2 *(industrial)*	1.425	77.30 %	0.98 (Pb), 0.60 (Cu)
Bay of Cadiz			
Factor Number	Eigenvalue (>1)	Cumulative Percentage	Factor Loading
1 *(urban)*	2.12	35.58 %	0.94 (Zn), 0.39 (Cu)
2 *(industrial)*	1.12	53.97 %	0.96 (Pb), 0.31 (Cu)
3 *(hydrochemical)*	1.08	72.00 %	0.99 (pH)

a) Winter data



a) Summer data

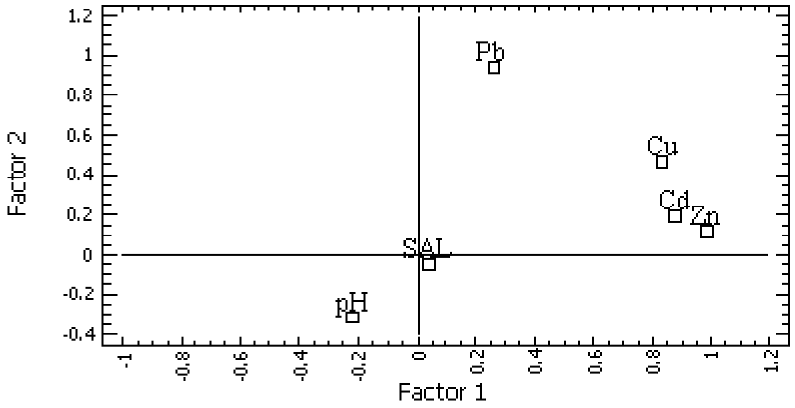
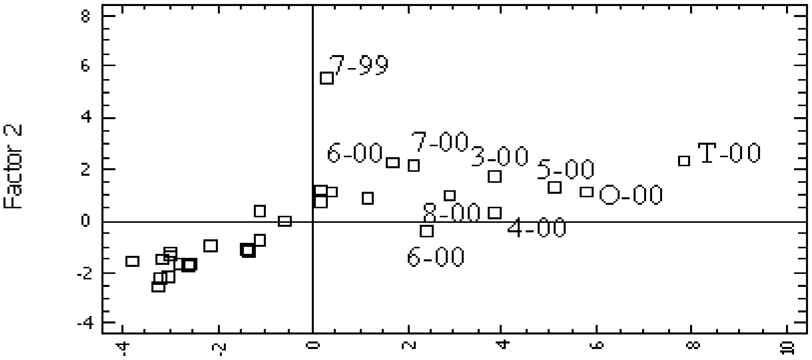


Fig. 6. Plot of factor loadings of data of Huelva (variables: pH, salinity (SAL), Cu, Cd, Zn and Pb)

a) Winter data



a) Summer data

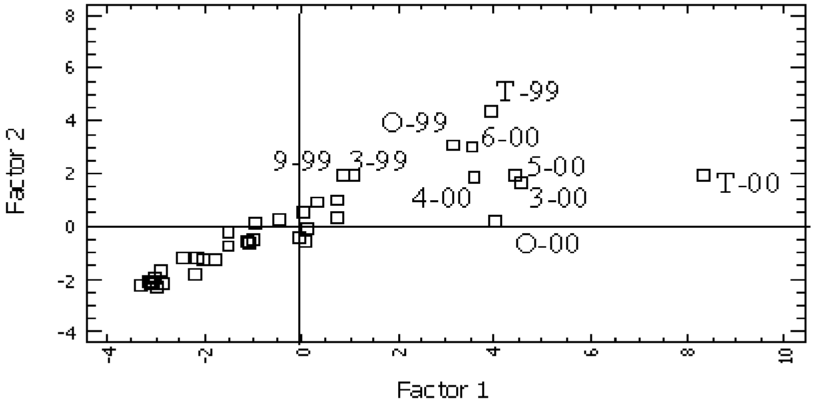
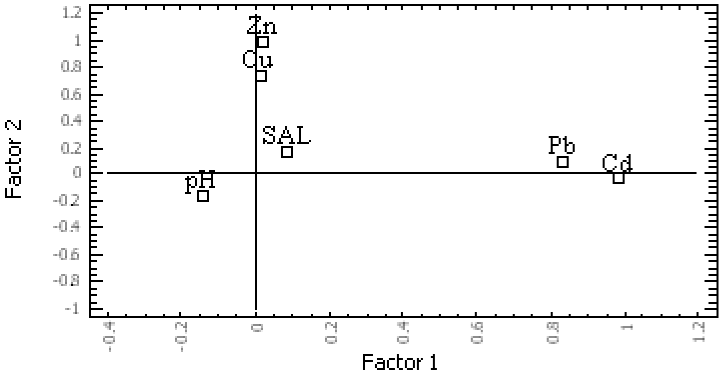


Fig. 7. Scatterplot from the factor analysis of data of Huelva (samples codes: a) sampling site (number: 1-18; O: point 1; and T: point 2); and b) year (year 1999: 99; year 2000: 00))

Seasonal variations were found for metal source in water from Cadiz. During summer, the factor 1 related with urban sewage principally included Zn; in addition, all metal showed a similar behavior related to F2 with low factor loadings. It meant that when the population increased, the industrial source were no significant (Fig. 8 b); in winter the metal came from two different origins: industrial activities (F1 including Pb and Cd, two toxic metals that could be produced by the industrial activities) and waste waters (F2 including Zn and Cu) (Fig. 8 a). The higher factor scored from the following samples showed this mentioned behavior (Fig. 9). Therefore for summer, the scatterplot of samples (Fig. 9) includes points Z and 16 from San Fernando City, point 12 near from Puntales in Cadiz and point G from estuary of Guadalete for F1. All these points have nearby effluents of urban wastewater. In winter, the industrial point as 4 (near the Puerto de Santa María Harbour) was included in F1 (with Pb and Cd). For this season, the urban influence was less, being significant only for the point Z. Points 1 and 5 showed a strange behavior for Cu and Zn, being included in F2 but with no urban influence. Probably, an external metallic influenced from the known high concentrations of metal pollutants reported for the Gulf of Cadiz and surrounding areas produced this fact (Baringer & Price, 1997; Johnson, Sanford & Baringer, 1994; Johnson, Lueck & Sanford, 1994). The histological alterations produced by pollutants in different organs and/or tissues of fishes, can be used as environmental contamination biomarkers (Van der Oost, Beyer & Vermeulen, 2003).The comparative study of the histopathological modifications in *Solea Senegalensis* from Huelva and Cadiz specimens points out that these modifications was more severe in the first area where the concentrations of contaminants are higher, with ratios for the different heavy metals oscillating from 3 (Pb) to 20 (Zn) (Table 8).

Although the cause-effect relationship between heavy metal concentrations and the appearance of pathological disorders was not clearly established in this study, several authors have emphasized that the exposition of the fishes to heavy metals is associated to structural damages in different tissues. Hepatic parenchyma vacuoles, steatosis, aneurisms and lamellar

a) Winter data



a) Summer data

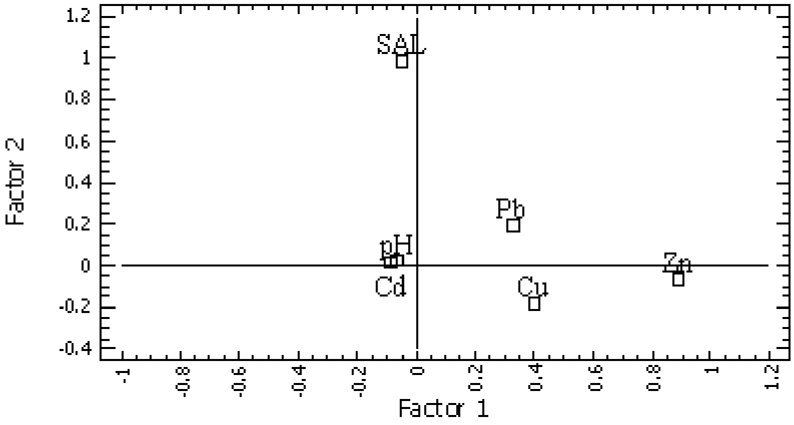


Fig. 8. Plot of factor loadings of data of Cadiz (variables: pH, salinity (SAL), Cu, Cd, Zn and Pb).

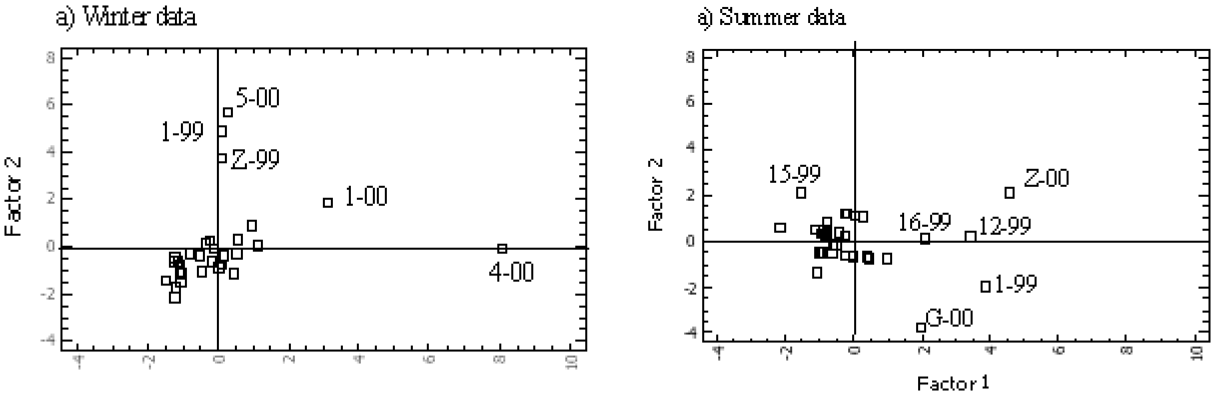


Fig. 9. Scatterplot from the factor analysis of data of Cadiz (samples codes: a) sampling site (number: 1-18; Z: point 17; and G: point 18); and b) year (year 1999: 99; year 2000: 00)

Table 8. Average metal concentration in water (nmol/L) for Huelva and Cadiz sampling sites

Sampling Sites	Parameter	Zn	Cd	Pb	Cu
Cadiz Bay	Mean	104.5	2.2	11.3	54.4
	Max	192.6	4.6	23.8	78.4
	Min	57.7	0.8	7.4	33.2
	SD	55.09	1.54	7.08	20.02
Ria of Huelva	Mean	2104.7	14.1	31.2	572.0
	Max	5071.1	29.8	46.2	1100.4
	Min	286.0	1.3	9.7	139.7
	SD	1787.42	12.07	13.46	337.63
Huelva/Cádiz Mean Concentration Ratio		20.1	6.4	2.8	10.5

fusion are frequent histopathological alterations and described by several authors as a result of the fish exposition to the different heavy metals which can be present in aquatic environments (Oliveira *et al.*, 2000; Moiseenko & Kudryavtseva, 2001; Oliva *et al.*, 2013).

High concentrations of copper and cadmium affects especially to gills (in close contact with the external environment) and liver (a metabolically active organ) because they are considered the target organs for heavy metals and other contaminants which can be present in the aquatic environment. The copper and cadmium cause a wide variety of physiological and histopathological effects in the fishes (Oliva *et al.*, 2013; Arellano, Storch & Sarasquete, 1999; Szbedinszky *et al.*, 2001).

Baker (1979) described how the secondary lamellas in specimens of *Pseudopleuronecter americanus* exposed to copper were much dilated. In specimens of *Solea senegalensis* exposed to sublethal concentrations of copper, via water, histopathological alterations in liver and gills have been observed when the exposition time to the contaminant increases. On the other hand, different histopathological studies on the effect of the copper in the liver and kidney reveal degenerative pathological signs such as the ones described after the treatment of different species of fish to copper concentrations varying between $1.57 \cdot 10^4$ nmol/L and $5.03 \cdot 10^4$ nmol/L, although specimens exposed to copper concentrations of $1.57 \cdot 10^3$ nmol/L did not show important alterations (Eisler & Gardner, 1973; Bunton & Frazier, 1989; Segner & Braunbeck, 1998).

Nevertheless, Arellano et al. (1999) observed in specimens of *Solea senegalensis* treated with the same concentration, an increase of the lipid-like vacuoles and blood infiltration in the sinusoids.Oliva et al. (2009) observed in juvenile of *S. senegalensis* exposed to 0.01, 0.1 and 1 mg/L of copper that histopathological alterations increased which the copper concentration. Considerable changes were observed only after extended exposure times at 1 mg/L copper. At 0.01 mg/L copper, the gill alterations were mild or non-existent. At 0.1 mg/L copper concentration the secondary lamellae became seriously affected and at the maximum concentration completely altered. Generalized hypertrophy and hyperplasia of secondary lamellae as well as hypertrophy of the cartilage were observed. Lamellae tended to shrink and disappear in some cases. Additional observations included numerous aneurysms in gill filaments, lifting of gill epithelia as well as a decrease of the mucus secretion and empty mucous cells. Liver of juvenile *S. senegalensis* specimens showed an increase of the histopathological alterations with the copper concentrations. Considerable changes were observed only after extended exposure times at 0.1 mg/L copper. At 0.01 mg/L copper, the liver alterations were mild or non-existent. At 0.1 and 1 mg/L copper concentration, a general disorganization of hepatic parenchyma was observed. A disorganization of hepatic parenchyma was related to the increase of exposure time to copper. Necrotic zones, dilatation of the blood capillaries and high concentration of sanguineous cells in the blood vessels were also observed. The hydropic vacuolization was observed in the totality of tissue, this vacuolization increased with the copper concentration. The hepatocytes presented a diffuse degeneration of the cytoplasm and several zones with lipid degeneration were observed. Moderate steatosis was observed in some zones of tissue. Hepatocytes showed diffuse degeneration of cytoplasm.

Oronsaye et al. (1989) observed the effects of cadmium in *Gasterosteus aculeatus*. Kidneys of fish exposed to 6 mg/L Cd^{2+} showed the disintegration of the tubules line from apical regions of the columnar cells. Evidence of tissue vacuolization was also obvious. Gills showed swelling and lifting up of epithelial layers of the secondary lamellae of the gill filament. Thophon et al. (2003) in *Lates calcarifes* exposed to cadmium observed at 96 h exposure, hypertrophy and hyperplasia of chloride and mucous cells, blood congestion and fusion of secondary lamellae in gills. Large lipid droplets and dark granules in hepatocytes and hypertrophy of tubules with hyaline droplets in tubular cells in kidney were also observed. Wangsongsak et al. (2007) observed in *Puntius gonionotus* exposed chronically to 2.4 mg/L Cd^{2+}, hyperplasia of pillar and epithelial cells in gills, necrotic areas in kidney and lipid vacuoles in liver after 3 months exposure. Similar effects were observed by Giari et al. (2007) in *Dicentrarchus labrax*.

Skidmore and Tovell (1972) in rainbow trout, *Oncorhynchus mykis*, exposed to zinc ($6.11 \cdot 10^5$ nmol/L) observed oedemas and a great dilatation of the secondary lamellas. Roncero et al. (1990) observed in gills of *Tinca tinca* exposed to 75 mg /L lead during 48 h oedema of the respiratory epithelium and epithelial proliferation of the lamellae. Martínez et al. (2004) observed in *Prochilodus lineatus* exposed to 98 mg/L lead a lifting and hyperplasia in the lamellar epithelium. In *Oncorhynchus mykiss* exposed to 10 mg/L lead, Suiçmez et al. (2006) observed degeneration of hepatic tissue, vacuolization, tubular degeneration and necrosis in liver and dilation of blood vessels and hypertrophy of epithelial cells in gills.

Similar histological alterations were observed in *S. senegalensis* from Ria de Huelva. Alterations in epidermal and chloride cells, hyperplasia and lamellar fusion of the secondary lamellae became apparent. In liver we observed an increase in the number of lipidic droplets based on the presence of great clear vesicles occupying the totality of hepatic cytoplasm and pushing the nucleus to the periphery and occlusion and disintegration tubular in kidney.

Other contaminants as pesticides, hydrocarbons, etc. produce similar alterations described previously in the target organs, it would be necessary to realize a study to establish correlations between concentration of heavy metals in tissues and histological alterations. Otherwise, the histopathological observed in *S. senegalensis* from the different sites can give general information on health of the two aquatic systems.

CONCLUSION

The dissolved heavy metal concentrations in the two studied ecosystems showed clear differences. Ria of Huelva showed the higher dissolved metal concentrations and it would be expected several adverse effects on marine life by Cu, Zn and Cd. The comparative study among *Solea Senegalensis* specimens of Huelva and Cadiz pointed out that systematically the histopathology in organs and tissues are more severe in the first area. The discrimination between Huelva and Cadiz samples was mainly based on the copper concentration of water. The seasonal variation was the most important source of variability of metal concentrations in both aquatic ecosystems. The observations were classified by salinity and Zn concentration in Cadiz and by Zn and Cu concentrations in Huelva. The variability between stations was more important in Cadiz, outstanding the existence of polluted spots, principally of wastewater. Factor analysis showed two factors identifying in Huelva the behaviour of the variables: fluvial and industrial factors, and three in Cadiz: urban, industrial and hydrochemical factors. Histopathological effects were observed in fish from environments with low metal concentrations but with long metals exposure time i.e. areas from Ria of Huelva. So, this study can show a causal relationship between occurrence of histopathological disorders in fish and contaminated waters but it is not possible to establish a clear relation between heavy metal concentrations in water and alterations in fish due to the possible presence of other pollutants in water and the complexity of the chemical mixtures in the aquatic environment.

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